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Troubleshooting and Diagnostics Tips and Tricks

Cerdanyola del Vallès, Enero 2014

Troubleshooting and diagnostics

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

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 the column, mobile phase, flow rate, temperature and can vary greatly with different methods

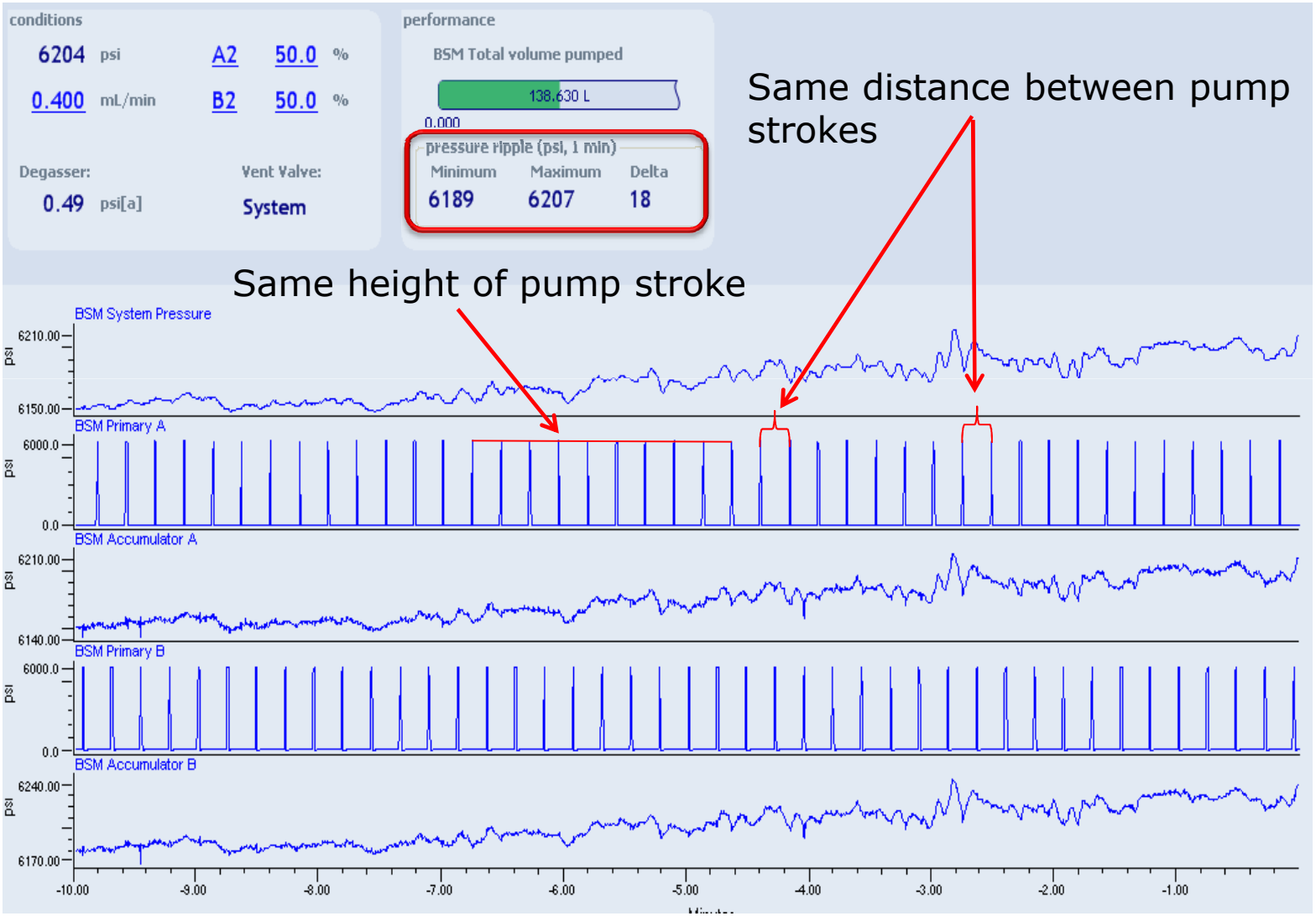
System Pressure problems

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

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*
  - *Air in solvent lines. Bottle filters dirty*
    - *Remove the bottle filters*
  - *Air in solvent lines. Not enough degas*
    - *Degas the mobile phase*
  - *Problem with check valves*
    - *Sonicate or replace the check valves*
  - *Problem with seals or plungers*

System pressure problems /Correct pressure



System Pressure problems

- Overpressure
  - *Check if pressure has risen gradually or suddenly.*
    - *If pressure has risen gradually particulates are accumulated in in-line filter, columns frits or column.*
    - *If pressure has risen suddenly something could be a obstruction in system or column.*

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 loosen fittings beginning with the last connection in line and working backward to the pump*
    - *After loosening each fitting observes if pressure stays the same or reduces.*
    - *Replace or clean the appropiate part*

Caution: Do not loosen fittings under high pressure

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. Not enough solvent in bottle*
    - *Replace the solvent bottle*
  - *Problem with check valves*
    - *Sonicate or replace the check valves*
  - *Problem with seals or plungers*

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Retention Time

- Retention time changed to a new constant value
- Erratic retention time
- Increasing retention time
- Decreasing retention time

Retention Time

- Retention time changed to a new constant value
  - *Check column, mobile phase, temperature, method, flow rate*
- Increasing/decreasing retention times
  - *Column contaminated, degraded*
  - *System not equilibrated*
  - *Mobile phase contaminated*
- Erratic retention times
  - *Check if system also has erratic pump pressure/pressure fluctuations*
  - *System not equilibrated*
  - *Check for leaks*
  - *Improper solvent blending*
  - *Temperature fluctuations*

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Loss of precision

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- Incorrect peak integration
- Check loss of precision is for all peaks in the cromathogram.
  - 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

Loss of precision

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- Check injector wash solvents. Are appropriate for the method?
- Check injection volume is inside system specifications
- Are area values erratics or are increasing or decreasing?
- Injector problem. Pass injector test
- It is important to have a system suitability to check the system.
- In case you don`t have one, Waters supplies specific solutions for each system to check the system. This solution is called ASR or QCRM.

Troubleshooting and diagnostics

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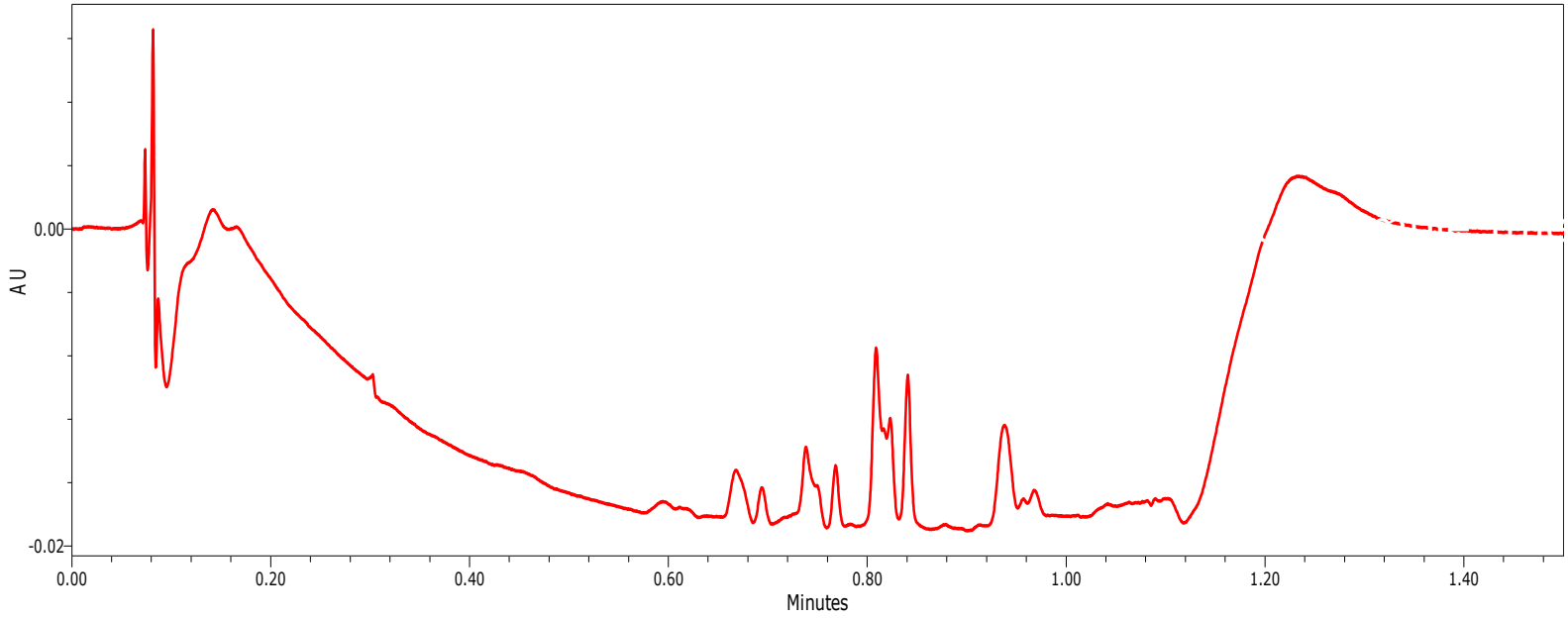
Definitions

- 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



Background and Contamination

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- Do these two terms mean the same thing?
- If they are different, how are they different?
- Is background ever zero?

Background and Contamination

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- Do these two terms mean the same thing?
  - *Not exactly*
- If they are different, how are they different?
  - *Background is the baseline noise and will always be present.*
  - *Contamination is background at an unacceptably high level.*
- Is background ever zero?
  - *No.*
  - *The sample and liquids used in HPLC and LC/MS are chemicals which the detectors may detect.*

Definition of Background

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- What is background in chromatography for UV and for mass spectrometry?
- How does it appear in a chromatogram?
- When does it appear?

Definition of Background

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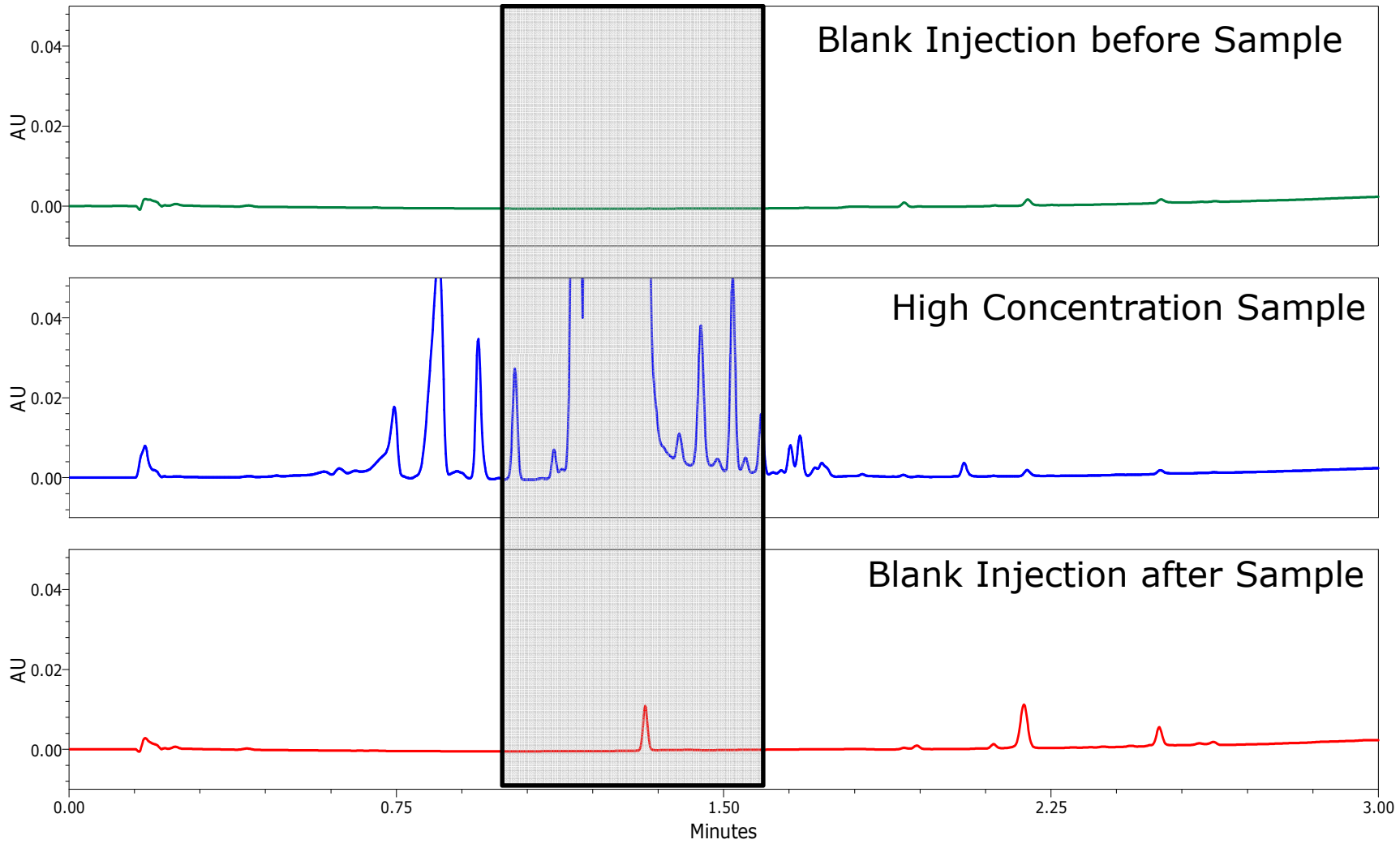
- What is background in chromatography for UV and for mass spectrometry?
  - *Changes in baseline*
- How does it appear in a chromatogram?
  - *Deviation from zero response*
- When does it appear?
  - *Always present.*
  - *The mobile phases are chemicals and contain additives or contaminants.*

Define Carryover

- What is carryover?
  - *Analyte remaining from a previous injection that appears as peaks in subsequent injections and compromises quantification.*
- How do you measure carryover?
  - *Sample or standard followed by one or more blanks*
- Does every equipment have a carryover specification?
  - *Yes. It should be define at the equipment user guide.*
  - *Compound and detector specific.*
- Is the injector always the problem when there is carryover?
  - *No*

Example of Carryover



How to Measure Carryover?

- Blank Injection(s)
  - Sample diluent/matrix without the analyte
  - Follow the injection of the sample with a blank injection
    - Carryover is expressed as the response observed in the blank divided by the response of the sample injection and expressed as a percentage
  - Follow the injection of the sample by multiple blank injections
    - Does the response in the blanks diminish with each injection or does it remain constant?
      - If the response does not decrease with multiple blank injections, the problem is likely contamination

Critical Factors Affecting Carryover

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- 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?

Contamination

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It is easier to prevent contamination than to remove it.

- Sources of information
- Source of contamination
- Prevention
- Diagnostic scheme
- Cleanup

Potential Sources of Contamination

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- 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

Questions to Ask

- Should you assume the solvents are free of contaminants?
  - *No*
- Should you assume the equipment is clean?
  - *No*
- You should not assume anything
  - *First determine if the solvents or their bottles contain any contamination.*
  - *Then determine if the equipment is contributing contamination.*

Contamination from the Sample

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

Contamination Diagnostic: Injector-Pump

- "Zero Volume Injection"
  - Have the injector go through the injection sequence without injecting any volume
    - Vial contamination

- "No Injection" or Disconnect the autosampler and connect pumps to detector
  - Run gradient without injection ("Inject Immediate" samples in Empower)
  - If the contamination peak is present without an injection, it is not from the injector
    - System contamination
    - Solvent contamination
    - Method Carryover (column)

- Change pump solvents to check the pump
  - If profile changes, contaminations could be related to the pump.

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 reequilibration 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

Solvent Quality

- What is good quality solvent?

- What is good quality water?

Solvent Quality

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- What is good quality solvent?
  - *Low background for the detector to be used*
  - *What is good for UV may not work for MS*
  - *Particle-free*
    - *Filtered by supplier to 0.2µm* filter before bottling
    - *Do not filter again because filters will add contamination.*
- What is good quality water?
  - *Is on-demand water purification system (e.g. Milli-Q) good?*
  - *Is bottled water better?*
  - *Never stored for long periods of time (things grow in it)*

Mobile Phase Quality

- Highest quality of solvents available
- Highest quality of additives
  - Buffer salts
  - Acids
  - Bases
  - Use additives at the lowest concentration that gives good results
- *WARNING* – Failure to use the highest quality solvents leads to higher backgrounds, contamination, loss of sensitivity

Equipment Cleanup

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- What should you do before starting the cleaning process?
  - *Cleaning solvents must be contaminant-free. Check them!*
  - Do not start to clean the UPLC/HPLC system until you prevent future contamination
- New and/or clean bottles
  - Never wash UPLC/HPLC glassware in the dishwasher
  - Rinse only with the HPLC-grade solvents
- If the contaminant is known, what determines your choice of solvents?
  - *Solvents must be compatible with the UPLC/HPLC materials of construction (no THF with PEEK parts)*
  - *Solvents and additives must be compatible with the required detector or they will become "contaminants".*

UPLC/HPLC Cleanup
Choices of cleanup solvent mixtures

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- Acetonitrile and isopropanol dissolve different things
- Some compounds require a mixture of water and organic
- pH will affect solubility
- Strong acids or bases for cleaning can cause other problems
  - 6N Nitric acid could dissolve particles from bottle filters. Do not use with these filters. Don´t use 6N Nitric acid for UPLC
  - If necessary, use 30% phosphoric acid with UPLC.
  - 1% Ammonium hydroxide will dissolve silica and glass releasing Si ions

UPLC/HPLC Cleanup
Several Mixtures

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- 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. Nitric acid for HPLC.
  - Use a last resort. Removal of all traces of acid with water is required.

UPLC/HPLC Cleanup
Cleaning the injector

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- Cleaning suggestion
  - Remove column.
  - Put wash lines in the cleaning mixture.
  - Fill a vial with cleaning mixture.
  - Inject multiple full loops with the cleaning mixture.
- Replace the parts if the cleaning of the injector does not work.

Summary

- Prevent contamination is easier than troubleshooting and cleaning up.

Troubleshooting and diagnostics

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- Troubleshooting
  - Pressure related Problems
  - Retention Time
  - Reproducibility
  - Carryover/Contamination
  - Split and Distorted Peaks
  - Baseline Noise

Split and distorted Peaks

Why does my column yield split/distorted peaks?

- As in traditional liquid chromatography, several possible factors could be contributing to a split or distorted peak:

- Poor tubing connections
  - can result in voids forming, giving distorted peaks.
- Wrong system volume. Check tube diameters and length.
- Blocked in-line filter
- What are you using as your needle wash?
  - when choosing the needle wash use an apropiate composition

Split and distorted Peaks

- What are the specific wash volumes, set in the instrument method?
  - If any strong wash is leftover, peak distortion can occur
- What is the sample diluent?
  - It might need to be similar to the mobile phase
- What is the injection volume?
- Is the sample overloaded?
- Are you using a mobile phase pre-heater for UPLC?
  - Thermal mismatch of mobile phase and column
- Have you allowed for proper column equilibration?

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

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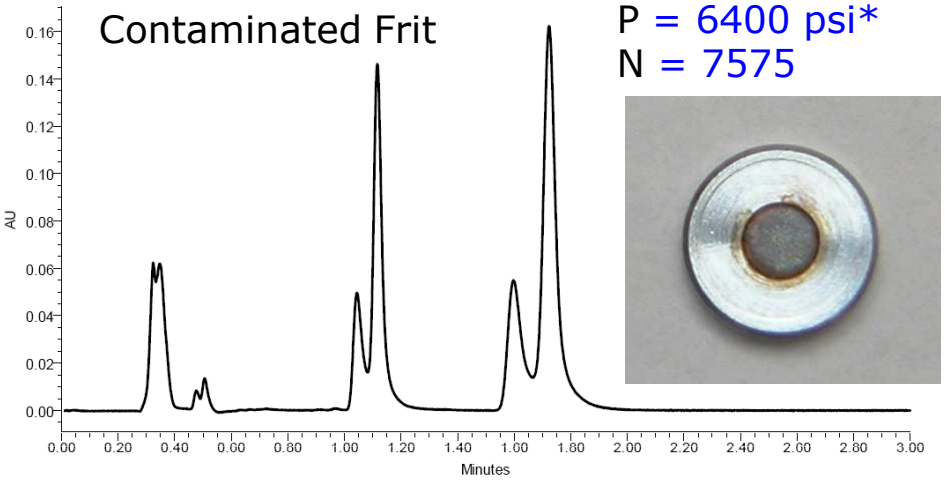
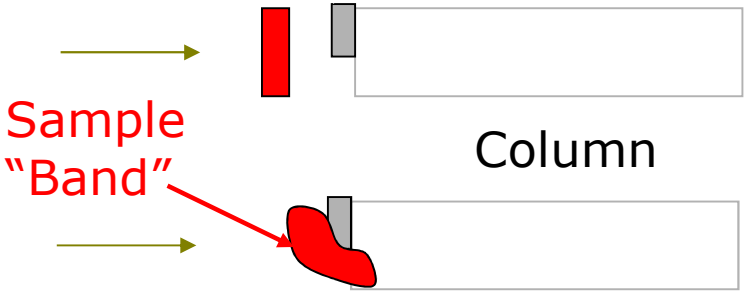
Debris from seal shedding, particulates from buffer, particulates from sample

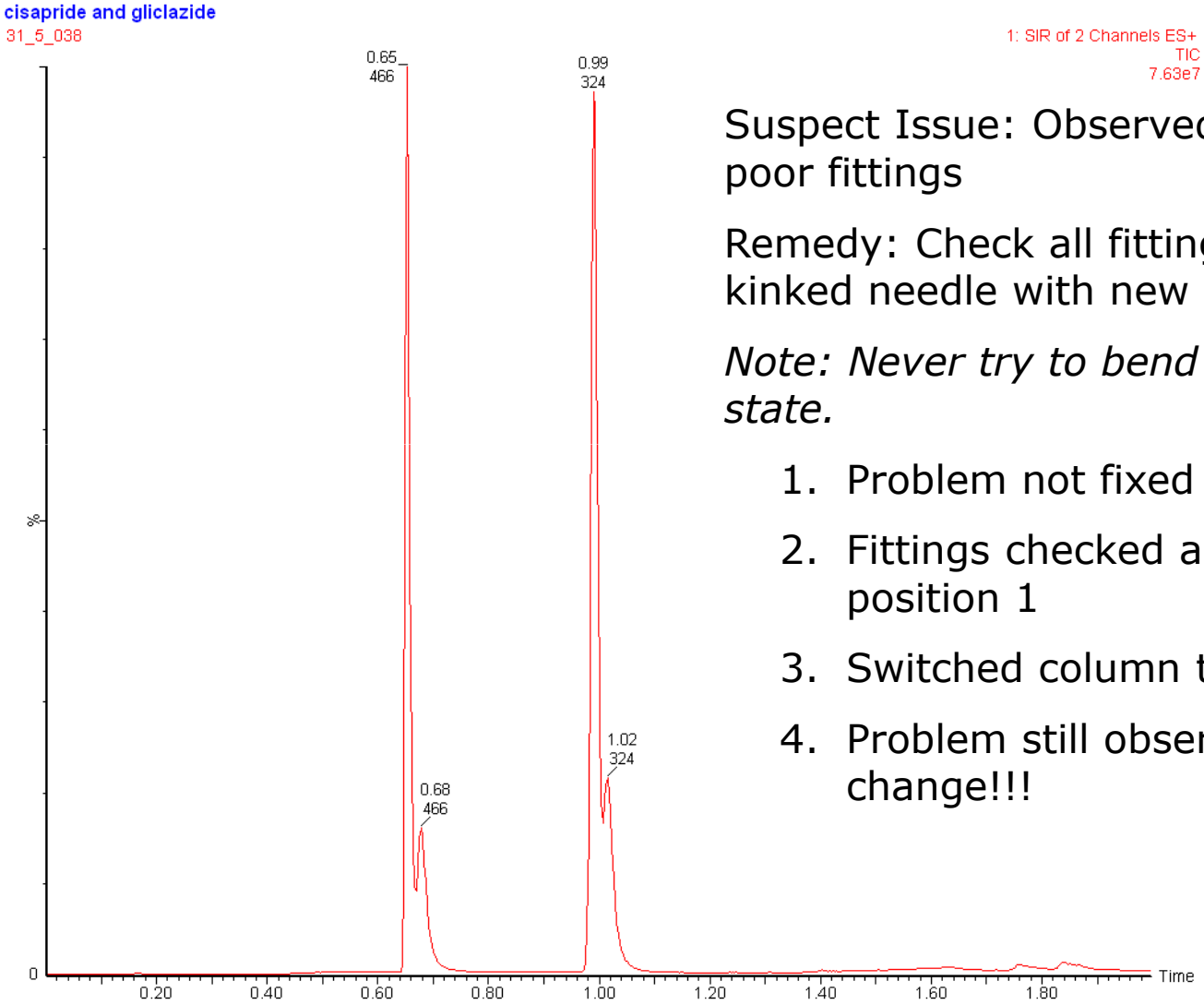


New Frit

P = 5100 psi*
N = 9349



Effect of a Poor Fitting/Poorly Cut Peak Tubing



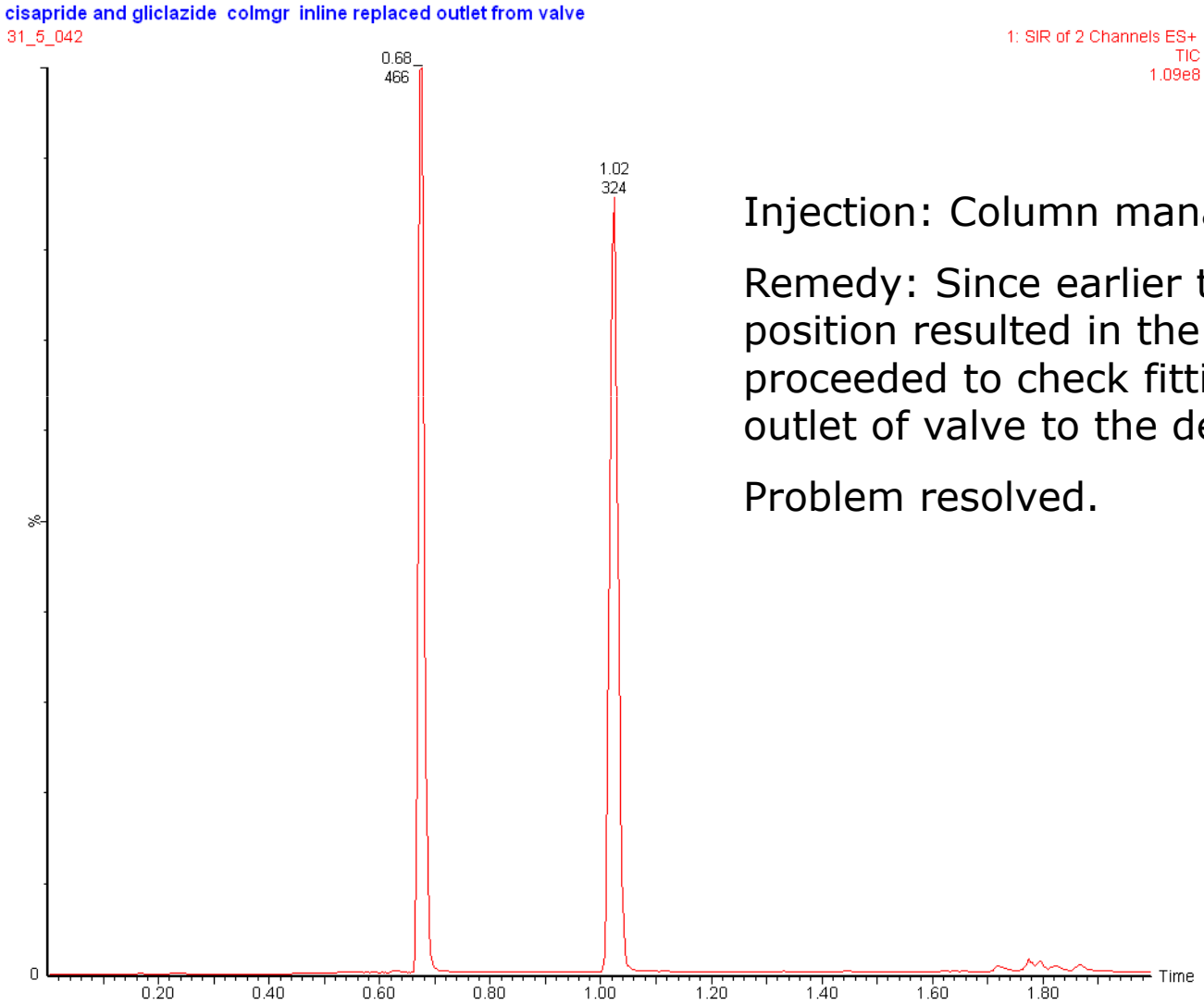
Suspect Issue: Observed kink in needle or poor fittings

Remedy: Check all fittings and Replace kinked needle with new needle

Note: Never try to bend needle to original state.

1. Problem not fixed with new needle
2. Fittings checked and tested on column position 1
3. Switched column to position 2 thru 4
4. Problem still observed during each change!!!

Effect of a Poor Fitting/Poorly Cut Peak Tubing

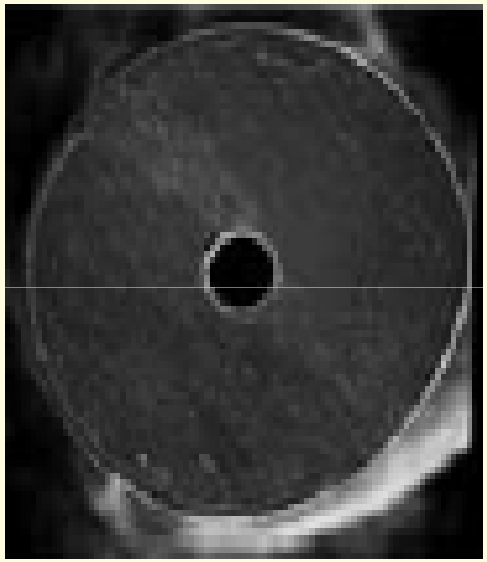


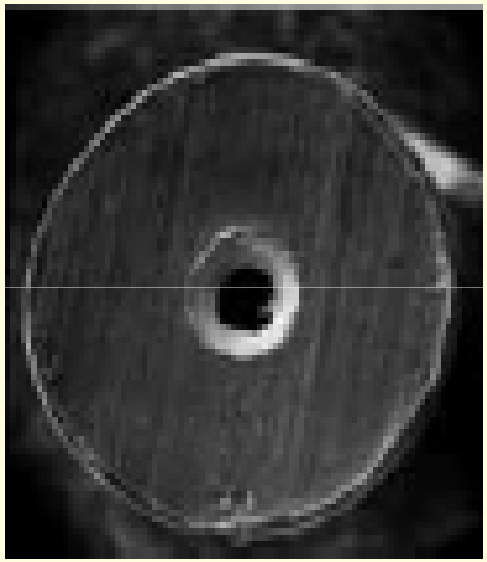
Injection: Column manager left valve in-line

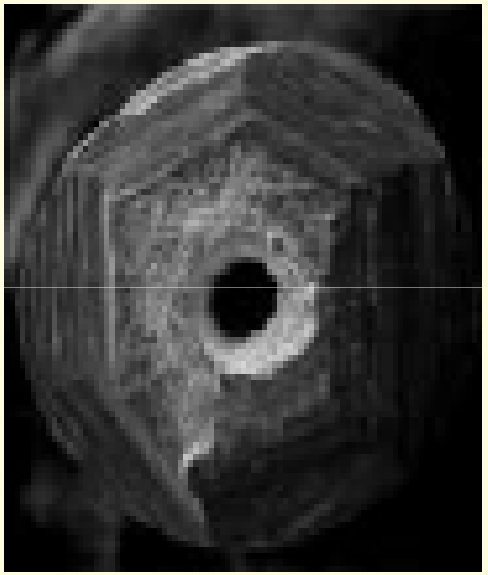
Remedy: Since earlier tests on each column position resulted in the split peaks, proceeded to check fitting and tubing on outlet of valve to the detector

Problem resolved.

Poorly Cut Peak Tubing





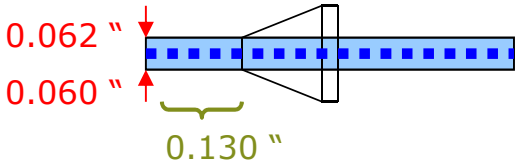


UPLC Tube

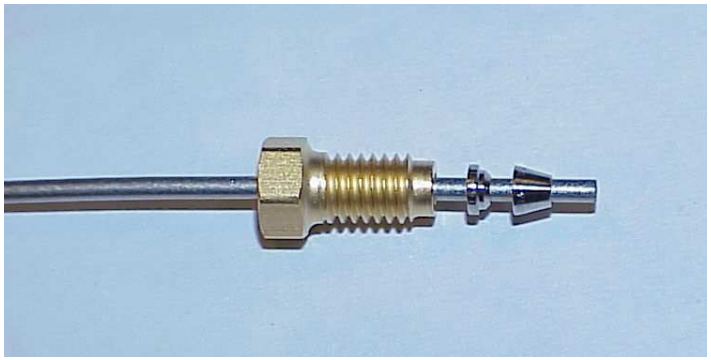
Poorly Cut Peak Tubing

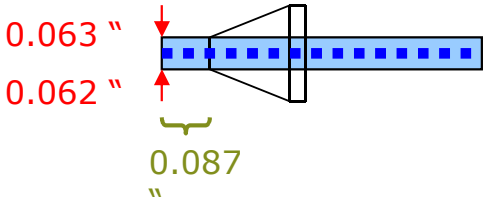
HPLC/UPLC Fittings

- Classic Waters HPLC fittings



- UPLC fittings

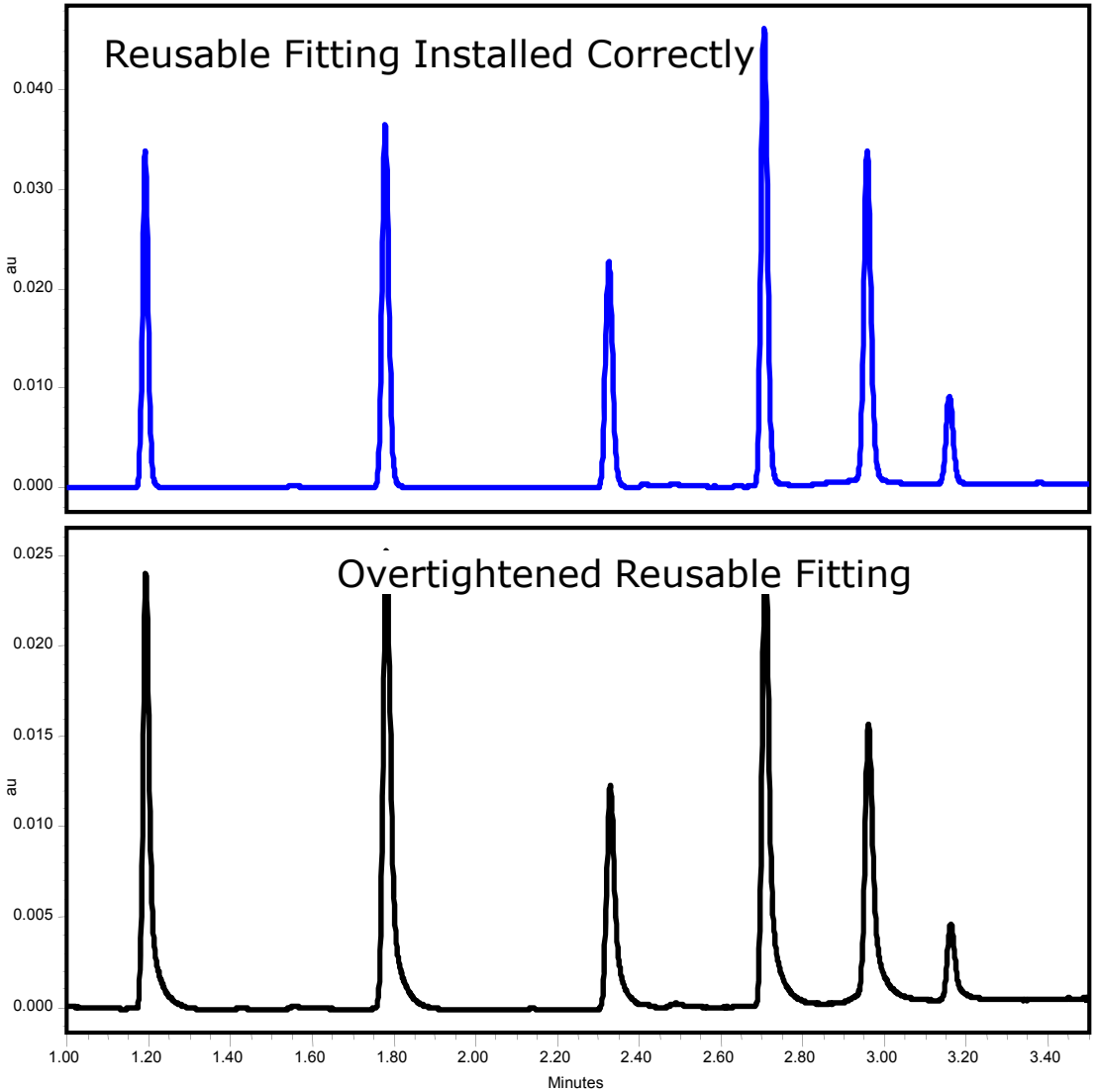




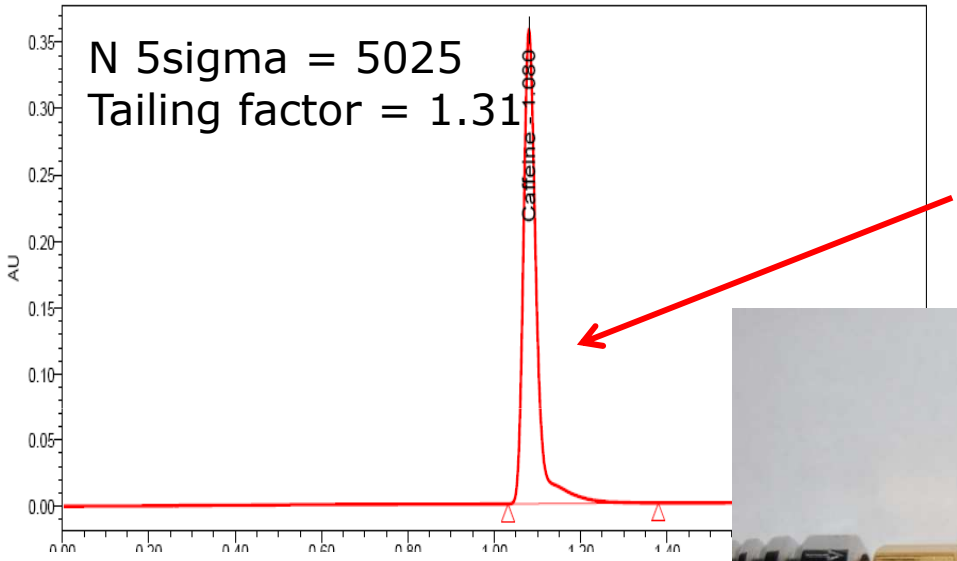
Over tightened Finger tight Fittings

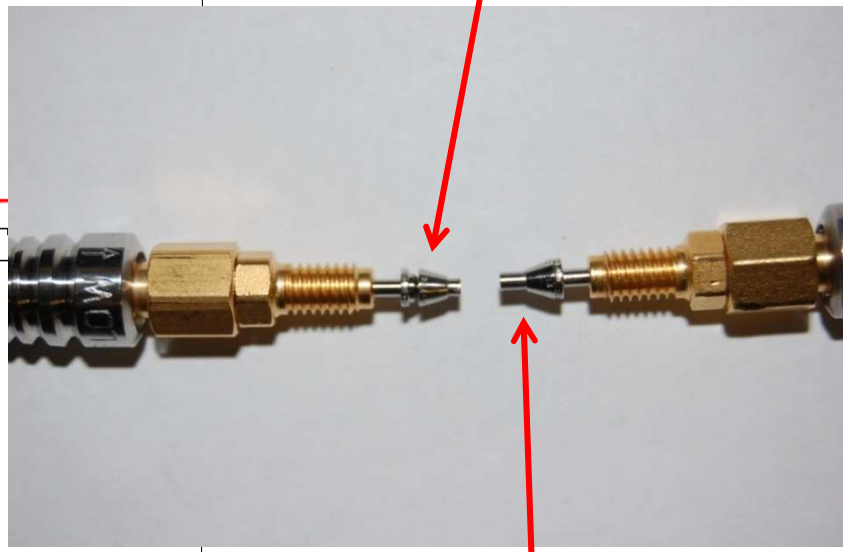
Normal Ferrule

Deformed Ferrule

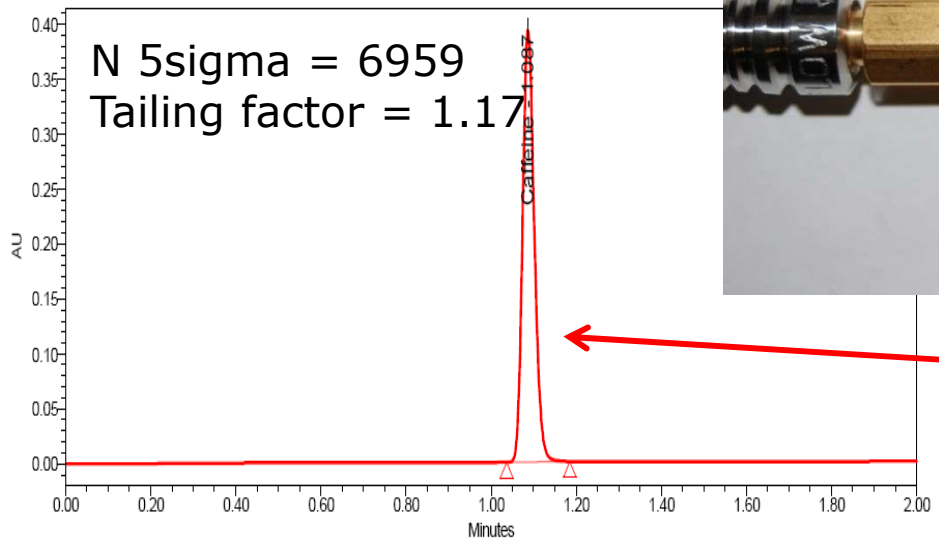


In-line filter installation problem





Wrong position of ferrule



Correct position of ferrule

Troubleshooting and diagnostics

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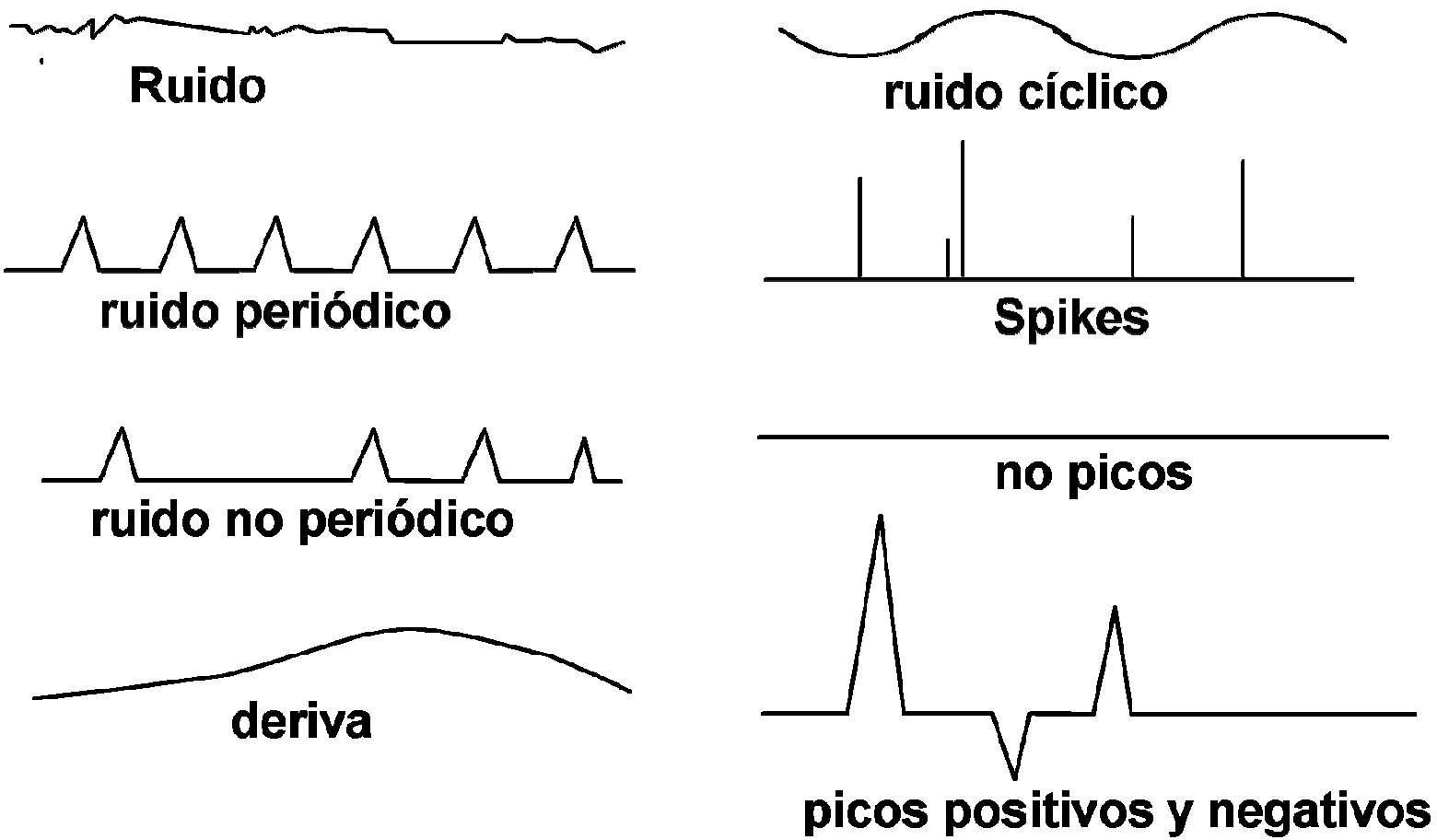
Baseline noise



Baseline noise characterization

- Non-cyclic (erratic) baseline noise
- Cyclic (synchronous) baseline noise
  - Short term cycling
  - Long term cycling
- Baseline drift
- Noise spikes on baseline

Troubleshooting procedure

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

- Stop the flow
- 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.

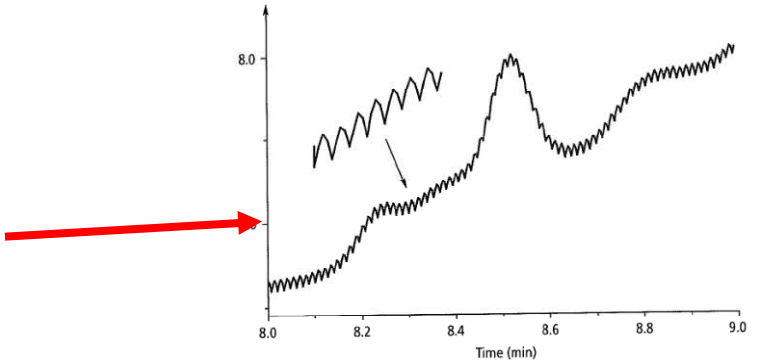
*Some flow sensitive detectors (such as RI, electrochemical) may require a significant time to stabilize once flow is stopped.

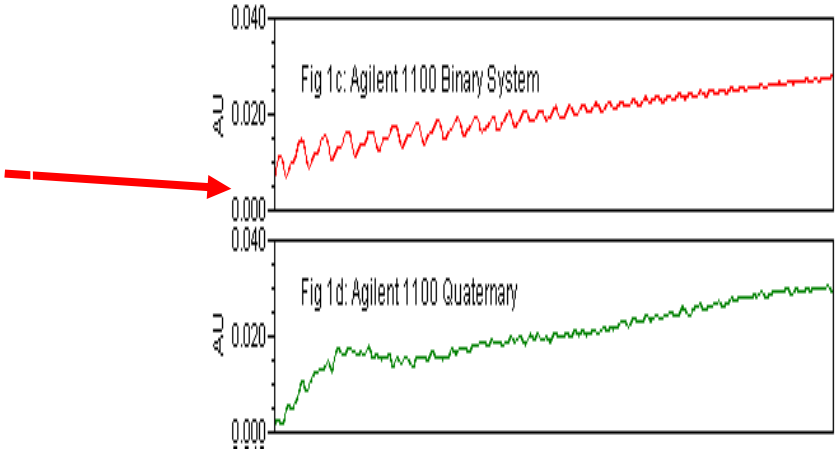
Fluid path-related noise

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 volume





Fluid path-related noise

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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

Fluid path-related noise

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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.

Fluid path-related noise

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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

Fluid path-related noise

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Baseline drift :

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

Fluid path-related noise

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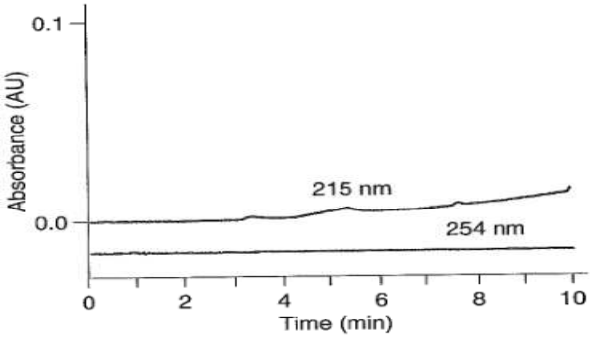
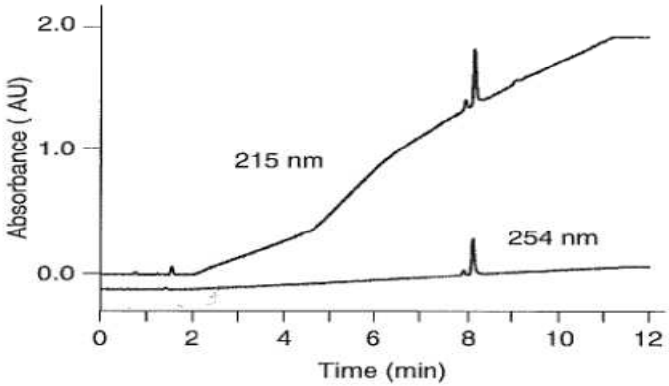
Baseline drift :

- 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 (trifunctional)
- Function of gradient and difference in UV absorbance of solvents
  - Does drift follow gradient curve/profile ?
    - Use higher wavelength
    - Replace methanol w. acetonitrile

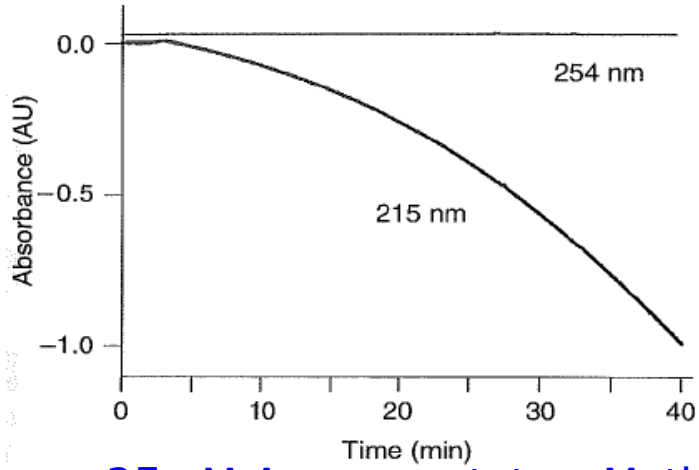
Gradient baseline drift



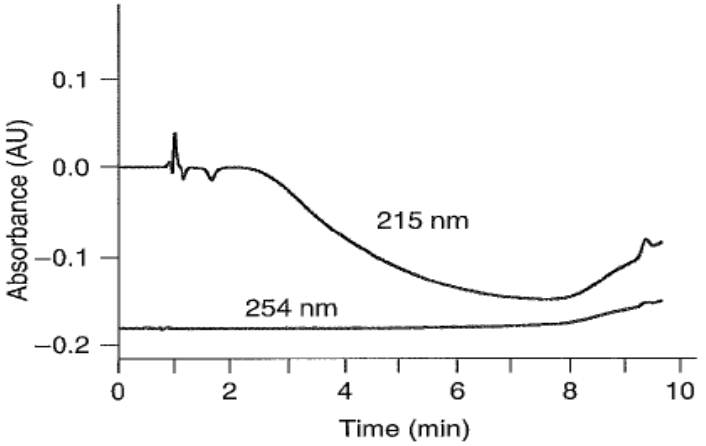
10mM Phosphate - Methanol gradient 5-80% in 10 min.



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



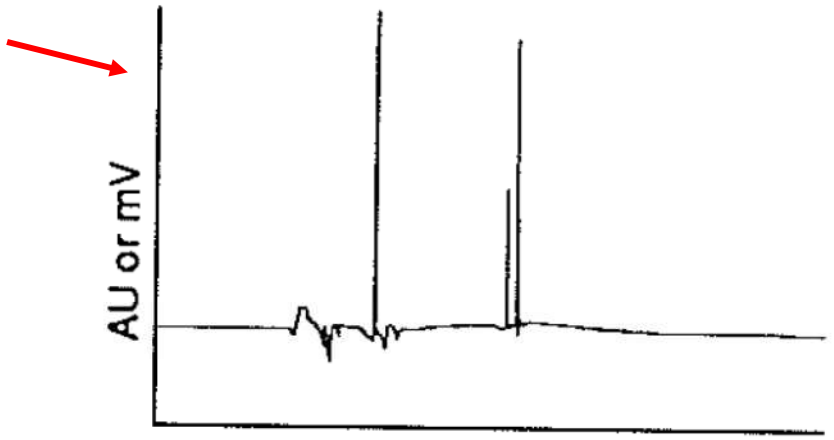
25mM Amm. acetate - Methanol gradient 5-80% in 40 min.



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

Detector related noise

- Spikes on the baseline
  - Defective lamp
  - Air bubble in flow cell
- Non-cyclic noise
  - Contaminated flow cell



Detector Tips and Tricks

Optimizing Noise and Resolution Performance

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

- Detector set up
  - Data rate
  - Filter constant

Importance of Sampling Rate

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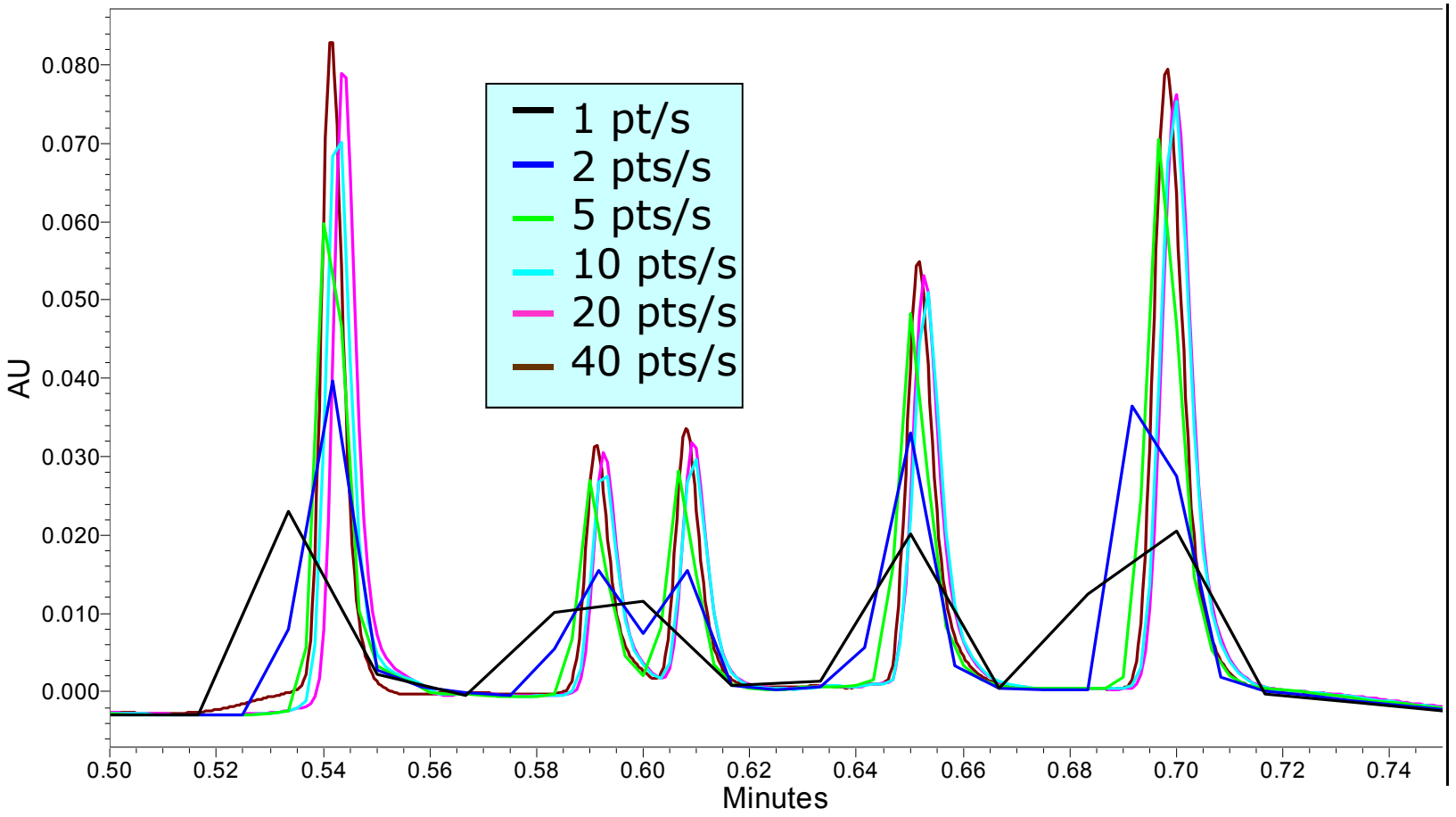
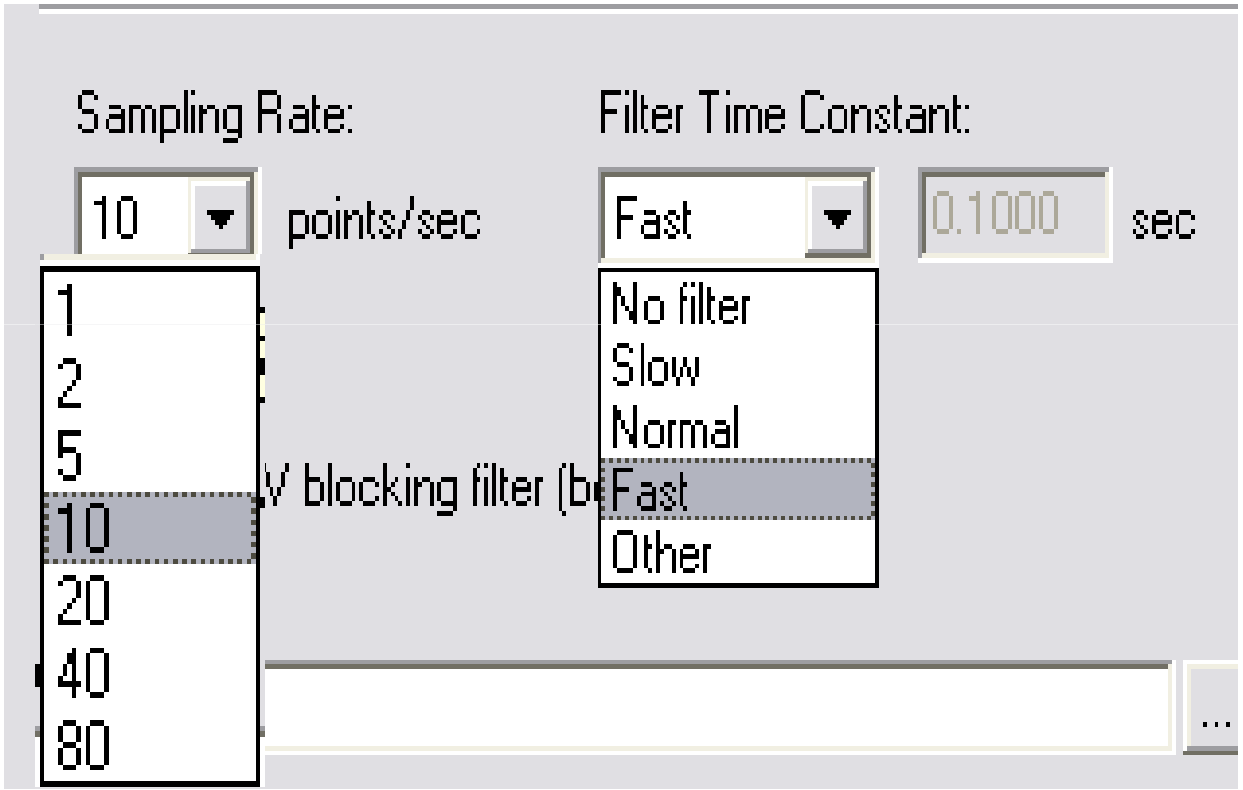
- Must ensure enough points are collected across a peak to adequately define the peak shape.
- Peak detection algorithms require a minimum number of points across a peak to distinguish it from baseline noise and correctly determine peak lift off and touch down.
- A peak which does not have enough data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

Effect of Sampling Rate



La Cuantificación Reproducible Requiere un Mínimo de 15-20 Puntos por Pico

Importance of Sampling Rate



What is Digital Filtering?

- Digital Filtering is a mathematical algorithm applied to a data set that smoothes out higher frequency noise.
- The desired result is reduced baseline noise with minimal impact on peak intensities so as to increase signal-to-noise.
- The degree of filtering (time constant) is crucial, too much filtering can dramatically impact peak shapes and resolution.

Optimizing Digital Filter Settings

- Ensure that the data rate is set to collect 25 – 50 points
- Start with the FAST, NORMAL, or SLOW settings depending upon the requirements for resolution versus sensitivity
- Optimize for the method
  - Start with the inverse of the sampling rate

    Filtering Time Constant = 1/Sampling Rate
  - Is increased sensitivity needed? Is baseline noise interfering with integration?

    ↑ *Filtering Time Constant*
  - Is increased resolution needed?

    ↓ *Filtering Time Constant*

Effect of Filter Time Constant Setting

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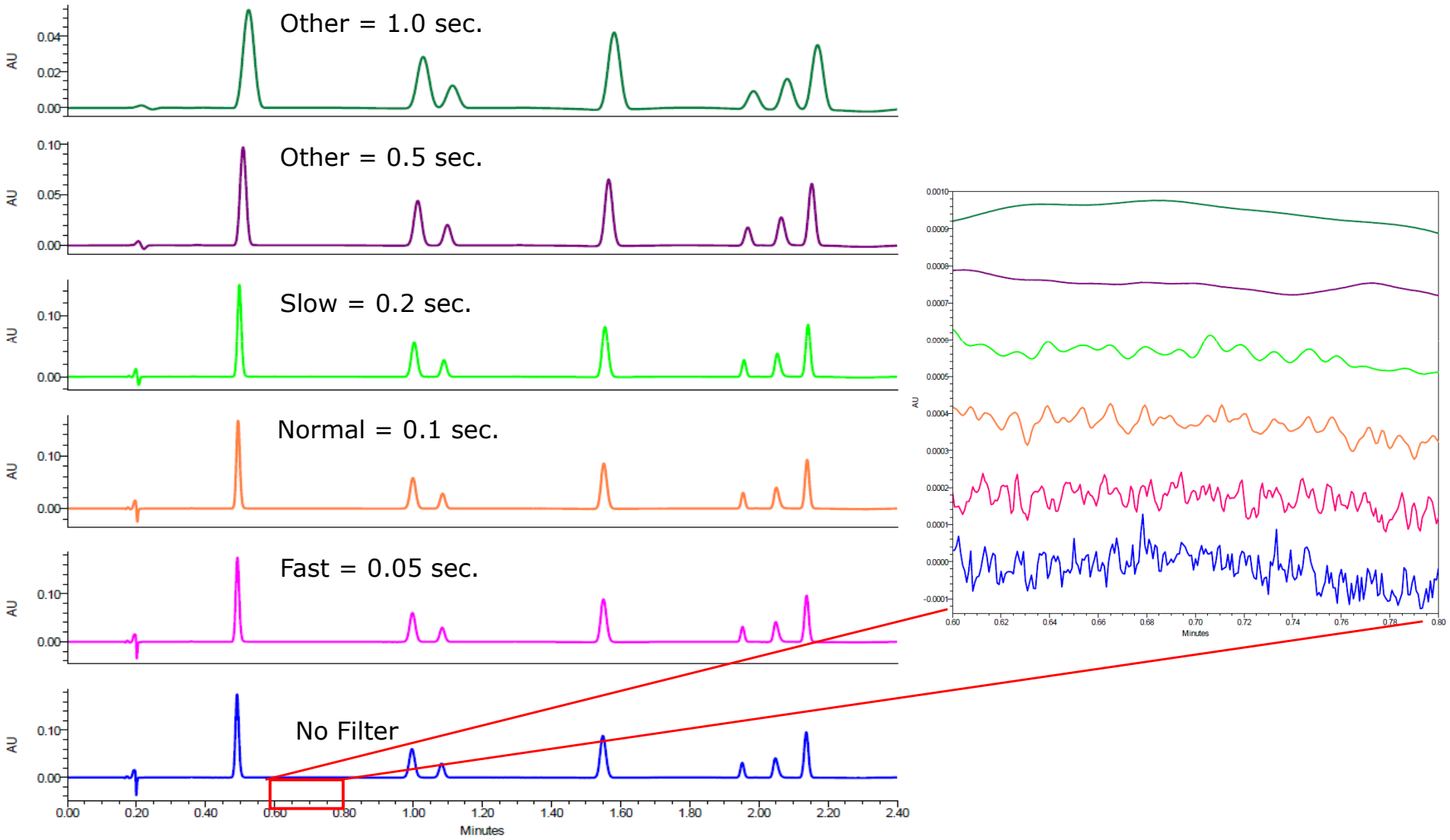

- Tips and tricks to prevent problems
  - Solvent considerations

Tips and Tricks
General Recommendations - Mobile Phase Preparation

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- Use High quality organic solvents, buffers and additives
  - Don´t use metallic container solvents
  - Always make sure that there are no insoluble particles in the eluents
  - Usually ***High Grade*** organic solvents are filtered through a membrane (read the label on the bottle)
  - Use high quality salts to prepare buffered eluents
- Use fresh Milli-Q water
- Change aqueous mobile phases often. Every 24–48 hr

Tips and Tricks

General Recommendations - Mobile Phase Preparation

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- All bottles must be capped
  - Do not use Parafilm
- Use clean glassware (bottles and for filtration)
  - Who takes care of this? How is it managed?
    - *Never use communal, department dishwashers*
    - *Never use any detergents for cleaning, especially for mass spectrometer applications*
  - What is the quality of glass? Is it compatible with the mobile phase (pH)?
  - Bottles must be capped
  - Do not top-off the bottles(Can promote microbial growth)

Tips and Tricks

General Recommendations - Mobile Phase Preparation

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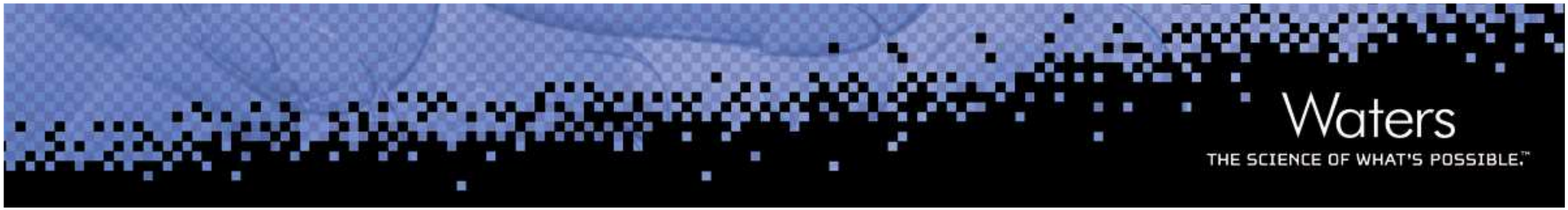
- Flush buffers out of system with water after use (after cleaning use 10-20% organic in water for storage).
- Keep all solvent lines primed (use 10-20% organic in water for unused lines, MeOH or IPA are fine)
- Keep seal wash primed. (90-95% water)
- Re-prime solvent lines before starting



Questions ?