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Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography

Yong Guo *, Sheetal Gaiki

Global Chemical and Pharmaceutical Development, Johnson & Johnson Pharmaceutical Research and Development, L.L.C., 1000 Route 202, P.O. Box 300, Raritan, NJ 08869, USA

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Abstract

Retention characteristics of four polar stationary phases (i.e., amide, amino, silica and sulfobetaine) were studied by using a group of small polar compounds in hydrophilic interaction chromatography (HILIC). Different polar stationary phases shared certain degrees of similarity, but also exhibited differences in retentivity and selectivity for the model compounds. Among the four columns studied, HILIC Silica column had the least retention for the model compounds, but also showed different selectivity from other three columns. Experimental data also provided some evidences that functional groups on the stationary phases might have certain degrees of influence on selectivity possibly through secondary interactions with the model compounds. The retention of the acids on the amino phase decreased with increasing salt concentration in the mobile phase due to the ion-exchange effect, and the retention process was endothermic as opposed to exothermic on other phases. This study also systematically investigated the effect of various experimental factors on the retention of the polar stationary phases, such as acetonitrile content, column temperature, buffer pH, salt type and concentration in the mobile phase.

Keywords: HILIC; Stationary phase; Retention; Selectivity; Aspirin; Cytosine

1. Introduction

Small polar compounds are often very challenging to method development due to lack of retention on conventional reversed-phase columns. Although very effective in separating polar compounds, normal-phase liquid chromatographic (NPLC) methods are generally not desirable for routine QC applications in the pharmaceutical industry due to poor reproducibility and difficulty in interfacing with mass spectrometric (MS) detectors. The so-called "polar embedded" or "polar end-capped" stationary phases can enhance the retention of polar compounds with a mostly aqueous mobile phase in reversed-phase liquid chromatography (RPCL), but this approach is not feasible for the compounds with low aqueous solubility. In addition, the sensitivity of MS detectors may be significantly reduced with low level of organic solvents in the mobile phase in LC/MS experiments. Hydrophilic interaction chromatography (HILIC) provides an alternative approach to effectively separate small polar compounds on polar stationary phases. Similar to NPLC, polar compounds are more strongly retained in HILIC, but non-aqueous mobile phase in NPLC is replaced with an aqueous–organic mixture with water being the stronger solvent [1]. This feature not only helps to eliminate the problem associated with low aqueous solubility, but also makes HILIC more amenable to MS detection and improves the MS sensitivity [2–6]. Early HILIC applications mainly focused on the area of carbohydrates and peptides analysis [1,7–17]. Recent applications have expanded to include many small polar compounds, such as drugs, toxins, plant extracts, and other compounds important to food and pharmaceutical industries [2–6,18–26].

HILIC separation employs polar stationary phases such as silica, amino or cyano phases traditionally used in NPLC [2,4,6,13,18,21]. Other types of stationary phases such as

* Corresponding author. Tel.: +1 908 704 4309; fax: +1 908 704 1612.
E-mail address: yguo2@prdus.jnj.com (Y. Guo).

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amide and aspartamide have also been used for small polar compounds [1,3,6,10,14,17,22,26,27]. More recently, some column manufacturers have started to market stationary phases specifically designed for HILIC, notably, Atlantis HILIC Silica and ZIC HILIC columns [2,25]. The availability of different stationary phases provides analytical chemists with more choices to meet specific needs; at the same time it is also challenging to choose the "right" column for the compounds of interest. Most literature reports on HILIC focused on a single column, and only a few studies evaluated multiple columns for the purpose of selecting the appropriate column for the compounds of interest [3,4,6,21]. Obviously, there is a need for more studies on different types of polar stationary phases in order to gain better understanding of HILIC separation.

A major objective of this study was to investigate the retention characteristics of polar stationary phases and hopefully gain some insights into chromatographic process of HILIC. To this end, four different polar stationary phases were selected including amide, amino, silica, and sulfobetaine that contained different functional groups on the silica surface. A group of polar compounds including salicylic acid and derivatives, some nucleosides and nucleic acid bases were selected as the model compounds because they were traditionally very difficult to retain on reversed-phase columns.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile was obtained from EM Science (Hawthorne, NY), and HPLC grade water from a Millipore Milli-Q Gradient purification system (Bedford, MA). Ammonium salts (i.e., acetate, formate and bicarbonate) were of ACS grade from Aldrich (Milwaukee, WI). Salicylamide, salicylic acid, 4-aminosalicylic acid, acetylsalicylic acid (aspirin), 3,4-dihydroxyphenylacetic acid, uracil, adenosine, cytosine, guanosine, uridine and cytidine were obtained from Aldrich. YMC-Pack NH_2 and Atlantis HILIC Silica columns were purchased from Waters (Milford, MA). TSKgel Amide-80 column was obtained from Tosoh Bioscience (Montgomeryville, PA), and ZIC HILIC column was purchased from SeQuant AB (Sweden) through The Nest Group (Southborough, MA). All columns were equilibrated with mobile phases prior to use and washed with acetonitrile and water (60/40, v/v) after use.

2.2. Equipment and method

All experiments were conducted on an Agilent HPLC system (Model HP1100), which was equipped with a quaternary pump (Model G1311A), a DAD detector (Model G1315B), a degasser (Model G1322A), a column heater (Model G1316A), and an auto-injector (Model G1313A). The ChemStation software (Rev. A.09.01) was used for data acquisition and analysis.

Mobile phases were prepared by mixing appropriate volumes of acetonitrile and stock salt solutions to reach desired organic levels and salt concentrations. The stock solution of buffer salt was made by dissolving appropriate amounts of various salts (e.g., ammonium acetate, formate and bicarbonate) in water. The pH of the salt solution was not adjusted before mixing with acetonitrile in most cases, except for the pH effect study. In this case, a stock solution of 100 mM ammonium formate was first prepared (pH ~ 6.5), then the pH of the stock solution was adjusted to desired pH values (pH 4.8, 4.0 and 3.3) by adding formic acid. The salt concentration in the text refers to the final concentration of the salt in the mobile phase.

The acid mixture including salicylamide was prepared by dissolving the acids in acetonitrile/water (90/10, v/v) at the following concentrations: salicylamide ~0.5 mg/mL, salicylic acid ~1 mg/mL, 4-aminosalicylic acid ~1 mg/mL, aspirin ~1 mg/mL, and 3,4-dihydroxyphenylacetic acid ~2 mg/mL. The nucleoside/base mixture was prepared at ~1 mg/mL in acetonitrile/water (80/20, v/v).

3. Results and discussion

3.1. Selectivity of polar stationary phases

Four commercially available columns representing different polar stationary phases were selected for this study. The columns are all silica-based and have the same dimensions and similar basic properties in terms of particle size, pore size and surface area as presented in Table 1, except that the ZIC HILIC column has a relatively larger pore size and smaller surface area. The surface chemistry of the stationary phase in these columns, however, is very different. The HILIC Silica column contains unmodified silica phase, which unlike that used in NPLC, is packed and stored in acetonitrile. The YMC-Pack NH_2 column has an aminopropyl phase without endcapping and is also stored in a mixture of water and ace-

Table 1
Properties of polar stationary phases

Column name	Phase type	Dimension (mm)	Particle size (μm)	Surface area (m^2/g)	Pore size (Å)
HILIC Silica	Silica	4.6 × 250	5	345	93
YMC-Pack NH_2	Amino	4.5 × 250	5	335	120
TSKgel Amide-80	Amide	4.6 × 250	5	313	80
ZIC-HILIC	Sulfobetaine	4.6 × 250	5	135	200

tonitrile instead of normal-phase solvents (e.g., hexane). The amide phase in the TSKgel Amide-80 column consists of carbamoyl groups attached to the silica surface through an aliphatic carbon chain [27]. In addition, the stationary phase in the ZIC-HILIC column is zwitterionic with sulfobetaine groups covalently attached to the silica surface [28].

A group of salicylic acid derivatives was separated on the four columns under HILIC conditions, as shown in Fig. 1. All the components in the acid mixture were well resolved, but the retention and elution order varied from column to column. The acids were most retained on the YMC-Pack NH_2 column; however, all peaks were relatively broad and had small tailing. Since the amino phase was positively charged under the mobile phase condition, it was possible that the negatively charged acids might interact with the amino phase through ion exchange, resulting in stronger retention as well as different selectivity towards the acids. The ion-exchange effect of the amino phase on the acidic compounds has also been observed by Olsen [21]. In comparison, the acids had weaker retention on the TSKgel Amide-80 column, and aspirin and 4-aminosalicylic acid were only partially resolved. It was also noted that 3,4-dihydroxyphenylacetic acid eluted as a split peak with a severe tailing. The fact that peak splitting was only observed on the amide phase indicated that this phenomenon might be specific to the amide phase, possibly due to some secondary interactions with the functional groups. The resolution of aspirin and 4-aminosalicylic acid was much improved on the ZIC-HILIC column, where aspirin was less retained and 4-aminosalicylic acid was more retained than on the TSKgel Amide-80 column. This might be the result of electrostatic interaction between the acids and negatively charged sulfonate groups on the sulfobetaine phase. In comparison, the HILIC Silica column had the least retention for the acids, however, the elution order was very similar to that on the YMC-Pack NH_2 column. Different elution patterns of the acids on the four columns indicated that the polar stationary phases had significant difference in retention and selectivity. Specific interactions between the acids and surface functional groups were most likely responsible for the selectivity difference, for example, the ion-exchange effect on the YMC-Pack NH_2 column and electrostatic effect on the ZIC-HILIC column.

Another test mixture consisting of uracil, cytosine, adenosine, cytidine, guanosine and uridine was also separated on four HILIC columns under the same HILIC conditions. As shown in Fig. 2, the model compounds displayed stronger retention on the TSKgel Amide-80 column than on the YMC-Pack NH_2 column, but elution patterns were very similar with adenosine and uridine co-eluting on both columns. In comparison, the ZIC-HILIC column showed less retention for the model compounds, but completely resolved adenosine and uridine. It was also interesting to note that the HILIC Silica column was least retentive among the four columns; however, all the components in the mixture were nearly baseline separated with an elution pattern quite different from that on other three columns. The elution order of adenosine and uridine was reversed and guanosine eluted even before cytosine, indicating different selectivity of the silica phase. Surface silanol groups have been shown to influence the retention and selectivity of nucleosides and nucleic acid bases on silica based reversed-phase columns [29]. It was reasonable to assume that the silanol groups might also interact with the model compounds in HILIC, thus leading to the unique selectivity of the silica phase. More studies are in progress to further understand the interactions on the silica phase.

3.2. The effect of acetonitrile content on retention

Similar to reserved-phase separation, HILIC separation commonly employs water and acetonitrile as the mobile phase, but requires much higher organic content (>60%) to ensure significant hydrophilic interaction [1]. The level of organic solvent in the mobile phase is probably the factor that has the largest influence on retention. In this study, the effect of acetonitrile content on retention was investigated by varying the percentage of acetontrile in the mobile phase while keeping ammonium acetate concentration constant at 5 mM. Low salt concentration was necessary to accommodate low solubility of salt in the mobile phase with high acetonitrile content (e.g., 95%). The logarithmic capacity factors of two model compounds (i.e., aspirin and cytosine) were plotted against the acetonitrile content in the mobile phase for the four columns as shown in Fig. 3. Both aspirin and cytosine exhibited typical HILIC behaviors of decreasing retention with increasing water content in the mobile phase on the TSKgel Amide-80, ZIC-HILIC and HILIC Silica columns. However, aspirin displayed an unusual behavior on the YMC-Pack NH_2 column. Its retention decreased initially as the acetonitrile content decreased from 90 to 80%, but leveled off when the acetonitrile content further decreased to 65%. Similar behaviors were also observed for other acids. This unusual behavior of the acids on the YMC-Pack NH_2 column was probably related to the ion-exchange interaction between the acids and the amino phase.

3.3. The effect of column temperature on retention

Column temperature is also an important parameter that affects the retention of polar compounds in HILIC separation. The relationship between capacity factor (k') and column temperature (T) in RPLC is often described by van't Hoff equation:

$$\ln k' = -\frac{\Delta H^{o}}{RT} + \frac{\Delta S^{o}}{R} + \ln \phi$$

where ΔH^{o} and ΔS^{o} are retention enthalpy and entropy, R gas constant and ϕ phase ratio. If the retention of polar compounds in HILIC is through partitioning between the mostly organic mobile phase and a water-rich liquid layer on the packing surface as proposed by Alpert [1], the van't Hoff equation should apply to HILIC. In this study, the temperature effect on retention was investigated by varying column

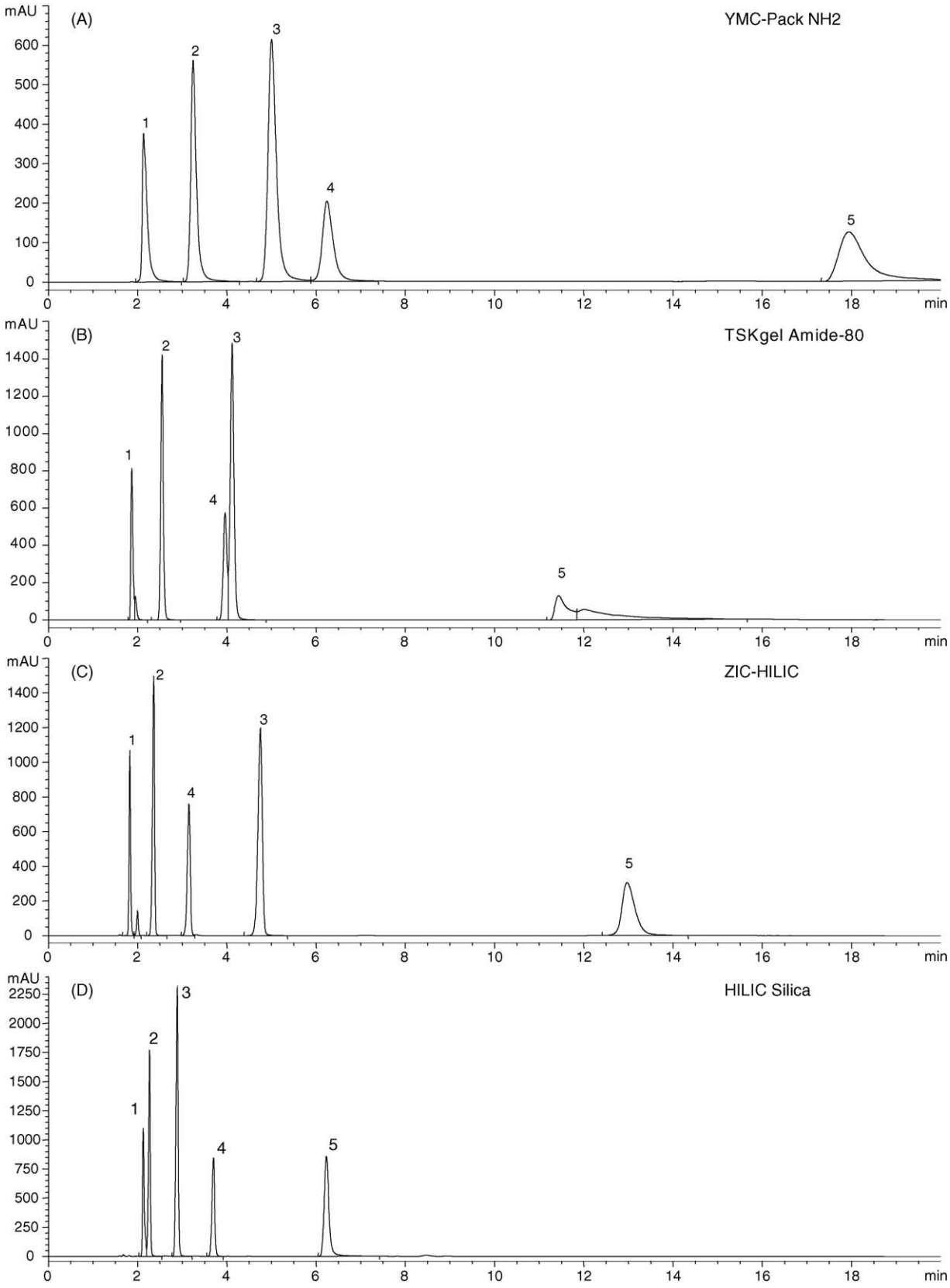

Fig. 1. Separation of acidic compounds on: (A) YMC-Pack NH_2; (B) TSKgel Amide-80; (C) ZIC-HILIC; and (D) HILIC Silica columns. Mobile phase: acetonitrile/water (85/15, v/v) containing 20 mM ammonium acetate. Column temperature: 30 °C. Flow rate: 1.5 mL/min. UV detection at 228 nm. Compounds: (1) salicylamide, (2) salicylic acid, (3) 4-aminosaliclyic acid, (4) acetylsalicylic acid, and (5) 3,4-dihydroxyphenylacetic acid.

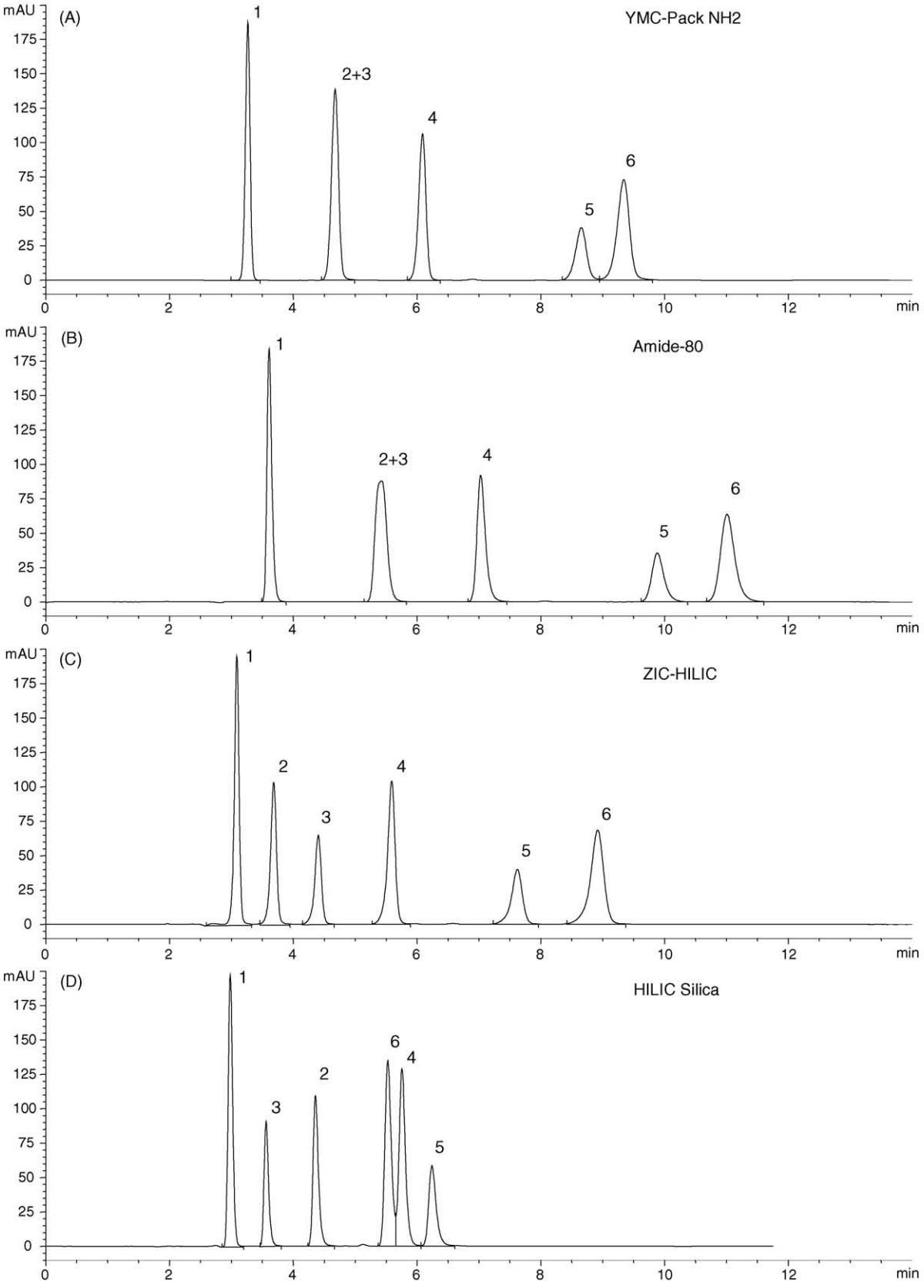

Fig. 2. Separation of nucleic acid bases and nucleosides on: (A) YMC-Pack NH_2, (B) TSKgel Amide-80, (C) ZIC-HILIC, and (D) HILIC Silica columns. Mobile phase: acetonitrile/water (85/15, v/v) containing 10 mM ammonium acetate. Column temperature: 30 °C. Flow rate: 1.5 mL/min. UV detection at 248 nm. Compounds: (1) uracil, (2) adenosine, (3) uridine, (4) cytosine, (5) cytidine, and (6) guanosine.

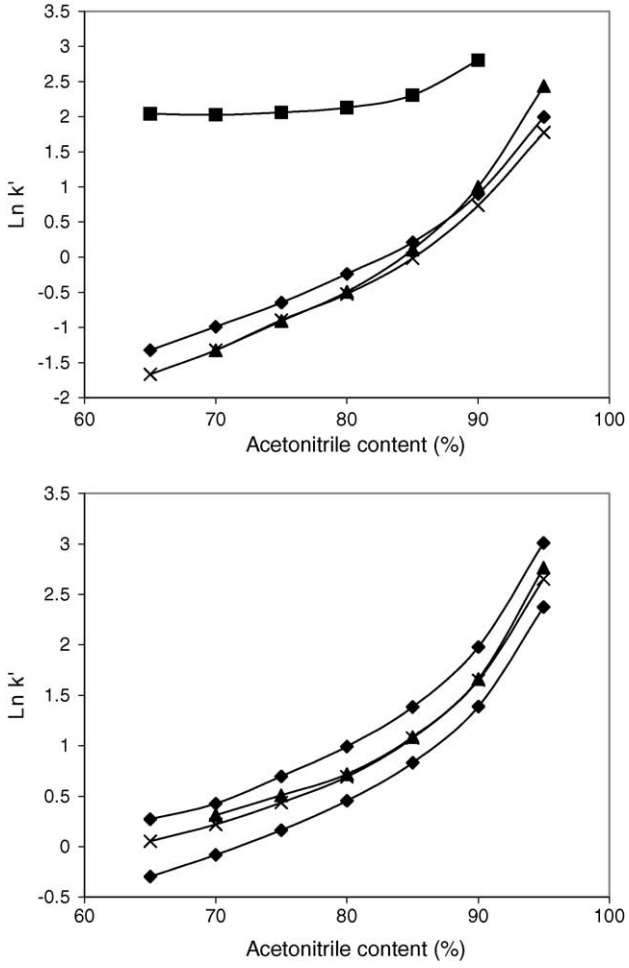

Fig. 3. The effect of acetontrile content on the retention of aspirin (top) and cytosine (bottom) on (♦) TSKgel Amide-80, (■) YMC-Pack NH_2, (▲) HILIC Silica, and (×) ZIC-HILIC columns. Column temperature was 30 °C and the mobile phase contained 5 mM ammonium acetate.

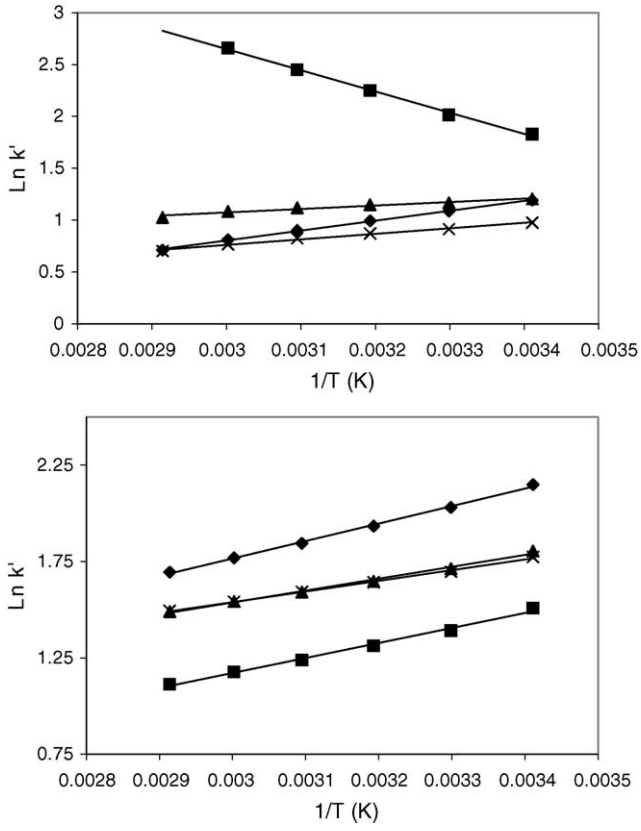

Fig. 4. The van't Hoff plots for aspirin (top) and cytosine (bottom) on (♦) TSKgel Amide-80, (■) YMC-Pack NH_2, (▲) HILIC Silica, and (×) ZIC-HILIC columns. Mobile phase: acetontrile/water (90/10, v/v) containing 10 mM ammonium acetate.

temperature from 20 to 70 °C, and the retention data for the model compounds were used to construct van't Hoff plots for the four columns. Fig. 4 shows the van't Hoff plots for aspirin and cytosine on the four columns. A decrease in retention was observed for cytosine on all four columns and for aspirin on three columns as the column temperature increased. Interestingly, the retention of aspirin increased with the temperature on the YMC-Pack NH_2 column. This phenomenon was also observed for other acidic compounds (e.g., salicylic acid and 4-aminosalicylic acid), but not for nucleosides and nucleic acid bases. In addition, a linear relationship between $\ln k'$ and $1/T$ was observed in the temperature range of 20–70 °C for the model compounds on all four columns with r^2 ranging from 0.960 to 0.998. This is similar to what is usually observed in RPLC [30,31] even though non-linear van't Hoff plots have also been reported and attributed to the temperature dependency of both enthalpy (ΔH^o) and phase ratio (ϕ) in RPLC [32,33]. It is not clear whether non-linear behavior exists in HILIC based on the data from this study. More detailed studies in a wider temperature range are currently under way to gain better understanding of thermodynamics in HILIC.

Based on the slope of the van't Hoff plots, the retention enthalpy values were calculated for three model compounds on the four columns as presented in Table 2. With the exception of salicylic acid and aspirin on the YMC-Pack NH_2 column, all three model compounds exhibited negative retention enthalpy ranging from −0.3 to −8.0 kJ/mol on the four columns, indicating an exothermic process of transfer-

Table 2
Retention enthalpy of test compounds on different columns[a]

	YMC-Pack NH_2	TSKgel Amide-80	HILIC Silica	ZIC-HILIC
Salicylic acid	21.0 ± 1.2	−6.2 ± (−0.4)	−7.9 ± (−0.3)	−0.3 ± (−0.2)
Aspirin	7.7 ± 0.1	−8.0 ± (−0.2)	−2.8 ± (−0.3)	−4.4 ± (−0.2)
Cytosine	−5.0 ± (−0.1)	−7.5 ± (−0.2)	−5.1 ± (−0.2)	−4.6 ± (−0.1)

[a] Mobile phase: acetonitrile/water (90/10, v/v) with 10 mM ammonium acetate.

Table 3
Retention time of the model compounds with different ammonium salts[a]

Column	Ammonium Salt	Salicylic acid (min)	Aspirin (min)	Cytosine (min)
TSKgel Amide-80	Acetate	2.39	3.65	7.19
	Formate	2.47	3.64	7.05
	Bicarbonate	1.39	1.68	6.99
YMC-Pack NH_2	Acetate	4.78	11.56	6.04
	Formate	6.26	16.35	6.26
	Bicarbonate	2.51	4.57	5.87
HILIC Silica	Acetate	2.06	3.51	5.78
	Formate	2.16	3.82	5.59
	Bicarbonate	1.28	1.57	5.87
ZIC-HILIC	Acetate	2.44	3.22	5.59
	Formate	2.50	3.28	5.61
	Bicarbonate	1.37	1.56	5.63

[a] Mobile phase: acetonitrile/10 mM ammonium salt solution (85/15, v/v). Column temperature: 30 °C. Flow rate: 1.5 mL/min.

ring solutes from the mobile phase to the stationary phase. Despite of large differences in stationary phase structure, the enthalpy values of the model compounds were similar on different columns, except salicylic acid on the ZIC-HILIC column (−0.3 kJ/mol). These data might imply that there were not very strong specific interactions between the solutes and functional groups on the stationary phases in HILIC. This interpretation is consistent with the retention model proposed by Alpert [1]; however, more data are needed to extract meaningful information regarding the retention mechanism in HILIC. In addition, positive retention enthalpy was found for the acids on the amino phase, indicating an endothermic process of transferring solutes from the mobile phase to the stationary phase. Further investigation also found that the enthalpy value of salicylic acid on the YMC-Pack NH_2 column decreased from 21.0 ± 1.2 kJ/mol to 11.1 ± 1.1 kJ/mol when the acetonitrile content was reduced from 90 to 75% (v/v) at 10 mM ammonium acetate. The enthalpy value dropped further to 7.6 ± 0.2 kJ/mol upon increasing the salt concentration to 40 mM in 75% acetonitrile. The positive enthalpy of salicylic acid was another evidence for the ion-exchange effect of the amino phase, and both ion-exchange and hydrophilic partition contributed to the retention of the acids on the amino phase. The large decreases in the enthalpy value of salicylic acid possibly reflected the change in the degree of relative contribution to the overall retention from two types of interactions.

3.4. The effect of salt type and concentration

Many salts typically used in reversed-phase chromatography are not suitable for HILIC due to poor solubility in the mobile phase containing high level of acetonitrile. In addition to ammonium acetate, other salts with relatively high solubility at high organic levels have also been used for HILIC, such as ammonium formate and bicarbonate salts, triethylamine phosphate and sodium perchlorate, but the last two are not compatible with MS detection. This study investigated the effect of different ammonium salts, namely, acetate, formate and bicarbonate on the retention of the model compounds on the polar stationary phases. The mobile phases used in this study contained 85% acetonitrile and 10 mM ammonium salts. The pH of the salt stock solution was 6.5 for ammonium formate, 6.9 for ammonium acetate and 7.9 for ammonium bicarbonate and was not adjusted before mixing with acetonitrile. Table 3 shows the retention time of the model compounds (i.e., salicylic acid, aspirin and cytosine) on the four columns in the mobile phases containing three ammonium salts. For cytosine, there was virtually no change in the retention time using different ammonium salts on all four columns. However, different ammonium salts had significant different impact on the retention time of the acids on different columns. For example, replacing the acetate with formate salt resulted in little change in the retention time of the acids on the TSKgel Amide-80, HILIC Silica and ZIC-HILIC columns, but led to a significant increase in the retention time on the YMC-Pack NH_2 column possibly due to different eluting strength of the competing ions (formate versus acetate) in the ion-exchange interaction. In addition, ammonium bicarbonate in the mobile phase caused drastic decrease in the retention time of the acids on all the columns, leading to deteriorated separation. Ammonium bicarbonate has been shown to cause a decrease in the retention time on the amide phase [6]; however, it was a little surprising that the bicarbonate salt destroyed the separation of the acids

In addition to salt type, the effect of salt concentration on the retention was also investigated by varying ammonium acetate concentration from 5 to 20 mM in the mobile phase of acetonitrile/water (85/15, v/v). Further increase in the salt concentration was not possible due to solubility limitation in the mobile phase. The retention time of three model compounds (i.e., aspirin, salicylic acid and cytosine) was obtained on the four columns at three concentration levels, as presented in Table 4. For aspirin and salicylic acid, the retention time increased by about 20–40% on the TSKgel Amide-80, HILIC Silica and ZIC-HILIC columns, but decreased sharply on the YMC-Pack NH_2 column. As discussed in previous sections, the ion-exchange interaction on the amino phase contributed

Table 4
Retention time of the model compounds at different ammonium acetate concentrations in the mobile phase[a]

Column	Concentration (mM)	Salicylic acid	Aspirin	Cytosine
TSKgel Amide-80	5	2.07	3.06	6.84
	10	2.39	3.65	7.19
	20	2.61	4.14	8.01
YMC-Pack NH_2	5	7.59	20.21	6.03
	10	4.72	11.50	6.10
	20	3.56	7.17	6.45
HILIC Silica	5	1.78	2.94	5.51
	10	2.06	3.51	5.78
	20	2.49	4.21	6.62
ZIC-HILIC	5	2.16	2.78	5.52
	10	2.44	3.22	5.59
	20	2.64	3.55	5.98

[a] Mobile phase: acetonitrile/ammonium acetate solution (85/15, v/v). Column temperature: 30 °C. Flow rate: 1.5 mL/min.

significantly to the retention of the acids. Higher salt concentrations increased the eluting strength of the mobile phase, thus leading to decreasing retention. Interestingly, no ion-exchange effect was observed for the acids on the ZIC-HILIC column, which contains positively charged quaternary amine groups and has been used for the separation of inorganic anions by ion chromatography [28]. It was possible that unlike smaller inorganic anions, electrostatic repulsion from negatively charged sulfonate groups on the sulfobetaine phase prevented the relatively lager acid molecules from reaching the quaternary amine groups located closer to the silica surface. Meanwhile, higher salt concentrations weakened the electrostatic repulsion, thus leading to stronger retention of the acids. The electrostatic repulsion could potentially explain the retention increase on the HILIC Silica column since the silica phase was negatively charged due to deprotonation of surface silanol groups under the experimental condition [34]. Theoretically, the amide phase could also bear negative charges due to ionization of residual surface silanol groups. However, the electrostatic interaction with the acids would be much weaker on the amide phase, compared to the silica or sulfobetaine phases. Nevertheless, the increase in retention on the percentage basis was very similar on the amide and silica phases. This indicated that the electrostatic repulsion was probably not the only factor that caused the increase in retention. In addition, the data in Table 4 indicated that increasing salt concentration resulted in a smaller, but significant (8–20%) increase in the retention time of cytosine on all four columns. The electrostatic effect was not applicable since cytosine was not charged in the mobile phase. The fact that the retention increase was observed on the stationary phases with different functional groups indicated that the retention increase might be related to hydrophilic partitioning process instead of any specific interactions with the functional groups on the stationary phases. The partitioning model for HILIC assumed the presence of a water-rich liquid layer on the packing surface [1]. High levels of organic content in the mobile phase made the salt prefer to be in the water-rich liquid layer. Higher salt concentration would drive more solvated salt ions into the water-rich liquid layer. This would result in an increase in volume or hydrophilicity of the liquid layer, leading to stronger retention of the solutes.

3.5. The effect of buffer pH

Mobile phase pH can have very significant impact on retention and selectivity in reversed-phase chromatography by influencing solute ionization in the mobile phase. Similarly, mobile phase pH also plays an important role in affecting selectivity in HILIC. The effect of the mobile phase pH on the HILIC separation was investigated in this study by changing the pH of stock salt solutions before mixing with acetonitrile. Ammonium formate was selected over ammonium acetate for its lower buffering range (pH 2.8–4.8). The pH of the stock solution (100 mM ammonium formate, pH ~ 6.5) was adjusted with formic acid to pH 4.8, 4.0 and 3.3. The apparent pH of the mobile phase was not directly measured, but would presumably deviate from the pH of the stock solution [35]. Table 5 shows the retention time of the model compounds obtained on the four columns in the mobile phase made of formate buffers of various pHs. For salicylic acid with a pK_a ~2.8 [36], the retention time remained essentially unchanged on all the columns because there were no significant changes in ionization in the pH range. However, the retention time of aspirin gradually decreased with the buffer pH on the TSKgel Amide-80, HILIC Silica and ZIC-HILIC columns. Aspirin has a pK_a ~3.5 in water [36] and may be slightly higher in the mobile phase containing high levels of acetonitrile [37]. When the mobile phase was acidic

Table 5
Retention time of the model compounds at different buffer pHs[a]

Column	pH	Salicylic acid	Aspirin	Cytosine	Cytidine
TSKgel Amide-80	6.5	3.18	5.53	11.95	20.78
	4.8	3.18	5.33	11.92	20.74
	4.0	3.23	4.58	12.39	21.88
	3.3	3.16	3.05	12.37	21.52
YMC-Pack NH_2	6.5	6.08	9.77	16.50	17.03
	4.8	5.98	9.69	15.56	16.82
	4.0	5.86	9.98	12.00	17.49
	3.3	5.75	6.57	9.74	16.94
HILIC Silica	6.5	2.66	6.02	8.70	9.71
	4.8	2.64	5.67	8.44	9.25
	4.0	2.68	4.89	8.73	9.53
	3.3	2.64	3.29	8.49	8.91
ZI-HILIC	6.5	2.89	4.49	8.23	13.65
	4.8	2.83	4.25	8.00	13.03
	4.0	2.87	3.71	8.29	13.66
	3.3	2.82	2.59	8.33	13.43

[a] Mobile phase: acetonitrile/10 mM ammonium formate (90/10, v/v). Column temperature: 30 °C. Flow rate: 1.5 mL/min.

enough, aspirin was protonated and became less hydrophilic, thus leading to a decrease in retention. In comparison, the retention time of aspirin remained relatively unchanged in the buffer pH from 6.5 to 4.0, but dropped sharply when the buffer pH was lowered to 3.3 on the YMC Pack-NH_2 column. The slight increase at the buffer pH 4.0 might be due to small differences in the acetonitrile content in the mobile phase as the retention time was very sensitive to the acetonitrile content. As discussed earlier, there was a significant ion-exchange effect on the amino phase. When the mobile phase pH was above its pK_a (3.5), the ion-exchange effect contributed significantly to the overall retention; therefore the retention time did not change significantly in the pH range of 4.0–6.5. However, when the mobile phase pH dropped below its pK_a (3.5), aspirin was protonated and the ion-exchange effect no longer existed, leading to a sharp decrease in retention. For cytosine and cytidine, the retention time only fluctuated slightly on the TSKgel Amide-80, HILIC Silica and ZIC-HILIC columns in the pH range used in this study. However, the retention time of cytosine decreased significantly, especially from pH 4.8 to 4.0 on the YMC Pack-NH_2 column. Cytosine has two pK_a values, pK_{a1} ~4.6 and pK_{a2} ~12.2 in water [36]. At the mobile phase pH below pK_{a1}, cytosine bore positive charges, which induced the electrostatic repulsion from the similarly charged amino groups. Hence, the electrostatic repulsion from the amino phase reduced the retention time of cytosine significantly at lower pH.

4. Conclusion

This study revealed different retention characteristics of four different polar stationary phases in HILIC separation. The amino phase was found to have stronger retention for acidic compounds due to the ion-exchange effect, but showed similar selectivity for nucleosides and bases to the amide phase. On the other hand, the silica phase was least retentive among the four phases and exhibited unique selectivity for the nucleosides and bases possibly due to secondary interactions between the solutes and surface silanol groups. The studies on the effect of buffer salt concentration and pH on retention provided experimental evidences that the ion-exchange effect was responsible for the strong retention of the acidic compounds on the amino phase and was probably related to other unusual phenomena, for example, the flattening $\ln k'$ versus ACN% curve and positive enthalpy values. The study on the effect of buffer salt concentration also found an across-the-board increase in retention with the salt concentration in the mobile phase, except for the acids on the amino phase. This was in sharp contrast to the reversed-phase chromatography, where the retention was usually not significantly affected by the buffer salt concentration, especially for uncharged compounds [38]. Unexplained by any specific interactions with surface functional groups (e.g., electrostatic interaction), the increase in retention time with salt concentration provided an indirect evidence to support the Alpert retention model for HILIC.

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