Part 1. General Chromatographic Theory

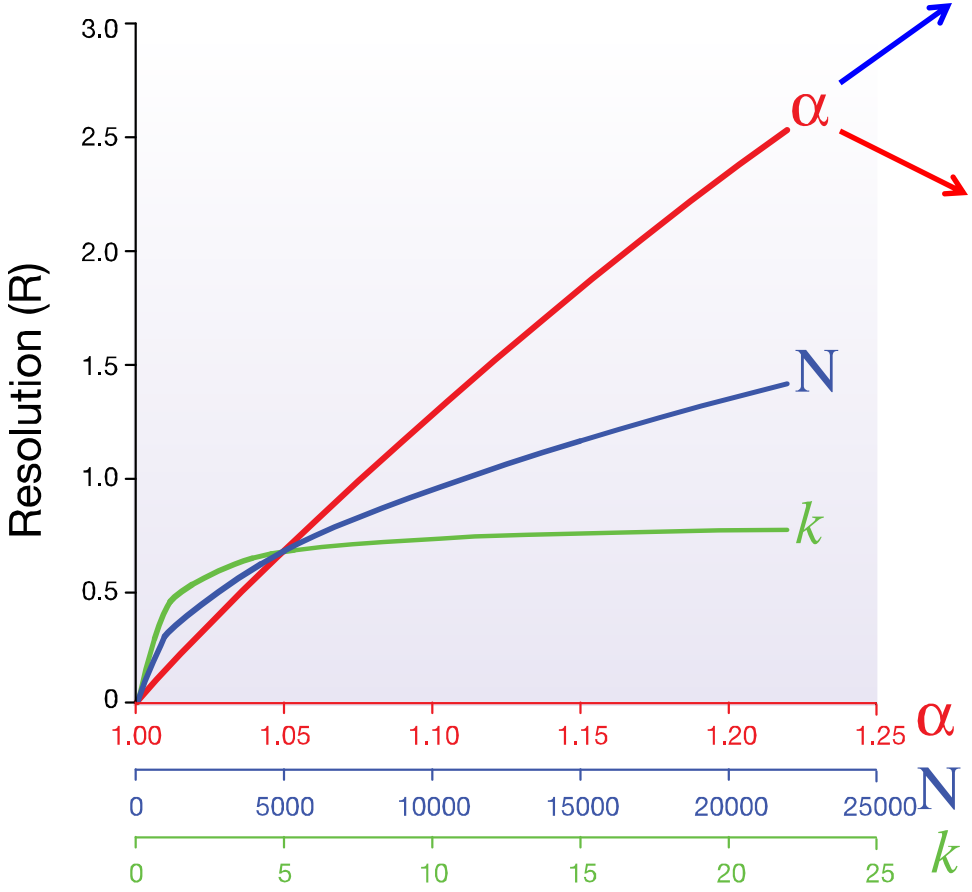
Part 2. Overview of HPLC Media

Part 3. The Role of the Mobile Phase in Selectivity

Part 4. Column Care and Use

Reversed-Phase Solvents

Solvents for RP Chromatography



Stationary Phase:

- Bonded phase (C18 vs Phenyl)
- Particle (Core-shell vs Monolith)

Mobile Phase:

- *Choice of solvent (ACN vs MeOH)*
- *Mobile phase pH*
- *Temperature*
- *Gradients*

Solvents for RP Chromatography

Mobile phase selection is much more challenging that stationary phase selection because the options are limitless. However, in practical method development, we can dramatically narrow down the options to focus on those conditions which will give us the highest likelihood of success.

Typical RP Solvents:

Weak Solvent: **Water/Buffer**

Strong Solvent: **Acetonitrile (64)**
Methanol (34)
Composite mixtures (1)
THF (1)

Frequency of use

Solvents for RP Chromatography

The **solvent strength** of a solvent will depend upon its hydrophobicity. The solvent strengths will determine the amount of solvent needed to elute a given compound.

Reversed Phase **Solvent Strengths**:

- Water 0
- **Methanol 2.6**
- **Acetonitrile 3.1**
- **THF 4.4**

Solvent Strength

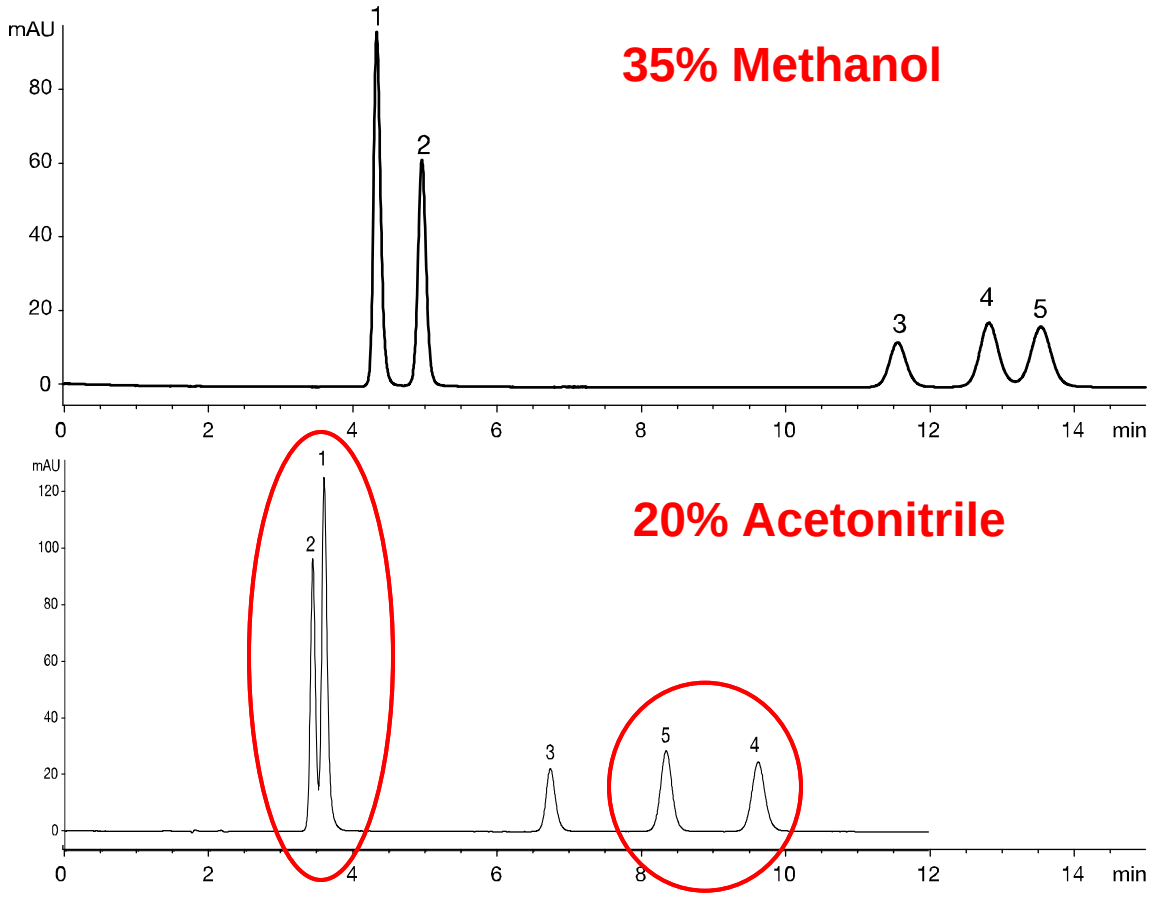
Other considerations when selecting solvents:

- **Methanol** – high viscosity may limit use of smaller particle size or longer columns at elevated flow rates
- **Acetonitrile** – relatively high cost
- **THF** – UV absorbance at low wavelengths; high viscosity

Solvent Strength

Column: Gemini 5 µm C6-Phenyl, 150 x 4.6mm
Mobile phase: 20mM KH_2PO_4, pH 2.5; % organic as noted
Flow rate: 1.0 mL/min
Detection: UV @ 220nm

1. Saccharin
2. p-Hydroxybenzoic Acid
3. Sorbic Acid
4. Dehydroacetic Acid
5. Methylparaben

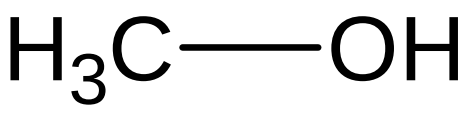


- **Analytes elute earlier** when using acetonitrile (even at lower % ACN)
- **Change in elution** order when switching to ACN

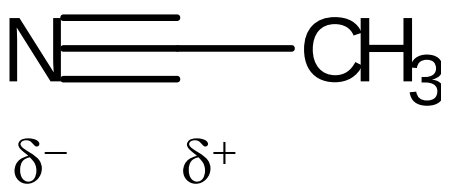
Solvent Selectivity

The elution strength of a given solvent is determined by its hydrophobicity (e.g. heptane would be stronger than hexane because it is more hydrophobic). The selectivity of a solvent, however, is determined by its **polar characteristics** (e.g. heptane and hexane would have the same solvent selectivity).

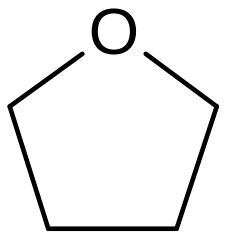
Methanol is a strong proton donor and a strong proton acceptor in hydrogen bonding.



Acetonitrile has a dipole moment but is only a very weak proton acceptor in hydrogen bonding.

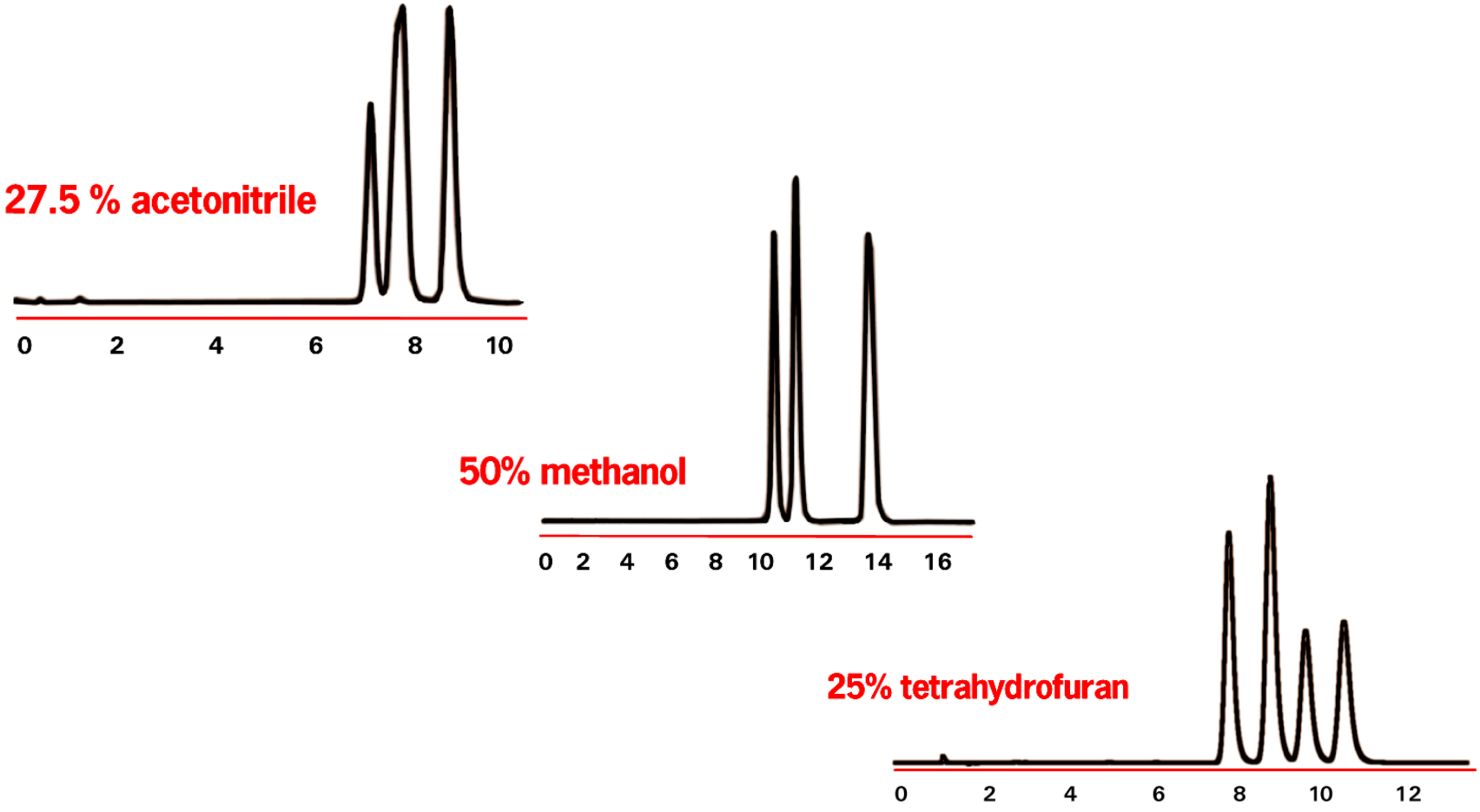


Tetrahyrofuran is able to accept a proton in hydrogen bonding but cannot donate a proton.



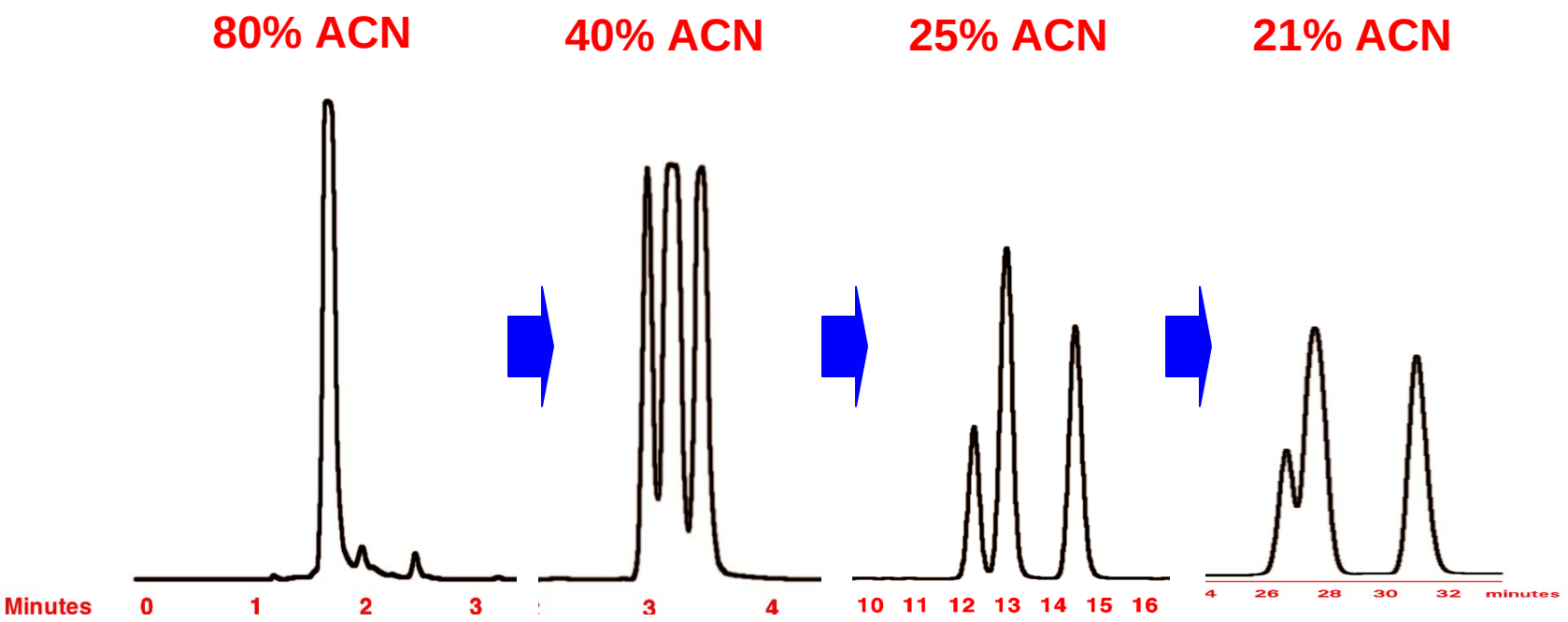
Solvent Selectivity

Optimum Separation of 4 Steroids in Different Solvents:



Solvent Screening for Isocratic Methods

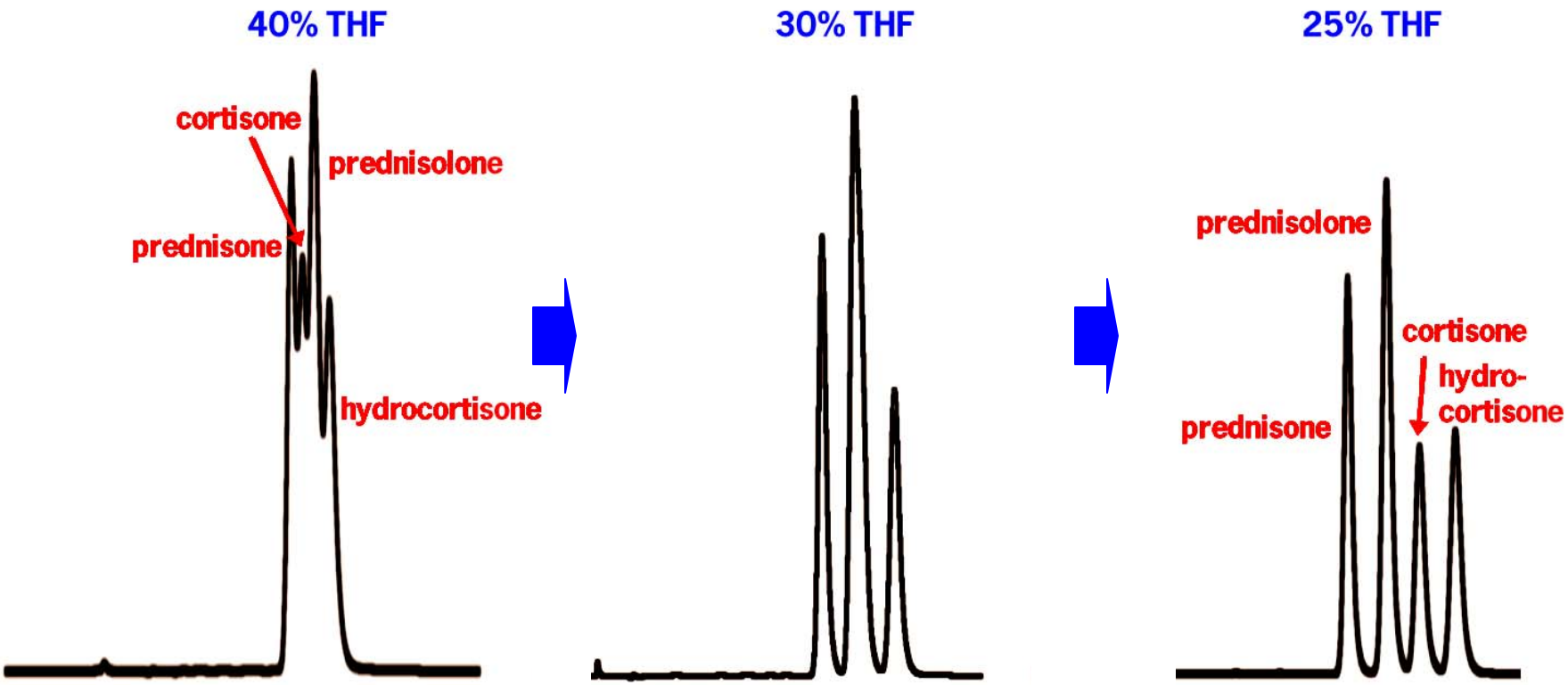
1. Start at high %acetonitrile and work backwards until k' is 2-10 (if possible)



k' = 0 k' ~ 0.8 k' ~ 6 k' ~ 11

Solvent Screening for Isocratic Methods

2. Repeat with alternative solvent:

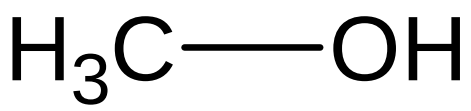


Solvents and Phenyl Selectivity

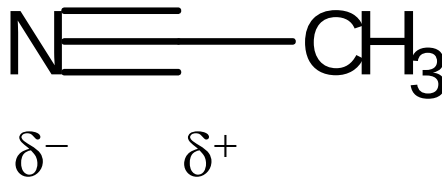
For any reversed-phase method, the choice of acetonitrile or methanol will have a significant effect on the final selectivity of the method. However, when using phenyl phases (e.g. Luna Phenyl-Hexyl; Synergi Polar-RP), you will find that **methanol is a much more effective solvent** for bringing out the unique *pi-pi* selectivity of the phenyl phase.

This is most likely due to the fact that the pi electrons of the nitrile bond in acetonitrile is able to disrupt interactions between the pi electrons of analyte molecules and the stationary phase phenyl ring pi electrons, while methanol is unable to do this as effectively.

Methanol is a strong proton donor and a strong proton acceptor in hydrogen bonding.

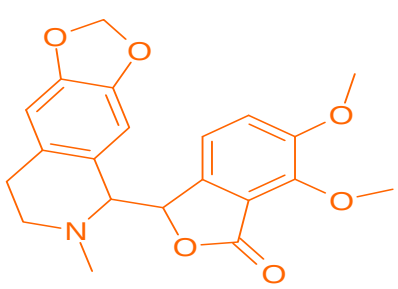


The nitrile bond in **Acetonitrile** may disrupt pi-pi interactions between phenyl rings in the stationary phase and analyte molecule.

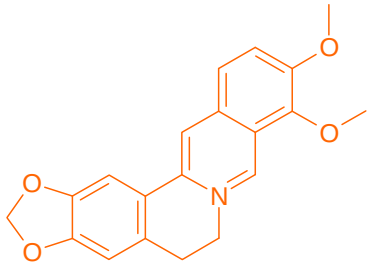


Solvents and Phenyl Selectivity

Columns:	5 µm **C18(2)** 150x4.6 mm 5 µm **Phenyl-Hexyl** 150x4.6 mm
Mobile phase:	A: 20 mM Potassium phosphate pH 2.5 B: 27% Acetonitrile **or** 50% Methanol
Flow rate:	1.0 mL/min
Components:	Extract from Goldenseal: 1. Hydrastine 2. Berberine



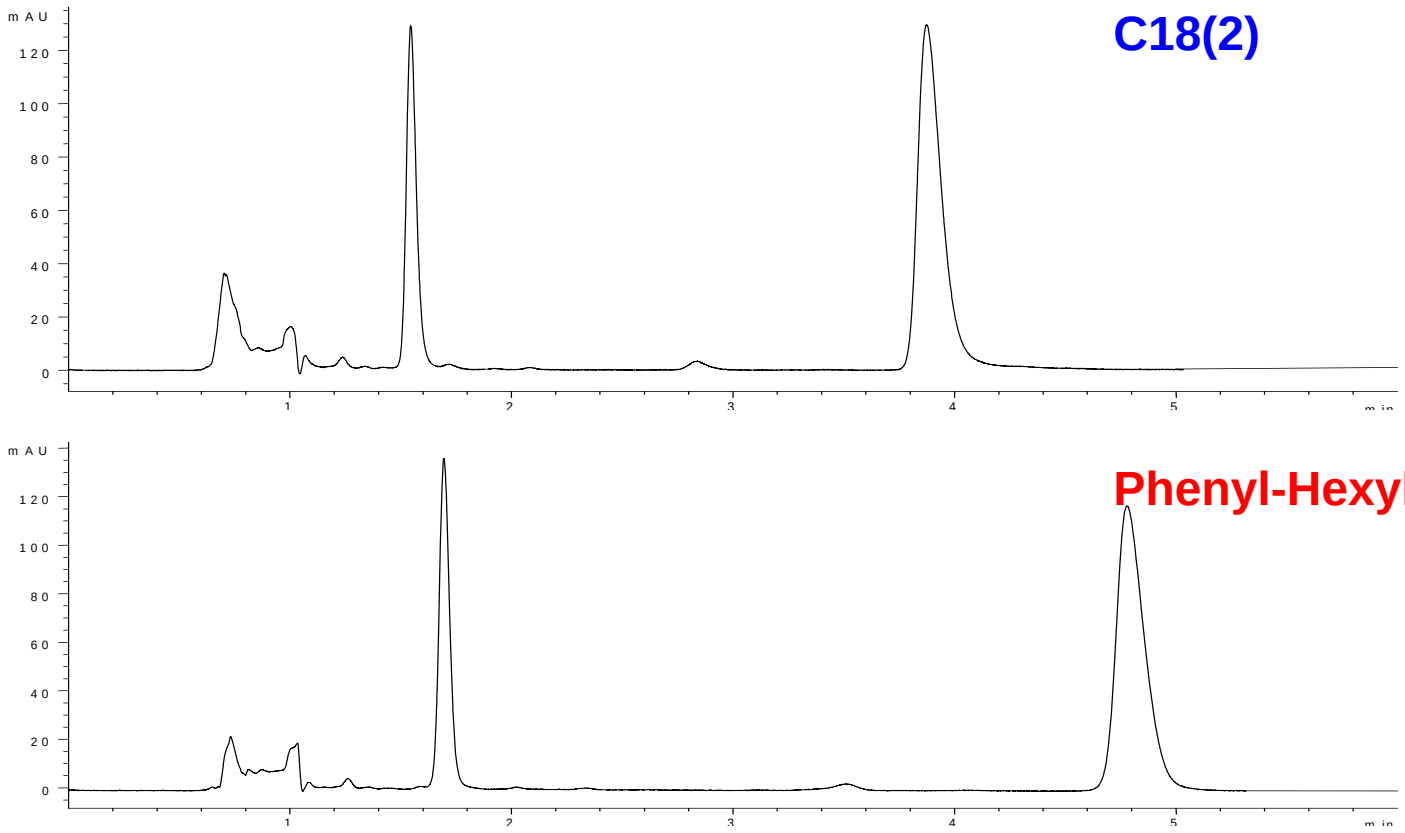
Hydrastine



Berberine

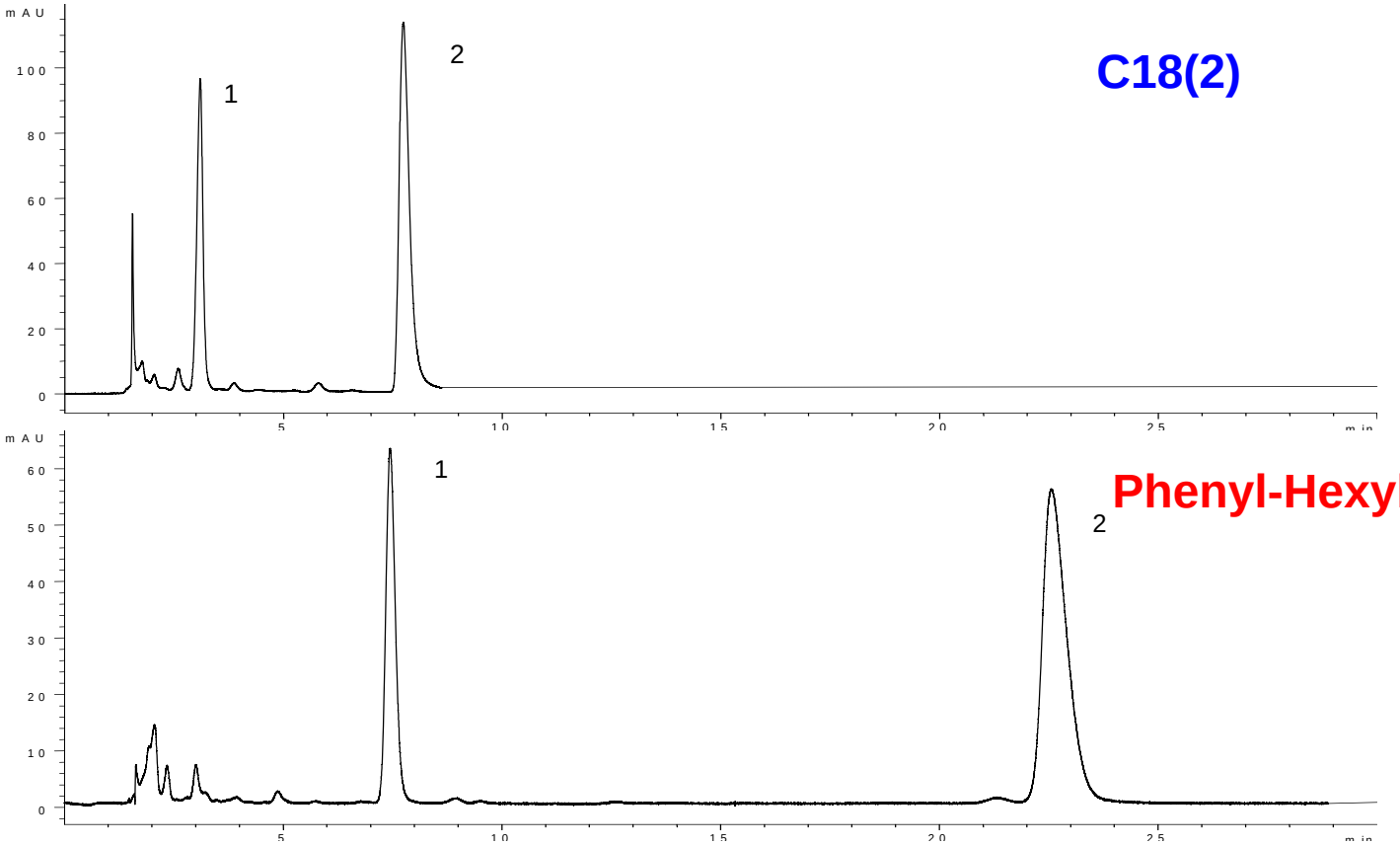
Solvents and Phenyl Selectivity

27:73 Acetonitrile : 20 mM Potassium Phosphate pH 2.5



Solvents and Phenyl Selectivity

50:50 Methanol : 20 mM Potassium Phosphate pH 2.5



Buffers and the Role of Mobile Phase pH

Buffer Selection for RP-HPLC

Choosing the correct buffer for HPLC method development can seem very intimidating due to the vast number of buffers available.
But it's really not, because the majority of methods use just a few buffers!!

Practical considerations when evaluating mobile phase pH:

1. Stability of target analyte(s)

2. Hydrolysis of stationary phase at low pH

- Acids stronger than TFA will cause loss of stationary phase
- Decrease in retention, exposure of silanols groups
- Stability limit will vary depending vendor/brand of media

3. Dissolution of silica at high pH

- "**Typical**" silica-based phases stable up to pH ~8
- **Protective bonding** (e.g. Luna®) increases stability to pH ~10
- **Organosilica hybrid** (e.g. Gemini®) increases stability to pH ~12

Buffer Selection for RP-HPLC

Buffer†	pK_a	Buffer Range (pH)	MS Compatible
Trifluoracetic Acid	< 2	< 2.5	•‡
Phosphoric Acid (pK_1)	2.1	1.1 - 3.1	
Citric Acid (pK_1)	3.1	2.1 - 4.1	
Formic Acid	3.8	2.8 - 4.8	•
Citrate (pK_2)	4.7	3.7 - 5.7	
Acetic Acid	4.8	3.8 - 5.8	•
Citrate (pK_3)	5.4	4.8 - 6.0	
Carbonate (pK_1)	6.4	5.4 - 7.4	•
Phosphate (pK_2)	7.2	6.2 - 8.2	
Triethanolamine	7.8	6.8 - 8.8	•

Buffer†	pK_a	Buffer Range (pH)	MS Compatible
TRIS	8.3	7.3 - 9.3	
Diethanolamine	8.9	7.9 - 9.9	•
Ammonia	9.2	8.2 - 10.2	•
Ethanolamine	9.5	6.5 - 10.5	•
Carbonate (pK_2)	10.3	9.3 - 11.3	•
Diethylamine	10.5	9.5 - 11.5	•
Triethylamine	11.0	10.0 - 12.0	•
Piperidine	11.1	10.1 - 12.1	
Phosphate (pK_3)	12.3	11.3 - 13.3	

= Typical for LC/UV

= Typical for LC/MS

Buffer Selection for RP-HPLC

Buffers for Low pH		
	pKa	Range
TFA	<2	<2.5
Phosphoric acid	2.1	1.1 - 3.1
Formic Acid*	3.8	2.8 - 4.8

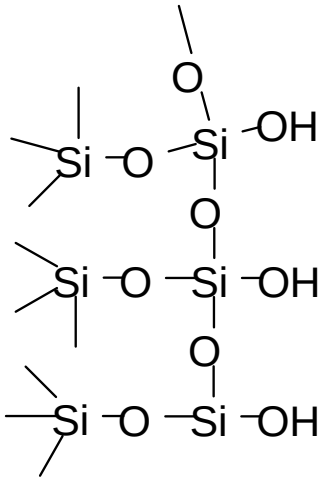
Buffers for High pH		
	pKa	Range
Bicarbonate (pK2)	10.3	9.3 - 11.3

Buffers for Neutral pH		
	pKa	Range
Phosphate (pK2)	7.2	6.2 - 8.2

Effect of pH on Base Silica

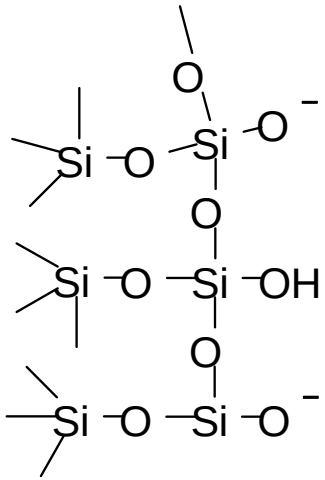
Any silica-based RP material will have some **residual silanols** left after bonding and end-capping. These Si-OH groups can be deprotonated at values above **pH ~3.5**. The deprotonated silanols are more likely to engage in ion-exchange with basic analytes, leading to peak tailing.

pH <3.5



- **Silanols protonated**
- **Less ion-exchange**
- **Less peak tailing**

pH >3.5

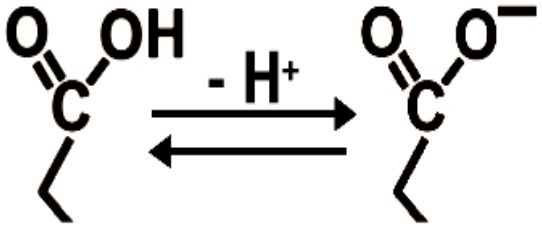


- **Silanols deprotonated**
- **Increased ion-exchange**
- **Increased peak tailing**

Effect of pH on Analyte Ionization

The primary mechanism of retention in RP chromatography is hydrophobic interaction. Ionizing compounds will cause them to behave as more polar species, and reduce their hydrophobic interaction with the stationary phase, leading to decreased retention.

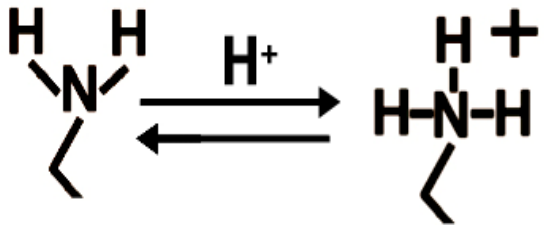
Acids



- More hydrophobic
- More strongly retained

- Less hydrophobic
- Less strongly retained

Bases



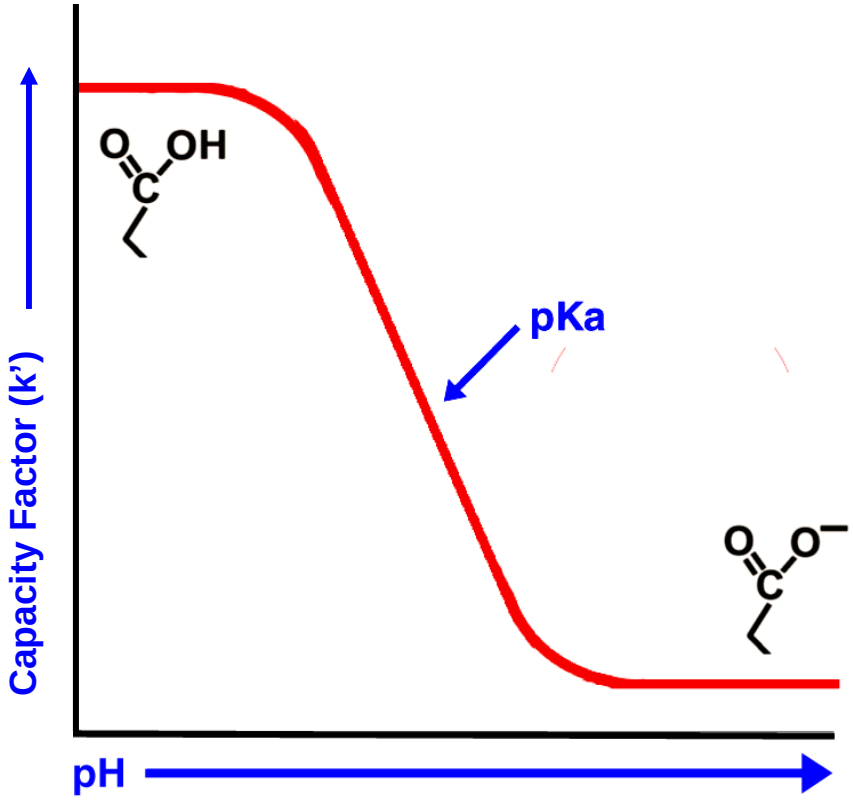
- More hydrophobic
- More strongly retained

- Less hydrophobic
- Less strongly retained

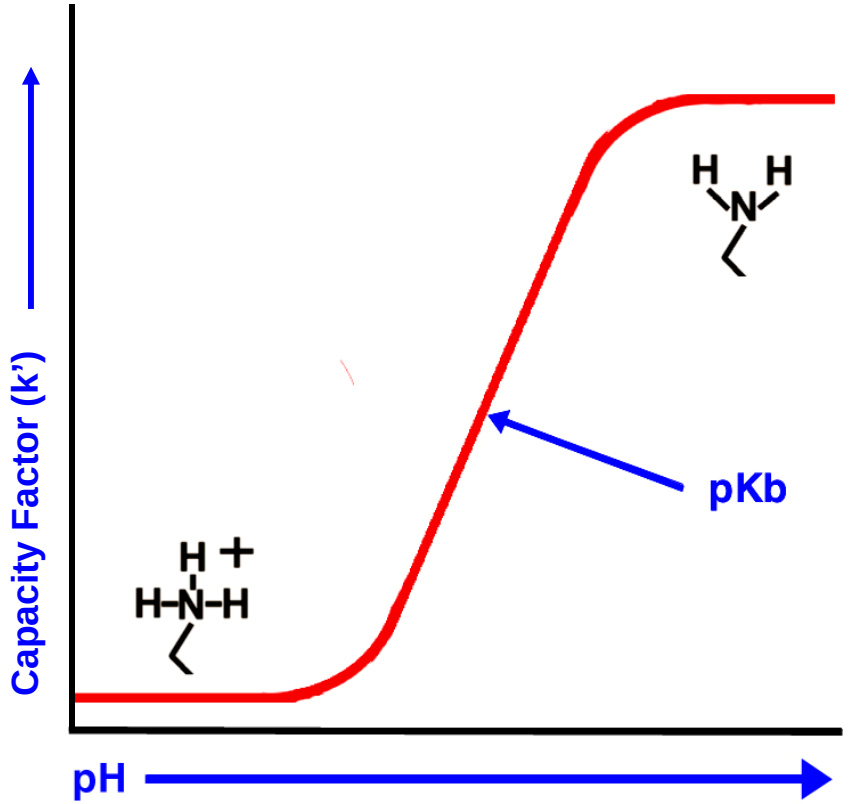
The ionization state of a molecule will be determined by the pH of the mobile phase. Therefore, **mobile phase pH will dictate retention behavior of analytes with ionizable functional groups**.

Effect of pH on Analyte Ionization

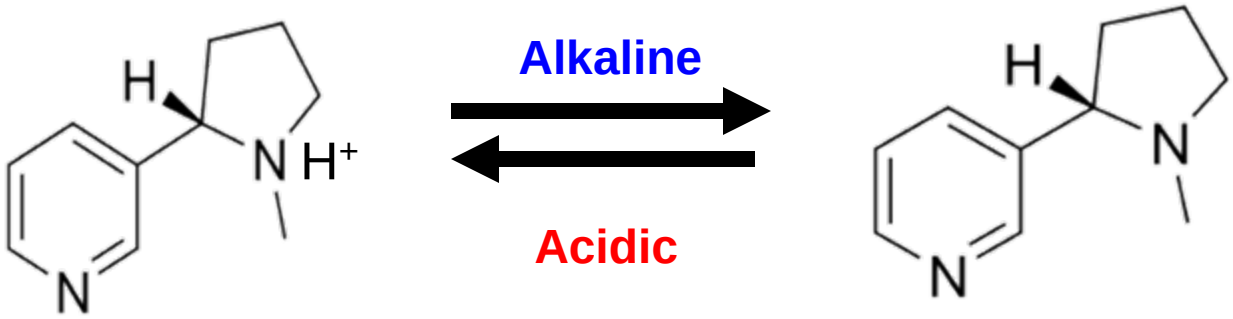
Acidic Compounds:

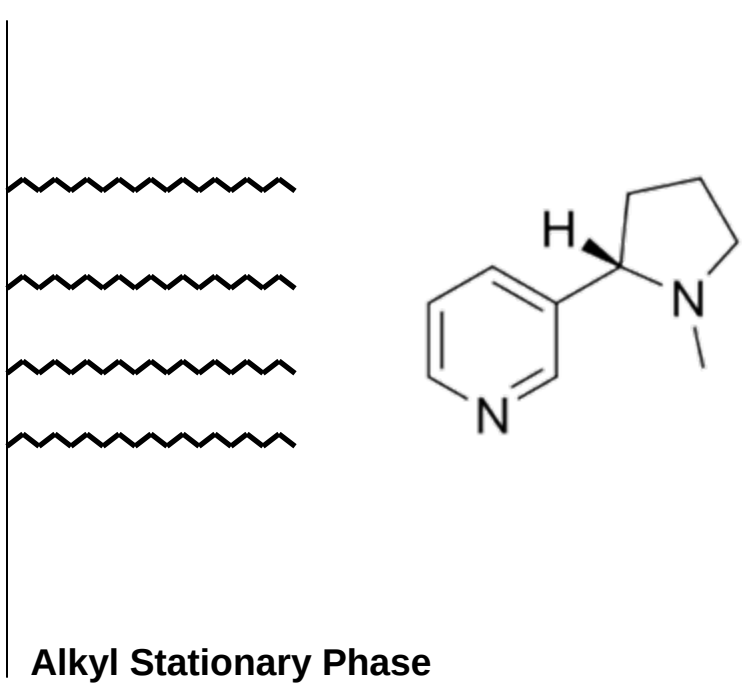


Basic Compounds:

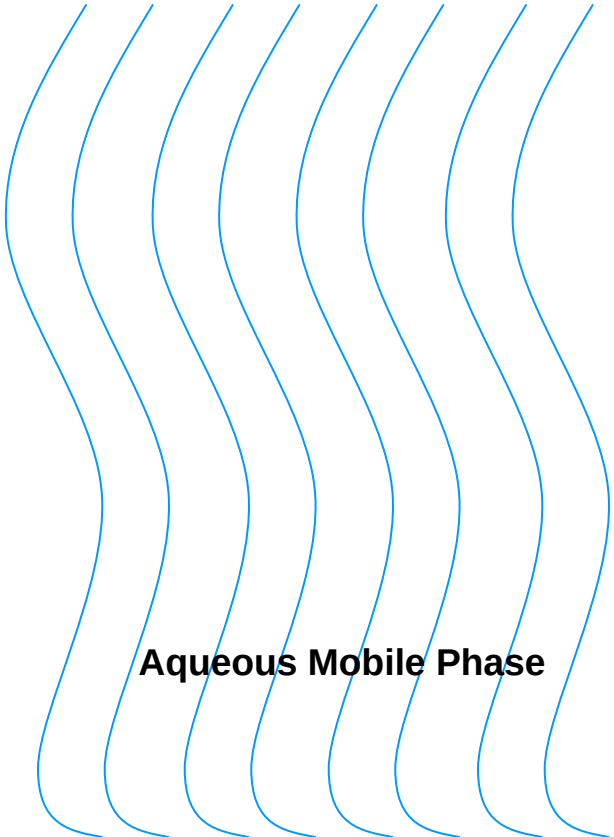


Effect of pH on Analyte Ionization



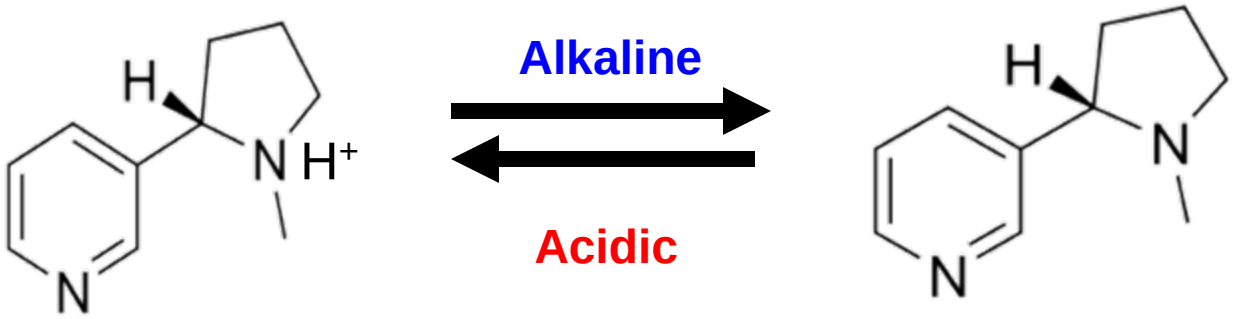


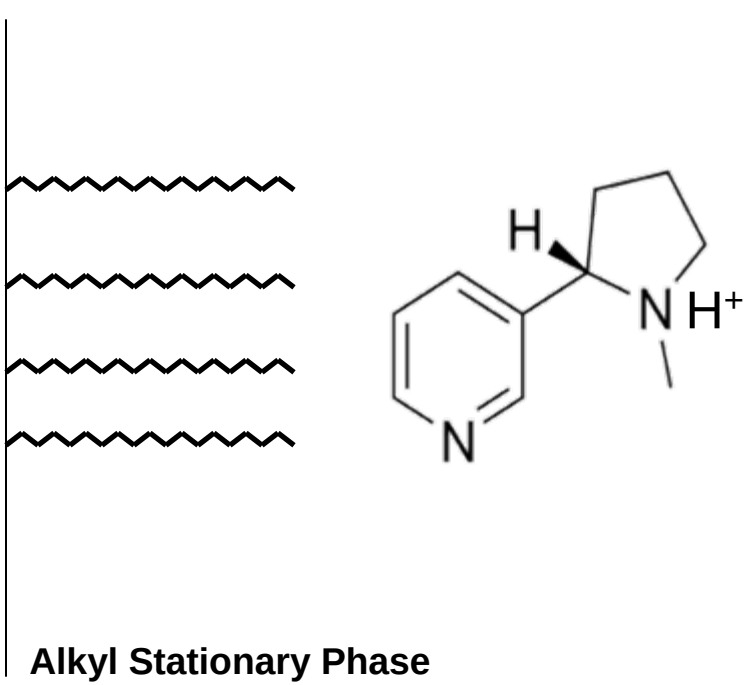
Alkyl Stationary Phase



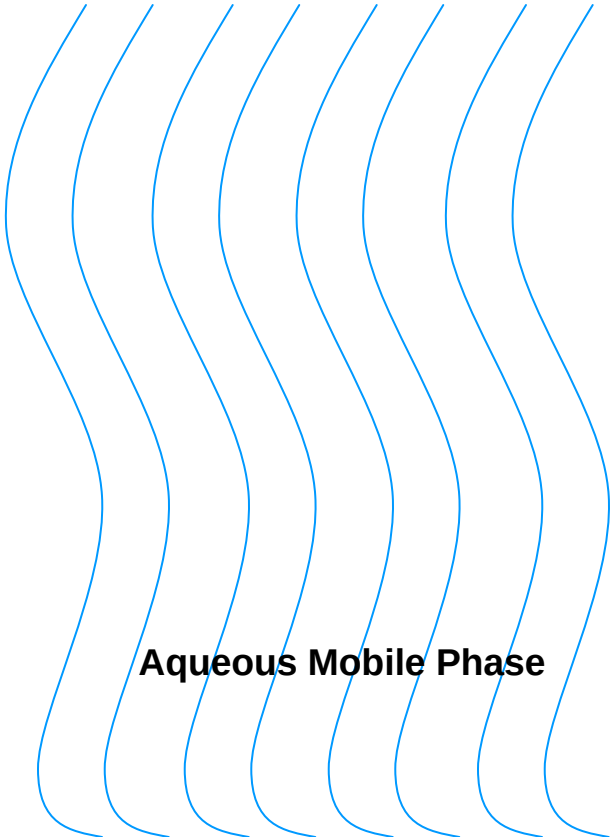
Aqueous Mobile Phase

Effect of pH on Analyte Ionization





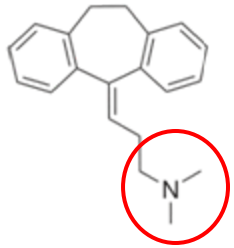
Alkyl Stationary Phase



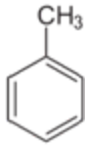
Aqueous Mobile Phase

Effect of pH on Analyte Retention

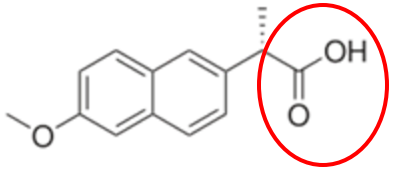
Amitriptyline (pKa 9.4) = (B)ase

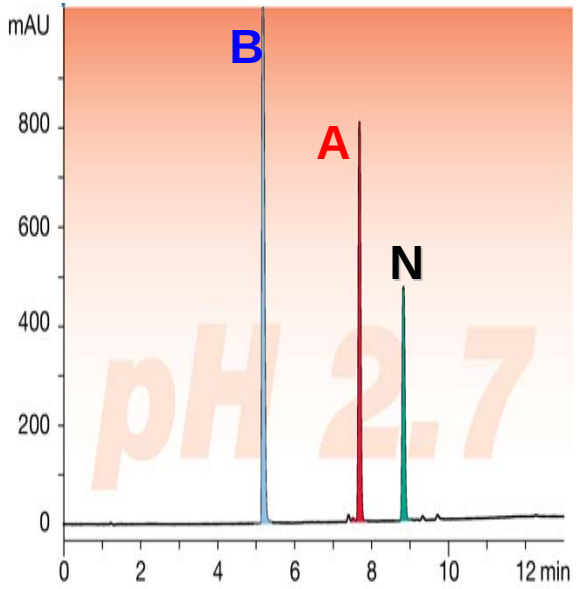


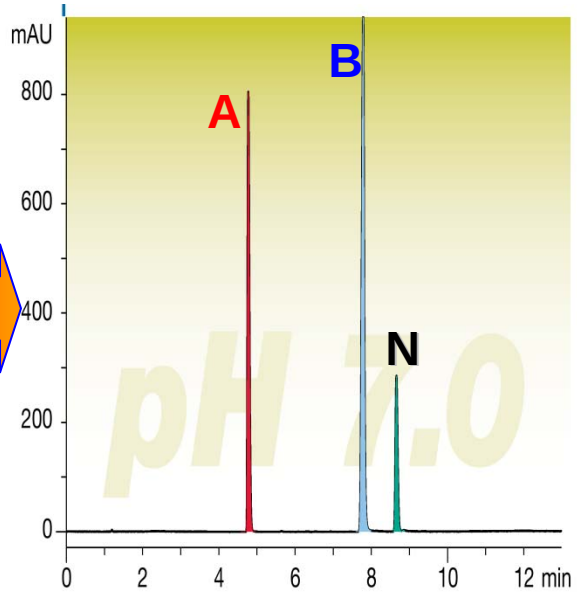
Toluene = (N)eutral

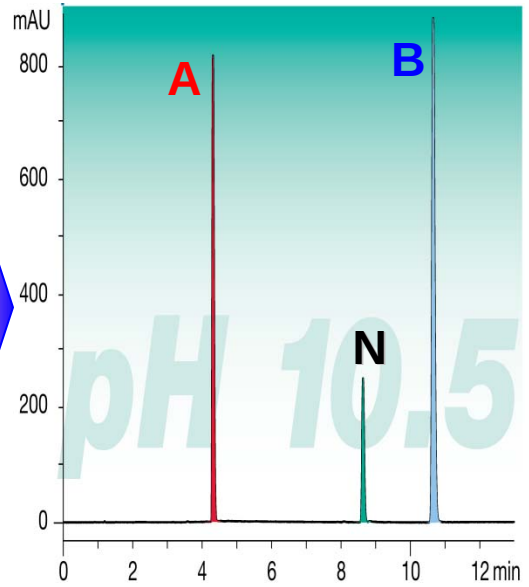


Naproxen (pKa 4.5) = (A)cid

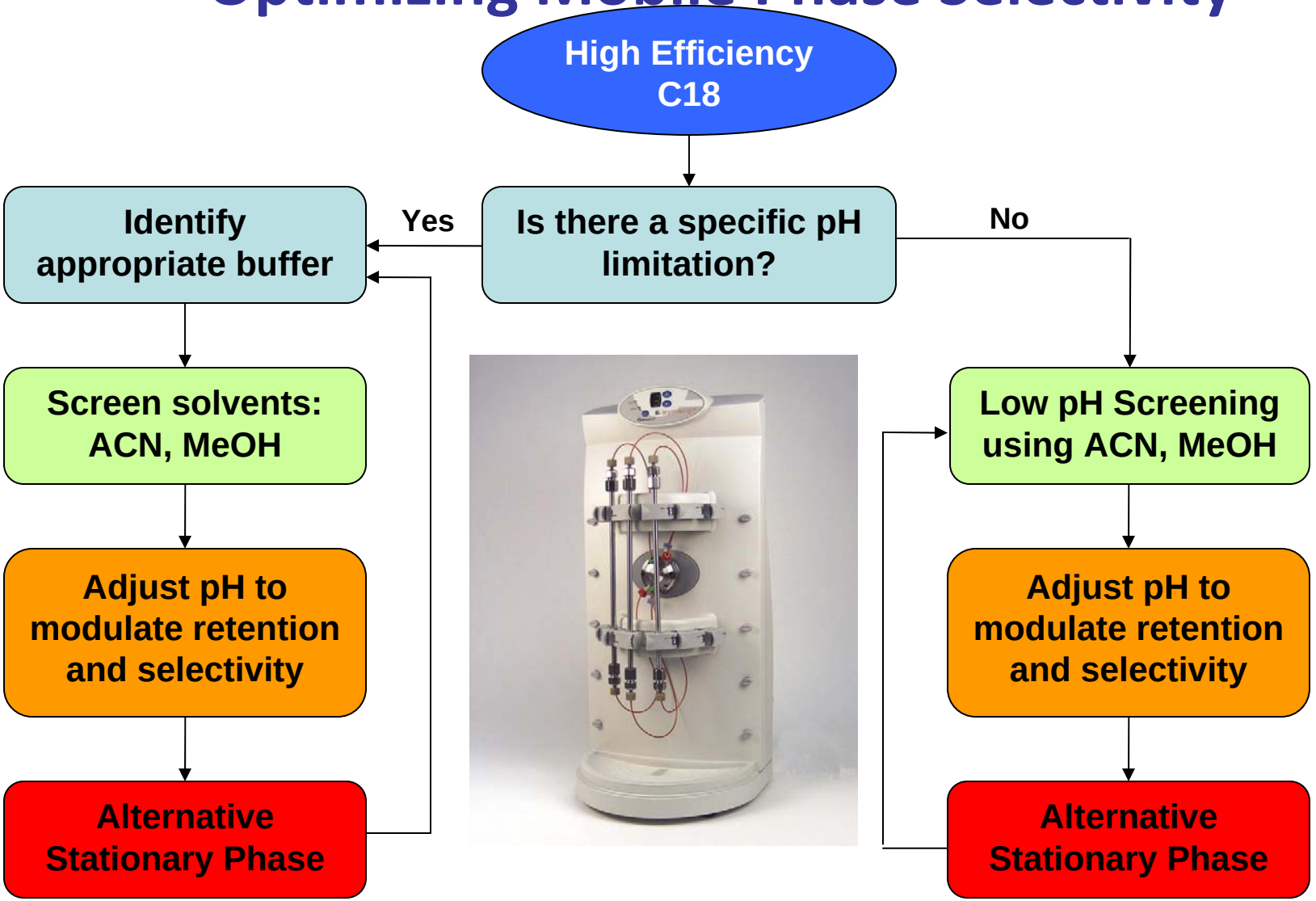








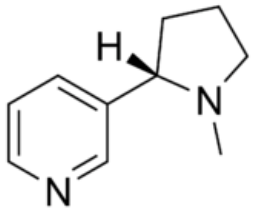
Optimizing Mobile Phase Selectivity



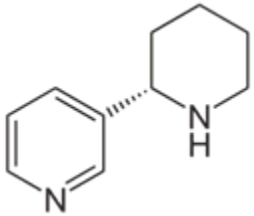
Method Development Exercise 3: Optimizing Mobile Phase and Stationary Phase

Optimizing Mobile and Stationary Phase

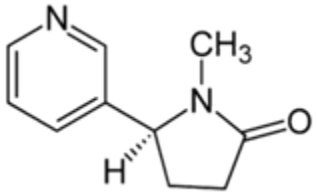
Analysis of **nicotine** and metabolites:



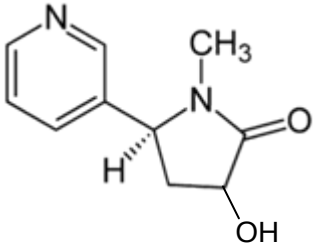
Nicotine
(pKa ~8)



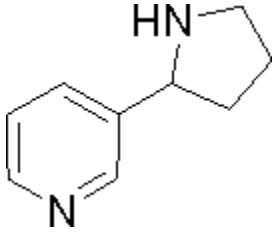
Anabasine



Cotinine

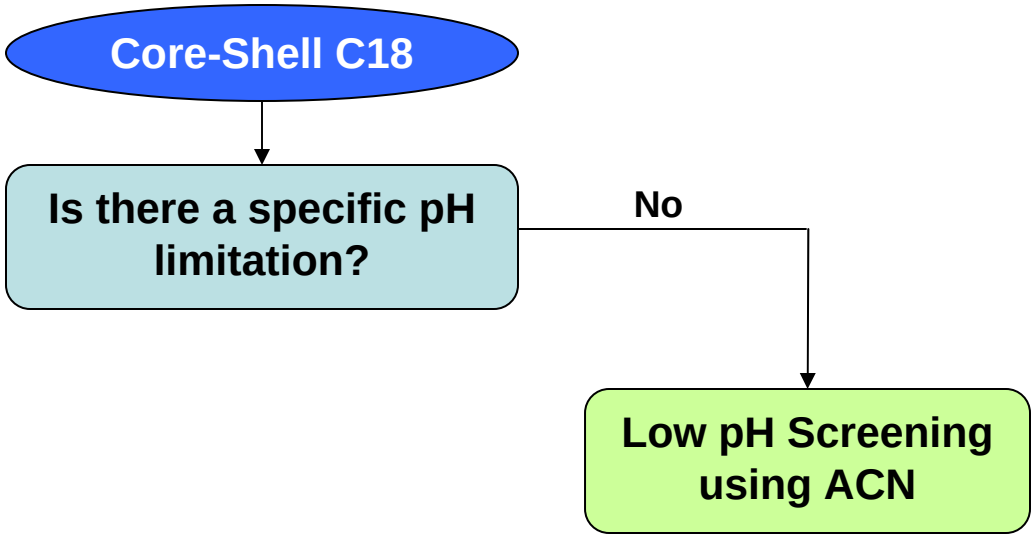


Hydroxycotinine



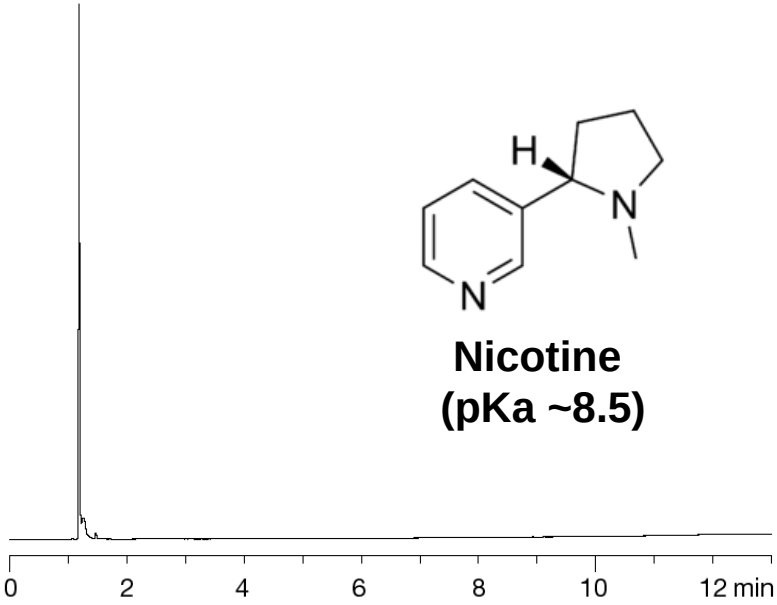
Nornicotine

Optimizing Mobile and Stationary Phase

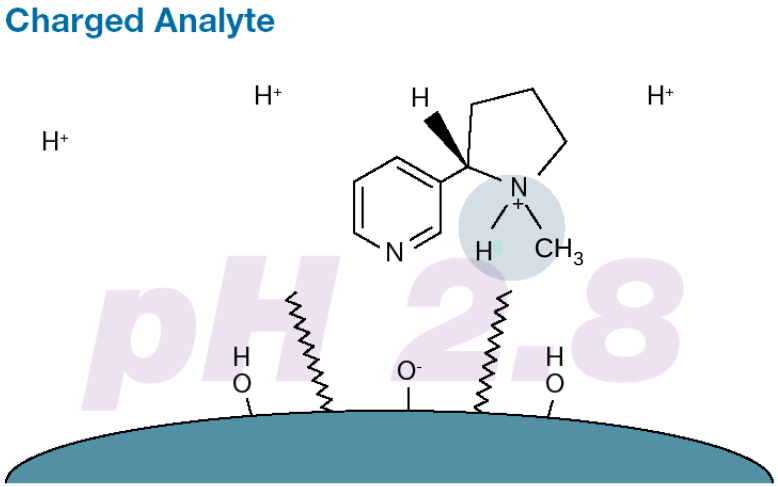


Optimizing Mobile and Stationary Phase

Mobile phase: A = 0.1% Formic acid in water
B = 0.1% Formic acid in acetonitrile
Gradient 5% to 95% in 10 min
Flow rate: 1.5 mL/min
Detection: 254 nm
Components: Nicotine (0.1%), 1 µL injection

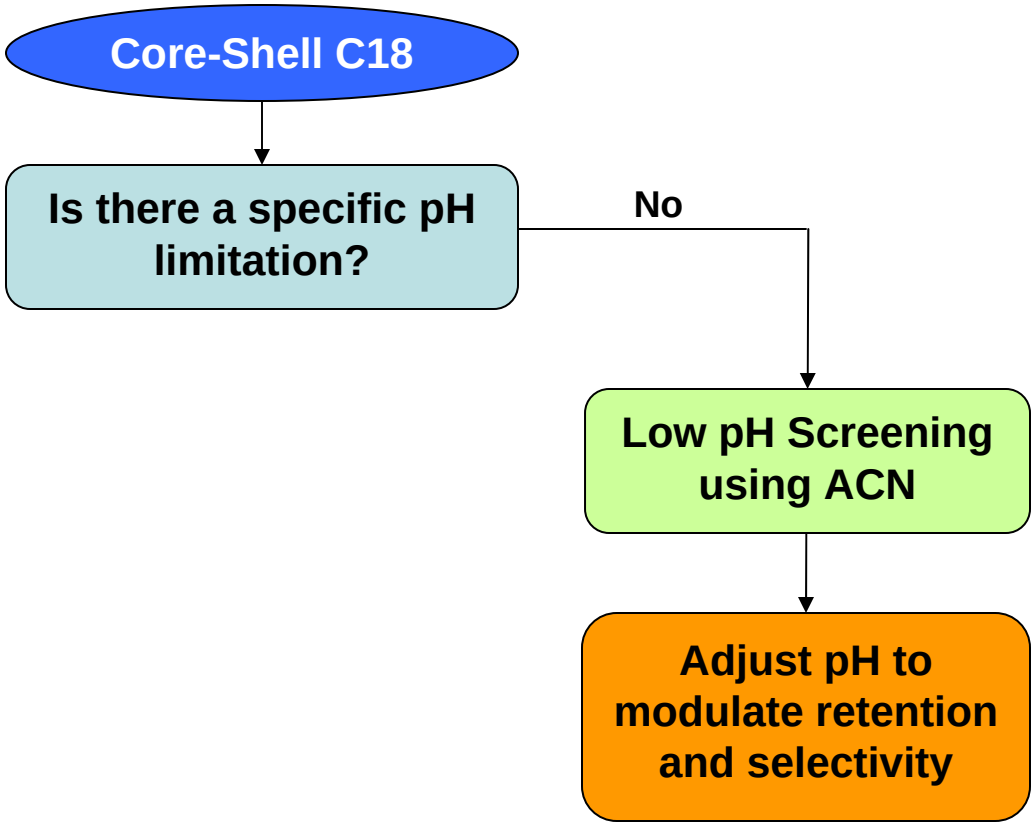


Nicotine
(pKa ~8.5)



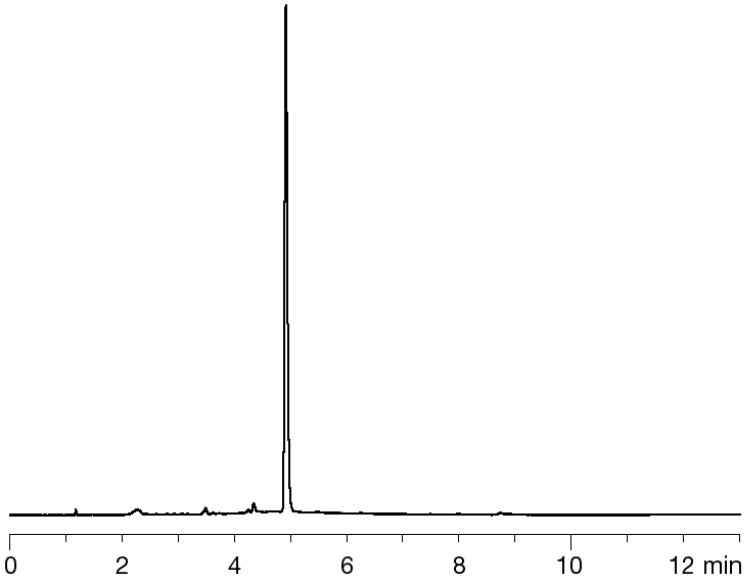
Poor retention at low pH due to ionization

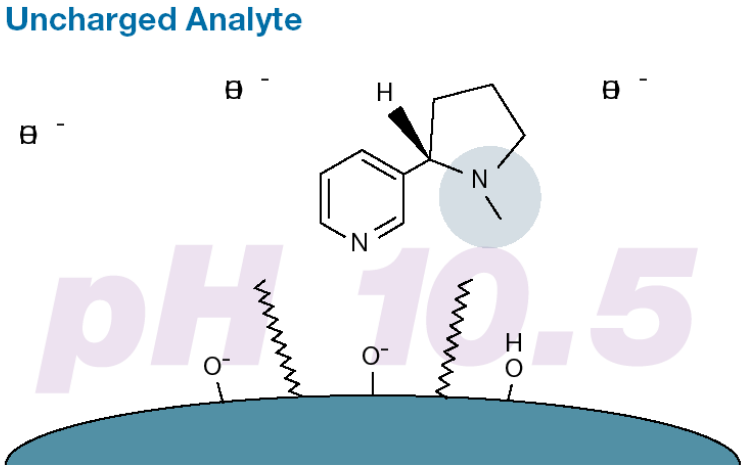
Optimizing Mobile and Stationary Phase



Optimizing Mobile and Stationary Phase

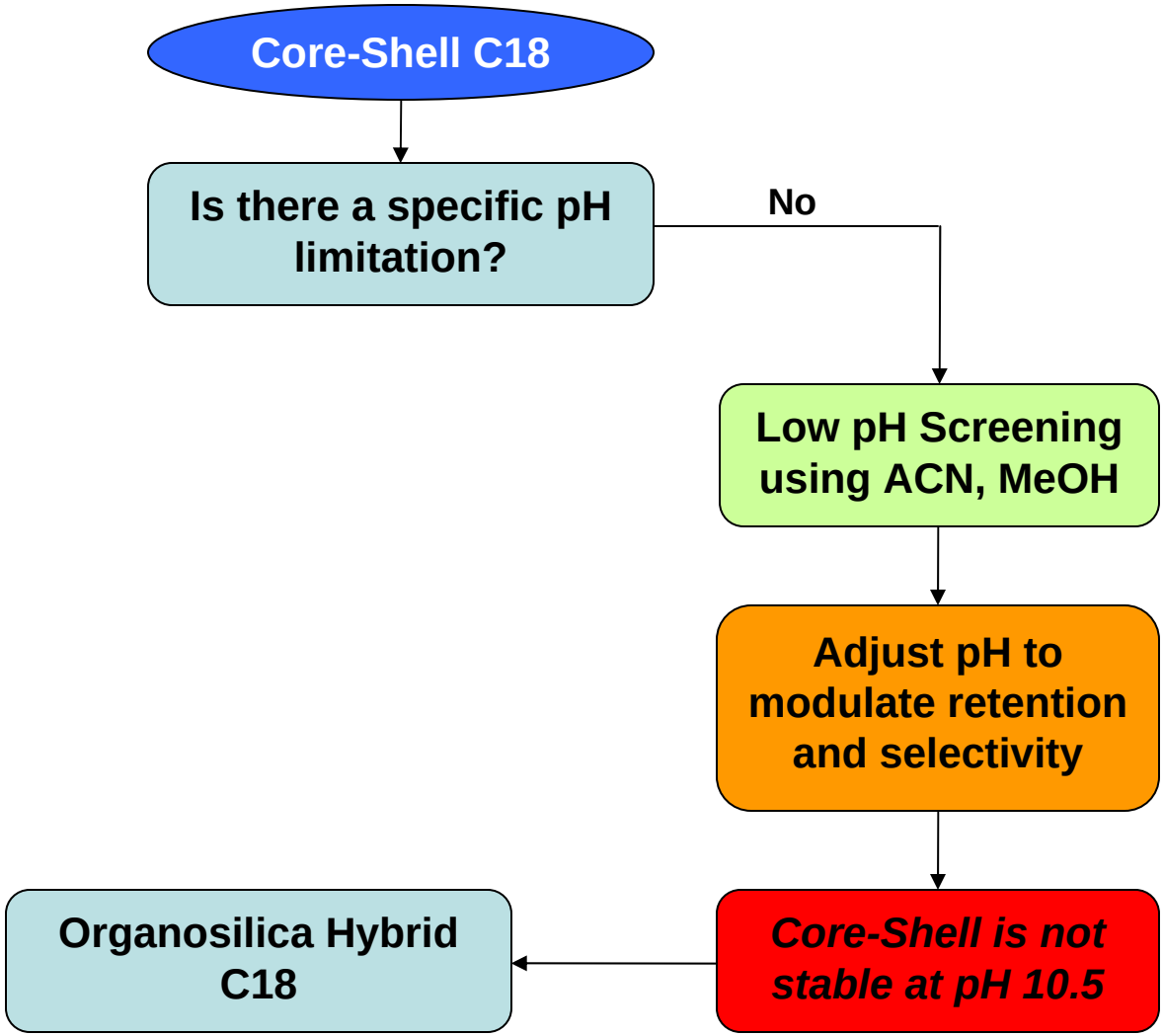
Mobile phase: A = 10mM ammonium bicarbonate pH 10.5
B = acetonitrile
Gradient 5% to 95% in 10 min
Flow rate: 1.5 mL/min
Detection: 254 nm
Components: Nicotine (0.1%), 1 µL injection





Improved retention at high pH

Optimizing Mobile and Stationary Phase



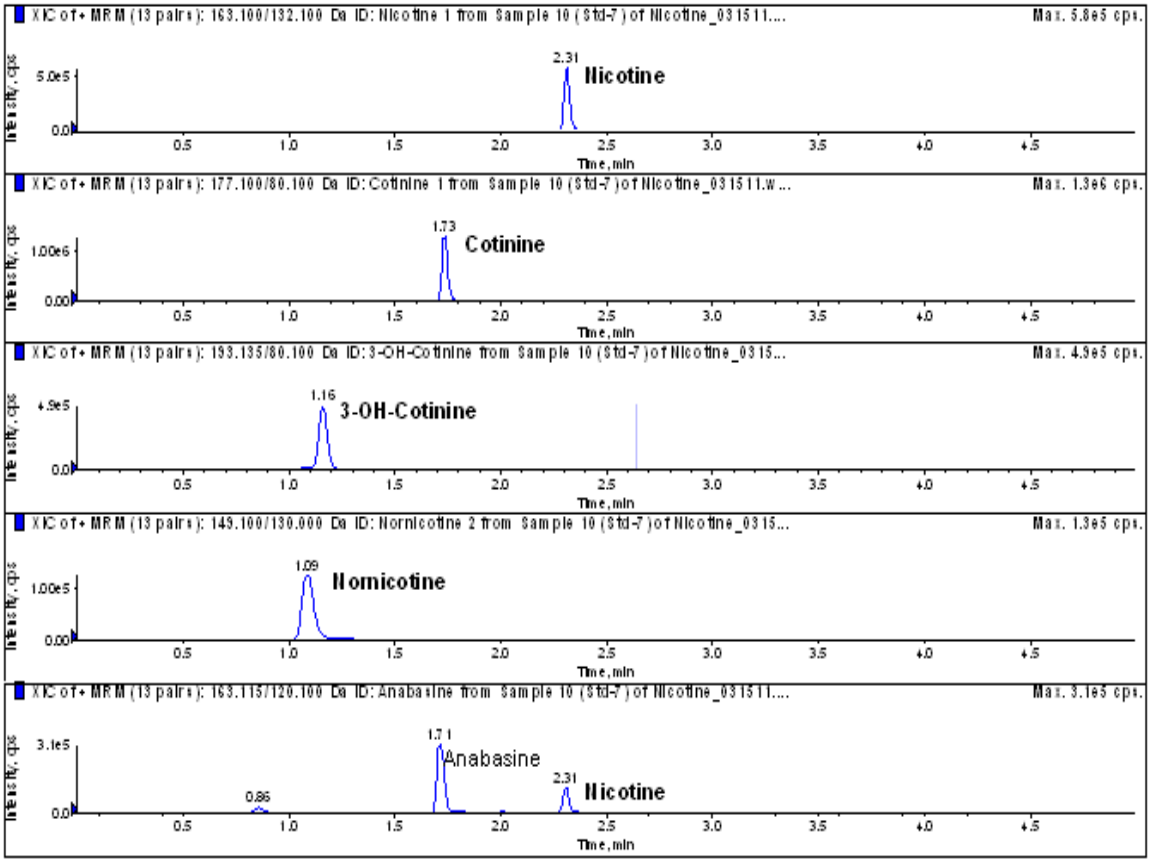
High pH using Organosilica Hybrid

Column: Gemini-NX C18, 3 µm 50 x 2.0 mm
Mobile Phase: A: 20 mM Ammonium Bicarbonate
B: 100% Acetonitrile

Gradient:

Time (min)	B (%)
0.00	10
3.00	75
3.10	10
5.00	10

Flow Rate: 0.5 mL/min
Injection Volume: 10 µL
Temperature: 25 °C



Gradient Analysis

Gradient Analysis

The purpose of gradient elution is to separate in the same chromatography run, compounds which differ widely in hydrophobicity, and which would not elute in a reasonable amount of time using isocratic elution.

Gradient elution:

- **Sharpens peaks** for better quantitation
- **Improves the detection** of small, later eluting peaks
- Is useful to **clean and regenerate the column** after each run
- Is useful for **scouting** analytical conditions

Columns:	3 µm C18(2) 50x4.6 mm
Mobile phase:	70:30 0.1% TFA in Water : 0.1% TFA in Acetonitrile
Flow rate:	2.0 mL/min
Components:	1. Thiourea (t_0 marker) 2. Caffeine 3. Phenol 4. Acetophenone 5. Dimethylphthalate 6. Butyrophenone 7. Valerophenone

Gradient Analysis

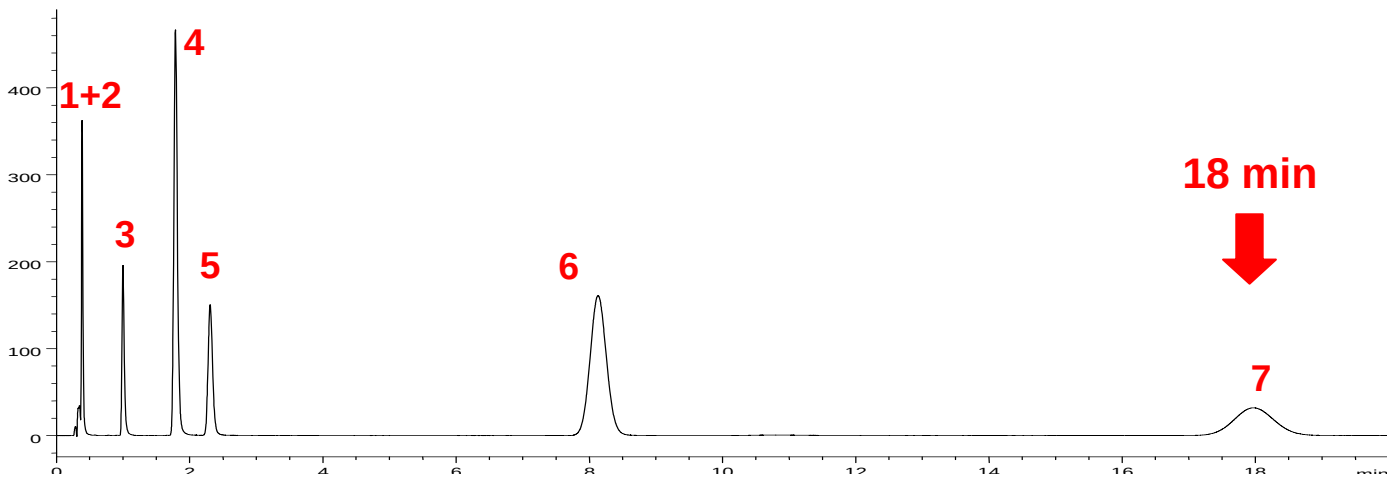
3 µm C18(2) 50x4.6mm

70:30 0.1% TFA in Water :

0.1% TFA in Acetonitrile

2.0 mL/min

1. Thiourea (t_0 marker)
2. Caffeine
3. Phenol
4. Acetophenone
5. Dimethylphthalate
6. Butyrophenone
7. Valerophenone



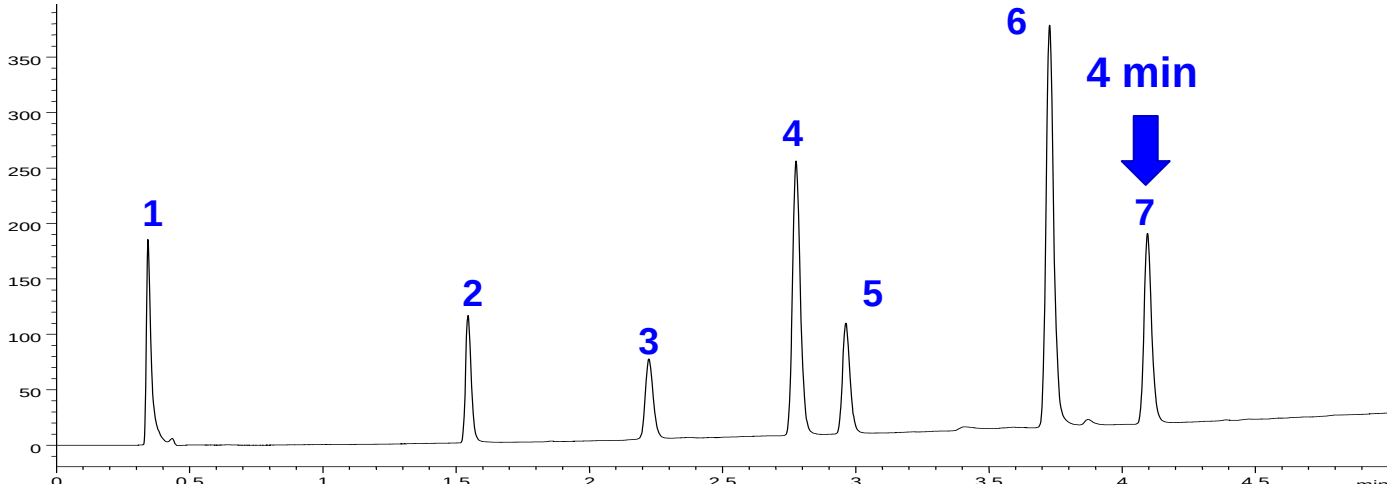
3 µm C18(2) 50x4.6mm

A = 0.1% TFA in Water

B = 0.1% TFA in Acetonitrile

5 to 100% B in 5 min

2.0 mL/min



Gradient Analysis

The **gradient slope** is analogous to solvent strength in isocratic elution.

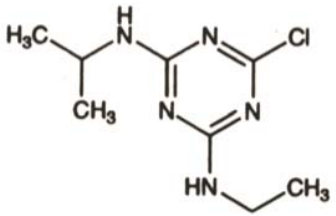
Isocratic Solvent Strength:		Gradient Slope:
Increasing the solvent strength reduces analysis time but also reduces resolution.	→	Increasing the gradient slope reduces analysis time but also reduces resolution.
Decreasing the solvent strength increases resolution at the cost of increased analysis time.	→	Decreasing the gradient slope increases the resolution at the cost of increased analysis time.
Solvent strength sometimes affects selectivity	→	Gradient slope sometimes affects selectivity

The goal of gradient elution is to optimize resolution while minimizing analysis time.

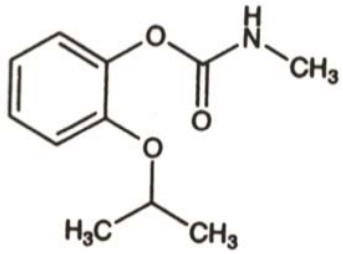
Gradient Analysis

Example: Five herbicides

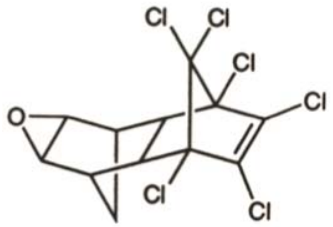
Column used: C18 150 x 4.6mm



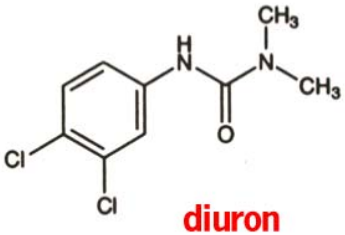
atrazine



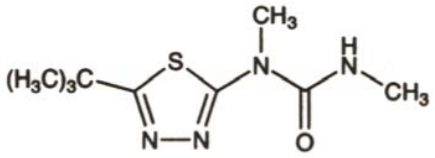
baygon



dieldrin



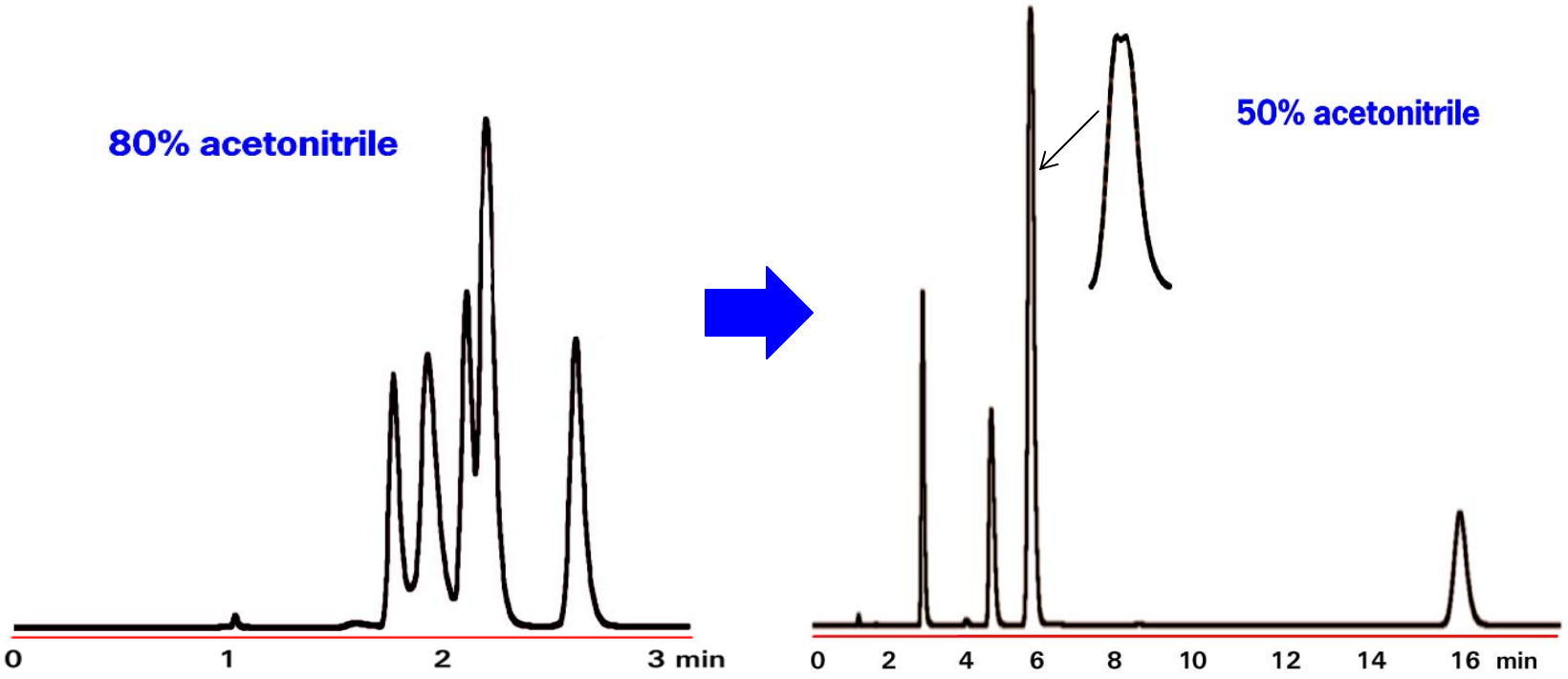
diuron



tebuthiuron

Gradient Analysis

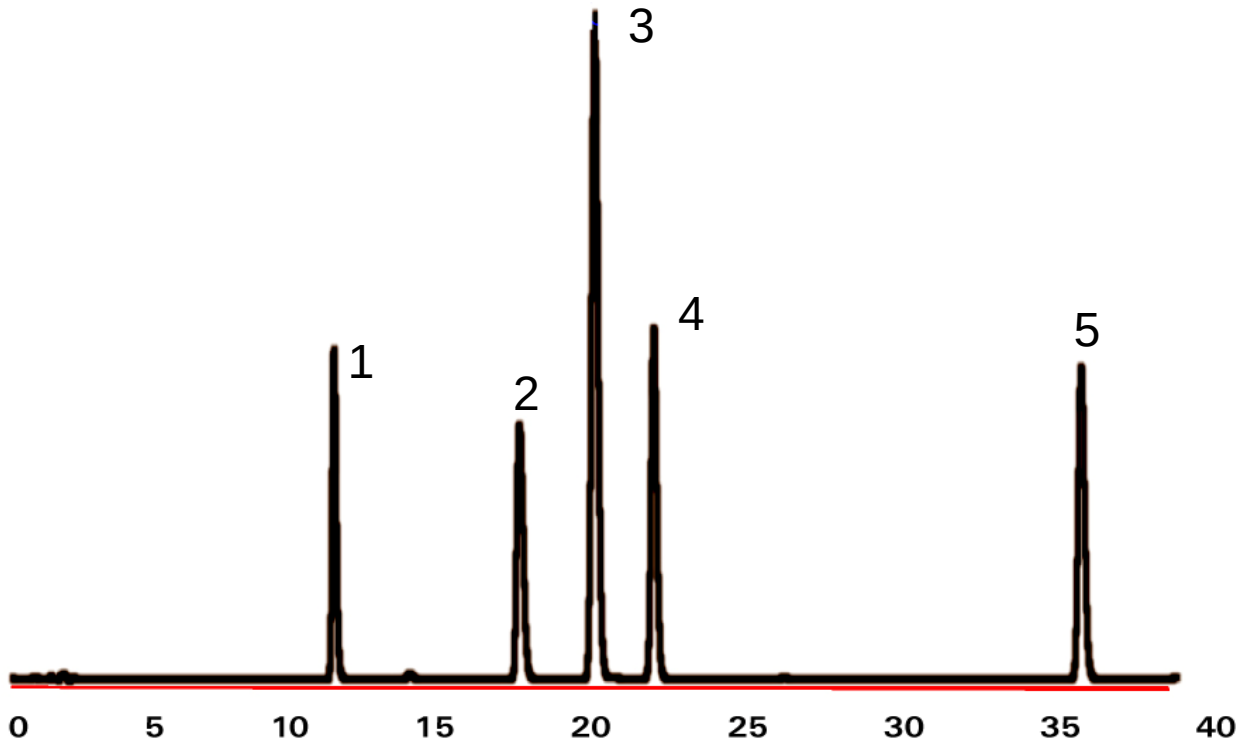
Five herbicides in isocratic elution mode:



Effect of Gradient Rate on Retention

Gradient slope: 1% / minute

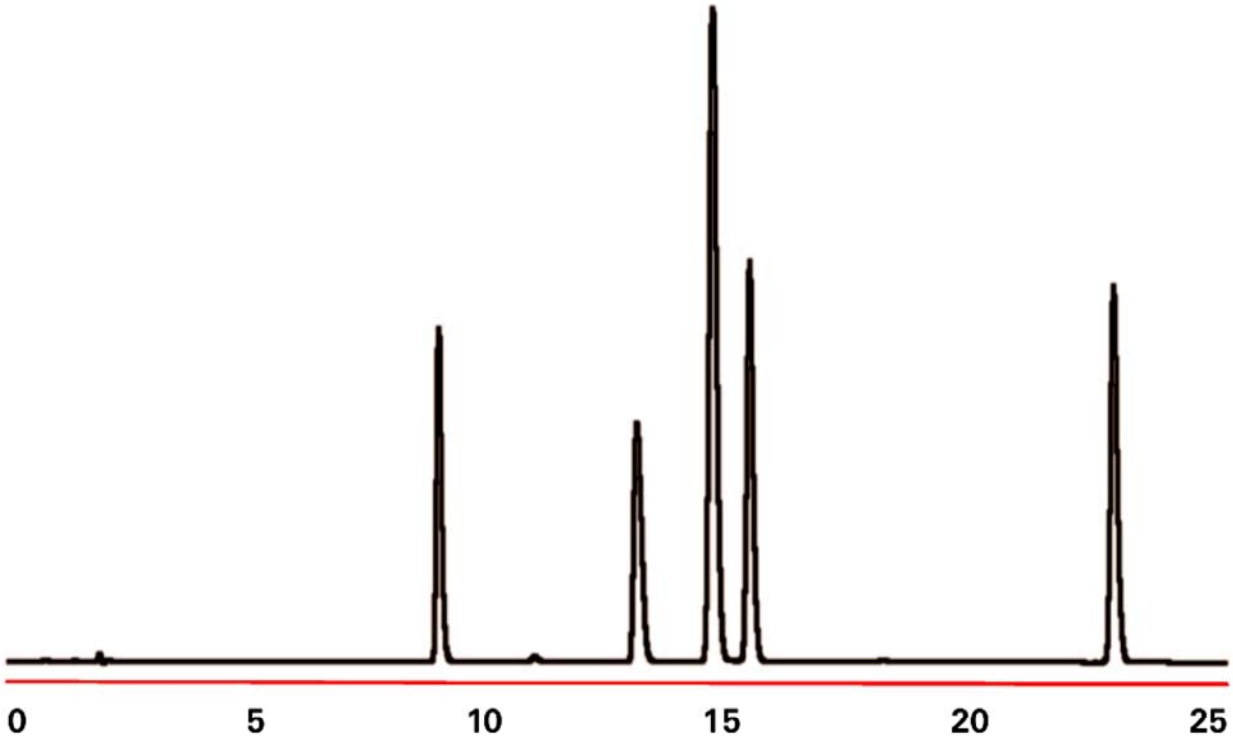
20 - 80% ACN over 60 minutes



Effect of Gradient Rate on Retention

Gradient slope: 2% / minute

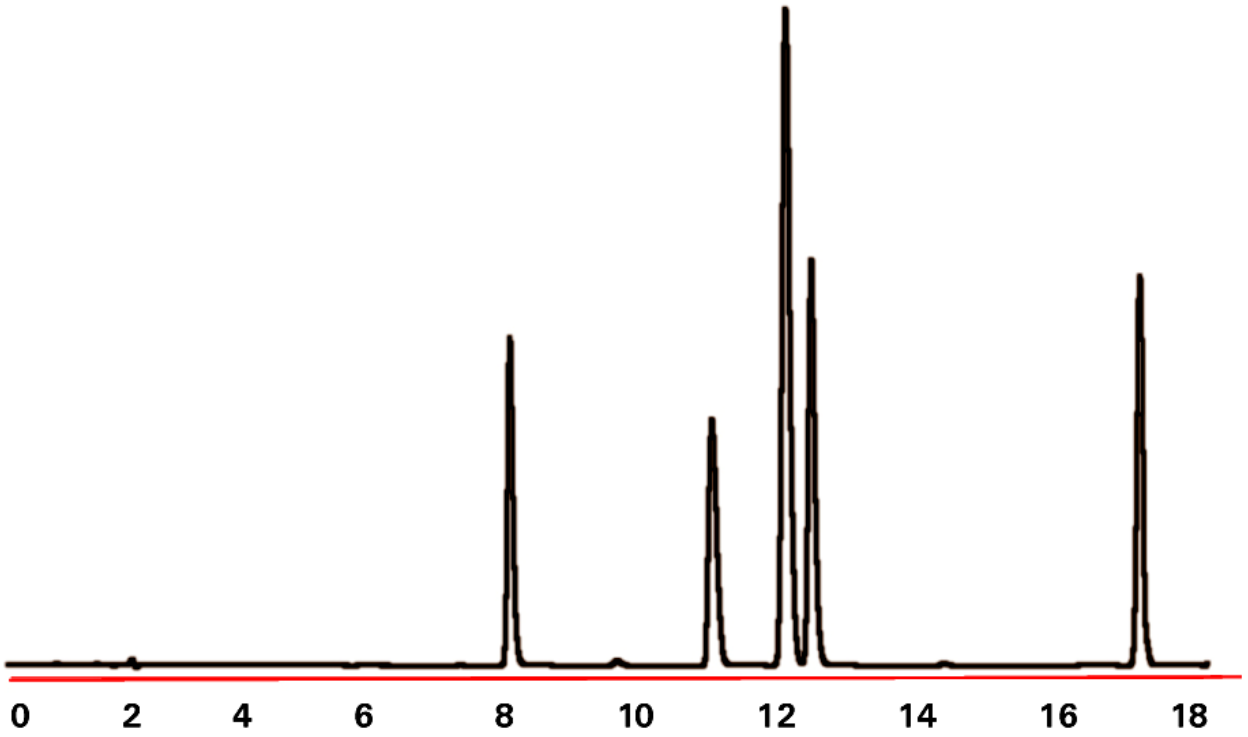
20 - 80% ACN over 30 minutes



Effect of Gradient Rate on Retention

Gradient slope: 3% / minute

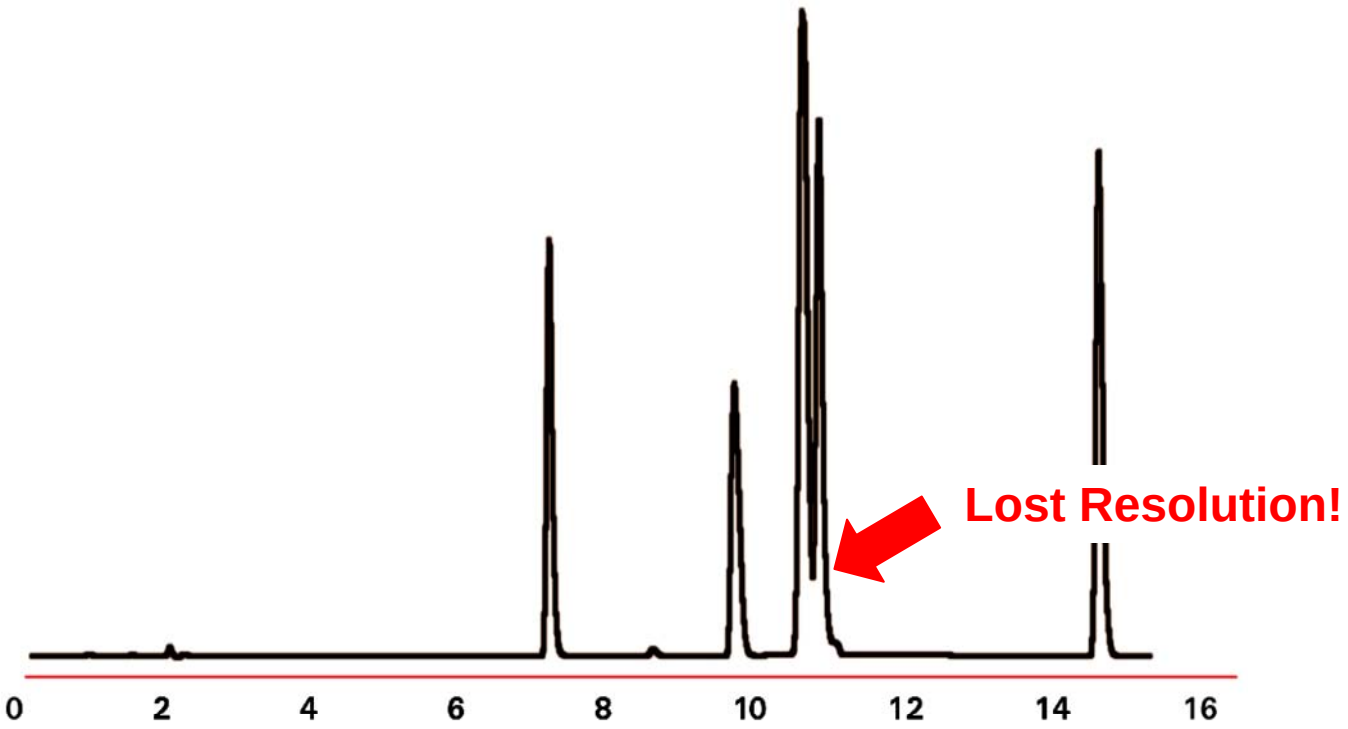
20 - 80% ACN over 20 minutes



Effect of Gradient Rate on Retention

Gradient slope: 4% / minute

20 - 80% ACN over 15 minutes

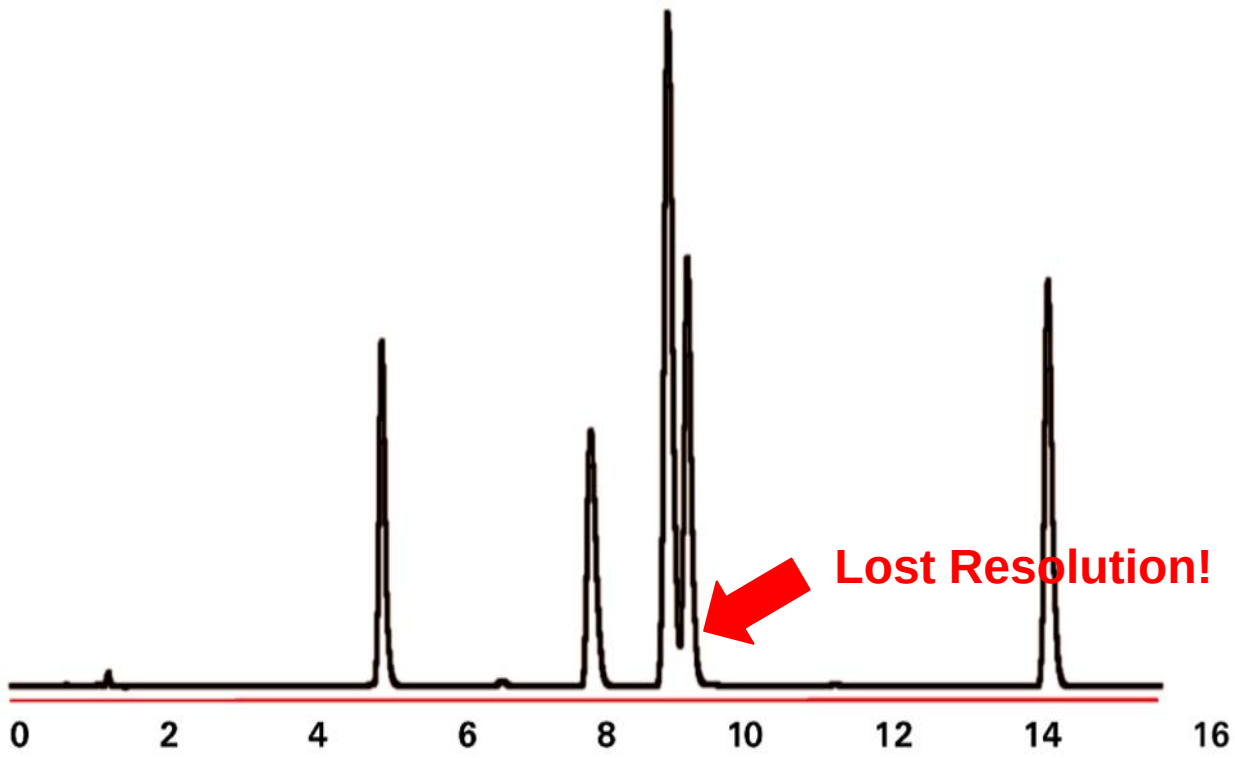


***Increasing the gradient slope will decrease overall retention and also decrease resolution**

Effect of Starting %Organic

Gradient slope: 3% / minute; Initial Strong Solvent = 30%

30 - 90% ACN over 20 minutes



***Increasing the amount of starting strong solvent will decrease overall retention and resolution**

Gradient Method Summary

1. Begin with **"scouting" gradient** to see analyte elution times:
   - 5-95% organic over X min (1 min per cm of column length)
   - 150x4.6 mm = 5-95% B over 15 min

2. Make adjustments to starting % organic to **accommodate early-eluting** components
   - isocratic hold at 3% organic for x min

3. **Adjust gradient slope** to optimize resolution or critical pairs
   - Shallower to improver R_s (5-95% B over 20 min)
   - Steeper if you have excess R_s (5-95% B over 12 min)

4. Optimize ending % organic for **clean-up**
   - Stop gradient at 65% B

5. **Adjust starting % organic** to reduce run time (if not limited by polar components)
   - 5-65% B over 18 min
   - 10-70% B over 18 min
   - 15-75% B over 18 min

Effect of Temperature

Temperature in HPLC Methods

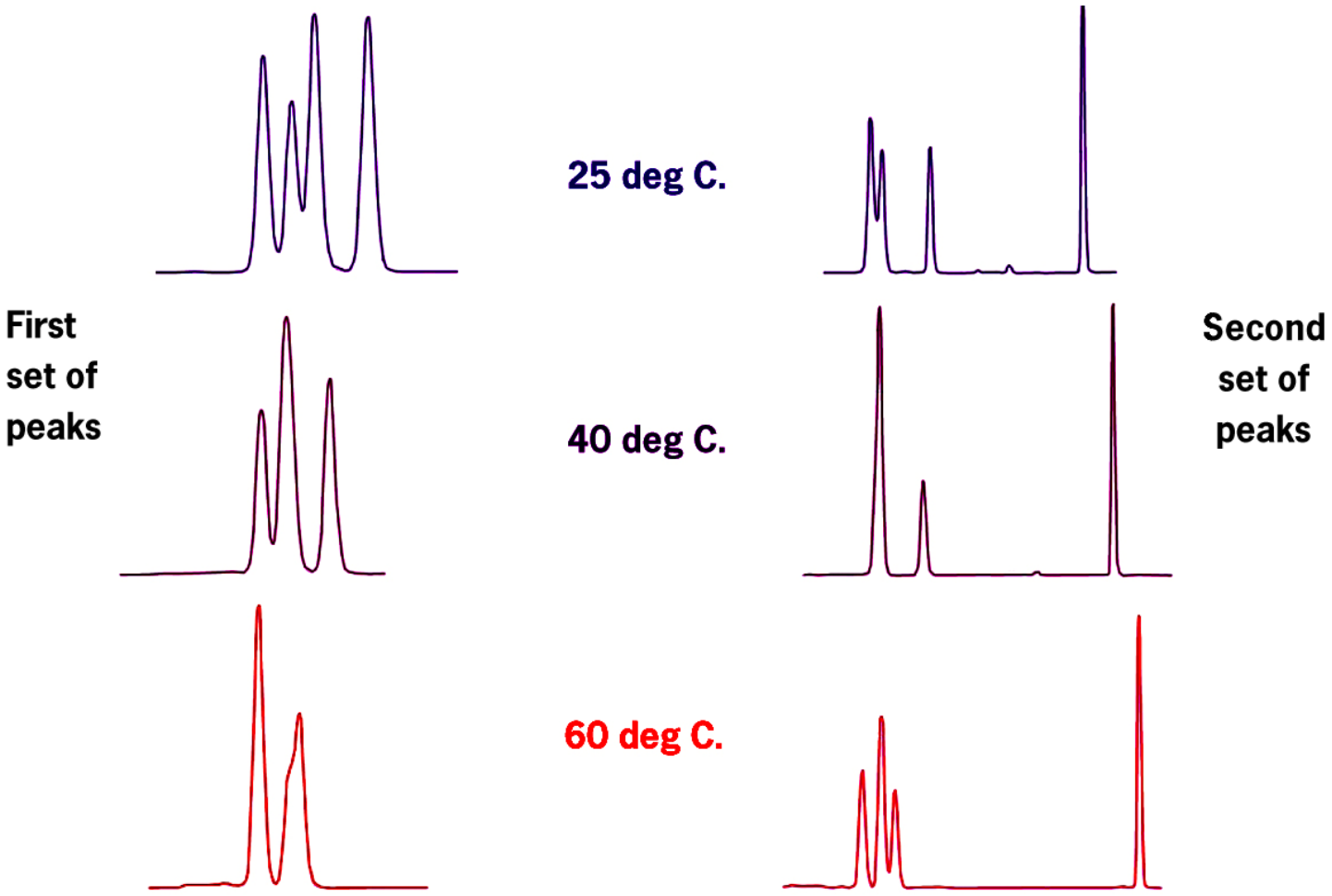
The use of temperature in HPLC method development presents a challenge because it can have unpredictable effects on selectivity.

The use of elevated temperatures will:

1. **Reduce mobile phase viscosity** and back-pressure. This can allow you to operate at higher flow rates, or to use longer columns/smaller particle sizes.
2. **Reduce elution time**.
3. Improve method **reproducibility** (as opposed to operating at room temperature).

However, it is impossible to determine if the use of elevated temperatures will help or hinder a specific separation. For complex separations, improvements in one portion of the chromatogram are almost always accompanied by decreases in another part of the same chromatogram.

Temperature in HPLC Methods



Temperature in HPLC Methods

In our method development work:

1. Initial method development is performed at 30 ºC.
    - Column screening
    - Mobile phase selection and optimization

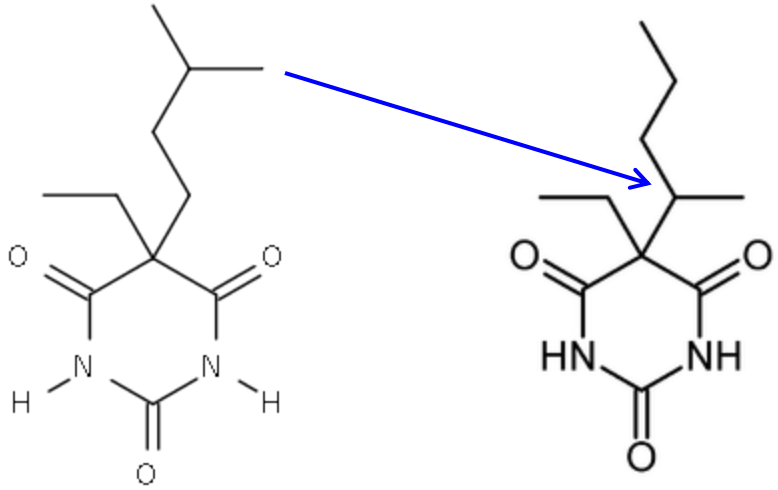
2. Higher temperatures are investigated only when:
    - We need to reduce back-pressure (usually with increasing flow rate or using a longer column length)
    - Unable to achieve required resolution at 30 ºC

Method Development Exercise 3: Gradient Optimization and Phase Screening

Gradient Method Optimization

Barbiturates are CNS depressants, and have been used to induce anaesthesia, and treat anxiety and insomnia, but are also subject to abuse.

The challenge with LC/MS analysis of these compounds is that amobarbital and pentobarbital are isomers with the same mass and must be separated chromatographically.



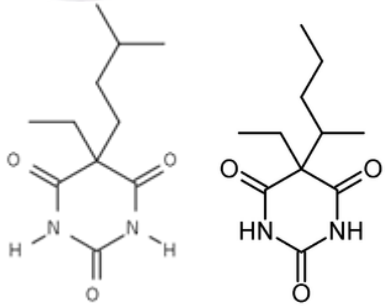
Amobarbital
pKa = 7.8

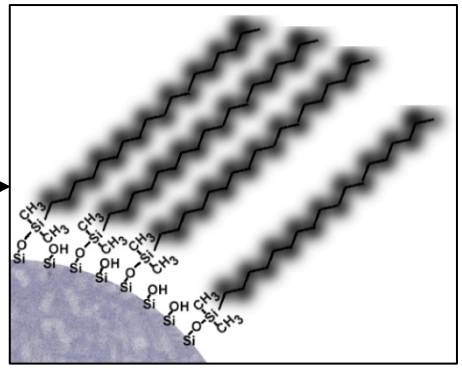
Pentobarbital

Optimize Mobile Phase and Stationary Phase

Basic Sample Info:
No pH restriction
Clean Sample

Goal of separation:
Resolution of
Amo/Pentobarbital





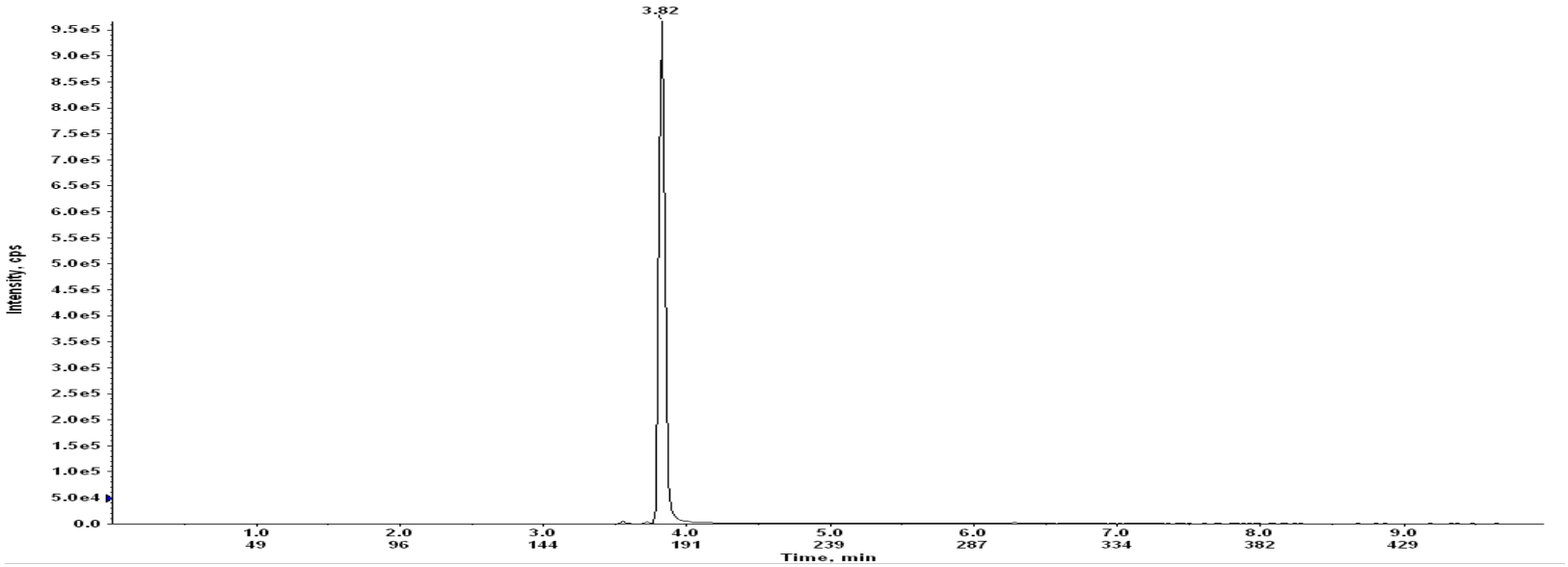
Solvent? → **ACN for low viscosity**

Buffer? → **Neutral (ammonium acetate) for negative ionization**

Scouting Gradient

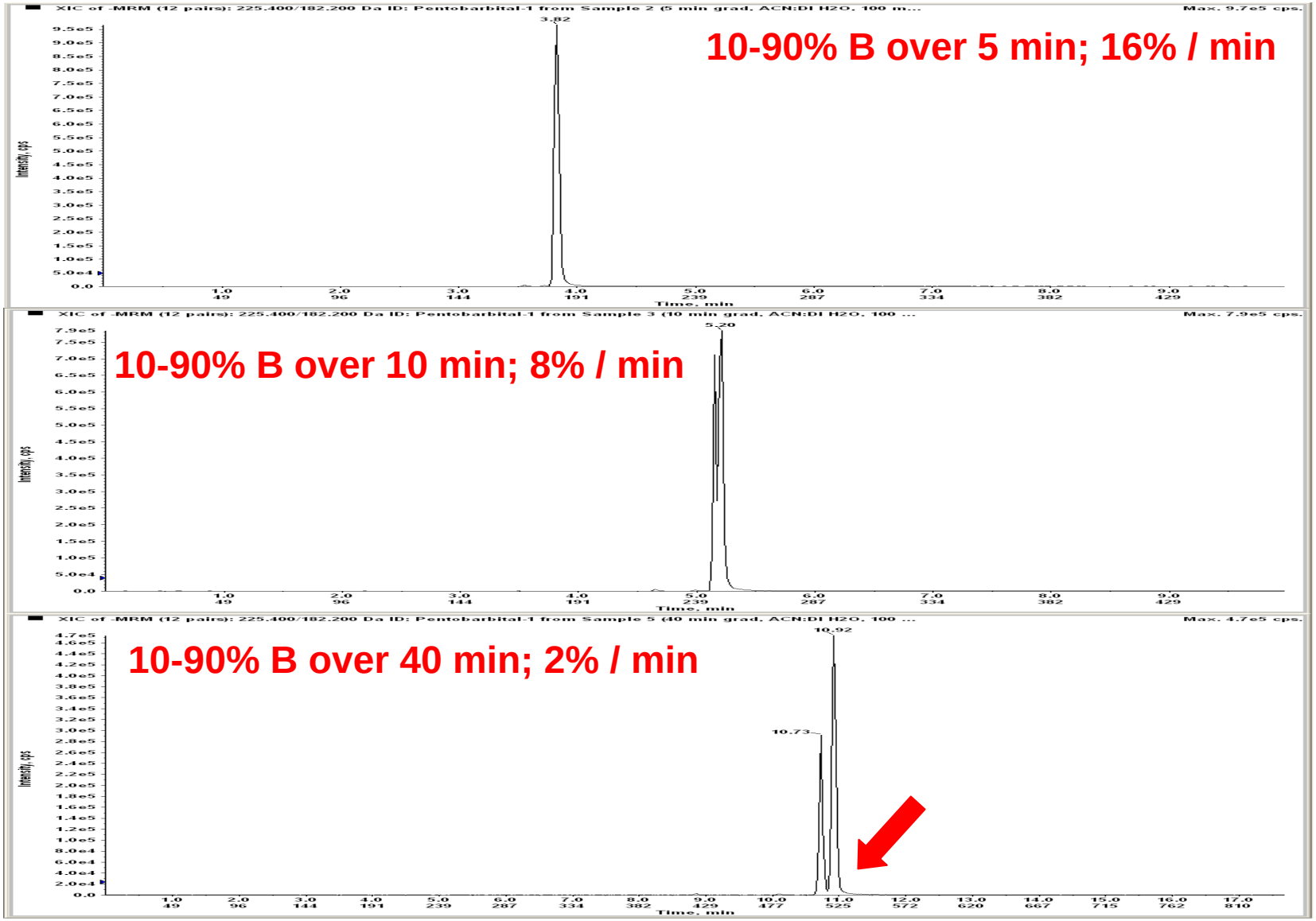
1. Rapid, steep gradient slope to determine general behavior of analytes:

10-90% B over 5min; 16% / min



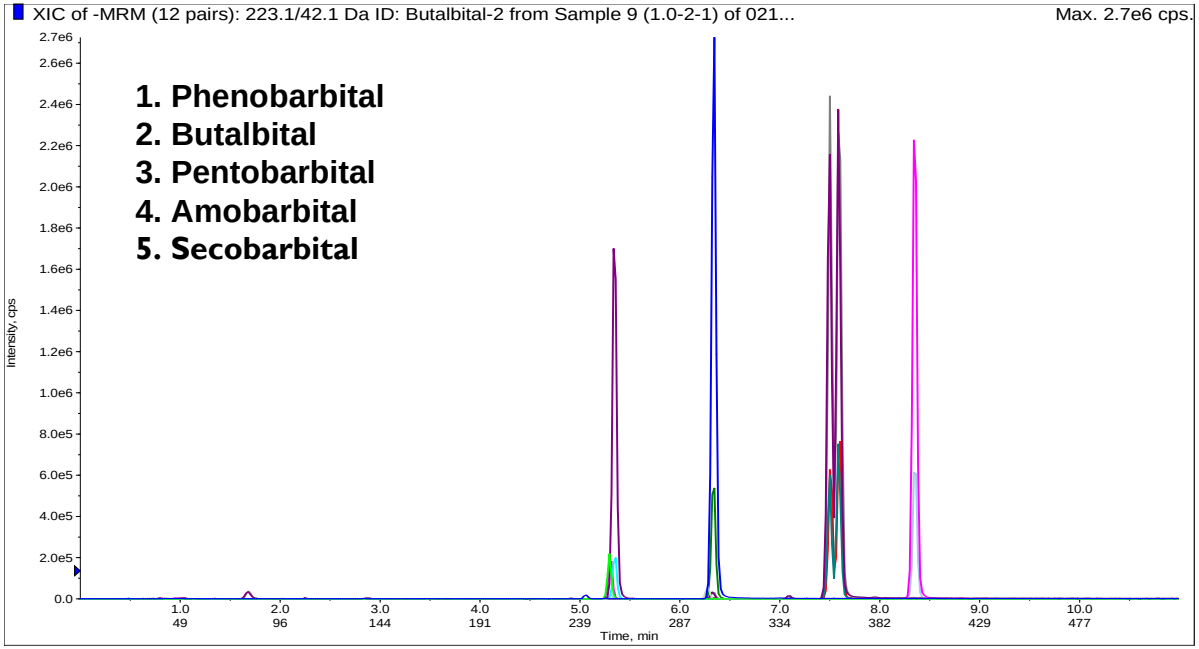
- *Good retention*
- *Good peak shape* → *Reduce gradient slope*
- *No separation*

Gradient Optimization



Final Barbiturate Method

Final Method:



Running conditions

- 2.6 µm Core-Shell C18 100x2.1 mm
- A = 5mM ammonium acetate
- B = Acetonitrile
- 500 µL/min
- 10-45% B over 10 min

- *Reasonable* resolution in ~10 minute run time
- Need to balance adequate resolution with sample throughput

End of Part III