Taking the Trouble Out of Troubleshooting

Is It The Column?

Is It The Method?

Is It The System?

Agilent ASTS 2017
IT MUST BE THE COLUMN!!! Right?!
Typical Case History: Lack of Efficiency on Poroshell 300

Agilent Publication

Customer Results

1100 quaternary pump
Typical troubleshooting questions are:

- What is wrong with the column?
- What is wrong with the instrument?

But separations are influenced by more than just the column or the instrument. A better question is:

“Why doesn’t the separation work as expected?”

The answer could be a problem with the column, the instrument or something else (mobile phase, sample, etc.)
General Troubleshooting Approach

![Troubleshooting Flowchart]

1. Problem
2. Recognition
3. Control
4. Analysis
5. Correction
6. Problem Solved
Categories of Column and System Problems

- Pressure
- Peak shape
- Detection
- Retention
<table>
<thead>
<tr>
<th>Problem</th>
<th>Percentage of Respondents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Back Pressure, Plugged Frits</td>
<td>24%</td>
</tr>
<tr>
<td>Poor Reproducibility</td>
<td>16%</td>
</tr>
<tr>
<td>Sample Recovery</td>
<td>14%</td>
</tr>
<tr>
<td>Loss of Resolution</td>
<td>13%</td>
</tr>
<tr>
<td>Instability</td>
<td>11%</td>
</tr>
<tr>
<td>Voids</td>
<td>8.1%</td>
</tr>
<tr>
<td>Leaks, Fittings</td>
<td>4.8%</td>
</tr>
<tr>
<td>pH Range</td>
<td>2.0%</td>
</tr>
<tr>
<td>Low Plate Count</td>
<td>2.0%</td>
</tr>
<tr>
<td>Column Overload</td>
<td>2.0%</td>
</tr>
<tr>
<td>Cost</td>
<td>1.7%</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>15%</td>
</tr>
</tbody>
</table>
# Pressure

<table>
<thead>
<tr>
<th>Low Pressure</th>
<th>Leaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wrong Flow Rate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>High Pressure</th>
<th>Plugged Frits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plugged Columns</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluctuating Pressure</th>
<th>Check Valves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pump Problems</td>
</tr>
</tbody>
</table>
Determining the Cause and Correcting High Back Pressure

Many pressure problems relate to blockages in the system. Check system pressure with / without column

If column pressure is high:
- Back flush column (care regarding future performance)
- Clear blocked frit (reverse flush with strong solvent)
- Wash column
- Eliminate column contamination and clear blocked packing
- Remove high M\text{w} / adsorbed compounds
- Clear precipitate introduced from the sample or buffer
Effect of Water Contaminants on LC

**PARTICLES**
- Damage pump and injector
- Plug column and frits
- Increase back pressure

**BACTERIA**
- Plug column and frits
- Increase back pressure
- Release ions and organics *(see effect of ions and organics)*

**ORGANICS**
- Lead to ghost peaks, baseline drift, poor repeatability
- Interfere with analytes
- Reduce column lifetime
- Increase MS background
- Suppress signal

**IONS**
- Form adducts
- Suppress signal
- Complicate mass spectra
Pictures of particulates and bacteria on LC

Plug column and inlet frits → results in high back pressure

UNFILTERED aqueous mobile phase

“AGED” aqueous mobile phase

leading to
- distorted peak shapes
- poor repeatability
Changing a Frit May Not Be a Good Idea

May not be possible with new generation columns
May damage high performance columns

Tip: Prevention is a Much Better Idea!

Results in a Void in the Column !!!
Inexpensive Filters Prevent Column Frit Plugging

Regenerated Cellulose (RC) **Recommended**
- Universal hydrophilic membrane, compatible with most solvents - aqueous and organic
- High purity, extremely low extractables and binding
- More Uniform Surface
- *Different than Other Cellulose Filters!!*

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
- LC Flow Path Filtration Points – Frits/Sieves to Replace on a regular basis.

  • Solvent Reservoir

  • Pump

  Replace PTFE frit in the purge valve.

  • In-line filters

    Normally placed between the autosampler and LC column

  • Column

  1. Difficult to replace column inlet frit (not recommended and can create a void)

  2. May be able to reverse column flow and dislodge blockage (flow to waste), but may not completely restore column performance.

  3. Most manufacturers use 2 um frits for 3 to 5 um particle columns, 0.5 um frits for sub 2 um particle columns. Match in-line frit to column frit size.
### Plugged Packing

<table>
<thead>
<tr>
<th>Particle Size</th>
<th>Area (μm²)</th>
<th>Diameter (μm)</th>
<th>Frit Porosity (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>2.7</td>
<td>0.9</td>
<td>2.0</td>
</tr>
<tr>
<td>3.5</td>
<td>1.3</td>
<td>0.6</td>
<td>2.0</td>
</tr>
<tr>
<td>2.7</td>
<td>1.0</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>1.8</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>1.7</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Beware of buffered mobile phases
Buffers usually contain insoluble material – filter
Buffer solubility decreases with increasing % organic* - avoid 100%B with buffer salts

Buffer solubility

This is an estimate of the soluble concentration of the least soluble buffer (potassium phosphate at pH 7.0) in three organic co-solvents.
Effect of Flushing on MS background

Milli-Q® water on Monday (after weekend)

Fresh Milli-Q® water after discarding several liters
Determining the Cause and Correcting High Back Pressure

Check pressure with/without column - many pressure problems are due to blockages elsewhere in the system.

If Column pressure remains high:

Rinse column (remove detector from flow path!)
  – Eliminate column contamination and plugged packing
  – high molecular weight/adsorbed compounds
  – precipitate from sample or buffer

Back flush column – may clear plugged column inlet frit

Install New column

Eliminate pressure issues – add a disposable 0.2, 0.5 or 2 um in-line filter to system.
Column Cleaning

Flush with stronger solvents than your mobile phase.

Reversed-Phase Solvent Choices in Order of Increasing Strength

Use at least 25 mL of each solvent for analytical columns

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

This Is Time Consuming
Often Performed Offline

Must Reverse to Re-Equilibrate

*Tip: When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.
Common Peak Shape Issues

Split Peak(s)

Peak Tailing/Fronting

Broad Peak

Many peak shape issues can be combinations i.e. peak tailing/fronting can also have shifts in retention

Symptoms can any number of peaks in the sample

Each problem can have multiple causes
Peak Splitting Caused By Disrupted Sample Path

- Flow Path Disrupted by Void
- Sample Allowed to Follow Different Paths Through Column
- Poorly Packed Bed Settles in Use
- High pH Dissolves Silica

Split or Double Peaks

Tip: Similar Effect Can be Caused by Partially Plugged Frit
Split Peaks from Injection Solvent Effects

Column: StableBond 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. Injection Solvent 100% Acetonitrile

B. Injection Solvent Mobile Phase

Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening
Trick: Keep Organic Concentration in Sample Solvent ≤ Mobile Phase
Peak Tailing, Broadening, and Loss of Efficiency

Secondary Interactions

Contamination

Aging

Overloading

Extra Column Effects
Peak Tailing

**Causes**

**Some Peaks Tail:**
- Secondary - Retention Effects.
- Residual Silanol Interactions.
- Small Peak Eluting on Tail of Larger Peak.

**Chemistry Problem**

**All Peaks Tail:**
- Extra-Column Effects.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.

**System Problem**
Peak Tailing
Identifying “Secondary Interactions”

Tip: Mobile phase modifier (TEA = triethylamine) competes with sample molecule for surface ion exchange sites at mid-range pH values

Additional Peak from TEA!
Low pH minimizes Secondary Interactions

Tip: Reducing mobile phase pH reduces interactions with silanols and peak tailing.
Raising pH Can also eliminate “Secondary Interactions”

Peak Shape and Retention of this sample of basic compounds improves at high pH where column has high IEX activity. Why?
Peak Tailing-Column Contamination

Tip: Quick Test to Determine if Column is Dirty or Damaged

Trick: Reverse Column and Run Sample – If Improved, Possible Cleaning Will Help - No improvement - Column Damaged and Needs to be Replaced

Contamination at head of column
Peak Shape Broadening

All Peaks Broadened:
- Loss of Column Efficiency.
- Column Void.
- Large Injection Volume.

Some Peaks Broadened:
- Late Elution from Previous Sample (Ghost Peak).
  - High Molecular Weight.
  - Sample - Protein or Polymer.
Test For Injection Volume Robustness

- Varying injection volume can sometimes reveal lack of robustness for resolution and peak shape.

**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
Unknown “Phantom Peaks”

**Sample 1:** Chlorpheniramine maleate
   Peak 1: maleate

**Sample 2:** Chlorpheniramine maleate and Pseudoephedrine
   Peak 1: maleate
   Peak 2: pseudoephedrine
   Peak 3: chlorpheniramine (from 1st injection)

**Plates**
1. 5922
2. 9879
3. 779

"Phantom" peak from first injection

Tip: The extremely low plates for moderately retained peaks are an indication of a very late eluting peak from a preceding run.
Peak Broadening
Extra-Column Volume

Column: StableBond SB-C18, 4.6 x 30 mm, 3.5 μm  Mobile Phase: 85% H₂O with 0.1% TFA: 15% ACN  Flow Rate: 1.0 mL/min

10 mL extra-column volume

50 mL extra-column volume (tubing)
Column Connections

Wrong ... too long
Ferrule cannot seat properly

If Dimension X is too long, leaks will occur

These poor connections cause:
• Poor efficiency
• Peak tailing
• Leaking

Wrong ... too short
Mixing Chamber

If Dimension X is too short, a dead-volume, or mixing chamber, will occur
Influence post-column capillary connections

One bad capillary connection!

Fixed!

130 mAU

160 mAU
Prefect Fitting Every Time

1300 Bar Rating

Finger Tight

600 Bar Finger Tight
1300 Bar with a ¼ turn
Changes in Retention (k)

Column Aging
Column Contamination
Insufficient Column Equilibration
Poor Column/Mobile Phase combination
Change in Mobile Phase
Change in Flow Rate
Change in Column Temperature
Instrument Issues
## Changes in Separation Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>New Range</th>
<th>Old Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate</td>
<td>+/- 1%</td>
<td>+/- 1% Tr</td>
</tr>
<tr>
<td>Temp</td>
<td>+/- 1 deg C</td>
<td>+/- 1 to 2% Tr</td>
</tr>
<tr>
<td>%Organic</td>
<td>+/- 1%</td>
<td>+/- 5 to 10% Tr</td>
</tr>
<tr>
<td>pH</td>
<td>+/- 0.01%</td>
<td>+/- 0 to 1% Tr</td>
</tr>
</tbody>
</table>
Other Causes of Retention Time Changes

1. Chelation properties of solute
2. Column “aging”
3. Determine pH sensitivity for ionizable compounds– use of buffers
4. Dwell volume (gradient delay volume) of instrument in gradient elution
5. Method and detection issues
6. Contact your supplier for additional support
Metal Sensitive Compounds Can Chelate

Hint: Look for Lone Pair of Electrons on :O: or N which can form 5 or 6 membered ring with metal.

Salicylaldehyde + $M^{+2}$ → 6-membered ring complex

8-hydroxyquinoline + $M^{+2}$ → 5-membered ring complex

α-benzoinoxomine + $M^{+2}$ → 5-membered ring complex
Acid Washing Can Improve Peak Shape

Before Acid Wash

After Acid Wash

50 – 100 mLs 1% H₃PO₄

Columns: ZORBAX SB-Phenyl
        4.6 x 150 mm
Mobile Phase: 75% 25 mM ammonium phosphate buffer
             25% ACN
Flow Rate: 1.0 mL/min.
Temperature: RT
Sample Size: 5 mL

Tf: 3.7

Tf: 1.2

• A 1% H₃PO₄ solution is used on SB columns, 0.5% can be used on endcapped columns.
Column Aging/Equilibration Causes Retention/Selectivity Changes

- The primary analyte was sensitive to mobile phase aging/conditioning of the column
- The peak shape was a secondary issue (metal chelating compound) resolved by “de-activating” the active metal contamination
Mobile Phase pH and pH Buffers
Why They Are Important in LC

• 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
Using Buffers Can Minimize Variability in Retention/Selectivity

Evaluate:

Method Ruggedness

- Type of Buffer Used
- Ionic Strength of Buffer
- Buffer Capacity

pH Sensitivity

- Sample
- Column
Case Study: Lot-to-Lot Selectivity Change Due to pH Choice.

- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot
- Indication of poorly controlled ionization
Why Worry About pH? pH, pKa and Weak Acids

\[
\text{RCOOH} \quad \xleftrightarrow{\text{K}_a} \quad \text{RCOO}^- + \text{H}^+ \quad \text{K}_a = \frac{[\text{RCOO}^-][\text{H}^+]}{[\text{RCOOH}]} \\
\]

\[
K_a = 6.4 \times 10^{-5} \quad \text{pK}_a = 4.2
\]

At pH 4.2 – the sample exists as benzoic acid and the benzoate ion in a ratio of 1:1. Peak shape can be poor.

At pH 5.2 – 91% of the sample exists as the benzoate ion. RP retention decreases.

At pH 3.2 – 91% of the sample exists as benzoic acid. RP retention increases.
Effect of pH on Peak Shape at or Near the Sample pKₐ

Column: ZORBAX SB-C8 4.6 x 150 mm, 5 mm
Flow Rate: 1.0 mL/min.

Mobile Phase: 40% 5 mM KH₂PO₄; 60% ACN
Temperature: RT

pH 4.4

pH 3.0

Ibuprofen
pKₐ = 4.4

● Inconsistent and tailing peaks may occur when operating close to an analyte pKₐ and should be avoided.
Why Worry About pH?

\[ R_3NH^+ \rightleftharpoons R_3N + H^+ \]

\[ K_a = \frac{[R_3N][H^+]}{[R_3NH^+] \rightleftharpoons R_3N + H^+} \]

\[ K_a = 1 \times 10^{-9} \]
\[ pK_a = 9 \]

At pH 9 – the sample exists as protonated and unprotonated diphenhydramine in a ratio of 1:1. Peak shape can be poor.
At pH 10 – 91% of the sample exists as unprotonated diphenhydramine.
At pH 8 – 91% of the sample exists as protonated diphenhydramine.
pH vs. Selectivity for Acids and Bases

Column: Nucleosil-C18
Mobile Phase: 45% ACN/55% phosphate buffer
Sample: Bile Acids

Column: mBondapak-C18
Mobile Phase: 60% 25 mM phosphate buffer
40% Methanol

1. Salicylic acid
2. Phenobarbital
3. Phenacetin
4. Nicotine
5. Methamphetamine

- Retention and selectivity can change dramatically when pH is changed.
Don’t Forget - Match Column to pH of Mobile Phase for Maximum Column Lifetime
High pH and Room Temperature (pH 11 RT)

Mobile Phase: 50%ACN: 50% Water : 0.2% TEA (~ pH 11)

After 30 injections

Tip: This column was not recommended for pH values above 9.0; Use Columns Designed for chosen pH (i.e. 11)
Tip: Dwell Volume Differences Between Instruments Can Cause Changes in Retention and Resolution

Column: ZORBAX Rapid Resolution Eclipse XDB-C8
4.6 x 75 mm, 3.5 μm

Mobile Phase: Gradient, 0 - 100 %B in 52.5 min.
A: 5/95 methanol/25 mM phosphate
pH 2.50
B: 80/20 methanol/25 mM phosphate
pH 2.50

Flow Rate: 0.5 mL/min

Temperature: 25°C

Injection: 5 μL
Detection: 250 nm

Sample: Mixture of antibiotics and antidepressants

Upper trace simulates actual run data entered into DryLab® 3.0 software
Lower trace is simulated chromatogram for larger $V_D$. 
Measuring System Dwell Volume ($V_D$)

\[ V_D = t_D \times F \]

- Intersection of the two lines identifies dwell time ($t_D$)
- Dwell volume is equal to product of the flow rate and the dwell time.
Detection Issues
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.
Ghost Peaks

Ghost Peaks - Peaks which appear even when no sample is injected.

Problem - Dirty Mobile Phase

20% - 100% MeOH Gradient
No Sample Injected
Noisy Baselines

Possible Causes:
- Dirty Flow Cell
- Detector Lamp Failing
- Pulses from Pump if Periodic
- Temperature Effects on Detector
- Air Bubbles passing through Detector
Drifting Baselines

- Gradient Elution
- Temperature Unstable (Refractive Index Detector)
- Contamination in Mobile Phase
- Mobile Phase Not in Equilibrium with Column
- Contamination Bleed in System
Chromatographic Results with “Wrong” Lamp at 214 nm Wavelength

Tip: Could also be a symptom of aging lamp
Expanded View of Chromatographic Results
Generic Source Lamp at 214 nm Wavelength

Tip: Poor S/N makes it difficult to detect low level impurities
**Detectors**

For narrow peaks, high data rates!!!

Maintaining Resolution at High Analysis Speed

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**80Hz versus 10Hz (20Hz) Data Rate**

- Peak Width: $-55\%$ ($-30\%$)
- Resolution: $+90\%$ ($+30\%$)
- Peak Capacity: $+120\%$ ($+40\%$)
- App. Column Eff.: $+260\%$ ($+70\%$)

<table>
<thead>
<tr>
<th>Data Rate</th>
<th>Peak Width</th>
<th>Resolution</th>
<th>Peak Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 Hz</td>
<td>0.300</td>
<td>2.25</td>
<td>60</td>
</tr>
<tr>
<td>40 Hz</td>
<td>0.329</td>
<td>2.05</td>
<td>55</td>
</tr>
<tr>
<td>20 Hz</td>
<td>0.416</td>
<td>1.71</td>
<td>45</td>
</tr>
<tr>
<td>10 Hz</td>
<td>0.666</td>
<td>1.17</td>
<td>29</td>
</tr>
<tr>
<td>5 Hz</td>
<td>1.236</td>
<td>0.67</td>
<td>16</td>
</tr>
</tbody>
</table>

Sample: Phenones Test Mix

Column: Zorbax SB-C18, 4.6x30, 1.8um

Gradient: 50-100%ACN in 0.3min

Flow Rate: 5ml/min
Different UV Data Collection Rates and MS Scan Rates in Scan Mode

Column: ZORBAX RRHD SB-C18, 2.1 x 100mm, 1.8um, 1200 bar
Sample: Green Tea

<table>
<thead>
<tr>
<th>UV Data Collection Rate</th>
<th>MS Scan Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.12 s</td>
<td>25 ms</td>
</tr>
<tr>
<td>PW$_{1/2}$ = 0.018</td>
<td>S/N = 39</td>
</tr>
<tr>
<td>0.25 s</td>
<td>50 ms</td>
</tr>
<tr>
<td>PW$_{1/2}$ = 0.018</td>
<td>S/N = 43</td>
</tr>
<tr>
<td>0.5 s</td>
<td>100 ms</td>
</tr>
<tr>
<td>PW$_{1/2}$ = 0.019</td>
<td>S/N = 86</td>
</tr>
<tr>
<td>1.0 s</td>
<td>250 ms</td>
</tr>
<tr>
<td>PW$_{1/2}$ = 0.025</td>
<td>S/N = 118</td>
</tr>
<tr>
<td>2.0 s</td>
<td>500 ms</td>
</tr>
<tr>
<td>PW$_{1/2}$ = 0.042</td>
<td>S/N = 109</td>
</tr>
</tbody>
</table>
1. First the green dots: the peak is defined by 24 values and shows a well-integrated profile.
2. Second the red dots/line: the peak is only described by six measurements, the peak area is smaller (limit of detection is smaller) and the calculated efficiency is only 89% of the blue-lined peak.
3. Third the yellow dots/line: the peak is severely distorted, retention time and peak area are unreliable and a calculated efficiency shows only 39% of the reality.

- Savitzky-Golay (1964): 12-15 points per peak
- Dyson (1998): 100 points per peak
Conclusions

HPLC column problems are evident as
- High pressure (prevention better than the cure)
- Undesirable peak shape
- Changes in retention/selectivity

Often these problems are not associated with the column and may be caused by instrument and chemistry (method) issues.
- pH of mobile Phase
- Instrument Connections
- Detector Settings
- Metal Contamination

Start With the Correct Questions
- Find the Answers
- The Answers will Lead to Solutions