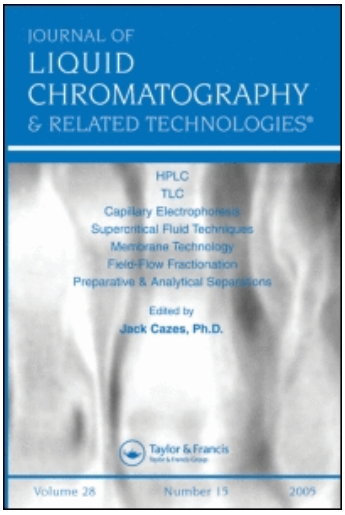

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FUNDAMENTAL CHROMATOGRAPHIC PARAMETERS IN COUNTERCURRENT CHROMATOGRAPHY: INFLUENCE OF THE VOLUME OF STATIONARY PHASE AND THE FLOW-RATE

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ABSTRACT

Two CCC (Countercurrent Chromatography) devices were compared using a test mixture of saturated fatty acids (myristic, palmitic and stearic acids) and the two-phase heptane/acetic acid/methanol solvent system. One device, defined by Dr. ITO as a HDES Type J, is known as a HSCCC (High Speed CCC) apparatus and the other one is defined as a CDCCC (Centrifugal Droplet CCC) HSES. The influence of the rotational speed and the flow-rate on the retention of stationary phase was studied. The variations of capacity factors and resolution against the retention of stationary phase were then plotted and we checked that conventional chromatographic equations can be applied to these curves. Finally a comparison was held between the plots of various chromatographic parameters *against* the flow-rate between each apparatus and also the ordinary chromatography curves. We also compared the calculated partition coefficients for the fatty acids on each device with the values obtained by the "Dual Mode" method on the CDCCC apparatus and by a scintillation detector used with radioactive fatty acids.

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INTRODUCTION

Countercurrent Chromatography (CCC) is a unique method based on the separation of solutes as they partition between two immiscible phases. Two liquid phases are continuously mixed and separated in the column by a centrifugal force field. One phase is stationary and the other phase is mobile, percolating through the stationary phase. Two devices are studied here. The first one is equipped with a separation column made of a Teflon tube, wound around a cylinder. This coil undergoes a planetary motion and the distribution of the two liquid phases is due to hydrodynamic phenomena operating under centrifugal forces. This technique was discovered and extensively studied by Y. ITO of the National Heart, Lung and Blood Institute (National Institutes of Health, Bethesda, MD, USA). As the rotational speed of the device is high, this variant of CCC is also called HSCCC (High Speed Countercurrent Chromatography) [1]. The other system is made of a rotor in which cartridges are installed, which thus undergo a circular motion. The distribution of the two-phase system is based on hydrostatic phenomena. This system is called CDCCC (Centrifugal Droplet Countercurrent Chromatography) or CPC (Centrifugal Partition Chromatography) [2,3].

An important feature of CCC is the variable retention of stationary phase in the column. Various parameters such as the internal diameter of the tube, the rotational speed of the device, the physico-chemical properties of the solvent system or the flow-rate may lead to a great variation in this feature. So we studied the influence of each factor separately and tried to give explanations about the observed behaviors.

Although the variation in the retention of stationary phase can be advantageous under certain conditions, it is troublesome in others. Therefore, the volume of stationary phase retained in the column was determined to study its influence on basic chromatographic parameters such as capacity factor, resolution and separation factor. They were calculated from the chromatograms of a saturated fatty acids test mixture (myristic, $C_{14}H_{28}O_2$, palmitic, $C_{16}H_{32}O_2$ and stearic, $C_{18}H_{36}O_2$ acids). The solvent system was n-heptane/acetic acid/methanol (1:1:1, v/v). The variations were then compared with the chromatographic relations of ordinary chromatography, such as Gas Chromatography, Liquid Chromatography or High Performance Liquid Chromatography, modified to involve the volume of stationary phase [4].

Finally we were able to compare the variations in the usual chromatographic parameters against the flow-rate between the two systems (HSCCC and CDCCC devices) and also with ordinary chromatography. The partition coefficients for the solutes were also calculated and compared with values obtained according to two different ways.

EXPERIMENTAL

Apparatus

Two different devices were used. The HSCCC apparatus consisted of two Gilson Model 303 pumps, Villiers-le-Bel, FRANCE, for pumping the organic and the aqueous phases. The pumps were connected to a P.C.Inc. system, Potomac, MD, USA, equipped with a single multilayer coil which was made of a continuous piece of 1.60 mm i.d. PTFE tubing to give a total volume of 300 mL. A counterweight is used to counterbalance the effect of the column filled with stationary and mobile phases. The β values ranged from 0.57 to 0.85 (β = r/R, where r is the distance from the coil to the holder axis and R, the distance from the holder to the central axis of the centrifuge). The rotational speed of the apparatus was checked by stroboscopic observations, using a Model STROB 1 stroboscope, AOIP, Paris, FRANCE.

The other apparatus was a Model LLN Centrifugal Partition Chromatograph (CPC), manufactured by Sanki Engineering Ltd., Yamazaki, JAPAN. It was used with six Type 250W cartridges, providing an internal volume of 125 mL. A Sanki pump (LBP II type triple plungers) was used to pump both aqueous and organic phases.

The radioactivity detector used to determine the partition coefficients was a Liquid Scintillation System Model LS3801 from Beckman Instruments, Nuclear Systems Operations, Irvine, CA, USA.

Reagents

All organic solvents were of HPLC grade. Methanol, acetic acid and heptane were purchased from Prolabo, Paris, FRANCE. They were filtered before use. The stearic acid ($C_{18}H_{36}O_2$) was purchased from Prolabo and the myristic

($C_{14}H_{28}O_2$) and palmitic ($C_{16}H_{32}O_2$) acids from Merck. The radioactive myristic acid (4.0 mCi/mmol) was purchased from Nuclear Research Chemical Inc., Orlando, Florida, USA, the radioactive palmitic acid (55 mCi/mmol) from Amersham International Ltd., Amersham, United Kingdom and the radioactive stearic acid (2.52 mCi/mmol) from Nuclear Instrument and Chemical Corporation, Chicago, Illinois, USA. All were labeled at the carbonyl carbon with ^{14}C. A standard ^{14}C sample (Toluene-^{14}C, calibrated at 4.24×10^5 dpm/mL) was purchased from New England Nuclear, Boston, Massachusetts, USA.

Solvent systems and sample solutions

The same solvent system and stationary phase were used on both devices. It consisted of heptane/acetic acid/methanol (1:1:1, v/v) two-phase mixture. The stationary phase was the heavier one (acetic acid/methanol phase saturated in heptane). The sample solution was prepared by dissolving the three fatty acids in the mobile phase (lighter, i.e. heptane saturated in acetic acid/methanol), providing for each fatty acid a 1 g/L concentration. The sample solution used on CDCCC apparatus contained only the stearic and myristic acids.

Separation procedure

Just before the experiment, the solvent two-phase mixture was thoroughly equilibrated in a separatory funnel. Three different procedures were followed for the HSCCC device.

The first one is a standard method. The column of the HSCCC device was first entirely filled with the stationary phase and the apparatus was rotated at 750 rpm. Then, the mobile phase was pumped into the column at the desired flow-rate in the tail-to-head elution mode. After equilibration, an 0.5 mL sample solution was injected into the column via a Rheodyne Model 7125 injection valve. Thus, the apparatus is equilibrated at a determined flow-rate F and the whole separation is achieved at this flow-rate.

The second procedure was intended to obtain a constant volume of stationary phase independent of the flow-rate. We chose a maximum 9.5 mL/min flow-rate F_0 to ensure no loss of stationary phase during the separation. The equilibration was achieved after 9.3 min (leading to a 0.70 retention of stationary

phase) but the pump was only stopped after 11 min. The flow-rate F was then chosen on the pump ($F \leq F_0$). At 11.2 min, the pump was started at a F flow-rate and the fatty acids sample was injected at 14 min.

The third procedure allowed to fix the volume of stationary phase retained in the column below the maximum value obtained with the first procedure. For the heptane/acetic acid/methanol system, the mobile phase (lighter one) was pumped into the tail of the column. Then the pump was stopped after a chosen time and the stationary phase (heavier one) was pumped until the first drops of mobile phase were seen at the head of the column. The HSCCC apparatus was then rotated at 60% of its power (750 rpm) and the mobile phase pumped at the tail of the column at a 6 mL/min flow-rate. The retention of stationary phase was also checked when emptying the device.

Three procedures were followed for the CDCCC apparatus. The first one is the usual method. The column was first filled with stationary phase, before being rotated at the desired rotational speed. It was always set at 600 rpm and checked by the stroboscope. A 1.0 mL sample solution was introduced via a SANKI injection valve (Model FCU II valve connection unit) in the device. The mobile phase was then pumped into the cartridges in the ascending mode (the "ascending" mode referring to the flow of the mobile lighter phase through the channels in an inward direction [5], opposite to the direction of the centrifugal force).

The second procedure, similar to that used with the HSCCC unit, allowed to obtain a constant volume of stationary phase retained in the column whatever the flow-rate may be. The equilibration was achieved at a F_0 flow-rate (4mL/min) and 600 rpm. The pump was then stopped and a 1.0 mL sample solution was introduced. The chosen flow-rate F ($F \leq F_0$) was settled on the pump, which was afterwards started on the ascending mode.

The third procedure was intended to vary the volume of stationary phase retained in the column. The column was first filled with the stationary phase (heavier one). The apparatus was then rotated at 600 rpm and the mobile phase pumped at a F flow-rate. After equilibration, the pump was stopped and the flow-rate fixed at a 0.5 mL/min flow-rate F_0 ($F_0 \leq F$). The 1.0 mL sample solution was then introduced and the pump was started.

Detection

The detection was based on evaporative light-scattering detection (ELSD) on-line with HSCCC apparatus [6]. The unit was a Sedex 45 ELSD system manufactured by Sédéré, Vitry-sur-Seine, FRANCE, designed for HPLC and used without modification. Its evaporation tubing temperature was 30°C, the nitrogen pressure equal to 2 bar and the gain equal to 7.

The nebulizer of the ELSD system was supplied by nitrogen, L'Air Liquide, Paris, FRANCE.

Measurement of chromatographic parameters [7]

A very important parameter for countercurrent chromatography is the volume of the stationary phase V_S retained in the column. The first measurement method is to estimate the volume of the stationary phase expelled from the column, V_e, by the mobile phase during the equilibrium period. Then V_{S1} is computed as $V_{S1} = V_t - V_e$, where Vt is the internal volume of the column. Another way is to measure the expelled volume of stationary phase, V_{S2}, when emptying the column with nitrogen gas. The comparison of the two volumes gives a good correlation so that it is possible to write $V_S = V_{S1} = V_{S2}$. The retention of the stationary phase S_F is then defined as

$$S_F = \frac{V_s}{V_t} \qquad (1)$$

The partition coefficient K for one solute is defined as

$$K = \frac{C_s}{C_m} \qquad (2)$$

where C_S is the concentration of the solute in the stationary phase and C_m its concentration in the mobile phase.

The holdup time t_0 is calculated as

$$t_0 = \frac{V_m}{F} \qquad (3)$$

where F is the flow-rate of the mobile phase and V_m the volume of the mobile phase in the column.

The capacity factor , k', is computed from the obtained chromatograms using the formula

$$t_R = t_0.(1 + k') \quad (4)$$

where t_R is the retention time of the solute. As k' is defined as the ratio of the quantity of solute in the stationary phase on its quantity in the mobile phase, the relation between k' and K is

$$K = k'.\frac{V_m}{V_s} = k'.(\frac{1}{S_F} - 1) \quad (5)$$

The number of theoretical plates N for one peak is computed using the conventional Gaussian peak dispersion equation

$$N = (\frac{4t_R}{w})^2 \quad (6)$$

where w is the peak base width expressed in time unit as t_R.
The resolution R_S between two peaks is calculated as

$$R_s = 2\frac{(t_{R_2} - t_{R_1})}{w_1 + w_2} \quad (7)$$

where w is expressed in time unit or, using the partition coefficients, as

$$R_s = 2\ V_s \frac{K_2 - K_1}{w_1 + w_2} \quad (8)$$

where w is expressed in volume unit.
The separation factor α, which is related to the solvent system selectivity, is defined as

$$\alpha = \frac{k'_2}{k'_1} = \frac{K_2}{K_1} \ ; \ \alpha > 1 \quad (9).$$

Measurement of partition coefficients using radioactive fatty acids

We describe below the method for one fatty acid; this is the same for the two others. First 10 mL of heptane, 10 mL of acetic acid and 10 mL of methanol were introduced in the beaker. Then 30 mg of one no radioactive fatty acid was

introduced in the beaker to lead to a 1 g/L concentration, as in the sample used for the CCC separations; 1 mL of the same radioactive fatty acid was added to the mixture. The beaker was let at room temperature during one night. Then 1 mL of the upper phase was added to 9 mL of the counting cocktail solution. The same preparation was made with the lower phase. The number of cpm (counts per minute) was then measured. As quenching occurred for these measurements, a standard ^{14}C was added in each solution. The cpm were thus measured for the second time. They allowed to compute the efficiencies for each solution, leading to their actual cpm. The partition coefficient was computed as the ratio of the number of cpm of the heavier phase to the number of cpm for the lighter phase.

Measurement of partition coefficients using "Dual Mode Centrifugal Chromatography" [8]

Described by S. J. GLUCK and E. J. MARTIN, this method was applied to the heptane/acetic acid/methanol solvent system. First, the CDCCC column was filled with the stationary phase, which was in this case the lighter one (heptane saturated with the methanol/acetic acid phase). Then it was rotated at 500 rpm and the mobile phase pumped in the descending mode [5] at a 2 mL/min flow-rate until the hydrostatic equilibrium was reached, leading to a retention of 65 mL of stationary phase in the column. The fatty acids sample was then injected and the mobile phase was pumped during the additional 20.5 min. The pumped volume of mobile phase after the injection is V_{m1} = 41 mL. The two fatty acids must not be eluted out (even if they are not retained in the stationary phase); V_{m1} must then be smaller than V_m, which is the volume of the mobile phase after the equilibrium in the column. Then the elution mode was reversed (t-0), the lighter phase was chosen as the mobile phase and was then pumped in the ascending mode. The pump was stopped after the detection of the two fatty acids. It was then possible to calculate the retention volume of each fatty acid. The stearic acid was eluted at 7.00 min and the myristic at 16.37 min, leading to $V_r(C_{18})$ = 14 mL and $V_r(C_{14})$ = 32.75 mL. Then the partition coefficients are obtained following the equation:

$$K = \frac{V_r}{V_{m_1}} \qquad (10)$$

RESULTS AND DISCUSSIONS

Parameters influencing the retention of the stationary phase

The retention of stationary phase is dependent on at least five parameters. Two of them are settled before the experiment: the physico-chemical properties of the solvent system [9] and the internal diameter of the column [10]. When such parameters are chosen for a given separation, it remains possible to set three others, which are the filling procedure, the rotational speed of the CCC apparatus and the flow-rate.

The first of those, described in part EXPERIMENTAL as the third procedure, enabled to change the retention of stationary phase without varying the flow-rate or the rotational speed with the HSCCC device. It was used to study the influence of the retention of stationary phase on chromatographic parameters such as capacity factors, separation factor and resolution.

The influence of the rotational speed, at constant flow-rate and with standard separation procedure, was studied on both HSCCC and CDCCC systems as shown in Figures 1, A and B respectively. Figure 1. A shows a linear variation (correlation coefficient: $r = 0.99$) within the range of ω. The retention of the stationary phase against ω is not linear for the CDCCC unit (Figure 1. B) as it increases faster for small values of rotational speed. On HSCCC and CDCCC devices, the retention of stationary phase is provided by the centrifugal force field. Increasing the rotational speed results in an increase of the centrifugal force field, leading to a higher retention of stationary phase. For both devices, attempts to alter the retention of the stationary phase by changing the rotational speed did not lead to reproducible results.

HSCCC and CDCCC systems do not show the same curve shapes when the retention of stationary phase is plotted against the flow-rate at constant rotational speed using the first separation procedures (Figures 2, A and B). For both devices, the values of the flow-rates were checked by measuring eluted volumes in a graduated vessel during timed periods. For the HSCCC apparatus (Fig. 2 A), S_F shows a linear decrease with F for the studied flow-rate range. The volume of stationary phase driven by a viscous drag force is larger when the flow-rate of the mobile phase is increased. The retention of stationary phase is then diminished. The same explanation applies to the CDCCC plot (Fig. 2 B). The great difference

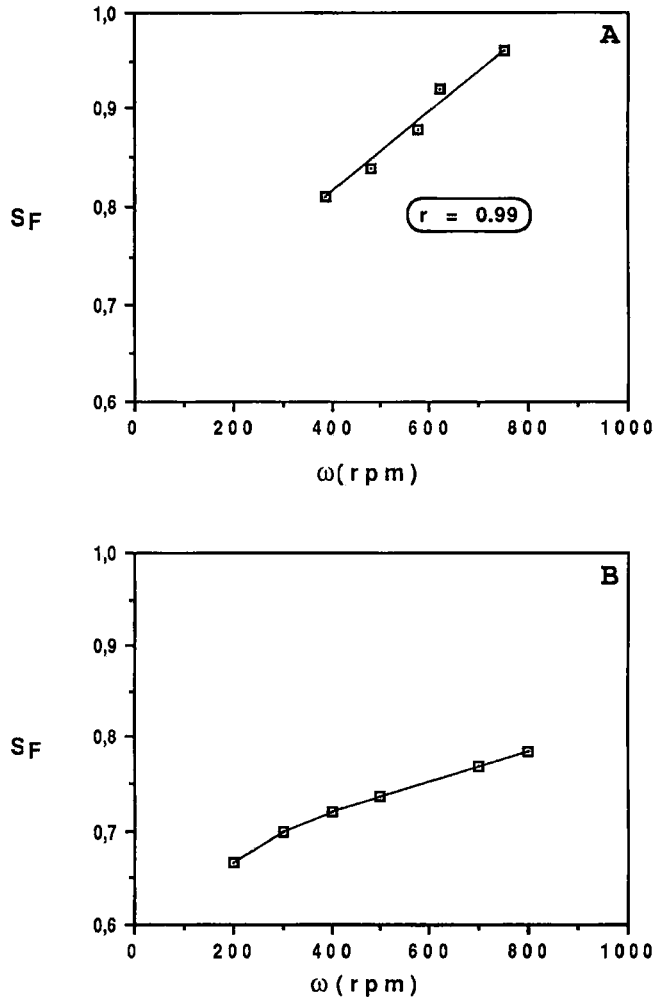

FIGURE 1. Effect of the revolution speed ω on the retention of stationary phase S_F on a HSCCC apparatus (A) and a CDCCC apparatus (B). (A) 4 mL/min flow-rate; (B) 2 mL/min flow-rate.

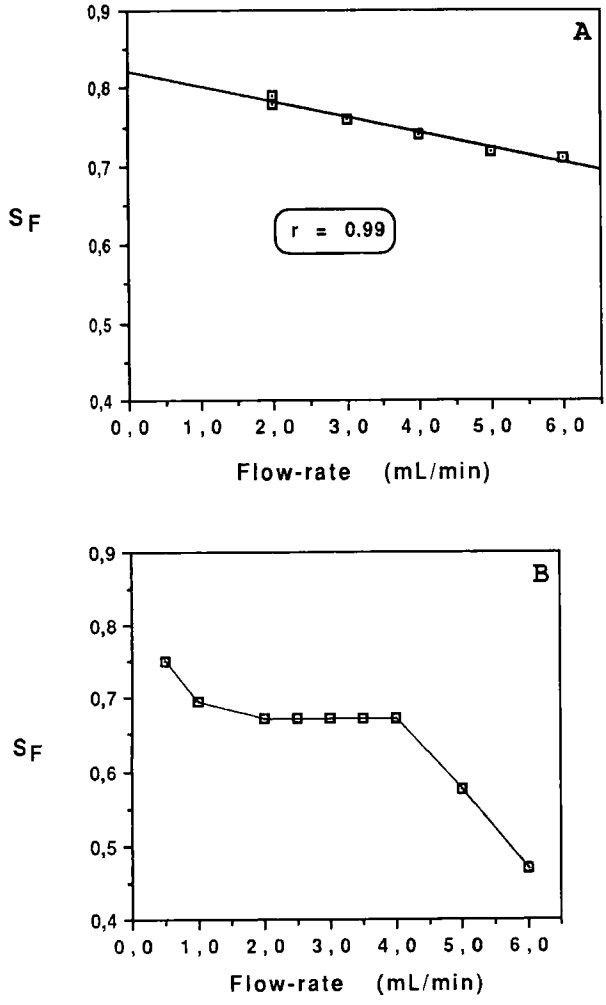

FIGURE 2. Effect of the flow-rate F on the retention of stationary phase S_F on a HSCCC apparatus (A) and a CDCCC apparatus (B).

is the plateau between 2 and 4 mL/min flow-rate. When using a flow-rate within this range, it is then possible to have a constant retention of stationary phase. The influence of the retention of stationary phase on various chromatographic parameters was studied on the CDCCC device using the third procedure described in part EXPERIMENTAL. One flow-rate used for the equilibration was chosen on the plateau (between 2 and 4 mL/min) and the other ones out of the plateau to ensure a variation in the retention of stationary phase.

Influence of the retention of stationary phase on various chromatographic parameters

Figure 3.1 displays the separation of three fatty acids on a HSCCC apparatus. The compounds are well separated ($R_{S1/2} \cong 1.2$, $R_{S2/3} \cong 1.55$) in a short time, due to a very high flow-rate of 21 mL/min. It was possible to use such a high flow-rate as the mobile phase was a low viscosity liquid (η - 0.38 cP). In fact column effluent was composed of 18 mL/min mobile phase flow-rate and of 3 mL/min stationary phase flow-rate (S_F indicated in the caption of Figure 3.1 was measured just before injection). In this case, separation was still possible since the separation time is short and the continuous loss of stationary phase was not sufficient to disturb the separation or the ELSD detection.

Figure 3.2 shows the separation of two fatty acids on a CDCCC apparatus. Only the stearic and myristic acids were used in this case because the resolution between stearic and palmitic acids did not permit accurate calculations of all chromatographic parameters. Separation of all three acids is possible with 12 cartridges (leading to a 250 mL internal volume column), at a 1000 rpm rotational speed and a 1 mL/min flow-rate [11].

Figure 4. shows the plots of the capacity factors against the retention of stationary phase. The curve shapes are the same for both HSCCC and CDCCC systems and for each fatty acid and are described by formula (5). This equation allowed to calculate the partition coefficients: K_{C18}=0.33 (±15%), K_{C16}=0.52 (±9%), K_{C14}=0.82 (±7%) with the HSCCC unit and K_{C18}=0.42 (±20%), K_{C14}=0.79 (±15%) with the CDCCC unit. The limited dispersion of the results validate the accuracy of our measurements.

The separation factors for each device show good reproducibility with S_F. For the HSCCC apparatus, $\alpha_{1/2}$ is equal to 1.62 (±8%) and $\alpha_{2/3}$ is equal to 1.57 (±4%). The CDCCC apparatus leads to a separation factor equal to 1.92 (±6%).

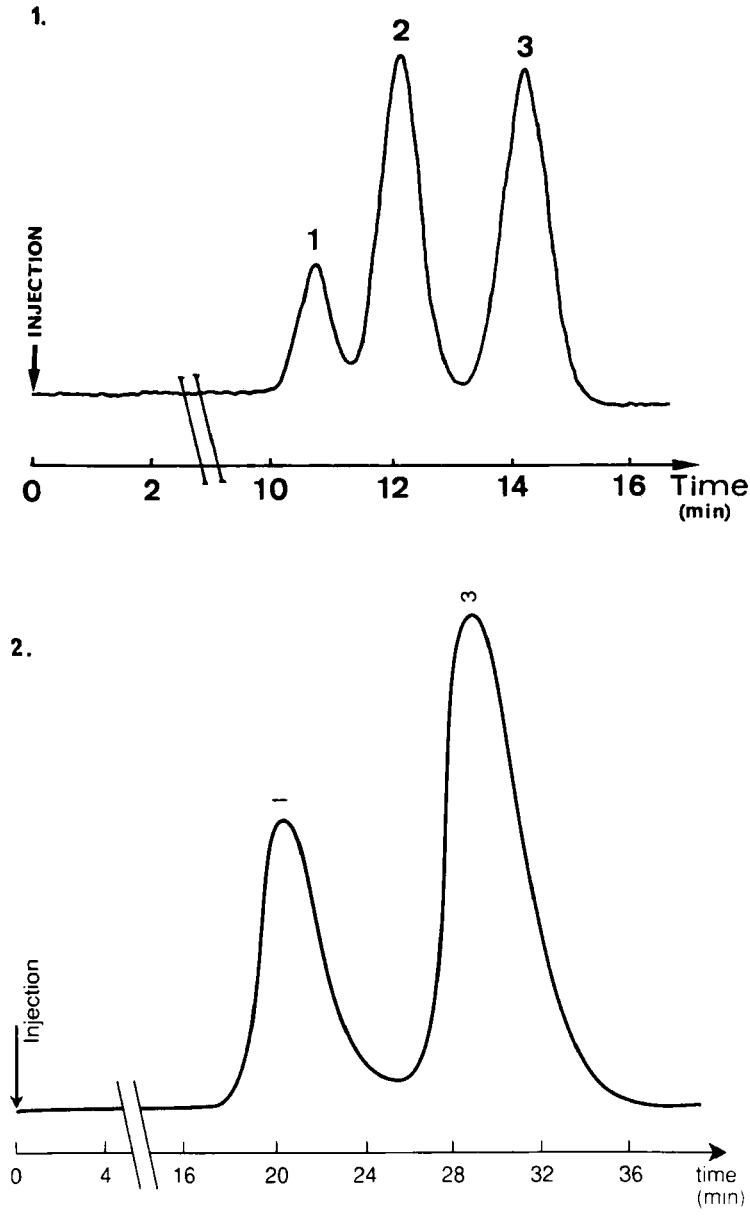

FIGURE 3. Chromatogram of: (1) three fatty acids (stearic (1), palmitic (2) and myristic (3) acids), separated on a HSCCC apparatus. Experimental conditions: 21 mL/min flow-rate. S_F=51%, $N_1 \cong 1900$, $N_2 \cong 1480$, $N_3 \cong 1480$, $R_{S1/2} \cong 1.2$, $R_{S2/3} \cong 1.55$; (2) two fatty acids (stearic (1) and myristic (3) acids), separated on a CDCCC apparatus. Experimental conditions: 3.5 mL/min flow-rate. S_F=65%, N_1=260, N_3=355, $R_{S1/3}$=1.34.

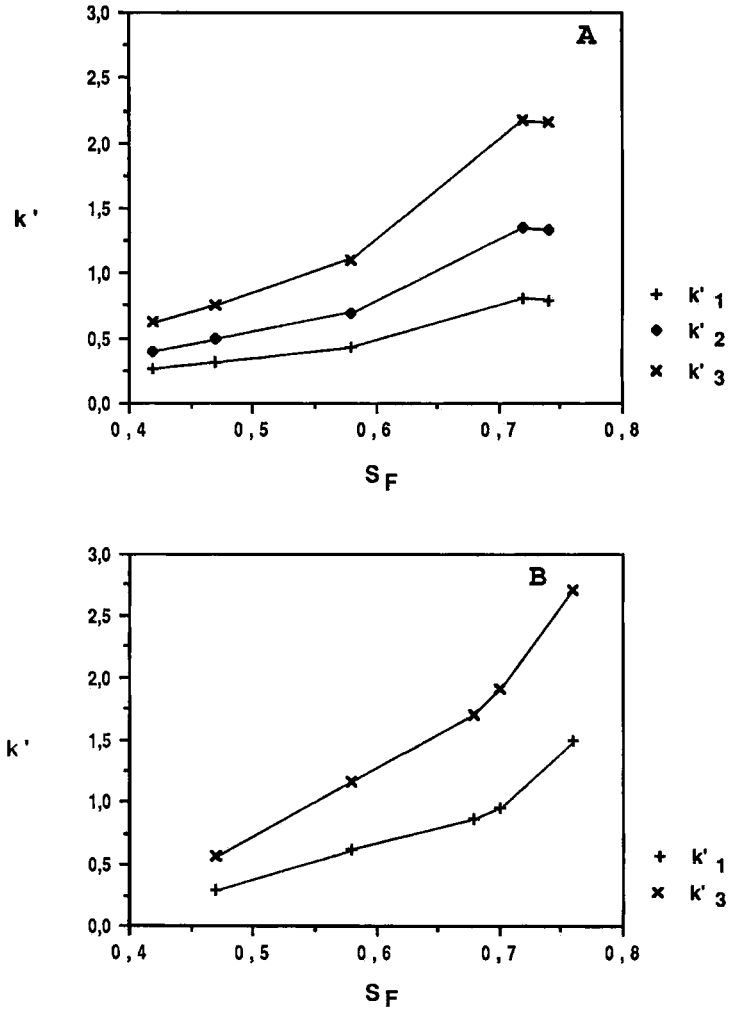

FIGURE 4. Effect of the retention of stationary phase S_F on capacity factors k' on a HSCCC apparatus (A) and a CDCCC apparatus (B).

The parameter $R_{S_{i/j}}(w_i + w_j)$, where $R_{S_{i/j}}$ is the resolution between peaks i and j, w_i and w_j the base widths of peaks i and j, is calculated for HSCCC and CDCCC systems. Figure 5. displays the variation of this value with the retention of stationary phase. The correlation coefficients are shown in the figures and their high values (0.98 and 0.99) demonstrate that R_S follows the linear formula (8).

All these studies demonstrate that chromatographic theoretical equations involving the volume of stationary phase apply very well to CCC. This highlights one of the interets of CCC which allows to fix the volume of the stationary phase retained in the column.

Variation of chromatographic parameters against the flow-rate

We compared the plots of efficiency (number of theoretical plates) against the flow-rate with Van-Deemter plots achieved for ordinary chromatography [12]. However these plots are obtained with constant capacity factors; so it was necessary to check the variation in capacity factor with flow-rate and to develop a procedure to try to keep this factor constant in the HSCCC system. Two procedures were used with the HSCCC apparatus and one with the CDCCC apparatus.

The first procedure used with the HSCCC device, described in part EXPERIMENTAL, is that usually employed. This leads to the usual variation in retention of stationary phase (Figure 2.A). The other procedure for the HSCCC device and the one for the CDCCC device are described in part EXPERIMENTAL as the second procedures. They were intended to keep the capacity factors constant by having a constant retention of stationary phase

Before comparing the plots of chromatographic parameters against the usual results achieved in ordinary chromatography, it is important to plot the variation of the capacity factors with the flow-rate (Figure 6.). The first procedure for the HSCCC unit (Figure 6. A1) leads to decreasing retention of stationary phase, involving decreasing capacity factors; so no real comparison with ordinary chromatography can be made. Figures 6. A2 and B emphasize the interest of the second procedures as capacity factors remain constant for both devices.

Comparison can thus be held between Van-Deemter plots and Figures 7. A2 and B. In both cases the shapes of the curves are far different from Van-Deemter plots, seen for ordinary chromatography. Instead of showing a maximum,

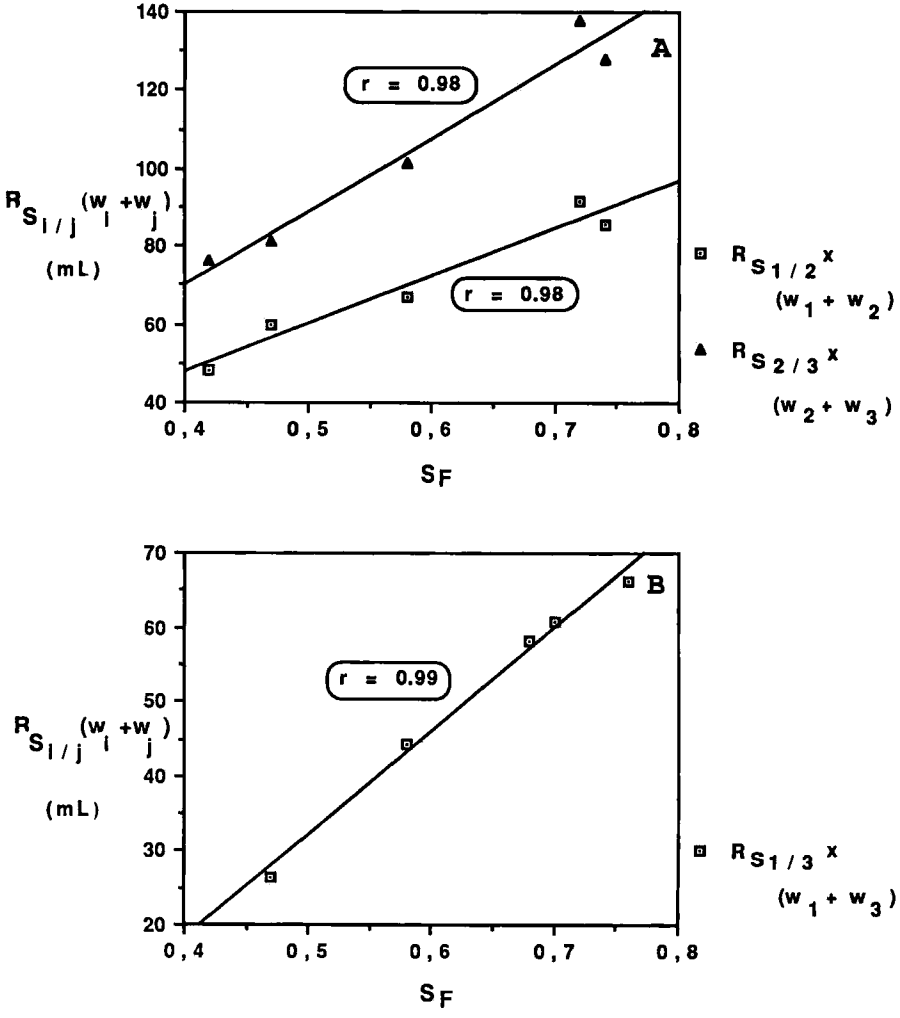

FIGURE 5. Effect of the retention of stationary phase S_F on $R_{Si/j}.(w_i+w_j)$ on a HSCCC apparatus (A) and a CDCCC apparatus (B).

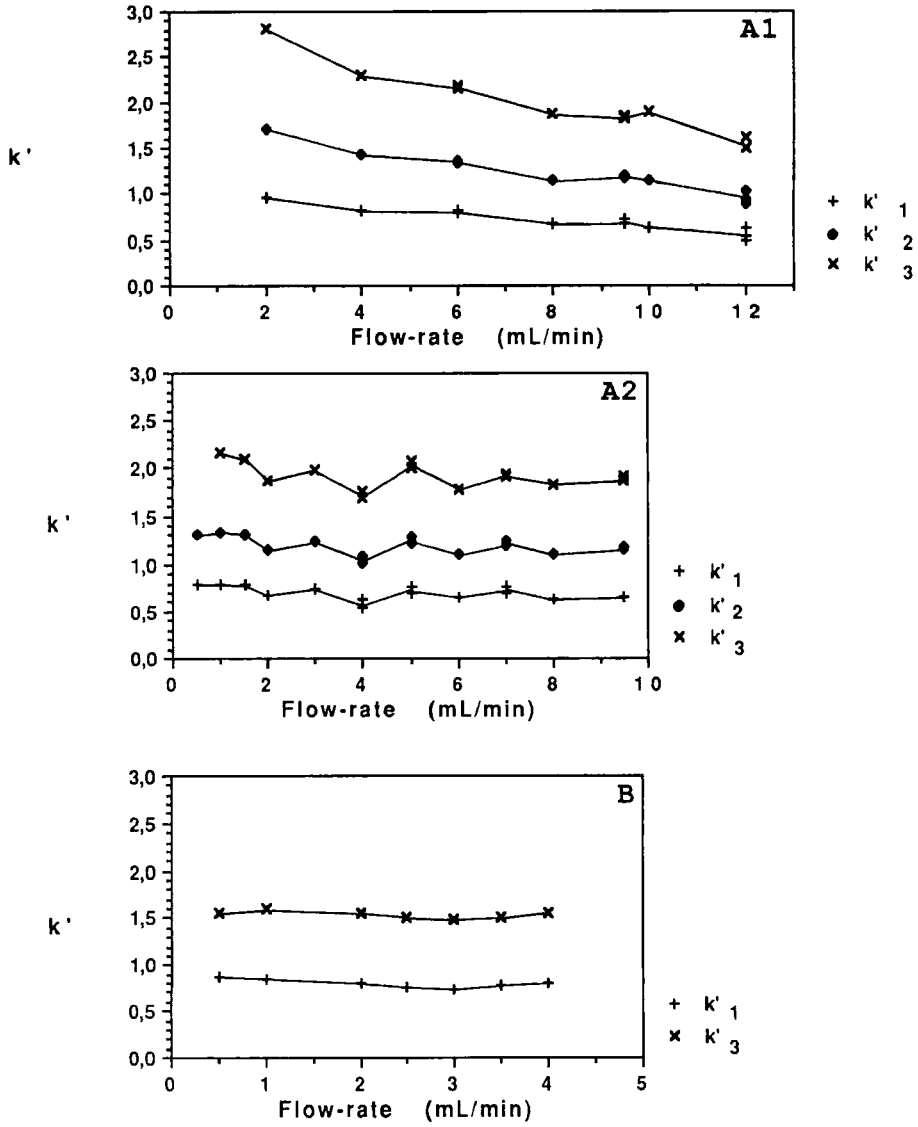

FIGURE 6. Effect of the flow-rate on the capacity factors k' on a HSCCC apparatus, (A1) using procedure 1, (A2) using procedure 2 and on a CDCCC apparatus (B). (A2) $S_F \cong 69\%$; (B) $S_F \cong 65\%$.

the number of theoretical plates always decreases with flow-rate for the HSCCC apparatus (Figure 7. A2) and shows a minimum for the CDCCC apparatus (Figure 7. B). For the latter, this shape was discussed and a theory developed [13], but for the HSCCC device, no complete explanation is available. Plotting N against F should be made with a constant retention of stationary phase. Otherwise, the shape of the curve (Figure 7. A1) is the same as that obtained on the CDCCC apparatus. It is plotted here only for myristic acid to simplify the figure but the shape of the curve is the same for the two other fatty acids.

The separation factors were then computed for the CCC devices. For the HSCCC apparatus with the first procedure, $\alpha_{1/2}$ is equal to 1.72 (±6%) and $\alpha_{2/3}$ is equal to 1.61 (±4%) and with the second procedure, $\alpha_{1/2}$ is equal to 1.72 (±5%) and $\alpha_{2/3}$ is equal to 1.62 (±4%). For the CDCCC, α is equal to 1.92 (±6%). The separation factors are thus approximately constant.

For both devices, the resolution R_S always decreases with the flow-rate, independently of the procedure. This is explained using the equation:

$$R_s = \frac{1}{4}(\alpha - 1)\sqrt{N}\left[\frac{K_1}{K_1\left(\frac{\alpha+1}{2}\right) + \left(\frac{1 - S_F}{S_F}\right)}\right] \quad (11),$$

given by CONWAY and ITO [14] who derived it from the definition of the resolution, given in formula (7). For the HSCCC apparatus, the separation factor and the retention of stationary phase can be considered as constant for the second procedure. The resolution R_S is then only dependent on the number of theoretical plates N. Thus the decrease of N against F leads to a decrease of R_S, as shown in Figure 8. A2. For the first procedure, α can be considered as constant and S_F is decreasing with the flow-rate. Thus the decrease of the number of theoretical plates N and also the decrease of the ratio $(K_1 / (K_1(1+\alpha)/2+(1-S_F)/S_F)$ with the flow-rate leads to a decrease in R_S against F, demonstrated in Figure 8. A1. For the CDCCC apparatus, the same explanation applies and the decrease is shown in Figure 8. B.

When the HSCCC device is equilibrated and operated for the separation at the same flow-rate, the mobile phase is distributed all along the column in an homogeneous way [15]. But when the apparatus is equilibrated at a higher flow-rate (F_0) than that used (F) to perform the separation, the distribution of the mobile phase in the column is not known. Because the volume of the mobile phase is

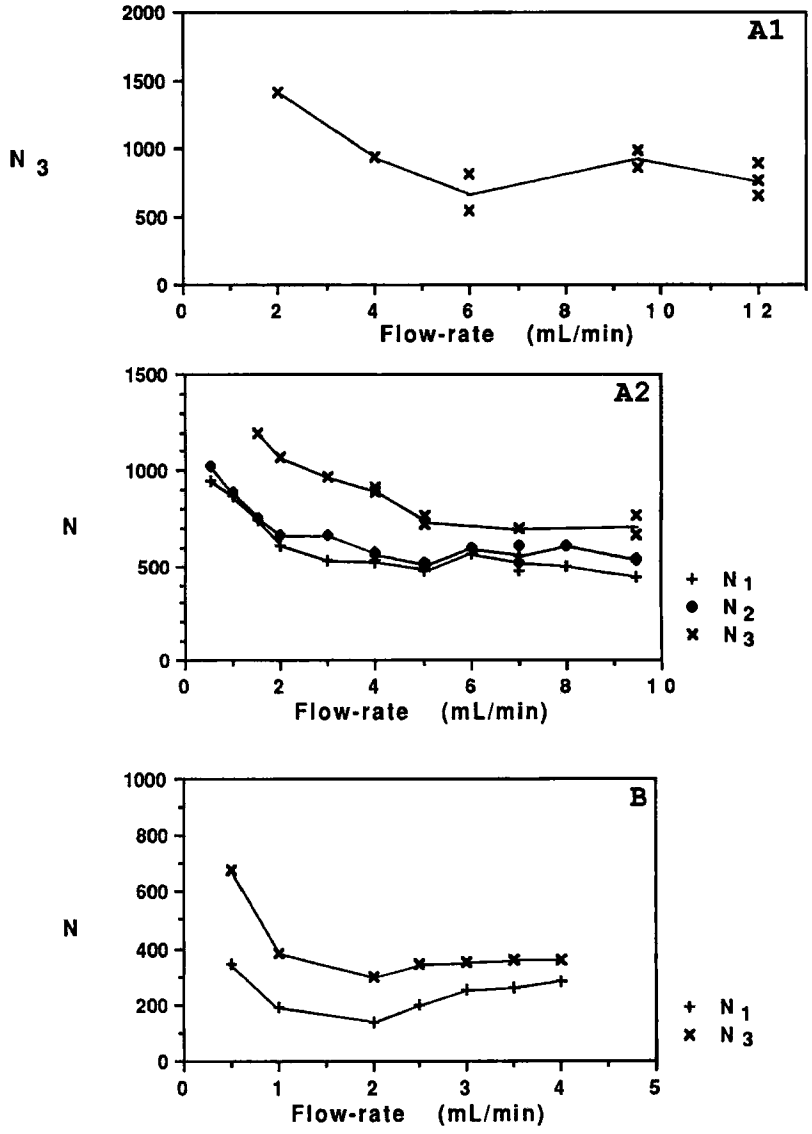

FIGURE 7. Effect of the flow-rate F on the numbers of theoretical plates N on a HSCCC apparatus, (A1) using procedure 1, (A2) using procedure 2 and on a CDCCC apparatus (B). (A2) $S_F \cong 69\%$; (B) $S_F \cong 65\%$.

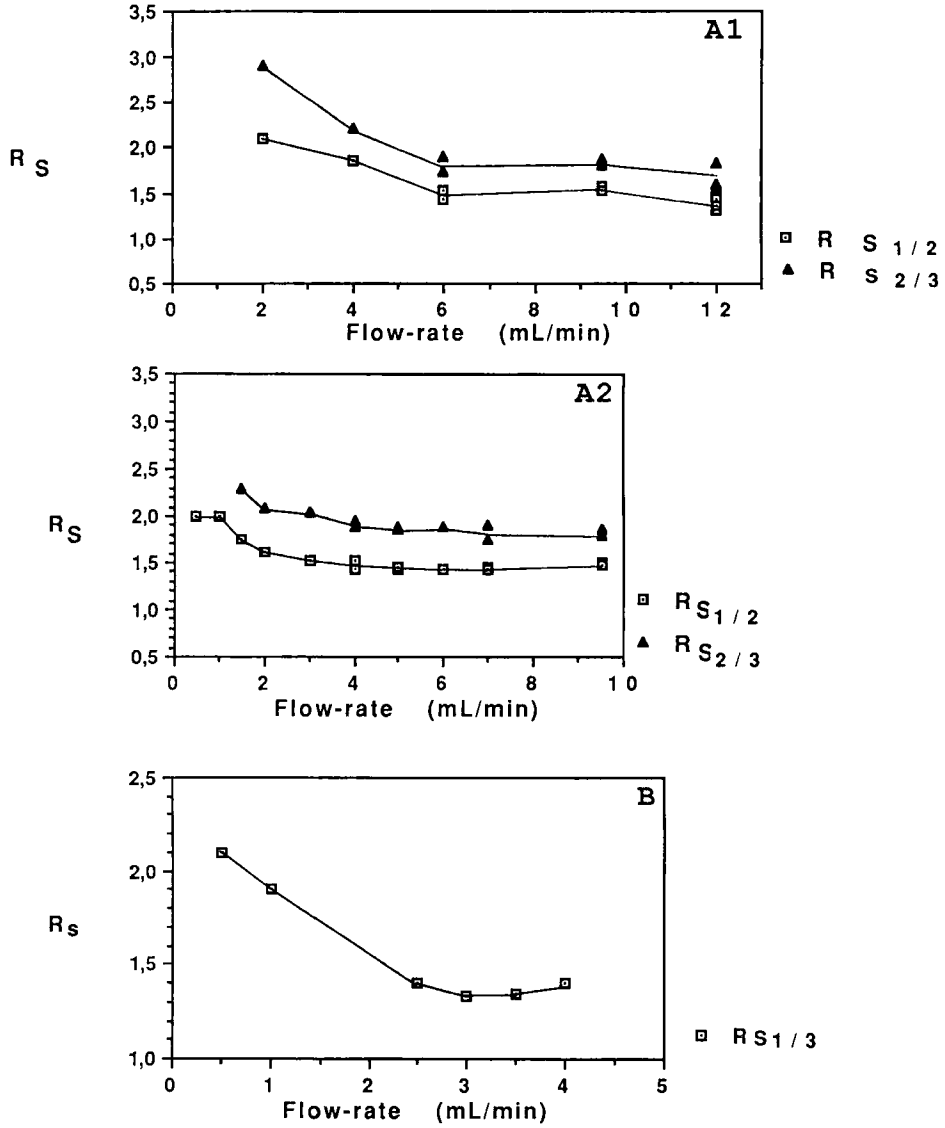

FIGURE 8. Effect of the flow-rate F on the resolutions R_S on a HSCCC apparatus, (A1) using procedure 1, (A2) using procedure 2 and on a CDCCC apparatus (B). (A2) $S_F \cong 69\%$; (B) $S_F \cong 65\%$.

TABLE 1. Comparison of the Actual Resolutions $R_{S1/2}$ and $R_{S2/3}$ for Typical Flow-rates and First and Second Procedures.

Flow-rate (mL/min)	$R_{S1/2}$ / S_F		$R_{S2/3}$ / S_F	
	first procedure	second procedure	first procedure	second procedure
2	2.69	2.33	3.72	3.01
4	2.54	2.14	3.00	2.76
8	2.55	2.24	3.54	2.99
9.5	2.25	2.11	2.79	2.57

artificially too high for the flow-rate F, there is an extra-volume of mobile phase. How this volume may be distributed in the column is unknown. It may be distributed homogeneously all along the column or accumulated in one part of the column, leaving another part filled only with mobile phase. To try to examine this distribution, we compared the resolutions for four different flow-rates, which were studied with both procedures. TABLE 1. summarizes these results. We actually calculated the ratio R_S/S_F, equal to $2V_t (K_2-K_1) / (w_2+w_1)$, to eliminate the influence of the retention of stationary phase between the two procedures. Our results show that the actual resolution R_S/S_F is lower for the second procedure than for the first one. The difference is significant as the gap between the first and the second procedures is on average equal to 15%. The values calculated for the 9.5 mL/min should be the same as this flow-rate is the one used for the second procedure to equilibrate the apparatus. Thus the observed difference is due to experimental errors (lower than 8%). This may validate the second hypothesis concerning the distribution of the mobile phase in the column: it may accumulate on the head side of the column in the same way as during a separation achieved with the first procedure. Thus a dead volume of mobile phase may exist on the head side of the column, leading to a relative decrease of resolution.

All the previous studies based on the first procedure were limited to a 12 mL/min flow-rate to ensure no loss of the stationary phase. But it is interesting to give some curves achieved at higher flow-rates. Figures 9. 1 and 2 show the variations of the number of theoretical plates and the separation factor for flow-

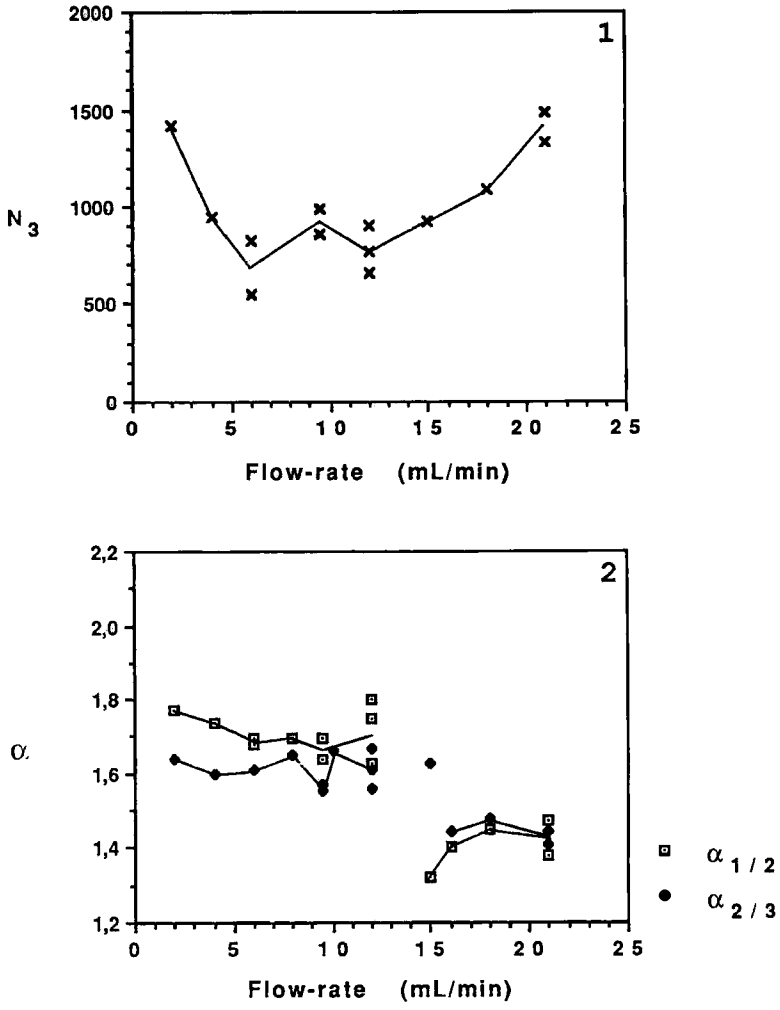

FIGURE 9. Effect of the flow-rate F on: (1) the numbers of theoretical plates N and (2) the separation factors α on a HSCCC apparatus, using procedure 1 up to 21 mL/min.

rates up to 21 mL/min. For flow-rates higher than 15 mL/min, a loss of stationary phase was always noticed. The increase of the number of theoretical plates during the loss of stationary phase is a well-known phenomenon and is displayed here in Figure 9. 1 at flow-rates higher than 15 mL/min. The plot is given only for the myristic acid to simplify the figure but the shape of the curve is the same for the two other saturated fatty acids. The smaller values of α, obtained at flow-rates higher than 15 mL/min (Figure 9. 2), could also be explained by the variation of retention of stationary phase during the separation. As defined by the first part of formula (9), α is the ratio of the capacity factors. So if a loss of stationary phase has to be considered, α is written:

$$\alpha = \frac{k'_2}{k'_1} = \frac{K_2}{K_1} \frac{\frac{1}{S_{F_1}} - 1}{\frac{1}{S_{F_2}} - 1} \qquad (12)$$

with $k'_2 > k'_1$ and $S_{F_2} < S_{F_1}$ (due to the loss of stationary phase between the elution of each fatty acid). Then the ratio $(1/S_{F_1}-1) / (1/S_{F_2}-1)$ is smaller than 1, leading to a worse separation factor α as it is smaller than the ideal ratio K_2/K_1.

Finally we calculated the partition coefficients for each fatty acid, using formula (5). Analysis of the measurements gives for the HSCCC unit with the first procedure K_{C18}=0. 26 (n=11, s=0.05), K_{C16}=0.45 (n=11,s=0.07), K_{C14}=0.74 (n=10, s=0.15), with the second procedure K_{C18}=0. 30 (n=15,s=0.03), K_{C16}=0.45 (n=15, s=0.05), K_{C14}=0.74 (n=14, s=0.07) and for the CDCCC unit with the second procedure K_{C18}=0.38 (n=11, s=0.04) and K_{C14}=0.74 (n=11, s=0.08).

We also used two other methods to obtain the partition coefficients. The first one is based on the "Dual Mode" procedure on the CDCCC device. The second method is the direct measurement of K using radioactive fatty acids. Both procedures are described in part EXPERIMENTAL. All the partition measurements from the two procedures with the HSCCC apparatus and the two methods with the CDCCC device are gathered in Table 2. to be compared with the values obtained from radioactivity measurements. The accuracy of the latter allows us to take them as references. The difference between chromatographic values and references decreases from the stearic acid to the myristic acid. One reason is the low capacity factor for the stearic acid which thus leads to higher experimental errors than for the two other fatty acids.

TABLE 2. Values of Partition Coefficients Obtained from HSCCC Chromatograms (first and second procedures), CDCCC Chromatograms, CDCCC "Dual Mode" Procedure and Direct Radioactive Measurement.

Apparatus/Method	Partition coefficients of the saturated fatty acids		
	stearic	palmitic	myristic
HSCCC first procedure	0.26	0.45	0.78
HSCCC second procedure	0.30	0.52	0.84
CDCCC second procedure	0.36	/	0.70
CDCCC "Dual Mode" procedure	0.34	/	0.80
Radioactivity measurements	0.26	0.58	0.74

The first HSCCC procedure leads to good results, as only the K value for the palmitic acid is different by 22% from the radioactive measurement. The second procedure is always different from the reference values by an average of 10%, which may be the result of a special solvent distribution in the column during the separation. The CDCCC second procedure leads to a bad result with the stearic acid, likely to be due the low capacity factor of this acid. The CDCCC "Dual Mode" procedure is always different from reference values. This may be due to the use of only one experiment for the special procedure leading to possible experimental errors. Moreover our CCC devices were not thermoregulated; this is another explanation of the observed differences with the references.

CONCLUSION

The use of a test sample of myristic, palmitic and stearic acids allowed us to compare two CCC devices, one based on the planetary motion (HSCCC) and the

other one on a rotational motion (CDCCC). For both devices, the usual chromatographic equations apply but the variation in the retention of the stationary phase, which is a major feature of both techniques, interferes with the determination of capacity factor, separation factor, resolution and efficiency. The CDCCC apparatus allows a more constant volume of stationary phase in the column within a range of flow-rates. This is not possible with the HSCCC device unless a special separation procedure, such as that described above, is used. However the latter may alter the distribution of the mobile and stationary phases along the column and thus the column length available for the partition process.

Efficiency plotted against the flow-rate shows a minimum for the CDCCC apparatus while there is only a decrease for the HSCCC apparatus without any loss of stationary phase. Consideration of the above factors with each CCC apparatus permitted the determination of the partition coefficients of the fatty acids and the comparison of their abilities to give accurate values. Procedures used with both HSCCC and CDCCC devices give good results when they are compared with accurate radioactivity measurements.

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