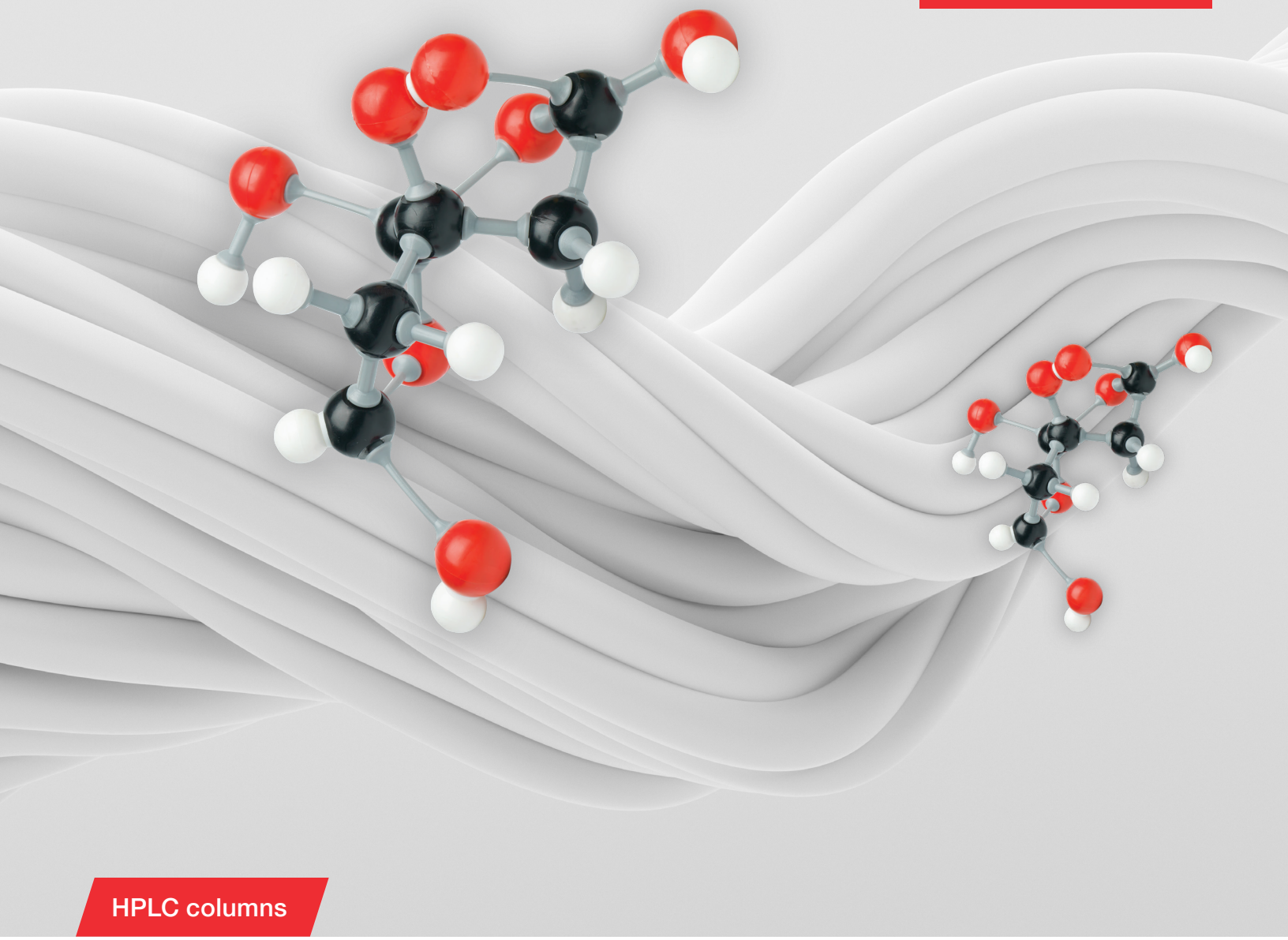
ThermoFisher SCIENTIFIC



HPLC columns

HILIC separations

Practical guide to HILIC mechanisms, method development and troubleshooting

thermo scientific

HILIC separations

Hydrophilic Interaction Liquid Chromatography (HILIC) is the most successful approach for the retention and separation of polar compounds. HILIC has seen a substantial increase in popularity over the last decade, driven by the increase in the development of polar drugs within the pharmaceutical industry and also the growing field of metabolomics, which primarily involves the analysis of polar molecules.

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Introduction

Early history

This technique has been described as “reversed ‘reversed phase’”,[1] since in HILIC the stationary phase is polar and the aqueous portion of the mobile phase acts as the stronger solvent, which is the opposite of conventional reversed phase chromatography. There are many stationary phases that can be used in a HILIC mode and many phases are generically described as HILIC, independently of their chemistry. There is also a variety of misinformation regarding the use of this technique. So what column should be used? What are the best mobile phase starting conditions? What are the common issues in HILIC method development that need to be addressed? These are the type of questions that HILIC users face and will be addressed within this guide. A range of applications will also be described which will highlight the use of this technique and how it can be successfully implemented into a working laboratory.

The HILIC mode of separation can be traced back to 1951, when Gregor et al.[2] described a water-enriched layer on an ion-exchange resin surface. The following year, Samuelson and Sjöström analyzed monosaccharides on an ion-exchange column.[3] Then, in 1954, Rückert and Samuelson postulated that a stagnant water layer could be responsible for the uptake of analytes.[4] Several years later, in 1975, the analysis of sugars was accomplished on amino columns.[5, 6] Nowadays, the existence of an semi-immobilized water-enriched layer on the polar stationary phase, combined with a partitioning equilibrium of analytes into the mobile phase, are accepted as the dominant HILIC mechanisms.[1, 7] However, it was not until 1990 that the acronym HILIC was first introduced by Alpert.[7] Hemström and Irgum[1] have written a comprehensive review on the subject matter which supplies an excellent background to the technique and how it has developed.

Typical HILIC stationary phases and applications

In spite of its early beginnings, HILIC did not become widely recognized as a distinct chromatographic mode until it was 'rediscovered' by the scientific community in the early 2000's.[8] The rising popularity of HILIC coincided with a wider availability of specifically designed HILIC stationary phases with diverse functionalities, which could offer different selectivity and higher retention for polar compounds.[8] However, unmodified bare or hybrid silica materials are still the most popular phases. Silica columns specifically intended for HILIC have been developed; these are packed and stored in aqueous/organic, which contrasts the more conventional use of silica in normal phase chromatography where the mobile phase would be a mixture of hexane and IPA solvents. Silica materials have also become available in sub-2 µm fully porous particles, in superficially porous particles and as monolithic columns. Approximately 35% of the reported applications are being developed on bare silica, as illustrated in the Figure 1.

HILIC phases vs % of applications

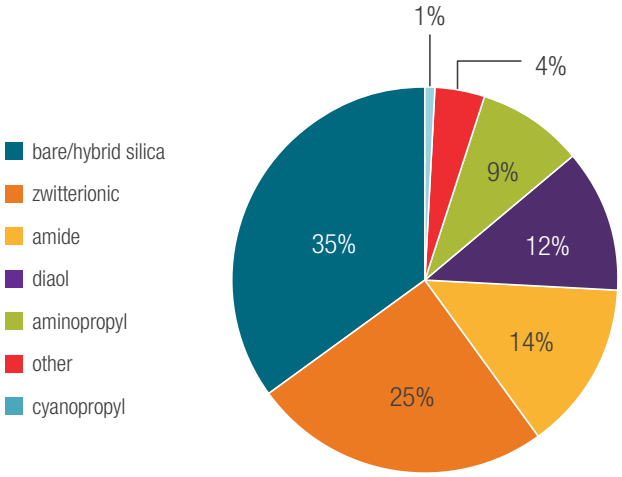


Figure 1. Based on Scifinder Scholar 2007 search of the Chemical Abstracts database 2003–2012

Typical HILIC stationary phases and applications (continued)

From the data above, it can be seen that the second most employed type of HILIC material are the zwitterionic phases, a typical example of which is the sulfobetaine phase. The sulfobetaine zwitterion has both positive (quaternary ammonium) and negative (sulfonic acid) groups in a 1:1 ratio, so that the net surface charge is zero, as illustrated in the Figure 2.

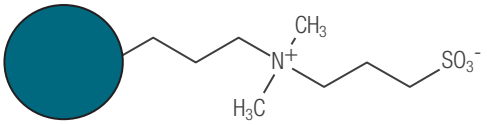


Figure 2. Sulfobetaine structure

The sulfoalkylbetaine zwitterionic functionality was originally introduced into polymeric supports by Irgum *et al.* to prepare ion-exchange materials for the analysis of inorganic compounds and proteins.[1] Subsequently, a similar functionality was immobilized on silica substrates. It must be pointed out that, although the net surface charge is zero, the negative charge of the sulfonic acid at the distal end of the phase may introduce some degree of electrostatic interaction with charged analytes.[9] Irgum and his group have discussed a new type of zwitterionic phase, with phosphorylcholine groups grafted onto a polymeric substrate. This material has a positively charged ammonium group at its distal end.[10] Zwitterionic phases are successfully used for the analysis of charged and neutral species, regardless of the possible electrostatic interactions that can arise.

Amide-bonded silica phases are another popular choice in HILIC. The amide functionality, which is attached to the silica surface either through a propyl or proprietary linker, cannot be charged in the pH range typical for HILIC mobile phases. Retention is therefore less affected by ion-exchange interactions. Additionally, amide phases are less prone to irreversible adsorption (a problem often associated with aminopropyl phases).[11] Amide columns also show good reproducibility and stability.

Other popular HILIC phases include:

1. Diol material, which exhibit a considerable degree of hydrophilicity

2. Aminopropyl materials are used for HILIC separations, especially for carbohydrate analysis. They have, however, shown irreversible adsorption of analytes (at least for reducing sugars), due to the reactive nature of amino functionalities[11]

3. Cyano materials find limited applications in HILIC, as insufficient retention of most polar compounds is generally experienced. This is due to the fact that cyano groups do not have hydrogen bond donor capabilities and therefore are not very hydrophilic

To monitor HILIC trends, Pontén et al.[12] conducted a literature survey for which the results are summarized below. It can be seen that the majority of the HILIC applications are found in the pharmaceutical and clinical markets with 46% of the research papers being published coming from these areas. Another contributing factor to the growing success of HILIC was its suitability for a large range of bioanalytical compounds that started to be considered at the time by the pharmaceutical industry because of their pharmacodynamic properties.[1]

A significant number of publications (18%) relate to biotechnology applications, in particular to proteomics, metabonomics and glycomics. 25% of the applications are distributed between a variety of areas, with food analysis being a major contributor.

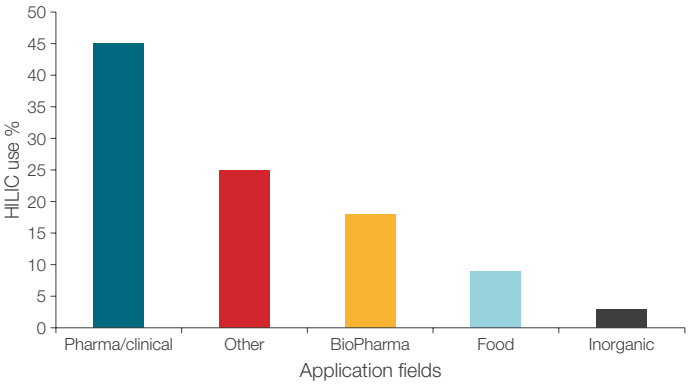


Figure 3. HILIC application field distribution

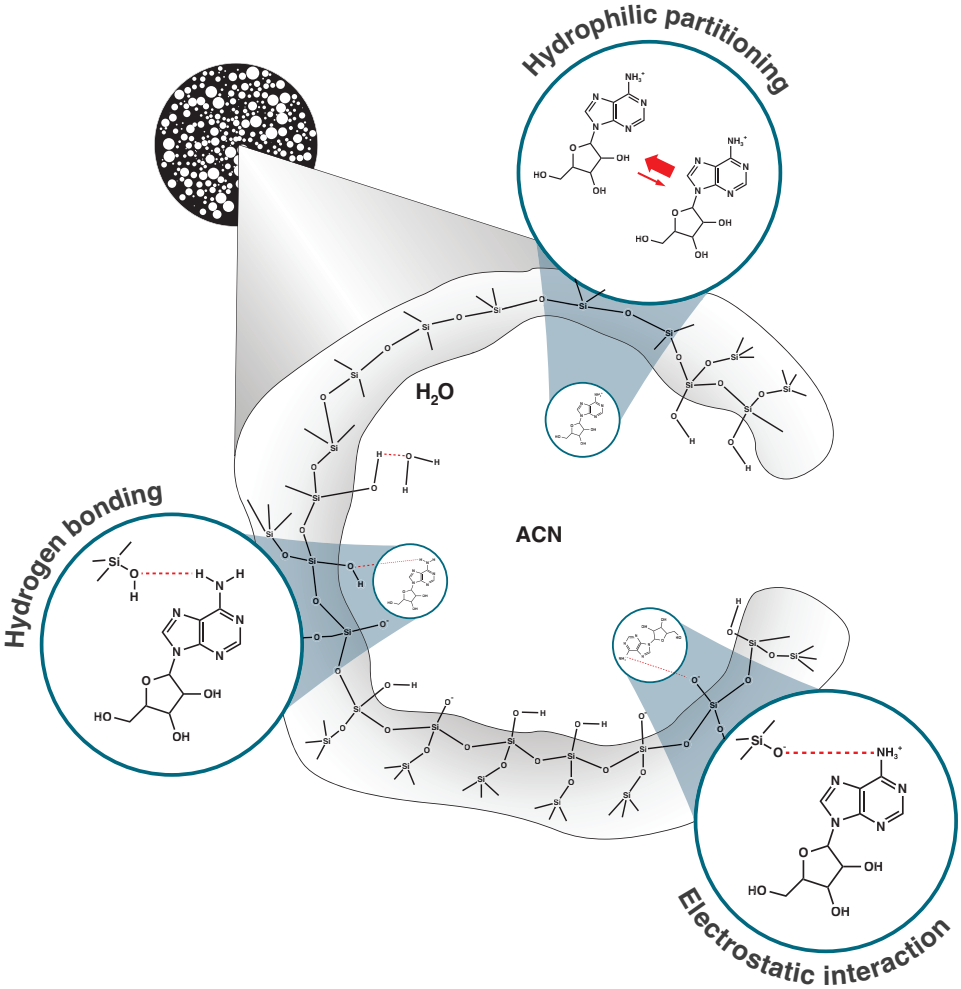
HILIC retention mechanism

HILIC can be described as a variation of reversed phase liquid chromatography (RPLC) performed using a polar stationary phase. The mobile phase employed in HILIC is highly organic in nature (>60% solvent, typically acetonitrile) containing a small percentage of aqueous solvent/buffer or other polar solvent. The water in the mobile phase forms an aqueous-rich layer absorbed to the polar surface of the stationary phase.

Polar analytes preferentially partition into this aqueous rich layer and evidence suggests that they are retained through a complex, combination of:

- Hydrophilic partitioning of the analyte between the aqueous-rich layer and the bulk of the mobile phase
- Hydrogen bonding between polar functional groups and the stationary phase
- Electrostatic interactions on ionized functional groups

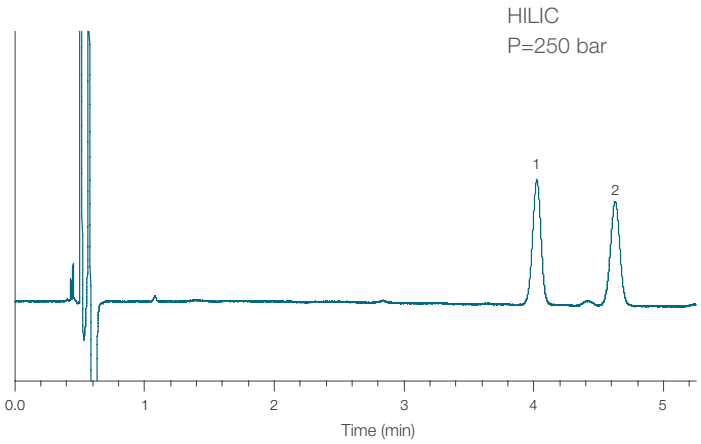
Additionally, under the appropriate experimental conditions, van der Waals interactions between the hydrophobic portions of the bonded ligands of the stationary phase (or the siloxane groups, at very low organic solvent concentrations), and the non-polar part of the analytes can also be present.[13]



HILIC retention mechanism (continued)

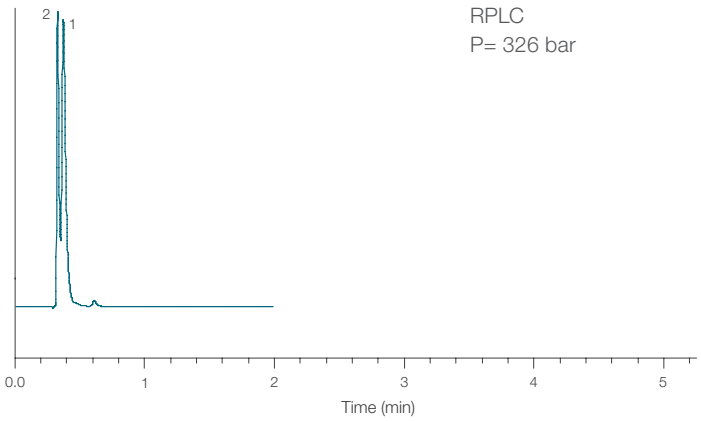
The resulting elution order in HILIC is roughly the opposite of that observed in a reversed-phase separation[14], as in the following chromatograms.

Elution under HILIC conditions



Thermo Scientific™ Accucore™ HILIC column, 2.6 µm, 150 × 4.6 mm	
Mobile phase	92:8 acetonitrile: 100 mM ammonium formate, pH 3.2
Flow rate	3.5 mL/min
Injection volume	10 µL
Temperature	40 °C
Detection	UV at 230 nm
Sample	1. Epinephrine
	2. Norepinephrine

Elution under RPLC conditions



Thermo Scientific™ Accucore™ aQ column, 2.6 µm, 100 × 2.1 mm	
Mobile phase	5 mM ammonium formate, pH 3.2
Flow rate	0.6 mL/min
Injection volume	2 µL
Temperature	30 °C
Detection	UV at 230 nm

In the examples given above the analytes were retained under both RPLC and HILIC modes. However, when operating under RPLC conditions the analytes were only marginally retained and not fully resolved. When working in HILIC the analytes are baseline resolved with the added advantage of a considerably lower back pressure.

Introduction (continued)

HILIC: Advantages

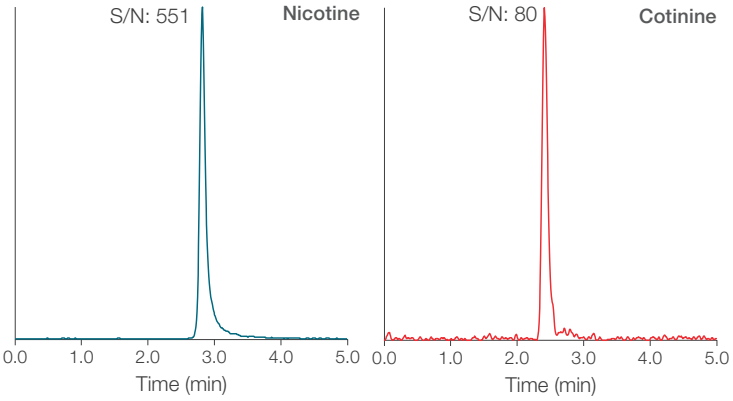
The main benefits of HILIC can be summarized as follows:

- Retention of hydrophilic compounds that are difficult to retain in RPLC without the use of an ion pair agent. Elution order is based primarily on analyte hydrophilicity, as opposed to hydrophobicity, so HILIC selectivity is complementary to RPLC selectivity
- Mobile phases have high organic content which generally allows improved signal/noise ratio, (S/N) with MS detection

The use of HILIC is often highlighted as advantageous for use with electrospray ionization mass spectrometry (ESI MS) detection by virtue of the fact that the highly organic mobile phases ensure efficient desolvation, which in turn leads to lower detection limits. In the example shown below, in order to retain nicotine and cotinine using reversed phase chromatography, a highly aqueous mobile phase is required (in this case 98%). This results in low S/N ratio, even after adjustment of the electrospray conditions.

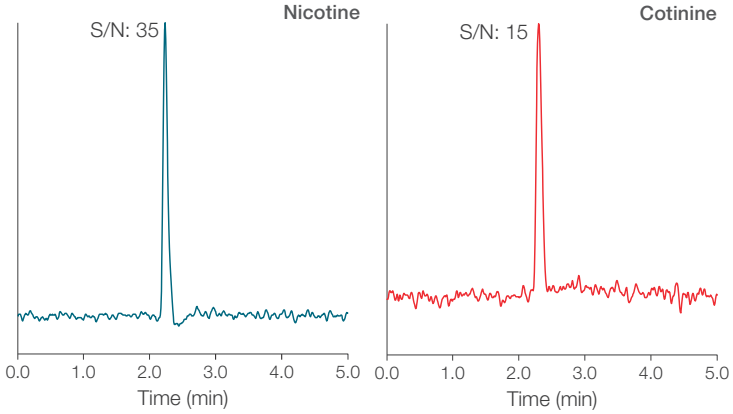
When the same two compounds are analyzed using HILIC, the S/N ratio for nicotine is 15 times higher than in RPLC; the S/N ratio for cotinine is 5 times higher in HILIC than in RPLC, as illustrated in the following chromatograms.

Extracted mass chromatograms for nicotine and cotinine in HILIC



Thermo Scientific™ Hypersil GOLD™ HILIC column, 5 µm, 150 × 2.1 mm	
Mobile phase	10:90 (v/v) ammonium formate 50 mM pH 3.5/acetonitrile
Detection	+ESI

Extracted mass chromatograms for nicotine and cotinine in RPLC



Thermo Scientific™ Hypersil GOLD™ column, 5 µm, 150 × 2.1 mm	
Mobile phase	98:2 (v/v) water/acetonitrile + 0.1 % formic acid
Detection	+ESI (spray conditions adjusted for higher aqueous content of mobile phase)

Introduction (continued)

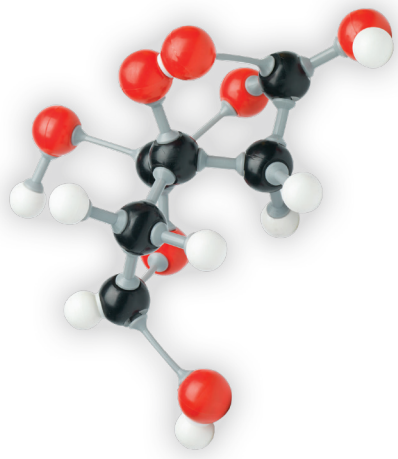
HILIC: Advantages (continued)

Other advantages that can be associated with this technique include:

- Low back pressures due to the low viscosity of the organic-rich mobile phases. This is especially relevant when using sub-2 µm particles; lower back pressure allows for the use of high flow rates for fast analysis (a word of warning: the optimum performance may become compromised as it has been observed[13] that in HILIC higher efficiencies are achieved at lower flow rates than in RPLC. This phenomenon will be illustrated further on in the guide). Lower back pressure also means that the frictional heating effect – which could be detrimental when using sub-2 µm particles in RPLC conditions – is not an issue in HILIC, since the pressure is generally 2–3 times less than in RPLC.[15]

- Less peak tailing upon column overloading. This phenomenon is observed when peaks for very polar analytes (eluted under HILIC conditions) are compared to the peaks that can be obtained for these compounds on silica columns using highly aqueous conditions (in per aqueous liquid chromatography, PALC). Good retention factors can be achieved under PALC conditions (by taking advantage of the hydrophobic character of siloxane groups at the surface of the silica), however the underlying adsorption mechanism is heterogeneous and involves activeadsorption sites. This results in column overloading being experienced when analyzing strongly retained samples ($k > 2$).[16]

- Compatibility with SPE extracts, if in 100% aprotic organic solvent. These can be directly injected without the need to dry down and reconstitute in mobile phase. Since the organic solvents are weak eluents under HILIC conditions, polar analytes are accumulated on a narrow zone near the column inlet (phenomenon described as ‘sample on-column’ focusing).[17]

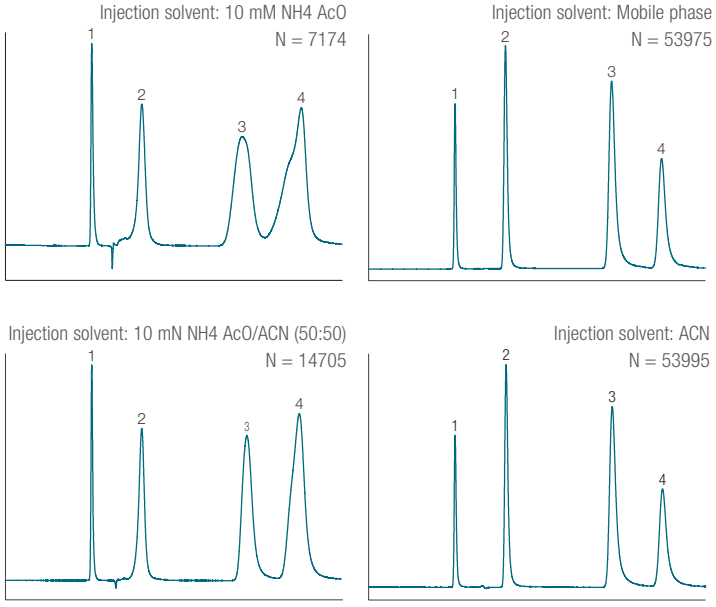


Introduction (continued)

HILIC: Limitations

One of the most common challenges in HILIC is distortion of peak shape that arises from mismatch of sample solvent and mobile phase as shown below.

Effect of sample solvent on peak shape, under HILIC conditions



Hypersil GOLD HILIC column, 5 µm, 150 × 4.6 mm	
Mobile phase	10:90 (v/v) ammonium acetate 10 mM, pH 5.0/acetonitrile
Flow rate	0.6 mL/min
Injection volume	1 µL
Temperature	30 °C
Detection	UV at 258 nm
Sample	1. Benzophenone
	2. Uracil
	3. Cytosine
	4. Acyclovir

Using an aqueous sample solvent (with high elution strength), which impairs the partitioning of the analytes into the stationary phase, is detrimental to the chromatographic peak shapes. Other negative 'side effects' that could be experienced if using an aqueous sample solvent are easy column overload and reduced retention and resolution. To ensure good chromatographic performance it is recommended that the sample solvent has an organic content greater than 50%.[17] However, this can cause solubility issues with highly polar analytes. Johnson *et al.* addressed this problem by using an injection loop partially filled with a central sample plug, surrounded by the focusing organic solvent.[18]

Other challenges associated with HILIC are:

- HILIC mechanisms are kinetically slower than the common mechanisms in RPLC, resulting in longer column equilibration times (typically two to four times longer than in RPLC)
- Accurate measurements of mobile phase pH cannot be made when using high proportions of organic solvent. McCalley[19] showed that acetonitrile/ammonium formate 85/15 (v/v), where the aqueous buffer pH was 3, presented in fact a pH of about 5.2 to the column and analytes*

There is a common misconception that all HILIC columns will retain polar compounds in a similar manner. Indeed, one of the most recurrent uncertainties in HILIC method development is the difficulty in identifying the most suitable column to use.

*Although mentioned here, this issue will not be discussed further in this guide. When reference to mobile phase pH is made, we always refer to the pH of the aqueous part.

Introduction (continued)

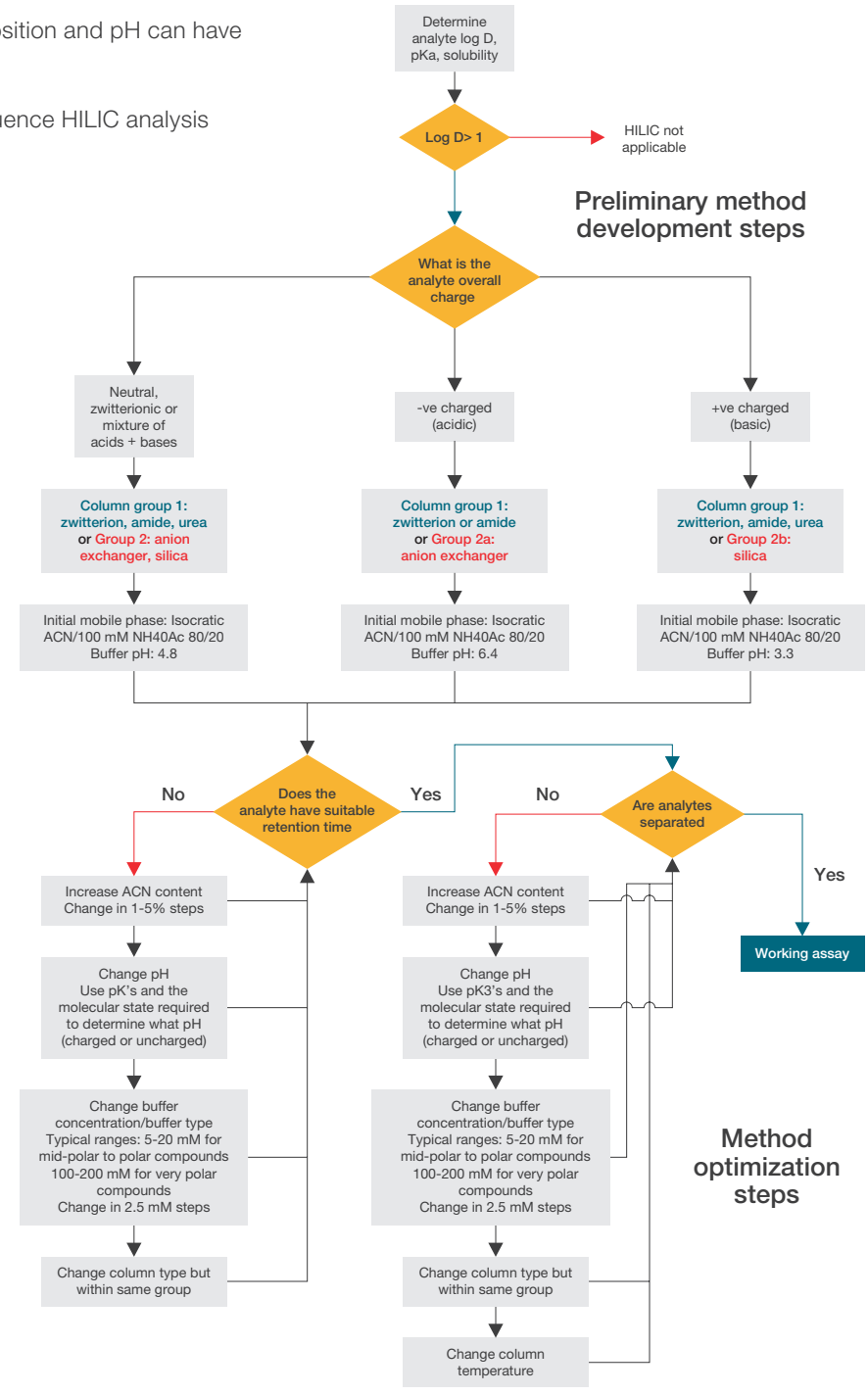
HILIC: Limitations (continued)

A systematic approach to method development should be taken when developing a method in HILIC. Method parameters should be considered and adjusted sequentially, one at the time.

This section provides some guidelines for:

- Which type of stationary phase is the most appropriate to use
- What effects the mobile phase composition and pH can have on the separation
- How the column temperature can influence HILIC analysis

The HILIC method development process can be summarized in the flow chart below (and on page 30), which outlines the necessary sequential method development steps in a logical manner.



HILIC method development

Know your analyte for HILIC method development

Knowledge of the compound structure and the physiochemical properties of the molecules under investigation will aid the choice of column, as it will determine the possible retention mechanisms. Fine-tuning of the separation by optimizing the organic solvent content, the buffer concentration and the mobile phase pH should follow.

Understanding the physiochemical properties of the analyte is therefore essential to the selection of a suitable HILIC column.

Firstly, gather information on the hydrophobic character of the analyte, provided by its log P (or log D) value.

The partition coefficient, log P, is used for neutral compounds, or where the compound exists in a single form. Log P can be calculated from the ratio of concentrations of an un-ionized analyte in the two phases of a mixture of immiscible solvents (octanol and water being the most commonly used):

$$\log P_{oct/water} = \log \frac{[Analyte]_{octanol}}{[Analyte]_{water}}$$

Log P indicates the degree of hydrophobicity of a compound.
A log P> 0 denotes a relatively hydrophobic compound.
A log P< 0 denotes a more hydrophilic compound.

HILIC is generally recommended for hydrophilic compounds with negative log P values.

Another important analyte parameter is the pK_a (-log K_a); K_a gives us an indication of the acidity of a molecule. Ka is the equilibrium constant for a chemical reaction and in the context of acid-base reactions is known as dissociation constant.* The equilibrium can be written symbolically as:

$$HA \rightleftharpoons A^- + H^+$$

Where HA is a generic acid that dissociates by splitting into:

A^-, the conjugate base of the acid.

H^+, the hydrogen ion or proton, which, in the case of aqueous solutions, exists as the solvated proton.

The chemical species HA, A^- and H^+ are said to be in equilibrium when their concentrations do not change with the passing of time. The dissociation constant is the ratio of the equilibrium concentrations (in mol/L), denoted by [HA], [A^-] and [H^+]:

$$K_a = \frac{[A^-]\,[H^+]}{[AH]}$$

The negative logarithmic measure of K_a is generally used, due to the many orders of magnitude spanned by K_a values.

The larger the pK_a value, the weaker the acid.

For ionizable solutes, the compound may exist as several different species in each phase at any given pH. The most appropriate descriptor for ionizable compounds is the distribution coefficient, log D, since it is a measure of the pH-dependant differential solubility of all species in the octanol/water system.

Log D is the ratio of the sum of the concentrations of all forms of the analyte in each of the two phases:

$$\log D_{oct/water} = \log \frac{[ionized\ Analyte]_{octanol} + [un\text{–}ionized\ Analyte]_{octanol}}{[ionized\ Analyte]_{water} + [un\text{–}ionized\ Analyte]_{water}}$$

Log D provides a good indication of the ionization state of a compound in solution at a specific pH and is more representative of the hydrophobic character in buffered conditions than log P.

Knowledge of the combination of log P, log D and pK_a data for the analytes will help to guide the stationary phase selection process.

*Note: k_b, the equilibrium constant for a base gives us an indication of the basicity of a molecule. Historically, k_b has been defined as the association constant for protonation of the base B, to form the conjugate acid, HB^+.

$$B + H_2O \rightleftharpoons HB^+ + OH^-$$

$$K_b = \frac{[HB^+] + [OH^-]}{[B]}$$

$$pk_b \approx 14\text{-}pk_a$$

HILIC method development (continued)

Stationary phase selection

Once the physiochemical properties of the analyte are known, it is then suggested to match the analyte log D values to the degree of polarity of the HILIC phases. In general terms, the more negative the log D value for an analyte, the greater the degree of stationary phase polarity required to retain it. The following chart, which illustrates the relative hydrophilicity and ion-exchange properties for Thermo Scientific™ HILIC columns, can be used as a guide in stationary phase selection at this stage:

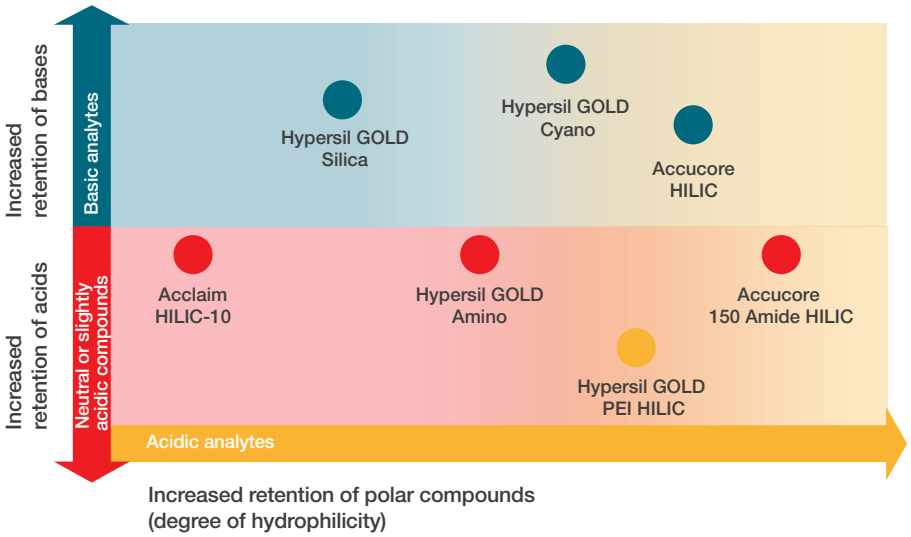


Figure 4. Relative polarity and ion-exchange characteristics for various HILIC phases (based on study illustrated in next section)

Stationary phase materials with a higher degree of hydrophilicity are shown on the right-hand side of the chart. So, for example Thermo Scientific™ Accucore™ 150-Amide-HILIC, being the most polar of these phases should be chosen for very polar analytes (which may not get enough retention on a lesser polar phase).

Although in HILIC the predominant retention mechanism is partitioning, secondary electrostatic interactions can play an important role in the separation.

With charged analytes it is possible to use the ion-exchange properties of the stationary phase to one's advantage. Materials with cation-exchange properties are shown above the X-axis, whilst the phase with negative ion-exchange properties (an anion-exchanger) is shown below. So, for example, Thermo Scientific™ Syncronis™ silica is a material with considerable cation-exchange characteristics, and can be recommended for the analysis of basic compounds. However, this material will not be suitable for the retention of acidic compounds.*

On the other hand, Thermo Scientific Hypersil GOLD HILIC, which has anion-exchange properties, can be considered for acids but will show reduced retention for basic compounds.

The materials with no ion-exchange properties, Thermo Scientific™ Acclaim™ HILIC-10, Thermo Scientific™ Accucore™ Urea-HILIC, Thermo Scientific™ Syncronis™ HILIC and Thermo Scientific™ Accucore™ 150-Amide-HILIC are suitable for both charged and neutral analytes. These phases are therefore particularly useful where there may be a requirement to separate a mixture of acidic, basic and neutral species. Column selection is discussed in more detail in the following section.

* Note: this might not be the case for the separation of a mixture of acids

HILIC method development (continued)

HILIC phase characterization: understanding the column chemistry

As we have seen in the previous section the stationary phases used in HILIC are quite diverse and users are often under the misconception that 'HILIC columns' are interchangeable.

A rigorous testing regime, such as that proposed by Tanaka et al.[20] is essential for the characterization and understanding of HILIC phases and should help to overcome this issue.

Understanding the possible HILIC retention mechanisms involved for a particular column-solvent-analyte system is crucial in the process of selecting the right HILIC stationary phase leading to a successful application. HILIC stationary phases should be characterized for:

- Degree of hydrophilicity
- Selectivity for hydrophilic-hydrophobic groups
- Selectivity for positional and conformational isomers
- Evaluation of electrostatic interactions
- Evaluation of the acidic-basic nature of the stationary phases

The data from this study show structure-selectivity relationships for the various HILIC phases and are discussed in the following sections.

HILIC selectivity

Thermo Scientific columns for HILIC are available in both solid core and fully porous materials, with a variety of chemistries. The data sets generated from the characterization testing regime (as developed by Tanaka)[20] and mentioned above, highlighted important retention characteristics and differences. Stationary phase characteristics have been visually illustrated by radar graphs,[20, 21] which allow to express multi-dimensional data in a two-dimensional format and ultimately allow to visually assess and compare columns. The separation factors obtained in the course of the assessment for the HILIC columns were therefore arranged in radar plots. Each axis of the radar plots represents one of the following parameters:

1. α (CH2) – degree of hydrophobicity
2. α (OH) – degree of hydrophilicity
3. α (V/A) – separation factor for configurational isomers
4. α (2dG/3dG) – separation factor for positional isomers
5. α (AX) – degree of anion-exchange interactions
6. α (CX) – degree of cation-exchange interactions
7. α (Tb/Tp) – acidic-basic nature of the stationary phase
8. k U – absolute retention

Note that a selectivity value of 1 corresponds to no selectivity (since $\alpha = k_2/k_1$, $\alpha = 1$ when $k_1 = k_2$).

The ion-exchange properties have a major impact on the shape of the radar plots, and this allows for a separation of the HILIC stationary phases into two main groups:

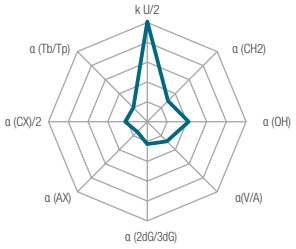
Group 1: For phases which have little ion-exchange activity. These materials can be used for neutral, zwitterionic and mixtures of acids and bases.

Group 2: For phases which have considerable ion-exchange activity. Group 2 can be divided further in two subcategories:

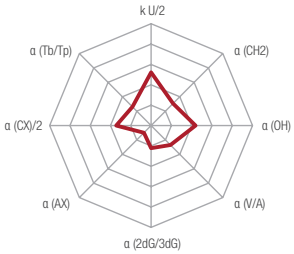
1. Anion exchange phases; recommended for the analysis of acids
2. Cation exchange phases; recommended for the analysis of bases

HILIC method development (continued)

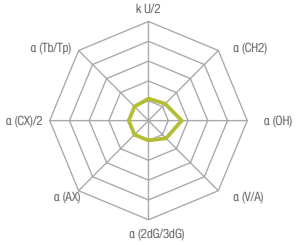
Group 1 – Radar plots for HILIC stationary phases with no ion-exchange properties



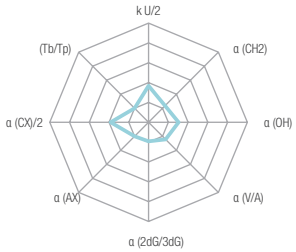
Syncronis HILIC



Accucore
150-Amide-HILIC

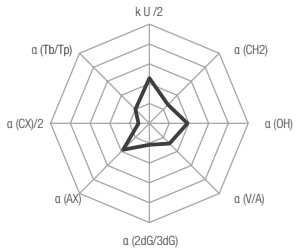


Accucore Urea HILIC



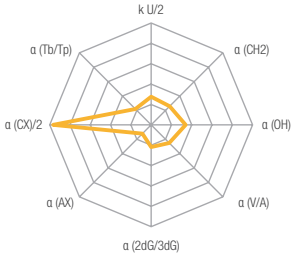
Acclaim HILIC-10

Group 2a – Radar plot for HILIC stationary phase with anion-exchange properties

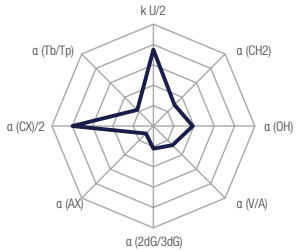


Hypersil GOLD HILIC

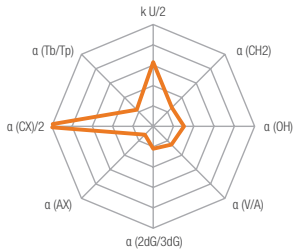
Group 2b – Radar plots for HILIC stationary phases with high cation-exchange properties



Hypersil GOLD Silica



Accucore HILIC



Syncronis Silica

Note: Further information on the data derived from this characterization testing regime, and on the individual column chemistries is provided in the 'Column characterization detail' section.

HILIC method development (continued)

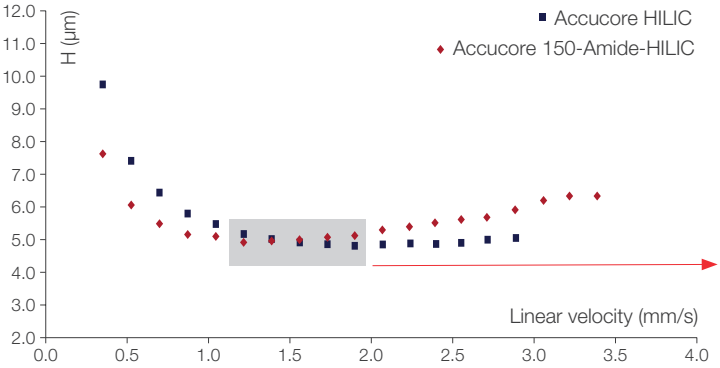
Column geometry

As a general recommendation, for HILIC standard operations select a 100 or 150 mm long column, packed with 3–5 µm particles and with a 4.6 mm id, if using UV detection.

If HILIC is coupled to MS or CAD detection, use a smaller column, e.g. 100 × 2.1 mm or 50 × 2.1 mm, packed with 3–5 µm particles (the reduction in column length should be undertaken after having considered sample complexity and detector selectivity). For higher separation efficiency and resolution we suggest either sub 2 µm fully porous particles or solid core materials or longer columns.

The optimum flow rate is dependent on the column internal diameter and particle size. Some investigations carried out into the kinetic performance of 100 × 4.6 mm HILIC columns highlighted that for the same particle size and particle type the optimum flow rate also varies with the column chemistry.

The following van Deemter graphs – where plate height, H, is plotted versus linear flow velocity - report the results of this investigation.



Accucore 150-Amide-HILIC column Accucore HILIC column 2.6 µm 100 × 4.6 mm	
Mobile phase	90/10 acetonitrile/100 mM ammonium formate (pH 3.5)
Flow rate	Accucore 150-Amide-HILIC, 0.7–1.0 mL/min Accucore HILIC, 0.9–1.7 mL/min
Sample	Atenolol

HILIC method development (continued)

Column geometry (continued)

With respect to the two solid core 2.6 µm particle size materials (Accucore 150-Amide-HILIC and Accucore HILIC), it can be seen above that the bare silica has a flatter profile (lower value of the C term in the van Deemter equation) and hence a better preservation of high efficiency at high flow rates than the amide material. The optimum flow rate for the bare silica (Accucore HILIC) is in the range of 0.9–1.7 mL/min; however, even above 1.7 mL/min efficiency is not compromised as the increase in H is minimal.

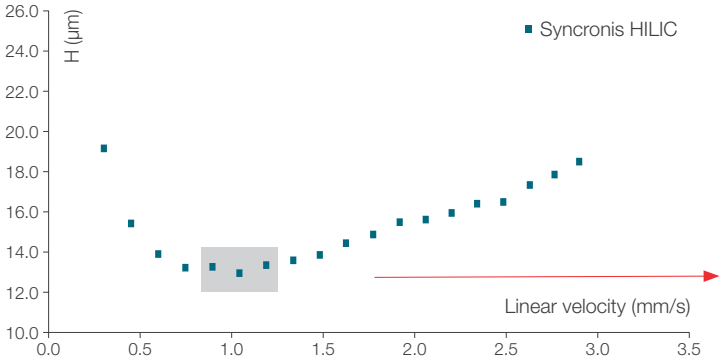
The amide phase (Accucore 150-Amide-HILIC) demonstrated an optimum flow rate range of 0.7–1.0 mL/min.

Within its optimal flow rate range, the bare silica phase demonstrated slightly lower H values (plate height) than the amide material. Lower H values correspond to higher plate counts, making the bare silica material more efficient.

The zwitterionic phase (5 µm particle size) investigated showed a prominent variation in efficiency with flow rate.

The zwitterionic phase showed relatively high H and mass transfer term values. The minimum plate height is similar to the values reported by McCalley[13] and Hemström and Irgum.[1] According to Hemström and Irgum, the relative thickness of the zwitterionic interactive layer could be the major contributor to the high mass transfer term (although the authors were referring to a polymeric rather than a monomeric coating).

From the van Deemter plot reported above, it can be seen that the best performance on the zwitterionic phase is achieved at flow rates between 0.6–0.8 mL/min.

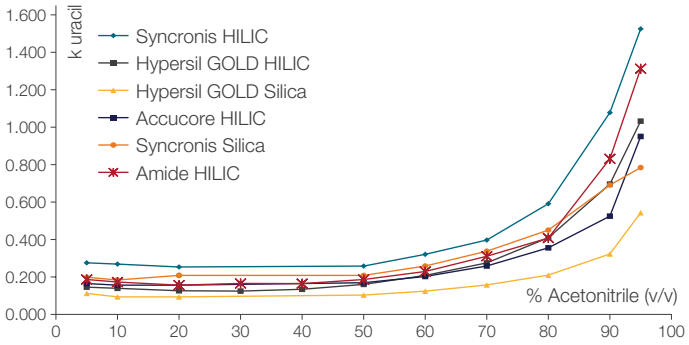


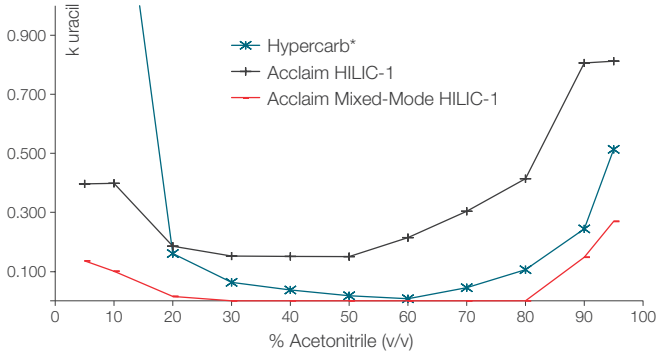
Syncronis HILIC column, 5 µm, 100 × 4.6 mm	
Mobile phase	90/10 acetonitrile/100 mM ammonium formate (pH 3.5)
Flow rate	Syncronis HILIC, 0.6–0.8 mL/min
Sample	Atenolol

The mobile phase in HILIC

Organic content

In HILIC the mobile phase is highly organic (generally 60–70%; at least 3% water is required). It has been demonstrated that besides the selection of a suitable column chemistry, the organic modifier/aqueous ratio is a major factor controlling the separation selectivity. An increase in the percentage of organic solvent leads to an increase in retention; this phenomenon is illustrated in the figures below.





*Although Hypercarb is not a polar stationary phase, it was included in this study because of its dual behavior (RP and HILIC), depending on the percentage of organic in the mobile phase, which allows its use for the retention of polars in RP and HILIC.

For most columns the test compound has little retention (mean k of 0.2) when the acetonitrile content is less than 60%. Acclaim HILIC-10, Acclaim Mixed Mode HILIC-1 and Thermo Scientific™ Hypercarb™, as seen in the plot on the right, display both typical reversed phase and HILIC mode retention characteristics, depending on the percentage of organic solvent in the mobile phase. When acetonitrile concentrations are between 60–90%, uracil retention increases with the percentage of acetonitrile (HILIC mode of retention); between 5–30% acetonitrile, uracil retention decreases as the concentration of acetonitrile becomes greater (a reversed-phase retention behavior). Interestingly, Acclaim Mixed Mode HILIC-1 column does not retain uracil when the percentage of acetonitrile is between 30–80.

The mobile phase in HILIC (continued)

Organic solvent type

Although acetonitrile is the most popular solvent used in HILIC, several other polar, water-miscible organic modifiers can be used. The elutropic strength is generally the inverse of that observed in RPLC, with the relative solvent strength summarized in the following table.

Solvent	Chemical formula
Aprotic solvents	
Tetrahydrofuran (THF)	/–CH_2–CH_2–O–CH_2–CH_2–\
Acetone	CH_3–C(=O)–CH_3
Acetonitrile (ACN)	CH3–C≡N
Protic solvents	
Iso-propanol (IPA)	CH_3–CH (–OH)–CH_3
Ethanol (EtOH)	CH_3–CH_2–OH
Methanol (MeOH)	CH_3–OH
Water	H–O–H

Increasing solvent elutropic strength in HILIC

In HILIC, it is generally advised to use aprotic solvents (solvents which cannot exchange a proton). However, it has been demonstrated that alcohols can be used as the weak eluent,[22] although they will generally cause a decrease in the strength of the partitioning, leading to less retentivity; this is due to their hydrogen bonding interactions with the water molecules and hence the competition between the two to solvate the surface of the HILIC stationary phase.[17, 23] Periat et al. unequivocally proved that large amounts of alcohols caused drastic reduction in retention.[15] Methanol and IPA – when used in mixtures with acetonitrile (80/20 ACN/MeOH or ACN/IPA) – are not able to provide significant changes in selectivity and lead to significant loss in kinetic performance and peak broadening.[17] In some instances, where weakly polar analytes would precipitate in water-containing mobile phases, protic solvents replaced water (technique known as 'non-aqueous HILIC').[24]

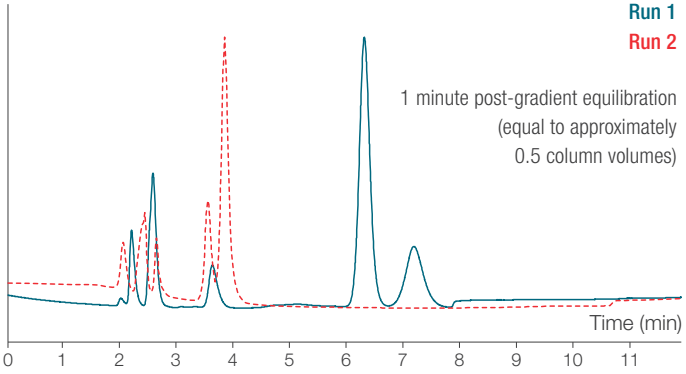
As outlined in the flow chart, the starting mobile phase we suggest consists of 80/20 acetonitrile/aqueous buffer; the elution strength is then adjusted until acceptable retention is achieved. Alternatively, a generic gradient can be run, starting from 95% acetonitrile, holding for 2 minutes – or longer, according to column length – (to establish whether compounds are weakly retained in HILIC mode) and gradually increasing the aqueous buffer percentage to about 40% over 15 minutes (for a 100 mm long column). A two minute hold should follow to establish whether compounds are strongly retained in HILIC.

If using a gradient, it is vital to have a sufficiently long post gradient re-equilibration stage, to allow for the volume of the water layer (as advocated by the HILIC partitioning model) to re-establish its initial conditions. We generally recommend a post gradient re-equilibration of approximately 20 column volumes.

The mobile phase in HILIC (continued)

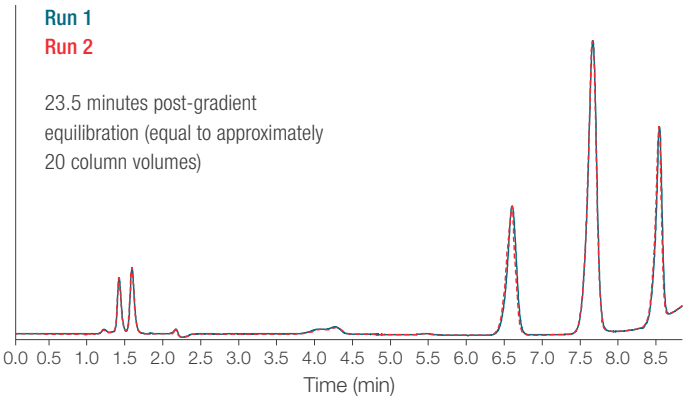
Organic solvent type continued

The following chromatogram illustrates how irreproducible chromatography, with drifting retention times is obtained when a post-gradient equilibration of 1 minute is carried out in between runs.



Accucore 150-Amide-HILIC column, 2.6 µm, 100 × 2.1 mm	
Mobile phase A	acetonitrile
Mobile phase B	100 mM ammonium formate (pH 6.6) delivered in a 5–40% B gradient over 15 minutes at 0.1 mL/min, followed by 1 minute equilibration time

On the other hand, reproducible chromatography is obtained when a suitable post-gradient equilibration is carried out in between runs. This is illustrated in the figure below, where a post-gradient equilibration time of 23.5 minutes (corresponding to 20 column volumes) leads to perfectly overlaid chromatograms.



Syncronis HILIC column, 5 µm, 100 × 2.1 mm	
Mobile phase A	acetonitrile
Mobile phase B	100 mM ammonium formate (pH 6.6) delivered in a 5–0% B gradient over 15 minutes at 0.2 mL/min, followed by 23.5 minutes equilibration time

The mobile phase in HILIC (continued)

Do I need a buffer?

As a general guideline, buffers are added to the mobile phase to reduce peak tailing and/or retention of charged analytes. Early research indicated that the absence of buffer salts resulted in excessively long retention times and very broad peaks on amide, cyclodextrin, cyano and amino-based columns.[25]

Due to their good solubility in organic solvents, ammonium salts of acetic and formic acids are recommended buffers for HILIC.

These buffers also have the advantage of being volatile for use with mass spectrometry and charged aerosol detection. Other salts with relatively high solubility in high organic levels are bicarbonate salts, triethylamine phosphate and sodium perchlorate, but the last two are not MS compatible.

Apart from these, most buffers used in reversed phase should be avoided in HILIC, due to their poor solubility in highly organic mobile phases.* Generally, negatively or positively charged stationary phases require higher concentrations of buffers than neutral or zwitterionic phases.

Electrostatic interactions are secondary forces which can have important contributions to the retention in HILIC, since some polar compounds can be charged at the mobile phase pH conditions typically used.[8] The presence of buffers in the mobile phase can reduce electrostatic interactions (both attractive and repulsive) between charged analytes and the stationary phase.

When using a gradient, we recommend adding the buffer in both mobile phases to allow constant buffer strength to be maintained throughout the run. For example, mobile phase A is made up of 100% aqueous buffer, and mobile phase B should contain 95% acetonitrile and 5% aqueous buffer. Both mobile phases could be prepared by adding 5% of concentrated aqueous buffer to either water or acetonitrile. We also suggest that the concentrated aqueous buffer should be a maximum of 10 mM but not above to avoid this solution crashing out when added to acetonitrile. The use of a lower concentration buffer will also reduce any suppression effects that can be observed with electrospray sources either in CAD or in MS.

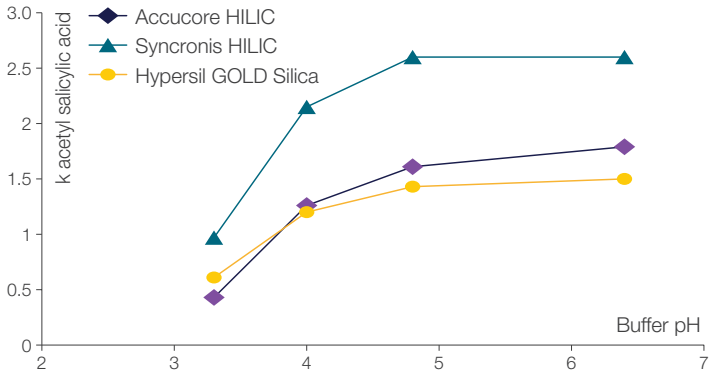
* Please note that these are general recommendations. There are specific cases – e.g. USP methods for sugars–which suggest the use of phosphate buffers in 75% acetonitrile.

The mobile phase in HILIC (continued)

Mobile phase buffer pH

In general, charged compounds are more hydrophilic, and therefore are more retained in HILIC. The plot below shows the retention factor of acetylsalicylic acid increasing with the buffer pH, on bare silica and zwitterionic phases.

The effect of mobile phase buffer pH on the retention of acetylsalicylic acid



Accucore HILIC column, 2.6 µm 100 × 4.6 mm Syncronis HILIC and Hypersil GOLD Silica columns, 5 µm 100 × 4.6 mm	
Mobile phase	90/10 acetonitrile/100 mM ammonium formate (the mobile phase buffer pH was measured before the addition of acetonitrile)
Flow rate	1.0 mL/min
Injection volume	5 µL
Temperature	30 ˚C
Detection	228 nm

Acetylsalicylic acid has a pK_a of 3.5; as the buffer pH increases (pH between 4–6.5), it becomes deprotonated and hence more retained. As the buffer pH drops below 4, the proportion of protonated acetylsalicylic acid increases, leading to less retention.*

The charge state of the stationary phase can affect HILIC retention of ionizable compounds, depending on the mobile phase buffer pH; electrostatic interactions between a charged stationary phase and a charged analyte can be critical, leading to drastic variations in retention. This concept was unequivocally demonstrated by Guo and Gaiki,[8] when analyzing cytosine on an amino column. When the mobile phase buffer pH approached the cytosine pK_{a1} value (~4.6), cytosine became positively charged, inducing electrostatic repulsion from the positively charged amino stationary phase and its retention dropped.

The mobile phase buffer pH can also affect the stationary phase charge state. This, for example, is the case for bare silica phases, where the silanol ionization varies with the mobile phase buffer pH. At pH >4-5, the silanols are deprotonated, making the silica surface negatively charged. This will have an effect on the retention of positively charged analytes. This phenomenon is illustrated below with the increased retention for cytidine.

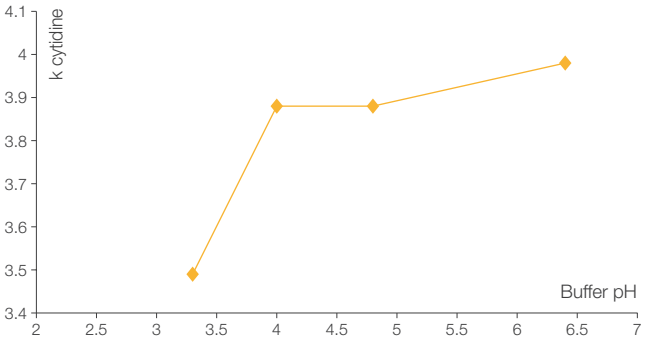
The retention of cytidine increases as the buffer pH increases from 3.3 to 6.4. As the mobile phase buffer pH increases the proportion of deprotonated silanol groups also increases, leading to increasing electrostatic attractions between the positively charged cytidine and the increasingly negatively charged silica surface. It is therefore very important to fine tune the mobile phase buffer pH when using bare silica phases for the analysis of basic compounds in order to avoid excessively long retention times and peak broadening.

* Interestingly there are two effects occurring with the retention of acetylsalicyclic acid, one is the increased polarity which gives an increase in retention and the second is the effect of the two charges of the stationary phase and also of the compound, which will have a slightly negative effect on the retention.

The mobile phase in HILIC (continued)

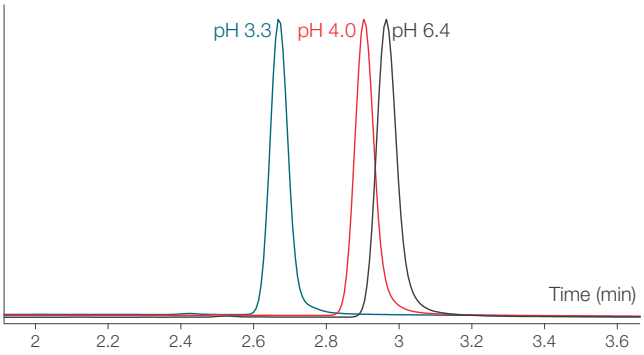
Mobile phase buffer pH (continued)

The effect of mobile phase buffer pH on the retention of cytidine on a bare silica phase



Hypersil GOLD Silica column, 5 µm 100 × 4.6 mm	
Mobile phase	90/10 acetonitrile/100 mM ammonium formate
Flow rate	1.0 mL/min
IInjection volume	5 µL
Temperature	30 ˚C
Detection	248 nm

Overlaid chromatograms relative to the separation of cytidine on a bare silica phase, showing an increased retention with the mobile phase pH



Hypersil GOLD Silica column, 5 µm 100 × 4.6 mm	
Mobile phase	90/10 acetonitrile/100 mM ammonium formate
Flow rate	1.0 mL/min
Injection volume	5 µL
Temperature	30 ˚C
Detection	248 nm

Silica-based neutral phases (e.g. amide phases) can bear negative charges, due to residual silanol groups when working within a 3–6.5 pH range.[26] This was demonstrated by the characterization testing carried out on the Accucore 150-Amide-HILIC, where some cation-exchange capabilities were observed. See the column characterization details on page 34 for further information.

The mobile phase in HILIC (continued)

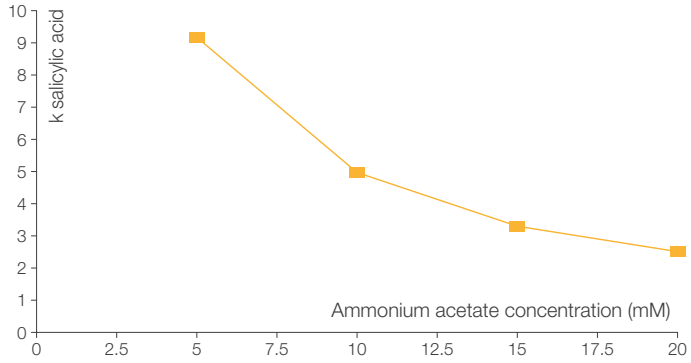
Mobile phase buffer type and concentration

The effect of different types of ammonium salts on the retention of acid and basic model compounds was investigated on various HILIC chemistries.[27] Ammonium formate provided longer retention times for acidic compounds on an amino phase, due to the weaker eluting strength of the formate ion in the ion-exchange interaction (as opposed to the acetate ion which has a greater neutralizing effect of the electrostatic attractions between the surface of the positively charged stationary phase and the negatively charged acid). Ammonium formate and ammonium acetate did not provide differences in retention of basic analytes on the amino column. No significant differences were observed between acetate and formate salt for neutral and zwitterionic phases.

As already pointed out, buffer salts in HILIC mobile phases are very important in reducing electrostatic interactions between charged analytes and the stationary phase.

When electrostatic interactions are prevalent, an increase in the salt concentration leads to a decrease in retention of charged solutes on the stationary phases of opposite charge. This phenomenon can be seen below, where the retention for salicylic acid (negatively charged) on Hypersil GOLD HILIC (positively charged) decreases as the buffer concentration increases.

The effect of ammonium acetate concentration on the retention of salicylic acid on Hypersil GOLD HILIC column (anion exchanger)

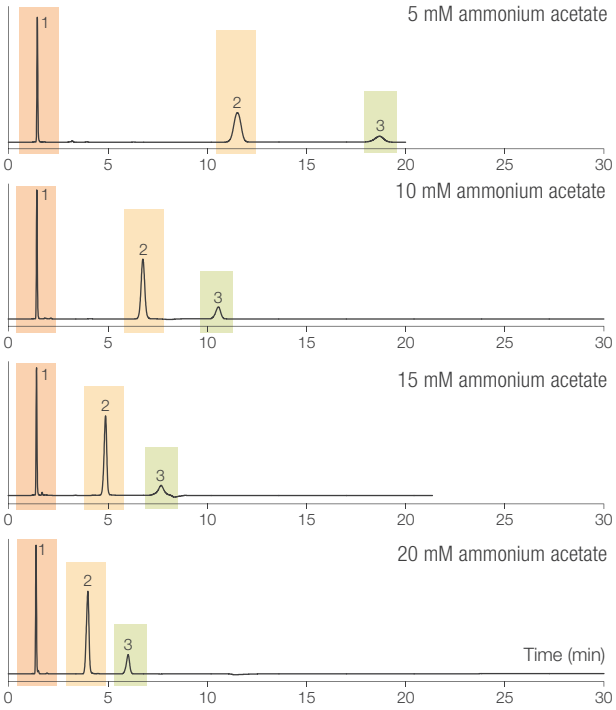


Hypersil GOLD HILIC column, 5 µm 100 × 4.6 mm	
Mobile phase	90/10 acetonitrile/ammonium acetate
Flow rate	1.0 mL/min
Injection volume	5 µL
Temperature	30 °C
Detection	228 nm

This is also illustrated by the following chromatograms, which show the separation of an acidic mixture, with retention of the anionic analytes decreasing as the concentration of ammonium acetate increases.

The mobile phase in HILIC (continued)

Mobile phase buffer type and concentration continued

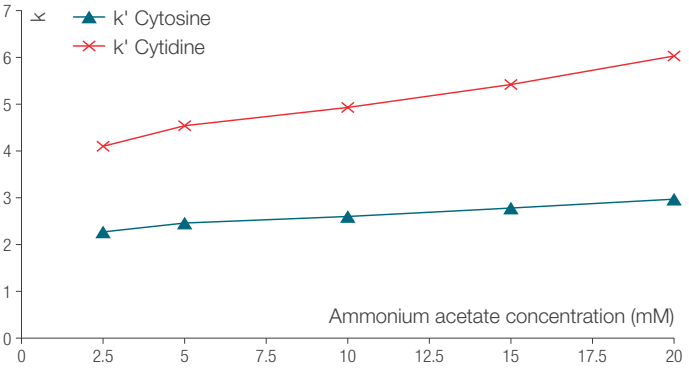


Hypersil GOLD HILIC column, 5 µm 100 x 4.6 mm	
Mobile phase	90/10 acetonitrile/ammonium acetate
Flow rate	1.0 mL/min
Injection volume	5 µL
Temperature	30 ˚C
Detection	228 nm
Sample	1. Salicylamide 2. Salicylic acid 3. Aspirin

From the chromatograms above it is also evident that the increased salt concentration is beneficial to the peak shape, with sharper peaks when 20 mM ammonium acetate is used.

Increased salt concentrations result in increased retention of positively charged solutes on stationary phases with same charge, as demonstrated below, where the retention of cytosine and cytidine on an anion-exchanger increases with the salt concentration. Enhanced hydrogen-bonding interactions (between the analyte and the stationary phase) are responsible for this behavior. The hydrogen-bonding interactions are facilitated by the increased population of solvated salt ions in the mobile phase (salting-out effect).

The effect of ammonium acetate concentration on the retention of cytosine and cytidine on Hypersil GOLD HILIC column (anion exchanger)

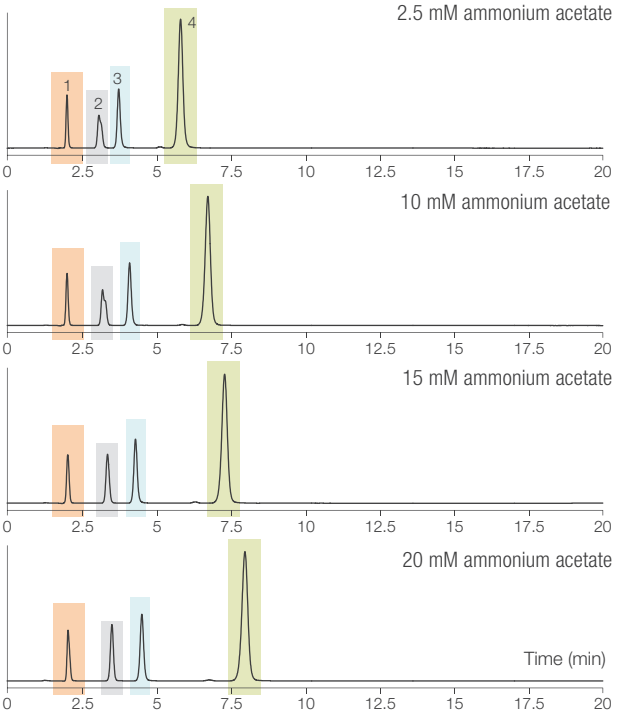


Hypersil GOLD HILIC column, 5 µm 100 x 4.6 mm	
Mobile phase	90/10 acetonitrile/ammonium acetate
Flow rate	1.0 mL/min
Injection volume	5 µL
Temperature	30 ˚C
Detection	248 nm

The mobile phase in HILIC (continued)

Mobile phase buffer type and concentration (continued)

Separation of a mixture of bases on Hypersil GOLD HILIC column (anion exchanger)



Hypersil GOLD HILIC column, 5 µm 100 × 4.6 mm	
Mobile phase	90/10 acetonitrile/ammonium acetate
Flow rate	1.0 mL/min
IInjection volume	5 µL
Temperature	30 ˚C
Detection	248 nm
Sample	1. Uracil 2. Uridine 3. Cytosine 4. Cytidine

In summary, the recommendations we offered for mobile phase selection for HILIC applications are:

- Use acetonitrile or other polar, water-miscible organic modifiers. Remember that the elutropic strength is inverse to what observed in RPLC. Aprotic solvents give longer retention than protic solvents
- Have a high organic content, between 60 to 97%; a minimum of 3% water is necessary to ensure sufficient hydration of the stationary phase
- An increase in organic solvent will lead to an increase in retention
- Use buffer salts such as ammonium acetate and ammonium formate to avoid peak tailing and to control retention times of charged analytes
- Buffer salts concentrations are 2–20 mM, although 20 mM is recommended for organic content of below 90% only. Higher concentrations are not soluble in high levels of organic and could impair MS or CAD signals
- When using gradients, buffer both mobile phases, do not run buffer gradients
- Do not run gradients from 100% organic to 100% aqueous
- The charge state of the stationary phase can affect HILIC retention of ionizable compounds, depending on the mobile phase pH

The mobile phase in HILIC (continued)

The effect of column temperature on HILIC retention

Column temperature is an important parameter that can also affect retention of polar analytes in HILIC.[27] The equation that is often used is derived from chemical thermodynamics, where the equilibrium point is related to the temperature, and is referred to as the van't Hoff equation. In a chromatography sense, the relationship between column temperature and retention factor is often described by the following:

$$\ln k = -\Delta H°/RT + \Delta S°/R + \ln \Phi$$

Where:

- $\Delta H°$ = enthalpy of interaction between stationary/mobile phase and analyte
- $\Delta S°$ = entropy of interaction between stationary/mobile phase and analyte
- R = universal gas constant
- T = column temperature in Kelvin
- Φ = phase ratio

The van't Hoff equation should also apply to HILIC if the retention mechanism is consistent, in which case the relationship between ln k and 1/T is linear.[27]

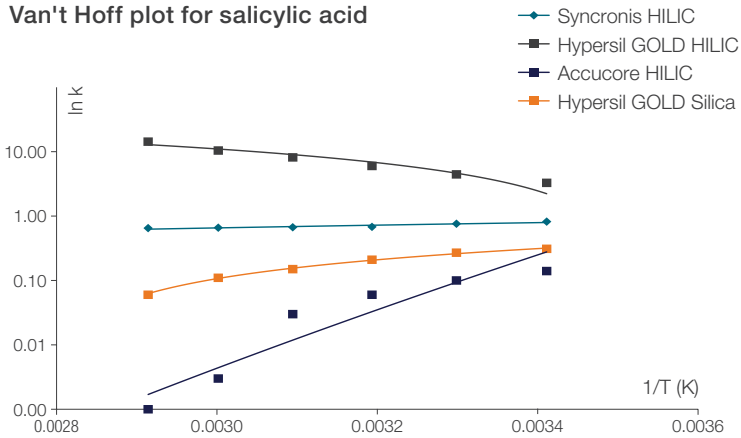
Using the experimental plots of ln k versus 1/T, covering a temperature range between 20–70 °C, we calculated the enthalpic and entropic contributions to the chromatographic retention of a series of test compounds (deriving – $\Delta H°$ from the slope and $\Delta S°$ from the intercept respectively). Temperature was observed to affect the retention and separation in different fashions dependent on the column being tested.

On the next page are van't Hoff plots for salicylic acid and cytosine, on four different columns. It can be seen that a decrease in retention for salicylic acid – indicating a negative heat of enthalpy and hence an exothermic reaction – is observed as the column temperature is increased on the two bare silica materials: Accucore HILIC and Hypersil GOLD Silica columns (although on Accucore HILIC the relationship is not linear, suggesting the presence of multiple retention mechanisms). On the zwitterionic phase–Syncronis HILIC–the retention of salicylic acid is more or less independent of temperaturetemperature. This behavior suggests low enthalpic contribution and high entropic contribution to the retention. Interestingly, the retention increases with the temperature on Hypersil GOLD HILIC. Positive enthalpy was obtained for this anion-exchanger, indicating an endothermic process of transferring salicylic acid from the mobile phase to the stationary phase. For Hypersil GOLD HILIC, the positive enthalpy of salicylic acid could also be the evidence of both ion-exchange and partitioning processes taking place.

The mobile phase in HILIC (continued)

The effect of column temperature on HILIC retention (continued)

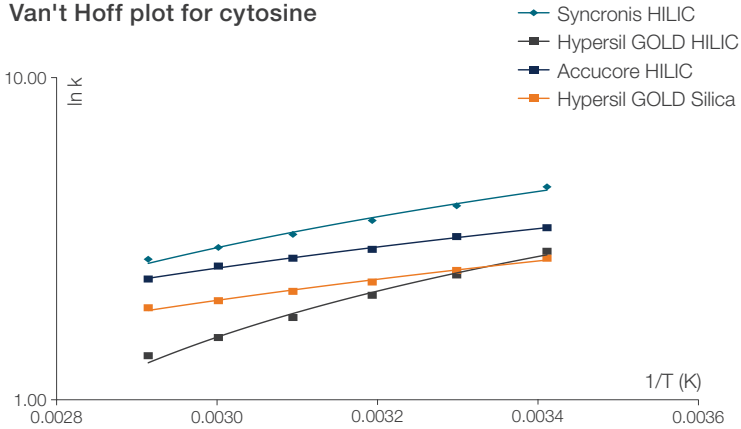
Van't Hoff plot for salicylic acid



Mobile phase	90/10 acetonitrile/100 mM ammonium acetate
Flow rate	1.0 mL/min
Injection volume	5 µL
Detection	228 nm

Shown below is a decrease in retention for cytosine on the four stationary phases as the column temperature is increased. Linear van't Hoff plots resulted from this study. Negative retention enthalpy values were obtained, indicating an exothermic process of transferring cytosine from the mobile phase to the stationery phase.

Van't Hoff plot for cytosine



Mobile phase	90/10 acetonitrile/100 mM ammonium acetate
Flow rate	1.0 mL/min
IInjection volume	5 µL
Detection	248 nm

Independently of its effect on the retention of test compounds, column temperature often positively affects the chromatographic efficiency due to lower viscosity and higher diffusivity. Our study demonstrated lower back pressures could be achieved when the temperature was raised, with average pressure dropping from 113 to 77 bar as the temperature was taken from 20 to 70 °C.

The mobile phase in HILIC (continued)

Start-up guide

A systematic method development approach is provided in the main section of this guide and we suggest referring to it for a comprehensive strategy.

As a general recommendation:

- Choose the column chemistry according to the nature of the interactions between analyte and stationary phase
- For standard operations, select a 100 or 150 mm long column with a 4.6 mm ID and packed with 3–5 µm particles. The column length can be changed if resolution needs optimizing.
- Smaller column diameter is recommended if HILIC is coupled to MS detection

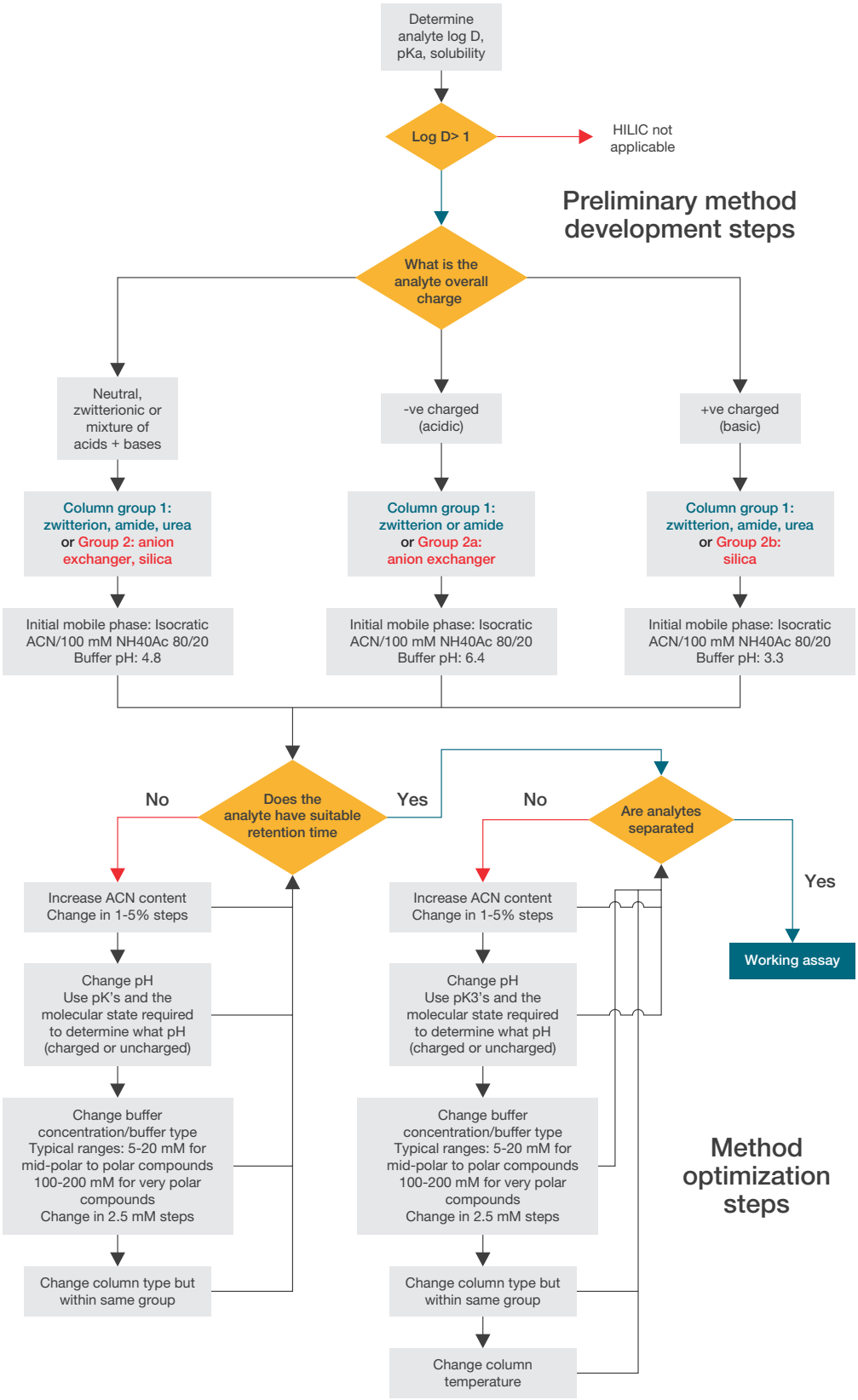
Optimum flow rate varies from phase to phase and column id. Some investigations carried out into the kinetic performance of 100 x 4.6 mm HILIC columns highlighted that the bare silica material has a wider range of optimal flow rate conditions (0.9-1.7 mL/min) than the amide or the zwitterionic materials (0.7–1.0 and 0.6–0.8 mL/min, respectively).

As outlined in the flow-chart reported on the next page, the starting mobile phase we suggest consists of 80/20 acetonitrile/aqueous buffer. Adjust the elution strength with small (<5%) changes in organic/aqueous ratio until acceptable retention is achieved.

Alternatively, a generic gradient can be run (especially when separation of analytes with differing retention factors has to be achieved), starting from 95% acetonitrile, holding for 2 minutes (to establish whether compounds are weakly retained in HILIC mode) and gradually increasing the water percentage to about 40% over 15 minutes (for a 100 mm long column). A 2 minute hold should follow, to establish whether compounds are strongly retained in HILIC. We discourage to use fast gradients or gradients that run from 100% organic to 100% aqueous, as it would be very difficult to reach a dynamic equilibrium with reproducible retention times and besides, the latter would fall outside the HILIC realm. We would also suggest that the buffer concentration is maintained constant during the gradient (by adding the buffer to the organic portion as well as the aqueous), unless using charged stationary phases where increasing the buffer concentration during the gradient would disrupt electrostatic interactions with the analyte.

If using gradients, it is vital to have a sufficiently long post gradient re-equilibration stage to allow for the volume of the water layer (as advocated by the HILIC partitioning model) to re-establish its initial conditions. It is recommended that a post gradient re-equilibration of approximately 20 column volumes is used. The same level of equilibration is also recommended for the initial column conditioning prior to the start of analysis. Failure to sufficiently equilibrate HILIC columns will result in irreproducible chromatography, with drifting retention times.

The mobile phase in HILIC (continued)



Troubleshooting

This section is intended as an aid in troubleshooting specific chromatographic problems that could occur during the running of HILIC separations. It is not meant to be an exhaustive guide to HPLC column and instrument troubleshooting. Advice specific to your column will be found in the Product Manual.

The following topics will be covered:

- Solubility
- Allowable buffers
- Retention reproducibility (peak retention drift)
- pH extremes affecting column stability
- Injection solvent
- Injection volume
- Syringe wash

Broad peaks

Possible cause	Corrective action
RunInjection solvent (sample solvent) too strong for the mobile phase	To ensure good chromatographic performance, it is recommended for the sample solvent to be as close as possible to the initial mobile phase conditions, or at least to have an organic content greater than 50%. Using aqueous sample solvent, with high elution strength – which impairs the partitioning of the analytes into the stationary phase – is detrimental for the chromatography, resulting in peak broadening. This in turn could lead to column overload, reduced retention and loss in resolution. Unfortunately polar analytes often have low solubility in organic solvents, in this case it is recommended to substitute water with methanol. In extreme cases of solubility issues, even the aqueous portion of the mobile phase can be replaced by polar non-aqueous solvents, in which case the technique is referred to as 'non-aqueous HILIC chromatography'.
Syringe/needle wash does not match mobile phase	The solution used for washing the syringe and the injection needle should be matched to the mobile phase composition but without the buffers. Undesired band broadening will result if too much water is used in the wash. Pure organic solvents should be avoided as well, as they are not polar enough to remove the analytes.
Injection volume too large	Injection of excessive sample volumes may cause column overloading, resulting in broad/tailing peaks or, in extreme cases flattened peaks. The recommend injection volumes are 0.5–5 µL, for a 2.1 mm ID column and 5–50 µL, for a 4.6 mm ID column.

Troubleshooting (continued)

Peak retention drift

Possible cause	Corrective action
Column not fully equilibrated	HILIC columns are less tolerant of short equilibration times than RPLC columns, mainly because the water in the aqueous layer within the stationary phase derives from the mobile phase and takes time to establish itself. This is particularly true for gradient conditions; post-gradient re-equilibrations of approximately 20 column volumes are recommended. Fast HILIC gradients and gradients that run from 100% organic to 100% aqueous are to be discouraged. New columns need conditioning too and we recommend to run approximately 20 column volumes of mobile phase.
Column contamination	A shift in peak retention or resolution, associated with a sudden increase in operating pressure beyond expected levels indicate column contamination. Reversing the flow direction of the column may be attempted to remove debris on the inlet frit (column effluent should go to waste, to avoid HPLC system contamination). To remove strongly retained materials from the column, flush the column in the reverse direction with very strong (HILIC) solvents such as 50:50 methanol:water. Strong buffers may be needed to remove ionic contaminants.

Allowable buffers

Possible cause	Corrective action
Solubility/MS compatibility	We recommend the use of buffered eluents in HILIC in order to minimize electrostatic interactions between charged analytes and deprotonated silanol groups of the stationary phase. A concentration range 5–20 mM is generally sufficient for most analytes. Ammonium acetate and ammonium formate are mostly recommended due to their high solubility in mobile phases with high organic content; their buffering range – 3.8–5.8 for ammonium acetate and 2.7–4.7 for ammonium formate – are suitable for most HILIC applications and additionally they are compatible with MS detection due to their volatility. If higher pH is required, ammonium hydroxide and ammonium carbonate can be considered. Formic acid and acetic acid are valuable alternatives, due to their solubility even in very high concentrations of organic solvents. Phosphate buffers should not be used, as they are liable to precipitate, and tend to foul MS sources.

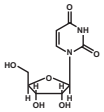
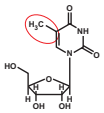
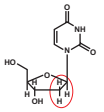
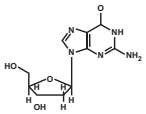
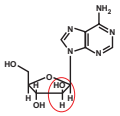
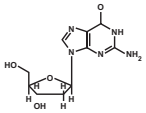
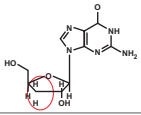
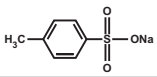
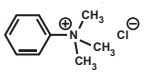
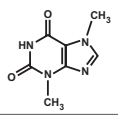
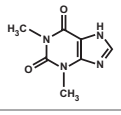
Column care in HILIC

As with any other column, to maximize HILIC column life ensure that samples and mobile phases are particle-free. We also recommend the use of guard columns or inline filters between the switching valve and the column.

Description	
pH stability	Thermo Scientific HILIC columns are silica based. A suitable pH range is 2–8.
Washing conditions	Prior to storage, buffered solutions used during HILIC analysis should be washed out of the column (and the HPLC system). A suitable wash solution is 60% organic solvent in water.
Storing conditions	HILIC columns should be stored under HILIC conditions. A mixture of organic solvent and water in a 90:10 ratio is recommended for storage. The Product Manual will give specific advice. Ensure the column end-fittings are sealed with end-plugs to prevent the stationary phase from drying.

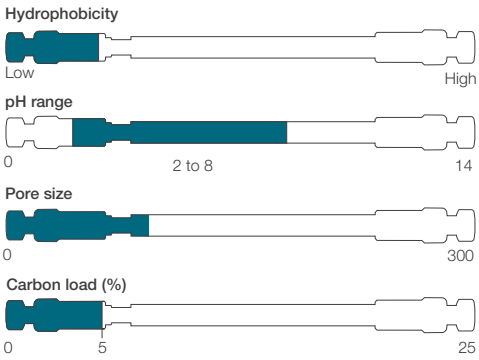
Stationary phase characterization details

Details of characterization test solutes and their physiochemical properties (pK_a and log D values obtained from chemspider.com). Chromatographic conditions as follows: mobile phase 90:10 (v/v) acetonitrile:ammonium acetate (20 mM on the column, pH 4.7); flow rate: 0.5 mL/min; UV detection: 254 nm; Injection volume: 5 µL; column temperature: 30 °C

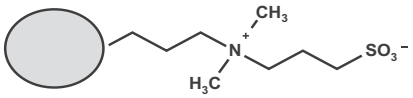
Chromatographic probes	Molecular Structure	Variable	pK_a	Log D	Separation factors
Toulene		t_o marker	na	2.72	all
Uridine		Hydrophobic/ hydrophilic interaction	12.6	-1.58	α(CH_2), α(OH), k(U)
5-Methyluridine		Hydrophobic interaction	12.0	-1.02	α(CH_2)
2'-Deoxyuridine		Hydrophobic interaction	13.9	-1.26	α(OH)
Adenosine		Configurational isomers selectivity	13.9	-1.03	α(V/A)
Vidarabine		Configurational isomers selectivity	13.9	-1.02	α(V/A)
2'-Deoxyguanosine		Positional isomers selectivity	13.5	-1.14	α(2dG/3dG)
3'-Deoxyguanosine		Positional isomers selectivity	13.5	-1.14	α(2dG/3dG)
Sodium p-toluenesulfonate		Anion exchange selectivity	na	0.88	α(AX)
N,N,N-trimethylphenylammonium chloride		Cation exchange selectivity	na	-2.31	α(CX)
Uracil		Hydrophilic interaction	13.8	-1.08	α(AX), α(CX)
Theobromine		Acidic-basic nature of stationary phase	10	-1.06	α(Tb/Tp)
Theophylline		Acidic-basic nature of stationary phase	8.6	-0.5	α(Tb/Tp)

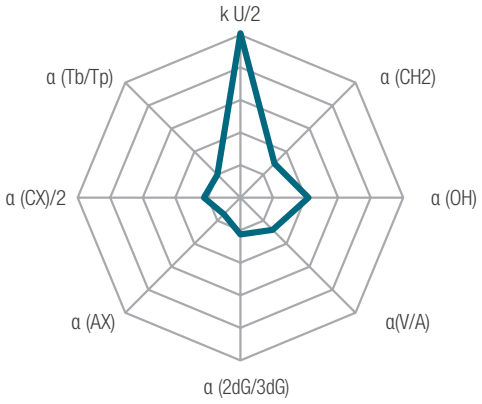
Stationary phase characterization details

Syncronis HILIC column



Particle size 1.7 µm, 3 µm, 5 µm

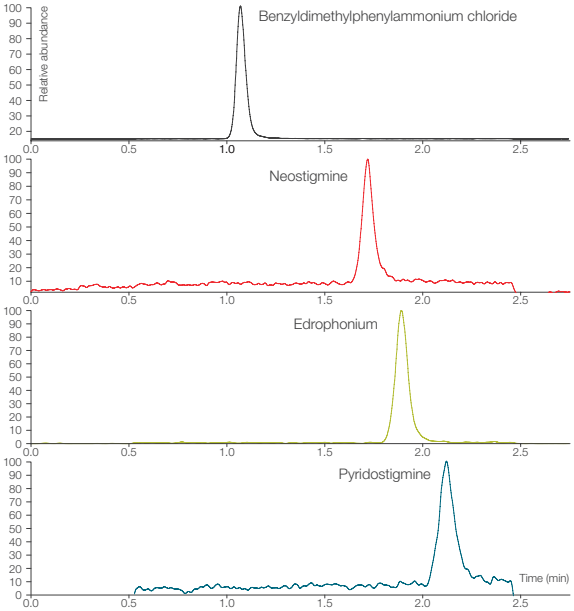




The zwitterionic material offers the highest absolute retention and good hydrophobic, hydrophilic and isomeric selectivity.
Low ion-exchange properties are demonstrated by this neutral material.

Syncronis HILIC provides enhanced retention of charged and neutral polar compounds. Outstanding peak shape and sensitivity are demonstrated on this phase.

Syncronis HILIC stationary phase carries a zwitterionic functional group of the sulfobetaine type, monomerically bonded to highly pure, high surface area silica.



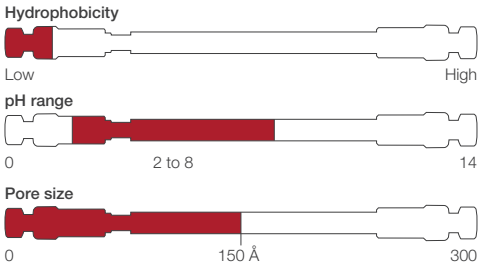
Analysis of edrophonium, neostigmine and pyridostigmine from plasma by LC-MS/MS

The sulfobetaine zwitterion has both positive (quaternary ammonium) and negative (sulfonic acid) groups in a 1:1 ratio, so that the net surface charge is zero. Due to counterbalanced electrostatic forces, which lead to weaker secondary electrostatic interactions with the analyte, lower buffer concentrations are required to disrupt these interactions. The charge density of this phase is pH-independent and the optimization of the mobile phase pH is solely dictated by the analyte.

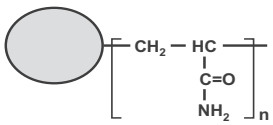
Syncronis HILIC, 1.7 µm, 100 mm × 2.1 mm	
Cat. no.	97502-102130
Mobile phase	Acetonitrile/ammonium formate, 100 mM, pH 3.3 (90:10 v/v)
Gradient	5–55% B in 60 min
Flow rate	500 µL/min
Injection volume	10 µL
Temperature	25 °C
Detection	MS

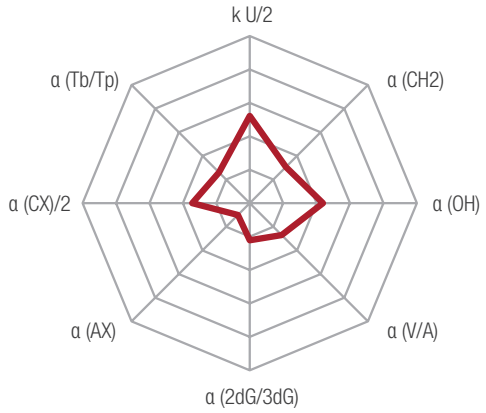
Stationary phase characterization details

Accucore 150-Amide-HILIC column



Particle size 2.6 µm

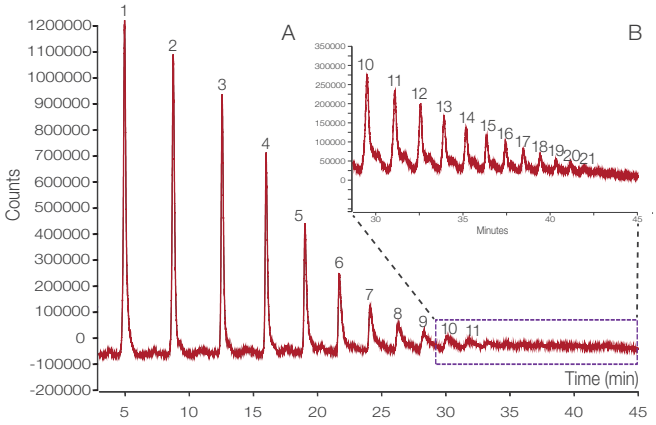




This material offers good hydrophobic, hydrophilic and isomeric selectivity and some cation-exchange properties

- Amide phase bonded onto 150 Å pore diameter solid core particles
- High retention of a broad range of hydrophilic analytes in HILIC mode
- Recommended for hydrophilic biomolecules such as glycans

The amide bonded phases provide strong hydrogen bonding interaction between the stationary phase and the analytes, resulting in unique selectivity compared to other HILIC phases.



2-AB labelled dextran ladder

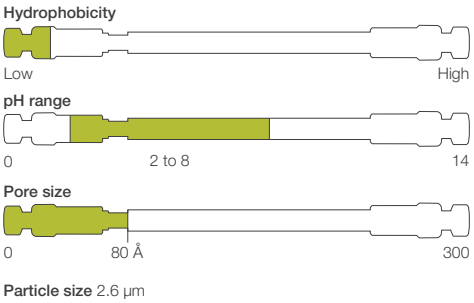
Combined with larger pore size of the solid core particles, Accucore 150-Amide-HILIC is well suited for separating a variety of hydrophilic molecules, including carbohydrates and peptides. As a result the Accucore 150-Amide-HILIC is an excellent choice for glycan separations.

A slight amount of cation-exchange properties are demonstrated by this slightly acidic phase, possibly due to the presence of residual deprotonated silanol groups (under the mobile phase pH conditions used for the characterization testing).

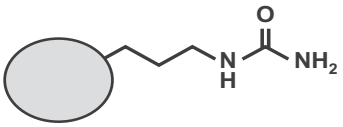
Accucore 150-Amide-HILIC, 2.6 µm, 100 × 2.1 mm	
Cat. no.	16726-102130
Mobile phase A	Acetonitrile
Mobile phase B	50 mM ammonium formate (pH 4.5)
Gradient	Time (min) B% 0 20 40 50 45 50 45.5 20 50 20
Flow rate	500 µL/min
Injection volume	2 µL 2 µL
Temperature	25 °C
Detection	Fluorescence excitation wavelength: 330nm Fluorescence emission wavelength: 420 nm
Sample	2-aminobenzamide labelled dextranladder
Backpressure	110 bar

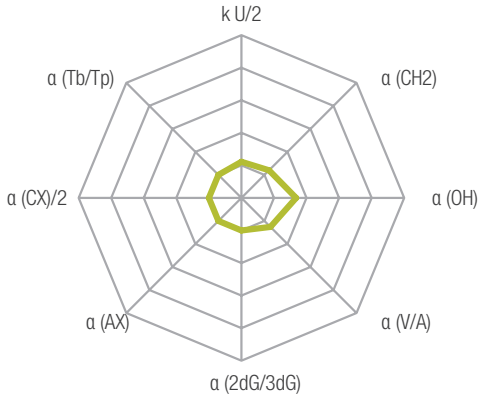
Stationary phase characterization details

Accucore Urea HILIC column



Particle size 2.6 µm

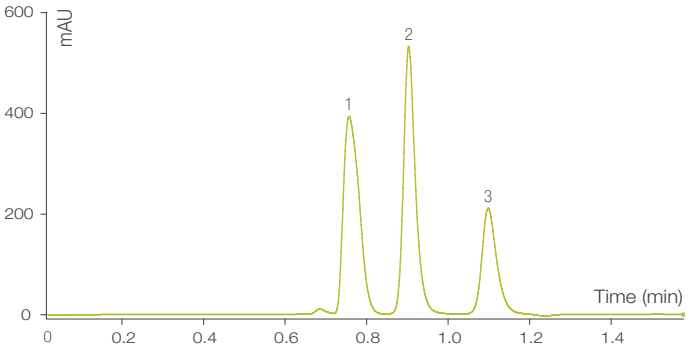




This neutral material offers good hydrophilic selectivity and low ion-exchange activity

The urea phase bonded onto solid core particles provides a unique selectivity for polar analytes.

Accucore Urea-HILIC provides good retention for charged and neutral polar compounds.



Analgesic compounds

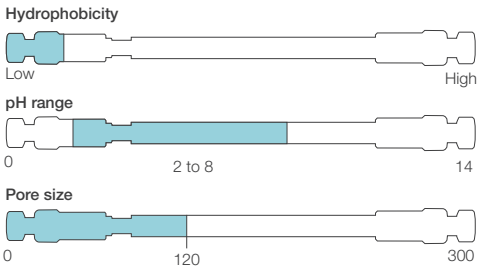
	Acetami-nophen		Salicylic acid			Aspirin		
	t_R	A_s	t_R	A_s	R_s	t_R	A_s	R_s
Mean	0.760	1.474	0.908	1.303	2.359	1.100	1.318	3.264
CV %	0.00	1.17	0.48	0.92	0.49	0.00	0.63	0.48

Accucore Urea HILIC, 2.6 µm, 100 × 2.1 mm	
Cat. no.	27726-102130
Mobile phase	Composition 10:80:10, A : B : C A: water B: acetonitrile C: 100 mM ammonium acetate adjusted to pH 4.9
Flow rate	300 µL/min
Injection volume	2 µL into 10 µL partial loop mode
Injection wash solvent	water:acetonitrile 20:80
Temperature	35 °C
Detection	UV at 230 nm
Backpressure	71 bar
Run time	2 minutes

Data from eight replicate analyses of a mixture of acetaminophen, salicylic acid and aspirin. Retention time (t_R), peak asymmetry (A_s), peak resolution (R_s)

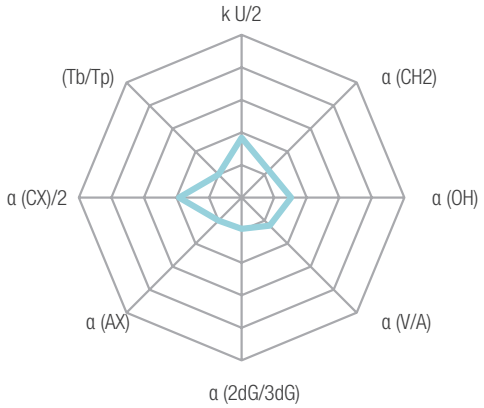
Stationary phase characterization details

Acclaim HILIC-10 column



Particle size 3 µm, 5 µm

Functionality: Proprietary hydrophilic layer



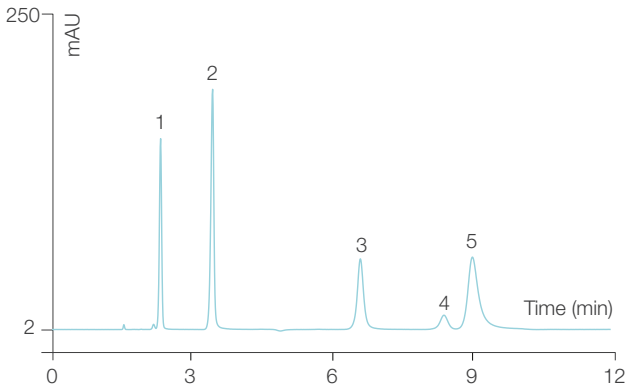
This material offers good hydrophilic selectivity. Some amount of cation-exchange properties are demonstrated by this slightly acidic phase.

Acclaim HILIC-10 is based on high purity, fully porous silica covalently modified with a proprietary hydrophilic layer.

The cation-exchange properties identified on this material are due to the presence of residual deprotonated silanol groups (under the mobile phase pH conditions used for the characterization testing).

Acclaim HILIC-10 provides good retention for charged and neutral polar compounds.

This phase shows dual mode retention behavior, with a ''U-shaped'' retention profile as a function of acetonitrile content in the mobile phase. Increasing retention is demonstrated at both low and high levels of acetonitrile (RPLC and HILIC modes, respectively).

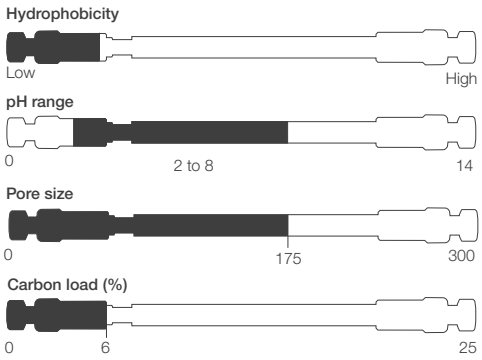


Pharmaceuticals

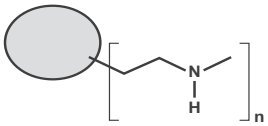
Acclaim HILIC-10, 3 µm, 150 mm × 4.6 mm		
Cat. no.	**074257**	
Mobile phase	90/10 v/v acetonitrile/10 mM (total) ammonium acetate, pH 5	
Flow rate	1 mL/min	
Injection volume	2 µL	
Temperature	30 °C	
Detection	UV at 230 nm	
Sample	1. Acetaminophen 2. Salicylic acid 3. Aspirin 4. Penicillin G 5. Metformin	0.1 mg/mL 0.1 0.2 0.1 0.1

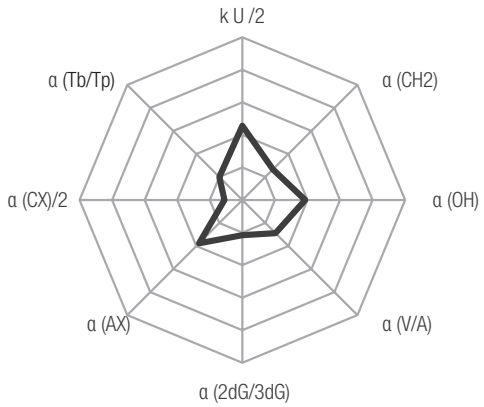
Stationary phase characterization details

Hypersil GOLD HILIC column



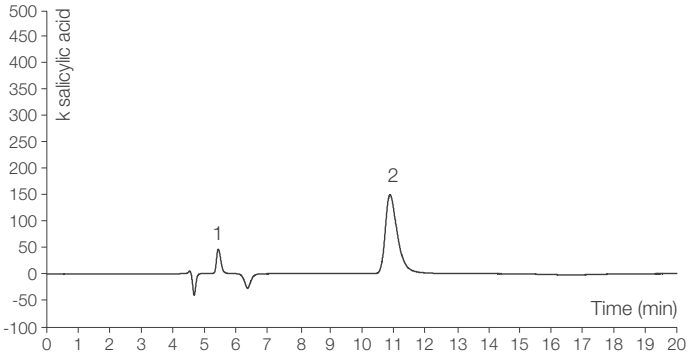
Particle size 1.9 µm, 3 µm, 5 µm





The tertiary amine functionality from the polyethyleneimine bonding of this phase shows anion-exchange activity with good hydrophilic selectivity

Hypersil GOLD HILIC is recommended for the analysis of acidic or neutral polar compounds.

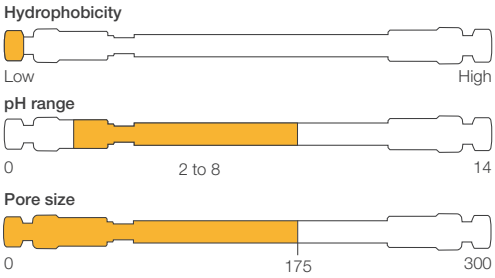


Melamine and cyanuric acid

Hypersil GOLD HILIC, 5 µm 150 × 4.6 mm	
Cat. no.	26505-154630
Mobile Phase	50 mM ammonium acetate buffer (pH 5)/ acetonitrile (10:90)
Flow rate	0.4 mL/min
Injection volume	10 µL
Temperature	30 °C
Detection	205 nm
Sample	1. Cyanuric acid 2. Melamine

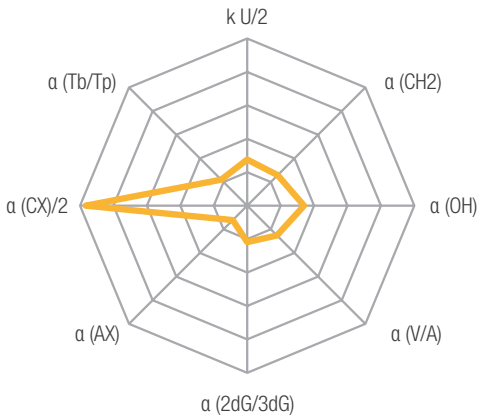
Stationary phase pharacterization details

Hypersil GOLD Silica column



Particle size 1.9 µm, 3 µm, 5 µm

Functionality: Unbonded silica



This bare silica material offers good hydrophilic selectivity and some isomeric selectivity. Very strong cation-exchange properties are demonstrated by this acidic phase.

- Highly pure base deactivated silica media
- Outstanding peak shape and sensitivity

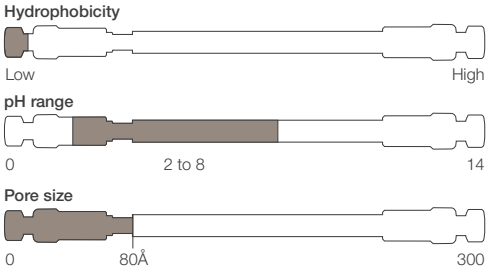
Besides being the underlying support for all Hypersil GOLD phases, Hypersil GOLD Silica has wide applications under both HILIC and normal phase conditions.

The cation-exchange properties identified on this material are due to the presence of residual deprotonated silanol groups (under the mobile phase pH conditions used for the characterization testing).

Hypersil GOLD Silica is recommended for the analysis of basic or neutral polar compounds.

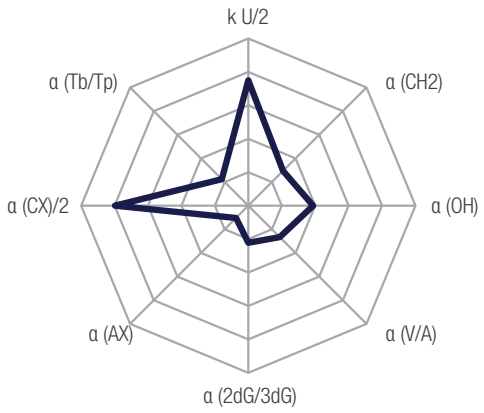
Stationary phase characterization details

Accucore HILIC column



Particle size 2.6 µm

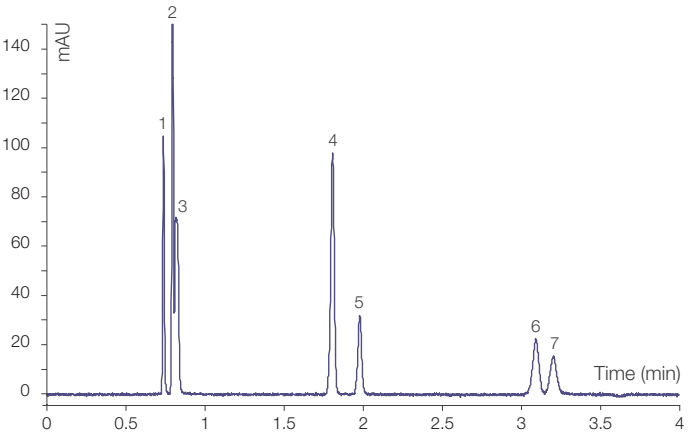
Functionality: Unbonded silica



This bare silica material offers good hydrophobic and hydrophilic selectivity. Good absolute retention and some isomeric selectivity are demonstrated. Very strong cation-exchange properties exhibited by this acidic phase.

The cation-exchange properties identified on this material are due to the presence of residual deprotonated silanol groups (under the mobile phase pH conditions used for the characterization testing).

Accucore HILIC is recommended for the analysis of basic or neutral polar compounds.

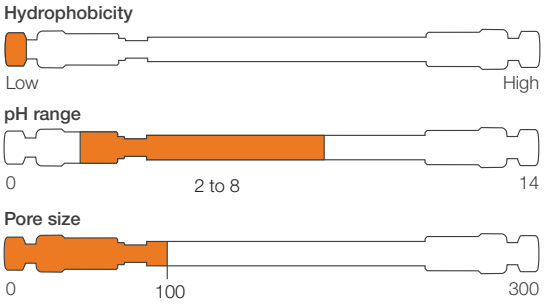


Catecholamines

Accucore HILIC 2.6 µm, 50 × 2.1 mm	
Cat. no.	17526-052130
Mobile phase	85:15 (v/v) acetonitrile/100mM ammonium formate, pH 3.2
Flow rate	2 mL/min
Injection volume	5 µL
Temperature	40 °C
Detection	UV at 280 nm
Backpressure	157 bar
Analytes	1. Catechol 2. 5-HIAA 3. DOPAC 4. Serotonin 5. L-tyrosine 6. Dopamine 7. L-DOPA

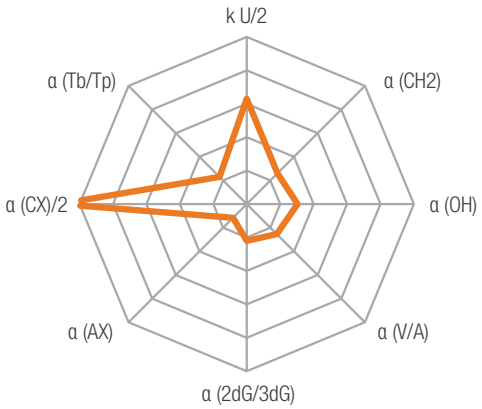
Stationary phase pharacterization details

Syncronis Silica column



Particle size 1.7 µm, 3 µm, 5 µm

Functionality: Unbonded silica



This bare silica material offers good absolute retention, good hydrophobic and hydrophilic selectivity and some isomeric selectivity. Very strong cation-exchange properties are demonstrated by this acidic phase.

Syncronis Silica offers highly pure, high surface area silica.

Serves as a powerful and efficient tool for the chromatography of moderately polar acids or neutral compounds in both HILIC and normal phase mode.

The cation-exchange properties identified on this material are due to the presence of residual deprotonated silanol groups (under the mobile phase pH conditions used for the characterization testing).

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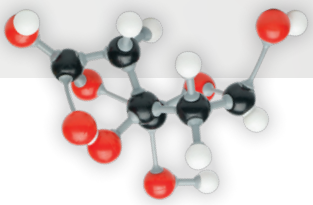
Syncronis Silica columns	
Hypersil GOLD HILIC columns	



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