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The Retention Behavior of Reversed-Phase HPLC Columns with 100% Aqueous Mobile Phase

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Abstract

The retention behavior of the reversed-phase was evaluated under 100% aqueous conditions. It is commonly said that reversed-phases, such as C18 (ODS) and C8, show a decrease in the retention time under 100% aqueous conditions. It was found that 100% aqueous mobile phase was expelled from the pores of the packing materials, so that the stationary phase in contact with the mobile phase decreased and the retention time decreased. Some parameter, such as the pore size, length and ligand density of the alkyl group of the stationary phase, the amount of residual silanol groups of the stationary phase, salt and ion-pair reagent concentration in the mobile phase, temperature and back pressure of the column, were shown to influence of the decrease in retention. Furthermore, the wettability between the C18 stationary phase and water as a mobile phase was analyzed. It was concluded that the retention behavior could be explained by capillarity, and reversed-phase separation could be carried out under 100% aqueous conditions, even if a mobile phase can not wet the stationary phase. Finally, these phenomena were applied to reversed-phase separation using C18 stationary phase and the mobile phase with less than 70% methanol and more than 30% water.

Keywords: retention; reversed-phase; water; capillarity

1 Introduction

Currently, alkyl group bonding of carbon number 1 to 18 is widely used as packing material for the chemical bonding of reversed-phase HPLC columns. Among these carbon numbers, the number C18 (ODS) is the most commonly used bonded phase due to its wide range of applications and superior durability compared to other alkyl group bonding. It is necessary to use mobile phases containing organic solvent as low as possible when analyzing highly hydrophilic compounds using C18 packing material. However, it has been considered that is essentially impossible to use a 100% aqueous mobile phase, mobile phases buffered with salt or acids, or aqueous mobile phases with a few percent of organic solvents with general alkyl group bonded reversed phases due to the instability of sample retentions and decrease in retention as time progresses. These phenomena were caused by the ligand collapse of a stearyl group (C18) and it was reported that this collapse even changed the selectivity of stationary phases[1-3]. In order to solve this problem, stationary phases, such as an alkyl group including highly polar amide[4-8] or carbamate[9] groups, that were bonded to silica gel, or that bonds polar groups as an endcapping to reduce the residual silanol groups on silica gel after stearyl group bonding, or that is less hydrophilic achieved by reducing the density of alkyl group bonding[10] have been used as packing material. Essentially, it has been considered that the reproducibility in retention under 100% aqueous mobile phase was achieved by having polar groups within stationary phases and making a structure that is less likely to cause ligand collapse of the alkyl group, such as stearyl group. However, these stationary phases show different separation performances due to the selectivity changes influenced by these polar groups or lower which includes polar groups that change selectivity due to the influence from these polar groups or lowered durability. Occasionally it improves separation, however, there are problems to separate compounds caused by short retention time at the same time.

In this study, we confirm that retention behavior on normal reversed phases drastically shifts[11-14] under 100% aqueous mobile phase by changing various conditions, such as stationary phases, mobile phases and temperature. While C18 packing material with large pore size showed stable retention and reproducibility, C18 packing material with small pore size decreased in retention, and even trimethylsilyl silica (TMS, C1), which is impossible to cause the ligand collapse of alkyl group, reduced in retention when its pore size was 6 nm, resulting in phenomena that are unexplainable by the previously believed ligand collapse of the alkyl group. Based on these phenomena, we confirm that the process of retention decrease occurs not by the ligand collapse of alkyl group but capillarity[15]. We also report that reversed phases without the previously mentioned polar groups—that is, stationary phases which have been considered impossible to use with 100% aqueous mobile phase—can obtain reproducibility with sufficient retention by using 100% aqueous mobile phase when using stationary phases that satisfy certain conditions or changing analytical conditions. Furthermore, not only 100% aqueous mobile phase, but also mobile phases that contain organic solvents of more than 30% shows similar phenomena, and this mechanism will also be described.

2 Problems when using 100% aqueous mobile phase

It is well known that the retention time of a C18 phase is reduced under 100% aqueous mobile-phase conditions. Conventionally, mobile phases that contain more than 90% of water, especially mobile phases with more than 95% of water, were considered to be avoided as they exhibit poor reproducible retention. Figure 1 shows the retention behavior of sodium nitrite and 2-propanol when using aqueous mobile phase on a 10-nm pore size C18 phase at 40 °C. We used sodium nitrite to measure the unretained time (t_0). We changed the solvent in a C18 column to water and applied pressure at the postcolumn outlet while retention was measured; the reason for applying pressure will be explained later in this report. After the column became stable the sample was injected, and the chromatogram obtained is shown in (A). Pumping was then stopped for one hour and restarted, and the chromatogram obtained is shown in (B).
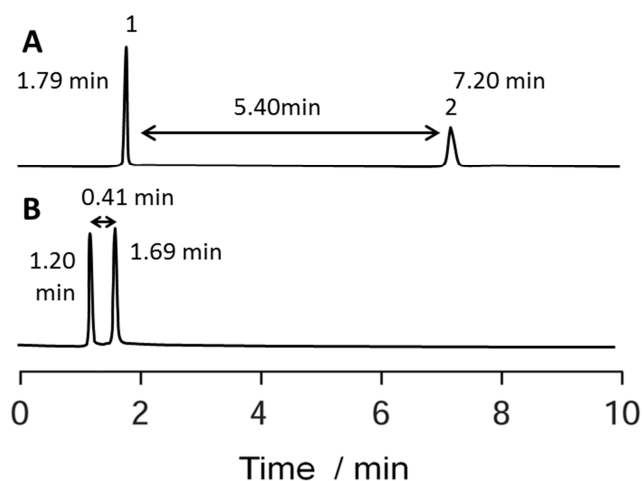
Fig. 1 Retention behavior of a C18 column under 100% aqueous mobile phase conditions
Shown are (A) initial results and (B) results after stopping the pump for 1 h and then starting it again. Column, 150 mm x 4.6 mm, 5 μm dp C18, 10-nm pore size; mobile phase, water; flow rate, 1.0 mL/min; column temperature, 40 °C; detection, refractive index; applied pressure 1.7 MPa at the postcolumn outlet. Peaks, 1 = sodium nitrite (t_0), 2 = 2-propanol.

Stopping the flow for one hour caused shorter retention times for both sodium nitrite (t_0) and 2-propanol. The difference in retention time between sodium nitrite and 2-propanl became the essential retention of 2-propanol in this case. After no flow for one hour, the t_0 decreased from 1.79 to 1.20 min and the retention time of 2-propanol decreased from 7.2 to 1.61 min. As shown in Table I, the essential retention of 2-propanol decreased to 7.6% compared to the initial result. Generally, a decrease of t_0 is unexpected.

We measured the column weight at the same time. The column was sealed at both ends with plugs as soon as the column pressure was reduced to 0 MPa to measure its initial weight. We then removed the plugs and one hour later sealed the column again to measure the weight after stopping the flow for one hour. As shown in Table I, the decreases in t_0 and column weight were 0.59 mL and 0.6 g. The values were almost the same because the specific gravity of water is 1.0 g/mL. Furthermore, we observed that 0.6 mL of water went out of the column during the hour when no mobile phase flowed through the column once the pump showed 0 MPa. Out of this 0.6 mL of water, approximately 0.3 mL flowed out in the first minute and the rest, 0.3 mL, flowed out over a period of more than 10 minutes. Before obtaining the initial chromatogram, sufficient amount of mobile phase, a 70:30 (v/v) acetonitrile/water solution was pumped each time to return the column to its initial state.

These results prove that the aqueous mobile phase used with C18 phase flows out of the column. 5 μm of C18 silica gel is packed into the column. The water that flowed out was either between the packing materials or in the pores of the packing material. As we describe later in the paper, if capillarity is causing water expulsion, the water should be coming out of the pores of the packing material.

3 Influential Parameters on Retention Reproducibility

3·1 Influences of Stationary Phases
3·1·1 Pore size of packing materials
Table 2 shows the characteristics of the stationary phases examined in this study. The alkyl group of each stationary phase was generated with excess monofunctional reagent with relatively high bonding density, and each phase was endcapped

Table 1 Retention time and weight of a C18 column under 100% aqueous mobile phase conditions

	t_0	$t_{2\text{-propanol}}$	$t_{2\text{-propanol}}-t_0$		Mass of Column
	(min or mL)	(min or mL)	(min or mL)	(%)	(g)
Initial	1.79	7.20	5.41	100	62.0
After stopping	1.20	1.61	0.41	7.6	61.4
Initial - after	0.59	5.59	5.00	92.4	0.6

Table 2 Characteristics of the stationary phases examined in this work[a]

Stationary Phase	Specific Surface	Pore Volume	Mean Pore	Carbon	Ligand Density
	($m^2 g^{-1}$)	($mL\, g^{-1}$)	(nm)	(%)	($\mu mol\, m^{-2}$)
TMS(Trimethylslyl silica)	223	0.93	12.9	4.7	4.8
TMS	302	0.91	9.3	5.0	4.3
TMS	331	0.65	6.5	6.3	4.1
C8	128	0.90	22.4	5.6	3.1
C8	142	0.88	19.0	7.2	3.3
C8	169	0.84	15.1	8.4	3.1
C8	199	0.74	11.3	10.6	3.3
C18	113	0.80	21.9	11.4	3.4
C18	123	0.73	17.9	12.8	3.2
C18	139	0.65	13.9	13.9	3.0
C18	163	0.58	10.4	18.4	3.2
C30	176	0.60	10.4	18.0	1.8
C30	219	0.53	7.2	19.7	1.2
C30	210	0.43	6.2	21.2	1.0

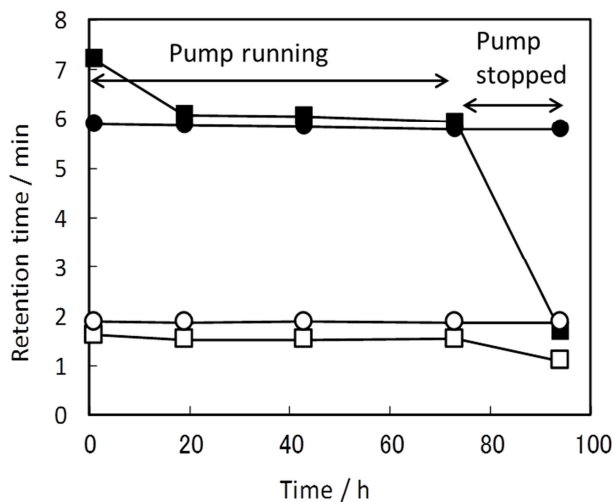a. All measurements were performed on bonded phases.

Fig. 2 Effect of the pore size on retention under 100% aqueous mobile phase conditions
Column, 150 mm x 4.6 mm, 5 μm dp C18, 10-nm and 22-nm pore size; mobile phase, 10 mM phosphate buffer (pH7.0); flow rate, 1.0 mL/min; column pressure, 6.0 MPa; temperature, 40 °C; detection, UV at 254 nm. Sample, □ = C18 (10-nm pore size) – sodium nitrite, ○ = C18 (22-nm pore size) – sodium nitrite, ■ = C18 (10-nm pore size) – thymine, ● = C18 (22-nm pore size) – thymine.

Fig. 3 Relationship between pore size and relative retention time under 100% aqueous mobile phase
Column, C30 (○), C18 (□), C8 (♦), and TMS (Trimethylsilyl silica) (△); column dimensions, 150 mm x 4.6 mm; sample, thymine. Other conditions were the same as in Fig. 2. Relative retention time was calculated as the ratio of the decrease in retention versus initial retention time.

with trimethylsilyl after bonding. Figure 2 shows the retention behavior of C18 with two different pore sizes of 10-nm and 22-nm. 10 mM of phosphate buffer (pH 7) was used as mobile phase, and the retention and elution time of thymine and sodium nitrite were measured at the column temperature of 40 °C. No pressure was added to the postcolumn outlet. With the 10-nm C18, the retention time of thymine decreased by 16% from after one hour to 19 hours later, and it was stable for 72 hours until pumping stopped. The retention time of thymine has greatly decreased by 58% when no pressure was added around the packing material in the column once pumping stopped after 72 hours. Similar changes to sodium nitrite elution time—that is, considered not retained at all—were observed. On the other hand, the retention times of thymine and sodium nitrite were unchanged on the 22-nm C18. The only difference between these two C18 silicas is pore size. When the pore size was around 22 nm, the retention time did not change. Figure 3 shows the influence of particle pore size of packing materials on the retention time for TMS (trimethylsilyl silica), C8, C18 and C30. Conditions were the same as shown in Figure 2. The horizontal axis shows the pore diameter of each stationary phase, and the vertical axis shows the relative retention time measured one hour after pumping stops against the initial retention time. With all the stationary phases, the retention time decreased as the pore diameter became smaller.

3·1·2 Alkyl chain length
As shown in Figure 3, different lengths of alkyl chain change the relative retention time against pore diameters and the decreasing ratio on retention time also differs. When comparing stationary phases using the same pore size of 15 nm, C8 decreased in retention time by 80% and C18 by 35%. However, C30 and TMS did not decrease the retention time. The longer the length of ligand for more than C8, the smaller the decrease in retention is with smaller pore diameters. That is, the longer alkyl chain is more stable on its retention even with small pore diameters. Generally the pore diameter of approximately 10 nm is used as
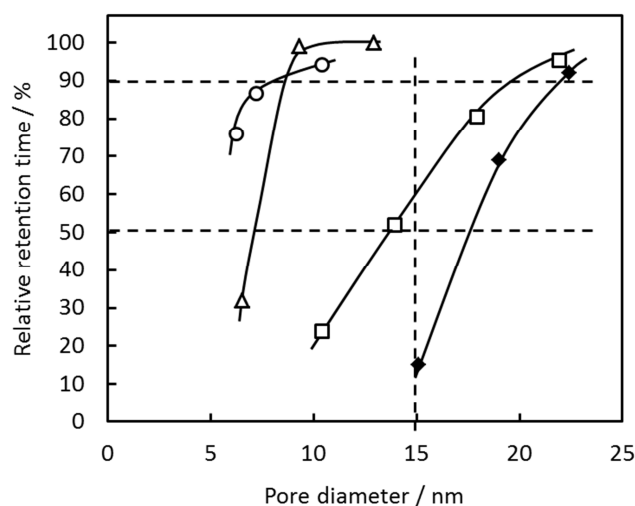
packing material. However, the ligand length of C30 enables the analyses with stable retention time even using aqueous mobile phase. Although TMS is in the methyl group and has a short ligand, it gains high retention with a pore diameter of 9 nm. However, with 7 nm of pore diameter, the retention decreases by greater than 50%. On the silica surface of C8, C18 and C30, the alkyl group is bonded and the silica is endcapped with trimethysilyl (TMS). However, as shown in Table 2, the ligand density of TMS is less than 4.8 μmol/m2, and considering the theory that the residual silanol group on the silica gel surface is 8 – 9 μmol/m2, it cannot be assumed that the endcapping with TMS completely silylates residual silanol group. In other words, this is not a comparison of alkane with different lengths of carbon chains, such as octane C_8H_{18}, octadecane $C_{18}H_{38}$ and triacontane $C_{30}H_{62}$. Rather, this is the result about the lengths of alkyl chains as the major constituent of a stationary phase that is bonded on the silica surface as a single-layer reversed phase with the thickness of 1 nm – 2 nm.

The result of the influence from the pore diameters of packing materials and the lengths of alkyl chains on retention points out several conflicts on the theories for ligand collapse that was previously believed as the cause of retention decrease. First of all, does the ligand collapse occur with small pore diameters and not with large diameters? As shown in Table 2, the ligand density of 10-nm C18 and 22-nm C18 is 3.2 μmol/m2 and 3.4 μmol/m2, which are not greatly different. Although the decreasing ratio of retention time differs largely depending on pore diameter, it is unlikely that ligand collapse depends on pore diameter. Additionally, when the pore diameter is small, TMS, which has a short alkyl group and that ligand collapse must not physically occur with, experienced a decrease in retention time. Therefore, ligand collapse cannot be explained by pore diameter.

3·1·3 Ligand density
Figure 4 shows the result of the impact to the ligand density of alkyl group (stearyl group, C18) on retention. The conditions were the same as shown in Figure 1, and the decreasing ratio of retention of 2-propanol one hour after pumping stopped was
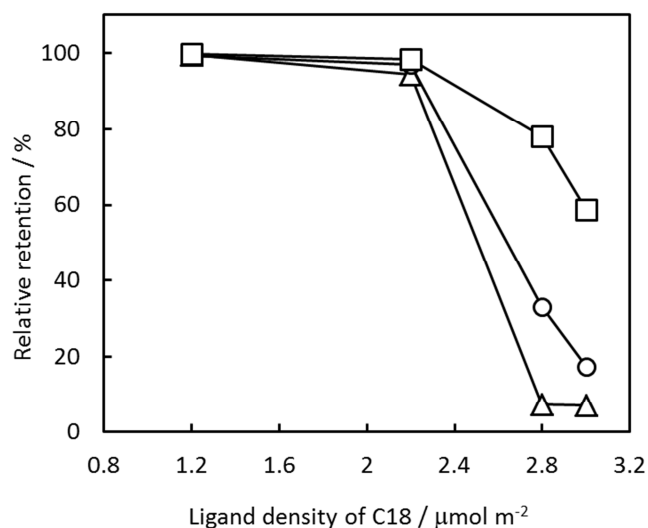
Fig. 4   Relationship between C18 ligand density and relative retention of 2-propanol under 100% aqueous mobile phase
Column, Develosil ODS-5 (3.0 μmol/m2), Develosil ODS-K-5 (2.8 μmol/m2), Develosil ODS-N-5 (2.2 μmol/m2), Develosil ODS-P-5 (1.2 μmol/m2); column dimension, 150 mm x 4.6 mm; temperature, Δ = 40 ℃, ○ = 30 ℃, □ = 20 ℃. Other conditions were the same as in Fig. 1.

Fig. 5 Effect of concentration of salt in the mobile phase
Column, 150 x 4.6 mm, 5 μm dp C18, 10-nm pore size; mobile phase, Δ = water, □ = 10 mM ammonium acetate (pH7.0), ○ = 100 mM ammonium acetate (pH7.0). Other conditions were the same as in Fig. 1.

measured. The same series of C18 phase from Nomura Chemical was used. The ligand densities were modified at 3.0 μmol m-2, 2.8 μmol m-2, 2.2 μmol m-2 and 1.2 μmol m-2, and C18 was bonded on silica gel with the same physical specification. After bonding C18 for all the silica gel, TMS was used for endcapping on residual silanol by an identical method. The comparison of influences from column temperatures at 40 ℃, 30 ℃ and 20 ℃ will be discussed later in this paper. When the column temperature was 40 ℃, C18 with high density, such as 3.0 μmol m-2 and 2.8 μmol m-2, decreased retention by around 90%. However, the C18 with 2.2 μmol m-2 showed only a few percent decrease in retention, and the C18 with 1.2 μmol m-2 did not show a decrease in retention. In short, the ligand density of C18 greatly impacts reproducibility in retention, and the lower the density was, the lower the decrease in retention was. The C18 with low density has more TMS used for endcapping. Therefore, the stationary phase should be considered as a mix of C18 and TMS rather than C18, and this stationary phase is considered to have similar behavior as 10 nm TMS that does not change its retention.

3·1·4 Residual silanol groups
The effect of residual silanol groups was investigated by using C18 with different endcapping. Table 3 shows the effect of residual silanol groups on three types of C18 columns. One column was not endcapped, and the others were single- and double-endcapped. Columns used were Develosil ODS-A-5, Develosil ODS-T-5 and Develosil ODS-HG-5 by Nomura Chemical. The residual silanol group amount was measured based on the separation factor of caffeine and phenol using a mobile phase of 30:70 (v/v) methanol-water. The larger the retention of caffeine and separation factor of caffeine and phenol, the more residual silanol groups exist. The retention was measured under the same conditions as shown in Figure 4. It proved that C18 stationary phase with more residual silanol groups showed fewer changes in retention time. Thus, silanol groups stabilize retention.

3·2 Effect of Mobile Phase
3·2·1 Concentration of salt
Figure 5 shows the retention time of three kinds of mobile phases with different salt concentrations. Water, 10 mM ammonium acetate (pH 7) and 100 mM ammonium acetate (pH 7) were used as mobile phases. Other conditions were the same as shown in Figure 1. The horizontal axis shows the time that pumping stops and the vertical axis shows relative retention having the retention before pumping stops as 100%. In the first 10 minutes after pumping stops, the retention greatly decreased and then gradually decreased over 60 minutes. When the column temperature was at 40 ℃, more than 80% of the total retention decrease was observed in the first 10 minutes. Among these three kinds of mobile phases, water with 0 mM of salt concentration showed the least relative retention. The higher the concentration of salt, the greater the increase in relative

Table 3 Effect of residual silanol groups[a]

Column	Endcapping	Hydrogen-bonding Capacity[b]	Relative Amount of Residual Silanol Groups	Final Retention Time Ratio[c] (%)
Develosil ODS-A-5	No	1.42	Much	97.5
Develosil ODS-T-5	Single	0.53	Little	58.2
Develosil ODS-HG-5	Double	0.38	Very little	39.9

a. Conditions as shown in Fig. 3.
b. Hydrogen-bonding capacity: separation factor of caffeine and phenol.
c. The final retention time ratio is the final retention when the retention time decreased to the initial retention time.
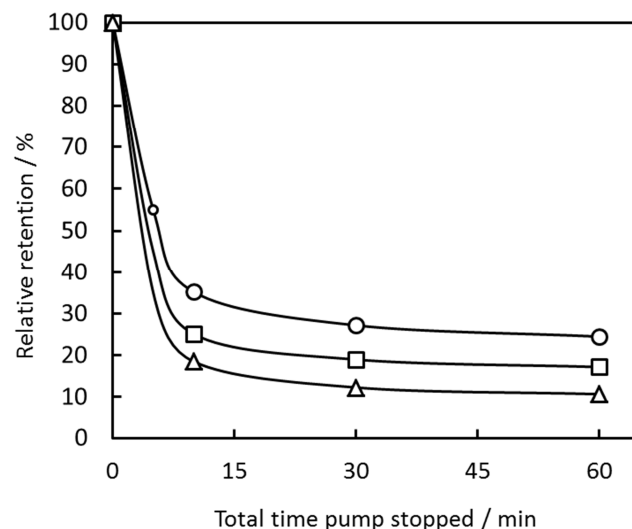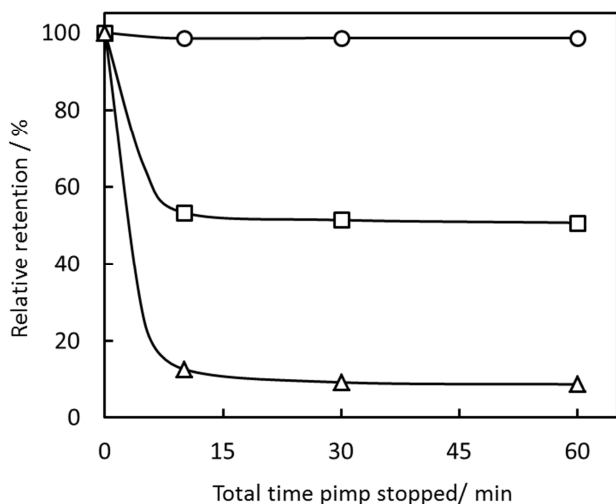
Fig. 6 Effect of concentration of the ion-pairing reagent in the mobile phase

Column, 150 mm x 4.6 mm, 5 μm dp C18, 10-nm pore size; mobile phase, Δ = 10 mM sodium phosphate (pH7.0), □ = 10 mM sodium phosphate-1 mM sodium octanesulfonate (pH7.0), ○ = 10 mM sodium phosphate-5 mM sodium octanesulfonat (pH7.0). Other conditions were the same as in Fig. 1.
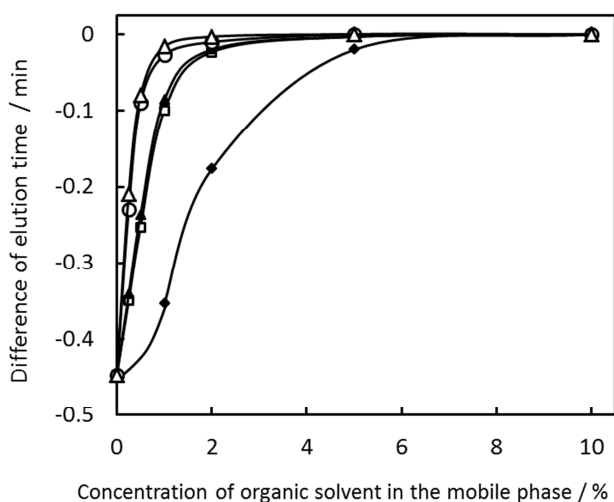

Fig. 7 Effect of concentration of organic solvent in the mobile phase

Column, 150 mm x 4.6 mm, 5 μm dp C18, 10-nm pore size; mobile phase, ♦ = methanol/water, □ = acetnitrile/water, ▲ = ethanol/water, ○ = dimethylformamide (DMF), Δ = 2-propanol; sample, sodium nitrite. Other conditions were the same as in Fig. 1.

retention and the lower retention decrease ratio.

3·2·2 Concentration of ion-pairing reagent

Figure 6 shows the effect of the concentration of ion-pairing reagent. Three kinds of mobile phases were prepared, which were 10 mM sodium phosphate (pH 7), 10 mM sodium phosphate with 1 mM sodium octanesulfonate and 10 mM sodium phosphate with 5 mM octanesulfonate. The retention behavior was plotted having the horizontal axis as pumping stop time the same as Figure 5. Other conditions were the same as shown in Figure 1. With a mobile phase without ion-paring reagent, the relative retention decreased to around 10% in 10 minutes. Although a mobile phase with 1 mM ion-paring reagent showed drastic change in retention and the relative retention decreased to approximately 50%, it became stable after 10

minutes. On the other hand, a mobile phase with ion-paring reagent of normal concentration, which is 5 mM, the retention hardly changed and showed almost 100% of relative retention. Thus, it was confirmed that adding ion-paring reagent reduces retention changes and there became no change in retention at a concentration of 5 mM.

3·2·3 Concentration of organic solvent

So far, the changes in retention time using water or phosphate buffers were discussed. Now, we will discuss the concentration of organic solvent. Fig. 7 shows different elution time of sodium nitrite as unretained compound using mobile phases of water added following solvents: methanol, acetonitrile, ethanol, dimethylformamide (DMF) and 2-propanol. The difference of elution time on the vertical axis shows times after the elution time was being deducted at 10% concentration from elution time of each concentration. The smaller values show the faster elution times. In other words, as described in the article discussing problems to use 100% aqueous mobile phase, the different elution times correspond to negative values of the mobile phase amount that was expelled from the packing material pores. The differences of elution times, that is, the expelled amount of mobile phase from pores of packing materials, greatly varied depending on the kinds of organic solvents. We confirmed that mobile phases were expelled from the packing material pores with less than 5% methanol, less than 2% acetonitrile and ethanol, and less than 1% 2-propanol. Although 1% of 2-propanol shows stable retention, the polarity of 2-propanol solution and 5% methanol solution is almost the same and therefore polar samples such as thymine would show almost the same retention time even using these mobile phases. Even though the retention of 2-propanol with low concentration to 1% is stable, the separation is almost as same as 5% methanol. Therefore, there is no merit to using 2-propanol. Put simply, the polarity of mobile phases with any organic solvents are the same as the concentration level of organic solvents when mobile phases are being expelled from packing material pores, and retention cannot be greater by changing the kinds of organic solvents under stable retention conditions.
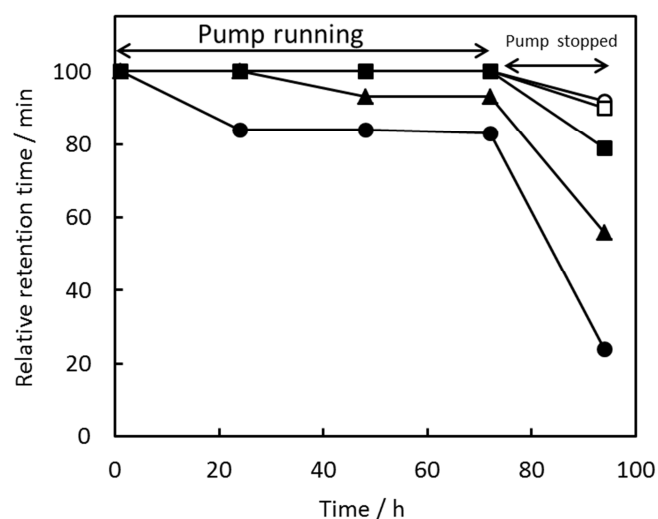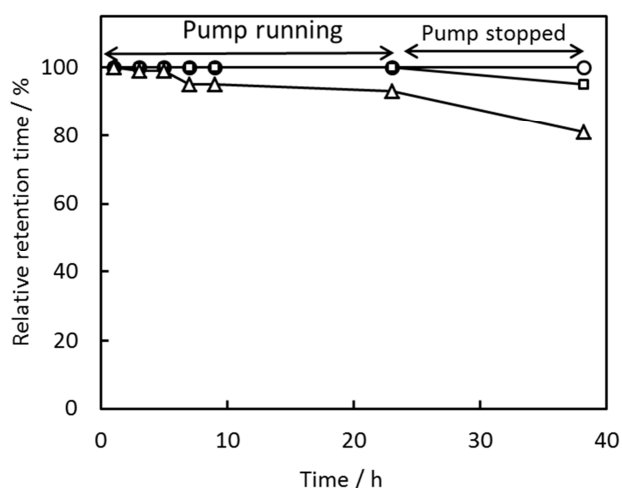

Fig. 8 Effect of temperature on C18 column (10-nm pore size) under 100% aqueous mobile phase conditions.

Column dimensions, 150 mm x 4.6 mm; temperature, ○ = 5 ℃, □ = 10 ℃, ■ = 20 ℃, ▲ = 30 ℃, ● = 40 ℃. Other conditions were the same as in Fig. 2.

3·3 Temperature

Figures 8 and 9 show the changes in retention time of 10-nm C18 and 10-nm C30 at different temperatures. Conditions were the same as Figure 2 except for stationary phases and temperatures. With 10-nm C18, the retention time decreased at 30 ℃ and 40 ℃ even while pumping. However, at 20 ℃, 10 ℃ and 5 ℃, retention time did not change while pumping and it decreased only after pumping stopped. Furthermore, the lower the temperature was, the lower the decreasing retention time ratio. At the temperature lower than 10 ℃, the decreasing retention time ratio was less than 10% even after stopping the pump, and therefore it is possible to use this phase. Moreover, 10-nm C18, which was considered to be impossible to use, can realize separations with high reproducibility by setting column temperature below 30 ℃, switching to aqueous mobile phase and continuously pumping. This result matches the changes in retention time at different temperatures shown in Figure 4. 10-nm C30 shows similar behavior to 10-nm C18, however the temperature range differs. 10-nm C30 did not change in retention time at 30 ℃ even after pumping stopped. At 40 ℃, the retention time decreased by 5% after pumping stopped, and at 80 ℃, it decreases even while

pumping and showed further decrease after pumping stopped. Compared to 10-nm C18, 10-nm C30 showed changes in retention at the higher temperature range of 40 ℃.

3·4 Back pressure after the column

As aqueous mobile phase is expelled from the packing material pores, how retention would change was investigated when there is pressure around the packing material. Figure 10 shows the result. The condition is the same as Figure 1. 10-nm C18 was used to measure the retention of 2-propanol at 40 ℃ under aqueous mobile phase. Retention has completely decreased one hour after pumping stops. A tubing of ID 0.1mm was connected to the column outlet and back pressure was applied to the postcolumn. The back pressure was controlled by gradually extending the tubing from 0.2 m to 3 m without stopping pumping. The retention did not change when the back pressure was increased to 7.5 MPa after the retention decreased once. However, the retention suddenly increased between 10 MPa and 16 MPa, and gradually increased up to 30 MPa. The back pressure decreased from 30 MPa to 0 MPa without stopping the pump. According to the results in Figure 2, 8 and 9, the retention changes over 10 hours during pumping. Therefore, the retention was measured after pumping more than 10 hours once the back pressure decreased. Up to 5 MPa, the retention did not change and decreased below 5 MPa. Figure 10 also shows the condition of the aqueous mobile phase that is inside the packing material pores within the column. It is considered that the retention increase and the increase of packing material with aqueous mobile phase permeating its pores occur at the same time. Aqueous mobile phase permeates inside of pores at a pressure of about 16 MPa. What needs to be looked at from this result is that greatly different curves were obtained when increasing and decreasing back pressure. This means that an extremely large hysteresis exists. Similarly, aqueous mobile phase sometimes permeates packing material pores at a back pressure of 5 MPa. This is determined by the condition before a back pressure of 5 MPa is applied. When the aqueous mobile phase permeates packing material pores, the mobile phase stays inside of the pores. On the other hand, when the aqueous mobile phase is not inside of the packing material pores, it does not permeate the pores even if a back pressure of 5 MPa was applied.

Hysteresis such as this exists in nature, but the hysteresis effects on the aqueous mobile phase and C18 stationary phase is quite

Fig. 9 Effect of temperature on C30 column (10-nm pore size) under 100% aqueous mobile phase conditions.
Column dimensions, 150 mm x 4.6 mm; temperature, ○ = 30 ℃, □ = 40 ℃, Δ = 80 ℃. Other conditions were the same as in Fig. 2.
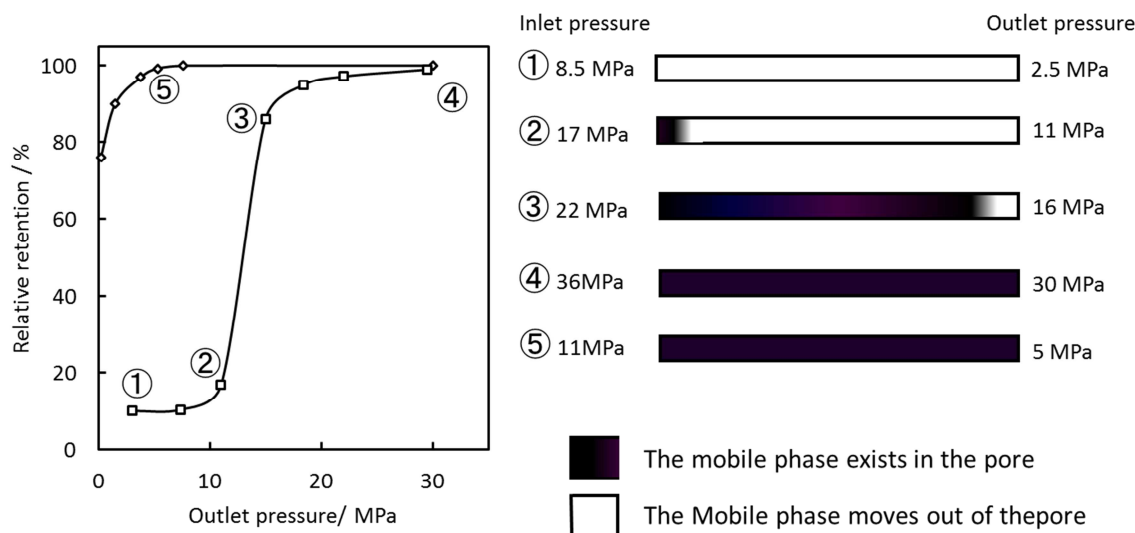

Fig. 10 Effects of the outlet pressure on the relative retention and water distribution in the column
Column, 150 mm x 4.6 mm, 5 μm d_P C18, 10-nm pore size. Other conditions were the same as in Fig. 1.

large. In order to measure pore size distribution of silica gel, an instrument, mercury intrusion porosimeter, is used. As mercury cannot be wet by any substance, a certain level of pressure is required to have it permeate pores. Pore size distribution can be determined by measuring the pressure and volume that mercury permeates pores. This relation is known as Washuburn's[16] equation shown in formula (1).

$$Pr=-2\gamma\cos\theta \qquad (1)$$

The r is radius, γ is surface tension, θ is a contact angle and P is pressure.

In fact, the relationship between the C18 stationary phase and water is the same as silica gel and mercury mentioned above. Comparable curves can be obtained by shortening the column length, making the pressure difference close to zero between the column inlet and outlet, and differentiating the curve when back pressure is increasing under the same condition as shown in Figure 10. When we measured the pore size distribution of silica material of 10-nm C18 stationary phase using this mercury intrusion porosimeter, the curve showing the relationship between the volume of mercury permeate pores and pressure was obtained, and this curve differed when pressure was increasing and decreasing. Thus, hysteresis exists. Both this hysteresis and the one shown in Figure 10 were measured with the pores of the same silica gels and therefore there should not be a large difference on its pore shapes and distribution. However, it was confirmed that the hysteresis in Figure 10 was much larger.

4 Wettability of the stationary phase and capillarity
4·1 Capillarity
Capillarity[17] is a phenomenon that when a thin tube (capillary) is set in liquid, the liquid surface elevates higher than the tube or is depressed. Depending on the magnitude relationship between the cohesion of liquid molecular and adhesion between the liquid and capillary wall, the liquid surface elevates when liquid is wetting the tube (great adhesion) and is depressed when not wet. Capillarity can be expressed in the following formula having the h as the height differences in and out of the tube, r as the radius of the tube, ρ as liquid density, γ as the surface tension of liquid, θ as a contact angle and g as the acceleration of gravity.

$$h=2\gamma\cos\theta/r\rho g \qquad (2)$$

As octadecane of carbon number 18 cannot solve with methanol or water, the stearyl of C18 stationary phase is considered to be mixed in the methanol and aqueous mobile phase[18-20]. Therefore, the surface of C18 stationary phase within the packing material pores has a similar physical property as octadecane and does not get wet with water. As a result, a force to move out of the pores functions due to the capillarity. The aqueous mobile phase that is expelled from packing material pores by capillarity is considered to cause the retention decrease of C18 stationary phase when using the aqueous mobile phase. The previously mentioned Washburn equation fits the capillarity formula by transforming it and shows the same phenomenon. When changing the liquid explained in this capillarity exclusively to mercury, it applies for the Washburn equation. Majors[21] and his associates explain the necessary pressure for water to permeate the pores of dried reversed phase Solid Phase Extraction (SPE) packing material by using the Laplace-Young equation. This equation is the same as the Washburn equation when the radius r moves to the left, and

shows the same as the capillarity formula. Originally, the Laplace–Young theory was derived from the observations of surface tension of substances, and as a result, their equation is the same as capillarity formula. Therefore, it is more appropriate to express the phenomenon that liquid being expelled from the pores in which the surface does not get wet as a capillary action is caused by capillarity rather than employing the theory of Laplace–Young.

Based on the result shown in Figure 10, the pressure that water permeates the pores of C18 stationary phase packing material with an average pore diameter 10 nm is approximately 16 MPa. Applying this value, the surface tension of 69.6 dyne cm-1 at 40°and 5.2 nm as the radius of pore diameter to the Washburn equation (1), the contact angle θ should be 126°. The contact angles can be calculated for C8 and C30 stationary phases under similar experiments. It is also possible to estimate contact angles from the data shown in Figure 3. Based on the formula (1), which is the Washburn equation, supposing that the cosine function values of each pore diameter and contact angle are proportional when the relative retention time in Figure 3 becomes 50%, the contact angles of the C8 and C30 stationary phases can be calculated based on the same C18 stationary phase. Pore diameters of C8, C18 and C30 stationary phases are 17.1 nm, 13.2 nm and 6.9 nm with 50% of relative retention time, and as the contact angle of C18 stationary phase is 126°, the same for C8 and C30 stationary phases would be 140° and 108°.

Retention changes with reversed-phase stationary phases under aqueous mobile phase are considered to be due to aqueous mobile phase that comes expelled from pores of packing materials because of capillarity, and parameters related to retention reproducibility can be explained based on capillarity. More aqueous mobile phase is expelled when pore diameters are small and less expelled when concentrations of salt and ion-pairing reagent are high with small liquid surface tension. It is also assumed that the contact angles change depending on alkyl chain lengths, ligand density of alkyl groups and the amount of residual silanol groups. As temperature increases, it is considered that liquid density, surface tension of liquid and contact angles change. Furthermore, it is thought that retentions recover by increasing the back pressure at the postcolumn outlet because higher back pressure is applied than the force that moves liquid out of the pores due to capillarity hence liquid moves back into the pores.

In 1992, Montgomery and his associates[2] fixed the C18 stationary phase on a glass surface and measured the contact angle of water and the C18 stationary phase fixed on the glass surface. They found that the contact angle was 93°and reported that water does not wet the surface of the C18 stationary phase. However, in the discussion paragraph of the literature, they concluded that, as in previous cases, the retention decreases of the C18 stationary phase under 100% aqueous mobile phase occurs due to the ligand collapse of alkyl groups after all. It can be easily assumed that water is expelled from the pores due to capillarity when the contact angle is greater than 90°, however they did not reach this conclusion. That they have reached the same conclusion can be considered so as the curves expressing the relationship between pressures and retentions when the pressure around the packing material increases and decreases to show how aqueous mobile phase moves in and out of pores is greatly different as described in the previous section, thus there is a large hysteresis.

4·2 Retention behavior of dried C18 column
When using the mixed solvent of organic solvent and water as a mobile phase, it is considered that the organic solvent in the
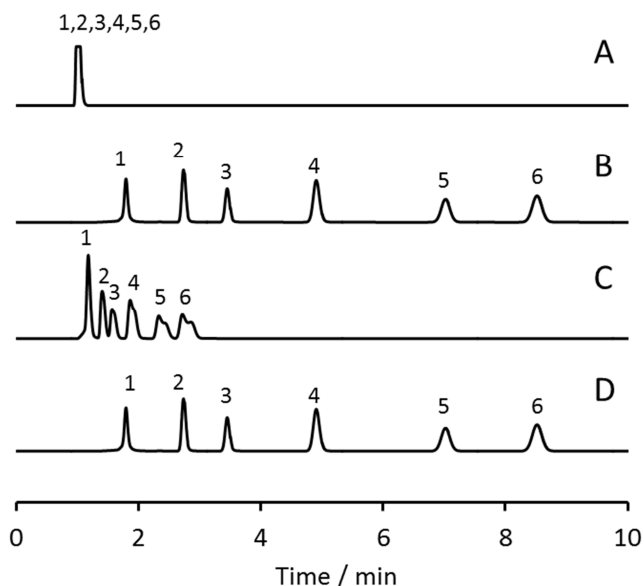
Fig. 11 Typical chromatograms of nitrite and nucleic acid bases
Column, 150 mm x 4.6 mm, 5 μm dp C18, 10-nm pore size; mobile phase, water; temperature, 40 ℃; detection, UV at 254 nm; flow rate, 1.0 mL/min; sample, 1 = sodium nitrite, 2 = cytosine, 3 = uracil, 4 = cytidine, 5 = uridine, 6 = thymine; A: C18 packing materials in a column were dried without plugs at 70 ℃ for 10 hours after chloroform flowed in a C18 column. Then, water as a mobile phase flowed at 40 ℃ at first and sample was injected into a column. B: Subsequently, 23 MPa of back pressure was added after outlet of a column without stopping flow. C: Flow was stopped for 1 hour, then flow was restarted. D: Successively, 23 MPa of back pressure was added after outlet of a column without stopping flow again.

mobile phase is solvated[19, 22-26] with stationary phases. Using C18 stationary phase after removing the solvated organic solvent, the retention reproducibility in aqueous mobile phase was investigated. Figure 11 shows the result. Filling the inside of a column with chloroform that is packed with 10-nm C18 opening both column ends, we evaporated the chloroform in a chamber at 70 ℃ to dry the packing materials in the column. Then, we pumped aqueous mobile phase at 40 ℃ into this column filled with dried C18 and injected the mixed samples of sodium nitrite and 5 kinds of nucleic acid bases. The chromatogram A shows the first injection of the sample after the baseline became stabilized and the 6 kinds of components eluted without being separated. From this condition, a tubing of ID 0.1 mm was connected to the postcolumn outlet without stopping the pump and applied the back pressure of 23 MPa to the column outlet. It is now assumed that aqueous mobile phase mixed with air came out of the column and residual air that existed in pores of the dried C18 packing materials was expelled. The chromatogram B shows the result of injection after the baseline stabilized. The retention time of thymine was approximately 8.5 minutes and all the six different of components were completely separated. Later, we stopped the pump for one hour and pumped again without applying pressure at the postcolumn outlet. The obtained chromatogram is shown as C, and the retention decreased by around 70%. Then 23 MPa of back pressure was applied again at the postcolumn outlet with continuously pumping. The first time, air came out of the column, however we could not confirm if there was air in the mobile phase that came out of the column the second time. Chromatogram D, which was obtained once the baseline became stabilized, was almost the same as chromatogram B. There was air in the pores of the C18 packing

material with chromatogram A and approximately pressure of 6 MPa was applied at the column inlet side. However, with this pressure, aqueous mobile phase did not permeate the pores of the packing material and it is considered that it only came out between packing materials. Therefore, the interaction with more than 99% of C18 stationary phase in the pores did not occur and almost all the components were eluted without being retained. The elution time of sodium nitrite that is usually not retained is approximately 1.8 minutes. However, it was eluted at 1.1 minutes from chromatogram A proving the elution without permeating the pores of the packing material based on the elution time. As chromatogram B shows, by applying back pressure of 23 MPa, the aqueous mobile phase permeates pores, and the retention and elution time of sodium nitrite increased. Even if the aqueous mobile phase permeated the pores of the packing material, it was expelled from the pores once pumping stopped, and pressure around the packing material decreases to atmospheric pressure and the retention decreased as shown in chromatogram C. The retention increased again when 23 MPa back pressure was applied to the postcolumn outlet, but no air was expelled. This means that no air was in the pores of the packing material after the aqueous mobile phase moved out. Considering that the pressure that arises due to capillarity is more than a few MPa, it is easily estimated that the inside of the pores is close to a vacuum, which is -1 atmosphere. Therefore, as the water exits out of the pores, there is water vapor in the packing material pores and the water vapor pressure is believed to be the temperature.

4·3 Wettability on the surface of the C18 stationary phase
Figure 12 shows the dispersion state of 10-nm C18 packing material to a mixed solvent of methanol and water. Image C shows that 10-nm C18 packing material dispersed at 70% methanol and was wet. As the 70% methanol wets the surface of C18, the solvent permeates the pores due to capillarity. Image B shows that the packing material did not disperse by agitation only at 50% methanol but partially dispersed by applying ultrasonic vibration. As shown in Image A, the packing material did not disperse and wet at 30% methanol even when applying
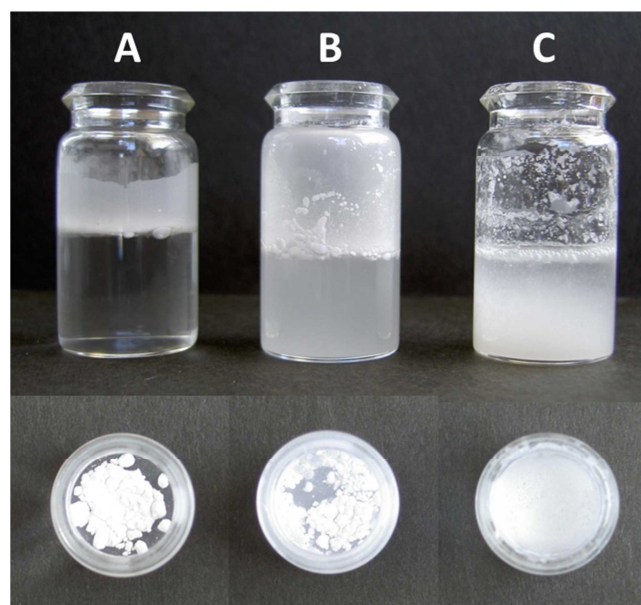

Fig. 12 Photograph of C18 packing materials mixed with methanol/water mixture solution with ultrasonic vibration
Packing material, 5 μm dp C18, 10-nm pore size; Solution, A = methanol/water(30:70), B = methanol/water(50:50), C = methanol/water(70:30).

ultrasonic vibration. From this result, we confirmed that methanol wets the surface of C18 at more than 70% but not less than 50%.

4·4 Comparison of the C18 stationary phase that is wet by methanol and dried

We pumped methanol to a dried C18 column for 30 minutes to wet the C18 stationary phase and applied solutions of 10:90, 30:70, 50:50, 70:30 and 90:30 (v/v) methanol to water as mobile phases to investigate the elution time (t_0) of sodium nitrite and uracil that are believed to not be retained under the said condition. Sodium nitrite was used as a sample to measure each t_0 when methanol concentration of the mobile phase was 10% and uracil was used when it was higher than 30%. In the meantime, we also investigated the elution time (t_0) of sodium nitrite and uracil by applying a solution of 10:90, 30:70, 50:50, 70:30 and 90:30 (v/v) methanol to water as mobile phases to a dried C18 column. The amount of mobile phase filling the spaces between particles and in the packing material pores corresponds to the value that the elution time was multiplied by the flow rate. The relative unretained time was sought by dividing the elution time of the dried C18 column by the same of the wet C18 column with methanol. Figure 13 shows the relationship between the methanol concentration ratio on the mobile phase and relative unretained time. Once methanol was pumped, C18 stationary phase becomes wet with methanol and methanol permeates the packing material pores by capillarity. When directly replacing this condition to mobile phases of 10:90, 30:70, 50:50, 70:30 and 90:30 (v/v) methanol to water, it is known that there is enough retention reproducibility with each mobile phase and the retention does not decrease as time goes by. That is, these mobile phases are permeating the C18 packing material pores. That the ratio of methanol is more than 70% and relative unretained time is 100% means that the solvent with more than 70% methanol that is supplied to a dried C18 column wets the surface of C18 stationary phase and permeates the packing material pores by capillarity. However, with the solvent of less than 50% of methanol ratio, the relative unretained time falls by 80%. This means that even if solvent with less than 50% methanol was
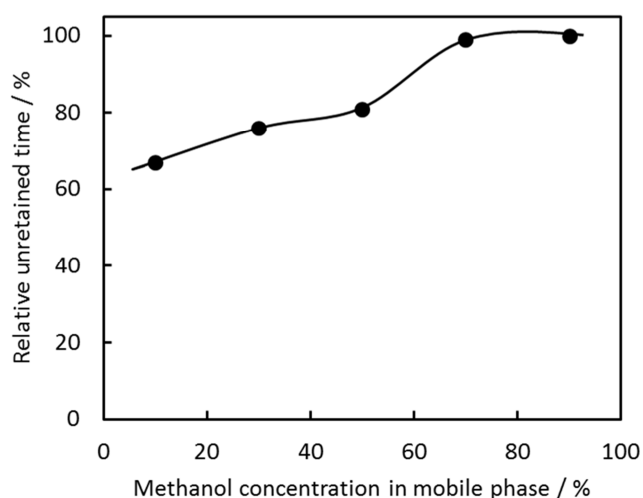

Fig. 13 Relationship between methanol concentration in mobile phase and relative unretained time

Column, 150 mm x 4.6 mm, 20 μm dp C18, 10-nm pore size; mobile phase, methanol/water = (10:90), (30:70), (50:50), (70:30), (90:10); temperature, 40 °C; detection, UV at 254 nm; flow rate, 0.2 mL/min; column pressure, 0.1 MPa; sample, sodium nitrite.

pumped to a dried C18 column, this solvent does not wet the C18 stationary phase and nor permeate the packing material pores. That is, the elution time, t_0, increased and the relative unretained time decreased as the solvent does not permeate the pores of the C18 packing materials. Furthermore, the reason why the relative unretained time is large with a higher methanol ratio even if it is decreasing is because there is a column pressure of 0.1 MPa at the column inlet side even if using large packing particle sizes and this pressure is believed to push more solvent with higher methanol ratio into the packing material pores.

Once the mobile phase is expelled from the packing material pores, it is necessary to pump the mobile phase that wets the stationary phase, such as a methanol to water ratio of more than 70% methanol concentration to permeate the packing material pores again. When a mobile phase permeates the packing material pores, a mobile phase, such as 10:90 (v/v) methanol to water, can be replaced without being expelled from the pores if it was under atmospheric pressure.

4·5 Capillarity of C18 stationary phase when using mixed organic solvent and water mobile phases

As described previously, methanol-water mobile phase with less than 50% methanol concentration does not wet the C18 stationary phase. That is, there is a force to expel the mobile phase from the packing material pores. However, usually retention reproducibility is not a problem with methanol-water mobile phase with more than 10% methanol concentration. As shown in Figure 10, a large hysteresis exists and therefore a pressure of more than 16 MPa is necessary for water to permeate the C18 packing material pores. However, when water already permeates the pores, the force to expel water is weak. Considering this hysteresis, although a higher pressure than atmospheric pressure is necessary for methanol to water mobile phase with less than 50% methanol concentration to permeate the C18 packing material pores, if the mobile phase was already in the pores, then the pressure to expel it is estimated as less than atmospheric pressure (0.1 MPa). We made a comparison of the mobile phase weights that were expelled from a C18 column by using a vacuum pump to decrease the pressure around the C18 column from atmospheric pressure down to 0.01 MPa. Figures 14 and 15 show the result. Figure 14 shows the relationship between the pressure around the column and the column weight. We used a 4.6 x 250 mm column packed with 10-nm C18 stationary phase. We replaced the solvent in the column from methanol to 90:10 (v/v) methanol to water, left the column for 20 minutes in a vacuuming chamber at 40 °C and plotted the decreased column weights on the vertical axis with negative displays. Figure 15 shows the weight changes against methanol concentration. Under 0.01 MPa pressure, we plotted the decrease in column weight after 20 minutes when methanol concentration in the column became 10%, 30%, 50%, 70% and 80% as shown in Figure 14. Figure 14 also shows 90:10 (v/v) methanol to water solvent was not expelled from the C18 packing materials pores at 1 MPa, but it moved out at below 0.05 MPa. Moreover, Figure 15 explains that although there was no change in the weight of the methanol concentration of more than 70% that wets C18 stationary phase at under 0.01 MPa and solvent stayed in the pores, the column weight decreased with the methanol concentration of less than 50% that does not wet C18 stationary phase and solvent was expelled from the pores. Based on this result, we confirmed that the mobile phase containing methanol from 10% to 50% does not wet C18 stationary phase and there is a force to expel the mobile phase from the pores due to capillarity, but the mobile phase stays in the pores under
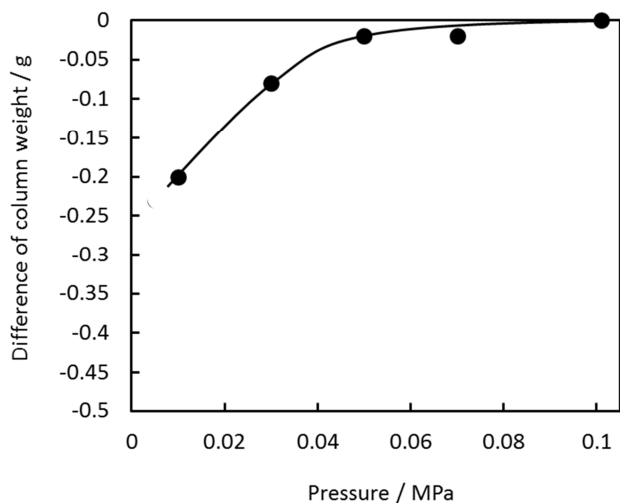
Fig.14 Relationship between pressure around the column and column weight
Column, 250 mm x 4.6 mm, 5 μm dp C18, 10-nm pore size; enclosed solution, methanol/water = (10:90); temperature, 40 ℃. Column weight was measured at the initial and after the pressure was reduced for 20 min.
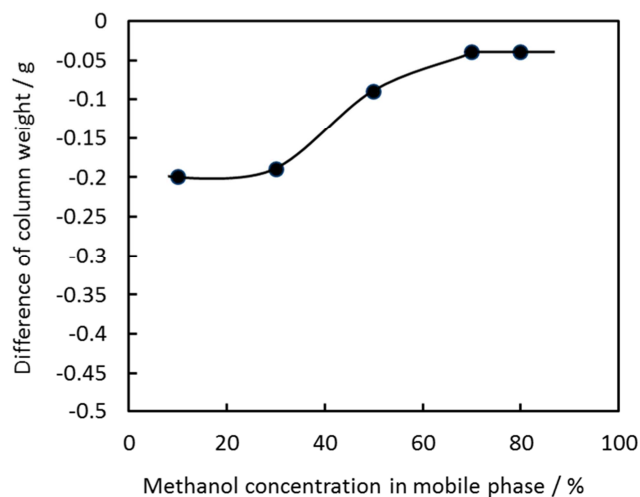

Fig. 15 Relationship between methanol concentration in mobile phase and column weight
Column, 250 mm x 4.6 mm, 5 μm dp C18, 10-nm pore size; enclosed solution, methanol/water = (10:90), (30:70), (50:50), (70:30), (80:20); temperature, 40 ℃. Column weight was measured at the initial and after the pressure was reduced to 0.01 MPa for 20 min.

atmospheric pressure and retentions are reproducible as this force is smaller than atmospheric pressure (0.1 MPa).

As described previously, the larger packing material pores realize higher retention reproducibility. There are no significant differences in the wettability of the aqueous mobile phase against the stationary phase surfaces even when the pore diameters are different, and it is believed that wettability does not change due to the sizes of the pores. That is, as the expel force due to capillarity decreases as the pore sizes increase and is weaker than the atmospheric pressure, it is assumed that the aqueous mobile phase stays in the packing material pores and therefore the retention does not decrease. Although the retention reproducibility increases with longer alkyl chains even with the same pore diameters, it is not that water wets C30 stationary phase but that the contact angle between the stationary phase and water decreases when alkyl chains get longer from C8 to C30. However, this contact angle does not decrease to less than 90°, which means wettability; rather, it means that although the contact angle is larger than 90°, it is getting close to 90°for less wettability. It is therefore assumed that the aqueous mobile phase stays in the packing material pores as the force to expel the aqueous mobile phase from the pores decreases and becomes less than atmospheric pressure as a result. Stationary phases that contain polar groups in its alkyl chains or reversed-phase stationary phases that high-polar endcapping was modified with realize stable retention even under aqueous mobile phase. This is also not because the stationary phase surfaces are wet but because the aqueous mobile phase stays in the pores due to atmospheric pressure being the same as stationary phases with long alkyl chain groups.

As discussed above, it is believed that the stationary phase surfaces of most of the reserved-phase would not become wet by aqueous mobile phase. It has been believed that sufficient separations could not be achieved unless the mobile phase wets the stationary phase. However, as shown in Figure 11, sufficient separations could be achieved with C18 stationary phase by applying a back pressure at the postcolumn outlet. These mobile phases do not wet stationary phases and are instead thought to

contact each other on the interface with a space of 0.2 nm to 0.3 nm. As using hexane and water, which do not mix together, for the liquid-liquid partition of solute, C18 stationary phase and aqueous mobile phase can separate solute for each phase as long as they are in contact with each other at a certain interface. It is possible to retain and separate solute once solute is partitioned to the stationary phase. A theory that has been conventionally believed "separations cannot be achieved unless the mobile phase wets the stationary phase" should be expressed as "separations cannot be achieved unless the mobile phase exists in the packing material pores".

It is believed that water does not wet the surfaces of silica based C18 stationary phase, however, water molecules exist in the stationary phase including its surfaces. For example, C18 packing materials were dispersed in a solvent of 80:20 (v/v) methanol-water. Later it was washed by acetone and hexane and dried sufficiently at 100 ℃, then applied to a vacuum dry at 180 ℃. During the vacuum dry, water that corresponds to approximately 1% of the weight of the C18 packing material was trapped. That is, it can be assumed that water molecules are adsorbed in the siloxane and silanol groups that are on the surfaces of the roots of C18 alkyl chains. This is a micro point of view for the inside stationary phases. In the meantime, the discussion on the wettability of C18 stationary phase is viewed with a macro issue that is the existence condition of water in the packing material pores. Therefore, it is possible that water exists as molecules in C18 stationary phase but does not wet the surface of C18 stationary phase.

5 Problems using 100% aqueous mobile phase and the solution

Once the causes are understood, it is possible to determine countermeasures against the problem, which is the decrease of retention, when using aqueous mobile phase or aquatic mobile phase containing less than 5% concentration of organic solvent with reversed-phase stationary phases. The retention decreases when using 100% aqueous mobile phase as the mobile phase is expelled from the packing material pores due to capillarity.

Therefore, it is effective to enlarge the packing material pore diameters, use a stationary phase with long alkyl chains, increase the mobile phase salt concentration, add ion-pairing reagent, and lower the column temperature so the pressure from capillarity decreases below atmospheric pressure. Although usable columns and mobile phases might be limited under these conditions, almost all kinds of reversed-phase stationary phases can solve this problem by applying a back pressure at the postcolumn outlet. As shown in Figure 10, a large hysteresis exists when mobile phase permeate the packing material pores and is expelled from them. By increasing the back pressure at postcolumn outlet to 30 MPa and then decreasing to 5 MPa, the mobile phase fills the packing material pores with the pump pressure increasing by only 5 MPa, hence separations with highly reproducible retentions become possible[27].

6 Conclusion

In this study, we investigated the retention decreases when using water and buffers as mobile phases with reversed-phase stationary phases and clarified the cause, which is that mobile phase was expelled from the packing material pores. We also found that capillarity functions on mobile phases containing organic solvents as on aqueous mobile phase and that there is a force to expel mobile phase from the packing material pores. The phenomena observed on reversed-phase chromatography that were not theoretically discussed have been recognized as empirical rules so far. However, now many of these empirical rules can be explained by capillarity studied in this work. We hope that our study contributes to the analyses of highly polar compounds with reversed-phase stationary phases which use mobile phases with no organic solvent.

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