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.

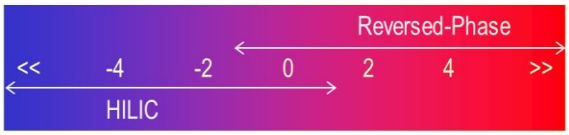

Figure 1: Schematic of logP values and typical chromatographic mode of separation.

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.

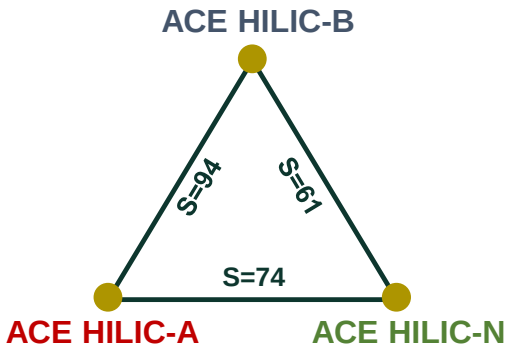

Figure 2: Selectivity triangle for the ACE HILIC range using HILIC probes on 10 mM ammonium formate pH 4.7 MeCN/H_2O (90:10 v/v)

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.

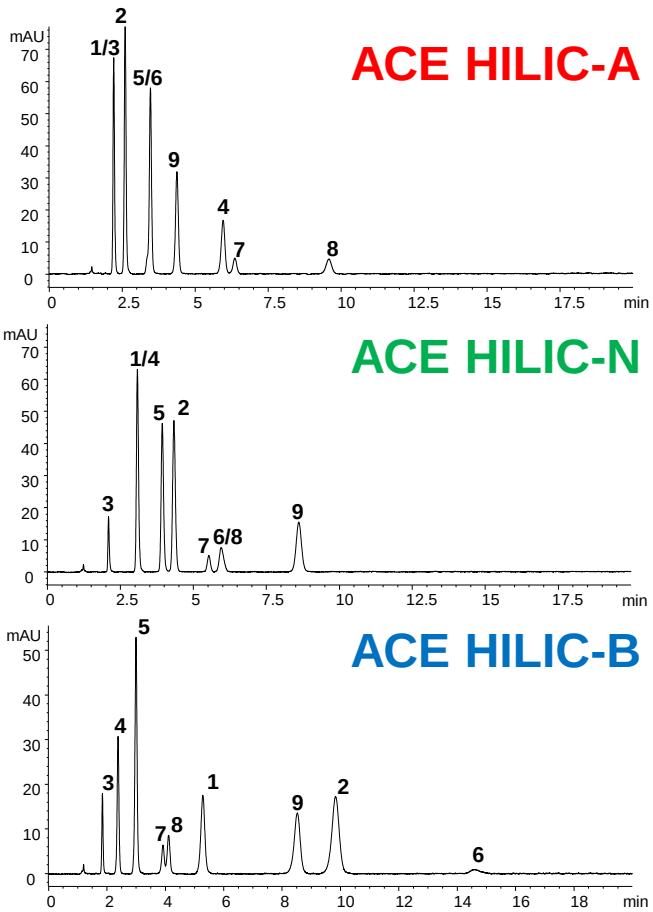

Figure 3: Comparison of elution order on the three ACE HILIC stationary phases. Column format: 150 x 4.6 mm, 5 µm. Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v). Flow rate 1.5 mL/min. Temperature: 25 °C. Detection: UV, 230 nm. Sample: 1. p-aminobenzoic acid, 2. 4-Hydroxybenzoic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

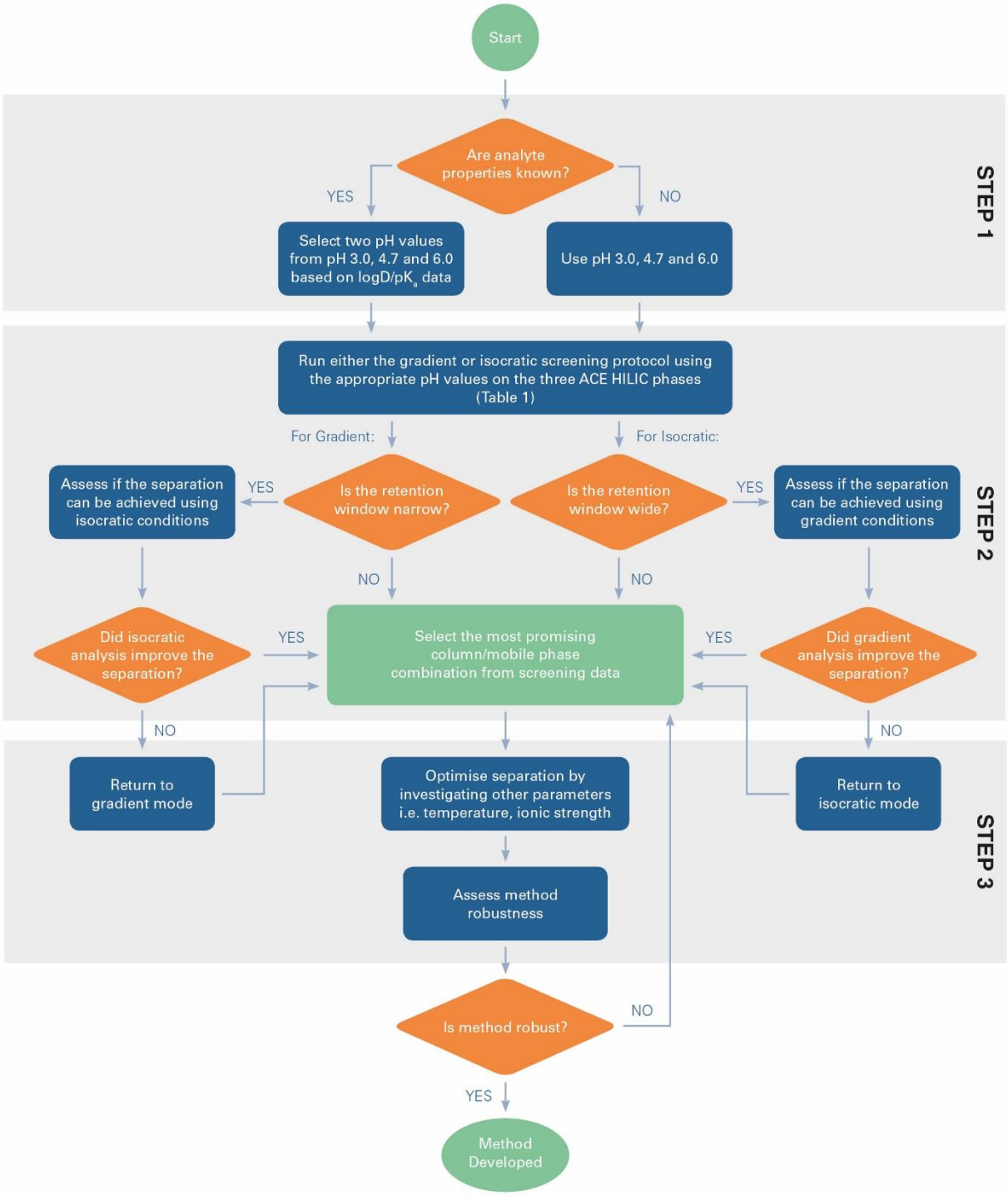

Figure 4: Flow diagram for step-by-step HILIC Method Development protocol.

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.