Part I: Polar Compound Retention

Tuesday, October 11 2016 – 3 pm CET / 9 am EST

Understanding What Works
and What Doesn’t Work
In Reversed-Phase Polar Compound Retention
Overview

- Introduction
  - The Problem
- Background
- Challenges for Reversed-Phase HPLC for Polar Molecules
- Columns for Polar Compound Retention
- Summary
Introduction
The Problem

- Conventional reversed-phase columns often do not provide adequate retention and separation of highly polar compounds

- Screening and Discovery
  - Gradient - High-throughput screening (HTS) does not work for polar compounds
    - Elute un-retained in the void volume
    - Co-elute at the beginning of the run – if retained at all

- Method Development
  - Isocratic - Many columns cannot be run under the 100% aqueous conditions necessary for polar compound retention – columns “dewet” (also termed - hydrophobic collapse)
Overview

- Introduction

- Background
  - Polar molecules
  - Chromatographic methods for retaining polar compounds

- Challenges for Reversed-Phase HPLC for Polar Molecules
- Columns for Polar Compound Retention
- Summary
What is a Polar Molecule?

- General chemistry definition:
  - A molecule whose centers of positive and negative charges do not coincide
  - The degree of polarity is measured by the dipole moment of the molecule
- Dipole moment is the product of the charge at either end of the dipole times the distance between the charges
  - The unequal sharing of electrons within a bond results in a separation of positive and negative electric charge.
- Polarity is dependent on the electronegativity difference between molecular atoms and compound asymmetry
Examples of Polar Organic Molecules

Thiourea (N)

Thymine (N)

Cytosine (B)

5-Fluoroorotic acid (A)

Acetone (N)

Epinephrine (B)

Ascorbic acid (A)
### Comparison of Chromatographic Methods for Retention of Polar Compounds

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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</table>
| **Reversed-Phase** | ▪ Familiar technique  
▪ High efficiency  
▪ Rapid equilibration  
▪ Wide selection of columns | ▪ Dewetting under aqueous conditions  
▪ Poor retention of polar compounds |
| **HILIC**       | ▪ High % organic mobile phases give higher sensitivity in MS  
▪ Eliminate evaporation of SPE eluents | ▪ Sample solubility problems  
▪ Not well understood  
▪ Not widely applicable |
| **Ion-Pairing** | ▪ Retains ionizable compounds  
▪ Ion-pairing and reversed-phase chromatography use same columns | ▪ Long equilibration times  
▪ Difficult to run gradients  
▪ Not compatible with MS  
▪ Difficult method development |
| **Ion-Exchange** | ▪ Can retain any ionizable compound | ▪ Buffer salts or pH gradients not compatible with mass spectrometry  
▪ Compounds must be ionic |
Overview

- Introduction
- Background

- Challenges for Reversed-Phase HPLC for Polar Molecules
  - What is Dewetting
  - What does not work for Polar Compound Retention

- Columns for Polar Compound Retention
- Summary
Challenge for Reversed-Phase Chromatography of Polar Compounds

- To retain polar compounds on a non-polar surface we reduce the amount of organic in the mobile phase
  - make mobile phase weaker (100% aqueous mobile phase)

- **PROBLEM:** Risk of dewetting the stationary phase particle surface

What is “dewetting” and why does it happen?

The non-polar pore surface expels the aqueous, polar mobile phase
(The chromatographic pores dry-out!!)
Proper Wetting of Bonded Chromatographic Surface

Mobile phase must be allowed into the pore in order for chromatographic retention of the analyte to take place.

The particles are very porous, like the pores of a sponge – 99% of chromatographic surface is inside the pores.

Mobile phase must be allowed into the pore in order for chromatographic retention of the analyte to take place.

If the pores are dry, the analyte cannot get into the pores and it will not be retained by the chromatographic surface.

What influence does this have on chromatography?
The observed reduction in $V_0$ and sudden loss in retention after a pressure release, indicates that material pores expel the aqueous solvent.

**ACQUITY UPLC® BEH C$_{18}$**

10% Reduction in Void Volume

23% Loss in Retention

Chromatographically Speaking: What is Dewetting?

Atlantis T3
4.6 x 100 mm, 3 µm

< 7% Thymine Dewet

Zorbax® Eclipse Plus C18
4.6 x 100 mm, 3.5 µm

Note enhanced retention

Thymine Dewet: ~100%

100% retention loss with conventional C18

Components:
1. Thiourea
2. 5-Fluorocytosine
3. Adenine
4. Guanosine-5’-monophosphate
5. Thymine

Comparative separations may not be representative in all applications.
Conventional C$_{18}$ Column Dewetting

Mobile phase: 0.1% Acetic Acid

Amoxicillin

1,500 psi

Initial

(Column was wetted first with organic)

After Flow Stoppage

(Pores “dewet” 100%)

$V_0$: No retention of analyte

The column is not broken -- It just stopped working

Why?
Pore Dewetting Mechanism

Flow stoppage relieves the pressure that was forcing the aqueous mobile phase into the pores. When pressure is reduced (e.g., pump stopped), the hydrophobic pore surface can expel the polar mobile phase and “dewet” the pore.

At flow, with pressure on the mobile phase.

Stopped flow with no pressure on the mobile phase.

Pores de-wet – restart flow – pores still dewetted and analytes never enter pores – resulting in no retention.

Remember: Most of the surface area (> 95%) is inside the silica pores!
Stationary Phase Wetting/Dewetting

**Water on C\textsubscript{18}:**
- \( d = 100\text{Å} \)
- \( g = 72.8 \text{ dynes/cm} \)
- \( \theta = 110.6^\circ \)

\( P_c = 1,500 \text{ psi} \)

**Methanol on C\textsubscript{18}:**
- \( d = 100\text{Å} \)
- \( g = 22 \text{ dynes/cm} \)
- \( \theta = 39.9^\circ \)

\( P_c < 0 \text{ psi} \)

\( \theta \)

Equation of Young and Laplace

\[ P_c = \frac{2 \gamma}{r} \cos \theta \]

Where:
- \( P_c \) = Capillary pressure
- \( \gamma \) = Surface tension
- \( r \) = Capillary radius
- \( \theta \) = Contact angle

Reducing the contact angle forces water back into the pore

Note: Self-wetting – No pressure required

Re-wetting a Stationary Phase

- Use a mobile phase containing > 40% methanol or other polar organic solvent (other organic solvents may vary in % required for wetting)
  - This works by reducing the contact angle ($\theta$)

- The use of pressure alone cannot force aqueous mobile phase back into silica pores
  - Not practical because column outlet is at atmospheric pressure – not all pores see required pressure (1,500 psi)
Dewetting Summary

- Dewetting - not “hydrophobic collapse” causes the sudden loss of retention under highly aqueous conditions

- $V_0$ reduction and sudden loss in retention after a pressure drop indicate that the pores expel aqueous solvent (vs. ligand chains “folding” or “matting” as described by hydrophobic collapse)

- Rewetting with organic solvent can rewet the pores by reducing the contact angle and surface tension

Ideally, it is best to avoid dewetting altogether.

How have chromatographers attempted to do this?
Embedded Polar Groups: What Doesn’t Work

- Embedded polar group columns designed to:
  - Improve peak shapes for basic compounds
  - Provide complementary selectivity as compared to straight-chain alkyl stationary phases

- Examples of embedded polar groups include carbamate, ether, amide, urea, etc.

- Another feature of embedded polar groups is 100% aqueous mobile phase compatibility

This feature is confused with enhanced polar compound retention.

Why?
Embedded Polar Groups: What Doesn’t Work

- Embedded polar groups resist pore dewetting by increasing the water layer at the surface of the pores.

Embedded polar group behaves like a “Polar Hook” – holding onto water.

Polar compound retention requires these very weak polar mobile Phases *i.e.*, highly aqueous mobile Phases with little-or-no organic modifier.

This aqueous compatibility is confused with polar compound retention. **This is false!**
Embedded Polar Groups: What Doesn’t Work

- Atlantis T3
  - $V_o = 1.3\text{min}$

- Zorbax Bonus RP (amide)
  - $V_o = 1.2\text{min}$

- GL Sciences Inertsil ODS EP
  - $V_o = 1.2\text{min}$

- Phenomenex Synergi Polar RP
  - $V_o = 1.3\text{min}$

Comparative separations may not be representative in all applications.
Embedded Polar Groups – What Doesn’t Work

- Atlantis® T3
  - $V_o = 1.3\text{min}$
- Supelco ABZ+ (amide)
  - $V_o = 1.3\text{min}$
- Metachem Polaris C18-A
  - $V_o = 1.3\text{min}$
- Dionex Acclaim® Polar Advantage
  - $V_o = 1.3\text{min}$

Comparative separations may not be representative in all applications.
**Embedded Polar Groups: What Doesn’t Work**

Why less retention of polar compounds with embedded polar group columns?

**Reason 1**
Water layer “shields” silanols and reduces cationic interactions and H-bonding.

This “water shield” results in less retention of polar compounds - These are also the reasons for the excellent peak shape observed for bases.

**Reason 2**
Shorter alkyl chain length in order to obtain higher ligand density.
Embedded Polar Groups: Not the Answer

- Embedded polar groups – summary
  - Embedded polar groups were designed for improved peak shape
  - Embedded polar groups provide aqueous compatibility
  - For some compounds, embedded polar groups can provide increased retention (e.g., polyphenolics)
  - For highly polar compounds, however, embedded polar groups produce less retention

Now that we’ve defined what is **not** an ideal column for polar retention, let’s define what an ideal column is for polar compound retention.
Overview

- Introduction
- Background
- Challenges for Reversed-Phase HPLC for Polar Molecules

- Columns for Polar Compound Retention
  - Designing the ideal column
  - Particle and Bonding Considerations
  - Introduction to the T3 Column Family

- Summary
Retaining Polar Analytes Using Reversed-Phase HPLC

How can a column simultaneously retain and separate analytes of different polarities?

Flow

Polar Mobile phase

Analyte X
Polar

Non-polar Stationary phase

Well retained (like attracts like)

Analyte Y Non-Polar

Poorely Retained (like attracts like)
Optimization of pore size, ligand type, ligand density and endcapping results in a good balance among peak shape, stability, dewetting and polar retention.
Designing the Ideal Column

Q: How can reversed-phase columns designed for polar compound retention be improved?

- Increase retention
- Improve column lifetime at low pH
- Provide better peak shape at neutral pH
- Little-to-no MS bleed
- Seamless scalability from UPLC-UHPLC-HPLC-Preparative separations
Bonded Phase Hydrolysis: Limitations of Monofunctional Ligands

Monofunctional = Attached to silica at one point
Highly susceptible to low pH hydrolysis (column bleed)
Making a Bonded Phase Material: $dC_{18}$ Difunctional Synthesis

\[ \text{C}_8 \text{ Dichlorosilane Ligand} \]

\[ + \quad \text{Synthesis} \]

\[ + \quad \text{HCl} \]

- Difunctional = Attached to silica at two points
- Less susceptible to low pH hydrolysis (column bleed)
Making a Bonded Phase Material: T3 Trifunctional Synthesis

C₈ Trichlorosilane Ligand

\[ \text{Synthesis} \]

\[ \text{Three siloxane bonds} \]

\[ + \text{HCl} \]

**Trifunctional** = Attached to silica at **three** points

**Much less** susceptible to low pH hydrolysis (column bleed)

Not quite as simple as this…. 

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Effect of Ligand Phase Functionality on Synthesis

- Trifunctional C₁₈-Bondings
  - 1-2 surface silanol group reacts with one silane ligand
  - More resistant to hydrolysis at pH <2
  - Reproducible, but more difficult reaction (polymeric side-reaction)
  - Higher silanol activity (on ligands)
  - Capable of higher ligand density

![Chemical structures of trifunctional C₁₈-bondings]
Low pH (0.1%TFA) Lifetime Study
*T3 Bonding - Atlantis T3 columns*

Compounds:
1. Adenine
2. Acetaminophen
3. Caffeine
4. 2-acetamidophenol
5. Acetanilide

Excellent stability during entire study with *little-to-no RT loss*

1000 injections over 2 weeks (35000 mL/14590 c.v. of mobile phase)
T3 Bonding Technology:
Longer Column Lifetimes at Low pH

% Loss in Retention Factor for Methyl Paraben after Exposure to 0.5% TFA at 60°C

- Atlantis T3
- XSelect HSS T3
- CORTECS T3
- CORTECS Shield RP18
- XBridge BEH Shield RP18
- Synergi Hydro-RP
- Synergi Polar-RP
- HALO RP Amide
- Atlantis dC18
- Kinetex EVO C18
- Phenomenex Aqua C18
- Zorbax Eclipse Plus C18
- Inertsil ODS-SP

% Loss in Retention Factor (Methyl Paraben)
How Do T3 Columns Work?

- T3 columns are designed to maximize the dominant reversed-phase (van der Waals forces – hydrophobic attraction) retention mechanism
  - Retention maximized using 100% aqueous mobile phases
  - Retention maximized by using reduced C\textsubscript{18} coverage
    - Polar analytes can “fit” between C\textsubscript{18} ligands and interact with surface silanols and alkyl chains

- Secondary interactions due to residual silanols that are more accessible due to reduced C\textsubscript{18} coverage
  - Cation-exchange interactions
  - Hydrogen bonding interactions
How Does T3 Bonding Work for both Nonpolar and Polar molecules?

- **NonPolar**
  - Retention mechanism is classic reversed-phase, hydrophobic interaction with the C$_{18}$ stationary phase

- **Polar**
  - Dominant retention mechanism is still reversed-phase
    - Retention maximized using 100% aqueous mobile phases (enabled by having the wider pore diameter)
    - Retention maximized by using reduced C$_{18}$ coverage
      - Polar analytes can “fit” between C$_{18}$ ligands and interact with surface silanols and alkyl chains
  - Secondary interactions due to residual silanols that are more accessible due to reduced C$_{18}$ coverage
  - Cation-exchange interactions
  - Hydrogen bonding interactions
T3 Particle Technology

- **T3 bonding** is a combination of a tri-functionally bonded, intermediate ligand density ligand and a proprietary endcap
  - C18 column chemistry designed to provide superior retention of small, water-soluble polar organic compounds

- Highest efficiency
- Greater Speed and Throughput
- Reduced backpressure

- High retention
- Seamless Scalability
- Compatible with UPLC operating pressures

- Exceptional loading capacity
- Superior basic peak shape
- Low pH Stability

Full aqueous compatibility and excellent peak shape across the entire usable silica pH 2 – 8 range
Atlantis T3 Columns:
Superior Polar Compound Retention

Aqueous Separation of Polar Compounds for Atlantis T3
vs. Conventional High Coverage C_{18} Column

**Conditions**
- Columns: 4.6 x 150 mm, 5 µm
- Mobile Phase: 10 mM NH_{4}COOH, pH 3.0
- Flow rate: 1.2 mL/min
- Injection volume: 7 µL
- Detection: UV@254 nm

**Compounds**
1. Thiourea
2. 5-Fluorocytosine
3. Adenine
4. Guanosine-5’-monophosphate
5. Thymine

Comparative separations may not be representative in all applications.
Atlantic T3 Columns: Superior Retention and Peak Shape

Compounds:
- Thiourea
- 5-Fluorocytosine
- Adenine
- Guanosine-5'-monophosphate
- Thymine

Comparative separations may not be representative in all applications.
pH 7 Peak Shape & Selectivity Test
MeOH/20mM $K_2HPO_4/KH_2PO_4$, pH 7 (65/35)

1) uracil, 2) propranolol, 3) butylparaben, 4) naphthalene, 5) dipropylphthalate, 6) acenaphthene, 7) amitriptyline

Comparative separations may not be representative in all applications.
**Superior Peak Shape and Retention: Superior Polar Compound Loading**

**Atlantis® T3**
5 µm, 4.6 x 100 mm

$V_0 = 1.5$ min

**Atlantis® Prep T3 OBD™**
5 µm, 19 x 100 mm

**Atlantis® Prep T3 OBD™**
5 µm, 30 x 100 mm

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration in DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>40 mg/mL</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>200 mg/mL</td>
</tr>
<tr>
<td>Propranolol</td>
<td>200 mg/mL</td>
</tr>
</tbody>
</table>

**Mobile phase A**: 0.1% formic acid in water

**Mobile phase B**: 0.1% formic acid in acetonitrile

Comparative separations may not be representative in all applications.
T3 Bonding Technology: High Strength Silica (HSS) Particles

- What is an HSS Particle?
  - **ACQUITY UPLC and XSelect High Strength Silica (HSS)** particles are the only 100% fully porous silica particles that are certified for use in applications up to 18000 psi/1240 bar

<table>
<thead>
<tr>
<th></th>
<th>CORTECS T3</th>
<th>ACQUITY HSS T3</th>
<th>XSelect HSS T3</th>
<th>Atlantis T3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intended Use</strong></td>
<td>High Efficiency</td>
<td>Wide Retention</td>
<td></td>
<td>Maximum Loading</td>
</tr>
<tr>
<td><strong>Particle Type</strong></td>
<td>Solid-Core Silica</td>
<td>Fully Porous High Strength Silica</td>
<td></td>
<td>Fully Porous Silica</td>
</tr>
<tr>
<td><strong>Maximum Rated Pressure</strong></td>
<td>18,000PSI (1240 bar)</td>
<td>18,000PSI (1240 bar)</td>
<td>6,000 PSI (400 bar)</td>
<td></td>
</tr>
<tr>
<td><strong>Available Particle Sizes</strong></td>
<td>1.6 µm, 2.7 µm</td>
<td>1.8 µm, 2.5 µm, 3.5 µm, 5 µm</td>
<td>3 µm, 5 µm, 10 µm</td>
<td></td>
</tr>
<tr>
<td><strong>Pore Diameter/Volume</strong></td>
<td>120 Å</td>
<td>100 Å</td>
<td>100 Å</td>
<td></td>
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<tr>
<td><strong>Pore Volume</strong></td>
<td>0.3 mL/g</td>
<td>0.7 mL/g</td>
<td>1.0 mL/g</td>
<td></td>
</tr>
<tr>
<td><strong>Surface Area</strong></td>
<td>100 m²/g</td>
<td>230 m²/g</td>
<td>330 m²/g</td>
<td></td>
</tr>
</tbody>
</table>
HSS T3 Technology

**Speed & Resolution – Without Compromise**

**HPLC**

2.1 x 150 mm, 5 µm

Rs (2,3) = 4.29

- Increase Speed
- Rs (2,3) = 4.29

**UPLC**

2.1 x 50 mm, 1.7 µm

Rs (2,3) = 4.28

- Increase Resolution
- Rs (2,3) = 4.28

**Fastest & Most Sensitive Methods**

**Same Resolution**

- Absorbance at 270 nm
- 0.00
- 0.26
- Minutes
- 0.40 0.80 1.20 1.60 2.00

**UPLC**

2.1 x 100 mm, 1.7 µm

Rs (2,3) = 6.38

- Faster & More Sensitive Methods
- Highest Resolution

- Absorbance at 270 nm
- 0.00
- 0.26
- Minutes
- 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00

**UPLC**

4.5X Speed

2X Sensitivity

1.5X Resolution

- Rs (2,3) = 6.38
- Absorbance at 270 nm
- 0.00
- 0.26
- Minutes
- 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00

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HSS T3 Bonding Technology: Comparative Chromatograms

Increased peak capacity!

**Atlantis T3**
- Average Peak Width @ 13.4% = 0.075
- Peak Capacity = 100

**XSelect HSS T3 XP**
- Average Peak Width @ 13.4% = 0.066
- Peak Capacity = 114

**CORTECS T3**
- Average Peak Width @ 13.4% = 0.058
- Peak Capacity = 129

Comparative separations may not be representative in all applications.

1. Propyl Gallate
2. 2,4,5-Trihydroxybutyrophenone
3. tert-Butylhydroquinone
4. Butylated Hydroxyanisole
5. 2,6 di-tert-butyl-4-hydroxymethylphenol
6. Octyl Gallate

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Catecholamines & Metabolites Transfer: Atlantis T3 to ACQUITY UPLC HSS T3

Compounds:
1. Norepinephrine
2. Epinephrine
3. Dopamine
4. 3,4- Dihydroxyphenylacetic acid
5. Serotonin (5-HT)
6. 5-Hydroxy-3-indoleacetic acid
7. 4-Hydroxy-3-methoxyphenylacetic acid (HVA)

Comparative separations may not be representative in all applications.
In Vitro Incubated Amitriptyline Hepatocyte Samples

2x increased retention compared to conventional C18!!

ACQUITY UPLC BEH C18
1.7 µm, 2.1x100mm

ACQUITY UPLC HSS T3
1.8 µm, 2.1x100 mm

Amitriptyline + 16 Metabolites
ACQUITY UPLC HSS T3 Columns: 
*Highest Retentivity – Polar Compounds*

Elution order: thiourea, 5-fluorocytosine, adenine, thymidine-5-monophosphate, thymine
10 mM NH₄COOH pH 3.0, 0.20 mL/min

Comparative separations may not be representative in all applications.
T3 Bonding Technology: CORTECS T3

- Solid core particle with
  - Larger pore diameter 120Å
  - **Lower ligand density** when compared to typical C$_{18}$ columns
  - Chemically stable at low pH and reproducible
  - LC/MS compatible

![Diagram of T3 bonding technology](attachment:image.png)

- **Balanced retention** of both polar and non-polar compounds under RPLC conditions
- **Increased retention for polar compounds** over typically bonded solid-core C$_{18}$ phases

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Increased CORTECS T3 Efficiency

57% Higher Efficiency
CORTECS UPLC T3 1.6 µm
compared to
ACQUITY UPLC HSS T3 1.8 µm

32% Higher Efficiency
CORTECS T3 2.7 µm
compared to
XSelect HSS T3 2.5 µm
More Efficiency per Unit Pressure
Measured N/ΔP*

- Faster flow rates for higher throughput
- Lower backpressures for 100% aqueous mobile phases
- Longer columns for added resolution

*Comparison of particles of equivalent particle size
T3 Particle Efficiency: 
*Atlantis T3 Versus CORTECS T3*

1. Propyl Gallate
2. 2,4,5-Trihydroxybutyrophenone
3. tert-Butylhydroquinone
4. Butylated Hydroxyanisole
5. 2,6 di-tert-butyl-4-hydroxymethylphenol
6. Octyl Gallate

**Atlantis T3**
4.6 x 150 mm, 5 µm

**CORTECS T3**
3.0 x 75 mm, 2.7 µm

Comparative separations may not be representative in all applications.
Summary: Reversed-Phase HPLC

- Reversed-Phase HPLC for Polar Compounds
  - Embedded polar groups were designed for peak shape, not polar compound retention
  - The sudden loss in retention observed under highly aqueous conditions is due to pore dewetting not hydrophobic collapse
  - A properly designed straight alkyl chain C18 column can provide the perfect balance of polar and non-polar compound retention

- T3 particle technology gives you:
  - Superior retentivity for polar compounds
  - Resistance to pore dewetting
  - Ultra-low MS bleed
  - Improved pH stability
  - Improved pH 7 peak shape
Thank You!

Live Q&A Session