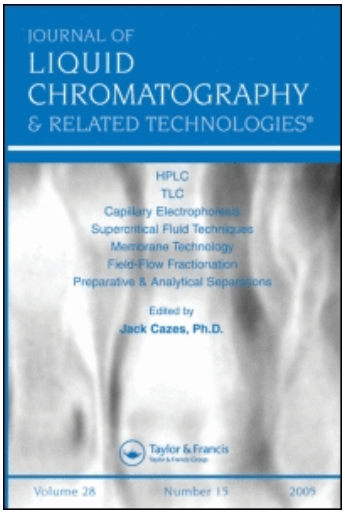

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Comparison of Several Aqueous two Phase Solvent Systems (ATPS) for the Fractionation of Biopolymers by Centrifugal Partition Chromatography (CPC)

Alain Foucault[a]; Koji Nakanishi[a]

[a] Department of Chemistry, Columbia University, New York, New York

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COMPARISON OF SEVERAL AQUEOUS TWO PHASE SOLVENT SYSTEMS (ATPS) FOR THE FRACTIONATION OF BIOPOLYMERS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY (CPC)

ALAIN FOUCAULT* AND KOJI NAKANISHI
Department of Chemistry
Columbia University
New York, New York 10027

ABSTRACT

Aqueous Two Phase Solvent Systems (ATPS) have been tested in a Centrifugal Partition Chromatograph (Sanki, Kyoto, Japan), to check for stability, backpressure, noiseless baseline and efficiency. From our experiments we conclude that ATPS are compatible with CPC, in particular the stationary phase is very stable and does not require a high rotational speed to stabilize it (1300 rpm was commonly used); but, the efficiency of the CPC apparatus decreases markedly, due to the high viscosity of ATPS as compared to classical biphasic solvent systems. Based on our results, new partition cartridges have been designed, which minimize the bulk volumes and considerably increase the interface, or favor the mixing step of the two phases by an *ad-hoc* design of both the inlet ducts and channels.

Introduction

Separation and extraction of macromolecules, organelles and cells by use of Aqueous Two Phase Solvent Systems (ATPS) originate from the work by Albertsson and are well documented[1,2]. It is only recently that

attempts have been made to use the aqueous two-phase systems in conjunction with countercurrent technologies, with the ultimate aim of producing a high resolution chromatographic method for fractionation and purification of biological materials. Among the various ATPS found in the literature, PEG/Dx [3] are frequently encountered, but Dextrans are expensive and their solutions are rather viscous. New, inexpensive polymers, with good physical properties recently appeared on the market as substitute for Dextran, namely Aquaphase PPT and Reppal PES; both are hydroxypropylated starch. We have used the centrifugal partition chromatograph to test some aqueous two-phase systems (polymer-salt and polymer-polymer); the main parameters (flow rate, rotational speed, temperature) have been varied systematically in order to try to get an efficient tandem CPC + ATPS.

Experimental

CPC separations :

A Centrifugal Partition Chromatography apparatus (Model CPC LLN) manufactured by Sanki Engineering (Nagaokakyo, Kyoto), and currently available from Sanki Laboratories (Sharon Hill, PA, USA) was used. It consists of a continuous-flow centrifuge containing 6 partition cartridges (type 250 W; total volume 128 ml), a constant flow pump (LBP V type triple pistons), a valve connection unit (FCU II) equipped with a 4 ml PTFE sample loop injector, an electric power supply unit (PCB II), a UV Vis detector (Linear, model 200) with a variable pathlength illuminated volume preparative scale flow cell (model 0203-7083) and a recorder (Fisher). Each partition cartridge contains four polychlorotrifluoroethylene plates, each engraved with 100 channels, giving a total of 2400 channels for 6 cartridges.

Materials

The aqueous two-phase systems we have tested are :

PEG 8000	Phosphate buffer
PEG 1000	Ammonium sulfate
PEG 8000	Industrial Dextran
PEG 8000	Aquaphase PPT
PEG 8000	Reppal PES 100
PEG 8000	Reppal PES 200

Polyethylene glycols and dextran come from Sigma (St Louis, MO, USA):

PEG 1000, No.P 3515, Approx. M.W. 1000

PEG 8000 (formerly listed as Mol. Wt. 6000), No.P 2139, Approx. M.W. 8000

Industrial dextran, No.D 7265, Lot 29F-0029, Average. M.W.: 298000, Lot 68F-0475, Average. M.W.:266000.

Aquaphase PPT, which is a hydroxypropylated starch, comes from Perstorp Biolytica, S-223 70 LUND, Sweden; the number average molecular weight is $M_n = 30000$.

Reppal PES, which is also a hydroxypropylated starch, comes from Reppe Glykos AB, S-352 50 VÄXJÖ, Sweden; the average molecular weight is :

Type	$\bar{M}_n = \frac{\sum n_i M_i}{\sum n_i}$ (number average M.W.)	$\bar{M}_w = \frac{\sum n_i M_i^2}{\sum n_i}$ (weighted average M.W.)
Reppal PES 100	10000	100000
Reppal PES 200	20000	200000

The tandem CPC + ATPS has been tested for efficiency and selectivity by injection of crude albumin, Sigma product No. A-5253, except for the first system PEG 8000 - phosphate buffer, which has been tested with smaller molecules *i.e.* phenol and pyridine.

We have used phase diagrams of the aqueous two-phase systems found in Albertsson's book, except for Aquaphase PPT and Reppal PES, for which we have used the literature provided by the manufacturers.

Results and discussion

PEG 8000 / Phosphate buffer

This system has been tested with phenol and pyridine as test samples. The system was : PEG 8000 = 7.6% w/w, Phosphate buffer = 10% w/w, and the tie line was very close to the binodial.

The temperature was maintained at 35°C; we used the lower phase as the mobile phase in the descending mode, with a rotational speed of 1500 rpm, and a flow rate of 0.35 ml/min. In these conditions, the mobile phase volume, Vo, was ≈ 40 ml, and the back pressure, ΔP, was 35 bars (≈ 500 psi).

152 mg of phenol and 30 µl of pyridine in 4 ml of mobile phase were injected.

Elution mode was reversed after 19 hours to elute phenol which was strongly retained by the upper phase. The corresponding chromatogram (Figure 1) shows two peaks very well defined; the stability of the system is remarkable, and it is not necessary to introduce a rise time constant for the detector to artificially lower the noise level.

System PEG 1000 / $(NH_4)_2SO_4$

This system has been used by K.E. Lentz *et al.* [4] with a Craig-Post apparatus. The binodial has not been reported, but it should be close to the one in Albertsson's book for PEG 1540. We have used the following system :

	PEG 1000, % w/v	$(NH_4)_2SO_4$ % w/v
Total system	19.2	14.5
Upper phase :	32.6	7.4
Lower phase :	5.7	22.9

We have measured the density of the upper and lower phases by weighing 50 ml of each phase at 22°C (see Table I), and have roughly estimated the tie line using anion exchange HPLC for SO_4^{2-} determination.

This system has been extensively tested because it seems physically very well suited for CPC experiments.

Figure 2 shows bare profile corresponding to an injection of 200 mg of crude ovalbumin, and the corresponding size exclusion HPLC control of the eluite; there are two major components coming soon after the void volume (void volume means, as in HPLC, volume of the mobile phase in the CPC).

Stability of the stationary phase versus flow rate and rotational speed:

Figure 3 shows the volume, Vo, occupied by the mobile phase when varying the rotational speed, Figure 4 the CPC profile for a rotational speed of only 500 rpm and a flow rate of 1 ml/min, and Figure 5 Vo versus flow rate for various rotational speeds. The stability of the stationary phase is remarkable, and we can see, for example, that a rotational speed of 1400 rpm with a flow rate of 4.5 ml/min can be

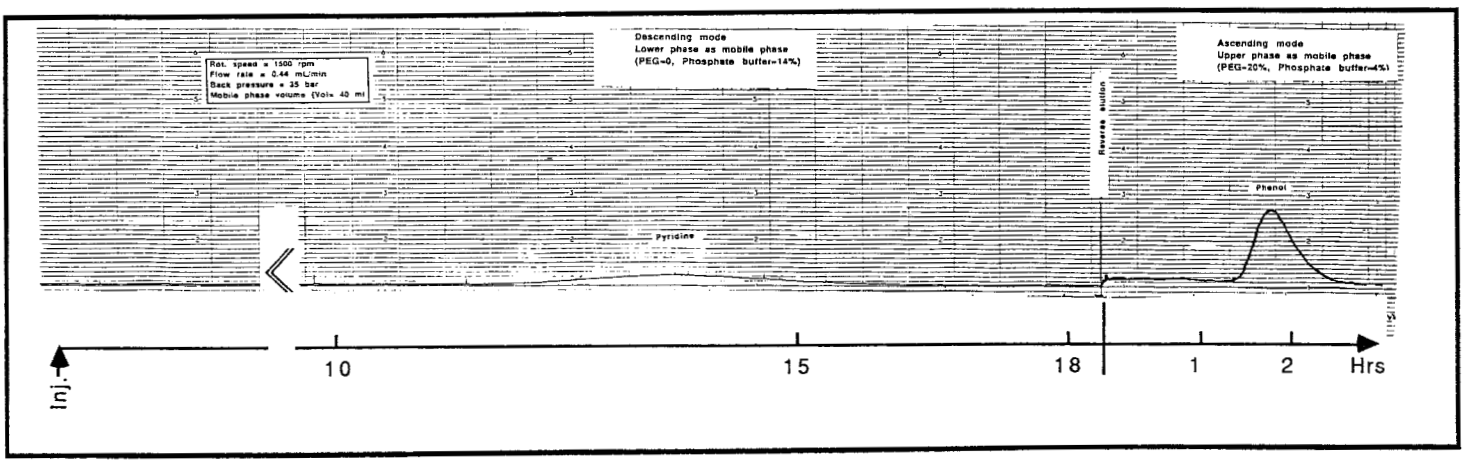


FIGURE 1. System PEG8000/potassium phosphate (7.6/10, %w/w) : elution of pyridine and phenol in the descending mode.
Flow rate : 0.44 ml/min
Rotational speed : 1500 rpm
Back pressure ≈ 35 bars (500 psi)
volume occupied by the mobile phase ≈ 40 ml
Ascending mode after 19 hours of elution in the descending mode.

TABLE I

Some properties of ATPS, and comparison with classic biphasic systems

System	Physical properties		CPC parameters (F=0.5 ml/min, ω = 1300 rpm)	
	Δρ density difference (g/cm^3)	Approx. settling time, min	Stationary phase (% tot. vol.)	ΔP, Asc. and Desc. mode (bar)
PEG 8000/Dx ind. gr.	0.064	17	50	20 and 50
PEG 8000/ Aquaphase PPT	0.059	12	54	22 and 31
PEG 8000/ Reppal PES 200	0.068	30	58	35 and 50
PEG 8000/ Reppal PES 100	0.055	30	54	25 and 30
PEG 1000/ $(NH_4)_2SO_4$	0.044	2	58	28 and 20
Hexane/ MeOH (1% H_2O)	0.046	0.2	62	40 (F=1ml/min)
$CHCl_3$/MeOH/H_2O	0.24 to 0.5	≈0.1	>60	≈50 or more
Various systems	0.1 to 0.2	<1		

reached without any loss of stationary phase. Even at low rotational speed (500 rpm, with F= 1 ml/min), the baseline is relatively noise-free (Fig. 4); the back pressure remains rather low regardless of the flow rate and the rotational speed.

Efficiency of the system versus flow rate and rotational speed:

Various combinations of flow rate and rotational speed have been tested to try to optimize the tandem CPC + ATPS, and efficiency has been estimated on the first peak, given by crude ovalbumin.

Figure 6 shows the number of plates versus the rotational speed for 0.5 and 1 ml/min; between 1000 and 1500 rpm, N remains between 450 and 550 plates, and there is no clear relationship when increasing the

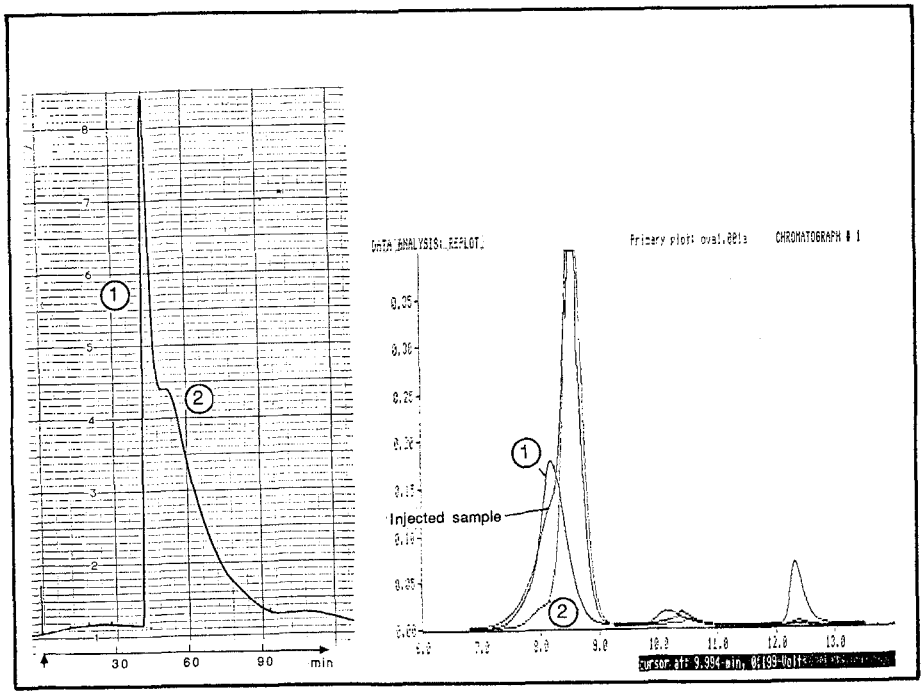


FIGURE 2. CPC profile :System PEG 1000/$(NH_4)_2SO_4$, 19.2-14.5 % w/v, pH=4.
ω = 1100 rpm, F=1 ml/min, Descending Mode, ΔP = 15 bars
Detection UV at 280 nm, 1 AUFS
Inj. : 200 mg of Ovalbumin in 4 ml

Size exclusion control
Column : Asahipac GS310,7.6 I.D. x 500 mm
Mobile phase : Phosphate buffer 0.1 M, pH = 6.8
Detection UV at 280 nm, 0.5 AUFS
Inj. 20 µl of CPC eluent, as indicated

rotational speed. If we examine Figures 7 and 8, which show the CPC profiles for two sets of experiments, we see that two combinations of flow rate/rotational speed give a better resolution for the two peaks given by crude ovalbumin, which are F = 0.45 ml/min with 1200 rpm, and F = 1.1 ml/min with 1100 rpm. These two sets have been run two times and give the same results. We must keep in mind that the flow rate

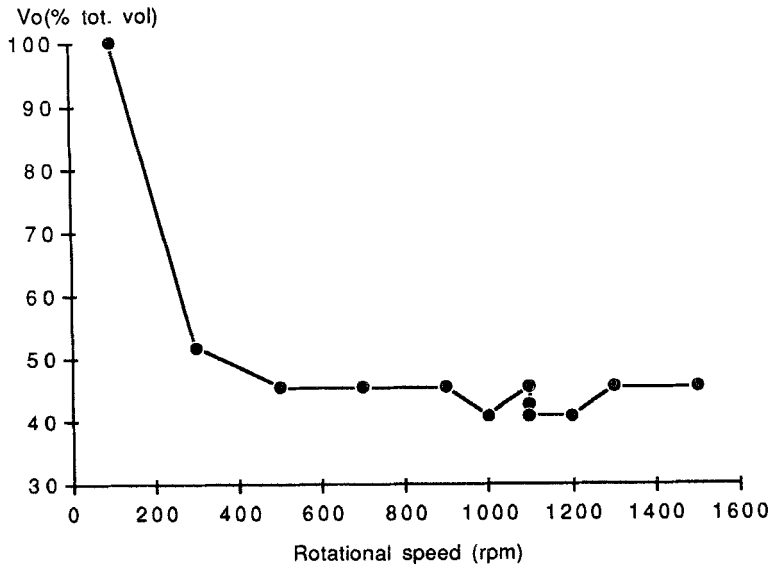


FIGURE 3. Volume (Vo) occupied by the mobile phase (as % total volume) versus rotational speed.
System PEG 1000-$(NH_4)_2SO_4$
19.2-14.5 % w/v,pH=4.
F ≈ 1 ml/min, descending mode
Solute : 200 mg OVA in 4 ml

determines the number of droplets/sec. which appear at the beginning of a channel, and the residence time of the mobile phase in that channel once the droplets are pooled at the end of the channel, while the rotational speed determines the velocity of the droplets in that channel.

Temperature effect :

Figure 9 shows the effect of temperature on CPC profiles. The efficiency of the system decreases markedly when the temperature decreases, and the second peak is affected more; the major reason for this effect is the increase of the viscosities of the two phases, which lowers the rate of mass transfer of a solute between them; the more a protein is partitioned, the more this effect is observed, and the CPC + ATPS conditions are then far removed from a steady state process.

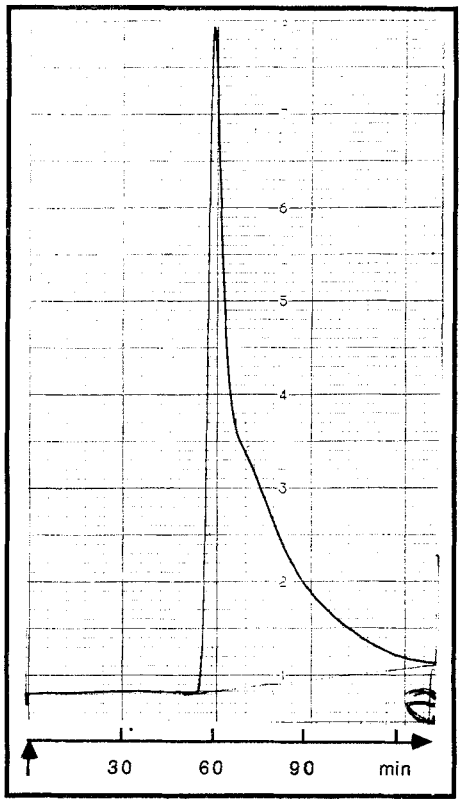


FIGURE 4. Stability of the stationary phase :
System PEG 1000/ $(NH_4)_2SO_4$
19.2% w/v 14.5% w/v
ω = 500 rpm, Flow rate = 1 ml/min, ΔP = 5 bars
T = 35°C, Descending mode
Solute : 200 mg OVA in 4 ml

When there is no problem of stability, aqueous two-phase systems must be used at high temperature to improve transfer of solutes between the two phases.

System PEG 8000 / Other Polymer (Dextran, industrial grade, or Aquaphase PPT, or Reppal PES).

Figure 10 gives the binodials for the four systems we have tested. Binodial for PEG 8000/Dx 37 has been found in Albertsson's book and used

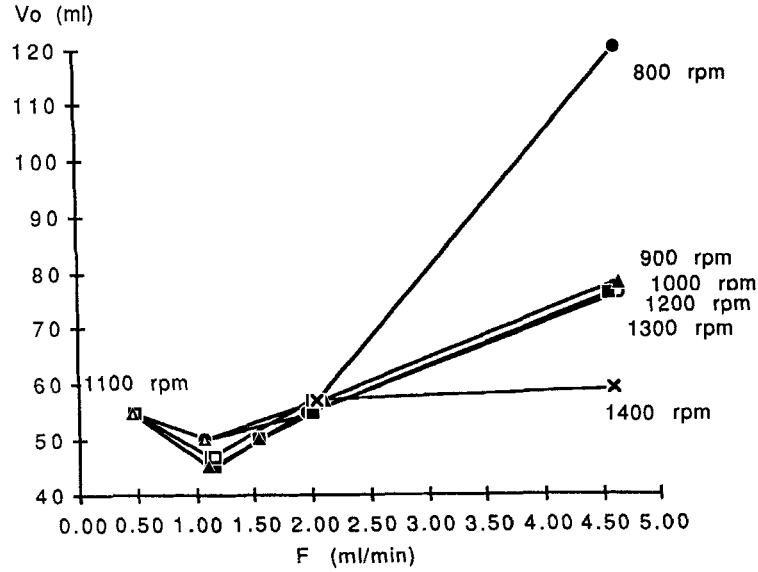


FIGURE 5. Stabitlity of the stationary phase (V_{tot}-Vo) versus flow rate and rotational speed
System PEG 1000 $(NH_4)_2SO_4$
19.2 14.5 % w/v, pH=4.
Descending mode
Solute : 200 mg OVA in 4 ml

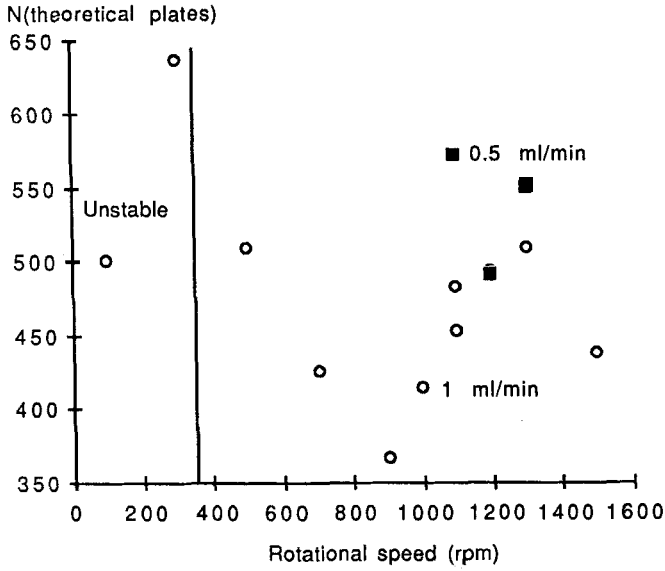
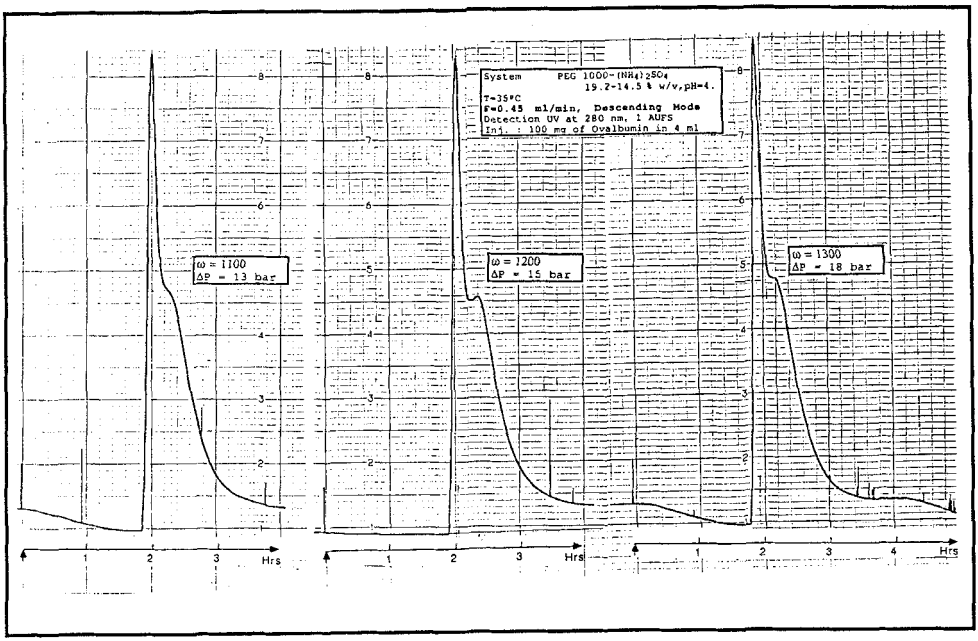


FIGURE 6. Efficiency versus rotational speed
System PEG 1000 $(NH_4)_2SO_4$
19.2 14.5 % w/v, pH=4
F = 1 & 0.5 ml/min, descending mode
Solute : 200 mg OVA in 4 ml



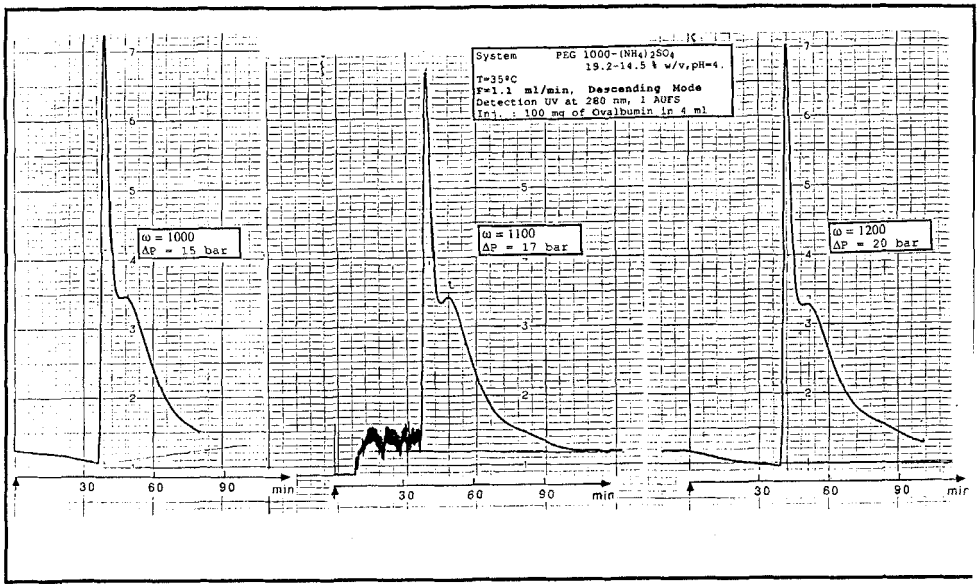


FIGURE 7. Influence of Flow rate and rotational speed on the CPC profiles of crude ovalbumin :
System PEG 1000 $(NH_4)_2SO_4$
19.2 14.5 % w/v, pH=4
Descending mode
Solute : 200 mg OVA in 4 ml
a : F = 0.45 ml/min, with ω = 1100, 1200, and 1300 rpm
b : F = 1.1 ml/min, with ω = 1000, 1100, and 1200 rpm

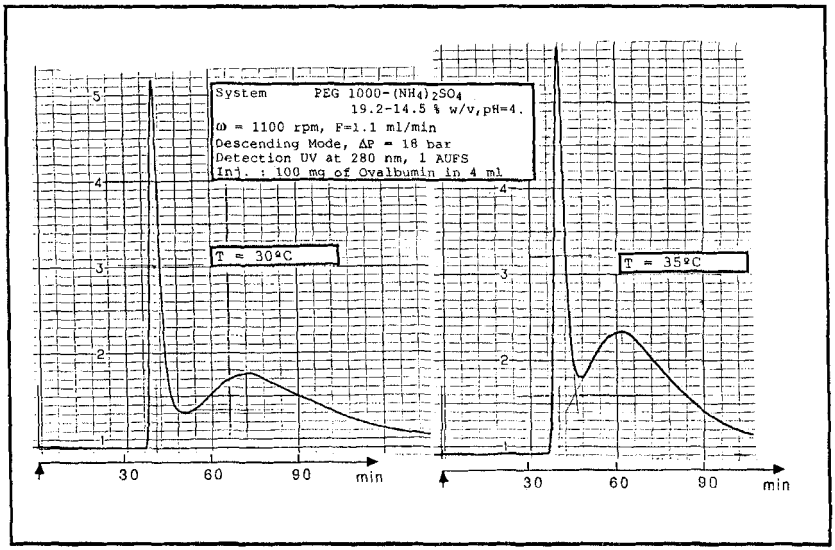


FIGURE 8. Effect of temperature.
System PEG 1000 $(NH_4)_2SO_4$
19.2 14.5 % w/v, pH=4
ω = 1100 rpm, Flow rate = 1.1 ml/min
Descending mode, ΔP = 18 bars
Solute : 200 mg OVA in 4 ml

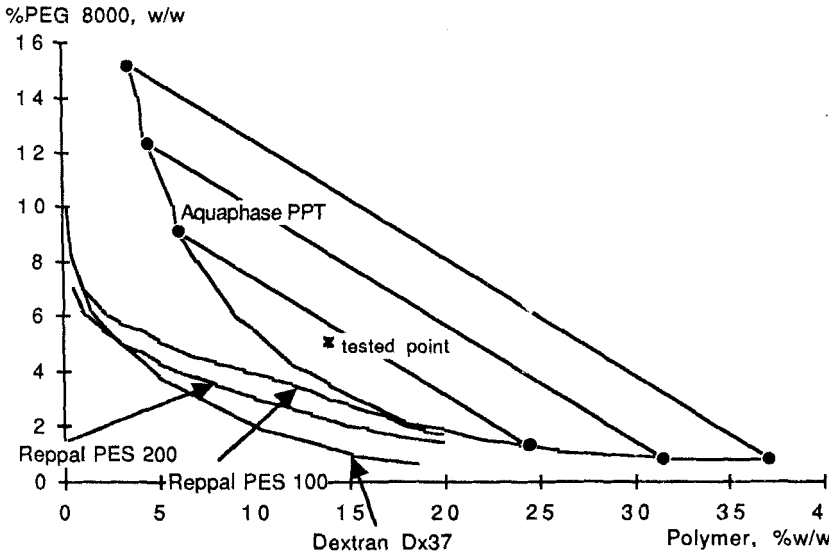


FIGURE 9. Comparison of phase diagrams of the systems PEG 8000/Aquaphase PPT, PEG 8000/Reppal PES 100&200, and PEG 8000/Dx 37.

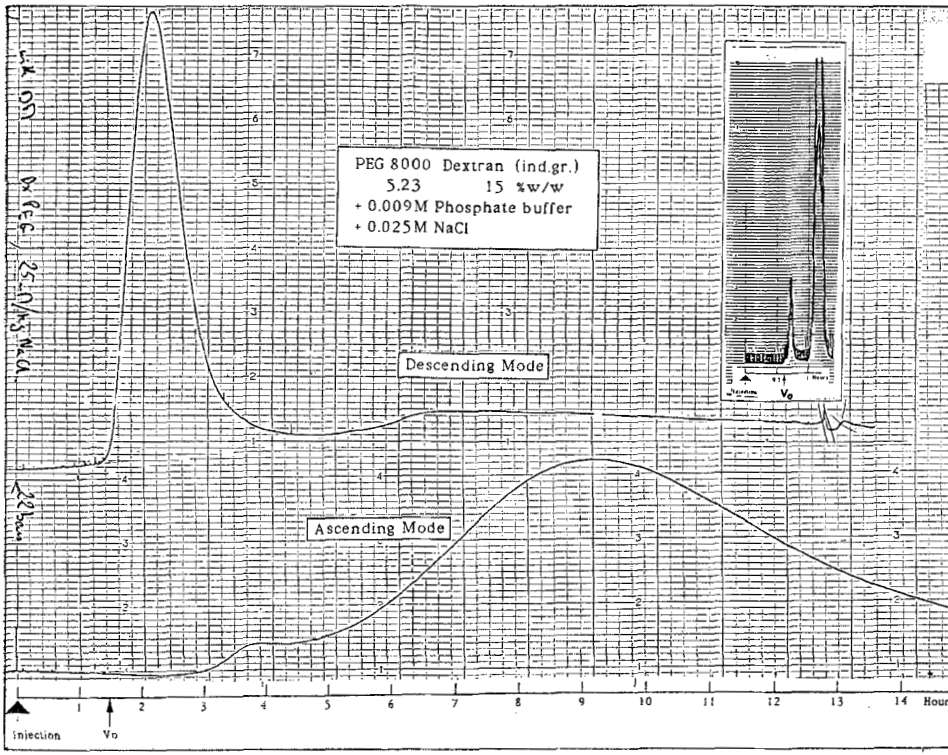


FIGURE 10. CPC profile of crude ovalbumin given by the system PEG 8000/ Dextran (ind. gr.)
Composition as shown
ω = 1200 rpm, Flow rate = 0.5 ml/min
T = 35°C
Solute : 200 mg OVA in 4 ml
The peak corresonding to the methyl ester of 2-naphthoic acid) eluted with the system Hexane/Methanol in similar conditions (1300 rpm, 1.1 ml/min) is shown at the same scale

as a good approximation to estimate the system PEG 8000/ Dx, ind. gr. (binodial not found). Binodial for Aquaphase PPT is given by the manufacturer, with the tie-lines, binodial for Reppal PES 100 and 200 are given by the manufacturer without tie-lines, and tie-lines for Reppal PES 200 have been recently determined[5].

Table I lets us compare some important parameters governing the properties of two phase systems (the density differences have been determined by weighing 50 ml of each phase at 22°C).

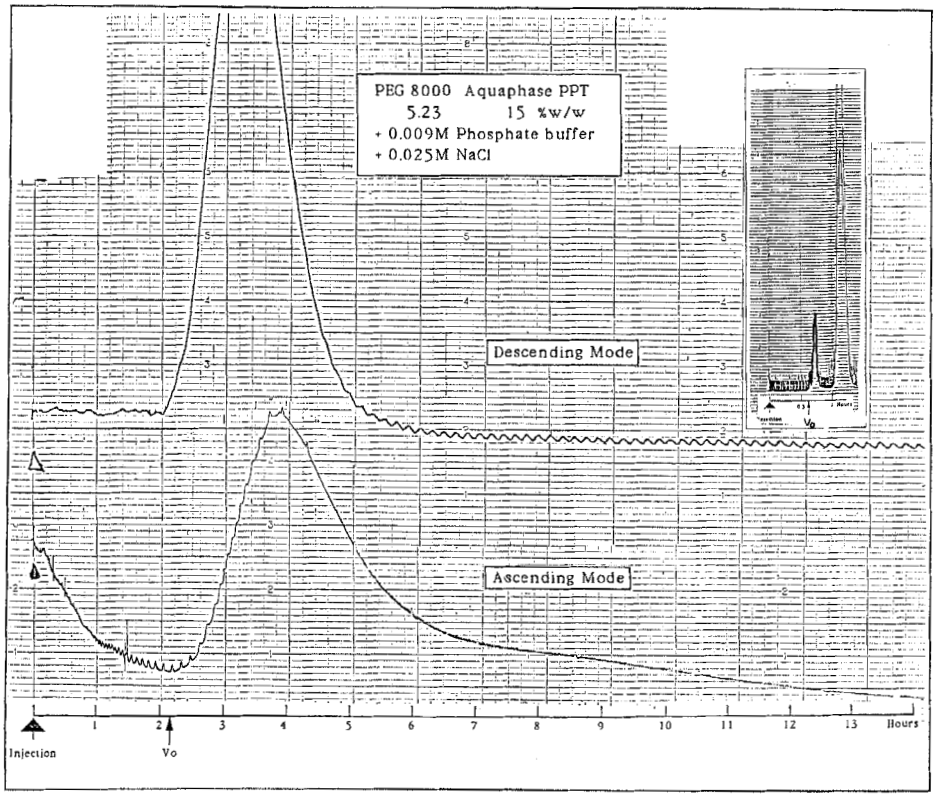


FIGURE 11. CPC profile of crude ovalbumin given by the system PEG 8000/ Aquaphase PPT
Composition as shown
ω = 1200 rpm, Flow rate = 0.5 ml/min
T = 35°C
Solute : 200 mg OVA in 4 ml
The peak corresonding to the methyl ester of 2-naphthoic acid) eluted with the system Hexane/Methanol in similar conditions (1300 rpm, 1.1 ml/min) is shown at the same scale

ATPS are compared to classical biphasic systems, and we can see that the system Hexane/Methanol has a density difference which is very close to that of ATPS, but a settling time which is ten times less than that of PEG/Salt system, and around one hundred times less than the PEG/Polymer system one.

Stability of the stationary phase:

Despite these very long settling times, PEG/Polymer systems are quite compatible with CPC requisites, which are medium pressure and good retention of the stationary phase. Moreover, the back pressure is rather small when compared to that produced by some common biphasic systems, mainly due to the very small density difference between the two phases.

Using the PEG/Dx system, we have increased the flow rate to check for stability; this increase does not affect the noise level given by the detector nor the back pressure, which remains quite small (18 bars for 3 ml/min and 1100 rpm, in the ascending mode), but the volume of the stationary phase decreases rapidly, and becomes null for 5 ml/min and 1100 rpm (it remains 36% of the total volume with the system Hexane/Methanol). This is mainly due to the high viscosity of the two polymer phases, as we can estimate the upper limit stationary phase volume, for a given flow rate, to be in the form $V_{stat.} = V_{tot.} - c \frac{\eta F}{\Delta\rho G}$, η being the viscosity of the stationary phase, F the flow rate, $\Delta\rho$ the density difference, and G the gravity field (based on the Stokes'law)[6].

Efficiency of the system :

Figures 11-14 give the profiles obtained for the injection of 200 mg of Ovalbumin by using the PEG/Polymer systems. As we can see, the efficiency of the CPC apparatus, which can be estimated by the chromatogram obtained by injecting a small molecule (Methyl ester of 2-naphthoic acid) eluted with the system Hexane/Methanol (shown on each figure at the same scale) disappears, and we get less than 30 theoretical plates produced by 2400 channels (six cartridges of 400 channels each).

The very poor mass transfer is the only reason for this loss of efficiency, as the droplets of mobile phase travel too fast in a channel, compared to the kinetic of transfer of the protein between mobile and stationary phase.

Three steps are required for highly efficient separations :

1- Mixing the 2 phases until equilibrium is reached, *i.e.* when the ratio of the concentration for a given protein is equal or very close to the partition coefficient; this step requires a long time (slow

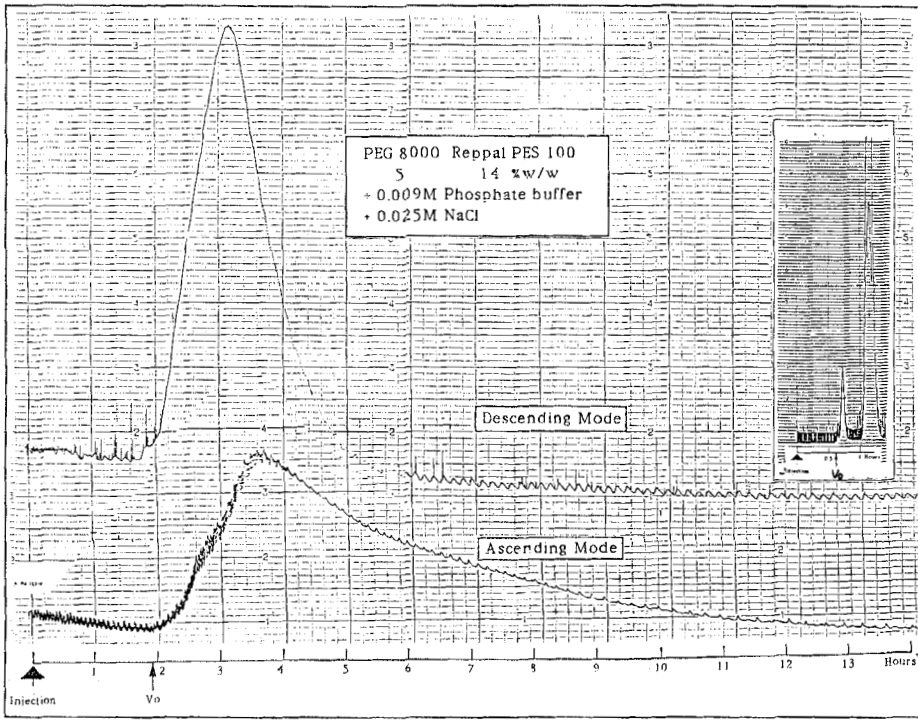


FIGURE 12. CPC profile of crude ovalbumin given by the system PEG 8000/ Reppal PES 100
Composition as shown
ω = 1200 rpm, Flow rate = 0.5 ml/min
T = 35°C
Solute : 200 mg OVA in 4 ml
The peak corresonding to the methyl ester of 2-naphthoic acid) eluted with the system Hexane/Methanol in similar conditions (1300 rpm, 1.1 ml/min) is shown at the same scale

diffusivity for proteins in a viscous solution) and/or a very large interface area compared to the bulk volumes of the two phases. The same problem has been encountered in classical chromatography, and has been solved first by using pellicular solid beads, then, later, small particles of silica with a high surface per mass unit.

Adequate shaking to assure complete mixing between phases and the material to be partitioned and to increase the area of interface is critical.

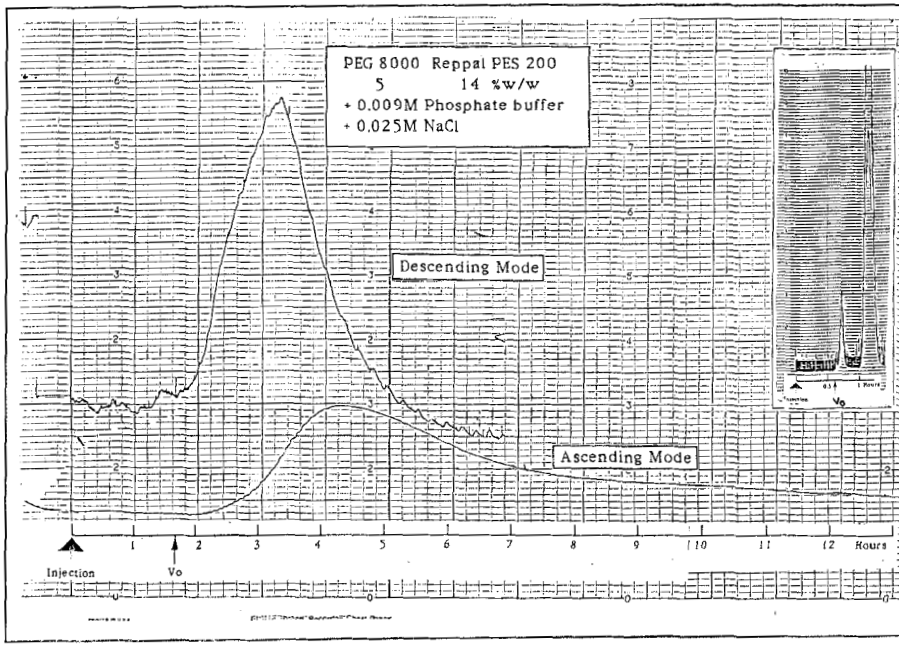


FIGURE 13. CPC profile of crude ovalbumin given by the system PEG 8000/ Reppal PES 200
Composition as shown
ω = 1200 rpm, Flow rate = 0.5 ml/min
T = 35°C
Solute : 200 mg OVA in 4 ml
The peak corresonding to the methyl ester of 2-naphthoic acid) eluted with the system Hexane/Methanol in similar conditions (1300 rpm, 1.1 ml/min) is shown at the same scale

2- Settling the 2 phases into 2 layers, which is readily achieved with a strong centrifugal field.

3- Transfer of the mobile phase to the next cell.

In CPC + ATPS tandem, steps 2 and 3 are easily obtained, while step 1 is not reached, because so far we cannot use sufficiently high flow rate to obtain good mixing in each cell.

Armstrong *et al.*[7] have studied the origin and mechanism of band broadening in CPC, and proposed the following equation :

$$-Ln(1-E) = A/F + B\,F^{b}$$

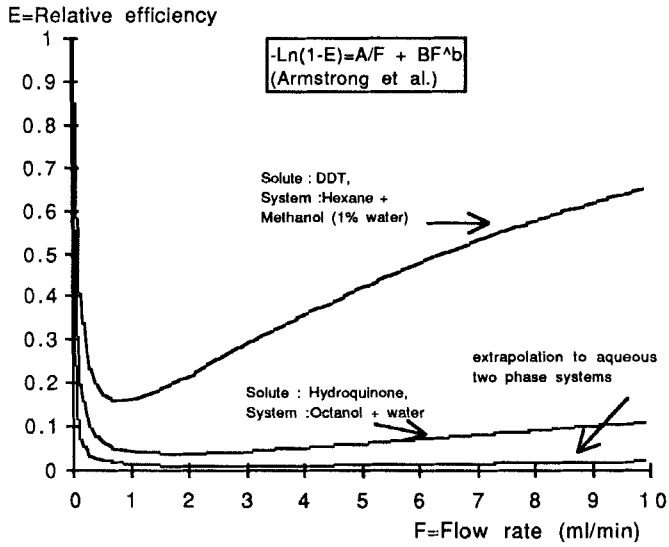


FIGURE 14. Variations of the CPC efficiency with flow rate, as defined by Armstrong et.al. (ref 7), and extrapolation to ATPS. Efficiency is defined as the ratio of the change in concentration of solute actually occuring in the mobile phase over the change that would occur in the mobile phase if the system were allowed to reach equilibrium

Data for the graph :

System	Solute	A	A/B	b
Hexane/Methanol	DDT	0.07	0.66	1
Octanol/Water	Hydroquinone	0.035	2.6	1
Extrapolation to ATPS	protein	0.01	5	1

where E is the relative efficiency of the system, F is the flow rate, A and B depend on the solute (rate of transfer, partition coefficient), as well as the solvent system (viscosity, density difference), and the geometry of the channels and conditions of the experiment.

Figure 15 shows the results obtained by Armstrong *et al.* and the extrapolation we can roughly predict for ATPS, using the same cartridges : for ATPS, the efficiency decreases quickly with F, and then

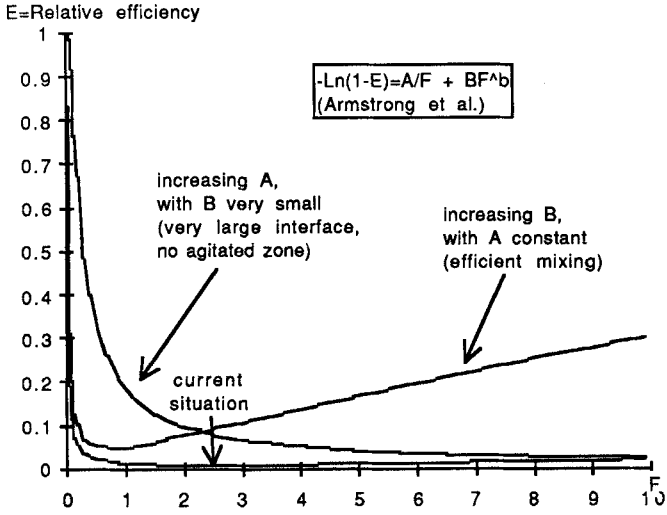


FIGURE 15. Evolution of Armstrong's curves with A and B

stays close to 0. As we cannot basically modify the viscosity of the phases nor the diffusion coefficients of proteins in these phases, the only way to try to enhance the CPC + ATPS tandem is to modify the design of the cartridges, to get an efficient mixing and/or a very large interface with a very small bulk volume.

The two terms of the Armstrong's equation can be assigned to the two mechanisms of partition in a channel, *i.e.* partition in an agitated zone starting from the inlet of the mobile phase ($B F^b$), and partition through the cross section of a channel, between the two phases, after the droplets are pooled in a quiescent zone, and before the resulting zone being transfered to the next channel (A/F).

We can see in Figure 15, the evolution of the Armstrong's curve when increasing A (very large interface, no agitated zone) or B (very efficient mixing zone, followed by decantation and transfer).

Conclusion

Use of CPC in conjunction with ATPS encounters the same problems as found in the early beginning of high performance liquid

chromatography, when the mass transfer was the limitating step of a more powerful system; pellicular packing, then smaller particles have overcome the problem and opened the way to modern HPLC. Reshaping the channels in a proper way to enhance the contact between the two phases and minimize the bulk volumes should give to CPC+ATPS more plates. The new polymers, Aquaphase PPT and Reppal PES, are very inexpensive substitutes for expensive Dextrans, and give better results (less back pressure, same or better efficiency); this could develop a low cost and efficient preparative method for protein isolation in the near future.

Acknoledgements

The studies were supported by grants NIH AI 10187.

References

[1] P. A. Albertsson, "Partition of cells particles and macromolecules", Wiley & sons Ed., 1986

[2] H. Walter, D.E. Brooks, D. Fisher, "Partitioning in aqueous two-phase systems", Academic Press, inc., 1985

[3] PEG is Polyethylene Glycol, Dx is Dextran

[4] K.E. Lentz, L.T. Skeggs, Jr., H. Hochstrasser, J.R. Kahn, "Countercurrent Distribution of Renin Substrate and Serum Protein in Polyethylene Glycol-salt Systems", Biochim. Biophys. Acta, 69 (1963) 263-270

[5] B. Mattiasson, T. Ling, Department of Biotechnology, Chemical Center, University of Lund, Sweden

[6] A. Foucault, K. Nakanishi, in preparation

[7] D.W. Armstrong, G.L. Bertrand, A. Berthod, "Study of the Origin and Mechanism of Band Broadening and Pressure Drop in Centrifugal Partition Chromatography", Anal. Chem., 60 (1988) 2513-2519.