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

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Advanced Chromatography Technologies Ltd

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Part 1

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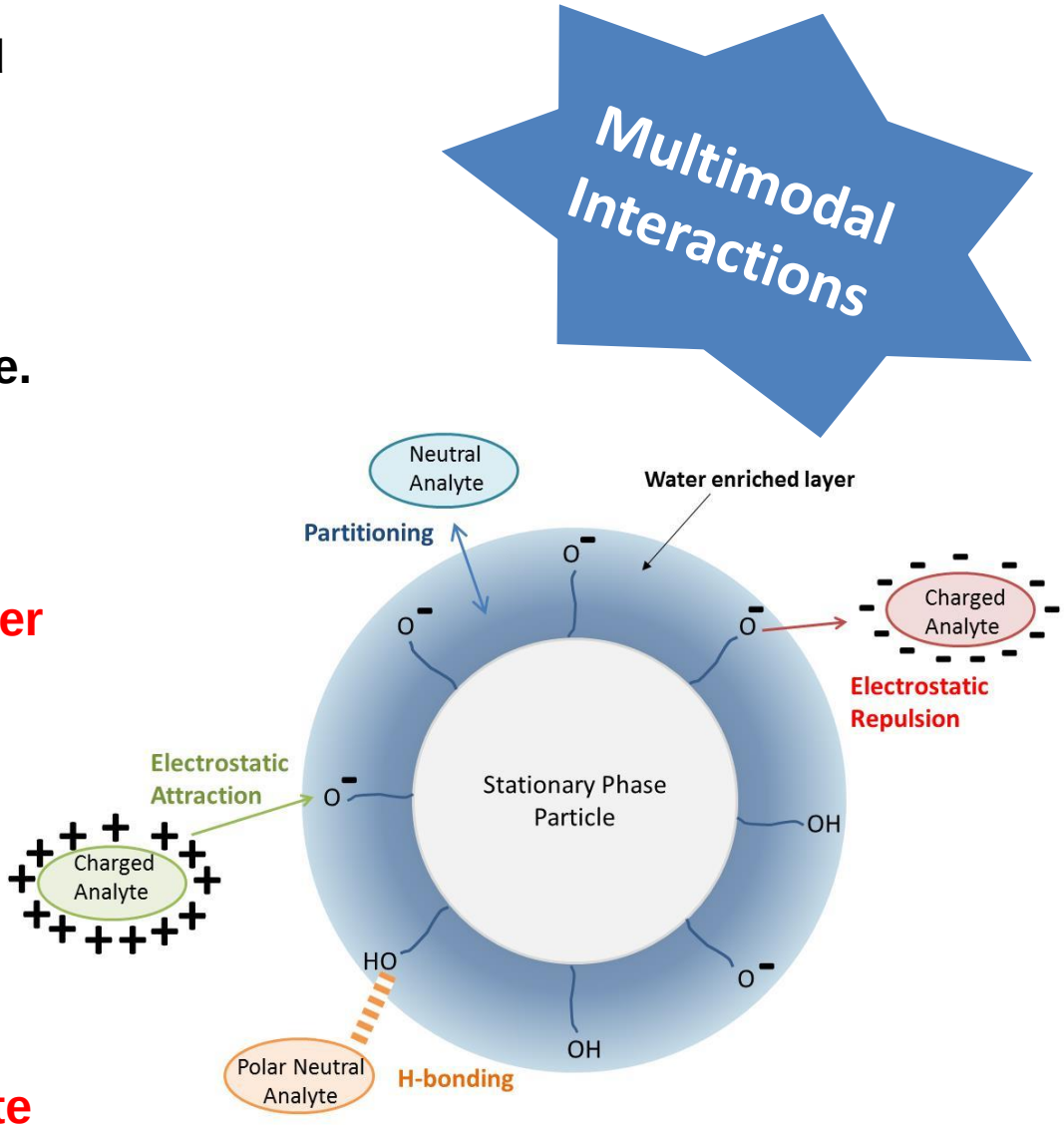
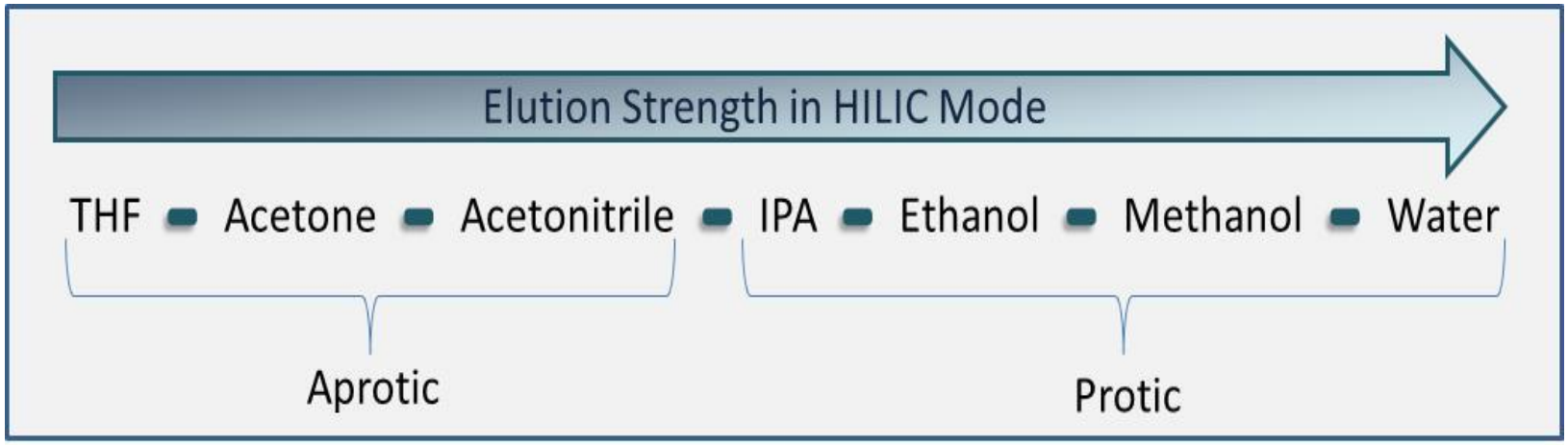
What is HILIC?

- 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

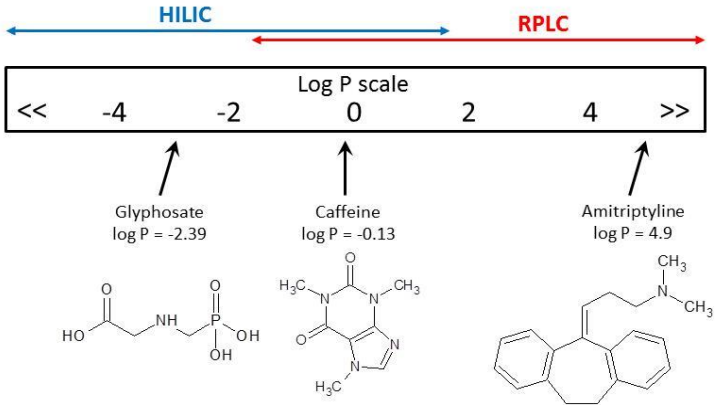


- **Bulk solvent = MeCN**
- **~3% minimum aqueous required to hydrate the water layer around particles for partitioning.**
- **(MeOH (other protic solvents) in the bulk solvent may disrupt the water layer leading to reduced retention repeatability).**

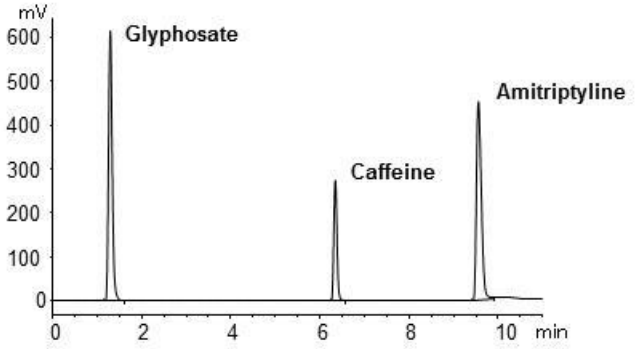
When Should Analysts Use HILIC?

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

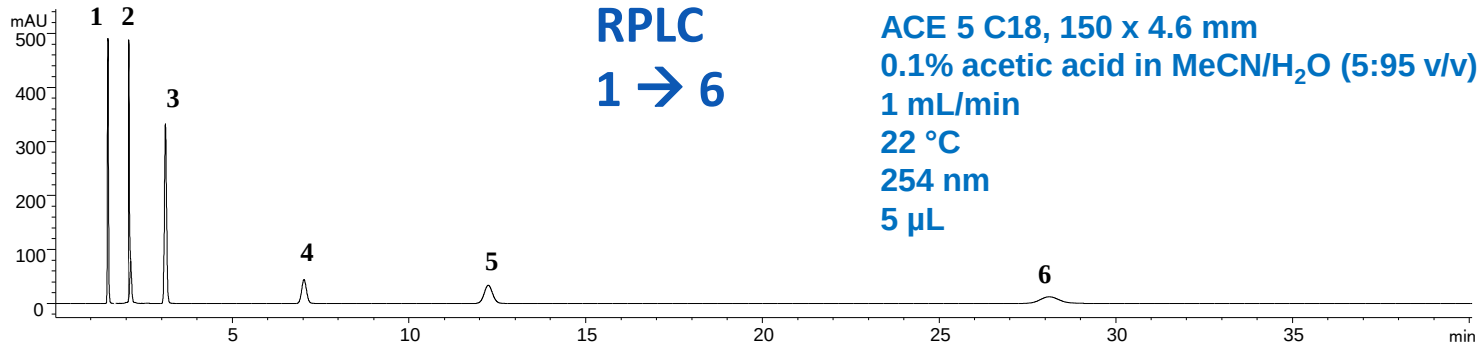
*** Reproduced from A P McKeown, *Chrom. Today*, Nov / Dec 2015, 8-12**

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.**

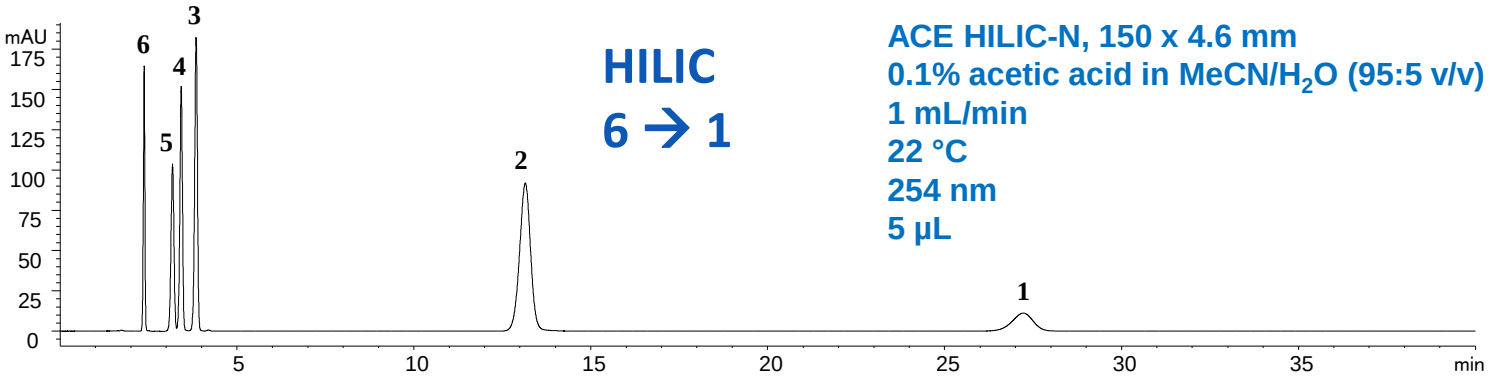
2. **HILIC provides orthogonal selectivity to RPLC.**

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



RPLC
1 → 6

ACE 5 C18, 150 x 4.6 mm
0.1% acetic acid in MeCN/H_2O (5:95 v/v)
1 mL/min
22 °C
254 nm
5 µL



HILIC
6 → 1

ACE HILIC-N, 150 x 4.6 mm
0.1% acetic acid in MeCN/H_2O (95:5 v/v)
1 mL/min
22 °C
254 nm
5 µL

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

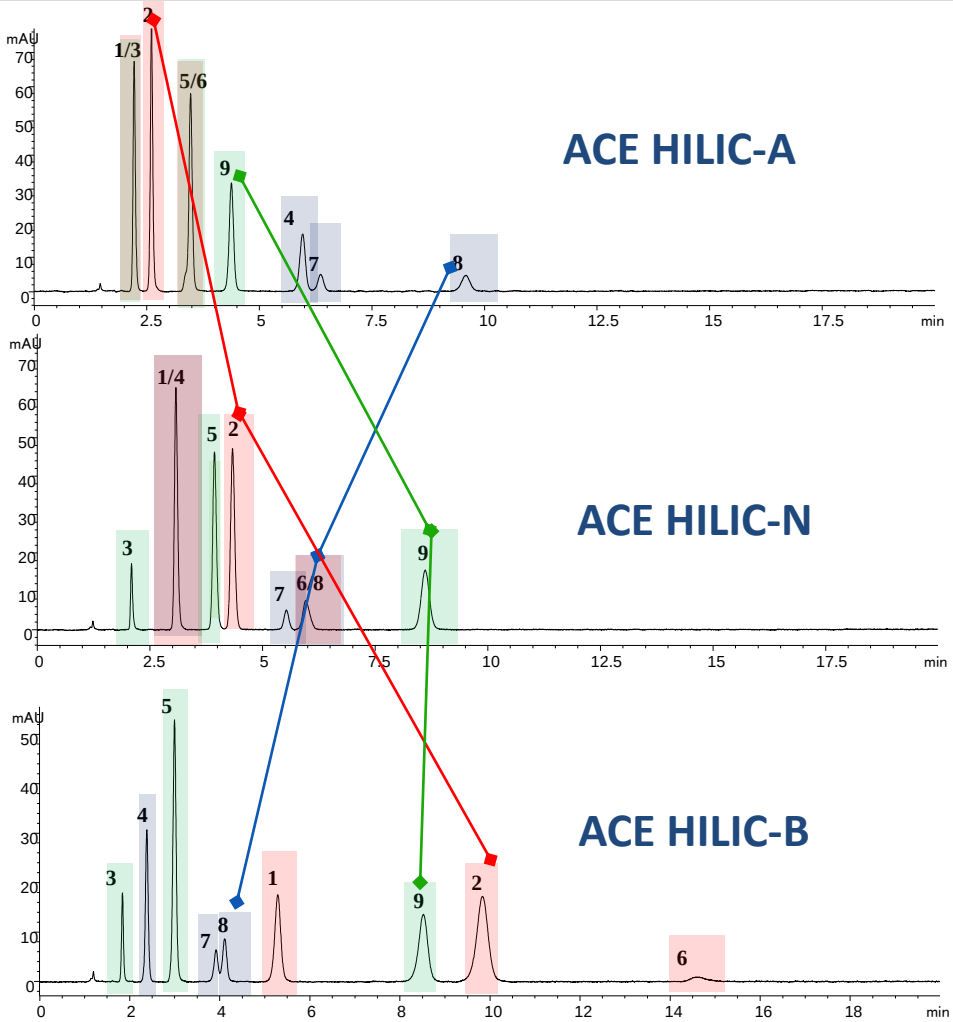
- HILIC stationary phases can be split into acidic, basic, neutral and 'other / novel' e.g. Zwitterionic / cyclodextrin etc.

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

Phase Character		Partitioning	Anionic Analyte Interactions		Cationic Analyte Interactions		H-bonding
			Attraction	Repulsion	Attraction	Repulsion	
Acidic	ACE HILIC-A	**	-	***	****	-	**
Basic	ACE HILIC-B	***	****	-	-	***	**
Neutral	ACE HILIC-N	****	-	-	-	-	****

Approximate value – determined by semi-quantitative mechanism weightings using 54 different polar analytes

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,

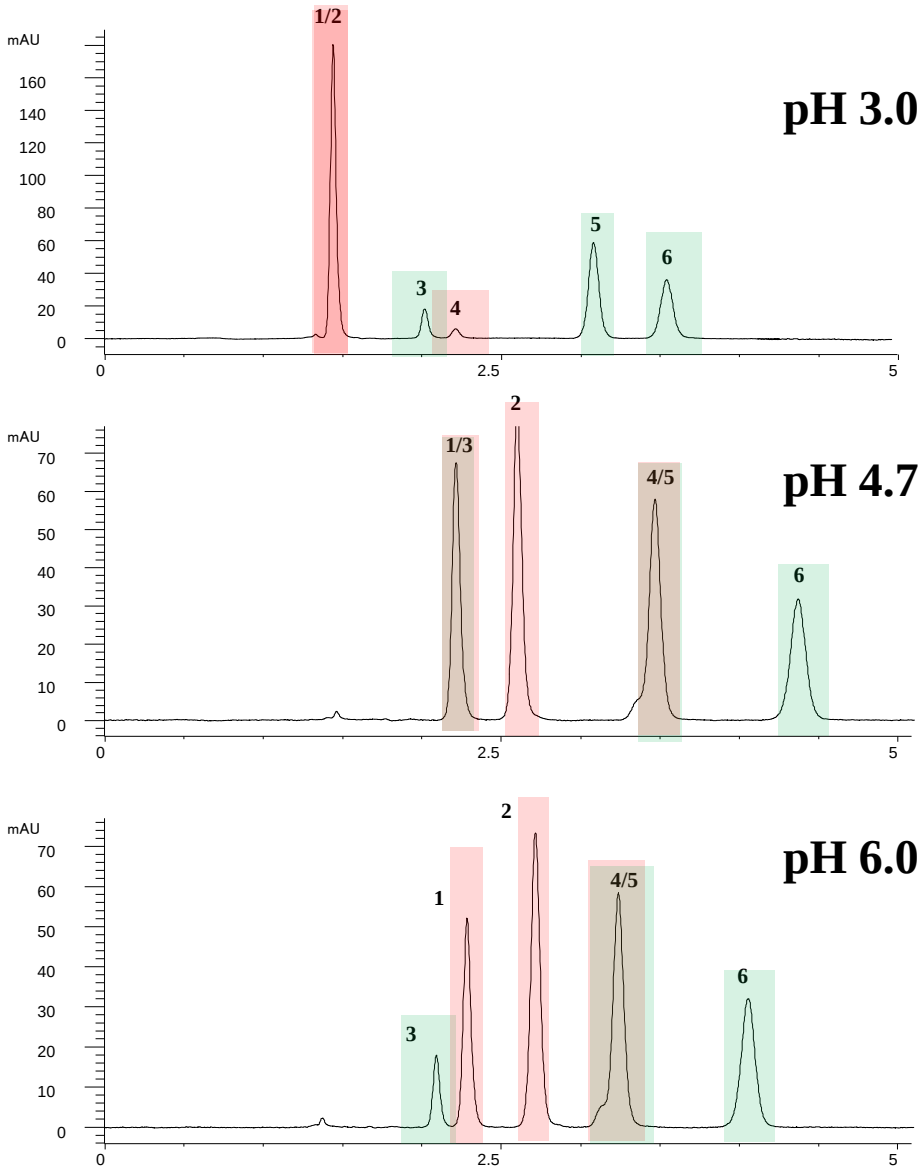
ACE® Specifications

June 2017 v1

PHASE	USP LISTING	FUNCTIONAL GROUP	ENDCAPPED	PARTICLE SIZE (µm)*	PORE SIZE (Å)	SURFACE AREA (m^2/g)	CARBON LOAD (%)	PH RANGE	100% AQ compatible
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	-
ACE NH_2	L8	Proprietary aminopropyl	Proprietary	1.7, 3, 5	100	300	4.0	2 – 7	YES
UltraCore SuperC18 (solid core)	L1	Octadecyl encapsulated	Encapsulated	2.5, 5	95	130 100	7.0 5.4	1.5 – 11.0	-
UltraCore SuperPhenylHexyl (solid core)	L11	Phenyl Hexyl encapsulated	Encapsulated	2.5, 5	95	130 100	4.6 3.6	1.5 – 11.0	-
CN-ES	L10	Cyano with extended alkyl spacer	Yes	1.7, 2, 3, 5, 10	100	300	12.6	2 – 8	YES
C18-Amide	L1 / L60	Polar embedded amide	Yes	1.7, 2, 3, 5, 10	100	300	17.0	2 – 8	YES
SuperC18	L1	Octadecyl encapsulated	Encapsulated	1.7, 2, 3, 5, 10	90	400	14.8	1.5 – 11.5	-
C18-AR	L1	Octadecyl with integral phenyl	Yes	1.7, 2, 3, 5, 10	100	300	15.5	2 – 8	YES
C18-PFP	L1	Octadecyl with integral PFP	Yes	1.7, 2, 3, 5, 10	100	300	14.3	2 – 8	YES
C18-HL	L1	Octadecyl	Yes	3, 5, 10, 15	90	400	20	2 – 8	-
C18 C18-300	L1 L1	Octadecyl Octadecyl	Yes Yes	1.7, 2, 3, 5, 10 3, 5, 10	100 300	300 100	15.5 9.0	2 – 8 2 – 8	-
C8 C8-300	L7 L7	Octyl Octyl	Yes Yes	2, 3, 5, 10 3, 5, 10	100 300	300 100	9.0 5.0	2 – 8 2 – 8	-
C4 C4-300	L26 L26	Butyl Butyl	Yes Yes	2, 3, 5, 10 3, 5, 10	100 300	300 100	5.5 2.6	2 – 8 2 – 8	-
CN CN-300	L10 L10	Cyano Cyano	Yes Yes	2, 3, 5, 10 3, 5, 10	100 300	300 100	5.5 2.6	2 – 7 2 – 7	-
Ph Ph-300	L11 L11	Phenyl Phenyl	Yes Yes	2, 3, 5, 10 3, 5, 10	100 300	300 100	9.5 5.3	2 – 8 2 – 8	-
AQ	L1	Proprietary	Yes	2, 3, 5, 10	100	300	14	2 – 8	YES
SIL	L3	Unbonded	No	2, 3, 5, 10	100	300	N/A	2 – 7	-

HILIC Eluent Parameters

Eluent pH: ACE HILIC-A (acidic character phase)

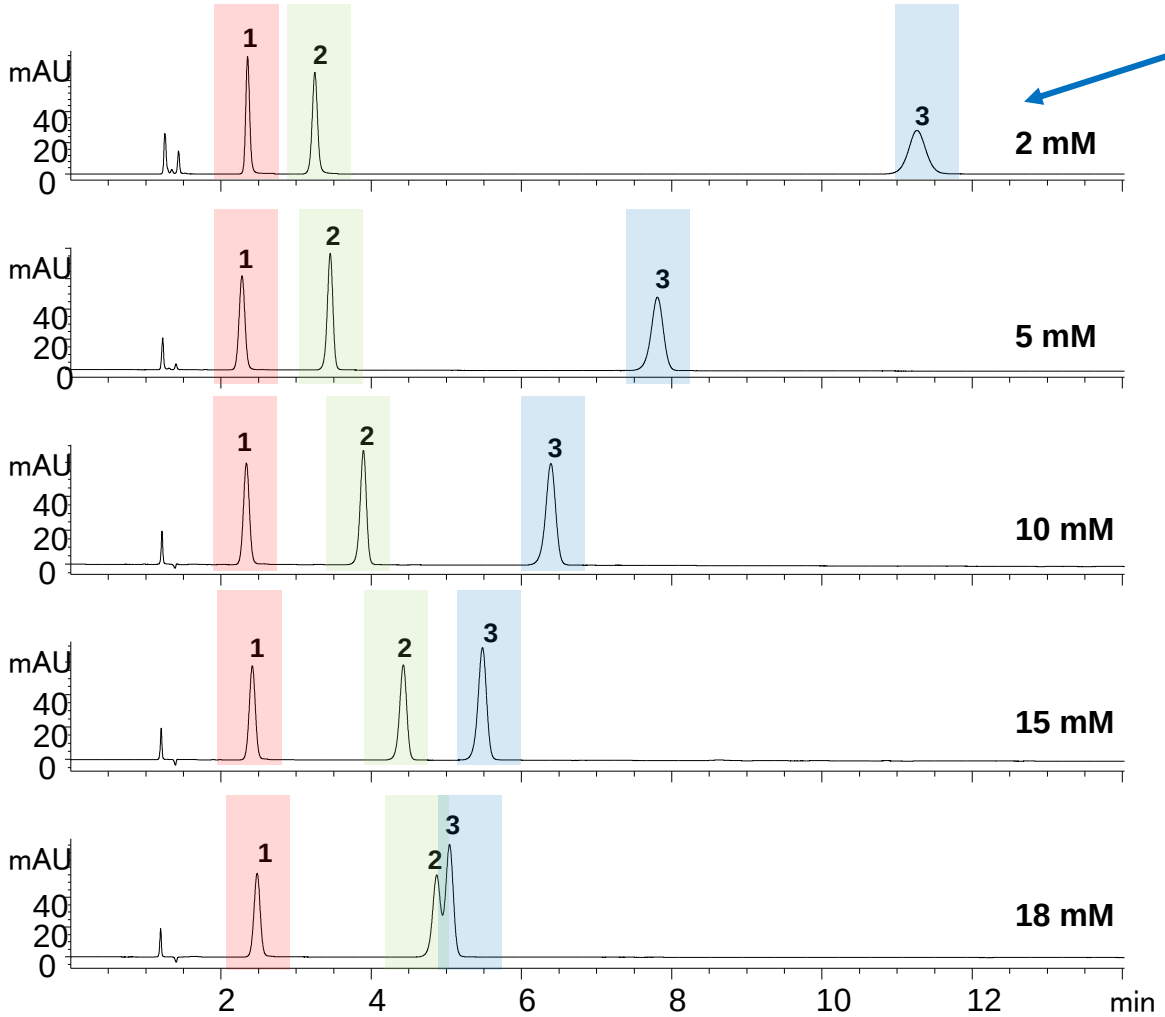


- **Eluent pH is a useful tool for method development.**
- **Eluent pH can change the ionisation state of the analyte.**
- **It can also change the ionisation state of the stationary phase**

1. p-Aminobenzoic acid, 2. 4-Hydroxybenzoic acid, 3. Nicotinamide, 4. Adenine, 5. Mandelic acid, 6. 2-Deoxyguanosine

Column: ACE HILIC-A, 10 mM ammonium formate in MeCN/H_2O (90:10 v/v), Flow: 1.5 mL/min, Detection: 254 nm, Temperature: 25 °C, Injection: 5 µL,

Effects of Buffer Concentration on Analytes with ACE HILIC-A



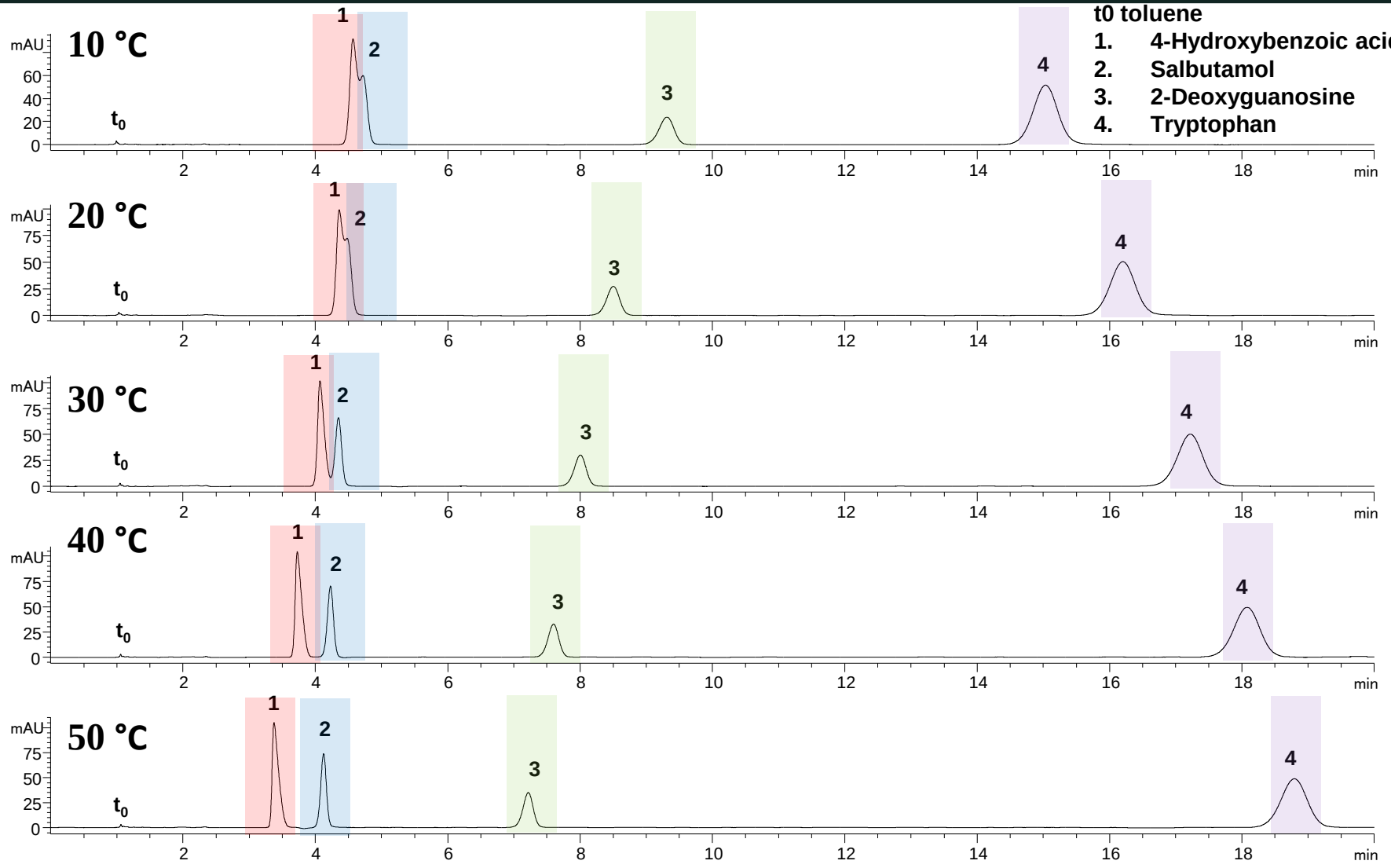
1. Pyridoxal **2. Cytidine** **3. Procainamide**

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: 1.5 mL/min, Detection: 254 nm, Temperature: 25 °C, Injection: 5 µL

Basic analyte retention changes are significant

- **Analyte – stationary phase interactions change**
- **Absorbed water layer properties may be effected**
- **Mechanisms of interaction likely to change (weightIngs of IEX / partitioning etc)**

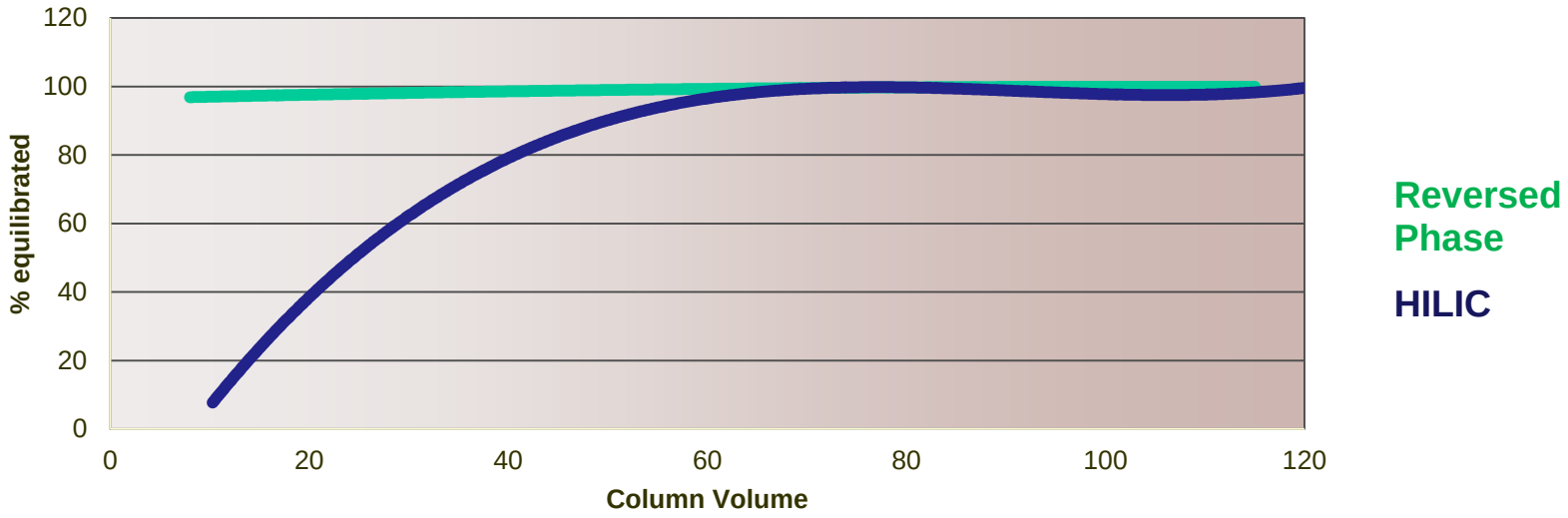
Influence of Temperature



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: 1.5 mL/min, Detection: UV, 230 nm, Injection: 5 µL

Key Topics

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 >>

Column Volumes and Equilibration Explained

- **Column volume is the amount of eluent in a column occupying the space between the particles and within the particle pores:**

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

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 such as pore size, surface area etc.**
  - **ACE 100Å porous columns, ε = 0.63**
  - **ACE 300Å porous columns, ε = 0.75**
  - **ACE 90Å solid core columns, ε = 0.55**

- **It is better to determine ε experimentally for the most accurate data**

- **Column volume is independent of flow rate and column dimension so is a better term to use to understand equilibration.**

Column Volumes and Equilibration Explained II

- **What does this all means practically?**

- **Typical column volumes:**

		Column lengths						
		20	30	50	75	100	150	250
	1.0	0.010	0.015	0.025	0.037	0.049	0.074	0.124
Column i.d.s	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

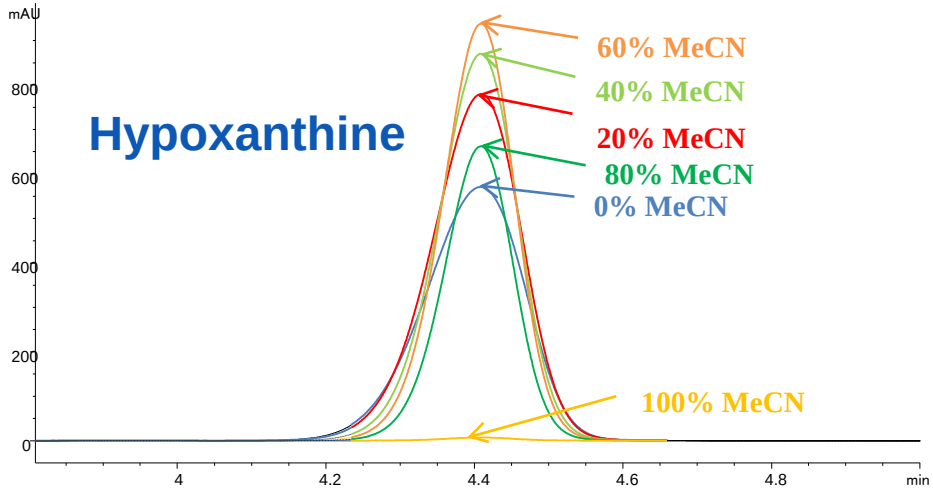
Table 1: Calculated ACE 100Å porous column volumes (mL)

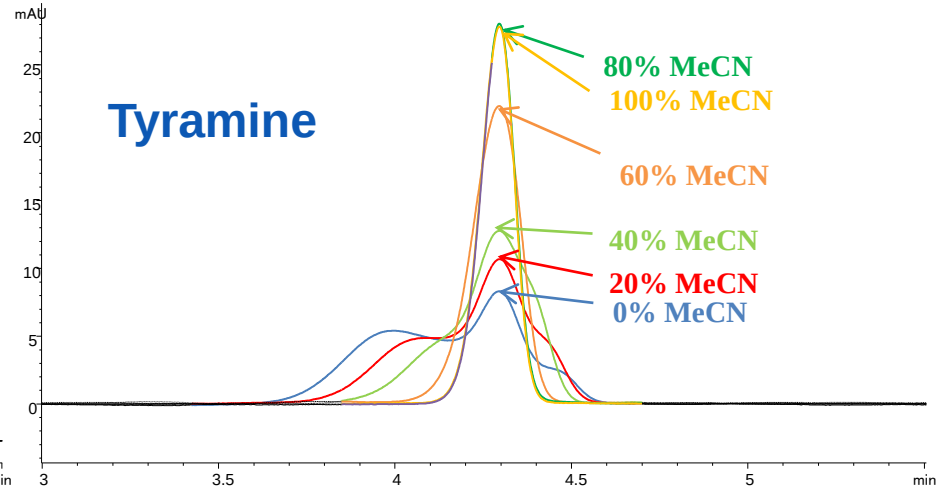
- **Equilibration times for example column dimensions / flow rates:**

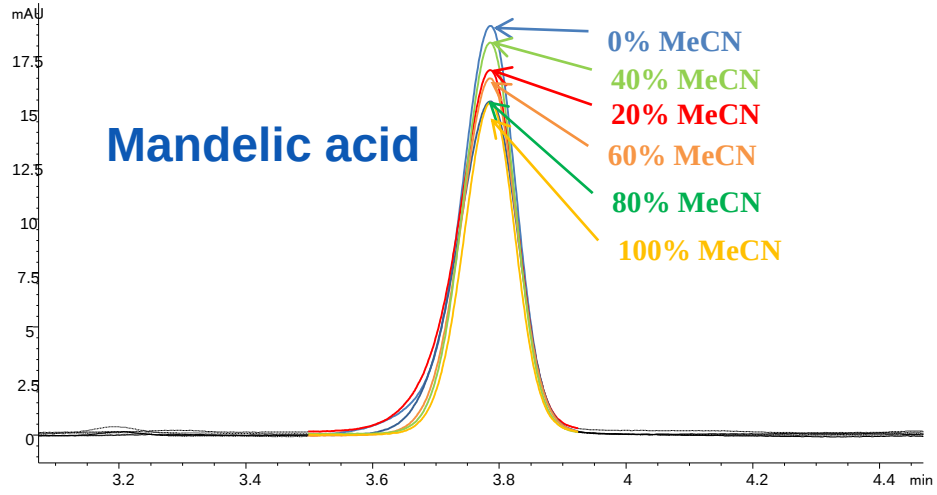
Column dimensions & particle size	Column Volume (mL)	Flow Rate (mL/min)	20 Col Vol Equilibration Time (mins)	60 Col Vol Equilibration Time From New (mins)
50x2.1mm, 1.7µm	0.109	0.21	10.4	31.2
50x2.1mm, 1.7µm	0.109	0.50	4.4	13.1
100x3.0mm, 3µm	0.445	1.00	8.9	26.7
150x4.6mm, 5µm	1.570	1.00	31.4	94.2
150x4.6mm, 5µm	1.570	2.00	15.7	47.1
250x4.6mm, 5µm	2.617	1.00	52.3	157.0
250x4.6mm, 5µm	2.617	2.00	26.2	78.5

Table 2: ACE 100Å porous column equilibration calculations

Sample Diluent on ACE HILIC-N

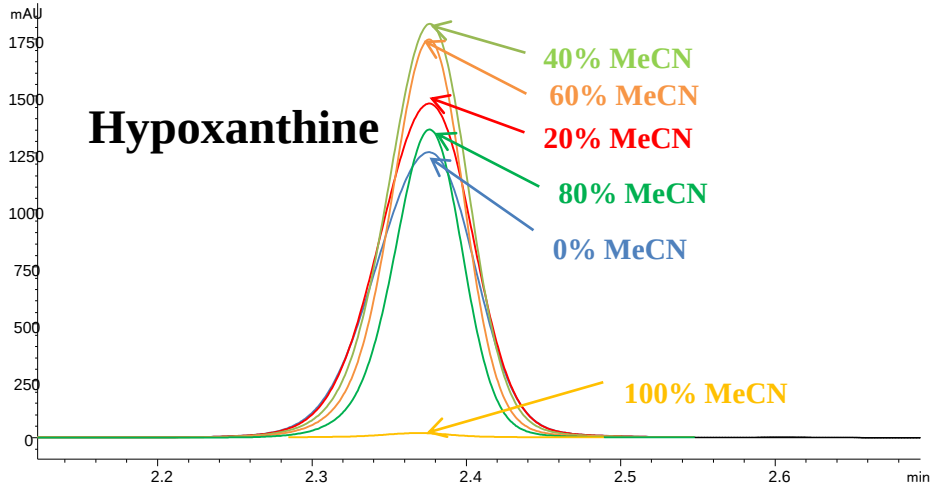


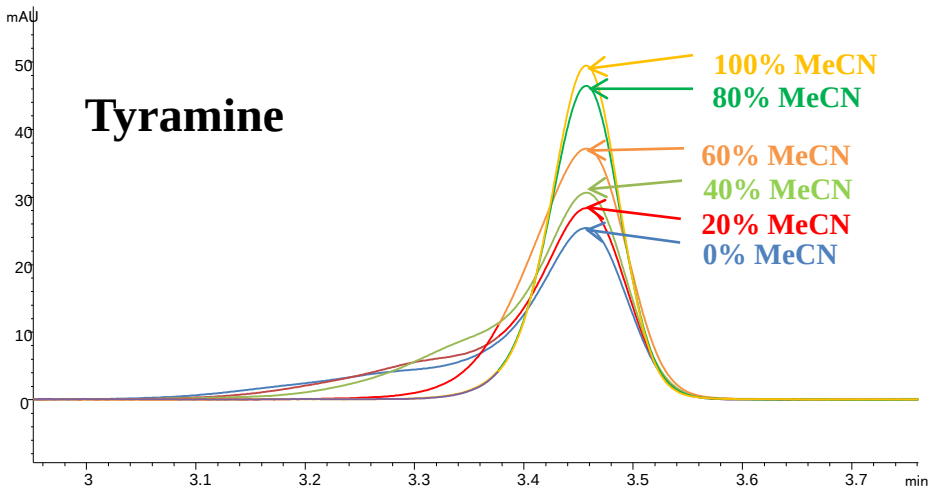


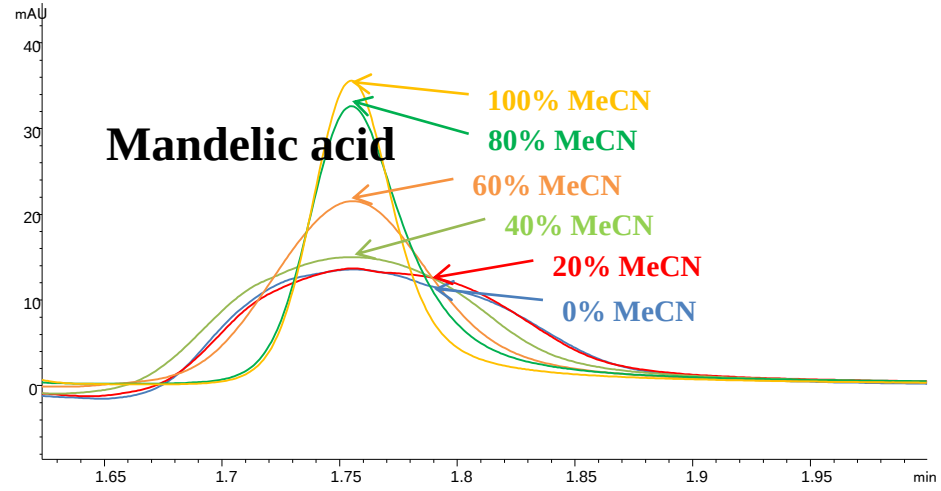


- **Sample solvent** is very important in HILIC
- **Too little MeCN** can result in split peaks/poor peak shape (i.e. tyramine)
- **Too much MeCN** and the sample might not fully dissolve (i.e. hypoxanthine)
- **Shine a laser pen through the vial**
- **Stationary phase** can also play a part

Sample Diluent on ACE HILIC-A

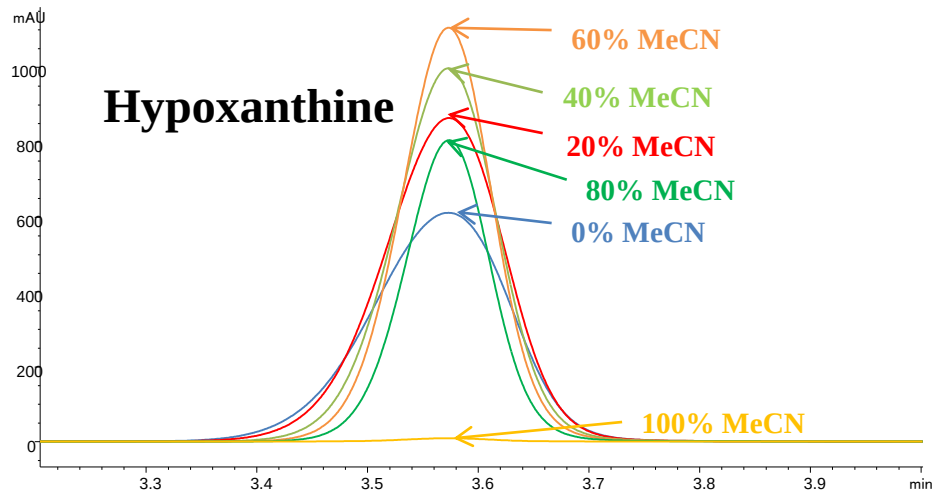


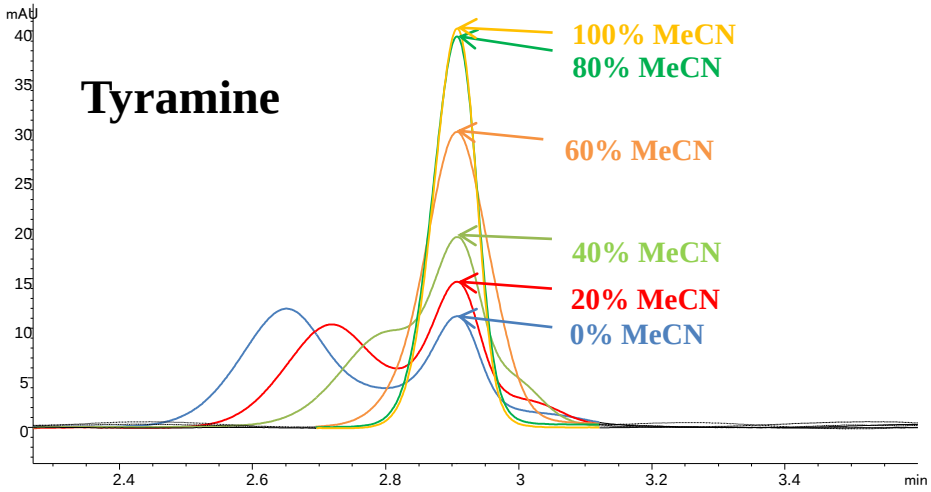


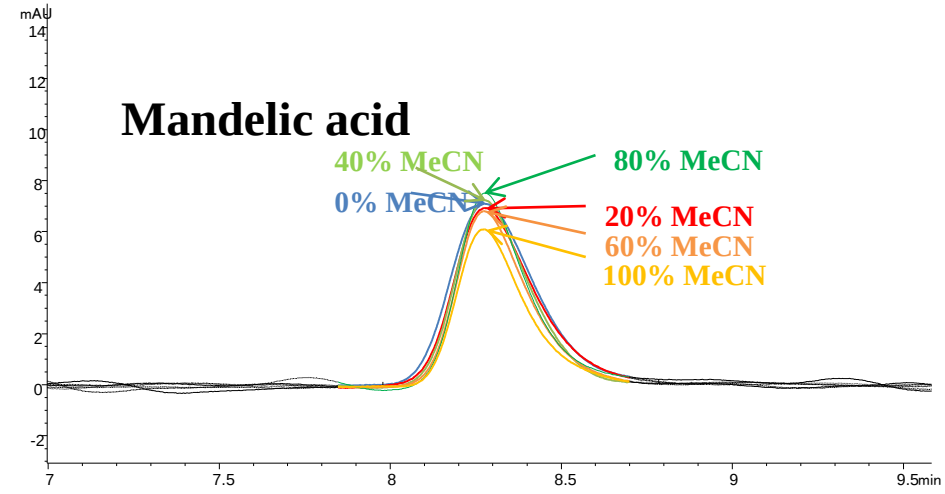


- **Stationary phase specific effects are also possible – ionic repulsion / overloading?**

Sample Diluent on ACE HILIC-B







- **Stationary phase specific effects** are also possible – **ionic repulsion / overloading**?
- Recommendation : **systematically** explore diluents of **20% - 80% volume fraction MeCN**

Hints and Tips

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

Providing Help for HILIC & 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**

↓

Understand the HILIC technique; useful hints & tips

↓

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

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

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

Contents – Part 2

Part 1

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  - Column Equilibration
  - Sample Diluent

Part 2

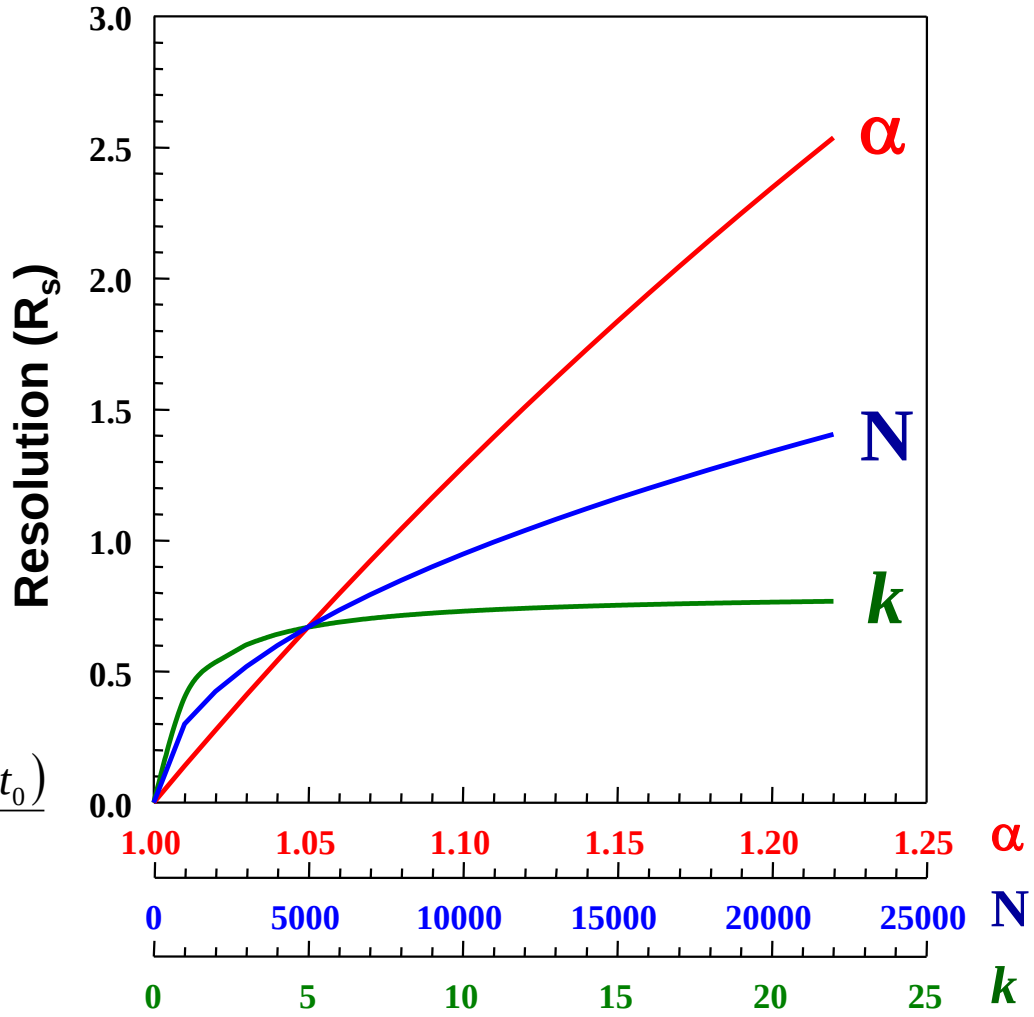
- **Resolution, Selectivity & Why They Matter**
- **Simple Method Development Approach**
- **Example – Caffeine and Related Compounds**

Chromatographic Peak Resolution

Efficiency | Selectivity | Retention

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

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



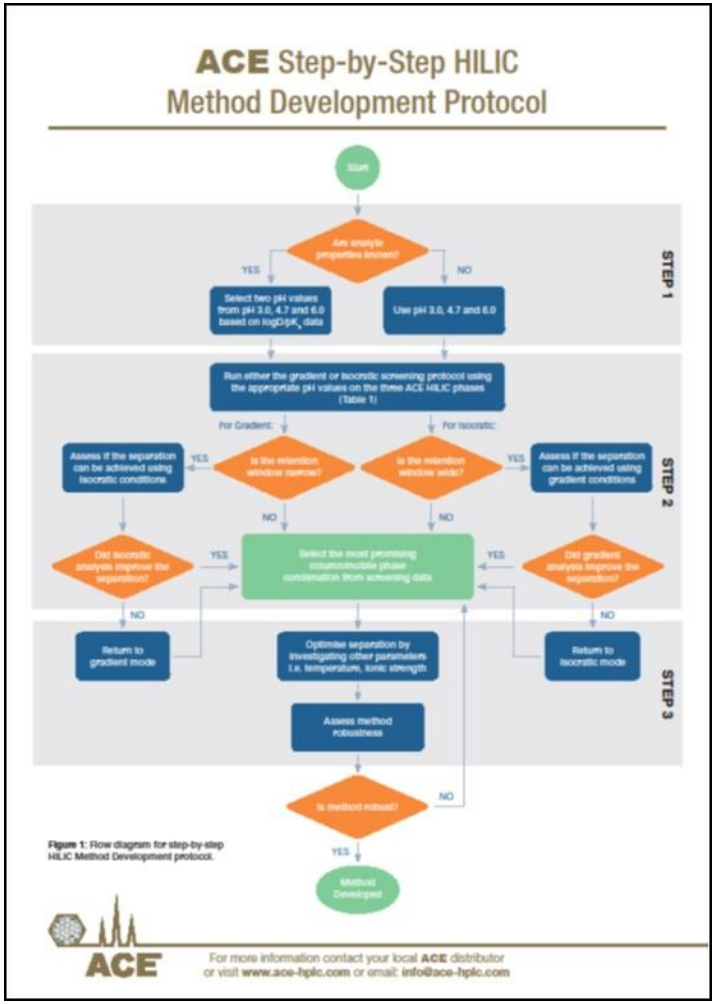
Zhao, J.H. and P.W. Carr. Analytical Chemistry, (1999) 71, 2623-2632

Simple Method Development Approach

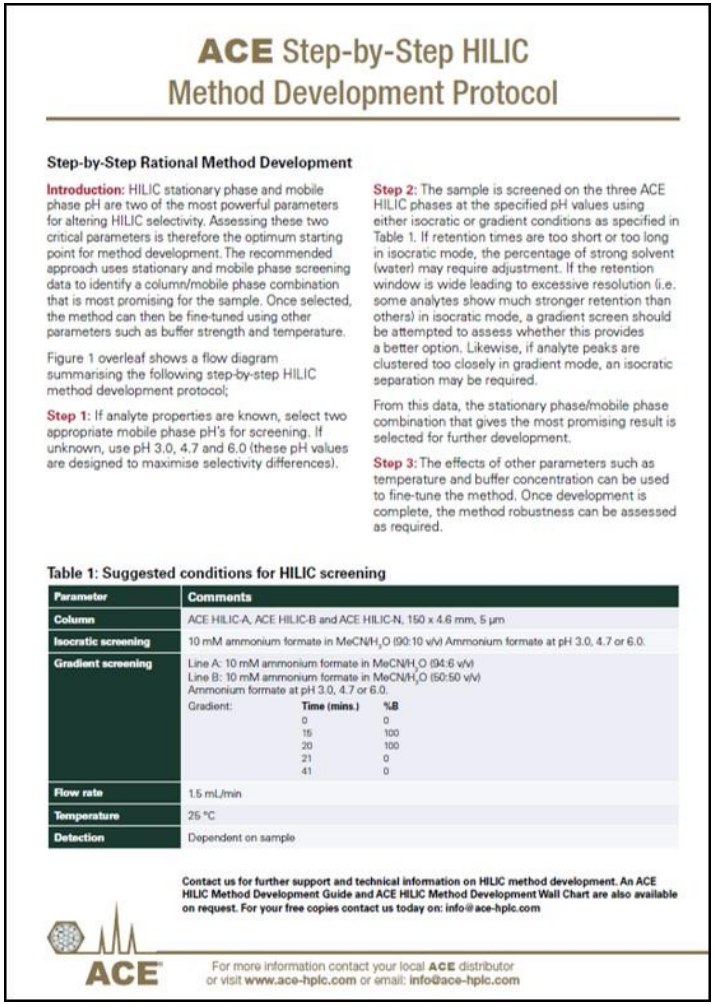
Overview of HILIC method development:

- **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**

ACE HILIC Meth Dev Flow Chart



Front



ACE Step-by-Step HILIC Method Development Protocol

Step-by-Step Rational Method Development

Introduction: HILIC stationary phase and mobile phase pH are two of the 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 1 overleaf shows a flow diagram summarising the following step-by-step HILIC method development protocol;

Step 1: If analyte properties are known, select two 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 as specified in Table 1. If retention times are too short or too 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 this 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.

Table 1: Suggested conditions for HILIC screening

Parameter	Comments
Column	ACE HILIC-A, ACE HILIC-B and ACE HILIC-N, 150 x 4.6 mm, 5 µm
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

Contact us for further support and technical information on HILIC method development. An ACE HILIC Method Development Guide and ACE HILIC Method Development Wall Chart are also available on request. For your free copies contact us today on: info@ace-hplc.com

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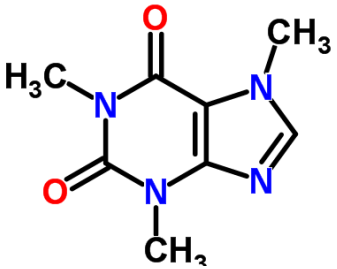
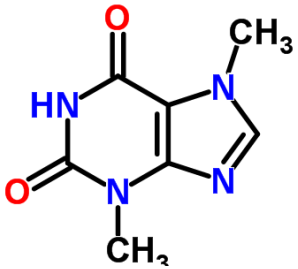
Example – Caffeine and Related Compounds

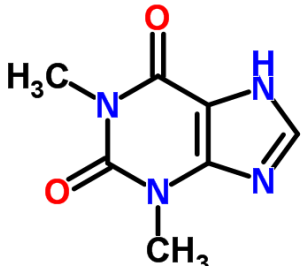
- **Good rule of thumb: If an analyte elutes before caffeine in reversed phase, HILIC might be suitable.**
- **Analytes: Having as much information as possible about the analytes of interest will help with method development. The Log P of caffeine and the related substances suggest they possess reasonable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



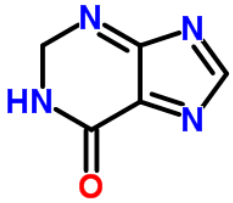
1. Caffeine
logP -0.13



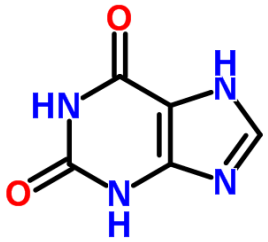
3. Theobromine
logP -0.72



2. Theophylline
logP -0.17



5. Hypoxanthine
logP -0.91



4. Xanthine
logP -0.81

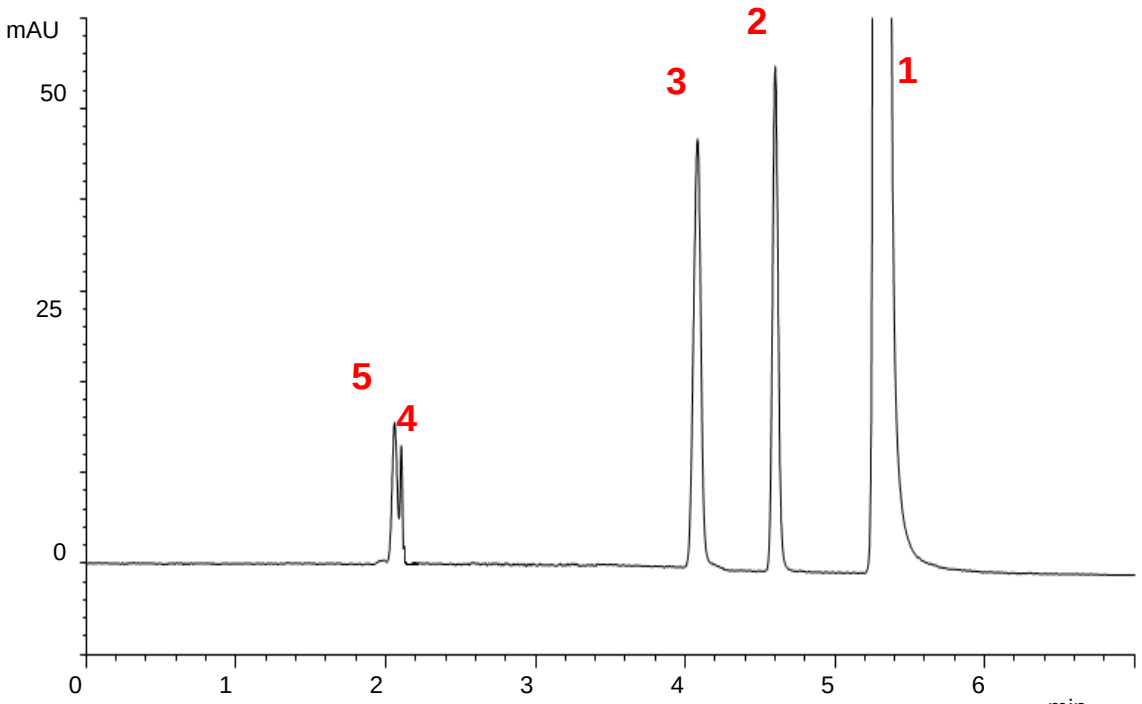
Example – Is HILIC Necessary?

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 factors (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 – 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)

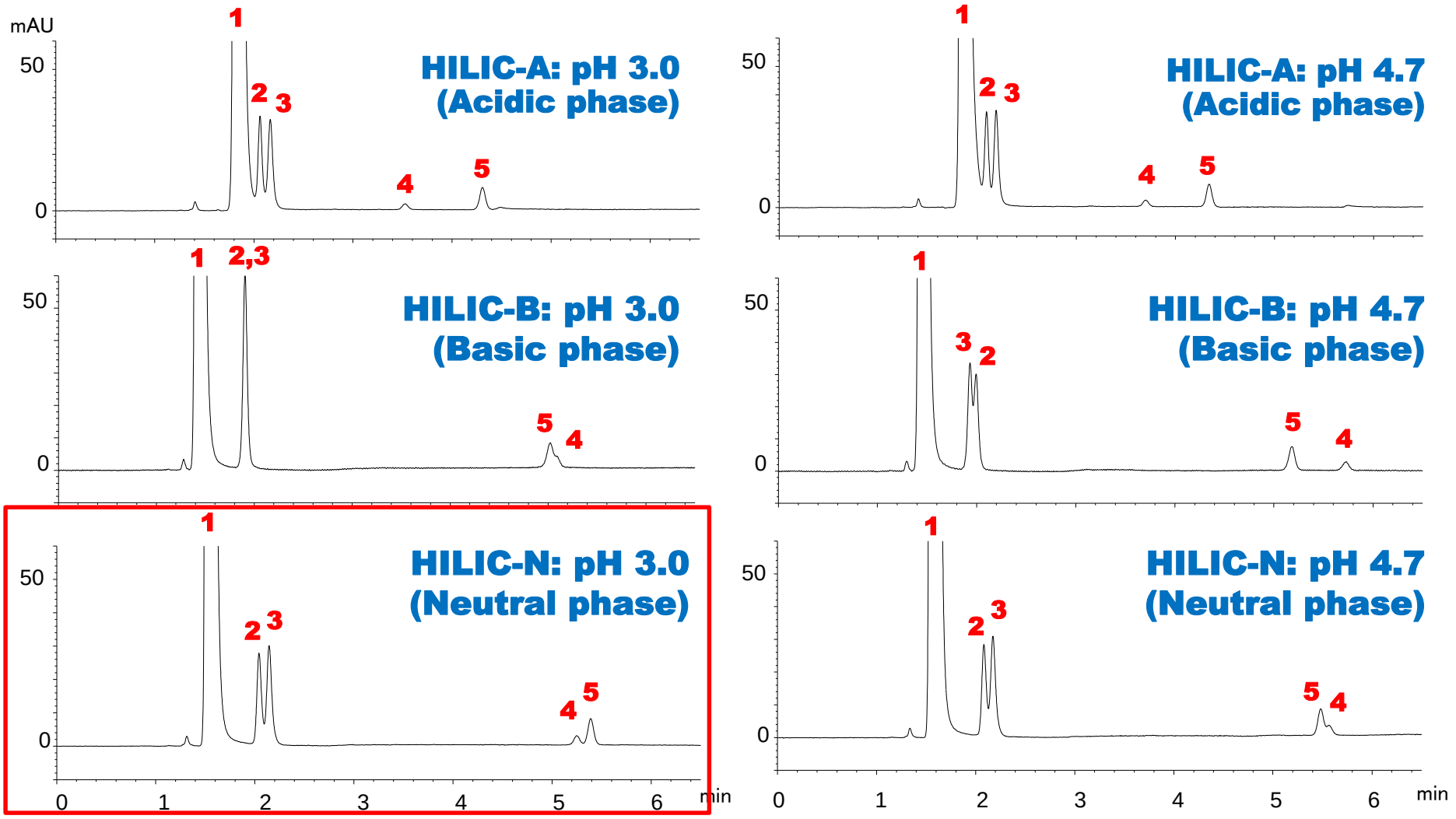
Columns: ACE HILIC-A, ACE HILIC-B and ACE HILIC-N (150x4.6mm, 5µm)
Mobile Phase A: 10 mM ammonium formate pH 3.0 & 4.7 in MeCN/H_2O (96:4 v/v)
Mobile phase B: 10 mM ammonium formate pH 3.0 & 4.7 in MeCN/H_2O (50:50 v/v)
Gradient:

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

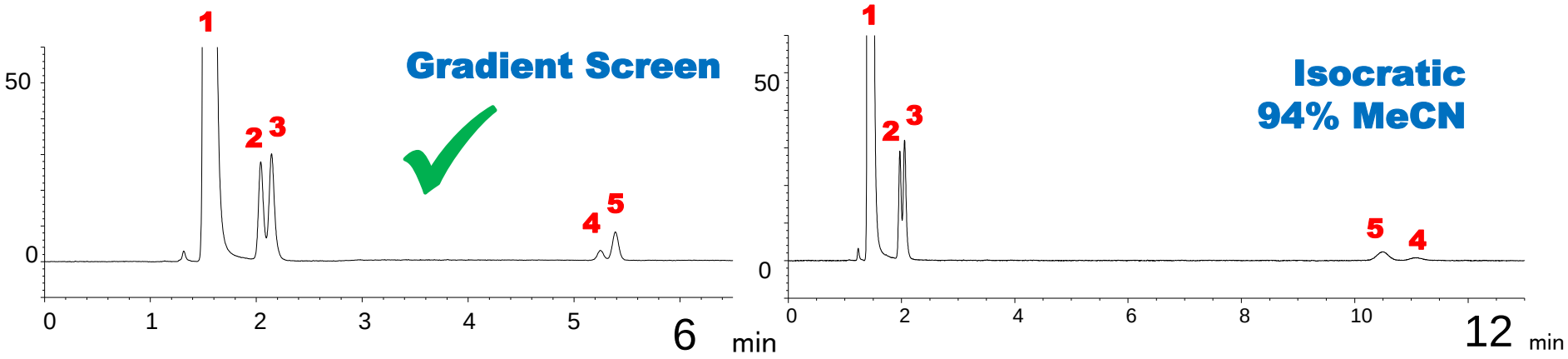
The screen suggests HILIC-N at pH 3.0 is should be developed further.



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

Example - Optimisation

Gradient or Isocratic?

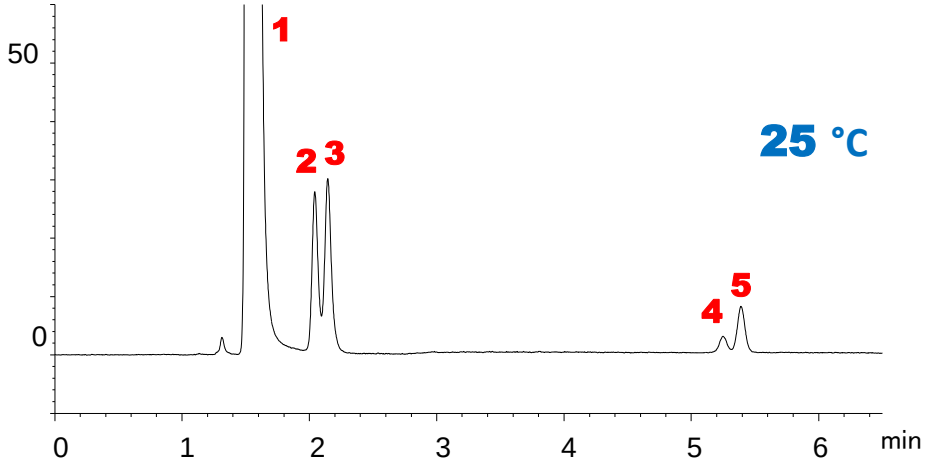


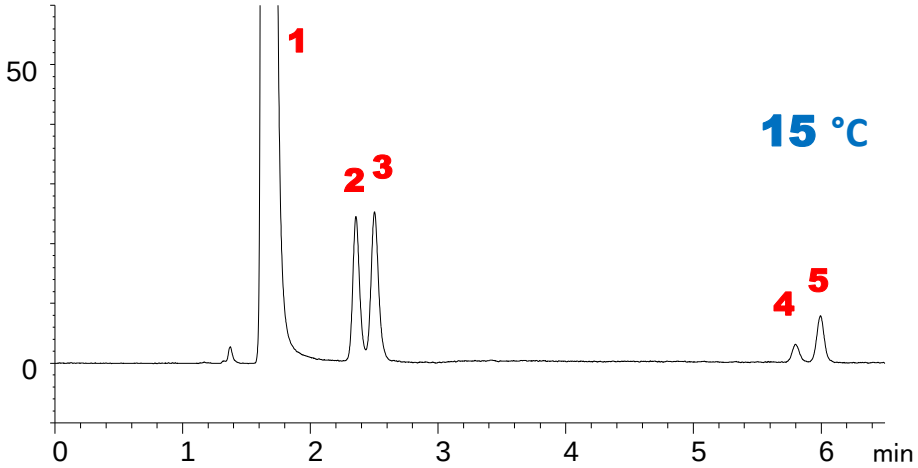
Isocratic greatly retained peaks 4 and 5, but failed to resolve peaks 2 and 3. Therefore, isocratic is not possible with this sample.

Further optimisation must be performed using gradient analysis.

Example - Optimisation

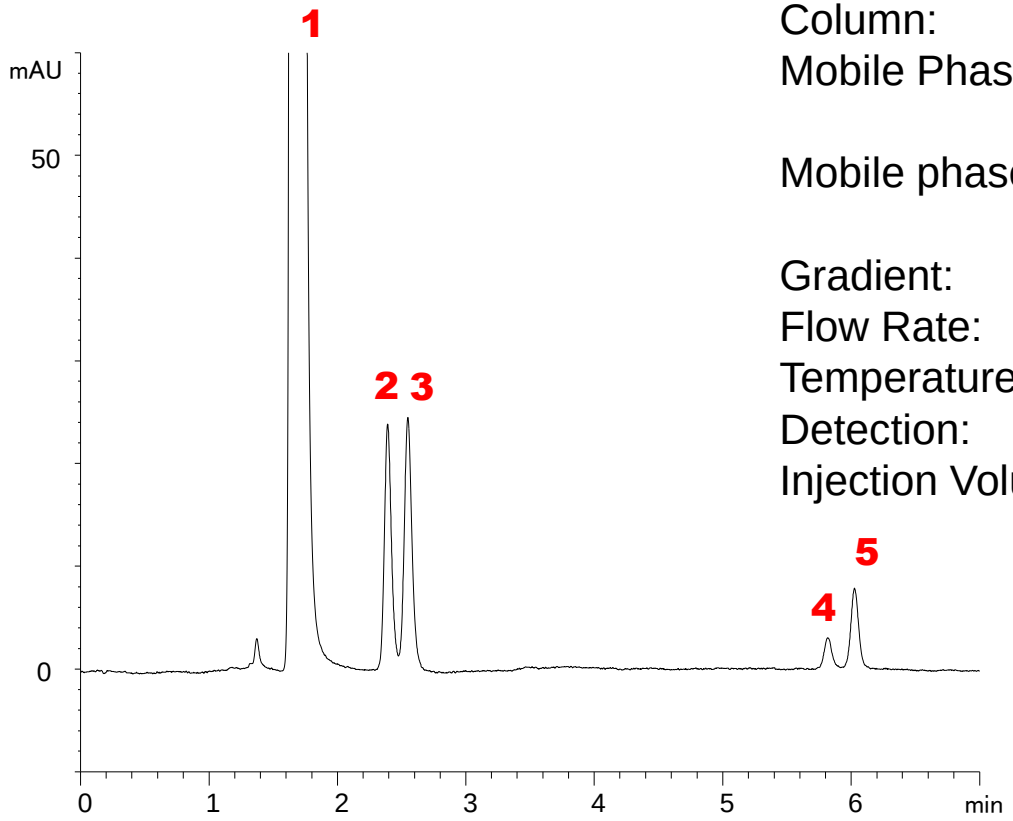
Effect of Temperature?





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



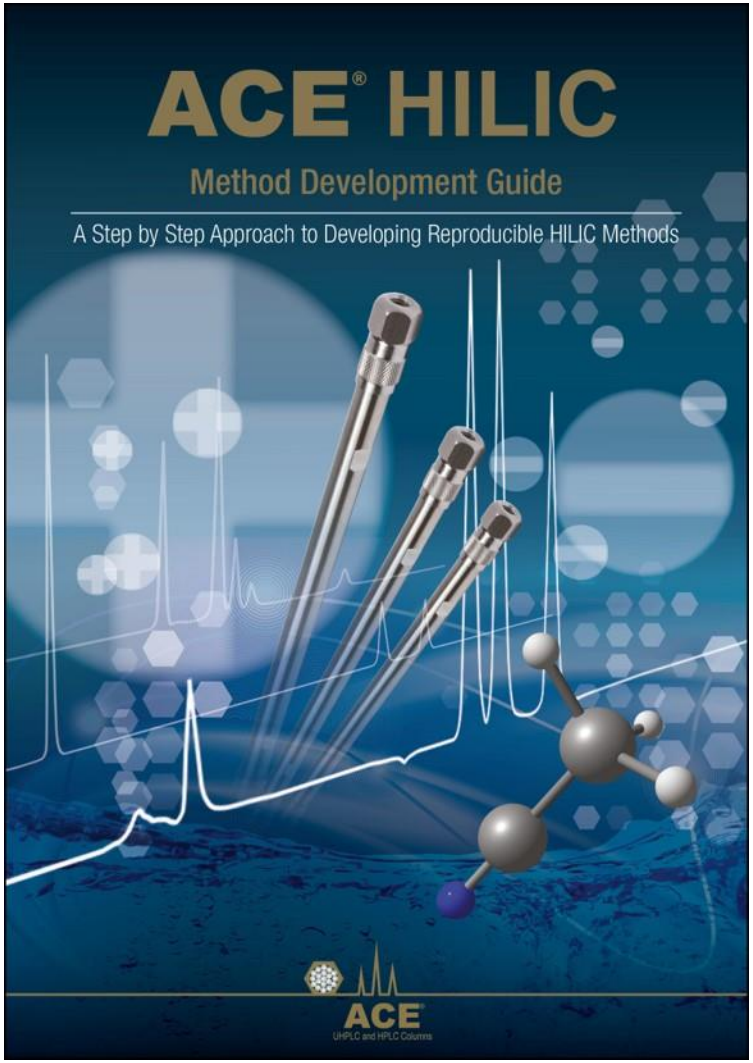
Final Method

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

ACE HILIC Method Development Guide



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ACE Knowledge Notes for HILIC

ACE Knowledge Note #0021

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

ABSTRACT

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. Hydrophilic 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.

INTRODUCTION

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.

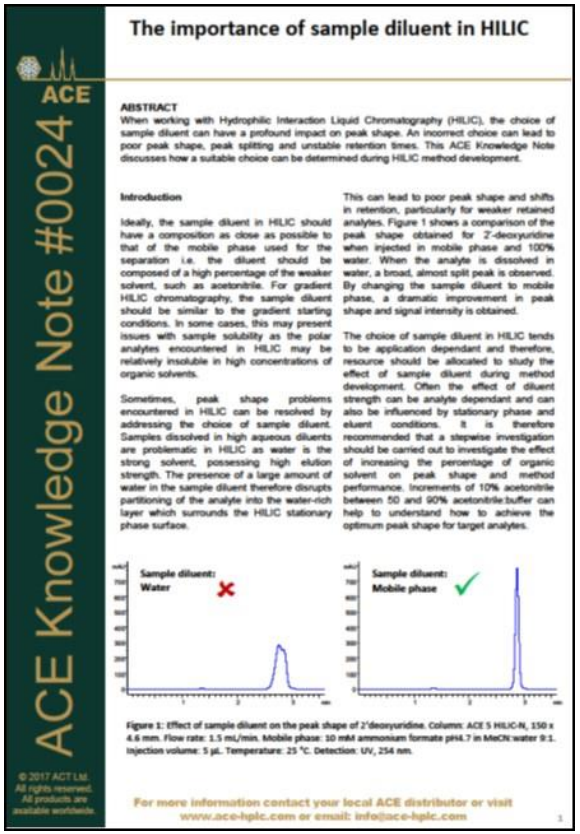
Figure 1 Schematic of logP values and typical chromatographic mode of separation.

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)



ACE Knowledge Note #0024

The importance of sample diluent in HILIC

ABSTRACT

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.

Introduction

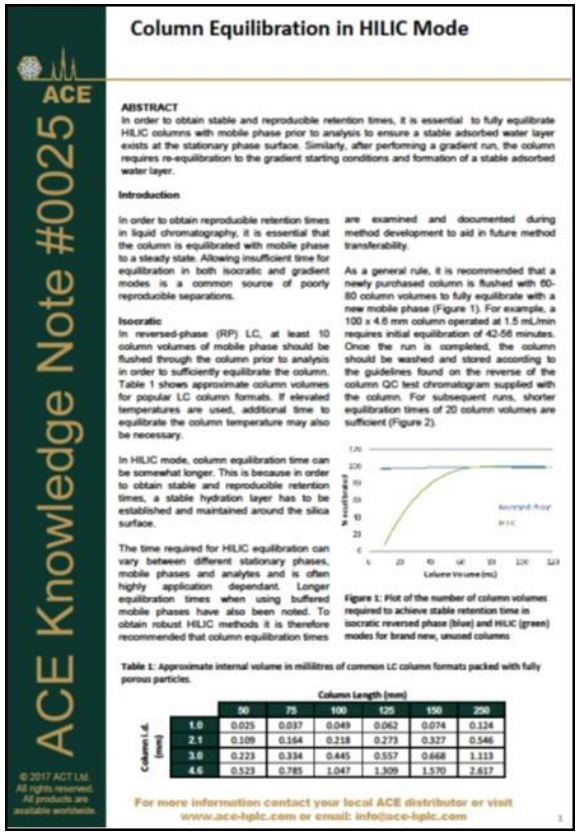
Ideally, the sample diluent in HILIC should have a composition as close as possible to that of the mobile phase used for the separation i.e. the diluent should be composed of a high percentage of the weaker solvent, such as acetonitrile. For gradient HILIC chromatography, the sample diluent should be 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 weaker 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.



ACE Knowledge Note #0025

Column Equilibration in HILIC Mode

ABSTRACT

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.

Introduction

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

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

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

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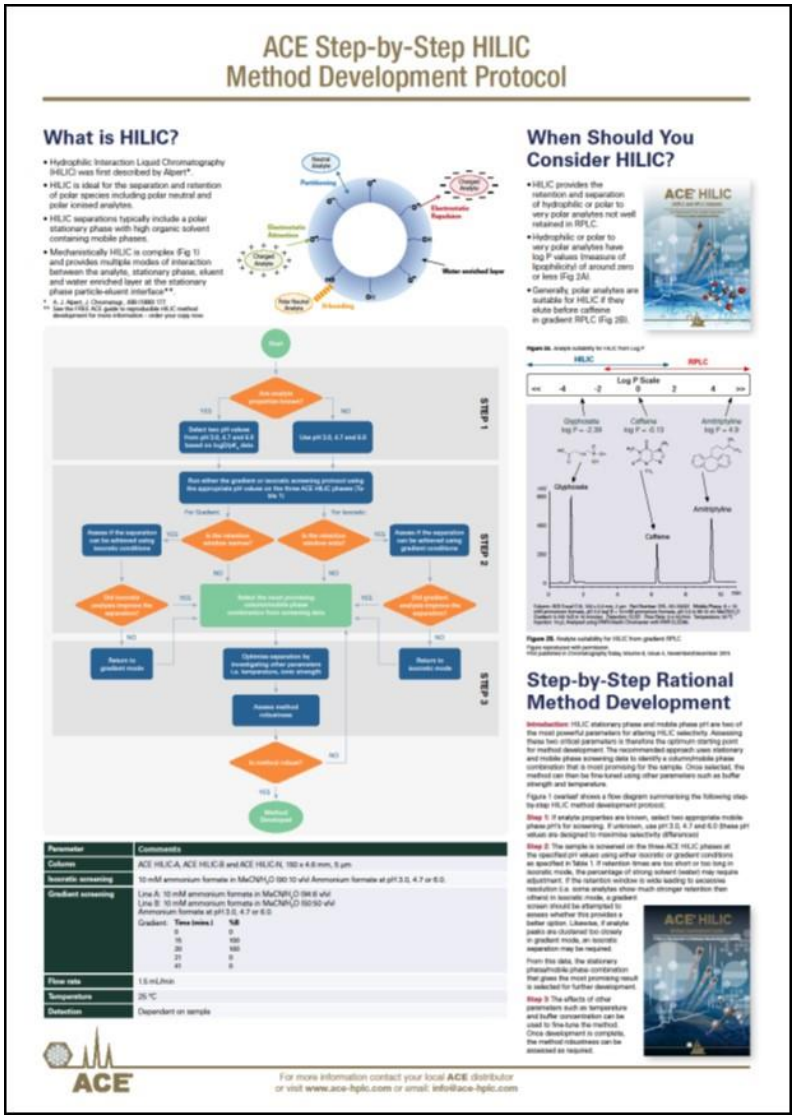

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Summary

- HILIC can provide polar analyte retention and alternative selectivity to reversed phase chromatography
- HILIC can be mechanistically complex
- A screening protocol with complementary phases can be helpful
  - Selectivity is the key to chromatographic resolution
- Carefully Consider your equilibration time and sample diluent

Thank You For Your Attention

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