A Simple Step-by-Step Protocol for HILIC Method Development

AKN0021

ACE Knowledge Note #0021

Introduction

The ever increasing demands on chromatographers to rapidly produce fit-for-purpose separations requires more efficient and methodical working processes. Method development workflows are generally well established for reversed phase liquid chromatography. Hydrophillic Interaction Liquid Chromatography (HILIC) is less well understood practically and mechanistically, meaning that the method development process tends to be less systematic and well defined. This ACE Knowledge Note details a simple, rationally designed protocol for HILIC method development using the three ACE HILIC phases.

Method Development

Efficient method development procedures require a logical exploration of key chromatographic parameters leading to identification of a robust method on a suitable column/mobile phase combination. Rationally designed method development procedures assess key parameters which affect chromatographic selectivity (e.g. stationary phase, pH etc.) and enable analysts to make well informed decisions, whilst reducing the risk of developing sub-standard, non-robust methods. By following a step-by-step process, method development can be streamlined thereby increasing laboratory productivity.

The approach outlined here is based on a logical assessment of the most powerful parameters affecting HILIC selectivity.

Understanding Analyte Properties

As a starting point, an understanding of the physico-chemical properties of analytes can be invaluable for selecting appropriate analytical conditions. The logP value of an analyte (octanol – water partition coefficient) allows an appropriate separation mode to be selected. As shown in Figure 1, an analyte with a logP of < 0 is suitable for HILIC, whilst a logP > 0 is more suited to reversed phase. In the region of overlap between the two modes, either could be used and the decision is typically application driven. As a general rule of thumb, if an analyte elutes before caffeine in RPLC (logP ~0), it may be better suited to HILIC mode.

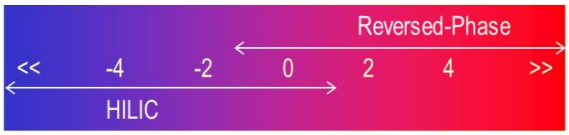


Figure 1: Schematic of logP values and typical chromatographic mode of separation.

Analyte pK_a can be used to determine the ionisation state of a molecule at a given pH. Basic species will be mostly ionised below their pK_a and vice versa for acids. Analyte pK_a can therefore be used to select an appropriate mobile phase pH. For robust method development, it is recommended to work two pH units away from an analyte's pK_a.

Screening Complementary Stationary Phases with Different Selectivity

The HILIC stationary phase is a powerful parameter for influencing selectivity. Column screening is a convenient and commonly applied practice used to identify a suitable column for method development. The ACE HILIC range has been specifically designed to deliver large differences in selectivity, ideal for method development screening. The range consists of acidic (ACE HILIC-A), basic (ACE HILIC-B) and neutral (ACE HILIC-N) phases and are applicable to a wide range of HILIC application areas. Screening a sample on the three HILIC stationary phases is therefore a very effective starting point for method development.

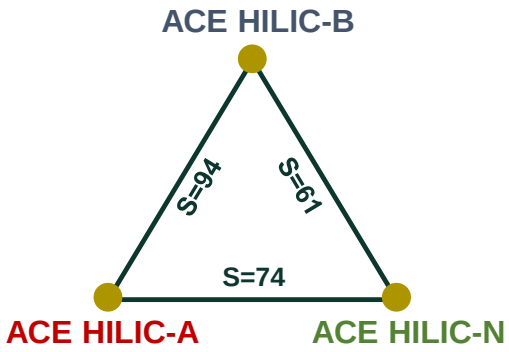


Figure 2: Selectivity triangle for the ACE HILIC range using HILIC probes on 10 mM ammonium formate pH 4.7 MeCN/H_2O (90:10 v/v)

Column selectivity can be experimentally assessed based on the Neue selectivity descriptor (S value). By comparing retention times of specific analyte probes, a correlation coefficient can be produced and used to generate an S value. A value of 0 indicates identical selectivity between phases, whilst a value of 100 denotes complete orthogonality. Selectivity data at pH 4.7 for the three ACE HILIC phases is shown in Figure 2. The high S values demonstrate the orthogonality of these three phases. In practical terms, this means that very different selectivity can be obtained by varying the stationary phase (Figure 3). By screening these three phases, the analyst can quickly assess a wide range of column selectivities and identify a suitable column for their application.

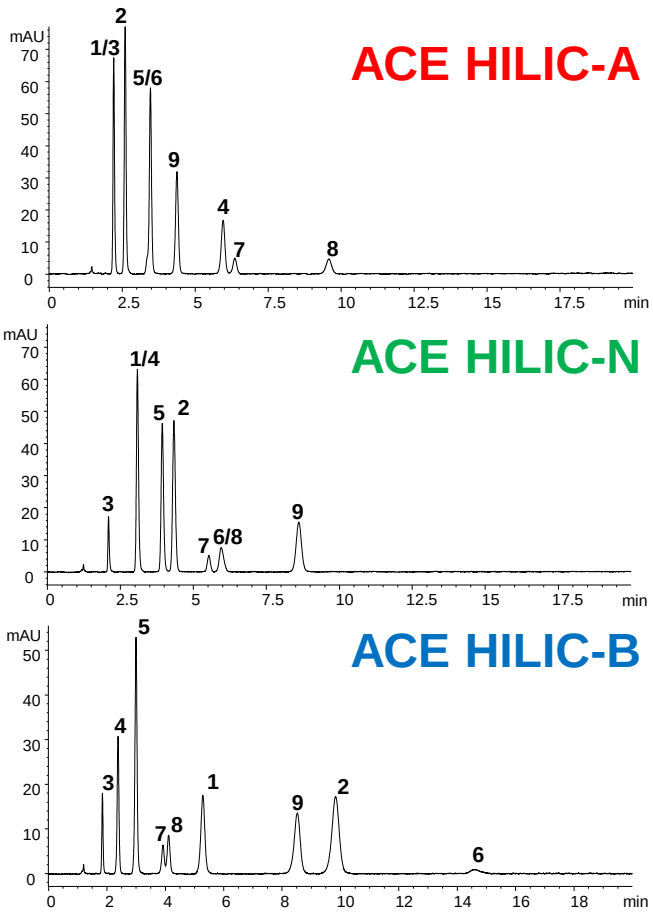


Figure 3: Comparison of elution order on the three ACE HILIC stationary phases. Column format: 150 x 4.6 mm, 5 µm. Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v). Flow rate 1.5 mL/min. Temperature: 25 °C. Detection: UV, 230 nm. Sample: 1. p-aminobenzoic acid, 2. 4-Hydroxybenzoic acid, 3. Nicotinamide, 4. Acebutolol, 5. Adenine, 6. Mandelic acid, 7. Tyramine, 8. Atenolol, 9. 2-Deoxyguanosine.

Selecting Appropriate Mobile Phase Conditions

The predominant retention mechanisms involved in a HILIC separation include partitioning, adsorption, electrostatic interactions and hydrogen bonding. In order for all of these retention mechanisms to operate, an aqueous rich environment must be established around the stationary phase surface. The solvents used for HILIC are similar to those used for reversed phase, with mixtures of acetonitrile and water typically used. Polar solvents such as methanol and IPA have been used as components of the aqueous fraction in order to adjust selectivity. A high proportion of the weaker solvent (usually acetonitrile) is required (>60%) with at least 3% aqueous necessary in order to suitably hydrate the stationary phase.

Mobile phase pH is also a powerful parameter for varying chromatographic selectivity in HILIC mode. For ionisable analytes, the mobile phase pH will determine the degree of ionisation of the analyte and therefore its polarity. In addition, the pH will also affect the polarity of the stationary phase surface, additionally influencing retention mechanisms. This makes mobile phase pH a powerful method development parameter which should be assessed during method development. The screening pH values recommended for HILIC are pH 3.0, 4.7 and 6.0 in order to affect the ionisation of both
the analyte (if acidic or basic) and the stationary phase.

As with reversed phase, it is good practice to buffer the mobile phase when varying pH. Ammonium salts are recommended for use in HILIC due to their solubility in high acetonitrile concentrations and buffering capacity at low pH. Buffer concentrations of 2-18 mM are typically used.

Step-by-Step Rational Method Development

HILIC stationary phase and mobile pH are the two most powerful parameters for altering HILIC selectivity. Assessing these two critical parameters is therefore the optimum starting point for method development. The recommended approach uses stationary and mobile phase screening data to identify a column/mobile phase combination that is most promising for the sample. Once selected, the method can then be fine tuned using other parameters such as buffer strength and temperature.

Figure 4 shows a flow diagram summarising a step-by-step HILIC method development protocol.

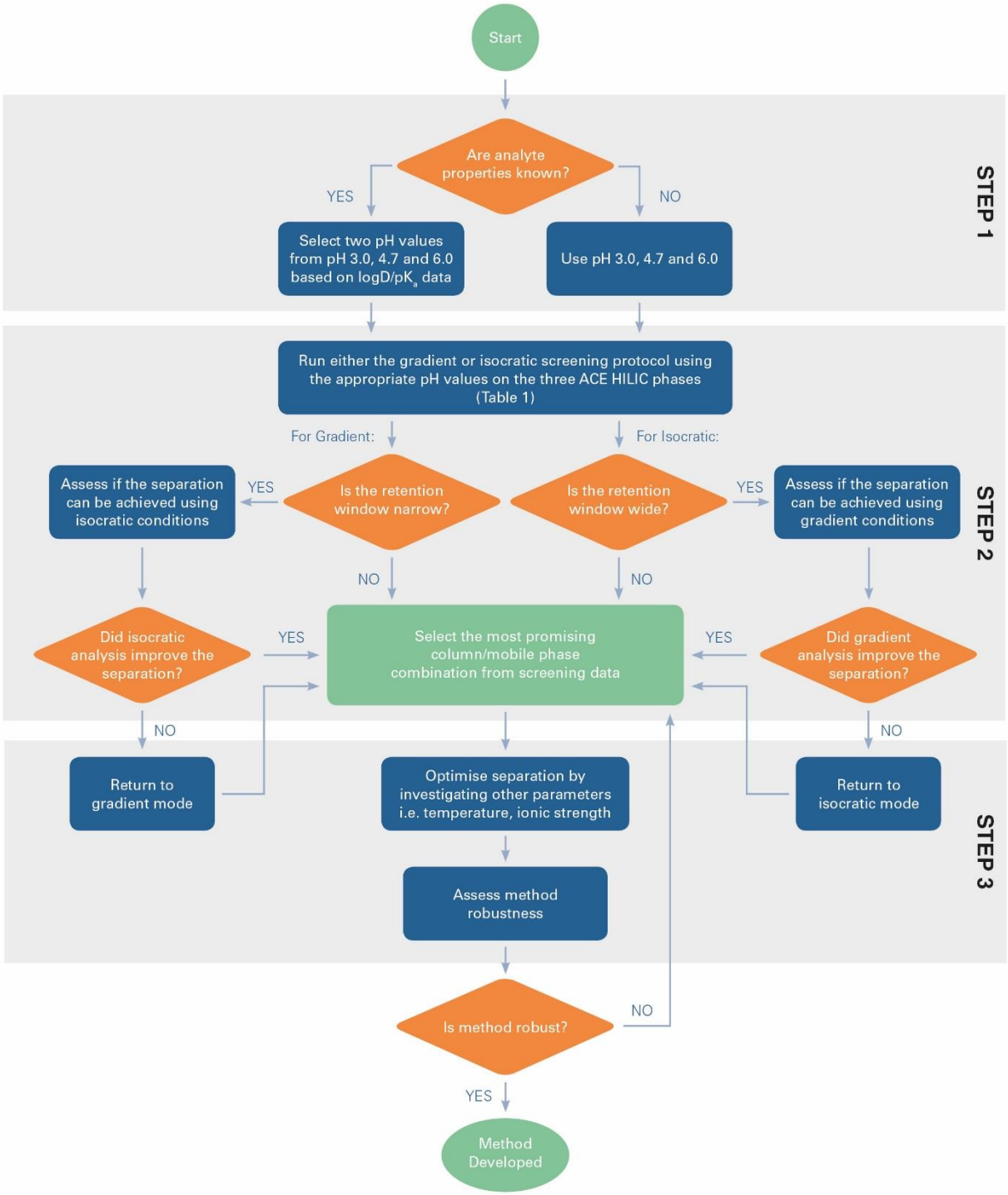


Figure 4: Flow diagram for step-by-step HILIC Method Development protocol.

Step 1: If analyte properties are known, select 2 appropriate mobile phase pH's for screening. If unknown, use pH 3.0. 4.7 and 6.0 (these pH values are designed to maximise selectivity differences).

Step 2: The sample is screened on the three ACE HILIC phases at the specified pH values using either isocratic or gradient conditions specified in Table 1. If retention times are too short or long in isocratic mode, the percentage

of strong solvent (water) may require adjustment. If the retention window is wide leading to excessive resolution (i.e. some analytes show much stronger retention than others) in isocratic mode, a gradient screen should be attempted to assess whether this provides a better option. Likewise, if analyte peaks are clustered too closely in gradient mode, an isocratic separation may be required.

From these data, the stationary phase/mobile phase combination that gives the most promising result is selected for further development.

Step 3: The effects of other parameters such as temperature and buffer concentration can be used to fine-tune the method. Once development is complete, the method robustness can be assessed as required.

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

Table 1: Suggested conditions for HILIC screening

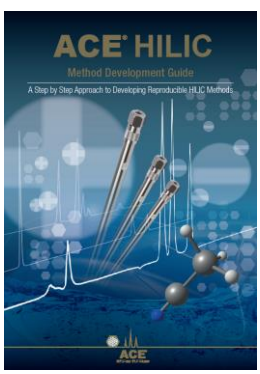
Hints and Tips

It is widely accepted that column equilibration times can be longer in HILIC mode than reversed phase. Many robustness issues can be solved by adequate equilibration of HILIC columns prior to use. During method development screening, it is therefore important to ensure that columns are fully equilibrated when switching between different buffered mobile phases. For HILIC gradient separations, appropriate equilibration between injections is also required (please see ACE Knowledge Note 0023 for further details).

Conclusion

This Knowledge Note has outlined a simple step-by-step process for HILIC method development. The approach demonstrated is based on screening the two most powerful parameters that affect HILIC selectivity, namely the stationary phase and mobile phase pH. The most promising combination can then be logically selected for further method development. Utilising this approach can remove a considerable amount of guess work from method development and help to streamline laboratory processes.

For a more comprehensive discussion of method development topics, please refer to the ACE HILIC Method Development Guide.



The Importance of Sample Diluent in HILIC

AKN0024

ACE Knowledge Note #0024

Introduction

When working with Hydrophilic Interaction Liquid Chromatography (HILIC), the choice of sample diluent can have a profound impact on peak shape. An incorrect choice can lead to poor peak shape, peak splitting and unstable retention times. This ACE Knowledge Note discusses how a suitable choice can be determined during HILIC method development.

Sample Diluent and Peak Shape

Ideally, the sample diluent in HILIC should have a composition as close as possible to that of the mobile phase used for the separation i.e. the diluent should be composed of a high percentage of the weaker solvent, such as acetonitrile. For gradient HILIC chromatography, the sample diluent should be similar to the gradient starting conditions. In some cases, this may present issues with sample solubility as the polar analytes encountered in HILIC may be relatively insoluble in high concentrations of organic solvents.

Sometimes, peak shape problems encountered in HILIC can be resolved by addressing the choice of sample diluent. Samples dissolved in high aqueous diluents are problematic in HILIC as water is the strong solvent, possessing high elution strength. The presence of a large amount of water in the sample diluent therefore disrupts partitioning of the analyte into the water-rich layer which surrounds the HILIC stationary phase surface. This can lead to poor peak shape and shifts in retention, particularly for weakly retained analytes. Figure 1 shows a comparison of the peak shape obtained for 2'-deoxyuridine when injected in mobile phase and 100% water. When the analyte is dissolved in water, a broad, almost split peak is observed. By changing the sample diluent to mobile phase, a dramatic improvement in peak shape and signal intensity is obtained.

The choice of sample diluent in HILIC tends to be application dependant and therefore, resource should be allocated to study the effect of sample diluent during method development. Often the effect of diluent strength can be analyte dependant and can also be influenced by stationary phase and eluent conditions. It is therefore recommended that a stepwise investigation should be carried out to investigate the effect of increasing the percentage of organic solvent on peak shape and method performance. Increments of 10% acetonitrile between 50 and 90% acetonitrile:buffer can help to understand how to achieve the optimum peak shape for target analytes.

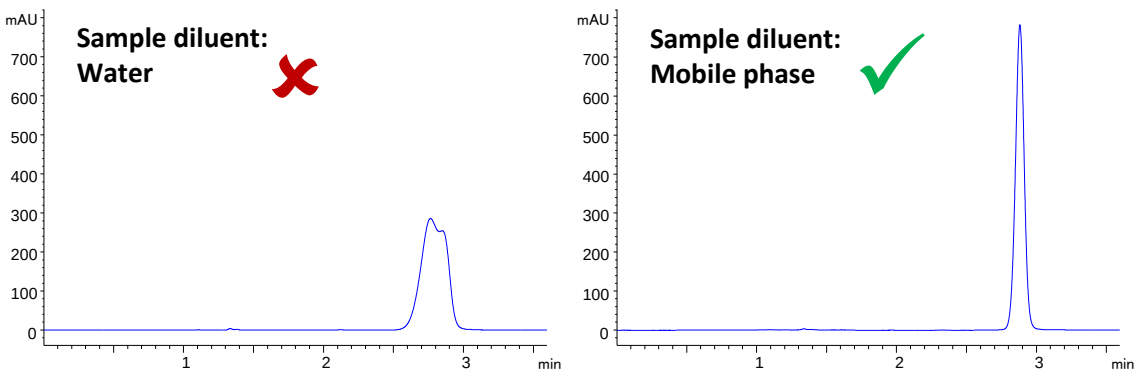


Figure 1: Effect of sample diluent on the peak shape of 2'deoxyuridine.
Column: ACE 5 HILIC-N, 150 x 4.6 mm. Flow rate: 1.5 mL/min.
Mobile phase: 10 mM ammonium formate pH4.7 in MeCN:water 9:1. Injection volume: 5 µL.
Temperature: 25 °C. Detection: UV, 254 nm.

Figure 2 shows the results of a study on the effect of sample diluent for basic, acidic and neutral analytes on the three ACE HILIC phases (ACE HILIC-A, HILIC-B and HILIC-N) at a mobile phase pH of 3.0. The sample diluent was a mixture of acetonitrile and water and the percentage of water was varied systematically between zero and 100%.

The peak shape of hypoxanthine (polar neutral) generally improved with increasing concentration of acetonitrile, with an optimum around 60-80% organic.

Tyramine (basic) gave a split peak on the HILIC-B and HILIC-N phases and poor peak shape on the HILIC-A at low percentage organic. Significant improvements in peak shape were obtained at acetonitrile concentrations greater than 60%. For mandelic acid, the peak shape was less affected by the diluent composition on the HILIC-B and HILIC-N phases, whilst on the HILIC-A, peak shape was poor at higher water concentrations. Overall, from this dataset a sample diluent composition of 60-80% is recommended for the application.

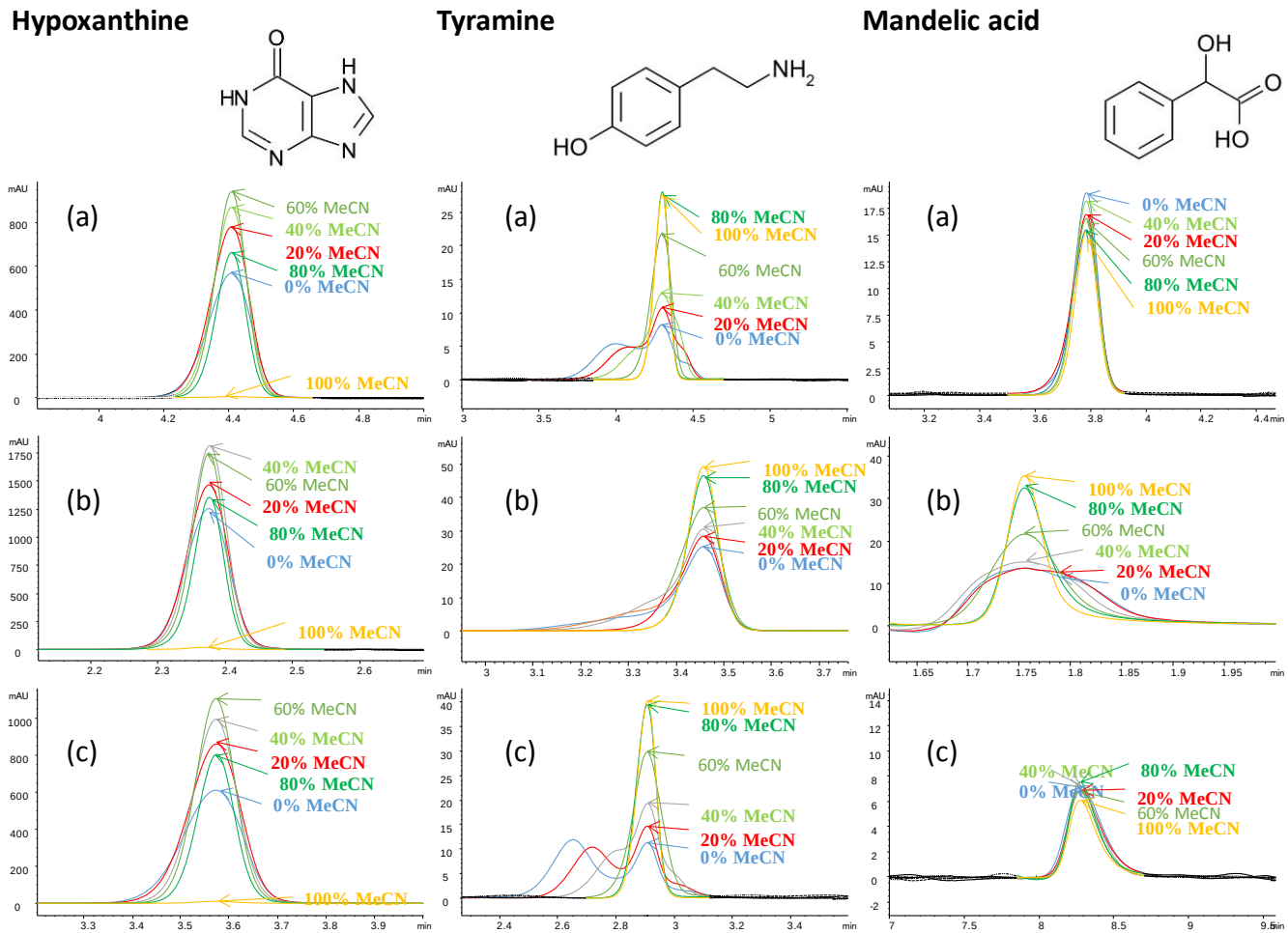


Figure 2: Hypoxanthine, tyramine and mandelic acid on (a) ACE 5 HILIC-N (b) ACE 5 HILIC-A and (c) ACE 5 HILIC-B, 150 x 4.6 mm. Ammonium formate pH 3.0 in MeCN/H_2O (90:10 v/v). Flow rate: 1.5 mL/min. Temperature: 25 °C. Detection: 254 nm. Injection volume: 5 µL.

Conclusion

The choice of sample diluent is important for peak shape and method performance in HILIC. In general, the sample diluent should be composed of as high a percentage of the weaker mobile phase solvent as possible (typically acetonitrile in HILIC). The use of high aqueous sample diluents should be avoided where possible. A systematic assessment of sample diluent for a given application can help to eliminate any undesirable peak distortion effects.

Column Equilibration in HILIC Mode

AKN0025

ACE Knowledge Note #0025

Introduction

In order to obtain stable and reproducible retention times, it is essential to fully equilibrate HILIC columns with mobile phase prior to analysis to ensure a stable adsorbed water layer exists at the stationary phase surface. Similarly, after performing a gradient run, the column requires re-equilibration to the gradient starting conditions and formation of a stable adsorbed water layer.

Equilibration

In order to obtain reproducible retention times in liquid chromatography, it is essential that the column is equilibrated with mobile phase to a steady state. Allowing insufficient time for equilibration in both isocratic and gradient modes is a common source of poorly reproducible separations.

Isocratic

In reversed-phase (RP) LC, at least 10 column volumes of mobile phase should be flushed through the column prior to analysis in order to sufficiently equilibrate the column. Table 1 shows approximate column volumes for popular LC column formats. If elevated temperatures are used, additional time to equilibrate the column temperature may also be necessary.

In HILIC mode, column equilibration time can be somewhat longer. This is because in order to obtain stable and reproducible retention times, a stable hydration layer has to be established and maintained around the silica surface.

The time required for HILIC equilibration can vary between different stationary phases, mobile phases and analytes and is often highly application dependant. Longer equilibration times when using buffered mobile phases have also been noted. To obtain robust HILIC methods it is therefore recommended that column equilibration times are examined and documented during method development to aid in future method transferability.

As a general rule, it is recommended that a newly purchased column is flushed with 60-80 column volumes to fully equilibrate with a new mobile phase (Figure 1). For example, a 100 x 4.6 mm column operated at 1.5 mL/min requires initial equilibration of 42-56 minutes. Once the run is completed, the column should be washed and stored according to the guidelines found on the reverse of the column QC test chromatogram supplied with the column. For subsequent runs, shorter equilibration times of 20 column volumes are sufficient (Figure 2).

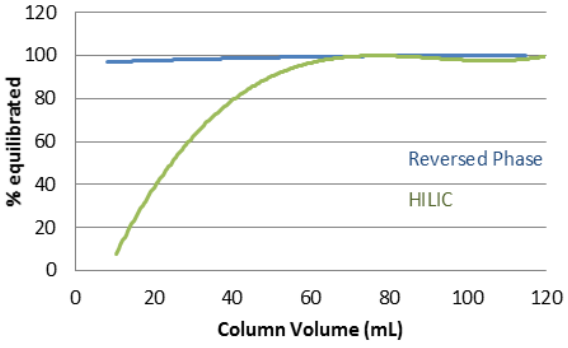


Figure 1: Plot of the number of column volumes required to achieve stable retention time in isocratic reversed-phase (blue) and HILIC (green) modes for brand new, unused columns

Column i.d. (mm) \ Column Length (mm)	50	75	100	125	150	250
1.0	0.025	0.037	0.049	0.062	0.074	0.124
2.1	0.109	0.164	0.218	0.273	0.327	0.546
3.0	0.223	0.334	0.445	0.557	0.668	1.113
4.6	0.523	0.785	1.047	1.309	1.570	2.617

Table 1: Approximate internal volume in millilitres of common LC column formats packed with fully porous particles.

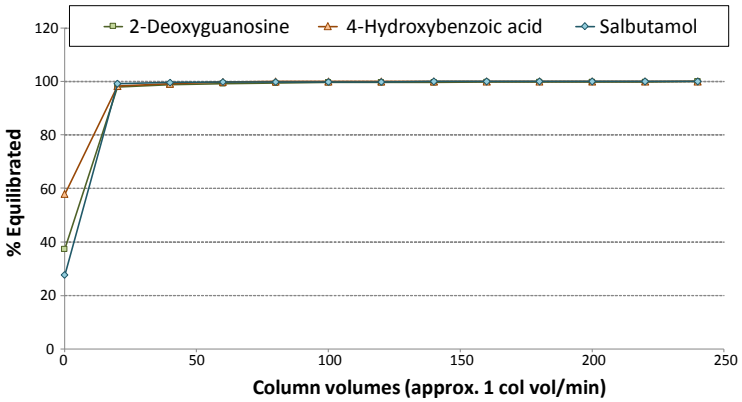


Figure 2: Plot of the number of column volumes required to achieve equilibration for second and subsequent analyses.
Column: ACE 5 HILIC-A, 100 x 3.0 mm, Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H2O (90:10 v/v), Flow rate: 0.43 mL/min, Temperature: 25 °C, Detection: UV 214 nm, Sample: 2-deoxyguanosine, 4-hydroxybenzoic acid, salbutamol.

Gradients

Similar rules apply for gradient HILIC chromatography. A new HILIC column should be equilibrated for at least 60-80 column volumes before the first injection. After performing a gradient analysis, a re-equilibration time of 10 column volumes is usually sufficient to re-establish the gradient starting conditions and obtain robust retention times (Figure 3). Gradient re-equilibration times are typically more critical to reproducible retention times for HILIC gradients than for RP gradients.

It is recommended that the re-equilibration time is thoroughly assessed during method development and then recorded accurately within official method documentation to ensure reliable future use of the method.

When using buffered mobile phases in gradient HILIC mode, it is also advisable to maintain a constant buffer strength throughout the gradient. This is easily achieved by incorporating the same concentration of buffer in both solvent lines. Note that care should be taken to avoid buffer precipitation at high % organic.

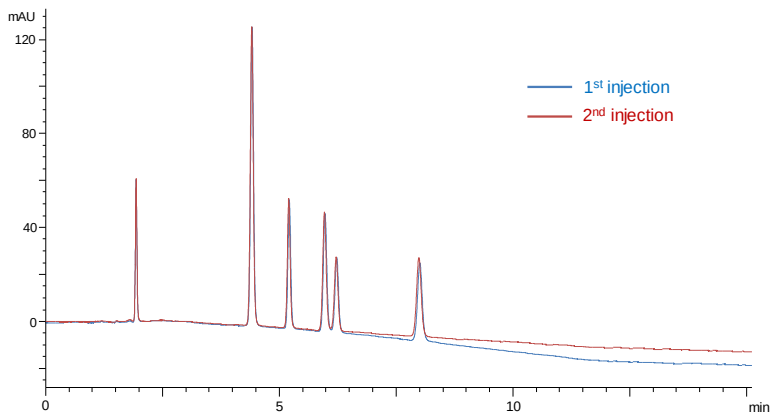


Figure 3: Repeat injections on a HILIC gradient method with re-equilibration time corresponding to 20 column volumes.
Column: ACE 5 HILIC-A, 150 x 4.6 mm, Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O, Gradient: 94 to 70% MeCN in 15 minutes, Flow rate: 1.5 mL/min, Temperature: 25 °C, Detection: 254 nm, Sample: theophylline, hypoxanthine, acebutolol, guanine, cytosine, cytidine.

Conclusion

HILIC methods typically require longer equilibration times than reversed-phase methods to obtain reproducible analyte retention. It is recommended that equilibration time is thoroughly assessed during method development. By following the guidelines outlined in this ACE Knowledge Note, reliable and reproducible HILIC methods can be developed using ACE HILIC columns.