Journal of Chromatographic Science 2013;51:684–693
doi:10.1093/chromsci/bmt015 Advance Access publication March 13, 2013

Review Article

Main Interactions and Influences of the Chromatographic Parameters in HILIC Separations

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Received 1 October 2012; revised 7 January 2013

Hydrophilic interaction liquid chromatography (HILIC) is a popular technique for the separation of polar compounds, which are poorly retained by reversed-phase liquid chromatography. Despite the versatility and the potentiality of this technique, many analysts still feel uncomfortable when approaching it. The HILIC retention mechanism is not completely elucidated and the availability of many different stationary phases may be confusing during method development. Understanding the principles that drive the separation and how they can be influenced by the selection of both stationary phase and chromatographic conditions enhances the range and the quality of possible applications. For this purpose, the review discusses the primary interactions at the basis of HILIC separations and presents an overview of the most common HILIC stationary phases. The effects of the stationary phase type and chromatographic parameters (i.e., organic solvent and salt content, mobile phase pH and column temperature) on each specific interaction are highlighted.

Introduction

Hydrophilic interaction liquid chromatography (HILIC) is now considered to be a consolidated and established analytical tool for the separation of polar compounds. Initially developed as a method for carbohydrate separation (1, 2), HILIC has more recently received a second successful life, in which the growing number of scientific publications and application fields are widely recognized as clear evidence of the potentiality of this technique (3).

HILIC provides excellent results in the separation of both small and large hydrophilic and very polar molecules, which include carbohydrates, amino acids, peptides and proteins, glycoproteins, nucleosides, vitamins, phenols, pesticides, toxins and a wide range of other hydrophilic metabolites occurring in food, water, human fluids and human tissue extracts (4–11). HILIC is becoming a routine technique in proteomic, glycomic and metabolomic research, and it is increasingly contributing to the fields of pharmaceutical, environmental and food chemistry (4, 12–15).

The reason for the popularity of HILIC principally resides in two factors. HILIC shows a complementary selectivity to reversed-phase liquid chromatography (RPLC), giving an elution order opposite to that obtained in RPLC, and in most cases, it represents the best choice for the separation of compounds, which are not sufficiently retained under RPLC conditions (16, 17). The second advantage is the use of water-miscible organic rich mobile phases, generally a mixture of water and acetonitrile (4). The HILIC mobile phases are compatible with atmospheric pressure mass spectrometric (MS) analysis and the presence of a high content of the organic solvent increases the detection sensitivity because of the higher efficiency of the spraying and desolvation processes (17). Other practical positive aspects of the use of these liquid phases are the lower back pressure than RPLC mobile phases, owing to the minor viscosity of the organic rich eluents, and the feasibility to inject samples in organic solvents (15, 17, 18).

Despite these interesting characteristics, many analysts are still suspicious and sometimes confused about what HILIC is and how to use it. The huge and growing number of commercially available HILIC stationary phases and lack of a versatile stationary phase, such as the C18 is in RPLC, contribute significantly to this disorientation. The retention mechanism that drives the HILIC separation is more complicated than that in RPLC, simultaneously comprising several kinds of interactions (3, 4, 17, 19). Identifying which is the prevailing interaction can be difficult, and the theory at the basis of HILIC mechanism is still under investigation (3, 4, 17, 19–21). The different interactions that contribute to the overall HILIC retention are strictly dependent on the stationary phase and can be variously modulated with the selection of the mobile phase (3, 17, 19).

In 2006, Hemström and Irgum published an excellent and comprehensive review about HILIC, with particular emphasis on the history and the theory of the retention mechanism (3). Following reviews have covered the advancements of the technique and discussed specific aspects in more detail, such as the development and selectivity of different HILIC stationary phases (15, 18, 19) and the impact of mobile phase composition and column temperature on HILIC selectivity (18, 22).

However, a simple description of the primary interactions at the basis of HILIC retention and the ways an analyst approaching to this new technique can practically act on them have not yet been presented. A deeper comprehension of the HILIC retention mechanism is fundamental in the selection of the appropriate column and can provide a guide during the optimization of a HILIC separation.

This review discusses the primary interactions at the basis of the HILIC retention, the most popular retention models, the principal stationary phases and how the variation of chromatographic parameters (i.e., organic solvent and salt content, mobile phase pH and column temperature) affect each specific interaction.

HILIC Retention Mechanism

HILIC is often presented as a variation of normal-phase liquid chromatography (NPLC), because both methods are based on the use of polar stationary phases and the retention increases

with the increase of analyte polarity (3, 7, 19). In NPLC, non-aqueous apolar mobile phases are employed, generally consisting of hexane, and the elution is promoted by the increase of a polar solvent, such as ethyl acetate (3). Unlike NPLC, HILIC mobile phases are mixtures of water–water miscible organic solvents, usually acetonitrile. Although HILIC mobile phases are more similar to those in RPLC, the solvent strength is opposite. Indeed, the starting setting in HILIC requires a high percentage of organic solvent and elution is promoted by increasing the water content in the mobile phase (4).

Whereas RPLC is related to liquid–liquid chromatography and can be satisfactory described with partition models, NPLC retention is based on surface adsorption (3, 23–25).

Despite some similarities with RPLC and NPLC, HILIC has to be considered a new type of chromatography; frequent comparisons with other separation techniques can be misleading in the comprehension of HILIC retention mechanisms.

The mechanism of HILIC separation is more complex and is still waiting for a theoretical description. It is often discussed as a mixed-mode mechanism. Partitioning of the analyte between the organic-rich mobile phase and the water enriched layer partially immobilized on the stationary phase is considered to be the primary retention mechanism (4). Also, other important interactions such as electrostatic interactions (attraction or repulsion) (26, 27) and hydrogen bonds contribute significantly to HILIC separation (20, 28).

Water layer

Polar stationary phases, such as the ones used in HILIC, are known to strongly retain water (29, 30). In the presence of an aqueous mobile phase at high acetonitrile content, there is the creation of an eluent gradient, which ranges from an acetonitrile-rich bulk to a water-rich layer near the hydrophilic surface of the phase (4). Recently, Wikberg and colleges have experimentally proven that in HILIC conditions, the water layer is immobilized and adsorbed onto the surface of the hydrophilic stationary phase (31).

The acetonitrile-rich bulk and the water-rich layer are two liquid phases of different polarity and can be regarded as a liquid–liquid separation system.

This supports the view that the HILIC separation mechanism is primarily governed by the partitioning of the analyte between these two phases on the basis of the relative solubility. As a consequence, polar hydrophilic analytes are preferentially solubilized into the water layer, and thus, strongly retained (3, 4, 19).

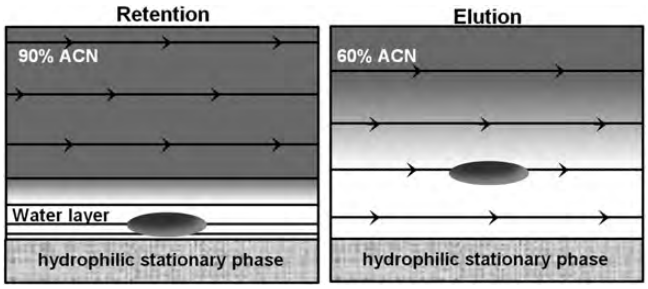


Figure 1. Scheme of the HILIC partitioning of a general polar analyte into the water layer adsorbed on the surface of the hydrophilic phase.

A minimum of 2–3% of water in the mobile phase is necessary for the creation of the water layer (16, 32). The retention of polar analytes is sustained at a high organic content in the mobile phase, because in these conditions water interacts more strongly with the surface of the polar stationary phase (15, 33, 34).

Increasing the water content has the effect of decreasing the difference in polarity between the bulk and the adsorbed layer. This results in the solubilization of the analyte in the mobile bulk phase, with ensuing elution (3, 4, 19).

A schematic description of the retention of a generic hydrophilic analyte in the water layer at high acetonitrile content and its elution with the increase of the water content in the mobile phase is provided in Figure 1, in which the influence of the mobile phase composition on the water layer is represented.

Electrostatic interactions

Charged analytes are generally ideal compounds to be separated in HILIC mode, because they are more hydrophilic than their uncharged forms; therefore, they are more retained under HILIC conditions (19). In this case, the presence of ionized groups or, for example, underivatized ionized silanol groups, on the stationary phase may lead to the creation of electrostatic interactions with charged analytes (6, 17, 35). These interactions can be attractive or repulsive, depending on the charged state of both the analyte and the stationary phase. Electrostatic attractions between positively charged compounds with negatively charged stationary phases, and vice versa, produce the increase of retention times, whereas electrostatic repulsion between analytes and phases with same charges have the opposite effect (6, 26).

The mechanism is partly similar to that observed in ion-exchange chromatography (IEC). However, several studies have proved that the retention behavior of charged analytes eluted under HILIC conditions is not completely described by conventional IEC retention models (20, 36, 37). Indeed, when electrostatic interactions can occur, they may also be considerable, but always represent only one contribution to the overall HILIC retention. Particularly interesting is the case of solutes that have the same charge as the stationary phase. They can still be retained through hydrophilic interactions, owing to a proper orientation of the molecule, which aims to minimize the electrostatic repulsions (Figure 2). This combination has recently been described by Alpert and is termed electrostatic repulsion–hydrophilic interaction chromatography (ERLIC) (38).

Hydrogen bonds

In HILIC, the possibility of direct interactions, i.e., hydrogen bonds, of the analyte with the stationary phase in addition to

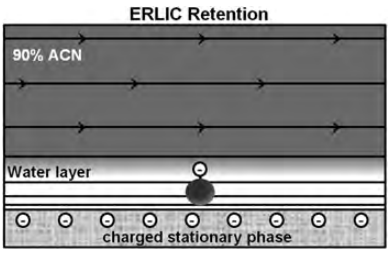


Figure 2. Retention of a schematic negatively charged compound on a negative charge stationary phase in ERLIC mode.

partitioning into the water layer has been debated for long time.

To gain some insights into the different contributions to the HILIC retention mechanism, quantitative structure-retention relationship (QSRR) approaches have been employed by several research groups. Among QSSR models, the linear solvation energy relationship (LSER) model has been widely used to investigate the factors affecting retention in chromatographic systems [detailed discussions are provided by Chirita *et al.* (36), Kozlík *et al.* (39), Abraham *et al.* (40), Quiming *et al.* (41) and Jandera *et al.* (42)]. LSER relates the retention of a given analyte to its physicochemical and structural parameters, using the solvatochromic descriptors provided by Abraham *et al.*, which include hydrogen bond acidity and basicity parameters (40).

The high values found for these parameters have proved that molecules with hydrogen-donor or hydrogen-acceptor functionalities can interact through the hydrogen bond with the stationary phase. This kind of interaction is evidently dependent on the possibility of the phase to create hydrogen bonds and becomes particularly important in case of uncharged analytes, when electrostatic interactions cannot support the retention (6, 36, 39).

The study of the retention of several hydroxybenzoic and aminobenzoic acids on a widespread HILIC phase has confirmed the importance of hydrogen bonds to the HILIC mechanism. Aminobenzoic acids, which have a hydrogen acceptor $-NH_2$ group, were less retained than their related hydroxybenzoic acids with a hydrogen donor $-OH$ functionality (20). Figure 3 shows a scheme of the hydrogen bond interaction between the phase material and the $-OH$ group of 4-hydroxybenzoic acid versus the lack of such interaction with 4-aminobenzoic acid.

HILIC retention models

Retention models are used in chromatography to predict the behavior of analytes under different conditions; they can be particularly useful during method development. In HILIC, the percentage of water in the eluent is the primary factor affecting the selectivity of the separation and several equations have been investigated that relate the retention to the water content in the mobile phase. Many works have indicated that the HILIC retention mechanism is very complex, in which both partitioning of the analytes between the bulk mobile phase and the water layer and adsorption-like interactions (hydrogen bonds or dipole–dipole) can occur. In particular, adsorption processes may become more significant at high contents of organic solvent in the mobile phase when the water layer is quite thin, allowing a direct interaction of solutes with the stationary phase (3, 17, 19).

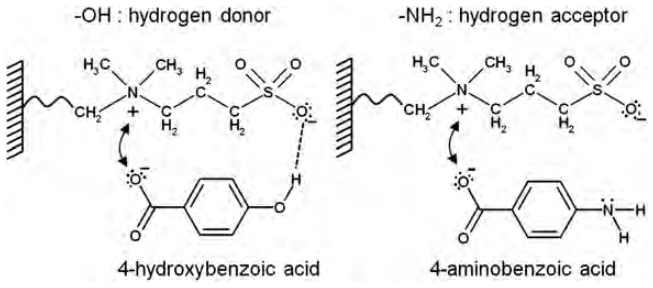


Figure 3. Phase material: sulfobetaine stationary phase (ZIC-HILIC). Schematic representation of the hydrogen acceptor activity of the sulfonate group with hydrogen donor or hydrogen acceptor analytes.

Initially, retention models developed for other kinds of liquid chromatography were applied to HILIC separations. The empirical equation established for partitioning in RPLC is (3, 23):

$$\log k' = \log k'_A - S\varphi \quad (1)$$

where k' is the solute retention factor, φ is the volume fraction of the stronger solvent in the mobile phase (water, in HILIC) and k'_A is the retention factor of the solute when the weaker solvent (ACN, in HILIC) is used as pure. Retention in NPLC is based on localized adsorption and can be satisfactorily described by the following equation (3, 24, 25):

$$\log k' = \log k'_B - n \log \varphi \quad (2)$$

where k'_B is the retention factor with pure stronger solvent as eluent.

In most cases, plots of log k' versus φ or log φ display curvatures, indicating that neither Eq. (1) nor Eq. (2) completely describes the HILIC retention mechanism. However, both equations are still very useful during the development and optimization of isocratic and gradient separations, and for the characterization of new stationary phases (37, 43, 44).

Recently, Liang and co-workers have introduced a new equation for describing HILIC retention (21):

$$\ln k' = a + b \ln \varphi + c\varphi \quad (3)$$

This retention model has a theory foundation and simultaneously considers both the adsorption of the solute on the stationary phase and its interaction with the solvent, so it should be more comprehensive and simultaneously account for both partition and adsorption processes. The constant a is related to the molecular volume of the solute and the interaction energy of the solute with the stationary phase and the mobile phase, the coefficient b is related to direct analyte–stationary phase interaction and c is related to the interaction energy between solute and solvent.

The retention behavior of nucleosides, water soluble vitamins and phenols on different stationary phases has been accurately described by this model (6, 20, 21). However, the usefulness of the model described by Liang and colleagues has not yet been completely elucidated and further investigations are required. In some cases, the presence of an additional regression parameter in comparison to Eqs. (1) and (2) may be responsible for the better fitting of the retention data (20).

The partition mechanism in RPLC, in addition to Eq. (1), can be also described by the following three-parametric equation:

$$\log k' = A\varphi^2 + B\varphi + C \quad (4)$$

This equation can also provide a good prediction of hydrophilic retention (20, 21).

In HILIC, chromatographic parameters other than the water content in the mobile phase can significantly influence the separation. For this reason, the employment of tools for experimental design is an interesting approach to study the simultaneous effect of different parameters on the retention behaviour of

solutes. The retention data as a function of two parameters (i.e., acetonitrile content and buffer concentration in the mobile phase, or buffer concentration and column temperature) can be represented as response surfaces in three-dimensional plots [detailed discussions are provided by Guo *et al.* (45), Jovanović *et al.* (46) and Rakić *et al.* (47)].

HILIC Stationary Phases

Any polar stationary phase that can retain water may be used in HILIC mode. The first applications were simply performed on NPLC phases, as amino and silica phases, and non-bonded silica is still among the most employed phases for HILIC separations. With the growing interest in polar compound analysis, many new functionalized stationary phases have been developed, which are suitable for a wide range of applications (18). Many HILIC bonded phases are silica-based, which are prepared by derivatization of the silica gel surface with polar groups.

The retention of an analyte cannot easily be predicted, because it depends on the characteristic of the stationary phase and on the specific interactions that can drive the separation (3, 19). First of all, the hydrophilicity of the stationary phase, and thus, its capacity to hold water, influences the thickness of the water layer in which the partition of the analyte can take place. In addition, the presence of charged groups or atoms, such as oxygen or nitrogen with lone pairs, may contribute to electrostatic and hydrogen bond interactions (6, 20).

The HILIC stationary phases are conveniently classified on the basis of the charged state of the functional groups, and they can be divided in neutral, charged and zwitterionic phases. Some selected HILIC phases with their relative properties are described in Table I.

Neutral stationary phases

Neutral stationary phases contain polar functional groups that are not in charged form in the range of pH 3–8, usually used for the mobile phase in HILIC; thus, they cannot provide ion-exchange interactions with any type of analyte. As consequence, the HILIC retention with the neutral phases is primarily supported by hydrophilic interactions. It has to be considered that at pH above 4–5, silica-based phases may contain deprotoned residual silanol groups that bring a negative charge and can contribute to electrostatic interactions. The majority of HILIC stationary phases belongs to this category, which comprises a large variety of functional groups such as amide, aspartamide, diol, ciano, cyclodextrine and saccharides (15, 19, 48).

Amide phases are produced by functionalization of the silica gel surface with carbamoyl or amide groups, linked through an alkyl spacer. They have found many applications for the separation of oligosaccharides and peptides (49, 50). Among the amide phases, the TSKgel Amide-80 is one of the most popular phases. It is extremely hydrophilic and neutral compounds are more highly retained than on other charged and non-charged phases (19).

Diol phases typically contain 2,3-dihydroxypropyl ligands, which show both hydrogen donor and hydrogen acceptor activities via the –OH group (7). In this case, hydrogen bond interactions take place, in addition to the hydrophilic partitioning, for polar analytes with hydrogen donor or acceptor functionalities. Diol phase are used for the analysis of proteins, polar metabolites and vitamins (51–53).

A particular type of diol phase is represented by cross-linked diol phases, such as the Luna HILIC column. Diol groups are connected through ether linkages and form a polymer layer on the silica surface containing both oxyethylene and hydroxy groups. These phases are more stable than classical diol phases and the polymer layer shields the residual silanols, so that cross-linked diol phases are completely independent from the pH of the mobile phase. In contrast, these phases result less hydrophilic and polar compounds are less retained under HILIC conditions (7, 19). Applications with cross-linked diol phases include the separation of phenols, oligonucleotides and estrogen metabolites (7, 54, 55).

Charged stationary phases

Charged stationary phases contain polar functional groups, which bear a positive or a negative charge that is usually dependent on the pH of the mobile phase. In this case, the separation of charged analytes is largely based on ion exchange mechanisms, in combination with hydrophilic partitioning (35).

Amino phases are among the first charged phases used for HILIC separations. The functional group is an aminopropyl ligand bonded to the silica support. The primary amino group is positively charged and shows high affinity for anionic acid compounds, which can be occasionally irreversibly adsorbed (56). Hydrophilic interactions prevail in the retention of uncharged polar compounds, whereas charged analytes are primarily retained through an anion exchange mechanism (56).

Amino phases are widely employed in proteomic and metabolomic fields. Some applications include the separation of polar metabolites, nucleosides and biomarkers in human and animal samples and natural sweeteners in plant extracts (49, 57–59).

Amino phases with ligands containing secondary or tertiary amino groups have the advantage over aminopropyl phases in that they cannot form Schiff bases with the carbonyl compounds; thus, they can be applied for the analysis of reducing sugars (60–61). This may also result in longer lifetimes of the columns (18).

Unmodified bare silica gels (Type A and B) are hydrophilic phases with silanol groups and siloxane bridges, which can display hydrogen donor and acceptor activities. At a mobile phase pH above 4–5, silanol groups are deprotonated and ion exchange may play an important role in the retention of charged compounds. The separation is achieved through a cation exchange mechanism in which positively charged basic analytes are strongly retained, whereas negative analytes are poorly retained due to electrostatic repulsions.

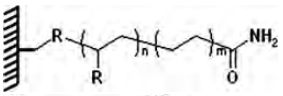
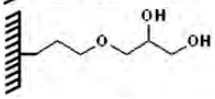
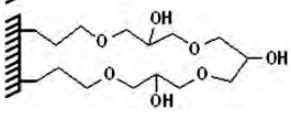
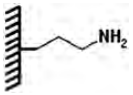
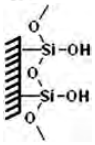
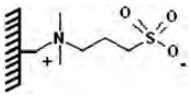
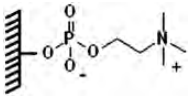
Type C silica gels are a class of less hydrophilic silica phases. They are obtained for hydrosilation of the silica surface and the majority of Si–OH silanols are converted in Si–H groups, which cannot contribute to either hydrogen bonds or electrostatic interactions.

Silica phases are among the most popular phases for HILIC, especially for the analyses of metabolites, toxic contaminants, and drugs in food and biological fluids (62–64).

Zwitterionic stationary phases

Zwitterionic stationary phases contain equal amounts of oppositely charged groups bonded in close proximity to the silica surface. The zwitterionic ligand generally comprises a strongly acidic and strongly basic functionality not sensitive to pH; such

Table I
Polar Stationary Phases Used in HILIC Separations

Phase name	Structure of stationary phase	Hydrophilicity*	Electrostatic interactions	Hydrogen bonds
Neutral phases				
Amide (TSKgel Amide-80)		+++	/	Hydrogen donor and acceptor
Diol		++	/	Hydrogen donor and acceptor
Cross-linked diol (Luna HILIC)		–	/	Hydrogen donor and acceptor
Charged phases				
Amino		+++	Strong (+)	Hydrogen donor
Silica		++	Strong (–) at pH>4–5	Hydrogen donor and acceptor at pH<4. Hydrogen acceptor at pH>4–5
Zwitterionic phases				
Sulfobetaine (ZIC-HILIC)		++	Weak (–)	Hydrogen acceptor
Phosphorylcholine (ZIC-cHILIC)		++	Weak (+)	Hydrogen acceptor

*Very high (+++), high (++) or low (–) hydrophilicity. Data are based on the study by Jandera (18).

phases have been initially developed for the separation of inorganic cations and anions in IEC. A description of the zwitterionic phases, their retention properties and the primary applications in IEC are topics of a recent review by Nesterenko and colleagues (65). An increasing number of manufacturers are placing new zwitterionic phases on the market. Among them, two peculiar zwitterionic columns, known as ZIC-HILIC and ZIC-cHILIC, are largely used for HILIC applications. ZIC-HILIC ligands are sulfobetaine groups, constituted of positively charged quaternary ammonium and negatively charged sulfonate groups separated by a short alkyl spacer. The presence of oppositely charged groups in a 1:1 ratio should impart a net surface charge equal to zero. However, the sulfonate group at the distal end of the phase gives a significant net negative charge to the ZIC-HILIC material (17, 20, 66). The ZIC-cHILIC phase with phosphorylcholine-type functionalities shows the opposite behavior. The different spatial dispositions of the charged groups, with the negative charged phosphate group bonded to the silica support and the positively charged quaternary ammonium in external position, are responsible for the positive charge of the phase (18, 67).

Zwitterionic ligands have a strong ability of binding water to the surface, and partitioning of hydrophilic analytes into the water layer remains the primary retention mechanism (20). Both sulfobetaine and phosphorylcholine ligands have hydrogen acceptor functionalities and adsorption-like interactions with hydrogen donor analytes may occur (20). In addition, the possibility of electrostatic interactions has a great influence in the elution order. Electrostatic interactions with the zwitterionic material are weaker than those with the charged phases, and compounds carrying either a positive or a negative charge can be retained by both types of zwitterionic phases. The different arrangements of the charged groups affect the separation selectivity, so that sulfobetaine phases show higher retention for basic compounds, whereas phosphorylcholine phases show higher retention for acid analytes (68).

Obelisc R and Obelisc N are other types of zwitterionic stationary phases, whose opposite charged groups are separated by a long chain. The two phases differ in the position of the charged groups and in the hydrophobicity of the spacer. In particular, Obelisc R has the negative group in external position linked to a hydrophobic spacer, whereas Obelisc N has the positive group in external position linked to a more hydrophilic spacer. The nature of the zwitterionic linkages is not specified by the manufacturer.

Zwitterionic phases are particularly versatile and have found several applications in the analysis of acid, basic and zwitterionic compounds. Specific exemplary separations include peptides, dietary phenols, metabolites in human fluids and drugs (10, 20, 67–71).

Effects of Chromatographic Parameters on HILIC Retention

The specific interactions between a given analyte and a HILIC phase are largely dependent on the chromatographic conditions;

the selection of the chromatographic parameters results in great differences in the retention. Several studies have investigated the retention behavior of selected analytes under different chromatographic conditions to elucidate the retention mechanism of many HILIC phases (6, 17, 19–20).

The following paragraphs present the effects of each chromatographic parameter (i.e., organic solvent and salt content, mobile phase pH and column temperature) on the various contributions to the HILIC mechanism. An insight into the relation between the chromatographic parameters and the HILIC interactions may permit a wiser modulation of the chromatographic conditions and finer optimization of the HILIC separation method.

Effect of organic solvent

The mobile phase in HILIC is constituted by an aqueous–organic mixture. As previously discussed, at high contents of organic solvent in the mobile phase, water is strongly adsorbed on the surface of the hydrophilic stationary phase. When the amount of water is above 5% of the mobile phase, the adsorbed water layer is thick enough to create a liquid–liquid partition system with the bulk organic mobile phase.

HILIC retention is strictly dependent on the composition and thickness of the water layer. At lower percentages of water in the mobile ($< 5\%$) direct interactions of the solute with the stationary phase are more likely to occur. This is reflected in a deviation from the partition mechanism, with consequent changing in the selectivity.

To ensure a better separation of the water layer from the bulk eluent, the ideal organic solvent should be miscible with water, but without hydrogen donor or acceptor functionalities, which can compete with water in the solvation of the stationary phase surface. Acetonitrile, which embraces all of these characteristics, is the most often used solvent for HILIC separations. Other types of water-miscible organic solvents that are occasionally employed include alcohols and cyclic ethers. Protic solvents, such as alcohols, provide the lowest retentions and are preferred in case of strong interactions with the column. Among the alcohols, the retention increases with the increase of the carbon chain (increased hydrophobicity) and the decrease of the acidity (decreased hydrogen donor activities) (72). Water-miscible cyclic ethers used in chromatography are tetrahydrofuran and dioxane. They display hydrogen acceptor activities that can interfere in the hydrogen bond interactions with the stationary phase and provide lower retention than acetonitrile. They are preferred for particular applications, such as the online coupling of HILIC to inductively coupled plasma mass spectrometer (73).

In general, the elution strength for common HILIC solvents follows the order: methanol > ethanol > isopropanol > tetrahydrofuran > acetonitrile.

The type of organic solvent may affect the retention and the elution order.

Independently from the stationary phase and the analyte, an increase in the retention is observed after increasing the acetonitrile content in the mobile phase, which represents a useful rule for identifying a HILIC mechanism.

Figure 4 shows the retention behavior of 2-hydroxybenzoic acid (2-HB) and 4-hydroxybenzoic acid (4-HB) on neutral, charged and zwitterionic columns (diol, amino and sulfobetaine phases, respectively) under different chromatographic conditions. 2-HB and 4-HB show typical HILIC behavior on all three columns; the retention of both is significantly decreased after doubling the water content from 10 to 20%.

Unlike in RPLC, water in HILIC represents the stronger eluting solvent. A classical gradient elution starts from 95% acetonitrile containing 5% of water and the water content is increased up to 40%. HILIC is more sensitive than RPLC to small changes of eluent composition and requires longer equilibration times to ensure complete column equilibration. The time varies significantly with column chemistry, but typically requires 15–20 column volumes.

Effect of salt

Salts are usually added to the mobile phase to control electrostatic interactions between charged analytes and the stationary phase. Salts commonly used in HILIC are ammonium acetate, formate and bicarbonate, due to their good solubility in mobile phases with high levels of organic solvent and compatibility with mass spectrometric detection. In several cases, the eluting strength of the ammonium salts follows the order: bicarbonate > formate ~ acetate.

The majority of HILIC separations are achieved with mobile phases containing ammonium formate or acetate. At the same concentrations, the use of one salt instead of the other may have a great impact on the retention, depending on the specific case (6, 26).

Increasing the salt concentration has the general effect of decreasing the electrostatic interactions of charged analytes on charged or zwitterionic columns. In the case of electrostatic attractions, this leads to decreased retention, whereas in the case of electrostatic repulsions, it leads to increased retention.

Typical salt concentrations are in the range of 5–100 mM. For better reproducibility and peak shape, it is recommended to maintain a minimum buffer concentration of at least 5 mM in the final mobile phase. At 95% acetonitrile in the mobile phase, the salt concentration has to be below 15 mM, due to limited solubility (20), and the salt should be solubilized in water before adding the organic solvent.

In addition to the effect on the electrostatic interactions, the salt concentration modifies the thickness of the water layer, also affecting the retention of uncharged compounds, for which electrostatic interactions are not present. A high percentage of organic solvent in the eluent causes salt to partition preferentially into the water layer. Increasing the salt concentration leads to an increase of the water layer, with ensuing stronger retention.

This double effect of the salt concentration can be observed when analyzing the retention of 2-HB and 4-HB on the three columns (Figure 4). At neutral pH, both analytes are in their charged anionic forms due to deprotonation of the carboxylic functionality. Increasing the concentration of ammonium acetate from 10 to 15 mM at a constant level of 90% acetonitrile in the mobile phase leads to a significant increase of their retention on the neutral diol column due the increase of the water layer. Their retention can partly be caused by a decrease in electrostatic repulsions with underivatized silanol groups. The increased retention observed on neutral phases at higher salt concentrations is also attributed to a salting-out effect, for which water molecules are involved in the solvation of salt ions;

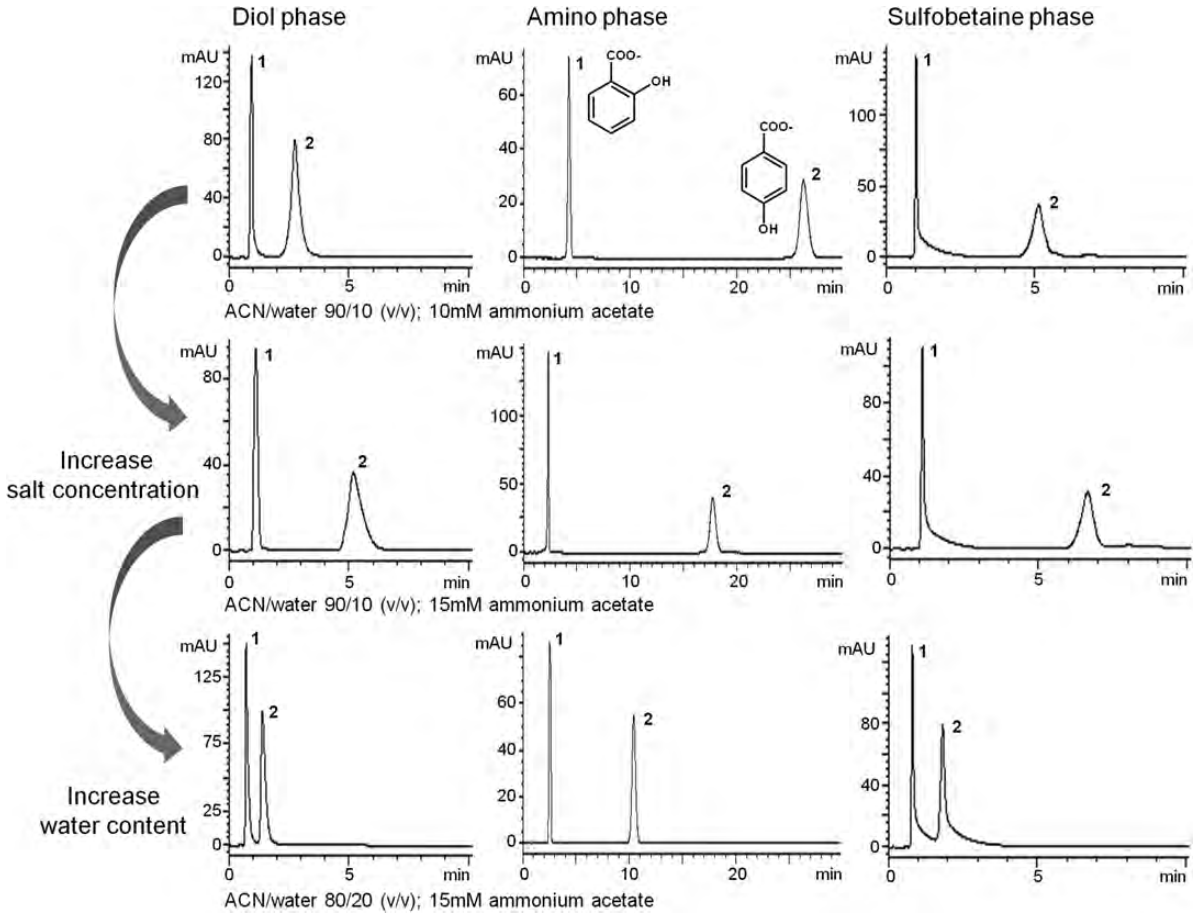


Figure 4. Separation of 2-HB (peak 1) and 4-HB (peak 2) under different chromatographic conditions. Columns: YMC-Diol (150 × 2.1, 5 μm); Luna-NH2 (150 × 2.0, 5 μm); ZIC-HILIC (150 × 2.1, 5 μm). Eluent: acetonitrile–water, 90:10 or 80:10 (v/v), containing ammonium acetate at final concentration of 10 or 15 mM. Column temperature: 25°C. Flow rate: 0.6 mL/min. Ultraviolet detection at 280 nm.

therefore, direct interactions between analytes and stationary phase are favored (18).

In the case of the positive charged amino column, electrostatic attractions with the anionic 2-HB and 4-HB play an important role in the retention. Increasing the salt concentration causes a decrease in the electrostatic attraction, with a consequent decrease in the retention. As previously discussed, in the zwitterionic column, the anionic group in the external position leads to preferential interactions; thus, in case of negatively charged 2-HB and 4-HB electrostatic repulsion prevails on electrostatic attractions with the internal cationic group. Even if the increase of the salt concentration diminishes both kinds of interactions, the primary effect is the decrease in the electrostatic repulsions, so that the two analytes are strongly retained.

Effect of mobile phase pH

The primary effect of the pH of the mobile phase is on the charged state of the analyte. It is preferable to select the pH to bring the analytes in their charged form, because they are usually more hydrophilic than their neutral forms and more retained in HILIC. However, other kinds of considerations, such as the selected ionization mode in electrospray ionization (ESI)-MS detection, or strong electrostatic attraction between analytes and stationary phases of oppositely charged states, may suggest that different pH conditions will be preferable.

The majority of HILIC separations are performed with mobile phases in the pH range of 3–8 and formic and acetic acids are the usual acid additives due to their volatility and MS compatibility.

In general, HILIC stationary phases are independent from the pH of the mobile phase. The primary main exception is represented by silica phases, in which the surface silanol groups are deprotonated at pH above 4–5, making the phase strongly negatively charged. Electrostatic interactions with positive charged analytes, such as amino compounds, are dramatically increased on silica phases, increasing the pH of the mobile phase (32). Also, silica-based phases may contain underivatized silanol groups, whose pH-dependent charged state may affect the retention and selectivity in HILIC.

Effect of column temperature

A recent study investigated the simultaneous effect of acetonitrile content, ammonium acetate concentration and column temperature on the retention of hydrophilic compounds in HILIC (45). The primary effects were observed for the modification of the chromatographic parameters in the following order: acetonitrile content > ammonium acetate concentration > column temperature.

Column temperature has a minor impact on retention and its effect is primarily related to the enthalpy of the analyte transfer between mobile phase and stationary phase. Generally in HILIC, a decrease in the retention time is observed, increasing column temperature (18, 45, 74). This phenomenon has been interpreted under the consideration that the transfer of hydrophilic analytes from the bulk mobile phase at high acetonitrile content to the water layer is an exothermic process, favored at lower temperatures. Basic analytes on silica columns or acid analytes on amino columns may show different behaviors when changing column temperature. When the retention is primarily governed by strong electrostatic attractions between charged analytes and charged stationary phases, increasing the column temperature leads to an increase in the retention (22, 26).

In this case, the formation of an ion pair between two oppositely charged groups is an endothermic process, supported at higher temperatures.

Conclusion

An increasing number of analysts are discovering the potentiality of HILIC, especially for the analysis of small and high polar compounds, which is a task that can be challenging with other conventional chromatographic techniques. A better understanding of how to modulate the water layer at the basis of HILIC retention through the choice of an appropriate stationary phase or how to change the composition of the mobile phase are the keys for effective method development. Additionally, it is important to consider whether electrostatic interactions or hydrogen bonds can occur between the stationary phase and the analytes. Knowledge about how to support or hinder them with the salt concentration or mobile phase pH leads to faster optimizations.

Acknowledgments

This work was in part supported by a financial grant from the HWP Program "Equal Opportunities for Women in Research and Academic Teaching," TU München, that covered Dr. G. Greco's fellowship. Merck SeQuant, YMC and Phenomenex are kindly acknowledged for providing HILIC columns as a gift.

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