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# Toroidal coil counter-current chromatography study of the mass transfer rate of proteins in aqueous–aqueous polymer phase system

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**Abstract**

The cause of excessive band broadening of protein samples in polymer phase partitioning by counter-current chromatography (CCC) was investigated. A simple rotary device was constructed to measure the mass transfer rates of five samples including potassium dichromate, methylene blue, lysozyme, ovalbumin and human serum albumin. The results indicated that the mass transfer rates of these samples are closely correlated with their molecular masses: the higher the molecular mass, the lower the mass transfer rate. These findings are also consistent with the partition efficiencies of these samples in the same solvent system by CCC. The beneficial effect of the Coriolis force demonstrated in protein separations by the toroidal coil centrifuge may be reasonably explained on the basis of the mass transfer resistance of protein molecules through the interface: we speculate that when the Coriolis force acts parallel to the effective coil segment it can produce large interfacial areas by dispersing the mobile phase into the stationary phase, thus accelerating the mass transfer rate of protein samples. © 1998 Elsevier Science B.V.

*Keywords:* Counter-current chromatography; Mass transfer; Proteins

## 1. Introduction

Partitioning of proteins can be performed using polymer biphasic systems that were introduced by Albertsson in the 1950s [1]. Traditionally, the partitioning is carried out using either a series of separatory funnels or Albertsson's thin-layer counter-current distribution apparatus [1]. In both methods the analytes are fully equilibrated with the polymer phase system in each partition step. Consequently, the partition efficiencies in these equilibrium systems are predetermined by the number of partition units.

More recently, counter-current chromatography (CCC) has been successfully applied to the partitioning of proteins using polymer phase systems. Using a cross-axis coil planet centrifuge [2–4] and a toroidal coil centrifuge [5], various protein samples including various recombinant enzymes have been separated using polymer phase systems composed of polyethylene glycol (PEG) and potassium phosphate at various concentrations. In these CCC techniques, analytes are subjected to a continuous partition process in a long tubular path under a centrifugal force field. Thus the partition efficiencies of these non-equilibrium systems vary according to the degree of mixing of the two phases and the mass transfer resistance of the analytes in the solvent system. Further studies revealed that the proteins

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separated with these polymer phase systems tend to produce substantially broader peaks compared with those from small molecules partitioned under identical experimental conditions [4]. This adverse phenomenon in the separation of proteins may have several explanations including (1) steadily changing three-dimensional structures of protein molecules during separation, (2) partially denatured components present in the sample, (3) the slow mass transfer rate of large protein molecules through an interface of the polymer phase system, and (4) combinations of the above.

This paper is focused on the study of the mass transfer rate of proteins through the interface of a PEG–potassium phosphate system. A simple rotary devise is constructed and used to measure the mass transfer rates of various samples through the interface of the polymer phase system. The results are correlated with the partition efficiencies of these analytes in CCC separation by the toroidal coil centrifuge.

## 2. Experimental

### 2.1. Reagents

PEG 1000 was purchased from Sigma (St. Louis, MO, USA) and dibasic potassium phosphate and potassium dichromate both of reagent grade from Fisher Scientific (Fair Lawn, NJ, USA). Protein samples including cytochrome *c*, myoglobin (horse skeletal muscle), lysozyme (chicken egg), ovalbumin (chicken egg) and human serum albumin are obtained from Sigma and methylene blue chloride from Martman-Leddon (Philadelphia, PA, USA).

### 2.2. Preparation of a polymer phase system

The polymer phase system was prepared by dissolving PEG 1000 and dibasic potassium phosphate each at 12.5% (w/w) in distilled water. The mixture was well stirred in a glass bottle at room temperature and the two phases were used for the CCC separation after clear layers were formed.

### 2.3. Measurement of mass transfer rates

A prototype of the simple rotary device (see Fig. 1) is designed to measure mass transfer rates of samples through the polymer phase system in a test tube. The apparatus consists of a motor which drives a rotary shaft symmetrically supporting a set of five plastic centrifuge tubes (15 ml capacity). Each centrifuge tube accommodates a glass test tube (100×13 mm O.D.) containing a polymer phase system to be tested. The rotary shaft is inclined at 18° against the horizontal line and the rotation is optimized at 30 rpm with a speed regulator.

In each measurement, the sample was dissolved in 2 ml of the lower phase of the above polymer phase system which was then delivered into a glass test tube. The sample concentrations ranged from 0.1 mg to 5 mg per milliliter depending on the molecular

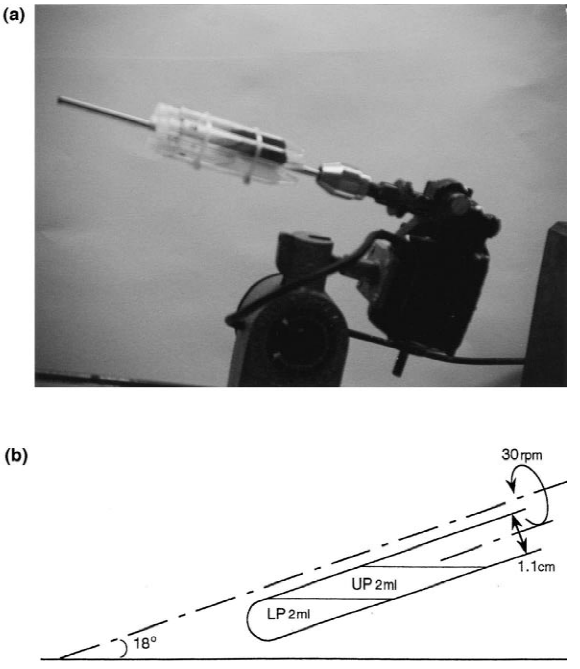


Fig. 1. Simple rotary device for measuring the mass transfer rate of analytes. (a) Overview of the device; (b) schematic diagram indicating the orientation and motion of the rotating tube showing an interface horizontally located between the two phases. The actual interface contributing the mass transfer is the sum of the elliptic interface between the two layers (3.04 $cm^2$) and the semicylindrical interface formed over the tube wall by rotation (5.85 $cm^2$).

mass and solubility of the sample in the polymer phases. Then 2 ml of the upper phase was gently layered over the lower phase.

A set of five tubes was accommodated into the centrifuge tubes and rotated at 30 ml/min. At intervals of 5, 10, 15, 20, 30 and 50 min, the rotation was interrupted and a small aliquot (50 μl) of the upper and lower phases was pipetted from each tube for spectrophotometric analysis at 280 nm (Zeiss PM 6, Zeiss, Hanover, MD, USA).

### 2.4. CCC procedure

The CCC separations of various samples were performed using a toroidal coil centrifuge. The design and principle of the apparatus have been reported earlier [6] so only brief description is given here. The apparatus is a modified commercial floor-model centrifuge (CRU-5000, Damon/IEC Division, Needham Height, MA, USA). The centrifuge head is newly designed so that continuous elution is possible without the use of a conventional rotary seal device [7]. A long toroidal coil is accommodated around the periphery of the flat centrifuge bowl (34 cm diameter) by making multiple turns forming a doughnut shaped configuration. The column was prepared from a single piece of PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) of about 60 m×0.55 mm I.D. which is wound around a 7 m of nylon pipe of 1.5 mm O.D. making about 10 000 turns. The total capacity measures about 13 ml.

Each experiment was initiated by filling the separation column with either upper or lower phase as the stationary phase. This was followed by injection of the sample solution (0.5 ml if otherwise indicated) containing 0.1 to several mg of analyte(s). Then the other phase was introduced through the inlet of the column while the apparatus was rotated at 1200 rpm (270 *g*). The effluent from the outlet of the column was continuously monitored at 280 nm and collected at 0.4 ml per tube using a fraction collector (LKB, Stockholm, Sweden). In order to facilitate clear tracing of the elution curve, the effluent was continuously diluted with an equal volume of distilled water before entering the flow cell using a syringe pump (Pump 22, Harvard Apparatus, South Natick, MA, USA) and a tee-junction inserted on the flow line near the inlet of the UV monitor. The collected fractions were also manually analyzed their absorbance at 280 nm using a PM6 Zeiss spectrophotometer.

### 2.5. Analysis of protein samples

The purity of all protein samples was checked by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

## 3. Results and discussion

### 3.1. Mathematical treatment of mass transfer rates

We followed the theoretical approach described by Fedotov et al. [8] who analyzed the mass transfer rates of metal ions in a propeller-driven mixing chamber. For our studies a simple rotary system was devised to measure the mass transfer rates of various samples through the interface of the polymer phase system. Fig. 1b shows a test tube containing the polymer phase system forming an interface, the lower phase (LP) containing an analyte at a concentration of $C_0$ and the upper phase (UP) is free of analyte. The tube is tilted at 18° against the horizontal plane and rotated at 30 rpm as indicated in the diagram. This slow rotation results in mixing of each phase while preserving the shape of the interface between the two phases. Consequently, the analyte present in the lower phase is gradually transferred into the upper phase through the interface until the distribution equilibrium is reached between the two phases. If the analyte concentration in the lower phase is $C_t$ at time $t$, and the mass transfer rate, $R$, is assumed to be proportional to the difference between $C_t$ and $C_\infty$ (equilibrium concentration of the analyte in the lower phase), the following relationship will hold:

$$-\mathrm{d}C_t = (A/V)R(C_t - C_\infty)\mathrm{d}t \quad (1)$$

where $A$ is the interfacial area and $V$, the volume of the lower phase. Integrating Eq. (1) gives

$$-\ln\left[(C_t - C_\infty)/(C_0 - C_\infty)\right] = R(A/V)t \quad (2)$$

If Eq. (2) is valid, plotting the left term, $-\ln$

$[(C_t - C_\infty)/(C_0 - C_\infty)]$, against time $t$ on the diagram should produce a straight line where $R$ is computed from its slope provided that $A/V$ is known.

### 3.2. Mass transfer rates of various samples

Using the rotary device, a series of experiments was performed to measure the mass transfer rates of five samples including potassium dichromate ($M_r$ 294), methylene blue ($M_r$ 374), lysozyme ($M_r$ 14 000), ovalbumin ($M_r$ 45 000) and human serum albumin ($M_r$ 68 000). At various times of 5, 10, 15, 20, 30 and 50 min the rotation was stopped and an aliquot (50 μl) of each phase was taken for spectrophotometric analysis. A typical result is shown in Fig. 2a where $-\ln [(C_t - C_\infty)/(C_0 - C_\infty)]$ is plotted against the time in minutes for the above five samples.

All samples show some exponential trend deviating from a straight line indicating that the mass transfer process is more complicated than assumed. The results however, clearly show that the mass transfer rate is closely correlated with the molecular mass of the sample: potassium dichromate ($M_r$ 294) reaches near saturation (over 3) within 5 min while human serum albumin ($M_r$ 68 000) fails to reach the same level even after 50 min of rotation.

From Fig. 2 the mass transfer rate of these analytes may be approximated from the slope of an early, nearly straight portion of the curve for each sample. Using Eq. (2), actual values (cm/s) are obtained by inserting 2 ml for $V$ (volume of the lower phase) and 8.9 $cm^2$ (interface area formed between the two phases) for $A$ as well as the slope of each curve in Fig. 2a. The results are shown in Fig. 2b where the mass transfer rate is plotted in ordinate against the molecular mass in a logarithmic scale on the abscissa. The diagram indicates that the mass transfer rate decreases as the molecular mass of the analytes increases. Here it is interesting to note that the data points of all organic molecules including methylene blue and three proteins fall on a straight line (dotted). On the other hand, potassium dichromate which represents an inorganic molecule exhibits an extremely high mass transfer rate of $2.67 \cdot 10^{-3}$, suggesting that the size of the molecule may play a more important role in the mass transfer process than the molecular mass.

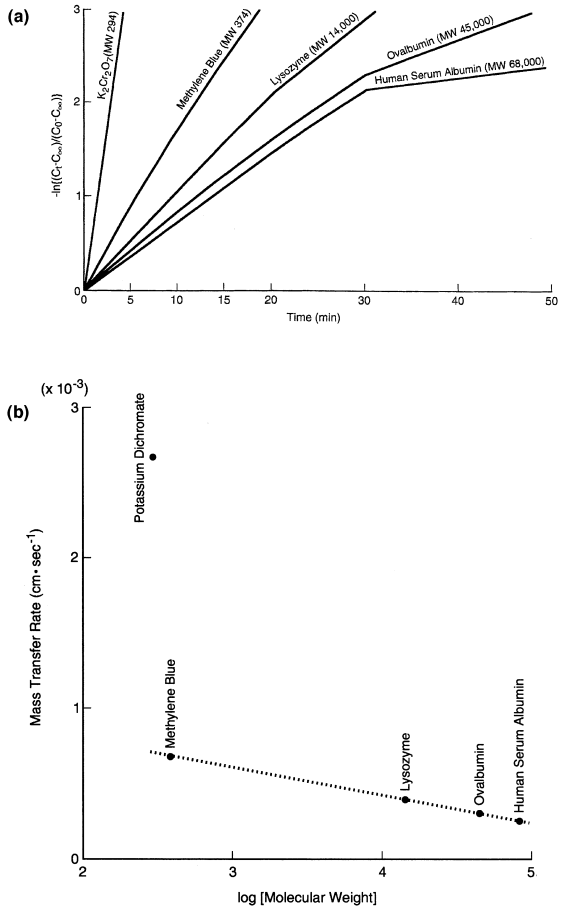


Fig. 2. Mass transfer rates of various analytes measured by the rotary device. (a) $-\ln [(C_t - C_\infty)/(C_0 - C_\infty)]$ vs. time plot (MW= molecular mass); (b) mass transfer rate (cm/s) vs. log (molecular mass) of the analytes.

These differences in the mass transfer rates of various samples should be reflected in the partition efficiencies of the same sample in CCC separation and this is described in Section 3.3.

### 3.3. CCC experiments

The first series of studies demonstrates the difference in mass transfer rate between the small molecules and the proteins used in the above basic study. Four samples including potassium dichromate, methylene blue, lysozyme and human serum albumin, were individually partitioned in the polymer phase system using the toroidal coil centrifuge. From

the resulting chromatograms the partition efficiencies were estimated and expressed in terms of theoretical plate number. The ovalbumin sample was eliminated from this study because it formed two closely associated bands in the SDS–PAGE and in this case the measurement of the theoretical plates for each component would require extremely high peak resolution.

A set of data in Table 1 shows that, consistent with the results obtained from the rotating tube studies, the partition efficiency of the analytes decreases as their molecular mass is increased. Importantly, the data indicates that among the two separations for each analyte the first elution mode (parallel to the Coriolis force) always produces higher theoretical plate numbers than those obtained from the second elution mode (crossing the Coriolis force). A similar phenomenon has been found in the separation of myoglobin and lysozyme with a 1 mm I.D. toroidal coil [9]. In both cases, the retention of the stationary phase appears to play no significant role in the improved partition efficiency. The present study further reveals that the effect of this Coriolis force is more pronounced in the higher-molecular-mass compounds such as human serum albumin than small molecules such as potassium dichromate and methylene blue.

During the centrifugation, the Coriolis force acts on the moving droplets to influence their flow direction. As illustrated in Fig. 3 (bottom), the Coriolis force produced by rotation at $\omega$ as indicated acts toward the right on the descending droplets (the heavier phase) and toward the left on the ascending droplets (the lighter phase). This results in shifting the motion of these droplets from the radial path toward the corresponding direction. When this Coriolis flow is nearly parallel to the coil segment (Fig. 3A), the mobile phase may be dispersed into the stationary phase to form multiple droplets, thus providing a larger surface area for the mass transfer of analytes. On the other hand, when the Coriolis flow forms an angle to the effective coil segment, the mobile phase tends to stream along the tube wall, providing much less interface area for the mass transfer of analytes. The fact that the Coriolis force produces the greatest effect on human serum albumin among all the samples applied is consistent with the earlier finding from the rotary tube experiment that higher-molecular-mass compounds have greater mass transfer resistance. In the second series of experiments, the effect of the Coriolis force is further demonstrated on CCC separations of a mixture of cytochrome *c*, myoglobin and lysozyme in the polymer phase system at various flow-rates. The results are shown in Fig. 4 where six chromatograms are arranged according to the acting mode of the Coriolis force and applied flow-rates. The partition efficiencies expressed in terms of theoretical plate

Table 1
Partition efficiencies of various samples in CCC separation by toroidal coil centrifuge

| Sample ($M_r$) | Mass (mg) | $K$ | Mobile phase[a] | Coriolis force[b] | TP | TP ratio Para/cross | Retention (%) |
|---|---|---|---|---|---|---|---|
| $K_2Cr_2O_7$ | 1 | 1.72 | UP | para | 1350 | 1.29 | 37.7 |
| (294) | 1 | 1.72 | UP | cross | 1050 | | 35.9 |
| | 1 | 0.58 | LP | para | 1930 | 1.15 | 31.0 |
| | 1 | 0.58 | LP | cross | 1700 | | 36.1 |
| Methylene | 0.1 | 0.63 | UP | para | 910 | 1.25 | 33.3 |
| blue (374) | 0.1 | 0.63 | UP | cross | 730 | | 39.6 |
| Lysozyme | 2.5 | 0.67 | UP | para | 376 | 1.16 | 39.2 |
| (14 000) | 2.5 | 0.67 | UP | cross | 323 | | 39.2 |
| Human serum | 1.5 | 0.48 | UP | para | 300 | 1.95 | 39.2 |
| albumin (68 000) | 1.5 | 0.48 | UP | cross | 154 | | 39.2 |

[a] UP: Upper phase; LP: lower phase.
[b] Para: parallel; cross: crossing.

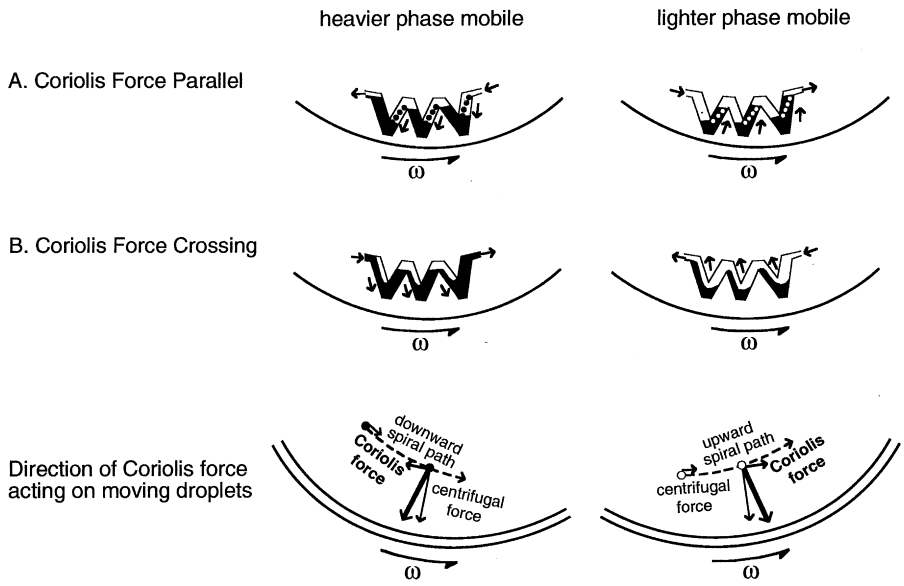


Fig. 3. Coriolis force acting on the moving droplets on the rotating body (bottom) and its effect on the flow pattern of the mobile phase in the effective coil segments. (A) Droplet flow of the mobile phase produced by the Coriolis flow nearly parallel to the effective coil segments; (B) streaming flow of the mobile phase produced by the Coriolis flow forming an angle to the effective coil segments.

numbers (TPs) and peak resolution ($R_s$) in these separations are indicated in Table 2 together with the retention % of the stationary phase.

At the lowest flow-rate of 50 μl/min (left chromatograms), all components were well resolved in both elution modes. As the flow-rate is increased to

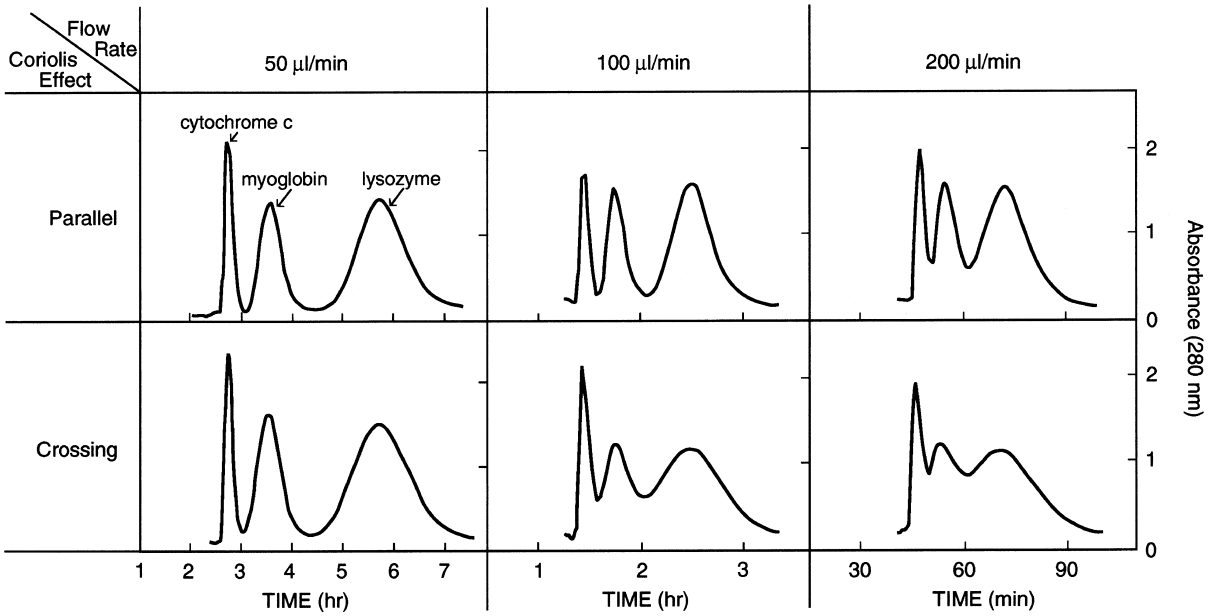


Fig. 4. Effect of the Coriolis force on CCC separation of three proteins at three different flow-rates.

Table 2
Effects of Coriolis force on partition efficiencies of three stable proteins

| Flow-rate (μl/min) | Analyte peak | TP (para/cross) | $R_s$ (para/cross) | Retention (%) (para/cross) |
|---|---|---|---|---|
| 50 | Cytochrome *c* | 1860/1490 | 1.62/1.39 | 29.2/32.0 |
| | Myoglobin | 365/266 | 1.66/1.40 | |
| | Lysozyme | 156/104 | | |
| 100 | Cytochrome *c* | 1760/821 | 1.27/0.86 | 30.0/30.3 |
| | Myoglobin | 433/172 | 1.39/0.84 | |
| | Lysozyme | 172/63 | | |
| 200 | Cytochrome *c* | 1296/– | 0.84/– | 22.8/21.3 |
| | Myoglobin | 330/– | 0.92/– | |
| | Lysozyme | 123/– | | |

100 μl/min (center), the lower chromatogram (Coriolis crossing mode) shows substantial loss in peak resolution while the upper chromatogram (Coriolis parallel mode) still maintains good resolution for all peaks. At the highest flow-rate of 200 μl/min (right) which produced significant loss of the stationary phase, both elution modes show loss of peak resolution but especially in the lower chromatogram (Coriolis crossing mode). Throughout all flow-rates the upper chromatograms (Coriolis parallel mode) yield substantially higher partition efficiencies than the lower chromatogram (Coriolis crossing mode) in both theoretical plate number (TP) and peak resolution ($R_s$). Here again there is no significant difference in the retention of the stationary phase between the two elution modes.

## 4. Conclusions

The mass transfer rate through an interface of polymer phase systems closely correlates with the molecular mass of the analytes: the higher the molecular mass, the lower the mass transfer rate. This finding explains the excessive band broadening of proteins in CCC separation as well as the beneficial effect of the Coriolis force on these proteins, especially for high-molecular-mass compounds.

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## References

[1] P.A. Albertsson, Partition of Cells and Macromolecules, Wiley-Interscience, New York, 1986.
[2] Y. Shibusawa, Y. Ito, J. Chromatogr. 550 (1991) 695.
[3] Y. Shibusawa, in Y. Ito and W.D. Conway (Editors), High-Speed Counter-current Chromatography, Wiley-Interscience, New York, 1996, Ch. 13, p. 385.
[4] K. Shinomiya, J.-M. Menet, H.M. Fales, Y. Ito, J. Chromatogr. 644 (1993) 215.
[5] L. Qi, Y. Ma, Y. Ito, H.M. Fales, J. Liq. Chromatogr. 21 (1998) 83.
[6] Y. Ito, R.L. Bowman, Anal. Biochem. 85 (1978) 614.
[7] Y. Ito, J. Suaudeau, R.L. Bowman, Science 189 (1975) 999.
[8] P.S. Fedotov, T.A. Maryutina, V.M. Pukhovskaya, B.Ya. Spivakov, J. Liq. Chromatogr. 17 (1994) 3491.
[9] Y. Ito, Y. Ma, J. Liq. Chromatogr. 21 (1998) 1.