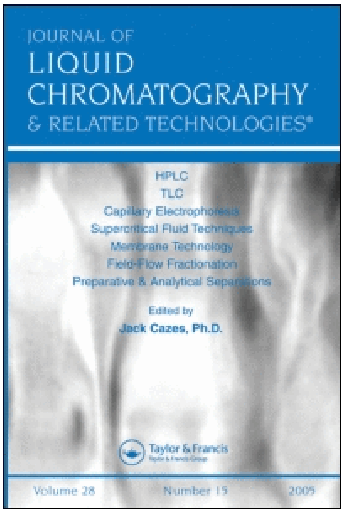

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A Fast, Effective Method of Characterizing New Phase Systems in CCC

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A Fast, Effective Method of Characterizing New Phase Systems in CCC

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ABSTRACT

This paper describes two simple tests that will characterize the hydrodynamics of a given phase system in a given CCC unit. Furthermore, it shows that, in practice, only one of these tests is necessary and the other can be predicted from the first. It goes on to show that retention information can be accurately obtained using a marker peak for the solvent front, so that both retention and resolution data can be collected in one simple test. While stationary phase retention (and, hence, peak elution) was found to behave quite predictably and could be modelled, resolution was not so predictable. Nevertheless, for analytical CCC, it was found that resolution of benzyl alcohol (BA) and *p*-cresol (PC) only reduced from 2.3 to 1.4 as flow increased 7-fold from 0.3 mL/min to 2 mL/min. Increasing the speed from 1000 to 2000 rpm (a 4× increase in "g" field) only increased the resolution a factor of 1.4×, whereas it was expected (from

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the number of mixing/settling cycles) to double. It is concluded that, for fast analytical CCC, it is worth running at as high a flow as possible (compensating for loss of resolution by increasing coil length) and increase speed only if it becomes necessary to obtain a reasonable retention.

Key Words: Countercurrent chromatography; Liquid stationary phase retention; Fundamental theory; Resolution; Flow rate.

INTRODUCTION

The two main variables affecting stationary phase retention for a given phase system are mobile phase flow rate and operation speed. For many years, Ito[1] has characterized phase systems by plotting how their stationary phase retention characteristics change with speed. More recently, Du has shown that the stationary phase retention parameter, S_f, decreased linearly with the square root of the mobile phase flow rate:[2] $S_f = A - \mathrm{B}\sqrt{F}$. Wood et al.[3] has since shown that this linear relationship can be explained by considering the coil planet centrifuge as a constant pressure pump. As a consequence of Wood's work,[4] it has been found that the Gradient B[2] is proportional to $1/\omega$ where ω is the rotational speed (in rpm). It is now possible, therefore, to construct a theoretical curve relating retention and speed once the B gradient is known.

This paper will describe two simple experiment procedures for obtaining the stationary phase retention parameter (S_f) in response to both changes in mobile phase flow and rotational speed for a heptane–ethyl acetate–methanol–water phase system (1.4 : 0.6 : 1 : 1). It will demonstrate how the retention of stationary phase plotted against rotational speed (S_f vs. ω) can be predicted from the retention of stationary phase plotted against the square root of mobile phase flow (S_f vs. $\sqrt{F}$) and how closely practice relates to theory. The paper will also show that, by using a simple test system comprising three known substances, one of which is a marker for the solvent front, it is possible to obtain both retention and resolution information as flow and speed varies in these two quick experiments—data which is essential for judging the performance of a system.

EXPERIMENTAL

Experimental Apparatus

A new Milli-CCC® apparatus,[5] with a rotor radius of $R = 50\,\mathrm{mm}$, speed range $\omega = 500-2100\,\mathrm{rpm}$ ($g = 14-247$ with $1\,\mathrm{g} = 10\,\mathrm{m\,s^{-2}}$) with temperature controlled to ±1°C from 20–30°C, was used for all the experimental work. The coil was wound from stainless steel with a $d = 0.76\,\text{mm}$ bore, coil volume $V_c = 4.6\,\text{mL}$ and inlet/outlet lead extra-coil volume of $V_{\text{ext}} = 0.3\,\text{mL}$ giving a total system volume of $V_s = 4.9\,\text{mL}$. The total coil length $L = 10.2\,\text{m}$ with 44 loops and β range of 0.68–0.79. The coil is wound from head (center) to tail (periphery) when rotating clockwise.

Phase System

A heptane–ethyl acetate–methanol–water (1.4 : 0.6 : 1.0 : 1.0) phase system (4B) was used throughout this study. This is an intermediate phase system, from the hydrophobicity point of view, with a relatively high density difference between the phases ($\rho_u = 0.708\,\text{g/cm}^3$, $\rho_1 = 0.938\,\text{g/cm}^3$), a significant viscosity difference ($\mu_u = 0.35\,\text{cP}$ and $\mu_1 = 1.35\,\text{cP}$) and a relatively low interfacial tension ($\gamma_i = 6.2\,\text{mN/m}$).

Test Analytes

Chemically pure organic solvents were applied without additional treatment. Uracil (2,4-dixydroxypyrimidine, 99%, Sigma) was used as a $K_D = 0$ marker peak. The chosen test system was benzyl alcohol (99%, Sigma) and *p*-cresol (4-methylphenol, 99%, Sigma). The model mixture of uracil (UR), benzyl alcohol (BA), and *p*-cresol (PC) were made up in mobile phase in the concentration ratio 1 : 7.5 : 2.5 (i.e., 0.5 mg/mL UR, 3.7 mg/mL BA and 1.2 mg/mL PC). The sample was injected in the column using a Rheodyne injection loop of 50 μL volume (1% of coil volume V_c).

Experimental Procedure for Obtaining a S_f Versus $\sqrt{F}$ Plot

The experimental set up is shown in Fig. 1. Solvent reservoirs are maintained at a controlled temperature with a Grant Type 81 water bath (Grant Instruments UK). The following equipment is required: a constant flow pump (Gilson model 302, Anachem, Luton), a syringe for filling the coil with stationary phase, a control valve for selecting which flow stream should pass into the coil, a nitrogen supply for pumping out the coil contents. An Antarctica Quality Cooler (Whitlenge, Halesowen, UK) was used as a cooling supply for the coil planet centrifuge thermal control system, and a measuring cylinder.

The coil system is initially filled with the phase intended to be the stationary phase (in this study the upper organic phase). The rotor is then set at the desired speed of rotation for the planned S_f vs. $\sqrt{F}$ plot (in this case 1800 rpm). The pump is set to the minimum flow (in this case 0.25 mL/min)

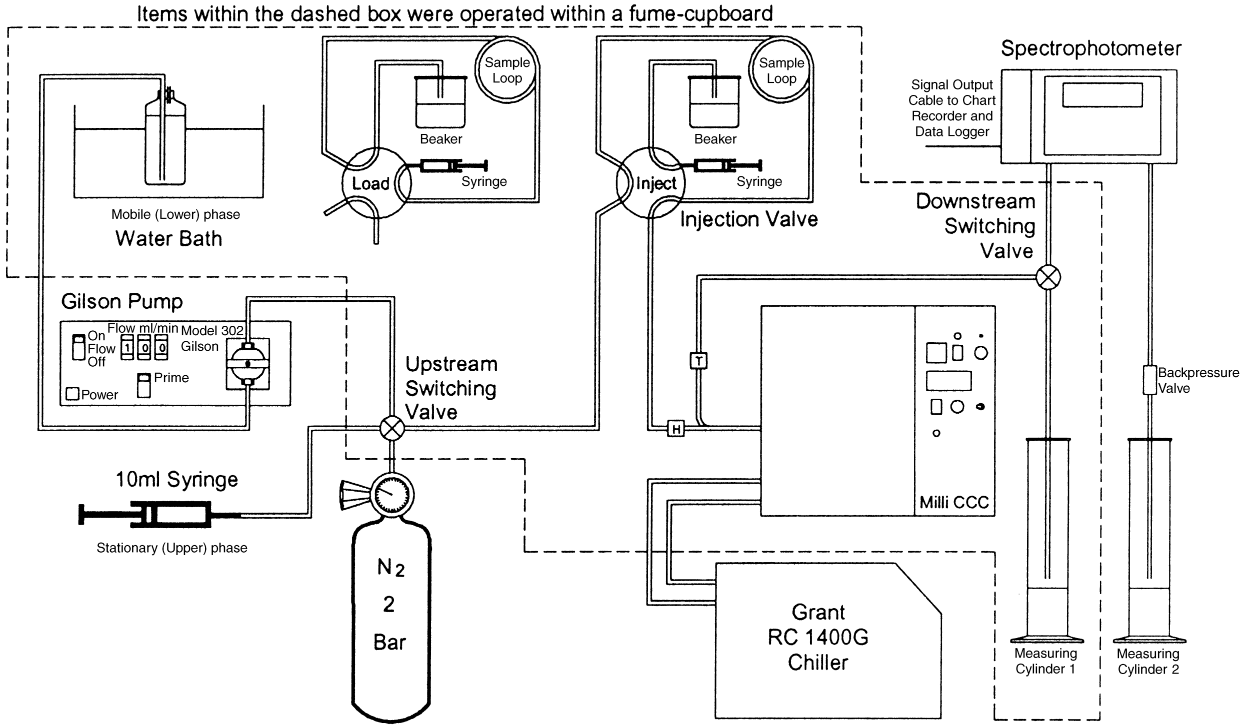

Figure 1. Schematic of experimental set-up.

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and the exit flying lead placed into a sufficiently accurate measuring cylinder. The pump is then switched on synchronously with a stop watch. The mobile phase will displace a fixed amount of stationary phase (V_{dis}) from the coil, which is equivalent to the volume of the inlet outlet leads (V_{ext}) plus the volume of mobile phase in the coil (V_m):

$$V_{\text{dis}} = V_{\text{ext}} + V_m \tag{1}$$

A check is made that equilibrium has been reach (i.e., there is no carry over of stationary phase). Then, at a noted time, the mobile phase flow is doubled. This will result in a new equilibrium being established with more stationary phase being eluted. Once equilibrium is reached the process of flow doubling can be repeated until between 50 and 100% of the stationary phase has been displaced. The recording of timed flow changes enables accurate measurements of the flow at each stage to be measured and plotted against either the displaced volume or the retention of stationary phase in the coil. Wood et al.[6] has shown that plotting V_{dis} against the square root of mobile phase flow will give an accurate measure of the extra-coil volume. This is particularly important when dealing with large bore tubing where the extra-coil volume can include more than just the input/output leads:

$$V_{\text{dis}} = V_{\text{ext}} + \mathrm{B}\sqrt{F} \tag{2}$$

The volume of the mobile phase in the coil can be calculated by subtracting the extra-coil volume from the displaced volume of stationary phase in Eq. (1). Then, the volume of stationary phase (V_s) can be calculated if the coil volume (V_c) is known accurately:

$$V_s = V_c - V_m \tag{3}$$

The percentage retention of stationary phase (S_f) then follows:

$$S_f = \frac{100 V_s}{V_c} \tag{4}$$

Once the retention of stationary phase is known it can be plotted against the square root of flow and the S_f vs. $\sqrt{F}$ plot established.

Experimental Procedure for Obtaining S_f vs. ω

The procedure is the same as for the previous section, above, except that the flow rate is fixed and the speed is set to the centrifuge's maximum value first (in this case, 2100 rpm). After reaching equilibrium, the volume of stationary phase eluted and the elapsed time are noted and the speed reduced by 300 rpm. Slowing the coil planet centrifuge will reduce the "g" field and allow more stationary phase to be displaced from the coil by the same flow rate and a new equilibrium established. This process is repeated until between 50–100% of the coil volume has been displaced.

Experimental Procedure for Measuring Retention and Resolution from a Chromatogram

The experimental set up for the resolution tests is the same as in Fig. 1, with the addition of a 50 μL Rheodyne injection loop inserted between the switching valve and the input flying lead and a flow through cell spectrophotometer placed in the outlet line. Note that the extra-coil volume increases from 0.3 mL for the retention tests using a measuring cylinder to 0.8 mL for the resolution tests, due to the volume of the extra lead to the spectrophotometer and the flow through cell. This was allowed for when calculating resolution, retention and distribution ratios.

A switching valve is used in the outlet line between flow or speed changes so that any displaced stationary phase is not passed through the spectrophotometer.

A typical chromatogram is shown in Fig. 2. Injection of the sample mixture is at $t = 0$. The UR peak elutes first, followed by the BA ($K_D = 0.33$)

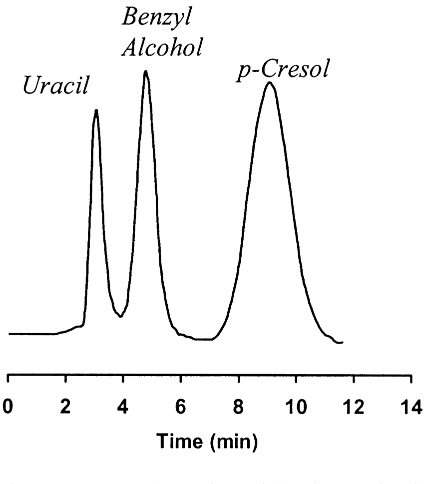

Figure 2. Analytical CCC separation of model mixture by hexane–ethyl acetate–methanol–water solvent system (1.4 : 0.6 : 1.0 : 1.0) obtained at $\omega = 2100$ rpm and $F = 0.6$ mL/min.

and PC ($K_D = 1.13$) peaks. The elution times (t_0, t_1, and t_2) and baseline peak widths (w_0, w_1, and w_2) of all the peaks are measured from the trace and entered on a spreadsheet from which retention, resolution, and distribution ratio data can be calculated as follows:

$$\mathrm{Rs}_{12} = 2\frac{(t_2 - t_1)}{w_1 + w_2} \tag{5}$$

$$V_{\mathrm{dis}} = \frac{t_0}{F} \tag{6}$$

where t_0 is the elution time of the UR solvent front marker peak, F the mobile phase flow rate, and V_{dis} is the calculated displaced volume.

$$V_m = V_{\mathrm{dis}} - V_{\mathrm{ext}} \tag{7}$$

where V_{ext} is the revised extra-coil volume and V_s and S_f obtained from Eqs. (3) and (4), respectively.

THEORY

Du et al. observed that plotting the retention of stationary phase against the square root of the mobile phase flow resulted in a linear plot.[2] The linear regression equation obtained from such a plot[2] can be used to predict how retention changes with rotational speed. This is done as follows:

$$S_f = 100 - \mathrm{B}\sqrt{F} \tag{8}$$

where the gradient B is a non-numeric constant with dimensions $(\mathrm{min/mL})^{0.5}$. It is known, from Wood's modelling the coil planet centrifuge as a constant pressure pump and analysis of flow by Hagen-Poiseuille[3] that:

$$S_f = 100 - \frac{100}{V_c}\sqrt{\frac{8\pi\mu_m L^3}{\Delta P}}\sqrt{F} \tag{9}$$

where $\mathrm{B} = 100/V_c\sqrt{(8\pi\mu_m L^3/\Delta P)}$, μ_m is the viscosity of the mobile phase, L the length of the coil and ΔP the pressure drop across the coil. Wood has also shown that the pressure drop across the coil is related to the density difference of the phases ($\Delta\rho$), the acceleration field ($R\omega^2$) and the length (L) of the tubing,[4] as follows:

$$\Delta P = KR\Delta\rho\omega^2 L \tag{10}$$

Substituting for ΔP in Eq. (9) and for $V_c = A_c L$ gives:

$$S_f = 100 - \frac{100}{\omega A_c}\sqrt{\frac{8\pi\mu_m}{KR\Delta\rho}}\sqrt{F} \tag{11}$$

where K is a constant depending on the CCC apparatus.

From the point of view of predicting the variation of retention with rotational speed from the S_f vs. $\sqrt{F}$ plot with the same phase system and the same geometry, where μ_m, R, $\Delta\rho$, and $A_c = \pi d_c{}^2/4$ are constant, it is sufficient to know that $\mathrm{B} \approx 1/\omega_N$ where ω_N is the rotational speed in rev/min (rpm) as follows:

$$S_f = 100 - \left(\frac{1800}{\omega_N}\right)\mathrm{B}\sqrt{F} \tag{12}$$

Then, substituting the value of flow (F) used for the S_f vs. ω_N plots, the theoretical prediction becomes:

$$S_f = 100 - C/\omega_N \tag{13}$$

where $C = 1800\mathrm{B}'\sqrt{F}$ and $\mathrm{B}' = \mathrm{B}$ at $\omega_N = 1800\,\mathrm{rpm}$.

Rearranging:

$$100 - S_f = C/\omega_N \tag{14}$$

Note that $100 - S_f$ is the percentage of the coil volume occupied by the mobile phase. Plotting this against the reciprocal of the rotational speed should give a linear response with slope C.

RESULTS AND DISCUSSION

The variation of stationary phase retention (S_f) with the square root of mobile phase flow (F) is shown in Fig. 3, with data taken from two different methodologies. Note that each data set is obtained from only one experiment with flow being incrementally increased from the lowest value (high retention of stationary phase) to its highest value in a stepwise manner as described in the experimental procedures section. Stationary phase retention is calculated using Eqs. (1)–(7). The first (diamond points) use displacement volumes (V_{dis}) calculated from the elution of the UR solvent marker peak. The second (triangular points) are from independent conventional stationary phase retention studies, collecting and measuring the displaced volume of

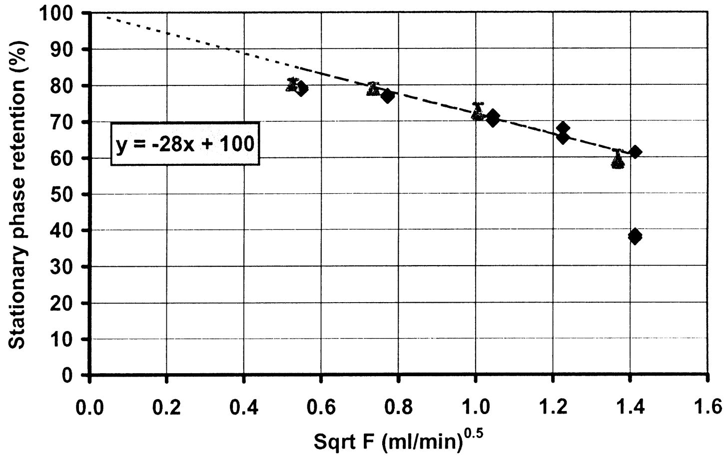

Figure 3. Variation of stationary phase retention (S_f) with the square root of flow ($\sqrt{F}$) at $\omega = 1800\,\text{rpm}$ with data taken from two independent methodologies. The triangular data points with error bars are the average of three conventional retention tests. The diamond data points are retentions calculated from the elution of the uracil peak, and the dotted line is the theoretical best fit.

stationary phase (V_{dis}). Each experiment was repeated three times with error bars added at one standard deviation. It can be seen that both sets of data are in good agreement. Note that, at high flow, the retention of stationary phase can start to drop off and become unstable (i.e., there can be non-equilibrium resulting in carry over of the stationary phase). This can be seen on the high flow chromatograms where carryover of droplets of stationary phase can produce a "spiky" trace (not shown). Note, also, that retentions at low flow were not as high as expected. This is not found with larger bore tubing and one conclusion would be that this non-linearity is due to the predominance of surface or threshold effects at these low flows. The middle range data points ($\sqrt{F} = 0.6 \gg 1.3$) was used for the theoretical best fit ($S_f = 100 - 28\sqrt{F}$), which was in turn used to predict, from Eq. (13), the retention/rotational speed characteristic in Fig. 4.

Figure 4 shows the variation of the retention of stationary phase (S_f) with rotational speed (ω_N) at a flow of $F = 0.6\,\text{mL/min}$. The data set shown calculates the retention of stationary phase (S_f) from the volume of stationary phase eluted which, in turn, is calculated from the elution time of the UR peak. It can be seen that the measured data and the prediction from the S_f vs. $\sqrt{F}$ plot of Fig. 3 are in close agreement.

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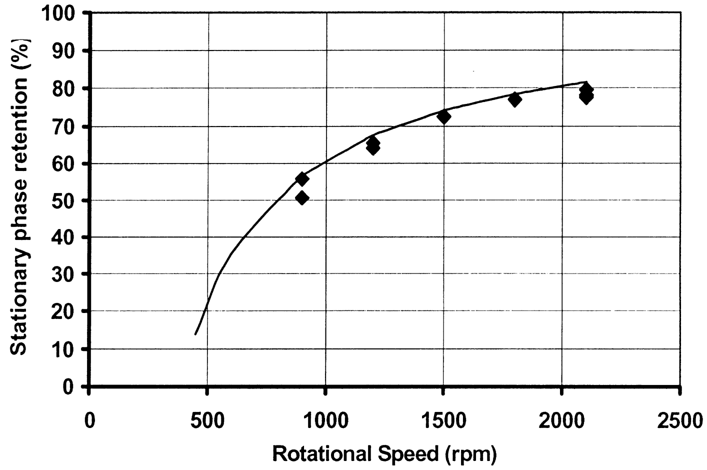

Figure 4. Variation of stationary phase retention with rotational speed. Retention data (diamond points) calculated from the elution of the uracil solvent front marker peak from the $F = 0.6\,\mathrm{mL/min}$ chromatograms. The theoretical prediction (dotted line) is made from the B gradient of Fig. 3 and Eq. (13).

When the same data is replotted (Fig. 5) in the form of Eq. (14) as the percentage of mobile phase in the coil (V_m) against the reciprocal of rotational speed ($1/\omega_N$), then a linear plot results, confirming Wood's hypothesis[4] that retention is proportional to $1/\omega$.

The variation of resolution (Rs) with mobile phase flow is plotted in Fig. 6. Each data point is the average and standard deviation of three sample injections, with measurements taken from three chromatograms. The significance of this result is that the resolution has only dropped from 2.27 to 1.37 as the flow has increased nearly 7-fold, from 0.3 to $2\,\mathrm{mL/min}$. This result shows that, for high speed CCC, it is better to increase the flow as much as possible and then restore resolution by increasing the length of the coil. It is known that doubling the length of the column will increase the resolution by $\sqrt{2}$.[7] In this example, increasing the flow from $0.3\,\mathrm{mL/min}$ to $2\,\mathrm{mL/min}$ reduced the resolution by a factor $1.66 \times (2.27/1.37)$. The separation time for the $F = 0.3\,\mathrm{mL/min}$ chromatogram was 22 min compared to 3 min for the $F = 2\,\mathrm{mL/min}$ chromatogram. If the coil length was increased by a factor $1.66^2 = 2.75$ then the same resolution as obtained with the $F = 0.3\,\mathrm{mL/min}$ flow on a $V_c = 4.63\,\mathrm{mL}$ coil would be obtained in 8.2 min with a $V_c = 12.75\,\mathrm{mL}$ coil and a flow rate of $2\,\mathrm{mL/min}$. This is a factor three improvement in separation time and gives separation times comparable to

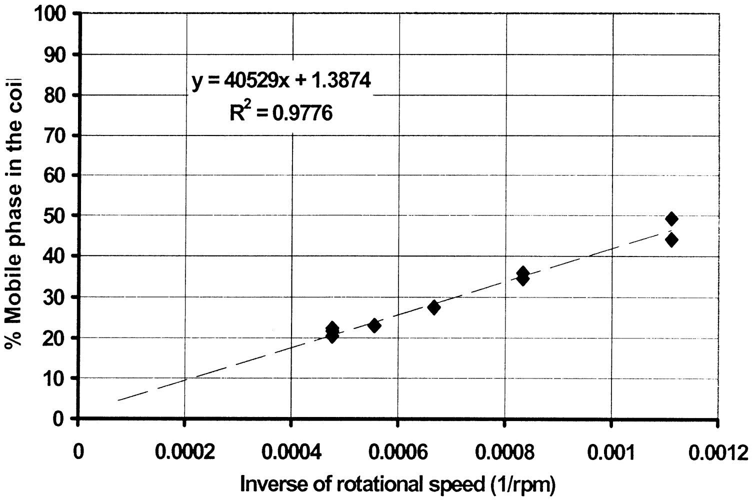

Figure 5. Variation of the percentage of mobile phase in the coil with the inverse of rotational speed for a flow of $F = 0.6\,\mathrm{mL/min}$. The dotted line is the best fit linear regression, demonstrating the linear relationship predicted by Eq. (14).

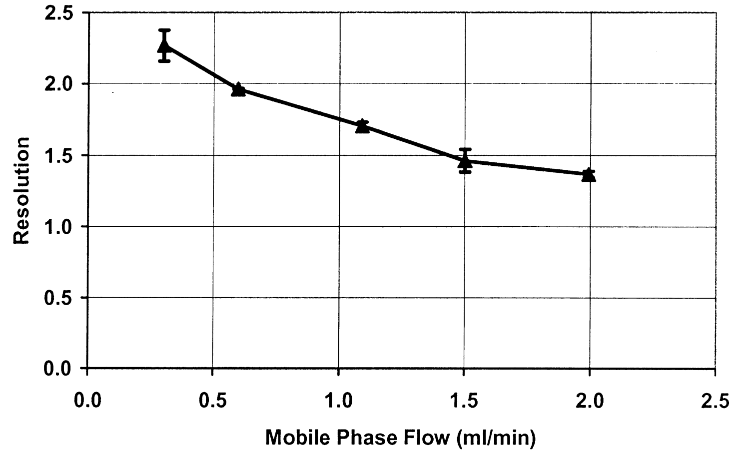

Figure 6. Variation of the resolution between benzyl alcohol and *p*-cresol with flow at a constant rotational speed of $\omega_N = 1800\,\mathrm{rpm}$.

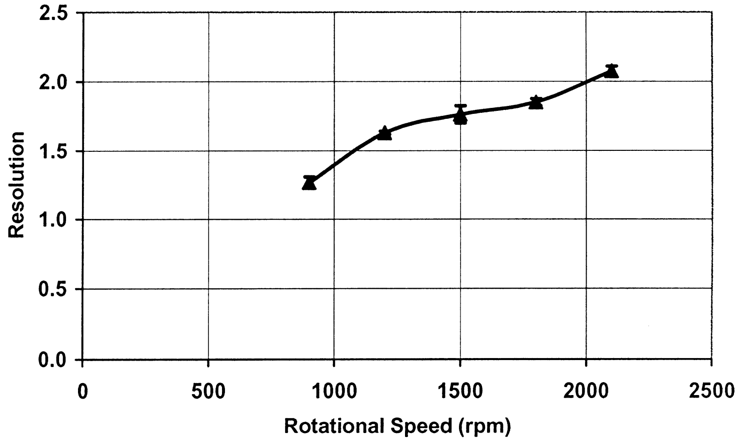

Figure 7. Variation of the resolution between benzyl alcohol and *p*-cresol with rotational speed at a constant flow of 0.6 mL/min.

HPLC. Furthermore, if higher resolution was required, then flow could be reduced to achieve it. This strategy will only work if the kinetics allow it to work, which they clearly do for the 4B phase systems used here.

The variation of resolution with rotational speed is shown in Fig. 7 for a mobile phase flow of 0.6 mL/min. Increasing the speed from 1000 rpm to 2000 rpm, which increases the "g" field by a factor of 4×, increases the resolution by only a factor 1.4× or $\sqrt{2}$. The equivalent effect can be obtained by increasing the coil length by a factor 2×. Of course, it might be necessary to increase the speed in order to obtain the retention for these high flows but, if this is not necessary, then it would appear to be simpler from the mechanical point of view (bearing wear, heat, etc.) to run at 1200 rpm (80 g). One conclusion is that the increased speed is good for retention but not so good for resolution. Resolution should increase linearly with speed, as the number of mixing and settling stages is proportional to rotational speed. It can only be concluded that mixing and, hence, mass transfer, becomes inhibited as the "g" field increases. The "g" field increases with the square of the speed while the number of mixing and settling steps is only proportional to speed. Nevertheless, it is interesting to note that resolution still increases with speed and has not reached an optimum value.

CONCLUSION

This research has shown that two very simple tests can characterize a CCC phase system in terms of retention and resolution as function of both mobile phase flow and rotational speed. It has also shown, for a heptane–ethyl acetate–methanol–water phase system, that retention speed characteristics can be predicted from the B gradient[2] of S_f vs. $\sqrt{F}$ plot, which characterises the hydrodynamics of the phase system by showing how stationary phase retention changes with the square root of mobile phase flow.

These same tests also show how resolution of a test system changes with operational variables, such as mobile phase flow and rotational speed, which can affect the hydrodynamics. It is concluded that high flow is important for rapid CCC separations and increasing the length of the coil can more than compensate for the loss of resolution. This will result in separation times in minutes as opposed to hours, leading to rapid-CCC separation in times comparable to HPLC.

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