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Counter-Current Chromatography

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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JOURNAL OF CHROMATOGRAPHY

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edited by **E.J. Karjalainen,** *Helsinki, Finland*

(Data Handling in Science and Technology, 6)

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Ion Chromatography

Principles and Applications

by **P.R. Haddad,** *University of New South Wales, Kensington, N.S.W., Australia and* **P.E. Jackson,** *Waters Chromatography Division, Milford, MA, USA*

(Journal of Chromatography Library, 46)

Ion chromatography (IC) was first introduced in 1975 for the determination of inorganic anions and cations and water soluble organic acids and bases. Since then, the technique has grown in usage at a phenomenal rate. The growth of IC has been accompanied by a blurring of the original definition of the technique, so that it now embraces a very wide range of separation and detection methods, many of which bear little resemblance to the initial concept of ion-exchange separation coupled with conductivity detection.

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SPECIAL ISSUE

COUNTER-CURRENT CHROMATOGRAPHY

Guest Editors

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(Amherst, NY)

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Journal of Chromatography, 538 (1991) 1
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PREFACE

Counter-current chromatography (CCC) can be broadly defined as any chromatographic process in which (1) a liquid phase is retained in a column by gravitational or centrifugal force, while (2) a second immiscible liquid phase continuously passes through it and (3) no solid supporting matrix is employed. Since solute adsorption is precluded by the absence of a solid support, retention of solutes is determined solely by their partition coefficients.

The origins of CCC can be traced at least as far back as the continuous-flow counter-current distribution apparatus which Cornish at Berkeley described in 1934. The modern era of CCC was inaugurated in 1965 when Yoichiro Ito introduced a helical coil of plastic tubing as the chromatographic column in the coil planet centrifuge, marketed as the CPC analyzer by Sanki Engineering (Kyoto, Japan). His subsequent studies of CCC have described about 30 types of counter-current chromatographs. Particularly noteworthy are the droplet CCC developed in the early 1970s and the multilayer coil planet centrifuge introduced in the early 1980s. The centrifugal droplet counter-current chromatograph described by Nunogaki and colleagues in the early 1980s and marketed by Sanki Engineering as the centrifugal partition chromatograph represents an alternative style of CCC based on a multichambered rotor.

The applicability of CCC to polar, labile and readily adsorbed materials led to its rapid adoption in the 1970s by natural product chemists. This collection of 24 papers, the first special issue on CCC to appear in the *Journal of Chromatography*, was compiled and edited by Drs. Yoichiro Ito, Martha Knight, Y. W. Lee and myself. The wide range of topics by authors from six countries testifies to the continuing refinement of CCC technology and its application to an increasing variety of separation problems.

WALTER D. CONWAY

Journal of Chromatography, 538 (1991) 3–25
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Review

Recent advances in counter-current chromatography

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ABSTRACT

During the past several years, counter-current chromatography (CCC) technology has been advanced to cover a broad spectrum of applications, from large-scale preparative to analytical-scale separations. These advances include liquid–liquid dual CCC, foam CCC and partition of macromolecules with aqueous–aqueous polymer phase systems. For these developments the synchronous coil planet centrifuge scheme has been used, which relies on a relatively simple mechanical design. Future developments in CCC may be focused on the improvement of the more intricate non-synchronous coil planet centrifuge scheme which has a greater potential for the separation of biopolymers and cell particles.

CONTENTS

1. DEVELOPMENTAL BACKGROUND OF COUNTER-CURRENT CHROMATOGRAPHY

Since the first introduction of counter-current chromatography (CCC) in 1970 [1], the method has been steadily improved to shorten elution times so that efficient chromatographic separations can be completed within a few hours.

Early developments in hydrostatic CCC systems such as helix CCC [1–3], droplet CCC [2–4] and locular CCC [2,3] have been superseded by more efficient

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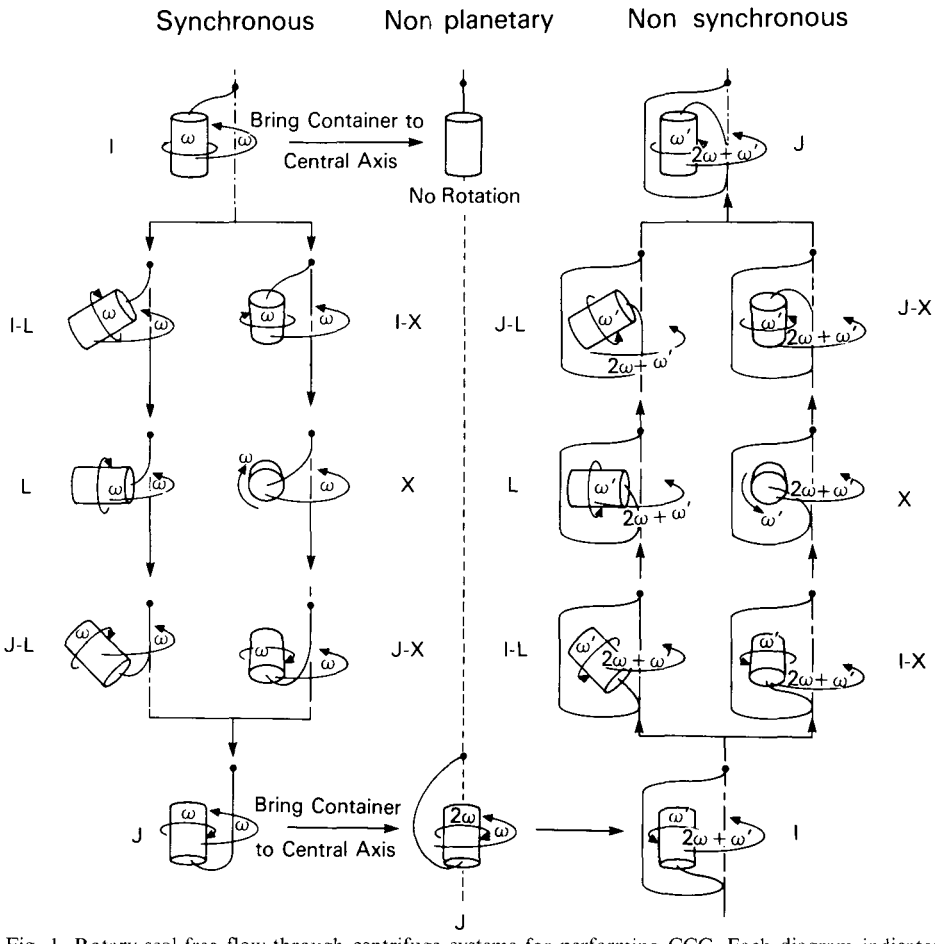

Fig. 1. Rotary-seal-free flow-through centrifuge systems for performing CCC. Each diagram indicates orientation and motion of a cylindrical column holder with a bundle of flow tubes, the other end being tightly supported on the central axis of the centrifuge. These centrifuge systems are divided into three categories according to their mode of planetary motion as indicated at the top of the diagram. In the type I synchronous planetary motion, the holder revolves around the central axis of the centrifuge and simultaneously counter-rotates about its own axis at the same angular velocity. The counter-rotation of the holder steadily unwinds the twist of the tube bundle, thus eliminating the need for the rotary seal. This principle works equally well with other synchronous systems in tilted (I-L and I-X), horizontal (L and X), dipping (J-L and J-X) and finally inverted (J) orientation of the holder. Here, the I–L–J series is obtained by tilting the holder radially toward the central axis of the centrifuge whereas the I–X–J is formed by rotating the holder tangentially relative to the revolutional orbit. This type J is a transitional form to the non-planetary system J. When the holder of type I synchronous planetary motion is moved to the central axis as indicated by an arrow, the holder becomes stationary due to cancellation of the rotation of the holder by revolution. However, when the same treatment is applied to the type J synchronous planetary motion, the holder gains the angular velocity by ω, because the revolution of the holder is added to the rotation. Thus, the holder rotates at 2ω around the central axis of the centrifuge. This non-planetary system provides a base for the non-synchronous systems. The holder of the non-planetary system is again moved away from the central axis to undergo synchronous planetary motions of types I, L, X, J and their hybrids. Although these planetary motions are synchronous with respect to the base, the net revolutional speed of the holder is the sum of the revolution rates of the top and the base. Since the revolution of the base is independent of the top planetary motion, the rotation/revolution ratio of the holder becomes freely adjustable.

hydrodynamic CCC systems which utilize synchronous planetary motion of the coiled column to produce efficient mixing of the two solvent phases promoting the partition process. Fig. 1 illustrates a series of flow-through centrifuge systems developed for performing CCC [5]. All these systems permit continuous elution through a rotating column without the use of a rotary seal device (see figure caption for details). These rotary-seal-free flow-through centrifuge systems provide various advantages such as leak-free elution under high back pressure, use of multiple flow channels without cross-contamination, elimination of the risks associated with heating or clogging of channels at the site of the rotary seal, and negligible dead space responsible for band broadening. Early designs based on these centrifuge systems have demonstrated that synchronous schemes I [6,7], I-L [8] and L [9,10] produce efficient analytical-scale separations, while synchronous scheme J [11,12] yielded excellent preparative-scale separations. However, in all these schemes the volume of the stationary phase retained in the separation column was usually limited to less than 50% of the total column capacity and the application of a high flow-rate of the mobile phase sharply decreased the retention volume of the stationary phase, resulting in serious loss in peak resolution.

In the early 1980s a great advance in the CCC technology was made by the discovery of a new hydrodynamic phenomenon in a rotating coiled tube [13]. When a coil containing two immiscible solvent phases is rotated around its horizontally placed axis at a critical angular velocity (*ca.* 100 rpm), the two solvent phases are unilaterally distributed along the length of the coil in such a way that one phase entirely occupies the head side and the other phase the tail side of the coil. Here, the head–tail orientation of the coil is referred to an Archimedean screw force which drives all objects in a rotating coil competitively toward the head of the coil. This particular hydrodynamic behavior of the two solvent phases can be used for performing CCC to retain a large volume of the stationary phase in the coiled column as described later in this article. A similar hydrodynamic phenomenon has been observed in the coil coaxially mounted on the holder in the scheme J synchronous coil planet centrifuge [14]. The application of the centrifugal force field has enhanced the hydrodynamic motion of the two solvent phases resulting in an excellent chromatographic separation in a short period of time [15,16]. More recently, it was found that this high-speed CCC system can also be obtained from other synchronous planetary motions such as schemes J-L [17,18] and X [19,20].

2. RECENT DEVELOPMENTS IN COUNTER-CURRENT CHROMATOGRAPHY

2.1. Large-scale preparative counter-current chromatography

The feasibility of large preparative CCC has been examined using a slowly rotating coiled tube [21]. A simple rotary coil assembly was used to study hydrodynamic distribution of the two immiscible solvent phases in a rotating glass coil (Fig. 2).

In each experiment, the coil was filled with about equal volumes of the two phases. In order to facilitate observation of the interface between the two phases, a small amount of a dye was added to color one of the phases. Then, the coil was sealed at both ends and rotated at a desired speed. After a steady state hydrodynamic equilibrium was established, rotation was stopped to allow the two phases to settle in

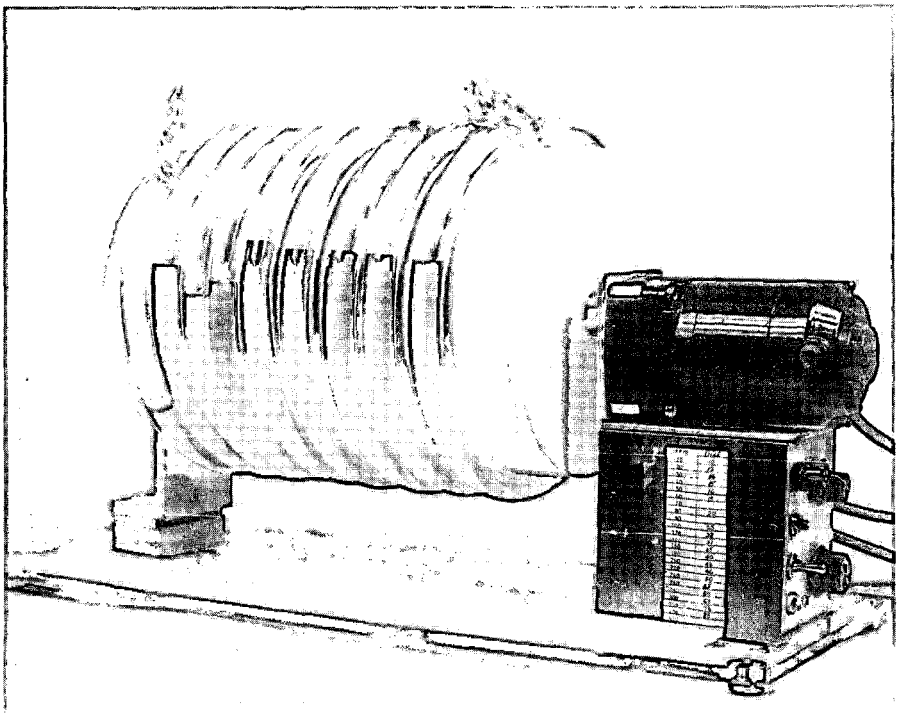

Fig. 2. Rotating coil assembly.

each turn of the coil. The volume of each phase occupying the head side of the coil was then measured. The experiment was repeated after changing the rotational speed of the coil.

The results of the experiments are summarized in Fig. 3 where a set of phase distribution diagrams are arranged according to the applied two-phase solvent system (top) and the coil dimensions, including internal diameters and core diameters (left), as indicated in the figure. In each diagram, the percentage of the heavier phase occupying the head side of the coil was plotted against the rotation speed of the coil. Experiments were performed with various two-phase solvent systems in 1 cm I.D. (upper panel) and 2 cm I.D. (lower panel) coils with helical diameters ranging from 2.5 to 20 cm. The solid line was obtained from the plain glass coil and the dotted line from the silicone-coated glass coil.

The results clearly show that all solvent systems display a unilateral phase distribution (100% or 0%) at a critical rpm where the head side of the coil is entirely occupied by one phase. This critical range is rather insensitive to the helical diameter of the coil in all the solvent systems. This critical rpm can be used in various ways, as illustrated in Fig. 4 where several coiled tubes are schematically drawn uncoiled to indicate the overall volume distribution of the two solvent phases along the length of the coil.

The top diagram (A) shows a bilateral phase distribution established in an

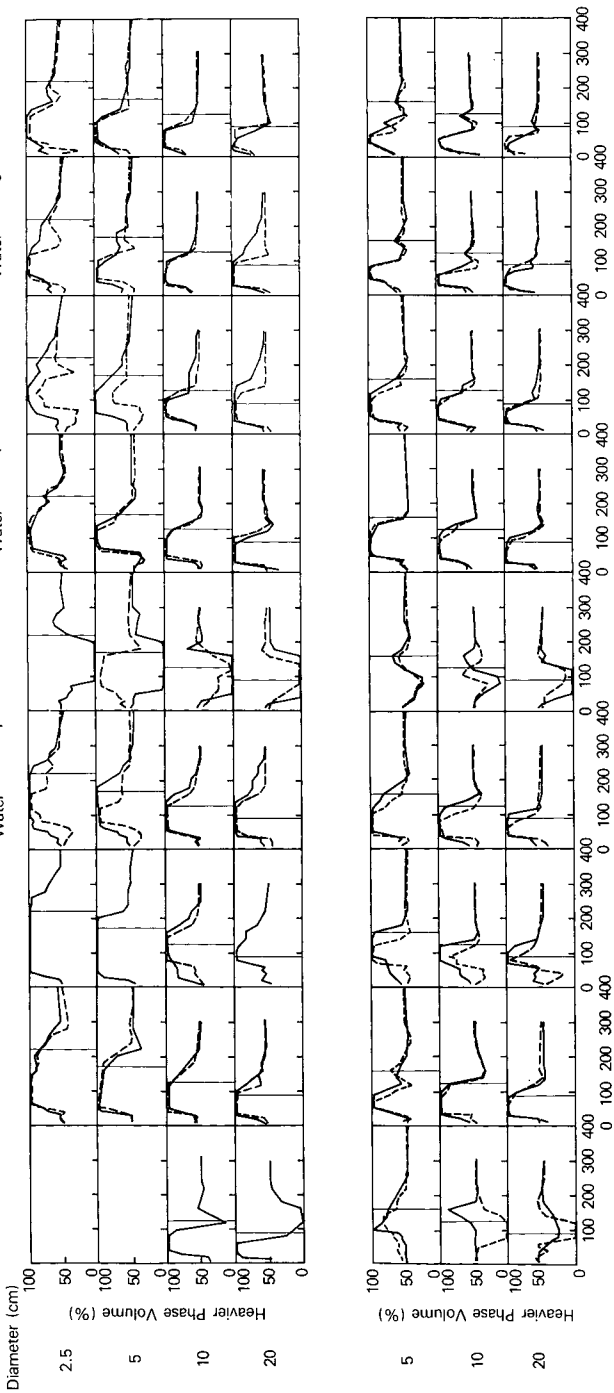

Fig. 3. Hydrodynamic phase distribution of the two immiscible solvent phases in slowly rotating coils. The coil dimensions are expressed in terms of the I.D. and core diameter as indicated on the left margin.

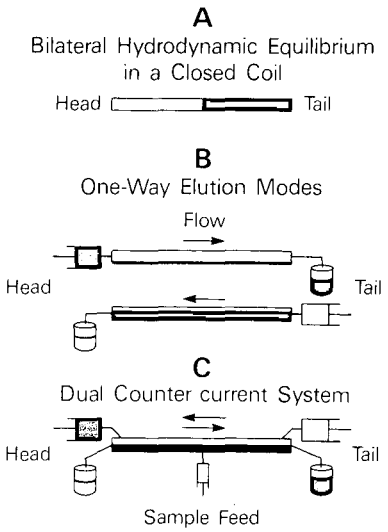

Fig. 4. Mechanism of high-speed CCC.

end-closed rotating coil with the white phase in the head side and the black phase on the tail side. This hydrodynamic distribution was previously called "unilateral" since one of the phases occupies the head side of the coil [22,23]. The white phase occupying the head side is the heavier phase in the present system, but it usually becomes the lighter phase in the centrifugal CCC schemes such as high-speed CCC. In the one-way elution mode (B), the coil is first entirely filled with the white phase followed by elution with the black phase from the head toward the tail. Alternatively, the coil is initially filled with the black phase followed by elution with the white phase from the reverse direction. In either case, the system can retain a large volume of stationary phase in the coil while efficient mixing is produced by rotation of the coil.

In the dual counter-current operation (C), the two solvent phases are simultaneously introduced into the coil through the respective terminals. This operation requires an additional flow tube at each end of the coil and, if desired, a sample feed port is made at the middle portion of the coil. This dual CCC system permits continuous sample feeding as well as batch sample loading. The rotary-seal-free multichannel flow-through system illustrated in Fig. 1 (non-planetary motion J) can be used to avoid various complications arising from the use of conventional multichannel rotary seal devices.

All these systems allow preparative CCC at a relatively slow rotation of the coil using the effect of the unit gravity. Consequently, this simple CCC scheme can be safely applied to a large-capacity multilayer coiled column without mechanical constraint and the system is easily automated for separations of hazardous materials such as radioactive samples.

2.2 Preparative counter-current chromatography

For laboratory-scale multigram separations, we have developed a new coil

planet centrifuge called the cross-axis synchronous flow-through coil planet centrifuge (X-axis CPC) [19,20]. It was designed according to the scheme X synchronous planetary motion previously described (Fig. 1).

Fig. 5 shows the orientation and planetary motion of the column holder of the X-axis CPC. The holder revolves around the main axis of the centrifuge while it simultaneously rotates about its own axis at the same angular velocity in the indicated direction. The bundle of flexible flow tubes supported at the central axis of the centrifuge does not twist because the synchronous rotation of the holder steadily unwinds each twist caused by revolution. Thus, the system eliminates the need for the rotary seal.

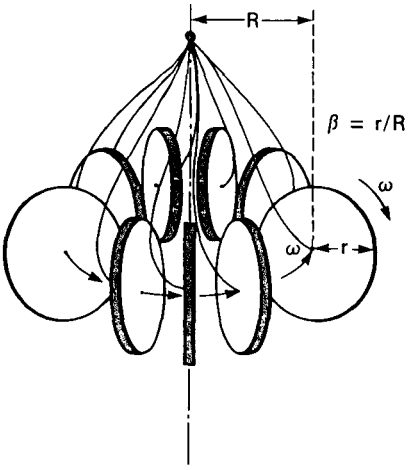

Fig. 5. Orientation and motion of the column holder in the cross-axis synchronous flow-through coil planet centrifuge.

The acceleration generated by this unique planetary motion has been mathematically analyzed [19]. The results indicate that the system produces a three-dimensional fluctuation of the centrifugal force field. The radial force field around the holder axis resembles that in the scheme J synchronous planetary motion, producing a bilateral phase distribution in the coil. The laterally-acting force field parallel to the holder axis is unique to the present system and promotes mixing of the two solvent phases across the diameter of the tube without undesirable longitudinal spreading of the sample bands.

Fig. 6 shows the original prototype of the X-axis CPC used in performing preparative separations [20]. The motor drives the rotary frame around the central axis of the centrifuge. The rotary frame consists of a pair of side plates rigidly bridged with links supporting a column holder and a counter-weight holder in symmetrical positions at a distance of 10 cm from the central axis of the centrifuge. A set of miter gears mounted at the bottom of the centrifuge drives a pair of toothed pulleys, and this motion is conveyed to each holder with a toothed belt.

The column is a multilayer coil prepared from a single piece of PTFE (polytetrafluoroethylene) tubing with a total capacity of approximately 400 ml. The

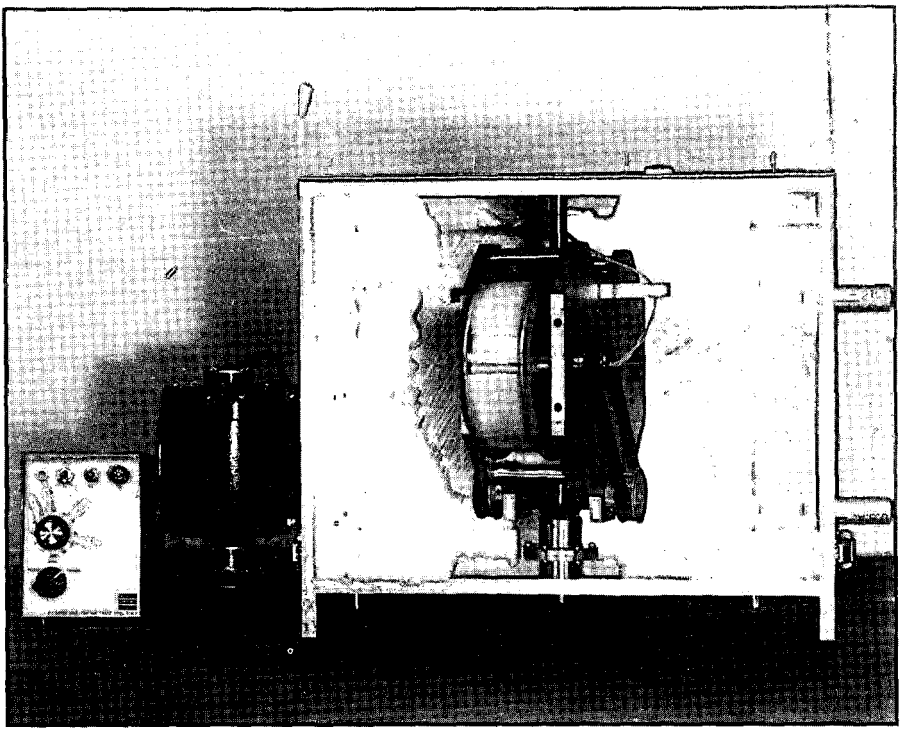

Fig. 6. Original cross-axis synchronous flow-through coil planet centrifuge.

preparative capability of this original apparatus was successfully demonstrated in separation of gram-quantities of dinitrophenyl amino acids and dipeptides [20].

In order to extend the capacity of the system, we have designed a second prototype of the X-axis CPC which has twice the revolution radius and is equipped with a long column holder measuring 25 cm in width [24–26]. A series of studies on the hydrodynamic distribution of the two solvent phases in a short coil revealed, interestingly, that the retention of the stationary phase is largely affected by the position of the coil on the holder. In the lateral coil position on the holder, the direction of the planetary motion further produced a strong effect on the stationary phase retention. Thus, with a proper combination of the planetary motion and the head–tail elution mode, the system produced extremely high retention of the stationary phase which greatly exceeded that obtained from the central coil position. The lateral coil position on the holder shaft also provides an additional advantage in that the apparatus can accommodate a pair of large diameter column holders which extend over the central axis of the apparatus. A pair of multilayer coils symmetricaly mounted on the rotary frame can be connected in series with a flow tube to double the column capacity.

In light of the above findings, a third prototype of the X-axis CPC was constructed [27,28]. Fig. 7 shows the apparatus which holds a pair of large multilayer coils in the lateral positions at 10 cm from the center of the holder shaft which in turn is

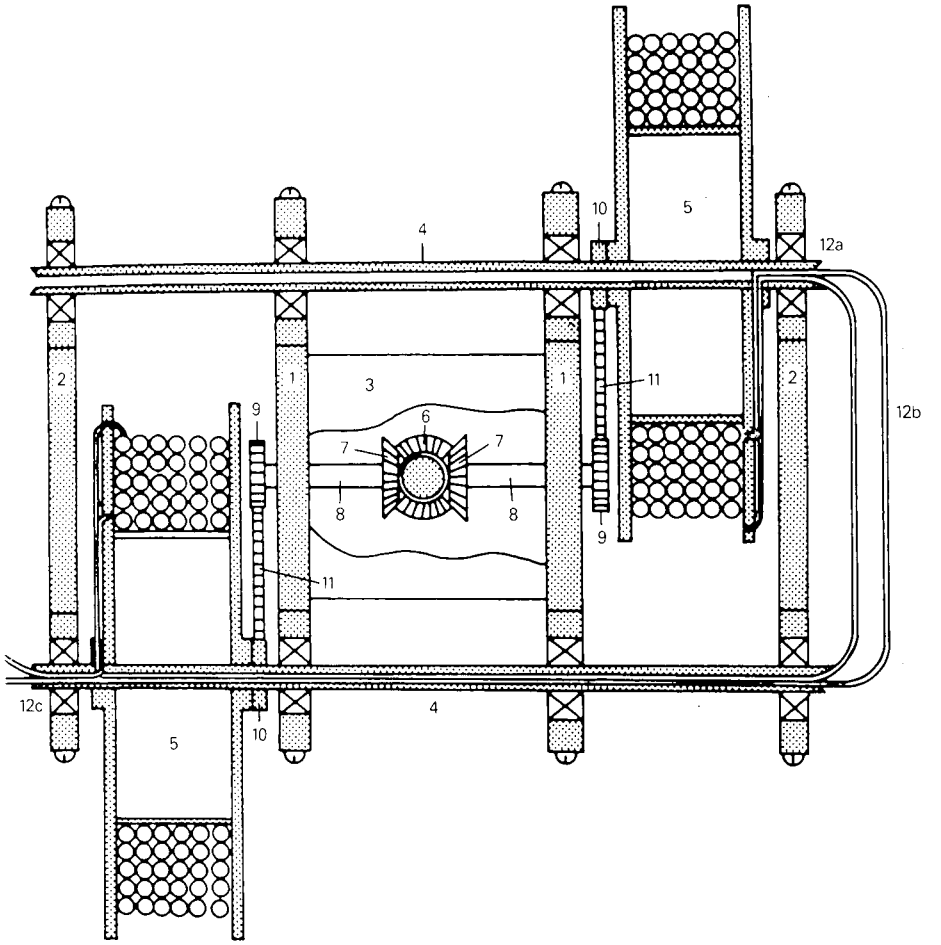

Fig. 7. Horizontal cross-sectional view of the improved cross-axis coil planet centrifuge. 1,2 = Side plates; 3 = bottom plate; 4 = column holder shafts; 5 = column holders; 6 = stationary miter gear; 7 = planetary miter gears; 8 = countershafts; 9,10 = toothed pulleys; 11 = toothed belts; 12a–c = flow tubes.

located at a distance of 10 cm from the central axis of the centrifuge. These two columns are serially connected to make up a total capacity of about 1.6 l.

The preparative capability of the apparatus was demonstrated in multi-gram separations of various biological samples [29]. Fig. 8 shows a chromatogram of crude synthetic steroids using the X-axis CPC. A 2.4-g quantity of the crude reaction mixture was efficiently separated into multiple peaks in 15 h. Five steroids corresponding to peaks 1–5 were analyzed by NMR as illustrated on the right side of the chromatogram. The desired product was found at peak 5 and over 300 mg of the crystalline material was obtained in high purity.

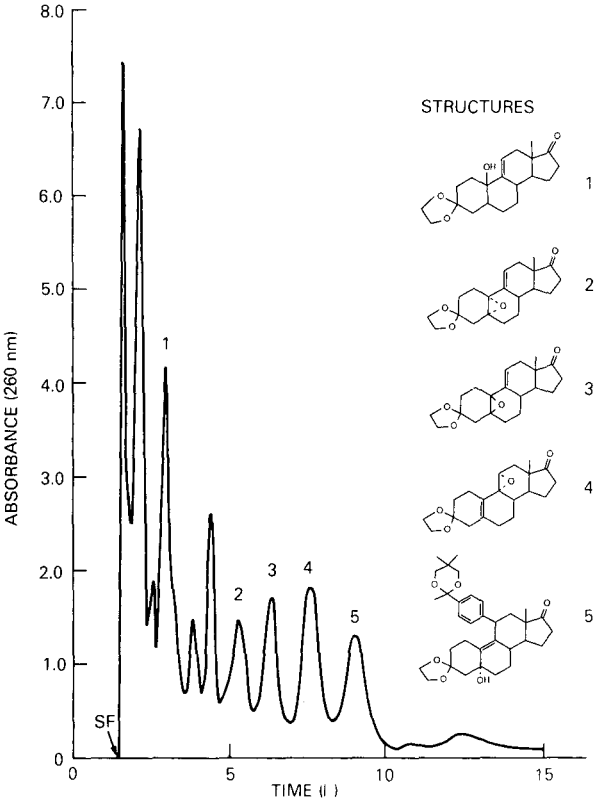

Fig. 8. Chromatogram of synthetic steroid intermediates obtained by the cross-axis coil planet centrifuge. Apparatus: cross-axis CPC, 20 cm radius; column: multilayer coils, 2.6 mm I.D., 1600 ml capacity; sample: crude steroid intermediates, 2.4 g; solvent system: hexane–ethyl acetate–methanol–water (6:5:4:2); mobile phase: lower aqueous phase; elution mode: head → tail; flow-rate: 240 ml/h; revolution: 450 rpm (P_1); retention: 71.3%.

2.3. Semi-preparative high-speed counter-current chromatography

The high-speed CCC centrifuge developed in the early 1980s has been successfully commercialized by P.C. Inc. (Potomac, MD, U.S.A.) and Pharma-Tech Research Corporation (Baltimore, MD, U.S.A.). Recently, we have made two important improvements on the performance of this apparatus.

Fig. 9 shows the principle of the first innovation [30]. The standard high-speed CCC centrifuge shown at the top holds a column holder on one side of the rotary frame and has a counterweight on the other side to balance the centrifuge system. The new design shown at the bottom eliminates the counterweight and accommodates two or more identical column holders symmetrically around the rotary frame. These holders can be interconnected with flow tubes in series so that both partition efficiency and the sample loading capacity are improved. In addition, the system totally eliminates the need for tedious adjustment of the counterweight mass and achieves perfect balancing of the centrifuge system once the column is equilibrated with the two solvent phases.

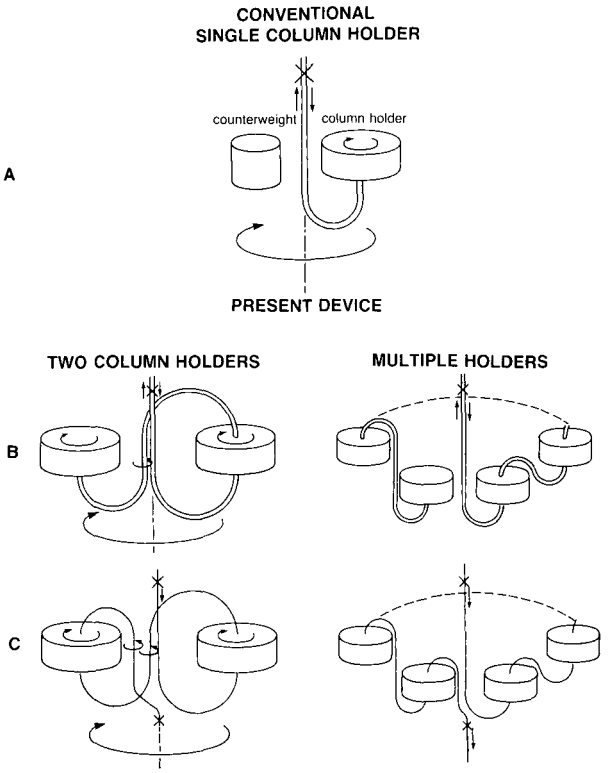

Fig. 9. Design principle of the multiholder coil planet centrifuge (type J synchronous planetary motion). (A) The conventional coil planet centrifuge equipped with a single holder and a counterweight. (B) Multiholder coil planet centrifuge systems with double passage of the flow tubes. (C) Multiholder coil planet centrifuge systems with a single passage of the flow tubes.

Fig. 10 shows our most recent high-speed CCC centrifuge equipped with three multilayer coils connected in series. This unit can be operated at 1250 rpm and yields high partition efficiencies of several thousand theoretical plates [31–33]. The capability of the apparatus has been demonstrated in separations of various samples including indole plant hormones, tetracycline derivatives, bacitracin components, flavonoids from a crude sea buckthorn extract, triterpenoic acids, and rare earth elements.

A second improvement in the apparatus involved the detection system of the effluent [34]. Since the CCC technology utilizes a two-phase solvent system, carryover of small droplets of the stationary phase from the separation column tends to disturb the uv detector. The two-solvent phases are always in a subtle equilibrium state, and any change of the ambient temperature may produce turbidity of the mobile phase due to formation of micro-droplets of the stationary phase. In addition to the above complications inherent to CCC, the pressure drop at the periphery of the flow passage may generate gas bubbles from the effluent disturbing the recording of the elution curve.

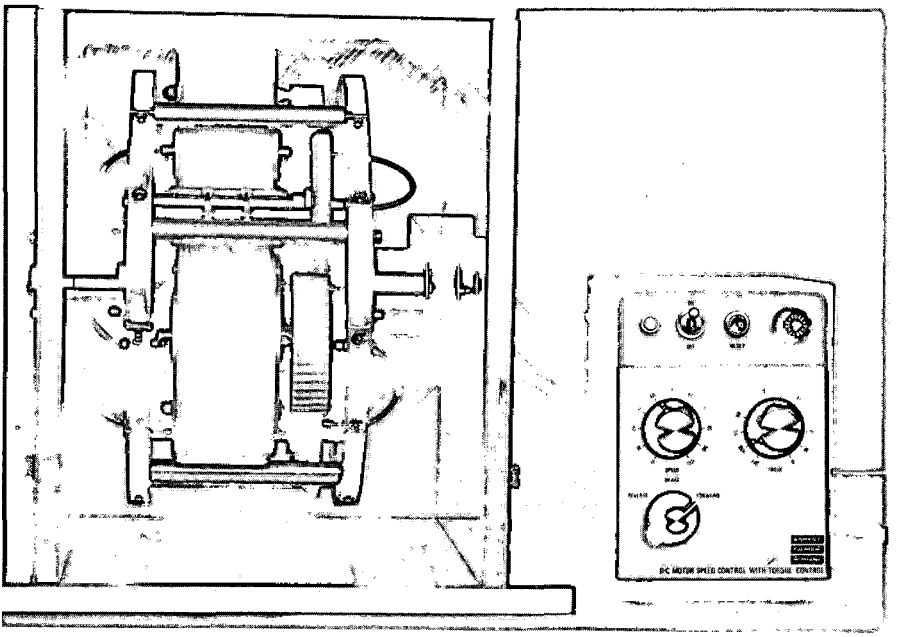

Fig. 10. High-speed CCC centrifuge equipped with three multilayer coils connected in series.

Fig. 11A shows a typical chromatogram obtained by the online UV tracing in high-speed CCC. In the chloroform solvent system, slightly altered ambient temperature in the flow cell produced turbidity of the mobile phase causing noise. This was superimposed on a higher frequency noise produced from gas bubbles generated under a reduced pressure.

These complications were totally eliminated by heating the effluent at 30°C at the inlet of the UV monitor and attaching a narrow-bore tube at the outlet of the monitor to prevent a sudden pressure drop in the flow cell. Fig. 11B shows an improved chromatogram which is comparable in quality with those obtained from high-performance liquid chromatography.

2.4. Analytical counter-current chromatography

In the past, development of the CCC instruments has been mainly directed toward preparative applications because of its long elution time. However, the recent advent of high-speed CCC has considerably shortened the separation times without sacrificing the peak resolution. Several different types of the high-speed CCC centrifuges are now available for analytical-scale separations.

Fig. 12A shows the original analytical high-speed CCC centrifuge with a 5 cm revolutional radius which can be operated up to 2000 rpm [35]. The analytical capability of the apparatus was demonstrated in separation of indole auxins at partition efficiency of 1600 theoretical plates (TP). The apparatus has been successfully interfaced to a mass spectrometer with a thermospray capillary device [36].

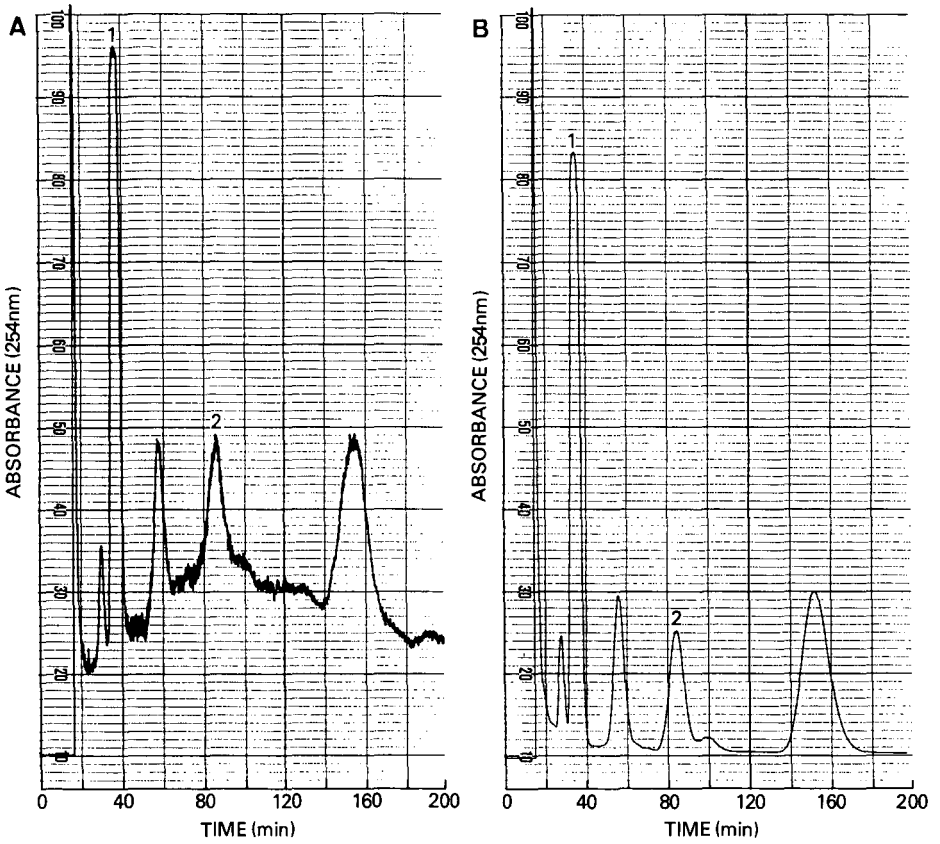

Fig. 11. High-speed CCC separation of flavonoids recorded by on-line UV monitoring with the conventional method (A) and improved method (B).

Because the narrow thermospray capillary required to limit flow into the mass spectrometer creates a high back pressure, direct interfacing is difficult. Therefore, the effluent from the outlet of the CCC column was split into two streams with the smaller flow proceeding into the mass spectrometer. Fig. 12B shows a total ion chromatogram of an alkaloid mixture obtained by high-speed CCC–mass spectrometry using a thermospray device in the mass spectrometer. This chromatogram revealed an isomer of vincine which was not resolved in high-performance liquid chromatographic analysis [36].

Fig. 13A shows a compact model of the analytical high-speed CCC centrifuge with a 2.5 cm revolutional radius [37]. This unit can be operated at a maximum speed of 4000 rpm while the capacity of the multilayer coil (0.85 mm I.D.) was limited to about 10 ml. The apparatus can yield efficient microgram-quantity separations as demonstrated in a rapid separation of flavonoids in a crude sea buckthorn extract (Fig. 13B).

Most recently, we have reevaluated the analytical performance of the toroidal coil centrifuge (helix CCC) by mounting a long coiled column made of narrow-bore (0.3–0.4 mm I.D.), thick-wall PTFE tubing. In order to speed the separation, mobile

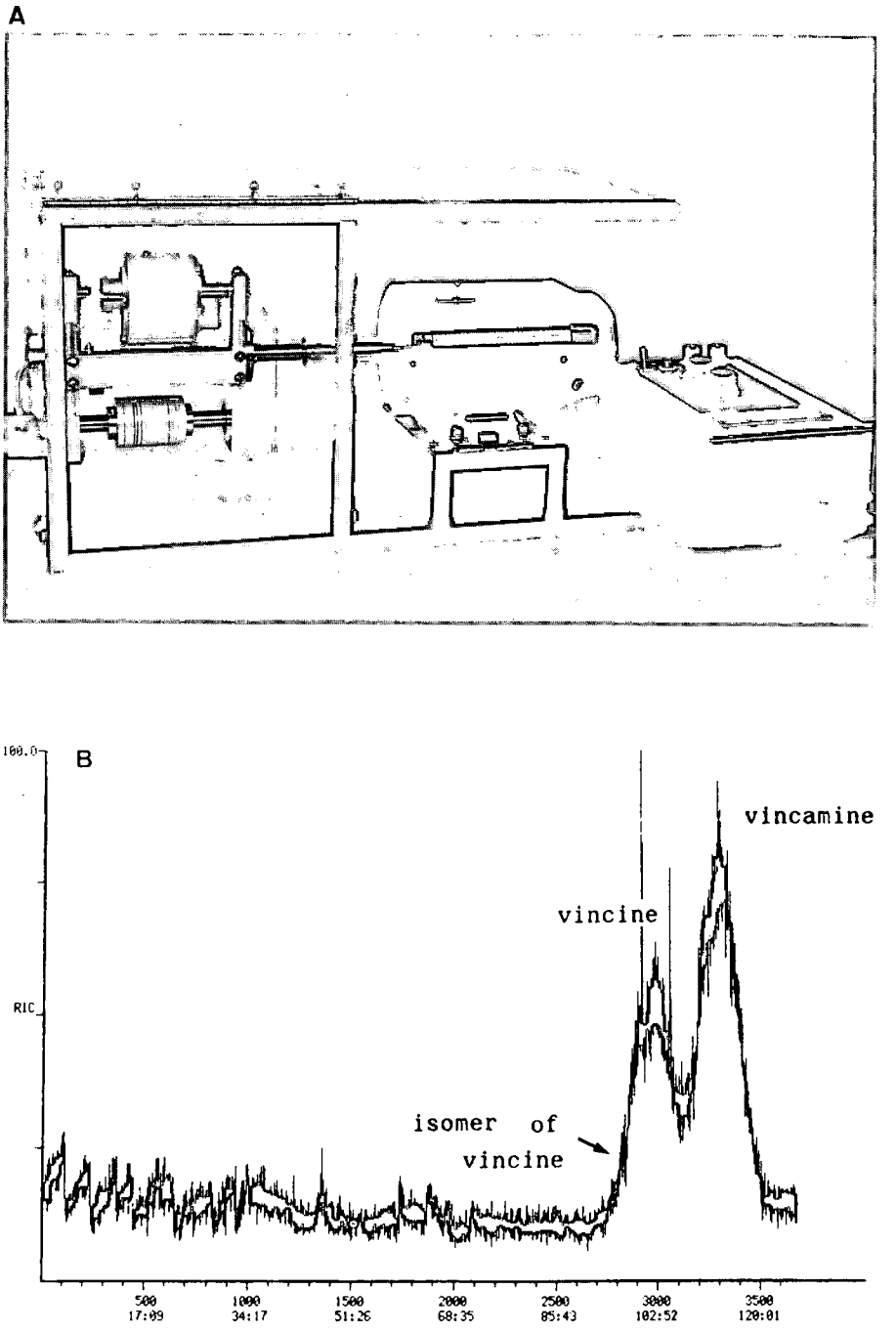

Fig. 12. (A) Original analytical high-speed CCC centrifuge. (B) Total ion chromatogram of alkaloids obtained by high-speed CCC–mass spectrometry with a thermospray capillary device; sample: vincamine–vincine mixture; horizontal scales: top, scan No.; bottom, time in min:s.

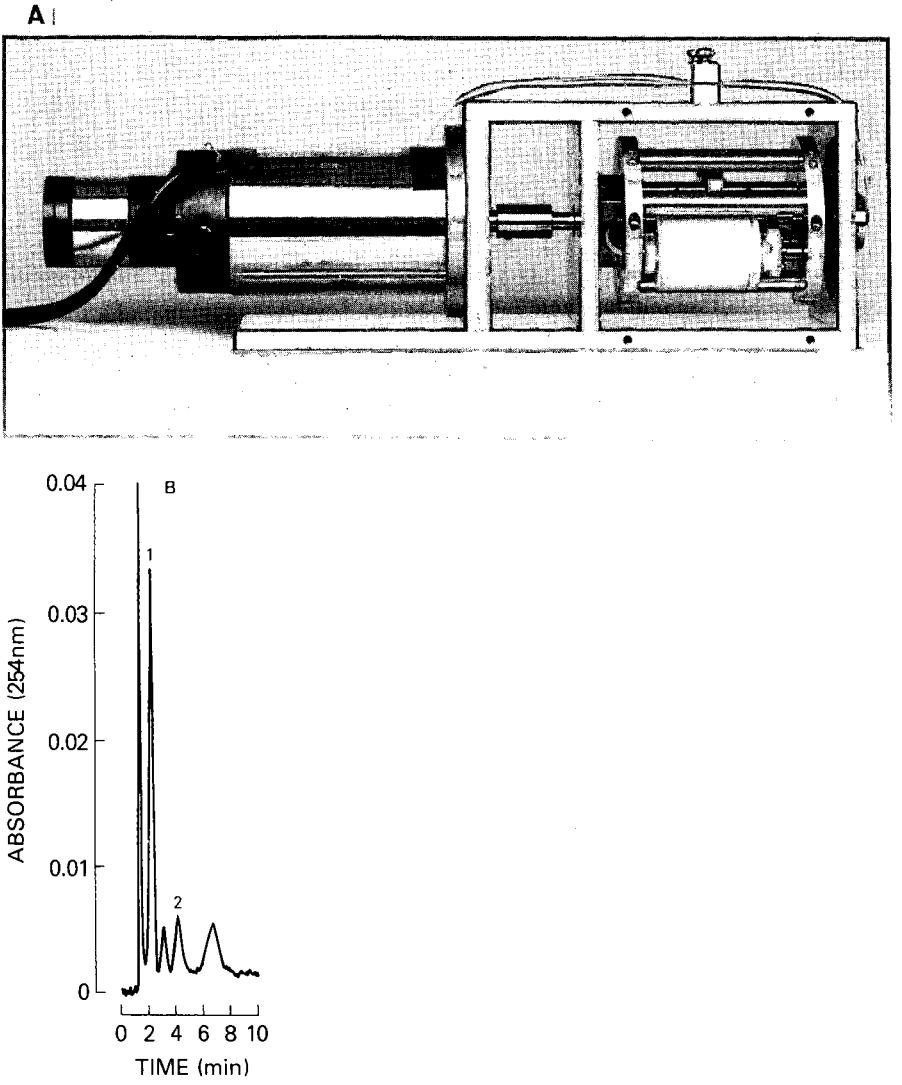

Fig. 13. (A) Analytical high-speed CCC centrifuge with 2.5 cm revolution radius. (B) Chromatogram of flavonoids obtained by the analytical high-speed CCC centrifuge. Peaks: 1 = isorhamnetin; 2 = quercetin. Conditions: apparatus, high-speed CCC centrifuge with 2.5 cm revolution radius, single multilayer coil, 0.85 mm I.D. and 8 ml capacity; sample, flavonoids from crude ethanol extract of *H. rhamnoides*, 210 μg; solvent system, chloroform–methanol–water (4:3:2, v/v/v); mobile phase, lower non-aqueous phase; flow-rate, 2 ml/min; revolution, 3500 rpm.

phase was pumped at a flow-rate of 1 ml/min while the column was subjected to a strong centrifugal force field (190 *g*) in order to retain a satisfactory volume of the stationary phase in the coil. The method produced rapid and efficient separations of an indole auxin mixture which were comparable to those obtained from the existing analytical high-speed CCC centrifuges in both partition efficiency and analysis time

[38]. Although the toroidal coil centrifuge tends to create a high hydrostatic pressure up to 1000 p.s.i., often damaging the plastic tubes leading to the column, it provides a stable tracing of the elution curves. It will be a better candidate for analytical CCC if its pressure problem can be overcome.

2.5. Dual counter-current chromatography

One of the most remarkable features of high-speed CCC is its ability to operate in a dual fashion where two immiscible phases undergo true counter-current movement through a narrow coiled tube. Appropriate solutes introduced in a sample at the middle portion of the coil, either batchwise or in a continuous mode, potentially can be separated and collected from each end according to their partition coefficients between the two phases. The method may be divided into two categories: liquid–liquid dual CCC and foam CCC. Since the recent development of liquid–liquid dual CCC has been reviewed separately by Lee [39] only foam CCC is elaborated here.

Foam separation [40] is based on the unique parameter of foaming capacity or foam affinity of samples in an aqueous solution. While it has great potential for application to biological samples, utility of the method has been extremely limited mainly due to its poor efficiency. In conventional instrumentation, foam separation was performed in a short column under unit gravity. Recently, we have developed a foam CCC centrifuge (scheme J synchronous planetary motion shown in Fig. 1) which provides a strong centrifugal force field to induce a rapid counter-current movement between the gas and liquid phases through a long narrow coiled tube [23]. The system conveniently utilizes the standard rotary-seal-free elution mechanism that permits the use of multiple flow tubes without risk of leakage and cross-contamination.

Fig. 14 illustrates a schematic layout of the foam CCC column equipped with five flow channels: the liquid is fed from the liquid feed line at the tail and collected from a liquid collection line at the head. Nitrogen gas is simultaneously introduced at the head and discharged through the foam collection line at the tail. The sample solution is introduced through the sample feed line at the middle portion of the coil either batchwise or in a continuous mode. A typical foam CCC column consists of 10 m × 2.6 mm I.D. PTFE tubing with a total capacity of about 50 ml. In the past the method was demonstrated in our laboratory in the separation of dyes with ionic surfactants [23,41,42], as well as separation of proteins in a phosphate buffer solution to minimize denaturation [23].

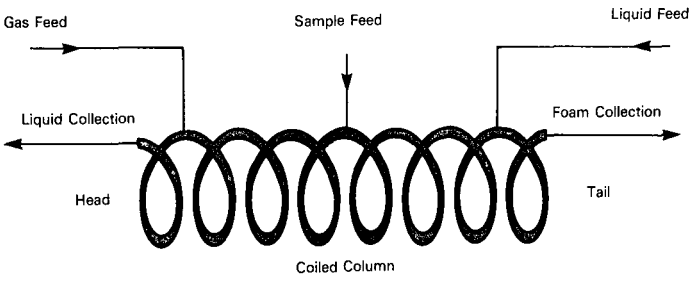

Fig. 14. Column design of foam CCC.

Recently, we have succeeded in separation of bacitracin components using only nitrogen gas and distilled water without the use of surfactants or any other additives [43,44]. Separation of the bacitracin components was initiated by introducing distilled water from the tail and nitrogen gas from the head at 80 psi while the column was rotated at 500 rpm. After a steady state counter-current equilibrium was established, the sample solution was injected through the sample feed line. During injection, the liquid flow was interrupted for about five minutes to allow the solutes to be distributed along the length of the coil according to their foaming capacity. Then, the pumping was resumed to collect the foam and liquid fractions from the respective terminals at 15-s intervals. These fractions were analyzed with high-performance liquid chromatography and the results are summarized in Fig. 15.

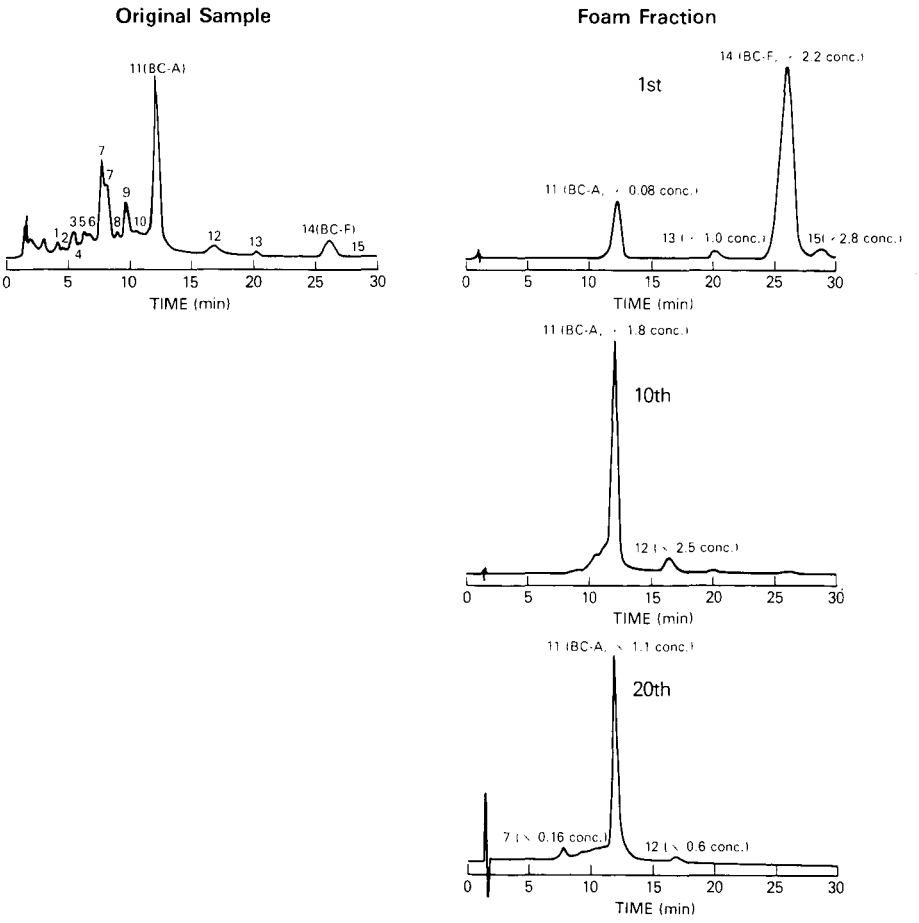

Fig. 15. Foam CCC of bacitracin. High-performance liquid chromatographic analysis of (left) the original sample and (right) the foam CCC fractions. High-performance liquid chromatography conditions: column, Capcell Pak C_{18}, 150 × 4.6 mm I.D., 5 μm; mobile phase, methanol–0.04 M Na_2HPO_4 (62:38), 1 ml/min; detection at 234 nm.

The chromatogram on the left was obtained from reversed-phase high-performance liquid chromatographic analysis of the original bacitracin sample which shows over 20 UV absorbing components. In this chromatogram, the most hydrophilic component eluted first and the remaining components followed in increasing order of hydrophobicity. Three chromatograms on the right were obtained from the foam fractions. In the first fraction, bacitracin F was enriched and in the 10th fraction, bacitracin A was almost completely separated from other components. At the 20th fraction, peak 7 appeared, while more polar components were not detected. The above results indicate that foam CCC separates the bacitracin components according to their hydrophobicity. On the other hand, in the liquid fractions the bacitracin components elute in decreasing order of polarity but with much less efficient separation than in the foam fractions [44].

More recently, this method has been successfully applied to continuous separation of bacitracin components. The results showed over one thousand-fold enrichment of foam active components as reported by Oka *et al.* [45].

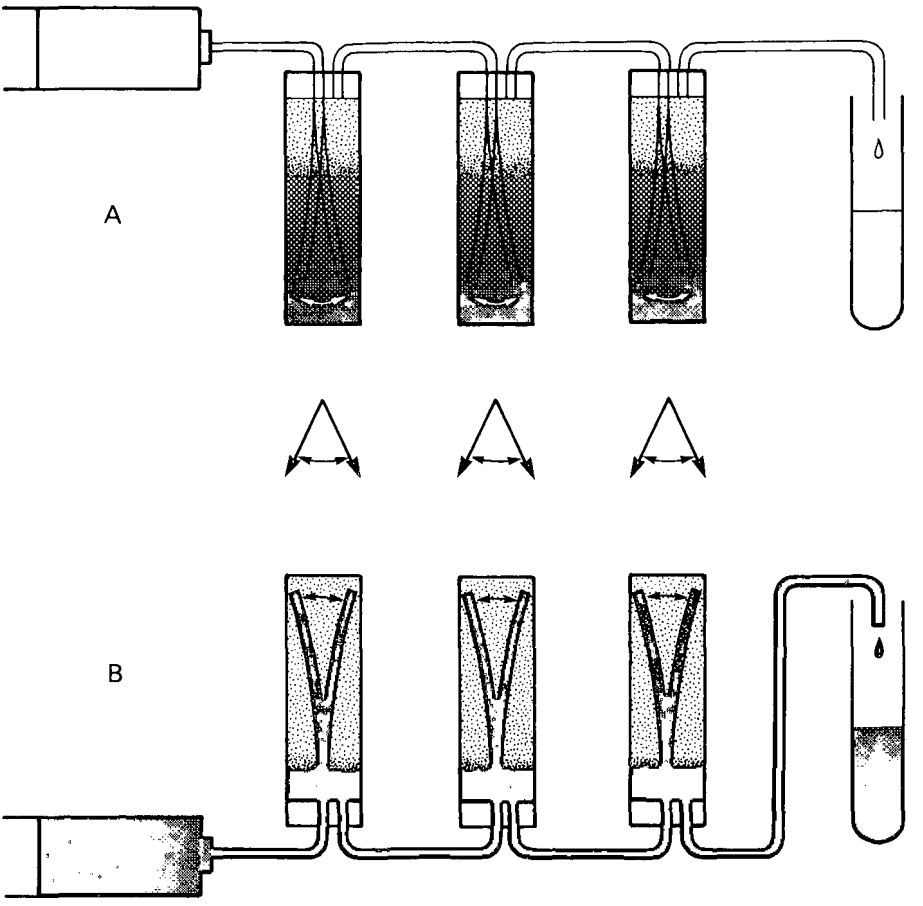

Fig. 16. Mechanism of multistage mixer-settler planetary centrifuge. A: Upper phase mobile; B: lower phase mobile.

2.6. Partition of macromolecules with polymer phase systems

Partition of macromolecules requires a special caution to prevent denaturation; the conventional organic-aqueous two-phase solvent systems are generally unsuited for this purpose [46]. The most commonly used solvent systems for partition of macromolecules contain one or more polymers (polyethyleneglycol, dextran, ficol, etc.) and various salts (sodium chloride, sodium or potassium phosphate, ammonium phosphate, etc.). These possess characteristic physical properties of high viscosity, low interfacial tension and relatively small density difference between the two phases [46]. Consequently, they require long settling times, and they have a strong tendency to emulsify. This causes a problem in maintaining stable retention of the stationary phase in the separation column with a continuous partitioning system like CCC. However, the above difficulty has been overcome by the use of various centrifugal CCC systems such as the angle rotor coil planet centrifuge [8], the elution centrifuge [10], the toroidal coil centrifuge [47,48], the toroidal coil planet centrifuge [49], the non-synchronous flow-through coil planet centrifuges [50–53], and the centrifugal droplet CCC apparatus (centrifugal partition chromatograph).

Recently, two new CCC instruments have been introduced for partition of macromolecules with polymer phase systems. Fig. 16 shows the principle of the first scheme where a series of cylindrical partition units is interconnected with transfer tubes. The mobile phase is delivered through a long flexible mixer tube extending toward the bottom of the partition unit. Under a fluctuating centrifugal force field provided by a scheme J synchronous coil planet centrifuge (see Fig. 1), the mixer tube vibrates to mix the contents of each partition unit to promote the partition process. This mixing effect is reduced at the outlet of the partition unit where the mobile phase is separated and transferred to the next unit. Thus, the system can maintain a steady state hydrodynamic equilibrium between the mobile and stationary phases during the continuous elution. Consequently, the solutes locally introduced at the inlet of the column are subjected to an efficient partition process in each partition unit and separated according to their partition coefficients. Fig. 17 shows a cross-sectional sketch of the planetary centrifuge equipped with a multistep partition column assembly [54].

The second scheme utilizes a set of four multilayer coils which are serially connected with flow tubes and arranged around the holder shaft of the similar coil planet centrifuge as shown in Fig. 18 [55]. Because of the eccentric orientation of the multilayer coils relative to the column holder axis, each helical turn of the coil retains satisfactory amounts of the stationary phase while the planetary motion of the coil produces efficient mixing of the two phases. Although the present system is similar to the original horizontal flow-through coil planet centrifuge [11,12], the shortened column holder tolerates much higher revolution speed (1000 rpm) to produce more efficient mixing effect and at the same time provide better retention of the polymer phase systems.

The above CCC system has been used to separate cytochrome *c* and lysozyme in a polymer phase system composed of 12.5% polyethyleneglycol in 1 *M* potassium phosphate aqueous solution (Fig. 19). The first scheme (see above) is suitable for gram quantity separations and the second scheme for semipreparative separations ranging from micrograms to several hundred milligrams. An improved model of the eccentric multilayer coil planet centrifuge has recently become available from Peptide Tech-

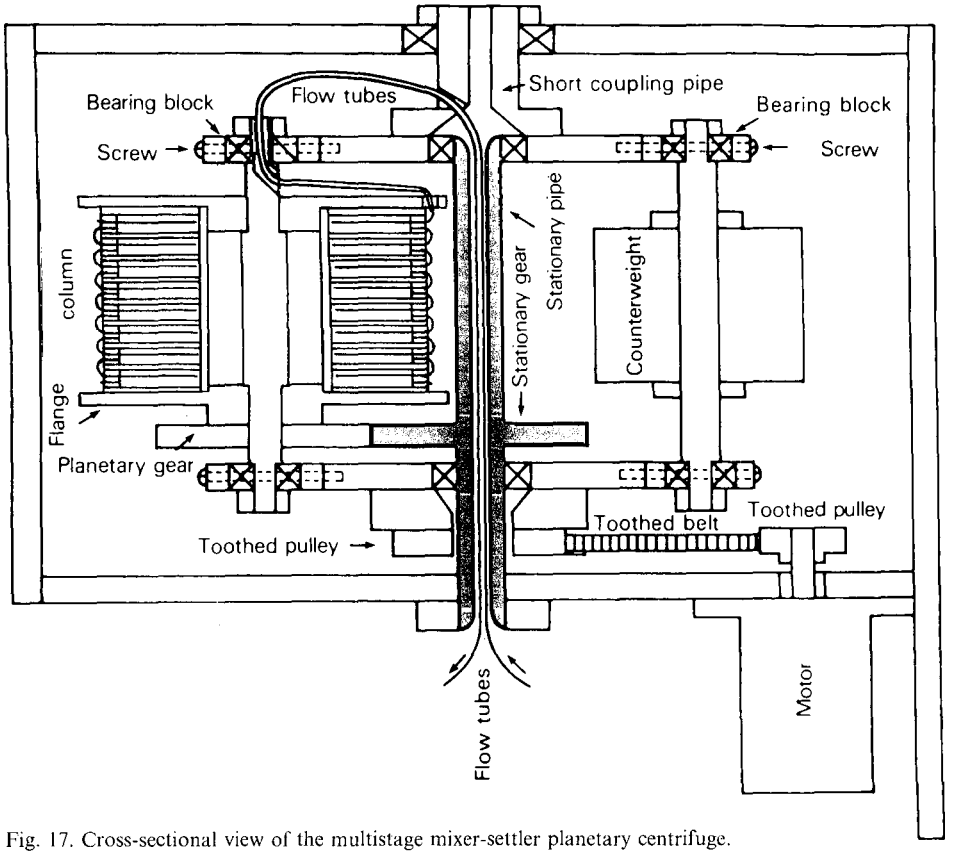

Fig. 17. Cross-sectional view of the multistage mixer-settler planetary centrifuge.

nologies Corporation (Washington, DC, U.S.A.) and Varex Corporation (Burtonsville, MD, U.S.A.) [56].

3. FUTURE DEVELOPMENT OF COUNTER-CURRENT CHROMATOGRAPHY

As discussed, recent work in our laboratory has been focused mainly on the synchronous coil planet centrifuge system. Various commercial models currently available have been successfully utilized for separation and purification of many natural and synthetic products [22].

Future advances in CCC technology may be expected from the development of the nonsynchronous coil planet centrifuge scheme (Fig. 1, right column) which has been neglected thus far due to its complex mechanical design. As described in the figure caption, the non-synchronous scheme has a unique feature in that the rotation and revolution rates of the coil are each independently adjustable. Consequently, choosing a suitable combination of slow coil rotation and high speed revolution will produce stable retention of the stationary phase for hydrophilic two-phase solvent systems such as aqueous–aqueous polymer phase systems used for partition of macromolecules and cell particles [46]. Although further development and refinement of the non-

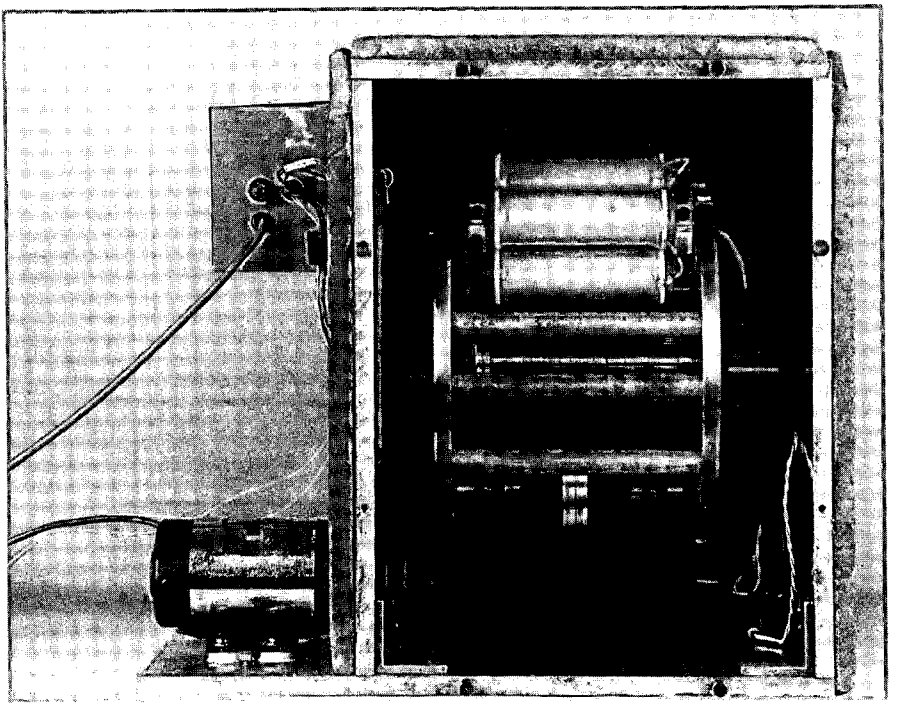

Fig. 18. Eccentric multilayer coil planet centrifuge.

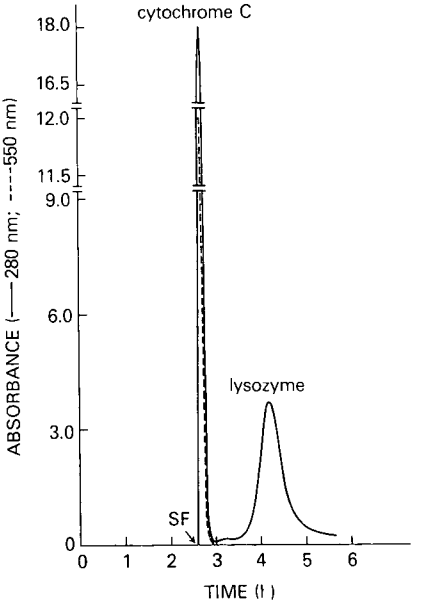

Fig. 19. Separation of proteins by the eccentric multilayer coil planet centrifuge. Conditions: apparatus, eccentric multilayer coil planet centrifuge with 10 cm revolution radius; a set of four multilayer coils were connected in series, 1.6 mm I.D. and 200 ml total capacity; sample, cytochrome *c* + lysozyme, each 100 mg; solvent system, 12.5% (w/w) polyethyleneglycol 1000 + 12.5% (w/w) anhydrous dibasic potassium phosphate in distilled water; mobile phase, lower phase; flow-rate, 1 ml/min; revolution, 800 rpm; SF = Solvent front.

synchronous coil planet centrifuge may require substantial time and cost, it may be advantageous for the separations of various biological substances important in biotechnology including proteins, nucleic acids, polysaccharides and cell particles.

4. ACKNOWLEDGEMENT

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Review

Counter-current chromatography

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ABSTRACT

The simplicity of counter-current chromatography (CCC) is sometimes overshadowed by a complex description of its mechanical and chromatographic attributes. The universal features of chromatographic theory relevant to CCC are summarized here, using a partition coefficient elution scale to present the chromatogram in a general, readily visualized, format. The principal types of CCC apparatus are summarized, along with selected applications and an indication of the type of apparatus best suited for some specific applications.

CONTENTS

1. INTRODUCTION

Counter-current chromatography (CCC) refers to the process of liquid–liquid chromatography (LLC) carried out without the aid of a solid supporting matrix to retain the stationary liquid in the chromatographic column. Although preceded by early forms of discontinuous counter-current distribution (CCD), CCC can be traced

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to at least 1934, when Cornish *et al.* [1] described a locular CCC column and applied it to the purification of oil-soluble vitamins (see ref. 2).

A renaissance in the development of efficient, laboratory-scale CCC apparatus has followed the description by Ito *et al.* [3] in 1966 of a coil planet centrifuge counter-current chromatograph containing a column consisting of a helical coil of plastic tubing. The principal techniques in current use are: (1) droplet counter-current chromatography (DCCC) [4], (2) centrifugal droplet counter-current chromatography (CDCCC) [5], also referred to as centrifugal partition chromatography (CPC), and (3) CCC with the multilayer coil planet centrifuge (MLCPC) [6]. Reviews of CCC instrumentation and applications have recently been published [1,7–12], including some in this thematic issue of the *Journal of Chromatography*.

The term counter-current chromatography usually refers to the process in which one liquid phase is maintained by gravitational or centrifugal forces as a stationary bed, distributed longitudinally in a column, while a second immiscible liquid phase flows through the stationary bed. However, true counter-current chromatography, in which both phases do flow in opposite directions relative to the columnn, has been carried out in the MLCPC apparatus [13]. The following theoretical discussion, which summarizes a more detailed presentation in ref. 2, refers to the simple CCC process, with one phase stationary.

2. CCC THEORY

Since solute adsorption is precluded by the absence of a solid support, retention in CCC can be accurately predicted from the solute partition coefficient, K, and the relative volumes of mobile and stationary phase, V_m and V_s. The process of CCC is illustrated schematicallyin Fig. 1. A mobile upper phase is illustrated, but, in general, either phase can be employed as mobile phase. The partition coefficient, as is customary in chromatography, is defined as

$$K = C_s/C_m \tag{1}$$

where C_s and C_m represent solute concentrations in stationary and mobile phase respectively.

2.1. The centrality of K = 1

The focal point of the countercurrent chromatogram (and, indeed, of any LLC chromatogram) is the emergence of the solute with a partition coefficient of unity.

Neglecting correction for any extra-column dead volume, solutes with $K = 1$ will always emerge when one column volume, V_c, of mobile phase has passed through the column. If the retention volume of a solute with $K = 1$ is designated as V_1, then $V_1 = V_c$. The elution of $K = 1$ at V_c, which is independent of the relative phase volumes and the flow-rate, is the hub of the counter-current chromatogram.

2.2. Retention times

Non-retained solutes, for which $K = 0$, will have eluted one stationary phase volume; V_s, earlier (Fig. 1B).

Solutes with integral partition coefficients greater than zero are eluted at

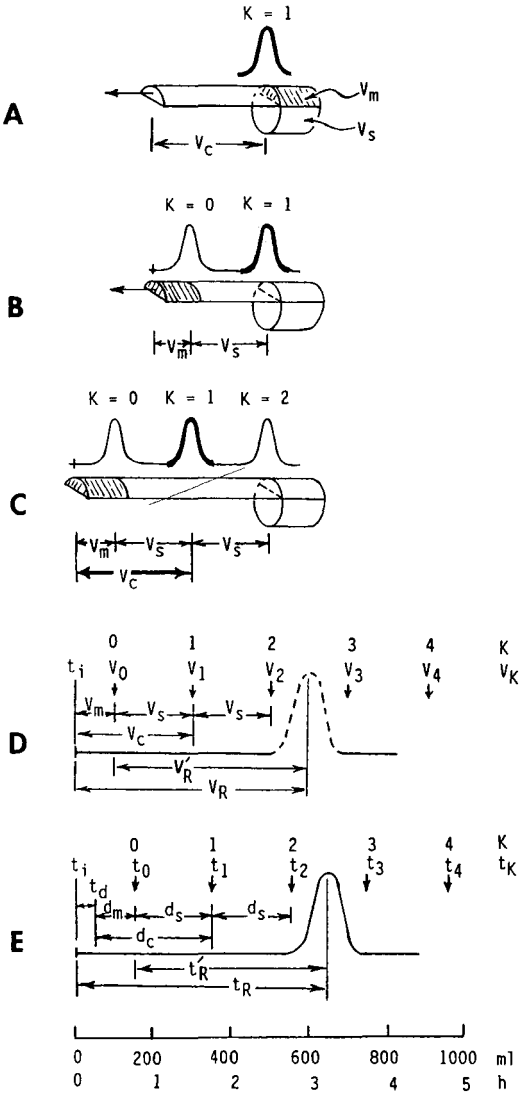


Fig. 1. Generalized counter-current chromatograms. (A, B, C) Elution of solutes with $K = 1, 2$. (D) Indexing the chromatogram by volume, dead volume negligible. (E) Parameters indicated as time and corresponding distances, d, dead volume included. The elution scale corresponds to V_c 300 ml, V_s 200 ml, V_d 50 ml, and flow-rate 200 ml/h.

multiples of the stationary phase volume (Fig. 1C). The general expression for the adjusted retention volume, V'_R, of a solute can then be written as

$$V'_R = V_R - V_m - V_d = KV_s \tag{2}$$

where V_R is the retention volume and V_d is a correction for extra-column dead volume, which is usually negligible in preparative applications of CCC.

2.3. Indexing the chromatogram

It is useful to employ the elution volumes of solutes with unit partition coefficients to index the countercurrent chromatogram, as illustrated in Fig. 1D. The symbols V_0, V_1, V_2, ...V_K are convenient. No standards are required. One needs to know only V_c, V_s and, if not negligible, V_d, to fix these points. When a loop is used for sample injection, adding half the injection volume, V_i, to the feed-line volume, V_f, improves the estimate of V_d for large samples, $V_d = V_f + V_i/2$. (If the Ito injection procedure [2] is employed, whereby one injects the sample directly into the column prior to starting mobile phase flow, half the injection volume is subtracted instead as $V_d = V_f - V_i/2$. This will result in a negative V_d for large sample volumes). Partition coefficients of solutes are readily apparent by visual inspection of the indexed chromatogram. For instance, the broken-line peak in Fig. 1D obviously represents a solute with a K of about 2.5.

The chromatogram indexed with a temporal scale is illustrated in Fig. 2E. The corresponding retention times, t_1, t_2, ...t_K, are obtained by dividing the volumes by the mobile phase flow-rate, f, $t_0 = V_0/f$. The effect of extra-column dead volume in shifting the chromatogram is illustrated in Fig. 1E. The dead time is $t_d = V_d/f$ and t_R and t'_R represent the retention time and adjusted retention time, respectively. The symbols d_m, d_s and d_c represent distances on the chromatogram corresponding to passage of V_m, V_s and V_c volumes of mobile phase, respectively.

Use of the symbols V_0 or t_0 and V_1 or t_1 for indexing is preferable to V_m or t_m and V_c or t_c, respectively, since the latter terms are ambiguous when the system contains dead volume. It is convenient to simply designate the time intervals corresponding to V_m and V_s, on the temporal chromatogram to avoid use of the symbol t_s which is widely used to indicate time spent by solute in the stationary phase.

2.4. Estimating V_1, V_0 and V_s

The position of V_1 is calculated from the known volumes in the system

$$V_1 = V_d + V_c = V_f \pm V_i/2 + V_c \tag{3}$$

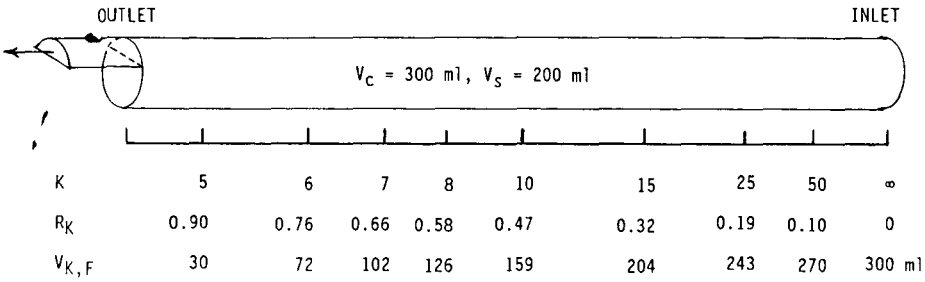
The term $V_i/2$ is + for loop injection but − when the Ito or on-column injection technique is used. In starting up the chromatograph, it is common practice to first fill the column completely with stationary phase. Collection of the volume of stationary phase "carried over", V_{co}, when the mobile phase first passes through the column, provides a measure of the mobile phase volume plus any dead volume in the system, $V_{co} = V_m + V_d \approx V_0$.

Alternatively, one can add a non-retained marker to the sample to obtain a direct indication of V_0.

V_s is obtained by subtracting V_0 from V_1. The positions of V_2, V_3, etc. are then estimated by marking off the distance corresponding to V_s on the chromatogram.

2.5. Solutes retained in the column

A major advantage of CCC is the ability to easily recover solutes retained in the stationary phase by extruding the column contents. The longitudinal position of



K	5	6	7	8	10	15	25	50	∞
R_K	0.90	0.76	0.66	0.58	0.47	0.32	0.19	0.10	0
$V_{K,F}$	30	72	102	126	159	204	243	270	300 ml

Fig. 2. Distribution of retained solutes along the column. Illustrated for the 300-ml column, with V_s of 200 ml, in Fig. 1D, after passage of 1000 ml of mobile phase.

solutes of partition coefficient K, within the column, is indicated by the parameter R_K, which is similar to the R_F value in planar chromatography. R_K is calculated as

$$R_K = \frac{d_K}{L} = \frac{C}{1 + S_F(K - 1)} \tag{4}$$

where S_F indicates the fraction of column volume occupied by stationary phase,

$$S_F = V_s/V_c \tag{5}$$

and C is the number of column volumes of mobile phase passed through the column. R_K is the distance, d_K, moved by the solute with partition coefficient K, divided by the column length, L. An example corresponding to the chromatogram in Fig. 1D, following development with 1000 ml of mobile phase, is given in Fig. 2. Solutes recovered by extruding the column in the forward direction (the direction of development) may be characterized by their forward extrusion volumes, $V_{K,F}$,

$$V_{K,F} = (1 - R_K)V_c \tag{6}$$

some of which are indicated in Fig. 3 for a 300-ml column with V_s of 200 ml.

2.6. Resolution

The contributions of the separation factor, α, column efficiency, N, partition coefficient, K, and stationary phase fraction, S_F, to resolution, R_s, are summarized in the equation

$$R_s = \tfrac{1}{4}(\alpha - 1)\sqrt{N}\ \frac{K_1}{K_1\left(\frac{\alpha + 1}{2}\right) + \left(\frac{1 - S_F}{S_F}\right)} \tag{7}$$

where K_1 is the partition coefficient of the first solute of the pair to be eluted. The separation factor is defined as $\alpha = K_2/K_1$ and will, therefore, always be greater than unity. The first part of the equation is identical to the Knox equation, which is commonly used to describe resolution in high-performance liquid chromatography. However, the bracketed term indicates that the fraction of stationary phase, S_F, in the

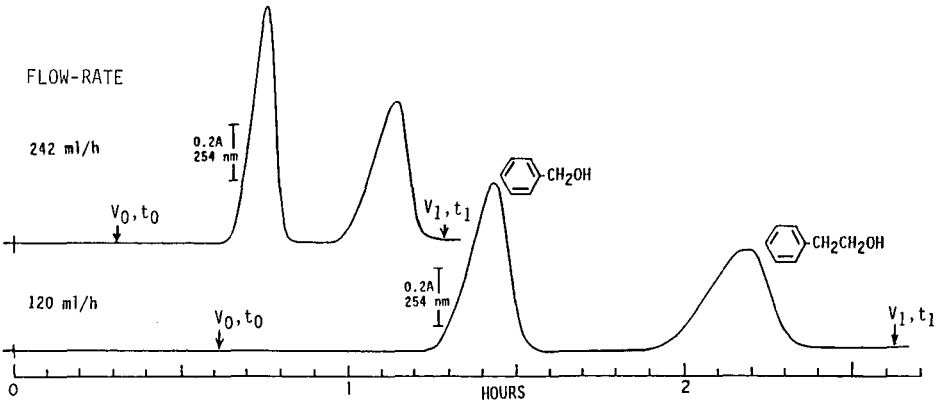


Fig. 3. CCC separation of 20 mg each of benzyl alcohol and phenylethanol, showing the effect of mobile phase flow-rate. Solvent system is heptane–(25% 2-propanol in water) (1:1, v/v) with a mobile aqueous phase. Instrument is the P.C. Inc. MLCPC with a 1.68 mm PTFE column, V_c 315 ml. Adapted from Fig. 9-4 of ref. 2.

column makes a major contribution to resolution. The ratio $(1 - S_F)/S_F$ is equivalent to the phase volume ratio V_m/V_s. The effect of changing V_s can be visually ascertained by examining Fig. 1C. If V_s is imagined to decrease, as may occur when the mobile phase flow-rate is increased, V_m will increase, bringing $K = 0$ closer to the invariant position of $K = 1$. At the same time, solutes with higher K values also approach $K = 1$ until, in the extreme, when $V_s = 0$, the column contains only a single phase and all solutes emerge at V_c. On the other hand, as V_s is increased, the corresponding distance on the chromatogram, representing resolution, increases, approaching a maximum as V_s approaches the column volume V_c, corresponding to S_F approaching unity. With modern apparatus, S_F is in the range of 0.5 to 0.9.

CCC typically provides an efficiency, N of 350 to 1000 theoretical plates. As in other forms of chromatography, selectivity, $\alpha = K_2/K_1$, which is determined by the solvent system, contributes dramatically to resolution. An advantage of CCC is its ability to employ a wide range of solvent systems.

The separation of benzyl alcohol and phenylethanol, shown in Fig. 3, illustrates the resolving power of CCC for a 40-mg sample. A sample of 800 mg is still baseline resolved at the slower flow-rate. Peak symmetry is much better than that obtained in adsorption chromatography.

2.7. Choosing a solvent system

The predictability of CCC performance makes it advantageous to search for solvent systems by measuring K values of sample constituents using analytical techniques other than CCC itself. A micro shake-flask approach coupled with thin-layer chromatography densitometry, high-performance liquid chromatography, gas–liquid chromatography or simple ultraviolet absorptiometry is generally convenient. Aiming for a system providing a K of 1 for the solute of interest is usually desirable. A K of 1 normally provides adequate resolution from more and less polar components but still permits chromatography in a reasonable time. With a typical column volume of 300 to 400 ml and a flow-rate in the range of 100 to 200 ml/h, a solute

with $K = 1$ requires 1.5 to 4 h for elution. As seen from Fig. 1E, it is impractical to elute components with K greater than about 3 within a typical workday. These components can still be recovered either by extrusion of the column contents or by inverting the mode of chromatography by pumping the formerly stationary phase in the anti-development direction. Comparable flow-rates can be employed in centrifugal apparatus of the MLCPC and CDCCC type, but permissible flow-rates in gravitational DCCC apparatus are much slower, requiring separation times up to about 72 h, though still providing good resolution.

The gravitational DCCC system is limited to solvents which form droplets. Various mixtures of chloroform–methanol–water have been most widely used [14,15]. The centrifugal apparatus do not require droplet formation and accommodate a broader variety of solvent systems, ranging in polarity from hexane–water to ethyl acetate–water, 1-butanol–water and 2-butanol–water. Solute partition coefficients can be systematically shifted by varying the content of a third or fourth component. The system hexane–ethyl acetate–methanol–water, which forms two phases over a wide range of compositions, is particularly versatile. Partitioning of solutes into the organic layer is usually favored by increasing the ethyl acetate content, while transfer to the aqueous phase is promoted by adding methanol. Since hexane and water are readily saturated by many organic compounds, sample capacity is usually greater in systems high in ethyl acetate and methanol.

2.8. The effect of pH

Variation in pH can be exploited to shift the partition coefficients of compounds which ionize. In the region where they are ionized (above the pK_a for acids, below the pK_a for bases), the partition coefficient can be expected to change 10-fold for a unit change in pH. Therefore pH must be carefully controlled and sufficient buffering capacity must be provided to accommodate the solute concentration encountered when large amounts of sample are chromatographed.

2.9. Polar and non-polar compounds

CCC offers obvious advantages for polar samples, which do not dissolve in common high-performance liquid chromatography solvent systems, and for labile compounds, which might be altered or adsorbed on contact with silica or other solid supports. The technique is therefore widely used by investigators in the field of natural products, including antibiotics.

CCC is equally applicable to lipophilic compounds. Of many organic solvents immiscible with hexane, the hexane–methanol and hexane–acetonitrile systems are most widely used. The latter system was recently applied to the CCC separation of a 300-g mixture of the ethyl esters of steric, oleic, linolenic and linoleic acids [16]. Formamide and ethylene glycol exhibit good dissolution properties for organic compounds. They function well as the stationary phase in CCC. Formamide forms two-phase systems with solvents as polar as diethyl ether, and ethylene glycol is relatively immiscible with ethyl acetate as well. The system ethylene glycol–chloroform was recently used to isolate 2,4-dinitrophenylvaleric acid from a crude reaction mixture [17]. Traces of ethylene glycol in the chloroform eluent were easily removed by washing the fractions with water prior to evaporation.

Crude sample mixtures, which might readily foul a high-performance liquid

chromatography column, are acceptable in CCC. Good solubility in both phases is required for high sample capacity, since the limit will be reached as saturation is approached in the phase providing lower solubility. A capacity of about 1 g is commonly obtained with a 400-ml column by dissolving the sample in up to 30 ml of either phase or in a mixture of both phases. Better resolution is obtained with a smaller sample volume.

2.10. Normal-phase or reversed-phase CCC

Most solvent systems permit the choice of either phase as the mobile phase. Both aqueous and non-aqueous systems employing the more polar phase as stationary phase can be considered to represent normal-phase CCC. The converse, with the less polar phase stationary, corresponds to the reversed-phase mode.

To simplify application of the chromatographic equations, in which K is defined as C_s/C_m (eqn. 1), it is convenient to define two partition coefficients:

$$K_N = C_{\text{non-polar}}/C_{\text{polar}} \tag{8}$$

and

$$K_P = C_{\text{polar}}/C_{\text{non-polar}} = 1/K_N \tag{9}$$

These symbols apply equally well to aqueous and non-aqueous systems. The subscript N for non-polar also infers the non-aqueous phase in aqueous systems, where water is always the more polar phase. The appropriate partition coefficient for application of the retention equation (eqn. 2) or the resolution equation (eqn. 7) will then be K_N for the reversed-phase mode (nonpolar stationary phase) and K_P for the normal-phase mode (polar stationary phase).

In cases where the chromatographer has a choice of either the normal- or reversed-phase chromatographic mode, eqn. 7 predicts that resolution of a particular pair of solutes will be greater for the mode in which the first solute to be eluted has the higher partition coefficient. The order of elution is, of course, reversed in the two modes. Note that the value of α will be the same in either mode and the judgement just made assumes S_F to be the same in each mode.

For instance, if the partition coefficients for the solutes 1 and 2 are $K_{N1} = 0.5$ and $K_{N2} = 1.0$, then $K_{P1} = 1/0.5 = 2.0$ and $K_{P2} = 1.0$. Since K_{P2} is greater than K_{N1}, eqn. 7 predicts greater resolution in the normal-phase mode, which employs a polar stationary phase.

3. OTHER CCC APPARATUS

This overview summarizes the principles, apparatus and a few applications for the counter-current chromatographic purification of soluble solutes. This use is the most extensively developed at the present time. From its inception however, Ito has demonstrated the applicability of CCC to the separation of a variety of samples. These include particulate (latex particles, cells and subcelluar organelles) separations by sedimentation–elutriation CCC using a single-phase eluent or by partitioning between

two-phase aqueous polymer systems. Proteins can be separated using the latter process as well as by foam CCC.

Ito's extensive research on the optimization of CCC instrumentation has resulted in several devices individually suited for a particular type of sample. For example, a non-synchronous horizontal flow-through coil planet centrifuge is ideal for separation of particulates by sedimentation–elutriation CCC [18,19]. Two-phase aqueous systems have been employed in the non-synchronous CCC as well as in several other units, including the helix CCC and the toroidal coil planet centrifuge [20–24]. Use of two-phase aqueous systems of the salt/polymer type for the separation of proteins has been demonstrated in the MLCPC and CDCCC apparatus and recently in a compact horizontal flow-through coil planet centrifuge with eccentric multilayer coils [25]. Columns have been described which readily adapt MLCPC apparatus to foam CCC [26–28] and true CCC [13,29].

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Review

Dual counter-current chromatography —its applications in natural products research

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ABSTRACT

Dual counter-current chromatography is a newly developed separation method which allows the performance of classic counter-current distribution in a highly efficient manner. The principles of CCC and its capabilities, as illustrated by several applications in natural products isolations, are reviewed.

CONTENTS

1. INTRODUCTION

The development in the 1980s of modern counter-current chromatography (CCC) based upon the fundamental principles of liquid–liquid partition has caused a resurgence of interest in the separation sciences. The advantage of applying continuous liquid–liquid extraction, a process for separating of a multicomponent mixture according to differential solubility of each component in two immiscible solvents have long been recognized. In spite of the limitations of the traditional counter-current distribution methods which prevailed in the 1950s and 1960s [1], liquid–liquid partition was used succesfully to fractionate commercial insulin into two subfractions differing by only one amide group in a molecular weight of 6000 [1].

In recent years, significant improvements have been made to enhance the performance and efficiency of liquid–liquid partitioning [2–8]. The high-speed CCC tech-

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nique utilizes a particular combination of coil orientation and planetary motion to produce a unique hydrodynamic, unilateral phase distribution of two immiscible solvents in a coiled column. These hydrodynamic properties can effectively be applied to perform a variety of liquid–liquid partition chromatographies including high-speed CCC (HSCCC) [2], foam CCC [8,9] and dual CCC (DuCCC) [10,11]. The name of DuCCC is redundant, however, it is useful to distinguish between HSCCC which ought to be called high-speed epicyclic partition chromatography, because only one solvent phase is mobile and the other solvent phase is stationary [2]. Strictly speaking, DuCCC should have two solvents counter-crossing each other from opposite directions and there is no stationary phase involved.

DuCCC has several advantages which are common to many types of liquid–liquid partition chromatography, such as no limitation of the two phase solvent system which can be employed and no sample losses due to irreversible adsorption or decomposition on the solid support. In addition, DuCCC is extremely powerful in separation of crude natural products which usually consist of multicomponents with a wide range of polarities. In a standard operation, the crude sample is fed through the middel portion of the column. The extreme polar and non-polar components are readily eluted from the opposite ends of the column followed by components with decreasing orders of polarity in one phase solvent and increasing order of polarity in the other phase solvent. A component with a partition coefficient equal to 1 will remain inside the coiled column. Essentially, the DuCCC permits a highly efficient performance of classic counter-current distribution.

Below, the principles, instrumentation of DuCCC, and its capabilities in natural products isolation are reviewed.

2. PRINCIPLES AND MECHANISM

The fundamental principle of separation for modern DuCCC is identical to classic countercurrent distribution. It is based on the differential partitions of a multicomponent mixture between two counter-crossing and immiscible solvents. In general, the crude sample is applied to the middle of the coiled column and the extreme polar and non-polar components are readily eluted by two immiscible solvents to

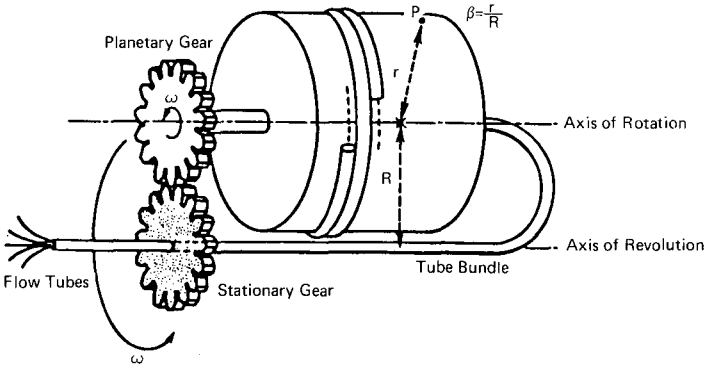

Fig. 1. Epicyclic rotation of DuCCC column holder.

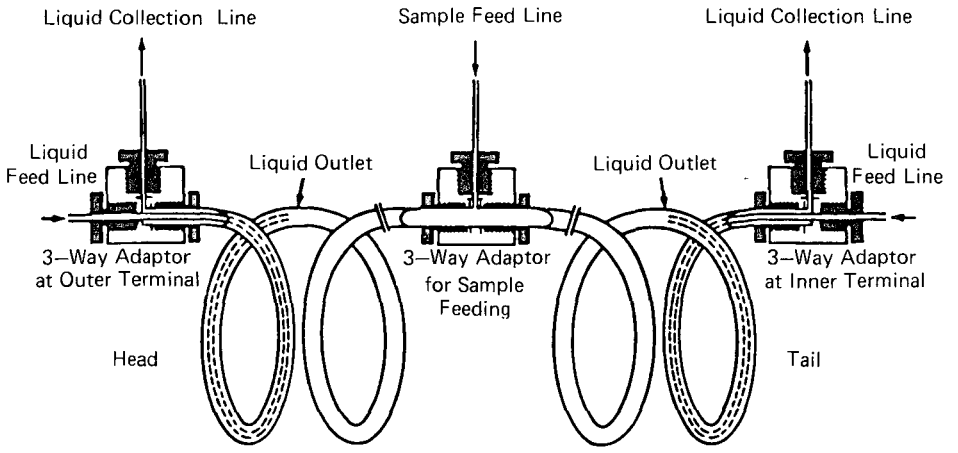

Fig. 2. Column design for DuCCC.

opposite outlets of the column and the component(s) with partition coefficient(s) of 1 will remain in the column. However, in comparison to classic countercurrent distribution, modern DuCCC allows the entire operation to be carried out in a continuous and highly efficient manner. Modern DuCCC is based on the ingenious design of Ito [8] and is illustrated in Figs. 1 and 2. In Fig. 1, a cylindrical coil holder is equipped with a planetary gear which is coupled to an identical stationary sun gear (shaded) placed around the central axis of the centrifuge. This gear arrangement produces an epicyclic motion; the holder rotates about its own axis relative to the rotating frame and simultaneously revolves around the central axis of the centrifuge at the same angular velocity as indicated by the pair of arrows. This epicyclic rotation of the holder is necessary to unwind the twist of the five flow tubes caused by the reovlution, eliminating the use of rotary seals to connect each flow tube. This unique design enables the performance of DuCCC using five flow channels connected directly to the column, as shown in Fig. 2. When a column with a particular coil orientation is subjected to a epicyclic rotation, it produces a unique hydrodynamic phenomenon in the coiled column, in which one phase entirely occupies the head side and the other phase occupies the tail side of the coil column. This unilateral phase distribution enables the performance of DuCCC in an efficient manner. A theoretical calculation of the hydrodynamic forces resulting from such an epicyclic rotation is very complicated and has not yet been elucidated.

3. METHODS AND APPARATUS

The DuCCC experiments are performed with a table top model high-speed planet centrifuge equipped with a multilayer coiled column connected to five flow channels. The multilayer coiled column is prepared from 2.6 mm I.D. PTFE tubing by winding it coaxially onto the holder to a total volume capacity of 400 ml. The multilayer coiled column is subjected to an epicyclic rotation at 500–800 rpm. The fractions are collected simultaneously from both ends of the column and analyzed by

thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) [8,12].

4. APPLICATIONS

In the past decade, the rapid development of sophisticated spectrosocpic techniques, including various 2D-NMR methods, automated instrumentation and routine availability of X-ray crystallography has greatly simplified structural elucidation in natural products investigations. Consequently, the challenge to today's chemists has shifted to one's capability of isolating the bioactive components from crude extracts of either plants or animals. The extract of crude natural products usually is comprised of hundreds of components over a wide range of polarities. In isolating these natural products, it is essential to preserve the biological activity while performing chromatographic purifications. DuCCC represents one of the mild methods of isolation.

DuCCC has several advantages over HSCCC or centrifugal partition chromatography (CPC) [13] in dealing with crude natural products. One distinct feature of DuCCC is the capability of performing normal-phase and reversed-phase elutions simultaneously. This provides a highly efficient method which cannot otherwise be achieved easily in the separation of crude natural products. In many instances, fractions eluted from DuCCC are pure enough for recrystallization or structural study. For example, Fig. 3 shows an analytical HPLC trace of the crude ethanol extract of *Schisandra rubriflora*. Because the major bioactive lignan, schisanhenol 6 is closely eluted with its acetate 5, it has been a major problem to isolate the pure lignan 6. The fractions collected from DuCCC after injection of a crude ethanol extract of *S. rubriflora* (125 mg) were analyzed by TLC (Fig. 4). Fig. 5 shows the reversed-phase HPLC

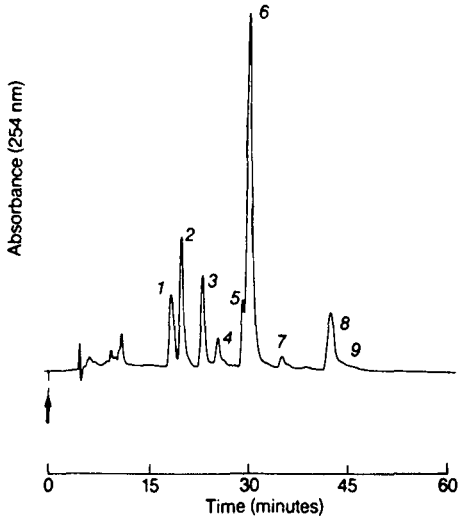

Fig. 3. Analytical HPLC trace of crude ethanol extract from *Schisandra rubriflora*. Column: Zorbax ODS 250 × 4.6 mm I.D.; detection: UV, 254 nm; mobile phase: methanol–water (75:25); sample: ethanolic extract of the kernels of *Schisandra rubriflora* Rhed et Wils. Peaks: 1 = wuweizisu C; 2 = (−)-rubschisandrin; 3 = rubschisantherin; 4 = deoxyschisandrin; 5 = schisanhenol acetate; 6 = schisanhenol; 7 = schisanhanol B; 8 = gomisin O; 9 = pregomisin.

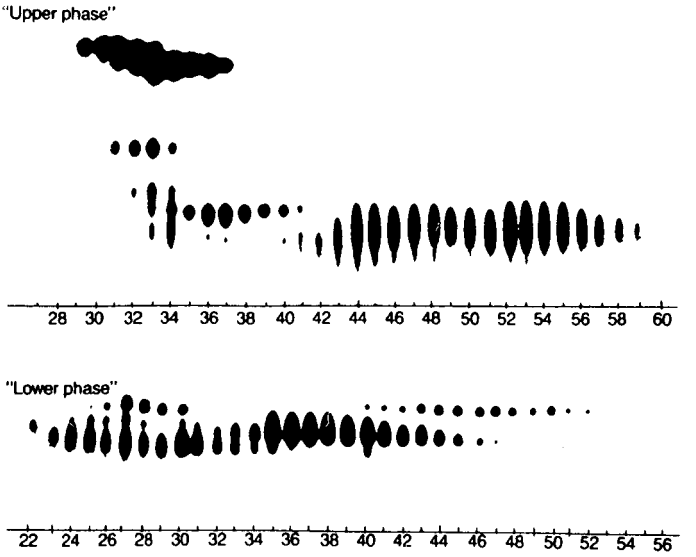

Fig. 4. DuCCC of crude ethanol extract from *Schisandra rubriflora*. Solvent system: hexane–ethyl acetate–methanol–water (10:5:5:1); flow-rate: 2 ml/min; sample crude extract of *Schisandra rubriflora* Rhed et Wils, 125 mg. Detection: TLC, acetone–dichloromethane (5:95). Numbers in figure are fraction Nos.

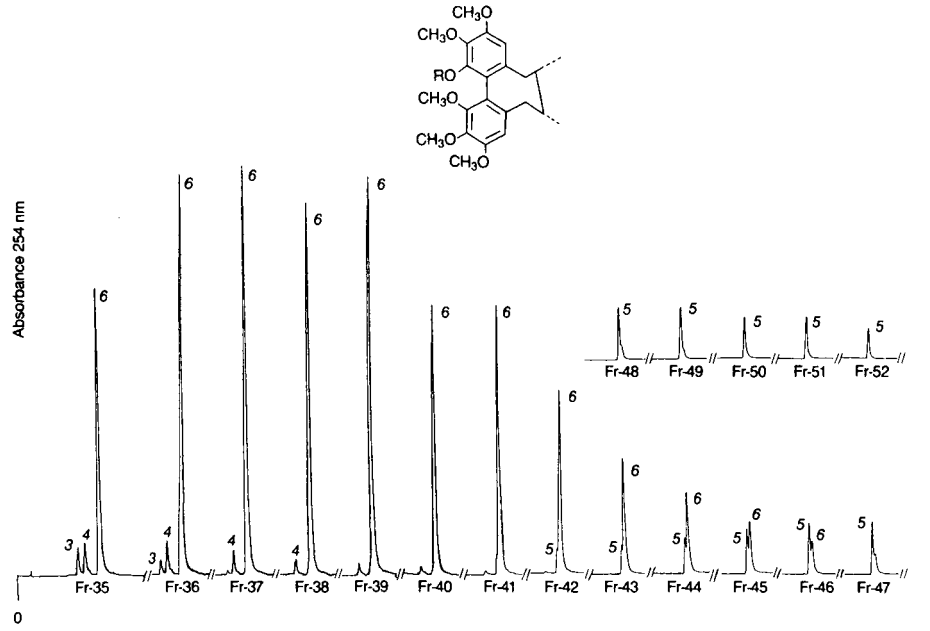

Fig. 5. HPLC traces of the fractions from DuCCC. Column: Zorbax ODS, 254 × 4.6 mm I.D.; detector: UV, 254 nm; mobile phase: methanol–water (75:25); sample: fractions from true counter-current chromatography. 5, R = CH_3CO; 6, R = H. Fr = fraction.

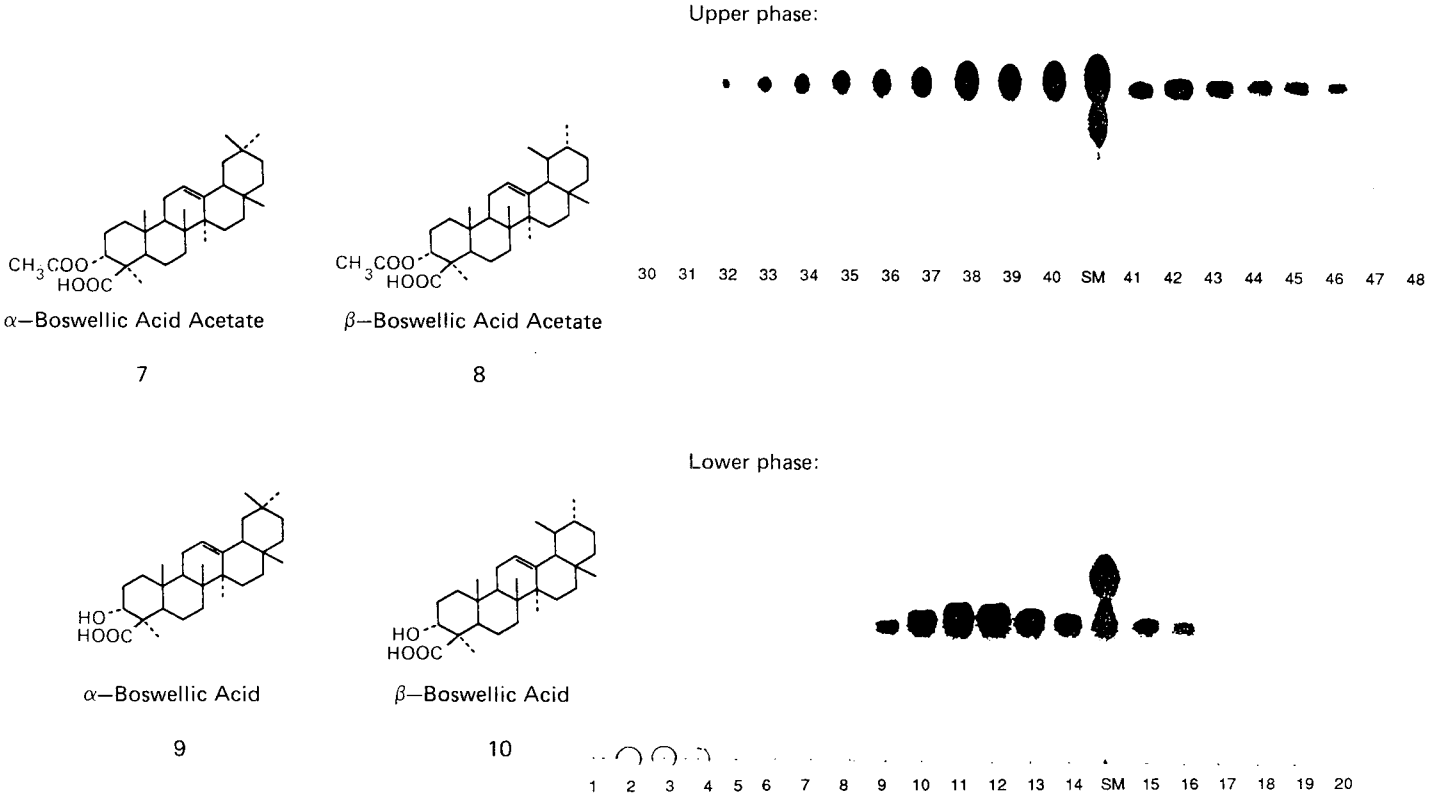

Fig. 6. DuCCC of triterpenoic acids. Sample: mixture of *Boswellic carterii* extract. (400 mg); solvent system: hexane–ethanol–water (6:5:1); flow-rate: upper phase, 2.0 ml/min; lower phase, 2.0 ml/min; rotational speed: 800 rpm; detection: TLC. SM = Starting mixture. Numbers in DuCCC are fraction Nos.

analyses of DuCCC fractions eluted from the lower phase. The solvent system employed for DuCCC was hexane–ethyl acetate–methanol–water (10:5:5:1). The upper phase, being less polar than the lower phase, results in a sequence of elution similar to normal-phase chromatography, while the lower phase provides a sequence of elution resembling reversed-phase chromatography. The bioactive components, schisanhenol acetate 5 and schisanhenol 6, were eluted in the lower phase. Reversed-phase HPLC analyses of fractions 36 to 40 accounted for 32 mg of almost pure schisanhenol 6. A total of 4 mg of schisanhenol acetate 5 was also obtained from fractions 50 to 57. As evidenced by this experiment, DuCCC offers an excellent method for semi-preparative isolation of bioactive components from very crude natural products [11].

The isolation of the topoisomerases inhibitor, boswellic acid acetate from its triterpenoic acid mixture has also been acomplished by DuCCC [12]. As shown in Fig. 6, when an isomeric mixture of triterpenoic acids (400 mg) was subjected to DuCCC, using a hexane–ethanol–water (6:5:1:) as the solvent system, provided 215 mg of the boswellic acid acetes (7 + 8, α + β isomer) and 135 mg of the corresponding boswellic acid (9 + 10, α + β isomer). Some highly polar impurities were eluted immediately in the solvent front, from fraction 1 to 4. The isomeric boswellic acids (7 + 8) were eluted in the lower phase solvent and the less polar acetates (9 + 10) were eluted simultaneously in the upper phase solvent. Although the α and β isomers were only partially resolved by DuCCC, this experiment demonstrates that DuCCC is a highly efficient system for preparative purification.

5. CONCLUSION

The capability and efficiency of DuCCC in performing classic counter-current distribution has been demonstrated in the isolation of bioactive lignans and triterpenoic acids from crude natural products. Besides the resolution and sample loading capacity offered by DuCCC, the unique feature of elution the non-polar components in the upper phase solvent (assuming upper phase is less polar than the lower phase) and concomitant elution of the polar components in the lower phase results in an efficient and convenient preparative method for purification of crude natural products.

The capability of DuCCC has not yet been fully explored. For instance, a particular solvent system can be selected to give the desire bioactive component a partition coefficient of n1. This will allow the "stripping" of the crude extract with DuCCC to remove the impurities or inactive components. Consequently, the bioactive component will be concentration inside the column for subsequent collection. This strategy can also be applied to extract and concentrate certin metabolites in the biological fluids such as urine or plasma. A large amount of sample can also be processed by DuCCC because there is no saturation of the stationary phase.

DuCCC needs more studies in order to explore its capability in isolation of not only natural products but also biological macromolecules.

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Review

Applications of analytical high-speed counter-current chromatography in natural products chemistry

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ABSTRACT

Applications of analytical high-speed counter-current chromatography in natural products chemistry are reviewed and the potential of the method is discussed.

CONTENTS

1. INTRODUCTION

Counter-current chromatography (CCC) is widely used in the isolation and purification of natural products. Separations are based on liquid–liquid partitioning

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and are carried out without solid stationary phase material [1]. CCC methods have proven particularly helpful for the purification of unstable natural products, as illustrated by the isolation of hydrolyzable tannins [2], oxydizable blood pigments from a sea squirt [3], a lignan diester from *Phyllanthus acuminatus* [4], and by the purification of the prostaglandin endoperoxide PGH_2 [5]. High-speed CCC (HSCCC) [6] and centrifugal partition chromatography (CPC) [7,8] are the fastest and the most efficient CCC techniques currently available. These methods can be considered as complementary to preparative HPLC. Theory and applications of preparative CCC techniques have been extensively reviewed [9–13].

Surprisingly, little attention has been given to analytical applications of CCC. The analytical use of CCC had been suggested before [14] for natural products research and small-bore tubing had been used for the flow-through coil planet centrifuge [15], but only recently instrumentation for analytical HSCCC has become available through the work of Ito and Lee [16], and of Romanach and De Haseth [17]. The number of applications in analytical HSCCC is still small and the usefulness of the method to natural products research can not yet be fully assessed. In order to do this the following points need to be addressed: (i) is analytical CCC equivalent to HPLC?; (ii) is the technique suitable for methods development for preparative CCC?; and (iii) is analytical CCC instrumentation useful for small-scale isolation?

In this article the potential of analytical HSCCC for natural products chemistry is evaluated and applications are reviewed.

2. THEORY

Theoretical aspects of CCC have been discussed by Conway and Ito in two very comprehensive articles [18,19]. Parameters and equations presented in those articles and reproduced in this review apply to all CCC methods based on liquid–liquid partitioning. Fig. 1 shows a hypothetical separation of four compounds (**I–IV**) by analytical HSCCC assuming a coil volume of 40 ml, a flow-rate of 1 ml/min, and a stationary phase retention of 75% ($S_F = 0.75$, see equations 1 and 2). The separation of compounds **I–IV** is based entirely on differences in their partition coefficients K. The partition coefficient is defined as the ratio of the concentration of a solute in the stationary phase to the concentration in the mobile phase (eqn. 3). In this example (Fig.

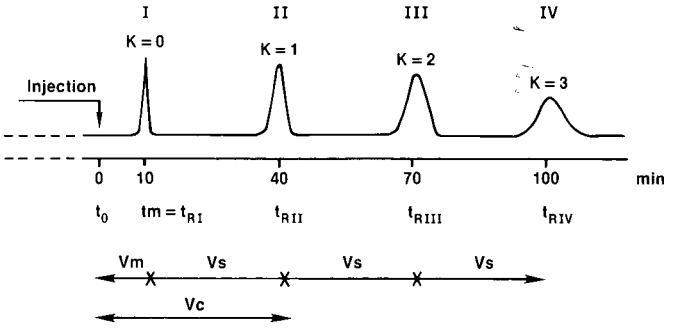


Fig. 1. Analytical HSCCC: hypothetical chromatogram and separation of compounds **I–IV** with partition coefficients of 0, 1, 2 and 3, respectively. (Modified and reproduced with permission from ref. 18).

1), compound **I** partitions completely into the mobile phase and is eluted with the "solvent front" at time $t_m = t_{RI}$. The corresponding elution volume V_m is equal to the amount of mobile phase in the coil. Compound **II** has a partition coefficient of $K = 1$ and elutes at its retention time t_{RII}. The retention time for compounds with $K = 1$ is equal to the time required for the elution of one coil volume (eqn. 4). For compounds with K values smaller or larger than 1, the retention time also depends on the stationary phase fraction S_F. Partition coefficients can be calculated from CCC chromatograms using eqn. 5.

The partition coefficient is by far the most important parameter to control in CCC. For analytical HSCCC a partition coefficient of around 1 is desirable. Theoretically, a higher K value provides higher resolution, but practical considerations such as increased run time and peak broadening due to diffusion are against solvent systems with $K > 3$. Partition coefficients < 1 result in faster separations and are indicated if less resolution is required. Partition coefficients can be calculated from CCC chromatograms (eqn. 3) or can be determined experimentally as part of the solvent selection process. Typically, a sample is distributed between equal volumes of upper and lower phases. The concentration in each phase can be determined by measuring the corresponding UV absorption or mass distribution. For complex samples, phase distribution and HPLC quantitation of individual compounds in upper and lower phases are recommended. Similarly, bioactivity can be partitioned between the upper and the lower phases [20]. Equal distribution of bioactivity corresponds to a partition coefficient of $K = 1$, assuming there is only one active component in the sample. Retention of stationary phase, expressed as stationary phase fraction (S_F), is another important parameter and can be determined directly after the run by displacing the column contents with a flow of nitrogen. The total volume of displaced stationary and mobile phases corresponds to the coil volume (V_c) and the retention of stationary phase is calculated using eqns. 1 and 2. Instead of using nitrogen, the column contents can be displaced by pumping additional mobile phase into the coil after its rotation has been stopped. In this case, the volume of displaced stationary phase is measured and S_F can be calculated (eqn. 2), if the coil volume is known. This more practical approach offers additional rinsing of the coil and is recommended when crude extracts are separated. The importance of a high stationary phase fraction in HSCCC has been demonstrated by Conway and Ito [19], who showed that resolution increases with higher stationary phase retention.

$$V_c = V_s + V_m \tag{1}$$

$$S_F = \frac{V_s}{V_c} \tag{2}$$

$$K = \frac{C_s}{C_m} = \frac{t_R - t_m}{t_m}\left(\frac{1 - S_F}{S_F}\right) = \frac{V_R - V_m}{V_m}\left(\frac{1 - S_F}{S_F}\right) \tag{3}$$

$$t_R = \frac{V_c}{f} \text{ if } K = 1 \tag{4}$$

$$t_R = \frac{V_c}{f}[1 + S_F(K - 1)] \tag{5}$$

where

C_s = concentration of solute in stationary phase
C_m = concentration of solute in mobile phase
f = flow-rate
K = partition coefficient
S_F = stationary phase fraction
t_0 = sample injection
t_m = "solvent front"
t_R = retention time
V_c = (total) volume of coil
V_m = volume of mobile phase fraction in coil
V_R = elution (or retention) volume
V_s = volume of stationary phase fraction in coil [time in min and volumes in ml]

3. INSTRUMENTATION

Instrumentation for analytical HSCCC currently available consists of a multilayer coil plant centrifuge usually equipped with 0.85 mm I.D. PTFE tubing [16,17]. Smaller bore tubing (0.38, 0.55 mm) was used by Romanach and De Haseth [17], but yielded insufficient retention of the stationary phase. Typical operating parameters are listed in Table I. Solvent–tubing wall interactions are increased in narrow-bore tubing and thus require higher centrifugal forces and higher rotational speeds (1500–2000 rpm) in order to maintain a sufficient stationary phase retention. The revolutionary radius [10] for analytical instruments was decreased compared to preparative HSCCC in order to accomodate the higher speed. Flow-rates are typically 0.5–1.0 ml/min and are limited by back pressure and stationary phase retention, depending on the solvent system selected. Higher flow rates have been reported for chloroform–methanol–water systems [21].

Most of the early studies in analytical HSCCC were carried out with user-built instruments, the latest example being an analytical multilayer coil planet centrifuge which can be operated at 4000 rpm maximum speed [22]. Performance of this instrument, equipped with 0.55 mm I.D. tubing, was compared with results obtained

TABLE I

TYPICAL PARAMETERS IN ANALYTICAL HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

Coil	Multilayer coil planet centrifuge
Coil volume	30–40 ml
Tubing	50–70 m × 0.85 mm I.D.
Counterweights	Fixed
Flow-rate	0.5–1.0 ml/min
Back pressure	<300 p.s.i.
Retention (S_F)	50–90%
Rotation	1500–2000 rpm
Sample	μg–10 mg
Run time	20–60 min

with an analytical toroidal coil centrifuge (TCC) with 0.3 mm I.D. tubing [23]. Presently, analytical HSCCC instruments are available from two manufacturers (see footnote to Table II). Plans also exist for the introduction of an analytical instrument for centrifugal partition chromatography [24].

4. DETECTION METHODS

4.1. UV–VIS

Carryover of droplets of non-retained stationary phase may cause high levels of detector noise if sensitive UV detectors such as photodiode array detectors are used. Most CCC chromatograms found in the literature have been redrawn from the original noisy tracings or were generated by tediously diluting and measuring the UV absorption of each of the fractions collected. An improvement has been suggested [25] by the use of a device as illustrated in Fig. 2. A solvent of intermediate polarity (methanol, isopropanol) is added to the coil effluent between the coil outlet and the detector by means of a mixing tee and a reactor coil. Droplets of non-retained stationary phase are thus "redissolved" and detector noise is significantly reduced. Applications of this method are shown in Figs. 3 and 4 by the separation of a mixture of standard compounds **1–4** and of phenolic natural products **5–8**, respectively. UV spectra of solutes were identical to those obtained with pure standards in methanol. UV spectra provide only limited structural information, but significantly enhance peak identification in analytical HSCCC.

In another approach to reducing detector noise, a piece of small-bore PTFE tubing (3 m × 0.46 mm I.D.) was inserted between the coil outlet and the UV detector [26]. This tubing was immersed in a water bath kept at 30°C. Experiments using a preparative HSCCC instrument suggested that the heating of the coil effluent significantly reduced levels of detector noise for selected solvent systems, *e.g.* chloroform–methanol–water. This method may also be applicable to analytical HSCCC.

4.2. Fourier transform infrared spectroscopy

Fourier transform infrared (FT-IR) spectroscopy has been suggested for CCC by Romanach and De Haseth [27]. Separations were carried out on a HSCCC instrument with 1.2 mm I.D. tubing (coil volume 160 ml) in chloroform–methanol–water (pH = 2) (3:1:3) and hexane–methanol–water (3:3:2) solvent systems. Flow cells with pathlengths ranging from 0.025–1.0 mm were used and solvent removal was not necessary. IR spectra were recorded on-line; however, relatively large sample sizes were required (0.2 to more than 1 mg per compound on the column). FT-IR

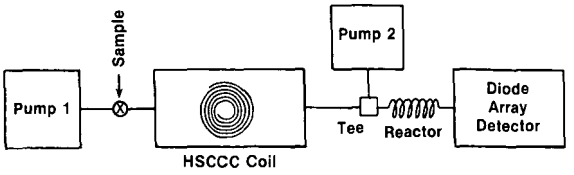


Fig. 2. Schematic diagram of analytical HSCCC with photodiode array detector and post-coil reactor.

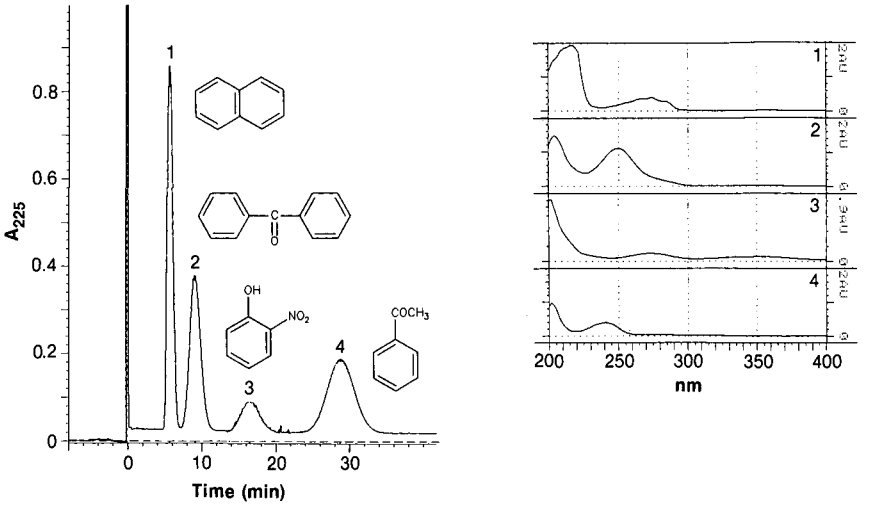


Fig. 3. Analytical HSCCC of naphthalene (**1**), benzophenone (**2**), *o*-nitrophenol (**3**), and acetophenone (**4**) (10–25 μg each); hexane–methanol–water (3:3:2), mobile phase = upper phase, 1.0 ml/min; additional flow of isopropanol at 0.5 ml/min, post-coil reactor. (Reproduced with permission from ref. 25).

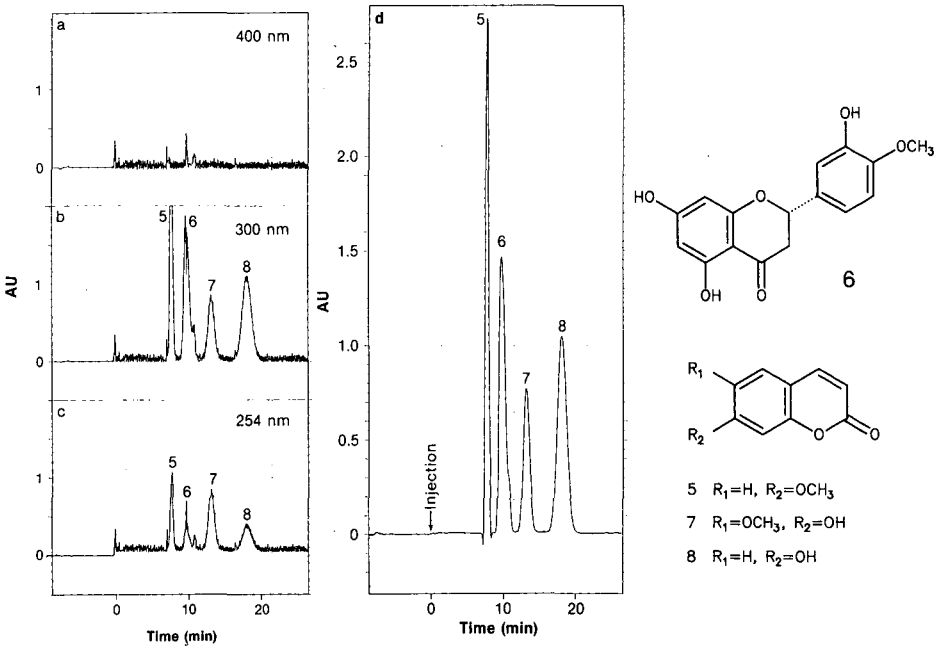


Fig. 4. Analytical HSCCC of herniarin (**5**), hesperetin (**6**), scopoletin (**7**), and umbelliferone (**8**), 50 μg each; chloroform–methanol–water (13:7:8), lower phase at 0.8 ml/min; post-coil reactor with additional flow of methanol (0.8 ml/min); original chromatograms (a–c) recorded with a photodiode array detector; chromatogram (d) obtained by subtracting (a) from (c) and smoothing. (Reproduced with permission from ref. 25).

spectroscopy represents an excellent method for the identification of solutes. On the other hand the large sample sizes make an application of CCC–FT-IR in natural products chemistry (*e.g.* separation of crude extracts/fractions) rather unlikely.

4.3. Mass spectrometry

The combination of analytical HSCCC with thermospray fast atom bombardment (FAB) mass spectrometry (MS) has been described by Lee and co-workers [28–30]. An experimental instrument for analytical HSCCC (planet centrifuge with 0.85 mm I.D. tubing) was interfaced with a thermospray quadrupole mass spectrometer. The high back pressure generated from the thermospray vaporizer was overcome by an additional high-performance liquid chromatography (HPLC)-type pump placed in-line between the CCC instrument and the mass spectrometer. Ion chromatograms and mass spectra allowed the specific detection of compounds in complex samples such as crude extracts (see applications).

5. SOLVENT SYSTEMS

Most of the solvent systems used so far in analytical HSCCC are hexane–water-based with a variable third, and occasionally fourth component (ethyl acetate, propanol, methanol; see Table II). Mixtures of chloroform–methanol–water represent the most frequently used solvent systems in CCC (see ref. 31 for a ranking of these solvents by polarity) and can also be used in analytical HSCCC. The solvent system chloroform–methanol–water (13:7:8) was used for the separation of phenolic compounds by analytical HSCCC and good retention (>80%) of the stationary (upper) phase was obtained [25]. Ito [32] studied the retention of stationary phase in analytical HSCCC (0.85 mm I.D. coil) as a function of the holder diameter, rotational speed, and flow-rate. Sufficient retention of the stationary phase was observed for hexane–water, hexane–ethyl acetate–methanol–water (1:1:1:1) and chloroform–water mixtures when flow-rates of less than 1 ml/min were used at speeds over 1500 rpm (holder diameter of 5 cm). Butanol–water systems were found to yield rather poor retention (< 50 %) even at flow-rates of less than 0.4 ml/min. Most recently, gradient elution has been suggested for CCC [33,34]. Gradient elution compensates for peak broadening of late-eluting compounds and shortens the run time in separations of samples which cover a wide range of polarity. So far, no experimental data are available for gradient elution in analytical HSCCC.

6. APPLICATIONS

Applications of analytical HSCCC in natural products chemistry are summarized in Table II. All separations were carried out on instruments using 0.85 mm I.D. tubing. Applications cover a wide range of classes of compounds, mostly from higher plants.

6.1. Alkaloids

An artificial mixture of the *Vinca* alkaloids vincamine (**9**) and vincine (**10**) was separated by analytical HSCCC in hexane–ethanol–water (6:5:5) and the separation was compared with results from analytical reversed-phase HPLC [28]. Baseline

TABLE 2

ANALYTICAL CCC OF NATURAL PRODUCTS

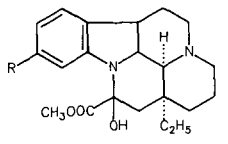
Compound	Source	Conditions[a]	Ref.
Alkaloids	*Stephania tetrandra* (Menispermaceae)	Hexane–ethyl acetate methanol–water (3:7:5:5), lower and upper, A	15
	Vinca minor (Apocynaceae)	Hexane-ethanol-water (6:5:5), lower, B	28
	Indole plant hormones	Hexane–ethyl acetate–methanol–water (3:7:5:5), lower, B	36
Anthraquinones	*Rheum palmatum* (Polygonaceae)	Hexane–ethanol–methanol–water (9:1:5:5), lower and upper, A	37
Coumarins	—	Chloroform–methanol–water (13:7:8), lower, C	25
Flavonoids	*Hippophae rhamnoides* (Elaeagnaceae)	Chloroform–methanol–water (4:3:2), lower, A	21
Lignans	*Podophyllum peltatum* (Podophyllaceae)	Hexane–ethyl acetate–methanol–water (1:1:1:1), lower, C	38
	Schisandra rubriflora (Schisandraceae)	Hexane–ethanol–water (6:5:5), lower, B	29,30,43
Macrolides	*Bugula neritina* (Bugulidae)	Hexane–isopropanol–methanol–water (4:1:1.6:0.4), lower, C	42

[a] Solvent system, mobile phase and instrumentation. A = CCC-2000 analytical high-speed counter-current chromatograph, *ca.* 70 m × 0.85 mm I.D. (*ca.* 40 ml coil volume); Pharma-tech, Baltimore, MD, U.S.A. B = Experimental instrument, 0.85 mm I.D. tubing, see corresponding references for further description. C = "Micro" high-speed counter-current chromatograph for analytical HSCCC, *ca.* 50 m × 0.85 mm I.D. (*ca.* 30 ml coil volume) operated at 1800 rpm; P.C. Inc., Potomac, MD, U.S.A.

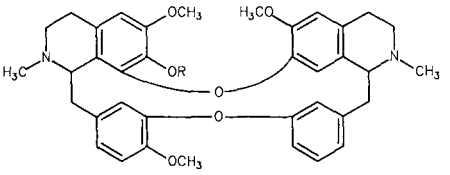
resolution was obtained with both methods and an isomer of vincine was separated by analytical HSCCC, but not by HPLC. The sample was also analyzed by CCC–MS. In another example [35], an artificial mixture of the bisbenzylisoquinoline alkaloids tetrandrine (**11**), fangchinoline (**12**), and the protoberberine type alkaloid cyclanoline (**13**) was separated in two *n*-hexane–ethyl acetate–methanol–water solvent systems. Alkaloids **11–13** were originally isolated from *Stephania tetrandra* S. Moore, a Chinese medicinal plant with antiinflammatory properties. Lee *et al.* [36] studied the separation of indole acetic acid and of three homologues. These plant growth hormones were resolved to baseline in hexane–ethyl acetate–methanol–water (3:7:5:5) with the lower phase as mobile phase.

6.2. Anthraquinones

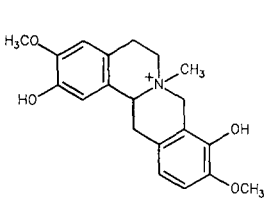
Five anthraquinones from *Rheum palmatum* L. were separated by analytical HSCCC using *n*-hexane–ethyl acetate–methanol–water (9:1:5:5) as the solvent system [37]. The sample mixture (1 mg of extract containing about 0.5% free hydroxy-anthraquinones) was analyzed in a 0.85 mm I.D./40 ml volume coil. The elution was first carried out with the lower phase as the mobile phase for 20 min at 1 ml/min, then the mode was reversed. Elution was then carried out with the upper phase for an



9: R = H
10: R = OCH_3



11: R = CH_3
12: R = H



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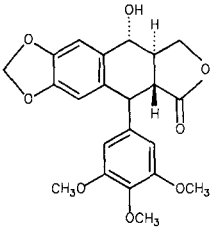

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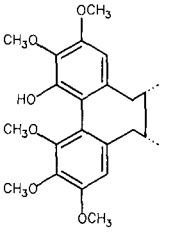

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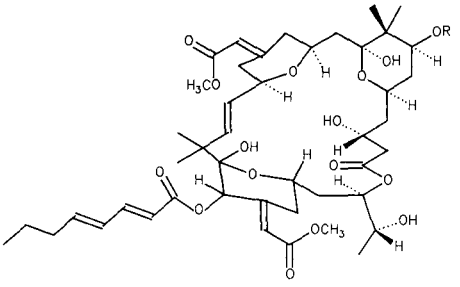

16: R = $COCH_3$
17: R = H

additional 50 min, thus eluting less polar anthraquinones which otherwise would have remained in the stationary phase. This example shows the advantage of reversed elution in the case of complex samples having a wide polarity range. Aloe-emodin, chrysophanol, emodin, physcion, and rhein were identified by MS but no information about peak purity was provided.

6.3. Lignans

Podophyllotoxin (**14**) is a major cytotoxic lignan of the mayapple, *Podophyllum peltatum* L. The dichloromethane extract of the rhizomes was separated (3 mg sample) by analytical HSCCC in hexane–ethyl acetate–methanol–water (1:1:1:1) [38]. Elution was carried out with the lower phase at 0.5 ml/min and fractions (1 min each) were collected. The thin-layer chromatographic (TLC) analysis of selected fractions is shown in Fig. 5.

Fraction 100 was a 1:1 mixture of podophyllotoxin (identified by HPLC, ^{1}H NMR, FAB-MS) with an unidentified lignan. Another compound (fraction 70) of

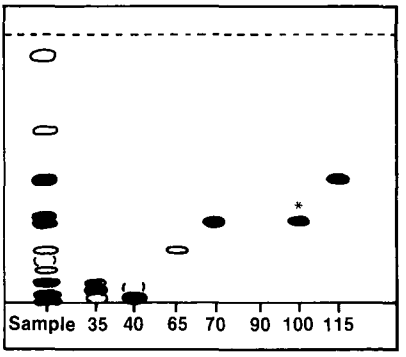

Fig. 5. TLC analysis of selected fractions from analytical HSCCC of *Podophyllum peltatum* extract (3 mg sample). Hexane–ethyl acetate–methanol–water (1:1:1:1), lower phase at 0.5 ml/min; fractions: 1 min; retention 50%. TLC: silica gel; dichloromethane–methanol–water (1:1:1, lower phase), UV 254 (empty circles) and anisaldehyde spray reagent (full circles); * marks a fraction containing podophyllotoxin.

almost the same R_F value as podophyllotoxin was easily separated by analytical HSCCC. Retention of the stationary phase was 50%.

The root extract of *Schisandra rubriflora* Rhed et Wils. was separated in hexanes–ethanol–water (6:5:5) with the lower phase being the mobile phase (0.8 ml/min). A thermospray mass spectrometer was used as a detector and schishanenol (**15**), along with five structurally related lignans were detected by means of selective ion chromatograms and mass spectra [29]. Schishanenol and its acetate derivative were separated by HSCCC, but not by reversed-phase HPLC with methanol–water. The lignans of *S. rubriflora*, a traditional Chinese medicinal plant for the treatment of hepatitis, were also isolated by true CCC, a promising new type of CCC [39].

6.4. Macrolides

Bryostatin 1 (**16**) is an antitumor macrolide isolated from the marine Bryozoan *Bugula neritina* [40] and currently in preclinical development. The detection of bryostatin 1 by reversed-phase HPLC in crude extracts or fractions of *B. neritina* requires several sample clean-up steps [41]. The extract (10 mg sample) was separated by analytical HSCCC using hexane–isopropanol–20% aqueous methanol (4:1:2) with the lower phase as mobile phase at 0.6 ml/min. Fractions eluting between 30–34 min contained bryostatin 1, but not bryostatin 2 (**17**) and were pure enough for direct HPLC analysis.

6.5. Phenolics

A mixture of three plant coumarins and one flavanone (see Figs. 2 and 4) was used to evaluate protodiode array detection for analytical HSCCC [25]. See section on Detection methods for further discussion.

Flavonoids from *Hippophae rhamnoides* L. fruits were studied by Zhang *et al.* [21] using a 0.85 mm I.D., 40 ml coil. The separation of the crude ethanolic extract (3 mg sample) by analytical HSCCC was accomplished with chloroform–methanol–water (4:3:2) within 100 min. Elution with the lower phase at 1 ml/min at 1800 rpm

yielded excellent retention of the stationary phase (86%). Isorhamnetin was detected as the major flavonoid present in the extract. The separation was repeated at different flow-rates and the analysis time was shortened to 15 min. A flow-rate of 5 ml/min provided sufficient resolution and acceptable stationary phase retention (68%). The authors also separated a sample (100 mg) on a preparative HSCCC instrument and obtained the same elution profile with similar resolution.

7. DISCUSSION

Typical instrumentation for analytical HSCCC consists of a multilayer coil planet centrifuge with 0.85 mm I.D. tubing and a total coil volume of 30–40 ml. High rotational speeds (1500–2000 rpm) are required to retain a high stationary phase fraction in the coil. Future instrumentation is expected to have smaller and shorter tubing and to be operated at even higher speeds [22]. Several detection systems have been used in conjunction with HSCCC, including UV [25,26], FT-IR [27] and thermospray MS [28–30]. Among those, CCC–MS is a very promising technique, although not readily available. Analytical HSCCC separations are based on differences in partition coefficients (K) between individual compounds and separations are therefore predictable. Partition coefficients represent the most important parameter to be controlled by the chromatographer. It is recommended to determine K values as part of the solvent selection process prior to carrying out separations. K values can also be calculated from chromatograms, using eqn. 5.

Applications of analytical HSCCC reviewed in this article emphasize a number of successful solutions to difficult natural products separation problems. Various biphasic solvent systems provide high resolution and relatively short separation time. An almost unlimited (but not yet fully explored) choice of solvent systems allows excellent control over selectivity. Theoretical plate counts are less than 2000 and suggest that analytical HSCCC at its present state can not compete with modern HPLC. Yet there are examples in which analytical HSCCC has given results which could not have been achieved on normal- or reversed-phase solid supports.

Analytical HSCCC represents a fast and reliable way of methods development for preparative CCC separations. This was demonstrated by Zhang *et al.* [21] by analytical and preparative HSCCC of flavonoids from *Hippophae rhamnoides*. Small-scale isolations of natural products in μg to mg quantities are yet another interesting application for analytical HSCCC instrumentation. Complex samples, *e.g.* crude extracts are conveniently fractionated with complete sample recovery. Enriched fractions or semipure compounds may be obtained in a single step, as illustrated by the separation of a crude *Podophyllum peltatum* extract [38] and by the enrichment of bryostatin 1 from *Bugula neritina* [42]. To our knowledge, analytical HSCCC has not yet been used for quantitative work with natural products.

Analytical HSCCC is ideal for methods development in preparative HSCCC (possibly also for other types of CCC) and represents a convenient method for micro- or small-scale purifications. This new analytical method is complementary to HPLC and most valuable in cases where resolution can only be obtained by changing the selectivity of the chromatographic system. A large number of applications of analytical HSCCC can be expected for natural products chemistry and for related areas where difficult separation problems are common.

8. ACKNOWLEDGEMENTS

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Cross-axis synchronous flow-through coil planet centrifuge (Type XLL)

I. Design of the apparatus and studies on retention of stationary phase

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ABSTRACT

The fourth prototype holds a pair of column holders in the lateral position at 15 cm from the center of the rotary shaft horizontally mounted on the rotary frame at 7.6 cm from the central axis of the apparatus. Using short coils of 2.6 mm I.D. PTFE (polytetrafluoroethylene) tubing with 7.6 cm and 24 cm helical diameters, retention of the stationary phase was measured in ten pairs of two-phase solvent systems under various experimental conditions. Satisfactory retention was obtained by choosing proper combinations of three factors, *i.e.*, the direction of planetary motion, head–tail elution mode, and inward–outward elution mode. The polar butanol solvent systems showed excellent retention from 65 to 80% in the 7.6 cm helical diameter left-handed coil.

INTRODUCTION

The cross-axis synchronous flow-through coil planet centrifuge (X-axis CPC) has a unique feature among coil planet centrifuges in that the column holder axis is perpendicular to the central axis of the centrifuge [1]. In the past, three different models of the X-axis CPC were fabricated and their capability for performing counter-current chromatography (CCC) was examined using various two-phase solvent systems [1–8]. Hydrodynamic studies on retention of the stationary phase in the coiled column indicated that the system provides more reliable retention of the stationary phase for viscous polar solvent systems compared with the high-speed CCC centrifuge based on the type J synchronous planetary motion [1,2]. Further studies have shown that the phase retaining capacity of the X-axis CPC is enhanced by

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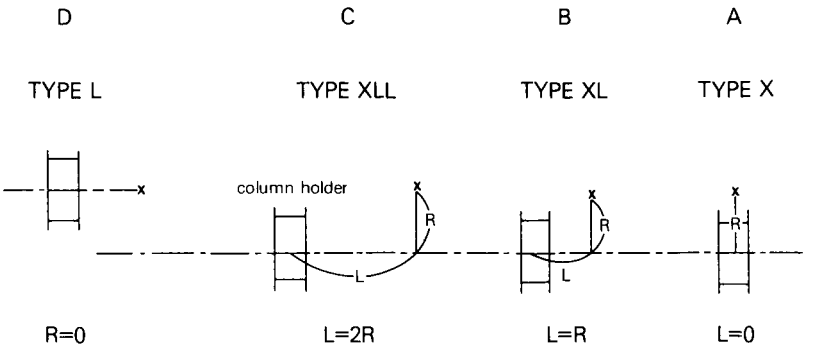

Fig. 1. Orientation of the column holder on the axis of rotation in four types of coil planet centrifuge. × = Axis of revolution; – – – – = axis of rotation; R = rotation radius; L = revolution radius.

laterally shifting the position of the column holder along the holder shaft, probably due to the asymmetry of the laterally acting force field between the upper and the lower halves of the rotating coil [3]. The degree of the lateral shift of the column holder may be conveniently expressed by L/R where L is the distance from the center of the holder shaft to the coil holder and R, the distance from the centrifuge axis to the holder shaft (revolution radius) (Fig. 1). The latest model [8] with $L/R = 1$ (Fig. 1B) has produced substantially higher stationary phase retention for the polar-solvent systems compared with the original model [1,2] with the central column position ($L = 0$, Fig. 1A). These results suggest that shifting the position of the column holder even further along the rotary shaft would permit the retention of highly viscous polar solvent systems including aqueous–aqueous polymer phase systems used for the partition of macromolecules and cell particles [9].

The present paper deals with the fourth model of the X-axis CPC equipped with a pair of column holders at $L/R = 2$ (Fig. 1C). In this paper we describe the design of the apparatus and hydrodynamic studies on retention of the stationary phase using short coils mounted on the column holders with two different hub diameters.

APPARATUS

The basic design of the apparatus has been reported earlier [7]. The present apparatus has $L/R = 2$ to provide a strong lateral force field to retain viscous polar solvent systems. Fig. 2 schematically illustrates the horizontal cross-section of the apparatus equipped with a pair of multilayer coils.

The motor (not shown in the diagram) drives the central shaft (1) and the rotary frame around the central axis of the centrifuge. The rotary frame consists of two pairs of side plates (2) rigidly bridged by a pair of horizontal plates (3) and holds a pair of rotary shafts (4) horizontally at a distance of 7.6 cm from the central axis of the centrifuge. A pair of column holders (5) are mounted one on each side of the rotary frame between the outer and inner side plates (2) at a distance 15 cm from the center of the rotary shaft. The desired synchronous planetary motion of the holders is produced as follows: The toothed pulley (6) mounted on each holder shaft is coupled

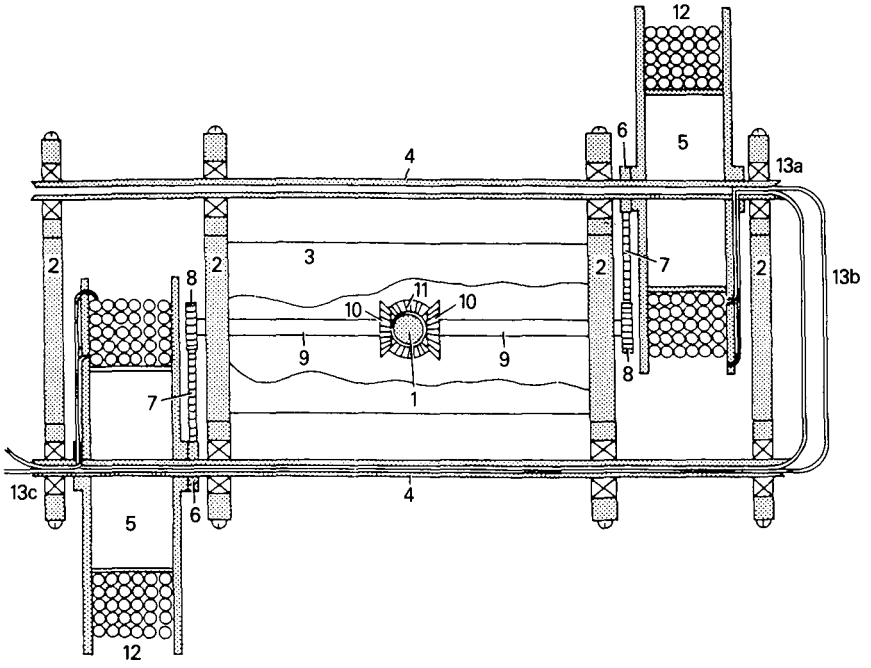

Fig. 2. Cross-sectional view of the type XLL coil planet centrifuge. 1 = Central shaft; 2 = side plates; 3 = bottom plate; 4 = rotary shafts; 5 = column holders; 6 and 8 = toothed pulleys; 7 = toothed belt; 9 = countershafts; 10 = planetary miter gears; 11 = stationary miter gear; 12 = multilayer coil separation columns; 13a–c = flow tubes.

with a toothed belt (7) to an identical pulley (8) on the countershaft (9) equipped with a miter gear (45°) (10) which is in turn engaged to the identical stationary sun gear (11) mounted on the bottom plate of the centrifuge. A pair of coiled columns (12) mounted on the holders are serially connected with flow tubes (13a–c) as illustrated in the diagram. The above mechanical arrangement prevents twisting of the flow tubes and continuous elution can be performed through the rotating column without the use of rotary seals. Three pairs of interchangeable coil holders were fabricated with hub diameters of 7.6, 15 and 24 cm, respectively. They can be easily removed from the rotary frame by loosening the screws on each bearing block.

The apparatus can be operated up to the maximum speed of 1000 rpm with a speed controller (Bodine Electric, Chicago, IL, U.S.A.).

EXPERIMENTAL

Reagents

n-Hexane, ethyl acetate, chloroform, methanol, *n*-butanol and *sec.*-butanol were all chromatographic grade and purchased from Burdick & Jackson Labs., (Muskegon, MI, U.S.A.). Acetic acid was reagent grade and obtained from Mallinckrodt (Paris, KY, U.S.A.).

Hexane Systems			Ethyl Acetate Systems		Chloroform Systems		Butanol Systems		
1	2	3	4	5	6	7	8	9	10
Hexane	Hexane	Hexane 1 Ethyl Acetate 1 Methanol 1	Ethyl Acetate	Ethyl Acetate 4 Acetic Acid 1 Water 4	Chloroform	Chloroform 2 Acetic Acid 2 Water 1	n-Butanol	n-Butanol 4 Acetic Acid 1 Water 5	sec.-Butanol
Water	Methanol	Water 1	Water		Water		Water		Water

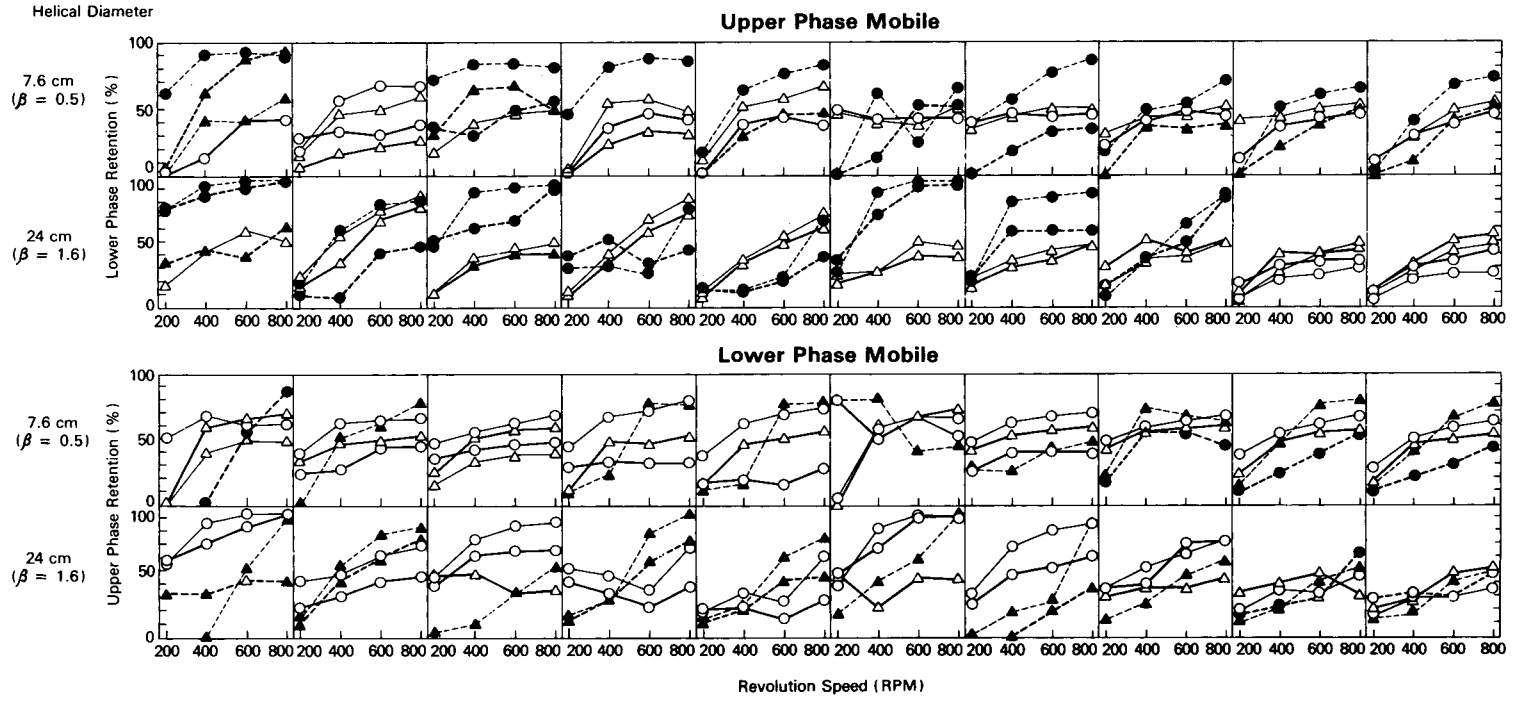

Fig. 3. A set of phase retention diagrams for ten pairs of two-phase solvent systems in two helical diameter coils. See Table I for symbols assigned for each elution mode.

Preparation of two-phase solvent systems

The following 10 pairs of solvent systems were then prepared: *n*-hexane–water, *n*-hexane–methanol, *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v), ethyl acetate–water, ethyl acetate–acetic acid–water (4:1:4, v/v/v), chloroform–water, chloroform–acetic acid–water (2:2:1, v/v/v), *n*-butanol–water, *n*-butanol–acetic acid–water (4:1:5, v/v/v) and *sec.*-butanol–water. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

Preparation of coiled columns

The present studies were performed with short coils of approximately 3–5 m long, 2.6 mm I.D. PTFE tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) wound directly around the holders of 7.6- and 24-cm hub diameters forming a single-layer coil with a total capacity of 20–30 ml. Both right-handed and left-handed coils were made for each holder. Each coiled column was firmly affixed on the holder with several pieces of fiber-glass reinforced adhesive tape. Each end of the column was connected to a 1 m × 0.85 mm I.D. PTFE flow tube, by inserting a series of smaller diameter PTFE tubing into one another.

Measurement of stationary phase retention

Experiments were performed according to the standard procedure described elsewhere [8]. In each measurement, the coil was first filled entirely with the stationary phase. Then the apparatus was rotated at the desired revolution speed while the mobile phase was pumped into the column at a flow-rate of 2 ml/min using a 50- or 20-ml capacity glass syringe driven by a syringe driver (Harvard Apparatus, Millis, MA, U.S.A.). The effluent from the outlet of the column was collected into a 25- or 50-ml graduated cylinder to measure the volume of the stationary phase eluted from the column as well as the total elution volume of the mobile phase. The elution was continued for 12–15 min until the total elution volume exceeded the column capacity. During the elution, the temperature inside the centrifuge was controlled at 22 ± 2°C by placing an ice-bag over the top plate of the centrifuge. Then the centrifuge was stopped and the column contents emptied into a graduated cylinder by connecting the inlet of the column to a pressured nitrogen line (*ca.* 80 p.s.i.). The column was then washed with several milliliters of methanol and finally flushed with several milliliters of the stationary phase which was to be used for the next experiment.

The experiments were performed with ten different two-phase solvent systems (Fig. 3). At the maximum speed of 800 rpm, the retention of each phase was measured in each coil under eight different experimental conditions, *i.e.*, all possible combinations of the planetary motion (P_I and P_{II}), the head–tail elution mode (head to tail and tail to head), and the inward–outward elution mode determined by the handedness of the coil (right-handed and left-handed) as indicated in Table I. Choosing two best conditions which produced high retention of the stationary phase in each handedness of the coil at 800 rpm, the retention was further measured under the reduced rpm (600, 400 and 200) to obtain the phase retention diagrams described below.

Phase retention diagram

From each measurement, retention of the stationary phase was expressed as a

TABLE I

EIGHT DIFFERENT ELUTION MODES IN X-AXIS CPC

Planetary motion	Head–Tail elution mode	Inward–Outward elution mode (handedness of coil[a])	Combined elution mode[b]	Symbolic signs in PDD[c]
P_I 	Head → Tail	Inward (R)	P_I-H-I	○——○
	Head → Tail	Outward (L)	P_I-H-O	○——○
	Tail → Head	Inward (L)	P_I-T-I	●----●
	Tail → Head	Outward (R)	P_I-T-O	●----●
P_{II} 	Head → Tail	Inward (L)	P_{II}-H-I	△——△
	Head → Tail	Outward (R)	P_{II}-H-O	△——△
	Tail → Head	Inward (R)	P_{II}-T-I	▲----▲
	Tail → Head	Outward (L)	P_{II}-T-O	▲----▲

[a] R: Right-handed. L: Left-handed.
[b] H: Head → Tail. T: Tail → Head. I: Inward. O: Outward.
[c] PDD: Phase Distribution Diagram (Phase Retention Diagram).

percentage relative to the total column capacity according to the expression $100(V_C, + V_F - V_S)/V_C$, where V_C is the total capacity of the coil; V_F free space in the flow tubes; and V_S the volume of the stationary phase eluted from the coil. From these retention data the hydrodynamic distribution of the two solvent phases in the coil was summarized in a phase retention diagram constructed by plotting percentage retention of the stationary phase as a function of revolutional speed for each mobile phase. A group of retention curves produced by different elution modes but otherwise identical experimental conditions can be illustrated in the same diagram. In order to distinguish each elution mode in the phase retention diagram, a set of symbolic designs was used to draw phase retention curves as illustrated in Table I.

RESULTS AND DISCUSSION

Phase retention diagrams

Fig. 3 illustrates a set of phase retention diagrams for ten different two-phase solvent systems with a broad range in hydrophobicity. Each column was obtained from the solvent system indicated at the top and arranged from left to right according to the degree of hydrophobicity of the major organic component. The upper panel shows the retention of the lower stationary phase and the lower panel, the retention of the stationary upper phase. In each panel, the first row was obtained from the coil mounted on the 7.6 cm diameter holder ($\beta = 0.5$) and the second row from the coil mounted on the 24 cm diameter holder ($\beta = 1.6$) as indicated on the left margin ($\beta = r/R$ where r is the distance from the holder axis to the coil and R, the distance from the holder axis to the central axis of the centrifuge).

In each diagram, four retention curves are drawn against the applied revolution speeds from 200 to 800 rpm. Among those four curves, two were obtained from the right-handed coil and the other two from the left-handed coil, both groups being selected among four possible combinations of the elution modes (see Table I for symbolic designs assigned for each elution mode). In general 50% retention is considered satisfactory, but at higher retention, better peak resolution is expected.

The results show that the majority of the retention curves rise with increased rpm approaching a plateau at the highest speed of 800 rpm. All solvent systems showed a satisfactory phase retention of 50% or greater at 800 rpm, regardless of the choice of the mobile phase or β values. The most important finding is that the hydrophilic and low interfacial tension solvent systems such as *n*-butanol–acetic acid–water (4:1:5) and *sec.*-butanol–water show excellent phase retention ranging from 65 to 80% in the 7.6 cm helical diameter coil ($\beta = 0.5$). These retention figures substantially exceed those obtained from the existing X-axis CPC including types X [1–6], XXL [3–6] and XL [7,8].

On some occasions, the effluent from the coil persistently showed cloudy appearance apparently due to steady carryover of the stationary phase. This phenomenon was more often observed in the solvent system with low interfacial tension, especially when the mobile phase was pumped in a tail-to-head elution mode. This continuous carryover of the stationary phase resulted in an uneven phase distribution in the coil, *i.e.*, gradual depletion of the stationary phase starting at the beginning of the coil. However, such carryover of the stationary phase does not significantly affect the actual separation, if the solute peak is eluted before a considerable loss of the stationary phase takes place. In the present experiments, the retention volume of the stationary phase was measured after the elution volume exceeded the total column capacity so that solutes with $K(\mathrm{Cm/Cs}) \geqslant 1$, if introduced at the beginning of the experiment, would be eluted before the end of the experiment. Here, $K(\mathrm{Cm/Cs})$ is the partition coefficient given by solute concentration in the mobile phase divided by that in the stationary phase.

The majority of the retention diagrams also show that the left-handed coil (thin lines) gives the highest retention levels especially in the 7.6 cm helical diameter coils. The comparison between the upper and lower panels reveals that two particular combinations of the elution mode dominate in each group, *i.e.*, P_I–T–I and P_{II}–H–I in the upper panel and P_{II}–T–O and P_I–H–O in the lower panel (see Table I for symbols), indicating that the inward–outward elution mode plays a more significant role than the head–tail elution mode in the retention of the stationary phase. It is important to note that all the optimum conditions are solely provided by use of left-handed coils (see Table I).

In practical use the multilayer coil column as shown in Fig. 2 (No. 12) usually consists of right-handed and left-handed coils alternating in each layer. In this configuration, efficient separations are obtained only if each coiled layer retains a satisfactory volume of the stationary phase at a given elution mode. However, the present experimental results clearly indicate that left-handed coils generally yield much higher retention levels than right-handed coils. In order to make the best use of this hydrodynamic trend the configuration of the conventional multilayer coil should be modified in such a way that all layers are also exclusively left-handed. This can be either done by connecting each layer with a narrow transfer tube or using a continuous piece of tubing which is directly returned to the starting position after completing each coiled layer. Although this modification may cause minor deformation of the multilayer coil by accommodating the connection flow tube between each layer, it promises a good retention of the stationary phase of viscous polar solvent systems such as butanol systems which tend to produce carryover problems in other types of the CCC apparatus. Because of the excellent phase retention obtained from the most viscous *sec.*-

butanol–water solvent system tested in the present study, the present system may also retain aqueous–aqueous polymer phase systems [9].

As described above, the results of the present studies provide useful information on a new configuration of the multilayer coil column used in XLL coil planet centrifuge. In the following article (Part II) [10], the phase retention data obtained in the present experiments are statistically analyzed to study the unique hydrodynamic mechanism involved in the present X-axis CPC system.

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Cross-axis synchronous flow-through coil planet centrifuge (Type XLL)

II[a]. Speculation on the hydrodynamic mechanism in stationary phase retention

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ABSTRACT

The hydrodynamic mechanism involved in the retention of stationary phase in the present ×-axis coil planet centrifuge system is discussed. A statistical treatment of the retention data disclosed important clues such as the effect of the inward-outward elution mode, the close correlation between the planetary motion and the head–tail elution mode, and the superior retention capacity of the left-handed coils. The combined effects of the radial and lateral centrifugal force field derived from the mathematical analysis of acceleration acting on the coil provide an explanation for the phenomena.

INTRODUCTION

As described in Part I, a series of experiments has been performed to measure the retention of the stationary phase with various two-phase solvent systems using the present cross-axis (X-axis) coil planet centrifuge (CPC). The results obtained from these studies may be summarized as follows:

(1) The phase retention is affected by three major factors, *i.e.*, the direction of the planetary motion, the head–tail and inward–outward elution modes.

(2) With a few exceptions, two particular combinations of these factors yield high percentage retention for each choice of the mobile phase.

(3) The optimum combinations occur almost exclusively with left-handed coils.

In Part II, efforts are made to explain the above hydrodynamic phenomena. A simple statistical method was devised to isolate the hydrodynamic effects produced by each factor, and a set of force distribution diagrams derived for the planetary motion. These results were combined and used to discuss the hydrodynamic mechanism involved in the present X-axis CPC system.

[a] For Part I, see ref. 8.

0021-9673/91/$03.50

TABLE I

RETENTION (%) OF STATIONARY PHASE IN 7.6 cm HELICAL DIAMETER COIL

Mobile phase	*Solvent System**																			
	1 *Hexane* H_2O		*2* *Hexane* *MeOH*		*3* *Hexane 1* *EtOAc 1* *MeOH 1* H_2O *1*		*4* *EtO Ac* H_2O		*5* *EtOAc 4* *AcOH 1* H_2O *4*		*6* $CHCl_3$ H_2O		*7* $CHCl_3$ *2* *AcOH 2* H_2O *1*		*8* *n-BuOH* H_2O		*9* *n-BuOH 4* *AcOH 1* H_2O *5*		*10* *sec.-BuOH* H_2O	
	*Condition***	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*
Upper	P_{II}-T-I	91.4	P_I-T-I	66.5	P_I-T-I	80.7	P_I-T-I	86.8	P_I-T-I	82.7	P_I-T-I	66.0	P_I-T-I	87.3	P_I-T-I	71.1	P_I-T-I	65.5	P_I-T-I	73.6
	P_I-T-I	88.3	P_{II}-H-I	59.4	P_{II}-H-I	55.3	P_{II}-H-I	48.2	P_{II}-H-I	66.0	P_I-T-O	52.3	P_{II}-H-I	51.3	P_{II}-H-I	50.8	P_{II}-H-I	53.8	P_{II}-H-I	54.8
	P_I-T-O	58.4	P_I-H-I	38.1	P_I-T-O	55.3	P_I-H-I	42.1	P_{II}-T-I	45.2	P_{II}-H-I	50.8	P_I-H-I	46.7	P_I-H-I	44.7	P_{II}-T-I	50.3	P_{II}-T-I	48.2
	P_I-H-I	41.1	P_{II}-H-O	25.4	P_{II}-T-I	49.2	P_{II}-H-O	31.5	P_I-H-I	38.1	P_I-H-I	43.1	P_I-T-O	35.5	P_{II}-T-I	38.1	P_I-H-I	47.7	P_I-H-I	47.2
	P_{II}-H-I	35.5	P_{II}-T-I	21.8	P_I-H-I	42.1	P_{II}-T-I	15.7	P_{II}-H-O	25.4	P_I-H-O	39.1	P_{II}-H-O	28.4	P_{II}-H-O	27.9	P_{II}-H-O	29.9	P_{II}-H-O	27.9
	P_{II}-H-O	25.4	P_{II}-T-O	1.1	P_I-T-O	34.5	P_I-H-O	5.1	P_{II}-H-O	9.6	P_I-T-O	35.5	P_{II}-T-O	24.4	P_I-H-O	25.9	P_I-H-O	16.2	P_I-H-O	7.6
	P_I-H-O	18.3	P_I-H-O	1.0	P_{II}-H-O	31.5	P_I-T-O	2.5	P_I-T-O	0	P_{II}-T-I	25.9	P_I-H-O	17.8	P_I-T-O	17.8	P_I-T-O	2.5	P_I-T-O	0
	P_I-T-O	0	P_I-T-O	0	P_I-H-O	29.9	P_I-T-O	0	P_I-T-O	0	P_{II}-H-O	24.9	P_{II}-T-I	10.2	P_I-T-O	1.0	P_I-T-O	0	P_I-T-O	0
Lower	P_I-T-O	86.3	P_{II}-T-O	77.2	P_I-H-O	68.5	P_{II}-H-O	79.2	P_{II}-T-O	78.6	P_{II}-H-O	73.6	P_I-H-O	71.1	P_I-H-O	68.0	P_{II}-T-O	80.2	P_{II}-T-O	77.2
	P_{II}-H-O	69.5	P_I-H-O	66.0	P_{II}-H-O	58.9	P_I-T-O	78.2	P_I-H-O	74.1	P_I-H-O	66.0	P_{II}-H-O	59.9	P_I-T-O	63.5	P_I-H-O	68.5	P_I-H-O	64.5
	P_I-H-O	61.9	P_{II}-H-O	52.8	P_I-H-I	48.7	P_{II}-H-O	52.3	P_{II}-H-O	55.8	P_{II}-T-I	55.8	P_{II}-T-O	48.7	P_{II}-H-O	60.9	P_{II}-H-O	56.3	P_{II}-H-O	54.3
	P_I-H-I	50.8	P_I-H-I	44.2	P_{II}-H-I	39.1	P_I-H-I	32.5	P_I-H-I	28.9	P_I-H-I	53.3	P_I-H-I	40.6	P_I-T-O	45.2	P_I-T-O	53.3	P_I-T-O	44.7
	P_{II}-H-I	48.7	P_I-T-O	12.2	P_I-T-O	27.9	P_I-T-O	30.5	P_I-T-O	21.3	P_I-T-O	45.7	P_{II}-H-I	38.1	P_{II}-H-I	42.6	P_I-H-I	33.0	P_I-H-I	29.9
	P_I-T-O	12.7	P_{II}-T-I	10.7	P_{II}-T-I	15.2	P_{II}-T-I	26.4	P_{II}-T-I	9.1	P_I-T-O	35.5	P_I-T-O	37.6	P_I-H-I	41.6	P_{II}-H-I	18.3	P_{II}-H-I	16.8
	P_{II}-T-I	3.0	P_{II}-H-I	0	P_{II}-T-O	14.2	P_{II}-H-I	17.8	P_{II}-T-I	5.1	P_{II}-H-I	20.8	P_{II}-T-I	35.5	P_{II}-T-I	9.6	P_{II}-T-I	10.2	P_{II}-T-I	10.2
	P_I-T-I	0	P_I-T-I	0	P_I-T-I	0	P_{II}-T-I	2.5	P_{II}-H-I	1.0	P_I-T-I	1.5	P_I-T-I	2.5	P_I-T-I	2.5	P_I-T-I	0	P_I-T-I	1.0

* MeOH = methanol; EtOAc = ethyl acetate; AcOH = acetic acid; BuOH = butanol.

** P_I = Planetary motion  ; P_{II} = Planetary motion  ;
H = Head to tail elution; T = Tail to head elution; I = Inward elution; O = Outward elution
Plain background = Right-handed coil; Shaded background = Left-handed coil.

ANALYSIS OF PHASE RETENTION DATA

A set phase retention data obtained from the coil mounted on the two different holders at 800 rpm are listed in Table I (7.6 cm diameter holder) and II (24 cm diameter holder). Each table shows percentage retention of the stationary phase for ten pairs of two-phase solvent systems with both upper and lower phases used as the mobile phase. Each column contains eight retention data obtained from the indicated solvent system under the experimental conditions expressed by combinations of three factors, *i.e.*, the direction of planetary motion (P_I and P_{II}) (see Table I bottom for definitions), the head–tail elution mode (H: head to tail and T: tail to head), and the direction of elution along the column axis (I: inward and O: outward). These data are arranged from top to bottom in decreasing order of phase retention. The results obtained from the left-handed coils are shaded.

The overall results indicate that all eight experimental conditions yield somewhat different levels of retention in each solvent system and that the data from the left-handed coils (shaded) dominate in both tables. However, the interrelation of these factors is quite complex and difficult to rationalize without additional statistical treatment.

In order to elucidate the hydrodynamic mechanism involved in the present system, it is highly desirable to isolate the effects produced by each individual factor on the retention of the stationary phase. This can be done by considering the three sets of diagrams shown in Figs. 1–3. Each diagram shows the retention with one combination of the three factors plotted on the horizontal axis against another combination with only one factor different on the vertical axis. This is done for each of the three factors, namely, the planetary motions P_I vs P_{II} (Fig. 1); the head-to-tail elution *vs.* tail-to-head elution (Fig. 2); and the inward elution *vs.* outward elution (Fig. 3). Each set of these figures includes a total of four graphs showing the phase retention in both 7.6 cm (A) and 24 cm (B) helical diameter coils each with the upper (left) and the lower (right) phases used as the mobile phase.

Fig. 1A shows a pair of diagrams obtained by plotting the percentage phase retention of P_I *vs.* P_{II} for the lower (left) and the upper (right) phases. For convenience each diagram is divided by three lines; thin horizontal and vertical lines divide the whole area into four equal squares where the upper right square indicates satisfactory retention from both planetary motions; the right lower and upper squares indicate satisfatory retention from P_I and P_{II}, respectively; and the left lower square, unsatisfactory retention regardless of the applied mode of planetary motion. Each diagram is also divided into two equal triangles by a thick diagonal line, which provides the most important information in the present analysis: The data points located on or near the diagonal line indicate that the planetary motion produces no significant effect on the retention. The deviation of the point above the line indicates a positive effect from P_{II}, or negative effect from P_I, or the combination on the phase retention, and *vice versa* if the point is below the diagonal line. The distance of the points from the diagonal line indicates the relative magnitude of the effect.

Each diagram contains 40 data points with specific symbols assigned to four combinations between the remaining two factors, *i.e.*, open circles for the head-to-tail elution and solid circles for the tail-to-head elution each with arrows directing toward the left to indicate outward elution and toward the right to indicate inward elution. All

TABLE II

RETENTION (%) OF STATIONARY PHASE IN 24 cm HELICAL DIAMETER COIL

Mobile phase	*Solvent System**																			
	1 *Hexane* *H_2O*		*2* *Hexane* *MeOH*		*3* *Hexane 1* *EtOAc 1* *MeOH 1* *H_2O 1*		*4* *EtOAc* *H_2O*		*5* *EtOAc 4* *AcOH 1* *H_2O 4*		*6* *$CHCl_3$* *H_2O*		*7* *$CHCl_3$ 2* *AcOH 2* *H_2O 1*		*8* *n-BuOH* *H_2O*		*9* *n-BuOH 4* *AcOH 1* *H_2O 5*		*10* *sec.-BuOH* *H_2O*	
	*Condition***	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*	*Condition*	*%*
Upper	P_I-T-I	96.5	P_{II}-H-I	84.8	P_I-T-I	93.3	P_{II}-H-I	83.0	P_{II}-H-I	70.0	P_I-T-I	95.8	P_I-T-I	87.3	P_I-T-I	84.8	P_{II}-H-I	49.8	P_{II}-H-O	55.5
	P_I-T-O	94.3	P_I-T-I	82.7	P_I-T-O	90.5	P_I-T-I	74.6	P_I-T-I	68.9	P_I-T-O	93.3	P_I-T-O	58.0	P_I-T-O	83.0	P_{II}-H-O	44.5	P_{II}-H-I	48.1
	P_{II}-H-I	52.3	P_{II}-H-O	77.4	P_{II}-H-I	49.8	P_{II}-H-O	71.4	P_{II}-H-O	60.8	P_{II}-H-I	46.3	P_{II}-H-I	48.1	P_{II}-H-O	51.2	P_I-H-I	35.7	P_I-H-I	43.8
	P_{II}-H-O	42.4	P_I-T-O	46.6	P_{II}-H-O	41.0	P_I-T-O	44.5	P_I-T-O	39.6	P_{II}-H-O	38.9	P_{II}-H-O	43.5	P_{II}-H-I	48.8	P_{II}-H-O	30.4	P_I-T-O	31.1
	P_{II}-T-I	30.4	P_I-H-I	24.4	P_{II}-T-I	18.7	P_I-H-I	28.6	P_I-H-I	31.4	P_{II}-T-I	20.5	P_I-H-I	12.7	P_{II}-T-I	21.2	P_I-T-I	22.6	P_{II}-H-O	26.9
	P_I-H-I	1.0	P_I-H-O	6.4	P_I-H-I	12.7	P_I-H-O	10.2	P_I-H-O	15.2	P_I-H-I	4.6	P_{II}-T-I	12.7	P_I-H-I	20.5	P_I-T-O	19.8	P_{II}-T-I	19.8
	P_I-H-O	0	P_{II}-T-I	0	P_I-H-O	6.4	P_{II}-T-I	0	P_{II}-T-I	11.7	P_I-H-O	1.1	P_I-H-O	8.8	P_I-H-O	14.1	P_{II}-T-I	16.3	P_I-T-I	9.9
	P_I-T-O	0	P_{II}-T-O	0	P_{II}-T-O	0	P_{II}-T-O	0	P_{II}-T-O	0	P_{II}-T-O	0	P_{II}-T-O	0	P_{II}-T-O	1.4	P_{II}-T-O	0	P_I-T-O	0
Lower	P_I-H-O	93.6	P_{II}-T-O	83.7	P_I-H-O	86.9	P_{II}-T-O	94.0	P_{II}-T-O	75.3	P_I-H-O	94.7	P_{II}-T-O	87.6	P_I-H-O	73.5	P_I-T-O	64.0	P_{II}-H-O	52.3
	P_{II}-T-O	90.5	P_{II}-T-I	74.6	P_I-H-I	65.7	P_{II}-T-I	72.8	P_I-H-O	61.5	P_{II}-T-O	94.0	P_I-H-O	86.6	P_I-H-I	72.8	P_{II}-T-O	53.4	P_{II}-T-O	50.9
	P_I-H-I	90.5	P_{II}-H-O	68.9	P_{II}-T-O	53.4	P_I-H-O	69.3	P_{II}-T-I	46.6	P_I-H-I	91.2	P_I-H-I	62.2	P_{II}-T-O	58.7	P_I-H-O	47.0	P_I-T-O	48.1
	P_{II}-T-I	45.9	P_I-H-I	46.3	P_{II}-H-O	36.4	P_I-H-I	38.9	P_I-H-I	28.6	P_{II}-H-O	44.5	P_{II}-T-I	38.2	P_{II}-H-O	44.9	P_{II}-H-O	32.2	P_I-H-O	37.1
	P_{II}-H-O	38.9	P_I-T-O	15.5	P_{II}-H-I	32.1	P_I-T-O	24.0	P_I-T-O	25.8	P_{II}-H-I	29.0	P_{II}-H-O	31.1	P_{II}-H-I	32.2	P_{II}-T-I	30.4	P_{II}-H-I	29.0
	P_{II}-H-I	20.1	P_I-T-I	6.4	P_{II}-T-I	18.0	P_I-T-I	10.2	P_I-T-I	13.4	P_{II}-T-I	25.8	P_{II}-H-I	28.6	P_{II}-T-I	28.6	P_{II}-H-I	28.6	P_{II}-T-I	19.8
	P_I-T-O	0	P_{II}-H-O	4.2	P_I-T-O	9.2	P_{II}-H-O	5.6	P_{II}-H-O	12.7	P_I-T-O	6.7	P_I-T-O	9.9	P_I-T-O	16.3	P_{II}-T-I	17.0	P_{II}-T-I	18.4
	P_I-T-I	0	P_{II}-H-I	1.1	P_I-T-I	3.0	P_{II}-H-I	0	P_I-H-I	0	P_I-T-I	3.2	P_I-T-I	3.5	P_I-T-I	8.5	P_I-H-I	14.1	P_I-H-I	15.9

* & ** See caption in TABLE I.

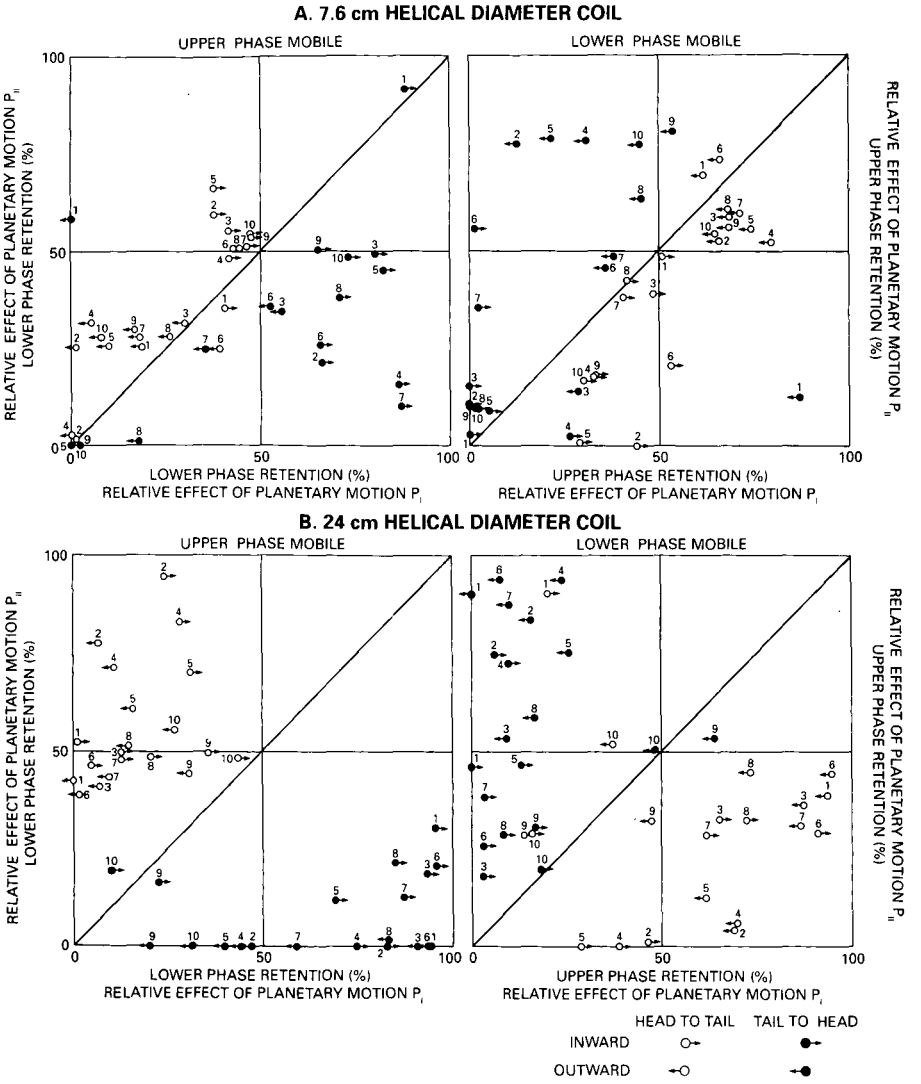


Fig. 1. Effects of planetary motion P_I and P_{II} on the retention of the stationary phase. (A) 7.6 cm helical diameter coil; (B) 24 cm helical diameter coil; left: upper phase mobile; right: lower phase mobile.

these points are also numbered from 1 to 10 to indicate the applied two-phase solvent systems (see Table I for identification of the solvent systems).

Observation on Fig. 1 clearly reveals that the data points are divided into two groups by the diagonal line. With a few exceptions, the open circles are distributed above the diagonal line and the solid circles below the line if the upper phase is mobile (left), this relationship is reversed if the lower phase is mobile (right). This indicates that the retention of the lower phase is enhanced by the combination of either planetary motion P_{II} and the head-to-tail elution (P_{II}-H) or planetary motion P_I and the

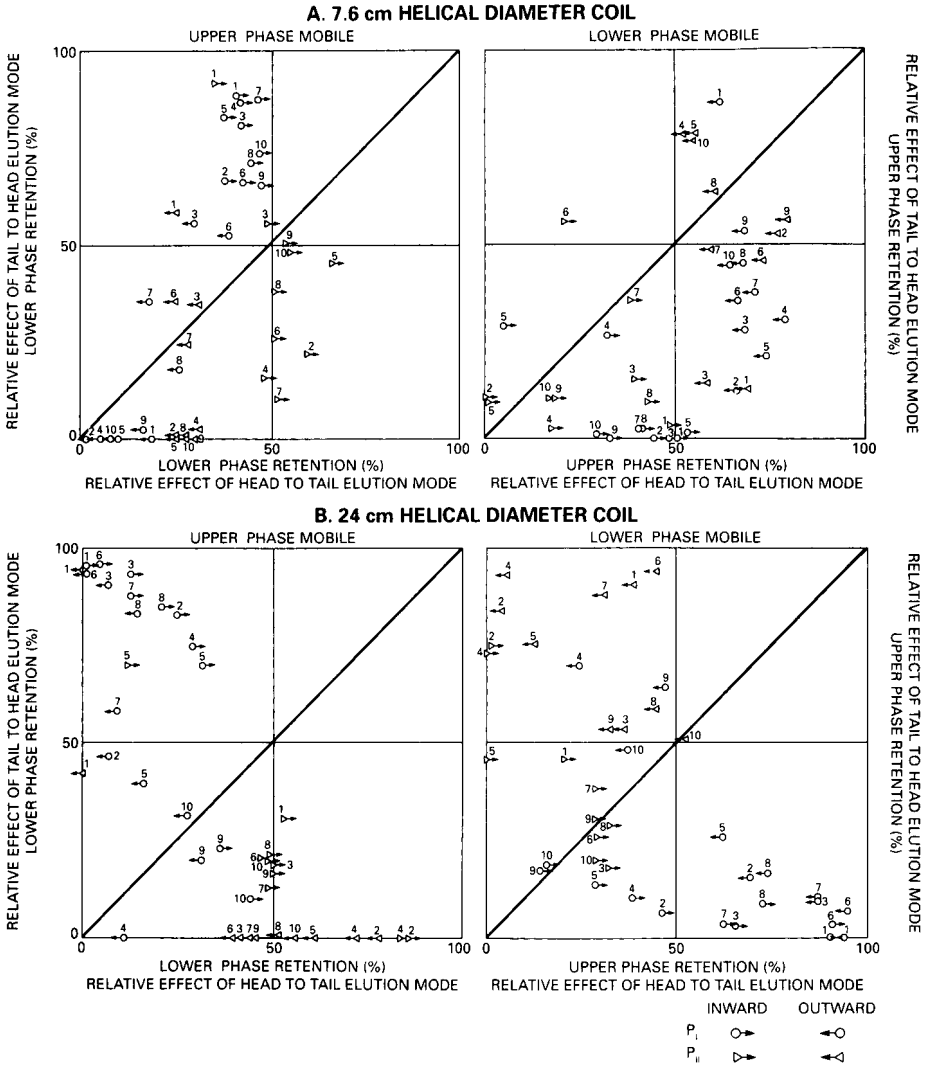


Fig. 2. Effects of the head–tail elution mode on the retention of the stationary phase. (A) 7.6 cm helical diameter coil; (B) 24 cm helical diameter coil; left: upper phase mobile; right: lower phase mobile.

tail-to-head elution (P_I-T) whereas the retention of the upper phase is similarly enhanced by the combination of either planetary motion P_I and the head-to-tail elution (P_I-H) or planetary motion P_{II} and the tail-to-head elution (P_{II}-T). In either case, the retention tends to increase in the larger diameter coil as evidenced by the greater deviation of each point from the diagonal line in the 24 cm helical diameter coil. These results indicate a close correlation between the planetary motion and the head-tail elution mode in the stationary phase retention while no significant correlation is observed between the planetary motion and the inward-outward elution mode.

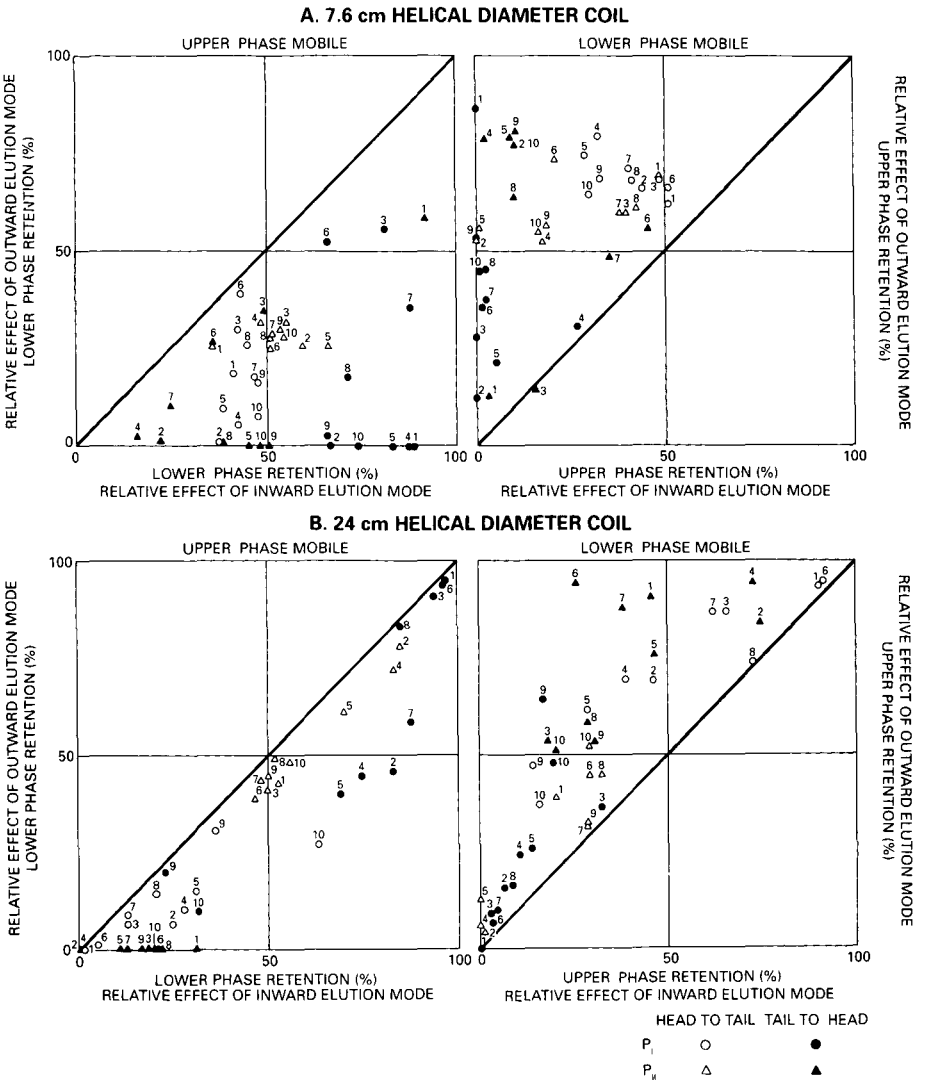


Fig. 3. Effects of the inward–outward elution mode on the retention of the stationary phase. (A) 7.6 cm helical diameter coil; (B) 24 cm helical diameter coil; left: upper phase mobile; right: lower phase mobile.

However, there are some exceptions; the correlation between the planetary motion and the head-tail elution mode is relatively weak among the viscous low interfacial tension butanol solvent systems (9 and 10) as indicated by location of these points near the diagonal line in the 24 cm helical diameter coil. Also, the high interfacial tension hexane–water system (1) shows an aberrant retention pattern in 7.6 cm helical diameter coil.

Figs. 2A and B similarly illustrate the effects of the head-tail elution mode on the stationary phase retention where the 40 data points are plotted with symbols assigned to four different combinations of the planetary motion and the inward-outward

elution mode as indicated at the bottom of the diagrams. These points are also numbered to facilitate identification of the applied two-phase solvent systems (see Table I).

In both 7.6 and 24 cm helical diameter coils combinations P_I-I (open circles with arrows directing toward the right) are mostly located in the left upper squares for the lower phase retention (left) while combinations P_{II}-O (open circles with arrows directing toward the left) dominate the right lower square for the upper phase retention (right). Here again the close correlation between the planetary motion and the head–tail elution mode is confirmed. The deviation of the points from the diagonal is also enhanced in the large helical diameter coils. In the 24 cm helical diameter coil (Fig. 2B right), combinations P_{II}-O (open triangles with arrows directing toward the left) also dominate in the left upper square indicating that the retention of the upper phase is remarkably improved by the combination of P_{II} and the outward elution in the large helical diameter coil.

Finally, Figs. 3A and B illustrate the effects of the inward–outward elution mode on the stationary phase retention where the 40 data points are similarly plotted with symbols corresponding to the four different combinations as specified at the bottom of the diagrams. As in other diagrams, all points are numbered to facilitate identification of the two-phase solvent systems used.

In contrast with other diagrams (Figs. 1 and 2), all the present diagrams show unilateral distribution of the data points with respect to the diagonal line. Except for a single data point, all points are located below the diagonal line if the upper phase is mobile (left) and above the diagonal line if the lower phase is mobile (right), indicating that eluting either the upper phase inward or the lower phase outward enhances the stationary phase retention under any combination with other factors in both 7.6 and 24 cm helical diameter coils. The deviation of each data point from the diagonal line is much greater in the 7.6 cm helical diameter coil, suggesting that this tendency is enhanced in the small helical diameter coils.

The overall results of the above analyses indicate that all three factors play important roles in the retention of the stationary phase in the present X-axis CPC. The hydrodynamic effects of the planetary motion and the head-tail elution mode are closely interrelated in such a way that the high retention levels are obtained by choosing the particular combinations of these two factors, *i.e.*, P_I-T or P_{II}-H for the upper phase mobile and the P_{II}-T or P_I-H for the lower phase mobile. On the other hand, the third factor of the inward–outward elution mode is rather independent of the above two factors and exclusively related to the choice of the mobile phase: the high percentage retention was achieved by eluting either the upper phase inward or the lower phase outward through the coil.

Consequently, the highest retention levels are generally provided from two combinations (among the eight possible combinations) for each choice of the mobile phase: P_I-T-I and P_{II}-H-I for the upper mobile phase and P_{II}-T-O and P_I-H-O for the lower mobile phase. As shown in Table I, these four combinations are exclusively provided by the use of left-handed coils.

FORCE DISTRIBUTION DIAGRAMS

Fig. 4 shows the orientation and motion of the coil holder in the present X-axis

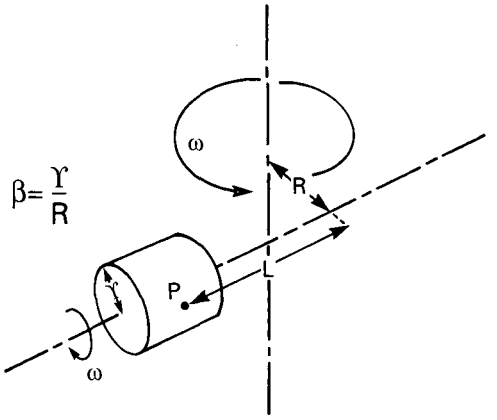


Fig. 4. Planetary motion of the coil holder in the present X-axis CPC.

CPC system. Analysis of acceleration acting on an arbitrary point, P, on the holder was previously performed by the aid of a three-dimensional coordinate system [1,2]. The acceleration acting on the holder was finally transferred to the x_b–y_b–z_b body coordinate system and expressed in the following equations:

$$\alpha_{xb} = -R\omega^2 (1 - 2\beta \cos \theta) \quad (1)$$

$$\alpha_{yb} = -R\omega^2 \beta \sin \theta \quad (2)$$

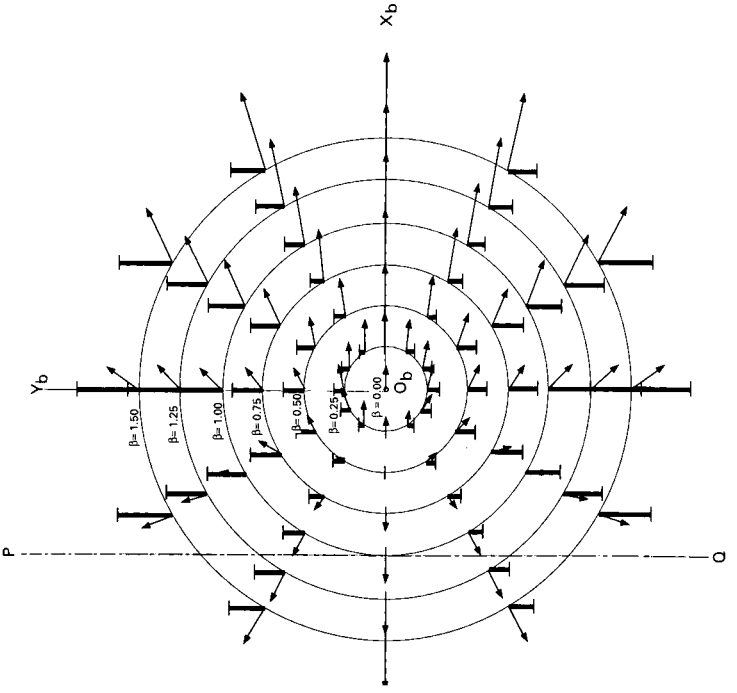
$$\alpha_{zb} = -R\omega^2 2\beta \sin \theta + L\omega^2 \quad (3)$$

where α_{xb}, α_{yb}, and α_{zb} are acceleration components acting along the indicated coordinates; R is the radius of revolution; ω is the angular velocity ($\theta = \omega t$); $\beta = r/R$ where r is the distance from the holder axis to the coil; and L is the lateral disposition of the coil expressed by the distance from the center of the holder shaft to the coil holder.

Using these equations, the centrifugal force vectors (same magnitude as the acceleration but acting in the opposite direction) at various locations on the holder were computed for three types to planetary motions, *i.e.*, type X ($L = 0$), type XL ($-L = R$), and type XLL ($-L = 2R$) and diagrammatically illustrated in Fig. 5A–C. In these force distribution diagrams, the three-dimensional distribution of the centrifugal force vectors are expressed on a two-dimensional diagram by combining the two force vectors, $-\alpha_{xb}$ and $-\alpha_{yb}$, into a single arrow forming various angles from the x_b-axis, whereas the third force vector, $-\alpha_{zb}$, which acts perpendicularly to the x_b–y_b plane, is drawn as a vertical column along the y_b-axis. The ascending column indicates the force acting upward ($z_b > 0$) and the descending column, the force acting downward ($z_b < 0$). Several concentric circles around point O_b (the axis of the holder) indicate locations on the holder corresponding to parameter β indicated in the diagram. The distribution of the centrifugal force vectors in each diagram is fixed to the x_b–y_b–z_b body coordinate system and every point on the holder rotates around point O_b (z_b-axis) in either clockwise or counterclockwise direction as determined by the planetary motion of the holder.

In these diagrams, the radially directed centrifugal force vectors (expressed by arrows) display an asymmetrical distribution pattern along the x_b-axis which is

A. FORCE DISTRIBUTION AT L = 0 (TYPE X)



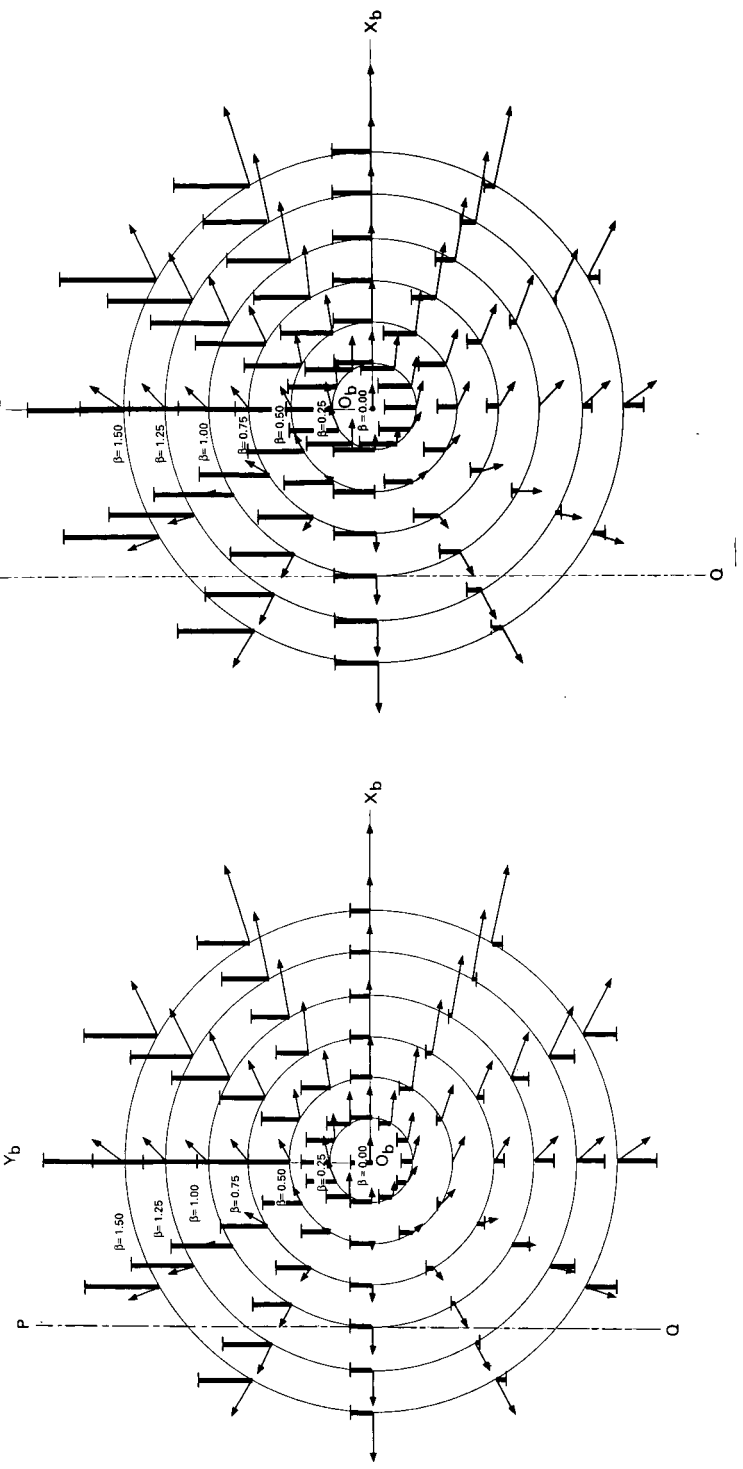


Fig. 5. Force distribution diagrams of the X-axis CPC. (A) Type X; (B) type XL; (C) type XLL. PQ: Axis of revolution (centrifuge axis).

unaltered by the change of the L values. The vertically acting centrifugal force vectors (expressed by columns), on the other hand, show a symmetrical distribution pattern at $L = 0$ (Fig. 5A), but the pattern changes according to the L values. At $-L = R$ (Fig. 5B), the force vectors acting upward on the upper half of the holder gain their magnitude whereas those acting downward at the lower half of the holder lose their strength, resulting in an asymmetrical distribution of the force vector along the z_b-axis. The degree of the asymmetry becomes greater at $-L = 2R$(Fig. 5C) representing the present XLL system.

These force distribution diagrams may serve as a useful guide for discussion of the hydrodynamic effects occurring in the present X-axis CPC system.

SPECULATION OF HYDRODYNAMIC MECHANISM

The analysis of acceleration described above indicated that the centrifugal force field acting on the rotating holder is divided into two groups; the radial force field expressed by arrows and the lateral (tangential) force field expresed by vertical columns. For convenience, the following discussion may be divided into two parts, *i.e.*, the effects of the individual force fields and the combined effects of the two on the retention of the stationary phase.

The effects of the radial force field on the retention of the stationary phase have been extensively studied with the type J synchronous coil planet centrifuge which produces a similar asymmetric radial centrifugal force field without lateral components [3–7]. A series of studies has shown that two immiscible solvent phases are unilaterally distributed in the coil according to the physical properties of the solvent system. Hydrophobic binary solvent systems such as *n*-hexane–water and chloroform–water always distribute the upper phase toward the head side of the coil whereas the hydrophilic low-interfacial-tension solvent systems such as *n*-butanol–acetic acid–water (4:1:5) and *sec.*-butanol–water show the opposite hydrodynamic trend, distributing the lower phase toward the head side of the coil. In other solvent systems with intermediate physical properties, the head phase is determined by the β values of the coil; at large β values the upper phase is distributed to the head side while in small β values the lower phase is distributed on the head side. In the X-axis CPC, the strength of the radial force field is reduced by separation of the Coriolis force which is directed laterally along the axis of the holder.

On the other hand, the individual hydrodynamic effects produced by the lateral force field have not been well investigated. However, it is apparent that with the lateral coil position such as in the present X-axis CPC system the centrifugal force gradient formed along the holder axis may facilitate the retention of the stationary phase if the heavier phase is eluted outward (toward the left) or the lighter phase inward (toward the right) as observed in the present studies (see Fig. 3A and B). Consequently, in the conventional multilayer coil configuration, every other layer can hold a greater volume of the stationary phase in a given elution mode. However, in order to understand the superior retention capacity of the left-handed coil to the right-handed coil (see Tables I and II), one must consider the complex hydrodynamic interaction between the radial and lateral centrifugal force fields.

Fig. 6 schematically illustrates the hydrodynamic interaction between the radial and lateral force fields acting on the coils undergoing planetary motions P_1 (left) and

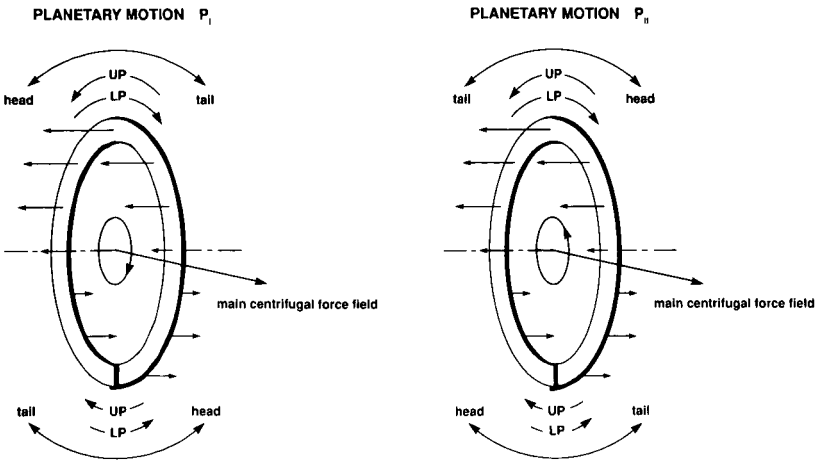


Fig. 6. Hydrodynamic effect of the radial and lateral centrifugal force fields in the present X-axis CPC. UP = Upper phase; LP = lower phase.

P_{II} (right) [2]. Because the directions of rotation and revolution are simultaneously reversed, these two planetary motions produce the identical centrifugal force field whereas reversed rotation of the coil holder causes reversal of the head-tail orientation of the coil. Under the main centrifugal force field directing radially toward the right as indicated by a large arrow, the upper (lighter) phase is driven toward the left and the lower (heavier) phase toward the right in major portions of the coil.

In Fig. 6 (left), planetary motion P_I determines the coil rotation, hence the head-tail orientation of the coil as indicated by a pair of curved arrows at the top and the bottom of the diagram. Owing to the asymmetric lateral force field between the

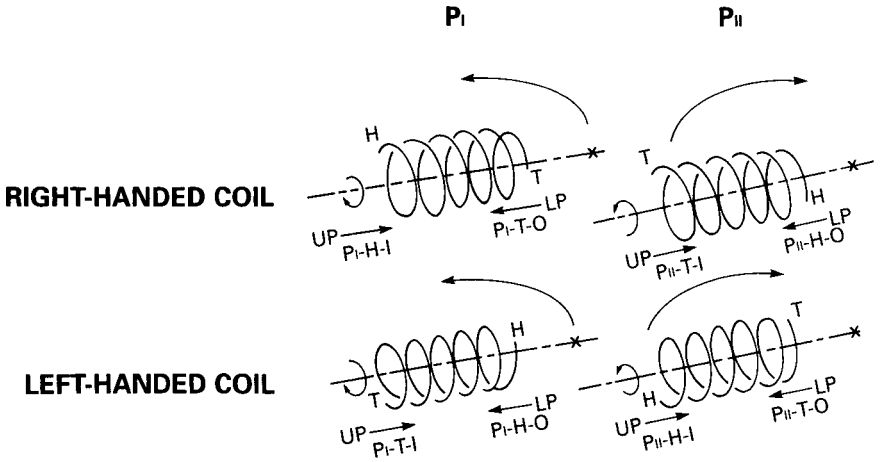


Fig. 7. Relationship between the planetary motion, handedness and head–tail orientation of the coil. H = Head; T = tail; UP = upper phase; LP = lower phase. I = inward elution; O = outward elution; × = the center of the rotary shaft.

upper and lower halves of the coil, the counter-current movement of the two solvent phases is accelerated in the upper portion of the coil owing to suppressed emulsification while the movement is decelerated in the lower portion of the coil owing to enhanced emulsification. Consequently, in this situation the tail-to-head elution (P_I-T) of the upper phase and the head-to-tail elution (P_I-H) of the lower phase results in enhanced retention of the stationary phase.

In Fig. 6 (right), planetary motion P_{II} reverses both the rotation and the head-tail orientation of the coil as illustrated. Owing to the asymmetric lateral force field left unaltered, the counter-current movement of the two solvent phases is similarly accelerated on the upper portion of the coil and decelerated in the lower portion of the coil. Therefore, in this case the head-to-tail elution (P_{II}-H) of the upper phase and the tail-to-head elution (P_{II}-T) of the lower phase result in enhanced retention of the stationary phase.

The above hydrodynamic mechanism provides reasonable explanation for the close correlation between the planetary motion and the head-tail elution mode observed in the present studies (Figs. 1 and 2). The radial force field may also play an additional role in stationary phase retention. For example, in the 7.6 cm helical diameter coil, the best retention of the lower phase is obtained by P_I-T-I, probably due to the effect of the radial force field which distributes the upper phase toward the head of the coil. An atypical retention pattern of hexane–water in the 7.6 cm helical diameter coil may indicate a possible inhibitory effect of the lateral force field on the counter-current flow of particular two-phase solvent systems with extremely high interfacial tension.

Fig. 7 shows the relationship between the planetary motion, handedness and head–tail orientation of the coils undergoing the XLL planetary motion as indicated by curved arrows. As illustrated by four different diagrams, the head-tail orientation of the coil is determined by the combination of handedness and planetary motion. Since the stationary phase retention is enhanced by eluting either the upper phase inward or the lower phase outward through the coil as indicated by straight arrows, the desirable combinations of P_I-T-I and P_{II}-H-I for the lower phase retention and those of P_I-H-O and P_{II}-T-O for the upper phase retention are all available only from the left-handed coils.

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Note

Improved high-speed counter-current chromatograph with three multilayer coils connected in series

IV. Evaluation of preparative capability with large multilayer coils

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As previously reported [1–3], an improved design of the centrifuge for high-speed counter-current chromatography (CCC) has greatly increased its partition efficiency in separations where the sample size ranges from micrograms to several hundred milligrams. In order to further extend the preparative capability of the apparatus, we have constructed a new centrifuge which can hold much longer multilayer coils for the separation of multigram quantities of samples. The performance of the apparatus has been successfully demonstrated in the separation of dinitrophenyl (DNP) amino acids using a two-phase solvent system composed of chloroform–acetic acid–0.1 *M* hydrochloric acid at a volume ratio of 2:2:1. The partition efficiencies obtained from the present apparatus are compared with those from the semianalytical and semipreparative columns previously reported.

EXPERIMENTAL

Apparatus

The design of the apparatus is almost identical to that of the original model [1] except that the revolutional radius is increased from 7.5 cm to 10 cm and the width of the column holder spool from 5 cm to 11 cm. Fig. 1 shows the photograph of the present high-speed CCC centrifuge equipped with a set of three large mutilayer coils around the rotary frame. Each multilayer coil consists of about 100 m of 2.6 mm I.D. PTFE (polytetrafluoroethylene) tubing (Zeus, Raritan, NJ, U.S.A.) forming seven layers of coil between a pair of flanges spaced 11 cm apart. Three columns are serially

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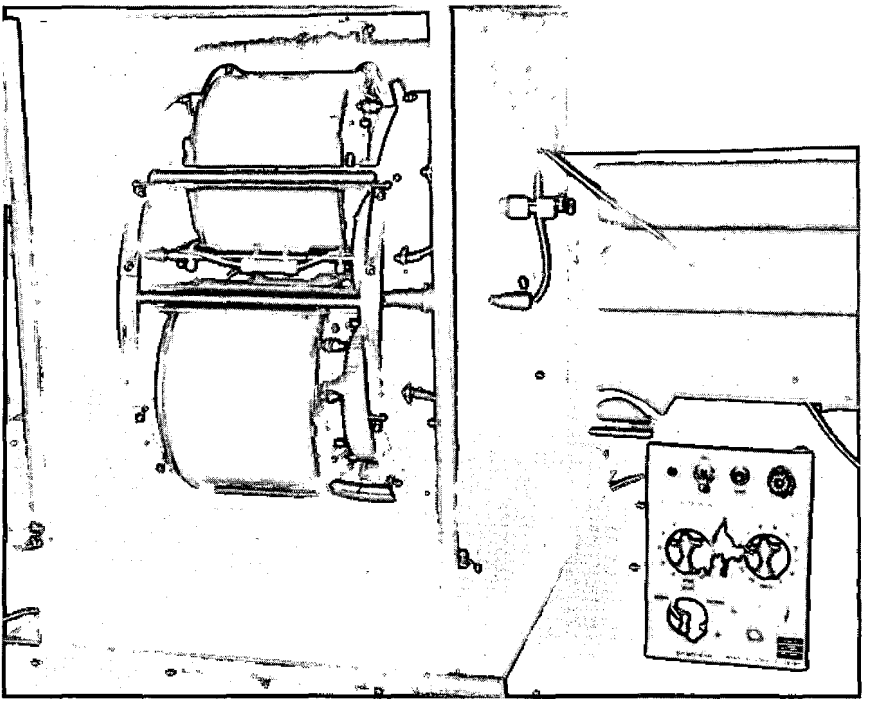

Fig. 1. Photograph of the apparatus.

connected with flow tubes to make up a total capacity of 1.6 l. The apparatus can be operated up to 1000 rpm with a speed controller (Bodine, Chicago, IL, U.S.A.).

Reagents

In the present study employed were chloroform and methanol of glass-distilled chromatographic grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), reagent-grade glacial acetic acid (Mallinckrodt, Paris, KY, U.S.A.), 1 *M* hydrochloric acid (Sigma, St. Louis, MO, U.S.A.) and DNP-amino acid samples (Sigma) including N-2,4-DNP-D,L-glutamic acid (DNP-glu), N,N-di(2,4-DNP)-L-cystine (diDNP-$(cys)_2$), N-2,4-DNP-L-alanine (DNP-ala) and N-2,4-DNP-L-valine (DNP-val).

Preparation of two-phase solvent system and sample solution

The two-phase solvent system used in the present study was composed of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel by repeated shaking and degassing, and the two phases were separated shortly before being applied to the column.

The sample solution was prepared by dissolving 1 g each of DNP-val and DNP-ala, 0.2 g of diDNP-$(cys)_2$ and 2 g of DNP-glu (total weight 4.2 g) in 80 ml of the stationary upper aqueous phase.

Separation procedure

The DNP-amino acid separation was performed in the following manner: The entire column was first completely filled with the upper aqueous stationary phase; this was followed by sample injection through the sample port. The column was then rotated at the optimum speed of 740 rpm while the lower non-aqueous mobile phase was pumped into the column at a flow-rate of 7 ml/min in a head-to-tail elution mode. Here, the head-tail relationship of the rotating coil refers to an Archimedean screw force which drives all objects in the coil competitively toward the head of the coil. Effluent from the outlet of the column was monitored by absorbance at 275 nm with a UV monitor (Uvicord S; LKB, Uppsala, Sweden) and then fractionated with a fraction collector (Ultrorac, LKB). After all peaks were eluted, the apparatus was stopped and the column inlet was connected to a pressured nitrogen line (80 p.s.i.) to collect the column contents into a graduated cylinder. The volume of the stationary phase retained in the column was determined to compute the percent retention relative to the total column capacity. The column was slowly rotated in the tail-to-head elution mode to accelerate the collection of the column contents. Finally, the column was washed with about 200 ml of methanol and then dried with nitrogen.

Analyses of CCC fractions

A 20-μl aliquot of each fraction was mixed with 3 ml of methanol and the absorbance wss determined with a Zeiss PM 6 spectrophotometer at 430 nm.

RESULTS AND DISCUSSION

The performance of the present preparative high-speed CCC centrifuge was evaluated by separation of a standard set of DNP-amino acid samples. Fig. 2 shows a typical chromatogram obtained from 4 g of a mixture of DNP-amino acids using a two-phase solvent system composed of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v). The separation was performed at a flow-rate of 7 ml/min using the lower non-aqueous phase as the mobile phase at a revolution speed of 740 rpm. The solvent front emerged in 75 min followed by substantial amounts of carryover of the stationary phase. Retention of the stationary phase measured 46% and the maximum pressure at the outlet of the pump was 70 p.s.i. All four components were well resolved and eluted within 8 h. The skewed peak of DNP-glu was apparently caused by a non-linear isotherm due to the high solute concentration in the sample solution.

The partition efficiencies of the separation can be computed from the chromatographic chart according to the conventional gas chromatographic formula

$$N = (4R/W)^2 \tag{1}$$

where N denotes the partition efficiency expressed in terms of theoretical plate number (TP); R the retention time or volume of the peak maximum; and W the peak width expressed in the same unit as R. The results give high partition efficiencies ranging from 1800 TP for the first peak to 1000 TP for the fourth peak. However, the absolute TP alone cannot be directly used for evaluating the performance of the apparatus since other parameters such as a length of the column, separation time, number of helical turns, etc. should also be considered. More useful expressions of the partition

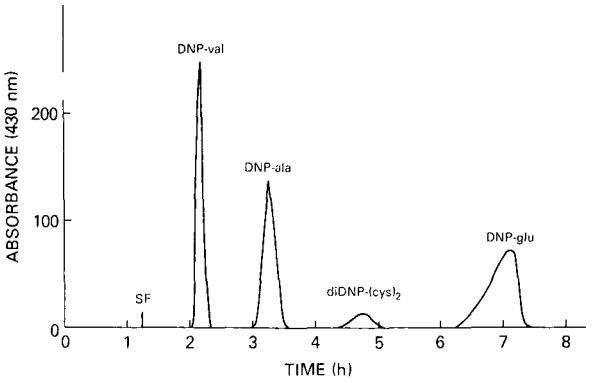

Fig. 2. Chromatogram of DNP-amino acids obtained with the present apparatus. SF = Solvent front. The experimental conditions were as follows: high-speed CCC coil planet centrifuge with 10 cm revolution radius; column, 3 multilayer coils, 2.6 mm I.D. and 1.6 l capacity with $\beta = 0.5$–0.8; β is a ratio 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); solvent system, chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v); mobile phase, lower non-aqueous phase; flow-rate, 7 ml/min; sample size, 4.2 g; revolution, 740 rpm; retention, 46%; pressure, 70 p.s.i.

efficiency, which incorporate these parameters, include s/TP (time required for one TP), cm/TP (a length of the column required for one TP) and TP/turn (the average TP value obtained from one helical turn of the column). Among these, s/TP is computed from the chromatogram by dividing the retention time (s) of the solvent front with the TP value of each peak.

Table I lists various partition efficiency values obtained from the present preparative column (2.6 mm I.D.) together with those similarly obtained from the semi-preparative (1.6 mm I.D.) and semi-analytical (1.07 mm I.D.) columns as previously reported in part I [1] and part III [3]. In the present preparative column, one TP is produced by a 20.5 cm length of tubing in every 3.5 s, yielding an average of 2.1 TP in each helical turn. As shown in the table, these figures are somewhat lower than those obtained from the smaller I.D. columns. The ratios in partition efficiencies between the preparative and smaller columns range from 1/2 to 1/6 in s/TP and 1/1.2 to 1/1.5 in cm/TP. The value for TP/turn for the preparative column is slightly improved over that in the semi-preparative column, apparently due to the use of a larger diameter

TABLE I

PERFORMANCE OF THREE DIFFERENT MULTILAYER COILS IN DNP-AMINO ACID SEPARATION

Multilayer coils (set of three)					Sample size	Partition efficiencies			
Type	I.D. (mm)	Length (m)	Capacity (ml)	No. of turns	(mg)	TP	s/TP	cm/TP	TP/turn
Preparative	2.6	300	1600	670	4200	1400	3.5	20.5	2.1
Semi-preparative	1.6	200	400	600	250	1125	1.7	17.8	1.9
Semi-analytical	1.07	300	270	1200	10	4000	0.6	13.3	3.3

holder spool in the preparative column which increased the length of tube per turn. However, the loss of partition efficiency in the present preparative column over the smaller I.D. columns discussed above is quite acceptable if one considers an increase in sample size from 10 mg and 250 mg to 4.2 g in the preparative column which amounts over 16 to 400 times scale up of the sample loading capacity.

CONCLUSION

The overall experimental results clearly demonstrate an excellent preparative capability of the above design. A compact bench-top model (1.5 ft. × 1.5 ft. × 1.0 ft.) of the apparatus performs multigram separation of samples at a high partition efficiency of over 1000 TP in 8 h. The present method should be useful in preparative separations of various natural and synthetic products on a laboratory scale.

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Note

Laser-light-scattering detection for high-speed counter-current chromatography

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High-speed counter-current chromatography (HSCCC) is a powerful separation technique increasingly used in natural products chemistry [1]. Analytical HSCCC instrumentation has recently become available [2] and applications in natural products chemistry have been reviewed [3]. Several types of detectors have been coupled to analytical HSCCC instruments including UV [4], IR [5] and mass spectrometric (MS) [6] detectors. These systems can provide important structural information about solutes, yet their application to HSCCC is limited by the complexity of instrumentation (MS), high noise levels (UV) or sensitivity (IR).

The use of light-scattering has been suggested as an alternative detection principle in liquid chromatography [7]. Evaporative laser light-scattering detection (ELSD) represents a universal detection method based on the following principle: the eluate [*e.g.* from a high-performance liquid chromatographic (HPLC) column] is nebulized and vaporized, leaving fine particles of solute in a carrier gas stream. These particles pass through a laser beam, scatter the light, and the scattered light is detected. The response is a function of the mass of solute passing through the detector. ELSD has proven particularly useful for carbohydrate and lipid analyses by HPLC [8,9].

In this paper we report the coupling of an ELSD system with analytical HSCCC.

EXPERIMENTAL

Solvents were HPLC grade (Burdick & Jackson) and water was purified with a Milli-Q system (Millipore). Reference compounds were purchased from C. Roth Inc. (Karlsruhe, F.R.G.). The "micro" high-speed counter-current chromatograph with a 30-ml coil volume and 0.85-mm I.D. PTFE tubing was obtained from P.C. Inc. (Potomac, MD, U.S.A.); solvents were pumped with a Milton Roy laboratory pump;

the ELSD system was obtained from Varex (Burtonsville, MD, U.S.A.); chromatograms were recorded on a Varian Model 9176 chart recorder (1 mV full scale).

The HSCCC coil was first filled with stationary phase, then rotation was started while stationary phase was still being pumped. As 1800 rpm was reached, the mobile phase was introduced through the head inlet. When coil equilibrium was reached (effluent changed from displaced stationary phase to mobile phase), the flow-rate was adjusted to 0.8 ml/min. Next, the coil outlet was connected to the light-scattering detector. Complete evaporation of solvent was indicated by a smooth baseline. Compounds **1–4** (see Fig. 1) were dissolved in the stationary phase and the sample solution was injected (time 0 in Fig. 1) by means of a Valco six-port valve and a 0.25-ml sample loop. Quantities injected were 300 μg (**1**), 10 μg (**2**), 35 μg (**3**) and 20 μg (**4**). Back-pressure was below 200 p.s.i., and a stationary phase retention of 90% was determined after the run. The detector was preheated for 20 min; the time constant was 1 s. A back-pressure of 32 p.s.i. nitrogen carrier gas was the result of an arbitrary flow setting of 8 on the detector.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a mixture of standard compounds **1–4** [4] by analytical HSCCC. The separation was carried out with the solvent system chloroform–methanol–water (13:7:8) where the lower phase was used as the mobile phase at a flow-rate of 0.8 ml/min. The coil effluent was directly led into the nebulizer compartment of the ELSD system. Nebulizer and exhaust temperatures were set at 110 and 80°C, respectively. This relatively high temperature setting was selected in order to evaporate small amounts of water in the mobile phase and possible droplets of non-retained (aqueous) stationary phase. Carry-over of aqueous stationary phase did not affect the chromatogram. A strong detector response was observed for compounds **2–4** (melting points over 200°C) at 10–35 μg per compound per injection. Compound **1** on the other hand induced a relatively weak detector signal, even at amounts of over 100 μg per injection. A possible explanation for this is that herniarin (**1**) which melts at 117°C, passes through the detector beam as liquid droplets from which incident laser light is not effectively scattered. Signal-to-noise ratios were better than obtained with a photodiode array detector when the same sample mixture was separated [4]. The chromatogram in Fig. 1 shows a smooth baseline, whereas stronger and unexplained detector noise was observed during peak elution.

ELSD is a sensitive and non-specific mass detection method with detection limits in the upper nanogram range [7,8]. However, substances with melting points equal to or smaller than the nebulizer temperature may be difficult to detect, as illustrated in the present application. This has to be taken into consideration if complex samples such as plant extracts are analyzed. Since ELSD is a destructive technique, a flow splitter installed in the eluate line would be needed in order to use this detection method in preparative HSCCC. Detector noise due to carry-over of stationary phase, typical of UV detectors, is eliminated. From our preliminary results it appears that ELSD is an ideal, easy-to-use detection method for analytical HSCCC. Further evaluations using non-UV-absorbing compounds and solvents with high UV cut-off are currently in progress in our laboratory.

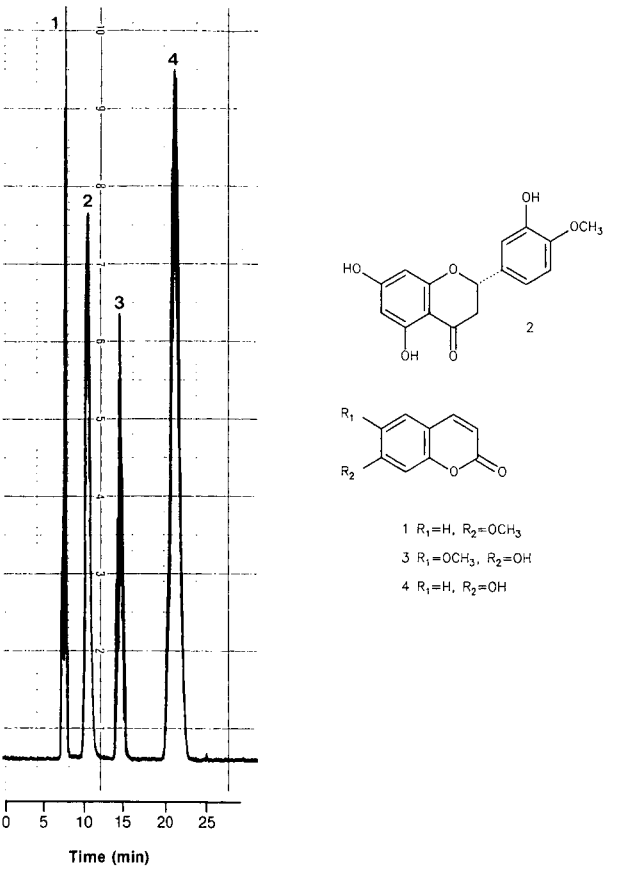

Fig. 1. Analytical HSCCC-ELSD separation of herniarin (**1**), hesperetin (**2**), scopoletin (**3**) and umbelliferone (**4**). Conditions: multilayer coil planet centrifuge (30 ml; 0.85 mm I.D.); solvent, chloroform–methanol–water (13:7:8) with the lower phase as the mobile phase at a flow-rate of 0.8 ml/min.

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CHROM. 22 722

Improvement of on-line detection in high-speed counter-current chromatography: UV absorptiometry and evaporative light-scattering detection

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ABSTRACT

Improvements in analytical high-speed counter-current chromatography involve reduction of the column volume and a high revolution speed in order to decrease the time of analysis. Continuous detection should be performed instead of laborious fraction collection before reading the UV absorbance of the fractions. In this work on-line UV absorptiometry was performed by adding a "make-up" solvent to the column effluent in order to obtain a homogeneous medium before the detection is carried out. The first attempt to use an evaporative light-scattering detector is discussed.

INTRODUCTION

Recently, counter-current chromatography has been radically improved because high-speed centrifugal apparatus has been developed [1–10] in order to increase efficiency and to reduce the separation time. This involved reduction of the column volume by using 0.85 mm I.D. tubing and increasing the revolution speed to 2000–4000 rpm to sustain the stationary phase in such a small diameter column [9,10]. Thus, high-speed countercurrent chromatography (HSCCC) can perform analytical-scale separations with speeds and resolutions approaching those achieved by high-performance liquid chromatography (HPLC) [3].

In order to become truly efficient, continuous detection has to be achieved on-line with HSCCC. Thermospray mass spectrometry [11,12], Fourier transform IR [13] and UV detection have already been used to monitor the column effluent. In a general way, UV detection is performed after laborious collection of fractions because problems can occur in direct UV detection, as has already been described and classified into four categories by Oka and Ito [14]: (1) carryover of the stationary phase due to improper choice of operating conditions or (2) to overloading of the sample, vibrations or fluctuations of the revolution speed; (3) turbidity of the mobile phase due to the difference in temperature between the column and the detection cell; and (4) gas bubbling after reduction of the effluent pressure. Some of these problems can be solved by optimization of the operating conditions, control of the temperature of the mobile

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phase [9] and addition of some length of capillary tubing after the detector to prevent bubble formation. Unfortunately, in case of stationary phase carryover (2% per hour in centrifugal partition chromatography (CPC) [15]), detection problems may still occur; these can be solved by adding to the column effluent prior detection a solvent which is miscible with both the stationary and mobile phases.

This paper also describes an attempt to use evaporative light-scattering detection (ELSD) on-line with HSCCC because this detector is known to permit evaporation of the mobile phase prior to detection in HPLC [16–18] or in supercritical fluid chromatography [16–20]. After a suitable evaporation step, in the worst case of segmented or emulsified mobile phase the column effluent should always be an aerosol of the solutes before reaching the detection cell.

PRINCIPLE OF THE ELSD

A thorough evaluation of the theory has been given in several papers [21–23]. ELSD involves atomization of the column effluent into a gas stream via a Venturi nebulizer, evaporation of the solvents by passing it through a heated tube to yield an aerosol of non-volatile solutes and finally measurement of the intensity of light scattered by the aerosol. The processes by which the path of electromagnetic radiation can change direction when passing through a medium containing a suspended particulate phase are related to the size of the particles, which can be varied in HPLC by altering parameters such as the density, viscosity, surface tension and velocity of the mobile phase. The radius of the particles also depends on solute concentration and temperature during evaporation.

EXPERIMENTAL

Apparatus

The HSCCC apparatus (Fig. 1) consisted of two Shimadzu Model LC 5 A reciprocating HPLC pumps (Touzart et Matignon, Vitry sur Seine, France) and a Constametric II G (Milton-Roy, Villepinte, France) for pumping the organic and the aqueous phases, respectively.

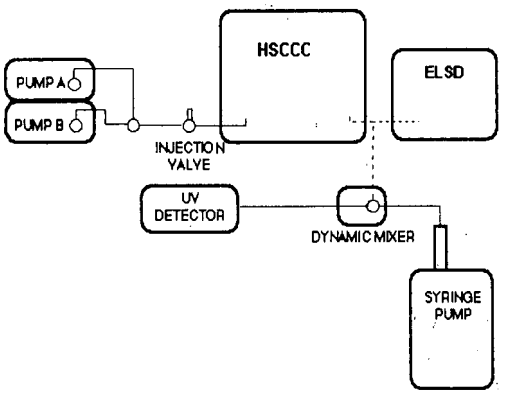

Fig. 1. Schematic diagram of the HSCCC apparatus.

The pumps were connected to a Model CPHV 2000 HSCCC system (SFCC, Neuilly-Plaisance, France) equipped with three identical and independent columns symmetrically arranged around the central axis of the centrifuge. Each column was prepared from 93.5 m × 0.80 mm I.D. PTFE tubing wound onto a holder to give a capacity of 47 ml. The columns underwent synchronous planetary motion and revolved around their own axis at the same angular velocity as the revolution around the central axis, avoiding twisting of the column flow tubes. The maximum speed attainable was 2000 rpm. The β value (ratio of the rotational radius to the revolution radius) ranged from 0.32 at the internal terminal to 0.7 at the external terminal. The separation utilized only one column connected to the pump using 0.8 mm I.D. PTFE tubing. The other two columns were only used to counterbalance the centrifuge after filling with an appropriate mixture of stationary and mobile phases. Samples were injected into the column via a Rheodyne Model 7125 injection valve equipped with a 315-μl loop. Injections of the samples dissolved in the mobile phase were carried out into the mobile phase after filling the column with the stationary phase.

Prior to the ELSD, a Model 2550 UV detector (Varian, Orsay, France) was used to monitor the absorbance of the column effluent. When only UV detection had to be used, isopropanol was continuously added to the column effluent using a Model 8500 syringe pump (Varian) before detection via a Model 811 dynamic mixing chamber (Gilson, Villiers-le-Bel, France) in order to reduce the background level in case of carryover of the stationary phase.

The Sedex 45 ELSD system (Sédéré, Vitry sur Seine, France) manufactured for HPLC was used without modification.

Solvent system

A two-phase solvent system composed of chloroform–methanol–water (3:1:3, v/v/v) was used to separate a test mixture of phenols. The solvent mixture was equilibrated at room temperature and the phases were separated shortly before use.

All organic solvents were of HPLC grade. Alcohols were purchased from Prolabo (Paris, France), other organic solvents from Rathburn (Chromoptic, Montpellier, France). Solvents were filtered before use. Water was doubly distilled. Nitrogen (L'Air Liquide, Paris, France) supplied the nebulizer of the ELSD system.

RESULTS AND DISCUSSION

Investigation of HSCCC coupled with UV detection

In order to study the potential of our HSCCC apparatus, the separation of a test mixture of phenols was done with UV detection (Fig. 2). Using the organic phase as the mobile phase, the retention of the aqueous phase was 80% at 1850 rpm and flow-rate 0.5 ml/min. The partition efficiency was calculated according to the HPLC equation for Gaussian peaks, $N = 5.54\,(t_r/\delta)^2$, where N is the number of theoretical plates (TP), t_r the retention time of the solute used to determine N and δ the width at half-height of the considered peak. The efficiency ranged from 650 TP for the first-eluting peak to 1830 for the third peak and increased with retention. The extra-column dispersion of the chromatographic system was measured by using the linear extrapolation method [24]. The total variance, σ^2, was plotted against the square of the retention volume, V_r, for a 0.5 ml/min flow-rate of the mobile phase (Fig. 3). Line a was obtained by

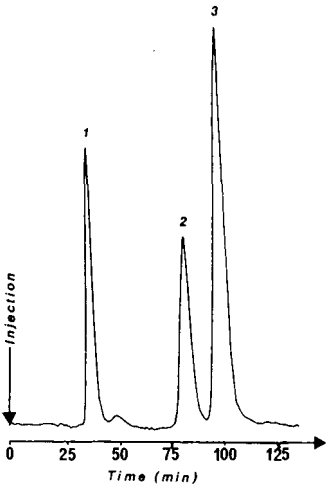

Fig. 2. On-line HSCCC–UV detection of a test mixture of phenols. Solvent, $CHCl_3$–CH_3OH–H_2O (3:1:3, v/v/v); retention of the stationary phase (aqueous phase), 80%; rotation speed, 1860 rpm; mobile phase flow-rate, 0.5 ml/min; isopropanol flow-rate, 0.5 ml/min (added to the column effluent); UV detection at 270 nm, 1 a.u.f.s. Solutes, 1 = *o*-nitrophenol; 2 = *p*-nitrophenol; 3 = phenol.

considering the mobile phase flow-rate (0.5 ml/min) without taking into account the isopropanol flow-rate (0.5 ml/min). Line b was obtained for the total flow-rate in the detector (mobile phase + isopropanol). The extra-column variance, σ_{ec}^2 was obtained from the intercept of the lines on the ordinate (Table I). For line b, the determined value of σ_{ec} (1.3 ml) was close to that calculated from the chromatogram in Fig. 2 ($\omega = 4\sigma$, where ω is the base width of the peak) and corresponded to the mixing chamber

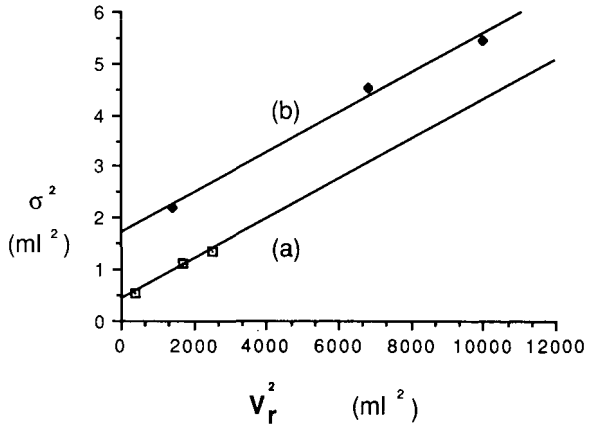

Fig. 3. Plot of total variance (σ^2) of the HSCCC–UV chromatographic system *versus* the square of the retention volume (V_r^2) of the phenolic compounds. Conditions as in Fig. 2. For line a, only the mobile phase flow-rate is considered in calculating V_r; for line b, V_r is calculated for a 1 ml/min flow-rate (mobile phase + isopropanol).

TABLE I

σ^2, σ AND N_c VALUES CALCULATED FROM THE DATA IN FIG. 3

Flow-rate (ml/min)	σ^2_{ec} (ml^2)	σ_{ec} (ml)	N_c (TP)
0.5	0.43	0.66	2600
1	1.7	1.3	2600

volume (1.5 ml). This means that the calculation made to obtain the line b is the correct approach to determine the extra-column variance for our system.

The true efficiency (N_c) of the column, 2600 TP, was obtained from the slope of the graph assuming the same efficiency for the compounds. The apparent and true values of the efficiency were fairly good for a single-column apparatus in comparison with HSCCC with two columns in series [1]. One can also establish that our apparatus permitted efficient counterbalancing of the centrifuge that minimized vibrations.

It must be pointed out that the sensitivity of UV detection was greatly improved by using 0.5 ml/min isopropanol "make-up" before detection; a range of less than 0.1 absorbance unit full scale (a.u.f.s.) was attainable instead of 2–20 a.u.f.s. because the background noise was reduced. Other types of mixing chambers are under investigation in order to reduce band broadening.

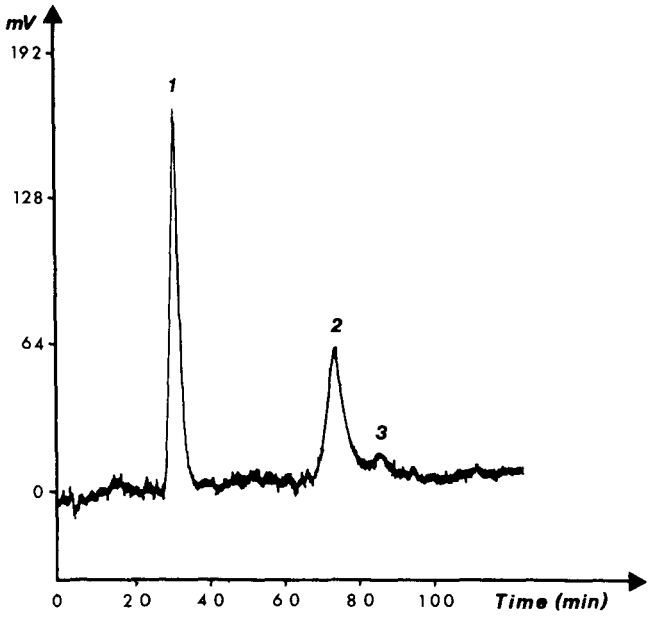

Fig. 4. On-line HSCCC–ELSD of a test mixture of phenols. HSCCC conditions as in Fig. 2. ELSD conditions: evaporation tubing temperature, 82°C; nitrogen pressure, 2 bar.

TABLE II

ELSD RESPONSE FOR VARIOUS COMPOSITIONS OF THE MOBILE PHASE PUMPED AT 1 ml/min

The first column is the mobile phase used after saturation with the stationary phase (in parentheses). The role of the two phases can be reversed.

Solvent system Mobile phase...saturated with...(stationary phase)		Detector signal (mV)
H_2O–CH_3OH	($C_2H_4Cl_2$)	8
$C_2H_4Cl_2$	(H_2O–CH_3OH)	1100
Heptane–ethyl acetate	(H_2O)	11
H_2O	(Heptane–ethyl acetate)	0
$CHCl_3$	(H_2O–CH_3OH)	>1339
H_2O–CH_3OH	($CHCl_3$)	−7
$C_2H_4Cl_2$	(HCOOH–CH_3OH)	1290
HCOOH–CH_3OH	($C_2H_4Cl_2$)	0
CH_3COOH–CH_3OH	(Heptane)	9
Heptane	(CH_3COOH–CH_3OH)	13
Heptane	(H_2O–CH_3OH)	−7
H_2O–CH_3OH	(Heptane)	−13
Acetone–HCOOH	(Methyl isobutyl ketone)	4
Methyl isobutyl ketone	(Acetone–HCOOH)	160
Heptane	(CH_3CN–CH_2Cl_2)	−2
CH_3CN–CH_2Cl_2	(Heptane)	37
n-Butanol	(H_2O)	415
H_2O	(*n*-Butanol)	35

On-line HSCCC–ELSD

This is the first report of on-line coupling of HSCCC and ELSD. Fig. 4 shows the HSCCC–ELSD result for the phenol mixture. Because sublimation of these compounds can occur during the evaporation of the mobile phase (82°C), phenol was difficult to detect. Work is being carried out with non-UV-absorbing molecules to show that ELSD can be used to advantage for detection in HSCCC.

The ELSD response was found to be noisy with the chloroform-containing mobile phase; Table II reports the values (in mV) of the signal of the detector for various CCC mobile phases in comparison with the signal recorded with water taken as a reference [25]. The data reported here are in good agreement with previous results [26], showing that chlorinated solvents give the highest signals. As has already been reported [27], the ELSD response is a function of the mean size of the droplets formed during the nebulization and depends on the properties of the solvents constituting the mobile phase. The response also varies with the refractive index of the scattering centre [27]: the higher the refractive index, the higher is the signal. In the worst case, *i.e.*, systems of solvents with chloroform, monitoring of the signals higher than 1300 mV required a decrease in the range of sensitivity of the photomultiplier. This has to be considered as a drawback because chloroform-containing mobile phases are often

used in CCC. The choice of a high quality of chloroform and solvents in general should be carefully investigated according to Dreux's experience [25].

CONCLUSION

UV detection can be performed on-line with HSCCC by using the experience gained by Oka and co-workers and by adding a make-up solvent in order to obtain a homogeneous phase prior detection. For molecules without chromophore or fluorophore groups or with mobile phases with a high UV cut-off (*e.g.*, toluene, acetone or ethyl acetate), ELSD can be useful but attention has to be paid to the quality of the solvents involved in the separation, especially when chlorinated solvents are used.

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Systematic search for suitable two-phase solvent systems for high-speed counter-current chromatography

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ABSTRACT

We have introduced two series of two-phase solvent systems which facilitate the systematic search for the solvent systems suitable for high-speed counter-current chromatography. The *n*-hexane–ethyl acetate–*n*-butanol–methanol–water systems provide a broad range of hydrophobicity, while the chloroform–methanol–water systems are extremely useful for separations of various natural products with moderate hydrophobicity. The practical use of these solvent series was demonstrated with several test samples which include dinitrophenyl amino acids, S-triazine herbicides, indole auxins, and non-ionic organic solvents.

INTRODUCTION

High-speed counter-current chromatography (HSCCC) developed in the late 1970's produces highly efficient chromatographic separations of solutes without the use of solid supports [1–3]. Thus, the method eliminates all complications caused by the soliud support matrix such as adsorptive loss and deactivation of samples, tailing of solute peaks, contamination, etc. Recently, HSCCC has been widely used for separation and purification of various natural products with excellent results.

As in other CCC schemes, HSCCC utilizes a two immiscible solvent phases, one as a stationary phase and the other as a mobile phase, and the separation is highly dependent on the partition coefficient values of the solutes, *i.e.*, the ratio of the solute concentration between the mobile and stationary phases. Therefore, the successful separation necessitates a careful search for the suitable two-phase system which provides an ideal range of the partition coefficient values for the applied sample.

Generally speaking, the two-phase solvent system should satisfy the following three requirements.

(i) The solvent system should provide nearly equal volumes of the upper and the lower phases. This facilitates the choice of the mobile phase without excessive waste of

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the solvent, and the two solvent phases are quickly separated into clear layers in a separatory funnel.

(ii) The two-phase solvent system should yield a reasonably short settling time (for the detailed description of the settling time, see Experimental). In HSCCC, the settling time should be shorter than 30 s for satisfactory retention of the stationary phase in the column [3,4].

(iii) The partition coefficient (K) of the desired compound should be close to one which gives the retention volume equal to the total column capacity. The smaller retention volume may result in a less efficient peak resolution while the greater retention volume tends to broaden the sample band by excessive mixing [5].

In the past, the search for the suitable two-phase solvent systems entirely relied on a laborious and time-consuming trial and error method which has often discouraged the users of CCC, while the method for the systematic solvent search has not been reported. This paper introduces two different series of two-phase solvent systems, one consisting of *n*-hexane–ethyl acetate–*n*-butanol–methanol–water and the other, chloroform–methanol–water, both of which lead to a systematic search for the suitable solvent systems. The search may be completed in several successive measurements of the partition coefficients by following the direction along the list of the two-phase solvent systems. Using these two-phase solvent systems, partition coefficient measurements were performed for the several groups of test samples with a broad range in hydrophobicity.

EXPERIMENTAL

Reagents

n-Hexane, ethyl acetate, *n*-butanol, methanol and chloroform were glass-distilled chromatographic grade and purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Acetone, 2-butanone and benzyl-alcohol were of reagent grade and obtained from Mallinckrodt (Paris, KY, U.S.A.), Eastman Kodak (Rochester, NY, U.S.A.) and Sigma (St. Louis, MO, U.S.A.), respectively. All dinitrophenyl (DNP) amino acids, dipeptides, and indole auxins were of reagent grade (Sigma), including N-2,4-DNP-δ-L-ornithine (DNP-orn), N-2,4-DNP-L-aspartic acid (DNP-asp), N-2,4-DNP-DL-glutamic acid (DNP-glu), N,N-di-(2,4-DNP)-L-cystine [diDNP-$(cys)_2$], N-2,4-DNP-β-alanine (DNP-β-ala), N-2,4-DNP-L-alanine (DNP-ala), N-2,4-DNP-L-proline (DNP-pro), N-2,4-DNP-L-valine (DNP-val), N-2,4-DNP-L-leucine (DNP-leu), L-tyrosyl–L-glycine (tyr–gly), L-tyrosyl–L-valine (tyr–val), L-tyrosyl–L-leucine (tyr–leu), indole-3-acetamide (IA), indole-3-acetic acid (IAA), indole-3-acetonitrile (IAN), indole-3-carboxylic acid (ICA), indole-3-acrylic acid (IAcA), indole-3-butyric acid (IBA), and indole-3-propionic acid (IPA). All S-triazine herbicides including atrazine, propazine, simazine, and trietazine were technical grade chemicals obtained from Ciba-Geigy (Greensboro, NC, U.S.A.).

Preparation of two-phase solvent systems

Twenty-seven volatile two-phase solvent systems shown in Tables I and II were prepared for the present studies. Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature by repeated shaking and degassing by opening the stopcock. Then, the solvent mixture was transferred to a graduated

TABLE I

PHASE COMPOSITION, TWO-PHASE VOLUME RATIO, AND SETTLING TIME OF *n*-HEXANE–ETHYL ACETATE–*n*-BUTANOL–METHANOL–WATER SOLVENT SYSTEMS

No.	*n*-Hexane–ethyl acetate–*n*-buthanol–methanol–water					Volume ratio (U/L)[a]	Settling time (s)
1	10	0	0	5	5	1.05	5
2	9	1	0	5	5	0.96	8
3	8	2	0	5	5	0.88	14
4	7	3	0	5	5	0.82	20
5	6	4	0	5	5	0.77	22
6	5	5	0	5	5	0.74	26
7	4	5	0	4	5	0.80	28
8	3	5	0	3	5	0.86	30
9	2	5	0	2	5	0.93	30
10	1	5	0	1	5	0.92	30
11	0	5	0	0	5	0.88	32
12	0	4	1	0	5	0.91	20
13	0	3	2	0	5	0.99	15
14	0	2	3	0	5	1.09	12
15	0	1	4	0	5	1.16	14
16	0	0	5	0	5	1.22	17

[a] Volume of the upper phase divided by that of the lower phase.

cylinder to measure the volume ratio of the two phases. The solvent mixture was then returned into the separatory funnel where the two phases were stored until use.

Measurement of settling time [4]

Using the above equilibrated solvent phases, the settling time was measured as follows. A 2-ml volume of each phase, the total volume was 4 ml, was delivered into

TABLE II

PHASE COMPOSITION, TWO-PHASE VOLUME RATIO, AND SETTLING TIME OF CHLOROFORM–METHANOL–WATER SOLVENT SYSTEMS

Methanol–water (%)	Chloroform–methanol–water			Volume ratio (L/U)[a]	Settling time (s)
0	10	0	10	0.98	8
10	10	1	9	1.00	8
20	10	2	8	1.03	12
30	10	3	7	1.06	13
40	10	4	6	1.10	12
50	10	5	5	1.16	10
60	10	6	4	1.37	11
70	10	7	3	2.05	22
80[b]	10	8	2	–	–

[a] Volume of the lower phase divided by that of the upper phase.
[b] The solvent mixture formed a single phase.

a 5-ml capacity graduated glass cylinder which was then sealed with a glass stopper. The solvent in the cylinder was gently mixed by inverting the cylinder 5 times and the cylinder was immediately placed on a flat table in an upright position. Then, the time required for the solvent mixture to settle into two clear layers was measured. The experiment was repeated several times to obtain the mean value.

Measurement of partition coefficient

In the present study, the partition coefficient is expressed by $K_{(org/aq)}$: the solute concentration in the organic phase divided by that in the aqueous phase. The partition coefficient value for each component was determined as follows: For the non-volatile test samples, approximately 0.1 ml of methanol or water containing about 1 mg of the material was evaporated with air stream in a 13-mm diameter test tube. Then, exactly 2 ml each of the upper and the lower phases were pipetted into the test tube. For the volatile liquid samples such as acetone, 2-butanone, and benzylalcohol, 10 μl of each was separately delivered into a test tube and immediately added with 2 ml each of the upper and the lower phases. The test tube was stoppered with a teflon-lined cap, shaken vigorously for 1 min to thoroughly equilibrate the sample with the two phases, and briefly centrifuged to obtain clear layers of the two phases. Then, a 0.5-ml volume of each layer was transferred to the second test tube containing 2 ml of methanol, and the contents were thoroughly mixed. The absorbance of each solution was determined at a suitable wavelength with a Zeiss PM6 spectrophotometer.

RESULTS

n-Hexane–ethyl acetate–n–butanol–methanol–water solvent system

This solvent series provides 16 two-phase solvent systems which cover a wide range of hydrophobicity continuously from the non-polar *n*-hexane–methanol–water system to the polar *n*-butanol–water system. These solvent systems are numbered from 1 to 16 in the order of hydrophobicity and listed in Table I. All these solvent systems are volatile and yield a desirable two-phase volume ratio of near 1 so that either phase can be chosen as the mobile phase without excessive waste of the solvents. These solvent systems also give suitable settling times in which the longest value slightly exceeds the critical value of 30 s in the ethyl acetate–water system. This ensures a satisfactory retention of the stationary phase in HSCCC. The overall results indicate that all the solvent systems can be efficiently applied to any centrifugal CCC scheme.

Partition coefficient values of various test samples in these solvent systems are summarized in Fig. 1A, B, and C where the $K_{(org/aq)}$ of each component is plotted in a semilogarithmic scale against the applied volume ratio of the solvent system.

Among DNP amino acids tested (Fig. 1A), the most hydrophobic component of DNP-leu gives an ideal K value of 1.05 in solvent system 3 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 8:2:0:5:5) while the most polar component of diDNP-$(cys)_2$ gives the same K value in solvent system 14 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 0:2:3:0:5). Other DNP amino acids show similar K values in the solvent systems between the above two extremes. In the S-triazine herbicides (Fig. 1B), the hydrophobicity decreases in the order of trietazine, propazine, atrazine, and simazine. The first two components of trietazine and propazine give K values of over 1.5 even in the most hydrophobic solvent system 1 (*n*-hexane–ethyl acetate–*n*-butanol–

methanol–water, 10:0:0:5:5) whereas atrazine and simazine give the desired K values in solvent system 2 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 9:1:0:5:5) and solvent system 5 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 7:3:0:5:5), respectively. Three non-ionic solvents (acetone, 2-butanone, and benzylalcohol) (Fig.

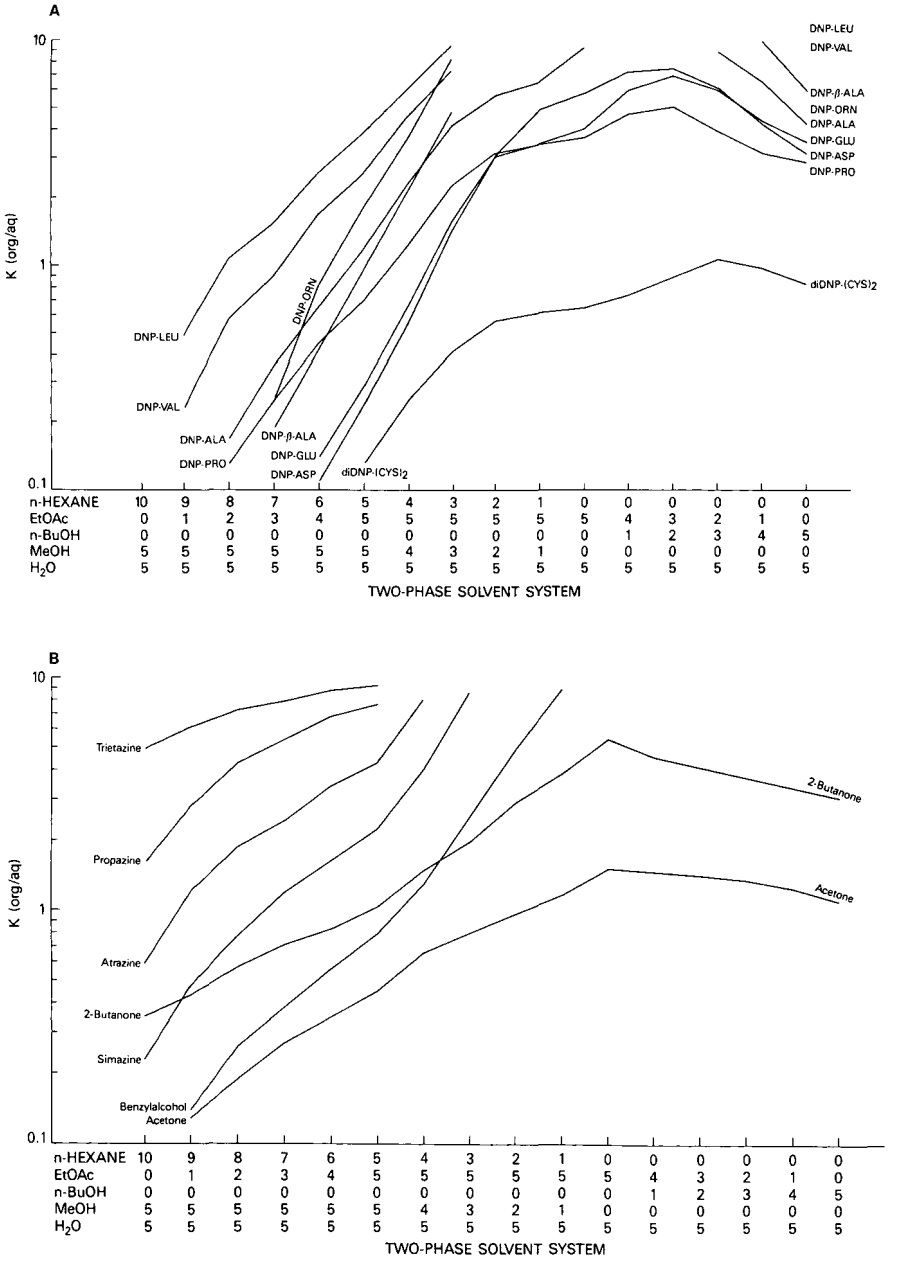

Fig. 1.

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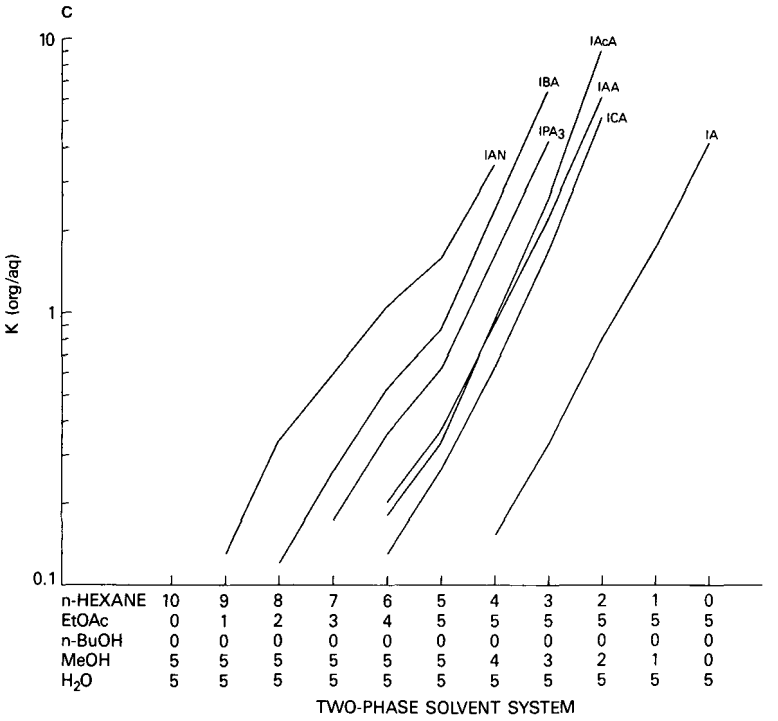


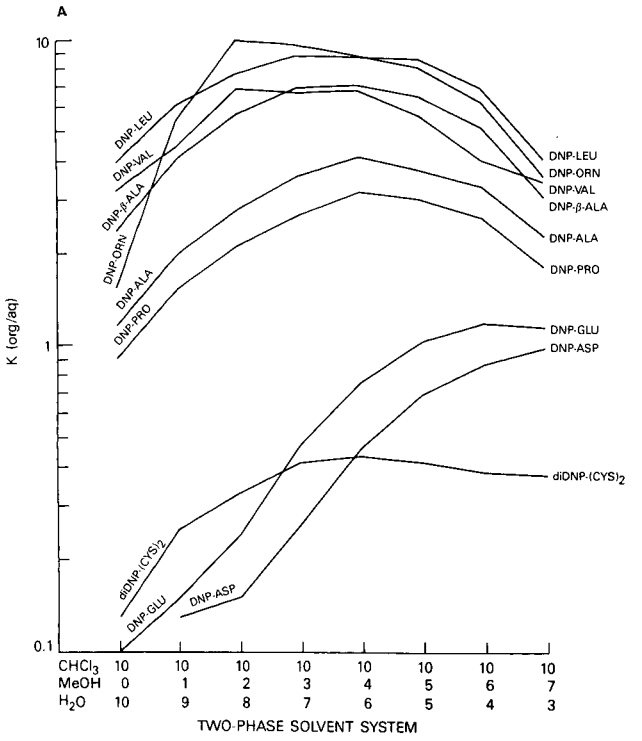
Fig. 1. (A) Partition coefficients, $K_{(org/aq)}$, of DNP amino acids in *n*-hexane–ethyl acetate–*n*-butanol–methanol–water system. (B) Partition coefficients, $K_{(org/aq)}$, of acetone, 2-butanone, benzylalcohol and S-triazine herbicides in *n*-hexane–ethyl acetate–*n*-butanol–methanol–water system. (C) Partition coefficients, $K_{(org/aq)}$, of indole auxins in *n*-hexane–ethyl acetate–*n*-butanol–methanol–water system. EtOAc = Ethyl acetate; *n*-BuOH = *n*-butanol; MeOH = methanol.

1B) and various indole auxins (Fig. 1C) give the desired K values each in the particular solvent system according to their polarity. All dipeptide samples tested showed low K values even in the most polar solvent system 16 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 0:0:5:0:5) and, therefore, more polar solvent systems are required for the separation of these compounds.

Chloroform–methanol–water solvent system

Listed in Table II are 11 chloroform–aqueous methanol (1:1) systems with various concentrations of methanol in water. The use of methanol concentration at 80% resulted in formation of a single phase, and 70% methanol concentration produced uneven volume distribution of the two phases. However, other solvent systems yielded near equal volumes of the two phases with excellent settling times of less than 15 s.

Partition coefficient values, $K_{(org/aq)}$, of various test samples in the chloroform solvent systems are illustrated in Fig. 2A, B, and C according to the format used in the *n*-hexane solvent systems. The partition coefficient values of the DNP amino acids (Fig. 2A) in these solvent systems varied greatly according to the hydrophobicity of the



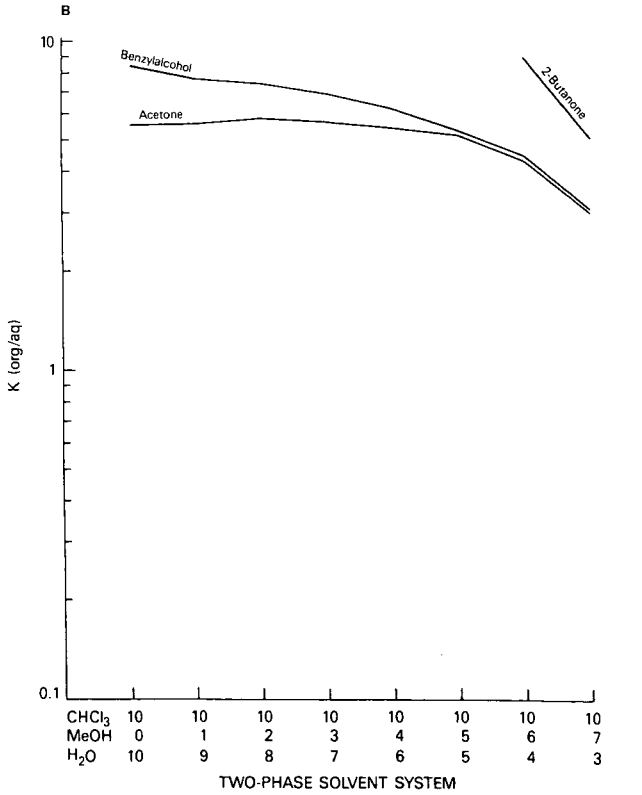


Fig. 2.

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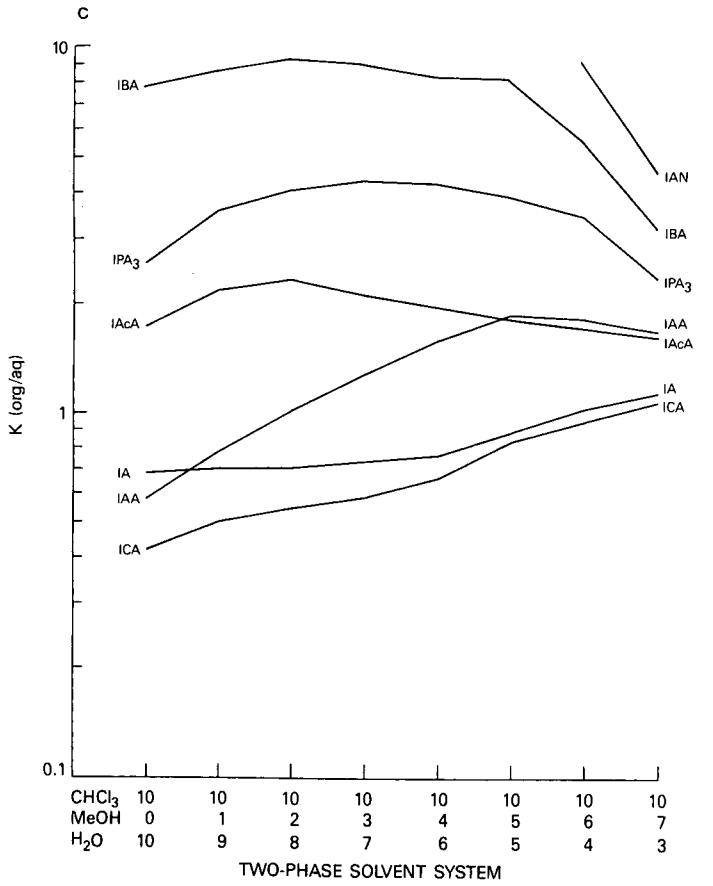

Fig. 2. (A) Partition coefficients, $K_{(org/aq)}$, of DNP amino acids in chloroform–methanol–water system. (B) Partition coefficients, $K_{(org/aq)}$, of acetone, 2-butanone, benzylalcohol in chloroform–methanol–water system. (C) Partition coefficients, $K_{(org/aq)}$, of indole auxins in chloroform–methanol–water system. $CHCl_3$ = Chloroform; MeOH = methanol.

sample. The hydrophobic components such as DNP-leu, DNP-val, DNP-orn and DNP-β-ala gave K values substantially greater than 1, while the hydrophilic components such as DNP-asp, DNP-glu, and diDNP-$(cys)_2$ gave K values smaller than 1. In most of the samples, K values became maximum at the 30–40% methanol concentration.

All chloroform solvent systems gave large K values for the non-ionic solvents (acetone, 2-butanone, and benzylalcohol) (Fig. 2B) and various S-triazine herbicides. Among the indole auxins (Fig. 2C), IAA, IA, and ICA gave K values of near 1 while other components showed much greater K values.

From the above results, it was observed that the hydrophobicity of the chloroform–methanol–water systems are corresponding to that of the *n*-hexane–ethyl acetate–*n*-butanol–methanol–water systems ranging in volume ratio between 1:1:0:1:1 and 3:5:0:3:5.

DISCUSSION

In order to facilitate the search for a suitable two-phase solvent system that provides an ideal range of the partition coefficient to the desired sample, we have selected two series of solvent systems: *n*-hexane–ethyl acetate–*n*-butanol–methanol–water and chloroform–methanol–water. In each solvent series, the partition coefficient of the sample can be finely adjusted by modifying the volume ratio of the components. The first series covers a broad range in both hydrophobicity and polarity continuously from *n*-hexane–methanol–water to *n*-butanol–water. The second series of chloroform–methanol–water provides moderate hydrophobicity and has proven extremely useful for separation of a variety of natural products simply by modifying the volume ratio between methanol and water. Most of these two-phase solvent systems provide near 1:1 volume ratios of the upper to the lower phases together with the reasonable range of settling times in 30 s or less so that they can be efficiently applied to HSCCC and other centrifugal CCC schemes.

To evaluate the usefulness of these two solvent series, we have examined the partition coefficients of the following 5 sets of test samples: 9 DNP amino acids with a wide range in hydrophobicity (which have been used as the standard samples in various CCC schemes); 7 indole auxins with a moderate range in hydrophobicity; 4 S-triazine herbicides with strong hydrophobicity; and 3 non-ionic solvents including acetone, 2-butanone, and benzylalcohol.

As described earlier, partition coefficient values, $K_{(org/aq)}$, of these test samples in the *n*-hexane and chloroform solvent systems are summarized in Figs. 1 and 2, respectively. These figures are extremely useful for selecting the proper solvent system for performing the HSCCC separations of the various sample mixtures. In these figures, the best separation will be obtained with the phase composition where the K values of the components are fairly evenly scattered around 1 at certain intervals. Some examples of the solvent selection from these figures are described below.

For the DNP amino acid separation, no single solvent system can achieve one-step separation of all compounds because of the wide range of the K values. However, the hydrophobic group, including DNP-leu, DNP-val, DNP-ala, and DNP-orn, can be separated with *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (6:4:0:5:5) (Fig. 1A) and the hydrophilic group, including DNP-pro, DNP-β-ala, DNP-glu, DNP-asp, and diDNP-$(cys)_2$, can be separated with the above solvent mixture with a volume ratio of 4:5:0:4:5 (Fig. 1A). The chloroform solvent system can also be used for the separation of relatively polar DNP amino acids: DNP-ala, DNP-pro, DNP-glu, DNP-asp and diDNP-$(cys)_2$ may be separated with chloroform–methanol–water (10:7:3) (Fig. 2A).

Similarly, one can select *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (3:5:0:3:5) for the separation of acetone, 2-butanone, and benzylalcohol and also the same solvent mixture (9:1:0:5:5) for the separation of S-triazine herbicides (Fig. 1B). The separation of the indole auxins may be successfully performed with *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (4:5:0:4:5) (Fig. 1C) except for IAcA and IAA which can be well resolved with chloroform–methanol–water (10:2:8) (Fig. 2C).

As described above, the two solvent series we have introduced can provide the suitable K values for the separation of various samples with a broad spectrum in hydrophobicity. For the sample mixture with an unknown nature, the search for the

suitable solvent system may be initiated with the partition coefficient measurement with *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (5:5:0:5:5) or chloroform–methanol–water (10:3:7). If the $K_{(org/aq)}$ value is too large, the search should be directed toward the more hydrophobic solvent systems in the hexane series and, if the K value is too small, the search should be directed toward the more hydrophilic solvent systems until the proper K values are obtained.

If the above solvent search reaches the solvent system 1 (*n*-hexane–methanol–water, 2:1:1, v/v/v) and a more hydrophobic solvent system is required, one may reduce the amount of water from the above solvent system and/or replace methanol with ethanol. Some useful solvent systems for the extremely hydrophobic compounds are *n*-hexane–ethanol–water (6:5:2, v/v/v) and *n*-hexane–methanol (2:1, v/v). On the other hand, if the solvent search ends at the solvent system 16 (*n*-butanol–water) and a still more hydrophilic solvent system is required, the above solvent system may be modified by the addition of an acid or salt: *n*-butanol–trifluoroacetic acid–water (1:0.01–0.001:1, v/v/v), *n*-butanol–acetic acid–water (4:1:5, v/v/v), and *n*-butanol–0.25 *M* ammonium acetate (1:1, v/v) have been successfully used for the separation of hydrophilic peptides [6]. Among those, the *n*-butanol–acetic acid–water (4:1:5, v/v/v) system exhibits a relatively long settling time and, therefore, the satisfactory retention of the stationary phase is obtained by applying a reversed elution mode, *i.e.*, eluting either the upper phase from the head toward the tail or the lower phase from the tail toward the head through a multilayer coil with β values less than 0.5 [4,7].

In the above experiments, the partition coefficient value of each component was obtained by measuring the absorbance of the pure sample in the upper and the lower phases. However, in practice, such a standard sample is not available, and the sample often contains various impurities or multiple components which interfere with the detection of individual components. In this case, the partition coefficient can be conveniently determined with high-performance liquid chromatography or thin-layer chromatography by chromatographing aliquots of the upper and the lower phases equilibrated with the sample. From the obtained pair of chromatograms, partition coefficient values for all resolved peaks can be determined by computing the ratio of the peak heights or areas between the corresponding peaks [8].

Although the present method has been devised for HSCCC, it may be effectively applied to other centrifugal CCC schemes.

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Solvent selection guide for counter-current chromatography

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ABSTRACT

Thirteen two-phases counter-current chromatography solvent systems (five not previously described for high-speed counter-current chromatography) were evaluated for relative polarity by using Reichardt's dye to measure solvachromatic shifts and by using solubility of index compounds. Three groups of systems were classified as lipophilic, polar or intermediate and a solvent selection guide was developed. Several intermediate systems appeared to have similar characteristics based on these evaluations. A method for making alternative solvent systems and determining composition of the two phases without concentration standards was described. Three of the lipophilic systems were tested for the separation of a 180-mg mixture of dimethylphthalate, dioctylphthalate and dodecyl acetate, and the hexane–acetonitrile (1:1) system was found to work best.

INTRODUCTION

Counter-current chromatography (CCC) is a rapidly progressing technique [1–3]. Although many two-phase solvent systems have been reported for CCC separations, there is no organized means for initially selecting one solvent system over others. Lists of CCC solvent systems [1,4] and separations of particular compounds are often arranged from "least" polar to "most" polar upper phase with no accurate measure of the polarity of the phases. Unless the compound to be separated by CCC is very similar to one already separated, there is difficulty in choosing a system.

To provide a framework for the initial selection of a CCC solvent system for a particular separation, we classified two-phase solvent systems by polarity of their upper and lower phases using Reichardt's dye [5]. Reichardt's dye is a resonance-stabilized charge-transfer compound that absorbs light in solution between 400 and 900 nm depending on the polarity of the solvent [5].

A secondary problem in making up two-phase solvent systems for CCC has been the lack of published data on composition of the two phases. Usually the phases of a four-solvent mixture are referred to as the "aqueous" phase or the "polar" phase without regard to true knowledge of their composition. Problems are encountered when the solvent mixture divides into two phases in an 80:20, 90:10, or similar disproportionate volume ratio. Filling the CCC column with one phase and then eluting with the second phase requires similar volumes of each phase and large amounts of solvent mixtures must be prepared to obtain sufficient volume of the smaller volume

phase if they separate disproportionately. Equal volume phases with similar composition to those that separate disproportionately can be devised if the percent composition of each phase is determined by our proposed method.

EXPERIMENTAL[a]

Materials

Solvent systems for study were chosen fromt he literature (systems 3, 4, 5, 7, 8, 10, 11, 12 in Table I) as indicated by Table I citations or developed at our laboratory (systems 1, 2, 6, 9 and 13 in Table I).

The least polar solvent in a system is listed first in each row of Table I. This is the solvent added incrementally to adjust the polarity of the overall system.

Dimethyl phthalate, dioctyl phthalate, dodecyl acetate, ethylene glycol, glycerol, methyl stearate and Reichardt's dye were obtained from Aldrich. Meadowfoam oil, a mixture of vegetable fatty acid triglycerides was supplied by Selim Erhan of our laboratory. Other chemicals are commercially available reagents.

TABLE I

TWO-PHASE SOLVENT SYSTEMS FOR COUNTER-CURRENT CHROMATOGRAPHY

System	Solvent and volume ratio	Ref.
1	Hexane–acetonitrile (1:1)	
2	Hexane–acetonitrile–chloroform (5:5:1)	
3	Hexane–ethanol–water (6:5:1:)	4
4	Hexane–ethyl acetate–acetonitrile–methanol (5:2:5:4)	3
5	Hexane–ethyl acetate–methanol–water (1:1:1:1)	4
6	Chloroform–methanol–water (13:7:2)	
7	Chloroform–methanol–water (1:1:1)	4
8	Chloroform–methanol–water (7:13:8)	4
9	Toluene–acetonitrile–water–ethanol (3:4:3:2)	
10	Chloroform–methanol–0.2 *M* acetic acid (1:1:1)	4
11	Ethyl acetate–ethanol–water (2:1:2)	
12	*n*-Butanol–acetic acid–water (4:1:5)	4
13	*n*-Butanol–ethyl acetate–water (4:1:4)	

Solvachromatic shift

A 1-mg amount of Reichardt's dye was added to 10 ml of one phase (upper or lower) of the two-phase solvent system and shaken. Using the solvent as background, the absorption spectrum of the dye solution was measured from 200 to 900 nm in a 1-cm pathlength quartz cell on a Beckman DU UV spectrophotometer. Samples were diluted to maintain maximum absorbance at or below 1.0 absorption unit. The maximum absorbance between 450 and 900 nm was determined automatically using instrument software and confirmed by inspection of the spectrum. Solvachromatic

[a] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

shifts were similarly obtained for low volatility liquids: dimethyl phthalate, dioctyl phthalate, ethanolamine and ethylene glycol.

Composition of the phases

Composition of the two phases was calculated from peak areas determined on a Hewlett-Packard Model 5790 gas chromatograph using a 15 m × 1.5 mm polydimethylsiloxane-coated capillary column from J & W Scientific. Flame ionization detection, a Hewlett-packard Model 3390A recording integrator and hydrogen carrier gas at 5 ml/min were used. Oven temperature varied depending on the nature of the solvent. When water was present in a system, its concentration was determined by the Karl–Fisher titration method on a Fisher Coulomatic Titrimeter Model 447. Using peak areas of individual solvent peaks for each solvent phase and the total amount of each solvent used, we calculated the concentration of each solvent in each phase. For example, if the total volume of four solvents mixed are A, B, C and D and the resulting volumes of upper and lower phases are V_U and V_L, respectively, then we can designate the volumes we wish to determine A_U, B_U, C_U, D_U, the volumes of solvents A to D in the upper phase, and A_L, B_L, C_L, D_L, the volumes of solvents A to D in the lower phase. A peak area response for each solvent in the upper phase was determined as described above by gas chromatography (GC) to be A_1, B_1, C_1 and D_1. The peak area determination was made three times and an average taken. The same size of injections (0.2 to 2.0 μl) were made for the upper and lower phases and average peak area responses designated A_2, B_2, C_2, D_2 for the lower phase. The fraction $A_1/A_2 \cdot V_U/V_L$ equals the ratio of the volume of solvent A in the upper phase to the volume of solvent A in the lower phase or $A_1/A_2 \cdot V_U/V_L = A_U/A_L$ (i). Since the total volume of solvent A equals the sum of A in the two phases we can write $A_U + A_L = A$ (ii). Substituting (i) in (ii) the volume of A in each phase can be derived. By repeating this calculation for all GC detectable peaks, the compositions of the two phases were determined. Water was determined separately by a Karl–Fisher titration as a volume percentage for each phase. Thus each phase could be made up separately in equal volumes and combined but retain their equilibrium compositions. Compositions of both phases were determined in those cases where the volume of one phase was substantially smaller than the other (systems 4, 6 and 8 in Table I).

Adjusting the solvent system polarity

To determine the effect of added non-polar solvent, either 1, 5 or 10 ml of the least polar solvent in the system was added to a 50:50 mix of upper and lower phase, shaken and the UV absorbance of Reichardt's dye in each phase was measured.

Partition coefficients

Sample (0.1 g) was shaken with 1 ml each of the upper and lower phases at room temperature. A 100-μl aliquot of each phase was dried under a flow of nitrogen at 50°C in an aluminum dish, weighed, and the partition coefficient (k) calculated as the ratio solute concentration in the lower phase to the solute concentration in the upper phase.

Solubility

Solubility of selected compounds, designated index compounds, were deter-

mined at the 2% concentration or lower level by weighing 0.100 g of the compound into a vial, adding 5.00 ml of one of the phases from the solvent mixtures and shaking to dissolve, then allowing it to stand at least 2 h. A 1-ml aliquot was taken to dryness under nitrogen at 50°C, weighed and the amount of the solute dissolved was determined.

CCC separation

Upper phase of a two-phase solvent system was pumped into a 30 m × 1.6 mm I.D. PTFE tube wound into a coil on one side of a Pharmatech revolving CCC apparatus. The solvent mixture was pumped into the end of the teflon tube starting from the center of the coil to the outside of the coil. A brass counterbalance was mounted opposite from the column on the revolving apparatus. After filling, the coil was rotated about its axis as it was simultaneously revolved about the axis between the coil and counterbalance at 750 rpm. The direction of revolution was set to go forward on the apparatus. This direction of revolution and rotation is such that the solvent in the coil is forced back toward the center of the coil, *i.e.*, the inlet. Pump pressure opposes this force and introduces more solvent. Next, lower phase was pumped onto the rotating column at 3.6 ml/min until no further upper phase was displaced at the column outlet. Sample (180 mg) dissolved in 3 ml of upper phase was introduced through a 5-ml sample loop. Lower phase continued to be pumped onto the column. Effluent from the column was sent through a 1 mm pathlength quartz cell in an Isco Model V4 recording UV detector set at 220 nm wavelength and minimum sensitivity. Fractions (14.4 ml) were collected in a Gilson Model GC-100 automatic fraction collector every 4 min for 90 min and then the revolution and pumping were stopped. Nitrogen was used to displace column contents and 14-ml fractions were collected as the column was emptied. The column was washed by pumping 100 ml ethanol through it and blowing it dry with nitrogen. Collected fractions were evaporated in a Haake Buchler Evapotec vortex evaporator at 50°C under vacuum. Identity of the effluent peaks were confirmed by their GC retention time and infrared spectra.

RESULTS AND DISCUSSION

Composition of the phases

A typical calculation of composition is shown for system 4 in Table II. Solvent systems 6 and 8 were similarly analyzed (Table III). Based on the composition in Table II, 2 l of upper and 2 l of lower phase were made and shaken together. Unfortunately, the two phases did not retain their original volumes of 2 l each, which indicates that some of our GC analyses are not accurate enough. Reproducibility of the GC injection and resolution of overlapping peaks are the probable sources of error. Nevertheless the goal of obtaining an alternative for solvent systme 4 was achieved. Instead of 4 l of a solvent system that separates into 441 ml and 3470 ml the alternative system separates into 1640 ml of upper phase and 2334 ml of lower phase but most importantly, solvachromatic shifts of the upper and lower phases of the alternative system 4 are nearly identical with those of the orignal system 4. Absorbance maxima of system 4 upper phase was 561 nm, system 4 lower phase was 533 nm and for alternative 4 upper, 558 nm and alternative 4 lower, 535 nm. We feel that this

TABLE II

PHASE COMPOSITION OF SOLVENT SYSTEM 4

	Solvent			
	Hexane	Ethyl acetate	Aceto-nitrile	Methanol
Volume mixed (ml)	1250	500	1250	1000
Phase volume (ml)				
Upper (U), lower (L), ratio U:L 441, 3470, 0.121				
GC peak area ratio U:L	3.41	1.19	0.0676	0.0726
Solvent volume ratio U:L	0.413	0.145	0.00818	0.00878
Solvent volume to make each phase[a]				
Upper	366	63.2	10.1	8.7
Lower	884	437	1240	991
Composition (%, v/v)				
Upper	81.7	14.1	2.25	1.95
Lower	24.9	12.3	34.9	27.9

[a] Volume of hexane in lower phase calculation: $H_U + H_L = 1250$ where H_U = volume hexane in the upper phase, H_L = volume of hexane in the lower phase. $H_U/H_L = 0.413$; $0.413\ H_L + H_L = 1250$ and $H_L = 1250/1.413 = 884$.

preliminary method can be refined, yet it provides an expedient alternative for the scientist working with two-phase systems that separate into disparate volumes.

Solvent selection guide

The relative polarity of each phase and some solvents, as determined by the solvachromatic shift method are shown in Fig. 1. The midpoint of the absorbance maxima for a solvent system is shown in the boxed-in area. This midpoint was used to arrange most systems from least polar (top of Fig. 1) to most polar (bottom of Fig. 1). Shifts of some solvent systems could not be measured by this method. Acids, salts, or low solubility of the dye prevented shift measurements in these cases. Therefore solvent systems also were classified by the partitioning and solubility of various solutes designated index compounds. Dashed-line boxes in Fig. 1 are systems placed in the chart based on partitioning and solubility of index compounds. Solute (0.1 g) was dissolved in each phase (5.00 ml) separately to determine the percent solubility of index compounds at low levels. Low concentrations were examined so as not to alter the overall polarity of the solvent mixture. If all the solute dissolved, that constituted >20 mg/ml solubility. Meadowfoam oil, methyl stearate and dodecyl acetate were chosen to represent non-polar solutes and dextrose, glycerol, and K_2SO_4 were chosen to represent polar solutes for ranking the various solvent mixtures. The results for

TABLE III

COMPOSITION OF PHASES IN SYSTEMS 6 AND 8

System		Upper %	Lower %
6	Chloroform–methanol–water (13:7:2)	6.7:53.4:39.9	64.3:29.7:6.0
8	Chloroform–methanol–water (7:13:8)	11.7:53.4:34.9	76.7:19.3:4.0

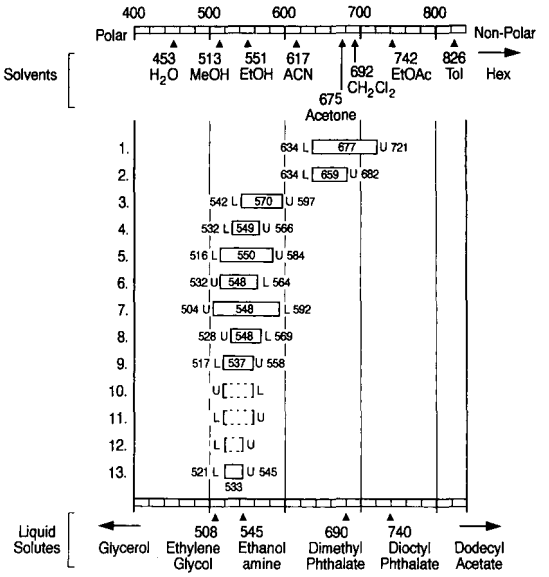

Fig. 1. A solvent selection guide for counter-current chromatography showing the absorbance maxima of Reichardt's dye (1 mg in 10 ml) in each phase of 13 two-phase solvent systems and 15 individual solvents and solutes. Dashed-line boxes were classified by solubility of index compounds, not Reichardt's dye. Midpoint values between upper and lower phase values are given inside the boxed-in spaces. Abbreviations: Hex = hexane; ACN = acetonitrile; MeOH = methanol; EtOH = ethanol; Tol = toluene; EtOAc = ethyl acetate; U = upper phase; L = lower phase. For solvent systems 1–13, see Table I.

these six compounds are shown in Table IV. Relative rankings of polar phases (P_P) from least polar (1) to most polar (13) for all two-phase systems were made using these rules for non-polar solutes (S_N): (1) if methyl stearate (MSt) and meadowfoam oil (MO) were soluble at >19.5 mg/ml phases were ranked by the highest dodecyl acetate (DDA) solubility being least polar; (2) after rule 1, phases in which MSt was soluble at 19.5 mg/ml or greater were ranked by meadowfoam oil solubility; (3) after the above, if MSt solubility <19 mg/ml and MSt, MO and DDA were $\neq 0$ the phases were ranked least polar by the highest sum of MSt, MO and DDA solubility; (4) after the above, if only MSt solubility = 0 the highest sum of MO plus DDA solubility was ranked most non-polar; (5) after 1 to 4, if MO or DAA solubility = 0 the sum of the other two were used to rank the systems; (6) finally, systems where solubility of MSt, MO and DDA = 0 were considered most polar in the ranking.

Relative rankings of non-polar phases, P_N, was made using the above rules for non-polar solutes, S_N. A similar set of rules using polar solutes (S_P) was used to rank the various phases. The four ranking combinations P_PS_N, P_PS_P, P_NS_N, P_NS_P resulted in Table V for the various phases of systems 9–13. Systems 10, 11 and 12 could not be ranked with Reichardt's dye. Table V results were thus used to place systems 10 and 11 very close to system 9 and system 12 very close to system 13.

There are three groups of systems —the lipohilic (systems 1–3 with 3 the least lipophilic), the polar systems (systems 9–13) and intermediate systems 4–8. Relative placement within each group may depend more on the difference in polarity between

TABLE IV

SOLUBILITY (mg SOLUTE/ml SOLVENT PHASE) OF 13 TWO-PHASE SOLVENT SYSTEMS

Solvent[a] system, phase	Solubility of					
	Methyl stearate	Meadowfoam oil	Dodecyl acetate	Glycerol	Dextrose	K_2SO_4
1U	>20	>20	16.1	0	0	0
L	11	0.32	18.9	17	0	0
2U	>20	>20	18.2	0	0	0
L	>20	3.1	9.9	14.7	0	0
3U	>20	19.1	12.9	1.0	0	1.7
L	13.9	0.84	11	>20	>20	0.24
4U	>20	19.9	13.3	0.30	0	0
L	16.0	12.5	13.0	>20	1.1	0.4
5U	>20	>20	11	1.0	0.18	0
L	0.30	2.2	2.8	>20	>20	1.7
6U	0	0	0	>20	>20	0.64
L	20	18.8	17.3	18.2	6.6	1.0
7U	0	0.54	0.18	>20	>20	2.9
L	19.6	>20	10.7	5.4	0.19	0.34
8U	0	0	0	>20	>20	1.4
L	18.8	19.8	8.9	15.6	2.12	0.32
9U	>20	9.9	>20	13.5	2.0	0
L	0.29	2.2	0	>20	>20	3.7
10U	0.50	0.20	0	>20	>20	2.62
L	>20	19.4	18.6	7.7	0.31	.07
11U	>20	3.5	19.1	12.9	4.0	.09
L	0.6	0.1	0.33	>20	>20	0.47
12U	19.6	3.5	18	>20	6.56	0.47
L	.21	0	0.25	>20	>20	19
13U	>20	2.32	17.4	17.4	8.7	
0.37	L	0.1	0.26	0	>20	>20
19.6						

[a] Make-up of solvent system as in Table I and Fig. 1. U = Upper phase; L = Lower phase.

TABLE V

RANKING OF PHASES IN SYSTEMS 9–13 BASED ON SOLUBILITY OF NON-POLAR SOLUTES (S_N) AND POLAR SOLUTES (S_P) IN UPPER (U) AND LOWER (L) PHASES[a]

Ranking least to most polar	Ranking criteria[a]				System	Average rank
	P_NS_N	P_NS_P	P_PS_N	P_PS_P		
1	10L	9U	11L	11L	9	2.0
2	9U	10L	9L	10U	10	2.0
3	11U	11U	10U	9L	11	2.0
4	12U	13U	12L	12L	12	4.25
5	13U	12U	13L	13L	13	4.75

[a] See discussion for applying solubilities in Table IV to determine relative polarities.

TABLE VI

SHIFT OF UV–VISIBLE ABSORBANCE MAXIMA OF REICHARDT'S DYE IN TWO-PHASE SYSTEMS WHEN NON-POLAR SOLVENT IS ADDED

System	Upper phase shift (nm)			Lower phase shift (nm)		
	+1 ml	+5 ml	+10 ml	+1 ml	+5 ml	+10 ml
3	−19	−12	−13	0	− 3	0
4	+ 5	0	+ 1	−2	− 3	+3
6	+10	+ 6	+ 6	+2	+ 2	+5
7	+ 5	0	+ 1	+1	+ 1	+3
8	+ 7	+ 2	+ 2	0	+ 1	+1
9	+ 1	+ 4	+ 6	+1	− 9	−9
13	0	− 1		−7	−13	

the upper and lower phase than on the average polarity of the system. The average polarity is similar by both the Reichardt's dye test and the index compound test. System 7 for example has a system polarity similar to system 8 but the upper and lower phases of system 7 are further apart in polarity than those of system 8. Partition coefficients of two natural products illustrate this effect. The partition coefficient of kampferol in system 8 is 3.40 and in system 7 is 4.03. The partition coefficient of rutin in system 8 is 2.04 and in system 7 is 2.25. This agrees with Fig. 1 and Table IV in that both are very similar in separation characteristics. However, he values for system 8 are closer to 1.0 than those of system 7 which reflects the closer polarity and more nearly equal partitioning of solute in the two phases.

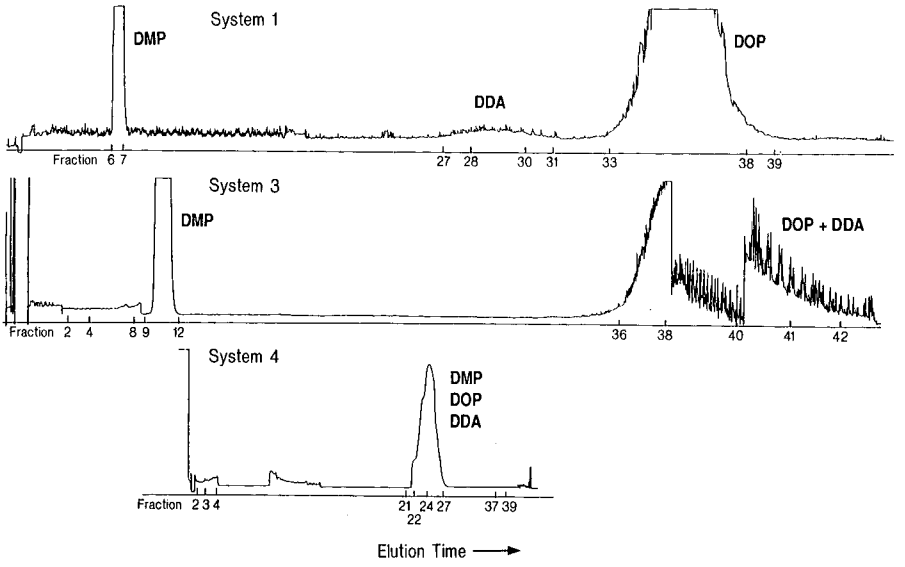

Fig. 2. Chromatograms from CCC Separations of dioctyl phthalate (DOP), dimethyl phthalate (DMP) and dodecyl acetate (DDA). Flow-rate 3.6 ml/min for 90 min, UV 220 nm, mobile phase: lower, 14.4-ml fractions collected. Column contents displaced with nitrogen after fraction 22.

Adjusting the solvent system polarity

Since so many systems have similar partitioning characteristics it seems doubtful that adding 1, 5 or 10 ml of the least polar solvent to a system would have much effect on the polarity of the two phases. Nonetheless, it is a well-known practice for adjusting CCC solvent system polarities. Using Reichardt's dye we tested the effect of adding 1, 5 or 10 ml of the least polar solvent, the first listed for each system in Fig. 1, to 100 ml of each system, 50 ml of each phase (Table VI). In some cases two phases were not maintained, but surprisingly some significant shifts in absorbance maxima did occur. The differences that these shifts make in partition coefficients and separations by CCC need to be investigated further.

CCC separations

The overall usefulness of classifying solvent systems by polarity for CCC separations is clear, but many separations using CCC are necessary to determine whether solvent systems with similar polarities are interchangeable for separations. If we use Fig. 1 and the interpretation above of Table IV data then we would expect that separation of dimethyl phthalate, dioctyl phthalate, and dodecyl acetate might be best accomplished in one of the systems from 1 to 4. Partition coefficients (not reported) and then actual separations as shown in Fig. 2 bore this out. The apparent changes in slope in the chromatogram from system 4 looked like possible separation so the fractions were reevaluated individually in a Hewlett-packard Model 8450A photodiode array UV–visible spectrophotometer linked to a Mod Comp Classic computer. Chromatograms were constructed at several wavelengths and intensities but no separation was evident for system 4 as shown in Fig. 3 for the reconstructed chromatograms at 254 nm. The separation in solvent system 1 is also evident in Fig. 3. We feel that this separation demonstrates the usefulness of the solvent selection guide for selecting a group of solvents. This group should be further examined by partition coefficients to select one best system for a particular separation. Much more research needs to be done on comparing several of the systems for nearly equivalent partition-

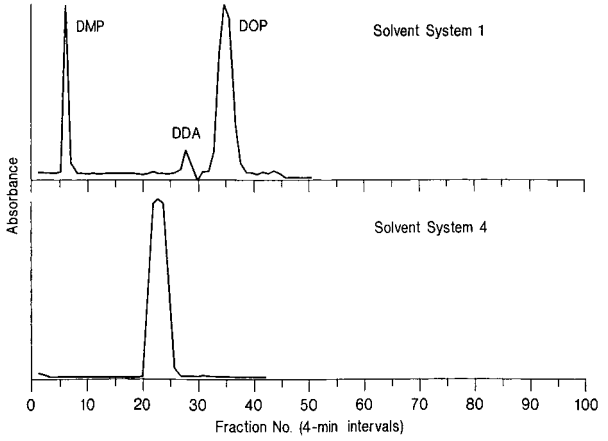

Fig. 3. Reconstructed chromatograms from fractions collected as in Fig. 2 but absorbance was remeasured at 254 nm.

ing ability. We find that many are similar, especially in the group from system 4 to system 8. Each system may have unique properties for a particular type of compound. However, deducing these differences will require that many partition coefficients be evaluated or that another method of evaluating mixed solvent polarity be used.

ACKNOWLEDGEMENTS

Expert technical assistance was rendered by Thomas Lee, Doris Palmer and Spencer Wang. Robert Peterson assisted in the generation of the reconstructed chromatograms in Fig. 3.

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Note

Toroidal coil centrifugal partition chromatography, a method for measuring partition coefficients

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Lipophilicity is a major physico-chemical parameter in quantitative structure–activity relationship (QSAR) studies [1,2]. A number of experimental methods are currently used to simulate partition processes in biological systems and to determine lipophilicity. The traditional shake-flask technique, using a biphasic liquid system, remains the method of reference for measuring partition coefficients (expressed as log P values), but suffers from a number of pratical limitations [3,4].

Solid–liquid partition chromatography has been applied as an alternative means for measuring lipophilicity, and the use of chromatographic retention parameters, in particular those obtained from reversed-phase high-performance liquid chromatography (RP-HPLC) [5], have become popular in replacing solvent–water partition coefficients in QSAR studies. However, the solid supports used in RP-HPLC, particularly octadecylsilane, interact with solutes in modes that in some instances may differ significantly from solute–solvent interactions in the shake-flask method [6,7], rendering difficult comparisons between data and the interpretation of lipophilicity–activity correlations.

Recently, centrifugal partition chromatography (CPC) has been explored as a novel approach for measuring liquid–liquid partition coefficients [8,9]. CPC has been shown to yield highly reproducible log P results, and various technical improvements have transformed it into a fast and efficient method [10,11]. To the best of our knowledge, the only limitation of the CPC method so far is a lipophilicity range that is still too restricted for medicinal use (log P from -3 to $+3$ [12], because the volume of the aqueous stationary phase cannot exceed 95% of the total coil capacity). Any CPC technique that is able, at least potentially, to extend this range is therefore worth investigating. In this paper, we report some preliminary results indicating that the technique known as toroidal coil CPC (TCCPC) can be used to determine partition coefficients, and we discuss its potential for measuring very low and very high log P values.

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The original design of the flow-through toroidal coil centrifuge (Fig. 1A) permits solvent flow in and out through the rotating column without the use of rotating seals, which often cause complications such as leakage, corrosion and contamination [13]. A helical column mounted in the periphery of the column container located on top of the rotor is shown in Fig. 1B. When a two-phase solvent system is introduced into the rotating column, the propagating eluent percolates continuously through the stationary phase retained in each turn of the coil. As a consequence, solutes introduced in either phase are subjected to an efficient partition process. This system has been demonstrated to separate nine dinitrophenylamino acids with efficiencies ranging between 4000 and 1250 theoretical plates [13]. The major difference between a system of this type and other CPC apparatus is that movement of solutes can be observed continuously through the transparent coil, provided that they are coloured; measurement of solute position becomes possible using a stroboscopic light source (see *Methods*).

EXPERIMENTAL

Chemicals

Pure Sudan III, phenol red and pararosaniline were obtained from Sigma (St. Louis, MO, U.S.A.). Crystal violet (94% purity) and *p*-nitroaniline were purchased from National Aniline Division (New York, U.S.A.) and Amend Drug & Chemical (New York, U.S.A.), respectively. 1-Octanol (99.3% purity) was from Aldrich (Milwaukee, WI, U.S.A.). All compounds and reagents were used without further purification.

Methods

The helical column was prepared by winding PTFE tubing (0.55 mm I.D.) (Zeus Industrial Products, Raritan, NJ, U.S.A.) around a nylon tube (110 cm × 4 mm O.D.) to make *ca.* 830 turns with a total capacity of 4.0 ml. The column was then filled with 1-octanol which had been saturated with 0.1 *M* phosphate buffer (pH 7.4). While the rational speed of the column container was adjusted to 1000 rpm, the mobile phase [0.1 M phosphate buffer (pH 7.4) saturated with 1-octanol] was pumped using a Shimadzu Model LC-6A pump at a flow-rate of 0.4 ml/min. When no stationary phase was observed in the eluate, the solute (dissolved in about 20 μl of 1-octanol phase) was injected from a Rheodyne injector.

The movement of the solutes was observed by stroboscopic illumination with a visible light source. To allow measurement of the solute position and propagation speed, the periphery of the column container was scaled into 35 units. Highly lipophilic solutes may take a very long time to elute, rendering almost impossible the direct measurement of their retention times (t_R). In such cases, hower, t_R can be calculated when the solute is still far from the column outlet by measuring the position of the centre of the solute band (X_t) at time t (Fig. 2):

$$t_R = t\,(X_R/X_t) \tag{1}$$

where X_R is the circumference of the support around which the coil is wound. It follows that log P values can be calculated from the predetermined flow-rate (F),

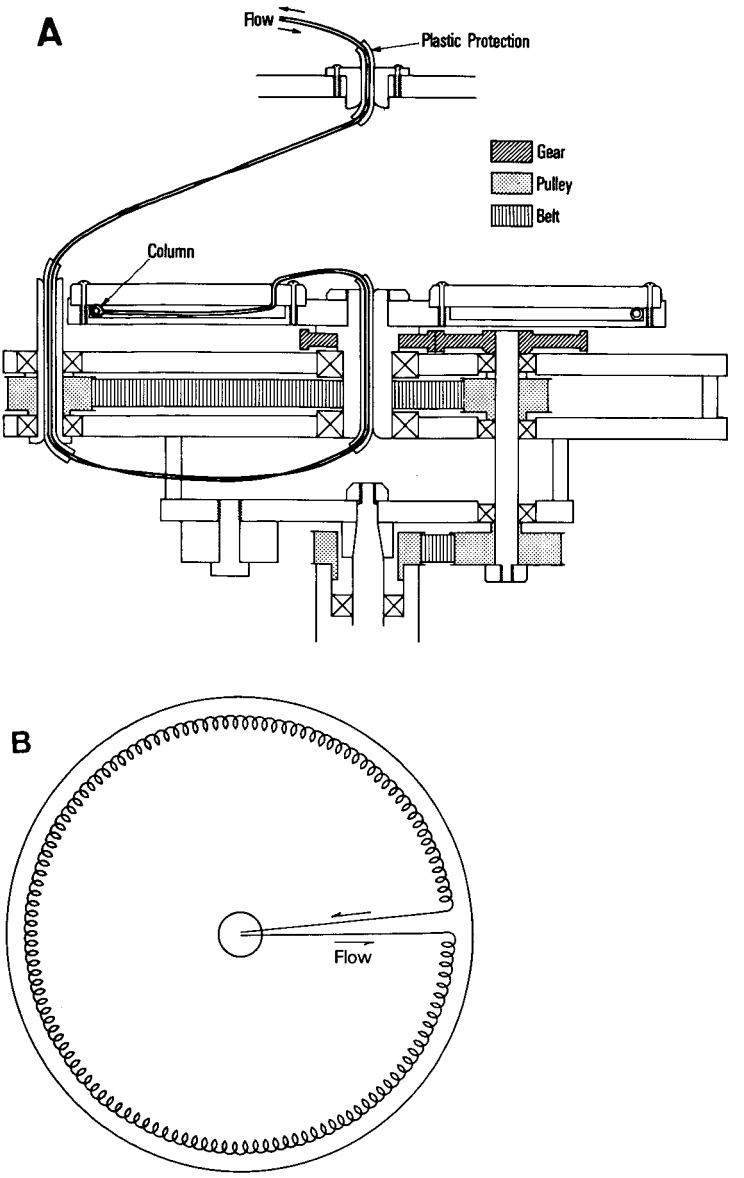

Fig. 1. Design of the rotor in the toroidal coil centrifugal partition chromatograph. (A) Overall view; (B) arrangement of the helical column.

mobile phase volume (V_m) and stationary phase volume (V_s) according to the equation

$$\log P = \log (t_R F - V_m)/V_s \quad (2)$$

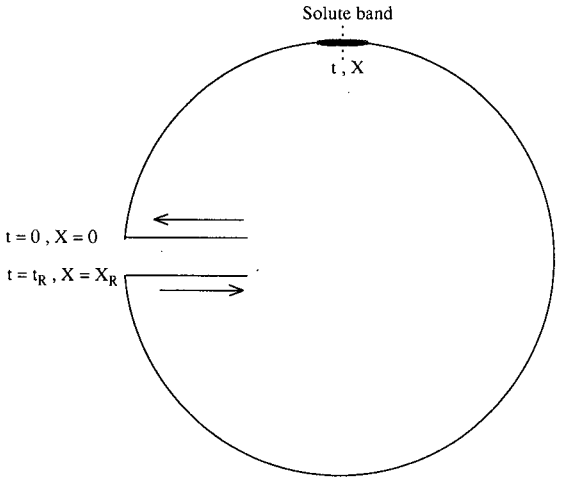

Fig. 2. Propagation of a solute band along the helical column at time $t = 0$ and at time $t = t_R$ (retention time of the solute when it has travelled the full length X_R of the coil).

RESULTS AND DISCUSSION

The time required for the solvent front to elute from the column, as measured by the retention time of potassium chromate, was found to be 5.0 min. The mobile phase volume (V_m) was thus calculated to be 2.0 ml ($V_m = 5.0$ min $\cdot F$) and that of the stationary phase to be 2.0 ml. The retention time of the investigated solutes and their calculated log P_{oct} values are listed in Table I. Note that the measured log P_{oct} value of *p*-nitroaniline (1.30) is close to the literature value of 1.39 [14].

The log P values measured here range from -0.21 to 3.02, but there is no reason why the method should not be applicable to solutes of much higher lipophilicity. Indeed, such compounds would be highly retained, but their X_t distance should become measurable after a sufficient elution time. For highly hydrophilic solutes, one would simply need to use water as the stationary phase and octanol as the eluent.

TABLE I

1-OCTANOL–WATER PARTITION COEFFICIENTS (LOG P_{oct}) MEASURED BY TOROIDAL COIL CENTRIFUGAL PARTITION CHROMATOGRAPHY (TCCPC)

Solute	Retention time, t_R (min)	Log P_{oct} [a]
Pararosaniline	8.1	−0.21
Crystal violet (hexamethylpararosaniline chloride)	21.0	0.51
p-Nitroaniline	105.0	1.30
Sudan III (1-{[4-(phenylazo)phenyl]azo}-2-hydroxynaphthalene)	653.3	2.11
Phenol red (phenolsulphonephthalein)	5250.0	3.02

[a] Measured at pH 7.4, not corrected for ionization.

The most obvious limitation of the method is solute detection, which in the present state of development is restricted to coloured compounds such as those investigated in this feasibility study. However, we believe that the method could be extended to all UV-active solutes by using a stroboscopic UV light source. This would certainly widen considerably the applicability and potential of TCCPC in measuring the lipophilicity of most drugs and many compounds of interest.

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Continuous separation of lanthanides by counter-current fractional extraction

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ABSTRACT

The application of a dual counter-current system which involves counter-current fractional extraction with continuous sample feeding is described for the separation of lanthanides. The separation of two model mixtures of samarium and gadolinium and of europium and gadolinium demonstrates the utility of the system. In addition, the column efficiency was determined.

INTRODUCTION

Various types of counter-current chromatography (CCC) have been developed and illustrated for the separation of both organic and inorganic compounds including biological samples and natural products [1]. Most of these techniques, however, involve a one-way elution mode, *i.e.*, one phase of a two-phase system proceeds through a column and the other phase is held stationary. In contrast, if the two phases could be continuously and simultaneously moved in opposite directions, then the system would allow for continuous introduction of the sample. Because CCC does not require a solid support, such a two-solvent counter-current extraction might easily be performed if a suitable column configuration could be devised.

Several dual countercurrent systems have been reported. For example, Ito [2] reported a dual counter-current system based on a foam separation method, which utilized a combined horizontal flow-through coil planet centrifuge with a liquid phase containing surfactant and a gas phase of nitrogen. The system was illustrated for two model mixtures of Rhodamine B–Evans blue and bovine serum albumin–sheep hae-

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moglobin. Kabasawa [3] reported on a continuous counter-current extraction system termed counter-current fractional extraction with continuous sample feeding (CFE). Fig. 1 shows a schematic diagram of the CFE method. The columns used in CFE are similar to those in rotation locular counter-current chromatography [4], which is segmented by a series of disks with a small hole in each disk. The columns are filled with the two phases, then held at an inclined position and rotated about its longitudinal axis. The lower phase is pumped down to the lower exit and simultaneously the upper phase is forced up the column toward the upper exit. The sample is fed continuously into the middle between the two columns. Recently, DL-valine could be completely separated into its two enantiomers by this device with an *n*-butanol–water system containing a copper(II) complex of *n*-dodecyl-L-hydroxyproline [5].

In this paper, we describe an application of CFE to the laboratory-scale separation of lanthanides. The capability of CFE is demonstrated for the separation of samarium (Sm) and gadolinium (Gd), and of Gd and europium (Eu) utilizing two-phase systems of dilute nitric acid and *n*-hexane containing di(ethylhexyl)phosphoric acid (DEHPA) as the extractant. The column efficiency is determined from the experimental data in conjunction with the theoretical equations of discontinuous dual counter-current distribution processes [6] such as counter double current distribution [7].

EXPERIMENTAL

Materials

Samarium chloride, europium chloride, gadolinium chloride, 2-hydroxyisobutyric acid and di(2-ethylhexyl)phosphoric acid were purchased from Tokyo Kasei (Tokyo, Japan) and were used without further purification. All other chemicals were of analytical-reagent grade and obtained from commercial sources.

Preparation of two-phase system

Equal volumes of *n*-hexane containing 0.5 *M* DEHPA and 0.55–0.8 *M* nitric acid were placed in a separating funnel and shaken vigorously. After the mixture was

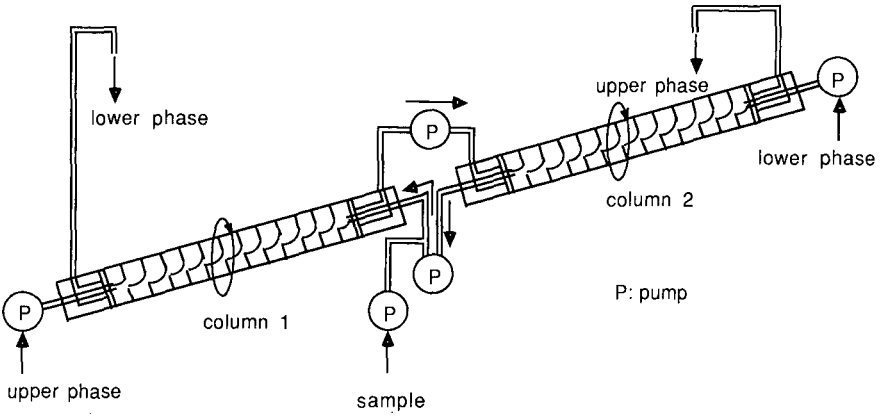

Fig. 1. Schematic diagram of CFE.

separated into two stable phases, these upper and lower phases were used in the following experiments.

Measurement of distribution ratios of lanthanides

The lower phase (2.5 ml) containing lanthanides (1 m*M* for each lanthanide) was agitated with the same volume of the upper phase for 2 min using a vortex mixer. After the phase separation, the lanthanides were determined by high-performance liquid chromatography (HPLC).

HPLC for the determination of lanthanides

A thermostated stainless-steel column (50 cm × 4 mm I.D.) packed with the ion-exchange resin IEX 215 (Tosoh, Japan) was employed. For the separation of Gd and Sm, the column temperature was adjusted to 45°C and 0.25 *M* 2-hydroxyisobutyric acid (pH 4.5) was used as the eluent. For the separation of Gd and Eu, 0.2 *M* 2-hydroxyisobutyric acid (pH 4.5) was used at 55°C. The eluent was pumped at 0.4 ml/min with a Kyowa (Japan) minipump. Samples (5 μl) were injected with a Tokyo Rikakikai Model 5010 injector. Lanthanides were detected by post-column derivatization with 0.1 m*M* 4-(2-pyridylazo)resorcinol dissolved in 3 *M* ammonium acetate (pH 10), which was fed at 0.8 ml/min by the same pump as that for the eluent. The absorbance was measured at 540 nm with a Uvidec-100-IV UV–VIS detector with a 16-μl flow-through cell (Jasco, Japan) and the data were recorded with a D-2000 data processor (Hitachi, Japan).

CFE

Five pumps (Kyowa, Japan) and two glass columns (both 150 cm × 2 cm I.D.) segmented by PTFE disks with a 6-mm hole were used. The number of cells per column was 130. The columns were equipped with end-caps which contained rotating seals together with an inlet for one phase and an outlet for the other phase, thus allowing for the possibility of dual counter-current separation. The details of the device has been described elsewhere [5]. To begin, the columns were in an upright position. The columns were filled with the upper phase, then fixed at 20° from the horizontal and rotated about the longitudinal axis at 100 rpm. The lower phase was introduced from the upper end of column 2 (Fig. 1) until it reached the lower end of the column 1, then the two phases were fed simultaneously at 0.72 ml/min. The sample feeding pump was also running (0.04 ml/min), however, the lower phase was pumped in. After a steady state had been established, the lanthanide mixture (10 m*M* of each) dissolved in the lower phase was introduced into the CFE system by the sample pump and eluent fractions were collected at appropriate intervals.

RESULTS AND DISCUSSION

Determination of concentration of nitric acid in the two-phase system of CFE

Although CFE is a continuous process, for theoretical purposes, it can be assumed that columns are divided into theoretical equilibration segments. Each segment gives complete equilibration between the two phases. Based on this assumption, theoretical treatment of the discontinuous counter-current distribution process can be applied to CFE.

In these experiments, our intention was to obtain the same amount per unit time for both components. For the separation of components i and j with the same throughput, the flow-rate ratio, r, can be determined by using the relationship $r = (D_iD_j)^{1/2}$, where D_i and D_j are the distribution ratios for the respective components, defined as the ratio of the concentration of the component in the organic phase to that in the aqueous phase. It has been demonstrated empirically that in the two-phase system of n-hexane and water, the same flow-rate for both phases ($r = 1$) gives the most stable counter-current stream. Therefore, the partitioning behaviours of Sm, Eu and Gd were examined in two-phase systems consisting of n-hexane and various concentrations of dilute nitric acid in order to obtain distribution ratios which gave a value of unity for r.

The distribution ratio was found to decrease with increasing concentration of nitric acid, but no significant change was observed for the separation factor, which is the ratio of the distribution ratios to be separated (Fig. 2). Thus, any nitric acid concentration between 0.55 and 0.8 M can be utilized without a decrease in the separation efficiency. A nitric acid concentration of 0.65 M was selected for the separation of Sm and Gd and 0.75 M for the separation of Gd and Eu.

Continuous separation of lanthanides

The continuous separation of Gd and Sm by CFE showed a satisfactory performance. Sm and Gd were obtained continuously over a 2-week period with purities of higher than 99.5% (Fig. 3). Purity is defined as the ratio of the component concentration to the total concentration of all components multiplied by 100. The complete separation of Gd and Eu could not be achieved. At steady state, the separation gave a 97% purity of Gd in the upper phase, whereas the lower phase was only 93% pure in Eu (Fig. 4).

If CFE consists of theoretical equilibration segments in series, the number of theoretical equilibration segments on the left side of the sample feed segment (m for

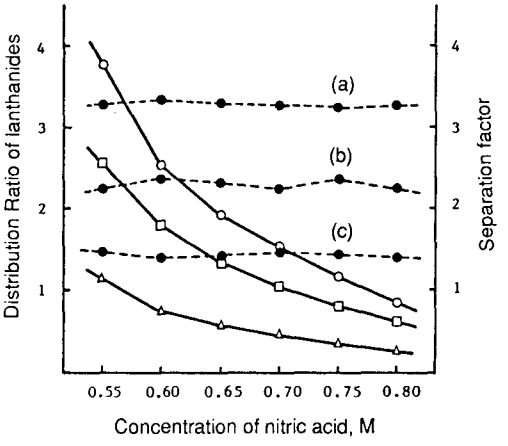

Fig. 2. Effect of nitric acid concentration on distribution ratios and separation factors of Sm, Eu and Gd. Solid lines, distribution ratios of (△) Sm, (□) Eu and (○) Gd; dashed lines, separation factors of (a) Gd/Sm (b) Eu/Sm and (c) Gd/Eu.

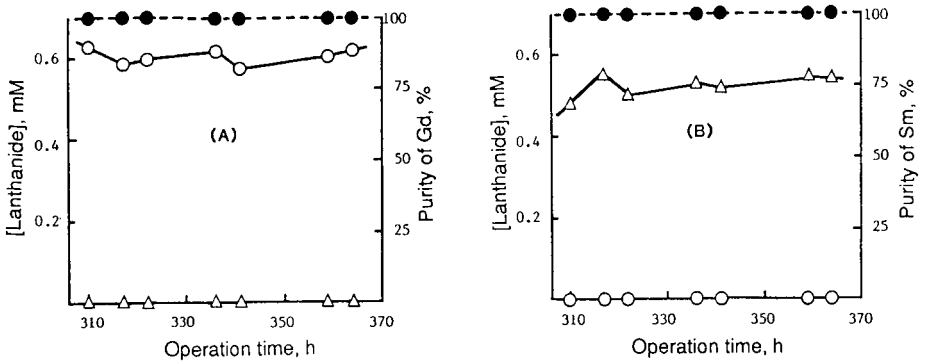

Fig. 3. Concentration and purity of Sm and Gd obtained with continuous separation: (A) upper phase; (B) lower phase. (△) Sm concentration; (○) Gd concentration; (●) purity. The two-phase system used was 0.5 *M* DEHPA in *n*-hexane and 0.65 *M* nitric acid.

column 1) and that on the right side (*n* for column 2) is approximated by solving the equation below using the experimental data. The feed segment is counted twice, hence the total number of segments is $m + n - 1$. The extraction factor (e) of a component is defined as the ratio of the amount of component in the aqueous phase to that in the organic phase; e_1 is the extraction factor in the column 1 and e_2 is that in the column 2. Because the ratio of flow-rates of the two phases (r) is equal to the ratio of the volumes of the two phases, $e = r/D$.

$$\psi = \frac{\text{amount of a component in the upper phase (mol/min)}}{\text{initial amount of a component fed (mol/min)}}$$

$$= \frac{(e_1 - 1)(e_2^m - 1)}{(e_1^{n+1} - 1)(e_2 - 1)e_2^{m-1} + (e_2^{m-1} - 1)(e_1 - 1)}$$

if m and n are assigned valves of 21 and 16, respectively, the calculated values were consistent with the experimental data (Table I). Hence it is estimated that the CFE

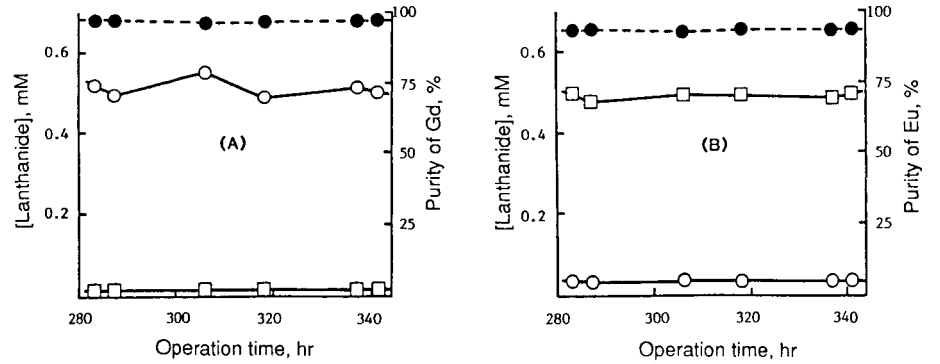

Fig. 4. Concentration and purity of Eu and Gd obtained with continuous separation: (A) upper phase; (B) lower phase. (□) Eu concentration; (○) Gd concentration; (●) purity. The two-phase system used was 0.5 *M* DEHPA in *n*-hexane and 0.75 *M* nitric acid.

TABLE I

COMPARISON OF EXPERIMENTAL VALUES WITH CALCULATED RESULTS FOR THE SEPARATION OF EUROPIUM AND GADOLINIUM BY CFE

Calculation condition: $m = 21$, $n = 16$, $r_1 = 1$, $r_2 = 1.06$, $D_{Eu} = 0.81$, $D_{Gd} = 1.17$, $\psi_{Eu,obs.} = 0.025$, $\psi_{Gd,obs.} = 0.925$, feed amount of each lanthanide $= 4.0 \cdot 10^{-7}$ mol/min.

	Experimental (mol/min)		Calculated (mol/min)	
	Gd	Eu	Gd	Eu
Upper phase outlet	$3.7 \cdot 10^{-7}$	$1.0 \cdot 10^{-8}$	$3.7 \cdot 10^{-7}$	$1.0 \cdot 10^{-8}$
Lower phase outlet	$2.7 \cdot 10^{-8}$	$3.7 \cdot 10^{-7}$	$3.0 \cdot 10^{-8}$	$3.9 \cdot 10^{-7}$

consists of 36 theoretical equilibration segments with each segment being about seven cells of the CFE column. The discrepancy in m and n could be explained by the difference in the flow-rate of lower phase between columns 1 and 2; the flow-rate of the lower phase in the column 1 was 0.76 ml/min because the sample was introduced into the lower phase.

The counter current process consists of two concurrent sub-processes: extraction and washing. For a component to be extracted, one of two solvents involved is used for extraction and the other is used for washing. The component is extracted between the feed point and the inlet of the extraction solvent, and concurrently the undesirable component is washed (back-extracted) between the feed point and the washing solvent inlet. For the other component, the situation is reversed. A given component with $e > 1$ is recovered in the aqueous phase and a component with $e < 1$ is recovered in the organic phase. Because e is a function of r and D, such extraction behaviour is controllable. Unlike conventional CCC, these processes occur simultaneously in the columns. Therefore, a reasonably high purity of extracts can be obtained with a limited number of theoretical equilibration segments. It is obvious from the nature of CFE that a sample can only be separated into two fractions. Thus, for the separation of multiple components in a sample, the number of sets of CFE needed to isolate each component is one less than the number of components.

CFE employs rotation of columns to promote the partitioning of components and the two phases completely fill the columns. This design prevents both the formation of emulsions and the oxidation of sample components, which often happen in discontinuous counter-current systems involving phase dispersion by vigorously mixing to reach equilibrium. The throughput of lanthanides in our experiments was $4 \cdot 10^{-7}$ mol/min. Although further experiments are necessary for preparative-scale separations, this preliminary application demonstrated certain advantages of CFE. Other factors which influence the column efficiency are currently being investigated and further applications of CFE are in progress.

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Separation of rare earth elements by high-speed counter-current chromatography

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ABSTRACT

Besides being widely used in electronic and glass industries, rare earth elements have recently been found to have important biological effects including the ability to stabilize and enhance interferon activity [J. J. Sedmak and S. E. Grossberg, *J. Gen. Virol.*, 52 (1981) 195]. In this paper, the rare earth elements have been separated using a high-speed counter-current chromatography (HSCCC) centrifuge equipped with three multilayer coils connected in series. Two-phase solvent systems were composed of *n*-heptane containing di-(2-ethylhexyl)phosphoric acid (stationary phase) and dilute hydrochloric acid (mobile phase) where the partition coefficient of each can be optimized by selecting the proper hydrochloric acid concentration. The mobile phase was eluted through the column at a flow-rate of 5 ml/min, while the apparatus was rotated at 900 rpm. Continuous detection of the rare earth elements was effected by means of a post-column reaction with arsenazo III and the elution curve was obtained by on-line monitoring at 650 nm. Excellent isocratic separations of closely related rare earth elements were achieved at high partition efficiencies up to several thousand theoretical plates. Versatility of the present method was demonstrated in an exponential gradient elution of hydrochloric acid concentration where fourteen rare earth elements were all resolved in about 4.5 h.

INTRODUCTION

High-speed counter-current chromatography (HSCCC) is the most advanced form of CCC which we have applied to the separations of various organic samples [1–3]. It provides advantages over other CCC methods such as high partition efficiency, rapid separation and the ability to perform dual CCC [1]. Recently, highly efficient chromatographic separations have been achieved using a set of three multilayer coils [4–6].

In the present study, the use of this device is demonstrated in the separation of rare earth elements. In addition to their many uses in the electronic industry, rare earth elements such as lanthanum, cerium, neodymium, samarium, gadolinium, dysprosium, erbium and ytterbium have recently been found to stabilize and enhance interferon activity [7]. The difficulty in separating the lanthanoid elements is mainly due to their formation of trivalent cations with almost equal diameters [8].

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Preparative-scale separation by means of conventional multistage extraction requires a very long time, while efficient analytical-scale separations can be made using high-performance liquid chromatography (HPLC) [9,10] and ion chromatography [11]. Recently, micorgrams to milligram quantities of the rare earth elements have been separated by centrifugal droplet CCC (centrifugal partition chromatography) [12,13]. The results of the present method are compared with those obtained from the centrifugal droplet CCC.

EXPERIMENTAL

Apparatus

The design of the CCC apparatus employed in the present study has been described in detail previously [4]. The apparatus holds a set of three identical columns symmetrically distributed on the rotary frame at a distance of 7.6 cm from the central axis of the centrifuge. Each column holder is equipped with two planetary gears, one of which is engaged with an identical stationary sun gear mounted around the central stationary axis of the centrifuge. This gear arrangement produces a planetary motion of each column holder, *i.e.*, one rotation about its own axis per one revolution around the central axis of the centrifuge in the same direction. The other gear on the column holder is engaged with an identical gear on the rotary tube support mounted between the column holders. This gear arrangement produces counterrotation of the tube support to prevent twisting of the flow tubes on the rotary frame.

All column holders can be removed from the rotary frame by loosening a pair of screws on each bearing block, facilitating the mounting of the coiled column on the holder. Each multilayer coil was prepared from a single piece of approximately 100 m long, 1.07 mm I.D. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) by winding it directly onto the holder hub (15 cm diameter), making 13 layers of the coil between a pair of flanges spaced 5 cm apart. The β values range from 0.5 at the internal terminal to 0.75 at the external terminal. $\beta = r/R$, where r is the distance from the column holder axis to the coil and R the distance from the holder axis to the centrifuge axis. β is a parameter determining the hydrodynamic distribution of the two solvent phases in the rotating coil. Each multilayer coil consists of about 400 helical turns with approximately 90 ml capacity. The apparatus can be operated at speeds up to 1200 rpm.

The flow diagram of the present assembly is illustrated in Fig. 1. A Shimadzu LC-6A pump (pump I) was used to pump the mobile phase, another Shimadzu LC-6A pump (pump II) or stream splitter delivers a portion of the effluent to the spectrophotometer and a Rainin 1011/B-100-S pump (pump III) was used to add the post-column reagent to the effluent. A Shimadzu SPD-6AV spectrophotometer was used to monitor absorbance of the effluent and an LKB fraction collector (Ultrorac) to collect the effluent. At an early stage of development, it was found that the use of a peristaltic pump for pump II produced problems of excessive sample band broadening and damage to the tubing by exposure to *n*-heptane carried over from the stationary phase. These complications were all eliminated by replacing the peristaltic pump with the above Shimadzu LC-6A pump which provided a uniform flow with a small dead space. A Buchler Instrument gradient maker (Model KCI-23A2A1) was used for gradient elution of 14 lanthanoid elements.

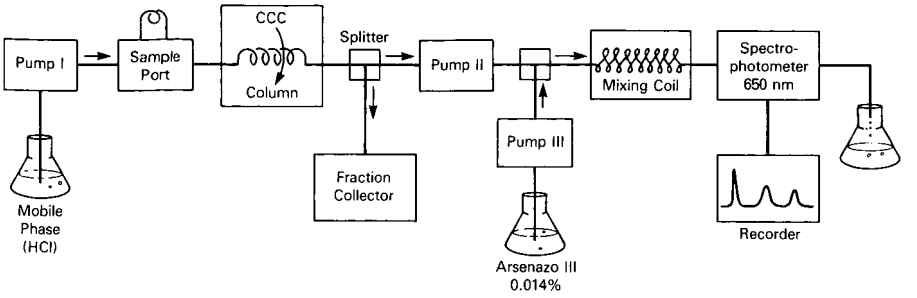

Fig. 1. Flow diagram of instrumentation assembly for separation of rare earth elements by high-speed CCC.

Reagents

n-Heptane of a glass-distilled chromatographic grade was purchased from Burdick and Jackson Labs., Muskegon, MI, U.S.A. Di-(2-ethylhexyl) phosphoric acid (DEHPA) was obtained from Sigma, St. Louis, MO, U.S.A.; 1 *M* hydrochloric acid from Fisher, Fairlawn, NJ, U.S.A. and arsenazo III from Aldrich, Milwaukee, WI, U.S.A. Lanthanoid chlorides including $LaCl_3$, $CeCl_3 \cdot 7H_2O$, $PrCl_3 \cdot 7H_2O$, $SmCl_3 \cdot 6H_2O$, $EuCl_3 \cdot 6H_2O$, $GdCl_3 \cdot 6H_2O$, $TbCl_3 \cdot 6H_2O$, $DyCl_3 \cdot 6H_2O$, $HoCl_3 \cdot 6H_2O$, $ErCl_3 \cdot 6H_2O$, $TmCl_3 \cdot 6H_2O$, $YbCl_3 \cdot 6H_2O$ and $LuCl_3 \cdot 6H_2O$ were obtained from Aldrich and $NdCl_3 \cdot 6H_2O$ from Fluka, Buchs, Switzerland.

Preparation of two-phase solvent and sample solutions

DEHPA was washed several times with 1 *M* hydrochloric acid followed by washing twice with de-ionized water and then dissolved in *n*-heptane (stationary phase). One molar hydrochloric acid was diluted with de-ionized water (mobile phase). Sample solutions were prepared by dissolving various lanthanoid chlorides in 0.02 *M* hydrochloric acid.

Partition coefficient measurement

In order to determine the optimal composition of the solvent system, the partition coefficient (K) for each rare earth element was measured by a simple test tube experiment. Rare earth elements were detected by means of the color reaction [8] with arsenazo III, a dye which forms a complex with rare earths shifting its absorbance maximum from 540 nm to 650 nm. Because quantitation with the color reaction is provided by the aqueous phase, the measurement was performed as follows: Equal volumes (about 3 ml) of the stationary non-aqueous phase (0.02 *M* DEHPA in *n*-heptane) and the mobile aqueous phase (dilute hydrochloric acid) were equilibrated in a separatory funnel at room temperature, the two phases were separated and the absorbance of the lower aqueous phase (A_O) was measured. Then a given amount of each rare earth element was dissolved in the lower phase and the solutions thoroughly equilibrated with an equal volume of the upper non-aqueous phase. The absorbance of the aqueous phase was measured before (A_T) and after (A_L) equilibration with the upper phase. From these absorbance values, the partition coefficient (K_T) obtained by the test tube measurement is given by:

$$K_T = (A_T - A_L)/(A_L - A_O) \quad (1)$$

The partition coefficient of each component was also calculated from the elution curve by:

$$K_E = (R - R_{SF})/(V_C - R_{SF}) \quad (2)$$

where R is the retention volume of the peak maximum, R_{SF} the retention volume of the solvent front and V_C the total column capacity.

Separation procedure

Each separation was initiated by filling the entire column with the stationary non-aqueous phase followed by injection of 100 μl sample solution containing 25 μg of each component through the sample port. Then the mobile aqueous phase was eluted through the column at a rate of 5 ml/min in the proper elution mode while the apparatus was rotated at 900 rpm. Continuous detection of the rare earth elements was effected by means of a post-column reaction with arsenazo III [9] and the elution curve determined by monitoring the effluent at 650 nm using a Shimadzu SPD-6AV spectrophotometer equipped with an analytical flow cell. The effluent was divided into two streams with a tee adapter and a low-dead-volume Shimadzu LC-6A pump (pump II) used to deliver a portion of the effluent at a flow-rate of 1.4 ml/min to the spectrophotometer (see Fig. 1). At the outlet of this pump, the arsenazo III–ethanol solution (0.014%, w/v) was continuously added to the effluent stream at a flow-rate of 2.7 ml/min with a Rainin metering pump (pump III). The resulting effluent was first passed through a narrow mixing coil (PTFE tube, 1 m × 0.55 mm I.D.) immersed in a water bath heated to *ca.* 40°C and then led through an analytical flow cell (1 cm light path) of a Shimadzu SPD-6AV spectrophotometer set to monitor the absorbance at 650 nm. The other effluent stream through the tee adapter was either collected or discarded (see Fig. 1).

Measurement of partition efficiencies

From each chromatogram the partition efficiency of the separation was calculated and expressed in terms of theoretical plates (TP) according to the equation:

$$N = (4R/W)^2 \quad (3)$$

where N is the partition efficiency in TP, R the retention time or volume of the peak maximum and W the peak width expressed in the same units as R.

The partition efficiency can also be expressed in terms of peak resolution using the formula:

$$R_s = 2(R_1 - R_2)/(W_1 + W_2) \quad (4)$$

where R_s is the resolution of two adjacent peaks measured in units of 4 δ in a Gaussian distribution, R_1 and R_2 are the retention times or volumes of two adjacent peaks ($R_1 > R_2$) and W_1 and W_2 are the widths (4 δ) of the corresponding peaks. When $R_s = 1.5$, baseline separation (99.7% pure) is indicated.

RESULTS AND DISCUSSION

The optimum range of the partition coefficient values (K_T) for neodymium, praseodymium, cerium and lanthanum was obtained with a solvent pair composed of 0.02 *M* DEHPA in *n*-heptane (stationary phase) and 0.02 *M* hydrochloric acid (mobile phase). Fig. 2 shows K_T values of these elements each plotted against the sample concentration from 0.005 to 0.025 m*M* in the mobile phase. As indicated in the diagram, the left chart was obtained from the ligand (DEHPA) treated with 1 *M* hydrochloric acid before being dissolved in *n*-heptane and the right chart was obtained from the ligand without the hydrochloric acid treatment. Using the above solvent systems, lanthanum, praseodymium and neodymium were separated at a flow-rate of 5 ml/min at a revolution speed of 900 rpm. Fig. 3A shows a chromatogram obtained from the solvent without the hydrochloric acid pretreatment. The three lanthanoid elements were well resolved in 2.5 h but each peak displays marked skewness as expected since K_T increases with decreased sample concentration (Fig. 2, right). This effect was largely eliminated by using the hydrochloric acid-treated ligand as illustrated in Fig. 3B. The maximum column pressure during these separations was about 300 p.s.i.

From these chromatograms, the partition coefficients (K_E) were computed for each peak using eqn. 2 and compared with the K_T values obtained from the test tube measurements (Fig. 2). As shown in Table I, the two partition coefficient values, K_E and K_T, are generally in a close agreement in both groups. With both methods the non-treated groups shows substantially higher K values than the hydrochloric acid-treated group, especially with lanthanum and praseodymium.

The partition efficiencies of the separation were determined from the chromatogram (Fig. 3B) using eqns. 3 and 4: These range from 5900 theoretical plates (TP) for the first peak (lanthanum) to 520 TP for the third peak (neodymium), while the peak resolution between the first and the second peak is 6.94 and that between the second and the third 1.74. Another set of lanthanoid elements (thulium, ytterbium and lutetium), which are most difficult to resolve [13], was also separated with the present method using a modified solvent composition of 0.003 *M* DEHPA in *n*-heptane as

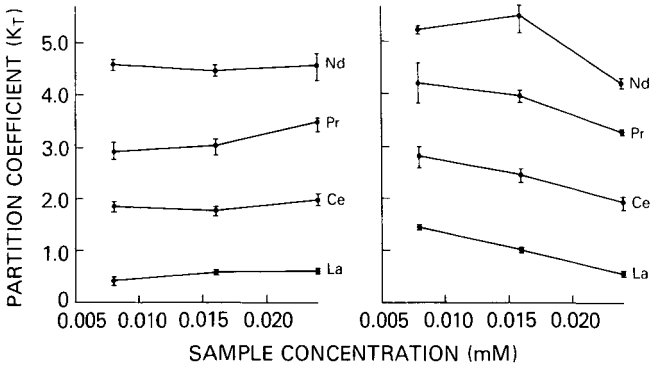

Fig. 2. Effects of hydrochloric acid treatment of ligand on the partition coefficients of light rare earth elements. (A) After washing with 1 *M* hydrochloric acid; (B) without pretreatment.

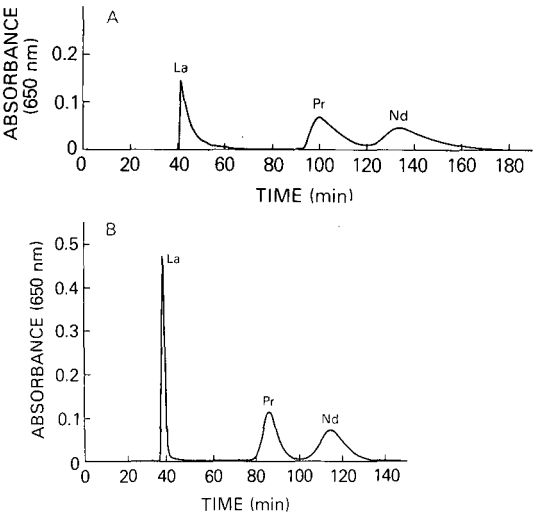

Fig. 3. Isocratic separation of lanthanum, praseodymium and neodymium with non-treated (A) and hydrochloric acid-treated (B) ligands by high-speed CCC. The experimental conditions were as follows: apparatus = HSCCC centrifuge with 7.6 cm revolution radius; column = three multilayer coils connected in series, 300 m × 1.07 mm I.D., 270 ml capacity; stationary phase = 0.02 *M* DEHPA in *n*-heptane; mobile phase = 0.02 *M* hydrochloric acid; sample = $LaCl_3$, $PrCl_3$ and $NdCl_3$ each 0.001 *M* in 100 μl of 0.02 *M* hydrochloric acid; revolution = 900 rpm; flow-rate = 5 ml/min; pressure = 300 p.s.i.

stationary phase and 0.1 *M* hydrochloric acid as mobile phase. The results showed much reduced partition efficiencies ranging from 170 TP for the first peak (thulium) to [illegible] TP for the third peak (lutetium).

In Table II these results are compared with those obtained by other CCC methods where N is the partition efficiency in TP, R_s is the peak resolution between the indicated two peaks and CDCCC stands for centrifugal droplet CCC (centrifugal partition chromatography). In both N and R_s groups, the middle column (CDCCC[b]) [12] indicates the efficiencies obtained using the same solvent pairs used in the present HSCCC method and the right column (CDCCC[c]) improved efficiencies with

TABLE I

PARTITION COEFFICIENT VALUES

Upper phase: 0.02 *M* DEHPA in *n*-heptane; lower phase: 0.02 *M* hydrochloric acid.

Element	K_E (average)		K_T	
	No washing[a]	Washing[b]	No washing[a]	Washing[b]
Lanthanum	0.5	0.3	0.48–1.40	0.33–0.59
Praseodymium	3.2	2.8	3.25–4.23	2.75–3.51
Neodymium	4.7	4.5	4.15–5.72	4.25–4.78

[a] DEHPA was dissolved in *n*-heptane without pretreatment.
[b] DEHPA was washed several times with 1 *M* hydrochloric acid before dissolving in *n*-heptane.

TABLE II

COMPARISON IN PARTITION EFFICIENCIES BETWEEN HSCCC AND PREVIOUS METHODS

Element	N^a			R_s^b		
	HSCCC[c]	CDCCC[d]	CDCCC[e]	HSCCC[c]	CDCCC[d]	CDCCC[e]
Lanthanum	5900	34	169.0	6.94	1.4	2.70
Praseodymium	770	54	85.8	1.74	0.44	0.77
Neodymium	520	41	86.2			
Thulium	170	–	19.5	1.24	–	–
Ytterbium	74	–	–	0.79	–	–
Lutetium	61	–	–			

[a] N: Theoretical plate numbers.

[b] R_s: Peak resolution.

[c] 0.02 M DEHPA in n-heptane (stationary phase) and 0.02 M hydrochloric acid (mobile phase) were used for the separation of lanthanum, praseodymium and neodymium. 0.003 M DEHPA in n-heptane and 0.1 M hydrochloric acid were used for the separation of thulium, ytterbium and lutetium.

[d] The values were obtained from centrifugal droplet CCC or centrifugal partition chromatography with the same solvent system [12].

[e] The values were obtained from centrifugal droplet CCC with 2-ethylhexyl-phosphonic acid mono-2-ethylhexyl ester (EHPA) at 55°C [13].

a modified stationary phase containing 2-ethylhexyl-phosphonic acid mono-2-ethylhexyl ester (EHPA) at a higher temperature (55°C) [13]. As seen from the table, the present method yields much higher efficiencies than those produced by the other CCC methods. The separation time was also much shorter in HSCCC (2 h) than in the other two methods (3–30 h).

To further demonstrate the present method, a one-step separation of all 14 lanthanoid elements was performed by applying an exponential gradient of hydrochloric acid concentration in the mobile phase. The main problem in gradient elution is that the optimum range of the ligand concentration in the stationary phase is substantially different between the lighter and heavier groups of the rare earth elements. Because the separation of the heavy elements including thulium, ytterbium and lutetium is more difficult, the ligand concentration in the present experiment was selected at 0.003 M for best resolution. Consequently, the resolution of the light lanthanoid elements such as lanthanum, cerium, praseodymium and neodymium became less efficient compared with that observed in the isocratic separation shown in Fig. 3B. Fig. 4 shows the chromatogram of all 14 lanthanoid elements resolved in less than 5 h.

As indicated in Table II, the HSCCC method radically improved the results compared to the other CCC methods. The higher performance of the HSCCC over the CDCCC may be explained on the basis of the hydrodynamics inherent to each system. In the hydrostatic equilibrium system such as the CDCCC, mixing of the two solvent phases relies entirely on the flow of the mobile phase [14]. Due to the high interfacial tension between the two solvent phases used in the present separation, lack of a mixing force tends to form large droplets of the mobile phase, limiting the partition efficiency. On the other hand, HSCCC is a typical hydrodynamic equilibrium system where two solvent phases are vigorously mixed by the effect of planetary motion of the coil [1].

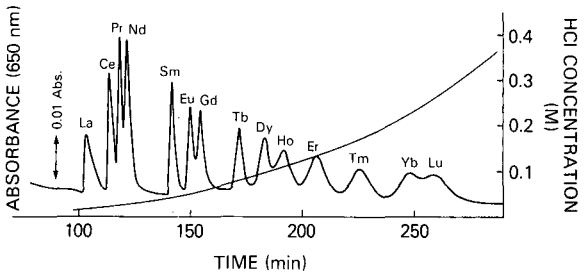

Fig. 4. Gradient separation of 14 rare earth elements obtained by high-speed CCC. The experimental conditions were as follows: apparatus = HSCCC centrifuge with 7.6 cm revolution radius; column = three multilayer coils connected in series, 300 m × 1.07 mm I.D., 270 ml capacity; stationary phase = 0.003 *M* DEHPA in *n*-heptane; mobile phase = exponential gradient of hydrochloric acid concentration from 0 to 0.3 *M* as indicated in the chromatogram; sample = 14 lanthanoid chlorides each 0.001 *M* in 100 μl water; revolution = 900 rpm; flow-rate = 5 ml/min; pressure = 300 p.s.i.

Small droplets of the mobile phase produced by the vigorous mixing reduce the mass transfer resistance between the two phases resulting in high partition efficiency. Compared with HPLC and ion chromatography, the present method will provide a higher capacity for preparative-scale separations.

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Purification of a synthetic myristylated peptide by counter-current chromatography

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ABSTRACT

A preparative purification of myristyl-Gly-Asn-Ile-Phe-Ala-Asn-Leu-Phe-Lys-Gly-Leu-Phe-Gly-Lys-Lys-Glu-NH_2 was accomplished using the multi-coil counter-current chromatograph. A partition coefficient was determined in the *n*-butanol–acetic acid–water (4:1:5) system. Chromatographic runs were made in this system and one modified with ethyl acetate. The peptide material showed anomalous elution behavior due to its surfactant properties. It was found that by loading the sample exclusively in the stationary phase, satisfactory retention of the compound occurred. Finally, conditions utilizing the upper phase as the mobile phase successfully separated the impurities.

INTRODUCTION

Modification of the amino terminal glycine by myristic acid has been shown to occur in a number of the protein components of signal transduction pathways (for a review see ref. 1). The role of the myristate moiety has been shown to be important in the reversible membrane association of these regulatory proteins [2]. The amino acid composition of peptides synthesized for their study are usually quite polar because of the presence of multiple lysine residues. However, the fatty acyl chain attached to the amino terminus renders the compound highly surfactant in solubility characteristics. The peptides, while being water soluble, are quite hydrophobic and are highly retained on reversed-phase columns. Over the years we have used semi-preparative reversed-phase chromatography for purification of synthetic myristylated peptides, but occasionaly we have had to resort to counter-current chromatography to effect final purification.

The peptide $CH_3(CH_2)_{12}CO$-Gly-Asn-Ile-Phe-Ala-Asn-Leu-Phe-Lys-Gly-Leu-Phe-Gly-Lys-Glu-NH_2, corresponding to the amino terminus of the ADP ribosylation factor [3], was synthesized for use in biochemical studies aimed at elucidating the role of the amino terminus in the activity and subcellular distribution of the 21 000-dalton GTP-binding protein. This sequence has a strong cationic charge, re-

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sulting from the three lysine side chains. The tetradecanoyl group and three phenylalanine residues make the peptide very hydrophobic, as well, Thus the peptide is only eluted from C_{18} columns in a high concentration of acetonitrile. When a preparative purification of 177 mg of this peptide was attempted on a 1-in reversed-phase column, less than 10 mg of material was recovered (unpublished results). The peptide, having been loaded in water on to the column, may have precipitated on the column in the conditions of the chromatographic run [4]. Therefore, we turned to counter-current chromatography in an effort to purify the compound with higher yield.

EXPERIMENTAL

Materials

The solvents used in the synthesis and chromatography are reagent grade or high-performance liquid chromatographic (HPLC) grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Water is purified through a Nanopure II system (Barnstead, Boston, MA, U.S.A.). Myristyl-Gly-Asn-Ile-Phe-Ala-Asn-Leu-Phe-Lys-Gly-Leu-Phe-Gly-Lys-Lys-Glu-NH_2 was synthesized by manual solid-phase synthesis [5] starting with 0.8 mmole Boc γ-benzyl-L-Glu *p*-methylbenzhydrylamine resin to which the *tert.*-butyloxycarbonyl (Boc) amino acid derivatives (Peninsula Labs., Belmont, CA, U.S.A. and Bachem, Torrance, CA, U.S.A.) were coupled for 2 h in 2.5 × molar excess over the first amino acid with equimolar dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt) which generate active esters *in situ*. The amino-protecting Boc groups are removed by 25% trifluoroacetic acid in dichloromethane (DCM) with indole, 1 mg/ml, for 30 min to prevent oxidation followed by DCM washes and neutralization with 10% triethylamine in DCM for 10 min. The steps are repreated as necessary to couple all the amino acids in sequence. 2-Chloro-carbobenzoxy-L-Lys was the Boc derivative used for Lys and Boc-L-Asn was used without side chain protection. After coupling of the last amino acid and deprotection, one half of the resin was removed to isolate the unmyristylated peptide and the rest was reacted with 1 mmole of myristic acid (Sigma, St. Louis, MI, U.S.A.) with DCC and HOBt in dimethyformamide (Burdick & Jackson, Muskegon, MI, U.S.A.). The couplings were monitored by the qualitative ninhydrin test [6]. The peptide resin was submitted to hydrogen fluoride, 10 ml/g resin, in the presence of anisole, 1 ml/g, for 45 min at 0°C. This reaction cleaved the peptide and simultaneously removed the side-chain-protecting groups. After evaporation of the hydrogen fluoride the peptide resin was washed with diethyl ether and dried. The peptide was extracted in water and lyophilized to give 1.15 g powder. The product and purified peptide were analyzed by HPLC as described below and amino acid analysis using a dedicated HPLC for ion-exchange chromatography and post-column fluorescence detection (St. Johns Assoc., Beltsville, MD, U.S.A.) [7]. The molar ratios found for the peptide are Asp, 1.75; Glu, 1.12; Gly, 2.99; Ala, 0.94; Ile, 1.04; Leu, 2.18; Phe, 3.30; Lys, 2.67.

Analytical chromatography

Analytical chromatography was conducted in Waters/Millipore equipment (Milford, MA, U.S.A.) including a U6K injector, dual extended flow 510 pumps, Model 481 variable-wavelength detector, Model 680 gradient controller and a SE 120 recorder. Analytical columns used were YMC-Pack 5-μm spherical, ODS, 200 Å, 150

× 6 mm I.D. (YMC, Morris Plains, NJ, U.S.A.) or μBondapak C_{18}, 250 × 3.9 mm I.D. (Waters, Milford, MA, U.S.A.). The separations of 10–50-μg sample were made in 0.1% aqueous phopshoric acid and gradients of acetonitrile at a flow-rate of 0.8 ml/min with detection at 215 nm or 254 nm according to previously published procedures [8].

Counter-current chromatography

Preparative chromatography was carried out in the multi-coil counter-current chromatograph (Peptide Technologies Corp., Washington, DC, U.S.A.) equipped with eight multi-layer coils in series on two column holders each carrying four coils mounted opposite each other across the axis of rotation. The apparatus has been described previously [9,10] and is shown in Fig. 1. The coils of continuous 1.6 mm I.D. PTFE tubing are filled with the stationary phase of a two-phase solvent system previously equilibrated in a separatory funnel and separated. The solvents used in these experiments are *n*-butanol–acetic acid–water (BAW) (4:1:5, v/v and ethyl acetate–*n*-butanol–acetic acid–water (3:1:1:5, v/v). The sample dissolved in equal volumes of the upper and lower phases is loaded into the tubing, then the rotation is started at 500 rpm and the mobile phase is pumped using a minipump (LDC/Milton

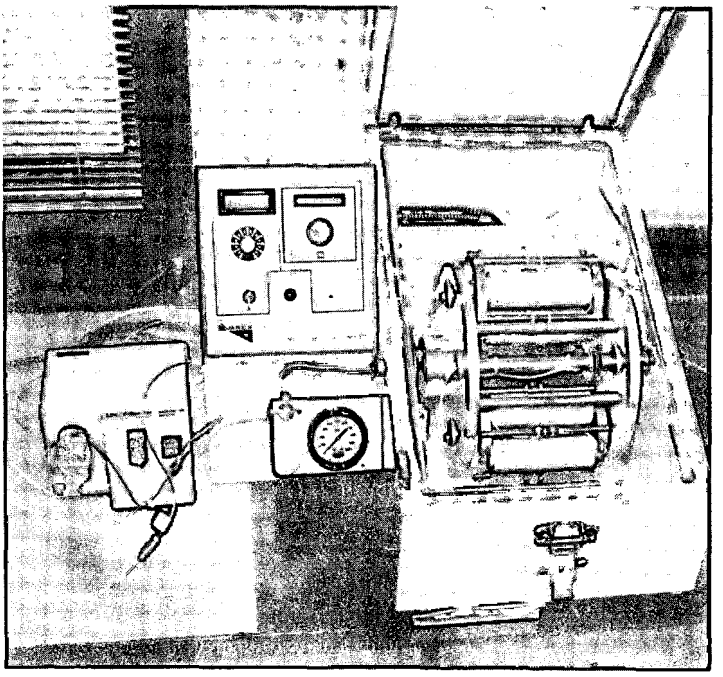

Fig. 1. The multi-coil countercurrent chromatograph that is an Ito coil planet centrifuge with eight multi-layer coils connected in series with four mounted on one column holder counterbalanced by the other four on the opposite side. Tubing enters left side with a "T" connection to a pressure gauge through the center shaft to coiling system and exits at the right side. The tubing is connected to a fraction collector. Solvent delivery is by a minipump (LDC/Milton Roy). The table-top apparatus was built at Varex Corporation, Burtonsville, MD, U.S.A.

Roy, Riviera Beach, FL, U.S.A.) at 1 ml/min. Fractions of 15 min are collected in a fraction collector (LKB, Gaithersburg, MD, U.S.A.) and presence of peptide is determined by reading the absorbance at 254 nm manually in a diode array spectrophotometer (Hewlett-Packard, Mountain View, CA, U.S.A.). After elution of a few column volumes the contents are pushed out with helium or nitrogen pressure, 20 p.s.i., and fractions collected. A small amount of acetone, approximately 50 ml, is pumped in and finally the tubing is dried by a stream of helium or nitrogen. Fractions containing peptide are analyzed by HPLC to determine purity. Those collected are evaporated in a rotary evaporator and lyophilized from water to yield a white powder.

RESULTS AND DISCUSSION

The product from the solid-phase synthesis of the myristylated peptide appeared heterogeneous by analytical HPLC due to difficulty in the coupling of some amino acids. Steric hindrance in the coupling was caused by the combination of the presence of particular amino acids that are difficult to couple, such as Asn, and the overall structure being hydrophobic with the tendency to interact with the resin or become involved in β bends. Nevertheless, a major component was present in the synthetic product as seen in the analytical chromatogram (Fig. 2). Fig. 2 shows the analysis of the sample in the two phases of the BAW solvent system. The ratio of the heights of the major peak in the upper phase to that of the lower phase is 1.4, an estimation of the partition coefficient. It was therefore decided to chromatograph this peptide in this system with the lower phase mobile. The peptide would be expected to elute well after the solvent front in these conditions.

The result of the counter-current chromatography of approximately 200 mg of crude peptide combined with peptide previously purified by HPLC was that the sam-

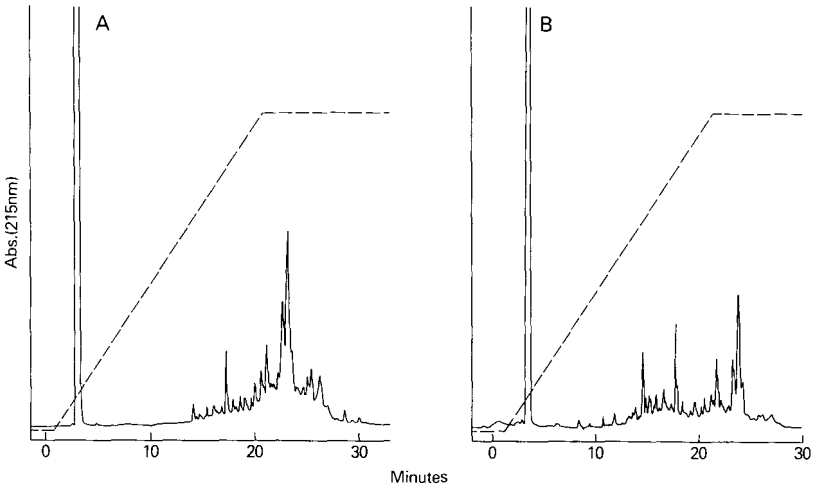

Fig. 2. Analytical HPLC of unpurified peptide distributed in upper phase (A) and lower phase (B) of the *n*-butanol–acetic acid–water (4:1:5) two-phase solvent system. Approximately 20 μg sample applied to the YMC-Pack 5-μm ODS column in 0.1% aqueous phosphoric acid and gradient of acetonitrile (broken line) from 10% to 70% in 20 min at 0.8 ml/min. Detection is at 215 nm at 0.5 a.u.f.s.

ple was eluted with the solvent front. There was some fractionation of the material. Some fractions contained more hydrophilic impurities, but no fraction contained the completely purified peptide (not shown). Also a lot of mass that was very heterogeneous was separated from the peptide and remained in the coil. Apparently, the peptide comprises a minor part of the total crude mass. Thus, this may be the reason for the observed low mass recovery of the desired peptide from the column chromatography. Another solvent system was tried, one modified with ethyl acetate to lower the partitioning of the compound into the upper phase with the lower phase being run again as the mobile phase. Poor fractionation resulted again with the sample at the solvent front. This solvent system was tried again with the upper phase mobile. The compound eluted very late as shown in Fig. 3. Since the absorbance values were low, the presence of peptide was verified by HPLC. Peptide was distributed from fractions 100 to 115 but with a major impurity as shown in Fig. 4. From this run there was 10 mg of 86% pure peptide and 13.2 mg of less pure peptide.

It was hypothesized that the elution of the peptide was affected by how it was dissolved prior to loading. In the previous runs the sample was dissolved in both phases and introduced into the tubing. Since the peptide is both highly cationic and hydrophobic it is probably lowering the surface tension of the solvent system and traveling at the interface. Therefore, it was decided to re-chromatograph 26 mg of a previous fraction 36 of the first counter-current chromatographic run. The conditions were in the original BAW system with the upper phase as the mobile phase and dissolving the compound only in the lower stationary phase. Thus, the coil was filled with lower phase, the sample charged in approximately 5 ml of the aqueous phase, then the upper phase was chromatographed. The result is shown in Fig. 5. A peak was

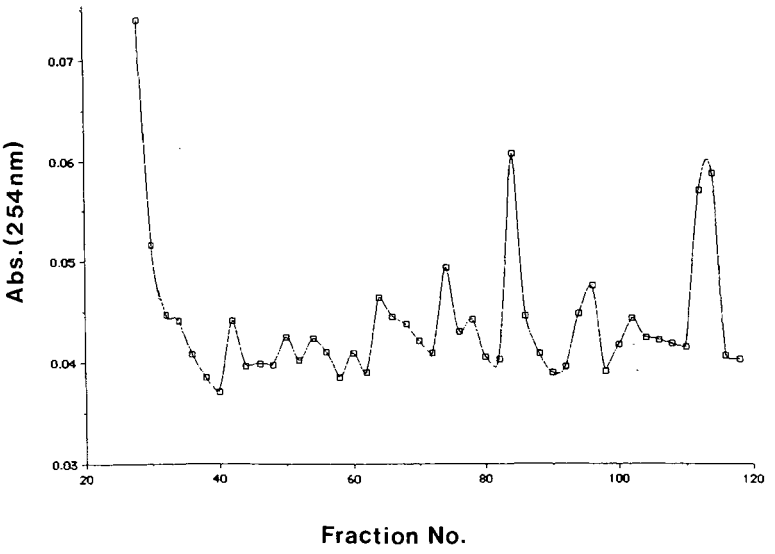

Fig. 3. Counter-current chromatography of sample recovered from a previous separation in the solvent system ethyl acetate–*n*-butanol–acetic acid–water (3:1:1:5) with the upper phase mobile. Aliquots of the fractions collected were diluted in 50% aqueous methanol and absorbance at 254 nm was read. Peptide was contained in fractions 100 to 115.

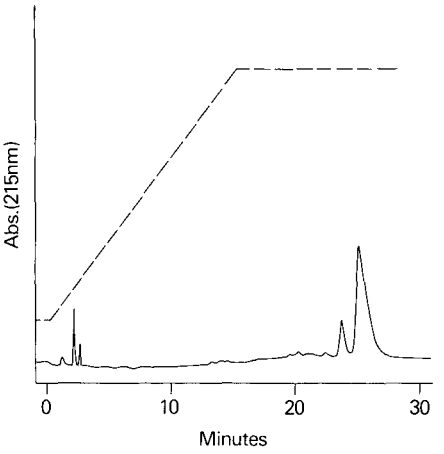

Fig. 4. Analytical chromatography of fraction 109 which contained 10 mg peptide. Separation is on a μBondapak C_{18} column in the same solvent system but with a gradient of 20% to 60% acetonitrile at 0.8 ml/min in 15 min. This was the purest fraction which appeared 86% pure.

eluted at fractions 32–40, after the solvent front which occurred at fraction 27. The material in the major part of the peak had far less contaminant and most of the material was recovered. Pure peptide was 18 mg out of 24.7 mg recovered in all the fractions. In Fig. 6 is shown the analytical HPLC of the recovered peptide compared with the sample loaded. The partition coefficient calculated from the elution volumes of the run is 1.05. From this experience it may be important to consider loading the sample in the stationary phase instead of both phases if there is a possibility that the compound has surfactant properties.

Since there is no more crude peptide left it cannot be determined whether these final conditions would have purified the compound in one step. But it is evident from this work that solid-phase synthesis products of hydrophobic peptides have a significant amount of heterogeneous side-product mass that is conveniently removed by

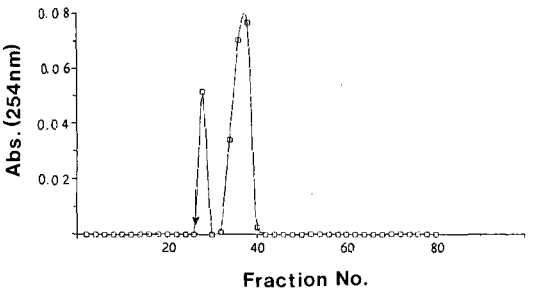

Fig. 5. Counter-current chromatography of 26 mg of a semi-purified sample (shown in Fig. 6) in the BAW system with the upper phase mobile. The solvent front emerged at fraction 27, indicated by arrow, and purified peptide was contained in fractions 33–37. The remainder of the peak contained more hydrophillic impurities.

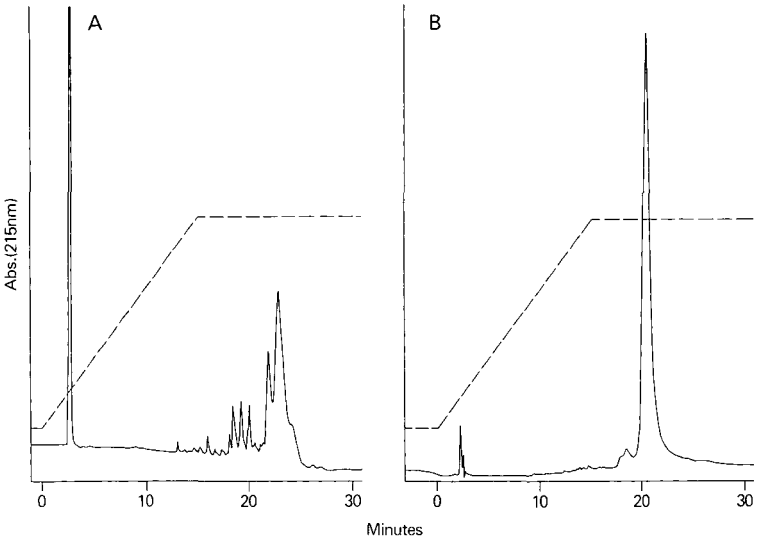

Fig. 6. (A) Analytical HPLC of sample loaded in the counter-current chromatography of Fig. 5. (B) Analysis of peptide recovered from the chromatography of Fig. 5. Conditions are as described in the legend to Fig. 4, except that the flow-rate in B was 1 ml/min.

counter-current chromatography instead of being deposited on expensive column packing.

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Purification of Food Color Red No. 106 (Acid Red) using high-speed counter-current chromatography

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ABSTRACT

High-speed counter-current chromatography (HSCCC) has been successfully applied to the separation of the components of Food Color Red No. 106 (R-106). The separation was performed using 25 mg of the sample with a two-phase solvent system composed of *n*-butanol and 0.01 *M* trifluoroacetic acid (1:1, v/v). Analyses by thin-layer chromatography, high-performance liquid chromatography and fast atom bombardment mass spectrometry confirmed that HSCCC was effective in the purification of the components of R-106. The separation gave 21 mg of a 99.9% pure main component (Acid Red) and 0.9 mg of 98.0% pure subsidiary dye which is probably a des-ethyl derivative.

INTRODUCTION

Many synthetic dyes are widely used for coloring foods, drugs and cosmetics. However, these synthetic colors have impurities deriving from the reactants and side products during the manufacturing process [1–4]. In the investigation of colorless organic impurities, main and subsidiary dyes are required for toxicological and structural evaluation for the purposes of sanitation and good manufacturing practice (GMP). High-performance liquid chromatography (HPLC) has been shown to be a powerful technique for the determination of main dyes and also subsidiary dyes in commercial colors [5–8], foods [9,10] and cosmetics [11,12]. Unfortunately, highly pure components of colors as reference standards are not commercially available for

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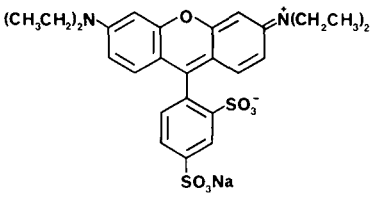

Fig. 1. Structure of the main component (Acid Red) in Food Color Red No. 106. Molecular weight: 580.

chemical analysis. Recently, however, the successful application of counter-current chromatography, a liquid–liquid partition method developed by Ito *et al.* [13], has been reported for the purification of synthetic colors [14–16].

In this study, high-speed counter-current chromatography (HSCCC) [13] was used for the purification of Food Color Red No. 106 (Acid Red, Color Index No. 45 100), which contains a synthetic xanthene-class color (Fig. 1) and at least one major subsidiary dye. Further analysis using HPLC and fast atom bombardment mass spectrometry (FAB-MS) served for the identification of the main and subsidiary dyes.

EXPERIMENTAL

Reagents

Acetonitrile, *n*-butanol, ammonium acetate, sodium chloride, potassium chloride, glycerol and trifluoroacetic acid (TFA) were of analytical-reagent grade and were purchased from Wako (Osaka, Japan). Food Color Red No. (R-106) was obtained from San-ei Chemical Industries (Osaka, Japan).

High-performance liquid chromatography

A chromatograph equipped with a constant-flow pump (LC-6A; Shimadzu, Kyoto, Japan) was used with a variable-wavelength UV detector (SPD-6A; Shimadzu) operated at 254 nm and a chromatographic data system (C-R3A; Shimadzu). The separation was performed on Wakosil $5C_{18}$ (5 μm) (250 × 4.6 mm, I.D.) (Wako) with 0.01 *M* TFA–acetonitrile (70:30, v/v) as the mobile phase at a flow-rate of 1.0 ml/min.

Thin-layer chromatography (TLC)

After applying a sample to a C_{18}-modified silica gel TLC plate (E. Merck, Darmstadt, F.R.G.; 15389), the plate was developed with 1 *M* sodium chloride–acetonitrile (67:33, v/v).

Measurement of partition coefficient

Approximately 1 mg of the test sample was weighed in a 10-ml test-tube and 2 ml of each phase of pre-equilibrated two-phase solvent system was pipetted in. The test-tube was stoppered and shaken vigorously for 1 min to equilibrate the sample thoroughly with the two phases. The resulting upper and lower phases were analysed by HPLC. Each partition coefficient was determined by dividing the corresponding peak area of the upper phase by that of the lower phase.

High-speed counter-current chromatography

The apparatus used was a Shimadzu HSCCC-1A prototype multi-layer coil planet centrifuge with a 10-cm orbital radius which produces a synchronous planetary motion at 800 rpm. The multi-layer coil was prepared by winding a *ca.* 160 m length of PTFE tubing onto the column holder with a 10-cm hub diameter and a 15-cm hub length, making six coiled layers with a total capacity of about 300 ml. The two-phase solvent system used was *n*-butanol–0.01 *M* TFA solution (1:1), which was thoroughly equilibrated in a separating funnel by repeated vigorous shaking and degassing at room temperature. The column was first entirely filled with the upper non-aqueous stationary phase, then 25 mg of the sample dissolved in 2 ml of both phases was loaded. The centrifuge was rotated at 800 rpm, while the lower aqueous mobile phase was pumped into the head of the column (the head–tail relationship of the rotating coil is conventionally defined by the Archimedean screw force, where all objects of different densities are driven toward the head of the coil) at a flow-rate of 2 ml/min by HPLC pump (LC-6A). The effluent from the outlet of the column was continuously monitored with an SPD-6A UV detector at 254 nm and then fractionated into test-tubes at 2 ml per tube with a fraction collector (DF-2000; Tokyo Rikakikai, Tokyo, Japan). When separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas combined with slow rotation of the coil in the tail-to-head elution mode. A 0.2-ml volume of the contents of each test-tube was diluted with distilled water and the absorbance was determined with a Ubest-50 UV–visible spectrophotometer (Japan Spectroscopic, Tokyo, Japan) at 254 nm.

Fast atom bombardment (FAB) mass spectrometry

The FAB mass spectra were obtained on a JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan). A xenon ion gun was operated at 6 kV. The matrix used was glycerol and the samples were dissolved in distilled water.

RESULTS AND DISCUSSION

Selection of two-phase solvent system

The partition coefficient (K) of the solute is the most important factor in selecting a suitable solvent system for HSCCC, because it determines the retention time and the resolution of the solute peak [13]. In general, the K value is determined simply by measuring the UV absorbance of the solute in both phases after partitioning in the two-phase solvent system [15–17]. However, when the sample is a mixture of various components such as Food Color Red No. 106 (R-106), a precise K value of each component cannot be determined by the above method. Although TLC could separate the orginal R-106 into only three spots, as shown in Fig. 2A, HPLC was able to separate it into nine components under the conditions described under Experimental. The components corresponding to peaks 7 and 9 are assumed to be a major subsidiary dye and Acid Red, respectively. After partitioning with the two phase solvent system, the resulting upper and lower phases were analysed by HPLC and the K values of peaks 7 and 9 could be determined.

Acid Red has a dipolar ion structure and is freely soluble in water, but it is difficult to dissolve it in organic solvents such as chloroform and ethyl acetate. First,

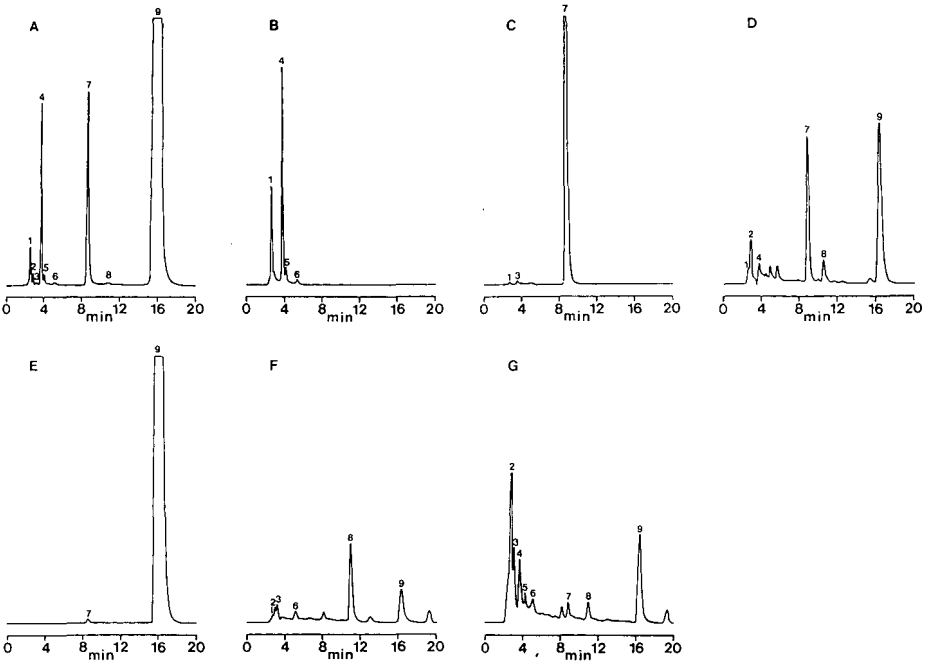

Fig. 2. HPLC separation of the components of Food Color Red No. 106. (A) Original Food Color Red No. 106; (B) fraction I; (C) fraction II; (D) fraction III; (E) fraction IV; (F) fraction V; (G) fraction VI.

we tried to use a mixture of *n*-butanol and water, which has been frequently applied as the solvent system in the separation of hydrophilic compounds [15,16]. However, the *K* value of the component corresponding to the peak 9 was 0.23, as shown in Table I. This indicates that the component is dissolved mostly in the lower aqueous phase and suggests that it is impossible to obtain a satisfactory separation using this solvent system, because the best separation is attained when the solute is partitioned evenly into both phases, namely $K = 1$ [13]. Next, *n*-butanol–0.01 *M* ammonium acetate solution (1:1), which is often used for the separation of synthetic peptides, was tested

TABLE I

PARTITION COEFFICIENTS (*K*) OF THE COMPONENTS OF FOOD COLOR RED NO. 106

K = Peak area of upper phase divided by peak area of lower phase.

Solvent system	Peak No.								
	1	2	3	4	5	6	7	8	9
n-Butanol–water (1:1)	0.89	0.19	0.84	0.00	0.00	0.00	0.13	∞	0.23
n-Butanol–0.01 *M* ammonium acetate solution (1:1)	1.45	0.19	1.46	0.00	0.02	0.00	0.34	∞	0.53
n-Butanol–0.01 *M* TFA solution (1:1)	0.05	0.15	11.70	0.00	0.00	0.00	0.65	∞	1.10

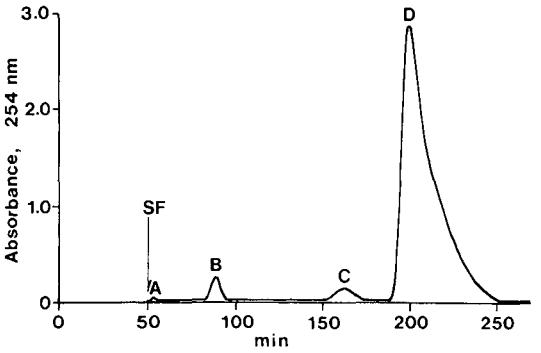

Fig. 3. Separation of Food Color Red No. 106 by HSCCC. SF = solvent front.

[18,19] but it still showed insufficient K values. In order to partition the components more readily into the upper organic phase, aqueous TFA solution was used [19]. The resulting k values were much improved using n-butanol–0.01 M TFA (1:1). These K values indicate that peaks 1, 2, 4, 5 and 6 are first eluted at the same time, peaks 3 and 8 remain in the column, but peaks 7 and 9 can be completely separated and eluted. Therefore, this system was selected as the solvent system for the purification of the components of R-106.

Purification of the components of Food Color Red No. 106 using HSCCC

A 25-mg amount of R-106 was separated using the selected solvent system. The retention of the stationary phase was 54.7%. The separation took 4.5 h and the elution volume of the entire run was 540 ml. The elution curve of the R-106 components at 254 nm is shown in Fig. 3. The components were separated into four peaks and each fraction and each phase of the column contents were analysed by TLC and

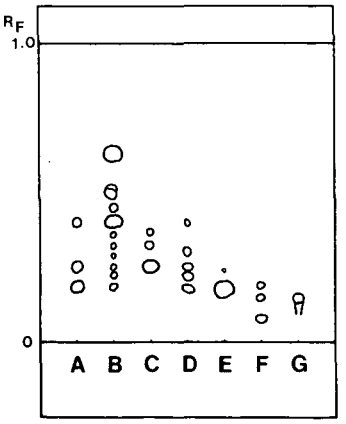

Fig. 4. TLC separation of the components of Food Color Red No. 106. (A) Original Food Color Red No. 106; (B) fraction I; (C) fraction II; (D) fraction III; (E) fraction IV; (F) fraction V; (G) fraction VI.

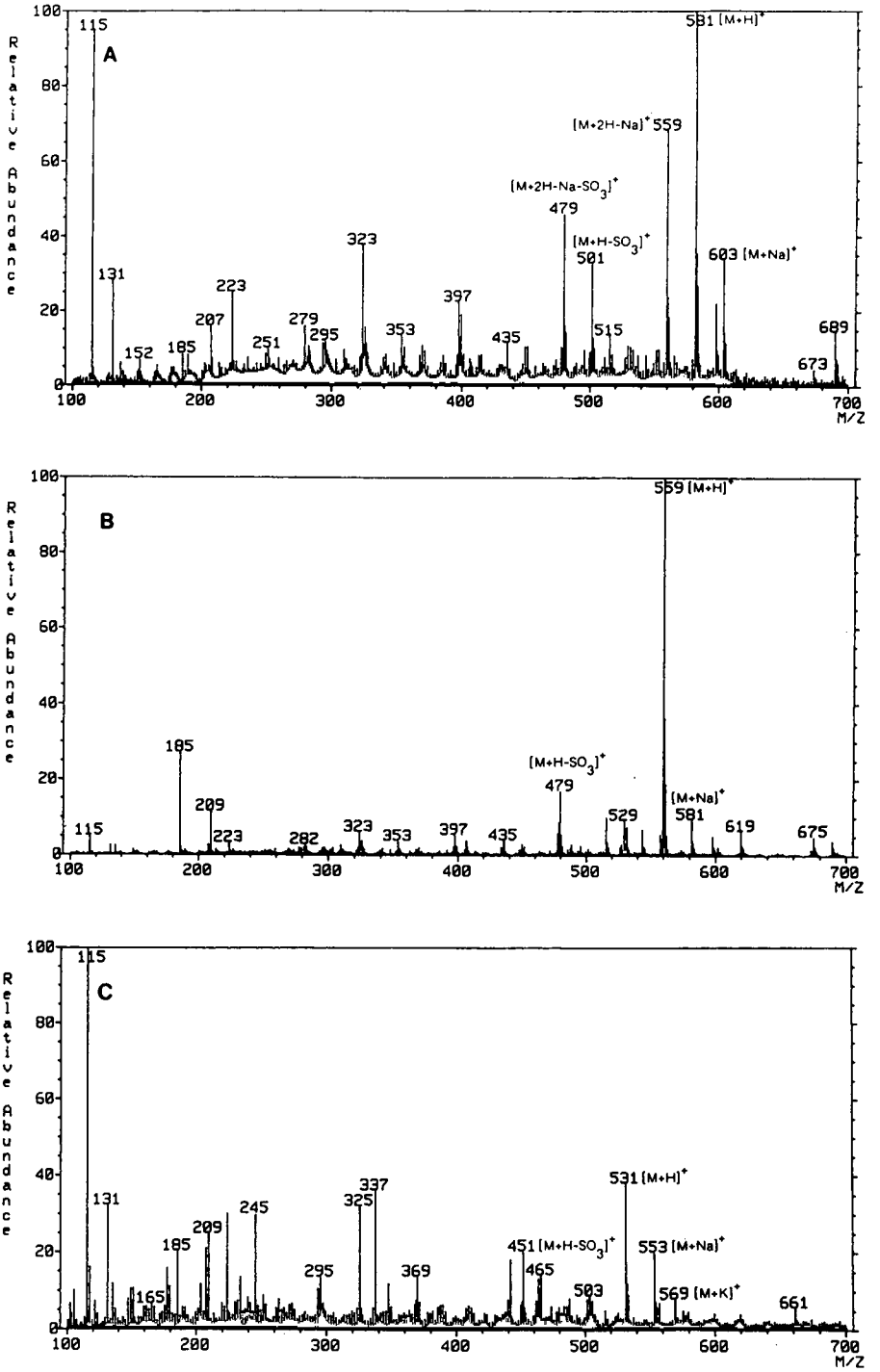

Fig. 5. FAB mass spectra of the components of Food Color Red No. 106. (A) Original Food Color Red No. 106; (B) fraction IV; (C) fraction II.

HPLC. At peak A on the elution curve, more hydrophilic compounds than the component corresponding to peak 1 were eluted and these compounds were hardly visible in HPLC and TLC of the original sample owing to the low concentrations. The compounds corresponding to the peaks 1, 2, 4, 5 and 6 were eluted at the same time at peak B. Peaks 7 and 9 were eluted at peaks C and D, respectively, on the elution curve, and peaks 3 and 8 remained in the column contents.

All contents in the fractionated test-tubes and column were combined to make six fractions on the basis of the results of TLC and HPLC analyses. Figs. 2B–G and 4 show the HPLC and TLC results, respectively. Although fractions I (test tube No. 50-15), III (test tube No. 183-188), V (upper phase of column contents) and VI (lower phase of column contents) contained various small amounts of the components, the components corresponding to peak 7 in fraction II (test tube No. 156-182) and peak 9 in fraction IV (test tube No. 189-290) were almost isolated from other components. In HPLC analyses, peaks 7 and 9 constituted about 2 and 95% of the total peak area at 254 nm, respectively, in the original sample, but after only one purification run by HSCCC the purity of peak 7 in fraction II (Fig. 2C) and peak 9 in fraction IV (Fig. 2E) increased to over 98.0 and 99.9%, respectively. Peak 9 was 21 mg of a pure compound and peak 7 was 0.9 mg of a pure compound. The results from TLC and HPLC analyses clearly indicate that the components of R-106 are easily purified by HSCCC.

Identification of acid red and an impurity by FAB-MS

The FAB mass spectra of the original sample, the compounds in fractions II and IV corresponding to peaks 7 and 9, respectively, are shown in Fig. 4 A–C. Acid Red has a molecular weight of 580 and the two molecular ion species, $[M + H]^+$ and $[M + Na]^+$, are clearly observed at m/z 581 and 603, respectively, and $[M + H - SO_3]^+$ also appears at m/z 501 in the spectrum of the original sample (Fig. 5A). The prominent ion at m/z 559 is assigned to $[M + 2H - Na]^+$. In the spectrum of the compound in fraction IV, the base peak is observed at m/z 559 together with the characteristic ions at m/z 479 and 581 (Fig. 5B). Further, when potassium chloride was added to the sample solution, the $[M + K]^+$ appeared at m/z 597. Hence the molecular weight was determined to be 558 and this compound in fraction IV is Acid Red, which possesses a free sulphonic acid instead of the sodium salt.

Fig. 5C shows the spectrum of the compound in fraction II. The three related molecular ions, $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$, are observed at m/z 531, 553 and 569, respectively. The last ion was much enhanced when potassium chloride was added, so the molecular weight was determined to be 530, which is 28 less than that of the parent food dye. The $[M + H - SO_3]^+$ is also observed at m/z 451. These results indicate that the structure of the subsidiary dye in fraction II is a des-ethylated derivative of R-106.

CONCLUSION

We were able to purify the components of R-106 using HSCCC. The HSCCC separation of the components was performed using 25 mg of sample with the two-phase solvent system *n*-butanol–0.01 *M* TFA (1:1). The confirmation studies by TLC, HPLC and FAB-MS showed that HSCCC is successful in the purification of the

major components of R-106. We obtained 21 mg of 99.9% pure main component (Acid Red) and 0.9 mg of a 98.0% pure subsidiary dye whose structure was shown to be a des-ethylated derivative. Hence the present HSCCC methodology can provide a useful separation technique for the study of the chemistry, composition and manufacture of synthetic colors.

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Preparative purification of tetrabromotetrachlorofluorescein and Phloxine B by centrifugal counter-current chromatography

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ABSTRACT

A centrifugal counter-current chromatographic method for preparative purification of commercial tetrabromotetrachlorofluorescein and Phloxine B (D&C Red Nos. 27 and 28, respectively) was developed. Ethyl acetate–*n*-butanol–0.01 *M* ammonium acetate (1:1:2) was used as the two-phase solvent system. Each purification trial involved 50 mg of sample and yielded 22 mg (±2 mg) of pure dye. The purity of the product was measured by high-performance liquid and thin-layer chromatography and was found to be 99.9%. The partition coefficients of these compounds were found to be highly concentration-dependent in the two-phase solvent system used. If this problem can be circumvented, then the counter-current chromatographic method can be extended for use with gram quantities of dye.

INTRODUCTION

Tetrabromotetrachlorofluorescein [**1**, Colour Index (1971) No. 45410:1, Fig. 1] and its disodium salt, Phloxine B (**2**, Colour Index (1971) No. 45410, Fig. 1], are dyes of the xanthene class. They are used primarily for coloring drugs and cosmetics [1], histological counter staining, and selective staining in some bacteriological and haematological techniques [2,3].

Tetrabromotetrachlorofluorescein is manufactured by condensing resorcinol with tetrachlorophthalic anhydride and brominating the reaction product [4]. Phloxine B is manufactured by alkaline hydrolysis of **1**. The reaction products (**1** and **2**) contain various organic impurities, including residues of the starting materials, side-reaction products and lower-halogenated subsidiary dyes. Compounds **1** and **2** are listed in the U.S. Code of Federal Regulations (CFR) for use in drugs and cosmetics and are designated as D&C Red No. 27 and D&C No. 28, respectively [1]. They are subject to batch certification by the Food and Drug Administration (FDA) to assure compliance with specifications and other requirements set forth in the CFR. The specifications include limitations for intermediates, subsidiary colors and organic

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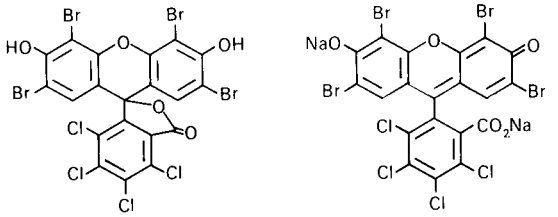

Fig. 1. Structures of tetrabromotetrachlorofluorescein (left, **1**) and Phloxine B (right, **2**).

side-reaction products. If present near the specification levels, these contaminants would represent a total of up to 8.3% of D&C Red No. 27 or 28.

In the development and validation of a high-performance liquid chromatographic (HPLC) method for analyzing batches of D&C Red Nos. 27 and 28 submitted to FDA for certification, pure samples of **1** and **2** were needed for use as standards. Pure dyes are desirable also for histological staining purposes to prevent the anomalous histochemical staining results explained by the presence of dye impurities as reported in the literature [5].

No preparative purification method was found in the literature for **1**; however, two methods were found for the purification of Phloxine B. One method uses acid precipitation [6], and the other uses gel chromatography [7]. Acid precipitation does not separate the lower-halogenated subsidiary colors from Phloxine B. The gel chromatographic method, while very effective, separates relatively small quantities (10–30 mg) of dye in each trial. For this study, a well-established method of purification, centrifugal counter-current chromatography (CCC) [8,9], was chosen as an alternative. CCC was previously shown to be successful in the preparative separation of polar compounds [10] and in the purification of other water-soluble dyes [11–13]. More significantly, two of these earlier studies [12,13] involved relatively large quantities of dyes (approximately 500 mg).

While preparative HPLC is a proven method for purification of gram quantities of compounds [14], it was not chosen for use in this case. From a study of the analytical-scale HPLC analysis of D&C Red No. 28 in progress at FDA, it was observed that the dye is adsorbed on the solid support of each of four different reversed-phase columns under evaluation. Since CCC involves liquid–liquid partition chromatography without a solid supporting matrix, the possibility of dye adherence to the column packing is eliminated.

This paper describes a simple and rapid method for the preparative purification of commercial tetrabromotetrachlorofluorescein and Phloxine B, using centrifugal counter-current chromatography.

EXPERIMENTAL

Materials

Tetrabromotetrachlorofluorescein and Phloxine B were selected from samples of commercial batches submitted for certification to the FDA. The tetrabromotetrachlorofluorescein (D&C Red No. 27) samples were hydrolyzed with Na_2CO_3 in accordance with the method used for solubilizing the lactone forms of Rose Bengal and

Eosin Y [15]. For HPLC measurements, stock solutions of commercial dyes and of CCC-purified dyes were prepared in 0.1 *M* ammonium acetate–methanol (3:1) at concentrations of 1 mg/ml and 0.5 mg/ml. The stock solutions were stored in a dark environment. Ammonium acetate (HPLC reagent) and ammonium hydroxide (30%) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.); 95% aqueous ethanol was obtained from Pharmco (Dayton, NJ, U.S.A.) and butylamine was purchased from Fisher Scientific (Springfield, NJ, U.S.A.). All other solvents used were HPLC or chromatographic grade.

Centrifugal counter-current chromatography

CCC separations were performed using a horizontal flow-through coil planet centrifuge equipped with three column holders. The basic design of the apparatus has been reported earlier [16]. The apparatus holds three sets of composite coiled-column assemblies around the rotary frame at a distance of 10 cm from the central axis of the centrifuge. Each coil assembly consists of eight identical coils, each prepared from 1.6 mm I.D. PTFE (polytetrafluoroethylene) tubing by winding it onto a 12 cm × 1.25 cm O.D. aluminum pipe, forming two layers of coils with approximately 70 helical turns and 9 ml capacity. These dual-layer coils are connected in series and mounted around the column holder parallel to, and at a distance of, 4 cm from the central axis of the holder. The three coil assemblies are connected in series on the rotary frame to make up a total column capacity of approximately 230 ml. The apparatus can be rotated up to 1000 rpm with a speed controller. Similar equipment is commercially available from either Peptide Technologies (Washington, DC, U.S.A.) or Pharma-Tech Research (Baltimore, MD, U.S.A.).

Based on the partition coefficient measurements, *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2, adjusted to pH 9 by addition of ammonium hydroxide to the lower phase) was selected as the two-phase solvent system for purifying **1** and **2**. The solvent mixture was thoroughly equilibrated in a separatory funnel and the two phases separated shortly before being applied to the column. The upper and lower phases were each used separately as the mobile phase in different runs. In each separation, the column was first filled entirely with the stationary phase followed by injection of the dye solution (50 mg in 5 ml of the lower phase). The mobile phase was then pumped into the column at a flow-rate of 60 ml/h while the apparatus was rotated at 800 rpm. When the lower phase was used as the mobile phase, the effluent from the outlet of the column was continuously monitored with an ultraviolet detector (Uvicord S; LKB, Stockholm, Sweden) at 276 nm and fractionated (3 ml or 6 ml/test tube) with a fraction collector (Ultrorac, LKB). When the upper phase was used in that capacity, an air bubble in the flow cell disrupted the UV monitoring. Therefore, an aliquot of each fraction was mixed with 3 ml of methanol and the absorbance determined at 546 nm with a Zeiss PM6 spectrophotometer.

Recovery of the pure dye

After separating the fractions with the highest absorbance values (*e.g.*, fractions 85–96, Fig. 7), the solvent (upper phase or lower phase) was removed by rotary evaporation. The purity of the dye was confirmed by HPLC and thin-layer chromatography (TLC). A typical trial yielded 23 mg of purified material.

High-performance liquid chromatography

The system consisted of a Model 8800 ternary pump, Model 4270 integrator, Model 8780 autosampler, Model 8500 dynamic mixer (all by Spectra-Physics, San Jose, CA, U.S.A.). The autosampler was equipped with a Model 7010 injector (Rheodyne, Cotati, CA, U.S.A.) with a 200-μl sample loop and Model 490 dual wavelength UV–VIS detector (Waters Assoc., Milford, MA, U.S.A.). A Hypersil MOS-1 RPC-8, 5 μm particle size column (250 mm × 4.6 mm I.D.) (Keystone, Bellefonte, PA, U.S.A.) was used throughout. A detector wavelength of 546 nm was used for monitoring the lower halogenated subsidiary dyes, and a wavelength of 254 nm was used for monitoring starting materials (uncombined intermediates) and side-reaction products. The solutions were filtered through LID/X syringeless filters AQOR.45 (Genex, Gaithersburg, MD, U.S.A.) prior to injection.

The chromatographic conditions were as follows: mobile phase, 0.1 *M* ammonium acetate (A)–methanol (B) with linear gradients from 25% to 90% B over 25 min and from 90 to 100% B over the next 5 min. This was followed by an isocratic column wash with 100% B for 6 min. The column was re-equilibrated with 25% B for 14 min. Other conditions were: injection volume, 50 or 100 μl; detector sensitivity, 1 a.u.f.s.; chart speed, 0.5 cm/min.

Thin-layer chromatography

The purity of the commercial samples and the CCC-purified fractions was determined by TLC according to the method used for the determination of lower-halogenated subsidiary dyes in D&C Red Nos. 27 and 28 [17]. Solid material (2 mg) from the original samples or obtained from the CCC separation was dissolved in 95% ethanol–water–30% ammonium hydroxide (5:5:0.1) (1 ml). A total of 1 mg of dye was streaked onto a silica gel G 20 × 20, 250 μm plate (Fisher Scientific). The plates were developed with acetone–chloroform–butylamine (66:24:4.5). After the band was scraped and the material corresponding to subsidiary dyes was extracted with 15% ammonium hydroxide (8–10 ml), the solution was examined spectrophotometrically (400–700 nm). No scraping was required for the CCC-purified fractions since no bands corresponding to lower-halogenated subsidiary dyes were observed.

RESULTS AND DISCUSSION

The results of the study demonstrate the ability of the CCC method to separate the impurities of **1** and **2**. Confirmation of adequate purification was provided by HPLC and TLC analyses. Each separation experiment involved 50 mg of commercial **1** and **2** and yielded 22 mg (±2 mg) of purified dye. Figs. 2 and 3 show HPLC chromatograms of typical samples of **2** and **1**, respectively, before (I) and after (II) purification. In both cases, integration of the HPLC–UV peak areas indicates that the desired separated dye is 99.9% pure (at 254 nm and 546 nm). TLC analyses corroborated the HPLC results in yielding only one spot each for purified **1** and purified **2**.

It should be noted that CCC purification was also attempted with larger quantities (210 mg and 2 g) of commercial Phloxine B. In each of the two trials, an anomalous double-peak elution curve was obtained, suggesting that the dye was separated into two different components (Fig. 4). HPLC analyses revealed that the two peaks (fractions 75 and 84, Fig. 4) of the elution curve represented identical, partially puri-

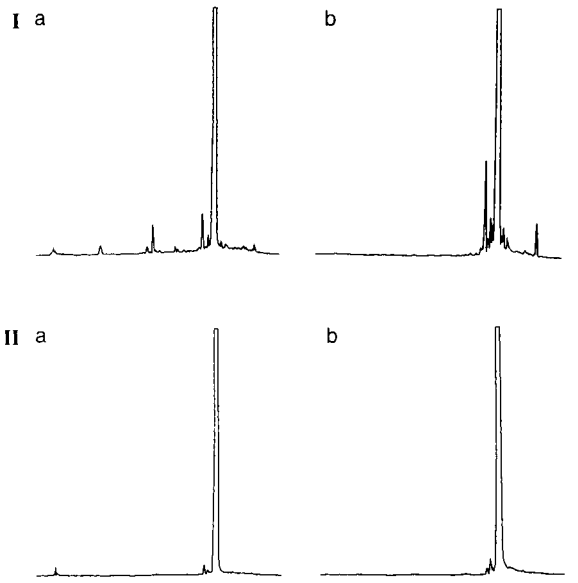

Fig. 2. HPLC chromatograms of typical commercial (I) and CCC-purified (II) Phloxine B. (a) 254 nm, (b) 546 nm. Each of the injections (100 μl) contained 50 μg of dye. Other chromatographic conditions are described in the Experimental section.

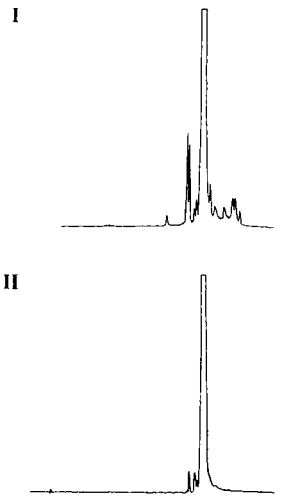

Fig. 3. HPLC chromatograms of typical commerical (I) and CCC-purified (II) tetrabromotetrachlorofluorescein at 546 nm. Each injection (100 μl) contained 50 μg of dye. Other chromatographic conditions are described in the Experimental section.

fied dye. It is suggested that this unusual result is related to the fact that the partition behavior of the dye is highly concentration-dependent. The effect of concentration on the partition coefficient in the two-phase solvent system of *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2) is illustrated in Fig. 5, where the abscissa indicates the dye concentration and the ordinate, the partition coefficient expressed as the solute concentration in the upper phase divided by that in the lower phase or $K(C_u/C_l)$, both plotted on a logarithmic scale. As shown in the diagram, at a high concentration the dye is partitioned primarily into the lower phase; when diluted, it moves into the upper phase. This non-linear isotherm is considered to produce a significant effect on the elution curve. Introduction of the two highly concentrated dye solutions into the CCC column thus yielded the double-peak elution profile.

Fig. 6 shows a chromatogram of **2** obtained by on-line monitoring of the effluent at 276 nm using the described CCC centrifuge. The separation was performed by eluting the lower aqueous phase at a flow-rate of 1 ml/min at 800 rpm. A 50-mg dye sample was eluted in approximately 3 h. Because of the non-linear isotherm of the partition coefficient mentioned above, the peak of the pure dye is markedly skewed toward the left while most of the impurities are fairly well separated from the pure dye because they eluted near the solvent front or remained on the column.

Fig. 7 shows a chromatogram of the same sample of **2** as that used for Fig. 6. In Fig. 7, the separation was obtained by eluting the upper non-aqueous phase. Elution

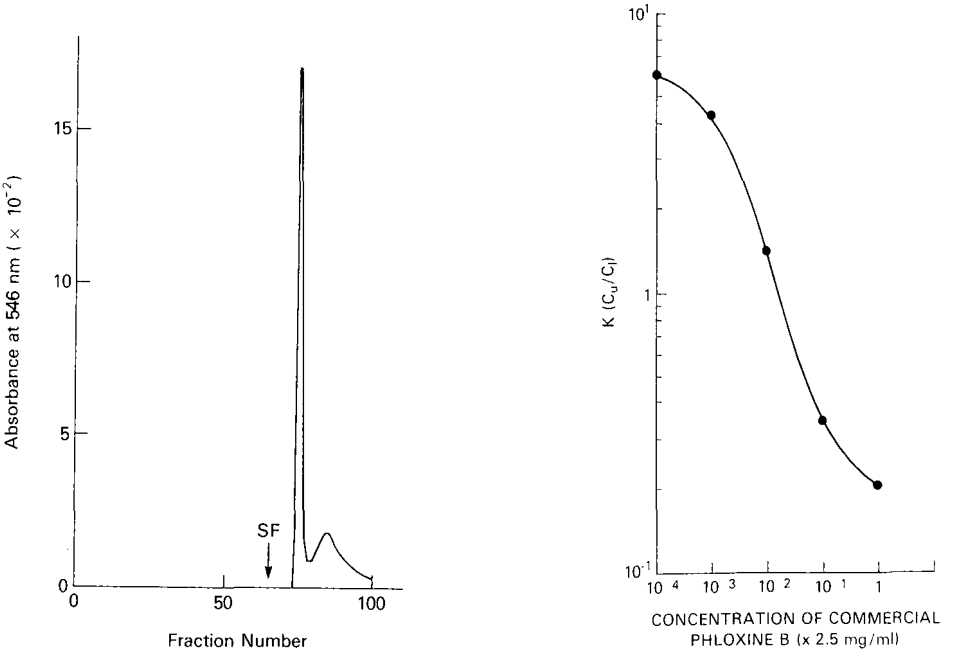

Fig. 4. Double-peak CCC elution curve obtained in a purification trial involving a 2-g sample of commercial Phloxine B. SF = Solvent front.

Fig. 5. Partition coefficient at various concentrations of commercial Phloxine B in *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2).

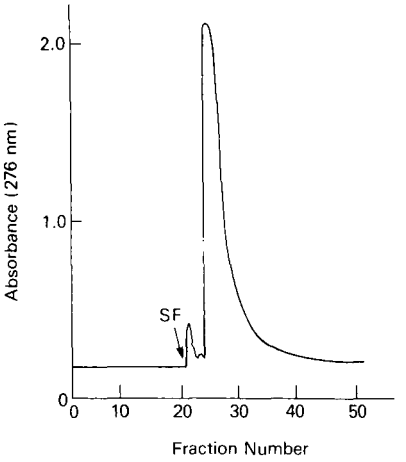

Fig. 6. Typical CCC elution curve for commercial Phloxine B when the lower aqueous phase of the *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2) solvent system was used as the mobile phase. SF = Solvent front. Other experimental conditions are as follows: flow-rate: 1 ml/min; fractionation: 6 ml/tube; revolution: 800 rpm; retention of the stationary phase: 28%; pressure: 300 p.s.i.

with the upper phase, however, produced a problem in on-line monitoring of the effluent. Introduction of the effluent into the flow cell from the bottom in an upward direction trapped the carried-over stationary phase in the flow cell. By reversing the flow, air bubbles in the flow cell were trapped. For this reason, the elution curve was manually drawn by measuring the absorbance of each fraction at 546 nm with a PM6 Zeiss spectrophotometer. This reversed elution mode provides an advantage over the normal elution mode (Fig. 6) in that the dye is retained longer in the separation column, yielding fractions of higher purity.

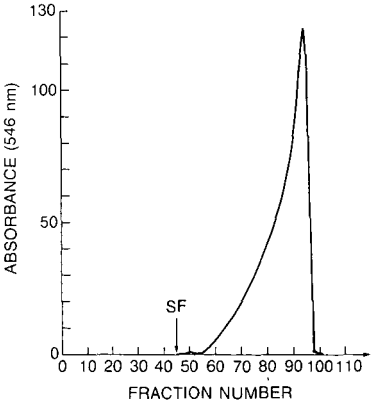

Fig. 7. Typical CCC elution curve for commercial Phloxine B when the upper non-aqueous phase of the *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2) solvent system was used as the mobile phase. SF = Solvent front. Other experimental conditions are as follows: flow-rate: 1 ml/min; fractionation: 3 ml/tube; revolution: 800 rpm; retention of the stationary phase: 27%; pressure: 300 p.s.i.

CONCLUSIONS

The CCC method was demonstrated in this study to be an effective method for purifying dyes of the xanthene class. It is therefore worthwhile to determine a means of circumventing the double-peak problem so that the CCC method can be applied to the purification of larger quantities of dyes. Specifically, a different solvent system must be found, and investigations toward this end are currently in progress. Research efforts are also being directed toward the identification and quantification of the various impurities found in commercial tetrabromotetrachlorofluorescein and Phloxine B. The data characterizing the impurities, together with improved purification techniques, will upgrade the quality and efficiency of FDA's certification process and contribute to more accurate histochemical staining uses of the dyes.

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Synthesis and characterization of N-bromoacetyl-3,3′,5-triiodo-L-thyronine

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ABSTRACT

N-Bromoacetyl-3,3′,5-triiodo-L-thyronine and carrier-free [3′-^{125}I]-N-bromoacetyl-3,3′,5-triiodo-L-thyronine, to be used for affinity labeling of thyroid hormone receptors, were synthesized using a one-step procedure: a solution of the thyroid hormone 3,3′,5-triiodo-L-thyronine and bromoacetyl bromide in ethyl acetate was refluxed for an optimal period of time which depends on the amount of hormone processed. The bromoacetylated hormone thus obtained was then fractionated by high-speed counter-current chromatography which yielded N-bromoacetyl-3,3′,5-triiodo-L-thyronine that was pure by the criteria of high-performance liquid chromatography and thin-layer chromatography with different solvent systems. The pure product was well separated from all contaminants including one which in high-performance liquid chromatography was not easily separated from N-bromoacetyl-3,3′,5-triiodo-L-thyronine. The latter was characterized by ^{1}H nuclear magnetic resonance, plasma desorption mass spectrometry, thin-layer chromatography, high-performance liquid chromatography, UV spectrophotometry, and melting point. Amounts of 3,3′,5-triiodo-L-thyronine ranging from picograms, including carrier-free ^{125}I-labeled triiodothyronine, to 200 to 300 mg can be processed with the equipment used in the present investigation.

INTRODUCTION

Labeled N-bromoacetyl-3,3′,5-triiodo-L-thyronine (BrAcT3) has been widely used as an affinity label for the study of thyroid hormone binding to a variety of receptor sites. This affinity label as well as its congener N-bromoacetyl-L-thyroxine (BrAcT4) has usually been prepared by a two-step reaction: coupling of bromoacetic acid with N-hydroxysuccinimide by means of dicyclohexylcarbodiimide and reaction of the N-hydroxysuccinimide bromoacetate formed with ^{14}C- or ^{125}I-labeled 3,3′,5-triiodo-L-thyronine (T_3) or L-thyroxine (T_4) [1–8]. For the synthesis of unlabeled BrAcT3, the second step was carried out with unlabeled T3. However, no physicochemical data have been published to prove unequivocally the identity of the synthesized products and several labeled or unlabeled bands or peaks are always obtained when the crude reaction product is being analyzed by thin-layer chromato-

graphy (TLC) or by high-performance liquid chromatography (HPLC). It is not clear which one of these bands or peaks represents BrAcT3 or BrAcT4. We now report a simple one-step synthesis of BrAcT3 and of carrier-free BrAc[^{125}I]T3. (One-step syntheses of BrAcT4 and of carrier-free BrAc[^{125}I]T4 which require a different methodology will be reported later).

The synthesis of BrAcT3 is based on the reaction of bromoacetyl bromide (BrAcBr) with T3 or [^{125}I]T3. The crude reaction produced is fractionated by means of high-speed counter-current chromatography (HSCCC) [9]. The most prominent absorption or radioactivity peak obtained in this fractionation is shown below (Experimental) by plasma desorption mass spectrometry (PD-MS) and ^{1}H NMR to represent unlabeled or labeled BrAcT3.

EXPERIMENTAL

Materials

The following materials were used: T3, 99% pure (Aldrich); [3′-^{125}I]T3, carrier-free (2200 Ci/mmol) and [3′,5′-^{125}I]T4, carrier-free (4400 Ci/mmol) (DuPont/NEN 110X and 111X, respectively); BrAcBr, 98+% (Aldrich); methanol, hexane, acetonitrile (HPLC grade); acetic acid, ammonium acetate, reagent grade; ethyl acetate, (reagent grade for bromoacetylation or HPLC grade for HSCCC); water, Milli-Q (Millipore); boiling chips (amphoteric alundum granules) (Hengar).

Synthesis of BrAcT3

A round-bottom flask equipped with a short reflux condenser was used for the processing of more than 0.01 mmol T3. Thus, 0.1 mmol T3 was mixed with 25 ml of ethyl acetate and 500 μl BrAcBr were added which causes dissolution of T3. The mixture, after addition of a very small boiling chip, was refluxed for 10 min. The top of the condenser was connected via an empty safety bottle to a wash bottle containing 1 *M* NaOH in 50% ethanol to absorb any escaping BrAcBr or HBr. The reaction mixture was cooled in ice water, then 500 μl of methanol were added to destroy excess BrAcBr.

The reaction mixture was concentrated to a small volume (*ca.* 0.5 ml) in a rotating evaporator (Büchi Rotovapor R) under reduced pressure (20–30 Torr) using a water bath not exceeding 30°C.

For the processing of amounts of T3 exceeding 0.1 mmol the amounts of reagents and solvent were changed proportionately. For the processing of less than 0.01 mmol T3, including that of carrier-free [^{125}I]T3, the round-bottom flask was replaced with a glass tube fashioned from a 99447 Corning culture tube and a 19/22 joint. In that case, refluxing with a metal block heater (Multi-Blok heater No. 2090; Labline Instruments) was reduced to 5 min. In a few experiments, the rotating evaporator was replaced with a SpeedVac concentrator (Savant) after short precooling of the reaction mixture to avoid bumping. The amounts of BrAcBr, ethyl acetate and methanol were always 50 μl, 2.5 ml and 50 μl, respectively, independent of the amount of T3.

Since [^{125}I]T3 is obtained from the manufacturer as a solution in aqueous propanol, that solvent must be eliminated before bromoacetylation. This is achieved by brief lyophilization for μl amounts or by SpeedVac concentration for ml amounts.

HSCCC fractionation

A commercial model of the HSCCC centrifuge (P.C. Inc., Potomac, MD, U.S.A.) was used in this study [10]. The apparatus holds a multilayer-coil separation column and a counterweight symmetrically on the rotary frame at a distance of 10 cm from the centrifugal axis of the centrifuge. The column consists of approximately 165 m × 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing with a total capacity of approximately 330 ml. The revolution speed can be regulated up to 1000 rpm with a speed controller.

The two-phase solvent systems composed of hexane–ethyl acetate–methanol–15 m*M* ammonium acetate (pH 4.0) (1:1:1:1 and 4:5:4:5, v/v) were selected on the basis of partition coefficient values of BrAcT3 as summarized in Tables I and II. The solvent mixture was thoroughly equilibrated in a separatory funnel and the two phases were separated shortly before use.

The sample solution (*ca.* 0.5 ml, ethyl acetate) was first mixed with a proper volume of each of the other solvents (hexane, methanol and 15 m*M* ammonium acetate) to adjust the phase composition and finally brought to approximately 4 ml by adding equal volumes of the upper and lower phases used for separation.

In each centrifugation, the separation column was first entirely filled with the upper, organic (stationary) phase and the sample solution was injected through the sample port. Then, the apparatus was rotated at 800 rpm while the lower, aqueous (mobile) phase was pumped into the column at a flow-rate of 3 ml/min using a metering pump (Milton Roy minipump; LDC Analytical, Riviera Beach, FL, U.S.A.). The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S; LKB Instruments, Stockholm, Sweden) at 276 nm and fractionated with a fraction collector (Ultrorac, LKB Instruments) to obtain 3-ml fractions. After the desired peak (BrAcT3) was eluted, the apparatus was stopped and the column contents were fractionated by elution with methanol at a flow-rate of 6 ml/min.

Analysis of HSCCC fractions

Fractions which were of interest because they showed either high absorbance (A_{276}) or high radioactivity were analyzed by PD-MS, HPLC, or ^{1}H NMR. Depending on the concentration of solute, small aliquots of a single fraction could be used. With very little solute, as for the analysis of carrier-free preparations of BrAc[^{125}I]T3, ten or more fractions had to be combined and concentrated. As expected, unlabeled and labeled BrAcT3 (A_{278} and cpm, respectively) coeluted.

The radioactivity of fractions was assessed with a γ scintillation counter (Auto-Gamma 5000 series, Packard). For the determination of the yield of unlabeled BrAcT3, a small amount (20 μl) of [3'-^{125}I]T3 (DuPont/NEN 110X) was evaporated to dryness and mixed with unlabeled T3. The BrAc[^{125}I]T3 formed upon bromoacetylation served as an internal standard. Furthermore, small duplicate samples (2 μl) of the same [3'-^{125}I]T3 preparation were counted alongside the BrAc[^{125}I]T3 in order to determine the yield as well as mechanical losses.

HPLC analyses were carried out with a Waters chromatograph using a 280-nm absorption detector in conjunction with an Omniscribe absorption recorder (Houston Instruments) and a FRAC-100 fraction collector (Pharmacia). Conditions were: 15-cm Nova C_{18} column; solvent A, 15 m*M* ammonium acetate, pH 4.0, prepared by adding acetic acid to an aqueous solution of 1.2 g ammonium acetate until pH 4.0 is

reached and bringing the solution to a total volume of 1 l with water; solvent B, acetonitrile; linear gradient (started 1 min after injection), 20–60% B in 40 min; flow-rate, 1 ml/min; sensitivity, 0.1 absorbance units/chart width in most instances and 0.2 or 0.5 absorbance units/chart width in some cases. Higher sensitivities (0.05 or 0.01) were required for the detection of carrier-free BrAc[^{125}I]T3.

T_4 (10^{-3} *M*) was always used as an internal standard. With a sensitivity setting of 0.1, 5 μl were injected together with the sample.

Mass spectra

PD-MS spectra were measured using a spectrometer built for the National Institutes of Health (NIH) by Professor R. Macfarlane of Texas A & M University and modified by one of us (H.M.F.) and L.K. Pannell. An accelerating voltage of 10 kV was used with a flight path of 42 cm. Samples were electrosprayed onto aluminized mylar films and spectra were allowed to accumulate for at least 1 h before processing on a Perkin-Elmer 3220 data system.

Absorption spectra

Ultraviolet spectra were determined with a Cary 219 spectrophotometer using quartz cells with a 1-cm light path.

Melting point

The melting point of BrAcT3 was taken using a Kofler hot stage microscope.

RESULTS AND DISCUSSION

A simple one-step method of synthesizing BrAcT3 consists of refluxing T3 and BrAcBr in ethyl acetate for an optimal period of time which depends on the amount of T3 processed. The reaction mixture is then fractionated by means of HSCCC (see Experimental) which results in a resolution superior to that which can be achieved with TLC or even with HPLC.

In the case of BrAcT3 the purification by HSCCC is highly dependent not only on temperature (a generally observed phenomenon), but also on solute concentration (non-linear isotherm). Table I shows the effect of temperature and concentration, using the solvent system hexane–ethyl acetate–methanol–15 m*M* aqueous ammonium acetate, pH 4.0 (1:1:1:1, v/v). The partition coefficient K (C_L/C_U) (see Tables I and II) increases with increasing temperature and with decreasing concentration. The dependence on concentration (Table I) is probably due to reversible aggregation. The table shows that the solvent system used is appropriate for a BrAcT3 concentration of 1 mg/ml, but results in excessive K values for lower concentrations. We have used this solvent system for concentrations of 1 mg/ml and greater.

For use with much lower solute concentrations, including those prevailing when carrier-free BrAc[^{125}I]T3 is to be purified, the effect of changing solvent volume ratios on K values was investigated (Table II).

The ethyl acetate and aqueous ammonium acetate buffer ratios within the total solvent system were kept constant, but the volumes of hexane and of methanol were gradually decreased. A decrease of the hexane volume results in an increase of polarity of the solvent system, while a decrease in methanol volume serves mainly to maintain

TABLE I

EFFECTS OF TEMPERATURE AND SOLUTE CONCENTRATION ON THE PARTITION COEFFICIENT (K) OF BrAcT3 IN HEXANE–ETHYL ACETATE–METHANOL–15 mM AMMONIUM ACETATE (pH 4.0) (1:1:1:1, v/v)

$K = C_L/C_U$, *i.e.*, solute concentration in the lower phase divided by that in the upper phase.

Temperature (°C)	K			
	Solute concentration (mg/ml)			
	1	0.1	0.01	<0.001
10	2.1	–	4.7	4.8
20	3.0	6.0	6.0	–
30	4.2	–	7.4	8.1

TABLE II

EFFECT OF VOLUME RATIO OF HEXANE–ETHYL ACETATE–METHANOL–15 mM AMMONIUM ACETATE (pH 4.0) ON THE PARTITION COEFFICIENT OF BrAcT3

$K = C_L/C_U$, solute concentration in the lower phase divided by that in the upper phase. Experimental conditions: temperature, 20°C; solute concentration: 0.01 mg/ml.

Hexane–ethyl acetate–methanol–15 mM ammonium acetate	K
5:5:5:5	5.7
4:5:4:5	1.5
3:5:3:5	0.21

the interfacial tension between the two phases. A decrease in interfacial tension, which would occur if the methanol volume were kept constant, would result in loss of peak resolution due to a decrease of the volume of the stationary (organic, upper) phase retained in the column. For the purification of BrAcT3 present in low concentration (0.01 mg/ml or less) including carrier-free BrAc[^{125}I]T3 we have chosen a hexane–ethyl acetate–methanol–15 mM ammonium acetate volume ratio of 4:5:4:5 because Table II shows that this ratio results in a K value not too far from unity.

Fig. 1 shows absorbance (276 nm) and radioactivity profiles in HSCCC. The yield of BrAcT3 in this and similar bromoacetylations, as described above (Experimental) was about 54%. It was somewhat lower when very small amounts of T3 (<0.01 mmol) were used. Derivatization of carrier-free [3'-^{125}I]T3 (Fig. 2A) resulted in yields of 30–40%. This lower yield is presumably due to mechanical losses such as adsorption of BrAcT3 on the PTFE wall of the HSCCC column and to more pronounced side reactions when only trace amounts of T3 are being processed.

PD-MS of the byproduct eluting from the column at fractions 37–39 in Fig. 1 indicated that it consists largely of acetyl-T3 presumably formed by aminolysis of ethyl acetate. Similarly, PD-MS of the very hydrophobic material eluting in fractions 170–190 (Fig. 1B) and consisting of a mixture of two or more compounds strongly

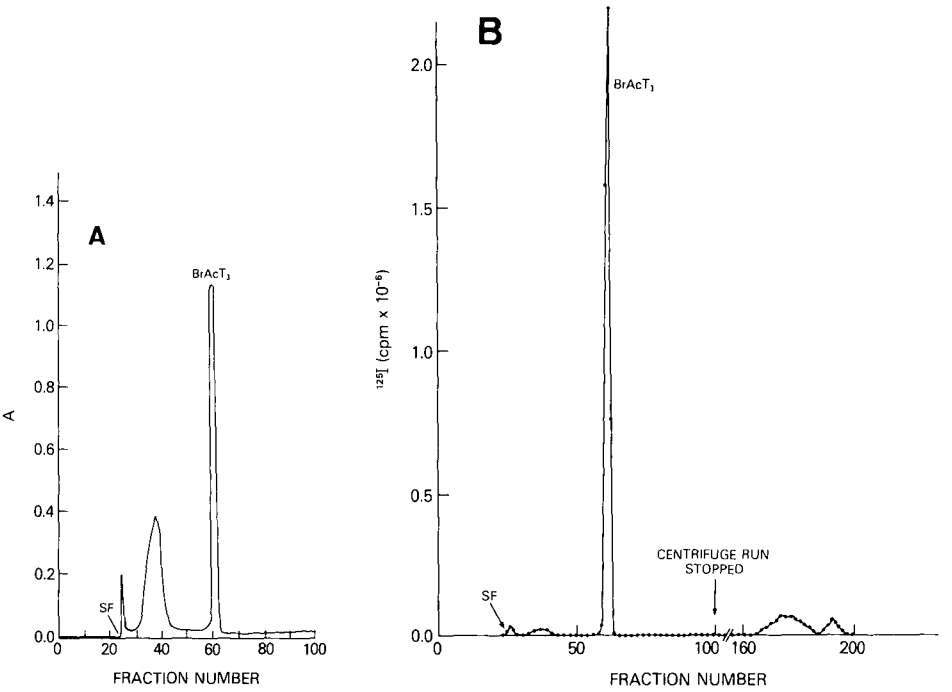

Fig. 1. Chromatograms (HSCCC) of crude BrAcT3 obtained by reacting T3 with BrAcBr. Chromatograms were monitored by UV absorbance at 276 nm (A) and by radioactivity of a ^{125}I-labeled internal marker (B). Sample No. 180A; solvent system, hexane–ethyl acetate–methanol–15 m*M* ammonium acetate, pH 4.0 (1:1:1:1); mobile phase, lower (aqueous) phase; flow-rate, 3 ml/min; 3-ml fractions; revolution, 800 rpm; retention of stationary phase, 77% of column capacity (330 ml); maximal column pressure, 65 p.s.i. SF = solvent front.

suggests that one of these is BrAcT3 ethyl ester presumably arising from transesterification of BrAcT3 with ethyl acetate. While acetyl-T3 cannot be directly linked covalently to T3 receptors, BrAcT3 ethyl ester should be able to react with nucleophiles in T3 receptors to establish a covalent bond. The amounts of all byproducts increase with refluxing beyond the optimal period of time. However, refluxing for much shorter periods of time results in incomplete conversion of T3 to BrAcT3. The small peak at the solvent front in Fig. 1A and B is caused by unreacted T3.

A sample of N-acetyl-T3 obtained as a byproduct in HSCCC (sample No. 137A, fraction 31) was checked against the PD-MS pattern of authentic N-acetyl-T3. It showed $(M+H)^+$, $(M+Na)^+$, and $(M+2Na-H)^+$ ions at *m/z* 691, 714 and 736, *i.e.*, *ca.* 79 a.m.u. less $(-Br+H)$ than BrAcT4 (see below). Ions are also present at *m/z* 588 and 610 for the losses of HI from the sodiated species.

A sample apparently containing BrAcT3 ethyl ester (sample No. 163A, fraction 136) showed $(M+Na)^+$ and $(M+H)^+$ ions at *m/z* 824 and 801, *i.e.*, 28-29 a.m.u. higher than the corresponding ions in BrAcT4. Ion A (Fig. 3) is also present at *m/z* 606 showing that the excess mass is confined to the carboxyl group, presumably as the ethyl

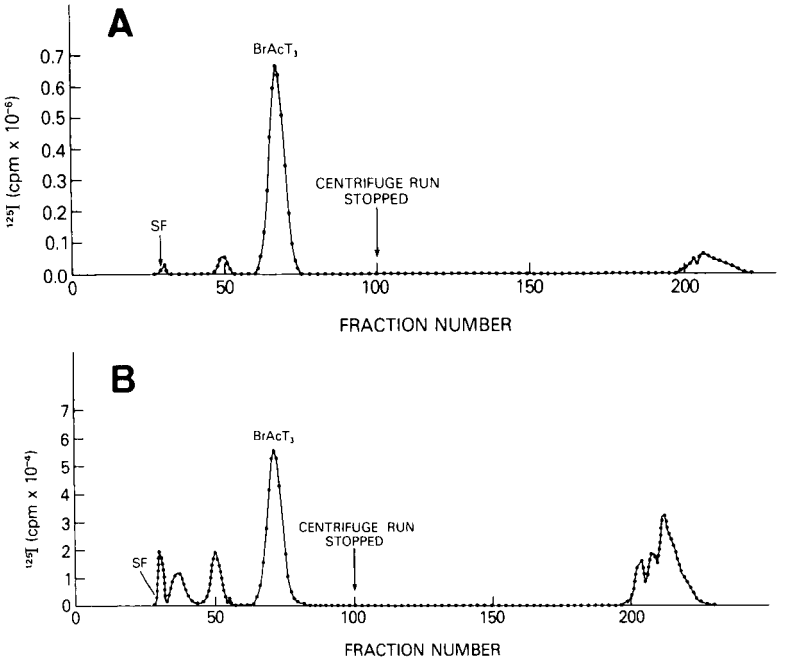

Fig. 2. Chromatograms (HSCCC) of crude ^{125}I-labeled BrAcT3 obtained by reacting carrier-free [^{125}I]T3 with BrAcBr. Chromatograms were monitored by radioactivity. Samples No. 171A, freshly prepared (A) and sample No. 175A, stored for 4.2 months in ethyl acetate at −20°C (B); solvent system, hexane–ethyl acetate–methanol–15 m*M* ammonium acetate, pH 4.0 (4:5:4:5). Other conditions as in Fig. 1.

ester. This is in agreement with its long retention by the non-polar (organic) phase in HSCCC.

The main HSCCC peak was clearly identified as BrAcT3 by PD-MS as well as by ^{1}H NMR. It is a very narrow peak when milligram amounts of T3 are being processed (fractions 59–63 in Fig. 1A and B) and broader when carrier-free T3 is being used (fractions 61–74 in Fig. 2A). A sample of BrAcT3 purified by HSCCC (sample No. 123A, fraction 37) produced the mass spectrum shown in Fig. 3. As is common for acidic and hydroxylic groups in PD-MS, the most abundant peak in the molecular weight region at *m/z* 818 (calculated 816.9) corresponds to the $(M+2Na-H)^+$ ion with smaller peaks for the $(M+H)^+$ and $(M+Na)^+$ ions at *m/z* 772 and 796. Losses from all three of these species of HBr and HI are evident while the $(M+H)^+$ ion also loses formic acid and bromoacetamide to give the corresponding styrene ions. Benzylic cleavage (ion A, Fig. 3) and formation of the imine ion B, Fig. 3, are also prominent. At lower masses the only important ions are at *m/z* 173 and 219 due to $(Na_2I)^+$ and the phenolic ring, respectively. Ions at *m/z* 322, 363 and 413 were variable in intensity and may represent impurities.

The NMR spectrum of a second sample of the same retention volume (sample No. 135A, fraction 50) in [$^2H_4$]methanol showed a quartet at δ 3.83 ($J=11.2$) from geminal coupling of the hydrogens of the CH_2 of the N-bromoacetyl group, the asymmetry arising from the proximity of the α-amido acid carbon. Likewise, the two

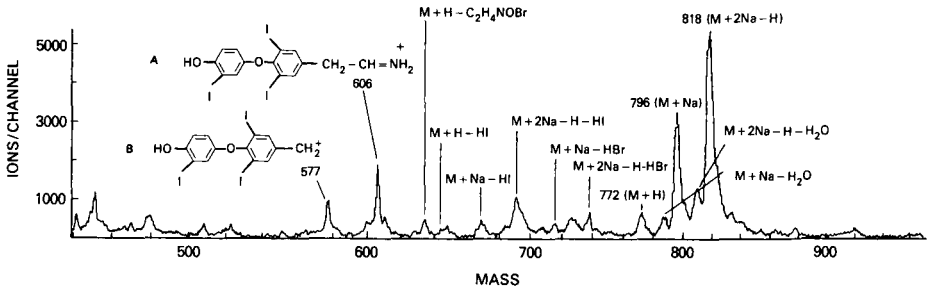

Fig. 3. Mass spectrum (PD-MS) of BrAcT3. Sample No. 123A, HSCCC fraction 37.

benzylic protons were seen at δ 2.95 and 3.24 ($J = 12.8$) coupled further to the α proton at δ *ca.* 4.65 ($J = 8.8$ and 4.8) as shown by decoupling.

When a preparation of crude carrier-free BrAcT3 (prior to HSCCC) was kept in ethyl acetate in a well-sealed container at −20°C for over 4 months, partial decomposition of BrAcT3 took place as a comparison of Fig. 2A and B shows, but even after this period of time pure BrAcT3 can still be obtained in somewhat reduced yield by HSCCC. Satisfactory conditions for long-term storage of BrAcT3 are now being investigated.

For the resolution of crude BrAcT3 (or of crude BrAcT4), HSCCC is far superior to TLC and also to HPLC. For example, a main disadvantage of TLC is that several consecutive runs are required to obtain a fairly pure BrAcT3 preparation and this causes considerable losses due to irreversible adsorption to the silica gel. It is difficult to obtain reasonable amounts of unlabeled BrAcT3 as required for competition studies and as reference material.

When crude preparations of BrAcT3 were analyzed by HPLC, the BrAcT3 peak was always accompanied by a satellite peak eluting immediately before the main peak. The two peaks were not resolved when preparative HPLC was used. The HSCCC peak material eluted in fractions 37–39 in Fig. 1A and B (eluted long before BrAcT3) produced an HPLC peak having a retention time identical to that of the satellite peak (not shown). Crude BrAcT3 preparations obtained by the two-step method mentioned in the Introduction also showed in HPLC a satellite peak with identical retention time. Therefore, the satellite material presumably consists at least in part of acetyl-T3 (see above).

HPLC elution patterns of crude BrAcT3 (sample No. 180A, prior to HSCCC) and of pure BrAcT3 (sample No. 180A, after HSCCC, fraction 61) are shown in Fig. 4A and B. Pure BrAcT3 eluted very reproducibly 2.5 min after T4 (4B). The 35.90-min peak in Fig. 4A and the smaller peak with still longer retention time presumably correspond to two components of the very hydrophobic HSCCC double peak (Fig. 1B, fractions 166–196). T3 eluted before T4 (not shown), not only under our experimental conditions but also under those used by Mol *et al.* [11] who report that their BrAcT3 (prepared by treatment of unlabeled T3 + [^{125}I]T3 with N-bromoacetyl chloride, followed by Sephadex LH-20 liquid chromatography) eluted before T3. We have no explanation for the discrepancy between their results and ours.

In earlier work [1–6] TLC (silica gel plates, acetic acid–ethyl acetate, 1:9, v/v) was used, while HPLC was used in some later work [7,8,11]. Before switching to HSCCC in

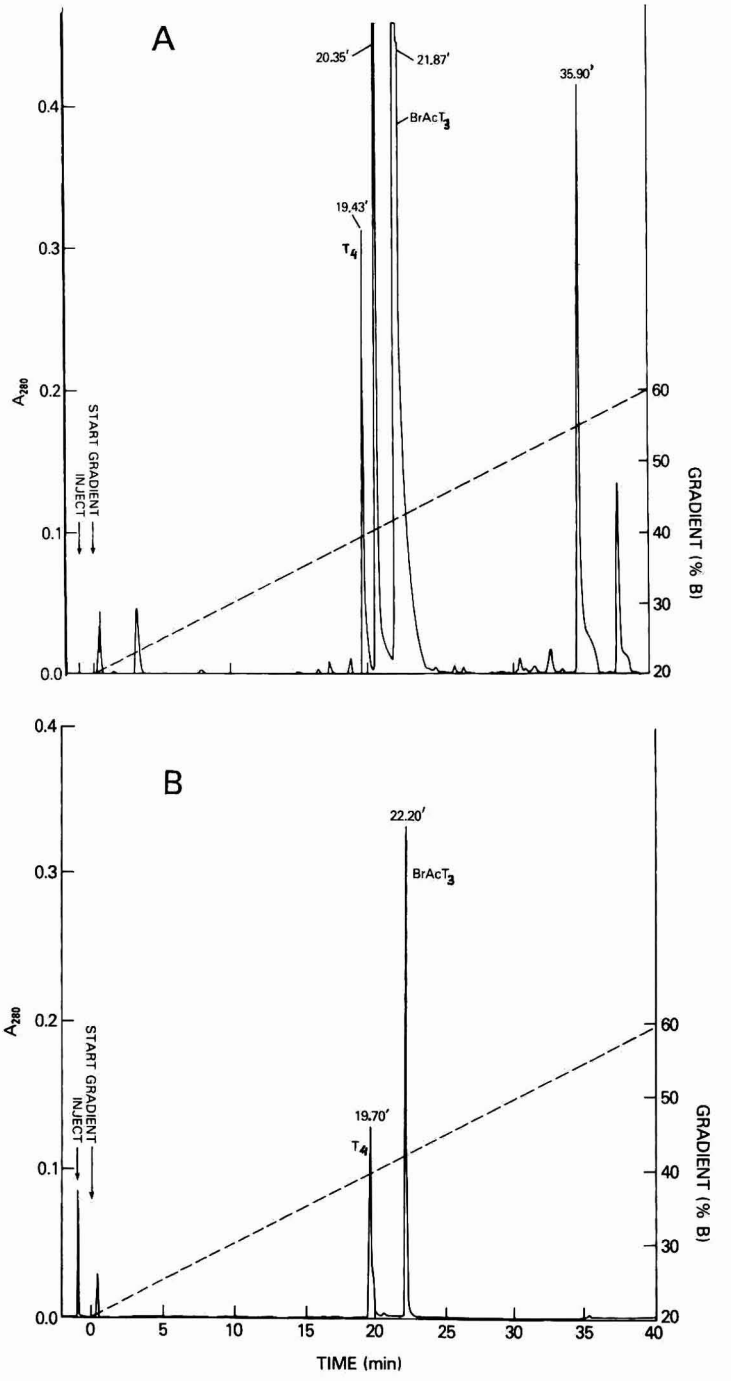

Fig. 4. HPLC of crude BrAcT3 (prior to HSCCC), sample No. 180A (A) and of purified BrAcT3, sample No. 180A, HSCCC fraction 61 (B). Waters chromatograph; 15-cm C_{18} Nova column (Waters); flow-rate, 1 ml/min; sensitivity, 0.5 absorbance units/chart width; solvents, 15 m*M* ammonium acetate, pH 4.0 (solvent A) and acetonitrile (solvent B); linear gradient (started 1 min after injection), 20–60% B in 40 min; internal standard, 20 μl of 10^{-3} *M* T4. An internal standard (10 μl of 10^{-3} *M* T4) was injected together with 2 μl of the sample.

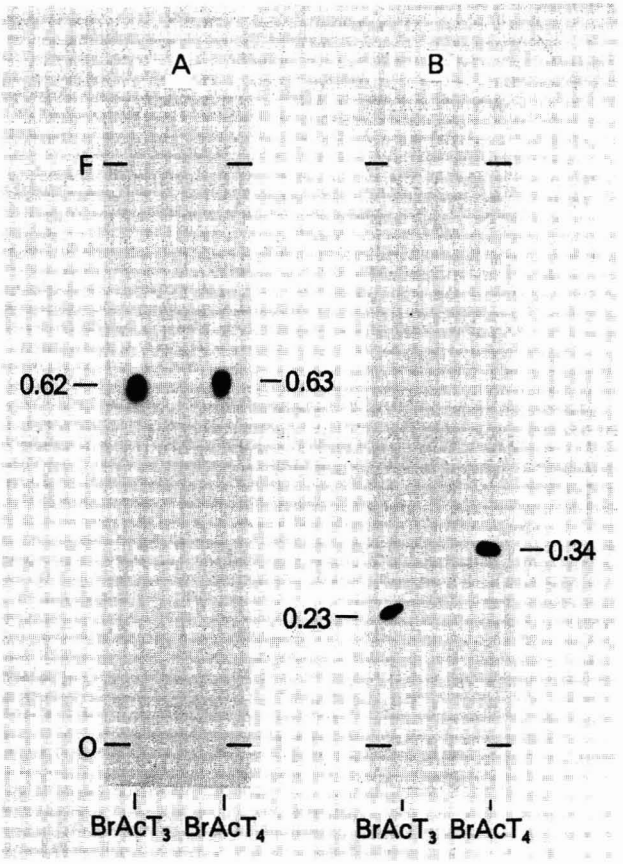

Fig. 5. TLC of BrAcT3 and of BrAcT4 in two solvent systems: acetic acid–ethyl acetate (1:9, v/v) (A) and acetic acid–methanol–chloroform (1:4:45), freshly prepared (B). Sample No. 180A, HSCCC fraction 61; Whatman K6F silica gel plates, 20 × 5 cm. O = Origin; F = front.

conjunction with HPLC, we also used the solvent system shown in the legend to Fig. 5B because it has a superior resolving power. Fig. 5 shows the chromatograms of HSCCC-purified BrAcT3 and BrAcT4 in the two solvent systems. The R_F values shown in Fig. 5 are reproducible only with the same type of TLC plates (Whatman K6F, 250 μm layer); R_F values obtained with preparative plates (Whatman PLK5F, 1000 μm layer) are considerably lower. Also the presence of a large amount of impurity changes R_F values.

Pure BrAcT3 has a melting point of 217.5–218.5°C with apparent decomposition since the melt is brownish, while the BrAcT3 used was a white solid material. It was obtained by concentrating HSCCC sample No. 173A, fraction 67 to one half of its volume in a SpeedVac concentrator (Savant) and then precipitating BrAcT3 by adding water. The precipitated material was collected by filtration, dried in a vacuum desiccator, and stored at room temperature in an amber container.

The UV absorption spectrum of this 94.4% pure BrAcT3 (HPLC) in 0.01 *M* NaOH containing 0.5% ethanol, showed the typical two peaks of 3′-iodo-4′-

hydroxydiphenyl ethers having a side chain in position 1. One has λ_{max} at 320 nm (molar absorption coefficient, $\varepsilon = 5500$ l mol^{-1} cm^{-1}), the other one at 225 nm ($\varepsilon = 49\,300$ l mol^{-1} cm^{-1}). In an acidic solvent (lower phase of HSCCC solvent 1:1:1:1) and in 1-butanol the higher-wavelength peaks were at 297 and 299 nm, respectively. Values for T3, T4 and other iodoamino acids have been summarized by one of us (H.J.C.) [12]. 3,3',5'-Triiodo-L-thyronine ("reverse T3") has its higher-wavelength peak at 322 nm ($\varepsilon = 6150$ l mol^{-1} cm^{-1}) in 0.01 *M* NaOH (unpublished data).

It can be concluded from our investigations that HSCCC is the method of choice for the purification of crude BrAcT3, conveniently prepared by a simple one-step bromoacetylation of T3.

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Semi-preparative purification of an endogenous ligand for brain serotonin-2 receptors by coil planet centrifuge counter-current chromatography

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ABSTRACT

A horizontal flow-through coil planet centrifuge equipped with a rotatory frame holding three sets of composite column assemblies was used for purification of an endogenous ligand (ketanserin binding inhibitor) for the [^{3}H]-ketanserin (^{3}H-KET) recognition site. The protein mixture containing the endogenous material was successfully resolved by using a two-phase solvent system consisting of 95% ethanol–31.5% ammonium sulphate (1:2). The active fractions on ^{3}H-KET binding obtained after counter-current chromatography (CCC) were further purified through a C_{18} μBondapak reversed-phase high-pressure liquid chromatographic column. The introduction of this advanced CCC technique represents an important step in the application of CCC for the separation of polar proteins from protein mixtures.

INTRODUCTION

In recent years, counter-current chromatography (CCC) has been used mostly for purification of substances with relatively high hydrophobicity [1]. Only recently, however, this technique has been applied to highly polar biological mixtures such as endogenous substances in brain [2] and anti-trypanosomal factor from *Pseudomonas fluorescens* [3]. Purification of proteins and peptides from brain and other tissues has been carried out classically using a wide variety of chromatographic methods including gel filtration, ion-exchange chromatography, affinity chromatography, CCC, electrophoresis, high-performance liquid chromatography (HPLC), etc. [4]. The recent introduction of an advanced centrifugal CCC technique in our laboratory represents an important step in the application of CCC to the separation of protein mixtures.

Previous studies from our group, as well as from other laboratories [5–7], have led to the conclusion that serotonin might not be the primary transmitter for the so-called serotonin-2 (5-HT-2) receptor. If this assumption holds true, an endogenous physiological chemical signal to be transduced by the 5-HT-2 receptor might exist.

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This endogenous ligand would function as a modulator or co-transmitter of the serotonergic receptors located at the post-synaptic site.

Binding techniques provide an easy and sensitive approach to study ligands for the 5-HT-2 recognition site and may be used to monitor the purification of endogenous putative ligands for 5-HT-2 receptors. Using [^{3}H]ketanserin (^{3}H-KET) as a radioligand probe to label the 5-HT-2 site, it was possible to extract, isolate, and purify ketanserin binding inhibitor (KBI), an endogenous compound that selectively inhibits ^{3}H-KET binding.

EXPERIMENTAL

Materials

Frozen rat brains were obtained from Zivic-Miller (Allison Park, PA, U.S.A.). Ammonium sulphate was purchased from Baker (Phillipsburg, NJ, U.S.A.). Glacial acetic acid and organic solvents were obtained from Aldrich (Milwaukee, WI, U.S.A.). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.). Bio-Gel P-10 resin was purchased from Bio-Rad (Richmond, CA, U.S.A.). The C_{18} μBondapak HPLC column was purchased from Waters (Milford, MA, U.S.A.). Ketanserin was obtained from RBI Labs. (Natick, MA, U.S.A.). ^{3}H-KET was purchased from New England Nuclear (Wilmington, DE, U.S.A.).

Protein purification

Routinely 360 g of rat brain were homogenized in 6 volumes of 0.1 *M* hot (80°C) acetic acid and centrifuged at 27 000 *g* for 15 min at 4°C. The clear supernatant was lyophilized and resuspended in 15 ml of 20% methanol–0.1 *M* acetic acid solution and applied to a Bio-Gel P-10 column (60 cm × 2.5 cm) with an eluting solvent consisting of 20% methanol–0.1 *M* acetic acid. Fractions of 2 ml were collected and aliquots of 50 μl were lyophilized and resuspended inTris–HCl buffer at pH 7.4 to determine its inhibitory activity of ^{3}H-KET binding. The fractions that inhibited ^{3}H-KET binding were then pooled and lyophilized. The material was resuspended in 0.1 *M* acetic acid and precipitated with 60% ammonium sulphate. After a 30-min stirring period, the sample was centrifuged at 40 000 *g* for 30 min and the supernatant was desalted by eluting a C_{18} Sep-Pak column with a 0.1 *M* TFA–60% acetonitrile solution and lyophilized. This material was injected directly into the CCC column.

Radioligand binding

^{3}H-KET binding was assayed on twice washed cortical synaptic membranes using 0.5 n*M* radioligand in 50 m*M* Tris–HCl buffer, pH 7.4. Incubation was performed at 4°C during 75 min. The reaction was stopped by filtration as previously described [6,8]. Non-specific binding was determined using 1 μM ketanserin.

Counter-current chromatography

Purification of KBI was carried out using a horizontal flow-through coil planet centrifuge equipped with three column holders (Model CCC-1000) (Pharma-Tech Research Corp., Baltimore, MD, U.S.A.). The rotatory frame of the apparatus holds three sets of composite column assemblies. Each assembly consists of a series of coiled columns which were prepared by winding two layers of PTFE tubing (1.6 mm I.D. ×

0.5 mm wall) onto ten stainless-steel rods (10 cm × 6.35 mm O.D.). The three composite columns were connected in series and mounted symmetrically on the column holder at a distance of 7.6 cm from the holder axis. The total capacity of the coiled column measured 150 ml. The two-phase solvent system was prepared by mixing 95% ethanol and 31.5% ammonium sulphate solution at a 1:2 volume ratio. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use. In a typical experiment, the stationary phase (upper non-aqueous phase) was introduced into the column at a flow-rate of 5 ml/min using a Model 300 LC perfusion pump (Scientific Systems, State College, PA, U.S.A.). After introduction of the stationary phase, the column was then rotated at 1000 rpm, and elution with the mobile phase (lower aqueous phase) started at a flow-rate of 1 ml/min. After perfusing 30 ml of mobile phase, the sample (2 ml total volume equilibrated in the two-phase solvent system) was introduced into the column through a four-way injection valve. Fractions of 2 ml were collected in a Model FRAC-100 fraction collector (Pharmacia, Piscataway, NJ, U.S.A.). After injection of the sample, the elution continued for 180 min. The volume of the stationary phase retained in the column averaged about 26% of the total column capacity. The elution profile was monitored using a Model SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan) coupled to a Model B5117-5 I Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). A 300-μl aliquot of each fraction was desalted through C_{18} Sep-Pak columns, lyophilized, and tested for its activity on ^{3}H-KET binding.

HPLC analysis

Further purification of the material was carried out using a Spectra-Physics liquid chromatographic system equipped with a Spectra-Physics Model 8700 solvent delivery system. The proteins were detected by monitoring absorbance at 210 nm. The material from the C_{18} μBondapak column previously equilibrated with a solution of 10% methanol–0.15 *M* acetic acid was eluted with a 0–60% gradient of a solution of 70% acetonitrile–15% methanol–0.1 *M* acetic acid. Fractions of 1 ml were collected and a 200-μl aliquot of each fraction was lyophilized and tested for activity on ^{3}H-KET binding.

RESULTS

Determination of the partition coefficient (K) of KBI in different two-phase solvent systems

As shown in Table I, the *K* values of KBI were determined using four different two-phase solvent systems. For these experiments, partially purified KBI obtained after Bio-Gel P-10 and 60% ammonium sulphate precipitation was used. After desalting and lyophilization, the material was dissolved in the solvent system under study. After equilibration, similar aliquots of the upper and lower phases were lyophilized and tested for their activity on ^{3}H-KET binding. The *K* value was determined by calculating the ratio between the percentage of ^{3}H-KET specific binding displacement by the upper phase (*a*) and that by the lower phase (*b*): $K = a/b$.

The first two solvent systems tested (*n*-butanol–acetic acid–water, 4:1:5, and *n*-butanol–phosphate buffer, 1:1) showed *K* values of 0, indicating that KBI was

TABLE I

PARTITION COEFFICIENT (K) VALUES OF KBI ON DIFFERENT TWO-PHASE SOLVENT SYSTEMS

Abbreviations: ($\%I_u/\%I_l$) = percentage inhibitory activity of KBI in the upper phase/percentage inhibitory activity in the lower phase; R = retention volume; V_c = total column capacity; R_{sf} = retention volume of the solvent front.

Solvent system	Direct analysis ($\%I_u/\%I_l$)	HPLC ($\%I_u/\%I_l$)	CPC-CCC $\frac{R - R_{sf}}{V_c - R_{sf}}$
n-Butanol–acetic acid–water (4:1:5)	0	–	–
n-Butanol–2 M phosphate buffer (1:1)	0	–	–
12.5% PEG 1000 in 1.5 M potassium phosphate buffer (pH 6.9)	1.7	–	–
1-Propanol–acetic acid–43% ammonium sulphate[a] (1:1:3.5)	0.97	–	Peak A: 0 Peak B: 0.15
95% Ethanol–31.5% ammonium sulphate[a] (1:2)	0.84	0.77	Peak 2: 0.34 Peak 3: 0.46

[a] Upper (organic) phase: stationary phase.

present only in the lower aqueous phase (Table I). Evaluation of a third solvent system containing 12.5% polyethyleneglycol (PEG) 1000 in 1.5 M phosphate buffer yielded a K value of 1.7 (Table I). This result prompted us to determine the feasibility of this solvent system for CCC separation and purification of KBI. Using PEG-rich upper phase as the stationary phase and the lower phosphate buffer as the mobile phase, CCC separation yielded multiple protein peaks by monitoring the absorbance profile at 210 nm. The use of this solvent system for purification of KBI, however, was hampered by the difficulty in eliminating the PEG 1000 present in each sample which interfered with the ^{3}H-KET specific binding assay.

The fourth solvent system analyzed (1-propanol–95% ethanol–43% ammonium sulphate, 1:1:3.5) yielded a K value of 0.97. When this system was applied to CCC using the organic upper phase as the stationary phase and the lower aqueous phase as the mobile phase, the absorbance profile monitored at 210 nm showed four partially resolved peaks eluted immediately after the solvent front. The first two peaks (peaks A and B, Table I) were active on binding while the third and fourth peaks presented little activity. Graphic analysis of the partition coefficients of peaks A and B showed K values different from those anticipated by the two-phase distribution of KBI in the same solvent system (Table I). The inability of this solvent system to resolve the active peaks from the bulk of proteins eluted near the solvent front led us to discard this system.

After evaluation of the eluotropic, solubility, and hydrophilicity parameters of a series of organic solvents it was concluded that a combination of 95% ethanol and high salt concentration represented a suitable solvent system for the separation of these highly hydrophilic compounds. The solvent system formed by 95% ethanol–31.5% ammonium sulphate (1:2) yielded a K value close to 0.8. This value was

constant either after determination of the partition coefficient through direct analysis of the two-phase distribution of KBI or after injection of equal volumes of each layer of the two-phase solvent system on a C_{18} μBondapak reversed-phase HPLC column. When this solvent system was used for CCC analysis, the K values differed from those anticipated using the methods mentioned above (Table I).

The efficient separation provided by this solvent system and the reproducibility of the absorbance profiles monitored at 210 nm led us to pursue the purification of KBI using a mixture of 95% ethanol–31.5% ammonium sulphate (1:2).

Physical properties of the 95% ethanol–31.5% ammonium sulphate (1:2) solvent system

As shown in Table II, all the physical parameters so far tested indicate that the two-phase solvent system used in the purification of KBI can be included within the hydrophilic systems. Both viscosity and density values are highest among the hydrophilic solvent systems (Table II and ref. 9). Similarly, both interfacial tension difference ($\Delta\gamma$) and settling time values (T and T') are also among the highest when compared to other hydrophilic systems (Table II and ref. 9). These particular physical properties led us to choose the present CCC centrifuge instead of a high-speed CCC centrifuge because the latter would not retain this highly hydrophilic solvent system possessing a long settling time.

Purification of KBI through a coil planet centrifuge

The active material on ^{3}H-KET binding obtained after acid extraction, Bio-Gel P-10 chromatography and ammonium sulphate precipitation represents a complex mixture of proteins of molecular weight below 20 000. For separation by CCC, an amount of material equivalent to 40–60 mg of protein is injected. Consequently, a high concentration of the solute in the two-phase system considerably alters the two-phase equilibrium composition and the partition coefficient values of the sample components. This equilibrium point may change according to the amount of material injected and, in turn, this could result in changes in the elution profile. To overcome this problem, the material was dissolved with a saturating concentration of ammonium sulphate (95% ethanol–40% ammonium sulphate 1:2). In this condition, the excess of the salt was precipitated from the sample solution and eliminated by centrifugation at 9400 g for 5 min at room temperature. The resultant supernatant (2-ml volume) was injected through an injector 30 min after starting the elution of the mobile phase (31.5% ammonium sulphate). This delay in the sample injection proved crucial for the successful separation of the active material.

As observed in Fig. 1, the elution profile shows multiple UV-absorbing peaks under the conditions selected. Determination of the activity of each single fraction after desalting 300-μl aliquots on C_{18} Sep-Pak columns indicated that the fractions active on ^{3}H-KET binding do not always correspond to the UV-absorbing peaks observed in the elution profile. The pattern of inhibitory activity of the eluted material on ^{3}H-KET binding after CCC clearly shows five different peaks, indicated as peaks 1–5 (Fig. 1.).

HPLC purification of KBI after coil planet centrifuge CCC

Fractions corresponding to peaks 1–5 underwent lyophilization, and the result-

TABLE II

COMPARISON OF THE PHYSICAL PROPERTIES OF SOME HYDROPHILIC TWO-PHASE SOLVENT SYSTEMS

Abbreviations: η_u/η_l = upper phase viscosity/lower phase viscosity; η = mean viscosity of the two phases; ρ_u/ρ_l = upper phase density/lower phase density; Δ_ϱ = density difference between the two phases; T = settling time after gentle mixing; T' = settling time after vigorous shaking.

Solvent system[a]	Interfacial tension, $\Delta\gamma$ (dyne/cm)	Viscocity (cp)		Density (g/cm^3)		Settling time at 22°C (s)	
		η_u/η_l	η	ρ_u/ρ_l	Δ_ϱ	T	T'
n-Butanol–acetic acid–water (4:1:5)	<1	1.63/1.40	1.52	0.90/0.95	0.05	38.5	37.5
n-Butanol–acetic acid–1 *M* NaCl (4:1:5)	1	1.69/1.26	1.48	0.88/1.05	0.16	26.5	24.5
sec.-Butanol–water	<1	2.70/1.67	2.19	0.87/0.97	0.10	57.0	58.0
sec.-Butanol–1 *M* NaCl (1:1)	3	1.91/1.29	1.60	0.84/1.03	0.19	34.0	33.5
95% Ethanol–31.5% ammonium sulphate (1:2)	3	2.90/2.82	2.86	.95/1.15	.20	41.0	40.0

[a] Obtained from ref. 9.

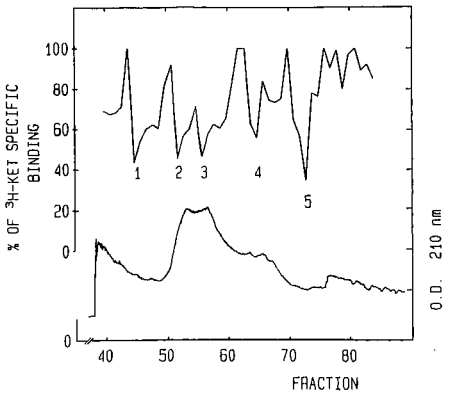

Fig. 1. Counter-current chromatography elution profile of KBI: UV absorbance and percentage inhibition of ^{3}H-KET specific binding.

ing material was redissolved in 2 ml of a 0.15 *M* acetic acid–10% ethanol mixture. Aliquots of 2–3 mg were applied to a semipreparative C_{18} μBondapak reversed-phase HPLC column previously equilibrated with a mobile phase A (0.15 *M* acetic acid–10% methanol) and eluted with a mobile phase B (0.10 *M* acetic acid–15% methanol–70% acetonitrile) gradient of 0–60% during 60 min. Evaluation of the inhibitory activity of peaks 1–5 after HPLC showed that only the material obtained from peak 2 and, to a much lesser degree, peak 3 was active on ^{3}H-KET binding. The inhibitory activity on ^{3}H-KET binding of peaks 1, 4 and 5 was no longer observed after HPLC purification. Fig. 2 shows the comparison on the elution profile and activity pattern after HPLC purification of KBI before (A) and after (B) (peak 2) CCC. A clear reduction in the absorbance peak is observed in the sample after CCC with almost no changes in the activity of the material on ^{3}H-KET binding.

DISCUSSION

The combination of different purification procedures including acid extraction, gel filtration of the supernatant and ammonium sulphate precipitation enabled us to obtain a partial purification of KBI. These high-capacity purification steps, however, did not allow enough purification on a semi-analytical or analytical low-capacity system (HPLC). At this point, the use of an intermediate capacity purification approach became necessary.

Analysis of the distribution pattern of KBI in the different solvent systems and the elution profile in CCC using the solvent system 1-propanol–95% ethanol–43% ammonium sulphate (1:1:3.5) suggested that this material had high polarity (see Results). While polymer phase systems are described as the most suitable approach for separation of hydrophilic compounds [10], difficulty in eliminating the PEG 1000 from single fractions hampered the use of this system.

The discrepancy between the partition coefficient values obtained after distribution of KBI in the two-phase solvent systems and after CCC separation reinforces the idea that *K* values in the sample mixture should be determined by HPLC analysis on each layer of the two-phase solvent system [11]. In the studies using the 95% etha-

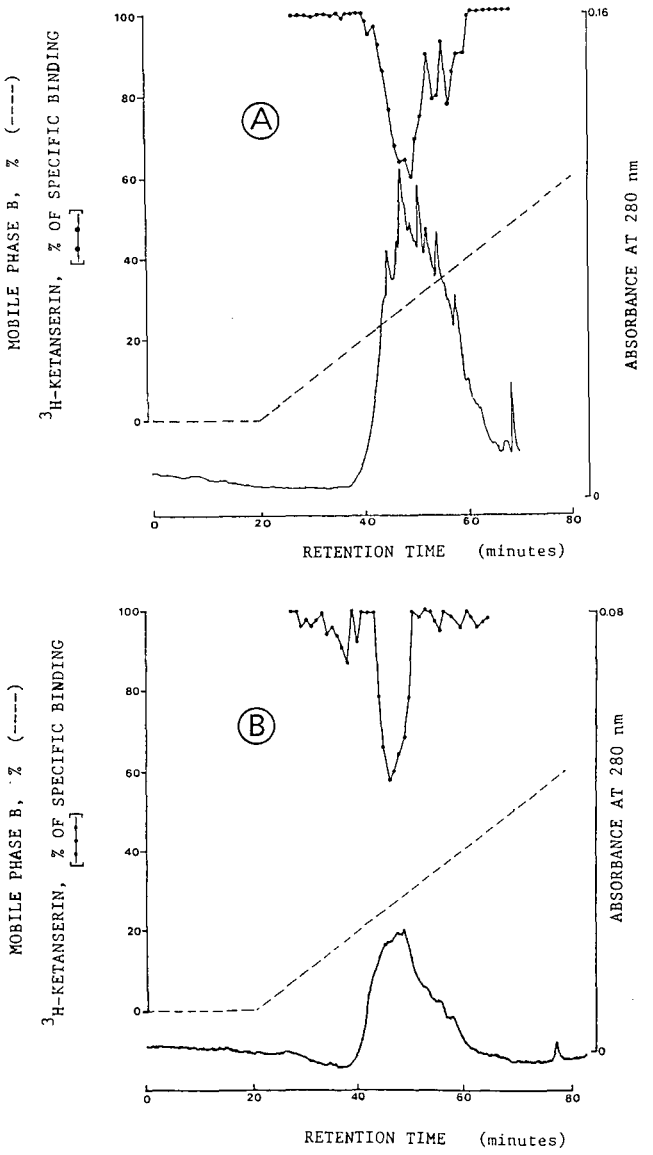

Fig. 2. Reversed-phase HPLC of KBI before (A) and after (B) counter-current chromatography: UV absorbance and percentage inhibition of ^{3}H-KET specific binding.

nol–31.5% ammonium sulphate (1:2) solvent system, however, it was observed that the *K* values were similar either after direct analysis of each layer of the two-phase solvent system on ^{3}H-KET binding or after analysis of the activity profile on ^{3}H-KET binding following the injection of each layer of the two-phase solvent system on a C_{18} μBondapak reversed-phase HPLC column. The inability of the reversed-phase HPLC system, under the present experimental conditions, to resolve the different

peaks of activity on ^{3}H-KET binding, as observed after CCC, could provide a rational basis for explaining the failure of HPLC analysis to provide accurate data on partition coefficient values in the complex sample mixture.

The physical properties of the 95% ethanol–31.5% ammonium sulphate (1:2) system place this two-phase solvent system in the framework of hydrophilic solvent systems [9]. However, the low interfacial tension and high viscosity make this solvent system unsuitable for high-speed CCC [12] due to the carry-over of the stationary phase from the column, as observed with the polymer phase system. The high polarity of the mixture, however, enables the separation of highly hydrophilic protein mixtures without the problems observed with the polymer phase system.

A highly effective separation was obtained when the upper (ethanol) phase was used as a stationary phase. When the lower (ammonium sulphate) phase was used as the stationary phase, fluctuations in the percentage of stationary phase retention produced inconsistent separation profiles from run to run. Further analysis of peaks 1–5 through reversed-phase HPLC revealed that only the second and, in a very small percentage, the third peak contained KBI activity. No significant KBI activity was detected after HPLC of peaks 1, 4 or 5. The ability of CCC to separate active material on ^{3}H-KET other than KBI (serotonin, etc.) provides further evidence on the efficiency of this system for semi-preparative purification of KBI. Moreover, comparison of the HPLC elution profiles before and after CCC (Fig. 2) provides reasonable information on the increase of specific activity of KBI.

In conclusion, the present semi-preparative CCC represents an important complementary approach in the purification of KBI. In this particular case, the coupling of CCC to HPLC significantly improved the purification of our material.

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Note

Recent applications of counter-current chromatography to the isolation of bioactive natural products

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In our continuing study of biologically active natural products, counter-current chromatographic (CCC) methods have been demonstrated to be useful, especially in the isolation of polar compounds. Thus, droplet counter-current chromatography (DCCC) and rotation locular counter-current chromatography (RLCCC), have an inherently large sample capacity, and yet a typically low solvent consumption. More importantly, CCC require no absorbent which often causes the irreversible absorption of polar compounds.

In general, when the fractionation is guided by bioassay to isolate active principles from crude extracts, the first step is to partition the extracts between water and organic solvents such as *n*-hexane, diethyl ether (or chloroform), ethyl acetate and *n*-butanol to narrow the spectrum of chemical constituents and concentrate the biological activity. If the biological activity is found in polar fractions such as the ethyl acetate and/or *n*-butanol fractions, CCC may be considered a practical application. Although the isolation of natural products usually involves a combination of various chromatographic methods, some compounds have been isolated using only CCC methods. We have previously reported on our isolation of various phytochemicals by DCCC and RLCCC [1–4]. The present paper is limited to a few additional phytochemicals recently reported in our laboratory. For example, efficient and simple methods for the isolation of various polar phytochemicals are described, such as (1) a flavone glycoside, rutin, from the leaves of *Esenbeckia pumila* (Ruraceae) by RLCCC, (2) two stilbene glycosides, rhaponticin and 4′-O-methylpiceid, from the roots of *Rheum palmatum* (Polygonaceae) by DCCC and (3) two antifungal steroidal glycoalkaloids, solasonine and solamargine, from the ripe fruits of *Solanum incanum* (Solanaceae) by a combination of RLCCC and DCCC.

APPLICATIONS

Flavone glycoside from Esenbeckia pumila

The dried powdered leaves of *E. pumila* (Rutaceae) (600 g), collected near Sãn Paulo, were extracted with dichloromethane followed by methanol at room tempera-

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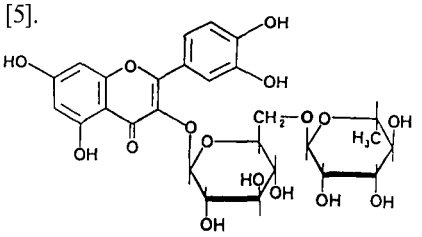
ture. Insect growth inhibitory activity against several lepidopterans was found in the methanol extract. The methanol extract was evaporated to dryness under 40°C to give a dark brown residue (15 g). A part of this residue (2.0 g) was suspended in a mixture of water and a small amount of methanol. The water suspension was partitioned with chloroform to remove non-polar components. The water layer was concentrated *in vacuo* to give a syrup (400 mg), which was dissolved in 20 ml of water. The water solution was injected into an RLCCC sample loop after filtration. A stationary phase of water, a mobile phase of water-saturated diethyl ether (500 ml) and water-saturated ethyl acetate (500 ml) and the upper layer of ethyl acetate–propanol–water (10:1:2 and 4:1:1, v/v, 1 l) proved to be a successful solvent system in the ascending method. The flow-rate of the mobile phase was adjusted to 1 ml/min, and the eluent was collected in 25-ml fractions. Each fraction was checked by thin-layer chromatography (TLC) with a vanillin–sulfuric acid spray reagent. Fractions 37–57 yielded 30 mg of an insect growth inhibitory yellow compound, which was then crystallized from methanol and water. The yellow needles were identified as rutin (**1**) by comparison of their spectroscopic data [UV, IR, fast atom bombardment mass spectrometry (FAB-MS), ^{1}H NMR and ^{13}C NMR] with those of an authentic sample. An efficient and simple method for the isolation of rutin by RLCCC using only a gradient elution as the mobile phase has been established [5].



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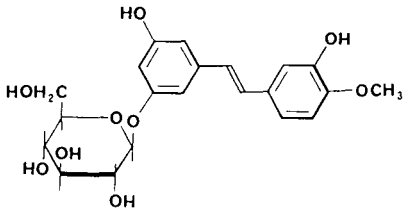

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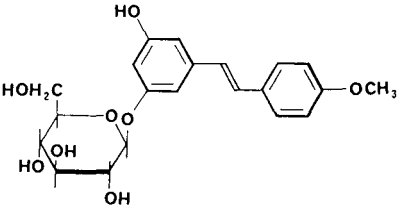

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The isolation of rutin has been reported from many plants by various methods. However, these reported methods use solid packing materials which often cause irreversible absorption (at least some if not all) of polar compounds such as rutin. In addition, all of these methods are time-consuming. In contrast, RLCCC is faster without using solid packing materials.

In addition, although an example is not provided in this paper, RLCCC can be used for the efficient initial fractionation using a gradient elution without forming troublesome emulsions that are frequently encountered in separatory funnel-type partitions.

Stilbene glycosides from Rheum palmatum

In Indonesia the roots of *R. palmatum* (Polygonaceae), known as "kelembak", are used to treat malaria and tropical cough. The roots of *R. palmatum* (397 g) were extracted with methanol at room temperature. This methanol extract inhibited β-glucosidase activity. Following suspension of a portion of the extract (4 g) in water, the water-insoluble portion was removed by filtration, and the suspension was partitioned into *n*-hexane, chloroform, ethyl acetate and water-soluble fractions, yielding 102, 147, 1108 and 1310 mg, respectively. The bioactive yellow precipitate was obtained from the ethyl acetate fraction after concentration of the volume to one twentieth, and a part of this (1 g) was dissolved in 14 ml of the stationary and mobile phase (1:1, v/v) for DCCC. The solvent system, chloroform–methanol–water (7:13:8, v/v) was chosen for DCCC separation in the descending method based on TLC analysis. The flow-rate of the mobile phase was adjusted to 21 ml/h. Each 21-ml fraction was collected into test tubes and monitored by TLC with use of a vanillin–sulfuric acid spray reagent. A total of 141 fractions were collected. Fractions 28–35 afforded 98.5 mg of compound **2** and fractions 87–141 yielded 184.5 mg of compound **3** (Fig. 2). Following crystallization from dichloromethane and ethanol, needles of compounds **2** and **3** were obtained. They were identified as rhaponticin, 3,5,3′-trihydroxy-4′-methoxystilbene 3β-D-glucopyranoside (**2**), and 4′-O-methylpiceid, 3,5-dihydroxy-4′-methoxystilbene 3β-D-glucopyranoside (**3**) [6], respectively, by spectroscopic data (UV, IR, FAB-MS, ^{1}H NMR and ^{13}C NMR) [7].

In contrast to RLCCC, typical DCCC separation is much more time-consuming since it operates at low flow-rates and pressures. The separation of 1 g of the above stilbene glycosides by DCCC required five to seven days, even though the flow limitation was reduced in part by a modification of the commercial instrument [8]. Another limitation of DCCC is selection of solvents which must form droplets [9–11]. Incidentally, this also limits the maximum elution flow-rate. Despite these limitations, DCCC is still very useful because of better resolution compared to RLCCC. An attempt to continuously inject the sample without washing the vertical glass columns of the DCCC system, to reduce the time between injections,failed due to the surfaces of the glass becoming wettable. Thus, washing the glass columns after each injection, at least in the case of the above separation, seems essential to maintain good resolution.

Steroidal glycoalkaloids from Solanum incanum

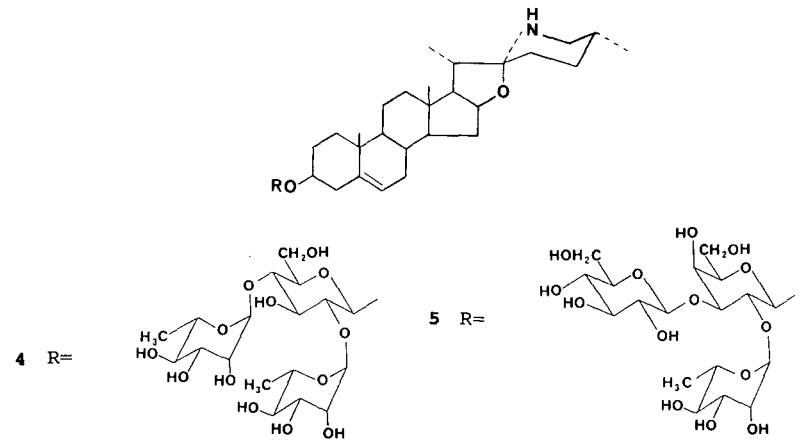
A shrub *S. incanum* (Solanaceae), known as the "sodom apple", is common in the tropics, especially in waste land. In East Africa the fruits of the shrub are used to treat skin diseases [12]. In our routine antimicrobial screening, the methanol extract of the ripe fruits of *S. incanum* exhibited antifungal activity.

Fresh ripe fruits of *S. incanum* (550 g) were extracted with methanol at room temperature. Following solvent partitions, the water-insoluble portion was removed, and the suspension was partitioned into *n*-hexane, chloroform, ethyl acetate and water-soluble fractions, yielding 0.7, 0.4, 0.5 and 16.6 g, respectively. The biological activity was found in the water-soluble fraction. The aqueous fraction was concentrated to 30 ml and injected directly into the sample loop of an RLCCC apparatus. For the successful RLCCC solvent system, water was used as a stationary phase, and a gradient elution of mobile phase was carried out in an ascending method. The first elution solvent, consisting of water-saturated ethyl acetate (1.5 l) was employed to

remove the non-polar components, as monitored by TLC with a vanillin–sulfuric acid spray reagent. The subsequent mobile phase, the upper layer of ethyl acetate–butanol–water (2:1:1, v/v), yielded crude antifungal alkaloid fractions consisting primarily of two components positive to Dragendorff's reagent.

A portion of the crude alkaloid fraction (400 mg) obtained from RLCCC was dissolved in 10 ml of the stationary and mobile phases (1:1, v/v) for DCCC. The solution was filtered and injected into a sample loop of a DCCC apparatus. The solvent system, chloroform–methanol–water–propanol–ammonium hydroxide (34:65:40:5:1, v/v), was chosen for DCCC separation in the ascending method based on TLC analysis of the fractionated alkaloids. The flow-rate of the mobile phase was adjusted to 3.0 ml/h. Each 9-ml fraction was collected into test tubes and monitored by TLC with vanillin–sulfuric acid and Dragendorff's spray reagents. A total of 95 fractions were collected. Fractions 41–51 afforded 84.3 mg of compound **5** and fractions 54–59 yielded 84.8 mg of compound **4**.

Following crystallization from methanol and water, fine needles of compounds **4** and **5** were identified as solamargine (**4**) and solasonine (**5**), respectively, by



comparison of their spectroscopic data (IR, FAB-MS, ^{1}H NMR and ^{13}C NMR) with those of authentic samples. Thus, the semi-preparative isolation of two bioactive steroidal glycoalkaloids, solamargine (**4**) and solasonine (**5**), was performed efficiently by the combination of RLCCC and DCCC [13].

Combining RLCCC with DCCC could exploit advantages of both techniques and, in our case, led to the isolation of the two steroidal glycoalkaloids from *S. incanum*. RLCCC was employed with a gradient elution for large-scale separation of a crude extract into several impure fractions prior to applying to DCCC. The subsequent application of DCCC, with its higher resolution, gave the pure steroidal glycoalkaloids on a large scale. This combined CCC technique accomplished the separation with solvent–solvent partition chromatography without solid packing materials. Thus, irreversible absorption of compounds to absorbents could be avoided. This method, therefore, might be generally applicable for the isolation of polar and/or unstable natural products.

CONCLUSION

The aforementioned, all-liquid separation techniques were completed without using any solid packing materials which might cause the irreversible absorption of large amounts of these polar compounds.

Although RLCCC is advisable to use prior to DCCC for initial fractionations to narrow the spectrum of chemical constituents for subsequent bioassay [1], RLCCC alone sometimes led to the isolation of pure compounds [1,3,14]. Usually, however, combining RLCCC with DCCC could maximize the advantages of both techniques.

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Applications of high-speed counter-current chromatography for the separation and isolation of natural products

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ABSTRACT

High-speed counter-current chromatography (HSCCC) with a Ito multi-layer coil separator–extractor was applied to perform efficient separations of natural products and to isolate pure compounds from crude plant extracts. The high performance of the system was demonstrated in the separation and purification of natural antioxidants from rosemary and sage extracts, bitter principles from hops and aroma-relevant constituents, such as phthalides, from celery and parsley roots. In addition, the evaluation of a gas chromatographic-compatible non-aqueous solvent system, suitable for the separation of flavour extracts, is described.

INTRODUCTION

In recent years there has been a renaissance of liquid–liquid chromatographic (LLC) methods, especially in the separation of natural products. These developments mainly involved the emergence of different counter-current chromatographic (CCC) techniques [1]. Further, the advent of high-speed CCC (HSCCC) led to considerable improvements in terms of separation efficiency, analysis time and handling of the apparatus compared with, *e.g.*, droplet counter-current chromatography (DCCC) [2–5].

In this paper we report investigations with an Ito multi-layer coil separator–extractor (a centrifugal CCC system) and several successful applications concerning the preparative isolation of antioxidant principles [6,7] as pure compounds from crude rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) solvent extracts and describe the evaluation of a novel non-aqueous biphasic system with good hydrodynamic properties which permits the preparative isolation of bitter principles from hops and aroma-relevant constituents from celery (*Apium graveolens* L.) and parsley [*Petroselinum crispum* (Mill.) Nyman syn. *P. sativum* Hoffm.] roots.

Additionally, it is shown that the present method has the advantage over, for example, adsorption chromatographic methods that the artefact formation of

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sensitive compounds is prevented and therefore compounds could be isolated that were not detectable by adsorption chromatography [8].

EXPERIMENTAL

All separations were carried out with a multi-layer coil planet centrifuge called the Ito multi-layer coil separator–extractor (obtained from (P.C. Inc., Potomac, MD, U.S.A.), equipped with a 160 m × 1.6 mm I.D. coil and with a total column capacity of *ca.* 325 ml. For solvent delivery the chromatograph was connected to a Gilson 303 single-piston pump with pressure module. The effluent from the outlet of the column was continuously monitored at 254 and 280 nm using a Uvicord III 2089 UV detector (LKB, Bromma, Sweden) connected to an LKB 2210 two-channel recorder, and fractionated into test-tubes with an LKB Ultrorac 7000 fraction collector. Samples were dissolved in the respective mobile phase and injected by means of a six-way valve and a 4.5-ml sample loop.

In each experiment the non-rotating multi-layer coiled column was first entirely filled with stationary phase, then the mobile phase was pumped into the column while the apparatus was run at the optimum speed of 800 rpm. After equilibrium had been reached, as indicated by a clear mobile phase eluting at the column outlet (effluent was collected in a graduated cylinder), the sample solution was injected. The percentage of the stationary phase retained in the column could easily be determined from the volume collected in the cylinder having been displaced by the mobile phase.

The appropriate elution mode used depended on the whether the upper or the lower phase of the biphasic solvent system applied was chosen as the stationary phase. When the stationary phase was the lower phase, mobile phase (upper phase) was pumped into the "tail" end of the coil (tail-to-head elution mode), and when the mobile phase was the lower phase, it was pumped into the "head" end of the coil (head-to-tail elution mode). The two-phase solvent mixtures applied for the different separations were thoroughly equilibrated in a separating funnel at room temperature and separated before use.

Monitoring of the fractions of interest was carried out by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Gynkotek 300B double-piston pump, a Gynkotek 250B low-pressure gradient former and a Philips PU 4021 diode-array detector, connected to a Shimadzu CR-3A integrator. HPLC separations were performed on a 250 mm × 4 mm I.D. LiChrospher 100 RP-18 (5-μm) column (Merck, Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Separation of antioxidants from rosemary and sage

Various crude solvent extracts of rosemary and sage were resolved by HSCCC with hexane–ethyl acetate–methanol–water (70:30:14:8) as solvent system (Fig. 1). The selection of the three different extracts mentioned in Fig. 1 was based on how far the solvent polarity influences the extraction of the antioxidants. All three extracts showed a strong antioxidative activity [8].

According to a rapid thin-layer chromatographic method for the choice of a two-phase aqueous solvent system that is applicable to the separation of a given

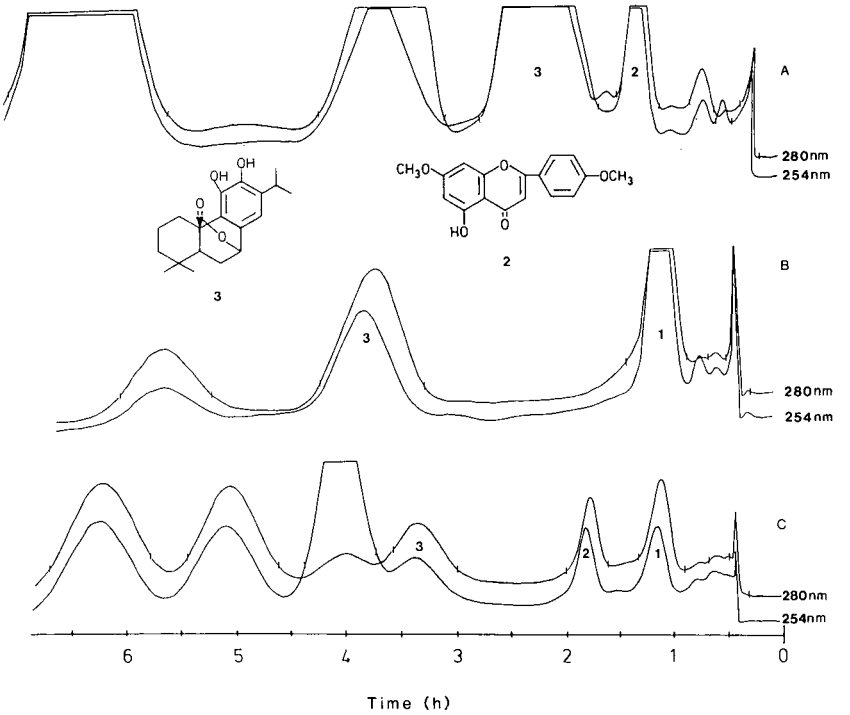

Fig. 1. HSCCC of (A) rosemary methanol, (B) sage hexane and (C) rosemary pentane extracts. Solvent system, hexane–ethyl acetate–water–methanol (70:30:8:14); flow-rate, (A) 1.6, (B) and (C) 1.5 ml/min; rotational speed, 800 rpm; sample, (A) 715, (B) 750 and (C) 235 mg. Peaks: 1 = unknown; 2 = genkwanin 4′-methyl ether; 3 = carnosol.

mixture or extract, described by Hostettmann *et al.* [9], the chosen solvent mixture appeared to be the most suitable, particularly in terms of separation efficiency. The separations were performed in the tail-to-head elution mode, whereby the lower phase was used as stationary phase and the upper phase was eluted through the column at flow-rates of 1.5 and 1.6 ml/min.

As shown in Fig. 1, the applied chromatographic conditions facilitate the separation of co-extracted spice constituents such as chlorophylls and carotenoids (the first three peaks in all chromatograms) from the relevant antioxidants, which makes it possible to isolate the antioxidant principles, genkwanin 4′-methyl ether (5-hydroxy-7,4′-dimethoxyflavone) (peak 2), carnosol (3) and a still unknown component (1), as pure compounds. Although large amounts of crude extract (see Fig. 1) were charged into the multi-layer coil, the compounds of interest were baseline separated and no peak overlap with other spice constituents occurred. Collecting only the fractions at the peak maximum, for example, the separation of the methanol extract of rosemary in a single run provided carnosol in amounts up to 66 mg and genkwanin 4′-methyl ether up to 6 mg with a purity of *ca.* 95%. The structure of the pure antioxidants was checked by spectroscopic methods (mass, IR, NMR). The spectra obtained were compared

with literature data. The 90% purity of the unknown component (peak 1) enabled us to perform gas chromatographic–mass spectrometric (GC–MS) analyses without further clean-up steps.

The results show clearly that this CCC method facilitates the direct isolation of pure antioxidants from crude spice extracts. Compared with the classical adsorption chromatography it is not necessary to clean up the extract (*e.g.*, by solvent washing, bleaching with active carbon) [10] before isolating the antioxidants. The applied solvent system in combination with a proper elution mode permits the direct efficient separation of undesirable constituents (*e.g.*, chlorophylls and carotenoids) in a single separation step. Apart from this advantage, preparative HSCCC offers the possibility of large sample loads without a decrease in separation efficiency (compare Fig. 1A with Fig. 1C) and provides a sample recovery of up to 99%.

Another aspect concerns the problem of artefact formation of sensitive compounds (*e.g.*, polyphenolic substances like the above-mentioned antioxidants) during work-up procedures. Compared with adsorption chromatographic separations, the lack of a solid support in CCC prevents artefact formation, based on interactions between solute molecules and sorbents. Comparative investigations of the same extract by CCC and adsorption chromatography furnish information about which constituents were originally in the extract and which have to be regarded as work-up artefacts (*e.g.*, produced via adsorption chromatographic separations).

In these studies, the not yet completely identified, highly antioxidative compound that gives peak 1 in Fig. 1 apparently only occurs in non-polar solvent extracts of rosemary and sage [8]. It seems to be a "precursor compound" of the known antioxidants such as carnosol, rosmanol, rosmaridiphenol and carnosic acid [6,10,11]. Different treatments and derivatization reactions of this substance, comparable to the work-up procedures with rosemary extracts described in literature [6,10], revealed the formation of the above-mentioned antioxidants [8]. As this compound could not be isolated or detected in fractions separated by adsorption chromatography on silica gel columns, the applied HSCCC method also appears to be a useful separation technique for investigations concerning artefact formation of sensitive compounds.

Separation of bitter principles from hops

Our interest was to isolate pure humulones from a crude carbon dioxide hop extract for use in metabolic studies. The separation of hop bittering compounds presents a certain analytical problem, as the humulones and lupulones consist of a number of methyl homologues and are closely related in their structures (Fig. 2). In order to achieve a efficient chromatographic separation, we sought an appropriate non-aqueous CCC solvent system, because the solubility of humulone in aqueous solutions is very poor.

Tests on the hydrodynamic properties of different possible two-phase systems according to Ito [4] showed that hexane and acetonitrile, mixed with *tert.*-butyl methyl ether as "modifier", are well suited, and the system hexane–*tert.*-butyl methyl ether–acetonitrile (10:1:10) proved to be promising. The retention of the stationary phase, which mainly determines the quality of a separation [4], was of the order of 80–90% in the reversed elution mode (head-to-tail elution mode).

Fig. 3 shows the chromatogram of a small-scale separation (sample loading *ca.* 24 mg) of bitter principles from the crude carbon dioxide hop extract. The first large

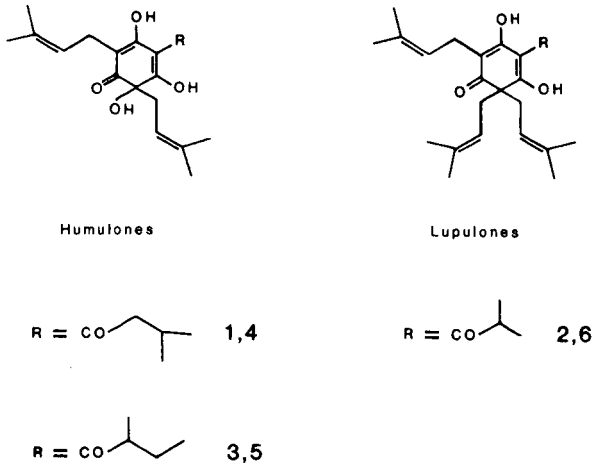

Fig. 2. Structural formulae of important hop bittering compounds. 1 = Humulone; 2 = cohumulone; 3 = adhumulone; 4 = lupulone; 5 = colupulone; 6 = adlupulone.

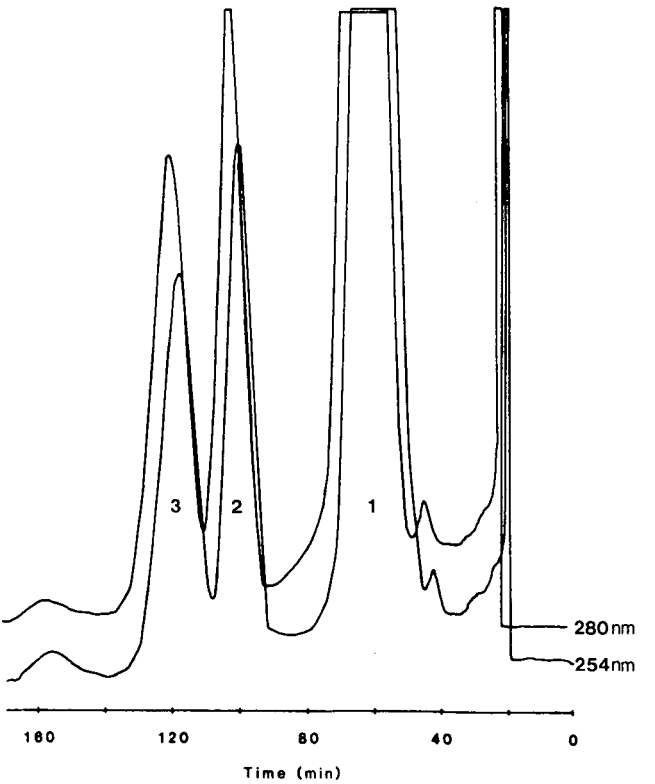

Fig. 3. HSCCC of a hop extract. Solvent system, acetonitrile–*tert.*-butyl methyl ether–hexane (10:1:10); flow-rate, 2.0 ml/min; rotational speed, 800 rpm; sample, 24 mg. Peaks: 1 = cohumulone, humulone, adhumulone; 2 and 3 = lupulones.

peak, marked 1, indicates cohumulone (ascending peak side), adhumulone and humulone, and the following two peaks (2 and 3) show the corresponding lupulones. Although this solvent system only separates groups of constituents (humulones and lupulones), the isolation of pure compounds was achieved. During the first part of a preparative HSCCC run with a sample loading of 500 mg, 1.5-ml fractions were taken, increasing to 5 ml in the last part. The components of these fractions were monitored by reversed-phase high-performance liquid chromatography (RP-HPLC) [12], using pure humulones and lupulones as references. UV detection in the HPLC of the relevant fractions showed only one peak. Although there is only the small difference of a single methyl group, 30 mg of cohumulone of purity >95% and 98 mg of humulone with the same purity could be isolated in a single run. The isolation of pure adhumulone was not tried and seems to be impossible with the configuration used, as it is with virtually any LC system. The separation concerning the lupulones was slightly worse and would require additional optimization of the biphasic system, but this task was beyond the scope of this study.

Compared with HPLC separations of hop extracts, where two RP 18-columns have to be connected in series [13] in order to achieve the separation efficiency required and a solvent is used that contains acid (eluent pH 2.0) to improve the elution profile, which is not desirable for preparative purposes in order to avoid possible acid-catalysed reactions, HSCCC also in this instance provides a mild, time-saving separation technique.

Separation of flavour compounds

A special task in separating flavour substances by LC is to find a system that is compatible with GC, which of course represents the state of the art for the analysis of such components. LC on silica gel, in principle well compatible with GC with respect to the solvents usually applied, is not useful for the separation of more polar compounds, because sample losses can occur owing to irreversible adsorption. Therefore addition of polar modifiers (*e.g.*, methanol) is required at the expense of selectivity and resolution [14]. The widely used reversed-phase chromatography is based, at least for the range of polarities covered by most flavour compounds, on aqueous solvent mixtures, a fact that necessitates an additional extraction step before GC analysis.

Accordingly, the non-aqueous biphasic system hexane–acetonitrile (1:1) was selected with respect to its GC compatibility as a basis for the following optimizations. A set of biphasic systems, obtained by addition of different amounts of the modifiers *tert.*- butyl methyl ether and dichloromethane, was evaluated, using a mixture of test compounds (95% purity) with different functional groups. The components were mixed in equal amounts. For better UV detectability, the aromatic compounds benzyl alcohol, benzyl methyl ketone, benzyl acetate and ethyl benzoate were chosen as test substances. For every phase system the number of theoretical plates N and the resolution R_s were calculated [15]. The results are summarized in Table I.

All separations were carried out in the head-to-tail elution mode with the upper phase as stationary phase. The pure hexane–acetonitrile system shows the maximum difference in polarity between the two phases, resulting in the smallest capacity factors for the test substances used. The system acetonitrile–*tert.* butyl methyl ether–hexane (10:1:10) exhibited good stability (retention of stationary phase) and a good separation power with an average theoretical plate number of 950. Increasing the modifier content

TABLE I

SEPARATION OF A POLAR TEST MIXTURE IN DIFFERENT NON-AQUEOUS BIPHASIC SOLVENT SYSTEMS

Solvent system[a]		Peak No.							
		P-1[b]		P-2		P-3		P-4: N	$\bar{N}$
		N[c]	$R_s(1,2)$[c]	N	$R_s(2,3)$	N	$R_s(3,4)$		
I	A–H (1:1)	1390	2.3	890	2.0	705	4.9	1000	1000
II	A–B–H (10:1:10)	1000	1.6	1025	2.0	865	4.3	900	950
III	A–B–H (10:3:10)	790	0.4	870	1.9	920	3.8	860	860
IV	M–H (1:2)	1085	3.7	1310	2.3	1220	1.7	1210	1205
V	A–D–H (7:3:13)	1180	2.9	1280	1.9	1060	3.9	1380	1225
VI	A–D–H (3:2:7)	Not stable during the run							

[a] A = acetonitrile; B = *tert.*-butyl methyl ether; D = dichloromethane; H = hexane; M = methanol.

[b] For identity of peaks, see Fig. 4.

[c] Theoretical plate number N and resolution between neighbouring peaks $R_s(x,y)$ were calculated according to Conway and Ito [15].

to a ratio of 10:3:10 markedly reduced the hydrodynamic stability and also the separation efficiency. Replacement of the ether modifier with dichloromethane brought a further increase in separation efficiency. The average plate number reached 1225, with clear baseline resolution between the individual peaks (Fig. 4B). The polarities of the two phases were obviously relatively close to the pure hexane–acetonitrile (1:1) system (Fig. 4A), as judged by the rapid elution of the compounds. An only very slight increase in the modifier content, however, led to an enormous deterioration of the stationary phase retention. This effect is even more drastic if the percentages of the solvents in both biphasic systems are compared: system V, 30.5% A, 13.0% D, 56.5% H; system VI, 25.0% A, 16.7% D, 58.3% H.

Solvent system II in Table I was successfully applied for the separation and preparative isolation of phthalides and other sensorially active constituents from celery and parsley roots. The first application was the isolation of phthalides, the main components and responsible for the typical flavour of these roots, in order to obtain pure compounds for reference spectra (NMR, IR). The samples were commercially available celery oils and also extracts that were prepared by cold solvent extraction of plant material which was ground under liquid nitrogen. The above solvent system was used for several preparative HSCCC runs in the reversed elution mode with sample loadings of *ca.* 500–750 mg. In this context it is noteworthy that an increase in the sample loading to 750 mg did not influence the separation of the two groups of phthalides.

Fractions collected at the peak maximum of the relevant peaks (see Fig. 5) provided sedanenolide and sedanolide with a purity of *ca.* 85% in amounts ranging from 1 to 30 mg. 3-*n*-Butyl phthalide and the two butylidene phthalides were isolated with purities of about 60%. With regard to the separation of these constituents the

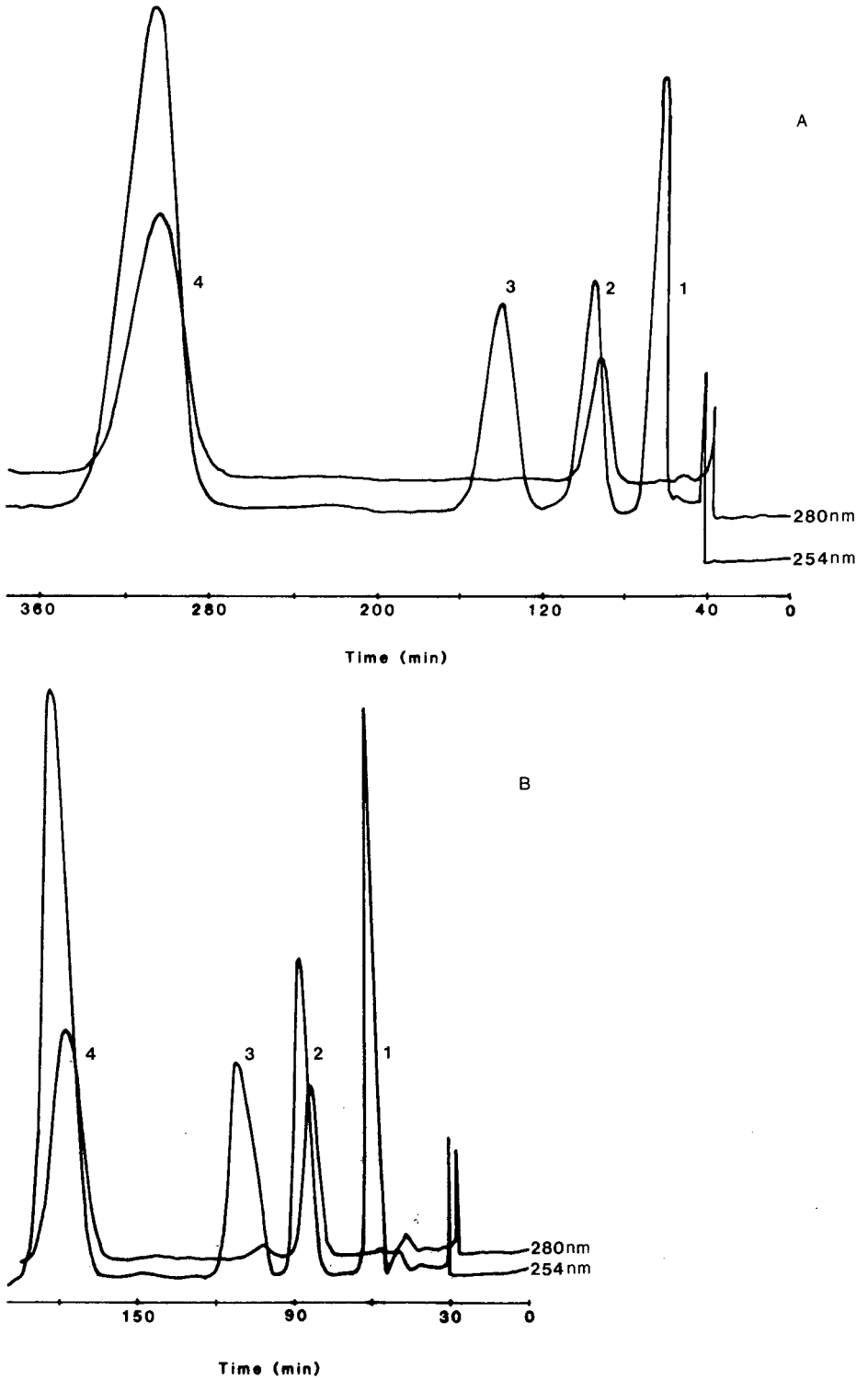

Fig. 4. Separation of (1) benzyl alcohol, (2) benzyl methyl ketone, (3) benzyl acetate and (4) ethyl benzoate by HSCCC. Solvent systems, (A) acetonitrile–hexane (1:1) and (B) acetonitrile–dichloromethane–hexane (7:3:13); flow-rate, 1.5 ml/min; rotational speed, 800 rpm.

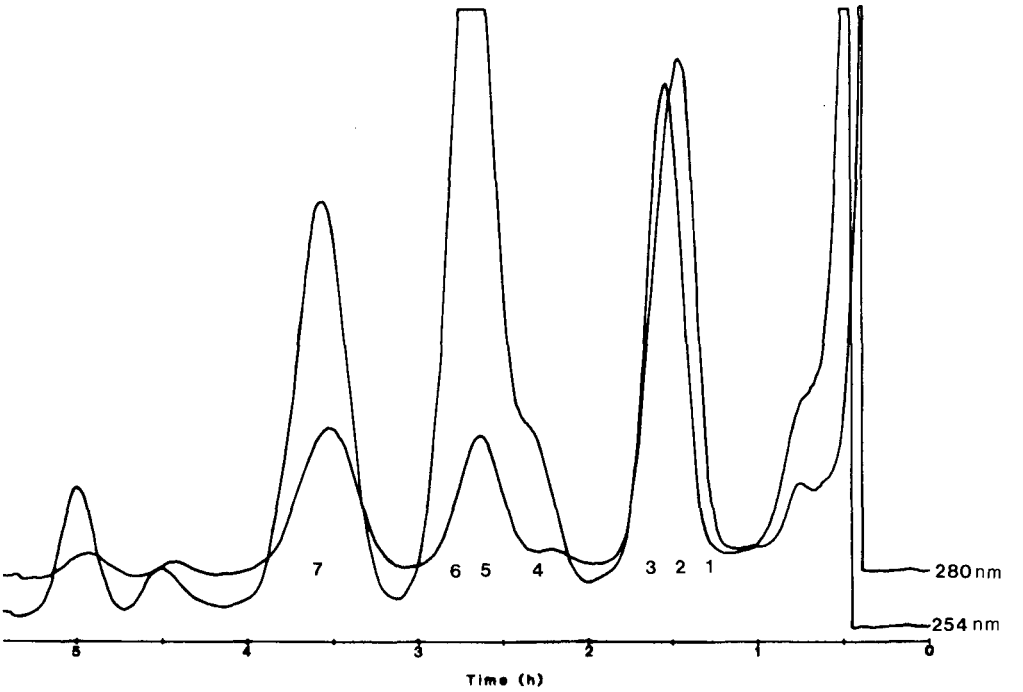

Fig. 5. HSCCC of a commercial celery oil. Solvent system as in Fig. 3; flow-rate, 1.0 ml/min; sample, 200 mg; rotational speed, 800 rpm. Distribution of single components: 1 = celery-like trace component; 2 = sedanenolide; 3 = 3-*n*-butyl phthalide; 4 = (*E*)-butylidene phthalide; 5 = sedanolide; 6 = (*Z*)-butylidene phthalide; 7 = benzyl benzoate.

solvent system has to be optimized. The fractions obtained were simultaneously analysed by GC and GC–MS to identify the components. Structural confirmaton was achieved by comparison with literature mass spectra.

As a consequence of the large amounts of sample being separated in a single run, the localization of sensorially active components in individual fractions was easily performed by checking the smell of the fractions. In this way a trace component (see 1 in Fig. 5) possessing a very strong celery-like odour could be detected. The appropriate fractions were collected and the desired component could be enriched from a barely detectable level in the original oil to a concentration that facilitated subsequent identification and structural elucidation of this substance [16].

In the same way, two unknown C_{17}-polyacetylenic alcohols possessing very similar mass spectra could be isolated from a parsley root solvent extract. The separation of 350 mg of extract was also carried out in the head-to-tail elution mode, using solvent system II in Table I. The first compound, a diol, eluted in fractions 6–9, whereas being relatively polar, the second compound, with only one hydroxyl group, eluted in fractions 50–55 and could be isolated in a yield of 35 mg with a purity of 98%. The structure was elucidated by means of spectroscopic methods such as NMR, FT-IR and mass spectrometry [17].

CONCLUSION

Liquid–liquid chromatographic methods such as DCCC have already demonstrated their potential especially in the field of natural product isolation. The recently developed HSCCC method brought about a great improvement in LLC techniques in terms of separation and partition efficiency and separation time, required for preparative fractionations of complex mixtures. As a consequence of the increased flow and mass transfer rates, however, the appropriate solvent system has to meet the demands of special hydrodynamics involved in the separation process. Therefore, most of the published biphasic systems for LLC have to be re-examined for their ability under HSCCC conditions. A great part of these solvent systems was collected under the special requirements of droplet-formation ability for DCCC, so that many new biphasic systems are conceivable that may be well suited for HSCCC but have never been mentioned in the literature on earlier LLC techniques. On the other hand, great potential for the HSCCC method could lie in the separation of essential oils or flavour extracts, using non-aqueous solvent systems that are compatible with GC such as the described acetonitrile–hexane mixture.

Another important advantage of the CCC techniques is the absence of a solid sorbent. Hence a potential source of complications arising from irreversible sample adsorption and contamination from the sorbent is eliminated. Decompositon or denaturation, or in general artefact formation, is reduced to a minimum, as is well demonstrated by the isolation of the antioxidant principles of rosemary and sage.

In conclusion, HSCCC represents a valuable extension of chromatographic methods, particularly in the separation and purification of natural products.

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Isolation of bacitracins A and F by high-speed counter-current chromatography

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ABSTRACT

The major components of bacitracin were separated and purified using high-speed counter-current chromatography (HSCCC). A systematic search for optimum two-phase solvent systems resulted in two systems: chloroform–ethanol–methanol–water (5:3:3:4) and chloroform–ethanol–water (5:4:3). These were selected based on the determination of the partition coefficients of all the components and the settling time of the phases. HSCCC with these solvent systems separated two components, bacitracins A and F. Improvements in the flow-cell arrangement eliminated noise in detection, making in-line monitoring possible. A tandem mass spectrometric technique was used to characterize the isolated components.

INTRODUCTION

Bacitracins, which are peptide antibiotics produced by *Bacillus subtilis* and *Bacillus licheniformis*, exhibit an inhibitory activity against Gram-positive bacteria [1] and are among the most commonly used antibiotics as animal feed additives [2,3]. These compounds were discovered in 1943 and were originally considered to be one substance [4]. More recently, it has been demonstrated that they consist of over twenty components of differing antimicrobial activities [5,6]. The major antimicrobial components are bacitracins A and B, and bacitracin F is a degradation product [5,7] that shows nephrotoxicity [4]. Only the structures of bacitracins A and F (Fig. 1) have been determined to date [4,8–10].

The bacitracins are highly polar compounds composed of a cyclic heptapeptide and a branch containing a thiazole ring. In order to establish appropriate chromatographic methods for the separation of the components, it is necessary to consider various properties. The bacitracin complex has low solubility in benzene, chloroform, ethyl acetate and acetone and is soluble to the approximate limit of 20 mg/ml in water or methanol. When silica gel and Sephadex are used for preparative separation, the

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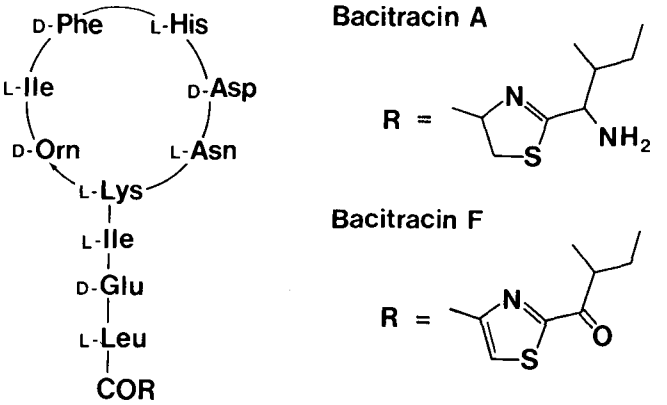

Fig. 1. Structures of bacitracins A and F.

recovery is very poor, probably because polar functional groups interact with silanol and other polar groups. Additionally, the antibiotics are thermally unstable and labile under basic conditions. We have also confirmed that bacitracin A decomposes thermally to give bacitracin F. These components have previously been separated by counter-current distribution methods [5,11–14], without complete resolution.

We have previously applied high-speed counter-current chromatography (HSCCC) to the preparative separation of six sporaviridin components, which are complicated basic glycoside antibiotics produced by *Streptosporangium viridogriseum* [15]. Here we describe the isolation of bacitracin components by HSCCC and their structural characterization by tandem mass spectrometry (MS–MS) under fast atom bombardment (FAB) conditions.

EXPERIMENTAL

Materials

Methanol, ethanol, *n*-butanol, isopropanol, acetone, diethyl ether, ethyl acetate, disodium hydrogenphosphate, sodium chloride, potassium chloride, ammonium chloride and ammonium acetate were of analytical-reagent grade. Bacitracin was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.).

Operation of HSCCC

The apparatus used was a Shimadzu (Kyoto, Japan) prototype of the coil planet centrifuge (HSCCC-1A) for HSCCC with a 160 m- length of 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing around a 10-cm diameter column holder making multiple coiled layers. The total capacity was 325 ml and the column was revolved at 800 rpm. Solvents were delivered with a Shimadzu LC-6A pump. After filling with the stationary phase, followed by sample injection, mobile phase was applied at 3 ml/min. UV detection at 254 nm was performed using a Shimadzu SPD-6A instrument. Eluates were collected in a Pharmacia (Uppsala, Sweden) FRAC-100 fraction collector.

High-performance liquid chromatography (HPLC)

HPLC was carried out using the following conditions: pump, Shimadzu LC-6A; detector, Shimadzu SPD-2A; column, Capcell Pak C_{18} (150 × 4.6 mm I.D.) (Shiseido, Tokyo, Japan); mobile phase, methanol–0.04 *M* aqueous disodium hydrogenphosphate (6:4).

Mass spectrometry

Liquid secondary ion mass spectrometry (SIMS) was performed using a Hitachi (Tokyo, Japan) M-80B mass spectrometer under the following operating conditions: primary ion, Xe^+; accelerating voltage, 8 kV (primary) and 3 kV (secondary). A mixture of glycerol and 10% aqueous oxalic acid was used as the matrix. MS–MS was carried out with a JEOL (Tokyo, Japan) JMS-HX110/HX110 system. The fast atom beam was operated at 6 kV using xenon gas and the spectrometer was operated at 10 kV accelerating potential. A mixture of thioglycerol, glycerol and 10% aqueous oxalic acid was used as the matrix. The collisionally activated dissociation (CAD) experiment was run using helium as the collision gas.

RESULTS AND DISCUSSION

As over twenty components are contained in the bacitracin complex, it is necessary to develop the chromatographic conditions carefully. We have already established an HPLC method for the analysis of the bacitracin complex, in which we used a Capcell Pak C_{18} column and an isocratic solvent system, methanol–phosphate buffer (pH 9.4) (6:4) [16]. As shown in Fig. 2, 22 peaks are separated. The peak numbers shown also applied to later HSCCC separations.

Precise separation by HSCCC is mainly dependent on the selection of the opti-

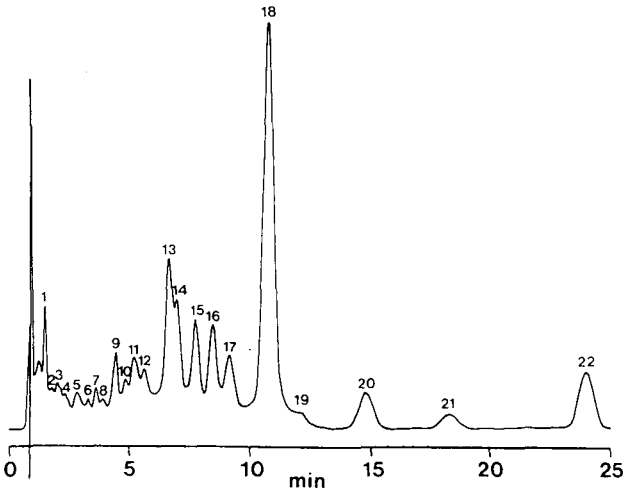

Fig. 2. Analytical HPLC of bacitracin components. Sample size, 3 μg; column, Capcell Pak C_{18} (5 μm) (150 × 4.6 mm I.D.); mobile phase, methanol–0.04 *M* disodium hydrogenphosphate (6:4); flow-rate, 1.3 ml/min; detection, UV (234 nm).

mum two-phase solvent system [17]. To select a suitable solvent system, the solubility of bacitracin components, partition coefficients (K) of the individual components and the settling time of the solvent system were carefully measured. A two-phase solvent system with a settling time of less than 30 s is desirable for high retention of the stationary phase in the HSCCC apparatus [18,19]. The partition coefficient was determined by a simple test-tube experiment using HPLC [16]. Partition coefficients were determined by dividing the corresponding peak area of components of the upper phase by that of components of the lower phase.

We selected *n*-butanol, ethyl acetate and chloroform as the organic components and then prepared two-phase solvent systems composed of one of these three solvents and water and/or methanol, which dissolve the bacitracin complex. Settling times and individual partition coefficients of peaks 13–22 using *n*-butanol and ethyl acetate systems are summarized in Table I. Ideally, partition coefficients of the bacitracin components should be dispersed around $K = 1$. For *n*-butanol solvent systems, peaks 13–18 have suitable partition coefficients, but those of peaks 20–22 are too high. These solvent systems also have the disadvantage of being laborious to evaporate.

TABLE I

PARTITION COEFFICIENTS OF BACITRACIN COMPONENTS WITH *n*-BUTANOL AND ETHYL ACETATE SYSTEMS

Solvent system[a]	Volume ratio	Settling time (s)	Peak No.							
			13,14	15	16	17	18	20	21	22
n-Bu–DE–W	10:0:10	15	0.63	1.52	0.63	1.48	0.75	13.9	UP[b]	23.14
n-Bu–DE–W	10:1:10	15	0.49	1.25	0.51	1.24	1.03	11.1	UP	15.47
n-Bu–DE–W	10:3:10	14	0.22	0.59	0.21	0.51	0.53	6.37	UP	9.95
EA–E–W	5:2:3	50	0.10	0.12	0.08	0.10	0.16	0.56	0.49	0.60
EA–i-P–W	4:2:3	60	0.07	0.14	0.06	0.13	0.15	0.74	0.68	0.85
EA–M–A–W	5:1:1:2	60	LP[c]	LP	LP	LP	0.01	0.08	LP	0.12
EA–E–i-P–W	4:1:1:4	60	0.06	0.13	0.05	0.10	0.12	0.57	LP	0.60
EA–n-Bu–M–W	4:1:1:4	40	LP	LP	LP	LP	0.03	0.50	LP	0.76
EA–n-Bu–M–W	5:1:1:4	30	LP	LP	LP	LP	0.02	0.29	LP	0.50
EA–E–i-P–W	4:1:1:4	60	0.06	0.13	0.05	0.10	0.12	0.57	LP	0.61
EA–E–i-P–10% aq. NaCl	4:1:1:4	30	0.07	(0.08)[d]		0.08	0.11	0.50	LP	0.67
EA–E–i-P–10% aq. KCl	4:1:1:4	30	0.21	(0.22)		0.15	0.33	0.88	0.85	1.25
EA–E–i-P–10% aq. NH_4Cl	4:1:1:4	32	LP	LP	LP	LP	0.07	0.45	0.64	0.64
EA–E–i-P–10% aq. NH_4OAc	4:1:1:4	35	–[e]	–	–	–	–	–	–	–
EA–M–A–W	5:1:1:2	60	LP	LP	LP	LP	0.01	0.08	LP	0.12
EA–M–A–10% aq. NaCl	5:1:1:2	30	LP	LP	LP	LP	LP	LP	LP	LP
EA–M–A–10% aq. KCl	5:1:1:2	30	LP	LP	LP	LP	0.01	LP	LP	0.15
EA–M–A–10% aq. NH_4Cl	5:1:1:2	30	LP	LP	LP	LP	LP	LP	LP	LP
EA–M–A–10% aq. NH_4OAc	5:1:1:1	40	LP	LP	LP	LP	LP	LP	LP	LP

[a] n-Bu = *n*-butanol; DE = diethyl ether; W = water; EA = ethyl acetate; E = ethanol; i-P = isopropanol; M = methanol; A = acetone; OAc = acetate.

[b] UP: bacitracins were exclusively partitioned into the upper phase.

[c] LP: bacitracins were exclusively partitioned into the lower phase.

[d] ( ): Both components were not separated.

[e] –: Decomposition.

TABLE II

PARTITION COEFFCIENTS OF BACITRACIN COMPONENTS WITH CHLOROFORM SYSTEMS

Solvent system[a]	Volume ratio	Settling time (s)	Peak No.							
			13,14	15	16	17	18	20	21	22
C–E–M–W	5:2:3:4	18	7.20	(4.92)[c]		2.46	4.17	0.64	0.65	0.48
C–E–M–W	5:2:1:4	19	UP[b]	UP	UP	UP	33.27	1.62	1.38	0.75
C–E–M–W[d]	5:3:3:4	27	3.35	1.59	4.43	1.40	2.37	0.57	0.47	0.45
C–E–W	5:3:3	15	11.14	(6.37)		3.20	5.34	0.32	0.35	0.27
C–E–W	5:4:2	20	3.19	(2.19)		1.05	2.00	0.25	0.26	0.21
C–E–W[d]	5:4:3	26	5.49	1.78	6.74	1.46	2.20	0.16	LP[e]	0.16
C–E–W	5:4:4	37	6.10	2.25	6.45	2.04	2.68	0.14	LP	0.10

[a] C = chloroform; E = ethanol; M = methanol; W = water.
[b] UP: Bacitracin components were exclusively partitioned into the upper phase.
[c] ( ): Both components were not separated.
[d] Optimum solvent systems.
[e] LP: Bacitracin components were exclusively partitioned into the lower phase.

Another disadvantage is that the ethyl acetate, alcohol and water system showed too long settling times. Addition of inorganic salts, such as sodium chloride, potassium chloride, ammonium chloride and ammonium acetate, to these systems did not improve the K values.

Chloroform, methanol and water systems have been extensively used in droplet CCC [20]. Direct application of these solvent systems to HSCCC was unsuccessful in bacitracin separation but the complete or partial replacement of methanol with etha-

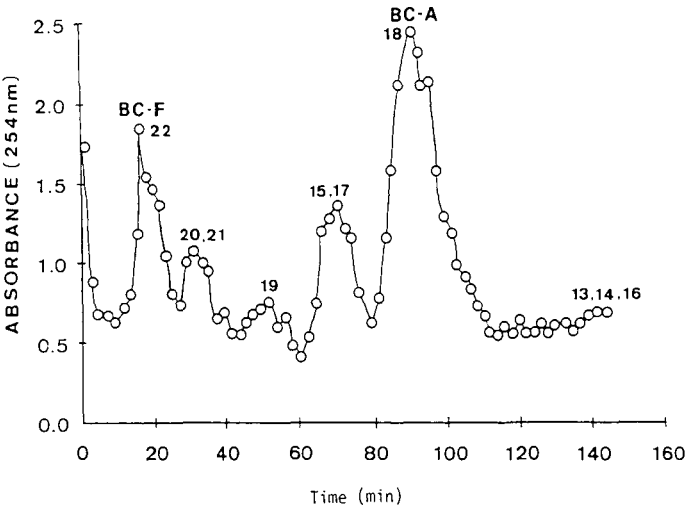

Fig. 3. Counter-current chromatogram of bacitracin components. Sample size, 50 mg; solvent system, chloroform–ethanol–water (5:4:3); mobile phase, lower phase; flow-rate, 3 ml/min; revolution speed, 800 rpm; fractionation volume, 3 ml per tube. The absorbance of fractions was determined manually.

nol gave good results. Other systems are summarized in Table II. The systems using chloroform–ethanol–methanol–water (5:3:3:4) and chloroform–ethanol–water (5:4:3) gave the best combination of partition coefficients.

Fig. 3 shows the counter-current chromatogram of bacitracin components using the chloroform system. The settling time was 26 s and the lower phase was delivered at 3 ml/min. A 50-mg amount of the complex was introduced and the apparatus was rotated at 800 rpm. The retention of the stationary phase was 74.1% and the experiment run was *ca.* 3 h. These components were eluted in order of their partition coefficients and peaks 18 and 22 could be almost completely separated.

Real-time UV monitoring showed a high degree of noise and an unstable baseline. These effects are due to turbidity of phase droplets in the mobile phase in the flow tube along with decavitation. It was necessary, therefore, to draw the elution curve manually after spectrometric analysis of individual fractions. To improve the in-line detection we modified the UV monitoring system by heating the effluent usually at 40°C near the inlet of the UV flow cell. A narrow-bore PTFE tube about 1 m long was attached at the outlet of the monitor [21]. The real-time UV detection in HSCCC is shown in fig. 4. From our previous work in separating the components by HPLC we have established that peaks 18 and 22 correspond to bacitracin A and F, respectively [16]. We were therefore able to compare the HSCCC and HPLC separations. Bacitracin A from HPLC was not always pure [16], whereas HSCCC gave bacitracin A in a pure state and the recovery was high. Similarly, bacitracin F was completely purified by HSCCC in the same run.

Liquid SIMS of the isolated bacitracins A and F was applied using glycerol and 10% oxalic acid as the matrix (Fig. 5). The protonated molecules $[M+H]^+$ are observed at m/z 1422 and 1419, correctly indicating their molecular weights. However,

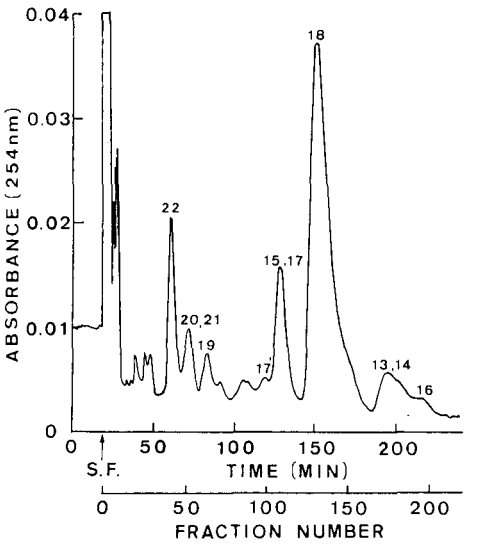

Fig. 4. Counter-current chromatogram of bacitracin components using improved in-line detection. Sample size, 50 mg; solvent system, chloroform–ethanol–methanol–water (5:3:3:4); mobile phase, lower phase; flow-rate, 3 ml/min; fractionation volume, 3 ml per tube; detection, UV (254 nm).

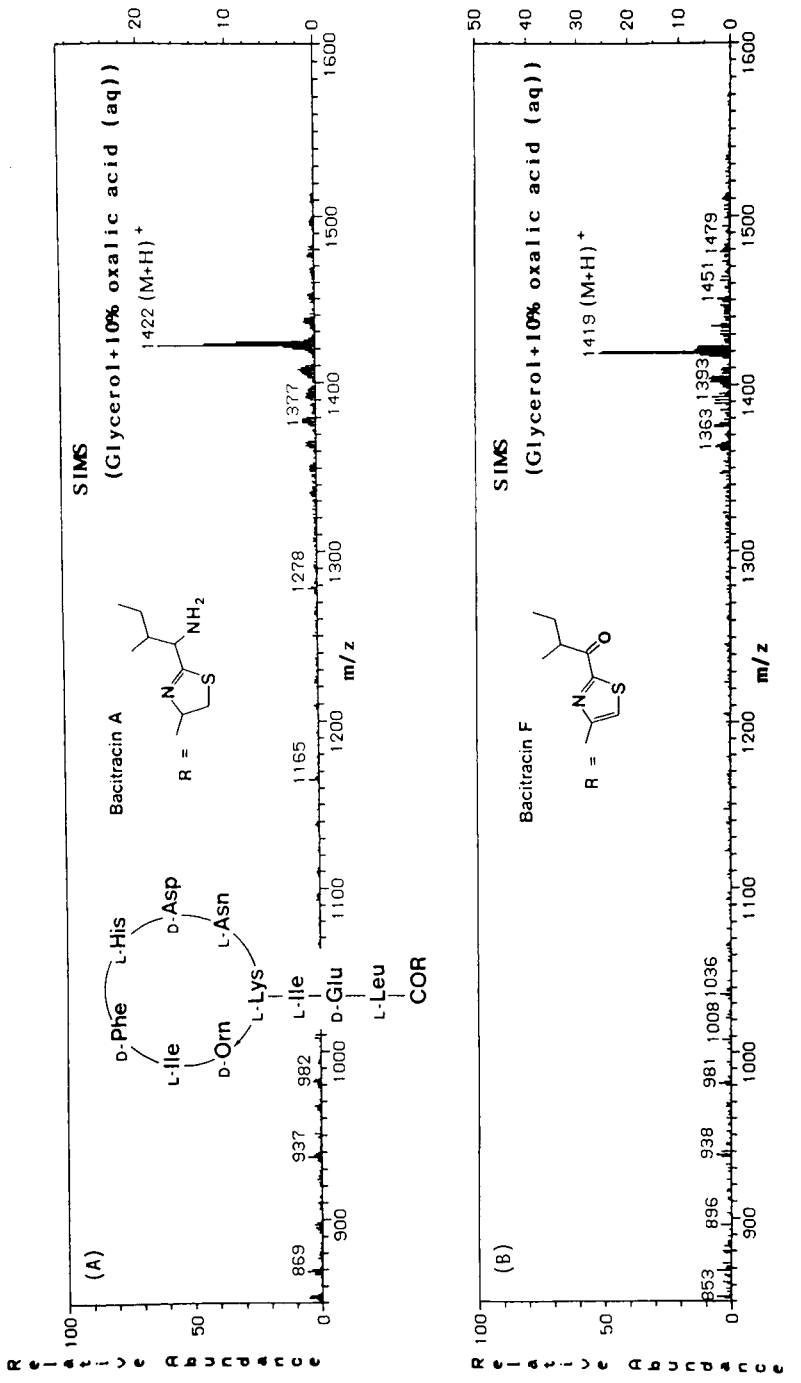

Fig. 5. Liquid SIMS of (A) bacitracin A and (B) bacitracin F using glycerol–10% oxalic acid as the matrix.

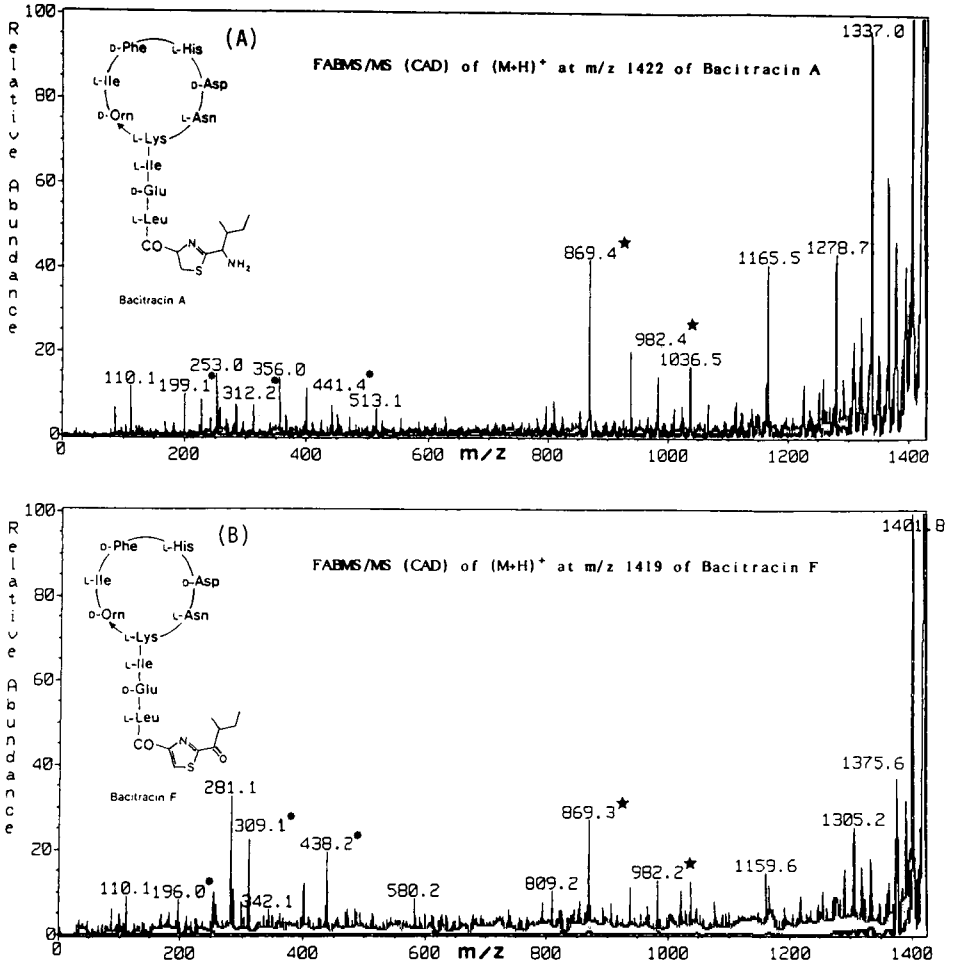

Fig. 6. Tandem mass spectra of protonated molecules of (A) bacitracin A and (B) bacitracin F.

little information concerning the peptide sequence was available from the data. MS–MS is frequently used for mixture analysis, identification of ion structure and elucidation of fragmentation. Usually CAD is applied to obtain fragment ions. Initially, the protonated molecules were formed under FAB conditions and selected as the precursor ions. Generally, peptides are cleaved at C–N bonds to yield C- and N-terminal fragment (daughter) ions which are available for sequence determination of constituent amino acids [22]. Fig. 6 shows the tandem mass spectra from the protonated molecules of bacitracins A and F. The fragmentation schemes of both components in the tandem mass spectra under CAD conditions are shown in Fig. 7. These daughter ions are classified into two groups of ions. The common fragment ions at *m/z* 982 and 869 are formed by cleavage at peptide bonds between Ile and Glu and between Lys and Ile, respectively, and are informative sequence ions including the cyclic peptide

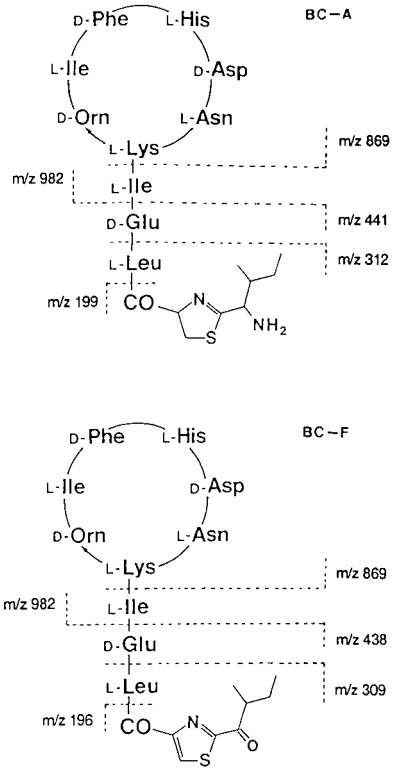

Fig. 7. Sequence ions from the tandem mass spectra of bacitracin A and B under CAD conditions.

moiety. Other ions are derived from the side-chain and there is a difference of 3 mass units in these ions between the components. These results confirm that bacitracins A and F [10] have the structures proposed by Ressler and Kashelikar [9] as shown in Fig. 1 and the data may be useful in the analysis of other unknown components of the bacitracins.

In conclusion, preparative separation and structural determination of the individual components of bacitracin were achieved. First we established HPLC for analysis and next optimized the operating conditions for HSCCC, so that bacitracins A and F were effectively isolated. These results indicate that HSCCC is useful for preparative separations. Other solvent systems are currently being examined to separate other components.

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Foam counter-current chromatography of bacitracin

II. Continuous removal and concentration of hydrophobic components with nitrogen gas and distilled water free of surfactants or other additives

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ABSTRACT

Foam counter-current chromatography has been successfully applied to continuous removal and concentration of hydrophobic bacitracin (BC) components from a large volume solution using nitrogen gas and distilled water free of surfactants or other additives. The experiment was initiated by introducing nitrogen at the head inlet of the coil rotated at 500 rpm. Then, a 2.5-l volume of the sample solution containing BC at 50 ppm was continuously introduced into the middle portion of the coil at 1.5 ml/min. The hydrophobic components produced a thick foam which was carried with the gas phase and collected from the tail end of the coil while other components stayed in the liquid stream and eluted from the head outlet of the coil. High-performance liquid chromatographic analysis of the foam fraction revealed that the degree of enrichment increased with hydrophobicity of the BC components. BC-A and -F were enriched 1400 and 2260 times, respectively, compared with the original concentration in the sample solution. These results clearly indicate that the present method will be quite effective for detection and/or isolation of a small amount of natural products present in a large volume of aqueous solution.

INTRODUCTION

Commercial bacitracin (BC) is known to contain the major component (BC-A), its degradation product (BC-F) and over 20 UV-absorbing minor components [1].

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Recently, efficient separations of these components have been performed with analytical high-performance liquid chromatography (HPLC) [1] and high-speed countercurrent chromatography (CCC) [2].

Our previous studies [3] have shown that the surfactant-free foam CCC method can separate the bacitracin components according to their hydrophobicity. The results encouraged us to conduct preparative-scale enrichment of the BC components in continuous sample feeding. Because the method exclusively utilizes inert nitrogen gas and distilled water, the harvested materials in the foam fraction can be easily recovered in a pure state without a risk of decomposition or deactivation which might occur in the process of eliminating the surfactants or other additives. Consequently, the method may be invaluable in detection and/or isolation of a minute amount of surfactant bioactive compounds present in a bulk of the biological fluids such as urine, blood and blood dialysate, tissue culture medium, fermentation mixture, etc.

EXPERIMENTAL

Reagents

The BC sample was purchased from Sigma (St. Louis, MO, U.S.A.). Glass-distilled chromatographic-grade methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Reagent-grade disodium hydrogenphosphate was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Apparatus

The design of the apparatus used in the present study having been described in detail in Part I [3] is briefly described here. The foam CCC centrifuge holds a column holder and a counterweight holder symmetrically on the rotary frame at a distance of 20 cm from the central axis of the centrifuge. The column holder revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction.

The separation column was prepared from approximately 10 m of 2.6 mm I.D. PTFE (polytetrafluoroethylene) tubing (Zeus, Raritan, N.J., U.S.A.) by winding it directly on the column holder hub of 15 cm diameter in double layers between a pair of flanges spaced 5 cm apart. The column design is schematically illustrated in Fig. 1.

The separation column is equipped with five flow channels: The liquid feed line and the foam collection line are connected at the tail end of the coil and the gas feed line and the liquid collection line at the head, while the sample feed line opens at the

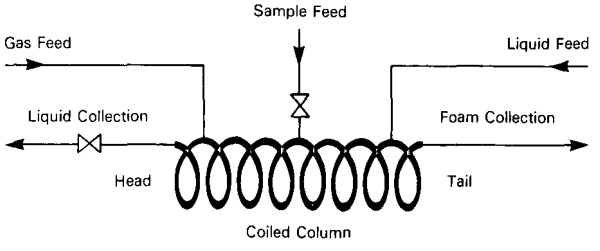

Fig. 1. Column design for foam CCC.

middle portion of the column. The liquid collection line and the sample feed line are each equipped with a needle valve (Washington Valve Co., Rockville, MD, U.S.A.) while the foam collection line is left open to the air. Nitrogen gas was fed through the gas feed line at 80 p.s.i. directly from a nitrogen cylinder. The sample solution was introduced through the sample feed line from a poly(vinyl chloride)-coated glass bottle pressured at 40 p.s.i. through another nitrogen cylinder. The liquid feed line was closed and not used in the present study.

General procedure of foam CCC

Each experiment was initiated by introducing nitrogen gas at 80 p.s.i. into the head of the column rotating at 500 rpm. Then, an aqueous sample solution containing bacitracin at a desired concentration was continuously fed into the column through the sample feed line which was connected to the reservoir pressured at 40 p.s.i. The opening of the needle valve on the liquid collection line was adjusted to obtain the desired liquid and foam outputs. Effluents from the foam and liquid collection lines were collected separately and subjected to HPLC analysis.

HPLC analysis

HPLC analyses of the foam and liquid fractions were performed in Shimadzu (Kyoto, Japan) equipment consisting of a Model LC-6A pump, a manual injector, A Model SPD-6A detector, and a Model C-R5A recording data processor using a Capcell Pak C_{18} column, 15 × 0.46 cm I.D. (Shiseido, Tokyo, Japan). The mobile phase, composed of 0.04 *M* disodium hydrogenphosphate (pH 9.4)–methanol (38:62, v/v), was isocratically eluted at a flow-rate of 1 ml/min and the effluent was monitored at 234 nm.

RESULTS AND DISCUSSION

As described elsewhere [1], BC consists of more than twenty components, including the major component BC-A, its oxidation product BC-F, and other minor components of unknown structure. Under the present reversed-phase HPLC conditions, BC components were eluted in the increasing order of hydrophobicity to yield over 15 resolved peaks as shown in Fig. 2A, where the polarity of each component may be judged from its retention time. In the present study, peaks, 3, 7, 11 (BC-A) and 14 (BC-F) are selected to study the degree of enrichment in the foam fraction.

The first series of experiments was conducted to optimize the operational conditions for continuous enrichment of the BC components. These studies were performed under a set of fixed conditions of sample volume (100 ml), sample feed rate (1.5 ml/min at 40 p.s.i.), nitrogen gas feed pressure (80 p.s.i.), and the rotational speed of the column (500 rpm) as indicated in Table I. Liquid was not fed from the liquid feed line, because it was found that liquid feeding dilutes the solutes, resulting in interruption of the steady foam elution. Effects of the needle valve opening at the liquid outlet on the enrichment of the BC components was investigated with a 100-ml sample volume by varying the BC concentration from 10 to 100 ppm. As shown in Table I, the results indicated that opening the needle valve less than 1.0 turn or over 5.0 turns yielded no foam fraction. However, when the needle valve was opened in an optimum range between 1 and 3 turns, peak 3 was enriched by 13 to 77 times; peak 7,

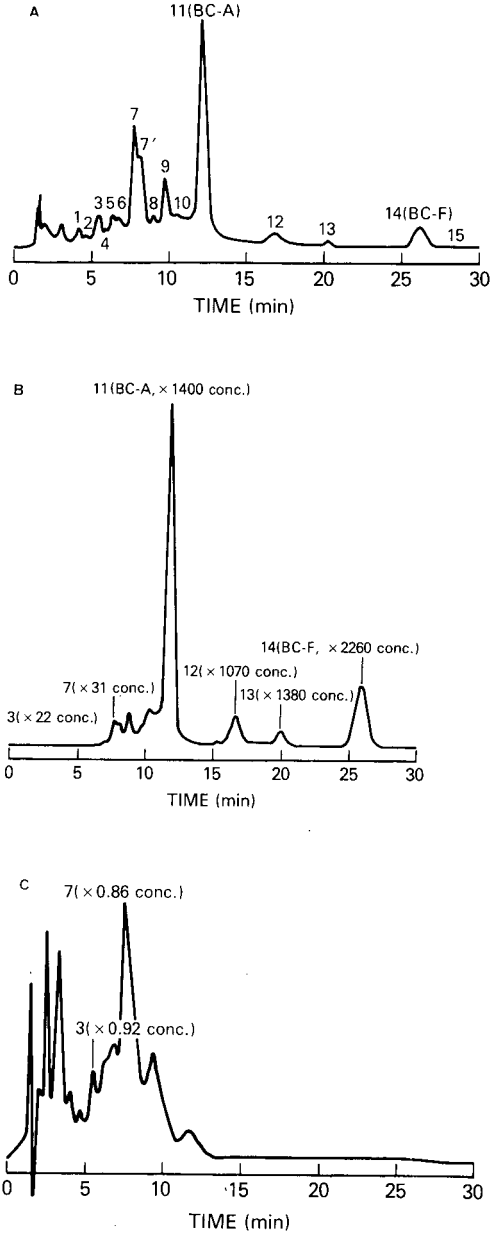

Fig. 2. HPLC analysis of bacitracin. (A) Chromatogram of original sample; (B) chromatogram of foam fraction; (C) chromatogram of liquid fraction. Experimental conditions: solvent: 0.04 *M* disodium hydrogenphosphate–methanol (38:62); flow-rate: 1 ml/min; detection: 234 nm.

TABLE I

OPTIMIZATION OF OPERATIONAL CONDITIONS FOR ENRICHMENT OF BACITRACIN ON CONTINUOUS SAMPLE FEEDING

Sample size: 100 ml in water; sample feeding-rate: 1.5 ml/min (40 p.s.i.); nitrogen gas pressure: 80 p.s.i.; liquid feeding-rate: 0.

Conditions	Enriched concentration (times)			
	Peak 3	Peak 7	Peak 11	Peak 14
Needle valve				
< 1.0 turn open	–[a]	–	–	–
1–*ca.* 3	13–77	52–180	180-4000	220–11 670
> 5.0	–	–	–	–
Sample concentration				
< 25 ppm	–	–	–	–
25	27–77	52–130	180–350	220–410
50	13–65	87–170	630–2000	1340–7700
100	55–77	94–150	280–4000	570–11 670

[a] No foam eluted.

52 to 180 times; peak 11, 180 to 4000 times; and peak 14, 220 to over 11 000 times. Studies on the sample concentration showed that no foam eluted if the concentration was less than 25 ppm. As the concentration was increased to 25 ppm, the foam began to emerge through the foam collection line with a substantial degree of enrichment of the BC components ranging from 27 to 410 times their concentration in the original sample solution, and further increase of the sample concentration at 50 ppm resulted in the enrichment of peak 11 by 2000 times and that of peak 14 by near 8000 times. Finally, at the 100 ppm sample concentration, peak 11 was enriched by 4000 times and peak 14 over 11 000 times. The above results clearly indicate that the yield of the solute concentration in the foam fraction increases with the hydrophobicity of the BC components and also with the original sample concentration within the applied range.

In the light of the results obtained from the above preliminary studies, large-scale enrichment of the BC components was performed as follows: The experiment was initiated by introducing nitrogen (80 p.s.i.) from the tail of the column rotated at 500 rpm; then, the dilute BC aqueous solution (50 ppm) was continuously fed through the sample feed line at a flow-rate of 1.5 ml/h from the reservoir bottle pressured at 40 p.s.i. The liquid feed line was closed, and the opening of the needle valve on the liquid collection line was adjusted at 2.0 turns to maintain the minimum foam output through the foam collection line. The run was continued for 28 h until the total 2.5 l volume of the sample solution was eluted. Both foam and liquid effluents were each separately pooled in a container and subjected to the HPLC analysis.

The results of the experiments are shown in Fig. 2B and C where the selected peaks are labelled together with the degree of enrichment over the original concentration in the sample solution. In the foam fraction (Fig. 2B), the yielded concentration increases with the hydrophobicity of the BC components as demonstrated in the preliminary studies described earlier: peak 3 was enriched by 22 times; peak 7, 31

times; peak 11 (BC-A), 1400 times; peak 12, 1070 times; peak 13, 1380 times; and peak 14 (BC-F), 2260 times. In the liquid fraction (Fig. 2C), the hydrophobic BC components corresponding to peaks 11 to 14 were undetected, while peaks 3 and 7 were eluted with no enrichment in concentration.

The overall results of the present studies clearly indicate that the foam CCC method is capable of concentrating a small amount of the hydrophobic BC components present in a large volume of the sample solution. The method may be effectively applied to detection and/or isolation of various natural products from a large volume aqueous solution.

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Studies on the preparative capability of the horizontal flow-through coil planet centrifuge and high-performance liquid chromatography in the separation of polar compounds from *Oxytropis ochrocephala* Bunge

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ABSTRACT

Horizontal flow-through coil planet centrifuge (CPC) and high-performance liquid chromatography (HPLC) techniques were used for separation of polar compounds from a crude ethanol extract of *Oxytropis ochrocephala* Bunge, a poisonous legume plant widely distributed in northwestern China. The performance of these two chromatographic methods was compared in terms of column efficiency, peak resolution, separation time, sample loading capacity, etc. The results indicated that two polar compounds in the crude extract were equally well separated by these two methods. HPLC gave comparable peak resolution in shorter separation time while its sample loading capacity was limited to the mg range. The CPC method required a long separation time, but yielded a higher purity of fractions with a much greater capacity.

INTRODUCTION

In the past, various chromatographic techniques have been developed to isolate milligram to gram quantities of pure substances from plant materials for structural studies, bioassays, pharmacological tests, etc. [1]. As a plant material often contains thousands of different chemical compounds, the choice of these chromatographic techniques needs serious consideration. Although conventional column chromatography and preparative thin-layer chromatography are both simple and inexpensive, they have a number of limitations, including low column efficiency, long separation time, irreversible adsorption loss of solutes onto the solid support, etc. Recently, high-performance liquid chromatography (HPLC) with micro-particulate column packings of a narrow size range has been widely used for preparative purposes. However, due to its low column capacity and high cost of the packing material,

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application of semi-preparative HPLC becomes impractical when a larger amount of pure compounds is desired.

For many years, preparative-scale separations of natural products have been performed with the horizontal flow-through coil planet centrifuge (CPC) which utilizes a rotating coiled column in a centrifugal force field. This counter-current chromatographic method is characterized by relatively high column efficiency, broad applicability of solvent systems and large sample-loading capacity [2,3]. Consequently, the method is useful in preparative separations of milligrams to gram range, especially for polar substances [4]. Although coil planet centrifugation is less efficient compared with the more recently developed high-speed counter-current chromatography [5–10], it provides more stable retention of the stationary phase for low-interfacial-tension solvent systems and is particularly suitable for preparative separations of natural products which often contain surface-active compounds.

The present paper describes separations of polar compounds from *Oxytropis ochrocephala* Bunge, a poisonous legume plant widely distributed in northwestern China, which causes chronic neurological disorders in livestock. The separations were performed with the horizontal CPC, semi-preparative and analytical HPLC and the results were compared in terms of resolution, separation time and sample-loading capacity. Two flavonoid glycosides were isolated for determination of their chemical structures as reported elsewhere [11].

EXPERIMENTAL

Apparatus

The horizontal CPC employed in the present study was fabricated at the Beijing Institute of New Technology Application, Beijing, China, the design of the apparatus being previously described in detail [4]. The apparatus holds a set of four coiled columns around the column holder, which is mounted on the rotary frame at a distance of 14 cm from the centrifuge axis. Each column unit consists of 1.6 mm I.D., 0.3 mm thick wall PTFE (polytetrafluoroethylene) tubing with a 30-ml capacity. Four column units are serially connected with flow tubes (0.85 mm I.D.) to make up a total capacity of 120 ml. The ratio (β value) of the distance between the coil and the holder axis to the distance between the holder axis and the centrifuge axis is about 0.26. The balance of the centrifuge system is maintained by mounting a counter-weight on the opposite side of the rotary frame.

The HPLC separations were performed using an HP-1090M Model (Hewlett-Packard, Waldbronn, F.R.G.) with three different columns: μBondapak C_{18} (300 mm × 3.9 mm I.D.) 10 μm, Herpsil ODS (100 mm × 2.1 mm I.D.) 5 μm and LiChrosorb RP-18 (200 mm × 10 mm I.D.) 10 μm.

Sample preparation

Above-ground parts of *O. ochrocephala* B. were collected in Qinhai Province of China and identified at the Institute of Plateau Biology, Academia Sinica. Milled raw plant tissue was extracted with hot ethanol under reflux over a water bath. The solvent was evaporated under vacuum, and the residue was dissolved in a 5% acetic acid aqueous solution. The supernatant was extracted with ether to remove chlorophylls and lipophilic compounds and then alkalized with ammonia to pH 9.0 for extraction of

alkaloids with dichloromethane. The upper aqueous phase was lyophilized to dryness and used as the sample.

Separation procedures

The CPC separation was performed with a two-phase solvent system composed of chloroform–ethyl acetate–methanol–water (2:4:1:4, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before being applied to the column. The separation was initiated by filling the entire column space with the upper aqueous stationary phase. This was followed by injection of the sample solution containing 60 mg of the crude extract in the upper phase. Then the lower non-aqueous mobile phase was eluted through the column at a flow-rate of 1 ml/min while the apparatus was run at 280 rpm. Effluent from the outlet of the column was continuously monitored with a UV monitor at 254 nm, and the peak fractions were manually collected for later analysis. After 270 ml of the mobile phase were eluted, the centrifuge run was stopped, and the column contents were slowly purged with nitrogen gas to collect the peak fractions retained in the column. The column was then washed with methanol and dried with nitrogen.

The HPLC separations were performed in a gradient-elution mode using methanol and water. The detailed gradient patterns are indicated under the captions of Figs. 2 and 4. The analytical columns were eluted at a flow-rate of 1 ml/min and the semi-preparative column at 4 ml/min. The effluent was monitored at 254 nm in all cases.

RESULTS AND DISCUSSION

CPC separation

According to the previous studies [12], the oxytropis species contains flavonoids which can be isolated by a solvent system composed of chloroform–methanol–water. The present study was focused on isolation of flavonoid glycosides with higher polarity, which required modifying the polarity of the above ternary solvent system by addition of ethyl acetate.

Fig. 1 shows a typical chromatogram of the crude extract of *O. ochrocephala* B. obtained with the CPC method. The separation was performed with a quaternary two-phase solvent system composed of chloroform–ethyl acetate–methanol–water (2:4:1:4, v/v) using the lower non-aqueous phase as the mobile phase. Multiple peaks were eluted in increasing order of polarity and six fractions corresponding to the main peaks, labeled 1–6 in the chromatogram, were manually collected and subjected to HPLC analysis.

HPLC separations

Because polar compounds are most conveniently separated by reversed-phase HPLC, all HPLC separations in the present study were performed with reversed-phase columns. Fig. 2 illustrates an analytical chromatogram of the crude *O. ochrocephala B.* extract obtained by a gradient elution of methanol in water using a μBondapack C_{18} column. Because of the reversed-phase HPLC, peaks were eluted in decreasing order of polarity in contrast to the CPC separation above. Among many compounds present in the crude extract, only two components corresponding to peaks 3 and 4 are obtained in relatively high concentrations.

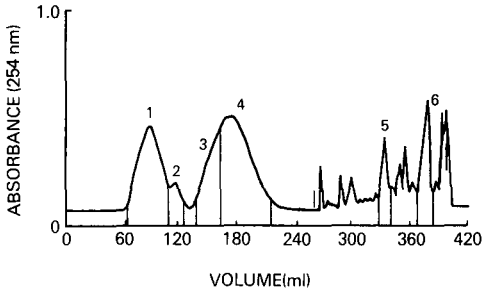

Fig. 1. CPC separation of ethanol extract of *O. ochrocephala* B. Experimental conditions: apparatus, horizontal coil planet centrifuge with 14 cm revolution radius, four coiled columns of 1.6 mm I.D. and 0.3 mm wall thickness and 120 ml capacity; sample size, 60 mg; solvent system, chloroform–ethyl acetate–methanol–water (2:4:1:4, v/v); mobile phase, lower non-aqueous phase; flow-rate, 1 ml/min; revolution, 280 rpm, detection at 254 nm.

Fig. 3 shows a semi-preparative separation of the same sample similarly obtained in a preparative mode using a 10-mm I.D. column operated under overloaded conditions with a 3-mg sample size. Four peak fractions indicated in Fig. 3 were manually collected, concentrated by evaporation in vacuum and subjected to HPLC analysis.

Comparison of separation efficiencies of CPC and HPLC

As described above, peak fractions obtained from the preparative separations (Figs. 1 and 3) were analyzed with HPLC under the same conditions applied to the separation shown in Fig. 2. The qualitative analyses revealed that the compounds found in CPC fractions 1, 3 and 5 (Fig. 1) correspond to those found in HPLC fractions 4, 3 and 1 (Figs. 2 and 3), respectively. It can be seen directly from Figs. 1, 2 and 3 that HPLC has a higher column efficiency, shorter separation time, but lower sample

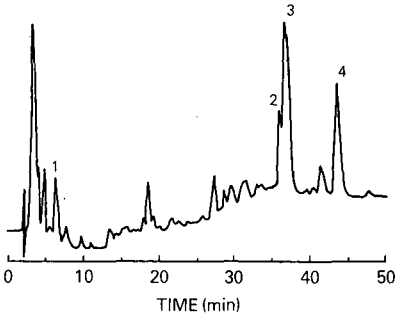

Fig. 2. HPLC analysis of ethanol extract of *O. ochrocephala* B. Experimental conditions: column, μBondapak C_{18} (300 × 3.9 mm I.D.), 10 μm; Mobile phase, water–methanol: initial composition, 100:0; changed between 0 and 10 min to 90:10; between 10 and 15 min to 80:20; between 15 and 30 min to 50:50; between 30 and 50 min to 30:70. Flow-rates, 1 ml/min; detection, HP-1040M photodiode array detector, 254 nm.

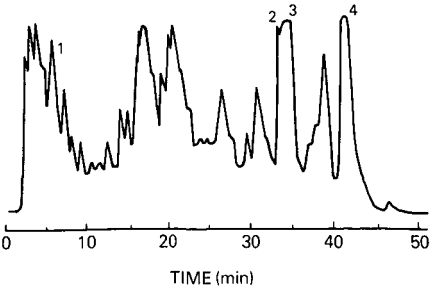

Fig. 3. Semi-preparative HPLC separation of ethanol extract of *O. ochrocephala* B. Experimental conditions: column, LiChrosorb RP-18 (200 × 10 mm I.D.), 10 μm; flow-rate, 4 ml/min; other conditions as in Fig. 2.

loading capacity. As indicated in Figs. 2 and 3, once the analytical HPLC conditions are optimized, the separations can be performed on a much larger scale with the semi-preparative column. However, the sample loading capacity of the semi-preparative column is still limited to a milligram range where further increase of the sample size would cause substantial loss in peak resolution.

In contrast, CPC has a lower column efficiency, requires a longer separation time, but provides a much greater sample loading capacity. Although the column efficiency of CPC is lower than that in the semi-preparative HPLC column, the method is still suitable for the separation of polar compounds such as those corresponding to peaks 1 and 3 in Fig. 1. Disadvantage of the longer separation time of CPC can be compensated by its larger sample loading capacity. For example, the CPC separation time in Fig. 1 is five times that of HPLC in Fig. 3, while the sample size in Fig. 1 is 20 times that applied in Fig. 3. The elution profile of the desired peaks in Fig. 1 suggests that the sample size may be further increased without a detrimental loss in peak resolution.

In order to compare the efficiency in preparative separations between CPC and semi-preparative HPLC, the purities of the peak fractions obtained from these two methods were quantitatively determined by analytical HPLC. The results are summarized in Fig. 4A–F where chromatograms on the left (I) were obtained from CPC fractions 1–6, and those on the right (II) were from semi-preparative HPLC fractions 4–1. In order to facilitate the comparison, these chromatograms are arranged from A to F in such a way that each matched pair represents analysis of the same main compound separated by the two methods. It can be seen that purities of the compound separated by CPC and HPLC are quite similar in Fig. 4A, but CPC yields substantially higher purity in Fig. 4C. Fig. 4E further indicates that the more polar compound in CPC fraction 5 shows a much higher purity than the same compound in semi-preparative HPLC fraction 1. The most polar compounds in CPC fraction 6 (Fig. 4F) contains a large amount of impurities, but it would neither be well separated by semi-preparative HPLC, as suggested from the elution profile in the chromatogram (Fig. 3).

Overall results of the above comparative studies on the preparative performance of CPC and HPLC indicate that CPC requires a longer separation time, but it has important advantages of yielding higher purity of fractions and providing a much

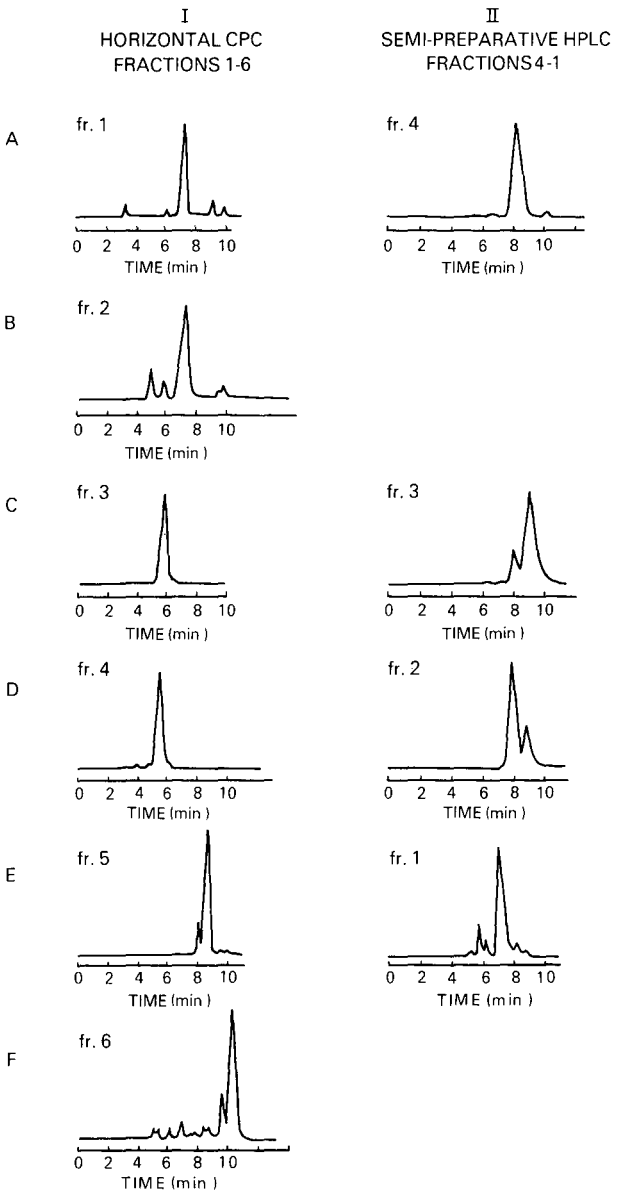

Fig. 4. HPLC analyses of peak fractions obtained from CPC (I) and semi-preparative HPLC (II). Fractions (fr.) 1–6 in I (left column) are corresponding to peak fractions 1–6 in Fig. 1 and fractions 4–1 in II (right column) to peak fractions 4–1 in Fig. 3. Matched pairs of chromatograms in A, C, D and E indicate analysis of the same components separated by the two different methods. Experimental conditions: I. Column: Herpsil ODS (100 × 2.1 mm I.D.), 5 μm; mobile phase: water–methanol: (A–D) initial composition 54:46; changed between 0 and 10 min to 32:68; (E,F) 95:5; otherwise same as described in Fig. 2. II. Mobile phase: water–methanol: (E) initial composition 100:0; changed between 0 and 10 min to 90:10; (C,D) 60:40; (A) initial composition 60:40; changed between 0 and 20 min to 40:60; other conditions are same as in Fig. 3.

greater sample loading capacity. These findings suggest that the isolation of natural products may be efficiently performed in two steps: CPC is used first to clean up the crude extract and HPLC is used next to effect the final purification of the desired compounds. Thus, the combined use of these two methods can be advantageous.

ACKNOWLEDGEMENTS

We are deeply indebted to Professor Lu Wan-Zhen and Dr. Long Yicheng for HPLC analysis of CPC fractions (Fig. 4-I) and to Hewlett-Packard for providing us an HP-1090M HPLC system. We also thank Dr. Henry M. Fales for his valuable suggestions in preparing the manuscript.

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Note

Separation of quassinoids from *Ailanthus altissima* by high-speed counter-current chromatography

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Quassinoids are bitter constituents found in most *Simaroubaceae* sp. and show interesting biological activities. In order to obtain standards of these compounds for the survey of metabolite production in *Ailanthus altissima* cell cultures [1,2], isolation of quassinoids from the root-bark of the plant was undertaken. The separation and purifiation of quassinoids have been usually acieved using combined chromatographic methods [column chromatography on silica gel or on reversed-phase silica gel, preparative tin-layer chromatography (TLC)]. High-speed counter-current chromatography (HSCCC) has not previously been used for the separation of this group of natural products.

EXPERIMENTAL

All chemicals were of analytical-reagent grade.

Apparatus

HSCCC was performed using an Ito multi-layer coil separator–extractor [3] (P.C., Potomac, MD, U.S.A.) equipped with a 2.6 mm I.D. column. An LDC Milton Roy (Riviera Beach, FL, U.S.A.) minipump was used to pump the solvents through the column. The rotational speed was 800 rpm. A manual sample injection valve (Lobar Column Accessories, Merck) equipped with a 10-ml loop was used to introduce the sample into the column. Fractions were collected using a LKB Ultrorac 7000 collector.

Preparation of sample

Powder of dried root-bark of *A. altissima* (350 g) was extracted with methanol and the methanolic extract was further suspended in light petroleum. The light petroleum-insoluble residue was dissolved in a small volume of methanol, absorbed on

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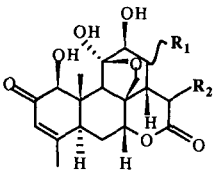
cellulose and chromatographed on a silica gel column. Elution was achieved with chloroform containing increasing amounts of methanol. The fractions eluted with chloroform–methanol (95:5) were evaporated under reduced pressure, chromatographed on a reversed-phase silica gel column and eluted with water–acetonitrile (80:20). Fractions containing ailanthone and a mixture of related quassinoids were evaporated and the residue (80 mg) was used for the HSCCC separation.

Separation procedure

A two-phase solvent system was prepared by equilibrating chloroform–methanol–water (5:6:4). After separation, the two phases were degassed in an ultrasonic bath. The upper phase, used as the stationary phase, was pumped into the column at 6 ml/min. The sample (80 mg) was dissolved in 6 ml of each phase and introduced through the injection port. The lower phase, used as the mobile phase, was then pumped into the column at 4 ml/min. The separation was performed at room temperature and 30 fractions of 15 ml were collected.

TABLE I

HSCCC OF THE QUASSINOIDS ISOLATED FROM A PREPURIFIED EXTRACT (80 mg) OF *A. ALTISSIMA* ROOT-BARK



	R_1	R_2
1 Chapparinone	····CH_3	H, H
2 Ailanthone	$=CH_2$	H, H
3 13,18-Dehydroglaucarubinone	$=CH_2$	OOC - C(OH)(CH_3) - C_2H_5, H
4 13,18-Dehydroglaucarubolone	$=CH_2$	OH

Fractions	R_F (TLC)	Constituent	Amount (mg)
1–3	–	–	
4	0.56	1	18
5–6	0.50–0.42	2+3	10
7–8	0.42	3	24
9–25	–	X[a]	17
26–30	0.25	4	10
		Total:	79

[a] X corresponds to a mixture of three compounds that were not identified; on the basis of co-chromatography with an authentic sample, the presence of chapparin in this mixture is likely.

Fractionation monitoring

The purity of fractions was checked by TLC on silica gel using chloroform–methanol (98:2) as the mobile phase; detection was achieved under UV radiation at 254 nm and also by spraying a 3% solution of sulphuric acid in methanol followed by heating for 5 min at 115°C.

RESULTS AND DISCUSSION

The solvent system was chosen on the basis of the partition coefficient of the quassinoids in the two solvent phases and TLC. The order of elution from the column corresponded to decreasing R_F values of the different compounds after TLC (Table I). Quassinoids 1, 3 and 4 were obtained in pure form and used directly without any further purification for structure determination (UV, mass and ^{1}H NMR spectrometry). No loss of constituents occurred during the separation, which was achieved within 2 h.

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END OF SPECIAL ISSUE

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Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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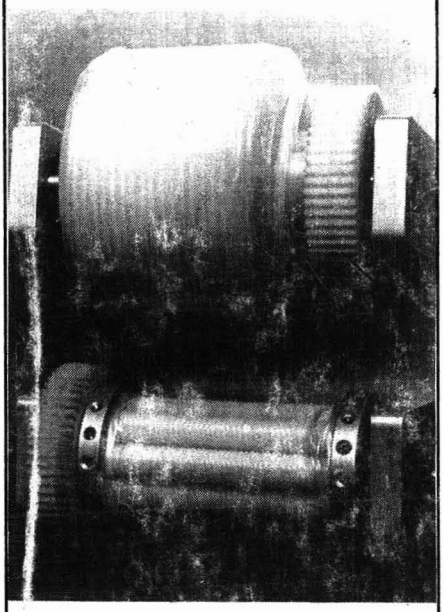

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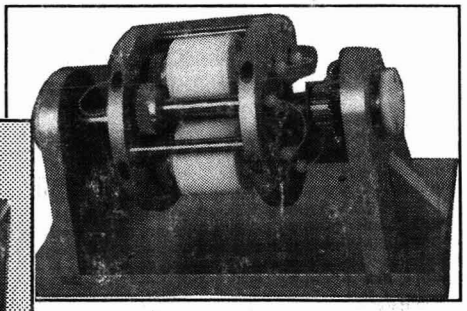

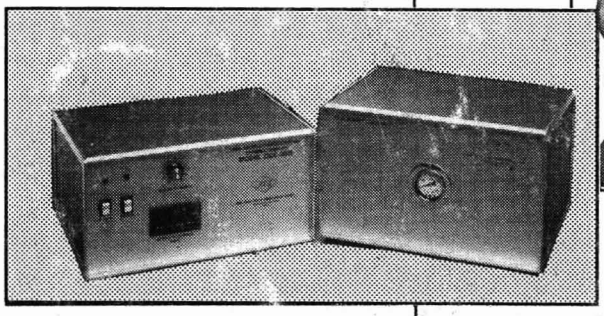

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