A Step-by-Step Approach for Method Development to Generate a Successful HILIC Separation

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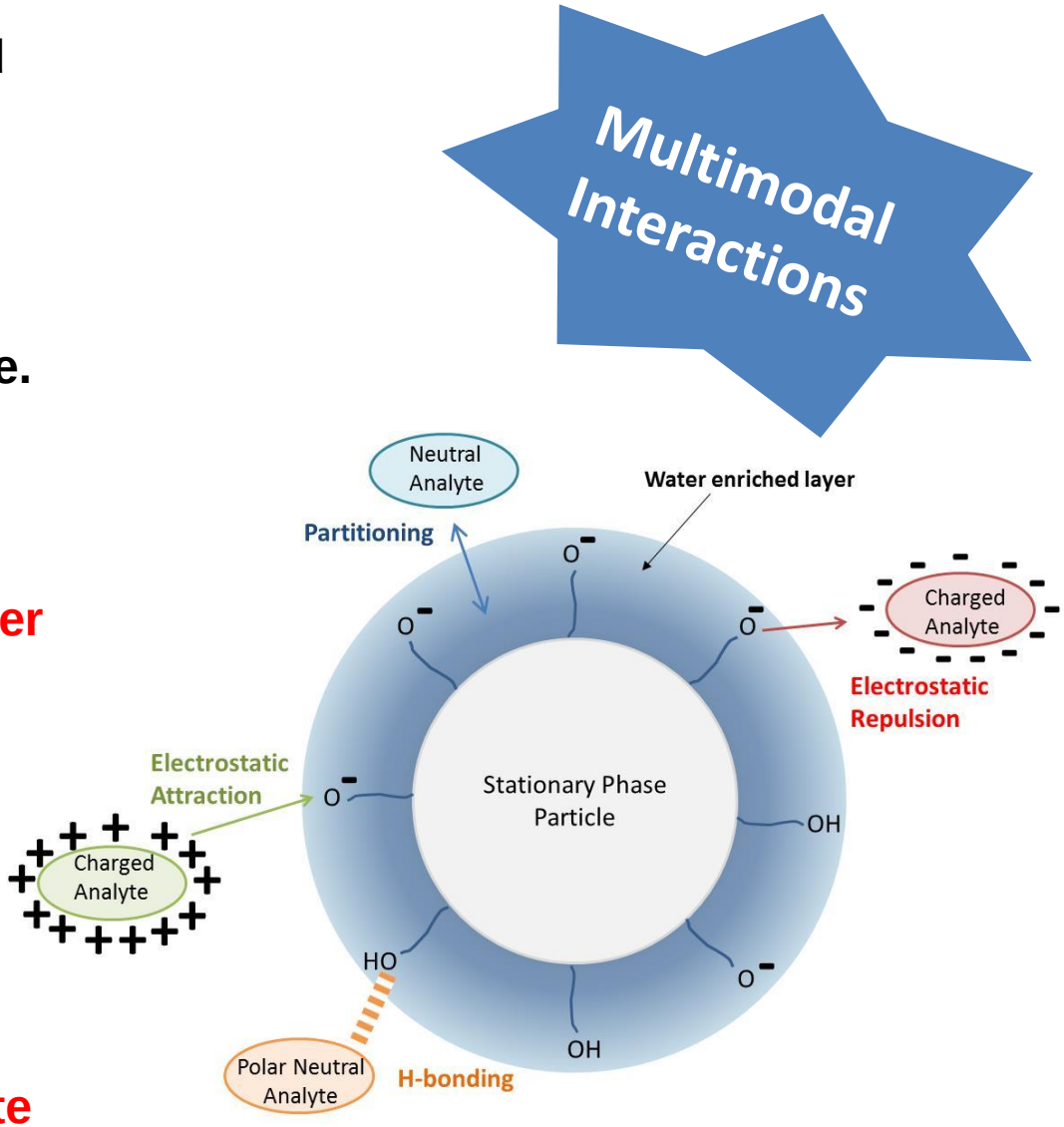
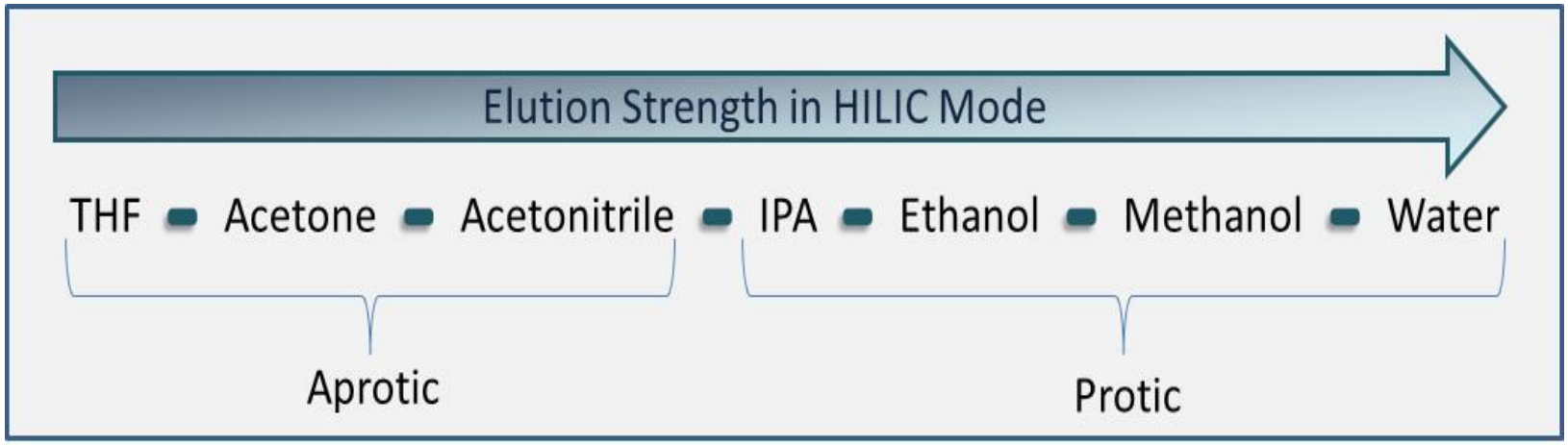
What is HILIC?

- Hydrophilic Interaction Liquid Chromatography (HILIC)*
- Ideal for retention of poorly retained or not retained at all analytes in reversed phase. i.e. polar neutral and ionisable polar species
- HILIC works by forming a water layer surrounding the silica particle. Analytes can then partition between the organic bulk layer and the preferred water layer
- Mechanisms such as IEX and H-bonding may also contribute



* A. J. Alpert, *J. Chromatogr.*, 499 (1990) 177.

HILIC Mobile Phases

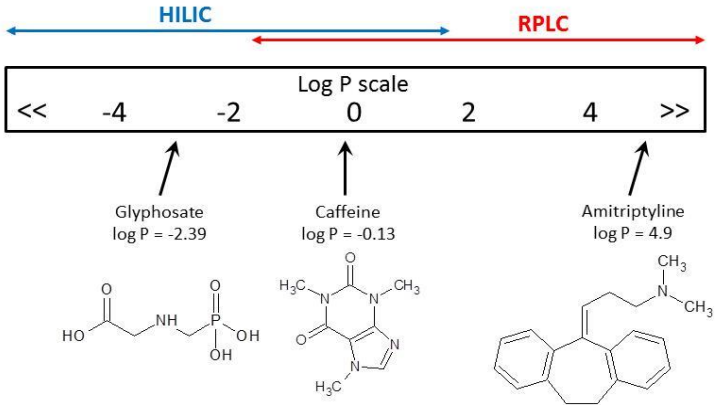


- **Bulk solvent = MeCN**
- **~3% minimum aqueous required to hydrate the water layer around particles for partitioning.**
- **(MeOH (other protic solvents) in the bulk solvent may disrupt the water layer leading to reduced retention repeatability).**

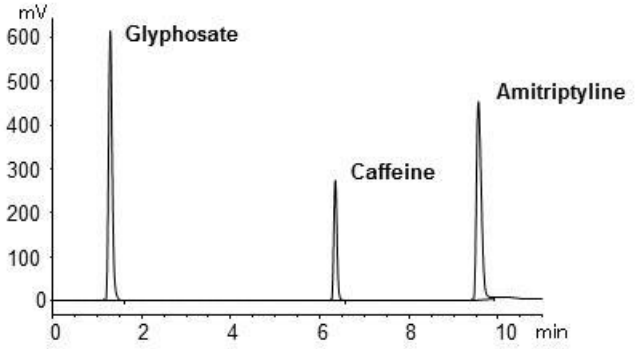
When Should Analysts Use HILIC?

1 If known, an analyte's **Log P value** (measure of **lipophilicity**) can indicate whether **HILIC is an option*:**

- Analytes with a **Log P of ~ ≤ 0** are typically **suitable for HILIC**



2 Alternatively, elution **before the caffeine peak retention** time on an ACE C18 with **gradient RPLC** can be a reasonable **'rule of thumb'**



Conditions:
Column = ACE Excel C18, 100 x 3.0mm, 2mm. Part number = EXL-101-1003U
A = 10mM ammonium formate, pH 3.0 (aq)
B = 10 mM ammonium formate, pH 3.0 in 9:1 v/v MeCN:H_2O
Gradient = 5-100%B in 10 minutes
ELSD detection, 0.4 mL/min, 30C, 10mL injection
Analysed using VWR-Hitachi Chromaster600-ELSD

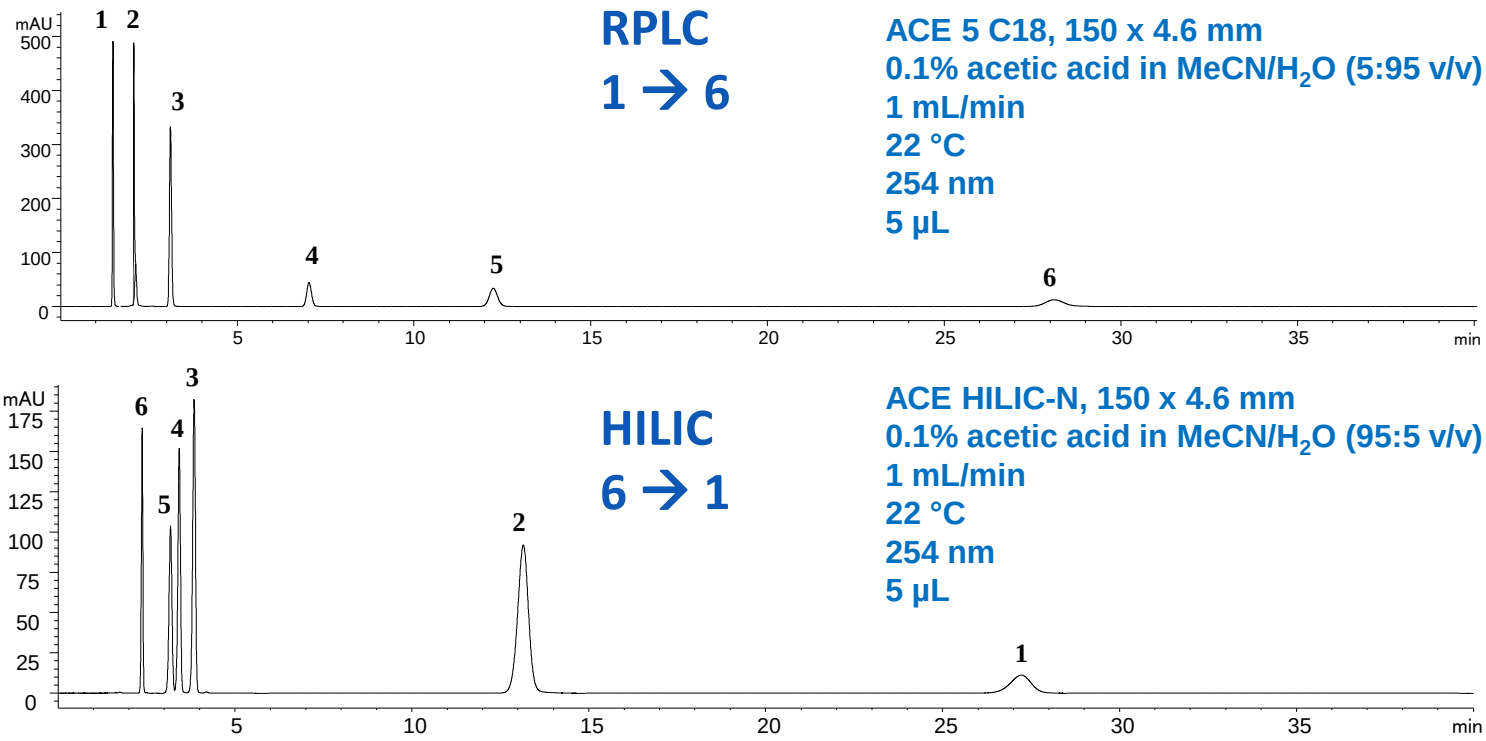
*** Reproduced from A P McKeown, *Chrom. Today*, Nov / Dec 2015, 8-12**

What Does HILIC Offer the Analyst?

1. **HILIC can provide retention and / or separation of polar analytes such as metabolites, polar pharmaceuticals, polar pesticides etc.**

2. **HILIC provides orthogonal selectivity to RPLC.**

1. Cytosine
2. Hypoxanthine
3. Thymine
4. Theobromine
5. Theophylline
6. Caffeine



Key Advantages of HILIC

- **Highly suited for the retention of polar to very polar analytes**
  - **No need for ion pairing reagents or additives as with RPLC, (which can come with its own challenges)**
- **The high volume fraction organic solvent eluents used has been showed to enhance mass transfer and lower the C term contribution in the van Deemter equation**
- **The high organic content also can aid ionisation for MS detection**
  - **Ideal for desolvation, ion formation and enhanced signal response**
- **The acetonitrile bulk solvent has low viscosity which translates to lower backpressures**
  - **Enables the chromatographer to move to higher flow rates, smaller particle sizes and therefore higher plate count**

Key Disadvantages of HILIC

- HILIC knowledge & experience is generally far lower than for RPLC with many analysts incorrectly using RPLC experience for HILIC
  - Key parameters less well understood
  - Stationary phase options far less
  - Operational guidelines are different to RPLC
- High volume fraction eluents can lead to challenges with solubility for hydrophilic analytes.
- Retention modelling can performed but predicting isocratic conditions from gradient models and vice versa has found to be unreliable (unlike RPLC)*. Conflicting success / failure has also been observed**.

* Anal Bioanal Chem. 2015 Dec;407(30):9135-52. doi: 10.1007/s00216-015-9079-2
** J Chromatogr A. 2014 Apr 11;1337:116-27. doi: 10.1016/j.chroma.2014.02.032

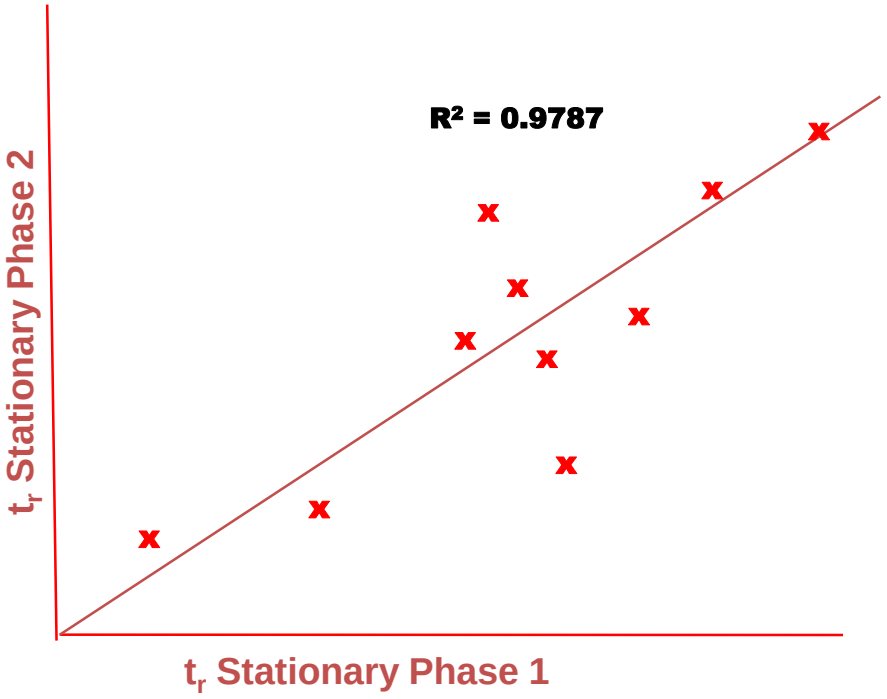
Method Development: HILIC Stationary Phases

- HILIC stationary phases can be split into acidic, basic, neutral and 'other / novel' e.g. Zwitterionic / cyclodextrin etc.

- To maximise selectivity, it is helpful to consider HILIC stationary phases with different character

Changing Stationary Phase Exploits Selectivity

Different stationary phase mechanisms may affect elution order and move peaks around in the chromatogram



Selectivity* = 100 x √ (1 – R²)

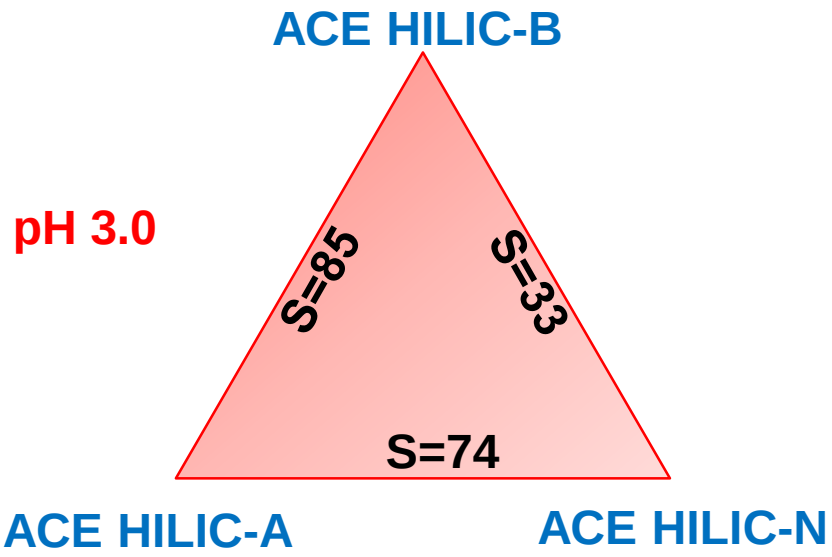
= 100 x √ (1 – 0.9787)
= ~ 15

Higher Selectivity values are better

* Neue, O'Gara, Méndez "Selectivity in Reversed-Phase Separations: Influence of the Stationary Phase", J. Chromatogr. A 1127 (2006), 161-174

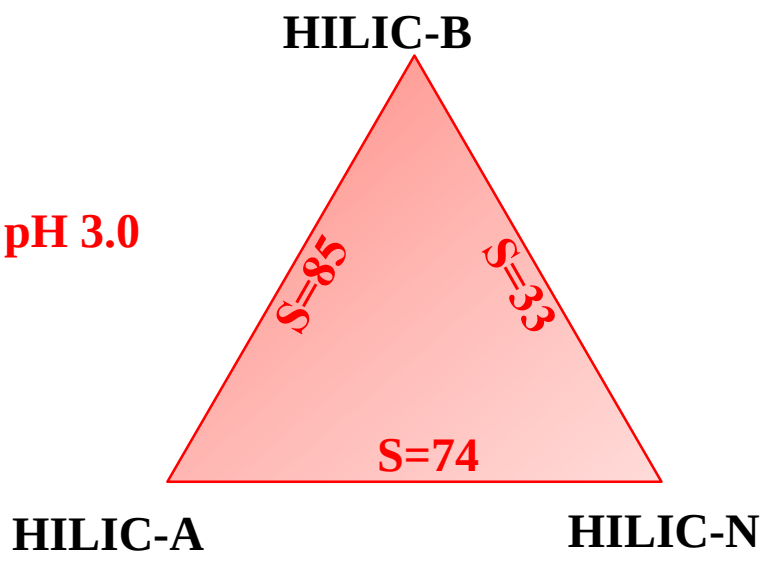
Stationary Phase Rationale - Selectivity

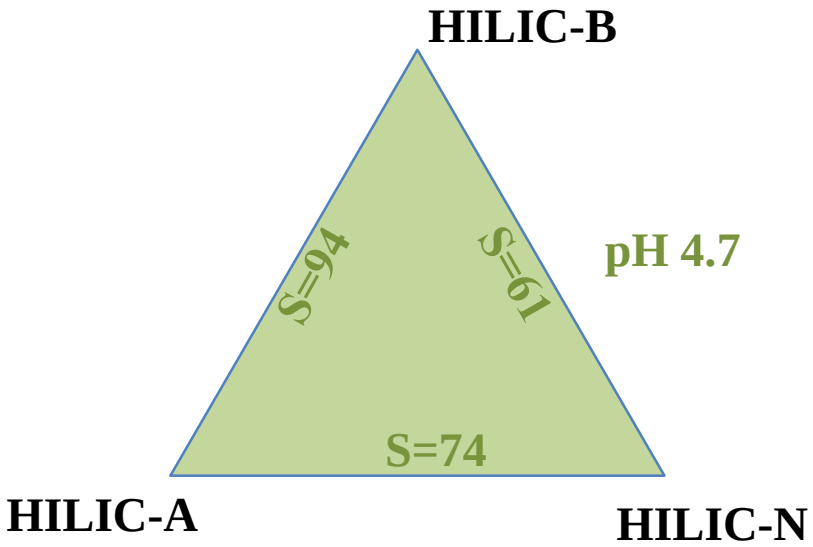
- Using a HILIC characterisation protocol with 54 polar analytes, Selectivity Descriptor values in HILIC mode were determined

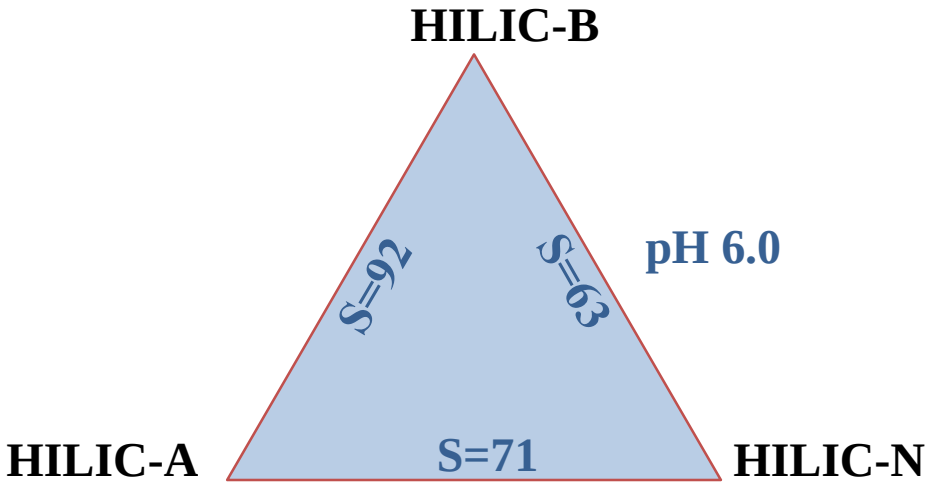


- These data indicate that phases of acidic, neutral and basic character offer suitable differences for HILIC method development activities.

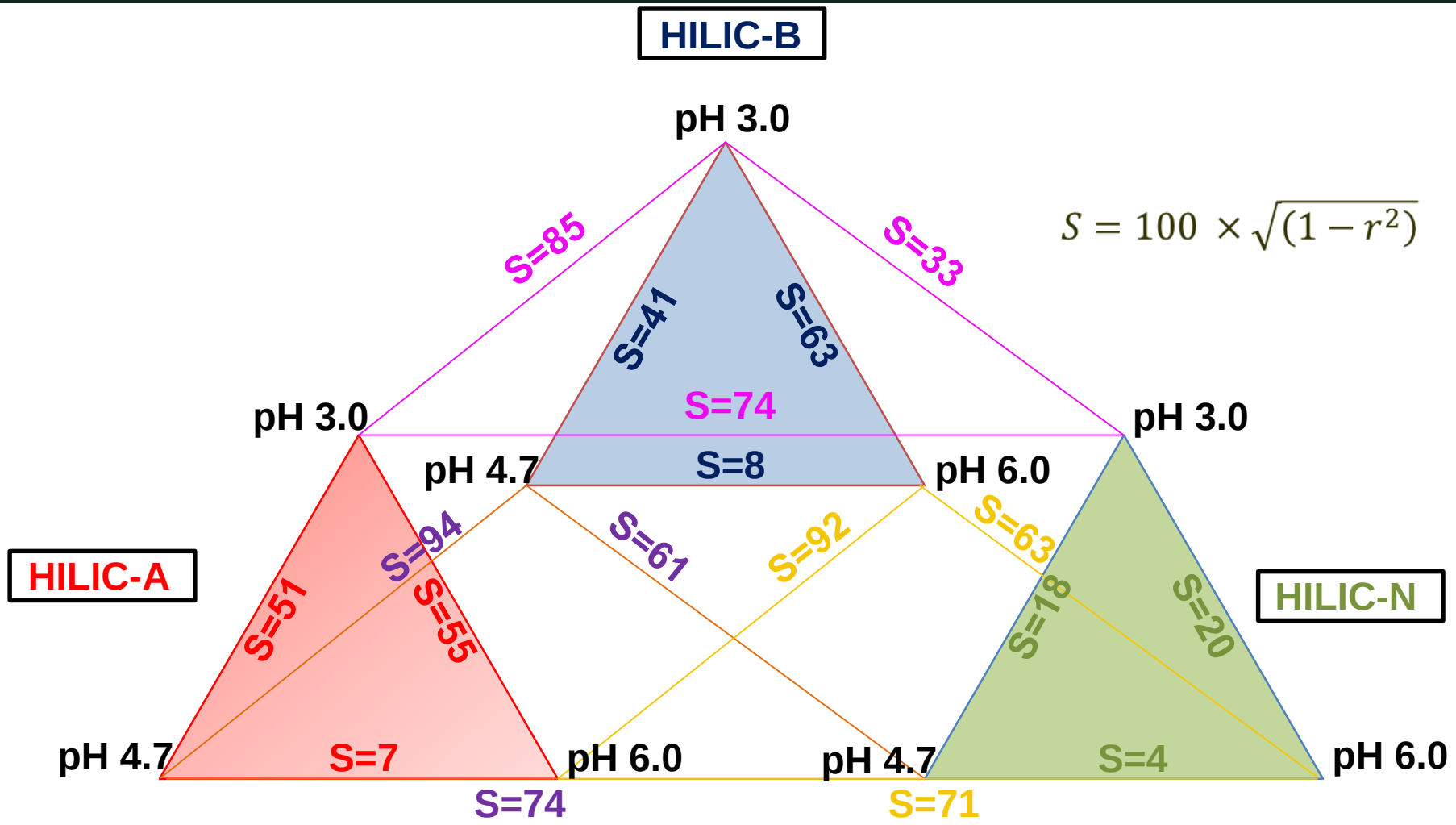
Influence of Eluent pH for Each Phase



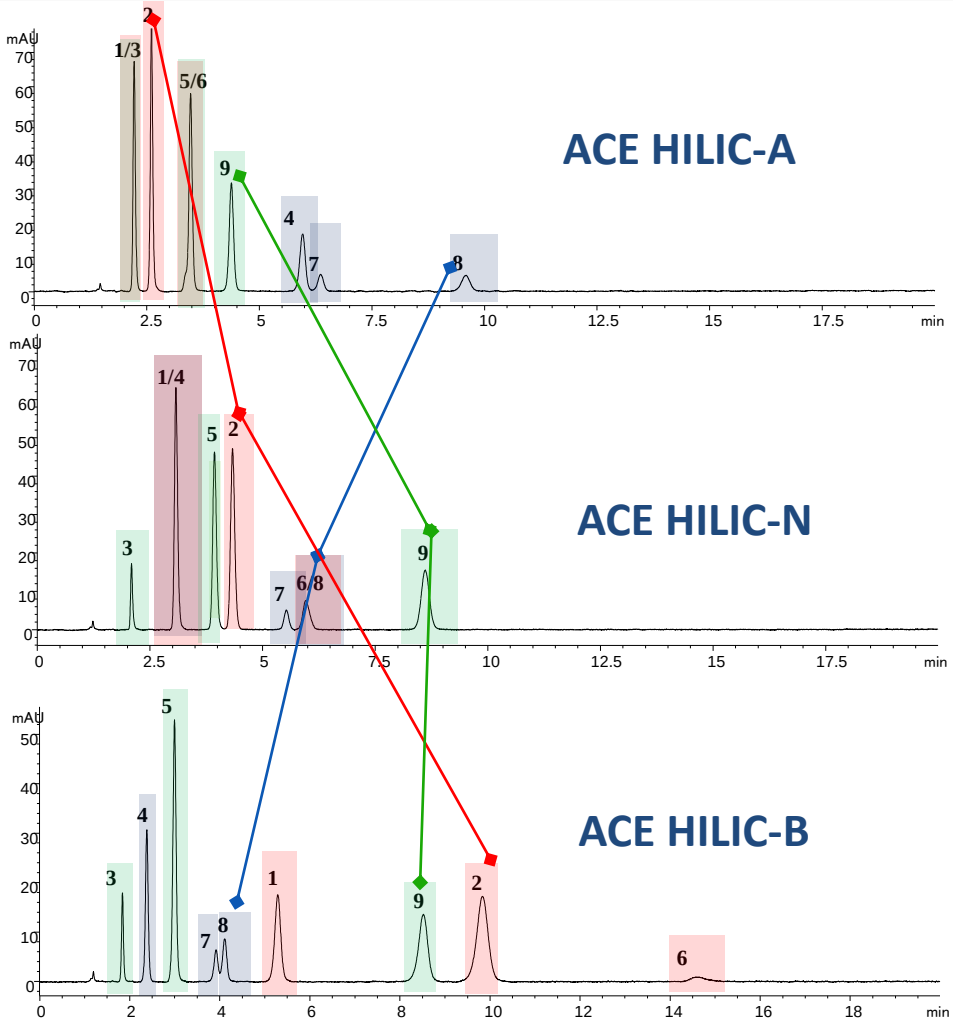




More Complex Selectivity Relationships…



HILIC Stationary Phases With Different Selectivity



Elution order

1/3, 2, 5/6, 9, 4, 7, 8

3, 1/4, 5, 2, 7, 6/8, 9

3, 4, 5, 7/8, 1, 9, 2, 6

1. p-Aminobenzoic acid, 2. 4-Hydroxybenzoic acid, 3. Nicotinamide, 4. Acebutalol, 5. Adenine, 6. Mandelic acid, 7. Tyramine, 8. Atenolol, 9. 2-Deoxyguanosine

Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v), Flow: 1.5 mL/min, Detection: 254 nm, Temperature: 25 °C, Injection: 5 µL,

HILIC Method Development Flow Chart

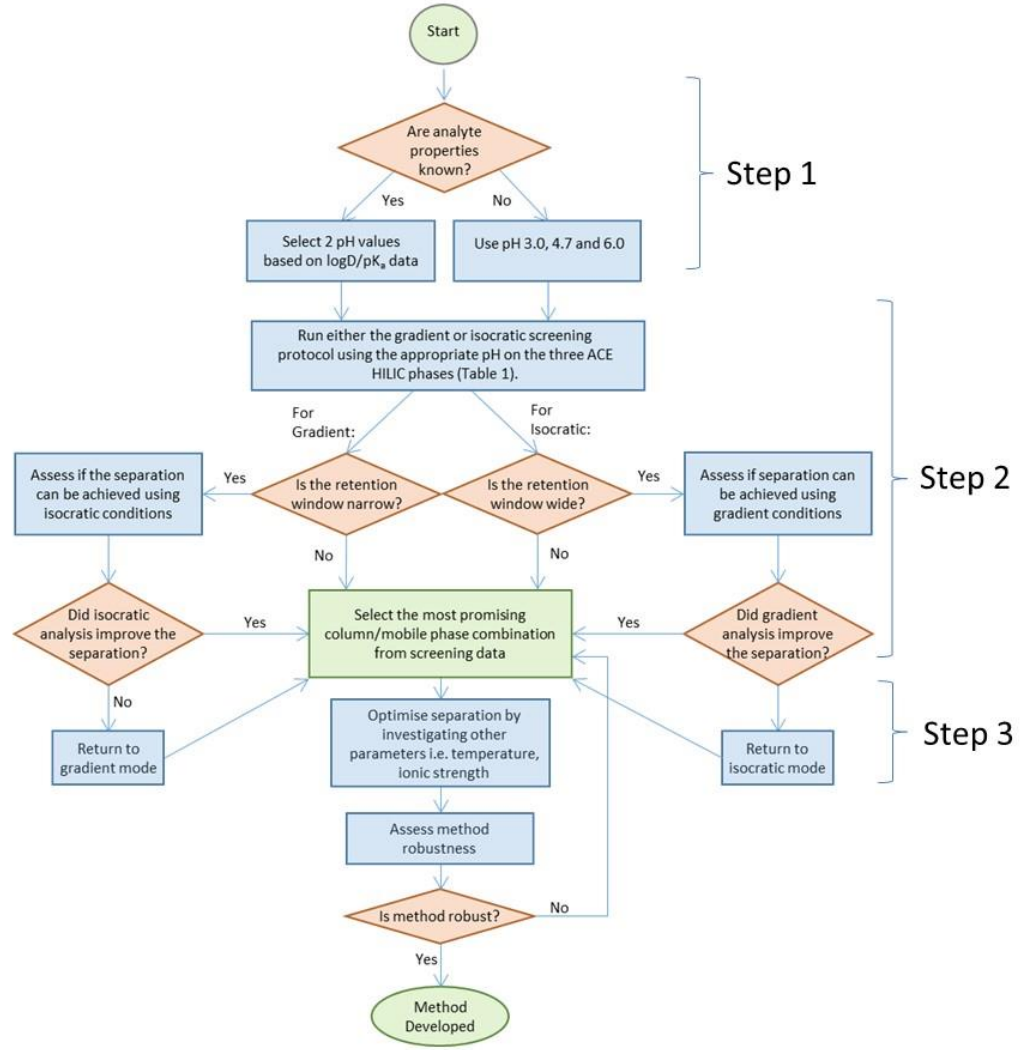


Figure 4: Flow diagram for step-by-step HILIC Method Development protocol.

Parameter	Comments
Column	ACE HILIC-A, ACE HILIC-B and ACE HILIC-N, 150 x 4.6 mm
Gradient mobile phase	A: 10 mM ammonium formate in MeCN/H_2O (96:4 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

Providing Help for HILIC Method Development

- **HILIC knowledge & experience is generally far lower than for RPLC with many analysts incorrectly using RPLC experience for HILIC**
  - **Key parameters less well understood**
  - **Stationary phase options far less**
  - **Operational guidelines are different to RPLC**

↓

Understanding selectivity in HILIC allows rationale stationary phase and eluent pH choices

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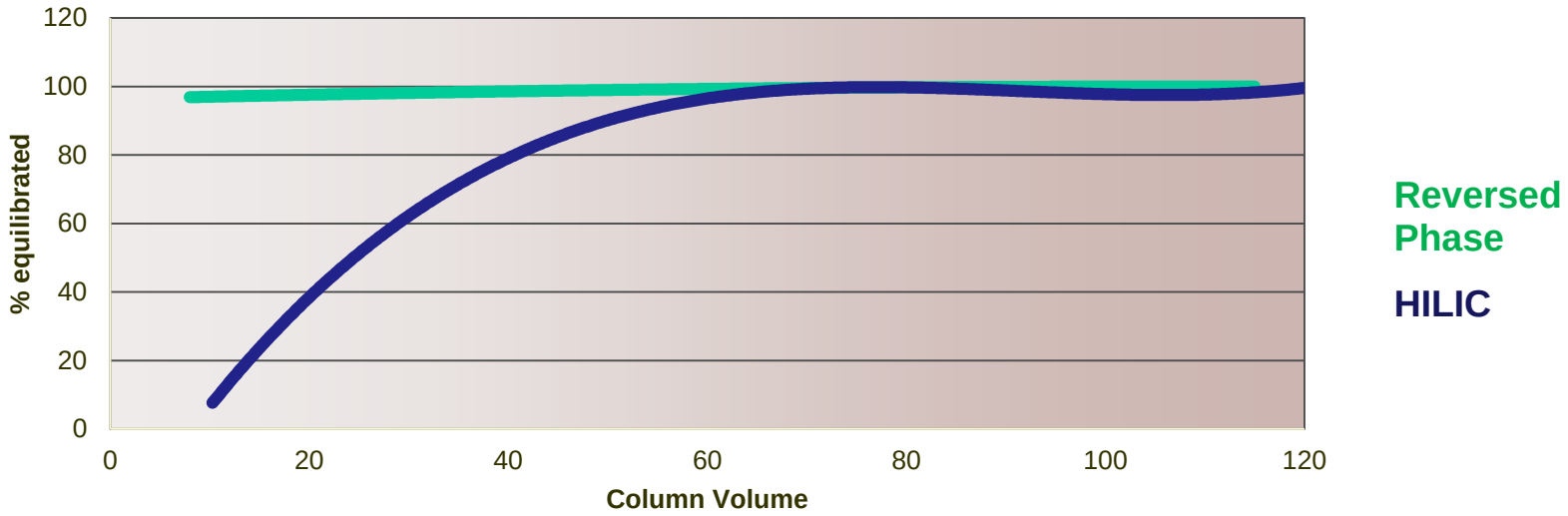
Screening samples with 3 x columns and 3 x eluent pH values explores the selectivity 'space'

(Removes initial 'tweaking'…the graveyard of method development)

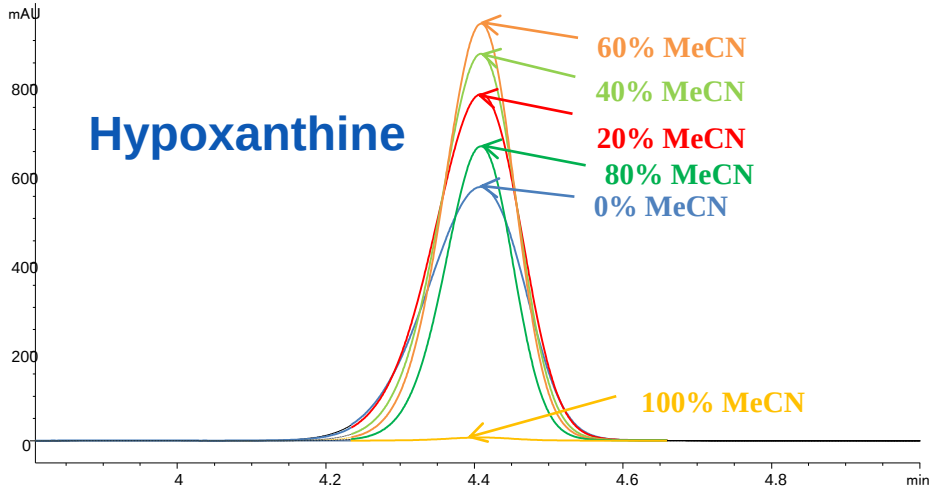
HILIC Equilibration Discussion

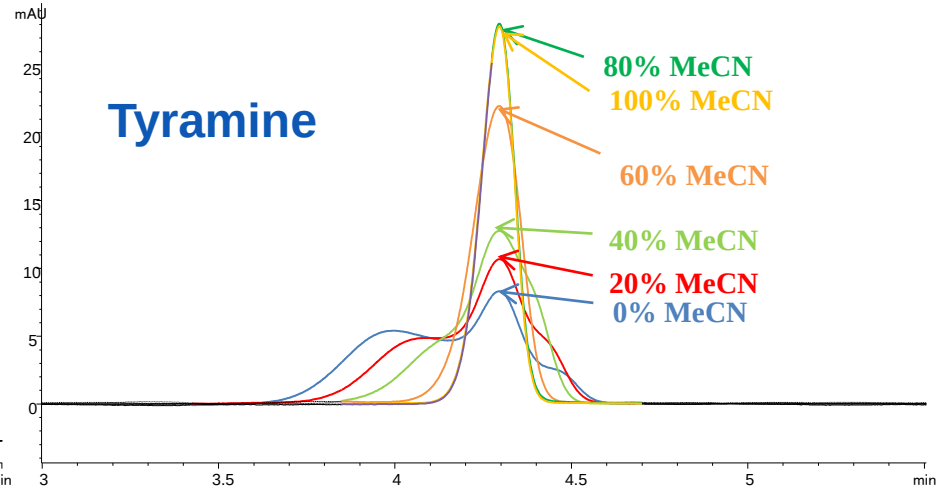


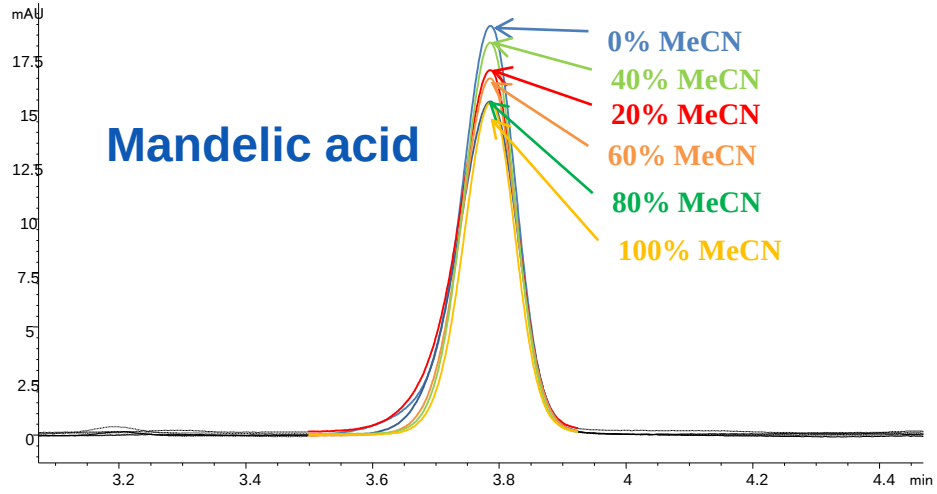
- **Equilibration** is key for **robust** chromatography
- **RPLC** typically requires **10 column volumes** for equilibration
- Minimum of **50-60 column volumes** **initially** is required for HILIC
  - Recent work has shown **subsequent equilibration much less (20 col vols?)**
  - For **isocratic HILIC methods** once the equilibration is complete, **stable retention**
  - For **gradient** HILIC methods **~10 column volumes** are required between injections for **reproducible retention times / separation**

<< APPLICATION AND CONDITION DEPENDENT >>

Sample Diluent on ACE HILIC-N







- **Sample solvent** is very important in HILIC
- **Too little MeCN** can result in split peaks/poor peak shape (i.e. tyramine)
- **Too much MeCN** and the sample might not fully dissolve (i.e. hypoxanthine)

Gradient HILIC Mobile Phases – Concentrations & Gradients

- **Many MPA & MPB lines have differing concentrations:**

X

  - MPA = 96:4 v/v MeCN:10 mM ammonium formate pH 3.0 — Actual concentration (0.4mM)
  - MPB = 1:1 v/v MeCN:10 mM ammonium formate pH 3.0 — (5mM)

- **Match the buffer concentration in MPA & MPB:**

✓

  - **MPA =** 10 mM ammonium formate pH 3.0 in MeCN/H_2O (96:4 v/v) — (10mM)
  - **MPB =** 10 mM ammonium formate pH 3.0 in MeCN/H_2O (1:1 v/v) — (10mM)

Ionic strength gradients lead to poor retention reproducibility – especially in HILIC

HILIC Hints and Tips Overview

- Balance buffer concentration in MPA and MPB for reproducible retention
- Ensure adequate equilibration (>~60 column volumes) when first setting up the HILIC method for isocratic or gradient HILIC work
- Include ~20 column volumes equilibration to re-establish the hydration later for subsequent column use
- Don't use high water containing sample diluents…but do experiment for your application what is best with the column
- Protic solvents in your HILIC mobile phases may disrupt the water layer and give poor retention robustness
- Don't use inorganic salts (precipitation worry) or ion-pair reagents (disrupt hydration layer) in HILIC methods
- Store ACE HILIC columns in IPA for maximum column lifetimes

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Part 2

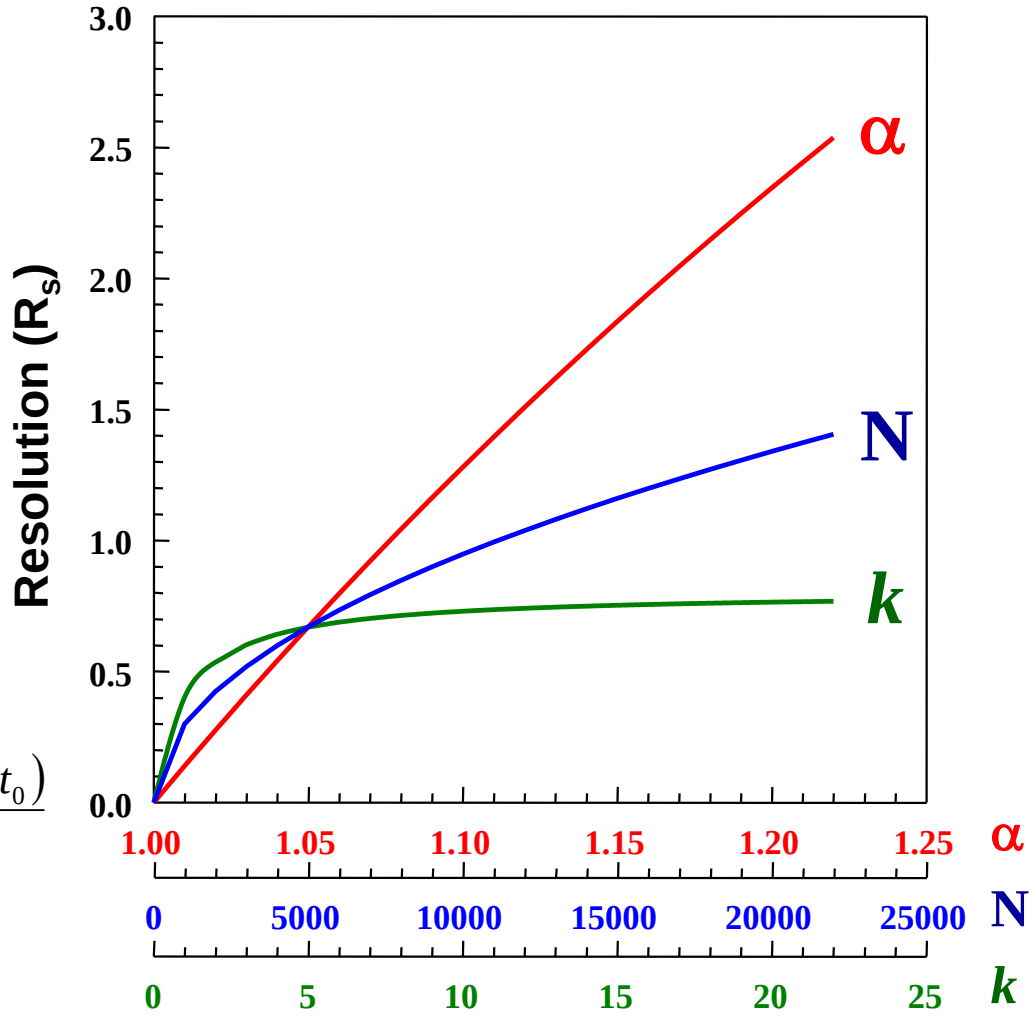
- **Resolution, Selectivity & Why They Matter**
- **Simple Protocol-based Method Development Approach**
- **Example – Caffeine and Related Polar Compounds**

Chromatographic Peak Resolution

Efficiency | Selectivity | Retention

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k}{1+k}$$

$$N = 5.54\left(\frac{t_r}{w_{0.5}}\right)^2 \qquad \alpha = \frac{k_2}{k_1} \qquad k = \frac{(t_R - t_0)}{t_0}$$



Zhao, J.H. and P.W. Carr. Analytical Chemistry, (1999) 71, 2623-2632

Simple Method Development Approach

Overview of HILIC method development:

- **Step 1: Assess analytes (if known)**
- **Step 2: Scouting runs – up to three pH values, three stationary phases, 1 gradient run**
- **Step 3: Optimise – gradient or isocratic, % organic, buffer concentration, temperature**
- **Step 4: Validate**
- **Step 5: Transfer / Implement**

HILIC Method Development Flow Chart

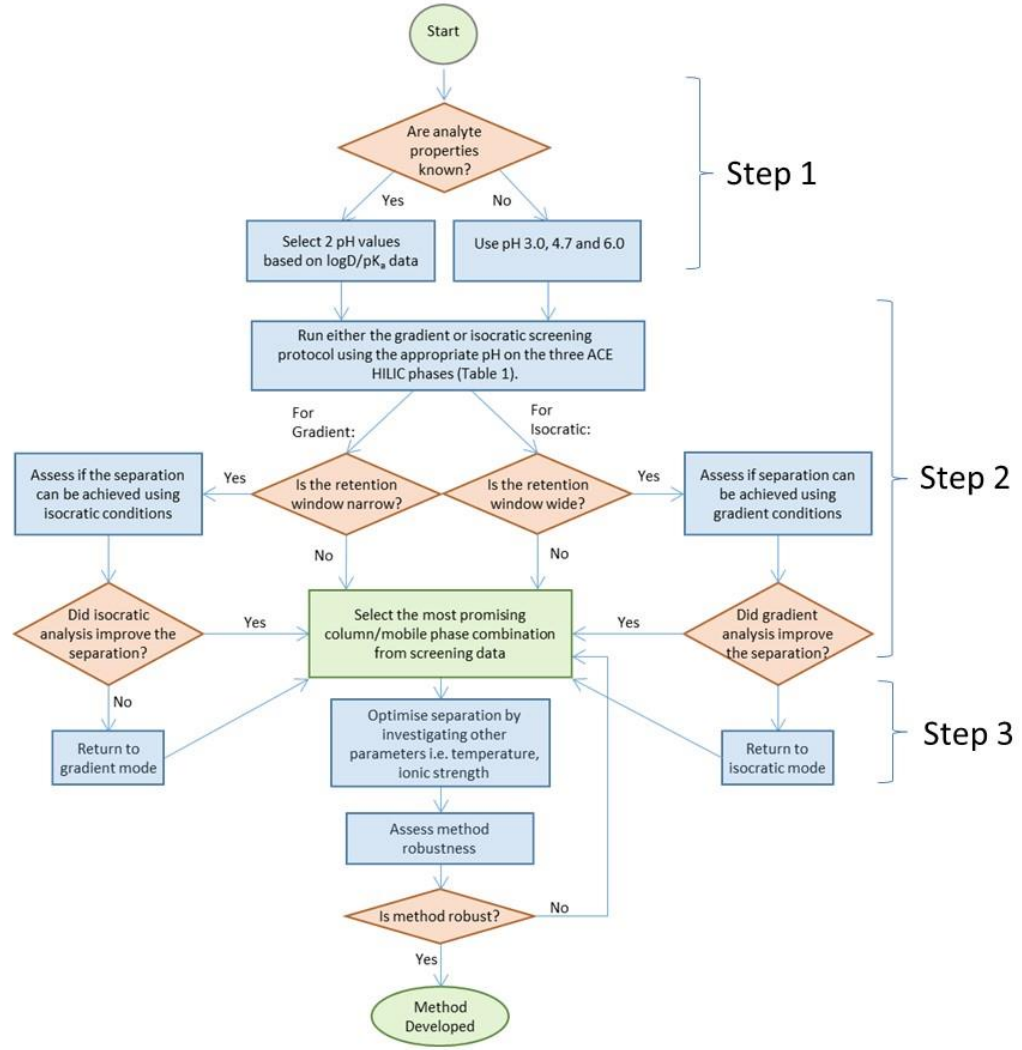


Figure 4: Flow diagram for step-by-step HILIC Method Development protocol.

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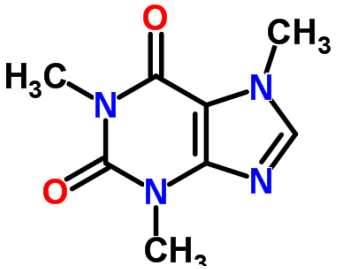
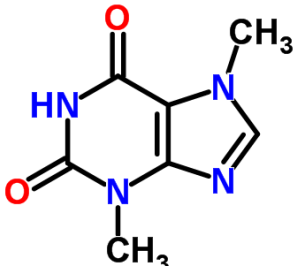
Gradient screen	
Time	**%B**
0	0
15	100
20	100
21	0
41	0
Isocratic mobile phase	10 mM ammonium formate in MeCN/H_2O (90:10 v/v) Ammonium formate is at pH 3.0, 4.7 or 6.0.
Flow rate	1.5 mL/min
Temperature	25 °C
Detection	Dependent on sample

Example – Caffeine and Related Compounds

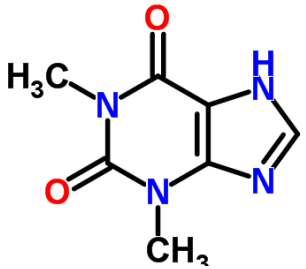
- **Good rule of thumb: If an analyte elutes before caffeine in reversed phase, HILIC might be suitable.**
- **Analytes: Having as much information as possible about the analytes of interest will help with method development. The Log P of caffeine and the related substances suggest they possess reasonable hydrophilicity therefore HILIC might be a suitable separation mode.**



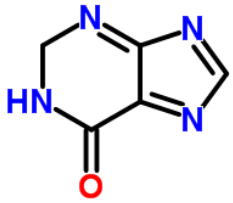
Caffeine
logP -0.13



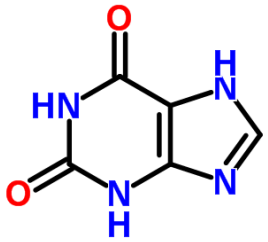
Theobromine
logP -2.08



Theophylline
logP -0.17



Hypoxanthine
logP -1.74



Xanthine
logP -1.99

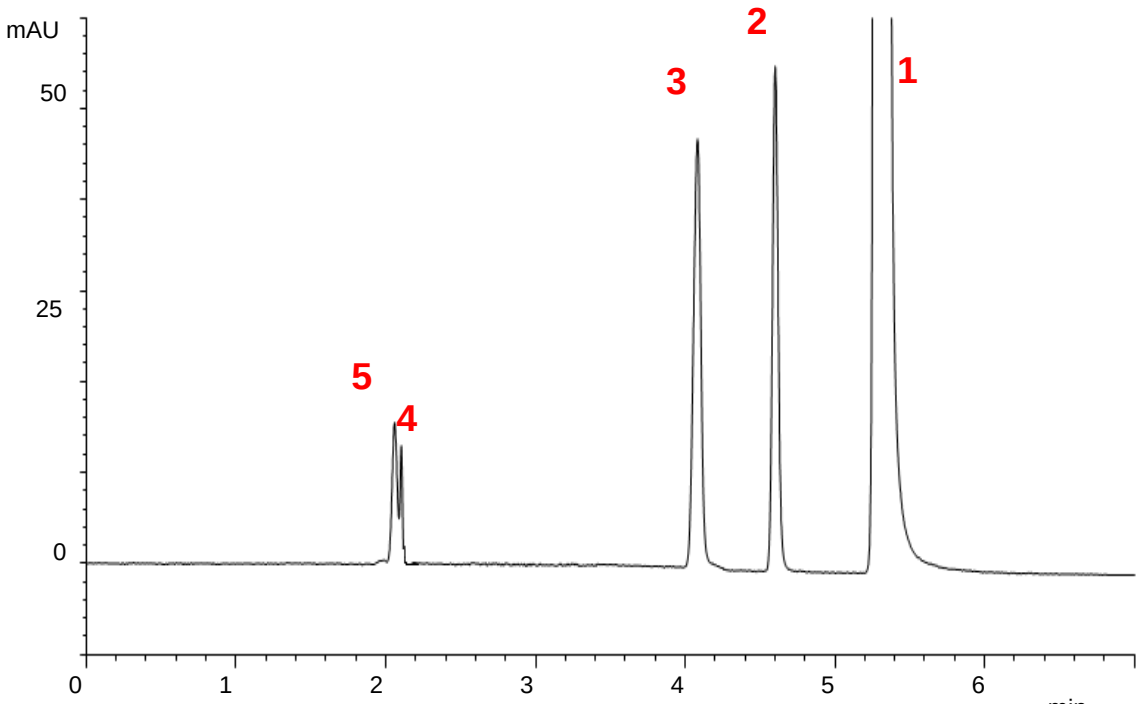
Example – Is HILIC Necessary?

Caffeine and related compounds (0.5% w/w) were chromatographed on an ACE C18 column using a generic gradient.

All analytes eluted before caffeine (Peak 1).

Two of the five analytes had low retention factor (k) and were unresolved.

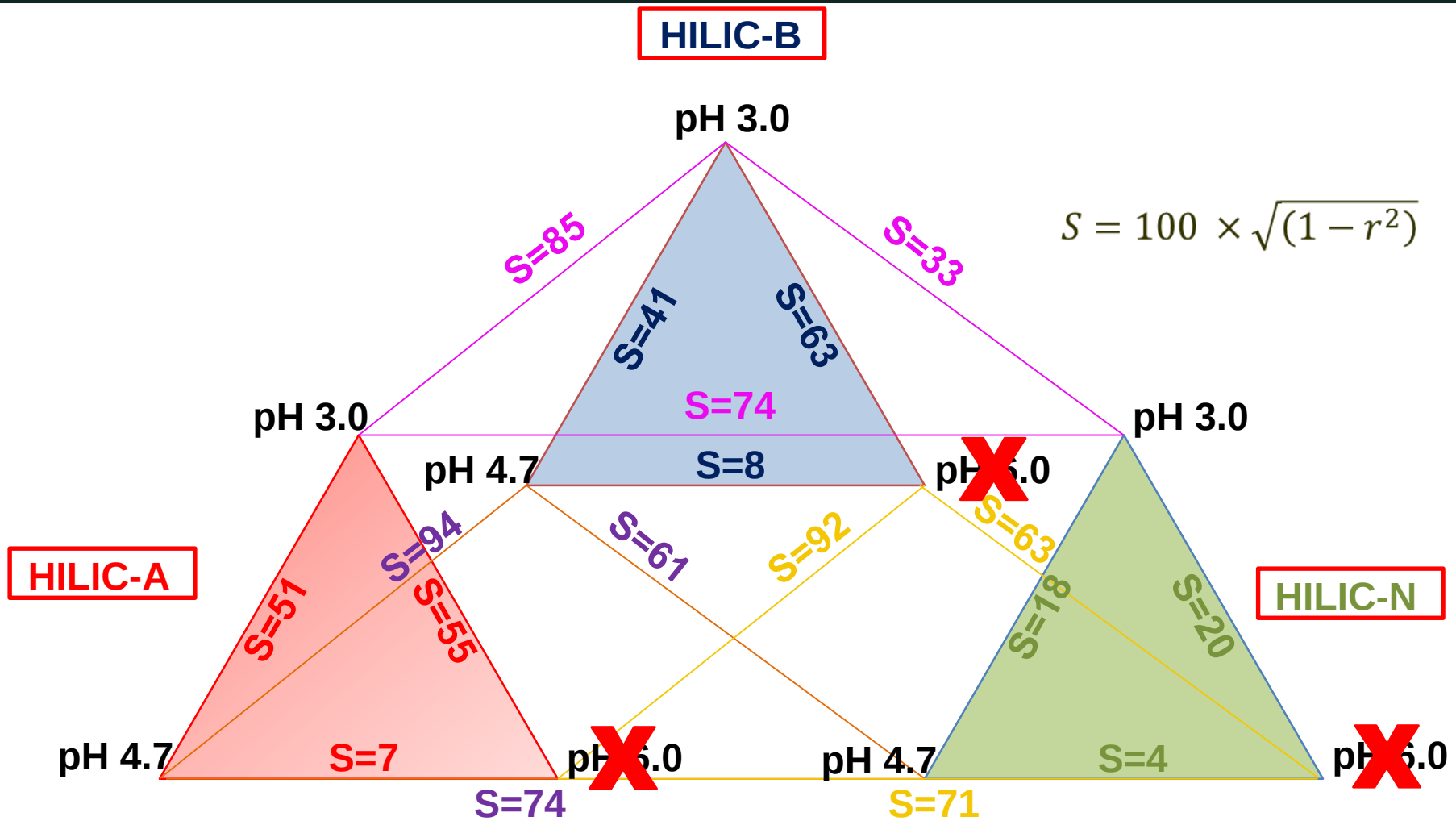
Therefore HILIC might provide a suitable alternative to RPLC



1. Caffeine, 2. Theophylline, 3. Theobromine, 4. Xanthine, 5. Hypoxanthine

Column: ACE 5 C18, 150 x 4.6 mm, Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O gradient – 5-95%MeCN in 15 mins, B: 10 mM ammonium formate pH 3.0 in MeCN/H_2O, Flow: 1 mL/min, Detection: UV, 275 nm, Injection: 2 µL,

Example – Selectivity Preparation



Caffeine and related substances are polar neutral compounds, therefore pH 6.0 is not required.
pH 3.0 and 4.7 on all three stationary phases will be screened using a generic gradient.

Example – Gradient Screening Conditions

Initial Column Equilibration: 80 column volumes for steady state

Sample: 25 mg/mL caffeine mixture with 0.5% w/w related substances in MeCN/H_2O (90:10 v/v)

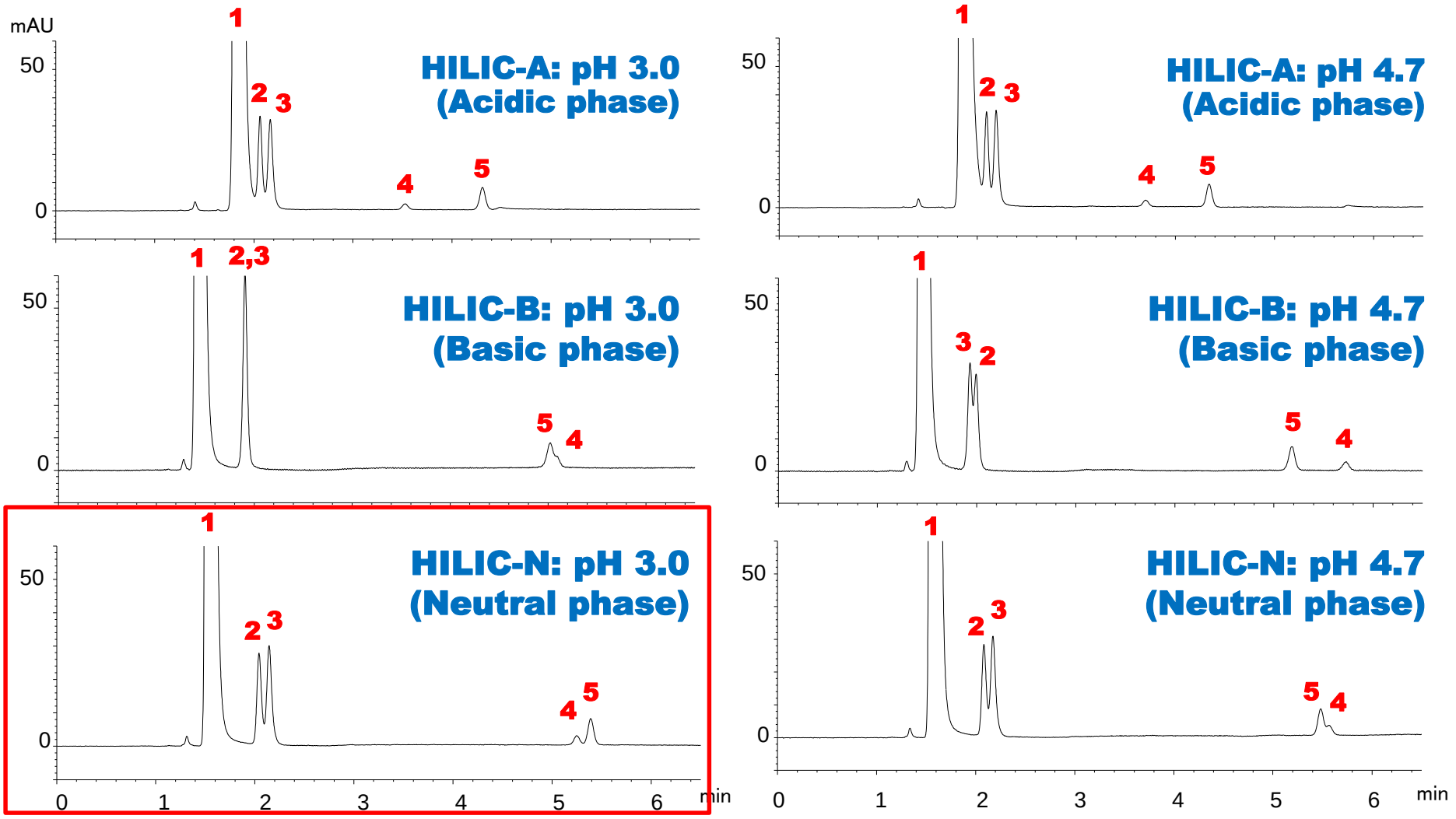
Columns: ACE HILIC-A, ACE HILIC-B and ACE HILIC-N (150x4.6mm, 5µm)
Mobile Phase A: 10 mM ammonium formate pH 3.0 & 4.7 in MeCN/H_2O (96:4 v/v)
Mobile phase B: 10 mM ammonium formate pH 3.0, 4.7 in MeCN/H_2O (50:50 v/v)
Gradient:

Time	%B
0	0
15	100
20	100
21	0
41	0

Flow Rate: 1.5 mL/min
Temperature: 25 °C
Detection: UV, 275 nm
Injection Volume: 2 µL

Example – Gradient Screening

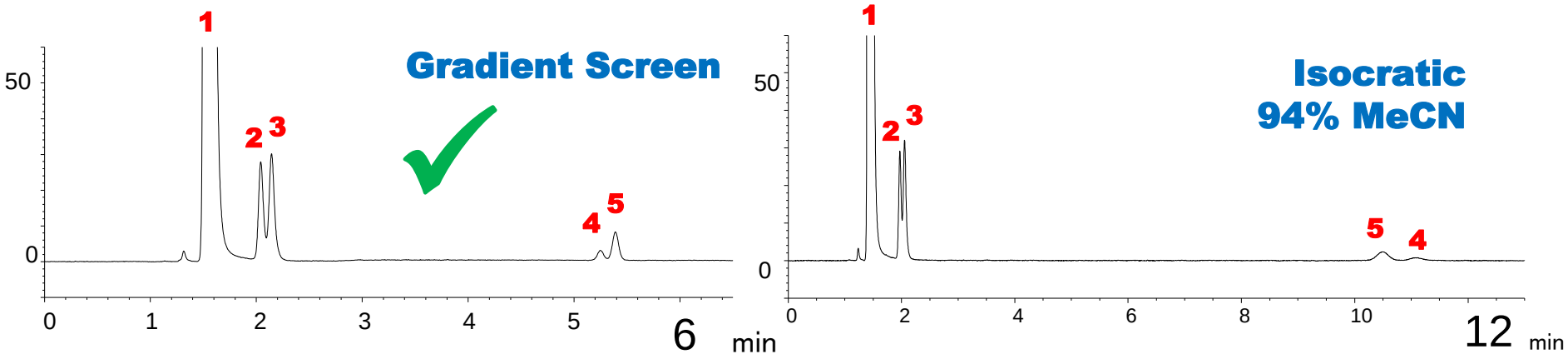
The screen suggests HILIC-N at pH 3.0 is should be developed further.



(1) Caffeine (2) Theophylline (3) Theobromine (4) Xanthine (5) Hypoxanthine

Example - Optimisation

Gradient or Isocratic?

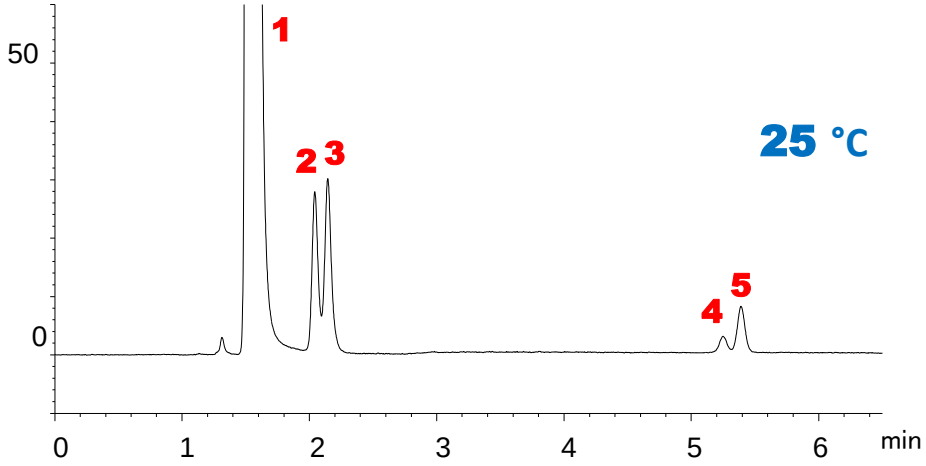


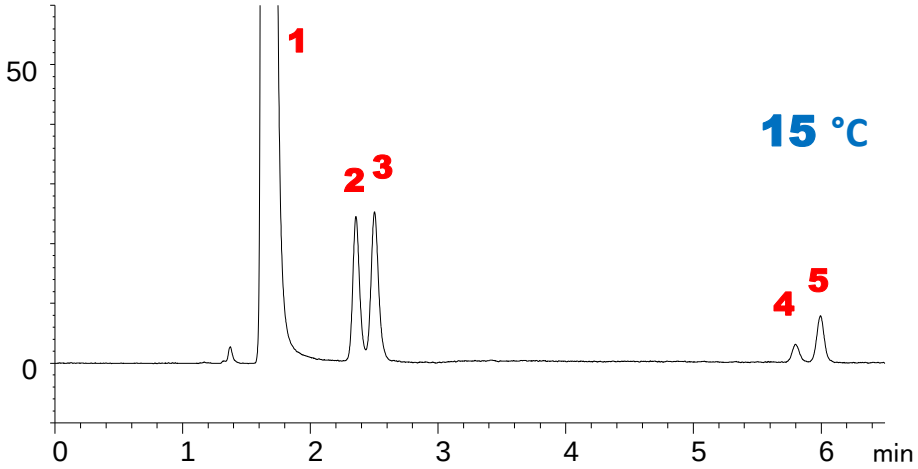
Isocratic greatly retained peaks 4 and 5, but failed to resolve peaks 2 and 3. Therefore, isocratic is not possible with this sample.

Further optimisation must be performed using gradient analysis.

Example - Optimisation

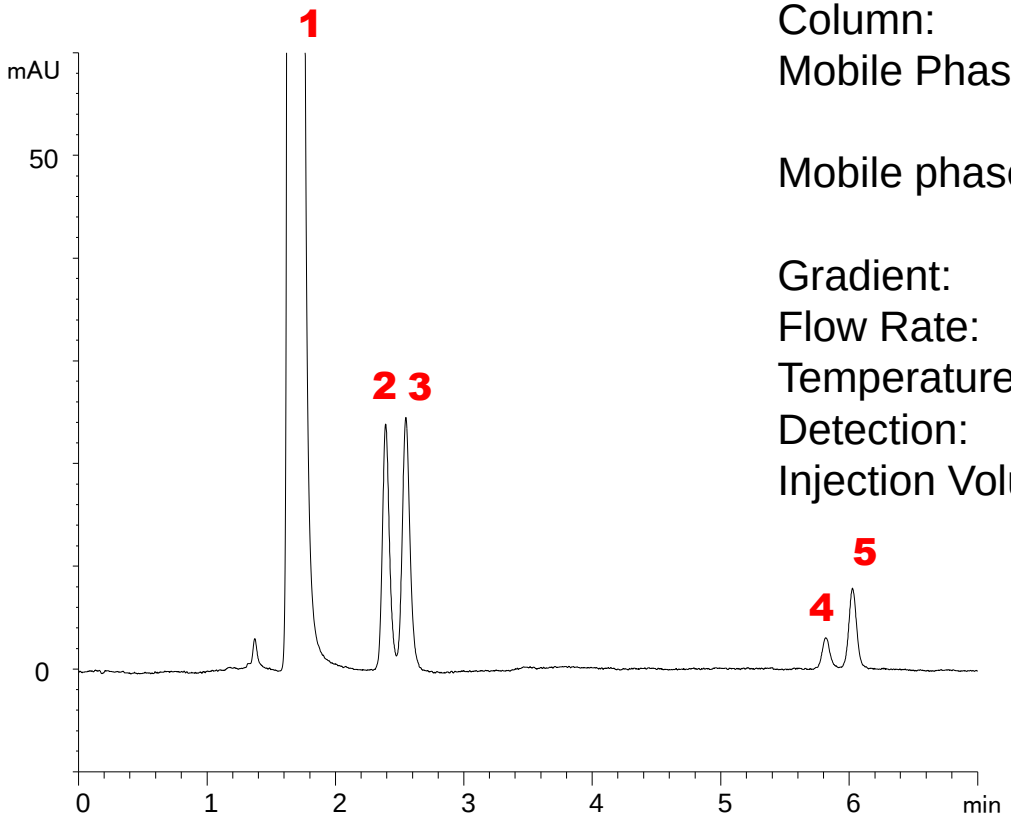
Effect of Temperature?





Decreasing the temperature has increased the resolution between the critical pair (Peaks 2 and 3) to within acceptable limits for this separation.

Example - Final Method



Final Method

Column:	ACE HILIC-N, 150x4.6mm, 5µm
Mobile Phase A:	10 mM ammonium formate pH 3.0 in MeCN/H_2O (96:4 v/v)
Mobile phase B:	10 mM ammonium formate pH 3.0 in MeCN/H_2O (50:50 v/v)
Gradient:	0-100%B in 15 minutes
Flow Rate:	1.5 mL/min
Temperature:	15 °C
Detection:	UV, 275 nm
Injection Volume:	2 µL

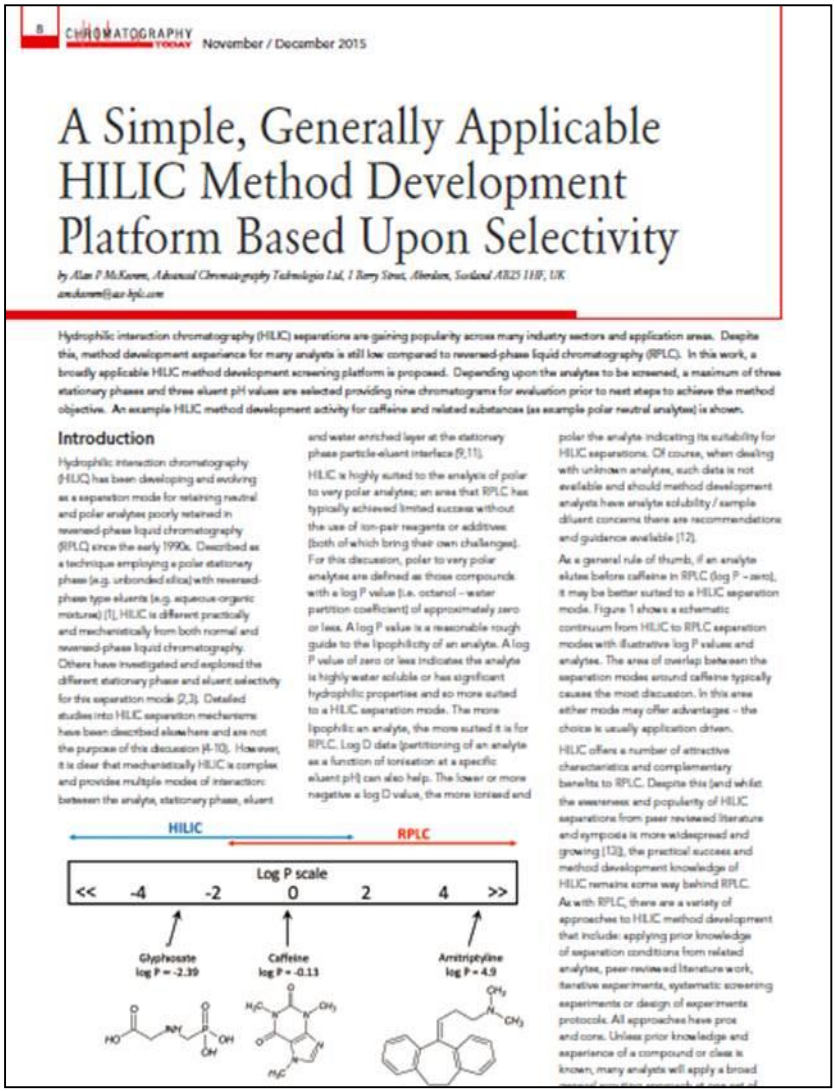
Method fit for purpose? ✓

(1) Caffeine (2) Theophylline (3) Theobromine (4) Xanthine (5) Hypoxanthine

Summary

- HILIC can provide alternative selectivity to reversed phase chromatography but can be mechanistically complex
- A screening protocol with complementary phases can be helpful
- Selectivity is the key to chromatographic resolution
- Eluent pH can be powerful in HILIC whether it is method development, screening or isolation activities
- Consider your sample and ensure adequate equil time

HILIC Method Development / Screening Protocol Article



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A Simple, Generally Applicable HILIC Method Development Platform Based Upon Selectivity

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Hydrophilic interaction chromatography (HILIC) separations are gaining popularity across many industry sectors and application areas. Despite this, method development experience for many analysts is still low compared to reversed-phase liquid chromatography (RPLC). In this work, a broadly applicable HILIC method development screening platform is proposed. Depending upon the analytes to be screened, a maximum of three stationary phases and three eluent pH values are selected providing nine chromatograms for evaluation prior to next steps to achieve the method objective. An example HILIC method development activity for caffeine and related substances (as example polar neutral analytes) is shown.

Introduction

Hydrophilic interaction chromatography (HILIC) has been developing and evolving as a separation mode for retaining neutral and polar analytes poorly retained in reversed-phase liquid chromatography (RPLC) since the early 1990s. Described as a technique employing a polar stationary phase (e.g. unbonded silica) with reversed-phase type eluents (e.g. aqueous-organic mixtures) [1], HILIC is different practically and mechanistically from both normal and reversed-phase liquid chromatography. Others have investigated and explored the different stationary phase and eluent selectivity for this separation mode [2,3]. Detailed studies into HILIC separation mechanisms have been described elsewhere and are not the purpose of this discussion [4-10]. However, it is clear that mechanistically HILIC is complex and provides multiple modes of interaction between the analyte, stationary phase, eluent and water enriched layer at the stationary phase particle-eluent interface [9,11].

HILIC is highly suited to the analysis of polar to very polar analytes; an area that RPLC has typically achieved limited success without the use of ion-pair reagents or additives (both of which bring their own challenges). For this discussion, polar to very polar analytes are defined as those compounds with a log P value (i.e. octanol – water partition coefficient) of approximately zero or less. A log P value is a reasonable rough guide to the lipophilicity of an analyte. A log P value of zero or less indicates the analyte is highly water soluble or has significant hydrophilic properties and so more suited to a HILIC separation mode. The more lipophilic an analyte, the more suited it is for RPLC. Log D data (partitioning of an analyte as a function of ionisation at a specific eluent pH) can also help. The lower or more negative a log D value, the more ionised and polar the analyte indicating its suitability for HILIC separations. Of course, when dealing with unknown analytes, such data is not available and should method development analysts have analyte solubility / sample diluent concerns there are recommendations and guidance available [12].

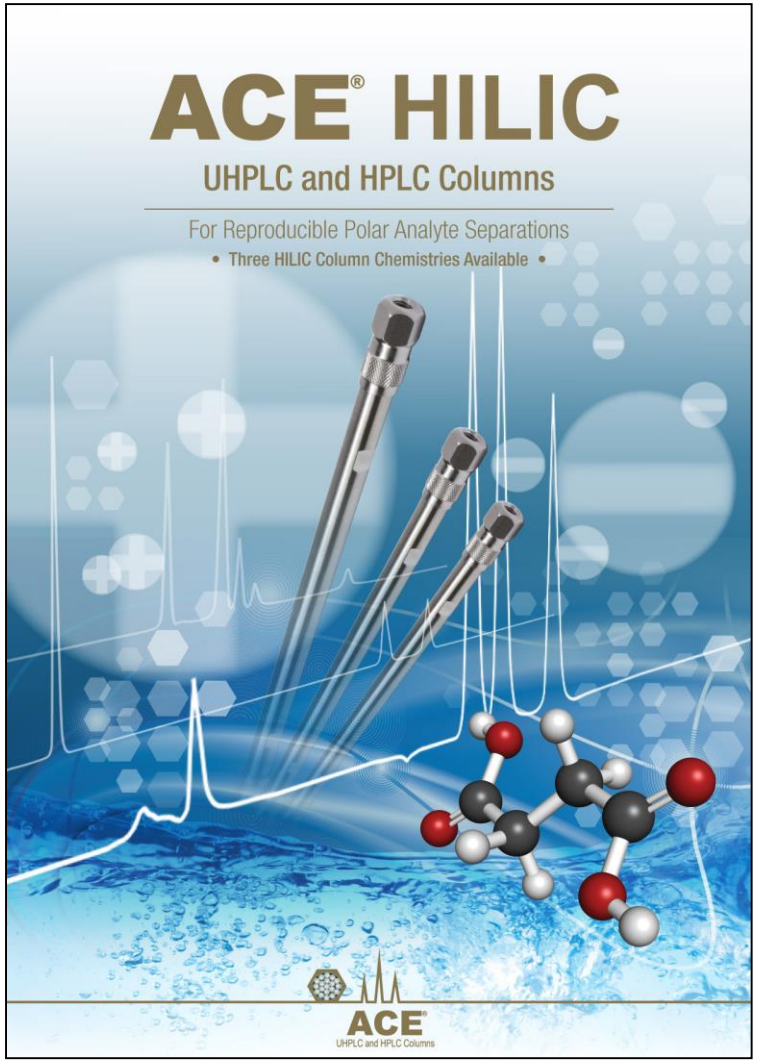
As a general rule of thumb, if an analyte elutes before caffeine in RPLC (log P ~ zero), it may be better suited to a HILIC separation mode. Figure 1 shows a schematic continuum from HILIC to RPLC separation modes with illustrative log P values and analytes. The area of overlap between the separation modes around caffeine typically causes the most discussion. In this area either mode may offer advantages – the choice is usually application driven.

HILIC offers a number of attractive characteristics and complementary benefits to RPLC. Despite this (and whilst the awareness and popularity of HILIC separations from peer reviewed literature and symposia is more widespread and growing [13]), the practical success and method development knowledge of HILIC remains some way behind RPLC.

As with RPLC, there are a variety of approaches to HILIC method development that include: applying prior knowledge of separation conditions from related analytes, peer-reviewed literature work, iterative experiments, systematic screening experiments or design of experiments protocols. All approaches have pros and cons. Unless prior knowledge and experience of a compound or class is known, many analytes will apply a broad

A P McKeown, *Chrom. Today*, Nov / Dec 2015, 8-12

ACE HILIC Brochure: Pre-order / Launched at HPLC2017



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