Method development HILIC white paper

An intelligent solution for hydrophilic interaction liquid chromatography method development

What is Hydrophilic Interaction Liquid Chromatography (HILIC)?

Hydrophilic Interaction Liquid Chromatography (HILIC) has evolved into a powerful chromatographic technique for the retention and separation of hydrophilic neutral and polar compounds, which are often difficult to retain by Reversed-Phase Liquid Chromatography (RPLC). HILIC utilises a polar stationary phase combined with an organic/aqueous mobile phase [1], typically containing a high percentage of the organic component (~>60% acetonitrile). In HILIC, water is the strong solvent and unlike RPLC, increasing the percentage of water in the mobile phase decreases analyte retention (Figure 1).

FIGURE 1. Solvent elution strength in HILIC mode.

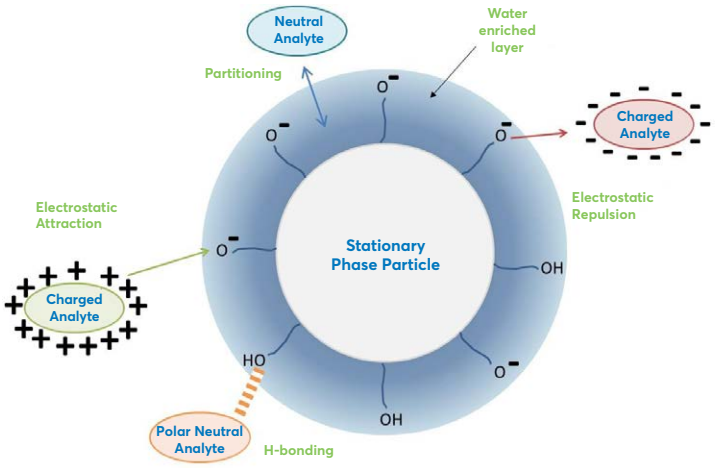
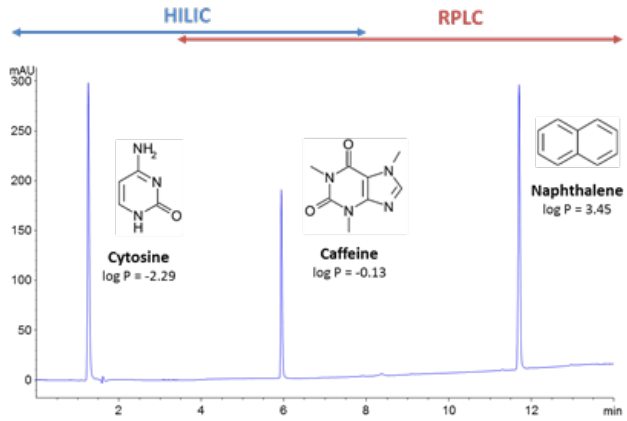


FIGURE 2. Schematic representation of retention mechanisms in HILIC.



Column:	ACE® Excel 2 C18, 100x3,0 mm
Mobile phase:	A: 10 mM ammonium formate pH 3,0 in H_2O
	B: 10 mM ammonium formate pH 3,0 in ACN/H_2O (90:10 v/v)
Gradient:	5 to 100% B in 10 minutes
Flow rate:	0,4 ml/min
Temperature:	30 °C
Detection:	UV, 254 nm

FIGURE 3. Practical illustration of reversed phase and HILIC operating ranges.

Mechanistically HILIC is complex, involving a combination of multiple modes of interaction between the analyte, stationary phase and eluent. In HILIC, a water-enriched layer is present at the stationary phase surface, as shown in Figure 2. A minimum of ~3% water is required in the mobile phase for formation of this layer. Analyte retention is often due to a combination of partitioning of the analyte into the water-enriched layer, together with hydrogen bonding and electrostatic interactions [2-5].

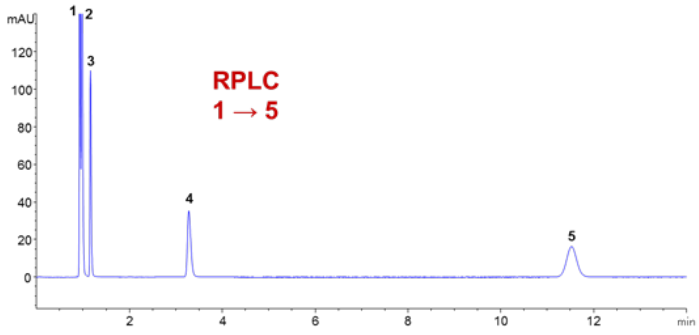
When to consider HILIC

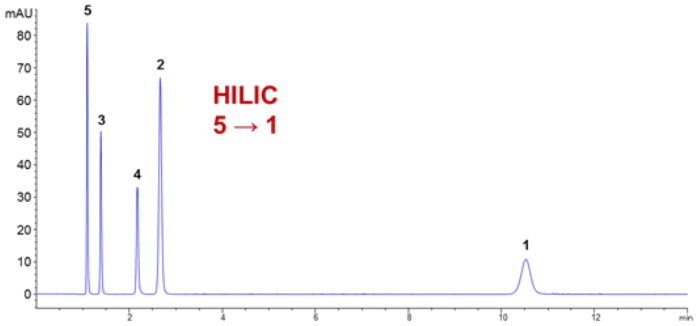
HILIC is applicable to hydrophilic analytes that are challenging to retain by RPLC. Analyte log P and log D values can provide an indication of the suitability of the analyte for retention by HILIC. Generally, if an analyte elutes before caffeine in RPLC (log P ~zero), it may be suitable for analysis by HILIC, whilst those eluting later may be better suited for RPLC. Figure 3 shows a chromatogram of three analytes separated using a RP gradient on an ACE® C18 column. Cytosine, being very hydrophilic, is poorly retained on the C18 column and is therefore, a suitable candidate for HILIC. The hydrophobic analyte, naphthalene, is well retained and best suited for RPLC. Caffeine is retained in RPLC mode, but can also be retained in HILIC mode. This region of overlap between RPLC and HILIC often causes discussion as either mode may offer advantages: The choice is usually application driven.

Advantages of HILIC

In addition to providing retention of polar analytes, the HILIC technique also offers other useful advantages. Figure 4 demonstrates how HILIC offers orthogonal selectivity to RPLC. In this example, the elution order for the mixture of analytes is completely reversed in HILIC mode compared to RPLC. Selectivity is the key to analyte resolution in chromatography, so maximising selectivity for polar to very polar analytes using HILIC is helpful, and a powerful tool to increase the likelihood that all species within a sample have been observed.

Unlike RPLC, HILIC does not require the use of mobile phase additives such as ion-pairing reagents (which can be challenging for other reasons) to retain polar compounds. The high volume fraction of acetonitrile in the eluent is advantageous as it generates lower back pressures and higher diffusivity. In practice, this allows the analyst to use smaller, more efficient particles and higher flow rates, leading to faster separations. Moreover, the high organic modifier content in HILIC mode leads to more effective aerosol droplet desolvation for LC-MS applications, which can result in improved ionisation and enhanced signal response.





Sample: 1: Cytosine 2: Uracil 3: Caffeine 4: Nortriptyline 5: Toluene

REVERSED PHASE

Column:	ACE 5 C18, 150x4,6 mm
Mobile phase:	10 mM NH_4COOH pH 4,7 in MeCN/H_20 (40:60 v/v)
Flow rate:	1.5 ml/min
Temperature:	30 °C
Detection:	UV, 254 nm

HILIC

Column:	ACE 5 C18, 150x4,6 mm
Mobile phase:	10 mM NH_4COOH pH 4,7 in MeCN/H_20 (92:8 v/v)
Flow rate:	1.5 ml/min
Temperature:	30 °C
Detection:	UV, 254 nm

FIGURE 4. Comparison of RPLC and HILIC for the separation of three polar analytes, one basic analyte and one hydrophobic analyte.

Despite its numerous advantages, the less well understood HILIC retention mechanism means that knowledge of, and experience with, HILIC is generally lower than for RPLC. Consequently, method development strategies tend to be less well defined. The new family of ACE HILIC phases, coupled with a systematic method development flow chart, is designed to offer a simplified approach to method development, and maximise the chances of generating a successful method.

Three phases for method development

To develop any chromatographic method, it is essential to adequately investigate the selectivity of a separation. This allows the chromatographer to optimise analyte resolution and develop more robust methods. The column stationary phase is one of the most powerful parameters for influencing selectivity in HILIC method development [6]. The new ACE HILIC column family is manufactured using ultra-inert porous silica to provide excellent peak shape, reproducibility and robustness. This column family consists of three novel stationary phases, which deliver substantially different selectivity to one another, and have been specifically designed to provide a total solution for method development. In addition to their availability as single columns, the three phases can also be purchased as a cost effective HILIC method development kits, which includes all three stationary phases for the price of a single column.

HILIC-A: An acidic stationary phase showing high cation exchange capability. Charged bases show electrostatic attraction to this phase.

HILIC-B: A basic character phase with reasonable anion exchange capacity for the retention of acidic analytes.

HILIC-N: A neutral bonded phase with low anion and cation exchange capabilities. Retention is governed mainly by polar interactions, adsorption and partitioning.

Phase	USP listing	Functional group	Endcapped	Particle size (µm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)	pH range
ACE HILIC-A	L3	Proprietary SIL	No	1,7; 3; 5	100	300	-	2 – 7
ACE HILIC-B	L8	Proprietary aminopropyl	No	1,7; 3; 5	100	300	4,0	2 – 7
ACE HILIC-N	Pending	Proprietary polyhydroxy	No	1,7; 3; 5	100	300	7,0	2 – 7

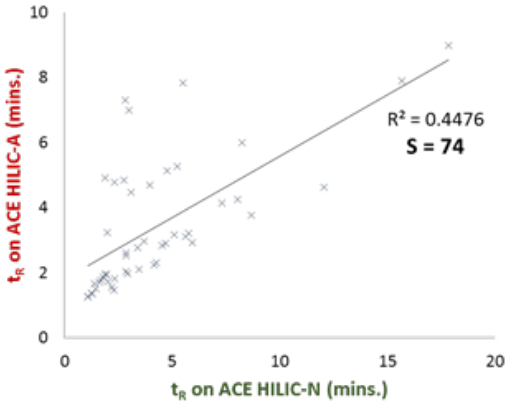


FIGURE 5. Comparison of retention times of 48 analytes on the HILIC-A and HILIC-N phases. For full details of experimental conditions, please refer to reference 6 and 8.

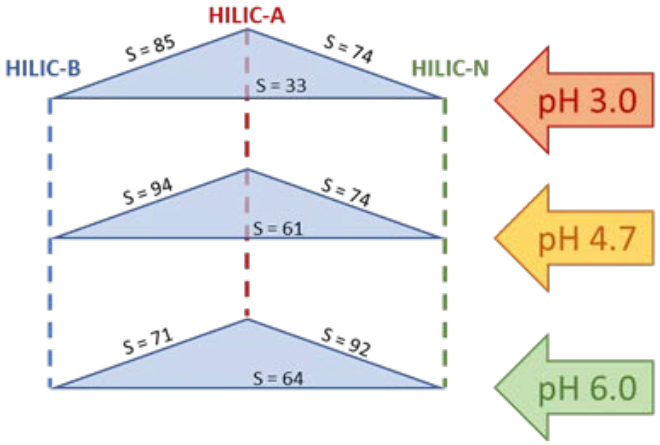


FIGURE 6. Selectivity triangles comparing the selectivity of the three HILIC phases at three different mobile phase pHs.

Maximise orthogonal selectivity for improved method development

The HILIC family of columns has been designed to provide three HILIC phases, that offer different selectivity from one another across a range of mobile phase compositions and pHs. It is possible to quantitatively assess how different two LC stationary phases are from one another, in terms of selectivity, by chromatographing a range of analytes under identical conditions, and examining the differences in the respective analyte retention times on the two phases.

By plotting the retention times obtained for the analytes on the two phases (see example in Figure 5), differences in selectivity can be identified by the scatter, as denoted by the coefficient of determination (R^2). A high degree of scatter (smaller R^2 value, farther away from 1,0) indicates the two phases offer very different selectivities. It is then possible to obtain the selectivity descriptor, S, per equation 1 [7].

$$S = 100 \times \sqrt{(1 - R^2)} \qquad \text{EQUATION 1}$$

To examine the selectivity differences offered by the three HILIC columns, a total of 48 compounds were screened under identical chromatographic conditions. The experiment was repeated for three mobile phase pH values (3,0; 4,7 and 6,0) to investigate the typical working pH range for HILIC. Figure 6 shows selectivity triangles comparing the three HILIC phases at each pH. The large S values obtained show that the three phases offer substantially different selectivity to one another at each pH value. These selectivity descriptor values demonstrate that the three phases will potentially offer different retention and elution order for a set of analytes at a given pH, which is ideal for method development screening. An S value close to 0 indicates the two phases offer identical selectivity, while a value of 100 indicates complete orthogonality.

An example of this orthogonal selectivity among the HILIC phases is demonstrated in Figure 7. A range of acidic, basic and neutral analytes were chromatographed on the three phases at pH 4,7. The neutral analytes show similar elution order on the three phases, although 2'-deoxyguanosine shows much greater retention on the HILIC-B and HILIC-N phases. Negatively charged acidic analytes showed low retention on the HILIC-A, intermediate retention on the HILIC-N, and are retained, on the HILIC-B. This is likely due to differing degrees of electrostatic interactions offered by each of the phases for this application. The positively charged basic analytes (peaks 4, 7 and 8) show the opposite trend, with shorter retention on the HILIC-B and much stronger retention on the HILIC-A. The net effect is that, as expected, the three phases demonstrate very different elution orders for this application,

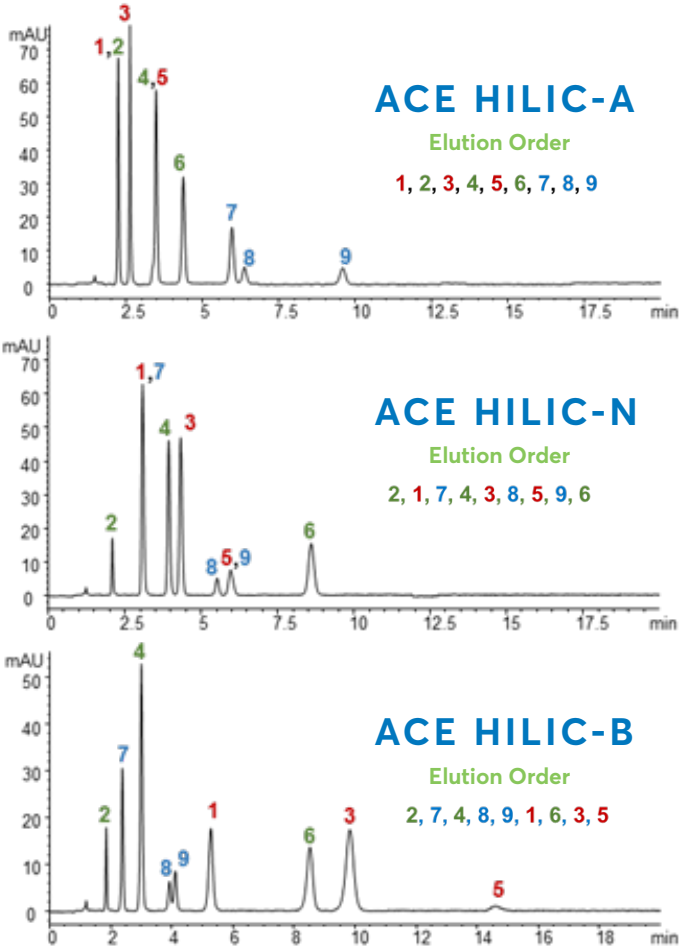


FIGURE 7. Comparison of a mix of acidic (red), basic (blue) and neutral (green) analytes on the HILIC phases. Column format: 5 µm, 150x4,6 mm, mobile phase: isocratic, 10 mM ammonium formate pH 4,7 in MeCN/H2O (90:10 v/v), flow rate: 1,5 ml/min, temperature: 25 °C, injection volume: 5 µl, detection: UV, 254 nm. Sample: 1. 4-amino benzoic acid, 2. nicotinamide, 3. 4-hydroxybenzoic acid, 4. adenine, 5. mandelic acid, 6. 2'-deoxyguanosine, 7. acebutolol, 8. tyramine,

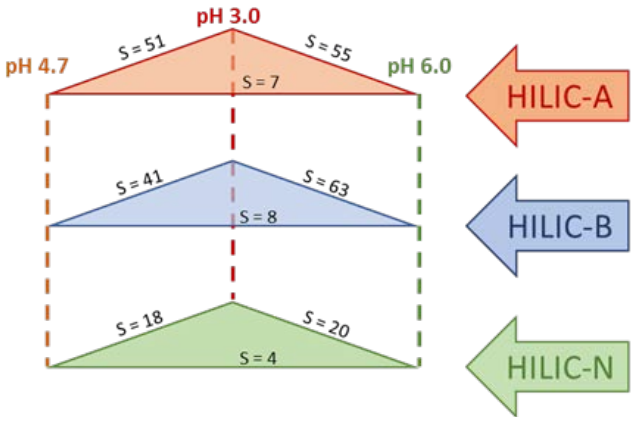


FIGURE 8. Selectivity triangles showing the effect of mobile phase pH on each of the three HILIC phases.

providing the chromatographer with a variety of options for method development.

Eluent pH: Further control over selectivity

After column stationary phase, the eluent pH is the next most powerful HILIC parameter for the control of selectivity. When a sample contains ionisable analytes, the degree of ionisation can often be controlled by varying the eluent pH. Acidic analytes will be neutral when chromatographed with eluent pH below their pK_a and will become ionised, and therefore, more polar at a pH above their pK_a. The opposite is then true for basic sample components.

The HILIC column family further enhances the effect of eluent pH, as the ionisation state of the HILIC-A and HILIC-B phases is also pH dependent. HILIC-A can carry a net negative surface charge. By increasing the pH, the degree of ionisation of the phase is enhanced to provide greater retention of cationic analytes. Similarly, eluent pH can affect ionisation of the HILIC-B phase, thereby varying the degree of positive character of the phase.

The selectivity descriptors for different eluent pHs can also be generated for each phase (Figure 8). Again, high S values were obtained for this exercise, particularly

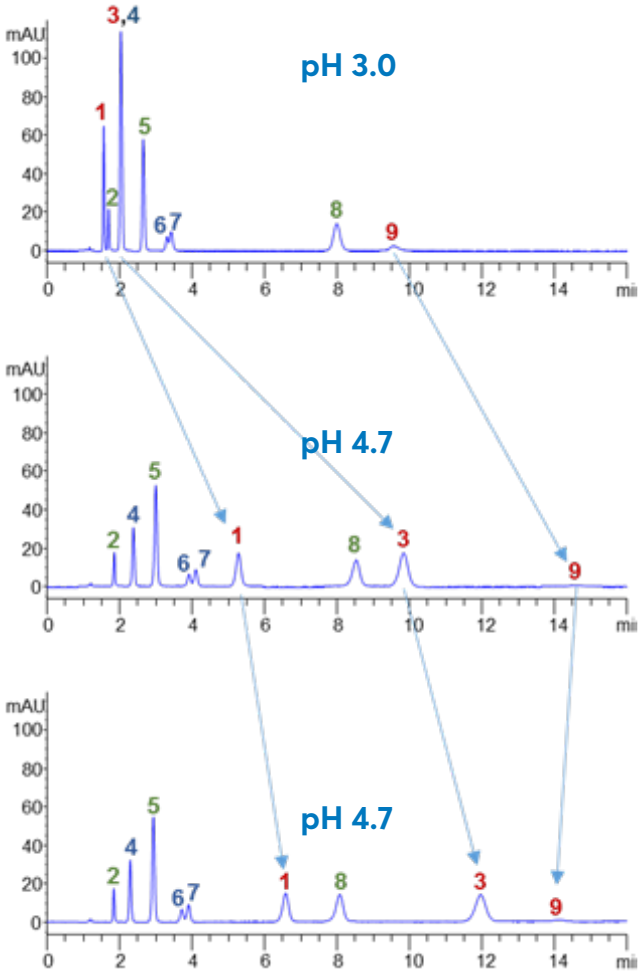


FIGURE 9. Comparison of a mix of acidic (red), basic (blue) and neutral (green) analytes on the HILIC-B phase at different mobile phase pHs. Column format: 5 µm, 150x4,6 mm, mobile phase: Isocratic, 10 mM ammonium formate in MeCN/H2O (90:10 v/v), flow rate: 1,5 ml/min, temperature: 25 °C, injection volume: 5 µl, detection: UV, 254 nm. Sample: 1. 4-amino benzoic acid, 2. nicotinamide, 3. 4-hydroxybenzoic acid, 4. acebutolol, 5. adenine, 6. tyramine, 7. atenolol, 8. 2'-deoxyganosine, 9. mandelic acid.

Figure 9 shows the same set of analytes chromatographed on the HILIC-B at three different mobile phase pHs. By altering the mobile phase pH, retention of the acidic analytes can be controlled, substantially altering the selectivity of the separation. It is clearly demonstrated that mobile phase pH can provide a powerful and practical route to optimise selectivity.

Streamlined HILIC method development strategies

From the data presented in this white paper, a powerful approach to method development in HILIC would be to examine both the type of HILIC stationary phase, and the mobile phase pH, to identify a suitable stationary phase/mobile phase combination. Figure 10 outlines a logical, step-by-step method development strategy based on these parameters. Initially (Step 1), it is recommended to gather as much analyte information as possible (e.g. pK_a, log P, log D etc). The sample can then be screened, using either isocratic or gradient conditions on the three HILIC phases at different mobile phase pHs, to identify a suitable column/pH combination for the separation (Step 2). Parameters such as % organic, temperature and buffer strength can then be optimised (Step 3) to produce the final HILIC separation. By employing a strategy such as this, it is possible to make well informed and, logical decisions during the method development process, and to develop robust methods using suitable column/mobile phase combinations.

Parameter	Comments	
Column	ACE HILIC-A, ACE HILIC-B and ACE HILIC-N, 150x4,6 mm	
Gradient mobile phase	A: 10 mM ammonium formate in MeCN/H_2O (96:6 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 (mins.)**	**%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	

TABLE 1. Suggested conditions for HILIC screening experiment.

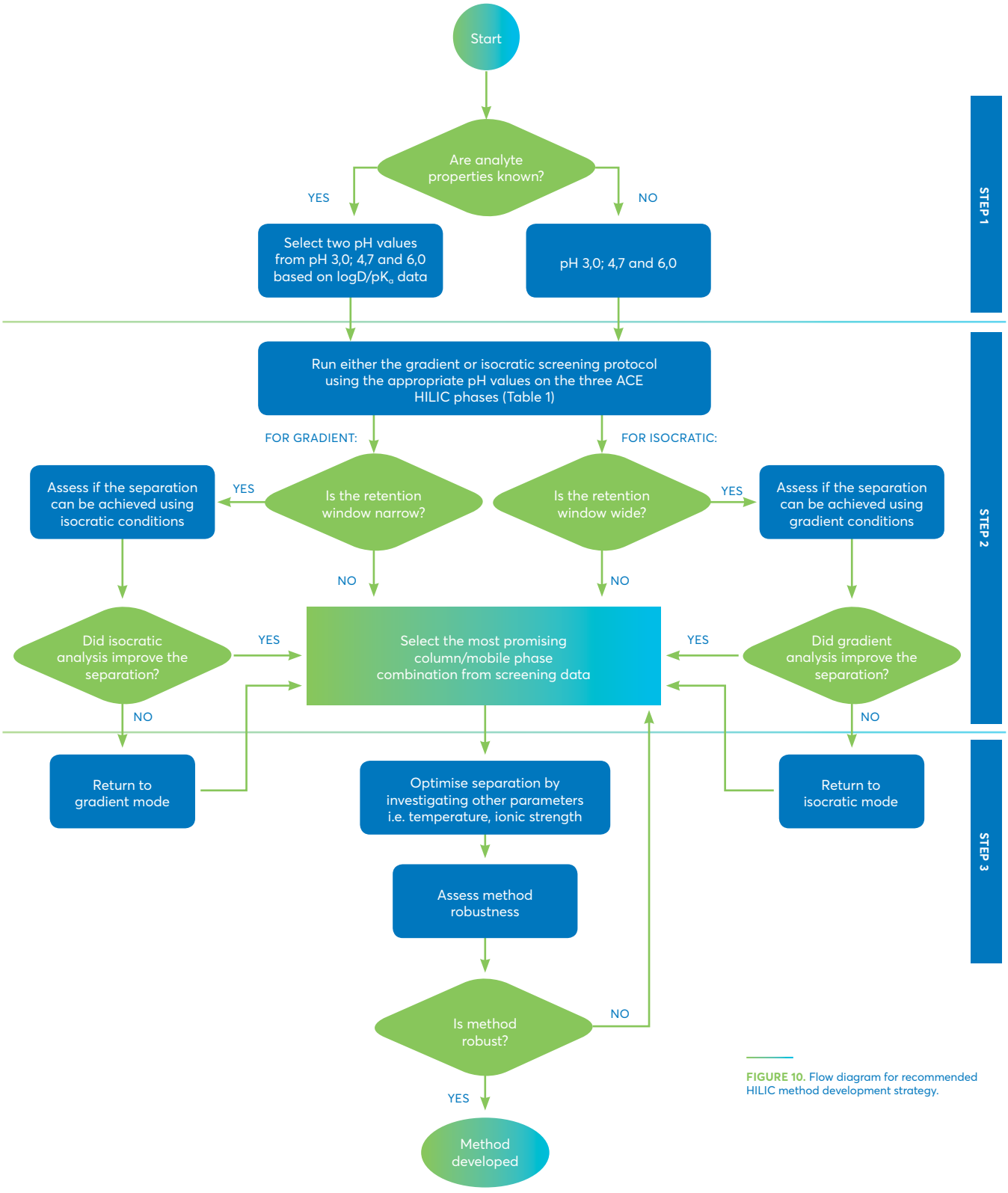


FIGURE 10. Flow diagram for recommended HILIC method development strategy.

Practical tips for successful HILIC methods – equilibration and sample diluent

Two of the most common causes of poor HILIC method performance and poor reproducibility are the sample diluent and column equilibration. In general, the sample diluent should be similar in composition to the mobile phase where possible, and high proportions of water (strong solvent in HILIC mode) in the diluent should be avoided. If poor peak shape is observed for a HILIC separation, the sample diluent should be assessed first.

The stationary phase always requires equilibration with the required mobile phase prior to use. Due to the requirement of a stable water-enriched region at the stationary phase surface, equilibration times in HILIC can be longer than in RPLC. In general, a new column should be equilibrated with 60 to 80 column volumes of mobile phase prior to its first use. After each use, the column should be flushed to remove any buffer and then stored as stated on the test chromatogram accompanying the column. For subsequent isocratic runs, 20 column volumes of mobile phase should be sufficient for equilibration prior to injecting the sample. The following equation can be used to estimate one column volume.

$$V_M = \frac{0.5c \times L \times d_c^2}{1000}$$

Where V_M is the column volume in ml, L the column length in mm and d_c the column internal diameter in mm. The time to elute one column volume of mobile phase in minutes is then given by the following (where F is the flow rate in ml/min):

$$t_{1V_M} = \frac{V_M}{F}$$

When working in gradient mode, it has been determined that 10 column volumes are required to re-equilibrate HILIC columns with the gradient starting conditions after every gradient run. In general, it is recommended that equilibration time is explored and accurately documented as a parameter in gradient HILIC method development to ensure method robustness and reproducibility. Further discussion of these two topics can be found here under educational information.

Conclusions

This white paper demonstrates that these three novel ACE HILIC phases provide very different and complementary selectivity to one another. The selectivity differences can be exploited, together with mobile phase pH, to thoroughly explore selectivity, and enable selection of an appropriate stationary phase/mobile phase combination during method development. This step-by-step approach to method development is recommended to aid the chromatographer to streamline method development processes in a practical manner.

References

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