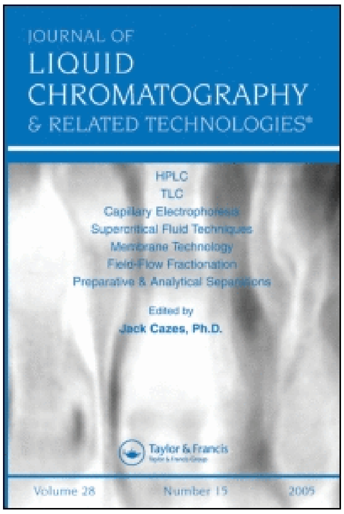

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INDUSTRIAL SCALE-UP OF COUNTERCURRENT CHROMATOGRAPHY

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FUNDAMENTAL INVESTIGATIONS AND TRENDS IN CCC

INDUSTRIAL SCALE-UP OF COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

The hydrodynamic, engineering, and chromatographic variables affecting scale-up of countercurrent chromatography (CCC) are examined. The predictable and linear scale-up from the current laboratory scale technology to industrial process scale, capable of kgm/month in the first phase, is demonstrated. Continued research will prepare the way for a new generation of tonne/annum capacity high throughput, high resolution CCC machines for pilot and plant scale separations of a range of bioprocess products.

INTRODUCTION

The inspiration for this work has arisen from one of the UK's BBSRC's focused research objectives to develop a "generation of new, robust, and usable techniques for bioprocess intensification and simplification" by providing high purity separations which are preparative, versatile, affordable, and applicable to a wide range of industrial applications.

The aim is to develop a generic high-resolution purification process that is as rapid as HPLC analysis, and yet can be linearly scaled up from laboratory to production scale. Current technology, like HPLC for example, is not a linear scale-up, uses large volumes of solvents, and products can become hydrolysed or react with the column.

CCC is a process that avoids these difficulties.[1,2] It is a form of liquid-liquid chromatography without a solid support, which separates soluble natural product substances on their partition, or differential solubility, between two immiscible solvents. The principle of separation (partition) is the same in both the laboratory and the production plant and is generic in that it can be applied to an extremely broad range of purification problems in many industries. Furthermore, because there is no solid support, there is 100% sample recovery and no need for any pre-purification.

The operational process is extremely simple. The system consists of a sample, a length of tubing, and two immiscible solvent phases. The tubing is initially filled with the solvent phase intended to be the stationary phase and the sample is injected with the mobile phase. After an appropriate period of time, fractions of the injected sample emerge from the downstream end of the tubing in the order of their partition coefficients. The tubing (usually PTFE) is wound on a drum, which is centrifugally rotated in planetary motion (Figure 1). This sets up alternating zones of mixing and settling along the length of the tube synchronous with the high and low "g" sides of the coil (Figure 2). Samples injected with the mobile phase undergo as many as 70,000 partitioning steps per hour resulting in

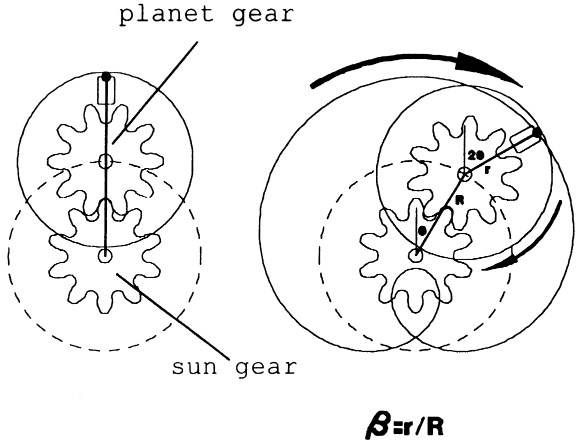

Figure 1. Planetary motion.

high-resolution separations with no sample adsorption onto solid supports. These zones of mixing and settling travel along the tube synchronously with the rotation. The mixing efficiency is excellent and the process is not limited by hydrostatic pressure.

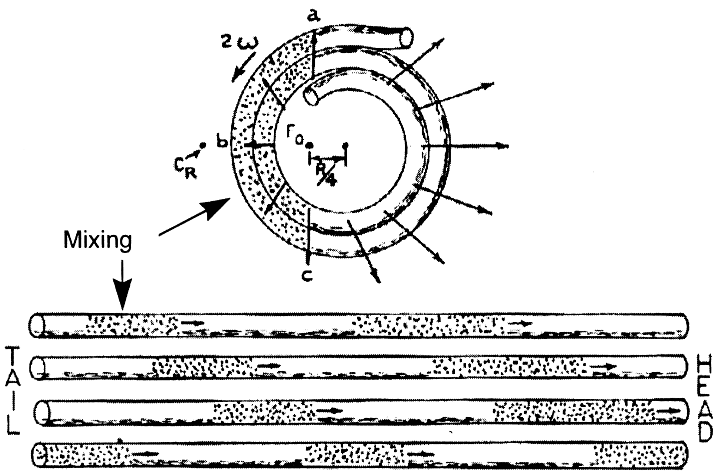

Figure 2. Variable force field and movement of mixing and settling zones toward the "Head" end of the coil, after Conway.[1]

Current Technology

A recent review on countercurrent chromatography as a preparative tool[3] described an extremely useful comparison of four different CCC approaches and concluded, "the real future belongs to the new generation of centrifugal instruments". It went on to highlight some of the key advantages of the technology:

1) it is possible to purify compounds in one step from a crude extract;
2) the method possesses considerable advantages over HPLC - high loading capacity without the need for sample clean-up procedures;
3) semi-pure mixtures and crude extracts can equally well be chromatographed without any material loss arising from irreversible adsorption on solid matrices;
4) samples with a wide range of polarities can be injected;
5) a high degree of versatility is possible with the solvents used, and
6) aqueous and non-aqueous systems are equally applicable.

They concluded that the way forward was

i) to develop improved and more reliable designs of current centrifuges;
ii) that there was a "need to accommodate higher loads on the 100g to 1kg scale," and
iii) that more work was needed to develop truly preparative instruments - "this will require a good deal of thought and a better understanding of the mechanisms of separation that actually occur in a separation coil".

Following this review Ito[4] has published his work on pH zone refining. pH zone refining offers a method of operating existing instruments preparatively when purifying ionizable compounds with 100x higher sample loadings. Sutherland et al.[5] have performed applications with the Quattro CCC machine demonstrating that preparative gram quantity separations of crude plant extracts use one tenth the volume of solvents compared to the equivalent prep-HPLC. They use a number of different applications to demonstrate the versatility of the process: 50mg to 5g capacity separations on one coil; switchable normal/reverse phase in one run; rapid method development and multi-stage extraction. Sandlin and Ito[6] have shown that CCC is feasible with tubing bore up to 5.5mm internal diameter and have successfully demonstrated fractionations in 750ml coils, but at relatively low flow, speed and β values. They have also investigated the effect on resolution of increasing sample volume and sample concentration.[7] Ito[7,8] and others[9] have described unit-gravity (non-centrifugal) slowly rotating coil devices, which would be suitable for large-scale CCC separations. Sutherland et al. have performed a preliminary review of the scale-up of CCC[10] and found it to be linear and predictable.[11]

The objective of this paper is to understand how critical scale-up variables affect the hydrodynamics and chromatographic integrity. For example:

1) how large can tubing diameter become before retention of the stationary phase is affected?
2) what are the limitations on flow rate or throughput?
3) is it advantageous to increase speed of rotation or "g" field?
4) will changing the ratio of bulk fluid inertia to wall effects change the retention characteristics?

This paper reviews the variables influencing scale-up, simplifying them to a few critical variables like speed, flow and tubing bore and then investigates how these critical variables affect the scale-up process.

THEORY

There are two major measures (or responses[12]) of prime importance in Countercurrent Chromatography (CCC) - "Retention (Sf - %) of the stationary phase", a measure of the hydrodynamic equilibrium of a given phase system in a given coil planet centrifuge and the "Resolution (Rs)" achieved chromatographically, a measure of the efficiency of the process in mixing and settling terms. There are a number of variables or factors that can influence these measures or responses and when first confronted with them they can be quite daunting.

These were partly listed recently by Goupy et al. [12] when conducting their experimental design methodology (EDM) for retention and have been defined earlier by Ito[13] and later Berthod[14]. A few more have been added to give the A to Z of CCC variables organised below as "*script alphabetic*" variables into five different control categories:

1) physical properties of the phase system,
2) operational variables controllable by the user,
3) fixed manufacturing variables
4) coil geometry, and
5) chromatographical variables - the usual symbols and units are appended in brackets

Phase System Physical Properties

A - Densities of upper & lower phases (ρ_u, ρ_l - kg/m^3)
B - Viscosity of upper & lower phases (μ_u, μ_l - Pas or Ns/m^2)
C - Interfacial tension (τ_i- mN/m)

Operational Variables

D - Speed (N - rpm, ω- rad/sec)
E - Flow (F - ml/min, Q_m - m^3/s)
F - Temperature (T - °C)
G - Acceleration field (ng)
H - Direction of rotation (head centre or periphery)
I - Direction of pumping (head to tail or tail to head)
J - Choice of pumped phase (upper or lower)

Rotor Geometry

K - Planetary radius (R - m)
L - Bobbin radius (r - m)
M - β -value range (r/R)
N - Bobbin width (w - m)

Coil Parameters

O - Coil tubing internal diameter (d - m)
P - Coil length (L - m)
Q - Helix Angle (α)
R - Spiral or axial winding
S - Left handed or right handed winding
T - Coil material
U - Number of loops or turns

Chromatographical Variables

V - Coil Volume (V_c - ml or m^3)
W- Volume of Mobile Phase (V_m- ml or m^3)
X - Partition Coefficients of Target Compounds (k_1, k_2 ….)
Y - Sample volume (V_i- ml or m^3)
Z- Sample concentration (c_i - kg/m^3)

Before attempting to review the critical variables or factors associated with scale up, it is important to put the above variables into their proper context. It is important to know which end of a coil is the “Head” and which end the “Tail” and to know that changing the direction of rotation reverses them. The “Head” is defined as the end a ball or bubble would move or wind to under Archimedean screw action. Slowly rotating the coil and holding a finger or pointer against the windings of the coil would reveal this. Research has now shown[15] that there are quite simple specific rules that can be followed for CCC to work and be optimised. These are as follows:

> For β values greater than 0.7 [Ref. 13 (page 367) and 15], the heavy (lower) phase always goes to the “tail” and, since it is a closed system, the lighter (upper) phase always goes to the Head.

The tail should be arranged to be at the periphery for the hydrostatic and Archimedean forces to be working together.[15]
Always pump the mobile phase in the direction it wants to go: heavy (lower) phase toward the "tail" and lighter (upper) phase toward the "Head"[15].

Once these rules are followed, half of the operational variables in the user's control (H, I, & J) and two of the coil parameters (R & S) disappear. Of the remaining variables A, B, & C are properties of the chosen phase system and can be fixed by running at constant temperature (F). As bearing friction can often lead to a gradual increase in operating temperature, it is best to operate above ambient temperature (say 30°C). This can be achieved by the instrument having a cooling circuit together with a temperature controlled heating system. In this way, any variation in hydrodynamic distribution due to the physical properties of the phase system changing is minimised.

The acceleration field (G) is a dependent variable. The tangential acceleration field is the same for all β values ($R\omega^2\sin\theta$) where R is the planetary radius (K), which is fixed, and ω is the angular rotation (D), which is in the control of the operator. Therefore, only two operator-controlled variables remain: speed (D) and flow (E).

The rotor geometry (K, L, M, and N) is fixed by the manufacturer and is machine specific. The operator can sometimes select the coil parameters. Different coils, of different length, volume, and even bore at differing values, are sometimes available on the same unit. Alternatively, some manufacturers tailor make coils for individual user requirements.

The major coil parameter is the tubing bore (O). Generally tubing is wound on a bobbin or drum in such a way that the helix angle (Q) is defined by the outside diameter of the tubing divided by the perimeter of one loop. The number of loops (U) that can physically be wound generally determines the length (P).

In summary, there are three major variables affecting scale-up: speed (D), flow (E) and tubing bore (O). The first two are in the control of the operator up to the limits of the performance of the equipment. The third (bore) can be controlled by the coil manufacturer. It should be noted, that the helix angle (Q) increases with tubing bore and so these two variables are not independent of one another. A fourth potential variable, tubing material (T), could affect retention and this needs to be assessed prior to the other three variables being systematically investigated.

Chromatographic Variables

Coil volume (V - Vc) can be derived from the tubing bore (O) and length of the tubing (P), but is listed as an important Chromatographical variable as, when divided by the flow, it gives the time of elution of the k=1 peak.[11] Once responses like retention (S_f) are known, then the volume of the mobile phase (W) in the coil can be calculated[11] and, hence, the time of elution of the k=0 point can be calculated by dividing the mobile phase volume by flow. The partition coefficients (X) of target compounds give well-defined elution points,[11] hence, predictions can be made. The main user controlled variables, which have been studied by Sandlin and Ito,[7] are the concentration (Z) and volume of sample added to the coil (Y).

Reynold's Number

The majority of terms mentioned so far have been described previously.[11,15,16] Reynold's number (Re), defined in equation (1) is an important parameter for process engineers as it is a non-dimensional ratio of inertia and viscous forces when liquids are flowing through pipes — giving a measure of throughput. It is assumed, here, that the retained phase is held stationary against the flow of the mobile phase and that the mobile lower phase occupies the outer segment of the coil, as shown in Figure 3. The mobile phase area will be bounded by the circular tubing wall on one side and the interface with the stationary phase on the other, forming a chord across the segment of tubing. A mean hydraulic diameter (d_m) is calculated for this shape for each retention measurement, as illustrated in Figure 3. A third degree polynomial best fit is calculated from the iterated solution, as described in equation (2) below.

Reynold's number of the mobile phase is calculated as follows:

$$Re_m = \rho_m u_m d_m/\mu_m = u_m d_m/\nu_m \qquad (1)$$

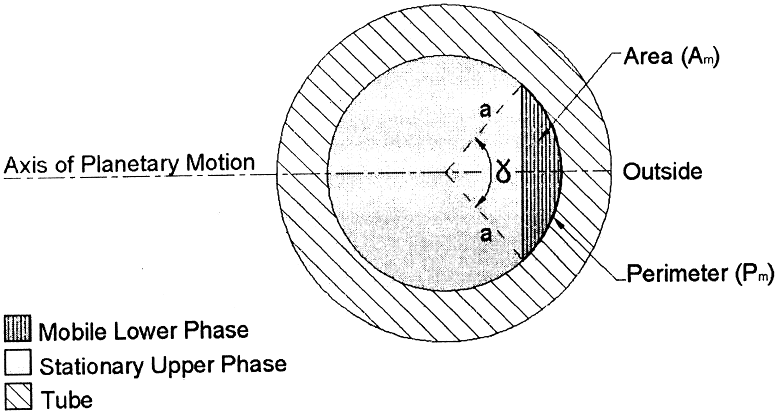

Figure 3. Cross section of tubing showing the lower mobile phase against the outer wall.

where $\nu_m = \mu_m / \rho_m$ is the dynamic viscosity and $u_m = Q_m/A_m$ is the linear velocity of the mobile phase.

The mean hydraulic diameter (d_m) is:

$$d_m = 4A_m/P_m \tag{2}$$

where A_m is the cross-sectional area of the pumped mobile phase, as distinct from the cross-sectional area of the tubing A_c, and P_m is the wetted perimeter of the mobile phase cross-section.

A_m can be calculated from the retention of the stationary phase (S_f) as follows:

$$S_f = V_s/V_c = A_s/A_c = 1- A_m/A_c \tag{3}$$

Re-arranging:

$$A_m = A_c(1-S_f) \tag{4}$$

Once A_m is known it is possible to work out the angle (γ) subtended at the centre of the tubing of radius (a) by the interface chord. From the geometry of Figure 3:

$$A_m = ½a^2[\gamma\text{-}\sin(\gamma)] \tag{5}$$

Perimeter (P_m) can also be calculated from the geometry of Figure 3:

$$P_m = a\gamma + 2a.\sin(\gamma)^2 \tag{6}$$

The mean hydraulic diameter of the mobile phase (d_m) can now be calculated from equation (2). In practice, the relationship between d_m and retention (S_f) is plotted and a 3rd order polynomial best fit used for calculating d_m for a given retention as follows:

$$d_m/d_c = 0.7126(A_m/A_c)^3\text{-}1.4915(A_m/A_c)^2 + 1.7589(A_m/A_c) + 0.0248 \tag{7}$$

where $A_m/A_c = V_m/V_c = (1\text{-}S_f)$.

EXPERIMENTAL

Experimental Apparatus

Three different sizes of CCC device were used in this study: 1) a high speed Process Scale CCC with 3.68mm bore tubing, β range of 0.63 to 0.87 and total coil volume of 928mL previously described;[11] 2) a standard Quattro Preparative

Scale CCC[5] with 1.6mm bore tubing, β range of 0.83 to 0.86 and coil volume of 95 mL; and 3) a high speed Analytical Scale CCC with a 0.76mm bore tubing, β value of 0.88 and coil volume of 25mL. All of these devices have the same planetary radius of R=110mm and can rotate at 800 rpm. The high-speed Process and high-speed Analytical units can operate faster at 1200 rpm and 1400 rpm, respectively. All three units are temperature controlled at 30°C and are operated in reverse phase mode, with the aqueous lower phase mobile pumping from Head (centre) to Tail (periphery). The methodology for performing the retention and resolution tests was previously described in detail.[11]

Phase Systems

A Heptane/Ethyl Acetate/Methanol/Water (1.4:0.1:0.5:1.0) phase system (abbreviation 4A) was chosen as a test system, with benzyl alcohol (k=0.28) and 2-phenyl-ethanol (k=0.46) as the sample mixture. This is a relatively high-density difference phase system (ρ_u=0.679, ρ_l=0.947) with a significant viscosity difference between the phases (ρ_u=0.52 and ρ_l=1.43) and a high interfacial tension (γ_i =16.9mN/m). The test conditions were described in detail.[11]

RESULTS AND DISCUSSION

The Effect of Tubing Material

Figure 4 shows a retention flow characteristic for two different tubing materials: PTFE and stainless steel. Each coil has a similar bore between 1.6 and 1.75 mm and the same coil geometry. Figure 5 shows the variation of resolution with flow for the same two tubing materials. These results show there is no significant difference between two very different materials. This suggests that retention and mixing are more a function of the hydrodynamics than a consequence of the properties of the tubing material. These tests were performed on spirally wound test coils on an experimental coil planet centrifuge[15] prior to the main variables being investigated as part of the above methodology.

The Effect of Flow

Du et al.[17] have found that there is a linear relationship between retention and the square root of flow. Figure 6 shows this relationship for the Process CCC using the Heptane:Ethyl Acetate:Methanol:Water phase system in reverse phase mode as described in the Methods section. Note how flow can be increased quite

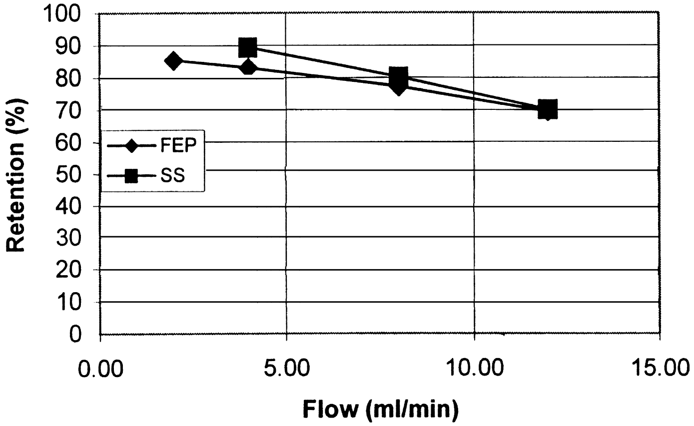

Figure 4. Variation of retention with flow for stainless steel and FEP tubing.

considerably, without too much loss of retention. It is now known,[16] there is a linear relationship between the square of the linear velocity and flow. It is possible, knowing the retention, cross sectional area, and the flow, to work out the linear velocity of the mobile phase and plot this against flow as shown in Figure 7.

It follows that, if there is a linear relationship between Retention (S_f) and the square root of flow, then there must be a linear relationship between retention

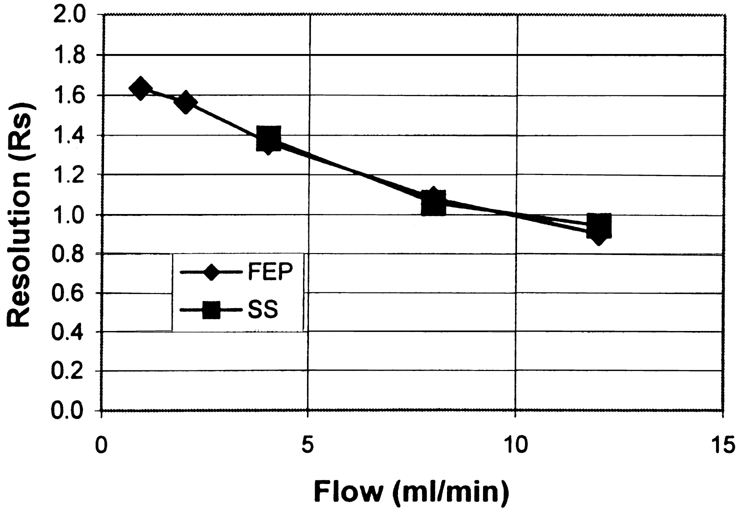

Figure 5. Variation of resolution with flow for stainless steel and FEP tubing.

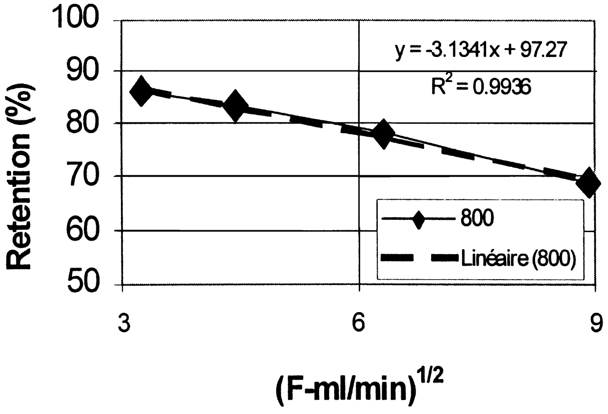

Figure 6. Variation of retention with square root of flow.

and linear velocity, provided the first parameter in the equation proposed by Du is unity.[17] This is shown in Figure 8 where linear velocity is plotted against mobile phase retention (100-S_f). Here, the ordinate gives a measure of the rate of throughput, which should be as high as possible for scale-up, and the abscissa gives the retention of mobile phase, which needs to be as small as possible to maximise peak separation. The higher this slope the more efficient the process in terms of throughput. Process engineers, however, prefer to use non-dimensional terms like Reynold's number in preference to linear velocity. Reynold's number

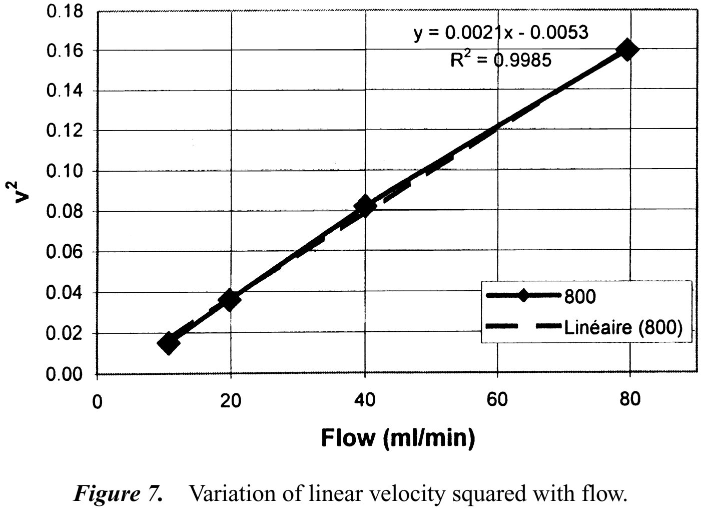

Figure 7. Variation of linear velocity squared with flow.

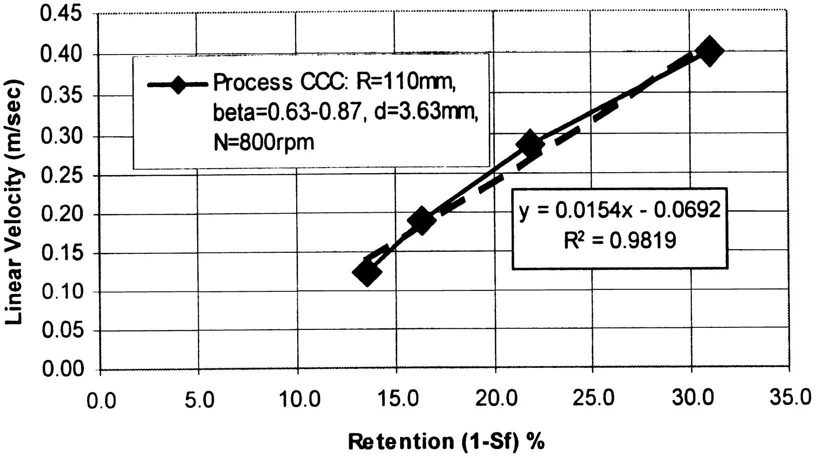

Figure 8. Variation of linear flow with retention of mobile phase.

can be approximated by assuming a mean hydraulic diameter for the mobile phase as outlined in equation (2). Reynold's number (Re) is plotted against Retention (S_f) in Figure 9.

The effect of flow on resolution is shown in Figure 10 for the Process CCC running at 1200rpm. Resolution reduces with increasing flow as would be expected, as the sample will have experienced fewer mixing and settling steps before it eluted. However, the increased flow appears to improve mixing as it can be seen that this reduction is only gradual. Doubling flow does not halve resolution and so it would appear advantageous to increase flow as much as possible when scaling up the process.

Effect of Speed

The effect on the linear velocity of the mobile phase of increasing the speed of rotation from 800 to 1200 rpm, is shown in Figure 9. Increasing speed increases the "g" field, leading to better stationary phase retention and the ability to maintain higher flows or linear velocities for a given retention. The slope of the Reynolds Number/Retention curve gives a non-dimensional measure of the Retention efficiency of a given device. The steeper the slope, the higher the throughput for a given change in percentage retention. Note, that the gradient of the Reynold's No/Retention curve increases in proportion to the increase in speed.

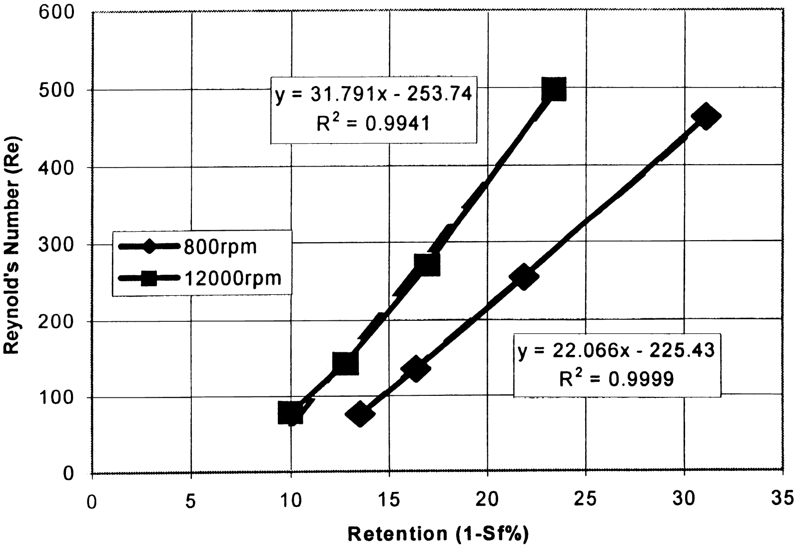

Figure 9. Variation of Reynolds Number with retention of mobile phase for different speeds.

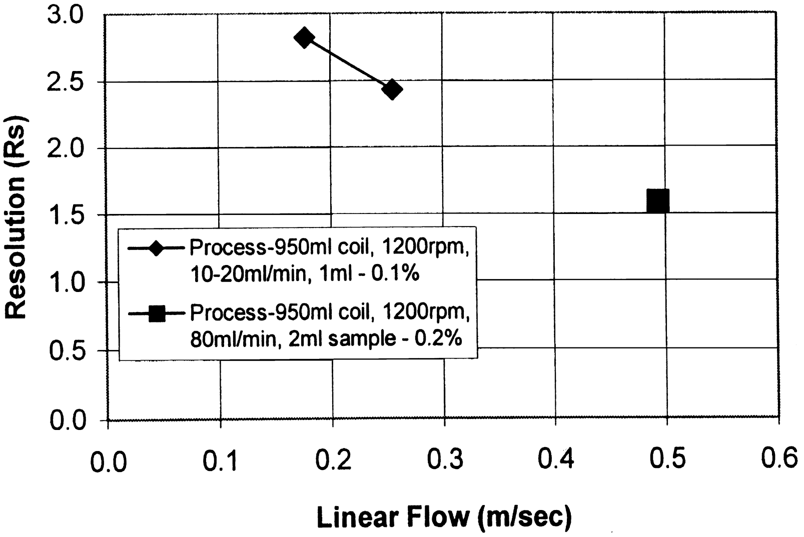

Figure 10. Variation of resolution with flow.

The effect of rotational speed on Resolution for two different sample volumes at a constant flow rate of 20 mL/min is given in Figure 11. It was expected that resolution would increase linearly, since there would be a proportional increase in the number of mixing and settling cycles. Similarly, an increase in retention leads to further increases in resolution. However, resolution remained approximately constant suggesting there could be a trade off between increased mixing due to the higher frequency of mixing and settling cycles against a damping effect due the increased "g" field.

Effect of Bore

The retention flow characteristics for the three units tested, the Process, Quattro, and Analytical, are given in Figure 12 and expressed as a Reynold's Number/Retention plot in Figure 13. Figure 12 dramatically illustrates how flow can be increased by increasing tubing bore size. All instruments are working in reverse phase (lower aqueous phase mobile) and give a clear linear relationship between Reynold's No. (Re) and mobile phase retention ($100\text{-}S_f$). As mentioned above, the slope of the Reynold's No/Retention curve gives a non-dimensional measure of the throughput that can be expected from a given change in retention. When this slope is plotted against tubing bore, there is a linear relationship between Reynold's No. (Re) for a given change in retention and the area of the tubing ($A_c=\pi d_c^2/4$):

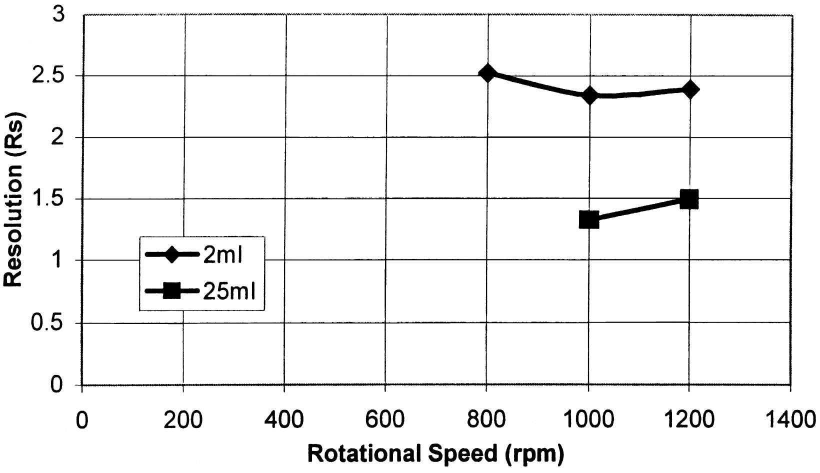

Figure 11. Variation of resolution with speed for two different sample volumes.

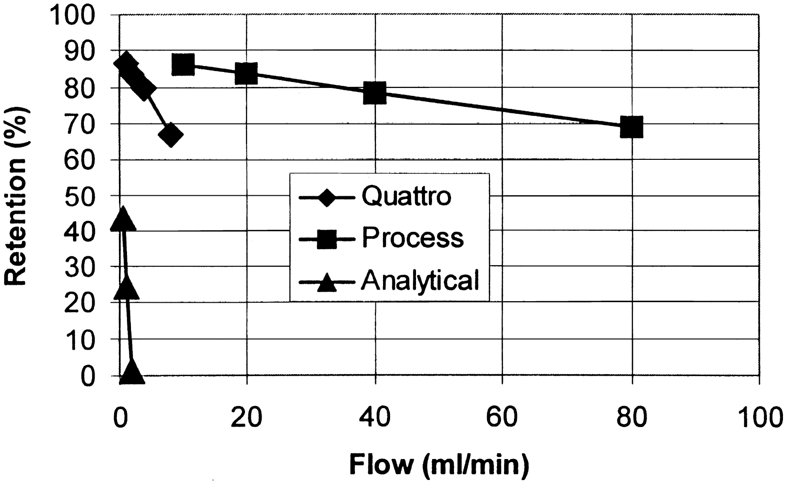

Figure 12. Variation of retention with flow for different tubing bore: an analytical CCC (0.76mm), for a Quattro (1.6mm) and a process CCC (3.68mm).

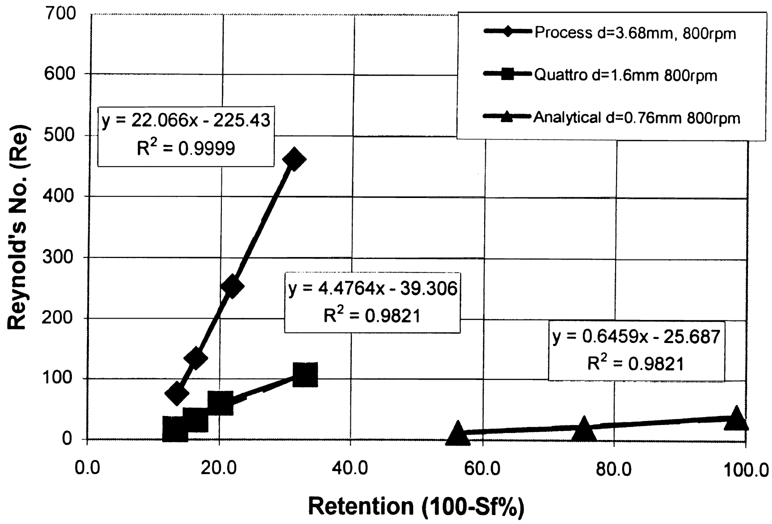

Figure 13. Variation of Reynold's Number with retention for three different bore sizes.

Slope of Re vs Sf = 1.6326 A_c $R^2=0.9993$

This indicates that further scale-up is possible, since the Reynold's No. has yet to reach a transition point.

The effect of bore on resolution has yet to be systematically studied. However, it has been noted that resolution does not reduce significantly as tubing bore increases for an equivalent linear flow, "g" field, and percentage sample size. For example, the Analytical CCC (25 mL capacity) running at 1,400 rpm with a flow rate of 1 mL/min and 0.5ml (2%) sample volume, gave a resolution of 1.7 for benzyl alcohol and 2-phenyl-ethanol in the 4A phase system. Whereas, the Process CCC (928 mL capacity) running at 1,200 rpm with a flow rate of 20 mL/min and 25 mL sample volume (2.5%), gave a resolution of 1.5 with the same sample and phase system._ It should be noted, that the Process scale result should be reduced by a factor $\sqrt{2}$ as the tubing is twice as long as the Analytical one. However, making allowances for the difference in speed and sample size, which would largely negate this reduction, it is evident that resolution can be maintained as cross-sectional area increases by a factor 20 and capacity increases by a factor of 40.

CONCLUSIONS

The critical scale-up variables affecting the hydrodynamics and chromatographical efficiency of the process have been examined for reverse phase operation with a relative high-density difference and interfacial tension phase system. It has been found, that retention increases predictably with rotational speed and tubing bore. Also, retention can be sustained using larger bore tubing for much higher linear flow rates than for the smaller bore devices. These results show there is further potential to scale-up CCC and that loss of retention is not a limitation, as the mean Reynold's No's have not yet reached the point where flow behaves unpredictably.

There was a fear that resolution could drop off significantly with scale-up as the bulk volume to surface area ratio increased with scale. However, resolution only halved as flow was increased an order of magnitude and did not decrease significantly as cross-sectional area was considerably increased. However, the increase in resolution with rotation speed was not as significant as expected.

The predictability of retention in reverse phase mode with this phase system and others[16. 17] means that it will be possible to predict the appropriate operating conditions for elution of material of a given partition coefficient.[11] Predicting how well the separation will resolve will be more difficult. More research is required on resolution with a broader range of phase systems to see if is possible to relate both retention and resolution behaviour to the physical properties of these systems, as well as the critical scale-up factors examined here.

The major outcome of this research is that the prospects for high-resolution scale-up of CCC are extremely good.

NOMENCLATURE

Symbols Used

α	Helix Angle
ß	The ratio r/R
γ	Angle subtended by interfacial chord at centre of tubing
μ_u,μ_l	Viscosity of upper phase & lower phases
θ	Angular position of rotor
ρ_u, ρ_l	Density of upper phase & lower phases
τ_i	Interfacial tension of phase system
ν	Dynamic viscosity (μ/ρ)
ω	Angular velocity of rotor
a	Internal radius of tubing
A_c	Cross-sectional area of tubing
A_m	Cross-sectional area of mobile phase in tubing
A_s	Cross-sectional area of stationary phase in tubing
c_i	Concentration of solute in the mobile phase
d_c	Internal diameter or bore of tubing
d_m	Mean hydraulic diameter of tubing ($=4A_m/P_m$)
F	Mobile phase flow rate
g	Earth's gravitational field
k	Partition coefficient
L	Total length of tubing in coil system
N	Rotational speed
r	Distance from the planetary axis to a given point on the planetary rotor (bobbin)
n	Scaling factor for "g" field
P_m	Perimeter of mobile phase cross-section
R	Distance from centre of main rotor to the planetary axis
Re	Reynold's Number ($=u_m d_m/\nu_m$)
R_s	Resolution between two peaks
S_f	Retention of stationary phase
T	Temperature of phase system
u_m	Linear velocity of mobile phase
V_c	Coil system volume

V_i	Sample volume injected
V_m	Volume of mobile phase in coil system
V_s	Volume of stationary phase in coil system
w	Bobbin width

Subscripts

C	Coil or whole system
l	Lower phase
m	Mobile phase
s	Stationary phase
u	Upper phase

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