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NEW CONTINUOUS EXTRACTION METHOD WITH A COIL PLANET CENTRIFUGE

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SUMMARY

A compact table-top model of the coil planet centrifuge simultaneously enables both preliminary purification and enrichment of samples from crude extracts or biological fluids. The method uses hydrodynamic behavior of two immiscible solvent phases in a rotating coiled tube to retain the stationary phase against a high flow-rate of mobile phase. Consequently, a small quantity of the sample present in a large volume of mobile phase is efficiently extracted into a small volume of the stationary phase within a short period of time and at a high recovery rate. The capability of the present method was demonstrated in the extraction of dinitrophenylamino acids (used as a comparative performance standard) with a set of two-phase solvent systems composed of ethyl acetate and 0.5 *M* NaH_2PO_4.

INTRODUCTION

Preliminary cleaning-up of crude extracts or biological fluids is often essential for purification of biological materials. When a small quantity of the material of interest is present in a relatively large quantity of the solvent, enrichment is also necessary. Conventional procedures such as repetitive extraction with a separatory funnel or a Craig countercurrent distribution method usually result in a large quantity of harvested solvent which necessitates further concentration.

The present paper introduces an efficient extraction method utilizing a coil planet centrifugation. The method enables both cleaning-up and enrichment in a short period of time and at a high recovery rate. The capability of the method was demonstrated on extraction of small amounts of dinitrophenyl (DNP) amino acids from several hundred milliliters of either aqueous or non-aqueous phase composed of ethyl acetate and 0.5 *M* sodium phosphate aqueous solution.

PRINCIPLE

The method takes advantage of the intriguing hydrodynamic behavior of two immiscible solvent phases in a coiled tube rotating in an acceleration field. When a

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coiled tube is slowly rotated around its horizontally oriented axis, particles present in the coil move toward one end of the coil. This end is defined as the head and the other end as the tail of the coil. Two immiscible solvents confined in such a tube are usually distributed from the head toward the tail at a particular volume ratio and any excess of either phase remains at the tail of the coil. While the distribution ratio of the two solvents varies with a number of parameters, the rotational speed of the coil becomes the major determinant for the phase distribution of a given pair of solvents. At a very slow rotational speed the two phases are distributed so that they are nearly in equal amount in each coiled turn. At a very high revolutional speed a strong centrifugal force field separates the two phases in such a way that the heavier phase occupies the outer portion and the lighter phase the inner portion of each helical turn. This results in the distribution ratio of the two phases in each helical turn being equal to the volume ratio originally present in the coil. However, when the rotational speed is between these two extremities, one of the phases occupies more space in the coil on the headside and in some cases the two phases are completely separated in the coil, *i.e.*, one phase entirely occupies the head side and the other phase the tail side of the coil. Ideal two-phase distribution for continuous extraction is represented by this complete phase separation at this intermediate rotational speed.

Let us assume a pair of immiscible solvent phases A and B where phase A is distributed on the head side and phase B on the tail side of the coil. Under this particular circumstance, continuous extraction is possible in three ways. In the first method, the coil is filled with phase A (stationary phase) followed by elution with phase B (mobile phase containing the sample) through the head end of the coil. Phase B then travels through phase A in the coil toward the tail. Consequently, the sample present in phase B is extracted into the stationary phase A and the stripped phase B is eluted through the tail of the coil. In the second operation, the coil is first filled with phase B (stationary phase) and phase A (mobile phase containing the sample) is pumped through the tail of the coil. Extraction process similarly takes place in the coil, the stripped phase A being eluted through the head of the coil. The third operation involves dual countercurrent extraction (not described in this paper) in which phases A and B are simultaneously introduced into the coil through the tail and the head, respectively. In this case the coil should be equipped with an additional pair of flow tubes at each end to collect both enriched and stripped phases. If desirable, the sample solution may be fed into the coil through another flow line connected at the middle portion of the coil.

As mentioned earlier, these extraction methods are perfected by providing an operational condition where the two phases are separated completely along the length of the coil. Use of the rotating coil in the gravitational field, however, usually fails to produce this ideal type of hydrodynamic behavior of the two phases. The search for a suitable extraction scheme which yields complete phase separation in a coiled tube has been successful in the utilization of a coil planet centrifuge. The apparatus provides a particular mode of the synchronous planetary motion to a coiled tube, *i.e.*, the coil revolves around the central axis of the centrifuge and rotates about its own axis at the same angular velocity and in the same direction[1–3].

The centrifugal force field produced by this planetary motion[3,4] is highly dependent upon the location of the point on the holder which is conveniently expressed by β, *i.e.*, the ratio between the radii of rotation and revolution. When the β value

exceeds 0.25, the centrifugal force vector is always directed outwardly from the holder while it oscillates in both amplitude and direction during each revolutional cycle of the holder. Though the motion of the two-phase solvent in the coil subjected to such a centrifugal force field is hardly predictable, the behavior of the two phases can be easily observed through the tube wall under stroboscopic illumination.

A series of observations has been made on various two-phase solvent systems having a wide spectrum of physical properties. The results so far obtained indicate that the distribution of the two phases is affected by three major factors, *i.e.*, wall affinity, relative density and viscosity of the two phases. The phase which has higher wall affinity, lower density and less viscosity tends to distribute itself toward the head of the coil. When all three requirements are satisfied, the upper phase will quickly move toward the head and the phase separation is completed in a short period of time. This ideal group of the solvent pair includes (if PTFE tube is used as the column) a number of useful extraction media such as hexane, ether, ethyl acetate, toluene, methyl ethyl ketone, benzene, etc., mixed with aqueous solution where various salts can be added to adjust the pH and ionic strength of the aqueous phase. Various third solvents such as methanol, acetic acid, etc., can also be added without altering the overall behavior of the two phases.

When the two solvent phases fail to meet the above requirements, complete phase separation may not occur; instead one of the phases usually dominates at the head side of the coil. Two typical solvent pairs in this group have been tested. In a *n*-butanol–water system the non-aqueous phase is more viscous than the lower aqueous phase. In this case the distribution ratio of the two phases is greatly affected by the β values. At $\beta = 0.25$, the aqueous phase is almost entirely distributed on the head side while at $\beta = 0.75$ the non-aqueous phase dominantly occupies the head of the coil especially under a high revolutional speed. In a chloroform–water system, the non-aqueous phase is much heavier than the aqueous phase. Probably due to this great difference in density, the aqueous phase always dominates on the head side regardless of the β values. In these non-ideal solvent pairs, application is limited to head–tail elution using the dominant phase as the stationary phase.

EXPERIMENTAL

Apparatus

The design of the apparatus used in the present studies is similar to the toroidal coil planet centrifuge which permits continuous elution without the use of rotating seals as described earlier[2,3]. Fig. 1 shows the cross-sectional view of the apparatus. The motor (Electro-Craft) drives the rotary frame around the horizontal stationary pipe (shaded) mounted on the axis of the centrifuge. The rotary frame consists of a pair of aluminum discs rigidly bridged together with multiple links (not shown in the figure) and holds a pair of rotary column holders in the symmetrical positions 10 cm away from the central axis of the centrifuge. The bottom holder has a diameter of 15 cm ($\beta = 0.75$) and the top holder of 10 cm ($\beta = 0.5$). The shaft of each holder is equipped with a plastic planetary gear which is coupled to an identical sun gear (shaded) mounted around the central stationary pipe. In order to provide mechanical stability, a short coupling pipe is coaxially mounted to the free end (right side) of the rotary frame while the other end of the coupling pipe is supported by a stationary wall member of the centrifuge through a ball bearing. The coiled column

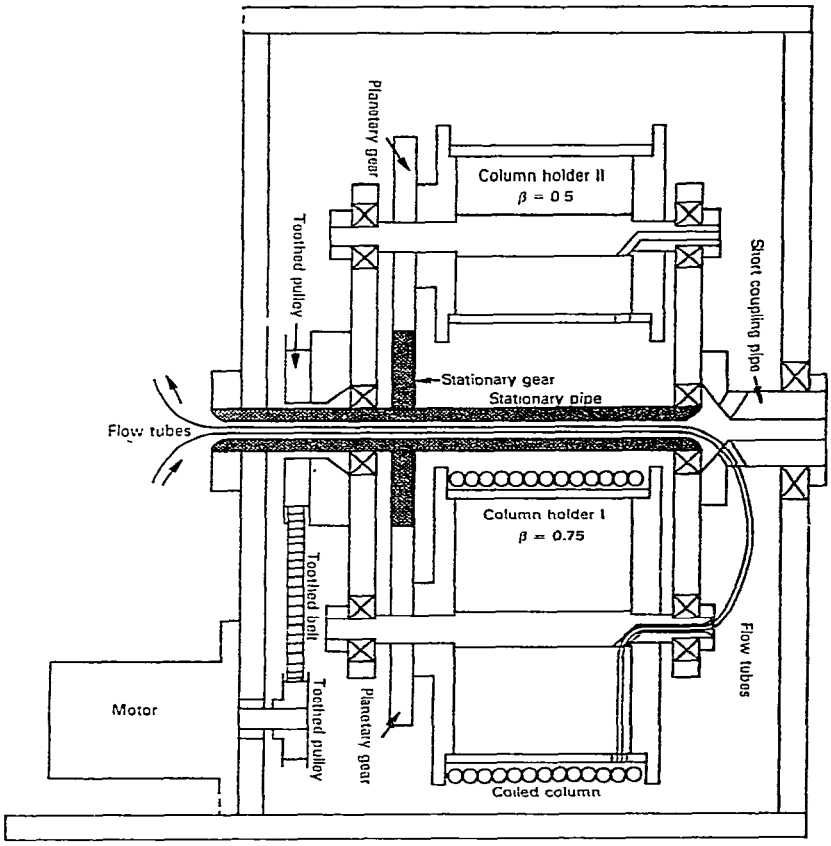

Fig. 1. Cross-sectional view of the apparatus.

was made by winding the desired length of a PTFE tube around one of the holders while a counterweight is applied on the other holder to balance the centrifuge. A pair of flow tubes from the coiled column is first passed through the center hole of the holder shaft and led through the side hole of the short coupling pipe to reach the opening of the central stationary pipe. These flow tubes are thoroughly lubricated with silicone grease and protected with a piece of plastic tubing at each supported portion to prevent direct contact with metal parts. The revolutional speed can be regulated up to 1000 rpm. The apparatus is a compact table-top model whose dimensions are *ca.* 16 × 16 × 17 in.

Studies on retention of the stationary phase

The capability of the present scheme in retaining a large amount of the stationary phase was demonstrated with a coiled column prepared from 2.5 m × 2.6 mm I.D. PTFE tube (Zeus Industrial Products, Raritan, NJ, U.S.A.) which was coiled around a holder having a β value of 0.75. The column consisted of five helical turns and had a total capacity of *ca.* 15 ml. Typical two-phase solvent systems composed of ethyl acetate–water and ethyl acetate–0.5 *M* sodium phosphate (pH 4.4) at a volume ratio of 1:1 were selected. Each two-phase system was equilibrated in a

separatory funnel and separated before use. In each operation the coiled column and the free space in the flow path were entirely filled with the stationary phase and the mobile phase was pumped into the column in the proper direction (head–tail elution for the aqueous phase and tail–head elution for the non-aqueous phase) while the apparatus was run at a given revolutional speed. The eluate through the outlet of the column was pooled in a graduated cylinder to measure the volume of the eluted stationary phase, V_s. From the predetermined figures of the total column capacity, V_c, and the free space in the flow path, V_f, the percentage retention, R, of the stationary phase relative to the total column capacity was calculated according to the formula: $R = 100\,(V_c + V_f - V_s)/V_c$. The experiments were performed under various revolutional speeds and flow-rates using both non-aqueous and aqueous phases as the stationary phase.

Continuous extraction experiments

A series of experiments has been performed to demonstrate the capability of the present scheme to extract a solute present in a large volume of the mobile phase into a small volume of the stationary phase retained in the coiled column. This requires a set of conditions such that the solute must favor partition to the stationary phase. With commonly used extraction media such as an ethyl acetate–aqueous system, partition coefficients of various biological materials can be conveniently adjusted by modifying the pH and/or ionic strength of the aqueous phase to meet the above requirement. For the present studies, a pair of DNP-amino acids (Sigma, St. Louis, MO, U.S.A.), N-DNP-L-leucine (DNP-Leu) and delta-N-DNP-L-ornithine (DNP-Orn), were selected as samples because they are readily observed through the column wall during the extraction process under stroboscopic illumination and also provide suitable partition coefficients for this present solvent system. The experiments were performed with the coiled column used in the previous retention studies. The overall experimental conditions in the following studies are summarized in Table I.

A typical extraction procedure may be divided into three steps, *i.e.*, extraction, cleaning, and collection. In each operation, the column was filled with the stationary phase and the mobile phase containing the sample was eluted through the column in the proper direction while the apparatus was run at 600 rpm. The extraction process was continued until 400 ml of the mobile phase was eluted. Then the mobile phase was replaced by the same phase but free of solute to wash the column contents. This cleaning process was continued until the additional 100 ml of the mobile phase was eluted. This would elute out all impurities having partition coefficients of 0.1 or greater. The sample extracted into the stationary phase in the coiled column was collected by eluting with the mobile phase in the opposite direction. This was done by switching the feed and return flow lines either by simply disconnecting the flow lines or the use of a four-way slide valve (Pierce, Rockford, IL, U.S.A.). The sample still remaining in the column was then washed out by eluting the column with the other phase originally used as the stationary phase.

Sample collection from the column may be performed in different ways. When the mobile phase is the aqueous phase, modification of the pH and/or ionic strength often results in a great shift in the partition coefficient of the solute in such a way that the solute favors partition into the aqueous phase. In this case either stepwise or gradient elution with such a modified aqueous phase produces a chromato-

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS AND RESULTS FOR CONTINUOUS EXTRACTION

Exp. No.	*Solvent system*	*Mobile phase*	*Stationary phase*	*Sample (P.C.)**	*Sample concn. in mobile phase (mg%)*	*Extracted mobile phase volume (ml)*	*Flow-rate (ml/h) (direction)*	*rpm*	*Collected stationary phase volume (ml)*	*Sample recovery (%)*
1	Ethyl acetate–0.5 *M* NaH_2PO_4(1:2)	Aqueous	Non-aqueous	DNP-Leu (<0.01)	4	400	516 (head–tail)	600	10.5	94
2	Ethyl acetate–0.5 *M* NaH_2PO_4(1:2)	Aqueous	Non-aqueous	DNP-Leu (<0.01)	0.4	400	516 (head–tail)	600	10.0	97
3	Ethyl acetate–0.5 *M* NaH_2PO_4(1:2)	Aqueous	Non-aqueous	DNP-Leu (<0.01)	0.04	400	516 (head–tail)	600	10.4	100
4	Ethyl acetate–0.5 *M* NaH_2PO_4(2:1)	Non-aqueous	Aqueous	DNP-Orn (<0.01)	0.4	400	516 (tail–head)	600	11.8	97
5	Ethyl acetate–0.5 *M* NaH_2PO_4(2:1)	Non-aqueous	Aqueous	DNP-Orn (<0.01)	0.04	400	516 (tail–head)	600	11.8	100
6	Non-equilibrium system	5% Ethyl acetate in 0.5 *M* NaH_2PO_4	Ethyl acetate	DNP-Leu (<0.01)	0.4	400	516 (head–tail)	600	6.1	99

* Partition coefficient (P.C.) is defined as solute concentration in the mobile phase divided by that in the stationary phase.

graphic separation of the solute which can be monitored by a conventional UV detector. This results in further purification of the solute retained in the column.

The degree of sample recovery was estimated by comparing the amount of the sample in the original mobile phase to that in the collected stationary phase. A Beckman DU spectrophotometer was used to measure the absorbance at 430 nm.

RESULTS AND DISCUSSION

The results of the retention studies are summarized in Fig. 2, where the percentage retention of the stationary phase was plotted against the applied revolutional speeds. The three lines drawn in each diagram indicate the effects of the different flow-rates applied. The flow-rate of 516 ml/h is the maximum rate available with the Beckman Accu Pump employed. The ideal retention level for extraction may be considered to be over 70% at or near the plateau of the curve, although much lower retention levels can be applied for extraction unless carryover of the stationary phase occurs. Fig. 2A and B show the retention curves of the solvent system com-

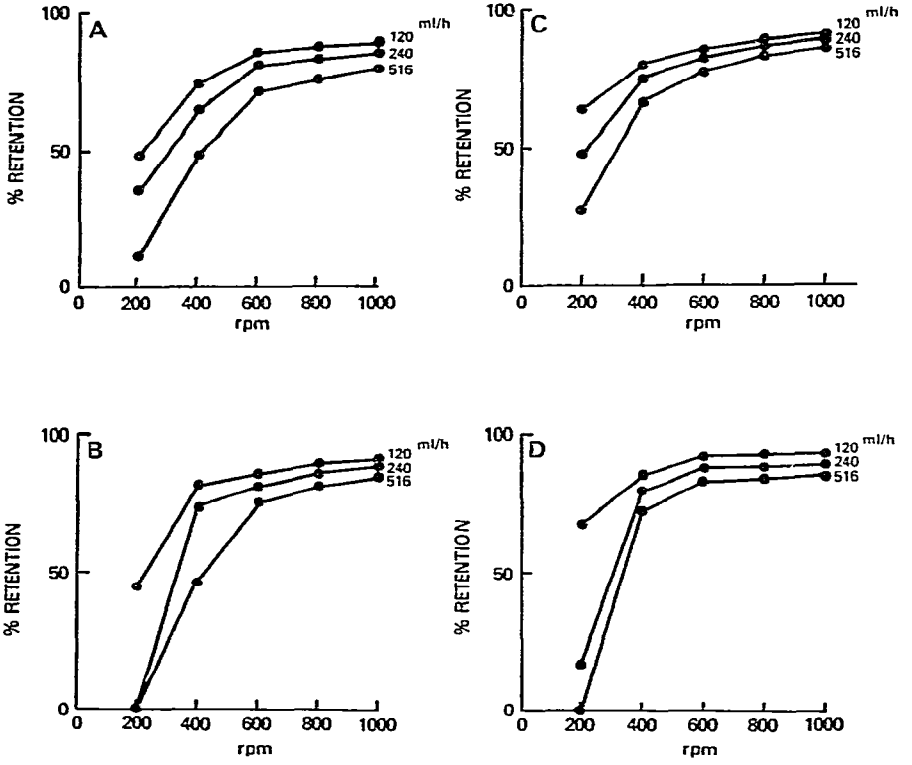

Fig. 2. Effects of revolutional speed and flow-rate on retention of the stationary phase. A, Phase system: ethyl acetate–water; stationary phase: upper non-aqueous phase; elution: head–tail. B, Phase system: ethyl acetate–water; stationary phase: lower aqueous phase; elution: tail–head. C, Phase system: ethyl acetate–0.5 *M* NaH_2PO_4 (1:1); stationary phase: upper non-aqueous phase; elution: head–tail. D, Phase system: ethyl acetate–0.5 *M* NaH_2PO_4 (1:1); stationary phase: lower aqueous phase; elution: tail–head.

posed of ethyl acetate and water, where both non-aqueous (A) and aqueous (B) phases were used as the stationary phase. In both cases the ideal retention levels are provided at the revolutional speed of over 600 rpm at all flow-rates applied. Comparison between A and B reveals that the retention levels of the aqueous phase in the tail–head elution always exceed those of the non-aqueous phase in the head–tail elution. This may be due to the higher wall affinity and lower viscosity of the non-aqueous phase which provides less resistance against the flow.

Fig. 2C and D show similar retention curves for the phase system composed of ethyl acetate–0.5 *M* NaH_2PO_4 (1:1). In both C and D, retention levels show much improvement over the previous phase system. Addition of salt to the phase system results in a greater density difference which promotes movement of the phases in the coil as described earlier. The overall results indicate that the system provides excellent retention under a broad range of operational conditions for both aqueous and non-aqueous stationary phases. The results also suggest that much higher flow-rates are applicable with high revolutional speeds.

In order to demonstrate the extraction capability of the present scheme, a series of model experiments has been performed with sets of solvent systems and DNP-amino acid samples as shown in Table I. In experiments 1–3 in Table I, DNP-Leu was dissolved in 400 ml of the aqueous phase at various concentrations and extracted into the non-aqueous phase retained in the column. The extracted sample was then cleaned by eluting the column with 100 ml of the clean aqueous phase and then collected from the column. The harvested stationary phase volume measured *ca.* 10 ml, containing over 90% of the original sample. A small amount of the sample still remaining in the column, usually a few percents of the total, was conveniently recovered by eluting the column with several milliliters of the non-aqueous phase. The total sample recovery is always well over 90%, as shown in the table. The reduction of the sample concentration from 4 mg% to 0.04 mg% somewhat improved the recovery rate, indicating that no significant sample loss occurs due to the adsorption effects and that further reduction of the sample concentration is feasible with high levels of recovery. The mode of elution that uses the non-aqueous phase as the stationary phase renders a great advantage in practical extraction in that the collected solvent is highly volatile and free of salts, facilitating further concentration. It also permits the stepwise or gradient elution of the sample by eluting the column with a modified aqueous phase to achieve further purification.

In experiments 4 and 5 in Table I, the DNP-Orn sample was dissolved in 400 ml of the mobile non-aqueous phase and extracted into the stationary aqueous phase by eluting the column from the tail toward the head. The retained aqueous phase in the column was then similarly cleaned with 100 ml of non-aqueous phase free of sample. The collected stationary aqueous phase measured *ca.* 12 ml in volume. This exceeds the volumes in experiments 1–3, as expected from the results of the retention studies. The sample still remaining in the column was eluted out with several milliliters of the aqueous phase. The sample recovery ranged over 95% with an improved figure at the reduced sample concentration as observed in the previous experiments.

In practice, application of the method to aqueous crude extracts or physiological fluids requires a preliminary adjustment of the solvent composition to provide a suitable partition coefficient of the desired material for the applied pair of solvents.

In this case pre-equilibration of the two phases is not essential. Experiment 6 in Table I shows an example of operation with such non-equilibrated solvents. The sample DNP-Leu was first dissolved in 400 ml of 0.5 *M* NaH_2PO_4 aqueous solution containing ethyl acetate at 5%, which is slightly below the saturation level of *ca.* 7%. The column was filled with ethyl acetate followed by elution with the above sample solution. Both extraction and cleaning processes were performed as in other experiments. The sample solution collected from the column measured slightly over 6 ml. This depletion of the stationary phase apparently resulted from use of the non-equilibrated solvent pair but without any effect on the sample recovery.

The overall results indicate a potential usefulness of the present method in processing a large amount of crude extracts or biological fluids in research laboratories. A small amount of the sample present in several hundred milliliters of the original solution can be enriched in 10 ml of the non-aqueous phase free of salt in 1 h at a high recovery rate.

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