Troubleshooting and Diagnostics Tips and Tricks

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Troubleshooting and diagnostics

- **Troubleshooting**
  - System Pressure Problems
  - Incorrect Retention time
  - Loss of precision
  - Carryover/Contamination
  - Split and Distorted Peaks
  - Baseline Noise

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Potential Sources of Chromatographic Problems

- Mobile Phase
- Injector
- In-Line Filter
- Column
- Detector
- Sample
- Pump
- Guard Column
- Connecting Tubing and Fittings
- Integrator/Recorder Software

Scientist/Analyst --
need for logical approach to save time

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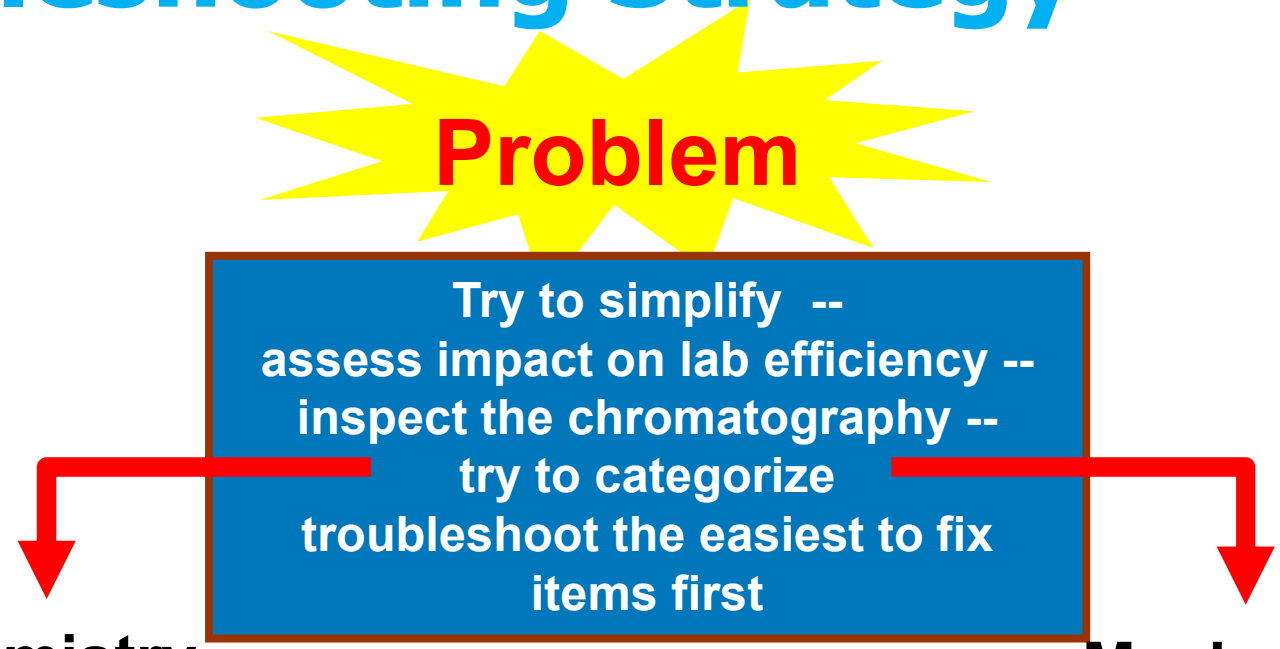
Troubleshooting Strategy



- COLUMN
  - GUARD COLUMN
- MOBILE PHASE
- SAMPLE

- PUMP
- INJECTOR
- DETECTOR
- DATA COLLECTION
- BAND SPREADING/ CONNECTIONS
- COLUMNS

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Troubleshooting and diagnostics

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System Pressure problems

- To identify a pressure change from ***normal operation***, create a pressure reference point

- System Pressure is affected by :
  - Column
  - Mobile phase
  - Flow rate
  - Temperature

  Can vary greatly with different methods

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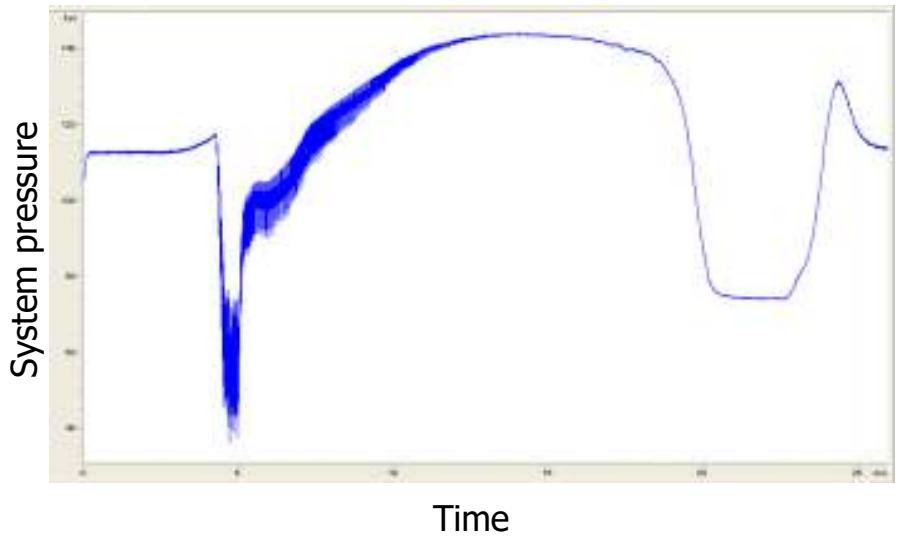
System Pressure problems

- Erratic flow rates/ pressure pulsations
- Overpressure
- No or low pressure



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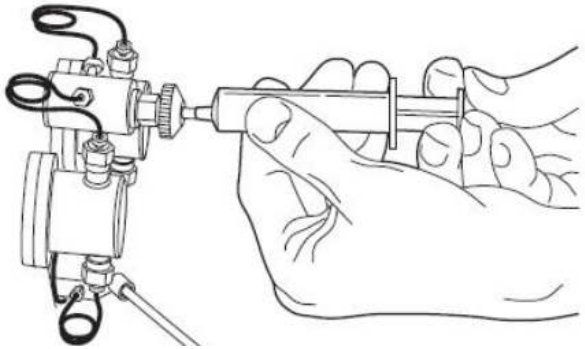
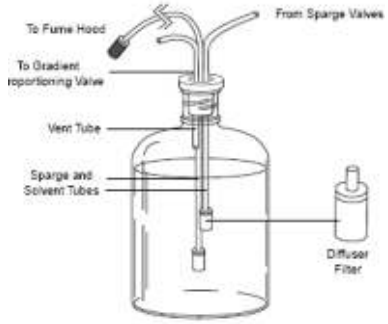
System Pressure problems

- **Erratic flow rates/ pressure pulsations**
  - ✓ **Air in system**
    Prime the pump (methanol or IPA to remove air)



  - ✓ **Air in solvent lines**
    Not enough solvent in bottle
    ➡ *Replace the solvent bottle*
    Bottle filters dirty
    ➡ *Remove the bottle filters / Replace*
    Not enough degas
    ➡ *Degas the mobile phase*



  - ✓ **Problem with check valves**
    ➡ *Sonicate or replace the check valves*
  - ✓ ***Problem with seals or plungers***

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System Pressure problems

- **Overpressure**
  - Check if pressure has risen *gradually or suddenly*

    ✓ *If pressure has risen gradually particulates are accumulated in inline filter, columns frits or column*

    ✓ *If pressure has risen suddenly something could be a obstruction in system or column*

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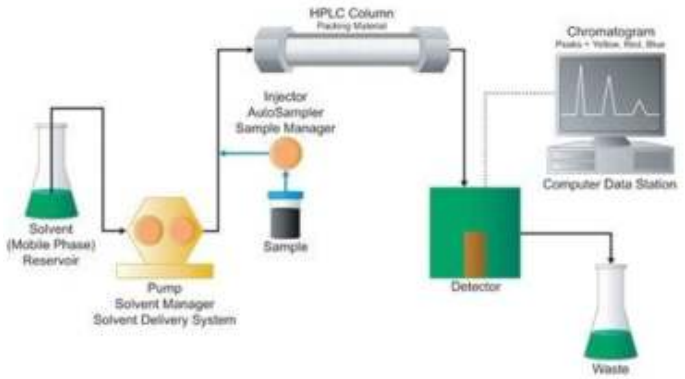
System Pressure problems



- **Overpressure**
  - *Check if something has changed (column, mobile phase,temperature)*
  - *If nothing has changed, remove the column and replace it with a union to check if the system pressure is the usual*
  - *If system pressure is high <u>loosen fittings beginning with the last connection in line</u> and working backward to the pump*
    - *After loosening each fitting observes if pressure stays the same or reduces*
    - *Replace or clean the appropriate part*

Caution: Carefully loosen fittings under high pressure

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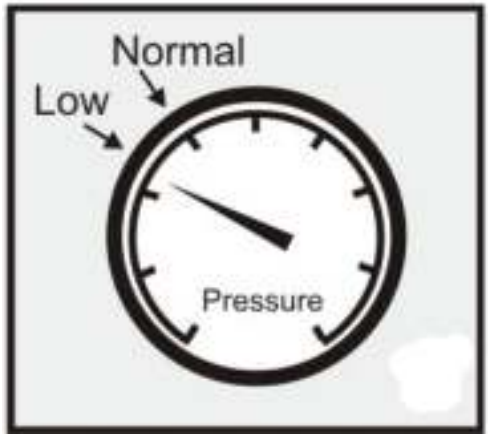
System Pressure problems

- **Low pressure**
  - *Check if something has changed (column, mobile phase, temperature, method)*
  - *If nothing has changed, check for leaks*
- **No pressure**
  - *Air in system*
    - ✓ *Prime the pump*

    (methanol or IPA to remove air)
  - *Air in solvent lines*
    - ✓ *Replace the solvent bottle*
  - *Problem of check valve*
    - ✓ *Sonicate or replace*
  - *Problem with seals or plungers*



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Troubleshooting and diagnostics

- **Troubleshooting**
  - System Pressure Problems
  - ***Incorrect Retention time***
  - Loss of precision
  - Carryover/Contamination
  - Split and Distorted Peaks
  - Baseline Noise

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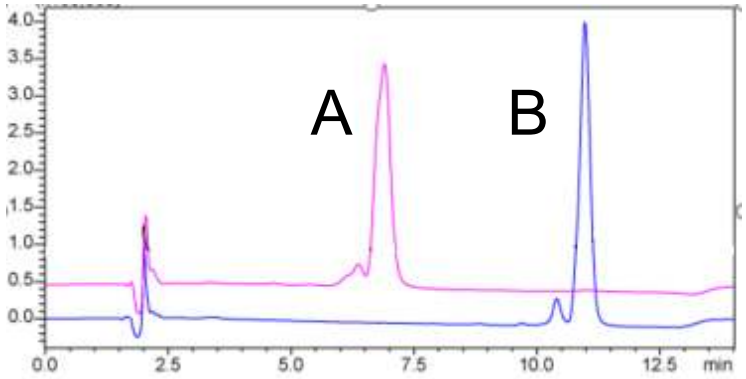
Retention Time

Retention time changed to a new constant value

- ***Less or more retention time : All Peak***
  - Pump flow rate problem
  - Wrong column type (C8 vs C18)
  - Temperature problem
  - % Organic in mobile phase



- ***Less or more retention time : Some Peak***
  - Chemistry problem
    - Wrong column type (CN vs C18)
    - Incorrect pH or un-buffered system (acid/bases compound)

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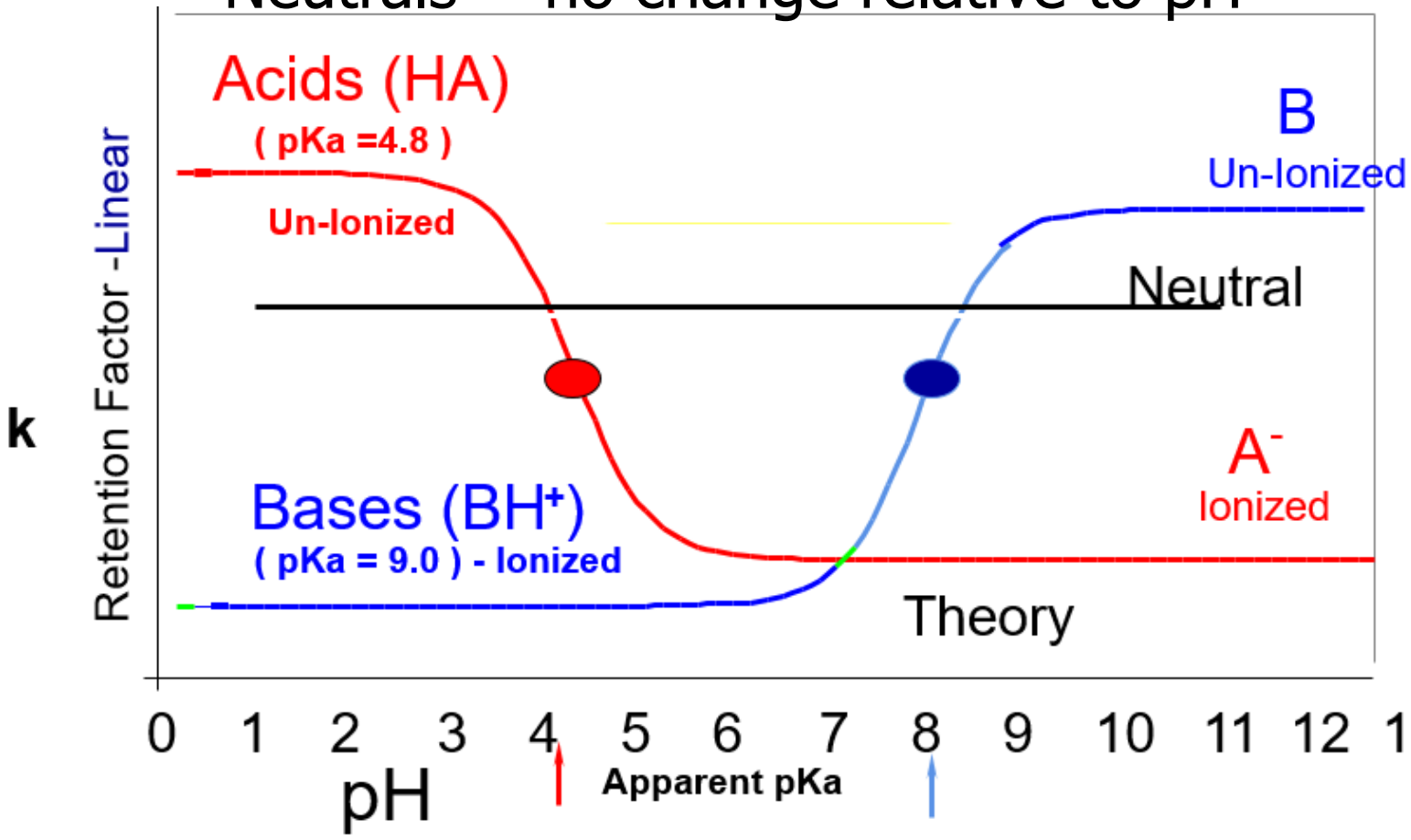
Dependence of retention by adjusting pH

retention maps - reversed-phase

Un-ionized form gives more retention,
Neutrals -- no change relative to pH



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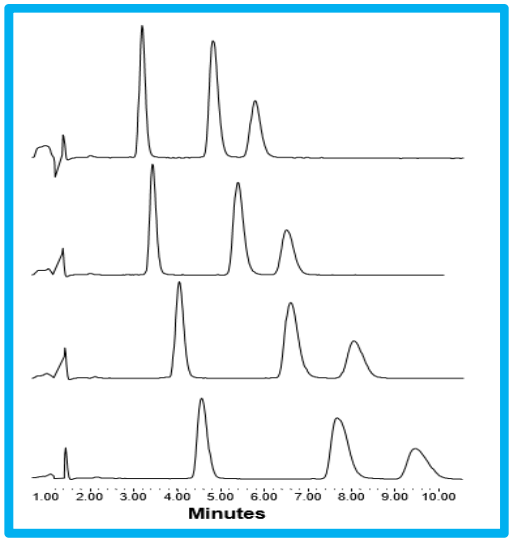
Retention Time

- **Erratic retention times**
  - *Column contaminated, degraded (C18 pH<2 or* >8*), not equilibrated (10-20 column volume)*
  - *Check if system also has erratic pump pressure/ Pressure fluctuations*
  - *Check for leaks*
  - *Improper solvent blending*
  - *Temperature fluctuations*
  - *Dewetting/Hydrophobic collapse (low organic<5%)*



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Troubleshooting and diagnostics

- **Troubleshooting**
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  - ***Loss of precision***
  - Carryover/Contamination
  - Split and Distorted Peaks
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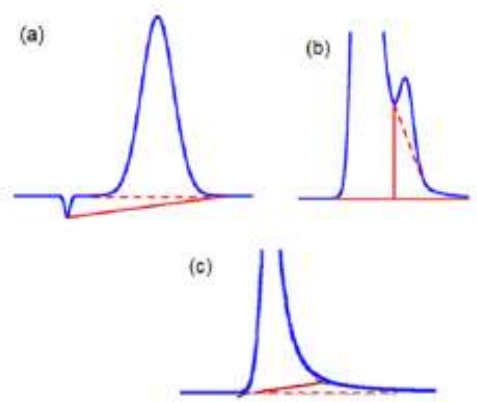
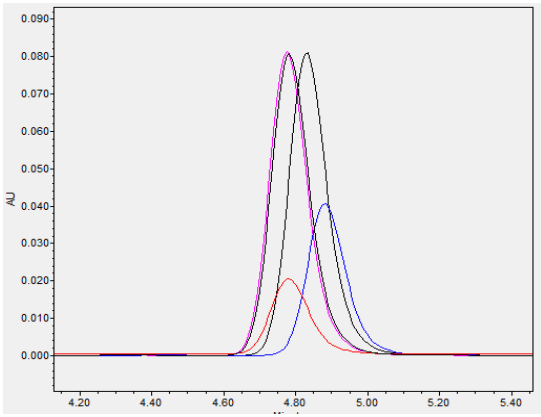
Loss of precision

- **Incorrect peak integration**
- **Check loss of precision is for all peaks in the chromatogram**
  - If it is only for some of them does not seem a injector problem



- **Check if reproducibility lack is for areas or also for Retention times**
  - Check for leaks
- **Check injection volume and sample concentration**
  - Don´t overload the column



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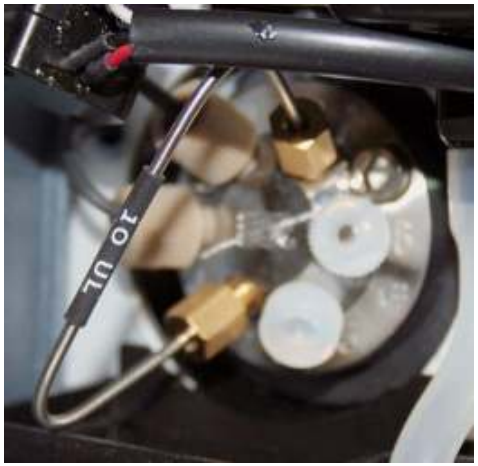
Loss of precision

- Check injector wash solvents. Are appropriate for the method?
- Check injection volume is inside system specifications
- Injector problem. Pass injector test
- It is important to have a system suitability to check the system



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Troubleshooting and diagnostics

- **Troubleshooting**
  - System Pressure Problems
  - Incorrect Retention time
  - Loss of precision
  - ***Carryover/Contamination***
  - Split and Distorted Peaks
  - Baseline Noise

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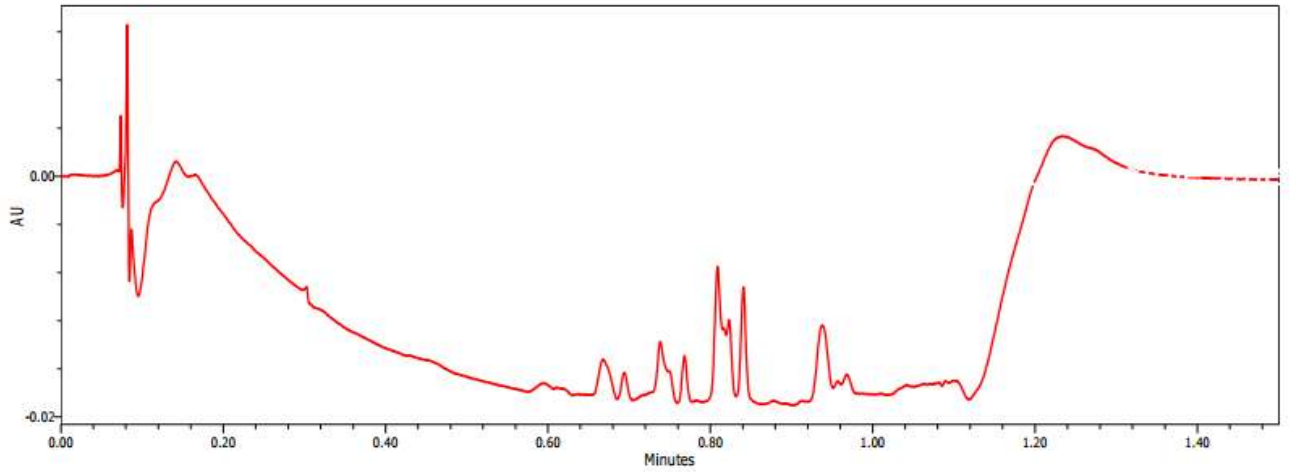
Definitions

- **Contamination**
  - The presence of any unwanted substance in a chromatographic system that appears either as peaks or high background noise
- **Carryover**
  - Is a specific type of contamination



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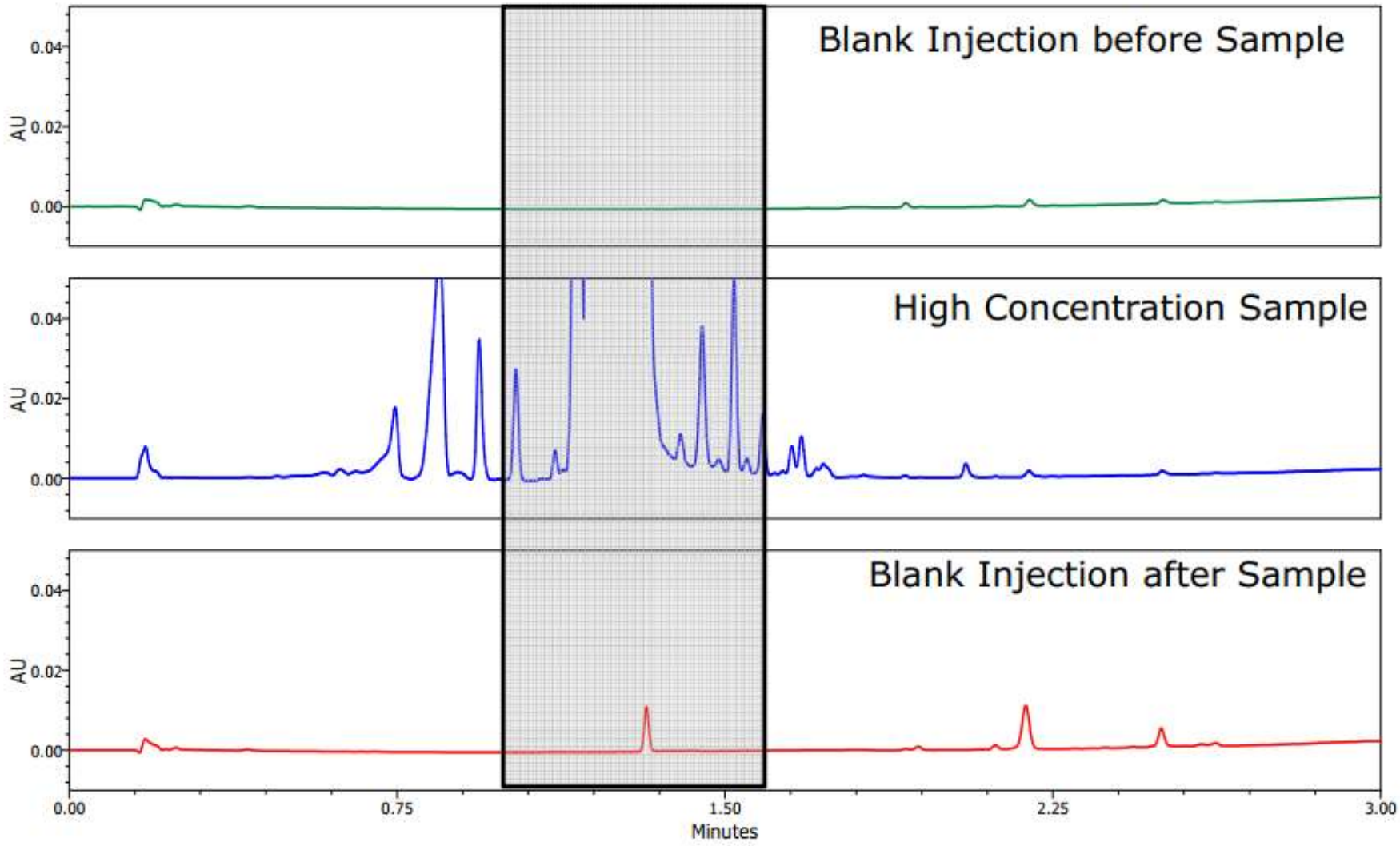
Example of Carryover



Critical Factors Affecting Carryover

- **Injection Type / Injection Technique / Wash Solvent Selection**
  - *Different injection modes*
  - *Choosing proper wash solvents*
- **Analytical Method**
  - *Column carryover ?*
  - *Precipitation in the injector ?*
- **Hardware Issues**
  - *Is something broken ?*
  - *Are there materials issues ?*

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Potential Sources of Contamination

- **Greater sensitivity means greater chance of seeing contamination**
- **Sources**
  - *Solvents and additives (water is a big problem)*
  - *Sample matrix*
  - *Sample preparation chemicals (detergents, salts)*
  - *Dirty glassware (Do not send solvent bottles to dish washer*
  - *Plastic containers or tubing*
  - *Detergents*
  - *HPLC systems, tubing*
  - *Hand creams*
  - *Manufacturing process*
- *WARNING* – Contaminates will adsorb and concentrate on C18 Columns

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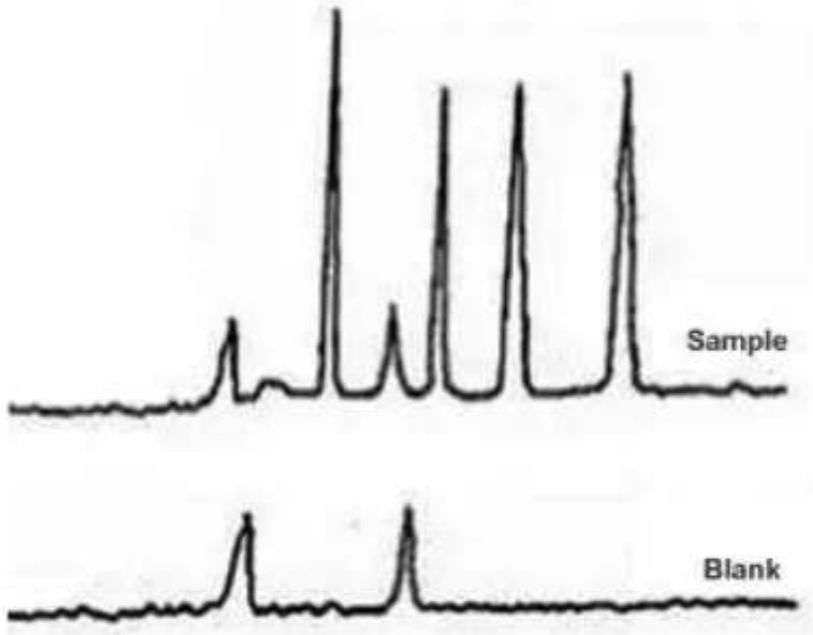
Contamination from the sample

- **Inject a pure standard**
- **Inject a blank of the sample diluent alone**
  - *Is it different from a water blank?*
- **Inject a sample with matrix**
  - *If there are other peaks they came from the matrix*
- **Inject volume zero**
  - *Injection 0 ul*



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Contamination Diagnostic: Injector-Pump

- **"Zero Volume Injection"**
  - *Have the injector go through the injection sequence without injecting any volume*
- **"No Injection" or Disconnect the autosampler and connect pumps to detector**
  - *Run gradient without injection*

  If the contamination peak is present without an injection, it is not from the injector

  System contamination, Solvent contamination, Column
- **Change pump solvents to check the pump**

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Solvent Contamination

- **With a C18 column in the system, a blank gradient is run and there are peaks in the chromatogram, there is contamination from somewhere**
  - *If it is in the water, the longer the re-equilibration at high aqueous, the larger the peaks*
- **Steps to eliminate**
  - *Find a better supply of solvents and or clean bottles*
  - *Strip column at 100% organic until baseline is low and stable*
  - *Run blank gradient again*
- **Organic solvent contamination, Change solvent bottles**

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UPLC/HPLC Cleanup Several Mixtures

- **Starting place**
  - *Isopropanol (IPA)*
  - *50 :50 acetonitrile-water + 0.1% formic acid*
- **Basic mixture – good for PEG, amides, esters**
  - *50:50 acetonitrile-water + 1% ammonium hydroxide*
- **Organic mixture – good for hydrophobic compounds**
  - *25:25:25:25 acetonitrile-methanol-isopropanol-water + 0.1% formic acid*
- **Organic mixture**
  - *Isopropanol-water + 1% acetic acid*
- **Acid cleanup**
  - *30% phosphoric acid (~4.4N) for UPLC,*

    6N Nitric acid for HPLC

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UPLC/HPLC Cleanup Cleaning the injector

- **Cleaning suggestion**
  - *Remove column*
  - *Put wash lines in cleaning mixture*
  - *Fill a vial with cleaning mixture*
  - *Inject multiple cleaning mixture*

- **Replace the parts if the cleaning of the injector does not work**

Prevent contamination is easier than troubleshooting and cleaning up

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Troubleshooting and diagnostics

- **Troubleshooting**
  - System Pressure Problems
  - Incorrect Retention time
  - Loss of precision
  - Carryover/Contamination
  - *Split and Distorted Peaks*
  - Baseline Noise

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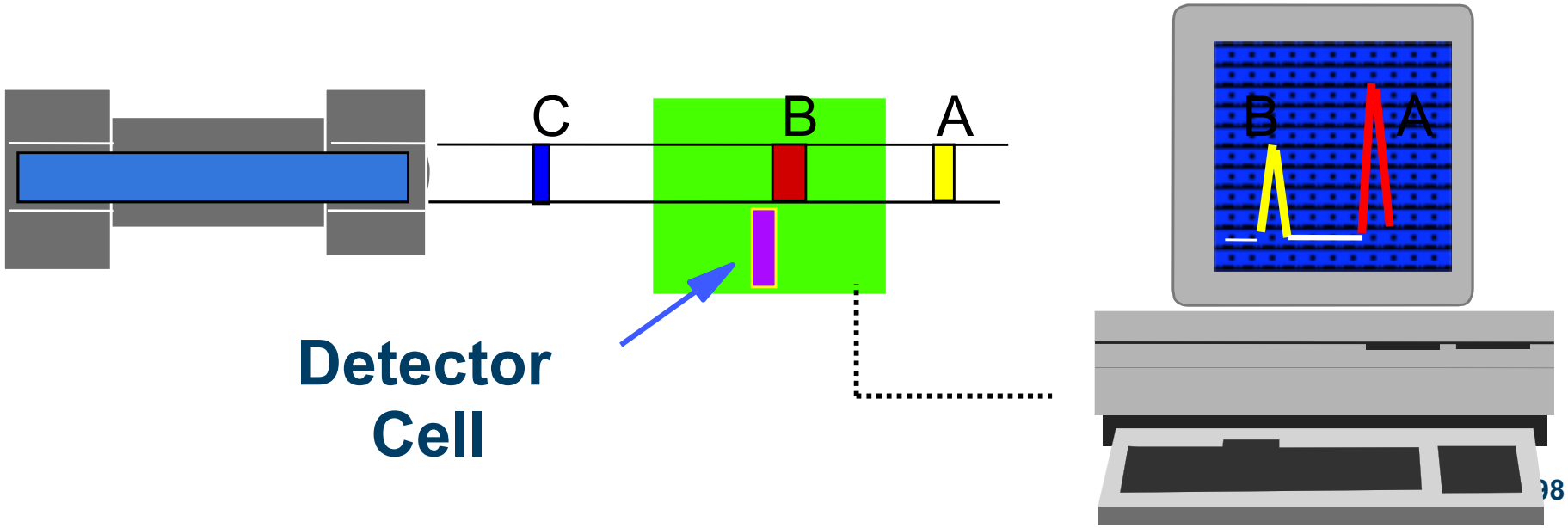
How to Categorize -- Inspect Chromatogram

- **How do you get sharp peaks with excellent resolution?**
  - Well Shaped Bands -- Well Separated

  (Good Mechanical And Chemical Performance)



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Why Do You Get Distorted Peaks?

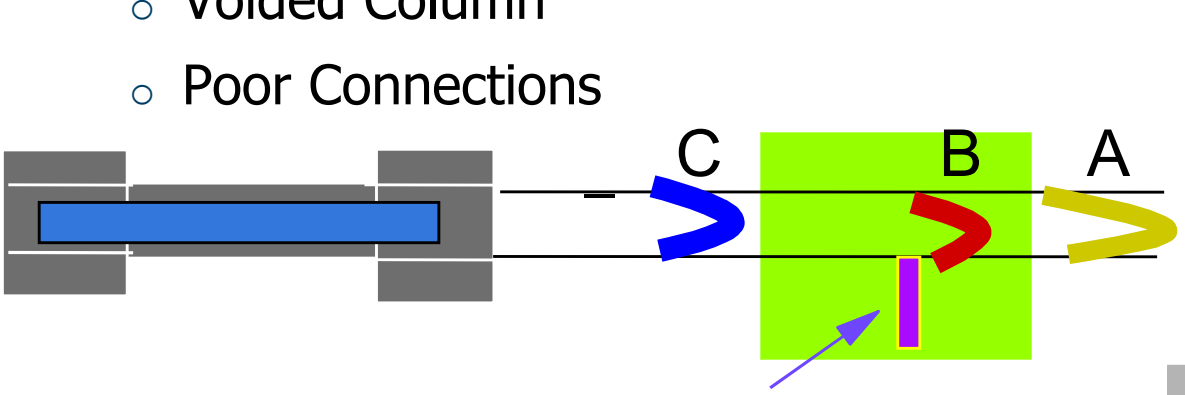
- **Why do you get all distorted peaks?**
  - **Distorted Bands –**

Mechanical Problem

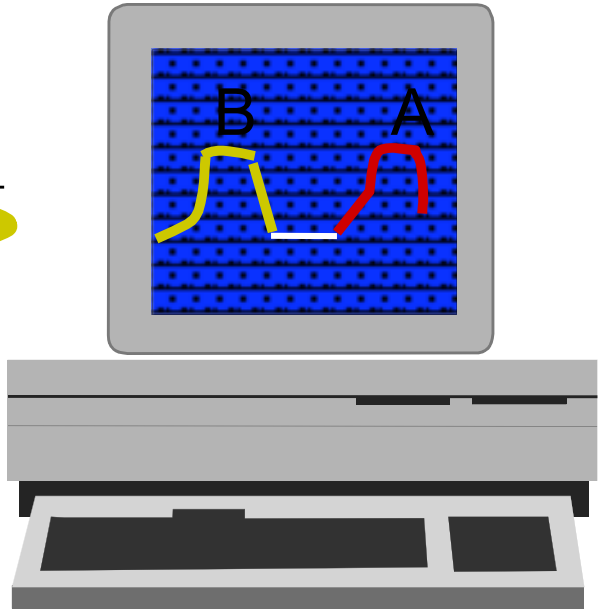
- Injector
- Voided Column
- Poor Connections

Chemical Problem

- Too Strong Sample Solvent



Detector Cell



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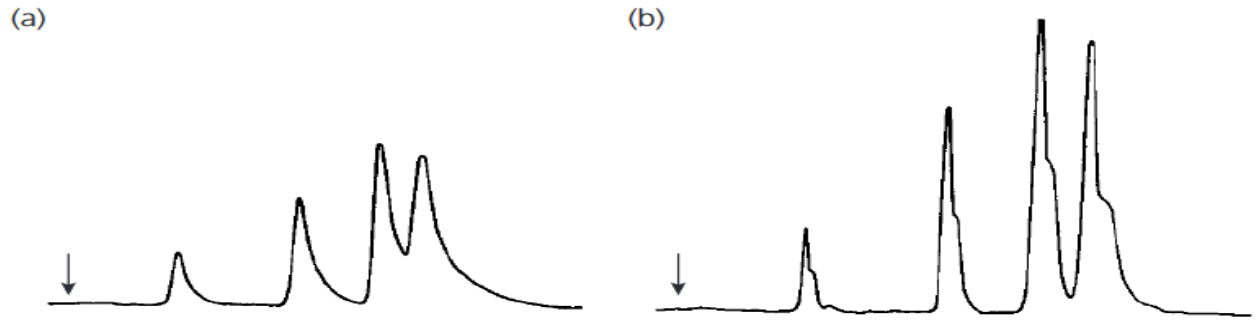
Split and distorted Peaks

- **Poor tubing connections**
  - *can result in voids forming, giving distorted peaks*
- **Blocked in-line filter**
- **What are you using as your needle wash ?**
- **What is the sample diluent ?**
  - *It might need to be similar to the mobile phase*
- **What is the injection volume ?**
- **Is the sample overloaded ?**
- **Have you allowed for proper column equilibration ?**



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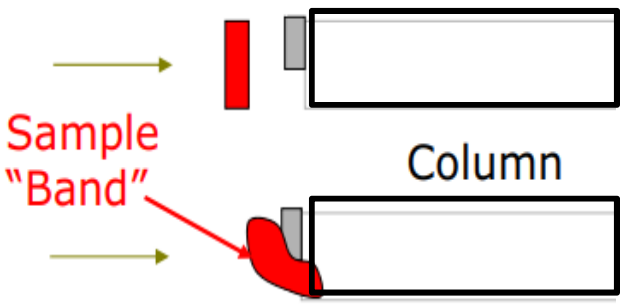
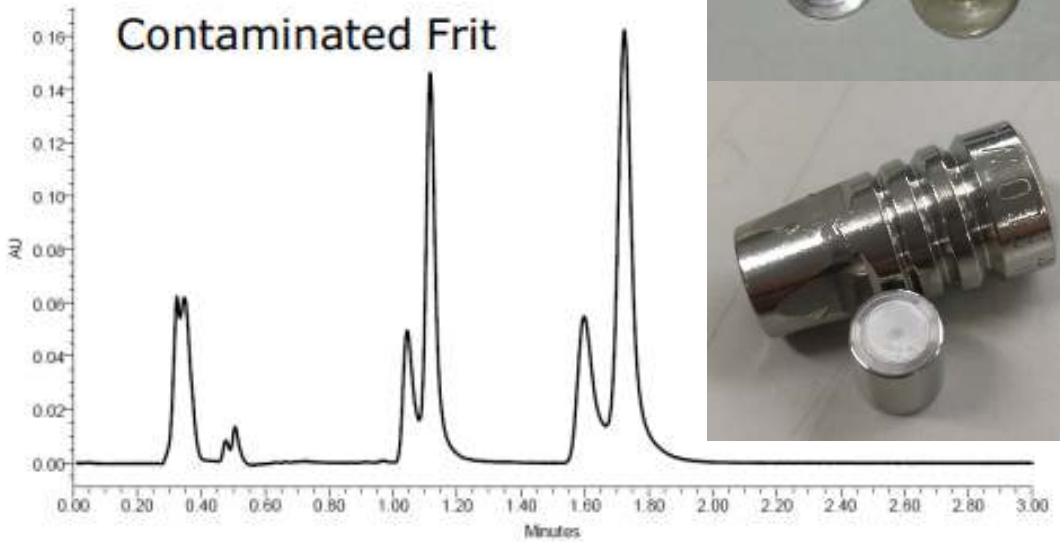
Effect of Contaminated/blocked In-line Filter on Peak Shape/Efficiency

Debris from seal shedding, particulates from buffer, particulates from sample





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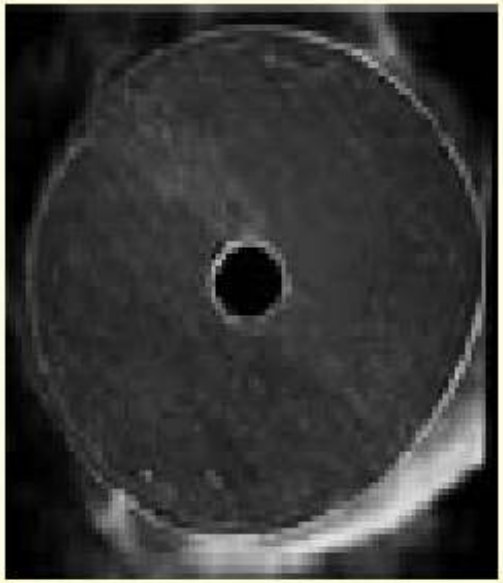
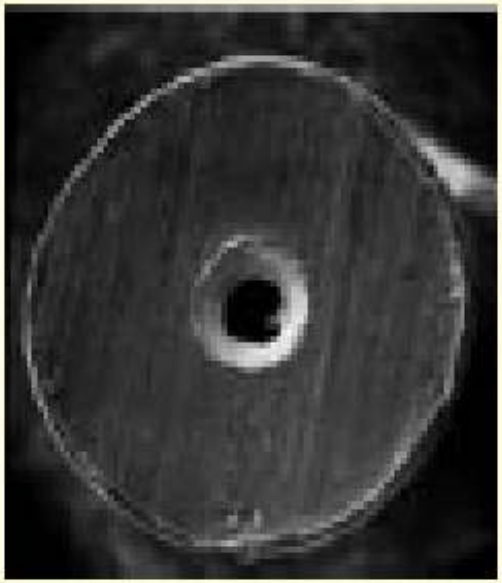
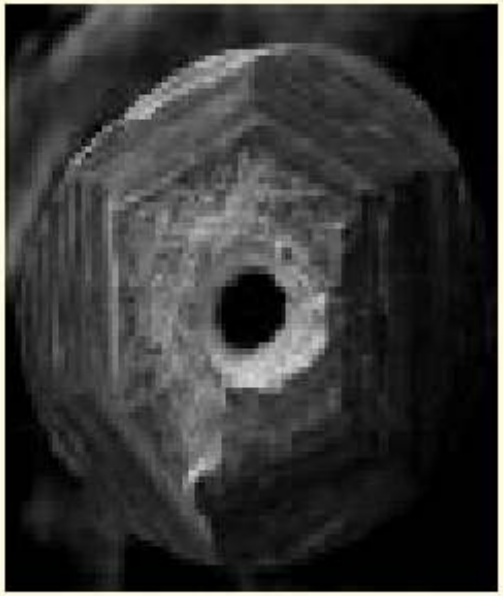
Poorly Cut Peak Tubing







UPLC Tube

Poorly Cut Peak Tubing

Stainless Steel
Tubing 1/16'' OD

PEEK 1/16in OD
x 0.005in ID

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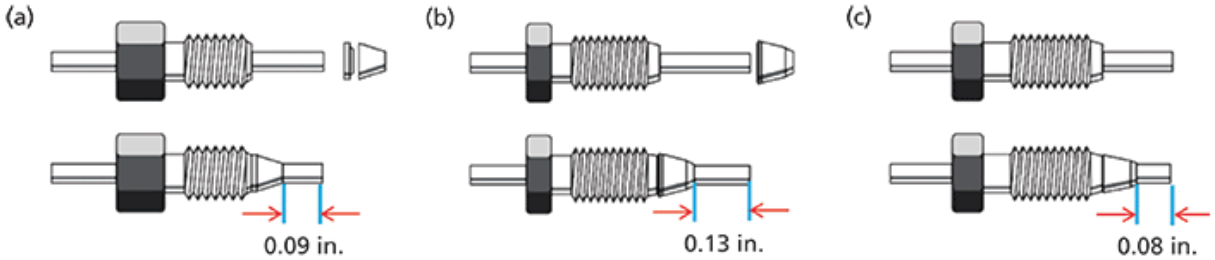
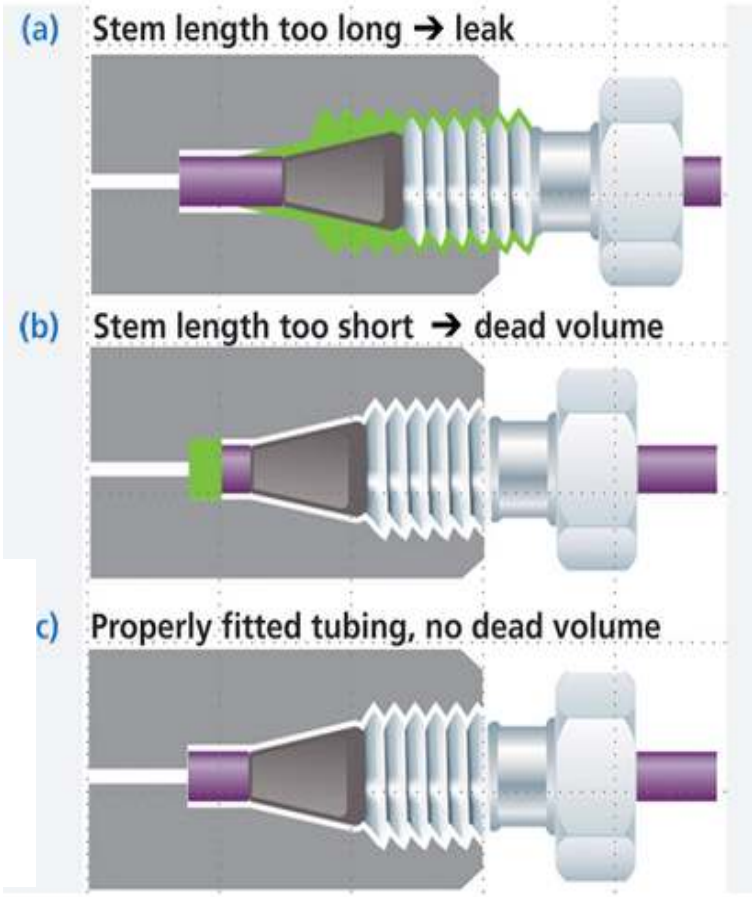
HPLC/UPLC Fitting

HPLC/UPLC 1/16” OD Fitting



Swagelok Waters Valco



Threading, geometry, sizes, material, and pressure requirements

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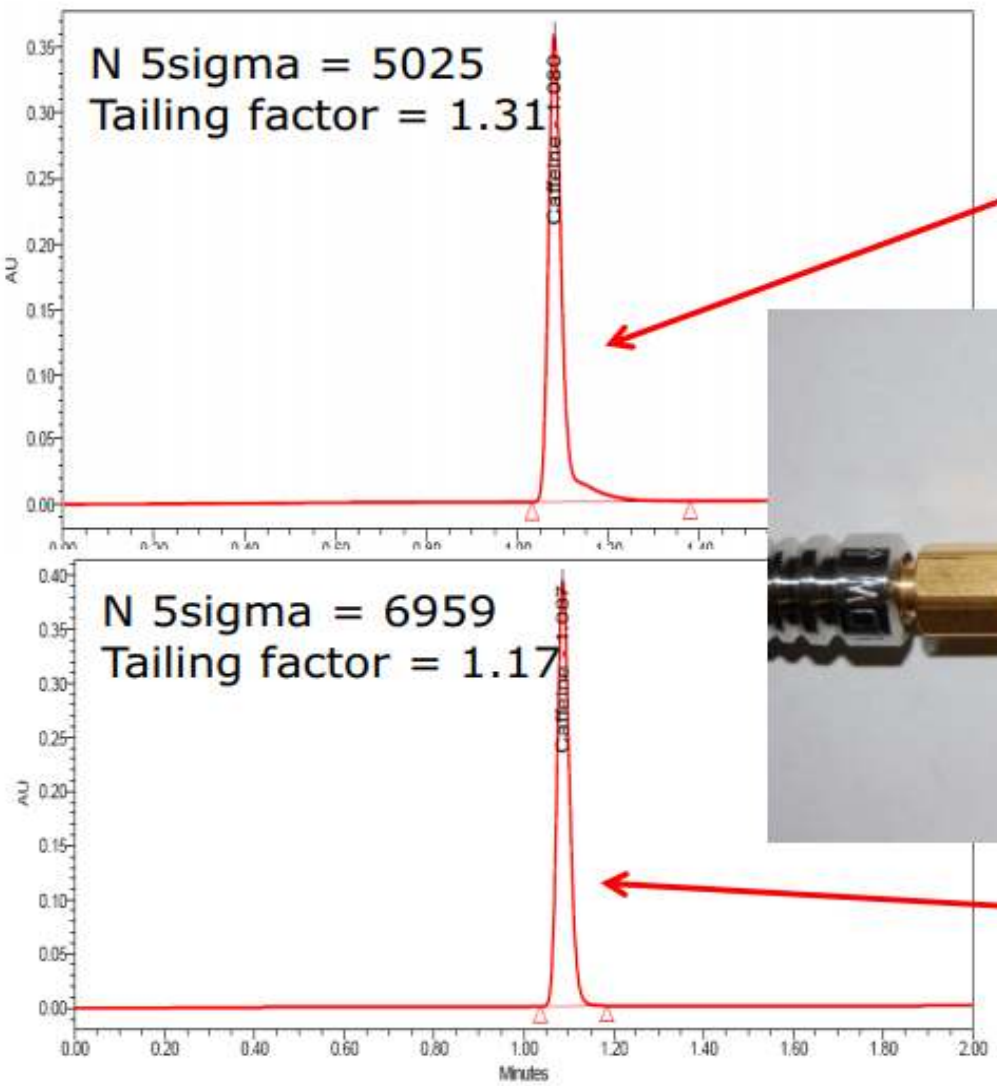
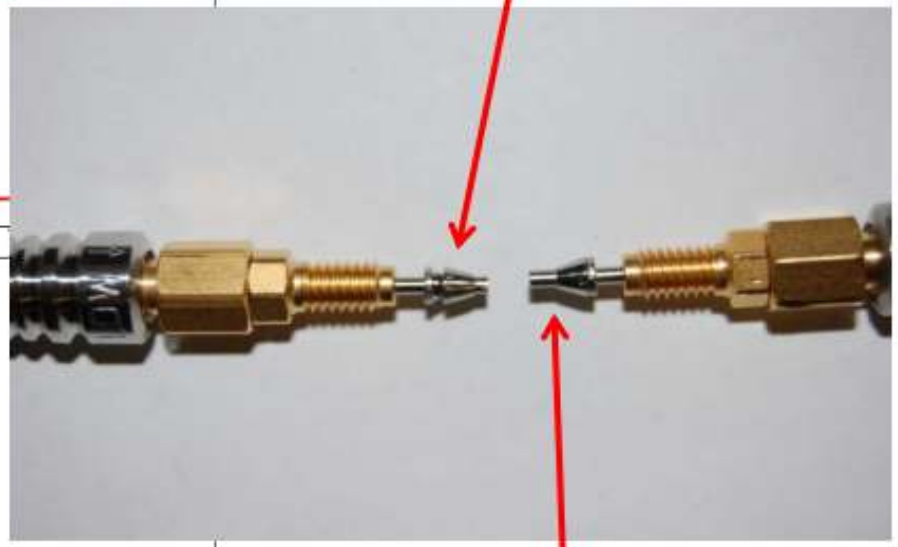
In-line filter installation problem





Wrong position of ferrule

Correct position of ferrule

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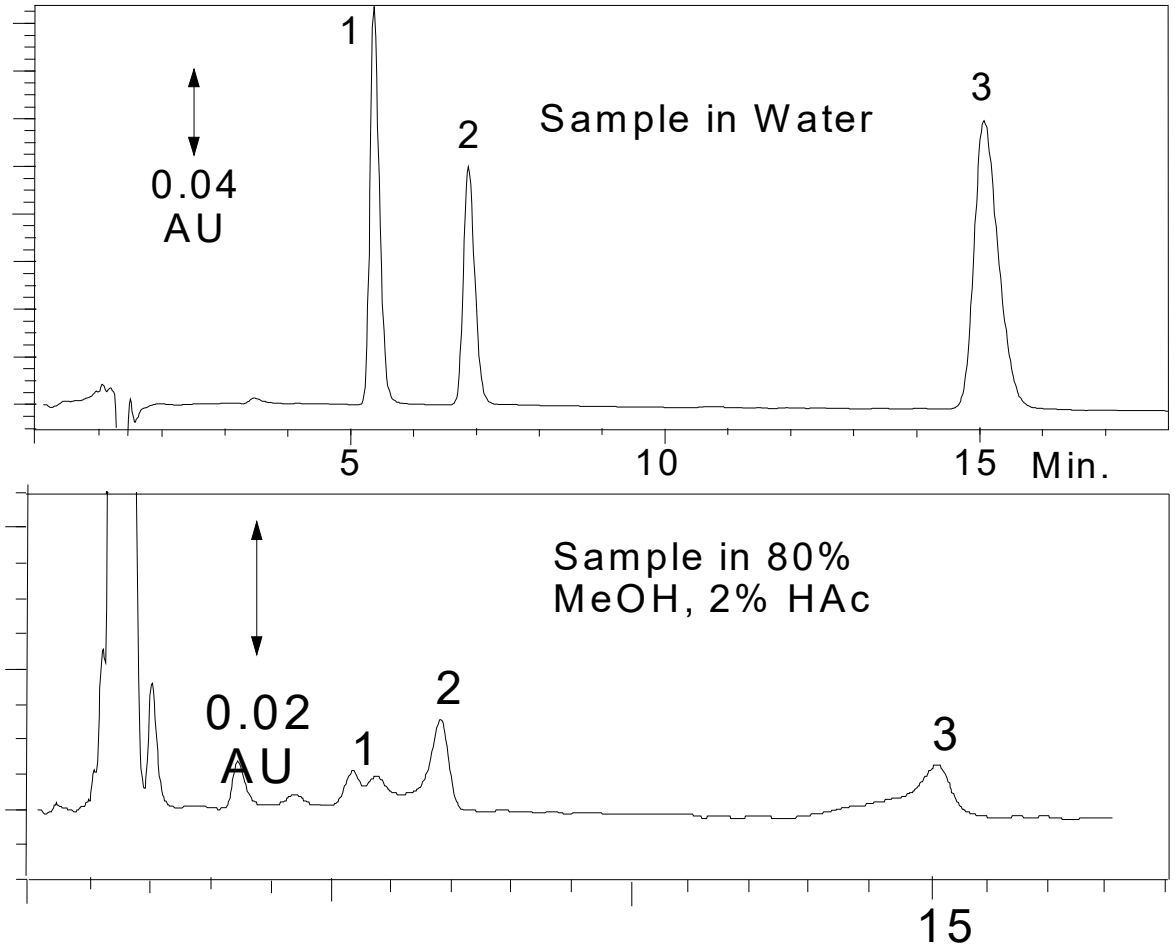
All Peaks Distorted – Chemical Problem



Sample Identification
1.EDDP
2. Diphenhydramine (IS)
3. Methadone

Column:	SymmetryShield™ RP18, 3.5 µm, 3.9 x 150 mm
Guard Column:	Sentry™ Guard Column SymmetryShield RP18, 5µm
Temperature:	30°C
Mobile Phase:	0.1% TFA:Methanol (60:40)
Detection:	UV at 210 nm
Flow Rate:	1 mL/min
Inj. Volume:	30 µL

Incorrect Sample Solvent – STRONGER than mobile phase

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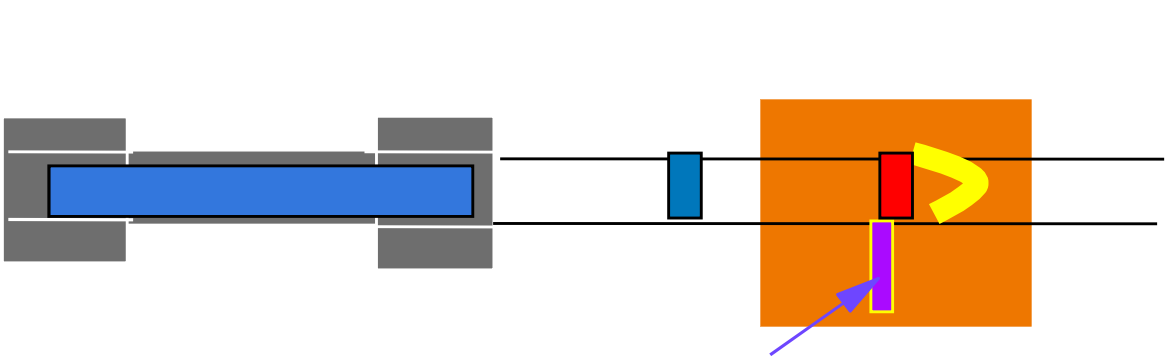
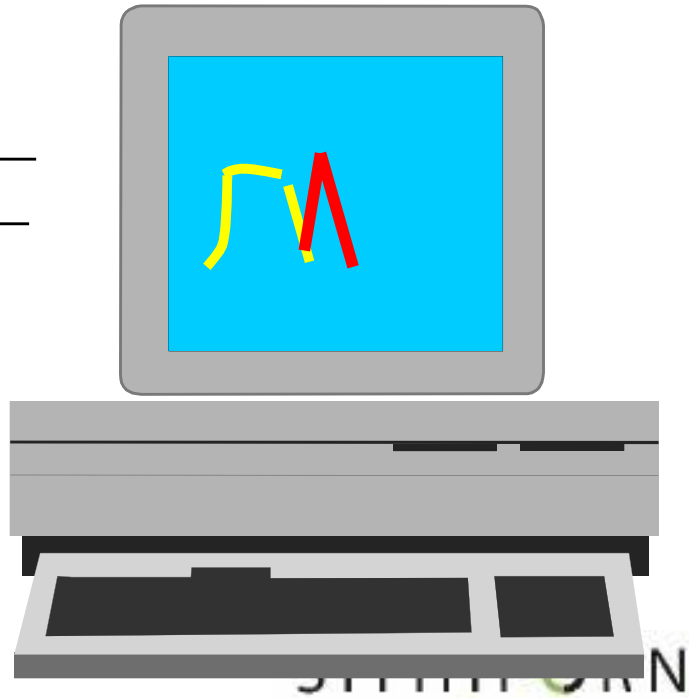
Why Do You Get One/Some Distorted Peaks?

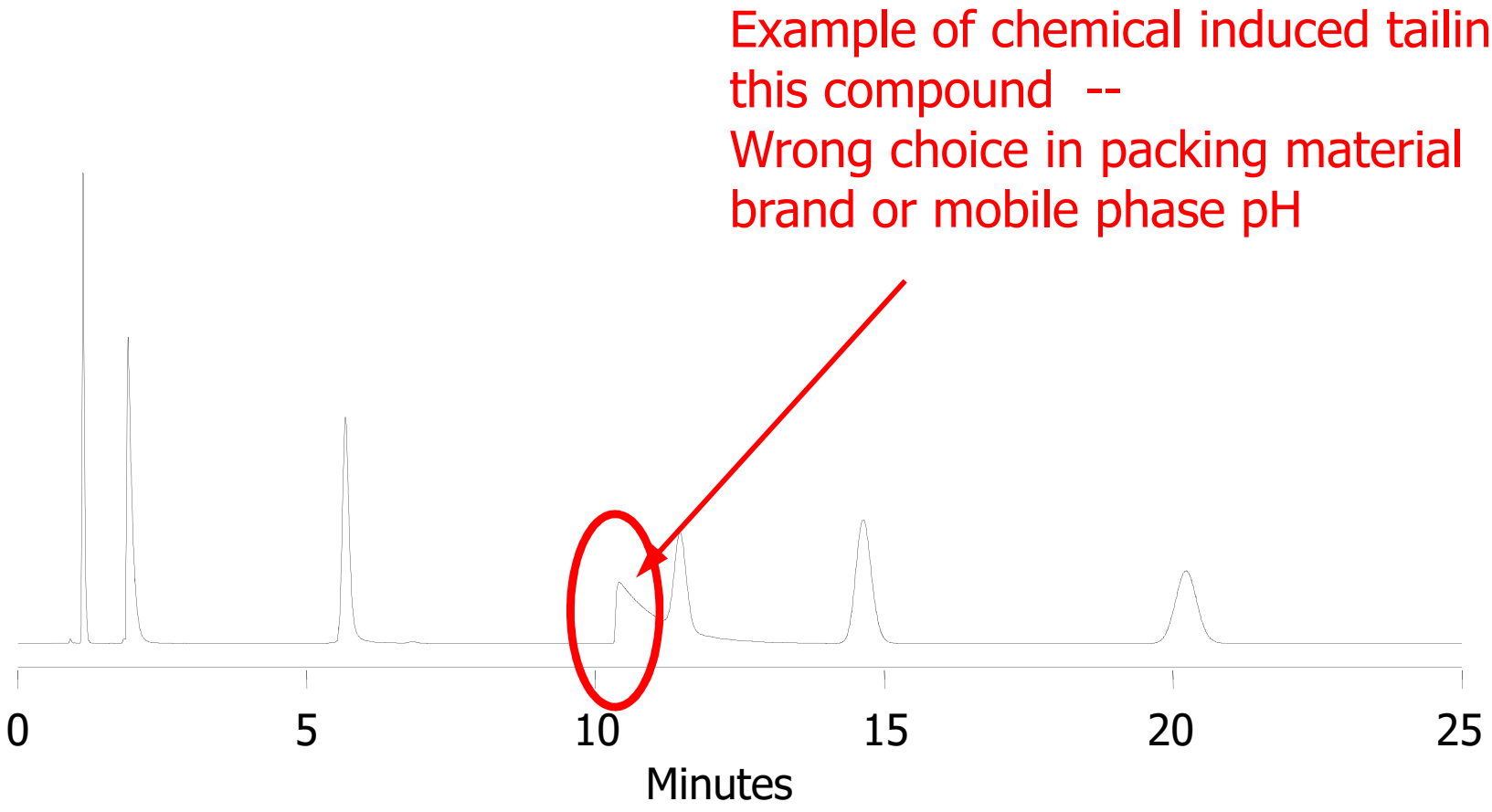
- **Why do you get one or some distorted peaks?**
  - Distorted Band – Chemical Problem
    - Cation exchange of one analyte to particle surface



Detector Cell



Great Peak Shape for Some Peaks, but Others Have Poor Peak Shape



Chemical Problem (Not Mechanical)

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Troubleshooting and diagnostics

- **Troubleshooting**
  - System Pressure Problems
  - Incorrect Retention time
  - Loss of precision
  - Carryover/Contamination
  - Split and Distorted Peaks
  - *Baseline Noise*

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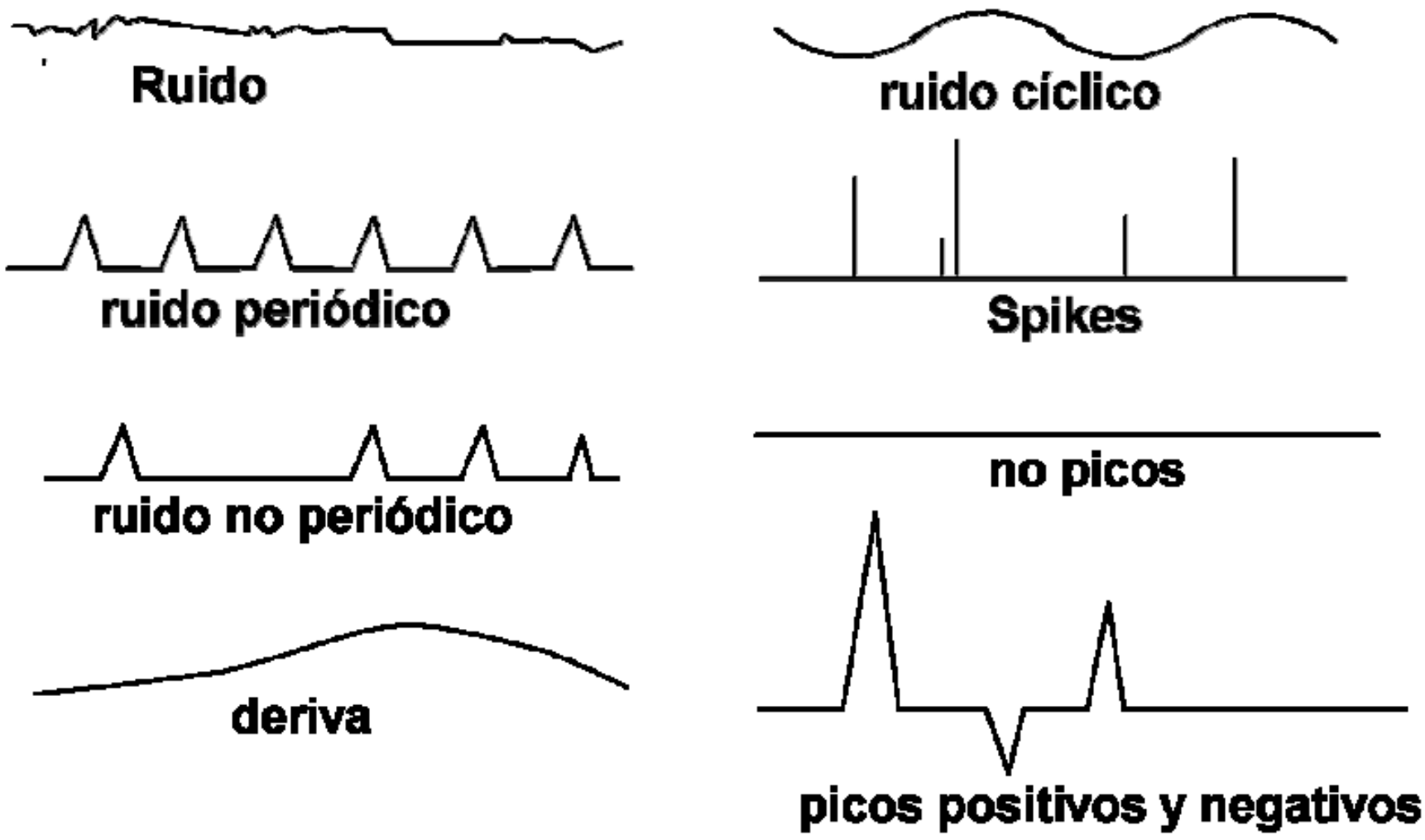
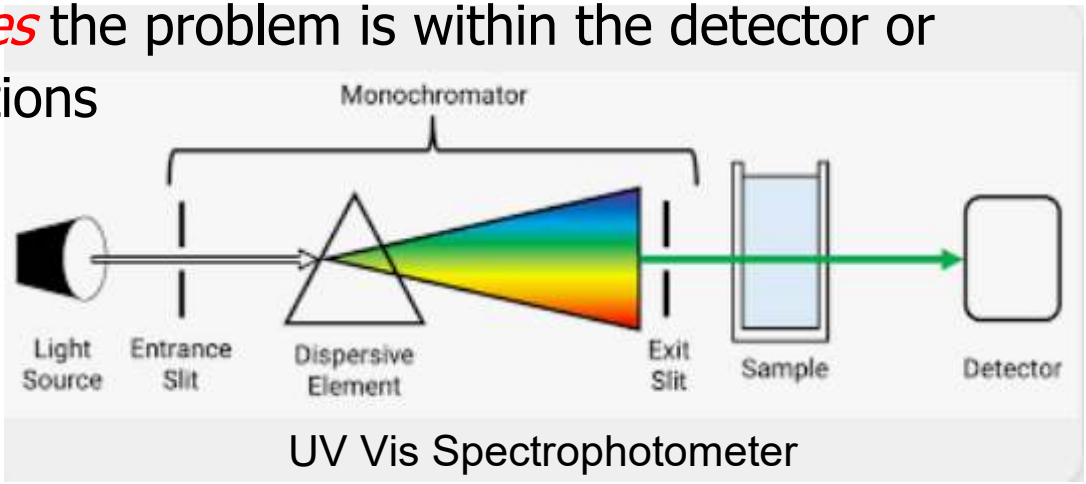
Baseline noise



Troubleshooting procedure

To isolate the source of the *baseline noise* (detector or not detector):

1. **Stop the flow**
2. **Monitor the baseline for a few minutes* and observe :**
   - If there is a *significant improvement* in the baseline noise the problem is within the fluid path (pump/mobile phase/flow path/column)
   - If the *noise continues* the problem is within the detector or its electrical connections



UV Vis Spectrophotometer

*Some flow sensitive detectors (such as RI, electrochemical) may require a significant time to stabilize once flow is stopped

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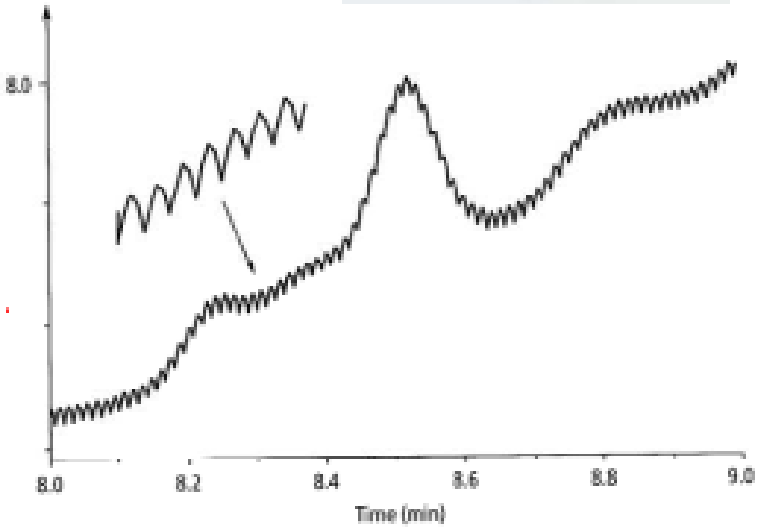
Fluid path-related noise

Short term (seconds to minutes) cyclic noise:
Most often related to pump pressure/flow fluctuation

- **Air in pump**
  - *Remove air – degas solvents*
- **Faulty check valve**
  - *Replace check valve*
- **Wrong plunger seals**
  - *Replace seals*
- **Broken plunger**
  - *Replace plunger*
- **Inadequate solvent blending**
  - *Increase mixing blending*



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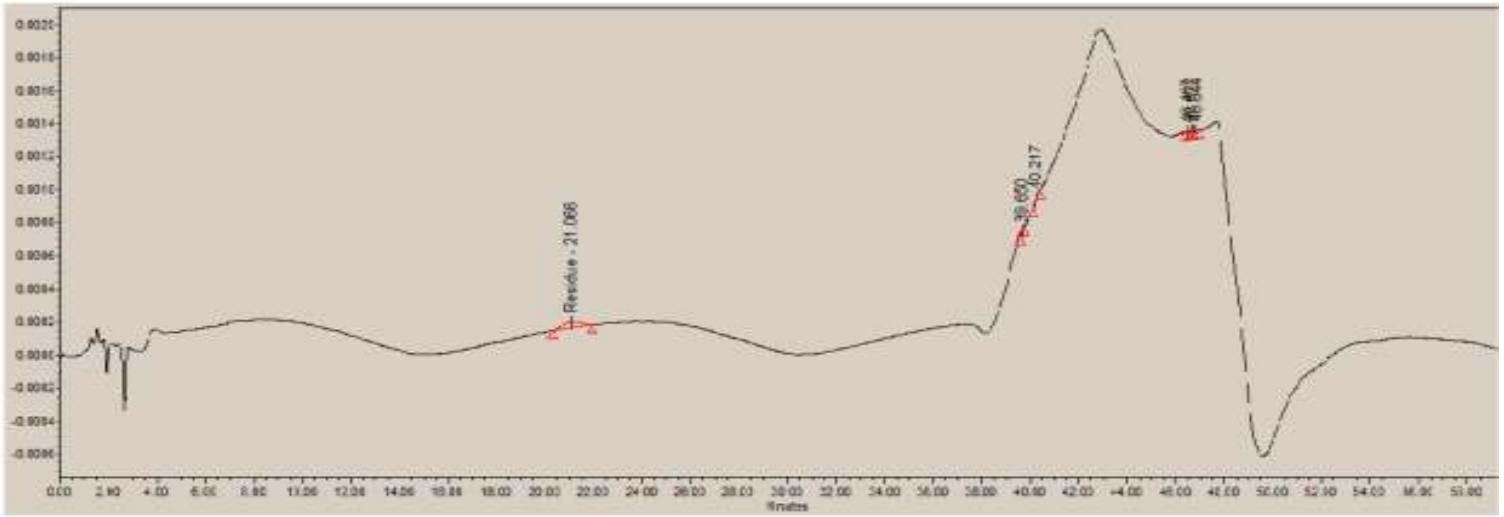
Fluid path-related noise

Long term (minutes to hours) cyclic noise:

- **Ambient temperature fluctuations**
  - *Stabilize column temp. 5°C > ambient temp*
- **Solvent recycling?**
  - *Avoid recycling if not absolutely necessary*



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Fluid path-related noise

Non-cyclic (erratic) noise:

- **Air bubble trapped in detector flow cell**
  - *Remove air in flow cell*
    - *To prevent air in flow cell add 50-100 cm of 0.23mm ID tubing to the detector outlet**
- **Small air bubbles traveling through the flow path**
  - *Degas mobile phase – remove air from pump*
- **System not stabilized**
  - *Equilibrate system*
- **Low Detector Energy**

** Keep in mind that not all detectors (such as Fluorescence, RI, Conductivity and Electrochemical) can tolerate backpressure on the flow cell. Consult the manual*

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Fluid path-related noise

Non-cyclic (erratic) noise (continued):

- **Mobile phase contaminated**
  - *Prepare fresh mobile phase. Clean solvent filters*

- **Detector flow cell leaking**
  - *Check for leaks - repair*
- **Column contaminated**
  - *Remove column and see if noise disappears*
    - *Replace/clean column*

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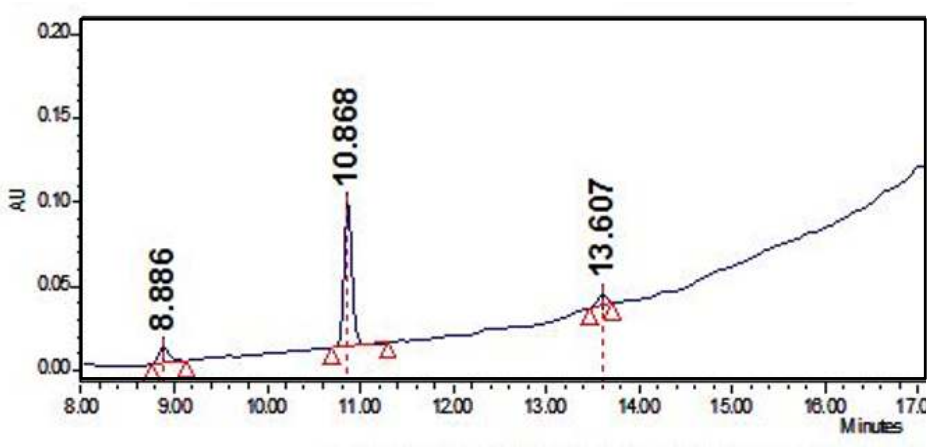
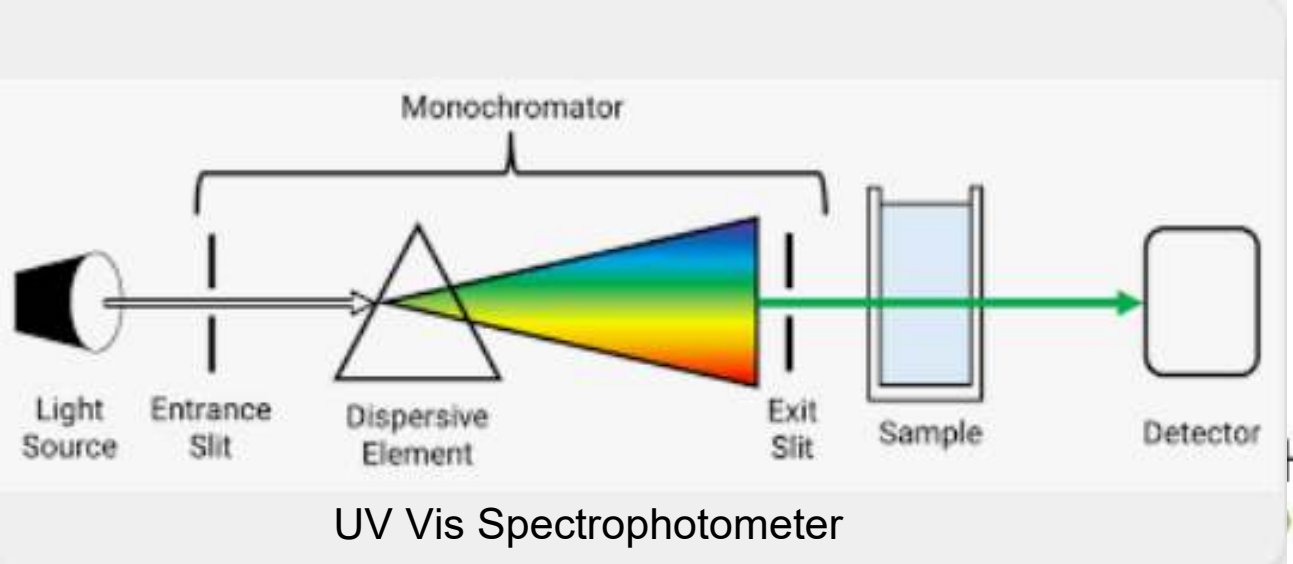
Fluid path-related noise

Baseline drift:

- **System not equilibrated**
  - *Equilibrate system*
- **Temperature fluctuations**
  - *Stabilize column temperature*
- **Mobile phase contamination**
  - *Prepare fresh mobile phase. Clean solvent filters*





UV Vis Spectrophotometer

Fluid path-related noise

Baseline drift:

- **Contaminated column**
  - *Remove column and see if noise disappears*
    - *Replace/clean column*
- **Stationary phase bleed (ligand hydrolysis)**
  - *Remove column and see if noise disappears*
    - *Check pH of mobile phase (<2 ?)*
    - *Select different pH*
    - *Select different column type (trifunctionl)*

- **Function of gradient and difference in UV absorbance of Solvents**
  - *Dose drift follow gradient curve/profile ?*
    - *Use higher wavelength*
    - *Replace methanol with acetonitrile*

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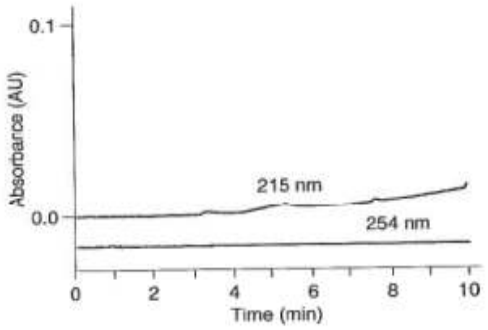
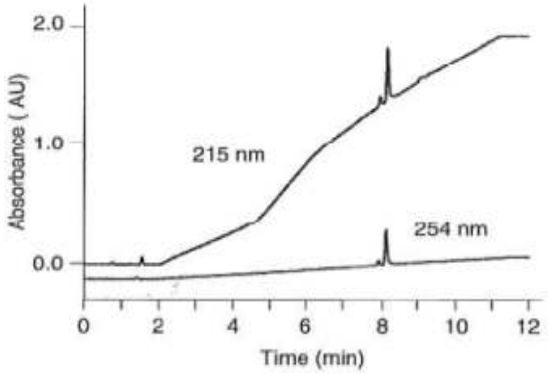
Gradient baseline drift

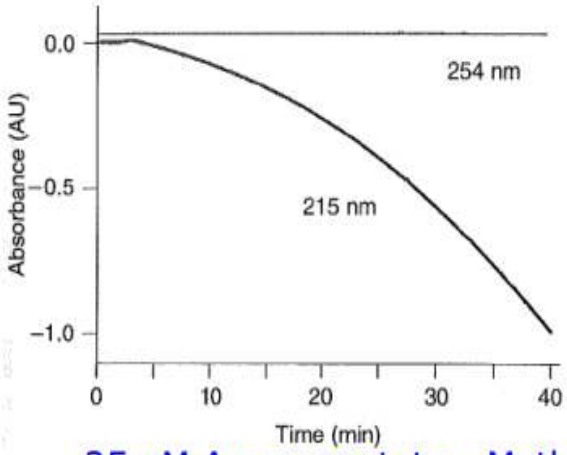


10mM Phosphate - Methanol
gradient 5-80% in 10 min.

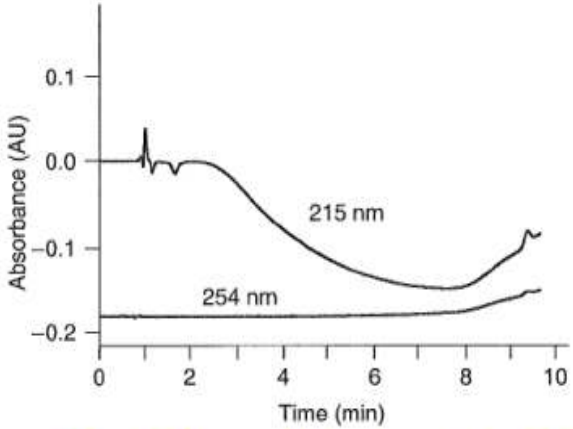


10mM Phosphate - THF
gradient 5-80% in 10 min.

Solvent	λ Cutoff [nm]
Acetic acid	260
Acetone	330
Acetonitrile	190
Chloroform	245
Cyclohexane	210
Dimethyl sulfoxide	265
Ethanol	210
Ethyl acetate	255
Heptane	197
Hexane	210
Methanol	210
2-Propanol	210
Tetrahydrofuran	220
Toluene	286
Water	191



25mM Amm. acetate - Methanol
gradient 5-80% in 40 min.



50mM Amm. bicarbonate - Methanc
gradient 5-60% in 10 min.

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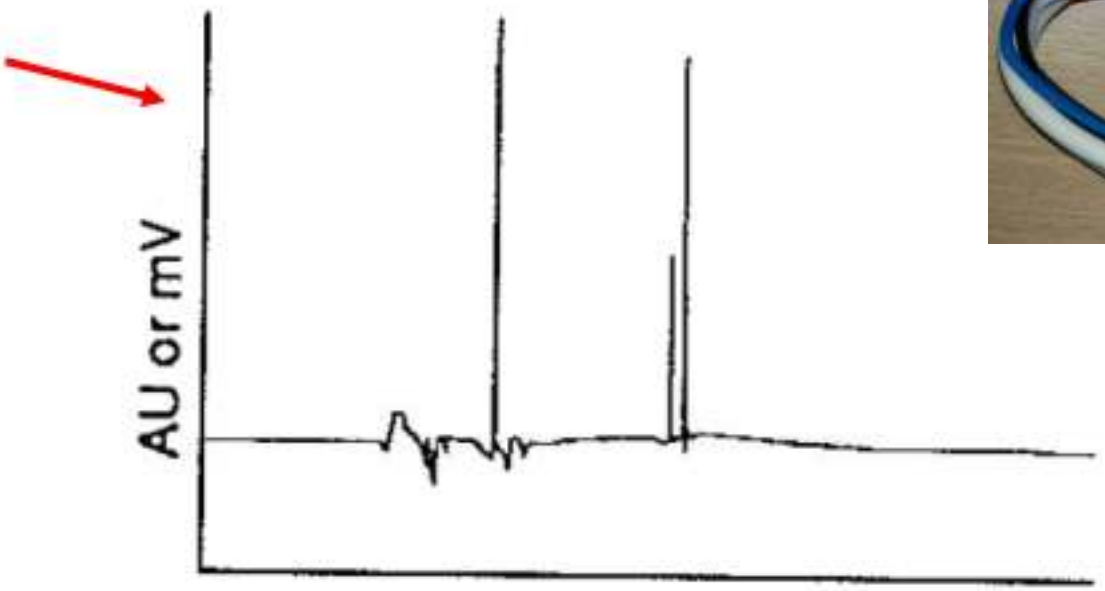
Detector related noise

- **Spike on the baseline**
  - *Defective lamp*
  - *Air bubble in flow cell*



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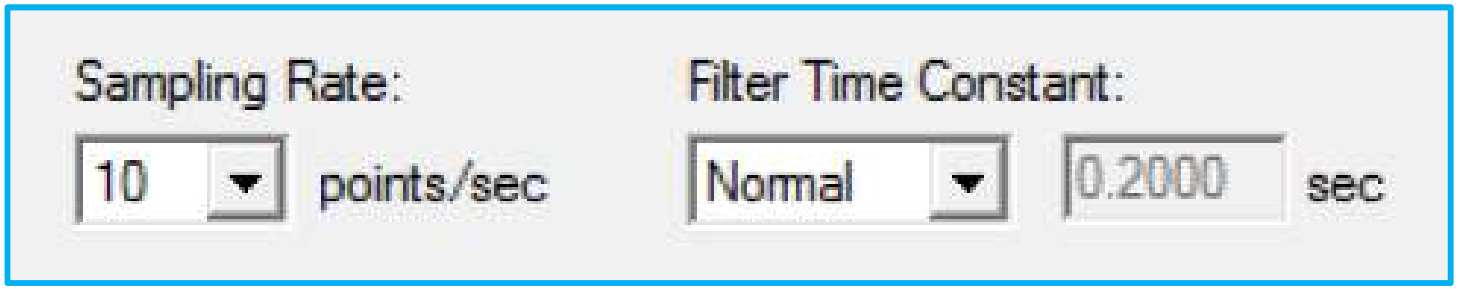
Detector Tips and Tricks

Optimizing Noise and Resolution Performance

- Independent optimization of data rates and digital filtering on detector allows for optimization of data rate without sacrificing resolution

- Detector setup
  - Data rate
  - Filter constant



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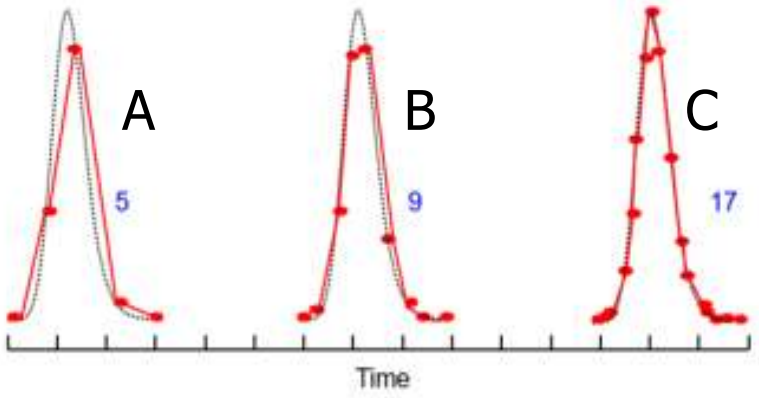
Importance of sampling rate

- Must ensure enough points are collected across a peak to adequately define the peak shape
- Peak detection algorithms require a minimum number of points across a peak to distinguish it from baseline noise and correctly determine peak lift off and touch down
- A peak which does not have enough data points will be difficult to integrate and therefore have irreproducible peak areas and heights
- We aim at collecting 25-50 points across a peak



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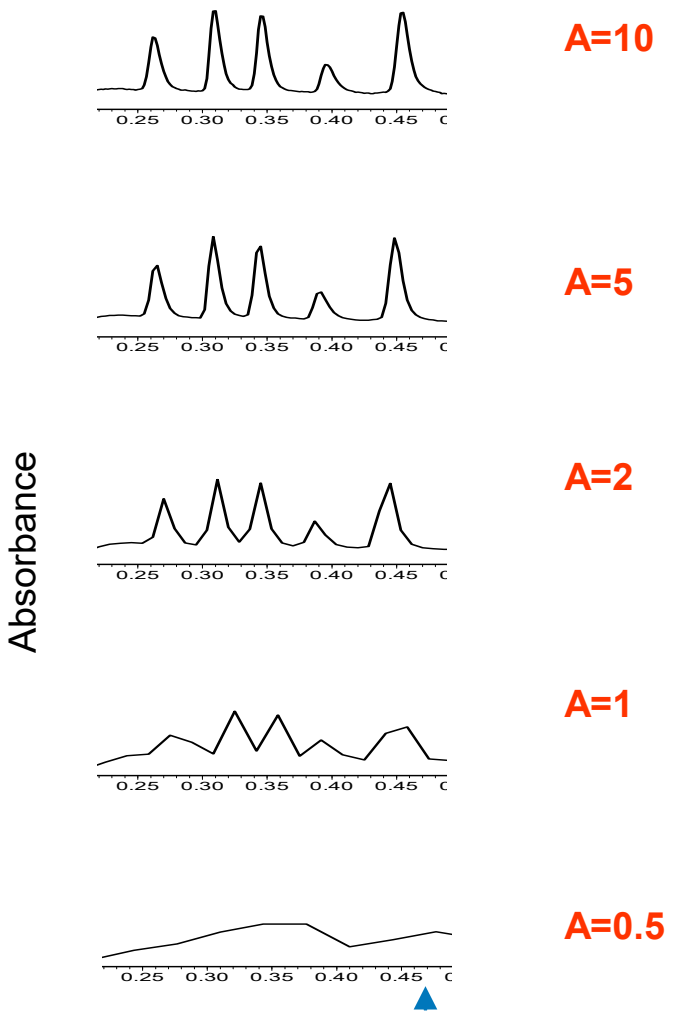
Practical Considerations - Data Acquisition Rate



Data Acquisition Rate Study
(pts/sec)
{for UltraFast Chromatography}

Sampling Rate:
When the peak width is only about 1 sec, use sampling rates of 10 pts/sec or faster.

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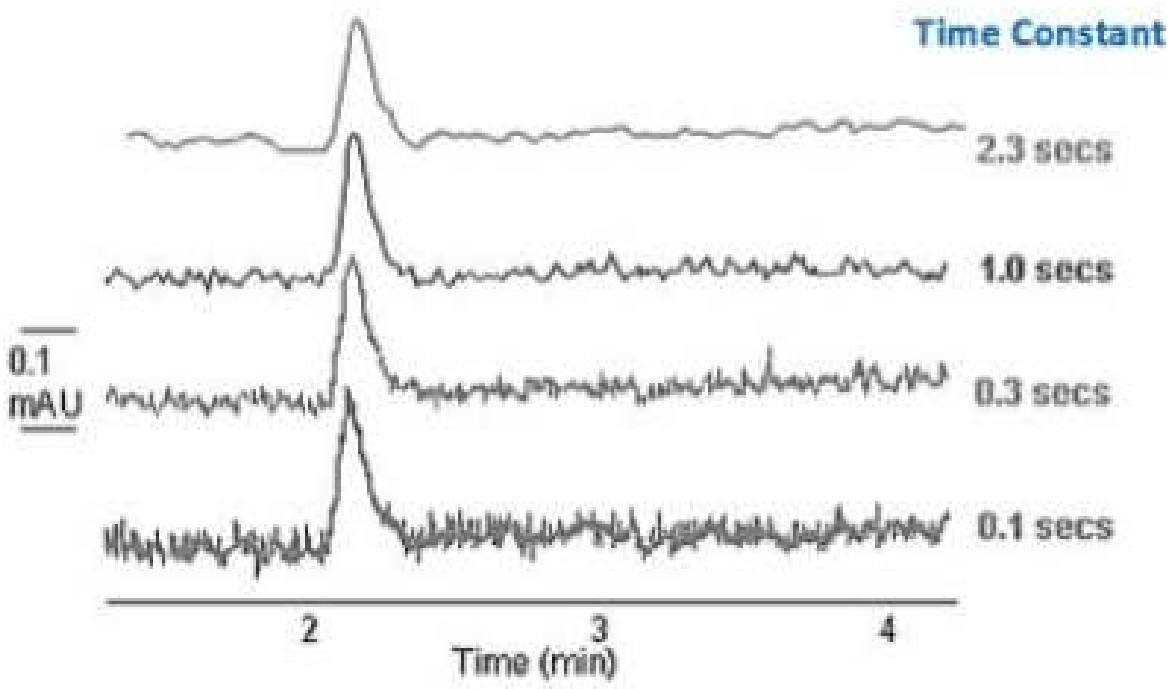
What is Digital Filtering?

- Digital Filtering is a mathematical algorithm applied to a data set that smoothes out higher frequency noise
- Reduced baseline noise to increase signal-to-noise
- Too much filtering can dramatically impact peak shapes and resolution



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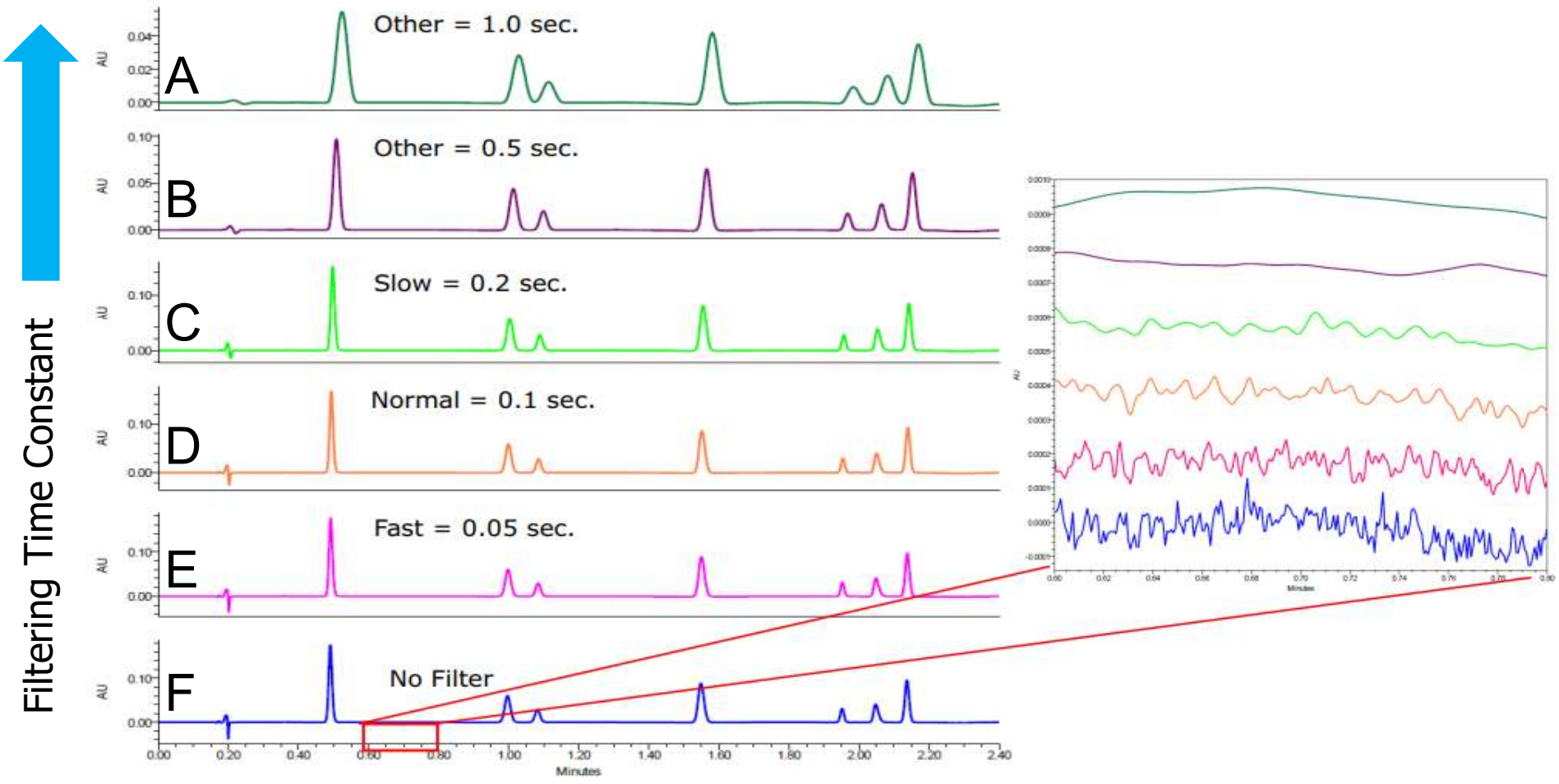
Effect of Filter Time Constant Setting



Filtering Time Constant ↑ Baseline noise ↓

Filtering Time Constant ↓ Resolution ↑

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Questions ?

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