HPLC Method Development: Standard Practices and New Columns

LC Columns and Consumables

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What are Some Standard Method Development Practices?

1. Follow preferred method development scheme and do “hands-on” method development – based on selectivity-changing parameters—e.g., pH, column, or mobile phase types
2. Use method development software – run a few predictive runs and get prediction for best method
3. Evaluate multiple columns/multiple mobile phases in a manual or automated fashion, determine best results (e.g., using valve switching to change between columns, software to track peaks etc.)

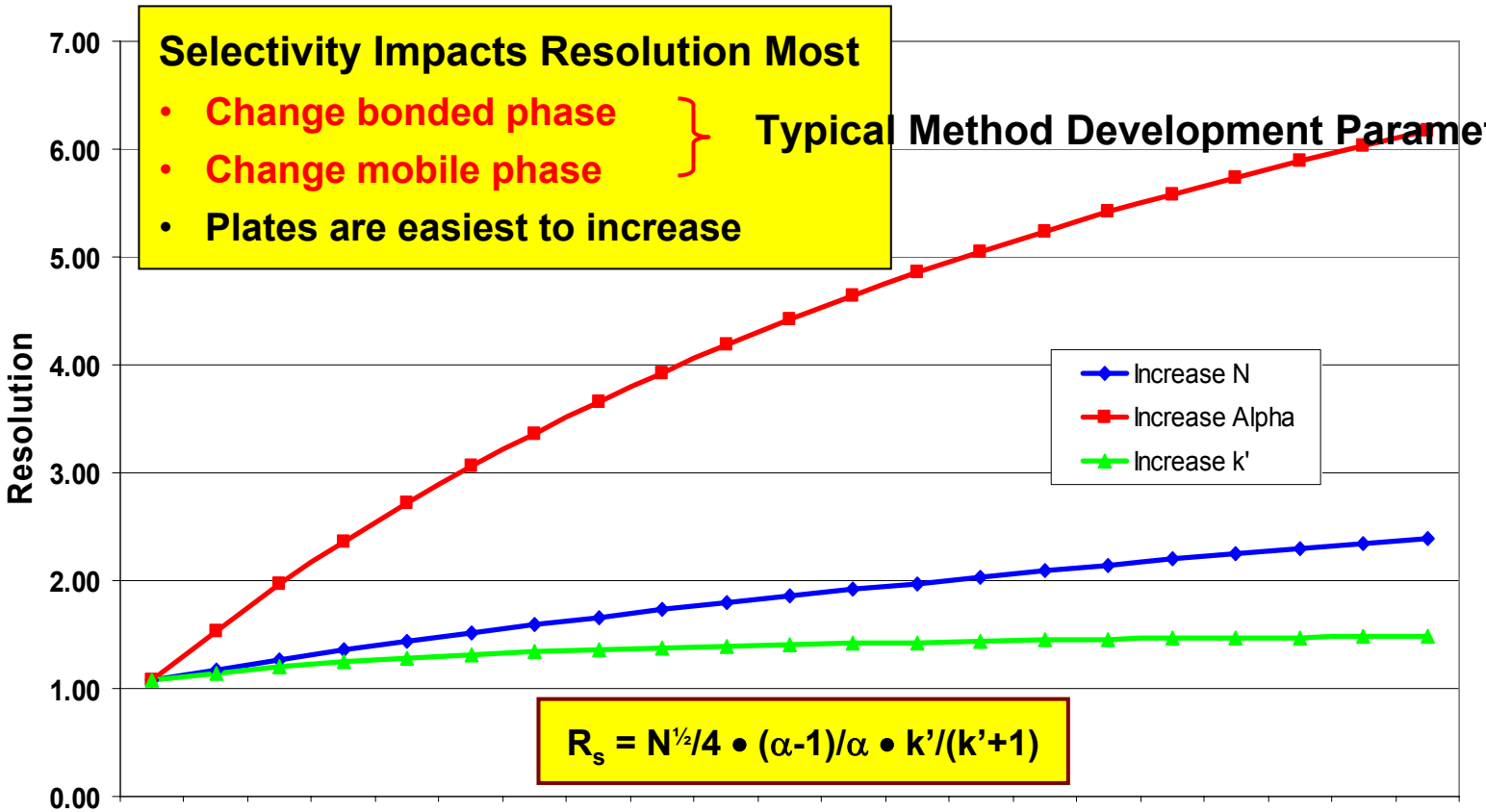
Practice #1 is used by many and incorporates “hands on” learning.

It requires nothing special to make it work, so we will work through a method development scheme for this practice.

What Makes a Good Starting Point for Hands-on Method Development?

1. Choose a C18 column – many choices, well known technologies
   a) A short column minimizes method development time
   b) Smaller particle sizes provide the resolution needed in a short time
   c) Newer C18 column choices can improve results – based on efficiency, and improved peak shape
2. Choose a simple mobile phase – reliable, works with many samples
   a) Phosphate buffer pH 3, TFA, or formic acid in aqueous portion
   b) Acetonitrile or methanol as organic modifier
3. Adjust mobile phase to get the desired retention and **resolution**
   a) Adequate resolution of all peaks, $R_s \geq 2.0$ – requires highest efficiency
   b) Retention of first peak preferred to be at least k = 1
   c) Analysis time below 20 minutes – shorter
   d) Newer column choices, short columns with small particle sizes can provide more efficiency and resolution in a very short time, thereby speeding up method development

Typical Method Development Parameters - Effects of Selectivity, Efficiency, and Retention on Resolution



Plates:	5000	10000	15000	20000	25000
Alpha:	1.10	1.35	1.60	1.85	2.1
k':	2.0	4.5	7.0	9.5	12.0

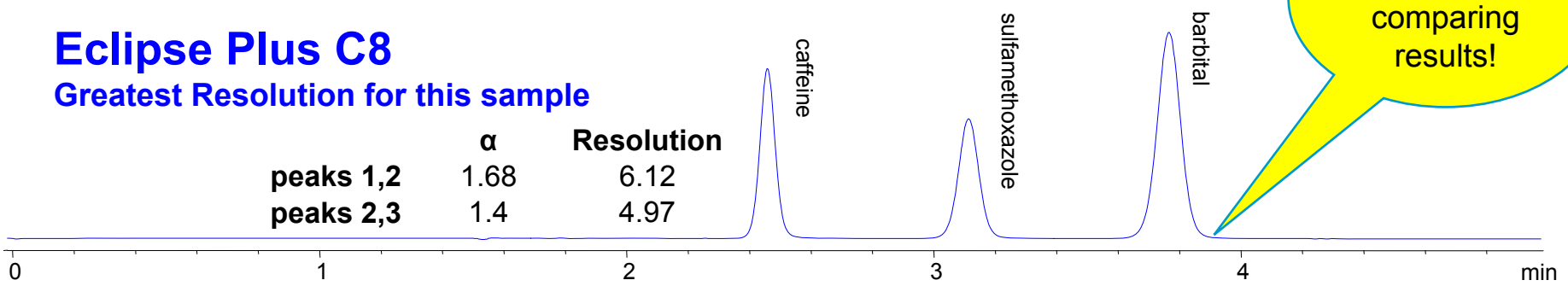
Small Differences in α – Selectivity - Can Provide Large Differences in Resolution

Remember to assess T_f when comparing results!

Eclipse Plus C8

Greatest Resolution for this sample

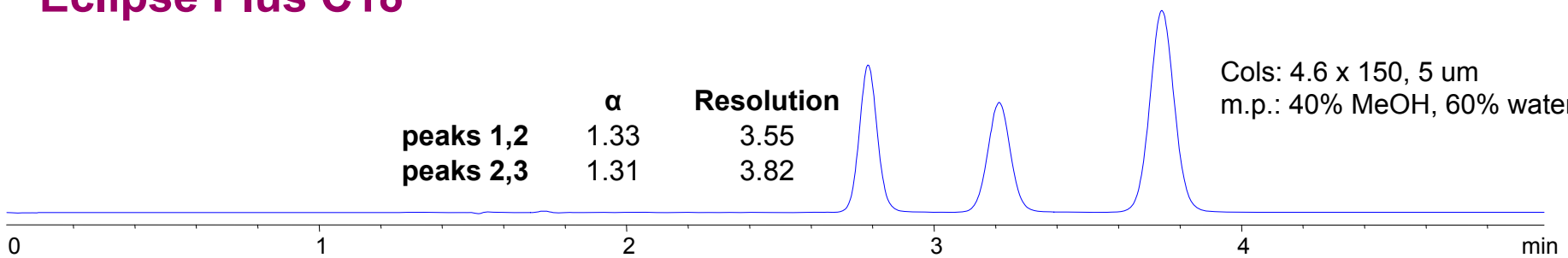
	α	Resolution
peaks 1,2	1.68	6.12
peaks 2,3	1.4	4.97



Eclipse Plus C18

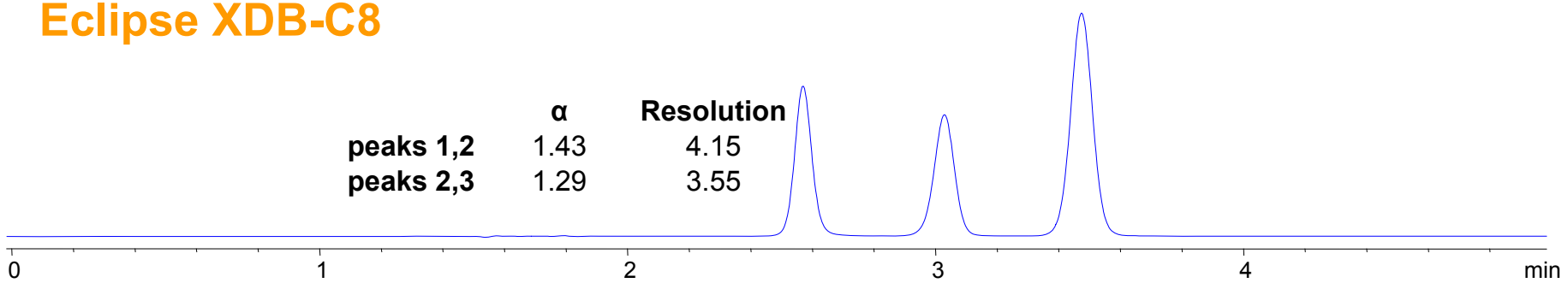
	α	Resolution
peaks 1,2	1.33	3.55
peaks 2,3	1.31	3.82

Cols: 4.6 x 150, 5 um
m.p.: 40% MeOH, 60% water



Eclipse XDB-C8

	α	Resolution
peaks 1,2	1.43	4.15
peaks 2,3	1.29	3.55



What Do We Want to Base Our Complete Method Development Scheme On? (Beyond the Start)

Changing selectivity to improve resolution with the two key parameters – mobile phase and bonded phase

Mobile phase (1st choice to change because it's easy)

- Mobile phase – organic modifier (ACN, MeOH, etc.)
- Mobile phase – pH – over a wide pH range – pH 1-12 if needed

Bonded phase (provides a lot of optimization potential)

- Many choices other than C18 – C8, Phenyl, CN (most orthogonal!)
- AQ for highly aqueous mobile phases
- Choose new columns with best peak shape performance

New Column Choices To Use for Standard Method Development

1. Shorter columns with smaller particle sizes

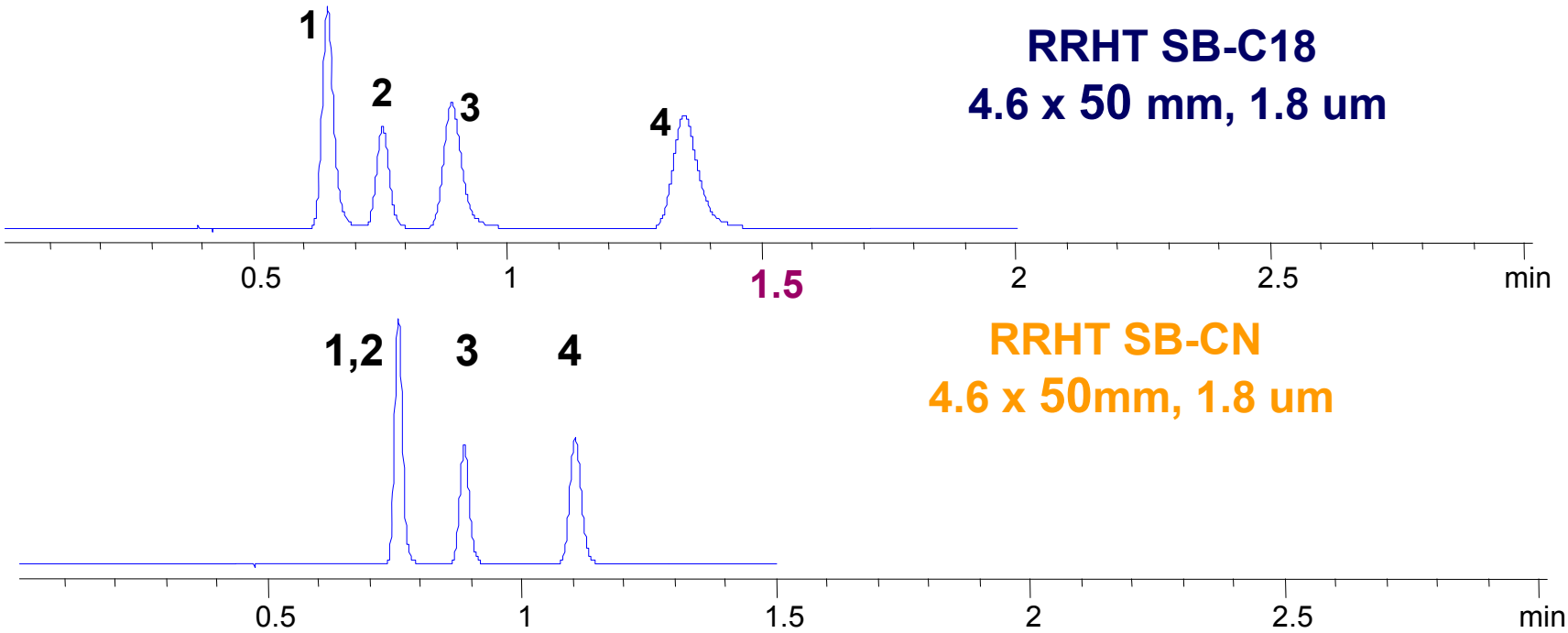
- Rapid Resolution High Throughput Columns (RRHT) with a 1.8 µm particle size – 50 mm or shorter for fastest method development
- Longer columns for maximizing efficiency
- Many bonded phase choices for a complete method development scheme

2. Improved bonded phases for superior performance

- Better peak shape and efficiency result in more resolution
- Critical to the best methods
- New Eclipse Plus columns can deliver improved performance

1. Start with Short RRHT C18 Columns with Highest Efficiency for Fastest Method Development

Column: 4.6 x 50mm, 1.8um Mobile Phase: A= 0.1% Formic Acid, B=ACN + 0.1%Formic Acid (95:5) Flow rate: 1.5 mL/min
Inj. Vol: 2 ul Sample: Xanthines: 1. 1-methylxanthine, 2. 1,3-dimethyluric acid, 3. 3,7-dimethylxanthine, 4. 1,7-dimethylxanthine

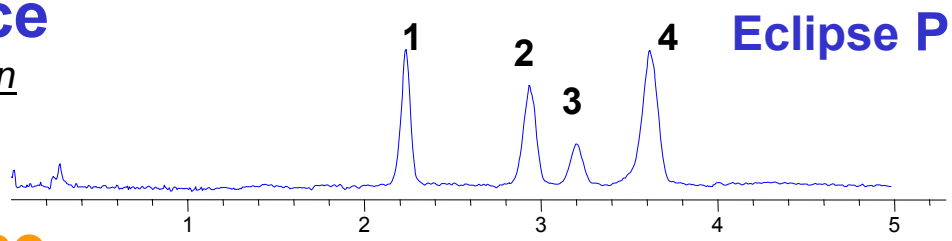


- **The C18 gives the most effective resolution – and with a 4.6 x 50 mm, 1.8um column the initial results are achieved in just a couple of minutes, with the highest efficiency.**
- **The 50 mm length is short and fast – methods can be approximated or completed in just minutes!!**

1. Start Method Development with RRHT Columns: Different ZORBAX RRHT C18 Bonded Phases for Max Selectivity

1st choice

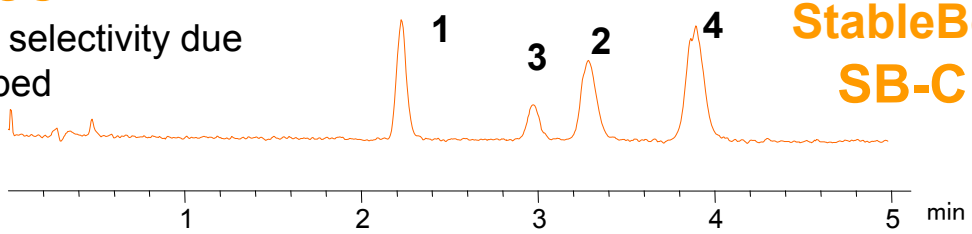
Best Resolution & Peak Shape



Eclipse Plus C18

2nd choice

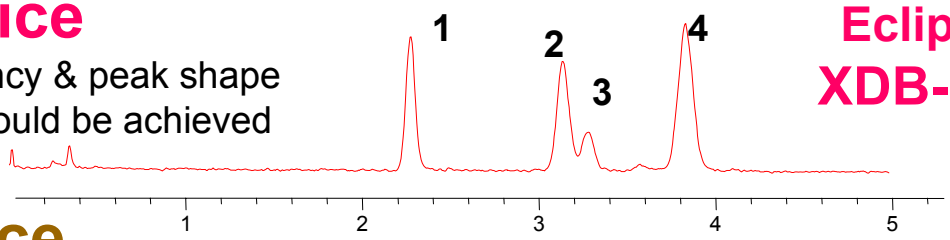
Good alternate selectivity due to non-endcapped



StableBond SB-C18

3rd choice

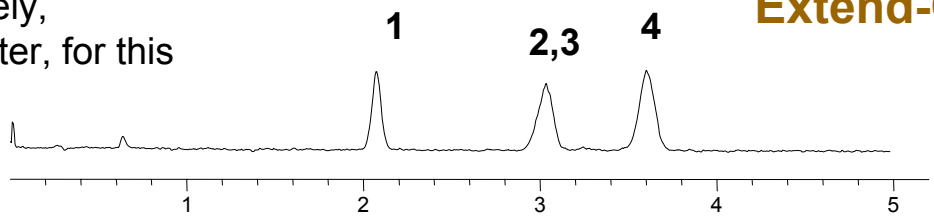
Good efficiency & peak shape
Resolution could be achieved



Eclipse XDB-C18

4th choice

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



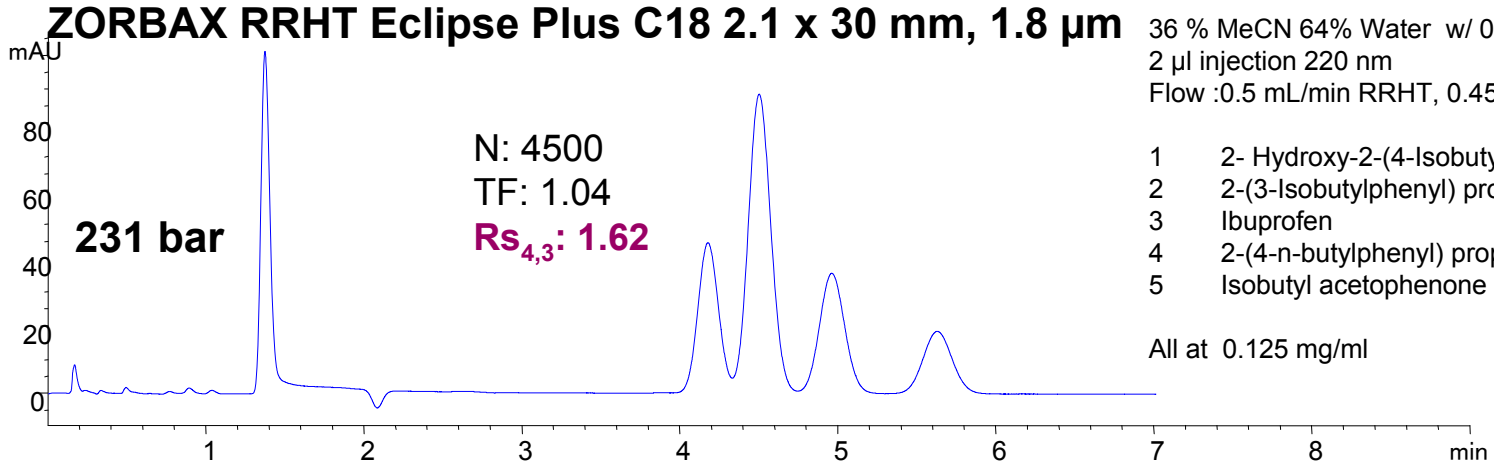
Extend-C18

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.

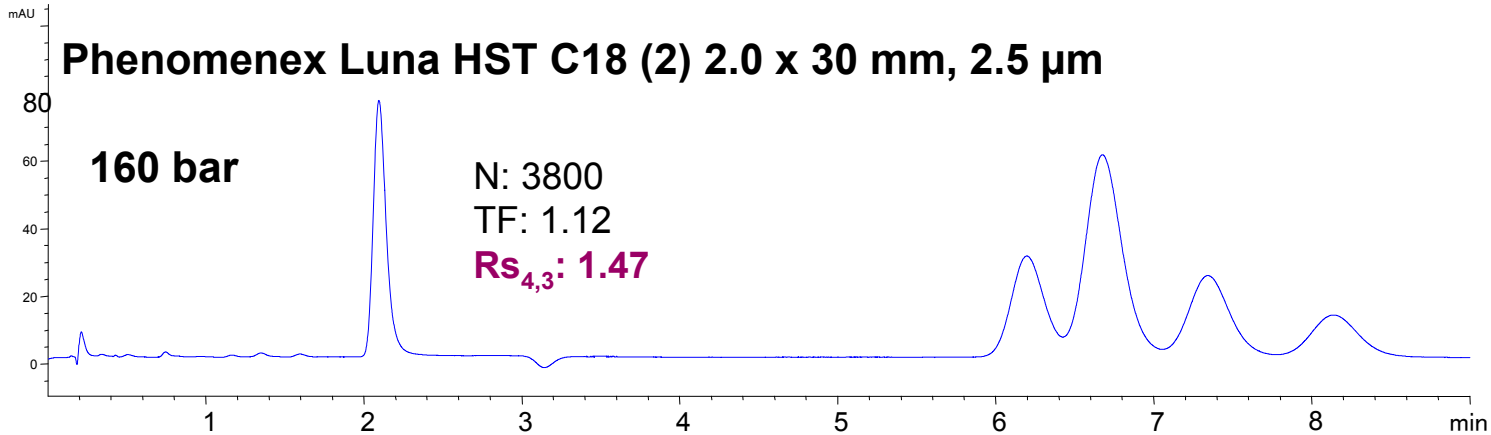
2. Agilent Rapid Resolution HT Eclipse Plus C18 Provides Superior Peak Shape and Efficiency



36 % MeCN 64% Water w/ 0.7 ml/L phosphoric acid
2 µl injection 220 nm
Flow :0.5 mL/min RRHT, 0.45 mL/min. (Luna)

1 2- Hydroxy-2-(4-Isobutylphenyl) proprionic acid
2 2-(3-Isobutylphenyl) proprionic acid
3 Ibuprofen
4 2-(4-n-butylphenyl) proprionic acid
5 Isobutyl acetophenone

All at 0.125 mg/ml

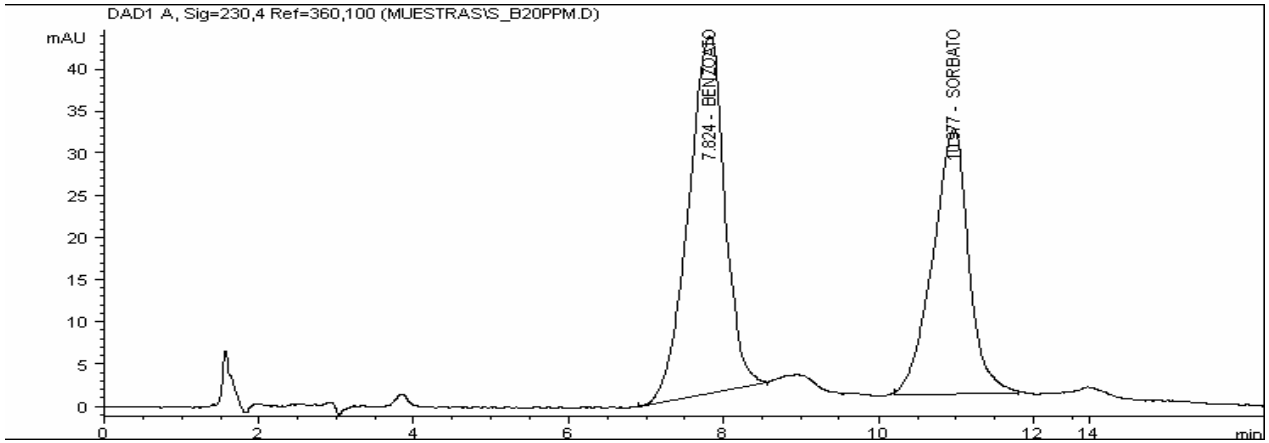


ZORBAX RRHT has at least 18% more efficiency than Luna HST C18(2)
That means greater than baseline resolution when needed.

2. Choose New Eclipse Plus C18 for Superior Efficiency and Peak Shape

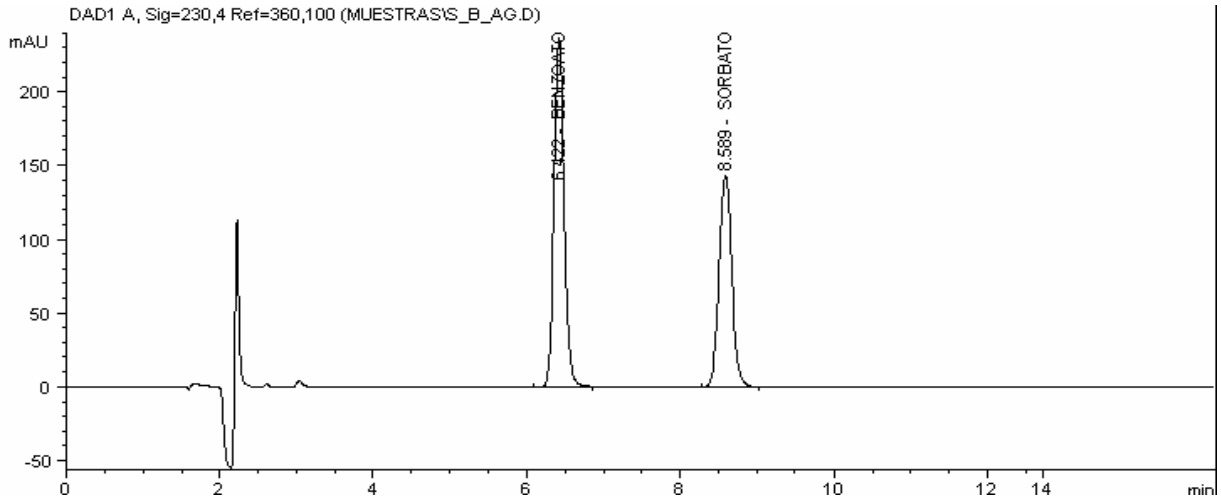
Analysis on Other C18

- Poor sensitivity
- Due to broad peaks
- Peaks do not tail, but are distorted.



Analysis on Eclipse Plus

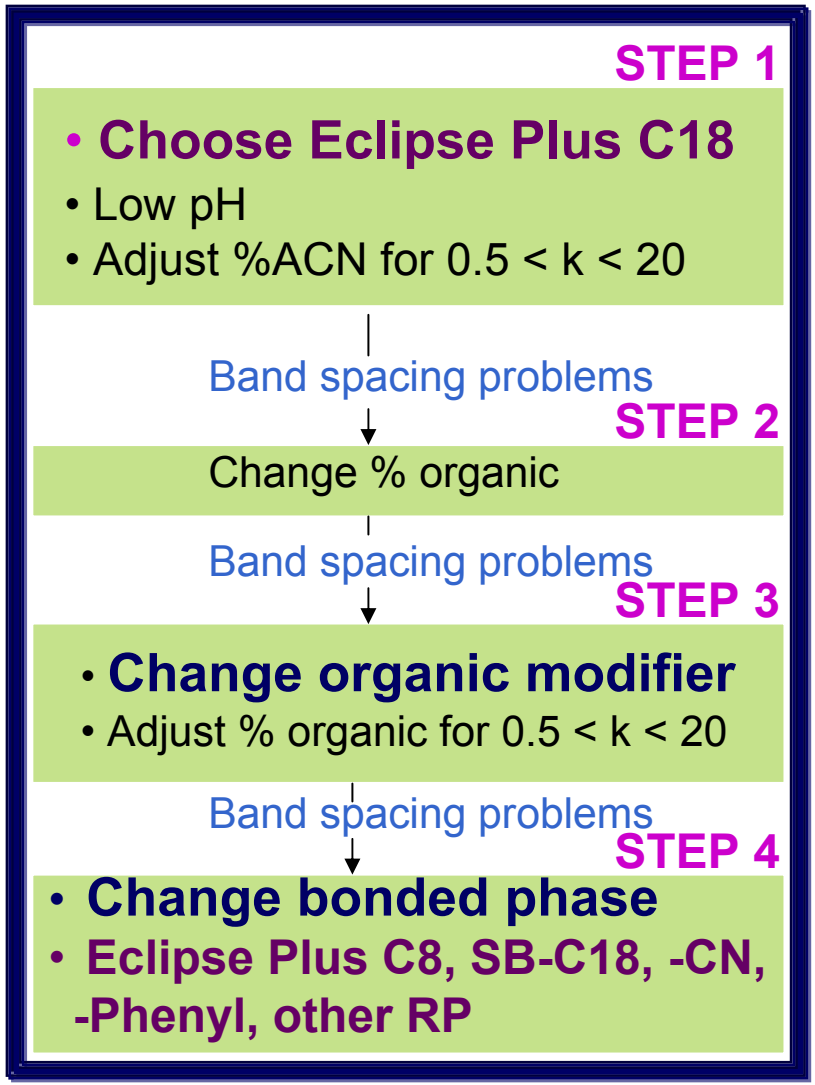
- Better Sensitivity
- Sharp, Efficient Peaks
- Excellent peak shape



How Do We Implement These New Column Choices in the Method Development Process?

- Follow a method development process around standard practices – **ONLY** when the first steps allow you to incorporate new products for fast, good results!
- A method development scheme based on simple strategies for optimizing selectivity and resolution can incorporate new products.
- **The 3 key parameters must be:**
  - **mobile phase**
  - **mobile phase pH**
  - **bonded phase**

First Steps in a Method Development Scheme

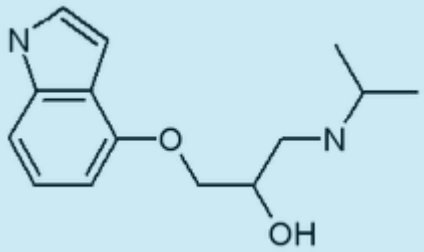


- **Select a high quality C18 or C8 bonded phase first for good peak shape, retention and resolution with typical acidic, basic and neutral samples.**
- **Optimize the organic component of the mobile phase to change selectivity**
- **Choose alternate bonded phases to completely optimize method, if needed**
- **Choose SB-C18 for pH 1-2**
- **With new RRHT column choices, these steps can be done quickly, making it possible to truly find the best method.**

Let's Look at Method Development of These Compounds

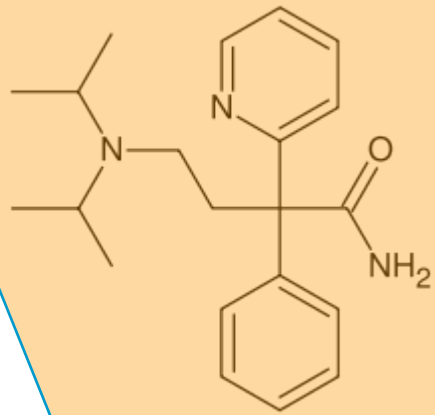
propranolol

pindolol

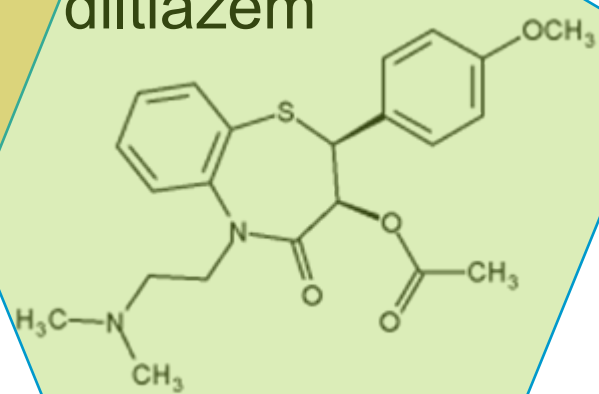


dipyridamole

disopyramide

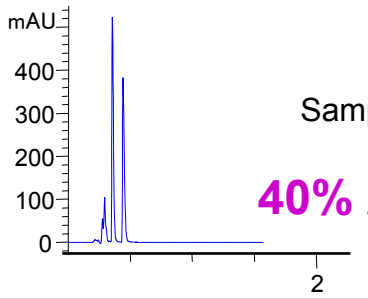


diltiazem



β-blocker
Anti-arrhythmic
Vasodilator
Ca^{+2} channel blocker

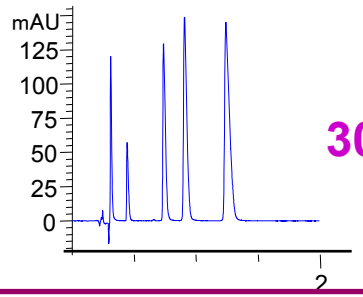
Start at Low pH, Adjust % Organic
Eclipse Plus C18 Cardiac Drugs with Acetonitrile



Column: ZORBAX RRHT Eclipse Plus C18, 4.6 x 50 mm, 1.8 µm
Mobile Phase: A: 25 mM NaH_2PO_4 , pH 3.0 B: ACN
Flow Rate: 2.0 mL/min Temperature: 30°C Detection: UV 240 nm
Sample: Cardiac Drugs 1.Pindolol 2. Di-isopyridamide 3.Propranolol 4.Dipyridamole 5. Diltiazem

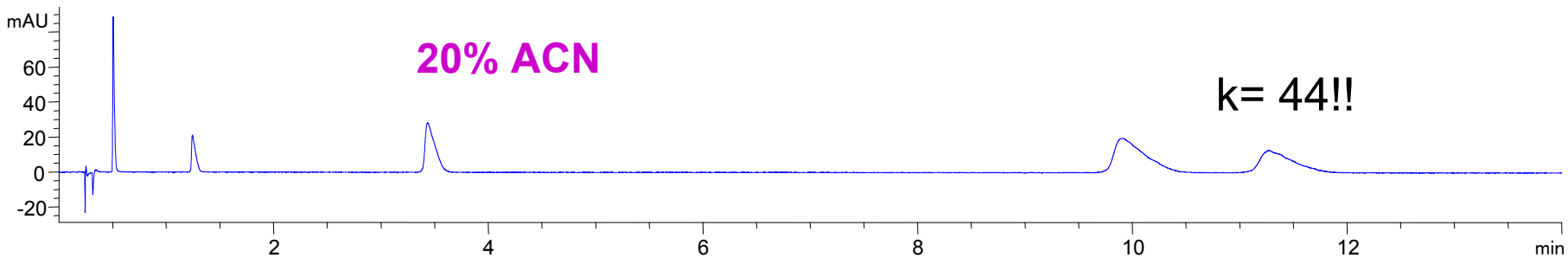
40% ACN

RRHT Eclipse Plus C18 allows for very fast optimization of % organic in mobile phase.



30% ACN

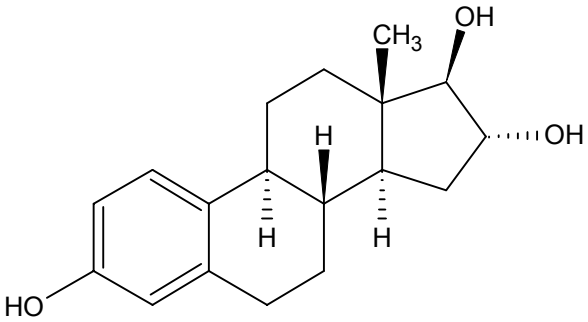
- **Good resolution**
- **Fast analysis**
- **No time wasted**



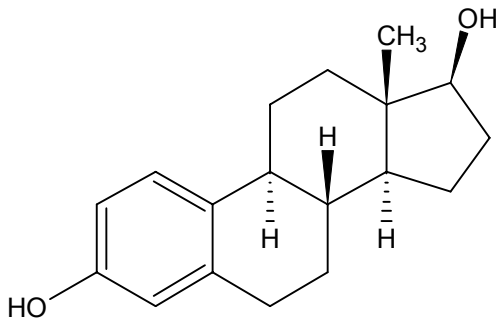
20% ACN

k= 44!!

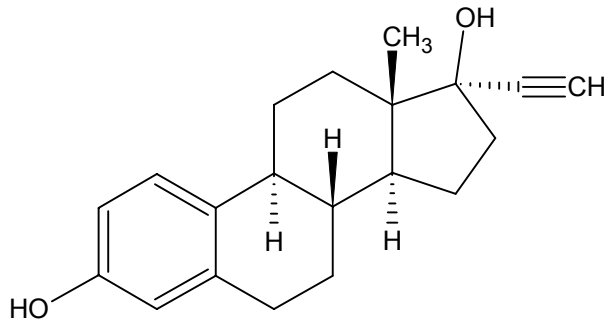
Steroidal Compounds



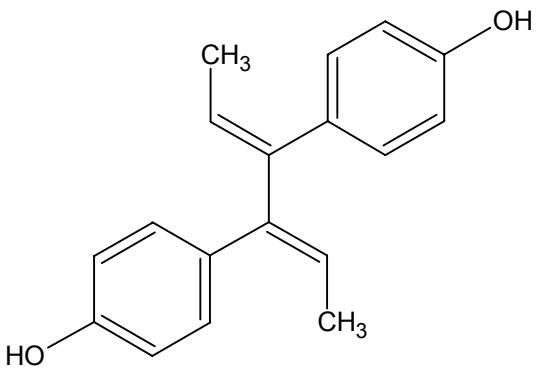
estriol



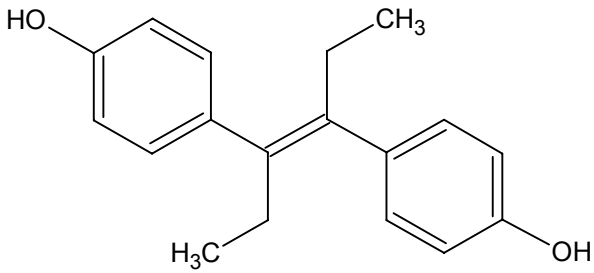
estradiol



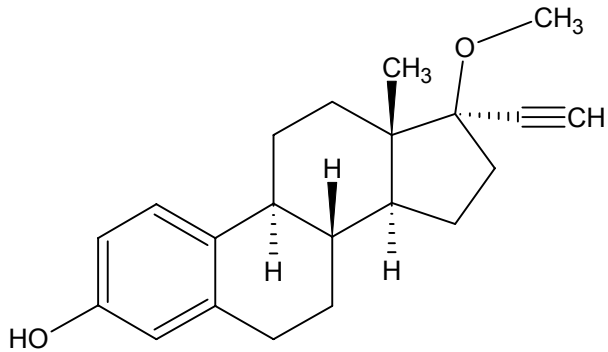
ethinyl estradiol



dienestrol

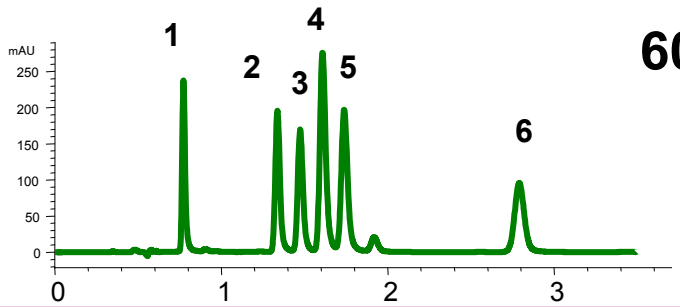


diethylstilbestrol



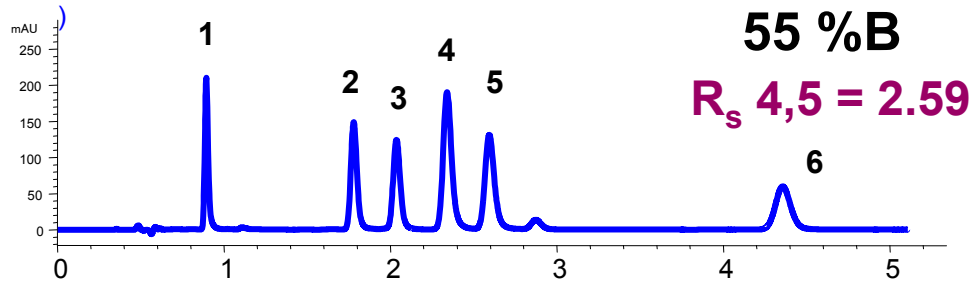
ethinyl estradiol 3-methyl ether

Mobile Phase Optimization on Short RRHT 4.6 x 50 mm, SB-CN Column



60 %B

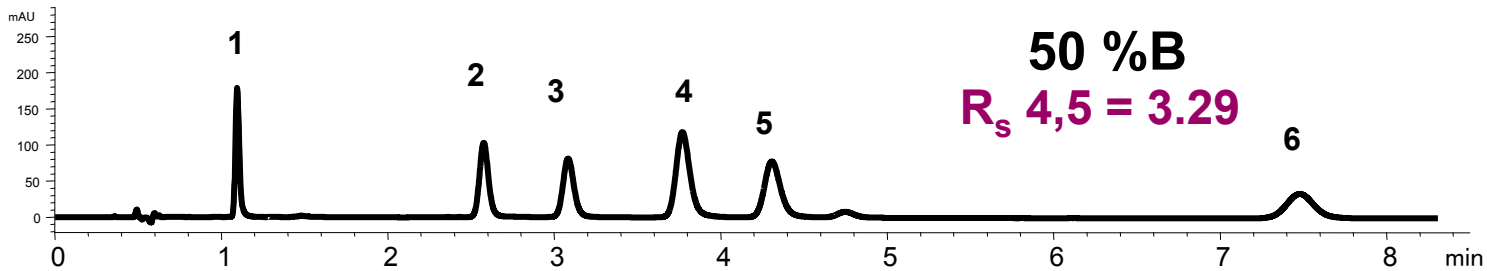
1. Estriol
2. Estradiol
3. Ethinyl Estradiol
4. Dienestrol
5. Diethylstilbestrol
6. Ethinyl estradiol methyl ether



55 %B

R_s 4,5 = 2.59

A: Water
B: 30 % MeOH/70 %MeCN
1 µl injection, 210 nm

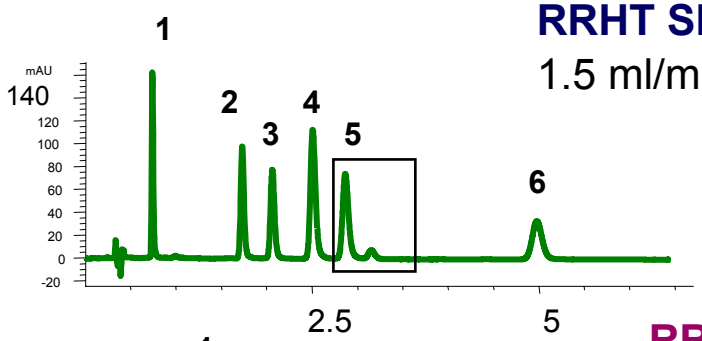


50 %B

R_s 4,5 = 3.29

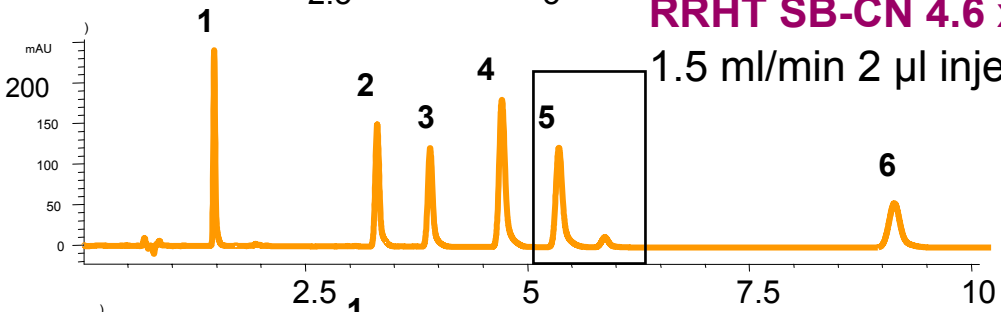
- **RRHT columns allow for very fast optimization of % organic in mobile phase.**

Comparison of Optimal Conditions On Columns with Different Dimensions – Length, Particle Size, Resolution



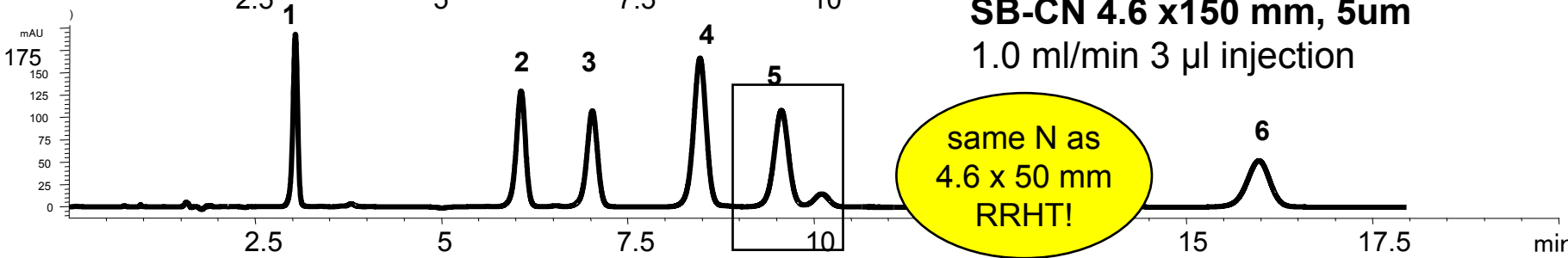
RRHT SB-CN 4.6 x 50 mm, 1.8um
1.5 ml/min 1 µl injection

1. Estriol
2. Estradiol
3. Ethinyl Estradiol
4. Dienestrol
5. Diethylstilbestrol
6. Ethinyl estradiol methyl ether



RRHT SB-CN 4.6 x100 mm, 1.8um
1.5 ml/min 2 µl injection

A: Water
B: 30 % MeOH/ 70 %MeCN
40 % B
Detection: UV 210 nm



SB-CN 4.6 x150 mm, 5um
1.0 ml/min 3 µl injection

same N as 4.6 x 50 mm RRHT!

RRHT Columns allow rapid resolution optimization during initial method development. Column lengths compared quickly.

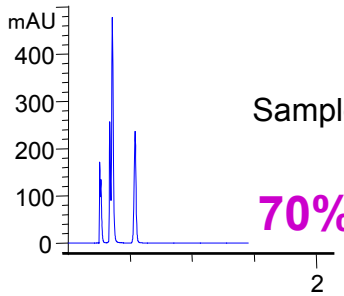
Why Do We Recommend Changing Organic Modifier Before Changing the Bonded Phase?

- It's easy – acetonitrile and methanol are readily available!
- It works on any bonded phase, so it gives you a tool to possibly optimize a separation no matter the column choice!

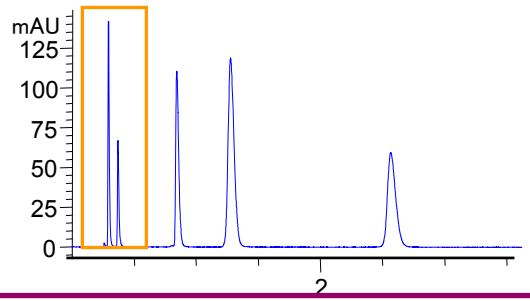
Start at Low pH on Eclipse Plus-C18 RRHT
Cardiac Drugs with Methanol, Changing Organic Modifier

Remember to use buffer!

Column: ZORBAX RRHT Eclipse Plus C18, 4.6 x 50 mm, 1.8 µm
Mobile Phase: A: 25 mM NaH_2PO_4 , pH 3.0 B: MeOH
Flow Rate: 2.0 mL/min Temperature: 30°C Detection: UV 240 nm
Sample: Cardiac Drugs 1.Pindolol 2. Di-isopyridamide 3.Propranolol 4.Diltiazem 5. Dipyridamole

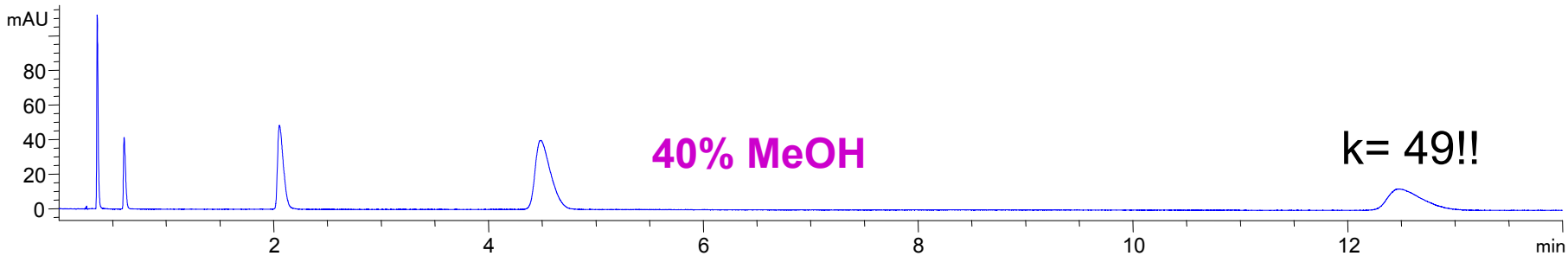


70% MeOH



50% MeOH

- **Adjusting [MeOH] influences retention like adjusting ACN**
- **Solvents can be compared quickly with RRHT columns**
- **MeOH changes selectivity and gives longer analysis time for these analytes**

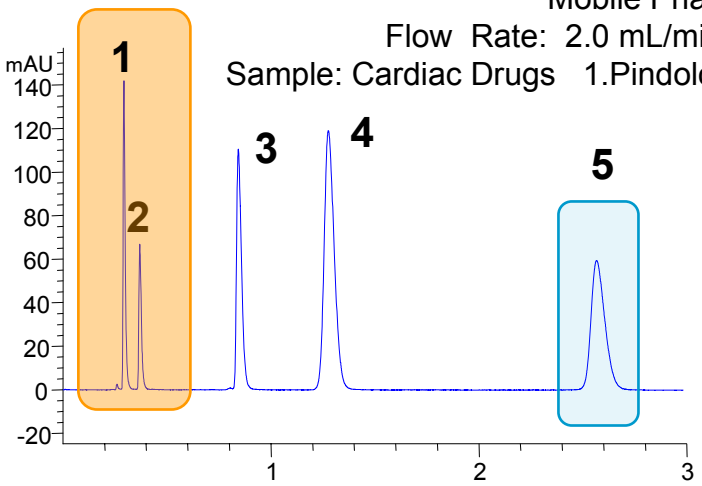


40% MeOH

k= 49!!

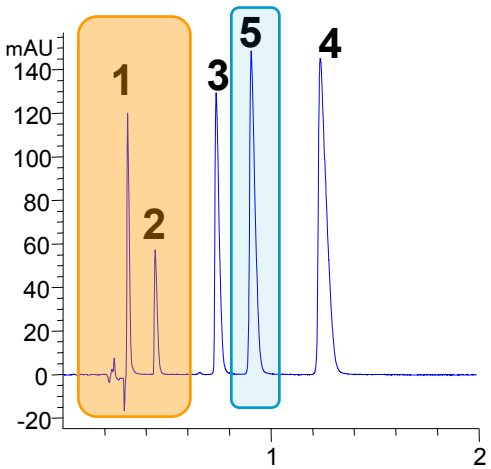
Method Development - Change Organic Modifier Comparison of Acetonitrile and MeOH

Column: ZORBAX RRHT Eclipse Plus C18, 4.6 x 50 mm, 1.8 µm
Mobile Phase: A: 25 mM NaH_2PO_4 , pH 3.0 B: organic
Flow Rate: 2.0 mL/min Temperature: 30°C Detection: UV 240 nm
Sample: Cardiac Drugs 1.Pindolol 2. Diisopyridamide 3.Propranolol 4.Diltiazem 5. Dipyridamole



50% MeOH

Resolution of pairs (2,3), (3,4), (4,5) is better in MeOH. Peak 5 selectivity shift



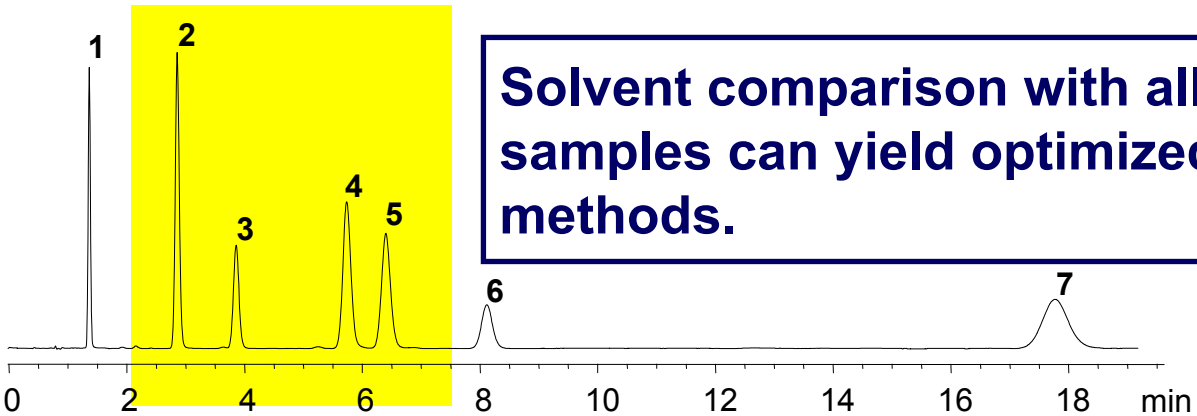
30% ACN

Resolution of critical pair (1,2), is better in ACN

Change Mobile Phase - Acetonitrile vs. Methanol on ZORBAX Eclipse XDB-CN with Estrogens

Column: ZORBAX Eclipse XDB-CN Column Dimensions: 4.6 x 150 mm, 5 µm Mobile Phase: As shown
Flow Rate: 2.0 ml/min Injection Volume: 2.00 µl Column Temperature: 25 °C Detector: UV, 210 nm

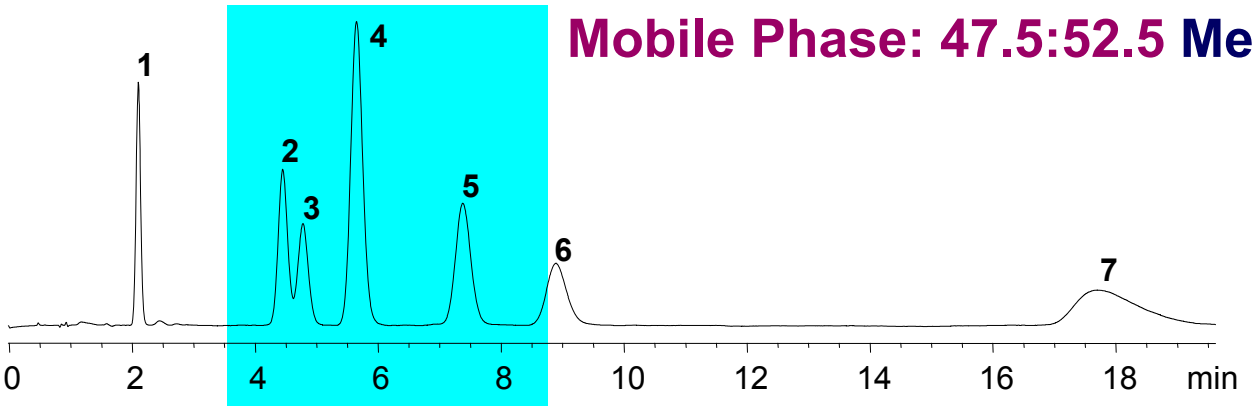
Mobile Phase: 35:65 ACN:Water



Solvent comparison with all samples can yield optimized methods.

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. Ethinylestradiol 3-methyl ether (0.00103 µg/µl)
7. Ethynodiol Diacetate (0.00139 µg/µl)

Mobile Phase: 47.5:52.5 MeOH:Water



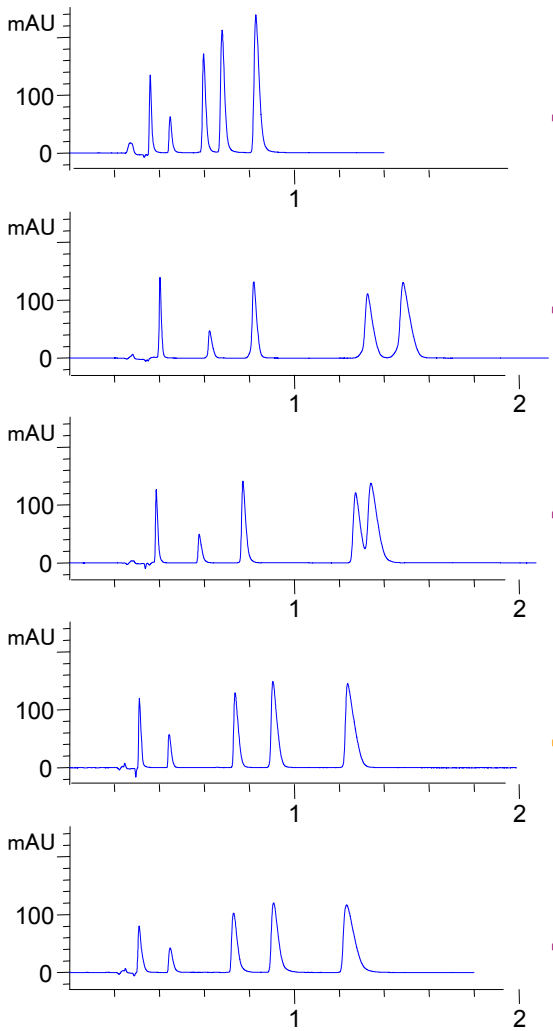
Why Is Changing the Bonded Phase Effective?

- Differences in interactions between polar and non-polar compounds.
- Other types of interactions with a bonded phase can be exploited (pi-pi interactions etc.)
- These all change with bonded phase!
- Changing the bonded phase can improve selectivity/ resolution, reduce analysis time

When you use RRHT (1.8um) columns, the comparison of bonded phases can be done quickly!!

- You must have multiple RRHT column choices available

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

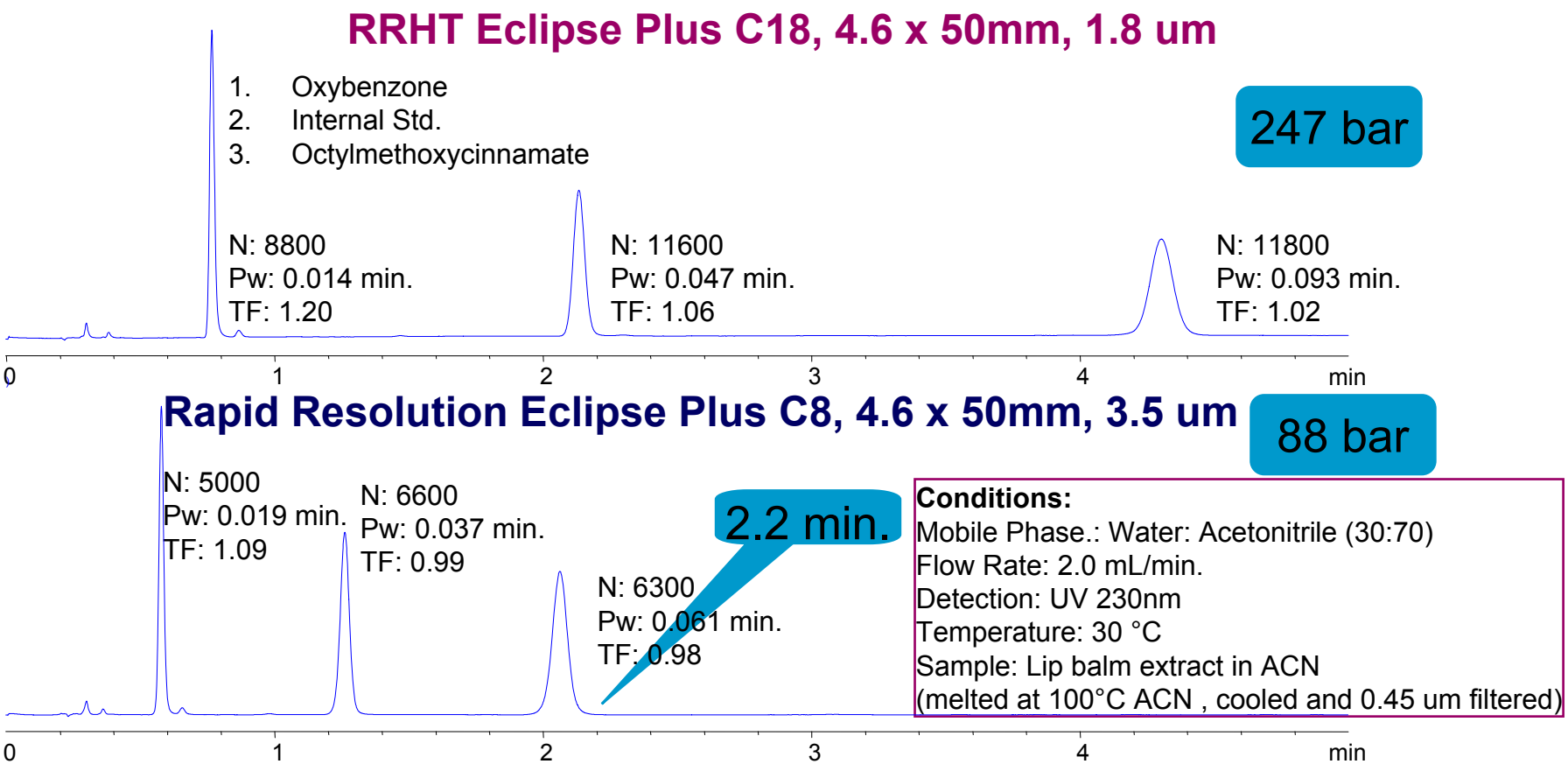
RRHT SB-Aq
4.6 x 50 mm, 1.8 µm

RRHT Eclipse Plus C18
4.6 x 50 mm, 1.8 µm

RRHT SB-C18
4.6 x 50 mm, 1.8 µm

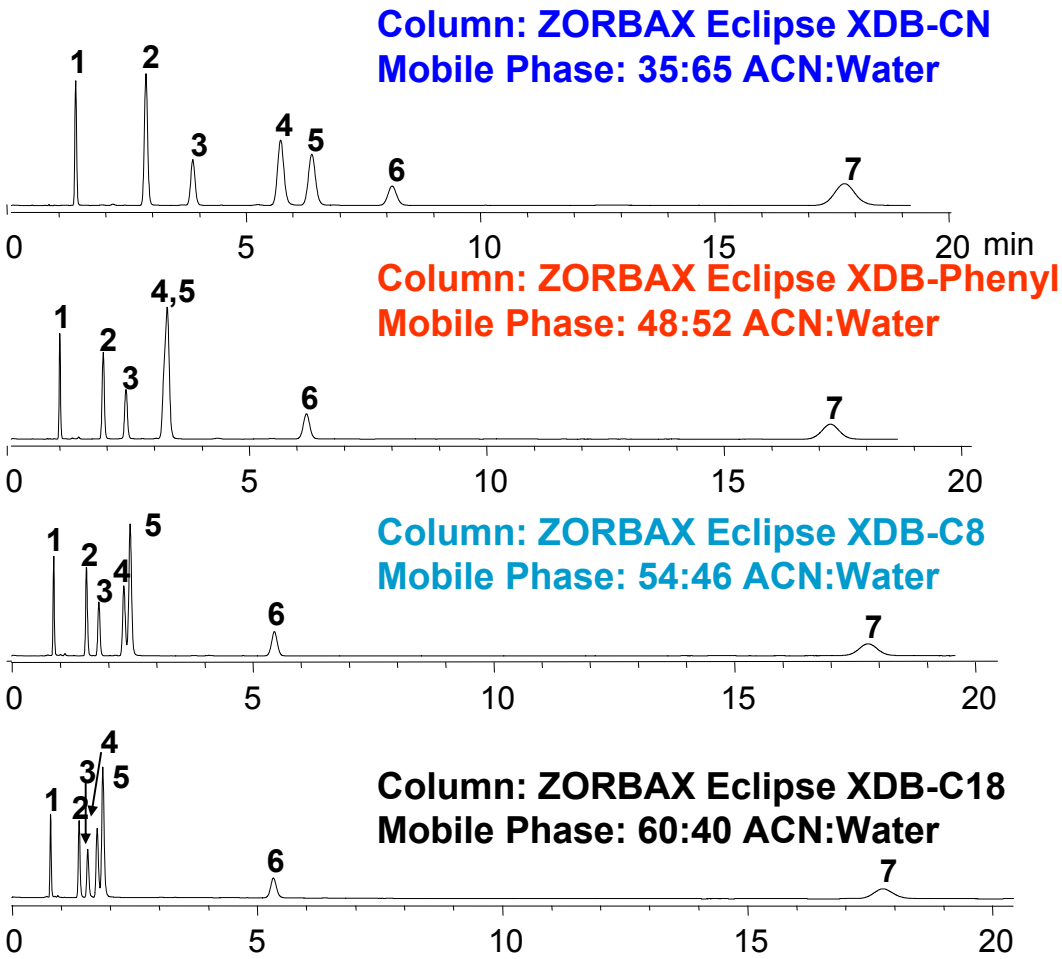
- 5 Different bonded phases compared
- Analysis time of each run is only 2 minutes
- Comparison among phases done in optimum % organic for SB-CN
- The fast runs mean a comparison can be done even if you have a good separation on the C18
- More chances to optimize!

Selectivity/Bonded Phase Choices:
Eclipse Plus C18 vs. C8 and RRHT vs. RR



Less retention can save significant time – the C8 is a good choice here.
The RRHT column is delivering the efficiency and resolution expected, but the C8 bonded phase may be the better choice.

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: 2.0 ml/min
Injection Volume: 2.00 μl
Column Temperature: 25 °C
Detector: UV, 210 nm

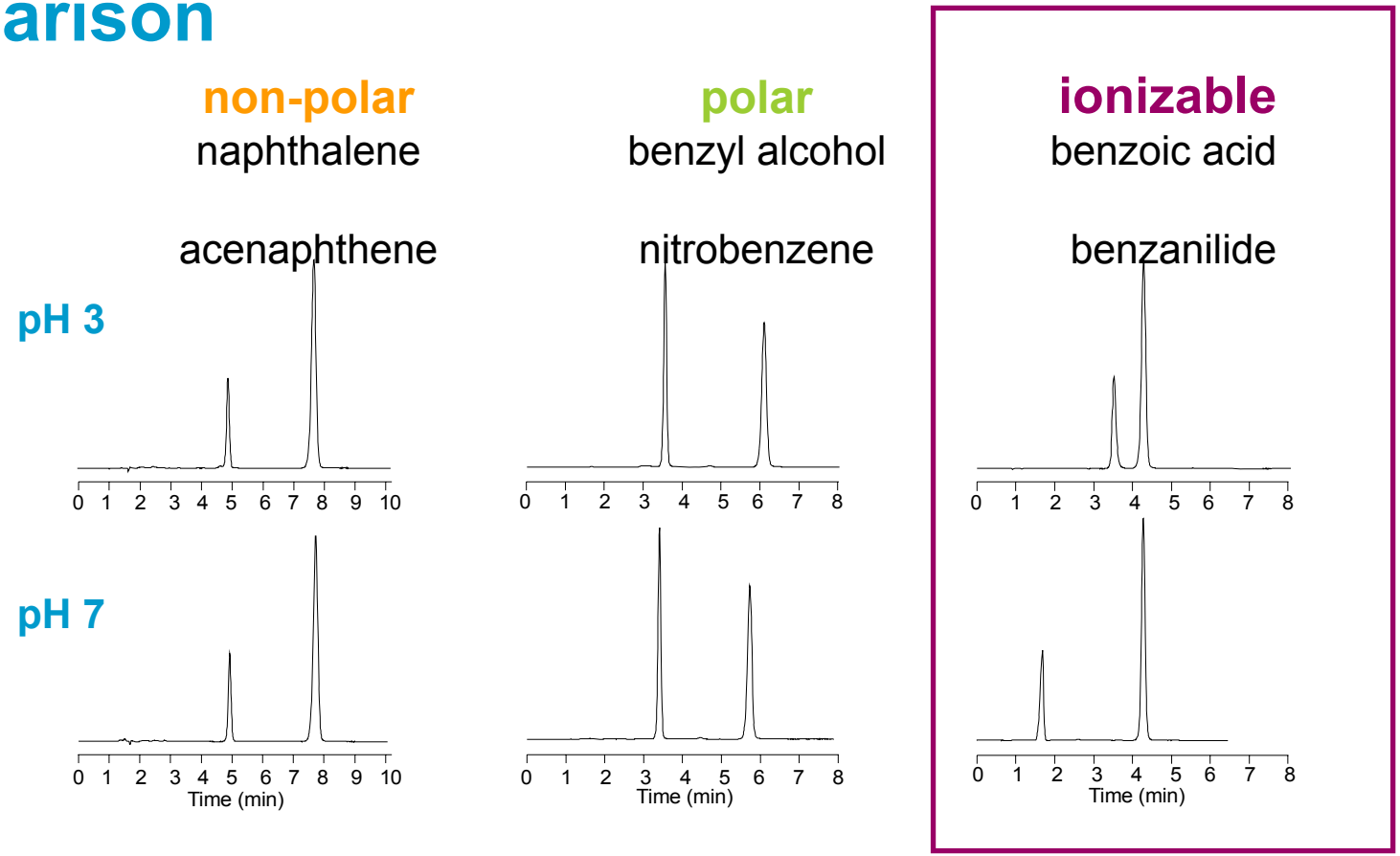
Sample:
1. Estriol (0.00130 μg/μl),
2. β-Estradiol (0.00130 μg/μl),
3. Ethinyl Estradiol (0.00147 μg/μl),
4. Dienestrol (0.00123 μg/μl),
5. Diethylstilbestrol (0.00128 μg/μl)
6. Ethinyl estradiol 3-methyl ether (0.00103 μg/μl)
7. Ethynodiol Diacetate (0.00139 μg/μl)

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

So then Why is pH Critical to Method Development?

- What compounds are pH sensitive?
- How does pH affect retention and resolution?
- How does pH influence method development?
- How does pH influence column choice?

When Does pH affect Resolution? Compound Type Comparison



- **Ionizable compounds – acids and bases can change retention and selectivity the most with changes in pH**

Change in Retention with pH for Ionizable Compounds is Key to Method Development

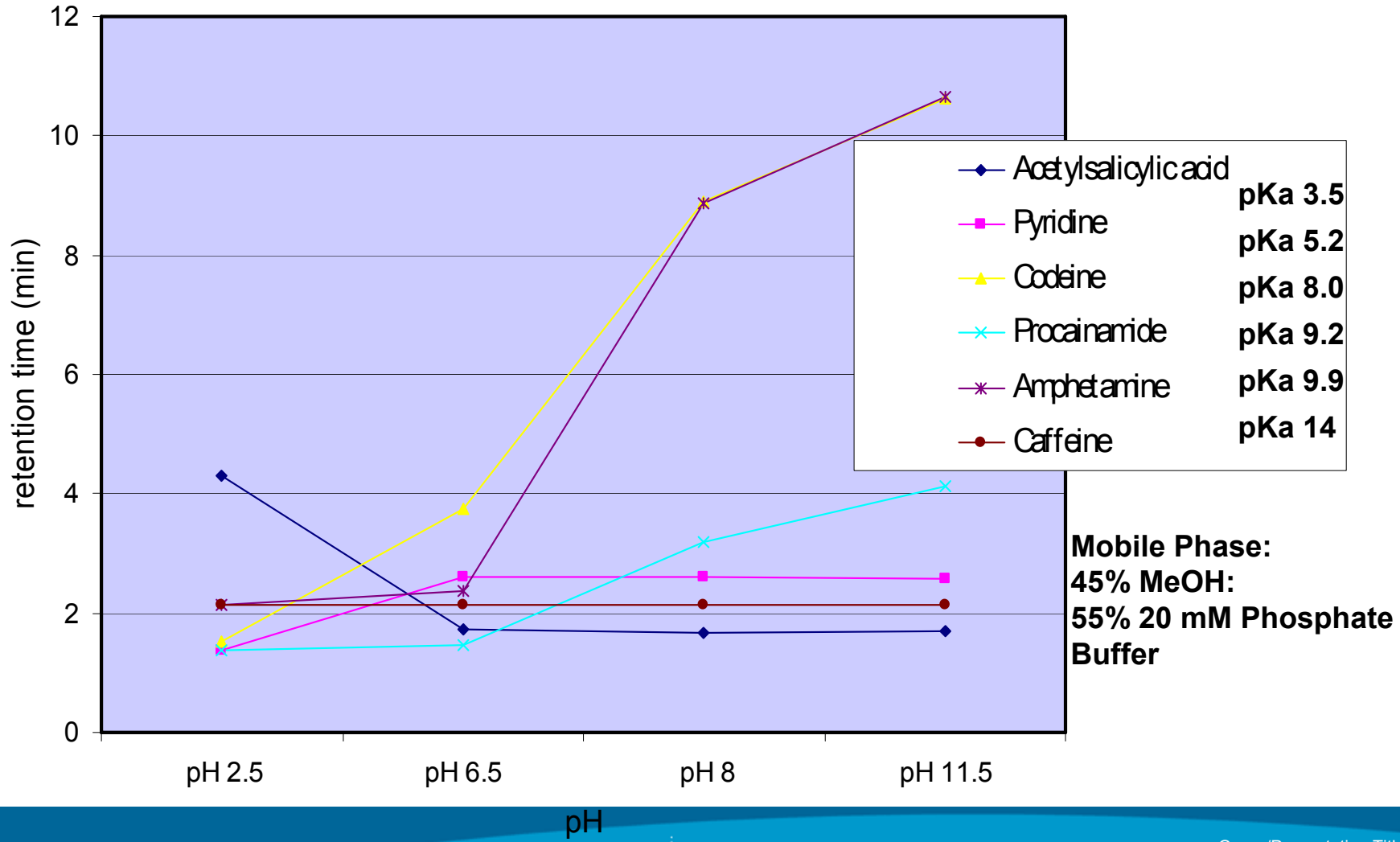
- Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)
- Silanols on silica ionize at mid-pH, increasing retention of basic analytes

(i.e possible ion-exchange interactions)

- Choose mobile phase pH to optimize retention and selectivity during method development
- Eclipse Plus can be used over a wide pH range
- Other choices exist for high pH

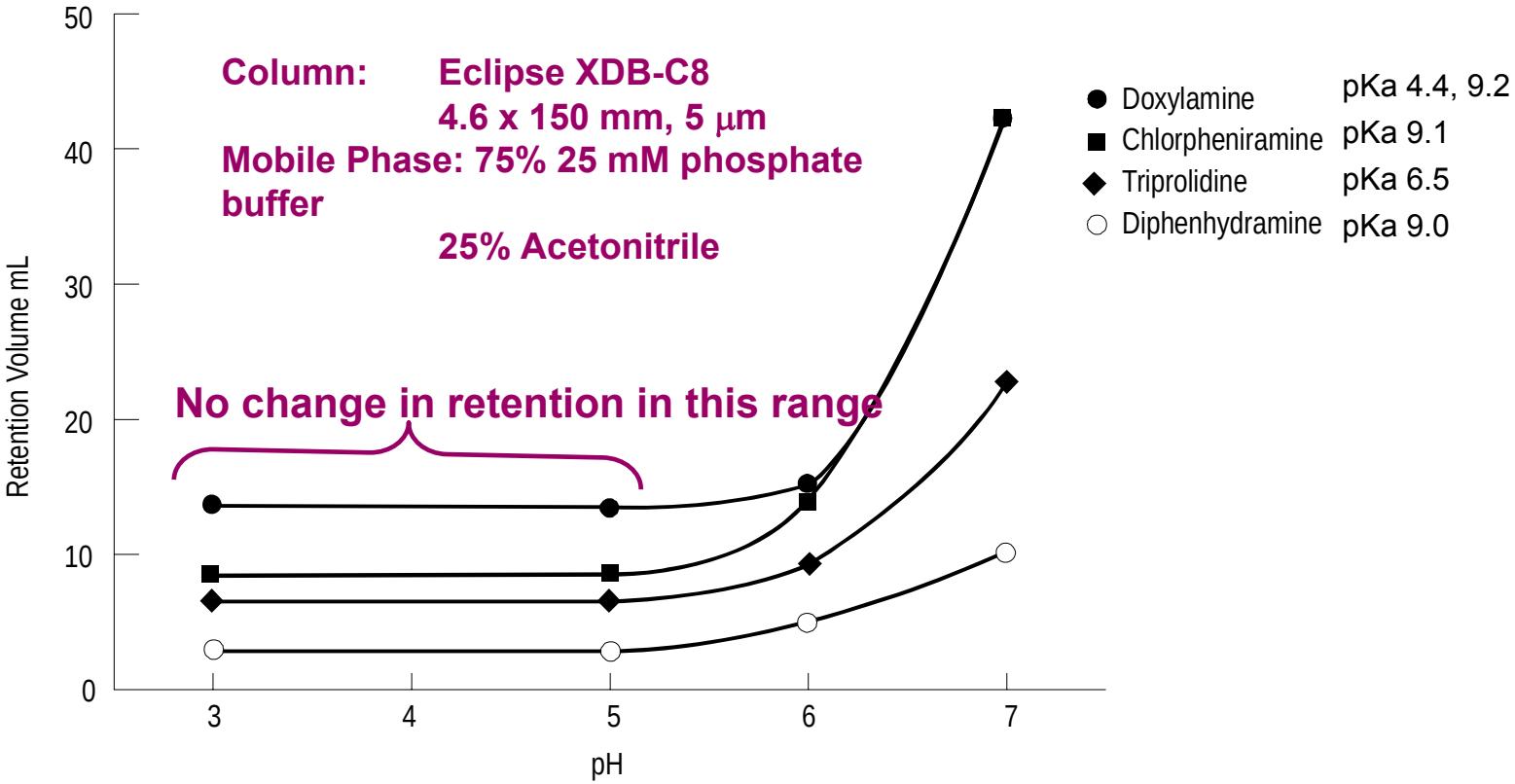
Change in Retention with pH for Ionizable Compounds is Compound Dependent

Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)



Mobile Phase:
45% MeOH:
55% 20 mM Phosphate Buffer

Retention vs. pH for Basic Antihistamines - Less Variation in Retention at Low pH



- Slight changes in pH may dramatically change retention and selectivity
- Always evaluate retention changes with pH during method development

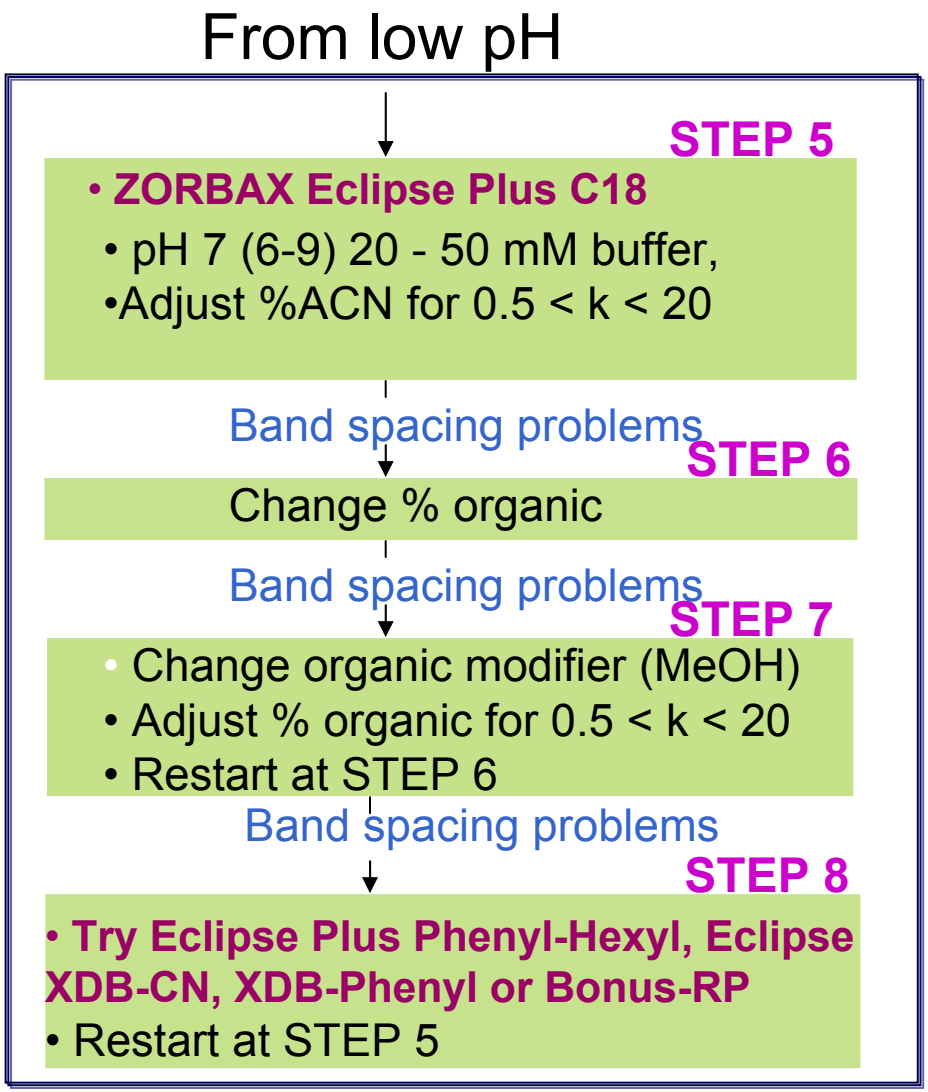
Therefore pH Should be Considered a Key Method Development Parameter

A single column can be used over a wide pH range making selectivity comparisons quick and reliable when pH is changed.

May have better selectivity in the pH range 3 – 8.

Other bonded phases can be reconsidered for mid pH, just like at low pH

Method Development Scheme – Adding Mid pH



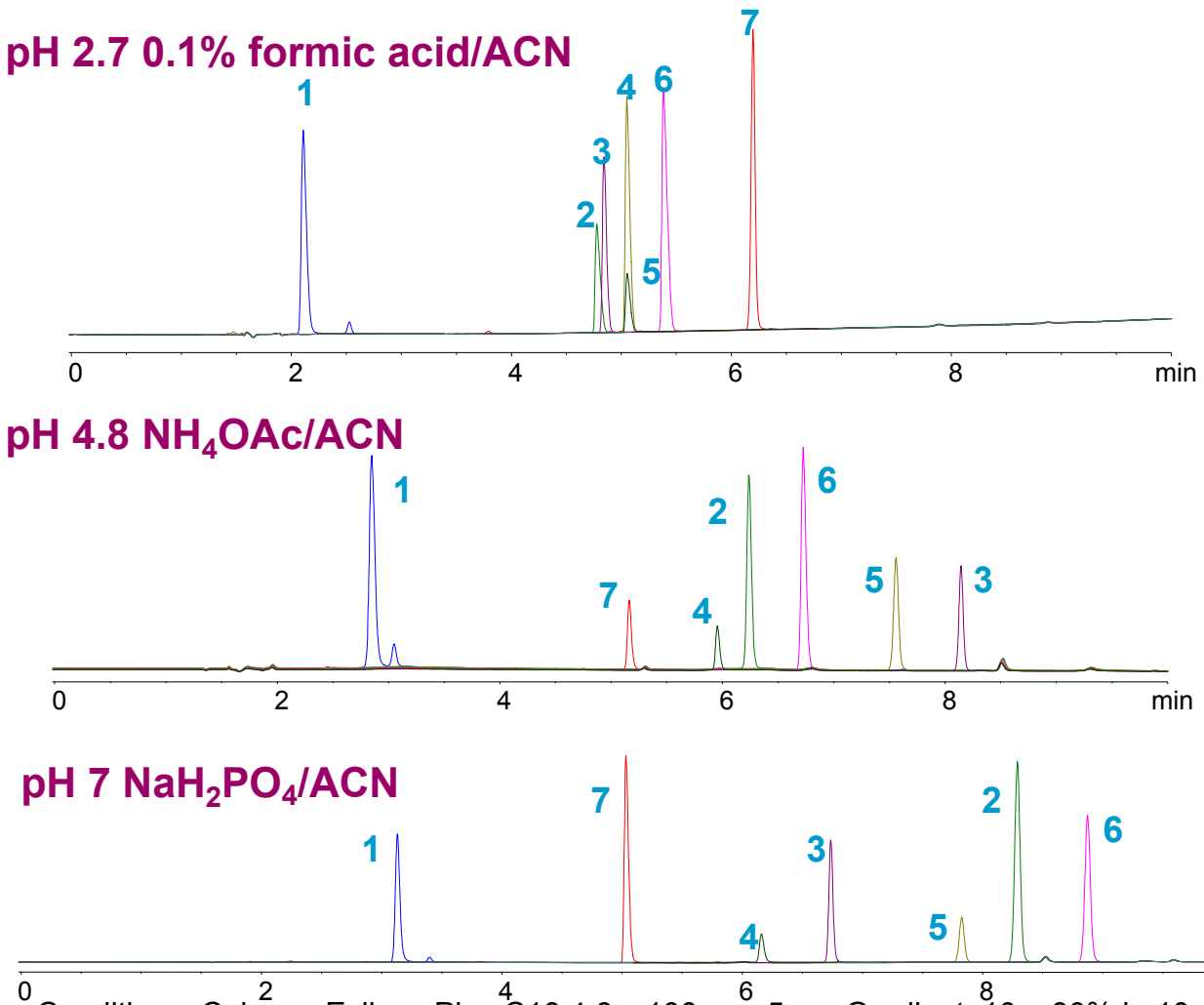
- **Mid pH can provide better selectivity**
- **It may be more compatible with your sample**
- **The process for investigating mid pH is the same as for low pH**
- **Eclipse Plus delivers outstanding performance at mid pH**
- **Alternate bonded phases should also be considered if improved selectivity is desired**

Eclipse Plus Columns Are Ideal for Method Development at Low and Mid pH

- Can be used from pH 2-9
- Accommodates method development in low and mid pH ranges
- Allows pH sensitive separations to be optimized

Excellent peak shape and efficiency achieved with new Eclipse Plus material

Eclipse Plus Can Be Used Over a Wide pH Range (pH 2 – 9) to Optimize Selectivity



1. procainamide
2. buspirone
3. pioglitazone
4. eletriptan
5. dipyridamole
6. diltiazem
7. furosemide

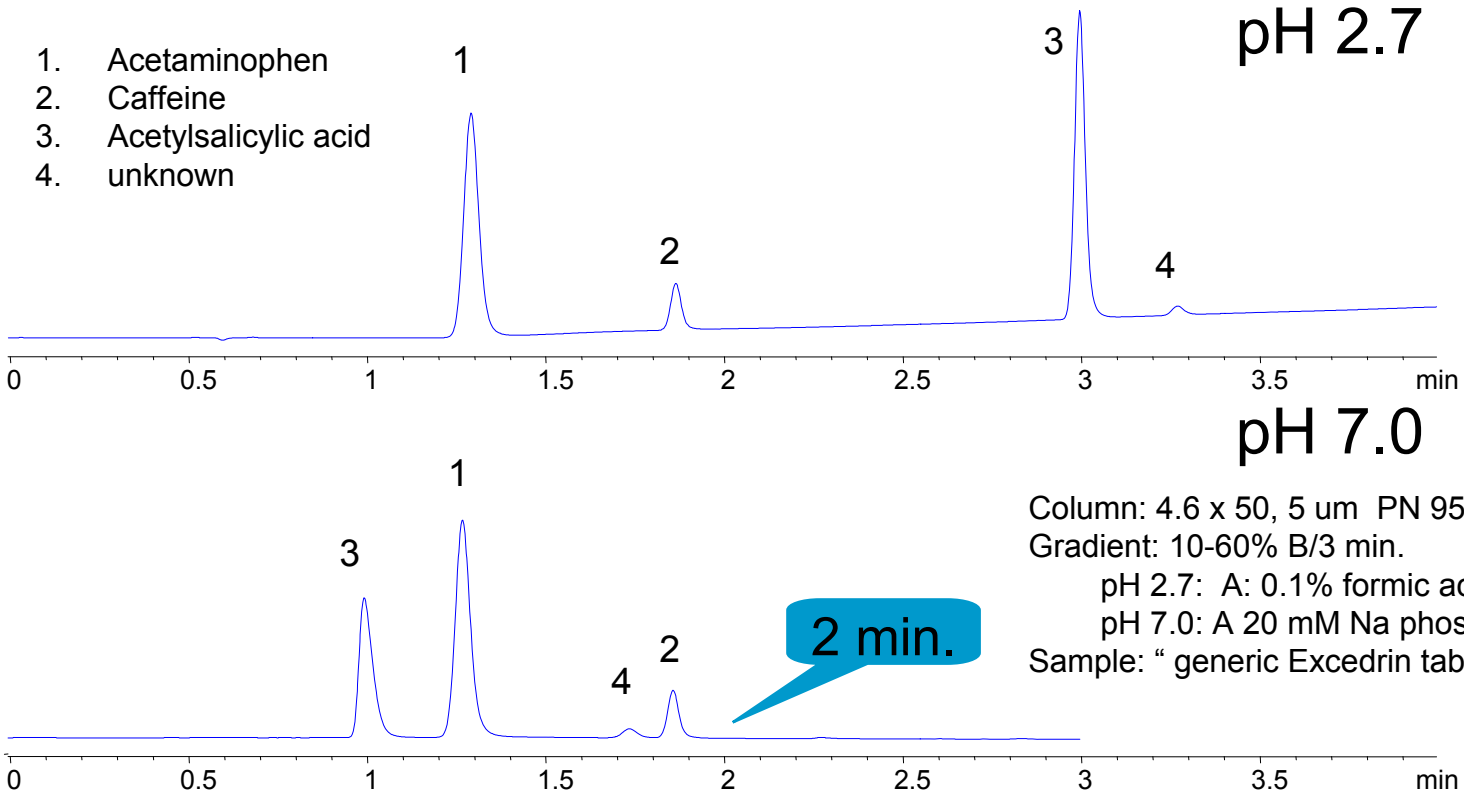
- Selectivity and resolution can change with pH
- Eclipse Plus can be used with many mobile phases and pHs

Conditions: Column: Eclipse Plus C18 4.6 x 100mm, 5um Gradient: 10 – 90% in 10 minutes Detection: UV 254 nm

Selectivity Differences at pH 2 and pH 7 Can Be Dramatic

Eclipse Plus C8 4.6 x 50mm, 5 um

1. Acetaminophen
2. Caffeine
3. Acetylsalicylic acid
4. unknown

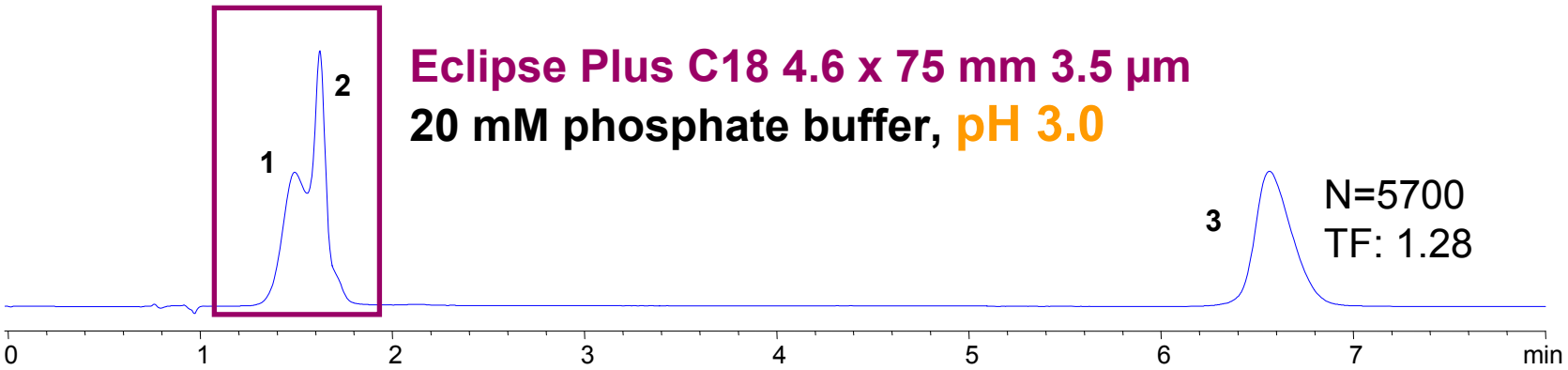


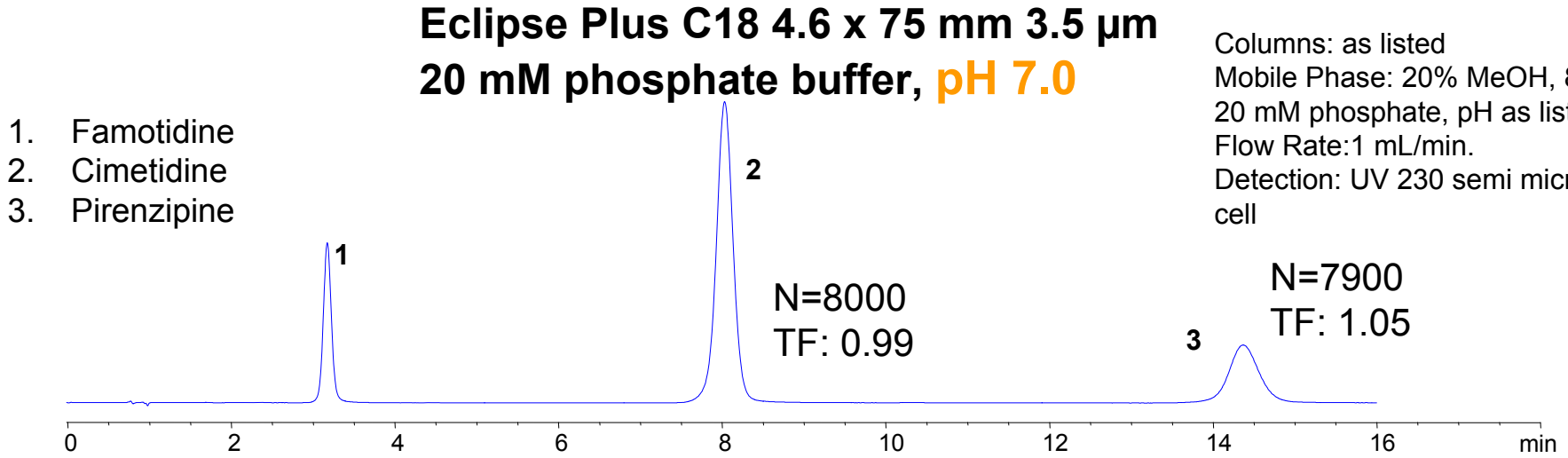
Column: 4.6 x 50, 5 um PN 959946-906
Gradient: 10-60% B/3 min.
pH 2.7: A: 0.1% formic acid B: 0.1% fa in ACN
pH 7.0: A 20 mM Na phosphate B: ACN
Sample: " generic Excedrin tablet"

- **Selectivity can change dramatically from pH 2 – 7 with many samples, such as for the ingredients in this analgesic tablet.**
- **Eclipse Plus can be used under both conditions**

Selectivity Differences at Mid pH Key to Resolution

Eclipse Plus Performs well at Low and Mid pH

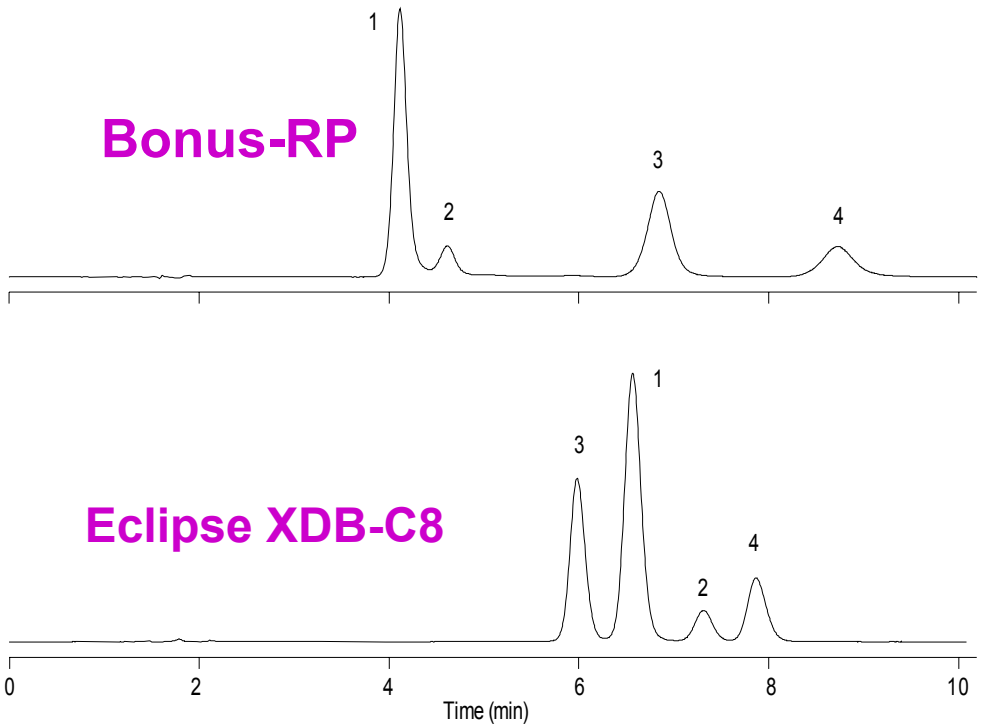




- For this sample resolution is not possible at pH 3, but is at pH 7
- Eclipse Plus was used to experiment at both pHs

Try an Alternate Selectivity at Mid pH if Needed

- One choice for alternate selectivity is Bonus-RP.
- The alkyl-amide bonded phase provides very different selectivity at mid-pH



Mobile Phase: 75% mM NaCitrate, **pH 6**
25% MeOH
Flow Rate: 1.0 mL/min
Temperature: Ambient
Detection: UV 254 nm
Injection Vol: 3 µL
Sample: Cephalosporins
1. Cephalexin
2. Cephaclor
3. Cephuroxime
4. Cephoxitin

Mid pH was the second pH region to consider.
High pH can also be considered if low or mid pH do not resolve all analytes.

Method Development at High pH – a Third Choice

From Mid pH

STEP 9

- **ZORBAX Extend-C18**
  - pH 10.5 (9-12) 5 mM ammonia, or TEA, or 10 – 50 mM organic or borate buffers
  - T = 25°C (ambient – 40°C)
  - Adjust MeOH for 0.5 < k < 20

Band spacing problems

STEP 10

- Change organic modifier (ACN or THF)
- Adjust for 0.5 < k <20

Try different HPLC mode

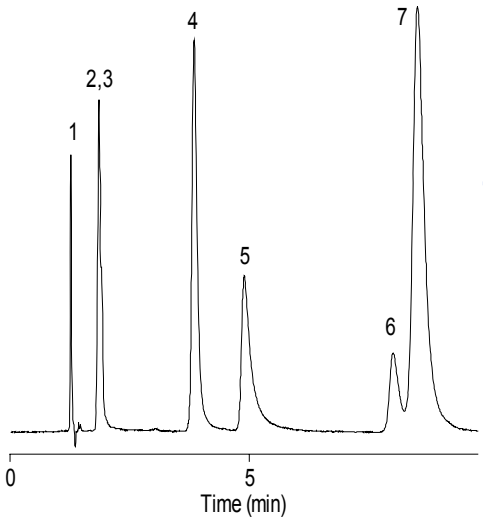
Reasons to Consider High pH

- **Increase retention of basic compounds by analyzing them in non-charged form**
- **Improve selectivity**

High pH Increases Retention of Antihistamines Extend-C18

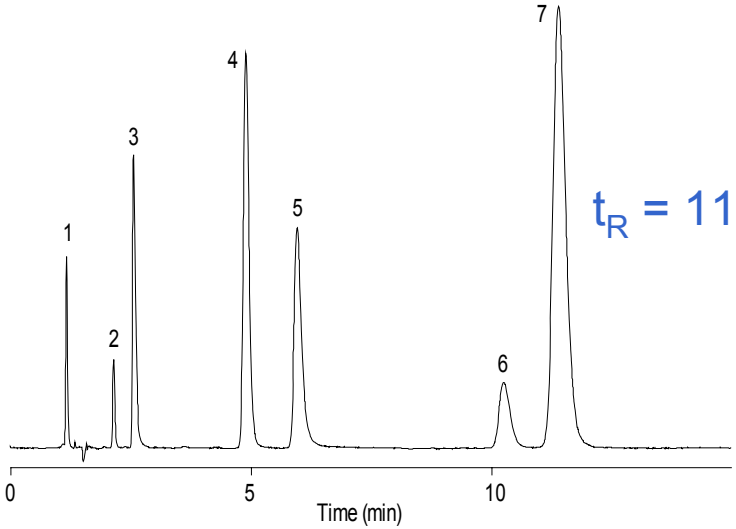
Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 µ m Mobile Phase: See Below Flow Rate: 1.0 mL/min
Temperature: RT Detection: UV 254 nm Sample: 1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7
30% 20 mM Na_2HPO_4
70% MeOH



t_R = 8.5

pH 11
30% 20 mM TEA (triethylamine)
70% MeOH



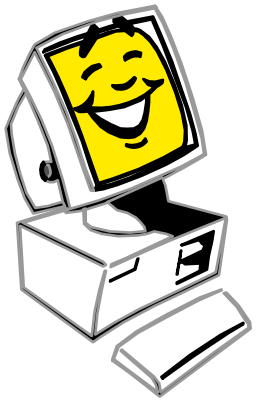
t_R = 11.4

- **The retention of the basic compounds in this sample increases at high pH.**

Conclusions

- ✓ New column choices, like 1.8um RRHT columns and Eclipse Plus bonded phases can enhance typical approaches to method development.
- ✓ These columns provide fast analysis, allowing more options to be considered quickly, and with superior performance, the best results are readily achievable.
- ✓ Since many compounds are ionizable, and retention and selectivity of these compounds are greatly impacted by pH, a method development scheme for LC methods should encourage varying pH.
- ✓ Any method development scheme should also include the use of different bonded phases. Different bonded phases and columns can be selected by pH as well.
- ✓ The third key aspect of method development is mobile phase and organic modifier and this is an effective tool for many separations.

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



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  - *Select option 4, then option 2*
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  - * *Select option 4, then option 1.*
  - www.agilent.com/chem

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