CHAPTER

1

SEPARATION MECHANISMS IN HYDROPHILIC INTERACTION CHROMATOGRAPHY

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1.1 INTRODUCTION

Hydrophilic interaction chromatography (HILIC) is a technique that has become increasingly popular for the separation of polar, hydrophilic, and ionizable compounds, which are difficult to separate by reversed-phase chromatography (RP) due to their poor retention when RP is used. HILIC typically uses a polar stationary phase such as bare silica or a polar bonded phase, together with an eluent that contains at least 2.5% water and >60% of an organic solvent such as acetonitrile (ACN). However, these values should not be regarded as definitive of the rather nebulous group of mobile and stationary phase conditions that are considered to constitute HILIC. Figure 1.1 shows the number of publications on HILIC between the years 1990 (when the term was first employed) and 2010 according to the Web of Knowledge [1] using the search terms "HILIC" or "hydrophilic interaction (liquid) chromatography." For the first 12 years or so, the number of publications remained between 1 and 15, but after this period, interest increased rapidly from 19 publications

Hydrophilic Interaction Chromatography: A Guide for Practitioners, First Edition.
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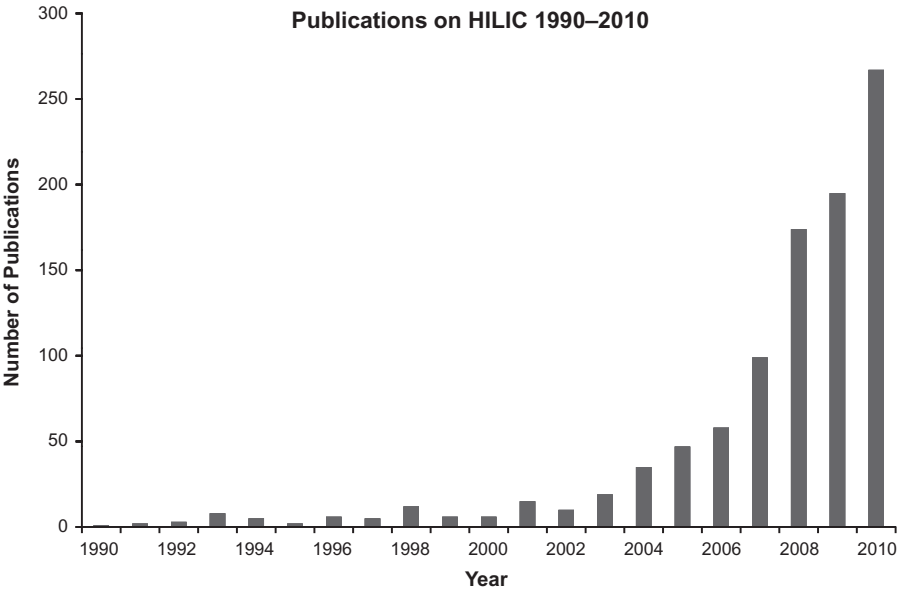

Figure 1.1. Yearly publications containing the terms "hydrophilic interaction chromatography" or "hydrophilic interaction liquid chromatography" or HILIC according to Thomson Web of Knowledge [1].

in 2003 to 267 in 2010. While HILIC has unique retention characteristics for hydrophilic compounds, this increase in interest also reflects the advantages of HILIC over RP methods in situations where either technique is applicable. These advantages result mostly from the high organic content of typical mobile phases and their resultant high volatility and low viscosity. A particular advantage is in coupling HILIC to mass spectrometry (MS) as mobile phases are more efficiently desolvated in interfaces such as electrospray, giving rise to better sensitivity than with RP methods. Thus, Grumbach and coworkers demonstrated sensitivity increases of 3–4 orders of magnitude when comparing the analysis of the drugs salbutamol and bamethan by HILIC on a bare silica column using a gradient analysis starting at 90% ACN with that on a C18 RP column using a gradient starting at 0% ACN [2]. Columns can be used at considerably lower pressures than in RP; the viscosity of 80–90% ACN mixtures with water as typically used in HILIC is only about half that of 20–30% ACN mixtures that might be used in RP separations [3]. Alternatively, longer columns can be used at pressures typically found in RP analysis, allowing high efficiencies to be obtained [4]. For example, when combining the low viscosity of HILIC with the efficiency gains shown by superficially porous (shell) particle columns, it is possible to generate column efficiencies in excess of 100,000 plates with reasonable analysis times, and using pressures that are well within the capabilities of conventional HPLC systems (pressure $<<$ 400

bar). Low viscosity also results in increased solute diffusion in the mobile phase, giving rise to smaller van Deemter C terms and improved mass transfer, and the possibility of operating columns at high flow rates with reduced losses in efficiency for fast analysis [5]. Surprisingly good peak shapes can be obtained for some basic compounds. For example, efficiencies of around 100,000 plates/m with asymmetry factors (A_s) close to 1.0 were reported for basic drugs such as nortritpyline (pK_a ~10) using a 5-μm particle size bare silica HILIC phase. In comparison, such solutes often give rise to peak asymmetry in RP separations.

A separate advantage of HILIC is its compatibility with sample preparation methods using solid-phase extraction (SPE). Some such methods incorporate an elution step that uses a high concentration of an organic solvent, which gives rise to a potential injection solvent of the eluate that is stronger than typical RP mobile phases [2]. This mismatch in solvent strengths can give rise to peak broadening or splitting, necessitating evaporation of the SPE eluate and reconstitution in the mobile phase. SPE eluates with high organic solvent concentrations can be injected directly in HILIC, as they are weak solvents in this technique. The combination of different retention mechanisms in sample purification and analysis steps (HILIC/RP) can be advantageous in giving extra selectivity compared with an RP/RP procedure, where in some cases the SPE column may act merely as a sort of filter for the analytical column [6].

While HILIC is simple to implement in practice, some recent papers have concluded that the separation mechanism is a complex multiparametric process that may involve partition of solutes between a water layer held on the surface and the bulk mobile phase, adsorption via interactions such as hydrogen bonding and dipole–dipole forces, ionic interactions, and even nonpolar retention mechanisms (similar to RP interactions), depending on the stationary and mobile phases [7–9]. In this chapter, we will consider in some detail the various mechanisms that contribute to HILIC separations.

1.2 HISTORICAL BACKGROUND: RECOGNITION OF THE CONTRIBUTION OF PARTITION, ION EXCHANGE, AND RP INTERACTIONS TO THE RETENTION PROCESS

The term “hydrophilic interaction chromatography” was coined in 1990 by Alpert [10]. He carefully avoided the acronym HIC to avoid confusion with the technique of hydrophobic interaction chromatography, the latter being an adaptation of the RP technique where decreasing salt concentrations are used to progressively elute large biomolecules from the stationary phase. However, it is possible that HILIC dates back to the earliest days of liquid chromatography, when Martin and Synge separated amino acids on a silica column using water-saturated chloroform as the mobile phase. These authors explained the separation mechanism as being the partitioning of the solutes between a water layer held on the column surface and the chloroform [11].

The silica was considered to act merely as a mechanical support. It later became clear that use of a solvent that is immiscible with water, such as chloroform, is not an essential requirement. Lindon and Lawhead [12] discussed the separation of sugars such as fructose, glucose, sucrose, melibiose, and raffinose on a micro-Bondapak carbohydrate column (an aminosilica column, 10 μm particle size). The mobile phase was ACN–water (75:25, v/v); the authors showed that increasing the concentration of water reduced the retention times of the sugars. The authors noted that while the α- and β-anomers of sugars are readily separated by gas-liquid chromatography, they were not separated by this LC method, removing an unnecessary complication. However, no explanation for this lack of separation, or for the retention mechanism, was presented. It was shown later that aminopropyl silica in the presence of ACN–water greatly increased the mutarotation rate of the sugars compared with the effect of bare silica [13]. This effect is due to the basic environment generated in the column pores by the presence of the amino groups [14]. With a refractive index detector, it was shown that water was retained on the aminopropyl silica when pumping mobile phases of ACN–water and that the volume fraction of water in the liquid associated with the stationary phase was much higher than that in the corresponding eluent. The extent of water enrichment in the stationary liquid was found to be relatively high when the eluent contained a low water concentration. The separation of the sugars was explained as being due to their partition between the water-rich liquid in the stationary phase and the bulk mobile phase. Using a similar experimental procedure, other workers showed a reduced uptake of water on an aminosilica column when methanol–water was used as the mobile phase compared with ACN–water, as the competition between water and methanol for polar sites on the column was increased [15].

While the reports on sugar analysis were clearly classical HILIC separations in their use of a polar phase together with an ACN–water mobile phase containing a high concentration of organic solvent, a number of other early papers using bare silica columns demonstrated separations that contain at least some of the mechanisms that are now considered contributory to HILIC. Bidlingmeyer and coworkers [16] separated organic amines on a silica column using "reversed-phase eluents" consisting typically of ACN–water (60:40, v/v) containing ammonium phosphate buffer, pH 7.8. They showed that increasing the salt concentration decreased the retention of ionized basic compounds, indicating the contribution of ionic retention to the overall mechanism. It was demonstrated that over the range 70–30% ACN, if buffer strength and pH were held constant, retention decreased with increasing proportion of ACN as would be expected in a reversed-phase separation. Good peak symmetry was obtained for basic compounds on these bare silica columns. The authors concluded from a comparison with RP that the key to good peak shapes with these solutes was not the presence or absence of silanols but more probably the accessibility of these surface groups. Nevertheless, the concentrations of

ACN employed in this work were at the lowest end of the range generally used for HILIC separations, and it is questionable whether the important partition element of the HILIC mechanism was involved to any extent in such separations, as the mobile phase becomes more hydrophilic and thus competitive with the stationary phase. Other early work by Flanagan and Jane also showed the separation of basic drugs on bare silica columns, but this time using nonaqueous ionic eluents [17,18]. The nonaqueous, primarily methanolic eluents, contained additives such as perchloric acid or ammonium perchlorate of appropriate pH and ionic strength. The authors demonstrated that the retention of quaternary compounds increased with eluent pH, particularly in the pH range of 7–9, whereas the retention of bases decreased steadily at a high pH, where they were unprotonated. The observations were consistent with an ionic retention mechanism on ionized silanols. However, in the absence of water in the mobile phase, the conditions are clearly not consistent with those of HILIC. Euerby and coworkers [19] separated a variety of basic analytes on bare silica columns of varying metal content using buffered methanol and ACN mobile phases of again rather low organic concentration (typically 20–40%). Their experimental conditions were somewhat similar to those of Bidlingmeyer (organic solvent concentrations were lower than classic HILIC conditions), and their conclusions were also that ionic and hydrophobic mechanisms were the main contributors to retention.

Cox and Stout [20] studied the retention of a set of nitrogenous bases such as thiamine and morphine on some bare silica columns. Their work was inspired by the difficulties that were encountered in the separation of basic compounds using typical RP columns available at that time (mid-1980s), which often gave long and variable retention, poor separation efficiency, and excessive peak tailing. While their studies indicated that ion exchange was a major contributor to retention in these systems, they reported that the mechanism appeared to be more complex, incorporating more than a single retention process. Linear plots of retention factor versus the reciprocal of buffer cation concentration (see Section 1.3.3.4) were obtained with retention decreasing as the concentration of the buffer increased, indicative of an ion-exchange mechanism. These experiments were performed at low concentrations of methanol (15% or 30%). These are not typical HILIC conditions, and little, if any, contribution of a classical HILIC partitioning mechanism seems likely. However, all of the plots showed a positive intercept on the *y*-axis. For a pure ion-exchange mechanism, straight lines passing through the origin should be generated. The positive intercept of the plot was cited as evidence for a competing mechanism, which existed at an infinite buffer concentration (i.e., when the reciprocal of the buffer concentration is zero). The authors first considered changes in the ionization of the solutes with addition of the organic solvent that could have influenced the results, but discounted this hypothesis on the basis that morphine was a strong base and should be completely ionized. The authors therefore concluded that some nonionic interaction of the solute with

silanol groups might occur. It seems that this contributory mechanism might in fact be of the same nature as that suggested in Bidlingmeyer's work; that is, it is hydrophobic in origin [16]. Most of this work was carried out with low concentrations of methanol, that is, remote from classical HILIC conditions. However, a plot of k derived from retention at an infinite buffer concentration for thiamine and morphine against methanol concentration from 15–75% v/v showed a U-shaped plot with retention maxima shown at 15% and 75% methanol, the maximum at 75% methanol in hindsight perhaps being indicative of the onset of a HILIC retention process.

While the paper of Alpert [10] was clearly not the first to demonstrate analysis using HILIC conditions, it was certainly a landmark publication because of the quality of the separations demonstrated for peptides, nucleic acids, and other polar compounds, and its careful discussion of the separation mechanism. Alpert showed that retention of peptides on hydrophilic columns, including a strong cation exchange material, PolySulfoethyl A, and a (largely) uncharged material, PolyHydroxyethyl A, increased dramatically when concentrations of ACN greater than 70% were used and that the order of their elution was from the least to the most hydrophilic, that is, the opposite from the order in RP separations. For the cation exchange material, electrostatic effects were superimposed on the HILIC mechanism. In agreement with the conclusions of previous workers [13,15], Alpert interpreted the earlier retention of sugars on amino columns as being not due to any electrostatic effects but caused by the hydrophilic nature of the basic column groups, demonstrating that the separation of carbohydrates could also be performed on the neutral PolyHydroxyl A phase, albeit giving elution in doublets corresponding to the α- and β-anomers. Clearly, this neutral phase could not generate the alkaline mobile phase environment required to speed up the mutarotation of sugars. However, the problem was overcome by addition of a small amount of amine to the mobile phase to speed up the mutarotation process. In analogy with the partition mechanism that had previously been suggested for the separation of sugars, Alpert proposed that the same mechanism could also explain the separation of other classes of polar solutes, such as peptides and amino acids. He also cited the relatively small differences obtained in the separations of peptides between uncharged and charged stationary phases as the organic content of the mobile phase was increased as further evidence that partition was the dominant mechanism. As the partition contribution to retention is increased, the proportional contribution of ion exchange to the total retention is reduced. It was, nevertheless, clearly shown on the cation exchange phase that ionic retention effects could be superimposed on HILIC retention and could give useful selectivity effects. Alpert noted distinct similarities in the separation of thymidilic acid oligomers between HILIC and classical partitioning systems, citing this result as being further indicative of a partition mechanism in the chromatographic technique. He speculated that some form of dipole–dipole interactions might be involved, although retention of sugars had been shown to correlate better with their hydration number than with their potential to form hydrogen bonds [15].

1.3 RECENT STUDIES ON THE CONTRIBUTORY MECHANISMS TO HILIC RETENTION

1.3.1 Overview

As HILIC retention depends on the hydrophilicity of the solutes, attempts have been made to correlate this retention with physical descriptors of this property. Log P values represent the log of the partition coefficient when a solute is distributed between an aqueous phase and *n*-octanol, which in simple terms (using concentrations as an approximation for activities) can be written as

$$\log \mathrm{P} = \log([C_\mathrm{o}]/[C_\mathrm{w}]),$$

where C_o is the concentration of the compound in octanol, and C_w is the concentration of the compound in water. Strictly, log P refers to the distribution of the nonionized form of ionogenic compounds. Alternatively, the distribution coefficient D is defined as the equilibrium concentration ratio of a given compound in both its ionized and nonionized forms between octanol and water. The use of log D instead of log P requires knowledge or estimation of the pK_a of the compound to calculate its ionization at a particular pH. Kadar and coworkers [21] studied the application of log D values produced by the ACD (Advanced Chemistry Development Inc.) calculation program to the prediction of a compound's suitability for HILIC analysis. The ACD program generates estimations of log D for mono- or polyprotic acids and bases over the pH range of 0–14 with increments of 0.1 pH units [22]. The authors tested the hypothesis that a relationship exists between the analyte's retention factor, *k*, and its log D at pH 3.0. The value of pH 3.0 was chosen due to the consideration that the majority of active pharmaceutical ingredients are basic amines that will be protonated under acidic conditions, and that these conditions are frequently used for their analysis. In this work the authors assumed that a partially immobilized layer of water existed on the phase and that the pH of this immobilized water layer was 3.0. The authors debated at some length whether aqueous pH data ($^{\mathrm{w}}_{\mathrm{w}}$pH) should be used rather than $^{\$}_{\mathrm{w}}$pH values (where the pH is measured in the aqueous–organic solution). There is a considerable difference in these quantities when large concentrations of ACN are utilized, as in typical HILIC separations. However, due to the paucity of data concerning pK_a values in aqueous–organic mixtures, the authors decided to use $^{\mathrm{w}}_{\mathrm{w}}$pH and $^{\mathrm{w}}_{\mathrm{w}}pK_\mathrm{a}$ data. A further consideration not mentioned by the authors is that if a water layer exists on the column surface in HILIC, it is possible that data measured in water are more appropriate. For this work, the authors selected 30 probe compounds representative of pharmaceutical compounds used in the therapeutic areas of anti-infectives, cancer, cardiovascular and metabolic disease, and the central nervous system. They determined the retention factor of each compound experimentally using a bare silica HILIC column and a mobile phase consisting of 85%, 90%, or 95%

ACN containing a total ammonium formate buffer concentration of 10 mM at pH 3.0. Linear regression analysis of log k versus log D produced correlation coefficients of 0.751, 0.696, and 0.689 for 85%, 90%, and 95% ACN concentrations, respectively, giving relationships (for example with 85% ACN) of the form

$$\log k = -0.132\ (\log \mathrm{D}) - 0.234.$$

These equations could then potentially be used to predict the value of k for a given calculated log D value. The authors interpreted the deviations of the correlation coefficients from unity as being due to secondary interactions in addition to the partitioning that was initially assumed in the hypothesis to be the only retention mechanism on the bare silica column. In particular, they considered that electrostatic interactions would occur with negatively charged silanols, giving increasing retention for charged basic compounds relative to that expected for a pure partition mechanism. Conversely, charged acidic compounds should experience repulsion and therefore give retention less than expected from a pure partitioning mechanism. Indeed, they showed that the predicted k from log D values of several compounds that contained at least one basic functional group that was fully protonated at the experimental pH was significantly underestimated compared with the experimentally measured retention. The authors concluded that there is a direct correlation between a compound's HILIC retention and its distribution ratio, although an accurate prediction of k could not be made due to these secondary interactions. They concluded that the work also supports Alpert's theory of a partition mechanism to describe HILIC separations. Bicker and coworkers [23], who studied the retention of nucleosides and nucleobases on a series of silica packings bonded with neutral trimethoxysilylpropylurea ligands, obtained variable results with prediction of retention based on log D values. They cautioned that these predictions should only be regarded as a simplistic concept for estimating the relative strength of HILIC-type interactions because the underlying molecular processes of retention and their correlations with solute polarity are not sufficiently understood as yet. They reported severe limitations of the predictions in the case of charged solutes, where other types of interaction than a partition mechanism come into effect. West and coworkers [24] acquired retention data for 76 model compounds using two zwitterionic phases and $\frac{w}{w}$ pH 4.4 ammonium acetate buffer in 80% ACN, with an overall salt concentration of 20 mM. The coefficients of determination (r^2) of 0.70 and 0.87 for ZIC-HILIC and a Nucleodor phase, respectively, gave evidence according to the authors that hydrophilic partitioning was only one of the mechanisms involved in HILIC separations, and thus log D values could only give a rough estimate of retention. They argued that the relatively high salt concentrations used should have suppressed some ionic interactions of ionized stationary phase groups, possibly improving the correlation with log D. No particular groups of solutes (neutral, anionic, cationic, or zwitterionic) appeared to be responsible for the poor

correlation, as all were scattered more or less uniformly about the regression line. However, it seemed that the fit was poorest for solutes with low retention, where the accuracy of the measurement could be a factor.

Some reports have shown the separation of the same mixture of solutes on a number of different stationary phases, in studies designed to contribute to elucidation of the separation mechanism. Guo and Gaiki examined the retention characteristics of four polar silica-based stationary phases (amide, amino, silica, and sulfobetaine—a zwitterionic phase containing quaternary amine and sulfonic acid groups) using small polar compounds as solutes. The solutes studied included salicylic acid and derivatives, some nucleosides and nucleic acid bases, selected because they are usually difficult to retain on RP columns [25]. Figure 1.2 shows the separation of the salicylic acid derivatives on the four columns using ACN–water (85:15 v/v) containing 20 mM ammonium acetate as the mobile phase. The retention and elution order clearly varied

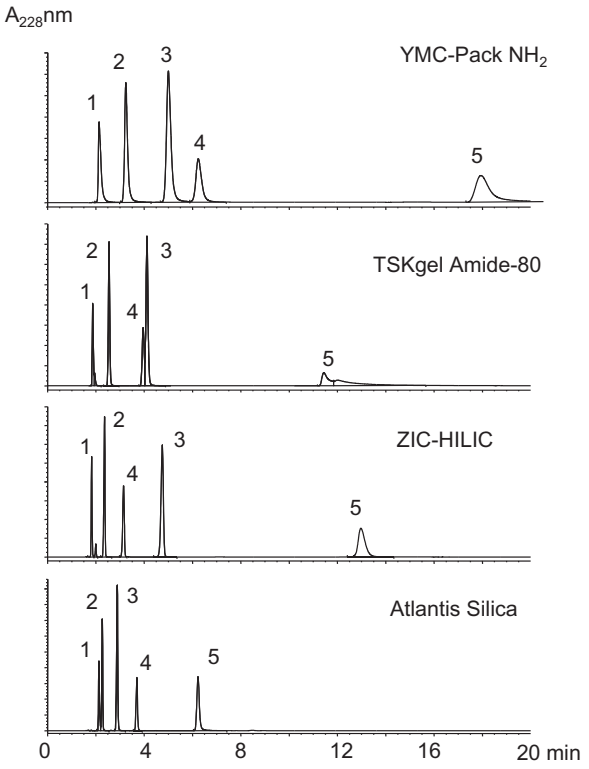

Figure 1.2. Separation of acidic compounds on four different columns. Mobile phase ACN–water (85:15, v/v) containing 20 mM ammonium acetate. Column temperature 30°C. Flow rate 1.5 mL/min. Ultraviolet (UV) detection. Compound identities: 1 = salicylamide, 2 = salicylic acid, 3 = 4-amino salicylic acid, 4 = acetylsalicylic acid, 5 = 3,4-dihydroxyphenylacetic acid. All columns 25 × 0.46 cm containing 5-μm particle size packing. Reprinted from Reference 25 with permission from Elsevier.

from column to column. The acids were most retained on the amino column. As this column contained positively charged groups with the mobile phase conditions used, the negatively charged acids could undergo ionic interactions, increasing their retention. The acids had weaker retention on the amide column, and aspirin and 4-aminosalicylic acid (peaks 4 and 3) were only partially resolved. In contrast, the resolution of these two solutes was greatly improved on the zwitterionic column, however with a reversed order of elution compared with the amino column. On the bare silica column, the peaks were also well resolved, but their elution order was more similar to that on the amino column. The authors considered that the different elution patterns of the acids on the four columns indicated that the polar stationary phases had significant differences in retention and selectivity. Similar differences in selectivity were noted for a mixture of nucleic acid bases and nucleosides on the four columns. Specific interactions between the solutes and surface functional groups were thought to be most likely to be responsible for these selectivity differences. Such interactions could not be considered under a pure partition model, nor would they be accounted for in predictions using log D values. The authors also investigated the contribution of ionic processes to the overall retention, examining the effect of different ammonium salts (ammonium acetate, formate, and bicarbonate) on the retention of the acid compounds. They showed some differences in retention of the solutes, which they attributed in part to different eluting strengths of the competing anions in ionic interactions with the positively charged column groups. They also investigated the effect of salt concentration by varying the concentration of ammonium acetate from 5 to 20 mM in a mobile phase of ACN–water (85:15, v/v). For salicylic acid and aspirin, they showed increases in retention on the amide, bare silica, and zwitterionic column of 20–40% as the buffer strength increased. The authors considered the possibility that an increase in the buffer strength could be reducing repulsive effects of the acids from negatively charged silanol groups on the silica-based phases. However, they observed smaller but significant (8–20%) increases in the retention time of cytosine on all four columns. As cytosine was not charged under the mobile phase conditions used, electrostatic effects could not contribute to retention increases for this solute. The authors concluded that in this case, the retention increase might be related to increased hydrophilic partitioning, instead of any specific interactions with the functional groups on the stationary phases. Higher salt concentrations should drive more solvated salt ions into the water-rich liquid layer on the column surface, resulting in an increase in volume or hydrophilicity of the liquid layer, leading to stronger retention of the solutes. The authors suggested that this experiment provided indirect evidence to support the retention mechanism of HILIC that had been proposed by Alpert [10]. Nevertheless, increases in the salt concentration produced considerable decreases in the retention of salicylic acid and aspirin on the amino column. The ion exchange interactions of the acids on this phase were reduced by increasing competition of the buffer ions. It was interesting to note that no such decreases in retention were observed

on the zwitterionic phase. The authors speculated that electrostatic repulsion from the negatively charged sulfonic groups was balanced by the influence of the quaternary amine groups on this phase.

A comparison of the retention properties of HILIC phases using a rather different set of solutes was performed recently by McCalley [26]. Figure 1.3 shows the separation of a mixture of two neutral compounds (phenol and caffeine) two strong acids (*p*-xylene-2-sulfonic acid and naphthalene-2-sulfonic acid) and four basic compounds (nortriptyine, diphenhydramine, benzylamine, and procainamide) on five different silica-based HILIC phases of the same dimensions and particle size (5 μm). The mobile phase was 5 mM ammonium formate, pH 3.0, in either 85% ACN (Fig. 1.3a) or 95% ACN (Fig. 1.3b). The structure of the bonded groups and the physical characteristics of

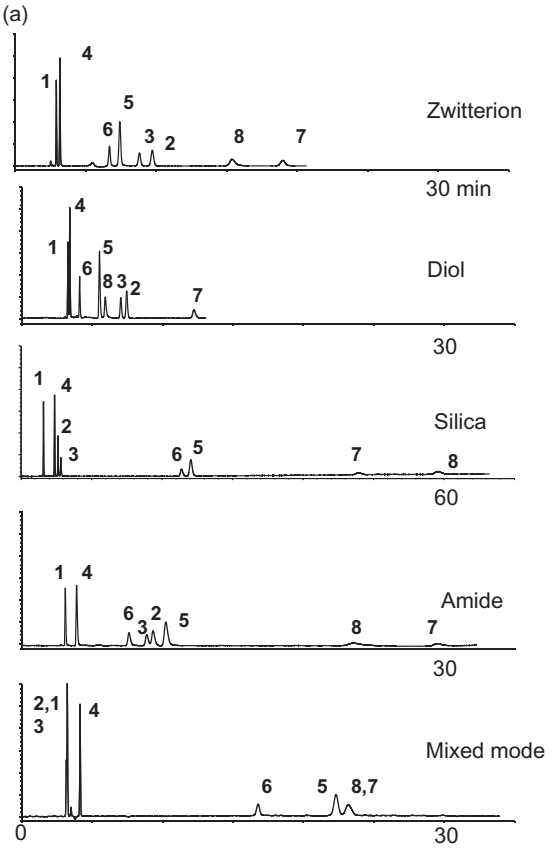

Figure 1.3. (a) Chromatograms of eight solutes on five different HILIC columns (all 25 × 0.46 cm, 5 μm particle size). Mobile phase ACN–water (95:5, v/v) containing 5 mM ammonium formate, pH 3.0, 1 mL/min. Peak identities: (1) phenol, (2) naphthalene-2-sulfonic acid, (3) *p*-xylenesulfonic acid, (4) caffeine, (5) nortriptyline, (6) diphenhydramine, (7) benzylamine, (8) procainamide.

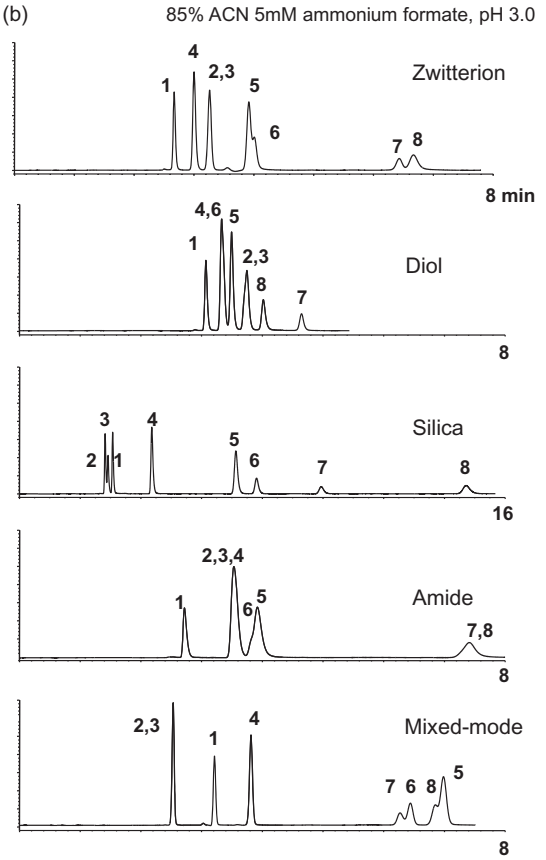

Figure 1.3. (b) Mobile phase ACN–water (85:15, v/v) containing 5 mM ammonium formate, pH 3. Reprinted from Reference 26 with permission from Elsevier.

these phases are given in Table 1.1. These were zwitterionic sulfobetaine, bare silica, diol, amide, and a mixed mode phase. The mixed mode phase was developed to exhibit both hydrophilic and reversed-phase characteristics [27], consisting of a long carbon chain with a diol grouping on the outlying carbon atoms. It was suggested that this phase has a dual operation mode. For example, the separation of cytosine and naphthalene could be achieved in the RP mode using ACN–ammonium acetate buffer pH 5 (52:48 v/v) with naphthalene eluting last, and in the HILIC mode using ACN–buffer (92:8, v/v) with naphthalene eluting first. It is immediately clear from this comparison that considerable differences exist in the selectivity of the various columns toward this group of solutes. For the basic solutes (peaks 5–8), the silica column is much more retentive than the other phases (note that the time axis is about double that for the other phases). This high retention is likely to result from ionic

Table 1.1. Specifications of the HILIC Columns Used

Column	Manufacturer	Bonded Group	Pore Diameter (Å)	Surface Area (m^2/g)	Void Volume (mL^b)
Zwitterion (Zilic)	Merck	~$CH_2N^+(CH_3)_2$–CH_2–CH_2–CH_2–SO_3^-	200	140	2.6
Silica	Phenomenex	~SiOH	100	400	3.0
Diol (Luna HILIC)	Phenomenex	~cross-linked diol/ethylene bridges	195	185	3.0
Amide	TSK	–$CONH_2$ nonionic carbamoyl	80	[a]	2.7
Acclaim mixed-mode HILIC-1	Dionex	~$Si(CH_3)_2C_9H_{19}CH(OH)CH_2$–OH	120	300	3.3

[a]Value not disclosed by manufacturer.
[b]Value measured using toluene in 90:10 ACN: water (v/v) containing 5 mM ammonium formate, pH 3.0.

interactions with ionized silanol groups. Nortriptyline, diphenhydramine, benzylamine, and procainamide all have pK_a values > 9 and thus should be protonated under the conditions of the experiment. In contrast, the bare silica phase shows low retention of the ionized acidic solutes *p*-xylene sulfonic and naphthalene 2-sulfonic acids (peaks 2 and 3). While ionization of solutes in general should increase their hydrophilicity and thus their retention by partitioning into the aqueous layer, low retention in this case can be explained by repulsion of these charged solutes from ionized column silanols. This low retention of acids is also shown on the mixed-mode phase. However, the relative retention of these acids is greater than that of the ionized base diphenhydramine (peak 6) on the zwitterionic, diol, and amide phases. On the diol phase, the acids show highest retention of all solutes apart from benzylamine, although the average retention of all solutes on this phase is rather low. The particular variety of zwitterionic, diol, and amide phases used in this study had a polymeric bonded phase layer [26], and it is possible that this shields the silanols from interaction with ionized solutes. A comparison of the separation of the probes when using either 85% or 95% ACN in the mobile phase, while maintaining the buffer concentration constant, shows some differences in selectivity. For example, the retention of benzylamine (peak 7) is increased relative to that of procainamide (8) on the amide column at the higher concentration of ACN, and the order of elution of the basic compounds (peaks 5, 6, 7, 8) on the mixed mode phase was also changed. These differences could indicate changes in specific interactions between the solutes and the column groups as the mobile phase is changed.

The differences in selectivity that occur between different columns when used with the same mobile phase as shown in these studies confirm that the

mechanism of separation in HILIC is complex and that the stationary phase gives a considerable contribution to retention. Thus, the stationary phase cannot be considered merely as an inert support for a layer of water into which solutes selectively partition. Ion-exchange processes have long been recognized as contributory to the overall mechanism. The same conclusions were reached recently by Bicker and coworkers [23], who cited three major retention mechanisms on bare silica, or columns bonded with the neutral ligand trimethoxysilylpropyl urea: (1) HILIC-type partitioning, (2) HILIC-type weak adsorption such as hydrogen bonding between solutes and the bonded ligands or the underlying silanols (which could be influenced by the experimental conditions), and (3) strong electrostatic forces for ionized solutes, which could be attractive or repulsive. They summarized that multi- or mixed mechanism separations seemed to be common under HILIC conditions, which are associated with useful selectivity effects.

These various contributors to retention in HILIC will be considered in more detail in the following sections.

1.3.2 Contribution of Adsorption and Partition to HILIC Separations

Studies on the retention of sugars showed that water from the mobile phase is retained on the surface of HILIC columns [13,15] and thus that the concentration of water is higher in the stationary phase than in the mobile phase, providing evidence for a partition mechanism. A more recent study [28] used the retention of benzene and toluene, which are unretained void volume markers in HILIC, to indicate more exactly the presence of a water layer on the stationary phase surface. Figure 1.4 shows the decrease in retention time of these solutes as the water concentration in an ACN–water mobile phase

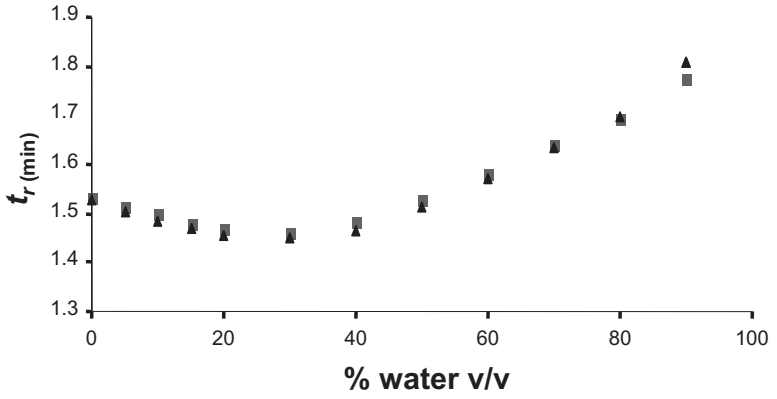

Figure 1.4. Retention time (t_r, min) of benzene (squares) and toluene (triangles) as a function of water content of aqueous ACN mobile phase. Flow rate 1.0 mL/min. Detection UV at 254 nm. Injection volume 5 μL. Column bare silica shell (2.7 μm particles, 15 × 0.46 cm). Reprinted from Reference 28 with permission from Elsevier.

was increased from 0% to about 30% v/v. Due to the limited solubility of these hydrophobic compounds in water, it can be assumed that they partition almost entirely into the bulk mobile phase and cannot penetrate the water layer. The difference between the retention volume of the probe in pure ACN and in a given mobile phase can be used to estimate the proportion of the pore volume occupied by water. However, the method breaks down at water concentrations of >30% v/v. The increasing retention of the probes at these higher water concentrations can be attributed to an RP-type retention mechanism on column siloxane groups, similar to that proposed originally by Bidlingmeyer [16]. The presence of a water layer has also been shown recently by the studies of Tallarek and coworkers [29] that involve molecular simulation dynamics using high-speed computers, modeling cylindrical silica pores that have a diameter of 3 nm. The simulations were performed with water/ACN mixtures of molar ratios 1/3, 1/1, and 3/1, which corresponded to approximate volumetric ratios of 10/90, 25/75, and 50/50 v/v. The results indicated that the water/ACN ratios in the pores were considerably higher at 1.5, 3.2, and 7.0 for the respective mixtures. The *relative* water fraction in the pores thus increased with decreasing water content of the bulk mobile phase. The simulations suggested a layer close to the surface (<0.45 nm) where water hydrogen bonds preferentially to silanol groups, with only scarce silanol–ACN bonds. The water molecules in this region appeared to be nearly immobilized to the silanol groups. Outside this immediate region close to the surface of the stationary phase, water–water hydrogen bonding was preferred, although some ACN–water hydrogen bonds were indicated.

Irgum and coworkers reported the use of ^{2}H nuclear magnetic resonance (NMR) spectroscopy for probing the state of water in a number of different HILIC phases including bare silica particles of pore size 60–100 Å, and in silica bonded with polymeric sulfobetaine zwitterionic functionalities [30]. They distinguished three types of water that could be present in polymer systems: free water that resembles ordinary bulk water, freezable bound water that has a slightly shifted transition temperature between the solid and liquid state compared with bulk water, and water that is bound within the polymeric network such that it does not freeze in the expected temperature range for bulk water. ^{2}H NMR was chosen rather than ^{1}H NMR because of the problems experienced when combining high-field NMR spectrometers with porous samples. Deuterated water enriched to 20–30 mL % was used for the study, which increased the freezing point of water by a small amount (0.8–1.2°C). The use of ^{2}H NMR enabled signals from liquid and frozen water to be clearly distinguished from each other. On freezing, the NMR line width increases significantly, spreading out over nearly a 300-kHz broad frequency range. Because the peak becomes so broad, the signal from frozen water effectively disappears from the NMR spectrum, allowing only the signal from water in the liquid state to be observed. For neat silica, the pore size had an influence on the depression of the freezing point of water, and thus on the percent of nonfreezable water, that is, water that was strongly associated with the stationary phase. This amount

decreased as the pore size of the silica increased. A 3-μm 100 Å silica was shown to contain 14% of nonfreezable water. The polymeric zwitterionic stationary phases were shown to contain higher amounts of water compared with the neat silica. A difficulty with these measurements is that they were apparently carried out under purely aqueous conditions, that is, in the absence of organic solvents like acetonitrile, and thus different from normal conditions used in HILIC.

While the existence of a water layer is a prerequisite for the partitioning model in HILIC, its existence does not preclude the occurrence of an alternative adsorption mechanism. Indeed, the different selectivities for some solutes on HILIC phases containing different polar bonded ligands could be interpreted as being caused by differential adsorption on these groups, as suggested in the previous discussion, even if the polar column groups are deactivated by the presence of water. It is possible that adsorption is a more likely contributor to the mechanism when low concentrations of water are present in the mobile phase. Hemström and Irgum [31] considered the relative contributions of adsorption and partition on the basis of fitting retention data to the relevant equations that describe these two mechanisms. Retention in adsorption chromatography can be described by the Snyder–Soczewinski equation:

$$\log k = \log k_B - n \log X_B \tag{1.1}$$

where X_B is the mole fraction of the strong solvent B (in this case water) in the mobile phase, k_B is the retention factor with pure B as the eluent, n is the number of B solvent molecules displaced by the solute. Alternatively, for a partition-like mechanism, the empirical equation

$$\log k = \log k_w - S\varphi \tag{1.2}$$

describes retention approximately, where φ is the volume fraction of the strong solvent B in the mobile phase, and k_w is the hypothetical retention factor when the mobile phase contains no B solvent (i.e., solely the weak solvent). Thus, a plot of log k versus log (mole fraction water) should yield a straight line for an adsorption mechanism, whereas a plot of log k versus (volume fraction water) should yield a straight line for a partition mechanism. The authors considered data from a number of studies (e.g., References 25, 32, and 33) to determine the relative linearity of these plots, although for the log–log plots they used the more approximate volume fraction instead of the mole fraction of water. Clearly, these quantities are not collinear. The original authors of Reference 32 had shown that for the separation of sugars on aminoethylenediamino and diethylenetriamino silicas, the mechanism appeared to be constant for all phases (the selectivity was the same, although the absolute retention increased as the number of amino groups on the stationary phase increased). They argued, therefore, that the bonded groups on the column were not directly involved in the separation, and served only to trap water; that is, this was strong evidence for a partition mechanism. However, Hemström and Irgum, in interpretation of the earlier data [32], showed considerably

better linearity in the log–log plots than the log–lin plots, consistent with an adsorption process. Nevertheless, their attempts to model other data showed equally poor fits using either type of plot. Overall, it was not possible to reach any firm conclusions from these studies as to whether partition or adsorption was likely to be the dominant mechanism.

Lindner and coworkers [9] constructed similar plots for the retention of cytosine and cytidine on some novel oxidized and nonoxidized 2-mercaptoethanol and thioglycerol phases, and some commercial diol phases. They found considerable intercolumn differences in the log–log and log–lin plots over the examined range (5–40% v/v water in the eluent). Similar trends were also found for other nucleobases and nucleosides examined. For example, with the mercaptoethanol phase, the log–lin plot was shown to give a better linear relationship, while for the more polar phases only the log–log model delivered adequately linear relationships. In this latter case, linear regression analysis of the log–log plots generated r^2 values for the oxidized phases of between 0.996 and 0.999. The authors suggested that the nonlinear behavior of the nonoxidized mercaptoethanol in the log–log but not in the log–lin plots indicated that partitioning was the prevailing mechanism under the specified elution conditions for the particular solutes examined. Furthermore, they showed that at low water concentrations, there was a transition to curvature in the log–lin plots for this column, which corresponded to a linear behavior in this range for the log–log plots. They interpreted this behavior as being indicative of adsorptive interactions that come into play when the water content of the mobile phase is low and the ACN content is high. However, this clear trend was not shown for the nonoxidized thioglycerol phase. With an increase in phase polarity produced by oxidation of these two phases, the authors surmised that adsorptive interactions become more relevant or even dominant for retention, as shown by the greater linearity of the log–log plots. They also noted that the change in selectivity of nucleoside separations when changing from the nonoxidized to the oxidized forms of the phases could not be explained satisfactorily on the basis of a pure partitioning process that is commonly invoked to describe retention in the HILIC mode. While a complete transition from a partitioning-dominated mechanism to an adsorption-driven mechanism was considered unlikely in the oxidized variants of these new phases, these data do indicate the potential impact of the nature of the stationary phase on the retention mechanism in HILIC. The conclusion of the authors was that a mixed-mode process for these new packings was operating that involved contributions of both partition and adsorption.

Li and coworkers [34] examined the retention of four zwitterionic tetracyclines on an amino bonded HILIC column, using buffered aqueous solutions in the range 10–50% v/v (90–50% ACN). They found r^2 values of the log–log plots of between 0.9953 and 0.9987 (the log of the volume fraction of water in the mobile phase was again used, rather than the mole fraction), whereas the log–lin plots were less linear, giving values from 0.9649 to 0.9978. However, the authors noted that r^2 for the log–lin plots improved to 0.9777–0.9915 when

the percentage of water was in the reduced range of 20–50%. They concluded that this result indicated that the relative contributions of partition and adsorption changed depending on the mobile phase composition.

A further study examined the relative linearity of log–log and log–lin plots for the same five columns and five of the eight solutes shown in Figure 1.3 [26]. The results are shown in Figure 1.5. This mixture of probes contained basic compounds that are also retained by ion-exchange as well as HILIC processes.

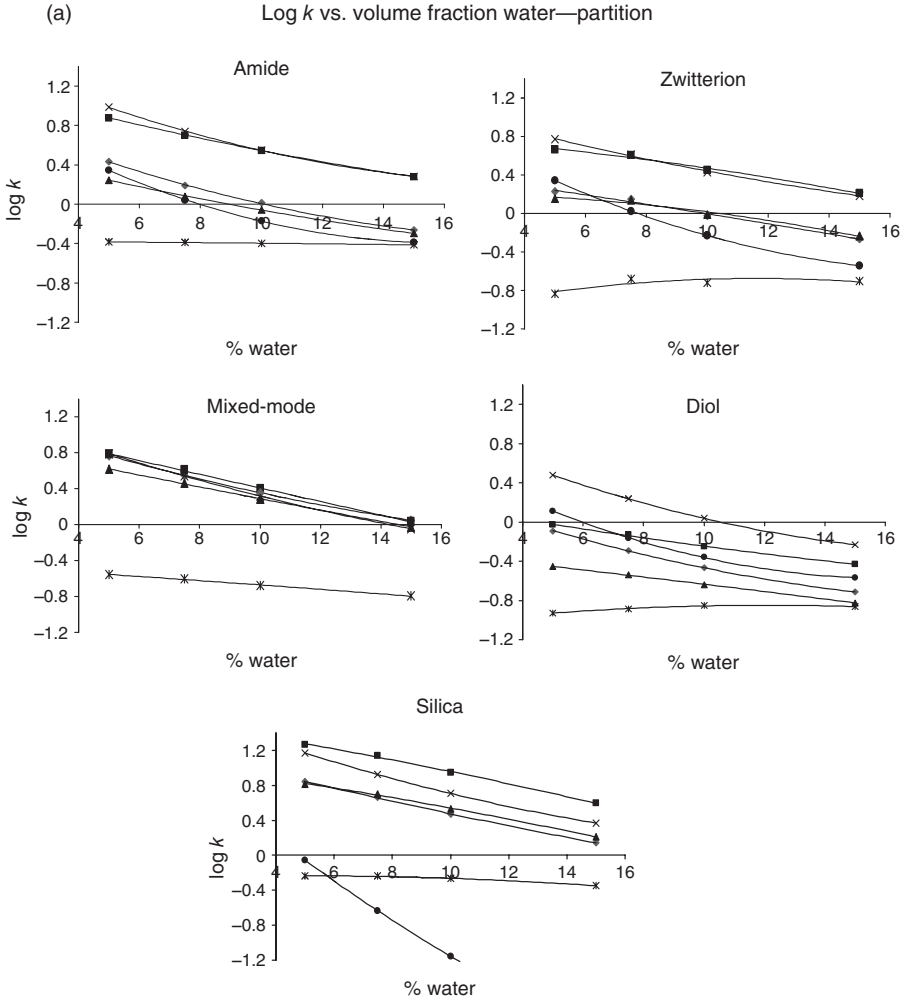

Figure 1.5. (a) Plots of log k versus volume fraction of water in the mobile phase and (b) plots of log k versus log mole fraction of water in the mobile phase. Solute identities: diamonds = nortriptyline, squares = procainamide, triangles = diphenhydramine, crosses = benzylamine, stars = caffeine, circles = p-xylenesulfonic acid. For other conditions see Figure 1.2. Reprinted from Reference 26 with permission from Elsevier.

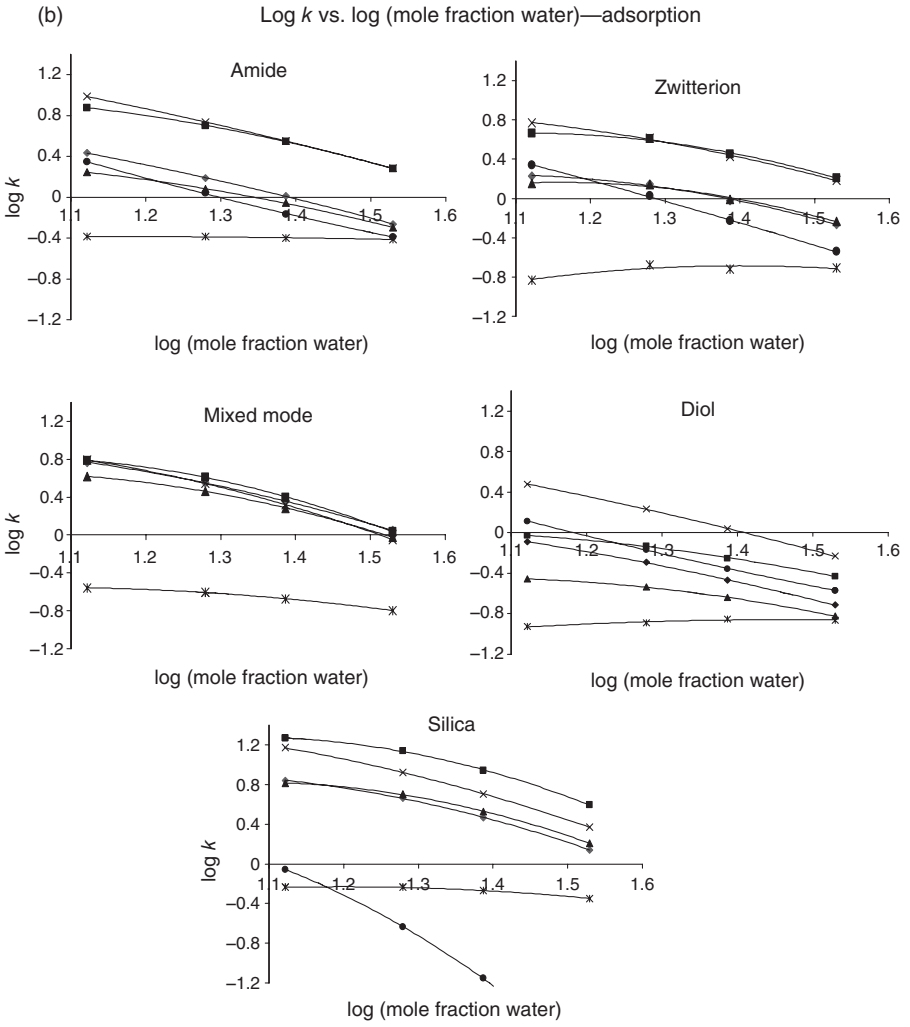

Figure 1.5. (*Continued*)

The overall buffer concentration was maintained constant at 5 mM and the ${}^{w}_{w}$pH of the aqueous component held fixed at 3.0 in an effort to maintain ionic interactions constant. However, the change in ACN concentration actually brought about a small change in the ${}^{s}_{w}$pH (the pH measured in the mobile phase, with the meter calibrated in aqueous buffers) from ${}^{s}_{w}$pH 6.1 in 95% ACN to ${}^{s}_{w}$pH 5.2 in 85% ACN, and it is conceivable that the contribution of ionic interactions could therefore change. Nevertheless, retention of these solutes was shown to be rather insensitive to small pH changes in this region in a previous study [5]. It is also debatable whether ${}^{s}_{w}$pH or ${}^{w}_{w}$pH is more appropriate to use in such a study, considering that the solutes are held in a water layer close

to the stationary phase. The plots show typical HILIC behavior, in that increasing concentration of water in the mobile phase results in decreased solute retention. A comparison of Figure 1.5a (log–lin plots) and Figure 1.5b (log–log plots) shows that these alternative plots were again rather inconclusive. For the bare silica and mixed mode phases, the log–lin plots seem to be more linear, indicating a partition mechanism, whereas for the diol and amide phases, the log–log plots appear to be more linear, indicating a partitioning mechanism. A complication of these plots is the possibility of the changing contribution of other mechanisms as the concentration of organic solvent changes (e.g., hydrophobic retention is possible at low concentrations of ACN; see Section 1.3.4). Furthermore, there are approximations involved with the equations themselves. We do not believe, therefore, that plots of this kind can give conclusive evidence of the predominance of either the partition or adsorption mechanism. Nevertheless, such plots can still be useful in indicating selectivity changes that occur as a function of organic solvent concentration. For example, Figure 1.5a,b indicate the increased relative retention of *p*-xylenesulfonic acid at low percent water on both the zwitterionic and amide phases.

It seems very likely that the predominant mechanism could change dependent on the stationary and mobile phase conditions. Irgum and coworkers [35] compared the performance of 22 commercial HILIC phases with a set of probe compounds designed to reflect the different possible contributions to the HILIC mechanism, evaluating the results using principal components analysis. They concluded that unmodified silica columns relied mainly on adsorption and oriented hydrogen bonding for selectivity. It was interesting in this respect that silica hydride phases, as prepared by Pesek and coworkers, appeared to exhibit similar behavior to conventional type A and type B silica phases [36]. Pesek and coworkers preferred the term "aqueous normal phase" (ANP) to describe separations with these phases. They argued that silica hydride has a lower hydrophilicity than type B silica, due to a lower concentration of silanol groups, and thus the hydration layer on the surface should be much thinner than for typical HILIC columns. If this is the case, then adsorption might have been expected to be the dominant mechanism for such columns. Irgum proposed that columns with highly hydrophilic polymeric interactive layers such as the zwitterionic column ZIC–HILIC generally showed a selectivity pattern that could be attributed to partitioning. Neutral and amino columns were stated to occupy an intermediate position between silica and zwitterionic columns.

1.3.3 Further Studies on the Contribution of Ionic Retention in HILIC

1.3.3.1 Introduction

As discussed previously, ionic retention has been recognized as contributory to retention on bare silica and other HILIC columns for many years. The contribution of ionic retention to total retention should increase as the

concentration of water in the mobile phase is increased, because the relative contribution of the HILIC mechanism (i.e., partition or adsorption) to the total retention should decrease. Ion exchange groups have been deliberately introduced into phases since the earliest days of HILIC. For instance, amino groups used in columns for the separation of sugars can also be used as anion exchangers and the PolySulfoethyl phase used by Alpert [10] gives retention of cations superimposed on HILIC retention effects in the separation of peptides. In Irgum's study of 22 different stationary phases [35], cation exchange was shown to be a very strong contributor to the selectivity of separations on bare silica columns. However, the group of columns studied included older type B silica phases that might be expected to show strong interactions of this type, due to the presence of acidic silanols, which are more readily ionized. The study was also carried out at neutral pH in ammonium acetate solutions, conditions under which silanols might be expected to be at least partially ionized. Zwitterionic columns such as the sulfobetaine phase ZIC–HILIC were also shown to exhibit cation exchange properties. Electrostatic interactions, however, were reported to be much weaker than for the (particular) underivatized silica columns examined in the study.

1.3.3.2 Mobile Phase Considerations for the Separation of Ionogenic Compounds

For the separation of ionic compounds, buffer solutions are preferred to stabilize the solute charge. Stabilization of the charge on column groups is also an important factor for ionized solutes. However, the charge on these groups might conceivably affect the formation of a water layer on the column and thus influence the separation even of uncharged compounds. Olsen [37], in a study of the separation of some pyrimidines, purines, and amides on silica and amino columns, concluded that mobile phases should contain a buffer of acid for pH control in order to achieve similar and reproducible results among columns from different sources. As HILIC is a particularly advantageous separation technique to use in conjunction with MS (see Section 1.1), volatile buffer solutions are often favored. Simple aqueous solutions of organic acids such as formic and acetic acids are also recommended by some column manufacturers for HILIC although sometimes at higher concentration (e.g., 0.2%) than used typically in RP separations. Figure 1.6d shows the analysis of 100 mg/L solutions of three neutral compounds (3-phenylpropanol, caffeine, and phenol), whereas Figure 1.6a shows three ionized compounds (2-naphthalene sulfonic acid, nortriptyline, and propranolol) at the same concentration, using an Atlantis silica column with acetonitrile–water (85:15, v/v) containing an overall concentration of 0.2% formic acid. While the neutral compounds gave excellent peak shape, the charged compounds gave broad fronting peaks and column efficiencies only about one tenth that for the neutral compounds. Reduction of the concentration of the ionized solutes, from 100 to 10 and 1 mg/L (Fig. 1.6b,c), gave improved peak shapes showing that the poor peak

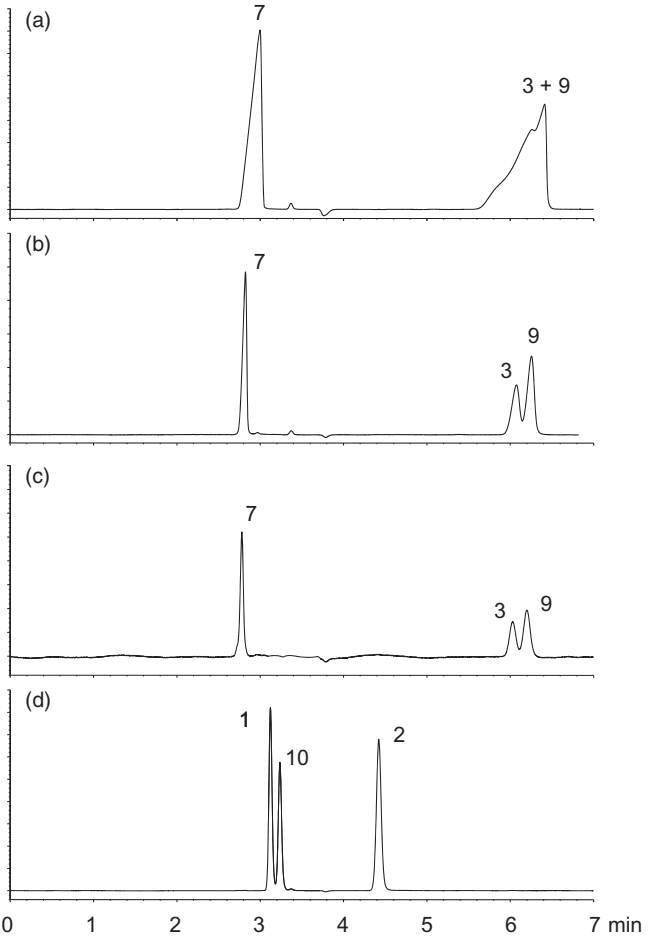

Figure 1.6. Analysis of selected compounds on Atlantis silica. Peak identities 1 = phenol, 2 = caffeine, 3 = nortriptyline, 7 = 2-naphthalenesulfonic acid, 9 = propranolol, 10 = 3-phenylpropanol. Solute concentrations (a) and (d) 100 mg/L; (b) 10 mg/L; (c) 1 mg/L. Mobile phase ACN–water 85:15, overall 0.2% formic acid. Reprinted from Reference 5 with permission from Elsevier.

shapes are due to some overloading effect. At 1 mg/L, efficiencies for ionized compounds approached those for neutral compounds, although some peak fronting was still shown (asymmetry factor A_s = 0.8–0.9). Eighty-five percent ACN causes formic acid to become such a weak acid that the ionic strength of this mobile phase is very low, which could cause overloading. In contrast, ammonium formate should be completely ionized even in high concentrations of ACN. Figure 1.3 has already shown the excellent peak shapes that can be obtained for these ionized acidic and basic compounds, when using mobile phases at similar pH containing ammonium formate. Clearly, analysis of

charged compounds is impractical in HILIC using solely formic acid as an additive. Problems of overloading with formic acid have also been reported in RP separations, although these are not so serious, as the ionization of the weak acid is not so greatly affected by the lower concentrations of ACN typically used in such separations [38,39]. Equally poor results in HILIC were also obtained with 0.2% acetic acid (results not shown), which is a weaker acid than formic acid.

Ammonium formate or ammonium acetate at low pH is often used as a buffer in HILIC separations; the latter is also quite frequently used [35] without further pH adjustment (pH of aqueous solutions is typically about 6.8). At neutral pH, ammonium acetate is not a buffer. However,no problems with reproducibility of retention times have been noted using this solution[35], at least not at the low solute concentrations used.

1.3.3.3 Ionization State of the Column as a Function of pH

Some studies, particularly with bare silica columns, have attempted to investigate the ionization of column groups by studying the retention of acids and bases as a function of the mobile phase pH. Figure 1.7 demonstrates the variation in the retention of a quaternary ammonium compound (benzyltriethylammonium chloride [BTEAC], always completely ionized under the conditions of the experiment) as a function of mobile phase pH on a bare silica column (Atlantis, Waters Associates). The mobile phase was 85% ACN containing 15 mM ammonia adjusted to various pH over the range $^{w}_{w}$pH 3.0 ($^{s}_{w}$pH 5.2) to $^{w}_{w}$pH 10.2 ($^{s}_{w}$pH 9.0). Figure 1.7 shows that retention increases only gradually as the pH is increased from $^{w}_{w}$pH 3.0 to $^{w}_{w}$pH 8.0 followed by a marked increase from $^{w}_{w}$pH 8.0 to $^{w}_{w}$pH 10.0, indicating a large increase in silanol ionization and thus increased ionic retention of this cationic species. Retention of the eight

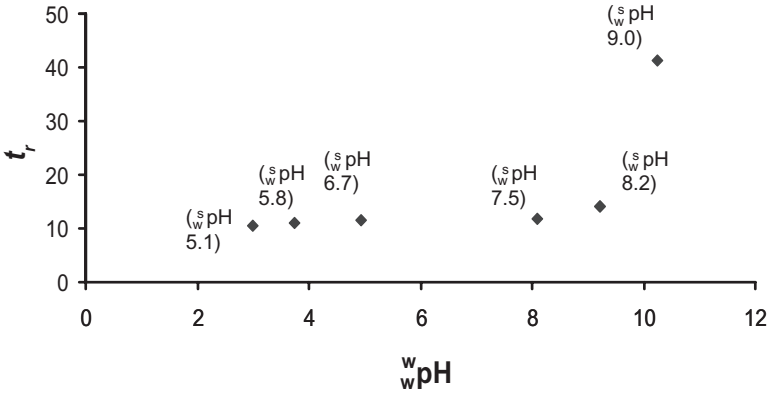

Figure 1.7. Retention of benzyltriethylammonium chloride (BTEAC) as a function of mobile phase pH on an Atlantis silica column (Waters). Mobile phase 85% ACN—0.1 M NH_3; pH adjusted with formic acid. Reprinted from Reference 5 with permission from Elsevier.

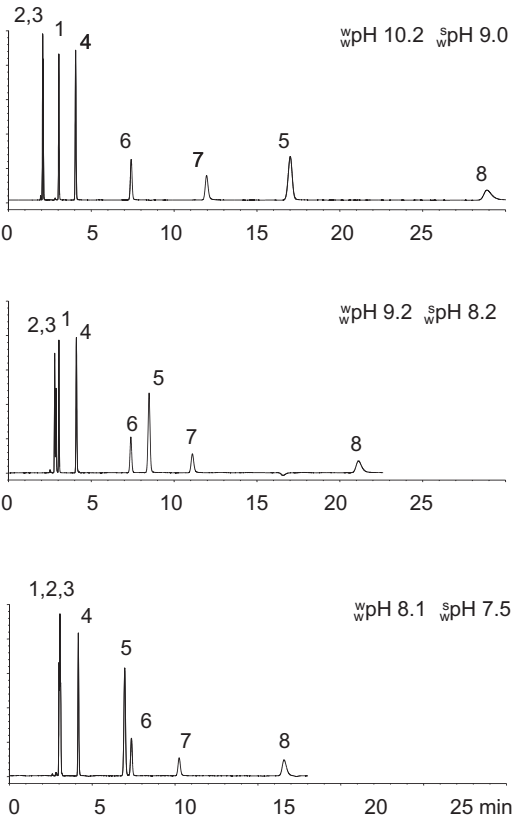

Figure 1.8. Analysis of test solutes on Atlantis silica. Mobile phase ACN—0.1 M $HCOONH_4$ $^{w}_{w}$pH 8.2 to 10.2 (85:15, v/v). Peak identities as for Figure 1.3. Reprinted from Reference 5 with permission from Elsevier.

test compounds used in Figure 1.3 was also investigated over the higher pH range of $^{w}_{w}$pH 8.1 to $^{w}_{w}$pH 10.2, as shown in Figure 1.8. Useful selectivity differences were demonstrated as the pH was varied. As pH increases, silanol ionization should increase, thus increasing the retention of cations. This effect was indeed shown by the strong base nortriptyline ($^{w}_{w}pK_a$ 10.2), whose retention follows the same pattern as BTEAC. However, the effect of decreasing solute ionization at higher pH was superimposed, particularly for the weaker bases (but not for the quaternary compound, which remains ionized), on that of increasing column ionization. Decreasing solute ionization reduces ionic retention, and retention might also be decreased by reduced solubility of the uncharged compound in the water layer associated with the silica. This effect is particularly shown by the weakest base, diphenhydramine ($^{w}_{w}pK_a$ 9.0), which elutes well before nortriptyline at $^{w}_{w}$pH 10.2 but after nortriptyline at $^{w}_{w}$pH 8.1 and below. However, ionic retention of cations occurs on this column even at

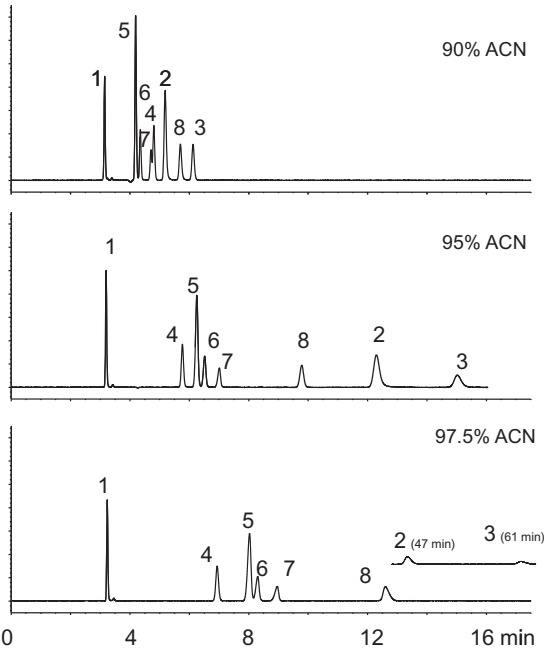

Figure 1.9. Analysis of test solutes on Atlantis silica. Mobile phase ACN–water containing overall 0.1% TFA. Peak identities as for Figure 1.3. Reprinted from Reference 5 with permission from Elsevier.

the lower pH studied ($^{w}_{w}$pH 3.0, see Section 1.3.3.4). The acids eluted below the column void volume at $^{w}_{w}$pH 10.2, presumably due to exclusion caused by the ionized silanols. The low retention of acids on a similar bare silica column at $^{w}_{w}$pH 3.0 has already been noted (see Fig. 1.3). In the same study, trifluoroacetic acid (TFA, overall concentration 0.1%) was used to determine whether the retention of the acids could be improved at a lower mobile phase pH than is obtainable with formate buffers. Figure 1.9 shows that this was indeed possible, and moreover a reversal in the order of elution of acids and bases on the Atlantis column was obtained with TFA, with the acids now eluting *after* the bases. Note that some variation in the order of elution of peaks was obtained by changing the ACN concentration from 90% to 95%. Increasing the ACN concentration further to 97.5% ACN gave retention for the acid *p*-XSA in excess of 1 h, whereas it eluted close to the void volume in ACN–ammonium formate buffer (compare results with Fig. 1.3). The change in elution pattern is rather surprising because the $^{w}_{w}$pH of 0.1% aqueous TFA is ~2.1, not vastly different from the $^{w}_{w}$pH 3.0 of aqueous ammonium formate as used previously. However, there are much larger differences in $^{s}_{w}$pH of these mobile phases, which were 1.35 for 85% ACN containing 0.1% TFA compared with $^{s}_{w}$pH 5.2 for 85% ACN–ammonium formate pH 3.0. The true thermodynamic pH ($^{s}_{s}$pH)

can be calculated from the pH measured after the addition of solvent, with the meter calibrated in aqueous buffers (${}^{s}_{w}$pH) according to the relationship:

$$ {}^{s}_{s}\mathrm{pH} = {}^{s}_{w}\mathrm{pH} - \delta \tag{1.3} $$

The δ term incorporates both the Gibbs energy for the transference of 1 mol of protons from the standard state in water to the standard state in the hydro-organic solvent at a given temperature, and the residual liquid junction potential (the difference between the liquid junction potential established during calibration in aqueous solutions, and that established in the hydro-organic mixture). Large negative δ values have been measured in aqueous–ACN mixtures with high ACN concentrations [40]. For example, δ ~−1.1 in 85% ACN and ~−1.6 in 90% ACN. Thus, the thermodynamic ${}^{s}_{s}$pH (calculated from Eq. 1.3), which is directly related to quantities such as the ionized fraction of the analyte, is ~6.3 in the ammonium formate buffer but ~2.5 for 0.1% TFA, both in 85% ACN. Clearly, formic acid becomes a very weak acid in high ACN concentrations, whereas the much stronger acid TFA is relatively unaffected. The differences in the ${}_{s}^{s}$pH of these mobile phases could explain the difference in the elution pattern of the solutes; silanol ionization could be almost completely suppressed at the low pH of the TFA solution, thus preventing stationary phase exclusion of acidic solutes. Suppression of ionic repulsion could facilitate HILIC retention of the acids (solubility of the acids in the stationary phase water layer). At the same time, the retention of the basic compounds by ionic processes would be reduced. However, the situation is complex: while the average pK_a of silanols is considered to be ~7 in purely aqueous solutions, it is unknown in the presence of such high concentrations of ACN. Silanols are weak acids, and as such their pK_a might increase in 85% ACN, counteracting the higher effective mobile phase pH compared with that in purely aqueous solution. However, as already mentioned, a major complication in these deliberations is that the presence of a water layer on the phase surface may indicate that ${}^{w}_{w}$pH and ${}^{w}_{w}$pK_a are more appropriate, when considering either the solutes or the silanol groups. It may be that while there is a population of silanols that become ionized over the range of ${}^{w}_{w}$pH 8 to ${}^{w}_{w}$pH 10, there may be a further population of silanols whose ionization is suppressed only at the low pH of TFA.

1.3.3.4 Quantitation of Ionic Retention Effects on Different Columns

While ionic retention has been shown in many studies to contribute to the retention of ionized solutes in HILIC, particularly on bare silica phases, quantitation of these effects would be of interest such that the relative magnitude of the contribution could be gauged for different stationary phases. This ionic contribution could arise from residual silanols on silica-based phases (relatively few HILIC columns are based on an organic polymer matrix) as well as from ionogenic ligands deliberately bonded to the phase. Ionic retention can be studied by examining retention as a function of the mobile phase buffer

concentration. Cox and Stout's studies of the retention mechanisms for basic compounds on bare silica columns under "pseudo-reversed-phase conditions" have already been mentioned [20]. The ion-exchange contribution to the retention of bases on silica can be expressed as

$$BH^+ + SiO^-M^+ \rightarrow SiO^-BH^+ + M^+ \tag{1.4}$$

where B is the base and M^+ represents the mobile phase counterions.

The ion-exchange equilibrium constant is:

$$K_{ix} = ([SiO^-BH^+][M^+])/([BH^+][SiO\text{-}M^+]). \tag{1.5}$$

The pH of the buffer controls the concentration of BH^+ through its ionization constant K_a

$$BH^+ \rightarrow B + H^+ \tag{1.6}$$

$$K_a = [B][H^+]/[BH^+] \tag{1.7}$$

Assuming that only the charged form BH^+ interacts with ionic sites on the stationary phase, the distribution coefficient between the stationary phase and mobile phase D_{ix} can be written as

$$D_{ix} = [SiO^-BH^+]/([BH^+] + [B]). \tag{1.8}$$

Rearranging Eq. 7 gives:

$$[B] = [BH^+]K_a/[H^+]. \tag{1.9}$$

Rearranging Eq. 5 gives

$$[BH^+] = ([SiO^-BH^+][M^+])/(K_{ix}[SiO^-M^+]). \tag{1.10}$$

Substituting Eq. 9 in Eq. 8 yields

$$D_{ix} = \frac{[SiO^-BH^+]}{[BH^+] + [BH^+]K_a/[H^+]} = \frac{[SiO^-BH^+]}{BH^+(1 + K_a/[H^+])}. \tag{1.11}$$

Substituting Eq. 10 in Eq. 11 gives

$$D_{ix} = \frac{[SiO^-BH^+]}{\frac{[SiO^-BH^+][M^+](1 + K_a/[H^+])}{K_{ix}[SiO^-M^+]}}. \tag{1.12}$$

This simplifies to

$$D_{ix} = \frac{K_{ix}[SiO^-M^+]}{[M^+]} \cdot \frac{1}{1 + K_a/[H^+]}. \tag{1.13}$$

Thus, the distribution coefficient, and the retention factor, which is directly proportional to the distribution coefficient through the phase ratio, varies with

the inverse of the counterion concentration in the mobile phase. A plot of the retention factor against the inverse of $[M^+]$ should be a straight line passing through the origin (assuming that no other retention mechanism exists), with the slope proportional to the ion-exchange equilibrium constant and the number of ionized sites (e.g., silanols on the silica surface). Alternatively, the presence of other retention mechanisms would be indicated by an intercept on the *k*-axis, which corresponds to an infinite competing ion concentration.

Figure 1.10 shows plots of *k* versus $1/[M^+]$ for each of the columns (used also in Fig. 1.3; specifications in Table 1.1) with 90% ACN containing overall buffer

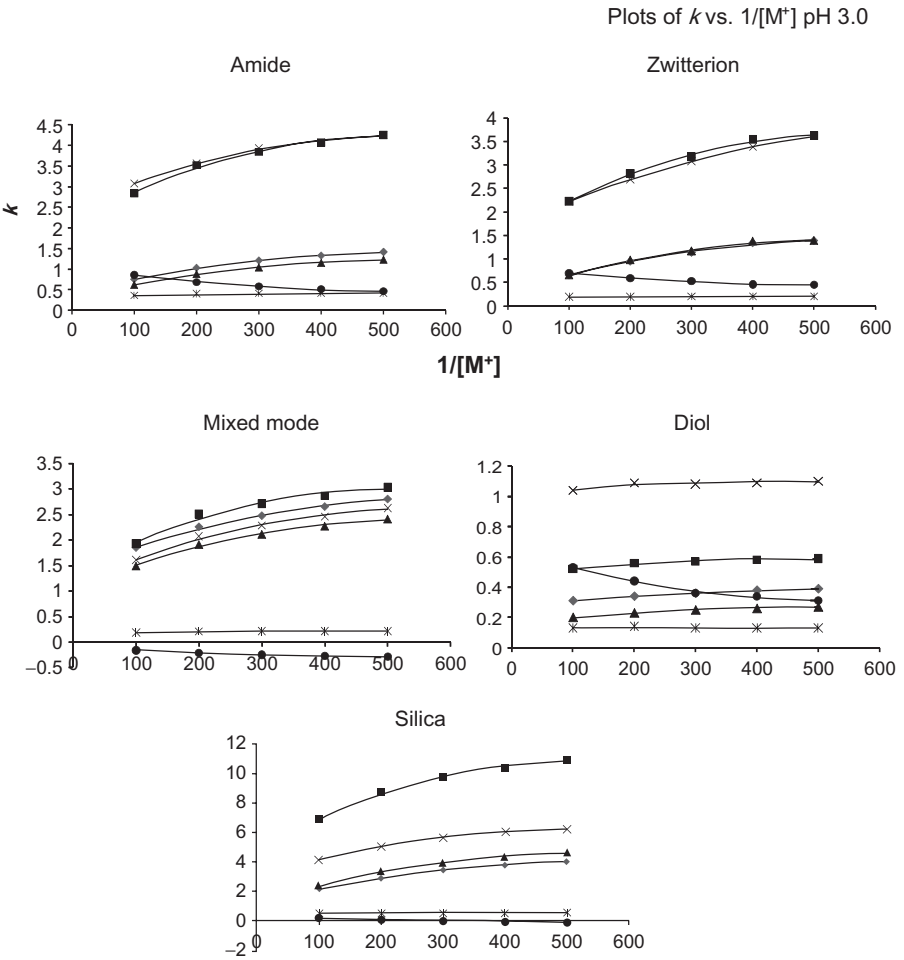

Figure 1.10. Plots of retention factor versus 1/[counter ion concentration] for five different HILIC columns. Solute identities: diamonds = nortriptyline, squares = procainamide, triangles = diphenhydramine, crosses = benzylamine, stars = caffeine, circles = *p*-xylenesulfonic acid. Mobile phase ACN–water (90:10, v/v) containing ammonium formate (concentration varied) $\frac{w}{w}$ pH 3.0. Reprinted from Reference 26 with permission from Elsevier.

Table 1.2. Percentage Contribution of Ion Exchange to *k* at Two Different Levels of Counterion Concentration for Four Basic Solutes

	Procainamide		Benzylamine		Diphenhydramine		Nortriptyline	
Column	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM
Silica	55	28	50	25	70	43	70	42
Diol	18	7	7	3	39	17	29	11
Zwitterionic	58	32	58	32	78	55	78	55
Mixed mode	54	27	56	27	55	27	49	22
Amide	49	24	41	19	73	46	72	47

concentration of 2–10 mM $^{w}_{w}$pH 3.0 as mobile phase, and using the 4 basic solutes procainamide, benzylamine, diphenhydramine, and nortriptyline together with the neutral compound caffeine. The bases all have pK_a > 9.0 and thus should be completely protonated under the conditions of the experiment [26]. The plots were curves rather than straight lines, indicating that the buffer concentration has some additional effect on the separation mechanism other than merely competing with solute cations for stationary phase ionic retention sites. The points were fitted to a second-order polynomial expression, and the equation of the curves (which produced excellent empirical fits) was extrapolated to yield *k* for each solute at an infinite buffer concentration, which corresponds to the retention due to mechanisms other than ion exchange (i.e., the HILIC partition or adsorption mechanism). From the experimentally measured values of *k* for a particular solute and buffer concentration, the percent contribution of ion exchange could be determined, with results shown in Table 1.2. The percent contribution of ion exchange is much greater at a low counterion concentration, as expected due to reduced competition from the buffer ions. Thus, for the bare silica column, 55% of the retention of procainamide is estimated to be due to ionic processes at a 2-mM counterion concentration compared with only 28% at a 10-mM concentration. Overall, ionic retention is of considerable importance for the bare silica column, accounting for 50–70% of the total retention for the four basic probes studied at a 2-mM counterion concentration. Somewhat surprisingly, the contribution of ion exchange at a 2-mM counterion concentration was high for all columns, except the diol column. For the zwitterionic phase, retention of solute cations on the sulfonic acid functionality of the phase could explain the high retention due to ionic processes. For the amide and mixed-mode columns, the ionic retention must be due to ionized silanols on the underlying silica of these phases. It is possible even that an acidic silica (i.e., type A silica) might deliberately be used for the preparation of some bonded HILIC columns to increase their retention properties, although this comment is speculative, as few details of column preparation are revealed by commercial manufacturers. In contrast to the other columns, the diol column gave a contribution of ionic retention of only 3–39% for the four basic test compounds at a 2-mM counterion concentration, indicating possibly that the cross-linked stationary phase layer

used in the particular type of column used in the study might give some shielding of the ionized silanols on the phase surface, or that the phase is bonded on a silica of low acidity.

Despite these considerations of the importance of ionic retention processes, it is clear that the retention on the silica column by nonionic processes is somewhat greater than the retention on the bonded-phase columns (k at an infinite buffer concentration is higher for the silica column as evidenced by the y-axis scales in Fig. 1.10). It is conceivable that there is a more extensive water layer on the silica column due to the greater concentration of polar silanol groups compared with the bonded silica phases; the surface area of the bare silica packing was also considerably higher than that of the other phases, as shown in Table 1.1. However, it seems likely that direct measurements of retention due to hydrophilic processes [35] will give a more accurate assessment of the situation.

Table 1.2 shows also that on all columns apart from the mixed-mode phase, the hydrophilic bases procainamide and benzylamine have a smaller proportion of their retention attributable to ion exchange than the hydrophobic bases diphenhydramine and nortriptyline. For example, on the zwitterion column, the percent contribution of ion exchange to retention was 58% for both benzylamine and procainamide using a 2-mM buffer concentration, but 78% for diphenhydramine and nortriptyline. For hydrophobic bases, there is likely to be smaller retention by conventional HILIC processes (e.g., solubility in a stationary phase water layer); thus, the contribution of ionic retention to overall retention on a given column will be greater. For the mixed-mode phase containing a long hydrocarbon chain, the hydrophilic and hydrophobic bases give rather similar percent contribution of ion exchange to total retention (range = 49–56%). It is likely that some hydrophobic retention of diphenhydramine and nortriptyline contributes to the overall retention of these compounds, making the ion-exchange contribution somewhat lower.

The question remains as to the cause of the curvature of the plots for the bases in Figure 1.10. The intercept on the k versus $1/[M^+]$ plots is indicative of a secondary retention mechanism (as discussed earlier), which could be partitioning into a water layer held on the column surface. If the secondary mechanism was simple and did not change with counterion concentration, then a straight line with a positive intercept should still result. It is possible that increasing the salt concentration might affect solute retention, for example, by increasing the thickness of a layer of water associated with the surface, in accord with the suggestions of Guo and Gaiki [25]. Thus, increasing salt concentration could be expected to increase the retention for all compounds.

It is interesting also to consider further the influence of the underlying base material of these bonded silica HILIC phases. Kumar et al. [6] studied the retention properties of catecholamines on a number of different phases. Catecholamines are biological amines released mainly from the adrenal gland in response to stress. The analysis of the compounds dopamine, epinephrine, and norepinephrine in biological fluids is important in hospital laboratories,

as elevated levels can be indicative of tumors of the adrenal gland or neural tumors. Good separations of these compounds were shown on a silica-based zwitterionic sulfobetaine phase (ZIC–HILIC). A phase with the same bonded zwitterionic groups is also available based on a polymer matrix (ZIC–pHILIC). The catecholamines were analyzed using a mobile phase consisting of ammonium formate buffer, pH 3.0, at various concentrations over the range of 3.7–25 mM in 75% ACN, and plots of *k* versus the inverse of the buffer cation concentration on each column are shown in Figure 1.11. The catecholamines

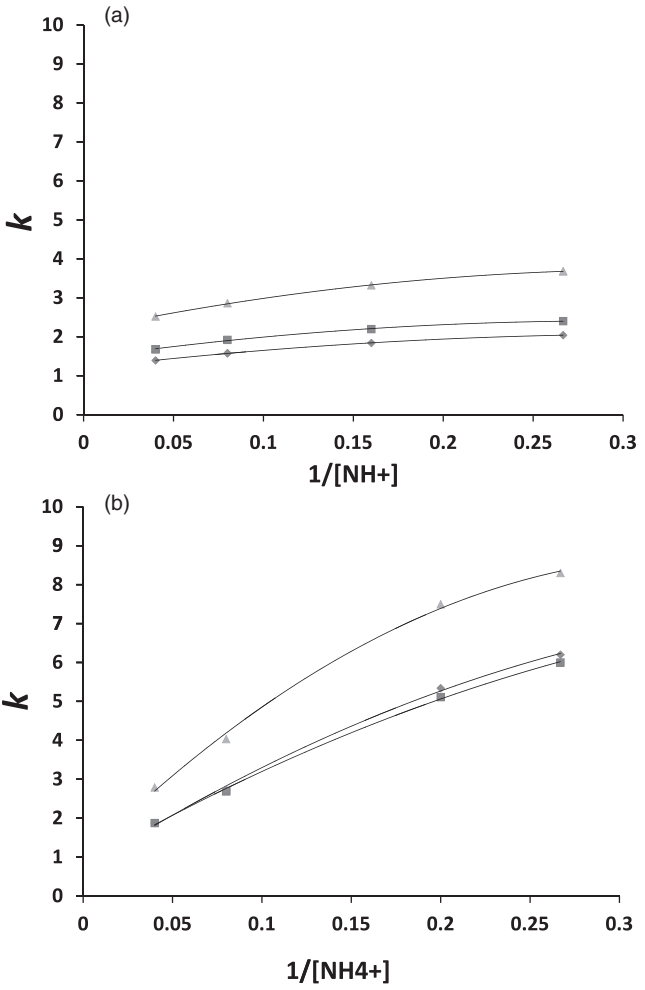

Figure 1.11. (a) Plots of *k* versus the inverse of buffer cation concentration using a ZIC–HILIC column. Mobile phases ammonium formate, pH 3.0 (3.7–25 mM) in 75% ACN. (b) As for (b) but using a ZIC–pHILIC column. Peak identities: diamonds = dopamine, squares = epinephrine, triangles = norepinephrine. Reprinted from Reference 6 with permission from Elsevier.

are strongly basic and should be protonated under the mobile phase conditions used. Figure 1.11 shows that retention decreases with increasing buffer strength on both columns, again giving curved plots, as had been shown for the basic solutes in Figure 1.10. Fitting the data to a second-order polynomial expression gave coefficients of determination $r^2 = 0.9984, 0.9978$, and 0.9991 for dopamine, norepinephrine, and epinephrine, respectively, using the polymer column, and similar fits were obtained for the silica-based column. However, it is clear that the slopes of the plots on the polymer column are much steeper than that for the silica column. Extrapolating the plots to an infinite buffer concentration allowed the HILIC contribution to retention to be determined. This contribution was estimated to be 8–14% for the three catecholamines on the polymer column using the lowest buffer concentration (3.75 mM), and 25–44% at the highest buffer concentration (25 mM). In comparison, for the silica-based column, the HILIC contribution was estimated to be 58–60% at a buffer concentration of 3.75 mM, and ~85% at 25 mM concentration. Thus, the HILIC contribution to retention of the polymer-based column was much smaller than that of the silica-based column, resulting in a much larger proportion of its retention being due to ionic processes. It seems likely that the ionic retention of the bases on the polymer column arises from interactions with the terminal sulfonic acid groups on the phase, although ionic contributions from the base polymer material itself cannot be completely discounted. Residues of catalyst material used in the production of the polymer can give rise to charged sites on polymers [41]. It is possible that the more hydrophobic matrix of the polymer column does not allow the formation of a water layer that is as extensive as that on a polar silica column. Thus, the contribution of HILIC retention to the overall process diminishes, leaving the influence of ion exchange to be more significant. However, much caution is necessary in such interpretations, as the exact details of column preparation are proprietary, and the silica and polymer columns are likely to differ in many other ways. A final practical consideration was that considerably lower efficiencies were obtained on the polymer column for the catecholamines (only about half those on the silica phase). This factor, which is also typical of the performance of RP columns, probably explains the predominance of silica-based columns in HILIC separations.

Of some further interest is the effect of the proportion of the ionic component of retention on efficiency. In RP separations, a mixed-mechanism process involving also ion exchange is generally considered detrimental to column efficiency and thus separation performance [42]. Figure 1.12 shows plots of column efficiency versus ammonium formate buffer concentration (2–10 mM at pH 3) for the hydrophilic base procainamide and the hydrophobic base nortriptyline on the five different columns of Table 1.1. The overall efficiencies shown were somewhat variable, with that of the amide column being lower than that of the other columns, which may be attributable to the polymeric nature of the bonded phase on this silica-based column. Nevertheless, there is little evidence on any of the columns of a detrimental effect of ionic retention on column efficiency for the solutes studied. This result may not hold, however, for other compounds. Efficiencies for the bare silica and mixed-mode (Dionex)

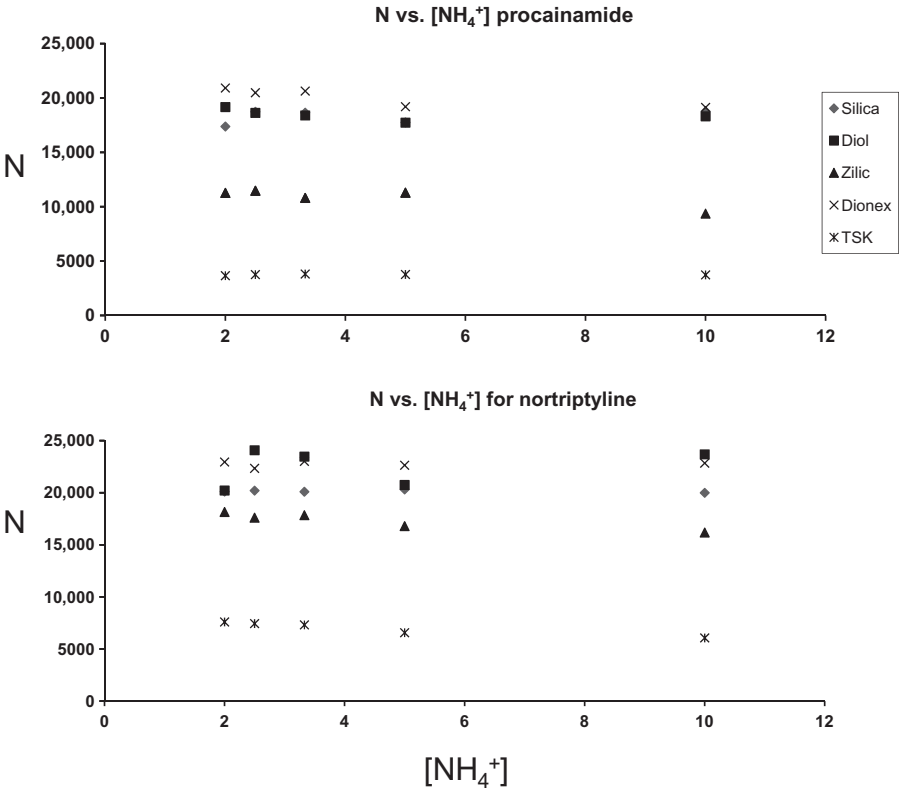

Figure 1.12. Plots of column efficiency against buffer cation concentration for five different HILIC phases (specifications detailed in Table 1.1). Mobile phase ACN–water (90:10, v/v) containing ammonium formate $^{w}_{w}$pH 3.0, various concentrations. Reprinted from Reference 26 with permission from Elsevier.

column were high, while these columns have a large proportion of retention due to ionic processes at low buffer concentrations (see Table 1.2). Efficiency was maintained even at a low buffer concentration, where the contribution of ionic processes to retention was highest. This result also indicates that low buffer concentrations can be used successfully for MS applications, where sensitivity can be compromised in concentrated buffers [43]. However, the effect of solute structure on efficiencies for other compounds is not well understood. For example, the catecholamines gave poor peak shapes on bare silica columns, but excellent results on the zwitterionic phase [6]. Thus, the effect of ion-exchange processes on column efficiency may be solute dependent.

1.3.4 RP Retention on Bare Silica

Evidence for reversed-phase retention on bare silica columns at relatively low concentrations of organic solvent has existed at least since the publication of Bidlingmeyer [16]. A separation of the same test solutes used in Figure 1.3 was

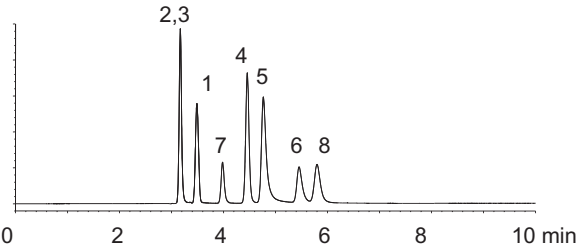

Figure 1.13. Analysis of test compounds using Atlantis silica. Mobile phase 10% ACN in 1.5 mM $HCOONH_4$, pH 3.0. Peak identities are the same as for Figure 1.3. Reprinted from Reference 5 with permission from Elsevier.

demonstrated on a bare silica column [5] using 10% ACN containing 1.5 mM ammonium formate, $^{w}_{w}$pH 3.0 (see Fig. 1.13). Reasonable peak shapes were still obtained, although some tailing was shown (e.g., the asymmetry factor for peak 5, nortriptyline was 2.1, compared with 1.0 for the silica column under true HILIC conditions in Fig. 1.3). The order of elution of the peaks differed from that obtained using the same column under HILIC conditions, showing lower retention of the hydrophilic base benzylamine (peak 7) and greater retention of caffeine (peak 4). Decreasing the concentration of ACN over the range of 10–2.5% ACN increased the retention of all solutes as would be expected for a RP-type mechanism, but in contradiction to changing its concentration when in the HILIC range (ACN concentration > 60%) [5]. These results may reflect RP retention on siloxane bonds of the bare silica, as suggested previously [16].

Lindner and coworkers [9] examined the separation properties of novel and commercial polar stationary phases in both the HILIC and the RP-LC mode. The novel phases included oxidized 2-mercaptoethanol and oxidized 1-thiogylcerol packings, as well as three commercial diol phases. These phases were all devoid of ionic stationary phase groups, although the authors noted that ionic interactions arising from the base silica could be present. The effect of the ACN fraction in the hydro-organic eluent was studied in the range of 5–95% v/v for solutes such as adenosine and uridine. Both solutes experienced markedly increased retention at higher concentrations of ACN (>60% v/v), in line with the supposition that a HILIC retention mechanism was operating at these levels. On the other hand, at low concentrations of ACN (<20% v/v), a significant RP type of retention was observed for the purine base adenosine, to a lesser extent also for guanosine, generating U-shaped curves of retention factor versus ACN concentration. This effect was not observed for the pyrimidine bases uridine and cytidine or the nucleobases cytosine and uracil. The effect was most pronounced with the nonoxidized and therefore less polar phases.

More recently, Sandra and coworkers have coined the term “per aqueous liquid chromatography” (PALC) to describe separations obtained on polar columns using eluents that are 100% aqueous or contain only low concentrations of organic solvents. These applications are recommended as examples of

"green chemistry" and overcome one of the main drawbacks of classical HILIC separations—the environmental cost of using toxic solvents [44]. While not strictly using HILIC conditions, these separations are of interest here because the retention mechanism may be contributory to that of HILIC, at least in situations where the organic content of the mobile phase is relatively low. Separations of some neurotransmitters on a bare silica column have been reported using this technique [44]. However, in later papers, Gritti and Guiochon (together with the original authors) showed that the surface of silica columns when operated with PALC mobile phases was seriously heterogeneous, with up to five different adsorption sites, including a small number of very strong sites [45,46]. They concluded from theoretical and practical considerations that better sensitivity, higher efficiency, and better resolution could be obtained in the conventional HILIC mode where the adsorption mechanism was found to be much more homogeneous. A problem with the PALC mode was found to be serious overloading of the few strong column sites even with very small amounts of some solutes, giving a poor peak shape. PALC separations with moderate solute k gave the worst column efficiencies, and only addition of ACN, resulting in very small solute k, and apparent blocking of these strong sites by this solvent, gave reasonable column efficiency. In a more recent study, Sandra and coworkers showed some separations on polyethyleneglycol (PEG) or diol columns with either 100% water or water containing 1% ACN or ethanol at 60°C giving efficiencies of up to 76,000 plates/m for solutes such as caffeine, acetophenone, aniline, phenol, toluene, and benzene. However, the authors reported some phase instability of the PEG column under PALC conditions [47].

1.3.5 Electrostatic Repulsion Hydrophilic Interaction Chromatography (ERLIC): A New Separation Mode in HILIC

In 2008, Alpert proposed a new variant of HILIC that has the potential to make significant contributions to the separation of biomolecules [48]. He noted that an elution gradient is often used to ensure that all solutes in a mixture elute in the same time frame. This is true particularly for the separation of biologically important molecules such as peptides, where the individual components of the mixture can have widely different retention times. However, an alternative strategy is to superimpose a second mode of chromatography on the primary separation mechanism that selectively reduces the retention of solutes that are usually the most strongly retained. The new mode uses coulombic effects superimposed on the usual HILIC separation mechanism and was termed ERLIC or electrostatic repulsion hydrophilic interaction chromatography. It uses an ion-exchange column of charge of the same sign as that on the solutes. In this paper, Alpert re-iterated the model for the retention mechanism of HILIC being mostly partitioning between the dynamic mobile phase and a slow moving layer of water with which the polar stationary phase is hydrated.

Alpert noted that gradients for HILIC involve increasing the polarity of the mobile phase, typically by decreasing the concentration of organic solvent. However, it is also possible to use increasing salt concentrations in a mobile phase containing 60–70% organic solvent. If a cation exchange column is used to separate acidic amino acids, the solutes will elute prior to the void volume of the column, as electrostatic repulsion prevents access of these solutes (which have the same charge as the column groups) to the full pore volume of the stationary phase. However, if the mobile phase contains >60% organic solvent, then acidic amino acids show almost the same retention on cation exchange columns as given by neutral columns, as had been shown by Alpert's original experiments using PolySulfoethylA and PolyHydroxyethyl phases [10]. The rationale given for this result was that hydrophilic interactions are independent of electrostatic effects. If sufficient organic solvent is used in the mobile phase, then hydrophilic interaction dominates solute retention. Another example discussed was that phosphate groups decrease the retention of basic histone proteins on a cation exchange column (presumably because the increased negative charge on the solute produces repulsion from the negatively charged column sites) in the absence of an organic solvent [49]. However, under HILIC conditions with the mobile phase containing 70% ACN, the phosphate groups lead to a net increase in retention of the protein. The hydrophilic interaction conferred by the phosphate groups acting to increase retention is stronger than the electrostatic repulsion, which decreases retention.

Alpert argued that basic solutes are usually the most retained in HILIC, followed by phosphorylated solutes [10]. Thus, gradients are necessary to separate samples that contain very basic peptides or strongly phosphorylated compounds such as adenosine triphosphate (ATP). However, if an anion exchange column was used in the HILIC mode, gradients should be unnecessary. An example of the application of this type of ERLIC separation is shown in Figure 1.14, which demonstrates the simultaneous separation of basic and acidic peptides using an isocratic method. Usually, basic peptides have much stronger retention than acidic peptides on a neutral HILIC column like PolyHydroxyethyl A. However, use of a PolyWAX LP column, a weak anion exchange material, gives repulsion of the positively charged basic compounds, reducing their retention to values similar to that of acidic peptides (whose retention is increased). Another possible application is the use of an anion exchange column at low pH, under which conditions tryptic peptides from protein digests are mostly uncharged at the carboxyl end, leaving peptides with a net positive charge. These peptides are repelled from the positively charged stationary phase, leaving peptides with phosphate groups or glycopeptides with sialic acid residues that retain negative charge under these conditions to be retained selectivity. Note that if a classical anion exchange column is used, the presence of a single phosphate still produces low retention of the peptide, due to repulsion of the positive ends of the peptide.

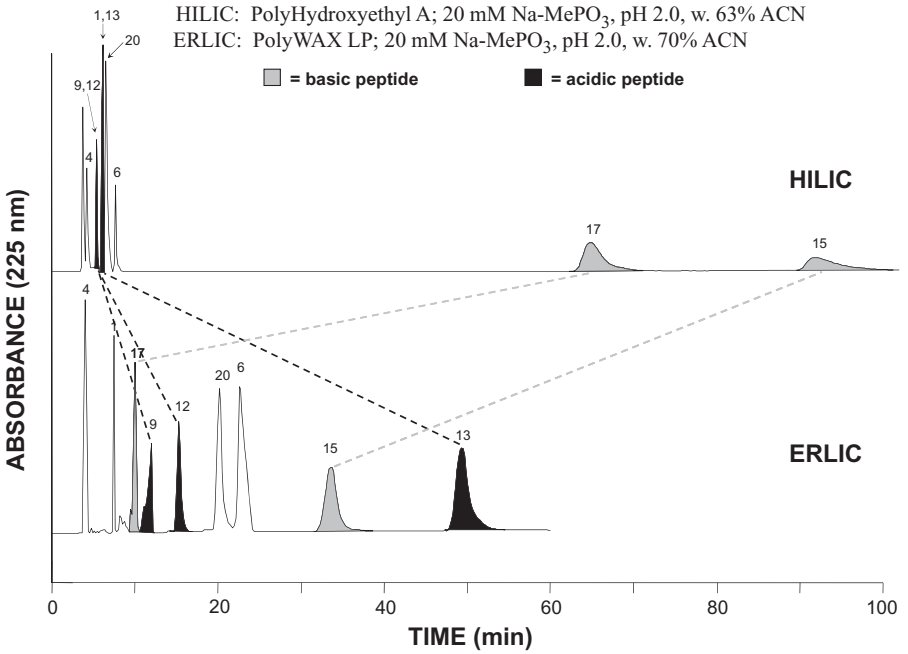

Figure 1.14. HILIC versus ERLIC separation of peptide standards. HILIC mode (top). Column: PolyHydroxyethyl A. Mobile phase: 20 mM Na-$MePO_4$, pH 2.0, with 63% ACN. Flow rate: 1.0 mL/min. ERLIC mode (bottom). Column: PolyWAX LP. Mobile phase: 20 mM Na-$MePO_4$, pH 2.0, with 70% ACN. Flow rate: 1.0 mL/min. Reprinted with permission from *Anal. Chem.* 2008; **80**: 62–76. Copyright (2008) American Chemical Society.

However, with the superimposed HILIC mechanism, retention of such compounds can be achieved.

Salt concentration is a critically important parameter in ERLIC separations in determining selectivity. Increasing levels of salt shield solutes from all electrostatic effects, both attractive and repulsive, and at high salt concentrations, the selectivity converges on that of HILIC. Using an anion exchange column in the ERLIC mode, retention of acidic peptides was shown to decrease as expected for acidic peptides (which undergo coulombic attraction with the column groups) but to increase for basic peptides (which undergo coulombic repulsion) with increasing salt concentrations. At the highest salt concentrations studied (120 mM $NaMePO_4$, pH 2.0) with 65% ACN, basic peptides once again became the most retained.

Alpert showed a number of other applications of ERLIC, including the separation of acidic, basic, and neutral amino acids, and the separation of nucleotides without recourse to gradients. Clearly, this new separation mechanism has much potential, particularly for the separation of molecules of biological significance.

1.4 CONCLUSIONS

Interest in HILIC separations has increased rapidly, in particular over the last 5 years. For the separation of polar, hydrophilic, or ionized compounds, HILIC shows many advantages over RP-LC. A better understanding of the mechanism of these separations is emerging, although the technique is not nearly so well understood as RP-LC. Contributory mechanisms are likely to be partition, adsorption, ionic interactions, and even hydrophobic retention depending on the experimental conditions. Although for samples to which it is applicable, HILIC has many advantages over RP-LC, the limitations of HILIC should also not be overlooked. These include problems with the solubility of some solutes, particularly in preparative separations, the longer time required for column equilibration than in RP, and the lack of applicability to the large number of solutes that are insufficiently polar. The lack of understanding of the HILIC method is also a barrier to the development of new analytical methods. Nevertheless, it seems that HILIC is a technique that is now firmly established as a complementary approach to RP analysis.

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