

A Look at Column Choices

Does one-size-fit-all?



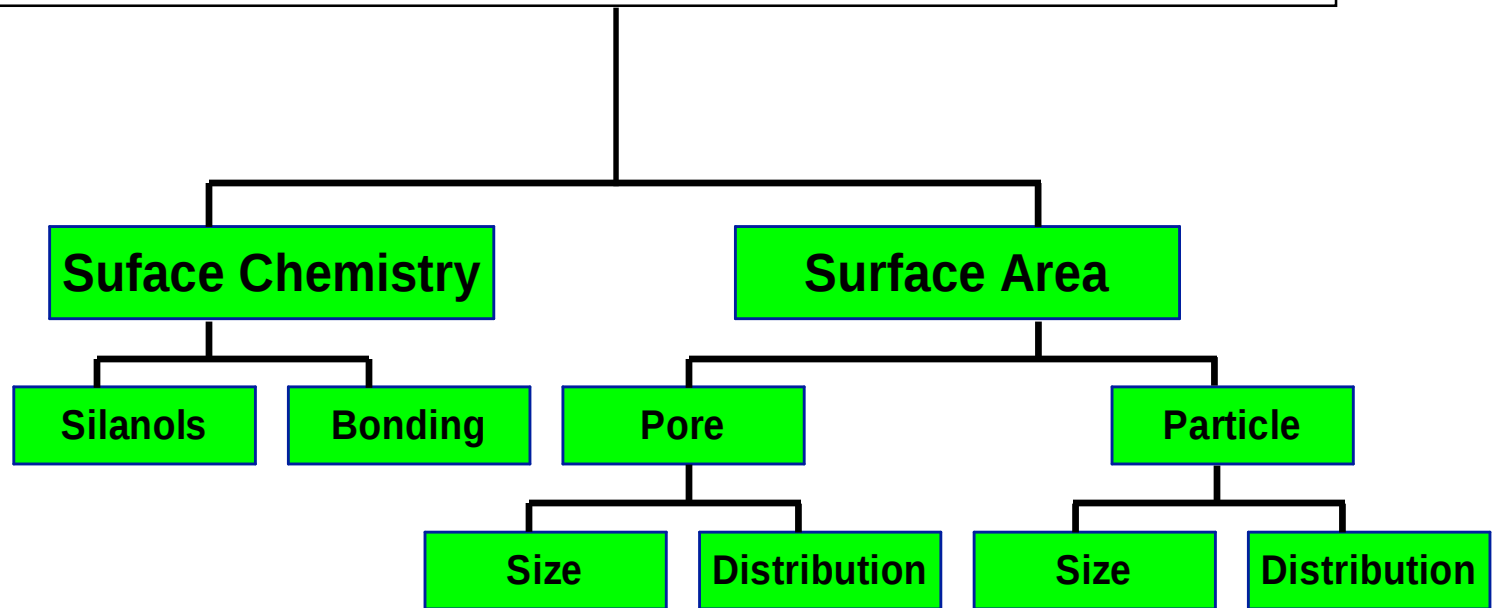
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Seminar Outline

- Silica Surface Chemistry & Physical Characteristics
- Silica Bonding Procedures
 - Special Column Chemistries
- Column Choices



Silica Column Characteristics

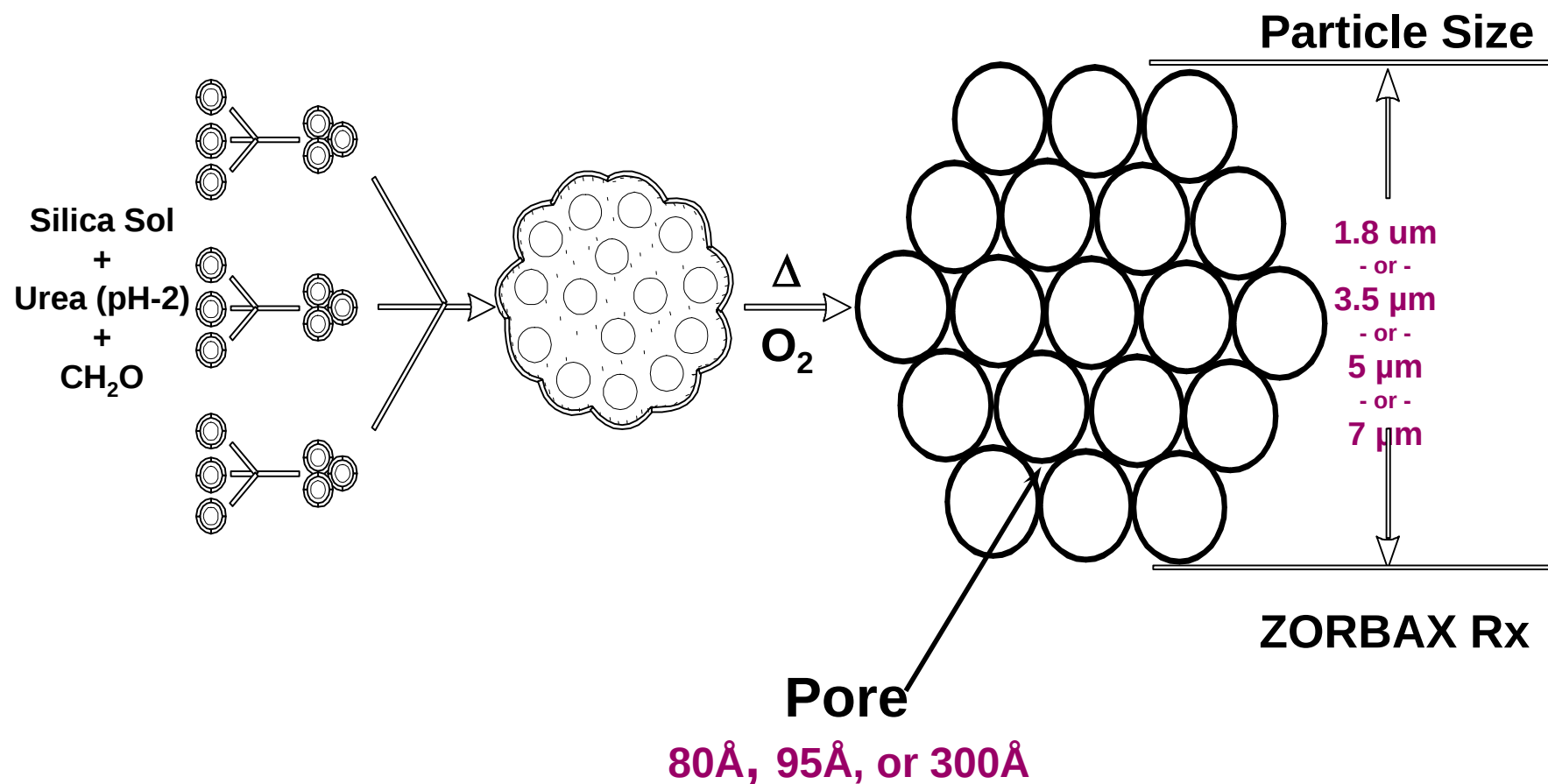


NOTE: SILICA IS STABLE AT $1.0 > \text{pH} < 11.0^*$

*** range depends on manufacturer's bonding**



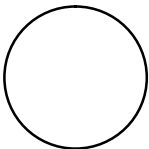
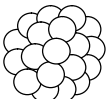
ZORBAX Porous Silica Particles



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History of HPLC Particle Development

Year(s) of Acceptance	Particle Size	Most Popular Nominal Size	Plates / 15cm
1950's		100µm	200
1967		57µm (pellicular)	1,000
1972		10µm	6,000
1985		5µm	12,000
1992		3.5µm	22,000
2003		≤2µm*	>30,000

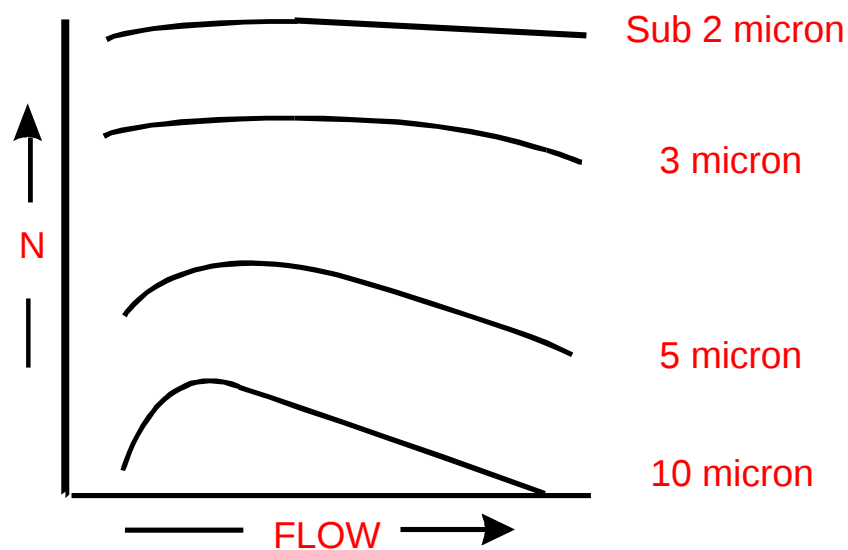
*Zorbax "Rapid Resolution HT", product launch 5/1/03



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Columns Packed with Smaller Particles Provide Higher Efficiency



Pore Size Recommendations

Use 60 - 80Å pore size column packings to separate small molecules equal to or less than 4000MW to maximize loading capacity and retention.

Use 95 or 300Å pore size columns for larger molecules like polypeptides and proteins (from 4000 to 500,000 MW) to maintain high efficiency.

Increase column diameter to increase loading capacity.



Chemistry Variations

Are All C-18s the Same?

1. Type of bonding
2. Completeness of bonding (Carbon Load)
3. Silica Chemistry
4. Bonding Chemistries
 - End-capping

NO!

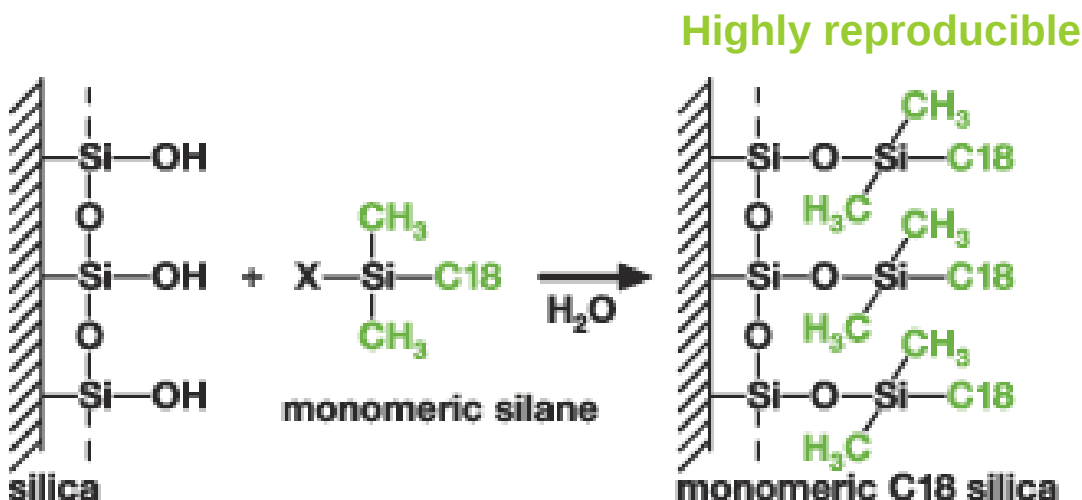


Monomeric vs. Polymeric Bonding

Monomeric bonding

Typical ZORBAX Bonding

- Eclipse Plus
- Eclipse XDB
- StableBond
- Bonus-RP
- Agilent HC/TC



Polymeric Bonding

Eclipse PAH

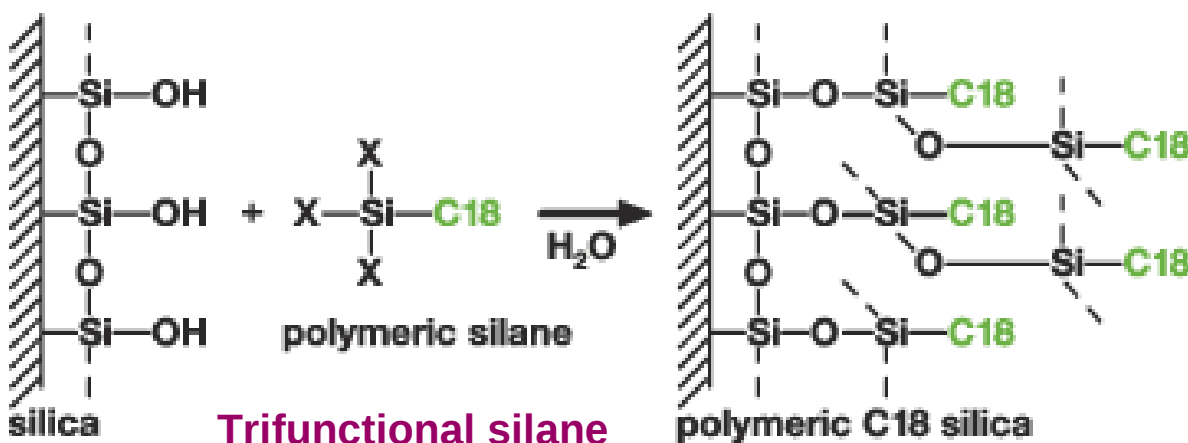


figure courtesy of Vydac



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2. Carbon Load

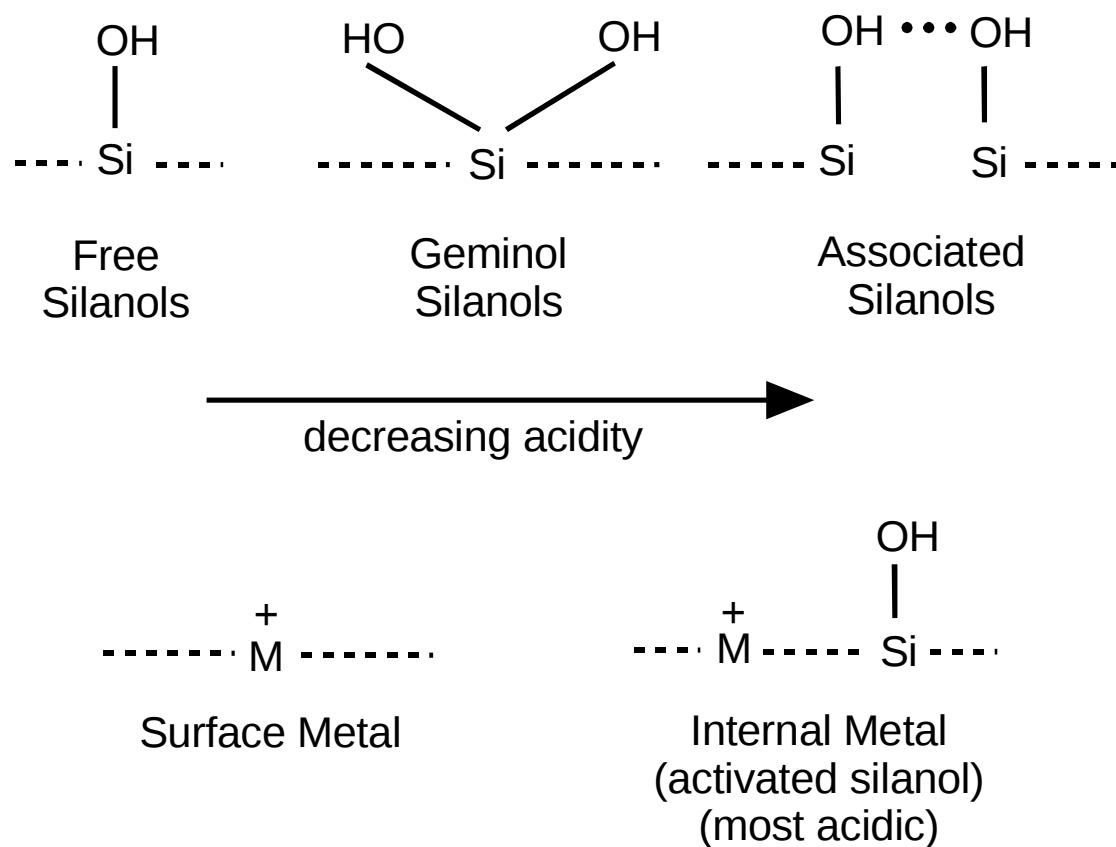
Carbon Load refers to the % carbon content of the silica bonded stationary phase.

Generally speaking, a high carbon load (example 18-25%) results in a more hydrophobic surface. The surface is also more resistant to high pH.

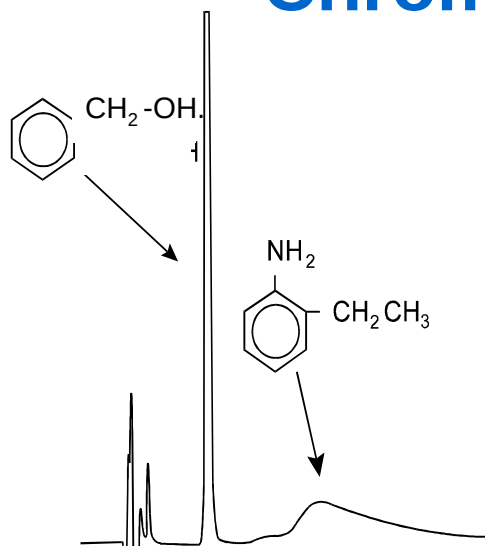
A high carbon load does not necessarily provide the best resolution.



3. The Surface of Silica Supports

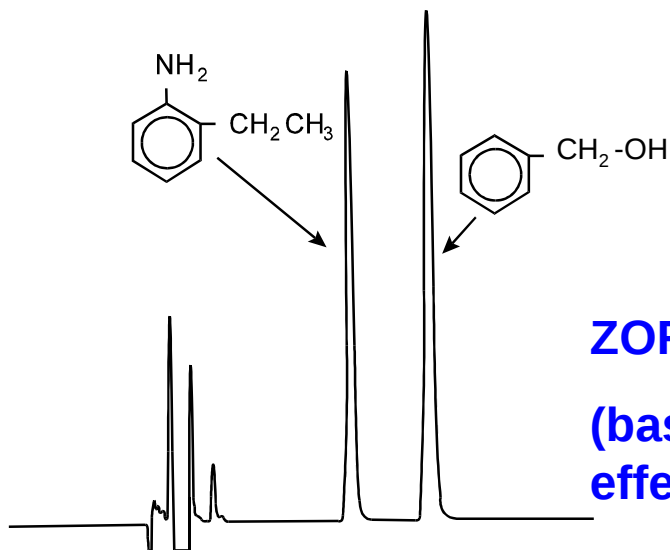


Chromatographic Improvement Using Highly Purified Zorbax Rx-Sil



Original ZORBAX, 1973 and other type A silicas
(basic compound can tail)

Conditions: Flow Rate: 2.0 mL / min.
Mobile Phase: 5% 2-Propanol in Heptane



ZORBAX Rx-Sil, 1987 and other Type B silicas
(basic compounds have less tailing; lower
effective silanol pKa)

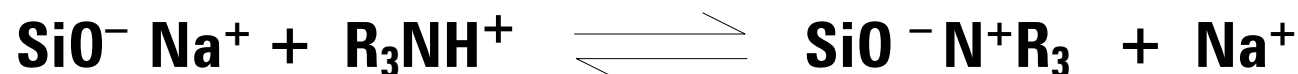


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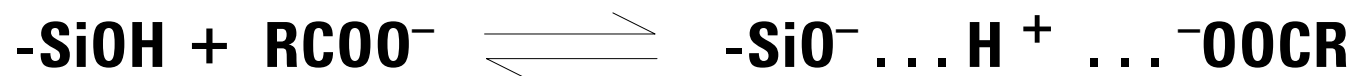
Potential Ion Exchange and Hydrogen Bonding Secondary Interactions

Ion-exchange



1. Ionized silanols (SiO^-) will ion-exchange with protonated bases (R_3NH^+) which can cause tailing and method variability. This occurs most often at mid pH where silanols are ionized.

Hydrogen Bonding



2. Unprotonated acids can compete for H^+ with protonated silanols. This can occur at low pH.

Some mobile phase additives can be added to the mobile phase to reduce these interactions and this will be discussed in the mobile phase section.



Zorbax StableBond with Rx-SIL Improves Peak Shape

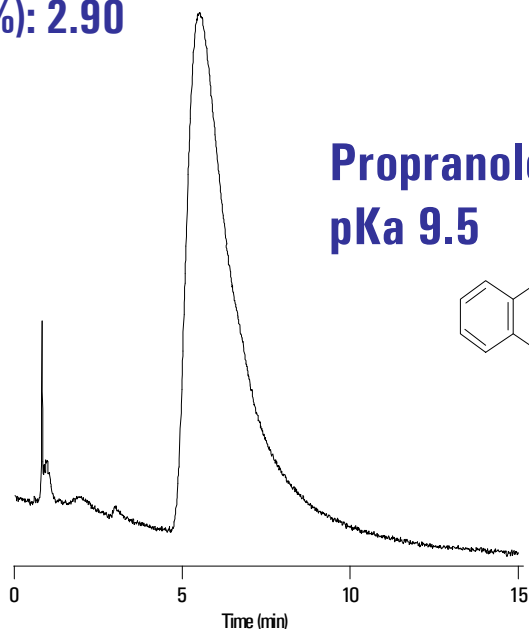
Mobile Phase: 75% 50 mM KH_2PO_4 , pH 4.4 : 25% ACN Flow Rate: 1.5 mL/min

Silica Type – More Acidic

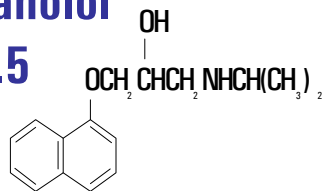
Column: ODS, 4.6 x 250 mm, 5 μm

Plates: 92

USP T_f (5%): 2.90



Propranolol
pKa 9.5

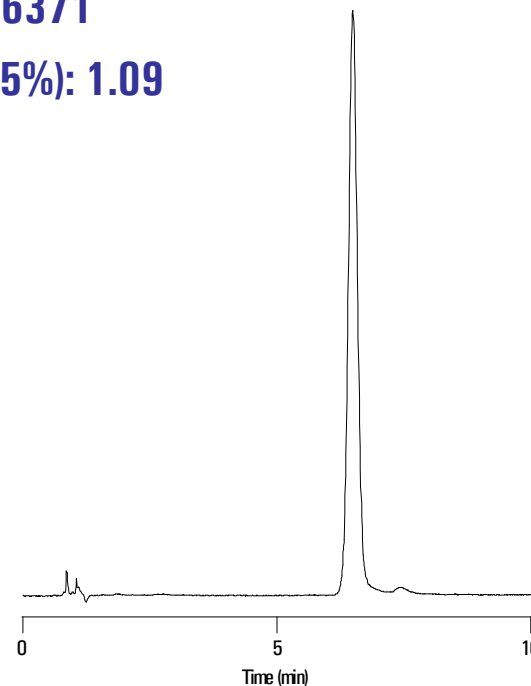


Silica Type – High Purity, Rx-Sil

Column: SB-C18, 4.6 x 150 mm, 5 μm

Plates: 6371

USP T_f (5%): 1.09



- The high purity Rx-SIL improves the peak shape dramatically on a C18 column



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So What Bonded-Phase Do I Choose?*

- C18** offers the most retention, maybe too much for some samples
- C8** less retentive than C18, but with similar selectivity in most cases
- Phenyl** significantly less retentive for non-polar compounds but retains polar compounds, so reduces analysis time for mixture if polar/non polar
- CN** least retentive, changes in selectivity, good for reducing analysis time with late eluters and avoiding gradient separations

* Greater than 20% Organic



So What Bonded-Phases Do I Choose?

High Aqueous* Mobile Phases

- C18 rarely offers retention advantages, and selectivity may not be optimum with little retention
- C8 often provides a little more retention than C18 with similar selectivity
- Phenyl most retentive with changes in selectivity
- C3 similar to Phenyl in retention but some changes in selectivity from C18, C8 and Phenyl
- CN least retentive, definite changes in selectivity, good resolution

* Below 20% Organic

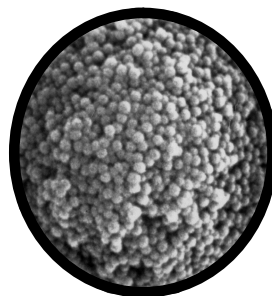


4. ZORBAX Bonding Technology

All Purpose Phases

- StableBond
- Eclipse XDB
- Eclipse Plus

Rx-Silica Support



- Ultra-pure
- Spherical, consistent pore size
- Fully-hydroxylated
- Sol gel maximizes strength and lifetime

Application Targeted Phases

- Bonus-RP
- Extend

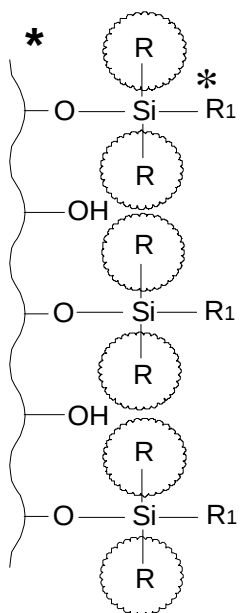


Match Method pH and Column Choice

Choose the Best Bonded-Phase for Each pH Range

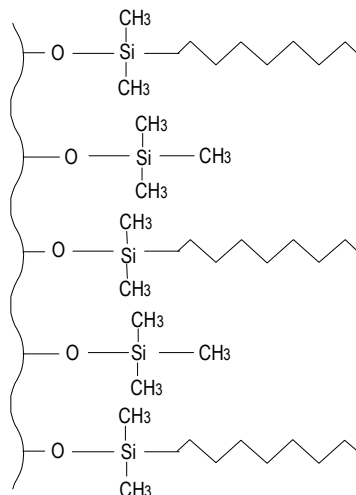
StableBond, pH 1-6

1. Uses bulky silanes
2. Non-encapped



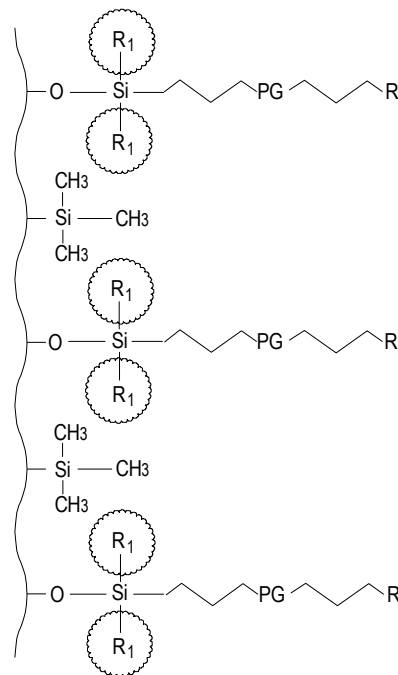
Eclipse Plus & XDB, pH 2-9

1. Proprietary double-endcapping



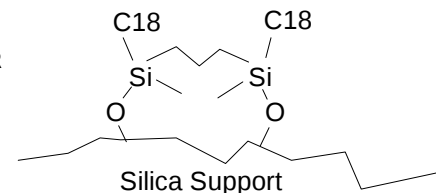
Bonus-RP, pH 2-8

1. polar alkyl phase
2. triple endcapped
3. uses bulky silanes



Extend-C18, pH 2-11.5

1. unique bidentate structure
2. double endcapped



ZORBAX StableBond Bonding

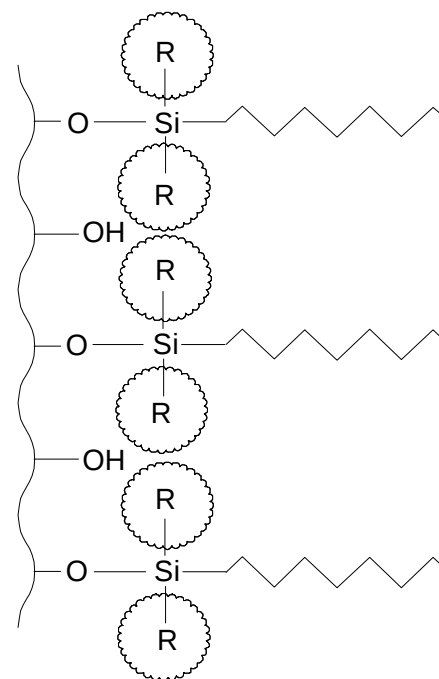
Superior low pH stability – down to pH 1

Non-endcapped for selectivity and lifetime

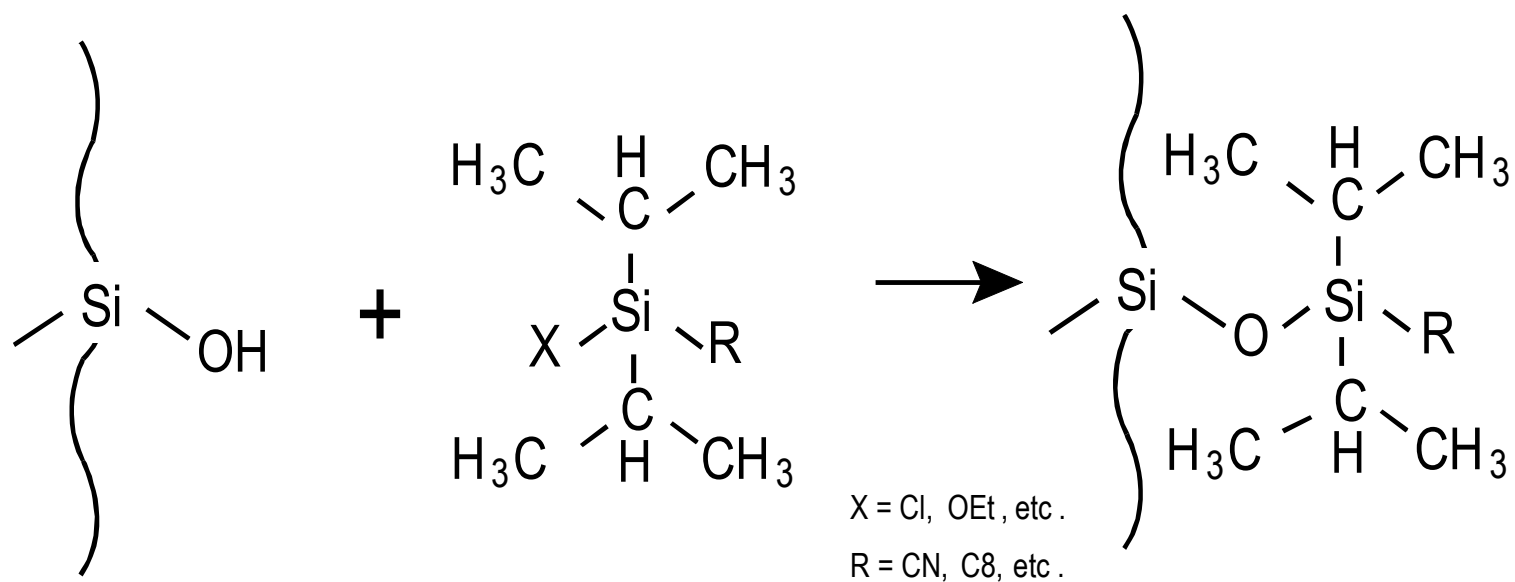
Patented sterically protecting bonding

5 different selectivities - C18, C8, CN, Phenyl, C3

Ideal for most sample types at low pH



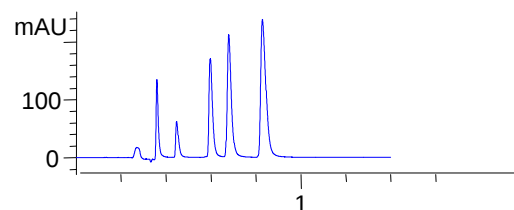
StableBond Reaction to Make a Sterically-Protected Surface



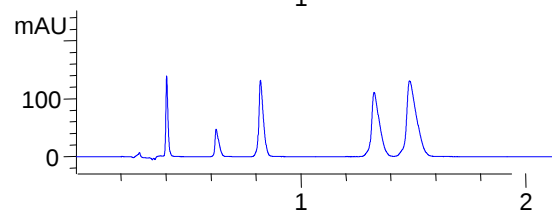
- **Diisopropyl silanes or diisobutyl silanes (C18)**



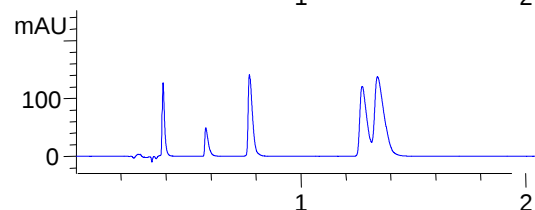
Bonded Phase Selectivity Differences in 30% ACN



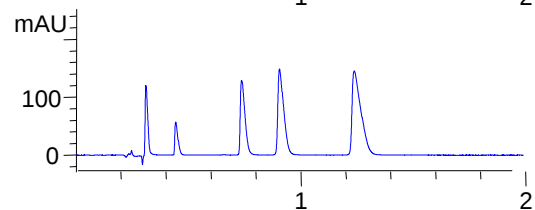
RRHT SB-CN
4.6 x 50 mm, 1.8 μ m



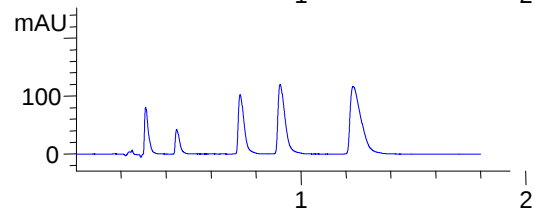
RRHT SB-Phenyl
4.6 x 50 mm, 1.8 μ m



RRHT SB-AQ
4.6 x 50 mm, 1.8 μ m



RRHT Eclipse Plus C18
4.6 x 50 mm, 1.8 μ m

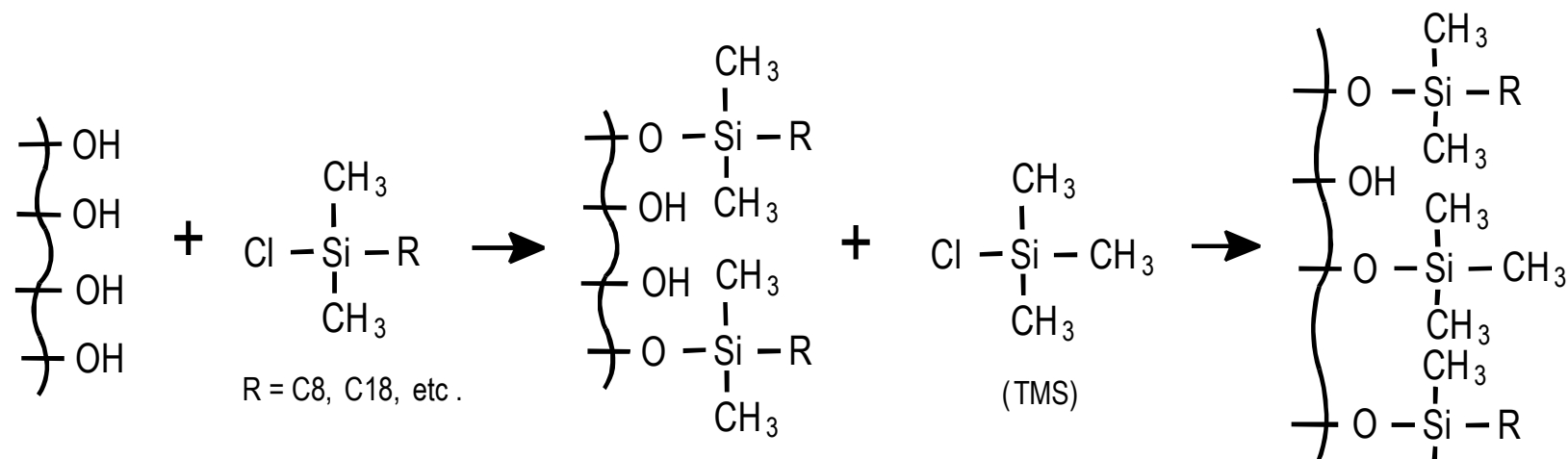


RRHT SB-C18
4.6 x 50 mm, 1.8 μ m

- 5 Different bonded phases compared
- Analysis time of each run is only 2 minutes
- Comparison done in optimum % organic
- The fast runs mean a comparison can be done even if you have a good separation on the C18
- More chances to optimize!



Traditional Stationary Phase Bonding and Endcapping Reaction



- Dimethyl silanes
- Endcapped with TMS



ZORBAX Eclipse Plus & XDB Columns

Improved peak shape for basic compounds – especially at intermediate pH

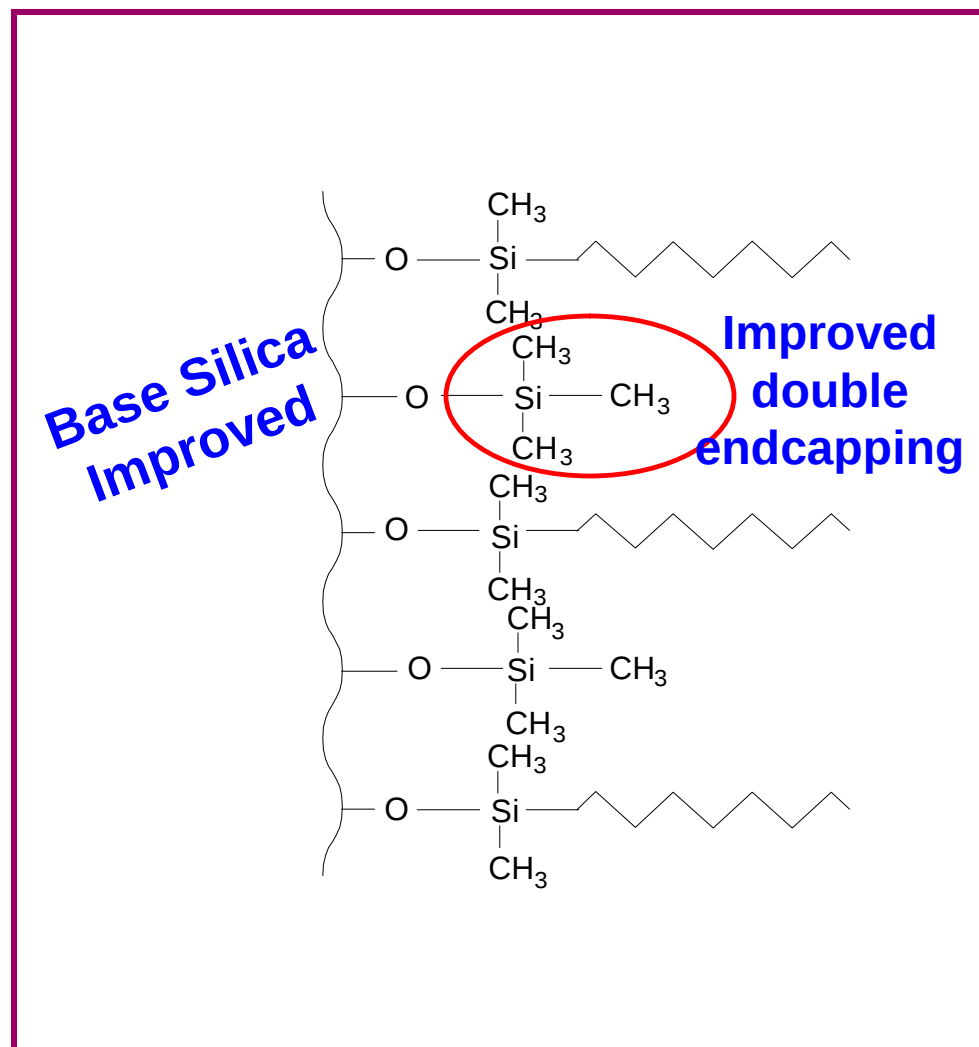
Good for all sample types – acid, base, neutral

Wide useable pH range, pH 2-9

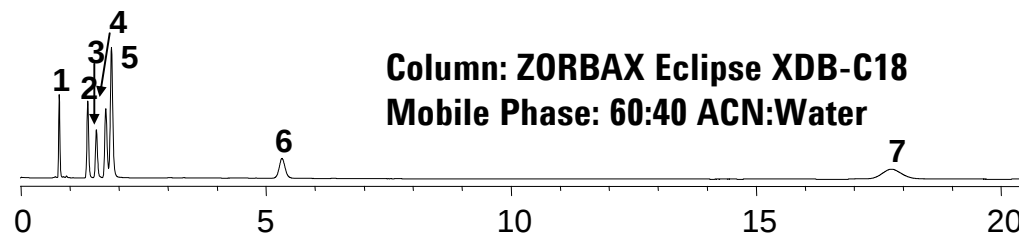
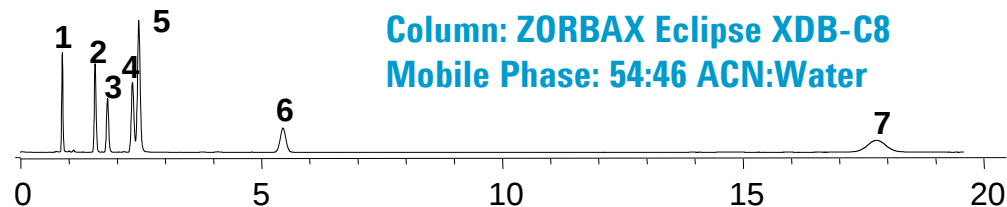
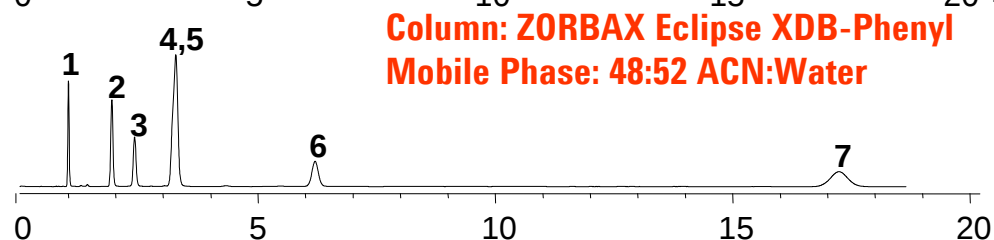
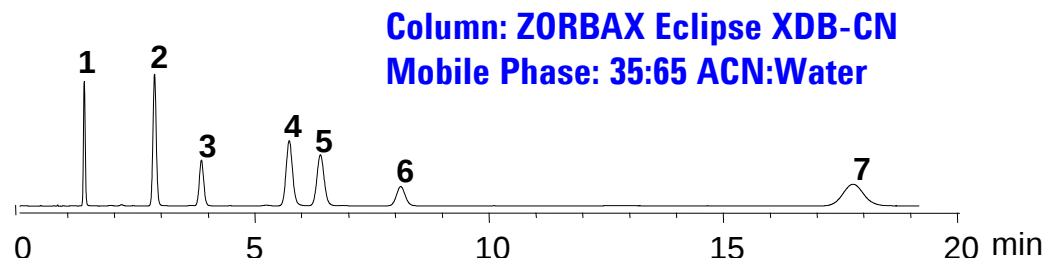
Double end-capped

Pore size:

XDB 80Å, **Plus 95Å**



Selectivity of Polar Phases Provides Optimum Separation of Steroids Versus Non-Polar C18/C8



Column Dimensions: 4.6 x 150 mm, 5 μ m
Flow Rate: 2.000 ml/min
Injection Volume: 2.00 μ l
Column Temperature: 25 $^{\circ}$ C
Detector: UV, 210 nm

Sample:

1. Estriol (0.00130 μ g/ μ l),
2. β -Estradiol (0.00130 μ g/ μ l),
3. Ethinyl Estradiol (0.00147 μ g/ μ l),
4. Dienestrol (0.00123 μ g/ μ l),
5. Diethylstilbestrol (0.00128 μ g/ μ l)
6. Ethynylestradiol 3-methyl ether (0.00103 μ g/ μ l)
7. Ethynodiol Diacetate (0.00139 μ g/ μ l)

➤ **Eclipse XDB-CN is the optimum bonded phase – the only one that can resolve all the components in under 20 minutes.**

ZORBAX Bonus-RP

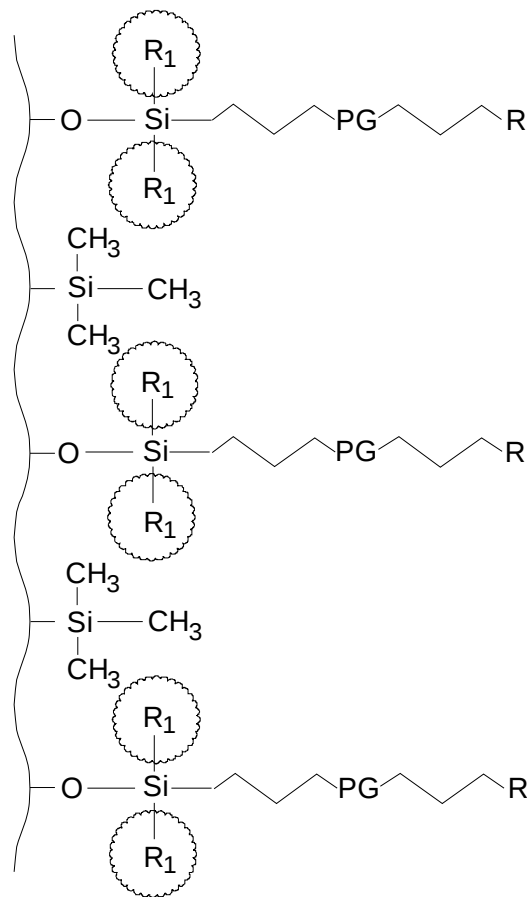
Good peak shape of basic compounds

Polar alkyl-amide bonded phase

Unique selectivity

Enhanced low pH stability with sterically protecting bonding

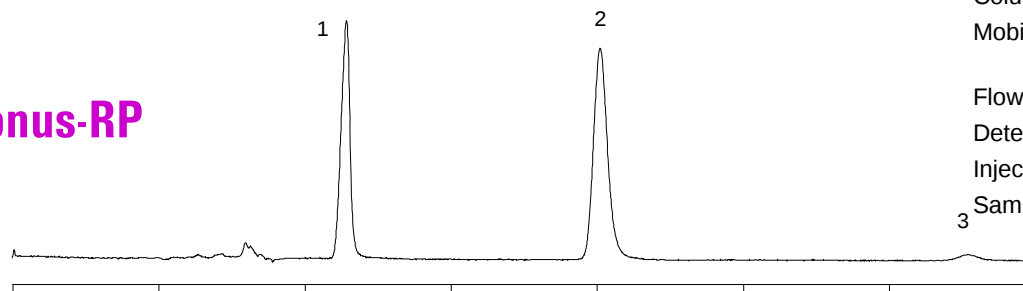
Triple endcapped



Improved Peak Shape of Basic Compounds

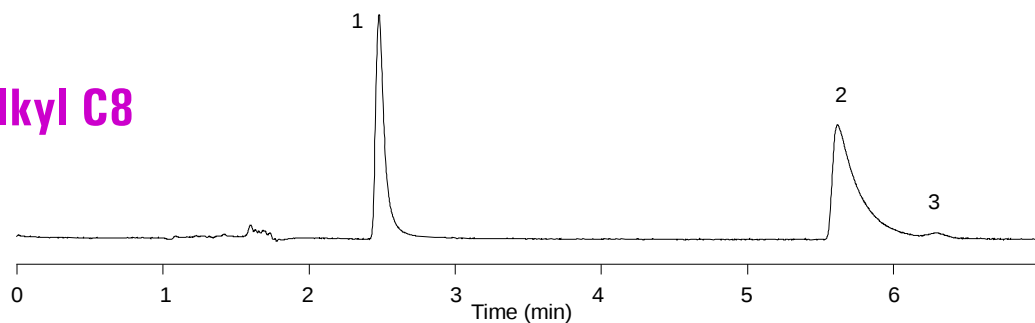
Separation of Small Molecule Anorectics on ZORBAX Bonus-RP and Traditional Alkyl Phase

Bonus-RP



Columns: 4.6 x 150 mm, 5 μ m
Mobile Phase: 45% 25 mM K_2HPO_4 , pH 7.2 : 55% (MeOH:ACN, 50:50)
Flow Rate: 1.0 mL/min.
Detection: 254 nm
Injection vol: 5 μ L
Sample: Anorectics ("Fen-phen")
1. Phentermine pKa 10.1
2. Fenfluramine pKa 9.1
3. Impurity

Alkyl C8



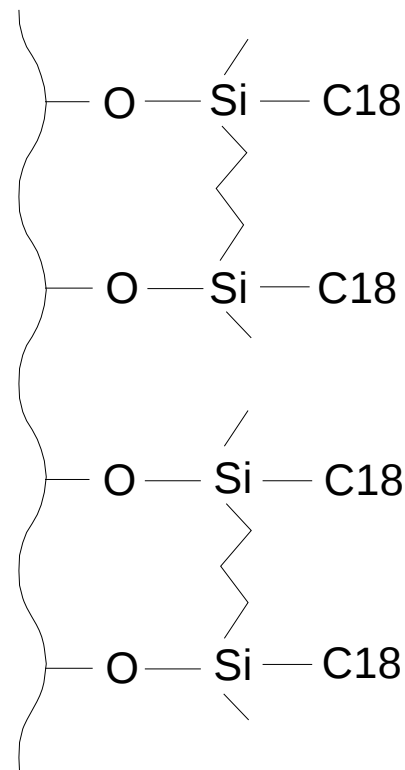
ZORBAX Extend-C18

Superior high pH stability – up to pH 11.5 with silica particles

Excellent reproducibility

Patented bidentate, C18 bonding

Double endcapping

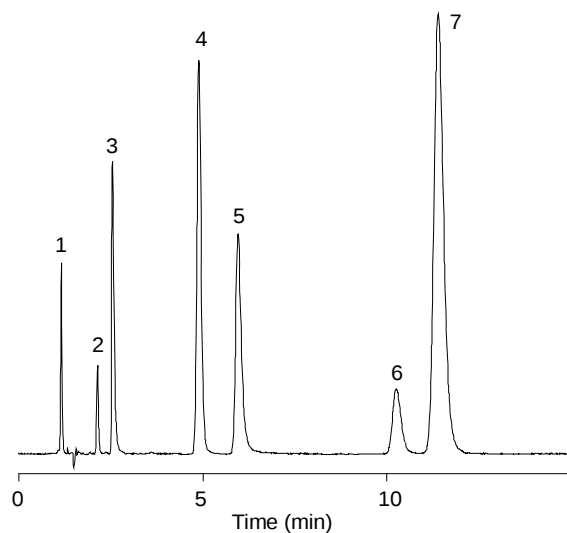


Improved Retention of Basic Antihistamines on ZORBAX Extend-C18

pH 11

30% 20 mM TEA

70% MeOH

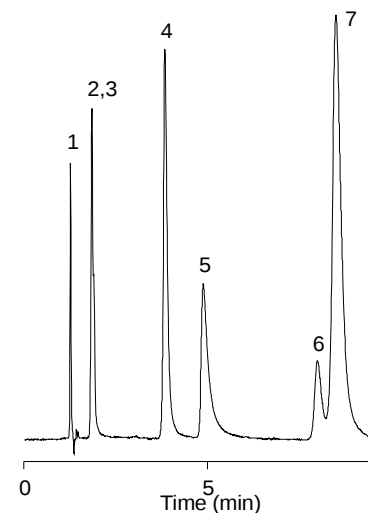


Column: 4.6 x 150 mm, 5 μ m
Mobile Phase: See Above
Flow Rate: 1.0 mL/min
Temperature: RT
Detection: UV 254 nm
Sample:
1. Maleate
2. Pseudoephedrine
3. Scopolamine
4. Doxylamine
5. Chlorpheniramine
6. Triprolidine
7. Diphenhydramine

pH 7

30% 20 mM Na_2HPO_4

70% MeOH



HPLC Columns

Within the Column is where separation occurs.

Proper choice of column is critical for success in HPLC

Column dimensions in HPLC:

- **Analytical** [internal diameter (i.d.) 1.0 - 4.6-mm; lengths 15 – 250 mm]
- **Preparative** (i.d. > 4.6 mm; lengths 50 – 250 mm)
- **Capillary** (i.d. 0.1 – 0.5 mm; various lengths)
- **Nano** (i.d. < 0.1 mm, or sometimes stated as < 100 μm)

Column Particle Sizes:

- **7, 5, 3.5 (RR), & 1.8 μm (RRHT)**

Materials of construction for the tubing

- **Stainless Steel** (the most popular; gives high pressure capabilities)
- **Glass** (mostly for biomolecules)
- **PEEK** polymer (biocompatible and chemically inert to most solvents)



LC Columns - analytical

Choose Column Configuration for Application

Column Type	I.D. (mm)	Lengths (mm)	Particle Sizes (um)	Flow Rate Ranges	Applications	Sensitivity Increase**
Nano	0.1, 0.075	150*	3.5	100 – 600 nL/min	Proteomics LC/MS	2000
Capillary	0.3, 0.5	35 – 250	3.5, 5	1 – 10 µL/min	Peptide Mapping LC/MS	100
MicroBore	1.0	30 – 150	3.5, 5	30 – 60 µL/min	High sensitivity LC/MS	20
Narrow Bore	2.1	15 – 150	1.8, 3.5, 5	0.1 – 0.3 mL/min	Sample limited, LC/MS	5
Solvent Saver	3.0	150, 250	1.8, 3.5, 5	0.3 – 1.0 mL/min	Analytical	2
Analytical	4.6	15 – 250	1.8, 3.5, 5	1 – 4 mL/min	Analytical	1
Semi-prep	9.4	50 – 250	5	4 – 10 mL/min	Small scale Protein purification	--
Preparative	21.2	50 – 250	5, 7	20 – 60 mL/min	CombiChem purification	--

** Based on 4.6 mm id columns

Resolving Power

Column Length (mm)	Resolving Power N(5 µm)	Resolving Power N(3.5 µm)	Resolving Power N(1.8 µm)	Typical Pressure Bar (1.8 µm)	Analysis Time*	
150	12,500	21,000	32,500	N.A.	Analysis Time	-33%
100	8,500	14,000	24,000	420		
75	6000	10,500	17,000	320		
50	4,200	7,000	12,000	210	Peak Volume	-67%
30	N.A.	4,200	6,500	126	Solvent Usage	-80%
15	N.A.	2,100	2,500	55		



Analysis Time

-33%

Peak Volume

-50%

-67%

Solvent Usage

-80%

-90%

* Reduction in analysis time compared to 150 mm column

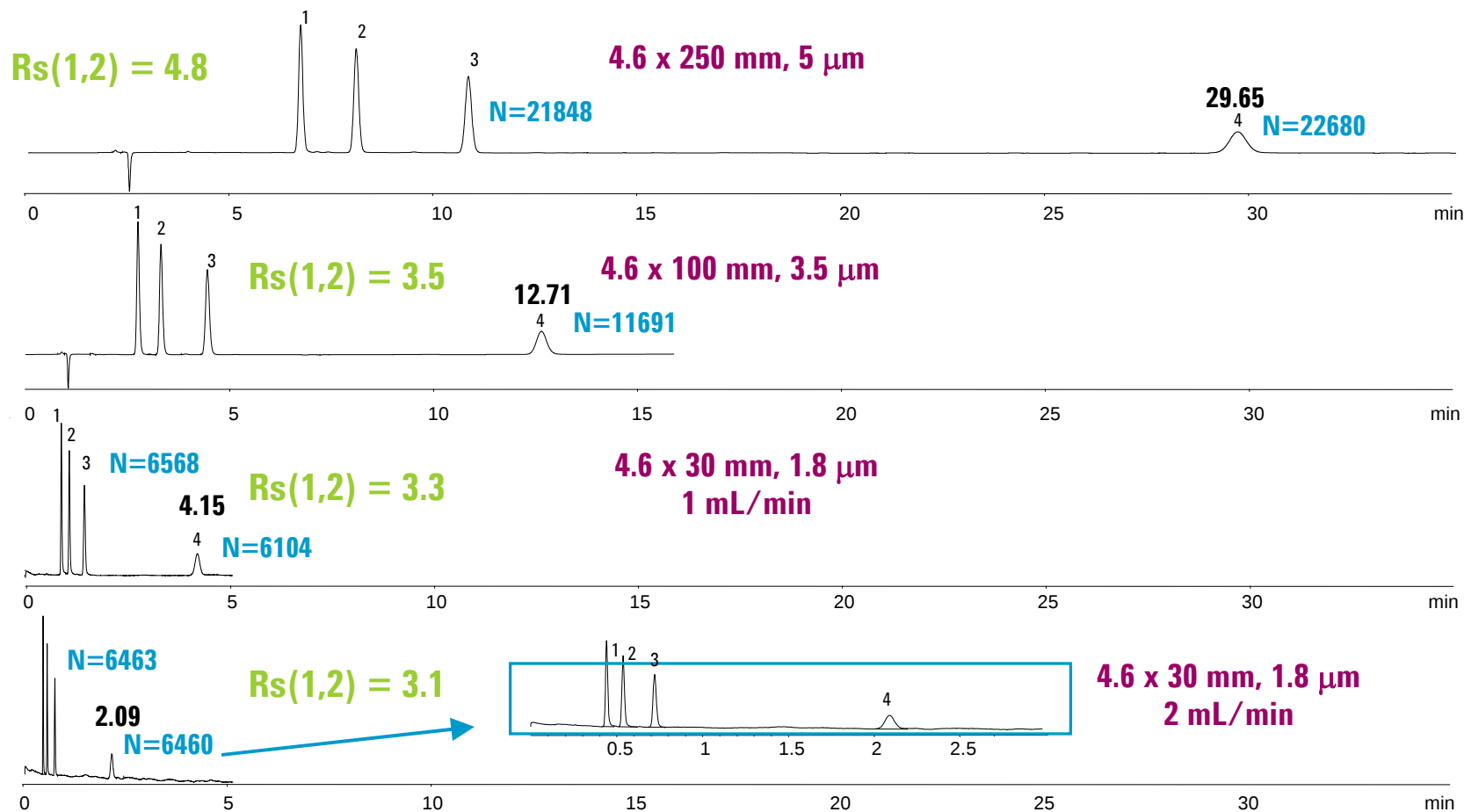
• pressure determined with 60:40 MeOH/water, 1ml/min, 4.6mm ID



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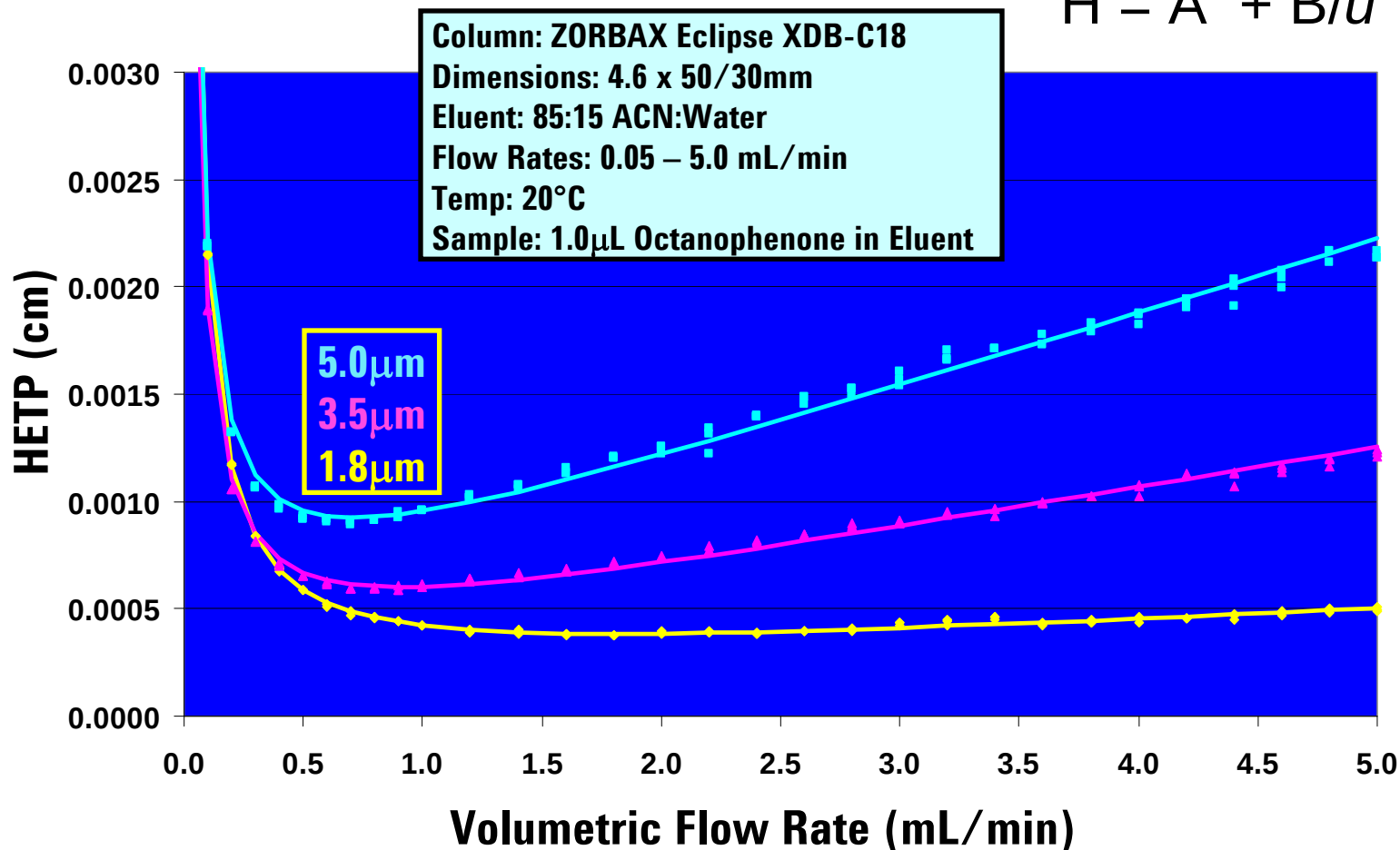
Pick the column and particle size to meet your needs



Columns: ZORBAX SB-C18 Mobile Phase: 50% 20 mM NaH₂PO₄, pH 2.8: 50% ACN Flow Rate: 1 mL/min Temperature: RT
 Detection: UV 230 nm Sample: 1. Estradiol 2. Ethynylestradiol 3. Dienestrol 4. Norethindrone

Smaller Particles Maintain Efficiency Over Wider Flow Rate Ranges

$$H = A + B/u + Cu$$

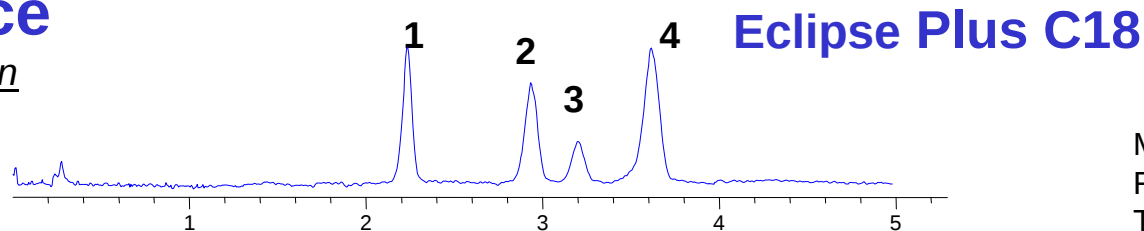


Smaller particle sizes yield flatter curves, minima shift to higher flow rates

Different C18 Bonded Phases for Max Selectivity

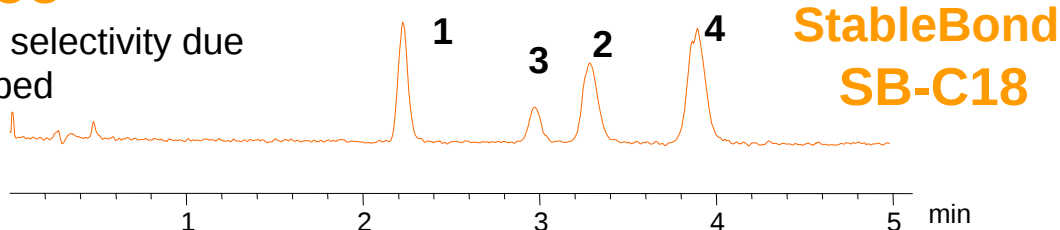
1st choice

Best Resolution
& Peak Shape



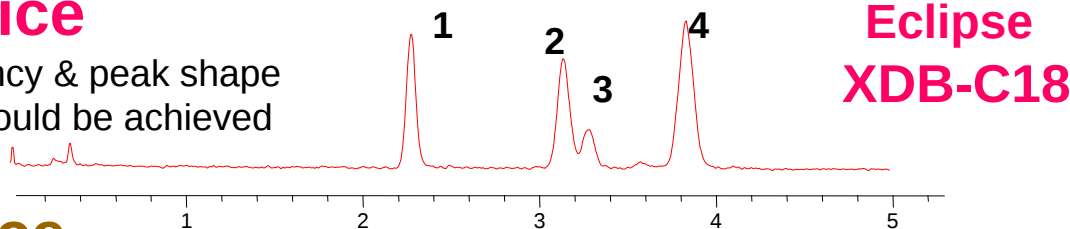
2nd choice

Good alternate selectivity due to non-endcapped



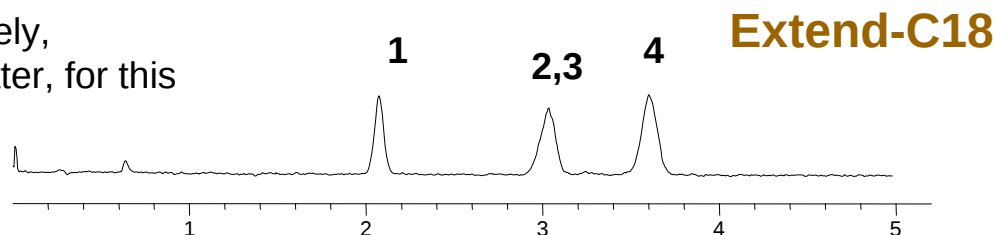
3rd choice

Good efficiency & peak shape
Resolution could be achieved



4th choice

Resolution not likely,
Other choices better, for this separation.



Mobile phase: (69:31) ACN: water
Flow 1.5 mL/min.
Temp: 30 °C
Detector: Single Quad ESI
positive mode scan
Columns: RRHT
4.6 x 50 mm 1.8 um

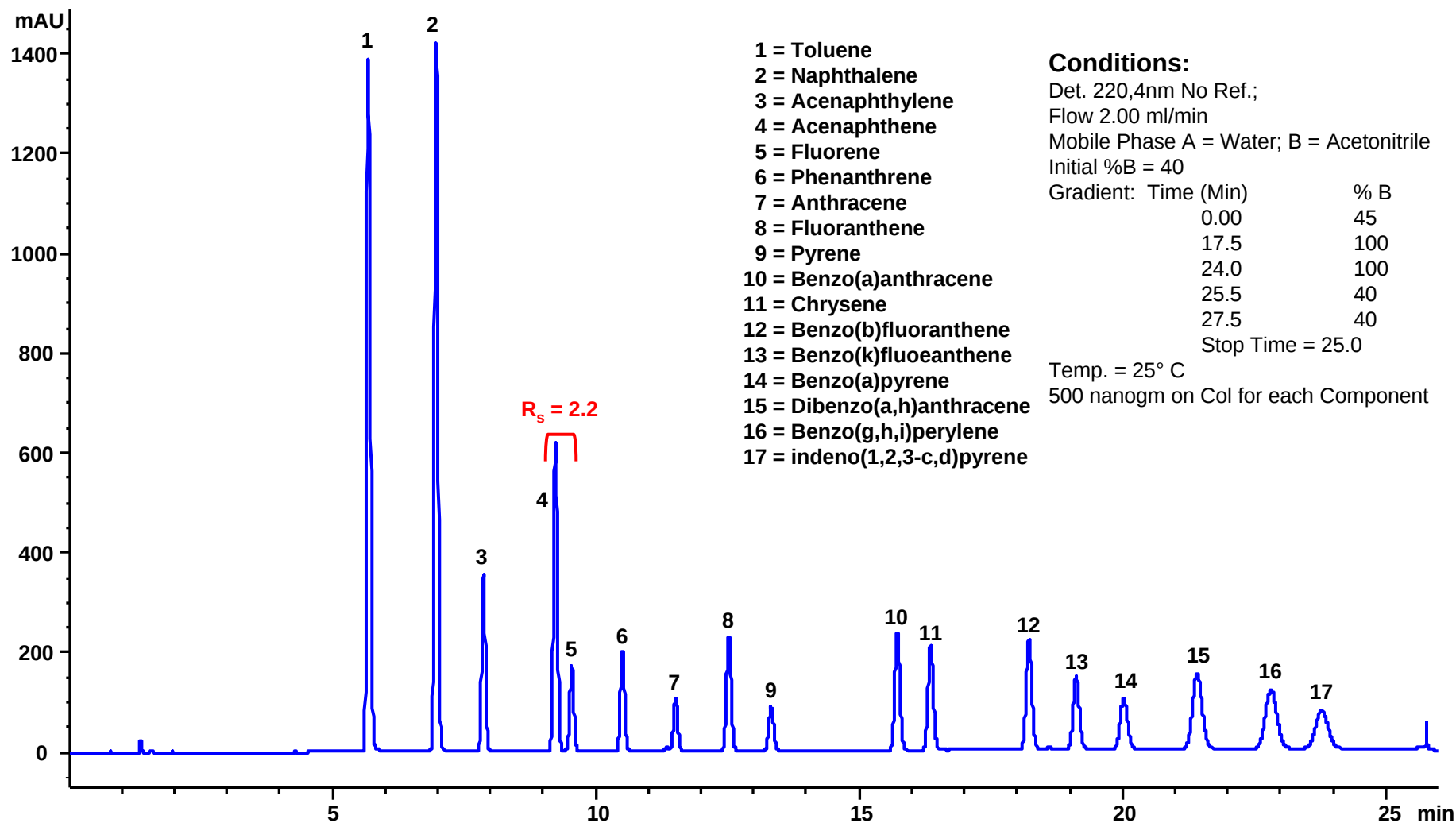
Sample:

1. anandamide (AEA)
2. Palmitoylethanolamide (PEA)
3. 2-arachinoylglycerol (2-AG)
4. Oleoylethanolamide (OEA)

Multiple bonded phases
for most effective method
development.
Match to one you are
currently using.

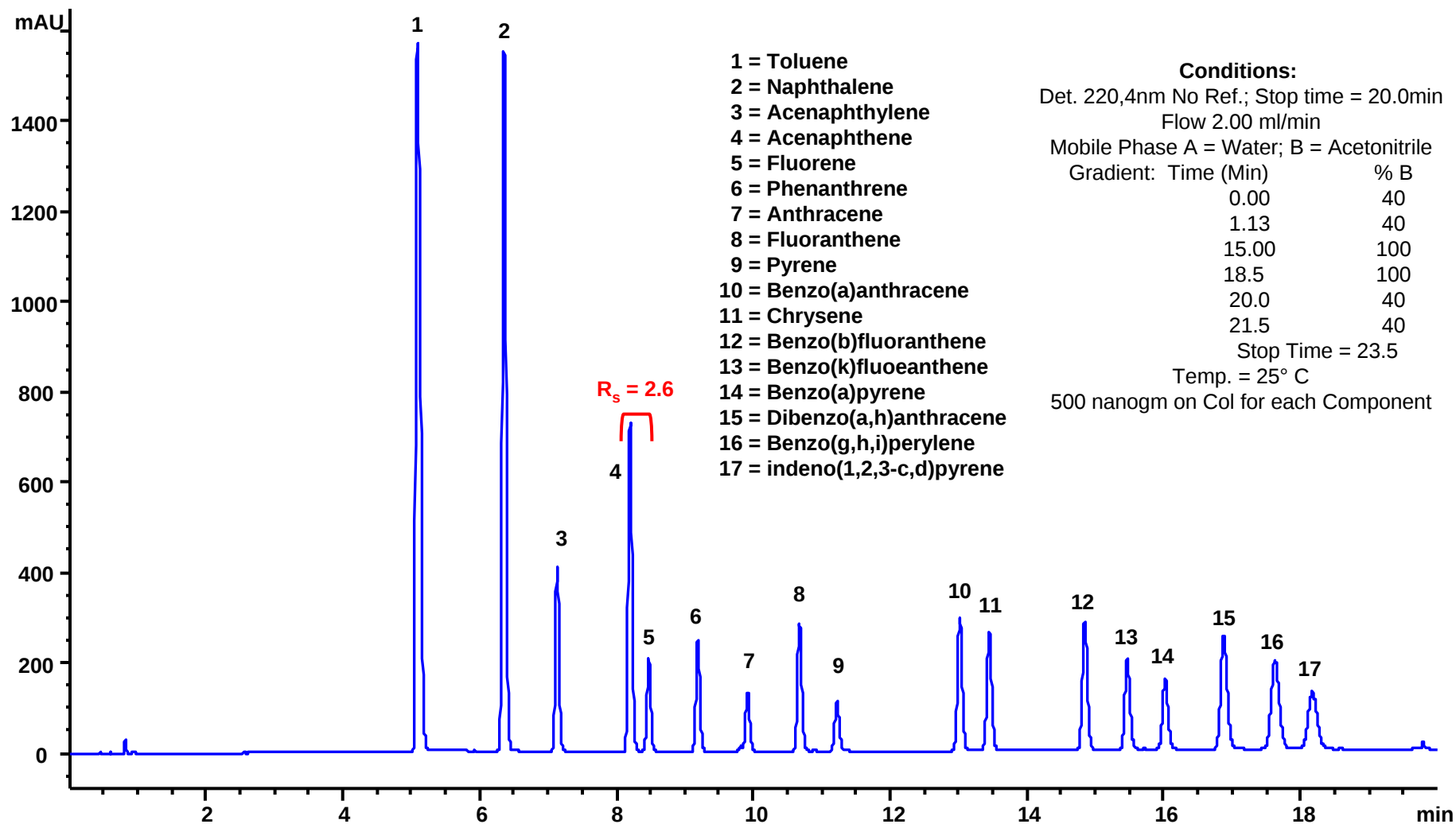


1 – Analytical 4.6x250mm, 5.0µm PAH Column – Separation of 16 PAH's in EPA 610

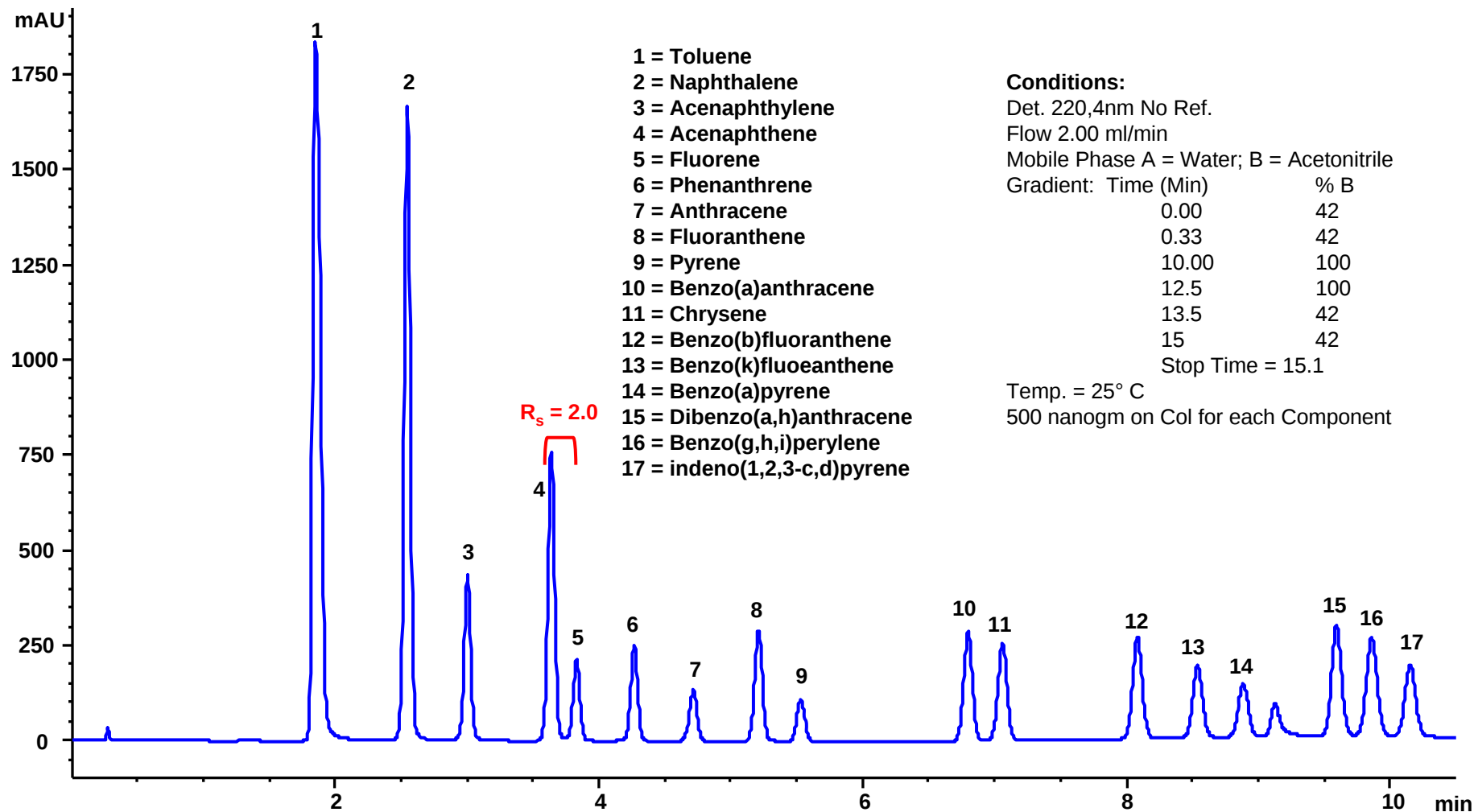


4 – Rapid Resolution – 4.6x150mm, 3.5µm PAH

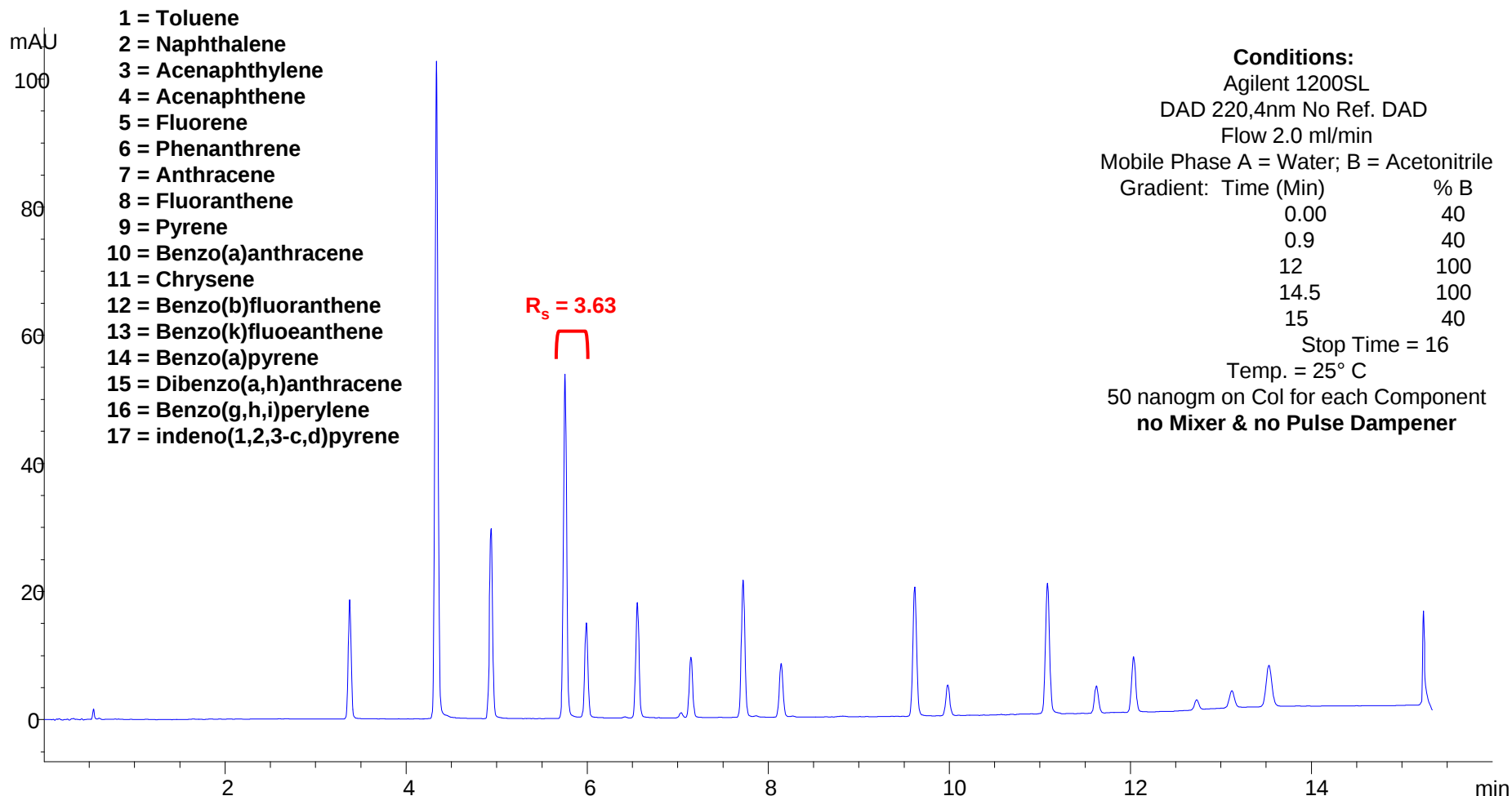
Column – Separation of 16 PAHs in EPA 610



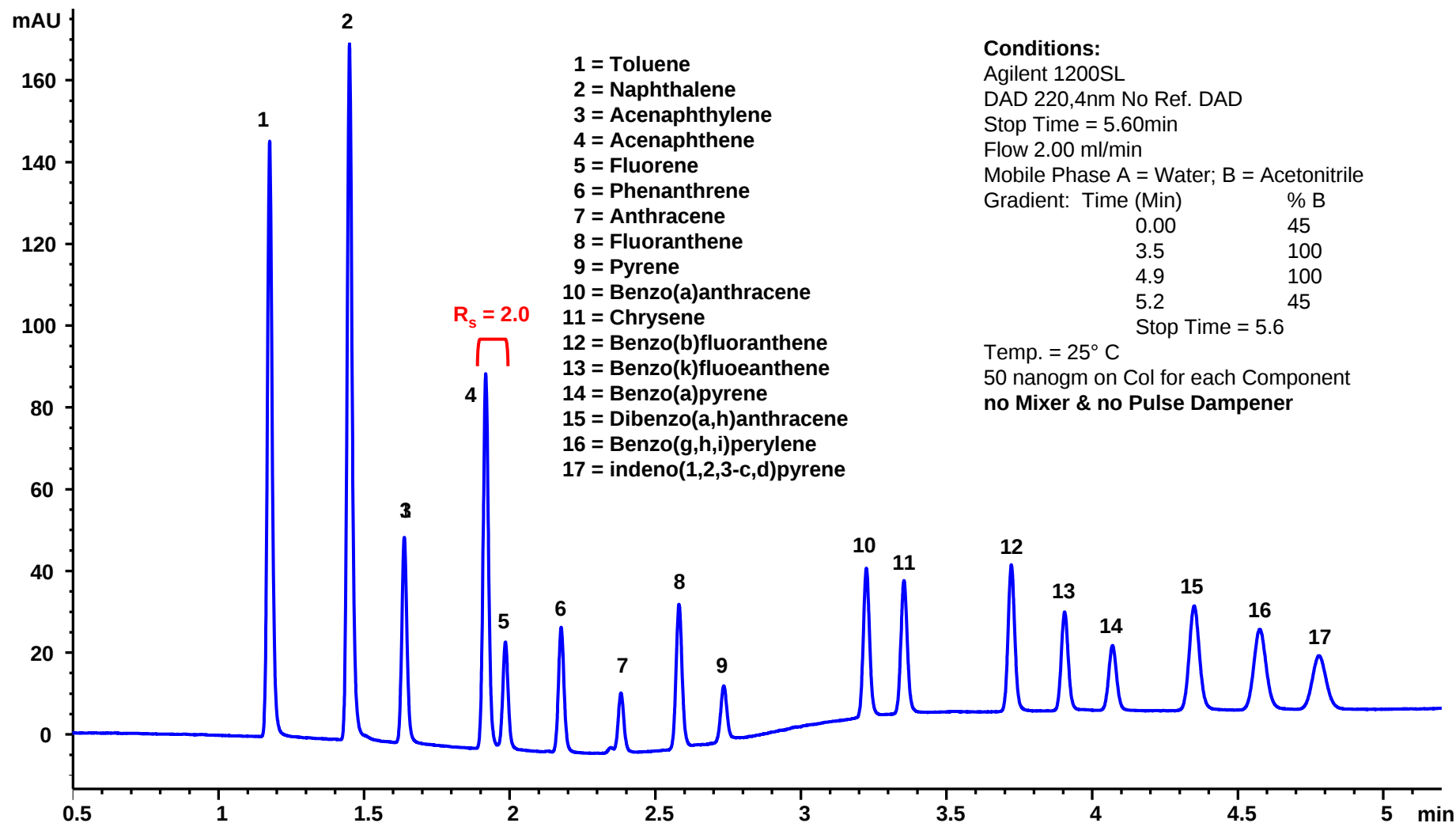
#6- Rapid Resolution Eclipse PAH 4.6x50mm, 3.5 μ m Column – Separation of 16 PAHs in EPA 610



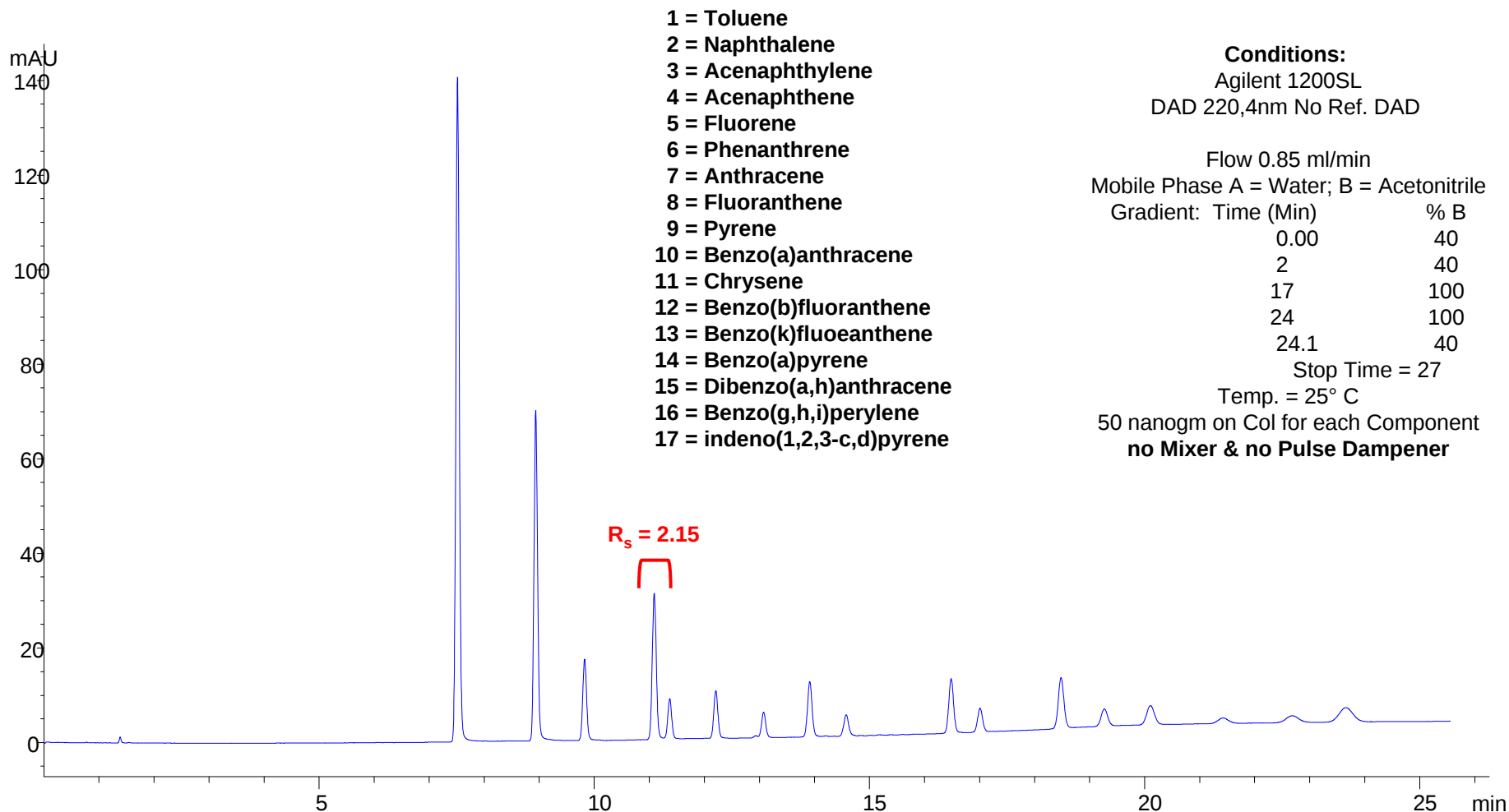
#7- Rapid Resolution HT Eclipse PAH 4.6x100mm, 1.8 μm Column - Separation of 16 PAHs in EPA 610



8 – Rapid Resolution HT Eclipse PAH 4.6x50mm, 1.8µm Column – Separation of 16 PAHs in EPA 610

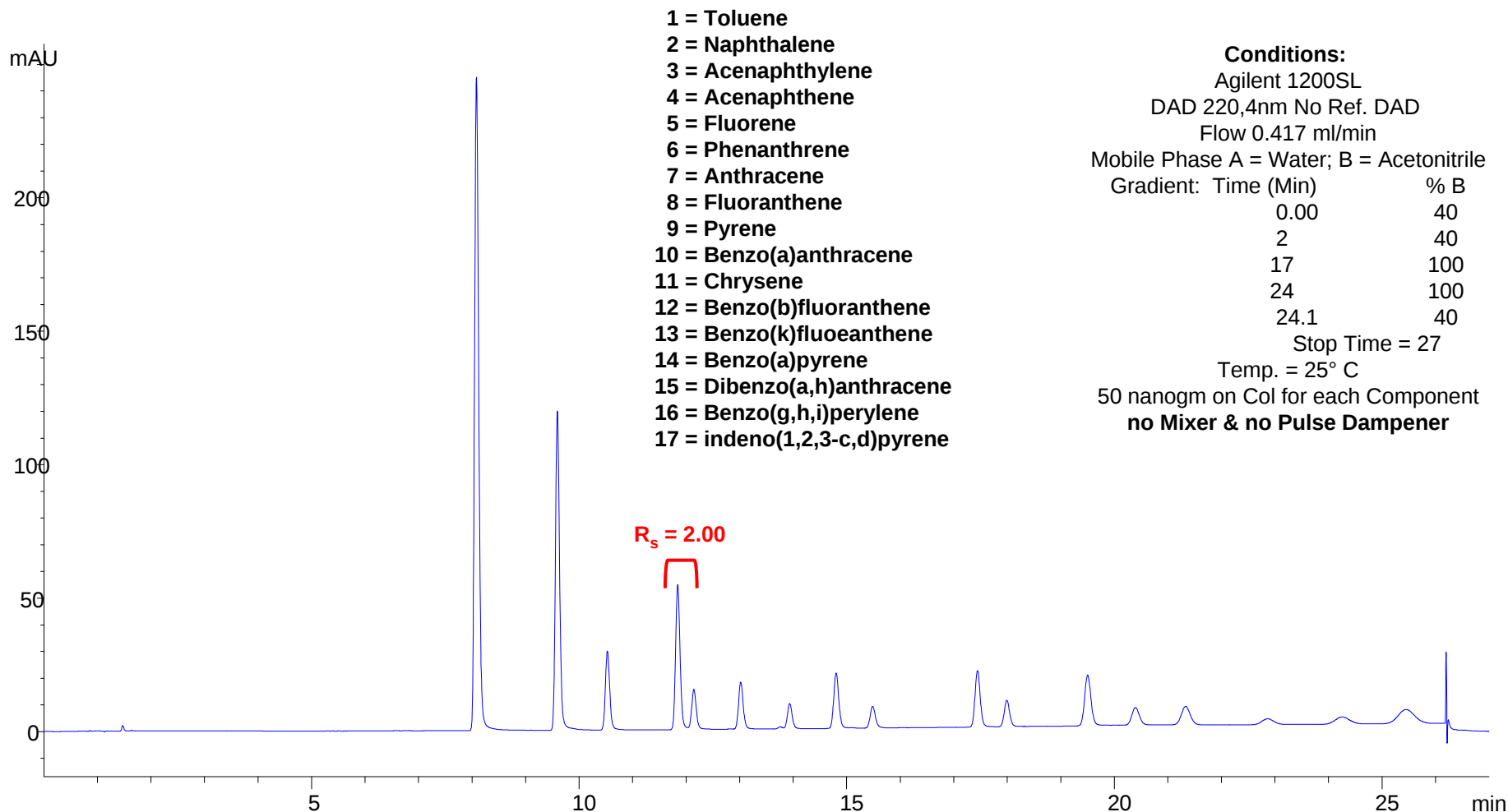


#10 Solvent Saver Eclipse PAH 3.0x250 mm, 5 µm Column - Separation of 16 PAHs in EPA 610



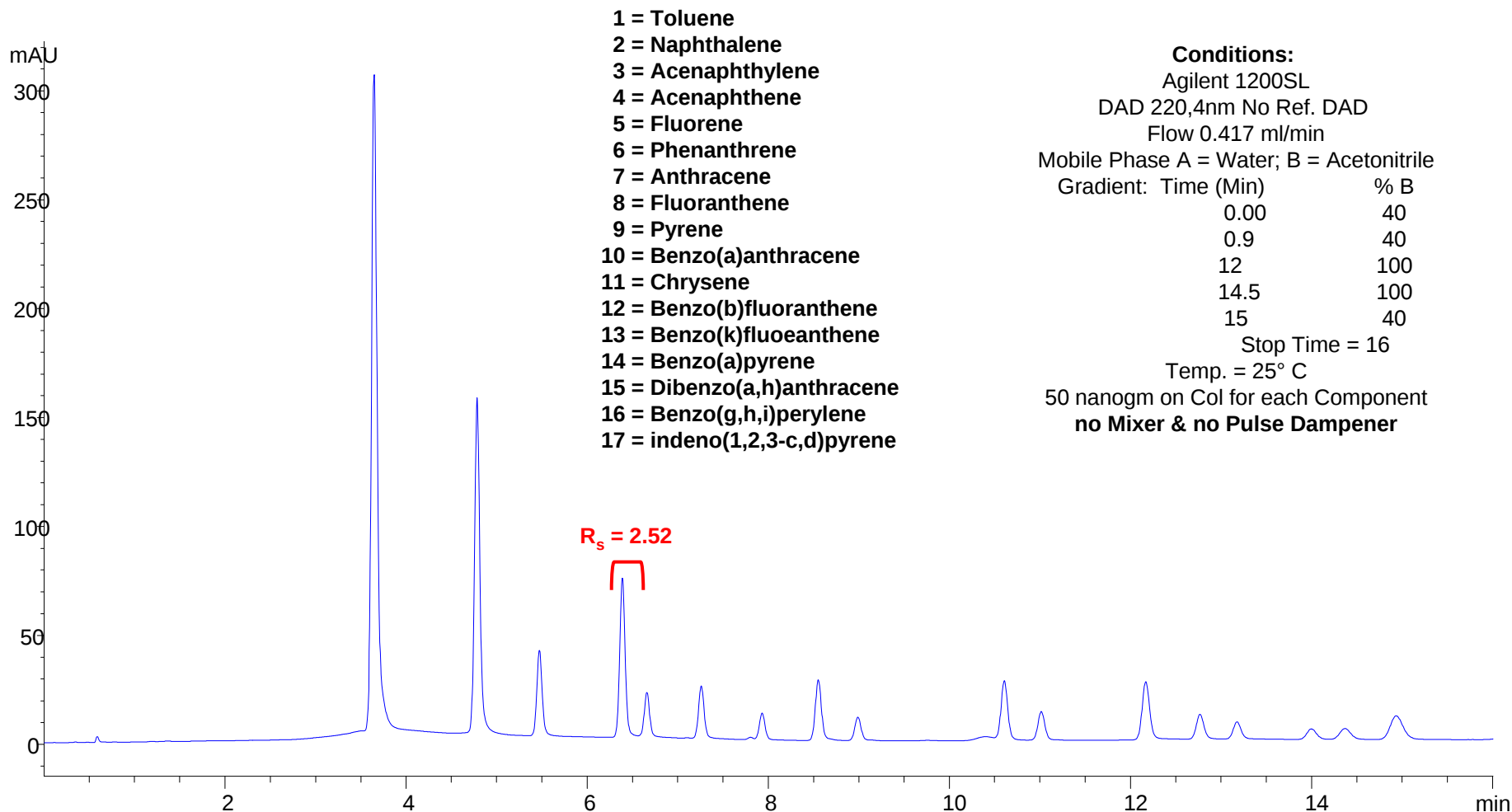
#11 Narrow Bore Eclipse PAH 2.1x250 mm, 5 μ m

Column - Separation of 16 PAHs in EPA 610



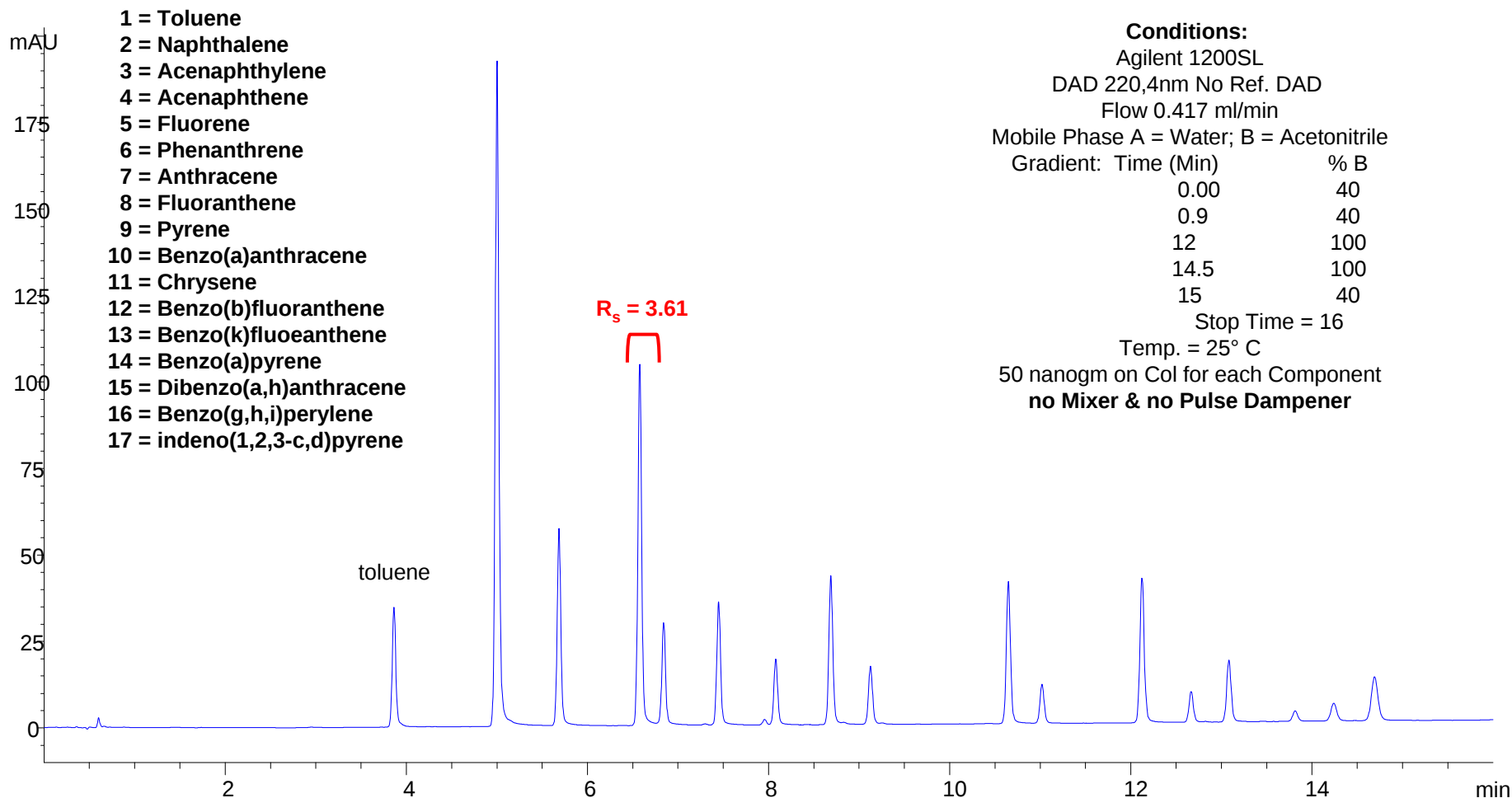
#13 Narrow Bore Rapid Resolution Eclipse PAH

2.1x100mm, 3.5 μ m Column - Separation of 16 PAHs in EPA 610

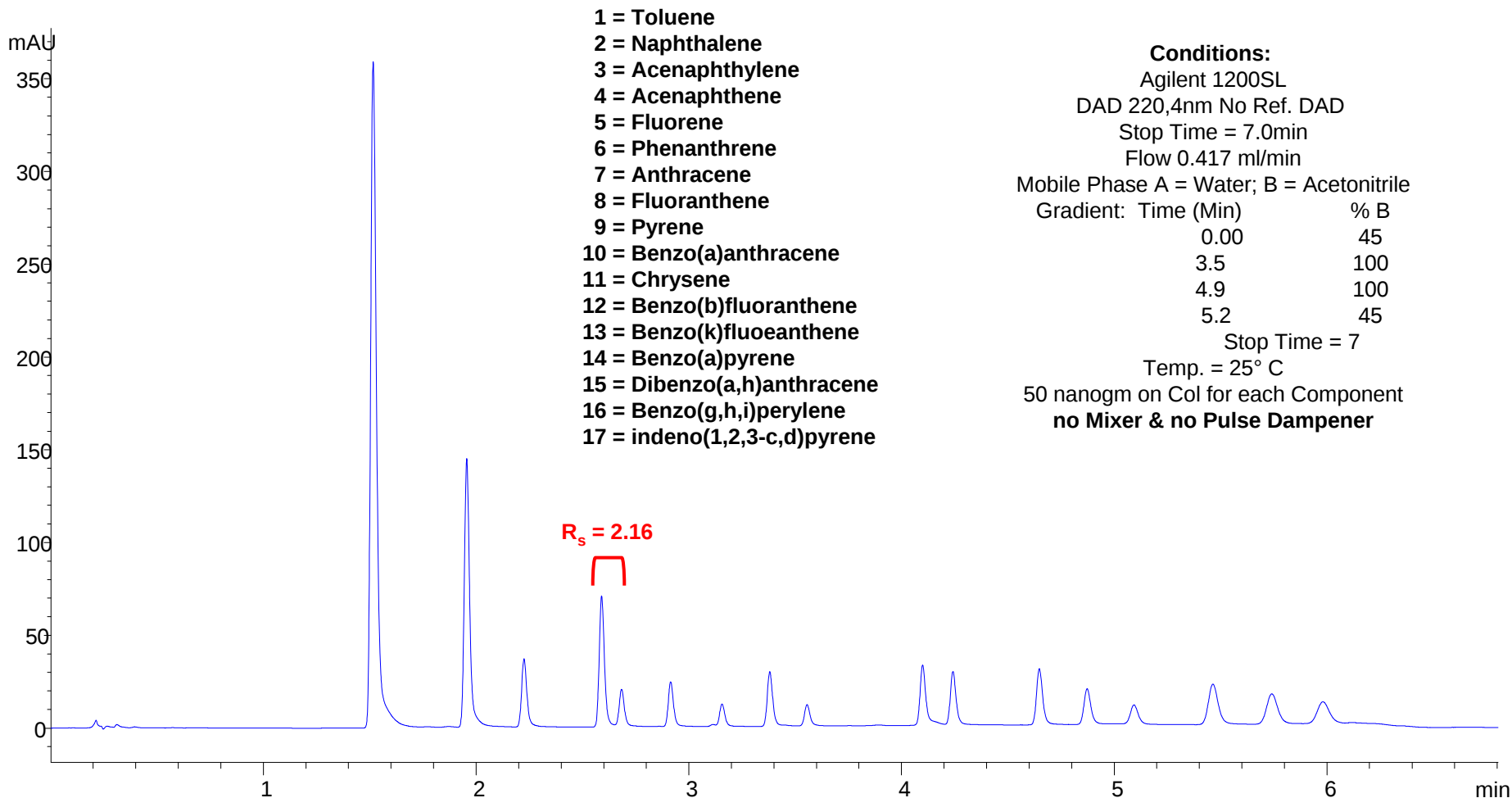


#14 – Narrow Bore RRHT Eclipse PAH 2.1x100mm

1.8 μm – Separation of 16 PAHs in EPA 610



#15 – Narrow Bore RRHT Eclipse PAH 2.1x50mm, 1.8µm PAH Column - Separation of 16 PAHs in EPA 610



How To “Match” a Column to a ZORBAX RRHT Column

General Phase Type	Starting ZORBAX Choice
Typical “endcapped” C18 or C8 bonded phases, newer columns	Eclipse Plus C18 or C8
Endcapped C18 or C8 columns, older generation	Eclipse XDB-C18 or C8
Non-endcapped columns	StableBond C18
Older types of columns	StableBond C18, C8 etc.
Aqueous “type” columns	SB-AQ
CN or Phenyl	SB-CN, SB-Phenyl

Conclusion

There is no one-size-fits all column

Choose the best for your application needs

