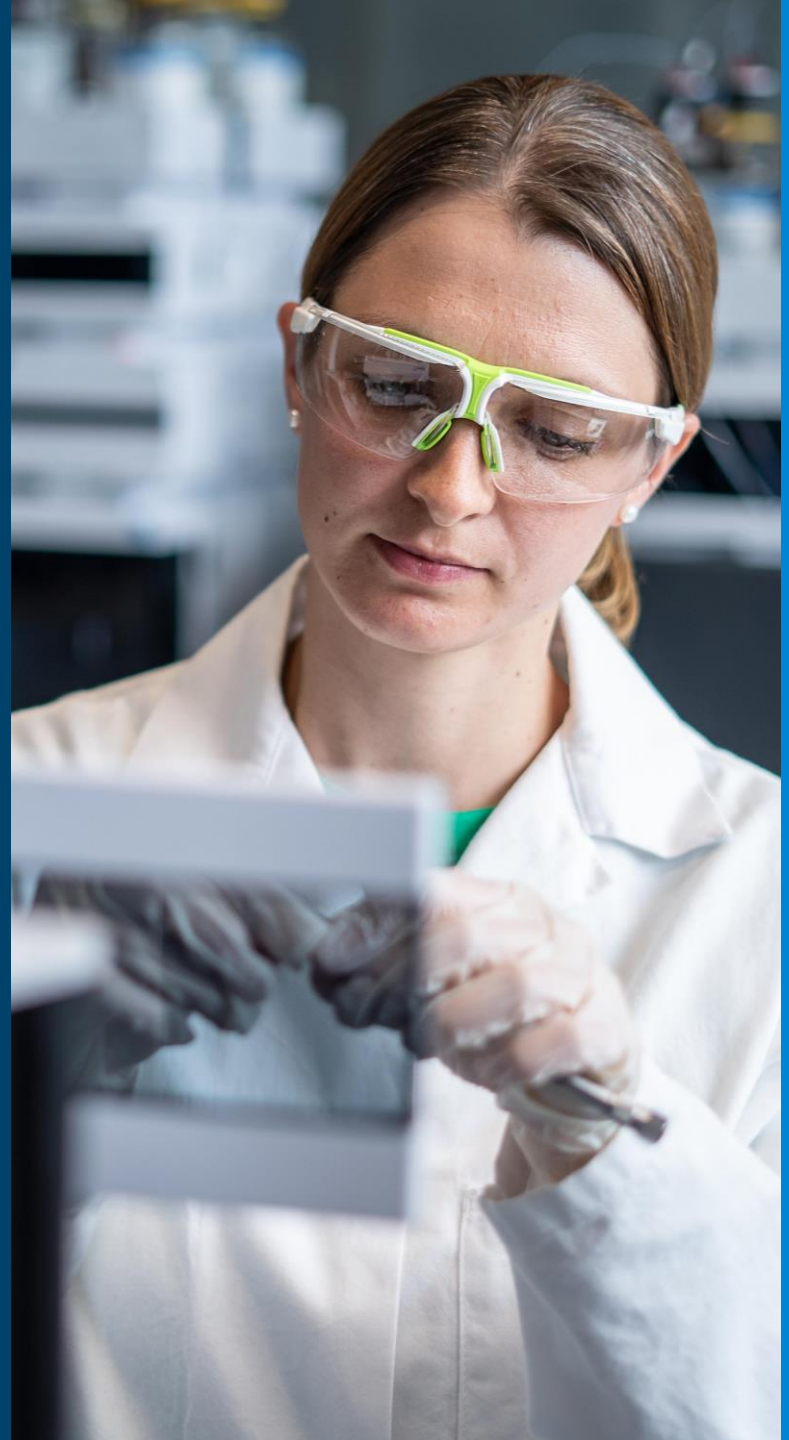
What Do I Need for Successful Method Development?

Mark Powell
Columns and Supplies Technical Support
December 10, 2020



Agilent
Trusted Answers

What is My Method Development Plan?

1. Smaller particles and superficially porous particles offer fast, efficient analysis
2. C18 column – general-purpose column choice
3. Simple mobile phase
   a) Formic acid or other additive in aqueous portion (buffer salts if necessary)
   b) Acetonitrile or methanol as organic modifier
4. Start with linear gradient (5% organic to 95% organic) for reversed-phase methods
5. Adjust mobile phase to get the desired retention and resolution
   a) Adequate resolution of all peaks, Rs ≥2.0
   b) Retention of first peak at least k=1
   c) Fastest analysis time with required resolution

Shorter columns with small particle sizes can provide more efficiency and resolution in a very short time, speeding up method development

What Column Do I Choose?

Smaller particle size offers

- Higher efficiency, shorter column, faster method
- Increased resolution
- Better sensitivity

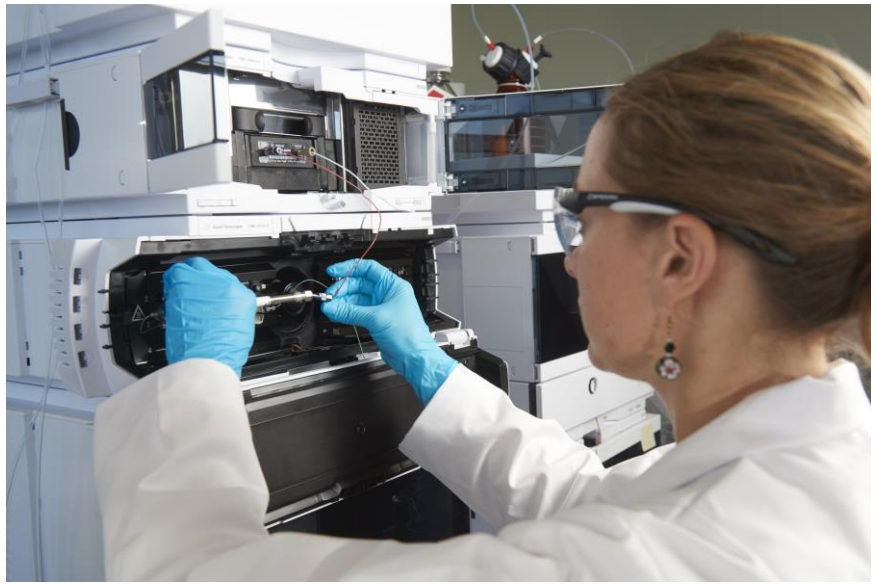
But make sure to consider pressure limit of instrument

Smaller diameter means

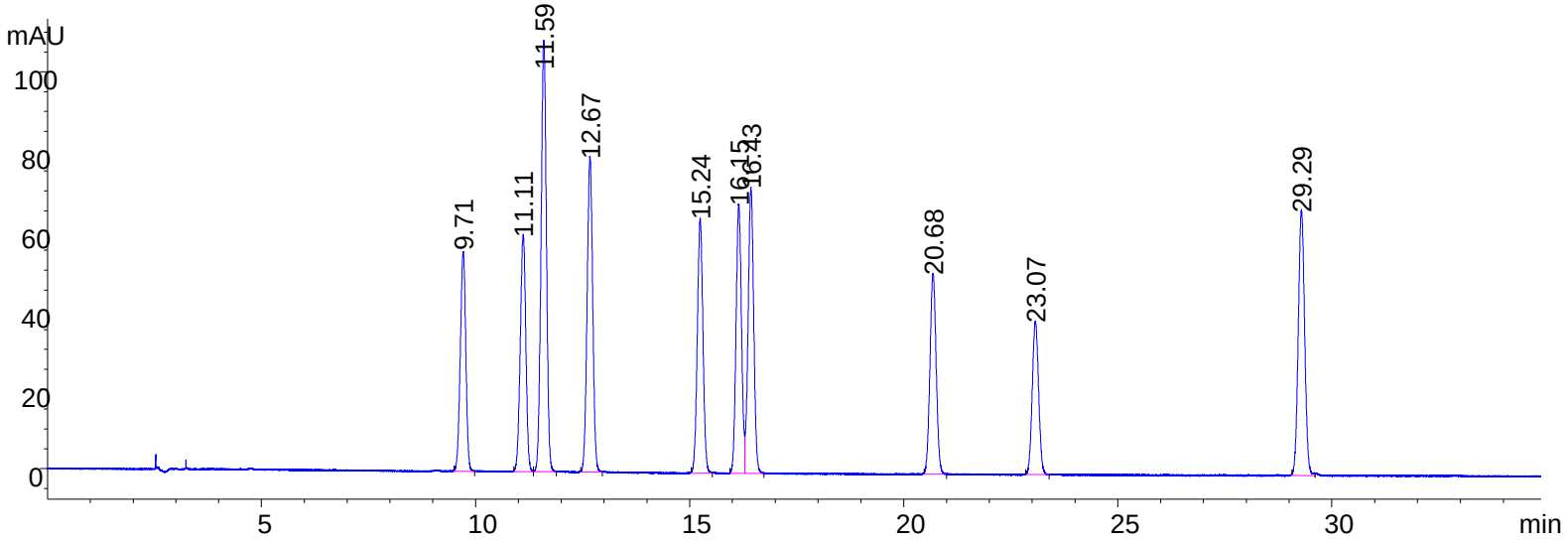
- Solvent savings

But this depends on instrument configuration and plumbing

- Bonded phase choices
  - Alternate selectivity
  - Match to pH of mobile phase
  - More robust column life



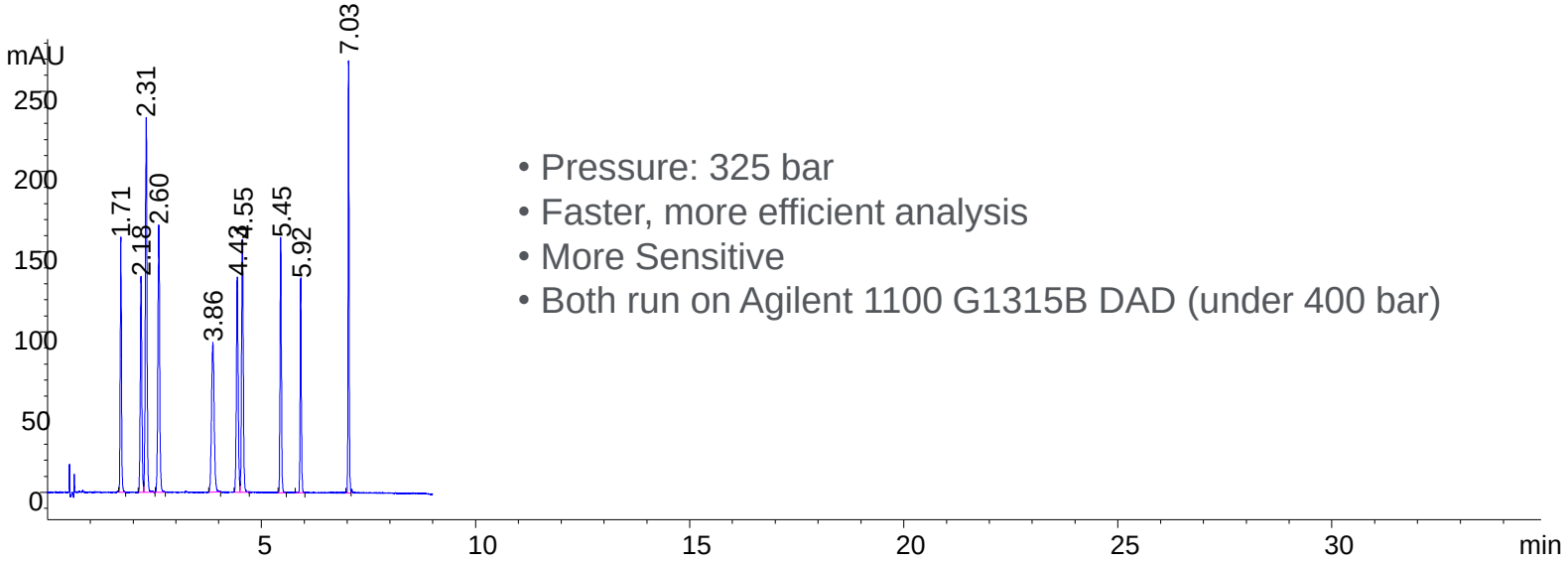
What Particle Do I Choose?



Totally Porous Particle

ZORBAX Eclipse Plus C18
4.6 x 250 mm, 5 µm

Runtime: 35 min



- Pressure: 325 bar
- Faster, more efficient analysis
- More Sensitive
- Both run on Agilent 1100 G1315B DAD (under 400 bar)

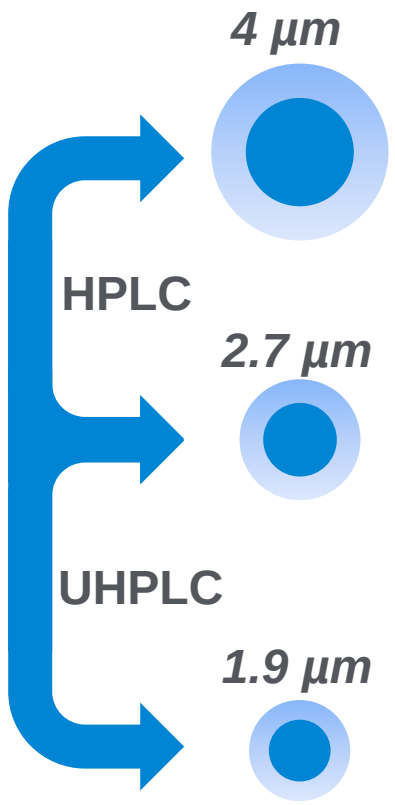
Poroshell Particle

InfinityLab Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 µm

Runtime: 9 min

A: 0.1% Formic Acid in water, B: ACN
Gradient: 8-33% ACN in 30 or 8 min
1 or 2 mL/min, 25 °C, 254 nm
Agilent App Note, 5990-5572EN

What Particle Size Do I Choose?



Improved HPLC performance
Pressure rating: 600 bar
Typical pressure: Often <200 bar
Efficiency: ~200% of 5 µm totally porous

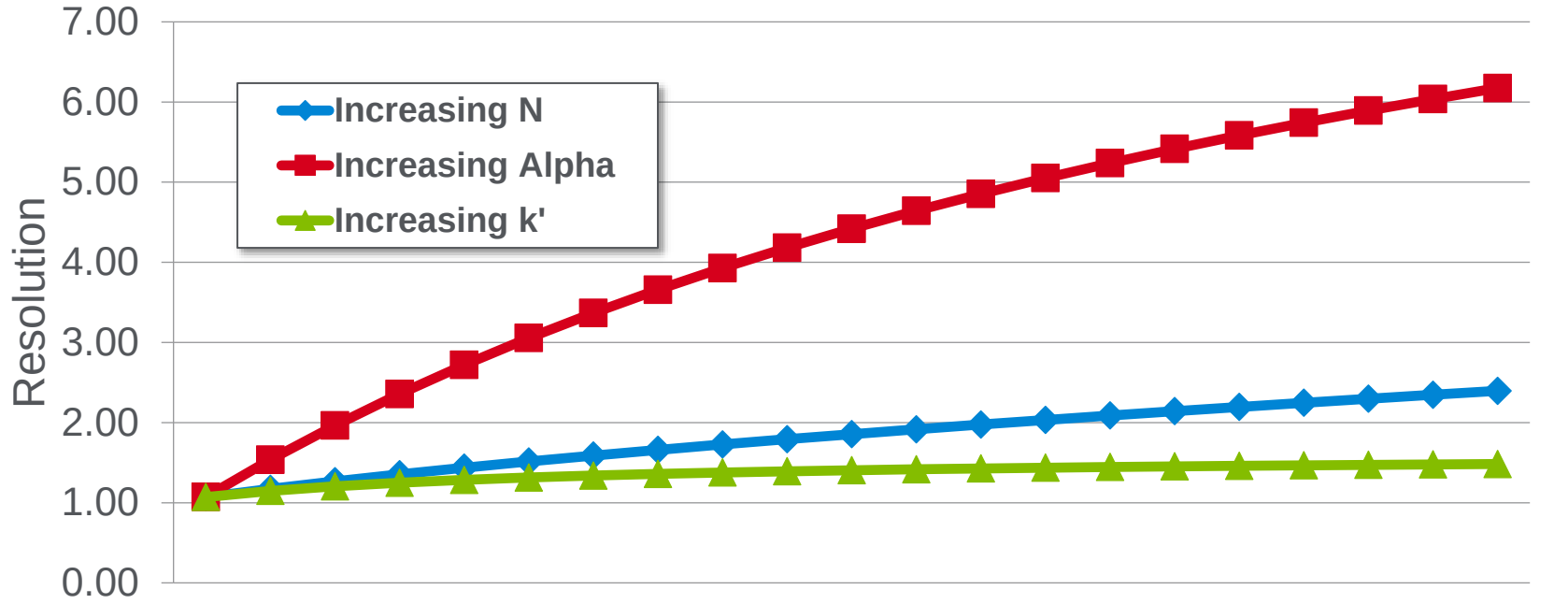
UHPLC performance at lower pressure
Pressure rating: 600 bar
Typical pressure: 50% of sub-2 µm totally porous
Efficiency: ~90% of sub-2 µm totally porous

Highest UHPLC performance
Pressure rating: 1300 bar
Typical pressure: Similar to sub-2 µm totally porous
Efficiency: ~120% of sub-2 µm totally porous

Factors that Affect Resolution

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution **Efficiency** **Selectivity** **Retention**



Plates:	**5000**	**10000**	**15000**	**20000**	**25000**
Alpha:	**1.10**	**1.35**	**1.60**	**1.85**	**2.1**
k	**2.0**	**4.5**	**7.0**	**9.5**	**12.0**

Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

Evaluate Different Bonded Phases

- Bonded phase affects selectivity (alpha)
- Different interactions for polar and non-polar compounds.
- Exploit other interactions with bonded phase (e.g., pi-pi)
- Changing the bonded phase can improve selectivity/resolution,
- May reduce analysis time
- Having different bonded phases available on the same particle makes development easier

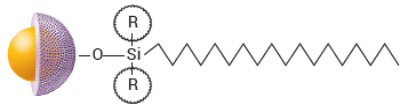
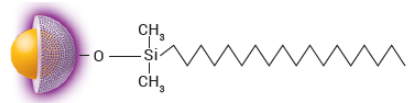
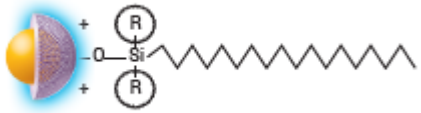
Evaluating different bonded phase chemistries early can save time in optimization and generate a more robust method

The Poroshell 120 Family

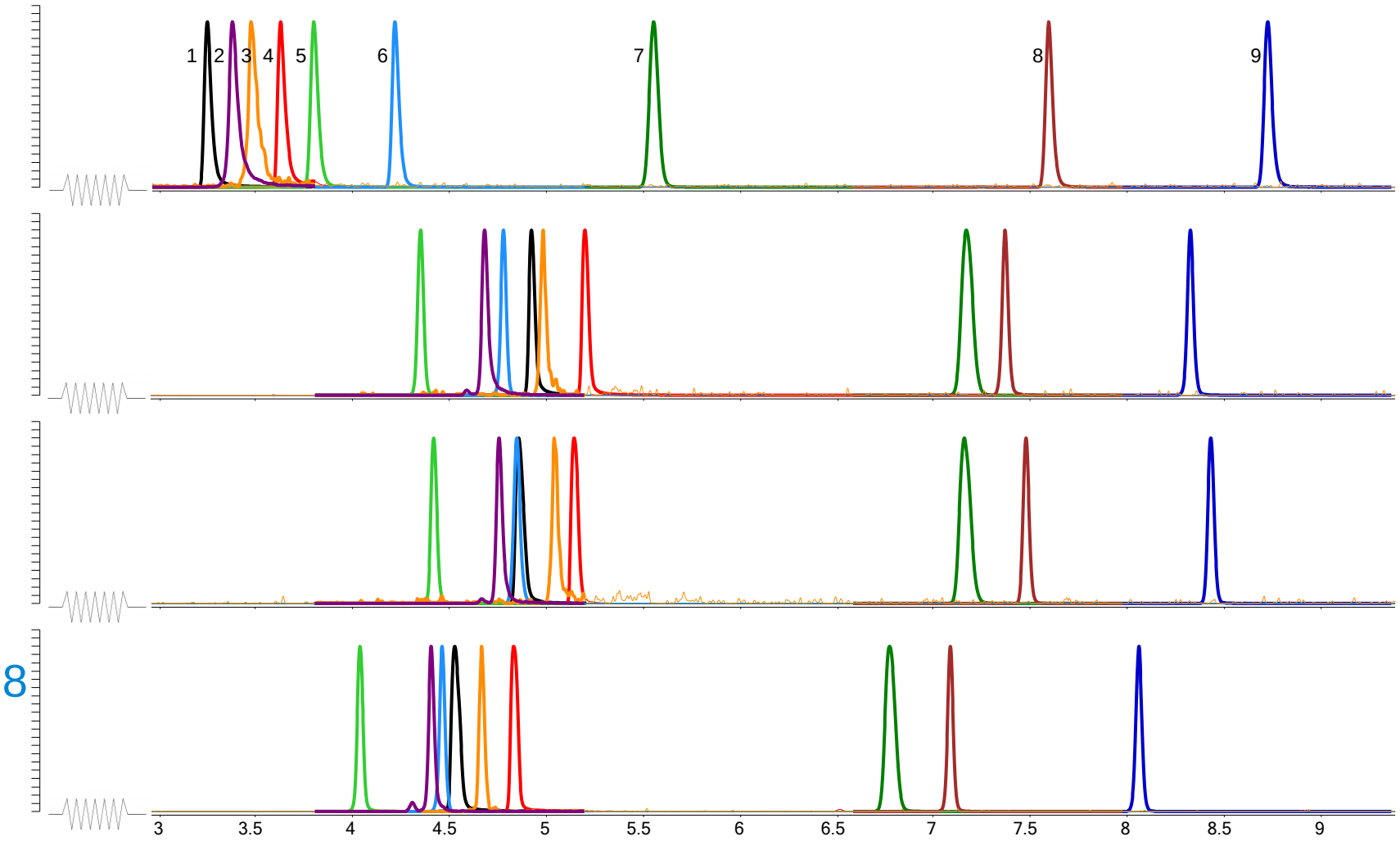
InfinityLab Poroshell 120 offers a broad portfolio to suit your needs

Best All Around	Best for Low pH Mobile Phases	Best for High pH Mobile Phases	Best for Alternative Selectivity	Best for More Polar Analytes	Chiral
EC-C18 1.9 µm, 2.7 µm, 4 µm	**SB-C18** 1.9 µm, 2.7 µm, 4 µm	**HPH-C18** 1.9 µm, 2.7 µm, 4 µm	**Bonus-RP** 2.7 µm	**SB-Aq** 1.9 µm, 2.7 µm, 4 µm	**Chiral-V** 2.7 µm
EC-C8 1.9 µm, 2.7 µm, 4 µm	**SB-C8** 2.7 µm	**HPH-C8** 2.7 µm, 4 µm	**PFP** 1.9 µm, 2.7 µm, 4 µm	**EC-CN** 2.7 µm	**Chiral-T** 2.7 µm
Phenyl-Hexyl 1.9 µm, 2.7 µm, 4 µm		**CS-C18** 2.7 µm		**HILIC** 1.9µm, 2.7 µm, 4 µm	**Chiral-CD** 2.7 µm
		New!		**HILIC-Z** 1.9 µm, 2.7 µm, 4 µm	**Chiral-CF** 2.7 µm
				HILIC-OH5 2.7 µm	

What C18 Bonded Phase?

InfinityLab Poroshell 120	Chemistry	Pore Size	Endcapped	Carbon Load	Surface Area	Best For
EC-C18 1.9 µm, 2.7 µm, 4 µm		120 Å	Yes	10%	130 m^2/g	General Purpose Excellent peak shape and efficiency for acids, bases, neutrals
SB-C18 1.9 µm, 2.7 µm, 4 µm		120 Å	No	9%	130 m^2/g	Low pH Excellent stability and peak shape in highly acidic conditions
HPH-C18 1.9 µm, 2.7 µm, 4 µm		100 Å	Yes	Proprietary	95 m^2/g	High pH Robust performance and long lifetimes
CS-C18 2.7 µm		100 Å	Yes	Proprietary	95 m^2/g	Alternate selectivity Improved peak shape and sample capacity for basic compounds with low ionic strength mobile phases High pH

Alternative Selectivity with InfinityLab Poroshell 120 C18s

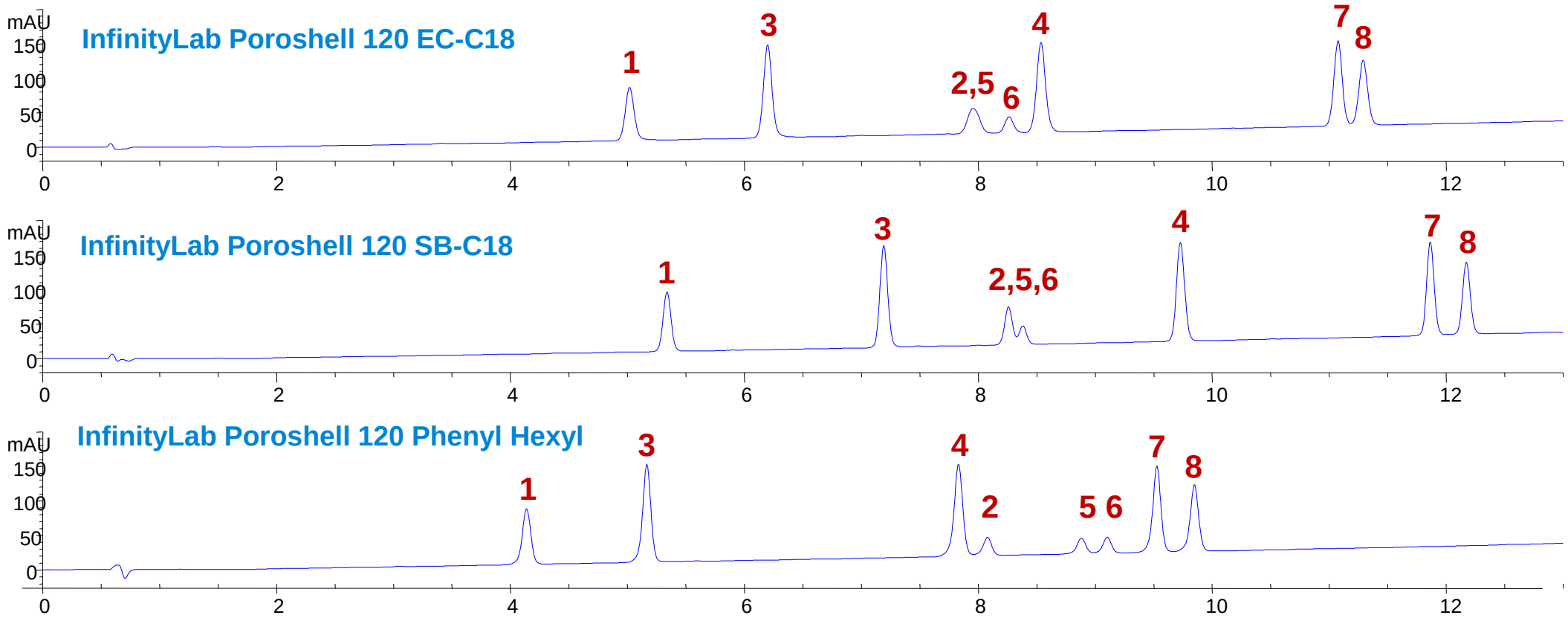


1	Ciprofloxacin	
2	Oxytetracycline	
3	Tetracycline	
4	Enrofloxacin	
5	Sulfamerazine	
6	Sulfamethazine	
7	Erythromycin	
8	Penicillin-G	
9	Oxacillin	

Method parameters:
A: 0.1% formic acid in water
B: acetonitrile
0.4 mL/min, 0-95% B in 15 min
0.05 µL injection
Sample: 0.1 mg/mL in water
Column: 30 °C, 2.1 x 100 mm, 2.7 µm
Detection: LC/MS, ESI+, dMRM

Agilent application note: 5994-2358EN

Selectivity Differences Across InfinityLab Poroshell Bonded Phases



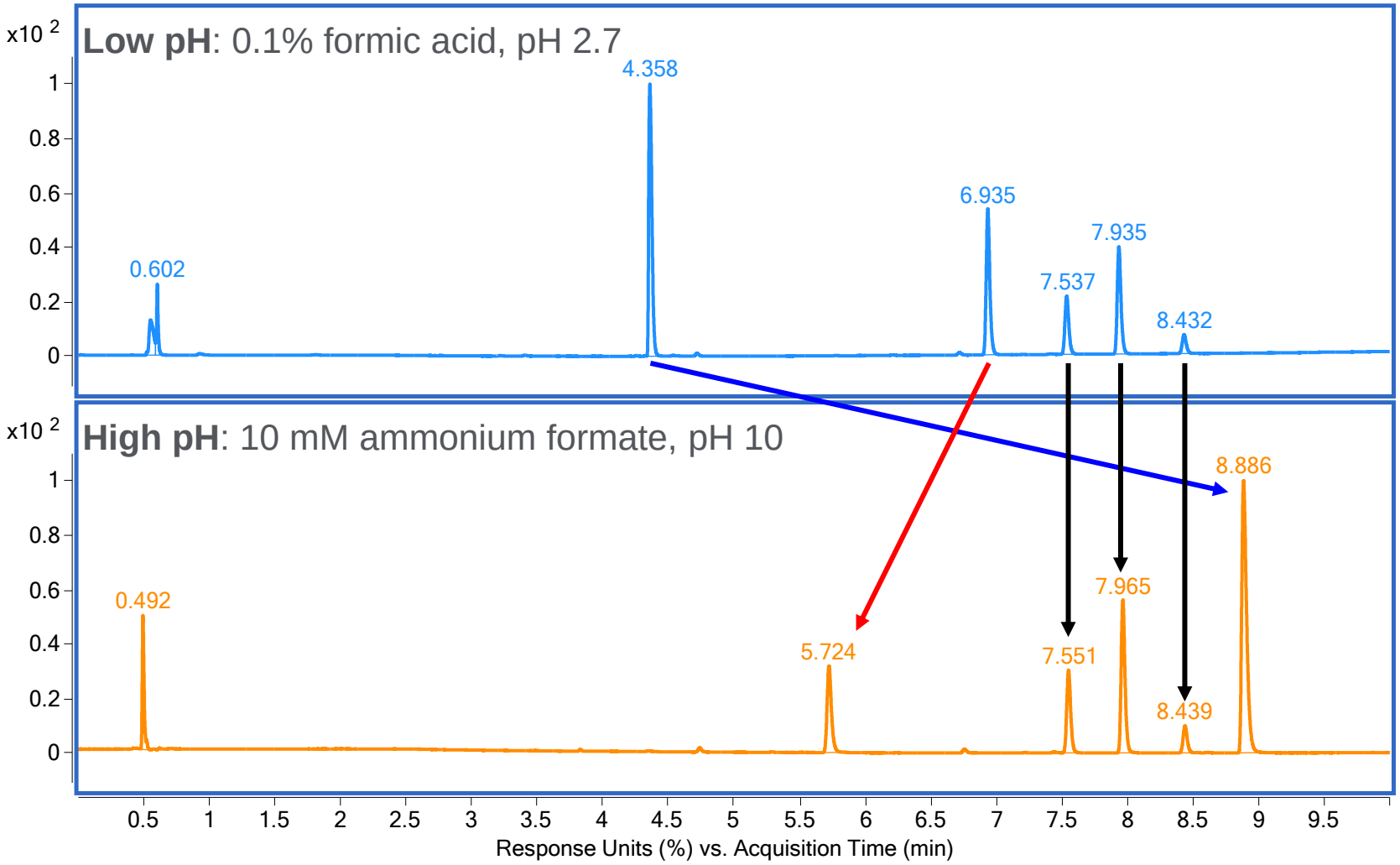
1. Hydrocortisone 2. β-Estradiol 3. Androstatriene-3,17-dione 4. Testosterone
5. Ethinyl estradiol 6. Estrone 7. Norethindrone acetate 8. Progesterone

40-80 % Methanol in 14 min, DAD 260, 80 nm 0.4 mL/min, 2.1 x 100 mm column, 40 C, 0.1% formic acid in water and methanol, Agilent 1260 Method Development Solution

Agilent InfinityLab Poroshell 120 CS-C18

Mobile phase pH is a method development tool for separating ionizable compounds

- With reversed-phase, ionizable analytes are more retained in their neutral state
- **Acids** are more retained at low pH
- **Bases** are more retained at high pH
- **Neutrals** are not affected by mobile phase pH



5-95% CH_3CN in 10 min, 4 min post run, mobile phase A varies, 0.4 mL/min, 2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 CS-C18, 30 °C, DAD: 254 nm, 80 Hz; Sample: uracil, amitriptyline, butyl paraben, dipropyl phthalate, acenaphthene

Agilent application note: 5994-2274EN

What Mobile Phase Modifiers Should I Try?

Mobile Phase	Useable pH range	Recommended for Silica-Based LC Columns?	Recommended for LC/MS Use?
TFA	<1.5	Limited	No
Phosphate	1.1-3.1	Limited	No
Formic acid	<2.8	Yes	Yes
Acetic acid	<3.8	Yes	Yes
Formate	2-8-4.8	Yes	Yes
Acetate	3.8-5.8	Yes	Yes
Carbonate	5.4-7.4	Yes	Yes
Phosphate	6.2-8.2	Limited	No
Bicarbonate	6.6-8.6	Limited	Yes
Ammonia	8.2-10.2	Limited	Yes
Phosphate	11.3-13.3	Limited	No

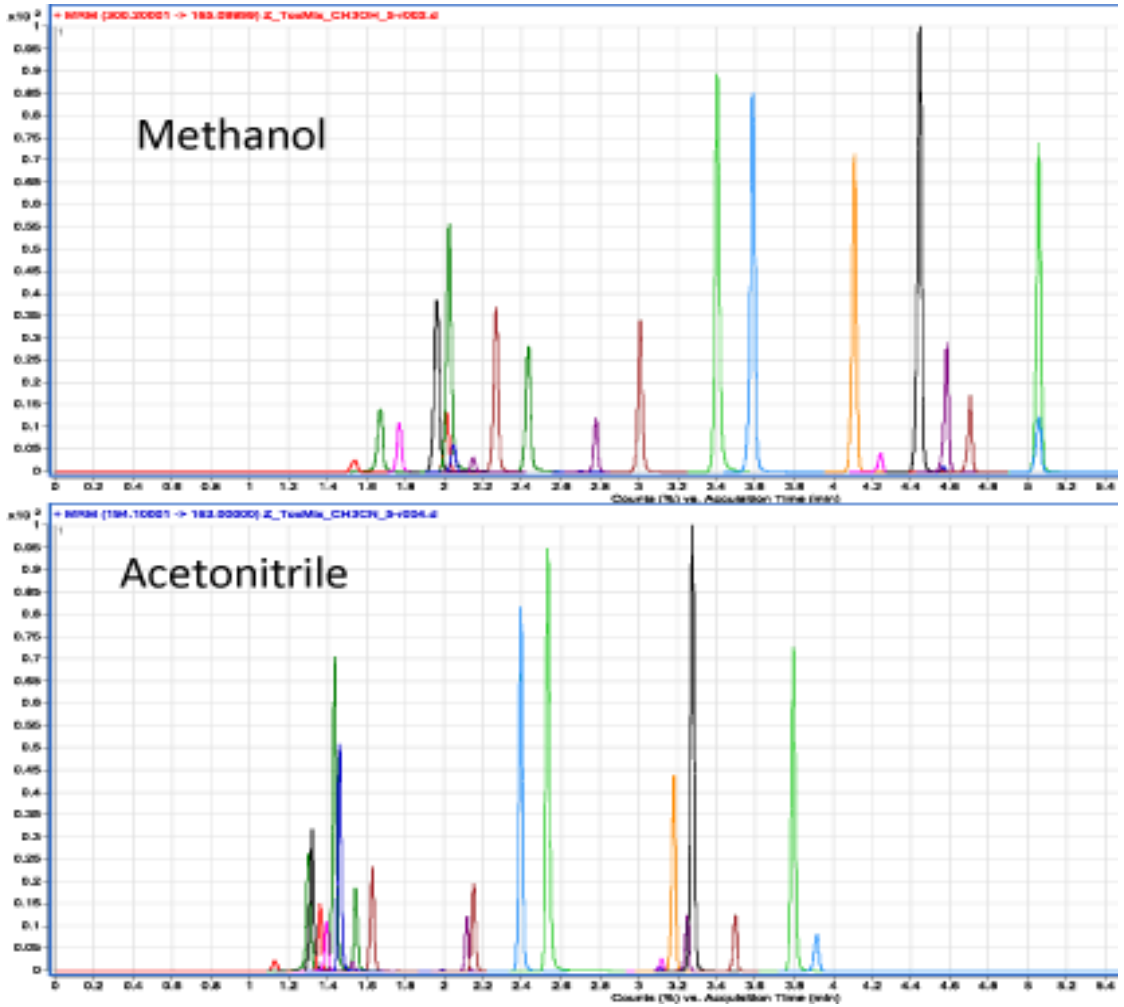
What Organic Solvent Should I Use?

Try both

- ACN and MeOH are readily available
- Works on any bonded phase – optimize separation no matter the column choice

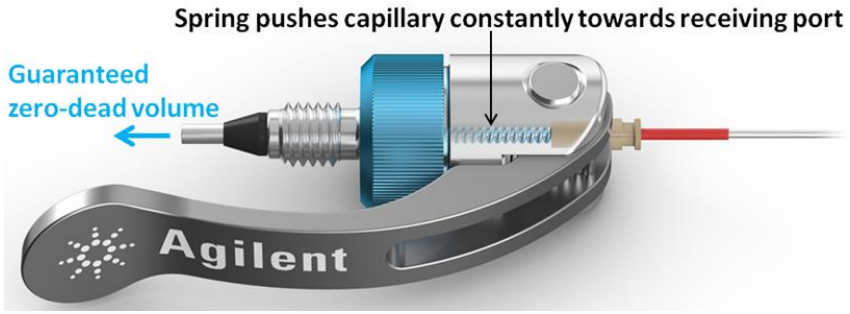
MeOH – Higher pressure, may give better peak shape with bases, protic solvent

Acetonitrile – Aprotic, wider UV window, stronger than MeOH



InfinityLab Quick Connect and Quick Turn Fittings

- Spring loaded design
- Easy, **no tools needed**
- Works for all column types
- Reusable
- Consistent ZDV connection



Quick connect fitting
- Finger tight up to 1300 bar
- Hand tighten the nut, then depress the lever

Quick turn fitting
- Finger tight up to 400 bar
- Up to 1300 bar with a wrench
- Compact design

Tips for Robust Methods

- Always start method development with a new column
- Select columns with robust properties at pH of method
- Choose a quality column with long lifetimes
- Consider batch-to-batch reproducibility
- Consider scalability of particle sizes and chemistries for downstream method transfer
- Make sure mobile phase preparation is documented and transferrable

Agilent employs end-to-end process control for quality LC columns

www.agilent.com/chem/qualitylc

What Should I Do with a New Column?

Performance Report

SERIAL NUMBER: USDAZ01333

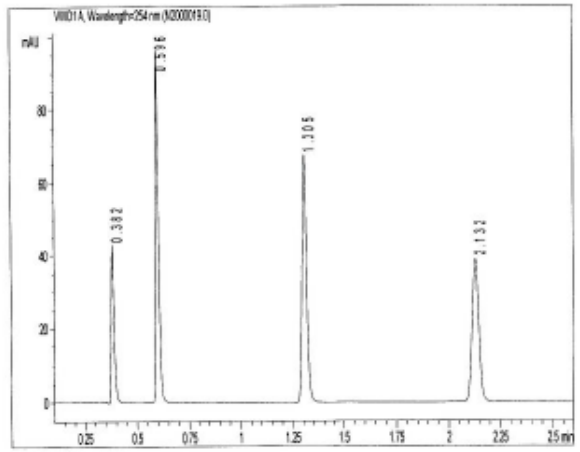
PART NUMBER: 959758-902
COLUMN TYPE: ZORBAX RRHD Eclipse Plus C18 2.1 x 100 mm, 1.8 µm
PACKING LOT #: B09089

TEST CONDITIONS

MOBILE PHASE	=	60% Acetonitrile / 40% Water
COLUMN PRESSURE	=	517.2 Bar
COLUMN FLOW	=	0.50 ml / min
LINEAR VELOCITY	=	0.436 cm / sec
TEMPERATURE	=	AMBIENT (Nominally 23 °C)
INJECTION VOLUME	=	1 µl

QUALITY CONTROL PERFORMANCE RESULTS FOR NAPHTHALENE

	TEST VALUES	SPECIFICATIONS
THEORETICAL PLATES =	22337	MIN = 21000
SELECTIVITY =	1.90	RANGE = 1.82 - 1.92
USP TAILING FACTOR = (@ 5% Peak Height)	1.08	RANGE = 0.98 - 1.20
k' =	4.58	



Sample components with concentrations diluted in mobile phase in the following elution order.

Peak #	Conc (ug/ml)	Sample Component
1	10	Uracil
2	400	Phenol
3	50	4-Chloro Nitrobenzene
4	80	Naphthalene

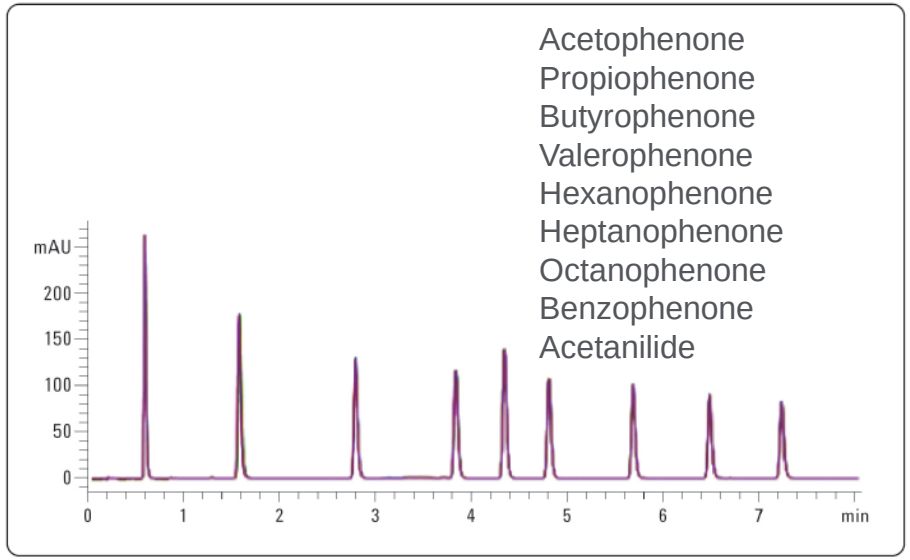
Manufacturing test chromatogram is done on a modified LC system to minimize ECV and will differ from a typical lab HPLC.

- Don’t expect to get the exact same result as the performance report
- Test column performance on your instrument to have as a reference

What Should I Do With a New Column?

Benchmark new column on your system

1. Standard mix; test mix (5188-6529, 01080-68704; QC reference material)
2. Criteria like retention time, peak area, peak tailing, resolution, response, and system pressure
3. Theoretical plates
   - Monitor column over time
   - Troubleshoot



Chromatographic conditions

Sample:	RRLC Checkout sample (p/n 5188-6529)
Column:	Agilent Poroshell 120 EC C18, 3 mm × 50 mm, 2.7 µm
Mobile phase:	A = Water B = Acetonitrile
Gradient:	0 min 20% B 8 min 80% B
Flow rate:	1.2 mL/min
Stop time:	8 min
Post time:	4 min
Injection volume:	1 µL
Column temperature:	30 °C
DAD:	245/10 nm Ref 400/100 nm
Flow cell:	10 mm
Peak width:	<0.025 min (10 Hz)

Mobile Phase Preparation

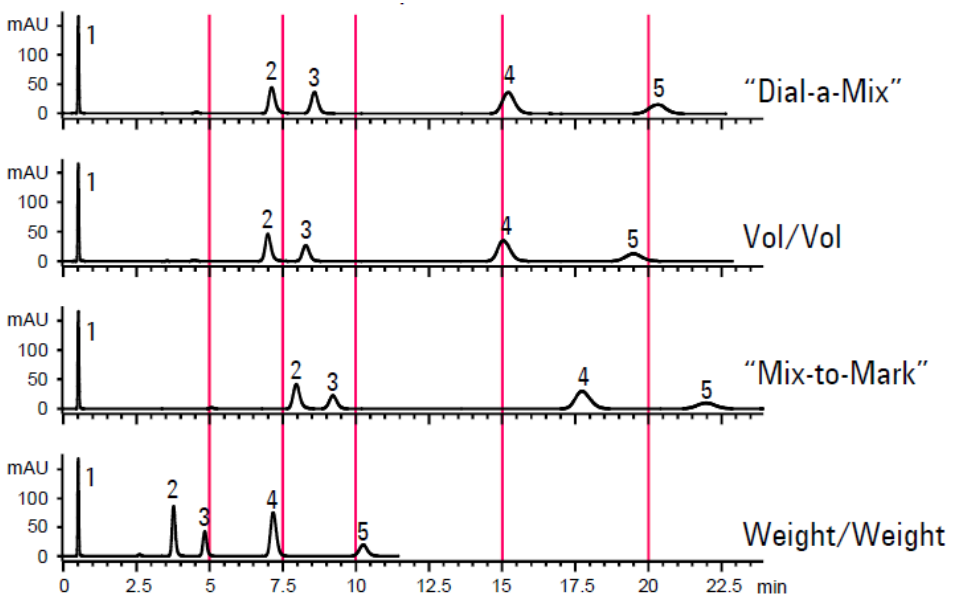
Specified volume ACN added to a 1 L volumetric and made to volume with H_2O

≠

Specified volume H_2O added to a 1 L volumetric and made to volume with ACN

≠

500 ml H_2O added to 500 ml ACN



HPLC System:	Agilent 1100 with quaternary pump
Column:	ZORBAX Eclipse XDB-C8 Rapid-Resolution (3.5µm), 4.6 x 50 mm Agilent Part No. 935967-906
Mobile Phases:	Dial-a-Mix= A: water B: MeOH, pump 50% B Vol/Vol=250 mL water + 250 mL MeOH, pump 100% Mix-to-Mark = 250 mL MeOH, fill to 500 mL with water, pump 100% Premixed (w/w) = 200 g MeOH + 200 g water, pump 100%
Detection:	UV 254 nm
Flow:	1 mL/ min.
Temperature:	ambient

1. Uracil
2. Butylparaben
3. Napthalene
4. Dipropylphthalate
5. Acenaphthene

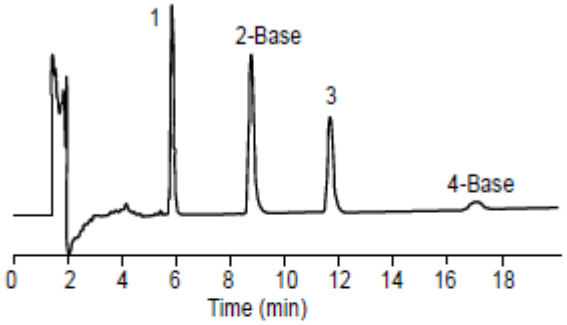
- Method used to prepare mobile phase can significantly affect the elution
- Be consistent and document the process

Effect of Mobile Phase Preparation on Chromatography, 5988-6476EN

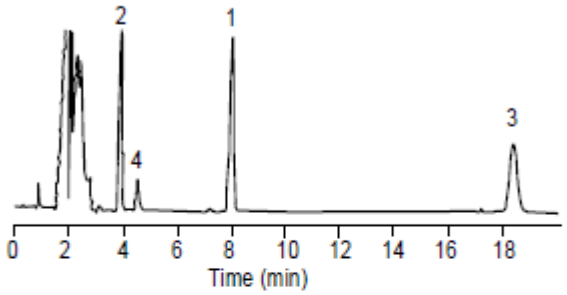
What Should I Test to Make a Robust Method?

pH 4.5 shows selectivity change from lot-to-lot for basic compounds

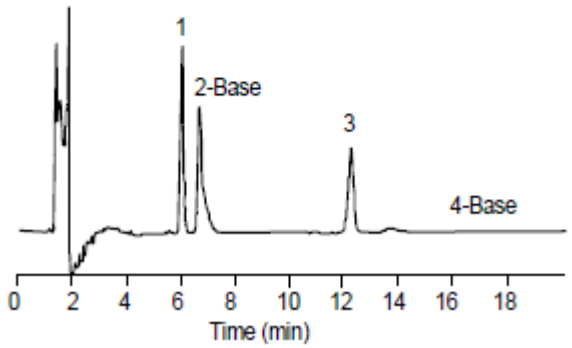
pH 4.5 - Lot 1



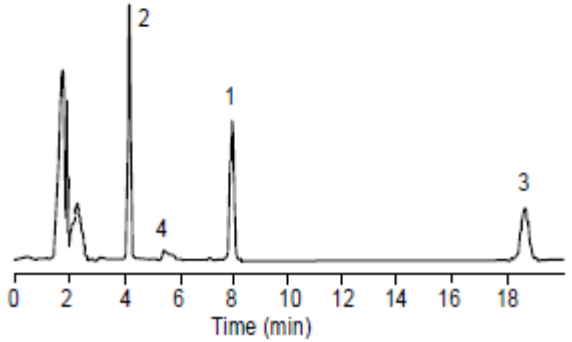
pH 3.0 - Lot 1



pH 4.5 - Lot 2



pH 3.0 - Lot 2



pH 3.0 shows no selectivity change from lot-to-lot

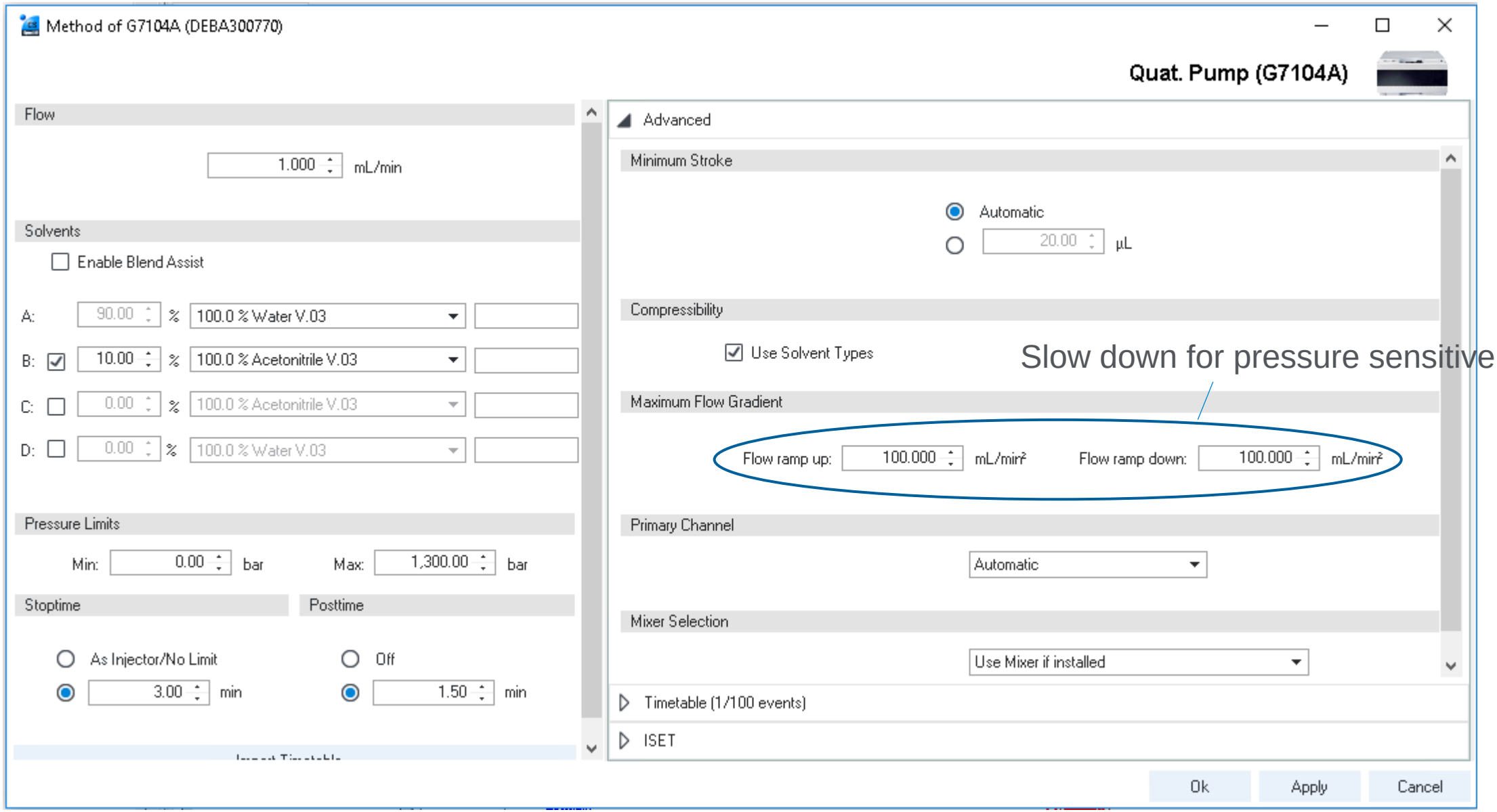
For method ruggedness

- Test three different column lots
- Compare R_s for the three lots
  - If ΔR_s is too large, modify method

Method Setup

- What method parameters should I optimize?
- Should I use default values?

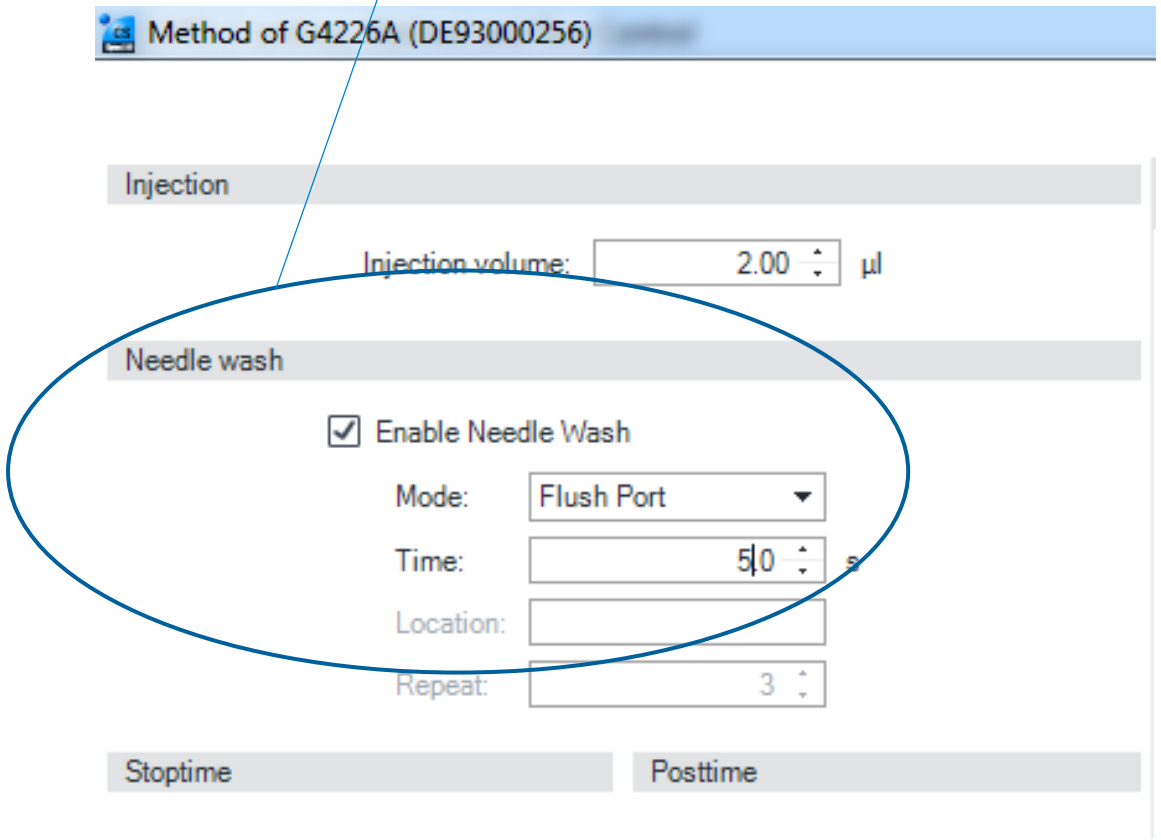
Pump Setting



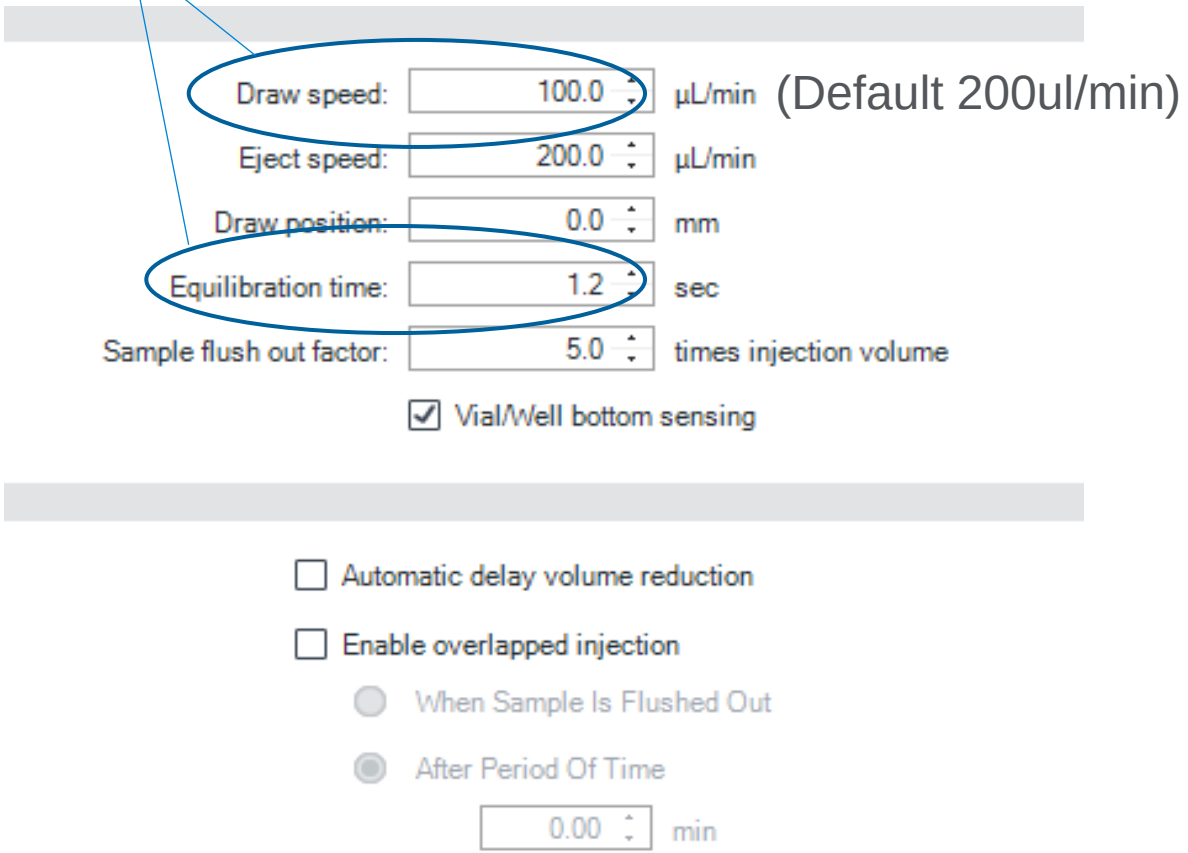
Slow down for pressure sensitive columns

Optimize Autosampler Performance

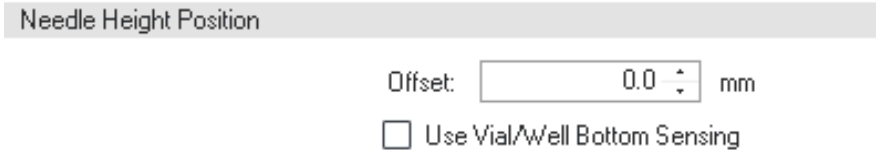
Reduce sample carryover

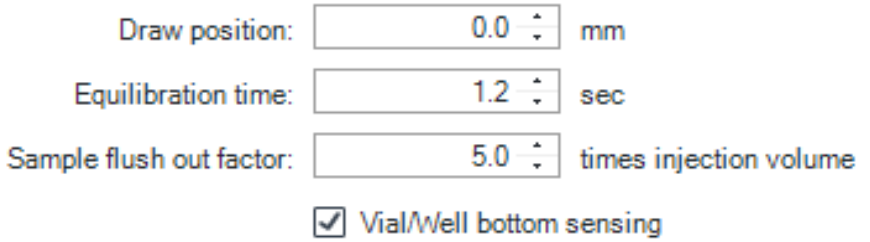


Improved accuracy for chilled samples

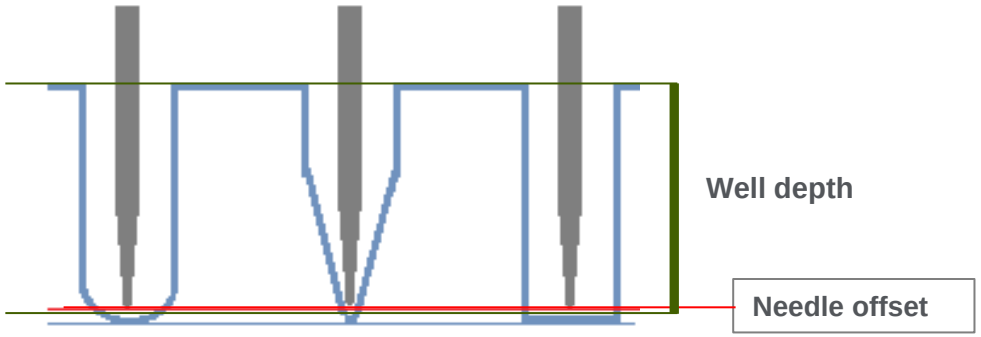


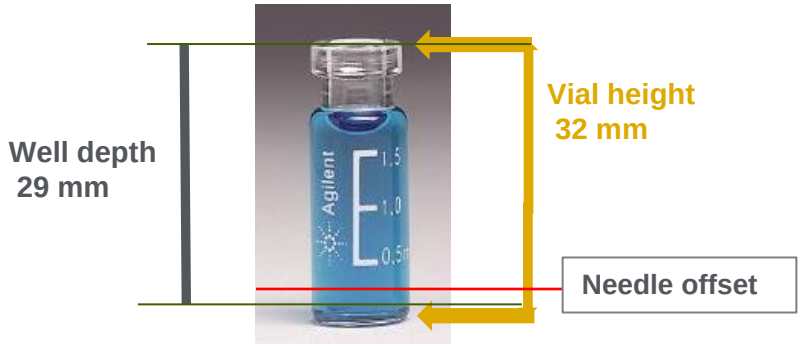
Optimize Autosampler Performance – Draw Position/Bottom Sensing



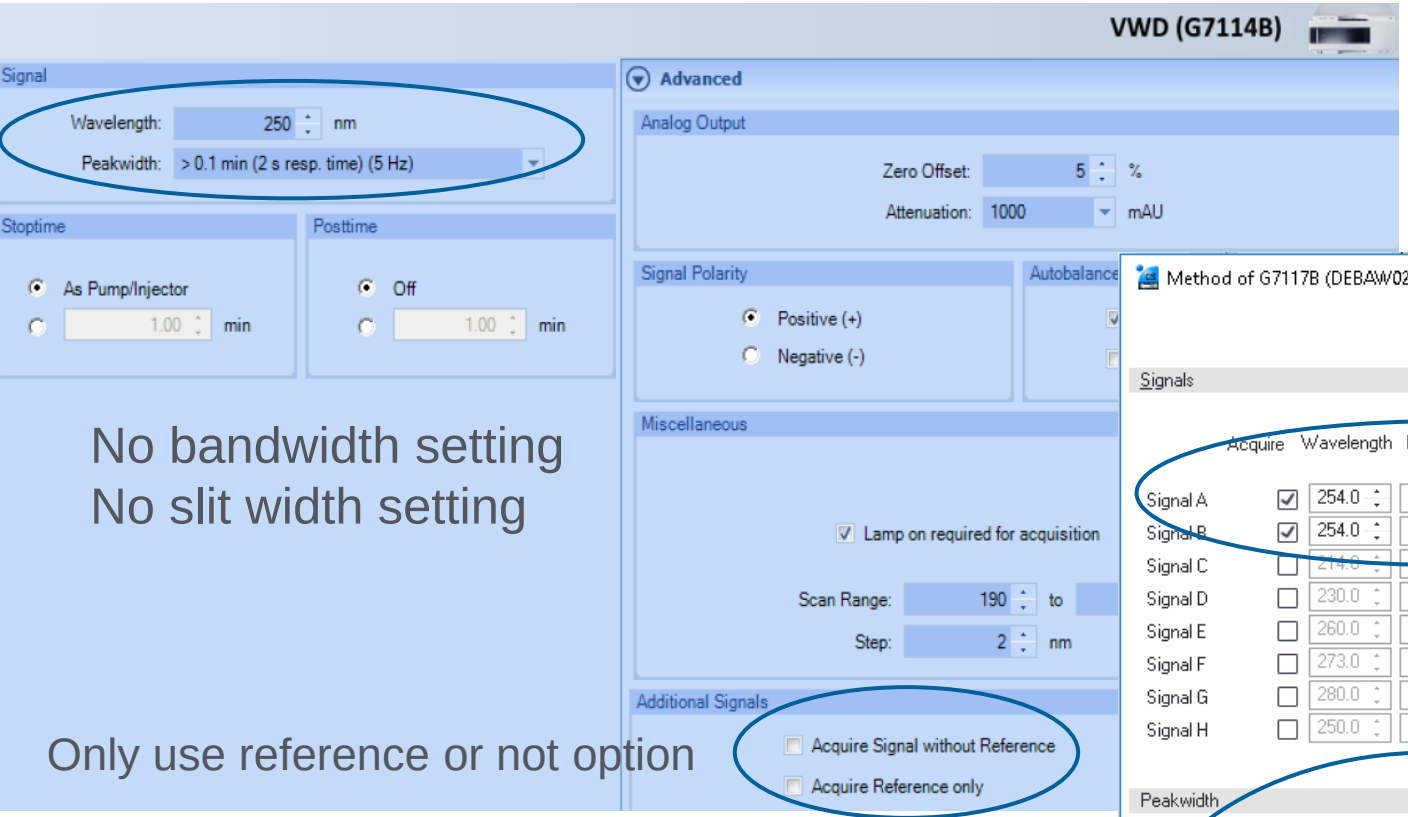


Draw Position/Needle Height Position Offset = 0	Vial Sampler G1329B/G7129A/B	Wellplate Sampler G1367E/G4226A	Multisampler G7167A/B
	2 mL vial (sample tray)	2 mL vial 54 vial tray	2 mL vial 54 vial tray
Without bottom sensing	2 mm	4 mm	5 mm
With bottom sensing	x	1 mm	2 mm



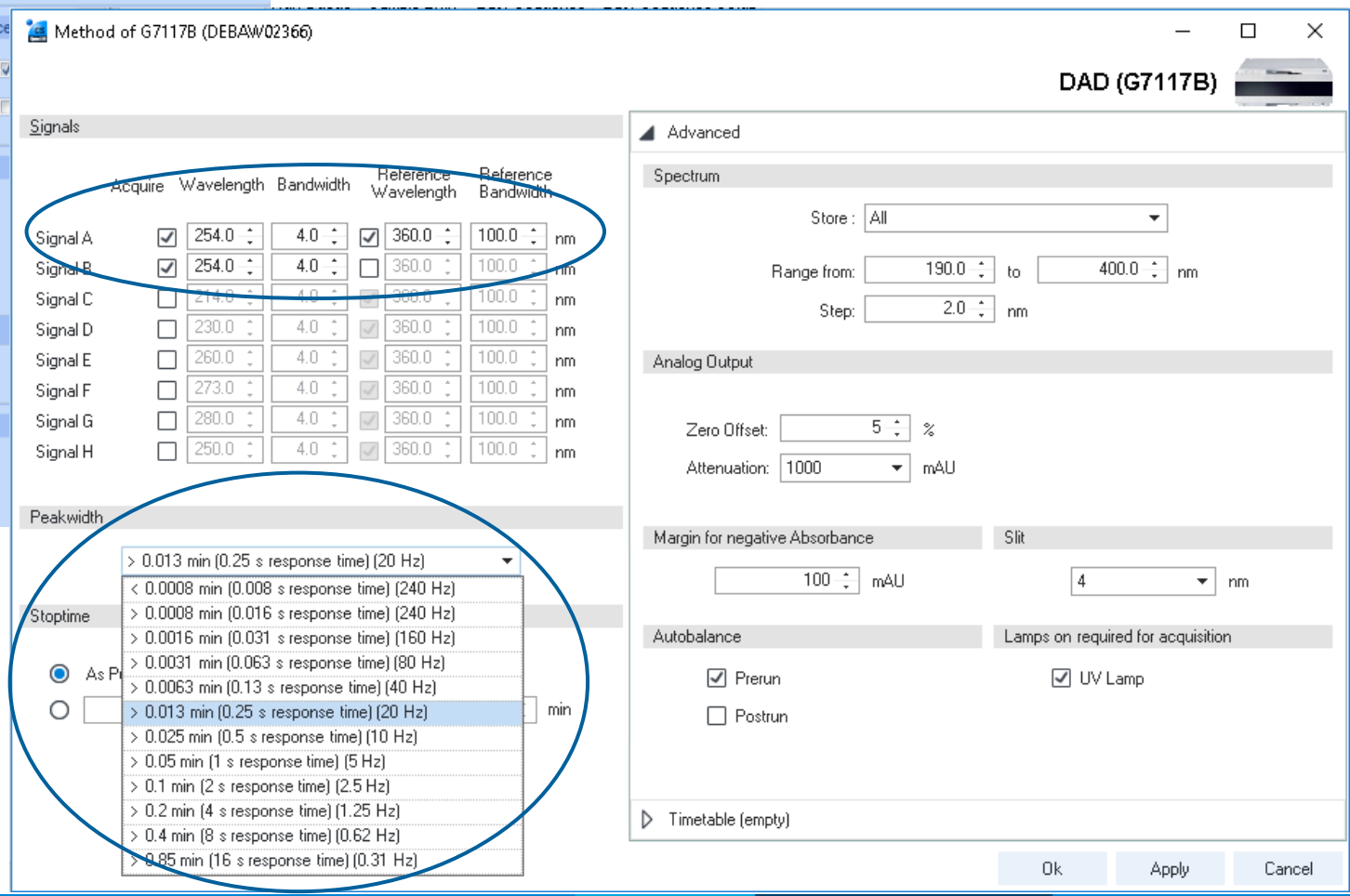


VWD and DAD Settings

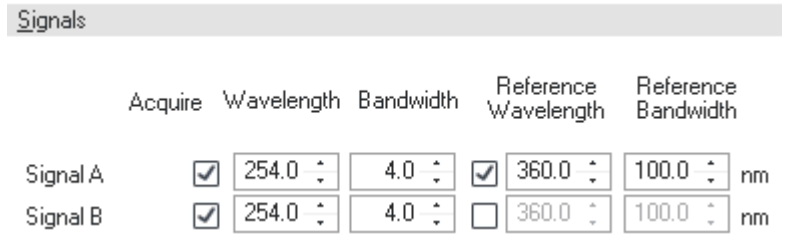


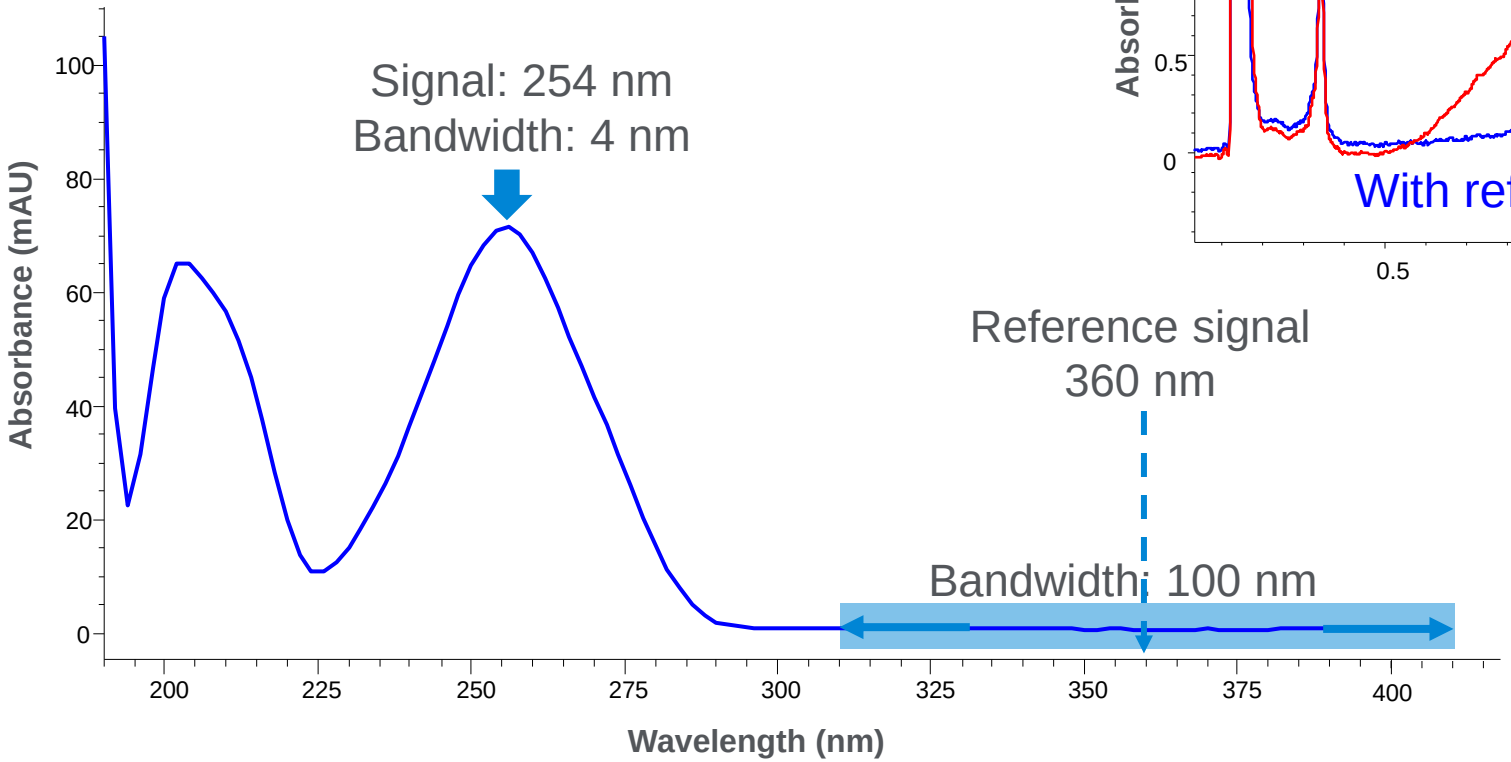
No bandwidth setting
No slit width setting

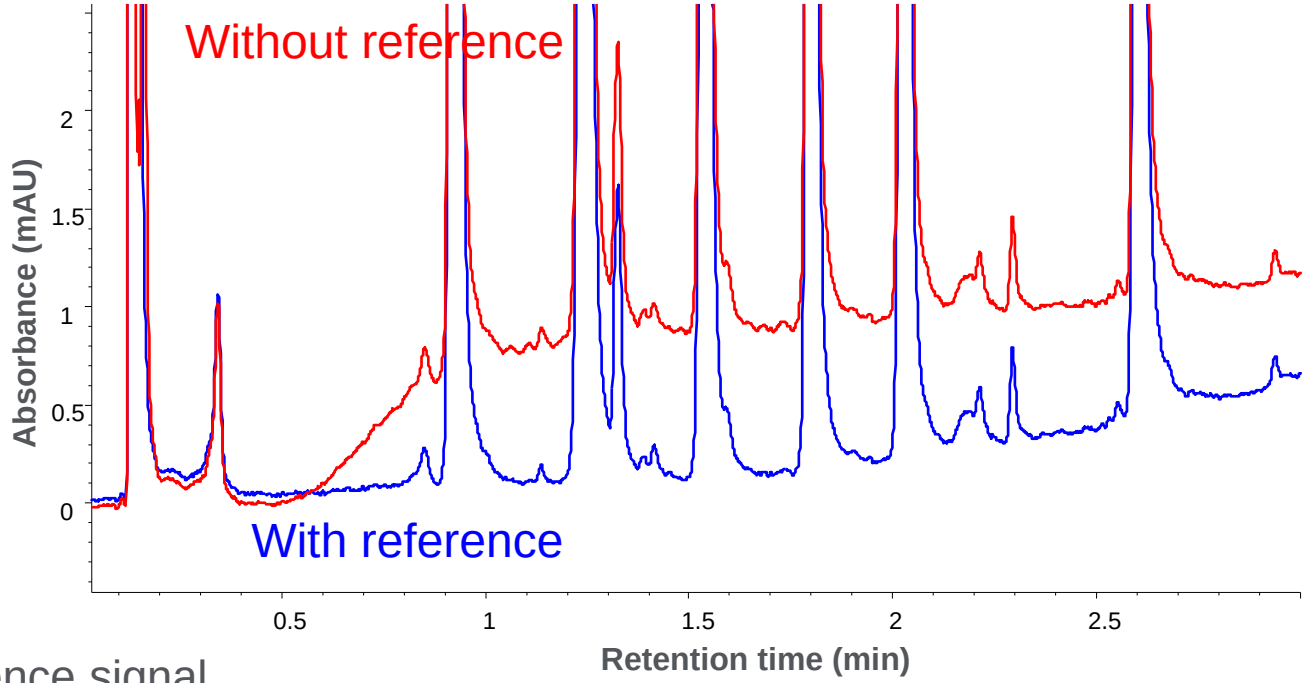
Only use reference or not option



DAD Setting – Choose the Right Signal and Reference

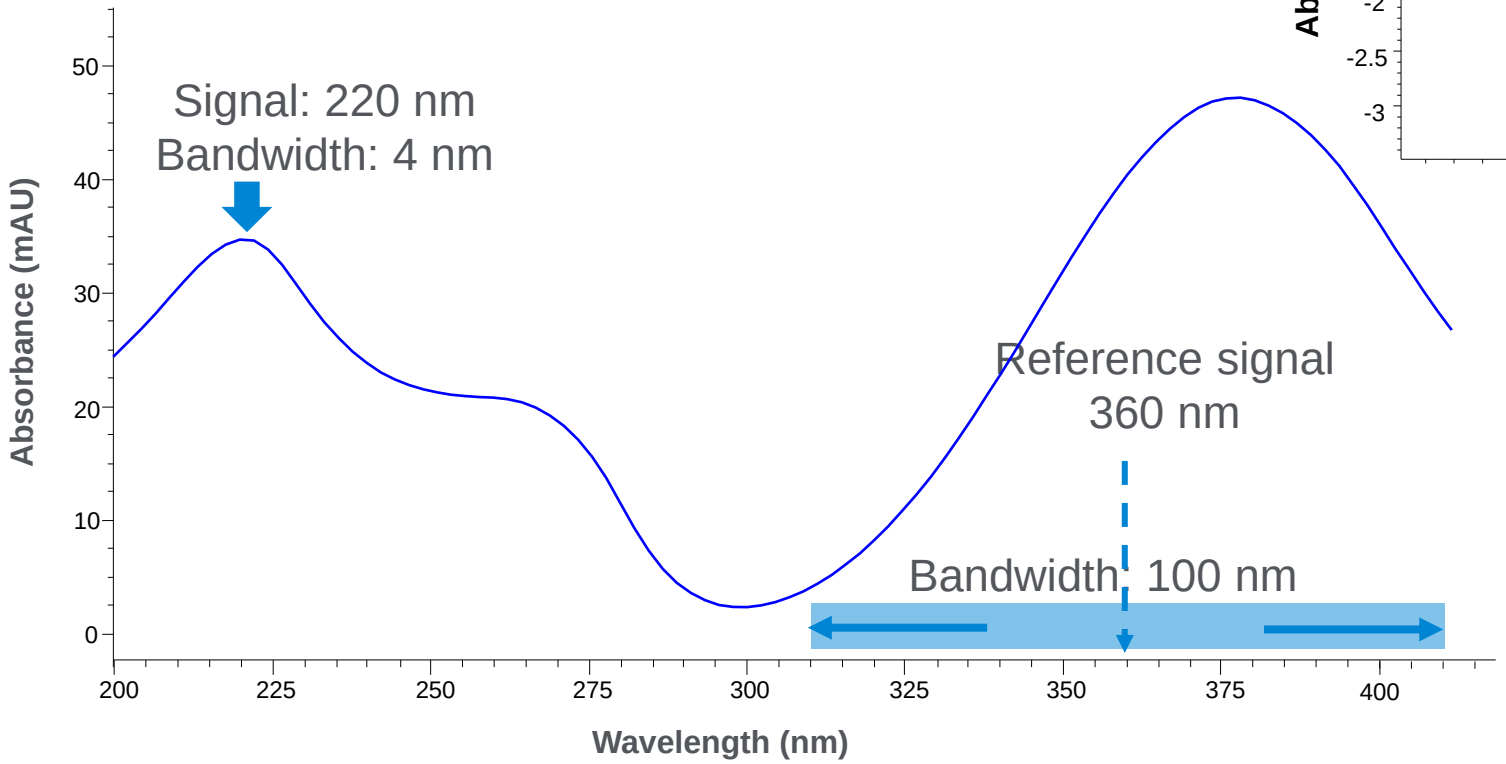


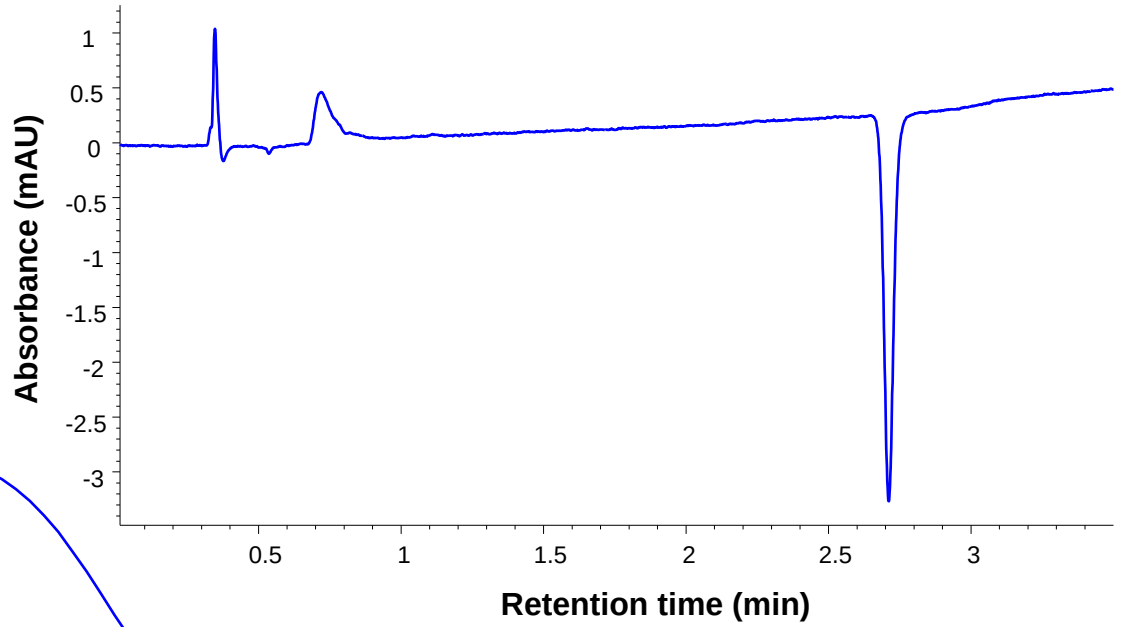




Gradient: 10-100% ACN in 3 min

DAD Setting – Choose the Right Signal and Reference



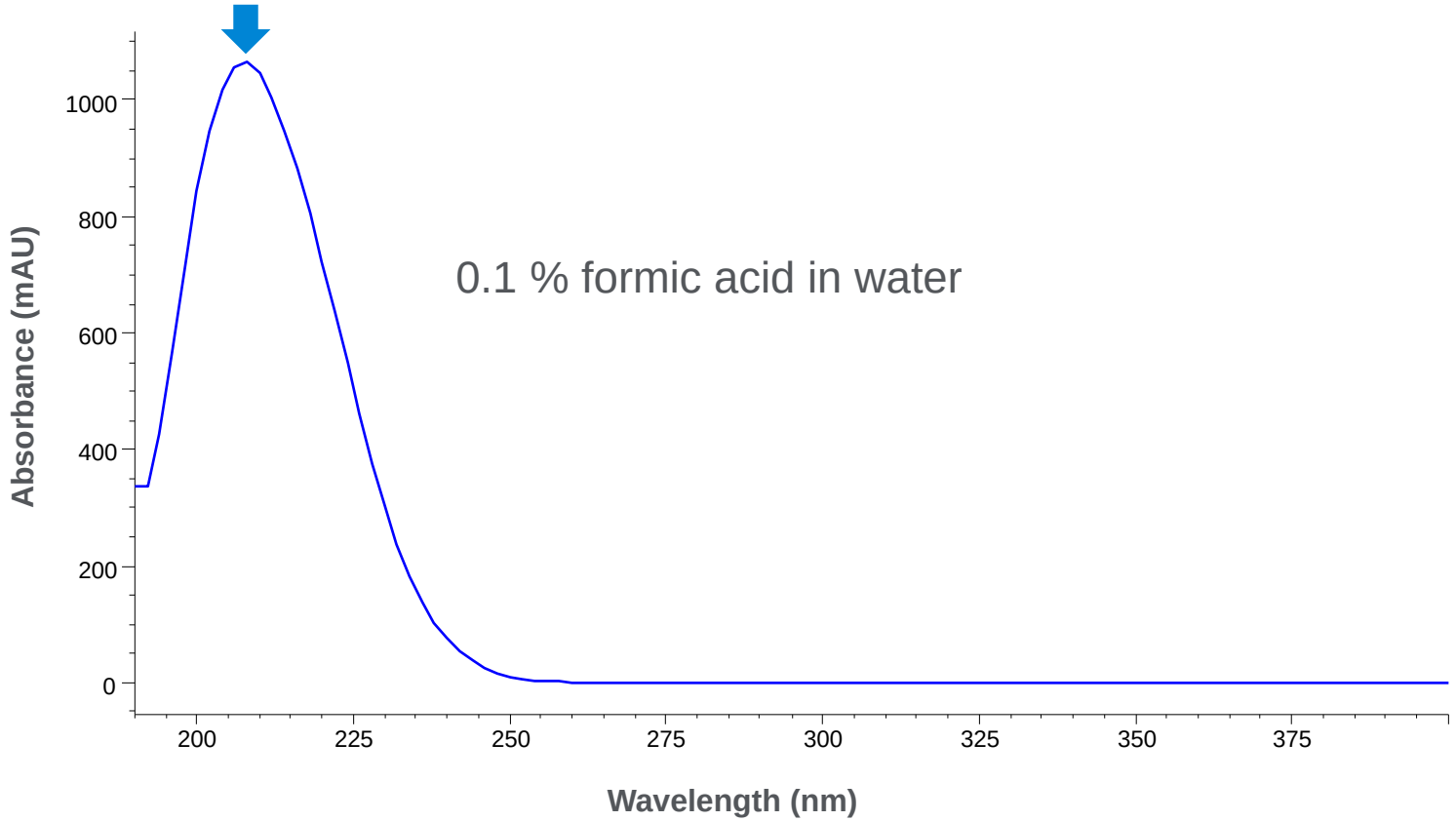


Reference signal may not be necessary

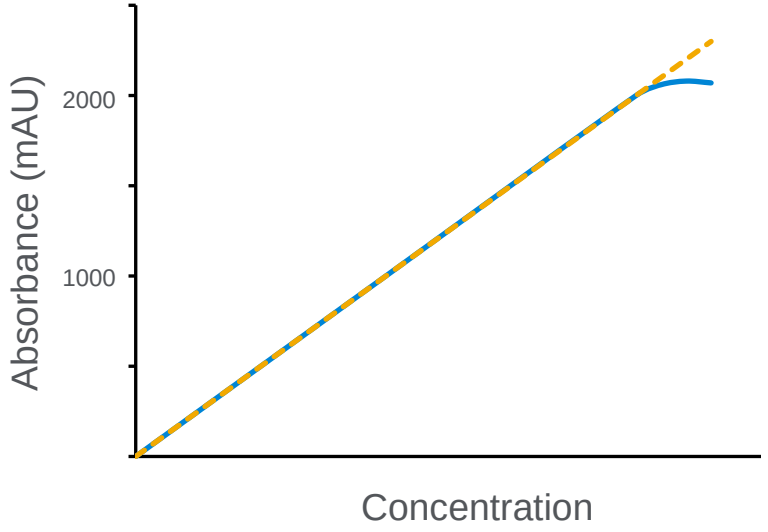
DAD Setting – Choose the Right Signal and Reference

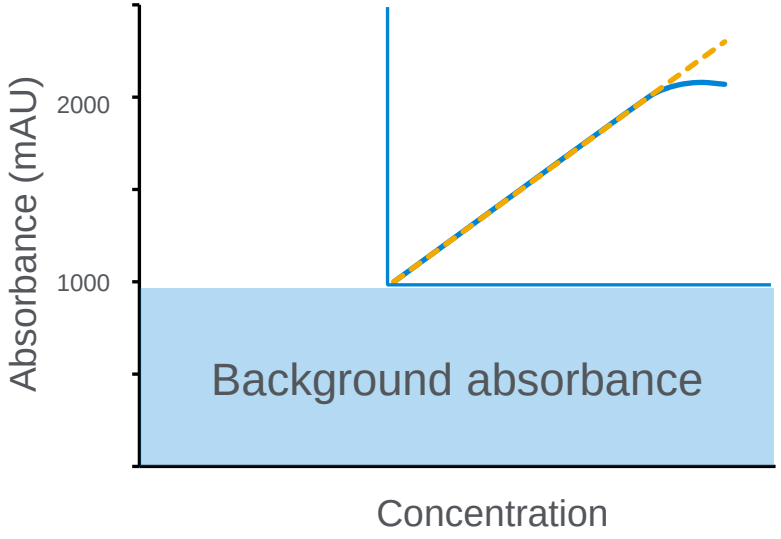
Move away from the UV cutoffs of mobile phase/additive

Signal: 210 nm



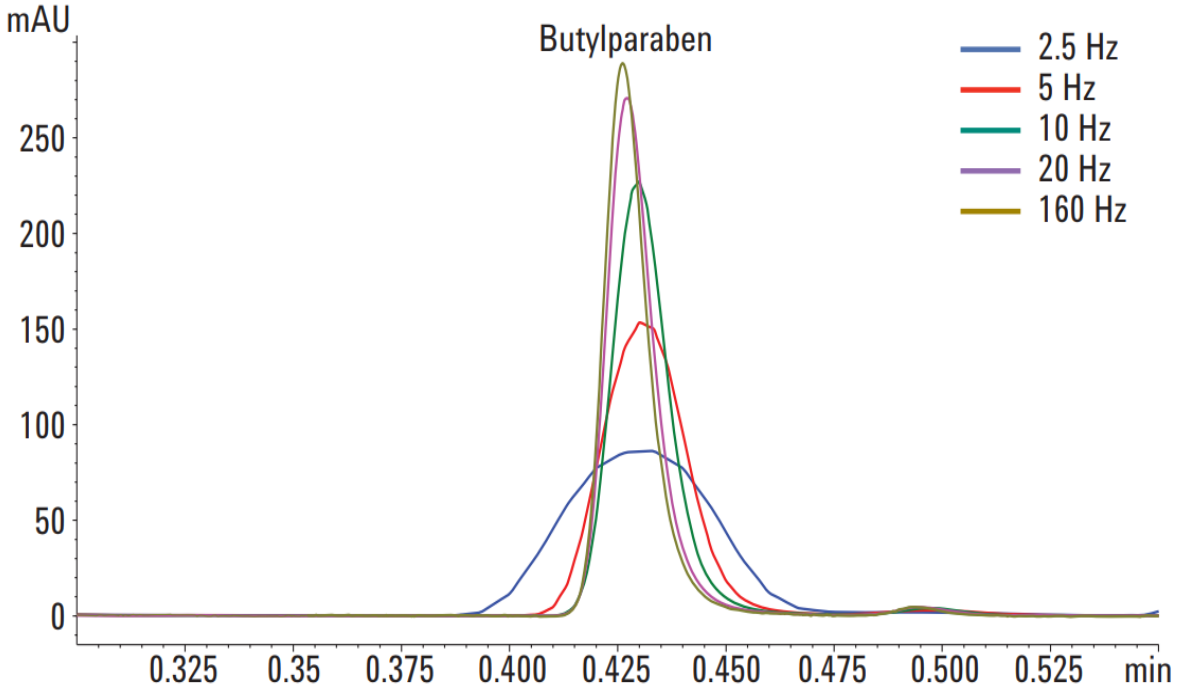
0.1 % formic acid in water



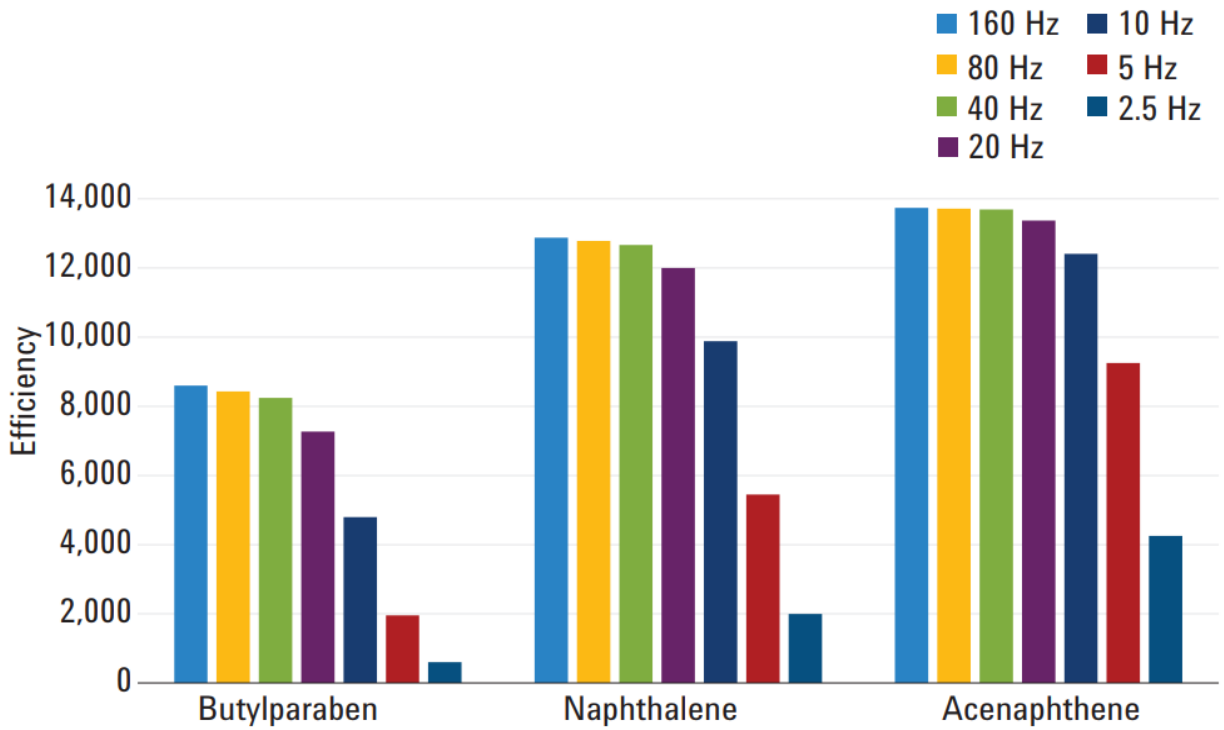


What Data Rate Should I Choose?

InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm
20 mM sodium phosphate pH 7 in water with acetonitrile premixed 40/60
0.5 mL/min

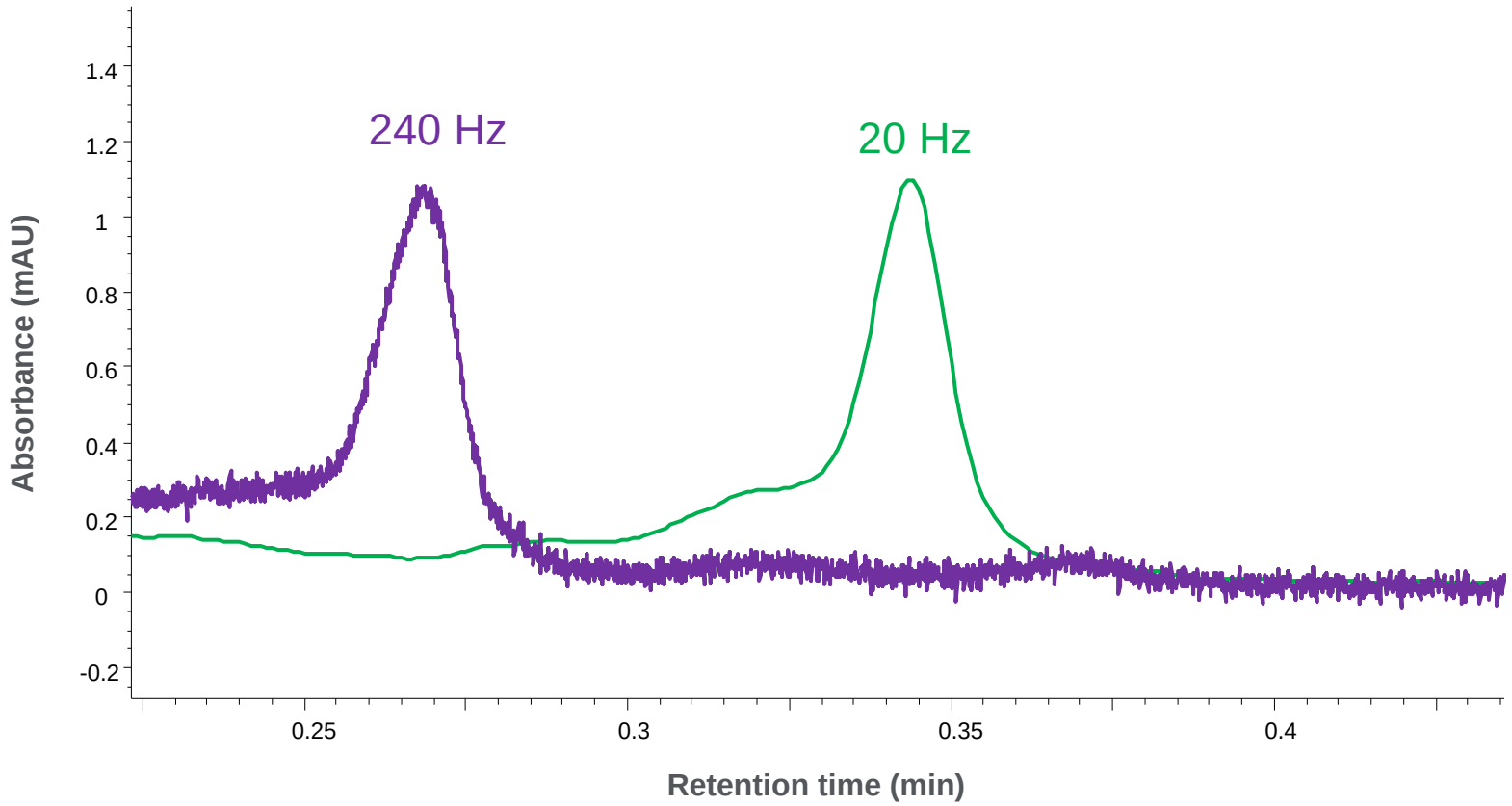


App note: 5991-7560EN

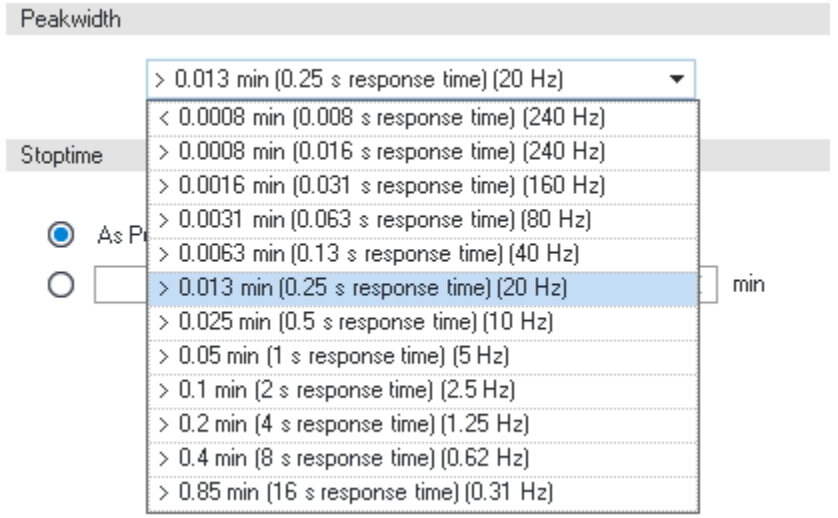


Fast data collection rates must be used with Agilent InfinityLab Poroshell 1.9 µm columns to accurately measure the efficiency of the column, especially for early eluting compounds such as butylparaben (k' = 1.3).

DAD Setting – Choose the Right Sampling Rate

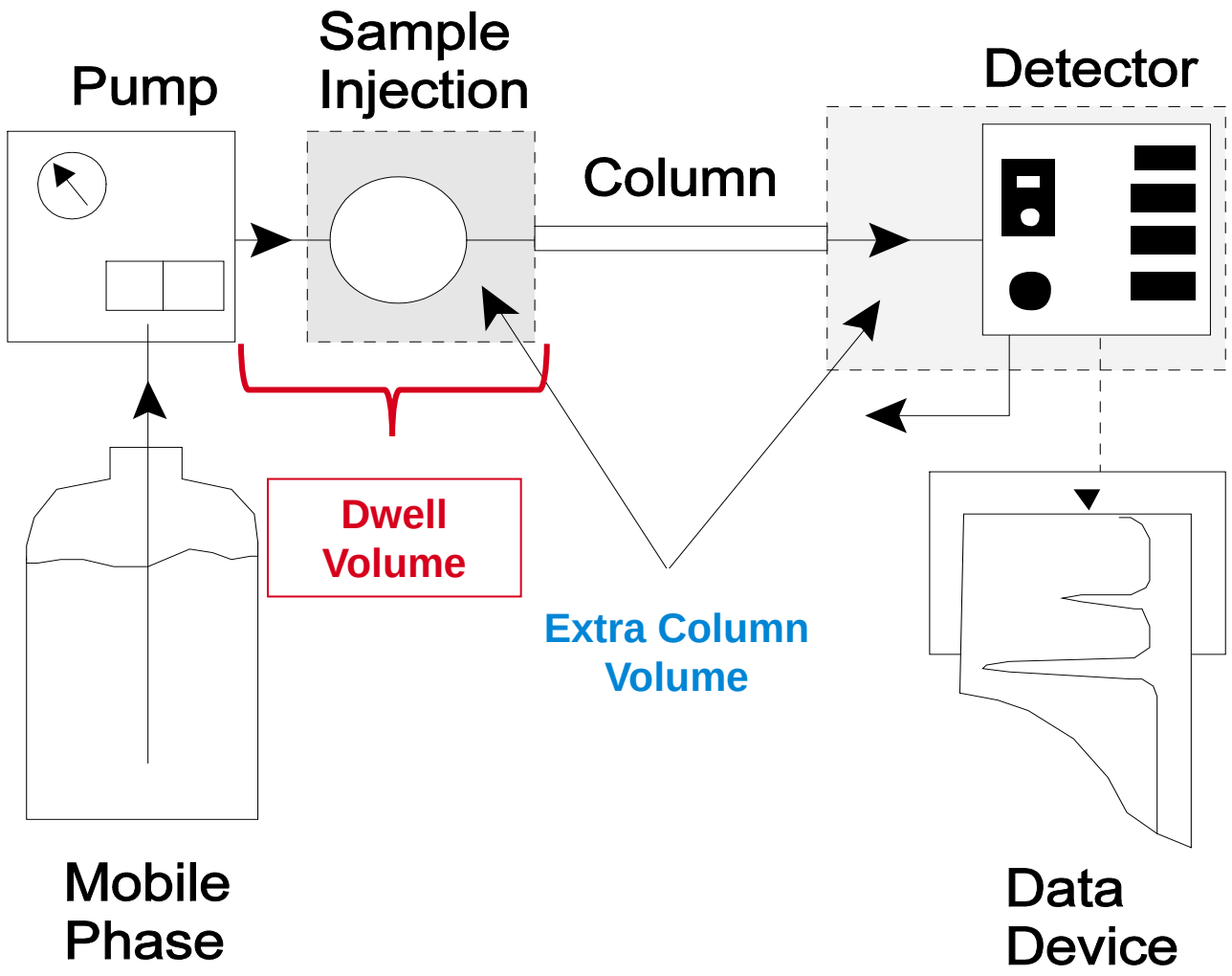


Do not use peak width smaller than necessary



Column: ZORBAX Eclipse Plus C18, 2.1 x 50 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

Instrument Configuration



Dwell Volume: from formation of gradient to top of column

-minimize for faster equilibration and more efficient gradient formation

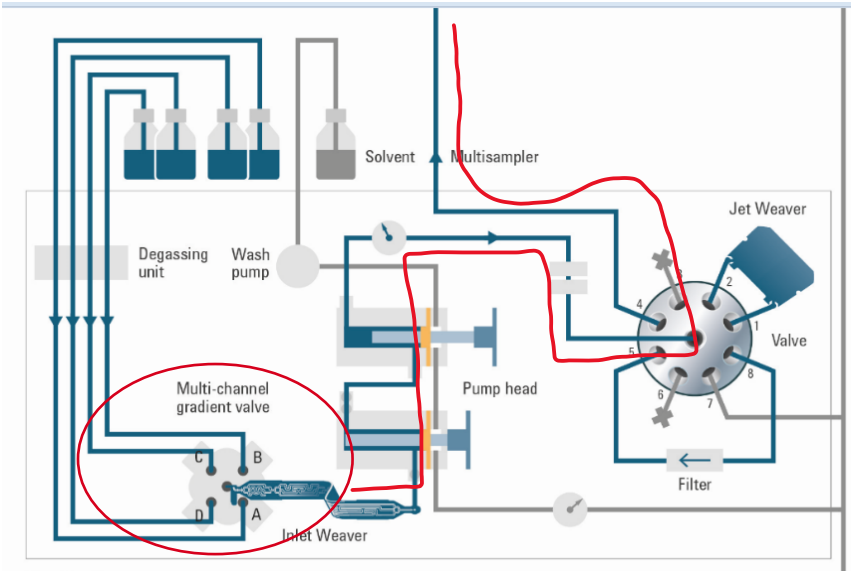
Extra column volume from injection to detector (flow cell) outside of the column

Minimize to reduce band broadening, for sharper peaks and better resolution

Comparison of Gradient Delay Volume (Dwell Volume)

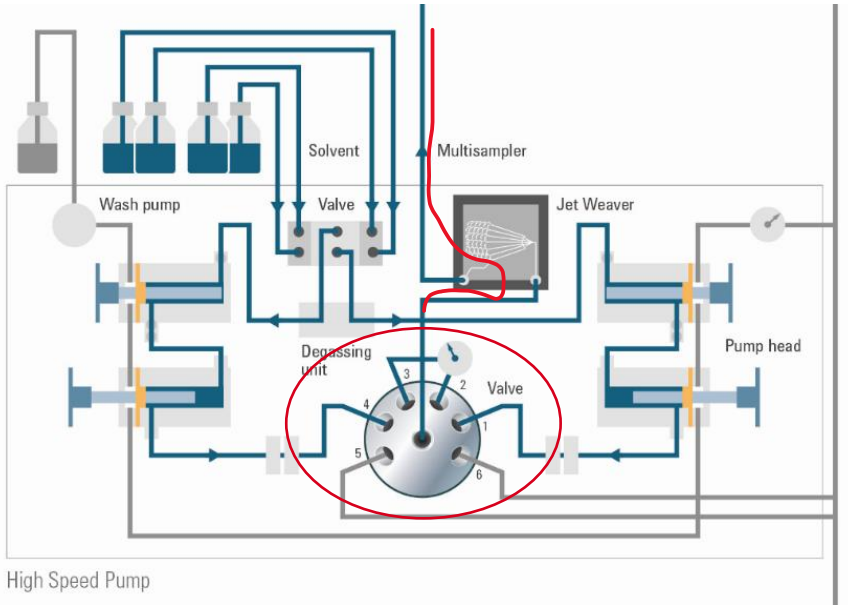
1290 Infinity II Flexible Pump (Quaternary)

- Integrated degasser
- Four solvent channels with concurrent mixing of all four channels
- Lower in price, typically, than binary pump



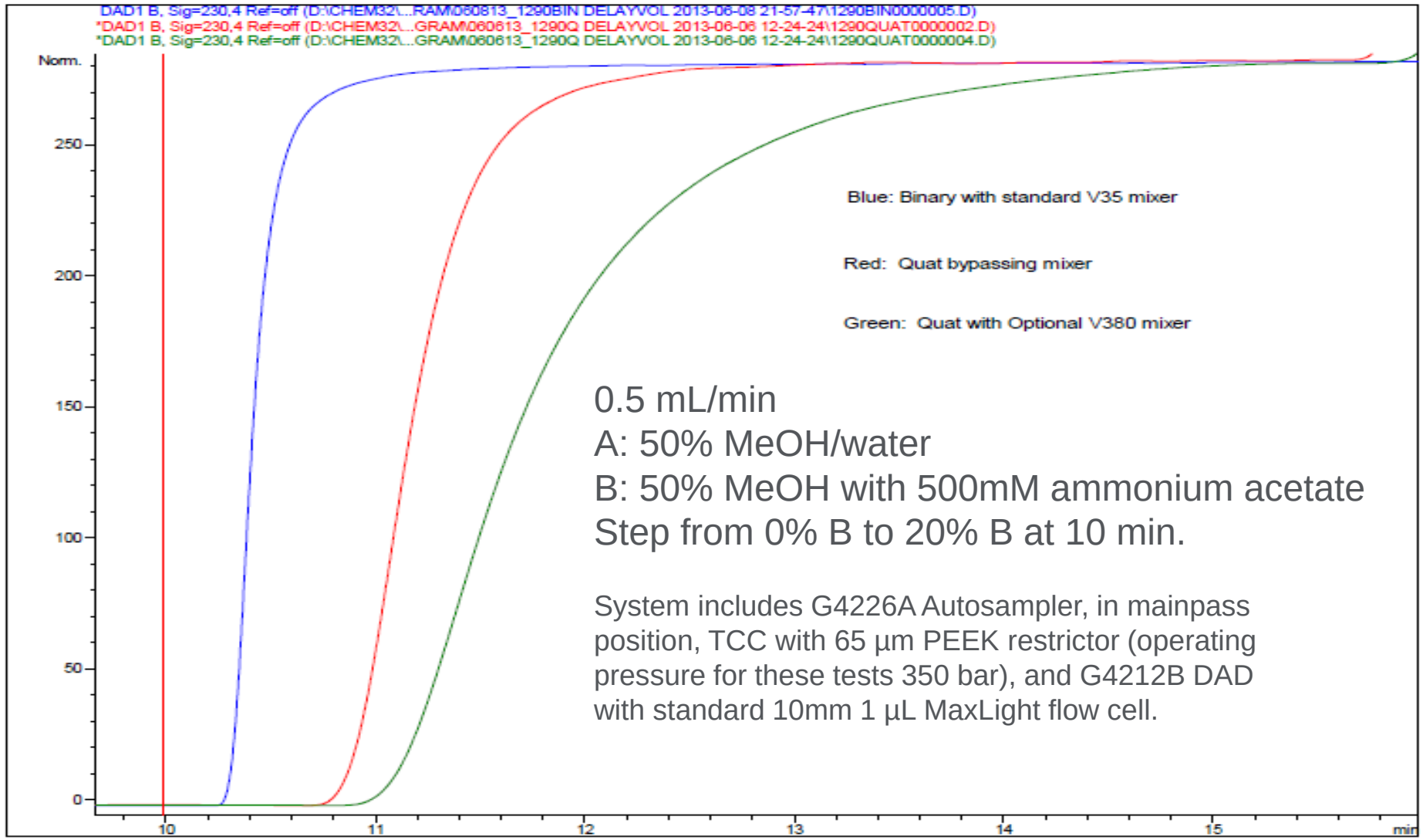
1290 Infinity II High Speed Pump (Binary)

- Integrated degasser
- Four solvent channels available, mixing of two channels possible
- Better performance concept is widely accepted
- Greater control over dwell volume compared to Quaternary pump

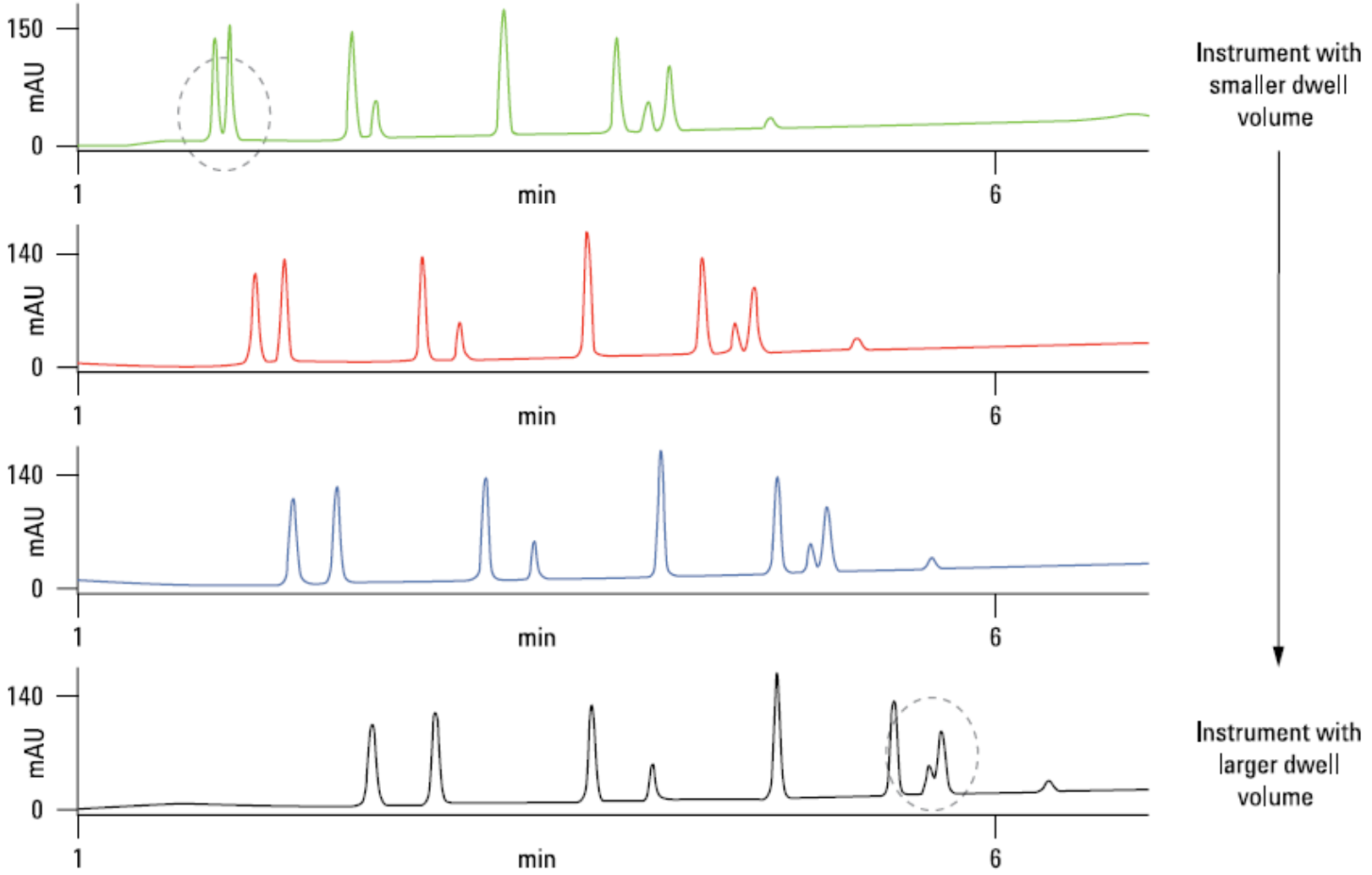


High Speed Pump

Delay Volume Profiles



Chromatographic Test Results with Different Delay Volumes



Dispersion Reduces HPLC Performance

What is dispersion?

- Original sample concentration being diluted as it is carried through the system plumbing (extra column volume)

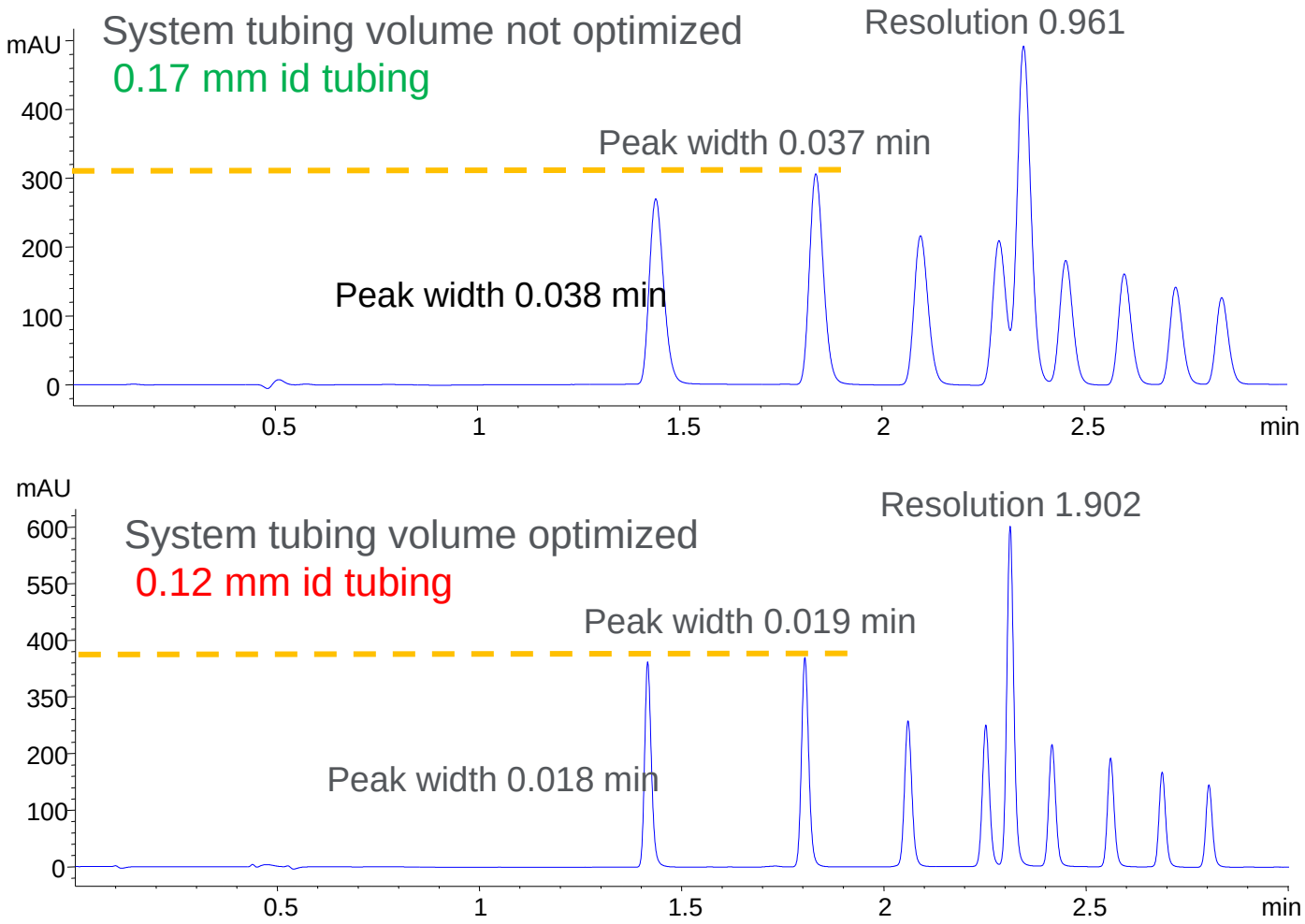
What increases dispersion?

- Connecting tubing that is too long
- Connecting tubing that is too large in diameter
- Connections that have gaps and form small mixing chambers

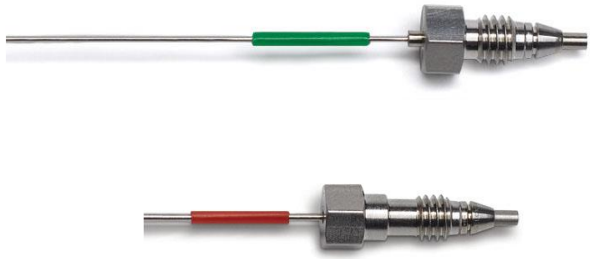
$$\sigma^2_{v,ext} = \frac{\pi\, d^4\, L_{cap}\, u_{cap}}{96 D_m}$$

$\sigma^2_{v,ext}$	is the volume variance
d	is the tubing diameter
L	is the tubing length
u	is the linear velocity of the liquid
D_m	is the molecular diffusion coefficient

Optimizing Connecting Tubing Volume For UHPLC Columns



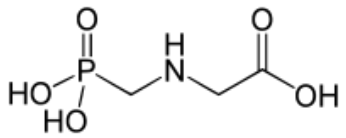
Length	10 mm	50 mm	100 mm	150 mm
Tubing id	Volume	Volume	Volume	Volume
0.17mm (green)	0.227 µL	1.1 µL	2.27 µL	3.3 µL
0.12 mm (red)	0.113 µL	0.55 µL	1.13 µL	1.65 µL



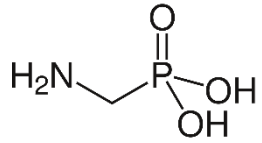
Passivation and Inert Hardware

- Steel has active sites that bind to certain classes of polar molecules
- **Most active molecules:**
  - Phosphorylated metabolites
  - Organophosphates and phosphonic acids
  - Di- and tricarboxylic acids and similar chelating acids
- **Commonly seen in:**
  - Pesticide analysis (glyphosate, AMPA, glufosinate)
  - Fermentation (citric acid cycle, organic acids)
  - Metabolomics (nucleotides, sugar phosphates, citric acid cycle)

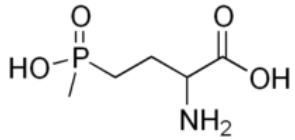
Organophosphates



Glyphosate

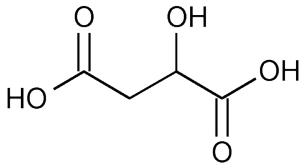


Aminomethylphosphonic acid (AMPA)

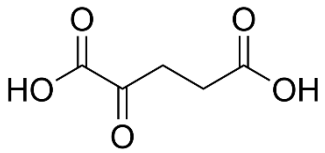


Glufosinate

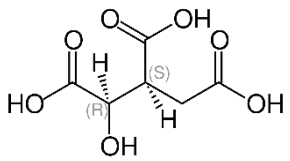
Chelating organic acids



Malic acid

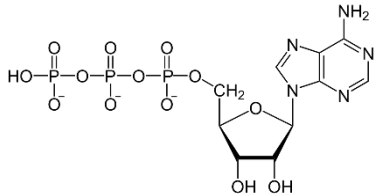


Alpha-Ketoglutaric acid

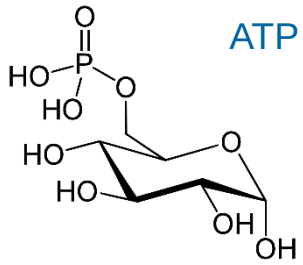


Isocitric acid

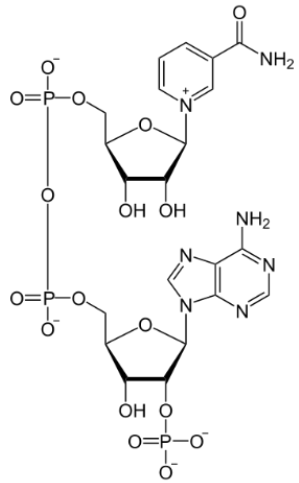
Phosphorylated metabolites



ATP



Glucose phosphates



NADP

Eliminating Sticking with Wash Step and Deactivator Additive

Example analysis conditions

Column: InfinityLab Poroshell 120 HILIC-Z, 2.1 x 50 mm (p/n: 689775-924)

Temperature = 30 °C

Injection volume = 1 µL

Flow rate = 0.25 mL/min

Mobile phase

A = 10 mM ammonium acetate in water at pH 9 + 5 µM deactivator additive

B = 10% 100 mM ammonium acetate in water at pH 9 + 90% acetonitrile + 5 µM deactivator additive

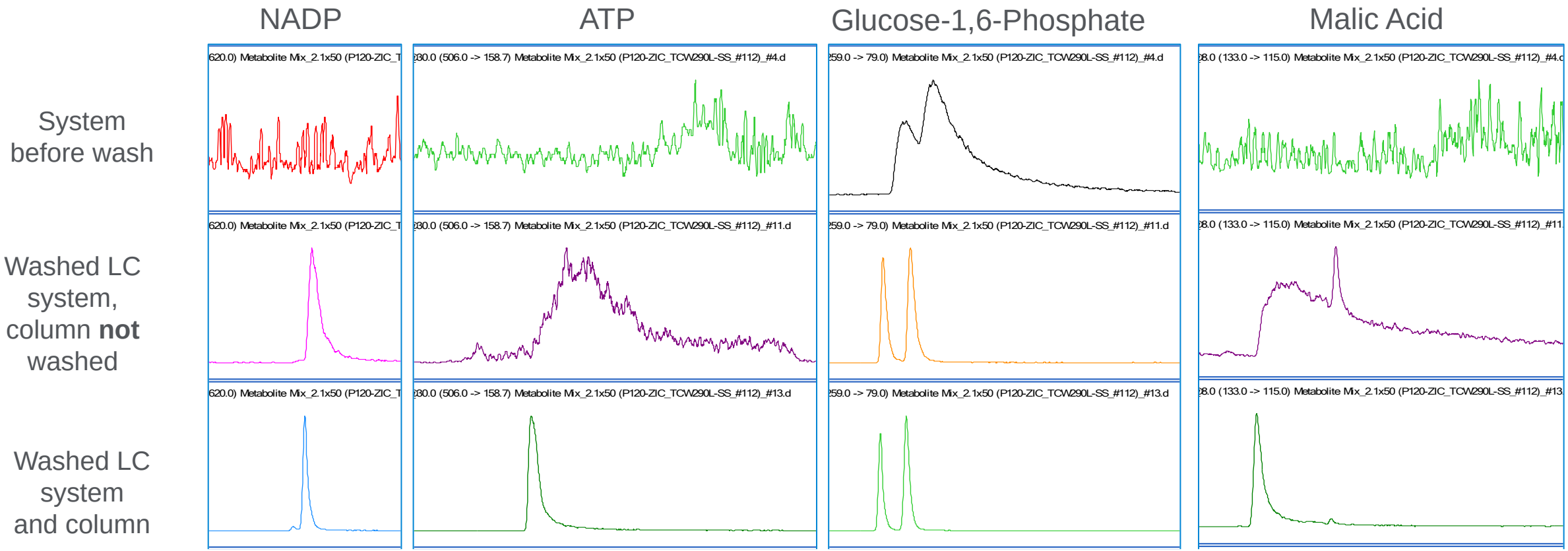
Total ionic strength – 10 mM for both mobile phases

Time (min)	Percentage A	Percentage B
0	10	90
2	10	90
12	40	60
13	10	90
21	10	90

Wash procedure

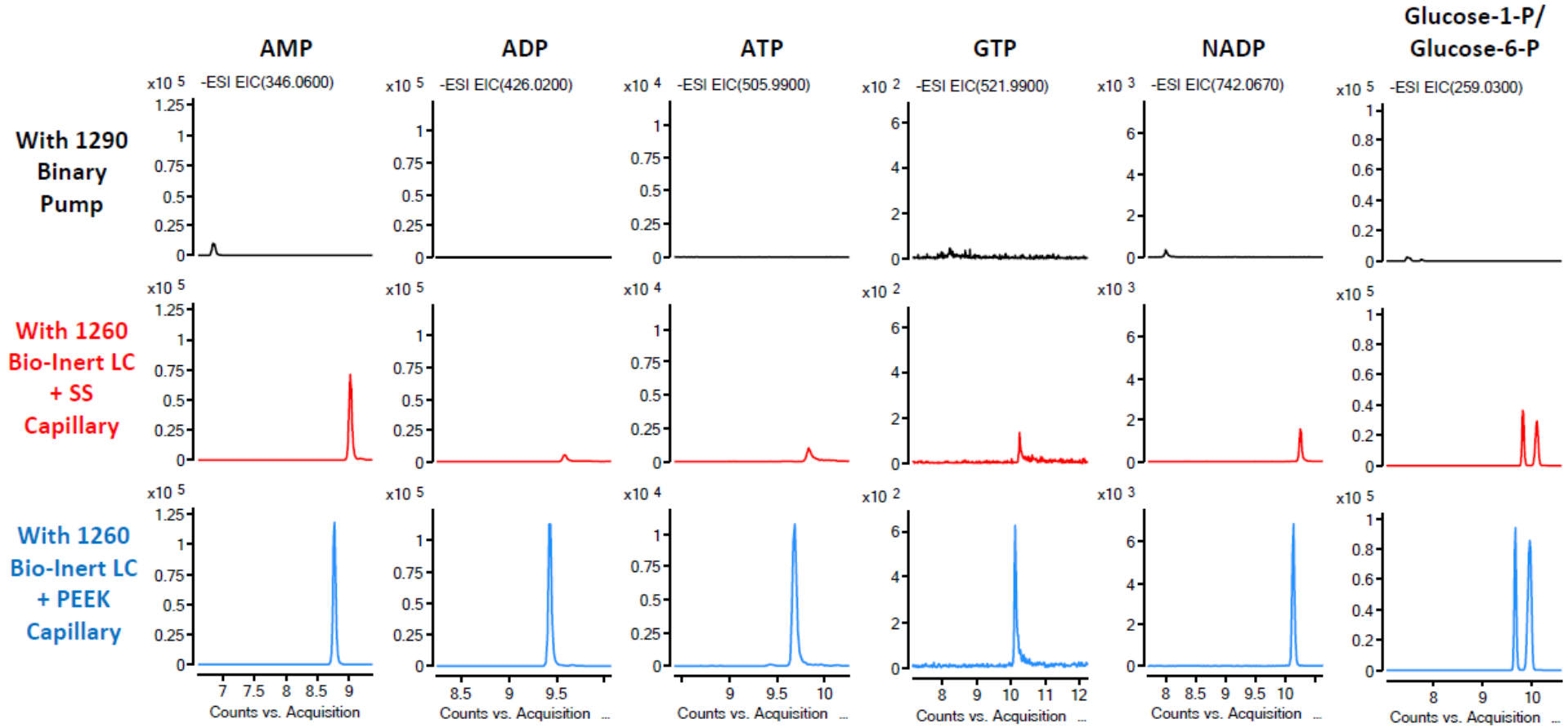
1. LC disconnected from MS and going directly to waste
2. IPA at 5 mL/min for 5 min
3. Water at 5 mL/min for 5 min
   - Flow at 0.5 mL/min for 1 hour
4. 0.5% phosphoric acid in 90% acetonitrile/10% water at 5 mL/min for 5 min
   - Flow at 0.1 mL/min overnight (at a minimum)
5. Water at 5 mL/min for 5 min
   - Flow at 0.5 mL/min for 1 hour
6. Mobile phase at 5 mL/min for 5 min
   - Flow at 0.25 mL/min for 1 hour
7. Reconnect LC to MS and proceed with analysis
   - Flow at 0.25 mL/min for 20 to 30 min

Improvements in Signal and Peak Shape



HILIC/MS Sensitivity with Bio-Inert LC

Nucleotide phosphates on a PEEK-lined Agilent InfinityLab Poroshell 120 HILIC-Z



Column used was Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 x 100 mm, 2.7 µm (PEEK-lined stainless steel); A: 10 mM ammonium formate pH 6.8 in water, B: acetonitrile + 10 mM ammonium formate pH 6.8, 95-30% B in 10 minutes, 0.25 mL/min, 0.2 µL injection (5 ng each on column), MS source: ESI, m/z 191.02, 346.06, 426.02, 505.99, 521.99, 742.067, 743.067, 259.03

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