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Review

Retention and selectivity of stationary phases for hydrophilic interaction chromatography

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ABSTRACT

More and more polar stationary phases have become available for the separation of small polar compounds in the past decade as hydrophilic interaction chromatography (HILIC) continues to find applications in new fields (e.g., metabolomics and proteomics). Bare silica phases remain popular, especially in the bio-analytical area. A wide range of functional groups (e.g., amino, amide, diol, sulfobetaine, and triazole) have been employed as polar stationary phases for HILIC separation. This review provides a survey of the popular stationary phases commercially available and discusses the retention and selectivity characteristics of the polar stationary phases in HILIC. The purpose of the review is not to provide a comprehensive overview of literature reports, but rather focuses on findings that demonstrate retention and selectivity of the polar stationary phases in HILIC.

Contents

1. Introduction

Twenty years after Alpert coined the term hydrophilic interaction chromatography (HILIC) [1], HILIC has been widely recognized as a distinct chromatographic mode and has enjoyed nearly a decade of rapid growth since its potential in separating very polar compounds was rediscovered by the scientific communities in the early 2000s [2–5]. HILIC has been applied to both small and large molecules and is becoming an increasingly important tool in proteomic and metabolomic research [6–8]. From a practical perspective, HILIC offers an attractive alternative to normal phase chromatography (NPC) to separate very polar compounds. The solvent used for the mobile phase in HILIC is similar to that in reversed-phase liquid chromatography (RPLC), thus eliminating the need to maintain dedicated instruments for normal phase methods. Secondly, organic solvents (e.g., acetonitrile) in HILIC mobile phases are more compatible with mass spectrometry, and the high organic content necessary to maintain retention in HILIC

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significantly increases ESI-MS sensitivity due to improved ionization efficiency [9,10]. Thirdly, some biological samples can be directly injected onto HILIC columns without the need for evaporation and reconstitution after protein precipitation with acetonitrile, thus simplifying sample preparation in bio-analysis. In addition, counter ions (e.g., Na^+, K^+, Cl^-, Br^-, amines and acids) that are commonly used to form pharmaceutical salts can also be retained and separated on HILIC columns [11,12]. Thus, counter-ion analysis of pharmaceutical salts can be performed using HILIC columns on regular HPLC instruments instead of using ion-exchange columns on specialized ion-chromatography (IC) instruments.

Along with increasing popularity of HILIC, stationary phases for HILIC have received a lot of attention from both academic researchers and column manufacturers, and many new stationary phases and columns have become commercially available in the past decade [2,13]. In HILIC, stationary phase chemistry is more diversified with a wide variety of functional groups employed (Table 1). Newer stationary phases have the potential to offer different retention and selectivity for polar compounds, and also provide method development chemists with opportunities to find an appropriate stationary phase for the desired separation. At the same time, it is challenging to select the optimal phase in a systematic manner in a short time.

HILIC has been the subject of a few excellent reviews in recent years [2,14–16]. Irgum's review in 2006 was comprehensive with special attention to the HILIC mechanism [2]. Tanaka published a review in 2008 focusing on the efficiency, and to a lesser extent, the retention of various columns used in HILIC [14]. Another review by Hao et al. systematically discussed the impact of column temperature and mobile phase components on the selectivity in HILIC [15]. However, there has not been a topical review focusing on the retention and selectivity of various stationary phases commonly used in HILIC. In light of recent development in HILIC stationary phases, it is fitting to review the subject. This review covers the commercially available stationary phases commonly used in HILIC, but is not intended to be comprehensive. Uncommonly used stationary phases are not included due to the lack of data to make comparison with the more commonly used ones. In this review, the HILIC stationary phases are classified based on the charge characteristics of their functional groups. This classification facilitates comparison of their retention and selectivity. Various retention models for HILIC are also discussed to further understanding of the difference in retention and selectivity of various stationary phases. In addition to the stationary phase, chromatographic parameters (e.g., organic solvent content, mobile phase pH, salt type and concentration, and column temperature) have significant effects on the retention and selectivity in HILIC. Although the effect of the chromatographic parameters on the retention and selectivity should be considered together and viewed as a whole, this review focuses on the effect of the mobile phase pH and salt concentration since organic solvents and column temperature have been extensively discussed in other reviews [2,15].

2. Stationary phases in HILIC

Similar to normal phase chromatography, polar stationary phases are typically used to retain polar solutes in HILIC. In fact, most HILIC separations were performed on normal phase columns (e.g., amino, cyano and silica phase) in the 1990s and early 2000s since only a few HILIC columns (e.g. amide and aspartamide phases) were commercially available [1,17–20]. Conventional silica and amino columns packed and stored in reversed-phase solvents are now commercially available. Many specialty phases with diverse functionalities have been developed exclusively for HILIC in recent years.

Underivatized silica remains a popular phase for HILIC, particularly in the bio-analytical field [16,21]. Irreversible adsorption of solutes and irreproducibility of retention have plagued silica columns in normal phase chromatography, but are not as problematic in HILIC due to the presence of significant levels of water in the mobile phase [22]. Many silica columns for normal phase chromatography have been tested for HILIC separations with varying degrees of success. Olsen reported a significant difference in retention among the silica columns (Type A and B silica) from different manufacturers [23]. This might be attributed to different purity of the silica material or different column preparation procedures. Many column manufacturers have developed silica columns specifically designed for HILIC, such as Atlantis HILIC, Zorbax HILIC plus and YMC pack silica columns. Silica columns promoted for HILIC applications are typically packed and stored in aqueous/organic solvents (e.g., water and acetonitrile) instead of normal phase solvents. In addition, the silica columns packed with sub-2 μm particles (e.g., Acquity 1.7 μm and Epic 1.8 μm silica columns) are also available for UHPLC applications [24]. The superficially porous silica column based on fused-core™ technology has been applied to HILIC separations and has been shown to have greater resistance to overloading by ionized basic compounds than silica-based reversed-phase columns [25].

In addition to underivatized silica columns, a wide variety of functional groups have been incorporated into the stationary phases for HILIC. The majority of the bonded phases are silica-based and prepared either as a monomeric phase or with a polymer coating covalently bonded to the silica. Table 1 shows the bonded stationary phases currently used for HILIC applications. These are referred to in this review by the conventional names of the functional groups, and representative columns are provided as an example of the corresponding phases. The stationary phases in Table 1 are classified into three categories based on the charge characteristics of the functional groups, namely, neutral, charged, and zwitterionic phases.

2.1. Neutral stationary phases

The functional groups in this category (e.g., amide, cyano, diol, and cyclodextrin) cannot be charged in the pH range typical for the mobile phase in HILIC. This category includes most bonded phases in HILIC and represents a wide variety of functional groups, which are all polar in nature. In this category the amide phase is one of the most popular and has found many applications in HILIC [26–29]. In addition, new amide phases have also been developed for HILIC recently, but few applications have been reported [30]. The amide moiety is attached to the silica surface either through a propyl linker or a proprietary linkage. The aspartamide phase is worth special mention since it was the first stationary phase especially developed for HILIC separations [1]. Its preparation has been discussed in detail in Ref. [1] and also in Irgum and Tanaka's reviews [2,14]. The aspartamide phase is a polymeric phase prepared by bonding a layer of polysuccinimide to aminopropylated silica, then treating the polymer with ethanolamine to generate the final stationary phase. The aspartamide phase has been applied to the separation of small polar compounds, peptides and proteins [31–33]. However, it is not as widely used as the amide phase, possibly due to lower efficiency and limited long-term stability.

Although the cyano and diol phases in this category are commonly used in normal phase separations, their applications in HILIC are still very limited. Both cyano and diol phases are monomeric phases directly attached to the silica surface through a propyl linker. Cyano groups lack hydrogen bond donor capability and are also less hydrophilic. This leads to insufficient retention for most polar compounds, and only a few special applications have been reported on the cyano phase [34]. In comparison, the diol

Table 1
Polar stationary phases commonly used in HILIC.

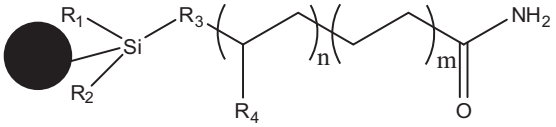
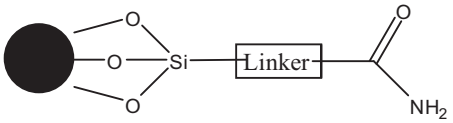
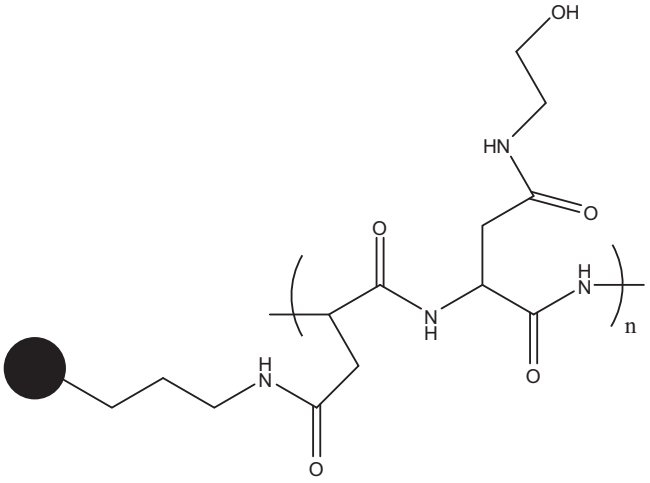
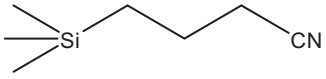
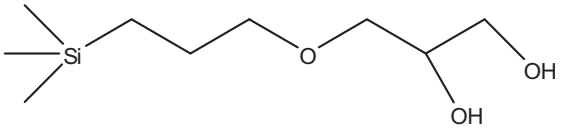
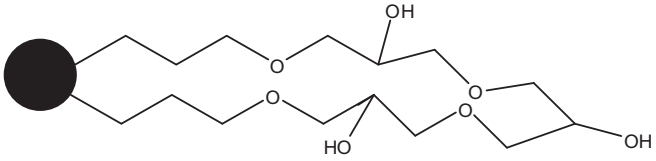
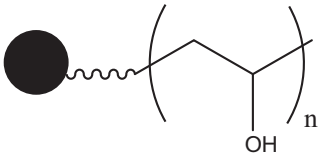
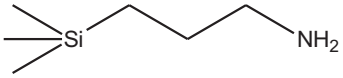
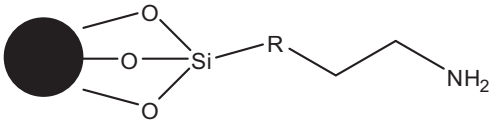
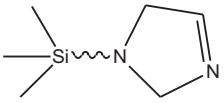
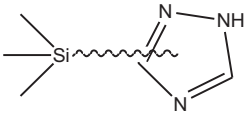
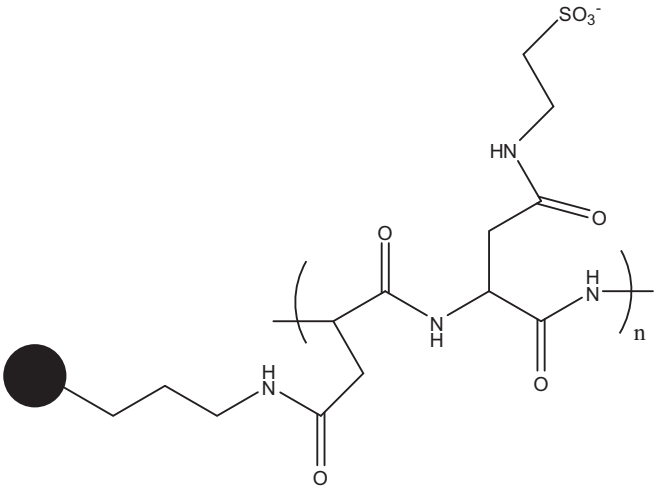
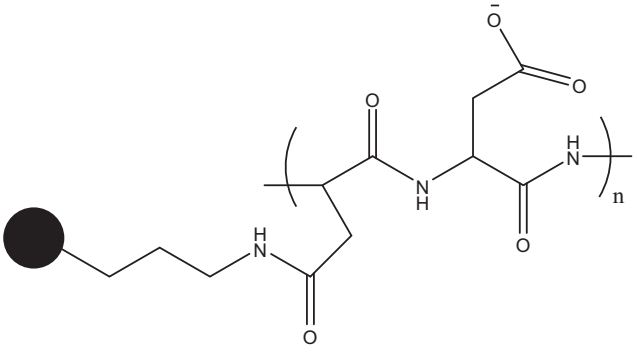
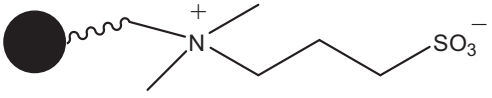
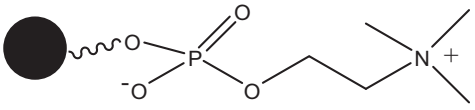
Phase type	Phase name	Functional group structure	Example column
Neutral	Amide		TSKgel Amide 80
			BEH Amide
	Aspartamide		Polyhydroxyethyl A
	Cyano		YMC-Pack Cyano
	Diol		YMC-Pack Diol, Inertsil HILIC
	Mixed-mode diol		Acclaim mixed-mode HILIC 1
	Cross-linked diol		Luna HILIC
	Polyvinyl alcohol (PVA)		YMC PVA-Sil
Charged	Amino		YMC-Pack Amino,
			TSKgel NH2-100

Table 1 (*Continued*)

Phase type	Phase name	Functional group structure	Example column
	Polyamine	NA	Luna NH2, YMC-Pack Polyamine II
	Imidazole		Sepax Polar-imidazole
	Triazole		Cosmosil HILIC
	Poly(2-sulfoethyl)		Polysulfoethyl A
	Poly(aspartic acid)		Poly CAT A
Zwitter-ionic	Sulfobetaine		ZIC-HILIC
	Phosphocholine		Shiseido PC HILIC
	Obelisc N		Sielc Obelisc N column

phase has found more HILIC applications, possibly due to more hydrophilic dihydroxyl groups as well as hydrogen bonding capabilities [35–38]. Unlike many silica columns specially packed for HILIC, most of the diol columns used in HILIC are packed for normal phase applications, and only a few diol columns are directly available for HILIC applications (e.g., YMC Pack Diol and GL Science HILIC). In addition to conventional diol phase, a mixed-mode HILIC column with a diol phase has also been developed by Dionex [38,39]. The diol groups are linked to the silica surface using a longer aliphatic chain, which renders this diol phase less hydrophilic, thus less retentive than the conventional diol phase.

Related to the diol phase are cross-linked diol, polyhydroxy and polyvinyl alcohol phases, which all have hydroxyl groups present on the surface of the polymer coating. The cross-linked diol phase is prepared by cross linking one of the diol groups through an ether linkage, thus forming a polymer layer on the silica surface [40]. The polyhydroxyl and polyvinyl alcohol phases are prepared by coating the silica surface with a layer of hydrophilic polymer, but

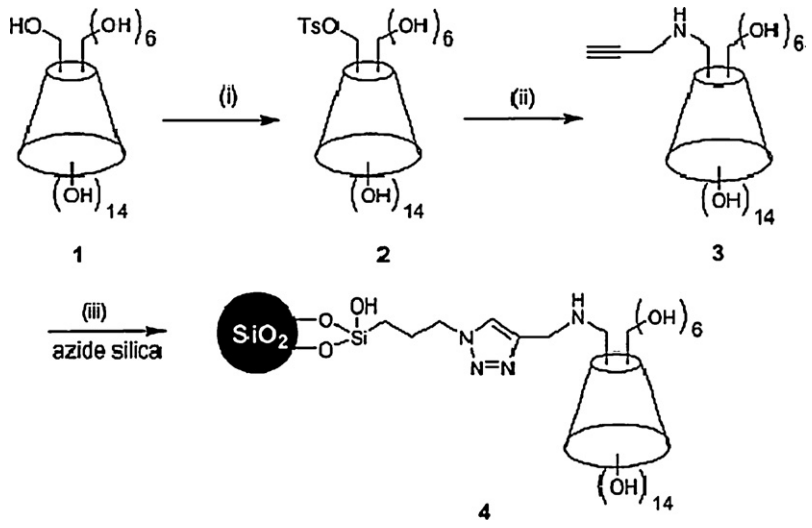


Fig. 1. Preparation of β-cyclodextrin-bonded phases using "click" chemistry: (i) TsCl, NaOH, 5–10 °C; (ii) propargylamine, 60–70 °C; (iii) azide-silica (CH_3OH/H_2O, 1/1, v/v), $CuSO_4$ (5 mol%), NaAsc (15 mol%), RT.

the polymer has not been disclosed by the column manufacturers. The three polymeric phases have not been investigated extensively, and only limited applications have been reported [41,70]. Based on limited data, the efficiencies of these polymer phases do not seem to be much lower than that of the monomeric diol phase. This will encourage the use of these polymer phases since they have the advantages of better stability at extremes of pH as well as reducing secondary interaction with residual silanol groups.

Cyclodextrins (CDs) have been widely used as chiral stationary phases for enantiomeric separations in either reversed-phase or normal phase mode [42]. Hydroxyl groups are present on the rims of the truncated cone structure of CDs, which make it possible to use the CD phase for HILIC separations [43]. The neutral CD phases have been used to separate oligosaccharides and polar compounds [44,45]. The CD phase has also been shown to be able to resolve polar chiral compounds in the HILIC mode [46,47]. In addition, the "click chemistry" concept has also been used to prepare the β-CD phase [48]. "Click chemistry" is a concept introduced by Sharpless to describe chemistry tailored to generate a substance quickly and reliably by joining small units together. Azide-alkyne Huisgen cycloaddition is one of the most popular reactions within the "click chemistry" concept [49]. As shown in Fig. 1, the silica surface is first modified with propylazide groups. Native β-CD is treated with p-tolylsulfonyl chloride to produce mono-6^A-(p-tolylsulfonyl)-β-CD, which is further reacted with propargylamine to finish functionalization of β-CD. The final β-CD phase is prepared by covalently bonding the functionalized β-CD with the modified silica via Huisgen [3+2] dipolar cycloaddition. The β-CD phase prepared via "click chemistry" is different from the conventional β-CD phase (e.g., cyclo-bond columns) in that the β-CD functional groups are attached to the silica surface through a linkage that contains a triazole and a secondary amine function. These groups, when ionized, can render the click β-CD phase positively charged, and potentially alter the selectivity of the phase. In addition, a maltose phase was also prepared by "click chemistry" and used for HILIC separations [50].

It should be noted that although the functional groups of neutral stationary phase are not charged, some columns (e.g., amide, cyano and diol) can carry negative charges at a mobile phase pH above 4–5 due to deprotonation of residual silanol groups. Surface silanol groups are better shielded with the polymeric phases, such as crosslinked diol, polyhydroxy and polyvinyl alcohol phases.

2.2. Charged stationary phases

The functional groups of the stationary phases in this category can bear charges in the pH range typically used in HILIC (e.g., pH 3–7). The amino phase is a typical example in this category and has been widely used for HILIC separations [2,5,11]. Significant differences in retention among various amino columns have been observed, possibly due to differences in the silica material and stationary phase preparation [11]. The CAPcell PAK NH2 column (Shiseido) exhibits appreciable better stability than conventional amino columns [51]. TSKgel NH2-100 (Tosoh Bioscience) is a new amino phase prepared in a step-wise fashion after end-capping as shown in Table 1. The new amino column (3 μm particle size) has been shown to have efficiency similar to the Amide-80 column prepared on 3 μm silica particles [52]. Polyamine phases, such as YMC Polyamine II, PolyWAX LP, and Luna NH_2 have also been used for limited HILIC separations [18,53,79]. YMC Polyamine II phase contains secondary and tertiary amine groups exclusively, and PolyWAX LP phase has a linear polyethyleneimine coating largely composed of secondary amines. The crosslinked amine phase (Luna NH_2) contains primary and secondary amine groups.

Two newer members in the charged phase category are imidazole and triazole phases. Both phases are monomeric type prepared on silica packing materials and can be positively charged in the typical pH range for HILIC separation. As shown in Table 1, the imidazole phase is attached to the silica surface through the secondary amine function. The triazole phase utilizes 1,2,4-triazole as the functional group, but the exact attachment point on triazole ring is not disclosed at this point. Since both the imidazole and triazole phases are relatively new, very limited applications of the two phases are found in literature [54].

In addition to positively charged phases, a couple of negatively charged phases have also been used for HILIC separation, such as poly(2-sulfoethylaspartamide), poly(aspartic acid) and sulfonated S-DVB phase. These phases are typically used as cation exchangers in ion-exchange chromatography, but have been used in the separation of peptide and basic drug compounds under HILIC conditions [55–57]. The separation is based on combined hydrophilic interaction and ion-exchange effects. The sulfonated S-DVB phase was demonstrated to separate ethylene glycol, propylene glycol and glycerol based on hydrophilic interaction, and the capacity factors were almost linearly related to the degree of sulfonation and the counter ions (H^+ *vs.* Ca^{2+}) [58]. In comparison to the positively

charged phases (e.g., amino and polyamine phases), the negatively charged phases are much less used in HILIC.

2.3. Zwitterionic phases

Zwitterionic phases are a relatively new class of polar stationary phases used for HILIC separation. A typical example is the sulfobetaine phase as shown in Table 1, which was initially developed for the separation of inorganic salts and small ionic compounds, but has found a wide range of applications in HILIC [2,5,16]. This might be attributed to the findings that the sulfobetaine zwitterions on the polymer strongly bind water to its surface [59]. The sulfobetaine phase is a grafted polymer with 3-sulfopropyldimethylalkylammonium functionality on either porous silica or polymer base and has both positively charged (quaternary ammonium) and negatively charged (sulfonic acid) groups in a 1:1 ratio, so the surface charge is ostensibly zero [60]. It is important to point out that the negative charge of sulfonic acid group at the distal end of the sulfobetaine phase may also introduce electrostatic interactions with charged solutes [61,79]. In addition to the sulfobetaine type of zwitterionic phase, a phosphorylcholine phase has also been reported [62]. Similar to the sulfobetaine phase, the phosphocholine phase is also based on a graft polymer with zwitterionic phosphorylcholine groups present on its surface. The major difference between the two zwitterionic phases is that the negatively charged sulfonate group is at the distal end of the sulfobetaine phase, while the positively charged quaternary ammonium group is at the distal end of the phosphocholine phase. Obelisc N (SIELC) phase is another type of zwitterionic phase that has been applied to HILIC separation, but the chemical nature of the positively and negatively charged groups has not been disclosed by the manufacturer [41,63].

2.4. Retention of stationary phases in HILIC

The ability to provide stronger retention of polar compounds and different selectivity from that of RPLC are major reasons for the increasing popularity of HILIC. As with any chromatographic mode, retention is determined not only by the type of stationary phase, but also by the mobile phase and column temperature.

3. Retention models in HILIC

Alpert suggested in his seminal paper that the retention was based on hydrophilic partitioning of solutes between bulk eluent and a water-rich layer immobilized on the surface of the stationary phase in HILIC [1]. He further pointed out that other interactions (e.g., hydrogen bonding and dipole–dipole interactions) could also play a role in HILIC separation. Orth and Engelhardt demonstrated that water was strongly retained on the surface of amino-, ethylenediamino-, and diethylenetriamino-modified silica and increased with length of the oligoethyleneimine chain [64]. Recently, McCalley and Neue demonstrated experimentally the existence and extent of the water-rich layer on the silica surface by measuring the retention time of benzene and toluene with a mobile phase of high acetonitrile content [65]. It was found that a significant percentage of the pore volume indeed was occupied by a water-rich layer under typical HILIC conditions, and the layer increased in thickness as the water content of the mobile phase increased up to 30% (v/v).

Hemström and Irgum carefully examined the published data using both the partitioning and surface adsorption models in their 2006 review, but found that the evidence was not conclusive, and the predominant retention mechanism was dependent on both the solute characteristics and stationary phases [2]. In a more recent study, McCalley investigated the retention mechanism of a mixture of neutral, strongly acidic and strongly basic compounds on the amide (TSKgel Amide-80), cross-linked diol (Luna HILIC), mixed-mode diol (Acclaim mixed-mode HILIC-1), silica and zwitterionic phase (ZIC-HILIC) [67]. The experimental data indicate a very complex mechanism, consisting of partitioning, adsorption, ionic interactions and sometimes even hydrophobic interaction. The contribution of ion exchange to the retention of strongly basic compound is found to be significant even at 10 mM buffer concentration on the amide, mixed-mode diol, silica and zwitterionic phases. The Luna HILIC phase seems to have less electrostatic interactions with the basic compounds, possibly because its surface silanol groups are better shielded by the layers of cross-linked diol chains.

A few recent studies further examined the relationship between the retention and water content in mobile phase in HILIC. Liang and co-workers investigated the retention change of nucleosides with the water content in the mobile phase on six different polar stationary phases including both commercial stationary phases (Atlantis HILIC silica, Venusil HILIC and BEH HILIC uncoated silica) and stationary phases synthesized in-house using "click chemistry" (single-hydroxyl, multi-hydroxyl and β-cyclodextrin phases) [68]. A linear relationship was not observed using either the partitioning model or surface adsorption model for adenosine on all six various stationary phases as shown in Fig. 2a and b. Stalikas and co-workers recently studied the retention behavior of water-soluble vitamins on a diol column in HILIC [69]. The retention data of water-soluble vitamins ($\log k'$) were plotted against both the volume fraction of water (φ) and $\log(\varphi)$ as shown in Fig. 3a and b. Most compounds exhibited curvature in the $\log k'$ *vs.* φ plots in the high organic range with the exception of nicotinamide. In comparison, some compounds had relatively linear relationship between $\log k'$ and $\log \varphi$, but others displayed curvature in the $\log k'$ *vs.* $\log \varphi$ plots in the range of high water content.

Based on the above observations, Liang and co-workers proposed a new equation to describe the retention behavior of polar solutes in HILIC:

$$\ln k' = a + b \ln C_B + cC_B$$

where C_B is the volume fraction of water in the mobile phase [68]. The constant a is related to the molecular volume of solutes, and b and c are related to direct analyte–stationary phase interaction and the interaction energy between solutes and solvents, respectively. Most of the nucleoside compounds had regression co-efficients higher than 0.99 on the six different columns used in the study, with the exception of the early eluting compounds (thymine and uracil) on the single-hydroxyl phase. The data of Stalikas and co-workers also demonstrated a good fit to Liang's model using the retention data of water-soluble vitamins on the diol phase [69]. The retention model proposed by Liang et al. illustrates that retention in HILIC is a complex process and may not be fully attributable to one mechanism (e.g., partitioning or adsorption). The predominant mechanism may depend on solute characteristics, the nature of the polar phases and mobile phase composition. Based on the retention behavior of water-soluble vitamins, Stalikas pointed out that hydrophilic partitioning might be the major retention mechanism in the mobile phase containing high levels of water, but other forces (e.g., adsorption, hydrogen bonding, and electrostatic interactions) may become predominant as water content decreases. This view is consistent with typical observations of retention change ($\log k'$) with the volume fraction of water in the mobile phase as seen in Figs. 2 and 3. In the higher water range, a linear relationship between $\log k'$ and the volume fraction of water in the mobile phase is typically observed for most polar solutes, indicating that hydrophilic partitioning may be the predominant retention mechanism. The findings of McCalley and Neue indicate that the

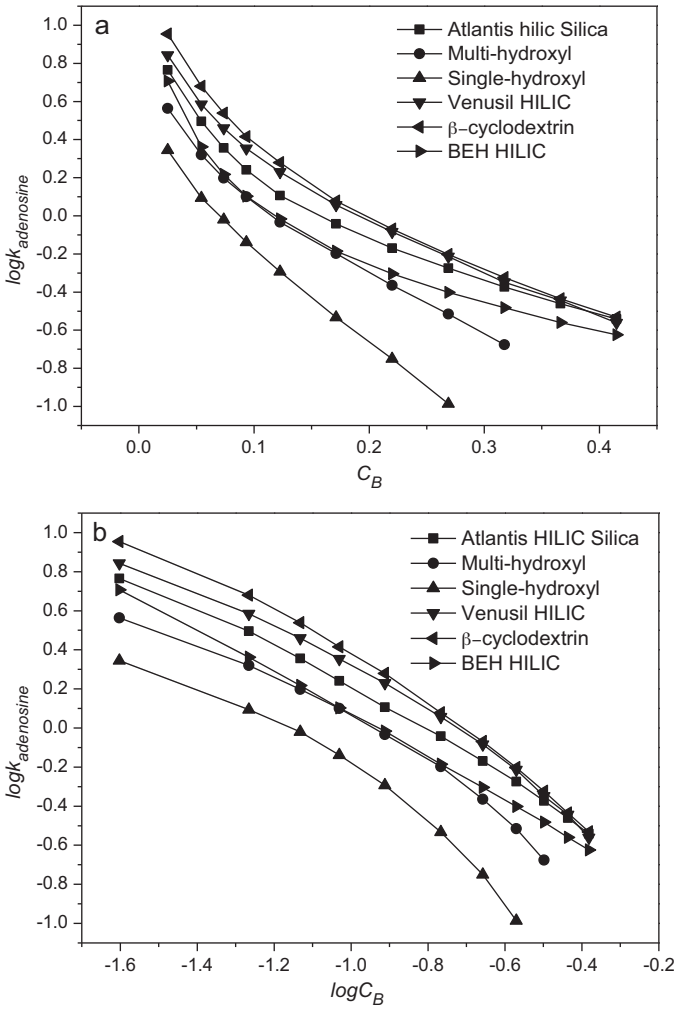


Fig. 2. Plots of log k *vs.* volume fraction (a) and logarithmic volume fraction (b) of water in the mobile phase for adenosine on (■) Atlantic HILIC silica column, (●) multi-hydroxyl column, (▲) single-hydroxyl column, (▼) Venusil HILIC column, (◄) β-cyclodextrin column and (►) BEH HILIC column.
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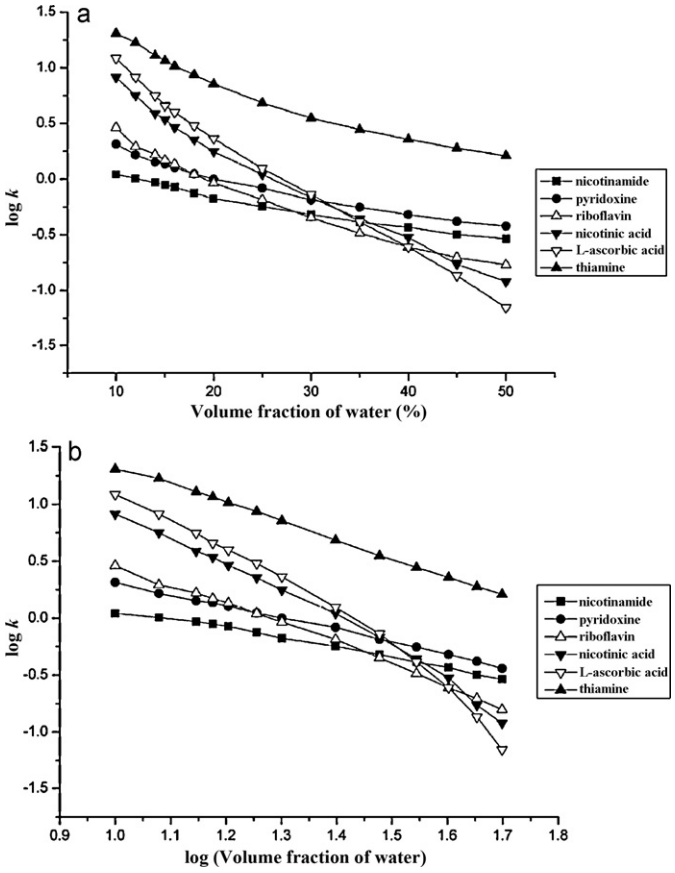

Fig. 3. Plots of log k *vs.* volume fraction (a) and logarithmic volume fraction (b) of water in the mobile phase for water soluble vitamins on Inertsil Diol column. Mobile phase: ACN-water, 10 mM ammonium acetate (pH 5.0). Flow rate: 0.6 mL/min. Column temperature: 25 °C.
Adapted from Ref. [69] with permission.

water-rich layer is relatively low in the mobile phase containing high acetonitrile and increases significantly as the water content increases [65]. This provides indirect evidence that direct interactions between the solutes and stationary phases are more plausible at lower water content because the water-rich layer might not be sufficient to sustain strong hydrophilic partitioning. It should be pointed out that other data support the contrary view that hydrophilic interaction increases as the water content in the mobile phase decreases, while other mixed-mode effects (e.g., electrostatic interactions) become less significant in the overall retention [79].

In addition to the model described in the above equation, a quantitative structure–retention relationship study provided useful insight into the retention mechanism in HILIC. Jinno et al. employed multivariate linear regression (MLR) approach to analyze the retention data of 20 adrenoreceptor agonists and antagonists on the silica, diol and PVA phases [70,71]. The acetonitrile content was selected as the mobile phase related predicator and the solutes were described by the molecular descriptors including the logarithm of the partition coefficient (log D), the number of hydrogen-bond acceptors (HBA), the number of hydrogen-bond donors (HBD), the dipole magnitude (DipolMag), the desolvation energy for octanol (FOct) and the total absolute atomic charge (TAAC). The relative effects of the mobile phase and solute descriptors on the retention of the model compounds were assessed by the signs and magnitude of the standardized coefficients (β) as shown in Table 2. The acetonitrile content has the highest β values, supporting the view that hydrophilic partitioning may be the predominant retention mechanism. This is also consistent with the negative β values of log D, which indicate negative correlation between hydrophobicity of the solutes and the retention on polar phases in HILIC. The β values of HDB suggest that hydrogen bonding contributes to the retention of the model compounds on the diol, PVA and silica phases.

3.1. Temperature effect on retention

Temperature can have significant influence on retention in both RPLC and HILIC due to its effects on mobile phase viscosity and solute diffusivity. Moreover, the relationship between retention and temperature can provide insights into the retention mechanism. The van't Hoff equation describes the relationship between retention and column temperature:

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

where ΔH° and ΔS° are the change of enthalpy and entropy between the mobile and stationary phases, R is the gas constant and ϕ is the phase ratio. The linear relationship between $\ln k'$ and $1/T$ is not only observed in RPLC where both partitioning and adsorption contribute to solute retention [72,73], but also in normal phase chromatography and ion-exchange chromatogra-

Table 2
Values of the standardized coefficients (β values) of the predictors.

Stationary phase	β values of the predictors						
	% ACN	log *D*	HBD	HBA	DipolMag	FOct	TAAC
Diol							
pH 3.0	0.798	−0.337	0.312	−0.080	–	–	−1.610
pH 4.0	0.815	−0.387	0.231	−0.152	–	–	−0.157
pH 5.0	0.823	−0.307	0.324	−0.131	–	–	−0.168
Unmodified silica							
pH 3.0	0.830	−0.605	–	−0.231	0.315	–	–
pH 4.0	0.768	−0.579	–	−0.212	0.308	–	−0.124
pH 5.0	0.679	−0.620	–	−0.244	0.322	–	−0.137
PVA-bonded							
pH 3.0	0.756	−0.249	0.579	–	–	0.295	−0.189
pH 4.0	0.728	−0.291	0.572	–	–	0.307	−0.187
pH 5.0	0.721	−0.264	0.599	–	–	0.343	−0.204

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phy where adsorption and electrostatic interaction are the major retention mechanisms [74]. Curvature in the van't Hoff plot typically implies a change in retention mechanism or multiple forces contributing to retention. Hao et al. reported the van't Hoff plots for six compounds (glycine, diglycine, triglycine, N-[1-deoxy-D-glucose-1-yl]-glycine, N-[1-deoxy-D-glucose-1-yl]-di-glycine, and N-[1-deoxy-D-glucose-1-yl]-tri-glycine) on an Atlantis Silica column [15]. As shown in Fig. 4, three of the compounds (glycine, diglycine and triglycine) had nearly perfect linear van't Hoff plots, but the van't Hoff plots for the other three compounds exhibited curvatures. All six compounds were positively charged under the experimental conditions. The curvature in the van't Hoff plots cannot be simply explained by the electrostatic interactions with deprotonated surface silanol groups since glycine, diglycine and triglycine would have electrostatic interactions similar to their glucose derivatives, but did not show any curvature. The curvature in the van't Hoff plots of the other three compounds is presumably due to the presence of the glucose moiety, which might induce specific interactions (e.g., hydrogen bonding). The retention change of salicyluric acid with column temperature was investigated on the amide and aspartamide phases using a mobile phase of acetonitrile/water (80/20, v/v) containing 15, 25 and 40 mM of ammonium acetate. As shown in Fig. 5a and b, the van't Hoff plots for salicyluric acid were mostly linear on the amide phase, but curvature was observed on the aspartamide phase [85]. The curvature in the van't Hoff plot is a strong indication that multiple forces are at play and various mechanisms (e.g., partitioning, polar interactions, electrostatic interactions) may contribute to the retention under the experimental conditions. In addition, Stalikas and coworkers observed deviation from a linear van't Hoff relationship for some water-soluble vitamins (nicotinic acid, L-ascorbic acid and thiamine) in a mobile phase of acetonitrile/water (90/10, v/v) containing 10 mM ammonium acetate on a diol column [69]. The authors attributed the deviation to the electrostatic interactions (repulsion for nicotinic acid and L-ascorbic acid, attraction for thiamine). What is noticeable is that linearity of the van't Hoff plots

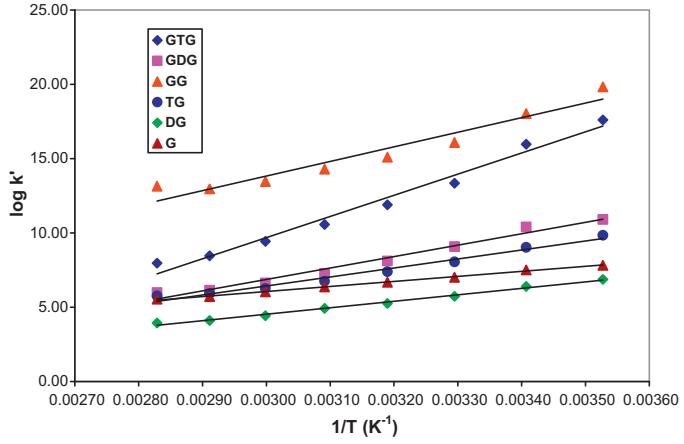

Fig. 4. The van't Hoff plots for glycine (G), diglycine (DG), triglycine (TG), N-(1-deoxy-D-glucose-1-yl)-glycine (GG), N-(1-deoxy-D-glucose-1-yl)-di-glycine (GDG), N-(1-deoxy-D-glucose-1-yl)-tri-glycine (GTG) on Atlantis silica column (50 mm × 2.1 mm, particle size 5 μm, flow rate 100 μL/min). Mobile phase: water/ACN (10/90, v/v) containing 0.4% formic acid.
Adapted from Ref. [15] with permission.

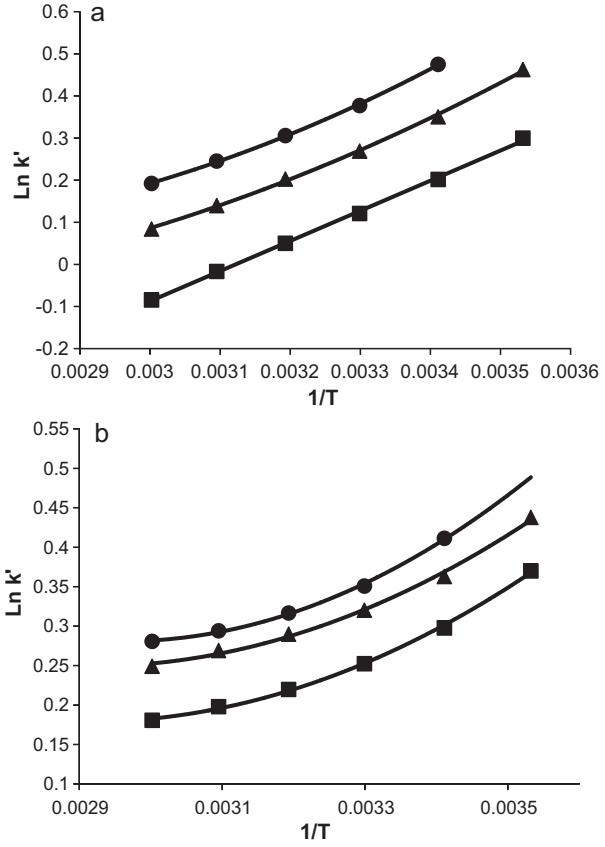

Fig. 5. The van't Hoff plots for salicyluric acid on amide phase (a) and aspartamide phase (b). Mobile phase: acetonitrile/water (80/20, v/v) containing 15 mM (■), 25 mM (▲) and 40 mM (●) ammonium acetate. Flow rate 1 mL/min and column temperature 30 °C.
Adapted from Ref. [85] with permission.

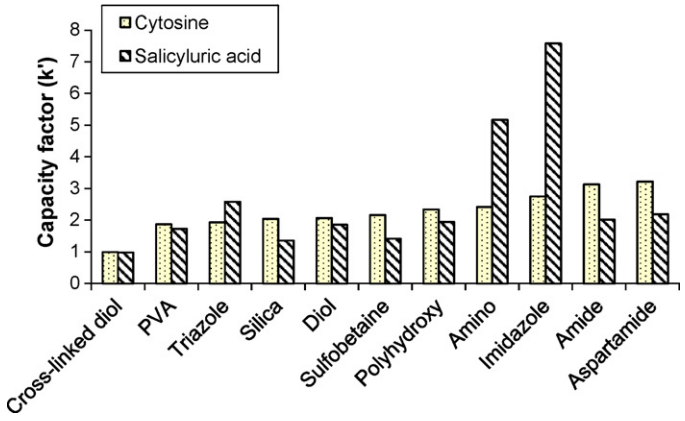

Fig. 6. Capacity factors of cytosine and salicyluric acid on various HILIC columns (250 mm × 4.6 mm ID, 5 μm particle size). Column temperature, 30 °C. Flow rate: 1.5 mL/min (cytosine) and 1.0 mL/min (salicyluric acid). Mobile phase: acetonitrile/water (85/15, v/v) containing 10 mM ammonium acetate (for cytosine) and 20 mM ammonium acetate (for salicyluric acid).
Adapted from Ref. [75] with permission.

for the three compounds was greatly improved ($R^2 > 0.99$) when the water content in the mobile phase was increased from 10% to 20%. The increase in water content of the mobile phase might facilitate partitioning, thus making partitioning the predominant mechanism under the experimental conditions.

3.2. Relative retentivity of stationary phases

In reversed-phase liquid chromatography (RPLC), the retention is highly dependent on the hydrophobicity of the bonded phase (e.g., C4, C8, C18, and phenyl). The relative retention on various stationary phases can be predicated simply based on the stationary phase structure, for example, C18 phase has stronger retention than C8 or phenyl phases for most compounds under the same chromatographic conditions. Alkylbenzenes (toluene and propylbenzene) are typically used as probe compounds to evaluate the retentivity of the stationary phases in RPLC. In HILIC, however, it is difficult to evaluate the relative retentivity of the polar stationary phases simply based on the bonded phase structure because first, it is difficult to compare the hydrophilicity of various polar phases based on the phase structure, and second, the retention might not be directly related to the hydrophilicity of the stationary phase itself. In addition, there has not been a commonly accepted probe compound that can be used to compare and evaluate retention of various polar phases in HILIC.

The relative retentivity of some commonly used stationary phases was investigated and compared using cytosine as the reference compound [75]. The capacity factors of cytosine on the selected polar phases, in the order of increasing retention, are graphically presented in Fig. 6. Electrostatic interaction does not contribute to retention since cytosine is uncharged under the experimental conditions. The bar chart in Fig. 6 clearly demonstrates different retentivity of various polar phases in HILIC. The cross-linked diol phase has the least retention for cytosine of all the phases. The amide and aspartamide phases have the strongest retention. Interestingly, most of the other polar phases exhibit relatively similar retention for cytosine. The diversity of the chemical structure in these phases does not seem to make a significant difference in the retentivity of the corresponding stationary phases. This tends to support hydrophilic partitioning as the retention mechanism for these stationary phases as opposed to strong specific interactions.

When salicyluric acid was used as the reference compound, as shown in Fig. 6, there are some similarities, but also noticeable differences in the relative retention of the polar phases [75]. The cross-linked diol phase is the least retentive phase for both cytosine and salicyluric acid. The other neutral phases (e.g., the diol, polyhydroxy and PVA phases) also show similar retentivity for salicyluric acid. However, the cationic phases, the amino and imidazole phase in particular, have much stronger retention for the acid, which can be attributed to the electrostatic attraction between the negatively charged acid and positively charged functional groups under the experimental conditions. In contrast, the silica and sulfobetaine phases seem to have reduced retention for the acid compared to the other phases. These phases carry negative charges, silica from deprotonated silanol groups and sulfobetaine from negatively charged sulfate groups. It is possible that the reduced retention of the acid on these phases is related to electrostatic repulsion of the negatively charged acid.

3.3. Selectivity of stationary phases in HILIC

In RPLC, stationary phase chemistry (e.g., C18 *vs.* phenyl phase) has been widely used to change selectivity and achieve desired separations. Although a variety of functional groups have been used as the stationary phase in HILIC (Table 1), there is still a need to better understand the selectivity of various polar phases. With the availability of more polar phases, this has become particularly important since knowledge about various HILIC phases is useful for selection of appropriate columns during method development.

4. Direct selectivity comparison of stationary phases

As in RPLC, selectivity in HILIC depends not only on the stationary phase, but also on the mobile phase as well as solute characteristics. The selectivity of various stationary phases needs to be evaluated under the same chromatographic conditions using the same set of model compounds [66,75]. For this purpose, a group of nucleic acids and nucleosides were used to compare the selectivity of 11 different polar stationary phases listed in Table 1. The model compounds were eluted isocratically using a mobile phase of acetonitrile and ammonium acetate solution (85/15, v/v). The selectivity of the amide, aspartamide, sulfobetaine and silica phases is well illustrated by the chromatograms shown in Fig. 7. The amide and aspartamide phases have somewhat similar selectivity, but the sulfobetaine phase shows different selectivity (e.g., adenosine and uridine). In comparison, the silica phase has a very different selectivity for the model compounds than do the amide, aspartamide and sulfobetaine phases. Fig. 8 shows the separation of the model compounds on the diol, cross-linked diol, polyhydroxy, and PVA phases, which all have hydroxy groups on the packing surface. These phases share relatively similar selectivity for the model compounds. The only difference is that the cross-linked diol and polyhydroxy phases seem to have better selectivity for cytidine and guanosine than the diol and PVA phases. In addition, it is interesting to note that the selectivity of the silica phase (Fig. 7) seems to be very different from that of the phases shown in Fig. 8, which indicates that the silica phase may have some specific interactions with the model compounds. The separation of the model compounds on the cationic phases (e.g., amino, imidazole and triazole phase) is shown in Fig. 9. The selectivity of the amino phase is somewhat similar to that of the amide and aspartamide phases, but very different from that of the sulfobetaine and silica phases shown in Fig. 7. The imidazole and triazole phases have a very different selectivity from each other, but also from other polar phases. For example, adenosine and uridine are barely resolved on the amino phase, but well separated on the imidazole and triazole phases with opposite elution order. It is also worth noting that the model compounds are not charged at the mobile phase pH used in this experiment. The difference in selec-

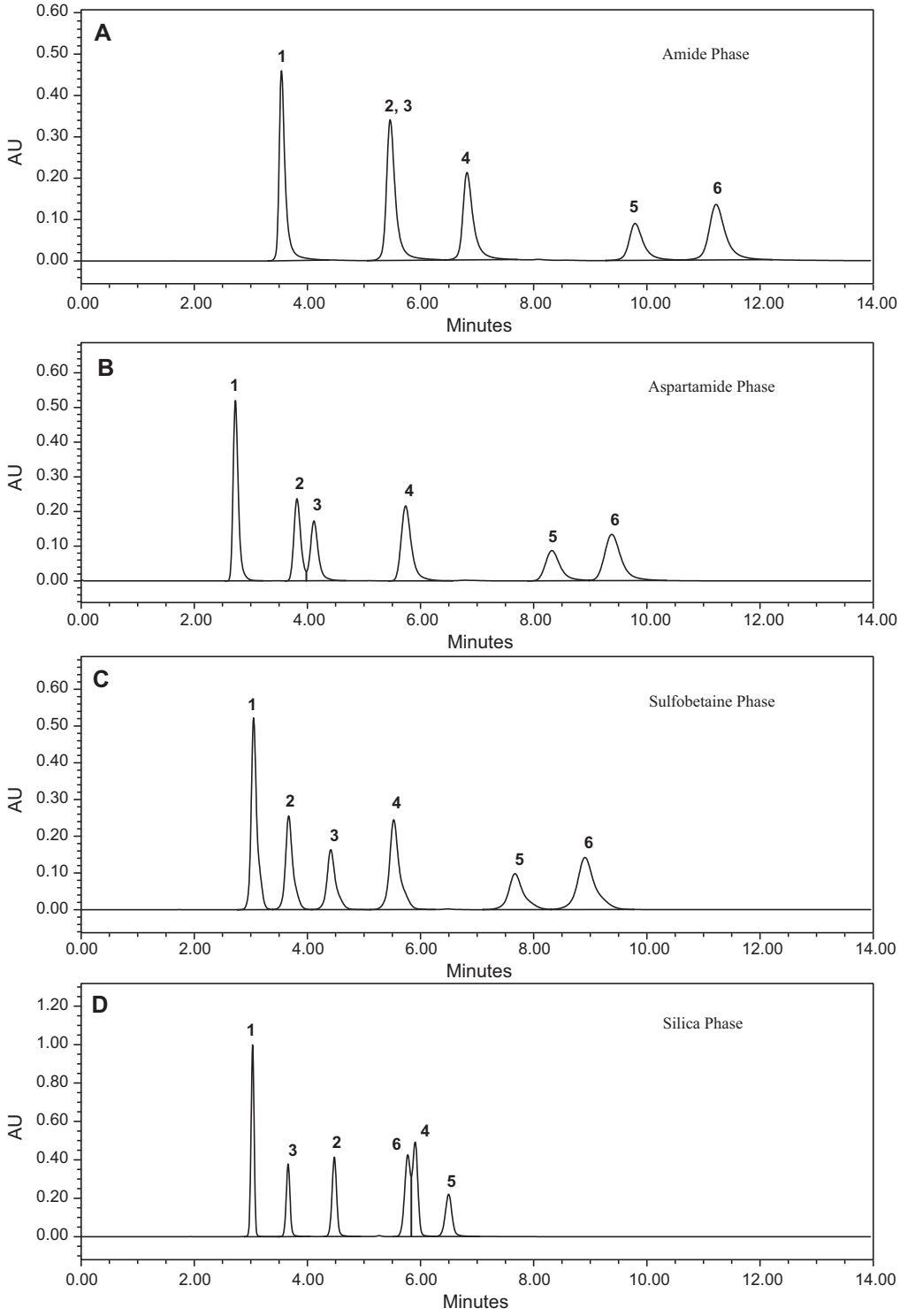

Fig. 7. Separation of selected nucleic acid bases and nucleosides on (A) amide, (B) aspartamide, (C) sulfobetaine, and (D) silica phase. Column dimension: 250 mm × 4.6 mm ID and 5 μm particle size. Mobile phase: acetonitrile/water (85/15, v/v) containing 10 mM ammonium acetate. Column temperature 30 °C. Flow rate 1.5 mL/min. Peak label: (1) uracil, (2) adenosine, (3) uridine, (4) cytosine, (5) cytidine, and (6) guanosine. Adapted from Ref [75] with permission.

tivity most likely results from specific interactions (e.g., hydrogen bonding, and dipole interaction) between the stationary phases and solutes.

Other published reports also compared the selectivity of various polar stationary phases used in HILIC. Fountain et al. recently evaluated the selectivity of three stationary phases of Waters BEH brand (i.e., BEH Amide, BEH Diol and BEH HILIC (uncoated silica)) [76]. As shown in Fig. 10, the BEH Amide phase is most hydrophilic, hence more retentive for the model compounds. The elution pattern is similar on the BEH Amide and Diol phases, but is very different on the BEH HILIC (uncoated silica) phase. It is interesting to note that the Atlantis HILIC silica phase is more hydrophilic, hence more retentive than the BEH HILIC (uncoated silica) phase, and its selectivity is also slightly different from that of the BEH

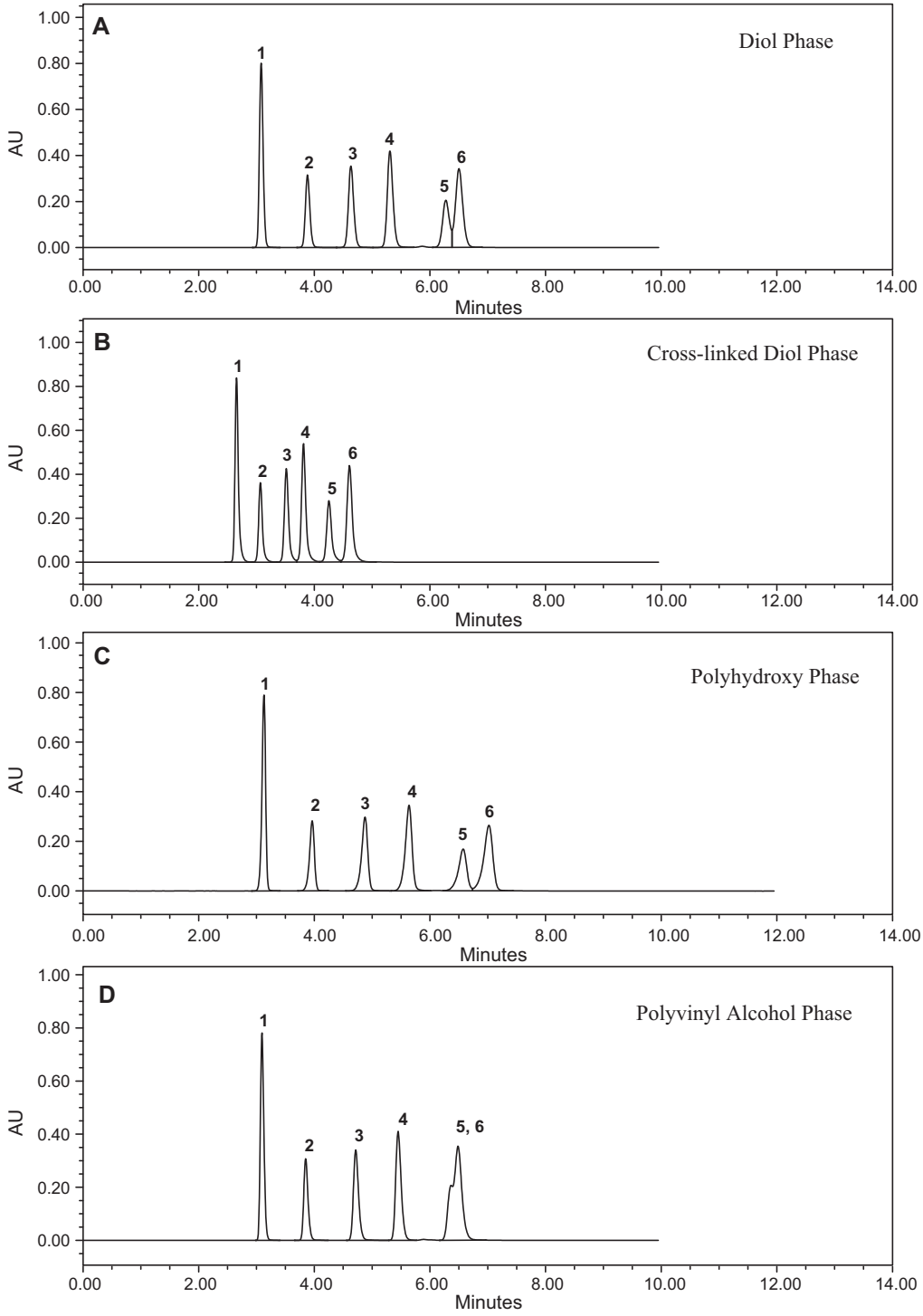

Fig. 8. Separation of selected nucleic acid bases and nucleosides on (a) diol, (b) cross-linked diol, (c) polyhydroxy, and (d) PVA phase. Other conditions are the same as in Fig. 7.

HILIC (uncoated silica) phase, especially towards peaks 3 and 5 Although both the Atlantis HILIC silica and BEH HILIC (uncoated silica) phases are based on unmodified silica, the BEH HILIC is a hybrid silica with ethylene bridges, which likely leads to differences in surface properties. Lämmerhofer et al. compared the selectivity of several common HILIC phases (amide, sulfobetaine, polyamine, and silica phase) and mixed-mode ion-exchange phases [77]. As shown in Fig. 11, the conventional HILIC phases (Amide-80 and ZIC-HILC) showed quite different selectivity than the ion-exchange phases (Luna NH_2 and Polysulfoethyl A). The silica phase (Chromolith Si) was also quite different from the other phases in terms of elution pattern. More recently, McCalley compared the selectivity of five HILIC stationary phases including amide (TSKgel Amide-80), cross-linked diol (Luna HILIC), silica, zwitterionic (ZIC-HILIC), and mix-mode diol (Acclaim mixed-mode HILIC-1) using a mixture of neutral, strongly acidic and strongly basic compounds [67]. As shown in Fig. 12, different stationary phases exhibit widely different retention and selectivity for the model compounds. Basic compounds seem to be more retained on all the phases, particularly on the silica phase.

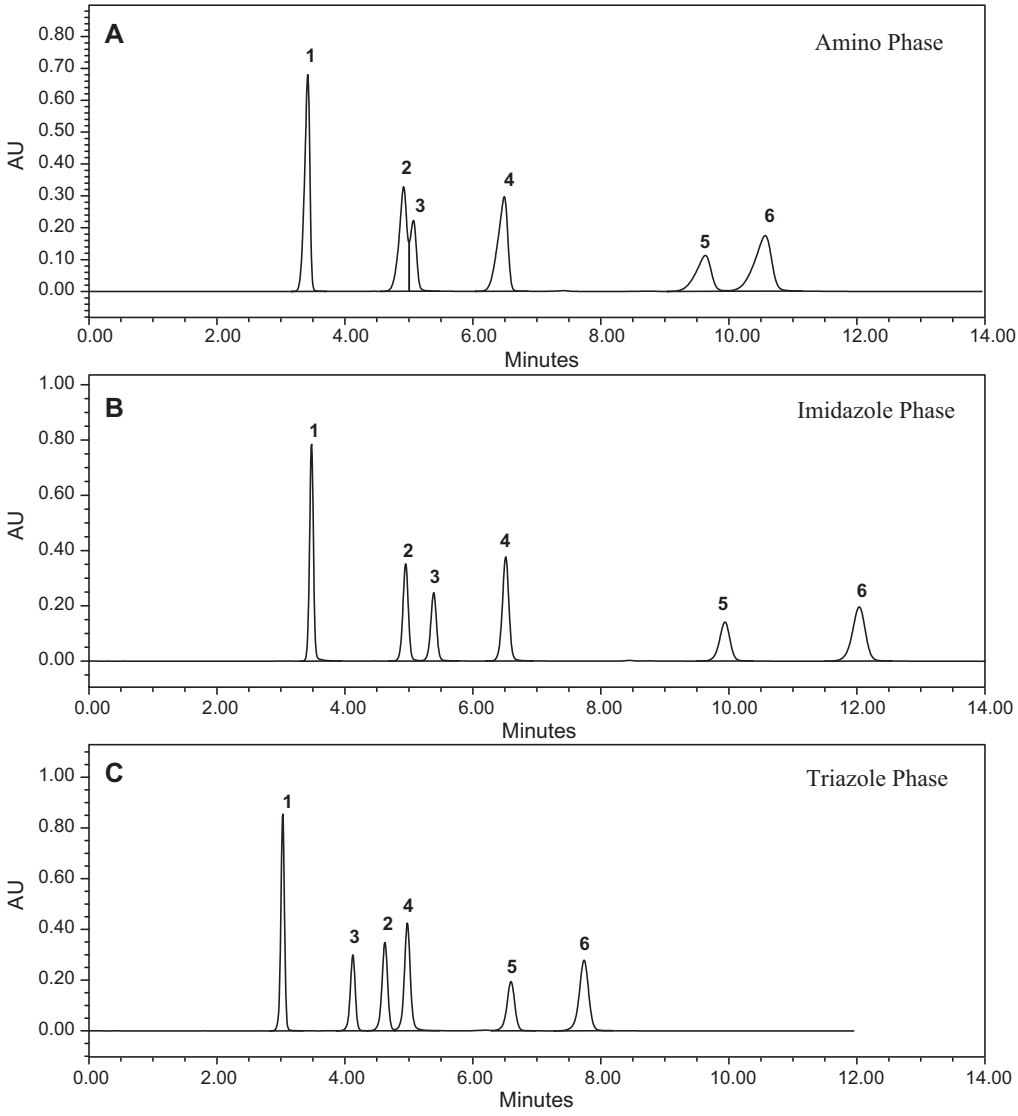

Fig. 9. Separation of selected nucleic acid bases and nucleosides on (a) amino, (b) imidazole, (c) triazole phase. Other conditions are the same as in Fig. 7. Adapted from Ref. [75] with permission.

4.1. Classification of stationary phases

As the polar stationary phases increase in both numbers and diversity, direct comparison of selectivity lacks the capability to provide the insight that is important to better understand the similarity and dissimilarity of various polar phases with distinct chemistry. Principal component analysis (PCA) has been used to evaluate the selectivity of various polar stationary phases for HILIC separations. Lämmerhofer et al. first employed the PCA approach to study the selectivity of 12 polar stationary phases including three mixed-mode phases (RP/WAX-AQ360, Acclaim WAX-1, and Primesep B2), three zwitterionic phases (ZIC-HILIC, Obelisc R and Obelisc N), two weak anion exchangers (Luna NH_2 and BioBasic AX), a cation exchanger (Polysulfoethyl A), a typical HILIC phase (TSKgel Amide-80) and a silica phase (Chromolith Performance Si) [77]. Based on the retention data of selected nucleosides on these phase, Lämmerhofer et al. found that the mixed-mode phases RP/WAX-AQ360 and Acclaim WAX-1 were similar in selectivity, but were different from the Primesep B2 phase. PCA indicates that the ZIC-HILIC and Obelisc N phases were different in their selectivity profiles, consistent with the elution pattern in Fig. 11. This can possibly be attributed to the difference in the aliphatic chain that links the permanent charges in these two phases. The silica phase (Chromolith Performance Si) was shown to be distinct from the other phases in this study, consistent with direct comparison in Fig. 11. It is interesting to note that the sulfobetaine (ZIC-HILIC), amide (TSKgel Amide-80) and Polysulfoethyl A phases were grouped together, indicating similar retention and selectivity in HILIC despite the differences in stationary phase chemistry and charge characteristics.

Chirita et al. also applied PCA to the classification of 11 polar stationary phases using 12 neurotransmitters as the test compounds [54]. Most stationary phases listed in Table 1 were investigated in this study, including 3 silica phases (Uptisphere HILIC, Pursuit XRs Si and Ascentis Express HILIC), 4 neutral phases, 3 positively charged phases and one zwitterionic phase (ZIC-HILIC). The neutral phases included a diol phase (Pursuit XRs Diol), a cross-linked diol phase (Luna DIOL), an amide phase (Amide-80) and a cyano phase (Uptisphere 5 CN). The positively charged phases included a triazole phase (Cosmosil HILIC), a polyamine phase (Astec apHera NH_2) and two aminopropyl phases (Polaris NH_2 and Uptisphere 12 AA NH_2). In general, the grouping of various polar phases depended on the test compounds. Score plots were generated for all the polar phases based on all test compounds (Fig. 13a), on acidic com-

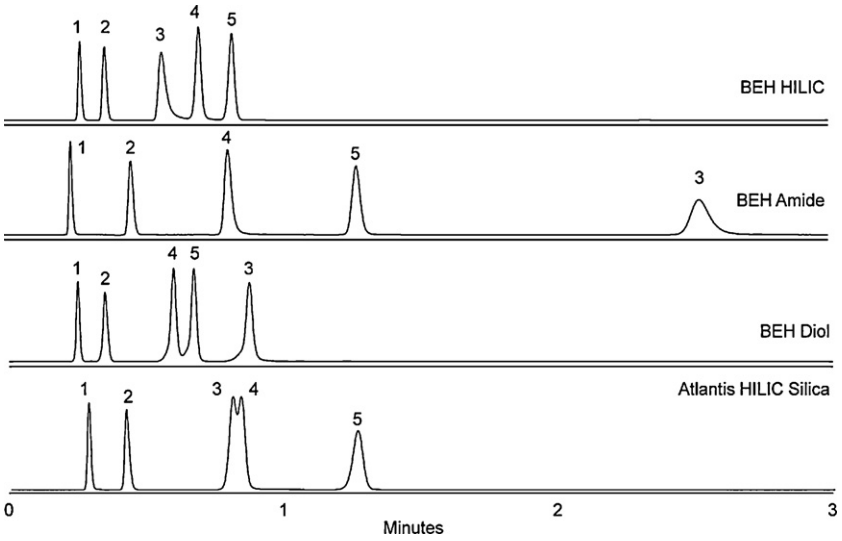

Fig. 10. Comparison of different HILIC stationary phases at pH 3. Column dimension: 50 mm × 2.1 mm ID and 1.7 μm particle size, 3 μm for Atlantis HILIC Silica. Mobile phase: ACN/water (90/10, v/v) containing 10 mM ammonium formate (pH 3). Flow rate 0.5 mL/min. Column temperature 30 °C. Peaks: (1) acenaphthene (void marker), (2) thymine, (3) 5-fluoroorotic acid, (4) adenine, and (5) cytosine.
Adapted from Ref. [76] with permission.

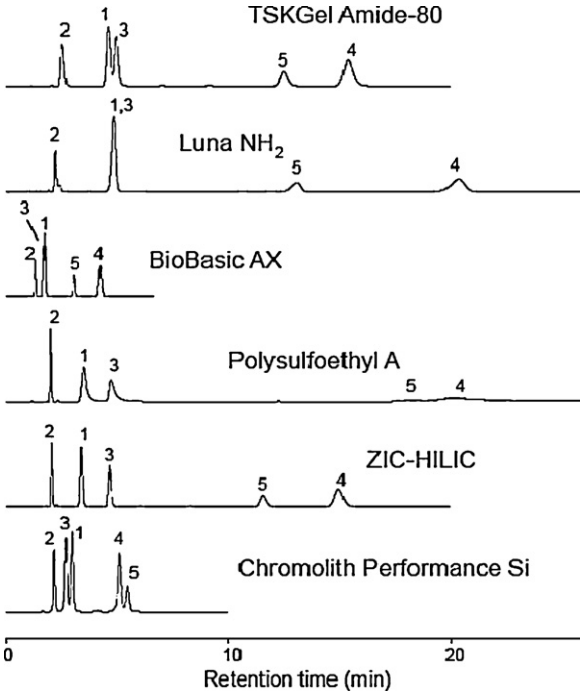

Fig. 11. Separation of nucleosides on various HILIC phases. Column dimension: 100 mm × 4 mm ID and 5 μm particle size, 100 mm × 4.6 mm ID for Chromolith Performance Si with a macropore diameter of 2 μm and a mesopore diameter of 13 nm. Column temperature 25 °C. Mobile phase: ACN/5 mM ammonium acetate buffer (90/10, v/v), apparent pH ~8. The flow rate was adjusted to the same linear velocity (1.7 mm/s). Peaks: (1) adenosine, (2) thymidine, (3) uridine, (4) guanosine, and (5) cytidine.
Adapted from Ref. [77] with permission.

pounds (Fig. 13b), on basic compounds (Fig. 13c) and on amino acids (Fig. 13d). With all the test compounds considered, PC1 and PC2 together accounted for about 85% of all the variance (Fig. 13a). All the positively charged phases were grouped together and essentially weighted by the acidic test compounds. On the other hand, the diol phase (Luna DIOL and Pursuit Diol), silica phase (Pursuit Si and Upt Si) and amide phase (TSKgel Amide 80) were grouped together. The sulfobetaine phase (ZIC-HILIC) and cyano phase (Upt CN) were

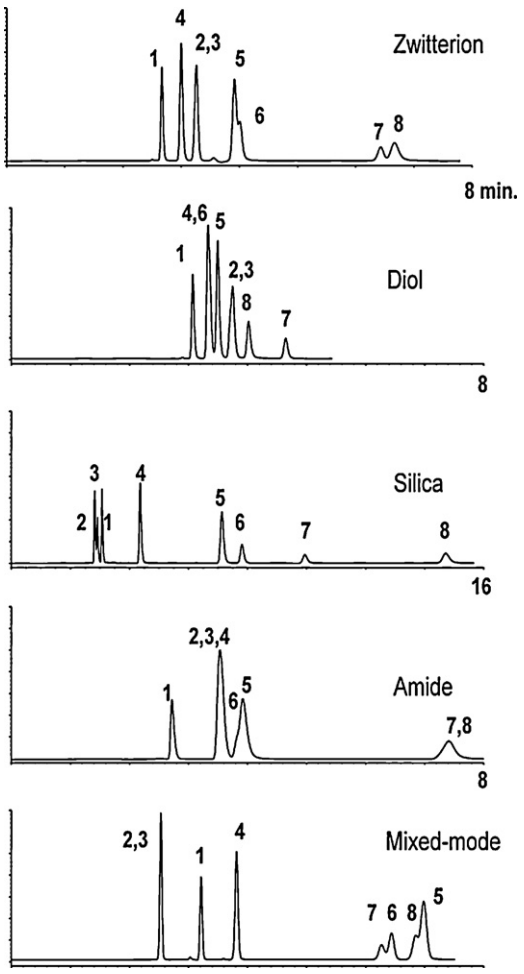

Fig. 12. Comparison of five HILIC columns. Column dimension: 250 mm × 4.6 mm ID and 5 μm particle size. Mobile phase: ACN/5 mM ammonium formate (85/15, v/v, pH 3.0). Column temperature 30 °C. Flow rate 1 mL/min. Peak identities: (1) phenol, (2) 2-naphthalenesulfonic acid), (3) p-xylenesulfonic acid), (4) caffeine, (5) nortriptyline, (6) diphenhydramine, (7) benzylamine, (8) procainamide.
Adapted from Ref. [67] with permission.

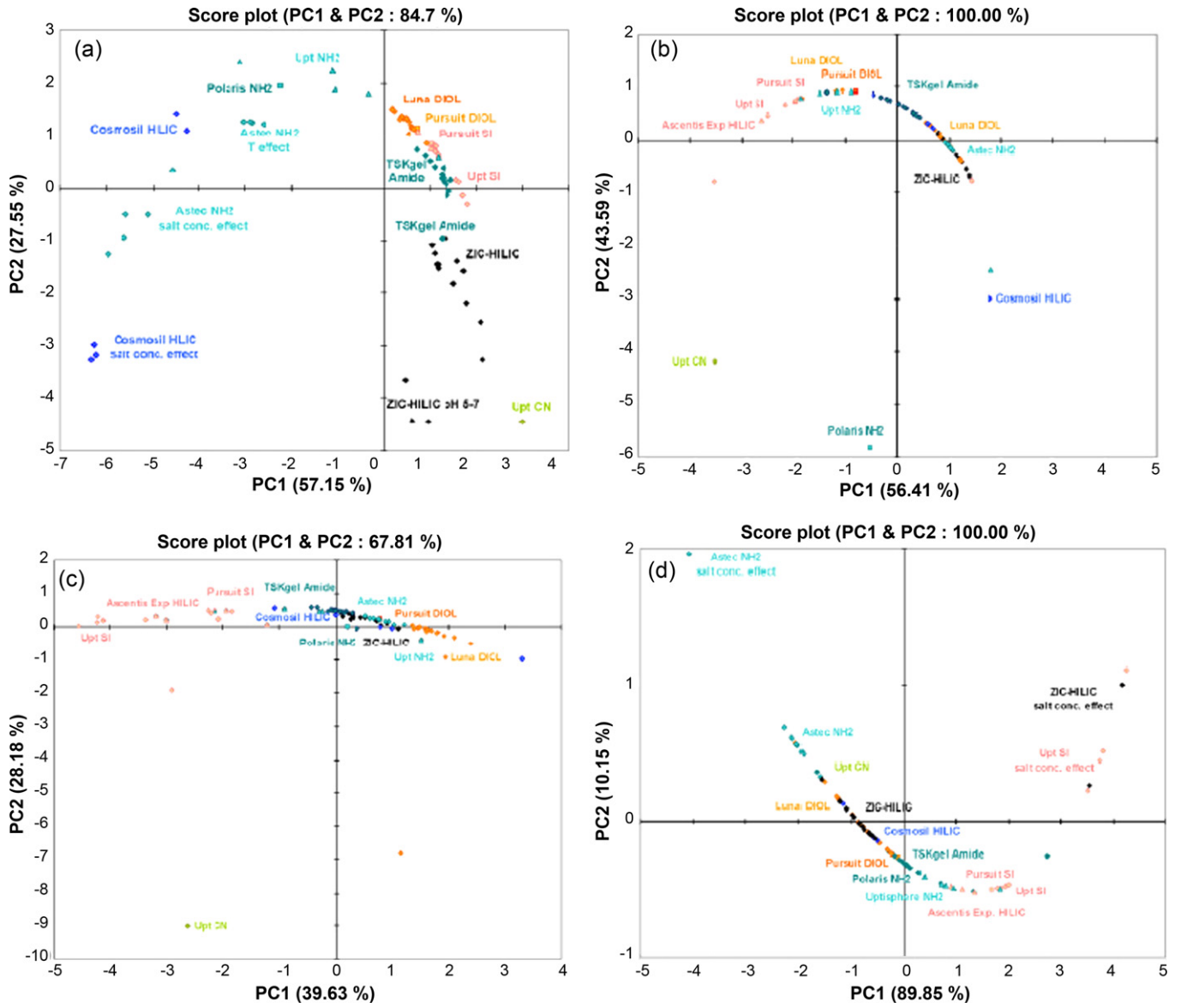

Fig. 13. Principal component analysis based on the retention data ($\log k'$) measured on 12 HILIC phases in all operating conditions: (a) PC1–PC2 score plot based on the retention data of all compounds (A, DA, S, HVA, DOPAC, 5HIAA, DOPA, Try and Trp), (b) PC1–PC2 score plot based on the retention data of anionic acidic compounds (HVA, 5HIAA and DOPAC), (c) PC1–PC2 score plot based on the retention data of cationic basic compounds (A, DA, S, 3-MT, NA and DHBA), and (d) PC1–PC2 score plot based on the retention data of zwitterionic amino acids (DOPA, Tyr and Trp).
Adapted from Ref. [54] with permission.

clearly separated from the other phases and also from each other. Grouping of the phases changed if PCA was performed on only the acidic or basic compounds. For example, the three positively charged phases (Upt NH_2, Cosmosil HILIC and Polaris NH_2) were separated, but all the other phases were more grouped together using the acidic compounds (Fig. 13b). When the basic compounds were used, the sulfobetaine phase (ZIC-HILIC) could be grouped with 2 diol phases (Luna DIOL and Pursuit DIOL) and 4 positively charged phases, but was separated from the silica phases on the first principal component (Fig. 11c). For amino acids, however, the cyano phase was less separated from the other neutral phases (the amide and diol phases) and the sulfobetaine phase (ZIC-HILIC); the silica phases were also less separated from the positively charged phases (Fig. 13d).

Dorpe et al. investigated column comparison and clustering of HILIC columns using peptides as test compounds [78]. The HILIC columns in this study covered a wide range of stationary phase chemistry, including amide phase (TSKgel amide-80), aspartamide phase (Polyhydroxyethyl A), diol phase (Grom Sapphire diol), sulfobetaine phase (ZIC-HILIC) and silica phase (Alltima HP HILIC and Atlantis HILIC). In addition to PCA, hierarchical clustering analysis (HCA) was also applied to the comparison and clustering of various polar phases. It should be pointed out that PCA and HCA were not performed directly on the retention data, but on various resolution measurements including geometric mean resolution, resolution product, time corrected resolution product and chromatographic response function (CRF). Other chromatographic measurements (asymmetry factor, LOD, plate number and peak capacity) were also used in PCA and HCA. Therefore, the comparison and clustering were based on the overall performance of the column, not only the selectivity of various polar phases. The dendrogram in Fig. 14(top) shows that the amide and diol phases are clustered together due to their similarity, but the sulfobetaine phase (ZIC-HILIC) is separated from the other polar phases. It is interesting to note that one of the silica phases (Alltima HILIC) is clustered more closely to the aspartamide phase than the other silica phase (Altantis HILIC).

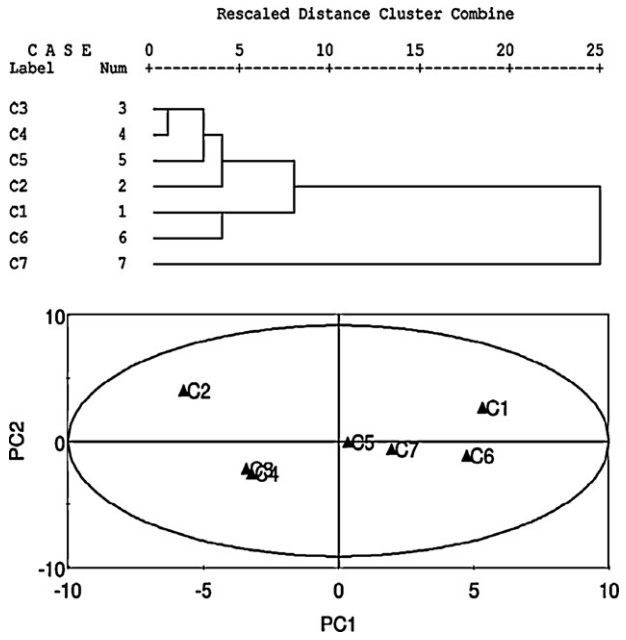

Fig. 14. Dendrogram based on the multiple responses using average linkage within groups from hierarchical clustering analysis (top), and PC1–PC2 score plot based on measured responses for 7 HILIC columns from principal component analysis (bottom). C1: TSKgel Amide-80 column (250 mm × 2.0 mm ID). C2: Alltima HP HILIC (250 mm × 2.1 mm ID). C3 and C4: Polyhydroxyethyl A (200 mm × 2.1 mm ID, 100 Å for C3 and 300 Å for C4). C5: Atlantis Silica (150 mm × 2.1 mm ID). C6: Grom Sapphire diol column (150 mm × 2.0 mm ID). C7: ZIC-HILIC column (150 mm × 2.1 mm ID).
Adapted from Ref. [78] with permission.

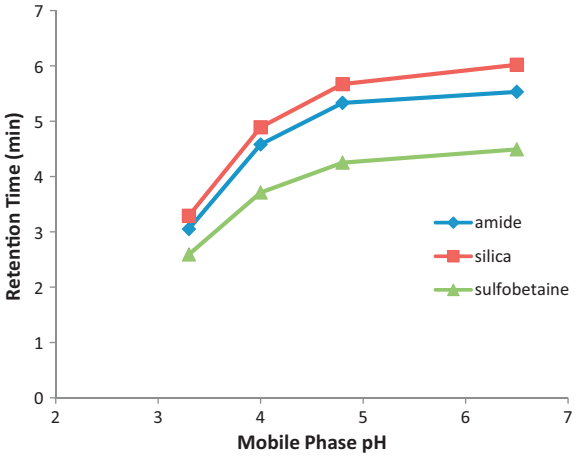

Fig. 15. The effect of mobile phase pH on the retention of acetylsalicylic acid on (♦) amide, (■) silica, and (▲) sulfobetaine phase. Mobile phase, acetonitrile/water (90/10, v/v) containing 10 mM ammonium formate. Mobile phase pH is the pH values of ammonium formate solutions. Column temperature 30 °C.
Adapted from Ref. [66] with permission.

In comparison, the score plot based on PCA as shown in Fig. 14 (bottom) displays distinct clustering of the polar phases. First, the Alltima HILIC silica and Polyhydroxyethyl A columns are apart from other columns and also from each other. Secondly, the Altantis HILIC silica column is close to the sulfobetaine phases (ZIC-HILIC). Third, the amide phase (TSK Amide-80) and diol phase (Grom Sapphire diol) could be grouped together, which is consistent with the clustering based on HCA.

4.2. Effect of experimental factors on retention

In addition to stationary phase chemistry, other experimental parameters (e.g., organic solvents, mobile phase pH, salt concentration, and column temperature) can also have significant impact on the retention and selectivity of polar compounds in HILIC. The effect of organic solvent content on the retention in HILIC has been well investigated in association with retention mechanism discussion [2]. Hao et al. also reviewed the effect of mobile phase composition and column temperature on the retention and selectivity in HILIC [15]. Since many polar compounds in HILIC separations can be charged at particular mobile phase pH values, electrostatic interaction is potentially a very important phenomenon in HILIC and could contribute significantly to the retention [66,79]. The electrostatic interaction can be modulated by the mobile phase pH and salt concentration. Therefore, this section focuses on the effect of mobile phase pH and salt concentration on the retention of polar compounds in HILIC.

5. Effect of mobile phase pH

Mobile phase pH is an important chromatographic factor since it can affect the charge state of both the stationary phase and polar solutes. Generally speaking, charged solutes are more hydrophilic than their neutral form and thus more strongly retained in HILIC. Fig. 15 shows the retention time of acetylsalicylic acid at various mobile phase pHs on the amide, silica and sulfobetaine phases [66]. The retention time of acetylsalicylic acid increased with the mobile phase pH from 3.3 to 4.8, and then leveled off. Acetylsalicylic acid has a pK_a of 3.5, and higher mobile phase pH resulted in more negatively charged species due to deprotonation, leading to stronger retention. The electrostatic repulsion between negatively charged acid and negative charges on the packing surface would result in a decrease in retention. Chirita et al. also observed a similar retention increase for some negatively charged neurotransmitters (homovanillic acid, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindole-3-acetic acid) on the amide, silica and sulfobetaine phases as the mobile phase pH increased from 3 to 6.5 [54]. Stalikas and co-workers investigated the effect of mobile phase pH on the retention of water soluble vitamins on a diol phase [69]. The charge state of nicotinamide, pyridoxine and riboflavin was not affected by the mobile phase pH, therefore the retention remained unchanged in the pH range used in the study (pH 3–6). An increase in retention was observed for nicotinic acid (pK_{a1} ~ 2.2) and L-ascorbic acid (pK_{a1} ~ 4.1) at higher pH due to increasing deprotonation. The mobile phase pH is also an effective means to achieve desired separation through changing solute selectivity [36]. As shown in Fig. 16, co-elution occurred for early eluting water soluble vitamins at lower pH 3 and 4, but the resolution was improved when the mobile phase pH increased to pH 5 and 6. It should be pointed out that the mobile phase pH in the above discussion refers to the pH values of the buffer solutions use to prepare the mobile phase. Both the mobile phase pH ($^{s}_{s}$pH) and solute pK_a ($^{s}_{s}$pK_a) are affected by the organic solvent (i.e., acetonitrile) added to the mobile phase [80,81]. In the pH range discussed above, the ionization of the acids is not likely to change significantly since both the mobile phase $^{s}_{s}$pH and solute $^{s}_{s}$pK_a are similarly affected by high acetonitrile content in the mobile phase. In addition to the charge state of the solutes, the charge state of the stationary phase can also be affected by the mobile phase pH, resulting in significant effects on the retention and selectivity in HILIC because the charged stationary phase can have electrostatic interactions (attractive or repulsive) with the charged solutes. Fig. 17 shows the retention time change with the mobile phase pH for acetylsalicylic acid and cytosine on the amino phase [66]. The retention time of cytosine decreased slightly from pH 6.5 to 4.8, but dropped significantly at the pH below 4.8. Cytosine has two pK_a values,

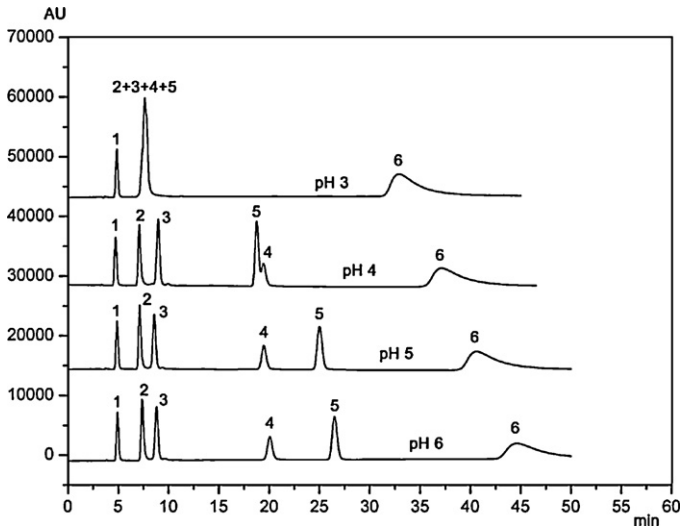

Fig. 16. The effect of pH on separation of selected water soluble vitamins on a diol phase (Inertsil HILIC, 150 mm × 4.6 mm ID, 5 μm particle size). Mobile phase: ACN/water (90/10, v/v) containing ammonium acetate 10 mM, with the aqueous buffer adjusted at various pH values. Flow rate 0.6 mL/min and column temperature 25 °C. Peaks: (1) nicotinamide, (2) pyridoxine, (3) riboflavin, (4) nicotinic acid, (5) L-ascorbic acid and (6) thiamine.
Adapted from Ref. [36] with permission.

$pK_{a1} \sim 4.6$ and $pK_{a2} \sim 12.2$ in water. At the mobile phase pH below pK_{a1}, cytosine became positively charged, inducing electrostatic repulsion from the positively charged amino phase and leading to reduced retention time below pH 4.8. For acetylsalicylic acid on the amino phase, the retention remained relatively unchanged in the range of pH 4–6.5; however, there was a large decrease in retention time below pH 4. With $pK_a \sim 3.5$, acetylsalicylic acid was negatively charged in the range of pH 4–6.5 due to deprotonation. There was presumably significant electrostatic attraction between the positively charged amino phase and negatively charged acid, which contributed to the overall retention. As the mobile phase pH approached its pK_a (3.3), the proportion of protonated acid increased, leading to reduced electrostatic attraction and retention. Moreover, the protonated acid was also less hydrophilic than the deprotonated form, which also resulted in reduced retention at low pH. Chirita et al. observed an increase in retention of the acidic neurotransmitters (homovanillic acid, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindole-3-acetic acid) on the positively charged amino and triazole phases when the mobile phase pH increased [54]. Presumably this observation reflects the same mechanism as with acetylsalicylic acid.

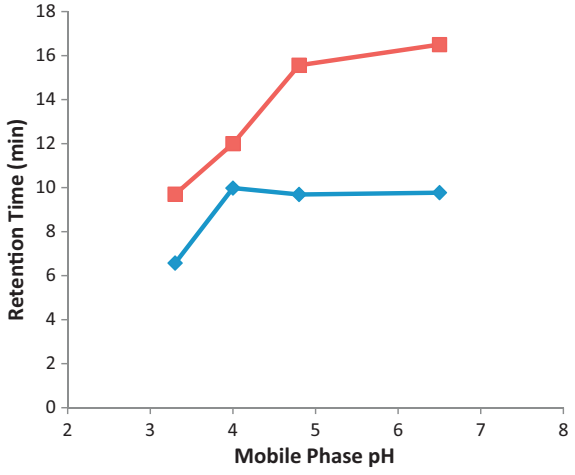

Fig. 17. The effect of mobile phase pH on the retention of acetylsalicylic acid and cytosine on an amino phase. Other conditions are the same as in Fig. 15. (■) Cytosine and (♦) acetylsalicylic acid.
Adapted from Ref. [66] with permission.

For the silica phase, the charge state can change depending on the mobile phase pH due to silanol ionization. At higher pH (pH > 4–5), the surface silanol groups are deprotonated, rendering the silica phase negatively charged. This change in the surface charge on the silica phase can have a significant effect on the retention of positively charged solutes in HILIC. For example, the retention of epirubicin and its analogues was found to increase from pH 2.4 to 4.2 [82]. As the mobile phase pH increased from 2.4 to 4.2, the proportion of deprotonated silanol groups increased, which led to increasing electrostatic attractions between the positively charged solutes and negatively charged silica surface. When the mobile phase pH increased further to 6.5, approaching the pK_a values of the solutes, the degree of positive charge on the solutes decreased. This resulted in reduced hydrophilicity of the solute and electrostatic attraction to the negative charged silica phase, leading to a decrease in retention. A similar observation was also made by Liu et al. for aminomethyl pyridine isomers on a silica phase [83]. Their retention increased as the mobile phase pH increased from 2.5 to 4.5 due to increasing electrostatic interaction between the positively charged solutes and increasingly negatively charged surface silanol groups. Chirita et al. observed not only longer retention time for basic neurotransmitters on the silica phase, but also peak shape deterioration with increasing mobile phase pH [54]. This phenomenon was attributed to the electrostatic interaction between the positively charged neurotransmitters and negatively charged surface silanol groups.

Silica-based neutral phases can also bear negative charge due to the presence of residual silanol groups on the silica surface. The amide phase has been found to possess negative charges in a particular range of mobile phase pH [66,79]. Consistent with this, neurotransmitters (both acidic and basic) were found to behave similarly on the amide phase and silica phase when the mobile phase pH was varied from 3 and 6.5 [54]. The diol phase was found to have significant silanophilic activity as well at pH 7 [38], indicating the presence of residual silanol groups on the silica surface [38,69]. As shown in Fig. 16, the retention of thiamine increased gradually on the diol phase as the mobile phase pH increased from 3 to 6 due to the presence of constant positive charge on thiamine. The electrostatic attraction between thiamine and deprotonated silanol groups increased, leading to an increase in retention as the level of deprotonation increased with increasing mobile phase pH. The retention of ascorbic acid was also found to increase in the same pH range, but this increase was likely due to increasing ionization of ascorbic acid ($pK_a \sim 4.1$) as the mobile phase pH increased from 3 to 6.

5.1. *Effect of salt type and concentration*

As discussed above, the presence of charges on the polar stationary phases can lead to significant electrostatic interactions with the charged solutes, which in turn can have significant impact on the retention and selectivity in HILIC. Early research indicated that the absence of buffer salts in the mobile phase resulted in excessively long retention and very broad peaks [84]. Organic soluble salts are needed, such as triethylammonium phosphate, sodium methylphosphate and ammonium acetate, due to the presence of high organic content in the mobile phase. The effect of different ammonium salts on the retention of polar compounds was investigated for acetylsalicylic acid and cytosine on various polar phases (amide, amino, silica and sulfobetaine phases) [66]. Ammonium acetate and formate did not show significant differences in the

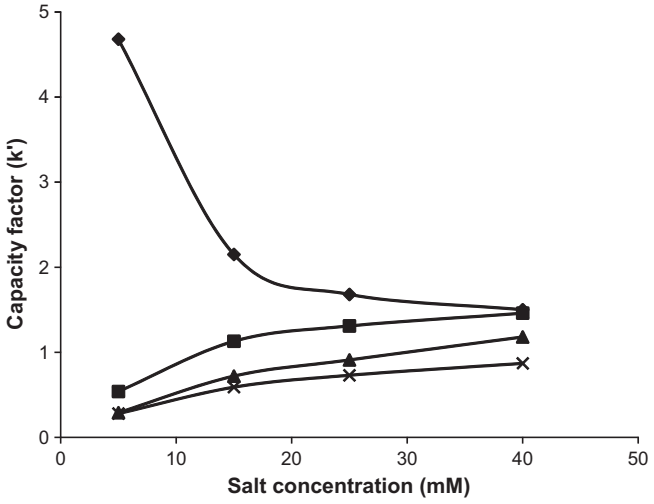

Fig. 18. The effect of salt concentration on the retention of salicyluric acid on (♦) amino, (■) amide, (▲) silica, and (×) sulfobetaine phase. Column temperature 30 °C. The mobile phase: ACN/water (80/20, v/v) containing 5–40 mM ammonium acetate. Adapted from Ref. [75] with permission.

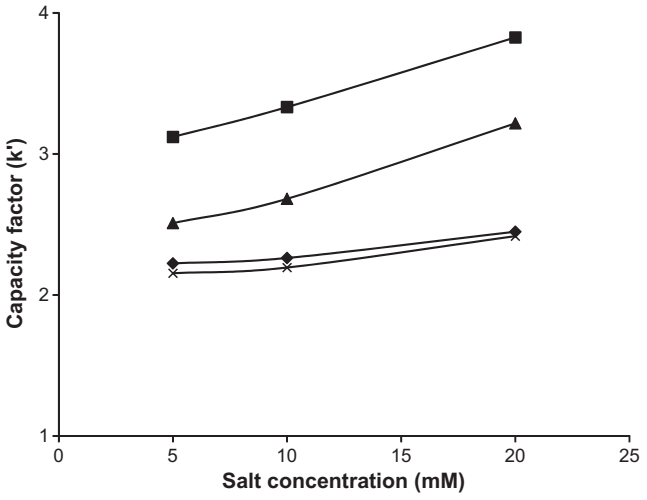

Fig. 19. The effect of salt concentration on the retention of cytosine on (♦) amino, (■) amide, (▲) silica, and (×) sulfobetaine phase. Column temperature 30 °C. The mobile phase: ACN/water (85/15) containing 5–20 mM ammonium acetate. Adapted from Ref. [75] with permission.

retention of cytosine on the four phases and acetylsalicylic acid on the amide, silica and sulfobetaine phases. However, ammonium formate provided significantly longer retention time for acetylsalicylic acid than ammonium acetate on the amino phase. This might be due to the difference in elution strength of formate and acetate since there was significant electrostatic attraction between the negatively charged acid and positively charged amino phase. Ammonium bicarbonate, on the other hand, was found to effect a considerable decrease in retention of acetylsalicylic acid, but has little effect on cytosine on the four phases. Significant differences between ammonium acetate and formate were also observed by Stalikas and co-workers [69]. Ammonium formate provided stronger retention for the acidic water soluble vitamins (i.e., nicotinic acid and L-ascorbic acid) than ammonium acetate on a diol phase due to different eluting strength of formate and acetate ions. Ammonium chloride was also found to reduce the retention of the acidic water soluble vitamins, possibly due to stronger competition by chloride ions than from deprotonated silanol groups for ammonium ions, resulting in stronger electrostatic repulsion. Chirita et al. found no differences between ammonium formate and acetate for catecholamines on various polar phases (i.e., polyamine, cyano, cross-linked diol, and triazole phases) [54]. However, small differences in selectivity were observed on the amide and sulfobetaine phases when the acetate salt was replaced with formate. More interestingly, no difference between the acetate and formate salt was found on one silica phase (Upisphere HILIC Silica), but significant differences were observed on the fused-core porous silica phase (Ascentis Expresss HILIC). The acetate salt caused longer retention and deterioration of the peak shape.

The presence of buffer salt in the mobile phase can effectively reduce the electrostatic interactions (both attractive and repulsive) between charged solutes and stationary phases in HILIC. In the case of electrostatic attraction, an increase in the salt concentration leads to reduced retention of charged solutes on stationary phases of opposite charge. The opposite effect is observed in the case of electrostatic repulsion: an increase in the salt concentration results in increased retention of charged solutes on stationary phases with the same charge. The salt concentration in this review refers to the final concentration in the mobile phase. Fig. 18 shows the effect of ammonium acetate concentration on the retention of salicyluric acid on the amide, amino, silica and sulfobetaine phases [75]. Retention on the amino phase decreased significantly as the salt concentration increased from 5 to 40 mM due to reduced electrostatic attraction between the negatively charged acid and positively charged amino phase. In contrast, a modest increase in the retention of salicyluric acid was observed on the amide, silica and sulfobetaine phases at least partially due to diminished electrostatic repulsion between the acid and negative charges on the surface of these stationary phases. Chirita et al. observed a decrease in the retention of noradrenaline on the amide phase and 3,4-dihydroxyphenylacetic acid on a polyamine phase (Astec apHera NH_2) when the ammonium formate concentration increased from 10 to 150 mM due to decreased electrostatic attraction [54]. A slight increase in retention was observed for 3,4-dihydroxyphenylacetic acid on the amide phase and for noradrenaline on the polyamine phase due to decreased electrostatic repulsion. Stalikas and co-workers reported a significant decrease in the retention of thiamine on the diol phase when the ammonium acetate concentration increased from 5 to 20 mM, again due to a decrease in electrostatic attraction [69].

In addition to modulating the electrostatic interactions between charged solutes and stationary phases, the salt concentration was also found to have an effect on the retention in a case where electrostatic interaction was apparently absent [66]. Fig. 19 shows the effect of ammonium acetate concentration on cytosine on the amino, amide, silica and sulfobetaine phases. A small increase in retention was found on all the phases when the salt concentration increased from 5 to 20 mM. Under the experimental conditions, cytosine was not charged, although the amino phase was positively charged and the amide, silica and sulfobetaine phases had negative charges. Stalikas and co-workers also reported a small increase in retention for riboflavin and pyridoxine on the diol phase [69]. Under the experimental conditions (pH ~ 5), riboflavin ($pK_{a1} \sim 1.9$ and $pK_{a2} \sim 10.2$) was in neutral form and pyridoxine ($pK_{a1} \sim 5.0$ and $pK_{a2} \sim 8.9$) would be partially positively charged. Obviously, electrostatic attraction could not explain the increase in the retention of pyridoxine on the diol phase. The cross-linked diol phase (Luna Diol) has been shown to have minimal silanophilic activity at pH 7 [54]. A small increase in retention was observed for both positively (tyrosine and noradrenaline) and negatively (3,4-dihydroxyphenylacetic acid) charged neurotransmitters on the cross-linked diol phase when the concentration of ammonium formate increased from 10 to 150 mM. In all the cases discussed above, the effect of the salt concentration on retention cannot be

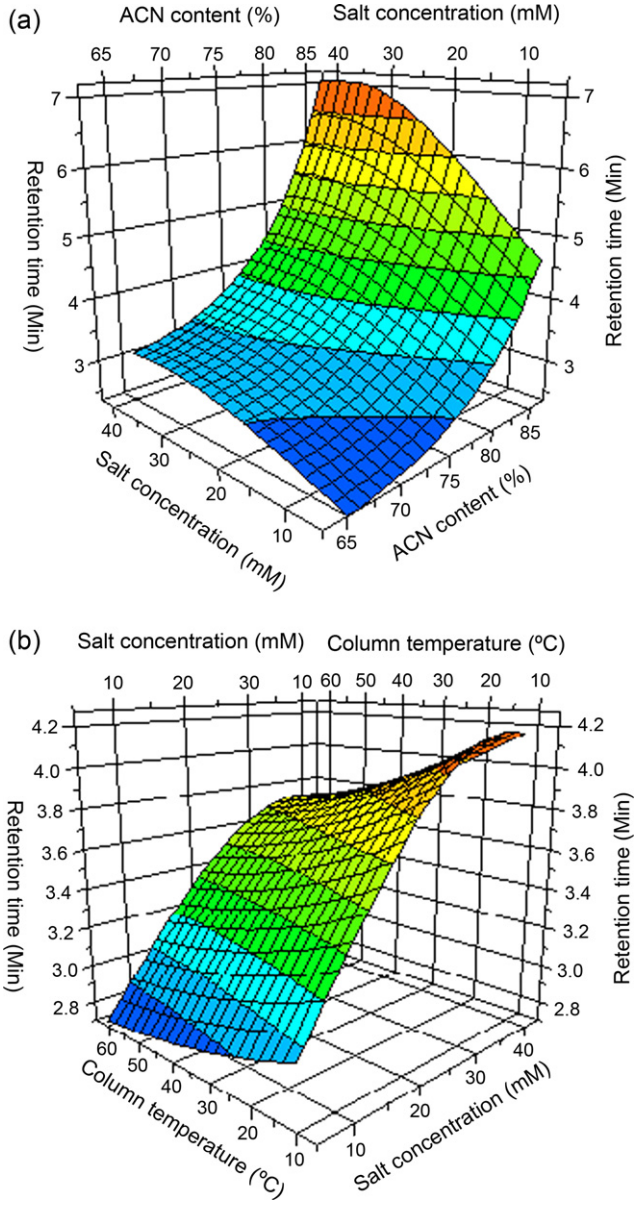

Fig. 20. Response surface for salicyluric acid on the aspartamide phase (Polyhydroxyethyl A column).
Adapted from Ref. [85] with permission.

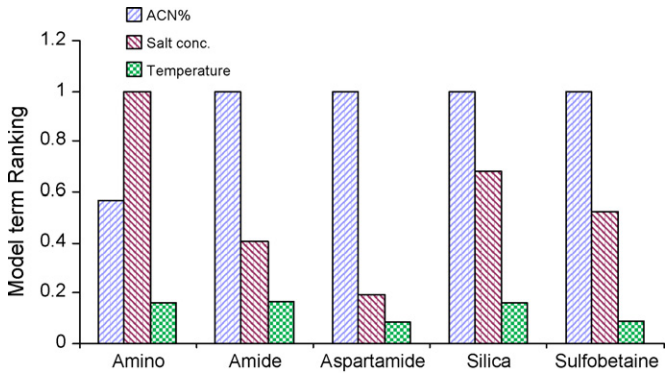

Fig. 21. Model variable ranking chart based on the retention data of salicyluric acid for various polar stationary phases.
Adapted from Ref. [85] with permission.

explained by electrostatic interaction. The increase in retention with the salt concentration seems to be a general trend in HILIC. It has been proposed that higher salt concentration might increase the volume of the immobilized liquid layer on the stationary phase, thus leading to stronger retention [66]. Although this hypothesis is consistent with the hydrophilic partitioning model, there is no direct experimental evidence to confirm the effect of increasing salt concentration on the water-rich liquid layer.

The investigation on the effect of salt concentration was typically conducted at a fixed column temperature using a mobile phase containing a fixed level of organic solvent, leaving the salt concentration as the only experimental variable. Such studies do not provide any insights into the inter-relationship between the salt concentration and other chromatographic factors, such as organic solvent content and column temperature. A design of experiment (DOE) approach has been applied to investigate the effect of multiple chromatographic factors (e.g., acetonitrile content, column temperature, and salt concentration) on the retention of polar compounds on various stationary phases [85,86]. Fig. 20 shows the response surfaces for salicyluric acid on the aspartamide phase. Similar response surfaces were obtained for the amino, amide, silica and sulfobetaine phases [85]. The response surfaces demonstrate that the effect of salt concentration on retention was not constant in the range of acetonitrile content and column temperature under investigation. The salt concentration seems to have more significant effects on retention at higher acetonitrile content and lower column temperature. Furthermore, the results from the DOE studies revealed the relative importance of the chromatographic factors (e.g., acetonitrile content, column temperature, and salt concentration) in HILIC. As shown by the variable ranking chart in Fig. 21, acetonitrile content had the most significant effect on the retention of salicyluric acid on the amide, aspartamide, silica and sulfobetaine phases, which implicates hydrophilic interaction as the major mechanism for retention. In contrast, the salt concentration was the most significant factor with the amino phase. Here, electrostatic interaction was a major factor in the retention of the acid. It is interesting to note that the salt concentration seems to be more significant than the column temperature in the retention of salicyluric acid on all the amide, aspartamide, silica and sulfobetaine phases. This is possibly due to electrostatic interaction between the acid and negative charges on the surface of these stationary phases, although the hypothetical effect of salt on the immobilized aqueous layer may play some role as well. Quiming et al. also found the salt concentration significant to the retention of uric acids on the diol phase, but less important than the column temperature, which might be attributed to the absence of electrostatic interaction between the neutral uric acids and any charge on the diol phase [86].

6. Conclusion

As more and more stationary phases become available for HILIC separation of polar compounds, it becomes increasingly important to have a better understanding of the retention and selectivity characteristics of these stationary phases. The relative retentivity of a stationary phase needs to be assessed experimentally since it is difficult to judge based on the structure of the functional group. The electrostatic interaction between charged solutes and stationary phases can alter the relative retentivity. The experimental data indicate that most of the stationary phases have similar retentivity towards neutral solutes despite large differences in the functional groups; however, the relative retentivity may change for charged solutes on charged stationary phases. The selectivity of the stationary phases can be very different for various types of solutes. This provides an opportunity to find the right stationary phase to achieve a desired separation.

In addition to identifying the right column, other experimental conditions also need to be optimized for desired methods. The type and content of organic solvents need to be evaluated and optimized first since this factor has the most significant effect on the retention and selectivity in HILIC. As demonstrated in this review, the mobile phase pH and salt concentration have a critical impact on retention and selectivity in HILIC since many polar compounds can be charged in a particular pH range. Special attention should be paid to the mobile phase pH and salt concentration in HILIC method development, especially when the solutes and/or stationary phases are charged. The experimental design approach can be used to optimize multiple experimental parameters and improve the robustness of the final method, but is rarely used in developing HILIC methods.

Most HILIC columns are packed with 5 μm particles, and the number of HILIC columns packed with smaller particles (3 μm) is still limited (e.g., Amide-80 and Polyhydroxyethyl A columns). Silica columns are not only available in the 3 μm particle size but also sub-2 μm particles (e.g., BEH HILIC column), which can provide faster separation when run on UHPLC instruments [24,87]. While the introduction of new stationary phases for HILIC continues, future development is likely to be more focused on reducing particle size and improving separation efficiencies in HILIC. As more and more reversed-phase columns are packed with sub-2 μm particles for UHPLC application, HILIC columns will not be far behind. In addition to underivatized silica phase, other types of stationary phases based on the fused-core silica particles are likely to be developed in the near future, thus providing more options for selectivity. Both silica-based and organic polymer monolithic columns have been developed for HILIC applications [88,89]. However, most of the applications employed capillary columns, which can limit the application of the monolithic columns. It is possible that some of the existing HILIC phases as shown in Table 1 will be developed in the wide-bore monolithic format, and new types of polymer based phases will continue to be developed in the monolithic columns. The development of HILIC columns with small particle size (sub-2 μm), the fused-core silica based stationary phases, and monolithic columns will make it possible to perform fast analysis while maintaining separation efficiency in HILIC.

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