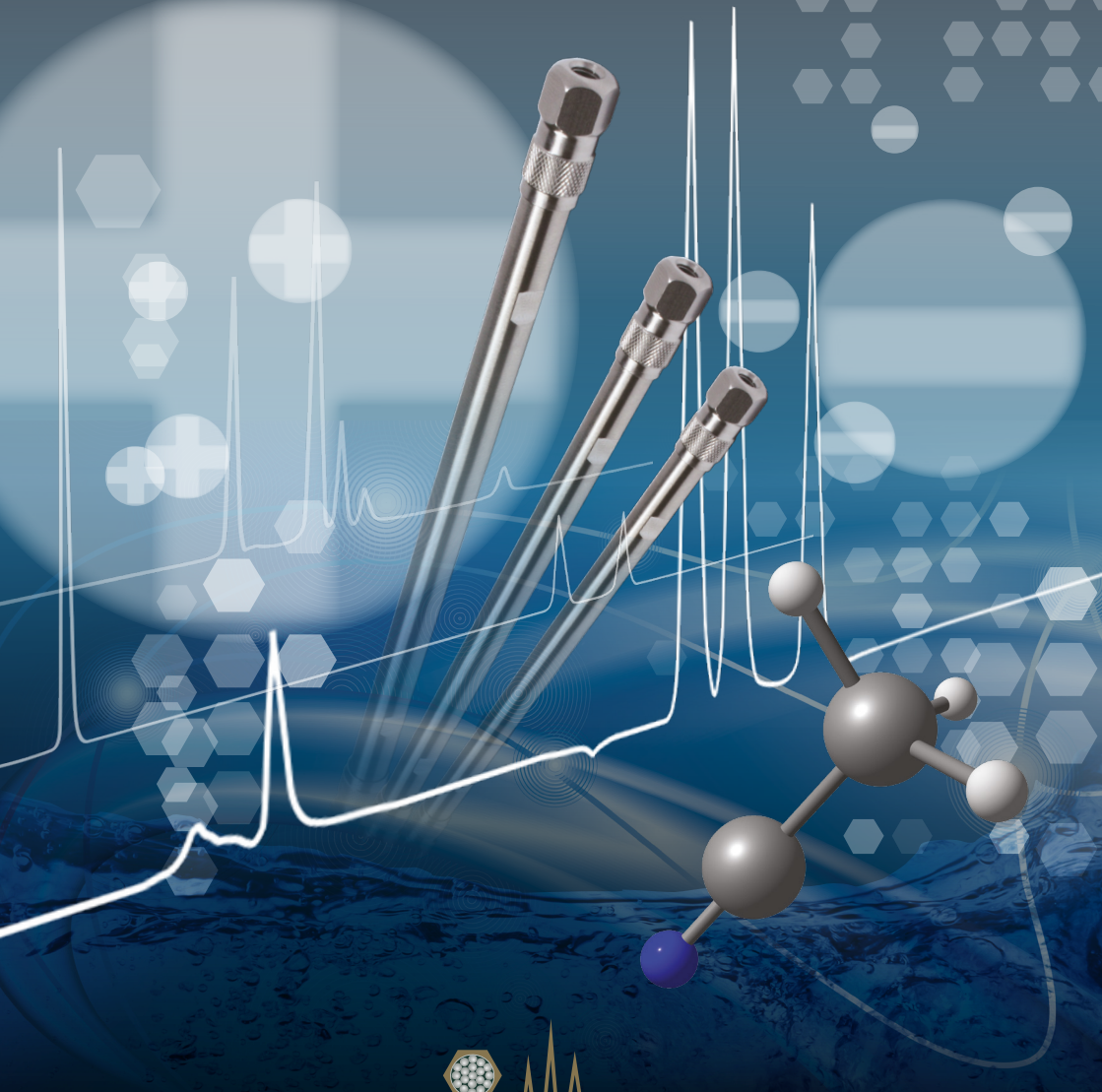
ACE® HILIC

Method Development Guide

A Step by Step Approach to Developing Reproducible HILIC Methods



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1.0 INTRODUCTION

Hydrophilic Interaction Liquid Chromatography (HILIC) uses similar eluents to Reversed-Phase Liquid Chromatography (RPLC) and provides a suitable retention and separation alternative for hydrophilic or polar to very polar analytes. The retention of these hydrophilic analytes (i.e. those species with a log P value of around zero or less) can be a challenge in RPLC, characterised by little or no retention. HILIC offers retention and alternative selectivity to RPLC for hydrophilic analytes without the use of additives such as ion-pairing reagents.

The use of high organic-containing eluents with aqueous components has been commonplace for many years but the term HILIC was first proposed in the early 1990s[1] to describe an organic solvent-rich eluent used for polar analyte retention and separation. As a technique HILIC has now been applied to almost all chromatography application areas and has proved useful for clinical analysis, environmental analysis, food & beverage applications, the pharmaceutical industry and proteomics, where the retention and separation of polar to very polar analytes can still be challenging.

This guide provides an overview of HILIC and useful practical advice for key operating parameters. There is a separate section on how to develop robust HILIC methods with practical examples.

1.1 Main advantages of HILIC for the chromatographer

HILIC provides retention of polar to very polar analytes

As a general practical rule of thumb, if an analyte elutes before caffeine in RPLC (log P ~zero), it may be better suited to a HILIC separation mode. If an analyte elutes after caffeine in RPLC, it may be better suited to RPLC.

Figure 1 shows a RPLC gradient chromatogram of a mixture containing glyphosate (log P = -2.39), caffeine (log P = -0.13) and amitriptyline (log P = 4.90). The top of figure 1 indicates an approximation of where HILIC and RPLC modes operate and overlap within the log P scale. It is clear that the very polar analyte glyphosate elutes near to the column void in RPLC and is suitable for HILIC. Caffeine can be retained in RPLC but HILIC can also be used. The area of overlap between RPLC and HILIC often causes discussion as either mode may offer advantages: the choice is usually application driven. Amitriptyline is a hydrophobic analyte best suited to RPLC.

If the log P properties of analytes are unknown, a broad scouting gradient run in RPLC as depicted in figure 1 can often help ascertain an approximate analyte hydrophilicity / hydrophobicity and indicate which separation mode may be most suitable.

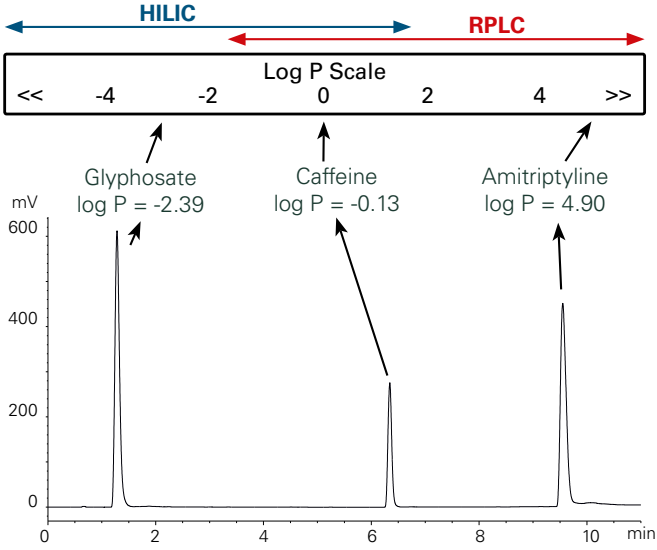


Figure 1

Practical demonstration of HILIC and RPLC operating ranges and overlap

Column: ACE 2 C18, 100 x 3.0 mm

Mobile Phase: A = 10 mM ammonium formate, pH 3.0 (aq) B = 10 mM ammonium formate, pH 3.0 in MeCN:H_2O (90:10 v/v)

Gradient: 5-100 %B in 10 min

Detection: ELSD

Flow rate: 0.4 mL/min

Temperature: 30 °C

Injection: 10 µL

Analysed using VWR-Hitachi Chromaster with VWR ELSD90

HILIC provides alternative selectivity to RPLC

A second attractive characteristic of HILIC is that it typically provides complementary or orthogonal selectivity to RPLC, as demonstrated in figure 2. The elution profile for the six polar analyte mixture in HILIC mode (figure 2b) is exactly the reverse of the same mixture analysed by RPLC (figure 2a), demonstrating the orthogonal selectivity of HILIC.

Figure 2

Demonstration of orthogonal selectivity between RP and HILIC

(a) Column: ACE 5 C18, 150 x 4.6 mm

Mobile phase: 0.1% acetic acid in MeCN/H_2O (5:95 v/v)

(b) Column: ACE 5 HILIC-N, 150 x 4.6 mm

Mobile phase: 0.1% acetic acid in MeCN/H_2O (95:5 v/v)

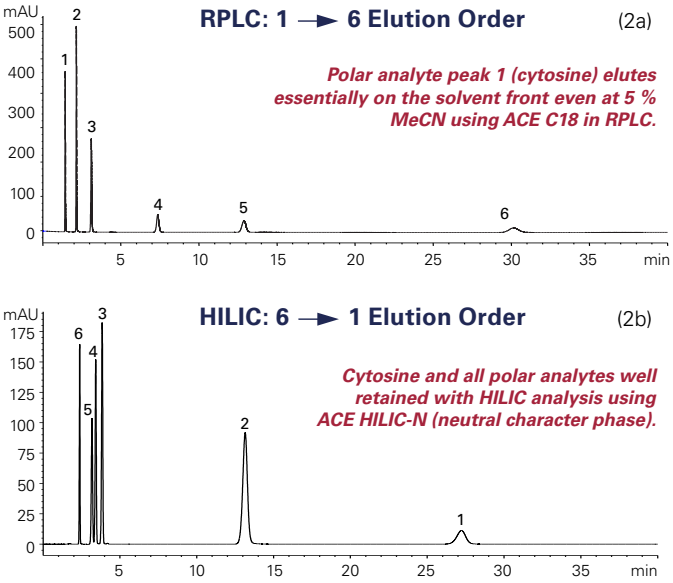
Flow rate: 1 mL/min

Temperature: 22 °C

Detection: UV, 254 nm

Injection: 5 µL

Sample:
1) Cytosine
2) Hypoxanthine
3) Thymine
4) Theobromine
5) Theophylline
6) Caffeine



Selectivity is the key to analyte resolution in chromatography, so maximising selectivity for polar to very polar analytes using HILIC is a helpful approach. Exploiting selectivity during method development is the first step to understanding analyte retention behaviour and a powerful tool to increase the likelihood that all species within a sample have been observed. The different and complex HILIC multi-mode retention mechanisms provide an ideal complementary selectivity separation mode to RPLC.

HILIC uses high organic containing eluents

The third advantage is derived from the high organic containing eluents used in HILIC. This has been shown to allow up to twice as fast diffusivity compared to RPLC, enhancing mass transfer and lowering the C term contribution to the van Deemter equation[2]. However, the mobile phase in HILIC must contain a minimum of 3% aqueous to form the adsorbed water layer around the stationary phase particles for partitioning. The aprotic organic solvent acetonitrile is typically used as the weaker solvent in HILIC. HILIC eluents are additionally helpful for MS detection[3]. The very high organic solvent content provides an ideal low surface tension, highly volatile liquid flow to the MS source, providing improved conditions for desolvation, ion formation and therefore enhanced signal response.

HILIC separations typically provide lower back pressure

In general, column (and system) back pressures are lower for HILIC than aqueous-organic RPLC eluents (especially when using predominantly acetonitrile, which is most popular). This offers the chromatographer an option to use smaller, more efficient particles at higher flow rates or even couple multiple columns together for increased plate counts or peak capacity with modest back pressures[4,5].

1.2 Main disadvantages of HILIC for the chromatographer

HILIC method robustness

This is a frequently discussed and reported topic with HILIC. Many robustness issues can be solved by adequate equilibration of HILIC columns prior to use. If the HILIC method includes a gradient, then appropriate equilibration between injections is also required. This topic is reviewed in more detail later in section 8.

Sample solubility

By their very nature, polar to very polar analytes can be challenging to dissolve and keep solubilised in organic rich diluents. This guide will provide hints and strategies to ensure samples remain suitable for HILIC analysis.

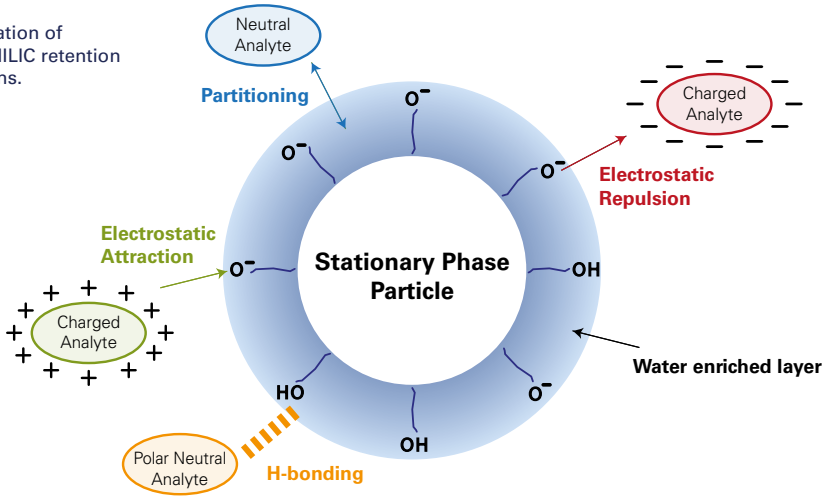
2.0 HILIC MECHANISM OF SEPARATION

HILIC is a complex separation technique with multiple modes of interaction contributing to retention. The weightings of these interactions are based on a combination of stationary phase, mobile phase and the physico-chemical properties of the analyte. To produce reliable and robust HILIC methods, it is helpful to understand the different interactions possible. This enables rational choices of columns and conditions for method development to be made.

The mobile phase in HILIC typically contains large quantities of acetonitrile (>70%) with a minimum of 3% aqueous. The aqueous component allows a hydrophilic environment to exist around the polar stationary phase and formation of an adsorbed water layer for analyte partitioning.The major HILIC retention mechanisms include analyte partitioning into the adsorbed water layer, various polar interactions and electrostatic (i.e. ion-exchange type) interactions (see figure 3). If a polar analyte is introduced into the HILIC environment, it may use any or all of these mechanisms for retention.

Figure 3

Representation of potential HILIC retention mechanisms.



Polar analytes typically contain various moieties capable of interacting with other polar groups, such as those on the stationary phase. Polar interactions include hydrogen bonding and dipole-dipole interactions amongst others. Charged analytes can also undergo electrostatic or ion-exchange type interactions, where the analyte may be attracted to or repelled from the stationary phase. For example, a negatively charged or acidic analyte may be repelled by a negatively charged (i.e. acidic) stationary phase. However, a positively charged or basic analyte may be retained by the negatively charged (acidic) stationary phase and vice versa.

3.0 STATIONARY PHASES

Historically, many HILIC stationary phases were simple unbonded silica. These acidic phases provided the necessary phase polarity to work well in HILIC mode with high organic containing eluents. Unbonded silica phases are useful and still widely used. More recently, a variety of bonded phases specifically designed for the HILIC environment have become commercially available. These new polar bonded phases can be broadly classed according to phase character and include acidic, basic, neutral and novel chemistry phases. The ACE HILIC portfolio currently contains acidic, neutral and basic stationary phases. These phases have been shown to provide different selectivity to each other. A structured approach to HILIC method development using these ACE HILIC phases is included in this guide.

The three ACE phases provide alternative selectivity to each other which can be seen in figure 4. The polar neutral and charged analytes in the nine component mixture show different retention and elution orders under the same conditions on the three different ACE HILIC phases. This clearly demonstrates the selectivity differences of each ACE HILIC stationary phase in HILIC mode making the three columns ideal for HILIC method development.

Figure 4

Comparison of elution order on the ACE HILIC stationary phase range

Column: 150 x 4.6 mm, 5 µm

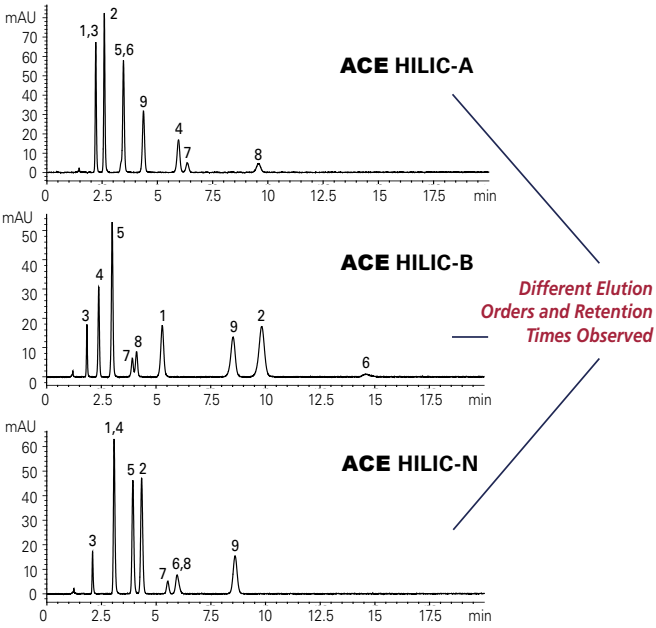
Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v)

Flow rate: 1.5 mL/min

Temperature: 25 °C

Detection: UV, 254 nm

Sample:
1) p-Aminobenzoic acid
2) 4-Hydroxybenzoic acid
3) Nicotinamide
4) Acebutolol
5) Adenine
6) Mandelic acid
7) Tyramine
8) Atenolol
9) 2'-Deoxyguanosine



3.1 **ACE** HILIC-A Phase

The ACE HILIC-A phase possesses the ability to form a negative charge and shows high cation exchange capacity. Charged bases undergo electrostatic attraction to the ACE HILIC-A phase, whilst charged acidic species are repelled.

The extent by which the ACE HILIC-A phase is charged is dependent on the mobile phase pH. The recommended pH limits for the ACE HILIC range are between pH 2.0 and pH 7.0. By increasing the pH, the negative charge will become more pronounced on the stationary phase, thereby providing greater retention of cationic analytes.

3.2 **ACE** HILIC-N Phase

The neutral ACE HILIC-N phase shows low ion exchange capacity for both anions and cations. The retention mechanisms for the ACE HILIC-N phase include polar interactions, adsorption and some degree of partitioning.

3.3 **ACE** HILIC-B Phase

The ACE HILIC-B phase has a reasonable anion exchange capacity leading to retention of acidic analytes, whilst basic analytes are repelled. Similarly to the ACE HILIC-A, the pH can affect the charge of the stationary phase, thereby reducing or increasing the positive character of the phase.

4.0 HILIC MOBILE PHASES

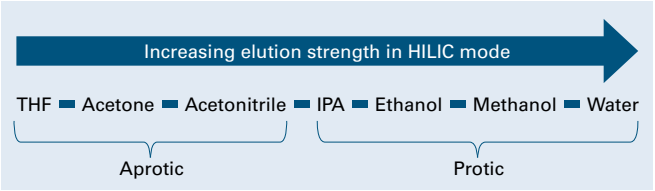
The solvents used as mobile phases in HILIC are similar to those used in RPLC. As discussed in Section 2.0, the mobile phase conditions are key contributors to the various HILIC retention mechanisms. By modifying the proportions of the organic and aqueous components in the eluent, analyte retention can be altered. In HILIC, a retention factor between 1.5 and 10 is recommended.

4.1 Organic Modifiers

The most popular organic modifier used in HILIC is the aprotic solvent, acetonitrile. Acetonitrile is used as the weaker elutropic solvent in combination with water (see figure 5). Unlike RPLC, increasing the % organic in the mobile phase increases analyte retention. Acetonitrile has further advantageous properties including low viscosity and a low UV cut-off.

Figure 5

Solvent elution strength in HILIC



Protic solvents such as methanol are normally avoided in the bulk solvent due to their ability to disturb the aqueous layer surrounding the stationary phase. These solvents can form hydrogen bonds and hence disrupt analyte partitioning.

There are, however, various examples where polar solvents such as methanol and 2-isopropanol (IPA) are used in combination with the aqueous portion to influence the selectivity of the separation. This is typically used as a last resort if all other parameters do not provide adequate separation[6].

4.2 Eluent pH

The pH of the eluent is a useful parameter for method development. Using a pH above or below the pK_a of an ionisable species changes the analyte ionisation state, which in turn affects its hydrophilicity. As such, this affects the potential interactions with the stationary phase, and impacts retention. The pH will also affect the polarity of the stationary phase surface, which additionally influences retention mechanisms.

Knowledge of the analyte's pK_a can greatly help pH selection. If possible, it is advisable to work 2 pH units away from the pK_a for method robustness. For acidic species, working below the pK_a will result in the analyte existing mostly in the unionised state. Working 2 pH units above the pK_a of the acidic analyte will result in the analyte being mostly ionised. The opposite is true for basic species.

For method development purposes, it is recommended to assess analyte mixtures on each ACE HILIC column at three different pH values: pH 3.0, 4.7 and 6.0. This approach explores the differences in selectivity when using different stationary phases at different eluent pH values and has found to be effective for HILIC method development. As an example, with the ACE HILIC-A phase, a mixture containing polar acidic and neutral species was analysed with isocratic conditions at pH 3.0, pH 4.7 and pH 6.0 (see figure 6). All other conditions were the same. The differences in retention and elution order (i.e. selectivity differences) can clearly be seen with the different pH values on this single HILIC phase.

Figure 6

Analysis of six polar analytes with ACE HILIC-A at different mobile phase pH values

Neutrals - Green
Acids - Red

Column: 150 x 4.6 mm, 5 µm

Mobile phase: 10 mM ammonium formate in MeCN/H_2O (90:10 v/v)

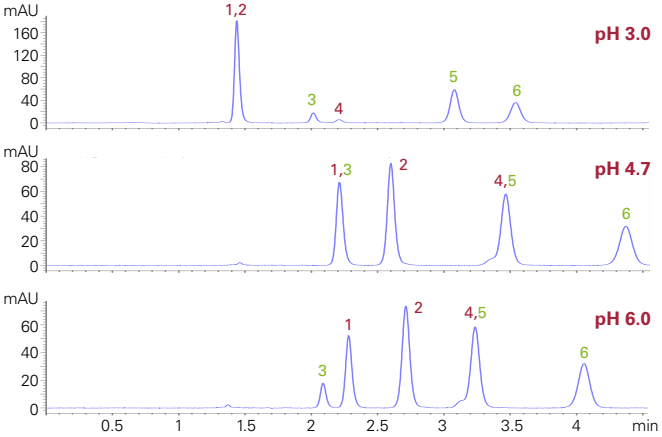
Flow rate: 1.5 mL/min

Detection: UV, 254 nm

Temperature: 25 °C

Injection: 5 µL

Sample:
1) 4-Aminobenzoic acid
2) 4-Hydroxybenzoic acid
3) Nicotinamide
4) Mandelic acid
5) Adenine
6) 2'-Deoxyguanosine



4.3 Buffer Concentration

A variety of buffer salts may be used in HILIC, however it is essential that the buffer must be soluble in high organic solvent containing eluents. This tends to rule out inorganic buffers such as phosphates. Ammonium formate buffers are favourable as they offer buffering capacity at the low pH range and good solubility in high acetonitrile containing eluents. HILIC typically uses a buffer concentration range of 2-18 mM.

The effect of buffer concentration on retention and selectivity depends on the properties of the analyte and the stationary phase. As an example, figure 7 shows the influence of ammonium formate buffer concentration at pH 3.0 on analyte retention for a three component mixture (polar acid, base and neutral analytes) using the ACE HILIC-A stationary phase. The low eluent pH means the acid pyridoxal (peak 1) is present in an ion suppressed or neutral form. Cytidine (peak 2) is a polar neutral analyte. Procainamide (peak 3) is a charged polar base. Under these conditions with the ACE HILIC-A phase, the polar base exhibits a significant reduction in retention as the buffer concentration increases. The polar base is likely to be predominantly retained by ion-exchange with the charged acidic stationary phase. The increase in buffer strength creates competition for the basic analyte and a subsequent reduction in retention as the ion-exchange mechanism depletes.

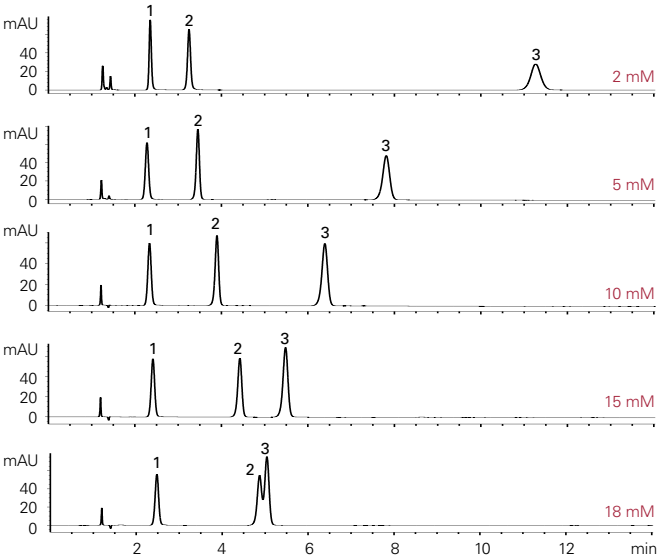


Figure 7

Effect of buffer strength on analyte retention

Column: ACE 5 HILIC-A, 150 x 4.6 mm

Mobile phase: ammonium formate pH 3.0 in MeCN/H_2O (90:10 v/v)

Flow rate: 1.5 mL/min

Detection: UV, 254 nm

Temperature: 25 °C

Injection: 5 µL

Sample:
1) Pyridoxal
2) Cytidine
3) Procainamide

5.0 SAMPLE DILUENT

Choosing the correct sample diluent in HILIC is often perceived to be difficult due to analyte solubility concerns and diluent mismatch with the HILIC eluent. Poorly optimised diluent choices can significantly reduce chromatographic performance and peak shape. In general, the effect of sample diluent can be different for each analyte and the behaviour varies depending upon the stationary phase choice and eluent conditions. As such, optimisation of sample diluent tends to be an application specific investigation.

In general, it is recommended that 20% increments of acetonitrile to buffer ratio between 20% and 80% are explored.

As an example, figure 8 (page 16) shows the peak shape of individual acidic, basic and neutral analytes on each of the three ACE HILIC phases at pH 3.0 where the sample diluent acetonitrile (MeCN) percentage was explored.

The peak shape of hypoxanthine (polar neutral) generally improved with increased organic concentration on all ACE HILIC phases. However, the analyte failed to dissolve in 100% acetonitrile. The most promising sample diluent for this analyte was 60-80% acetonitrile. At low concentrations of acetonitrile in the sample diluent, tyramine (basic analyte) showed a split peak with the ACE HILIC-B and ACE HILIC-N. This effect was less pronounced on the ACE HILIC-A, however, the peak shape was still poor. The peak shape improved with all ACE HILIC phases above 60% acetonitrile. The peak shape of mandelic acid was generally unaffected by sample diluent on ACE HILIC-B and ACE HILIC-N. However, on the ACE HILIC-A, the peak shape was poor at low concentrations of organic.

Based upon this dataset, a sample diluent of 60-80% acetonitrile is recommended for this HILIC application. Such systematic investigations are recommended for each HILIC application.

If possible, higher concentrations of analytes and smaller injection volumes are preferred. This can minimise disruptions to the adsorbed water layer. A balance with sample solubility and sample concentration is always challenging – especially with HILIC.

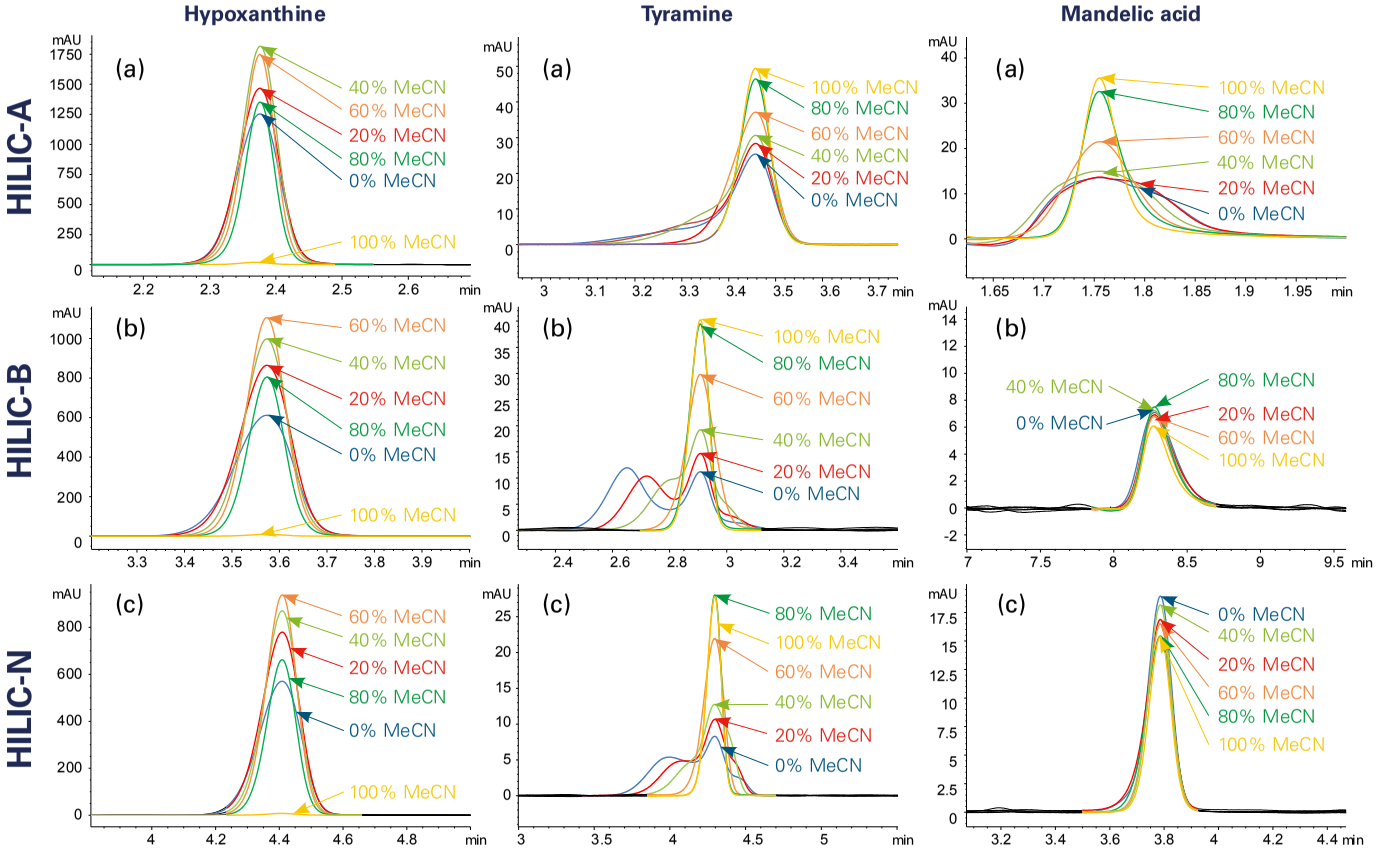


Figure 8

Effect of sample diluent on chromatographic peak shape for hypoxanthine, tyramine and mandelic acid on (a) ACE HILIC-A (b) ACE HILIC-B and (c) ACE HILIC-N.

Column: 150 x 4.6 mm, 5µm Mobile phase: ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v) Flow rate: 1.5 mL/min Temperature: 25 °C Detection: UV, 254 nm Injection: 5 µL

6.0 TEMPERATURE

Figure 9

Effect of temperature on analyte retention for a mixture of acidic, basic and neutral analytes on:
(a) ACE 5 HILIC-A
(b) ACE 5 HILIC-N
(c) ACE 5 HILIC-B

Column: 150 x 4.6 mm

Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v)

Flow rate: 1.5 mL/min

Detection: UV, 230 nm

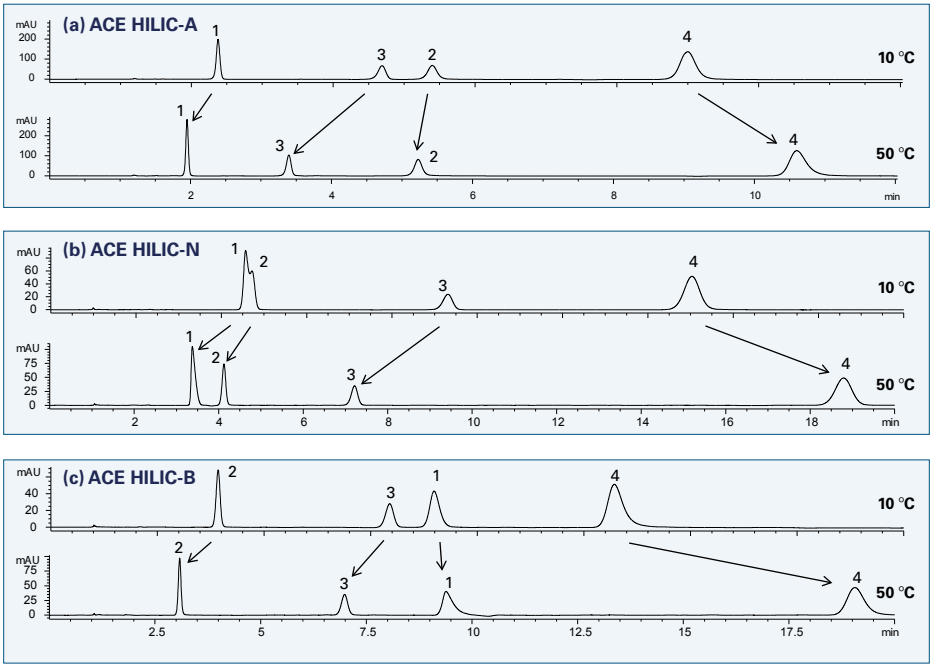
Injection: 5 µL

Sample:
1) 4-Hydroxybenzoic acid
2) Salbutamol
3) 2'-Deoxyguanosine
4) Tryptophan

Temperature can be a powerful parameter for use in method development. The extent to which temperature affects retention is dependent on the combination of stationary phase and analyte. For example, as the temperature changes, the pK_a of the analyte may change, affecting its degree of ionisation and retention.

It has been documented that for HILIC, it is possible to have both increases and decreases in retention with increasing temperature, as demonstrated in figure 9 for the separation of a mixture of polar acids, bases and neutral analytes.

It is recommended that temperature should not be used as a principal parameter to optimise selectivity and separation in HILIC method development. Its ability to alter retention in HILIC mode is minimal in comparison to parameters such as column chemistry, % organic or eluent pH.



7.0 DETECTORS

HILIC is a technique that interfaces well with numerous popular detectors. The choice of detection is therefore driven by a combination of detector availability, detection limits needed and by the analyte's physico-chemical properties, i.e. possession of a chromophore or charge.

7.1 UV-Vis Detectors

UV-Vis detectors are one of the most common found in analytical laboratories. Depending on instrumentation, multiple wavelengths can be recorded at once for optimal coverage of a range of analytes. Sometimes analytes in mixtures can be quickly identified by referencing to UV libraries. The example in figure 10 shows the separation of five β-blockers which can be clearly identified by their different spectra.

Figure 10

Chromatogram and UV spectra for five β-Blockers

Column: ACE 5 HILIC-N, 150 x 4.6 mm

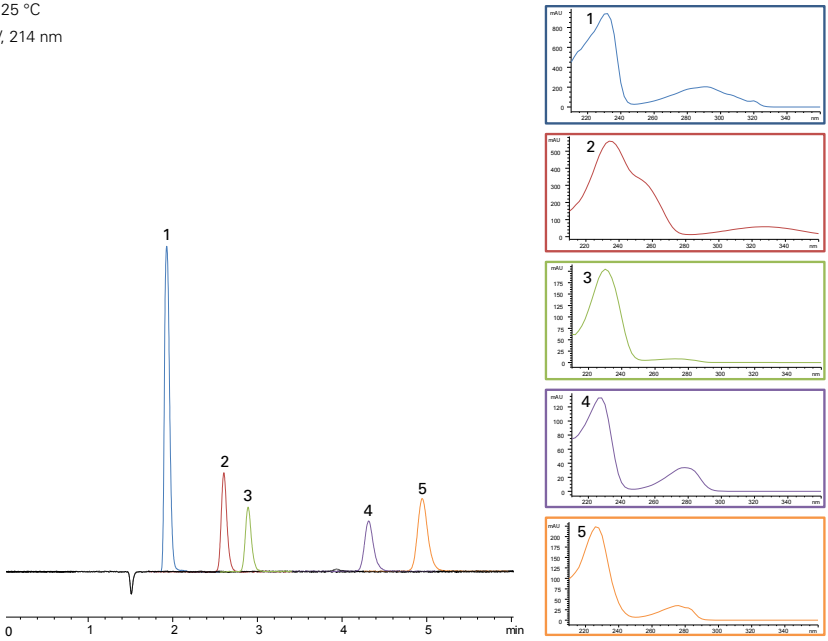
Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O (90:10 v/v)

Flow rate: 1.5 mL/min

Temperature: 25 °C

Detection: UV, 214 nm

Sample:
1) Propranolol
2) Acebutolol
3) Sotalol
4) Salbutamol
5) Atenolol



7.2 Refractive Index Detectors

Refractive index detectors (RID) measure the refractive index of an analyte peak relative to a reference solvent (i.e. the mobile phase) as it passes through the detector. If there is a difference between the two, a peak is observed in the chromatogram. The RID is useful for analytes which lack a UV-Vis active chromophore and is particularly popular for sugar analysis.

There are several disadvantages with RID. These include limited sensitivity and no peak information (individual standards are typically required to verify peak identities). Additionally, RIDs are limited to use with isocratic conditions, as eluent changes alter the refractive index response. As a physical measurement, refractive index is also highly affected by temperature meaning baseline noise and reproducibility can vary.

7.3 Evaporative Light Scattering Detectors

Evaporative light scattering detection (ELSD) works by spraying the output eluent from the LC with an inert gas (typically nitrogen) to form droplets which desolvate within a heated chamber. The desolvated analytes are hit by a light source which is scattered and then detected. The detector requires volatile eluents making HILIC highly suitable. ELSD is a good alternative to RID as it is ideal for non-chromophoric analytes, is more sensitive and is suitable for use with gradient chromatography. ELSD however, does not provide spectral information so peak identity can be challenging without standards.

For HILIC separations, ELSD can be advantageous for polar analytes which possess limited chromophores, such as methylmalonic acid (MMA) and succinic acid (figure 11). MMA is used as a biomarker for vitamin B12 deficiency but succinic acid is isobaric to MMA and must therefore be fully resolved to prevent false positive results. The HILIC method with ELSD also allows better quantification for the MMA than UV detection.

Figure 11

Chromatogram for methylmalonic acid and succinic acid

Column: ACE 5 HILIC-B, 150 x 4.6 mm

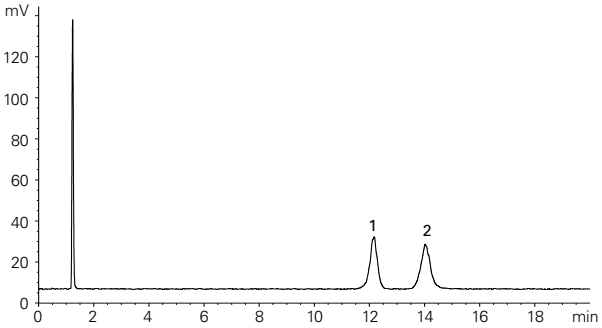
Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O (90:10 v/v)

Flow rate: 1.5 mL/min

Temperature: 25 °C

Detection: ELSD

Sample:
1) Succinic acid
2) MMA



7.4 Mass Spectrometers

Mass spectrometers (MS) are the most information rich detection technique for LC separations. The eluent flow from the LC is desolvated within a heated chamber usually with an inert gas (such as nitrogen) and ionised before detection. Different mass analysers such as quadrupole, time-of-flight (TOF), ion-trap and others can be used. The HILIC eluent with a high organic content is particularly suited for MS detection. There are multiple ionisation sources available, which are selected based on the application. Electrospray ionisation (ESI) is very popular but corona discharge and photo-ionisation sources are also used. MS can be highly specific as individual mass-to-charge ratios (*m/z*) can be tracked using Selected Ion Monitoring (SIM), leading to greater sensitivity. MS can also perform scans across mass ranges which can be helpful for unknown samples. MS scans, however, are typically far less sensitive.

MS can be useful to detect analytes which possess a weak or no chromophore. MS is also helpful as an identification tool in HILIC applications where analytes have similar UV spectral properties but different masses. A good HILIC example is nucleobases and nucleosides. The polar analytes adenine, adenosine and 2'-deoxyadenosine, have very similar UV spectra (figure 12).

Figure 12

Comparison of UV spectra for adenine, adenosine and 2′-deoxyadenosine

Blue trace: Adenine

Red trace: Adenosine

Green trace: 2′-Deoxyadenosine

Column: ACE 5 HILIC-N, 150 x 4.6 mm

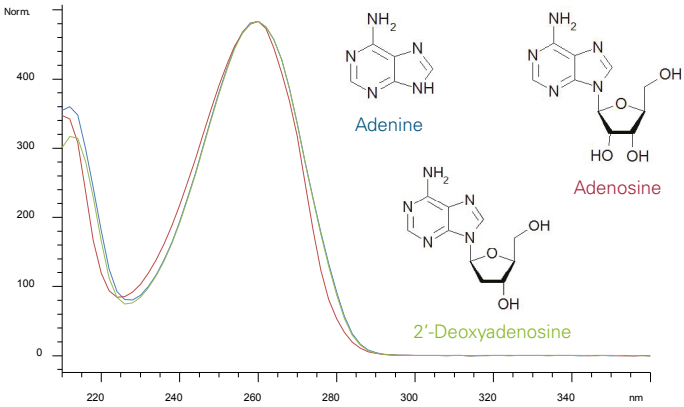
Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v)

Flow rate: 1.5 mL/min

Temperature: 25 °C

Detection: UV

Injection volume: 2 µL



The different mass to charge ratios of these analytes can be used by the MS for detection and peak identification (figure 13). SIM was applied to improve sensitivity.

Figure 13

Analysis of adenine and nucleosides on the ACE HILIC-N with tandem UV and MS detection

(a) UV chromatogram of
1) 2′-Deoxyadenosine (*m/z* 252.4)
2) Adenine (*m/z* 136.1)
3) Adenosine (*m/z* 268.3)
(b) confirmation of peak identities with MS SIM

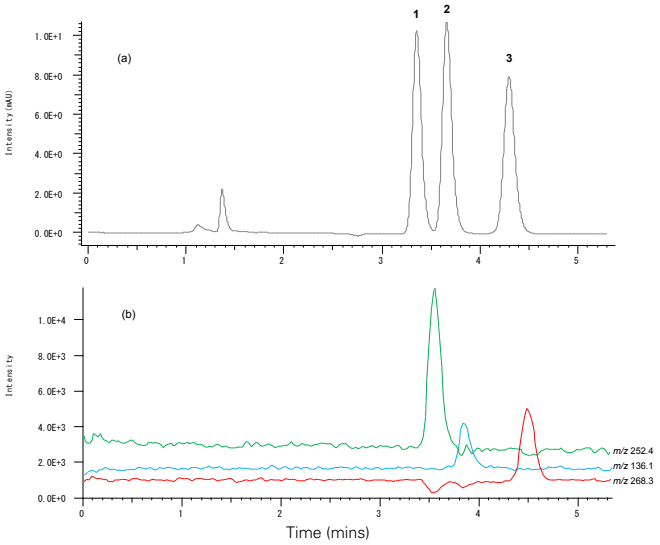
Column: ACE 5 HILIC-N, 150 x 4.6 mm

Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v)

Flow rate: 1.5 mL/min

Temperature: 25 °C

Detection: UV, 254 nm and MS



8.0 HILIC METHOD EQUILIBRATION TIMES

In RPLC, the typical column equilibration needed for reproducible retention is approximately 10 column volumes. This can be clearly seen in figure 14. (For an explanation of column volumes, see page 31).

For HILIC separations, however, it is different. HILIC columns typically take longer to equilibrate than RPLC columns. This is a feature of the technique and necessary for reproducible retention.

Establishing and forming a stable hydration layer around the stationary phase particles is central for reproducible HILIC chromatography methods. The time taken can also vary depending upon the phase character, eluent and analytes and is therefore application dependent. The guidelines below can be used as an approximation. It is important that equilibration is explored as a parameter in HILIC method development for each separation developed.

For brand new, unused columns, it is recommended that 60-80 column volumes are used to initially establish the water layer required for HILIC.

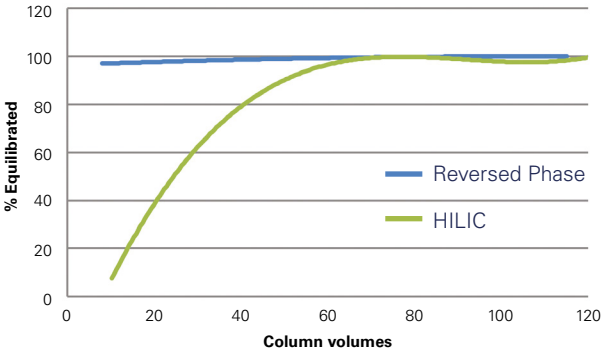


Figure 14

Comparison of the number of column volumes required in RPLC and HILIC to reach equilibration for brand new, unused columns

Once the water layer is established, reproducible and consistent retention and chromatography is possible.

Once the run is completed, the column should be washed and stored according to the guidance in Section 9. For second and all subsequent runs with the column, it is recommended that only 20 column volumes are required to achieve stable retention times, see figure 15.

Figure 15

Plot of the number of column volumes required in HILIC to achieve equilibration for second and subsequent analyses

Column: ACE 5 HILIC-A, 100 x 3.0 mm

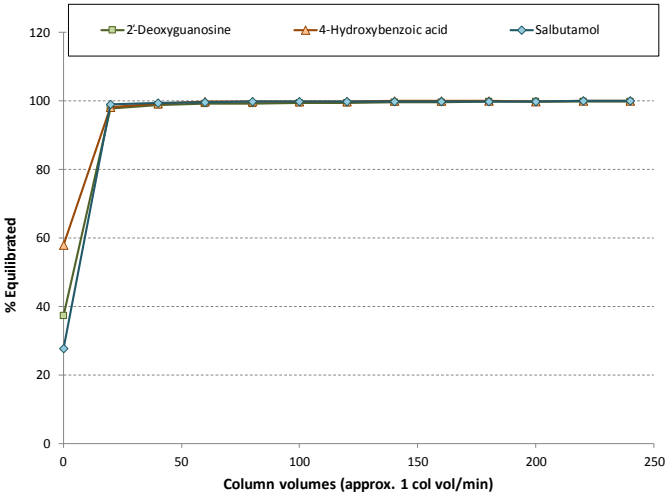
Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v)

Flow rate: 0.43 mL/min

Temperature: 25 °C

Detection: UV, 214 nm

Sample:
1) 2′-Deoxyguanosine
2) 4-Hydroxybenzoic acid
3) Salbutamol



8.1 Gradient HILIC Equilibration

Reproducible gradient HILIC methods with ACE HILIC phases are also possible. It has been determined that approximately 10 column volumes between injections are needed. The equilibration time between injections is lower than that required for a new column as it is only necessary to re-establish starting conditions. The equilibration for a new column is necessary to form the hydrated layer on the stationary phase particles. Figure 16 shows excellent retention reproducibility with gradient HILIC analysis for two successive injections using 20 column volume equilibration between injections.

Figure 16

Overlay of chromatograms for two successive gradient runs on the ACE HILIC-A

Column: ACE 5 HILIC-A, 150 x 4.6 mm

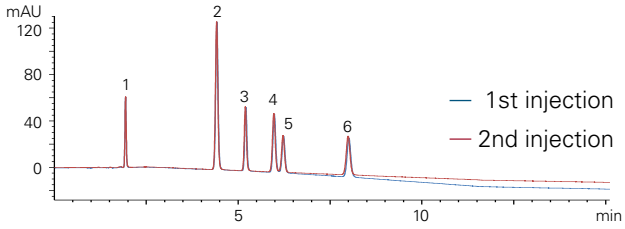
Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O, 94 to 70% MeCN in 15 minutes

Flow rate: 1.5 mL/min

Temperature: 25 °C

Detection: 254 nm

Sample:
1) Theophylline
2) Hypoxanthine
3) Acebutolol
4) Guanine
5) Cytosine
6) Cytidine



9.0 ACE HILIC COLUMN STORAGE

After use, the ACE HILIC column should be flushed with 7:3 v/v acetonitrile:water to remove all buffer salts. The column should then be flushed at a lower flow rate with 100% isopropanol for storage. The column end caps should be screwed back firmly and the column placed back in its box. It is recommended to establish a shutdown method to wash the column and then flush the column onto isopropanol after the end of each analytical run unless it is being used the following day.

10.0 ACE HILIC METHOD DEVELOPMENT PLATFORM AND WORKED EXAMPLES

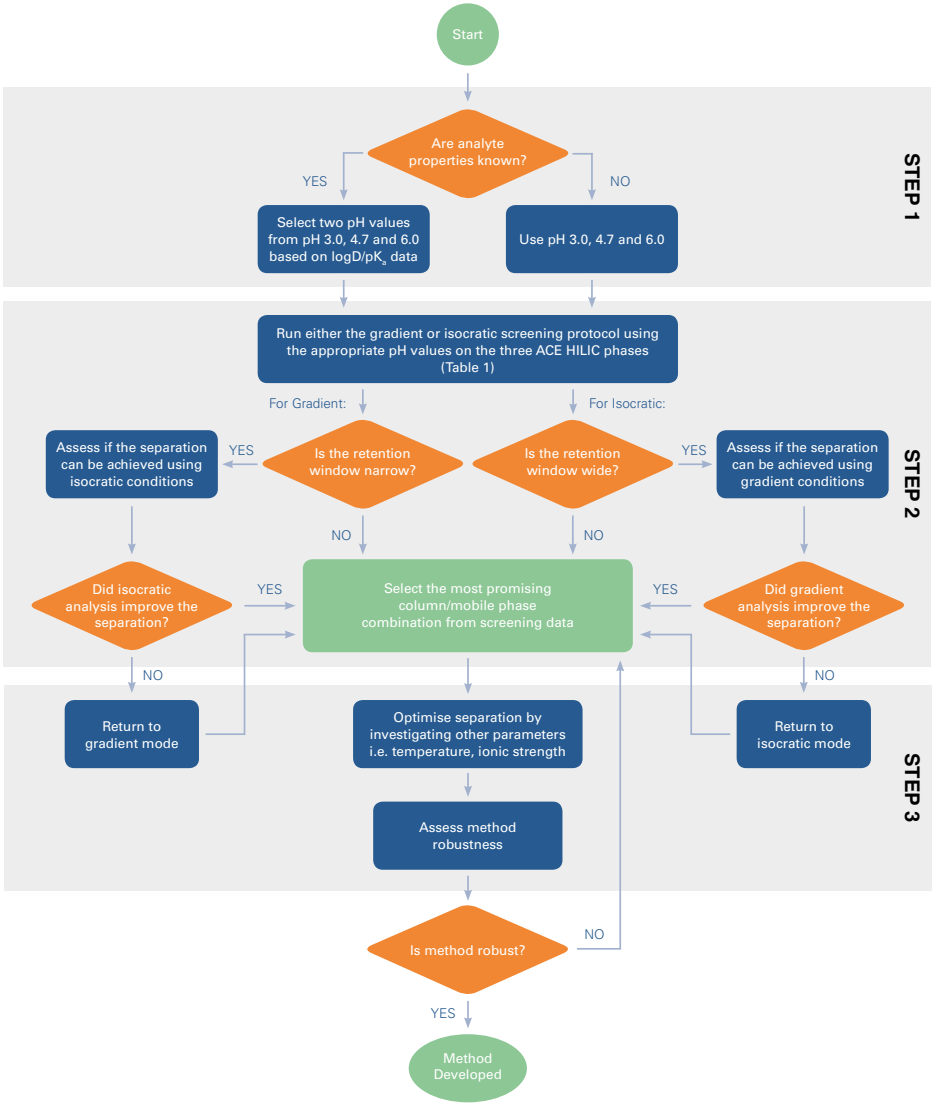
A flow diagram for HILIC method development is shown in figure 17.

The general approach is to gather information on the analytes (if known), execute gradient or isocratic HILIC screening experiments (depending upon the sample analytes hydrophilicity range) with the three ACE HILIC phases and different eluent pH values, before optimising the chromatography to produce an acceptable HILIC method.

The ACE HILIC screening conditions can be seen in table 1 (page 26). These have been devised to explore a wide selectivity range and offer a good starting point to achieve the required separation.

Figure 17

ACE HILIC method development flow diagram



(Flow diagram available separately at www.ace-hplc.com and as part of a FREE ACE HILIC wallchart/poster – contact your local dealer or request from info@ace-hplc.com)

Table 1

Conditions for ACE HILIC Screening Experiments

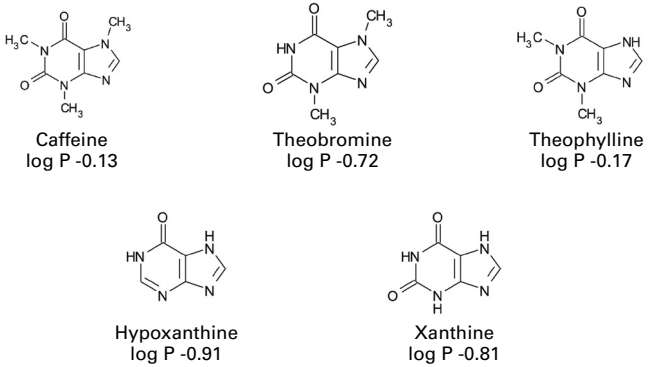
Parameter	Comments	
Column	ACE HILIC-A, ACE HILIC-B and ACE HILIC-N, 150 x 4.6 mm, 5 µm	
Gradient mobile phase	A: 10 mM ammonium formate in MeCN/H_2O (94:6 v/v) B: 10 mM ammonium formate in MeCN/H_2O (50:50 v/v). Ammonium formate is at pH 3.0, 4.7 or 6.0.	
Gradient screen	**Time**	**%B**
	0	0
	15	100
	20	100
	21	0
	41	0
Isocratic mobile phase	10 mM ammonium formate in MeCN/H_2O (90:10 v/v). Ammonium formate is at pH 3.0, 4.7 or 6.0.	
Flow rate	1.5 mL/min	
Temperature	25 °C	
Detection	Dependent on sample	

10.1 Example 1 – Caffeine and Related Compounds

Caffeine and four related compounds (theobromine, theophylline, hypoxanthine and xanthine) required method development. These compounds are all polar neutral species, with negative log P values indicating reasonable hydrophilicity and therefore are suitable for HILIC (figure 18).

Figure 18

Structure and log P data for caffeine and related substances



Caffeine and the related compounds are not ionisable at pH 3-6 and therefore the eluent pH will have little effect on the molecules directly. For this reason, pH 3.0 and 4.7 were selected. The stationary phase can be affected by the change in eluent pH, which is possibly advantageous. The change

in ionisation of the stationary phase will affect the hydration layer surrounding the particles, which will affect the degree to which analytes partition into the phase or form hydrogen bonds. Therefore, all three stationary phases were screened at pH 3.0 and pH 4.7, with the results seen in figure 19.

The new ACE HILIC columns were each equilibrated using 60 column volumes to establish the HILIC hydration layer around the particles. The mobile phase, gradient and temperature shown in table 1 were used.

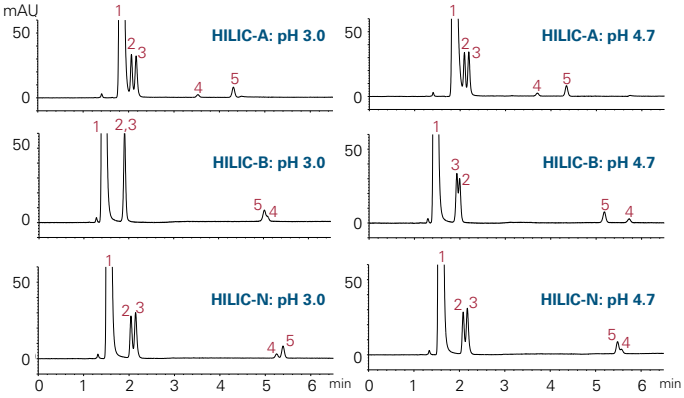
The screening results show there are some selectivity differences observed between the three stationary phases and two pH values. The most promising separation based upon the screening data is on the ACE HILIC-N at pH 3.0. These conditions were selected for further optimisation.

Figure 19

Gradient screens on ACE HILIC columns

Conditions as described in table 1 apart from detection at 275 nm. 2 µL injections of 25 mg/mL caffeine mixture (with the related substances spiked at 0.5% w/w in MeCN/H_2O (90:10 v/v) using pH 3.0 and pH 4.7 ammonium formate.

Sample:
1) Caffeine
2) Theophylline
3) Theobromine
4) Xanthine
5) Hypoxanthine



The retention window for the early eluting peaks is quite narrow, which suggested isocratic HILIC could be used to separate the analytes. 10 mM ammonium formate pH 3.0 in MeCN/H_2O (94:6 v/v) was selected as the isocratic conditions (figure 20). Peaks 4 and 5 were much greater retained under these conditions, however, peaks 2 and 3 were still unresolved. Based on the results of the gradient run, it appears a higher acetonitrile content would not improve resolution of peaks 2 and 3 so gradient HILIC analysis was progressed and temperature explored leading to a final method which can be seen in figure 21.

Figure 20

Isocratic analysis of caffeine and related compounds on the ACE HILIC-N

Column: ACE 5 HILIC-N, 150 x 4.6 mm

Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O (94:6 v/v)

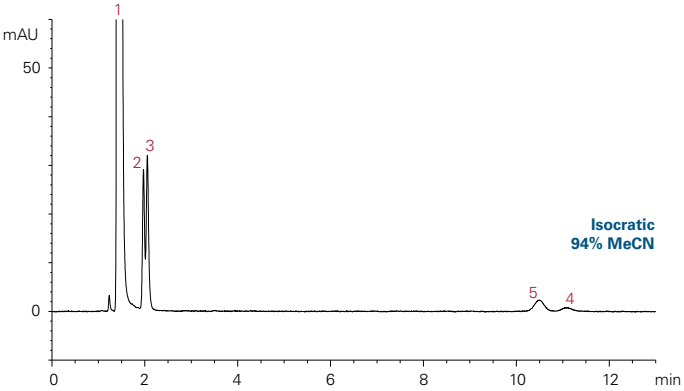
Flow rate: 1.5 mL/min

Temperature: 25 °C

Detection: UV, 275 nm

Injection: 2 µL

Sample:
1) Caffeine
2) Theophylline
3) Theobromine
4) Xanthine
5) Hypoxanthine



A decrease in temperature was found to improve the resolution between theophylline and theobromine, therefore the final method (figure 21) was considered fit for purpose.

Figure 21

Final developed method:

Column: ACE 5 HILIC-N, 150 x 4.6 mm

Mobile phase: A = 10 mM ammonium formate pH 3.0 in MeCN/H_2O (96:4 v/v)
B = 10 mM ammonium formate pH 3.0 in MeCN/H_2O (1:1 v/v)

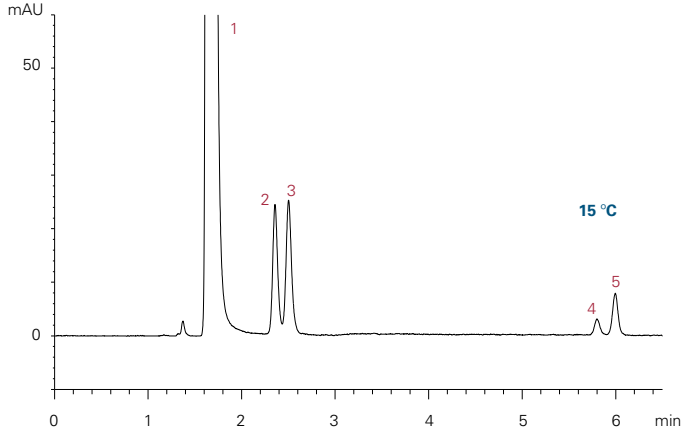
Gradient: 0-100 %B in 15 mins, 100 %B for 5 mins, 20 min hold at starting conditions for next injection

Flow rate: 1.5 mL/min

Temperature: 15 °C

Detection: 275 nm

Injection: 2 µL

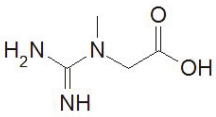


10.2 Example 2 – Creatine and Creatinine

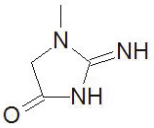
Creatine (figure 22) is an amino acid synthesised using glycine and arginine. It plays a major role in supplying energy to cells in the body, and forms the by-product creatinine. Creatinine is measured in the blood to determine if the kidneys are functioning correctly, where an increased level of creatinine indicates the kidneys might not be filtering waste products sufficiently.

Figure 22

Structures and log P data for creatine and creatinine



Creatine log P -1.88



Creatinine log P -1.83

Figure 23

Isocratic screen comparisons on the ACE HILIC range using pH 3.0, pH 4.7 and pH 6.0 ammonium formate

Sample:
1) Creatinine
2) Creatine

The two analytes were screened using isocratic conditions on the three HILIC stationary phases at all three pH values as described in table 1 (page 26). The results (figure 23) indicate that creatinine is suitably retained on all three ACE HILIC phases, however creatine did not elute within a reasonable timeframe due to excessive retention. From the flow diagram in figure 17, an excessive retention window suggests a gradient might be more appropriate.

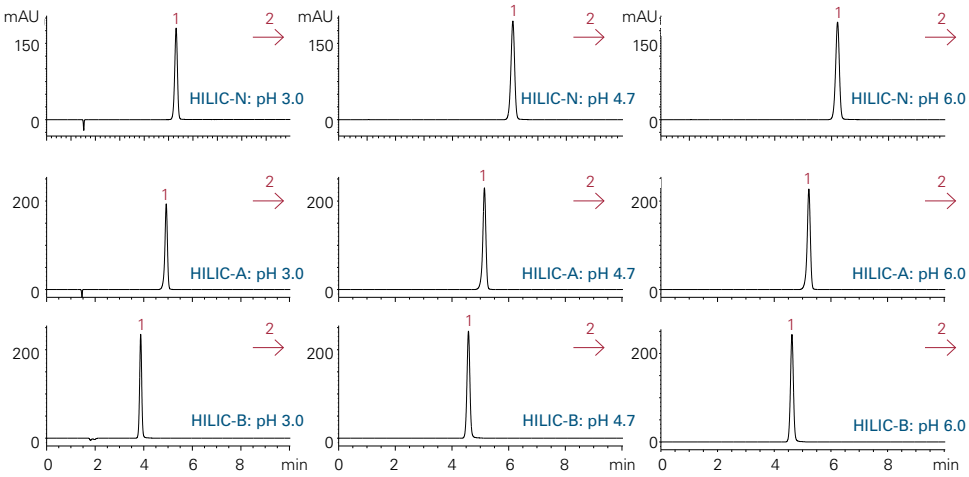


Figure 24

Final method with the ACE HILIC-A

Column: 150 x 4.6 mm, 5 µm

Mobile phase:
A: 2 mM ammonium formate pH 3.0 in MeCN/H_2O (90:10 v/v)

B: 2 mM ammonium formate pH 3.0 in MeCN/H_2O (50:50 v/v)

Gradient: 5-55 %B in 10 mins

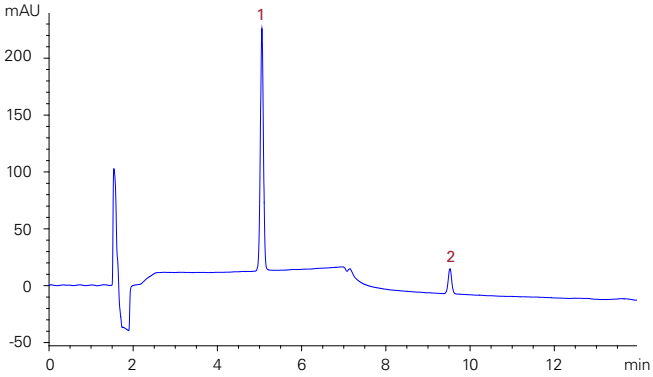
Flow rate: 1.5 mL/min

Detection: 230 nm

Injection: 5 µL

Sample:
1) Creatinine
2) Creatine

The ACE HILIC-A was selected at pH 3.0 to proceed with gradient analysis. The standard gradient eluted both analytes of interest within 10 minutes with excellent resolution (data not shown). Therefore, this allowed a further reduction of the gradient time to speed up the overall run time. The final method can be seen in figure 24.



11.0 CONCLUSION

HILIC is a versatile mode of chromatography for polar analytes. Mechanistically, it can be considered complex, but reproducible HILIC methods are possible if simple rules are followed. The three ACE HILIC phases have been designed to explore selectivity during HILIC method development and provide options to achieve the desired separation as rapidly as possible. The ACE HILIC method development protocol has been successfully used to develop a number of HILIC methods and should provide a structured approach to HILIC method development activities.

For further advice and technical information please email: info@ace-hplc.com

12.0 APPENDIX 1

Column volume explained

Column volume is defined as the volume within an LC column occupied by eluent (i.e. between particles and within the pores of particles). Column volume can be calculated according to:

$$V_m = \pi r^2 L \varepsilon$$

Where: V_m = column volume in mL r = column radius in cm
L = column length in cm ε = bed porosity value

The bed porosity value, ε, is dependent upon particle parameters (eg pore size, surface area etc) and vendor. For ACE 100 Å porous columns, the ε value is ~0.63; for ACE 300 Å porous columns, the ε value is ~0.75; whilst for ACE 90 Å solid core columns, the ε value is ~0.55.

When discussing column equilibration it is far more accurate to refer to column volumes required to reach a steady state, as it is independent of flow rate and column dimension. As an example, table 1a below shows the calculation of column volume values for a range of ACE 100 Å porous columns.

		Column Length (mm)						
		20	30	50	75	100	150	250
Column id (mm)	1.0	0.010	0.015	0.025	0.037	0.049	0.074	0.124
	2.1	0.044	0.065	0.109	0.164	0.218	0.327	0.546
	3.0	0.089	0.134	0.223	0.334	0.445	0.668	1.113
	4.6	0.209	0.314	0.523	0.785	1.047	1.570	2.617

Table 1a: Calculated ACE 100 Å porous column volumes in mL

Table 2a shows the time required at various flow rates to achieve 20 and 60 column volume equilibration for 4 different ACE 100 Å porous column dimensions.

Column Dimensions and Particle Size	Column Volume (mL)	Flow Rate (mL/min)	20 Column Volumes Equilibration Time (mins)	60 Column Volumes Equilibration Time For New Column (mins)
50 x 2.1 mm, 1.7 µm	0.109	0.21	10.4	31.2
50 x 2.1 mm, 1.7 µm	0.109	0.50	4.4	13.1
100 x 3.0 mm, 3 µm	0.445	1.00	8.9	26.7
150 x 4.6 mm, 5 µm	1.570	1.00	31.4	94.2
150 x 4.6 mm, 5 µm	1.570	2.00	15.7	47.1
250 x 4.6 mm, 5 µm	2.617	1.00	52.3	157.0
250 x 4.6 mm, 5 µm	2.617	2.00	26.2	78.5

Table 2a: ACE 100 Å porous columns equilibration calculations

13.0 PART NUMBERS

ACE HILIC UHPLC/HPLC and Microbore Columns Part Numbers

Matched guard cartridges (for HPLC columns) or prefilters (for UHPLC columns) are available – please enquire.

ACE HILIC Method Development Kits

(Contains 3 HILIC columns: ACE HILIC-A, ACE HILIC-B and ACE HILIC-N of specified dimensions)

(UHPLC/HPLC hardware format with 1000 bar/15000 psi pressure limit)			
Column Dimensions	**1.7 µm**	**3 µm**	**5 µm**
2.1 x 20 mm	MDKH-17-0202U	MDKH-3-0202U	MDKH-5-0202U
2.1 x 30 mm	MDKH-17-0302U	MDKH-3-0302U	MDKH-5-0302U
2.1 x 35 mm	MDKH-17-3502U	MDKH-3-3502U	MDKH-5-3502U
2.1 x 50 mm	MDKH-17-0502U	MDKH-3-0502U	MDKH-5-0502U
2.1 x 75 mm	MDKH-17-7502U	MDKH-3-7502U	MDKH-5-7502U
2.1 x 100 mm	MDKH-17-1002U	MDKH-3-1002U	MDKH-5-1002U
2.1 x 125 mm	-	MDKH-3-1202U	MDKH-5-1202U
2.1 x 150 mm	-	MDKH-3-1502U	MDKH-5-1502U
2.1 x 250 mm	-	MDKH-3-2502U	MDKH-5-2502U
3.0 x 20 mm	MDKH-17-0203U	MDKH-3-0203U	MDKH-5-0203U
3.0 x 30 mm	MDKH-17-0303U	MDKH-3-0303U	MDKH-5-0303U
3.0 x 35 mm	MDKH-17-3503U	MDKH-3-3503U	MDKH-5-3503U
3.0 x 50 mm	MDKH-17-0503U	MDKH-3-0503U	MDKH-5-0503U
3.0 x 75 mm	MDKH-17-7503U	MDKH-3-7503U	MDKH-5-7503U
3.0 x 100 mm	MDKH-17-1003U	MDKH-3-1003U	MDKH-5-1003U
3.0 x 125 mm	-	MDKH-3-1203U	MDKH-5-1203U
3.0 x 150 mm	-	MDKH-3-1503U	MDKH-5-1503U
3.0 x 250 mm	-	MDKH-3-2503U	MDKH-5-2503U
4.6 x 20 mm	-	MDKH-3-0246U	MDKH-5-0246U
4.6 x 30 mm	-	MDKH-3-0346U	MDKH-5-0346U
4.6 x 35 mm	-	MDKH-3-3546U	MDKH-5-3546U
4.6 x 50 mm	-	MDKH-3-0546U	MDKH-5-0546U
4.6 x 75 mm	-	MDKH-3-7546U	MDKH-5-7546U
4.6 x 100 mm	-	MDKH-3-1046U	MDKH-5-1046U
4.6 x 125 mm	-	MDKH-3-1246U	MDKH-5-1246U
4.6 x 150 mm	-	MDKH-3-1546U	MDKH-5-1546U
4.6 x 250 mm	-	MDKH-3-2546U	MDKH-5-2546U

ACE HILIC Microbore Method Development Kits

(Contains 3 HILIC Microbore columns: ACE HILIC-A, ACE HILIC-B and ACE HILIC-N of specified dimensions)

(HPLC hardware format with 400 bar/6000 psi recommended pressure limit)				
Column Dimensions	3 µm		5 µm	
	1/16" port	1/32" port	1/16" port	1/32" port
0.5 x 30 mm	MDKH-3-03005	MDKH-3-03005S	MDKH-5-03005	MDKH-5-03005S
0.5 x 50 mm	MDKH-3-05005	MDKH-3-05005S	MDKH-5-05005	MDKH-5-05005S
0.5 x 75 mm	MDKH-3-75005	MDKH-3-75005S	MDKH-5-75005	MDKH-5-75005S
0.5 x 100 mm	MDKH-3-10005	MDKH-3-10005S	MDKH-5-10005	MDKH-5-10005S
0.5 x 125 mm	MDKH-3-12005	MDKH-3-12005S	MDKH-5-12005	MDKH-5-12005S
0.5 x 150 mm	MDKH-3-15005	MDKH-3-15005S	MDKH-5-15005	MDKH-5-15005S
0.5 x 250 mm	-	-	MDKH-5-25005	MDKH-5-25005S
1.0 x 30 mm	MDKH-3-0301	MDKH-3-0301S	MDKH-5-0301	MDKH-5-0301S
1.0 x 50 mm	MDKH-3-0501	MDKH-3-0501S	MDKH-5-0501	MDKH-5-0501S
1.0 x 75 mm	MDKH-3-7501	MDKH-3-7501S	MDKH-5-7501	MDKH-5-7501S
1.0 x 100 mm	MDKH-3-1001	MDKH-3-1001S	MDKH-5-1001	MDKH-5-1001S
1.0 x 125 mm	MDKH-3-1201	MDKH-3-1201S	MDKH-5-1201	MDKH-5-1201S
1.0 x 150 mm	MDKH-3-1501	MDKH-3-1501S	MDKH-5-1501	MDKH-5-1501S
1.0 x 250 mm	-	-	MDKH-5-2501	MDKH-5-2501S

ACE HILIC-A Columns

(Individual HILIC Columns of specified dimensions)

(UHPLC/HPLC hardware format with 1000 bar/15000 psi pressure limit)			
Column Dimensions	**1.7 µm**	**3 µm**	**5 µm**
2.1 x 20 mm	HILA-17-0202U	HILA-3-0202U	HILA-5-0202U
2.1 x 30 mm	HILA-17-0302U	HILA-3-0302U	HILA-5-0302U
2.1 x 35 mm	HILA-17-3502U	HILA-3-3502U	HILA-5-3502U
2.1 x 50 mm	HILA-17-0502U	HILA-3-0502U	HILA-5-0502U
2.1 x 75 mm	HILA-17-7502U	HILA-3-7502U	HILA-5-7502U
2.1 x 100 mm	HILA-17-1002U	HILA-3-1002U	HILA-5-1002U
2.1 x 125 mm	-	HILA-3-1202U	HILA-5-1202U
2.1 x 150 mm	-	HILA-3-1502U	HILA-5-1502U
2.1 x 250 mm	-	HILA-3-2502U	HILA-5-2502U
3.0 x 20 mm	HILA-17-0203U	HILA-3-0203U	HILA-5-0203U
3.0 x 30 mm	HILA-17-0303U	HILA-3-0303U	HILA-5-0303U
3.0 x 35 mm	HILA-17-3503U	HILA-3-3503U	HILA-5-3503U
3.0 x 50 mm	HILA-17-0503U	HILA-3-0503U	HILA-5-0503U
3.0 x 75 mm	HILA-17-7503U	HILA-3-7503U	HILA-5-7503U
3.0 x 100 mm	HILA-17-1003U	HILA-3-1003U	HILA-5-1003U
3.0 x 125 mm	-	HILA-3-1203U	HILA-5-1203U
3.0 x 150 mm	-	HILA-3-1503U	HILA-5-1503U
3.0 x 250 mm	-	HILA-3-2503U	HILA-5-2503U
4.6 x 20 mm	-	HILA-3-0246U	HILA-5-0246U
4.6 x 30 mm	-	HILA-3-0346U	HILA-5-0346U
4.6 x 35 mm	-	HILA-3-3546U	HILA-5-3546U
4.6 x 50 mm	-	HILA-3-0546U	HILA-5-0546U
4.6 x 75 mm	-	HILA-3-7546U	HILA-5-7546U
4.6 x 100 mm	-	HILA-3-1046U	HILA-5-1046U
4.6 x 125 mm	-	HILA-3-1246U	HILA-5-1246U
4.6 x 150 mm	-	HILA-3-1546U	HILA-5-1546U
4.6 x 250 mm	-	HILA-3-2546U	HILA-5-2546U

ACE HILIC-A Microbore Columns

(Individual HILIC Microbore Columns of specified dimensions)

(HPLC hardware format with 400 bar/6000 psi recommended pressure limit)				
Column Dimensions	3 µm		5 µm	
	1/16" port	1/32" port	1/16" port	1/32" port
0.5 x 30 mm	HILA-3-03005	HILA-3-03005S	HILA-5-03005	HILA-5-03005S
0.5 x 50 mm	HILA-3-05005	HILA-3-05005S	HILA-5-05005	HILA-5-05005S
0.5 x 75 mm	HILA-3-75005	HILA-3-75005S	HILA-5-75005	HILA-5-75005S
0.5 x 100 mm	HILA-3-10005	HILA-3-10005S	HILA-5-10005	HILA-5-10005S
0.5 x 125 mm	HILA-3-12005	HILA-3-12005S	HILA-5-12005	HILA-5-12005S
0.5 x 150 mm	HILA-3-15005	HILA-3-15005S	HILA-5-15005	HILA-5-15005S
0.5 x 250 mm	-	-	HILA-5-25005	HILA-5-25005S
1.0 x 30 mm	HILA-3-0301	HILA-3-0301S	HILA-5-0301	HILA-5-0301S
1.0 x 50 mm	HILA-3-0501	HILA-3-0501S	HILA-5-0501	HILA-5-0501S
1.0 x 75 mm	HILA-3-7501	HILA-3-7501S	HILA-5-7501	HILA-5-7501S
1.0 x 100 mm	HILA-3-1001	HILA-3-1001S	HILA-5-1001	HILA-5-1001S
1.0 x 125 mm	HILA-3-1201	HILA-3-1201S	HILA-5-1201	HILA-5-1201S
1.0 x 150 mm	HILA-3-1501	HILA-3-1501S	HILA-5-1501	HILA-5-1501S
1.0 x 250 mm	-	-	HILA-5-2501	HILA-5-2501S

ACE HILIC-B Columns

(Individual HILIC Columns of specified dimensions)

(UHPLC/HPLC hardware format with 1000 bar/15000 psi pressure limit)			
Column Dimensions	**1.7 µm**	**3 µm**	**5 µm**
2.1 x 20 mm	HILB-17-0202U	HILB-3-0202U	HILB-5-0202U
2.1 x 30 mm	HILB-17-0302U	HILB-3-0302U	HILB-5-0302U
2.1 x 35 mm	HILB-17-3502U	HILB-3-3502U	HILB-5-3502U
2.1 x 50 mm	HILB-17-0502U	HILB-3-0502U	HILB-5-0502U
2.1 x 75 mm	HILB-17-7502U	HILB-3-7502U	HILB-5-7502U
2.1 x 100 mm	HILB-17-1002U	HILB-3-1002U	HILB-5-1002U
2.1 x 125 mm	-	HILB-3-1202U	HILB-5-1202U
2.1 x 150 mm	-	HILB-3-1502U	HILB-5-1502U
2.1 x 250 mm	-	HILB-3-2502U	HILB-5-2502U
3.0 x 20 mm	HILB-17-0203U	HILB-3-0203U	HILB-5-0203U
3.0 x 30 mm	HILB-17-0303U	HILB-3-0303U	HILB-5-0303U
3.0 x 35 mm	HILB-17-3503U	HILB-3-3503U	HILB-5-3503U
3.0 x 50 mm	HILB-17-0503U	HILB-3-0503U	HILB-5-0503U
3.0 x 75 mm	HILB-17-7503U	HILB-3-7503U	HILB-5-7503U
3.0 x 100 mm	HILB-17-1003U	HILB-3-1003U	HILB-5-1003U
3.0 x 125 mm	-	HILB-3-1203U	HILB-5-1203U
3.0 x 150 mm	-	HILB-3-1503U	HILB-5-1503U
3.0 x 250 mm	-	HILB-3-2503U	HILB-5-2503U
4.6 x 20 mm	-	HILB-3-0246U	HILB-5-0246U
4.6 x 30 mm	-	HILB-3-0346U	HILB-5-0346U
4.6 x 35 mm	-	HILB-3-3546U	HILB-5-3546U
4.6 x 50 mm	-	HILB-3-0546U	HILB-5-0546U
4.6 x 75 mm	-	HILB-3-7546U	HILB-5-7546U
4.6 x 100 mm	-	HILB-3-1046U	HILB-5-1046U
4.6 x 125 mm	-	HILB-3-1246U	HILB-5-1246U
4.6 x 150 mm	-	HILB-3-1546U	HILB-5-1546U
4.6 x 250 mm	-	HILB-3-2546U	HILB-5-2546U

ACE HILIC-B Microbore Columns

(Individual HILIC Microbore Columns of specified dimensions)

(HPLC hardware format with 400 bar/6000 psi recommended pressure limit)				
Column Dimensions	**3 µm**		**5 µm**	
	1/16″ port	**1/32″ port**	**1/16″ port**	**1/32″ port**
0.5 x 30 mm	HILB-3-03005	HILB-3-03005S	HILB-5-03005	HILB-5-03005S
0.5 x 50 mm	HILB-3-05005	HILB-3-05005S	HILB-5-05005	HILB-5-05005S
0.5 x 75 mm	HILB-3-75005	HILB-3-75005S	HILB-5-75005	HILB-5-75005S
0.5 x 100 mm	HILB-3-10005	HILB-3-10005S	HILB-5-10005	HILB-5-10005S
0.5 x 125 mm	HILB-3-12005	HILB-3-12005S	HILB-5-12005	HILB-5-12005S
0.5 x 150 mm	HILB-3-15005	HILB-3-15005S	HILB-5-15005	HILB-5-15005S
0.5 x 250 mm	-	-	HILB-5-25005	HILB-5-25005S
1.0 x 30 mm	HILB-3-0301	HILB-3-0301S	HILB-5-0301	HILB-5-0301S
1.0 x 50 mm	HILB-3-0501	HILB-3-0501S	HILB-5-0501	HILB-5-0501S
1.0 x 75 mm	HILB-3-7501	HILB-3-7501S	HILB-5-7501	HILB-5-7501S
1.0 x 100 mm	HILB-3-1001	HILB-3-1001S	HILB-5-1001	HILB-5-1001S
1.0 x 125 mm	HILB-3-1201	HILB-3-1201S	HILB-5-1201	HILB-5-1201S
1.0 x 150 mm	HILB-3-1501	HILB-3-1501S	HILB-5-1501	HILB-5-1501S
1.0 x 250 mm	-	-	HILB-5-2501	HILB-5-2501S

ACE HILIC-N Columns

(Individual HILIC Columns of specified dimensions)

(UHPLC/HPLC hardware format with 1000 bar/15000 psi pressure limit)			
Column Dimensions	**1.7 µm**	**3 µm**	**5 µm**
2.1 x 20 mm	HILN-17-0202U	HILN-3-0202U	HILN-5-0202U
2.1 x 30 mm	HILN-17-0302U	HILN-3-0302U	HILN-5-0302U
2.1 x 35 mm	HILN-17-3502U	HILN-3-3502U	HILN-5-3502U
2.1 x 50 mm	HILN-17-0502U	HILN-3-0502U	HILN-5-0502U
2.1 x 75 mm	HILN-17-7502U	HILN-3-7502U	HILN-5-7502U
2.1 x 100 mm	HILN-17-1002U	HILN-3-1002U	HILN-5-1002U
2.1 x 125 mm	-	HILN-3-1202U	HILN-5-1202U
2.1 x 150 mm	-	HILN-3-1502U	HILN-5-1502U
2.1 x 250 mm	-	HILN-3-2502U	HILN-5-2502U
3.0 x 20 mm	HILN-17-0203U	HILN-3-0203U	HILN-5-0203U
3.0 x 30 mm	HILN-17-0303U	HILN-3-0303U	HILN-5-0303U
3.0 x 35 mm	HILN-17-3503U	HILN-3-3503U	HILN-5-3503U
3.0 x 50 mm	HILN-17-0503U	HILN-3-0503U	HILN-5-0503U
3.0 x 75 mm	HILN-17-7503U	HILN-3-7503U	HILN-5-7503U
3.0 x 100 mm	HILN-17-1003U	HILN-3-1003U	HILN-5-1003U
3.0 x 125 mm	-	HILN-3-1203U	HILN-5-1203U
3.0 x 150 mm	-	HILN-3-1503U	HILN-5-1503U
3.0 x 250 mm	-	HILN-3-2503U	HILN-5-2503U
4.6 x 20 mm	-	HILN-3-0246U	HILN-5-0246U
4.6 x 30 mm	-	HILN-3-0346U	HILN-5-0346U
4.6 x 35 mm	-	HILN-3-3546U	HILN-5-3546U
4.6 x 50 mm	-	HILN-3-0546U	HILN-5-0546U
4.6 x 75 mm	-	HILN-3-7546U	HILN-5-7546U
4.6 x 100 mm	-	HILN-3-1046U	HILN-5-1046U
4.6 x 125 mm	-	HILN-3-1246U	HILN-5-1246U
4.6 x 150 mm	-	HILN-3-1546U	HILN-5-1546U
4.6 x 250 mm	-	HILN-3-2546U	HILN-5-2546U

ACE HILIC-N Microbore Columns

(Individual HILIC Microbore Columns of specified dimensions)

(HPLC hardware format with 400 bar/6000 psi recommended pressure limit)				
Column Dimensions	3 µm		5 µm	
	1/16" port	1/32" port	1/16" port	1/32" port
0.5 x 30 mm	HILN-3-03005	HILN-3-03005S	HILN-5-03005	HILN-5-03005S
0.5 x 50 mm	HILN-3-05005	HILN-3-05005S	HILN-5-05005	HILN-5-05005S
0.5 x 75 mm	HILN-3-75005	HILN-3-75005S	HILN-5-75005	HILN-5-75005S
0.5 x 100 mm	HILN-3-10005	HILN-3-10005S	HILN-5-10005	HILN-5-10005S
0.5 x 125 mm	HILN-3-12005	HILN-3-12005S	HILN-5-12005	HILN-5-12005S
0.5 x 150 mm	HILN-3-15005	HILN-3-15005S	HILN-5-15005	HILN-5-15005S
0.5 x 250 mm	-	-	HILN-5-25005	HILN-5-25005S
1.0 x 30 mm	HILN-3-0301	HILN-3-0301S	HILN-5-0301	HILN-5-0301S
1.0 x 50 mm	HILN-3-0501	HILN-3-0501S	HILN-5-0501	HILN-5-0501S
1.0 x 75 mm	HILN-3-7501	HILN-3-7501S	HILN-5-7501	HILN-5-7501S
1.0 x 100 mm	HILN-3-1001	HILN-3-1001S	HILN-5-1001	HILN-5-1001S
1.0 x 125 mm	HILN-3-1201	HILN-3-1201S	HILN-5-1201	HILN-5-1201S
1.0 x 150 mm	HILN-3-1501	HILN-3-1501S	HILN-5-1501	HILN-5-1501S
1.0 x 250 mm	-	-	HILN-5-2501	HILN-5-2501S

Important Note: ACE microbore columns (1.0 mm id and 0.5 mm id) are available with either standard 1/16" (10-32 thread) connections or 1/32" (6-40 thread) connections. For use with Eksigent micro and nano LC systems, order columns with 1/32" connections and use either ACE 6-40 fittings (part number ACE-MC3210, 10 pack) or Eksigent 6-40 fittings (part number 5019621).

For 1/16" HPLC column connections up to 6000 psi, PEEK™ 1/16" fingertight fittings (part number ACE-CC10, 10 pack) are recommended. For 1/32" microbore HPLC column connections up to 6000 psi, PEEK™ 1/32" (6-40 thread) fingertight fittings (part number ACE-MC3210, 10 pack) are recommended. For 1/16" UHPLC column connections up to 25000 psi, reuseable 1/16" fittings (part number EXL-CC10, 10 pack) are recommended. To further extend UHPLC and HPLC column lifetimes, ACE pre-column filters are recommended. For further details please contact your distributor or visit www.ace-hplc.com

Referances

1. A. J. Alpert, J. Chromatogr., 499 (1990) 177.
2. D. V. McCalley, J. Chromatogr. A, 1171 (2007) 46.
3. J. Heaton, N. Smith, Chromatography Today, May/June (2012) 44.
4. D. V. McCalley, J. Chromatogr. A, 1193 (2008) 85.
5. S. Louw, F. Lynen, M. Hanna-Brown, P. Sandra, J. Chromatogr. A, 1217 (2010) 514.
6. K. J. Fountain, J. Xu, D. M. Diehl, D. Morrison, J. Sep. Sci., 33 (2010) 740.

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