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Effect of the Silanization Conditions on Chromatographic Behavior of an Open-tubular Capillary Column Coated with a Modified Silica-gel Thin Layer

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The performance of an open-tubular capillary column coated with a modified silica-gel thin layer was investigated, particularly concerning the effect of the silanization process on it. Although the increase in the octadecyltriethoxysilane (ODTES) concentration in the silanization process could enhance the retention factor of naphthalene, its theoretical plate number was significantly reduced (ODTES, 5 to 50%; k, 0.2 to 4.3; N, 79600 to 2600 m^{-1}). Namely, the increase in the retention factor was accompanied by a decrease in the theoretical plate number. A similar phenomenon was also observed when octadecyldimethylchlorosilane (ODCS) was used as the silanization regent. However, increases in both the retention factor and the theoretical plate number could be achieved (sample, naphthalene; k, 0.05 to 0.09; N, 149000 to 220000 m^{-1}) by a NaOH treatment to the fabricated thin porous silica-gel layer before silanization with ODCS. The electrochromatographic separation of proteins and peptides by using the NaOH-treated column could obtain more peaks than electrophoretic separation.

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Introduction

Capillary electrochromatography (CEC) is a separation technique that combines high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE).[1-3] A capillary column for CEC can be divided into three types: a packed capillary column, a monolithic capillary column, and an open-tubular capillary column (OTCC). The packed capillary column is the most common, and can be packed with various packing materials for CEC and HPLC. The monolithic capillary column is currently widely used, and is employed in both HPLC and CEC.[4-6] The monolithic capillary column has high porosity and can operate at a low pressure. The OTCC can also operate at a low pressure, which is one of its advantages. One more advantage of the OTCC is the absence of eddy diffusion, which leads to an increase in the theoretical plate number. However, due to the small volume of the stationary phase, the retention factor when using an OTCC is significantly smaller than that when using a packed-column. Various methods have been developed to increase the volume of the stationary phase. For example, etching of the inner wall of the capillary is a frequency used method.[7,8] Liu *et al*. reported that the surface area could be enhanced by 1000 times by HF/NH_4F etching.[7] The sol-gel method has also been used to fabricate OTCC.[9-11] Guo and Colon developed a method to fabricate an organic-inorganic hybrid thin film onto the inner walls of a fused silica capillary with a single step, and achieved a separation efficiency of 500000 Nm^{-1} in some analytes.[9,10] Heyes *et al*. reported the separation of polycyclic aromatic hydrocarbons using an OTCC coated with a porous thin layer synthesized by the sol-gel method.[11]

In our previous study, we developed a preparation method for an OTCC by using silica oligomer to form a thin porous layer on the inner wall.[12] Our column was prepared with multiple steps in the fabrication of a thin porous layer and its silanization. Both processes are important to achieve good separation efficiency. In this study, we performed a further investigation on our OTCC preparation method to improve the separation efficiency, particularly in the silanization process. Further, the OTCC was applied for the separation of positively charged proteins and peptides.

Experiment

Column preparation

The column preparation procedure was almost the same as described in our previous report, except for some details.[12] First, the inner wall of a fused silica capillary (i.d. 30 μm, o.d. 375 μm, supplied from GL Science, Tokyo, Japan) was pretreated as follows: the capillary was filled with 1 M sodium hydrate, and both its ends were sealed using glue; it was kept at 80°C for 1 h. The capillary was then washed with distilled water for 30 min and dried under a nitrogen gas stream.

Second, a silica-gel thin layer was formed as follows. A 30% silica-oligomer (kindly donated by Piatech, Mie, Japan) ethanol solution containing 0.05% cyclohexane was filled in the capillary, following which the capillary was dynamically coated

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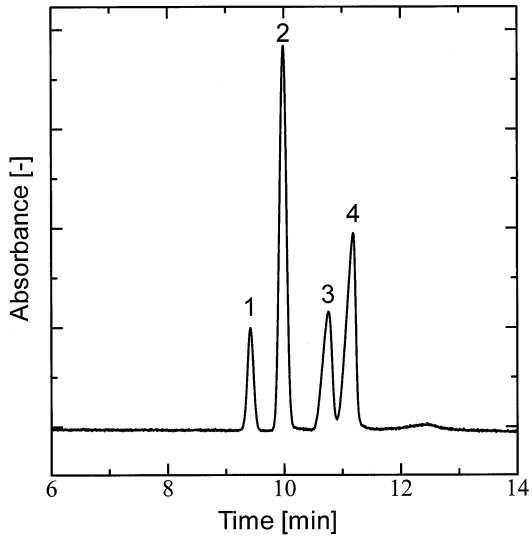

Fig. 1 Separation of neutral compounds by ODTES-modified OTCC. Column: i.d., 30 μm; whole length, 40 cm; effective length, 28 cm; modified with 5% ODTES; eluent, acetonitrile/10 mM phosphate buffer (30/70); applied voltage, 10 kV; detection, 210 nm; injection, 10 kV for 5 s. Peaks: 1, thiourea; 2, naphthalene; 3, biphenyl; 4, fluorene.

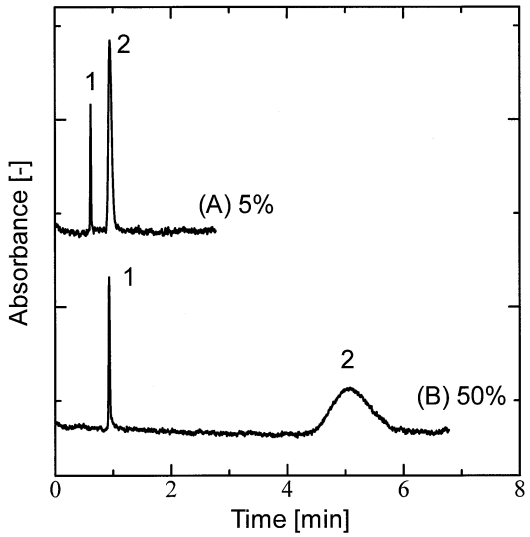

Fig. 2 Effect of the ODTES concentration on separation. Column: i.d., 30 μm; whole length, 20 cm; effective length, 10 cm; modified with (A) 5% and (B) 50% ODTES; eluent, methanol/10 mM phosphate buffer (30/70); applied voltage, 5 kV; detection, 210 nm; injection, 1 kV for 5 s. Peaks: 1, thiourea; 2, naphthalene.

under a nitrogen gas stream (0.2 MPa). A 5% ammonia aqueous solution was filled in the capillary to polymerize the silica-oligomer. The ammonia aqueous solution was flushed out using a nitrogen gas steam. The capillary was heated at 80°C for 16 min, after which it was programmed at a rate of 4°C/min until 180°C, at which it was kept for 16 min. The capillary was then dried at 120°C for 30 min under a nitrogen gas stream.

Finally, the silica-gel layer was silanized using octadecyltriethoxysilane (ODTES) or octadecyldimethylchlorosilane (ODCS). The capillary was filled with xylene containing ODTES or ODCS, and was kept at 150°C for 3 h. Then, the capillary was sequentially washed with xylene, acetone, 2-propanol, and an eluent.

Apparatus and CEC conditions

Two types of CEC systems were used in the experiment: a commercial CE system (CAPI-3100, Otsuka Electronics, Osaka, Japan) and a laboratory-made CEC system. The latter comprised a UV detector (UV-970, Jasco, Tokyo, Japan), a high-voltage power supply (HCZE-30PNO, Matsusada Precision, Shiga, Japan), and two laboratory-made reservoirs. CAPI-3100 was mainly used in this experiment, and the laboratory-made CEC system was used for a shorter capillary column of 10/20 cm (effective length/whole length).

A mixture of an organic modifier (methanol or acetonitrile) and 10 mM phosphate buffer (pH 6.1) was used as the eluent for the separation of thiourea, naphthalene, biphenyl, and fluorene. Thiourea was used as a t_0 marker. A mixture of 300 mM phosphoric acid and 190 mM Tris (pH 2.1) and acetonitrile (80:20) was used as an eluent for the separation of proteins and peptides. Details of the CEC condition are described in each figure caption. All chemicals were purchased from Wako Pure Chemical Industry (Osaka, Japan).

Results and Discussion

Effect of the ODTES concentration on CEC separation

A typical separation of neutral compounds (thiourea, naphthalene, biphenyl, and fluorene), obtained by using the OTCC modified with ODTES, is shown in Fig. 1. The retention factors and the theoretical plate numbers of neutral compounds in Fig. 1 are listed in Table 1 (5% ODTES). The theoretical plate numbers for all compounds were over 75000 m^{-1}. However, the retention factors were not sufficiently high. The effect of the ODTES concentration on the enhancement in the retention factor was investigated. Figure 2 shows the separations of thiourea and naphthalene by OTCCs modified with 10% and 50% ODTES, respectively. The retention factor of naphthalene increased significantly (0.6 to 4.3); however, the theoretical plate number was reduced by a great extent. The variations in the retention factor and the theoretical plate number of naphthalene with respect to the ODTES concentration are shown in Fig. 3. The retention factor was almost proportional to the ODTES concentration, and the theoretical plate number decreased with an increase in the ODTES concentration.

The theoretical plate number depends on various parameters, such as the flow velocity, retention factor, and injection volume. The reaction of ODTES with silanol groups reduced the electroosmotic mobility, as shown in Fig. 2 (the elution time of thiourea was reduced in OTCC modified with 50% ODTES). The flow velocities in OTCCs modified with 5, 10, 20, and 50% ODTES were 0.60, 0.46, 0.42, and 0.30 mm/s, respectively. The *H*–*u* plot for the OTCC modified with 5% ODTES yields the equation of H [μm] = $2.8/u + 22.4u$, where u is the flow velocity; the optimum flow velocity was 0.34 mm/s. The variation in the flow velocity from 0.30 to 0.60 mm/s may not significantly affect the theoretical plate number. In other words, the decrease in the theoretical plate number shown in Fig. 3 was not caused by the variation in the flow velocity.

The relationship between the theoretical plate height and the

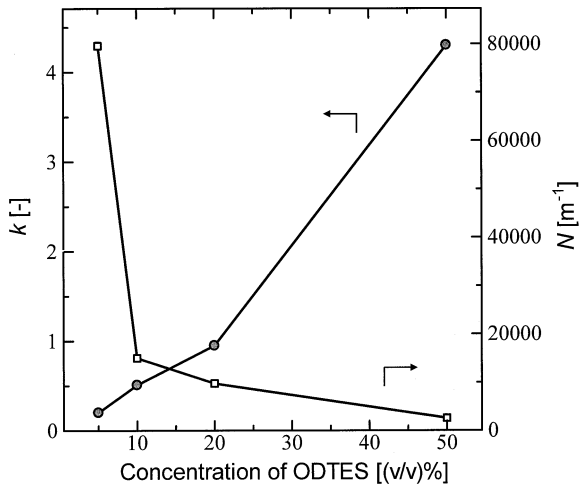

Fig. 3 Variation in the retention factor and theoretical plate number with respect to the ODTES concentration. Other conditions are the same as in Fig. 2.

retention factor in the OTCC was explained by Golay, and it was expanded in order to apply it for separation under electroosmotic flow:[1]

$$H = \frac{2D_m}{u} + \frac{k^2}{4(1+k)^2}\frac{r^2}{D_m}u + \frac{2k}{3(1+k)^2}\frac{d}{D_s}u, \quad (1)$$

where D, k, r, and d are the diffusion coefficient, retention factor, radius of the capillary, and thickness of the stationary phase, respectively. Subscripts m and s correspond to the mobile and stationary phases, respectively. As shown in Eq. (1), the theoretical plate number is a function of the retention factor. Particularly, the second term on the right side of Eq. (1) depends significantly on the retention factor. When the retention factor was increased from 0.2 (5% ODTES) to 0.5 (10% ODTES), the second term increased by 4 times. Therefore, the increase in the retention factor was one of the reasons for the decrease in the theoretical plate height. A high ODTES concentration has an advantage regarding the retention factor, but not for the theoretical plate number.

OTCC modified with ODCS

Since ODTES is a trifunctional reagent, a polymeric stationary phase can be formed when the ODTES concentration becomes high. The decrease in the theoretical plate number using OTCC modified with a high concentration of ODTES may have some relation with the polymeric stationary phase. The OECS is a monofunctional reagent, and cannot form a polymeric stationary phase. The separations of neutral compounds using OTCC modified with ODCS are shown in Fig. 4B. The retention factors decreased slightly in a 5%-ODCS-modified column compared to the 5%-ODTES-modified column (Fig. 4A), and the theoretical plate numbers increased (listed in Table 1). Namely, the increase in the theoretical plate number was provided by the decrease in the retention factor. In the case of OTCC modified with 50% ODCS (Fig. 4C), the retention factors were almost the same as those in the case of the OTCC modified with 5% ODCS, and the theoretical plate numbers were decreased, except for naphthalene. The insignificant increase in the retention factors may suggest saturation in the reaction of silanol with ODCS. Therefore, the increase in the retention factor in the OTCC modified with high concentration ODTES may be due to the formation of the polymeric stationary phase.

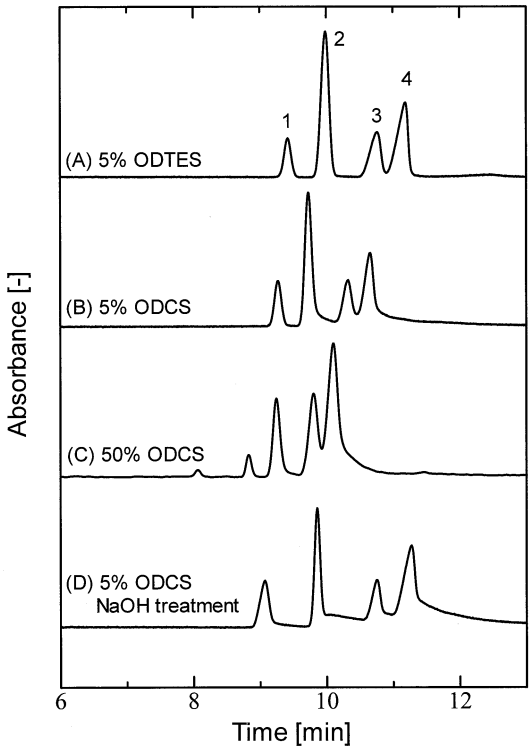

Fig. 4 Separation of neutral compounds by an ODCS-modified OTCC. Column: i.d., 30 μm; whole length, 40 cm; effective length, 28 cm; modified with (A) 5% ODTES, (B) 5% ODCS, (C) 50% ODCS, and (D) 5% ODCS after a NaOH treatment. Other conditions are the same as in Fig. 1.

Table 1 Effect of silanization on the retention factors (k) and theoretical plate numbers (N)

Silanization		Naphthalene	Biphenyl	Fluorene
5% ODTES	k [–]	0.06	0.14	0.19
	N [m^{-1}]	110000	76000	82000
5% ODCS	k [–]	0.05	0.11	0.15
	N [m^{-1}]	149000	126000	124000
50% ODCS	k [–]	0.05	0.11	0.15
	N [m^{-1}]	152000	100000	77000
5% ODCS after	k [–]	0.09	0.19	0.24
NaOH treatment	N [m^{-1}]	220000	134000	91000

The saturation in the reaction of silanol with ODCS might inhibit a increase in the retention factor, and an increase in the number of silanol groups on the surface of porous silica-gel layer is effective to increase the retention factor. A silica-gel layer was treated using 1 M NaOH for 5 min before silanization to increase the number of silanol groups on the surface. The separations of neutral compounds using the OTCC modified with 5% ODCS after a NaOH treatment is shown in Fig. 4D. The NaOH treatment could enhance the retention factors compared to the untreated OTCC. The NaOH treatment to a fabricated porous thin layer was effective to increase in the retention factor.

As can be clearly observed in Figs. 2 and 3, an increase in the retention factor is accompanied by a decrease in the theoretical plate number, and a similar phenomenon was also observed

between Figs. 4A and B. However, the NaOH treatment could provide increases in both the retention factor and the theoretical plate numbers of naphthalene and biphenyl, as listed in Table 1. A NaOH treatment to a fabricated porous thin layer before the silanization was effective to improve both the retention factor and the theoretical plate number.

Separation of peptides and proteins

OTCC treated with NaOH before silanization was used for the separation of peptides and proteins. A commercially available beverage containing soybean proteins and its degraded peptides (Powerade, Coca-Cola (Japan), Tokyo) was used as the sample. The beverage was diluted three times using an eluent. In this eluent (pH 2.1), almost all peptides and proteins may have positive charges, and the acidic condition suppresses the electroosmotic flow velocity. Therefore, migration of the analyte under the electric field was performed electrophoretically. Figure 5 shows the separations of peptides and proteins using CE and CEC. The sample was moderately separated by CE, and approximately 40 peaks were detected. In the case of CEC separation, the separation period was longer due to retention by the stationary phase. Further, the distances between the peaks increased, and approximately 50 peaks were detected. The C18 stationary phase could assist in the separation of positively charged proteins and peptides.

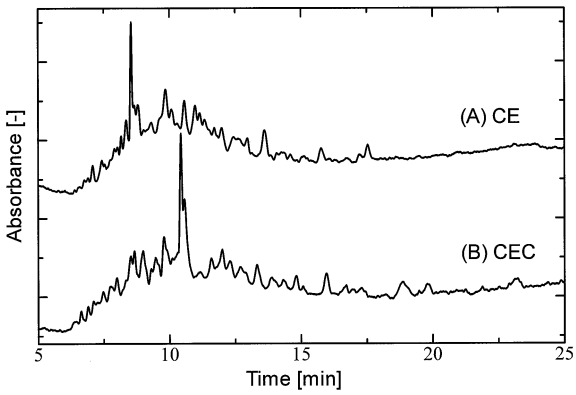

Fig. 5 Separation of proteins and peptides by CE and CEC. Column: i.d., 30 μm; whole length, 40 cm; effective length, 28 cm; modified with 5% ODCS after a NaOH treatment; eluent, acetonitrile/300 mM phosphoric acid and 190 mM Tris (pH 2.1) (80/20); applied voltage, 15 kV; detection, 208 nm; injection, 10 kV for 5 s.

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