An Introduction to HPLC Instrumentation

Part 1

Agilent HPLC Webinar Series

Lori Sandford, Applications Scientist
Agilent Technologies, Inc.
Wood Dale, IL

Agilent
Trusted Answers

DE.3915972222

Topics for Today's Seminar

Agilent 1260/1290 Specifications

Instrument Flowpath

The Pump

- General Hygiene of Solvents
- Pump Design
- Starting up the Instrument/Prime/Purge
- Pumping Parameters
- Compositional Accuracy

The Autosampler

- Injection Mechanism

The Column Compartment Settings and Proper Use

HPLC Detectors

- UV Detection
  - UV Settings
  - Sensitivity

Definitions of Basic Parameters for Optimization

- Delay Volume
- Dispersion

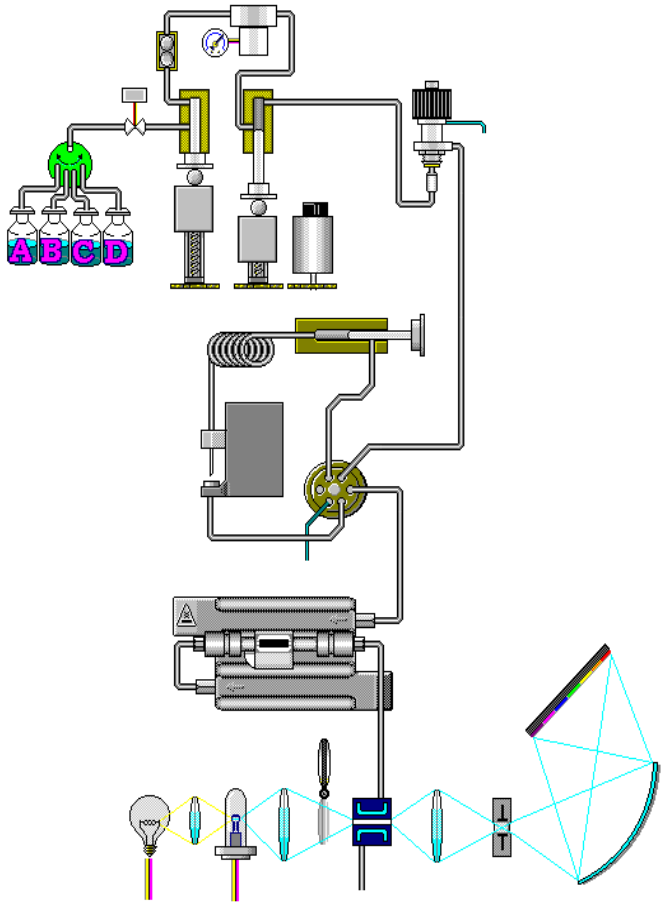
Typical HPLC System

Detector

Column Compartment

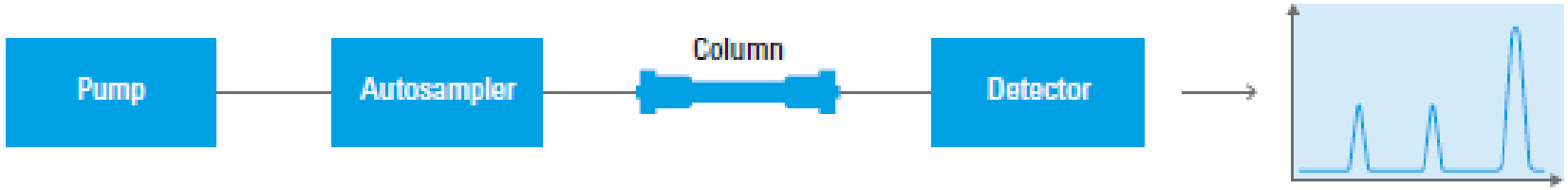
Autosampler

Pump & Vacuum Degasser



Typical HPLC System
Follow the Flowpath

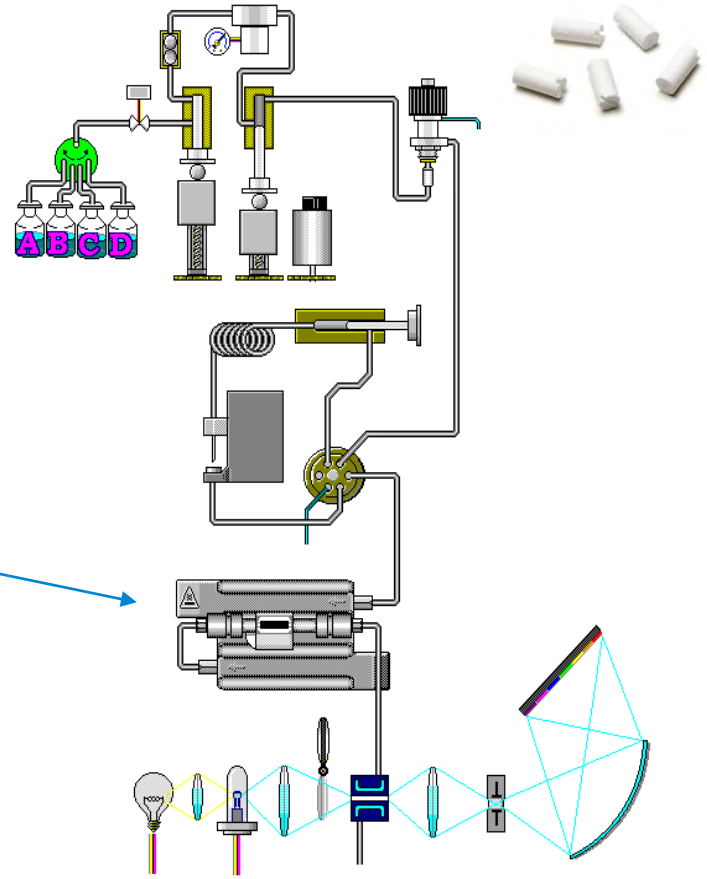
- Where are the moving parts?
- Where can blockages occur?
- Where are the consumable parts?
- Where can leaks occur ?



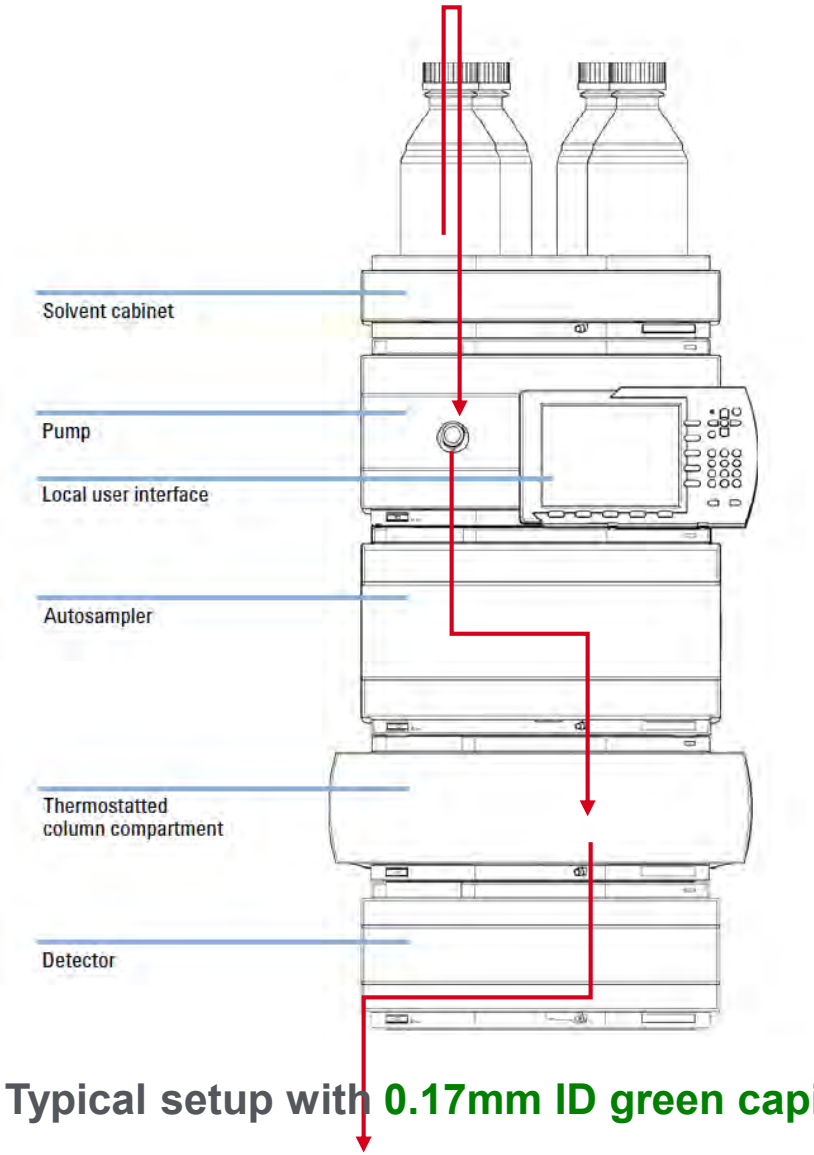
What can I do to eliminate, reduce or anticipate potential problems with the LC ?

Filters and Bottle necks for blockages
Reduce Downtime by Understanding Common Problems

- Solvent inlet filters in solvent bottles
  - glass: 20um – replace if needed!
  - SST: 12-14um – replace, opt sonicate
- Heat exchanger (bent? Connection?)
- PTFE frit in the purge valve at outlet of the quaternary pump head: replace
- Binary pump only: inline filter at outlet check valve
- Troubleshoot: Disconnect other modules behind pump



The HPLC Stack
Common Consumable Parts



Typical setup with 0.17mm ID green capillaries

Solvent bottles :

01018-60025 Solvent inlet filter **12-14um** SS

3150-0944 Glass filter, solvent inlet, 40um

5043-1218 A-Line Stay Safe cap (GL45), 2-port

9301-1450 Solvent bottle (GL45) amber, 1 L

9301-1421 Solvent bottle (GL45) clear, 1 L with cap

Pump (1260 Quat with integrated degasser):

G1311B Agilent 1260 Infinity Quaternary Pump
PTFE Frit (pack of 5) 01018-22707

Autosampler - Column Compartment:

G1367E Agilent 1260 Infinity High Performance Autosampler
G1329B Agilent 1260 Standard Autosampler

Column Compartment - Column:

G1330B Agilent 1260 Infinity Thermostatted Column Compartment
5067-5965 A-Line quick-connect LC fitting

Detector - waste:

G4212B 1260 Infinity Diode-Array Detector
G4212B #030 1260 Max-Light Hi-sensitivity cell
G314F Variable Wavelength Detector

Pumps

- General Hygiene and Housekeeping
- Preparing Pumps
- High and Low Pressure Mixing Pumps
- Compositional Accuracy
- Compressibility of Solvents
- Seal Wash

Performance Characteristics of the Pump

Important Characteristics	Influence on…
Common to isocratic and gradient pumps	
• Flow accuracy	• Retention time and peak area precision (system to system)
• Flow precision	• Retention time and peak area precision (within one system)
• Pressure pulsation	• Baseline noise
Common to gradient pumps only	
• Delay volume in low and high pressure mixing	• Gradient shape and precision
• Compositional accuracy	• Retention time and peak area precision (system to system)
• Composition precision	• Retention time and peak area precision (within one system)

Reduce LC problems by eliminating most common sources of flow blockage

- Filter or use HPLC grade solvents
- Particles leading to blockage can come from sources located both outside and inside the LC system:
  - Solvent, buffer
  - Microbial growth in solvent reservoirs
  - The Sample
  - Wear of LC components – piston seals, autosampler valve, etc.
- Debris will either be captured on the column frit or in-line filter

- Use 0.45 um filter for mobile phase components
- Replace aqueous solvents every two days
- Avoid exposure to direct sunlight
- Preventative Maintenance is the Key!
- Keep a log of maintenance to prevent downtime

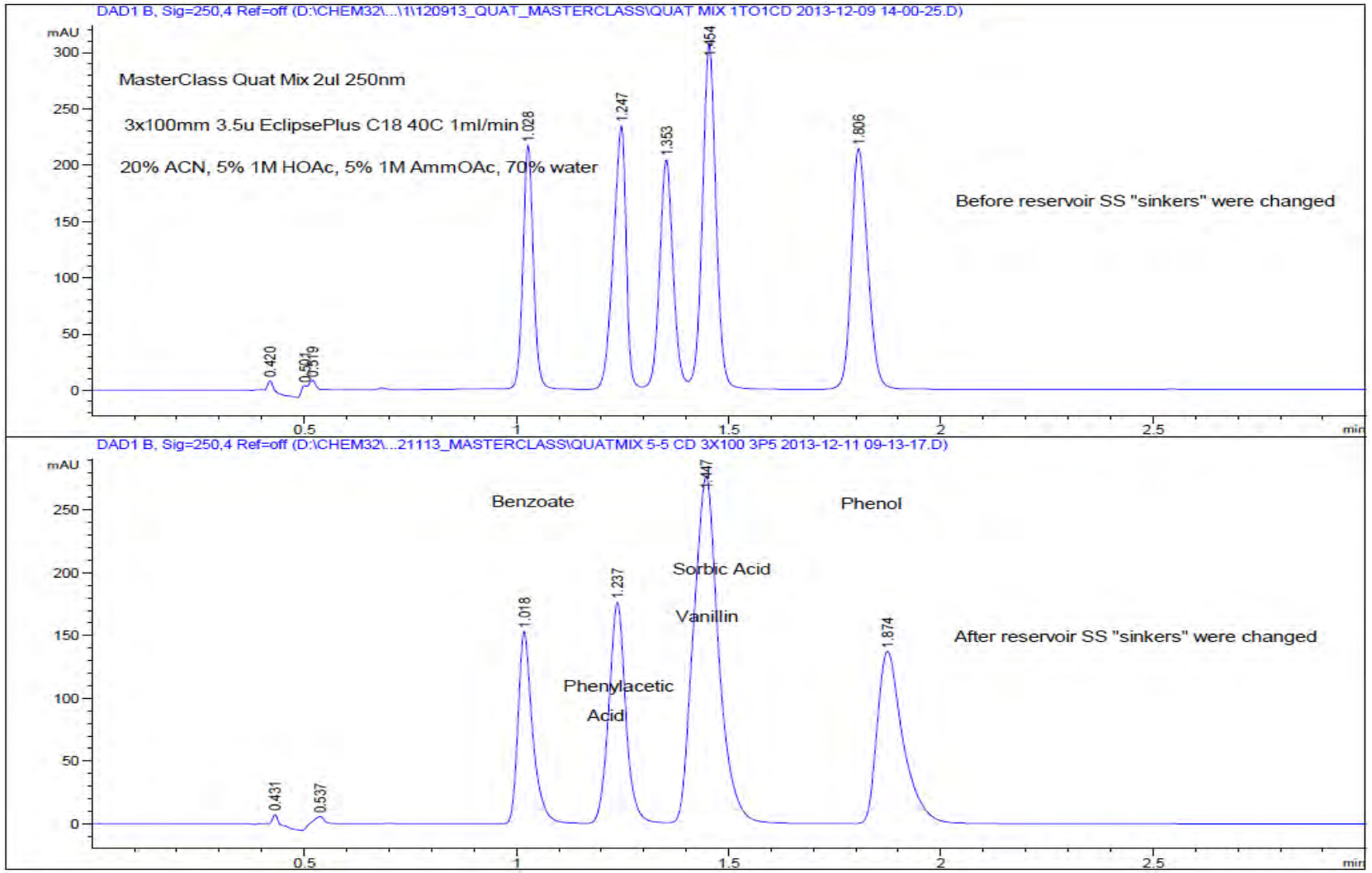
Examples: Used / Unused Filters

Glass filters: 3150 - 0944

Stainless Steel Filters: 01018 – 60025

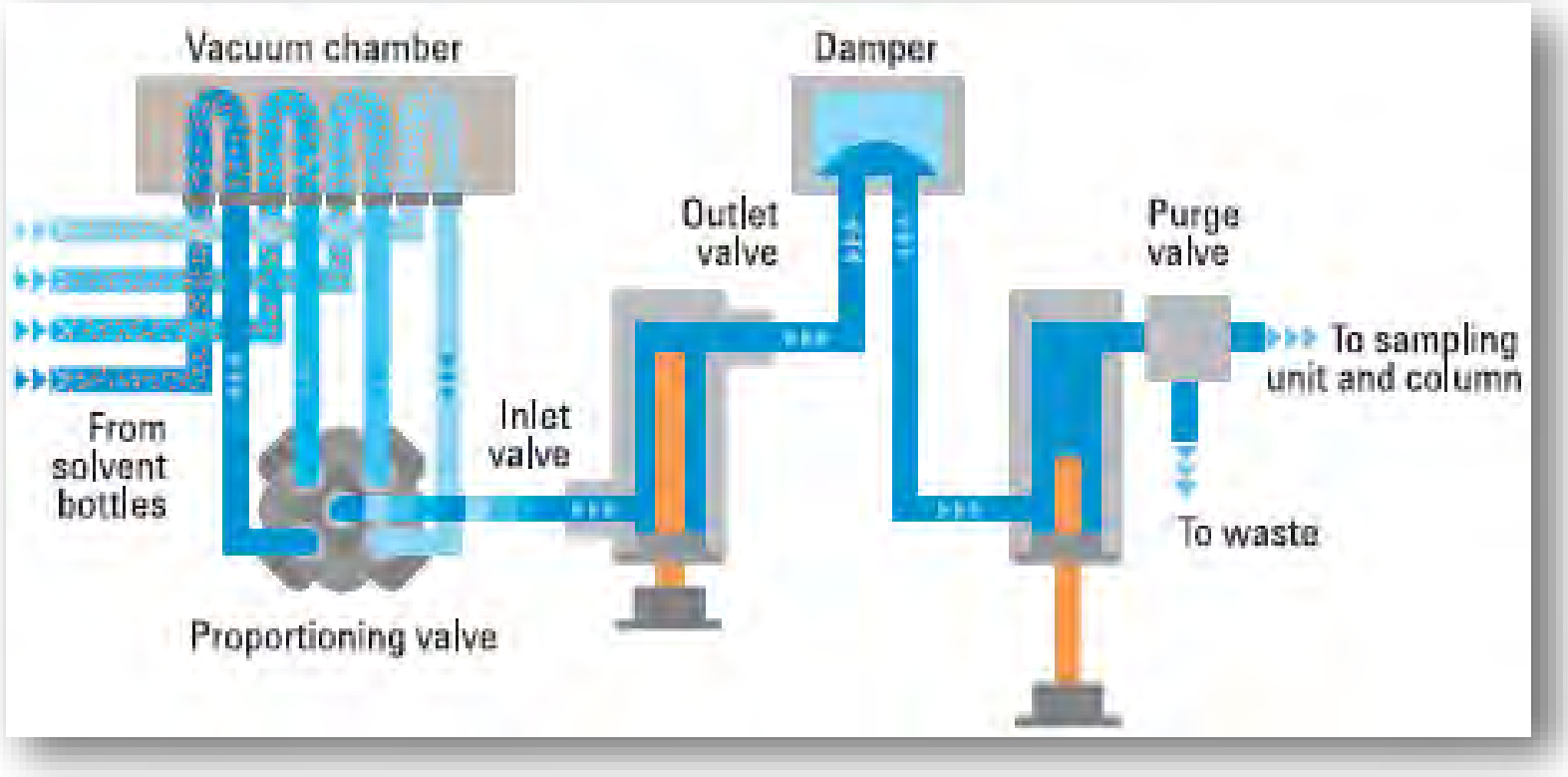
(less volume, no Na+ ions)

Clean or Replace the Solvent Inlet Filters



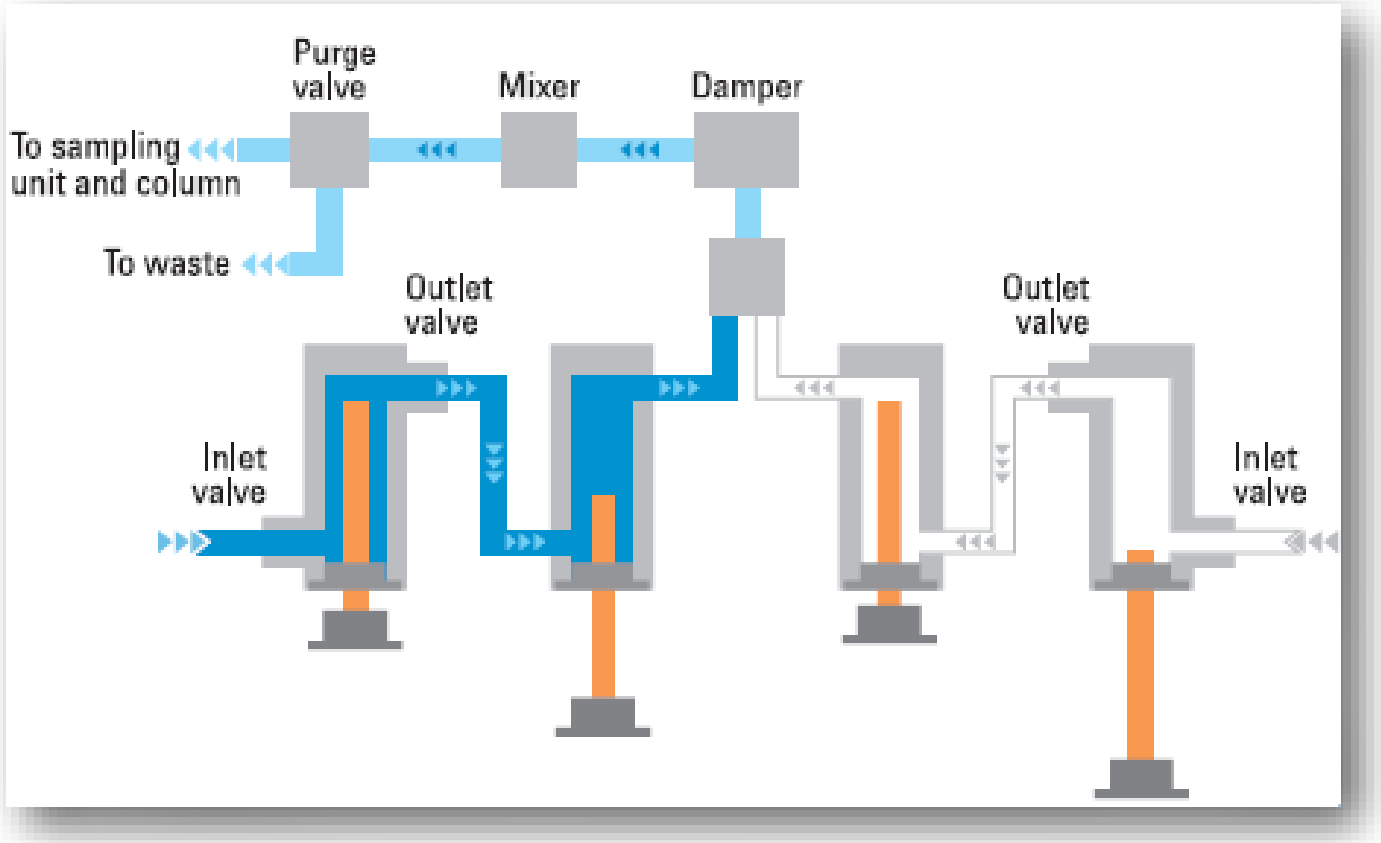
Clogged inlet filter let to cavitation which in one line causing pH change which changed selectivity

Agilent 1260 Infinity Quaternary Pump
Low-pressure mixing (LPM) principle



Mixing by low-pressure proportioning valve before the pump head

Agilent 1260 Infinity Binary Pump
High-pressure mixing (HPM) principle



Combination and mixing of mobile phases <u>after</u> the pump head

When to use purge, prime, condition?

Purge

Change solvents

When pump is refilled with new/different mobile phase the purge valves allows both pump heads (binary pump) to be connected to waste at the same time

Prime

When the pump is dry

When Purge and Condition still show exhausted pressure ripple

Condition

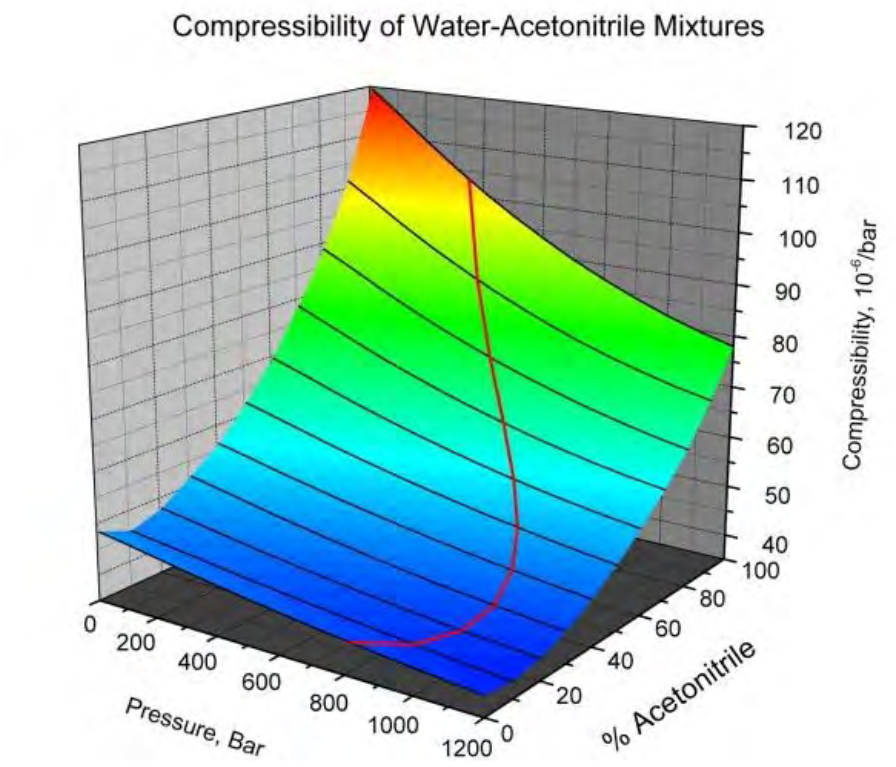
When first starting up for the day or after changing solvents

When pump pressure ripple or composition ripple is too high (mixing noise) air bubble is hidden in pump head (listen)

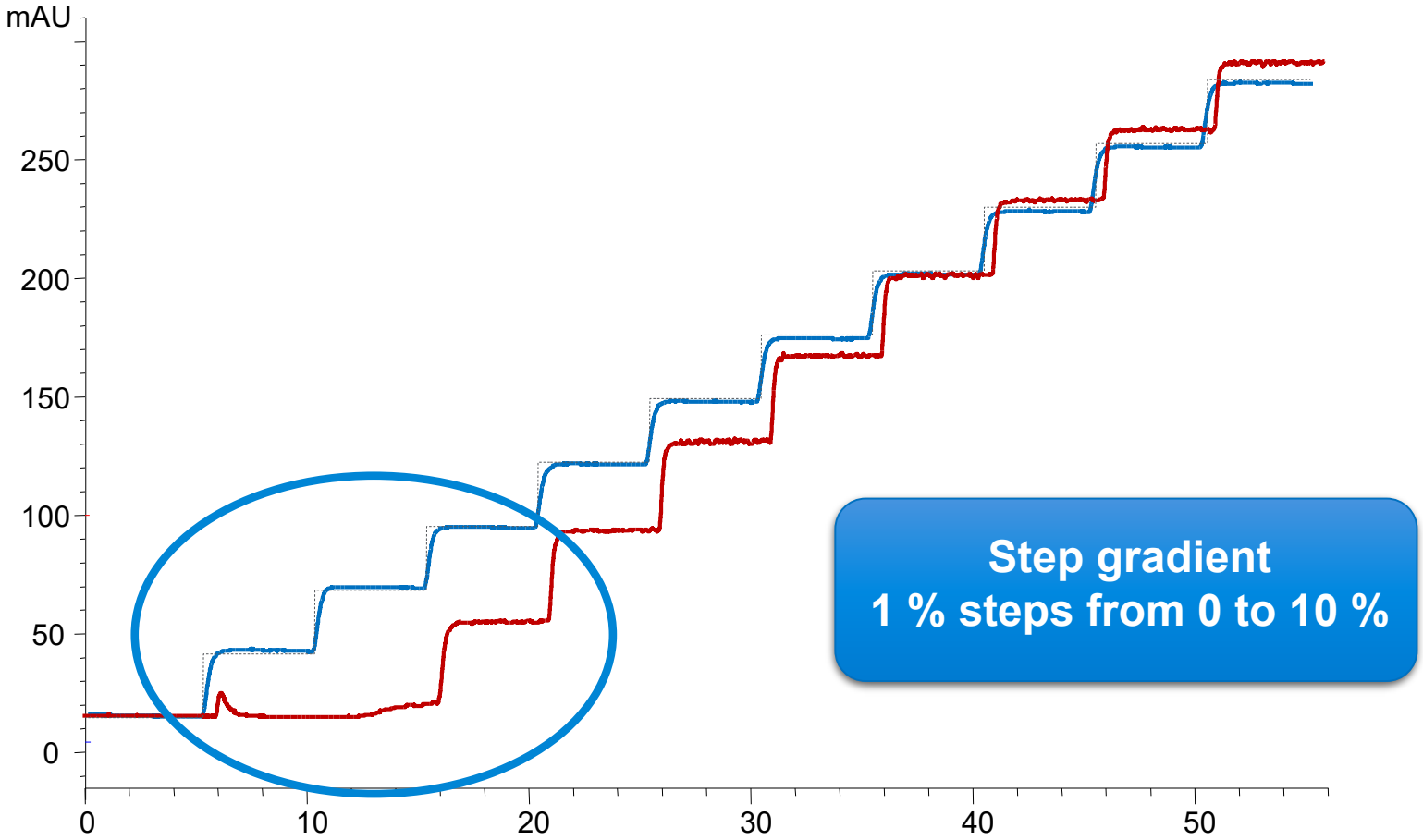
best once a day to condition for smooth operation

Solvent Compressibility

- Solvent compressibility must be accounted for, especially when running at UHPLC pressures >9000 psi
- Failure to account for compressibility will result in different flow rates at higher pressures

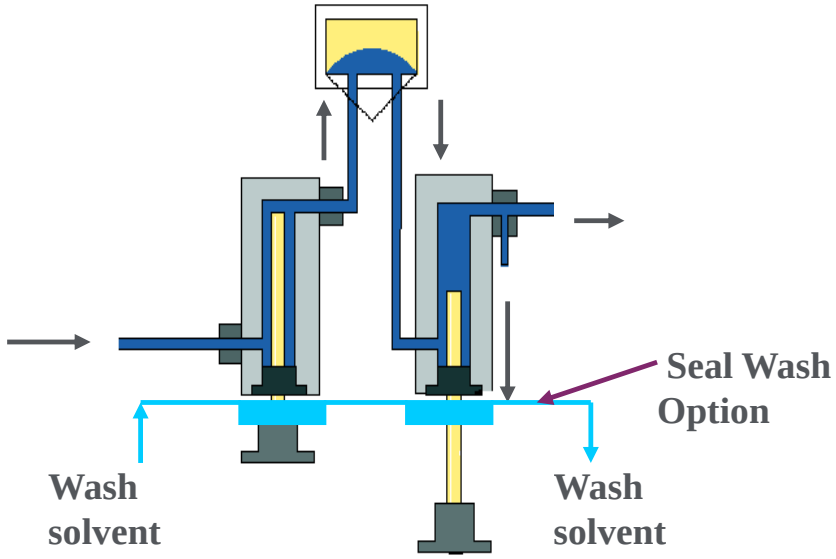


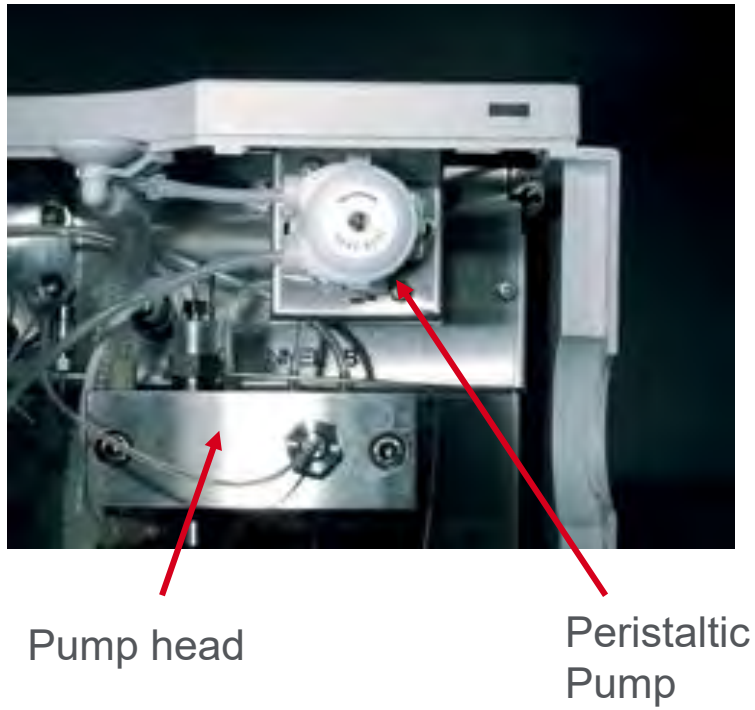
Compositional Accuracy
Select the correct solvent type in your pump parameters



Shallow gradient at low organic concentration. In this case the trace in red cannot perform the steps at low composition. This system should not be used for shallow gradients (<1% delta B / minute)

Many Pumps Have Seal Wash Option and an In-Line Filter



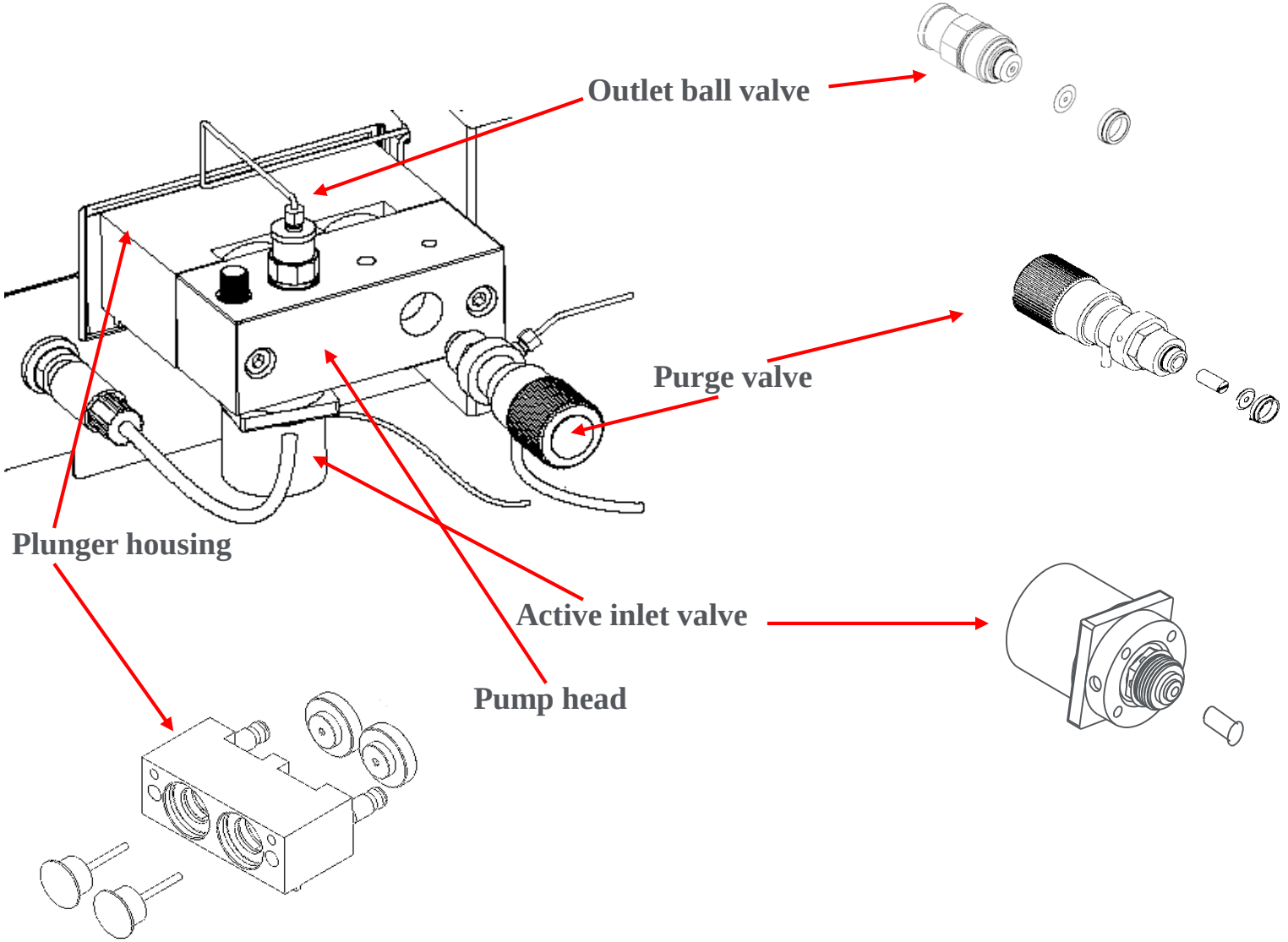


- Protects your pistons and seals from excessive wear
- Recommended when using aqueous buffer or salt solutions > 0.1M
- 10% isopropanol recommended as pump seal wash solvent
- Change solvent weekly (date on bottle)

Pump and Degasser Maintenance

- Clean the degasser lines by flushing with isopropanol.
- When using buffers, flush with water, then with isopropanol.
- Check for air bubbles in outlet lines.
- Be aware of the possibility of microbial growth in aqueous phases
  - flush aqueous lines weekly with IPA
  - Don‘t top off water/solvents, use fresh bottles that have been cleaned with solvent and dried
- Check for solvent compatibility and flush with appropriate solvents
- Unused channels should be left in isopropanol.

Pump Head – Main Components



Autosampler – the brain behind workflow automation logistics

- Sampler Settings
  - –Fixed Loop vs Flow-Through Autosamplers
  - –Principle of Operation

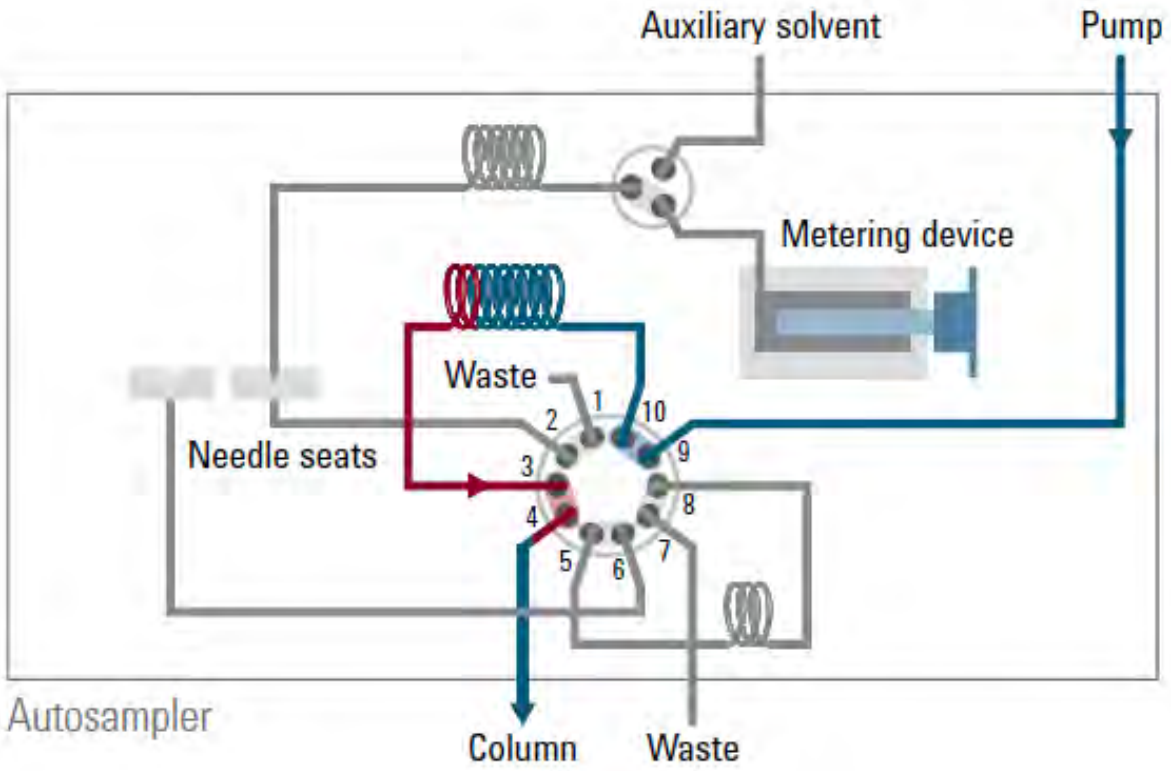
Performance characteristics of the Autosampler

Characteristic		Influences
• Injection volume precision	→	• Precision of peak area/height
• Wide linearity	→	• Accuracy of peak area/height (when using different injection volumes)
• Minimum carry over	→	• Precision of peak area/height
• Wide dynamic injection volume	→	• Versatility, application range

Fixed Loop Autosampler

Sample is drawn or pushed into a sample loop and this loop is switched into the sample flow-path

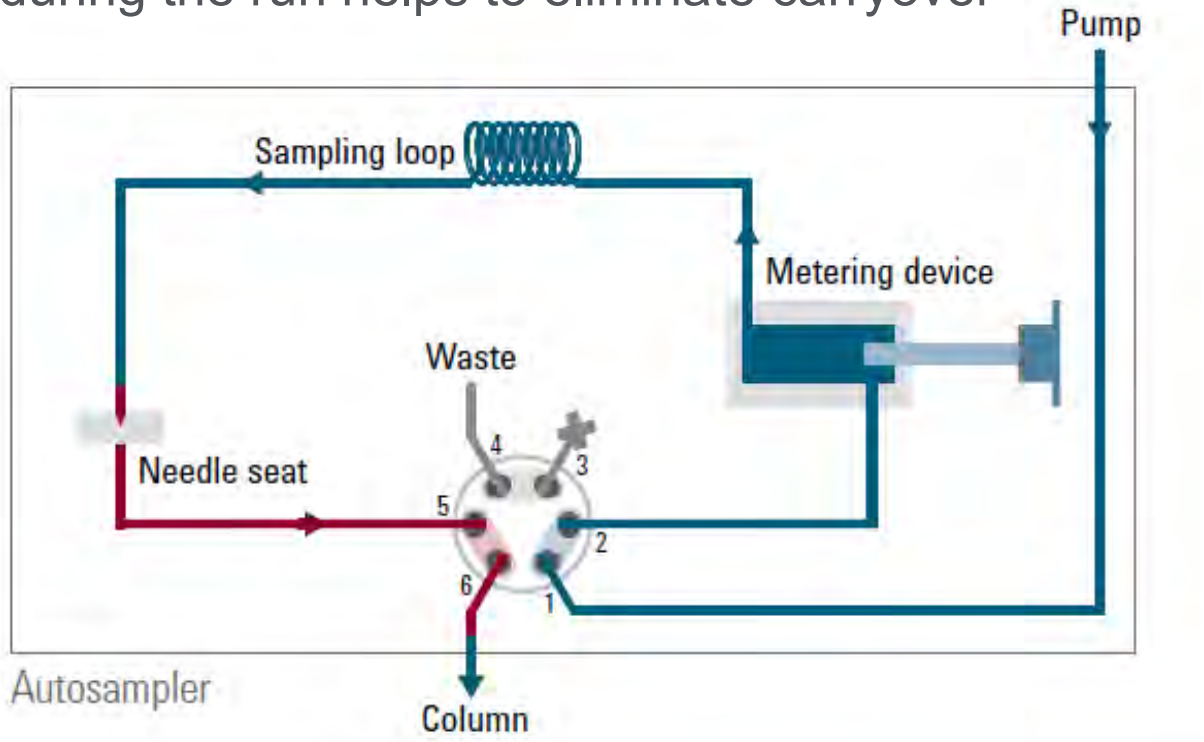
- To achieve highest reproducibility you must usually overload the sample loop. Partial filling of the loop is possible but reproducibility of this technique is poor.
- To inject smaller or larger volumes with a fixed-loop autosampler, you must typically change the sample loop.



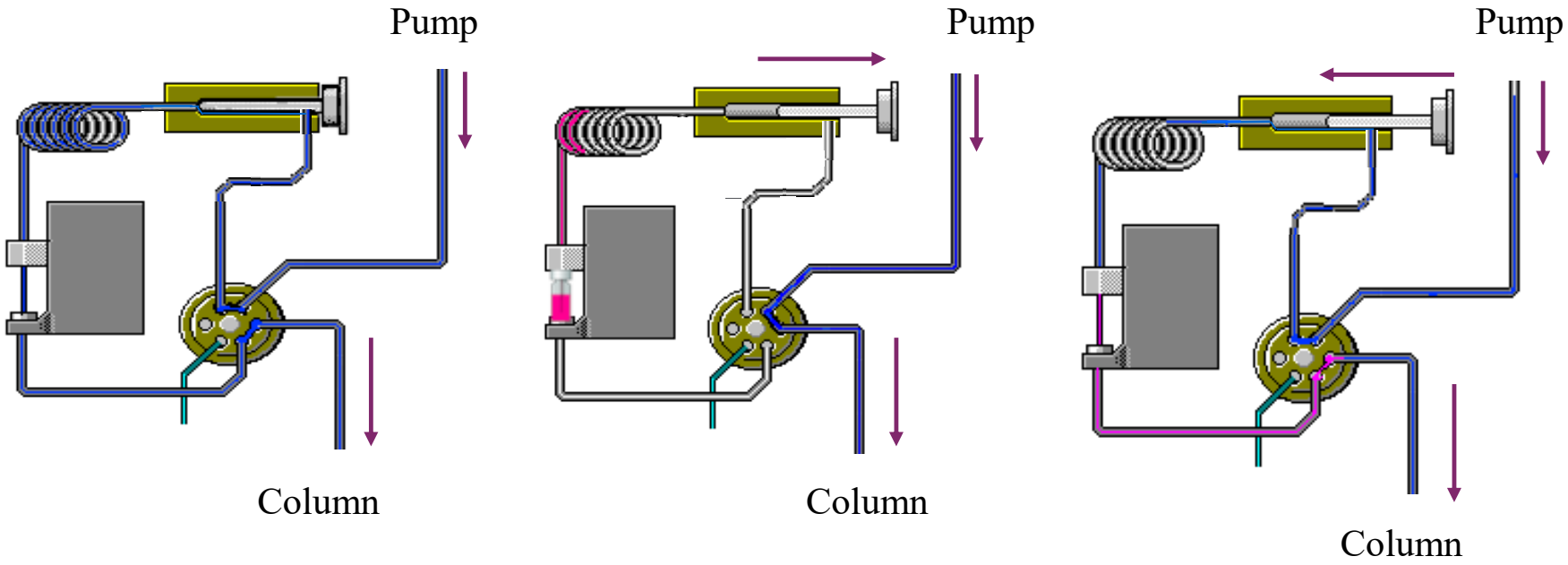
Flow-Through Autosampler
Most Agilent Autosamplers

The mobile phase passes through the sample loop as well through the metering device and injection needle.

- The major advantage of a flow-through design is more flexibility in terms of the injection volume range
- Flowing through the needle during the run helps to eliminate carryover



Principle of Operation



Prior to Injection

Valve in Mainpass Position

Draw Sample

Valve in Bypass Position

Injection and Run

Valve in Mainpass Position

Thermostatted Column Compartment

Important performance characteristics

Excellent temperature accuracy →

Excellent temperature precision →

Influence on...

- Elution order
- Peak identification

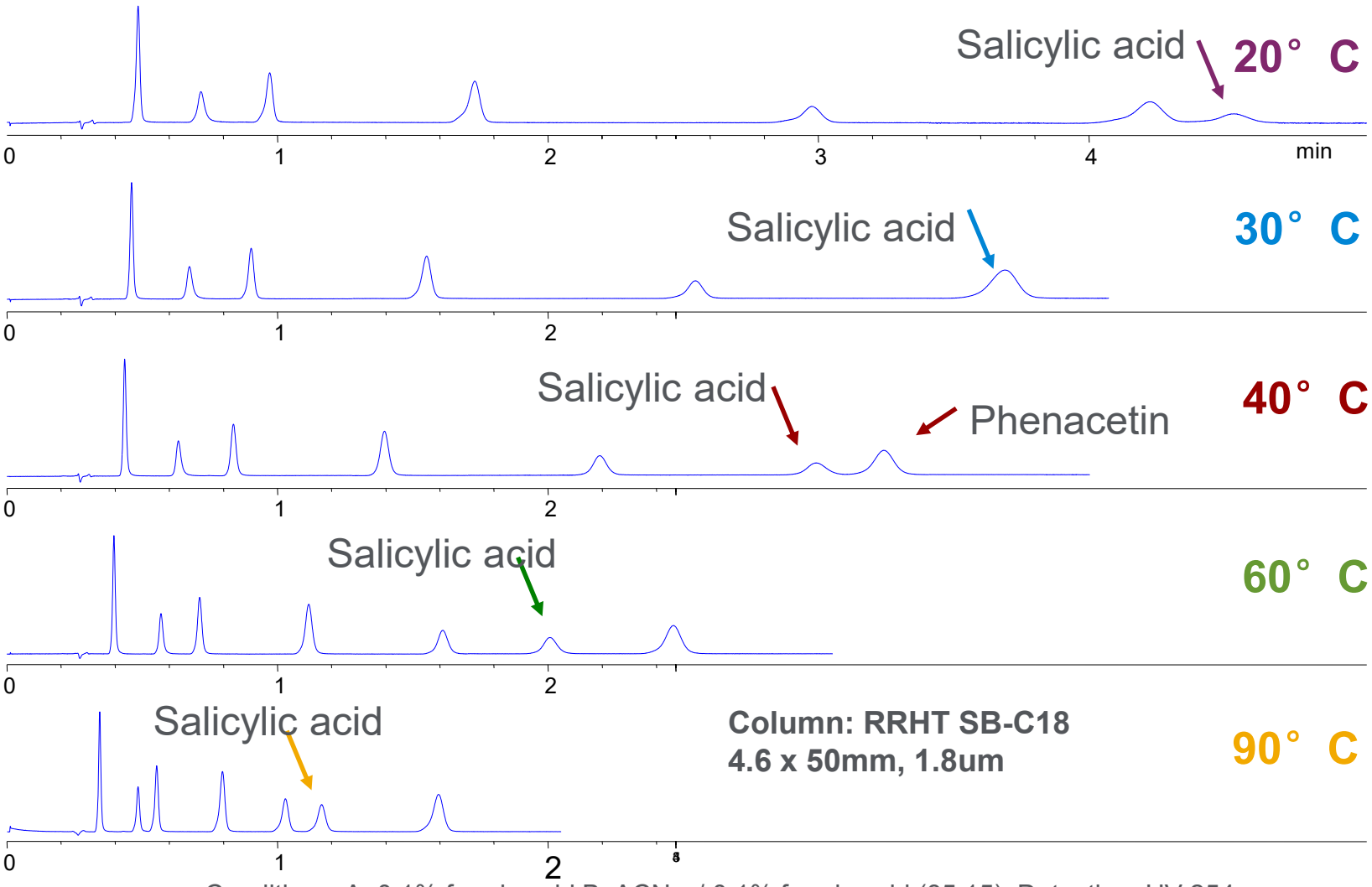
- Elution order
- Retention time precision
- Peak identification

Column Compartment

- Temperature Settings
- Precolumn Heating
  - Needed for analyses done at non-ambient condition
  - Columns with smaller IDs (<4.6mm) and shorter length (<250mm) will see greater temperature disparities from front to back of column due to insufficient mobile phase heating

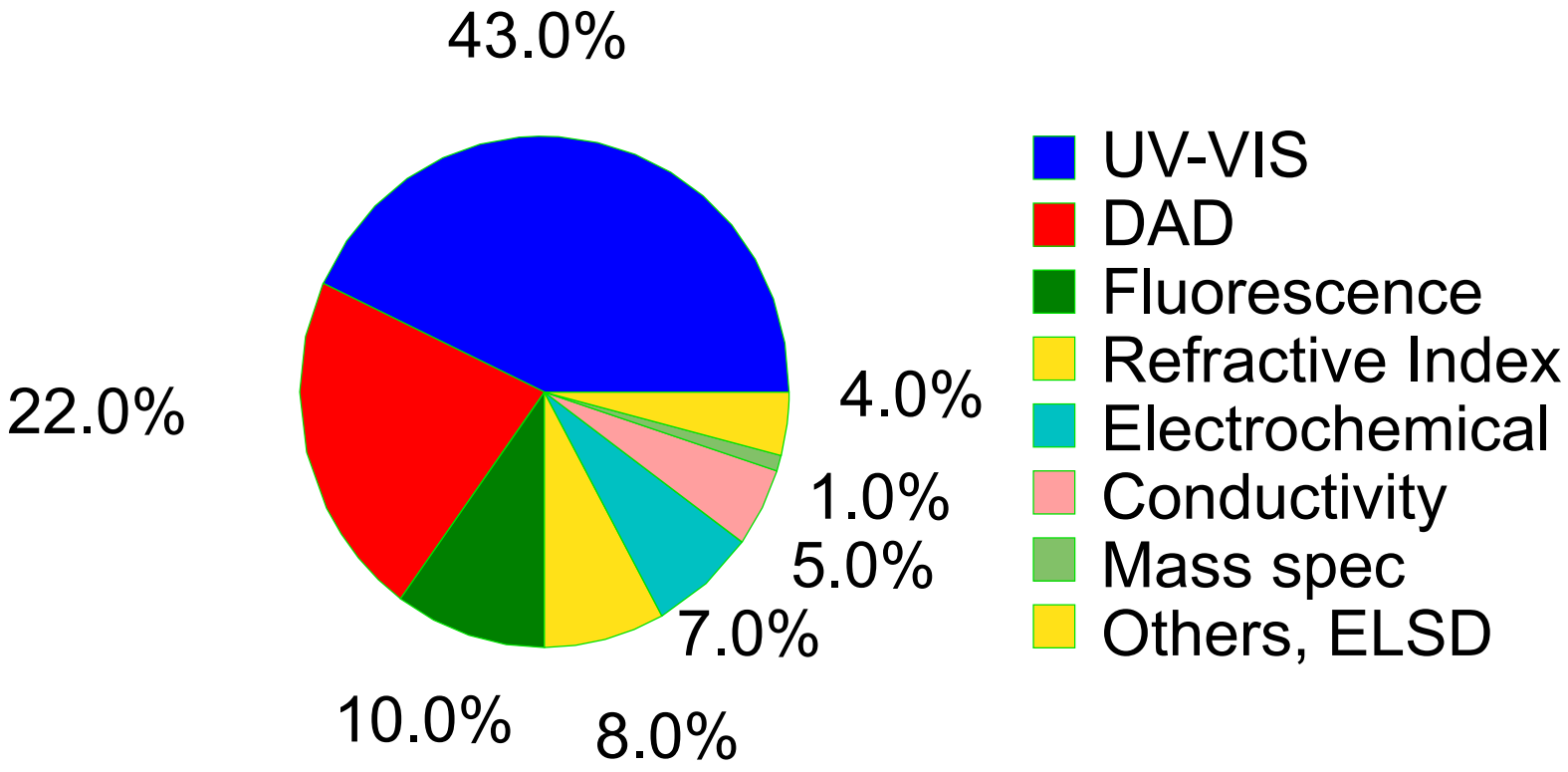
High Efficiency Heat Exchangers

Effect of Temperature on Separation



Conditions: A: 0.1% formic acid B: ACN w/ 0.1% formic acid (85:15) Detection: UV 254 nm

HPLC Detector Usage



HPLC Detector Characteristics

Detector Type	Sensitivity	Selectivity	Useful % of Compounds	Advantages
VWD	ng/pg	-	80	Low cost
DAD	ng/pg	++	80	Peak confirmation
Fluorescence	pg/fg	++	10	High sensitivity
Electro-chemical	pg/fg	+	>20	High sensitivity
Conductivity	ng/pg	-	10	Ion chromatography
Refractive Index	µg/ng	-	100	Universal resposne
Mass Spectrometer	ng/pg	++	<100	**MW structural information**

UV-Based Detectors

Fixed or Variable Detector

- One Wavelength or programmable Wavelength

Diode Array Detector

- Multiwavelength
- Spectra (Library, Peak Purity)

Variable Wavelength (VWD) UV/VIS Detector Optical System

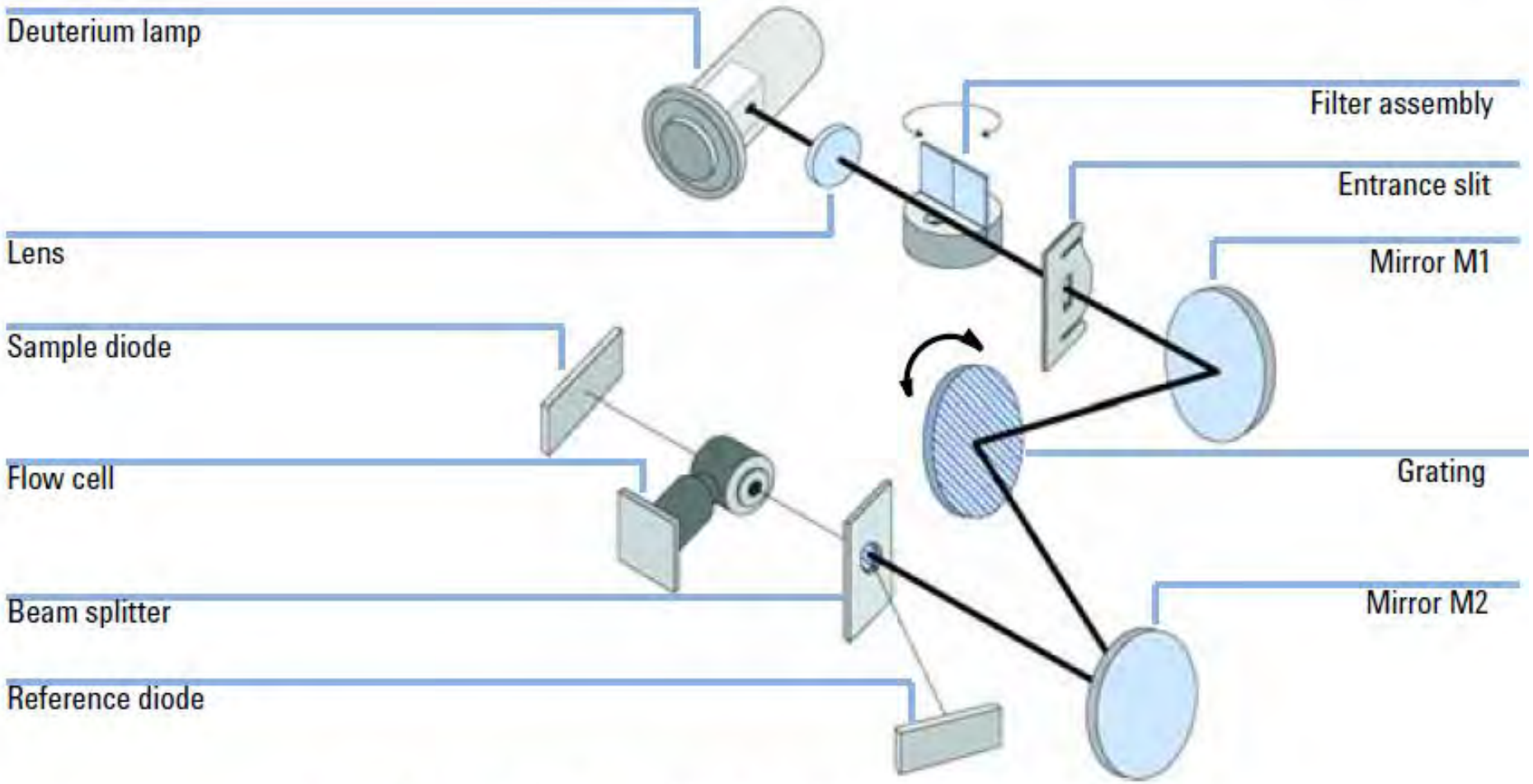
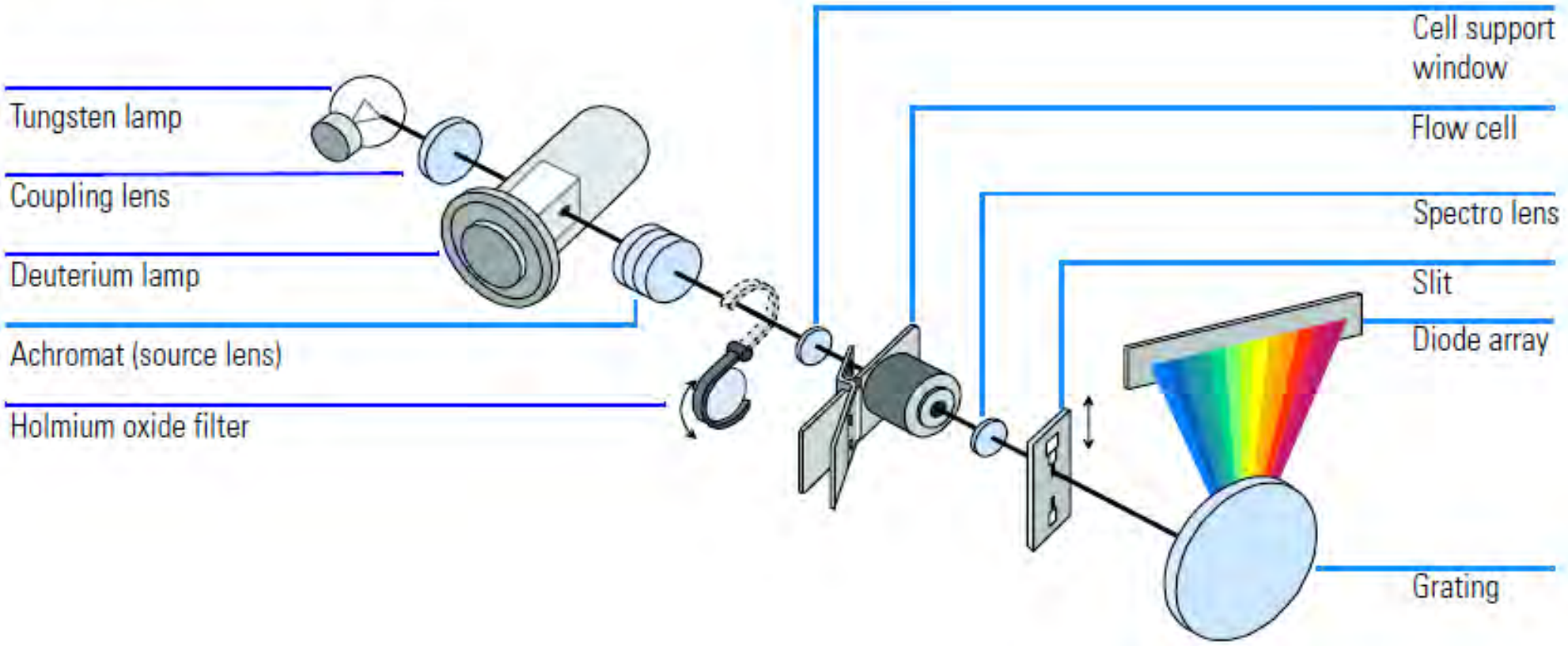


Figure 1 Optical Path of the Variable Wavelength Detector

Diode Array Detector (DAD) UV/VIS Optical System

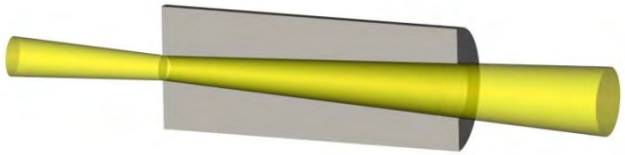
Figure 59 **Optical System of the Detector**



Detector – Baseline noise
Type and geometry of flow cell

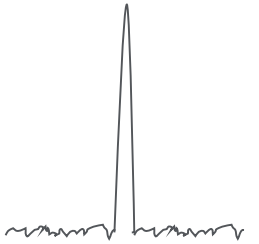
4.6 mm id column

+



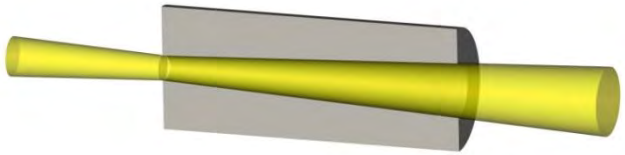
10 mm path length
13 µL volume

=



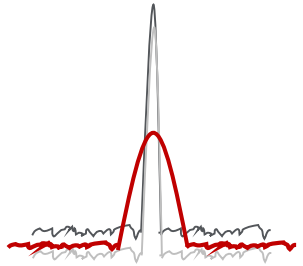
Short 2.1 mm id column

+



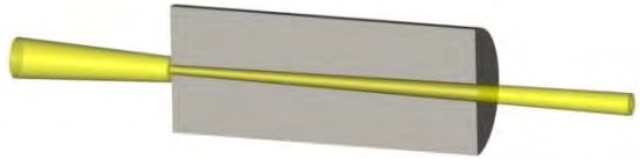
10 mm path length
13 µL volume

=



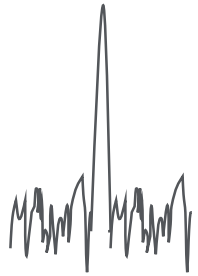
Short 2.1 mm id column

+

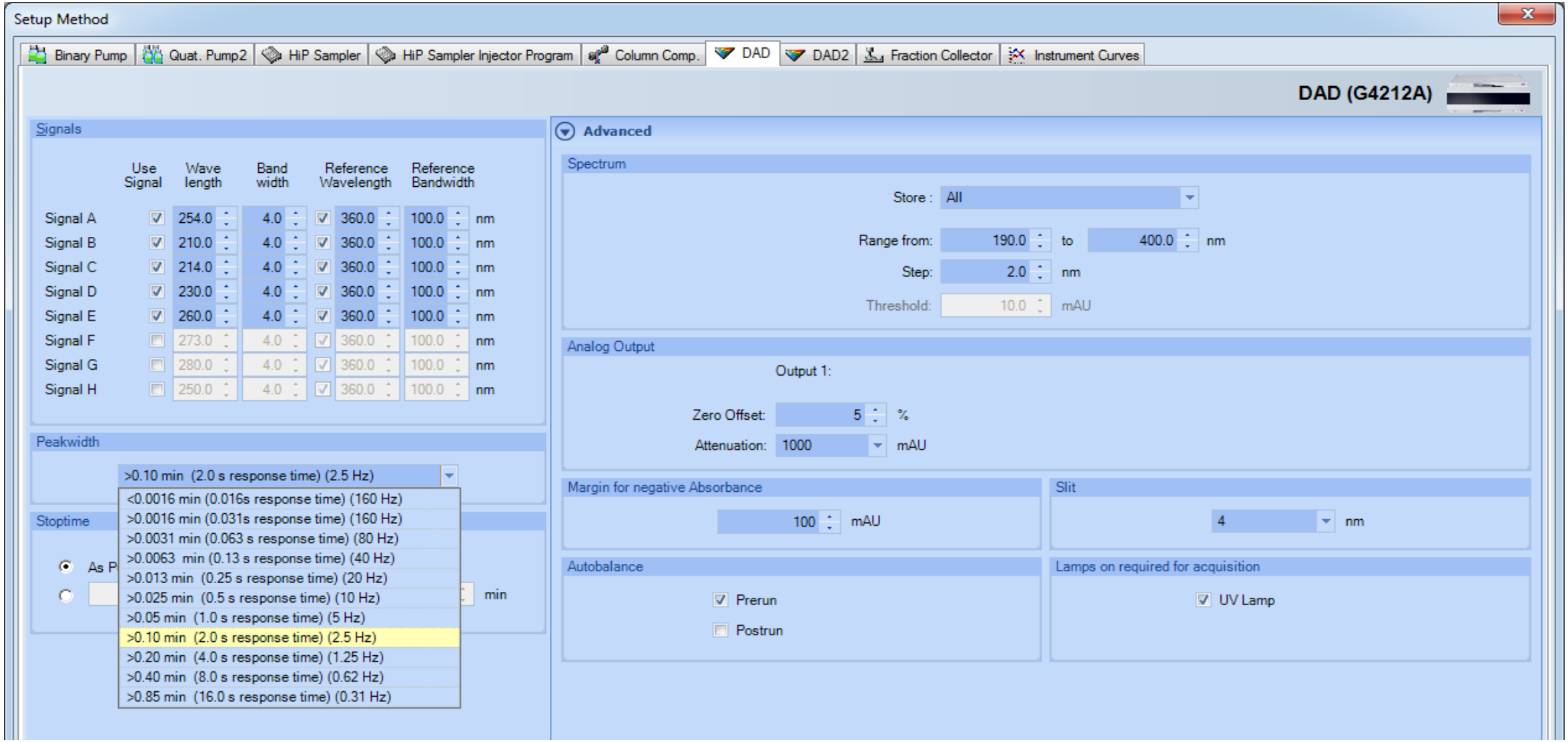


Long path length (10 mm, 0.5 µL)
Low light transmission. => high noise

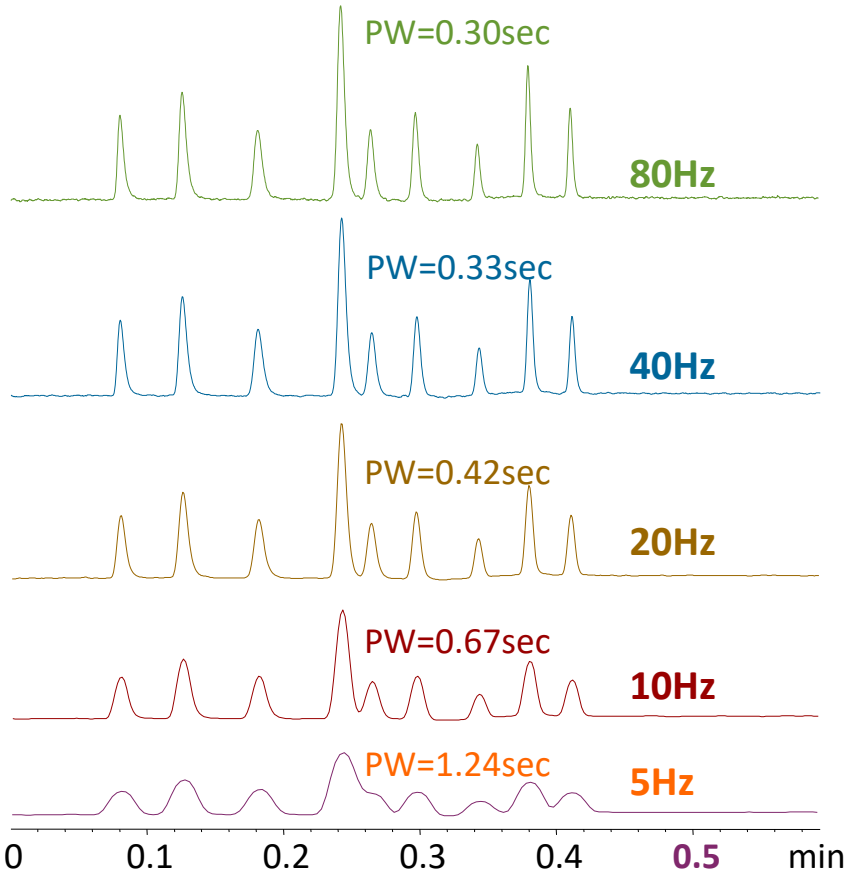
=



Data Rate Setting



Detectors
For narrower peaks, increase data rates!
Maintain Resolution at High Analysis Speed



80Hz versus 10Hz (20Hz) Data Rate

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

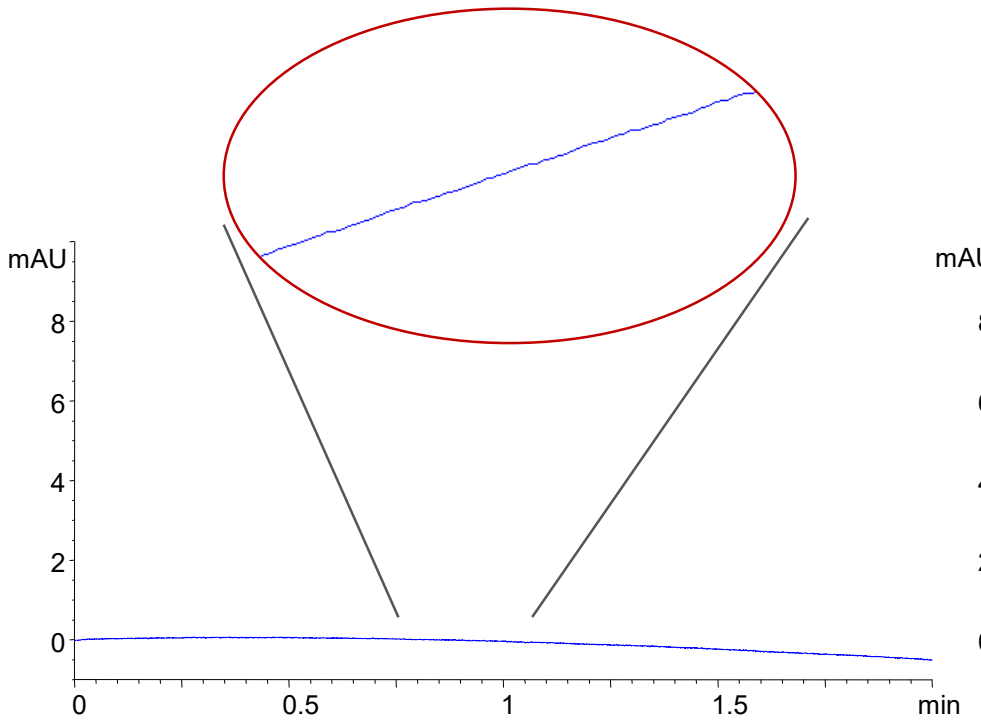
Data Rate	Peak Width	Resolution	Peak Capacity
80 Hz	0.300	2.25	60
40 Hz	0.329	2.05	55
20 Hz	0.416	1.71	45
10 Hz	0.666	1.17	29
5 Hz	1.236	0.67	16

Sample: Phenones Test Mix
Column: Zorbax SB-C18, 4.6x30, 1.8um
Gradient:: 50-100%ACN in 0.3min
Flow Rate: 5ml/min

Detector – Baseline noise
Data rate

Data rate = 10 Hz

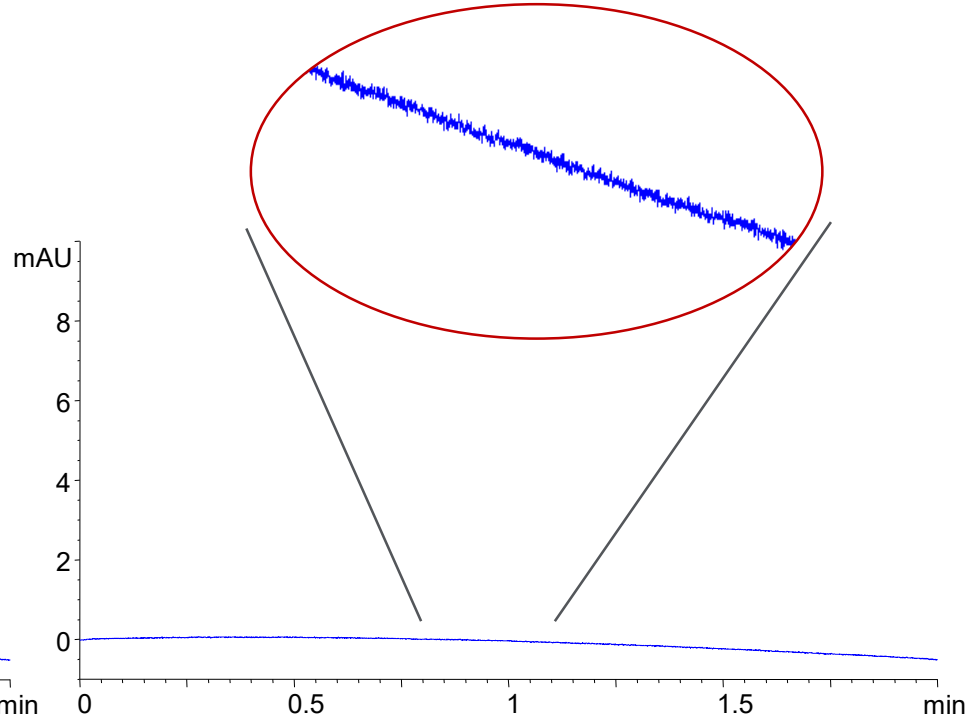
$Noise_{(PtoP,\ 0.5 – 1.5\ min)} = 1.48 \times 10^{-2}$



$File\ size_{(DAD1A.ch)} = 9\ KB$

Data rate = 160 Hz

$Noise_{(PtoP,\ 0.5 – 1.5\ min)} = 5.43 \times 10^{-2}$



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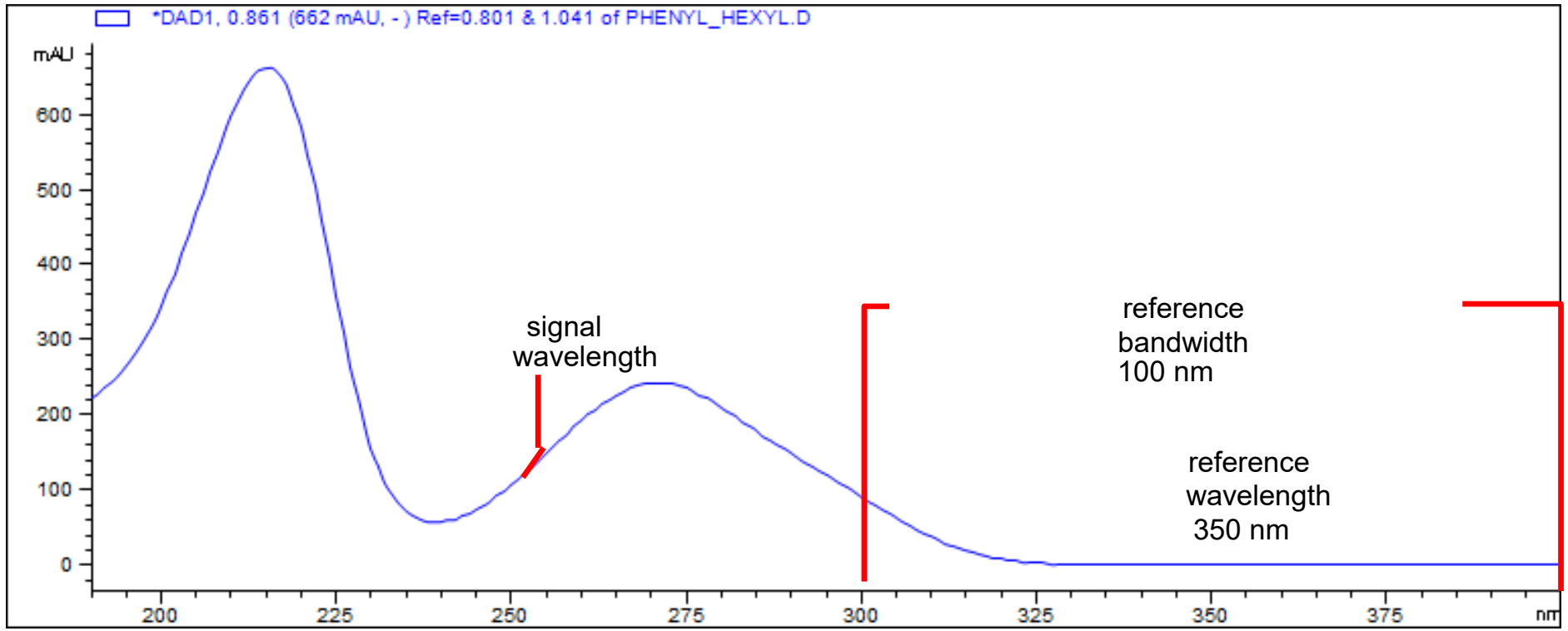
Total Signal with Diode Array Detection



$$\frac{\text{Absorbance}_{\text{Sample wavelength}} + \text{Absorbance}_{\text{averaged over Bandwidth}}}{\text{\# of wavelengths used}} - \frac{\text{Absorbance ref}_{\text{wavelength}} + \text{Absorbance}_{\text{averaged over Bandwidth}}}{\text{\# of wavelengths used}} = \textbf{Total Absorbance}$$

Care of Detector Flow Cells

Avoid the use of alkaline solutions with pH > 9.5 which can attack quartz and impair optical performance.

Prevent crystallization of buffers or salts which will lead to blockage and damage.

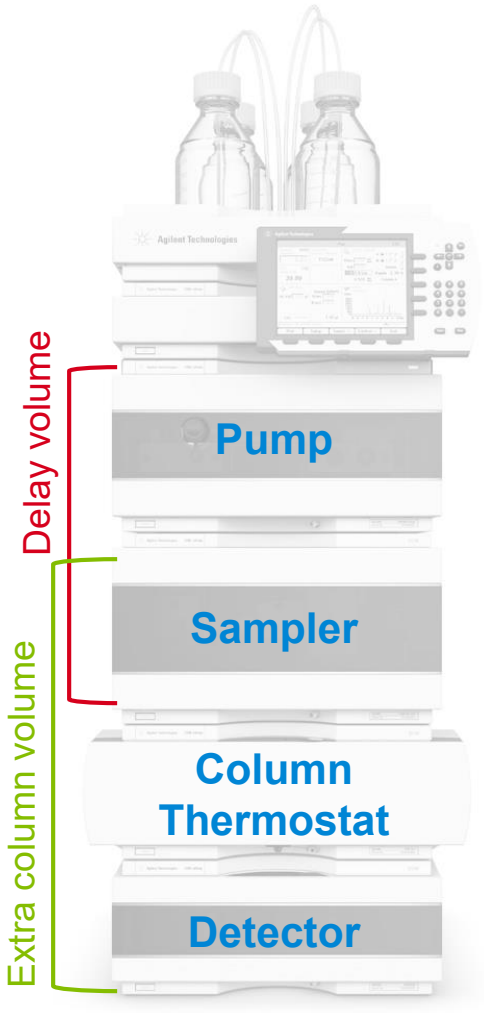
Aqueous solvents can allow algae growth. Don't leave 100% water standing in the flow cell. When leaving LC idle, pump a mobile phase with at least 5-10% of organic solvent.

Observe the pressure limits of flow cells. Be careful when using detectors in series or fraction collectors.

System Optimization
Definitions of Critical Parameters

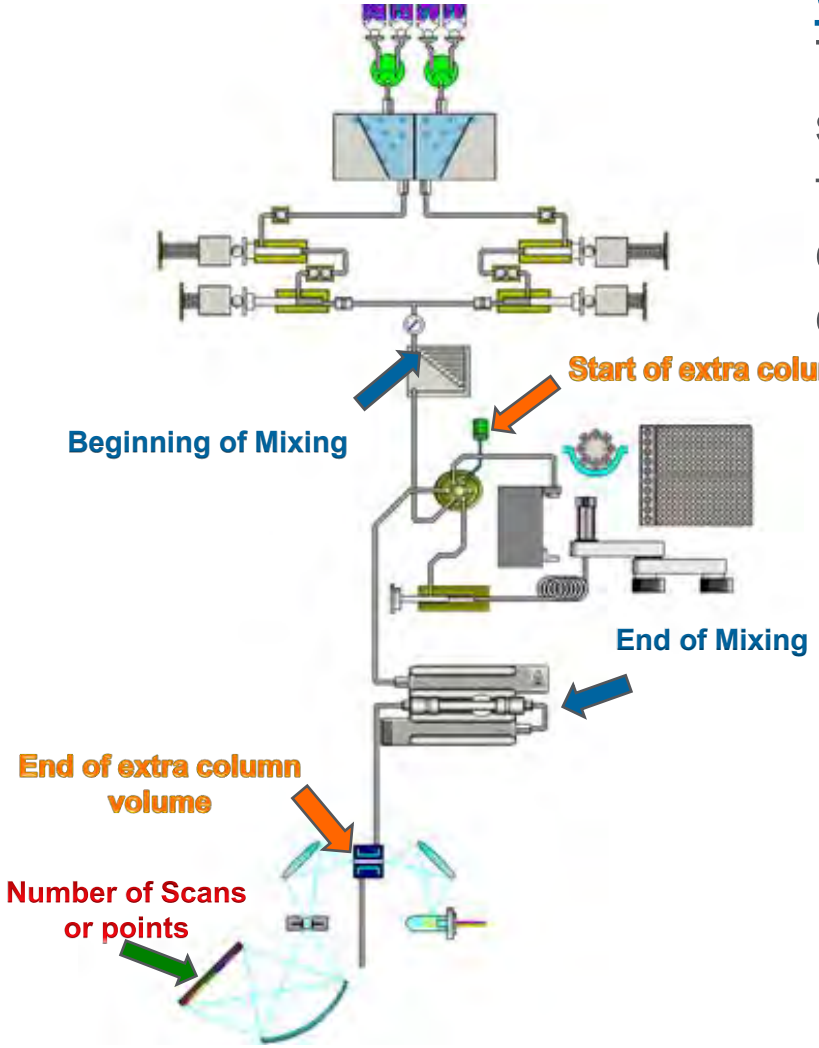
- Delay volumes
- Dispersion

Typical HPLC System -Important Parameters



Delay volume Gradient mixing behavior Pressure x flow rate	Retention Time Resolution
Delay volume Extra column volume Injection volume	Retention Time Resolution Sensitivity
Temperature profile Extra column volume	Retention Time Resolution
Data rate Extra column volume Path-length	Resolution Retention Time Sensitivity

Considerations for HPLC systems



Gradient Delay or Dwell Volume

The volume between the point of mixing of solvents (usually in the mixing chamber or at the proportioning valves in the liquid chromatograph) and the head of an LC column.

Extracolumn Volume

The volume between the effective injection point and the effective detection point, excluding the part of the column containing the stationary phase. It comprises the volumes of the injector, connecting lines and frits, and the detector. It determines the ***extracolumn effects.***

Conclusions

This Presentation was intended to be an introduction to The High Performance Liquid Chromatography Instrument. The HPLC is a widely used and powerful tool in analytical chemistry.

We discussed:

Typical HPLC Instrumentation

Understanding the Flow Path

- Pump
- Autosampler
- Column Compartment
- Common HPLC Detectors

General Maintenance and Housekeeping

General Settings

Resources for Support

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

Thank you!!!!

Questions?