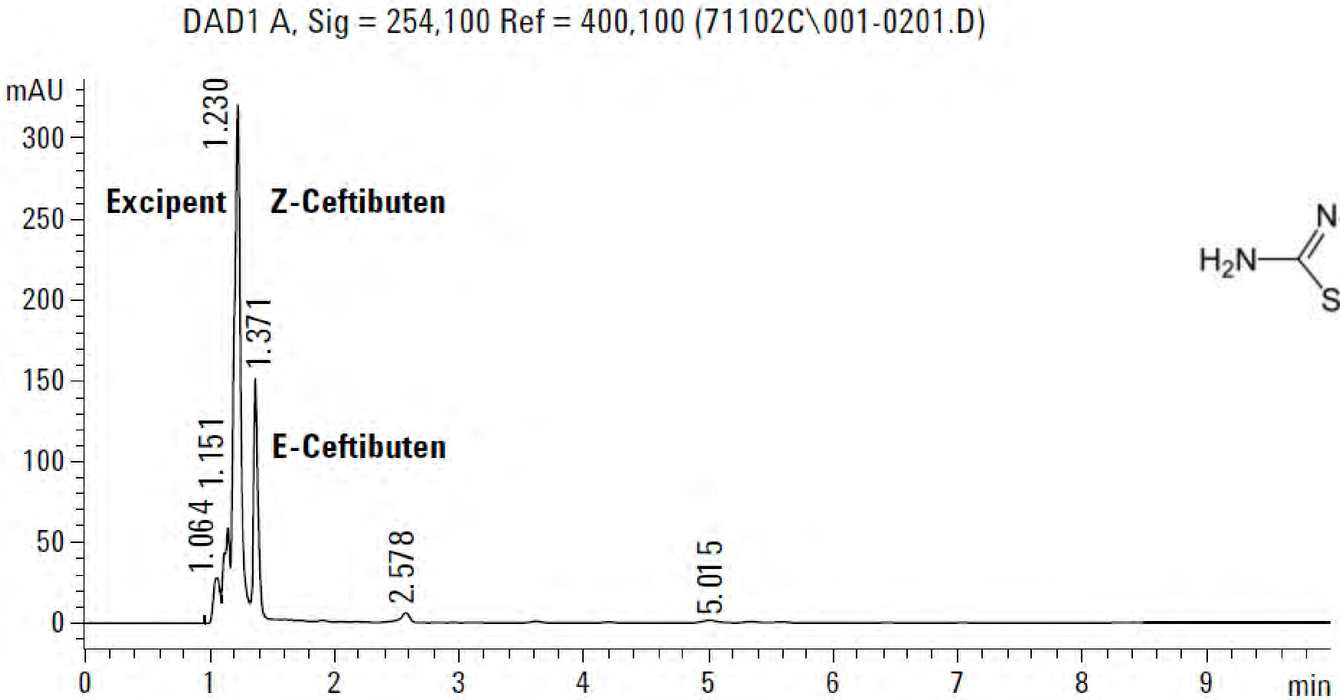
Polar Analytes: C18 Didn't Work, Now What?

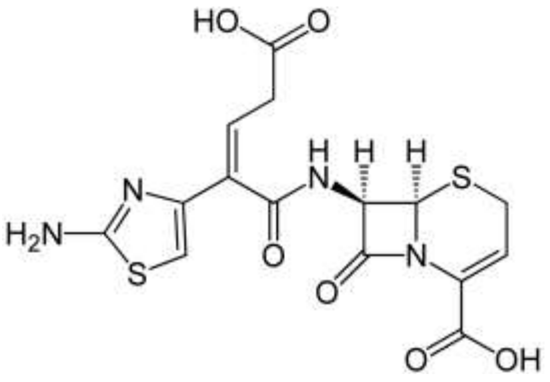
Mark Powell

Agilent Technologies

Technical Support

C18 Doesn't Always Work…





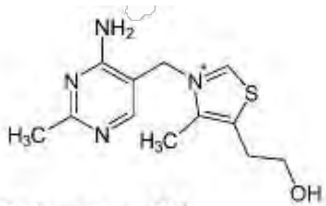
Instrument: Agilent 1100 Series HPLC; **Temp:** ambient; **Column:** Alkyl-C18, 4.6 × 150 mm, 5 µm; **Mobile phase:** 2% ACN, 98% 10 mM ammonium acetate, pH 5.4; **Flow rate:** 1 mL/min; **Injection volume:** 5 µL; **Diode array detector:** 254 nm; **Reference:** 400 nm; **Bandwidth:** 100 nm

Agilent Technologies

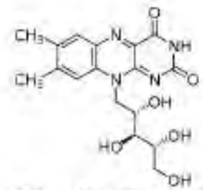
Pore Dewetting or Phase Collapse

- Alkyl phases such as C8 or C18 can exhibit poor retention or reproducibility of retention in low organic mobile phases
- Phenomenon known as pore dewetting or phase collapse
- Onset can be unpredictable
- A method robustness issue often mistaken as a column or lot issue
- See Przybyciel and Majors, *LCGC* **20**(6), 516-523 (2002).

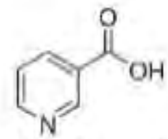
Water Soluble Vitamins



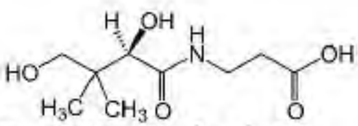
Thiamine, B1



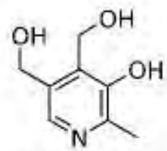
Riboflavin, B2



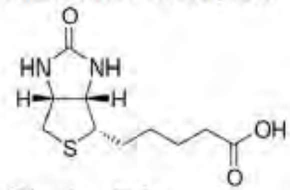
Nicotinic Acid, B3



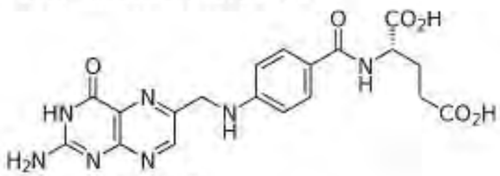
Pantothenic Acid, B5



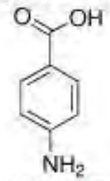
Pyridoxine, B6



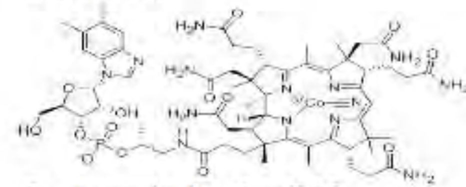
Biotin, B7



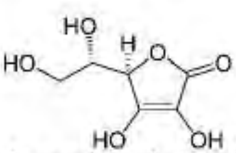
Folic Acid, B9



PABA, B10



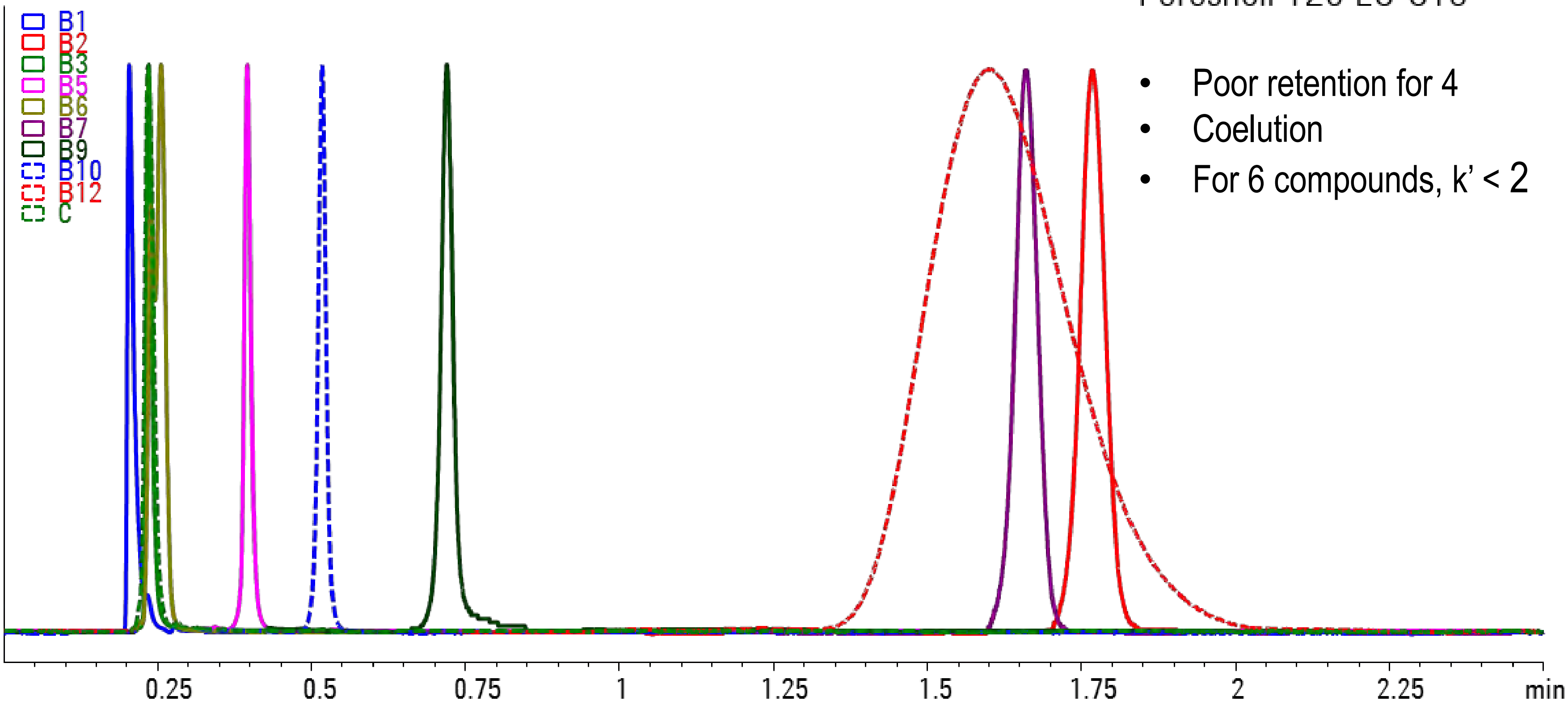
Cyanocobalamin, B12



Ascorbic Acid, C

Water Soluble Vitamins
C18 at low pH

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



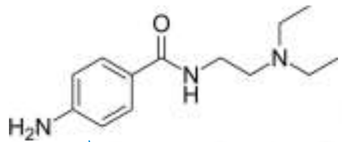
- Poor retention for 4
- Coelution
- For 6 compounds, k' < 2

Now What?

- **Adjust mobile phase pH**
- Ion-pair chromatography
- Alternate column choice
- HILIC (hydrophilic interaction chromatography)

Why try higher pH?

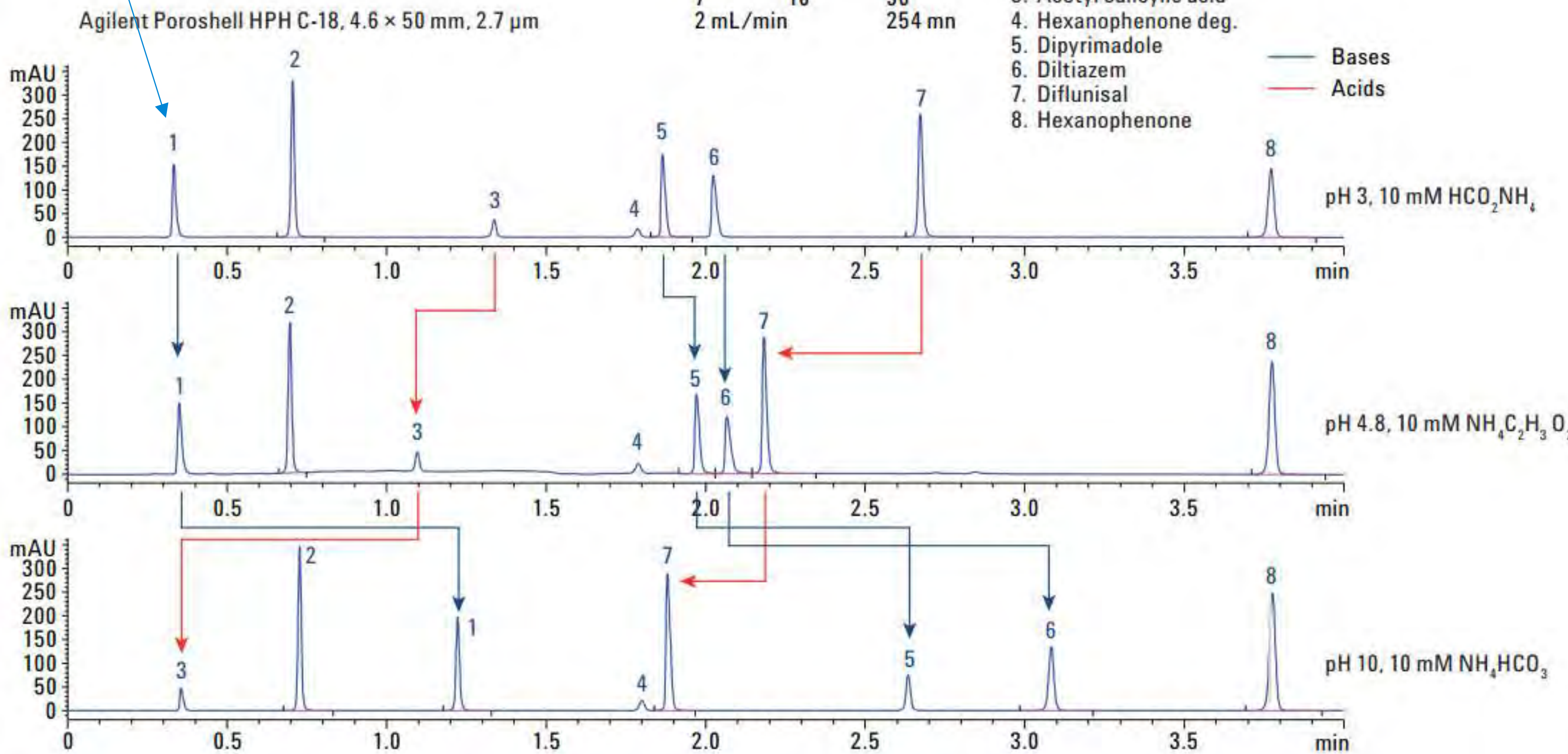
Poroshell HPH-C8 or C18



pKa = 9.2

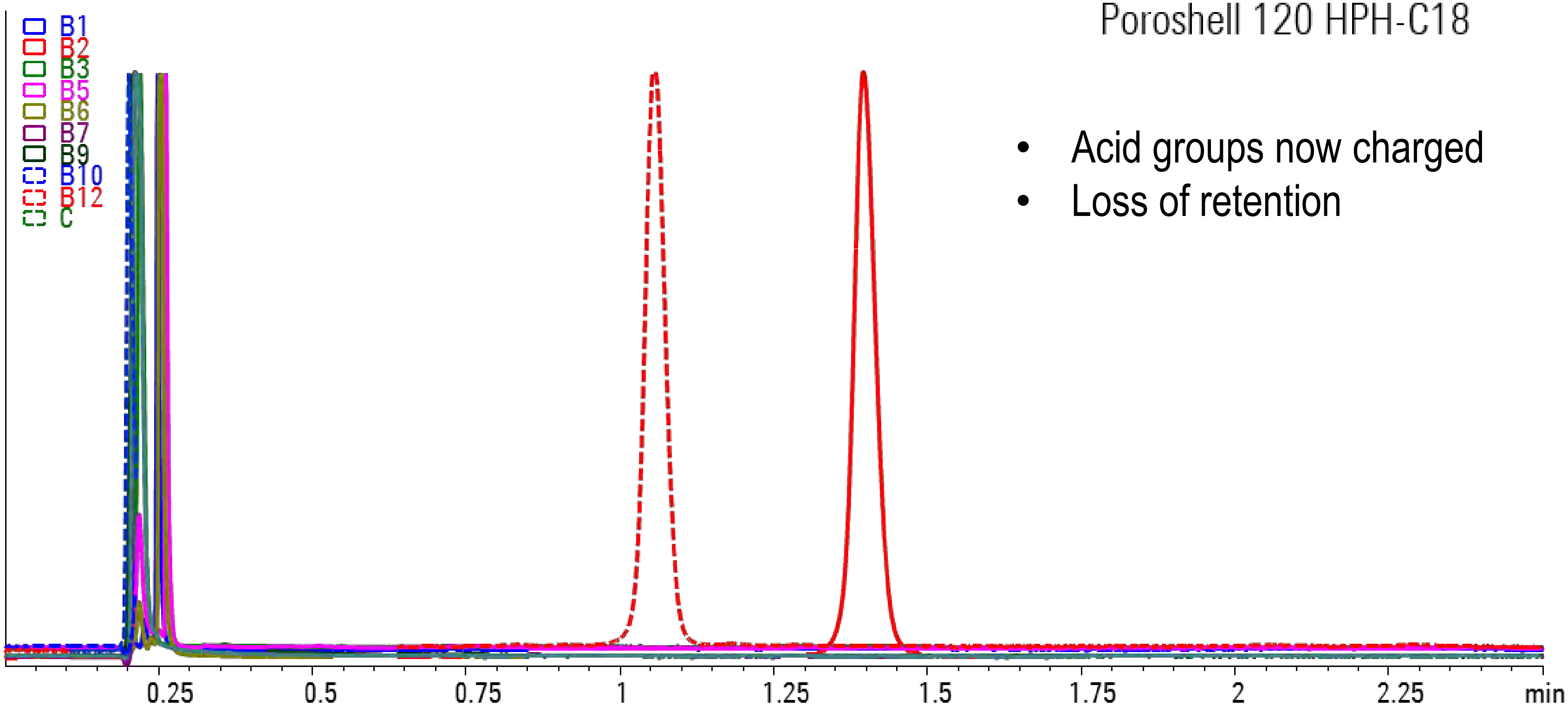
Time	% Buffer	% MeCN
0	10	90
5	90	10
7	10	90
2 mL/min		254 mn

Peak ID
1. Procainamide
2. Caffeine
3. Acetyl salicylic acid
4. Hexanophenone deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone



Water Soluble Vitamins C18 at pH 7.5

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



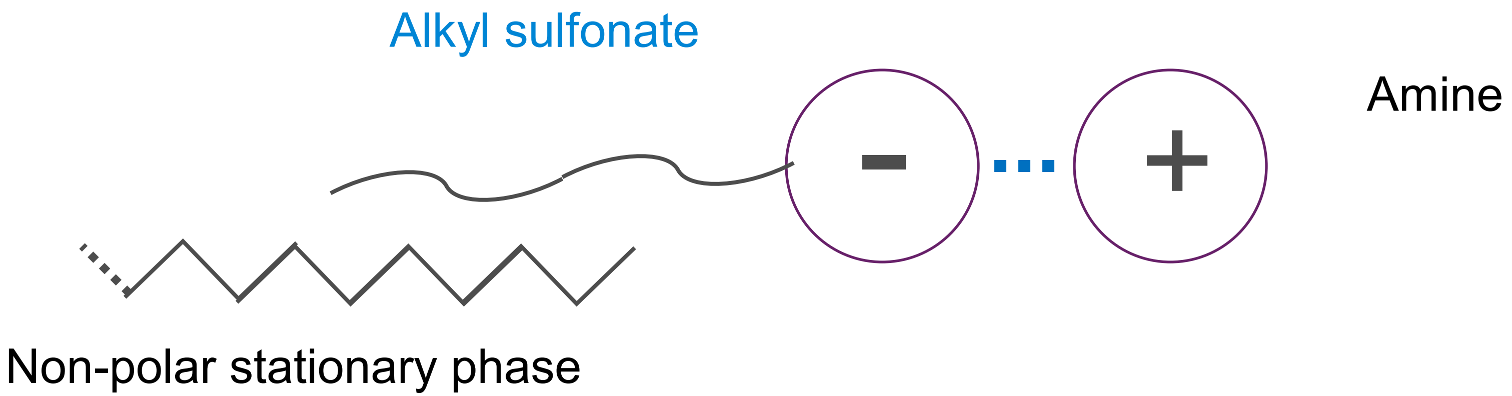
- Acid groups now charged
- Loss of retention

Now What?

- Adjust mobile phase pH
- **Ion-pair chromatography**
- Alternate column choice
- HILIC

Ion-Pair Chromatography

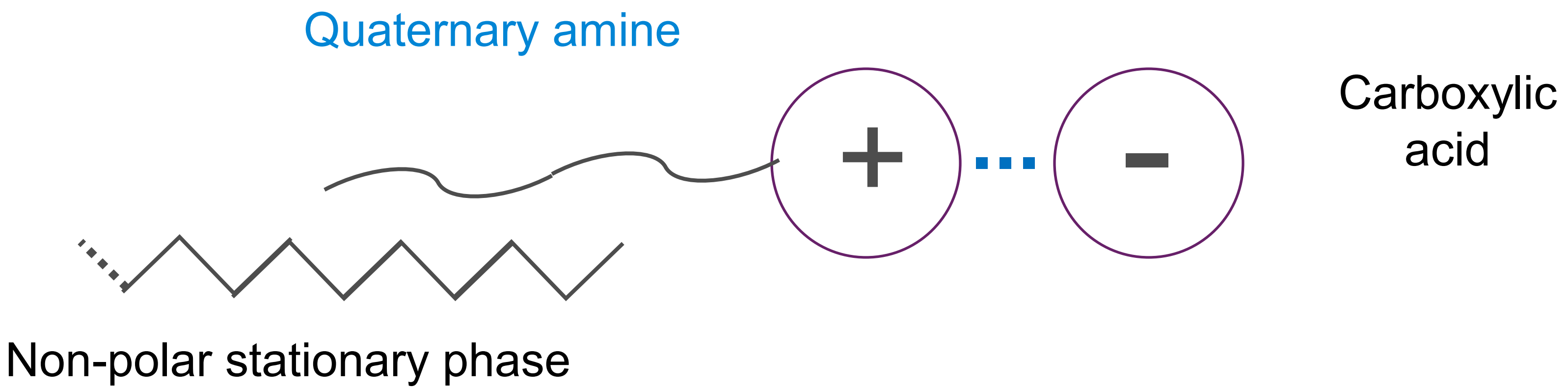
Similar to reversed-phase, but an ion-pairing reagent is added to the mobile phase



•Non-polar alkyl chain will adsorb into the non-polar stationary phase
•Polar part of the ion-pairing reagent will "stick-out" into the mobile phase

Ion-Pair Chromatography

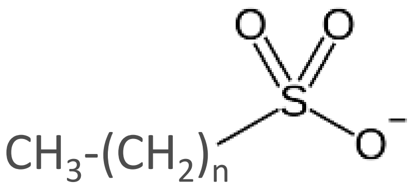
Similar to reversed-phase, but an ion-pairing reagent is added to the mobile phase



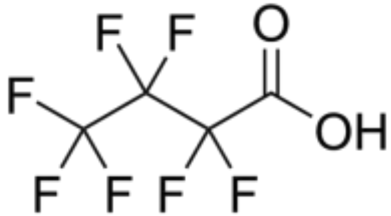
•Non-polar alkyl chain will adsorb into the non-polar stationary phase
•Polar part of the ion-pairing reagent will "stick-out" into the mobile phase

Some Common Ion-Pairing Reagents

Pairs with Cations

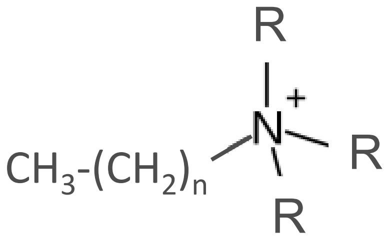


Alkyl sulfonates



Heptafluorobutyric acid
(HFBA)

Pairs with Anions



Quaternary amines

Ion-Pair Chromatography
Suggested Experimental Conditions

Column: C8 or C18

Mobile Phase:

- Organic – often methanol
- Aqueous - Buffered with appropriate IP reagent
- Temperature controlled between 35° and 60°C

<u>Cations – bases</u>

Buffer: 25 – 50 mM phosphate, pH 2- 3

IP reagent: 10-100 mM heptane sulfonate

<u>Anions – acids</u>

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

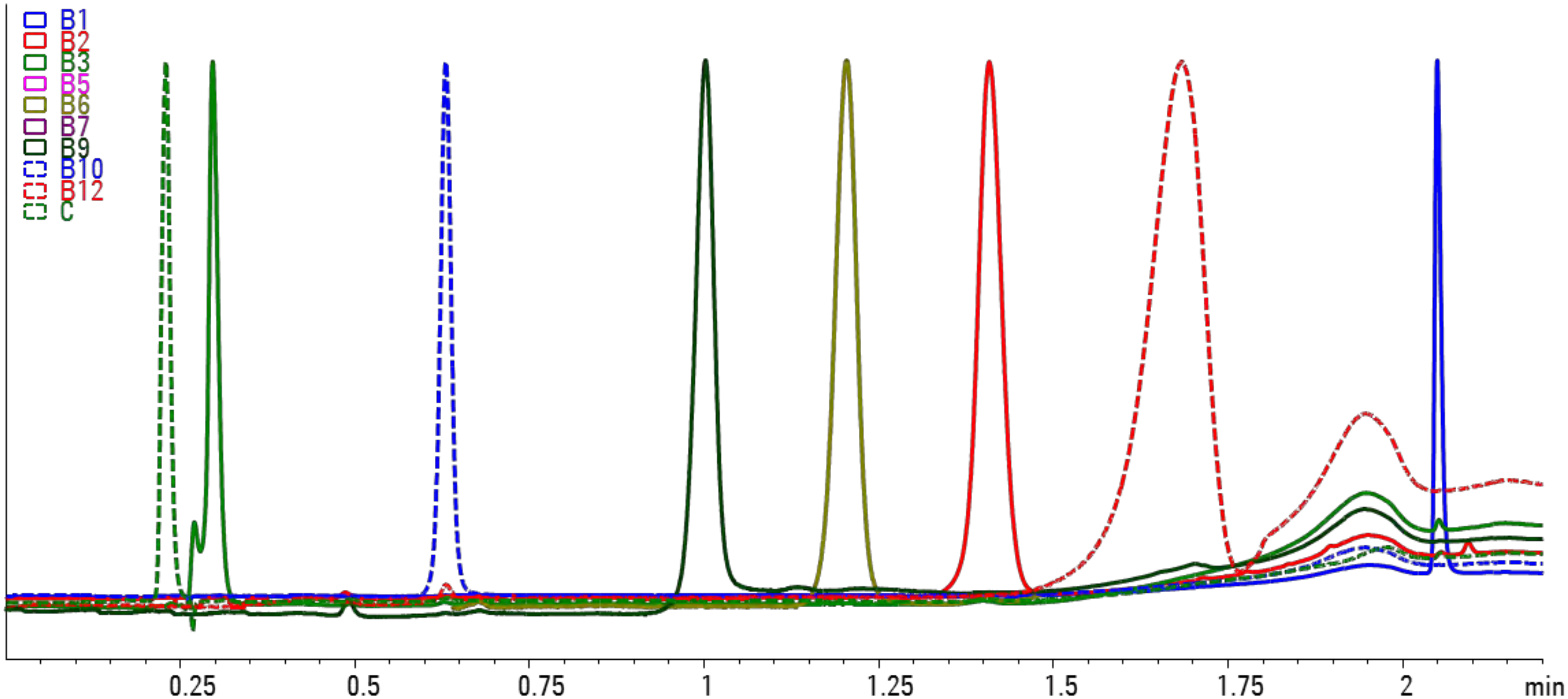
IP reagent: 10-40 mM tetrabutyl ammonium phosphate

Agilent Technologies
Page 13

Ion-Pair Chromatography Limitations

- Higher level of complexity than RP, so generally chosen only if needed
- Requires careful control of IP reagent, pH, temperature
- Gradient methods are more difficult than RP
- Equilibration is much slower than RP
- Column dedicated to IP
- IP reagent in the injection solvent

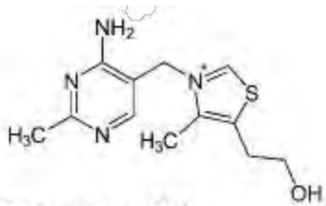
Water Soluble Vitamins
Ion Pair Conditions

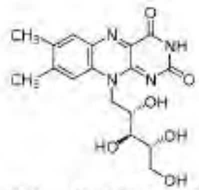


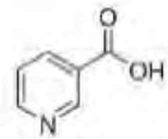
EC-C18
A: 1.5 g sodium 1-heptanesulfonate + 0.2 mL triethylamine + 7.5 mL acetic acid + 992.5 mL water
B: CH3CN
0.5 mL/min,10% B for 1 minute, then 10-40% B in 1 minute
injection volume: varies according to signal strength
TCC: 30 C
260, 8 nm Ref Off, 8 nm slit, 80 Hz

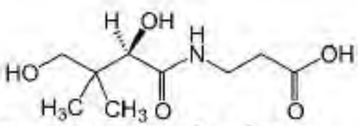
The ion pairing reagent increased retention for most compounds
- 6 compounds have k' > 2
- B5 and B7 could not be detected due to low signal and high background noise at 210 nm (not detectable at 260 nm)

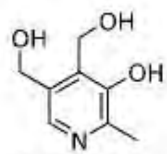
Water Soluble Vitamins

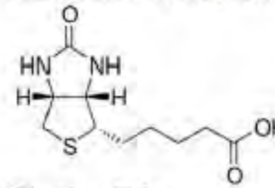

Thiamine, B1

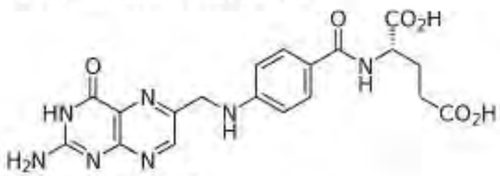

Riboflavin, B2

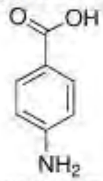

Nicotinic Acid, B3

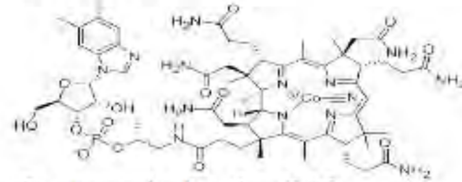

Pantothenic Acid, B5

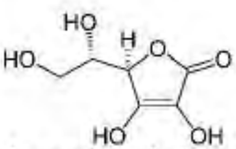

Pyridoxine, B6


Biotin, B7

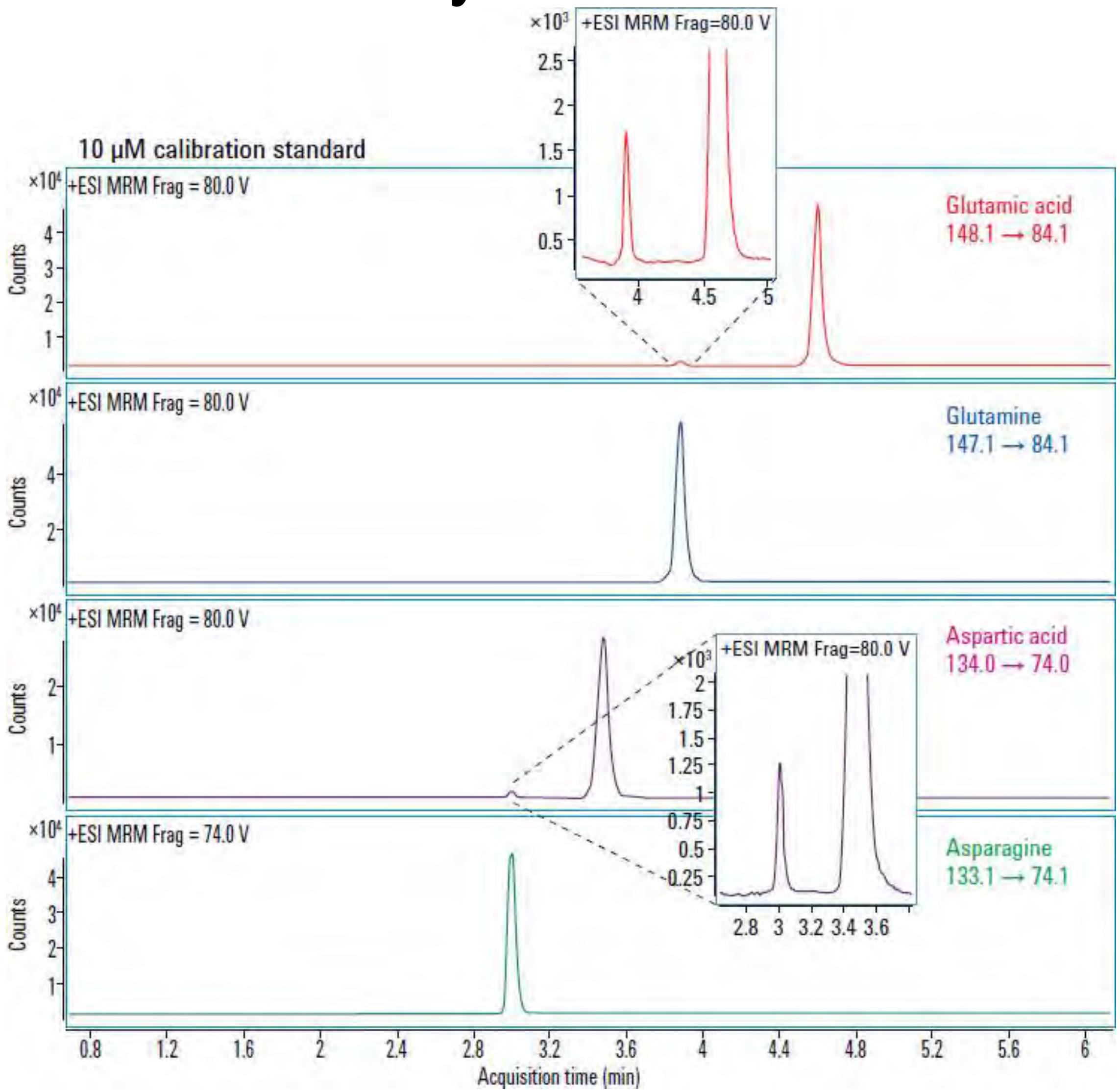

Folic Acid, B9

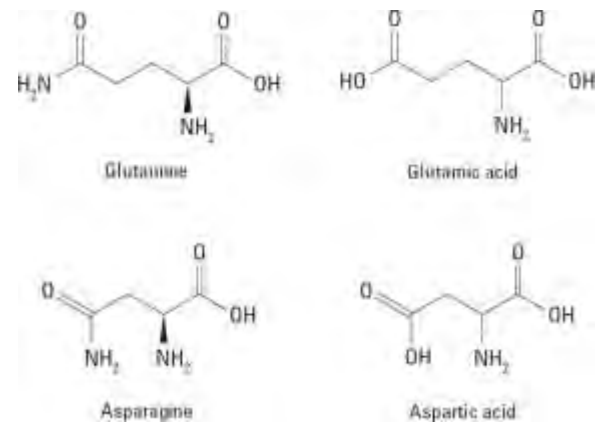

PABA, B10


Cyanocobalamin, B12


Ascorbic Acid, C

Amino Acids by Ion-Pair





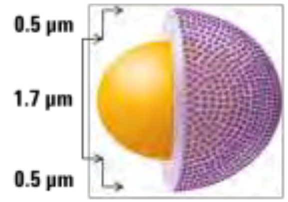
ZORBAX SB-C18 RRHT, 1.8 µm,
3 x 50 mm, 25 °C, 1 µL inj
0.4 mL/min
A: water/ 0.5 % FA + 0.3% HFBA
B: ACN/0.5% FA + 0.3% HFBA
0 to 5% B over 5 minutes

5991-0904EN

Now What?

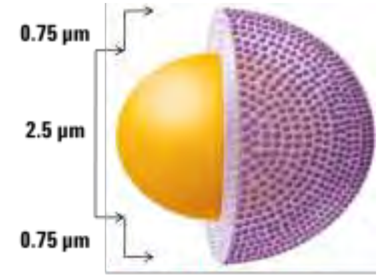
- Adjust method conditions
- Ion-pair chromatography
- **Alternate column choice**
- HILIC

Phase Choices



Poroshell 120 2.7 µm

Efficiency 90% of < 2 µm TPP
Pressure 50% of < 2 µm TPP
2 µm inlet frit



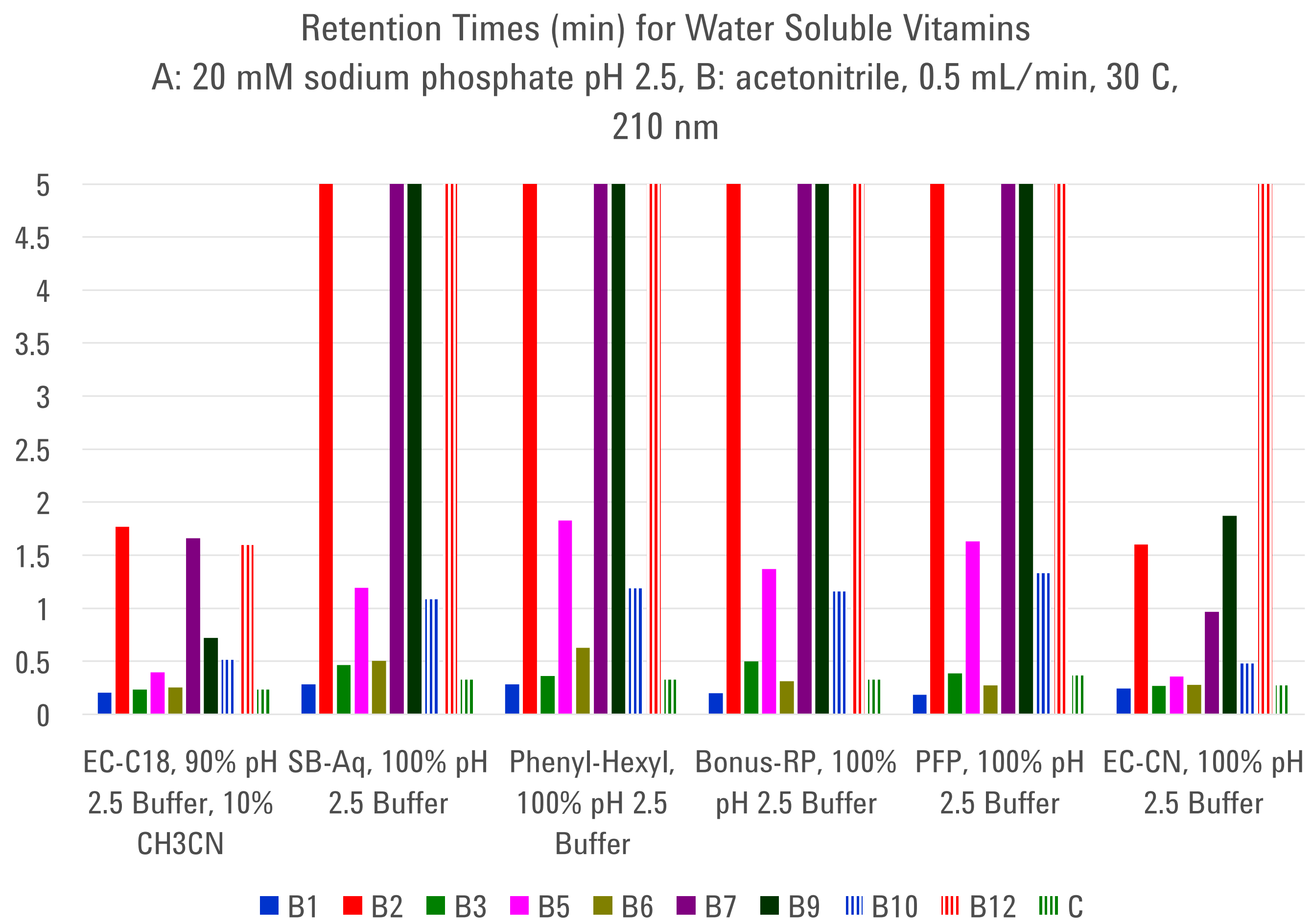
Poroshell 120 4 µm

Efficiency 2x 5 µm TPP
Pressure often below 200 bar
2 µm inlet frit

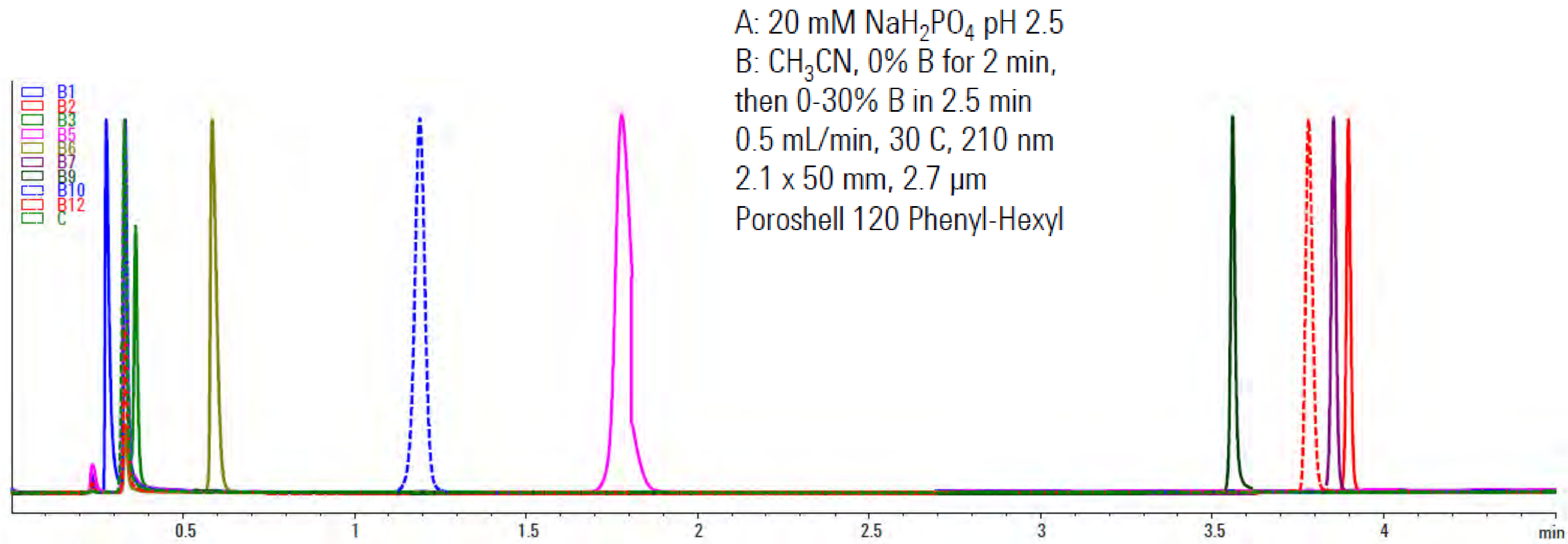
Best all around	Best for low pH mobile Phases	Best for high pH mobile phases	Best for alternative selectivity	Best for more polar compounds
Poroshell 120 **EC-C18** 2.7 µm, 4 µm	Poroshell 120 **SB-C18** 2.7 µm	Poroshell **HPH-C18** 2.7 µm, 4 µm	Poroshell 120 **Bonus-RP** 2.7 µm	Poroshell 120 **SB-Aq**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

These phases can be used with 100% aqueous mobile phases to improve retention of highly polar analytes in RPLC mode

Water Soluble Vitamins
Alternative Phases

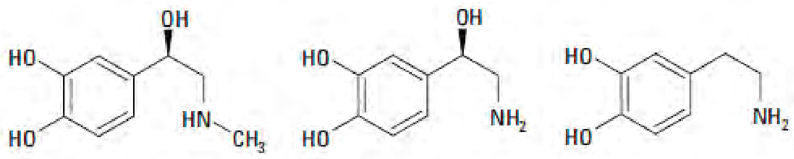


Water Soluble Vitamins
Phenyl-Hexyl



- Phenyl-Hexyl has the best retention
- 7 compounds have k'> 2;
- C18 analysis had only 4 compounds with k' > 2

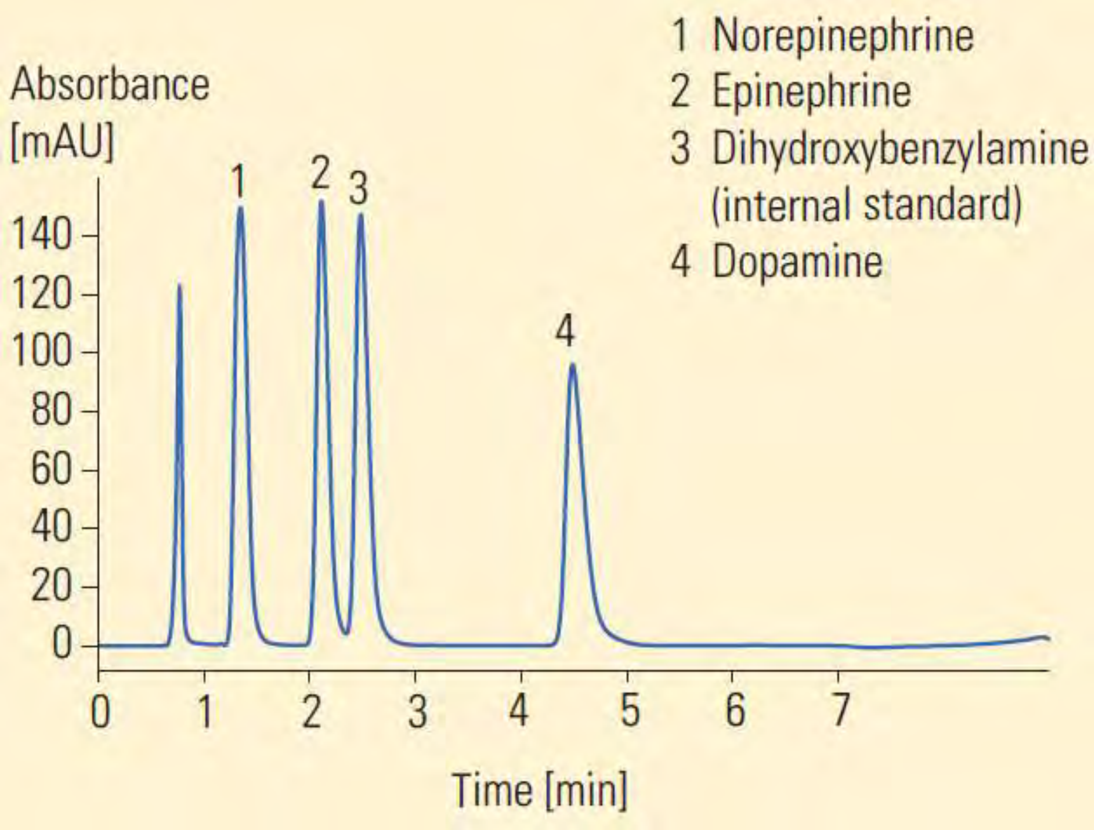
Catecholamines by Ion-Pair



Epinephrine
pKa = 8.55

Norepinephrine
pKa = 8.4

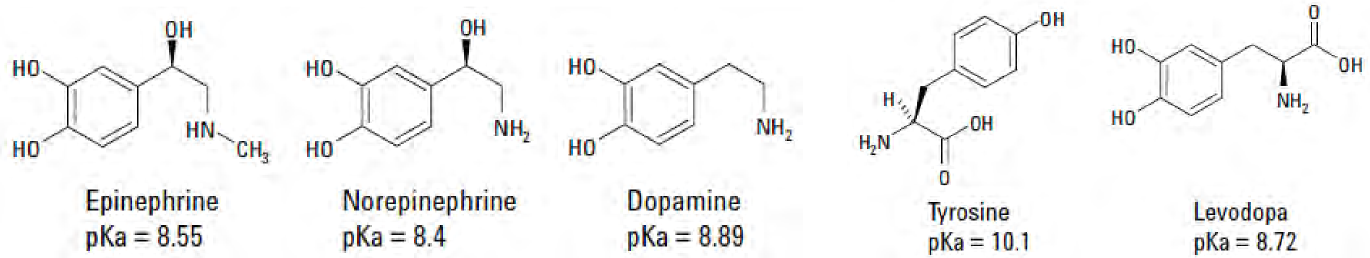
Dopamine
pKa = 8.89

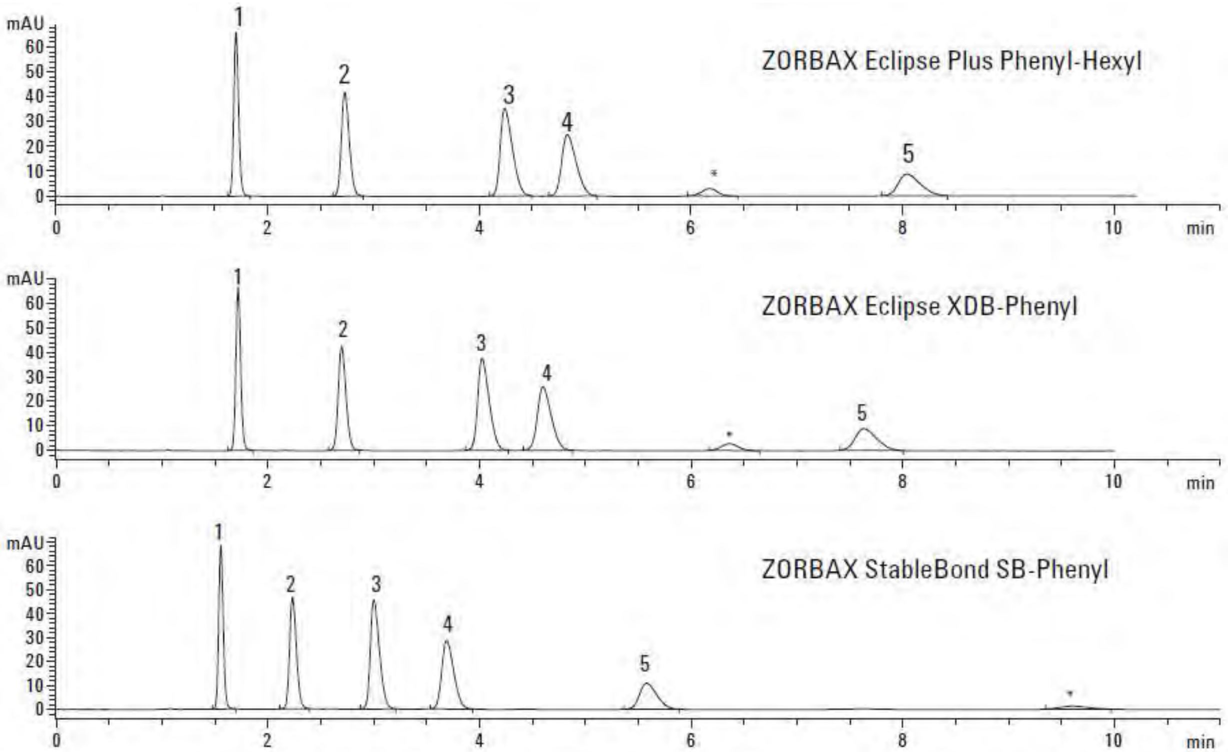


Column
4.6 x 75 mm Zorbax SB-C18 , 3.5 µm
Mobile phase
A = 0.025 M KH_2PO_4 + 0.3 mM heptanesulfonic acid in water (pH = 3), B = acetonitrile
Flow rate
1.0 ml/min
Gradient
at 0 min 1 % B
at 5 min 2 % B
at 7 min 15 % B
Column wash
at 8 min 1 % B
UV detector
variable wavelength detector
204 nm, standard cell
Column compartment temperature
30 °C
Injection volume 5 µl

5968-2966E

Catecholamines on Phenyl Phases

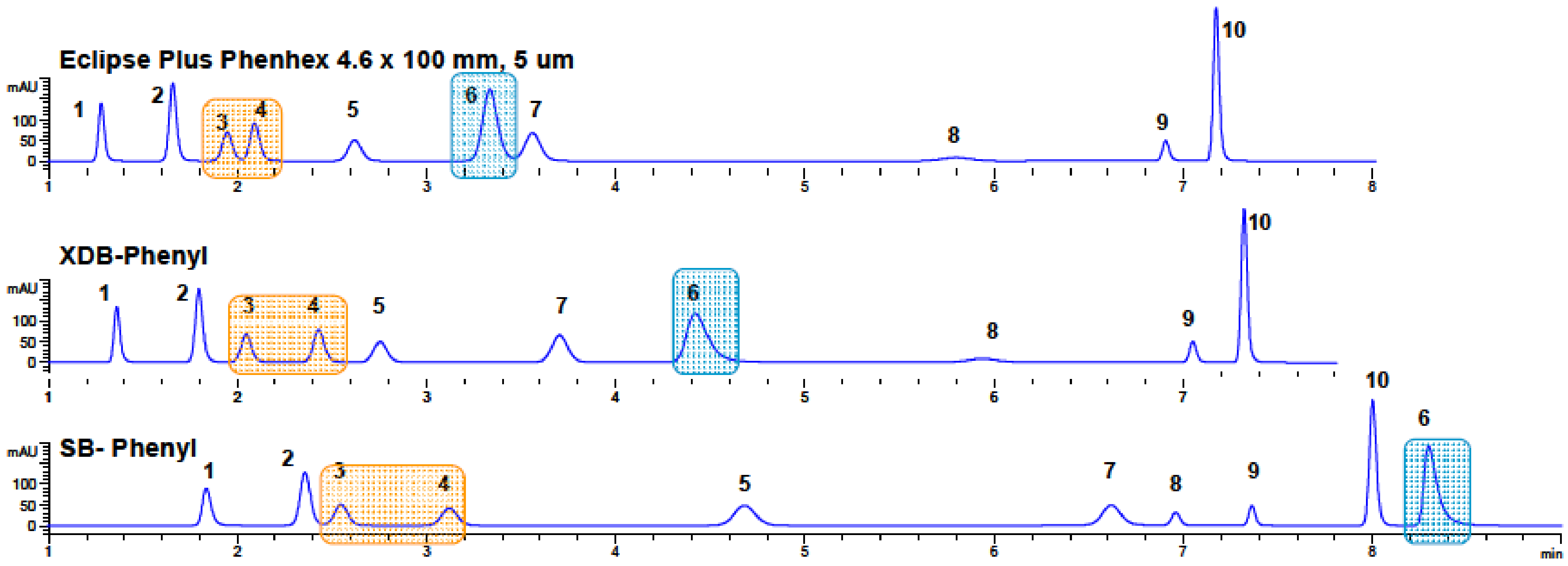




Norepinephrine, epinephrine, dopamine, levodopa, impurity*, tyrosine 0.2 mg/mL each 5 µL 4.6 mm × 100 mm, 5 µm columns.
Mobile phase = 0.1% TFA in water, 1 mL /min, 265 nm.

5990-3616EN

Nucleobases and Nucleosides

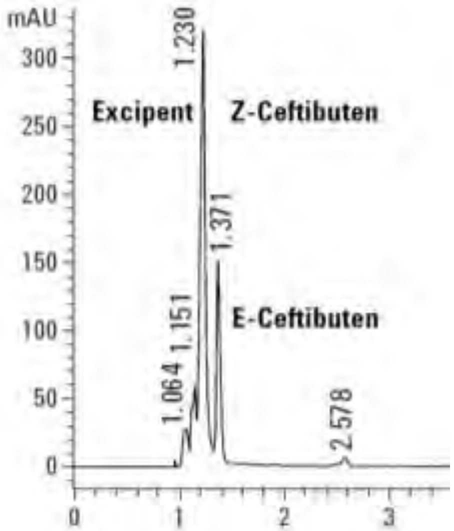


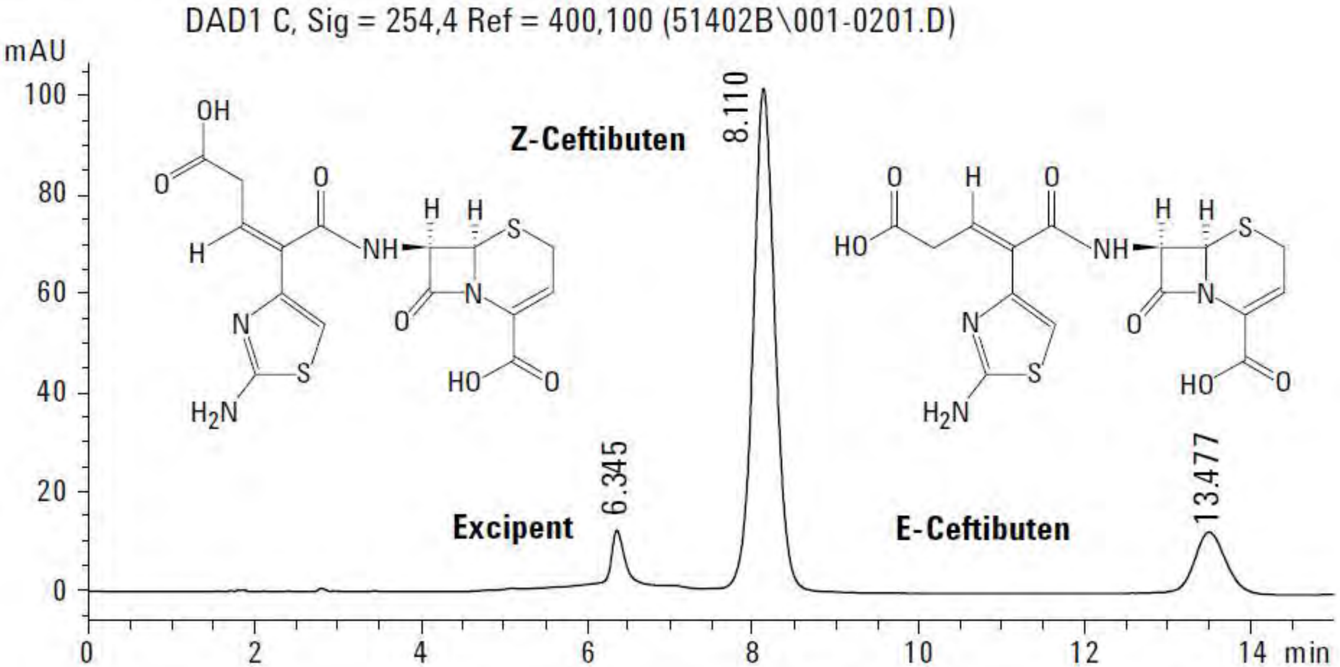
1.	Cytosine	6.	Adenine
2.	Uracil	7.	Thymine
3.	Cytidine	8.	Guanosine
4.	Guanine	9.	Thymidine
5.	Uridine	10.	Adenosine

A: 20 mM ammonium acetate, pH 4.5
B: methanol
1 mL/min, 254 nm

Time (min)	% B
0	1
4	1
6	50

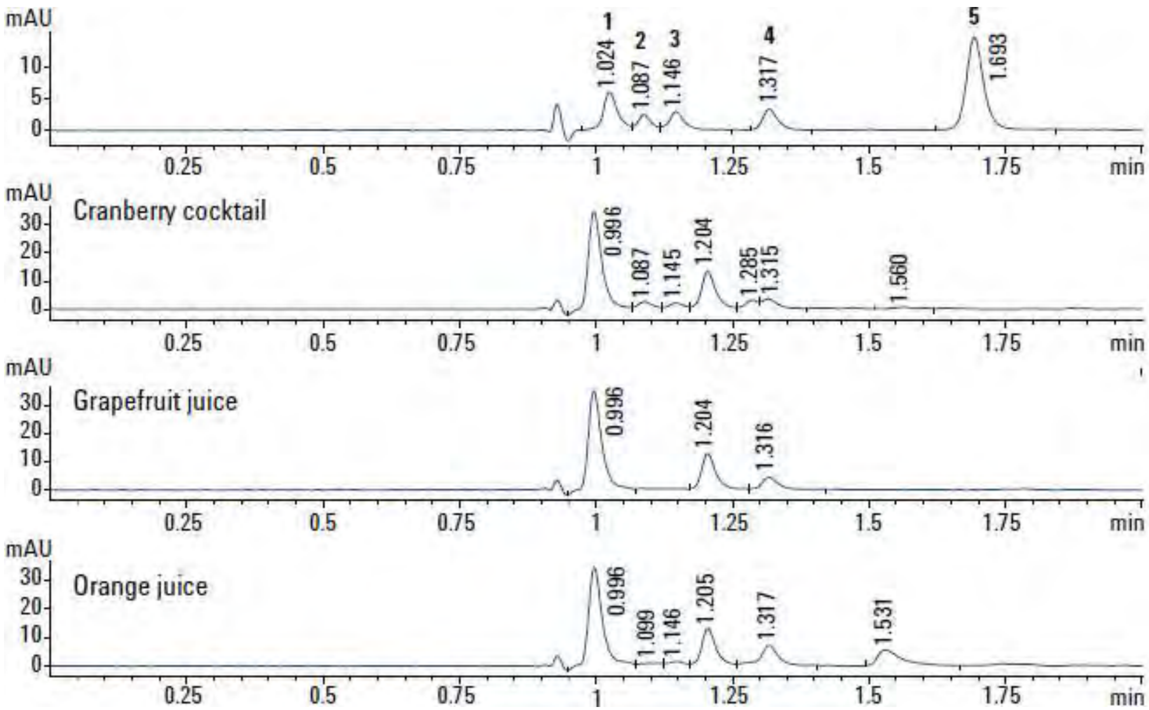
Our First Example, now with SB-Aq





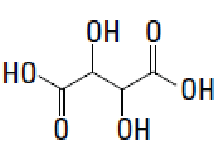
Instrument: Agilent 1100 Series HPLC; **Temp:** ambient; **Column:** Zorbax SB-Aq, 4.6 × 150 mm, 5 µm (part number 883975-914); **Mobile phase:** 100% 10 mM ammonium acetate, pH 5.4; **Flow rate:** 1 mL/min; **Injection volume:** 5 µL; **Diode array detector:** 254 nm; **Reference:** 400 nm; **Bandwidth:** 100 nm

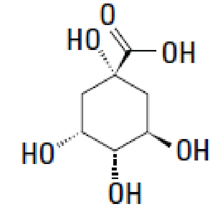
Aliphatic Acids
SB-Aq



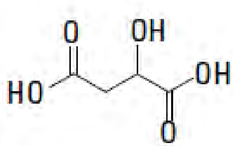
Peak ID

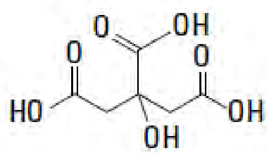
1. Tartaric acid
2. Quinic acid
3. Malic acid
4. Citric acid
5. Fumaric acid

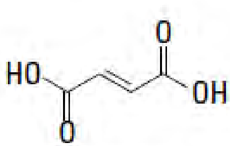

Tartaric acid


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

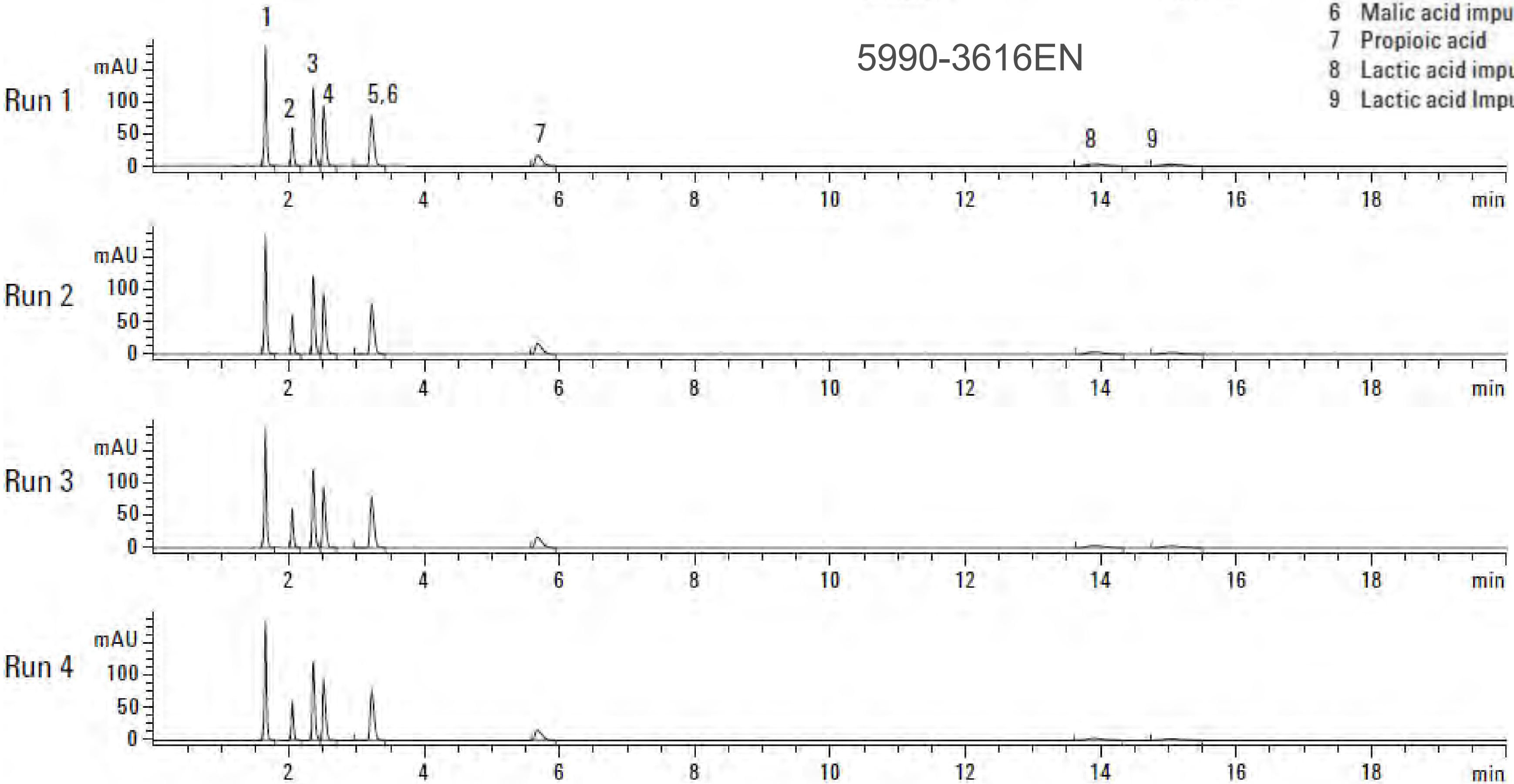
Aliphatic Acids
Phenyl-Hexyl

25 mM sodium phosphate buffer, pH = 2.5

Flow rate:	1.0 mL/min
Temperature:	25 °C
Injection volume:	5 µL
Detector:	220 nm

1 Tartaric acid
2 Malic acid
3 Lactic acid
4 Acetic acid
5 Citric acid
6 Malic acid impurity
7 Propioic acid
8 Lactic acid impurity
9 Lactic acid Impurity

5990-3616EN



1 and 2) Run successively with no pause. 3) Pump stopped 30 minutes and restarted. 4) Pump stopped 30 minutes and restarted ZORBAX Eclipse Plus Phenyl-Hexyl 4.6 mm × 150 mm 3.5 micron, p/n 959961–912.

What Now?

- Adjust method conditions
- Ion-pair chromatography
- Alternate column choice
- **HILIC (Hydrophilic Interaction Chromatography)**

HILIC
Hydrophilic Interaction Chromatography

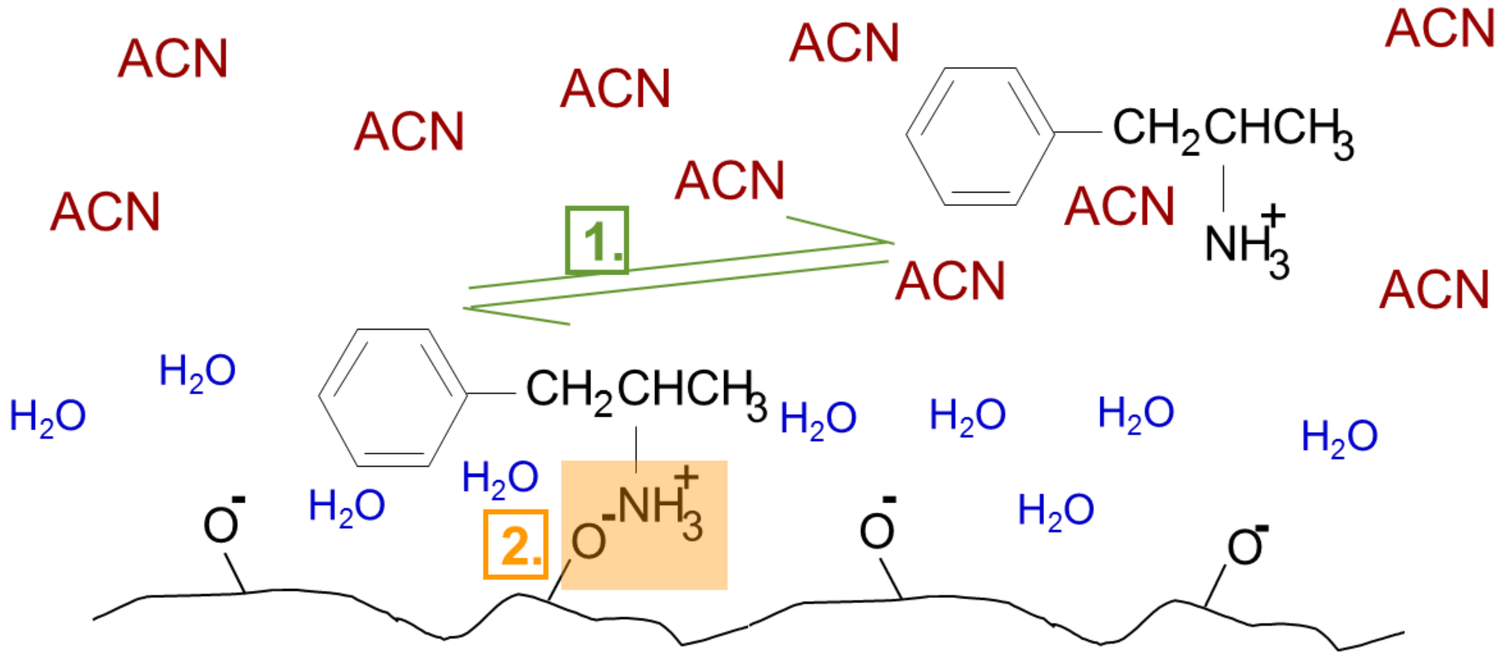
- Polar stationary phase:
  - Silica
  - Amine
  - Amide
  - Diol

HILIC
Hydrophilic Interaction Chromatography

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

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

How Does HILIC Work on Silica?



1. Partitioning in and out of adsorbed water layer
2. Ion exchange with silanols

HILIC Advantages

- Good peak shape for basic compounds where RP may give tailing and/or low efficiency
- Low viscosity mobile phases with high organic content allow the use of higher flow rates and/or long columns
- Enhanced detection sensitivity with MS
- Can directly inject ACN extracts from C18 SPE cartridges

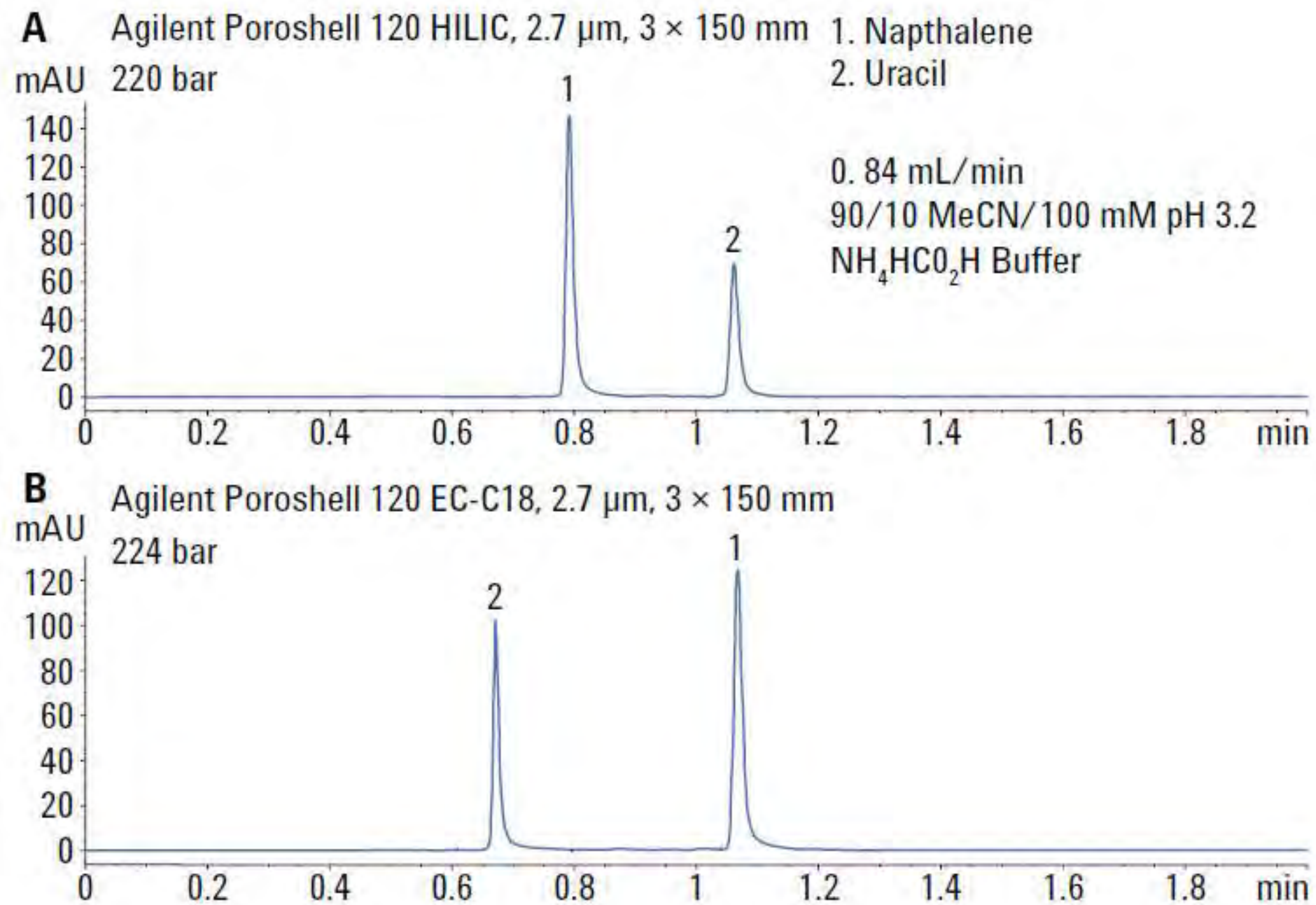
HILIC Challenges

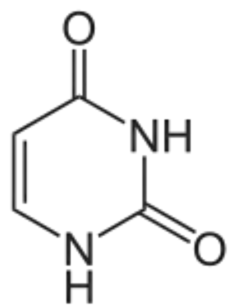
- Slower equilibration than RPLC
  - Particularly true for bare silica columns
  - Longer to equilibrate initially
  - Longer to equilibrate when mobile phase changes for gradients or method development are required
- Peak distortion with mobile phase / sample solvent mismatch

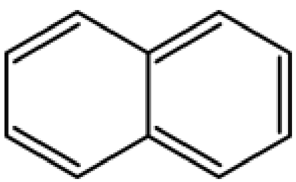
Typical Conditions

- Silica column (ZORBAX Rx-SIL, HILIC Plus, Poroshell 120 HILIC)
- Water (at least 2-3%, ~ 25%)/ACN
- Buffer (*e.g.*, ammonium acetate)
- pH control

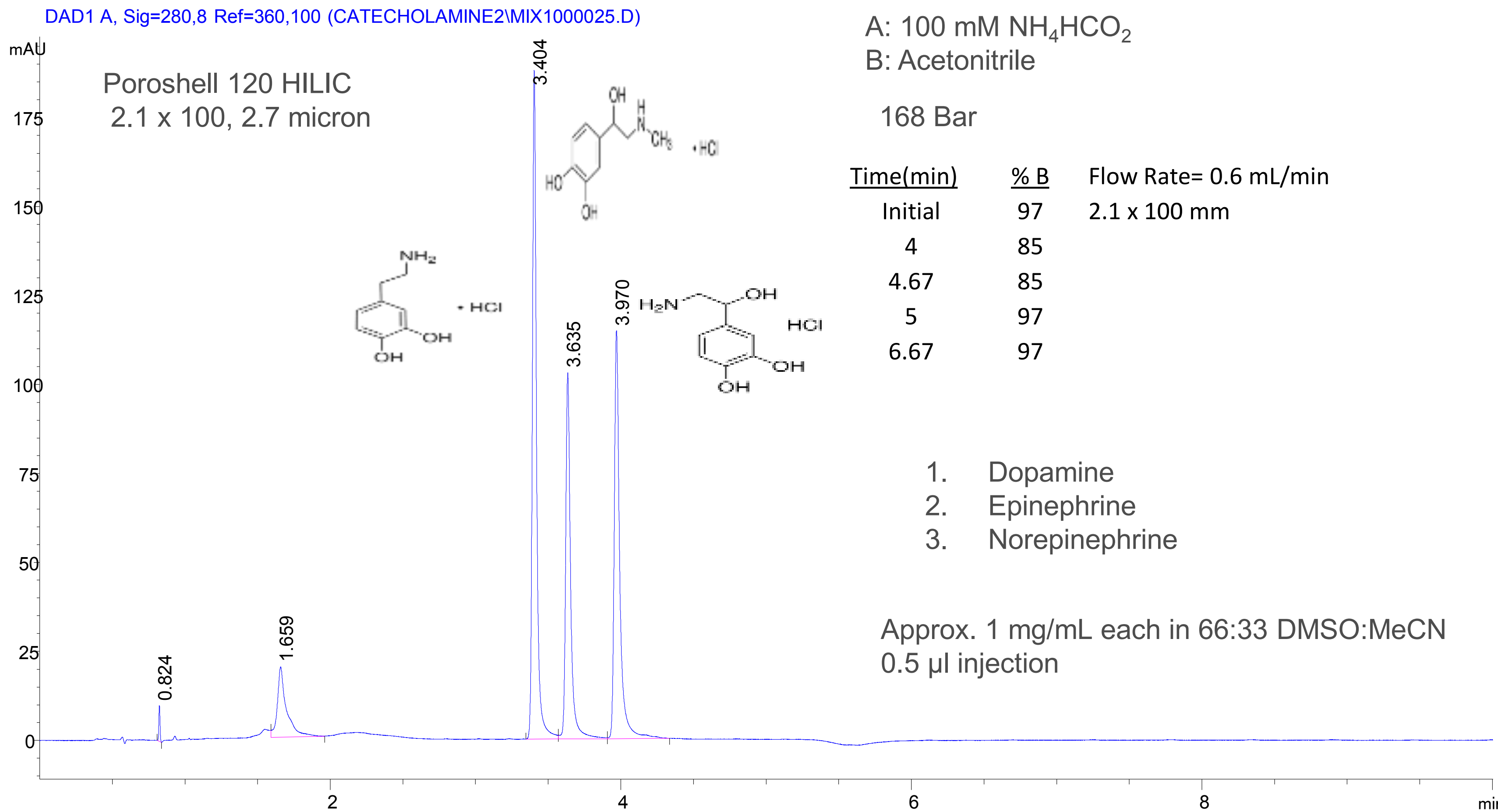
HILIC – comparison with C18







HILIC Separation of Catecholamines Poroshell 120 2.1 x 100, 2.7 micron



A: 100 mM NH_4HCO_2
B: Acetonitrile

168 Bar

Time(min)	% B
Initial	97
4	85
4.67	85
5	97
6.67	97

Flow Rate= 0.6 mL/min
2.1 x 100 mm

1. Dopamine
2. Epinephrine
3. Norepinephrine

Approx. 1 mg/mL each in 66:33 DMSO:MeCN
0.5 µl injection

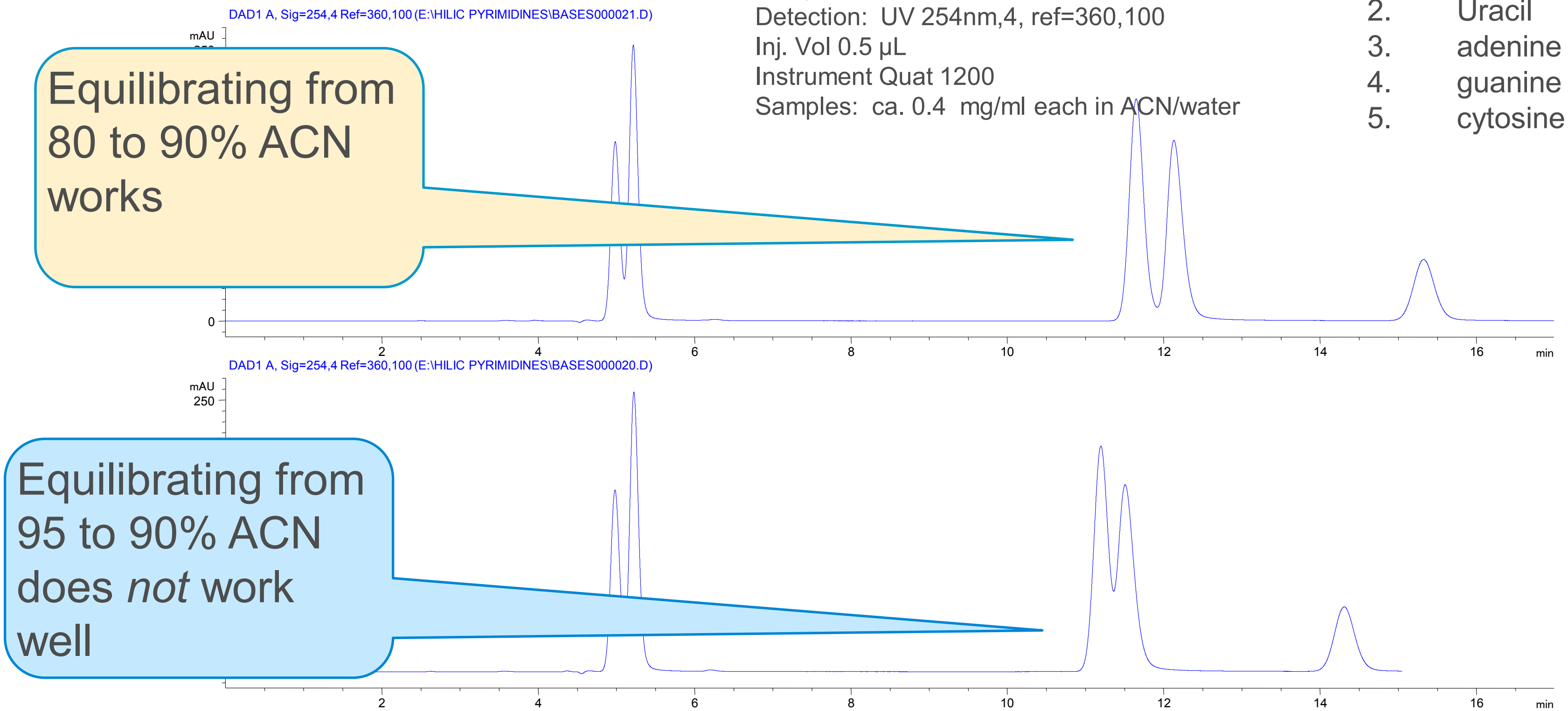
Equilibrate from high aqueous to low

Critical factor when changing mobile phases

ZORBAX Rx-Sil, 2.1 x 150, 5um

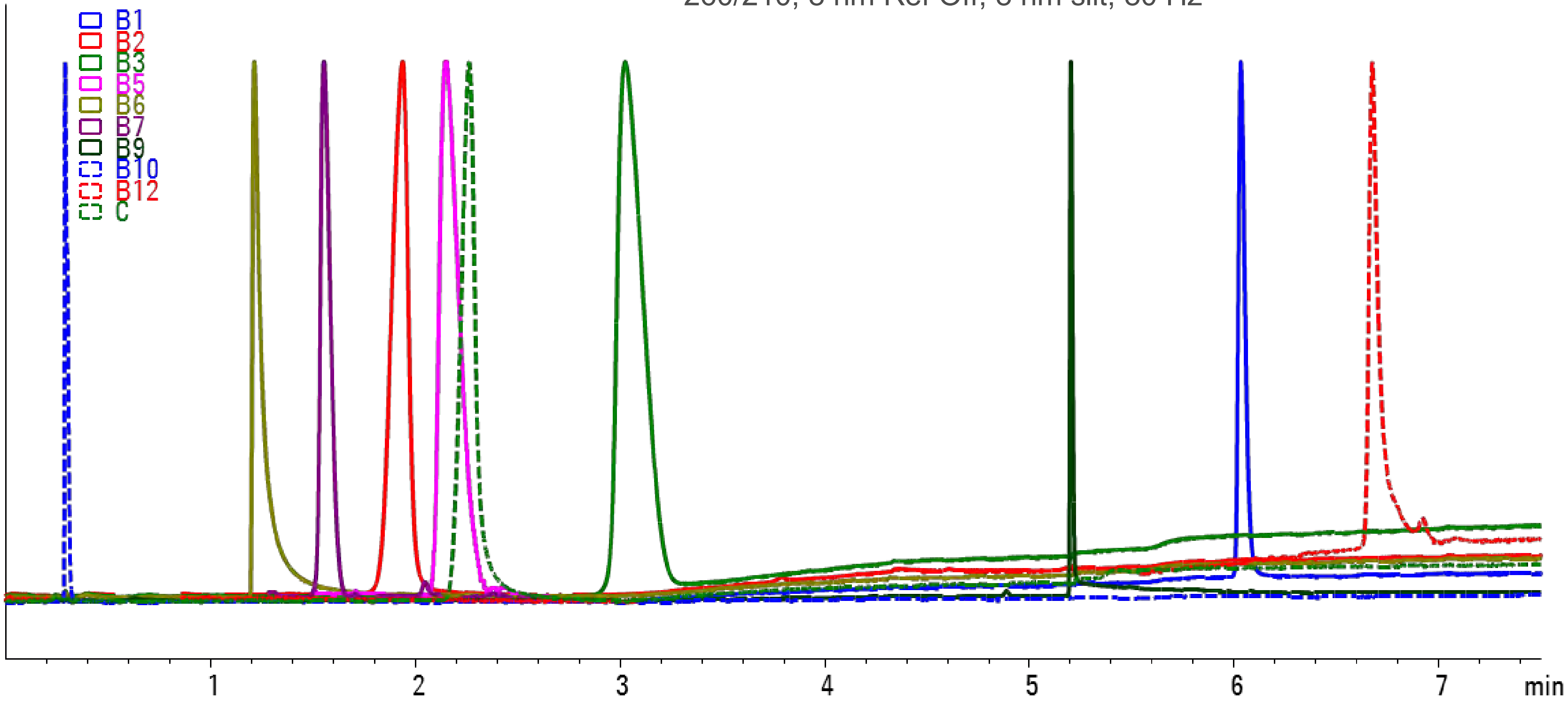
A: 25mM ammonium acetate with 2.5 mM ammonium formate
B: acetonitrile
10:90 A:B
Flow : 0.1 mL/min
Temp: 25 °C
Detection: UV 254nm,4, ref=360,100
Inj. Vol 0.5 µL
Instrument Quat 1200
Samples: ca. 0.4 mg/ml each in ACN/water

1. thymine
2. Uracil
3. adenine
4. guanine
5. cytosine

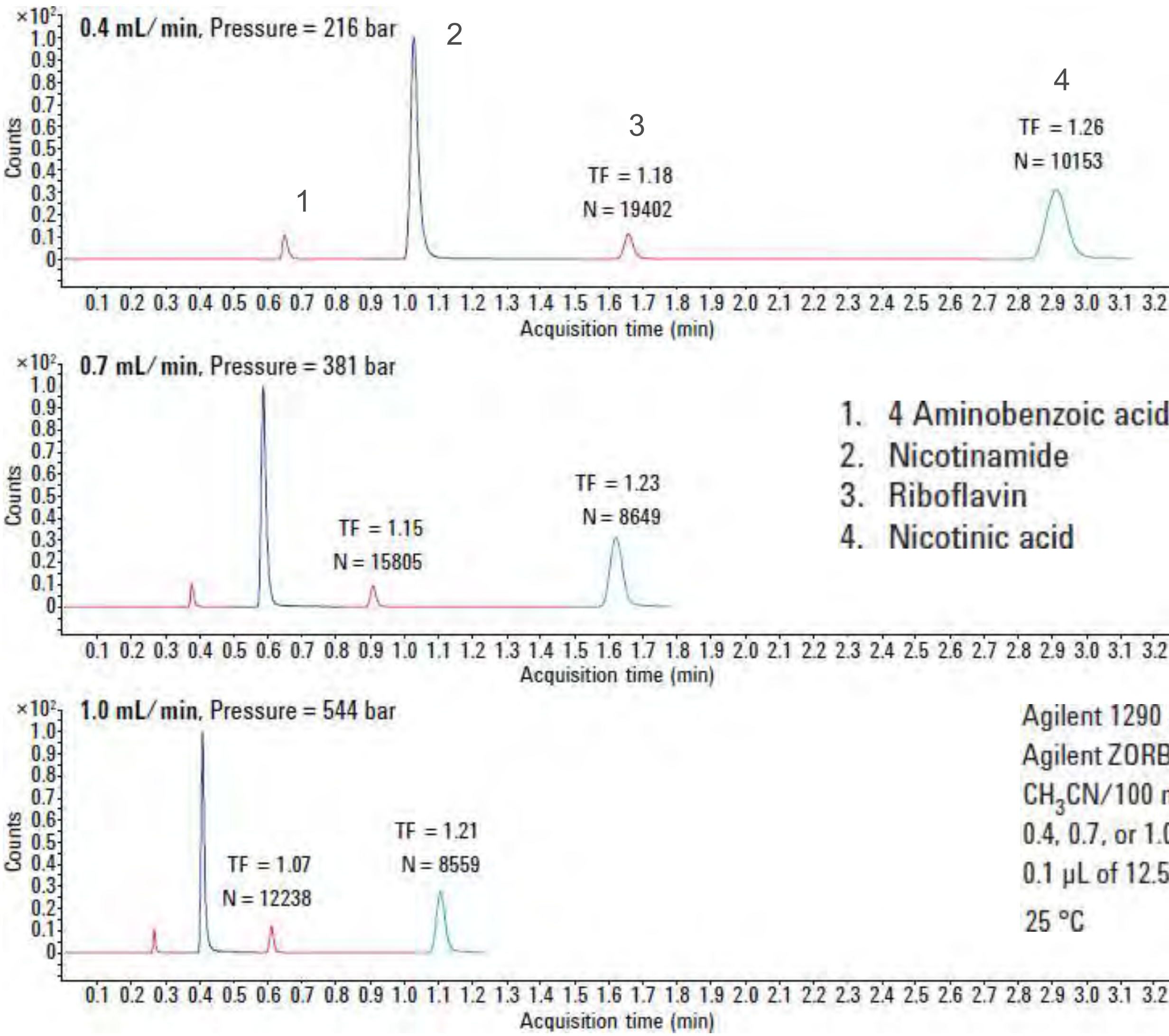


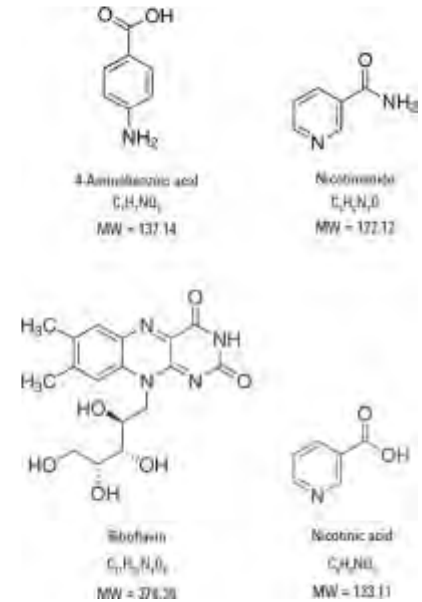
Water Soluble Vitamins
HILIC

Poroshell 120 HILIC
A: 100 mM Ammonium Formate in H2O pH 3.0 with Formic Acid
B: CH3CN
0.5 mL/min, 97% B for 2.5 minutes, then 97-60%D in 5 minutes
injection volume: varies according to signal strength
TCC: 30 C
260/210, 8 nm Ref Off, 8 nm slit, 80 Hz



HILIC Separation





Agilent 1290 Infinity LC System with an Agilent 6410A ESI+

Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm

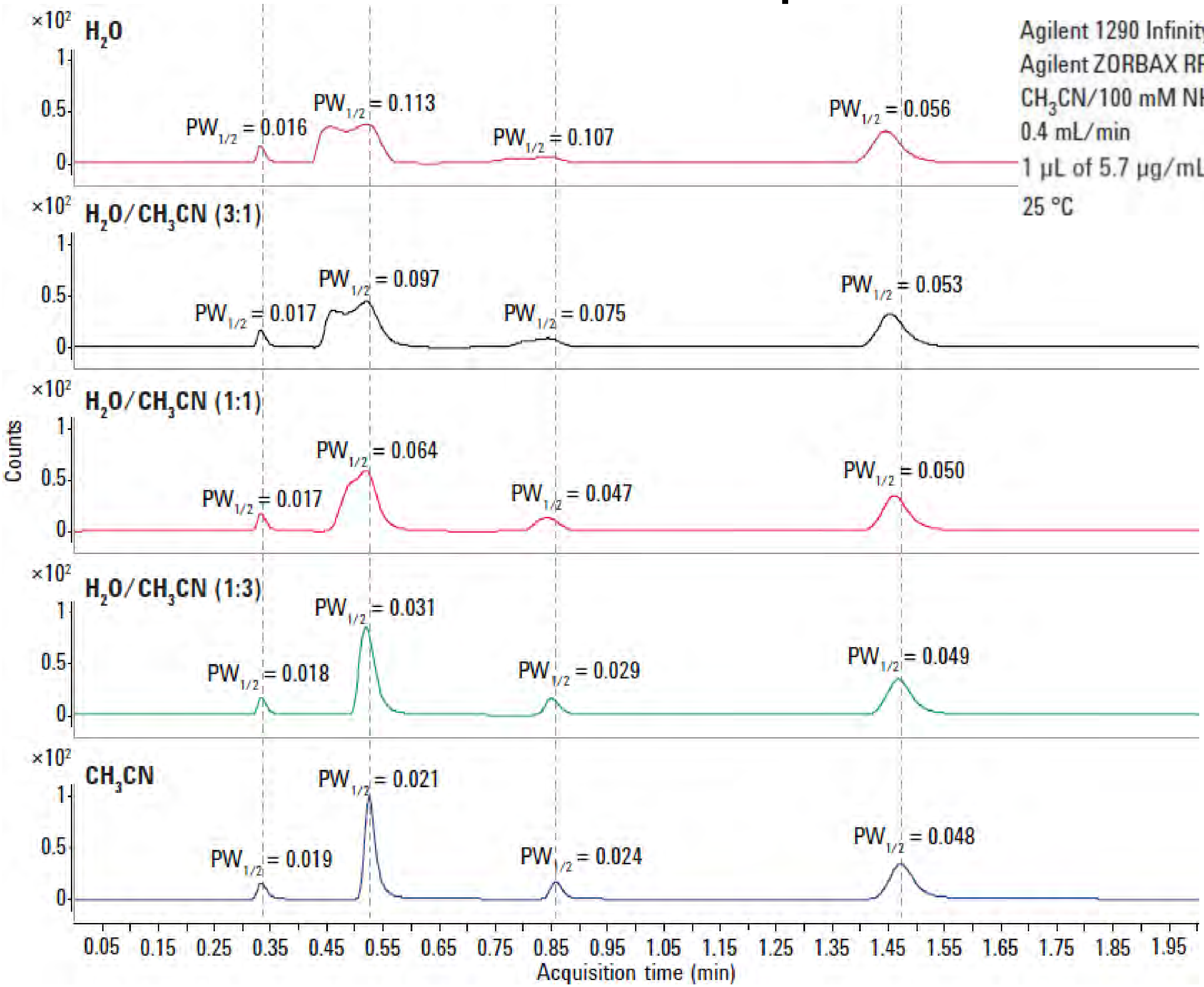
CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)

0.4, 0.7, or 1.0 mL/min

0.1 µL of 12.5 µg/mL each in CH_3CN

25 °C

HILIC and Choice of Sample Solvent



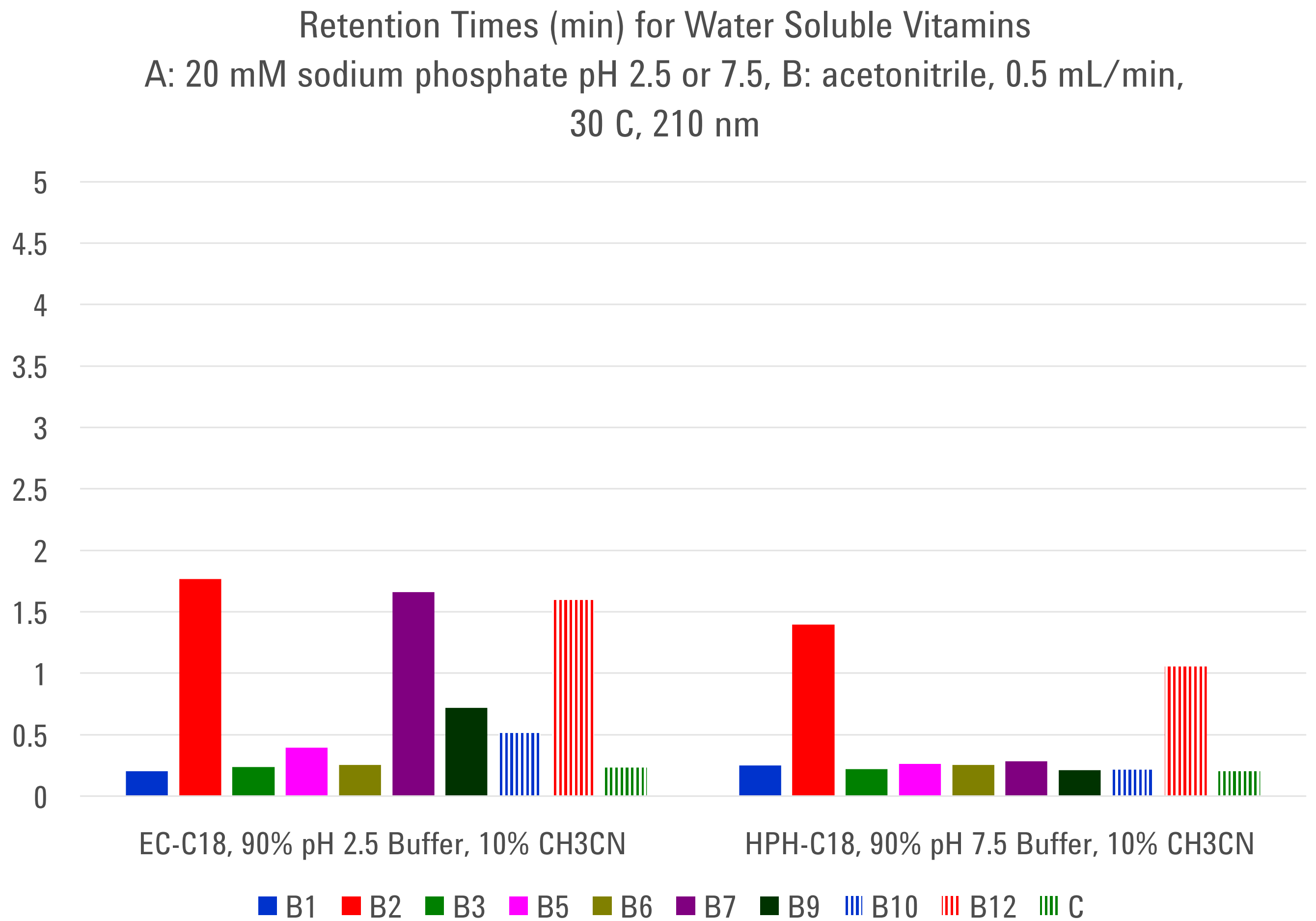
Agilent 1290 Infinity LC System with an Agilent 6410A ESI+
Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm
CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
0.4 mL/min
1 µL of 5.7 µg/mL each
25 °C

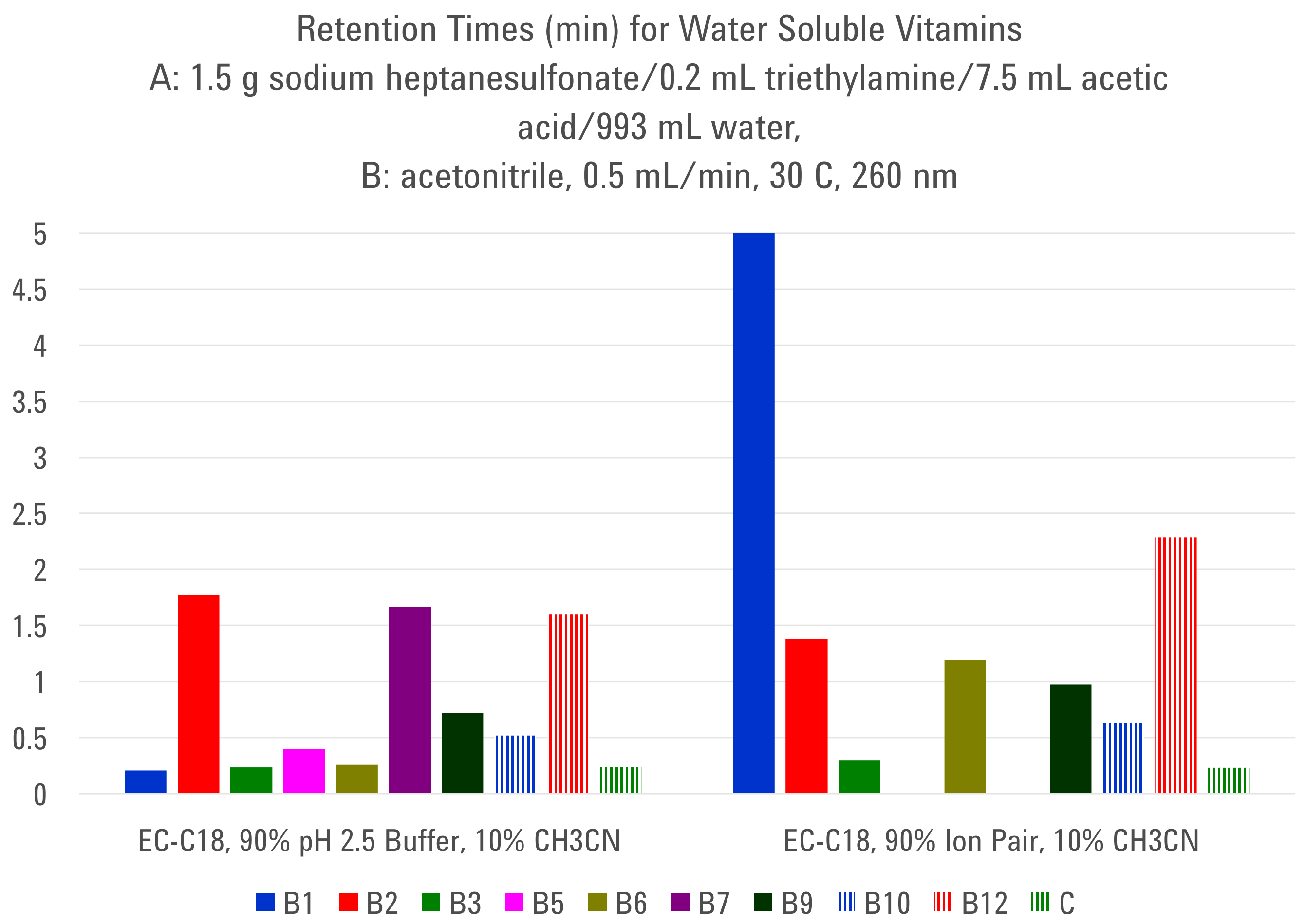
Summary

- What do you do when your analyte is too polar?
- Stick with reversed-phase but
  - Adjust pH of mobile phase
  - Phenyl-Hexyl, Bonus-RP, SB-Aq, EC-CN
- Consider HILIC
- Ion-pair chromatography

Additional slides

Water Soluble Vitamins
C18 at pH 2.5 vs 7.5




Retention Times (min) for Water Soluble Vitamins
A: 1.5 g sodium heptanesulfonate/0.2 mL triethylamine/7.5 mL acetic acid/993 mL water,
B: acetonitrile, 0.5 mL/min, 30 C, 260 nm
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
EC-C18, 90% pH 2.5 Buffer, 10% CH3CN
EC-C18, 90% Ion Pair, 10% CH3CN
B1 B2 B3 B5 B6 B7 B9 B10 B12 C

Ion-Pair Parameters

- IP reagent
  - Longer alkyl chain--more readily adsorbed by stationary phase
  - Choose alkyl length which gives best separation (more retention of amines with octanesulfonate than hexanesulfonate)
  - Select cationic ion-pairing reagent for anions (*e.g*., acids)
  - Select anionic ion-pairing reagent for cations (*e.g*., amines)
  - Not both together
- IP Concentration
  - Increase retention with increasing IP concentration
  - Increase concentration with %B - non-linear adsorption
- pH
- Buffer concentration
- Choice of organic modifier
- %B
- Temperature