ELSEVIER

Journal of Chromatography A, 790 (1997) 17–30

JOURNAL OF CHROMATOGRAPHY A

Dual-mode high-speed counter-current chromatography: retention, resolution and examples

M. Agnely[1], D. Thiébaut*

Laboratoire de Chimie Analytique de L'Ecole Supérieure de Physique et de Chimie Industrielles de la Ville de Paris, URA CNRS 437, 10 Rue Vauquelin 75231 Paris cedex 5, France

Received 27 February 1996; received in revised form 12 May 1997; accepted 30 June 1997

Abstract

Counter-current chromatography (CCC) is a very versatile technique offering high resolution power in recovering very pure compounds from complex matrices. Dual-mode CCC where the phase role is reversed during the separation is investigated here; it ensures elution of all the injected species from the column while, unlike backflush, the separation is still progressing after phase reversal; equations giving retention and retention factor are derived from the basic equations of chromatography. Compared to normal-mode CCC, it is shown that enhanced resolution in dual-mode CCC can be obtained in conditions derived from a theoretical model. The experimental section provides the validation of the retention prediction while resolution is also proven to be enhanced in dual-mode CCC. However, equations given in the theoretical section cannot fully explain the results obtained for resolution because they do not deal with kinetics. Dual-mode CCC has also been applied to separation of polyoxypropylene glycol polymers; separation can be achieved with a small number of experiments because all the injected compounds are eluted by reversing the phases. Dual-mode CCC also gave improved yields in the purification of antibiotics compared with previous results using normal-mode CCC. © 1997 Elsevier Science B.V.

Keywords: Counter-current chromatography; Retention prediction; Resolution; Polyoxypropylene glycol; Fatty acids; Antibiotics

1. Introduction

Modern counter-current chromatography (CCC) was introduced in the 1980s when a strong centrifugal force field was applied to a coiled column (type J coil planet centrifuge) or to channels engraved in the rotor of a centrifuge to lead respectively to high-speed CCC (HSCCC) and to centrifugal partition chromatography (CPC). Both use two liquid phases, the stationary and the mobile phases. Various applications have demonstrated their ability to perform preparative separations [1], partition coefficients determination [2] or extraction/separation of compounds from complex matrices [3].

Advantages over liquid chromatography come from the large volume of stationary phase available for partition and from the high selectivity provided by biphasic solvent systems. Owing to the liquid nature of the stationary phase irreversible retention is impossible because one can empty the column to

*Corresponding author.

[1]Present address: Rhône-Poulenc Aubervilliers, STP, 52 Rue de la Haie Coq, 93208 Aubervilliers, France.

0021-9673/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.
PII S0021-9673(97)00742-5

recover the solutes trapped in the stationary phase or reverse the elution mode, the initial mobile phase becoming the stationary phase and vice versa. The latter has been called dual-mode CCC [4–8] or reverse-development CCC [5]. Various applications showed its usefulness in reducing analysis time in preparative CCC [6] and in determining larger values of partition coefficients [7].

The theoretical development of partition coefficient determination using dual-mode CCC was provided by Menges et al. [8] and Gluck et al. [7] but equations expressing elution times were not provided. Furthermore distribution of the stationary phase in a HSCCC column was not studied.

This paper deals with the theoretical treatment of retention and resolution in dual-mode CCC. The results described here are valid for any CCC system. Nevertheless, owing to the shape of the column, retention of the stationary phase in a HSCCC apparatus is more dependent on operating conditions than in CPC. Thus, the effect of reversing elution mode during analysis on the stationary phase distribution into the column and validation of theoretical predictions is described for HSCCC. Validation is presented using two different coil planet centrifuge machines.

Dual-mode CCC is applied to separation of polyoxypropylene glycol polymers (this is one of the first applications of CCC to separate synthetic polymers) and to improve the yield in the purification of antibiotics compared to previous methods using normal-mode CCC.

2. Theory

In HSCCC, the stationary phase is retained in the column owing to the centrifugal force field generated by the planetary motion of the column. Retention of the stationary phase depends on various parameters such as density, viscosity, or polarity of the solvents used. In a two-phase system, rules have been given to properly pump the mobile phase in order to maximize the retention of the stationary phase: for example, many solvent systems lead to maximum retention of the stationary phase if the lighter mobile phase is pumped from tail to head or, if the system is reversed, when the heavier mobile phase is pumped from head to tail.

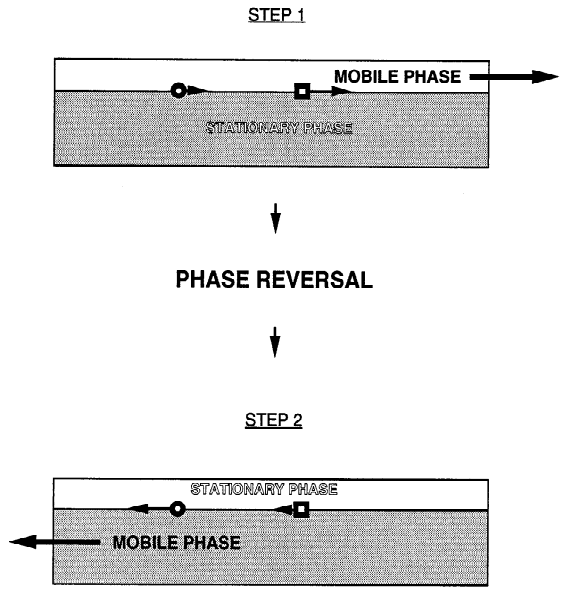

Fig. 1. Behavior of two solutes during the first (step A) and second step (step B) of a dual-mode CCC separation.

Fig. 1 shows the behavior of two solutes during the first step of the separation (step a) and during the second step (step b), after phase reversal. If K is the partition coefficient of a solute in the first step (a), the partition coefficient will be $1/K$ in the second step (b). The higher the retention of solute in step a, the lower its retention in step b (but, compared to LC backflush, separation is still progressing in step b because stationary phases are not the same during the two steps).

2.1. Prediction of retention time and K of solutes

In dual-mode CCC, calculations of partition coefficient have been described by Gluck and Martin [7] and Menges et al. [8] using pumped volumes of mobile phases. This model is used here to obtain retention volume using the following assumptions:

1. both phases (mobile and stationary phases) are uniformly distributed in the column.
2. phase distribution is not modified after phase reversal.

Notations used are:

$$k_{1,2} = \frac{[\text{solute}]_1}{[\text{solute}]_2}$$

$K_{1,2}$ is the partition coefficient of solute and $[\text{solute}]_1$ and $[\text{solute}]_2$ are the concentration of solute in phase 1 and 2 respectively. V_c (column volume) = V_1 (volume of phase 1) + V_2 (volume of phase 2)

Let us consider the dual-mode CCC separation:

2.1.1. Step a (first part of the separation)

Stationary phase:	Phase 1
Mobile phase:	Phase 2

V_{inv} is the volume of phase 2 pumped before phase reversal and V_{back} is the volume of phase 1 pumped after phase reversal. Menges et al. [8] and Gluck and Martin [7] have shown that:

$$K_{1,2} = \frac{V_{inv}}{V_{back}} \quad (1)$$

Total retention volume of solute, V, is given by:

$$V = V_{inv} + V_{back} \quad (2)$$

and using Eq. (1)

$$V = V_{inv}\left(1 + \frac{1}{K_{1,2}}\right) \quad (3)$$

If K_{inv} is the partition coefficient of a solute that is eluted at the end of step a, then starting from the classical retention equation in chromatography

$$V_r = V_m + KV_s \quad (4)$$

V_r is the retention volume of a solute, V_m the dead volume, K the partition coefficient and V_S the volume of the stationary phase in the column V_{inv} can be expressed as:

$$V_{inv} = V_2 + K_{inv}V_1 \quad (5)$$

that is:

$$K_{inv} = \frac{V_{inv} - V_2}{V_1} \quad (6)$$

Considering partition coefficients, step a leads to elution of solutes to which $K_{1,2}$ belongs $[0,K_{inv}]$ if pumped volume of phase 2 is higher than V_2; this implies $V_{inv} > V_2$.

2.1.2. Step b (second part of the separation):

During step b, solutes which have not been eluted during step a, i.e. which $K_{1,2}$ belongs to $[K_{inv}, +\infty]$ or $K_{2,1}$ belongs to $[0,1/K_{inv}]$, are eluted. V is calculated using Eq. (3) and belongs to $[V_{inv}\ (K_{2,1}=0),\ V_{max}\ (K_{2,1}=1/K_{inv})]$ with

$$V_{max} = \left(1 + \frac{1}{K_{inv}}\right)V_{inv} \quad (7)$$

Combining Eqs. (3) and (7) leads to:

$$V_{max} = \frac{V_{inv} - V_2 + V_1}{V_{inv} - V_2} \cdot V_{inv} \quad (8)$$

It must be pointed out that V_{max} does not depend on the partition coefficient of the solutes. It only depends on V_{inv} and on the composition of the column. If $V_{inv} = V_c$, $V_{max} = 2\,V_c$ and does not depend on V_1 and V_2. Consequently, whatever the solvent system and phase ratio, elution of all the solutes of a sample can be obtained in dual-mode CCC by pumping V_c of mobile phase 2 during step a and V_c of mobile phase 1 during step b. This corresponds to elution of solutes whose $K_{1,2} \in [0,1]$ during step A and solutes whose $K_{1,2}$ is higher than 1 during step B. Pumping $2\,V_c$ corresponds to usual V_r in CCC. This procedure can be used for fast optimization of solvent system as will be shown.

2.1.3. Remark

Plot of V_{max} versus V_{inv} exhibits a minimum $V_{max,min}$:

$$V_{max,min} = (V_1 + V_2) + 2\sqrt{V_1V_2} \quad (9)$$

It is reached when:

$$V_{inv,min} = V_1 + \sqrt{V_1V_2} \quad (10)$$

2.2. Resolution

Resolution between two adjacent peaks A and B can be calculated using

$$R_s = 2 \cdot \frac{t_{r,B} - t_{r,A}}{w_B + w_A} \quad (11)$$

$t_{r,B}$ = Retention time of the more retained solute of considered adjacent peaks: $t_{r,A}$ = retention time of the less retained solute of considered adjacent peaks; w_B

and w_A = base peak width of solutes B and A, respectively.

Let $\Delta V_r = V_r(B) - V_r(A)$

Resolution between two adjacent peaks can be enhanced either by reducing w at constant ΔV_r or by increasing ΔV_r at constant w. For a constant analysis time conditions giving a higher ΔV_r using dual-mode instead of single-mode separation will be expressed; w will be considered as constant because analysis time will be the same for single and dual-mode operation. Two cases will be considered:

2.2.1. First case

This will deal with a dual-mode separation which will end in the same way as the single-mode separation from the point of view of phase role. This means:
Single-mode separation:

Stationary phase	Phase 2
Mobile phase	Phase 1

Dual-mode separation:

Step a		Step b	
Stationary phase	Phase 1	Stationary phase	Phase 2
Mobile phase	Phase 2	Mobile phase	Phase 1

Two solutes A and B whose retention volumes are $V_r(A)$ and $V_r(B)$ ($V_r(A) > V_r(B)$) in the single mode and $V_r^{dm}(A)$ and $V_r^{dm}(B)$ in the dual-mode will be considered.

$$\Delta V_r = V_r(A) - V_r(B) \quad (12)$$

$$\Delta V_r^{dm} = V_r^{dm}(A) - V_r^{dm}(B) \quad (13)$$

Conditions for increased difference in retention volume in the dual-mode separation are:

(I) $\Delta V_r^{dm} > \Delta V_r$

and

(II) $V_r(A) = V_r^{dm}(A)$

for constancy of separation duration. $V_r(A)$ and $V_r(B)$ can be expressed as:

$$V_r(A) = V_1 + \frac{1}{K_{1,2}(A)} V_2 \quad (14)$$

$$V_r(B) = V_1 + \frac{1}{K_{1,2}(B)} V_2 \quad (15)$$

Combining Eqs. (12)–(14) leads to:

$$\Delta V_r = \left(\frac{1}{K_{1,2}(A)} - \frac{1}{K_{1,2}(B)} \right) V_2 \quad (16)$$

For dual-mode separation, using Eq. (3):

$$V_r^{dm}(A) = \left(1 + \frac{1}{K_{1,2}(A)} \right) V_{inv} \quad (17)$$

$$V_r^{dm}(B) = \left(1 + \frac{1}{K_{1,2}(B)} \right) V_{inv} \quad (18)$$

Replacing in Eq. (13) gives:

$$\Delta V_r^{dm} = \left(\frac{1}{K_{1,2}(A)} - \frac{1}{K_{1,2}(B)} \right) V_{inv} \quad (19)$$

Using Eqs. (16) and (19), condition (I) can be expressed as:

$$\left(\frac{1}{K_{1,2}(A)} - \frac{1}{K_{1,2}(B)} \right) V_{inv} > \left(\frac{1}{K_{1,2}(A)} - \frac{1}{K_{1,2}(B)} \right) V_2 \quad (20)$$

As $K_{1,2}(A) < K_{1,2}(B)$ Eq. (20) can be simplified to:

$$V_{inv} > V_2 \quad (21)$$

Requirement for the validity of Eq. (21) is, of course, non-elution of solutes A and B during the step A of dual-mode elution:

$$V_2 + K_{1,2}(A) V_1 > V_{inv} > V_2 \quad (22)$$

Condition (II) can be expressed as:

$$V_1 + \frac{1}{K_{1,2}(A)} V_2 = \left(1 + \frac{1}{K_{1,2}(A)} \right) V_{inv} \quad (23)$$

or

$$V_{inv} = \frac{V_1 + \frac{1}{K_{1,2}(A)}V_2}{\left(1 + \frac{1}{K_{1,2}(A)}\right)} \quad (24)$$

To sum up, for the same analysis time, considering constant solutes peak width, higher Δt_R (leading to higher resolution) could be obtained in dual-mode separation than in single-mode separation, if

$$V_{inv} > V_2$$

$$V_2 + K_{1,2}(A)V_1 > V_{inv}$$

and

$$V_{inv} = \frac{V_1 + \frac{1}{K_{1,2}(A)}V_2}{\left(1 + \frac{1}{K_{1,2}(A)}\right)}$$

2.2.2. Remark: can all these conditions be satisfied at the same time?

The choice of the inversion volume is imposed by Eq. (24). It is easy to check that Eqs. (21) and (24) can both be satisfied if $V_1 > V_2$. Similarly, it can easily be shown that Eqs. (22) and (24) can be satisfied at the same time if $V_2 + K_{1,2}(A)V_1 > 0$, which is always true. To summarize, if V_1 is larger than V_2, it will be possible to find operating conditions satisfying Eqs. (21), (22), (24).

2.2.3. Second case

This case will deal with a dual-mode separation which will start in the same way as the single-mode separation. This means

Single separation:

Stationary phase	Phase 2
Mobile phase	Phase 1

Dual-mode separation:

Step a		Step b	
Stationary phase	Phase 2	Stationary phase	Phase 1
Mobile phase	Phase 1	Mobile phase	Phase 2

Solute A is more retained than solute B in single-mode separation but A will elute before B in the dual-mode separation. Eq. (12) i.e.

$$\Delta V_r = V_r(A) - V_r(B)$$

is still valid but ΔV_r^{dm} must be defined as:

$$\Delta V_r^{dm} = V_r^{dm}(B) - V_r^{dm}(A) \quad (25)$$

Conditions for enhanced ΔV_r in dual-mode separation are:

(III) $\Delta V_r^{dm} > \Delta V_r$

and

(IV) $V_r(A) = V_r^{dm}(B)$

$V_r(A)$ and $V_r(B)$ are defined using Eqs. (14) and (15). But

$$V_r^{dm}(A) = [1 + K_{1,2}(A)]V_{inv} \quad (26)$$

$$V_r^{dm}(B) = [1 + K_{1,2}(B)]V_{inv} \quad (27)$$

must be used.

Combining Eqs. (25)–(27) gives:

$$\Delta V_r^{dm} = [K_{1,2}(B) - K_{1,2}(A)]V_{inv} \quad (28)$$

Considering Eqs. (16) and (28), (III) is equivalent to:

$$V_{inv} > \frac{V_2}{K_{1,2}(A)K_{1,2}(B)} \quad (29)$$

Again, the requirement for the validity of Eq. (29) is non-elution of solutes A and B during step a of dual-mode elution:

$$V_{inv} < V_1 + \frac{1}{K_{1,2}(B)}V_2 \quad (30)$$

Condition (IV) is equivalent to:

$$V_1 + \frac{1}{K_{1,2}(A)}V_2 = [1 + K_{1,2}(B)]V_{inv} \quad (31)$$

which can be written:

$$V_{inv} = V_1 + \frac{\frac{1}{K_{1,2}(A)}V_2}{[1 + K_{1,2}(B)]} \quad (32)$$

In this second case, conditions for enhanced resolution in dual-mode separation are Eqs. (29), (30), (32) i.e.

$$V_{inv} > \frac{V_2}{K_{1,2}(A)K_{1,2}(B)}$$

$$V_{inv} < V_1 + \frac{1}{K_{1,2}(B)}V_2$$

and

$$V_{inv} = V_1 + \frac{\frac{1}{K_{1,2}(A)}V_2}{[1 + K_{1,2}(B)]}$$

These conditions are more restrictive than in the first case as partition coefficients of both solutes have to be considered.

If efficiency remains similar to the single-mode operation, this model shows that improvement of separation resolution should occur in dual-mode HSCCC if the conditions given above are satisfied.

3. Experimental

3.1. HSCCC

The HSCCC systems used were the Ito multi-layer coil planet centrifuge from PC Inc. (Potomac, USA) and a tri-column apparatus, Model CPHV 2000 derived from Pharma-Tech model CCC 2000 (Baltimore, MD, USA). The Ito multi-layer coil planet centrifuge has a single column with a capacity of 320 ml made from 137 m×1.6 mm I.D. PTFE tubing wound around a spool. The column is balanced by a counterweight. The other unit is equipped with three identical columns connected in series and symmetrically arranged around the central axis of the centrifuge. The columns are prepared from 1.6 mm I.D. PTFE tubing to obtain a total capacity of around 130 ml. The connections between columns are made from 0.8 mm I.D. PTFE tubing. For all units, the columns undergo synchronous planetary motion and revolve around their own axis, avoiding twisting of the flow-tubes. Depending on the models, the rotation movement is either horizontal or vertical.

Besides the HSCCC system, the complete HSCCC apparatus consisted of a Shimadzu Model LC 5 A LC pump (Touzart et Matignon, Vitry-sur-Seine, France) and a Gilson pump, Model 302 (Villiers-le-Bel, France) used for the mobile and stationary phases. The columns were connected to the pumps by 0.8 mm I.D. PTFE tubing via a three-way valve. Samples were injected into the column via a Rheodyne Model 7125 injection valve.

Dual-mode was performed using a Rheodyne model 7010 valve to reverse column inlet and outlet (it acts as a LC backflush valve). The role of the 'backflush valve' is to provide an easy selection of the elution mode. While the initial mobile phase pump is switched off, the second pump is turned on and the valve is switched.

A Sedex 45 evaporative light scattering detection (ELSD) system (Sédéré, Vitry sur Seine, France) manufactured for LC was used without modification [9].

Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use. Solvents were provided by Prolabo (Paris, France) or SDS (Vitry-sur-Seine, France) and were of analytical grade.

3.2. Determination of phase distribution in the HSCCC column

Determination of phase distribution in the HSCCC column was done using compressed air to gently push out the column contents after stopping rotation and phase pumping. Column effluent was collected and fractions allowed measurement of stationary and mobile phase volumes. $S_f = V_s/V_c$ was used to express the fraction of stationary phase in the column.

3.3. Test separations

A synthetic mixture of fatty acids ranging from C_{14} to C_{22} was used as test mixture for experimental validation of the model. The solvent system used was heptane–methanol–acetic acid (1:1:1, v/v),

injection volume was 0.3–0.5 ml of 0.5 g/l solution of each fatty acid dissolved in heptane.

3.4. Polymers

Separations of polymers were performed using polyoxypropylene purchased from Aldrich (Strasbourg, France).

3.5. Separation of fermentation broth antibiotics

Ethyl acetate crude extract of fermentation medium of antibiotics was provided by Rhône-Poulenc Rorer (Centre de Recherche de Vitry-Alfortville). The solvent system was water–methanol–ethyl acetate–chloroform (2:3:1.5:2.5, v/v).

4. Results and discussion

4.1. Distribution of the solvent system in the HSCCC column

Distribution of stationary phase in the HSCCC column was studied using water–heptane (1:1, v/v) solvent system on the two HSCCC machines. Fig. 2 shows the plot of S_f for each point of the column in the single-mode operation performed after the hydrodynamic equilibrium is reached; for the given flow-rate and the rotation speed, S_f is maximum ($S_{f\ max}$) because it is the 'natural' hydrodynamic equilibrium; Fig. 2 shows the stationary phase is distributed throughout the whole column whatever the phase used as the stationary phase, except at the column inlet and outlet; after stopping rotation and pumping, while compressed air duct was connected to the column inlet instead of the pump, a slight motion of liquids in the column still appeared which may explain these deviations at the column inlet and outlet.

After phase reversal, the volume of mobile phase in step a becomes the stationary phase. Given $S_{f\ max}$ during step a, this volume is much lower than the maximum volume of stationary phase the column could contain in the step b. Fig. 3 shows the distribution of the stationary phase in step b after 100 ml (Fig. 3A) and 300 ml of mobile phase (Fig. 3B) have been pumped. Fig. 3B shows the step b stationary phase is found at the tail of the column, the ratio V_S/V_c being S_{fmax} in this region. The rest of the column does not contain stationary phase except a hitch at 60% and would not be involved in the separation process in a single-mode separation; this

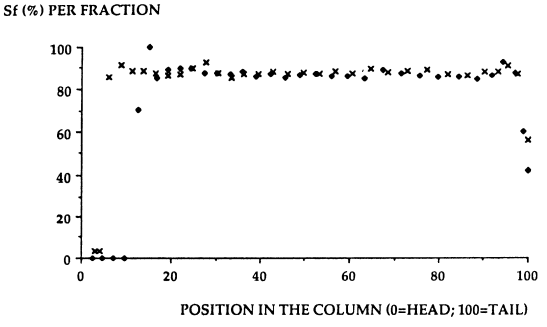

Fig. 2. Stationary/organic fraction of organic phase (S_f) versus the position in the column. PC chromatograph, 800 rpm; phase system water–heptane (1:1, v/v), mobile phase: water, flow-rate: 6 ml/min.

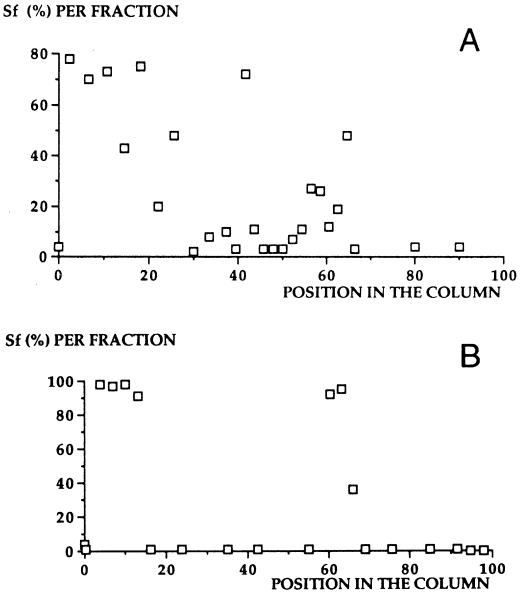

Fig. 3. Stationary/organic fraction of organic phase versus the phase ratio (S_f) versus the position in the column in dual-mode operation. PC chromatograph, 800 rpm; phase system water–heptane (1:1, v/v). Mobile phase flow-rate: 6 ml/min. Phases are reversed after 100 ml of initial mobile phase (water) have been pumped. Fractions are collected after 100 ml (A) or 300 ml (B) of the second mobile phase (heptane) have been pumped.

can be considered as a reduction of column length and addition of a dead volume [10]; the same situation is encountered in underload-mode HSCCC [10]. If only 100 ml of the mobile phase have been pumped after phase separation, the stationary phase is encountered in more than 60% of the column length (Fig. 3A); of course, $S_{f\ max}$ cannot be reached. This means that after the phases have been reversed, motion of the step a mobile phase to become step b stationary phase, distributed at one end of the column according to its behavior, requires some time (here more than 25 min). If solute partition coefficients in step b are low, separation will end before total organization of the step b stationary phase is reached and hypothesis of uniform distribution of the stationary phase in the column cannot totally be rejected; the model used for solutes retention time calculation will be valid.

Remark: if K, V_S and V_m are constant, retention factor (k') calculated as $k'=K\ V_s/V_m$ for a single-mode operation, will be constant whatever the distribution of the stationary phase in the column! The distribution of both phases will mainly depend on rotational speed and mobile phase flow-rate.

4.2. Validation of retention model

Figs. 4 and 5 show the plot of experimental retention time of fatty acids versus calculated retention time for various V_{inv} (or t_{inv}) using PC Inc. apparatus (Fig. 4) or CPHV apparatus (Fig. 5). For both machines, these plots demonstrate a very good agreement between calculated and experimental retention time (deviation is always lower than 15%); for multi-column apparatus, the behavior of one column is similar to the single column apparatus; the behavior of each column is independent of other columns.

The best results (deviation lower than 5% which is close to the repeatability of retention time in dual-mode HSCCC) are obtained if partition coefficient of solutes are greater than 1 in step a (Fig. 4A and Fig. 5A): if step a is performed at $S_{f\ max}$ for the whole column, step b duration is much shorter than step a because step b partition coefficients are lower than 1; thus relative error on total retention time will be low; in that case, retention times predicted are lower than experimental ones and the model reliability increases

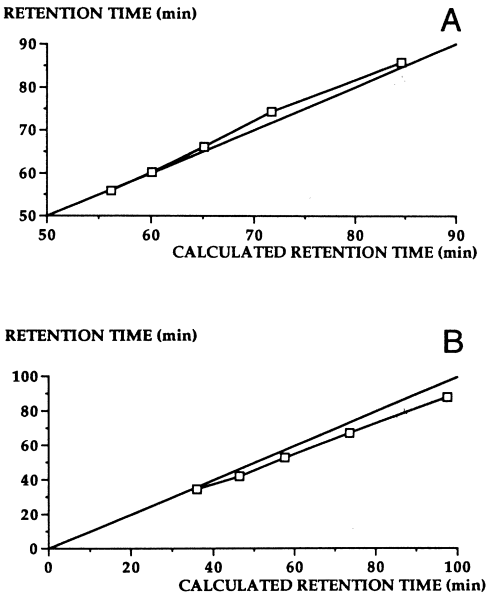

Fig. 4. Plot of experimental versus calculated retention times of fatty acids in dual-mode operation. PC 800 rpm; methanol–acetic acid–heptane (1:1:1, v/v) solvent system; flow-rate 6 ml/min; Solutes: saturated linear fatty acids C_{14}, C_{16}, C_{18}, C_{20}, C_{22}; injected volume: 0.5 ml of 0.5 g/l solution of each fatty acid; ELSD, nitrogen pressure 2000 hPa, 30°C, gain 6. Phases are reversed at 47 min (A) and 16 min (B). First mobile phase: polar phase (A) and heptane phase (B), for stationary phase at $S_{f\ max}$.

with S_f. On the contrary, when partition coefficients are lower than 1 in step a, the model overestimates the retention time (Fig. 4B, Fig. 5B,C) because the stationary phase is not distributed uniformly in the column: in the portion of column without stationary phase, migration of solutes is faster than in presence of stationary phase (the difference depends on the partition coefficient).

4.3. Application of phase reversal CCC to the separation of polyoxypropylene polymers

Dual-mode HSCCC (CPHV 2000) was applied to a mixture of polyoxypropylene polymers 725 and 3000 (POP) (Fig. 6) so elution of all the oligomers can be obtained (cf. Eq. (8)). V_{inv} was 120 ml ($V_{inv}=V_c$). The solvent system described for liquid–liquid fractionation of POP was cyclohexane–methanol–water (1:0.5:0.5, v/v) [11]. The separation started with the organic phase as the stationary

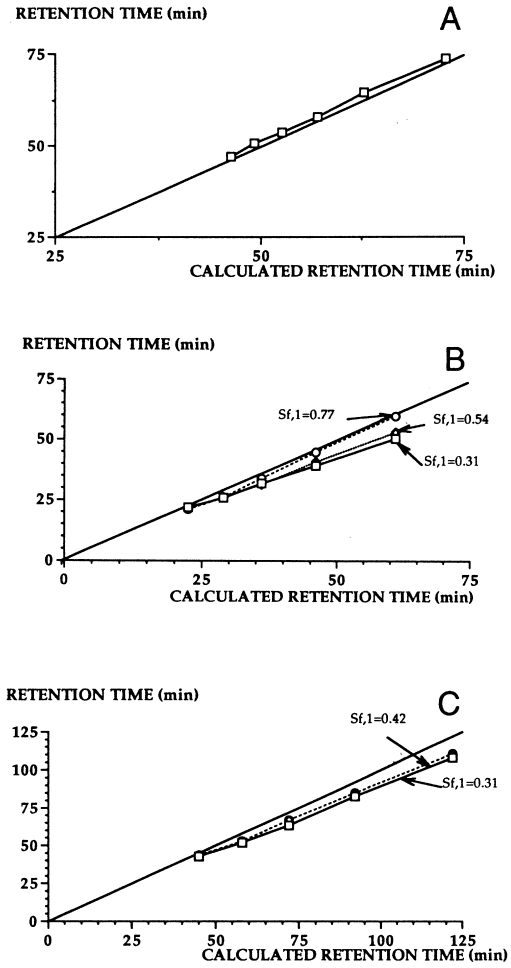

Fig. 5. Plot of experimental versus calculated retention times of fatty acids in dual-mode operation. CPHV chromatograph, 1400 rpm; other conditions as in Fig. 4 except phases are reversed at 41 min (A), 10 min (B) and 20 min (C). First mobile phase: polar phase (A) and heptane phase (B, C), for stationary phase at $S_{f\ max}$ (A) or at various S_f (B, C)

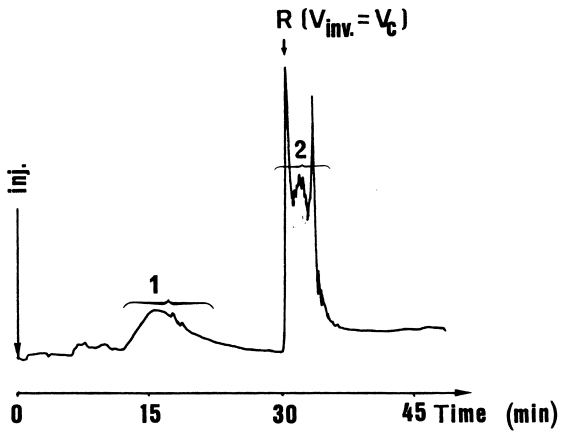

Fig. 6. Dual-mode separation of polyoxypropylene 725 (1) and 3000 (2) CPHV chromatograph, 1400 rpm; water–methanol–cyclohexane (0.5:0.5:1, v/v) solvent system; flow-rate 4 ml/min; injected volume: 1 mg each polymer in 0.5 ml cyclohexane; ELSD, nitrogen pressure 2000 hPa, 40°C, gain 6. Phases are reversed at 30 min. First mobile phase: cyclohexane for stationary phase at $S_{f\ max}$.

phase, the aqueous mobile phase allowing elution of POP 725 (peak 1); phase reversal permitted elution of POP 3000 in less than 1 h. Calculation of partition coefficient leads to *K* lower than 1 for the oligomers of POP 725 and *K* ranging from 8 to 30 for POP 3000 oligomers which means that the selectivity is higher than 8. ELSD was used to directly monitor the column effluent; however, the second peak eluted just after the phases were reversed and had a strange peak shape due to the re-organization of the phases and some spiking occurring sometimes with ELSD (this is certainly the case for the second spike at the end of peak 2). This separation in single-mode CCC would have required more than 15 h.

To our knowledge, this is the first application of HSCCC to the separation of non-biological polymers according to their molecular mass. Dual-mode HSCCC operation allowed faster elution of solutes than previous fractionation using the Craig's machine [12] while experimental condition selection was speeded up because all solutes can be eluted within a given time. Owing to the difficulty of selecting suitable solvent systems for polymer fractionation (it is reported that 100 systems were tried before separating PVC using continuous polymer fractionation [13]), dual-mode HSCCC offers unique versatility for quick experimentation with new solvent systems or new samples. Preparative capability of CCC techniques would be advantageous for production of standards of oligomers.

4.4. Increase of resolution

The influence of dual-mode operation versus single-mode on resolution was studied using fatty acid test samples. Conditions of dual-mode separations were set up using the model described in

Section 2.1 in order to obtain the same analysis time for both experiments. Two cases were studied and will be discussed here.

(I) First case — single separation:

Stationary phase:	Non-polar phase (heptane) as phase 2
Mobile phase:	Phase 1: polar phase methanol; fatty acid K values are greater than 1 to obtain a high enough resolution.

Dual-mode separation:

Part a:

Stationary phase:	Phase 1
Mobile phase:	Phase 2 ($K<1$)

Part b:

Stationary phase:	Phase 2
Mobile phase:	Phase 1 ($K>1$)

Part b duration is longer than that of part a.

Figs. 7 and 8 show the plot of resolution of adjacent fatty acids versus partition coefficient of the most retained of two adjacent peaks for various S_f during step a, for single-column (Fig. 7) and multi-column apparatus (Fig. 8).

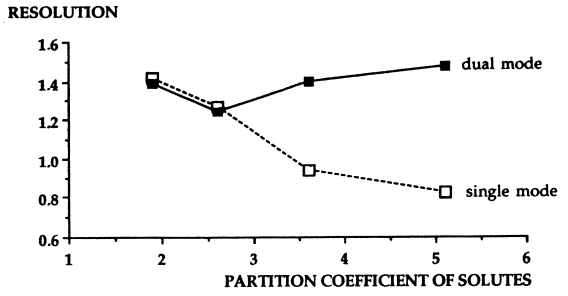

Fig. 7. Plot of resolution versus partition coefficient of the most retained solute of a pair of fatty acids for normal (single)-mode and dual-mode operation. Duration of both is fixed at 100 min. Conditions same as in Fig. 4B for dual-mode. Normal-mode: polar mobile phase at 6 ml/min, $S_f=0.23$ (elution order is from C_{14} to C_{22}).

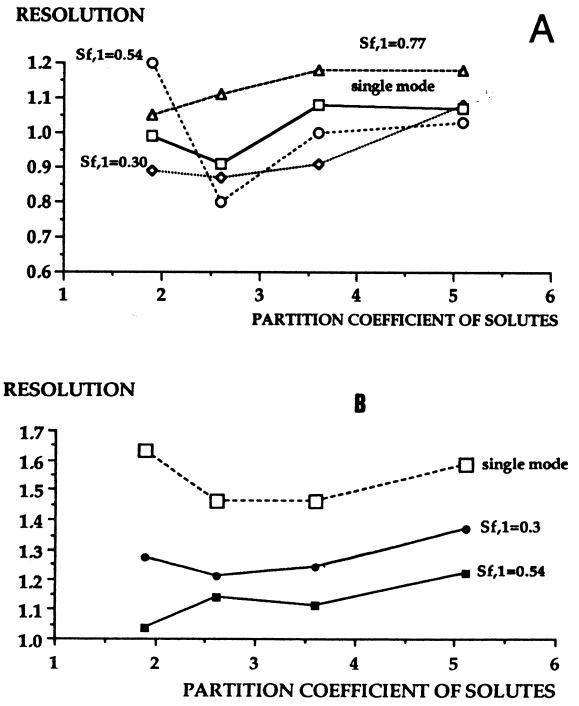

Fig. 8. Comparison of resolution versus partition coefficient of the most retained solute of a pair of fatty acids for normal-mode and dual-mode operation. Separation duration of both is fixed at 60 min (A) and 80 min (B) with a long second step in dual-mode (partition coefficients higher than 1); CPHV chromatograph dual-mode operation:, same conditions as Fig. 5B.

Dual-mode operation leads to an increase of resolution with multicolumn apparatus only for the shortest separation performed at the highest S_f during step a; on the other hand, the single-column apparatus operated in dual-mode always improved resolution. The explanation for these different behaviors are not trivial. Perhaps, in the single-column apparatus, the large volume of solvents involved in the separation (320 ml) which have to be reorganized after phase reversal is responsible for this phenomenon.

Thus, the possibility of increasing resolution in dual-mode CCC has been demonstrated, at least for large column volume apparatus. Using the PC machine, dual-mode operation provides higher resolu-

tion for same analysis time than single-mode operation or faster analysis at constant resolution.

(II) Second case

For the same single-mode separation as in the first case, the dual-mode conditions are:

Part a:

Stationary phase:	Phase 2
Mobile phase:	Phase 1 ($K>1$)

Part b:

Stationary phase:	Phase 1
Mobile phase:	Phase 2 ($K<1$)

Part a duration is longer than that of part b.

Fig. 9 shows fatty acid resolution for both apparatuses and operation modes. In dual-mode, an increase of resolution is obtained for both apparatuses, mainly for retained solutes. Chromatograms showing single- and dual-mode separations of fatty acids are presented in Fig. 10; dual-mode operation

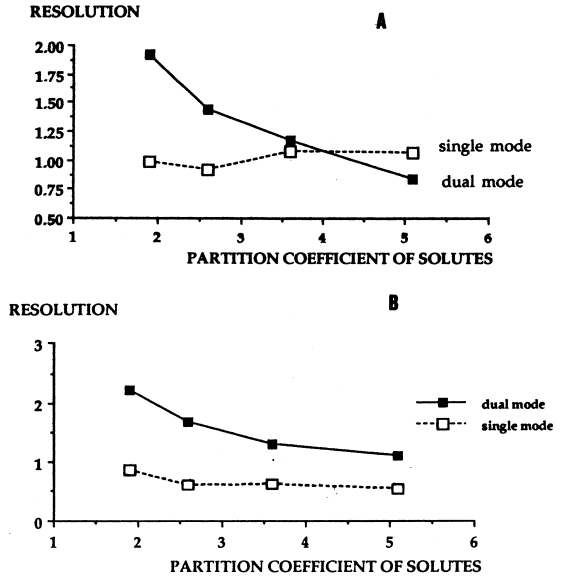

Fig. 9. Comparison of resolution versus partition coefficient of the most retained solute of a pair of fatty acids for normal-mode and dual-mode operation, separation duration of both is fixed at 60 min (CPHV chromatograph) (A) and 85 min (PC chromatograph) (B) and for fast second step in dual-mode operation (partition coefficients lower than 1). Dual-mode operation, same as Fig. 5B (A) and Fig. 4B (B). Single-mode operation: $S_f=0.23$, aqueous phase as the mobile phase; flow-rate, 4 ml/min (A) and 6 ml/min (B).

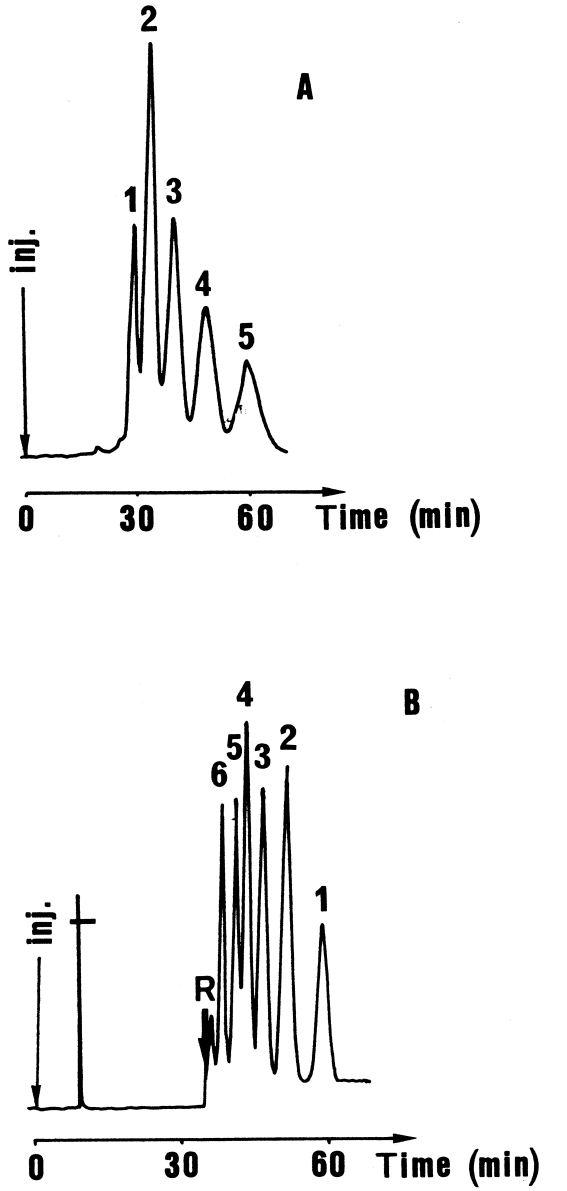

Fig. 10. Single- (A) and dual- (B) mode separations of fatty acids. CPHV chromatograph, same conditions as Fig. 5 except $S_f=0.23$ for non-polar stationary phase (A); $t_{inv}=35$ min, first stationary phase: heptane (B). Solutes: saturated linear fatty acids C_{14} (1), C_{16}(2), C_{18} (3), C_{20} (4), C_{22} (5) and C_{26} (6) only in the dual-mode operation; R: phase reversal.

provides higher efficiency and permits elution of C_{26} fatty acid which would require 30 min more in the single-mode operation. Increase of resolution comes from the increased efficiency because solutes are eluted in a two-fold smaller volume in the dual-mode operation. The model developed in the theory section cannot explain these results because it does not account for efficiency (yet, no satisfactory model for describing efficiency in underload-mode HSCCC is available. It has recently been shown that the usual expression for calculation of efficiency is not valid in underload HSCCC because part of the column does not contain stationary phase and acts as a dead volume. Work is being carried out to investigate the role of 'in column dead volume' in HSCCC on efficiency) [9].

To summarize, dual-mode HSCCC is proven to lead to an increased resolution in comparison to the single-mode operation. The model derived in 'theory' predicted this effect but experiments demonstrate that the strong effect of phase reversal on efficiency cannot be neglected for case B.

Whatever the mechanism involved, operation of HSCCC in dual mode is a new chromatographic parameter for improving separations resolution.

4.5. Application of dual-mode CCC to the improvement of resolution in preparative HSCCC of antibiotics

Optimisation of the solvent system for preparative scale separation of antibiotics has recently been reported. It was shown that 11 g of purified sample could be injected in one run to give more than 2 g of purified compound [14] using 1 l column apparatus in underload mode. Thus, it was concluded that similar results could be obtained on a smaller column volume machine such as PC Inc. model while time and solvents could be saved.

The HSCCC sample was ethyl acetate extract of fermentation broth dissolved in either the mobile phase or the stationary phase prior to injection. The solvent system was chloroform–ethyl acetate–methanol–water as previously reported [14,15]. Fig. 11 shows separations of antibiotics in single-mode operation (A), dual-mode operation (sample dissolved in chloroform) (B) and dual-mode operation

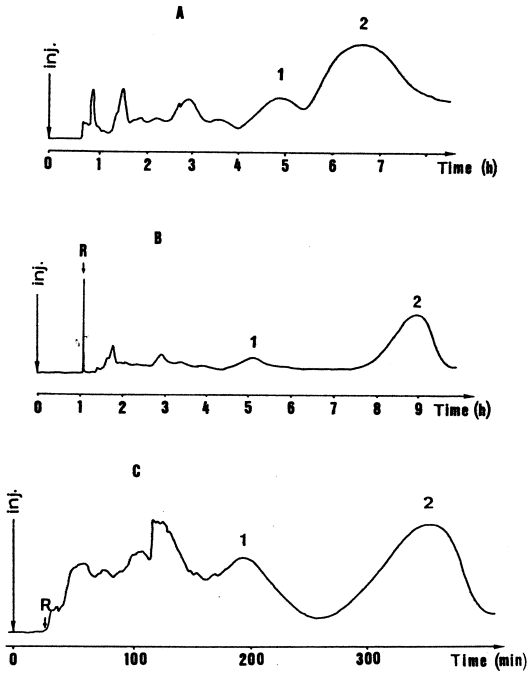

Fig. 11. Separation of antibiotics in single- (A) and dual- (B, C) mode operations. PC chromatograph, 800 rpm, solvent system water–methanol–ethylacetate–chloroform (2:3:1.5:2.5, v/v), aqueous mobile phase, 4 ml/min, $S_f = 0.5$ (A); injected quantity: 2 g of crude extract. (B) same conditions except reversal of phases at 60 min (R), first mobile phase: non-aqueous phase, injected quantity 5 g of crude extract dissolved in 5 ml of non-aqueous phase. (C) same conditions as (B) except reversal at 21 min and 5 g of crude extract injected in 100 ml of aqueous phase via a large syringe.

(sample dissolved in 100 ml of the aqueous phase) (C). Single-mode separation was carried out using the organic phase as the stationary phase. The comparison of the two different modes of separation exemplifies the increased resolution using case A dual-mode operation: it permits injection of a 5-g sample (Fig. 11B,C) instead of 2 g in the single mode (Fig. 11A) while the resolution is much higher in the dual-mode operation; chromatograms show that the difference between retention time of solutes is higher and the efficiency slightly higher in the dual-mode separation.

The influence of sample solvent on peak shape is evident: front tailing appears when the sample is prepared in chloroform; the first step of the sepa-

ration is performed using a mobile phase (organic phase) where the solutes are more soluble than in the second part; thus, saturation of the second step mobile phase occurs after phase reversal. Moreover, when large quantities are loaded, there is a discrepancy between predicted and experimental retention times (Fig. 11A,B) whereas this did not occur for analytical scale injections. An explanation can be found in the non-linearity of distribution isotherms of solutes in the two-phase solvent system [15] that can lead to discrepancy between theoretical and experimental retention times even in single-mode operations or in the modification of the solvent by the sample loaded (this type of fermentation extract includes polar and non-polar compounds and the studied antibiotics have surface active properties).

Separations of antibiotics obtained after injection of a 5 g mixture dissolved in 100 ml of aqueous phase led to retention times similar to those of the single-mode separations (Fig. 11A,C) and symmetrical elution peaks. As reported in Ref. [14], peak compression by a factor of 3 can be obtained by proper selection of sample solvent system; raw estimation of peak volume shows this does not occur here because the solutes are recovered in 3–5 times larger a volume than the injected volume; nevertheless, the resolution between the two major peaks is still greater than 1.2 and allows high purity fractions to be collected (purity was checked using the LC procedure described in Ref. [14] and was more than 99% for fractions 1–2). For such large volume injection, the sample was directly pushed in the CCC column using a syringe at a higher flow-rate than the mobile phase to reduce the sampling time.

As the resolution in Fig. 11B is high, we can reduce separation time by shortening the first step of separation (Fig. 12): t_{inv} in Fig. 12 is twice t_{inv} in Fig. 11B; thus, baseline separation of compounds 1 and 2 can be obtained while almost 4 h are saved.

Considering the data used for comparison of HPLC and HSCCC of these antibiotics [14], the separation sample input has been twice for a similar analysis time. But, we used an analytical machine instead of a preparative one and owing to the complexity of the sample injected here compared to the purified sample used for our initial experiments, dual-mode operation led to a real improvement of productivity because a time consuming (two days!) purification scheme is required prior to LC injections (also used for HSCCC in our first experiments); in the results presented here it is suppressed: one step permits purification and recovery of very high purity compounds from a very raw material. This confirms the potential of HSCCC for purification and isolation of solutes from complex matrices; moreover, analytical units such as ours provide separation capability exceeding several grams. The limitations arise from the sample itself which can be difficult to dissolve before injection or, when injected as a large volume (1/3 of the column volume in this work) can disturb the equilibrium of solvent system used for the separation.

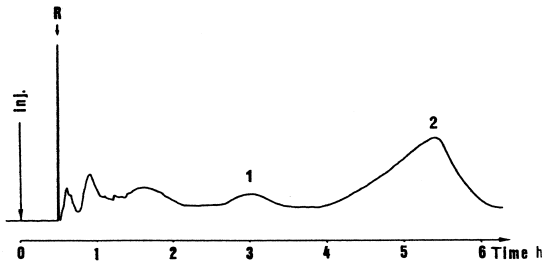

Fig. 12. Separation of antibiotics in dual-mode operation. Same conditions as for Fig. 11B except $t_{inv}=30$ min.

5. Conclusion

Models for calculating retention time and for increasing resolution in HSCCC have been derived.

Despite the fact that there is a motion of solvents after phase reversal prior to reaching a new equilibrium, calculated retention times have been demonstrated to fit the experimental ones; best results were obtained when the second part of the dual-mode separation was fast.

Prediction of retention time and partition coefficient was used for selection of operating conditions for first reported separation of non-biological polymers. Dual-mode operation in HSCCC is a simple means to reduce time consuming solvent selection in CCC and it does not require external techniques such as TLC, HPLC, spectrophotometry etc. for partition

coefficient determination prior to separation. Thus, dual-mode operation provides a unique way to reduce analysis time and to optimise separation.

Starting from the theoretical data showing that resolution should be higher in dual-mode than in single-mode operation, separation of fatty acids and of antibiotics demonstrated this could occur but, owing to the lack of a model for efficiency, results cannot be fully explained using our model. From a practical point of view, increased resolution has been found in many cases but results depend on solutes, sample volume and solvent system.

Finally, use of dual-mode HSCCC for preparative purification of antibiotics is highlighted by comparison with our previous results; purification of compounds is greatly simplified: direct injections of a fermentation extract and dual-mode operation provides higher resolution per unit of time; separation and purity of collected fractions were similar to those obtained previously using purified extract as injected sample; thus, raw comparison between LC and HSCCC separations of these compounds is much in favor of HSCCC from the point of solvent consumption, recovery yield and productivity as a single analysis provides sample treatment and recovery of high purity compounds from a very raw material. The limitations are non-linearity of distribution isotherms, injected volumes and by-products that can disturb the system equilibrium in the column and lead to leaks of the stationary phase.

References

[1] A. Marston, K. Hostettmann, J. Chromatogr. A 658 (1994) 315.
[2] A. Berthod, Y.I. Han, D.W. Amstrong, J. Liq. Chromatogr. 11 (1988) 1441.
[3] S. Drogue, M.-C. Rolet, D. Thiébaut, R. Rosset, J. Chromatogr. 593 (1992) 363.
[4] I. Slacanin, A. Marston, K. Hostettmann, J. Chromatogr. 482 (1989) 234.
[5] W. Murayama, Y. Kosuge, N. Nakaya, Y. Nunogaki, K. Nunogaki, J. Cazes, H. Nunogaki, J. Liq. Chromatogr. 450 (1988) 283.
[6] A. Marston, C. Borel, K. Hostettmann, J. Chromatogr. 450 (1988) 91.
[7] S.J. Gluck, E.J. Martin, J. Liq. Chromatogr. 13 (1990) 3559.
[8] R.A. Menges, G.L. Bertrand, D.W. Armstrong, J. Liq. Chromatogr. 13 (1990) 3061.
[9] S. Drogue, M.-C. Rolet, D. Thiébaut, R. Rosset, J. Chromatogr. 538 (1991) 91.
[10] Agnely, M., Thiébaut, D. in preparation.
[11] L.C. Case, Makromol. Chem. 41 (1960) 61.
[12] K.E. Almin, Acta Chem. Scand. 13 (1957) 1278.
[13] H. Geerissen, J. Roos, B.A. Wolf, Makromol. Chem. 186 (1985) 735.
[14] Drogue, S. Thèse de Doctorat, Université Paris VI, 1992.
[15] Rolet-Menet, M.-C., Thése de Doctorat, Université Paris VI, 1993.