The Secrets of Rapid HPLC Method Development

Choosing Columns for Rapid Method Development and Short Analysis Times

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Rapid Analysis Is More Than Run Time

- It is developing a method to meet a goal and developing and validating it quickly.
- The final method should minimize analysis time for the greatest sample throughput.
- It must be reproducible and robust.

Four Critical Aspects of Rapid Method Development and Analysis

- Rapid Sample Preparation – minimum steps for maximum effectiveness, use updated tools (combination filters) and multi-sample preparation equipment (SPE 96-well plates)
- Choose best bonded phases for high resolution – selecting from typical C18 and C8 bonded phases or those targeted to special sample types
- Choose the best column configuration for minimum analysis time with high efficiency and resolution – best column length, internal diameter, particle size
- Using your HPLC instrument to further reduce analysis time and increase sample throughput – optimizing the HPLC and using new features effectively

Bonded Phase Choice Drives Resolution

- Changing selectivity (α) influences resolution the most
- Bonded phase is the column choice that controls selectivity

$$Rs = \frac{1}{4} N^{1/2} \left[\frac{(\alpha - 1)}{\alpha} \right] \left(\frac{k}{1 + k} \right)$$

α= selectivity – increase by changing bonded phase and mobile phase

= retention – increase by changing bonded phase and mobile phase
does not improve Rs above k ≈10

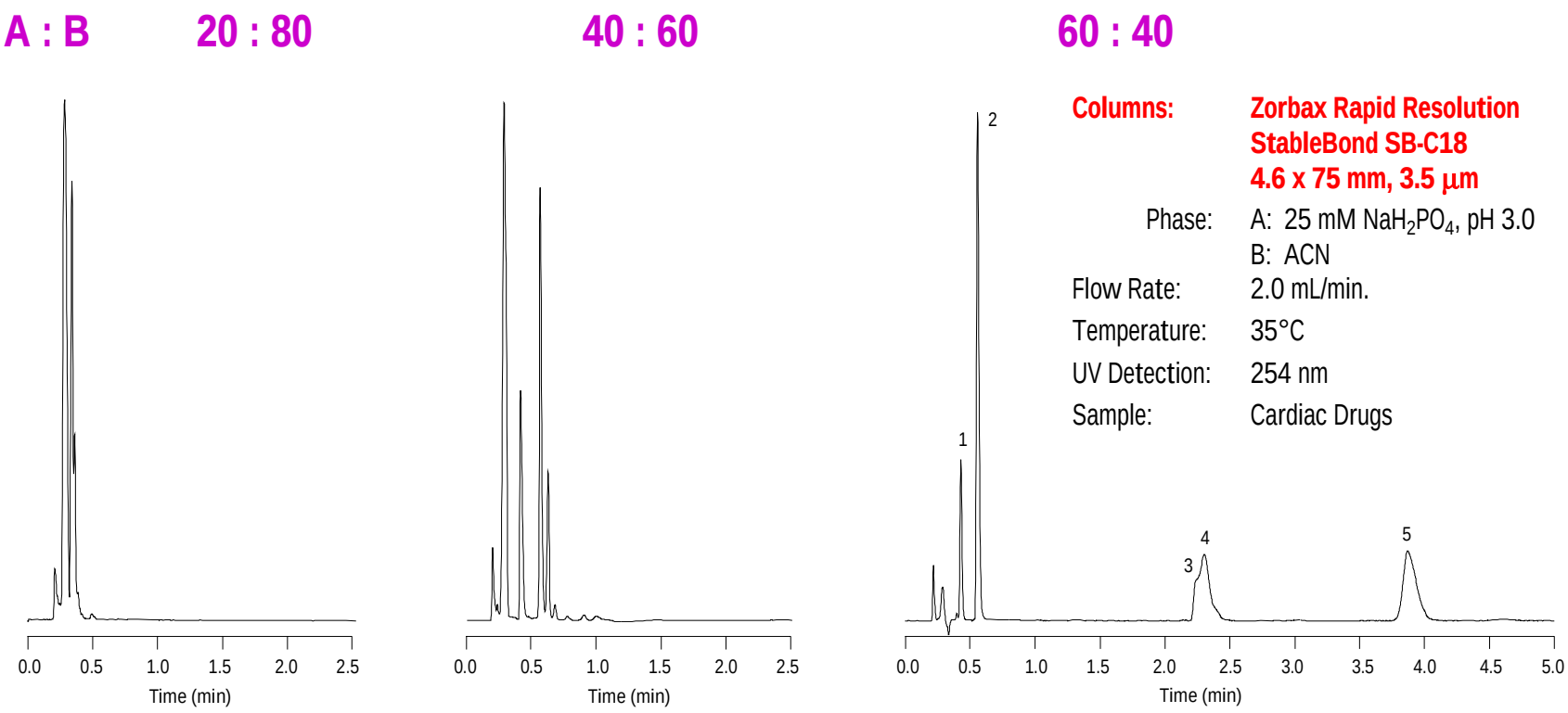
Selecting a Bonded Phase

- Choose columns known to have long lifetimes at operating mobile phase pH.
- Choose bonded phases on high purity, low acidity silica for best peak shape.
- Select a C18 or C8 bonded phase first for good retention and resolution with typical acidic, basic and neutral samples.
- If sample is expected to be difficult (i.e. very polar, difficult to retain, very basic) then select targeted bonded phases available for these types of samples.

Rapid Method Development Scheme – Low pH

- Start at low pH for best peak shape, retention and long-term reproducibility
  - Select starting conditions
    - StableBond C18 or C8 for maximum lifetime – Rapid Resolution columns
    - pH 1 – 3 with 20 – 50 mM buffer for best peak shape
    - Acetonitrile or methanol – start high to scout
    - Adjust organic for maximum resolution and retention
    - Change organic if resolution not achieved – MeOH or ACN
    - Change bonded phase if resolution not achieved – SB-CN, SB-Phenyl, SB-C3
- Use elevated temperature to reduce analysis time further

Rapid Method Development Scouting Chromatograms on StableBond-C18 Rapid Resolution Columns



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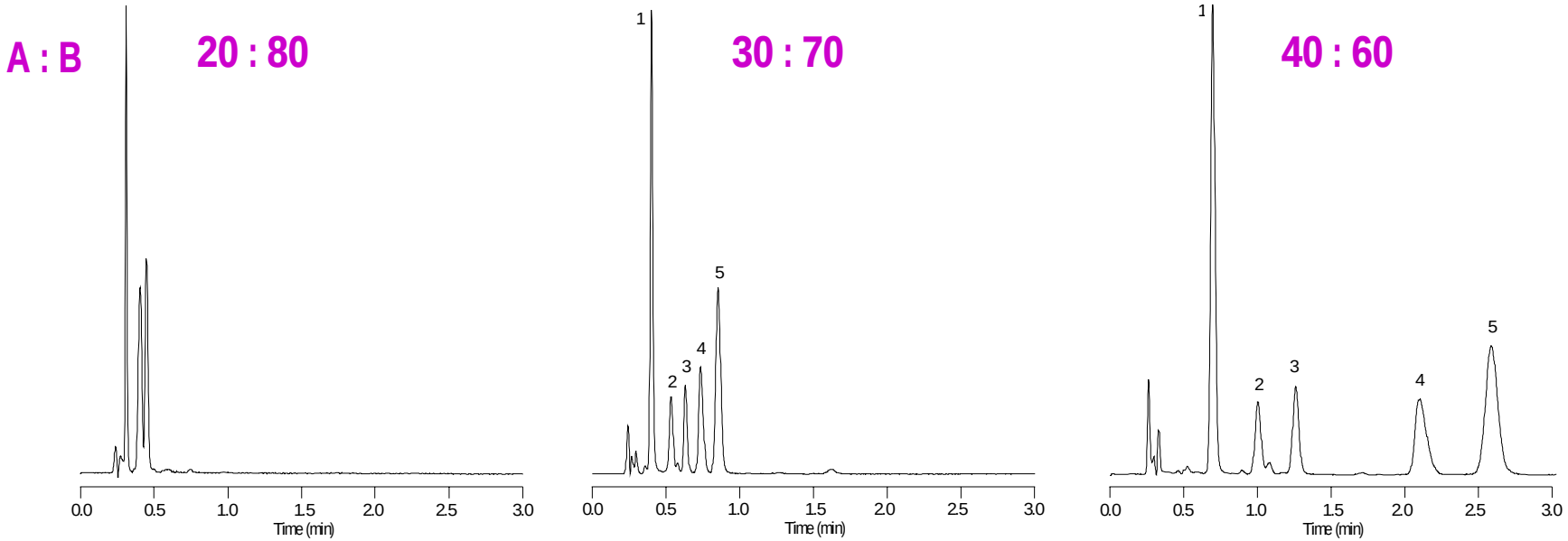
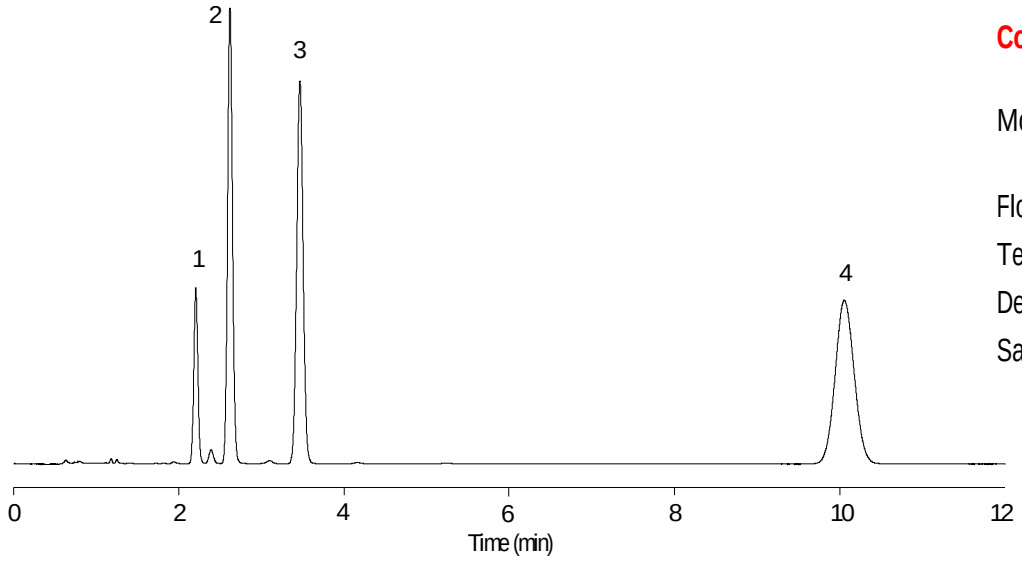
Rapid Method Development
Scouting Chromatograms
Change Organic Modifier

Column: Zorbax Rapid Resolution SB-C18, 4.6 x 75 mm, 3.5 µm Mobile Phase: A: 25 mM NaH_2PO_4 , pH 3.0 B: MeOH
Flow Rate: 2.0 mL/min Temperature: 35°C Detection: UV 254 nm
Sample: Cardiac Drugs 1. Diltiazem 2. Dipyridamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine



- Changing organic modifier can alter selectivity and improve peak shape.

Rapid Method Development Process Optimizes Separation on StableBond-C18 at Low pH



Column:	**Zorbax Rapid Resolution SB-C18, 4.6 x 75 mm, 3.5 µm**
Mobile Phase:	50% ACN 50% 20 mM NaH_2PO_4, pH 2.8
Flow Rate:	1.0 mL/min
Temperature:	RT
Detection:	UV 254 nm
Sample:	1. Estradiol 2. Ethynylestradiol 3. Dienestrol 4. Norethindrone

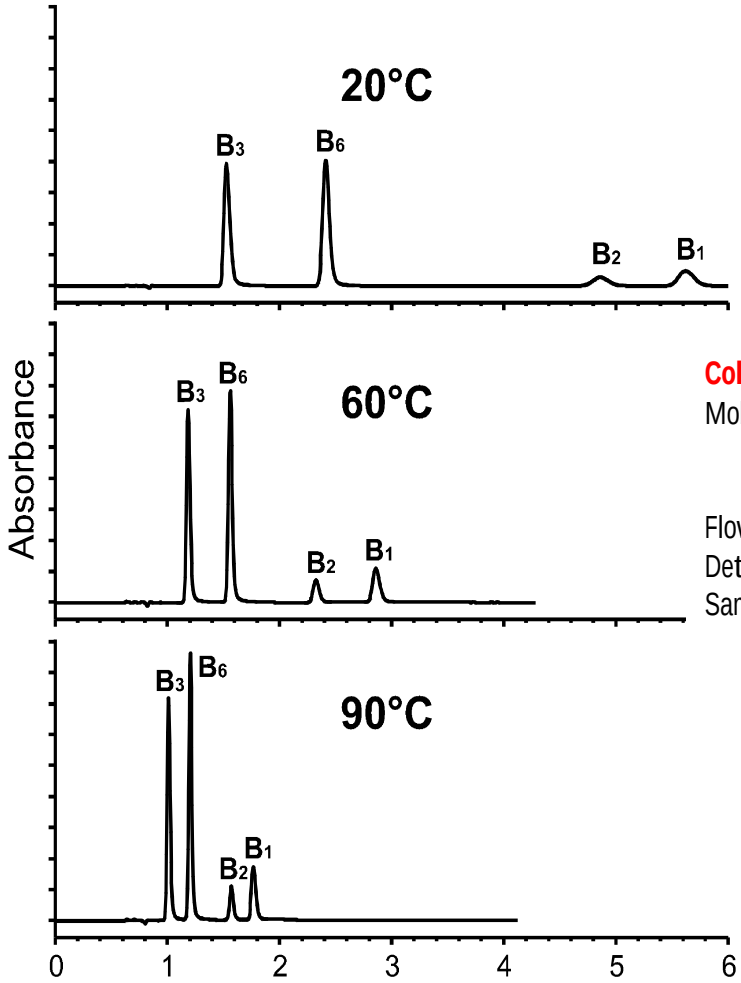
- A Rapid Resolution SB-C18 column at low pH was used to develop this thorough and rapid analysis of steroids and impurities following the rapid method development process.

Increase Temperature to Reduce Analysis Time

Low Temperature

Method Optimization

High Temperature



Column: **Zorbax SB-C18, 4.6 x 75 mm, 5 µm**
Mobile Phase: 74% 7.4 mM hexane sulfonate and 0.07% phosphoric acid
26% MeOH
Flow Rate: 1 mL/min
Detection: UV 280 nm
Sample: B Vitamins

- Agilent instruments provide reliable temperature control to 80 - 90°C allowing method development flexibility.

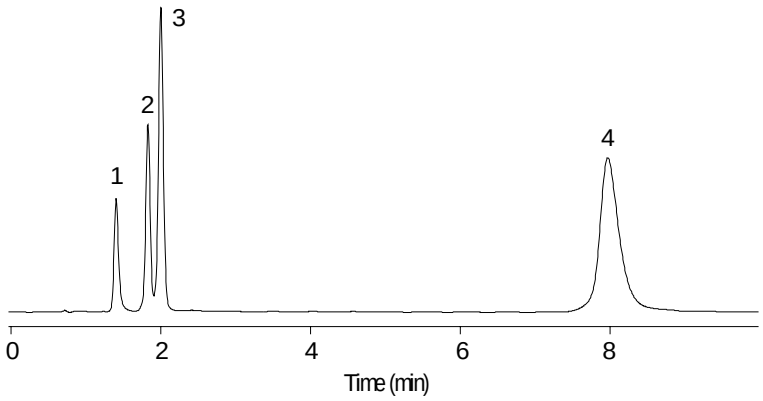
Rapid Method Development Scheme – Mid pH

- Try pH 7 (pH 6 – 9) with Eclipse XDB-C18 or C8 and follow same process, selecting Eclipse XDB-Phenyl as alternate bonded phase
- Use temperature to reduce analysis time further

Eclipse XDB-C18 is Bonded Phase Choice for High Resolution at Mid pH

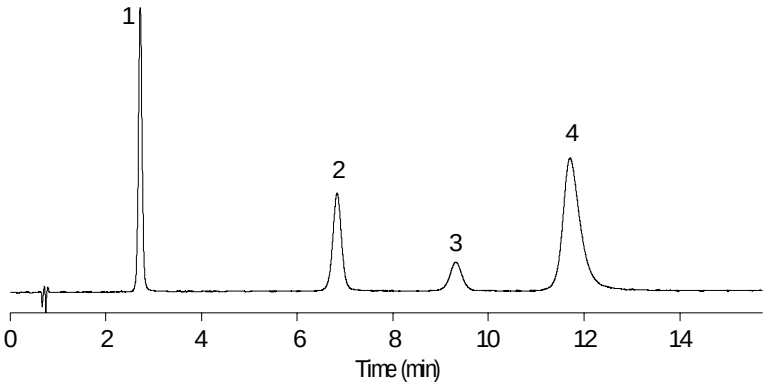
SB-C18, 4.6 x 75 mm, 3.5 µm

pH 3



Eclipse XDB-C18, 4.6 x 75 mm, 3.5 µm

pH 7



Mobile Phase: 20% Methanol: 80% 20 mM phosphate buffer + (10 mM TEA @ pH 7) Flow Rate: 1.0 mL/min Temperature: RT
Detection: UV 254 nm Sample: 1. Nizatidine 2. Famotidine 3. Cimetidine 4. Pirenzipine

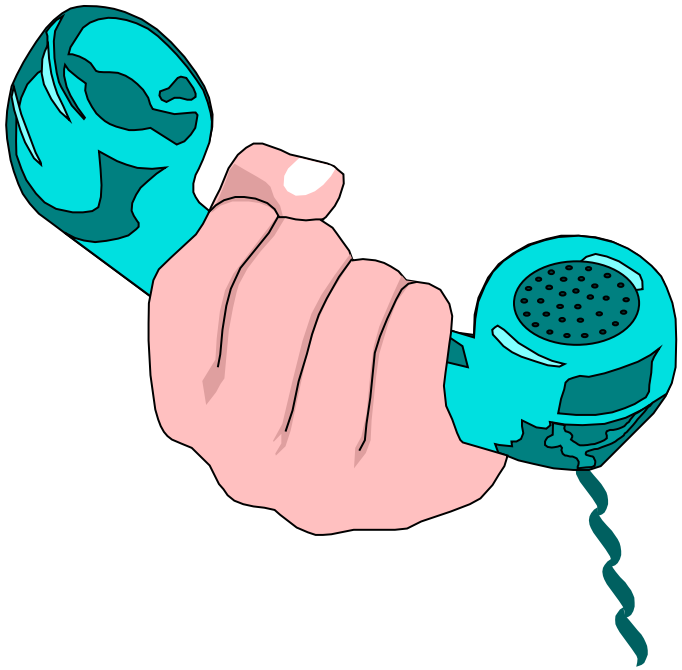
- This sample is only resolved at mid pH on Eclipse XDB-C18 following rapid method development process.

Break Number 1

For Questions and Answers

Press *1 on Your Phone to

Ask a Question



Choose Special Bonded Phases for Difficult Samples

- **Sample 1 – Highly basic compounds**
- May exhibit poor peak shape
- May be difficult to retain
- Column choice 1 – Bonus-RP for better peak shape
- Column choice 2 – Extend-C18 at high pH for better retention and peak shape

- **Sample 2 – Highly polar compounds**
- May be difficult to retain
- May require high aqueous mobile phases
- Column choice 1 – SB-Aq for better retention with high aqueous mobile phases
- Column choice 2 – Bonus-RP for use with high aqueous mobile phases

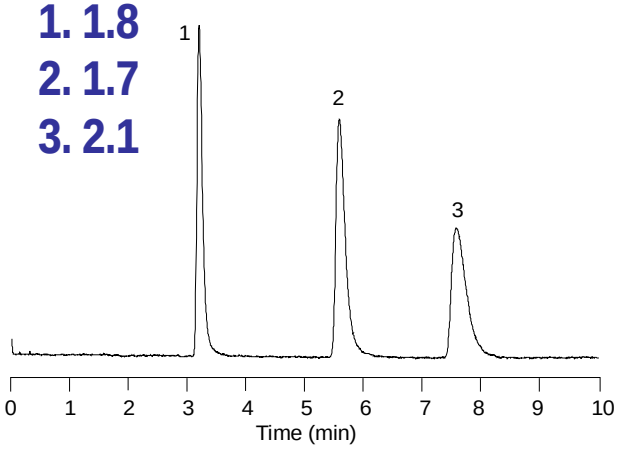
Sample 1: Highly Basic Compounds

Select Bonus-RP for Improved Peak Shape

Column: 4.6 x 150 mm, 5 µm Mobile Phase: A: 75% 25 mM NH_4Ac, pH 5.5 : 25% ACN B: 80% 25 mM NH_4Ac, pH 5.5 : 20% ACN
Flow Rate: 1.5 mL/min Detection: UV 254 nm Sample: 1. Doxylamine 2. Chlorpheniramine 3. Triprolidine

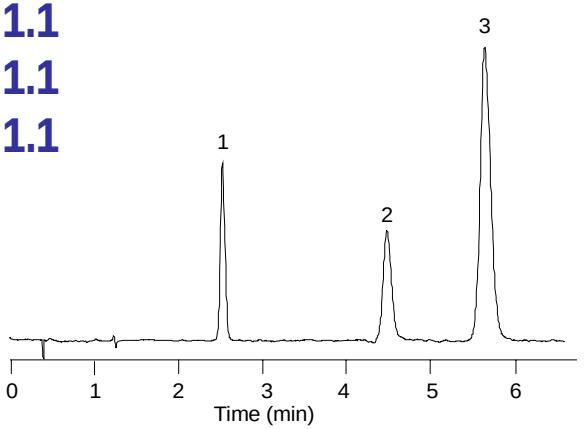
A. Alkyl C8

TF
1. 1.8
2. 1.7
3. 2.1



B. Bonus-RP

TF
1. 1.1
2. 1.1
3. 1.1



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Sample 1: Highly Basic Compounds

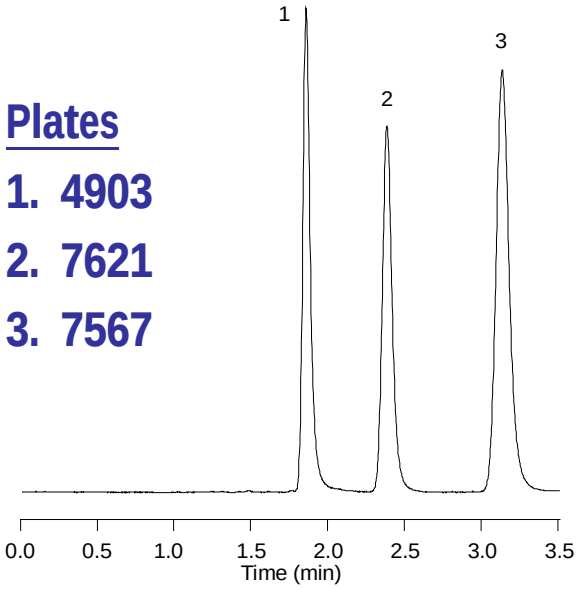
Extend-C18 Improves Retention and Efficiency of Procainamides at High pH

Column: Zorbax Extend-C18, 4.6 x 150 mm, 5 µm Mobile Phase: See Below Flow Rate: 1.0 mL/min Detection: UV 254 nm
Temperature: RT Sample: 1. Procainamide pKa 9.2 2. N-acetylprocainamide 3. N-propionylprocainamide

50% 25 mM Na_2HPO_4, pH 7.0 : 50% MeOH

Plates

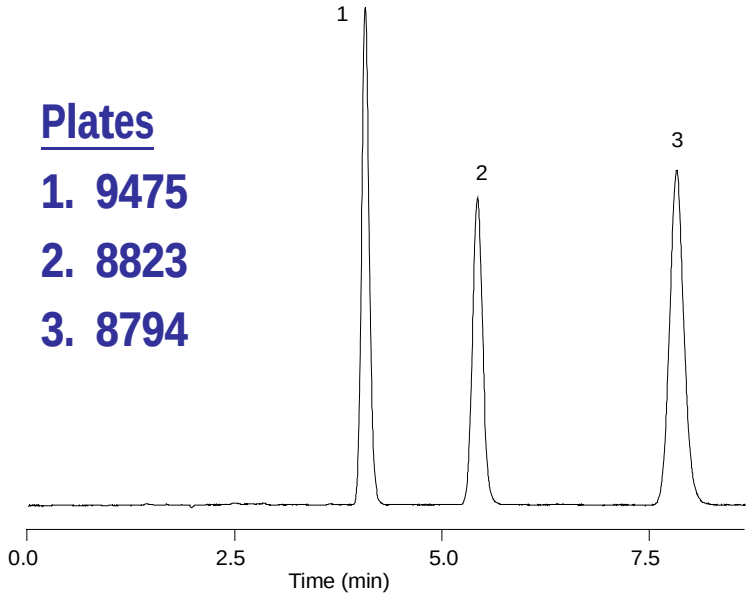
1. 4903
2. 7621
3. 7567



50% 20 mM TEA, pH 11: 50% MeOH

Plates

1. 9475
2. 8823
3. 8794

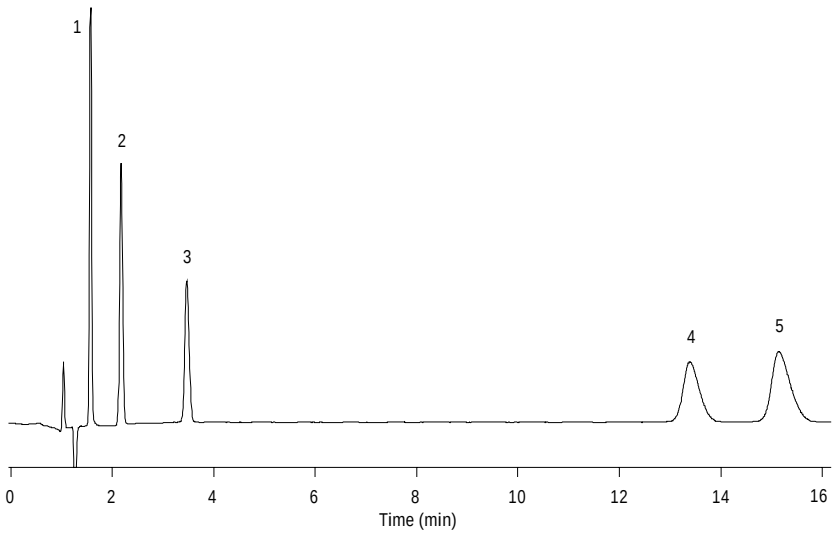


- High efficiency improves resolution and column lifetime and the increase in retention results in a more rugged method.

Sample 2: Highly Polar Compounds
Select New ZORBAX SB-Aq Columns for Method Development in High Aqueous Mobile Phases

- Good retention for polar compounds in high aqueous mobile phases
- Reproducible retention without “phase collapse”
- Different selectivity vs. conventional C18 columns
- Highly, stable at low pH and high temperature (up to 80°C)

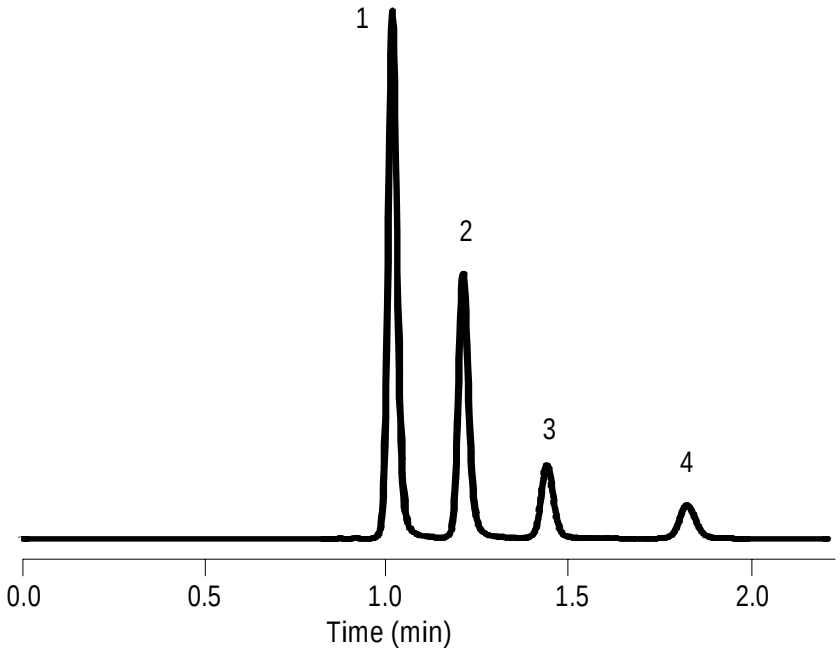
Sample 2: Highly Polar Compounds
ZORBAX SB-Aq Provides Good Retention of Polar Compounds



Columns:	**Zorbax SB-Aq, 4.6 x 150 mm, 5 µm**
Mobile Phase:	90% 0.2% TFA 10% ACN
Temperature:	25°C
Flow Rate:	1.5 mL/min.
Detection:	UV 254 nm
Sample:	1. Maleate 2. Phenylephrine 3. Phenylpropanolamine 4. Pyrilamine 5. Chlorpheniramine

- These small polar compounds are difficult to retain on most columns.
- The SB-Aq provides excellent retention with a 90% aqueous mobile phase.

Sample 2: Highly Polar Compounds
ZORBAX SB-Aq Provides Good Retention of Water Soluble Vitamins without Ion Pairing



Columns:	**Zorbax SB-Aq, 4.6 x 150 mm, 5 µm**
Mobile Phase:	95% 0.1% TFA 5% MeOH
Temperature:	35°C
Flow Rate:	2.0 mL/min.
Detection:	UV 254 nm
Sample:	1. Thiamine – B1 2. Nicotinic Acid 3. Pyridoxine – B6 4. Niacinamide – B3

- The SB-Aq column provides good retention of these polar compounds without ion pairing.
- The result is a simpler method without the reproducibility problems associated with ion pairing.

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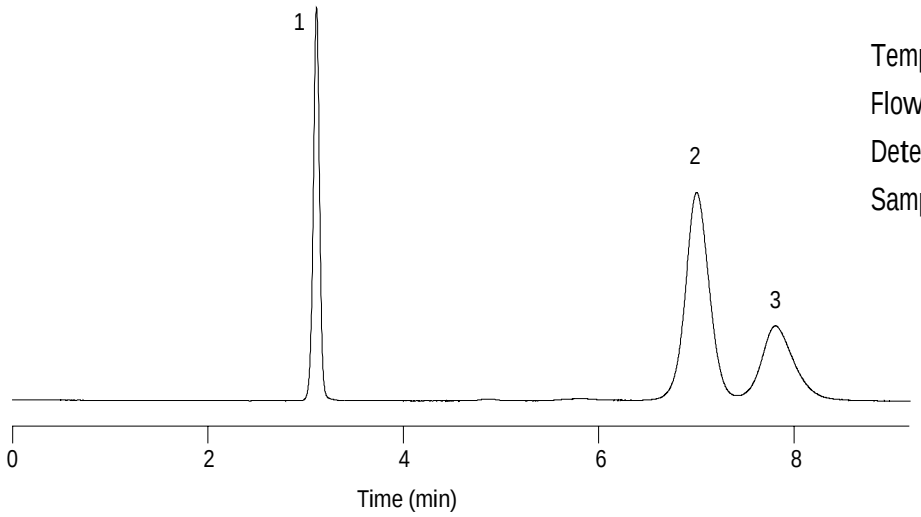
Sample 2: Highly Polar Compounds

ZORBAX SB-Aq Provides Good Retention of NADH/NAD$^+$ with LC/MS Compatible Mobile Phase



Columns:	**Zorbax SB-Aq, 4.6 x 150 mm, 5 µm**
Mobile Phase:	94% 10 mM ammonium acetate, pH 5.5 6% MeOH
Temperature:	25°C
Flow Rate:	1.0 mL/min.
Detection:	254 nm
Sample:	1. Uracil 2. NADH 3. NAD$^+$

- These coenzymes are difficult to retain and analyze by LC/MS.
- The SB-Aq provides baseline resolution without ion pairing.

Rapid Resolution Columns Reduce Method Development and Analysis Time

- Rapid Resolution columns (3.5 µm) are available in many configurations
  - Available in analytical, narrow bore, microbore, and capillary internal diameters for compatibility with any sample size.
  - Semi-preparative and preparative columns use 5 µm particles.
- Rapid Resolution columns reduce isocratic and gradient run times with:
  - Shorter column lengths
  - Higher flow rates
  - Optimized HPLC instrument

Choose Column Configuration for Application

Column Type	I.D. (mm)	Lengths (mm)	Particle Sizes (µm)	Flow Rate Ranges	Applications
Capillary	0.3, 0.5	35 – 250	3.5, 5	1 – 10 µL/min	Max sensitivity LC/MS
MicroBore	1.0	30 – 150	3.5, 5	30 – 60 µL/min	Higher sensitivity LC/MS
Narrow Bore	2.1	15 – 150	3.5, 5	0.1 – 0.3 mL/min	High sensitivity LC/MS
Solvent Saver	3.0	150, 250	3.5, 5	0.3 – 1.0 mL/min	Analytical
Analytical	4.6	15 – 250	3.5, 5	1 – 4 mL/min	Analytical
Semi-prep	9.4	50 – 250	5	4 – 10 mL/min	Small scale prep (mg)
Preparative	21.2	50 – 250	5, 7	20 – 60 mL/min	Large scale prep

High Resolution with Rapid Resolution Columns

Isocratic Separation of Aspartame

Column: Zorbax StableBond SB-C18 Mobile Phase: 85% Water with 0.1% TFA : 15% Acetonitrile Flow Rate: 1.0 mL/min.
Temperature: 35°C Sample: 1. Phenylalanine 2. 5-Benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame

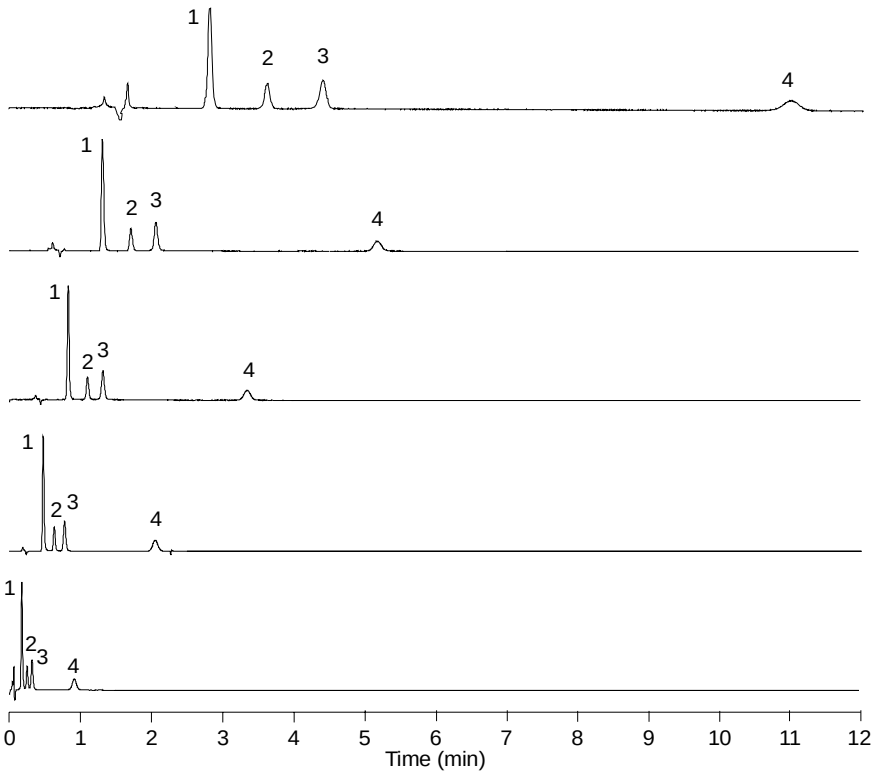
4.6 x 150 mm, 5 µm

4.6 x 75 mm, 3.5 µm

4.6 x 50 mm, 3.5 µm

4.6 x 30 mm, 3.5 µm

4.6 x 15 mm, 3.5 µm



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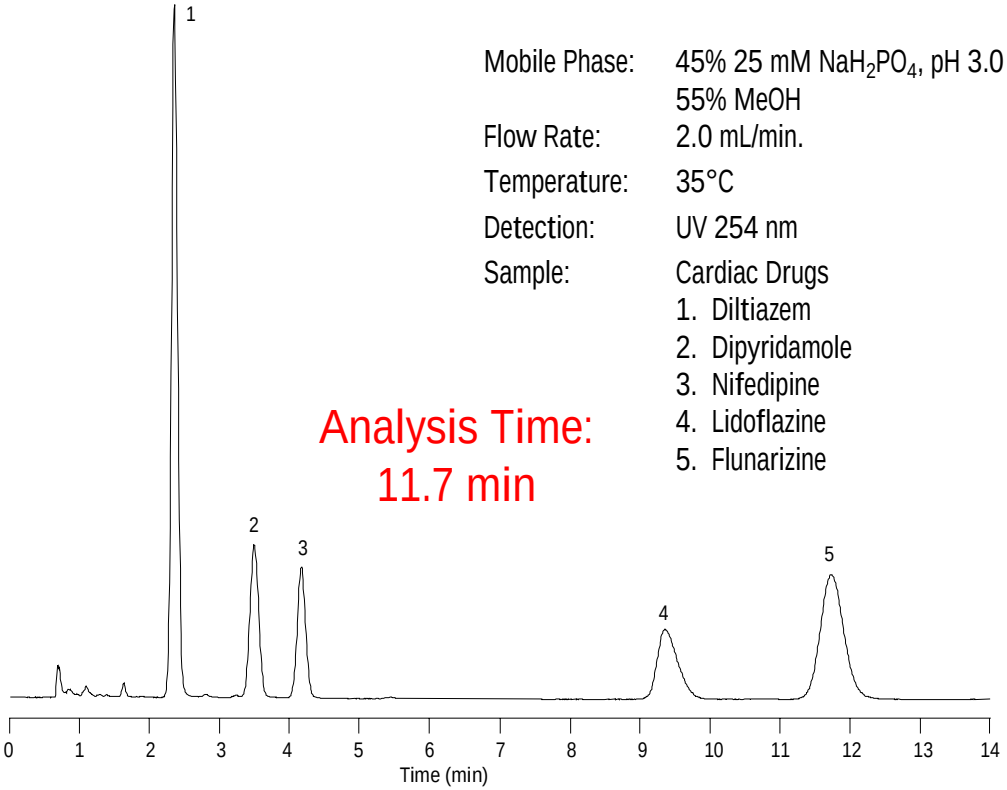
Rapid Resolution Columns Reduce Isocratic Analysis Time by 50% or More

StableBond SB-C18

4.6 x 150 mm, 5 µm

Mobile Phase:	45% 25 mM NaH_2PO_4, pH 3.0 55% MeOH
Flow Rate:	2.0 mL/min.
Temperature:	35°C
Detection:	UV 254 nm
Sample:	Cardiac Drugs 1. Diltiazem 2. Dipyridamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine

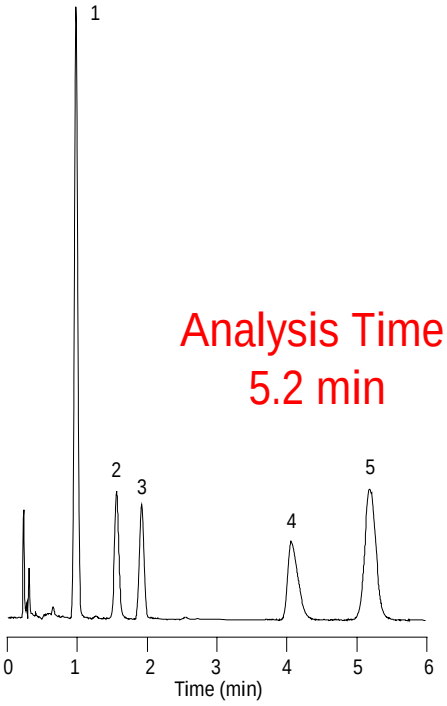
Analysis Time: 11.7 min



Rapid Resolution StableBond SB-C18

4.6 x 75 mm, 3.5 µm

Analysis Time: 5.2 min



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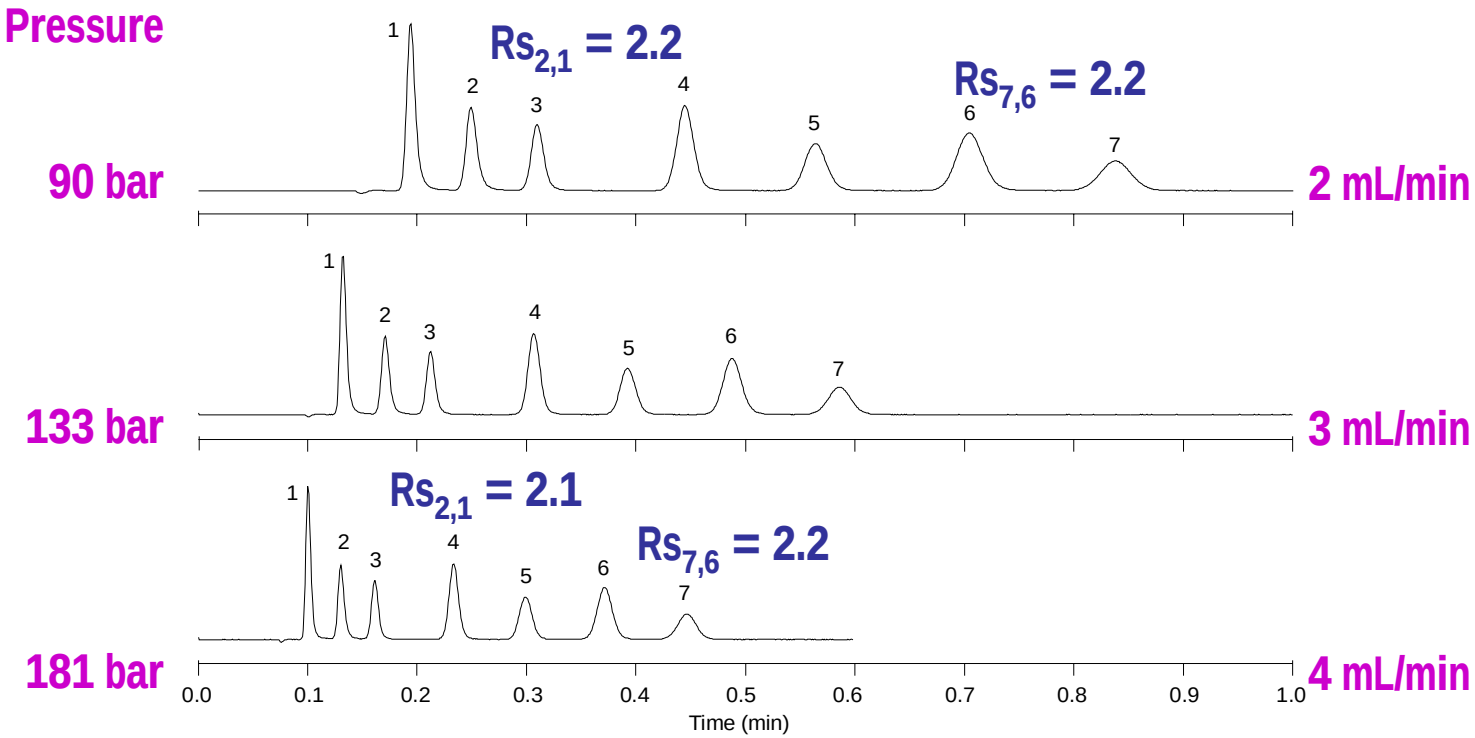
Higher Flow Rates Reduce Analysis Time with Rapid Resolution Columns

Column: Zorbax Rapid Resolution StableBond SB-C18, 4.6 x 30 mm, 3.5 µm

Sample: 1. Acetaminophen (4-acetamidophenol) 2. Caffeine 3. 2-Acetamidophenol
4. Acetanilide 5. Acetylsalicylic acid 6. Salicylic acid 7. Acetophenetidin



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Gradient Re-equilibration Times are Minimal on Short Rapid Resolution Columns

Column Volume (Vm) and Equilibration Time

Column Dimension (mm)	Internal Volume (Vm)	Equilibration Time at 1.0 mL/min (Vm x 10 x F)	
4.6 x 50	0.5 mL	5 min	
4.6 x 30	0.3 mL	3 min	
4.6 x 15	0.15 mL	1.5 min	
4.6 x 150	1.54 mL	15 min	
		at 0.2 mL/min	at 1.0 mL/min
2.1 x 50	0.10 mL	5 min	60 sec
2.1 x 30	0.06 mL	3 min	36 sec
2.1 x 15	0.03 mL	1.5 min	18 sec

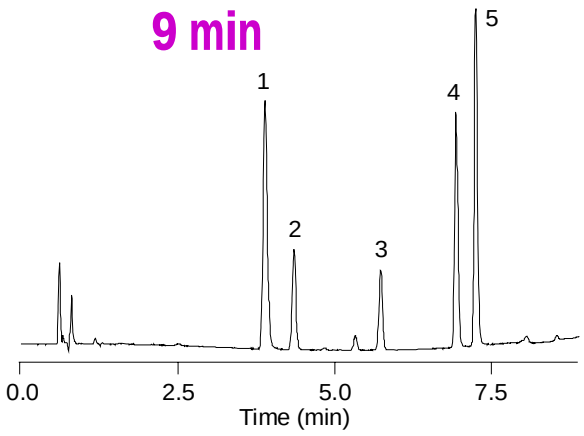
Gradient Analysis Time = Run Time + Equilibration Time (single step return)

Short, Rapid Resolution Columns Reduce Gradient Analysis Time

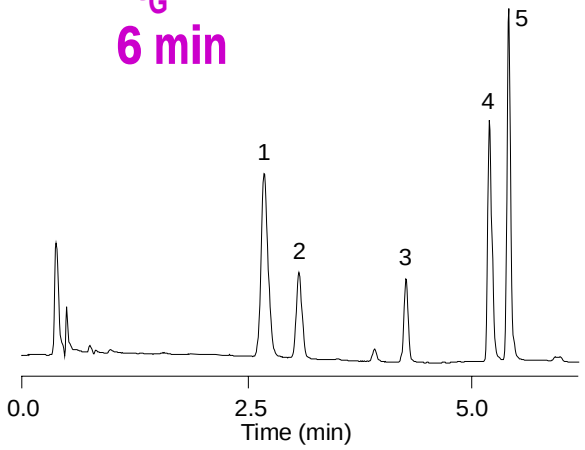
Gradient Time α Column Length

Column: Zorbax Eclipse XDB-C8, 4.6 mm i.d. Gradient: 45-90% B in t_G minutes Mobile Phase: A: 25 mM Na_2HPO_4, pH 3 B: Methanol
Temperature:35°C Flow Rate: 1.0 mL/min Sample: Cardiac Drugs: 1. Diltiazam 2. Dipyridamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine

Length = 75 mm, 3.5 µm
t_G = 9 min



Length = 50 mm, 3.5 µm
t_G = 6 min

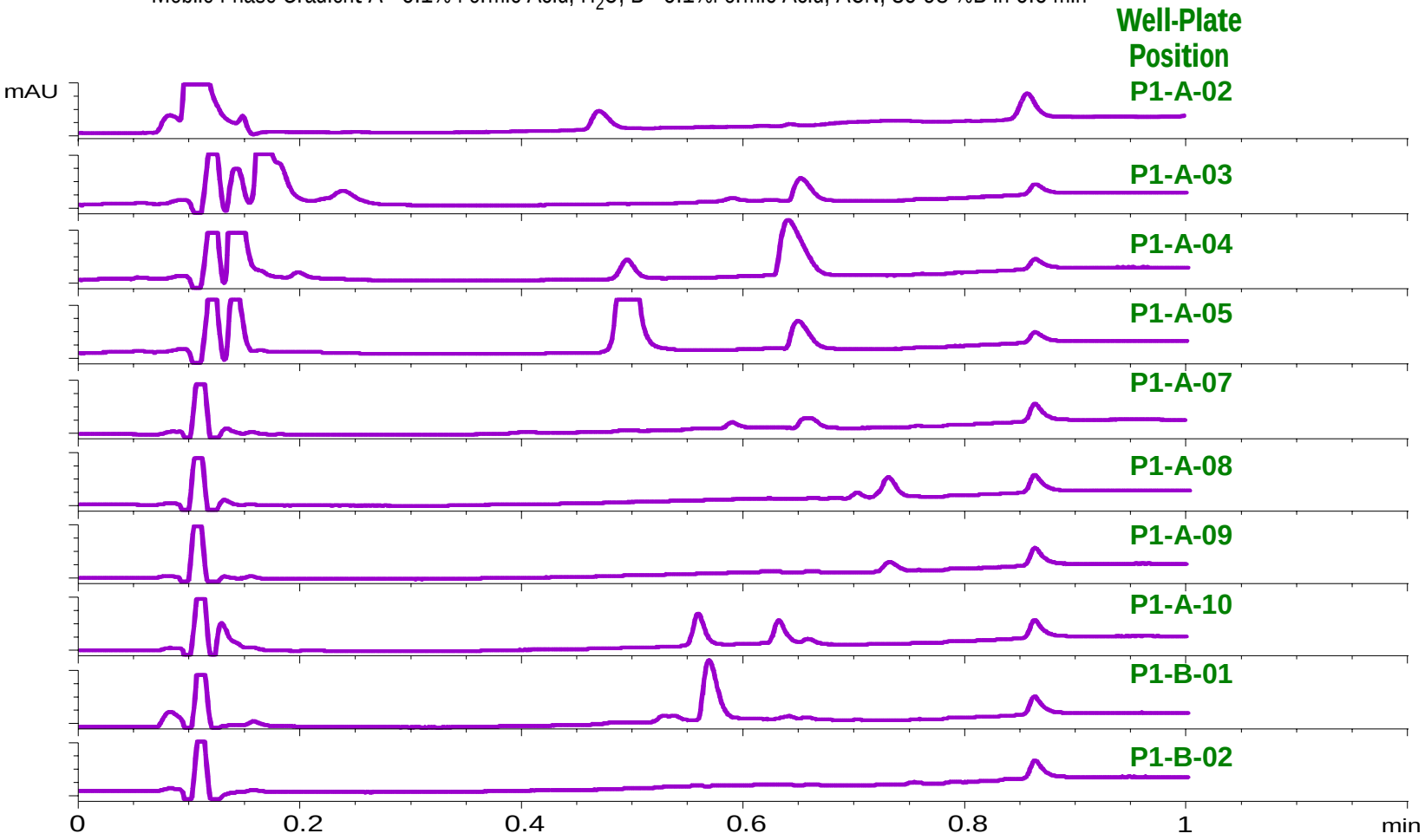


Rapid Gradient CombiChem Analysis

1.5 min Injection-to-Injection

Column: Zorbax SB-C18, 2.1 x 30 mm, 3.5µm Flow Rate: 0.832mL/min Temperature: 20°C

Mobile Phase Gradient A=0.1% Formic Acid, H_2O; B=0.1%Formic Acid, ACN; 30-98 %B in 0.6 min



1 min

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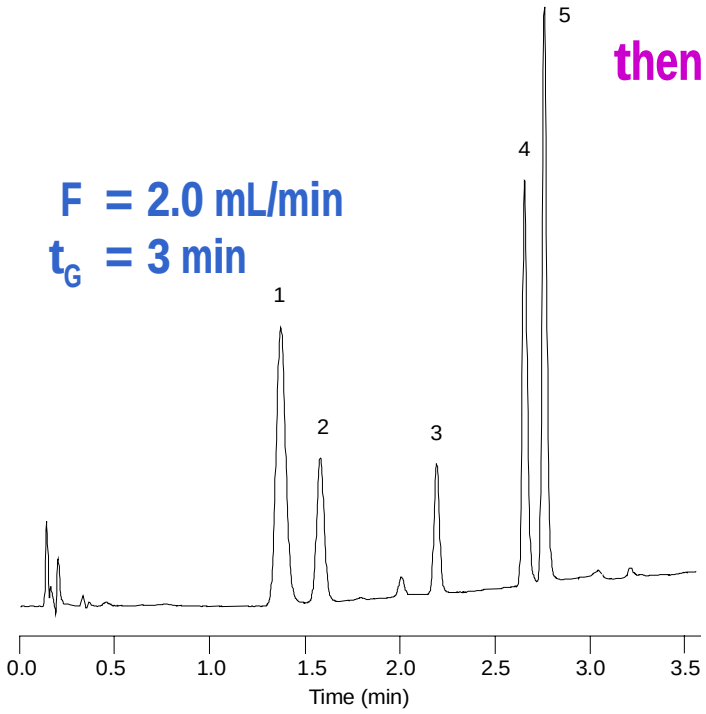
Increasing Flow Rate Reduces Gradient Analysis Time Further

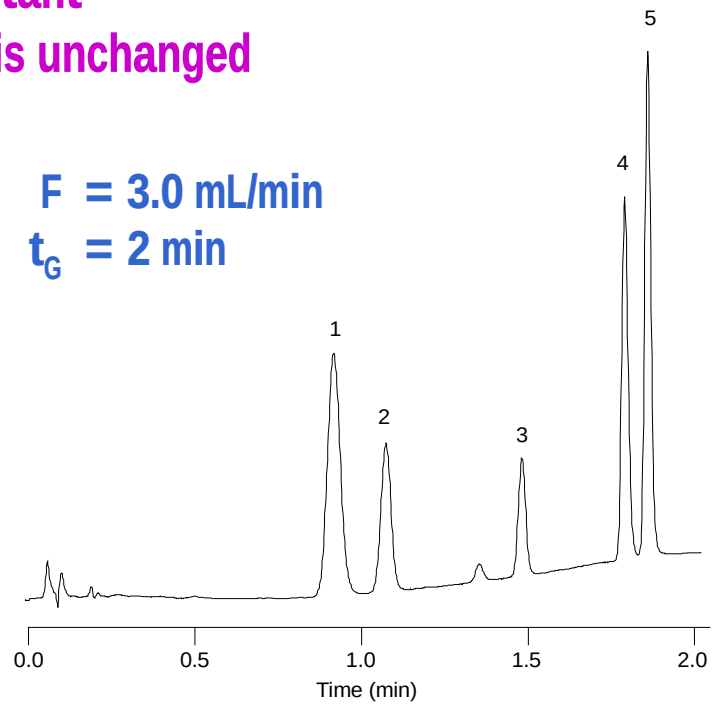
Column: Zorbax Eclipse XDB-C8 4.6 x 50 mm, 3.5 µm Gradient: 45-90% B in t_G minutes Mobile Phase: A: 25 mM Na_2HPO_4, pH 3 B: MeOH
Flow Rate: 1.0 mL/min Temperature: 35°C Sample: Cardiac Drugs 1. Diltiazam 2. Dipyradamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine

If t_G x F = constant
then the elution pattern is unchanged

F = 2.0 mL/min
t_G = 3 min



F = 3.0 mL/min
t_G = 2 min

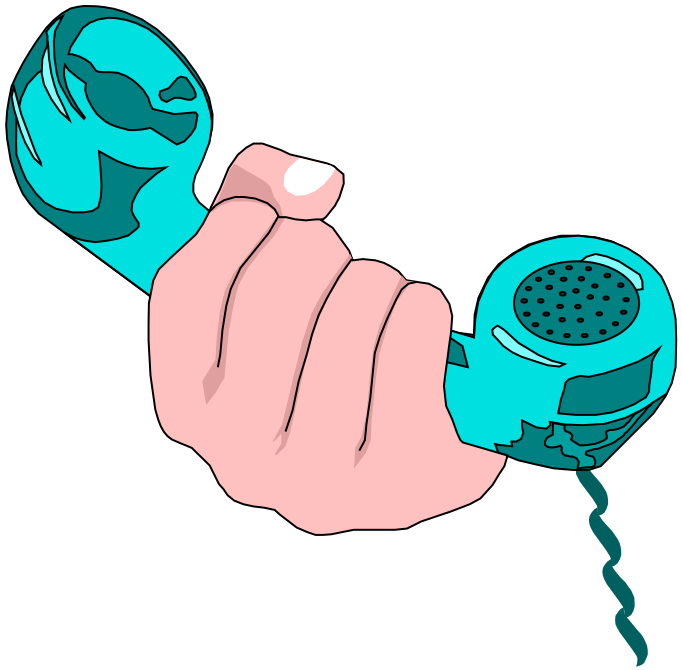


Break Time

For Questions and Answers

Press *1 on Your Phone to

Ask a Question



HPLC Instrument Optimization Increases Efficiency and Decreases Method Development and Analysis Time

- Choose the best column configuration for your instrument type and application (i.e. Capillary LC for highest sensitivity or compatibility with LC/MS)
- Instrument optimization can improve efficiency for isocratic and gradient separations
- New instrument features can help you reduce analysis time

Choose HPLC Instrument for Application

Agilent 1100 Type	Compatible Columns	Flow Rate Range	Applications	Why?
Capillary LC	Capillary, MicroBore, Narrow-bore, Solvent Saver, Analytical	1 µL/min – 2.5 mL/min	1. Sample limited 2. Most MS compatible	5 µL dwell volume, best low volume gradient reproducibility
LC	Narrow-bore, Solvent Saver, Analytical, Semi-prep	0.05 – 5 mL/min* binary 0.2 – 10 mL/min* isocratic/quaternary	1. Analytical 2. MS compatible	Standard analytical more semi-prep than low volume Work at 3- 4mL/min
Prep LC	Semi-prep, Prep	1 - 100 mL/min	1. Purification	Preparative and CombiChem prep

* Optimum range

Optimizing Results with Rapid Resolution Columns

Isocratic Separations

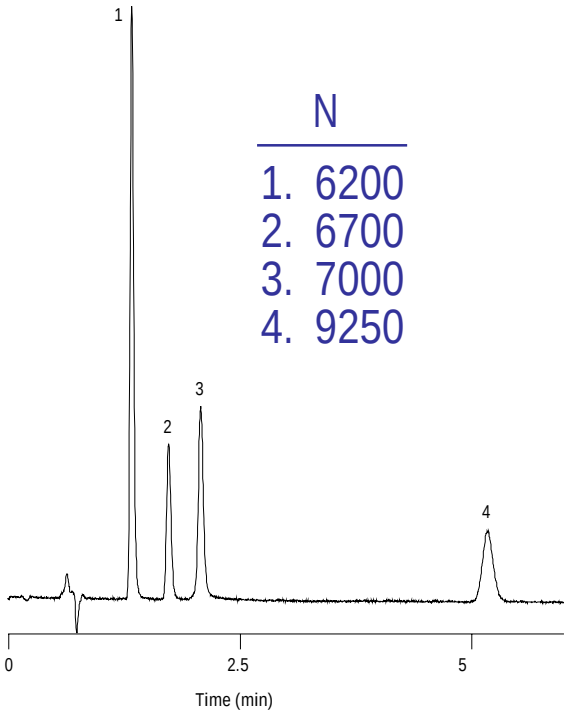
- Minimize extra column volume to minimize band broadening
  - Keep the injection volume small (< 5 µL)
  - Use semi-micro or micro detector cells (5 µL or less)
  - Use 0.12 mm i.d. tubing (0.005”)
- Prepare the sample in an injection solvent with the same or weaker solvent strength than the mobile phase
- Overlap injections
- Select the Agilent 1100 Capillary HPLC for capillary and microbore columns
- Select correct detector response time for rapidly eluting peaks

Effect of Detector Cell Volume on Peak Width

4.6 x 75 mm, 3.5 µm

Column: Zorbax StableBond SB-C18 Mobile Phase: 85% H_20 with 0.1% TFA : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylalanine 2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame

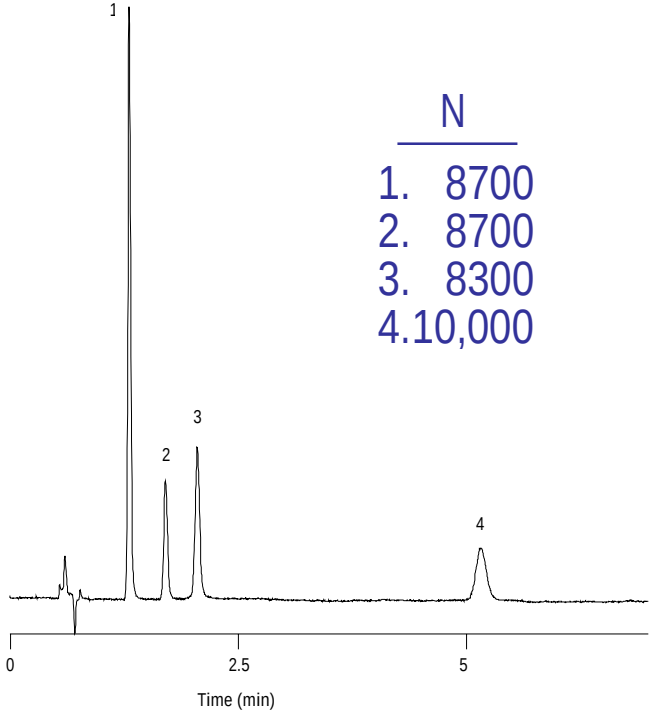
Standard Flow Cell - 8 µL



	N
1.	6200
2.	6700
3.	7000
4.	9250

	↓
1.	-29%
2.	-23%
3.	-16%
4.	-7%

Microflow Cell - 2.5 µL



	N
1.	8700
2.	8700
3.	8300
4.	10,000

Agilent Technologies

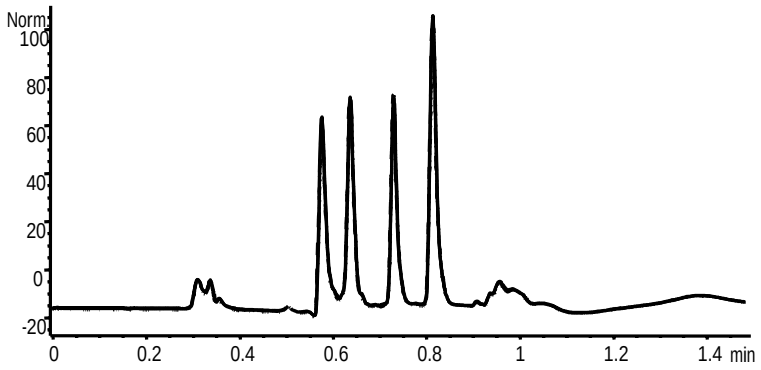
Dial 1- 904-779-4740 for e-Seminar Audio

Optimizing Results with Rapid Resolution Columns
Gradient Separations

- Minimize dwell volume – includes mixing volume and injector volume
  - Use high pressure mixing and reduce mixer volume
  - Run autosampler in bypass – reduce dwell volume by 300 µL
- Minimize extra column volume
- Overlap injections
- Select correct detector response time for rapidly eluting peaks
- Select Agilent 1100 Capillary HPLC with 5 µL dwell volume for capillary and microbore columns

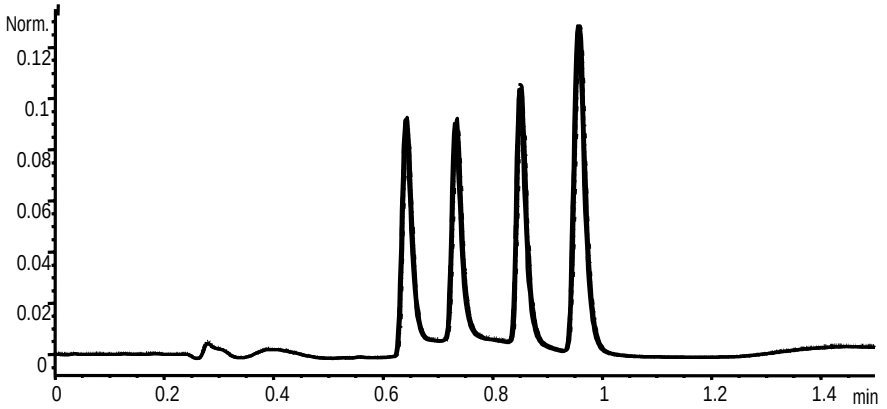
Reproducible, Fast Gradient Analysis with High Pressure Mixing

Agilent 1100, binary pump high pressure mixing



Compound	rsd RT for 10 runs	rsd Area for 10 runs
Dimethylphthalate	0.09	0.98
Diethylphthalate	0.10	0.87
Biphenyl	0.08	1.83
o-Terphenyl	0.07	0.90

Low pressure mixing, Brand X

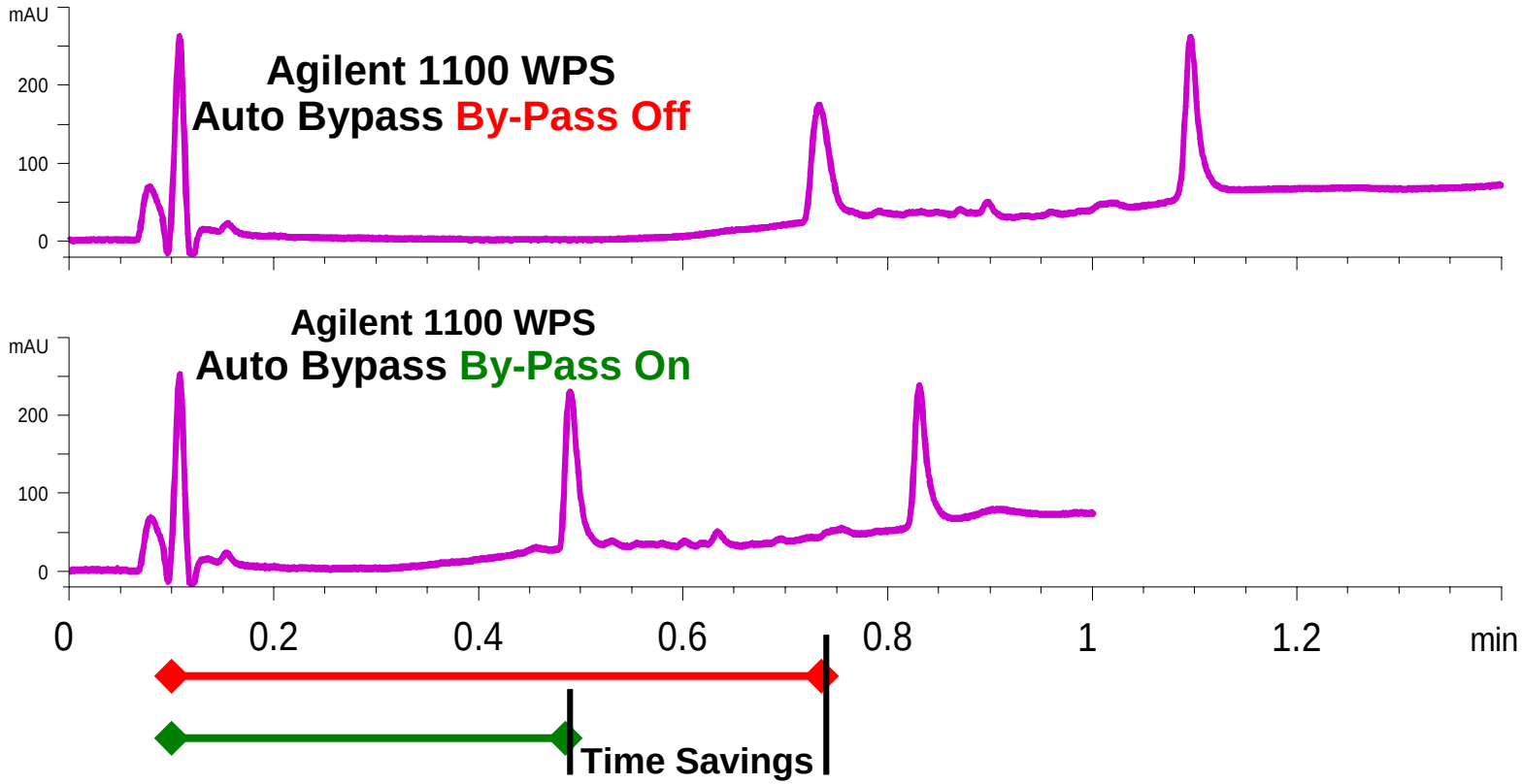


Compound	rsd RT for 10 runs	rsd Area for 10 runs
Dimethylphthalate	1.09	5.91
Diethylphthalate	0.55	5.64
Biphenyl	0.52	5.30
o-Terphenyl	0.43	6.87

Column: Zorbax StableBond C18, 2.1 x 50 mm

Reduce Gradient Analysis Time and Dwell Volume with Bypass of the Sample Loop

Column: Zorbax SB-C18, 4.6 x 30 mm, 3.5µm Flow Rate: 0.832 mL/min Temperature: 20°C
Mobile Phase Gradient: A= 0.1% Formic Acid, H_2O; B = 0.1% Formic Acid, ACN; 30-98 %B in 0.6 min



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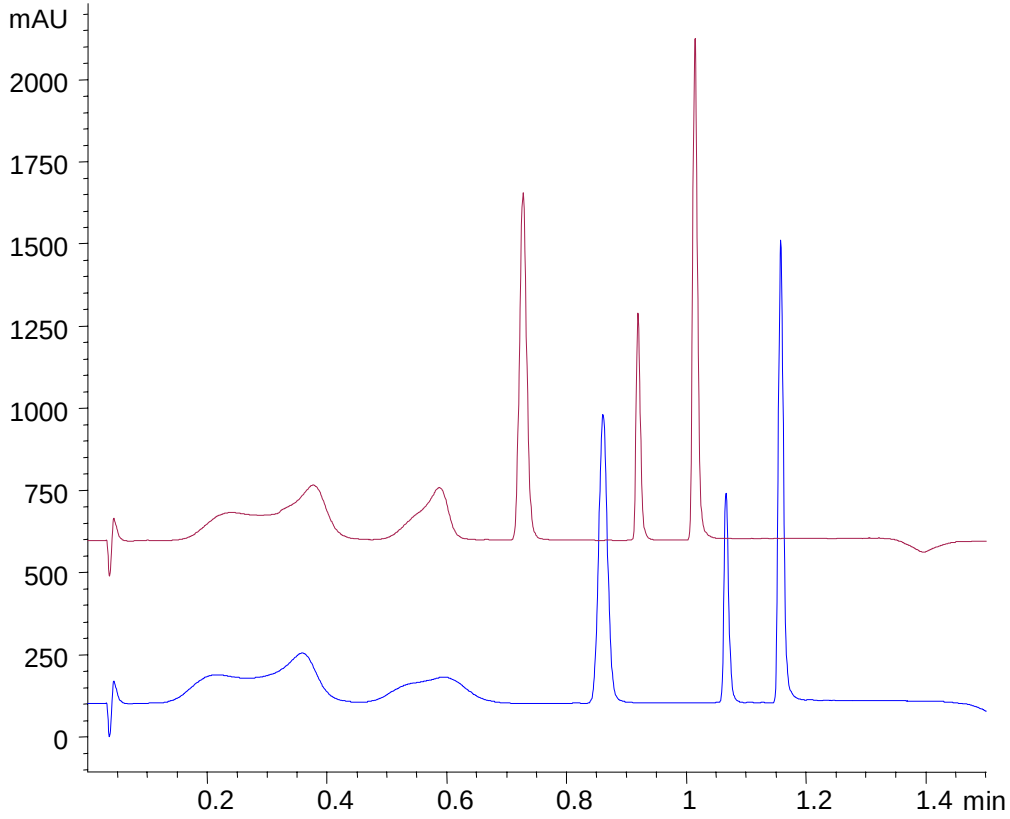
Overlapped Injection Reduces Analysis Time

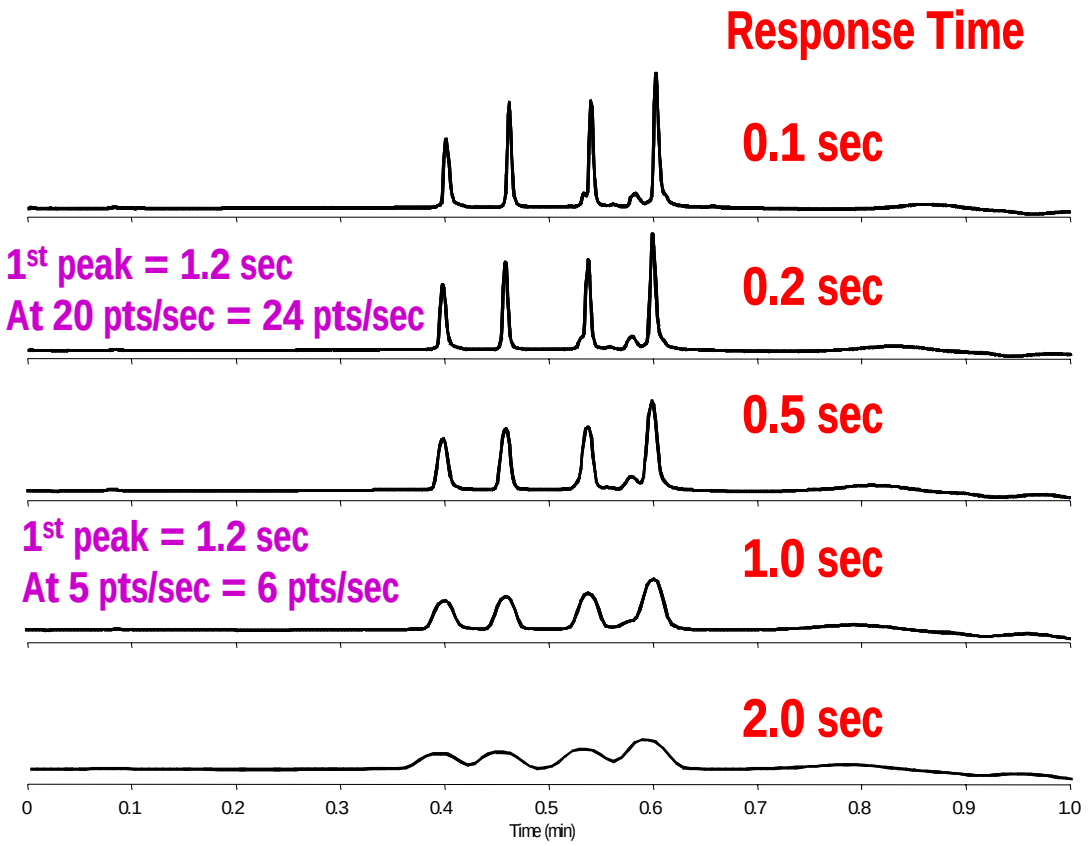


Overlapped Injection
Cycle time is ~ 2min 12sec
Total time for 20 runs: 44 min 22 sec

Standard Injection
Cycle time is ~ 2min 57sec
Total time for 20 runs: 58 min 20 sec

- Overlapped injections – sample is drawn up during previous injection – reduce gradient and isocratic run times.

Effect of Detector Response Time on Ultra-Fast Gradient Analyses



Agilent 1100 DAD
Agilent 1100 WPS with ADVR

Column: **Poroshell 300SB-C18**
2.1 x 75 mm, 5 µm

Mobile Phase:
A: 95% H_2O, 5% ACN with 0.1% TFA
B: 5% H_2O, 5% ACN with 0.1% TFA

Flow Rate: 2 mL/min

Temperature: 70°C

Detector: UV 215 nm

Piston stroke: 20

Sample:
1. Neurotensin
2. RNaseA
3. Lysozyme
4. Myoglobin

- You may have to adjust the response rate of your detector for rapid peak detection.

Summary

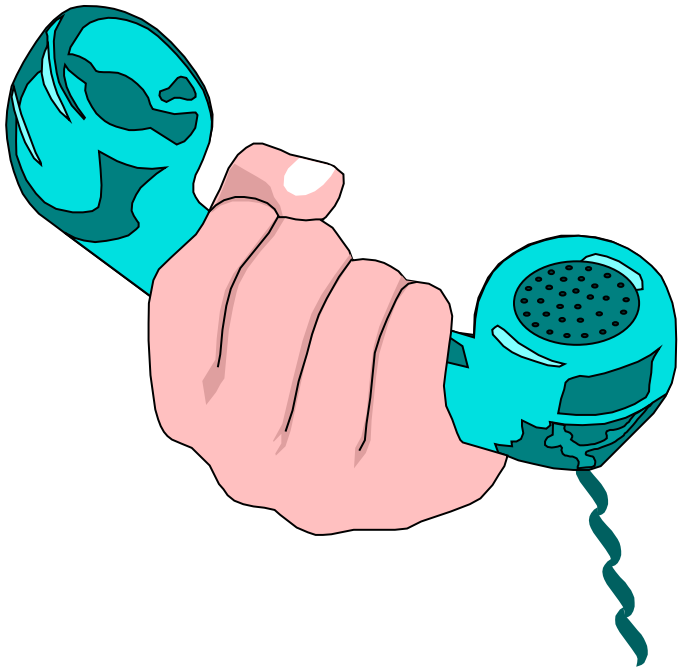
- C18 and C8 bonded phases are the best for initial rapid method development with typical sample types
- Choosing the most sample appropriate bonded phase and using special, targeted bonded phases, such as SB-Aq for polar, difficult to retain compounds can decrease method development time
- Rapid Resolution columns are needed to reduce method development time
- Rapid Resolution columns reduce both gradient and isocratic analysis time and permit high throughput rapid analysis
- Rapid Resolution columns can work effectively with your HPLC
- HPLC instruments may have additional capabilities to speed up method development and reduce analysis time

Break Time

For Questions and Answers

Press *1 on Your Phone to

Ask a Question



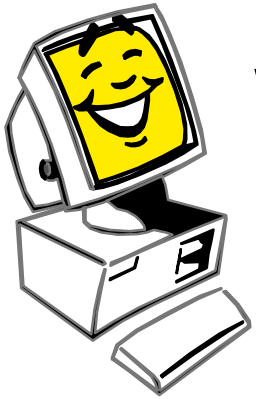
HPLC Column Technical Support

800-227-9770 (phone: US & Canada)*

302-993-5304 (phone)*

**** Select option 4, then option 2.***

916-608-1964 (fax)



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