Peak Shape

Why it matters and how to get good peak shape

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Agilent InfinityLab

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Agenda

- What is a good peak shape and why is it important?
- How is peak shape measured?
- Problems with peak shape
- Factors affecting peak shape
- Examples of peak shape problems
- Guidelines for improved peak shape

What is Good Peak Shape and Why is it Important?

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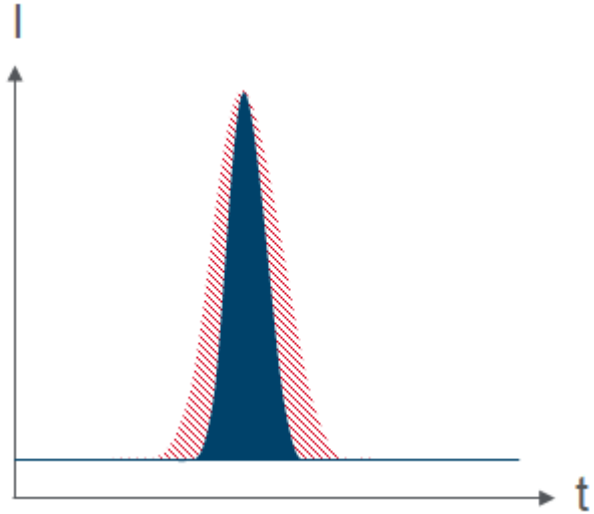
Good peak shape can be defined as symmetrical or Gaussian.

Good peak shape can be defined by:

- Tailing factor of 1.0
- High efficiency
- Narrow peak width

Good peak shape is important for:

- Improved resolution, sensitivity, and precision
- More accurate quantitation
- Longer usable column lifetime (based on system suitability criteria)



How is Peak Shape Measured?

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Measures:

- USP tailing factor – at 5% of peak height
- Asymmetry factor – at 10% of peak height

Indicators:

- Efficiency – plate number
- Peak width – peak width at ½ height

How is Peak Shape Measured?

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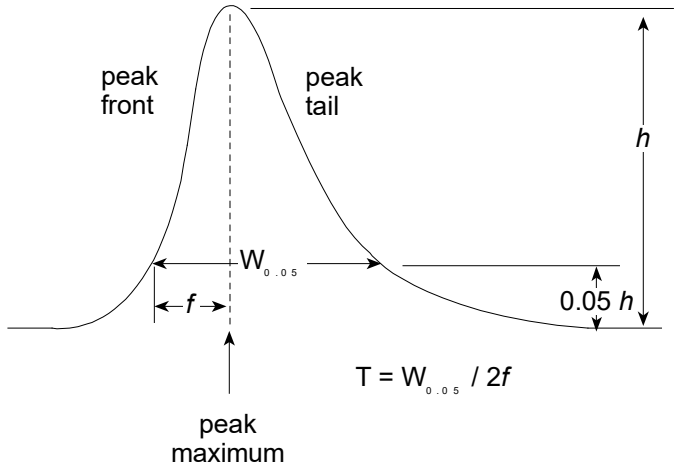
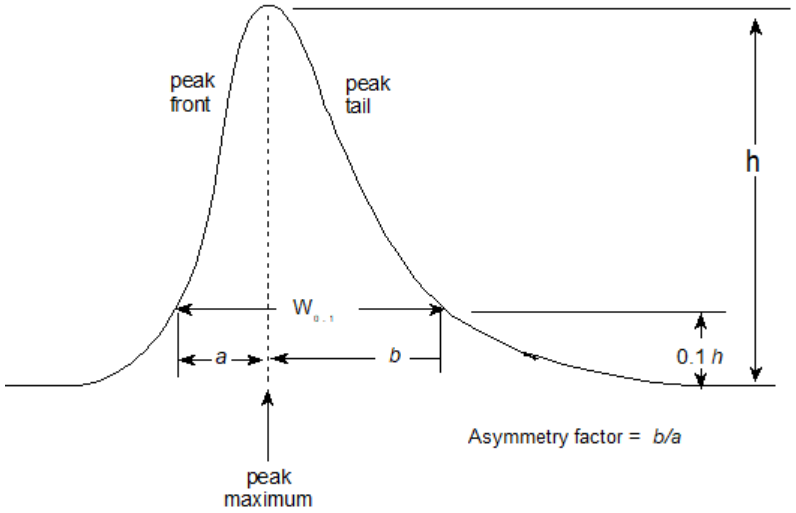
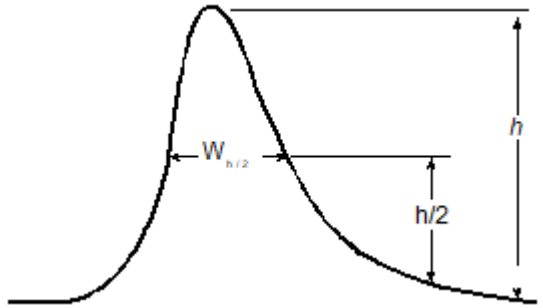
USP tailing factor at 5% height



Asymmetry factor at 10% height



Peak width at ½ height



Efficiency

$$N = 5.54 \left(\frac{t_R}{W_{h/2}} \right)^2$$

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How is Peak Shape Measured?

Column plate number as a function of experimental conditions

$$\mathrm{N(plate\ number)} = \mathrm{L(column\ length)/H(plate\ height)}$$

H varies with the linear velocity (u) of the mobile phase as it passes through the column ($u=L/t_0$).

$$H = A + \frac{B}{u} + Cu$$

Van Deemter equation

A, B, and C are constants for a particular compound and set of experimental conditions. Linear velocity (u) is variable.

- A is eddy diffusion
- B is longitudinal (axial) diffusion
- C is resistance to mass transfer

How is Peak Shape Measured?

Van Deemter plot

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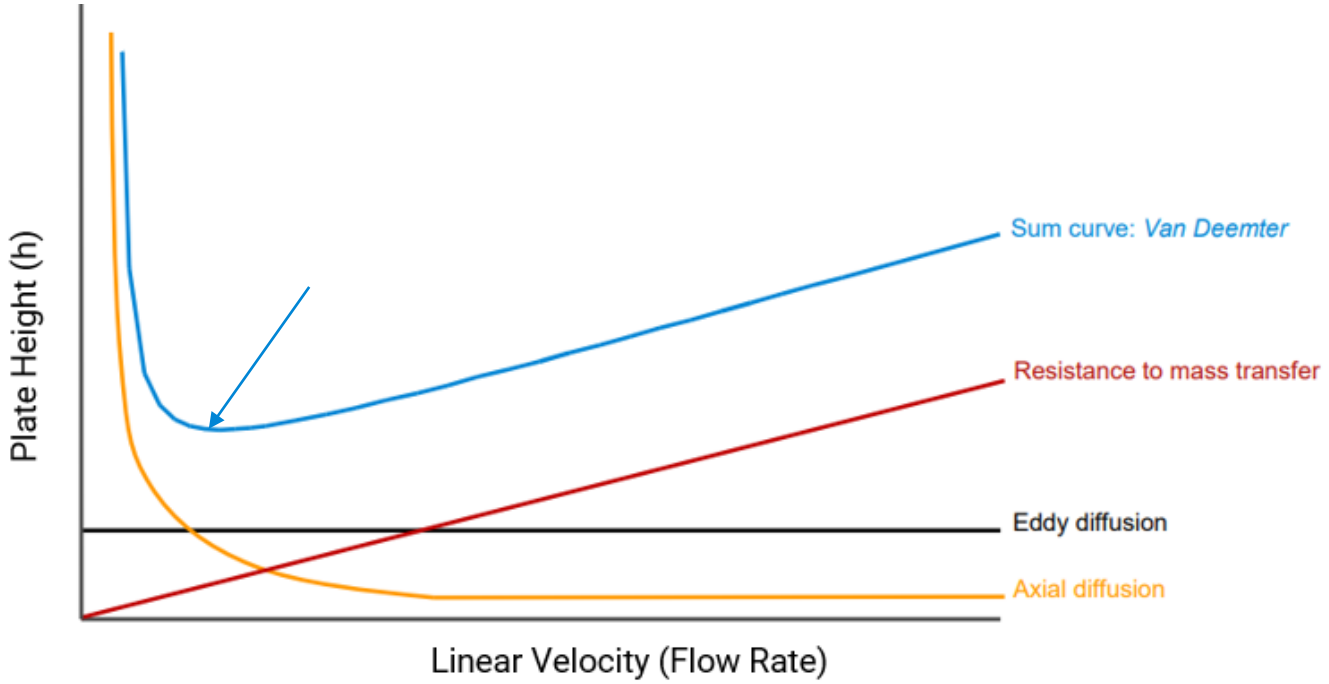
A plot of plate height versus linear velocity



The point where minimum plate height is reached is the “optimum” linear velocity, at which the maximum plate number is reached.

Optimum flow rate = u (optimum linear velocity) x s (cross section area of the column)

How is Peak Shape Measured?

Efficiency – column plate number as a function of experimental conditions

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Column plate number (N) increases with:

- Quality of column packing
- Column length
- Optimal flow rate
- Smaller particle size
- Use of superficially porous particles
- Use of appropriate pore size
- Lower mobile phase viscosity
- Higher temperature
- Minimized extracolumn effect

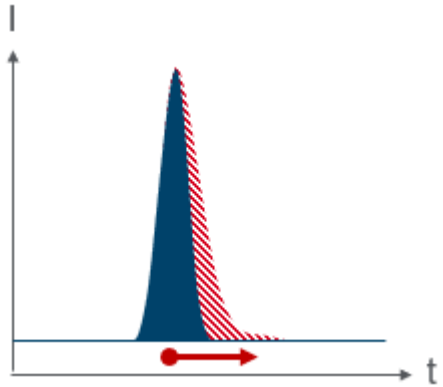
$$N = 5.54 \left(\frac{t_R}{W_{h/2}} \right)^2$$

t_R= band retention time

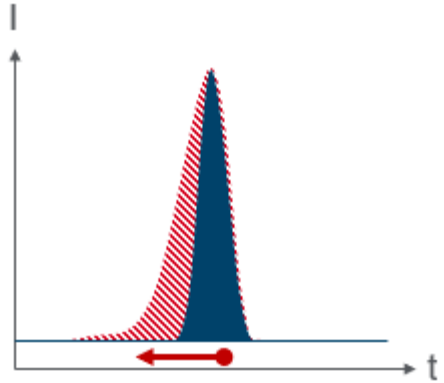
$W_{h/2}$= bandwidth at half-height

Problems with Peak Shape

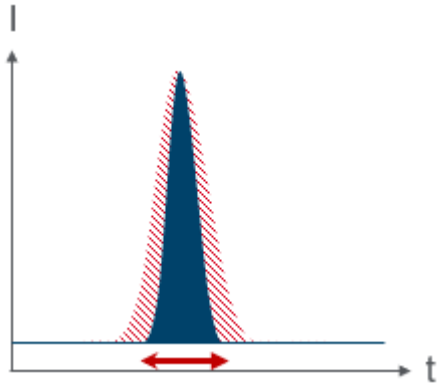
- Tailing
- Fronting
- Broadening
- Splitting/doubling



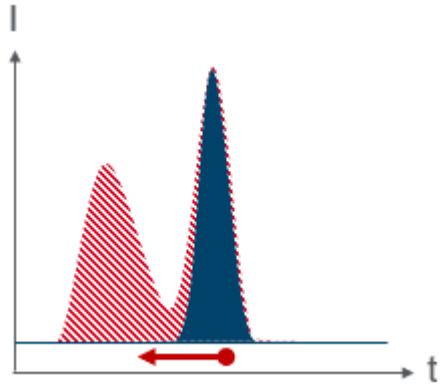
Tailing



Fronting



Broadening



Splitting/doubling

Factors Affecting Peak Shape

- Column
- Mobile phase
- Connecting capillaries and fittings
- System
- Sample

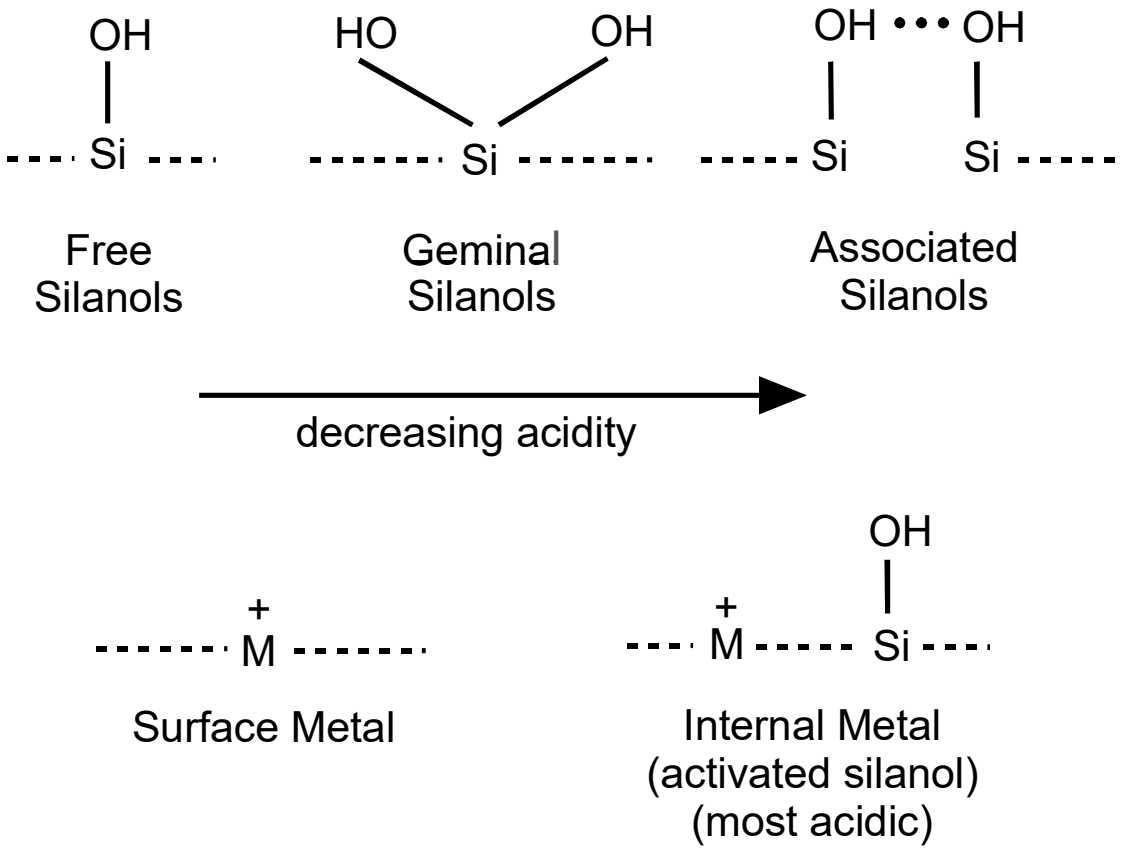
Factors Affecting Peak Shape

Column-related factors

- Silica type/acidity/metal content
- Column bonding and endcapping
- Column packing
  - Pore size/particle size/particle morphology
  - Formation of voids in the packed bed

Factors Affecting Peak Shape

Column-related factors – silica type



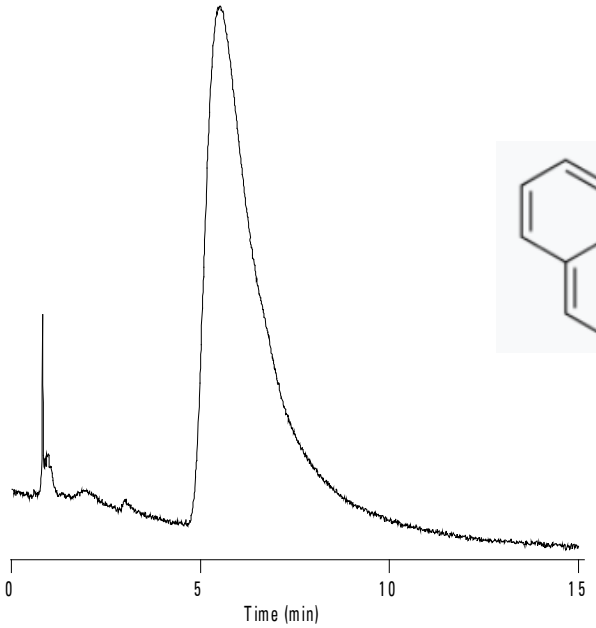
Fully hydroxylated and metal-free silica reduces acidity

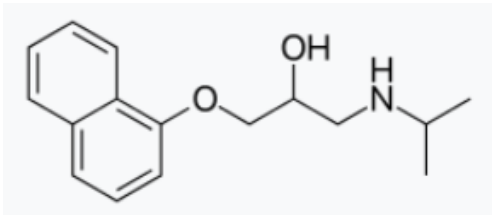
Factors Affecting Peak Shape

Column-related factors – silica type

Silica type – more acidic
Column: ODS, 4.6 x 250 mm, 5 µm
Plates: 92

USP Tf (5%): 2.90

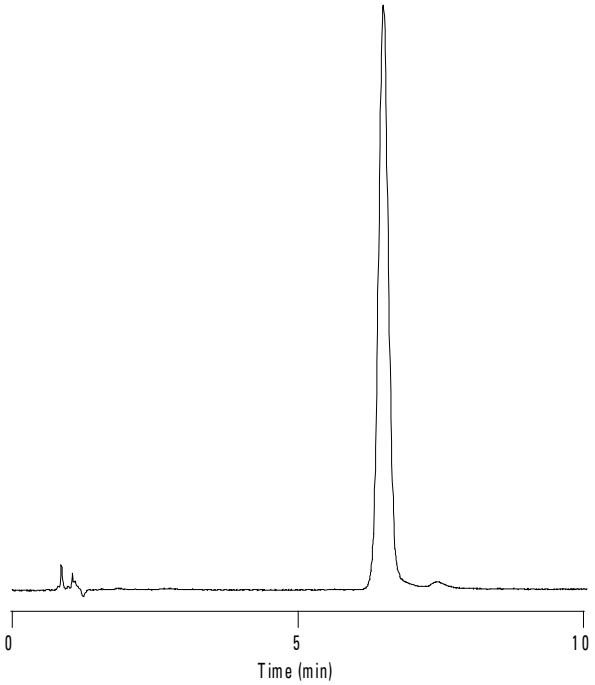




Propranolol
pKa 9.5

Silica type – high purity, Rx-Sil
Column: SB-C18, 4.6 x 150 mm, 5 µm
Plates: 6371

USP Tf (5%): 1.09

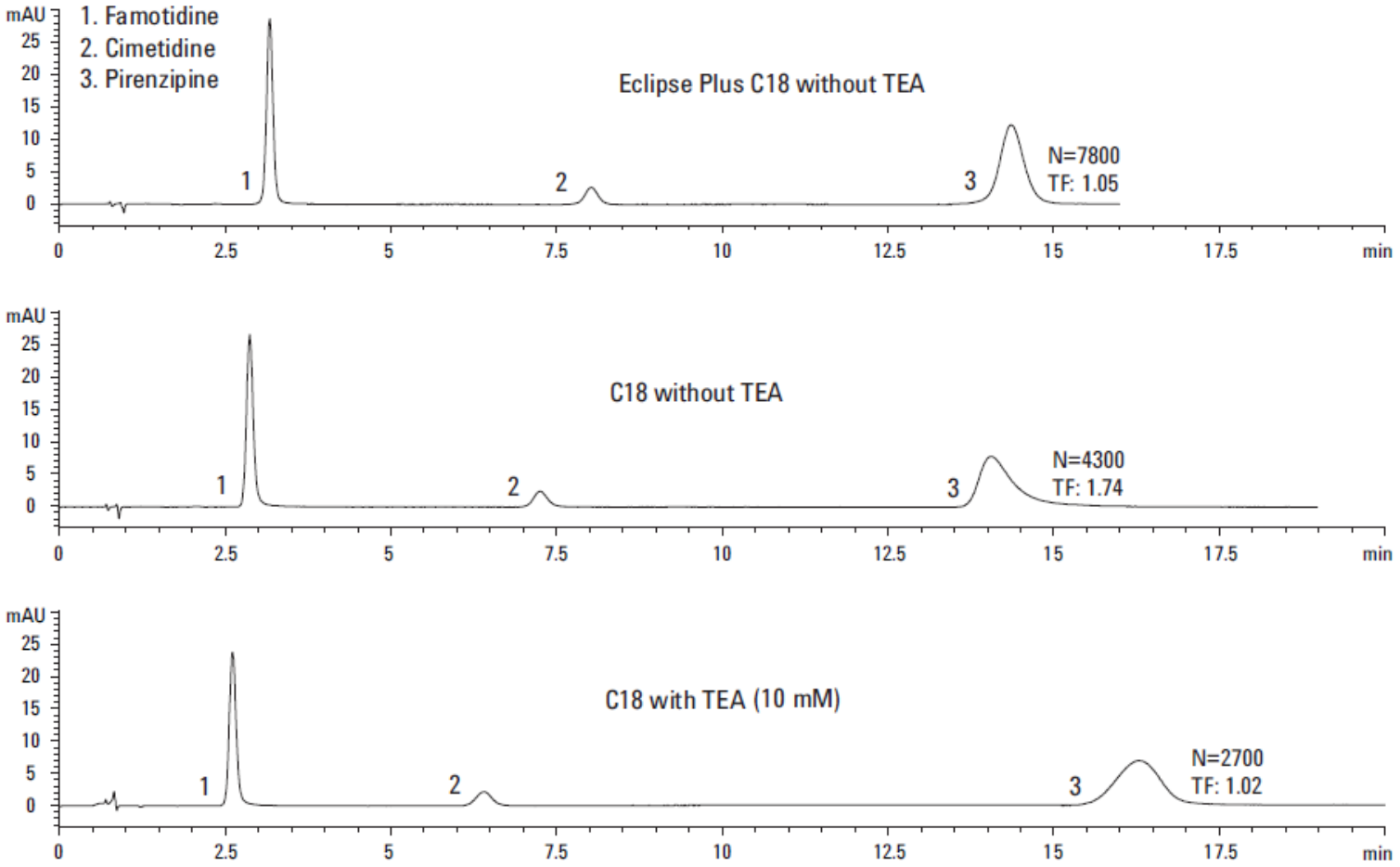


Mobile phase: 75% 50 mM KH_2PO_4, pH 4.4 : 25% ACN flow rate: 1.5 mL/min

ZORBAX StableBond with Rx-SIL improves peak shape

Factors Affecting Peak Shape

Column-related factors – silica type



Columns: 4.6 x 75 mm, 3.5 µm
Mobile phase:
20% MeOH, 80% 20 mM phosphate pH 7.0
Flow rate: 1 mL/min
UV 254 nm
Semimicro flow cell

Effect of ionized acidic silanols on peak shape of amine-containing ulcer medications. A comparison of Eclipse Plus C18 and another C18 column.

Factors Affecting Peak Shape

Column-related factors – column bonding and endcapping

Column bonding and endcapping

- Endcapping minimizes the number of free silanols and potential peak tailing interactions. Most Agilent columns are endcapped.
- Bonded phases that are stable at a high pH (Poroshell 120 HPH-C18, Poroshell 120 CS-C18, and ZORBAX Extend C18) minimize the interaction of basic compounds with free silanols, which reduces peak tailing.

Factors Affecting Peak Shape

Column-related factors – column bonding and endcapping

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InfinityLab Poroshell 120 HPH-C18 with hybridized particle surface and double endcapping is designed to withstand high pH with good peak shape

Conditions:

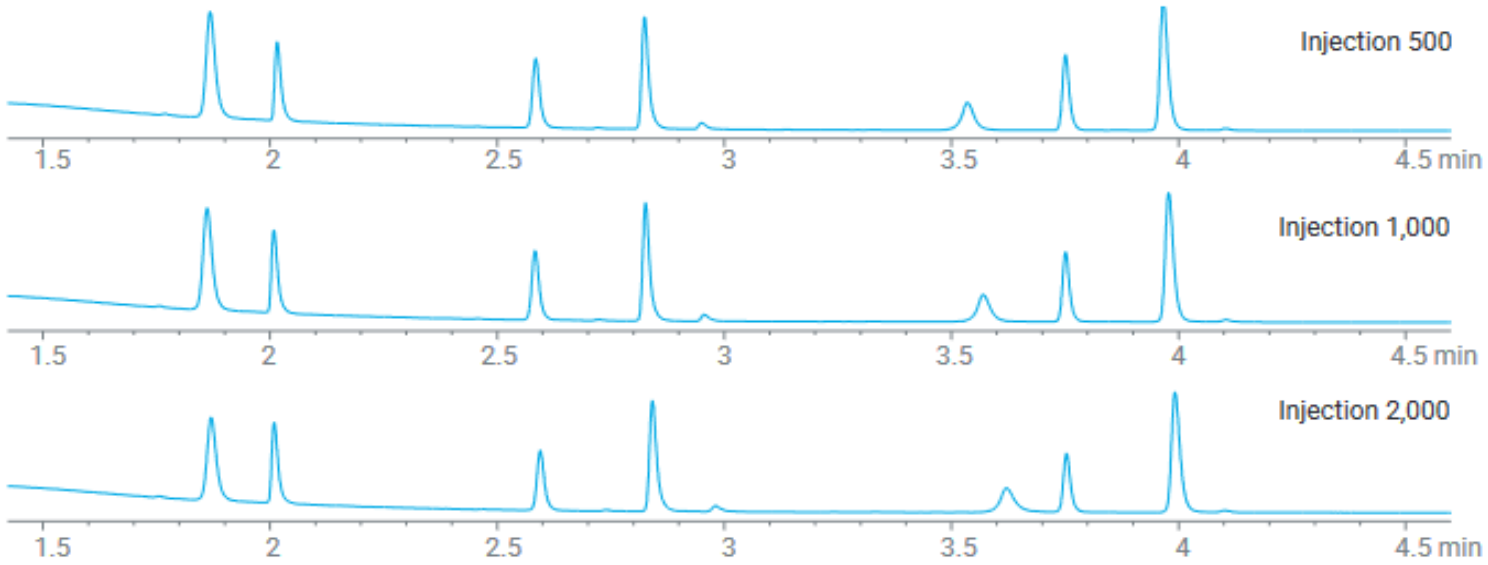
Instrument: 1260 Infinity II Binary LC
Mobile phase: A: 10 mM Ammonium bicarbonate adjusted to pH 10.0 in water
B: Acetonitrile
Flow rate: 0.4 mL/min
Gradient:

Time	%B
0	5
5	95
5.1	5

Sample:

1. Methyl salicylate
2. 4 Chlorocinnamic acid
3. Acetophenone
4. Quinine
5. Nortryptyline
6. Heptanophenone
7. Amitriptyline

InfinityLab Poroshell HPH-C18, 2.1 x 50 mm, 2.7 µm

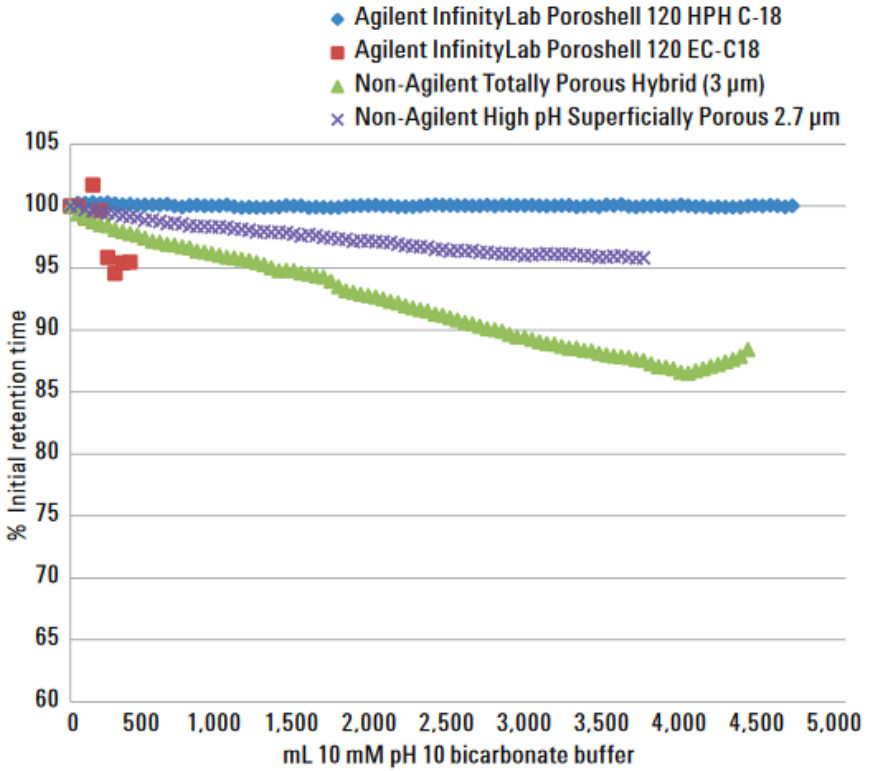


After 2,000 injections at pH 10, InfinityLab Poroshell 120 HPH-C18 showed no change in performance.

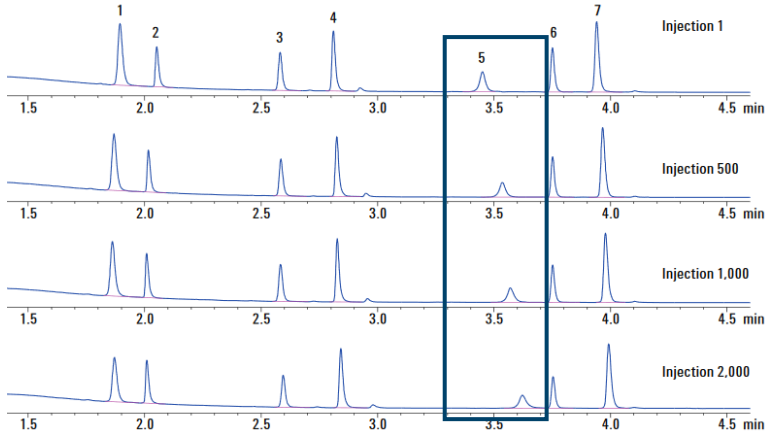
Factors Affecting Peak Shape

Column-related factors – column bonding and endcapping

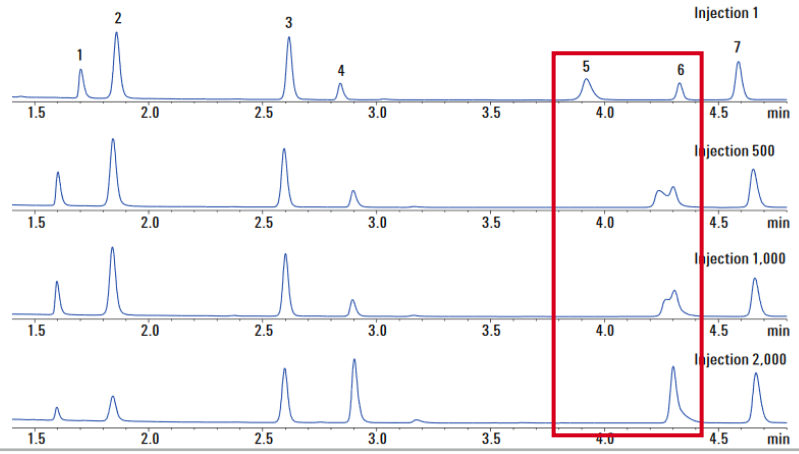
High pH lifetime study



Poroshell HPH-C18



Other hybridized surface column



Column: 2.1 x 50 mm, 2.7 µm
Sample:
1. Methyl salicylate
2. 4-Cholorcinnamic acid
3. Acetophenone
4. Quinine
5. Nortriptyline
6. Heptanophenone
7. Amitriptyline

Instrument: 1260 Infinity II Binary LC
Mobile phase:
A: 10 mM ammonium bicarbonate in water pH 10
B: Acetonitrile
Flow rate: 0.4 mL/min
Gradient method:

Time	%B
0	5
5	95
5.1	5

Factors Affecting Peak Shape

Column-related factors – column bonding and endcapping

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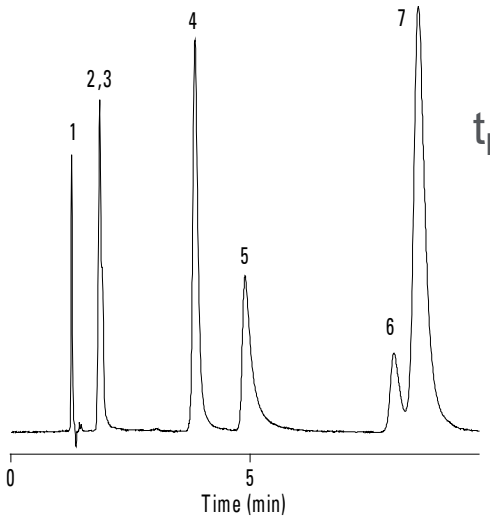
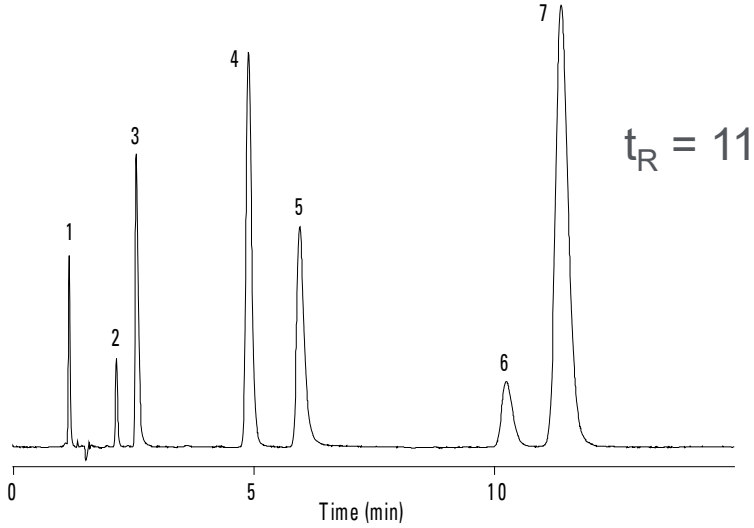
ZORBAX Extend-C18 at pH 7



t_R = 8.5 min

ZORBAX Extend-C18 at pH 11



t_R = 11.4 min

Mobile phase: 30% buffer: 70% MeOH; pH 7 buffer: 20 mM Na_2HPO_4; pH 11 buffer: 20 mM TEA
Flow rate: 1.0 mL/min; temperature: ambient; detection: UV 254 nm
Sample: 1. Maleate 2. Scopolamine pKa 7.6 3. Pseudoephedrine pKa 9.8 4. Doxylamine pKa 9.2 5. Chlorpheniramine pKa 9.1
6. Triprolidine pKa 6.5 7. Diphenhydramine pKa 9.0

Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 µm

Retention and peak shape of basic compounds is improved at high pH on ZORBAX Extend-C18

Factors Affecting Peak Shape

Column-related factors – column packing

Pore size/structure

To get good peak shape, select column pore size according to the size of analyte molecules.

- Wide-pore (300 Å and larger) columns can be selected for separating larger molecules such as proteins and peptides.
- Superficially porous Poroshell 120 columns can be used for small molecules and peptides for improved efficiency at higher flow rates.
- Small-pore totally porous particle columns can be used for small molecules.

Factors Affecting Peak Shape

Column-related factors – column packing

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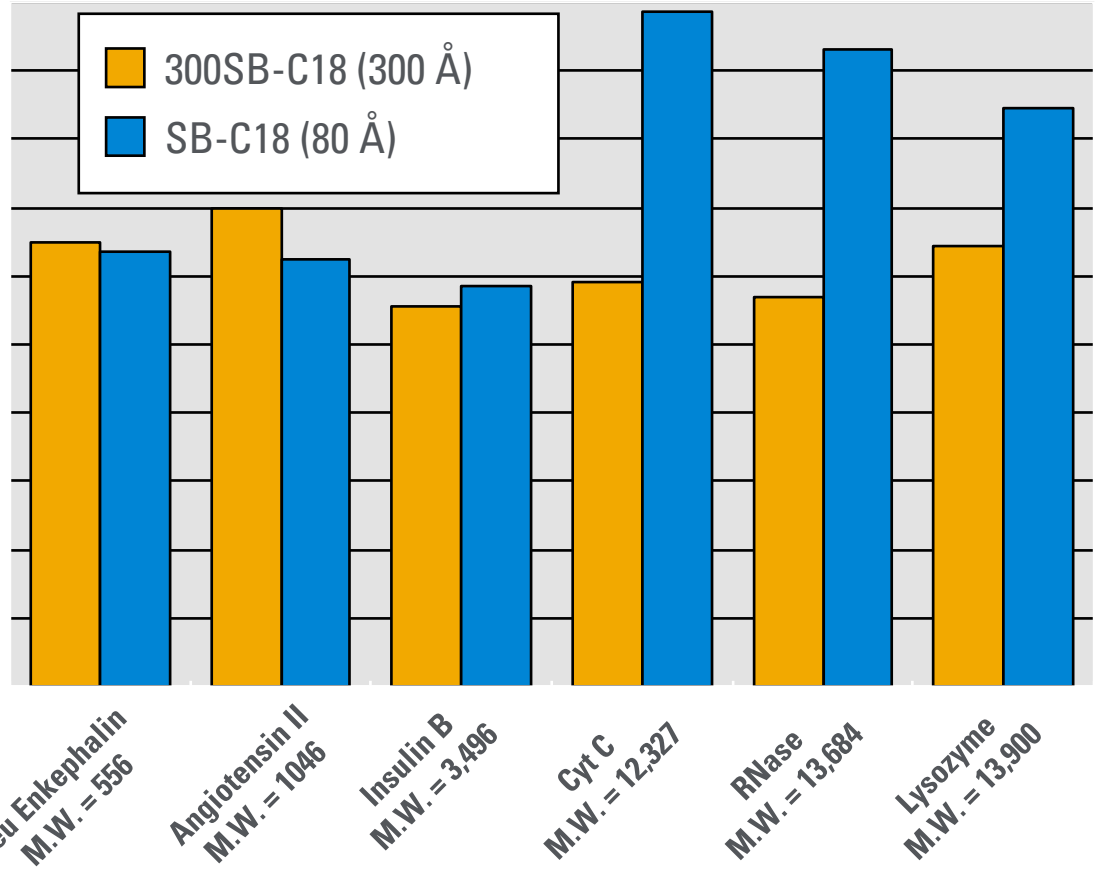
Effect of pore and molecular size on peak width – gradient separations



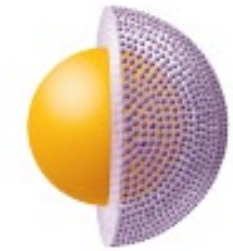
Proper pore size selection results in sharper peaks for large molecules

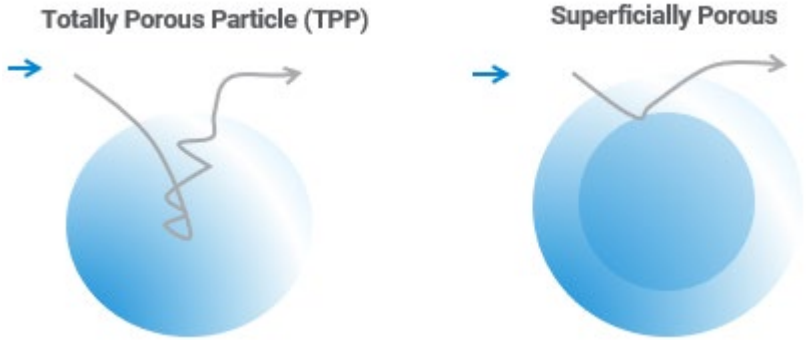
Factors Affecting Peak Shape

Column-related factors – column packing

Poroshell particle technology

- Superficially porous, solid core particles with a porous outer layer provide both improved throughput and higher resolution
- Superior peak shapes for faster, more accurate, results due to high-purity silica and advanced bonding chemistries
- Poroshell 120, 4 µm columns can provide higher efficiency at higher flow rates compared to 5 µm totally porous columns
- Poroshell 120 2.7 µm columns can achieve similar efficiencies as sub-2 µm totally porous columns with substantially less pressure
- Poroshell 120 1.9 µm columns can achieve superior efficiencies over totally porous sub-2 µm columns





InfinityLab Poroshell 120 RP 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

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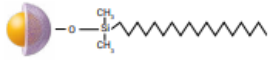
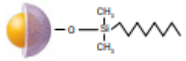
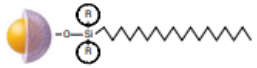
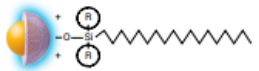
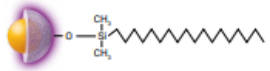
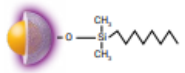
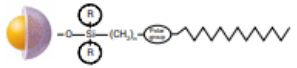
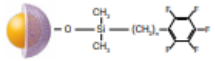
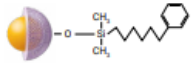
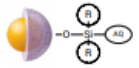
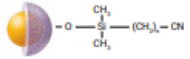
InfinityLab Poroshell 120 RP Bonded Phases

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

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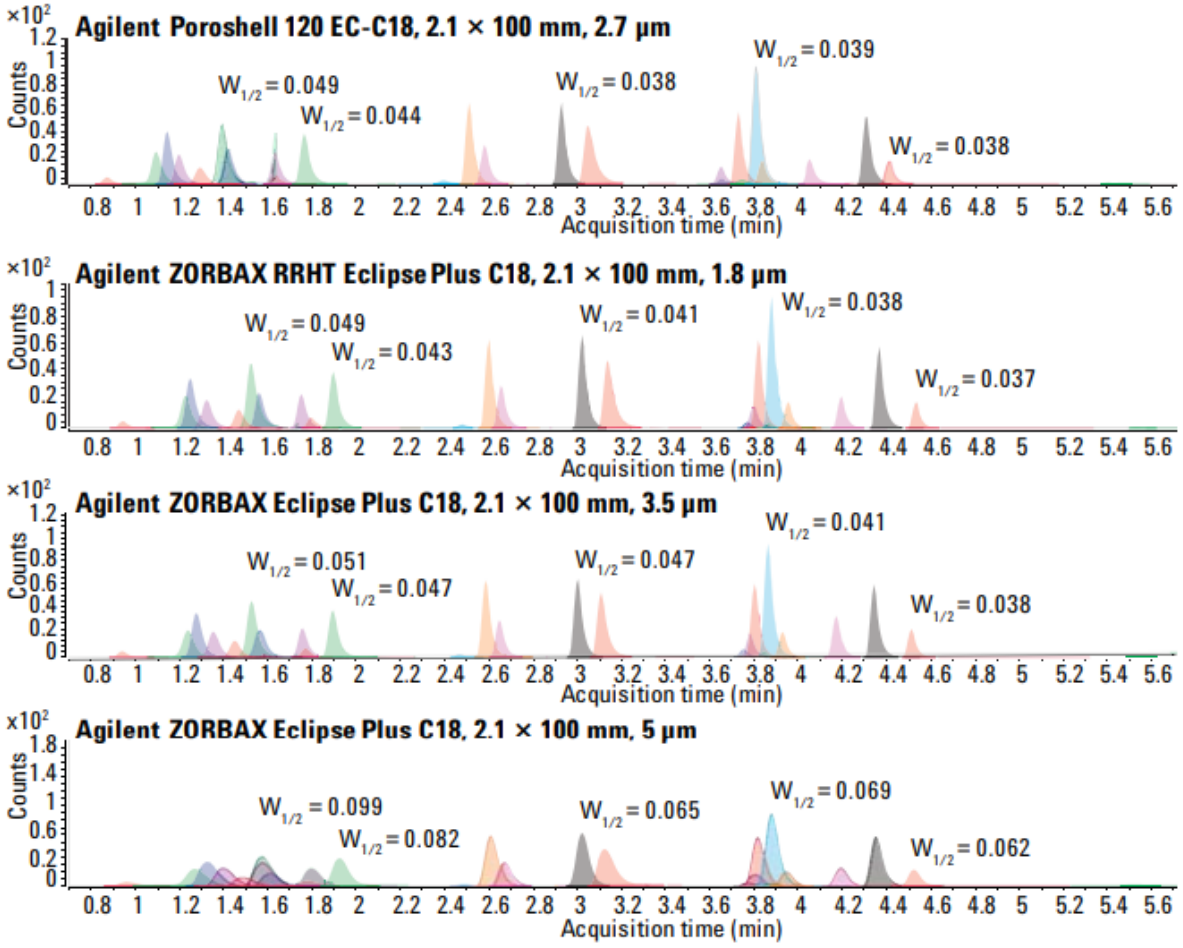
Factors Affecting Peak Shape

Column-related factors – column packing

Smaller particles and superficially porous particles provide sharper peaks



Instrument: Agilent 1200/6410 LC/MS/MS
A: 5 mM ammonium formate with 0.01% formic acid in water
B: Acetonitrile
Flow rate: 0.4 mL/min
Gradient method:

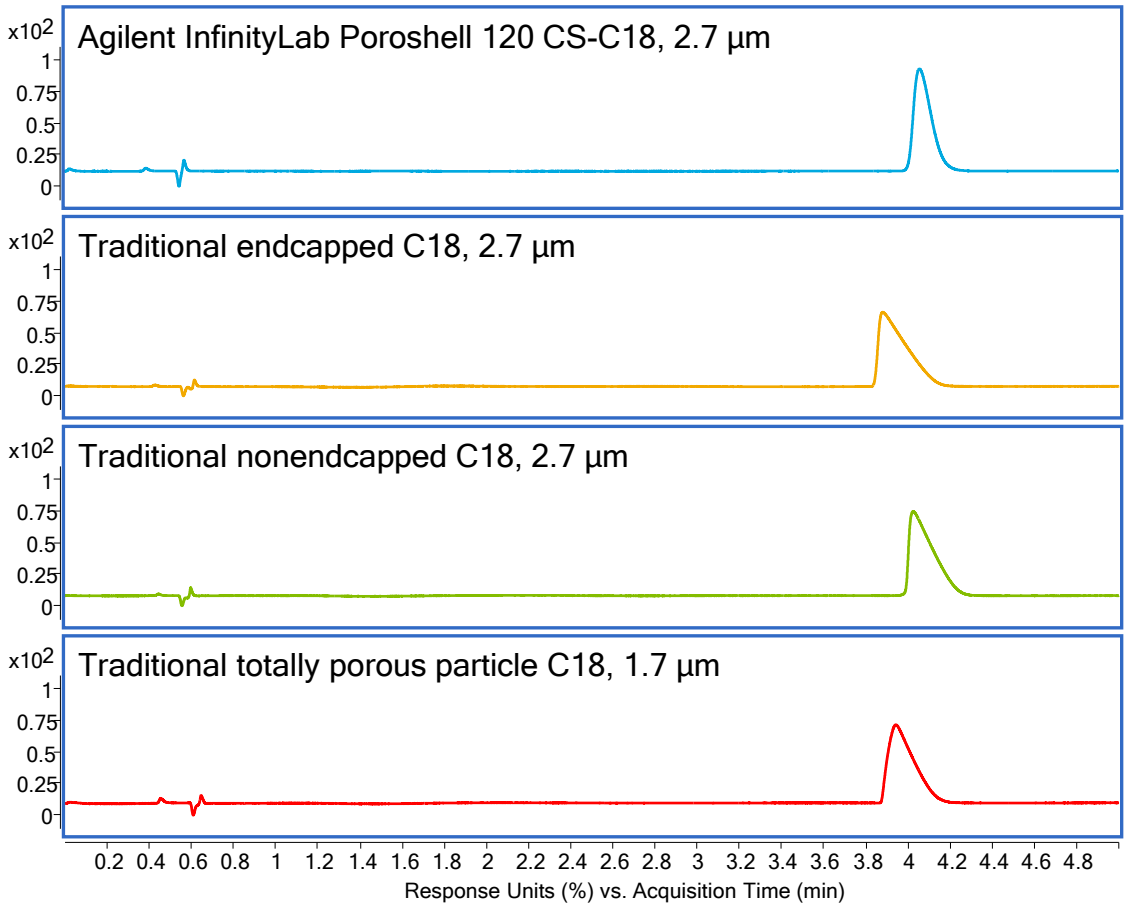
Time	%B
0	10
0.5	15
3.0	50
4.0	95
6.0	95

Stop time: 6 min
Postrun time: 2 min
Temperature: 60 °C
Injection volume: 5 µL
Sample: Agilent LC/MS test mix (p/n 5190-0470), diluted 1:10 in water

Factors Affecting Peak Shape

Column-related factors – column packing

Agilent InfinityLab Poroshell 120 CS-C18 provides better peak shape compared to other C18 columns



A: 0.1 formic acid
B: Acetonitrile; 0.4 mL/min isocratic
2.1 x 100 mm columns
0.5 µL injection
30 ˚C
LC/MS: ESI+, dMRM
Sample: 30 µg/mL amitriptyline

Agilent application note: 5994-2095EN

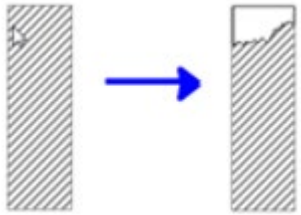
Factors Affecting Peak Shape

Column-related factors – column packing

The formation of a void in the column can result in bad peak shape

Initial

Mobile phase: 50% ACN: 50% water with 0.2% TEA (~ pH 11)



After 30 injections

Multiple peak shape changes can be caused by the presence of a void in the column.
In this case the void resulted from silica dissolved at high pH.

Factors Affecting Peak Shape

Column-related factors – body of the column

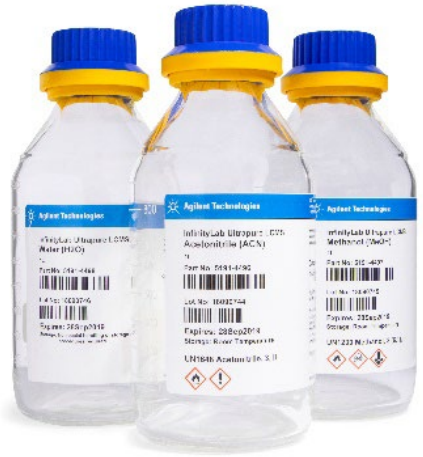
Metal – sensitive compounds can interact with the stainless-steel body of the column.

We will discuss this further, in the “metal-sensitive compounds” section.

Factors Affecting Peak Shape

Mobile phase-related factors

- pH
- Buffers
- Temperature
- Organic modifiers
- Mobile phase additives (TEA, TFA)

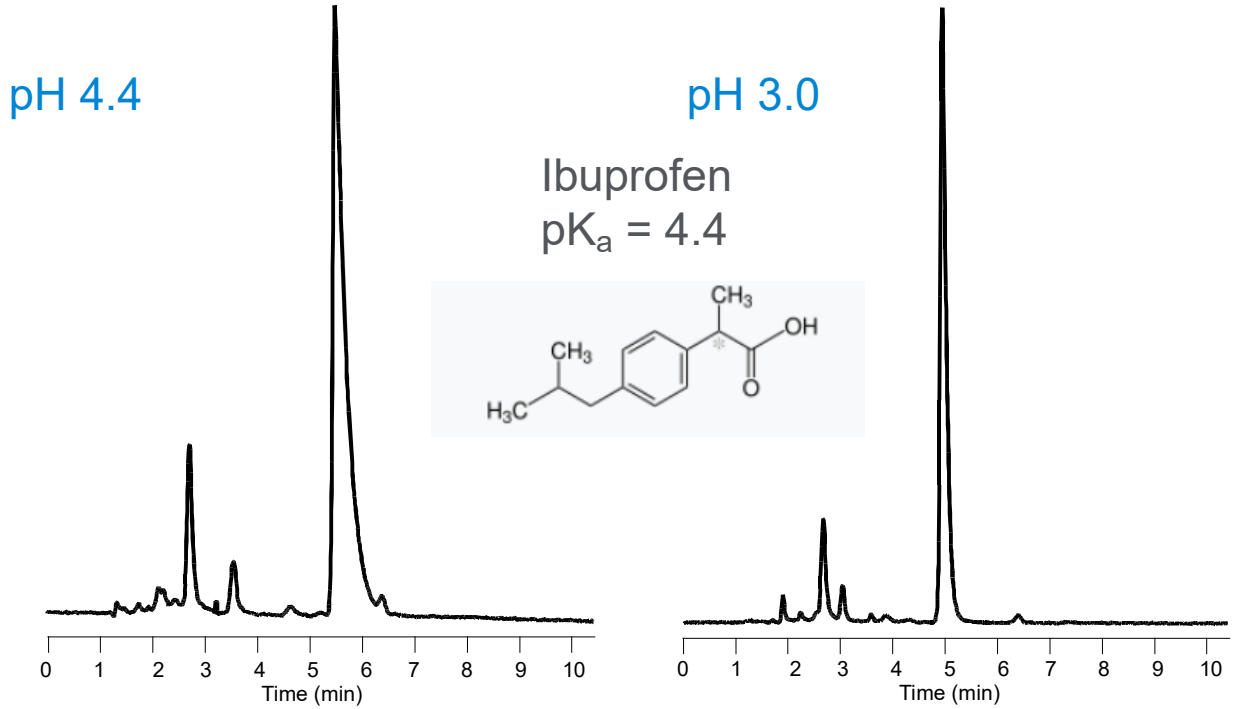


Factors Affecting Peak Shape

Mobile phase-related factors – pH

Agilent InfinityLab

Effect of pH on peak shape at or near the sample pKa

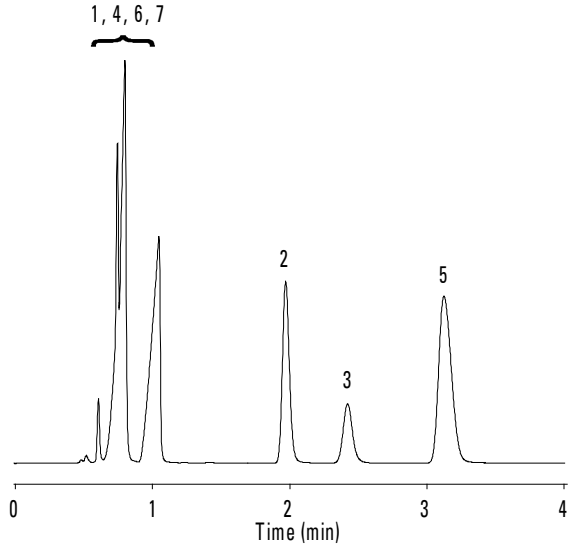


Column: ZORBAX SB-C8, 4.6 x 150 mm, 5 µm
Mobile phase: 40% 5 mM KH2PO4, 60% ACN
Flow rate: 1.0 mL/min.
Temperature: ambient

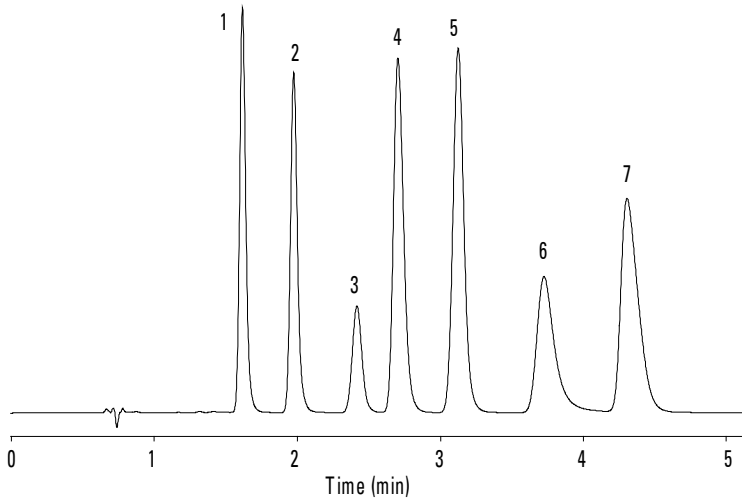
Factors Affecting Peak Shape

Mobile phase-related factors – buffer

A = pH 7.0 water



A = pH 7.0, **25 mM phosphate buffer**



Column: ZORBAX Rapid Resolution Eclipse XDB-C8, 4.6 x 75 mm, 3.5 µm Mobile phase: 44% A : 56% methanol
Flow rate: 1.0 mL/min Temperature: 25°C Detection: UV 250 nm
Sample: 1. Ketoprofen 2. Ethyl paraben 3. Hydrocortisone pKa 5.1 4. Fenoprofen pKa 4.5 5. Propyl paraben 6. Propranolol pKa 9.5 7. Ibuprofen pKa 4.4

Buffered mobile phases enhance retention, resolution, and peak shape

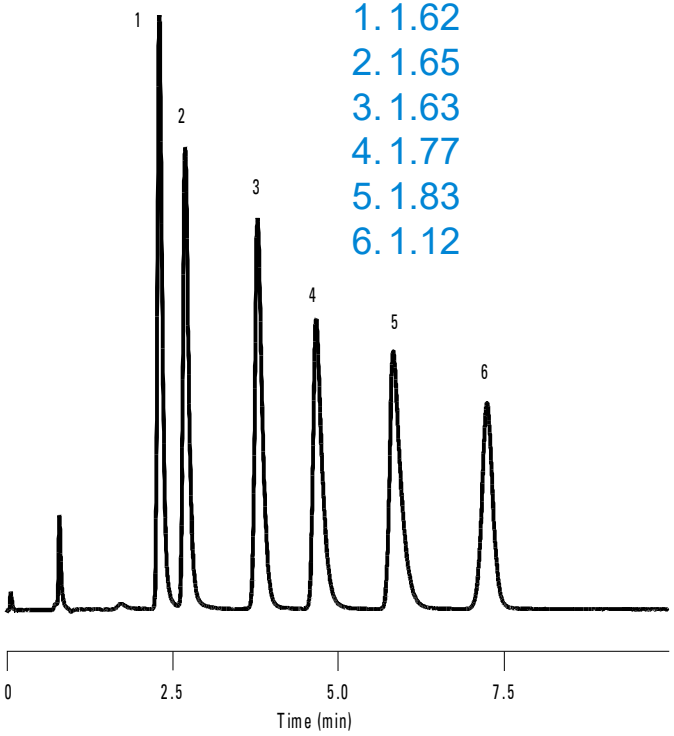
Factors Affecting Peak Shape

Mobile phase-related factors – buffer concentration

10 mM Phosphate, pH 7.0

USP Tf

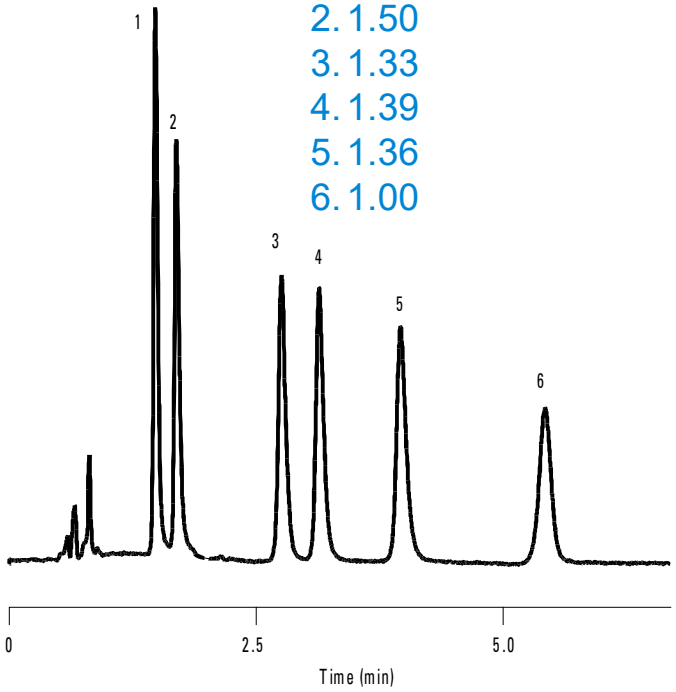
1. 1.62
2. 1.65
3. 1.63
4. 1.77
5. 1.83
6. 1.12



25 mM Phosphate, pH 7.0

USP Tf

1. 1.41
2. 1.50
3. 1.33
4. 1.39
5. 1.36
6. 1.00



Columns: Eclipse XDB-C8
4.6 x 150 mm, 5 µm

Mobile phase: 40% phosphate buffer, 60% ACN

Flow rate: 1.5 mL/min.

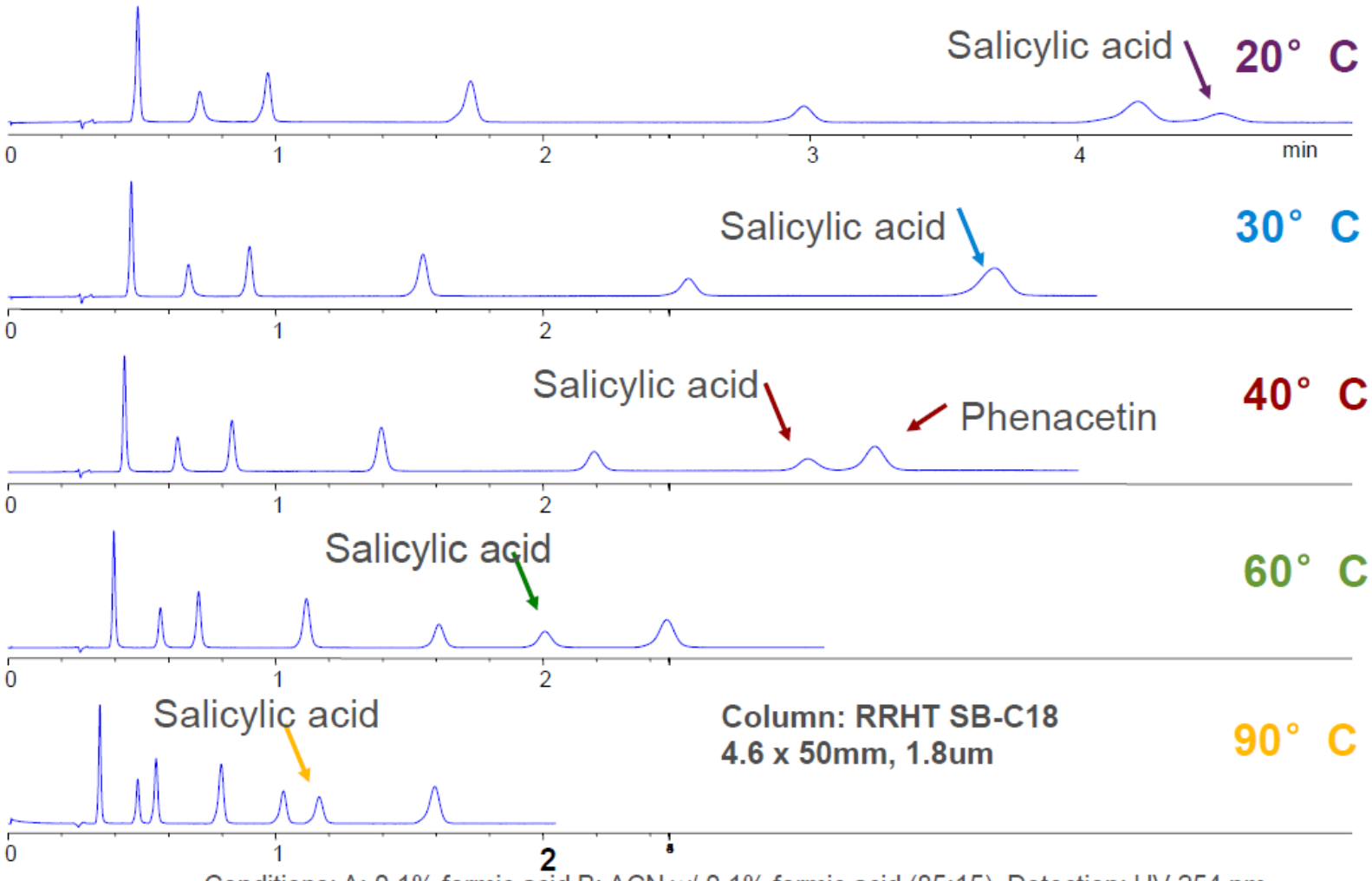
Temperature: 40 °C

Sample: Tricyclic antidepressants
1. Desipramine
2. Nortriptyline
3. Doxepin
4. Imipramine
5. Amitriptyline
6. Trimipramine

Increasing buffer concentration decreases tailing factor (Tf)

Factors Affecting Peak Shape

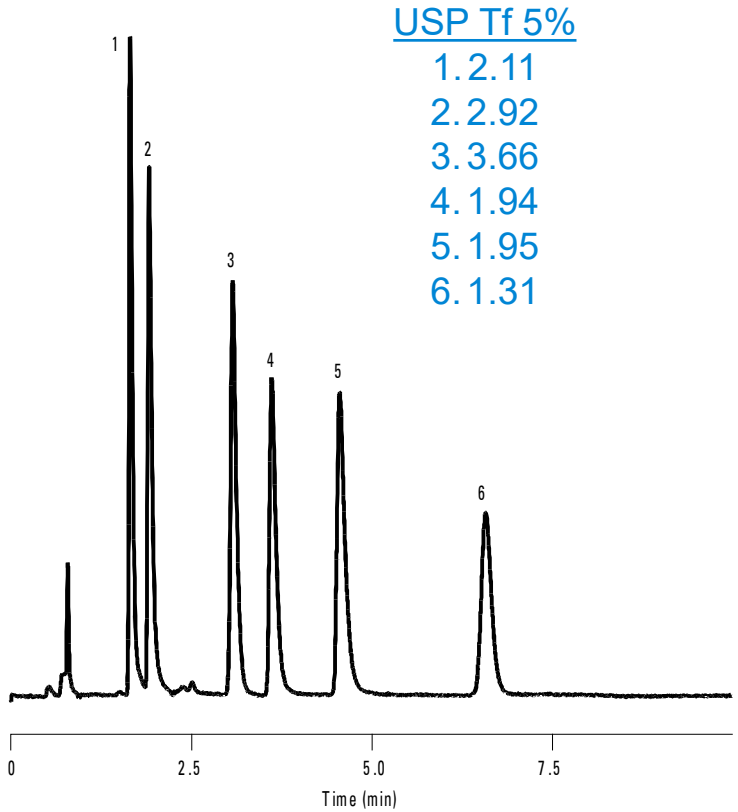
Mobile phase-related factors – temperature



Factors Affecting Peak Shape

Mobile phase-related factors – organic modifier

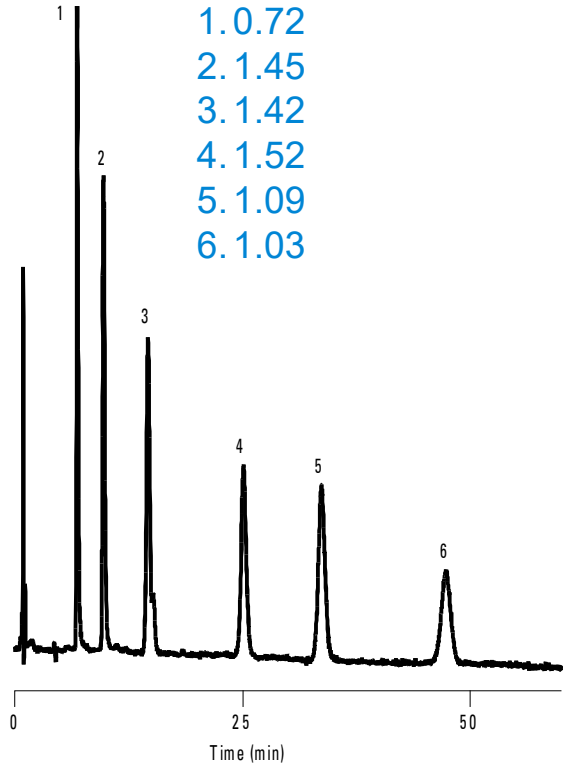
40% 25 mM Na2HPO4 pH 7.0
60% **ACN**



USP Tf 5%

1. 2.11
2. 2.92
3. 3.66
4. 1.94
5. 1.95
6. 1.31

40% 25 mM Na2HPO4 pH 7.0
60% **MeOH**



USP Tf 5%

1. 0.72
2. 1.45
3. 1.42
4. 1.52
5. 1.09
6. 1.03

Columns: Eclipse XDB-C8
4.6 x 150 mm, 5 µm

Flow rate: 1.5 mL/min.

Temperature: 40 °C

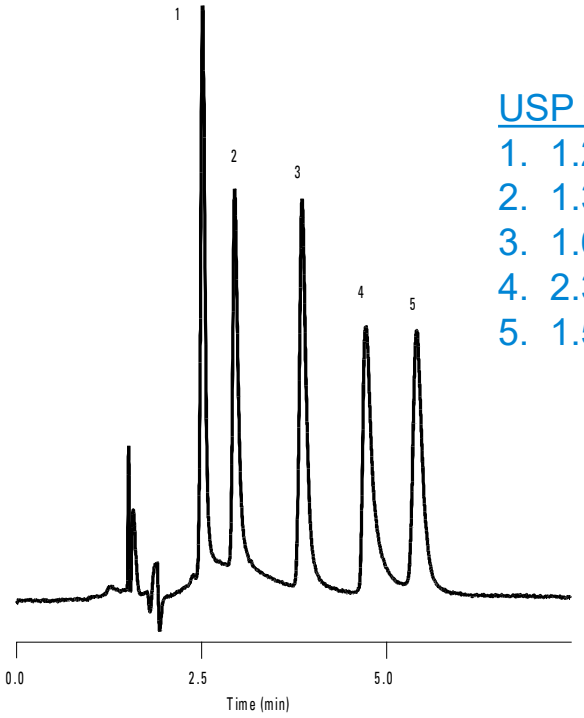
Sample: Tricyclic antidepressants:
1. Desipramine
2. Nortriptyline
3. Doxepin
4. Imipramine
5. Amitriptyline
6. Trimipramine

Acetonitrile versus methanol

Factors Affecting Peak Shape

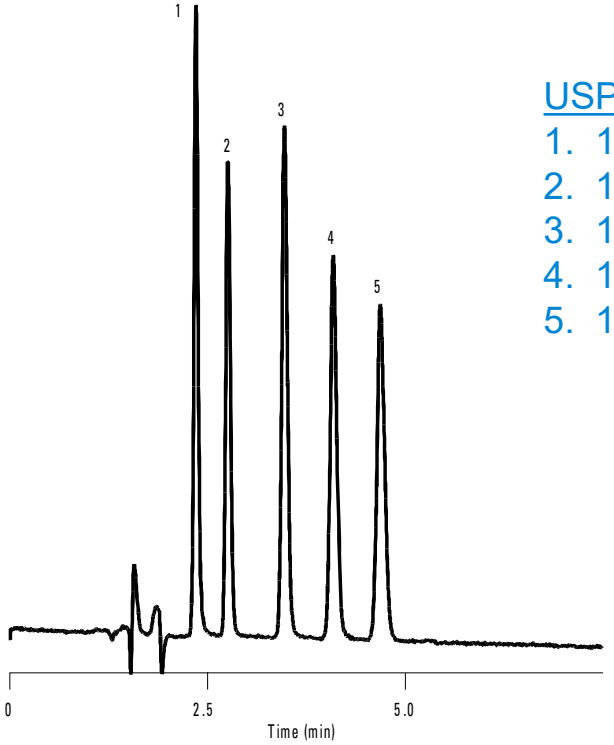
Mobile phase-related factors – mobile phase additives

No TEA



USP Tf 5%
1. 1.29
2. 1.33
3. 1.63
4. 2.35
5. 1.57

10 mM TEA



USP Tf 5%
1. 1.19
2. 1.18
3. 1.20
4. 1.26
5. 1.14

Columns: Eclipse XDB-C8, 4.6 x 150 mm, 5 µm Mobile phase: 85% 25 mM Na2HPO4 : 15% ACN pH: 7 Flow rate: 1.0 mL/min.
Temperature: 35 °C Sample: Amphetamines 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

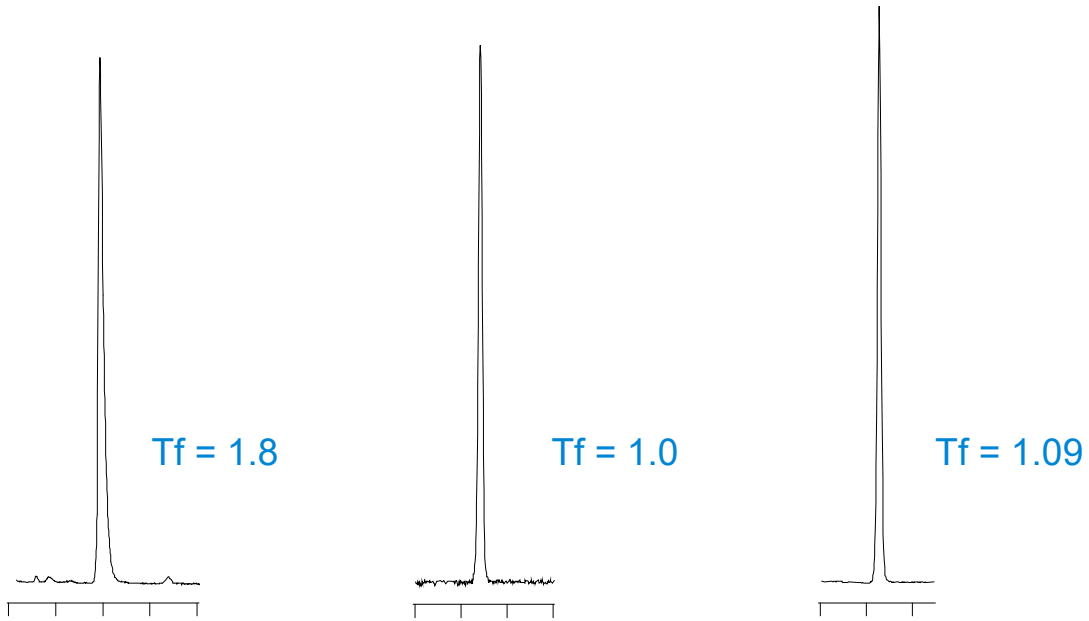
Effect of TEA on peak shape of basic compounds

Factors Affecting Peak Shape

Mobile phase-related factors – mobile phase additives

Mobile phase A:

pH 3 5 mM NaH2PO4	pH 3 5 mM NaH2PO4 **1% acetic acid**	pH 2.5 5 mM NaH2PO4 **0.1% TFA**



Column: StableBond SB-C18
4.6 x 150 mm, 5 µm
Mobile phase: 40% A: 60% ACN
Flow rate: 1.0 mL/min.
Temperature: Ambient
Sample: Ibuprofen, pKa 4.4

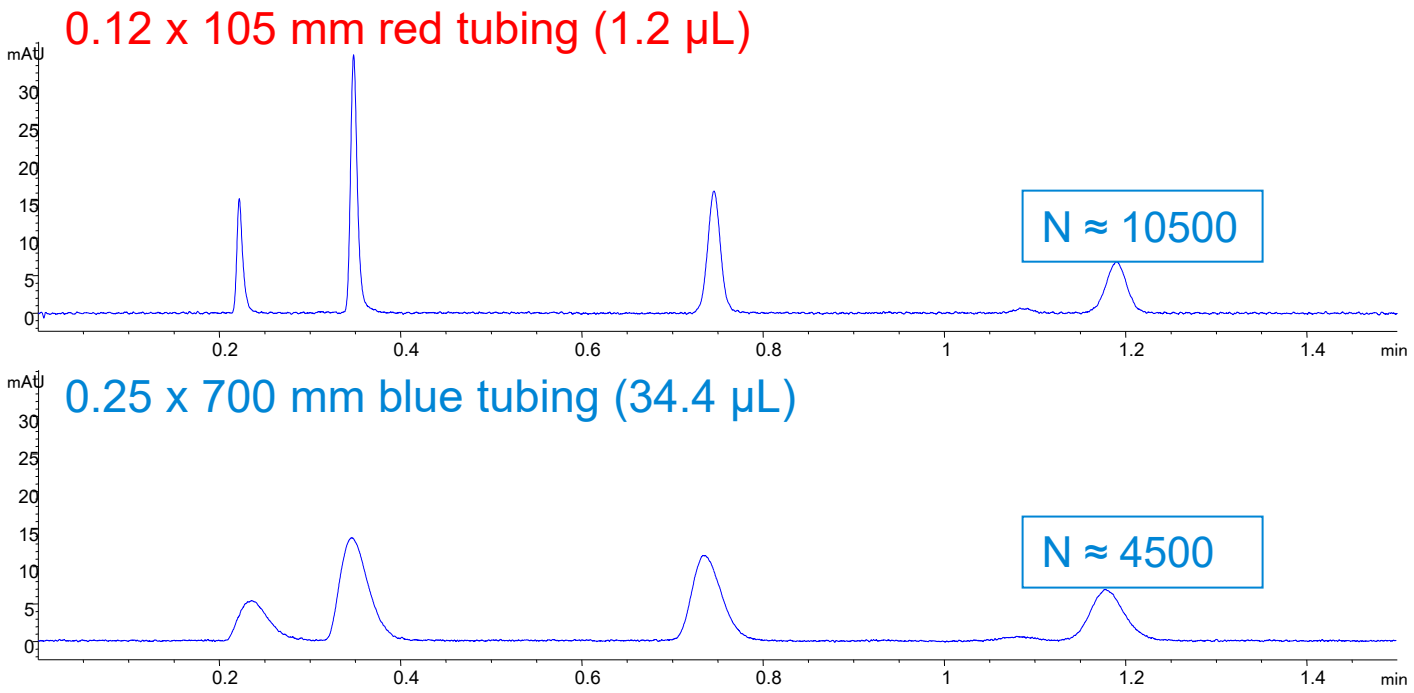
Effect of competing acids on the peak shape of acidic compounds

Factors Affecting Peak Shape

Connecting capillaries and fittings

Agilent InfinityLab

Capillary tubing dimensions can affect peak shape



QC test conditions:
55% ACN
45% H_2O
Isocratic, 0.6 mL/min
1 µL injection of QC mix
23 °C
254 nm

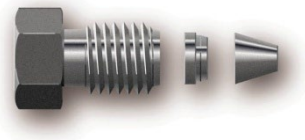
QC mix (in elution order):
1. 5 µg/mL uracil
2. 200 µg/mL phenol
3. 25 µg/mL 4-chloro-nitrobenzene
4. 40 µg mL naphthalene

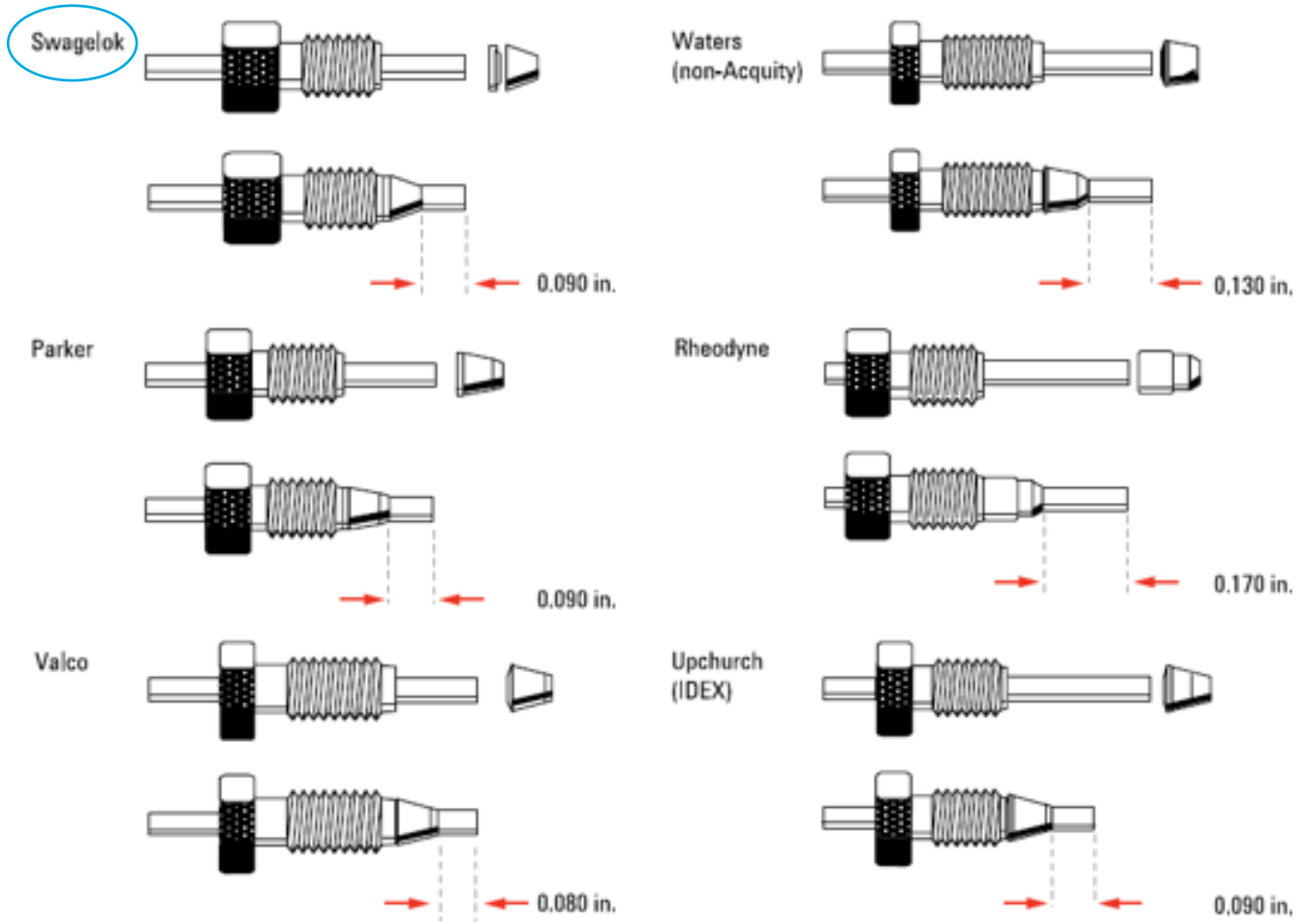
In 50/50 ACN/water

- 2.1 x 50 mm, 1.8 µm Eclipse Plus C18
- Peak broadening when larger volume tubing is installed between autosampler and column
- 43% of the efficiency is lost with too much extracolumn volume

Factors Affecting Peak Shape
Fittings

- Improper fittings can lead to broad, split, and tailing peaks
- Different manufacturers supply different types of fittings
- Use the fittings recommended for your system
- Agilent LC systems use Swagelok-type fittings for many instrument connections

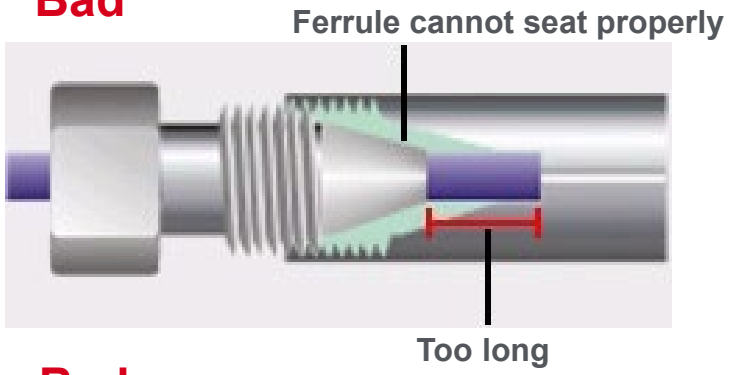




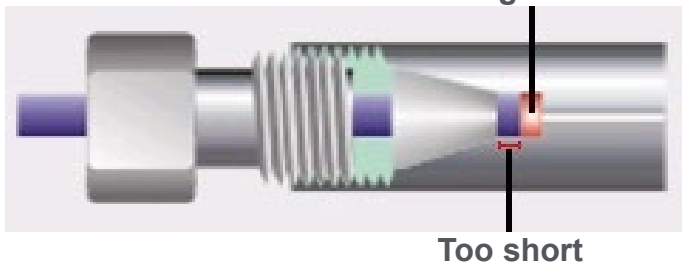
Different fitting types have different stem lengths

Factors Affecting Peak Shape
Fitting connections

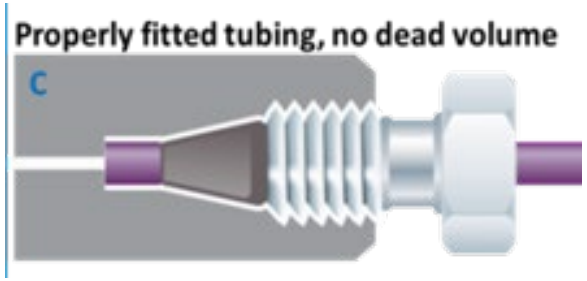
Bad



Bad

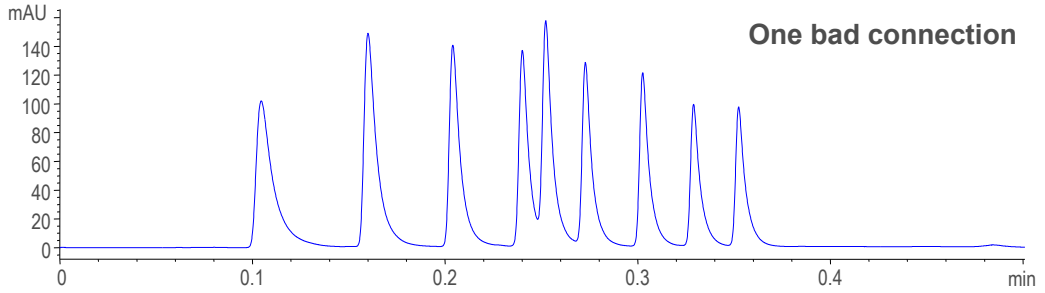


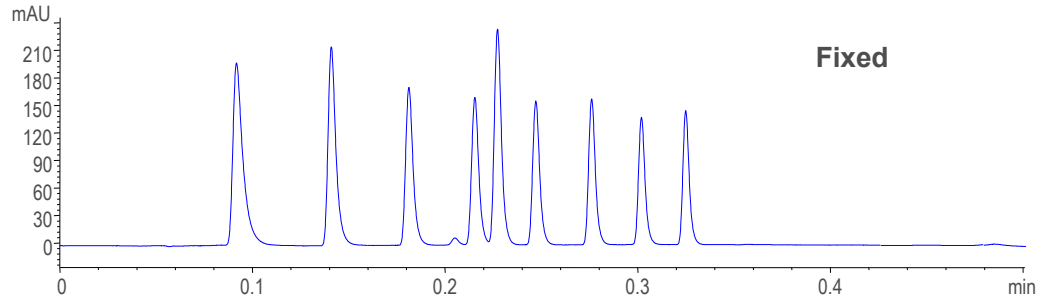
Good



Poor fitting connections

- Will broaden or split peaks or cause tailing
- Will typically affect all peaks, but especially early eluting peaks
- Can cause of carryover

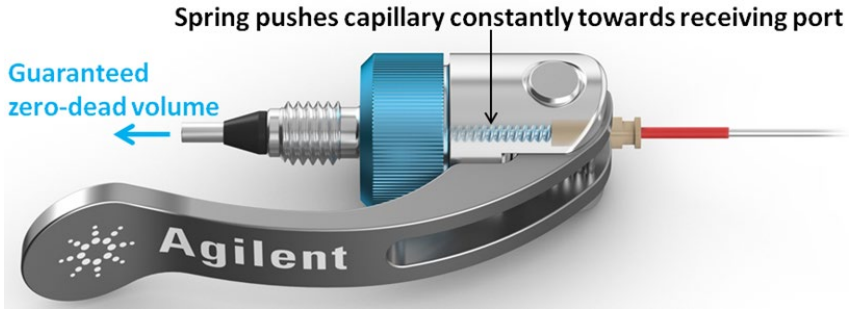




Factors Affecting Peak Shape

Fittings – InfinityLab Quick Connect and Quick Turn

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



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

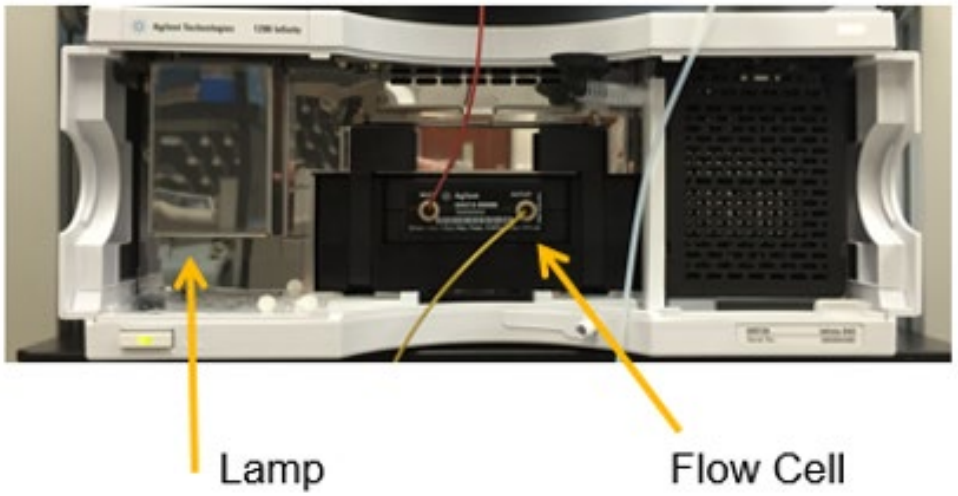
Quick Turn fitting
- Finger tight up to 400 bar
- Up to 800 bar with mounting tool
- Up to 1300 bar with a wrench
- Compact design, fits everywhere

Factors Affecting Peak Shape

System-related factors

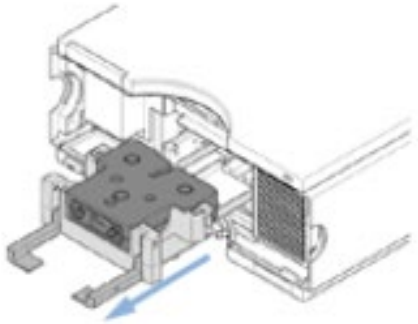
Detector

- Lamp
- Detector setting – response time/data collection rate
- Flow cell



Lamp and flow cells are available with RFID to track use and to predict replacement

Max-Light cartridge



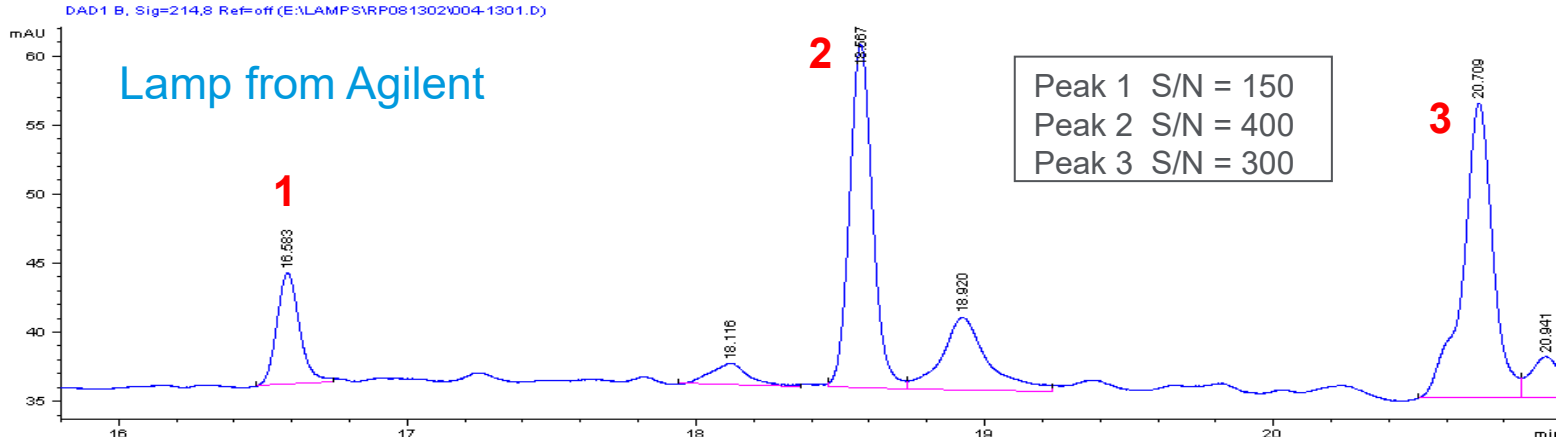
1290 Infinity and some 1260 Infinity systems

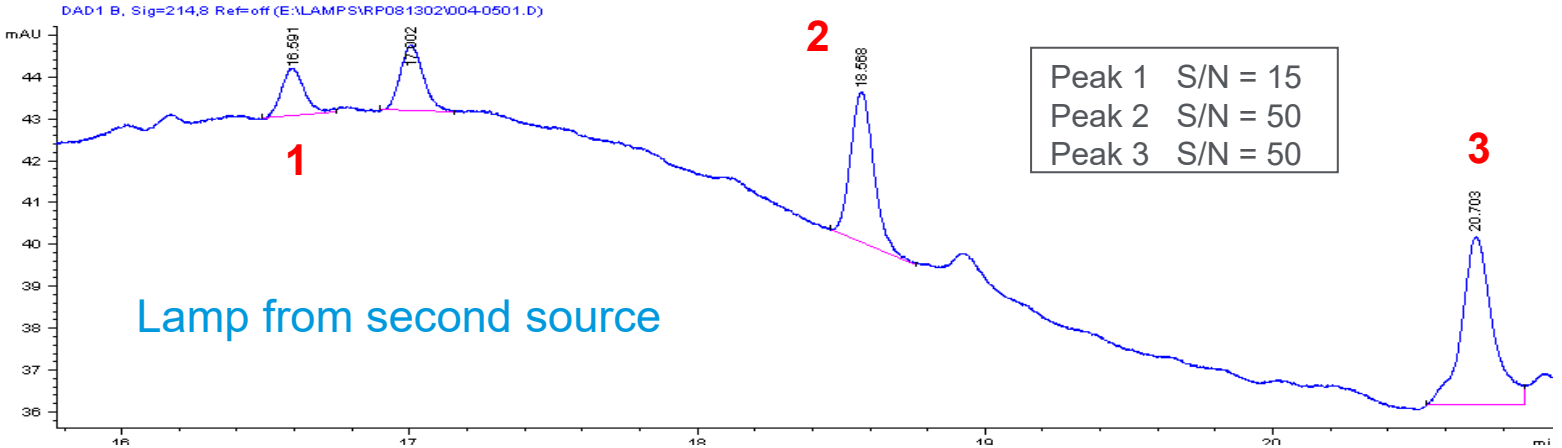
Factors Affecting Peak Shape

System-related factors – detector lamp

Agilent InfinityLab

Detector lamp performance



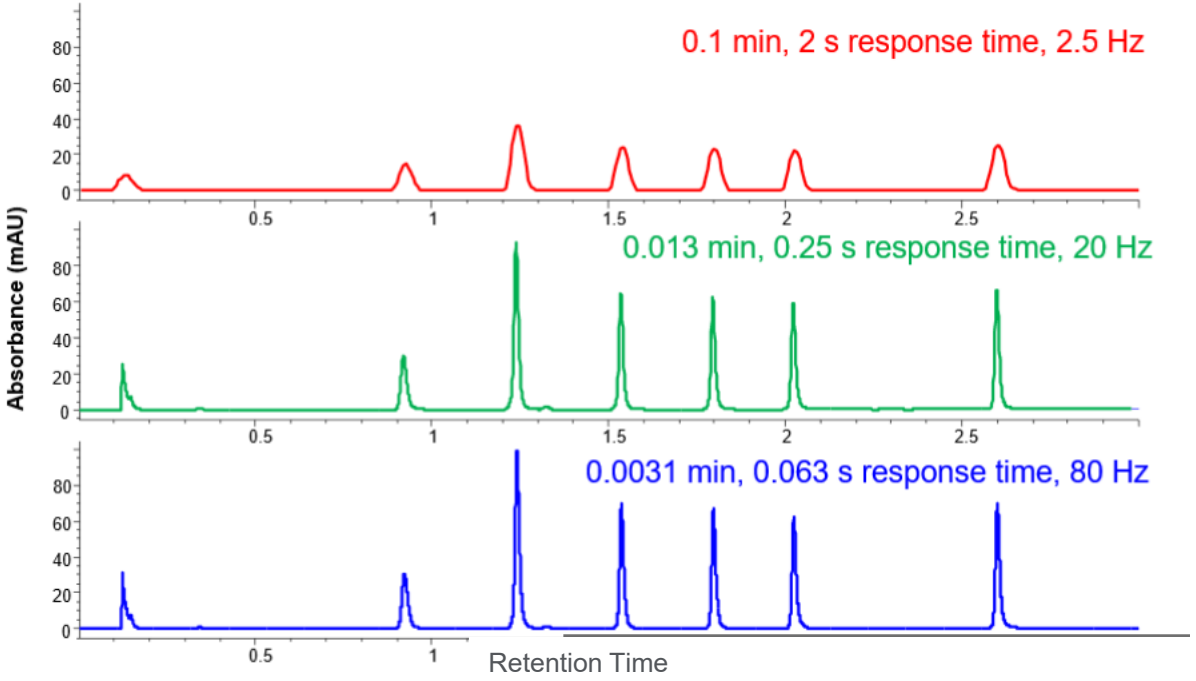


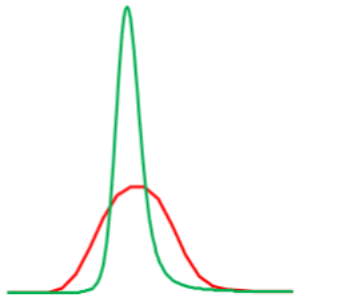
Agilent

Factors Affecting Peak Shape

System-related factors – detector setting

DAD setting – choose the right sampling rate





Changes in **Peak Width** and **Resolution**

Column: ZORBAX Eclipse Plus C18, 2.1x 50 mm, 1.8 µm
Column temperature: 35 °C
Flow rate: 1 mL/min
Gradient: 10-100% acetonitrile in 3 min
Signal: 254 nm, band width: 4 nm
Reference: 360 nm, band width: 100 nm

Factors Affecting Peak Shape

System-related factors – flow cells

Agilent InfinityLab

Match flow cell volume to chromatographic peak widths

Flow Cell Volume/Pathlength	Uv Signal /Noise	Chrom. Resolution*
13 µl / 10 mm	+++	+
5 µl / 6 mm	++	++
1.7 µl / 6 mm	+	+++

* Depends on analytical conditions and column dimension

13 µl Standard Flow Cell:
For highest sensitivity and linearity
4.6-3 mm ID, 2.7, 3.5, 5 µm columns

1.7 µl Micro Flow Cell:
For highest resolution
UHPLC, 1.8, 2.7 µm
2.1-1 mm ID columns

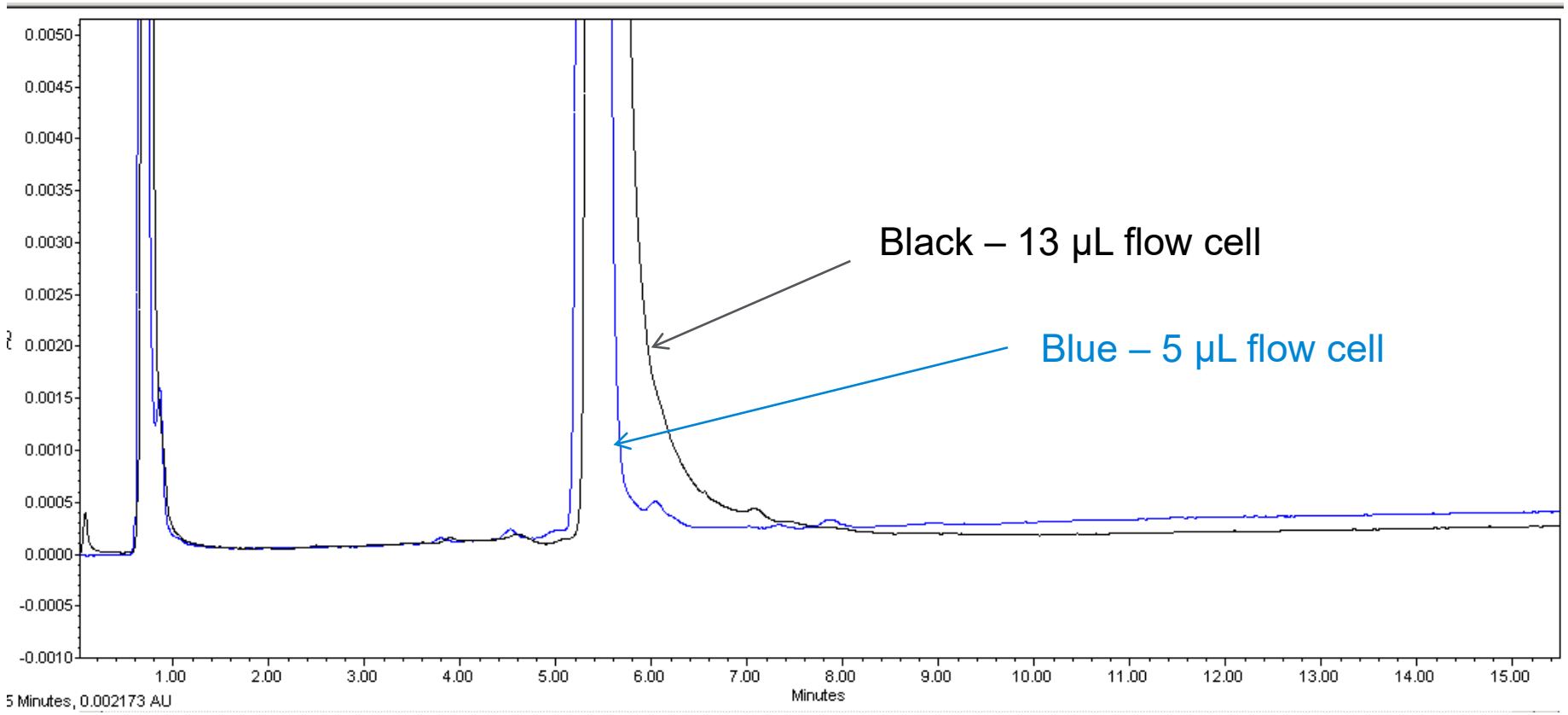
5 µl Semi-micro Flow Cell:
Best compromise of sensitivity & selectivity
HPLC/UHPLC, 1.8 to 5 µm
4.6 – 1 mm ID columns

Other flow cells include
Max-Light Cartridge cells for Infinity DAD
500 nL for capillary LC
80 nL for nano LC
0.6 mm for Prep LC

Factors Affecting Peak Shape

System-related factors – flow cell

To get good peak shapes, match the flow cell volume to the column



3 x 100 mm, 1.8 µm column

Factors Affecting Peak Shape

Sample-related factors

- Sample load
- Sample solvent strength
- Sample cleanliness
- Metal complexation – will be discussed in the “metal-sensitive compounds” section

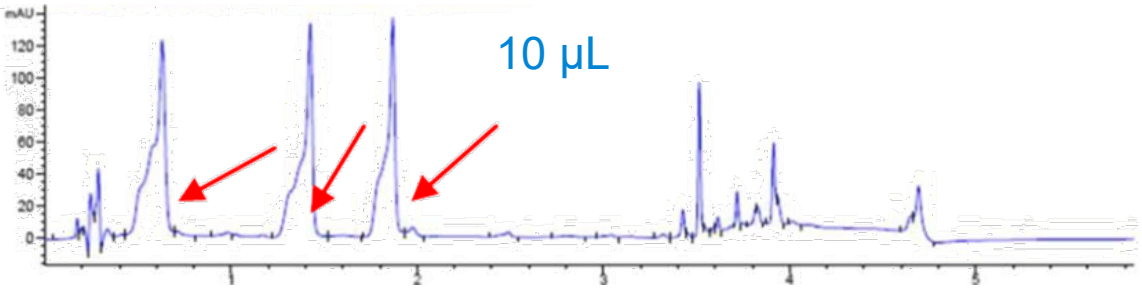
Factors Affecting Peak Shape

Sample-related factors – sample load

Sample overload may cause peak fronting/broadening/splitting/doubling

- Peak fronting from sample overload – more sample than can effectively partition, results in some sample preceding the rest of the peak

- Reduce sample load to eliminate the problem



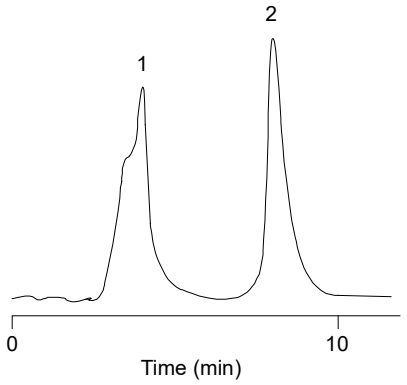
Factors Affecting Peak Shape

Sample-related factors – sample solvent strength

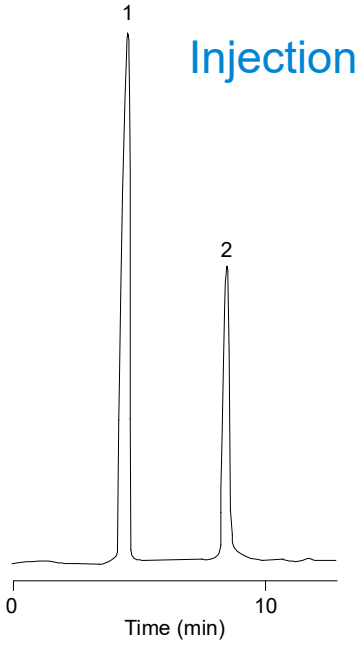
Agilent InfinityLab

Strong injection solvent may cause poor peak shape

Injection solvent: **100% acetonitrile**



Injection solvent: **mobile phase**

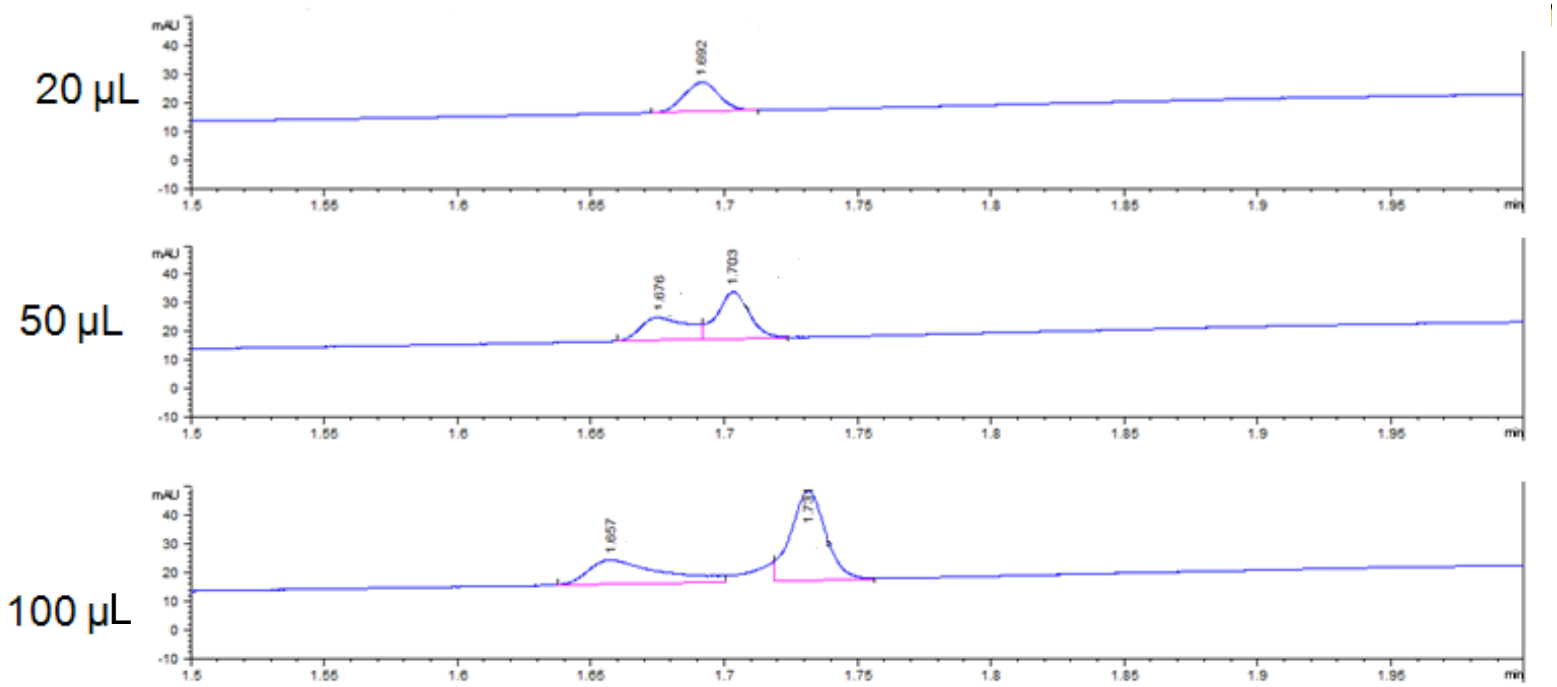


Column: StableBond SB-C8, 4.6 x 150 mm, 5 µm
Mobile phase: 82% H_2O : 18% ACN
Injection volume: 30 µL
Sample: 1. Caffeine 2. Salicylamide

Factors Affecting Peak Shape

Sample-related factors – sample solvent strength

Peak splitting when injecting a large volume of sample in a solvent stronger than the mobile phase



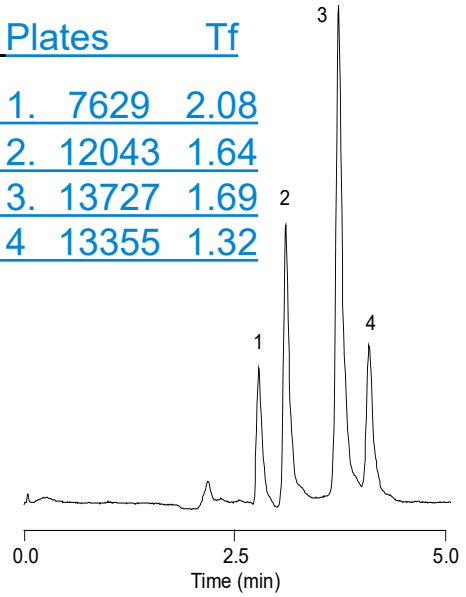
ZORBAX SB-C18, 4.6 x 50 mm, 1.8 µm
Mobile phase: 80% H_2O with 0.1% TFA; 20% ACN
Injection solvent; 40% H_2O, 60% ACN

Factors Affecting Peak Shape

Sample-related factors – sample cleanliness

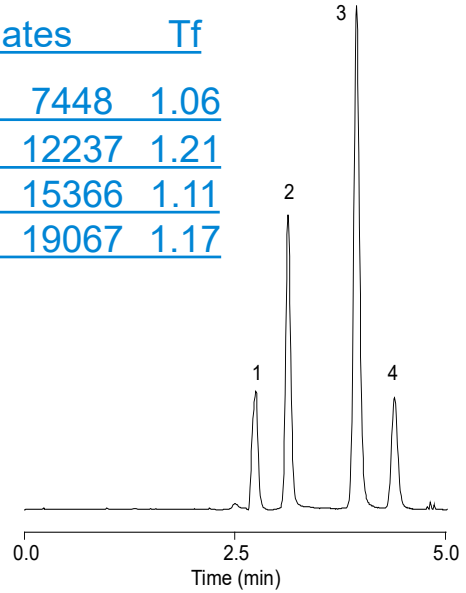
Column contamination from the samples causing peak tailing

	Plates	Tf
1.	7629	2.08
2.	12043	1.64
3.	13727	1.69
4	13355	1.32



QC test, contaminated column

	Plates	Tf
1.	7448	1.06
2.	12237	1.21
3.	15366	1.11
4	19067	1.17



QC test after cleaning the column

Column: StableBond SB-C8, 4.6 x 250 mm, 5 µm Mobile phase: 20% H_2O : 80% MeOH Flow rate: 1.0 mL/min
Temperature: ambient Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

Factors Affecting Peak Shape

Sample-related factors – sample cleanliness

Problem

- Dirty samples can partially clog the column inlet frit, causing split peaks.
- Chemical contamination from the sample can reside on the column and cause secondary interactions with analytes, resulting in peak tailing, broad peaks, or coelution with contamination peaks.

Solution

- Use an inline filter, guard, or online SPE
- Captiva line of physical and chemical filtration products
- Chem Elut S line of SLE products
- Bond Elut line of solid phase extraction products

Factors Affecting Peak Shape

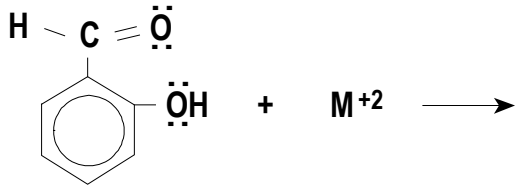
Metal-sensitive compounds

- Metals are present in the LC system, column, tubing, ferrules, and frits
- Metal-sensitive compounds, such as proteins, can interact with metal parts of the flow path
- Analytes that can complex with metals may show poor peak shape
- A column packed with high-purity silica eliminates silica as a source of metals

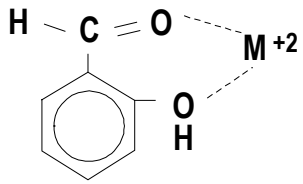
Factors Affecting Peak Shape

Metal-sensitive compounds

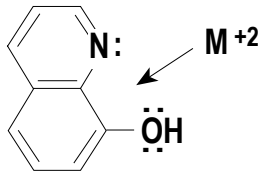
Some metal-sensitive compounds can chelate with metals



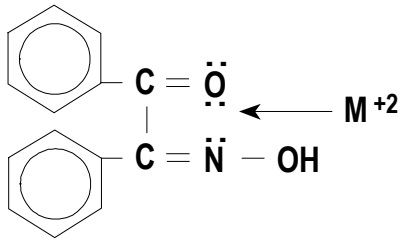
Salicylaldehyde



6-membered ring complex



8-hydroxyquinoline
5-membered ring complex



α-benzoinoxomine
5-membered ring complex

Hint: Look for lone pair of electrons on oxygen or nitrogen which can form a 5 or 6-membered ring with metal.

Factors Affecting Peak Shape

Metal-sensitive compounds

What to do to minimize these interactions?

- Use a PEEK-lined column
- Use a Bio-Inert LC system
- Use bio-inert capillaries and fittings
- Passivate the LC system
- Use InfinityLab deactivator additive in the mobile phase

Factors Affecting Peak Shape

Metal-sensitive compounds

For metal sensitive compounds, use the bio-inert LC system

- Metal-free sample flow path minimizing unwanted surface interaction
- Inert flow cells for UV and fluorescence detection
- Inert solvent and column selection valves
- Novel bio-inert capillaries, InfinityLab Quick Connect/Quick Turn Fittings
- High salt tolerance (2 M) and wide pH range (1–13, short term 14)

1260 Infinity II bio-inert LC system

Factors Affecting Peak Shape

Metal-sensitive compounds

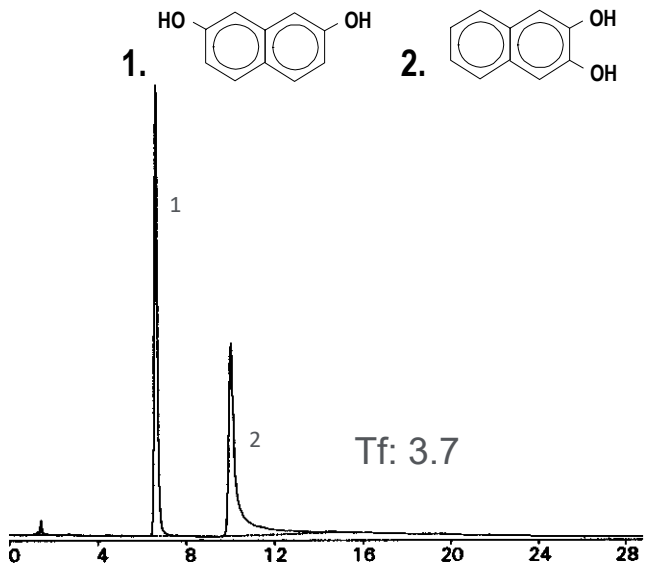
System passivation-acid wash

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

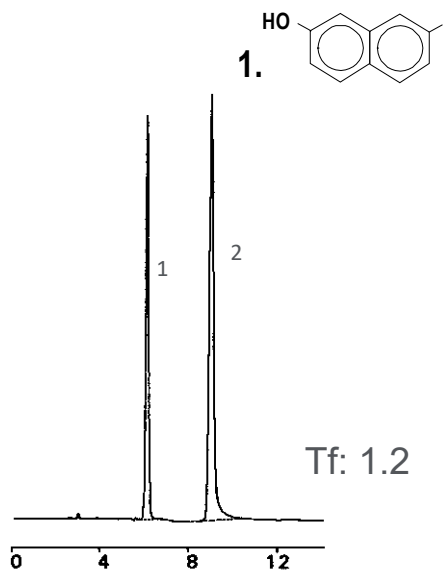
Factors Affecting Peak Shape
Metal-sensitive compounds

System passivation can improve peak shape of metal complexing compounds

Before passivation



After passivation



Columns: ZORBAX SB-Phenyl
4.6 x 150 mm

Mobile phase: 75%, 25 mM ammonium phosphate buffer,
25% acetonitrile

Flow rate: 1.0 mL/min

Temperature: ambient

Sample size: 5 µL

1% H_3PO_4 in 10% acetonitrile solution is used on SB columns

0.5% H_3PO_4 in 10% acetonitrile solution can be used for endcapped columns

Factors Affecting Peak Shape

Metal-sensitive compounds

InfinityLab deactivator additive for metal-sensitive compounds

Benefits	Improvement
Reduce metal-analyte interaction	• Chelate-free metals, covers exposed active sites in sample flow path, reducing unwanted metal-analyte interactions and allowing lower detection limits using LC/MS
Amenable to LC/MS use	• Optimized for use at a 5 µM (1:1000 dilution) with minimal ion suppression effects • Does not persist in the LC/MS system after use (unlike traditional ion pairing reagents)
Operational time and cost savings	• Saves the time needed to passivate your system • Can avoid derivatization • Can avoid potential system contamination from ion pairing agents • Limits of detection can be lowered for challenging compounds, such as phosphorylated metabolites, phosphate pesticides, and organic acids

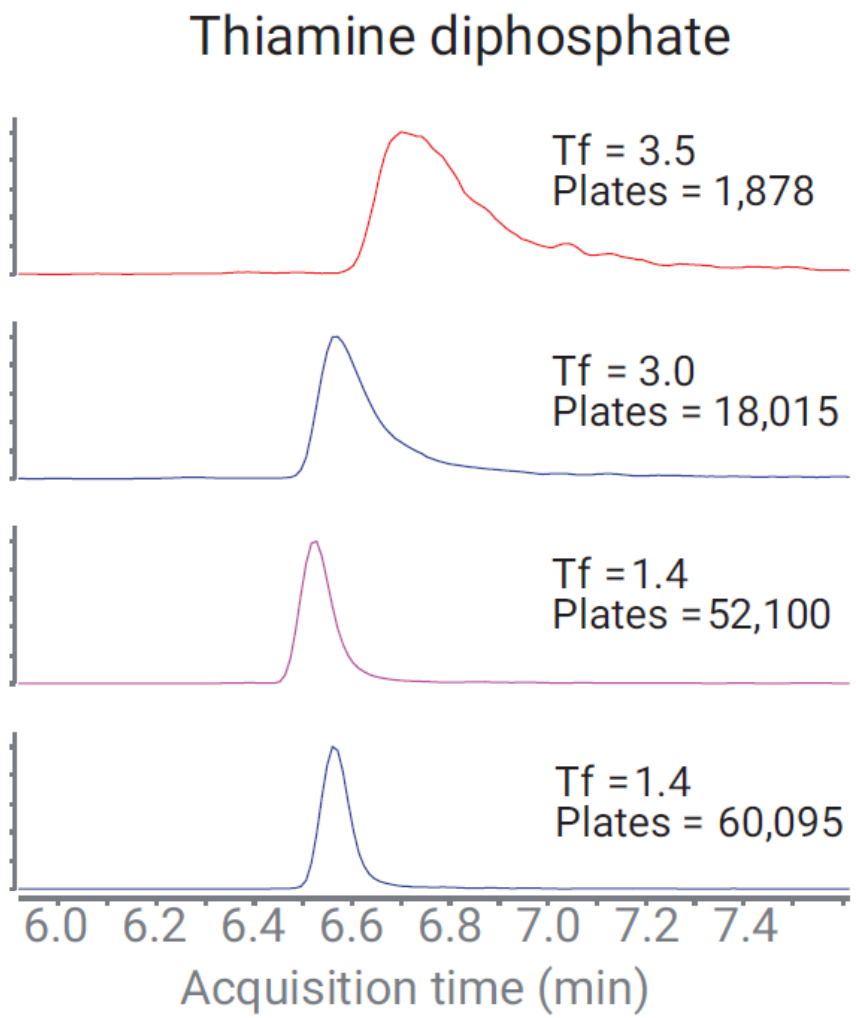
InfinityLab deactivator additive
50 mL: 5191-4506
25 mL: 5191-3940

Recommended read

More information can be found in the InfinityLab Deactivator Additive user guide 5991-9516EN.

Factors Affecting Peak Shape

Stepwise improvements for metal-sensitive analytes



Before system passivation

↓

After LC passivation with 0.5% phosphoric acid in 9:1 acetonitrile/water

↓

Added InfinityLab deactivator additive to mobile phase

↓

Installed PEEK-lined HILIC-Z column

Guidelines for Improved Peak Shape

- Select columns based on high-purity, fully hydroxylated silica, such as InfinityLab Poroshell line of columns, as well as ZORBAX Eclipse Plus, StableBond, Eclipse XDB, Bonus-RP and Extend-C18
- Select double or triple endcapped columns for mid pH or difficult basic compounds
- Select special bonded phases (InfinityLab Poroshell 120 HPH-C18 and CS-C18, ZORBAX Bonus-RP, ZORBAX Extend-C18) for better peak shape at mid and high pH
- Select wide-pore columns for high molecular weight analytes
- Use spring loaded fittings, such as InfinityLab Quick Connect and Quick Turn together with appropriate connecting capillaries
- Use buffered, low pH mobile phases to reduce secondary interactions
- Use 20 to 50 mM buffered mobile phases at every pH
- Use mobile phase additives when needed
- Do sample cleanup
- Check sample solvent and its strength
- Use optimized flow rate and data collection rate

Resources for Support

- Agilent University http://www.agilent.com/crosslab/university
- Tech support http://www.agilent.com/chem/techsupport
- Resource page http://www.agilent.com/chem/agilentresources
  - Quick reference guides
  - Catalogs, column user guides
  - Online selection tools, how-to videos
- InfinityLab LC Supplies catalog (5991-8031EN)
- LC handbook (5990-7595EN)
- Best Practices for Using an Agilent LC System (01200-90090)
- LC Troubleshooting poster (5994-0709EN)
- Your local FSE and specialists
- Youtube – Agilent Channel (maintenance videos)
- Agilent service contracts

Contact Agilent Chemistries and Supplies Technical Support

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, filtration, and QuEChERS

Option 4 for spectroscopy supplies

Option 5 for chemical standards

Available in the U.S. and Canada, 8-5 all time zones

gc-column-support@agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

chem-standards-support@agilent.com

advancebio.glycan@Agilent.com

WebChat: product pages of agilent.com

Appendix

Column Cleaning Procedure for Reversed Phase Columns

Flush with stronger solvents than your mobile phase

Reversed-Phase Solvent Choices
in Order of Increasing Strength

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile
- 75% Acetonitrile:25% Isopropanol
- 100% Isopropanol
- 100% Methylene Chloride*
- 100% Hexane*

Use at least 10 column volumes of each solvent for analytical columns

* When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.