Too Polar for Reversed Phase – What Do You Do?

June 20, 2013

Mark Powell

Columns and Consumables

Technical Support

C8 or C18 Doesn't Always Do the Job

- Typical reversed phase conditions involve water/buffer with 5 to 100% organic
- Some very polar acids and bases not well retained
- Increase retention with less organic
- Performance of C18 columns under highly aqueous conditions not always robust

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).

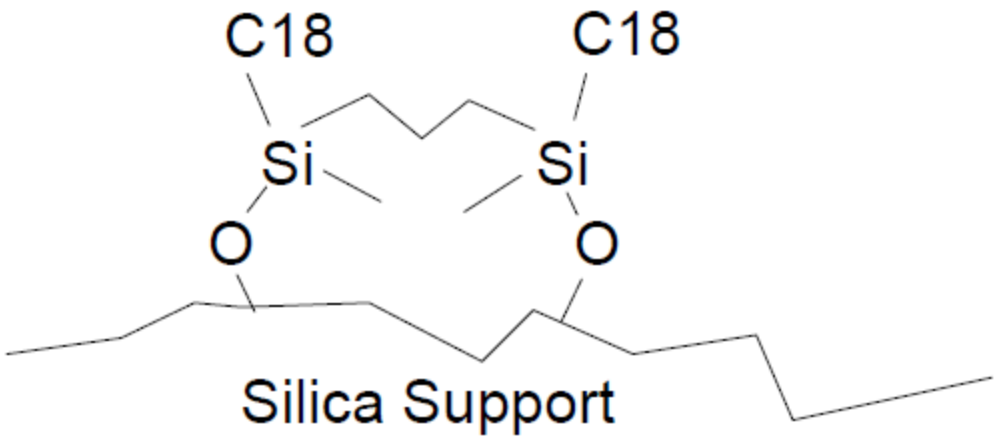
What Do You Do?

- Adjust method conditions
- Ion-pair chromatography
- Alternate column choice (polar modified)
- Normal phase
- HILIC
- Ligand exchange

Adjust Method Conditions

- Reversed-phase methods often use low pH
- Basic compounds will be charged
- Adjusting pH up (>8) will generally increase retention
- High pH can damage many silica based columns
- Choose ZORBAX Extend-C18 (to 11.5) or PLRP-S (to 14)

Extend-C18



- Bidentate structure
- Double endcapped
- pH 2 – 11.5 (at 40 °C)

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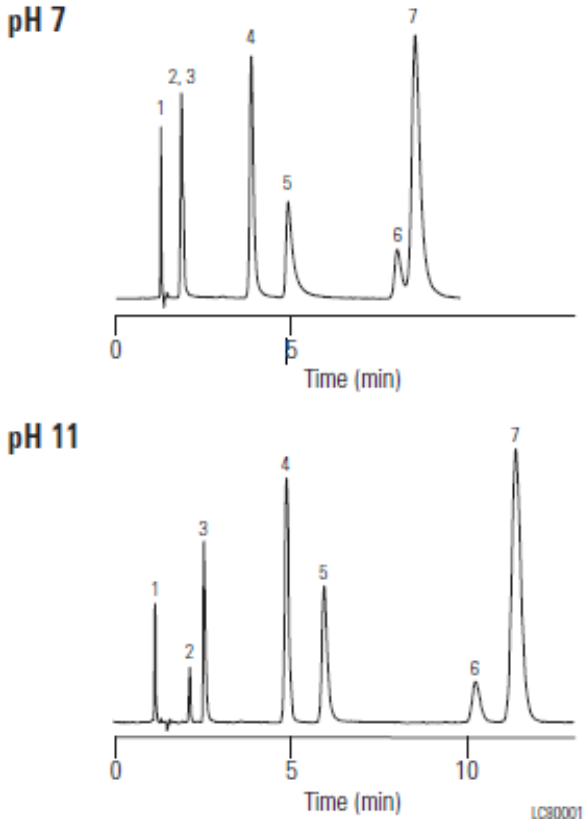
Basic Antihistamines at High pH

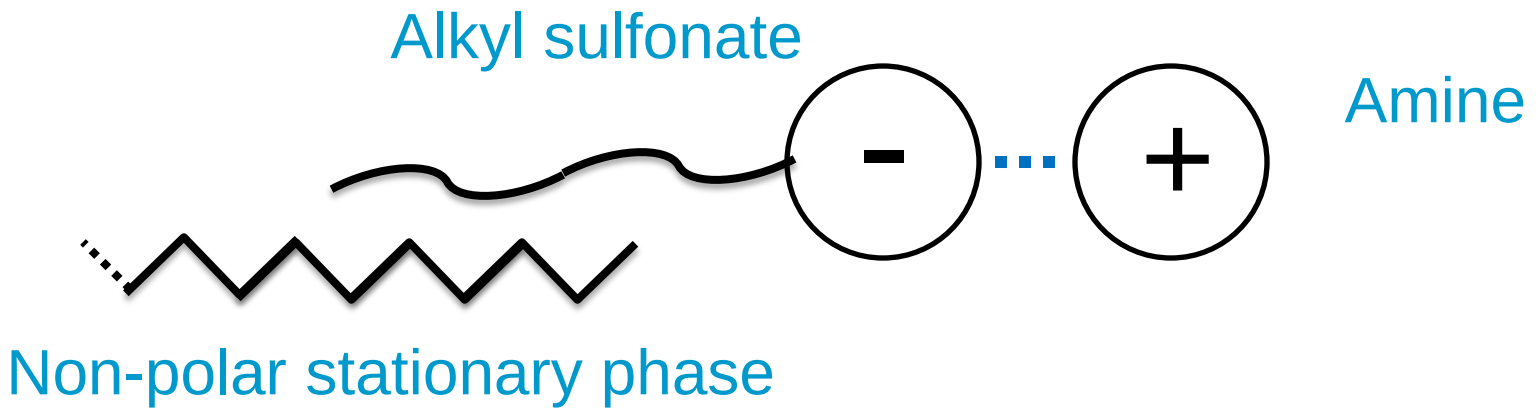
Column:	**ZORBAX Extend-C18** **773450-902** **4.6 x 150 mm, 5 µm**
Mobile Phase:	pH 7: 30% 20 mM Na_2HPO_4 70% MeOH pH 11: 30% 20 mM TEA 70% MeOH
Flow Rate:	1.0 mL/min
Temperature:	Ambient
Detector:	254 nm
Sample:	Antihistamines



1. Maleate
2. Scopolamine
3. Pseudoephedrine
4. Doxylamine
5. Chlorpheniramine
6. Triprolidine
7. Diphenhydramine

Ion-Pair Chromatography

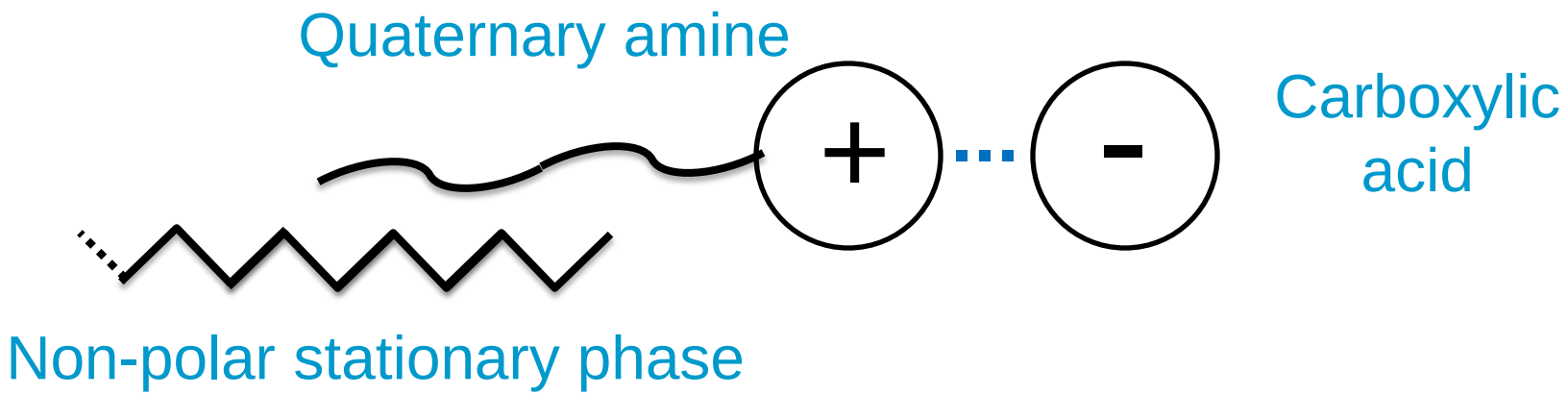
Similar to reversed-phase, but an ion-pairing reagent is added to the mobile phase



- *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

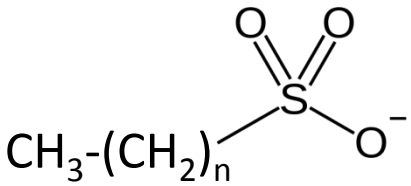
Similar to reversed-phase, but an ion-pairing reagent is added to the mobile phase



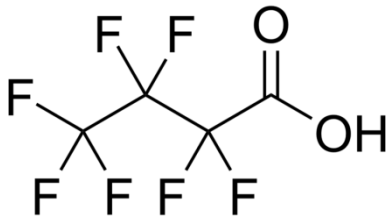
- *Non-polar alkyl chain will adsorb into the non-polar stationary phase*
- *Polar part of the ion-pairing reagent will "stick-out" into the mobile phase*

Some Common Ion-Pairing Reagents

Pairs with Cations

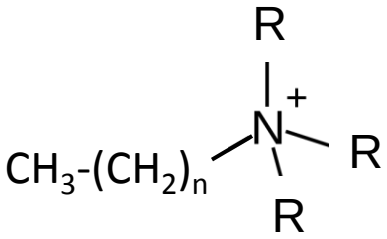


Alkyl sulfonates



Heptafluorobutyric acid (HFBA)

Pairs with Anions



Quaternary amines

Ion-Pair 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

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

Cations – bases

Buffer: 25 – 50 mM phosphate, pH 2- 3

IP reagent: 10-100 mM hexane sulfonate

Anions – acids

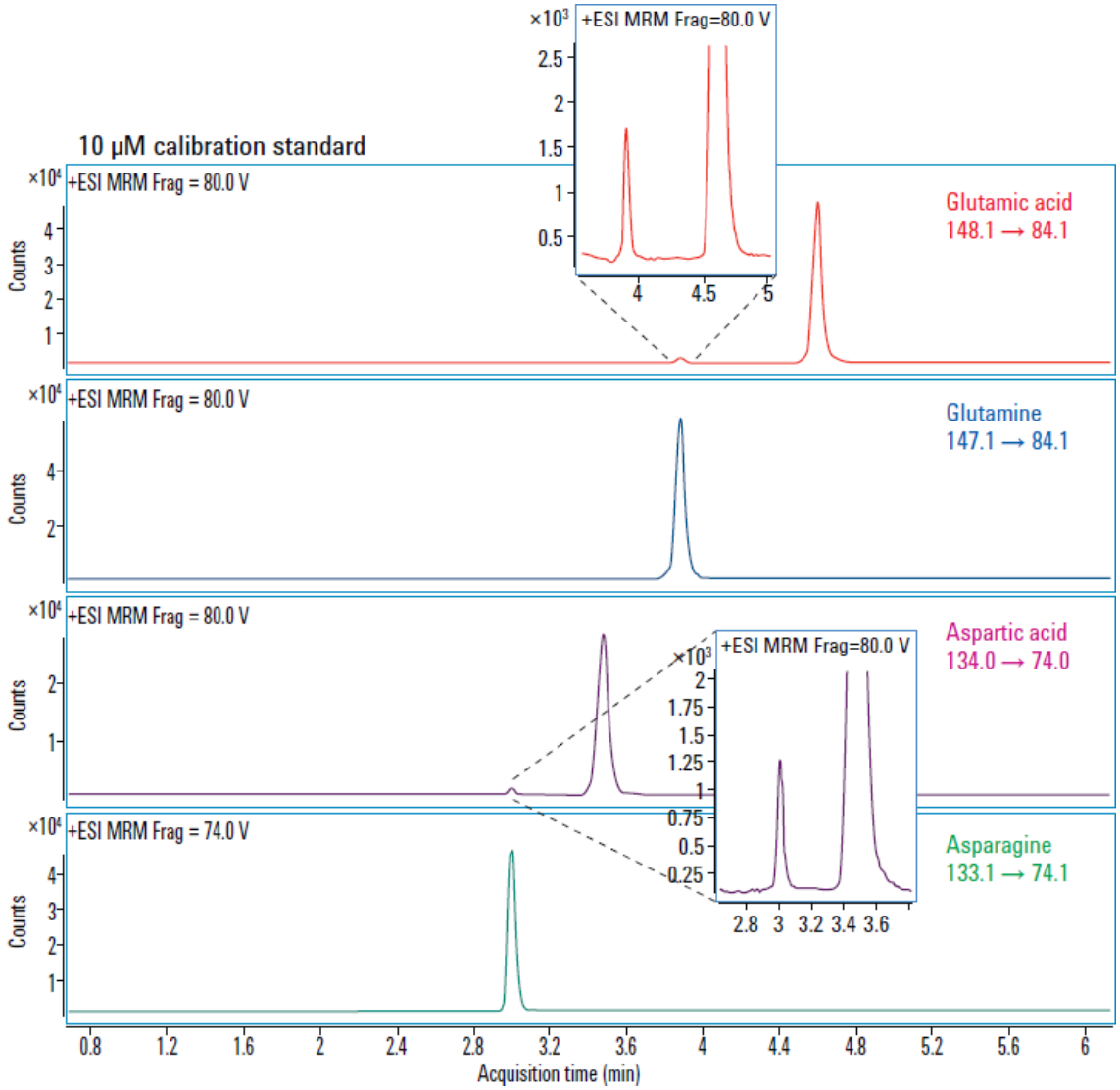
Buffer: 25 – 50 mM phosphate, pH 6 – 7

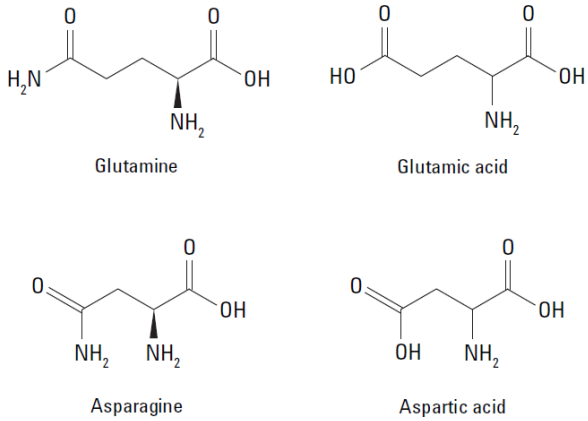
IP reagent: 10-40 mM tetrabutyl ammonium phosphate

Ion-Pair Chromatography Issues

- 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

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

Alternative Column Choices

- Some column phases resist “dewetting” for use at low organic or 100% aqueous conditions
- Options include:
  - Phenyl or Phenyl-Hexyl
  - Bonus-RP (polar amide embedded)
  - SB-Aq

ZORBAX Method Development Kits

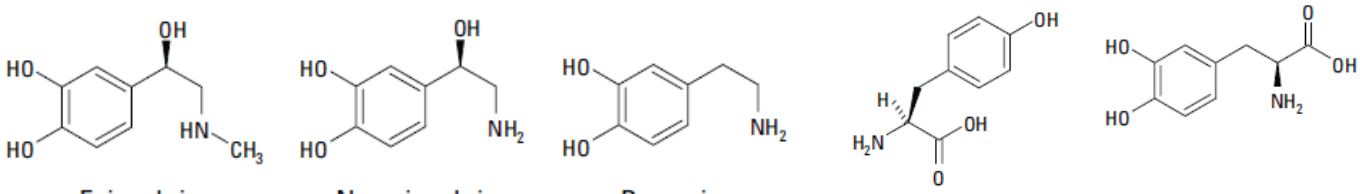
ZORBAX Method Development Kit Information

Kits (SAP Description)	Description	Dimension	Part No.
ZORBAX RRHD pH Method Development Kit	One of each: SB-C18, Eclipse Plus C18, and Extend-C18	2.1 x 50 mm	5190-6152
ZORBAX RRHD Eclipse Plus Method Development Kit	One of each: Eclipse Plus C18, Eclipse Plus C8, Eclipse Plus Phenyl-Hexyl	2.1 x 50 mm	5190-6153
ZORBAX RRHD Aqueous Method Development Kit	One of each: SB-Aq, Bonus RP, Eclipse Plus Phenyl-Hexyl	2.1 x 50 mm	5190-6154
Poroshell 120 Selectivity Method Development Kit	One of each: EC-C18, Phenyl-Hexyl, Bonus-RP	2.1 x 50 mm	5190-6155
Poroshell 120 Selectivity Method Development Kit	One of each: EC-C18, Phenyl-Hexyl, Bonus-RP	4.6 x 50 mm	5190-6156
Poroshell 120 Aqueous Method Development Kit	One of each: SB-Aq, Phenyl-Hexyl, Bonus-RP	2.1 x 50 mm	5190-6157
Poroshell 120 Aqueous Method Development Kit	One of each: SB-Aq, Phenyl-Hexyl, Bonus RP	4.6 x 50 mm	5190-6158
Poroshell 120 L1, L7, and L10 USP Method Development Kit	One of each: EC-C18, EC-C8, EC-CN	4.6 x 100 mm	5190-6159
Poroshell 120 L1, L7, and L10 USP Method Development Kit	One of each: EC-C18, EC-C8, EC-CN	3.0 x 100 mm	5190-6160

Phenyl Phases

- ZORBAX options include:
  - Eclipse Plus Phenyl-Hexyl
  - Eclipse XDB-Phenyl
  - StableBond SB-Phenyl
  - Poroshell 120 Phenyl-Hexyl
- Slight selectivity differences
- Choice of mobile phase important

Catecholamines on Phenyl Phases



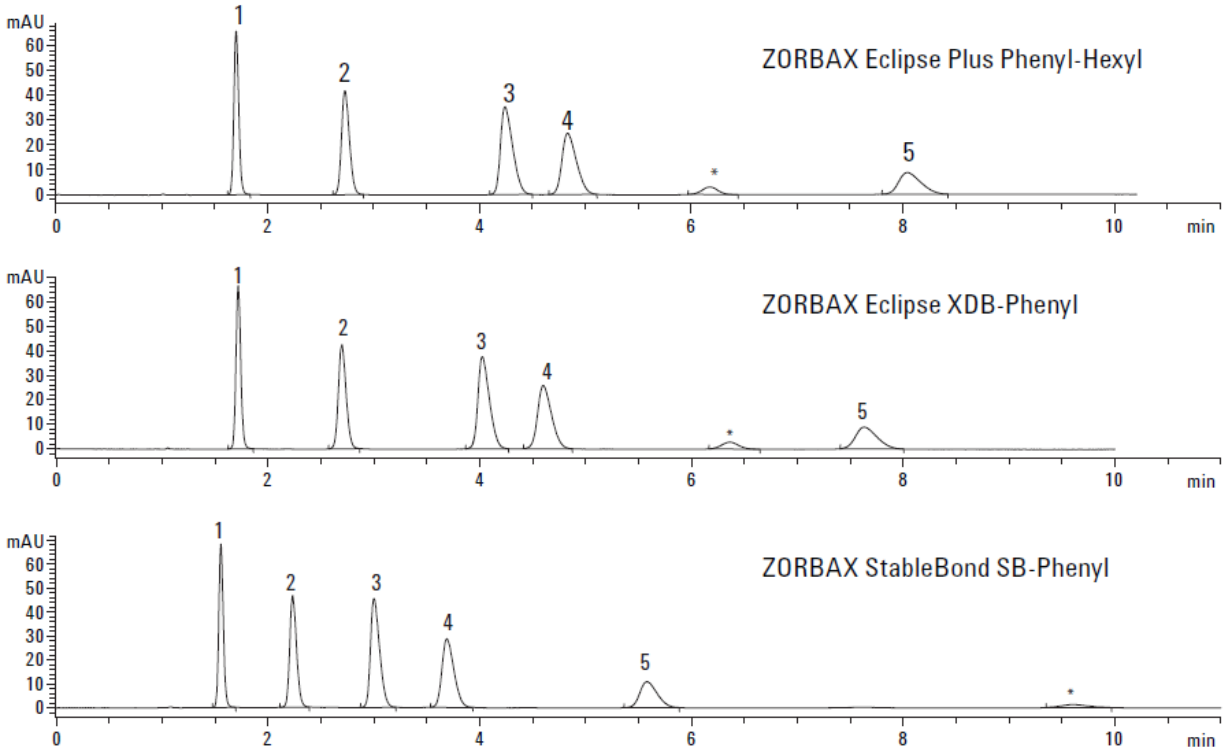
Epinephrine
pKa = 8.55

Norepinephrine
pKa = 8.4

Dopamine
pKa = 8.89

Tyrosine
pKa = 10.1

Levodopa
pKa = 8.72



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.

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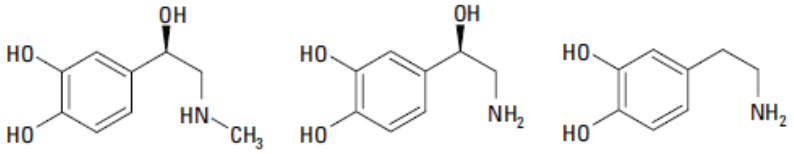
Catecholamines by Ion-Pair



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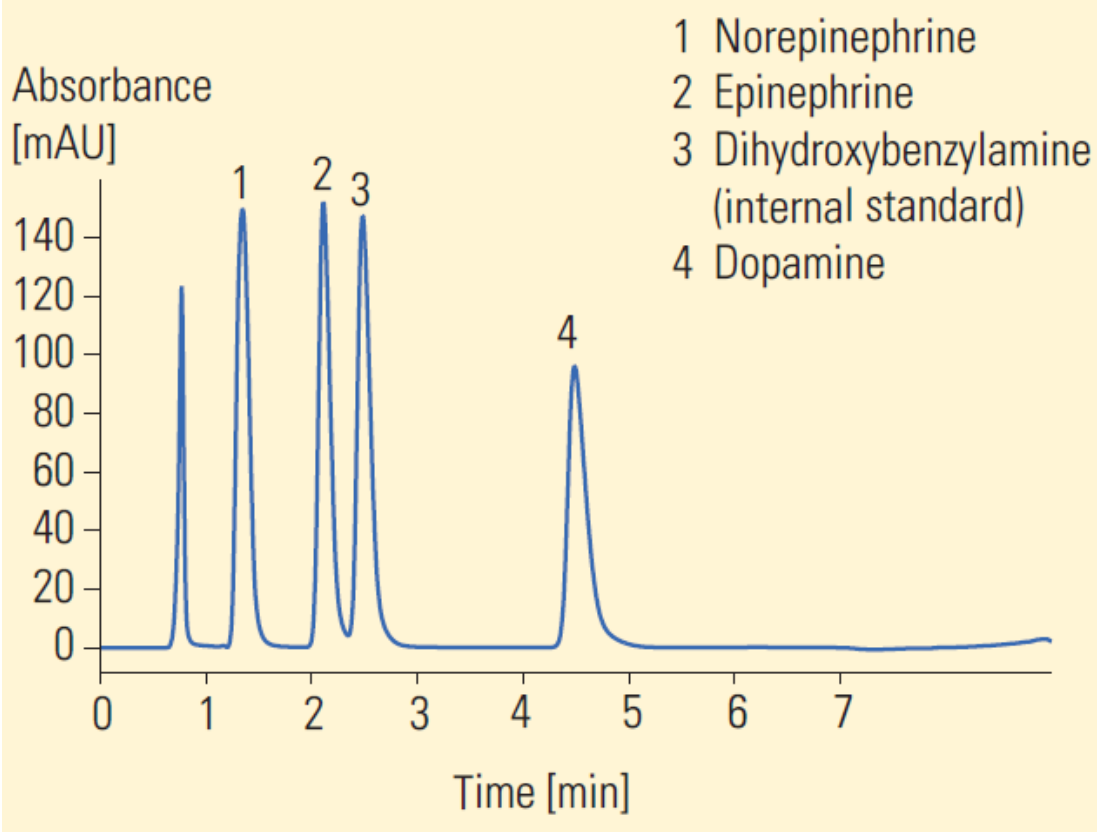

Column
4.6 x 75 mm Zorbax SB-C18 , 3.5 µm
Mobile phase
A = 0.025 M KH_2PO_4 + 0.3 mM heptanesulfonic acid in water (pH = 3), B = acetonitrile
Flow rate
1.0 ml/min
Gradient
at 0 min 1 % B
at 5 min 2 % B
at 7 min 15 % B
Column wash
at 8 min 1 % B
UV detector
variable wavelength detector
204 nm, standard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

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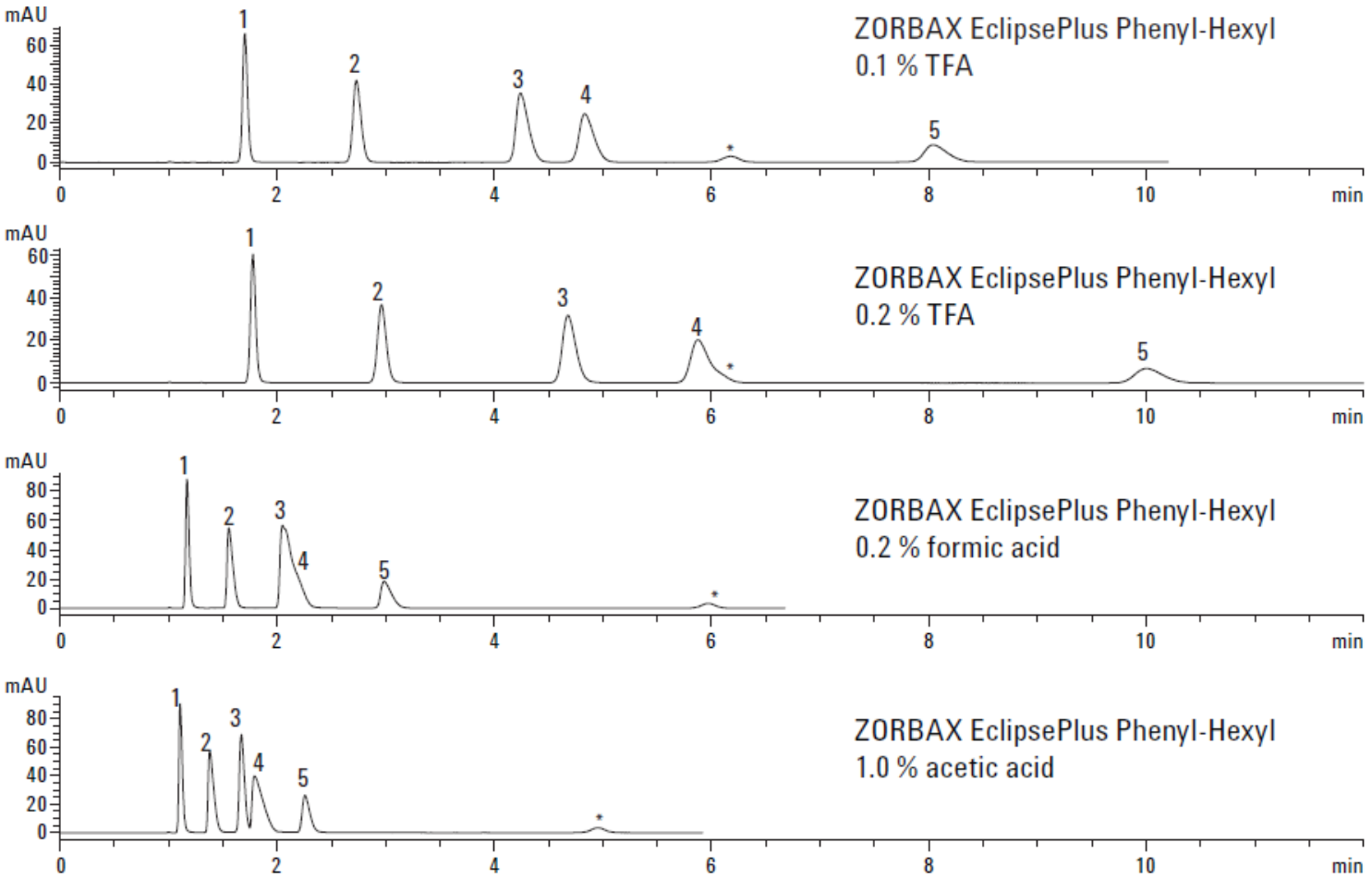
Catecholamines on Phenyl Phases



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.

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Aliphatic Acids

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

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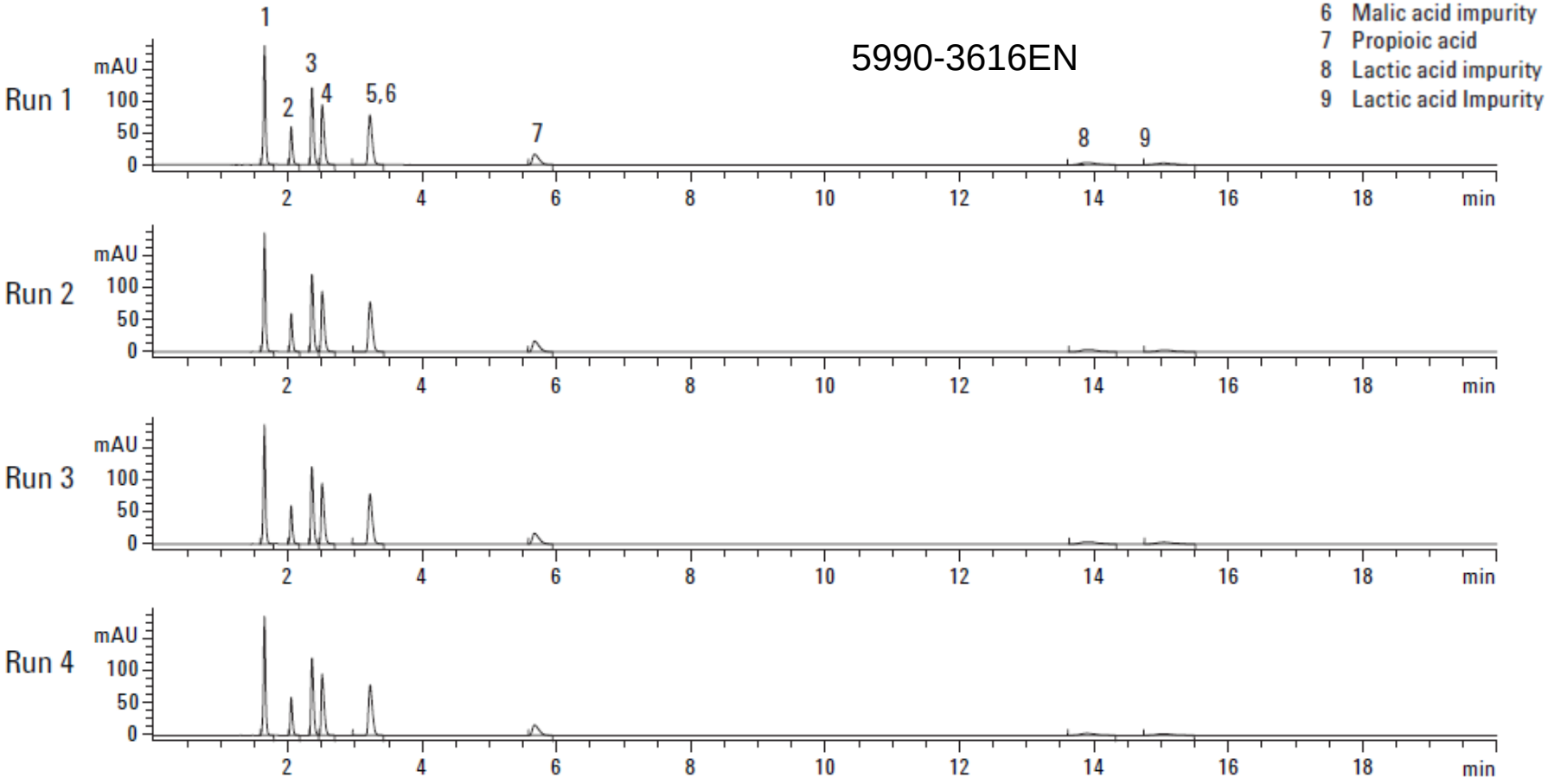
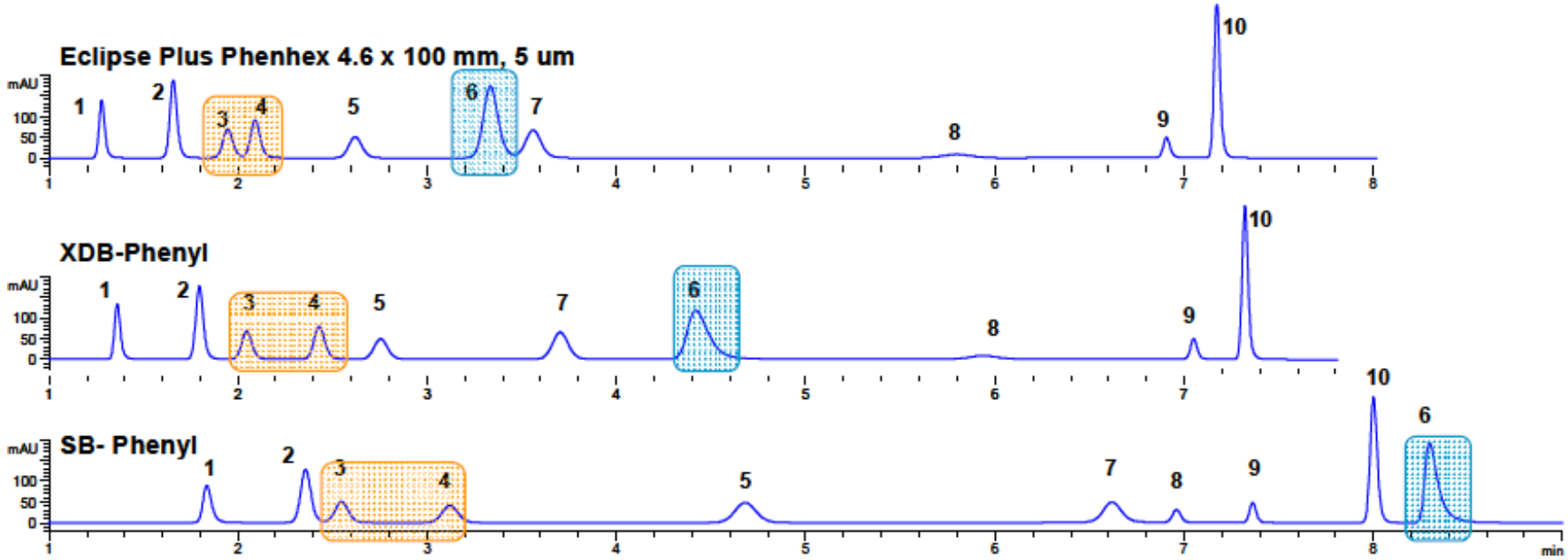


1 and 2) Run successively with no pause. 3) Pump stopped 30 minutes and restarted. 4) Pump stopped 30 minutes and restarted ZORBAX Eclipse Plus Phenyl-Hexyl 4.6 mm × 150 mm 3.5 micron, p/n 959961–912.

Nucleobases and Nucleosides

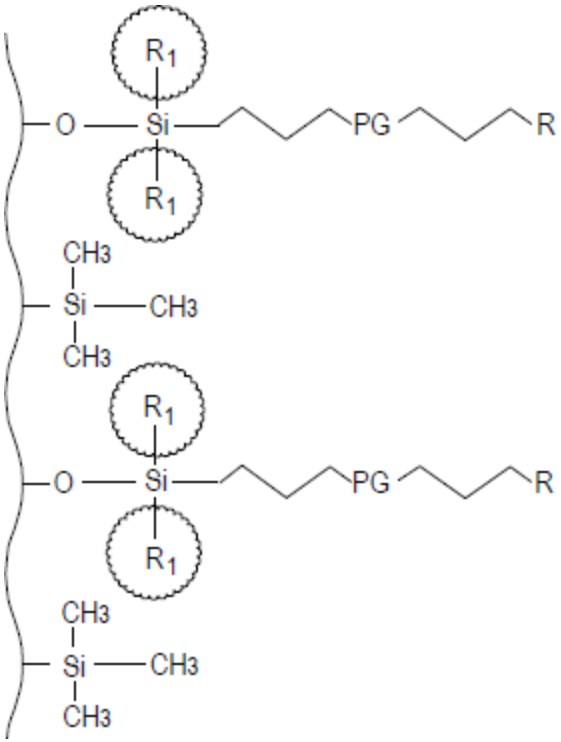


1. Cytosine
2. Uracil
3. Cytidine
4. Guanine
5. Uridine
6. Adenine
7. Thymine
8. Guanosine
9. Thymidine
10. Adenosine

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

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

ZORBAX Bonus-RP Phase



- Alkyl phase
- Polar group embedded
- Triple Endcapped
- pH 2 – 9

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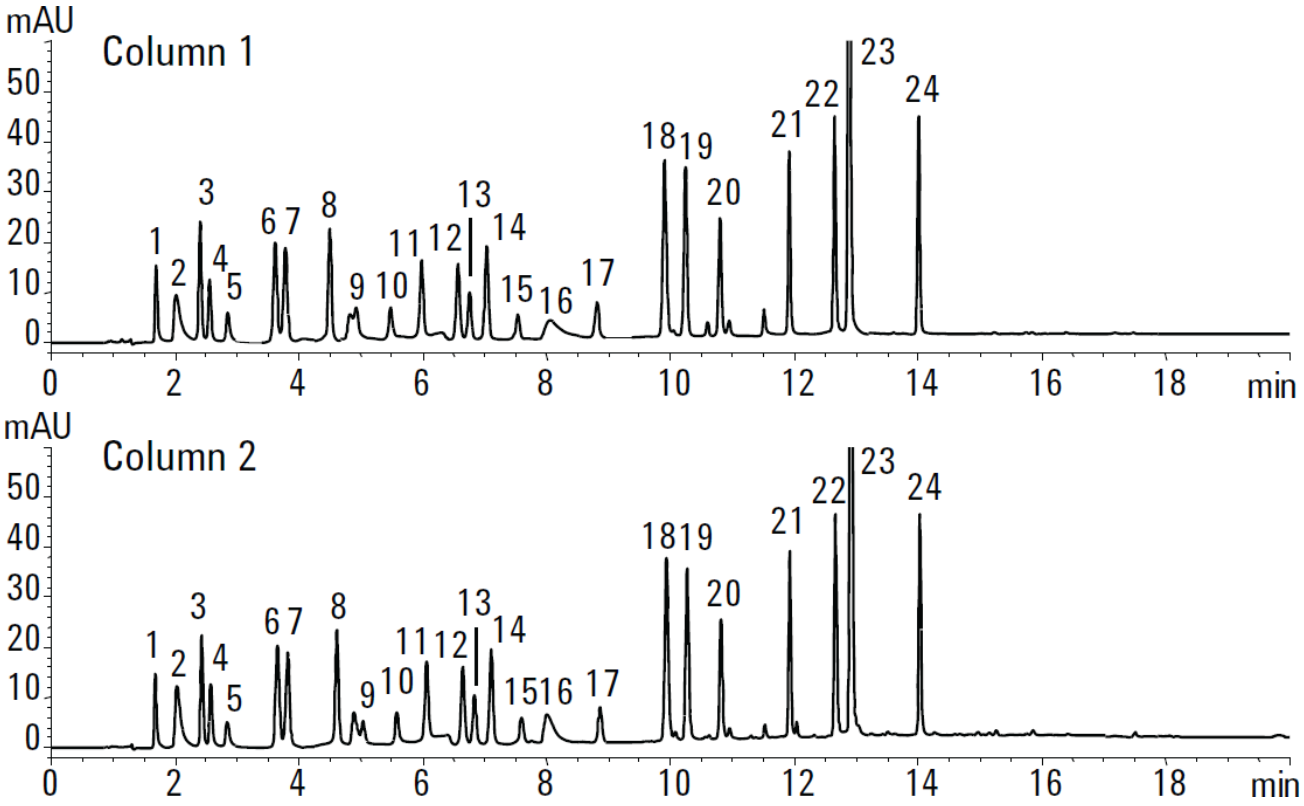
Hair Dye Analysis on Bonus-RP



Instrument:	Agilent 1290 Infinity LC System(installed with 1290 inline filter after injector valve, p/n 5067-4638)			
Column:	Agilent Poroshell 120 Bonus-RP, 3.0 × 100 mm, 2.7 µm			
Mobile phase:	A, 10 mM acetate; B, ACN			
Gradient:	0 min	0% B	Stop time:	20 min, post run 3 min
	2 min	0% B	Flow rate:	0.5 mL/min
	8 min	20% B	Injection:	1 µL
	15 min	70% B	Detector:	UV 280 nm
	18 min	80% B		

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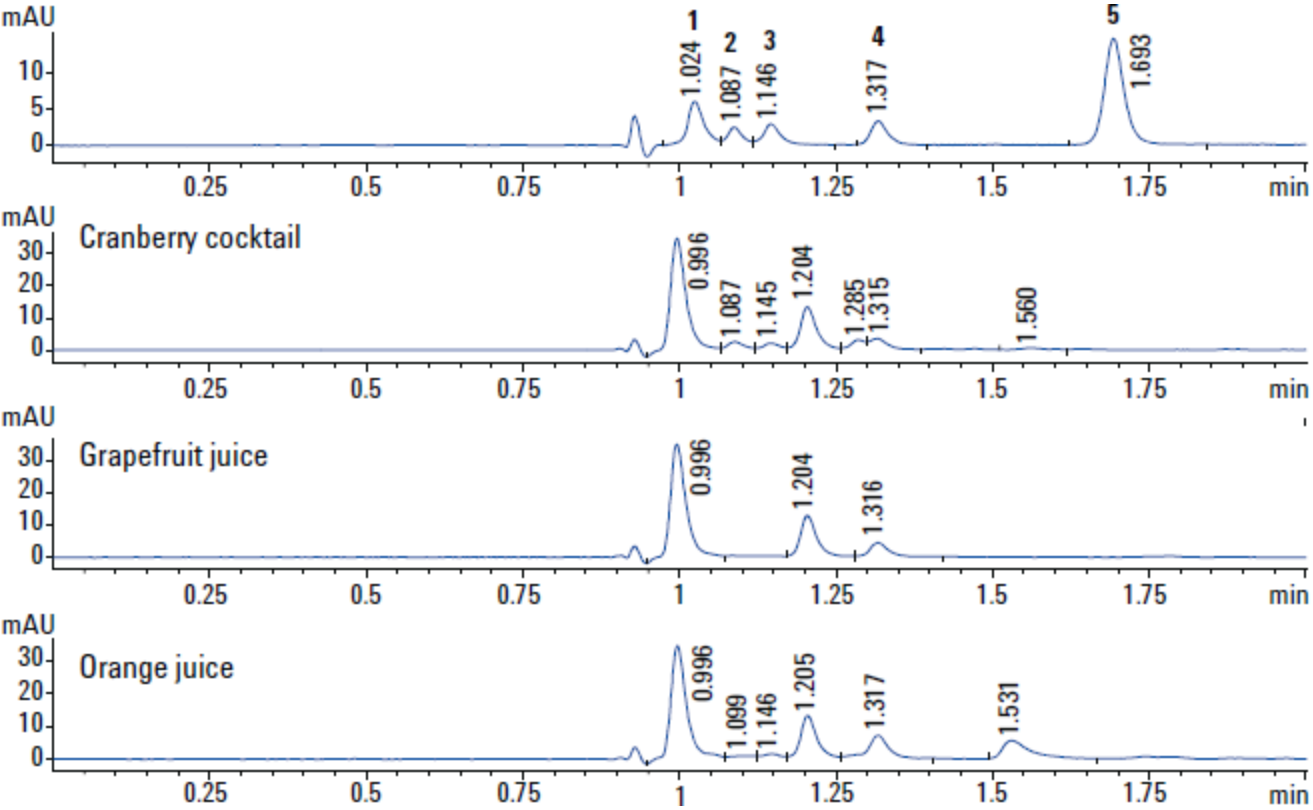
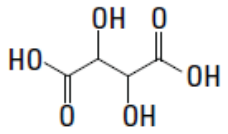
1. p-Phenylenediamine
2. 2-Amino-3-hydroxypyridine
3. m-Phenylenediamine
4. 4-Aminophenol
5. 2,5-Diaminotoluene sulfate
6. o-Phenylenediamine
7. 3-Aminophenol
8. Hydroquinone
9. 2-Chloro-1,4-phenylenediamine sulfate
10. 4-Methylaminophenol sulfate
11. Resorcine
12. 3,4-Diaminotoluene
13. 1,4-Diamino-2-nitrobenzene
14. 5-Amino-o-cresol
15. 2-Methylresorcinol
16. 6-Amino-m-cresol
17. 4-Nitro-o-phenylenediamine
18. 4-Amino-3-nitrophenol
19. 6-Hydroxyindole
20. 4-Chlororesorcinol
21. 2,7-Dihydroxynaphthalene
22. 1,5-Dihydroxy naphthalene
23. 4-Aminodiphenylamine
24. 1-Naphthol

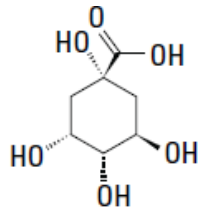
ZORBAX SB-Aq Phase

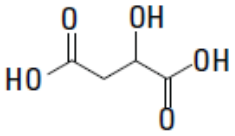


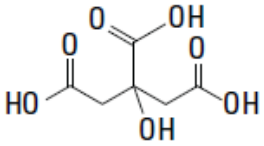
Peak ID

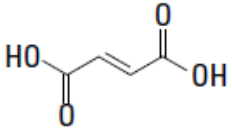
1. Tartaric acid
2. Quinic acid
3. Malic acid
4. Citric acid
5. Fumaric acid


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

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Normal Phase

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

- (Relatively) Non-polar mobile phase:
  - Typical solvent systems hexane/methylene chloride, hexane/ethyl acetate, methylene chloride/methanol, hexane/isopropanol, etc.

Normal Phase

- Very polar compounds will be well retained
- Reproducibility often an issue
- Important to control the amount of water in MP with silica column
- Slow equilibration of silica columns
- Tailing peaks
- Cyano phase – equilibration faster, gradients possible

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?

- Water layer must be adsorbed onto the stationary phase
- Polar analytes partition in and out of this adsorbed layer
- Charged polar analytes can also ion exchange with charged silica particles, *i.e.*, cation exchange with amines
- Combination of mechanisms drives retention in HILIC
- Retention/elution is from least to most polar – opposite of reversed-phase LC
- **HILIC offers more retention than reversed-phase for very polar bases**

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
  - Efficient spraying and desolvation in electrospray MS
  - As much as 3X sensitivity
- 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
- Mechanism not well understood

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, if necessary

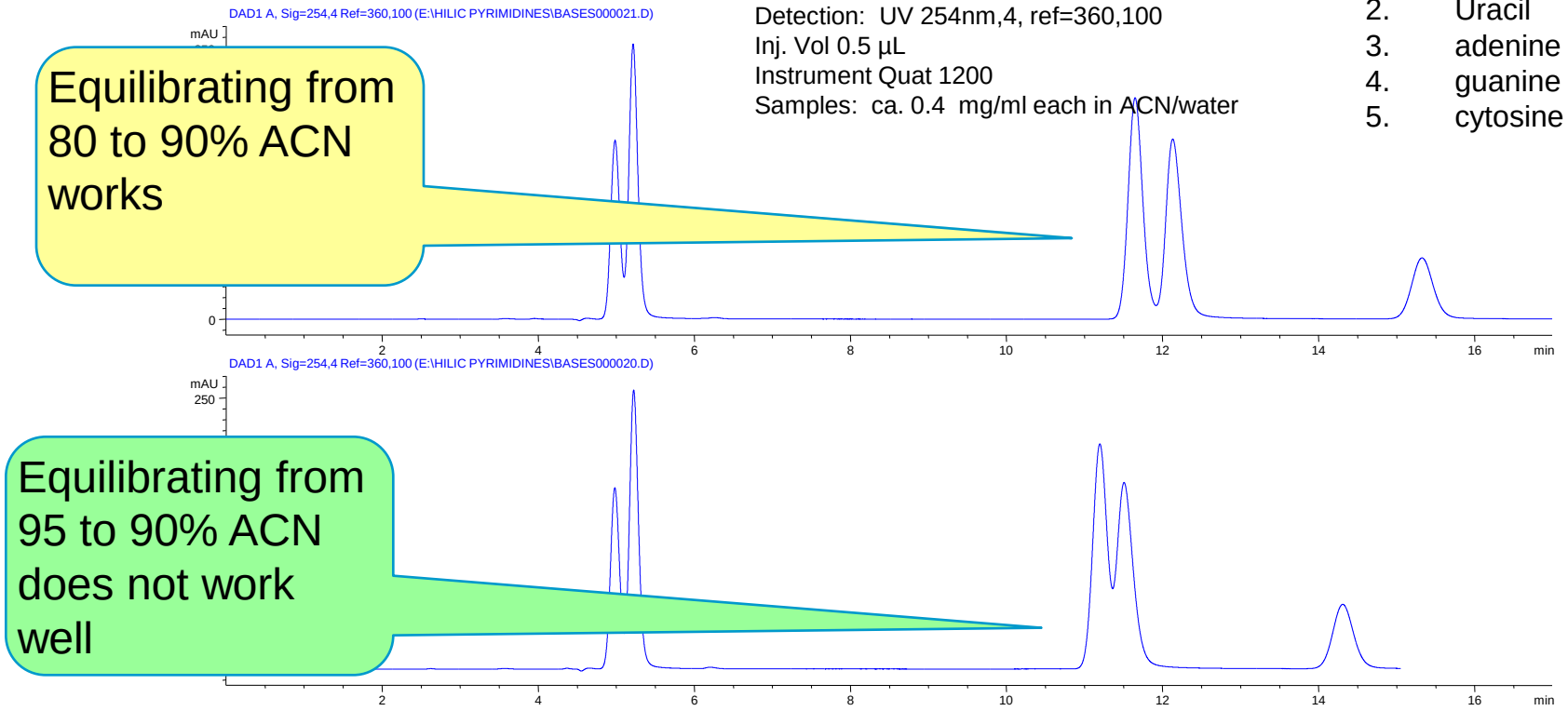
Equilibrate from high aqueous to low

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



Equilibrating from 80 to 90% ACN works

Equilibrating from 95 to 90% ACN does not work well

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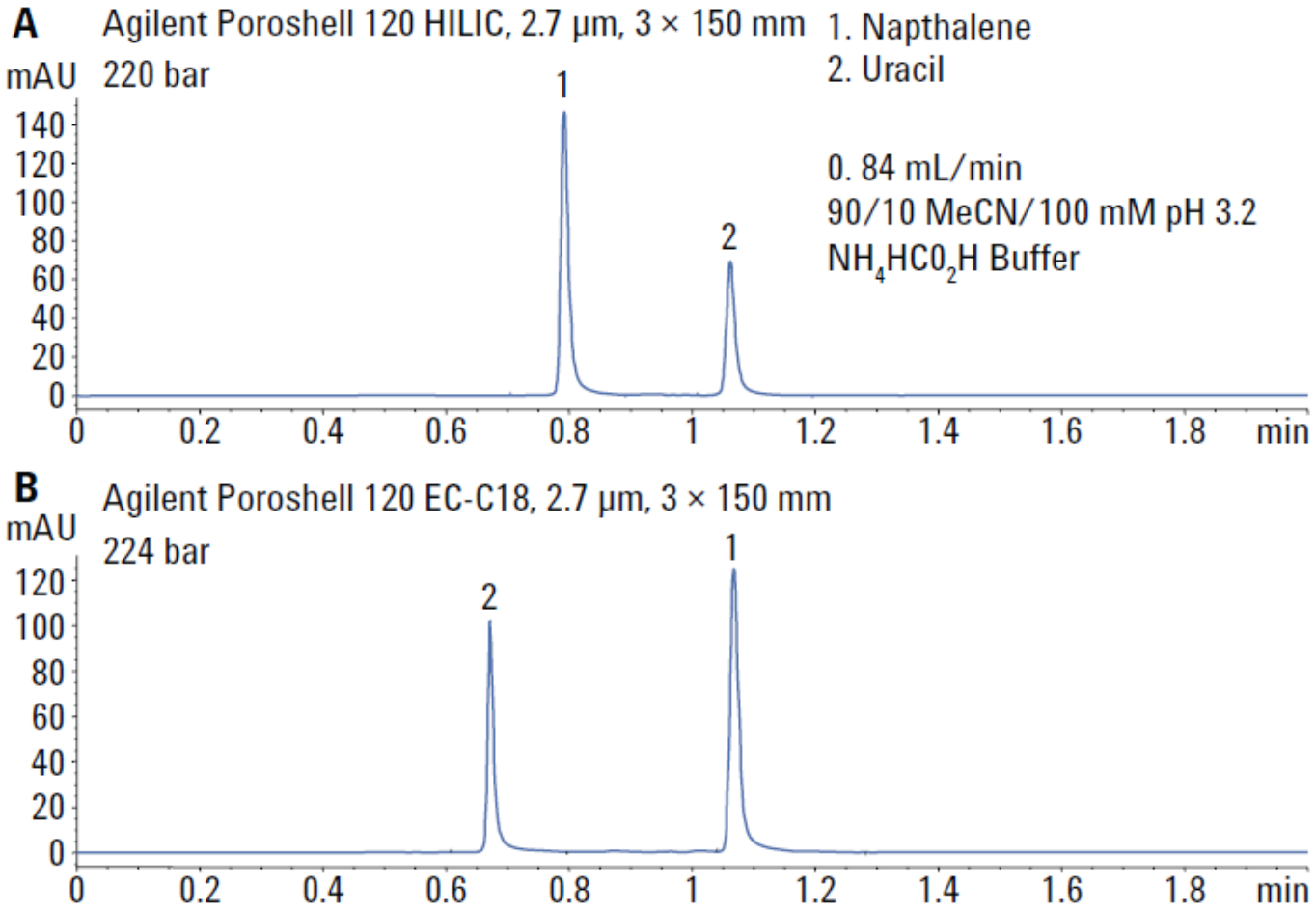
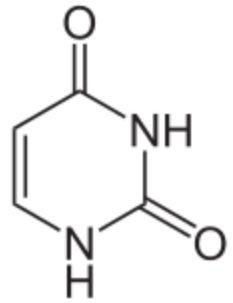
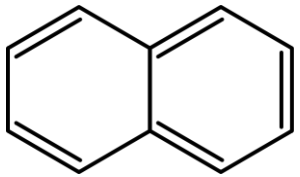
HILIC – comparison with C18

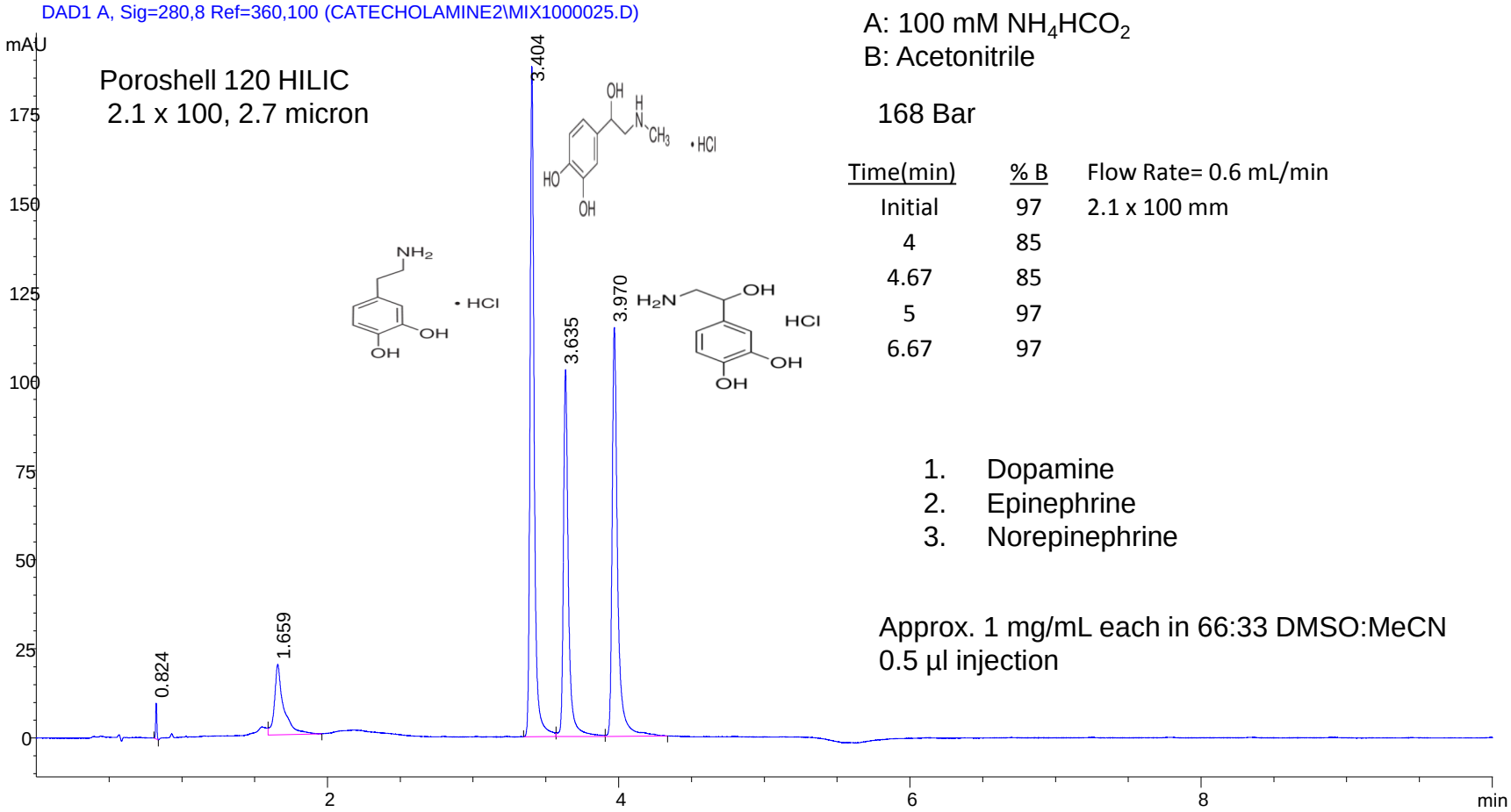






HILIC Separation of Catecholamines

Poroshell 120 2.1 x 100, 2.7 micron



A: 100 mM NH_4HCO_2
B: Acetonitrile

168 Bar

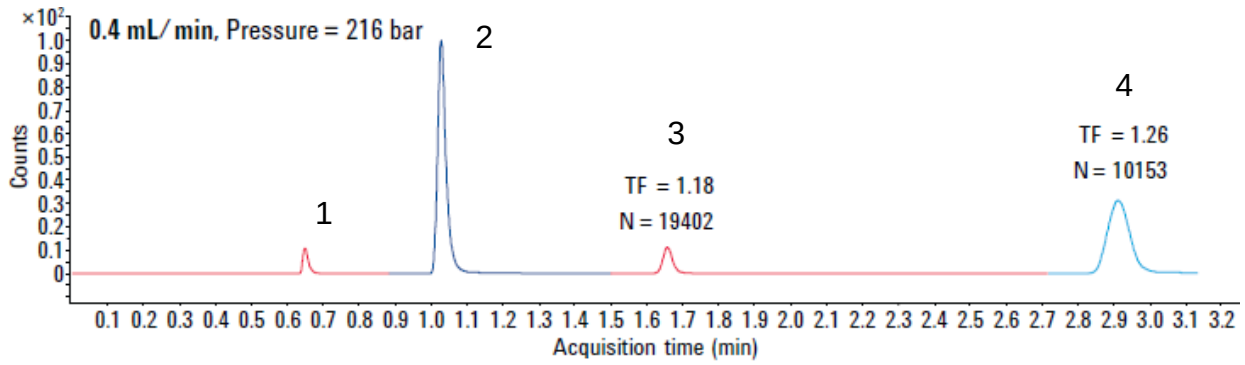
Time(min)	% B
Initial	97
4	85
4.67	85
5	97
6.67	97

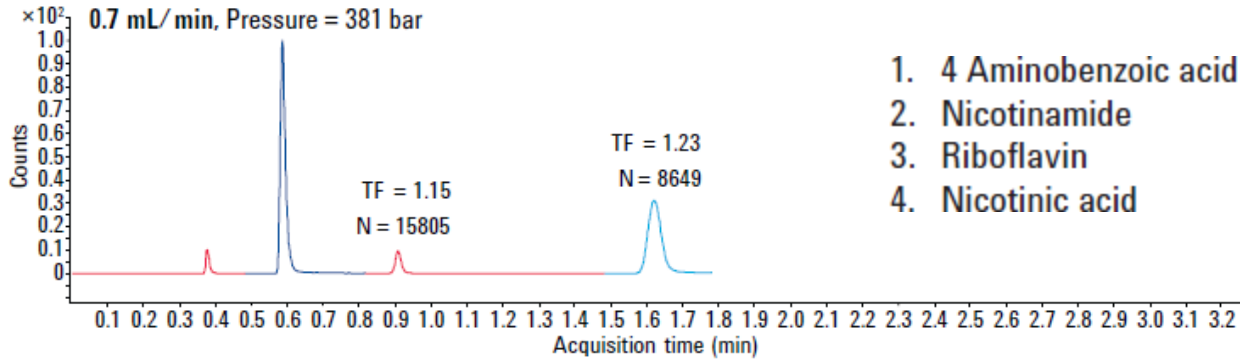
Flow Rate= 0.6 mL/min
2.1 x 100 mm

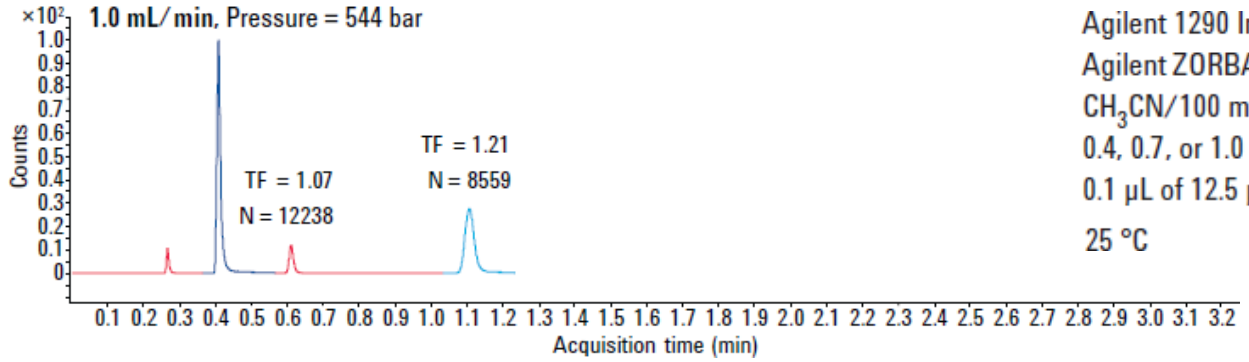
1. Dopamine
2. Epinephrine
3. Norepinephrine

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

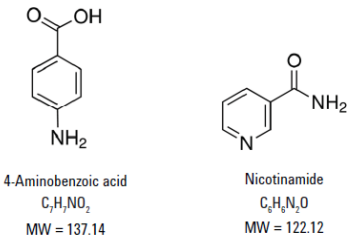
HILIC Separation of B Vitamins

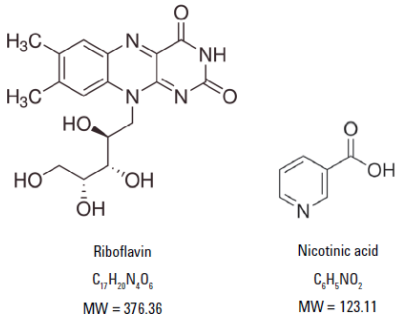






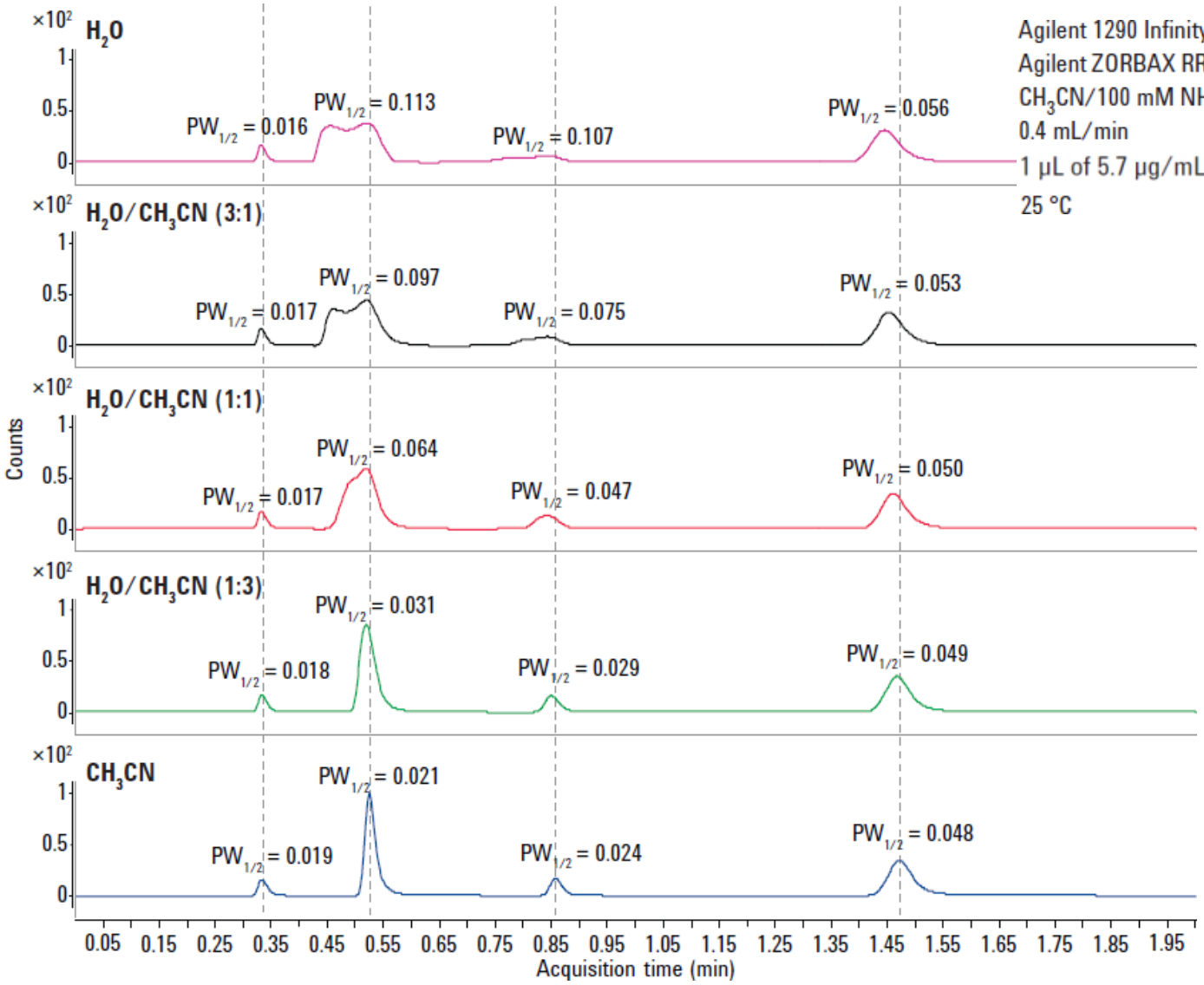
1. 4 Aminobenzoic acid
2. Nicotinamide
3. Riboflavin
4. Nicotinic acid





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

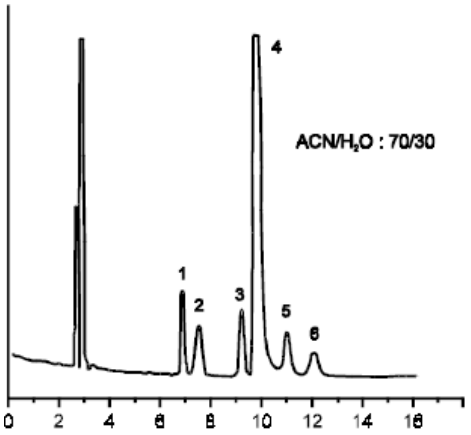
HILIC Separation of B Vitamins



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

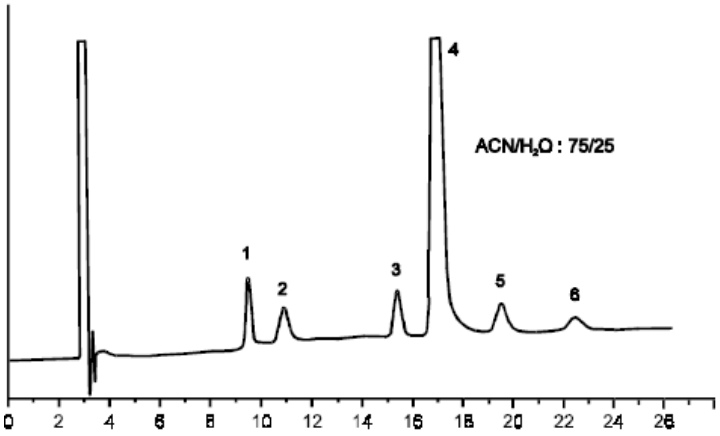
HILIC Separation of Sugars

ACN/Water: 70:30



1. Fructose
2. Glucose
3. Saccharose
4. Palatinose
5. Trehalulose
6. Isomaltose

ACN/Water: 75:25



ZORBAX NH_2 (4.6 x 250 mm) (Agilent Part No. 880952-708)
Mobile Phase: ACN : H_2O, as indicated
1 mL/min, Detect. = Refractive Index

Ligand Exchange (Hi-Plex)

- Used primarily for sugars, sugar alcohols, organic acids
- Sulfonated polystyrene/divinylbenzene particles
- Hydrogen form, or Ca, Na, K, Pb
- Positively charged ion associated with sulfonate
- Interacts with the slightly negative hydroxyls of sugars (ligand)
- Size-exclusion mechanism for oligosaccharides

Ligand Exchange (Hi-Plex)

Bonded Phase	Temperature Range	Flow Rate (mL/min)	Eluent
Hi-Plex Ca	80-90 °C	0.6	Water
Hi-Plex Ca USP L19	80-90 °C	0.3	Water
Hi-Plex Pb	70-90 °C	0.6	Water
Hi-Plex H for carbohydrates	60-70 °C	0.6	Water
Hi-Plex H for organic acids	40-60 °C	0.6	Dilute Acid
Hi-Plex Ca (Duo)	80-90 °C	0.6	Water
Hi-Plex K	80-90 °C	0.6	Water
Hi-Plex Na (Octo)	80-90 °C	0.6	Water, Sodium Hydroxide
Hi-Plex Na	80-90 °C	0.3	Water

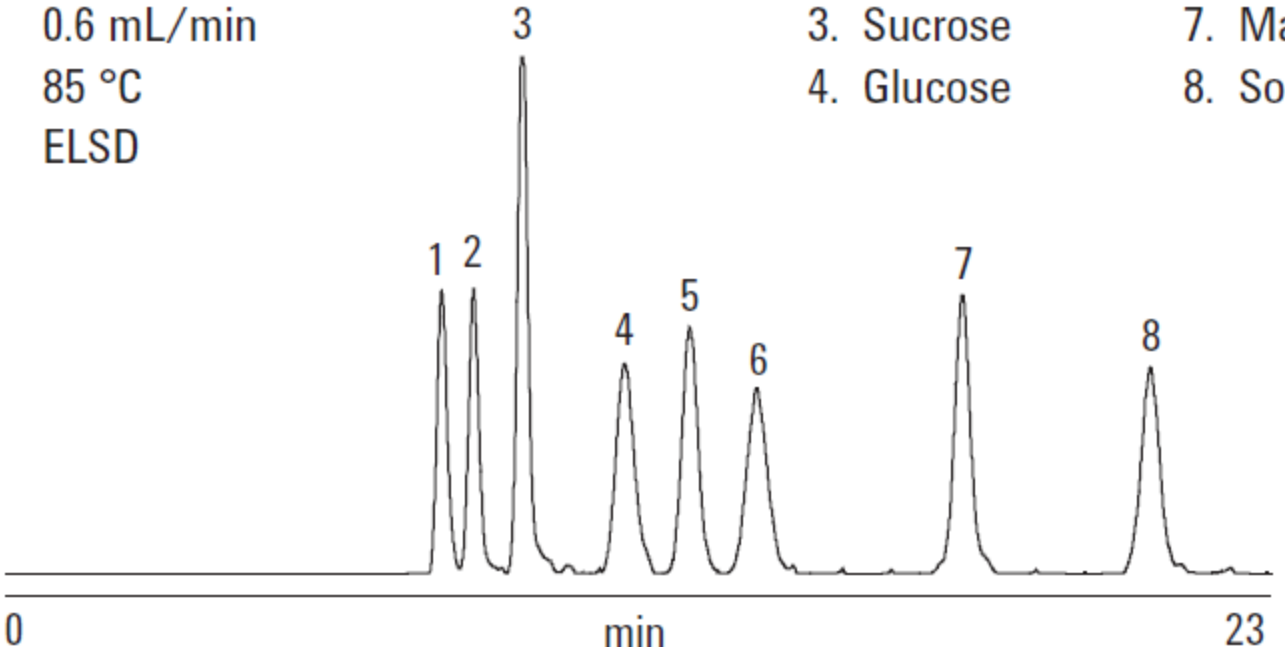
- Mobile phase is typically water (or dilute acid)
- Temperature is main variable for adjusting resolution

Sweeteners by Hi-Plex Ca

Column:	Agilent Hi-Plex Ca, 7.7 x 300 mm
Mobile Phase:	Water
Flow Rate:	0.6 mL/min
Temperature:	85 °C
Detection:	ELSD

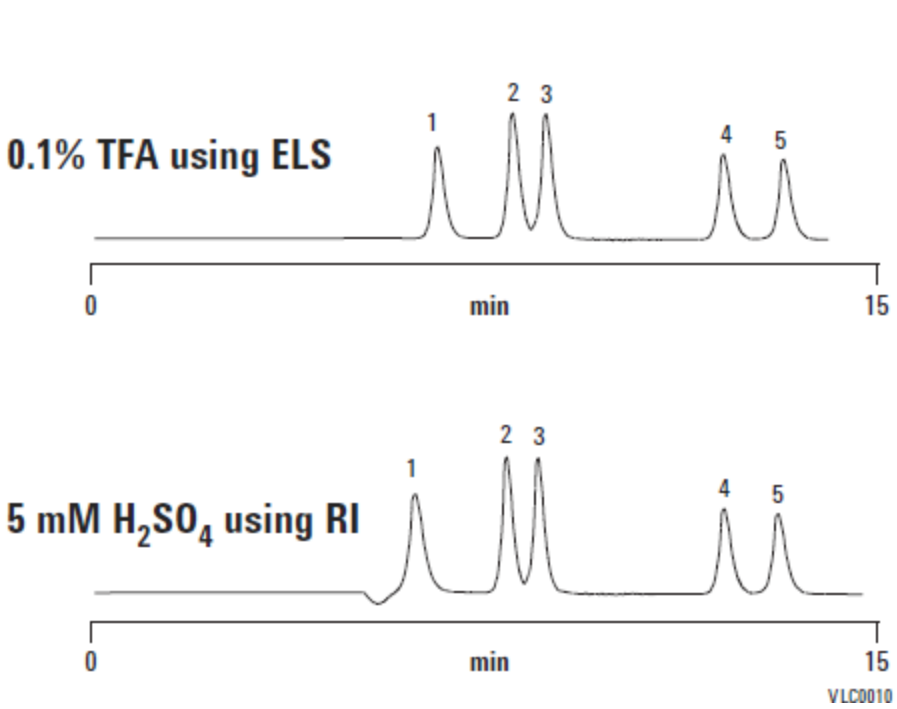
Peak Identification

1. Stachyose
2. Raffinose
3. Sucrose
4. Glucose
5. Galactose
6. Fructose
7. Mannitol
8. Sorbitol



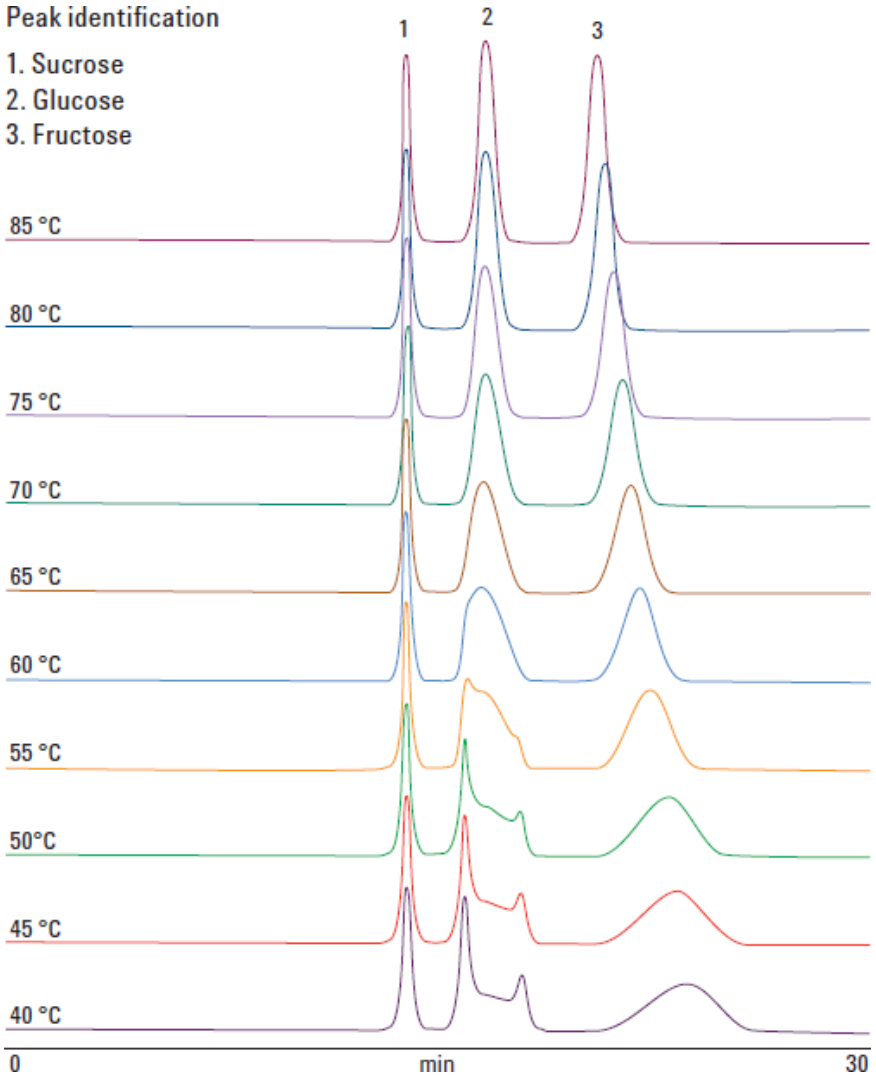
Organic acids by Hi-Plex H

Column:	**Hi-Plex H** **PL1170-6830** **7.7 x 300 mm, 8 µm**
Mobile Phase:	Water with acid as specified
Flow Rate:	0.6 mL/min
Temperature:	60 °C
Detector:	ELS (neb=80 °C, evap=90 °C, gas=0.7 SLM), RI



1. Oxalic acid
2. Citric acid
3. Tartaric acid
4. Succinic acid
5. Lactic acid

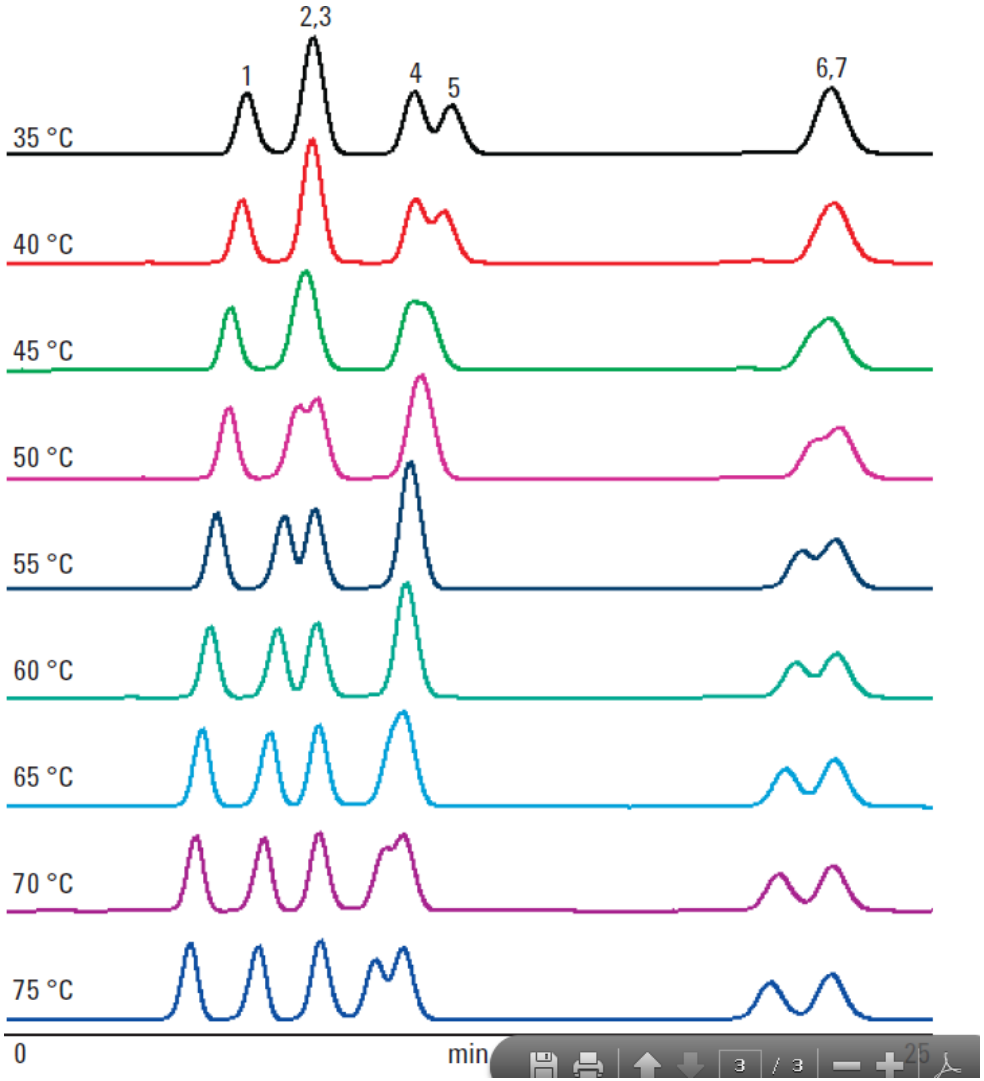
Temperature Effects with Hi-Plex



Conditions

Column	Agilent Hi-Plex Ca, 7.7 x 300 mm, 8 µm (p/n PL1170-6810)
Mobile phase	100% DI H_2O
Flow rate	0.4 mL/min
Temperature	Various
Detector	RI

Temperature Effects with Hi-Plex



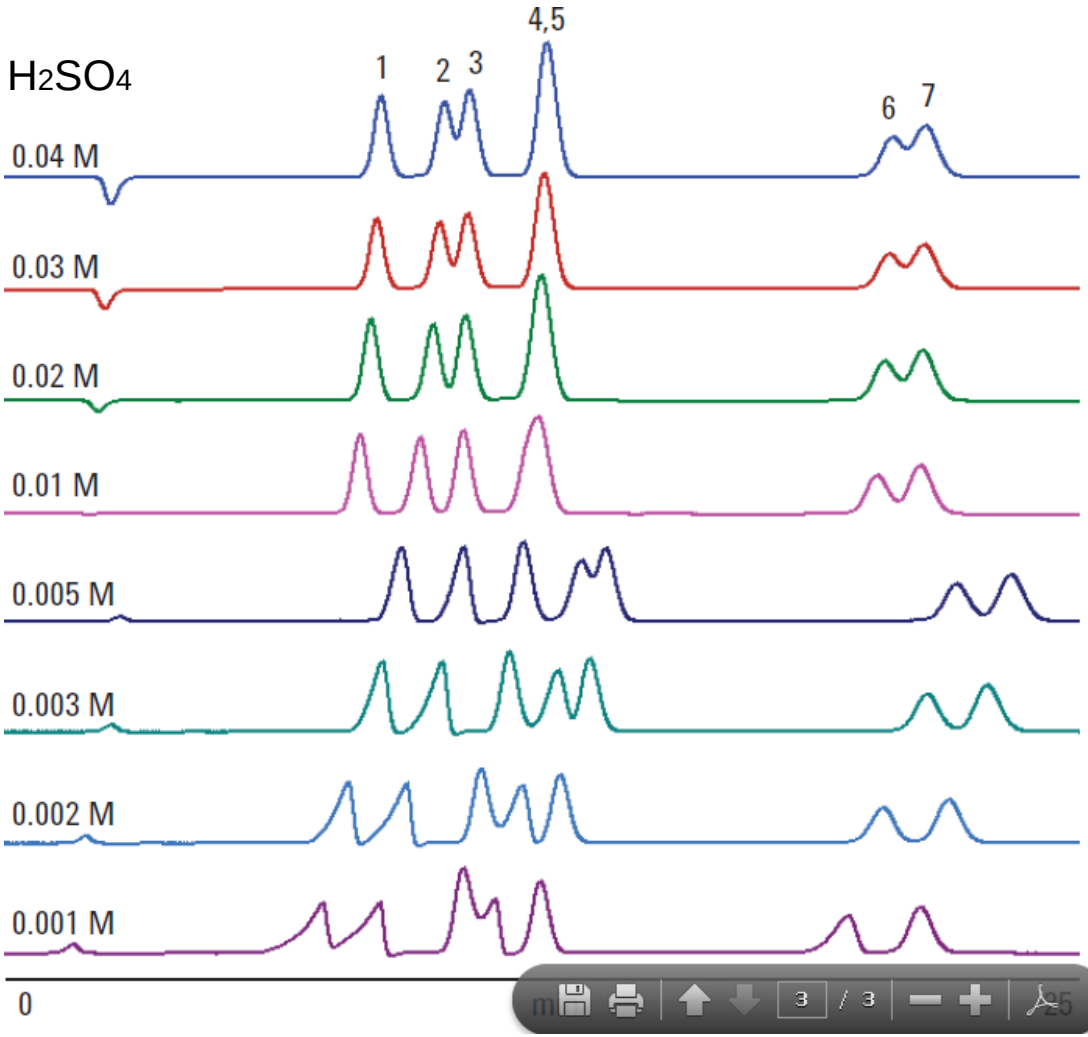
Peak identification

1. Citric acid
2. Tartaric acid
3. Glucose
4. Malic acid
5. Fructose
6. Lactic acid
7. Glycerol

Conditions

Column	Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830)
Mobile phase	0.01 M H_2SO_4
Flow rate	0.4 mL/min
Temperature	> 75 °C
Detector	RI

Mobile Phase Effects with Hi-Plex



Peak identification

1. Citric acid
2. Tartaric acid
3. Glucose
4. Malic acid
5. Fructose
6. Lactic acid
7. Glycerol

Conditions

Column	Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830)
Flow rate	0.4 mL/min
Detector	RI

Summary

- What do you do when your analyte is too polar?
- Stick with reversed-phase but choose a more polar phase
  - Phenyl, Phenyl-Hexyl, Bonus-RP, SB-Aq
- Consider HILIC
- Ion-pair chromatography
- Consider application specific phases:
  - Carbohydrate Analysis
  - Hi-Plex