

HPLC Guide to Troubleshooting



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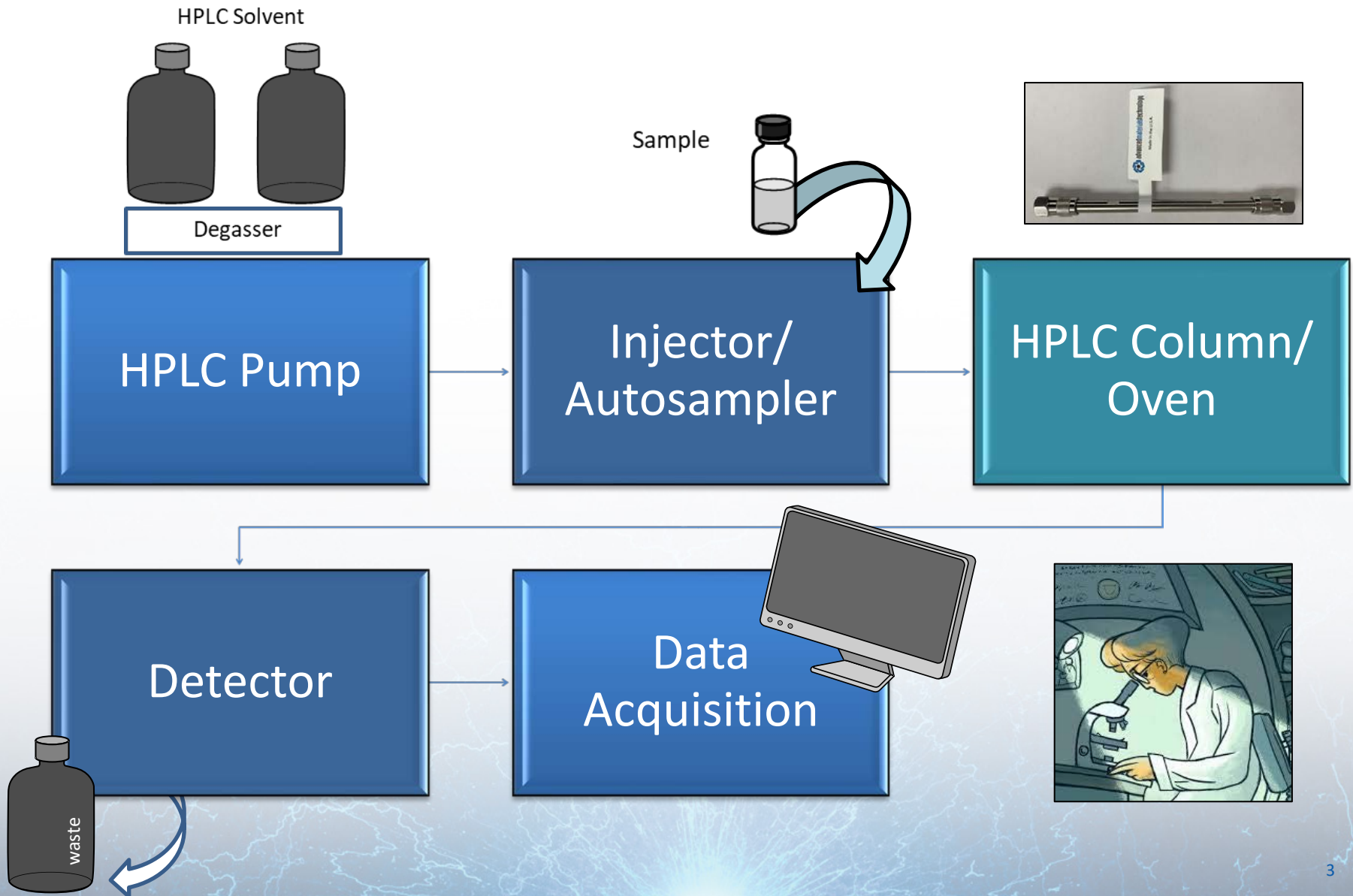


- ❖ HPLC Overview: Possible Sources for Chromatographic Problems
- ❖ Peak Broadening
- ❖ Baseline Issues
- ❖ Artifact Peaks
- ❖ Peak Area Variability
- ❖ Retention Time Variability
- ❖ Prevention of Problems
- ❖ Conclusion

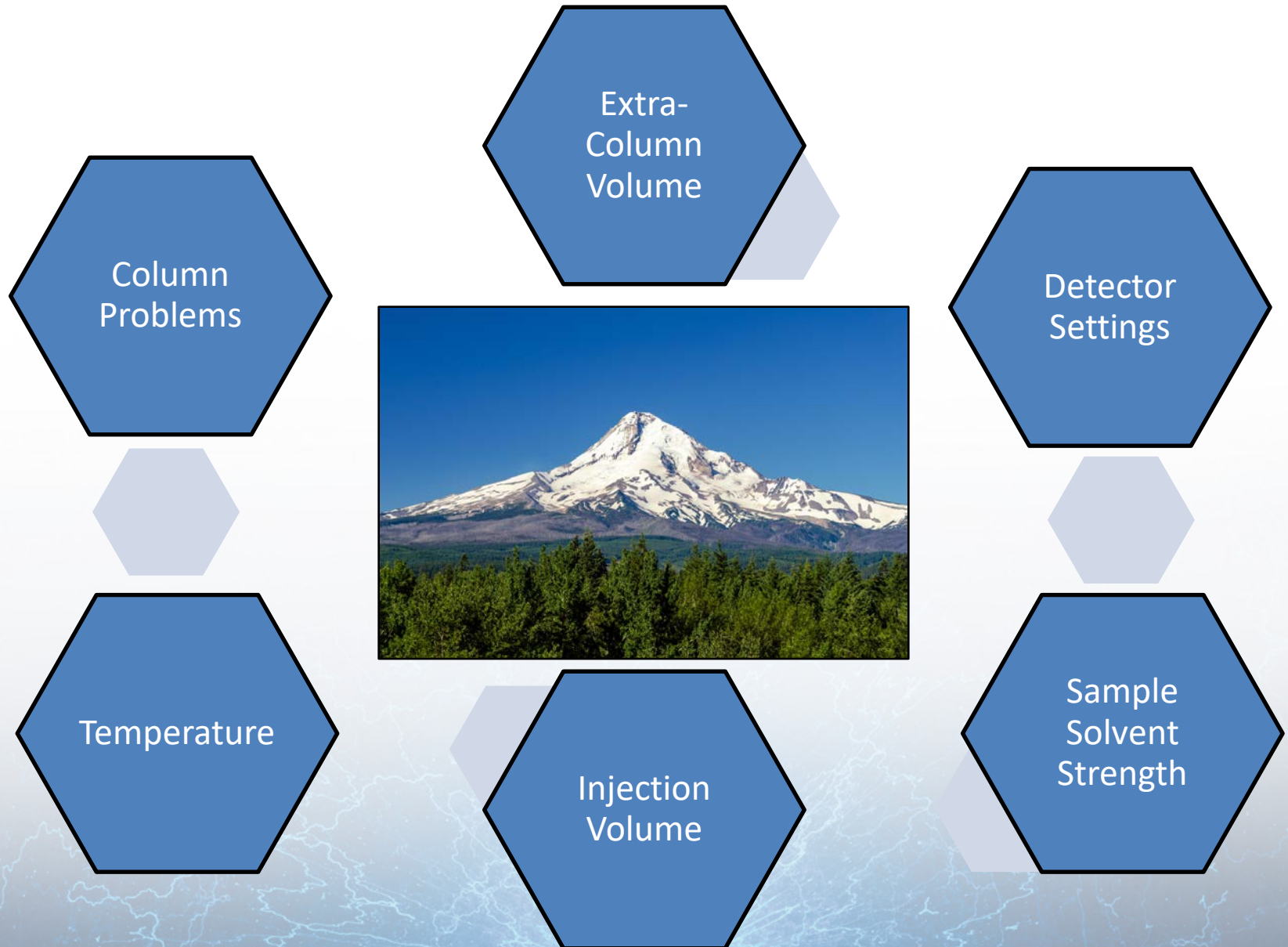


HPLC Overview

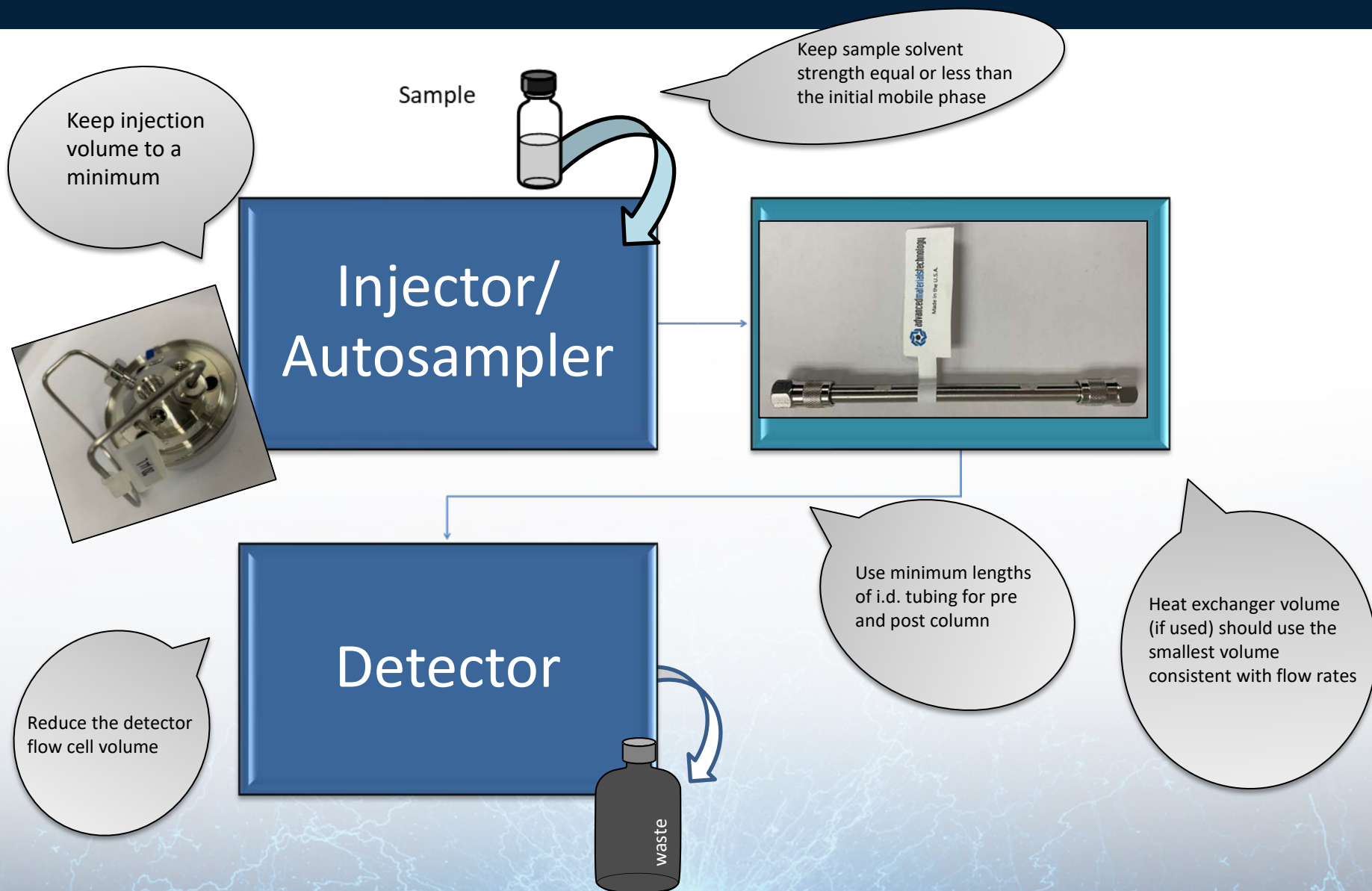
Possible Sources for Chromatographic Problems



Peak Broadening



Extra Column Volume



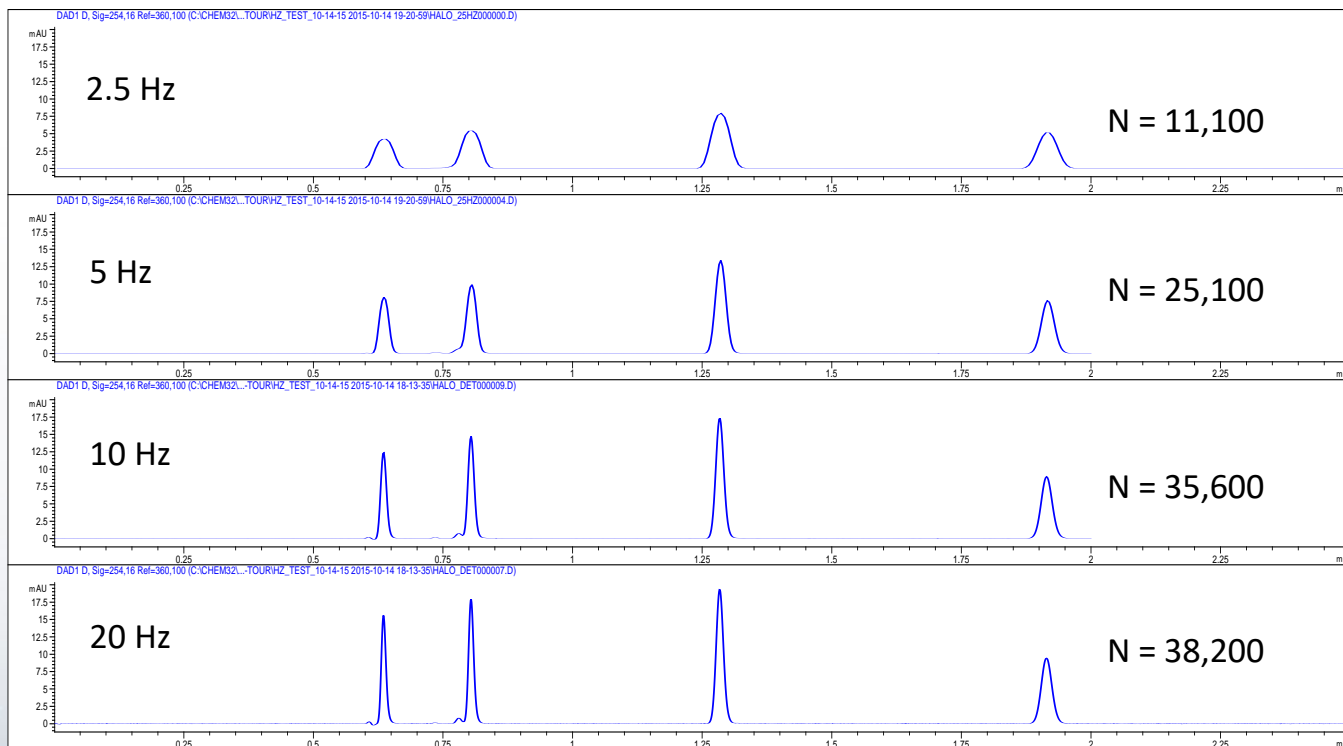
Effect of Data Acquisition Rate on Efficiency

System operation well, check your settings

HALO C18
4.6 x 150 mm 2.7 μ m
1.8 mL/min

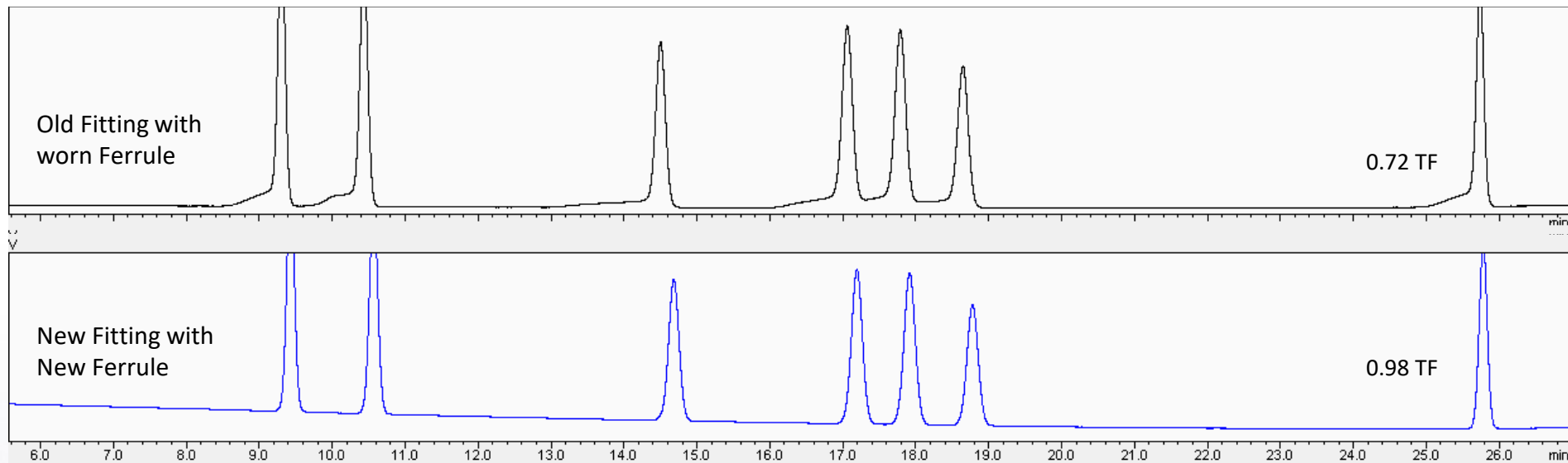
70/30 ACN/water
30 °C

Sample contains uracil, phenol, 1-chloro-4-nitrobenzene, and naphthalene



Column Fittings

Separation of Parabens using Chinese Pharmacopeia Method
(widely used preservatives in cosmetics and pharmaceutical products) on HALO C18, 2.7 μ m



TEST CONDITIONS:

Column: HALO 90Å C18, 2.7 μ m, 4.6 x 100mm

Part Number: 92814-602

Mobile Phase A: Water

Mobile Phase B: Methanol

Gradient: Time %B

0.0 40

23.0 55

28.0 70

Flow Rate: 1.2 mL/min

Initial Pressure: 403 bar

Temperature: 30°C

Detection: UV 252 nm, PDA

Injection Volume: 1.5 μ L

Sample Solvent: 50-50 Methanol-Water

Data Rate: 40 Hz

Response Time: 0.025 sec.

Flow Cell: 1 μ L

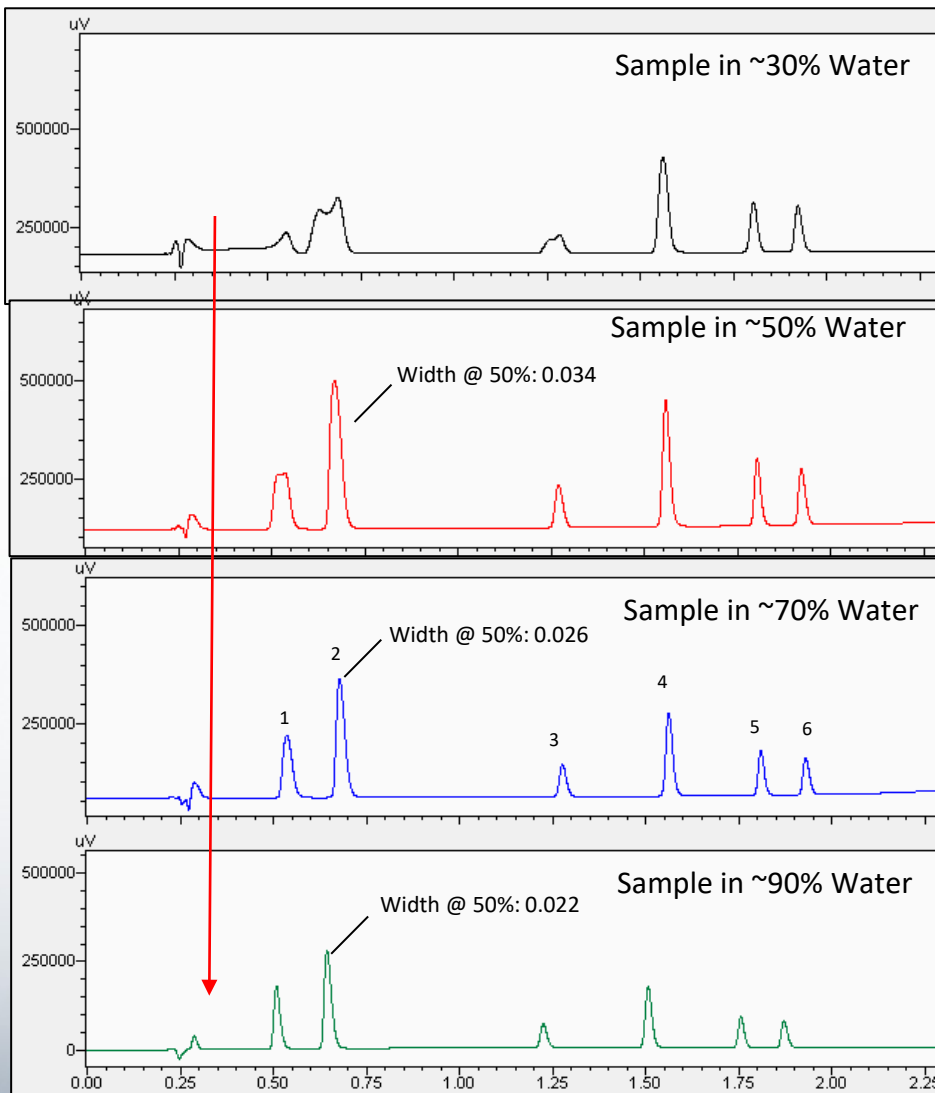
LC System: Shimadzu Nexera X2



PEAK IDENTITIES:

1. Isopropyl paraben
2. Propyl paraben
3. Phenyl paraben
4. Isobutyl paraben
5. Butyl paraben
6. Benzyl paraben
7. Pentyl paraben

Sample Solvent vs Initial MP



*Make sure your sample solvent is equivalent to the initial mobile phase being used in your test. This will save you time troubleshooting odd peak shapes in your chromatogram.

*A separation of beta blockers starting from 30% water in the sample solvent and increasing to 90% water in the sample solvent from top to bottom. Notice how much the peak shapes improve as your sample solvent gets closer to the initial mobile phase composition of 90%.

TEST CONDITIONS:

Columns: HALO 90 Å Biphenyl, 2 μ m, 2.1x50mm

Mobile Phase A: Water, 0.1% TFA

Mobile Phase B: Acetonitrile, 0.1% TFA

Gradient:

Time	% B
0.0	10
5.0	50

Flow Rate: 0.5 mL/min

Temperature: 35° C

Detection: UV 220 nm, PDA

Injection Volume: 1 μ L

Sample Solvent: Water

Data Rate: 40 Hz

Response Time: 0.025 sec.

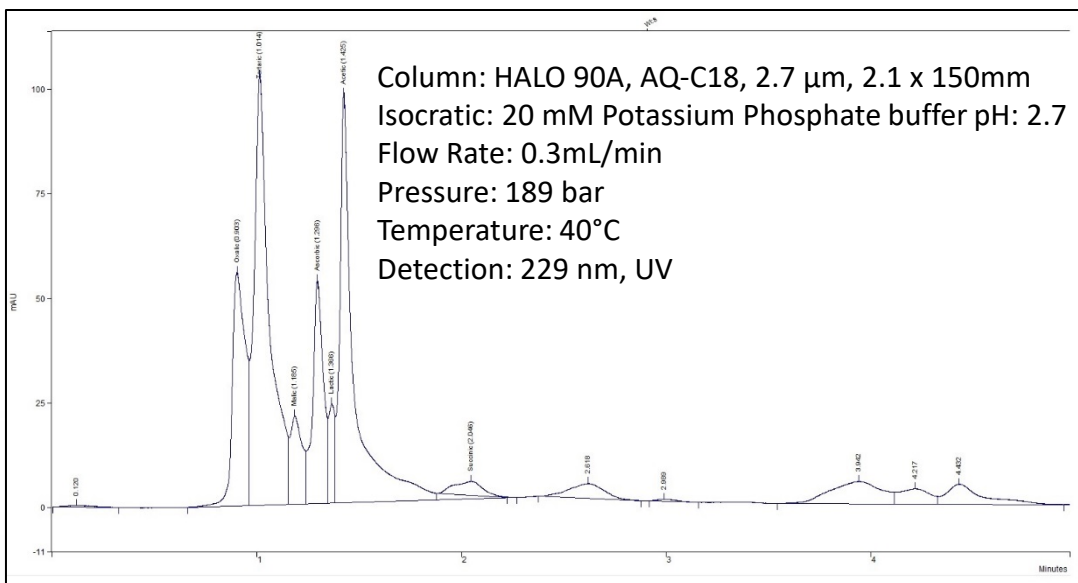
Flow Cell: 1 μ L

LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

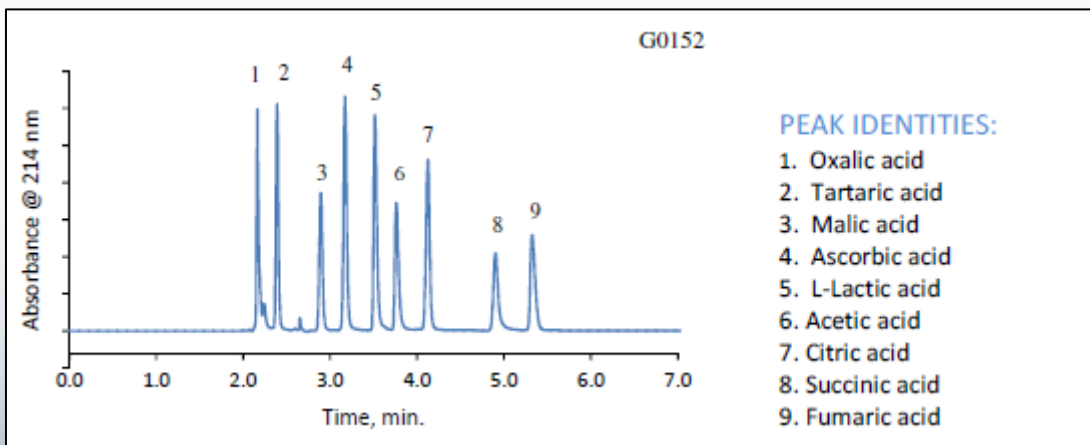
1. Atenolol
2. Sotalol
3. Nadolol
4. Pindolol
5. Acebutolol
6. Metoprolol

Overloading Column/ Loss in Performance



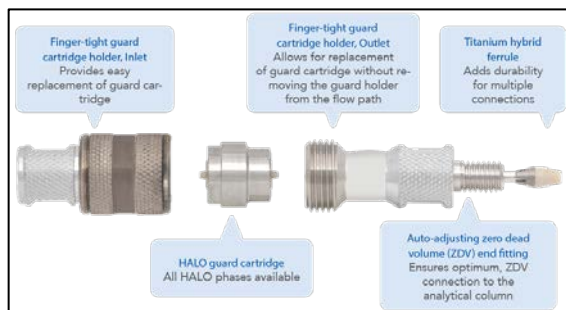
Column Overload?

Be careful not to inject too much sample or solute onto the column. Column overloading reduces the performance of the column.



Column: HALO 90A, AQ-C18, 2.7 μ m, 4.6 x 250mm
Isocratic: 20 mM Potassium Phosphate buffer pH: 2.7
Flow Rate: 1.0 mL/min
Pressure: 307 bar
Temperature: 40°C
Detection: 214 nm, PDA

Column Problems



Frit problem
(back pressure, split peaks)

- Filter samples
- Use a guard column/ inline filter

Column Aging
(reproducibility)

- Operate column within the recommended conditions
- Clean column regularly
- Store column properly

Column Void
(split peaks)

- Avoid pressure shocks
- Cap off columns when not in use (solvent evaporation)



Why Pore Size Matters

TEST CONDITIONS:

Column: HALO 1000Å ES-C18, 2.7µm, 2.1 x 150 mm

Part Number: 92712-702

A= Water, 0.1% TFA

B= 80/20 ACN/ Water, 0.085% TFA

Gradient:

Time (min.) %B

0.00 27

15.00 60

Flow Rate: 0.4 mL/min.

Pressure: 268 bar

Temperature: 60 °C

Injection Volume: 2 µL

Sample Solvent: Water/0.1% TFA

Detection: UV 280 nm, PDA

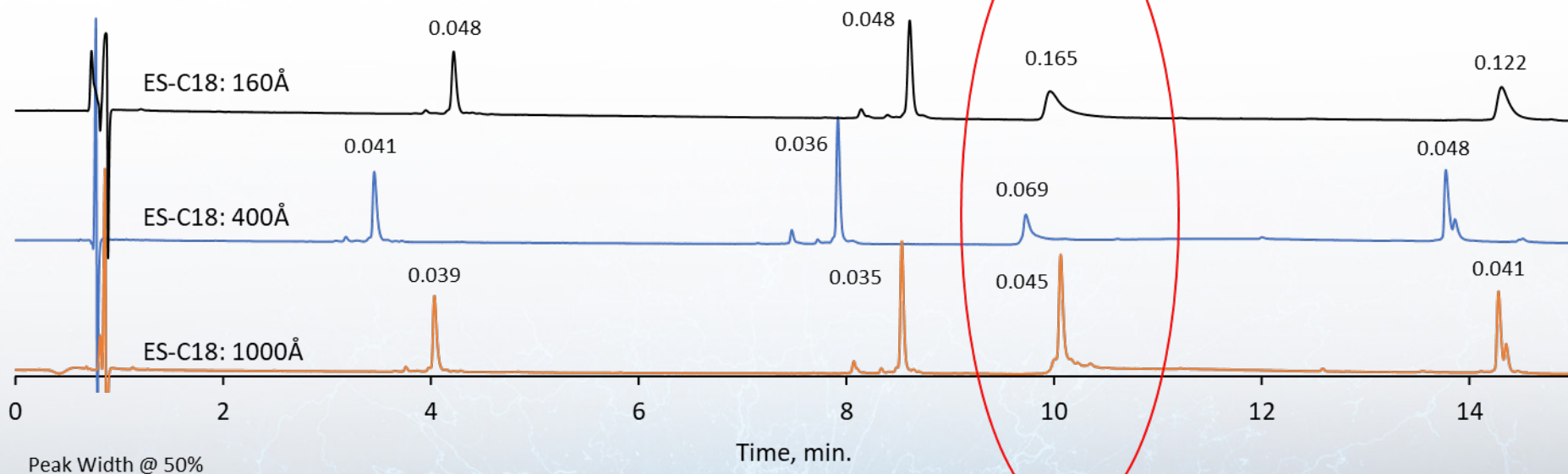


Molecule Size	Pore Size (Å)	Application	Particle Sizes (µm)	Column Family
SMALL (<5000 Da)	90	Small Molecules	2, 2.7, 5.0	HALO
SMALL (< 20 kDa*)	90	Glycans	2.7	HALO BIOCLASS
MEDIUM (100 Da < MW < 15 kDa)	160	Peptides	2, 2.7, 5.0	
LARGE (2 kDa < MW < 500 kDa)	400	Proteins	3.4	
LARGE (> 50 kDa)	1000		2.7	

* for glycan, glycopeptide, glycoproteins

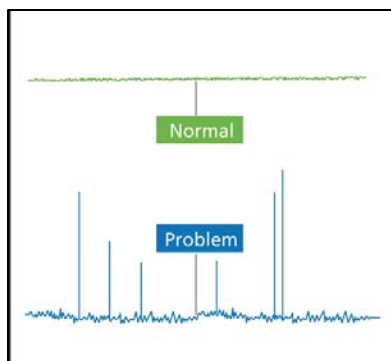
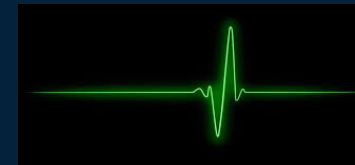
PEAK IDENTITIES:

1. Ribonuclease A 13.7 kDa
2. Lysozyme 14.3 kDa
3. SigmaMAb ~150 kDa
4. Enolase 46 kDa monomer

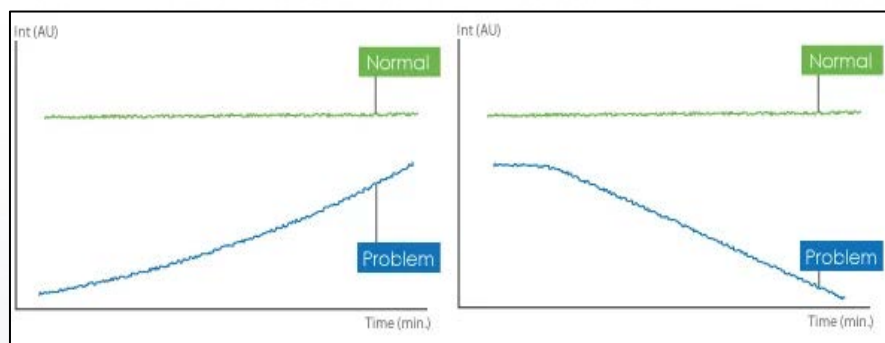




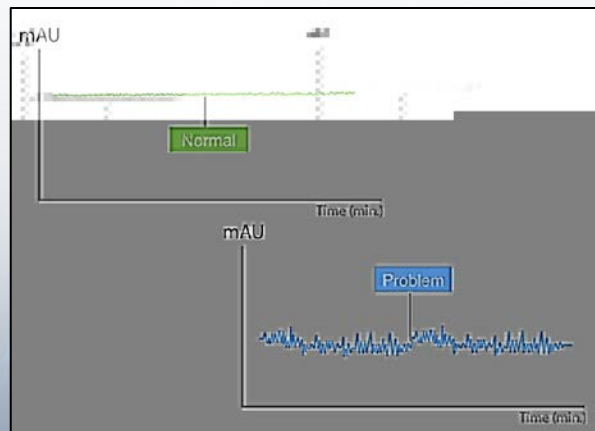
Baseline



Baseline Spikes



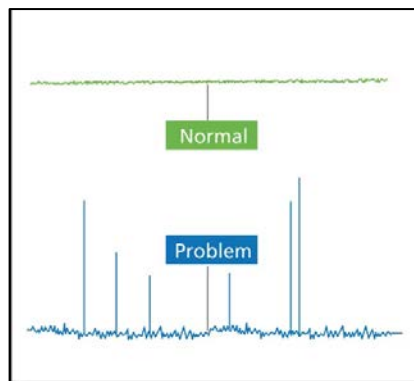
Baseline Drift



Noisy Baseline



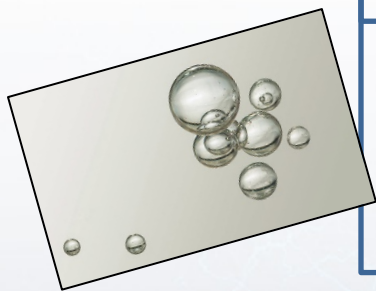
Baseline Spikes



Air Bubbles

Problem only
exists while
flowing

Purge lines



Dead Lamp

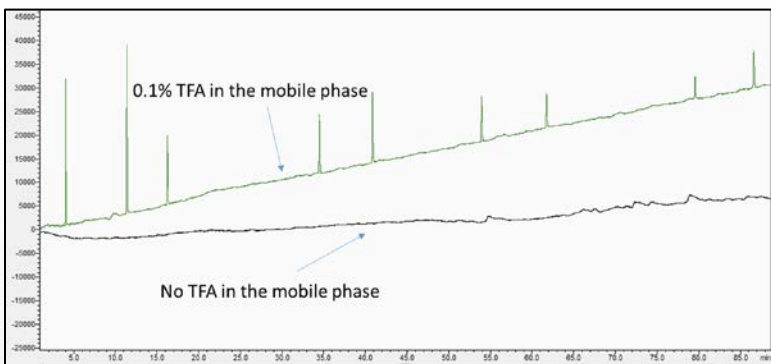
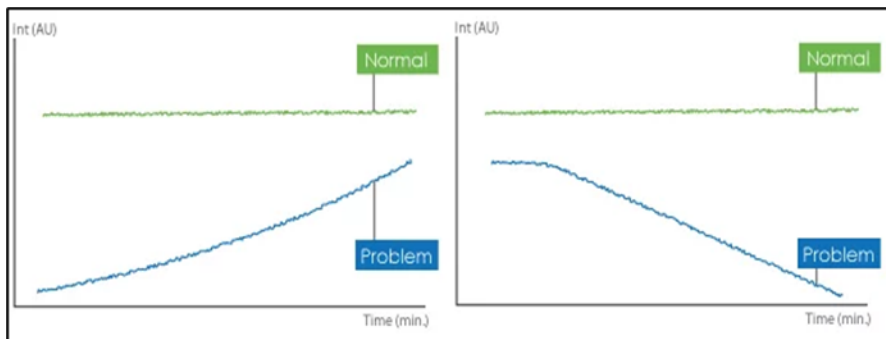
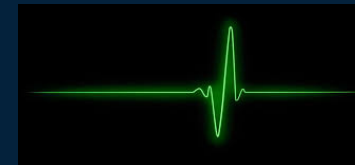
Problem
occurs with
no flow

Replace lamp





Baseline Drift



Negative/ Positive Drift

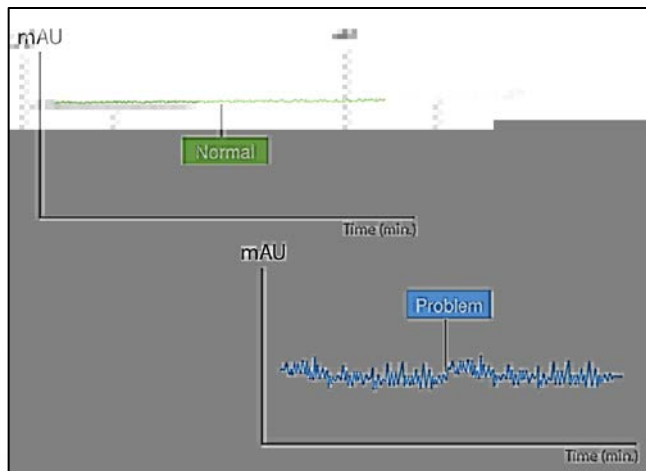
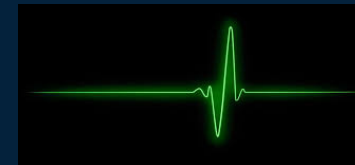
Modifier in
Mobile Phase/
Difference in
Absorbance

Add Modifier to
Mobile Phase/
Change
Wavelength

- Insufficient equilibration
- Baseline rise in gradient mode is normal
- Temperature fluctuation



Noisy Baseline



Old Lamp

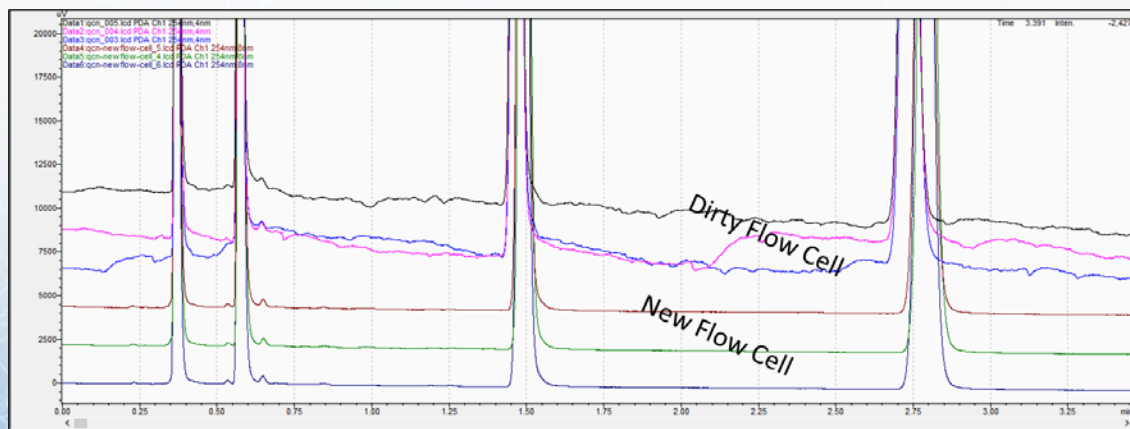
- Track lamp hours



- Insufficient degassing
- Air bubbles
- Poor mixing

Contamination

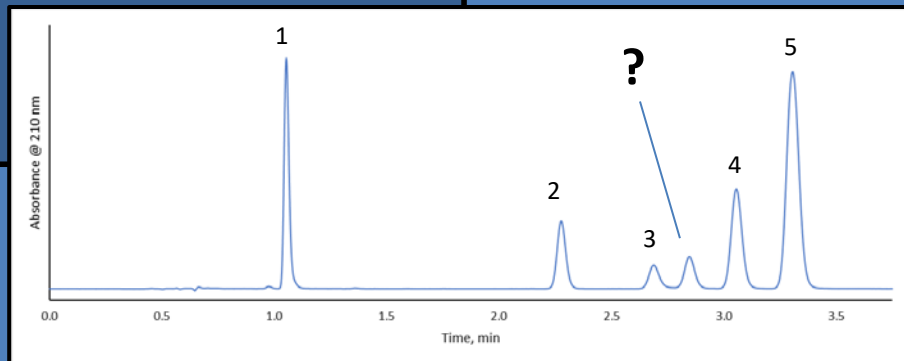
- Water
 - IPA
 - Water
- passivation may be required



Artifact Peaks

Late Elution Peaks

Carry-over Peaks

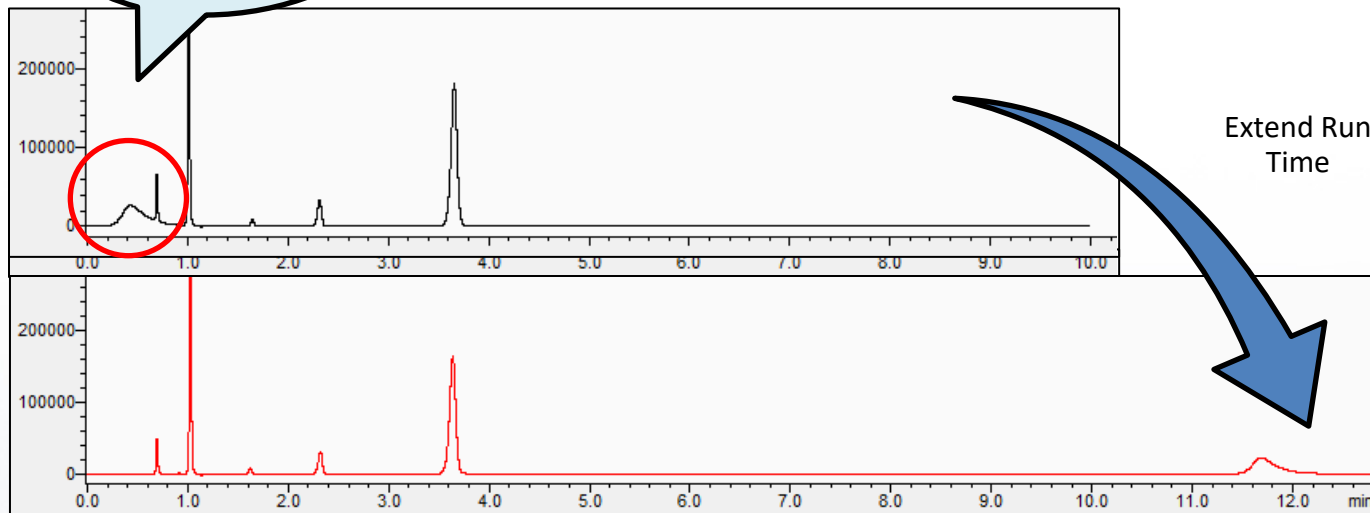


Contamination

Ghost Peaks

Late Elution Peak

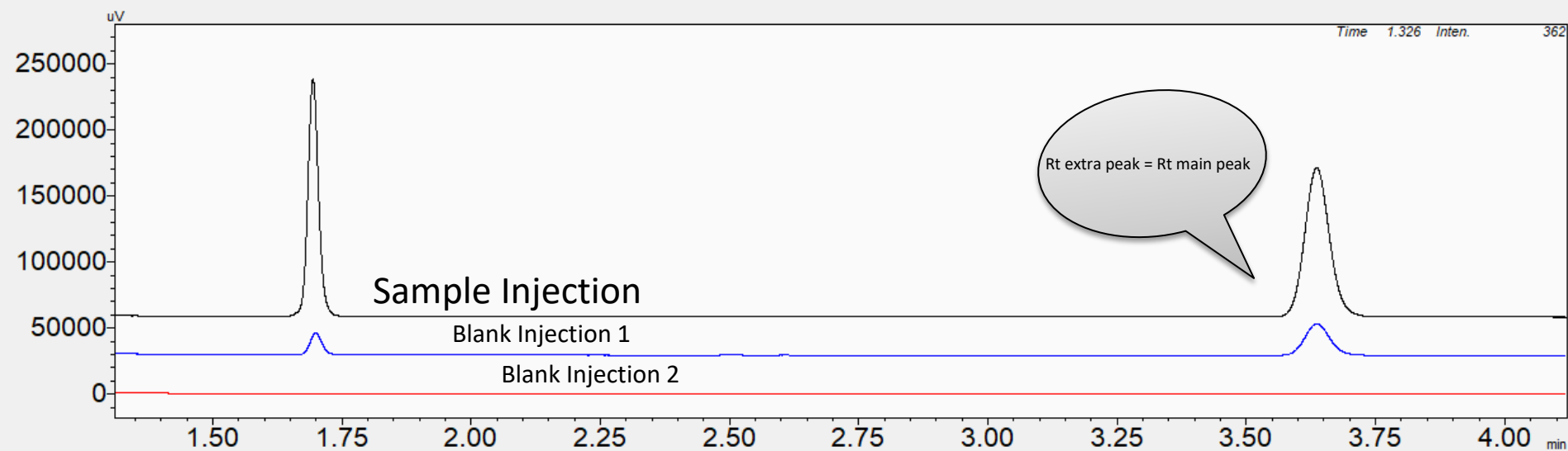
Broad peak not consistent with the width of nearby peaks?



- ☒ Extend Previous Injection
- ☐ Series of Blank Injections
- ☐ New Blank/ Sample
- ☐ Clean Solvents/ Acidic Modifiers



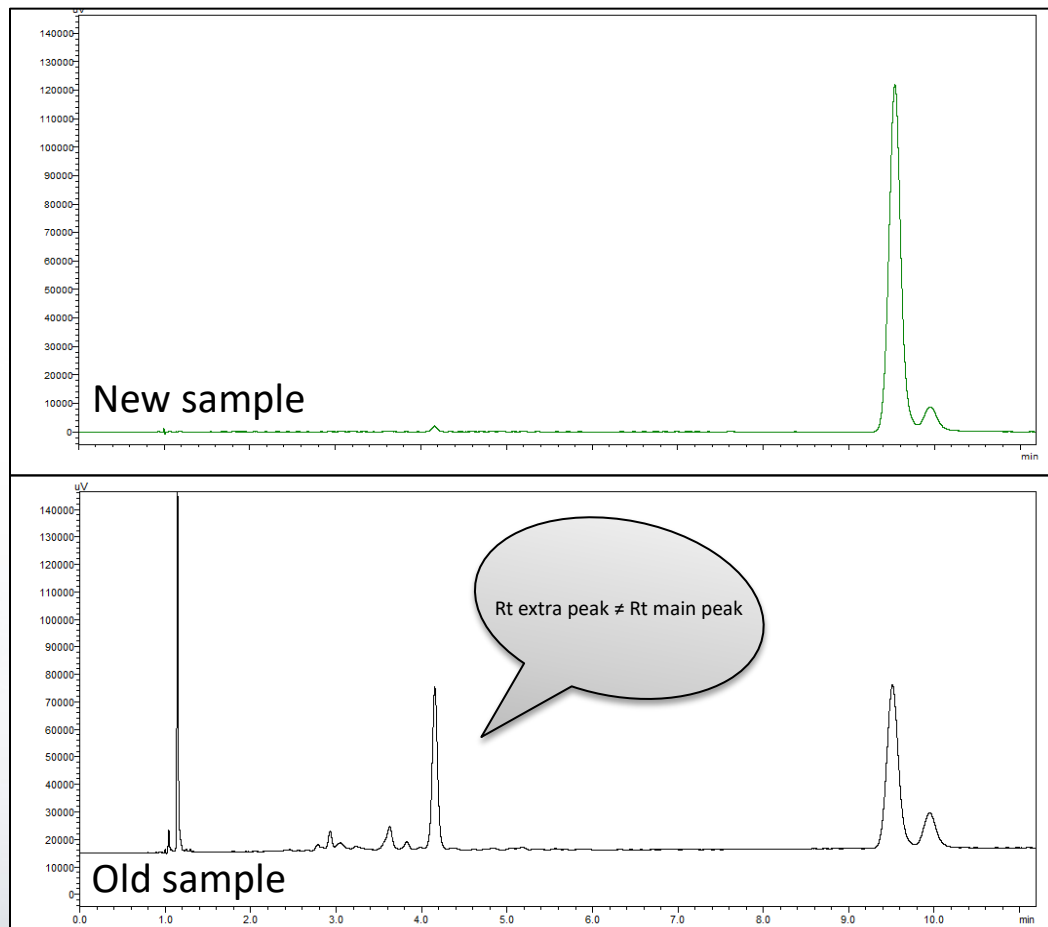
Carry-Over Peaks



- ☒ ~~Extend Previous Injection~~
- ☒ Series of Blank Injections
- ☐ New Blank/ Sample
- ☐ Clean Solvents/ Acidic Modifiers



Contamination: Sample



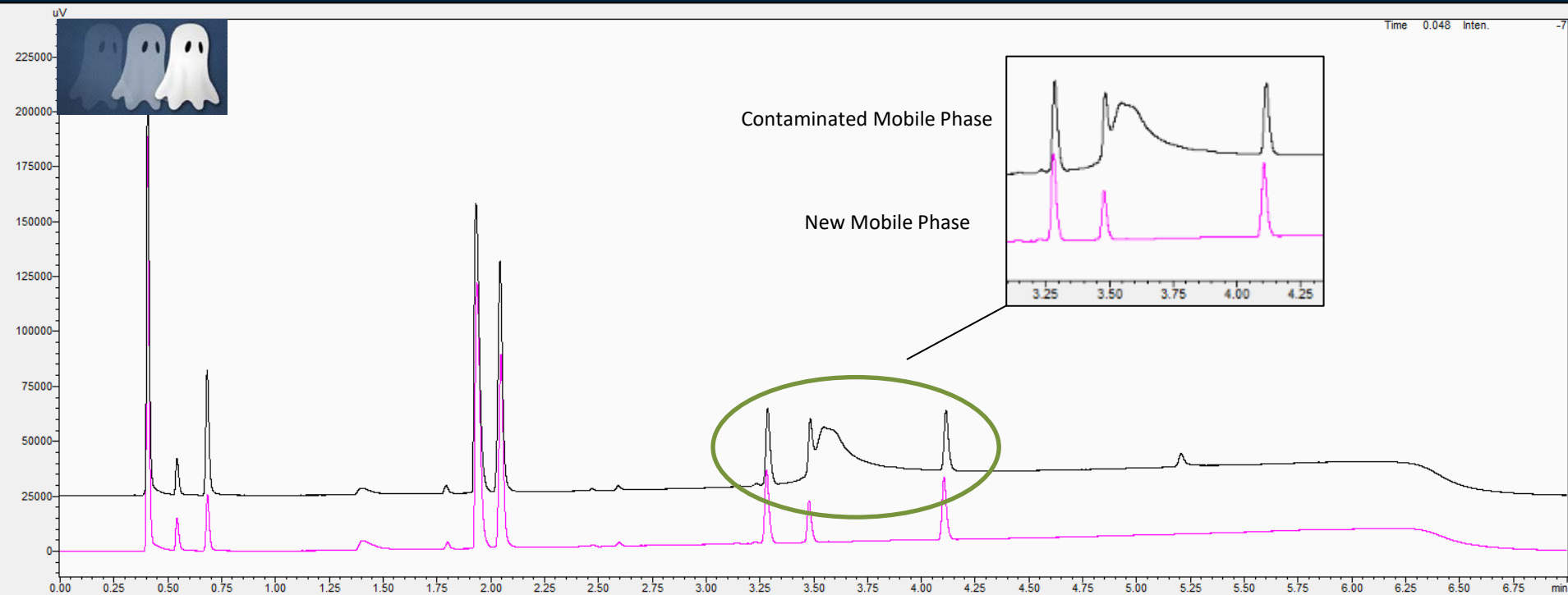
☒ ~~Extend Previous Injection~~

☒ ~~Series of Blank Injections~~

☒ New Blank/ Sample

☐ Clean Solvents/ Acidic Modifiers

Ghost Peak: Mobile Phase Contamination



☒ Extend Previous Injection

☒ Series of Blank Injections

☒ New Blank/ Sample

☒ Clean Solvents/ Acidic Modifiers



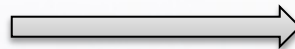
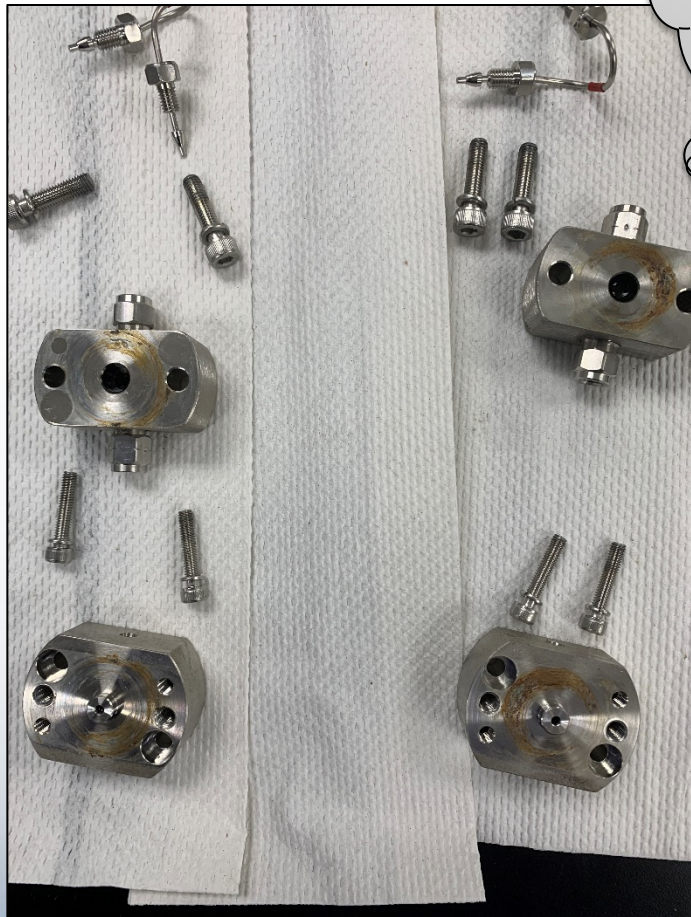
Mystery Peak?

Mystery peaks are often thought to be from sample carry over or some type of contamination. Don't forget about your mobile phases! In this example, a gradient is performed using water and acetonitrile mobile phases using formic acid as an acidic modifier. A mystery peak in the middle of the gradient turned out to be from a contamination with the acidic modifier.

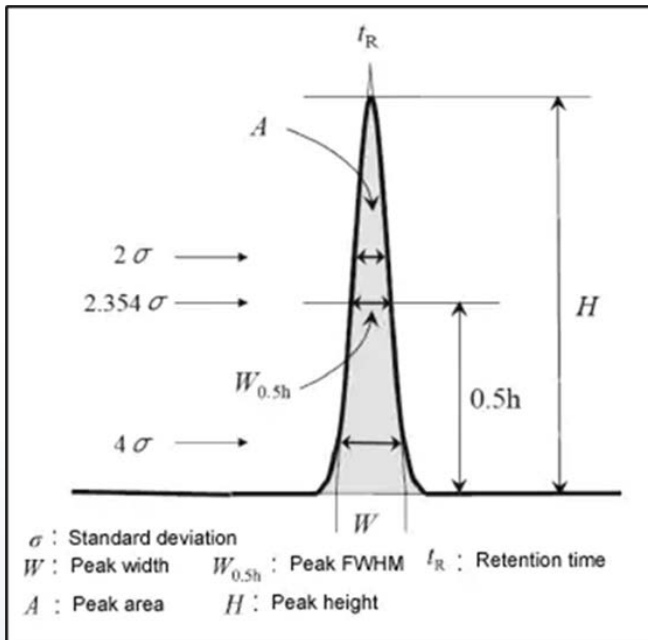


Side Note: Rust Maintenance

Nasty pump heads? Try using some aluminum oxide with a little bit of water to scrub them clean! Aluminum oxide is a great abrasive. Toothpaste works too.



Peak Area Variability

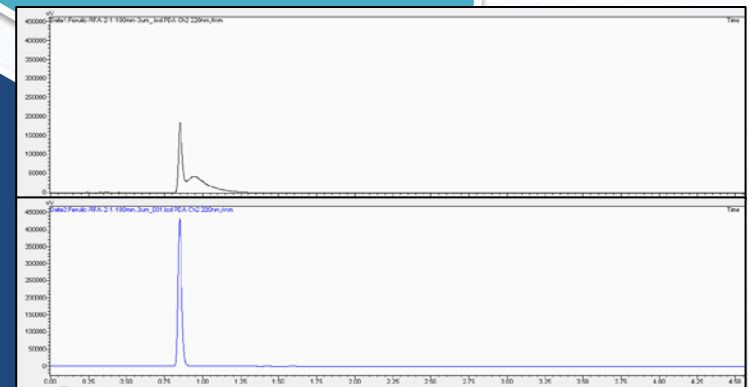
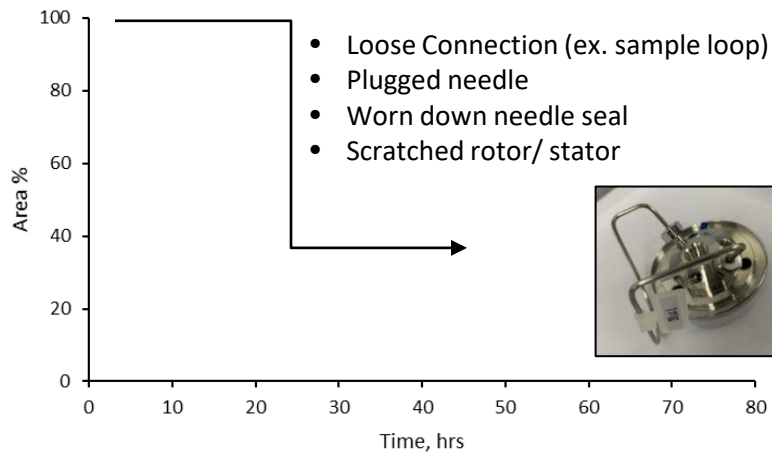
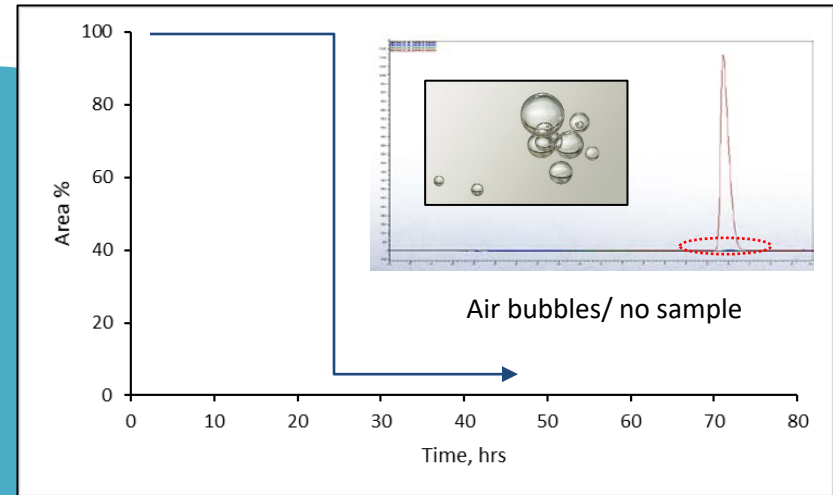
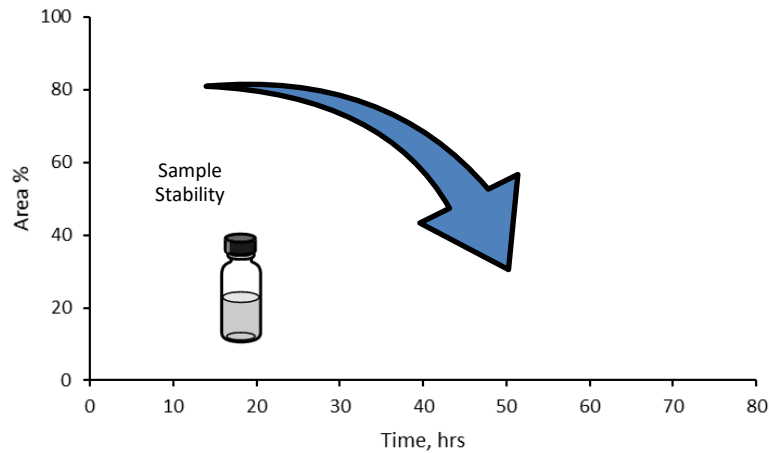


Decreases Over Time

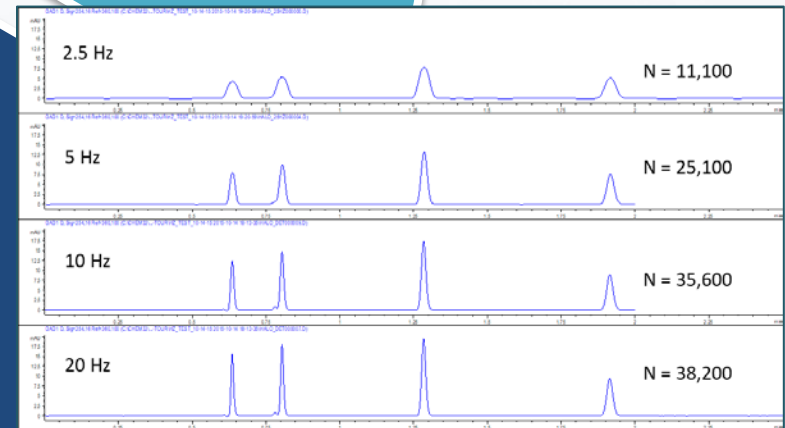
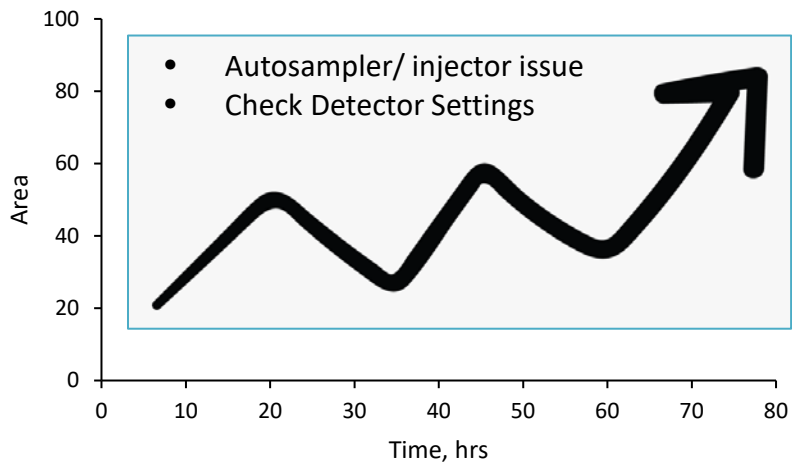
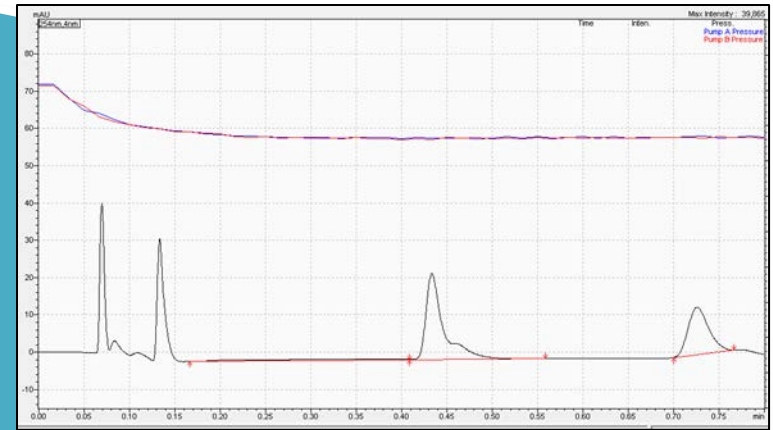
Random

Increases Over Time

Area: Decreasing



Area: Random

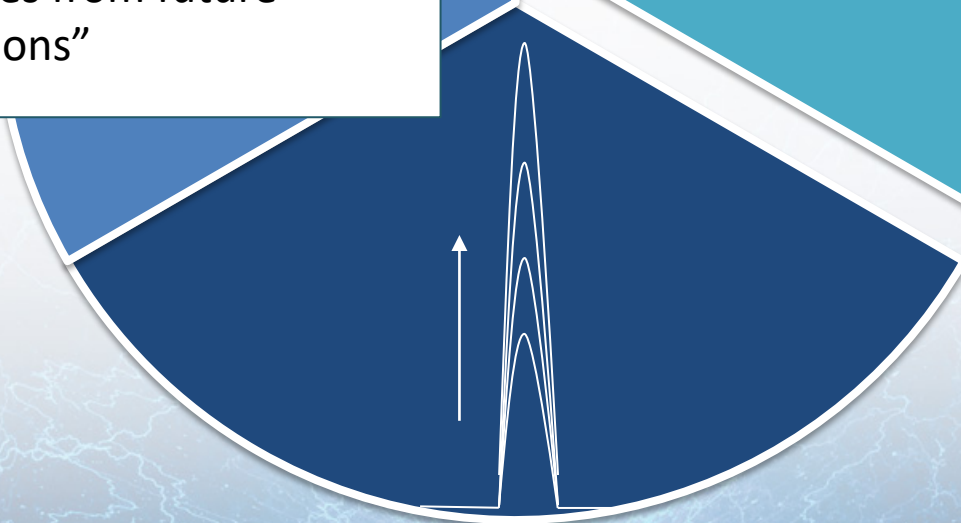
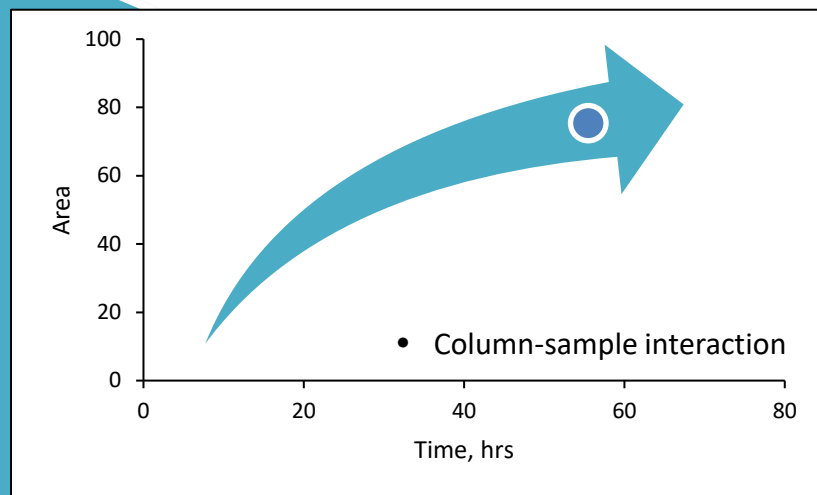


Area: Increasing

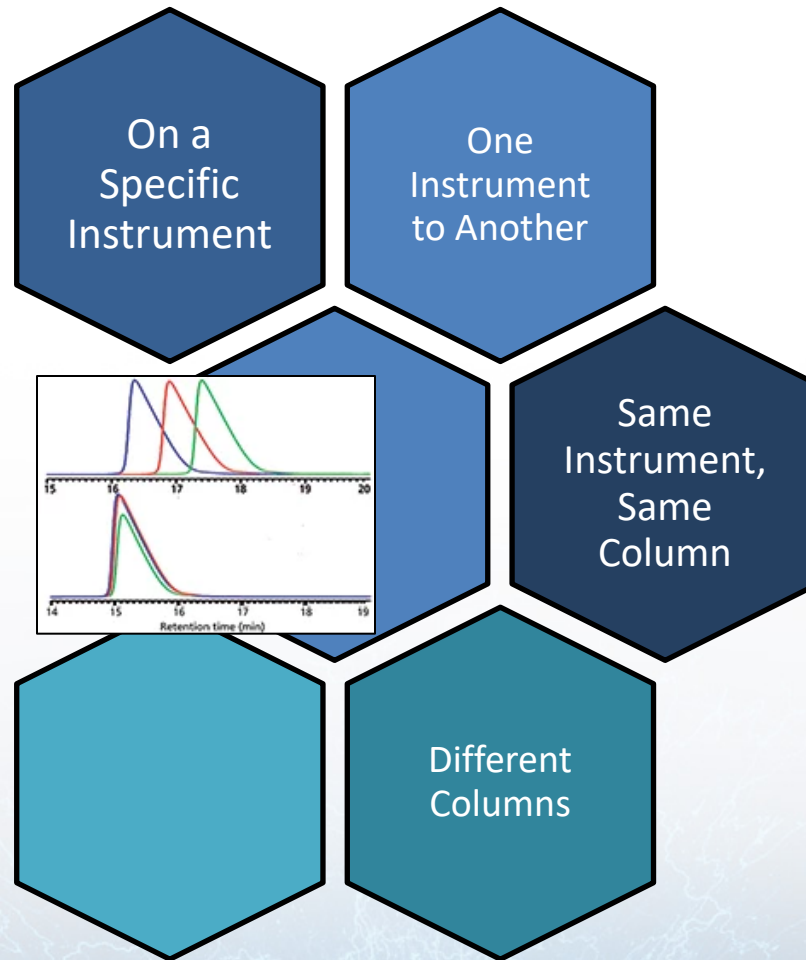
Column conditioning may need to occur, especially for biomolecules greater than 10,000 MW

“conditioning improves column performance by irreversibly binding to sites of specific adsorption, and blocking these sites from future nonideal interactions”

Powell and Timperman

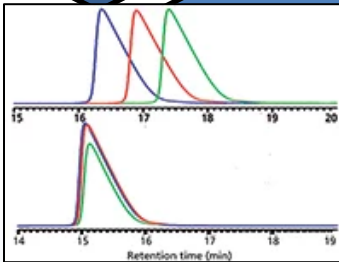


Retention Time Variability

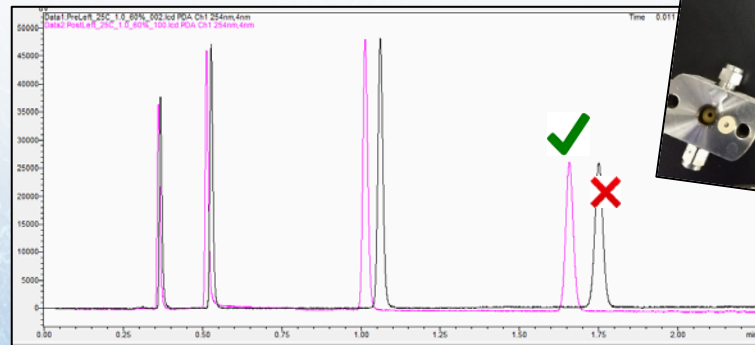
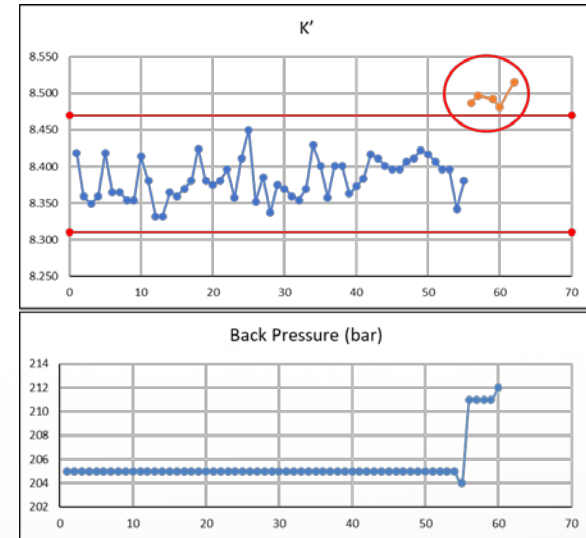


Retention Time Variability

On a
Specific
Instrument

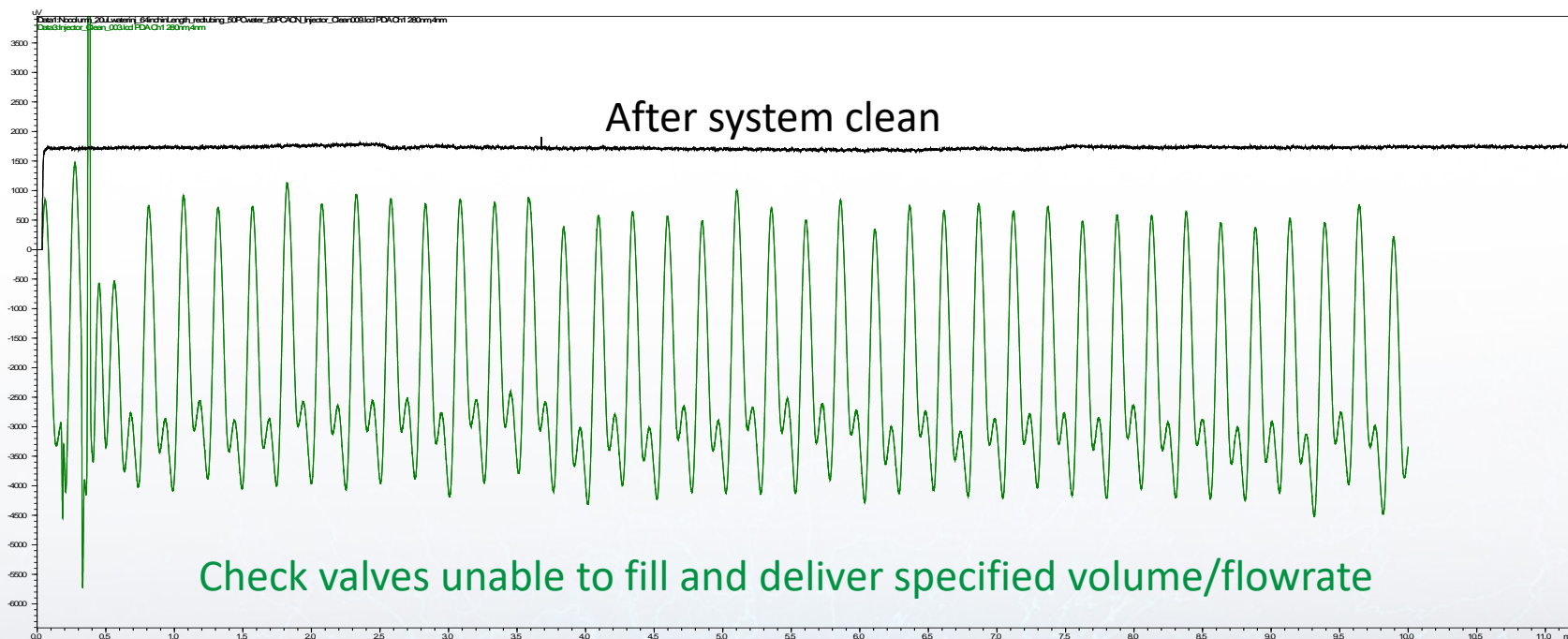


- Check for leaks
- Inspect back pressure (check valve)
- t_0 comparisons (seal/ flow rate)



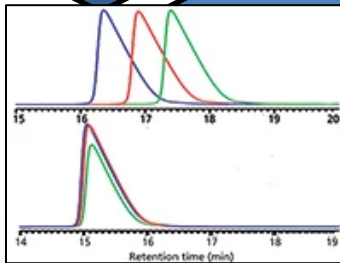
Check Valves: Unstable Back Pressure

- Erratic pressure trace: common problem caused by precipitation/ particulate build-up
- Take apart check valves, sonicate in Water/ MeOH or IPA
- Still no luck? Swap with new check valves



Retention Time Variability

One
Instrument
to Another



Dwell Volume: volume of the gradient mixer plus that of the mobile-phase flow path between the mixer and the column inlet

Dwell Volume Calculator

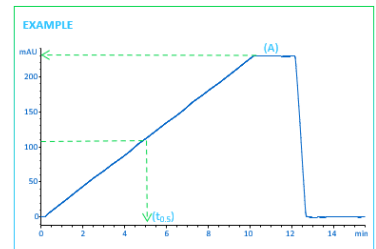
Avantor LC Translator

Method

Column:	ZDV Union	Gradient:	Time	%B
Line A:	Water		0.0	0
Line B:	0.1% acetone in water		10.0	100
Flow Rate:	2.00 mL/min		12.0	100
Temperature:	22 °C		12.5	0
Detector:	UV, 265 nm		15.5	0

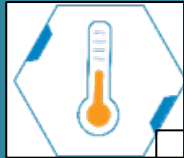
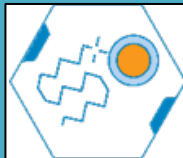
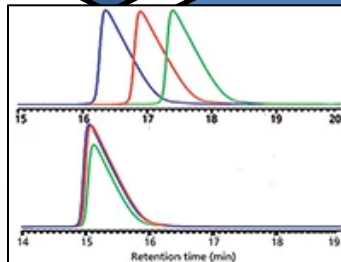
Results

Gradient Time (t_g)		mins
Flow Rate		mL/min
Endpoint of the gradient (A)		mAU
Midpoint of the gradient		mAU
Midpoint of the gradient ($t_{0.5}$)		mins
Dwell time (t_0)		mins
Dwell Volume (V_0)		mL



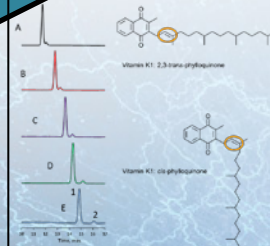
Retention Time Variability

Same
Instrument,
Same
Column



- Mobile phases
- pH/ Buffer Concentration
- Temperature
- Column (gradual change/ sudden?)

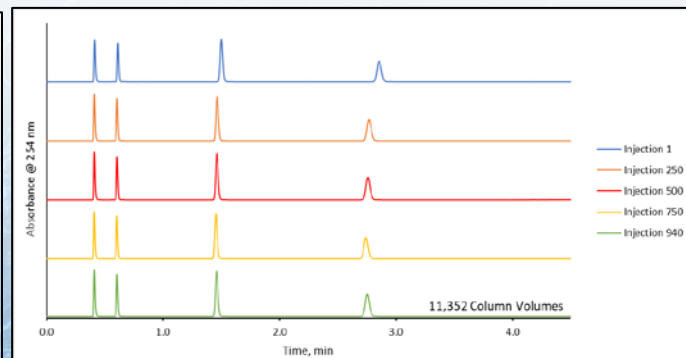
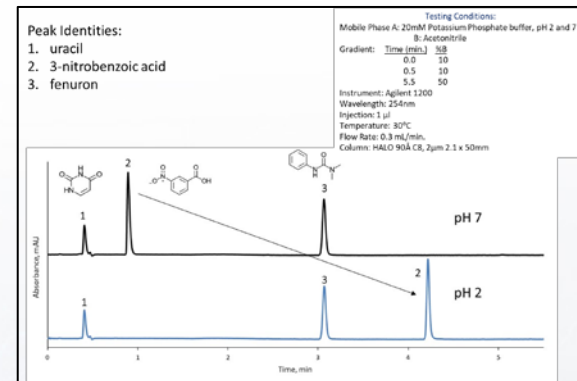
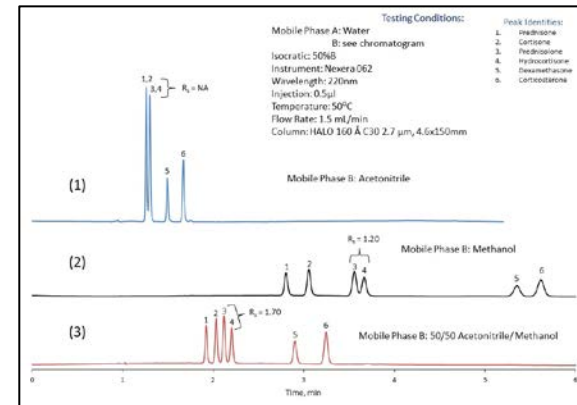
PEAK IDENTITIES:
1. 2,3-trans-phytyloquinone
(Vitamin K1)
2. cis-phytyloquinone (K2)



Column: HALO 100 Å C30, 2.7 µm, 4.6 x 150 mm
Part Number: 92114-730

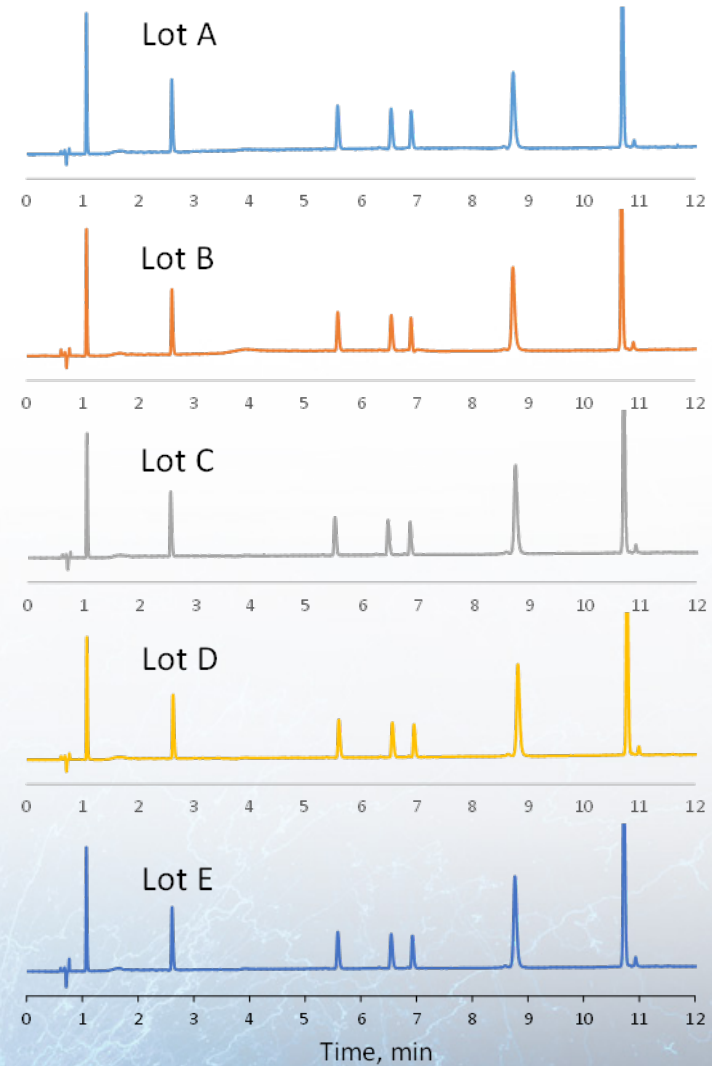
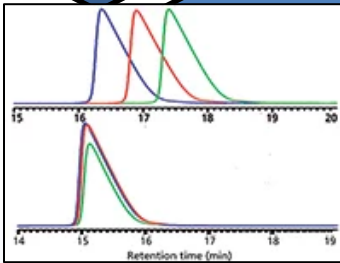
Mobile Phase A: Water
Mobile Phase B: Methanol
Isocratic: 95% B
Flow Rate: 1.5 mL/min
Initial HALO Pressure: 341 bar
Detection: 280 nm, PDA
Injection Volume: 1.0 µL
Sample Solvent: Methanol

	Temperature (°C)	Resolution
A	35	1.53
B	30	1.58
C	25	1.78
D	20	2.20
E	15	3.03



Retention Time Variability

Different
Columns

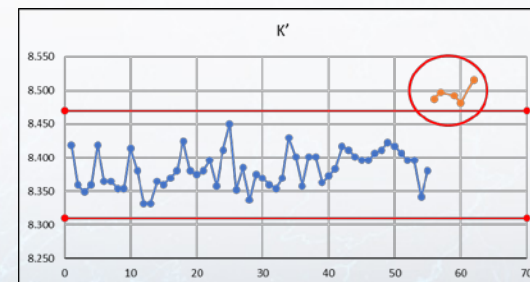


Prevention of Problems

A structured preventive maintenance program should be established



- **System Performance Test**
 - Gradient Performance Test (mixing accuracy)
 - Flow Rate Test
 - Retention Reproducibility Test (verifies flow, mobile phase)
 - Peak-area reproducibility (autosampler check)
- **Periodic Maintenance (repair/ replace parts with limited lifetime)**
 - Preventative Maintenance, help encounter fewer breakdowns
 - Labs that wait for failure usually spend more time and money than those who maintain systems regularly
- **System Suitability Test (test ran before batch of samples)**
 - Column Standard (run multiple injections)
 - Cheap standards
- **Repair and Maintenance Record**
 - The value of historical records in establishing instrument failure patterns and preventive maintenance programs cannot be underestimated
 - Know you system configuration! (tubing, parts, series #)
 - Keep repair log



Problem Isolating Strategies

- Keep safety in mind! (eye protection, gloves, lab coat)
- Change one thing at a time
- Make sure the problem happens more than once
- Have spare parts (and put them back)

Conclusion

Have a Preventative Maintenance plan to reduce instrument issues

- In house/ service engineer

Know your system

- Components/ tubing
- System pressure
- Standard column

Have spare parts

Keep a log book/
maintenance record

- Detailed records can help fix future issues quicker

References and Useful Links

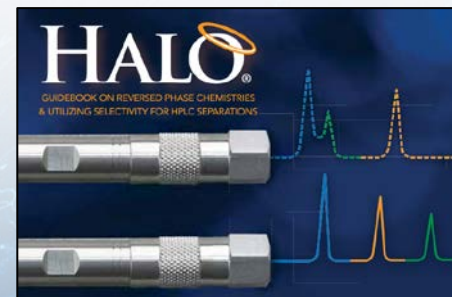
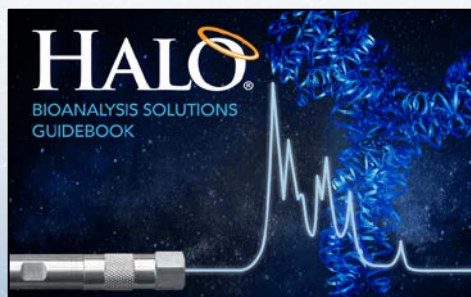
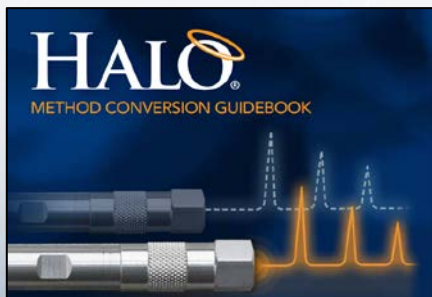
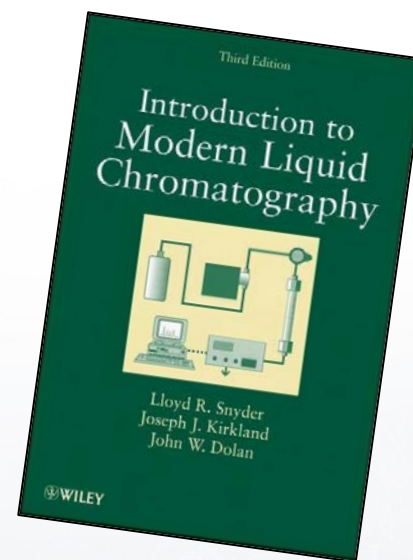
- Introduction to Modern Liquid Chromatography: Snyder, Kirkland, Dolan
- LC-GC: <https://www.chromatographyonline.com/> (John W. Dolan, Dwight R. Stoll)

LC|GC®

- Halocolumns.com
 - <https://halocolumns.com/technical-resources/>



- Quality Brochure
 - [AMT21](#) QB REV 0-Web-2.pdf (halocolumns.com)





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