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# Resolution studies on counter-current chromatography using supercritical fluid carbon dioxide

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## Abstract

Counter-current chromatography (CCC) is a form of liquid–liquid partition chromatography. It requires two immiscible solvent phases; the stationary phase is retained in the separation column, generally by centrifugal force, while the mobile phase is eluted. We recently replaced the mobile phase with supercritical fluid carbon dioxide (SF CO<sub>2</sub>). Since the solvent strength of SF CO<sub>2</sub> can be varied by changing the temperature and pressure of the system, separation adjustments are thus more versatile. We investigated the pressure and temperature effects on resolution using water and low-carbon alcohol mixtures as the stationary phases. It was demonstrated that these special properties of SF CO<sub>2</sub> were indeed beneficial to the optimization of separations. In addition, the phase retention ratio was examined in terms of separation resolution. The results appeared very similar to those obtained from conventional traditional CCC. This study should be helpful for the future development of SF applications in CCC. © 1997 Elsevier Science B.V.

**Keywords:** Counter-current chromatography; Mobile phase composition; Resolution; Naphthalene; Benzophenone; Acetophenone

## 1. Introduction

Counter-current chromatography (CCC) is a generic name for various liquid partition chromatographic methods in which the stationary phase is retained in the apparatus without porous or adsorptive matrices [1,2], thus eliminating irreversible adsorption, and minimizing the risk of sample denaturation using CCC as a preparative separation tool. Samples can be almost completely recovered. Modern CCC was introduced by Ito et al. in Japan [3]. In the past thirty years or so, great advances have been made in CCC instrumentation and meth-

odology. For example, recently two closely related techniques called pH-peak-focusing and pH-zone-refining have been developed as highly efficient preparative methods [4–13].

One of the major tasks CCC practitioners have to face is finding a solvent system that provides adequate partitioning of the analytes in the two phases. Selecting a two-phase solvent system for CCC is similar to choosing a column and eluent solvent for high-performance liquid chromatography (HPLC). Conventionally, both CCC phases are in liquid states. We reported on replacing the liquid mobile phase with supercritical fluid carbon dioxide (SF CO<sub>2</sub>) for the first time in a previous study [14]. In order to distinguish this from conventional liquid–

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liquid CCC (LL-CCC), we called this technique supercritical fluid/liquid CCC (SL-CCC). As is generally known, SF CO<sub>2</sub> has relatively high diffusivity, low viscosity and liquid-like solvent strength, as compared to ordinary liquids, and has been employed as the mobile phase in supercritical chromatography (SFC) [15–24]. It is believed that the higher mass-transfer rates of SFs, due to the high diffusivity, gives SFC an edge in separation efficiency over liquid chromatography (LC). However, it has yet to be determined whether it is also true for SL-CCC compared with LL-CCC.

Since CCC has been applied mainly to preparative chromatography, it can be an advantage to have the mobile phase SF CO<sub>2</sub> leave the column as a gas separated from the analytes. In the practice of LL-CCC, however, further concentration of the analytes is usually needed after separation.

We demonstrated the benefit of solvent strength modification using SF CO<sub>2</sub> as the mobile phase through the process of optimizing the separation of a mixture of naphthalene (Np), benzophenone (Bp) and acetophenone (Ap). Basically, it was accomplished with a small adjustment in the temperature and pressure applied to the system. The composition of the stationary phase still plays a major role in altering the partition coefficient of sample molecules in the mobile and stationary phases. Separation

optimization was also investigated in this study through some fine-tuning of the composition of the stationary phase. Finally, the phase retention ratio was analyzed with respect to the separation resolution.

## 2. Experimental

### 2.1. Set-up

The general features of the experimental set-up used in the present study were described previously [14]. The instrumental layout is shown in Fig. 1. The high-pressure pump and the absorbance monitor were respectively, an ISCO Model 260 syringe pump, and a V4 UV-Vis detector (Lincoln, NE, USA). Two Valco (Houston, TX, USA) six-port valves were used for flow-path switching and sample injection; one Swagelok metering valve was used to control the SF CO<sub>2</sub> flow-rate. All valves, including several two-way ones, were connected by 1.59 mm O.D.×0.25 mm I.D. polyether ether ketone (PEEK) tubing and Swagelok fittings. The PEEK tubing can endure pressures up to 41 mPa and is flexible enough to suit the anti-twisting mechanism well. A phase separator (total volume~0.5 ml) installed just before the detector was needed to remove occasional bleed-

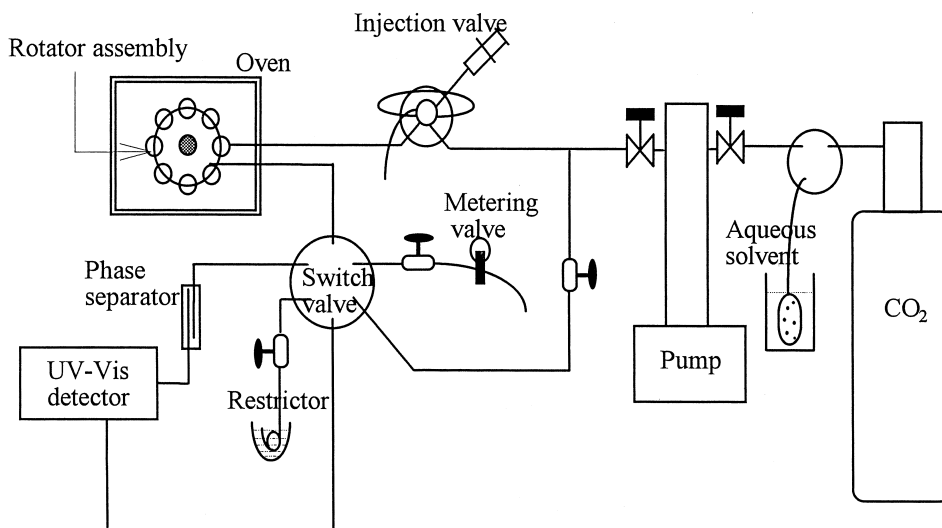


Fig. 1. Schematic diagram of the instrument layout.

ing solvent droplets in order to avoid light-scattering during monitoring. The linear restrictor used in this study was a 30 cm×50 μm I.D. fused-silica tubing.

The laboratory-made rotator assembly was a solar eccentric type using Adams anti-twisting mechanism which was better suited to this high-pressure system than a rotation seal would have been. In this study, eight evenly spaced rods (20 cm×1.78 cm O.D.) were wound in series from a 24 m long PEEK tube (3.18 mm O.D.×1.57 mm I.D.), that gave an approximate total separation-column capacity of 46 ml. This larger-bore tubing can withstand lower pressures, i.e., 20 mPa, than the smaller-bore tubes used as connection tubing. The inlet and outlet tubes (1.59 mm O.D.×0.25 mm I.D. PEEK), lubricated with silicone grease, were handled with great care to prevent them from cracking. Portions touching the frame during rotation were protected by flexible plastic sheaths to prevent abrasion from direct contact with metal parts. The revolution speed of the apparatus was motor-adjustable over a range of 0–450 rpm. The whole assembly was housed in an oven with a ±1.0°C tolerance.

## 2.2. Reagents

Reagent-grade acetophenone (Ap), benzophenone (Bp) and naphthalene (Np) were purchased from Sigma (St. Louis, MO, USA), HPLC-grade methanol from Mallinckrodt (Paris, KY, USA). Deionized water was supplied by Milli-Q (Millipore, St. Quentin-en-Yvelines, France) and SFC-grade CO<sub>2</sub> from Scott Specialty Products (Plumsteadville, PA, USA). The sample solutions were prepared in methanol. The injection volume for all separations was 100 μl. The aqueous stationary phases were degassed before use.

## 2.3. Procedure

The coiled column was first filled with the stationary phase at a flow-rate of 15 ml/min using the syringe pump, while the apparatus was rotated at about 60 rpm. The oven was then set at the desired experimental temperature, while the syringe pump was filled with CO<sub>2</sub> (the mobile phase) and compressed to the desired pressure. The assembly was then rotated at 420 rpm. After the experimental

conditions, such as the oven temperature and the pressure in the pump, of the system had stabilized, the mobile phase was pumped into the coiled column at constant pressure. Part of the aqueous stationary phase was gradually removed by the SF CO<sub>2</sub> flow through the metering valve. Ten minutes after the mobile phase began appearing at the outlet of the metering valve, the column outlet was connected to the capillary restrictor with an appropriate arrangement of the switch valve. Since the two phases had established hydrodynamic equilibrium, the sample solution was injected through the injection valve. The flow-rate measured at the pump head was kept at 0.5 ml/min for all of the separations. The phase separator was never overflowed with the stationary phase during the chromatographic runs. The effluent was continuously monitored by UV–Vis absorbance detection at 247 nm.

## 3. Results and discussion

The versatility offered by using SFs as the mobile phases in chromatography result from the fact that the solvent strength can be manipulated through applied pressure and temperature. In addition, the stationary phase composition also plays one of the major roles in separation. We demonstrated these effects by optimizing the separation of three components – Np, Bp and Ap.

### 3.1. Temperature effect

Temperature programming has long been used in optimizing SFC operations [25,26]. Two factors must generally be considered [27] in understanding solute retention due to temperature change. Under constant-pressure conditions, solute solubility depends on the density of the fluid, which varies as a function of temperature. When the temperature rises, the density of the fluid decreases, resulting in lower solubility and greater retention time. However, the vapor pressure of the solutes also changes along with the temperature. The association of these two factors eventually determines the retention of the solute. We investigated the temperature factor using a mixture of Np and Bp and a mixture of Np and Ap. Instead of temperature programming, separations under fixed

temperatures were conducted to clearly demonstrate the effect. The results are illustrated in Figs. 2 and 3, and show that a 5°C difference can result in considerable improvement in resolving the analyte peaks in both mixture separations.

It should be noted that the flow-rate of 0.5 ml/min was measured at the pump head at room temperature, and differed from the column temperature. Expansion occurred when the SF CO<sub>2</sub> flowed from the pump head to the column. Therefore the retention volume determined according to the retention time and the flow-rate could not be used to estimate the stationary-phase retention ratio. The phase-retention volume was measured directly by flushing the stationary phase out of the column at the end of each chromatographic run. Since the phase separator was never overflowed with the stationary phase in any chromatographic run, bleeding could essentially be ignored.

Note the Np retention time decreased as the

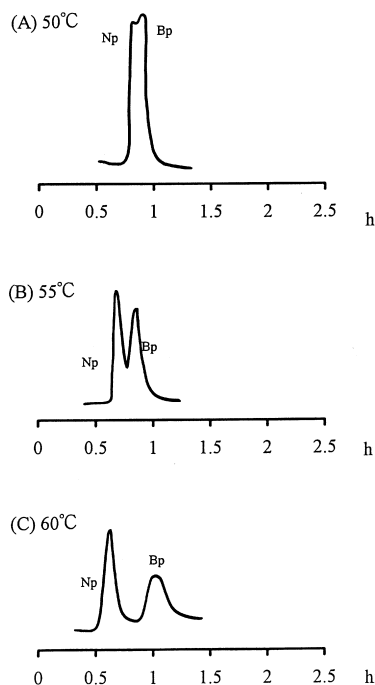


Fig. 2. Separation of Np and Bp at different temperatures. Experimental conditions: 105 bar, methanol–water (30:70), 1000 ppm for each component.  $S_F = 27.1, 28.3, 29.6\%$  for (a), (b) and (c), respectively.

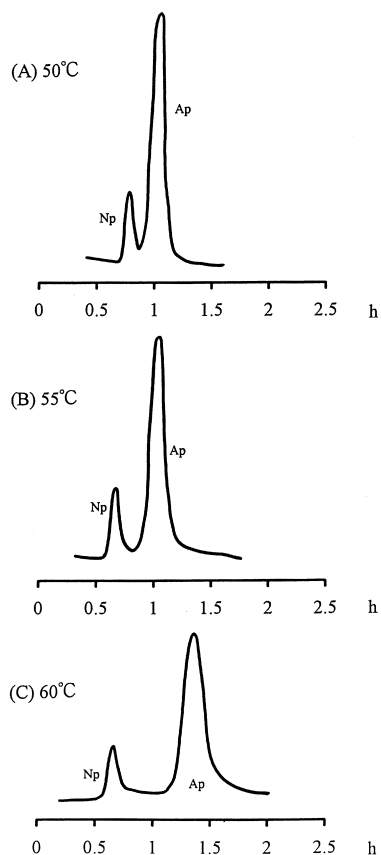


Fig. 3. Separation of Np and Ap at different temperatures. Experimental conditions: 105 bar, methanol–water (30:70), 1000 ppm for each component.

temperature rose, while that of the Bp reacted inversely. Solute solubility usually increases when the liquid solvent (i.e., the stationary phase) temperature increases. The decrease in retention time indicates, however, a higher partitioning of Np in the mobile phase. As pointed out above, the density of SF CO<sub>2</sub> and the vapor pressure of the solute play major roles in determining the capacity factor when temperature is varied. At the higher temperatures, the Np vapor pressure increase in this case seemingly overcame the decrease in solvent strength due to its lowered fluid density. In addition, one other factor must not be ignored. The methanol content in the SF CO<sub>2</sub> as well as the stationary at different temperatures would also affect the solvent strength of SF CO<sub>2</sub> for the analytes.

### 3.2. Stationary phase modification

In comparing Figs. 2 and 3 it seems that the mixture of Np, Bp and Ap would be resolved to certain extent at either 55 or 60°C. A sample mixture of these three compounds was prepared and chromatographed. The chromatograms are shown in Fig. 4. The overlapping of Bp and Ap was shown to be worse than predicted.

One of the characteristics of CCC is the ease of changing both the stationary and mobile phases, or commonly said, the solvent system. In this study, the mobile phase, i.e., the SF CO<sub>2</sub> was kept the same while the composition of the stationary phase was modified to investigate the resolution between the peaks. In addition to the methanol–water combination, small portions of ethanol or 2-propanol were added to the stationary phase. Under the same modifications, the chromatograms at 55°C gave better resolution than those at 60°C in general; see Figs. 5 and 6. The stationary phase with the composition of methanol–water (20:80), however, delivered better results at 60°C than at 55°C, again due to the shorter retention time of Np at the higher temperature. The coupling effect of solvent com-

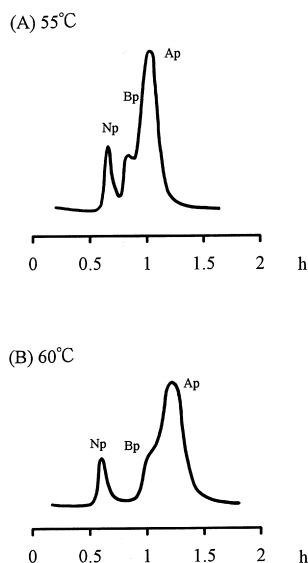


Fig. 4. Separation of Np, Bp and Ap at 55 and 60°C. Experimental conditions: 105 bar, methanol–water (30:70), analyte concentration: 645, 900, 450 ppm for Np, Bp and Ap, respectively.

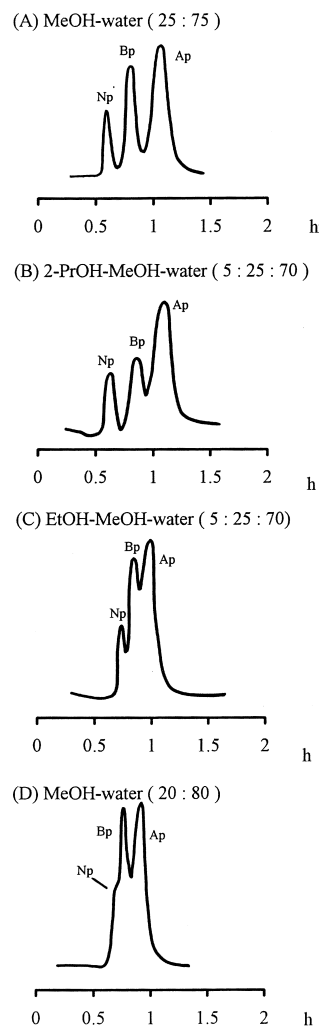


Fig. 5. Separation of Np, Bp and Ap with different stationary phase modifications. Experimental conditions: 105 bar, 55°C, analyte concentration: 645, 900, 450 ppm for Np, Bp and Ap, respectively.

position and temperature could make the separation results more difficult to predict, however in the meantime, it also adds versatility to the technique.

### 3.3. Pressure effect

In addition to temperature, the applied pressure is another factor that modifies the solvent strength of the mobile fluid. The density, which changes with

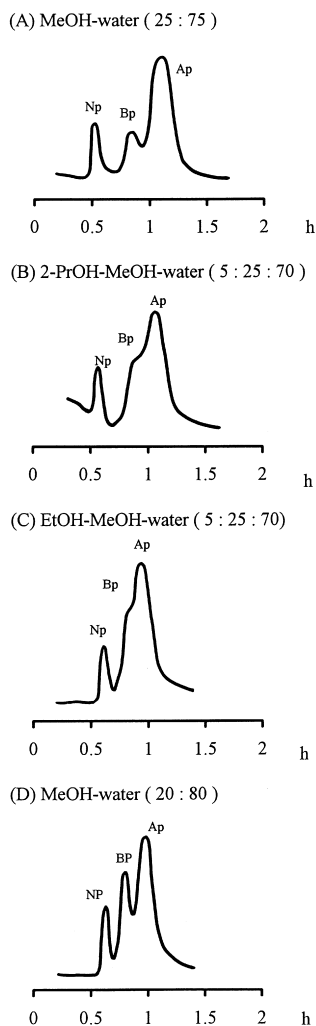


Fig. 6. Separation of Np, Bp and Ap with different stationary phase modifications. Experimental conditions: 105 bar, 60°C, analyte concentration: 645, 900, 450 ppm for Np, Bp and Ap, respectively.

the pressure, is actually the parameter that reflects the solvent strength. Since the compressibility of liquids is not to be compared with SFs, their densities (and also their solvent strengths) are considered the same in this study. Therefore, the separation resolution due to pressure changes in the elution is considered to be dependent only on the mobile phase in our study. Accordingly, the pressure factor is considered simpler than the temperature factor. Note from Figs. 2A and 3A that good resolution of the

three components apparently could not be achieved at 50°C and 105 bar even when the experiment was not performed. Experiments were performed under 100 bar using same stationary phase, i.e., methanol–water (30:70), at two different temperatures. An acceptable resolution was obtained just by lowering the pressure from 105 bar to 100 bar; see Fig. 7A. At 55°C (see Fig. 7B) the retention times of Bp and Ap became longer and the band broadening of both peaks eventually spoiled the resolution. Again, the temperature influenced the separation resolution as pointed out above, only more profoundly under these experimental conditions.

We also tried to improve two other separations. The experimental conditions shown in Figs. 4B and 6B were held the same, while the applied pressure was elevated from 105 bar to 115 bar. The outcomes are shown in Fig. 8A,B. Although the retention time of Np increased, it did not influence the resolution. On the other hand, narrower peaks due to the shorter retention times helped the resolution of Bp and Ap.

### 3.4. Phase-retention-ratio effect on separation resolution

The LL-CCC equation derived by Conway and Ito

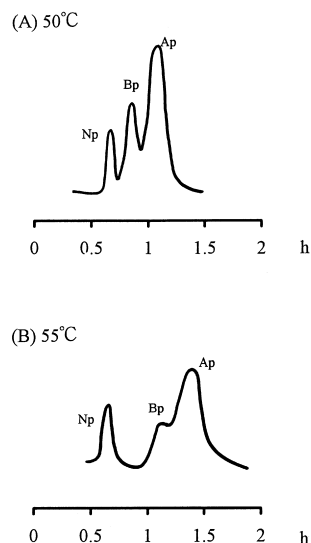


Fig. 7. Separation of Np, Bp and Ap at 50 and 55°C. Experimental conditions: analyte concentration: 645, 900, 450 ppm for Np, Bp and Ap, respectively, 100 bar, methanol–water (30:70).

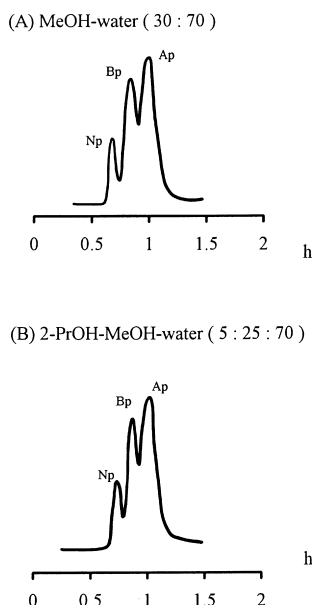


Fig. 8. Separation of Np, Bp and Ap at 60°C, 115 bar with different stationary phase modifications. Analyte concentration: 645, 900, 450 ppm for Np, Bp and Ap, respectively.

[28] to correlate the resolution of separation with the phase retention ratio, is shown below:

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

where  $\alpha$  is the selectivity factor,  $N$  the number of theoretical plates,  $K_1$  the solute partition coefficient and  $S_F$  the phase-retention ratio. We examined the relationship between phase retention and resolution on SL-CCC using this equation although it was originally derived for LL-CCC. Phase-retention ratio was found to be dependent on several experimental parameters, such as assembly rotation speed, temperature, pressure, flow-rate and stationary phase composition. Different phase-retention ratios were achieved using different flow-rates in this study. Once hydrodynamic equilibrium was achieved, separation was performed at a slower flow-rate to make certain no bleeding would occur. In other words, all the experiments were performed at the same flow-rate but different phase-retention ratios. The number

of theoretical plates,  $N$  was calculated as the average of the pairs of peaks considered in the resolution calculation. The precise value of  $K$  can be measured using a spectrophotometer [28]. In this study, the  $K$  value was estimated using the following equation [29] instead:

$$K = \frac{V_R - V_m}{V_m} \left( \frac{1 - S_F}{S_F} \right) \quad (2)$$

where  $V_R$  stands for the mobile phase volume from injection to peak emergence minus the dead volume contained in the feed lines,  $V_m$  the solvent front volume minus the dead volume contained in the feed lines and  $S_F$  the phase-retention ratio.  $V_R$  was estimated to be the retention volume from injection to the emergence of the individual peak.  $V_m$  was estimated as the mobile phase volume when the solvent front of the SF CO<sub>2</sub> appeared at the restrictor outlet during stationary phase displacement.  $S_F$  was obtained by collecting and measuring the stationary phase volume after the separation run was completed. Notice that volume measurements were done at room temperatures and pressures different from those in the column. However, the expansion coefficients and isothermal compressibilities of liquids [30] are very small, the volume change of stationary phases can be ignored. Accordingly,  $V_m$  and  $V_R$  were mobile phase volumes of the SF CO<sub>2</sub> measured consistently at the pump head while  $S_F$  was calculated from the volume of the liquid-state stationary phase. The calculated and experimental resolutions are listed in Table 1 for comparison. The experimental values were computed by the conventional formula in the following:

Table 1  
Resolution calculated using Eq. (1) compared with experimental results for various phase-retention ratios

$S_F$ (%)	Np/Bp		Bp/Ap	
	Calculated	Experimental	Calculated	Experimental
33.1	1.67	1.41	1.11	1.11
23.3	1.33	1.04	1.02	0.81
21.4	0.95	0.98	0.75	0.66

Np=Naphthalene, Bp=benzophenone, Ap=acetophenone. The partition coefficients ( $K$ ) calculated from Eq. (2) are 0.24, 1.02 and 2.04, respectively for Np, Bp and Ap. Experimental conditions: 105 bar, 55°C, 0.5 ml/min, methanol–water (25:75).

$$R_s = \frac{2(t_2 - t_1)}{w_{b1} + w_{b2}} \quad (3)$$

where  $t_1$  and  $t_2$  are retention times of the peaks, and  $w_{b1}$  and  $w_{b2}$  are corresponding peak widths measured at the baseline. Deviations between the experimental and calculated values are apparent. However, the plot of calculated values versus experimental values shows a reasonably linear relationship with the regression of 0.912 and 0.891 for Np/Bp and Bp/Ap pairs. This investigation demonstrated the validity of the trend derived from Eq. (1) for phase-retention ratio effect upon separation resolution.

#### 4. Conclusions

Using SFs as mobile phases in conventional CCC requires high-pressure tubing, tube fittings and valves, and precise temperature control. But due to the physical properties of the SFs, it also possesses some versatility advantages over LL-CCC. We demonstrated separation optimization processes through fine-tuning of the solvent strength of the mobile phase by changing the column temperature and the applied pressure, and through modification of the solvent systems. The phase-retention ratio was also briefly examined with regard to resolution. Our results appear to conform with behaviors similar to those of LL-CCC. It is therefore proven to be very useful to adopt knowledge from both techniques, i.e., LL-CCC and SFC, to make the operation of “marrying” them successful.

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