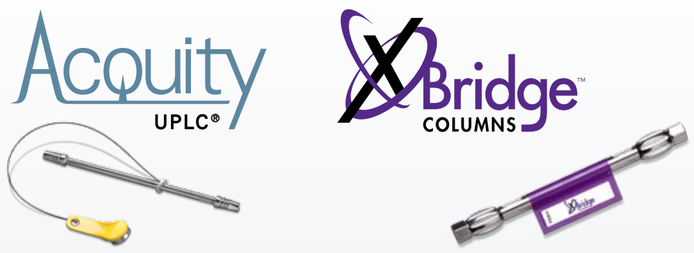
[www.waters.com/HILIC]

EFFICIENT HYDROPHILIC INTERACTION CHROMATOGRAPHY [HILIC] METHOD DEVELOPMENT STRATEGY



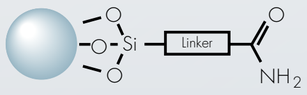
ACQUITY UPLC Columns and XBridge HPLC Columns

Ethylene Bridged Hybrid [BEH] Phases

BEH HILIC

- Unbonded Ethylene Bridged Hybrid [BEH] substrate
- Particle Size: 1.7 µm [UPLC]; 2.5, 3.5 and 5 µm [HPLC]
- Pore Size: 130Å; Surface Area: 185 m^2/g; Carbon Load: NA
- pH Range: 1-9; Low pH Temp. Limit: 45 °C; High pH Temp Limit: 45 °C
- Recommended Usage: Excellent for the retention of very polar, basic, water soluble analytes. Specifically designed and tested for HILIC separations using high organic mobile phases.

BEH Amide



- Trifunctional amide, bonded to Ethylene Bridged Hybrid [BEH] substrate
- Particle Size: 1.7 µm [UPLC]; 3.5 µm [HPLC]
- Pore Size: 130Å; Surface Area: 185 m^2/g; Carbon Load: 12%
- pH Range: 2-11; Low pH Temp. Limit: 90 °C; High pH Temp. Limit: 90 °C
- Recommended Usage: Rugged HILIC stationary phase designed to separate polar acidic, neutral and basic compounds. Particularly well suited for carbohydrate [saccharide] analysis using high concentrations of organic modifier, elevated temperature and high pH.

Atlantis HPLC Columns

Atlantis HILIC Silica

- Unbonded high purity silica substrate
- Particle Size: 3, 5 and 10 µm [HPLC]
- Pore Size: 100Å; Surface Area: 330 m^2/g; Carbon Load: NA
- pH Range: 1-5; Low pH Temp. Limit: 45 °C; High pH Temp Limit: 45 °C
- Recommended Usage: Excellent for the retention of very polar, basic, water soluble analytes. Specifically designed and tested for HILIC separations using high organic mobile phases.

ESSENTIAL INFORMATION FOR METHOD DEVELOPMENT

Sample solubility

- A mixture of 75% acetonitrile with 25% methanol is a good general purpose diluent for the best combination of sample solubility and peak shape. 0.2% formic acid or ammonium hydroxide can be added to further encourage analyte solubility.
- The best chromatographic performance will be observed when the sample is dissolved in the initial mobile phase composition.
- Peak distortion may occur when the sample is dissolved in a solvent of very different polarity and viscosity to that of the mobile phase.
- The impact of varying the injection volume should be investigated.

Chemical properties: functional groups

- Analytes will often exhibit the most retention when they are ionized [i.e., bases at low to mid pH, acids at mid to high pH], which is the opposite of reversed phase.
- For sample mixtures containing different types of analytes, use an intermediate pH ~ 5-7 [10 mM ammonium acetate with 0.02% acetic acid].
- Knowledge of the pKa value[s] of the analytes is helpful when developing a method.

Mobile phase preparation

- For the best gradient performance and reproducibility, a constant ionic strength of buffer [10 mM] or additive [0.2%] is recommended.

Column equilibration

- Condition new columns with 50 column volumes of 50:50 acetonitrile:water with 10 mM buffer or 0.2% additive solution. Store the column in these conditions.
- Equilibrate the column with 20 column volumes of initial mobile phase conditions before routine use [approximately 2 blank gradients].
- For gradient analysis, re-equilibrate with 5-8 column volumes between injections.

Needle wash solvents

- The needle wash solvent should closely match the initial mobile phase conditions [90-95% acetonitrile] to preserve retention and peak shape.

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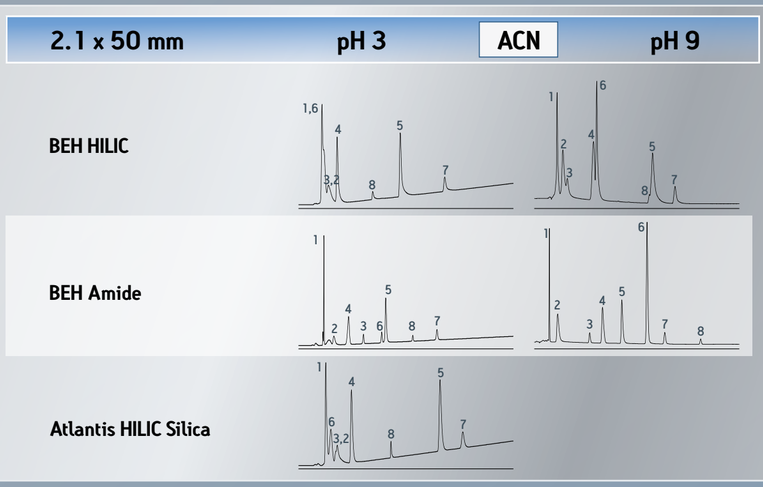
SYSTEMATIC SCREENING METHOD DEVELOPMENT PROTOCOL



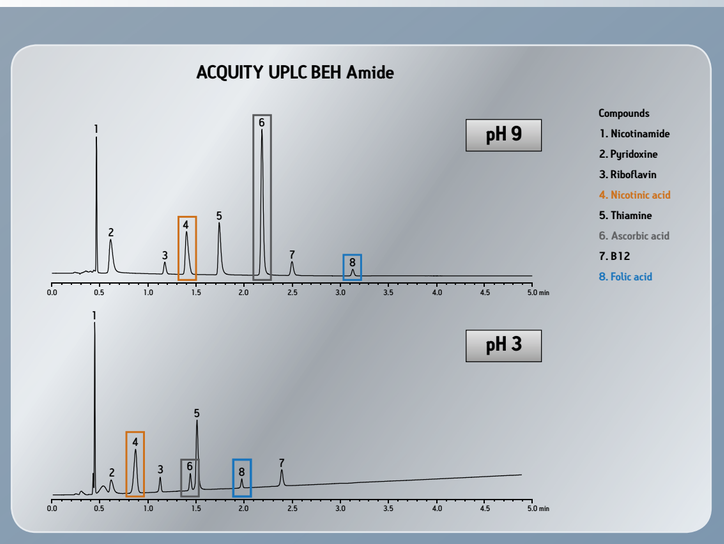
Chromatographic Conditions

Column Dimensions:	2.1 x 50 mm, 1.7 or 3/3.5 µm
Mobile Phase A:	water
Mobile Phase B:	acetonitrile
Mobile Phase C:	200 mM NH_4COOH and 2.5% HCOOH in H_2O, pH 3
Mobile Phase D:	200 mM CH_3COONH_4 and 0.8% NH_4OH in H_2O, pH 9
Flow Rate:	0.5 mL/min
Gradient:	Time (min) / Profile %A %B %C/D
	0.0 0 95 5
	5.0 45 50 5
	5.01 0 95 5
	6.0 0 95 5
Injection Volume:	5.0 µL
Needle Wash:	95% acetonitrile
Temperature:	30 °C
Instrument:	Waters ACQUITY UPLC® H-Class with Column Manager, ACQUITY® PDA and ACQUITY SQD

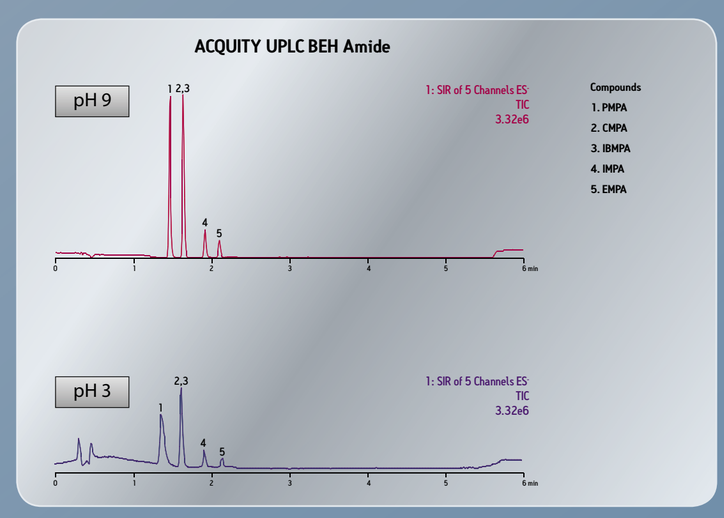
A systematic screening approach allows chromatographers to quickly determine which experimental parameters are most effective in manipulating the selectivity of a separation when little is known about the chemical properties of the analyte.

COLUMN SELECTION

THE IMPORTANCE OF MOBILE PHASE pH



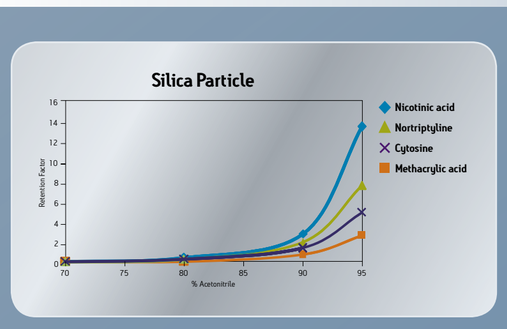
Mobile phase pH is a useful parameter to manipulate retention and selectivity. In HILIC, retention increases when the compound is ionized.



In addition to retention and selectivity differences, mobile phase pH can be manipulated to alter MS signal intensity.

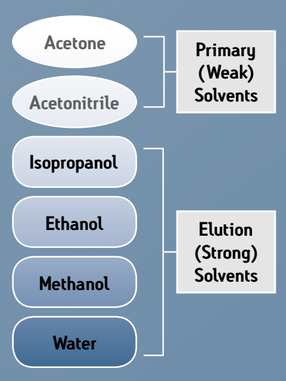
MOBILE PHASE pH

ORGANIC MODIFIER SELECTION

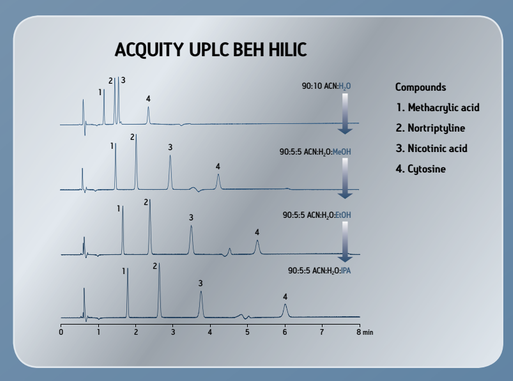


HILIC mobile phases generally contain greater than 70% acetonitrile in the mobile phase to facilitate retention.

At least 3% of the mobile phase must be a polar solvent [i.e., water, methanol, isopropanol] to maintain a hydrophilic layer on the stationary phase surface.



In terms of solvent elution strength in HILIC, acetonitrile is often the weak solvent and water is the strong elution solvent.



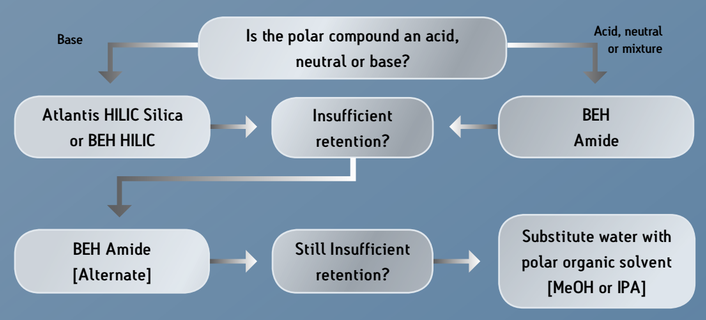
Subsituting a portion of water in the mobile phase with a weaker polar organic solvent may be useful to alter retention and selectivity.

ORGANIC MODIFIER

METHOD SELECTION AND OPTIMIZATION

Method Selection Based on Analyte Classification

The functional groups of analytes play a large role in determining the optimal conditions for analysis. If the chemical properties of the analytes are known, both column and mobile phase selection will be considerably easier.



Key

Atlantis HILIC Silica = low pH (pH 3), 10 mM ammonium formate with 0.2% formic acid in 95:5 ACN:H_2O

*BEH HILIC = mid pH (pH 5), 10 mM ammonium acetate with 0.02% acetic acid in 95:5 ACN:H_2O

*BEH Amide = high pH (pH 9), 10 mM ammonium acetate with 0.04% ammonium hydroxide 95:5 ACN:H_2O

*BEH Amide [Alternate] = mid pH (pH 5), 10 mM ammonium acetate with 0.02% acetic acid in 95:5 ACN:H_2O

*BEH represents either XBridge HPLC columns or ACQUITY UPLC BEH columns

METHOD SELECTION

Method Optimization Sequence

Once the stationary phase, organic modifier and mobile phase pH have been chosen, retention and selectivity can be further optimized by altering gradient slope and column temperature.

1. Adjust gradient slope
2. Adjust column temperature
3. Adjust column length and flow rate
4. Isocratic mode instead of gradient 95:5 ACN:H_2O with 10 mM buffer or 0.2% additive
5. Replace a portion of the water in the mobile phase with a less polar solvent [MeOH, EtOH or IPA]

Evaluate result after each step. Stop after criteria for success has been met. Consider injection solvent (sample diluent) if poor peak shape/resolution.

METHOD OPTIMIZATION

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