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COUNTER-CURRENT CHROMATOGRAPHY USING A NEW COIL PLANET CENTRIFUGE

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SUMMARY

Coil planet centrifugation has existed for a number of years, but its potential in superseding existing counter-current distribution and liquid–liquid chromatographic techniques has not been fully realised. The development of a new coil planet centrifuge that is capable of analytical or preparative separations of biological materials is described. Testing of solvent systems, fluid dynamic problems, and the running of a complete separation system are discussed, and the accuracy and repeatability of separations are demonstrated.

INTRODUCTION

Gas chromatography has been widely used as a fast, high-resolution analytical technique for a number of years. More recently, high-pressure liquid chromatography (HPLC) has been developed. Both of these procedures are capable for use on a preparative scale but with considerable increase in cost and complexity. The scaling-up procedures can also involve some sacrifice of the high resolution of sample components obtainable at the analytical level. Liquid–liquid chromatography of the type discussed in this paper, which does not use a solid support, is capable of giving separations that are mathematically predictable in terms of the physical properties of the sample components. The system can operate with large or small solvent volumes and consequently may be used analytically or preparatively.

A separation using liquid–liquid chromatography relies on a sample "partitioning" between two immiscible solvents. If a test tube containing two equal volumes of these solvents has a known amount of sample put in it, is shaken and allowed to settle, then the partition coefficient K is defined as

$$K = \frac{\text{Weight or concentration of sample in upper phase}}{\text{Weight or concentration of sample in lower phase}}$$

Craig[1] demonstrated that materials with closely similar physical properties can be separated, provided they have slightly different partition coefficients in two

immiscible solvents, by a repetitive process of mixing, settling and transferring of one of the phases relative to the other. He devised a machine to carry out these operations semi-automatically.

Inevitably, high-resolution separations of sample constituents using samples of slightly different partition coefficients are only possible with a large number of transfers. One transfer can take up to 10 min and as many as 1000 transfers may be needed for a good separation. The long operation times and the circumstance that small amounts of valuable sample may have to be handled in large volumes of mixed solvents, together with problems of sample stability and the initial problem of devising a suitable two-phase solvent system, may have inhibited the full exploitation of this potentially very powerful separation procedure. Even so, despite its obvious disadvantages, the Craig system of counter-current distribution has remained one of the few general techniques for separating closely related substances in pure form in a predictable fashion, and as such it has been widely used[2].

The coil planet centrifuge was invented by Ito and Bowman[3], who with others[4,5] have demonstrated its use for separating amino acids and peptides. Sutherland *et al.*[6] have discussed the versatility of the system when separating polyene and peptide antibiotics.

The centrifuge described can perform separations on either an analytical or a preparative scale in a few hours, using less than 1 l of solvent. This paper describes the development of the centrifuge and highlights some of the fluid dynamic problems that can be encountered when choosing a solvent system. The testing of solvent systems using the centrifuge and the operation of the overall system are also discussed. Finally, the separation of a particular polyene antibiotic demonstrates the accuracy and repeatability of the separation process.

FLUID DYNAMIC PROBLEM

Ito showed that a two-phase solvent system constrained to rotate in planetary motion in a helical coil of small-diameter tubing (I.D. < 1.0 mm) will, under certain conditions, segregate into alternating bands of upper and lower phase. The rotating acceleration vector acts so as to screw bands along the helix. If one of the solvent phases is pumped in the opposite direction to this motion then an equilibrium can develop between the inertia forces moving the bands one way and the flow moving them the other. The flow causes a series of dislocations of the pumped phase through the system without affecting the other phase. A block of upper phase, for example, pumped in one end will result in upper phase being displaced or eluted from the other end.

For larger-diameter tubing (I.D. > 1.0 mm) surface tension forces become small in comparison to the inertia forces so that multiple droplets of the two phases are formed. Theoretical studies of the behaviour of droplets in a two-phase liquid–liquid system undergoing planetary motion[7] predict that the droplets rotate in circles. The droplets reach a limit cycle whose diameter is a function of the machine rotational radius, droplet size and relative density. The angular velocity is that of the machine, and they will progress up the tube by means of a number of collisions against other droplets and the tube walls; the rate of progression is a function of the curvature of the tubing and the limit cycle diameter. If the tubing is straight, no pro-

gression will take place. In this way if the pumped or mobile solvent phase is flowing against the progression of the droplets, then droplets of the non-pumped phase will accumulate at the pump end of the column while the mobile solvent phase percolates past.

Droplet size is fundamental to the dynamic stability of the process. If the droplets are small, drag forces dominate inertia forces and carry-over, or "stripping", of the droplets with the mobile phase can occur. In a bad case emulsification of the phases can occur.

It is important therefore to maintain stable droplets of reasonable diameter (*ca.* 100 μm). Both the Reynolds number ($Re = \varrho vd/\eta$) and the Webber number ($We = \varrho v^2 d/T_i$) should be kept as low as possible to reduce turbulence and increase droplet stability. (ϱ and η are the mobile phase density and viscosity, respectively, and d is the droplet diameter.) This is achieved by keeping the droplet velocity (v) low and choosing a high interfacial tension (T_i).

The models for both large- and small-diameter tubing are similar from the chromatographic point of view. One of the solvent phases is held stationary while the other is pumped through the column as the mobile phase. The sample is injected with the mobile phase and its constituents are separated by the difference in their respective partition coefficients between the two phases.

APPARATUS

Constraints

The use of counter-rotating gravitational fields to produce suitable droplets of the two-phase solvent system and induce one solvent to remain within the column despite the flow through of the other introduces a number of constraints on the mechanical design of the apparatus. There is a need to produce the necessary "g" force, and the conflicting requirement of an adequately high gravitational force vector and a low Reynolds number, or flow fluid velocity. This requirement manifests itself in two ways. First, there is the need to provide for the radius of the action of the stack and its rotational speed to be varied over a wide range, and, secondly, there is the elimination of all unnecessary vibration.

Centrifuge

The apparatus developed for this purpose is shown in Fig. 1 and diagrammatically in Fig. 2. The basic centrifuge consists of two circular plates of approximately 300 mm diameter spaced 600 mm apart, rotating on a central column and driven through a belt drive onto the lower plate by a 250-W variable-speed motor at speeds up to 1500 rpm. The central column is mounted in a single rubber bush such as to give the complete centrifuge a natural frequency about that point of 2 Hz (120 rpm). To correct any out-of-balance, a number of balls with a total mass of 0.25 kg are placed in a circular track in the top plate of the centrifuge. As the centrifuge rotates the balls align themselves with the radial acceleration of the out-of-balance mass. Below resonance the resulting displacement is in phase with the out-of-balance mass; however, above the resonant speed the displacement is 180° out of phase so that the balls distribute themselves to oppose the out-of-balance mass. In this way self balancing is achieved at all rotational speeds greater than resonance,

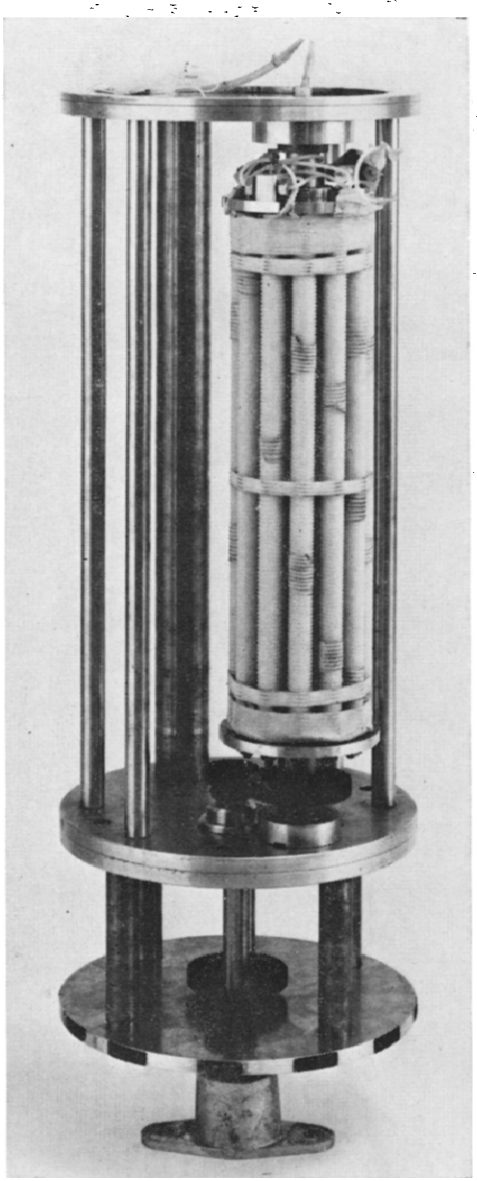

Fig. 1. Coil planet centrifuge.

and vibration is minimised and, most importantly, the need to rebalance the machine when using different solvent densities and different stacks is eliminated.

The self balancing also ensures that the centrifuge bearings carry little or no radial load during normal running.

Stack

The helical tubing in which the solvent distribution takes place (the stack) is supported on a stainless-steel tube rotating in self-aligning bearings in the upper and

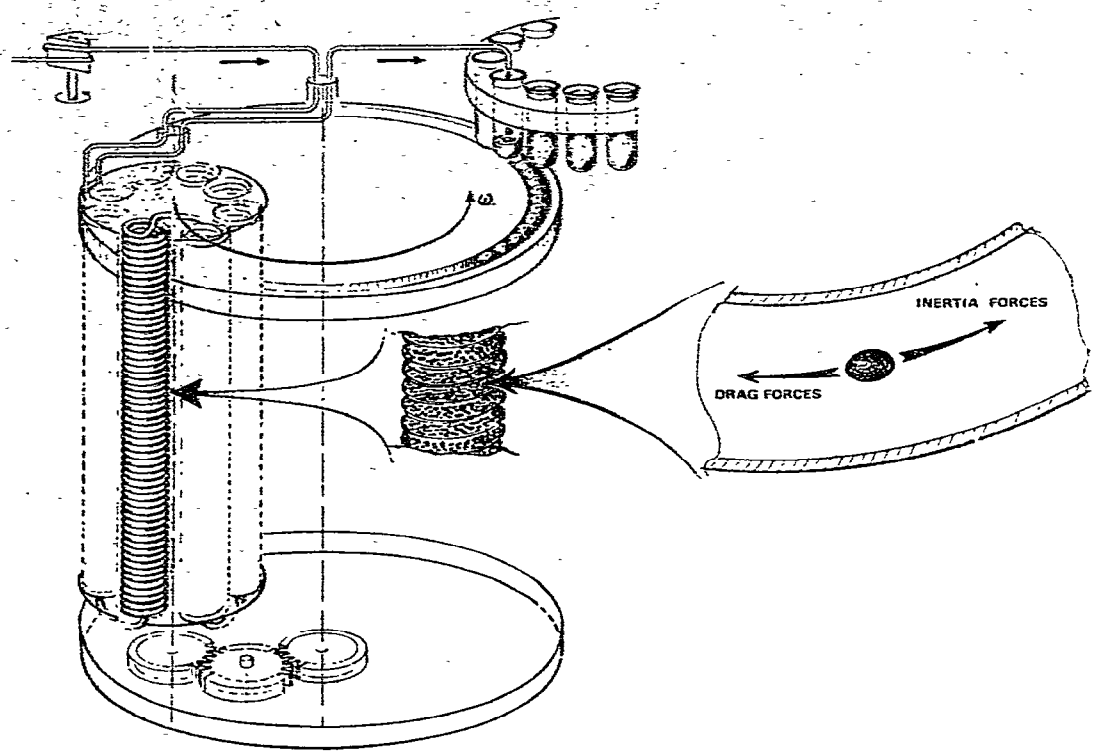

Fig. 2. Coil planet centrifuge (diagrammatic).

lower plates of the centrifuge. The planetary motion of the stack is obtained by gearing to the non-rotating support column of the centrifuge through an idler gear. The stack is counter-balanced by an equivalent mass mounted between the centrifuge plates geometrically opposite the stack. Six bearing positions are provided at radii between 6 and 100 mm and the design will accommodate stack assemblies up to 125 mm in diameter. The stack assembly may be quickly and easily replaced by the removal of the top plate of the centrifuge.

The stack consists of a number of coils of tubing, usually PTFE, tightly wound onto support rods surrounding the central tubular support rod. These coils may be connected in series to form the stack used for normal separations, or used in parallel during feasibility tests on different solvent systems. As the stack assembly does not rotate with respect to the framework, it is a relatively simple matter to make any number of flying connections. The stacks can be constructed using various sizes of tubing. Thick-wall tubing is generally preferred since it minimizes the permeability to solvents. Small-bore tubing is wound onto a former and secured to the side of the central tubular rod.

The ability of the machine to produce very large cyclical forces on the stack demands careful detail design of the stack and its bearings. (Acceleration of 250 g is possible with the machine, and a complete stack may have a mass of 15 kg when filled with liquid.)

TESTING SOLVENT SYSTEMS

Test-tube studies

If a sample has a partition coefficient of between say 0.2 and 5.0 in a given two-phase immiscible solvent system, then it is a relatively simple matter to examine the feasibility of performing a separation on the coil planet centrifuge.

A stoppered cylinder is partially filled with equal volumes of each solvent phase. The sample is then added to give a mean concentration that is equivalent to what is intended to be injected into the coil planet. The cylinder is then stoppered, shaken and the time taken for the two phases to settle is measured. If this time is less than 1 min, running that system on the coil planet is feasible. The longer this time, the more difficult phase distribution becomes, and it is increasingly necessary to plot a phase distribution diagram.

Phase distribution diagram

A phase distribution diagram maps the percentage of upper phase retained in the column for a range of operating speeds. Good separation conditions exist when the percentage of the two phases in the column lies between 30 and 70%.

A typical phase distribution diagram is shown in Fig. 3a, where the effect of interfacial tension is examined by using two different solvent systems: *viz.* (i) methanol–chloroform–borate buffer (4:4:3) ($T_i = 1.5$ dynes/cm) or (ii) methanol–chloroform–borate buffer (4:4:2) ($T_i = 0.5$ dynes/cm). All the other parameters are held constant. The open points refer to the upper phase as the mobile phase while the closed points refer to the lower phase as the mobile phase.

Phase distribution tests are performed with a short column of about 50–100 coils whose volume is V^*. The column and inlet and outlet tubes are initially pumped full of the phase intended to be the stationary phase. The total liquid volume in the system would then be $V_0 + V^*$, where V_0 is the volume of the inlet and outlet tubes. The inlet tube is then moved to the mobile phase and the centrifuge and pump are switched on.

An equilibrium occurs as the two phases mix together, and distribution of the two phases is established uniformly throughout the column. This equilibrium is reached when there is no further carry-over of stationary phase, which coincides with the first appearance of the mobile phase in the eluent. A further period of about 10% of the transit time of the mobile phase through the system is allowed before the pump and the centrifuge are switched off. The volume of the stationary phase collected in the measuring cylinder (V_s) will then be equivalent to the sum of the mobile phase in the stack (V_m) and the inlet and outlet tubes (V_0), so that

$$\frac{V_m}{V^*} = \frac{V_s - V_0}{V^*} \tag{1}$$

The proportion of upper phase in the column can be calculated from eqn. 1 in one of two ways, depending on which phase is the mobile phase, *viz.*

$$\frac{V_u}{V^*} = \frac{V_m}{V^*}$$

where the upper phase is the mobile phase or

$$\frac{V_u}{V_*} = 1 - \frac{V_m}{V_*} \quad (2)$$

where the lower phase is the mobile phase.

After these tests have been performed for different rotational speeds a phase distribution diagram (Fig. 3) can be plotted and an operating condition selected to give the highest stationary phase retention. In Fig. 3a, for example, an operating speed of 500 rpm would be chosen using the 4:4:3 solvent system, as either phase could then be used as the mobile phase. If the sample partitions more towards the upper phase, then this phase would be used as the mobile phase until most of the constituent peaks had been eluted when the mobile phase could be switched, so that the residue peaks could be eluted.

The fluid dynamics of the phase distribution diagram are still not fully understood. The two-phase flow goes through different regimes as rotational speed is increased. There is initially a region of "plug flow", where the pumped or mobile phase pushes out the stationary phase without any mixing. As speed is increased, there is a "spike" region between 200 and 300 rpm where there is a short band of increased retention followed by a sharp decrease. As speed is further increased, retention improves, but can decrease again at high speeds for a low interfacial tension system. This is due to the formation of very fine droplets and the consequential stripping of the stationary phase.

Dynamically a number of variables have been found to affect phase distribution. These are: interfacial tension, density difference, viscosity, speed, tubing bore, helix diameter, planet radius, and flow. The first three variables are set by the two-phase solvent system and in chosing a particular system a high interfacial tension (Fig. 3a) and density difference will give enhanced stationary phase retention. However, if a low interfacial tension system has to be run, then manipulating the machine variables, for example, reducing helix diameter and flow and increasing the planet radius (Fig. 3b), will guarantee a working phase distribution. Finally the tubing bore size can be increased with no significant loss in retention (Fig. 3c), which makes the scaling up of the process and preparative separations feasible, subject to the practical restraints of limiting helix diameter.

The sample injection volume for the 1.6-mm-bore tubing can exceed 2 ml without loss of resolution. The actual weight of the sample injected will, of course, depend on its solubility limit in the particular solvent system used; in fact, this could influence the choice of solvent system if preparative separations were required. Separations of up to 200 mg have been possible using the 1.6-mm-bore tubing and increases of a further order of magnitude are feasible using larger-bore tubing (Fig. 3c). Finally, one aspect of choosing a solvent system that should not be overlooked is the effect that the sample may have on the fluid dynamic properties, such as interfacial tension. A sample that lowers the interfacial tension of the two-phase solvent system on injection can lead to some carry-over of the stationary phase.

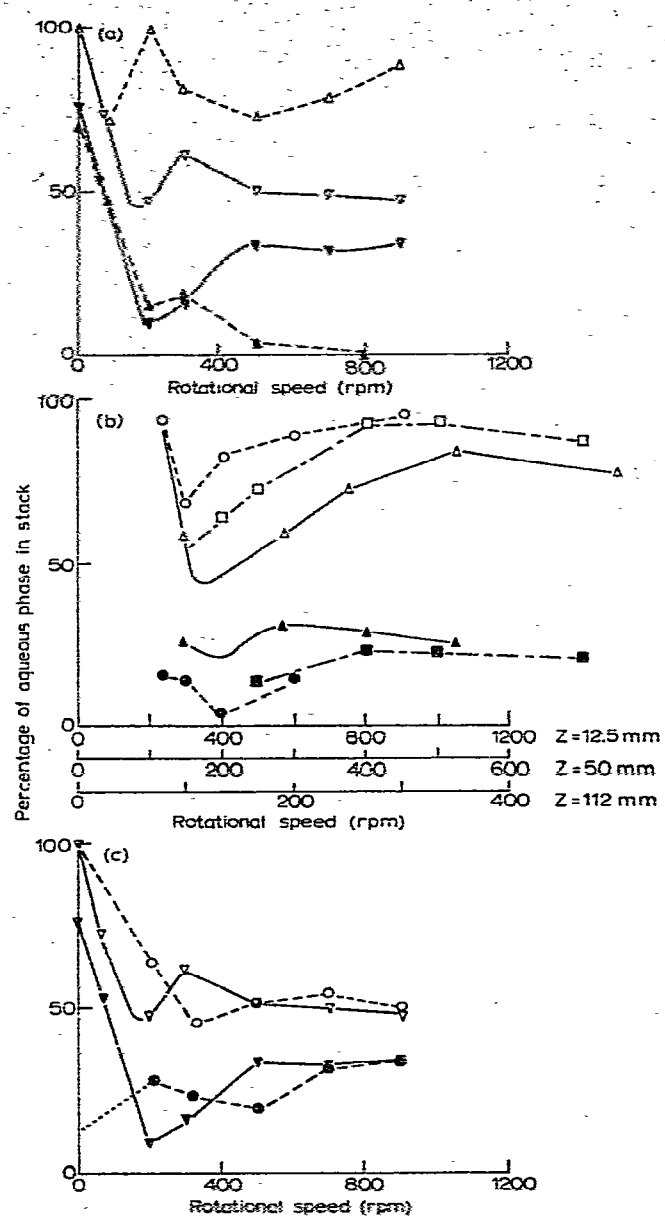

Fig. 3. Typical phase distribution diagrams showing the effects of: (a) interfacial tension (T_i), (b) planet rotational radius (Z), and (c) tubing bore. (a) Mobile phase chloroform–methanol–borate buffer: ▽—▽, 4:4:3, $T_i = 1.5$ dynes/cm or △—△, 4:4:2, $T_i = 0.5$ dynes/cm; I.D. = 3.2 mm; $Z = 95$ mm. (b) ○—○, $Z = 12.5$ mm; □—□, $Z = 50$ mm; △—△, $Z = 112$ mm; I.D. = 3.2 mm; $T_i = 0.5$ dynes/cm. (c) ▽—▽, I.D. = 3.2 mm; ○—○, I.D. = 1.6 mm; $Z = 95$ mm; $T_i =$ 1.5 dynes/cm. Open symbols, upper phase as mobile phase; closed symbols, lower phase as mobile phase.

OPERATING SYSTEM AND PROCEDURE

A practical system for counter-current chromatography is shown schematically in Fig. 4. The solvents forming the stationary and mobile phases are pumped by a variable displacement pump into and through the coil planet centrifuge from an open reservoir of both equilibrated phases. The output pressure of the pump is monitored and displayed on the multichannel chart recorder. The enclosure of the centrifuge may be held at any desired temperature in the range 10–35 °C within $\frac{1}{2}$ °C. The enclosure is cooled by a 250-W refrigerator unit operating continuously and maintained at temperature by an IR heater controlled by a commercial temperature controller. The temperature is also displayed on the chart recorder. Speed control of the centrifuge is provided with the range 0–1000 rpm to $\pm$ 1 % from a tachogenerator fitted to the shaft of the drive motor. The 250-W a.c./d.c. drive motor is controlled with reference to the tachogenerator by a motorised variable transformer. The tachogenerator output provides the third recorder output.

After having passed through the helically coiled stack in the planetary centrifuge, the mobile phase passes through a small flow-through cell in a spectrophotometer (Cecil CE 272) and into a suitable fraction collector. The absorption output from the spectrophotometer forms the fourth channel on the output recorder, whilst the number of fractions collected are indicated by an event marker enabling the fractions to be easily identified. A septum and sample injector are provided on the inlet to the centrifuge.

Normal operation of the apparatus is as follows: (1) The two liquid phases are prepared and allowed to equilibrate in the reservoir for a period of several hours. (2) The lower phase is pumped into the stack until the complete system is filled, and all air has been excluded. (3) With the centrifuge running at the desired speed the

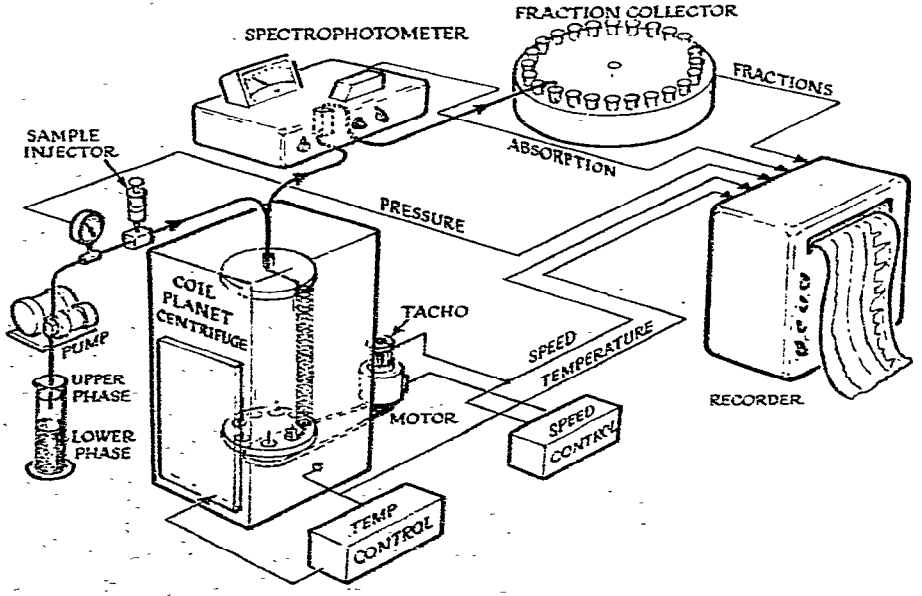

Fig. 4. Schematic arrangement of operating system.

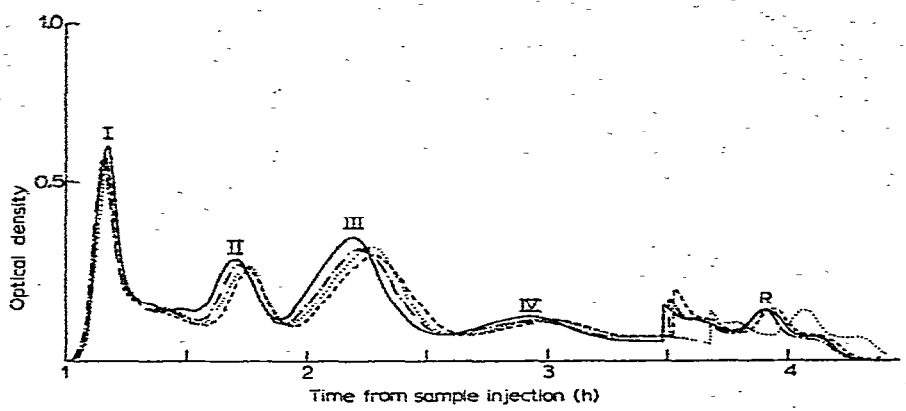

Fig. 5. Variation of optical density with time from sample injection for four loadings of the same Candicidin sample (Penick, Batch. No. 8461-NJF-1). Sample size, 0.3 ml; sample concentration, 1.0 mg/ml; rotational speed, 500 rpm; rotational radius (Z), 95 mm; I.D., 1.6 mm; O.D., 3.2 mm; helix diameter, 15.9 mm; column capacity, 110 ml; number of columns, 8; number of coils, 1024; flow-rate, 60 ml/h; operating temperature, 18 °C.

upper phase is now pumped through the system, displacing some of the lower phase from the stack until equilibrium is established. Measurement of the phase distribution with the stack in equilibrium may be made from the amount of lower phase carried over into the fraction collector during this period. The back pressure will increase steadily until it reaches a steady value when the equilibrium of the two phases has been established. (4) The spectrophotometer may be zeroed when the upper phase is passing through. (5) The sample can be dissolved in either phase and injected through the septum once a steady-state equilibrium condition exists in the stack. A typical recording obtained during a separation is shown in Fig. 5.

There are a number of other methods of operation: (a) The sample eluate constituent peaks may be selectively recycled to obtain greater resolution. (b) The mobile phase can be changed in the middle of a separation, in order to elute the constituents that remain in the stack. The discontinuity in the optical density traces of Fig. 5 is the new solvent front eluting following a change-over of the pumped phases. (c) It is possible to reverse the whole process and use the heavy, lower phase as the mobile phase for the complete separation, which would be preferable for a sample with a mean partition coefficient biased towards the lower phase.

RESULTS

The reproducibility of the system described can be analysed by comparing the results of four separations of the same macrolide antifungal polyene antibiotic, Candicidin (Penick, batch No. 8461-NJF-1). The solvent system used was chloroform–methanol–borate buffer (4:4:3), which was mixed and allowed to equilibrate for over 24 h before each run. The separations were spread over a time period of three weeks, with the same operating conditions and sample preparation techniques used on each occasion. The injection volume was restricted to 0.3 ml, at a sample concentration of 1 mg/ml, for the optical density record to be on a convenient scale. The mobile phase

TABLE I

P VALUES AND AREAS FOR THE FOUR CANDICIDIN SEPARATIONS SHOWN IN FIG. 5

$P = K/(K + 1)$; A = peak area as percentage of sample injected.

Peak No.		*Separations*							
		1		*2*		*3*		*4*	
		P	*A*	*P*	*A*	*P*	*A*	*P*	*A*
I		0.896	18.5	0.910	18.3	0.941	18.5	0.902	18.1
II	A	0.654	21.1	0.689	20.9	0.713	20.5	0.704	20.5
	B	0.541		0.526		0.528		0.528	
III		0.392	30.5	0.379	29.3	0.378	30.3	0.380	29.7
IV		0.278	16.3	0.271	17.9	0.269	15.8	0.275	17.7
R	A	0.223	13.5	0.221	13.5	0.222	14.8	0.227	13.9
	B	0.193		0.191		0.188		0.187	
	C	0.101		0.106		0.106		0.109	
	D	0.027		0.034		0.034		0.039	

was aqueous for the first part of the separation but was changed to the organic phase at $2\frac{1}{2}$ times the elution time from sample injection. This was difficult to judge exactly. The variation of optical density with time for the four separations is shown in Fig. 5, and it can be seen that there is very close agreement between each separation.

A qualitative comparison of partition coefficients (K) from one separation to the next is difficult when K ranges from zero to infinity. It is more convenient to use the proportion of peak material in the mobile phase, (P corrected, like K, for equal volumes), where $P = K/(K + 1)$. The partition coefficients and hence P values for all the peaks, including the residue peaks, can be calculated from the optical density traces[5], and are given along with peak areas in Table I.

The variation of both the P values and the peak areas for the four separations are within $\pm$ 2% for the major peaks and $\pm$ 5% for the measurable minor ones.

The prototype centrifuge used for these separations has now been in operation for more than a year, performing about 40 separations in 200 h of operation, and has functioned very reliably during this period.

CONCLUSIONS

A new coil planet centrifuge has been developed that: (i) is capable of analytical as well as preparative separations on any materials that partition in a two-phase solvent system; (ii) can recycle chosen peaks for increased resolution and purity; (iii) can elute constituents that are retained in the centrifuge, by changing the pumped phase; (iv) is reliable and free from vibration; (v) gives consistent and repeatable results.

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