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# Chromatographic characterization of hydrophilic interaction liquid chromatography stationary phases: Hydrophilicity, charge effects, structural selectivity, and separation efficiency

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ABSTRACT

Fourteen commercially available particle-packed columns and a monolithic column for hydrophilic interaction liquid chromatography (HILIC) were characterized in terms of the degree of hydrophilicity, the selectivity for hydrophilic–hydrophobic substituents, the selectivity for the regio and configurational differences in hydrophilic substituents, the selectivity for molecular shapes, the evaluation of electrostatic interactions, and the evaluation of the acidic–basic nature of the stationary phases using nucleoside derivatives, phenyl glucoside derivatives, xanthine derivatives, sodium *p*-toluenesulfonate, and trimethylphenylammonium chloride as a set of samples. Principal component analysis based on the data of retention factors could separate three clusters of the HILIC phases. The column efficiency and the peak asymmetry factors were also discussed. These data on the selectivity for partial structural differences were summarized as radar-shaped diagrams. This method of column characterization is helpful to classify HILIC stationary phases on the basis of their chromatographic properties, and to choose better columns for targets to be separated. Judging from the retention factor for uridine, these HILIC columns could be separated into two groups: strongly retentive and weakly retentive stationary phases. Among the strongly retentive stationary phases, zwitterionic and amide functionalities were found to be the most selective on the basis of partial structural differences. The hydroxyethyl-type stationary phase showed the highest retention factor, but with low separation efficiency. Weakly retentive stationary phases generally showed lower selectivity for partial structural differences.



## 1. Introduction

There is increasing focus of late on the hydrophilic interaction chromatography (HILIC) mode, and the number of publications on HILIC has been increasing dramatically since 2003. In 2008 and 2009, over 200 articles were published [1,2] although the term HILIC was first introduced by Alpert in 1990 [3]. Many researchers have used mixtures of acetonitrile (ACN) and water or buffer solutions as common mobile phases for HILIC, and methanol or ethanol can be used instead of ACN in some cases [1,4,5]. In addition, 2-propanol and tetrahydrofuran have been employed as organic modifiers for HILIC separation [6]. Organic solvents such as 1-propanol, 1,4-dioxane, and *N*,*N*-dimethylformamide were also suggested as alternatives for ACN, but the use of these viscous solvents led to a decrease in separation efficiency, albeit with an increase in the sensitivity of liquid chromatography–inductively coupled plasma mass spectroscopy (LC–ICP-MS) detection in the specific case of cisplatin and related compounds [7]. Recently, due to the shortage of ACN, different separation modes, *per* aqueous liquid chromatography (PALC) using mobile phases rich in water on the HILIC columns have been suggested by Sandra and co-workers [8], although such a mobile phase has been reported earlier for separations on bare silica columns [9,10]. Pesek and Matyska investigated the use of hydride-based silicas for aqueous normal phase (ANP) as the chromatographic mode [11]. In spite of these attempts to find alternative organic modifiers for HILIC, ACN remains the organic solvent used by most researchers. Although the organic modifier/aqueous portion ratio is the predominant factor in providing sufficient retention in HILIC, the aqueous buffer solution also has a great effect on the retention and selectivity, and the pH, type

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of buffer salts, and concentration of salts are significant issues for the optimization of the separation method [1,3,6,12–14]. Alpert has suggested a novel separation mode using highly polar compounds, electrostatic repulsion–hydrophilic interaction chromatography (ERLIC), which involved Coulombic attraction/repulsion interactions [12].

In addition to the selection of the mobile phase, researchers have to choose stationary phases for HILIC which are suitable for their separation targets. Elfakir and co-worker suggested a good scheme for column selection, and applied it to the analysis of neurotransmitters [15]. In terms of the retention characteristics and selectivity, their column selection method seems to be acceptable; however, the separation efficiency must be kept in mind to provide better resolution, *Rs*. In this decade, new types of HILIC stationary phases are being released continuously, and other "classic" stationary phases, such as bare silica and materials with amide, poly(succinimide)-derived, and sulfoalkylbetaine functional groups have been improved in their separation efficiency due to a down-sizing of the particles from 5 μm to 3 or 3.5 μm diameter in the case of bonded phases, and to 1.7 μm for a bare silica phase [16–18]. A superficially porous silica stationary phase of 2.7 μm particles, the Halo HILIC column, exhibited significantly higher separation efficiency in a 4.6 mm × 450 mm column [19]. However, the sample capacity of the column is not high, and in the case of samples of high concentration, peak fronting resulted in a significant loss of separation efficiency [19,20]. To select the proper stationary phases for a separation target, one has to know the retention, selectivity, and separation efficiency of HILIC columns for that specific application. In the case of HILIC separation, electrostatic interactions sometimes decrease the separation efficiency, which results in a lower *Rs* [18], and there seems to be a match–mismatch relationship between functional groups in the analytes and the stationary phases.

The characterization of HILIC stationary phases has been reported, but there is no test scheme to describe the structure-selectivity relationships for HILIC phases, in comparison with the well-accepted column tests for reversed-phase (RP) stationary phases [21–24]. For example, West and Lesellier reported the characterization of polar stationary phases, including bare silica, diol-, cyano-, amino-, poly(ethylene glycol)-, and poly(vinyl alcohol)-bonded phases, using substituted benzene and naphthalene compounds in supercritical fluid chromatography mode [25]. Their work was based on a quantitative structure-retention relationship (QSRR) [26], but the selectivity for differences in the structures of the analytes on each column was not discussed. Lämmerhofer et al. suggested a test scheme for mixed-mode stationary phases with a comparative study using RP and HILIC phases, including bare silica, amino-, amide-, zwitterionic, and sulfonate-bonded phases, using xanthines, nucleosides, and water soluble vitamins as test samples [27]. Veuthey and co-workers employed 15 model compounds, including nucleobase, saccharides, pharmaceutical compounds, and amino acids for a test of bare silica columns [28]. Nucleic bases and nucleosides were selected by Marrubini et al. as test samples for amide-bonded and zwitterionic based stationary phases [29]. These studies did not mention any partial structure selectivity, such as a selectivity for methylene group, $\alpha(CH_2)$, *k*(amylbenzene)/*k*(butylbenzene) or the selectivity for a planar structure in the RP system, such as $\alpha$(T/O), *k*(triphenylene)/*k*(*o*-terphenyl) [21] for a variety of HILIC phases that have much wider differences in functionalities bonded to the supporting materials as compared to RP stationary phases. In some cases, the peak shapes of the test samples were not good enough to discuss retention or selectivity [15].

Considering these circumstances, we suggest an inclusive test scheme of HILIC stationary phases using nucleosides, saccharides, xanthines, sodium *p*-toluenesulfonate, and trimethylphenylammonium chloride to describe the degree of hydrophilicity, the selectivity for hydrophilic and hydrophobic groups, positional selectivity, and the configuration of hydrophilic groups, the anion- and cation exchange properties, the local pH conditions on the stationary phases, and shape selectivity. It is possible to divide the HILIC phases into several groups with similar selectivity, and this would be helpful for selecting stationary phases when a target analyte possesses some of the structural characteristics discussed here. The 15 stationary phases examined included bare silica, amino-, amide-, zwitterionic-, diol-, cyclodextrin-, triazol-, sulfoethyl-, and hydroxyethyl-bonded phases, and they covered the common HILIC column functionalities with neutral, acidic/basic, and anion/cation exchange characteristics. The test samples are not pH sensitive, and the retention characteristics did not change under these pH conditions, except for the xanthine derivatives, which were selected to determine the local pH conditions on the stationary phases.

## 2. Experimental

### 2.1. Materials

EP grade acetonitrile (Wako Pure Chemical, Osaka, Japan) was used after a simple distillation. Water was purified with an Alium611 UV system (Sartorius, Göttingen, Germany). The buffer solution was prepared from analytical grade ammonium acetate and acetic acid (Nacalai Tesque, Kyoto, Japan).

### 2.2. Columns

The following particle-packed columns were employed: amide-bonded phases: Amide-80 (3 μm, 150 mm × 4.6 mm and 5 μm, 150 mm × 4.6 mm) from Tosoh (Tokyo, Japan), XBridge Amide (3.5 μm, 150 mm × 4.6 mm) from Waters (Milford, MA, USA); zwitterionic-bonded phases: ZIC-HILIC (3.5 μm, 150 mm × 4.6 mm and 5 μm, 150 mm × 4.6 mm) from Merck SeQuant (Umeå, Sweden), and Nucleodur-HILIC (3 μm, 150 mm × 4.6 mm) from Macherey-Nagel (Düren, Germany); amino-bonded phases: COSMOSIL HILIC (triazol-bonded phase, 5 μm, 150 mm × 4.6 mm), $NH_2$-MS (5 μm, 150 mm × 4.6 mm), and Sugar-D (5 μm, 150 mm × 4.6 mm) from Nacalai; poly(succinimide)-derived phases: PolyHYDROXYETHYL A (3 μm, 100 mm × 2.1 mm) and PolySULFOETHYL A (3 μm, 100 mm × 2.1 mm) from PolyLC (Columbia, MD, USA); cyclodextrin-bonded phase: CYCLOBOND I (5 μm, 250 mm × 4.6 mm) from Astec (Whippany, NJ, USA); bare silica phase: Halo HILIC (2.7 μm, 150 mm × 4.6 mm) from Advanced Materials Technology (Wilmington, DE, USA); and diol-bonded phase: LiChrospher 100 Diol (5 μm, 100 mm × 4.6 mm) from Merck (Darmstadt, Germany). A monolithic silica column, Chromolith Si (100 mm × 4.6 mm, Merck), was also examined.

### 2.3. Instrument and chromatographic measurements

The HPLC system consisted of a PU610 pump, CO630 column oven, and a UV620 detector with a semi-micro cell (1.5 μL), all from GL Science (Tokyo, Japan). The samples were injected using a sample injector (7725, Rheodyne, Park Court, CA, USA) with a 20 μL loop. Throughout these experiments, the columns were maintained at 30 °C. An EZChrom Elite-2.61 data processor (GL Science) was used for processing the chromatographic data. Principal component analysis (PCA) has been processed using a software, R version 2. 12. 1 (The R Foundation for Statistical Computing).

### 2.4. Samples

Nucleosides including adenosine, cytosine, cytidine, 2′-deoxycytidine, 2′,3′-dideoxyguanosine, guanine, 5-methyluridine,

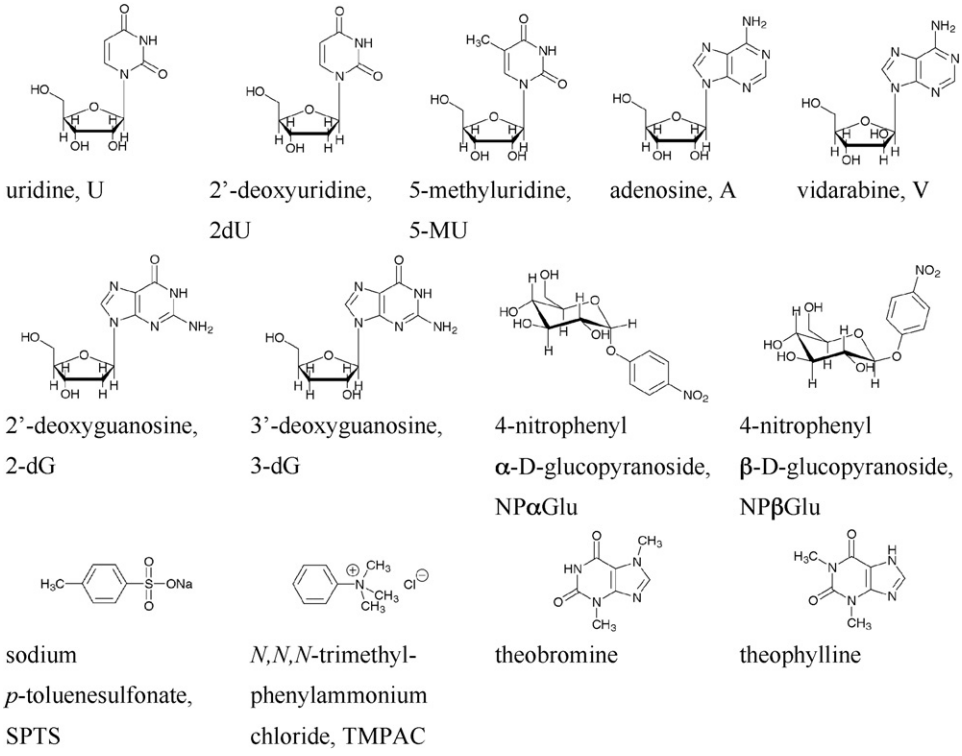


**Fig. 1.** Structure of test samples.

sodium *p*-toluenesulfonate, theobromine, theophylline, thymine, uracil, uridine (Wako Pure Chemical), 2′-deoxyadenosine, 2′-deoxyuridine, *N*,*N*,*N*-trimethylphenylammonium chloride, vidarabine (cytosine arabinoside) (Tokyo Kasei, Tokyo, Japan), 2′-deoxyguanosine, 3′-deoxyguanosine, thymidine, 2′,3′-dideoxycytidine, 2′,3′-dideoxyadenosine, 4-nitrophenyl α-D-glucopyranoside, 4-nitrophenyl β-D-glucopyranoside (Sigma, St. Louis, MO, USA), adenine, 3′-deoxythymidine, 2′,3′-dideoxyuridine (Aldrich, St. Louis, MO, USA), and guanosine (KOHJIN, Tokyo, Japan) were dissolved in the mobile phase at 0.1 mg/mL to obtain the nucleoside samples. The dead time ($t_0$) marker, toluene, was purchased from Nacalai and dissolved in the mobile phase at 1 mg/mL (Fig. 1).

## 3. Results and discussion

### 3.1. Selection of standard samples for the evaluation of HILIC separation

HILIC separation has been postulated to be determined by a combination of hydrophilic interaction, hydrophobic interaction, dipole–dipole interactions, and ion-exchange interactions [16]. In particular, the presence of ion-exchange interactions can sometimes result in peak broadening or tailing, probably due to stronger interactions between the samples and the stationary phases [30]. In order to select standard samples for the evaluation of HILIC separation to determine the degree of hydrophilic interactions, ion-exchange interactions would best be excluded. In HILIC separation, various pH and salt concentration (ionic strength) conditions are applied, and some samples change their net charges according to the pH of the mobile phase. These changes cause the differences in the degree of ion-exchange interactions. Many types of compounds are known to be retained on HILIC stationary phases, but in the case of Brønsted acids–bases, they can be involved in ion-exchange interactions in accordance with their p$K_a$ values and the separation conditions. Ideally, standard samples should have an adequate retention factor of up to 5 for a fast evaluation, and should be unaffected by the pH conditions to maintain their net charges. Saccharides are known to be well-retained by HILIC systems [31,32], but their native forms are not suitable for UV–Vis detection, and the presence of anomers can result in complex chromatograms. The problem with separation of anomers is easily overcome by choosing sugars or oligosaccharides that do not have a reducing end or which have an amide sugar that is not the reducing end. In that case, the oligosaccharide will bind in an orientation where the reducing end is not in contact with the stationary phase and anomer separation does not occur [31]. The labeling of saccharides by pyridylamino groups is useful for UV–Vis detection [33], but the amino functionality of the labeling agent can change between a salt and a free base, which can cause ion-exchange interactions. An alternative label for saccharides for UV–Vis detection is preferable.

Nucleosides are quite hydrophilic, showing negative log *P* values in the range from −1.8 to −2.8 [34] or −1.1 to −2.5 [29]. In general, they are well-retained on HILIC phases, and are detectable using UV–Vis detectors, but nucleobase ionization states change between salts and free acids–bases. The adenine and cytosine series possess lower p$K_a$ values, and at pH = 3.0 they are well retained on cation-exchange stationary phases, whereas uracil and guanine elute without much retention on the same column [35]. This phenomenon can be understood based on their p$K_a$ values; uracil, guanine, and thymine possesses p$K_a$ values in the range of 9.4–9.9, and under neutral and slightly acidic conditions, they exist in neutral forms. Thus, uracil, guanine, and thymine derivatives are suitable as standard samples for hydrophilicity tests, since their retentions are less influenced by ion-exchange interactions at acidic pH conditions.

Four HILIC stationary phases were examined using a mixture of 16 compounds, including toluene ($t_0$ marker), nucleobases (adenine, cytosine, guanine, thymine, uracil), nucleosides (adenosine, A, cytidine, C, guanosine, G, 5-methyluridine, 5-MU, uridine, U), and 2′-deoxynucleosides (2′-deoxyadenosine, 2dA, 2′-deoxycytidine, 2dC, 2′-deoxyguanosine, 2dG, thymidine, T, 2′-deoxyuridine, 2dU)

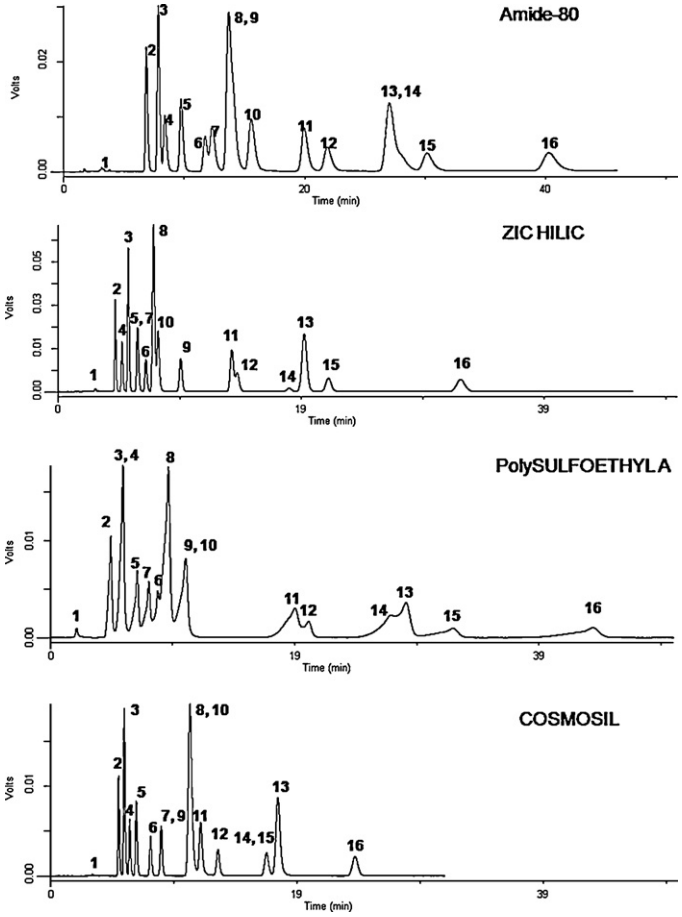


**Fig. 2.** The separation of nucleosides on four HILIC stationary phases. Column: Amide-80 (5 μm, 150 × 4.6 mm), ZIC-HILIC (5 μm, 150 mm × 4.6 mm), COSMOSIL HILIC (5 μm, 150 mm × 4.6 mm), PolyHYDROXYETHYL A (3 μm, 100 mm × 2.1 mm). Mobile phase: ACN–ammonium acetate buffer (20 mM in the aqueous portion, pH 4.7) (90:10, v/v). Flow rate: 0.1 mL/min for PolyHYDROXYETHYL A, 0.5 mL/min for others. Injection volume: 3 μL for PolyHYDROXYETHYL A, 5 μL for others. Temperature: 30 °C. Detection: UV–Vis, λ = 254 nm. Solute: 1, toluene; 2, thymine; 3, uracil; 4, thymidine; 5, 2′-deoxyuridine; 6, 5-methyluridine; 7, 2′-deoxyadenosine; 8, adenine; 9, uridine; 10, adenosine; 11, cytosine; 12, 2′-deoxycytidine; 13, guanine; 14, 2′-deoxyguanosine; 15, cytidine; 16, guanosine.

in an ACN–ammonium acetate buffer (pH = 4.7, 20 mM in the aqueous portion) (90:10, v/v), as shown in Fig. 2. The buffer concentration is not high [15], but this is desirable for researchers employing LC–MS analysis.

The degree of separation and the elution order of these compounds differed significantly on each column. On Amide-80, the separation of A–U and G–2dG–C were incomplete, whereas A and U were completely separated using the other three phases. The ZIC-HILIC phase separated G, 2dG, and C completely, although the other three phases could not. The separation of nucleosides were examined on the same stationary phases: the elution order of A, C, G, T, U was reported to be T–A–U–C–G on both Amide-80 and ZIC-HILIC [27], but was found to be T–U–A–C–G on Amide-80, and T–A–U–C–G on ZIC-HILIC in the present study, and also in a report by Marrubini et al. [29]. The differences in these results presumably reflect differences in the pH of the mobile phases in these systems, and adenosine can be charged in the pH range examined here. In addition, the salt concentration in the mobile phases in these systems is different. It is known that differences in the salt concentration can result in changes in retention in HILIC separations [15,36]. This means that any discussion of the elution order of A and U is meaningless if the pH conditions and salt concentration are not considered.

**Table 1**
Selectivities due to the structural differences of nucleosides on HILIC stationary phases.

| | α(ribose) | α(dribose) | α(ddribose) | α(OH3) | α(OH2) |
|---|---|---|---|---|---|
| *Amide-80* | | | | | |
| Thymine | 2.32 | 1.42 | 0.73 | 1.95 | 1.64 |
| Uracil | 1.81 | 1.40 | 0.71 | 1.97 | 1.68 |
| Adenine | 1.18 | 0.87 | 0.59 | 1.48 | 1.36 |
| Cytosine | 1.52 | 1.12 | 0.59 | 1.91 | 1.44 |
| Guanine | 1.49 | 1.05 | 0.63 | 1.66 | 1.48 |
| *ZIC-HILIC* | | | | | |
| Thymine | 2.53 | 1.34 | 0.57 | 2.35 | 1.89 |
| Uracil | 2.58 | 1.28 | 0.49 | 2.59 | 2.02 |
| Adenine | 1.08 | 0.74 | 0.46 | 1.62 | 1.45 |
| Cytosine | 1.71 | 1.04 | 0.42 | 2.46 | 1.64 |
| Guanine | 1.75 | 0.93 | 0.40 | 2.32 | 1.88 |
| *PolyHYDROXYETHYL A* | | | | | |
| Thymine | 2.37 | 1.35 | 0.63 | 2.14 | 1.76 |
| Uracil | 2.35 | 1.32 | 0.60 | 2.20 | 1.77 |
| Adenine | 1.19 | 0.79 | 0.49 | 2.18 | 1.51 |
| Cytosine | 1.73 | 1.06 | 0.45 | 1.76 | 1.62 |
| Guanine | 1.57 | 0.96 | 0.51 | 1.86 | 1.64 |
| *COSMOSIL HILIC* | | | | | |
| Thymine | 2.22 | 1.43 | 0.74 | 1.93 | 1.56 |
| Uracil | 2.12 | 1.38 | 0.71 | 1.93 | 1.57 |
| Adenine | 0.99 | 0.68 | 0.42 | 1.62 | 1.45 |
| Cytosine | 1.62 | 1.16 | 0.61 | 1.90 | 1.40 |
| Guanine | 1.40 | 0.94 | 0.55 | 1.70 | 1.49 |

α(ribose) = *k*(nucleoside)/*k*(nucleic base).
α(dribose) = *k*(2′-deoxynucleoside)/*k*(nucleic base).
α(ddribose) = *k*(2′,3′-dideoxynucleoside)/*k*(nucleic base).
α(OH3) = *k*(2′-deoxynucleoside)/*k*(2′,3′-dideoxynucleoside).
α(OH2) = *k*(nucleoside)/*k*(2′-deoxynucleoside).

Among the 2′-deoxy nucleoside moieties, the elution order of T–dU–dA–dC–dG was maintained, although dU and dA were not separated on the ZIC-HILIC phase. In general, 2′-deoxy nucleoside moieties exhibited less retention than corresponding nucleosides but their elution order was maintained, probably because retention is determined mainly via the base, which did not vary. As shown in Table 1, the number(s) of hydroxy groups in the ribose moiety has a great influence on retention factors of the nucleosides. The 2′,3′-dideoxynucleosides showed less retention than the corresponding nucleobase, which means that aliphatic methylene groups decrease retention on HILIC phases. In Table 1, the adenine series shows the lowest selectivity in many cases, suggesting that the purine base part makes a greater contribution to retention than the ribose moieties. Fig. 3 shows the retention factor, *k*, of each nucleoside on four

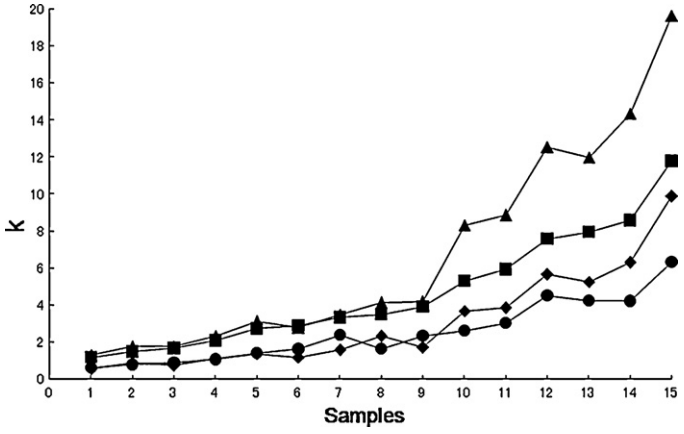


**Fig. 3.** Nucleoside samples and their retention factors. Separation conditions, see Fig. 2. Amide-80 (■), ZIC-HILIC (♦), COSMOSIL HILIC (●), PolyHYDROXYETHYL A (▲). Solute: 1, toluene; 2, thymine; 3, uracil; 4, thymidine; 5, 2′-deoxyuridine; 6, 5-methyluridine; 7, 2′-deoxyadenosine; 8, adenine; 9, uridine; 10, adenosine; 11, cytosine; 12, 2′-deoxycytidine; 13, guanine; 14, 2′-deoxyguanosine; 15, cytidine; 16, guanosine.

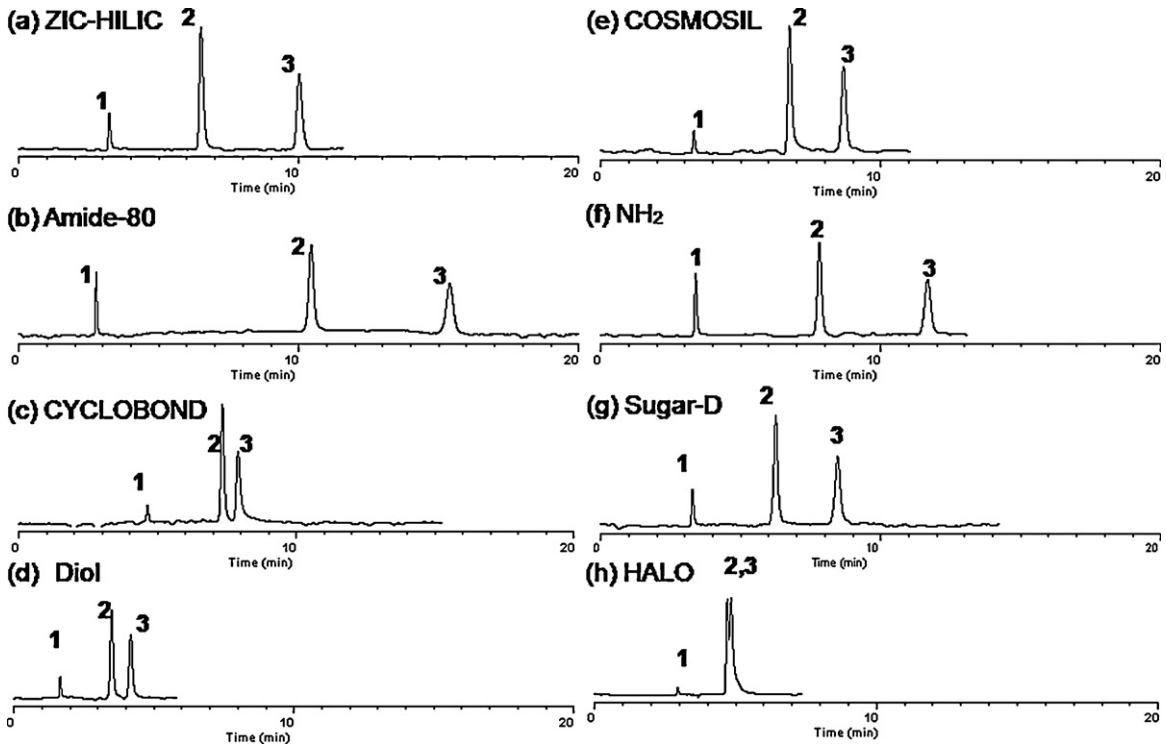


**Fig. 4.** Chromatograms for the test of methylene group selectivity, $\alpha(CH_2)$. Columns: (a) ZIC-HILIC (3.5 μm, 150 mm × 4.6 mm), (b) Amide-80 (3 μm, 150 mm × 4.6 mm), (c) CYCLOBOND I (5 μm, 250 mm × 4.6 mm), (d) LiChrospher Diol (5 μm, 100 mm × 4.6 mm) (e) COSMOSIL HILIC (5 μm, 150 mm × 4.6 mm), (f) $NH_2$-MS (5 μm, 150 mm × 4.6 mm), (g) Sugar-D (5 μm, 150 mm × 4.6 mm), (h) Halo HILIC (2.7 μm, 150 mm × 4.6 mm). Flow rate: 0.50 mL/min for (a), (c), (d), (e), (f), (g), and (h); 0.55 mL/min for (b). Backpressure: (a) 2.6 MPa, (b) 2.8 MPa, (c) 2.1 MPa, (d) 1.5 MPa, (e) 1.4 MPa, (f) 1.2 MPa, (g) 1.2 MPa, (h) 3.8 MPa. Injection volume: 4.0 μL. Temperature: 30 °C. Detection: UV–Vis, λ = 254 nm. Solute: 1, toluene; 2, 5-methyluridine; 3, uridine.

HILIC stationary phases. The adenosine and cytosine series should be avoided as standard compounds due to the possibility of charged structures when their p$K_a$ values are considered. Guanosine derivatives are useful to investigate differences among the stationary phases, but their appreciable retention is somewhat problematic: thus, a uridine series is the standard.

### *3.2. Separation factors provided by methylene α(CH2), and hydroxy groups α(OH)*

The selectivity for a methylene group, $\alpha(CH_2)$, is a useful parameter to know the degree of surface coverage of silica by hydrophobic groups in RPLC [21]. This parameter is available from a comparison of $k$(U) and $k$(5MU), and it should be useful for a discussion of the degree of hydrophobic interaction between the stationary phases and the test compounds. Similarly, the selectivity caused by the hydroxy group, $\alpha$(OH), can be defined as $k$(U)/$k$(2dU). Chromatograms of 8 representative stationary phases in ACN–ammonium acetate buffer (pH = 4.7, 20 mM in the aqueous portion) (90:10, v/v) are shown in Fig. 4 for $\alpha(CH_2)$ and Fig. 5 for $\alpha$(OH). The parameters of selectivity ($\alpha$), the height equivalent to the number of theoretical plates, $H$ (μm), and the peak asymmetry (Asym) of all columns are summarized in Table 2. Among the stationary phases investigated, Amide-80 (3 μm) showed the largest retention for U ($k$ = 4.58), with a small $H$ (9 μm) and good Asym (0.99) values. XBridge Amide also possesses amide functionality, but the $k$(U) was smaller than that of Amide-80, and peak tailing was observed. Both amide-bonded columns, Amide-80 and XBridge

**Table 2**
Selectivity for methylene groups $\alpha(CH_2)$ and hydroxy groups $\alpha$(OH).

| Column | U | | | 2dU | | | α(U/2dU) | 5MU | | | α(U/5MU) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k$ | $H$ (μm) | Asym | $k$ | $H$ (μm) | Asym | | $k$ | $H$ (μm) | Asym | |
| ZIC-HILIC (5 μm) | 2.11 | 25 | 1.34 | 1.04 | 26 | 1.40 | 2.03 | 1.26 | 26 | 1.39 | 1.67 |
| ZIC-HILIC (3.5 μm) | 2.10 | 12 | 1.26 | 1.02 | 15 | 1.34 | 2.07 | 1.23 | 14 | 1.26 | 1.71 |
| Nucleodur HILIC (3 μm) | 2.20 | 14 | 0.88 | 1.42 | 14 | 0.96 | 1.55 | 1.72 | 14 | 0.92 | 1.28 |
| Amide-80 (5 μm) | 3.30 | 26 | 1.37 | 1.98 | 29 | 1.44 | 1.67 | 2.60 | 31 | 1.29 | 1.27 |
| Amide-80 (3 μm) | 4.58 | 9 | 0.99 | 2.79 | 9 | 1.04 | 1.64 | 3.60 | 9 | 0.97 | 1.27 |
| XBridge Amide (3.5 μm) | 2.55 | 12 | 1.42 | 1.50 | 14 | 1.59 | 1.70 | 1.98 | 13 | 1.51 | 1.29 |
| PolySULFOETHYL (3 μm) | 1.58 | 62 | 1.11 | 0.74 | 167 | 1.97 | 2.13 | 1.07 | 85 | 1.49 | 1.48 |
| PolyHYDROXYETHYL (3 μm) | 3.92 | 61 | 0.99 | 2.04 | 103 | 1.28 | 1.92 | 2.88 | 45 | 0.92 | 1.36 |
| CYCLOBOND I (5 μm) | 0.70 | 18 | 1.73 | 0.58 | 12 | 1.17 | 1.21 | 0.62 | 13 | 1.36 | 1.13 |
| LiChrospher Diol (5 μm) | 1.50 | 17 | 0.98 | 1.10 | 17 | 1.13 | 1.36 | 1.30 | 17 | 1.06 | 1.15 |
| Chromolith Si | 0.31 | 12 | 1.00 | 0.31 | 13 | 1.01 | 1.00 | 0.28 | 13 | 1.06 | 1.12 |
| Halo HILIC (2.7 μm) | 0.64 | 8 | 1.47 | 0.60 | 6 | 1.36 | 1.08 | 0.56 | 7 | 1.53 | 1.16 |
| COSMOSIL HILIC (5 μm) | 1.60 | 12 | 1.11 | 1.00 | 13 | 1.43 | 1.60 | 1.40 | 13 | 1.18 | 1.14 |
| Sugar-D (5 μm) | 1.58 | 17 | 1.12 | 0.91 | 16 | 1.05 | 1.74 | 1.10 | 16 | 1.09 | 1.44 |
| $NH_2$-MS (5 μm) | 2.44 | 12 | 1.04 | 1.30 | 12 | 1.09 | 1.88 | 1.88 | 12 | 1.11 | 1.30 |

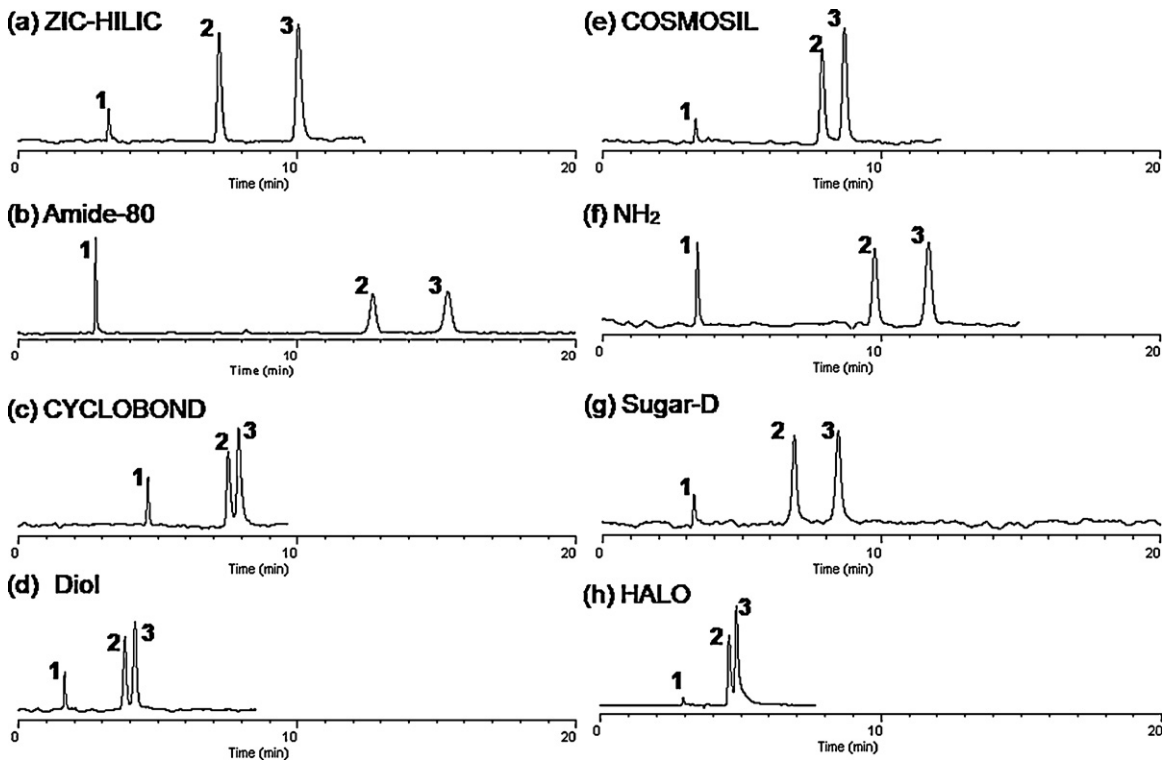


**Fig. 5.** Chromatograms for the test of hydroxy group selectivity, $\alpha$(OH). Separation conditions are the same as in Fig. 4. Solute: 1, toluene; 2, 2′-deoxyuridine; 3, uridine.

Amide, showed similar selectivity for $\alpha(CH_2)$ and $\alpha$(OH), which implies that the difference in silica supports or spacers between the silica surface and functional group had little effect on the selectivity. The retention factors for $k$(U), $k$(2dU), and $k$(5MU) on Amide-80 (3 μm) and Amide-80 (5 μm) are different as compared to those on the ZIC-HILIC columns. The two Amide-80 phases, based on 5 μm and 3 μm particles, might be functionalized by different procedures.

The ZIC-HILIC and PolyHYDROXYETHYL A phases exhibited greater $\alpha(CH_2)$ and $\alpha$(OH) selectivity than the amide-bonded phases, although the latter gave peak fronting under the separation conditions. The Nucleodur HILIC phase seems to contain a similar zwitterionic functionality as ZIC-HILIC, but its $\alpha(CH_2)$ and $\alpha$(OH) selectivities were smaller than those of ZIC-HILIC. The Nucleodur HILIC phase exhibited greater retention than the ZIC-HILIC phase for U, dU, and 5MU. This fact suggests that the degree of retention does not have a direct correlation with the selectivity, and this set of solutes can be used to describe the difference in these stationary phases, as shown in Table 2. Among the bonded phases, CYCLOBOND I showed the smallest $\alpha$(OH) selectivity and hydrophilicity, and the diol-bonded phase also possessed small $\alpha$(OH) selectivity. The bare silica phases provided small $k$(U) and $\alpha(CH_2)$ and $\alpha$(OH) values. Among the three HILIC phases with Brønsted base functionalities, the primary amine based phase $NH_2$-MS showed the largest $k$(U). The $\alpha(CH_2)$ and $\alpha$(OH) values did not show apparent correlation, but the COSMOSIL HILIC showed the smallest value among them.

PolyHYDROXYETHYL A and PolySULFOETHYL A showed poor separation efficiency, albeit high selectivity. Among the tested stationary phases, only these columns had a narrow inner diameter. To reduce the extra-column effects, the examinations were carried out at 1.0 mm/s linear velocity, the same as the other columns, using a semi-micro flow cell of 1.5 μL cell volume. These stationary phases have also been used in gradient modes, which should be a good way to obtain more symmetrical peaks [12,37,38].

### 3.3. Evaluation of the discrimination of regio and configurational isomers

HILIC has been shown to afford fine separation of biological samples having differences in hydrophilic properties, especially peptides [39–42] and saccharides [43–47]. These samples contain configurational and structural isomers to be separated. In order to describe the selectivity of the configurational and regio isomers, sets of vidarabine/adenosine, V/A (configurational isomers at the 2′ position of the ribose moiety), and 2′-deoxyguanosine/3′-deoxyguanosine, 2dG/3dG, were chosen as test samples. If ionic interactions are involved in these cases, they should be nearly canceled out by using the same nucleobase. Vidarabine is used as an antiviral to treat eye infections and encephalitis caused by herpes simplex virus, or shingles caused by the varicella zoster virus [48], and thus the fine separation of vidarabine would be meaningful. The configurational isomers A and V were separated as shown in the selected chromatogram in Fig. 6. The regio isomers 2dG and 3dG were separated as exhibited in Fig. 7. The whole set of retention, selectivity, HETP, and peak asymmetry data are summarized in Tables 3 and 4.

As shown in Tables 3 and 4, ZIC-HILIC provided the best selectivity for $\alpha$(V/A) and $\alpha$(2dG/3dG), although the $\alpha$(2dG/3dG) values were less than 1.13 for all stationary phases. The Nucleodur HILIC and Sugar-D phases also exhibited good selectivity for these samples, implying a zwitterionic functionality is useful for the fine separation of complex mixtures of saccharides. The test for the stationary phases with hydroxy groups as a hydrophilic functionality, CYCLOBOND I, LiChrospher Diol, and PolyHYDROXYETHYL A, resulted in different retention and selectivity from each, suggesting that the structure around the hydroxy groups, their quantity and the differences in the spacers between these stationary phases were responsible. In all stationary phases, 2dG was retained more than 3dG. Nucleodur HILIC, LiChrospher Diol, and PolyHYDROXYETHYL A phases tended to give peak fronting, whereas ZIC-HILIC, XBridge

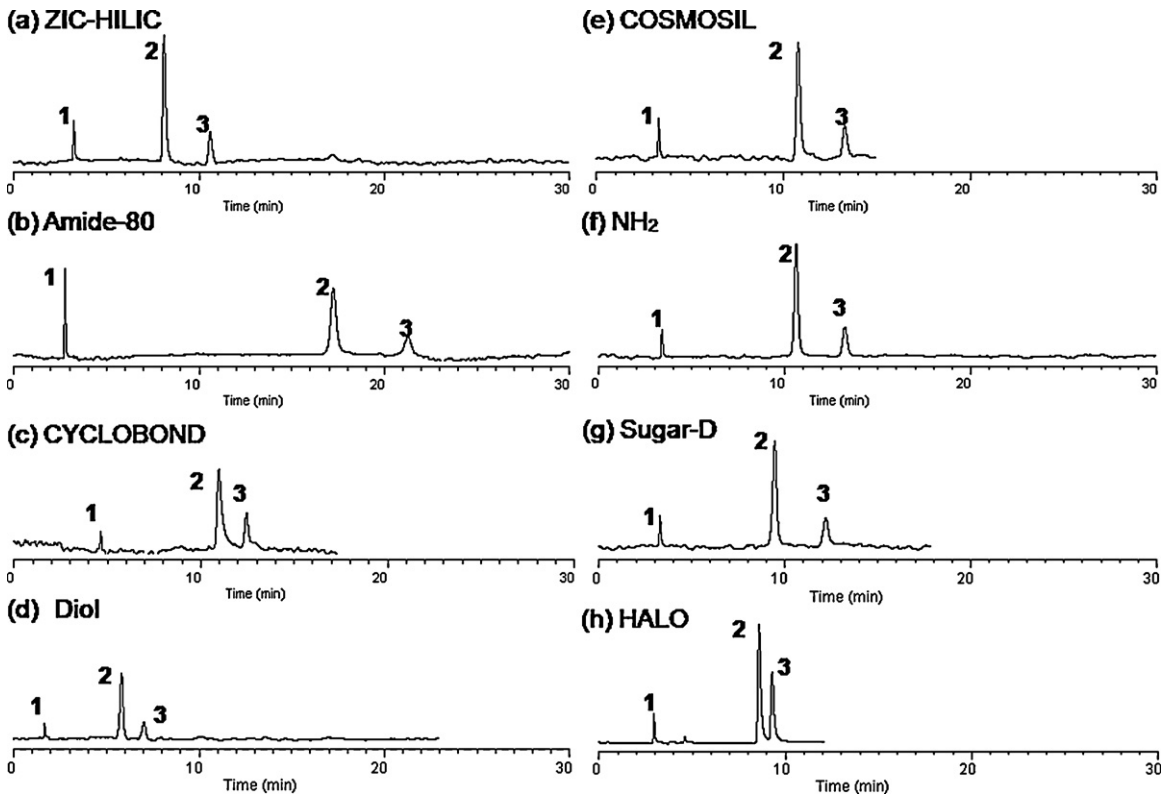


**Fig. 6.** Chromatograms for the test of selectivity for regio isomers. Separation conditions are the same as in Fig. 4. Solute: 1, toluene; 2, adenosine; 3, vidarabine.

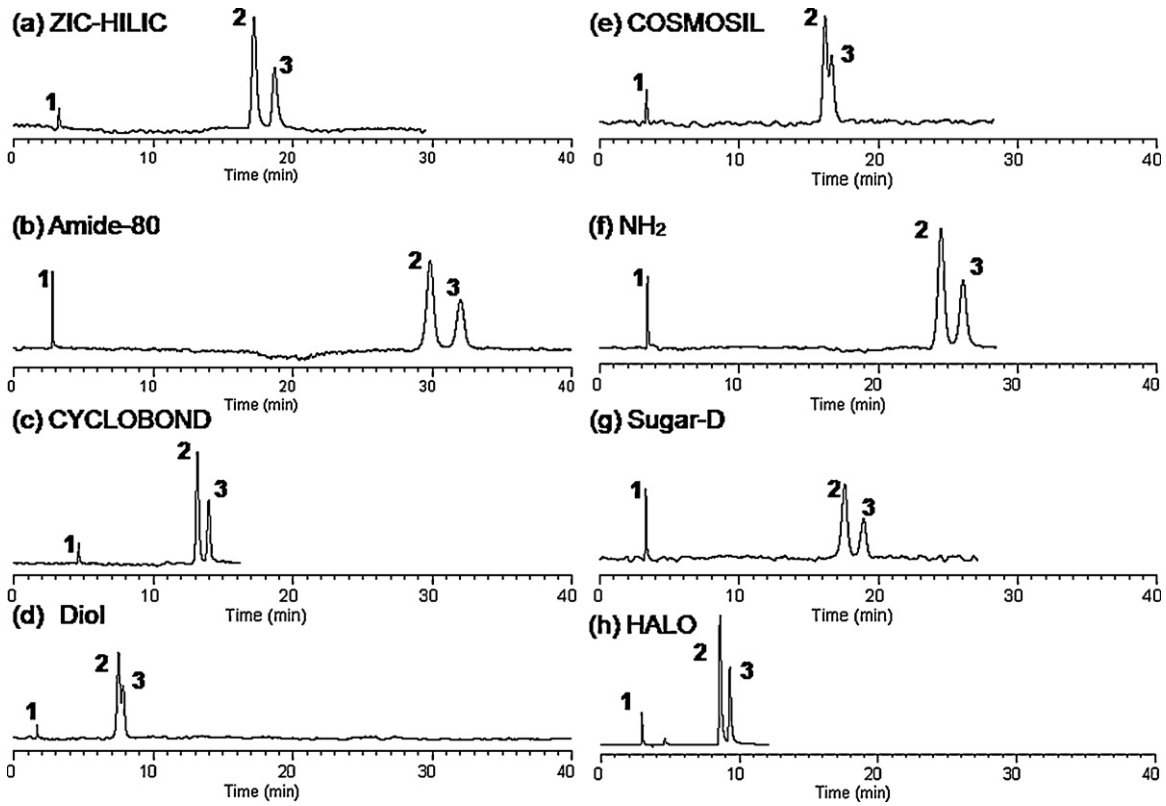


**Fig. 7.** Chromatograms for the test of selectivity of configurational isomers. Separation conditions are the same as in Fig. 4. Solute: 1, toluene; 2, 3′-deoxyguanosine; 3, 2′-deoxyguanosine.

**Table 3**
Selectivity for configurational isomers.

| Column | A | | | V | | | α(V/A) |
|---|---|---|---|---|---|---|---|
| | k | H (μm) | Asym | k | H (μm) | Asym | |
| ZIC-HILIC (5 μm) | 1.55 | 24 | 1.22 | 2.32 | 23 | 1.21 | 1.50 |
| ZIC-HILIC (3.5 μm) | 1.51 | 12 | 1.32 | 2.28 | 11 | 1.19 | 1.51 |
| Nucleodur HILIC (3 μm) | 2.33 | 14 | 0.97 | 3.40 | 16 | 0.97 | 1.46 |
| Amide-80 (5 μm) | 3.80 | 28 | 1.38 | 4.90 | 20 | 1.38 | 1.29 |
| Amide-80 (3 μm) | 5.26 | 11 | 1.07 | 6.72 | 9 | 1.21 | 1.28 |
| XBridge Amide (3.5 μm) | 2.81 | 11 | 1.23 | 3.64 | 11 | 1.24 | 1.30 |
| PolySULFOETHYL (3 μm) | 1.15 | 44 | 2.56 | 1.39 | 80 | 1.36 | 1.21 |
| PolyHYDROXYETHYL (3 μm) | 3.75 | 50 | 0.99 | 4.93 | 44 | 0.91 | 1.31 |
| CYCLOBOND I (5 μm) | 1.36 | 25 | 1.84 | 1.68 | 12 | 1.09 | 1.24 |
| LiChrospher Diol (5 μm) | 2.50 | 17 | 0.97 | 3.30 | 18 | 0.97 | 1.32 |
| Chromolith Si | 0.73 | 13 | 2.02 | 0.85 | 11 | 0.99 | 1.16 |
| Halo HILIC (2.7 μm) | 1.59 | 6 | 1.37 | 1.87 | 8 | 1.54 | 1.18 |
| COSMOSIL HILIC (5 μm) | 2.20 | 12 | 1.16 | 3.00 | 11 | 1.19 | 1.36 |
| Sugar-D (5 μm) | 1.88 | 15 | 0.98 | 2.72 | 17 | 1.08 | 1.45 |
| $NH_2$-MS (5 μm) | 2.13 | 11 | 1.09 | 2.90 | 11 | 1.07 | 1.36 |

Amide, CYCLOBOND I, and PolySULFOETHYL A were liable to yield peak tailing as shown in Tables 3 and 4. The Amide-80 (3 μm), COSMOSIL HILIC, $NH_2$-MS, and Sugar-D phases provided symmetrical peaks for these test samples.

### 3.4. Shape selectivity

The shape selectivity, such as α(triphenylene/*o*-terphenyl) can be used to interpret the density of the bonded groups on the surface of the stationary phases for reversed-phase HPLC [21]. The α(V/A) and α(2dG/3dG) values mentioned above also reflect shape selectivity, but larger differences in structures should also be tested. Here, 4-nitrophenyl α-D-glucopyranoside (NPαGlu) and 4-nitrophenyl β-D-glucopyranoside (NPβGlu) were employed, since one of them was used as a test sample for the $NH_2$-MS phase. These aryl glycosides possess ether linkages at the 2-carbon, fixing the configuration of the anomeric isomers. As shown in Table 5, the selectivity α(NPαGlu/NPβGlu) was in the range of 1.1–1.3. Interestingly, the highest α(NPαGlu/NPβGlu) values were obtained on bare silica columns, whereas ZIC-HILIC, Amide-80, LiChrospher Diol, and COSMOSIL HILIC gave small α(NPαGlu/NPβGlu) values. Apparently, the mobile phase conditions resulted in little retention, and in many cases, significant tailing of the peaks was observed. The separation efficiency was also worse than for the other test samples. The selectivity obtained here did seem larger than for the separation of nitrophenyl glycosides on monolithic silica columns possessing cyano groups [49]. Further investigation with the use of nitrophenyl ethers of di- or trisaccharides, or NPβGlu/NPβGlu in a 95% ACN mobile phase, high enough to induce appreciable retention, will provide more information for interpreting shape selectivity in HILIC separation.

### 3.5. Evaluation of ion-exchange interactions

Ion-exchange interactions can be important forces influencing retention on HILIC phases, particular when separating polar ionic compounds via hydrophilic interaction [12]. To evaluate the degree of the ion-exchange nature of the stationary phases, relatively hydrophobic organic ions, such as sodium *p*-toluenesulfonate (SPTS) for the anion-exchange property, AX, and *N*,*N*,*N*-trimethylphenylammonium chloride (TMPAC) for cation-exchange property, CX, were employed as standard samples. It is reasonable to postulate that these samples are also retained by hydrophilic interaction, so the retention factors of these samples, *k*(SPTS) and *k*(TMPAC), were divided by *k*(U) to account, at least partially, for the contribution of hydrophilic interactions. Thus, α(AX), and α(CX) were given by *k*(SPTS)/*k*(U), and by *k*(TMPAC)/*k*(U), respectively. The selectivity recorded in two types of mobile phases, 90% (v/v) ACN–ammonium acetate buffer (pH = 4.7) with 20 and 100 mM in the aqueous portion, is tabulated in Table 6.

The ion-exchange selectivity is significantly different on each column. In the vacant cell of Table 6 in the case of 20 mM buffer in the aqueous portion, SPTS or TMPAC eluted earlier than the $t_0$ marker, toluene, which suggested that the ionic ligands in the stationary phases excluded these samples from the pore volume by electrostatic repulsion [12]. The PolySULFOETHYL A and

**Table 4**
Selectivity for regio isomers.

| Column | 3dG | | | 2dG | | | α(2dG/3dG) |
|---|---|---|---|---|---|---|---|
| | k | H (μm) | Asym | k | H (μm) | Asym | |
| ZIC-HILIC (5 μm) | 4.34 | 22 | 1.21 | 4.80 | 23 | 1.28 | 1.11 |
| ZIC-HILIC (3.5 μm) | 4.30 | 12 | 1.27 | 4.80 | 12 | 1.31 | 1.12 |
| Nucleodur HILIC (3 μm) | 5.57 | 13 | 0.88 | 6.02 | 13 | 0.85 | 1.08 |
| Amide-80 (5 μm) | 7.10 | 25 | 1.30 | 7.70 | 25 | 1.28 | 1.08 |
| Amide-80 (3 μm) | 9.82 | 9 | 1.00 | 10.60 | 9 | 0.95 | 1.08 |
| XBridge Amide (3.5 μm) | 5.23 | 10 | 1.18 | 5.58 | 10 | 1.08 | 1.07 |
| PolySULFOETHYL (3 μm) | 3.43 | 61 | 1.11 | 3.64 | 57 | 0.99 | 1.06 |
| PolyHYDROXYETHYL (3 μm) | 10.20 | 28 | 0.71 | 10.90 | 34 | 0.79 | 1.07 |
| CYCLOBOND I (5 μm) | 1.83 | 12 | 1.11 | 2.01 | 12 | 1.07 | 1.10 |
| LiChrospher Diol (5 μm) | 3.50 | 15 | 0.96 | 3.70 | 15 | 0.92 | 1.06 |
| Chromolith Si | 0.91 | 12 | 0.96 | 1.01 | 11 | 0.93 | 1.11 |
| Halo HILIC (2.7 μm) | 1.92 | 8 | 1.51 | 2.16 | 8 | 1.48 | 1.13 |
| COSMOSIL HILIC (5 μm) | 3.90 | 12 | 1.12 | 4.00 | 12 | 1.07 | 1.03 |
| Sugar-D (5 μm) | 4.36 | 16 | 0.95 | 4.78 | 14 | 0.90 | 1.10 |
| $NH_2$-MS (5 μm) | 6.21 | 11 | 1.02 | 6.67 | 11 | 0.97 | 1.07 |

**Table 5**
Selectivity for molecular shapes.

| Column | NPαGlu | | | NPβGlu | | | α(α/β) |
|---|---|---|---|---|---|---|---|
| | *k* | *H* (μm) | Asym | *k* | *H* (μm) | Asym | |
| ZIC-HILIC (5 μm) | 0.67 | 24 | 1.46 | 0.59 | 26 | 1.46 | 1.14 |
| ZIC-HILIC (3.5 μm) | 0.67 | 24 | 1.35 | 0.59 | 25 | 1.29 | 1.14 |
| Nucleodur HILIC (3 μm) | 1.38 | 13 | 0.88 | 1.21 | 13 | 0.92 | 1.14 |
| Amide-80 (5 μm) | 1.45 | 32 | 1.36 | 1.23 | 32 | 1.41 | 1.18 |
| Amide-80 (3 μm) | 2.05 | 10 | 1.09 | 1.74 | 10 | 1.09 | 1.18 |
| XBridge Amide (3.5 μm) | 1.13 | 11 | 1.23 | 0.97 | 11 | 1.26 | 1.16 |
| PolySULFOETHYL (3 μm) | 0.78 | 140 | 1.15 | 0.63 | 112 | 1.28 | 1.24 |
| PolyHYDROXYETHYL (3 μm) | 2.02 | 70 | 1.18 | 1.67 | 86 | 1.24 | 1.21 |
| CYCLOBOND I (5 μm) | 0.54 | 11 | 1.13 | 0.45 | 10 | 1.13 | 1.20 |
| LiChrospher Diol (5 μm) | 1.08 | 17 | 1.02 | 0.92 | 17 | 1.11 | 1.17 |
| Chromolith Si | 0.21 | 14 | 1.5 | 0.16 | 14 | 1.74 | 1.31 |
| Halo HILIC (2.7 μm) | 0.44 | 7 | 1.37 | 0.34 | 7 | 1.44 | 1.29 |
| COSMOSIL HILIC (5 μm) | 1.12 | 12 | 1.04 | 0.99 | 12 | 1.29 | 1.13 |
| Sugar-D (5 μm) | 0.78 | 15 | 1.1 | 0.64 | 15 | 1.14 | 1.22 |
| $NH_2$-MS (5 μm) | 1.76 | 12 | 1.05 | 1.47 | 12 | 1.12 | 1.20 |

Chromolith Si phases exhibited significant CX character, whereas NH2–MS and Sugar-D phases exhibited appreciable AX character with 20 mM buffer in the aqueous portion. These results are as expected, because PolySULFOETHYL A is made for use as a CX phase, and the NH2–MS phase possesses primary amino groups, which work as an AX functionality at the experimental conditions of pH = 4.7. Among the three basic phases, the NH2–MS phase showed the greatest α(AX), 2.7-fold that of COSMOSIL HILIC and 1.5-fold greater than Sugar-D. Other phases exhibited much smaller α(AX) values, which meant the AX interaction was not important in these phases under the conditions employed.

When the concentration of salt was increased from 20 to 100 mM in the aqueous portion, the difference between columns decreased and PolySULFOETHYL A did not show significant CX interaction. This is presumably caused by greater competition for binding between the *N*,*N*,*N*-trimethylphenylammonium and ammonium cations than in the case of the dilute buffer. Even with 100 mM buffer in the aqueous portion, the Chromolith and HALO Si phases exhibited strong CX interaction, though the α(TMPAC/U) value became much smaller than in the case of 20 mM buffer. The AX interaction in the three amine-based phases was largely suppressed in the 100 mM buffer. Here, α(SPTS/U) of CYCLOBOND I, 4.73, became the highest value, reflecting a high *k* value for SPTS compared to that for U. This strong AX interaction with the CYCLOBOND I phase is difficult to understand: the column possesses ether linkage between the spacer and β-cyclodextrin, and ostensibly no amine and amide bonds are included, per the written information about the column. The possibility of non-ionic host–guest interaction between the β-cyclodextrin and toluene moieties might account for the high *k* value.

SPTS eluted earlier than the $t_0$ marker on Chromolith Si and Halo HILIC in the case of 20 mM buffer in the aqueous portion. Bare silica phases are known to possess CX ability due to their silanols (SiOH) functionality. The p$K_a$ of silanols is around 4.7; thus it exists here as $SiO^-$ at least in part, and the CX interaction can play an important role in HILIC retention on bare silica phases [1,50]. Even on "neutral" stationary phases, the remaining silanols could have an influence on the CX interaction [24]. Therefore the relatively low retention of SPTS could be understood as a result of electrostatic repulsion interactions. The zwitterionic phase, ZIC-HILIC, showed strong CX interactions among neutral stationary phases due to the sulfo group in the phase. The column manufacturer suggests using this column as an ion-exchange stationary phase as well, for example, using 10 mM phosphate buffer with NaCl (0–1 M) as a mobile phase.

In HILIC separation, basic samples are known to be strongly retained [14,15], and this can be regarded as a result of acid–base interactions with the remaining silanol groups in addition to partitioning into the water-rich stationary phase [3,12]. The XBridge Amide phase showed the smallest CX interaction among the phases investigated. The Amide-80 phase exhibited a larger value than the XBridge Amide phase, although the amide functionality of both phases is supposedly similar. The use of ethylene-bridged hybrid

**Table 6**
Selectivity for ionic compounds.

| Column | SPTS | | TMPAC | |
|---|---|---|---|---|
| | *k* | α(SPTS/U) | *k* | α(TMPAC/U) |
| ZIC-HILIC (5 μm) | 0.10 (0.69)[a] | 0.05 (0.33)[a] | 9.31 (3.32)[a] | 4.41 (1.57)[a] |
| ZIC-HILIC (3.5 μm) | 0.11 (0.56)[a] | 0.05 (0.27)[a] | 9.10 (3.45)[a] | 4.33 (1.64)[a] |
| Nucleodur HILIC (3 μm) | 0.33 (1.13)[a] | 0.15 (0.34)[a] | 7.60 (3.14)[a] | 3.46 (0.95)[a] |
| Amide-80 (5 μm) | 0.10 (0.89)[a] | 0.03 (0.19)[a] | 12.00 (4.57)[a] | 3.62 (1.00)[a] |
| Amide-80 (3 μm) | 0.26 (0.90)[a] | 0.06 (0.41)[a] | 12.90 (6.04)[a] | 2.82 (2.75)[a] |
| XBridge Amide (3.5 μm) | 0.22 (0.74)[a] | 0.09 (0.47)[a] | 3.02 (1.89)[a] | 1.18 (1.20)[a] |
| PolySULFOETHYL (3 μm) | –[b] (0.25)[a] | –[b] (0.06)[a] | 12.10 (1.38)[a] | 7.66 (0.35)[a] |
| PolyHYDROXYETHYL (3 μm) | 0.37 (0.87)[a] | 0.09 (0.34)[a] | 9.67 (3.34)[a] | 2.47 (1.31)[a] |
| CYCLOBOND I (5 μm) | 0.31 (3.32)[a] | 0.44 (4.73)[a] | 3.76 (0.45)[a] | 5.36 (0.63)[a] |
| LiChrospher Diol (5 μm) | 0.02 (0.95)[a] | 0.01 (0.63)[a] | 4.90 (1.73)[a] | 3.27 (1.16)[a] |
| Chromolith Si | –[b] (0.06)[a] | –[b] (0.09)[a] | 20.30 (5.25)[a] | 65.27 (8.21)[a] |
| HALO Si (2.7 μm) | –[b] (0.20)[a] | –[b] (0.64)[a] | 28.20 (9.03)[a] | 43.86 (29.03)[a] |
| COSMOSIL HILIC (5 μm) | 4.50 (1.28)[a] | 2.81 (0.80)[a] | 0.15 (0.78)[a] | 0.09 (0.49)[a] |
| Sugar-D (5 μm) | 8.18 (3.00)[a] | 5.18 (1.90)[a] | –[c] (0.39)[a] | –[c] (0.25)[a] |
| $NH_2$-MS (5 μm) | 18.40 (2.01)[a] | 7.54 (0.82)[a] | –[c] (0.69)[a] | –[c] (0.28)[a] |

[a] Data in parenthesis were obtained in a mobile phase ACN–ammonium acetate buffer (pH = 4.7, 100 mM in aqueous part) (90:10, v/v).
[b] SPTS eluted faster than the $t_0$ marker, toluene.
[c] TMPAC eluted faster than the $t_0$ marker, toluene.

**Table 7**
Test for pH on the surface of stationary phases.

| Column | Theophylline | | | Theobromine | | | $\alpha$(Tb/Tp) |
|---|---|---|---|---|---|---|---|
| | $k$ | $H$ (μm) | Asym | $k$ | $H$ (μm) | Asym | |
| ZIC-HILIC (5 μm) | 0.30 | 22 | 1.36 | 0.36 | 21 | 1.29 | 1.18 |
| ZIC-HILIC (3.5 μm) | 0.28 | 12 | 1.44 | 0.34 | 12 | 1.47 | 1.20 |
| Nucleodur HILIC (3 μm) | 0.52 | 16 | 1.00 | 0.52 | 16 | 1.00 | 1.00 |
| Amide-80 (5 μm) | 0.76 | 36 | 1.41 | 1.06 | 32 | 1.37 | 1.39 |
| Amide-80 (3 μm) | 1.08 | 9 | 1.07 | 1.43 | 10 | 1.15 | 1.32 |
| XBridge Amide (3.5 μm) | 0.52 | 20 | 3.26 | 0.71 | 24 | 1.37 | 1.38 |
| PolySULFOETHYL (3 μm) | 0.23 | 114 | 1.91 | 0.23 | 138 | 2.17 | 1.00 |
| PolyHYDROXYETHYL (3 μm) | 0.66 | 126 | 1.81 | 0.75 | 92 | 1.31 | 1.14 |
| CYCLOBOND I (5 μm) | 0.43 | 12 | 1.14 | 0.44 | 11 | 1.1 | 1.01 |
| LiChrospher Diol (5 μm) | 0.55 | 12 | 1.14 | 0.57 | 15 | 1.28 | 1.04 |
| Chromolith Si | 0.26 | 14 | 1.09 | 0.31 | 13 | 1.14 | 1.22 |
| Halo HILIC (2.7 μm) | 0.50 | 8 | 1.36 | 0.64 | 7 | 1.3 | 1.26 |
| COSMOSIL HILIC (5 μm) | 0.55 | 16 | 1.37 | 0.49 | 16 | 1.7 | 0.89 |
| Sugar-D (5 μm) | 0.59 | 22 | 1.11 | 0.31 | 15 | 1.44 | 0.52 |
| $NH_2$-MS (5 μm) | 0.80 | 12 | 1.15 | 0.43 | 12 | 1.18 | 0.54 |

particles in the XBridge Amide phase decreased the concentration of residual silanols, and that would reduce CX interactions. By using our simple test, the CX interactions of the stationary phases can easily be described. It was apparent that the use of the 20 mM buffer revealed the CX and AX nature of the stationary phases. These ion-exchange interactions were suppressed in the 100 mM buffer, and the values of $\alpha$(SPTS/U) and $\alpha$(TMPAC/U) had a smaller distribution compared to the case with the dilute buffer. The use of the dilute buffer may be meaningful for column characterization, though it is a somewhat lower concentration than is usual with HILIC separation.

### 3.6. pH on the surface of the stationary phases

The mobile phases in HILIC separation play important roles, and the pH conditions are generally controlled by buffer solutions with a 5–20 mM salt concentration. However, there has been little discussion of the pH conditions on the surface of the stationary phases, probably due to the lack of commercially available acidic stationary phases for HILIC. The PolySULFOETHYL A column was introduced prior to the HILIC concept; however, the use of the column as an acidic phase for HILIC has been limited [3]. The behavior of ionizable samples under CX conditions has been well discussed [15], but weak AX conditions are not often employed.

During the testing of the retention factors of various solutes on different stationary phases, it was observed that the xanthine derivatives, theophylline and theobromine, showed interesting retention behavior, $\alpha(k_{\text{theobromine}}/k_{\text{theophylline}})$, as shown in Table 7. The buffer capacity of 2 mM (in total) might be low to confer a significant amount of buffering capacity, so these amino materials probably have a high pH in the vicinity of the surface. Xanthine derivatives are often employed as test samples in HILIC [27,51]. Theophylline is used as a drug for asthma [52], and theobromine is also employed in the therapeutic field as a vasodilator and heart stimulant [53]. As metabolites of caffeine, the separation of these xanthine derivatives is considered to be important [54].

The p$K_a$ values of theophylline and theobromine have been reported as p$K_{a\,\text{theophylline}}$ = 8.6, and p$K_{a\,\text{theobromine}}$ = 10 [27,55]. Thus, theobromine is a stronger base than theophylline. Chromatograms with the eight representative stationary phases are shown in Fig. 8. The acidic phase, poly(acrylic acid)-bonded phase (PAA), showed an $\alpha(k_{\text{theobromine}}/k_{\text{theophylline}}) = 1.24$ (Table 8), and the ZIC-HILIC and Amide-80 phases also provided high $\alpha(k_{\text{theobromine}}/k_{\text{theophylline}})$ values. These phases are supposedly neutral in terms of the nature of the functional groups of the stationary phases, but they are known to show an acidic nature in terms of their retentions [12,30]. Under these conditions, theobromine is believed to exist in its protonated form. The zwitterionic phase Nucleodur did not separate them at all, and this difference in selectivity from ZIC-HILIC might be caused by differences in the technology of stationary phase preparation. The fact that theobromine is more strongly retained on acidic stationary phases is probably due to some other aspect of its structure. For example, if the chief location of positive charge in the molecule is at the nitrogen at position 1, substitution of a methyl group there, as in theophylline, would sterically hinder the ability of the molecule to orient itself with that nitrogen in proximity to the stationary phase. In addition, the possible charge heterogeneity in the molecules of theophylline and theobromine would be different, and this may have some influence on retention. Therefore, the difference in retention times of these two molecules may involve orientation effects on their access to the stationary phases, in addition to ion-exchange interaction [32].

Stationary phases with hydroxy groups that impart hydrophilic character, such as PolyHYDROXYETHYL A, CYCLOBOND I, and LiChrospher Diol, did not separate these xanthine derivatives. In the case of basic phases, such as COSMOSIL HILIC, $NH_2$-MS, and Sugar-D, the $\alpha(k_{\text{theobromine}}/k_{\text{theophylline}})$ value was smaller than 1.0, and the smallest value, on Sugar-D, was 0.52. On the surface of these basic phases, the deprotonation of theophylline should be an important process, because the retention of theobromine on these phases was comparable. COSMOSIL HILIC provides lower CX interactions, and that resulted in less retention of theophylline but a larger $\alpha(k_{\text{theobromine}}/k_{\text{theophylline}})$ value than $NH_2$-MS and Sugar-D.

**Table 8**
$\alpha(k_{\text{theobromine}}/k_{\text{theophylline}})$ values on monolithic and particle-packed columns.

| pH conditions of stationary phase | Stationary phase | $\alpha(k_{\text{theobromine}}/k_{\text{theophylline}})$ |
|---|---|---|
| Acidic | XBridge Amide | 1.39 |
| | Amide-80 (5 μm) | 1.39 |
| | Amide-80 (3 μm) | 1.32 |
| | Halo HILIC | 1.26 |
| | Chromolith Si | 1.25 |
| | PAA monolith[a] | 1.24 |
| | ZIC-HILIC | 1.20 |
| Neutral | PolyHYDROXYETHYL A | 1.14 |
| | PAAm monolith[a] | 1.11 |
| | LiChrospher Diol | 1.04 |
| | PolySULFOETHYL A | 1.00 |
| | CYCLOBOND I | 1.00 |
| | Nucleodur HILIC | 1.00 |
| Basic | COSMOSIL HILIC | 0.89 |
| | $NH_2$-MS | 0.54 |
| | Sugar-D | 0.52 |

[a] Data were obtained from monolithic silica columns prepared in house [56,57].

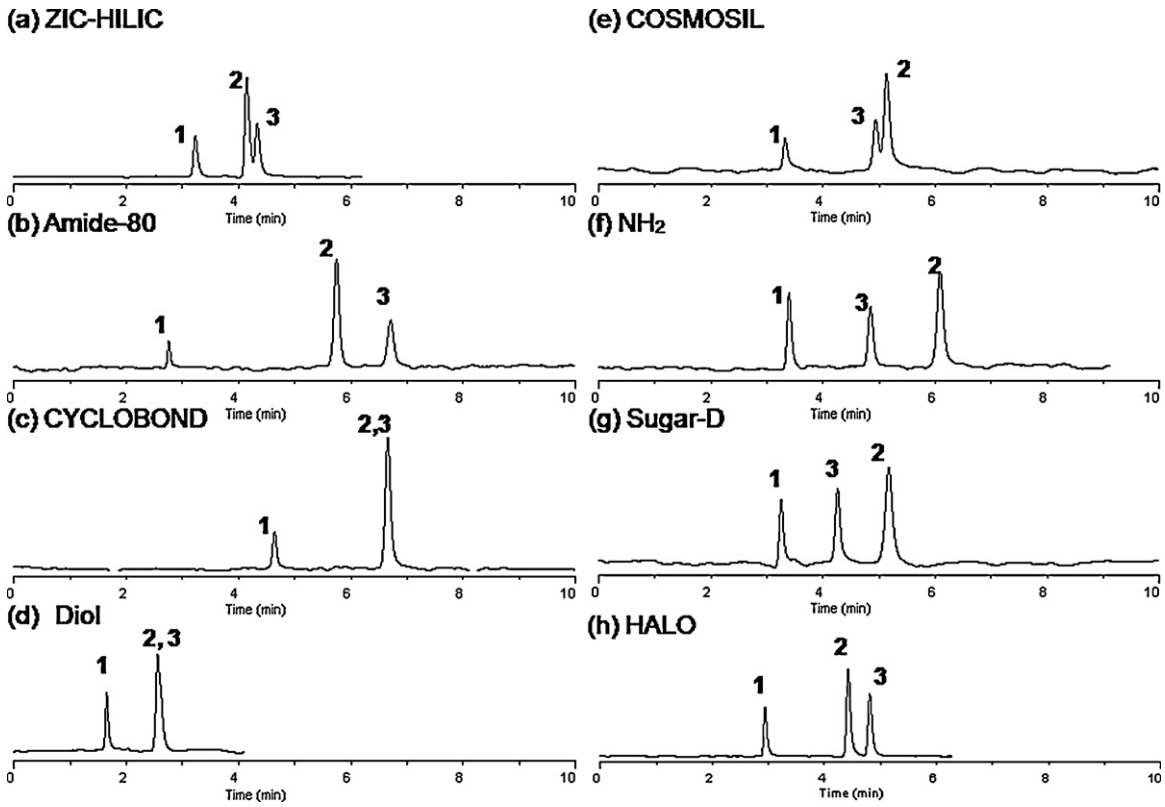


**Fig. 8.** Chromatograms for the test of pH on the surface of stationary phases. Separation conditions are the same as in Fig. 4. Solute: 1, toluene; 2, theophylline; 3, theobromine.

Monolithic silica columns functionalized by the polymerization of acrylic acid (PAA type) [56] and acrylamide (PAAm type) [57] were employed for the separation of theophylline and theobromine, respectively, and the results are summarized in Table 8 along with $\alpha(k_{theobromine}/k_{theophylline})$ values obtained with particle-packed columns. The PAA column showed $\alpha(k_{theobromine}/k_{theophylline})$ value larger than 1.00. The acidic (and CX) nature of the carboxylic acid in the PAA phase can result in the retention of basic and/or cationic compounds. Here, the tendency for the retention of theobromine on the PAA phase increased upon adding ion-exchange interactions to hydrophilic interactions, whereas the retention of theophylline was comparable to that of the PAAm column. Basic stationary phases gave $\alpha(k_{theobromine}/k_{theophylline})$ values smaller than 1 [27]. In the study by Lämmerhofer, the $\alpha(k_{theobromine}/k_{theophylline})$ of the Amide-80 and ZIC-HILIC phases were different from the present results. This difference may be caused by differences in the mobile phases in both experiments. Furthermore, basic stationary phases give $\alpha(k_{theobromine}/k_{theophylline})$ values <1, neutral phases give $\alpha(k_{theobromine}/k_{theophylline})$ values around 1, and acidic phases seem to give $\alpha(k_{theobromine}/k_{theophylline})$ values >1. The selectivity seemed to reflect surface pH conditions, while excluding ion-exchange interaction, since PolySULFOETHYL A, a typical CX stationary phase, could not separate these xanthine derivatives.

Many targets for HILIC separation possess ionizable functional groups, and knowing the acid–base properties of the stationary phases is important to controlling the analytical results. These findings have led to a test method to evaluate the degree of equilibration of the stationary phases. The equilibration of stationary phases containing amino groups is known to be slow [58], and a test to determine the completion of the column conditioning should be useful. A fresh $NH_2$-MS column was employed to separate theophylline and theobromine, and at intervals of 1, 3, 4, 12, and 13 h after starting the equilibrium. The test was repeated using a mobile phase ACN–ammonium acetate buffer (pH = 4.7, 20 mM in the aqueous portion) (90:10, v/v). As shown in Fig. 9, the retention of theophylline gradually decreased, and after 12 h of equilibration, the $\alpha(k_{theobromine}/k_{theophylline})$ became constant, meaning that the column conditioning was completed at that point. After the first use of the column, 3 h of equilibration was adequate for column conditioning. This test provides a quick and easy way to determine the endpoint of column conditioning for amine-containing stationary phases. The Sugar-D column was also tested the same way to detect the degree of equilibration. When a mobile phase ACN–ammonium acetate buffer (pH = 4.7, 100 mM in the aqueous portion) (90:10, v/v) was used, the period required for equilibration was shorter, and the $\alpha(k_{theobromine}/k_{theophylline})$ value had nearly reached 1 at the end.

### 3.7. Separation efficiency and peak asymmetry versus retention

In a previous review on separation efficiency in HILIC, the relationship between the separation efficiency, $H$, and the retention factor, $k$, was discussed [18]. The separation efficiency became slightly worse as retention increased in the separation of salicylamide, salicylic acid derivatives, and nucleobase. Based on the experience above, the separation efficiency can be supposed to be worse if the retention mechanism is predominantly one of ion-exchange interactions. Here, the influence of the ion-exchange interactions was reduced by choosing solutes less ionized under the experimental conditions. To investigate the relationship between $H$ and $k$, the data for U, 2dU, 5MU, A, V, 2dG, and 3dG samples on ZIC-HILIC, Nucleodur HILIC, Amide-80, XBridge-Amide, CYCLOBOND-I, LiChrospher Diol, COSMOSIL HILIC, Sugar-D, $NH_2$-MS, and Halo HILIC phases are plotted in Fig. 10. With the stationary phases with strong retention, $H$ tends to be constant in the case of phases based on 3–3.5 μm particles. Among the phases with weak retention, COSMOSIL HILIC and $NH_2$-MS could maintain constant $H$

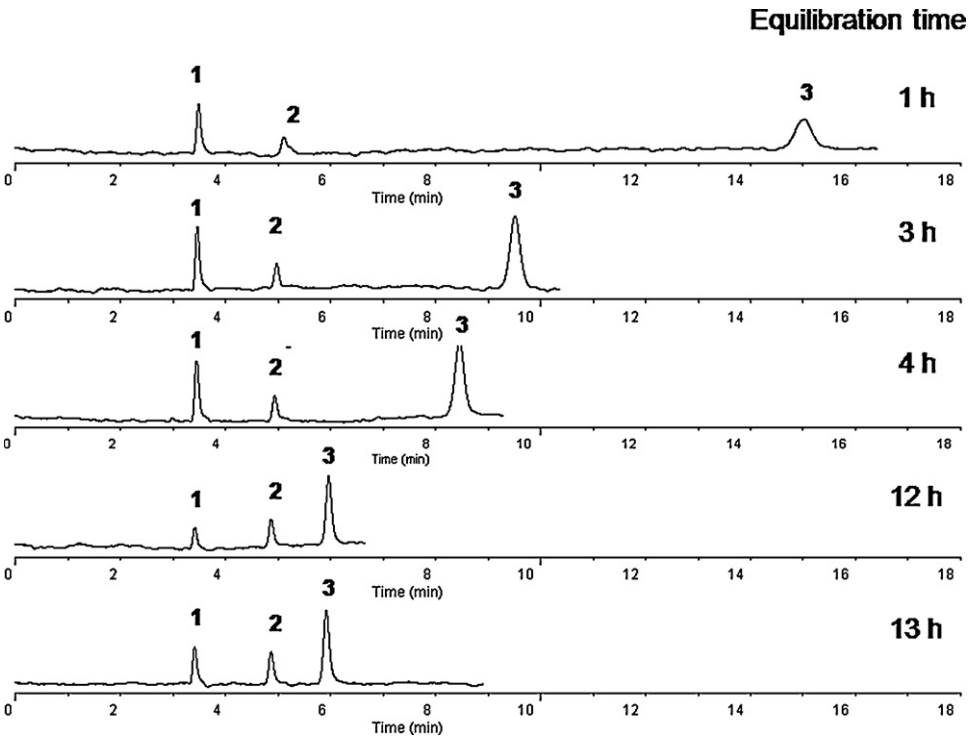


**Fig. 9.** Test of equilibration of $NH_2$-MS phase. Separation conditions are the same as in Fig. 8.

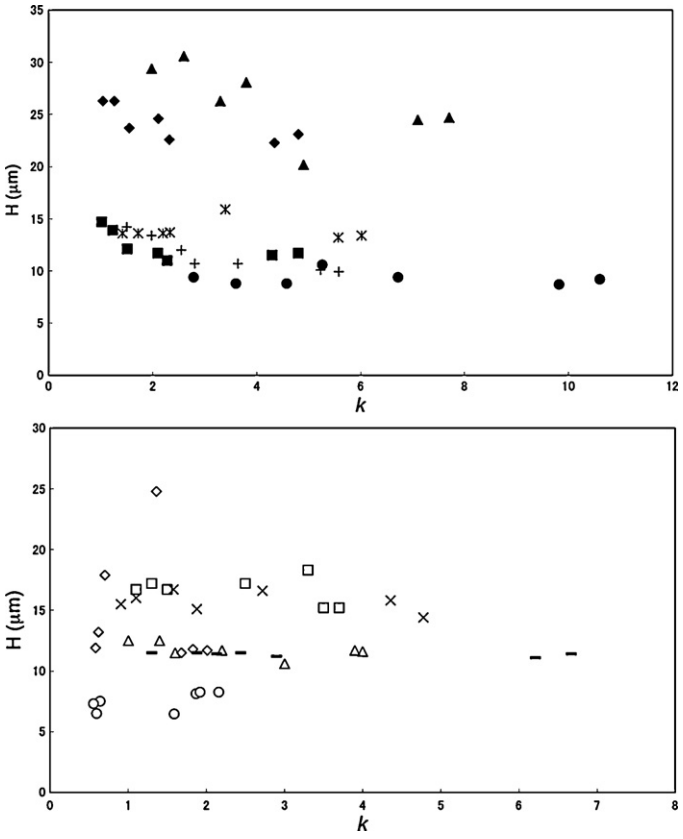


**Fig. 10.** Relationship between retention and separation efficiency. Column: ZIC-HILIC 5 μm (♦), ZIC-HILIC 3.5 μm (■), Amide-80 5 μm (▲), Amide-80 3 μm (●), Nucleodur (*), XBridge-Amide (+), CYCLOBOND I (◊), LiChrospher Diol (□), COSMOSIL HILIC (△), Sugar-D (×), MS-$NH_2$ (–), and Halo HILIC (○).

values over a wide region of *k*. The LiChrospher Diol and Sugar-D phases also showed acceptable reproducibility of the *H* values. The CYCLOBOND-I phase decreased the separation efficiency in less retentive region than $k = 1.5$. Apparently, the *H* did not increase with an increase in the *k*, and thus this result clearly showed that reducing ion-exchange interactions are important to obtain better column efficiency in HILIC.

The peak asymmetry, obtained for the same samples as in Fig. 10, was plotted against the *k* in Fig. 11. The Halo HILIC phase tends to give significant peak tailing. CYCLOBOND I also caused peak tailing for the less retained solutes, U, 5MU and A. Except for these phases, the HILIC phases with weak retentions provided good peak asymmetry. On the other hand, HILIC phases with strong retention tended to give peaks with tailing. For the test solutes, ZIC-HILIC gave peak asymmetry values >1.2, whereas Nucleodur HILIC exhibited peak fronting with an asymmetry <1. For these phases, the linear velocity might not be optimized, and the ZIC-HILIC phase would give better results when separations were carried out with a slower flow rate. Among the set of the columns tested, Amide-80 (3 μm) provided acceptable peak asymmetry. Although they were excluded from the plot, PolyHYDROXYETHYL A and PolySULFOETHYL A sometimes showed significant peak tailing.

### 3.8. Principal component analysis

The collected data matrix of the HILIC test (Tables 2–6) has been processed by principal component analysis (PCA) to obtain a profiling plot. The data sets of retention factor, *k*, and selectivity, $\alpha$, were employed, and these values for AX and CX selectivity in ACN–ammonium acetate buffer (pH = 4.7, 100 mM in the aqueous portion) (90:10, v/v) were chosen, since data in 20 mM buffer contained negative *k* values, probably due to electrostatic repulsion (Table 6). This method for comparing similarity and differences between HILIC or mixed mode phases was carried out by Lämmerhofer [27] and Elfakir [15] and their co-workers. The values of selectivity, $\alpha$, were in a relatively small range, except for AX

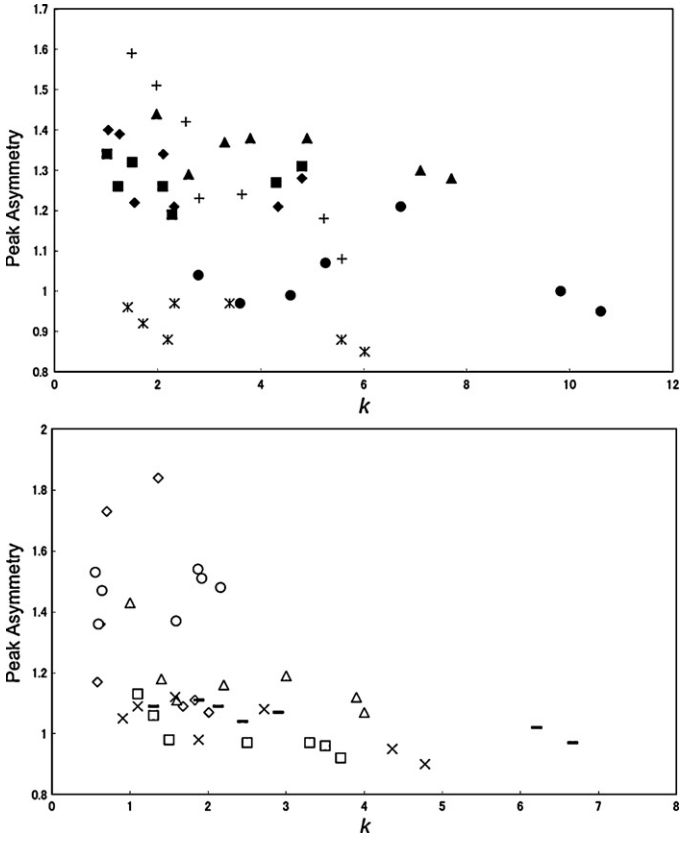


**Fig. 11.** Relationship between retention and peak asymmetry. Marks for columns are the same to Fig. 10.

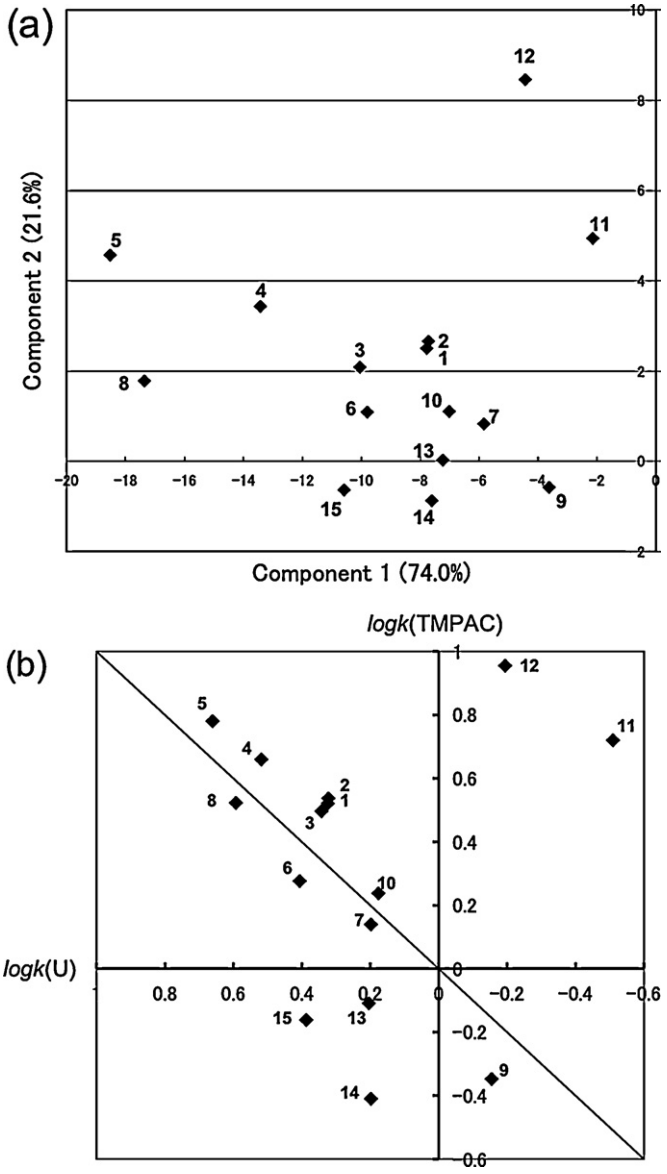


**Fig. 12.** Profiling of HILIC phases. (a) PCA profiling plot of the two principal components for the retention factor. (b) The log $k$(U)–log $k$(TMPAC) plot for the same data set as (a). The data set for TMPAC and SPTS was obtained in ACN–ammonium acetate buffer (100 mM in the aqueous portion, pH 4.7) (90:10, v/v), and other data was collected in ACN–ammonium acetate buffer (20 mM in the aqueous portion, pH 4.7) (90:10, v/v). Column: 1, ZIC-HILIC (5 μm); 2, ZIC-HILIC (3.5 μm); 3, Nucleodur HILIC (3 μm); 4, Amide-80 (5 μm); 5, Amide-80 (3 μm); 6, XBridge Amide (3.5 μm); 7, PolySULFOETHYL A (3 μm); 8, PolyHYDROXYETHYL (3 μm); 9, CYCLOBOND I (5 μm); 10, LiChrospher Diol (5 μm); 11, Chromolith Si; 12, Halo HILIC (2.7 μm); 13, COSMOSIL HILIC (5 μm); 14, Sugar-D (5 μm); 15, $NH_2$-MS (5 μm).

and CX selectivity; thus, these two components, (ion-exchange interaction) having significant covariance, were not so useful for categorizing these tested phases. When retention factor, $k$, was used in the data set, the two-dimensional profiling plot exhibited clusters of different categories of HILIC phases, as shown in Fig. 12a. The two principle components were found as $k$(U) (PC1) and $k$(TMPAC) (PC2) values, with the percentage of variance in the data matrix calculated as 74.0 (PC1) and 21.6% (PC2), respectively. It was apparent that there are at least three clusters of stationary phases, such as bare silica (in the upper right corner), amide-bonded phases (middle left), and amino-bonded phases (bottom). From the direction of right to left, $k$(U) value became greater, thus more hydrophilic, and from the direction of bottom to top, $k$(TMPAC) value became greater, with cation-exchange more prominent. Several phases such as ZIC-HILIC, LiChrospher Diol, and PolySULFOETHYL A were located in the middle of these three clusters. This plot is dimensionless, and a more concrete way of obtaining a scatter plot would be a log $k$–log $k$, with a consideration of these principal components. The log $k$(U) was plotted against log $k$(TMPAC) in Fig. 12b, with the direction of the axis arranged as in Fig. 12a. It is obvious that many phases fall on the line log $k$(TMPAC) = log $k$(U), with amine-based phases located in the lower area from the line, and bare silica phases located in the far upper area from the line. These two scatter plots are consistent with each other, and this kind of profiling is thus shown to be useful for categorizing HILIC phases.

### 3.9. Radar plots of the stationary phases

To classify the stationary phases and to illustrate the characteristics of each phase visually, radar shaped graphs were used in some cases [21,23]. Different from PCA, these plots are useful for expressing multi-dimensional data in a two-dimensional form. Even in the case of C18 modified stationary phases, there are significant differences in their radar plots. Among several patterns of data sets, $\alpha$(SPTS/U) and $\alpha$(TMPAC/U) collected in ACN–ammonium acetate buffer (pH = 4.7, 20 mM in the aqueous portion) (90:10, v/v) were shown to be useful to distinguish the selectivity of the HILIC phases. In the case of data in 100 mM buffer, the radar plot seemed similar except for several phases with very strong AX and CX properties. Here, HILIC phases modified with different functional groups were compared using similar plots in Fig. 13. To show the degree of selectivity, the $\alpha$ values were normalized to 1.0 as the greatest value, except for AX and CX axes, where the average values of $\alpha$(SPTS/U)

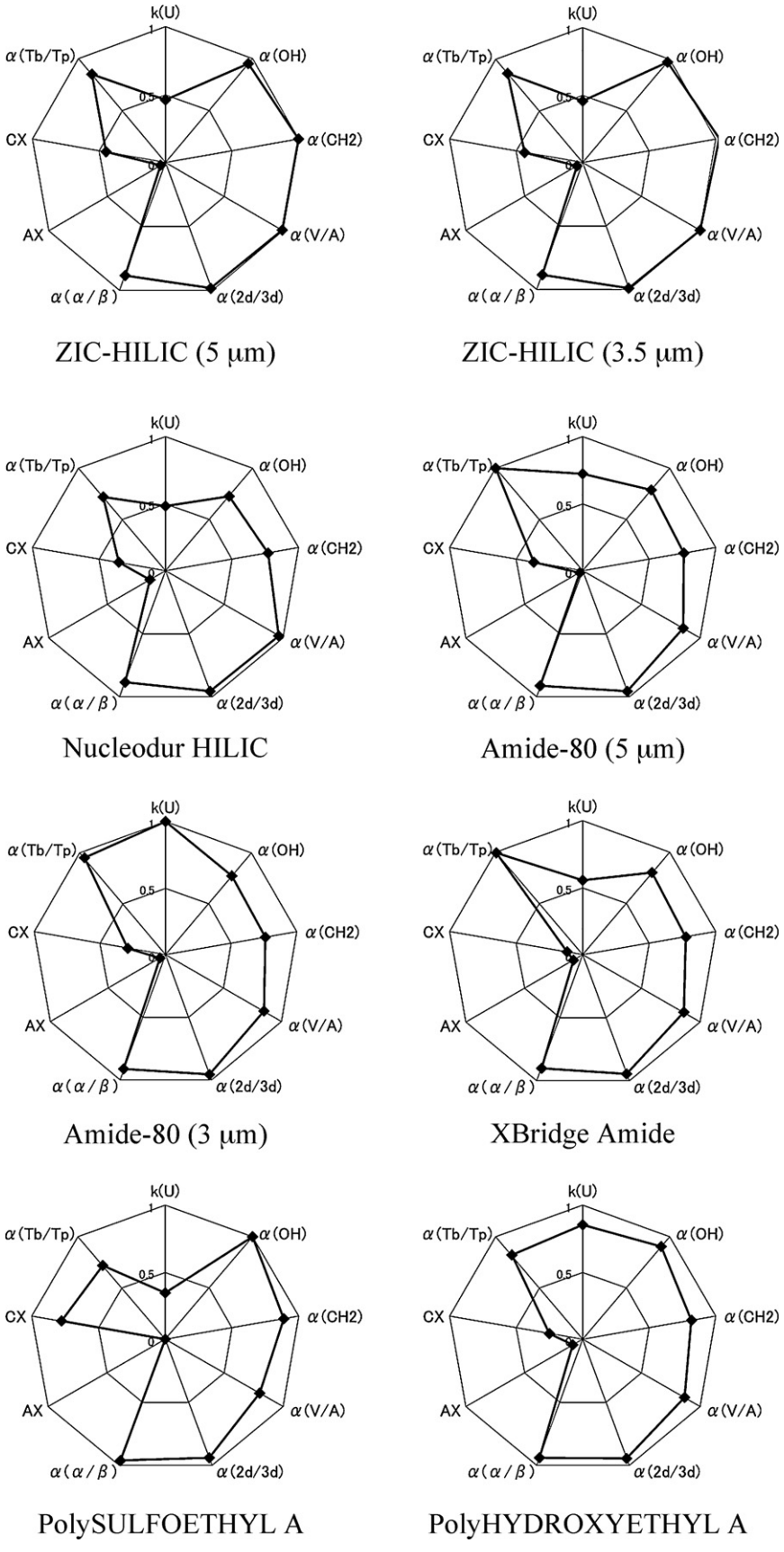


**Fig. 13.** Radar plots of HILIC phases. All data in the set was collected in ACN–ammonium acetate buffer (20 mM in the aqueous portion, pH 4.7) (90:10, v/v).

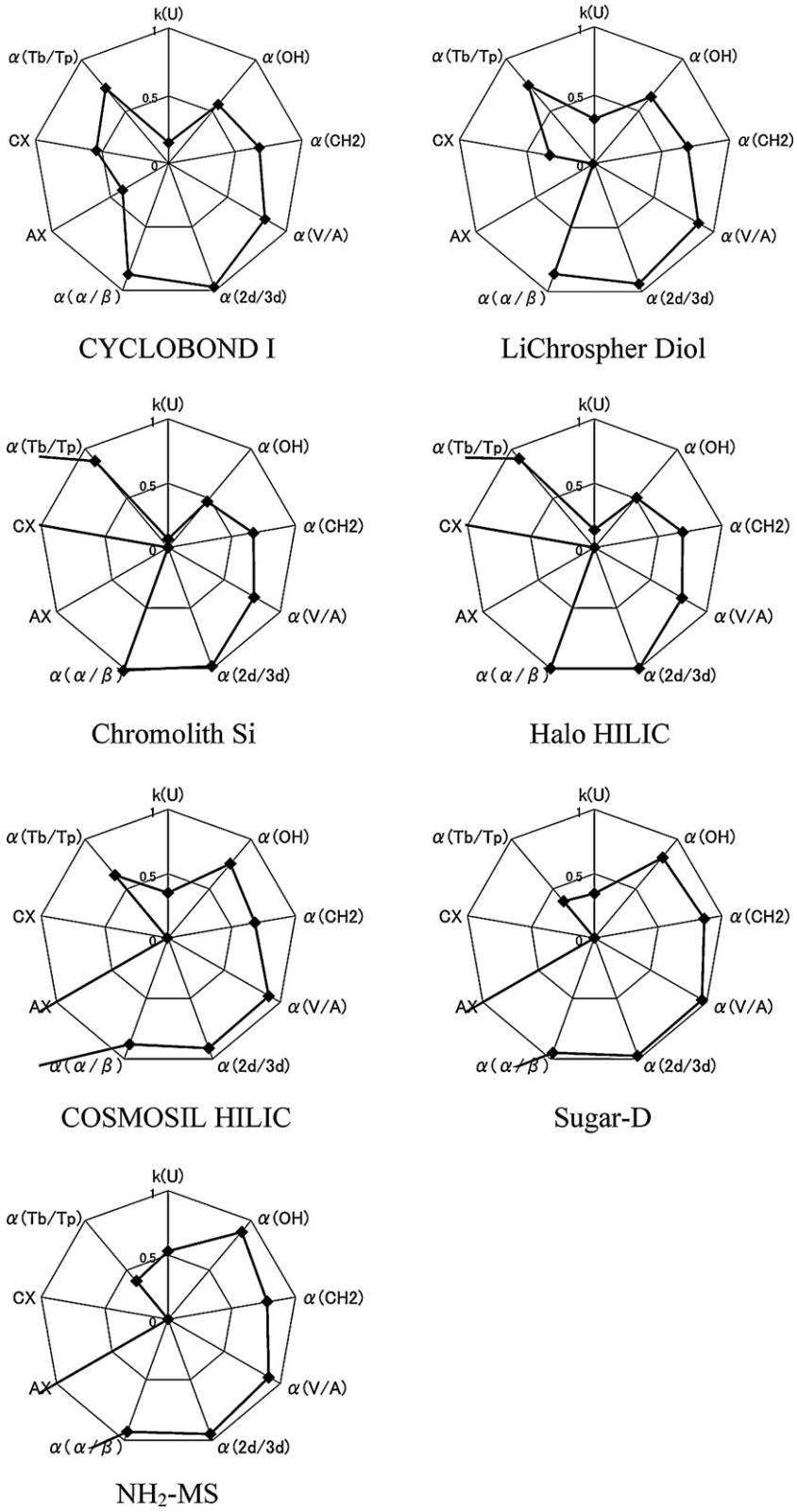


**Fig. 13.** (*Continued*).

and $\alpha$(TMPAC/U) were estimated as 1.0; thus, values larger than 1.0 were allowed.

As expected from the results on the C18 phases, pairs of phases with similar functional groups, such as ZIC-HILIC and Nucleodur HILIC, Amide-80 and XBridge Amide, Chromolith Si and Halo HILIC, and COSMOSIL, $NH_2$-MS and Sugar-D, did not generate the same shapes on the radar plots. The degree of the ion exchange interactions had a significant influence on the shapes of the plots. The HILIC phases tested here can be roughly separated into two groups. Phases containing amides, sulfonates, and zwitterionic groups, i.e. double bonds between carbon and hetero atoms, or between hetero atoms and hetero atoms, these being hydrogen-bond acceptors,

showed greater retention due to hydrophilic interactions, and better selectivity for the test compounds. On the other hand, phases containing hydroxy and amino groups, hydrogen-bond donors, and no functionality except for silanols, showed relatively low retention with little selectivity.

If the target compounds do not contain ion-exchanging functional groups, HILIC phases functionalized with amide, sulfonates, and zwitterionic groups should be employed for better separation. Special care should not be required, so that more retentive phases should be useful to provide sufficient retention with high selectivity. When samples are acidic, then the use of basic stationary phases will decrease the separation efficiency due to ion-exchange interactions. For the separation of these compounds, neutral phases would be better as long as retention is adequate. Diol-bonded or cyclodextrin-bonded phases would be acceptable if these phases provided adequate retention over ACN concentrations of 70–95%, and amide-bonded phases should be useful as well. The PolyHYDROXYETHYL A phase could be another choice, even though gradient separation is preferred in this case. The manner of column selection is basically similar to a routine method [15] as long as electrostatic effects are taken into account.

In samples with basic functionalities such as amino groups, controlling the pH of the mobile phase becomes very important to provide good separation with reproducibility. The use of bare silica phases or sulfonate-functionalized phases should be avoided to reduce acid–base interaction and CX interactions in addition to the hydrophilic partitioning. In column characterization using solutes with an ion-exchange nature, diol-bonded phases and silica phases are unique in terms of their separation behavior, and ZIC-HILIC lies somewhere between the diol and silica phases [15]. This finding is somewhat different from the present results, probably due to the presence of ion-exchange interactions in their experiments that would cause significant peak tailing.

## 4. Conclusions

HILIC phases have been characterized here in terms of selectivity for methylene ($CH_2$) and hydroxy (OH) groups, regio and configurational isomers, and molecular shapes. Ion-exchange interactions were examined with consideration for their reduction, since they can cause a decrease in separation efficiency. The pH conditions of the HILIC phases were also tested using a theobromine/theophylline sample set; $\alpha(k_{\text{theobromine}}/k_{\text{theophylline}})$ could be used as an index to determine if the phases were acidic, basic, or neutral. Judging from these tests, the HILIC phases could be separated into several groups in terms of their degree of hydrophilicity. Strongly hydrophilic phases include amide-bonded phases and zwitterionic phases; these types of columns are useful in terms of their selectivity. In particular, the zwitterionic phase ZIC-HILIC showed better selectivity for the test samples than the other phases. The amide-bonded phases exhibited little selectivity for regio and configurational isomers. Other phases such as cyclodextrin-, diol-, triazol- (COSMOSIL HILIC phase), amino-bonded phases and bare silica phases can be categorized as weakly hydrophilic phases. These phases exhibited less retention for the test samples under the conditions than amide-, zwitterionic-, and PolyHYDROXYETHYL A phases. As compared to the amine-bonded phases, the functionalization of stationary phases with hydroxy groups resulted in less retentive and less selective HILIC phases. This clustering of HILIC phases was also derived from PCA.

These findings were summarized as radar graphs; these graphs indicated the degree of the selectivity of the functional groups, and their structural differences. Not only that, they suggest the possibility of ion-exchange interactions that increase retention with loss of separation efficiency. These graphs will be helpful in choosing stationary phases for HILIC separation.

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