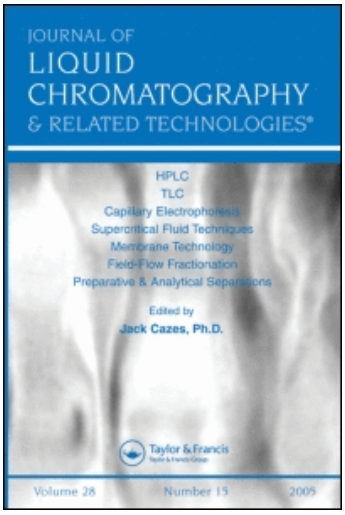

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Resolution in Countercurrent Chromatography

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RESOLUTION IN COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Using as an example the separation of DNP-glutamic acid and DNP-alanine in a nonplanetary countercurrent chromatograph, it was demonstrated that resolution, R_S, of a solute pair can be satisfactorily predicted from knowledge of the column efficiency, N, the fraction of column volume occupied by stationary phase, S_F, and the partition coefficients of the substances in the solvent system employed. Resolution is a function of the phase volume ratio and separation of rapidly eluted compounds is favored by an increase in S_F. The partition coefficients, K, the separation factor, α, and the column efficiency, N, are independent of S_F. Increasing S_F brings about an increase in R_S by increasing the capacity factor k'. For S_F of 0.4, characteristic of the horizontal flow-through CCC, baseline separation (R_S = 1.5) is obtained

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for values of K_1, the first eluted substance, of about 0.5 for N = 1000 or K_1 of 10 for N = 100, where α is 2. Increasing S_F to 0.8, characteristic of the multilayer coil, high speed CCC, favors resolution of rapidly eluted solutes, those with low K values. Baseline resolution is then obtained for K_1 of about 0.07 with N = 1000 or for K_1 of about 1.5 for N = 100.

INTRODUCTION

Chromatographic resolution, R_S, is conventionally defined as the peak separation divided by the average base width, eq. 1 (1). Assuming a Gaussian peak profile, the base width, W, is taken as the 4 σ width, estimated by drawing tangents to the peak inflection points and extrapolating these to the baseline. Both peak separation, shown in units of time, t, in equation 1, and W must be expressed in the same units.

$$R_S = \frac{2(t_2 - t_1)}{W_2 + W_1} \qquad \text{(eq. 1)}$$

If the entire area of the chromatographic peak is taken as 6 standard deviation units ($\pm 3\sigma$), then R_S will be 1.5 for baseline (99.9%) separation. The chromatographic efficiency, in terms of the theoretical plate number, N, is commonly expressed as

$$N = 16 \left(\frac{t}{W}\right)^2 \qquad \text{(eq. 2)}$$

The capacity factor, k', for a particular solute (eq. 2), may be expressed as

$$k' = \frac{Q_s}{Q_m} = \frac{C_s V_s}{C_m V_m} = K \left(\frac{V_s}{V_m}\right) = K \left(\frac{S_F}{1-S_F}\right) \qquad \text{(eq. 3)}$$

where Q and C represent the respective quantities and concentrations of a solute in volumes V of stationary, s, and mobile, m, phases contained in the chromatographic column. K is the solute partition coefficient expressed as

the ratio of concentration in the stationary phase to that in the mobile phase and S_F is the fraction of the column volume occupied by stationary phase.

The separation factor, α, is conventionally defined as

$$\alpha = \frac{K_2}{K_1} \quad , K_2 > K_1 \qquad \text{(eq. 4)}$$

The restrictions that $K_2 > K_1$, implying that $\alpha > 1$, and that K be defined as the ratio of concentrations in stationary over mobile phases, insure that α for a particular solute pair will be identical, regardless of whether a CCC system is run in the normal or reversed phase mode.

The quantities defined in equations 2,3 and 4 may be substituted into equation 1 to obtain

$$R_S = \tfrac{1}{2}\sqrt{N}\ \frac{\alpha-1}{(\alpha+1) + \frac{2}{K_1}\left(\frac{1-S_F}{S_F}\right)} \qquad \text{(eq. 5)}$$

Equation 1 is customarily employed to calculate the resolution of adjacent peaks on a chromatogram. However, equation 5 might be used to estimate the resolution expected for a column of N plates and stationary phase fraction, S_F, using values of K_1 and α determined by non-chromatographic means. The present study was undertaken to test the validity of this prediction using data obtained on the separation of DNP-glutamic acid and DNP-alanine in a nonplanetary countercurrent chromatograph using the solvent system chloroform/acetic acid/0.1N HCl (2:2:1).

EXPERIMENTAL

Apparatus

The countercurrent chromatograph was a slowly rotating nonplanetary, rotating-seal-free unit, employing a single-

layer, coaxial column (2). Three columns were made by successively rewinding a 4.2 m length of 0.55 cm i.d. FEP (fluorinated ethylene propylene) tubing (Galtek Corp., Jonathan Ind. Ctr., Chaska, MN) as a single layer onto cylindrical cores of 3,10 and 20 cm. diam. The respective columns consisted of 45,14 and 7.3 turns and had volumes of 100,112 and 113 ml. The smaller volume of the 3 cm column resulted from slight flattening of the tubing when wound on the narrow diameter core. Influent and effluent lines of 0.85 mm i.d. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ) were attached for continuous elution.

Solvent System

The two-phase solvent system studied consisted of chloroform without preservative (Burdick and Jackson Laboratories, Inc., NJ), glacial acetic acid and 0.1 N hydrochloric acid (Fisher Scientific Co., Fairlawn, NJ) in a volume ratio of 2:2:1. The solvent mixture was equilibrated in a separatory funnel at room temperature and separated before use.

Procedure

Prior to rotation, the column was filled with stationary phase, 0.5 ml of sample solution was injected, and rotation adjusted to the desired rate, which ranged from 40 to 200 rpm. Either upper or lower phase was used as stationary phase and the corresponding mobile phase was pumped in either the head to tail (H $\rightarrow$T) or tail to head (H $\leftarrow$T) direction at 120 ml/hr. The eluent stream was monitored at 280 nm using the LKB Uvicord S, 1.0 absorbance full scale, and an LKB recorder, chart speed 3 cm/hr.

The sample solution contained 0.5 g% of each of N-2,4-DNP-DL-glutamic acid and N-2,4-DNP-alanine (Sigma Chemical Co., St. Louis, MO) in the upper, aqueous, phase.

The resolution, R_S, referred to herein as the measured resolution, was determined from the chromatogram using equation 1.

After completion of the run, the apparatus was stopped and the column contents were forced out under nitrogen pressure (50 psi) and collected in a graduated cylinder to determine the fraction, S_F, of the column volume occupied by stationary phase. During emptying the column was slowly rotated so that the flow was in a tail to head direction to facilitate removal of final traces of solvent. The column efficiency, N, was evaluated for each DNP-amino acid using equation 2 and the mean efficiency calculated. The partition coefficients were determined spectrophotometrically after partitioning a small amount of each amino acid between measured volumes of each phase. These were 1.9 and 0.56 for DNP-glu and DNP-ala respectively, expressed as the concentration in upper phase divided by the concentration in lower phase, from which the separation factor, α, was determined to be 3.39. These values of S_F, mean N, K and α were used to calculate predicted values of resolution using equation 5.

RESULTS AND DISCUSSION

A total of 30 chromatograms were obtained under normal phase and reversed phase operation with a mobile phase flow rate of 120 ml/hr and rates of column rotation ranging from 40 to 200 rpm. In the normal phase mode, the upper, more polar, aqueous phase is stationary and mobile phase flow is from tail to head. In the reversed phase mode, the lower, less polar, organic phase is stationary and mobile phase flow is from head to tail. DNP-alanine elutes first in the normal phase mode while DNP-glutamic acid elutes first in the reversed phase mode.

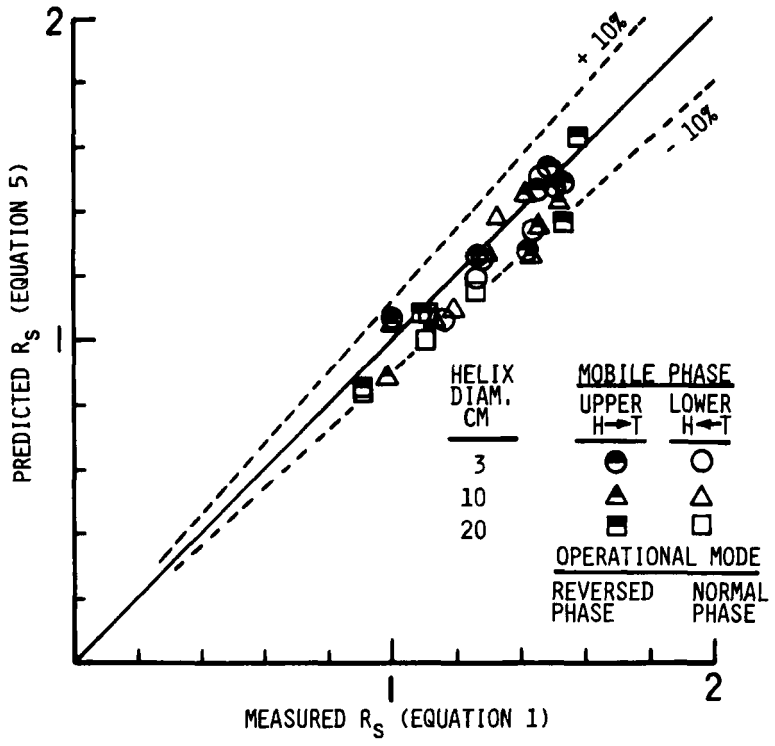

FIGURE 1. Predicted versus measured resolution for CCC separation of DNP-glutamic acid and DNP-alanine with the system $CHCl_3$/HOAC/0.1N HCl (2:2:1).

Column Efficiences

Column efficiencies, N, measured for 12 runs in the narrow (3 cm) helical diameter column ranged from 28 to 117 plates for the first peak eluted and from 32 to 75 plates for the second peak eluted, the highest efficiencies being obtained at 200 rpm with the reversed phase mode. Efficiencies for 8 runs in the widest (20 cm) diameter column ranged from 14 to 73 plates for the first peak and 15 to 50 plates for the second peak, the highest efficiencies again being observed

with the reversed phase mode. Intermediate values were obtained with the 10 cm helix.

Predicted versus Measured Resolution

Correlation of the predicted resolution (equation 5) with the measured resolution (equation 1) for the 30 chromatograms is summarized in Fig. 1. Predicted values range from 0.84 to 1.62 while measured values range from 0.90 to 1.58. The plot shows high correlation ($r = 0.929$), with the predicted values being usually slightly lower (slope = 0.919, y intercept 0.07) than those measured. The largest deviations differed by only 10% from the theoretical slope of unity. The most likely source of this bias is thought to be incomplete recovery of the column contents which would result in a slightly low estimate of S_F. Other possibilities are a bias in drawing the chromatogram baseline when estimating N or slight errors in measuring the partition coefficients. Nevertheless, for practical purposes, there is excellent agreement between the predicted and measured values of resolution which should greatly simplify the choice of solvent systems or column size when partition coefficients are known for the solutes to be separated.

Effect of S_F on R_S

The relationship of the phase volume ratio and resolution was examined for countercurrent distribution and liquid-liquid chromatography by Metzger, Barford and Rothbart (3). They concluded that for the withdrawal procedure, analogous to chromatography, resolution is improved by using the minimum mobile phase volume and at the same time, the mobile phase volume required to elute a peak is reduced. Both of these effects have been observed in countercurrent chromatography.

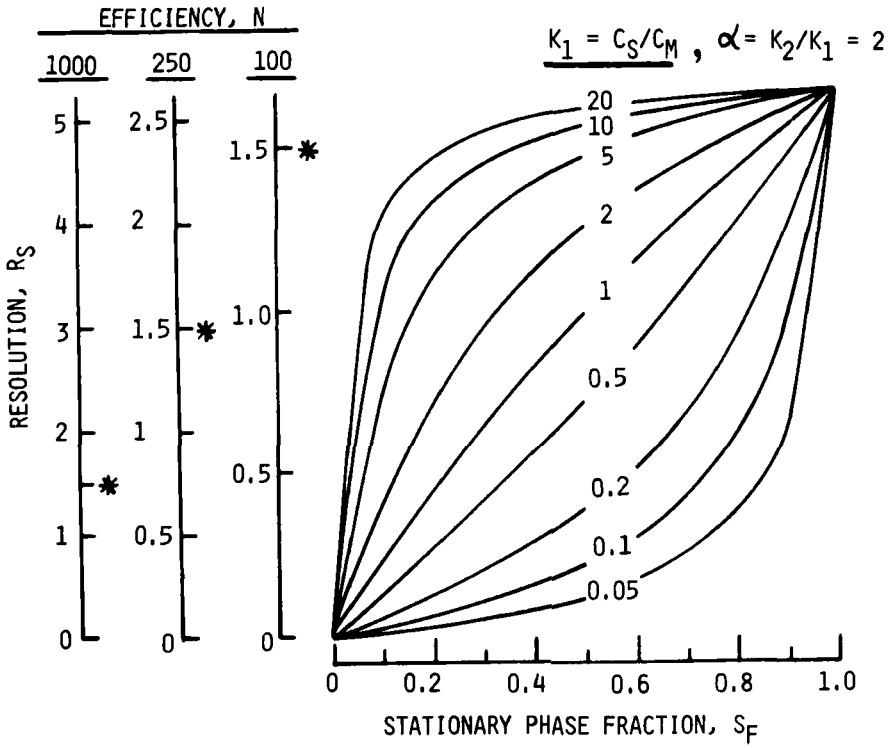

FIGURE 2. Predicted resolution as a function of stationary phase fraction, S_F, the partition coefficient of the first-eluted solute, K_1, and a constant separation factor, α, of 2, for columns containing 100, 250 or 1000 theoretical plates, N. Baseline resolution, R_S = 1.5, is indicated by an asterisk. K is expressed as concentration in stationary phase divided by concentration in mobile phase.

The effect of the stationary phase fraction, S_F, on R_S as calculated from equation 5, is summarized in Fig. 2 for a solute pair for which the partition coefficient, K_1, of the first eluted peak varies from 0.05 to 20 while α remains constant at a value of 2. Resolution is shown for columns

of 100, 250 and 1000 theoretical plates. Substances with high partition coefficients (K_1 of 1 to 20) are best resolved in long columns (1000 theoretical plates) with small stationary phase volume, S_F about 0.2. As S_F is further increased, resolution of solutes with high K_1 (10 to 20) improves only slightly, while resolution of substances with intermediate partition coefficients (K_1 of 0.2 to 2) increases appreciably. When S_F of 0.8 is reached, which is typical for the multilayer coil planet centrifuge (5,6), substances with partition coefficients almost as low as 0.05 can be resolved on long columns. Solute pairs with K_1 values above about 1 are predicted to be resolved on short columns of about 100 theoretical plates if S_F is 0.8.

Short columns having S_F in the region of 0.4, which are typical for the horizontal flow-through coil planet centrifuge (4), are predicted to be adequate for resolution of substances with K_1 values in the range of 5 to 20.

These estimates are based on a separation factor of 2 and the expected resolution under the same conditions will decrease as α is lowered and will increase with higher α.

Effect of S_F on k'

Since the partition coefficient, K, is defined as a ratio of concentrations, it and consequently also the separation factor, α, is independent of S_F. The column efficiency, N, is also not directly dependent on S_F. Increasing S_F therefore increases R_S by increasing the capacity factor k'. The terms are related as shown in equation 3, which indicates that for a solute with a given K, k' increases in direct proportion to the phase volume ratio, Vs/Vm, which is equivalent to $S_F/(1-S_F)$. This effect is illustrated graphically in Fig. 3 where, for example, a substance having a partition coefficient of 10 exhibits a k'

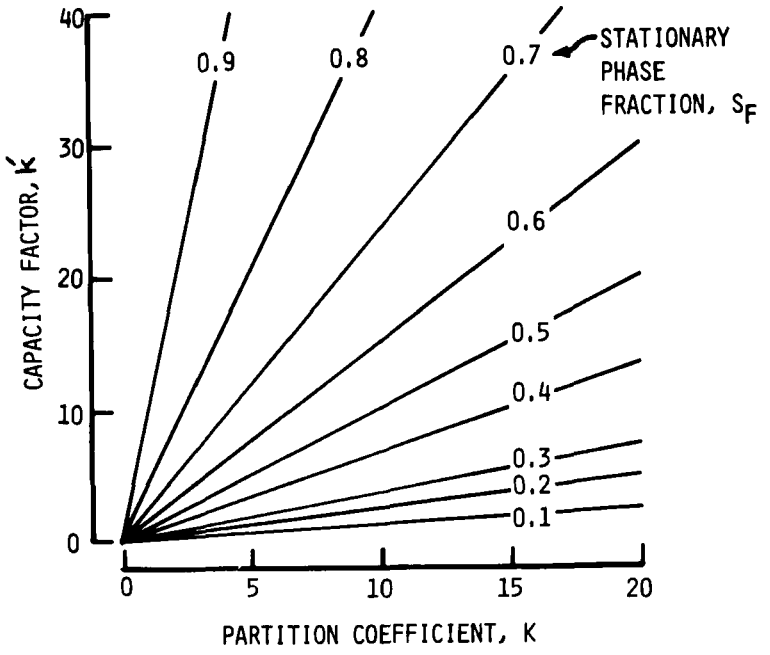

FIGURE 3. Influence of stationary phase fraction, S_F, on the capacity factor, k', as a function of the partition coefficient, K.

of 7 in a column with S_F of 0.4 but a k' of 40 when S_F increases to 0.8. This would result in about a 4-fold increase in retention time for columns of equal size.

Separation factor, α, required for resolution

Resolution of a solute pair becomes easier as the separation factor, α, increases. However, increasing α also increases the time required to elute the second member of the pair. The K_1 and S_F terms of equation 5 may be replaced by the following equivalence

$$\frac{1}{K_1}\left(\frac{1-S_F}{S_F}\right) = \frac{1}{k'_1} \qquad \text{(eq. 6)}$$

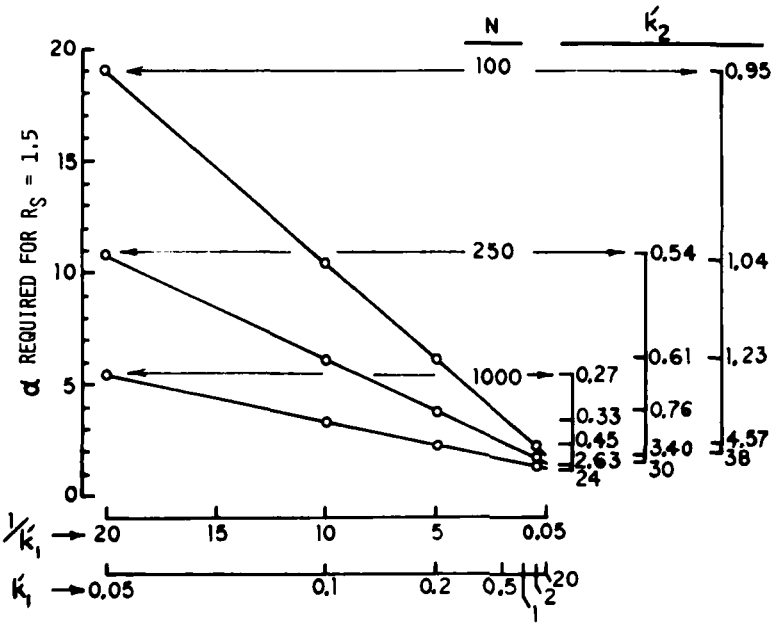

FIGURE 4. Separation factor, α, required for baseline resolution, $R_S = 1.5$, as a function of column efficiency, N, and the capacity factors k'_1, and k'_2. Subscript 1 indicates the solute eluting first.

and the resulting equation recast as follows to permit calculation of α required for various degrees of separation.

$$\alpha = \left(\frac{4R_s}{\sqrt{N} - 2R_s} \right) \frac{1}{k'_1} + \frac{\sqrt{N} + 2R_s}{\sqrt{N} - 2R_s} \qquad \text{(eq. 7)}$$

It is apparent from equation 7 that a plot of α vs $1/k'_1$ will be linear for a column with a fixed number of plates, N, and an arbitrarily chosen value of R_S.

Fig. 4 presents a plot of α required for baseline resolution ($R_S = 1.5$), versus the k'_1 value of the earliest

eluted solute of the pair for k'_1 values ranging from 0.05 to 20. Plots for columns of 100, 250 and 1000 plates are presented and the corresponding k'_2 value for the solute eluted last is presented on the right side of the figure. This plot is useful for chosing the minimum size column required for separating a solute pair for which estimates of α are available. While the required α is seen to decrease as k'_1 is increased, the elution time, which is proportional to k'_1 will also increase and becomes impractically long, particularly for the last eluted solute of the pair.

CONCLUSION

Resolution to be expected in CCC can be satisfactorily predicted from partition coefficients evaluated non-chromatographically, a knowledge of S_F for the chromatographic system and an estimate of the theoretical plates provided by the column. The parameters K, α and N are independent of S_F. Increasing S_F affects resolution in CCC by increasing k'. The effect on k' can be readily calculated and the result used to calculate the α value required for the desired degree of separation on a column providing a given number of theoretical plates.

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