Original Paper

Effect of Column Structure on Separation Efficiency in Low-Temperature HPLC Using Pure Liquid Carbon Dioxide as the Mobile Phase

Mina OTSUBO, Tomohiro MOTONO, Shinya KITAGAWA*, Hajime OHTANI

Graduate School of Engineering, Nagoya Institute of Technology, Gokiso, Showa, Nagoya 466-8555, Japan

Abstract
The effect of column structure and chromatographic conditions (flow rate, inlet pressure, and temperature) on separation behavior in low-temperature HPLC using pure liquid CO₂ mobile phase was investigated. Three types of packed columns (fully porous, small nonporous, and core-shell particles) and a monolithic column (fully porous) were used. The effect of temperature (-5 to -30°C), inlet pressure (5.3 or 6.3 MPa), and flow rate (1 or 2 mm/s) on chromatographic behavior was evaluated. The retention factors of the analytes (naphthalene, anthracene, and pyrene) were not affected by either inlet pressure or flow rate. Although the column temperature somewhat affected the separation efficiency, the typical relationship between temperature and separation efficiency was not observed. On the other hand, both inlet pressure and flow velocity affected the separation efficiency. In this study, the highest separation efficiency was achieved in the core-shell column with a flow rate of 1 mm/s and inlet pressure of 6.3 MPa. The best separation efficiency under this condition was 12 μm in theoretical plate height (85 000 N/m) for pyrene at -30°C. In addition, the supplied liquid CO₂ was found to solidify in the column at low temperatures and its behavior also depends on both the chromatographic conditions (particularly for flow rate) and the column structure. The typical relationship between solidification and chromatographic behavior was not observed clearly.

Keywords: Column efficiency; Column porosity; Column structure; Liquid carbon dioxide; Low-temperature; Solidification

1. Introduction
HPLC is a general separation method used in various research fields with a wide variety of separation modes developed in order to separate various analytes. In general, almost all HPLC separations are performed at approximately ambient temperature. However, HPLC separations at high and low temperatures have also been reported because temperature is one of the important factors in HPLC. HPLC at high temperature is often employed to achieve high performance separation since both the mass transfer rate between the mobile and stationary phases and the diffusion coefficient of the analyte are enhanced at high temperatures [1-4]. Moreover, Shibukawa et al. reported ion chromatography (IC) at high temperatures for investigating the solvation effects of ions [5,6].

In contrast, HPLC at low temperatures has also been reported. In early days, low-temperature HPLC was applied to analyze thermally labile compounds [7-9]. The preparative separations of both unstable synthetic and natural compounds [10,11] and the enantiomeric separations [12,13] in low-temperature HPLC have also been reported. As an alternative use of low temperature in HPLC, Okada et al. proposed ice chromatography, in which ice was employed as a stationary phase [14-16]. Consequently, HPLC at low temperatures is effective for the analysis of thermally labile compounds, the enhancement of separation efficiency, and the study of specific interactions at low temperatures.

In HPLC at low temperatures above -65°C, organic solvents (methanol, acetonitrile, hexane, dichloromethane, and etc.) and their mixture, and hydrophilic organic solvents (methanol, acetonitrile, and etc.) containing water were mainly used as the non-freezing mobile phases. In addition, low-temperature HPLC using liquefied gasses as the mobile phase has also been reported [17-20]. For example, Pirkle et al. demonstrated enantioselective separations at low...
temperatures above -47°C using mobile phases based on liquid CO₂ [17,18]. In 2016, Motono et al. demonstrated ultralow-temperature HPLC at -196°C using liquefied N₂ based mobile phases, which was the lowest temperature for HPLC separation ever reported [20]. Suitable liquefied gases have sufficiently low freezing points. In addition, liquefied gases have lower viscosities and higher diffusion coefficients despite the low temperature. Therefore, liquefied gases have high potential as a mobile phase for low and ultralow temperature HPLC.

Recently, we investigated the basic chromatographic behaviors of small organic compounds in low-temperature HPLC using a pure liquefied CO₂ mobile phase [19]. In that study, retention factors and separation efficiencies of analytes at low temperatures (-5 to -35°C) were discussed for both ODS and bare-silica columns packed with general fully porous spherical particles. Meanwhile, various advanced HPLC column technologies have recently been developed to achieve high performance separations, e.g., small size packing [21,22], core-shell type packing [22,23], and monolithic columns [24-29]. However, all previous studies for low-temperature HPLC using liquefied gases employed columns packed with general fully porous particles. Thus, in this paper, we investigated the effect of column structure on chromatographic behavior in low-temperature HPLC using pure liquefied CO₂ as a mobile phase under various separation conditions.

2. Experimental

2.1. Apparatus

The low-temperature HPLC used in this study was almost the same arrangement as our previous research [19]. The apparatus was composed of a CO₂ gas cylinder, an automated rotary valve injector (Valve Unite FCV-20AH2, Shimadzu, Kyoto, Japan), a backpressure regulator (KB1L0A422P20000, Swagelok, Solone, OH, USA), two pressure gauges (KDM30, Asone, Osaka, Japan), a gas flowmeter (Mass Flow Sensor Model 3810DSII, Kofloc Kyoto, Kyoto, Japan), a UV light source (high-power UV-Vis fiber light source L10290, Hamamatsu Photonics, Shizuoka, Japan) equipped with a band pass filter (detection wavelength, 254.66 ± 4.68 nm, VPF-25C-10-12-25370, Sigma Koki, Tokyo, Japan), fiber optics (CUV-CCE, Ocean Optics, Dunedin, FL, USA), a photomultiplier (H5783-06, Hamamatsu Photonics), and a low-temperature incubator (38 L JF-NU40G-S, Haier, Qingdao, China), in which the temperature was controlled by a heater set inside of the incubator. In this apparatus, the inlet pressure could be controlled by the regulator of the CO₂ cylinder. The flow rate (v₀) was adjusted by the pressure difference between the inlet and the outlet (ΔP = Pₐ₀-Pₜ₀) using the backpressure regulator. The t₀ based flow rate, v₀ = (column length) / (elution time of solvent peak), was used as a flow rate in this text. The average pressure, \( P_{av} = (P_{in}+P_{out})/2 \), was used for the characterization of CO₂ in the column at low temperatures. Columns packed with fully porous ODS particles of 5 μm d.p. (Chemcobond 5-ODS-H 0.10 × 15 cm, Chemco, Osaka, Japan), small nonporous ODS particles of 2 μm d.p. (Presto FF-C18, 0.10 × 3 cm, Imtakt, Kyoto, Japan), and core-shell ODS particles of 5 μm d.p. (Kinetex 5u C18, 0.21 × 15 cm, Phenomenex, Torrance, CA, USA) were used in the experiment. An ODS silica monolithic column (fully porous) (MonoBis low pressure type 11 nm, 0.10 × 15 cm, Kyoto Monotech, Kyoto, Japan) was also used.

2.2. Chemicals

Carbon dioxide (> 99.995% purity) for the mobile phase was purchased from Sogo Kariya Sanso (Nagoya, Japan). All analytes (naphthalene, anthracene, and pyrene) and acetonitrile, as a sample solvent, were purchased from Wako Pure Chemical Industries (Osaka, Japan). The concentration of the sample component was 1 mM each.
3. Results and discussion

3.1. Effect of packed particle type on retention factor

The effect of the temperature on the separation was investigated using three types of packed columns (fully porous (FP), small nonporous (SNP), and core-shell (CS)). Typical chromatograms at various temperatures under the condition of $P_{in} = 5.3$ MPa and $v_{t0} = 2$ mm/s using the FP and CS columns are shown in Fig. 1. The decrease in temperature induced an increase in retention of the analytes in both columns. On the other hand, the CO$_2$ mobile phase was often clogged, or solidified, in the SNP column. The reason for this clogging will be related with the relatively narrow flow channel in the SNP column (d.p. 2 $\mu$m) compared to the other columns (d.p. 5 $\mu$m). Therefore, evaluation of the chromatographic behavior of the SNP column was not performed in the following section.

As shown in Fig. 1, we decreased $P_{out}$ according to decrease in temperature to control the flow rate, i.e., $\Delta P$ increased due to an increase in the viscosity of liquid CO$_2$ at the lower temperature (0.11 to 0.17 mPa s for -5 to -30°C, respectively, at the corresponding $P_{av}$ [30]). The relationship between the column temperature and the retention factor, or van’t Hoff plot, at four conditions in the FP and CS columns are shown in Fig. 2. In this figure, the retention factors at four separation conditions ($v_{t0} = 1$ mm/s and $P_{in} = 5.3$ MPa, $v_{t0} = 2$ mm/s and $P_{in} = 5.3$ MPa, $v_{t0} = 1$ mm/s and $P_{in} = 6.3$ MPa, and $v_{t0} = 2$ mm/s and $P_{in} = 6.3$ MPa) were summarized. For all conditions, the retention factors of each compound increased with a decrease in the temperature, and the slope of the van’t Hoff plot changed at approximately -15°C ($1/T = 0.0039$). This behavior has been reported in our previous research [19]. As shown in
Fig. 2, the difference in the chromatographic conditions did not affect the analyte retention factors. The density of liquid CO2 could be regarded as approximately constant (0.97 to 1.09 g/cm³) at our experimental conditions [30]. In contrast to SFC with supercritical CO2 mobile phases [31], the effect of pressure on the retention factor was negligible in low-temperature HPLC using a liquid CO2 mobile phase.

3.2. Effect of packed particle type and chromatographic conditions on separation efficiency

The effect of temperature on separation efficiency for the FP and CS columns was investigated at the four chromatographic conditions. Here, a separation efficiency is evaluated using height equivalent theoretical plate (H) calculating by $H = L(5.545\times t_0/W_{1/2})^2$, where $L$, $t_0$, and $W_{1/2}$ are column length, elution time, and full width at half maximum, respectively. The separation efficiency was somewhat affected by the temperature as shown in Fig. 3. However, the typical relationships between temperature and column efficiency were not observed for either the FP or CS columns at any condition. Overall, the separation efficiency at $v_0 = 1$ mm/s was better than that at $v_0 = 2$ mm/s in all experimental sets. This phenomenon indicated that the C term of the equation of $H = A + B/t_0 + Cu$ dominated the separation efficiency at this condition. In addition, the obvious effect of $P_{in}$ on separation efficiency was not clearly observed. However, for both the columns, an increase in separation efficiency was found at the condition of $v_0 = 1$ mm/s and $P_{in} = 6.3$ MPa in particular for the CS column. For instance, at -30°C, the theoretical plate heights with the CS column for naphthalene, anthracene, and pyrene were 21, 18, and 12 mm, respectively, which are about 30 - 40, 25 - 50, and 50 - 70% of the other three conditions.

As shown in Fig. 1, the peak tailing, or asymmetric peak profile, was observed in both the columns. The tailing factors ($T_t$) for the FP column are 3.0/10.1/2.0 and 3.3/6.6/3.3 (naphthalene/anthracene/pyrene) at -5 and -30°C, respectively ($P_{in} = 5.3$ MPa, $v_0 = 2$ mm/s, Fig. 1A). In the CS column (Fig. 1B), these values are 1.1/4.6/2.5 (-5°C) and 1.5/2.1/2.6 (-30°C). At the condition of $P_{in} = 6.3$ MPa, $v_0 = 1$ mm/s, and -30°C, the $T_t$ values for the FP and CS columns are 2.1/1.4/1.4 and 1.4/1.7/2.1, respectively. It is obvious that the inlet pressure, flow rate, and temperature affect both the peak width (column efficiency) and its profile.

3.3. Evaluation of CO2 behavior in column

Because the liquid CO2 mobile phase clogged the SNP column by solidifying as described in section 3.1, the porosity ($\phi$), or void fraction, of each column was estimated using the following equations to evaluate the behavior of CO2 in the column.

$$\nu_{t0} = \frac{Q_L}{A_0\phi} \tag{1}$$

$$Q_L = \frac{Q_G \rho_G}{\rho_L} \tag{2}$$

where $A_0$ and $Q_L$ are the cross-sectional area of the void column and volumetric flow rate at liquid state (inside of column), respectively, and $Q_G$, $\rho_G$, and $\rho_L$ are the flow rate of gaseous CO2 measured by the mass flow meter at the column outlet (outside of column), the density of gaseous CO2, and the density of liquid CO2 at $P_{in}$, respectively.

The effect of temperature on the column porosity for both the FP and CS columns were investigated at the four conditions. As shown in Fig. 4, both the columns did not indicate any obvious dependence of the column porosity on temperature. In the FP column, the porosity significantly depends on both $P_{in}$ and $v_0$, in contrast to the CS column. This result suggested that the CO2 solidified easily in the FP column compared with the CS column. In the FP column, the porosity at $v_0 = 1$ mm/s is clearly smaller than that at $v_0 = 2$ mm/s, which would cause the solidification of the CO2.

Fig. 4. Relationship between temperature and the column porosity at four conditions with (A) FP and (B) CS columns. Error bars indicate standard deviation ($n = 3$).
The variation in the flow rate at -5°C and -30°C using (A) FP and (B) CS columns at various conditions. The solidification of CO₂ in the column would normally result in the heterogeneity of the column structure. However, as shown in Fig. 3, the separation efficiency at $v_{\text{in}} = 1 \text{ mm/s}$ is better than that at $v_{\text{in}} = 2 \text{ mm/s}$. Moreover, as shown in Fig. 2, the solidification of CO₂ did not affect the retention ability whereas the solidification would normally produce variation in the volume ratio between the mobile and stationary phases.

The variation in volumetric flow rate during the separation is shown in Fig. 5. In both columns, the flow rate at -5°C was unstable with $P_{\text{in}} = 6.3 \text{ MPa}$, indicating that the flow rate decreased with clogging but then increased when the clogging was solved. Interestingly, the flow rate at -30°C was clearly stable compared with that at -5°C whereas the lower temperature would enhance solidification. On the other hand, the relatively high $P_{\text{in}}$ (6.3 MPa) produced an unstable flow rate compared with $P_{\text{in}} = 5.3 \text{ MPa}$, which might be acceptable because the high pressure generally enhances the solidification. The variation in flow rate shown in Fig. 5 suggest that the clogging and unclogging dynamically cycled in the columns. The dynamic solidification and un-solidification may relate to the separation efficiency in low-temperature HPLC using liquid CO₂ mobile phase. Further study is necessary to reveal this complicated solidification behavior of CO₂ in the column and the relationship between the clogging behavior and the separation efficiency.

3.4. Comparison with a monolithic column

In this section, the chromatographic behavior for a monolithic column was studied for low-temperature HPLC. The separation efficiency of naphthalene, anthracene, and pyrene in the FP, CS, and monolithic columns were compared under the four chromatographic conditions at -30°C. As shown in Fig. 6, the separation efficiency of all compounds was affected by the separation conditions for
Moreover, the condition of the column with the highest separation performance was achieved for the CS column. In this study, the highest separation efficiency for all columns. In this study, the highest separation efficiency was not observed.

The comparison of the column porosity at various conditions is shown in Fig. 7A. The porosity of the monolithic column varied based on the separation conditions. Since the porosity of the CS column was the most stable among the three columns, this stability may be related to the higher separation efficiency for the CS column in a low-temperature HPLC using a liquid CO₂ mobile phase. The through pore of a monolithic column is significantly larger than the spatial gap between packed particles. Therefore, the risk of clogging in the flow channel will be reduced in the monolithic column. As shown in Fig. 7B, however, the flow rate in the monolithic column varied with the condition of \( v_0 = 2 \) mm/s and \( P_{in} = 6.3 \) MPa. Moreover, the condition of \( v_0 = 1 \) mm/s and \( P_{in} = 6.3 \) MPa also partially resulted in instability of the flow rate whereas the other conditions \( (P_{in} = 5.3 \) MPa) produced relatively stable flow rates. In other words, CO₂ in the monolithic column at 6.3 MPa was more easily solidified than at 5.3 MPa as similar to all columns used in this study.

4. Conclusions

In this paper, we investigated the effect of column structure and chromatographic conditions (flow rate, inlet pressure, and temperature) on the separation behavior in low-temperature HPLC using a pure liquid CO₂ mobile phase. In this study, the highest separation efficiency of theoretical plate height of 12 \( \mu \)m (85 000 N/m) for pyrene was achieved in the CS column with the condition of \( v_0 = 1 \) mm/s and \( P_{in} = 6.3 \) MPa at -30°C. Although the column temperature somewhat affected the separation efficiency, the typical relationship between temperature and separation efficiency was not observed.

The study of column porosity suggested that the CO₂ solidified in the columns dynamically depending on both the chromatographic conditions (particularly for flow rate) and the column structure. However, the solidification behavior is complicated and further study is necessary for understanding it in detail. The dynamic solidification and un-solidification of CO₂ in the columns will result in dynamic heterogeneity of the column structure. Investigation of the relationship between the solidification of CO₂ and the column efficiency, including the peak profile, is also necessary for the progress in low-temperature HPLC using a liquid CO₂ mobile phase. The addition of organic modifiers may be effective in suppressing the solidification of CO₂ and enhancing the separation efficiency. Further study of low-temperature HPLC using such a liquid CO₂-based mobile phase is currently ongoing in our laboratory.

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