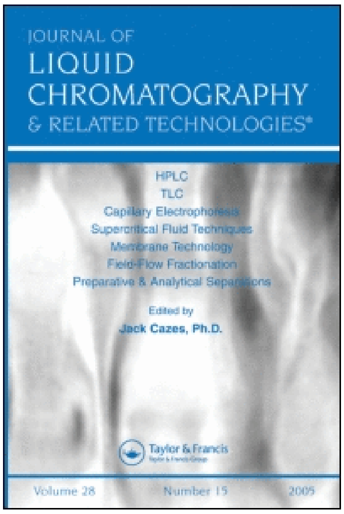

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Hydrodynamics of PEG-Phosphate Aqueous Two-Phase Systems in a J-Type Multilayer Countercurrent Chromatograph

I. Al-Marzouqi[a]; M. S. Levy[a]; G. J. Lye[a]
[a] The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, London, UK

To cite this Article Al-Marzouqi, I. , Levy, M. S. and Lye, G. J.(2005) 'Hydrodynamics of PEG-Phosphate Aqueous Two-Phase Systems in a J-Type Multilayer Countercurrent Chromatograph', Journal of Liquid Chromatography & Related Technologies, 28: 9, 1311 — 1332

To link to this Article: DOI: 10.1081/JLC-200054806

URL: http://dx.doi.org/10.1081/JLC-200054806

Journal of Liquid Chromatography & Related Technologies®, 28: 1311–1332, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1081/JLC-200054806

Hydrodynamics of PEG-Phosphate Aqueous Two-Phase Systems in a J-Type Multilayer Countercurrent Chromatograph

I. Al-Marzouqi, M. S. Levy, and G. J. Lye
The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, London, UK

Abstract: Recently, we have shown that countercurrent chromatography (CCC) is an effective method for the purification of plasmid DNA vaccines and gene therapy vectors.[1] As a basis for further work, this paper studies the hydrodynamics of various PEG-Salt aqueous-aqueous two-phase systems in a Brunel J-type countercurrent chromatograph. The degree of stationary phase retention, S_f, once a hydrodynamic equilibrium is achieved has been studied as a function of mobile phase flow rate (0.5–2.0 mL · min^{-1}), coil rotational speed (500–850 rpm), column volume (92.3 and 167.3 mL), choice of mobile phase (PEG or phosphate), and mobile phase pumping direction (Head → Tail or Tail → Head). Three different aqueous two-phase systems (ATPS) were studied, which consisted of PEG 300-K_2HPO_4, PEG 600-K_2HPO_4, and PEG 1000-K_2HPO_4, having density and viscosity ratios between 1.15–1.13 and 0.27–0.12, respectively. High S_f values in comparison to previous aqueous-aqueous studies with CCC were obtained of up to 73.7%. These high S_f values were obtained when the lower aqueous phase was pumped from Tail (periphery) → Head(centre), opposite to the direction normally recommended for most organic-aqueous systems in J-type CCC machines.[2] This is believed to be due to the high settling times and low density/high viscosity difference of aqueous-aqueous systems compared to organic-aqueous systems. Du[3] plots of the data showed the essential linear relationship between S_f and $\sqrt{F}$, provided that $S_f > 20\%$. Data obtained from Du plots for each phase system could also be used to satisfactorily

Address correspondence to Gary J. Lye, The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Plance, London WC1E 7JE, UK. E-mail: g.lye@ucl.ac.uk

predict S_f as a function of column rotational speed. This work gives an insight into the behaviour of aqueous phase systems in J-type CCC machines and is useful as a basis for process design and scale-up.

Keywords: Countercurrent chromatography, Hydrodynamics, PEG-phosphate aqueous two-phase system

INTRODUCTION

Countercurrent chromatography (CCC) is an emerging low pressure chromatographic technique, which separates molecules on the basis of different partition coefficients between two immiscible liquid phases.[4] Within the CCC column, one liquid phase (the stationary phase) is held in place by centrifugal forces created as a result of rotating a spirally wound coil i.e., the CCC column in planetary motion. A second liquid phase (the mobile phase) is then continuously pumped through the column until a hydrodynamic equilibrium is achieved. Rotation of the coil results in a variable 'g' field. This promotes mixing and separation of the two phases in the coil which facilitates solute transfer between the phases, and hence, separation of species with different partition coefficients. Due to the rotation of the coil a large number of stages can be generated in a single machine (spinning the column at 800 rpm for 1 hour will generate 4.8×10^4 mixing and settling stages[5]), thus promoting efficient separations. Organic-aqueous systems in different designs of CCC devices have been extensively studied[6,7] and the industrial scale-up of CCC has been shown to be achievable by Sutherland et al.[8] for a variety of applications. Work in our own laboratory has focused on the optimisation[9] and predictive scale-up of antibiotic fractionation using both model systems[10] and fermentation broth derived feeds.[11]

A key parameter in the operation and performance of CCC devices is the volume fraction of stationary phase retained in the coil once a hydrodynamic equilibrium is established. This is termed the stationary phase retention, S_f. Apart from the actual mechanical design of the CCC device, the two main operational variables affecting S_f for a given phase system are mobile phase flow rate and rotational speed. With regard to the hydrodynamic equilibrium of CCC, Du et al.[3] showed that there is a linear relationship between S_f and the square root of the mobile-phase flow rate, F_c, when using a range of organic-aqueous systems:

$$S_f = A - B\sqrt{F_C} \tag{1}$$

Where according to Du et al.,[3] the A parameter indicates the "difference in composition of the phases in any multiphase solvent system" and the B parameter indicates the "difference in the volume ratio of the solvent for the same composition of the solvent system". Using Equation (1), only two retention tests are required at different flow rates in order to predict S_f at any other intermediate flow rate. This linear relationship has been explained by considering the coil planet centrifuge as a constant pressure pump.[12] As a result of this, Ignatova et al.[13] were able to accurately predict the relationship between S_f and column rotational speed from the B values of Du plots[3] determined for heptane-ethyl acetate-methanol-water phase systems:

$$S_f = \mathrm{A} - \mathrm{C}/\omega_{\mathrm{N}} \tag{2}$$

Where the A parameter is the y-intercept of the initial Du plot, ω_{N} is the rotational speed of the column (rpm), $\mathrm{C} = w\ \mathrm{B}'\ \sqrt{F}$ and w is the column rotational speed (rpm) used for the initial Du plot, B' is the gradient obtained from S_f vs $\sqrt{F_C}$ plots at w and F is the mobile phase flow rate ($\mathrm{mL \cdot min^{-1}}$) used for the S_f vs ω_{N} plots.

Phase distribution in CCC has been extensively studied using organic-aqueous solvent systems by Ito[14–17] and Sutherland.[2] Ito observed the 'Head' and 'Tail' behaviour of a wide range of solvent systems in a number of horizontal coil planet centrifuge (incorporating helix and spiral columns) machines in order to predict the direction that a given mobile phase should be pumped to obtain the maximum S_f values. He hypothesised that the heavy phase moved towards the 'Tail' (periphery) for hydrophobic phase systems, to the 'Head' (centre) for hydrophilic ones, and could go either way for intermediate phase systems. Sutherland et al.[2] also studied various different phase systems and found that the lower phase prefers the 'Tail' end of the coil when the Tail is at the periphery of the coil planet centrifuge bobbin. Notable exceptions to this general rule were with hydrophilic and intermediate systems, and systems with a low density ratio (<1.15). In these cases, the lower phase was found to travel to the 'Head' end when the head is at the periphery. This was attributed to the Archimedean forces and hydrostatic forces operating in the rotating CCC coil opposing each other. The general hypothesis showed that the Archimedean screw force causes the lower phase to flow towards the 'Tail' end, and the hydrostatic forces causes the lower phase to flow towards the 'periphery'. When the 'Tail' is at the periphery, both forces will work in the same direction.

For applications involving the separation of biological polymers such as proteins and DNA, the use of organic-aqueous phase systems is not appropriate. In this case, aqueous two-phase systems (ATPS) must be used as they offer gentle, non-toxic environments[18–20] which promote preservation of biopolymer structure and function. We have recently shown, for example, that the use of ATPS with CCC enables the efficient separation of plasmid DNA from contaminant nucleic acids.[1] For ATPS, hydrodynamic studies have been previously performed in non-synchronous CPC,[21] cross-axis CPC,[22,23] horizontal CPC,[24] and eccentric multi-layer CCC[23] devices being applied to the separation of model protein mixtures. To date however, there has been no detailed study on the hydrodynamics and phase distribution of ATPS in multilayer J-type CCC devices.

This paper studies the hydrodynamics and phase distribution of three ATPS in a J-type multilayer CCC device. The ATPS comprised of 18% w/w PEG (average molecular weight 300–1000) and 18% w/w potassium-phosphate. The influence of several operational parameters on stationary phase retention was studied including mobile phase flow rate (0.5–2 $\mathrm{mL \cdot min^{-1}}$), column volume (92.3 mL or 167.2 mL), rotational speed (600 or 800 rpm), choice of mobile phase (upper or lower), and pumping orientation (Head → Tail, or Tail → Head). The results are presented as Du plots[3] and show that S_f values up to 73.7% can be achieved, but with different phase distribution behaviour than is generally seen with organic-aqueous systems. In addition, it is shown that the gradient and intercept of the Du plots[3] could be used to satisfactorily predict S_f at different rotational speeds.

EXPERIMENTAL

Experimental Apparatus

The CCC machine used was a Brunel Labprep, "J" type design (Dynamic Extraction Ltd, Uxbridge, UK). Full details of the design and operation can be found elsewhere.[9] The column used was made from 3.2 mm o.d., 1.6 mm i.d. PTFE tubing spirally wound onto the bobbin from the centre to the periphery in an anti-clockwise manner. The columns used had a total volume of 92.3 mL or 167.2 mL and β values (radial distance between the centre of the coil divided by the radial distance between the center of the sun gear and the planet gear) of between 0.83–0.86.[5] The rotor and coils were enclosed in temperature controlled housing, maintained at 25°C ± 0.5°C.

The CCC machine was operated with the 'Head' end of the coil at the centre and the 'Tail' end at the periphery.

Phase System Formation and Properties

Polyethylene glycol (PEG) of average molecular weight 300, 600, or 1000 and anhydrous K_2HPO_4 were obtained from Sigma (Poole, Dorset, UK) and were of the highest purity available. In all cases, the biphasic aqueous-aqueous systems were prepared by adding the components in the following order: R.O. water, salt, and PEG. The upper (V_L) and lower phase (V_H) volumes were measured and the phase volume ratio, V_R, calculated where $V_R = V_L/V_H$. Three different phase systems ($V_R = 1$) were chosen for use in CCC experiments: (1) 18% w/w PEG 1000-18% w/w K_2HPO_4;

(2) 18% w/w PEG 600- 18% w/w K_2HPO_4; (3) 18% w/w PEG 300-18% w/w K_2HPO_4. A number of phase systems were also prepared with different phase volume ratios using different compositions of PEG and phosphate: (4) 10% w/w PEG 300-25% w/w K_2HPO_4 ($V_R = 0.32$); (5) 30% w/w PEG 300:15% w/w K_2HPO_4 ($V_R = 2.14$); (6) 10% w/w PEG 600-25% w/w K_2HPO_4 ($V_R = 0.33$); (7) 30% w/w PEG 600:15% w/w K_2HPO_4 ($V_R = 1.71$).

Table 1 shows the physical properties of the phase systems used. Densities of PEG and salt phases were determined gravimetrically. The viscosity of each phase was measured using a DV-II viscometer (Brookfield, Stoughton, USA). All physical property measurements were made in triplicate at room temperature (22 ± 0.5°C). The maximum coefficient of variance in the determination of density and viscosity were 0.6% and 3.8%, respectively. The settling time, which is the time required for the biphasic mixture to be completely separated into two clear layers in a unit gravitation field was determined for each sample. This is done by inverting a 40 mL cylinder containing

Table 1. Physical properties of the phase systems used in this work. All measurements performed at 22°C. Interfacial tension taken from [19]

Phase and system physical properties	PEG 300 potassium phosphate[a]	PEG 600 potassium phosphate[a]	PEG 1000 potassium phosphate[a]
Density Upper phase (ρ_1—$kg \cdot m^{-3}$)	1000	1020	1030
Density Lower phase (ρ_2—$kg \cdot m^{-3}$)	1150	1160	1160
Density difference (ρ_2—ρ_1, $kg \cdot m^{-3}$)	150	140	130
Density ratio (ρ_2/ρ_1)	1.15	1.14	1.13
Viscosity Upper phase (η_1—$mPa \cdot s$)	5.23	10.73	18.3
Viscosity Lower phase (η_2—$mPa \cdot s$)	1.41	2.52	2.14
Viscosity difference (η_2—η_1, $mPa \cdot s$)	3.82	8.21	16.16
Viscosity ratio (η_2/η_1)	0.27	0.24	0.12
Kinematic viscosity ratio ($\eta_2\rho_1/\rho_2\eta_1$)	0.24	0.21	0.11
Interfacial tension ($mN \cdot m^{-1}$)	<1[19]		
Settling times (t_s—s)	90	70	51

[a] 18% w/w, each component.

the biphasic system five times and placing it on a flat table in an upright position. The volumes of upper and lower phases were both 8 mL ($V_R = 1$). The time required for the two phases to completely settle was then measured.

Experimental Method for Obtaining S_f vs $\sqrt{F_C}$ Plot

The selected stationary phase was initially pumped into the column. Once completely filled with stationary phase, rotation of the bobbins was started in the 'reverse' (anti-clockwise) direction. When a rotational speed of 600 or 800 rpm had been reached the mobile phase was pumped isocratically through the column at flow rates from 0.5-2 $\mathrm{mL.min^{-1}}$. Eluted stationary phase was collected in a graduated measuring cylinder and used to calculate the proportion, by volume, of stationary phase retained (S_f) once hydrodynamic equilibration of the phases had been achieved (typically after 140 and 240 minutes for the 92.3 mL and 167.2 mL columns respectively at 0.5 $\mathrm{mL \cdot min^{-1}}$). The subsequent calculation of S_f took into account the volume of the inlet and outlet leads as described elsewhere.[11] After each test, the volume of stationary phase retained in the column was pumped out with oxygen-free nitrogen gas (B.O.C., Surrey, UK) and added to the volume of stationary phase eluted to give an estimate of the total column volume. All results were accurate within ± 2 mL of the total column volume. Certain experiments were performed in triplicate and the maximum coefficient of variance for the calculation of S_f was 3%.

Experimental Method for Obtaining S_f vs ω_N Plots

The procedure for obtaining plots of S_f vs ω_N is described by Ignatova.[13] The method is similar to the one above except that the mobile phase flow rate is fixed and the rotational speed is initially set to a maximum value of 850 rpm. When hydrodynamic equilibrium is reached (typically after 140 minutes), the speed is reduced in a step-wise manner until a new equilibrium is achieved.

RESULTS AND DISCUSSION

Measurement and Optimisation of S_f

The degree of stationary phase retention is a critical parameter in the operation of CCC devices. High S_f values are generally considered to be necessary for efficient chromatography with values up to 95% being achievable[4] in J-type machines using organic-aqueous two-phase systems. Here, in order to obtain an understanding of the behaviour of ATPS in J-type machines, a wide range of operational variables have been examined using three different PEG-phosphate aqueous-aqueous phase systems. The results are presented in Figs. 1–5.

The highest S_f value determined was 73.7% and in all cases the measured S_f values decreased with increasing mobile phase flow rate as expected. In contrast to results with most organic-aqueous phase systems, however, markedly different phase behaviour was observed. Sutherland's[2] recommendation on mobile phase pumping direction, is that the lower salt phase should be pumped from Head → Tail (when the head is at the centre and tail at periphery) or that the upper PEG phase pumped from Tail → Head direction. In this way the Archimedean and hydrostatic forces should work

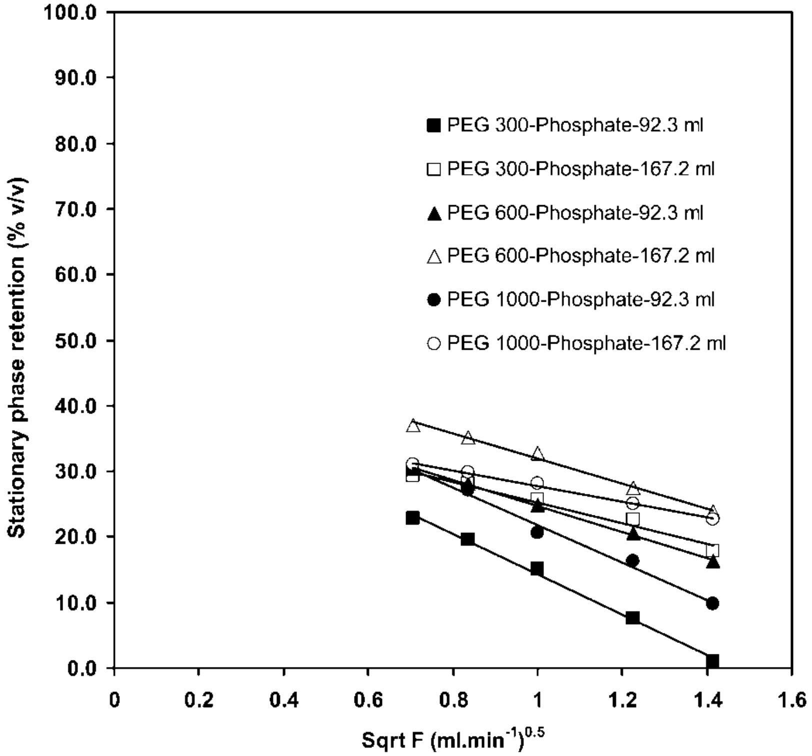

Figure 1. Measured stationary phase retention for various PEG molecular weights against square root of mobile phase flow rate (0.5–2.0 mL·min^{-1}) using column volumes of 92.3 mL (closed symbols) and 167.2 mL (open symbols). Rotational speed: 600 rpm. Mobile phase: K_2HPO_4 (18% w/w). Stationary phase: PEG (18% w/w). Direction: HEAD → TAIL. Phase systems prepared and experiments performed as described in Experimental Method for Obtaining S_f vs $\sqrt{F_C}$ Plot section. The solid lines are the best fit linear regression.

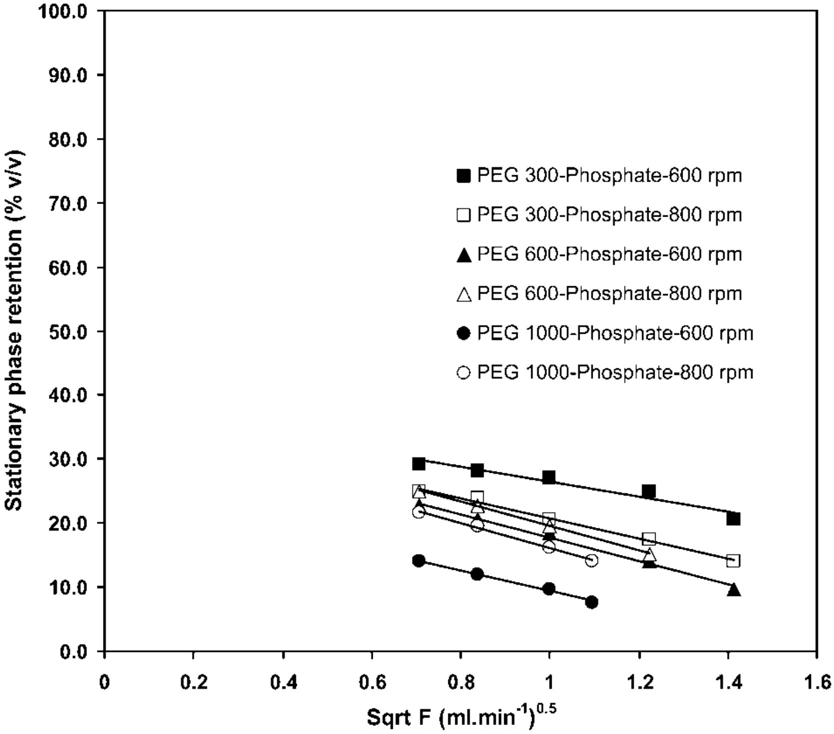

Figure 2. Measured stationary phase retention for various PEG molecular weights against square root of mobile phase flow rate (0.5–2.0 mL · min^{-1}) using a column volume of 92.3 mL and rotational speeds of 600 rpm (closed symbols) and 800 rpm (open symbols). Mobile phase: PEG (18% w/w). Stationary phase: K_2HPO_4 (18% w/w). Direction: HEAD → TAIL. Phase systems prepared and experiments performed as described in Experimental Method for Obtaining S_f vs $\sqrt{F_C}$ Plot section. The solid lines are the best fit linear regression.

together, thus giving the highest stationary phase retention. For the aqueous-aqueous systems used here the highest S_f values were generally obtained when pumping in the opposite direction. This is discussed further in the following sections.

Choice of Phase Systems and Mobile Phase

The degree of stationary phase retention as a function of mobile phase flow rate was studied for the three ATPS described in Table 1. For the PEG 300 system, a higher S_f was achieved when the upper PEG phase was pumped from Head (centre) → Tail (periphery) at a rotational speed of 600 rpm than when the lower salt phase was pumped in the same direction (compare

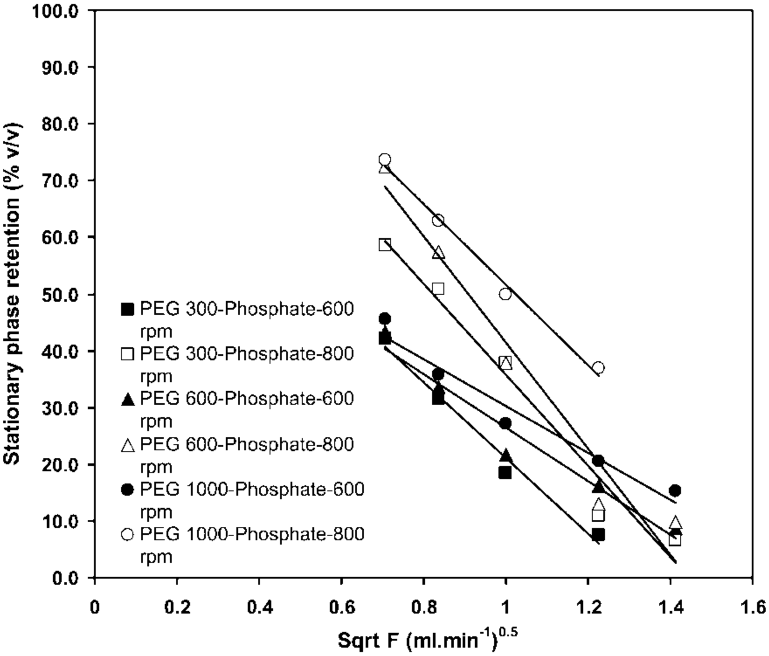

Figure 3. Measured stationary phase retention for various PEG molecular weights against square root of mobile phase flow rate (0.5–2.0 mL · min^{-1}) using column volumes of 92.3 mL and rotational speeds of 600 rpm (closed symbols) and 800 rpm (open symbols). Mobile phase: K_2HPO_4 (18% w/w). Stationary phase: PEG (18% w/w). Direction: TAIL → HEAD. Phase systems prepared and experiments performed as described in Experimental Method for Obtaining S_f vs $\sqrt{F_C}$ Plot section. The solid lines are the best fit linear regression.

Figs. 1 and 2). This is the opposite behaviour generally seen with organic-aqueous systems.[2] Very low density, high viscosity, and low interfacial tension differences exist between the phases as indicated in Table 1. These result in longer phase distribution settling times (90 seconds) compared to organic-aqueous systems where settling times are usually less than 30 seconds.[25] The results for the PEG 300 system show similarity to Ito's[14,16] hydrophilic systems (butanol-water) having a relatively high viscosity and setting time greater than 30 seconds. The results also show similarity to previous research on hydrophilic and intermediate organic-aqueous systems[14,16] and Heptane/Ethyl Acetate/Methanol/Water phase systems with density ratios of less than 1.15.[2] In general, PEG 300 is preferably used as the mobile phase since it results in higher S_f values at higher flow rates and any rotational speed compared to the use of a mobile salt phase (compare Figs. 2 and 3). This is only true for PEG 300.

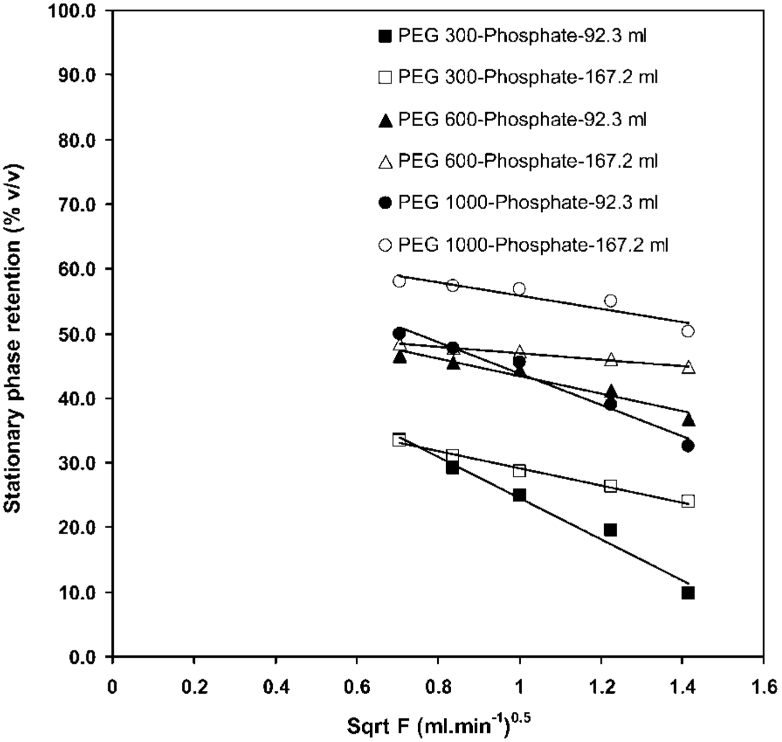

Figure 4. Measured stationary phase retention for various PEG molecular weights against square root of mobile phase flow rate (0.5–2.0 mL · min^{-1}) using column volumes of 92.3 mL (closed symbols) and 167.2 mL (open symbols). Rotational speed: 800 rpm. Mobile phase: K_2HPO_4 (18% w/w). Stationary phase: PEG (18% w/w). Direction: HEAD → TAIL. Phase systems prepared and experiments performed as described in Experimental Method for Obtaining S_f vs $\sqrt{F_C}$ Plot section. The solid lines are the best fit linear regression.

For the PEG 600 and PEG 1000 systems, using the upper PEG phase as the mobile phase results in lower S_f values, compared to a mobile salt phase due to the high viscosities of the PEG phases. This makes the use of the higher molecular weight PEG phases as a mobile phase undesirable (compare Figs. 1 and 2) and suggests that when operating the J-type CCC devices using either PEG 600 or 1000 systems, it is desirable to use the lower salt phase as the mobile phase.

Effect of Mobile Phase Pumping Orientation

The effects of pumping direction of the mobile phase on S_f were examined. For PEG 300, pumping the upper PEG phase from Tail → Head resulted in no

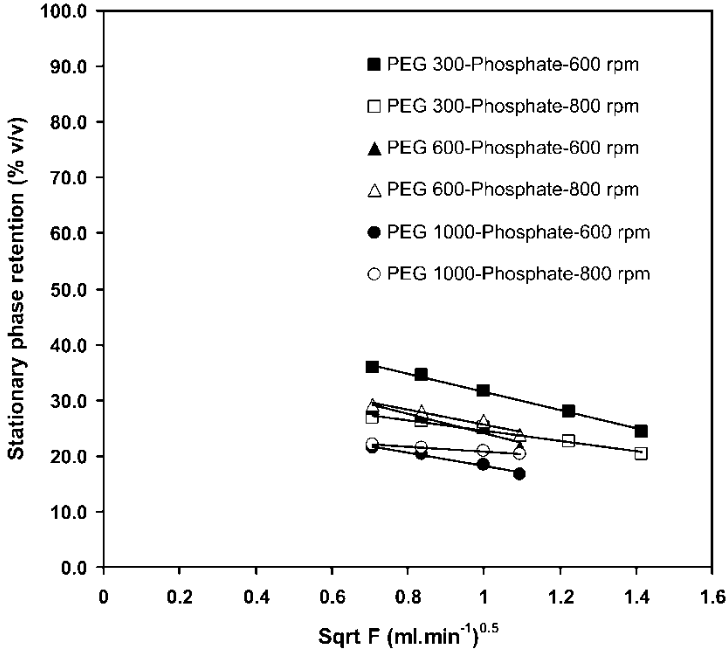

Figure 5. Measured stationary phase retention for various PEG molecular weights against square root of mobile phase flow rate (0.5–2.0 mL · min^{-1}) using column volumes of 167.2 mL and rotational speeds of 600 rpm (closed symbols) and 800 rpm (open symbols). Mobile phase: PEG (18% w/w). Stationary phase: K_2HPO_4 (18% w/w). Direction: HEAD → TAIL. Phase systems prepared and experiments performed as described in Experimental Method for Obtaining S_f vs $\sqrt{F_C}$ Plot section. The solid lines are the best fit linear regression.

retention (results not shown), which again suggests reversed phase distribution behaviour compared to most organic-aqueous phase systems. In addition, pumping the lower salt phase from Tail → Head greatly improves retention for low flow rates ($S_f = 42.3\%$ for 0.5 mL · min^{-1}, Fig. 3). For PEG 300 systems at lower rotational speeds (600 rpm), pumping the PEG phase as the mobile phase from the Head → Tail direction (Fig. 2) would be the best system to use since pumping the salt phase from the Tail → Head direction results in a rapid decrease in S_f values with an increase in flow rate (Fig. 3). These findings are in agreement with those for PEG 1000-phosphate systems used in single-spiral disks.[26]

Results for PEG 600 and PEG 1000 systems (Fig. 3) show that the heavy salt phase prefers moving towards the Head (centre) end of the coil. Surprisingly, with these systems very high retentions were achieved, depending on the pumping orientation of the mobile phase, which are comparable to those for organic-aqueous systems.[10] In the Choice of Phase

Systems Section, it was recommended to use the salt phase as the mobile phase. Therefore, it can be seen that to achieve maximum retention, the lower mobile phase should be pumped in a Tail → Head orientation (Fig. 3). Previous research using single-spiral disk J-type devices also yielded the best retention with this pumping orientation.[26]

Finally, the improvement in retention obtained when pumping the heavy salt phase towards the Head end rather than the Tail end for larger molecular weight PEG systems (600 and 1000) is not as great as for PEG 300 systems. It seems that systems with higher settling times and lower viscosity differences such as PEG 300 systems will have a direct effect on retention, making the choice of whether to pump the mobile lower phase towards the Head or Tail of greater importance.

Effect of Rotational Speed and Column Volume

The effects of rotational speed on S_f were studied for the three ATPS. An increase in rotational speed from 600 to 800 rpm generally increases S_f, when the upper phase is chosen as the mobile phase due to an increase in 'g' field. When PEG 300 was used as the mobile phase however (Figs. 2 and 5), S_f values were seen to decrease with increasing rotational speeds which could be a result of emulsification. For all other cases (compare Figs. 1 and 4), an increase in rotational speed increased stationary phase retention, with the highest increases obtained when the lower salt phase was pumped in the Tail → Head direction (Fig. 3).

The effect of increasing column volume on S_f was also tested and shown to result in an increase in stationary phase retention (compare Figs. 1 and 5) regardless of the choice of mobile phase or pumping direction. An improvement in retention with column volume using longer tube length while keeping the bore size constant using ATPS, was previously seen with different CCC devices.[27] Increasing flow rate when both higher rotational speeds (800 rpm) and column volumes (167.2 mL) were used (compare open symbols in Fig. 1 and Fig. 4) did not significantly decrease S_f values to such a great extent in all phase systems. This might indicate that there is a lower dependence of S_f on the phase physico-chemical properties. Higher rotational speeds were previously seen to be less dependant on phase system parameters for organic-aqueous systems.[28]

Du Plots Analysis

The relationship between S_f and F_C proposed by Du et al.[3] has previously been successfully applied to organic-aqueous systems but has not been extensively tested on aqueous-aqueous phase systems. The regression analysis between $\sqrt{F_C}$ and S_f for the data presented in Figs. 1–5 is summarised in Table 2.

Table 2. Regression analysis between $\sqrt{F_C}$ and S_f of PEG-phosphate systems.[a]

System	Mobile phase	Volume (mL)	Speed (rpm)	Direction	Equation of linear regression	Coefficient (R^2)
PEG 300 potassium phosphate[b]						
	Lower	92.3	600	H → T	$S_f = 45.18 - 30.84\sqrt{F_C}$	0.996
	Lower	92.3	800	H → T	$S_f = 56.93 - 32.55\sqrt{F_C}$	0.989
	Upper	92.3	600	H → T	$S_f = 38.08 - 12.07\sqrt{F_C}$	0.988
	Upper	92.3	800	H → T	$S_f = 36.41 - 15.69\sqrt{F_C}$	0.994
	Lower	167.2	600	H → T	$S_f = 41.01 - 15.74\sqrt{F_C}$	0.973
	Lower	167.2	800	H → T	$S_f = 42.28 - 13.15\sqrt{F_C}$	0.999
	Upper	167.2	600	H → T	$S_f = 47.86 - 16.36\sqrt{F_C}$	0.994
	Upper	167.2	800	H → T	$S_f = 33.98 - 9.42\sqrt{F_C}$	0.977
	Lower	92.3	600	T → H	$S_f = 88.12 - 67.09\sqrt{F_C}$	0.982
	Lower	92.3	800	T → H	$S_f = 116.22 - 80.35\sqrt{F_C}$	0.967
PEG 600 potassium phosphate[b]						
	Lower	92.3	600	H → T	$S_f = 44.68 - 19.90\sqrt{F_C}$	0.998
	Lower	92.3	800	H → T	$S_f = 56.92 - 13.94\sqrt{F_C}$	0.991
	Upper	92.3	600	H → T	$S_f = 35.93 - 18.15\sqrt{F_C}$	0.991
	Upper	92.3	800	H → T	$S_f = 38.47 - 18.99\sqrt{F_C}$	0.999
	Lower	167.2	600	H → T	$S_f = 51.07 - 19.09\sqrt{F_C}$	0.991
	Lower	167.2	800	H → T	$S_f = 52.02 - 5.01\sqrt{F_C}$	0.991
	Upper	167.2	600	H → T	$S_f = 41.10 - 16.98\sqrt{F_C}$	0.969
	Upper	167.2	800	H → T	$S_f = 39.33 - 13.88\sqrt{F_C}$	0.983
	Lower	92.3	600	T → H	$S_f = 73.58 - 47.12\sqrt{F_C}$	0.953

(*continued*)

Table 2. Continued

System	Mobile phase	Volume (mL)	Speed (rpm)	Direction	Equation of linear regression	Coefficient (R^2)
	Lower	92.3	800	T → H	$S_f = 135.03 - 93.48\sqrt{F_C}$	0.956
PEG 1000 potassium phosphate[b]						
	Lower	92.3	600	H → T	$S_f = 53.27 - 30.91\sqrt{F_C}$	0.984
	Lower	92.3	800	H → T	$S_f = 67.84 - 24.26\sqrt{F_C}$	0.983
	Upper	92.3	600	H → T	$S_f = 35.64 - 16.16\sqrt{F_C}$	0.991
	Upper	92.3	800	H → T	$S_f = 25.54 - 19.52\sqrt{F_C}$	0.997
	Lower	167.2	600	H → T	$S_f = 39.79 - 12.02\sqrt{F_C}$	0.996
	Lower	167.2	800	H → T	$S_f = 65.49 - 10.04\sqrt{F_C}$	0.974
	Upper	167.2	600	H → T	$S_f = 30.17 - 12.03\sqrt{F_C}$	0.979
	Upper	167.2	800	H → T	$S_f = 25.23 - 4.46\sqrt{F_C}$	0.991
	Lower	92.3	600	T → H	$S_f = 71.58 - 41.25\sqrt{F_C}$	0.956
	Lower	92.3	800	T → H	$S_f = 128.76 - 78.03\sqrt{F_C}$	0.991

[a] Values derived from Figs. 1–5; H = Head; T = Tail.

[b] 18% w/w, each component.

When the mobile phase was pumped in the Head → Tail direction, all the correlation coefficients were found to be greater than 0.96. This indicates that the linear relationship between $\sqrt{F_C}$ and S_f can be applied to PEG-phosphate systems. When the mobile phase was pumped in the Tail → Head direction, the linearity of the curves was limited to S_f values above 20% (Figs. 6 and 3). The results presented by Du[3] show that the intercept of the vertical axis (A term in Equation (1)) for most solvent systems tested is close to 100% at zero flow. However, this is not found to be the case for PEG-salt aqueous-aqueous systems (Table 2). For PEG 600 and PEG 1000 systems, pumping the lower salt phase from Head → Tail resulted in A values of approximately 50%. When the upper phase was pumped in Head → Tail direction, the A values decreased to 25–40%.

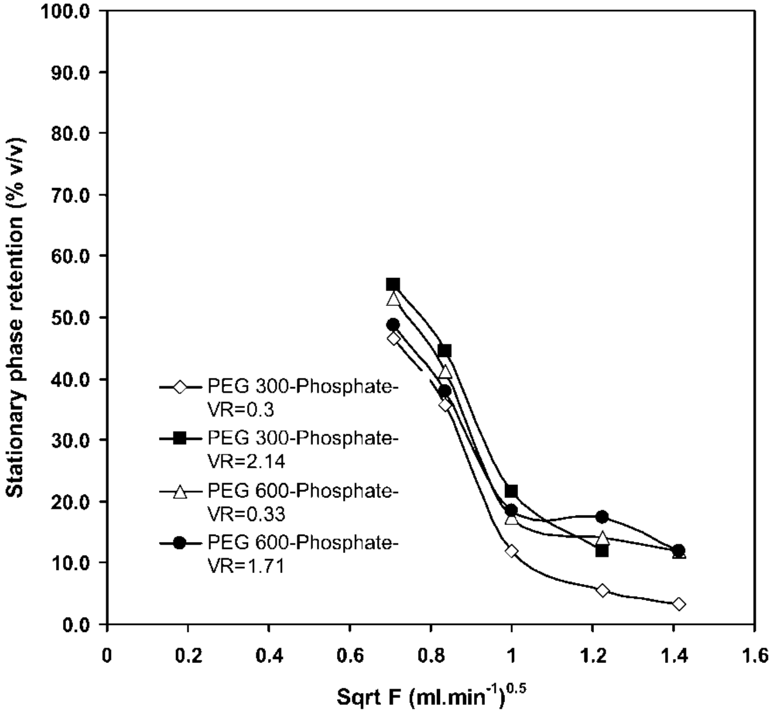

Figure 6. Measured stationary phase retention for various PEG molecular weights and phase volume ratios (V_R) against square root of mobile phase flow rate (0.5–2.0 mL · min^{-1}) using a column volume of 92.3 mL. Rotational speed: 600 rpm. Mobile phase: K_2HPO_4 (18% w/w). Stationary phase: PEG (18% w/w). Direction: TAIL → HEAD. V_R is calculated as described in Phase System Formation and Properties section, while phase systems prepared and experiments performed as described in Experimental Method for Obtaining S_f vs $\sqrt{F_C}$ Plot section.

Interestingly, when the heavy lower phase is pumped in the Tail → Head direction, the values of A and B increased greatly, with the A value much closer to 100%. Du et al.[3] state that A indicates the difference in the solvent composition. From our results, it can be seen that the A term is instead affected by all parameters tested, and most significantly, by the mobile phase pumping orientation. A possible explanation for this could be due to the different physical properties of ATPS compared to the solvent systems used previously. At higher rotational speeds (800 rpm), the A values become greater than 100 when the mobile phase is pumped from Tail → Head which is similar to Du's results for ethyl acetate-water (10:10) where the A coefficient is 132.59.[3] This system has a small density difference ($<40\,\mathrm{kg \cdot m^{-3}}$) compared to the other organic-aqueous solvent systems used and is similar to the PEG-phosphate systems used here (Table 1). For comparison, the results by other authors[23,24] using aqueous-aqueous phase systems in different CCC designs have been analysed and plotted according to Equation (1). The regression analysis for these is presented in Table 3. Again these results show $A \neq 100\%$.

The gradient of the Du relationship, the B term in Equation (1), represents the sensitivity of the stationary phase retention to the mobile phase flow rate. The steeper the gradient, the more sensitive the retention is to the mobile phase flow rate. Pumping the lower mobile phase in the Tail → Head results in much higher (negative) B values demonstrating that the mobile phase pumping orientation greatly affects S_f. Higher B values were also obtained for the smaller column volume and lower rotational speed, meaning retention is more sensitive to flow rates in these cases. Du reported that the B term may also indicate the difference in the phase volume ratio.[3] Tests using different volume ratios (Fig. 6) showed that B is indeed affected by volume ratio, however, other parameters such as mobile phase pumping direction and rotational speeds were also seen to affect the coefficient B.

Prediction of S_f at Different Rotational Speeds

Ignatova et al.[13] showed the retention-rotational speed characteristics of organic-aqueous phase systems can be predicted from the B gradient[11] of Du plots using Equation (2.) The work presented here using aqueous-aqueous systems showed this equation to be applicable. Given the results presented in Figs. 1–5, the A value of 100% was replaced by the actual value determined for the phase systems used as calculated in Table 2. The measured data and the prediction from S_f vs $\sqrt{F_C}$ plot gave a logarithmic relationship that is in close agreement regardless of the pumping orientation and phase systems. Fig. 7, for example, shows a plot of S_f vs rotational speed for the PEG 600 system at a fixed flow rate of $0.5\,\mathrm{mL \cdot min^{-1}}$. When Equation (2) was used with the $A = 100$, the theoretical prediction was

Table 3. Summary and Du plot analysis of previous studies using PEG-phosphate aqueous two-phase systems

CCC design	Phase system	β value	Speed (rpm)	Column volume (mL)	Equation of linear regression	Correlation coefficient (R^2)	Reference
Eccentric multi-layer CCC	PEG 1000 (12.5% w/w)-potassium phosphate (12.5% w/w)	0.44	600	430	$S_f = 50.36 - 16.51\sqrt{F_C}$	0.987	Lei & Hsu 1992
Horizontal CPC	PEG 3400 (10.1% w/w)-Potassium phosphate (10.9% w/w)	0.3	750	250	$S_f = 74.73 - 20.8\sqrt{F_C}$	0.987	Shibusawa et al. 1991

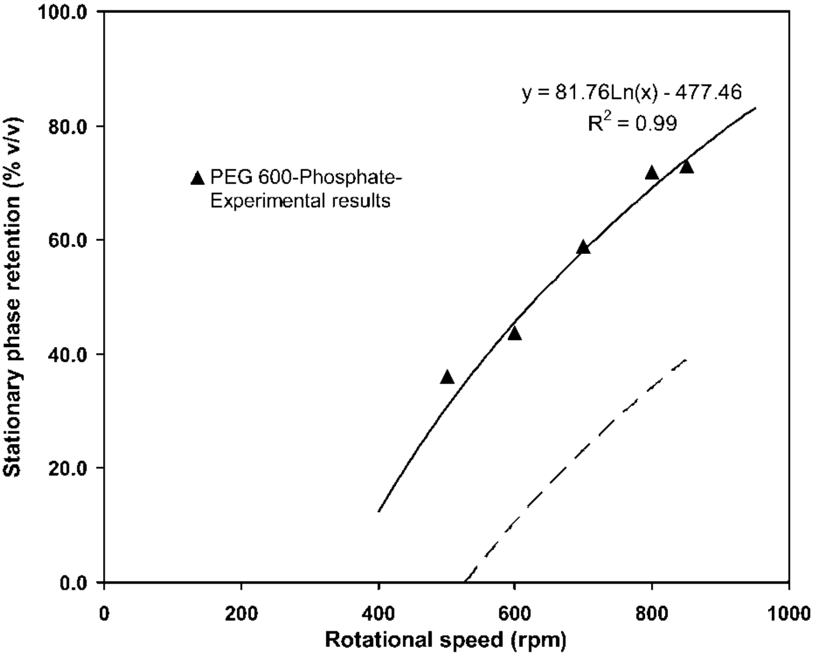

Figure 7. Measured and predicted stationary phase retention for a PEG 600 system against rotational speed (500–850 rpm) using a column volume of 92.3 mL. Mobile phase flow rate fixed at 0.5 mL · min^{-1}. Mobile phase: K_2HPO_4 (18% w/w). Stationary phase: PEG 600 (18% w/w). Direction: TAIL → HEAD. Experimental points as described in Experimental Method for Obtaining S_f vs · ω_N plots section. Solid line predicted as described in Prediction of S_f at Different Rotational Speeds section with A = 135.03 (from Table 2). Dashed line predicted with A = 100 as proposed by Ignatova et al.[13]

significantly less than found experimentally (Fig. 7). In contrast, when the A value was taken as 135.03 (as given in Table 2) very good agreement was found between experiments and predicted behaviour. Experiments using other phase systems and parameters showed similar results. This data was also used to plot (Fig. 8) the percentage mobile phase in the coil (V_m) against the reciprocal of rotational speed ($1/w_N$). This results in a linear plot, which means that retention is proportional to $1/w$ confirming Wood's hypothesis[12] that the coil planet centrifuge behaves as a constant pressure pump.

CONCLUSIONS

This work describes the first detailed study on the hydrodynamics of PEG-phosphate aqueous-aqueous two-phase systems in multilayer J-type CCC

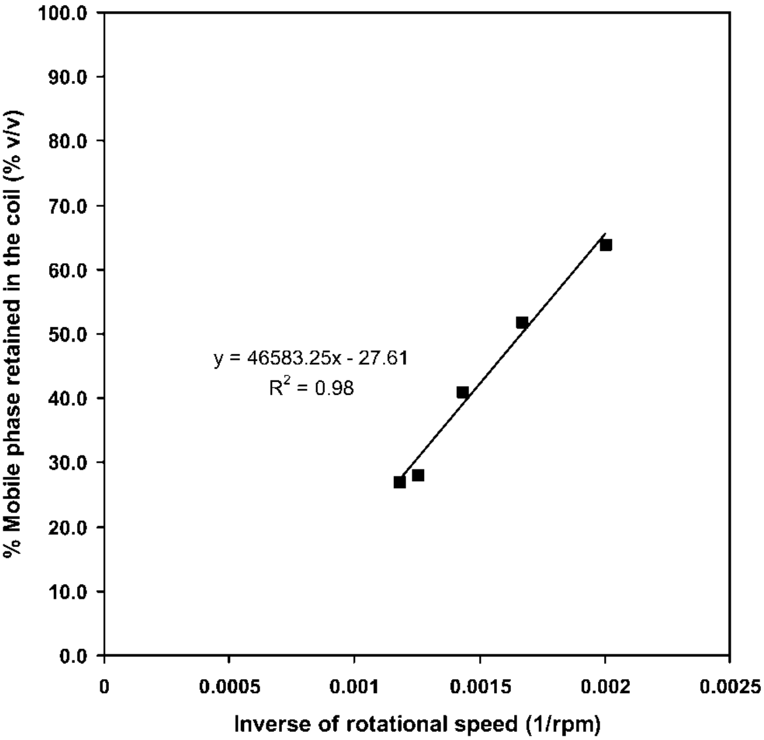

Figure 8. Plot of percentage mobile phase in the coil against the inverse of rotational speed for a mobile phase flow rate of $0.5\,\text{mL}\cdot\text{min}^{-1}$ using a column volume of 92.3 mL. Mobile phase: K_2HPO_4 (18%). Stationary phase: PEG 600 (18%). Direction: TAIL → HEAD. The solid lines are the best fit linear regression.

machines. It has been found that reasonably high S_f values of up to 73.7% can be obtained but that the phase distribution behaviour of such systems is reversed compared to most organic-aqueous phase systems, i.e., the lower salt phase generally prefers the head (centre) end of the coil. This reversal is believed to be due to the very low density and/or high viscosity difference between the phases and the longer phase settling times of aqueous-aqueous systems compared to most of the organic-aqueous systems previously studied. Du plots[3] of the data obtained showed good linearity for all PEG-potassium phosphate systems when S_f was $>20\%$. The retention-rotational speed characteristics of the three phase systems studied could also be successfully predicted from the results of the corresponding Du plots. Current work in this laboratory is examining the influence of phase system composition and CCC hydrodynamics on the efficiency of plasmid DNA separations.

ACKNOWLEDGMENTS

The support of the Engineering and Physical Sciences Research Council (EPSRC) Life Science Interface and Innovative Manufacturing Research Centre initiatives for the IMRC in Bioprocessing is gratefully acknowledged. The IMRC is part of The Advanced Centre of Biochemical Engineering, UCL with collaboration from a range of academic partners and biopharmaceutical and biotechnology companies. IAM would like to thank Gulf Pharmaceutical Industries (Julphar, UAE) and the Ministry of Higher Education & Scientific Research (UAE) for financial support. Prof. Ian Sutherland (Brunel University) and Dr Andrew Booth (UCL) are thanked for an initial reading of the original manuscript.

REFERENCES

1. Kendall, D.; Booth, A.J.; Levy, M.S.; Lye, G.J. Separation of supercoiled and open-circular plasmid DNA by liquid-liquid countercurrent chromatography. Biotech. Lett. **2001**, *23*, 613–619.
2. Sutherland, I.A.; Muytjens, M.; Prins, M.; Wood, P. A new hypothesis on phase distribution in countercurrent chromatography. J. Liq. Chromatogr. & Rel. Technol. **2000**, *23* (15), 2259–2276.
3. Du, Q.; Wu, C.; Qian, G.; Wu, P.; Ito, Y. Relationship between flow rate of the mobile phase and retention of the stationary phase in countercurrent chromatography. J Chromatogr A **1999**, *835*, 231–235.
4. Ito, Y. In *High-speed Countercurrent Chromatography*; Ito, Y., Conway, W.D., Eds.; Wiley Interscience: New York, 1996.
5. Conway, WD. *Countercurrent Chromatography: Apparatus, Theory and Applications*; ISBN 0-89573-331-5, VCH Publishers: New York, 1990.
6. Berthod, A.; Billardello, B. Test to evaluate countercurrent chromatographs: Liquid stationary phase retention and chromatographic resolution. J. Chromatogr. A **2000**, *902*, 323–335.
7. Maryutina, T.A.; Ignatova, S.N.; Sutherland, I.A. Organic phase retention in CCC: Effect of temperature, tubing material, tubing bore, mobile phase flow, and the addition of an extraction agent. J. Liq. Chromatogr. & Rel. Technol. **2003**, *26* (9/10), 1537–1550.
8. Sutherland, I.A.; Booth, A.J.; Brown, L.; Kemp, B.; Kidwell, H.; Games, D.; Guillon, G.G.; Hawes, D.; Hayes, M.; Janaway, L.; Lye, G.J.; Massey, P.; Preston, C.; Shering, P.; Shoulder, T.; Strawson, C.; Wood, P. Industrial scale-up of countercurrent chromatography. J. Liq. Chromatogr. & Rel. Technol. **2001**, *24* (11/12), 1533–1553.
9. Booth, A.J.; Lye, G.J. Optimisation of the fractionation and recovery of polyketide antibiotics by countercurrent Chromatography. J. Liq. Chromatogr. & Rel. Technol. **2001**, *24*, 1841–1861.
10. Booth, A.J.; Sutherland, I.A.; Lye, G.J. Modelling the performance of pilot-scale countercurrent chromatography: Scale-up predictions and experimental verification of erythromycin separation. Biotechnol. Bioeng. **2003**, *81* (6), 640–649.

11. Booth, A.J.; Ngiam, S-H.; Lye, G.J. Antibiotic purification from fermentation broths by countercurrent chromatography: Analysis of product purity and yield trade-offs. Bioproc. Biosystems Eng. **2004**, *27*, 51–61.
12. Wood, P.L.; Hawes, D.; Janaway, L.; Sutherland, I.A. Stationary phase retention in countercurrent chromatography: modeling the J-type centrifuge as a constant pressure drop pump. J. Liq. Chromatogr. & Rel. Technol. **2003**, *26* (9/10), 1373–1396.
13. Ignatova, S.N.; Sutherland, I.A. A fast, effective method of characterizing new phase systems in CCC. J. Liq. Chromatogr. & Rel. Technol. **2003**, *26* (9/10), 1551–1564.
14. Ito, Y. Speculation on the mechanism of unilateral hydrodynamic distribution of two immiscible solvent phases in the rotating coil. J. Liq. Chromatogr. & Rel. Technol. **1992**, *15*, 2639–2675.
15. Ito, Y. Experimental observations of the hydrodynamic behaviour of solvent systems in high speed countercurrent chromatography: I. Hydrodynamic distribution of two solvent phases in a helical column subjected to two types of synchronous planetary motion. J. Chromatogr. **1984**, *301*, 377–386.
16. Ito, Y. Experimental observations of the hydrodynamic behaviour of solvent systems in high speed countercurrent chromatography: II. Phase distribution for helical and spiral columns. J. Chromatogr. **1984**, *301*, 387–404.
17. Ito, Y.; Conway, W.D. Experimental observations of the hydrodynamic behaviour of solvent systems in high speed countercurrent chromatography: III. Effects of physical properties of the solvent systems and operating temperature on the distribution of two-phase solvent systems. J. Chromatogr. **1984**, *301*, 405–414.
18. Zaslavsky, B.Y. *Aqueous Two-phase Partitioning, Physical Chemistry and Bioanalytical Applications*; Marcel Dekker Inc: New York, 1995.
19. Albertsson, P.A. *Partition of Cell Particles and Macromolecules*, 3rd Ed.; Wiley: New York, 1986.
20. Kula, M.R.; Kroner, K.H.; Hustedt, H. Purification of enzymes by Liquid-Liquid-extraction. In *Advances in Biochemical Engineering, 24*; Fiechter, A., Ed.; Springer Verlag: Berlin, 1982; 73–118.
21. Shinomiya, K.; Kabasawa, Y.; Yanagidaira, K.; Sasaki, H.; Muto, M.; Okada, T.; Ito, Y. Protein separation by nonsynchronous coil planet centrifuge with aqueous-aqueous polymer phase systems. J. Chromatogr. A **2003**, *1005*, 103–112.
22. Shinomiya, K.; Menet, J-M.; Fales, H.M.; Ito, Y. Studies on a new cross-axis coil planet centrifuge for performing countercurrent chromatography: I. Design of the apparatus, retention of the stationary phase, and efficiency in the separation of proteins with polymer phase systems. J. Chromatogr. **1993**, *644*, 215–229.
23. Lei, X.; Hsu, J.T. Separation of proteins using aqueous two-phase systems in eccentric multi-layer coil planet centrifuge. J. Liq. Chromatogr. & Rel. Technol. **1992**, *15* (15/16), 2801–2817.
24. Shibusawa, Y.; Ito, Y. Protein separation with aqueous-aqueous polymer systems by two types of countercurrent chromatographs. J. Chromatogr. **1991**, *550*, 695–704.
25. Oka, F.; Oka, H.; Ito, Y. Systematic search for a suitable two-phase solvent system for high-speed countercurrent chromatography. J. Chromatogr. **1991**, *538*, 99–108.
26. Ito, Y.; Yang, F.; Fitze, P.E.; Sullivan, J.V. Spiral disk assembly for HSCCC: column design and basic studies on chromatographic resolution and stationary phase retention. J. Liq. Chromatogr. & Rel. Technol. **2003**, *26* (9/10), 1355–1372.

27. Shibusawa, Y.; Eriguchi, Y.; Ito, Y. Purification of lactic acid dehydrogenase from bovine heart crude extract by countercurrent chromatography. Chromatogr. B **1997**, *696*, 25–31.
28. Fedetov, P.S.; Thiébaut, D. Retention of the stationary phase in a coil planet centrifuge: effects of interfacial tension, density difference and viscosities of liquid phases. J. Liq. Chromatogr. & Rel. Technol. **1998**, *21* (1/2), 39–51.

Received December 1, 2004
Accepted December 29, 2004
Manuscript 6532