An Introduction to the Retention of Polar Analytes by Hydrophilic Interaction Chromatography (HILIC)

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Hydrophilic Interaction Chromatography (HILIC)
  — Challenges of polar analytes
  — Overview of HILIC

Tips for Method Development
  — Organic Modifier
  — Stationary Phase
  — pH
  — Method optimization

Troubleshooting
  — Injection solvent
  — Column equilibration
Hydrophilic Interaction Chromatography (HILIC)

- Challenges of polar analytes
- Overview of HILIC

Tips for Method Development

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- Stationary Phase
- pH
- Method optimization

Troubleshooting

- Injection solvent
- Column equilibration
- Polar compounds have charge separation or an overall dipole moment

- In the case of polarity, like attracts like
  - Polar ↔ polar; non-polar ↔ non-polar

- Polar and non-polar molecules are not attracted to each other
  - Classic example: oil and water
- Unfortunately, traditional reverse phase columns are very non-polar (oily)
  - High ligand densities and extensive end-capping to eliminate silanol activity
Unfortunately, traditional reverse phase columns are very non-polar

- High ligand densities and extensive end-capping to eliminate silanol activity

The result is poor retention for polar and charged analytes on a traditional reverse phase column.
Retention by reverse phase can be improved

$V_0 = 1.3\text{min}$

C18 High Ligand Density ex. Sunfire
Improved Retention of Polar Analytes

Retention by reverse phase can be improved
  — Reducing ligand density allows for the interaction of the analyte with the polar surface of the particle

![Graph showing retention times for different ligand densities](image-url)

- Column A: C18, High Ligand Density, Ex. Sunfire
  - $V_0 = 1.3\text{min}$

- Column B: C18, Lower Ligand Density, Ex. Atlantis
  - $V_0 = 1.3\text{min}$
Why are Polar Compounds Problematic?

- We need to think back to the basics of chromatography...

- We are create a competition between the stationary phase and our mobile phase for the analyte
  - Using solvents and stationary phases with opposite polarities
How fast the analyte moves through the column is dependent upon how much it “likes” the stationary phase as compared to the mobile phase.

- Example: high density C18 column with a gradient from 100% water to 100% ACN
  - Small alcohols will elute very quickly as they are polar and prefer water
  - Aliphatic hydrocarbons will elute later, with more ACN as they prefer the non-polar surface of the column to water.
- Very polar compounds such as sugars an elute with or close to the void marker for

- In some cases, using columns with lower ligand density can improve the retention of polar analytes
A compound can be too polar for reverse phase

- However, in other cases, no reversed phase column will provide the desired result

\[
V_0 = 0.65 \text{ min}
\]

Allantoin
A compound can be too polar for reverse phase

- However, in other cases, no reversed phase column will provide the desired result
  - We will need to investigate other technologies, such as HILIC

\[ V_o = 0.65 \text{ min} \]

\[ V_o = 1.15 \text{ min} \]
What is HILIC?

- **HILIC - Hydrophilic Interaction Chromatography**
  - Term coined in 1990 to distinguish from normal-phase*

- HILIC is a *variation* of normal-phase chromatography without the disadvantages of using solvents that are not miscible in water
  - “Reverse reversed-phase” or “aqueous normal-phase” chromatography

- Stationary phase is a POLAR material
  - Silica, hybrid, cyano, amino, diol

- The mobile phase is highly organic (> 80%) with a smaller amount of aqueous mobile phase
  - Water (or the polar solvent(s)) is the strong, eluting solvent

Benefits of HILIC:

- **Retention** of highly polar analytes not retained by reversed-phase
  - Less interference from non-polar matrix components

- Complementary **selectivity** to reversed-phase
  - Polar metabolites/impurities/degradents retain more than the parent compound
Complementary Selectivity to Reversed-Phase Atlantis® HILIC Silica

HILIC offers complementary selectivity to reversed-phase

1. Bamethan

2. Albuterol
Benefits of HILIC:

- **Retention** of highly polar analytes not retained by reversed-phase
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- Complementary **selectivity** to reversed-phase
  - Polar metabolites/impurities/degradents retain more than the parent compound

- Enhanced **sensitivity** in mass spectrometry
  - High organic mobile phases (> 80%) promotes enhanced ESI-MS response
  - Direct injection of PPT supernatant without dilution possible
    - Facilitates the use of lower volume samples

- Improved sample **throughput**
  - Direct injection of high organic extracts from PPT, LLE or SPE without the need for dilution or evaporation and reconstitution
When we need to improve
  — The retention of hydrophilic or ionizable compounds
  — The MS response for polar or ionizable compounds
  — Sample throughput for assays using organic extraction
**HILIC: How does it work?**

- Combination of partitioning, ion-exchange, hydrogen bonding
  - Polar analytes partition between mobile phase and the partially immobilized polar layer on the surface of the column
  - Secondary interactions can occur between surface functional groups and charged analytes
  - Hydrogen bonding occurs between positively charged analytes and any negatively charged silanols
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  — Challenges of polar analytes
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Troubleshooting
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  — Column equilibration
Creating Mobile Phase pH

Creating Selectivity

α

Column Chemistries

Organic Solvent
Solvent Strength

Weakest

Acetone

Acetonitrile

Isopropanol

Ethanol

Methanol

Water

Strongest

Primary [Weak] Solvents

Elution [Strong] Solvents

Use a less polar solvent to *Increase* retention of polar analytes

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Considerations for Retention: Solvent Polarity

Retention increases with decreasing solvent polarity.

Analytes:
1: methacrylic acid
2: cytosine
3: nortriptyline
4: nicotinic acid

10 mM ammonium acetate with 0.02% acetic acid

Creating Mobile Phase pH

Creating Selectivity

Column Chemistries

Organic Solvent

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HILIC Column Options

Hybrid HILIC Columns (pH range 1 – 11)

ACQUITY UPLC® BEH Amide
XBridge™ Amide

ACQUITY UPLC® BEH HILIC
XBridge™ HILIC

Silica HILIC Columns (pH range 1 – 5)

Atlantis® HILIC Silica
**Effect of Column Chemistries**

**ACQUITY UPLC BEH HILIC**
2.1 x 50 mm, 1.7 μm

*Unbonded hybrid with low silanol activity*

**ACQUITY UPLC BEH Amide**
2.1 x 50 mm, 1.7 μm

*Bonded hybrid*

**Atlantis HILIC Silica**
2.1 x 50 mm, 3 μm

*Unbonded silica with high silanol activity*

(1) acenaphthene  (2) thymine  (3) 5-fluoroorotic acid  (4) adenine  (5) cytosine; UV 254 nm
Effect of Column Chemistry

BEH Amide

BEH HILIC

Atlantis HILIC Silica

Compounds
1. Nicotinamide  50 µg/mL
2. Pyridoxine     50 µg/mL
3. Riboflavin     30 µg/mL
4. Nicotinic acid 50 µg/mL
5. Thiamine       50 µg/mL
6. Ascorbic Acid  50 µg/mL
7. B12           50 µg/mL
8. Folic Acid    25 µg/mL
Creating Mobile Phase pH

Creating Selectivity

α

Column Chemistries

Organic Solvent
Effect of pH

ACQUITY UPLC BEH Amide, 2.1 x 50 mm, 1.7 μm

Compounds
1. Methacrylic acid
2. Nortriptyline
3. Nicotinic acid
4. Cytosine

pH 3
Nicotinic Acid
pKa = 2.2, 4.8

Nortriptyline
pKa = 10

Cytosine
pKa = 12.2

Methacrylic Acid
pKa 4.58
Effect of pH

ACQUITY UPLC BEH Amide, 2.1 x 50 mm, 1.7 μm

Compounds
1. Nicotinamide
2. Pyridoxine
3. Riboflavin
4. Nicotinic acid
5. Thiamine
6. Ascorbic Acid
7. B12
8. Folic Acid

Folic acid

Ascorbic acid

Nicotinic acid
Common HILIC Mobile Phases

- Common buffers and additives
  - Ammonium formate, ammonium acetate
  - Formic acid, ammonium hydroxide, acetic acid
  - Phosphate salt buffers are NOT recommended due to precipitation in the highly organic mobile phase
- Recommended buffer concentration: 10 mM on-column
- Recommended additive concentration: 0.2 % on column

- Note that the actual pH of the mobile phase may be 1 pH unit closer to neutral due to the highly organic mobile phase
Additives vs. Buffers

**pH 9 Observations**
Acids are unretained without a buffered mobile phase
Selectivity shifts for basic compounds

**pH 3 Observations**
Poor peak shape and retention for bases without a buffered mobile phase
Selectivity shifts for acidic compounds

0.2% ammonium hydroxide pH 9
20 mM ammonium acetate pH 9
0.2% formic acid pH 3
20 mM ammonium formate pH 3

All contain 90:10 MeCN:H₂O
Method Optimization Steps

1. Adjust gradient slope

2. Adjust column temperature

3. Adjust column length and flow rate

4. Try isocratic mode instead of gradient
   - 95:5 ACN:H2O with 10 mM buffer or 0.2% additive

5. Replace water in the mobile phase with a less polar solvent
   - Keep at least 5% water in the mobile phase

- Evaluate your results after each step
- Consider changing your injection solvent if poor peak shape or resolution are observed
Optimization of Gradient

99.9% to 0.1% B in 5 min
SIR of 5 Channels ESI- TIC
3.32e6

BEH Amide, pH 9
Shallower gradient slope results in improved resolution

99.9% to 50% B in 5 min
SIR of 5 Channels ESI- TIC
4.18e6

99.9% to 90% B in 5 min
SIR of 5 Channels ESI- TIC
5.12e6

Compounds
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA

500 ng/mL each
Optimization of Temperature

30 °C

BEH Amide, pH 9
Shallow gradient

Increased temperature results in improved resolution

SIR of 5 Channels ESI-TIC 5.12e6

50 °C

SIR of 5 Channels ESI-TIC 5.08e6

Compounds
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA

500 ng/mL each
Optimization of Column Length

**2.1 x 50 mm**

- SIR of 5 Channels ES-TIC

**BEH Amide, pH 9**
- Shallow gradient, 65 °C
- 100 mm column results in improved resolution
- 50 mm column results in shorter run time

Select result that meets method criteria

Compounds:
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA

500 ng/mL each
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- **Troubleshooting**
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Like with reverse phase, sample diluent strongly influences solubility and peak shape.

Sample diluent should be at least 75% ACN or as close to the initial conditions as possible.

BUT polar analytes often have low solubility in organic solvent.

- 75:25 ACN:MeOH works well for most polar analytes
  - Offers a compromise between solubility and peak shape
  - Can add 0.2% formic acid to increase solubility
  - In some cases even 25% MeOH can be too polar
Effect of Diluent

ACQUITY UPLC® BEH HILIC
2.1 x 100 mm, 1.7 μm

Analytes
1. Methacrylic acid
2. Cytosine
3. Nortriptyline
4. Nicotinic acid

Peak shape improves as % ACN in the diluent increases.

What about alternative polar organic solvents?
Effect of Diluent

ACQUITY UPLC® BEH HILIC
2.1 x 100 mm, 1.7 μm

Analytes
1. Methacrylic acid
2. Cytosine
3. Nortriptyline
4. Nicotinic acid

Peak shape and solubility improve by replacing water with methanol

Peak shape improves as % ACN in the diluent increases.
- Brand new column
  - Run 50 empty column volumes of 50:50 ACN:water with 10 mM buffer or 0.2% additive solution

- Column equilibration
  - Equilibrate with 20 empty column volumes of your initial mobile phase conditions

- Gradient separations
  - Re-equilibrate after each gradient with at least 5 to 8 empty column volumes

- As with any column, insufficient equilibration can cause drifting retention times
Conclusions

- HILIC is used to retain polar analytes that cannot be retained by reverse phase
- ACN is the most commonly used weak solvent
- Water, MeOH, EtOH & IPA are strong solvents
- Stationary phase, organic modifier and pH can be manipulated during method development
  - Gradient slope and temperature can be adjusted to fine tune a method
- A HILIC column must be properly equilibrated before use and between injections for reproducible results
- The sample diluent should contain at least 75% ACN for solubility and peak shape
Questions?

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