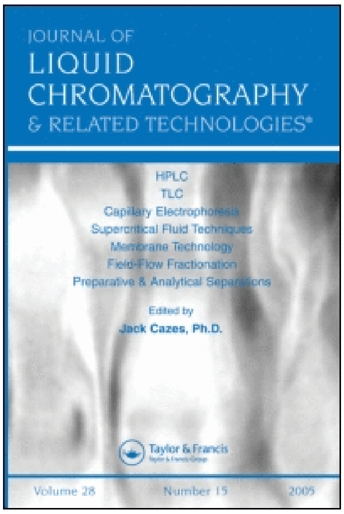

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DISCREPANCY BETWEEN THE THEORETICAL PLATE NUMBER (N) AND PEAK RESOLUTION (Rs) FOR OPTIMIZING THE FLOW RATE IN COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

This paper deals with a problem in optimizing the flow rate of the mobile phase in countercurrent chromatography (CCC). A set of data including theoretical plate number (N), resolution factor (Rs), % retention, etc. are obtained from the separation of three indole auxins in a two-phase solvent system of *n*-hexane / ethyl acetate / methanol / water (1:1:1:1) by applying the flow rates of the mobile phase at 0.5 - 8.0ml/min. The experimental results revealed a disagreement between the efficiencies expressed in parameters N and Rs at the high flow rates of the mobile phase in the CCC separation. Namely, the partition efficiency expressed in terms of Rs decreases with an increased flow rate of the mobile phase, while the N rises with increasing the flow rate. Since Rs represents the actual separation of the solute peaks, the results indicate that the N used in high performance liquid chromatography is not always a reliable measure in CCC separation. However, the application of a high flow rate yields better min/Rs (time in min required to yield Rs=1) than that of a low flow rate. On the other hand an extremely slow flow rate of the mobile phase results in an abrupt increase of the partition efficiency in terms of both N and Rs. Therefore, the present findings suggest new strategies to improve the separation efficiency in CCC.

INTRODUCTION

Countercurrent chromatography (CCC) is distinguished from other chromatographic methods by eliminating the solid support matrix from the separation column. The method employs an open tubular column where the partition process takes place between the flowing mobile phase and the stationary phase retained by the effect of gravity or a centrifugal force field. Consequently, the volume of the stationary phase retained in the column widely varies according to the experimental conditions including the flow rate of the mobile phase, applied force field, etc. Since the volume of the stationary phase in the column largely influences the resolution of solute peaks, it becomes an important variable in CCC for optimizing the experimental conditions.

Partition efficiency of the chromatographic column is usually expressed in two different parameters, i. e., the theoretical plate number (N) and peak resolution (Rs). The relationship between these two parameters are given by the following equation,

$$Rs = 0.25\,(\alpha - 1)N^{1/2}[k'_1 / (k'_1 + 1)] \qquad (1)$$

where $\alpha = k'_2 / k'_1$, k'_1 and k'_2 are the capacity factors of two solutes. For application to CCC, equation 1 is further modified as

$$Rs = 0.25\,[(K_2 - K_1) / K_1]\,N^{1/2}\,[K_1 (V_s / V_m) / \{K_1 (V_s / V_m) + 1\}] \qquad (2)$$

where $k'_1 = K_1 (V_s / V_m)$ and $k'_2 = K_2 (V_s / V_m)$; K_1 and K_2 are partition coefficients of the respective solutes; and V_s and V_m, the volumes of the stationary and mobile phases, respectively.

In high performance liquid chromatography (HPLC) where the amount of stationary phase (V_s) in the column is constant, the peak resolution is solely determined by N for a given pair of solutes. In other words, N becomes a reliable parameter to represent the peak resolution in HPLC. However, in CCC where the volume of the stationary phase (V_s) varies with the experimental conditions, the peak resolution of the solutes is determined by two parameters, N and the volume of the stationary phase retained in the column. Therefore, parameter N by itself may not be a reliable factor to evaluate the peak resolution, especially when the retention volume of the stationary phase varies in a wide range.

This paper deals with a problem in optimizing the flow rate of the mobile phase in CCC. A set of data including N, Rs, % retention, etc. is obtained from the separation of three indole auxins in a two-phase solvent system of *n*-hexane / ethyl acetate / methanol / water (1:1:1:1) by applying the flow rates of the mobile phase at 0.5 - 8.0ml/min.

EXPERIMENTAL

Apparatus

The CCC used was a Shimadzu HSCCC-1A prototype multilayer coil planet centrifuge (Shimadzu Corp., Kyoto, Japan) with a 10cm orbital radius which produces a synchronous planetary motion at 800rpm. The multilayer coil was prepared by winding a *ca.* 160m length of polytetrafluoroethylene (PTFE) tubing onto the column holder with a 10cm hub diameter and a 15cm hub length, making six coiled layers with a total capacity of about 300ml.

Reagents

All organic solvents, including *n*-hexane, ethyl acetate, and methanol, were glass-distilled chromatographic grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

Indole-3-acetamide (IA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A).

Preparation of solvent system and sample solution

A two-phase solvent system composed of *n*-hexane / ethyl acetate / methanol / water (1:1:1:1) was used in the present study. The solvent system was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking and degassing at room temperature and the two phases were separated before use.

The sample solution was prepared by dissolving 20mg of IA, 60mg of IAA, and 60mg of IBA in 10ml of the nonaqueous upper phase of the above solvent system.

Separation procedure

The column was first entirely filled with the upper nonaqueous stationary phase. Then, the column was rotated at the optimum speed of 800rpm, while the mobile phase was pumped into the head of the column (the head-tail relationship of the rotating coil is conventionally defined by the Archimedean screw force, where all objects of different densities are driven toward the head of the coil) at the desired flow rate using a Shimadzu Model 6A HPLC pump. After a steady state hydrodynamic equilibrium was reached, 0.5ml of the sample solution was loaded. The effluent from the outlet of the column was continuously monitored by the absorbance at 260nm using a Shimadzu Model SPD-6A detector.

RESULTS AND DISCUSSION

A series of experiments was conducted to study the relationship between various parameters such as N, Rs, and % retention at various flow rates of the mobile phase. Using a multilayer coil planet centrifuge, three indole auxins were separated in a two-phase solvent system composed of *n*-hexane/ethyl acetate/methanol/water at an equal volume ratio with the lower aqueous phase as the mobile phase. The results are summarized in Table I where % retention of the stationary phase; N computed from peaks IA, IAA and IBA; and Rs for IA/IAA and IAA/IBA are listed for various flow rates of the mobile phase ranging from 0.5 to 8.0ml/min. The Rs values computed from N using equation 2 are also shown in parenthesis. Using these data the effects of the flow rate of the mobile phase on N and Rs are presented in Figs. 1A and B, respectively.

In Fig. 1A, the height equivalent per theoretical plate (cm/N) is plotted against the applied flow rates of the mobile phase. The efficiency sharply decreases from 0.5 to 1.0 and 2.0ml/min where all three curves reach the lowest efficiency. Further increase in flow rate improves the

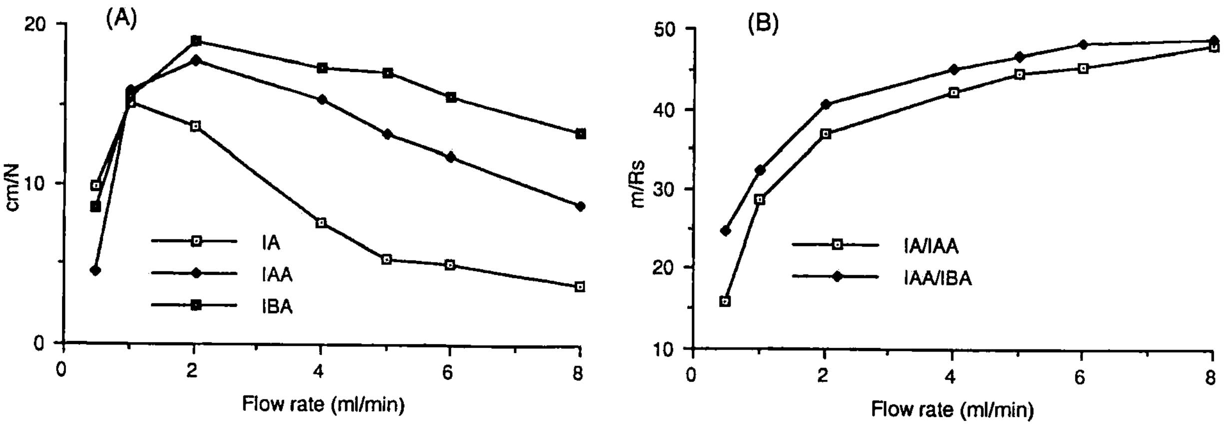

Fig. 1 The effect of the flow rate of the mobile phase on height equivalent per theoretical plate number (A) and resolution factor (B)

efficiency up to the maximum flow rate at 8.0ml/min. These curves are quite similar to those obtained from the centrifugal partition chromatograph reported by Armstrong *et al.* (1,2), showing an inverse relationship to the van Deemters equation (3).

Fig. 1B shows a similar diagram between the height equivalent per peak resolution (m/Rs) and flow rate of the mobile phase which was produced from the same set of the separation data in Table I. Both curves show the best efficiency at the lowest flow rate of 0.5ml/min and a further increase of the flow rate results in a steady exponential decrease in efficiency up to the maximum applied flow of 8.0ml/min.

The above two diagrams in Figs. 1A and B reveal a disagreement between the efficiencies expressed in parameters N and Rs at the high flow rates of the mobile phase in the CCC separation. Since Rs represents the actual separation of the solute peaks, the results indicate that N is not always a reliable measure in partition efficiency especially when a high flow rate such as 8.0ml/min is applied to the separation as in the present case. This discrepancy is certainly caused by a decrease in the retained stationary phase volume at the increased flow rate. As shown in Fig. 1A, this effect is enhanced on the solute with a smaller partition coefficient as IA.

The relationship between N and % retention of the stationary phase is illustrated in Fig. 2A where the N values for IA, IAA and IBA are plotted against % retention. All three curves show a steady decline up to 83% retention where they start a steep increase. This abrupt rise of the N values at the high retention may imply that the hydrodynamic pattern of the mobile phase is changed from a continuous stream flow to a

Table I

Theoretical plate numbers and resolution factors at various flow rates

Flow rate[a] (ml/min)	% Retention of stationary phase	Theoretical plate number, N			Resolution factor, Rs (Computed Rs value)[b]	
		IA	IAA	IBA	IA/IAA	IAA/IBA
0.5	83.8	1609	3587	1883	10.0 (11.4)	6.4 (13.2)
1.0	81.9	1063	1006	1037	5.6 (8.4)	4.9 (6.7)
2.0	75.3	1172	905	840	4.3 (6.4)	3.9 (5.5)
4.0	65.3	2116	1044	922	3.8 (5.7)	3.5 (4.5)
5.0	58.2	2940	1211	938	3.6 (5.1)	3.4 (4.0)
6.0	57.6	3136	1347	1024	3.5 (4.6)	3.3 (3.6)
8.0	51.9	4268	1820	1202	3.3 (4.2)	3.3 (3.7)

[a] Mobile phase: lower phase of *n*-hexane-ethyl acetate -methanol-water (1-1-1-1) .

[b] Computed Rs value from N using equation 2.

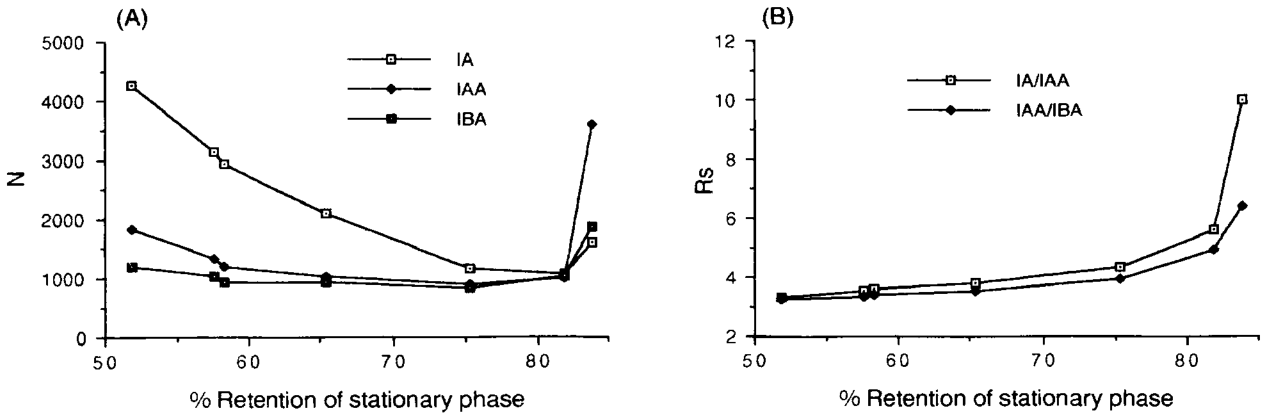

Fig. 2 Relationships between theoretical plate number (A) and resolution factor (B), and % retention of stationary phase

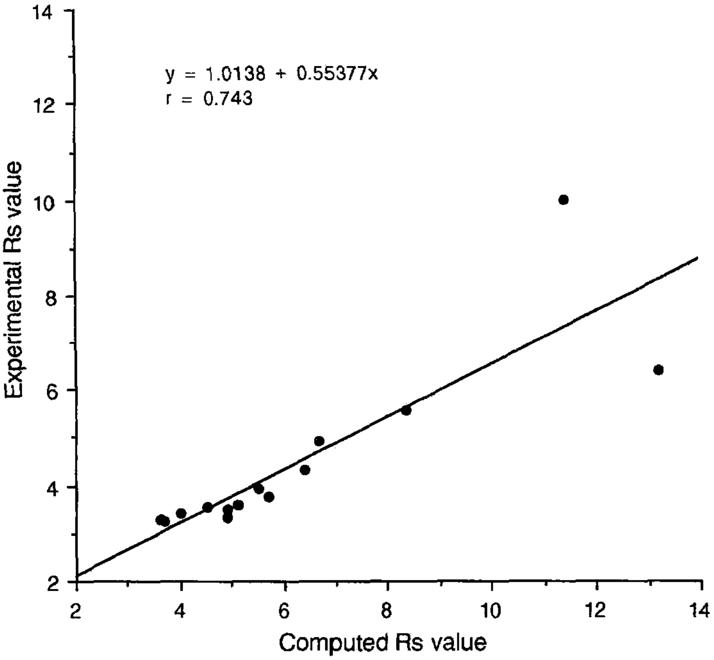

Fig. 3 Correlation between experimental and computed resolution factors

discontinuous droplet flow under the application of the extremely low flow rate.

The relationship between Rs and % retention of the stationary phase shows an opposite trend as shown in Fig. 2B. The Rs values for both IA/IAA and IAA/IBA rise with the increased retention of the stationary phase. Here again Rs values abruptly rise at the highest retention of 83% which was resulted from the lowest flow rate of the mobile phase.

These relationships between N, Rs and % retention of the stationary phase are predicted from equation 2 described earlier. The Rs values

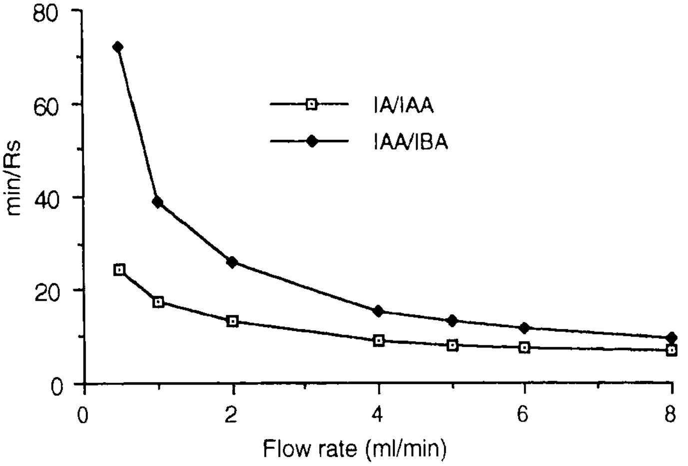

Fig. 4 Relationship between time equivalent per resolution factor and flow rate

computed from the equation closely correlated with the experimental data as shown in Fig. 3.

The overall results of the present study indicate that the resolution of solute peaks decreases as the flow rate of the mobile phase is increased. However, the application of a high flow rate provides a shortened separation time which may be advantageous in some applications. Fig. 4 shows the relationship between min/Rs (time in min which is required to yield Rs=1) and the applied flow rate. The min/Rs is improved with the increased flow rate, indicating that application of high flow rate is advantageous for the analytical works.

CONCLUSION

The results of the present studies with a multilayer coil planet centrifuge may be summarized as follows:

1. The partition efficiency expressed in terms of Rs decreases with an increased flow rate of the mobile phase.

2. The N used in HPLC is not always a reliable measure in CCC separation especially when the application of a high flow rate of the mobile phase results in a substantial depletion of the stationary phase in the separation column.

3. The application of a high flow rate provides better min/Rs (time in min required to yield Rs=1) than that of a low flow rate.

4. An extremely slow flow rate of the mobile phase such as 0.5ml/min would cause a change in the flow pattern from a stream flow to a more efficient droplet flow which results in an abrupt increase of the partition efficiency as indicated by the increase of the values of both N and Rs.

Finally, the present findings suggest novel strategies in CCC to improve the separation efficiency; the application of a high flow of the mobile phase through a long multilayer coil or the application of an extremely slow flow of the mobile phase through a short coil.

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