Analysis of Polar Analytes by LC
What are the Options?

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Applications Engineer
Columns and Supplies Technical Support
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Agilent
Trusted Answers

Options for Analysis of Polar Analytes by LC

- Try reversed phase C18. If needed, adjust method conditions (strength of mobile phase, pH)

- Ion-pair reversed-phase chromatography

- Alternative column choice – more polar RP phases are compatible with 100% aqueous mobile phase

- Alternative mode of separation – HILIC (hydrophilic interaction chromatography)
  - Silica
  - Amino
  - Amide
  - Zwitterionic
  - Diol

- Alternative mode of separation – ligand exchange
  - Hi-Plex (sulfonated polystyrene/divinylbenzene with different counterions) for analysis of organic acids and carbohydrates

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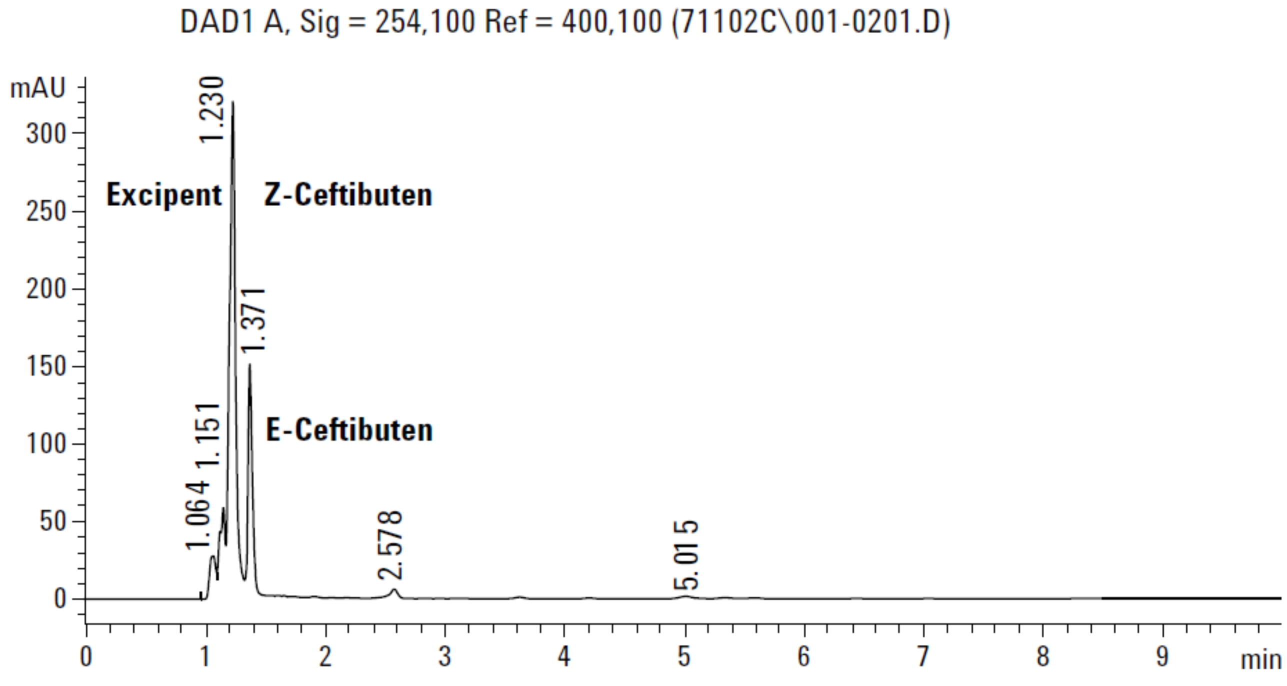
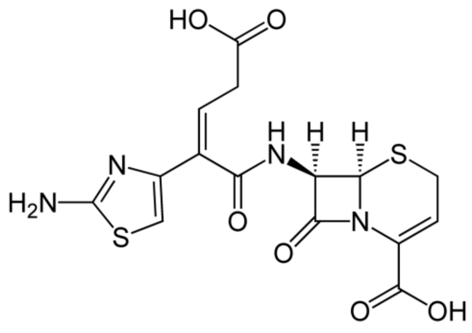
Options for Analysis of Polar Analytes by LC

Try reversed phase C18

What if it does not work? Decrease the strength of the mobile phase



Instrument: Agilent 1100 Series HPLC; **Temp:** ambient; **Column:** Alkyl-C18, 4.6 × 150 mm, 5 µm; **Mobile phase:** 2% ACN, 98% 10 mM ammonium acetate, pH 5.4; **Flow rate:** 1 mL/min; **Injection volume:** 5 µL; **Diode array detector:** 254 nm; **Reference:** 400 nm; **Bandwidth:** 100 nm



cephalosporin

Options for Analysis of Polar Analytes by LC

Pore dewetting or phase collapse of RP phases

- Alkyl phases such as C8 or C18 can exhibit poor retention or reproducibility of retention in low organic mobile phases
- This is a phenomenon known as pore dewetting or phase collapse
- It is a method robustness issue, which is often mistaken as a column or lot related issue
- The onset can be unpredictable
- See Przybyciel and Majors, *LCGC* **20**(6), 516-523 (2002)

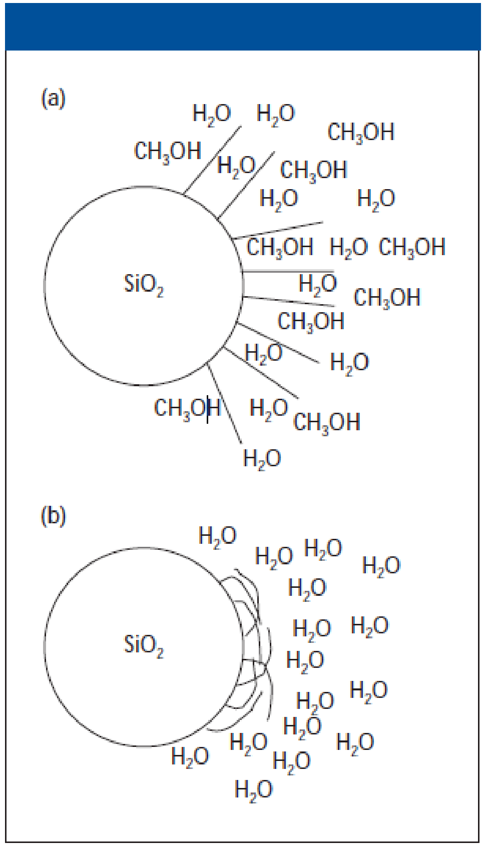


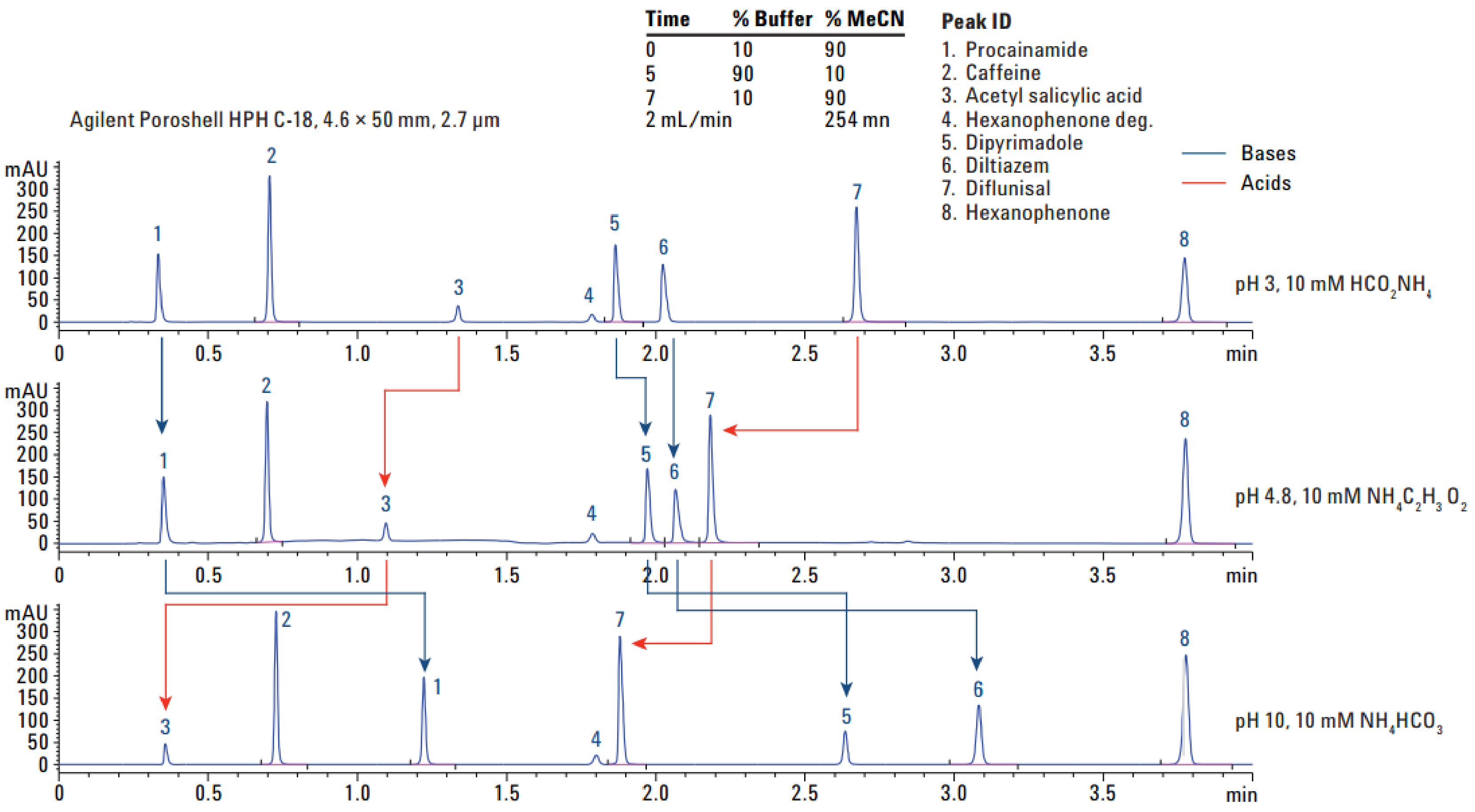
Figure 1: Illustration of the classic explanation of phase collapse in reversed-phase chromatography. Shown are the configurations of long-chain bonded alkyl phases (a) in water–methanol mixtures and (b) in 100% water.

Options for Analysis of Polar Analytes by LC

Try reversed phase C18

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

- In RPLC mode, 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 impacted by mobile phase pH



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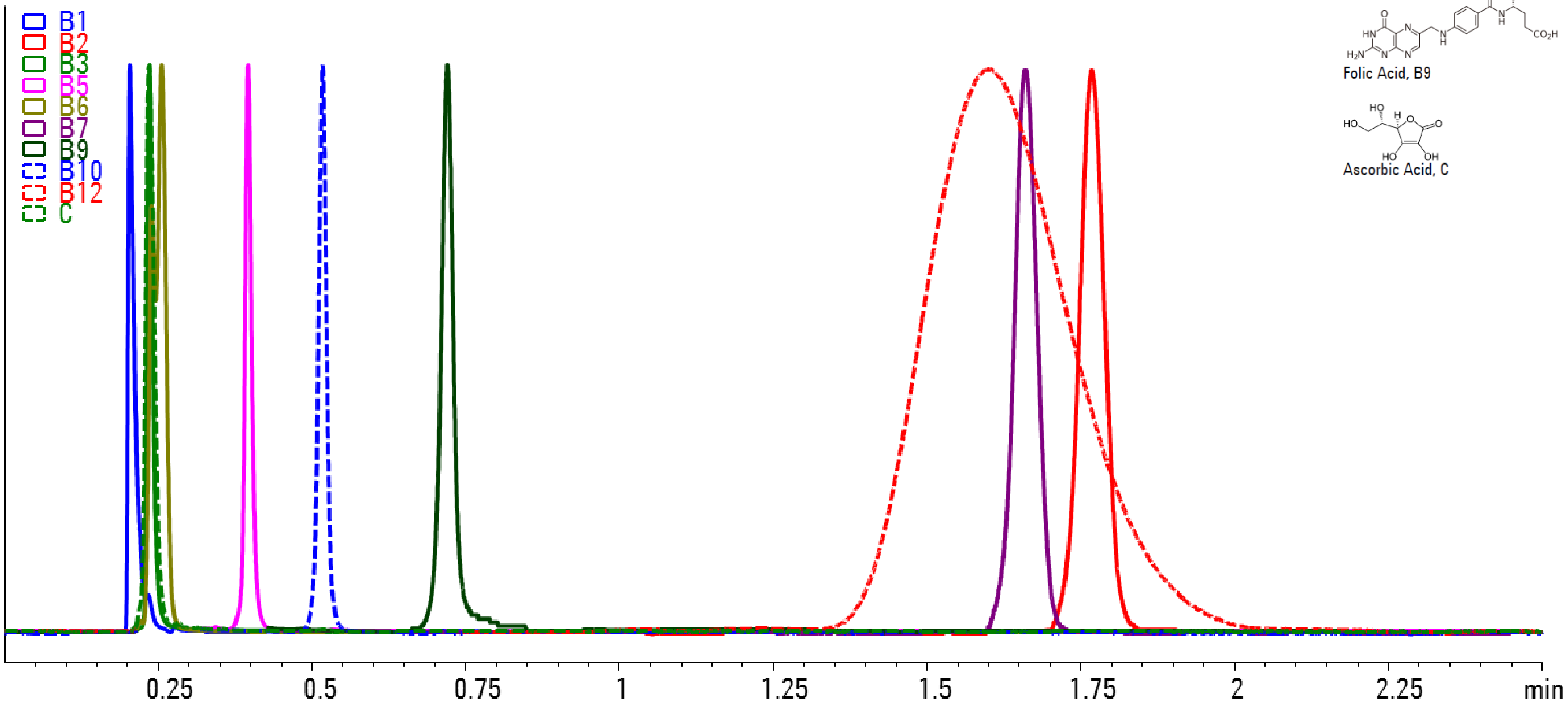
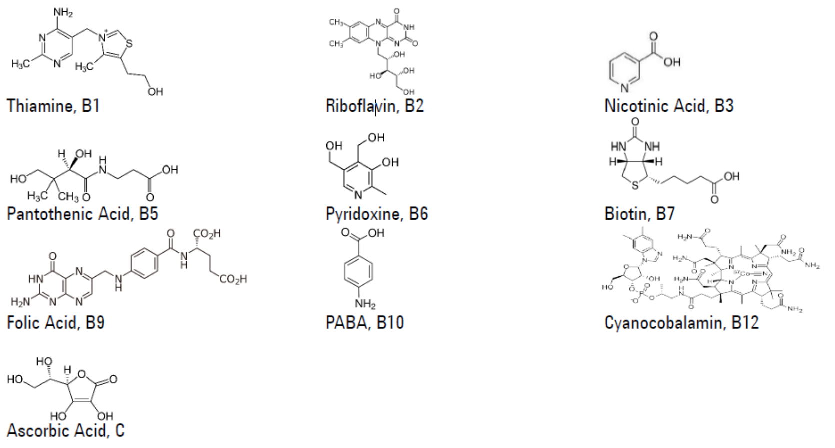
Options for Analysis of Polar Analytes by LC

Try reversed phase C18

Water soluble vitamins on C18 at low pH



Water soluble vitamins on a C18 column at low pH (2.5)



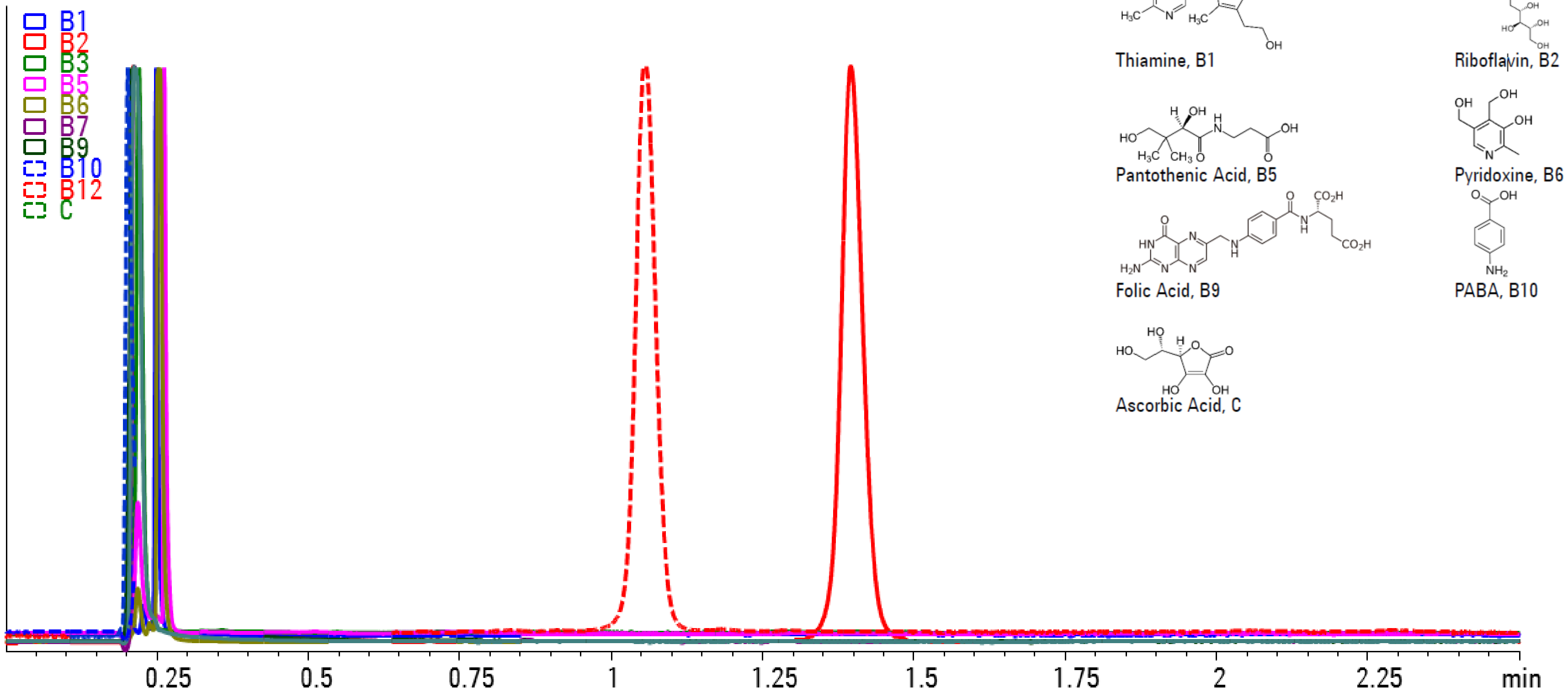
A: 20 mM NaH_2PO_4 pH 2.5
B: CH_3CN, 10% B isocratic
0.5 mL/min, 30 C, 210 nm
2.1 x 50 mm, 2.7 µm
Poroshell 120 EC-C18

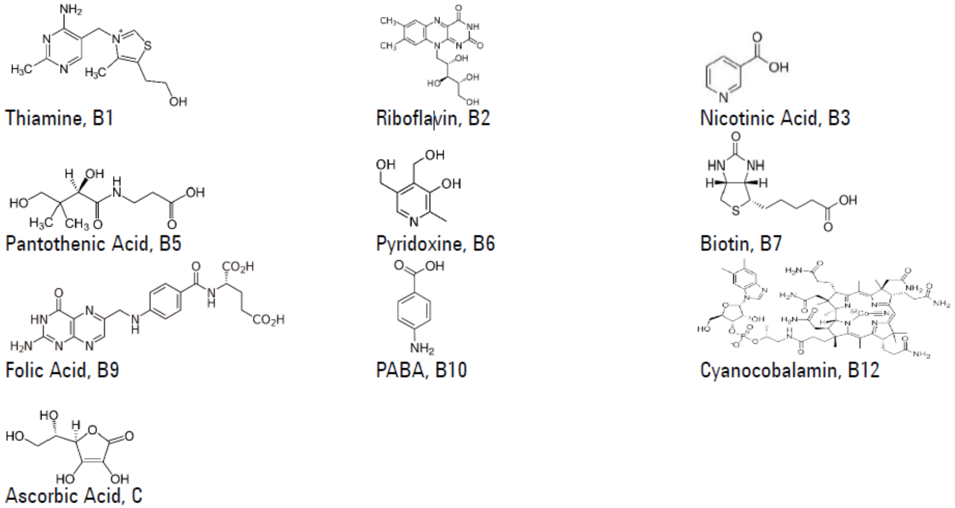
- Poor retention for four
- Coelution
- For six compounds,
- k' < 2

Options for analysis of Polar Analytes by LC

Try reversed phase C18

Adjust method conditions – pH modification





A: 20 mM Na_2HPO_4 pH 7.5
B: CH_3CN, 10% B isocratic
0.5 mL/min, 30 C, 210 nm
2.1 x 50 mm, 2.7 µm
Poroshell 120 HPH-C18

Water soluble vitamins on a C18 column at pH 7.5

- Acid groups now charged
- Loss of retention

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Options for Analysis of Polar Analytes by LC

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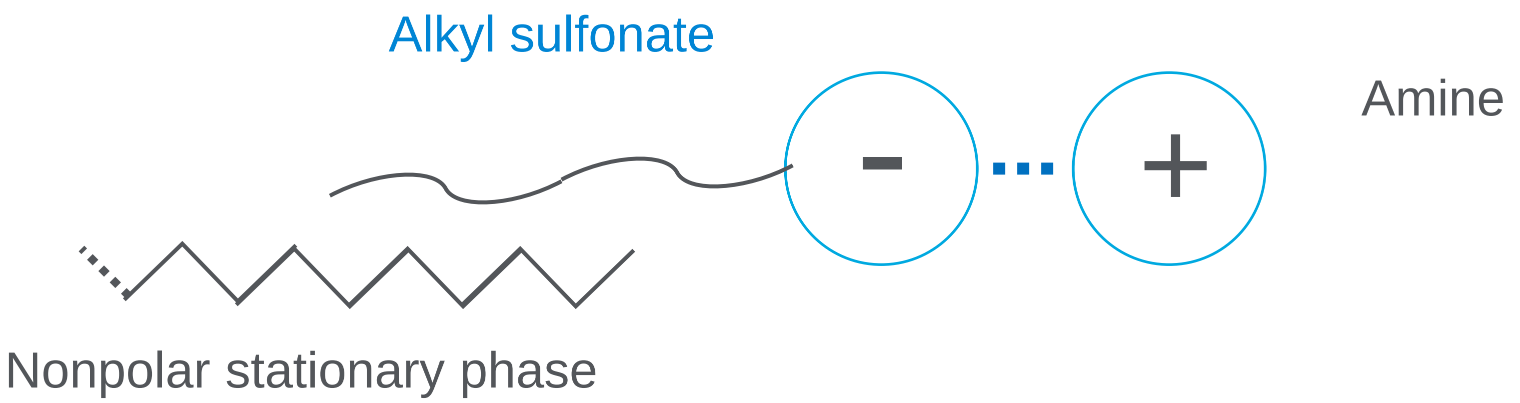
Options for Analysis of Polar Analytes by LC
Ion-pair reversed-phase chromatography

Similar to reversed phase, but an ion-pairing reagent is added to the mobile phase



- Nonpolar alkyl chain will adsorb into the nonpolar stationary phase
- Polar part of the ion-pairing reagent will "stick out" into the mobile phase

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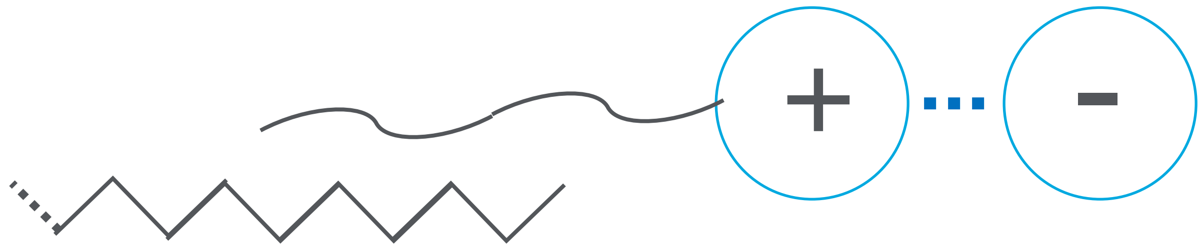

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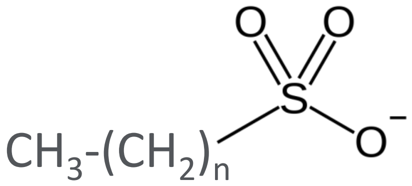
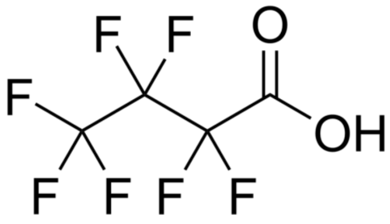
Options for Analysis of Polar Analytes by LC
Ion-pair reversed-phase chromatography

Common ion-pairing reagents

Pairs with cations

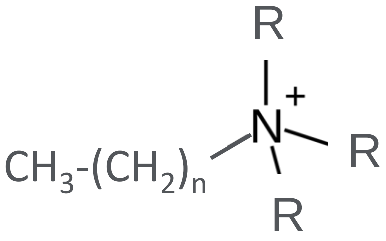


Alkyl sulfonates

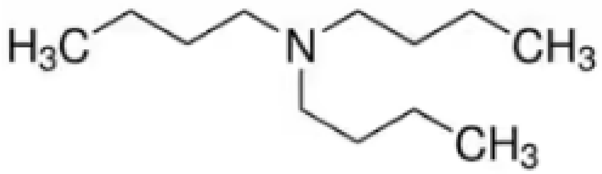


Heptafluorobutyric acid (HFBA)

Pairs with anions



Quaternary amines



Tributylamine (TBA)

Ion-Pair Chromatography
Suggested experimental conditions

Suggested experimental conditions

Column: C8 or C18

Mobile phase:

- Organic – often methanol
- Aqueous – buffer + appropriate IP reagent

Temperature controlled 35 to 60 °C

<u>Cations – bases</u>

Buffer: 25 to 50 mM phosphate, pH 2 to 3

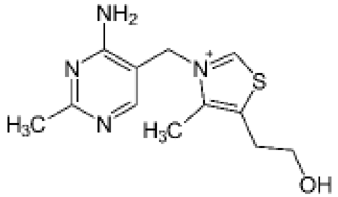
IP reagent: 10 to 100 mM heptane sulfonate

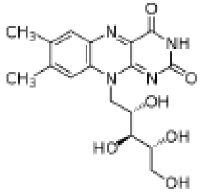
<u>Anions – acids</u>

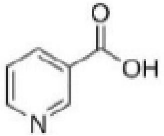
Buffer: 25 to 50 mM phosphate, pH 6 to 7

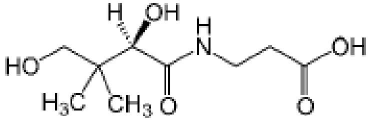
IP reagent: 10 to 40 mM tetrabutyl ammonium phosphate

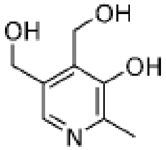
Water Soluble Vitamins

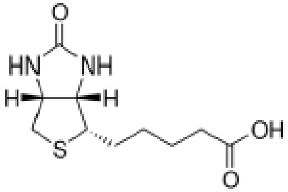

Thiamine, B1

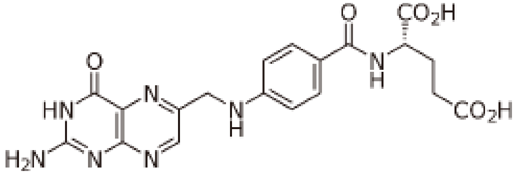

Riboflavin, B2

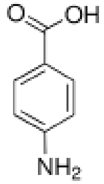

Nicotinic Acid, B3

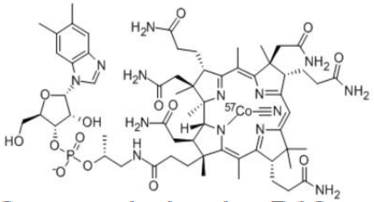

Pantothenic Acid, B5

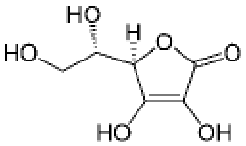

Pyridoxine, B6


Biotin, B7

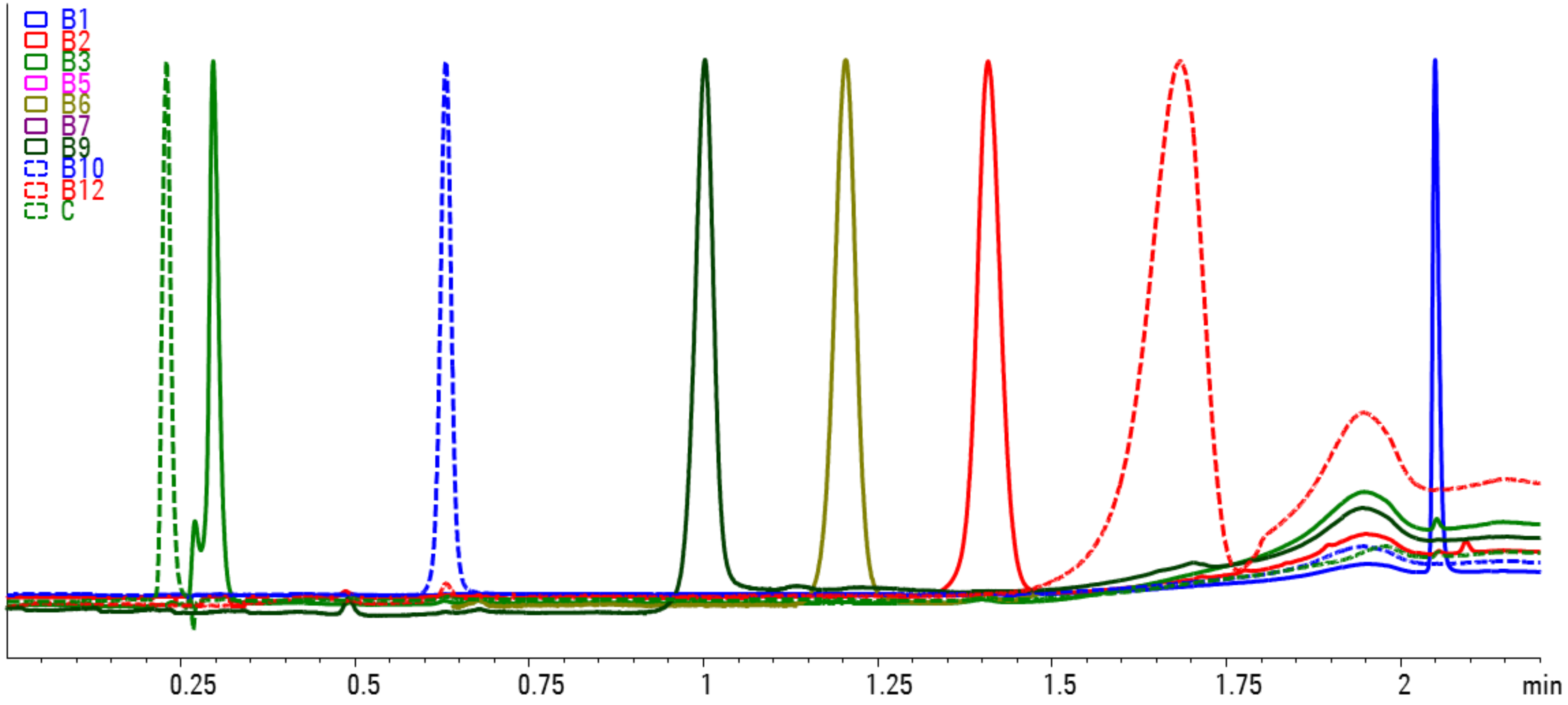

Folic Acid, B9


PABA, B10


Cyanocobalamin, B12


Ascorbic Acid, C

Water Soluble Vitamins
Ion pair conditions



EC-C18
A: 1.5 g sodium 1-heptanesulfonate + 0.2 mL triethylamine + 7.5 mL acetic acid + 992.5 mL water
B: CH_3CN
0.5 mL/min,10% B for 1 minute, then 10-40% B in 1 minute
injection volume, varies according to signal strength
TCC: 30 °C
260, 8 nm Ref Off, 8 nm slit, 80 Hz

The ion pairing reagent increased retention for most compounds
- Six compounds have k' > 2
- B5 and B7 could not be detected due to low signal and high background noise at 210 nm (not detectable at 260 nm)

Ion-Pair Chromatography
Limitations

- Higher level of complexity than RP, so generally chosen only if needed
- Requires careful control of IP reagent, pH, and temperature
- Gradient methods are more difficult than RP
- Equilibration is much slower than RP
- Column dedicated to IP
- IP reagent in the injection solvent
- IP reagents not desirable for MS detection

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Chemistries with Unique Selectivity

InfinityLab Poroshell 120 offers a broad portfolio to suit your needs

Best All Round	Best for Low pH Mobile Phases	Best for High pH Mobile Phases	Best for Alternative Selectivity	Best for More Polar Analytes	HILIC for Polar Analytes	Chiral
EC-C18 1.9 µm, 2.7 µm, 4 µm pH: 2-8	**SB-C18** 1.9 µm, 2.7 µm, 4 µm pH: 1-8	**HPH-C18** 1.9 µm, 2.7 µm, 4 µm pH 2-11	**Bonus-RP** * 2.7 µm pH: 2-9	**Aq-C18** * New! 2.7 µm pH: 1-8	**HILIC** 1.9µm, 2.7 µm, 4 µm pH: 0-8	**Chiral-V** 2.7 µm pH: 2.5-7
EC-C8 1.9 µm, 2.7 µm, 4 µm pH: 2-8	**SB-C8** 2.7 µm pH: 1-8	**HPH-C8** 2.7 µm, 4 µm	**PFP** * 1.9 µm, 2.7 µm, 4 µm pH: 2-8	**SB-Aq** * 1.9 µm, 2.7 µm, 4 µm pH: 1-8	**HILIC-Z** 1.9 µm, 2.7 µm, 4 µm pH:2-12	**Chiral-T** 2.7 µm pH: 2.5-7
Phenyl-Hexyl * 1.9 µm, 2.7 µm, 4 µm pH: 2-8		**CS-C18** 2.7 µm pH: 1-11		**EC-CN** * 2.7 µm pH: 2-8	**HILIC-OH5** 2.7 µm pH: 1-7	**Chiral-CD** 2.7 µm pH: 3-7
						Chiral-CF 2.7 µm pH: 3-7

*Can be operated at 100% aqueous mobile phase conditions to improve retention of highly polar analytes in RPLC mode.

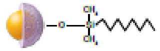
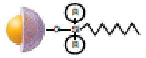
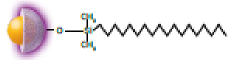
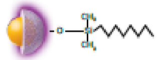
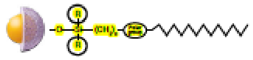
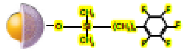
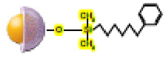
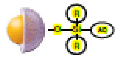
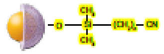
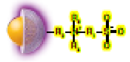
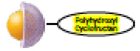
InfinityLab Poroshell 120 Column Specifications

InfinityLab Poroshell 120 column specifications

InfinityLab Poroshell 120	Pore Size	Temperature Limit	pH Range	Endcapped	Carbon Load	Surface Area	USP Designation
EC-C18	120 Å	60 °C	2.0–8.0	Yes	10%	130 m2/g	L1
EC-C8	120 Å	60 °C	2.0–8.0	Yes	5%	130 m2/g	L7
Aq-C18	120Å	90 °C	1.0–8.0	Yes	Proprietary	130 m2/g	L1
SB-C18	120 Å	90 °C	1.0–8.0	No	9%	130 m2/g	L1
SB-C8	120 Å	80 °C	1.0–8.0	No	5.5%	130 m2/g	L7
CS-C18	100 Å	90 °C	1.0–11.0	Yes	Proprietary	95 m2/g	L1
HPH-C18	100 Å	60 °C	2.0–11.0	Yes	Proprietary	95 m2/g	L1
HPH-C8	100 Å	60 °C	2.0–11.0	Yes	Proprietary	95 m2/g	L7
Bonus-RP	120 Å	60 °C	2.0–8.0	Yes	9.5%	130 m2/g	L60
PFP	120 Å	60 °C	2.0–8.0	Yes	5.1%	130 m2/g	L43
Phenyl-Hexyl	120 Å	60 °C	2.0–8.0	Yes	9%	130 m2/g	L11
SB-Aq	120 Å	80 °C	1.0–8.0	No	Proprietary	130 m2/g	L96
EC-CN	120 Å	60 °C	2.0–8.0	Yes	3.5%	130 m2/g	L10
HILIC-Z	100 Å	80 °C	2.0–12.0	No	Proprietary	95 m2/g	L114
HILIC	120 Å	60 °C	1.0–8.0	No	NA	130 m2/g	L3
HILIC-OH5	120 Å	45 °C	1.0–7.0	Proprietary	Proprietary	130 m2/g	L86
Chiral-V	120 Å	45 °C	2.5–7.0	Proprietary	Proprietary	130 m2/g	L88
Chiral-T	120 Å	45 °C	2.5–7.0	Proprietary	Proprietary	130 m2/g	L63
Chiral-CD	120 Å	45 °C	3.0–7.0	Proprietary	Proprietary	130 m2/g	L45
Chiral-CF	120 Å	45 °C	3.0–7.0	Proprietary	Proprietary	130 m2/g	NA

5991-9123EN

InfinityLab Poroshell 120 Bonded Phases

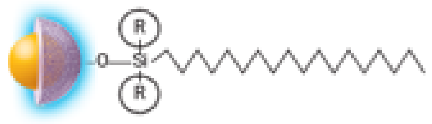
InfinityLab Poroshell 120	Chemistry	Particle Sizes	Benefits and Applications
EC-C18		1.9, 2.7, 4 µm	General purpose Excellent peak shape and efficiency for acids, bases, and neutrals
EC-C8		1.9, 2.7, 4 µm	General purpose Lower retention of hydrophobic analytes vs. C18
Aq-C18		2.7 µm	Enhanced retention for challenging polar compounds while also separating non-polar analytes. 100% aqueous mobile phase compatibility and low pH stability
SB-C18		1.9, 2.7, 4 µm	Low pH Excellent stability and peak shape in highly acidic conditions
SB-C8		2.7 µm	Low pH Excellent stability at low pH Lower retention of hydrophobic analytes vs. C18
CS-C18		2.7 µm	Alternate selectivity Improved peak shape and sample capacity for basic compounds with low ionic strength mobile phases High pH capable
HPH-C18		1.9, 2.7, 4 µm	High pH capable Robust performance and long lifetimes Improved retention, resolution, and peak shape of basic compounds
HPH-C8		2.7, 4 µm	High pH capable Robust performance and long lifetimes Lower retention of hydrophobic analytes vs. C18
Bonus-RP		2.7 µm	Alternative selectivity to C18 Improved peak shape for basic compounds, stable in 100% aqueous conditions
PFP		1.9, 2.7, 4 µm	Alternative selectivity Excellent peak shape for polar and nonpolar analytes Unique selectivity for aromatic and halogenated compounds
Phenyl-Hexyl		1.9, 2.7, 4 µm	Alternative selectivity with aromatic groups Highly nonpolar bonded phase takes advantage of pi-pi interactions
SB-Aq		1.9, 2.7, 4 µm	Alternative selectivity Excellent peak shape and retention of polar compounds using reversed-phase LC Exceptional stability under high-aqueous conditions, including 100% water
EC-CN		2.7 µm	Alternative selectivity Use in reversed phase for alternative selectivity of polar and midpolar compounds Use in normal phase for excellent peak shape and retention of nonpolar analytes
HILIC-Z		1.9, 2.7, 4 µm	Polar analytes Excellent retention of highly polar or charged compounds by HILIC Rugged performance at high pH or high temperature
HILIC		1.9, 2.7, 4 µm	Polar analytes Excellent retention of polar compounds by HILIC
HILIC-OH5		2.7 µm	Polar analytes Fructan bonded phase offers alternative selectivity to other HILIC phases

5991-9123EN

InfinityLab Poroshell 120 Aq-C18 Column

InfinityLab Poroshell 120 Aq-C18 features a proprietary endcapped stationary phase surface on a 120Å, 2.7µm superficially porous particle.

Built on Poroshell particle technology with controlled bonding density for use of 100% aqueous mobile phase conditions.



Agilent InfinityLab Poroshell 120 Aq-C18, 2.7 µm

Size	Part Number	Pressure
2.1 x 30	691775-742	600 bar
2.1 x 50	699775-742	600 bar
2.1 x 100	695775-742	1000 bar
2.1 x 150	693775-742	1000 bar
3.0 x 50	699675-742	600 bar
3.0 x 100	695675-742	1000 bar
3.0 x 150	693675-742	1000 bar
4.6 x 50	699975-742	600 bar
4.6 x 100	695975-742	600 bar
4.6 x 150	693975-742	600 bar

Aq-C18 2.7µm Fast Guards

Size	Part Number
2.1 x 5	821725-955
3.0 x 5	823750-953
4.6 x 5	820750-942

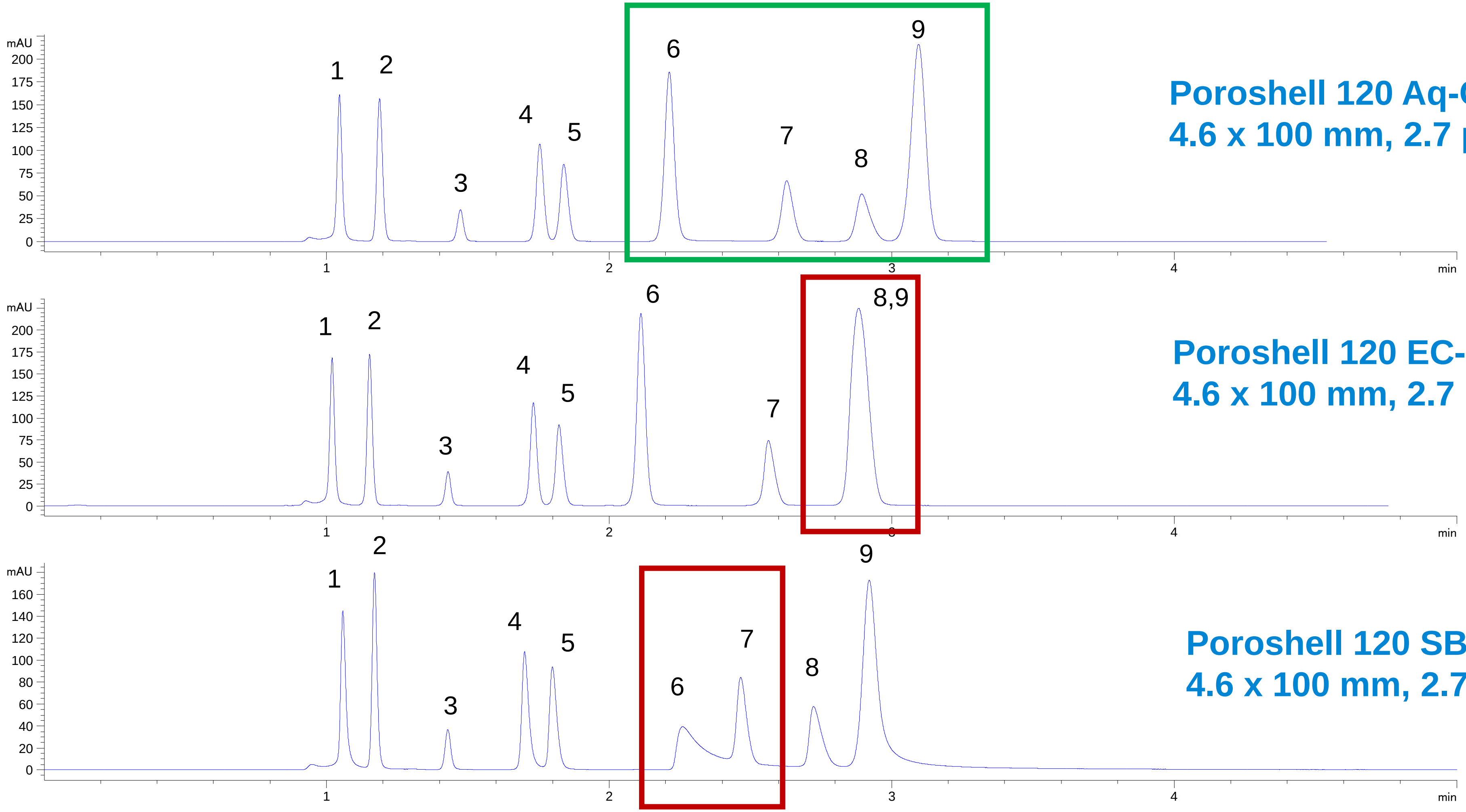
Attributes	Specifications
Particle Size	2.7 µm
Pore Size	120Å
Temperature Limit	90 °C
pH Range	1-8
Carbon Load	Proprietary
Surface Area	130 m^2/g
Pressure rating	See p/n table
Endcapped	Yes
USP Designation	L1

Provides improved performance for challenging polar compounds, as well as nonpolars, in one run.

Comparison of Aq-C18 with Existing C18 phases

Aq-C18 performs better than conventional C18 for polar organic acids

1. Oxalic acid
2. Tartaric acid
3. Malic acid
4. Lactic acid
5. Acetic acid
6. Maleic acid
7. Citric acid
8. Succinic acid
9. Fumaric acid



Poroshell 120 Aq-C18
4.6 x 100 mm, 2.7 µm

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

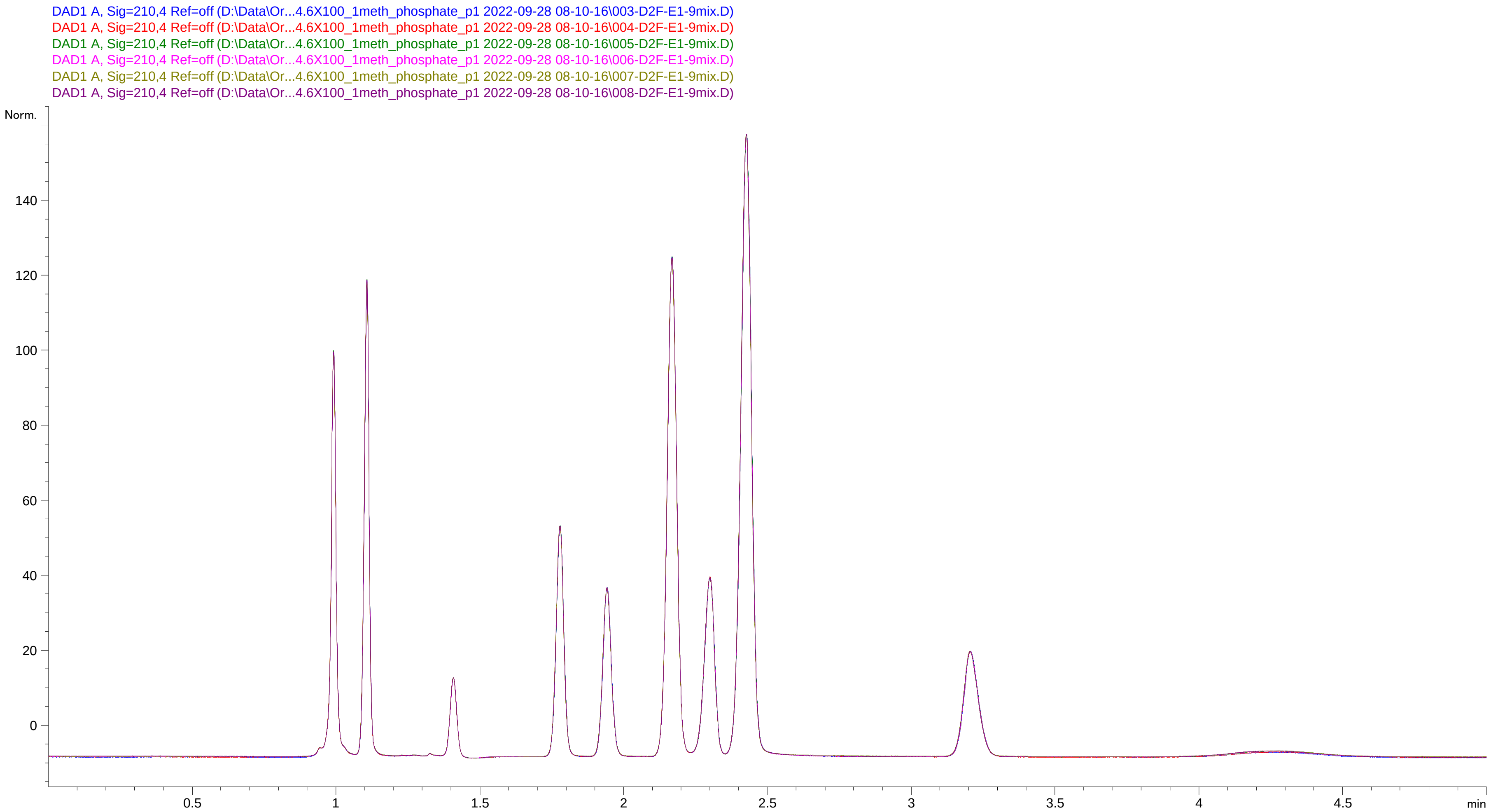
Poroshell 120 SB-C18
4.6 x 100 mm, 2.7 µm

- **MP: 97.5% 0.1%H_3PO_4/2.5% methanol; 1 mL/min; 210 nm; 2 µL; 25 °C**

Agilent

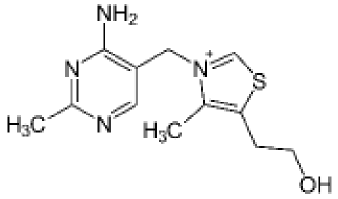
InfinityLab Poroshell 120 Aq-C18 Column

Good reproducibility of six injections using 99% phosphate buffer

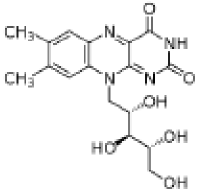


- **MP: 99% 50 mM NaH_2PO_4/1% methanol; 1 mL/min; 210 nm; 1 µL; 25 °C**

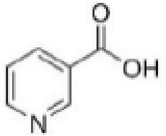
Water Soluble Vitamins



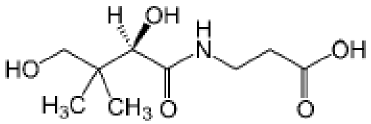
Thiamine, B1



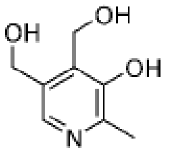
Riboflavin, B2



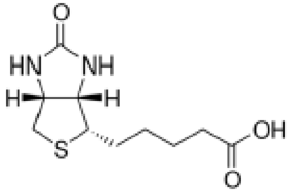
Nicotinic Acid, B3



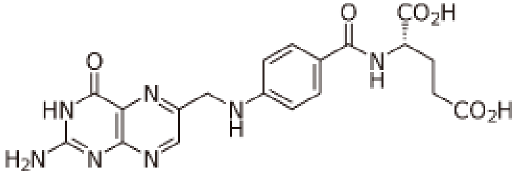
Pantothenic Acid, B5



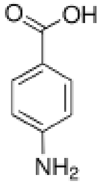
Pyridoxine, B6



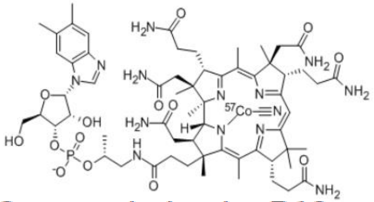
Biotin, B7



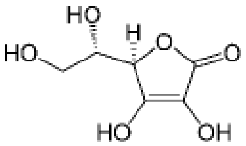
Folic Acid, B9



PABA, B10



Cyanocobalamin, B12

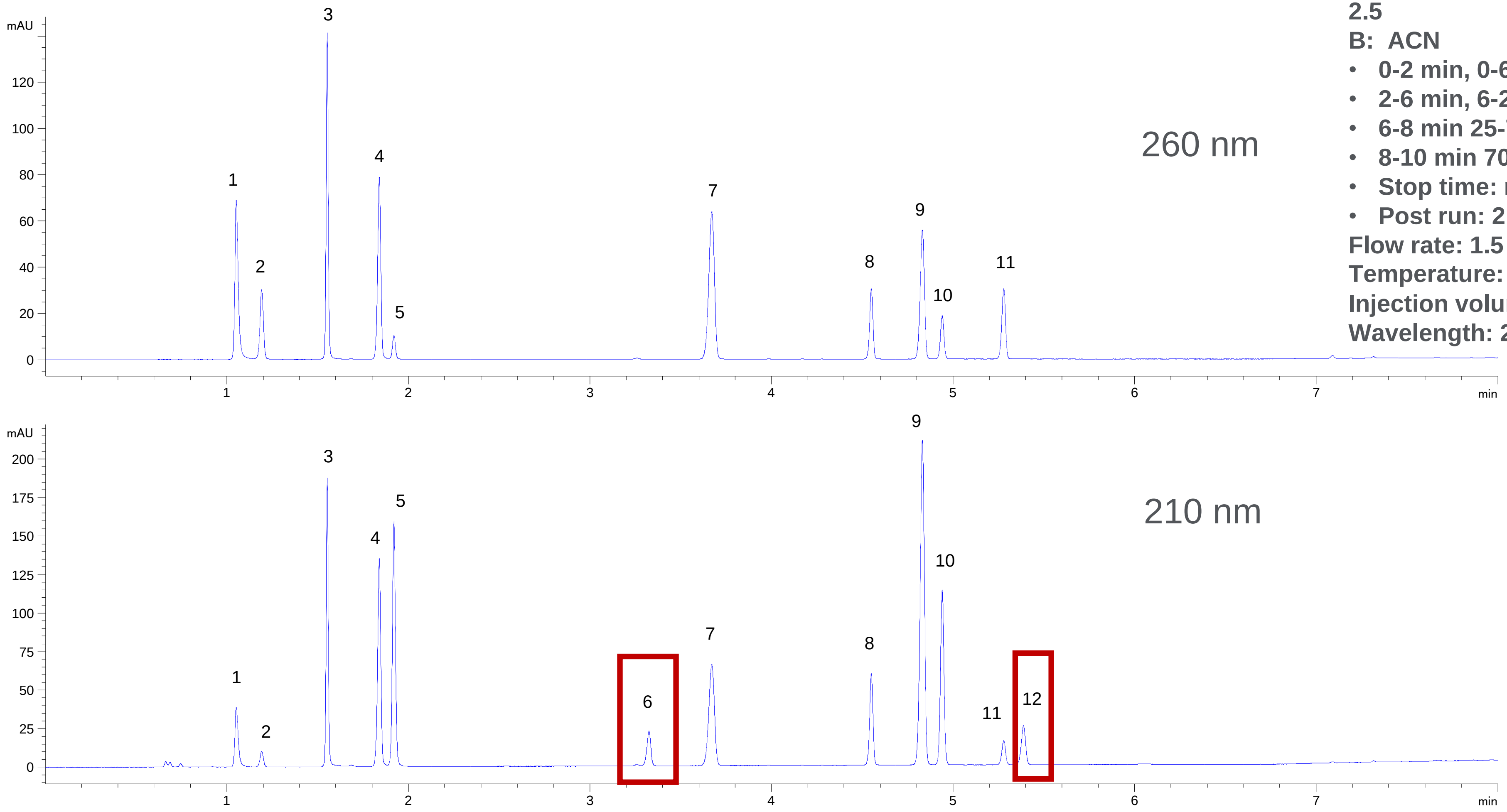


Ascorbic Acid, C

Water Soluble Vitamins Separation on Aq-C18

A gradient starts with 100% aqueous phosphate buffer

1. Thiamine
2. Ascorbic acid
3. Nicotinic acid
4. Nicotinamide
5. Pyridoxine
6. Pantothenic acid
7. Aminobenzoic acid
8. Folic acid
9. Caffeine
10. Cyanocobalamin
11. Riboflavin
12. Biotin



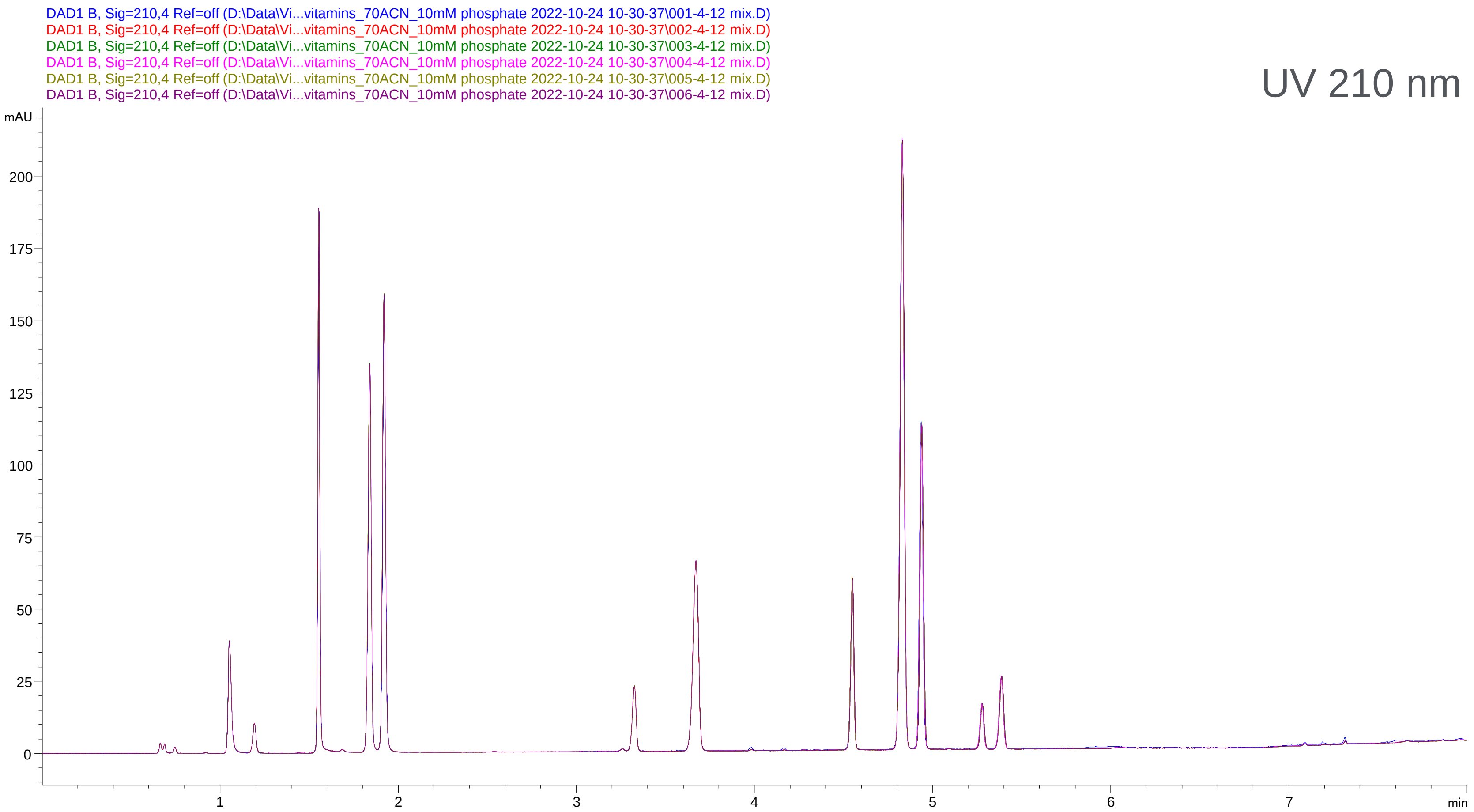
1290 Infinity II Binary pump
A: 10 mM NaH_2PO_4 buffer pH 2.5
B: ACN
- **0-2 min, 0-6% B**
- **2-6 min, 6-25%B**
- **6-8 min 25-70%B**
- **8-10 min 70%B**
- **Stop time: minute 10**
- **Post run: 2 min**

Flow rate: 1.5 mL/min
Temperature: 30 °C
Injection volume: 1 µL
Wavelength: 260 nm/210 nm

Excellent Reproducibility of Consecutive Six Injections on Aq-C18

Water soluble vitamins (gradient starting from 100% aqueous phosphate buffer)

UV 210 nm



InfinityLab Poroshell 120 CS-C18 Column

InfinityLab Poroshell 120 CS C18 is a hybrid, endcapped C18 stationary phase on a 100Å, 2.7 µm superficially porous particle column.

Built on Poroshell particle technology modified to have a positively charged surface, it eliminates interactions with polar amine-containing compounds.

Agilent InfinityLab Poroshell 120 CS-C18, 2.7 µm

Size	Part Number	PEEK-lined
2.1 x 50	699775-942	679775-942
2.1 x 100	695775-942	675775-942
2.1 x 150	693775-942	
3.0 x 50	699675-342	
3.0 x 100	695675-342	
3.0 x 150	693675-342	
4.6 x 50	699975-942	
4.6 x 100	695975-942	
4.6 x 150	693975-942	

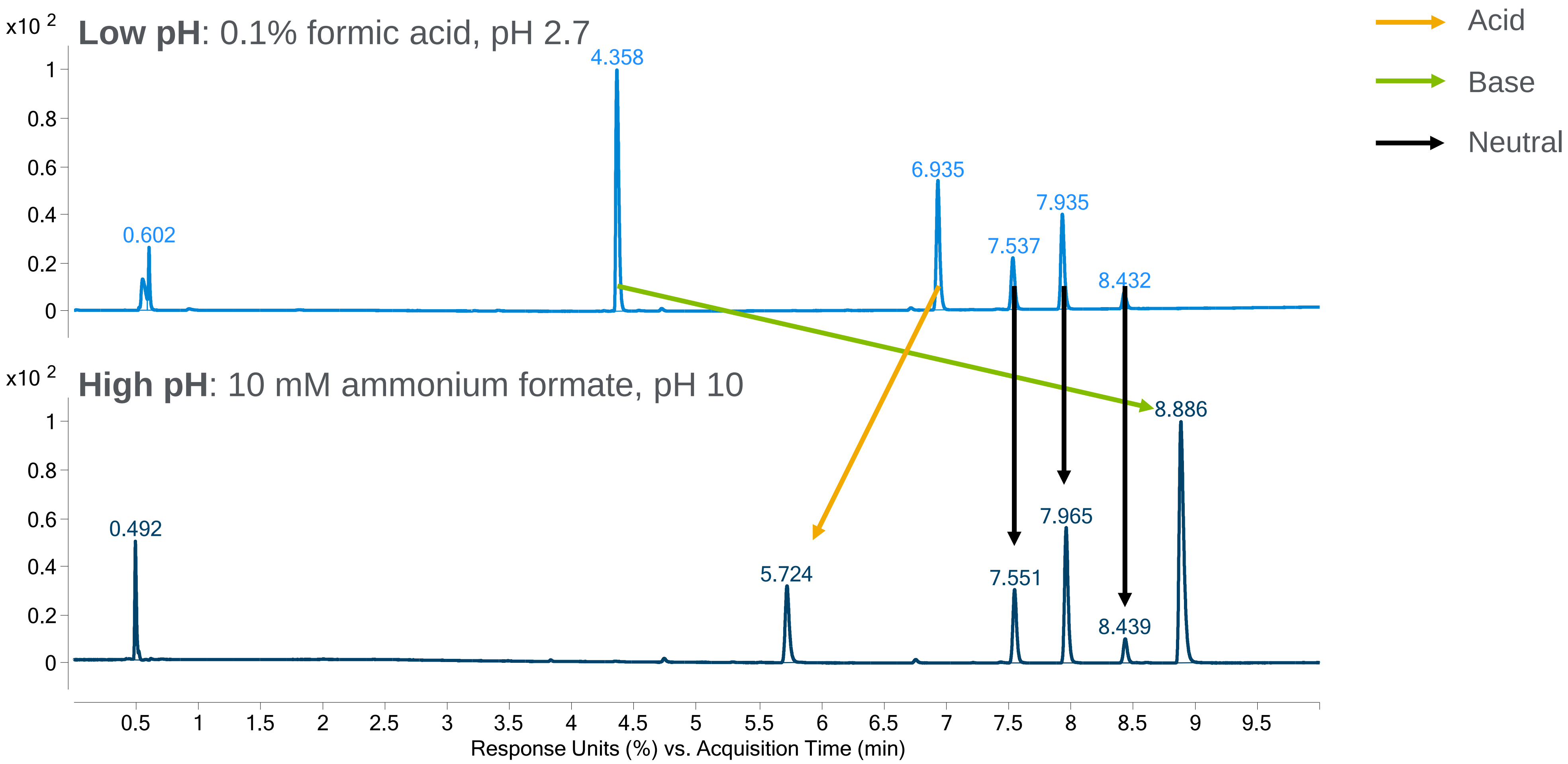
CS-C18 2.7µm Fast Guards

Size	Part Number
2.1 x 5	821725-953
3.0 x 5	823750-949
4.6 x 5	820750-939

Attributes	Specifications
Particle Size	2.7 µm
Pore Size	100Å
Temperature Limit	90 °C
pH Range	1-11
Carbon Load	Proprietary
Surface Area	95 m^2/g
Pressure rating	See p/n table
Endcapped	Yes
USP Designation	L1

Excellent peak shape with MS-friendly low ionic strength formic acid and acetic acid eluents, eliminating TFA.

A pH Change Can Strongly Affect Selectivity of CS-C18



5-95% CH_3CN in 10 min, 4 min postrun, 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

5994-2274EN

CS-C18 Retains and Separates Glyphosate and Other Polar Pesticides

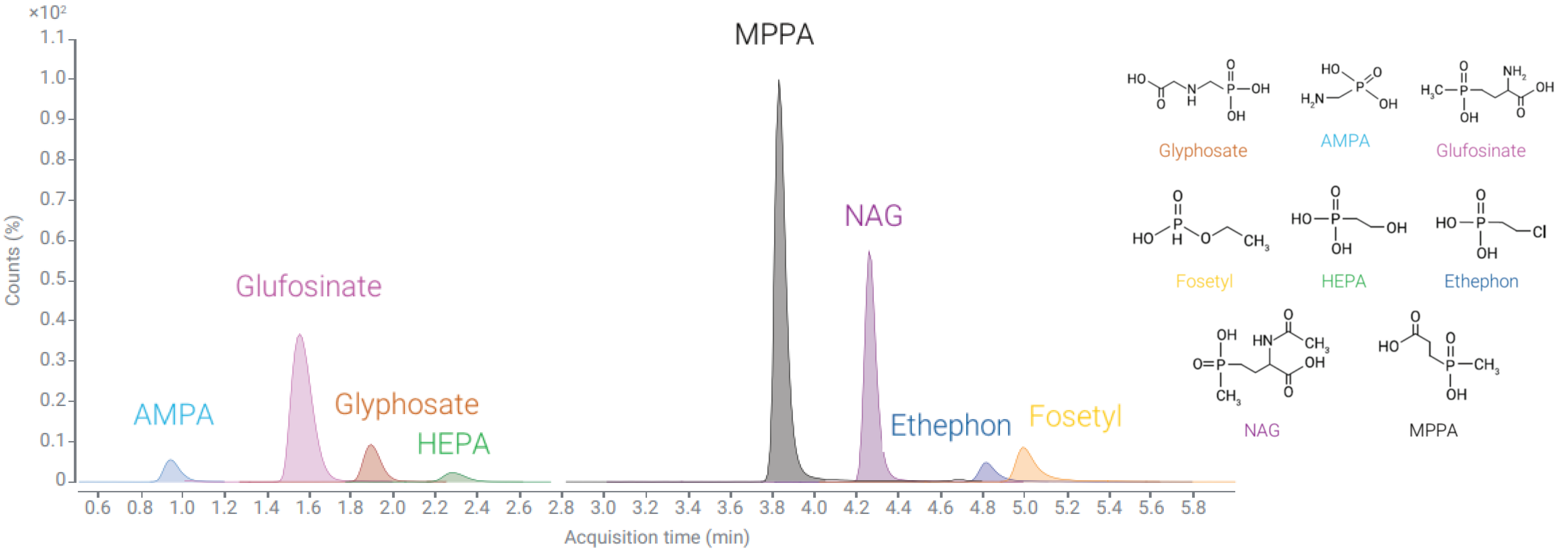


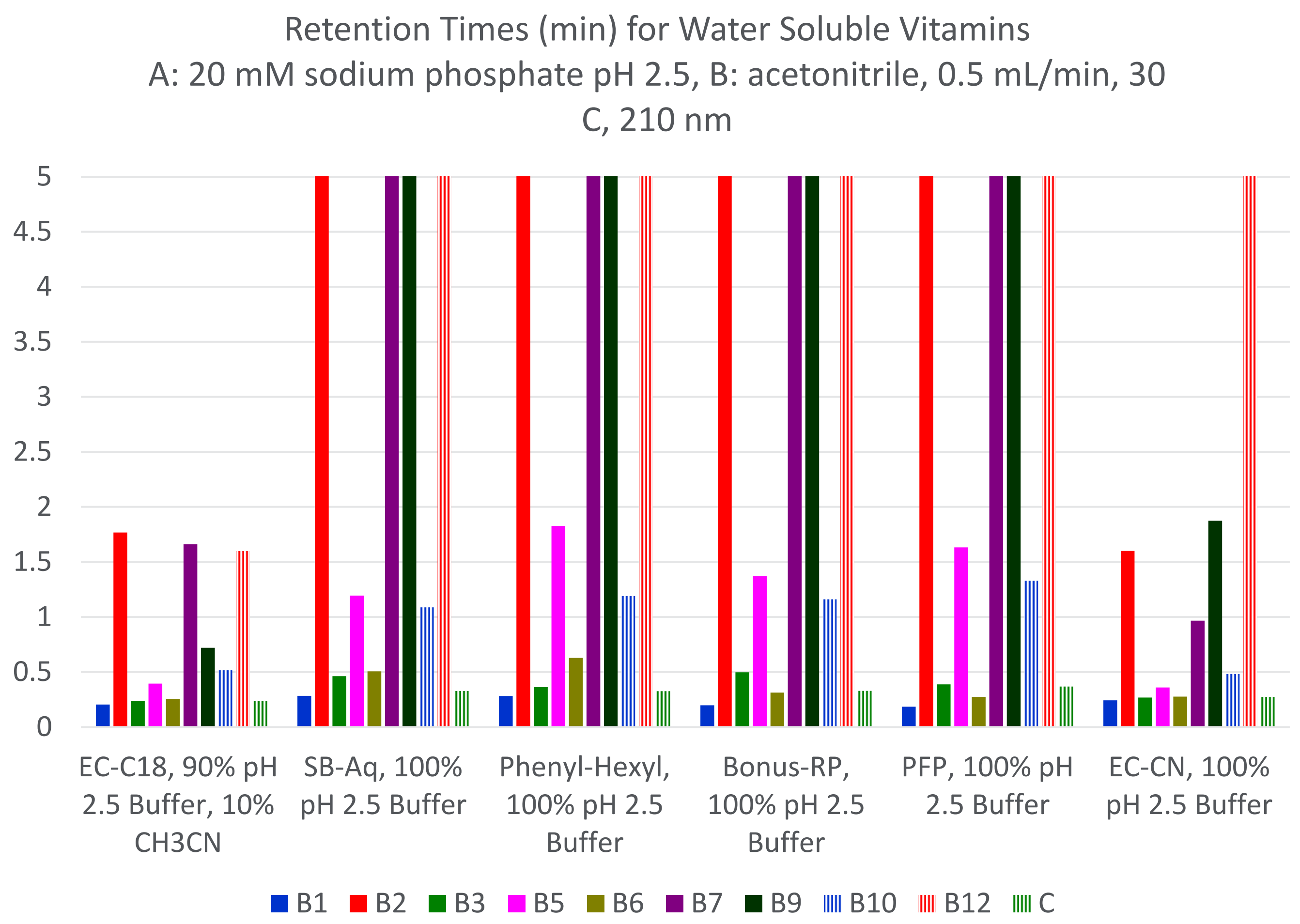
Figure 2. Typical chromatogram for the eight pesticides at 10 µg/L. Retention times are as follows: AMPA = 0.95 minutes, Glufosinate = 1.6 minutes, Glyphosate = 1.9 minutes, HEPA = 2.3 minutes, MPPA = 3.8 minutes, NAG = 4.3 minutes, Ethephon = 4.8 minutes, Fosetyl = 5.0 minutes.

A: 0.1% formic acid +5 µM deactivator additive in ultrapure water
B: 0.1% formic acid in methanol
Gradient 99.9% A → 60% A
Column: Agilent InfinityLab Poroshell 120 CS-C18, 2.1 × 150 mm, 2.7 µm (p/n 693775-942)

5994-2986EN

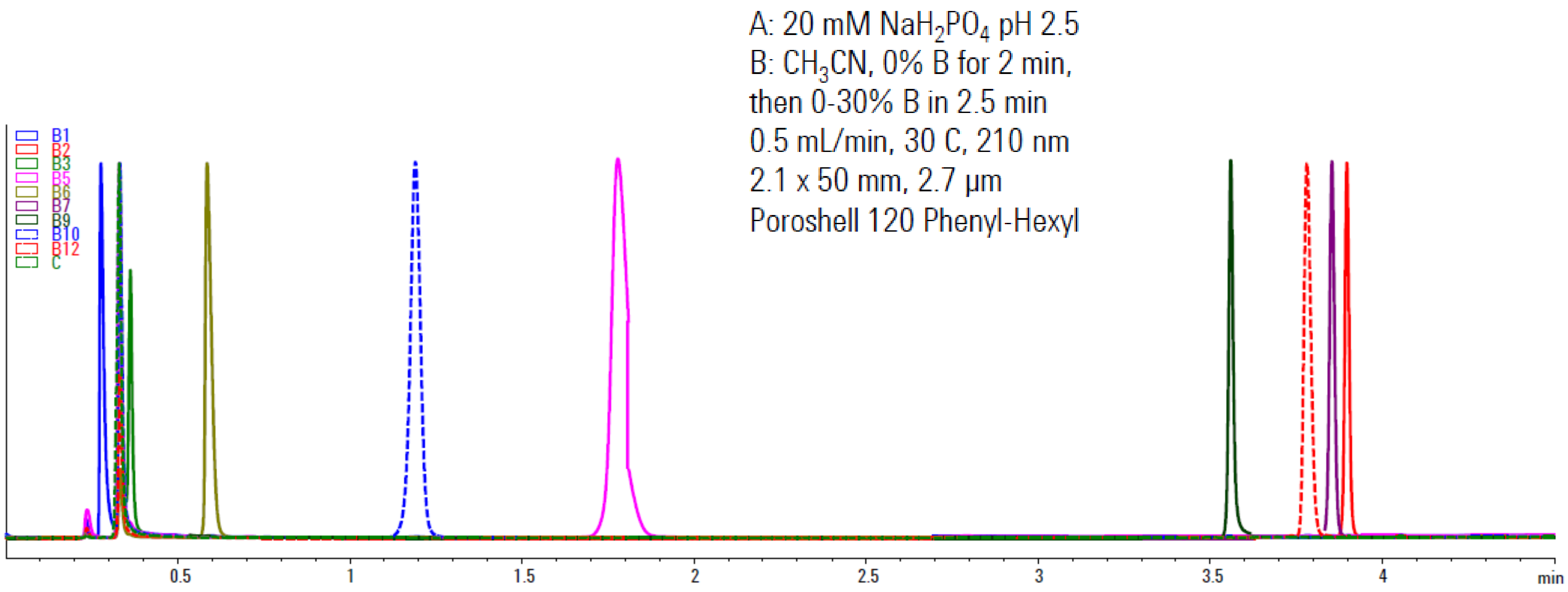
Water Soluble Vitamins

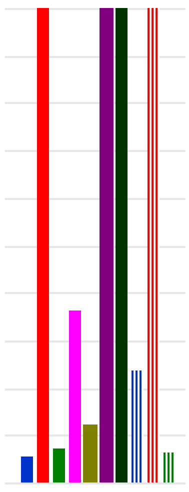
Alternative phases – comparison of retention



Water Soluble Vitamins

Phenyl-Hexyl

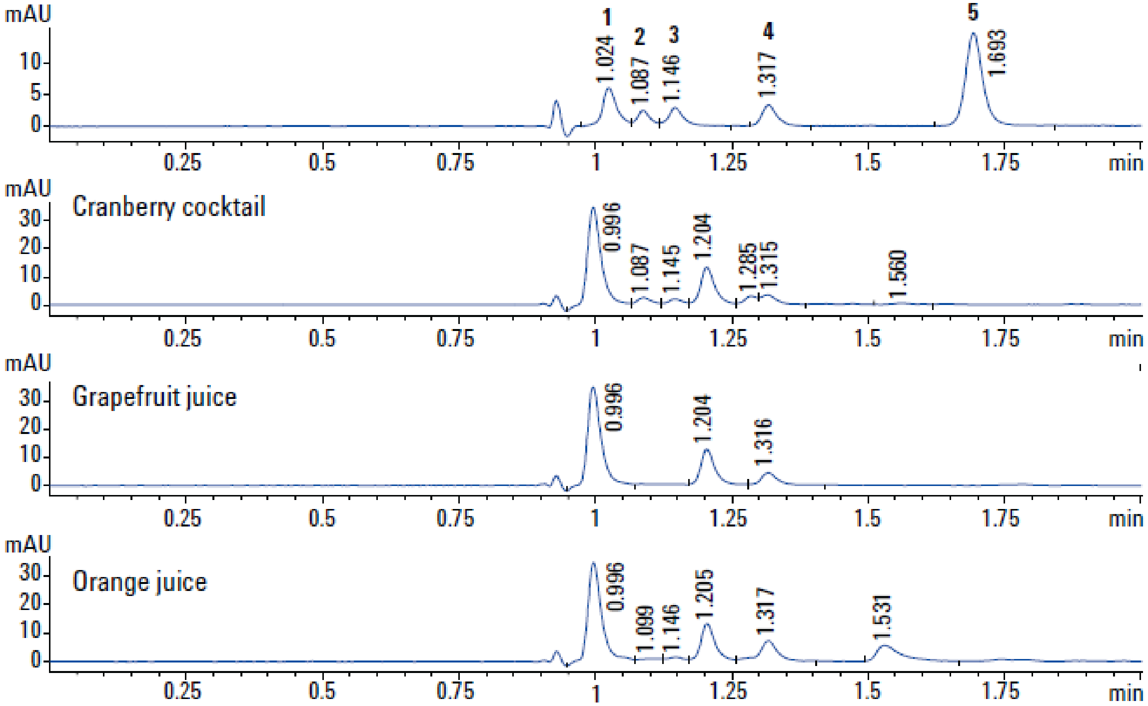




- Phenyl-Hexyl has the best retention
- Seven compounds have k'> 2;
- C18 analysis had only four compounds with k' > 2

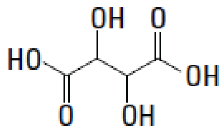
Organic Acids

SB-Aq

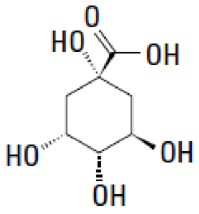


Peak ID

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

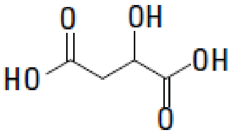


Tartaric acid

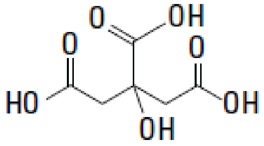


Quinic acid

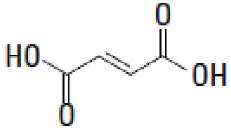
Column:	Agilent Poroshell 120 SB-Aq, 3 × 100 mm, 2.7 µm (p/n 685975-314)
Eluent:	100 mM Potassium phosphate buffer, pH 2.5
Injection volume:	5 µL
Flow rate:	0.5 mL/min
Temperature:	50 °C
Detector:	DAD, at 226 nm



Malic acid



Citric acid



Fumaric acid

5991-1992EN

Options for Analysis of Polar Analytes by LC

- Try reversed phase C18. If needed, adjust method conditions (pH and strength of mobile phase)

- Ion-pair reversed-phase chromatography

- Alternative column choice – more polar RP phases compatible with 100% aqueous mobile phase

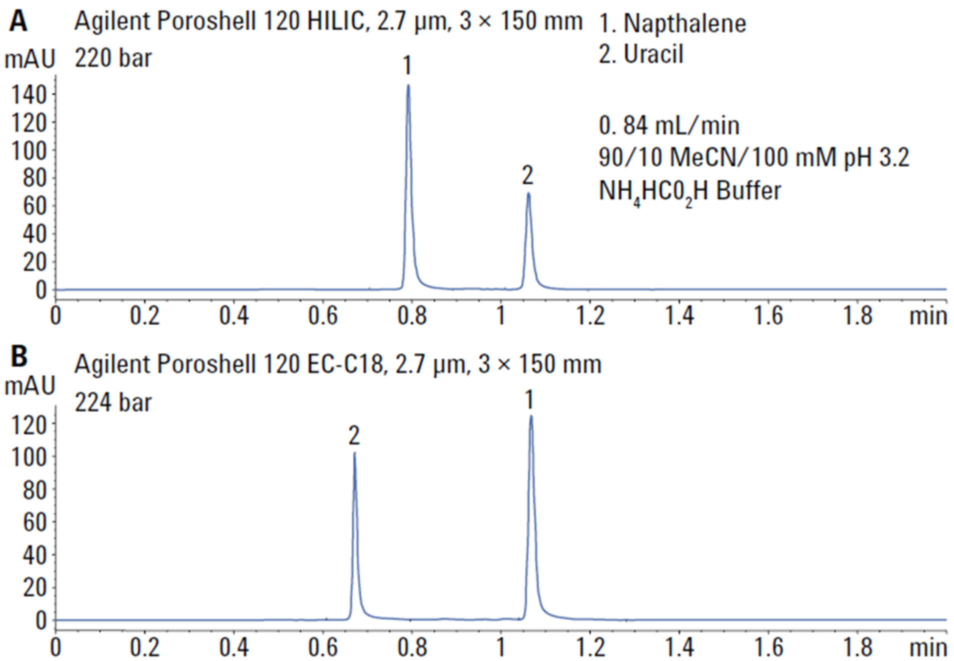
- **Alternative mode of separation – HILIC (hydrophilic interaction chromatography)**
  - **Silica**
  - **Amino**
  - **Amide**
  - **Zwitterionic**
  - **Diol**

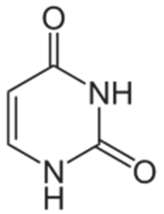
- Alternative mode of separation – ligand exchange
  - Hi-Plex (sulfonated polystyrene/divinylbenzene with different counterions) for analysis of organic acids and carbohydrates

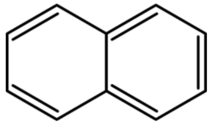
What is HILIC?

Hydrophilic Interaction Liquid Chromatography

- Retains hydrophilic compounds
- Polar stationary phase: silica, amino, diol/hydroxyl-based, zwitterionic
- Uses a mix of organic and aqueous mobile phases with a buffer



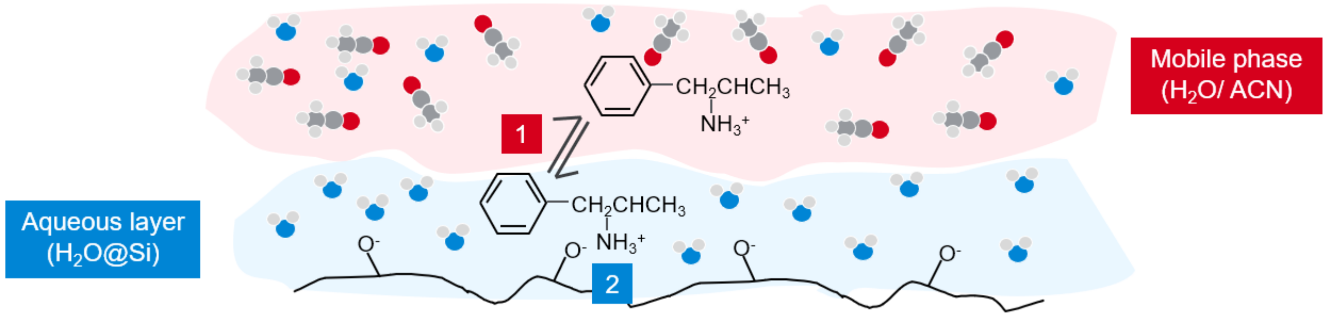




HILIC Mechanism

Retains moderate to highly polar analytes

- A water layer is adsorbed onto the polar silica surface, creating a liquid/liquid extraction system
- Polar analytes can **partition into and out of the water layer**, with more polar analytes having a stronger interaction (1)
- Charged polar analytes can also undergo **ion exchange with the silica surface** (2)

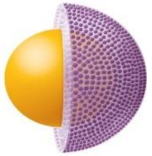


Key points to note:

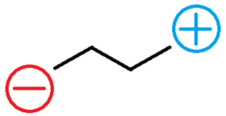
- Water is the strong solvent
- Elution is least polar to most polar, opposite of RPLC.
- Gradients run from **high organic to high aqueous** (10% aqueous to 50% aqueous is a common scouting gradient)
- Reversed-phase solvents (ACN/water)
  - Typically use a buffer like ammonium formate or ammonium acetate
  - Higher buffer concentration increases solvent strength, improves peak shape, and can change selectivity slightly

Solvent strengths in HILIC mode are: THF < acetone < acetonitrile < isopropanol < ethanol < methanol < water

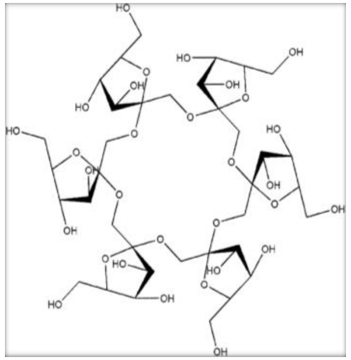
InfinityLab Poroshell 120 HILIC Columns – Specifications



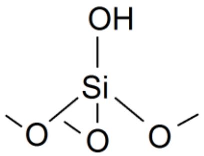
InfinityLab Poroshell Column	Bonded Phase	Particle	Particle Size	Pressure Limit	Pore Size	Temp Limit	pH Range	Dimensions (ID in mm)	Dimensions (Length in mm)
HILIC-Z	Proprietary zwittionic chemistry	Hybrid Poroshell superficially porous particle	2.7 µm	600 bar	100 Å	80 °C	2-12	2.1 3.0 4.6	5 (guard) 50 100 150
								PEEK-lined version available	
HILIC-OH5	Poly-hydroxy fructan chemistry	Poroshell superficially porous particle	2.7 µm	400 bar	120 Å	45 °C	1-7	2.1 3.0 4.6	50 100 150
HILIC	Bare-silica (unbonded)	Poroshell superficially porous particle	1.9 µm 2.7 µm 4 µm	1300 bar (1.9) 600 bar (2.7) 600 bar (4)	120 Å	60 °C	0-8	2.1 3.0 4.6	5 (guard) 50 100 150



HILIC-Z
Proprietary zwitterionic chemistry



HILIC-OH5
Polyhydroxy fructan chemistry



HILIC
Bare silica chemistry

Agilent

HILIC Method Development: Common LC Parameters

Type of stationary phase

- Different retention mechanism and selectivity
- Three phases on Agilent InfinityLab Poroshell 120 2.7 µm particles
  – HILIC-Z (also available in PEEK-lined), HILIC-OH5, HILIC

Mobile phase pH

- Controls ionization of silica and analytes
- Compounds are more retained in their charged state
  – Acids should be run at high pH, bases at low pH

Temperature

- Increasing temperature will decrease retention
- Increasing temperature will increase column efficiency
- Decreasing temperature can improve selectivity

HILIC Method Development: Mobile Phase and Sample Considerations

Organic solvent concentration

- Solvent strength in HILIC mode:
  - *THF < acetone < CH_3CN < IPA < EtOH < MeOH < H_2O*
- H_2O must be present — *need > 3% H_2O*

Ionic strength of buffer

- Concentration of (salt) buffer increases strength
- Different anions and cations may can also affect analyte retention

Type of buffer

- Acetates and formates are good, soluble in CH_3CN, and also MS friendly
- *Phosphate salts* have low CH_3CN solubility

Injection solvent

- Ideally 100% acetonitrile if there are no solubility issues

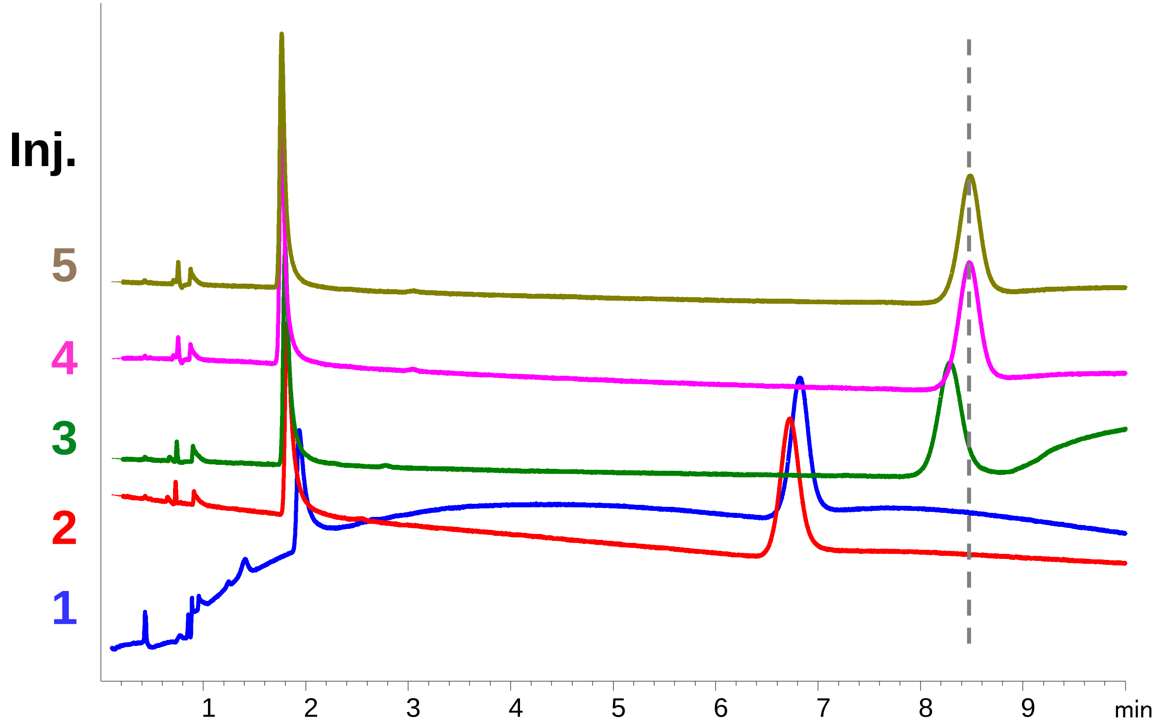
HILIC Method Development

HILIC column equilibration is faster with higher amounts of aqueous

B vitamins on Agilent InfinityLab Poroshell 120 HILIC-OH5 (2.1 x 100 mm, 2.7 µm)

4% aqueous, isocratic

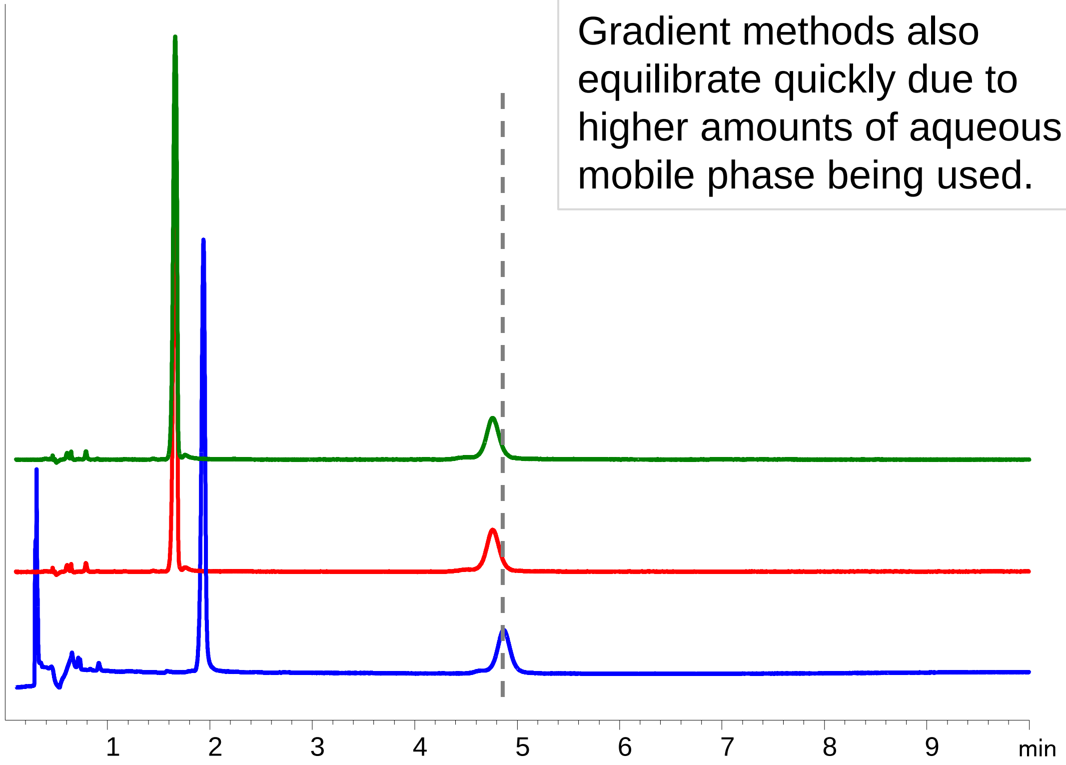
Equilibrated in 30 minutes (75 column volumes)



Column stored in 100% CH_3CN before analysis; A: 100 mM ammonium formate pH 3.0, B: CH_3CN, 96% B isocratic, 0.5 mL/min, 1 µL injection of B2+B6, 25 ˚C, 260 nm, 80 Hz

20% aqueous, isocratic

Equilibrated in <10 minutes (<25 column volumes)

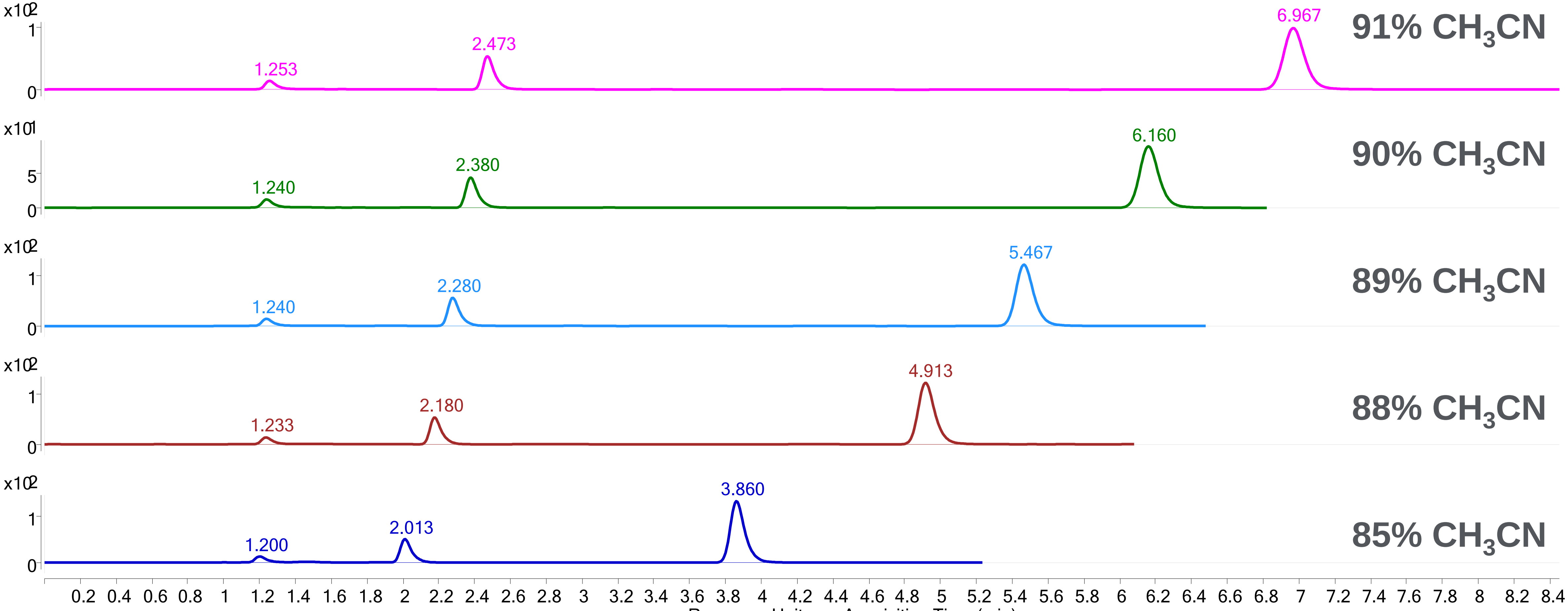


Gradient methods also equilibrate quickly due to higher amounts of aqueous mobile phase being used.

Column stored in 100% CH_3CN before analysis; A: 100 mM ammonium formate pH 3.0, B: CH_3CN, 80% B isocratic, 0.5 mL/min, 1 µL injection of B9+B12, 25 ˚C, 260 nm, 80 Hz

HILIC Method Development

Less CH_3CN makes a HILIC mobile phase stronger, causing less retention



Column used was 2.1 x 150 mm, 2.7 µm Agilent InfinityLab Poroshell 120 HILIC-Z (PEEK-lined); A: 100 mM pH 3 ammonium formate in water, B: acetonitrile, x % B, isocratic elution, 0.25 mL/min, 30 ˚C, 1 uL injection of toluene, cytosine, uracil QC mixture, 254 nm

HILIC Method Development – Injection Solvent Strength

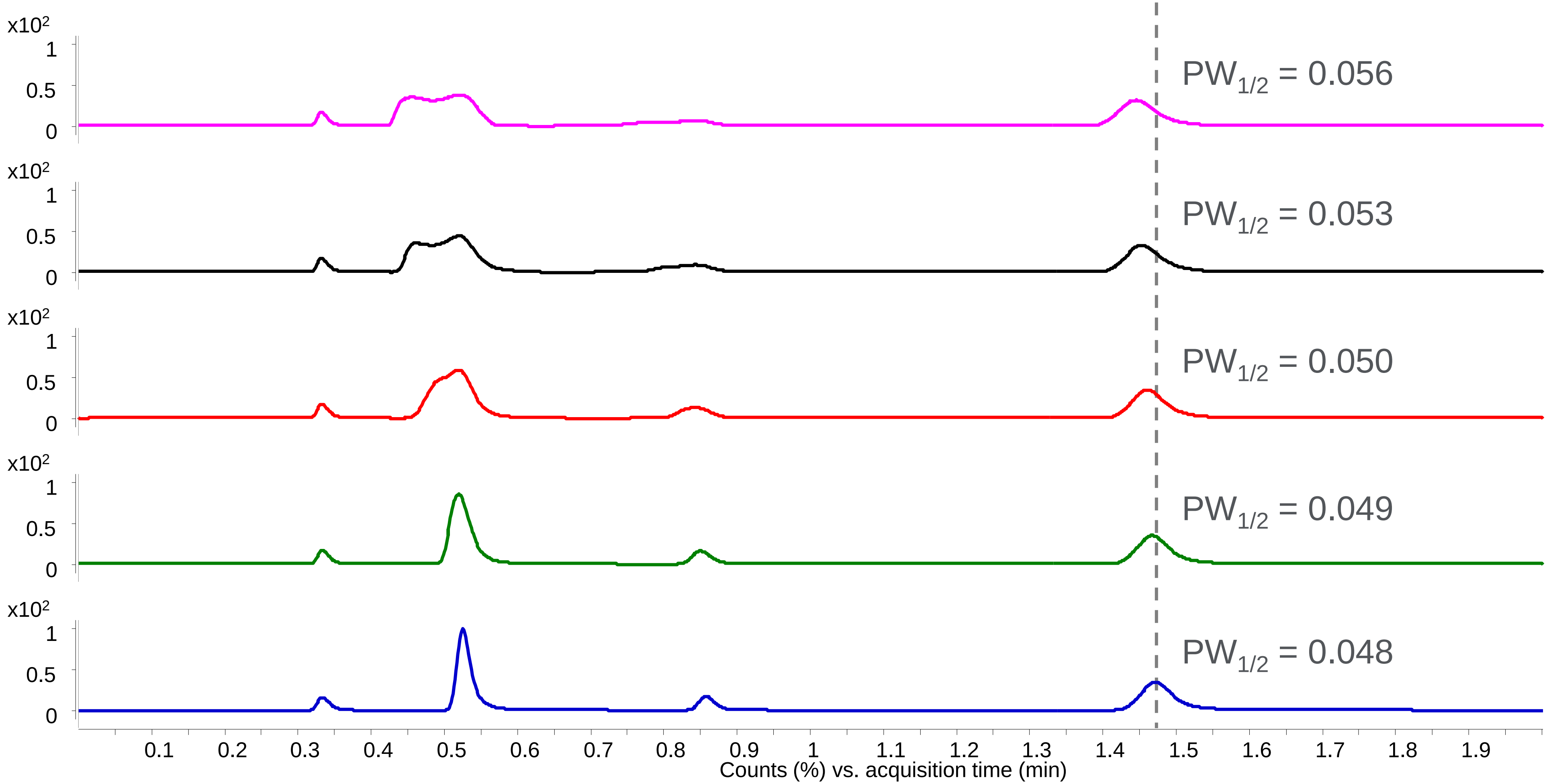
1 µL injection in H_2O

1 µL injection in H_2O/CH_3CN (3:1)

1 µL injection in H_2O/CH_3CN (1:1)

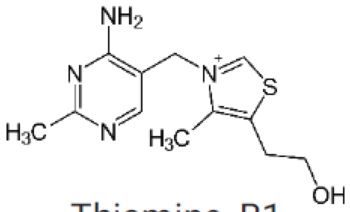
1 µL injection in H_2O/CH_3CN (1:3)

1 µL injection in CH_3CN

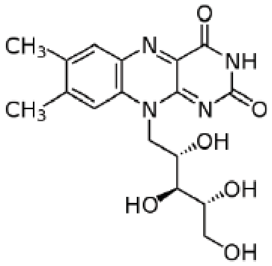


Agilent ZORBAX RRHD HILIC Plus 2.1 x 50 mm, 1.8 µm; mobile phase: acetonitrile/100 mM ammonium formate pH 3.2 in water (9:1), isocratic elution, 0.4 mL/min, 1 µL injection of 5.7 µg/mL each of 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid ; 25 °C, MS Source: ESI+, 200 °C, 10 L/min,, 30 psi, 4000 V; SIM: 138, 123, 377, 124

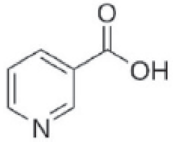
Water Soluble Vitamins



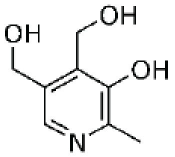
Thiamine, B1



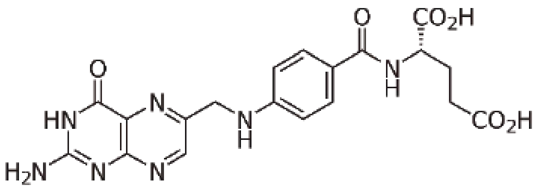
Riboflavin, B2



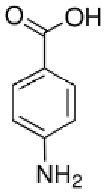
Nicotinic acid, B3



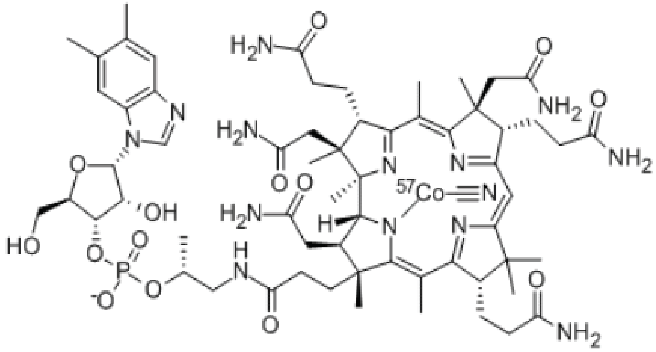
Pyridoxine, B6



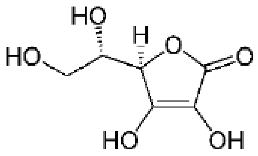
Folic acid, B9



PABA, B10

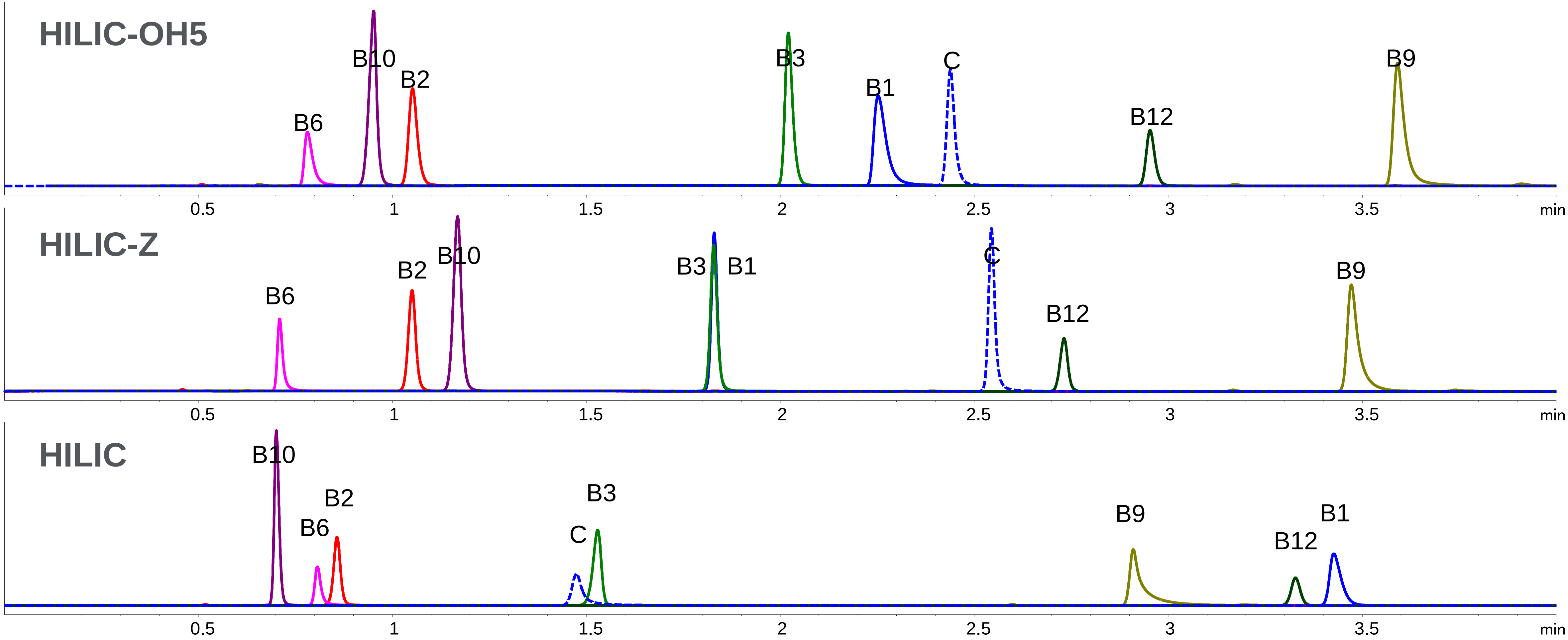


Cyanocobalamin, B12



Ascorbic acid, C

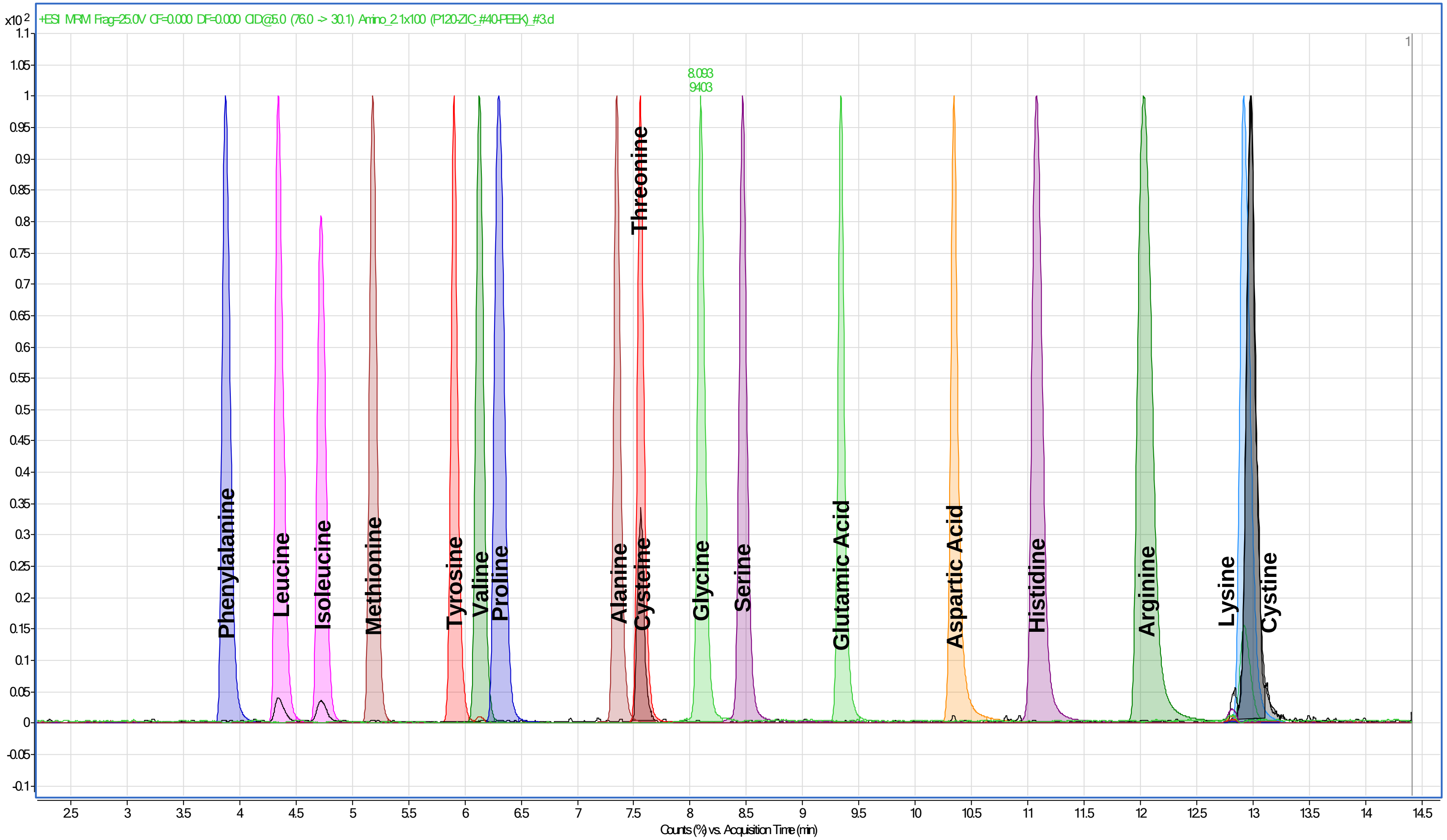
Water Soluble Vitamins by HILIC



Columns used were 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 of individual vitamin standards (0.1-0.4 mg/mL each), 40 ˚C, 260 nm, 80 Hz

Excellent Retention, Peak Shape and Sensitivity with HILIC-Z

Underivatized amino acids by LC/MS



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

A: 20 mM ammonium formate in H_20, pH 3
B: 9/1 ACN/H_20 with 2 0mm ammonium formate, pH 3

Gradient: 100%B to 70% B over 10 min, return to 100% B
Flow rate: 0.8 mL/min
Temp: 30 deg
MS detection: Agilent MS-QQQ, MS2 SIM mode

Sugar Analysis on Agilent InfinityLab Poroshell 120 HILIC-Z Using ELSD

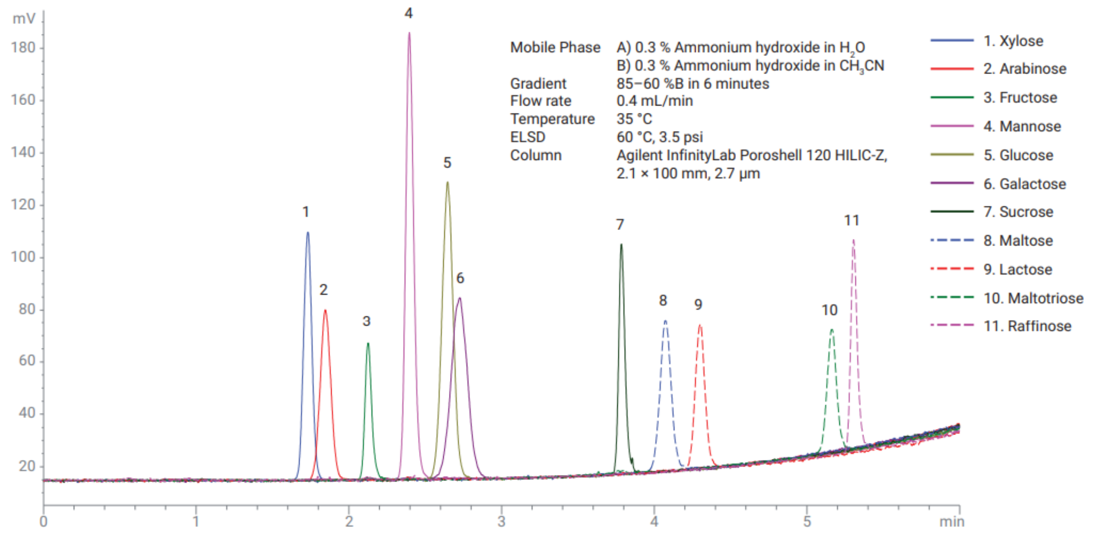


Figure 3. Gradient separation of 11 sugar compounds on an Agilent InfinityLab Poroshell 120 HILIC-Z LC column.

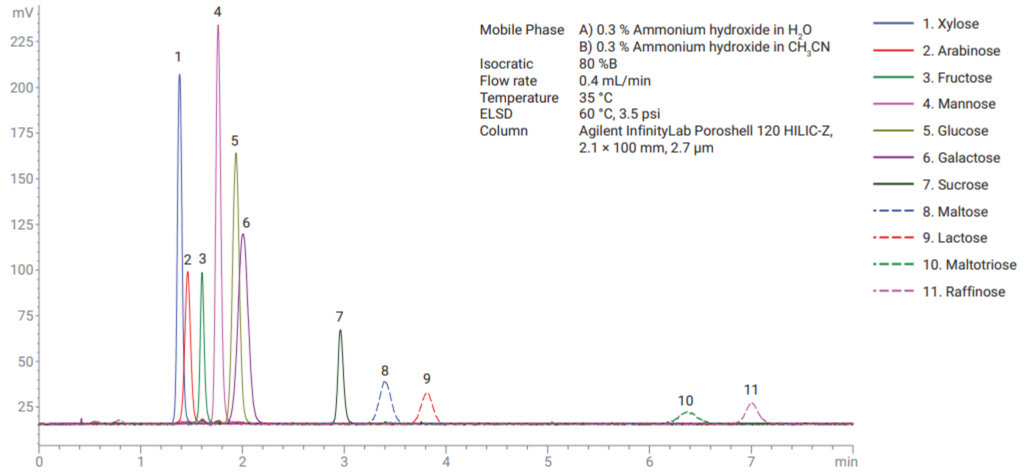


Figure 4. Isocratic separation of 11 sugar compounds on an Agilent InfinityLab Poroshell 120 HILIC-Z LC column.

Gradient method

Isocratic method

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Advantages of HILIC

- Retains polar analytes where reversed-phase methods may not
- Offers alternative selectivity to RPLC mode
- Can retain cations, anions, and polar neutrals in a single run
- Can improve peak shape for basic compounds
- Uses a standard LC system and common reversed-phase solvents
- Uses low viscosity mobile phases with high organic content
  - Fast methods with high flow rates
  - Longer columns for higher efficiency at lower pressures
- Enhanced detection sensitivity with MS compatible methods, in both positive and negative modes

Options for analysis of Polar Analytes by LC

- Try reversed phase C18. If needed, adjust method conditions (pH and strength of mobile phase)
- Ion-pair reversed-phase chromatography
- Alternative column choice – more polar RP phases compatible with 100% aqueous mobile phase
- Alternative mode of separation – HILIC (hydrophilic interaction chromatography)
  - Silica
  - Amino
  - Amide
  - Zwitterionic
  - Diol
- **Alternative mode of separation – ligand exchange**
  - **Hi-Plex (sulfonated polystyrene/divinylbenzene with different counterions) for analysis of organic acids and carbohydrates**

Hi-Plex Columns for Analysis of Organic Acids and Carbohydrates

- Ligand exchange
- Sulfonated cross-linked styrene-divinylbenzene gel in hydrogen form, or various metal cations with different selectivities (Ca, Pb, K, Na)
- Operates on a standard HPLC and does not require specialized equipment
- Simple operating conditions: mobile phase is water or dilute acid, isocratic conditions, compatible with up to 30% acetonitrile as a modifier
- Available in 8 µm and 10 µm particle sizes
- Comply with USP L17, L19, L22, L34, and L58
- Can be used with UV, RID, ELSD and MS detectors
- Must be run at elevated temperatures 40 to 90 °C
- Low back pressure columns, 50 bar pressure limit

Hi-Plex applications compendium: 5990-8801EN

Organic Acids on Hi-Plex H, LC/MS

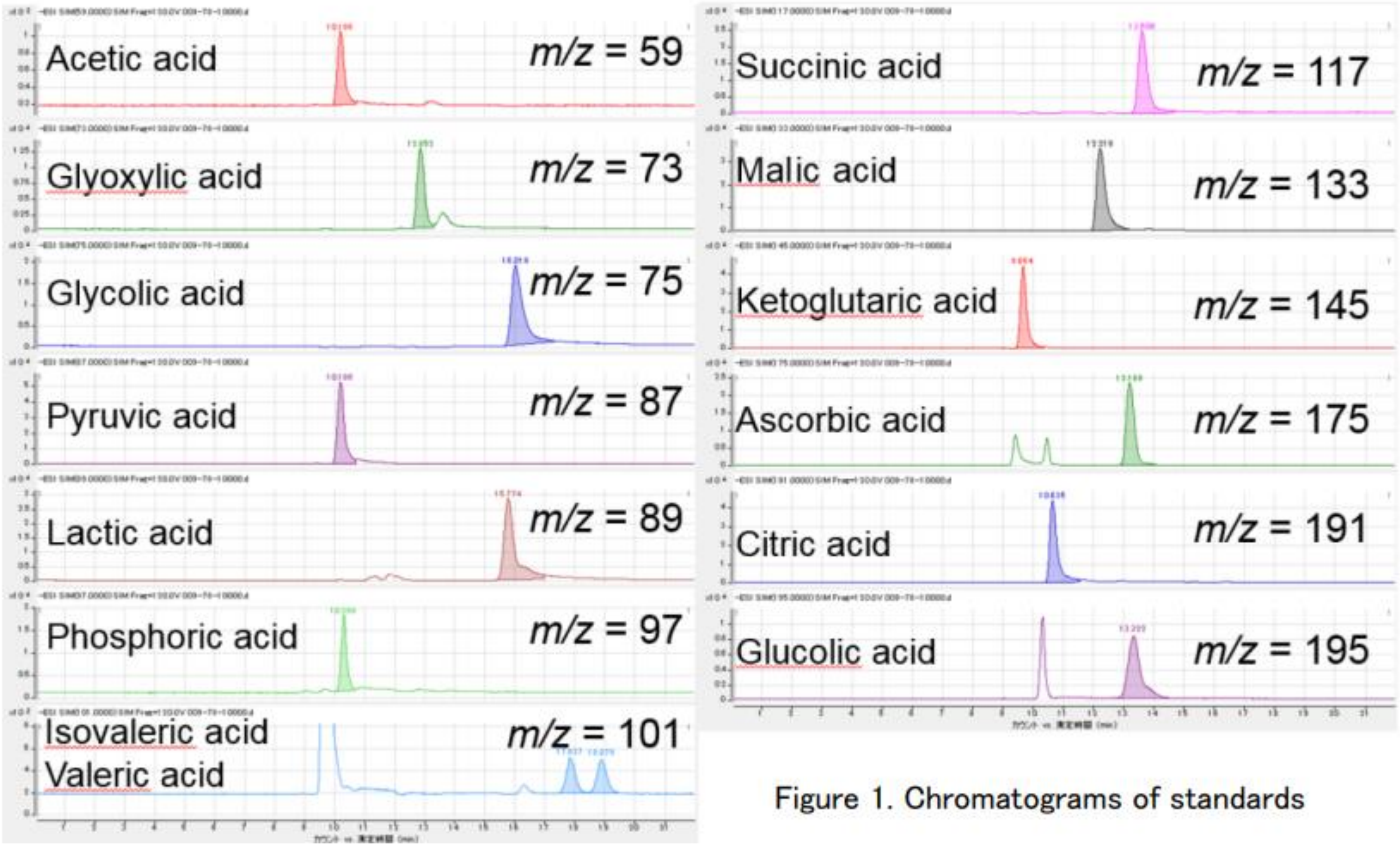

Figure 1. Chromatograms of standards

Column: Agilent Hi-Plex H, 300 x 7.7 mm
Mobile phase A: 0.01% aqueous formic acid
Mobile phase B: Acetonitrile
A/B: 70/30 (Isocratic)
Flowrate: 0.4 mL/min
Column temperature: 45 ℃
Injection volume: 10 µL
Analysis time: 22 min
MS ion source: ESI
Gas flow: N_2 , 12 L/min
Gas temperature: 200 ℃
Nebulizer pressure: 50 psi
Capillary voltage: 3000 V (negative mode)
MS signal: SIM negative

Poster

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Organic Acids on Hi-Plex H, RID

Conditions

Sample	Organic acids
Column	Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830)
Mobile phase	0.1 M H_2SO_4
Flow rate	0.6 mL/min
Temperature	50 °C
Detector	RI

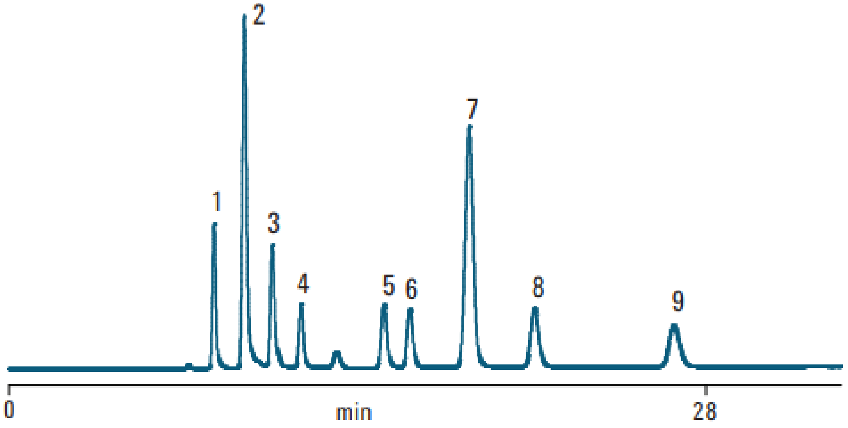

Figure 2. Separation of nine organic acids on an Agilent Hi-Plex H column with RI detection.

Peak number	Analyte	As. USP	10% Asymmetry	Plate count	Plates/m
1	Oxalic acid	1.23	1.12	19471	64904
2	*cis*-Aconitic acid	1.29	1.15	16122	53741
3	Tartaric acid	1.30	1.24	19272	64240
4	Malic acid	1.10	1.07	20153	67176
5	Lactic acid	1.16	1.10	21469	71563
6	Formic acid	1.08	1.05	22118	73726
7	Fumaric acid	1.05	1.03	15751	52504
8	Propionic acid	1.12	1.09	20492	68305
9	Butyric acid	1.15	1.13	18181	60603

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Organic Acids on Hi-Plex H, UV

Conditions

Sample	Organic acids
Column	Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830)
Mobile phase	100% 0.01 M H_2SO_4
Flow rate	0.6 mL/min
Injection volume	20 µL
Temperature	50 °C
Detector	UV, 210 nm

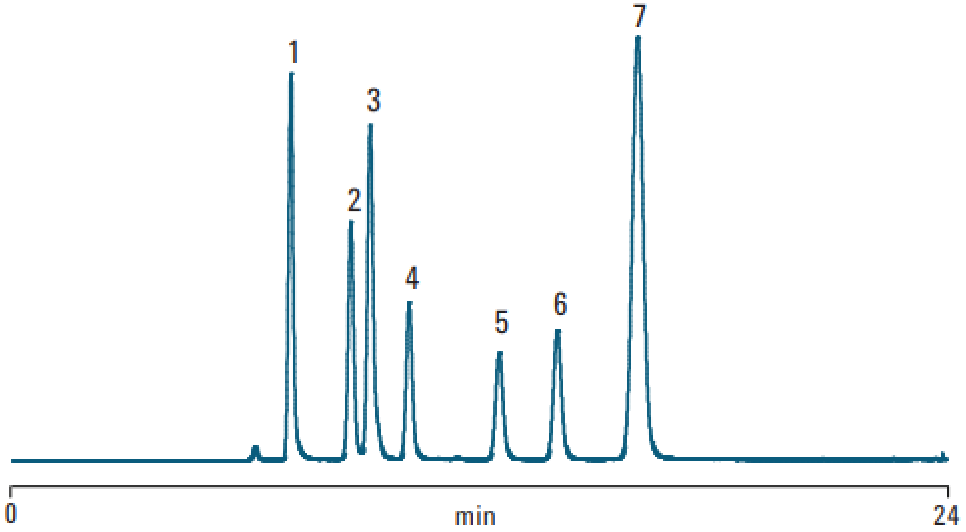

Figure 3. Separation of seven organic acids on an Agilent Hi-Plex H column with UV detection.

Peak number	Analyte	As. USP	10% Asymmetry	Plate count	Plates/m
1	Oxalic acid	1.13	1.02	17164	57212
2	Citric acid	1.11	1.07	17588	58626
3	Tartaric acid	1.30	1.23	19251	64170
4	Malic acid	1.11	1.07	20170	67233
5	Succinic acid	1.08	1.06	19705	65684
6	Formic acid	1.07	1.05	21991	73302
7	Fumaric acid	1.05	1.03	15139	50464

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Sugars and Organic Acids on Hi-Plex H, RID

Conditions

Sample	Sugars and organic acids
Column	Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830)
Mobile phase	100% 0.0085 M H_2SO_4
Flow rate	0.4 mL/min
Injection volume	20 µL
Temperature	65 °C
Detector	RI

Peak number	Analyte	As. USP	10% Asymmetry	Plate count	Plates/m
1	Citric acid	0.92	0.92	21207	70691
2	Tartaric acid	0.84	0.85	21475	71583
3	Glucose	1.04	1.04	21805	72684
4	Malic acid, fructose	0.93	0.93	10012	33372
5	Lactic acid	0.89	0.96	19685	65618
6	Glycerol	1.16	1.06	19070	63566

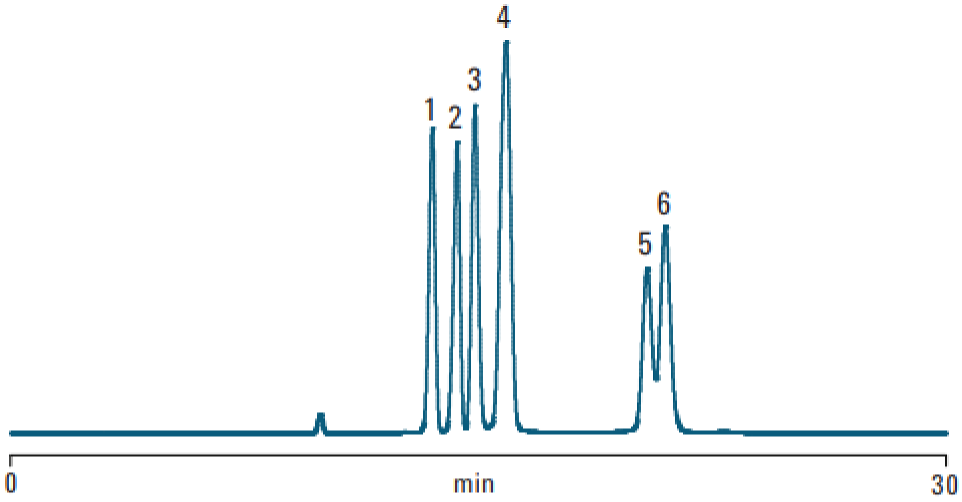

Figure 1. Separation of monosaccharides, organic acids, and glycerol on an Agilent Hi-Plex H column with RI detection.

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Sugars and Sugar Alcohols on Hi-Plex Ca, RID

Conditions

Sample	Sugars and sugar alcohols
Column	Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810)
Sample size	10 mg/mL
Mobile phase	100% DI H_2O
Flow rate	0.6 mL/min
Injection volume	10 µL
Temperature	85 °C
Detector	RI

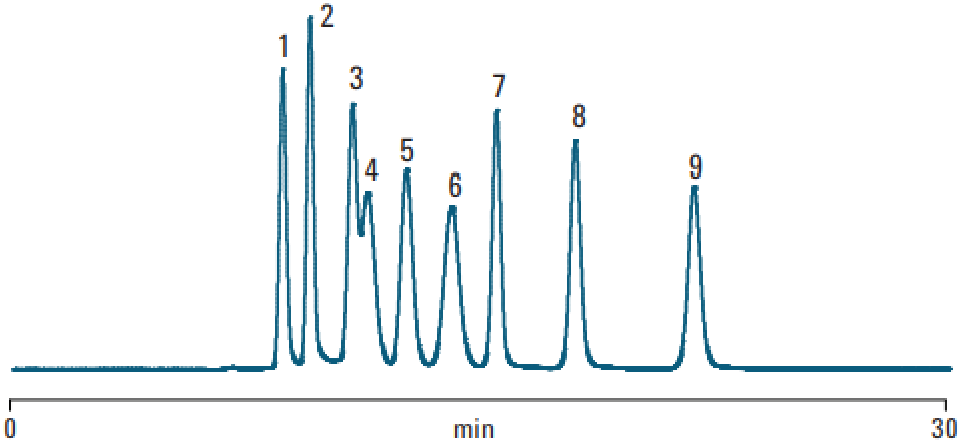

Figure 4. Separation of a mixture of sugars and sugar alcohols on an Agilent Hi-Plex Ca column.

Peak number	Analyte	As. USP	10% Asymmetry	Plate count	Plates/m
1	Raffinose	1.12	1.08	7138	23793
2	Sucrose	1.12	1.06	9389	31298
3	Lactulose	0.85	0.92	3858	12861
4	Glucose	1.79	1.59	2986	9955
5	Galactose	1.07	1.07	5008	16694
6	Fructose	1.01	1.01	3727	12423
7	Ribitol	1.00	1.00	14758	49194
8	Mannitol	1.04	1.04	13861	46204
9	Sorbitol	1.04	1.04	14170	47234

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Summary
What to do when your analyte is too polar

- Try reversed phase, adjust mobile phase composition
- Try ion pair
- Try more polar reversed-phase columns with 100% aqueous mobile phase
- Consider HILIC or ligand exchange

Contact Agilent Chemistries and Supplies Technical Support

Agilent InfinityLab

1-800-227-9770 option 3, option 3:

- Option 1 for GC and GC/MS columns and supplies
- Option 2 for LC and LC/MS columns and supplies
- Option 3 for sample preparation
- Option 4 for spectroscopy supplies
- Option 5 for chemical standards
- Option 6 for former Prozyme products

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