What’s Wrong With My Chromatography

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January 13, 2015
Outline of Presentation

I. Common Problems, Symptoms, and Solutions
   - Baseline Shifts/Noise
   - Peak Shapes
   - Pressure
   - Retention Shifts

II. Preventative Maintenance/Good Practices
   - In-line Devices
   - Sample Preparation
   - Operational Limitations
Common Problems
Noisy Baselines

Possible Causes

- Dirty flow cell
- Lamp failing
- Pulses from pump (if periodic)
- Temperature effects on detector
- Air bubbles passing through detector

Time (min.)
Non-spec Lamp
Chromatographic Results with Second Source Lamp at 214 nm

Lamp from Agilent Technologies
Peak 1  S/N = 150
Peak 2  S/N = 400
Peak 3  S/N = 300

Lamp from Second Source
Peak 1  S/N = 15
Peak 2  S/N = 50
Peak 3  S/N = 50
Drifting Baseline

Possible Causes

- Gradient elution
  - Mobile phase component
- Temperature unstable (RID)
- Contamination in mobile phase
- Mobile phase not in equilibrium with column
- Contaminant bleed in system
  - Hardware chemical compatibility
Effect of TFA on Baseline

A: 0.1% TFA in H$_2$O    B: 0.1% TFA in ACN    Temperature: 35°C

Eclipse XDB-C8
4.6 x 150 mm, 5 µm

Gradient: 5 - 100% B in 30 min  
Flow Rate: 2.0 mL/min

✓ Wavelength
• 215nm
• 254nm less impact

✓ Adjust TFA concentration in Solvent B to level baseline

➢ Know the UV Cutoff of your mobile phase components
Peak Shape

Ghost Peaks – Appear When No Sample is Injected

20% - 100% MeOH

No Sample Injected

Problem
- Dirty mobile phase
- Sample carryover
  - May imply poor recovery
- Peak from an early run
  (isocratic)
Solvent Contamination

Injections on Agilent 1100

- Solvent Source 1
- Solvent Source 2
- Solvent Source 3
- Solvent Source 4, Lot 1
- Solvent Source 4, Lot 2
Peak Shape
Double Peaks

Possible Causes

- Void in column
- Partially plugged frit
- Only one peak
- Sample solvent mismatch
Strong Sample Solvent Can Compromise Peak Shape

**Column:** ZORBAX SB-C8, 4.6 x 150 mm, 5 µm  
**Mobile Phase:** 82% H₂O:18% ACN  
**Injection Volume:** 30 µL  
**Sample:** 1. Caffeine  2. Salicylamide

A. Sample Solvent  
100% Acetonitrile

B. Sample Solvent Mobile Phase

![Graph A](image1)  
![Graph B](image2)
Peak Shape

Broad Peaks

All Peaks Broadened
- Loss of column efficiency
- Large injection volume/mass
- High viscosity mobile phase
- Sample solvent mismatch

Some Peaks Broadened
- Late elution from previous sample (isocratic)
- High MW sample – Protein or polymer
Injection Volume Robustness

Column: ZORBAX Rapid Resolution Eclipse XDB-C8
4.6 x 75 mm, 3.5 µm
Mobile Phase: 44% 25 mM phosphate, pH 7.00
56% methanol
Flow Rate: 1.0 mL/min
Temperature: 25°C
Detection: UV 250 nm
Sample: 1. ketoprofen
2. ethyl paraben
3. hydrocortisone
4. fenoprofen
5. propyl paraben
6. propranolol
7. ibuprofen

\( R_s(6,7) = 1.6 \)

\( R_s(6,7) = 2.1 \)

\( R_s(6,7) = 2.5 \)

✓ Varying injection volume can sometimes reveal lack of robustness for resolution and peak shape.
Extra Column Volume

Increasing extra-column volume

- Use short, small internal diameter tubing between the injector and the column and between the column and the detector
- Make certain all tubing connections are made with matched fittings
- Use a low-volume detector cell
- Inject small sample volumes
Peak Broadening
Differences in Detector Flow Cell Volume Can Affect N and $R_s$

Scenario: ZORBAX Rapid Resolution Column: 75 mm, 3.5-µm; Flow Rate: 1mL/min; $k = 3$

<table>
<thead>
<tr>
<th>Flow Cell Volume</th>
<th>Band Broadening* (4.6 mm)</th>
<th>Band Broadening* (2.1 mm**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 µL</td>
<td>0.3%</td>
<td>6%</td>
</tr>
<tr>
<td>8 µL</td>
<td>6%</td>
<td>138%</td>
</tr>
<tr>
<td>14 µL</td>
<td>19%</td>
<td>423%</td>
</tr>
</tbody>
</table>

*Versus 8571 theoretical plates (HPLC Calculations Assistant, Version 2.1, Savant Audiovisuals)
**Flow Rate, 0.2 mL/min
Analysis of Morphine and Metabolites by LC/MS/MS using an RRHD HILIC Plus Column

**Default Agilent 1290 Infinity LC System with 6410A Triple Quadrupole Mass Spec, 8.7 µL Extra-Column Volume**

- **A:** 10mM ammonium formate, pH 3.2
- **B:** acetonitrile/100mM ammonium formate, pH 3.2 (9:1)
- 0.4 mL/min
- t(min) 0.0 0.25 1.00
- %B 100 100 55
- 0.1 µL injection
- TCC: 25 °C
- MS: ESI+, dMRM, 250 C, 11 L/min, 30 psi, 4000 V, 200 V delta EMV, 40 ms cycle time
- Agilent ZORBAX RRHD HILIC Plus, 2.1 mm x 50 mm, 1.8 µm

**Optimized Agilent 1290 Infinity LC System with 6410A Triple Quadrupole Mass Spec, 3.1 µL Extra-Column Volume**

- 1. Normorphine
- 2. Morphine
- 3. Morphine-6-b-D-glucuronide (M6G)
- 4. Morphine-3-b-D-glucuronide (M3G)
Peak Shape
Tailing Peaks, >1.5

Causes

Some Peaks Tail
- Secondary effects
  - Residual silanol interactions
- Small peak eluting on tail of larger peak

All Peaks Tail
- Extra column effects
- Bad column
- Contamination
  - Column
  - Frit
- Metals
- Inappropriate sample size or solvent
Peak Tailing
Injector Seal Failure

Column: Bonus-RP, 4.6 x 75 mm, 3.5 mm  Mobile Phase: 30% H₂O : 70% MeOH  Flow Rate: 1.0 mL/min

Plates  USP TF (5%)
1. 2235  1.72
2. 3491  1.48
3. 5432  1.15

Plates  USP TF (5%)
1. 3670  1.45
2. 10457  1.09
3. 10085  1.00

Before

After replacing rotor seal and isolation seal

فضيليةٌ على الرطب يمكن أن تسبب مشاكل تشكّل الأطراف.

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Agilent Technologies
Peak Shape
Fronting Peaks, Symmetry <0.9

Causes
- Small band eluting before large band
- Column void
- Inappropriate sample solvent
Agilent 1290 Infinity LC System
Agilent 6410A LC/MS
Agilent ZORBAX RRHD HILIC Plus 2.1 x 50 mm, 1.8 µm

Acetonitrile / 100 mM ammonium formate pH 3.2 (9:1)
0.4 mL/min, Pressure: 135 bar
Isocratic elution
Injection Volume: 1 µL of 5 µg/mL sample
Column: 25 °C
MS: ESI+, SIM, 200 °C, 10 L/min, 30 psi, 4000 V, 15 ms dwell time

Sample:
4-Aminobenzoic acid, m/z 138 (Frag 110 V)
Nicotinamide, m/z 123 (Frag 130 V)
Riboflavin, m/z 377 (Frag 160 V)
Nicotinic acid, m/z 124 (Frag 130 V)

Strong injection solvents negatively affect peak shape and retention
Poorly Made Connections = Peak tailing/fronting

Wrong … too short

If Dimension X is too short, a dead-volume, or mixing chamber, will occur. This can broaden or split peaks and/or cause tailing. It will typically affect all peaks, but especially early eluting isocratic peaks.

For information on making proper connections check out The LC Handbook, Pub. No. 5990-7595EN

➢ New A-Line fittings and capillaries can prevent this
Peak Shape
Negative Peaks

**Causes**

- Absorbance of sample is less than the mobile phase
- Equilibrium disturbance when sample solvent passes through the column
- Normal with Refractive Index Detectors
- Indirect UV detection
Peak Shape Issues
“Usual Suspects”

✓ Partially blocked inlet frit
✓ Sample solvent strength
✓ Injection volume
✓ Sample load
✓ Secondary interactions
✓ Hardware failure (rotor, stator)
✓ Ghost peaks
✓ Metal interactions/chelation
✓ No or insufficient mobile phase pre-heating
✓ Extra column volume
✓ Mobile Phase
Troubleshooting Pressure

I. Continuously Increasing Pressure *With No* Injections
   - Pump Seals
   - Mobile Phase Particulates
   - Mobile Phase Solubility
   - Mobile Phase Unstable (polymerization)
   - Column Void Formation (use condition dependent)

II. Increasing Pressure *With* Sample Injections
   - Sample Particulates
   - Sample Not Soluble in Mobile Phase
   - Sample Components Irreversibly Bound to Stationary Phase
Determining the Cause and Correcting

➢ Check pressure with/without column - many pressure problems are due to blockages in the system or at the guard or in-line filter

If Column pressure is high:

• Wash column
  ✓ Eliminate column contamination and plugged packing
  ✓ high molecular weight/adsorbed compounds
  ✓ precipitate from sample or buffer

• Back flush column
  ✓ Clear plugged frit

• Change frit – Not recommended
Column Cleaning

Flush with stronger solvents than your mobile phase

Reversed-Phase Solvent Choices
in Order of Increasing Strength

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile
- 75% Acetonitrile:25% Isopropanol
- 100% Isopropanol
- 100% Methylene Chloride*
- 100% Hexane*

Use at least 10 column volumes of each solvent for analytical columns

* When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.
Protein/Peptide Samples

If precipitate might be present (i.e., aggregated protein, cellular material, polymer)

• Attempt to clear blockage with appropriate solubilizing solvent
  - 0.1% TFA / 80% acetonitrile, 6M Guanidine HCl, THF, HFIP
  - See Appendix for other suggestions
Microbial Growth

- **Potential problems**
  - Increased system pressure or pressure fluctuations
  - Increased column pressure, premature column failure
  - Can mimic application problems
  - Gradient inaccuracies
  - Ghost peaks

- **Prevent and/or Reduce Microbial Growth**
  - Use freshly prepared mobile phase
  - Filter
  - Do not leave mobile phase in instrument for days without flow
  - Always discard “old” mobile phase
    - Do not add fresh mobile phase to old
  - Use an amber solvent bottle for aqueous mobile phase
  - If possible, can add
    - 5% organic added to water can be used to reduce bacterial growth
    - Few mg/l sodium azide

*Check your instrument manual for guidelines*
Column Troubleshooting
Retention Shifts

All Peaks Shift to Lower Retention (acids, bases, neutrals)
- Loss of bonded phase
- Mobile phase unstable (less likely)
- Solvent delivery system (flow rate or mixing)

All Peaks Shift to Greater Retention
- Loss of organic solvent in aqueous/organic mix
- Column change (less likely)
- Solvent delivery system (flow rate or mixing)

Ionic Peaks Shift Retention
- Loss of volatile MP component (ionic strength, pH shift)
- Column Change (bonded phase or contamination)
Retention Shifts

Mobile Phase Related Problems
  - Make fresh, compare to aged
    - pH
    - conductivity
    - chromatographic test

Column Related Problems
  - Test new column
  - Test current column with test mixture or e.g., Toluene
  - "Wash" column and retest
  - Consider effect of sample matrix
Separation Conditions That Can Cause Changes in Retention*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Change</th>
<th>Retention</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate</td>
<td>+/- 1%</td>
<td>$t_R$</td>
<td>+/- 1%</td>
</tr>
<tr>
<td>Temperature</td>
<td>+/- 1 deg C</td>
<td>$t_R$</td>
<td>+/- 1 to 2%</td>
</tr>
<tr>
<td>% Organic</td>
<td>+/- 1%</td>
<td>$t_R$</td>
<td>+/- 5 to 10%</td>
</tr>
<tr>
<td>pH</td>
<td>+/- 0.01%</td>
<td>$t_R$</td>
<td>+/- 0 to 1%</td>
</tr>
</tbody>
</table>

Change in Volatile Buffer Concentration Can Cause Shifts in Retention Time and Peak Shape

30:70 ACN:Water with 0.1% TFA, pH 2

- Berberine: $T_f = 1.11$
- Imipramine: $T_f = 1.16$
- Amitriptyline: $T_f = 1.18$

30:70 ACN:Water with 0.01% TFA, pH 2.9

- Berberine: $T_f = 1.28$
- Imipramine: $T_f = 1.57$
- Amitriptyline: $T_f = 1.73$

Column: C18, 4.6x100mm, 5µm
Flow Rate: 2 mL/min,
Detection: UV 210nm Detection,
Temp: 25 °C
Inj Amt: 0.05µg each compound (2 µL Inj.)
Mobile Phase Preparation

- Small changes in mobile phase strength can have a large effect on retention

- HPLC grade or better
- Buffer prep procedure
  - Be consistent
    - Document process
  - See appendix

- Volume % of solvents can depend on preparation
  - Specified volume ACN added to a 1 L volumetric and made to volume with H₂O ≠
  - Specified volume H₂O added to a 1 L volumetric and made to volume with ACN ≠
  - 500 ml H₂O added to 500 ml ACN

- Degree of contraction is affected by the relative quantities of each
- Temperature
II. Preventing HPLC Column Problems

✓ In-line devices
  • Pre-column
  • In-line Filter
  • Guard Column

✓ Sample Preparation

✓ Operational Conditions
In-line Filters & Guard Columns

- **Mobile Phase from pump**
- **Solvent Filter**
- **Injector**
- **Guard column and/or In-line Filter**
- **Stripping or concentrating column (if used)**
- **Analytical Column**
- **To Detector**

**Pre-injector** - Acts on mobile phase

**Post Injection** – Acts on sample
- In-line filter
  - Particulates that can plug frit
- Guard column
  - Protects analytical column from sample contaminants that are strongly or permanently retained

[Diagram showing the flow of mobile phase through the system with labels and annotations for each component.]
II. Preventing HPLC Column Problems

✓ In-line devices

✓ Sample Preparation
  • SPE
    - Analyte Adsorption (Bind-Elute)
    - Matrix Adsorption (Interference Removal)
  • Filtration
    - Syringe
    - In-line

✓ Operational Conditions
Why is Sample Prep Required?

Current Sample - Unsuitable for further analysis

Why?
- Too dilute
  • Analyte(s) not concentrated enough for quantitative detection
- Too dirty
  • Contains other sample matrix components that interfere with the analysis
- Too dangerous
  • Contaminants can be ‘column killers’
Inexpensive Filters
Prevent Column Frit Plugging

E.g., Regenerated Cellulose (RC)

- Universal hydrophilic membrane, compatible with most solvents - aqueous and organic
- High purity, extremely low extractables and binding
- More uniform surface
- Choose appropriate pore size

In-line filters

- Easy to Use and replace
- Frits available in 0.2, 0.5 and 2.0µ porosity
- Much Less expensive than a Column
- Easier and Faster to Replace than a Column Frit
II. Preventing HPLC Column Problems

✔ In-line devices

✔ Sample Preparation

✔ Operational Conditions
Mobile Phase pH and Buffers
Why Are These So Important in HPLC?

- pH - Effects Ionization
  - Silica surface of column
  - Sample components of interest

- Buffers
  - Resist changes in pH and maintain retention
  - Improve peak shape for ionizable compounds

- Effects Column Life
  - Low pH strips bonded phase
  - High pH dissolves silica
Operational Conditions
Mobile Phase Effects on Column Life

Low pH (1-3) - Bonded phase loss by acid catalyzed hydrolysis

* Hydrolytically sensitive siloxane bond

Conventional

StableBond

* Hydrolytically sensitive siloxane bond

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Operational Conditions
Mobile Phase Effects on Column Life

High pH >7- Silica Dissolution

Non-endcapped

Endcapped and/or modified surface
Eclipse Plus, Poroshell 120 EC, HpH

Dissolution Rate Increases With
• pH increase
• Temperature increase
• Phosphate>Borate>Organic Buffers
Good Column Practices

- Filter buffers.
- Investigate effects of sample solvent on solubility and separation.
- Pretreat samples which contain strongly retained components of no interest.
- Awareness of column packing limits
  - pH
  - Temperature
  - Chemical compatibility
- Use fresh aqueous solutions, consider use of a bio-stat (sodium azide).
- Flush column periodically with strong solvent
- To store column, purge buffers and leave in appropriate solvent (ACN)
- Avoid physically mishandling columns: banging, dropping, or over tightening fittings
Troubleshooting Summary

Most HPLC column problems can be prevented provided proper precautions are taken:

- Mobile phase must be soluble and particulate free
- Use guard columns and in-line filters; change as needed
- Use appropriate sample preparation procedures
- Use appropriate column cleaning procedure
- Use appropriate columns for operating conditions (e.g., SB: pH 1-6, Poroshell 120 HpH: pH 3-11)
- Keep record of column backpressure & important chromatographic parameters (e.g. R, N, k ’)
- Store column in organic solvent (acetonitrile) or other recommended by manufacturer

Problems are not always associated with the column and may be caused by instrument or experimental condition issues.
Agilent Technical Support

800-227-9770 (US & Canada)
Options 3, 3, 2
Email: lc-column-support@agilent.com
www.agilent.com/chem
Appendix
Typical Column Problems

1. High back pressure
   a) Plugged frits

2. Reproducibility

3. Peak shape

4. Sample recovery

5. Loss of resolution

6. Lifetime

7. Leaks
Extra Column Volume and Dwell Volume

ECV = injection volume + connecting tube volume + fitting volume + detector cell volume

Dwell Volume = volume from formation of gradient to top of column
Solubilization Solvents for Proteins/Peptides

In Order of Weakest to Strongest

- Water / phosphate buffer
- Dilute Acid (TFA, HOAc or HCl)
- Neutral pH 6-8 M Guanidine-HCl or isothiocyanate
- 5% HOAc / 6 M Urea
- Dilute Acid + aqueous / organic solvents (ACN, MeOH, THF)
- Dilute Base (Ammonium Hydroxide)
- Neat Organic Solvents- ACN, MeOH, THF
- 99% Formic Acid
- HFIP or HFIP / aqueous mixtures
- 100% TFA
- DMSO or 0.1 - 1% TFA in DMSO
- Formamide
High Pressure In-line Filter Kit

For 2.1mm Frits use inserts with small cone

For 4.6mm Frits use inserts with big cone
Assembling the High Pressure Filter Kit

Put the first insert into the frit housing.

Place the frit on top of this insert.

Then place the second insert on top of the frit.
Assembling the High Pressure Filter Kit

Close the frit housing, screw finger tight

Slide the fitting, back and front ferrule onto the capillary. Insert the capillary into the frit housing bore, then tighten the fitting with your fingers. Push the capillaries all the way in into the bore. Connect the second capillary.

Ensure that both capillaries are still pushed all the way in into the bore. Then tighten both fittings at the same time with two ¼” spanners. This compresses the frit assembly and assures a leak tight connection.
SPE Modes

**Analyte Adsorption (Bind-Elute)**

Analyte(s) retained \((K_D \gg 1)\)

Matrix unretained \((K_D \approx 0)\)
and/or strongly retained \((K_D \gg 1)\)

Pre-concentration factor

Cleaner extracts

Load at 1-3 drops/sec (recovery \(\propto 1/\text{flow}\))

Capacity issues may be more important

**Matrix Adsorption (Interference Removal)**

Analyte(s) unretained \((K_D \approx 0)\)

Matrix retained \((K_D \gg 1)\)

No pre-concentration advantage

Eluates may not be as clean

Sample loading may be gravity fed

Used less often than analyte adsorption