The LC Column Playbook

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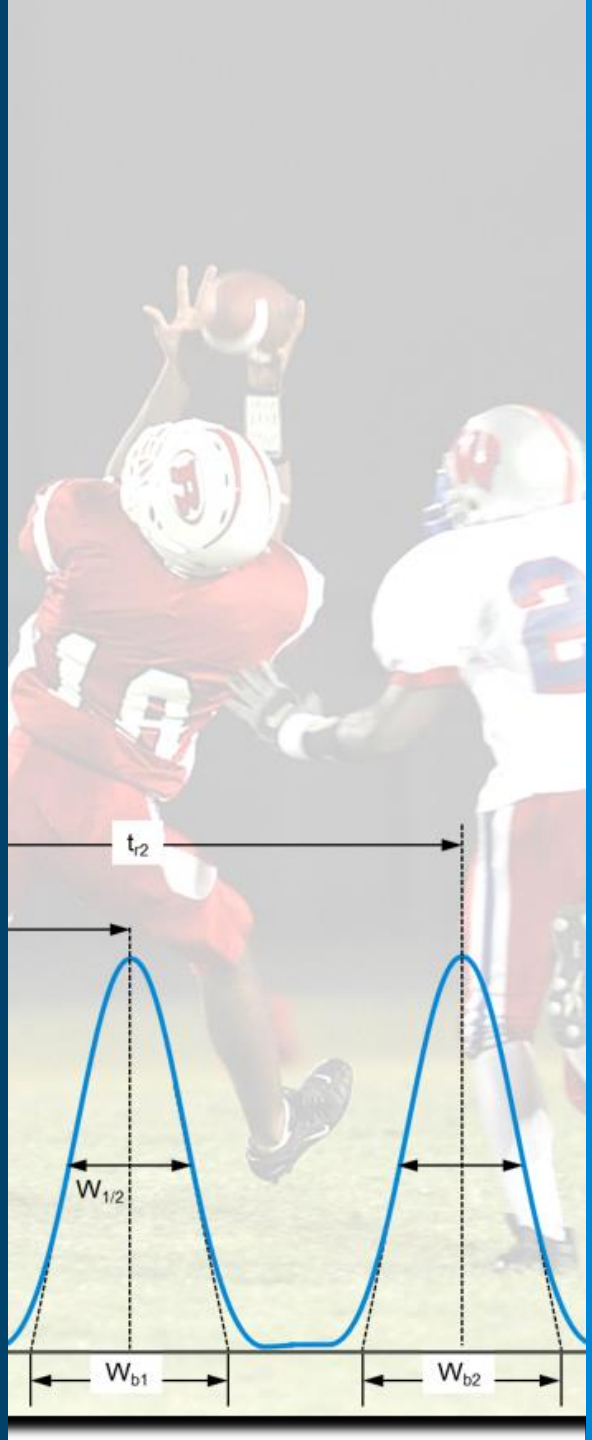
Technical Support
October 10, 2017

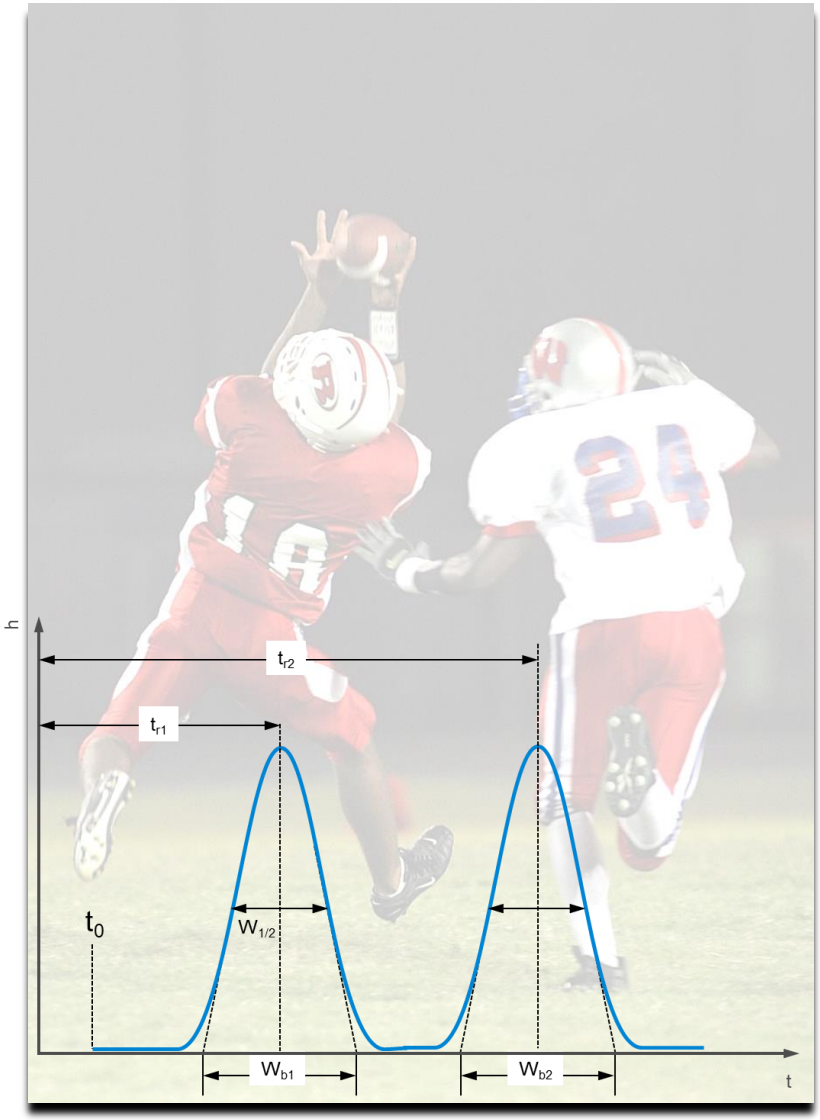


Agilent
Trusted Answers

Overview

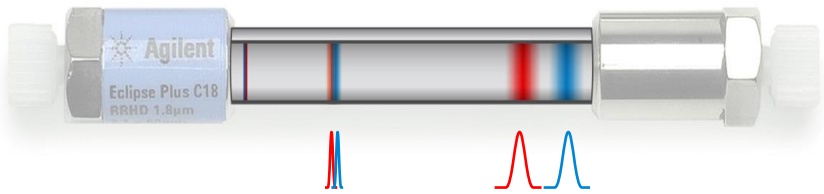
How do we achieve our goal: Resolution?

- Efficiency
  - Particle size
  - Column length
- Selectivity
  - Bonded phase
  - Mobile phase
- Retention
  - Polar compounds
  - Polar bonded phase
  - HILIC



Resolution

$$R_s = \frac{t_{r2} - t_{r1}}{1/2 \cdot (W_{b2} + W_{b1})}$$

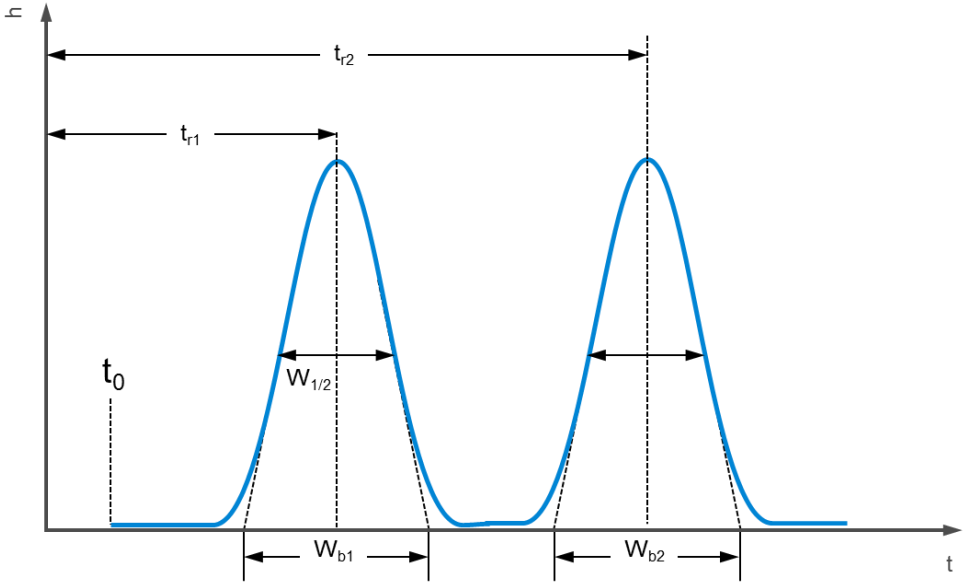


Separation t_{r2}-t_{r1}

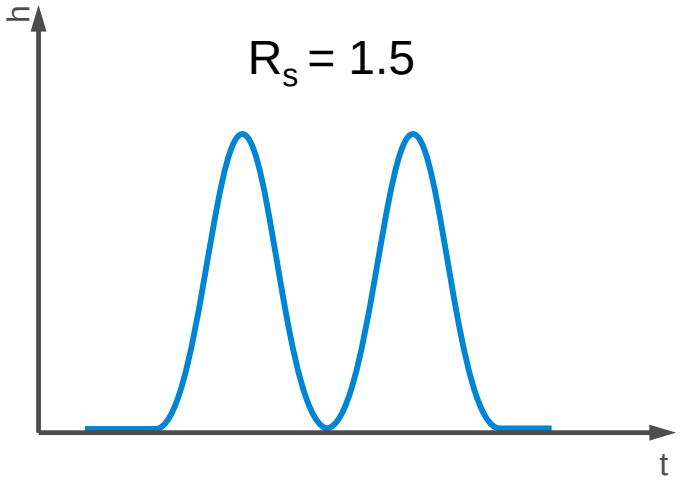
Peak width $W_{b1,2}$

Resolution describes the ability of a column to separate the peaks of interest.

Resolution describes whether you have achieved base line separation or not.



Resolution
Baseline Separations



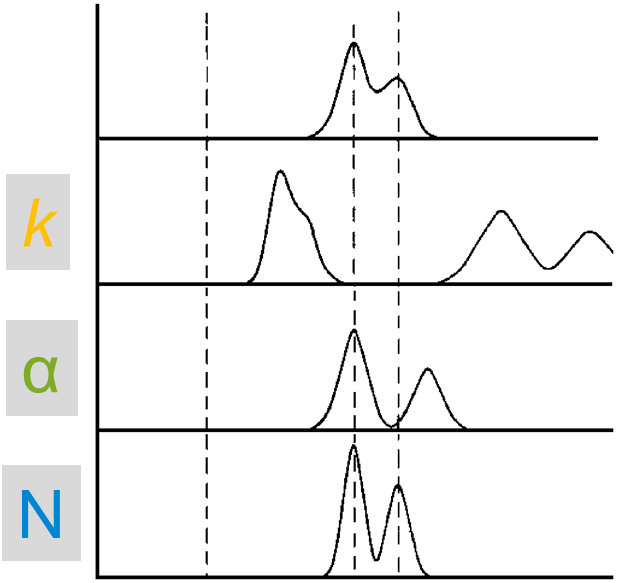
If we consider peaks of equal height:

- 1 - minimum for a measureable separation
- 0.6 - required to discern a valley between two equal-height peaks
- 1.5 - considered to be a baseline separation
- 1.7 or greater - desirable for rugged methods

Resolution Equation

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

Efficiency **Selectivity** **Retention**



Improve resolution by improving any of these parameters:

- Efficiency describes the separation power of the column
- Selectivity has the highest influence on the resolution. Small changes in selectivity can lead to big changes in resolution
- Retention has only a significant influence at small k values

Efficiency

$$N \propto \frac{L}{\mathrm{d}_p}$$

Parameters influencing column efficiency:

- Column length (increasing column length increases efficiency)
- Particle size (decreasing particle size increases efficiency)

Retention Factor

$$k = \frac{(t_R - t_0)}{t_0}$$

t_R = retention time for sample peak

t_0 = retention time for unretained peak

The retention factor measures the period of time that the sample component resides in the stationary phase relative to the time it resides in the mobile phase. It is calculated from the retention time divided by the time for an unretained peak.

Selectivity (Separation Factor)

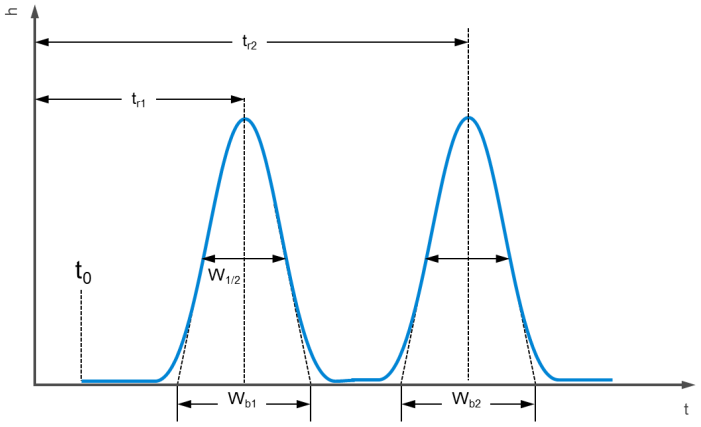
$$\alpha = \frac{k_2}{k_1}$$

α	Selectivity
k_1	Retention factor of 1st peak
k_2	Retention factor of 2nd peak

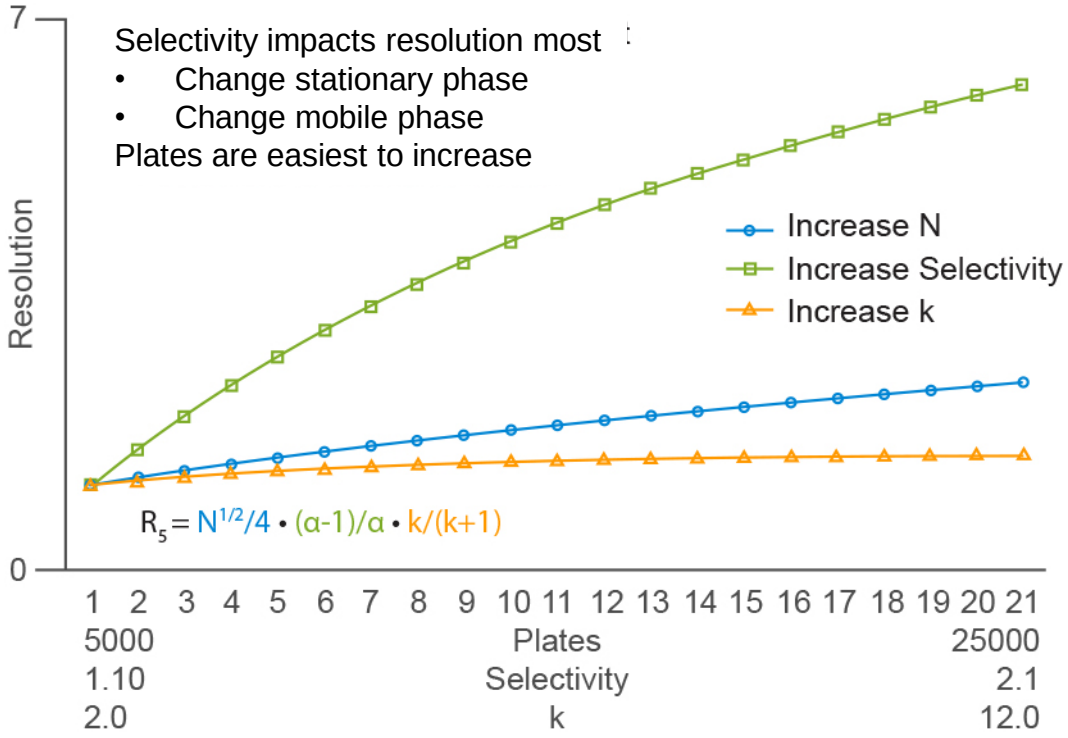
Selectivity is a measure of the time or distance between the maxima of two peaks. If α = 1, the two peaks have the same retention time and co-elute.

Parameters influencing selectivity:

- Stationary phase
- Mobile phase
- Temperature



Resolution



- Resolution as a function of selectivity, column efficiency or retention
- If you double the column length, you will obtain more theoretical plates
- But you only get a square root of 2, or 1.4x improvement in the resolution

How to Improve Resolution?

High plate number (***N***) provides:

- Sharp and narrow peaks
- Better detection
- Ability to resolve complex samples

But **resolution** increases only with the square root of the plate number

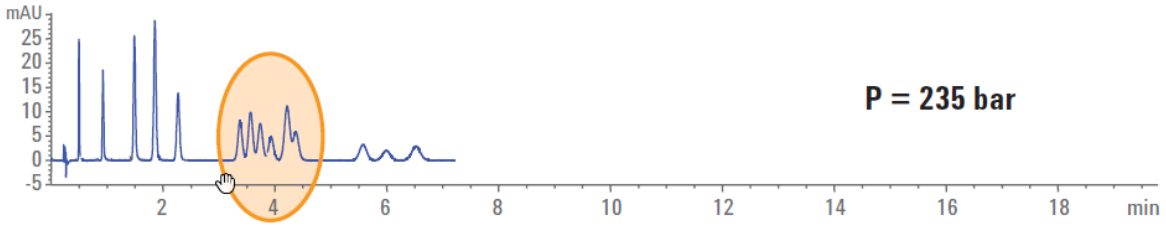
- $R_S \sim \sqrt{N}$

Plate number increase is limited by experimental conditions

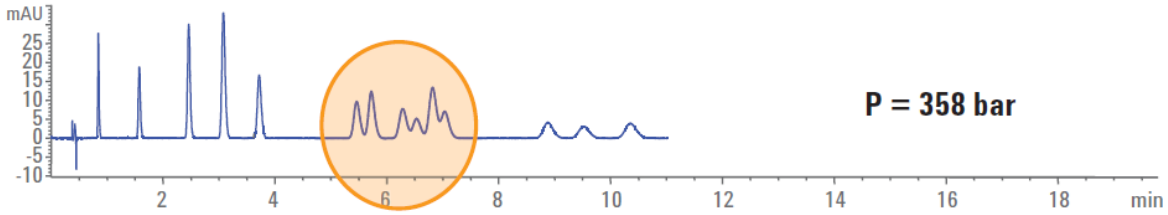
- **Time**
- **Pressure (instrument)**

How to Improve Resolution?
Increase N with longer column

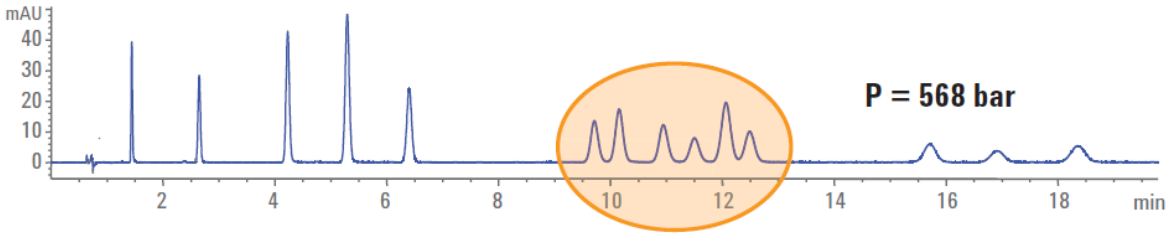
InfinityLab Poroshell 120 EC-C18, 4.6 x 50 mm (p/n 699975-902)



InfinityLab Poroshell 120 EC-C18, 4.6 x 100 mm (p/n 695975-902)



InfinityLab Poroshell 120 EC-C18, 4.6 x 150 mm (p/n 693975-902)



Balancing column length, resolution, and analysis time are important for any separation.

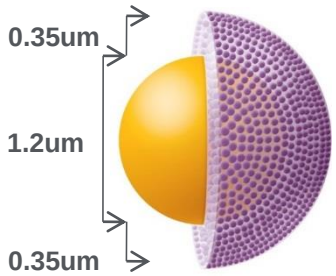
InfinityLab Poroshell 120 for HPLC and UHPLC comparison of EPA 8330 separation on short and long columns

Conditions:

Mobile phase:	25% Methanol, 75% water
Flow rate:	1 mL/min
Temperature:	44 °C

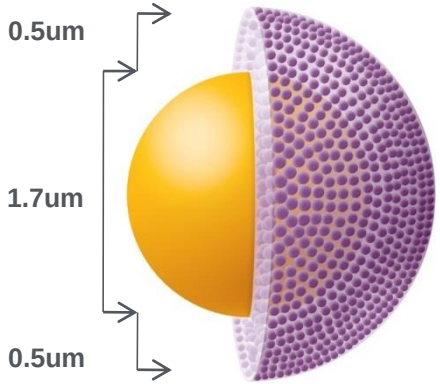
$$N \propto \frac{L}{d_p}$$

Poroshell 120 Particle Sizes



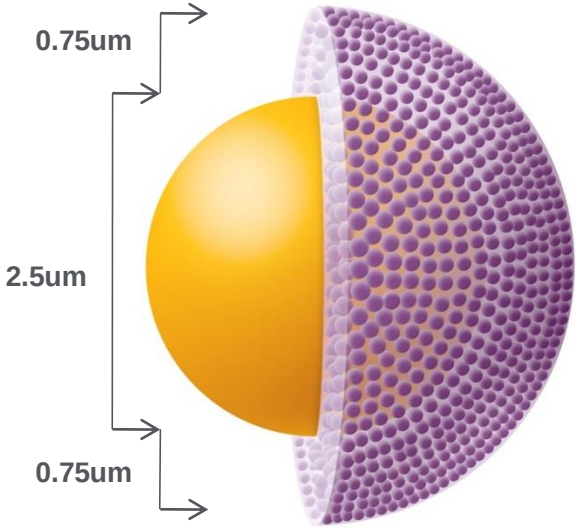
InfinityLab Poroshell 120
1.9 µm

Highest UHPLC performance



InfinityLab Poroshell 120
2.7 µm

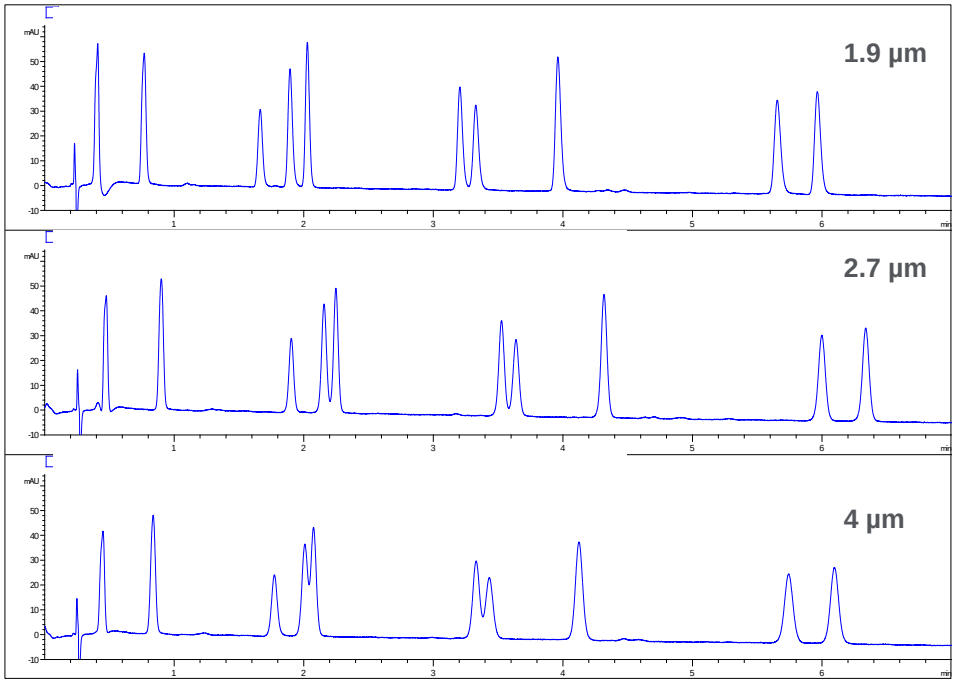
UHPLC performance at lower pressure



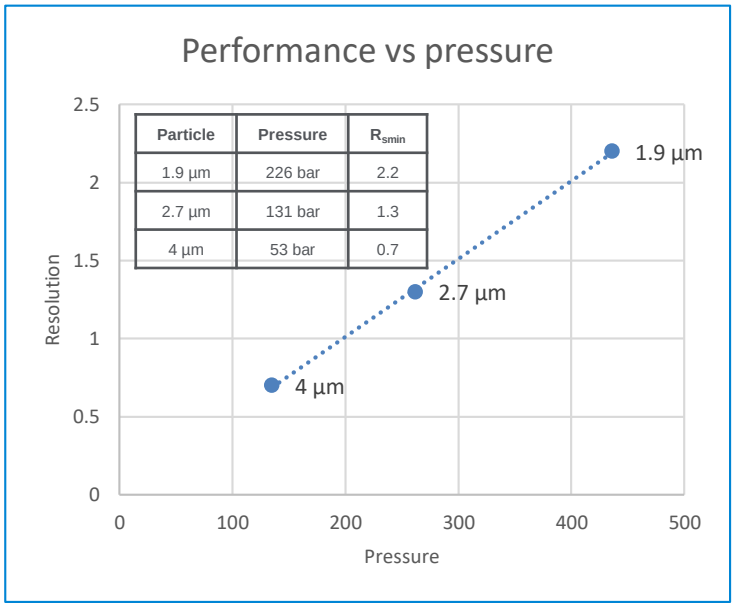
InfinityLab Poroshell 120
4 µm

Improved HPLC performance

Decreasing Particle Size Increases Efficiency



Columns:
InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 1.9 um
InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 2.7 um
InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 4 um
Mobile phase A: 0.2% formic acid in water
Mobile phase B: Acetonitrile
Gradient: 5-16% B in 7 min
Flow rate: 0.5 mL/min
Detection: 240 nm @ 80 Hz
Sample: 1 µL of 0.06 mg/mL each of gallic acid, gallocatechin, epigallocatechin, catechin, caffeine, epicatechin, epigallocatechin gallate, gallocatechin gallate, epicatechin gallate, catechin gallate

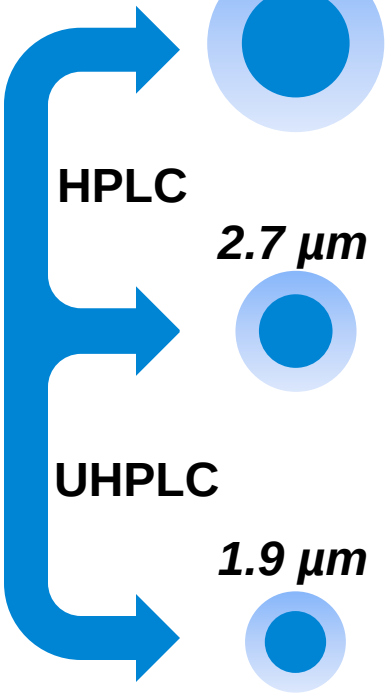


- Higher N improves resolution as particle size is decreased

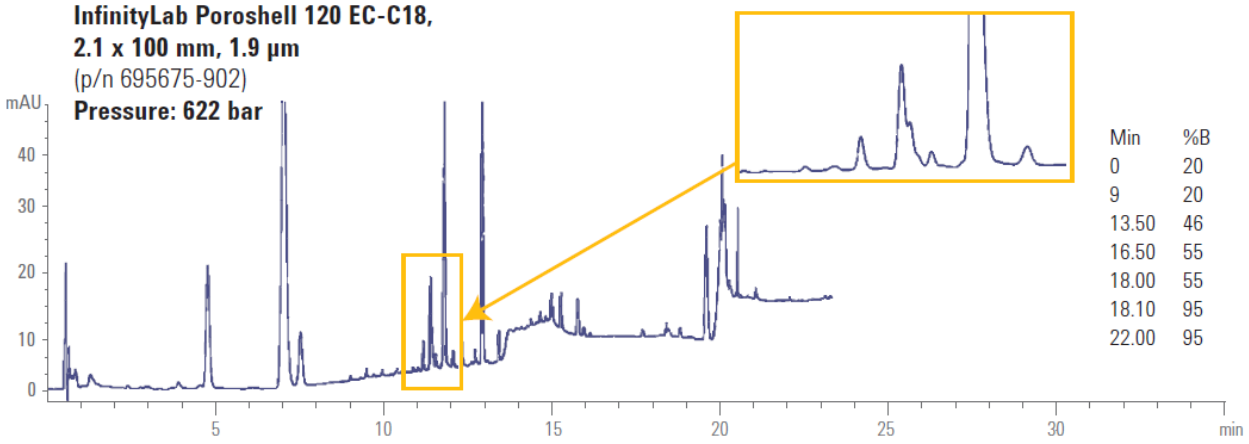
$$N \propto \frac{L}{d_p}$$

Particle Size: When to Use What Size

Technique / product		*Performance*
HPLC	*4 µm*	**Improved HPLC performance** Pressure rating: 600 bar Typical pressure: Often < 200 bar Efficiency: ~200% of 5 µm totally porous
HPLC	*2.7 µm*	**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
UHPLC	*1.9 µm*	**Highest UHPLC performance** Pressure rating: 1300 bar Typical pressure: Similar to sub-2 µm totally porous Efficiency: ~120% of sub-2 µm totally porous



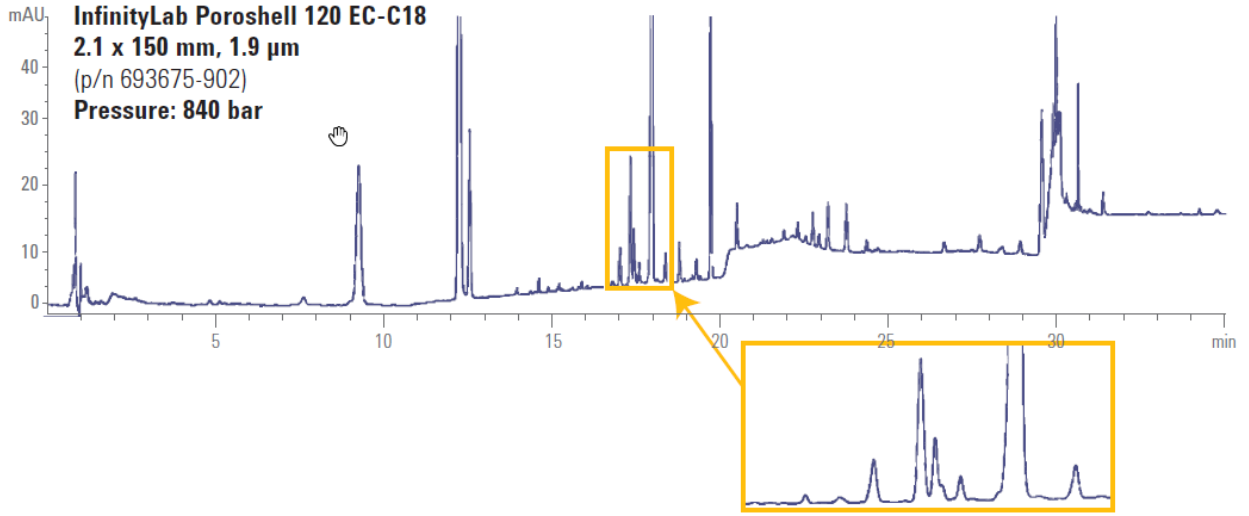
High Resolution Separations
Smaller Particle Size with Longer Columns



Min	%B
0	20
9	20
13.50	46
16.50	55
18.00	55
18.10	95
22.00	95

Conditions:

Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Gradient:	See chromatograms
Flow rate:	0.42 mL/min
Temperature:	25° C
Detection:	203 nm @ 80 Hz
Sample:	1.5 µL of Notoginsenoside R1, Ginsenoside Rg1, Ginsenoside Re, Ginsenoside Rb1, Ginsenoside Rd



Min	%B
0	20
9	20
20.25	46
24.75	55
27.00	55
27.10	95
33.00	95

How to Improve Resolution?

Change Selectivity:

Bonded phase

- Phases other than C18/C8
- Phenyl-Hexyl, Polar-embedded, CN, PFP, HILIC
- Different interactions with polar or non-polar compounds
- Other types of interactions with a bonded phase (π-interactions, etc.)

Mobile phase

- Mobile phase – organic modifier (ACN, MeOH etc.)
- Mobile phase – pH – over a wide pH range – pH 1-12 if needed

Temperature

Agilent InfinityLab Poroshell phases

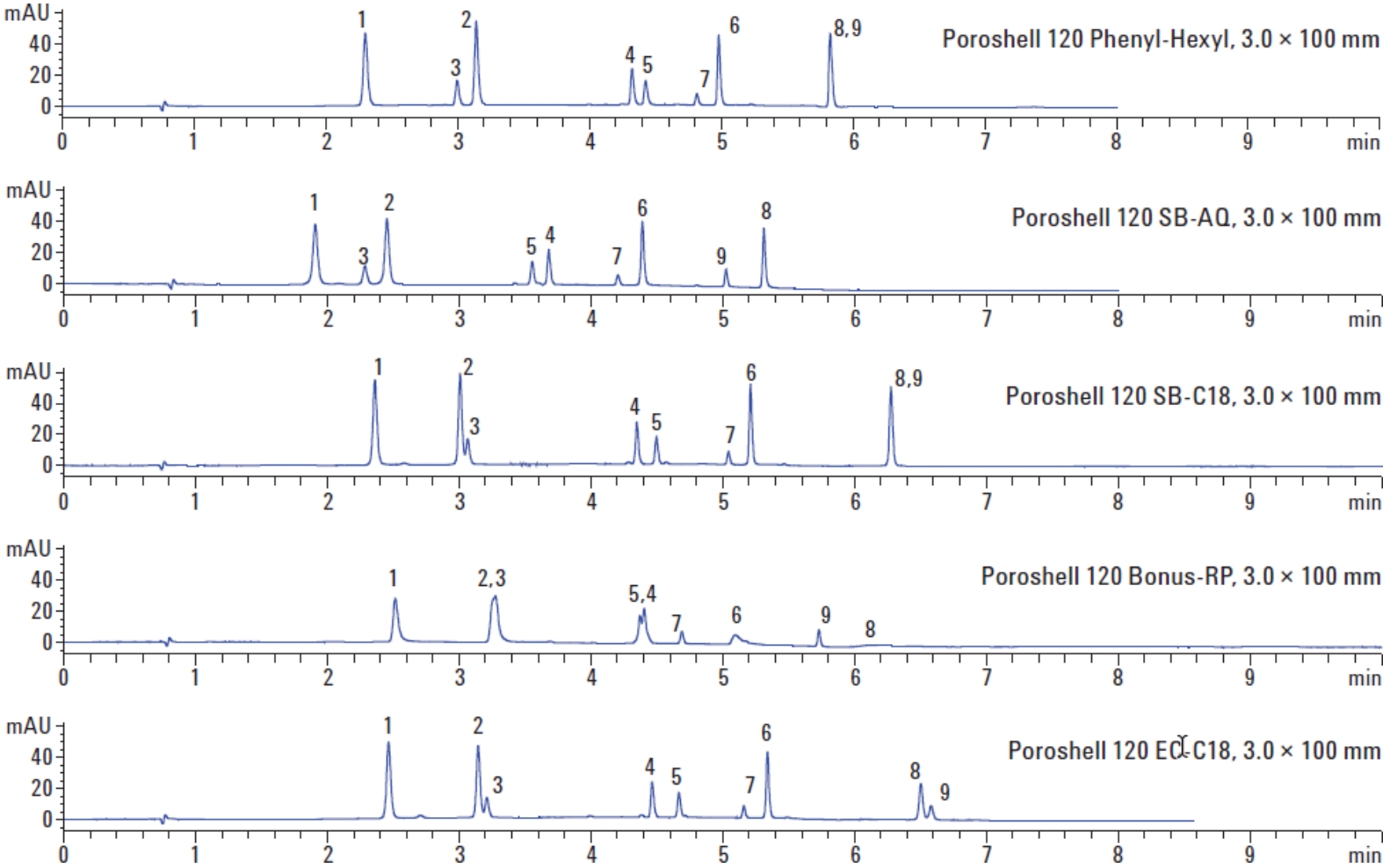
Best all around	Best for low pH mobile phases	Best for high and mid pH mobile phases	Best for alternative selectivity	Best for more polar compounds	HILIC for polar compounds
Poroshell 120 **EC-C18** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-C18** 2.7 µm	Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **Phenyl-Hexyl** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-Aq** 2.7 µm	Poroshell 120 **HILIC** 1.9 µm, 2.7 µm, 4 µm
Poroshell 120 **EC-C8** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-C8** 2.7 µm	Poroshell **HPH-C8** 2.7 µm, 4 µm	Poroshell 120 **Bonus-RP** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **EC-CN** 2.7 µm	Poroshell 120 **HILIC-Z** 2.7 µm
			Poroshell 120 **PFP** 2.7 µm		Poroshell 120 **HILIC-OH5** 2.7 µm

Why is Changing the Bonded Phase Effective?

- 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, reduce analysis time
- Having numerous different bonded phases available on the same particle makes development easier

  – Fast Poroshell methods make development faster

Poroshell Selectivity – Antioxidants (ACN)



Sample:
1. PG
2. THBP
3. TBHQ
4. NDGA
5. BHA
6. OG
7. Ionox-100
8. DG
9. BHT

Eluent:	A 1.5% acetic acid, B methanol
Injection volume:	2 µL of 10 ppm mixture in 10% methanol
Flow rate:	0.6 mL/min
Gradient:	Time (min) / % B: 0 / 40; 1 / 40; 5 / 80; 8 / 80
Temperature:	40 °C
Detector:	UV, 280 nm

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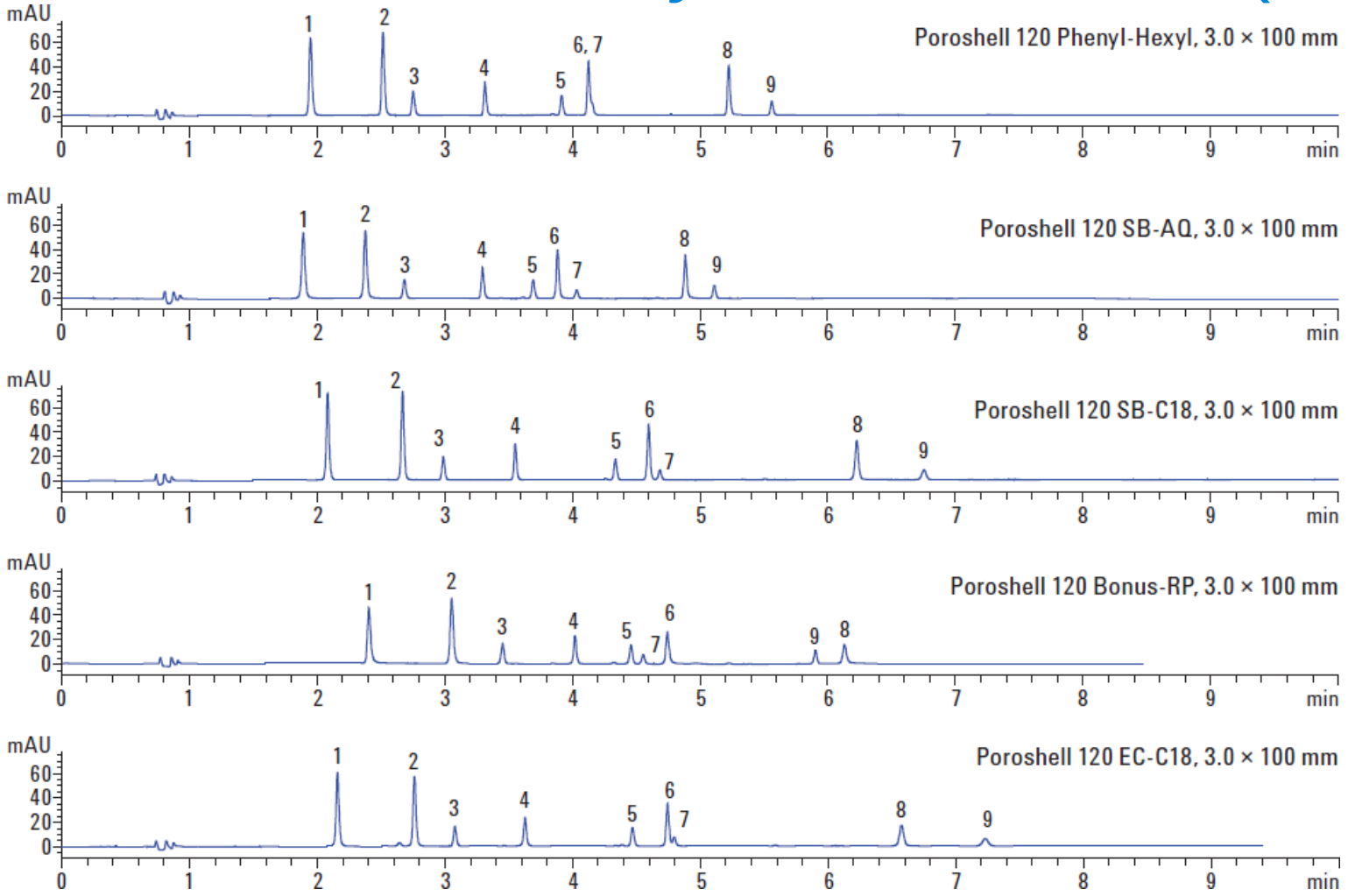
Poroshell Selectivity – Antioxidants (MeOH)



Sample:
1. PG
2. THBP
3. TBHQ
4. NDGA
5. BHA
6. OG
7. Ionox-100
8. DG
9. BHT

Eluent:	A 1.5% acetic acid, B ACN
Injection volume:	2 µL of 10 ppm mixture in 10% methanol
Flow rate:	0.6 mL/min
Gradient:	Time (min) / % B: 0 / 25; 0.5 / 25; 5 / 80; 8 / 80
Temperature:	40 °C
Detector:	UV, 280 nm

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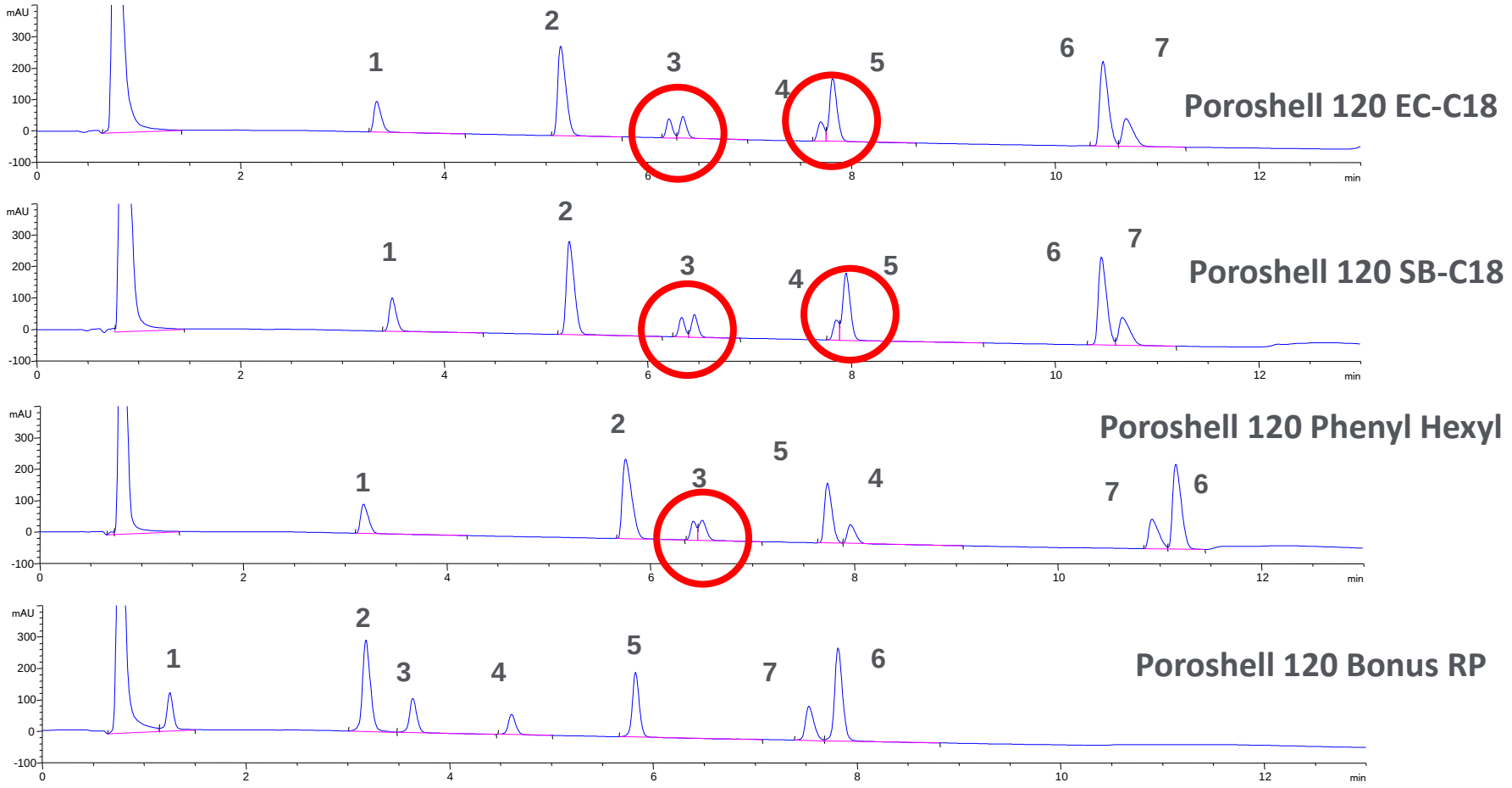
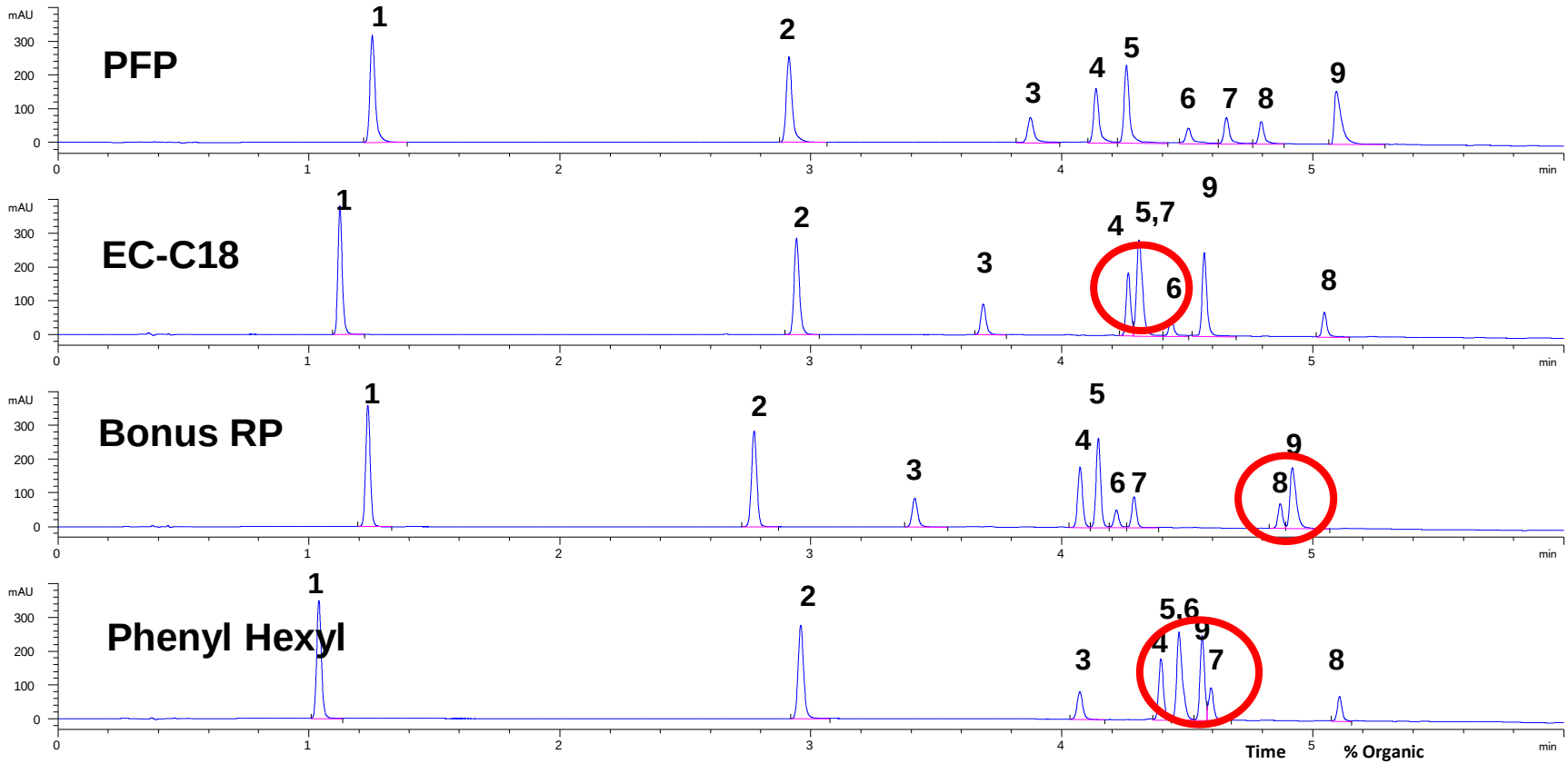
Poroshell Selectivity – Beta Blockers



1.Atenolol, 2. Pindolol, 3. Naldolol, 4. Metoprolol, 5. Acebutolol, 6. Propranolol, 7. Alprenolol

10 mM pH 3.8 NH_4HCO_2, Methanol; 90 % B to 30 % B over 12 min, 0.35 ml/min, 2.1 x 100 mm

NSAID Separation with a Methanol Gradient



1. APAP, 2. Phenacetin, 3. Piroxicam, 4. Tolmetin, 5. Ketoprofen, 6. Naproxen, 7. Sulindac, 8. Diclofenac, 9. Diflunisal

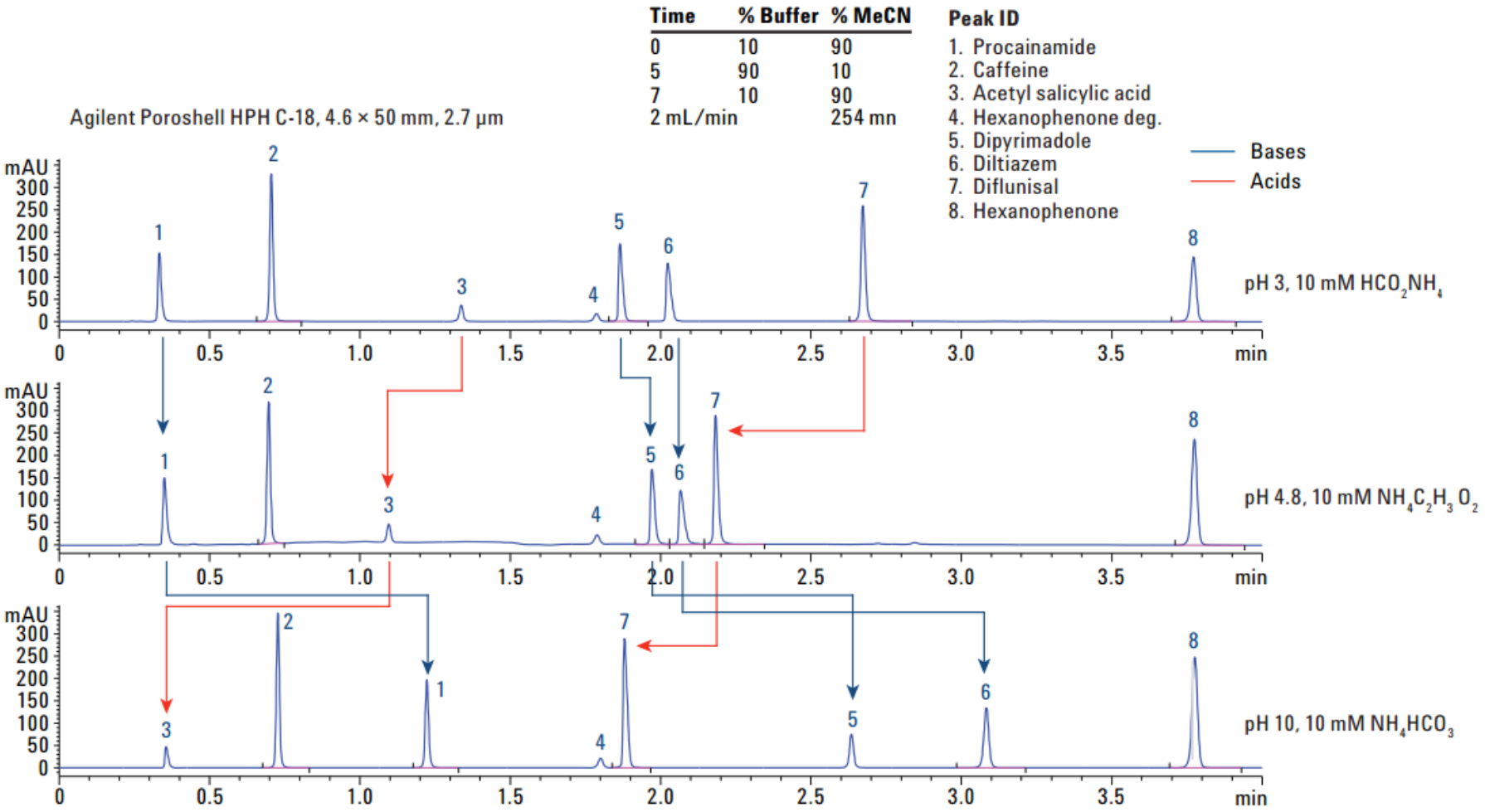
Time	% Organic
0	8
6	100
7	100
8	8

2mL/min 254 nm

Agilent Poroshell phases

Best all around	Best for low pH mobile phases	Best for high and mid pH mobile phases	Best for alternative selectivity	Best for more polar compounds	HILIC for polar compounds
Poroshell 120 **EC-C18** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-C18** 2.7 µm	Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **Phenyl-Hexyl** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-Aq** 2.7 µm	Poroshell 120 **HILIC** 1.9 µm, 2.7 µm, 4 µm
Poroshell 120 **EC-C8** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-C8** 2.7 µm	Poroshell **HPH-C8** 2.7 µm, 4 µm	Poroshell 120 **Bonus-RP** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **EC-CN** 2.7 µm	Poroshell 120 **HILIC-Z** 2.7 µm
			Poroshell 120 **PFP** 2.7 µm		Poroshell 120 **HILIC-OH5** 2.7 µm

Comparing Low, Mid, & High pH



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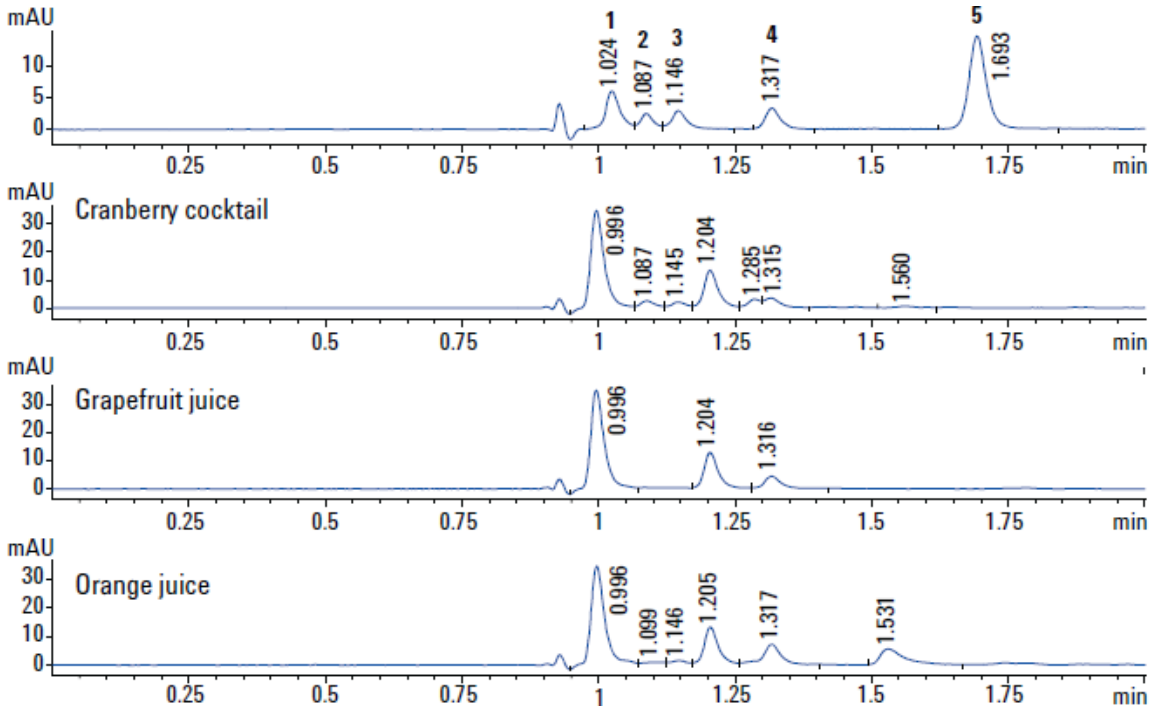
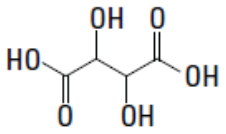
			Poroshell 120 **PFP** 2.7 µm		Poroshell 120 **HILIC-OH5** 2.7 µm

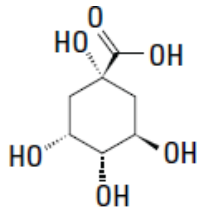
Aliphatic Acids on Poroshell SB-Aq

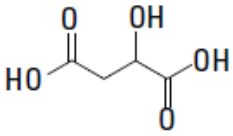


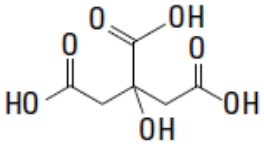
Peak ID

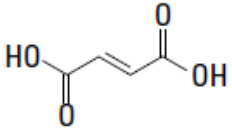
1. Tartaric acid
2. Quinic acid
3. Malic acid
4. Citric acid
5. Fumaric acid


Tartaric acid


Quinic acid


Malic acid


Citric acid


Fumaric acid

Column:	Agilent Poroshell 120 SB-Aq, 3 × 100 mm, 2.7 µm (p/n 685975-314)
Eluent:	100 mM Potassium phosphate buffer, pH 2.5
Injection volume:	5 µL
Flow rate:	0.5 mL/min
Temperature:	50 °C
Detector:	DAD, at 226 nm

5991-1992EN

HILIC
Hydrophilic Interaction Chromatography

- HILIC offers more retention than reversed-phase for very polar bases

- Polar stationary phase:
  - Silica
  - Amine
  - Amide
  - Zwitterionic
  - Diol
  - Others

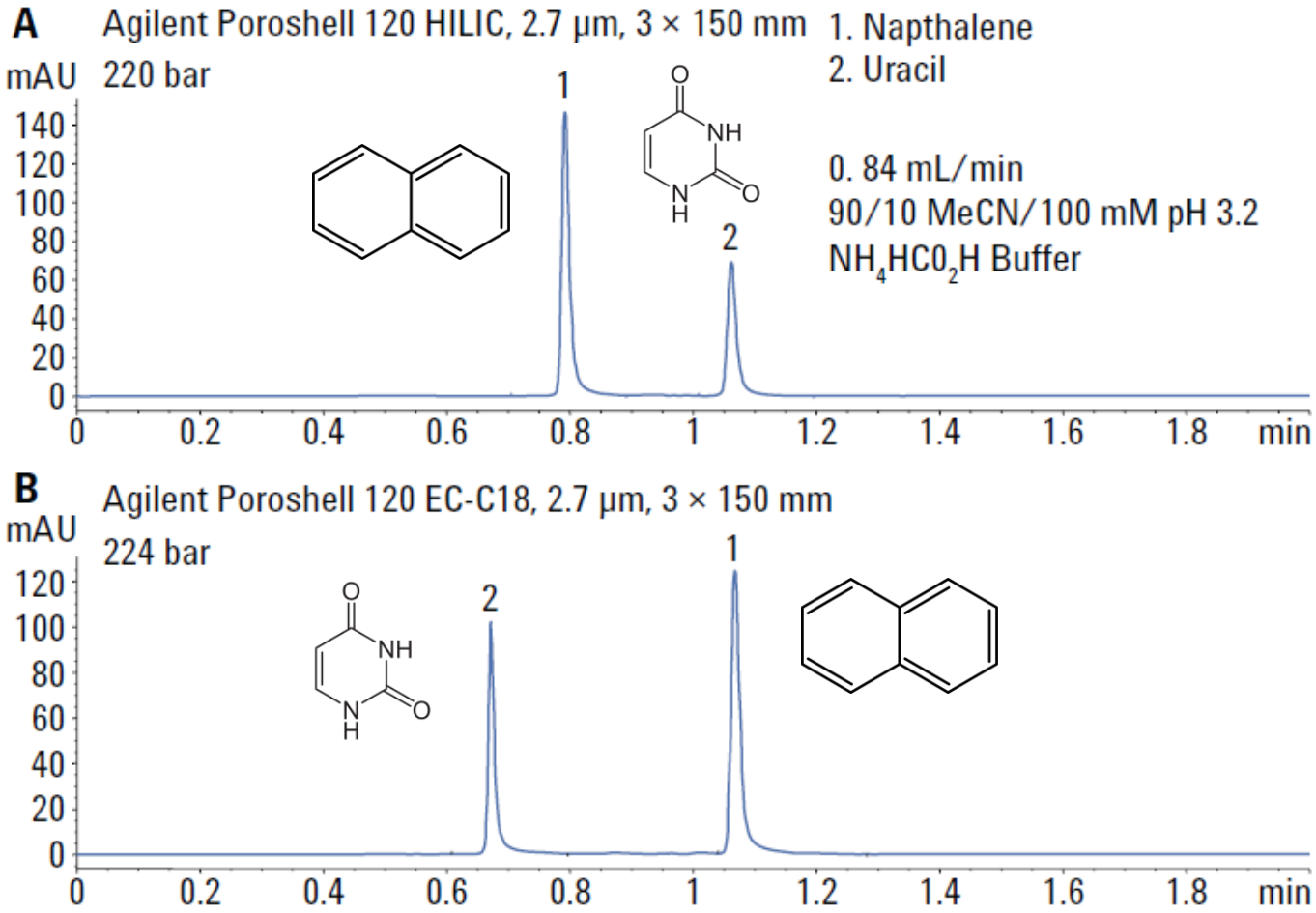
- Polar mobile phase:
  - Water is the strong solvent
  - Typically ACN/water
  - Buffer controls ionization of analyte and stationary phase
  - Typically ammonium acetate or ammonium formate

- Retention/elution is from least to most polar

Advantages to using HILIC

- Uses a standard LC system and common reversed-phase solvents
- Separates cations, anions and polar neutrals in a single run
- Retains polar analytes where reversed-phase methods may not
- MS compatible eluents and easy setup
- Improved ionization and sensitivity in MS mode
- Compatible with MS in both positive and negative analysis modes

HILIC vs C18



NEW INFINITYLAB POROSHELL 120 HILIC COLUMNS

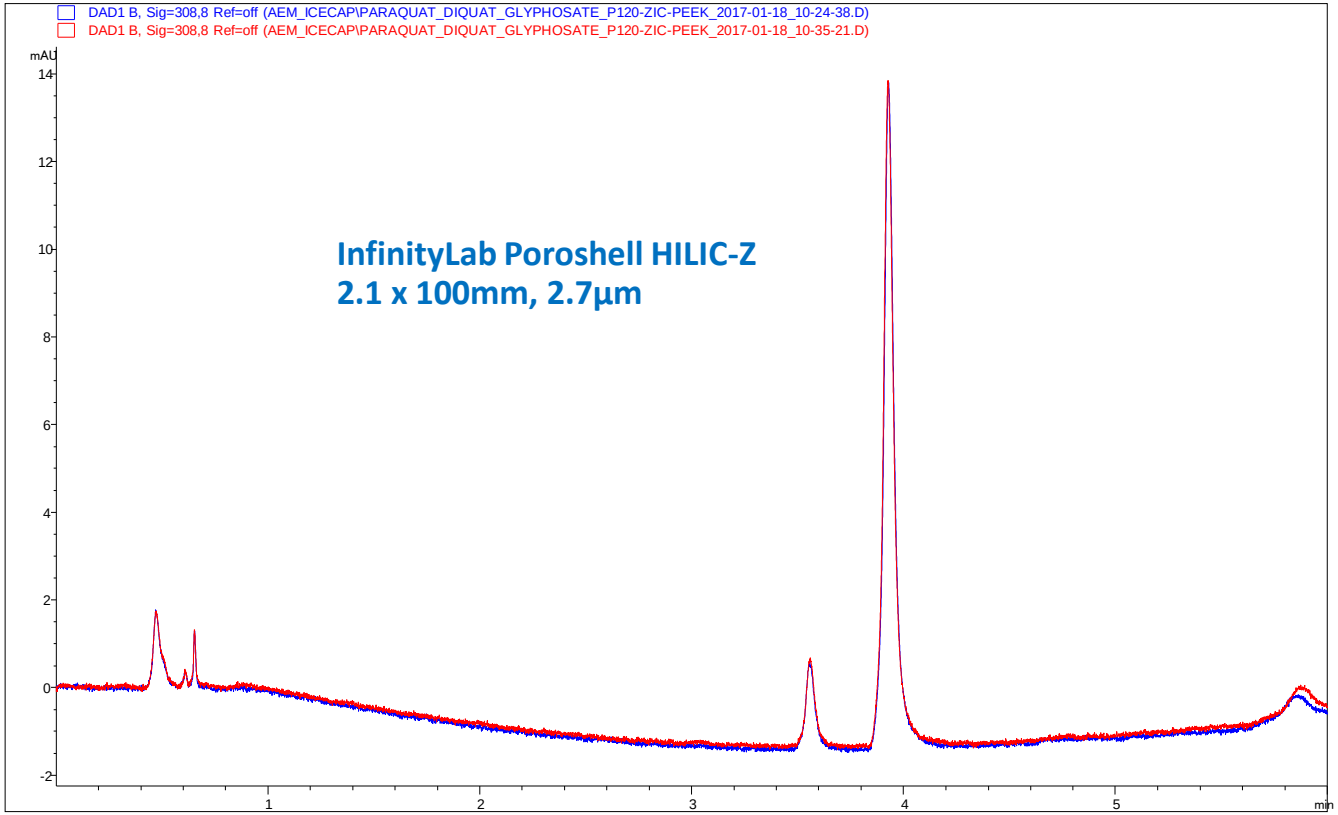
HILIC-Z: Zwitterionic stationary phase

- 2.7 µm Poroshell particles
- 2.1mm, 3.0mm and 4.6mm ID
- 50mm, 100mm and 150mm lengths

HILIC-OH5: poly-hydroxy fructan stationary phase

- 2.7 µm Poroshell particles
- 2.1mm and 4.6mm ID
- 50mm, 100mm and 150mm lengths

Fast re-equilibration and excellent peak shape
Polar pesticides

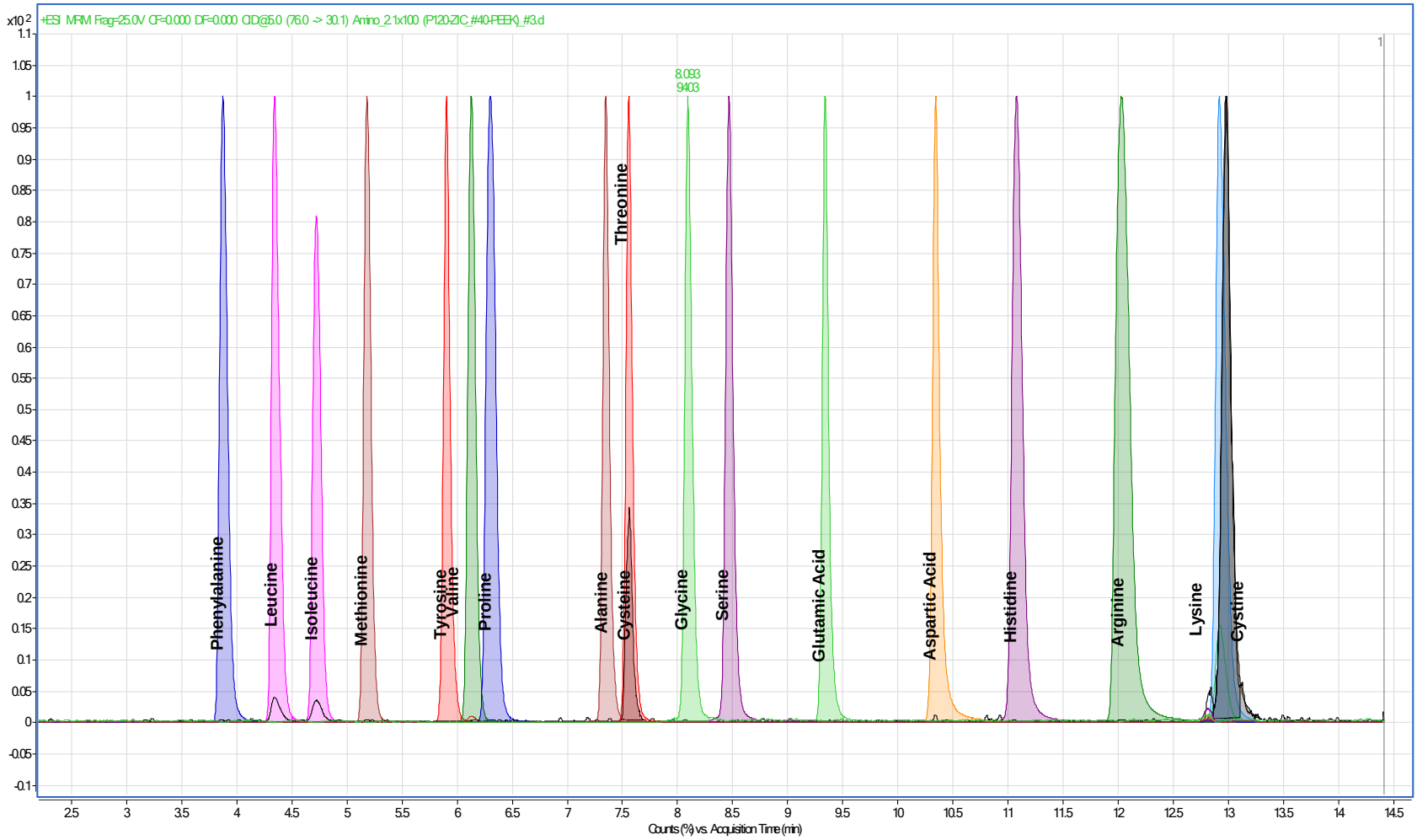


Repeat Injections (back-to-back)

Sample	Diquat and Paraquat
Gradient	6 min
Equilibration	4 min
Total Runtime	10 min

Excellent baseline reproducibility with only 1.5 min re-equilibration between injections!

Excellent retention, peak shape and sensitivity
Underivatized Amino Acids by LC/MS



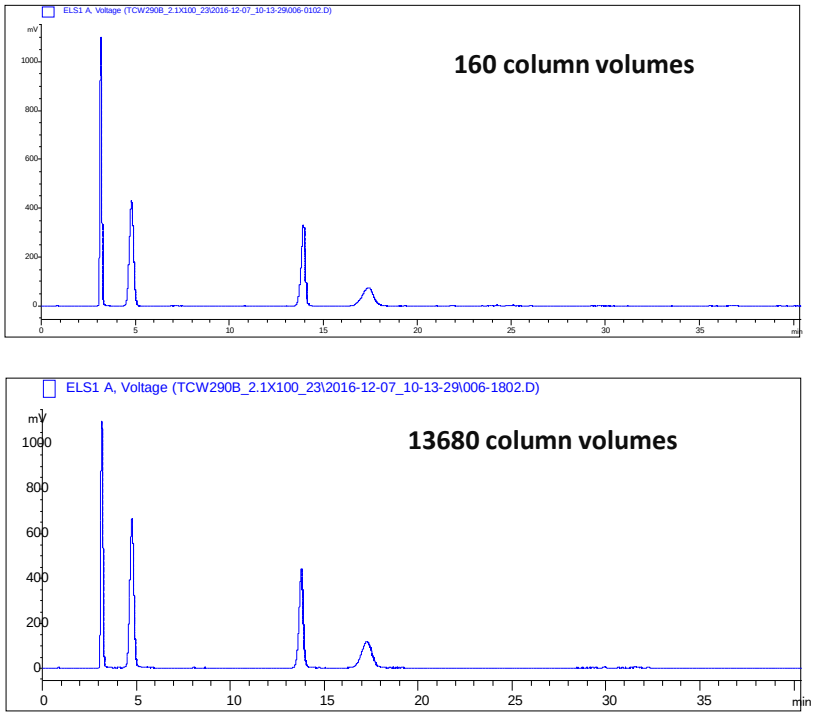
InfinityLab Poroshell HILIC-Z

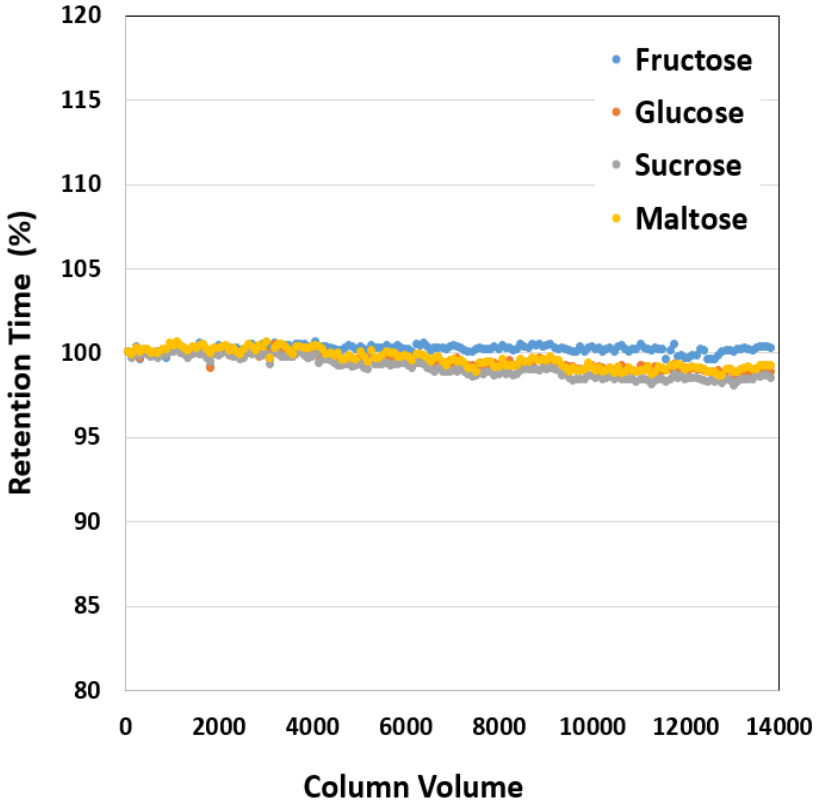
2.1 x 100mm, 2.7µm

A: pH 3.0 20mM ammonium formate
B: ACN

Gradient method on LC/QQQ

Consistent analytical reproducibility and long column lifetimes
High pH Sugar Analysis

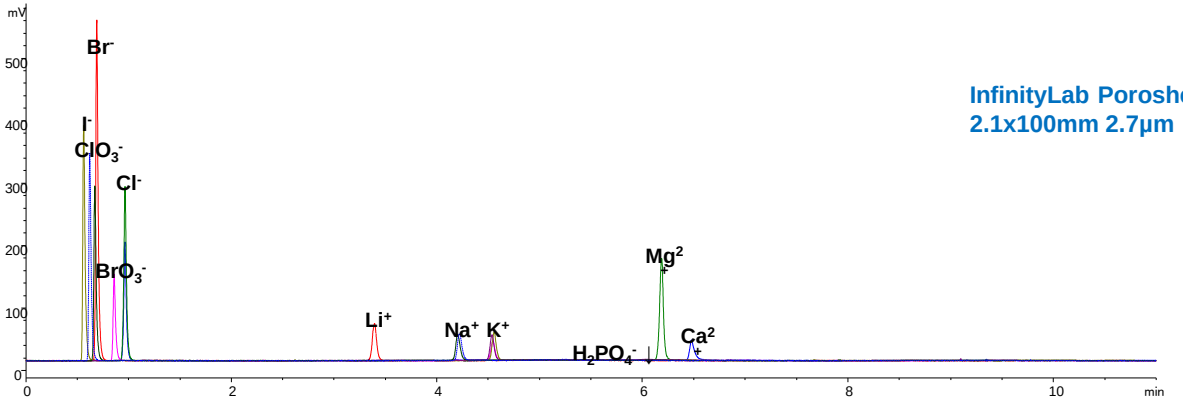




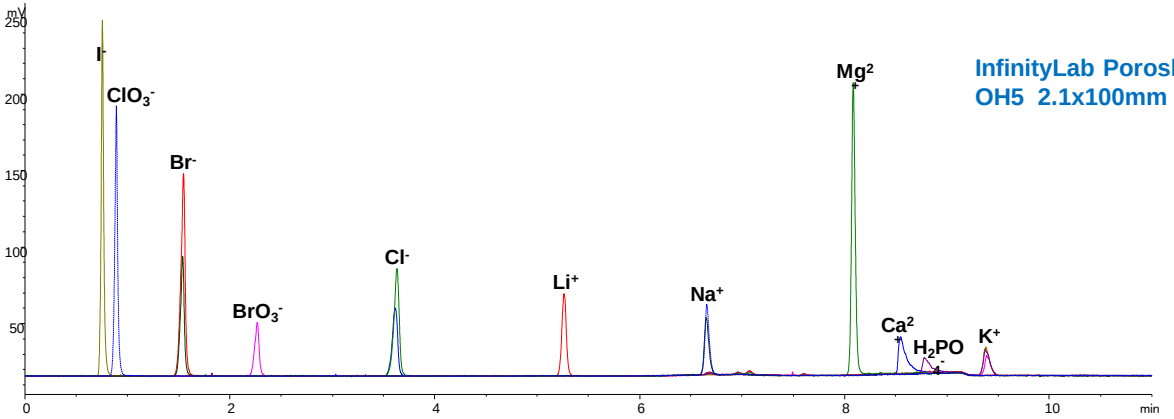
InfinityLab Poroshell HILIC-Z
2.1 x 100mm, 2.7µm

A: 1.0% Ammonium hydroxide pH 12.0
B: CH3CN
90%B for 40 min, 0.4 mL/min, 35˚C
ELSD: 60C/3.5psi

Alternate Selectivity of HILIC Phases - Retention of Inorganic Ions



InfinityLab Poroshell 120 HILIC 2.1x100mm 2.7µm



InfinityLab Poroshell 120 HILIC-OH5 2.1x100mm 2.7µm

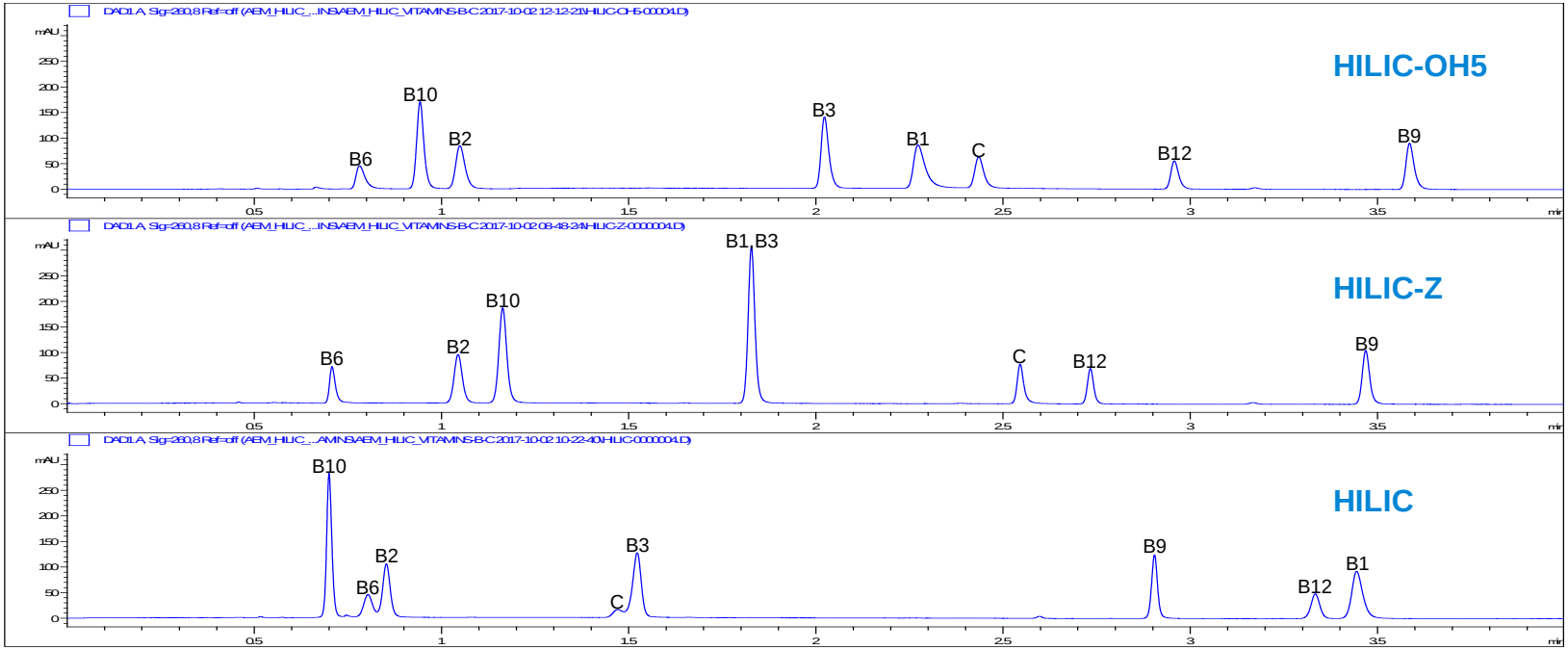
Calcium chloride
Lithium bromide
Magnesium chloride
Potassium bromate
Potassium iodide
Potassium phosphate
Sodium bromide
Sodium chlorate

Method conditions for both applications:

A: 100 mM pH 3 Ammonium Formate, B: Acetonitrile, hold 91% B for 1 min, 91-60% B in 10 min, 3 min re-equilibration,
0.4 mL/min, 30 C, 1 uL injection, ELSD 40C/3.5psi/30Hz

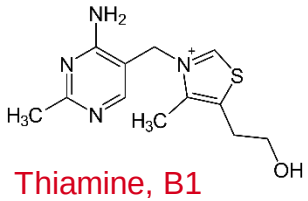
Selectivity Across HILIC phases – Water soluble vitamins

InfinityLab Poroshell 120 column 2.1 x 100 mm 2.7 µm

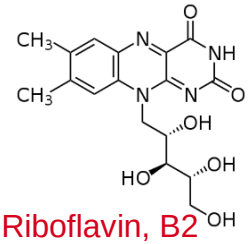


A: 100 mM Ammonium Acetate + 0.5% Acetic Acid (pH ~4.6) in H_2O, B: CH_3CN
0.5 mL/min, 87% B for 1 min, 87-50% B in 4 min, 3 min re-equilibration
1 µL injection, 40 °C, UV detection at 260 nm, 80 Hz

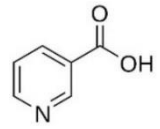
Water-Soluble Vitamins



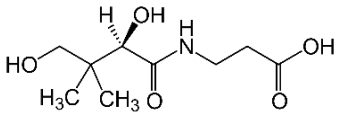
Thiamine, B1



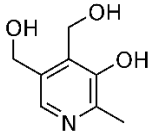
Riboflavin, B2



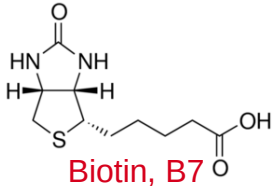
Nicotinic Acid, B3



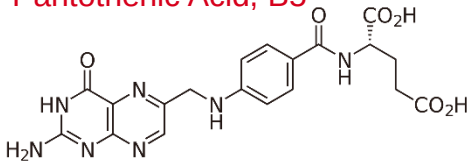
Pantothenic Acid, B5



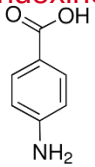
Pyridoxine, B6



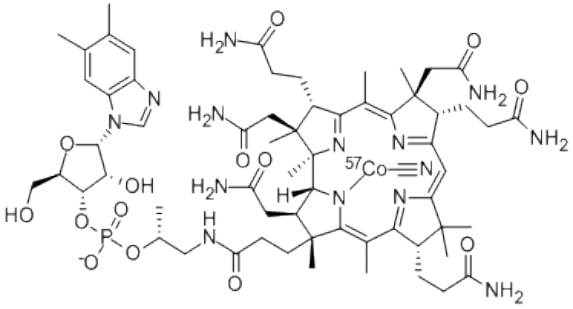
Biotin, B7



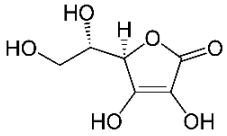
Folic Acid, B9



PABA, B10

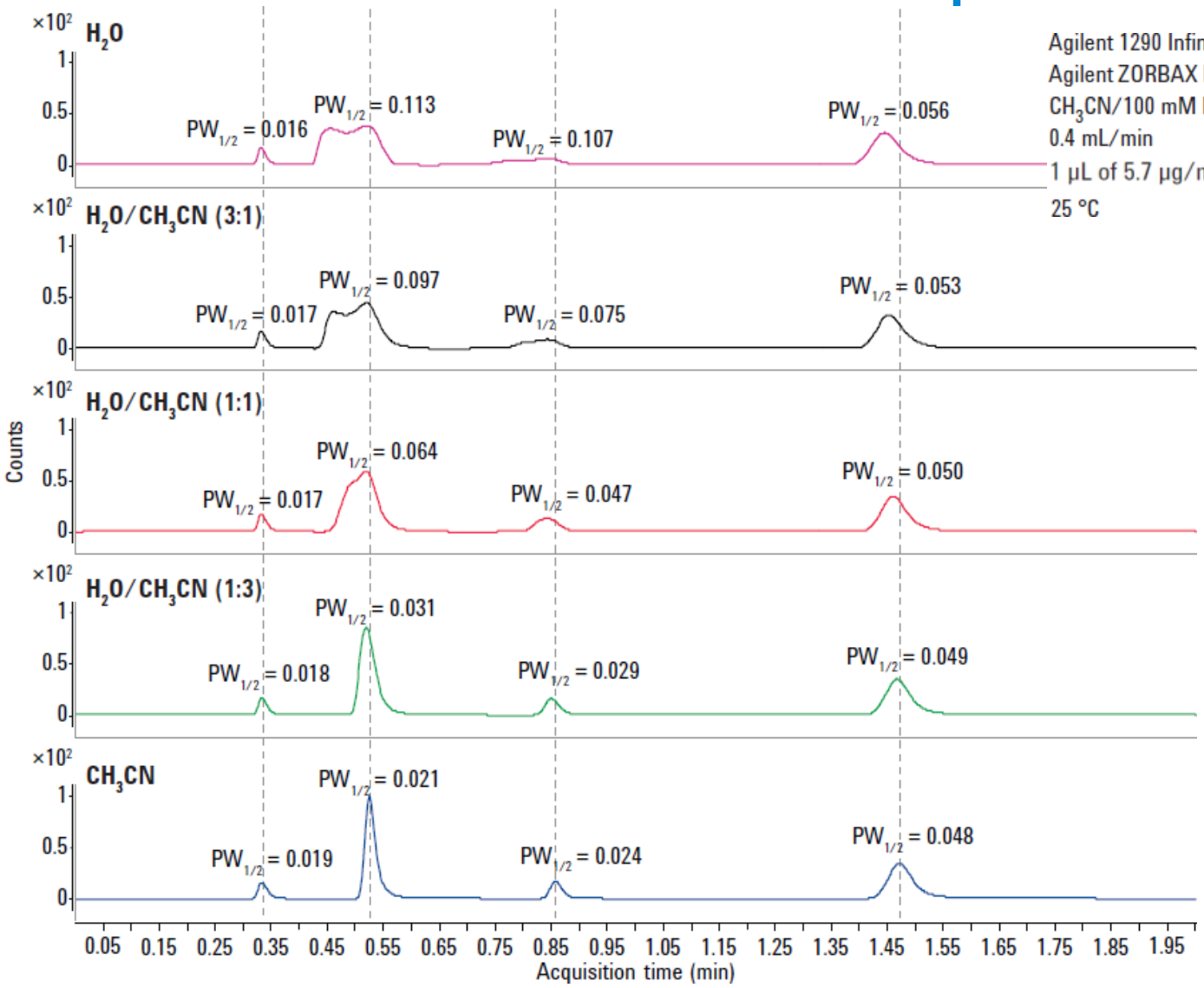


Cyanocobalamin, B12



Ascorbic Acid, C

HILIC and Choice of Sample Solvent



Agilent 1290 Infinity LC System with an Agilent 6410A ESI+
Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm
CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
0.4 mL/min
1 µL of 5.7 µg/mL each
25 °C

1. 4 Aminobenzoic acid
2. Nicotinamide
3. Riboflavin
4. Nicotinic acid

Resolution

How to reach your goal?

- Choose a column with the right balance of particle size and length
- Take advantage of selectivity changes
  - Mobile phase
  - pH
  - Bonded phase
- Take advantage of polar phases or HILIC for hard to retain analytes

Thank you!

LC-column-support@agilent.com