A Look at Column Choices

Does one-size-fit-all?

LC Columns and Consumables

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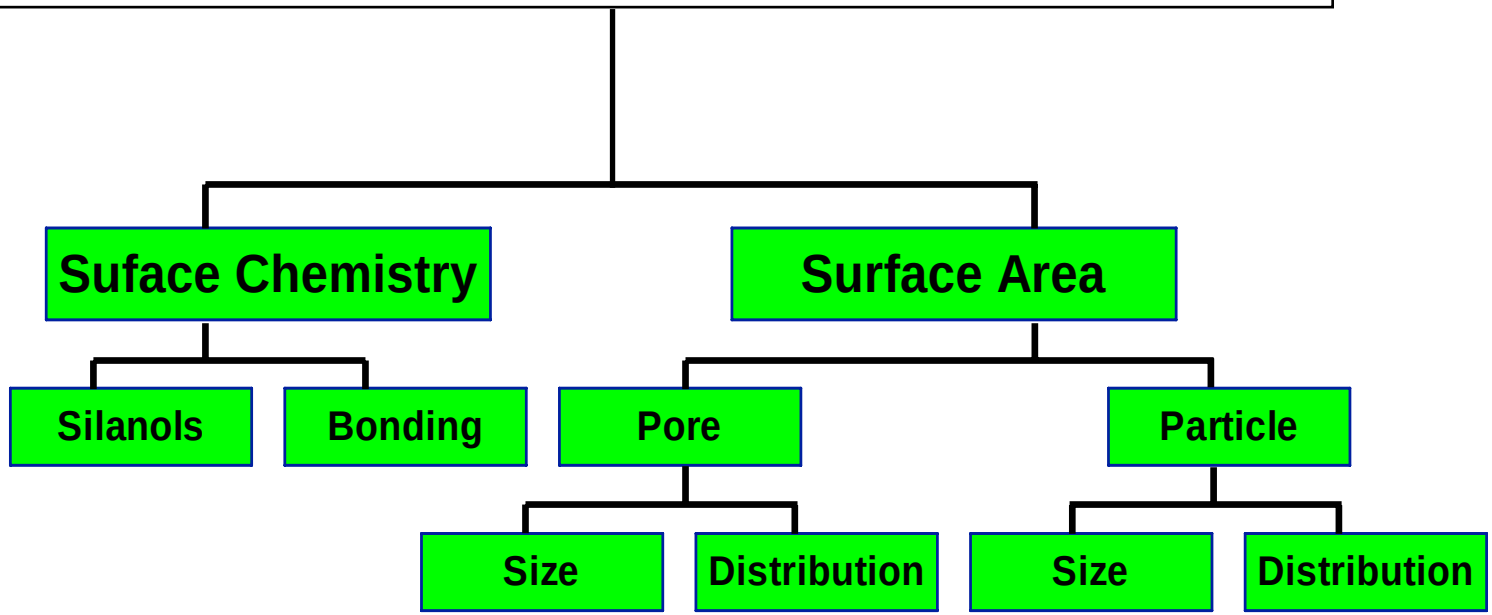
Seminar Outline

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

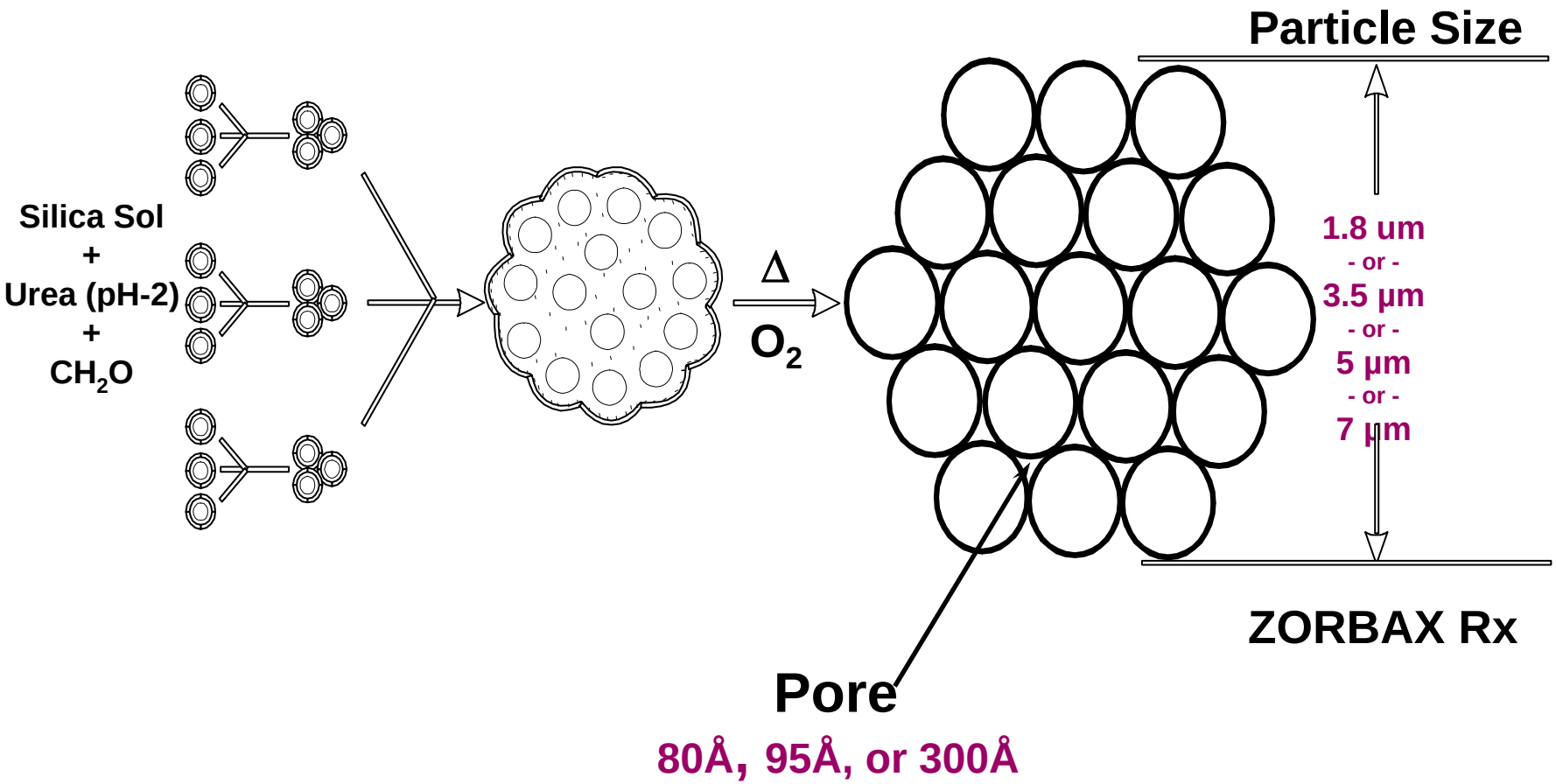
Silica Column Characteristics



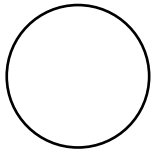
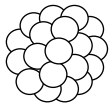
NOTE: SILICA IS STABLE AT 1.0 > pH < 11.0*

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

ZORBAX Porous Silica Particles

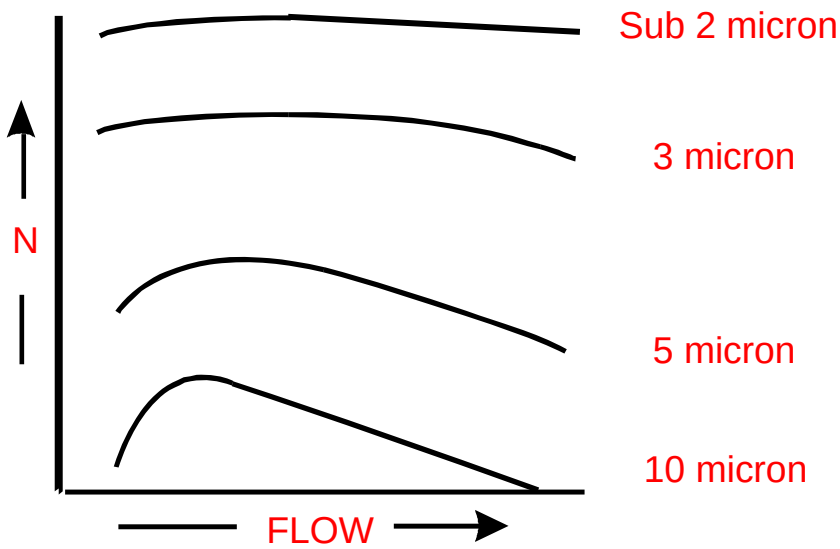


History of HPLC Particle Development

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

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 ad 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

Highly reproducible

Monomeric bonding

Typical ZORBAX Bonding

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

Polymeric Bonding

Eclipse PAH

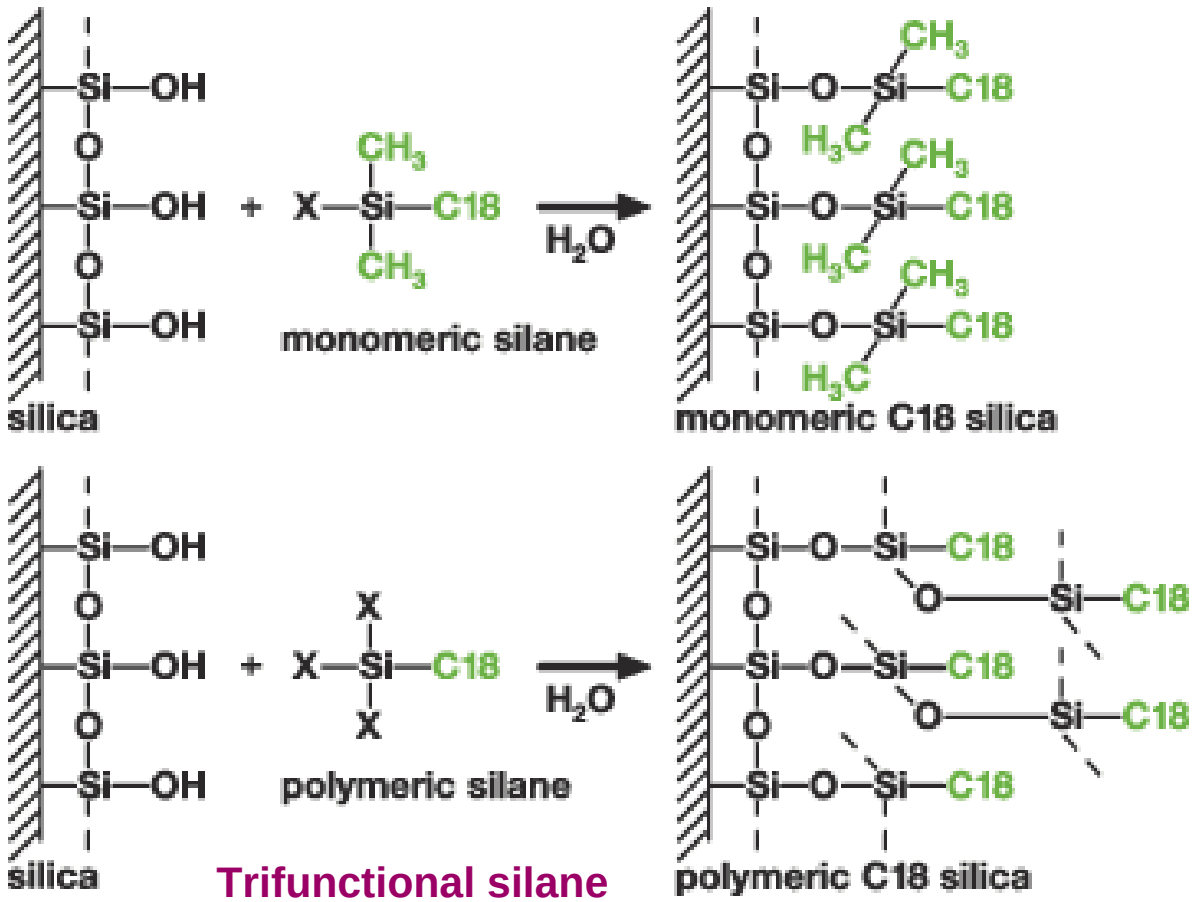


figure courtesy of Vydac

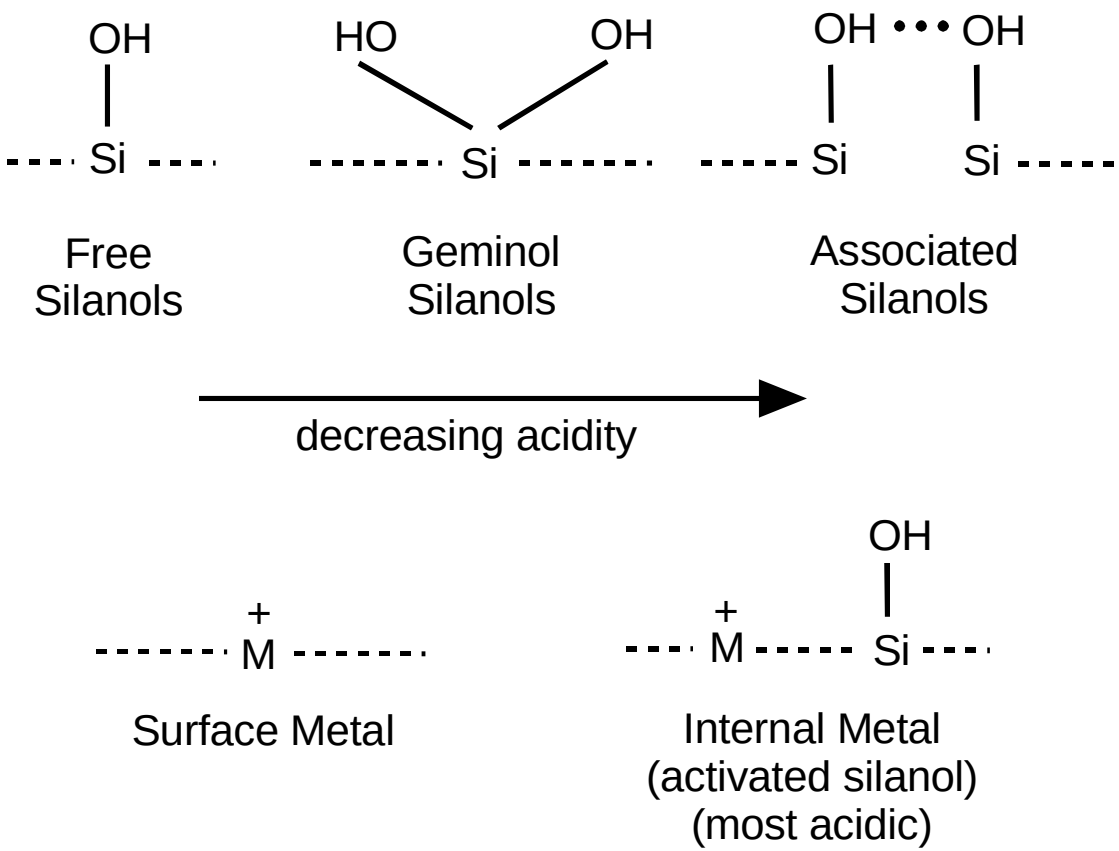
2. Carbon Load

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

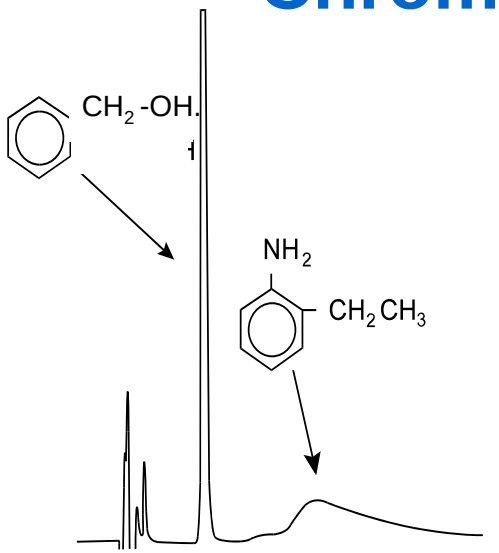
Generally speaking, a high carbon load (example 18-25%) results in a more hydrophobic surface. The surface is also more resistant to high pH.

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

3. The Surface of Silica Supports

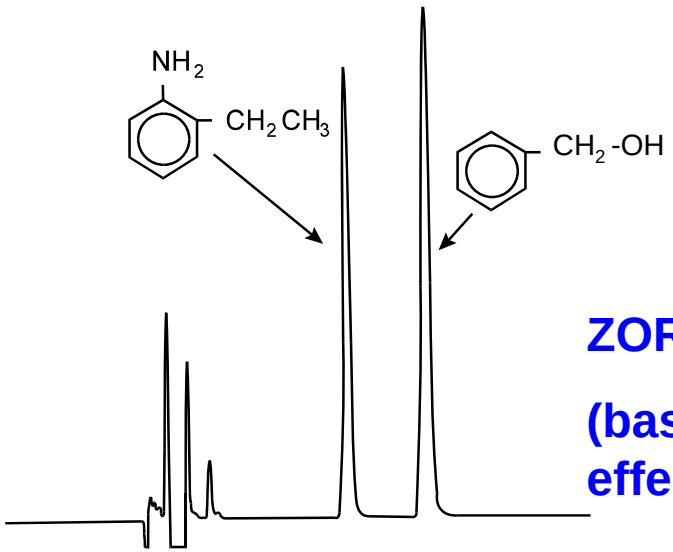


Chromatographic Improvement Using Highly Purified Zorbax Rx-Sil



Original ZORBAX, 1973 and other type A silicas

(basic compound can tail)



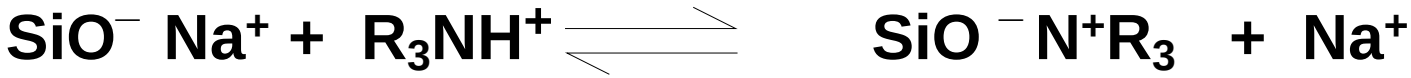
Conditions: Flow Rate: 2.0 mL / min.
Mobile Phase: 5% 2-Propanol in Heptane

ZORBAX Rx-Sil, 1987 and other Type B silicas

(basic compounds have less tailing; lower effective silanol pKa)

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

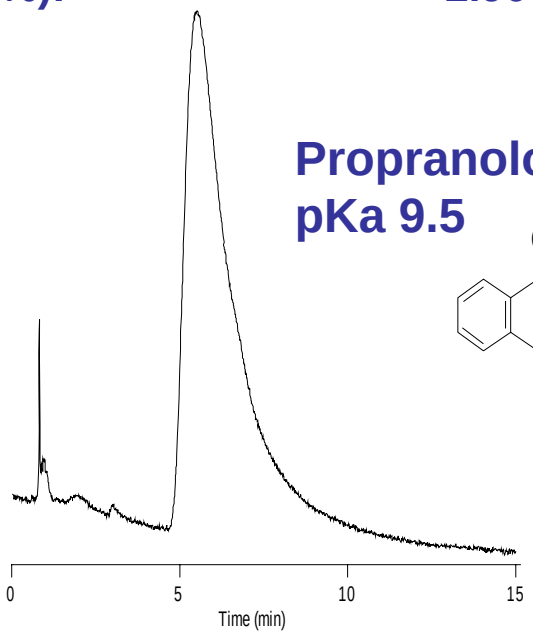
2. Unprotonated acids can compete for H^+ with protonated silanols. This can occur at low pH.

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.

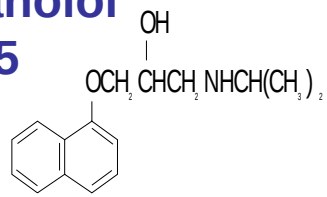
Zorbax StableBond with Rx-SIL Improves Peak Shape

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

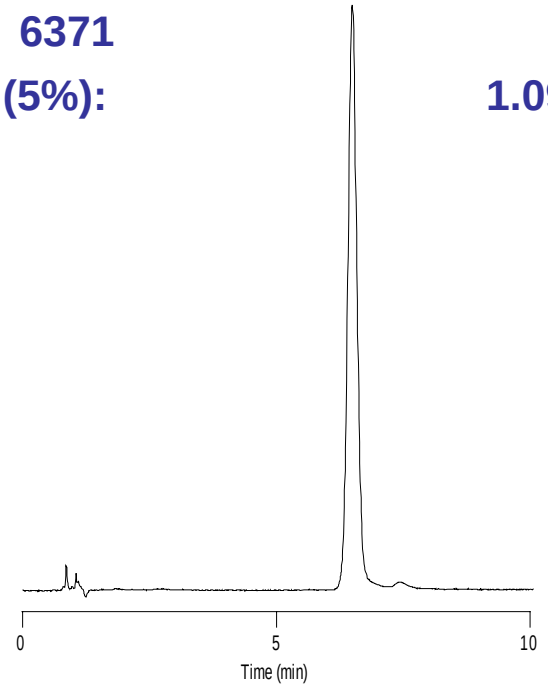
Silica Type – More Acidic
Column: ODS, 4.6 x 250 mm, 5 μm
Plates: 92
USP T_f (5%): 2.90



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

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

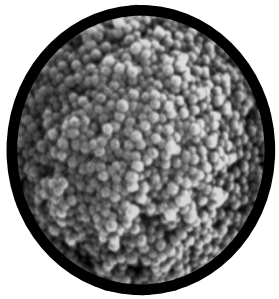
* Below 20% Organic

4. ZORBAX Bonding Technology

All Purpose Phases

- StableBond
- Eclipse XDB
- Eclipse Plus

Rx-Silica Support



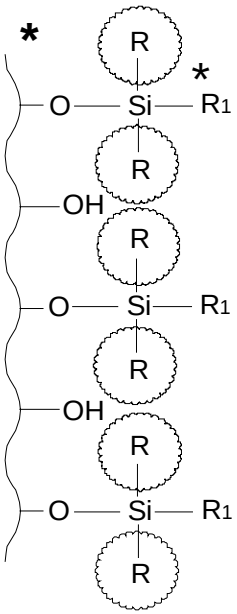
- Ultra-pure
- Spherical, consistent pore size
- Fully-hydroxylated
- Sol gel maximizes strength and lifetime

Application Targeted Phases

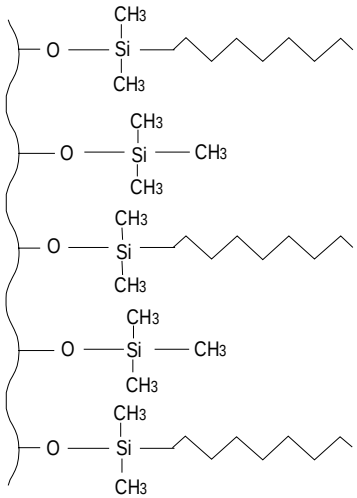
- Bonus-RP
- Extend

Match Method pH and Column Choice
Choose the Best Bonded-Phase for Each pH Range

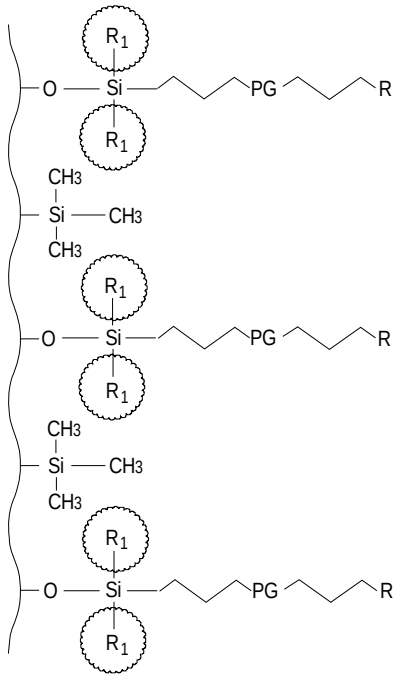
StableBond, pH 1-6
1. Uses bulky silanes
2. Non-endcapped



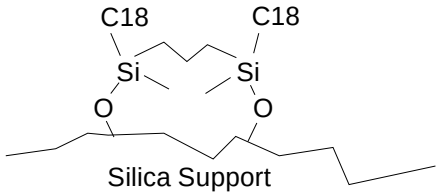
Eclipse Plus & XDB, pH 2-9
1. Proprietary double-endcapping



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

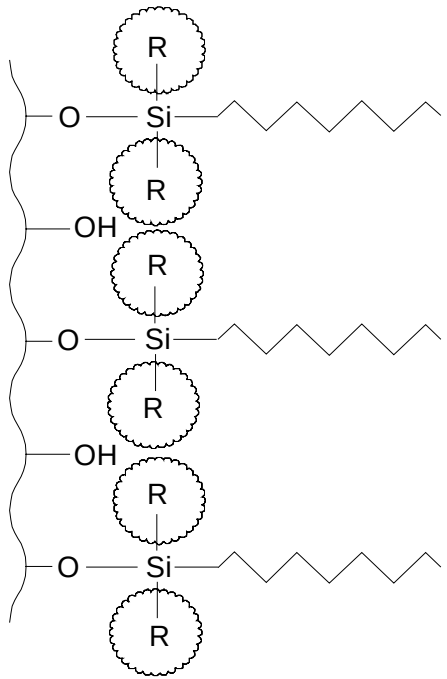
Superior low pH stability – down to pH 1

Non-endcapped for selectivity and lifetime

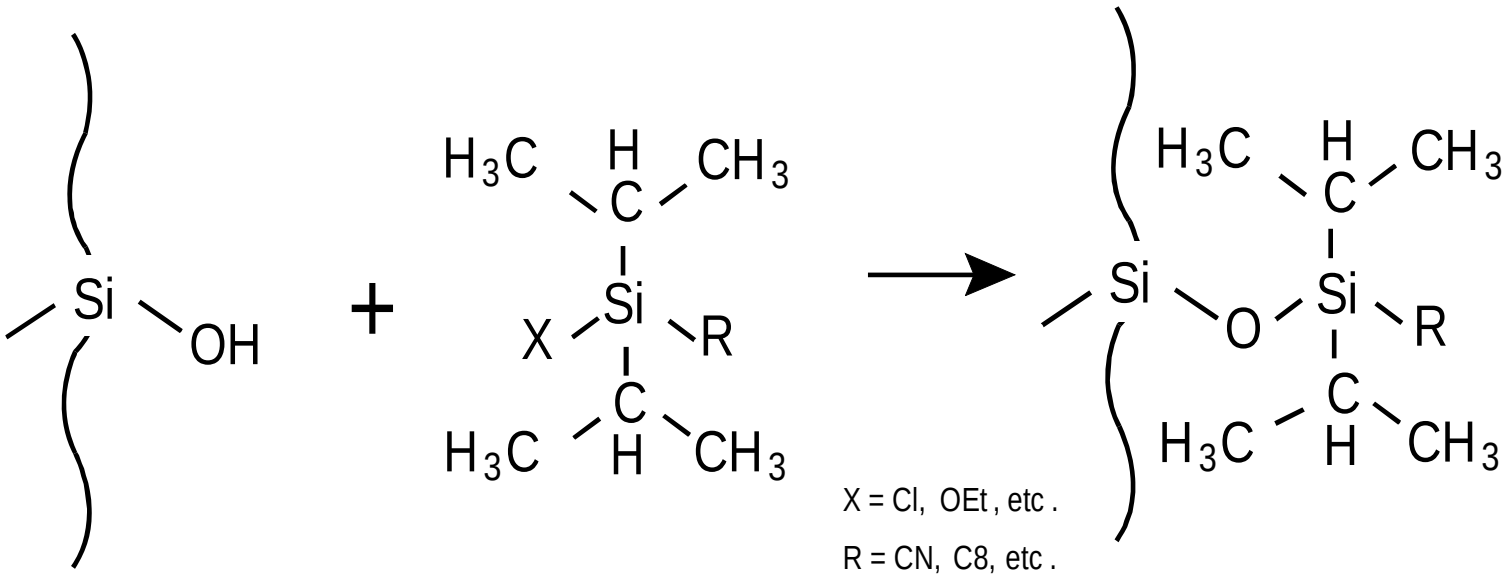
Patented sterically protecting bonding

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

Ideal for most sample types at low pH

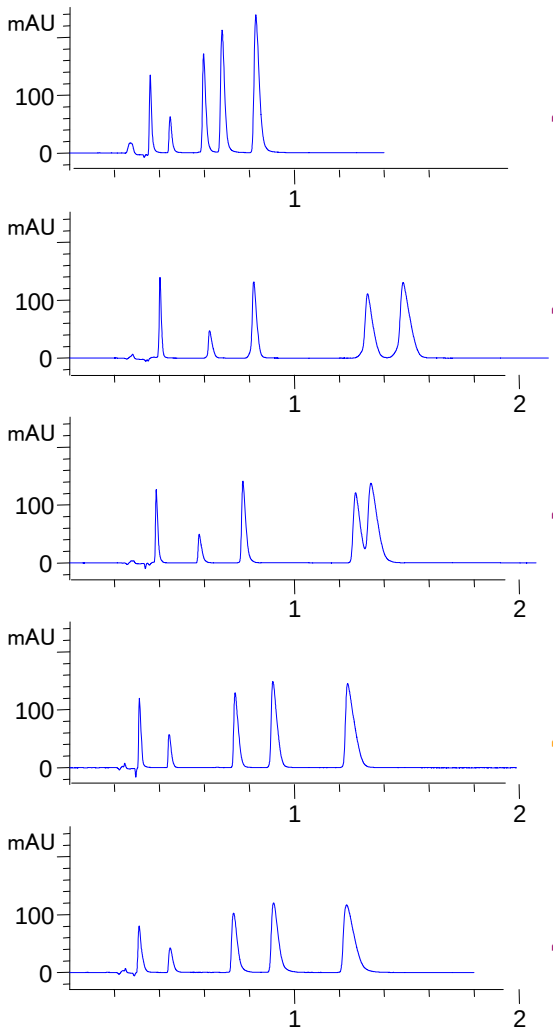


StableBond Reaction to Make a Sterically-Protected Surface



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

Bonded Phase Selectivity Differences in 30% ACN



RRHT SB-CN
4.6 x 50 mm, 1.8 µm

RRHT SB-Phenyl
4.6 x 50 mm, 1.8 µm

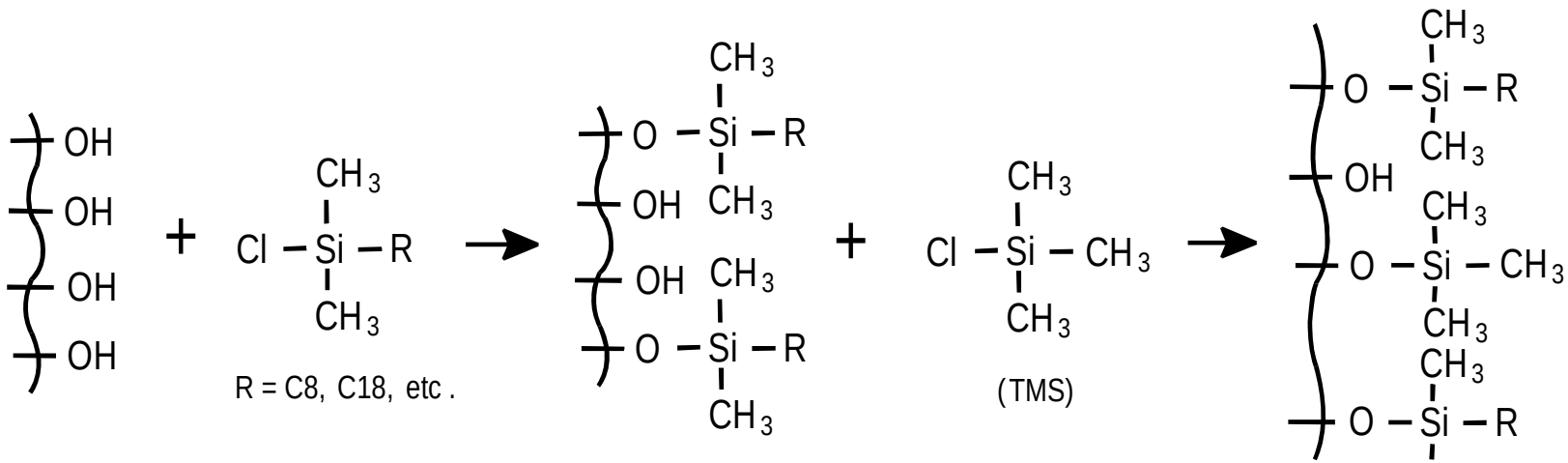
RRHT SB-AQ
4.6 x 50 mm, 1.8 µm

RRHT Eclipse Plus C18
4.6 x 50 mm, 1.8 µm

RRHT SB-C18
4.6 x 50 mm, 1.8 µm

- **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

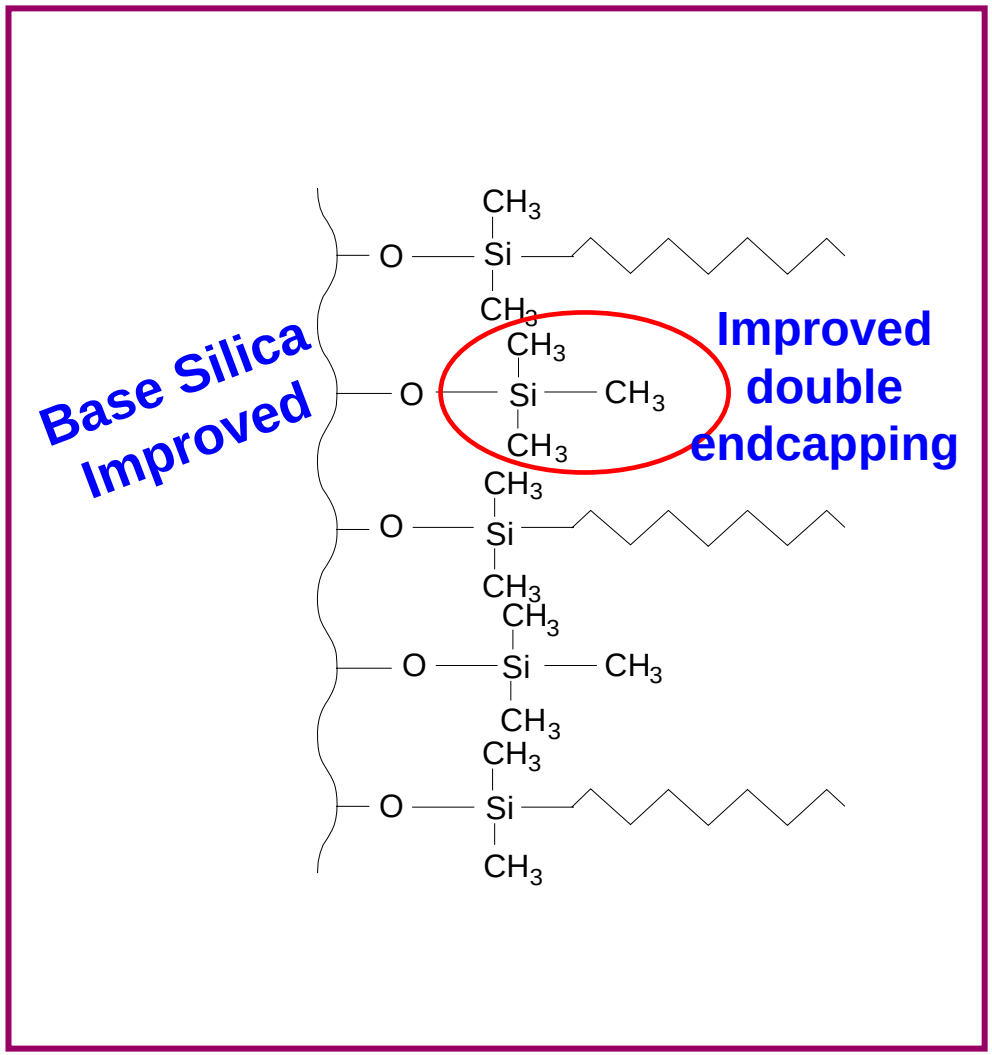
Good for all sample types – acid, base, neutral

Wide useable pH range, pH 2-9

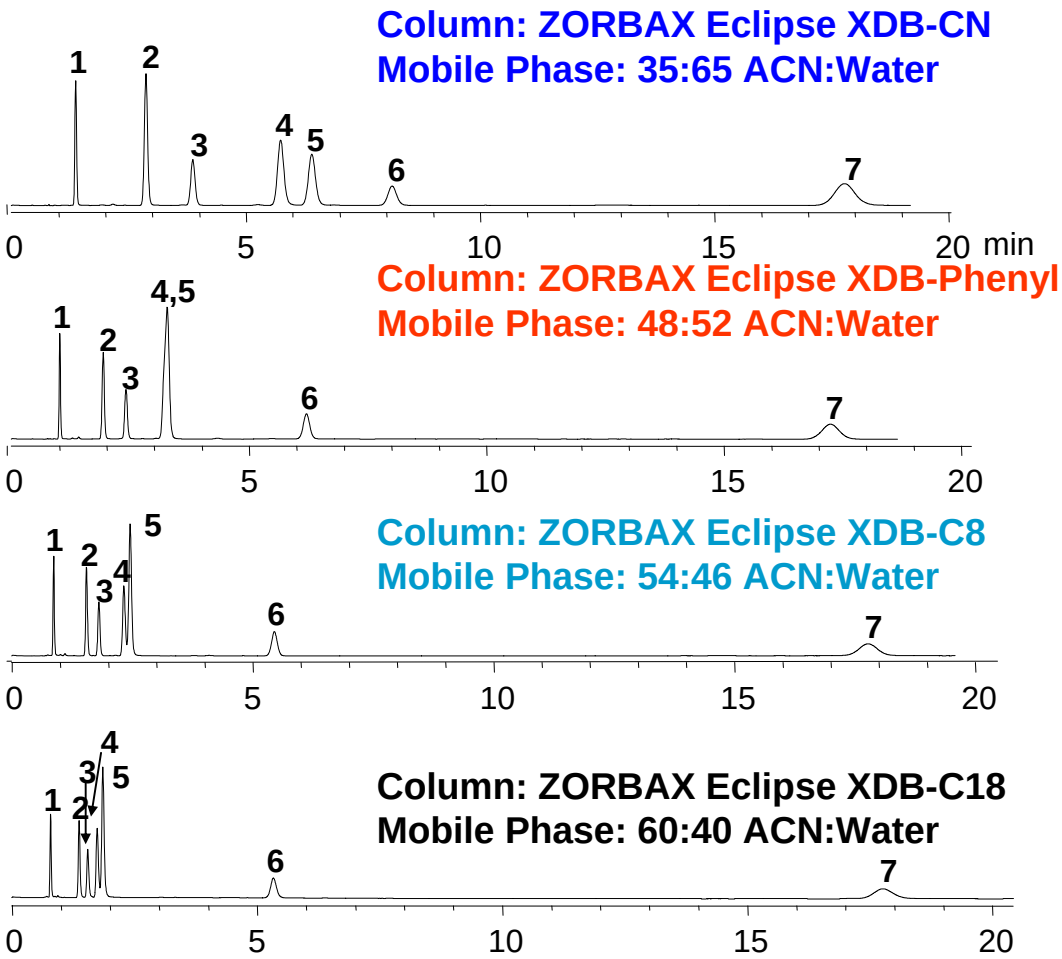
Double end-capped

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 μm
Flow Rate: 2.000 ml/min
Injection Volume: 2.00 μl
Column Temperature: 25 °C
Detector: UV, 210 nm

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

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

ZORBAX Bonus-RP

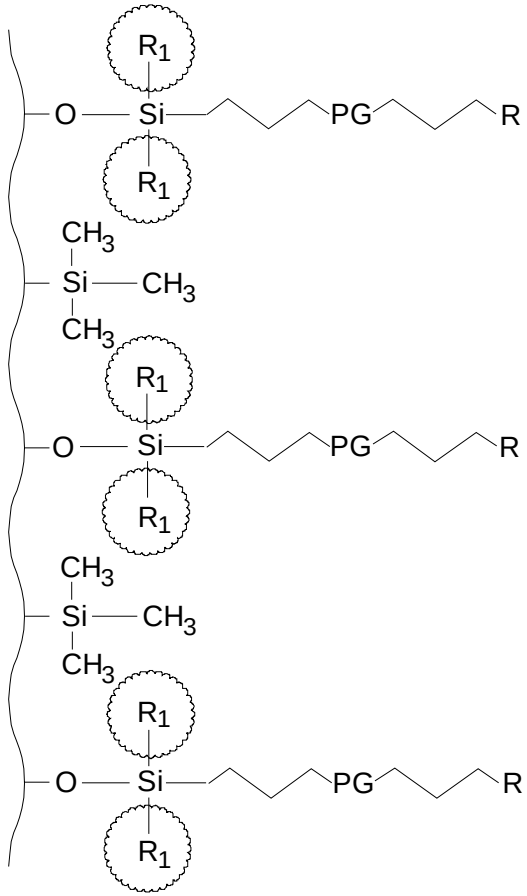
Good peak shape of basic compounds

Polar alkyl-amide bonded phase

Unique selectivity

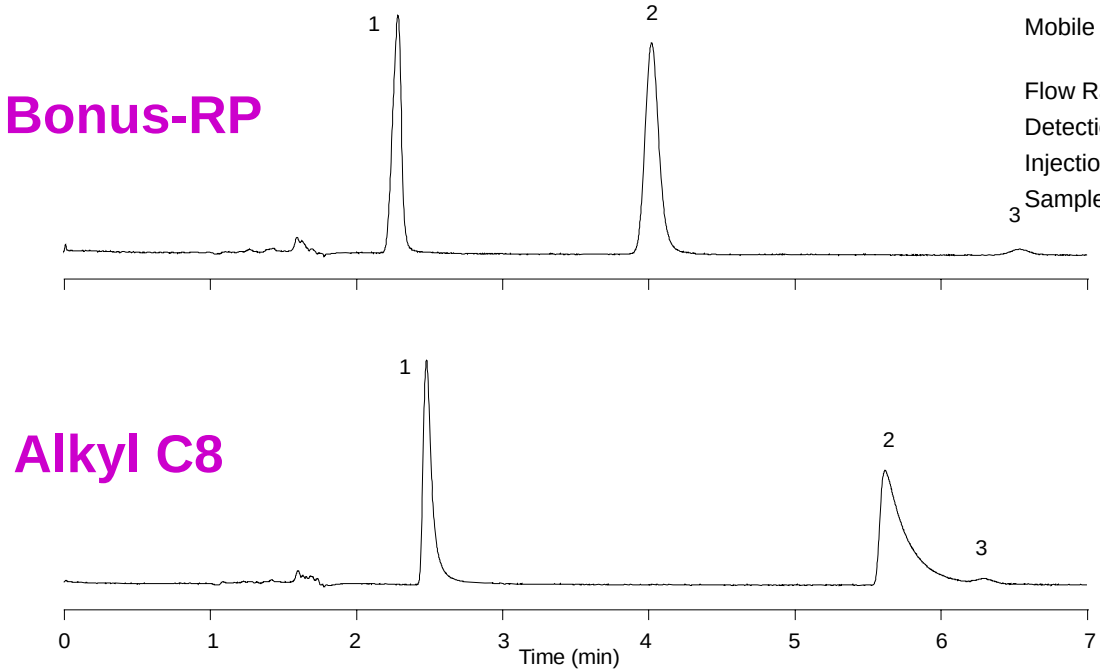
Enhanced low pH stability with sterically protecting bonding

Triple endcapped



Improved Peak Shape of Basic Compounds

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



Columns:	4.6 x 150 mm, 5 µm
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 µL
Sample:	Anorectics (“Fen-phen”) 1. Phentermine pKa10.1 2. Fenfluramine pKa 9.1 3. Impurity

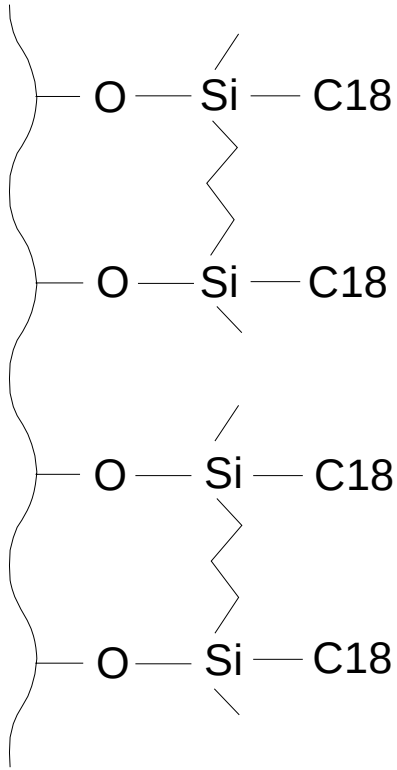
ZORBAX Extend-C18

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

Excellent reproducibility

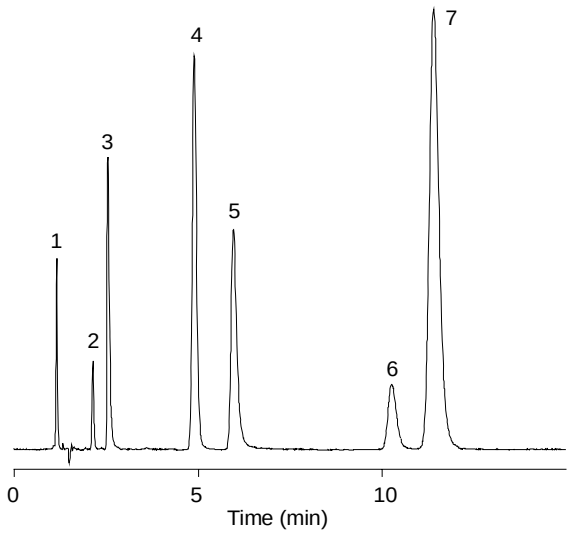
Patented bidentate, C18 bonding

Double endcapping



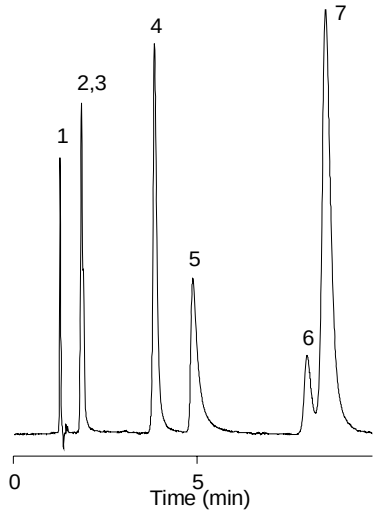
Improved Retention of Basic Antihistamines on ZORBAX Extend-C18

pH 11
30% 20 mM TEA
70% MeOH



Column:	4.6 x 150 mm, 5 µm
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)

LC Columns - analytical

Column Particle Sizes:

- **7, 5, 3.5 (RR), & 1.8 um (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)

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

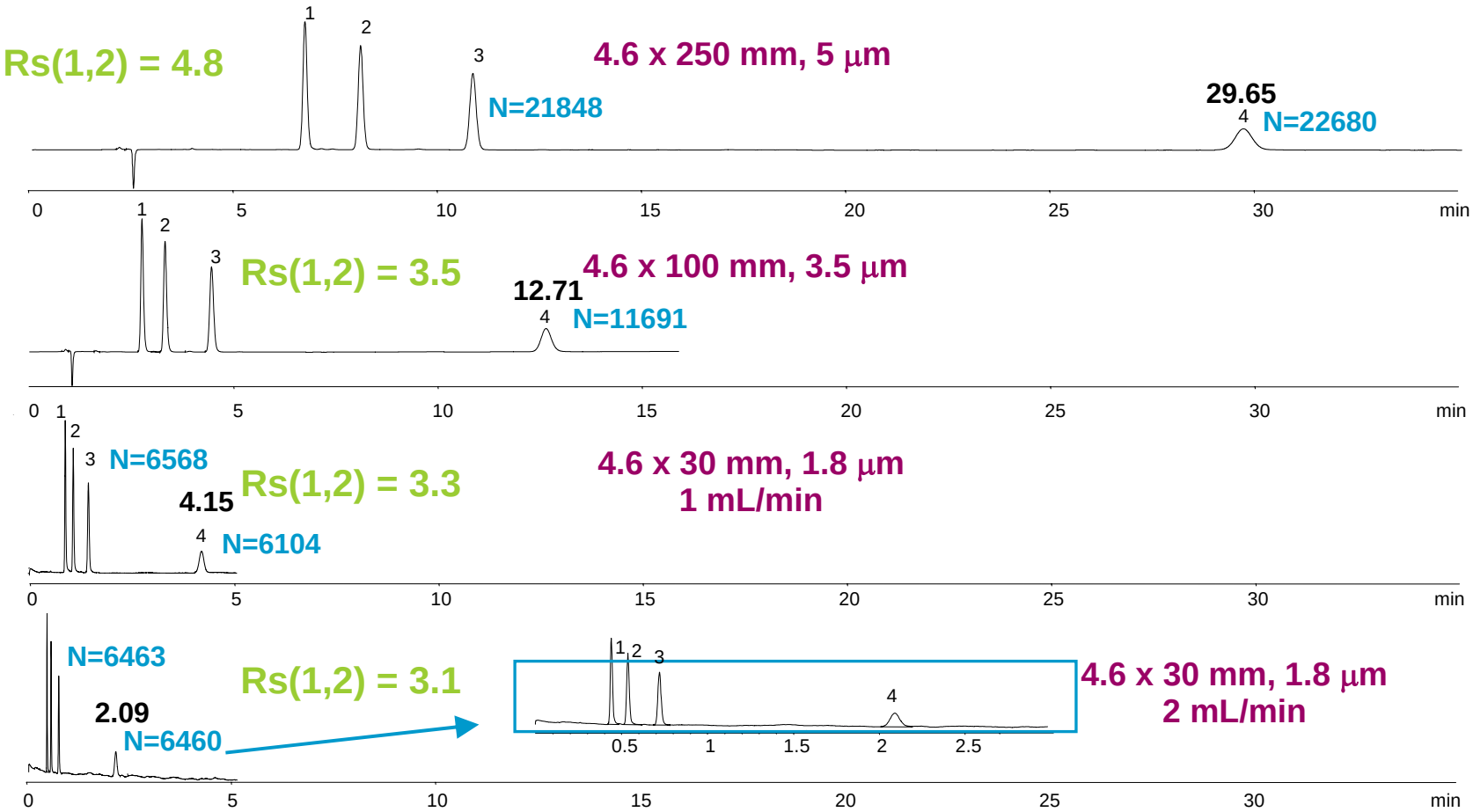
Column Length (mm)	Resolving Power N(5 µm)	Resolving Power N(3.5 µm)	Resolving Power N(1.8 µm)	Typical Pressure Bar (1.8 µm)
150	12,500	21,000	32,500	N.A.
100	8,500	14,000	24,000	420
75	6000	10,500	17,000	320
50	4,200	7,000	12,000	210
30	N.A.	4,200	6,500	126
15	N.A.	2,100	2,500	55

Analysis Time*	
Analysis Time	-33%
	-50%
Peak Volume	-67%
	-80%
Solvent Usage	-90%

* Reduction in analysis time compared to 150 mm column

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

Pick the Column and Particle Size to Meet Your Needs

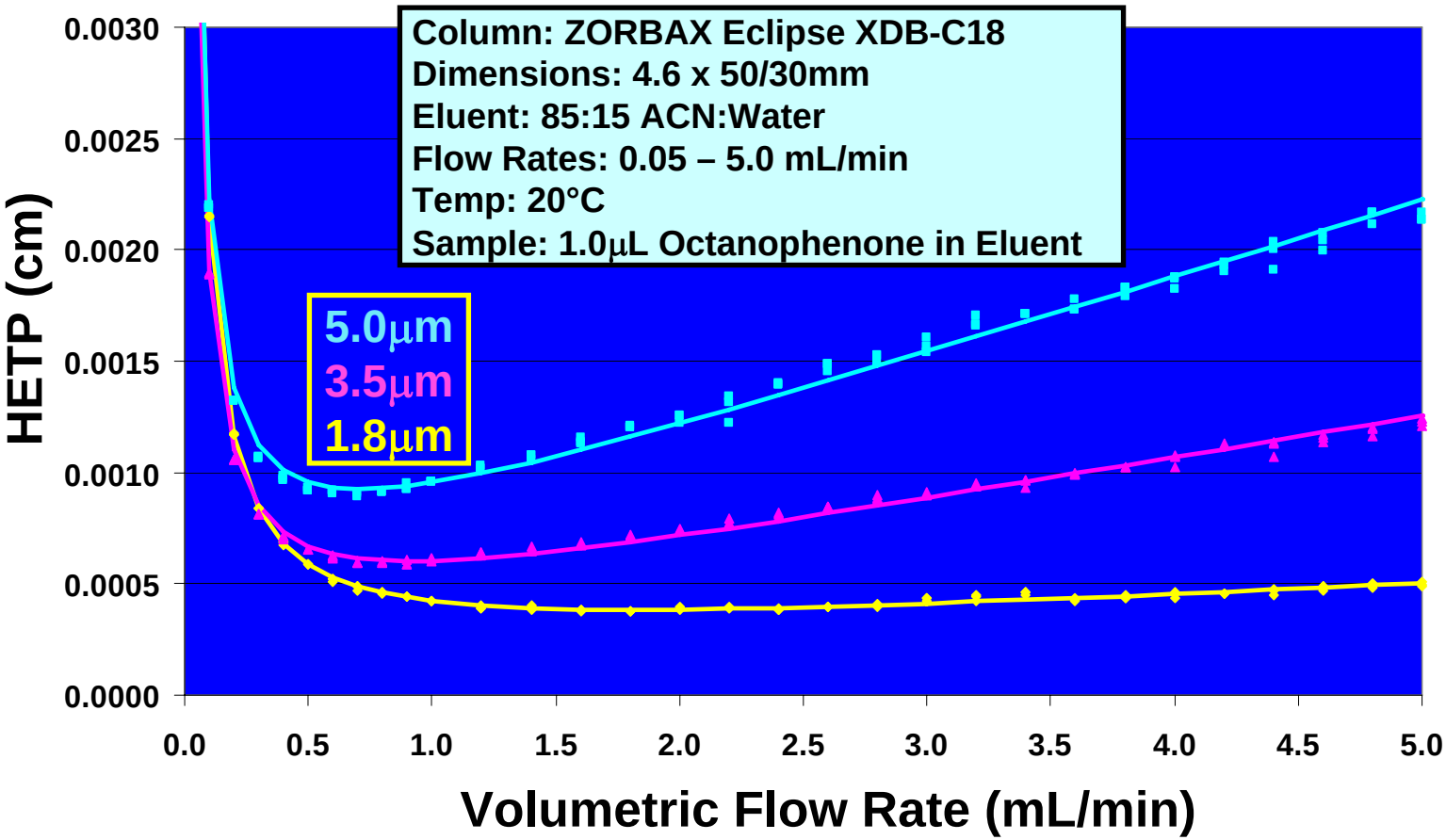


Columns: ZORBAX SB-C18 Mobile Phase: 50% 20 mM NaH_2PO_4, pH 2.8: 50% ACN Flow Rate: 1 mL/min Temperature: R

Detection: UV 230 nm Sample: 1. Estradiol 2. Ethynylestradiol 3. Dienestrol 4. Norethindrone

Smaller Particles Maintain Efficiency Over Wider Flow Rate Ranges

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

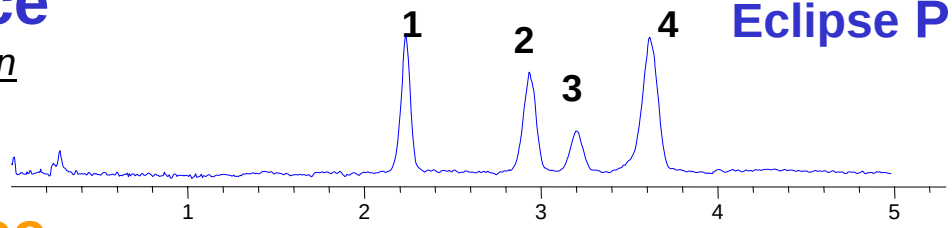


Smaller particle sizes yield flatter curves, minima shift to higher flow rates

Different C18 Bonded Phases for Max Selectivity

1st choice

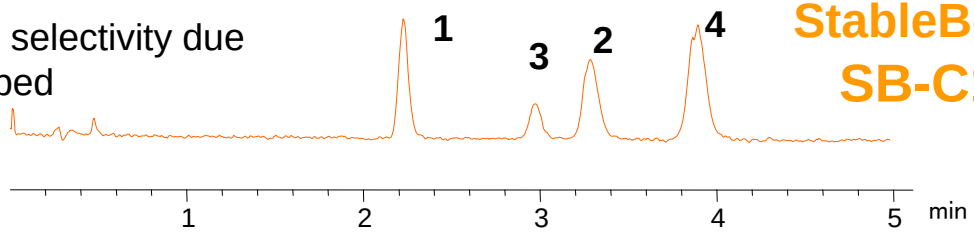
Best Resolution & Peak Shape



Eclipse Plus C18

2nd choice

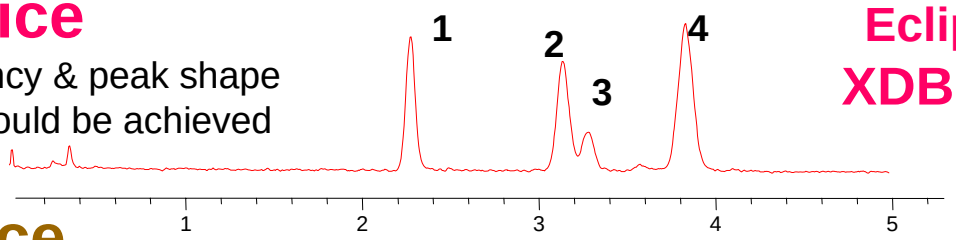
Good alternate selectivity due to non-endcapped



StableBond SB-C18

3rd choice

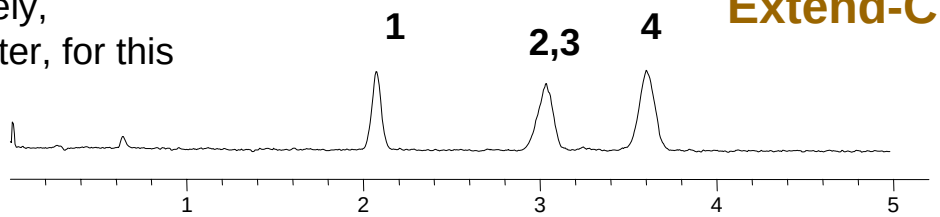
Good efficiency & peak shape
Resolution could be achieved



Eclipse XDB-C18

4th choice

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



Extend-C18

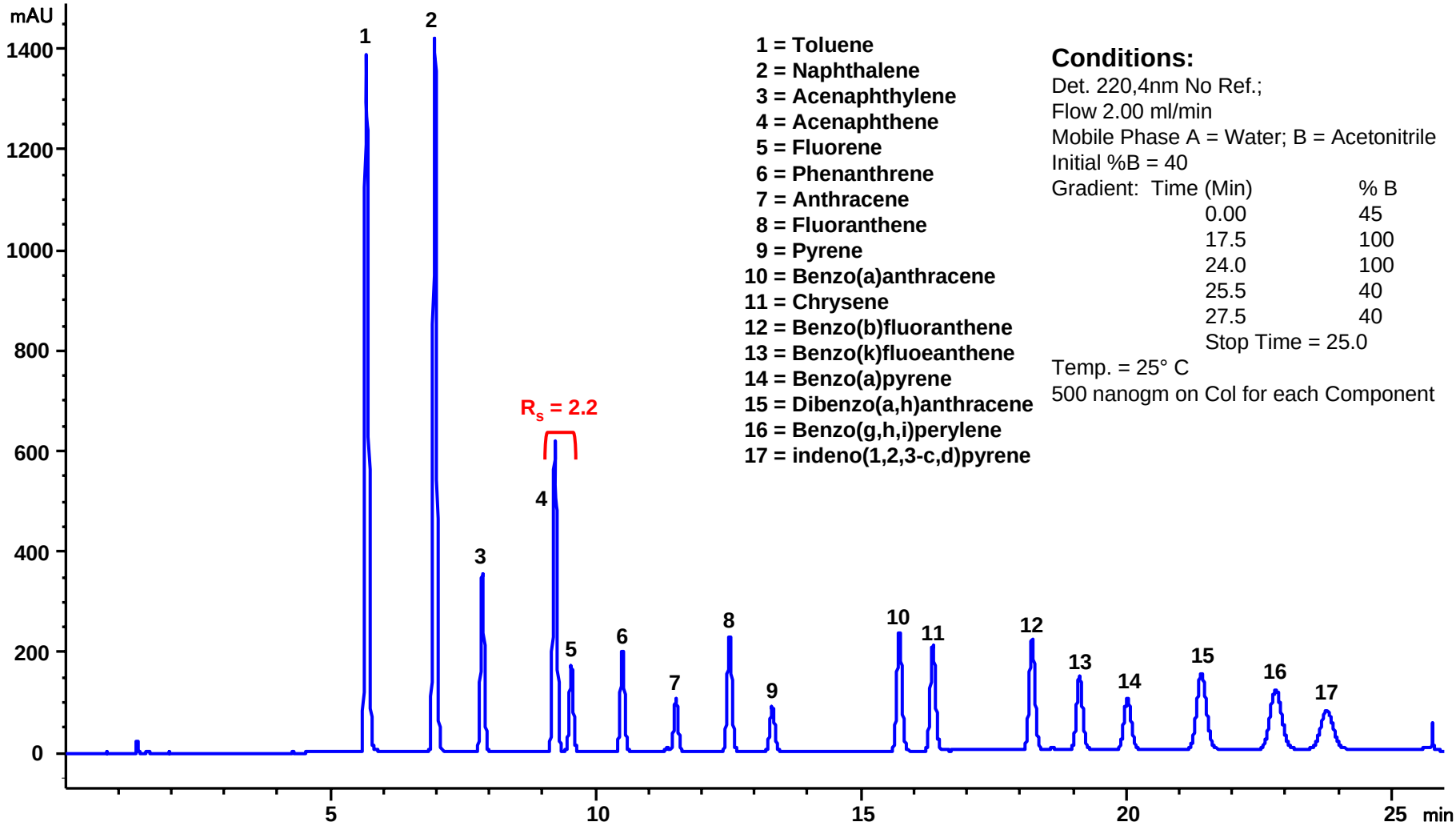
Mobile phase: (69:31) ACN: water
Flow 1.5 mL/min.
Temp: 30 °C
Detector: Single Quad ESI positive mode scan
Columns: RRHT 4.6 x 50 mm 1.8 um

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



1 = Toluene
2 = Naphthalene
3 = Acenaphthylene
4 = Acenaphthene
5 = Fluorene
6 = Phenanthrene
7 = Anthracene
8 = Fluoranthene
9 = Pyrene
10 = Benzo(a)anthracene
11 = Chrysene
12 = Benzo(b)fluoranthene
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

Conditions:

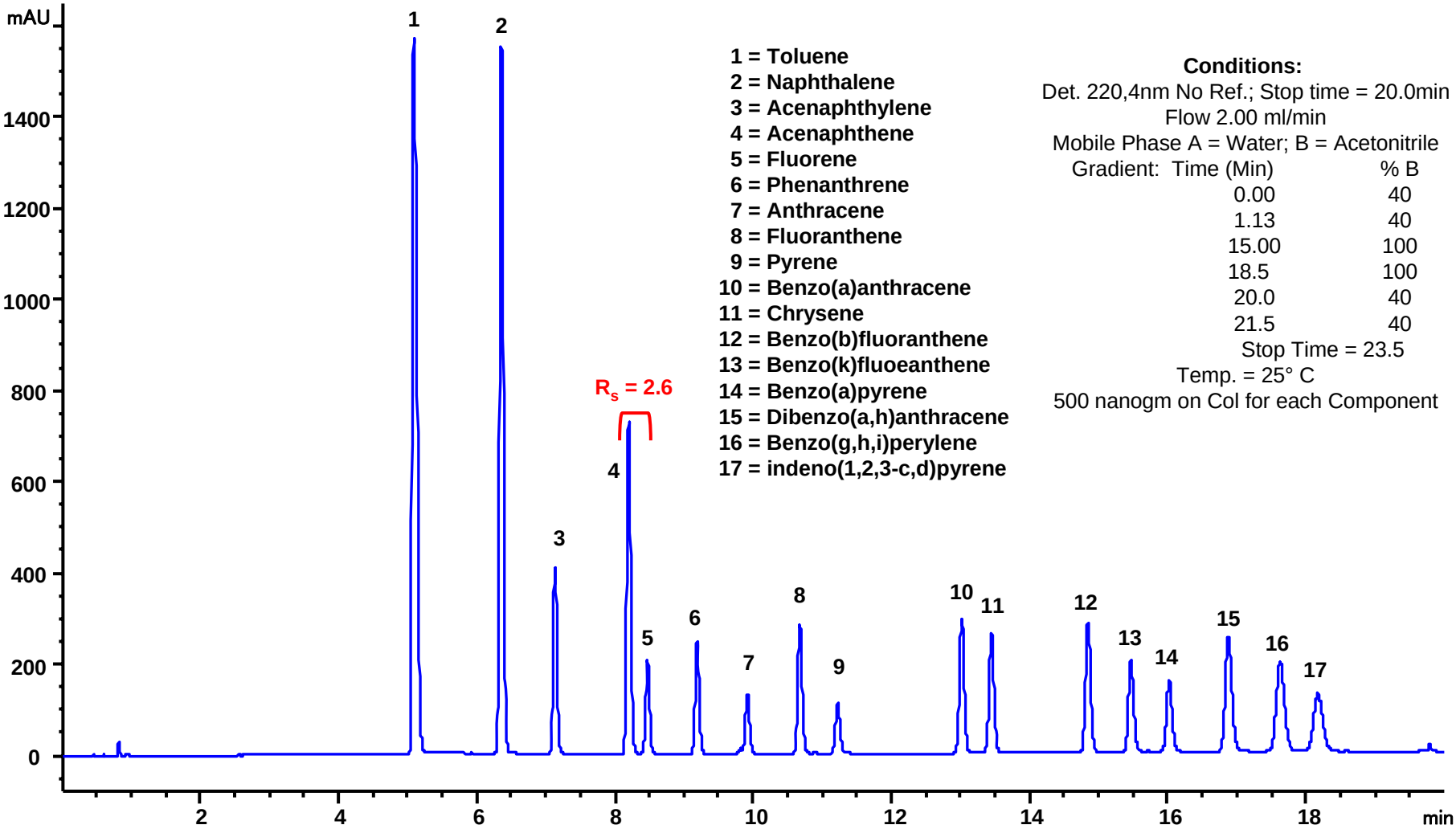
Det. 220,4nm No Ref.;
Flow 2.00 ml/min
Mobile Phase A = Water; B = Acetonitrile
Initial %B = 40
Gradient:

Time (Min)	% B
0.00	45
17.5	100
24.0	100
25.5	40
27.5	40

Stop Time = 25.0

Temp. = 25° C
500 nanogm on Col for each Component

4 – Rapid Resolution – 4.6x150mm, 3.5µm PAH Column – Separation of 16 PAHs in EPA 610



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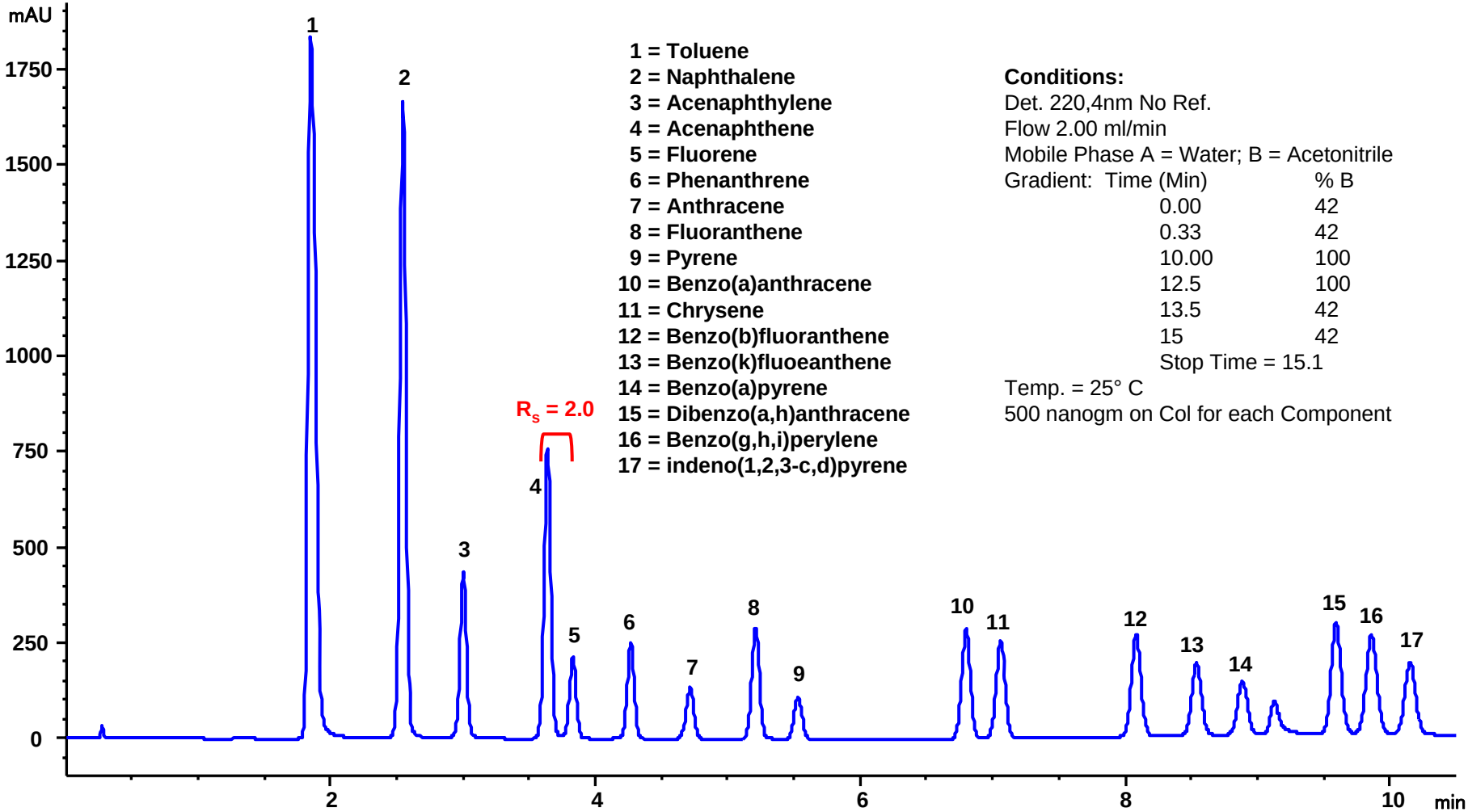
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

Conditions:
Det. 220,4nm No Ref.; Stop time = 20.0min
Flow 2.00 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	40
1.13	40
15.00	100
18.5	100
20.0	40
21.5	40

Stop Time = 23.5
Temp. = 25° C
500 nanogm on Col for each Component

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



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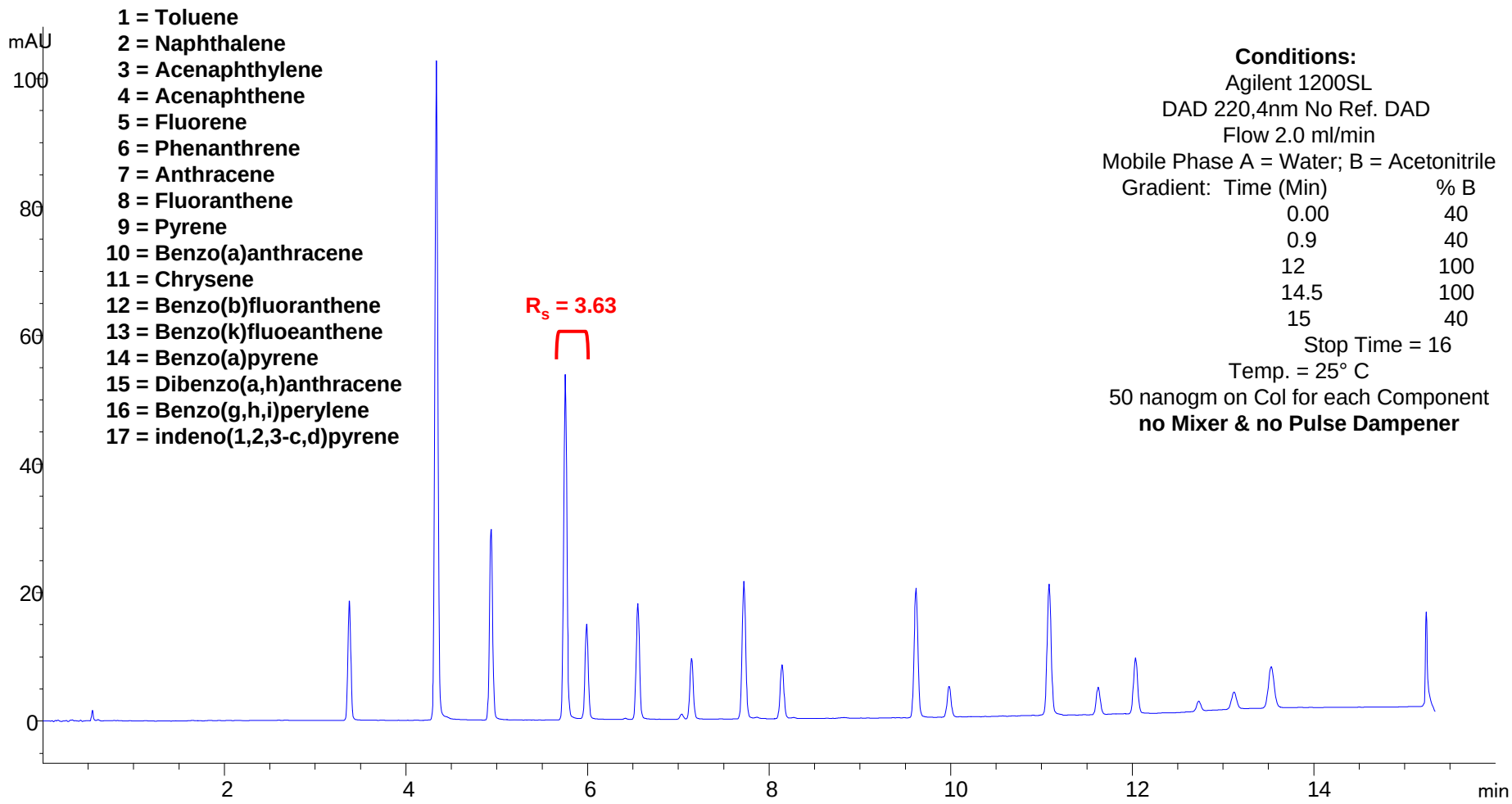
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

Conditions:
Det. 220,4nm No Ref.
Flow 2.00 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	42
0.33	42
10.00	100
12.5	100
13.5	42
15	42
Stop Time = 15.1	

Temp. = 25° C
500 nanogm on Col for each Component

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

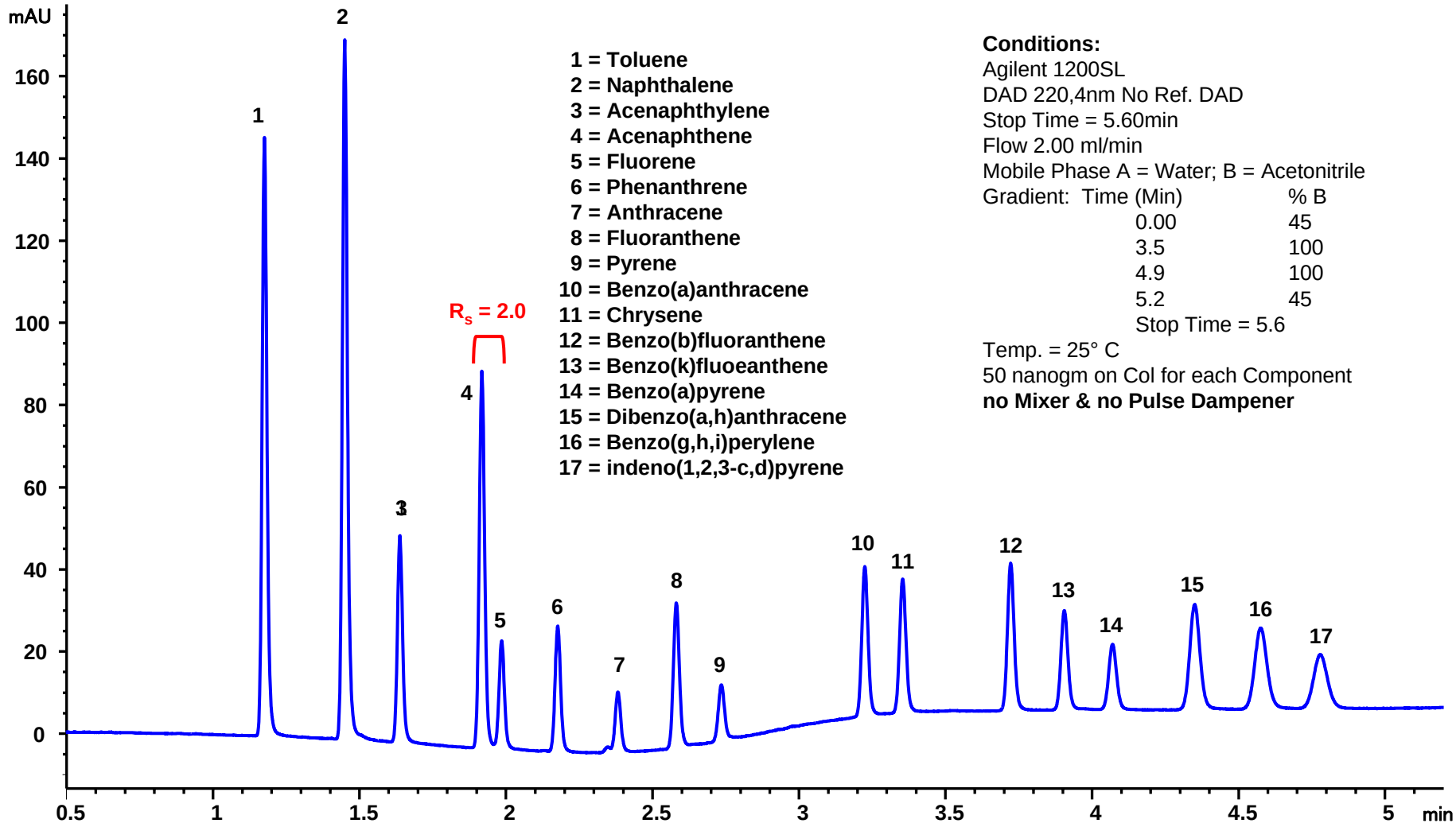


Conditions:
Agilent 1200SL
DAD 220,4nm No Ref. DAD
Flow 2.0 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	40
0.9	40
12	100
14.5	100
15	40

Stop Time = 16
Temp. = 25° C
50 nanogm on Col for each Component
no Mixer & no Pulse Dampener

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



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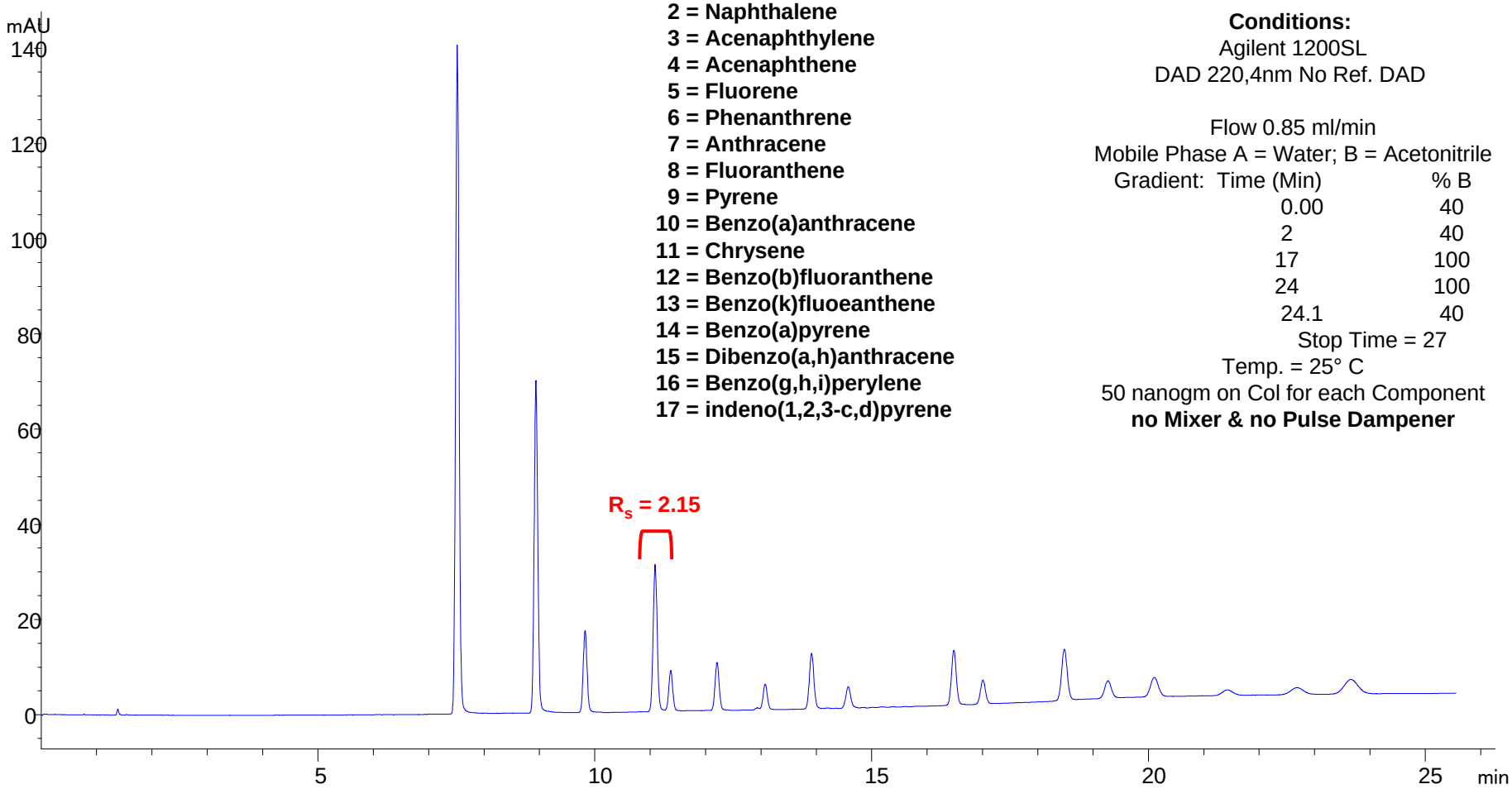
12 = Benzo(b)fluoranthene
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

Conditions:
Agilent 1200SL
DAD 220,4nm No Ref. DAD
Stop Time = 5.60min
Flow 2.00 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	45
3.5	100
4.9	100
5.2	45
Stop Time = 5.6	

Temp. = 25° C
50 nanogm on Col for each Component
no Mixer & no Pulse Dampener

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



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8 = Fluoranthene
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12 = Benzo(b)fluoranthene
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

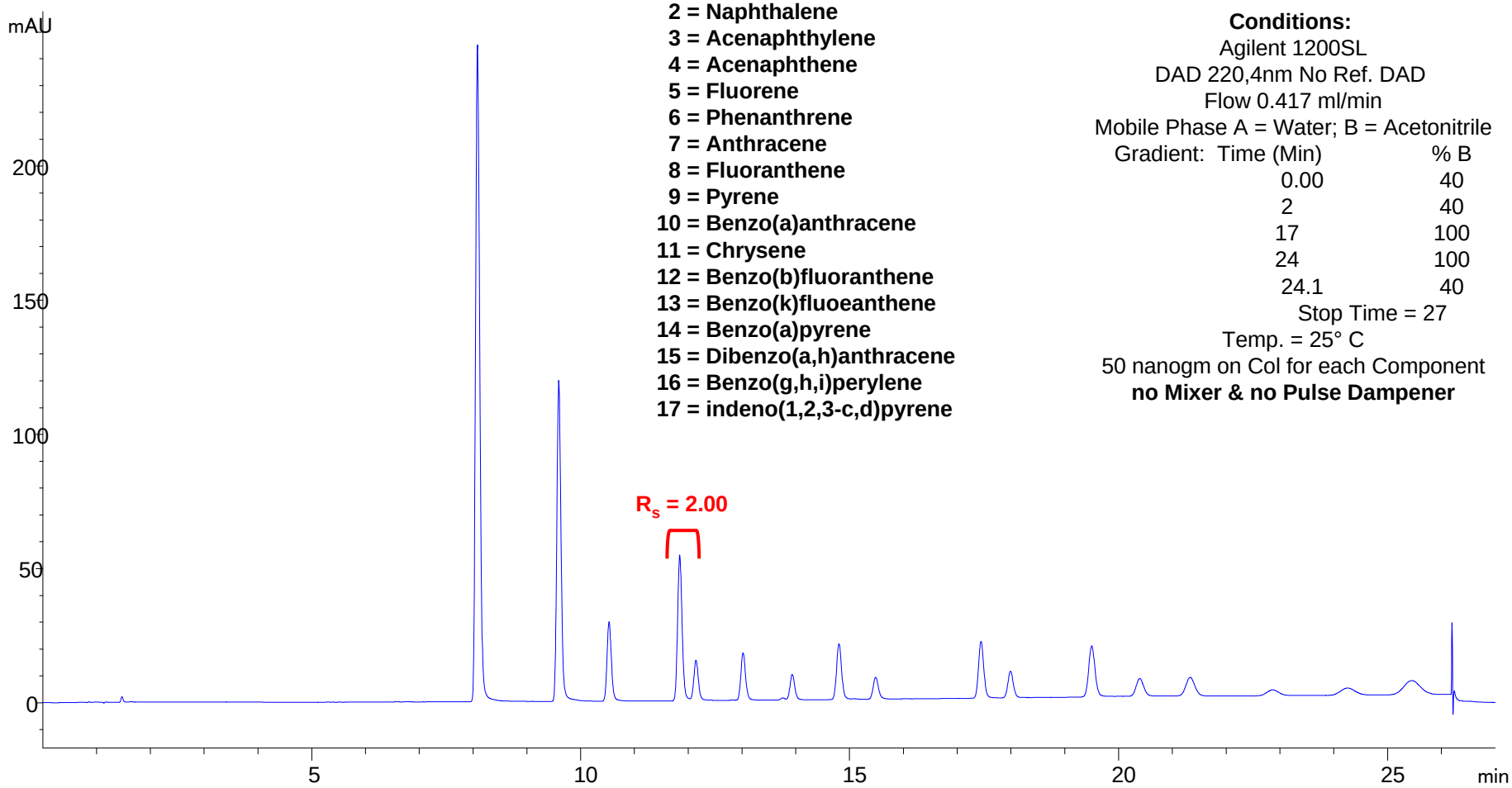
Conditions:
Agilent 1200SL
DAD 220,4nm No Ref. DAD

Flow 0.85 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	40
2	40
17	100
24	100
24.1	40

Stop Time = 27
Temp. = 25° C
50 nanogm on Col for each Component
no Mixer & no Pulse Dampener

#11 Narrow Bore Eclipse PAH 2.1x250 mm, 5 µm Column - Separation of 16 PAHs in EPA 610



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2 = Naphthalene
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5 = Fluorene
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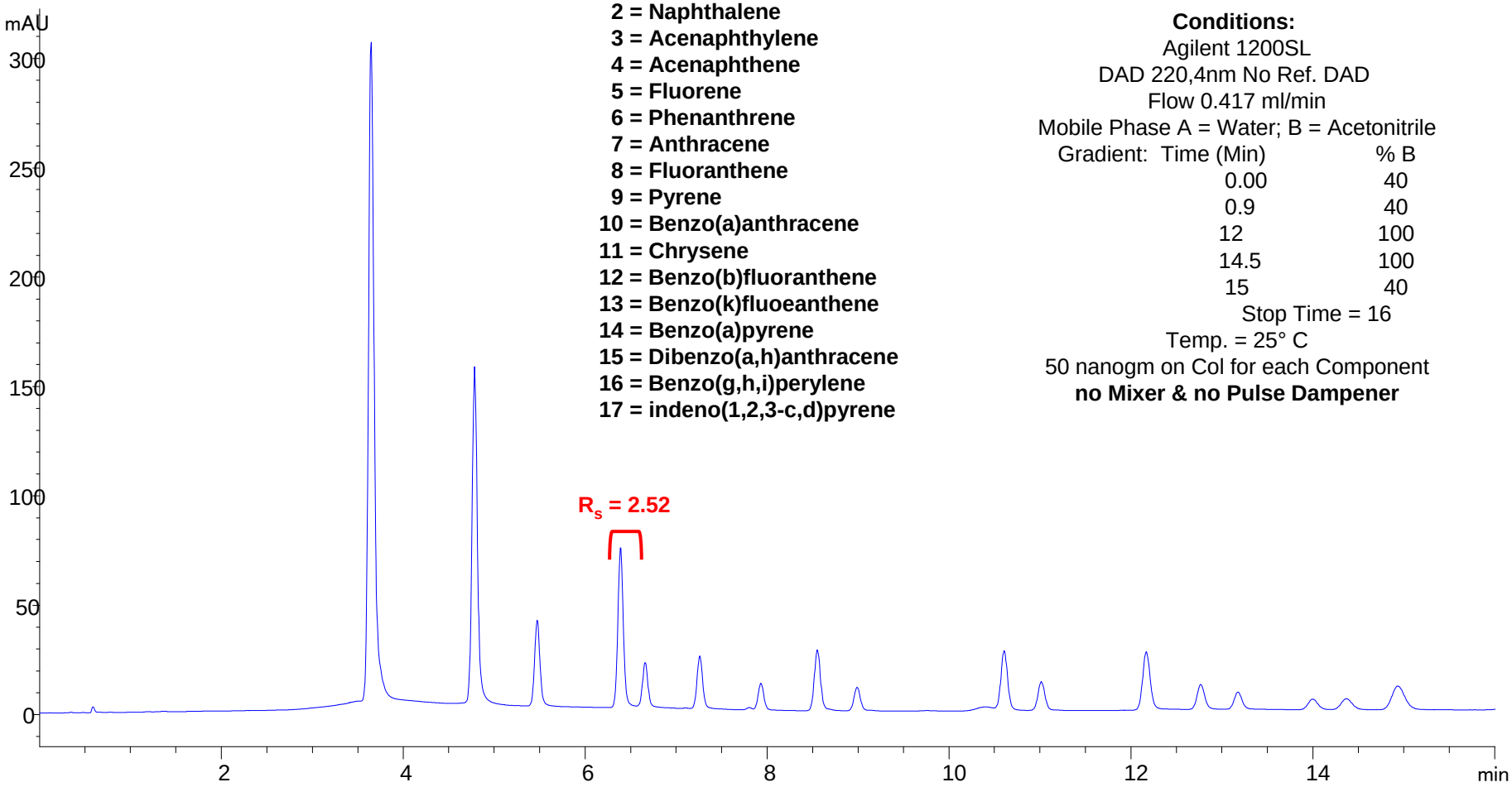
7 = Anthracene
8 = Fluoranthene
9 = Pyrene
10 = Benzo(a)anthracene
11 = Chrysene
12 = Benzo(b)fluoranthene
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

Conditions:
Agilent 1200SL
DAD 220,4nm No Ref. DAD
Flow 0.417 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	40
2	40
17	100
24	100
24.1	40

Stop Time = 27
Temp. = 25° C
50 nanogm on Col for each Component
no Mixer & no Pulse Dampener

#13 Narrow Bore Rapid Resolution Eclipse PAH 2.1x100mm, 3.5 µm Column - Separation of 16 PAHs in EPA 610



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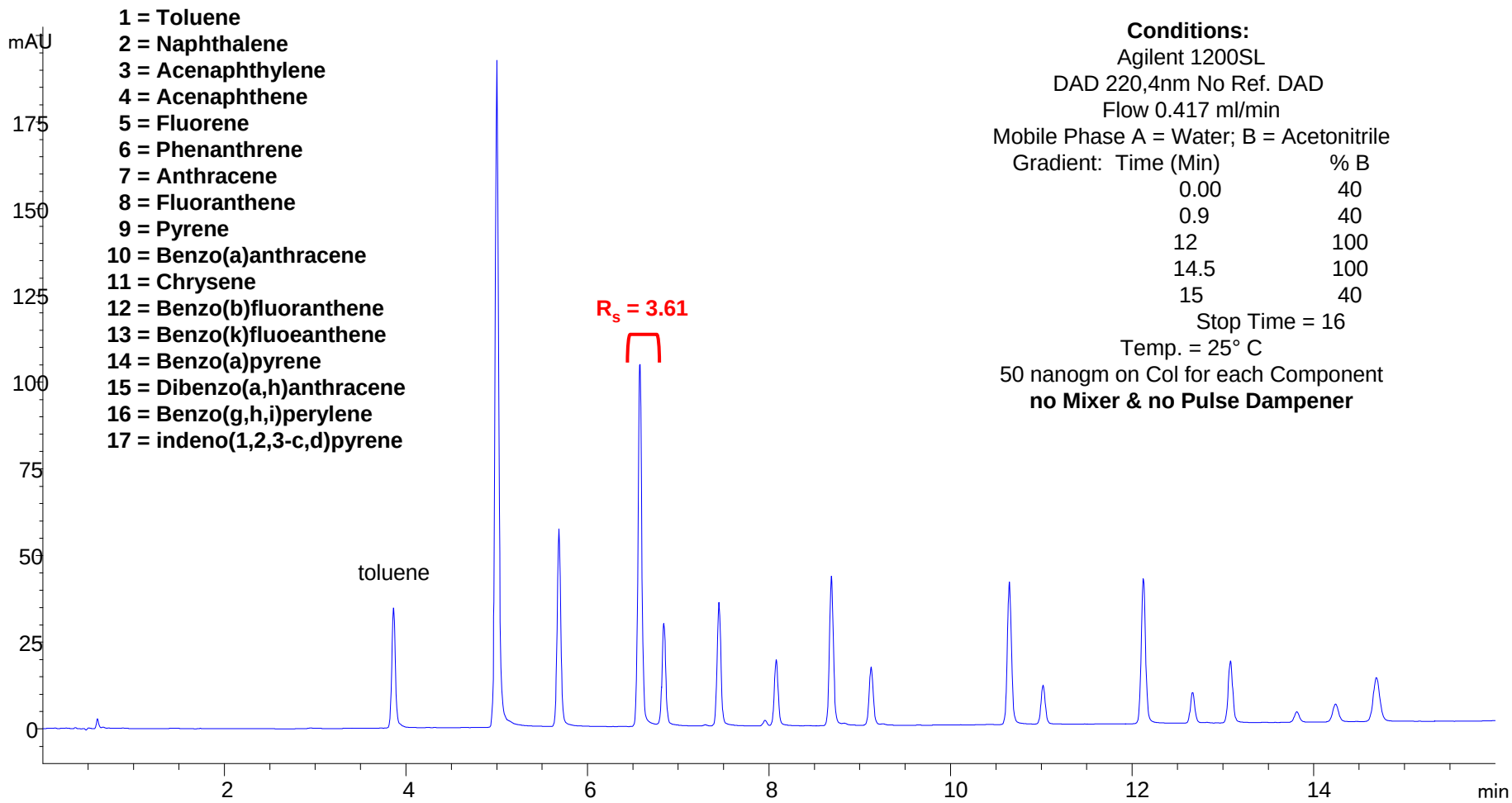
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

Conditions:
Agilent 1200SL
DAD 220,4nm No Ref. DAD
Flow 0.417 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	40
0.9	40
12	100
14.5	100
15	40

Stop Time = 16
Temp. = 25° C
50 nanogm on Col for each Component
no Mixer & no Pulse Dampener

#14 – Narrow Bore RRHT Eclipse PAH 2.1x100mm 1.8 µm – Separation of 16 PAHs in EPA 610

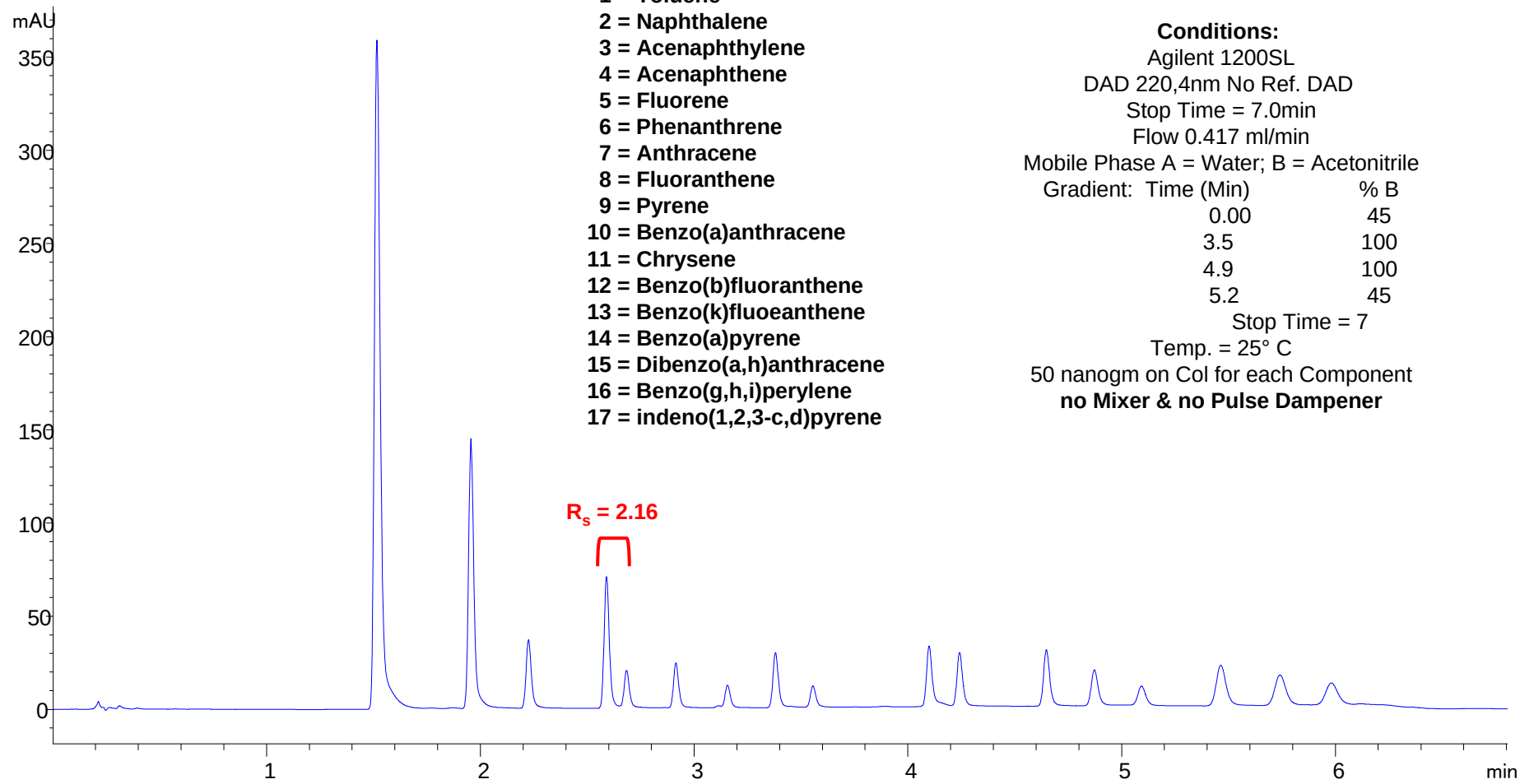


Conditions:
Agilent 1200SL
DAD 220,4nm No Ref. DAD
Flow 0.417 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	40
0.9	40
12	100
14.5	100
15	40

Stop Time = 16
Temp. = 25° C
50 nanogm on Col for each Component
no Mixer & no Pulse Dampener

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



1 = Toluene
2 = Naphthalene
3 = Acenaphthylene
4 = Acenaphthene
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6 = Phenanthrene
7 = Anthracene
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11 = Chrysene
12 = Benzo(b)fluoranthene
13 = Benzo(k)fluoeanthene
14 = Benzo(a)pyrene
15 = Dibenzo(a,h)anthracene
16 = Benzo(g,h,i)perylene
17 = indeno(1,2,3-c,d)pyrene

Conditions:
Agilent 1200SL
DAD 220,4nm No Ref. DAD
Stop Time = 7.0min
Flow 0.417 ml/min
Mobile Phase A = Water; B = Acetonitrile

Gradient: Time (Min)	% B
0.00	45
3.5	100
4.9	100
5.2	45

Stop Time = 7
Temp. = 25° C
50 nanogm on Col for each Component
no Mixer & no Pulse Dampener

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

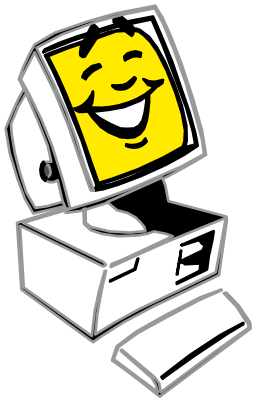
General Phase Type	Starting ZORBAX Choice
Typical “endcapped” C18 or C8 bonded phases, newer columns	Eclipse Plus C18 or C8
Endcapped C18 or C8 columns, older generation	Eclipse XDB-C18 or C8
Non-endcapped columns	StableBond C18
Older types of columns	StableBond C18, C8 etc.
Aqueous “type” columns	SB-AQ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

Conclusion

There is no one-size-fits all column

Choose the best for your application needs

Agilent LC Columns and Agilent J&W GC Columns Scientific Technical Support



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- **302-993-5304 (phone)**
  - **For LC columns**
  - ***Select option 4, then option 2***
  - ***For GC Columns***
- **** Select option 4, then option 1.***
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Introduction to Capillary GC

February 20, 2008 – 1:00 p.m. EST

Selection of a Capillary GC Column

March 13, 2008 – 2:00 p.m. EST

Method Development

March 18, 2008 – 2:00 p.m. EST