Eliminate your Application and Chromatography Challenges

LC Application Scientist Session

Information Contributed by USA HPLC Applications Scientist Team
Speaker: Mike Woodman, LC AE Chicago area

This Session will cover

Troubleshooting your Method, Application / Chromatography

- Sample prep considerations
- Troubleshooting by following the LC flow. Mobile Phase and System Hygiene
- Step through Method Setup to highlight parameters that are critical but often overlooked or misunderstood

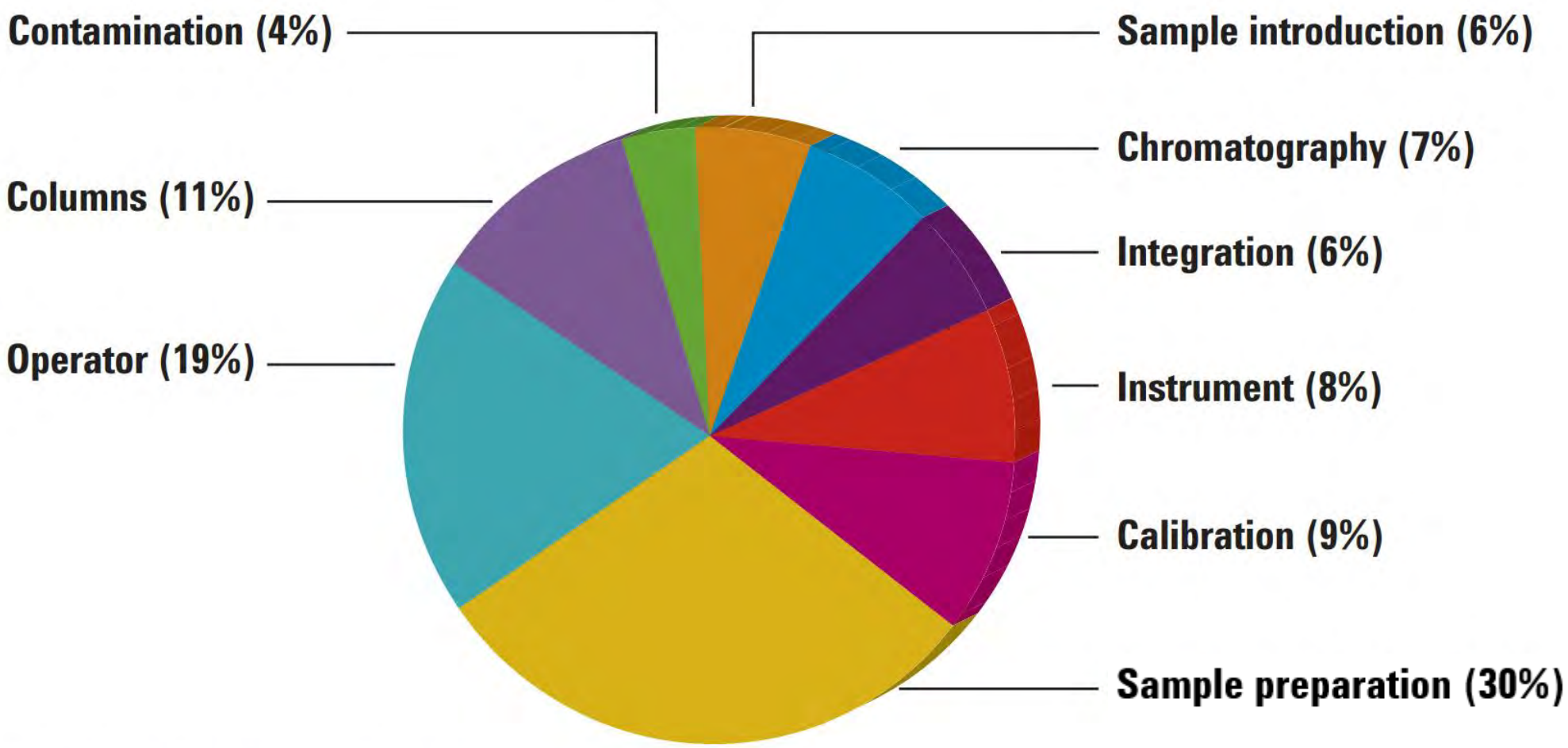
Optimizing your Application / Method Transfer considerations

- What needs to be considered when implementing App notes and transferring method between systems
- Delay volume, column void volumes
- Capillary selection and connections
- Column considerations (dimension and particle size)

Advancing your Application / Chromatography

- How to choose the appropriate LC system for the application that is going to be run.
- Where to find resources or info

Sources of Error Generated During Chromatographic Analysis

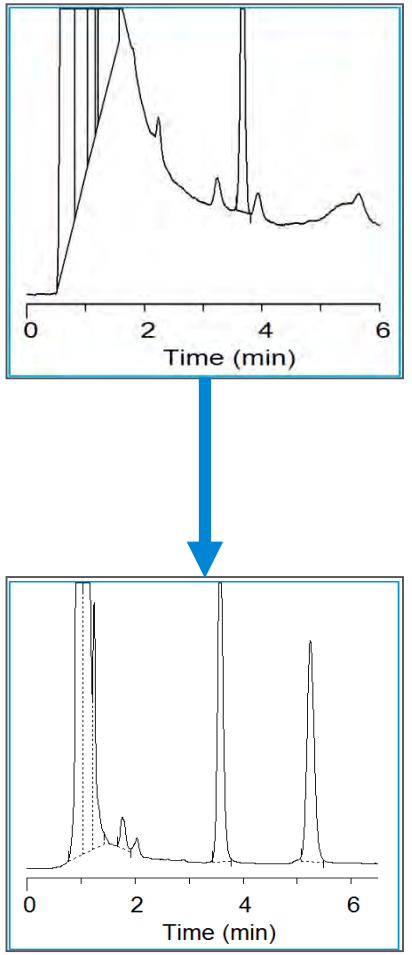


Data taken from Agilent Technologies survey

Sample Preparation Guidelines

Good sample preparation can:

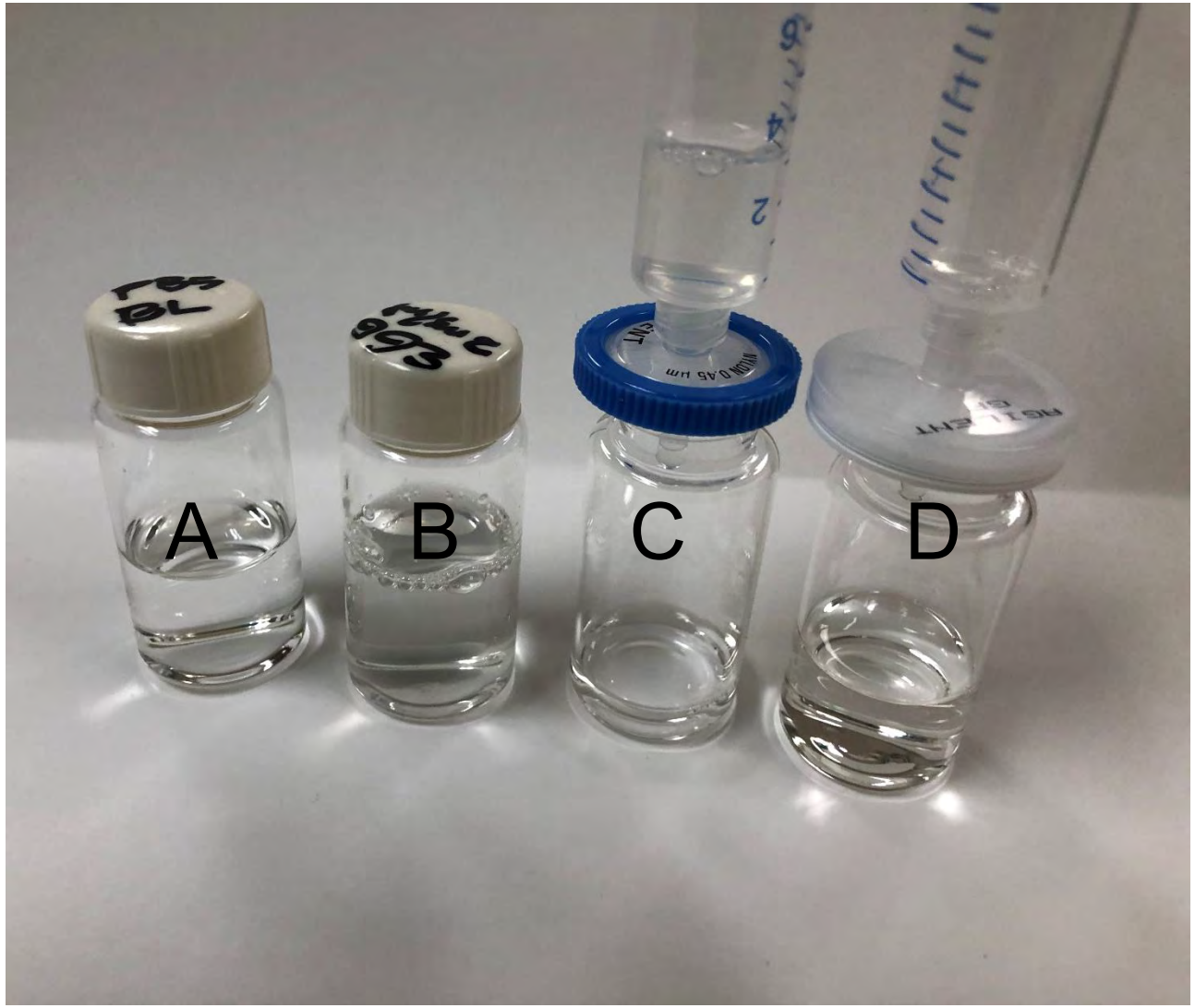
- Selectively remove interferences in the separation
- De-salt a sample (for LC/MS cleanliness)
- Enhance sensitivity via pre-concentration
- Simplify/speed the separation
- Increase column life by minimizing particulates and strongly retained sample contaminants



Options for Sample Preparation

- Filtration (particulate level)
- Ultrafiltration (molecular level)
- Centrifugation
- Drying or freeze-drying (lyophilized)
- Precipitation
- Liquid-liquid extraction
- Solid phase extraction (primitive prep LC)
- Derivatization
- .....and chopping, crushing, dissecting, etc.

Choose Your Sample Filter Wisely



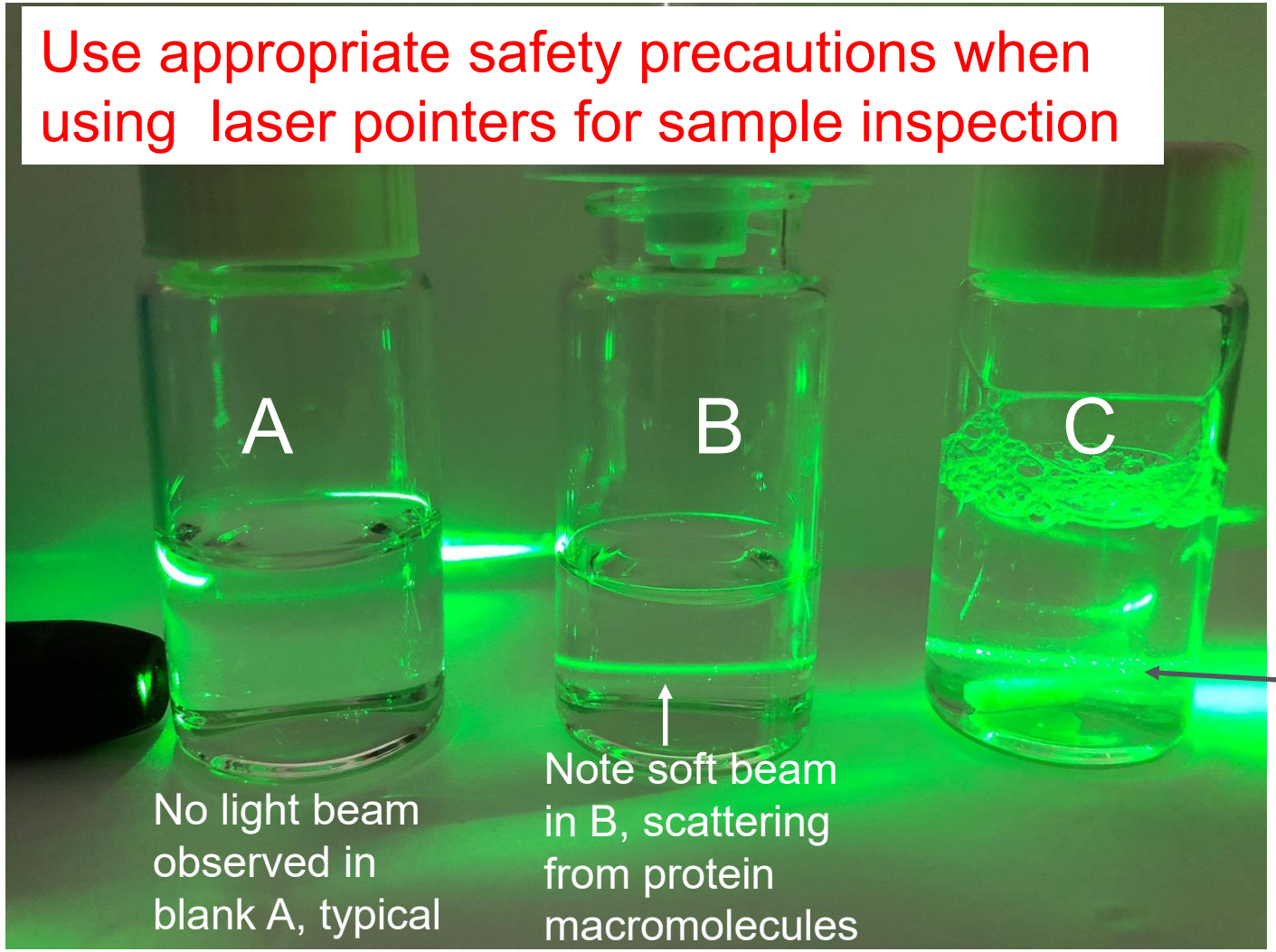
A. PBS diluent buffer

B. 2mg/ml overnight chilled dissolution of egg albumin in PBS

C. Protein sample dead-end filtration using 0.45u flat nylon membrane
-- plugged after ~2ml

D. 2mg/ml egg albumin after Captiva GF (glass over regenerated cellulose 0.45um) filtration with 100% solution recovery

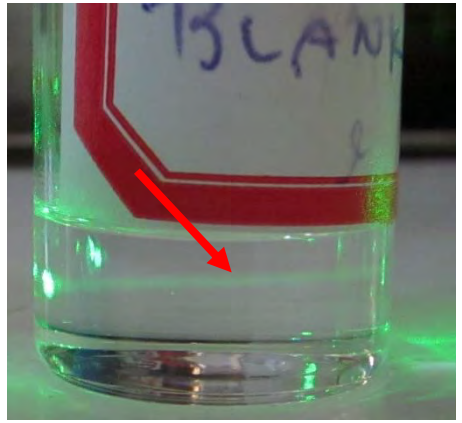
Think Through Sample Prep Options – Laser !



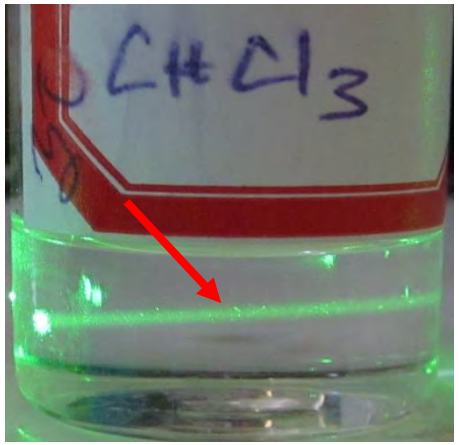
A. PBS diluent buffer
B. 2mg/ml egg albumin after GF (glass over regen. Cell.) filtration
C. 2mg/ml overnight chilled dissolution

Original solution, C, contains insoluble debris and agglomerated protein (seen as bright “sparkles”)

Three Polymer Examples w/ Chloroform – Laser Pointer Inspection



Most solvents exhibit minor light scattering. Sparkles in the beam are dust/particulate (none seen here) when seen in blank vials

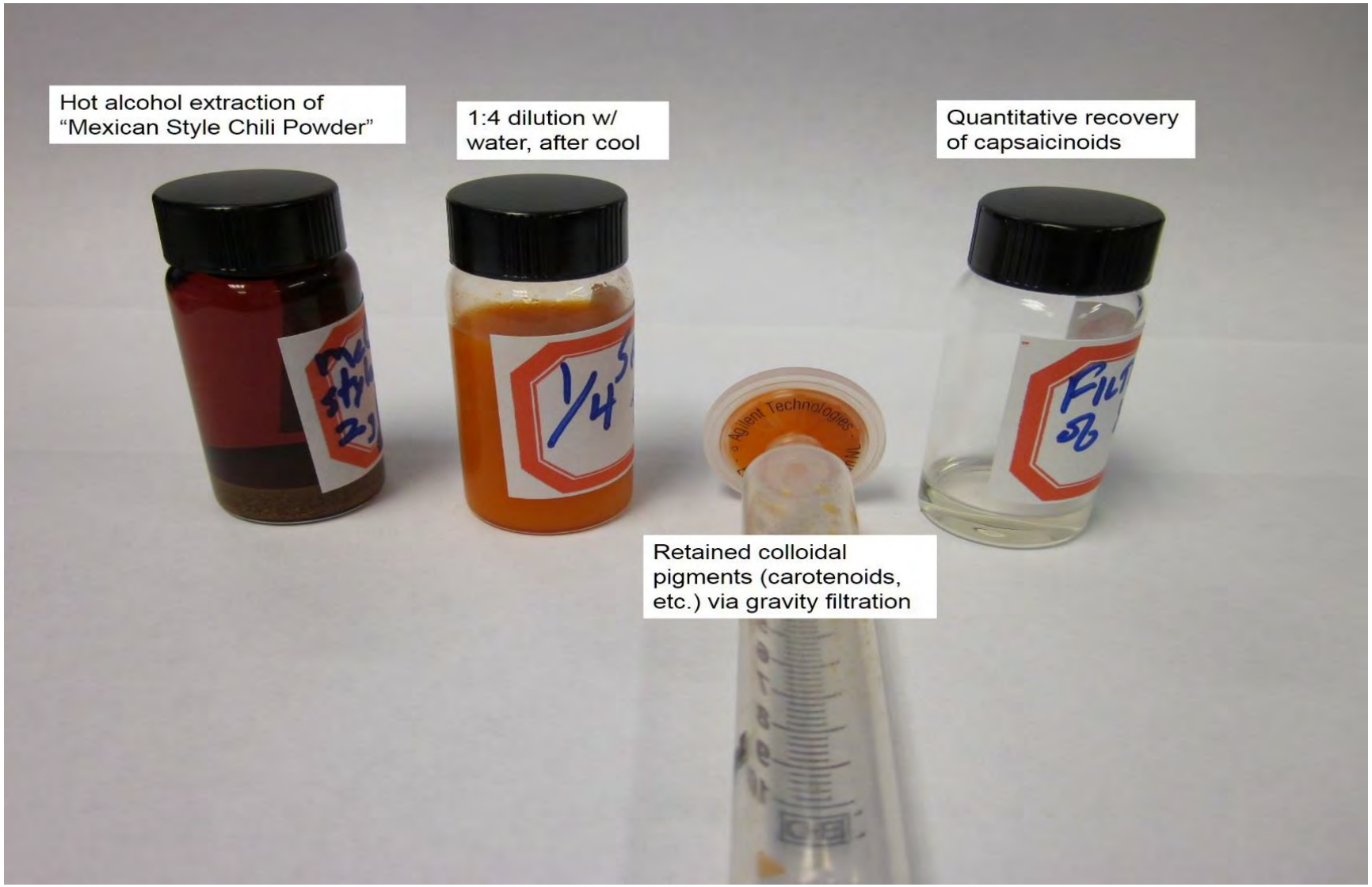


Polymer solution with minor amounts of particulate and/or gel present, unfiltered
Beam intensity increases with increasing molecular weight, too

A good polymer solution that has been gravity filtered through a 0.45 micron 25mm PTFE syringe filter

A bad polymer solution with lots of gel and thus little dissolved polymer, unfiltered

Removing Gel by Gravity Filtration, Here the Analysis of Heat Compounds in Chili Products



Alternate Approach for High Lipid Matrix

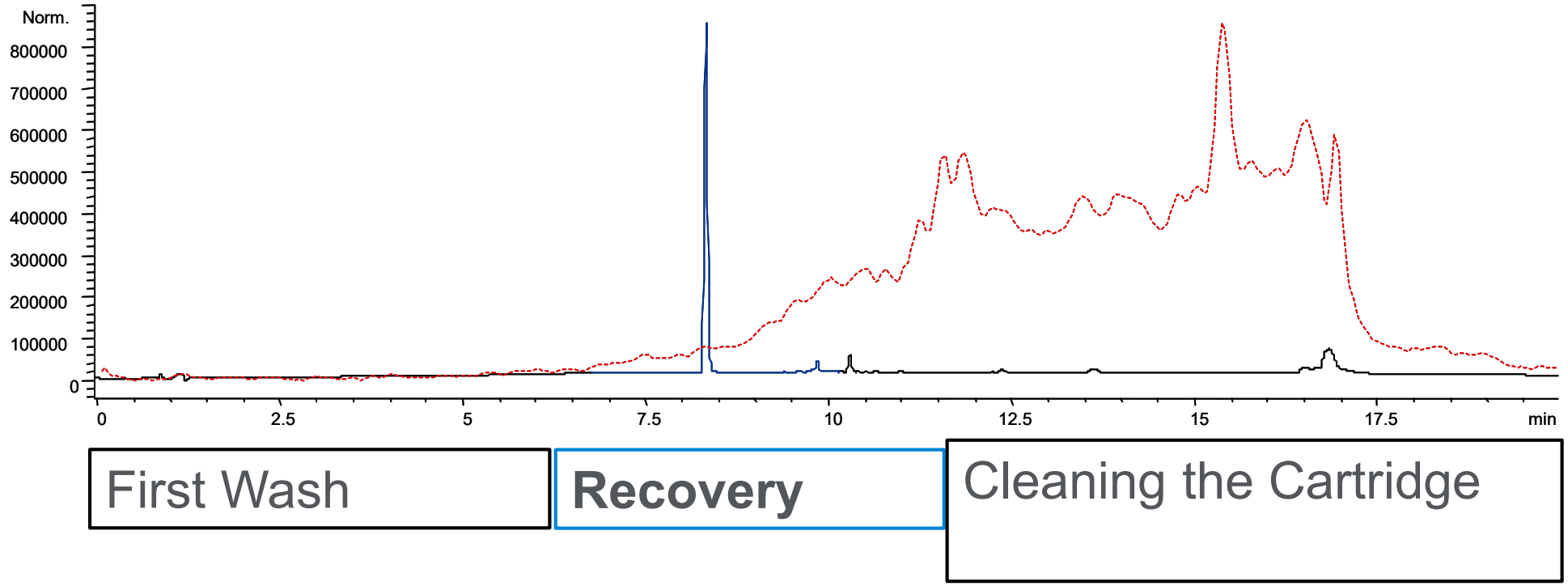
Methanol extract of chili product is applied directly onto a highly retentive SPE cartridge

BondElut ENV PS-DVB was in use here and proved suitable for neutral lipids from methanol extracts

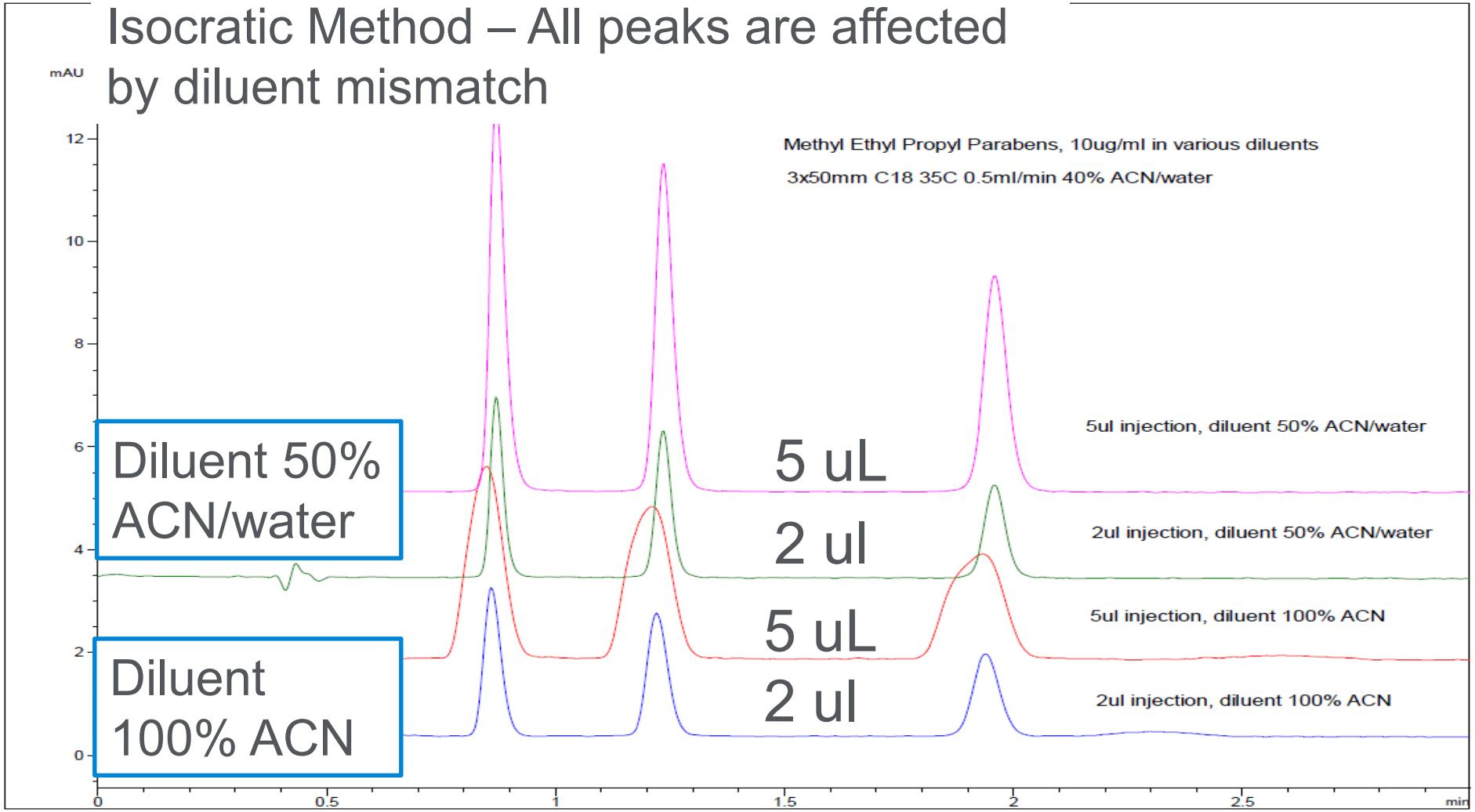
Agilent EMR (Enhanced Matrix Removal) SPE products directly target removal of more polar lipids (i.e. phospholipid) in biological samples

Example Sample Preparation – Solid Phase Extraction (SPE) Mode

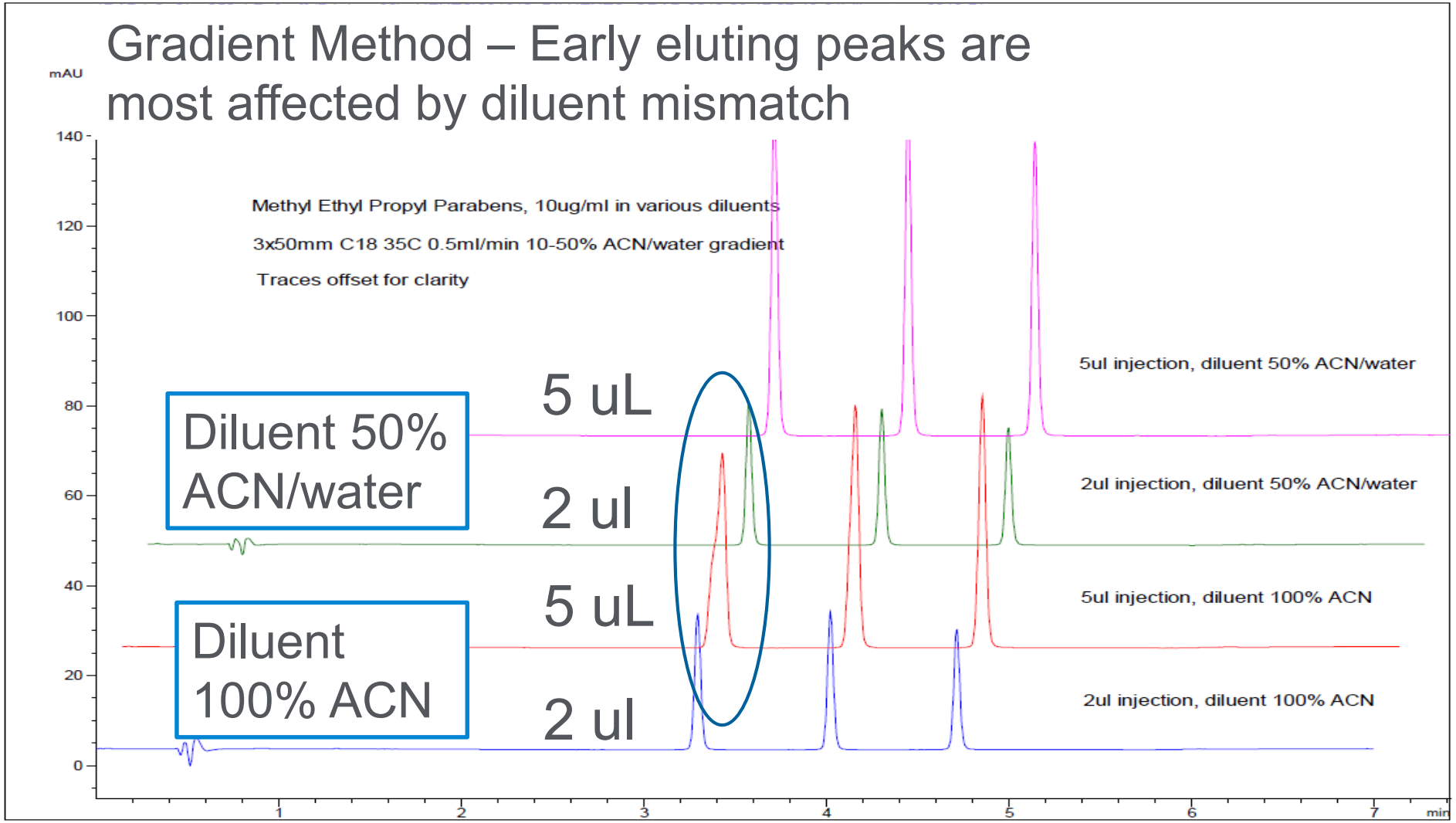
SPE (primitive prep LC):
pre-fractionates the sample to isolate the analyte region for analysis, others go to waste



Strong Diluents can Disrupt Equilibration – Isocratic Method

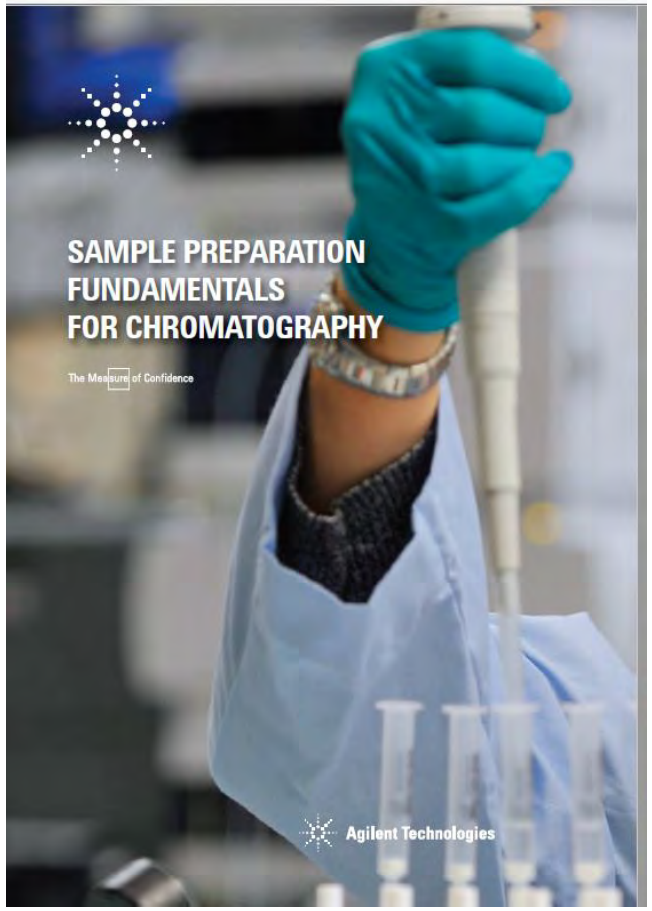


Strong Diluents can Disrupt Equilibration – Gradient Analysis



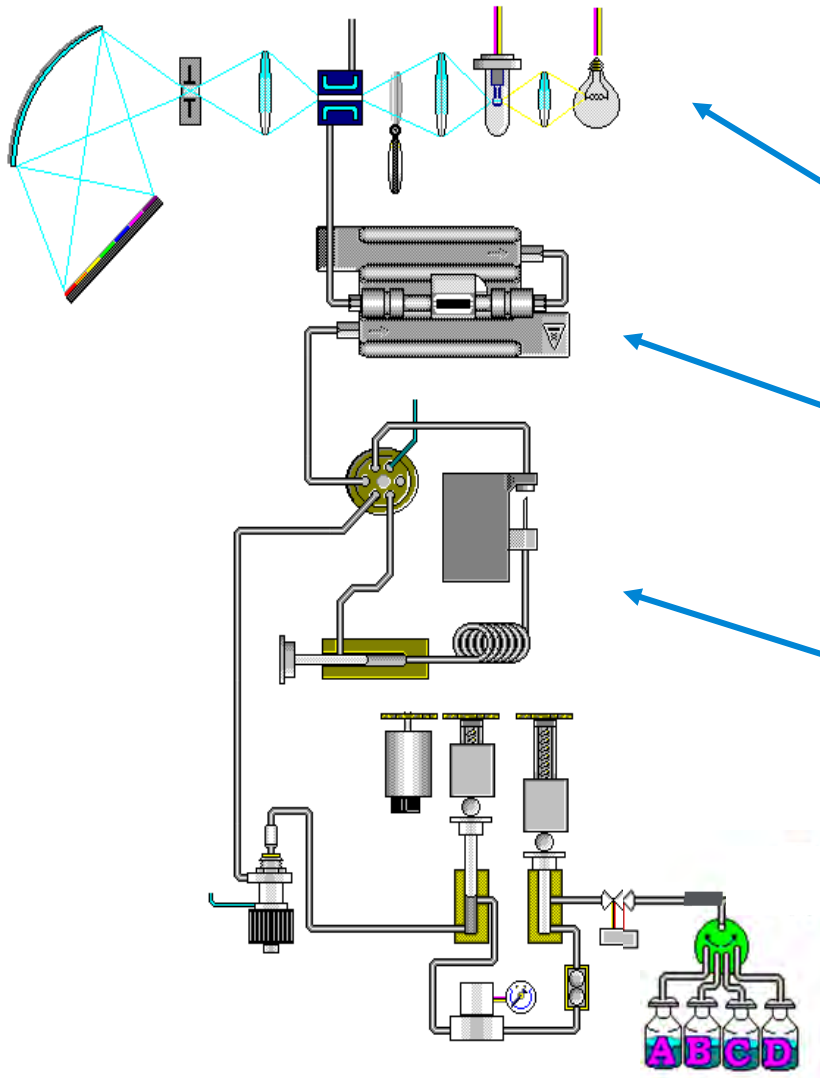
Sample Preparation Summary

- **Do only what is required**
  - Remove particulates, insoluble components as needed
  - Concentrate analytes for sensitivity enhancement
- **Losses and extra work are inherent in sample prep**
  - Use controls to track recovery
  - Simplify procedures for time, material cost
  - Consider automation, where possible
- **Take care with diluent mismatch**
  - Organic strength
  - Solution pH and molarity



https://www.agilent.com/cs/library/primers/public/5991-3326EN_SPHB.pdf

Understand Your LC System and Follow the Flow Path



Mobile Phase

Detector

Column Compartment

Autosampler

Pump

Mobile Phases Hygiene – Starting point of your LC flow path

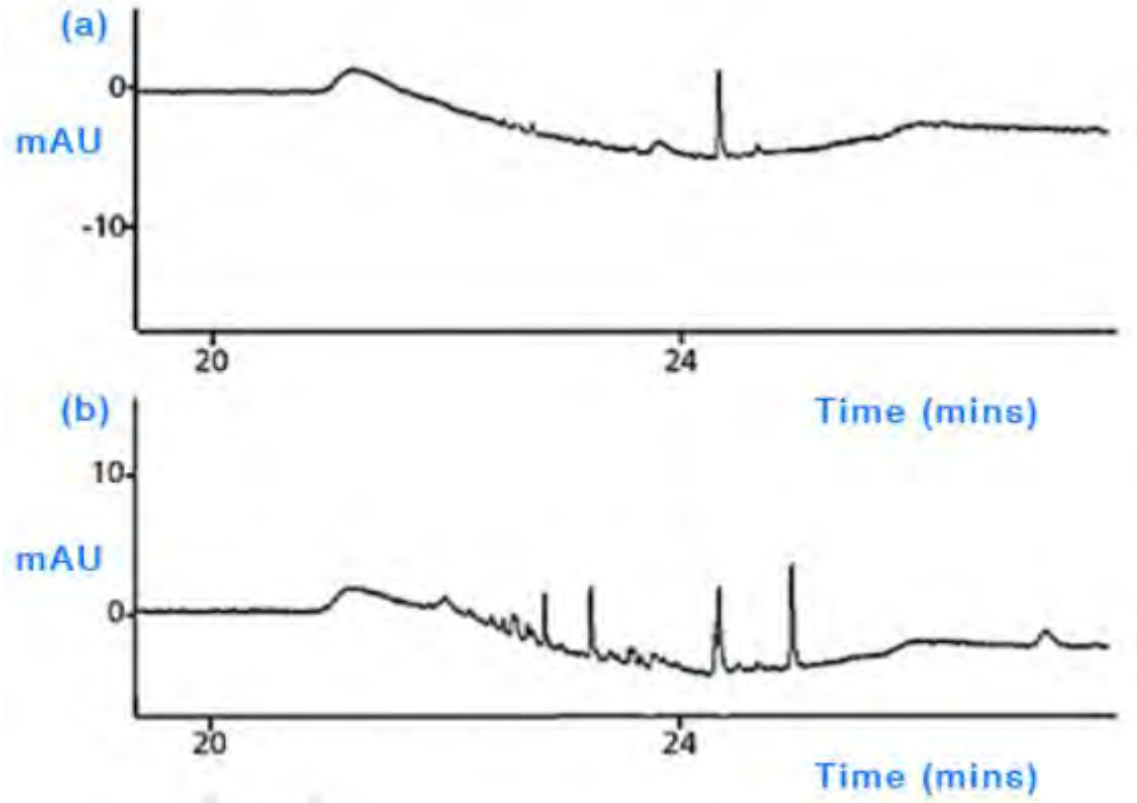
Contaminated Mobile Phases can cause

- Lower sensitivity
- Rising/drifting baselines
- Higher noise
- Ghost peaks on the chromatogram with gradient separations.

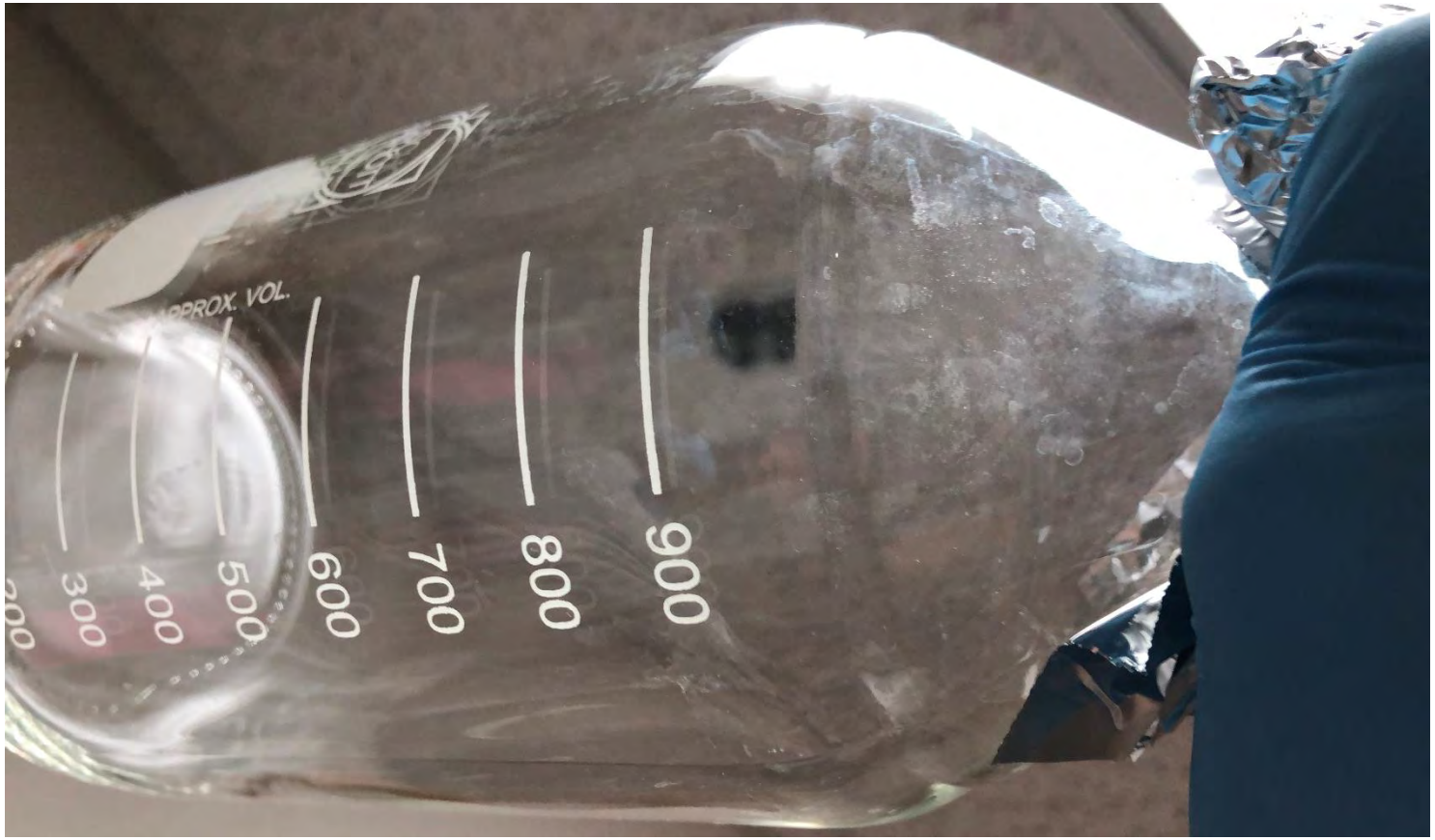
Often the issue is confused with Autosampler carryover.

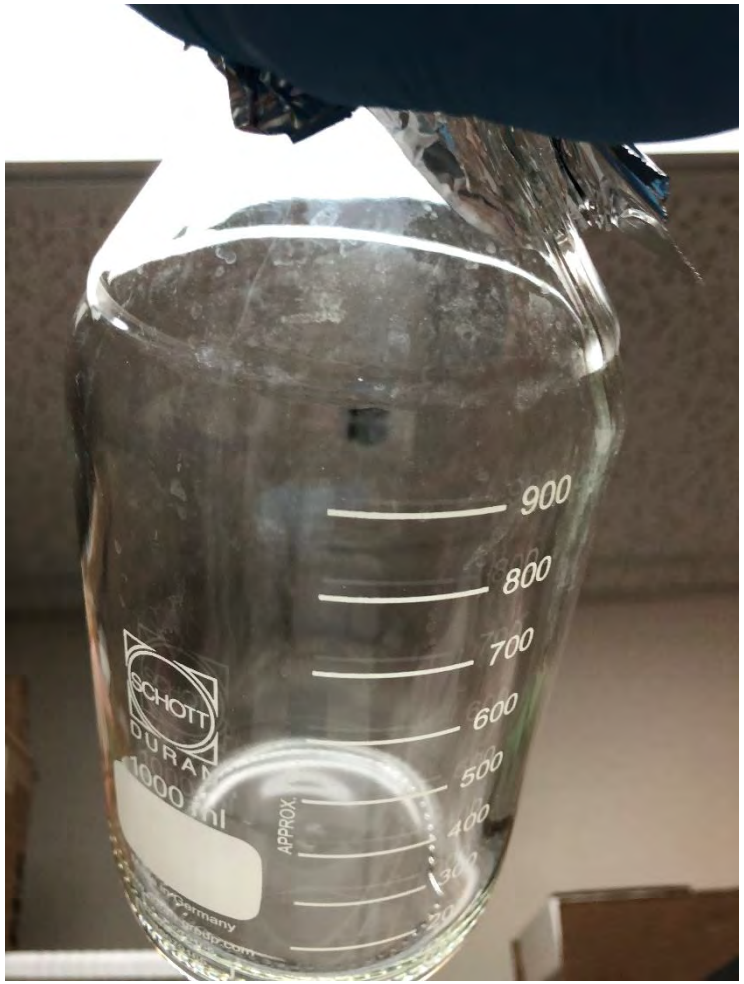
It can be identified by repeating the gradient run without sample injection - Same ghost peaks will be observed reproducibly.

Always run multiple blanks before standards or samples to distinguish gradient artifacts from possible carryover.



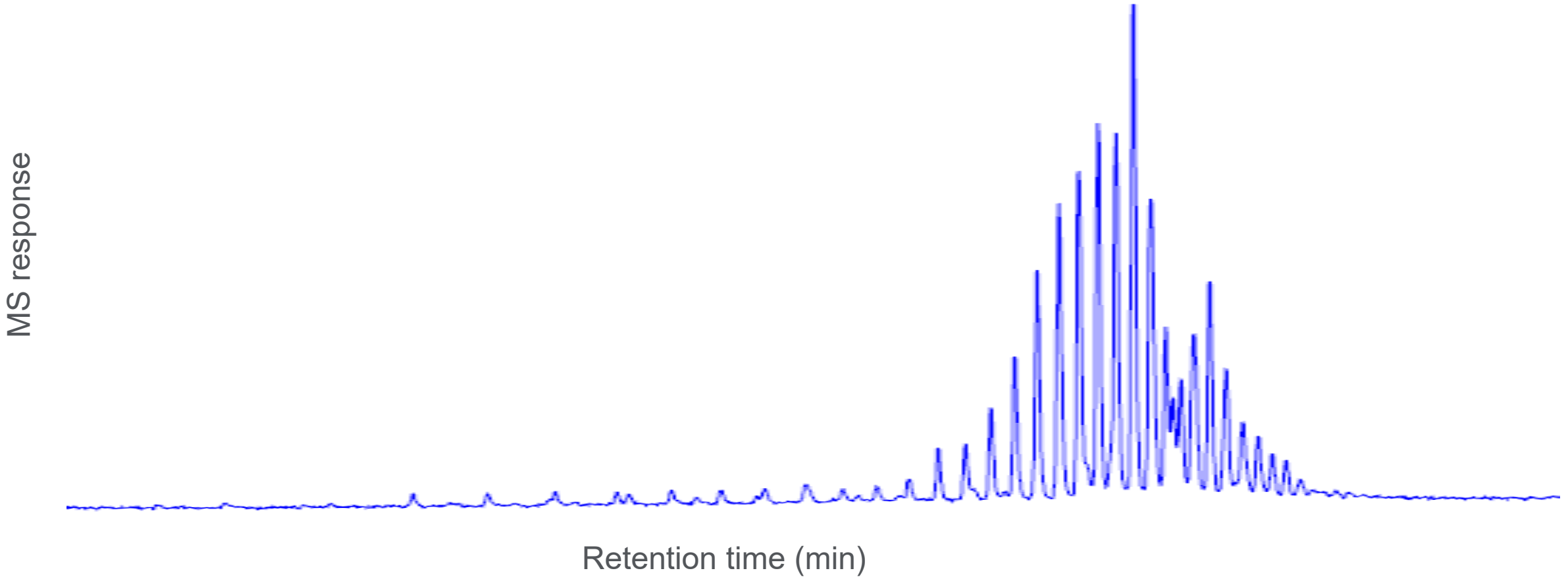
Hygiene – Do you want the hands that washed these bottles to touch your instrument? I don't think so…





Is it Solvent or System Contamination? Either way, it's trouble

PEG Chromatogram, system blank injection, water/ACN gradient on a C18 column



Mobile Phase Hygiene #1: Glassware Cleaning

Improper cleaning of solvent bottles can cause contamination of mobile phases and resulting in gradient artifacts!

- Wash solvent bottles with hot water, deionized water, and organic solvent (IPA or acetonitrile).
- Leave glassware inverted on paper towels on bench or on clean pegboard dowels to dry.
- Avoid using detergents! If it is necessary to use detergents to get glassware clean, re-wash with plenty of hot water and cold water so that all detergent residues are removed. Follow with deionized water and organic (IPA or acetonitrile) rinses.
- Store glassware inverted on shelves or in drawers, or cover openings

Identification Silicone ring (8/pk with 4 different colors) 9301-6529

5043-1192

This allows you to use the 4 liter glass bottles

Mobile Phase Hygiene #2: Solvent Purity and Buffer Preparation

- Use HPLC grade Organic mobile phases
- Use HPLC grade water or Milli Q DI water
- Use HPLC grade reagents including salts, ion pair reagents, and base and acid modifiers
- Always rinse pH electrode thoroughly when measuring/adjusting pH of mobile phase
- Prepare fresh buffers to avoid contaminants from the growth of bacteria or algae
- Filter your mobile phase buffer with 0.45um filter before use
- The solvent filters installed at the end of solvent lines should be replaced periodically.

…or use Milli-Cup -- from Millipore

Mobile Phase Preparation for Gradient Methods: Solvent Miscibility Test

- **Gradient methods are widely used in HPLC analyses**
- **Buffer solutions are also commonly used for pH adjustment to achieve desired separation.**

- **It is important to test the solubility of the mixture of the two solvents for the highest percentage of organic solvent necessary for elution of the analytes of interest.**

For testing, always add the organic solvent to the buffer with stirring, and not vice versa

Immiscible solvent flow can cause high system pressure and triggering system shutdown during acquisition. Small particles in mobile phases can permanently block capillaries in degasser.

Mobile Phase Preparation for Gradient Methods – Solvent Miscibility

In general, methanol is more miscible than acetonitrile with phosphate and other inorganic buffer. But methanol gives higher UV cutoff and higher back pressure.

Acetonitrile and phosphate buffers are mutually soluble up to approximately 80% with 20 mM phosphate. This percentage drops down to about 70% at 30 mM phosphate.

100% ACN

90%ACN+10% buffer (10mM phosphate)

Important Buffer Systems

Buffer Selection

Buffer	pK_a	pH Range	UV Cutoff (A > 0.5)
Trifluoroacetic acid	<<2 (0.5)	1.5-2.5	210 nm (0.1%)
KH_2PO_4/phosphoric acid	2.12	1.1-3.1	<200 nm (0.1%)
tri-K-Citrate/hydrochloric acid 1	3.06	2.1-4.1	230 nm (10 mM)
Potassium formate/formic acid	3.8	2.8-4.8	210 nm (10 mM)
tri-K-Citrate /hydrochloric acid 2	4.7	3.7-5.7	230 nm (10 mM)
Potassium acetate/acetic acid	4.8	3.8-5.8	210 nm (10 mM)
tri-K-Citrate /hydrochloric acid 3	5.4	4.4-6.4	230 nm (10 mM)
Ammonium formate	3.8 9.2	2.8-4.8 8.2-10.2	(50 mM)
Bis-tris propane•HCl/Bis-tris propane	6.8	5.8-7.8	215 nm (10 mM)
Ammonium acetate	4.8 9.2	3.8-5.8 8.2-10.2	(50 mM)
KH_2PO_4/K_2HPO_4	7.21	6.2-8.2	<200 nm (0.1%)
Tris•HCl/Tris	8.3	7.3-9.3	205 nm (10 mM)
Bis-tris propane•HCl/Bis-tris propane	9.0	8.0-10.0	225 nm (10 mM)
Ammonium hydroxide/ammonia	9.2	8.2-10.2	200 nm (10 mM)
Borate ($H_3BO_3/Na_2B_4O_7$•10 H_2O)	9.24	8.2-10.2	
Glycine•HCl/glycine	9.8	8.8-10.8	
1-methylpiperidine•HCl/1-methylpiperidine	10.1	9.1-11.1	215 nm (10 mM)
Diethylamine•HCl/diethylamine	10.5	9.5-11.5	
Triethylamine•HCl/triethylamine	11.0	10.0-12.0	<200 nm (10 mM)
Pyrollidine•HCl/pyrollidine	11.3	10.3-12.3	

Adapted from Practical HPLC Method Development, 2nd Edition, Snyder, L.R., Kirkland, J.J. and Glajch, J.L., page 299.

Mobile Phases – Instrument Compatibility

Some Mobile Phases are not compatible with your HPLC system.

- Extreme pH - corrosive to instrument and flow cell.
- Normally pH 2-11 for regular Agilent HPLCs
- Bio-inert HPLC can tolerate pH 1-13.
- Handling Normal phase solvents – specific system modifications are required.

- Some fluorinated solvents (such as Freon,Fluorinert, or Vertrel) – dissolving degasser and waste tubing causing leaks.

  - Bypass degasser and make sure compatible tubing including solvent lines are used if these solvents are to be used.

  - Limited life time for Hexafluoroisopropanol (HFIP). To ensure the longest possible life with HFIP, it is best to dedicate a particular chamber to this solvent.

HPLC Pump – First module on LC flow path

Deliver mobile phases with accurate flow successfully

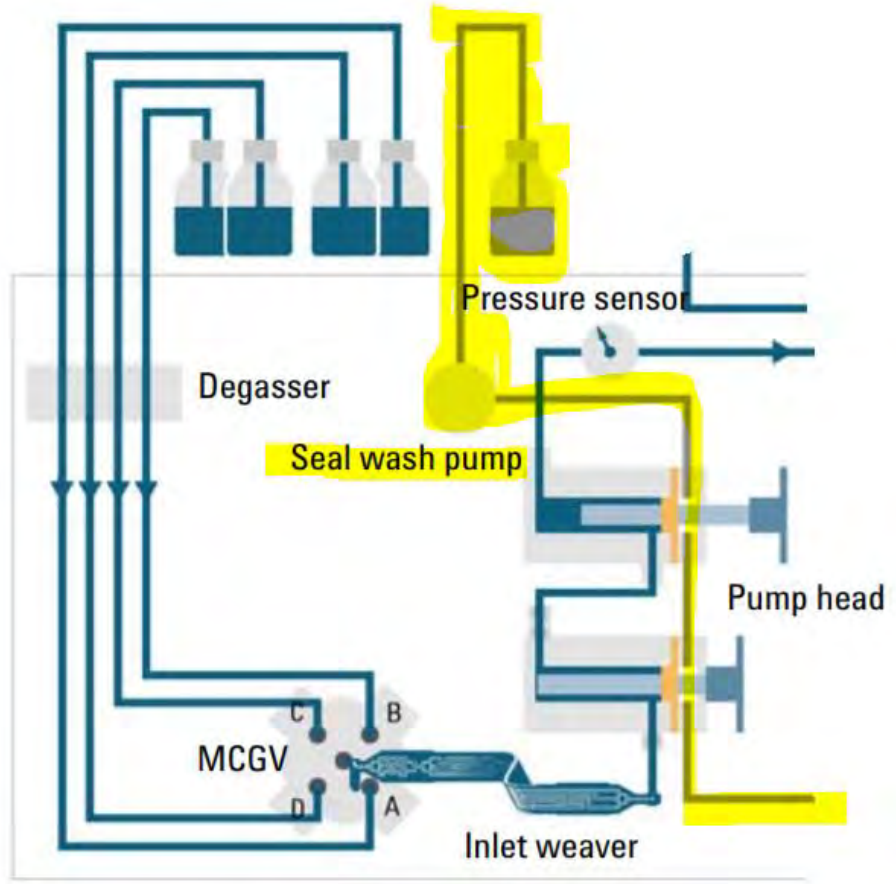
Knowing the limits and features of your HPLC pump

- flow range, pressure limits, pH limits

Pump seal wash option – required for running buffer/salt mobile phases

Using pump seal wash to prevent buffer salt build up on pump seals.

- Seal wash solvent – Water with 10% IPA is recommended for running salt buffer mobile phases.
- Seal wash should be set to run periodically regardless of instrument is acquiring data or not.
- Seal wash is set to run automatically for 1290 pumps
- Make sure seal wash solvent bottle is not empty



HPLC Pump – Shutdown State and Instrument Flushing

Shutdown State

Next day use—using same buffers

- Pump mobile phase very slowly (for example, 0.05 – 0.1mL/min) overnight

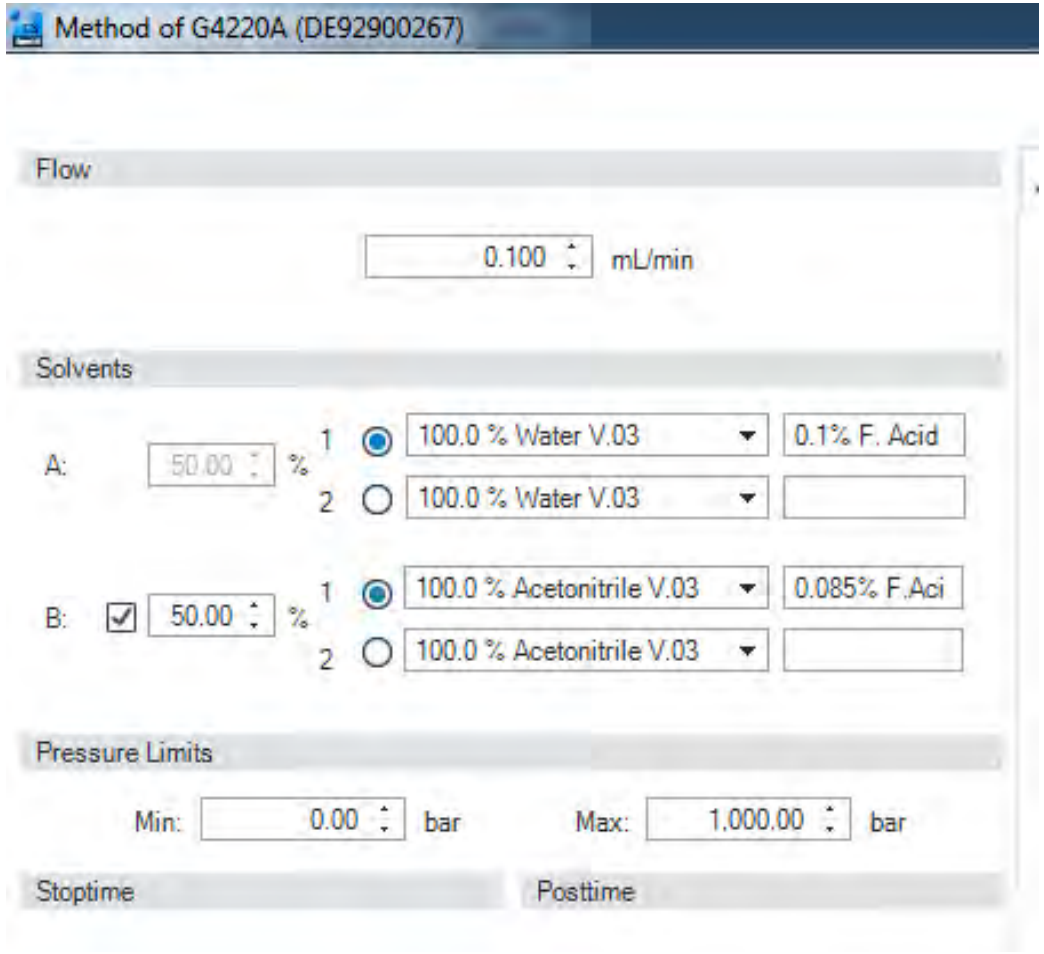
When flushing column or for longer term column storage

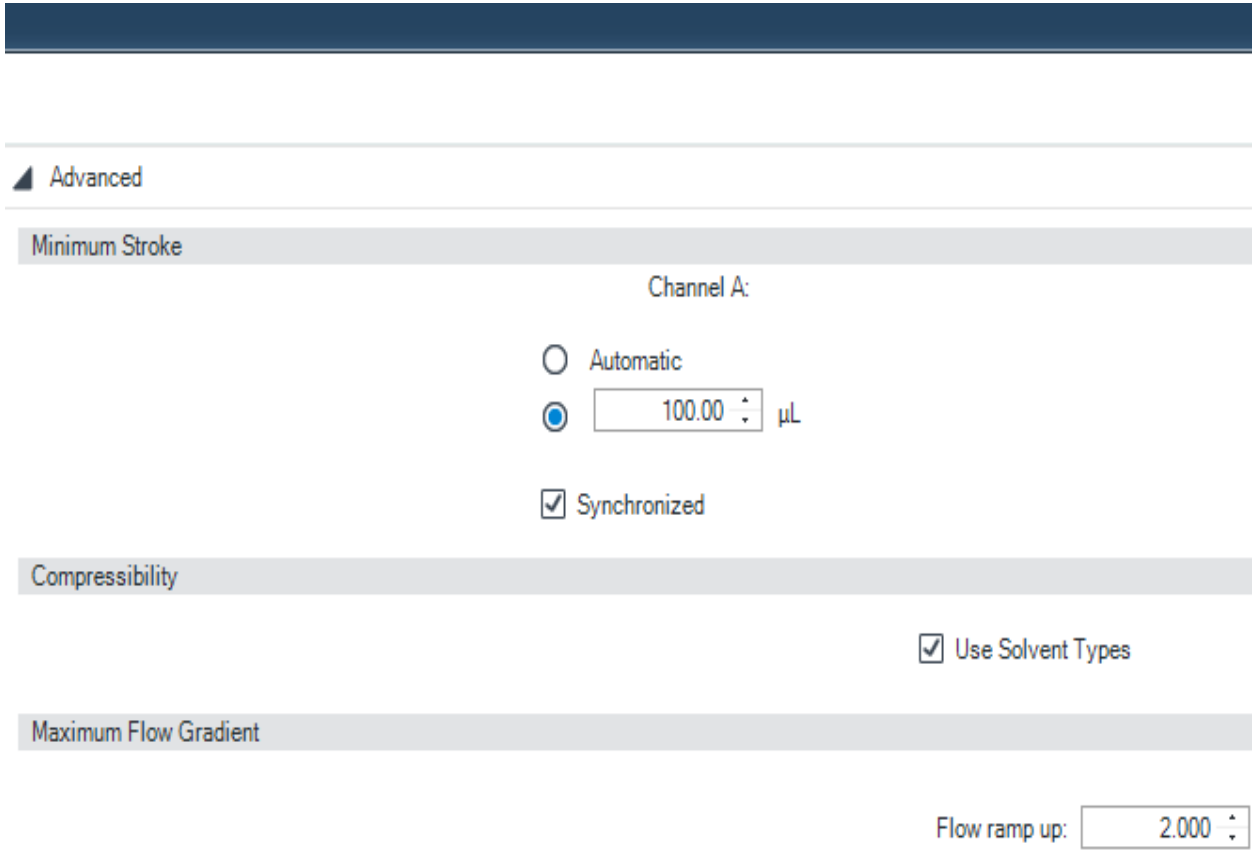
- Reverse Phase Column - Flush with 20/80 organic/water depending method/column used, then 80/20 organic/water or 100% organic.
- SEC/IEX columns– Flush with recommended Storage Buffer

Instrument flushing

- Replace column with capillary tubing. Leave disconnected from detector.
- Flush pumps with water, then connect capillary tubing to detector.
- Inject water 2-3 times at maximum injection volume setting.
- Flush all pumps with 100% organic for long term storage.

Extend Pump Seal Life, Ensure System Readiness with Low Flow Standby Methods 100 µL stroke

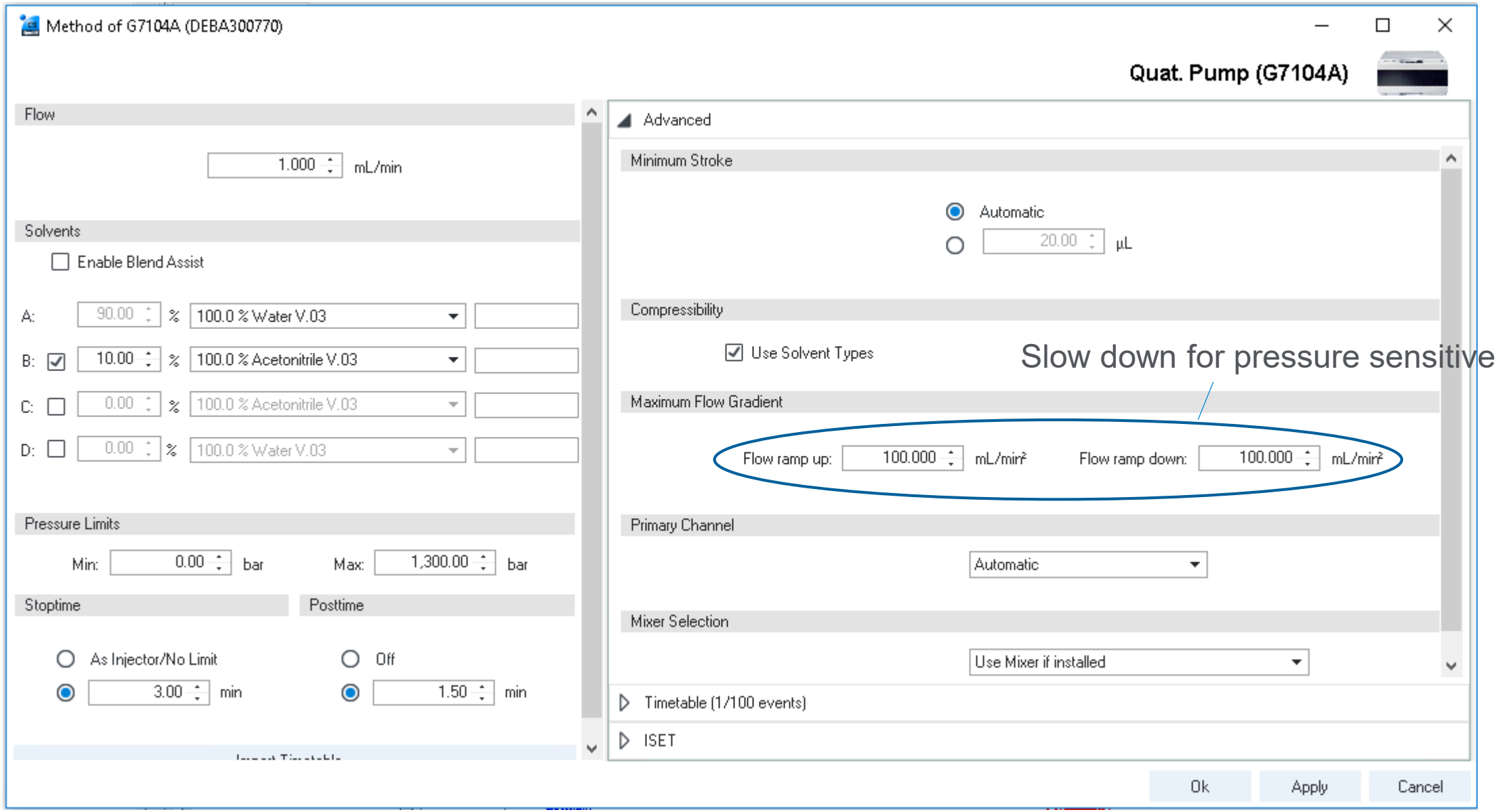




Method Setup

- Should we always use default values?
- When and how to optimize?

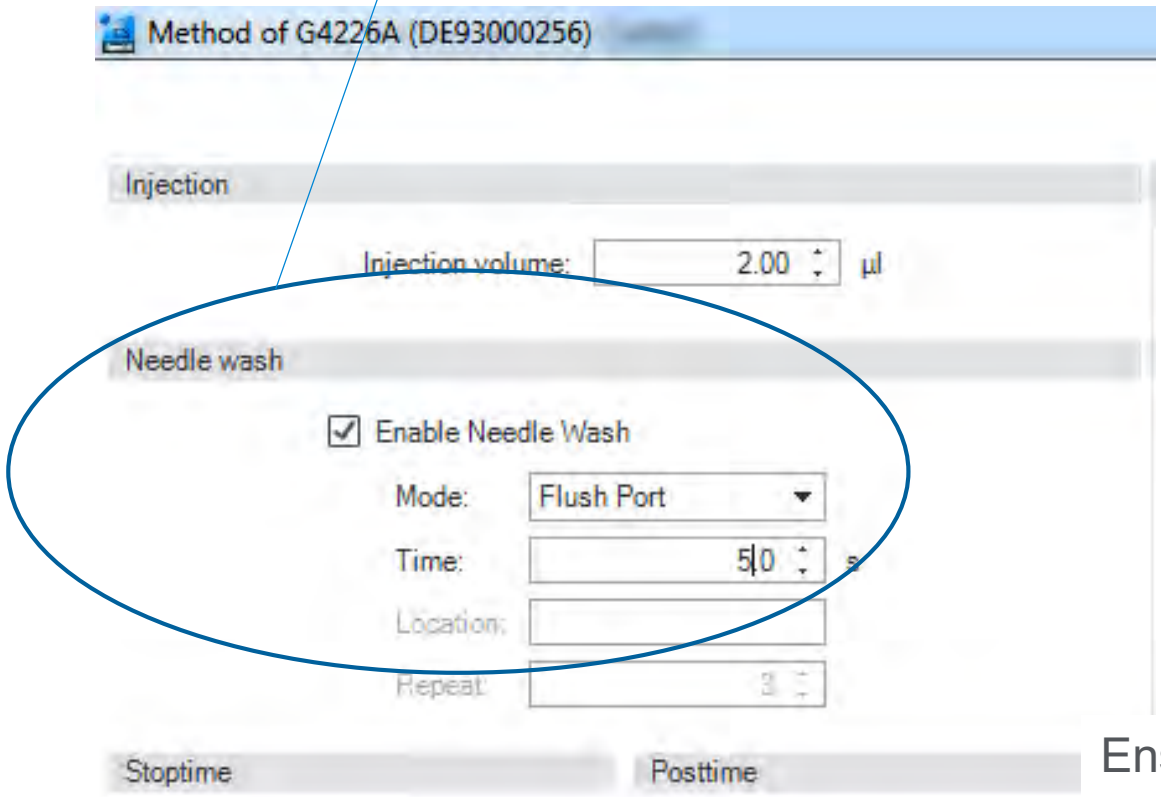
Pump setting



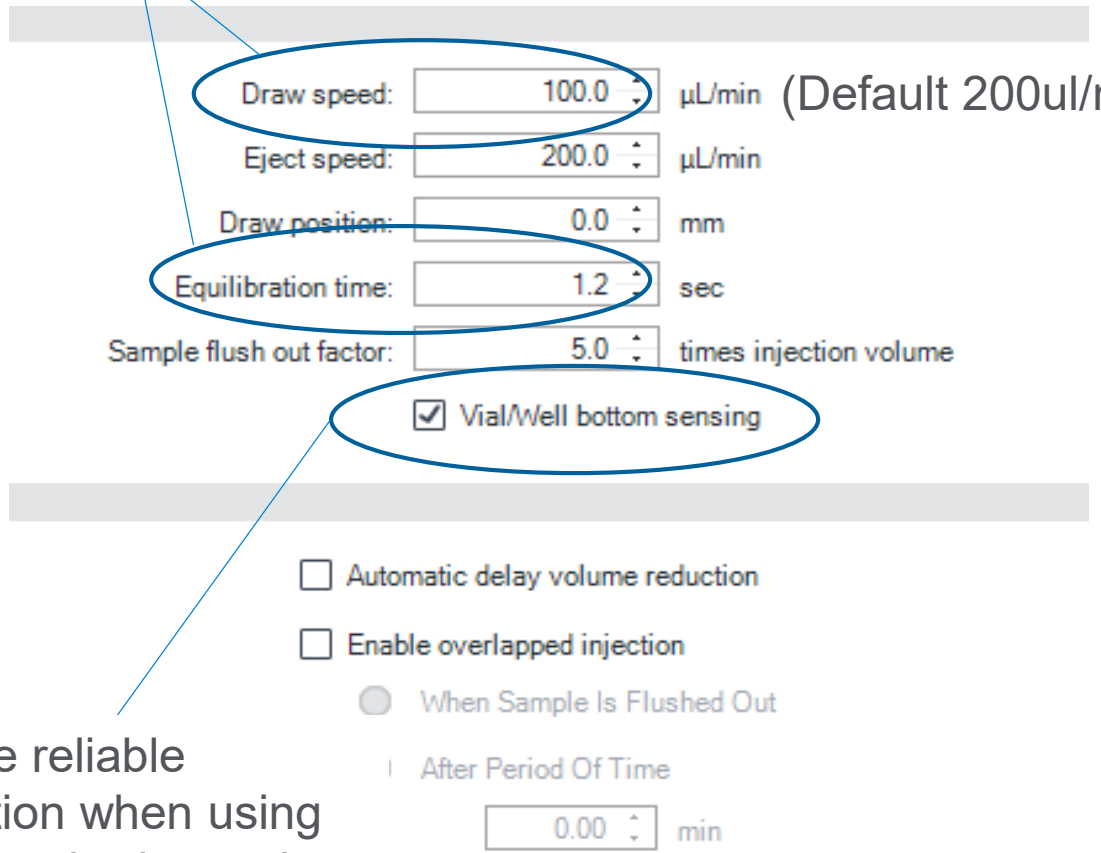
Slow down for pressure sensitive columns

Optimize Autosampler Performance

Reduce Sample Carryover



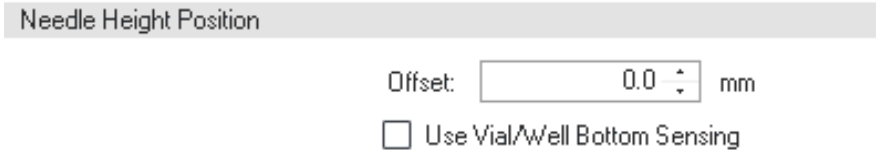
Improved Accuracy for Chilled Samples

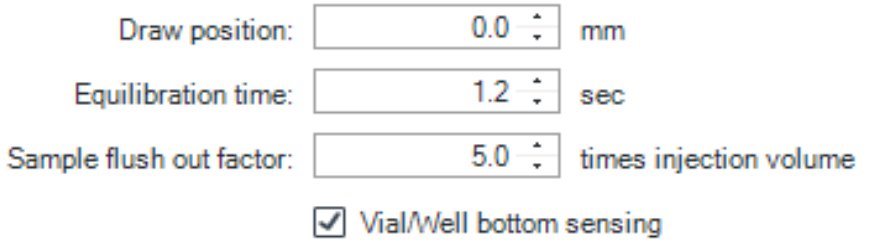


(Default 200ul/min)

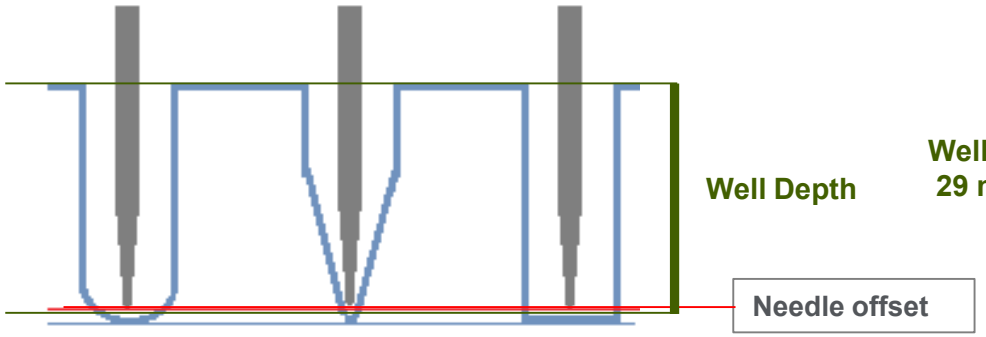
Ensure reliable operation when using non-standard sample containers

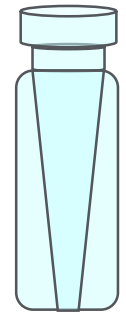
Optimize Autosampler Performance — Draw Position/Bottom Sensing





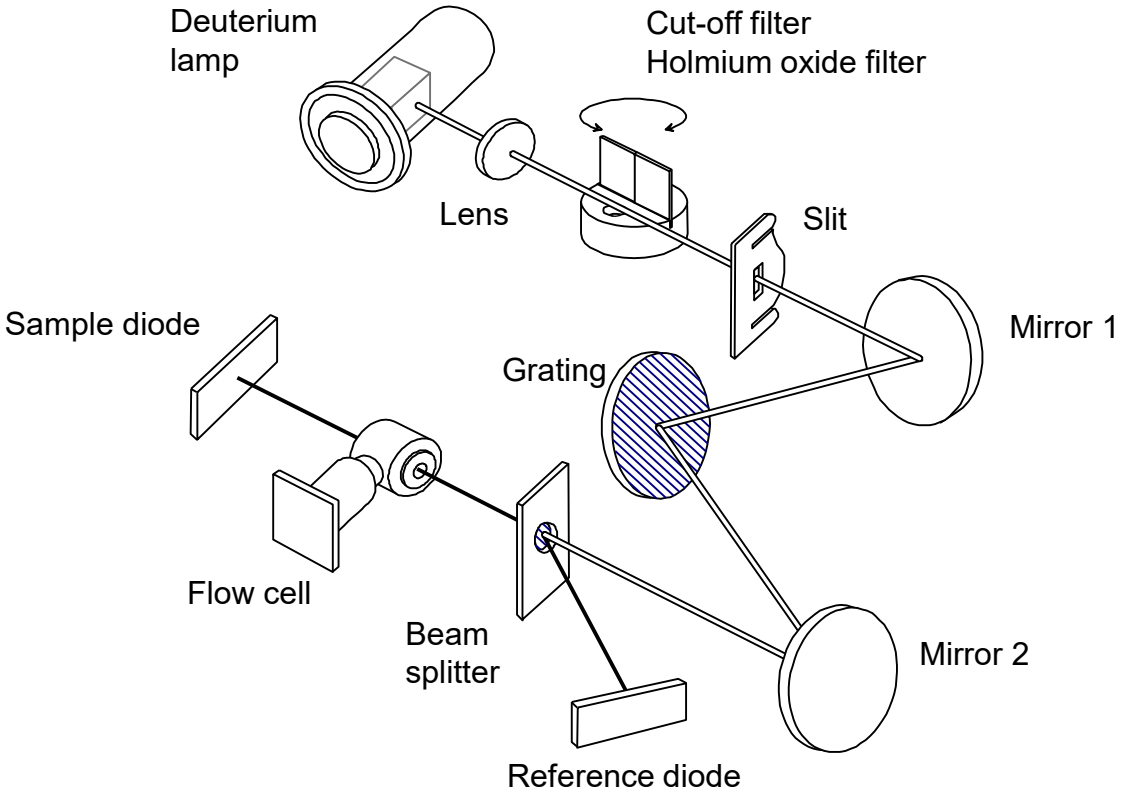
Draw Position/Needle Height Position Offset = 0	Vial Sampler G1329B/G7129A/B	Wellplate Sampler G1367E/G4226A	Multisampler G7167A/B
	2 mL vial (sample tray)	2 mL vial 54 vial tray	2 mL vial 54 vial tray
Without Bottom Sensing	2.0 mm	4 mm	5 mm
With Bottom Sensing	x	1 mm	2 mm



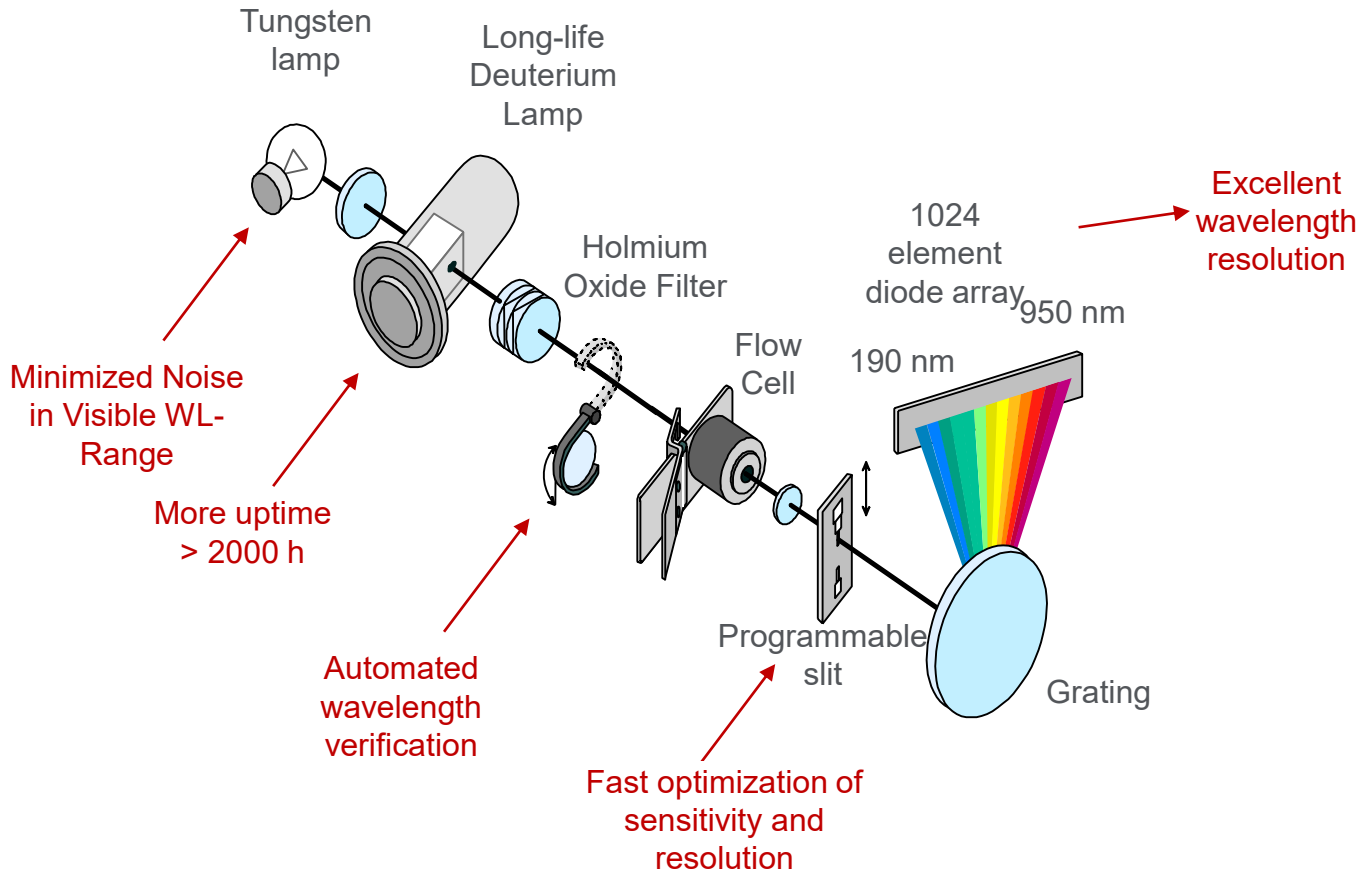


Technical Principles of VWD, MWD and DAD

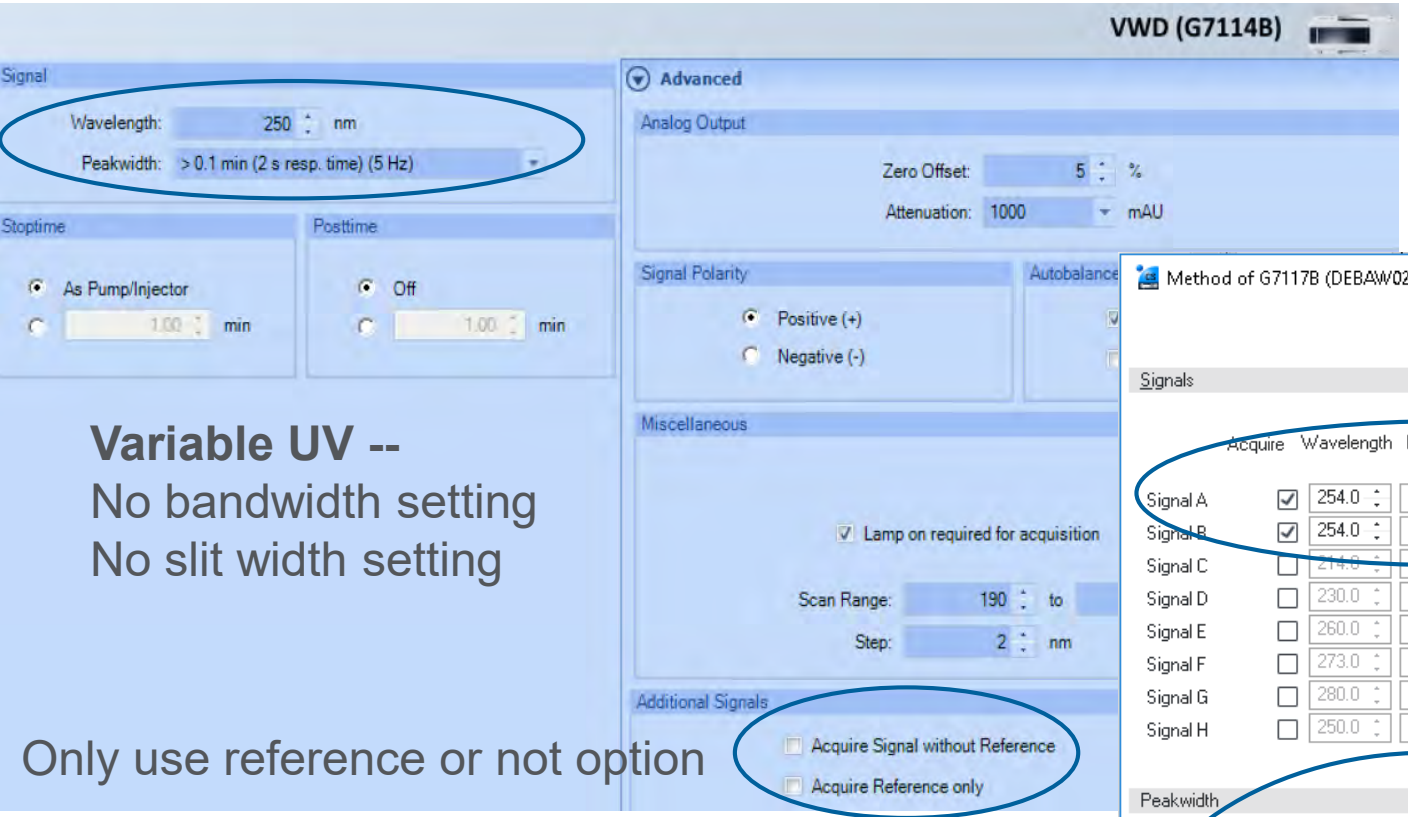
VWD, variable wavelength detector



MWD, multiple wavelength detector
DAD, diode array detector



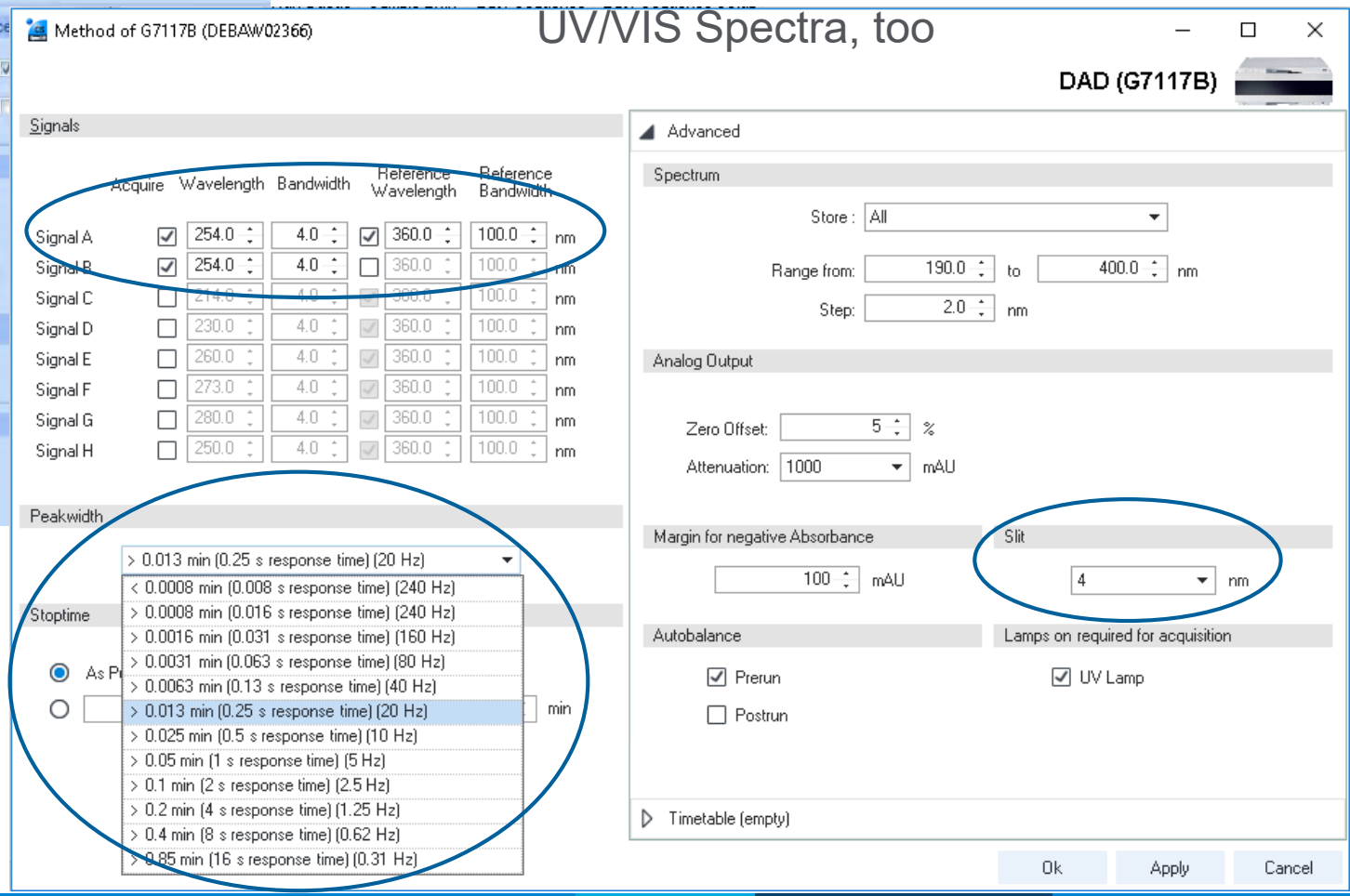
VWD and DAD Settings



Variable UV --
No bandwidth setting
No slit width setting

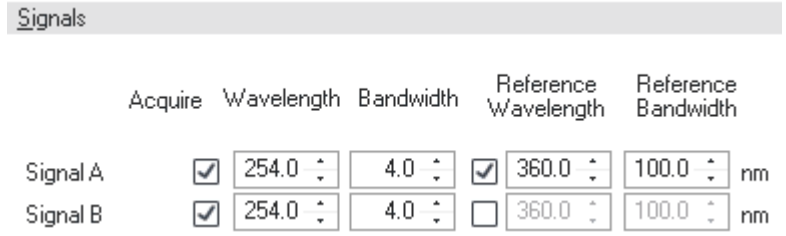
Only use reference or not option

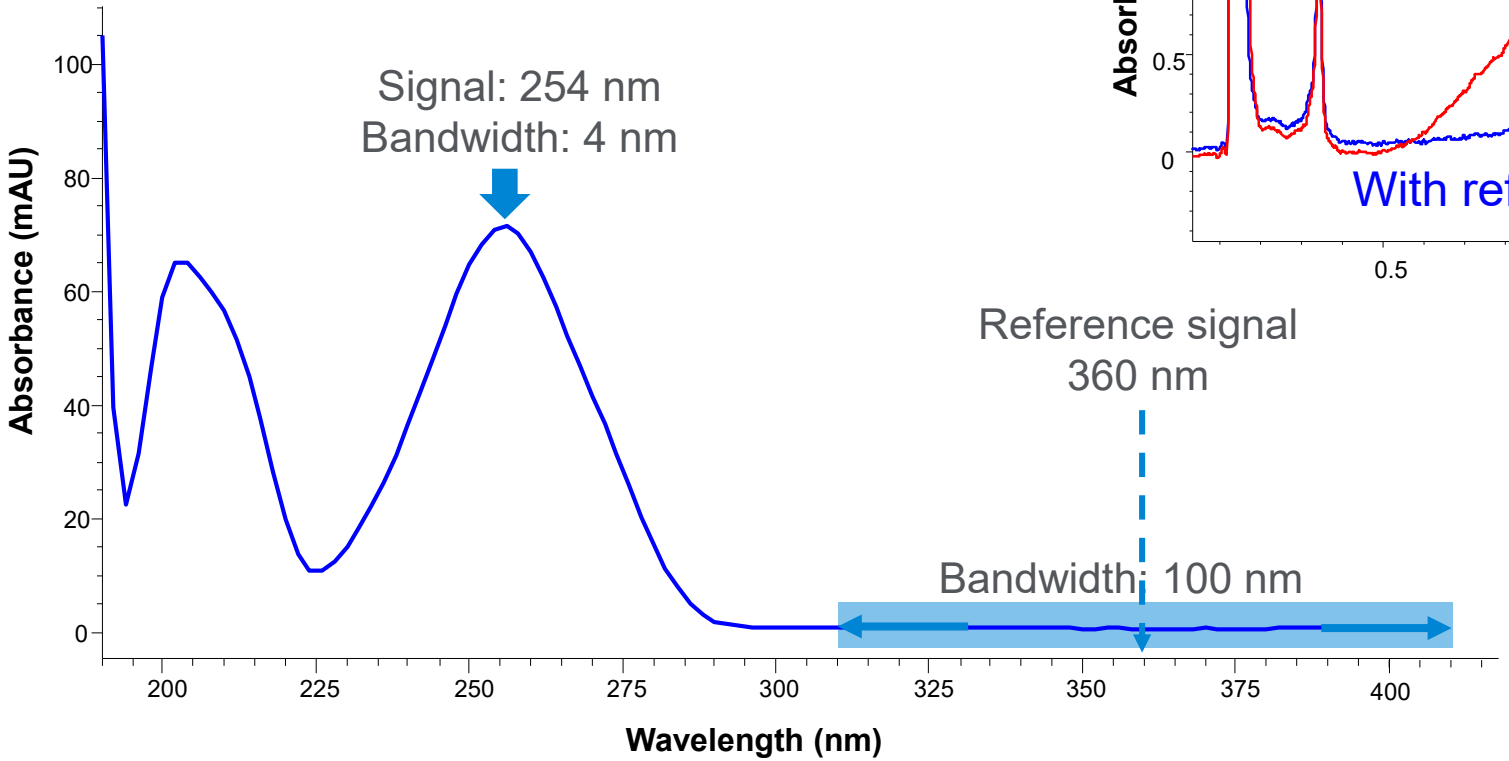
Diode Array UV or UV/VIS
Diode(s) bandwidth setting
Maybe optical slit setting
Multi-channel UV easy
UV/VIS Spectra, too

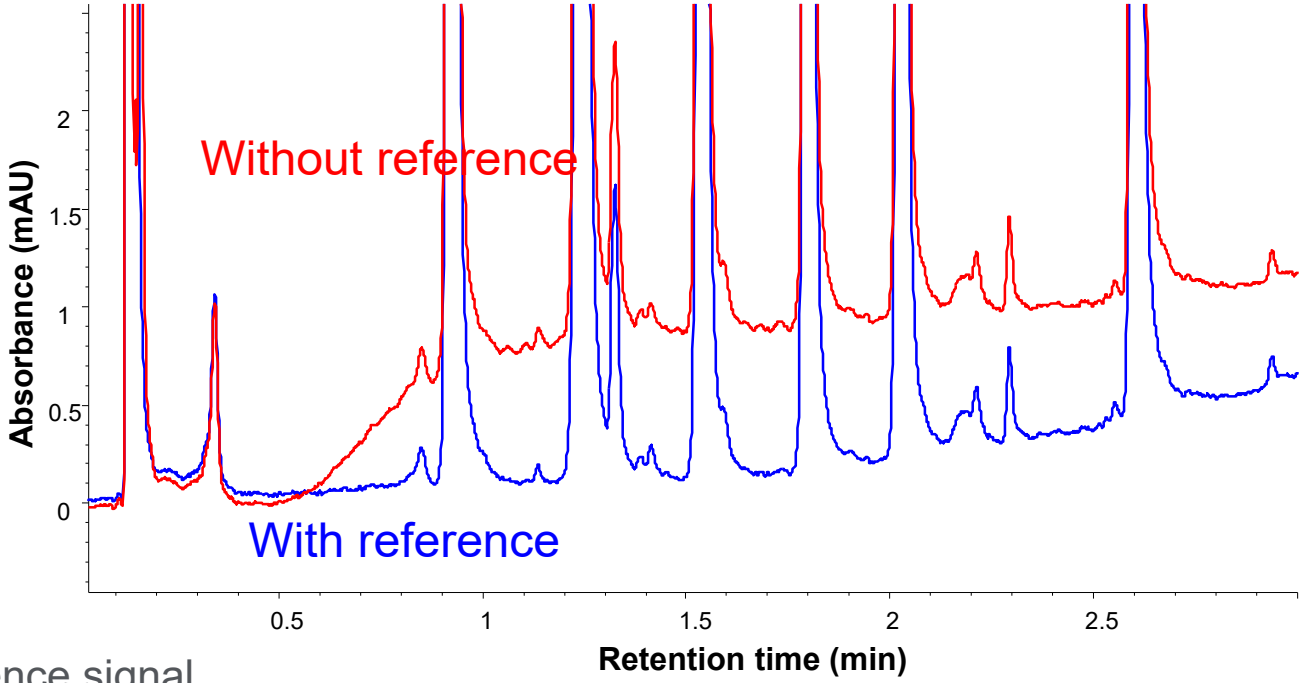


Different parameters

DAD Setting — Choose the right signal and reference

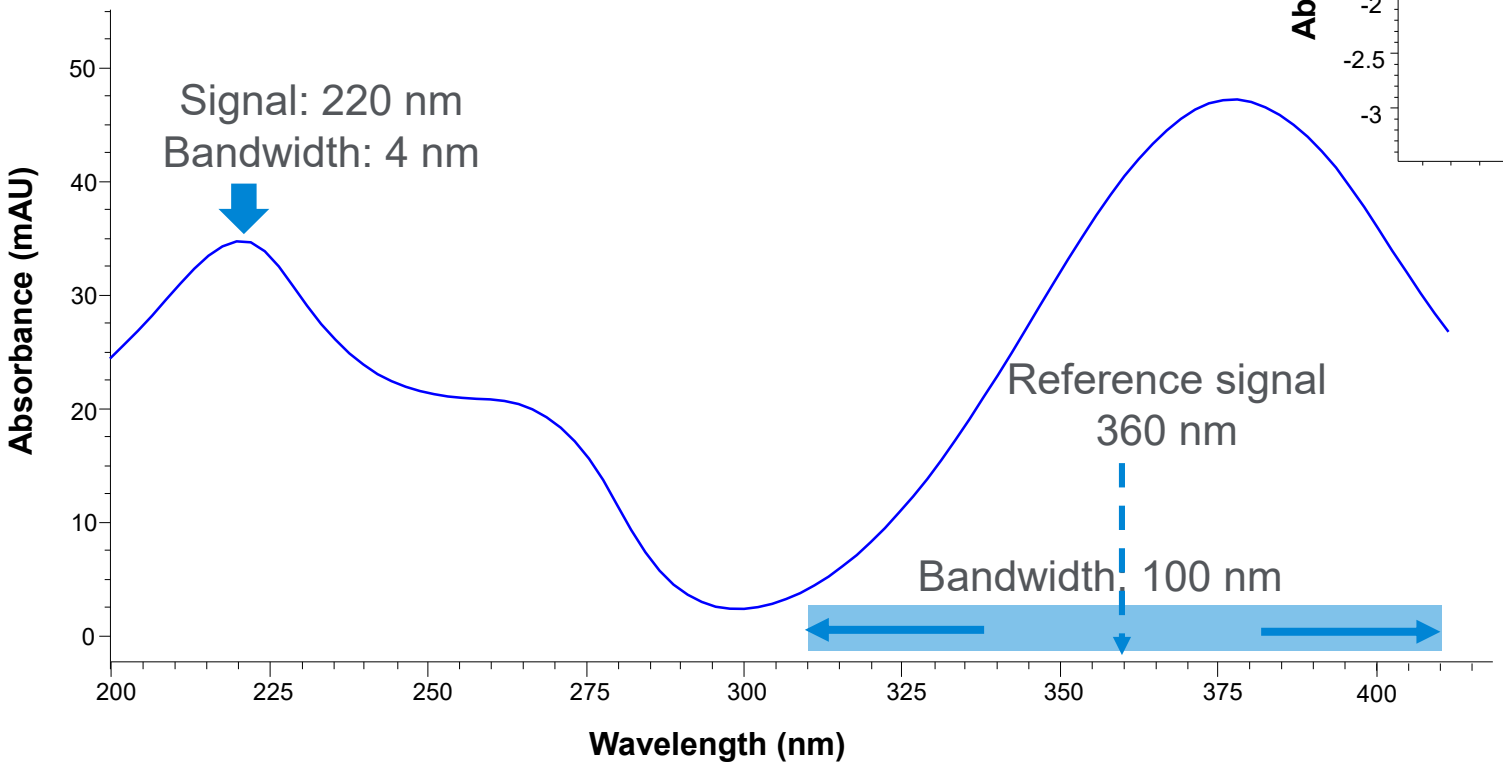


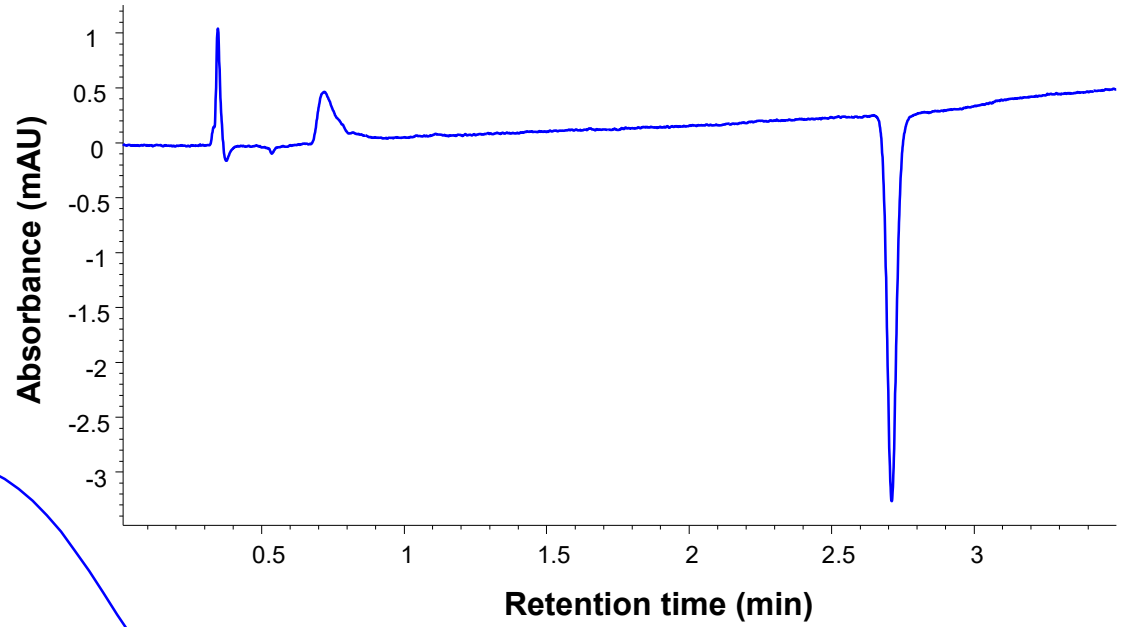




Gradient: 10-100% ACN in 3 min

DAD Setting — Choose the right signal and reference



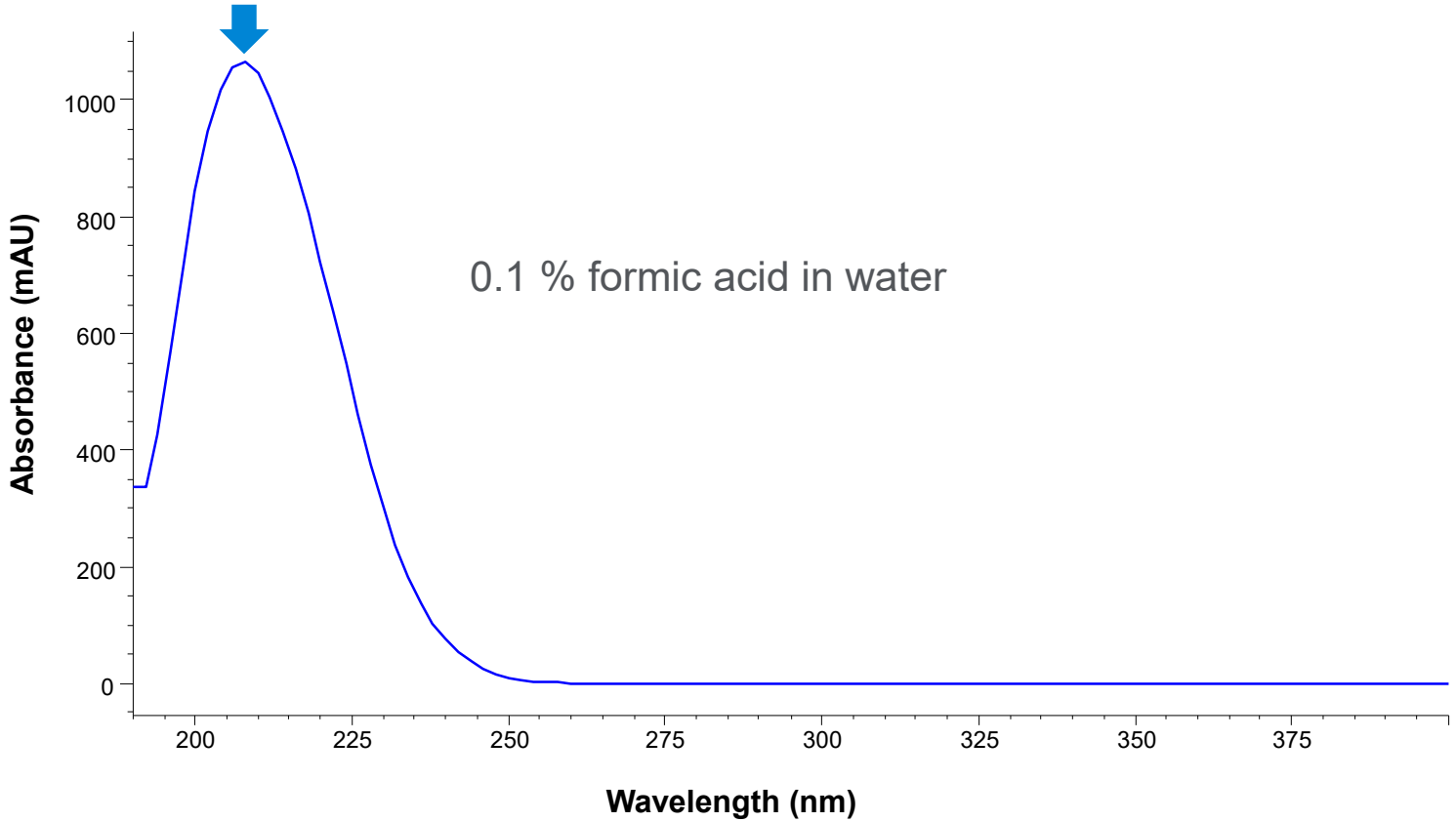


Reference signal may not be necessary

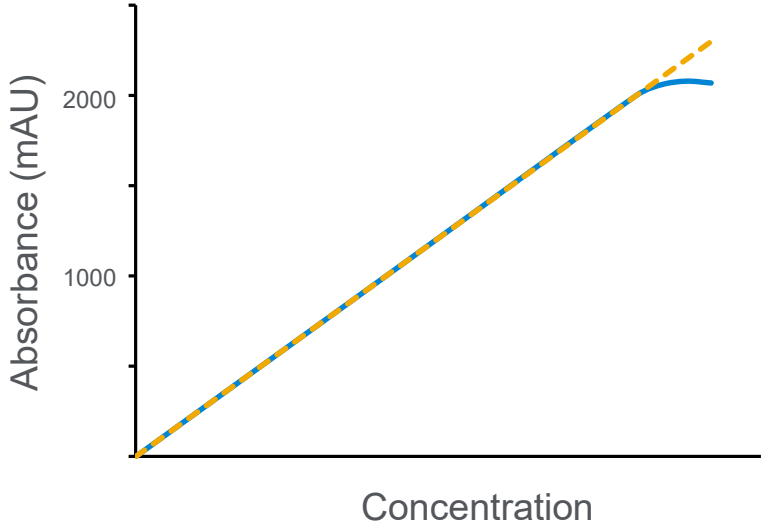
DAD Setting — Choose the right signal and reference

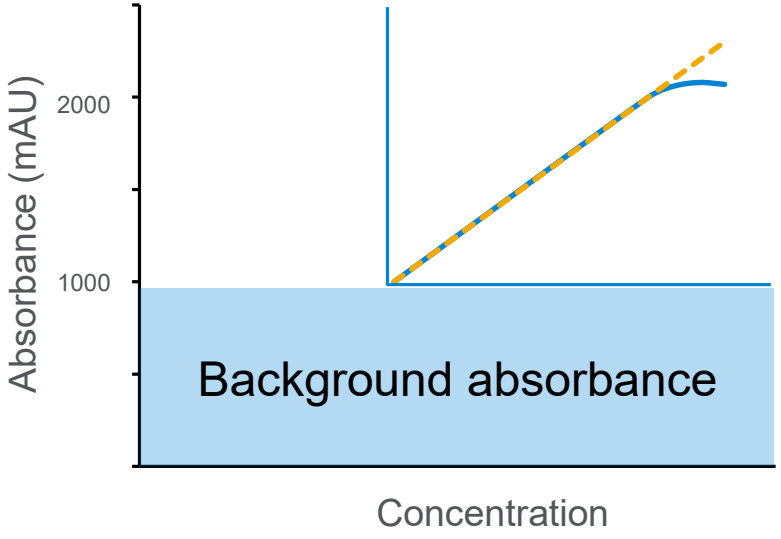
Move away from the UV cutoffs of mobile phase/additive

Signal: 210 nm

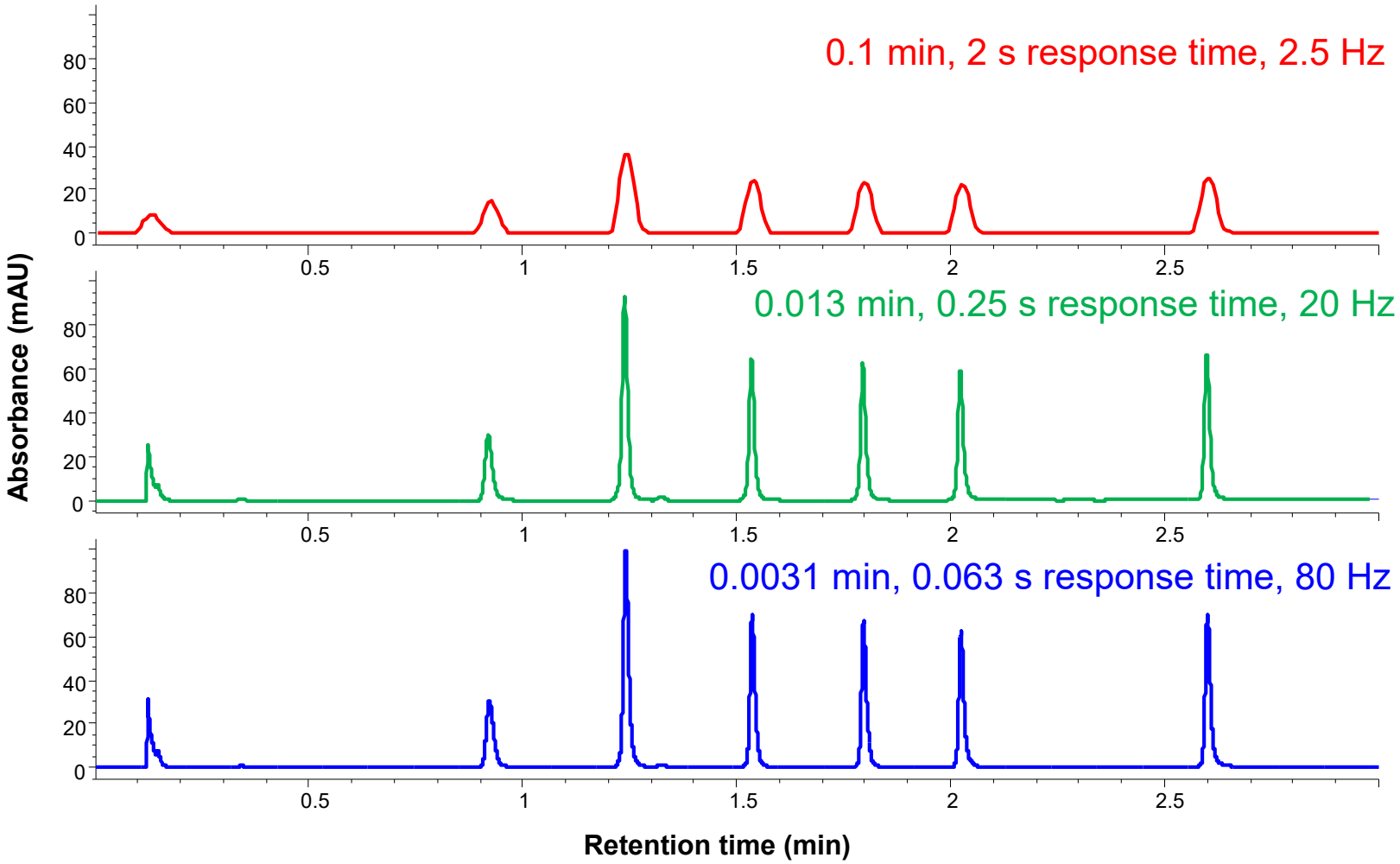


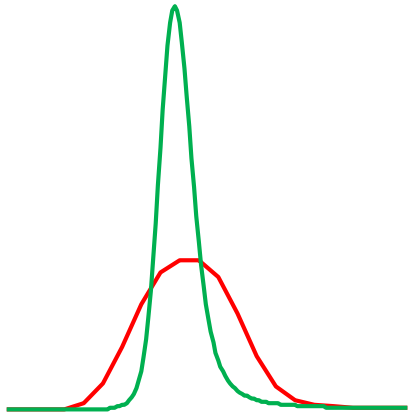
0.1 % formic acid in water





DAD Setting — Choose the right sampling rate

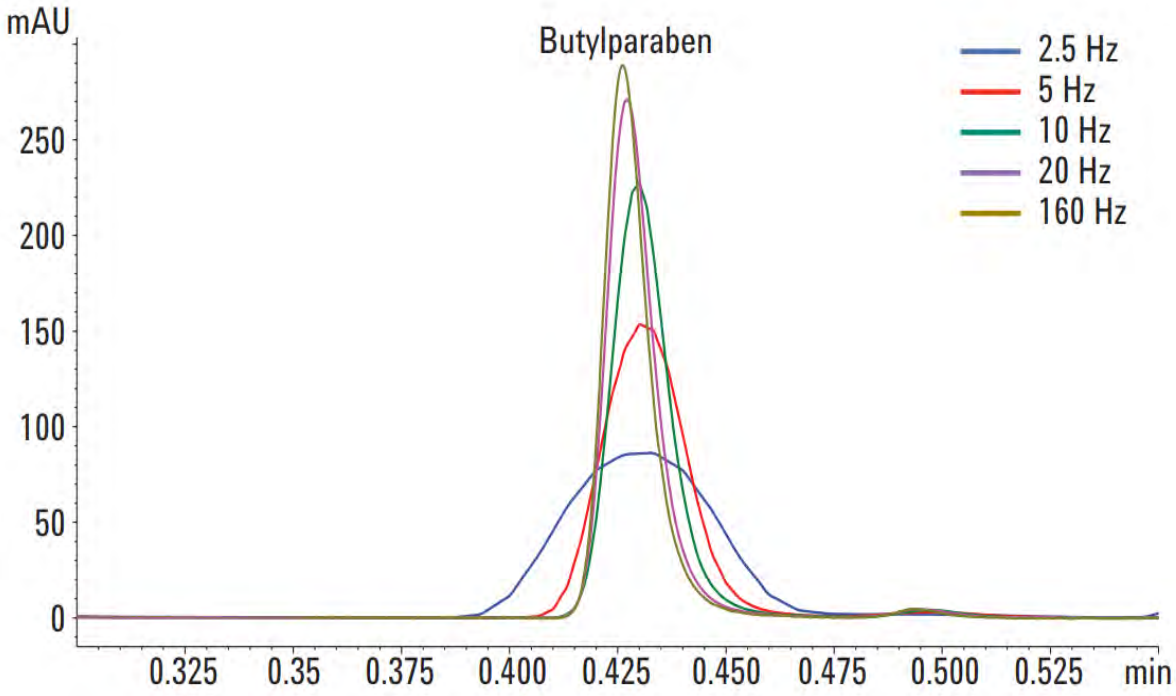


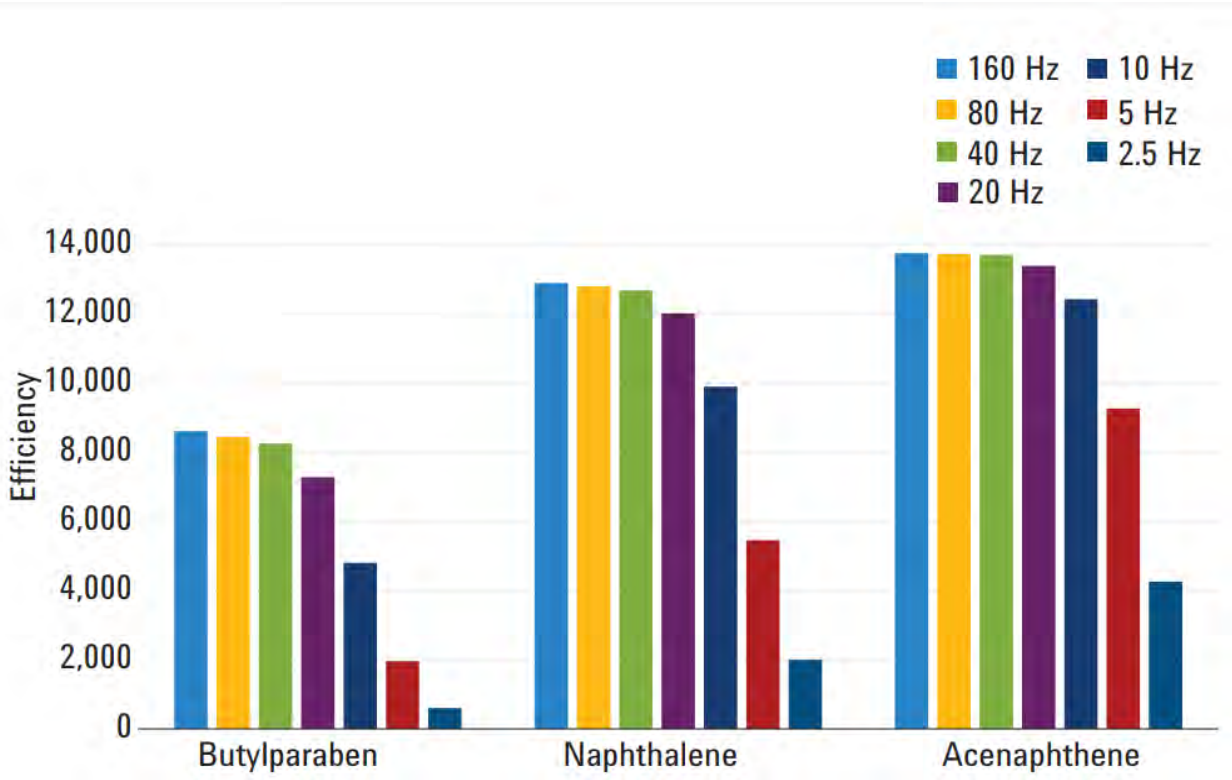


Changes in **Peak Width** and **Resolution**

Column: ZORBOX Eclipse Plus C18, 2.1x50 mm, 1.8 µm
Column temperature: 35 °C; Flow rate: 1 mL/min
Gradient: 10-100% ACN in 3 min
Signal: 254 nm, Bandwidth: 4 nm
Reference: 360 nm, Bandwidth: 100 nm

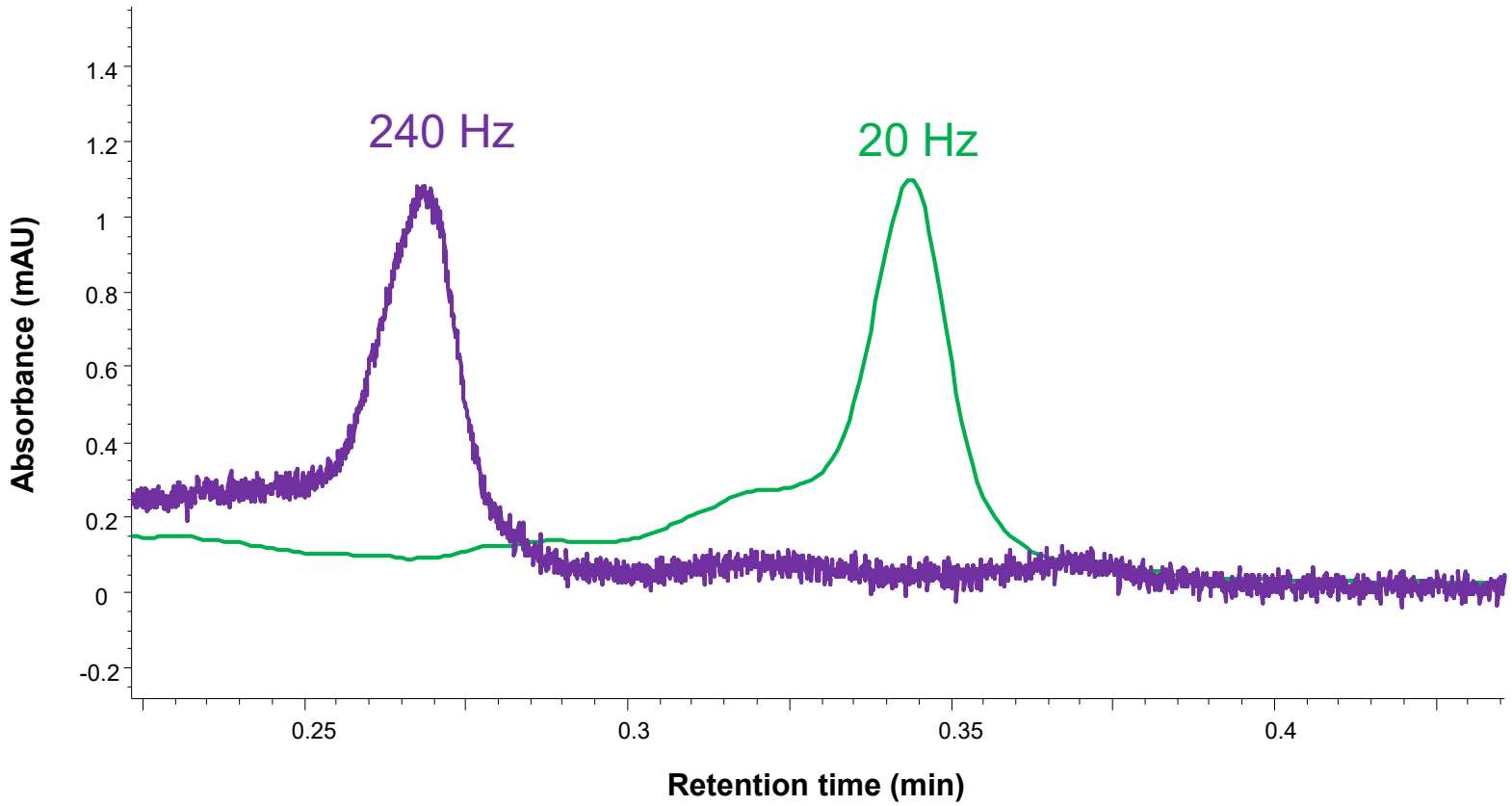
Data Rates May Impact Observed Resolution



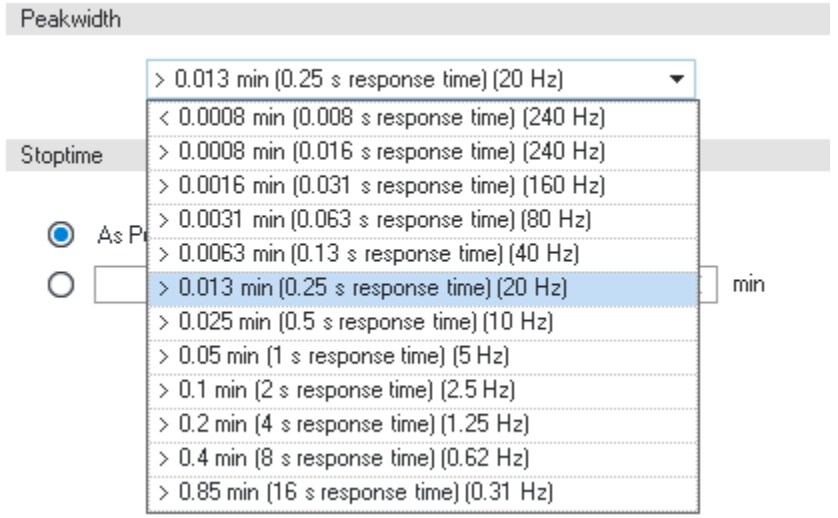


Fast data collection rates must be used with Agilent InfinityLab Poroshell 1.9 µm columns to accurately measure the efficiency of the column, especially for early eluting compounds such as butylparaben (k′ = 1.3).

DAD Setting — Choose the right sampling rate

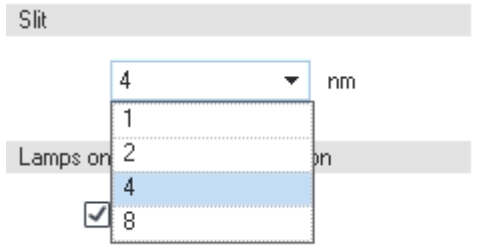


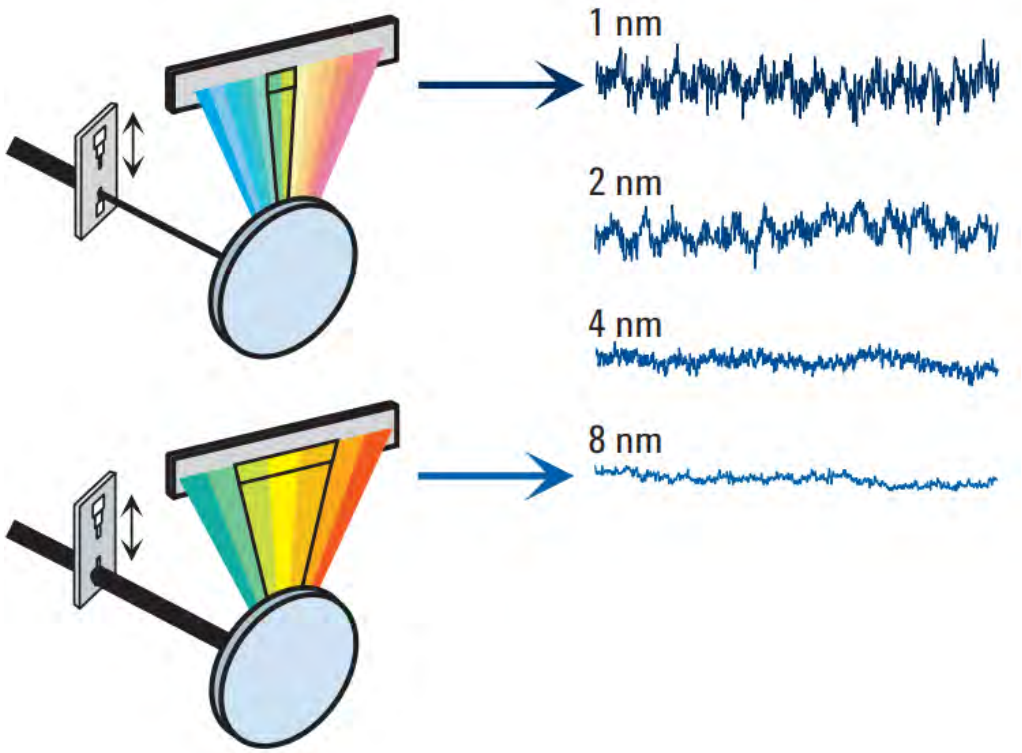
Do Not Use Peakwidth Smaller Than Necessary



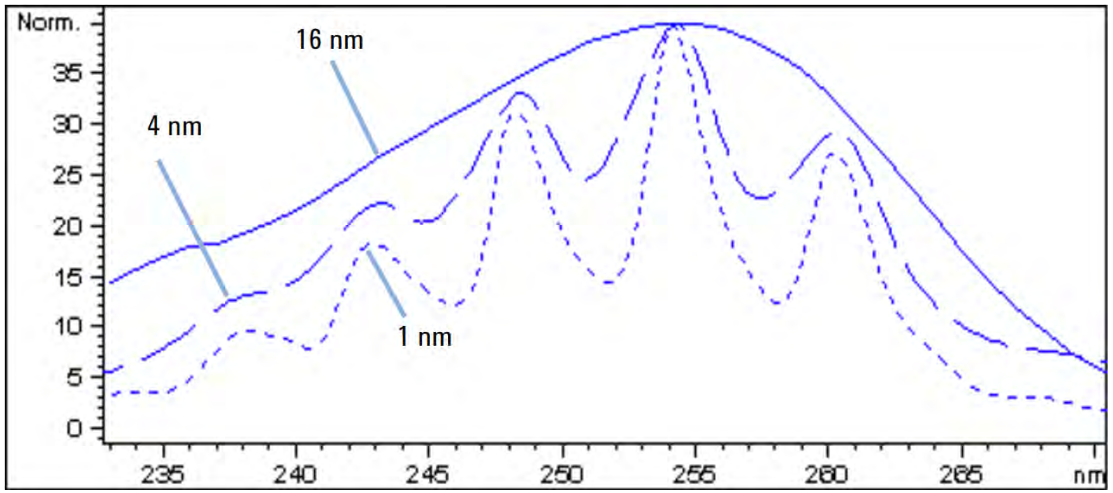
Column: ZORBOX Eclipse Plus C18, 2.1x50 mm, 1.8 µm
Column temperature: 35 °C; Flow rate: 1 mL/min
Gradient: 10-100% ACN in 3 min
Signal: 254 nm, Bandwidth: 4 nm
Reference: 360 nm, Bandwidth: 100 nm

DAD Setting — Choose the appropriate slit width





Benzene Spectral resolution



Resources — Primers

5990-7595EN
The LC Handbook
Guide to LC Columns and Method Development

5991-2359EN
Two Dimensional Liquid Chromatography

5990-3777EN
High Performance Capillary Electrophoresis

5991-5509EN
Supercritical Fluid Chromatography

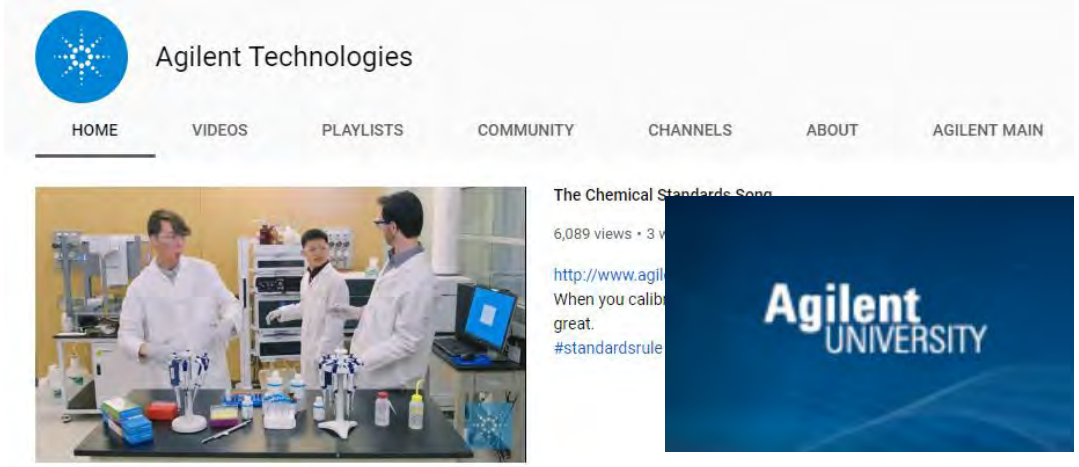
5989-6639EN
Principles in Preparative HPLC

5991-3326EN
Sample Preparation Fundamentals for Chromatography

5980-1397EN
Fundamentals of UV-visible Spectroscopy

Resources for Support

- Collection of LC resources: https://community.agilent.com/docs/DOC-1852-lc-insights-to-go#jive_content_id_LC_Troubleshooting
- Agilent support resources: https://community.agilent.com/community/resources
- Agilent University: http://www.agilent.com/crosslab/university
- Agilent resource center: http://www.agilent.com/chem/agilentresources
- InfinityLab Supplies Catalog (5991-8031EN)
- Your local FSE and Specialists
- Youtube – Agilent Channel

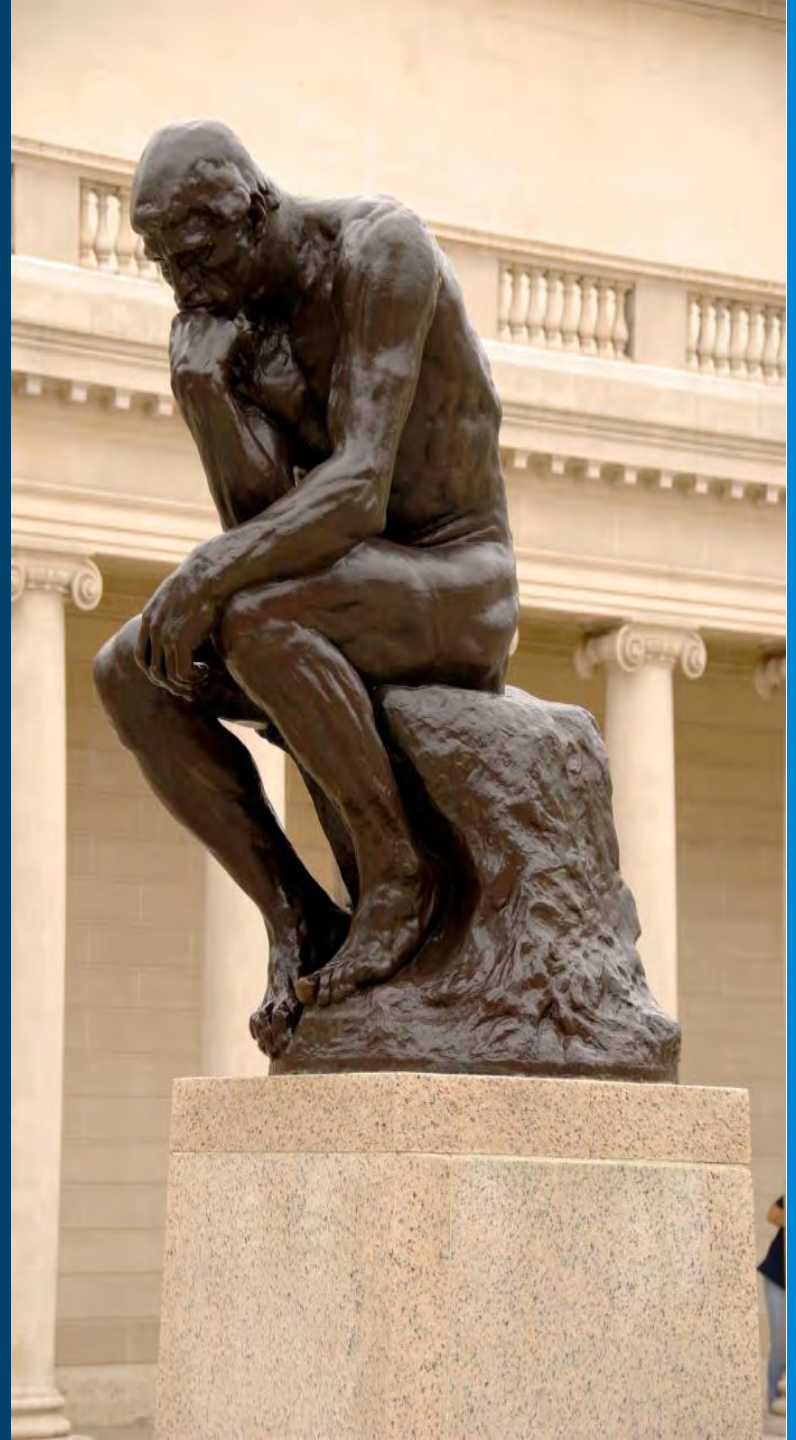


- Sales and support phone assistance (US and Canada):

1-800-227-9770 Phone Tree Navigation Assistance

gc-column-support@agilent.com
lc-column-support@agilent.com
spp-support@agilent.com
spectro-supplies-support@agilent.com

Thanks for your attention!



Agilent
Trusted Answers