

Reversed Phase: Mobile Phase Considerations

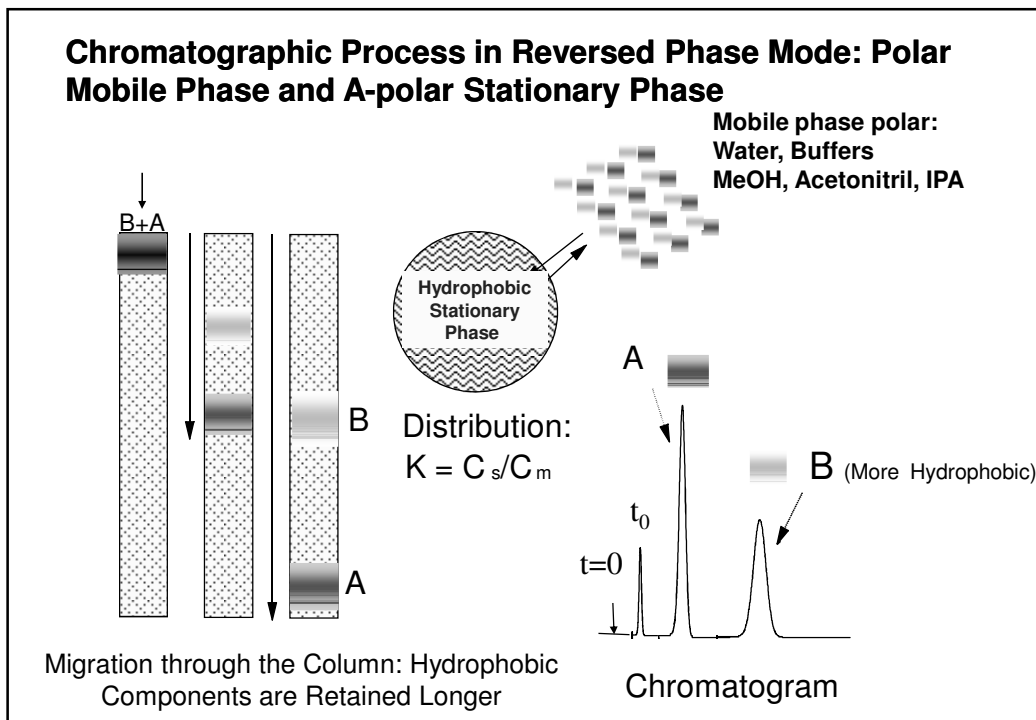
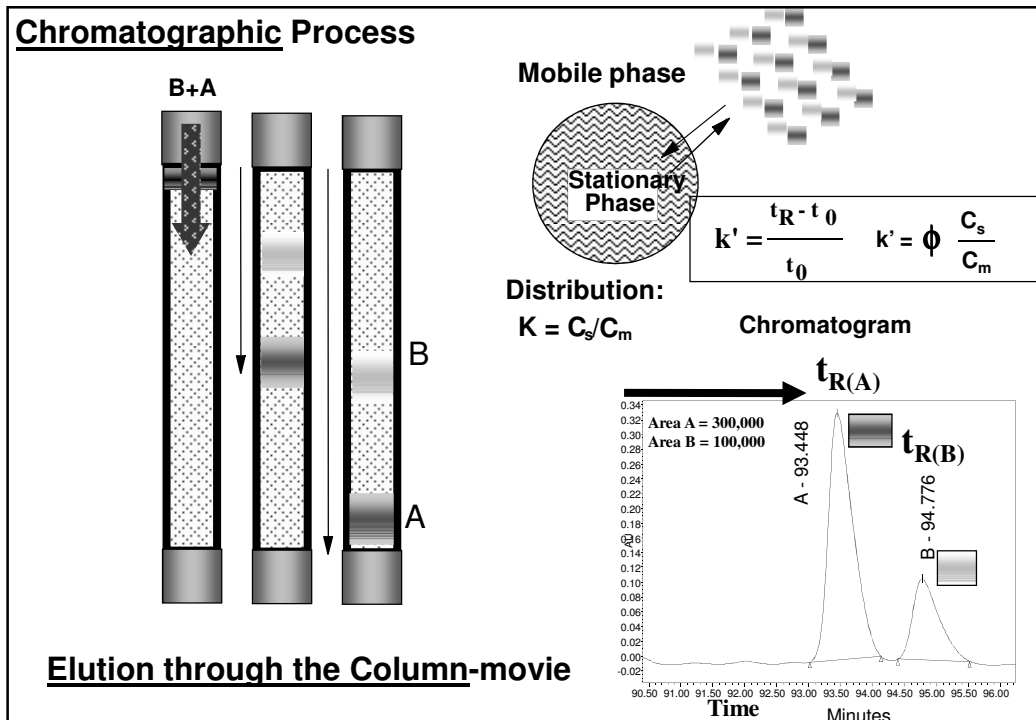
Dr. Shulamit Levin, Israel

www.forumsci.co.il/HPLC

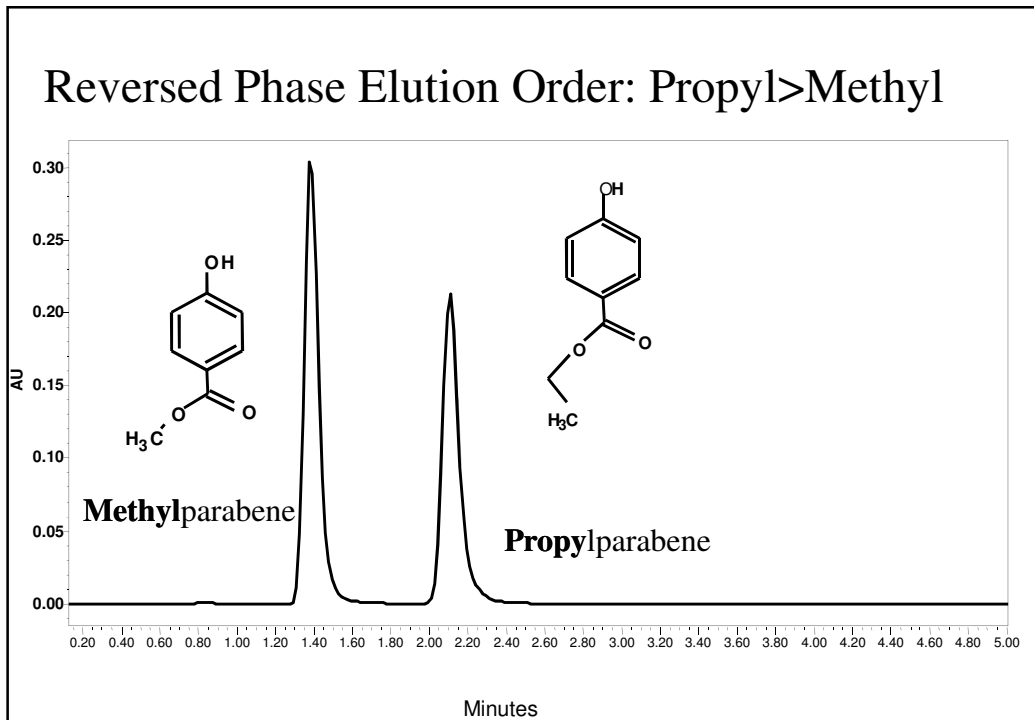
Example of a Reversed Phase Method with UV Detection

HPLC	Assay determination
Column & Packing:	Hypersil ODS, 50 x 4.6 mm, 3 μ m, P.N. 30103-054630, Thermo
Buffer	2.0 ml of phosphoric acid (85%) in 1L water, adjust to pH=3.0 with sodium hydroxide
Eluent	70 : 30, Buffer : Acetonitrile (v : v)
Sample volume:	100 μ L
Flow Rate:	3.0 mL/min
Detector:	220 nm
Column temperature:	15°C
Autosampler temperature	10°C
Diluent	30 : 70, Acetonitrile : water (v : v)

Reversed Phase Chromatography: Mobile phase Considerations



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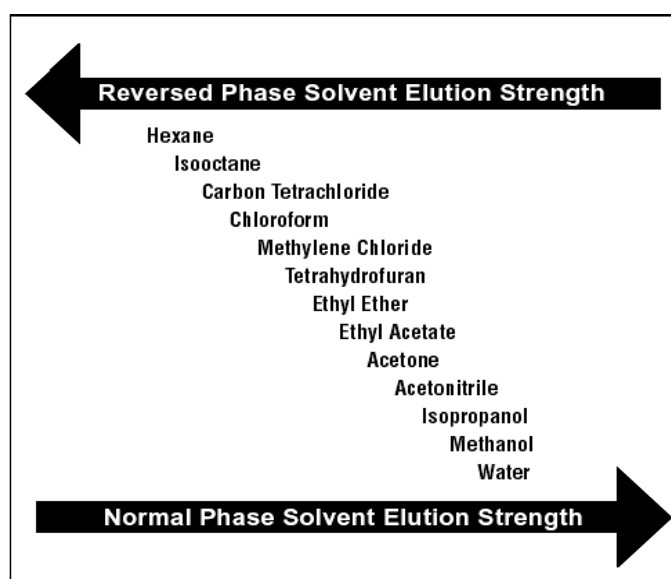
MOBILE PHASE Parameters that Affect Separation

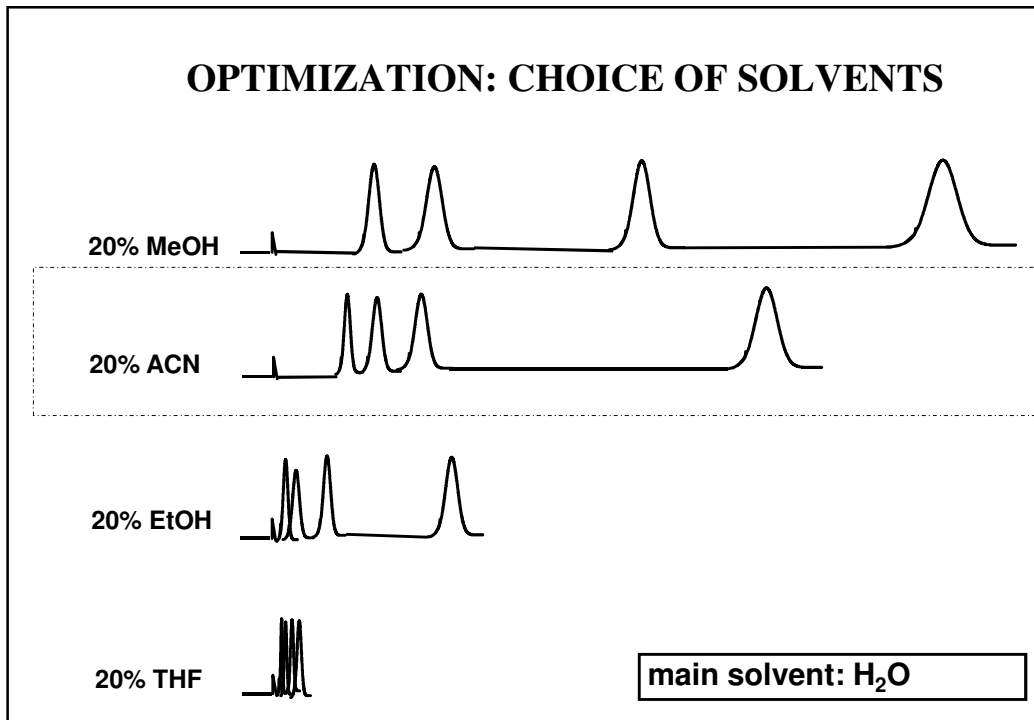
- * **TYPE OF MODIFIER (MeOH, ACN)**
- * **MOBILE PHASE COMPOSITION (% modifier)**
- * **pH**
- * **TYPE OF BUFFER (phosphate, acetate)**
- * **IONIC STRENGTH (Salts, buffer concentration)**
- * **ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)**

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Elution Strength





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SOLVENT STRENGTH

Analyte Retention as a Function of % Modifier: Negative Slope

k (retention) for each analyte changes independently as % Modifier changes.

Thus, the resolution between peaks changes.

$$k' = \frac{t_R - t_0}{t_0}$$

M. Gilar et al. / J. Chromatogr. A 958 (2002) 167-182

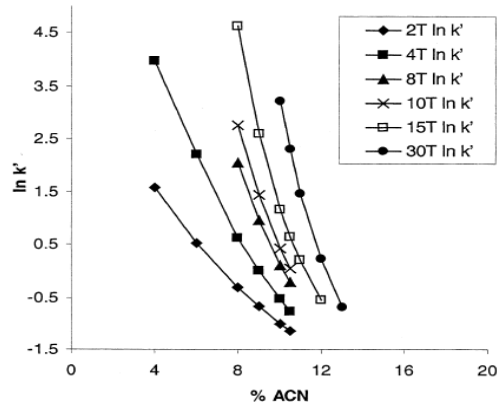
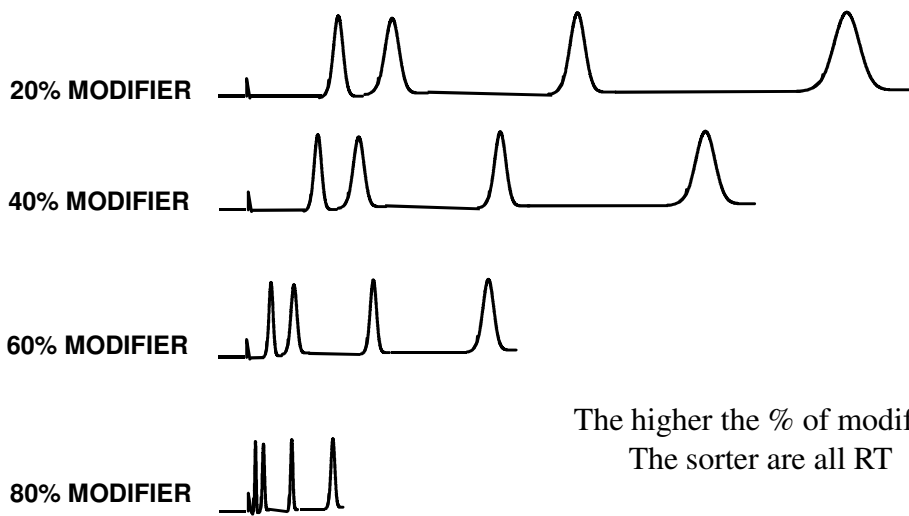


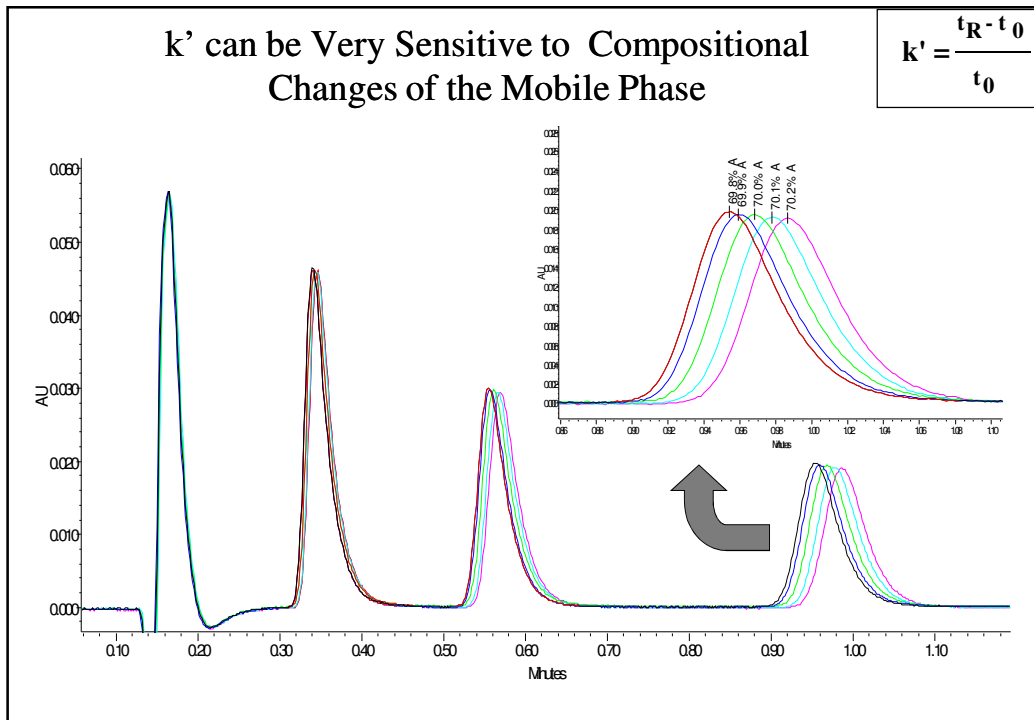
Fig. 2. Isocratic retention behavior of oligodeoxythymidines. Longer oligonucleotides show a sharper decrease in retention with the change of mobile phase strength. XTerra MS C_{18} 30×4.6 mm, $2.5 \mu\text{m}$; flow-rate 1 ml/min, 60°C . Mobile phase consisted of acetonitrile and 0.1 M TEAA, pH 7. Retention factor k for selected oligonucleotides was measured at 4, 6, 8, 9, 10, 10.5, 11, 12, and 13% (v/v) ACN.

OPTIMIZATION: % SOLVENTS



The higher the % of modifier
The shorter are all RT

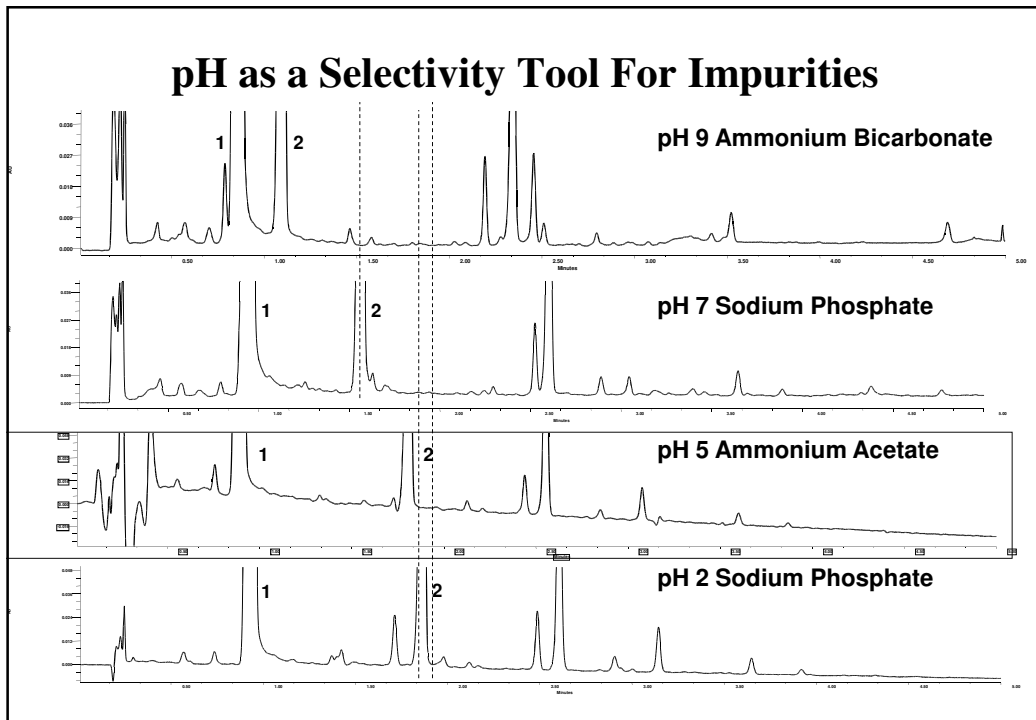
Reversed Phase Chromatography: Mobile phase Considerations



MOBILE PHASE

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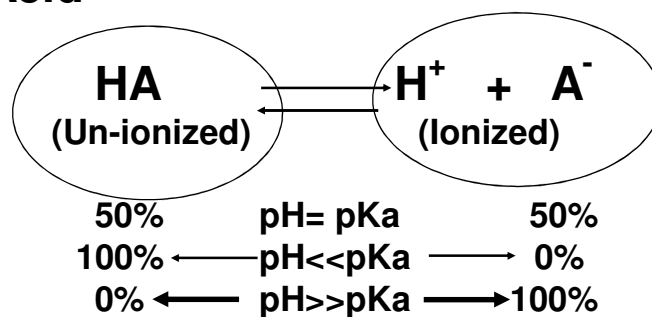
Reversed Phase Chromatography: Mobile phase Considerations



Ionization of Acids and Bases

Dissociation of Molecule

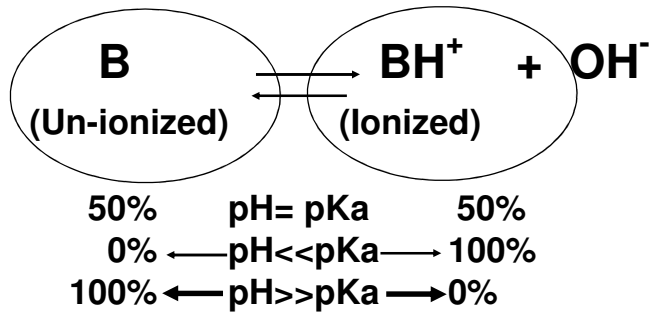
Acid



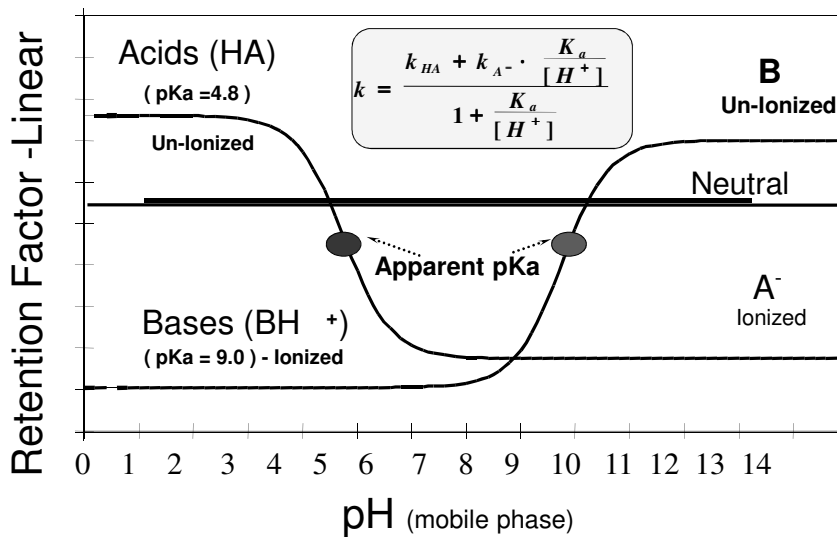
Ionization of Acids and Bases

Dissociation of Molecule

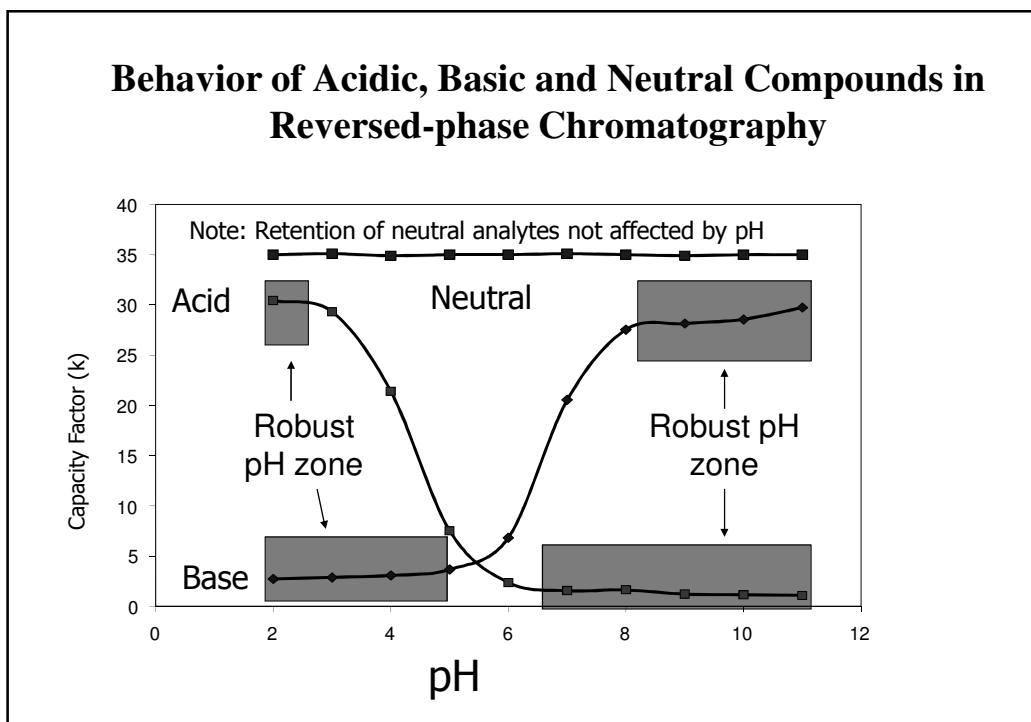
Base



Retention Factor versus pH for Acids, Bases and Neutrals



Reversed Phase Chromatography: Mobile phase Considerations



Impact of Organic Concentration on the pH of the Mobile Phase

- Aqueous buffers prepared from an acid and mixed with an organic solvent
 - pK_a of the acid increases
 - pH of the buffers increases

Acid	Aqueous pK_a	pK_a in 50% methanol
Phosphoric	2.11	3.21
Phosphoric	7.19	8.24
Formic	3.73	4.35
Acetic	4.77	5.54

Appendix: Methanol References 2 and 3

Impact of Organic Concentration on the pH of the Mobile Phase

- Aqueous buffer prepared from a base and mixed with an organic solvent
 - pK_a of the base decreases
 - pH of the buffers decreases

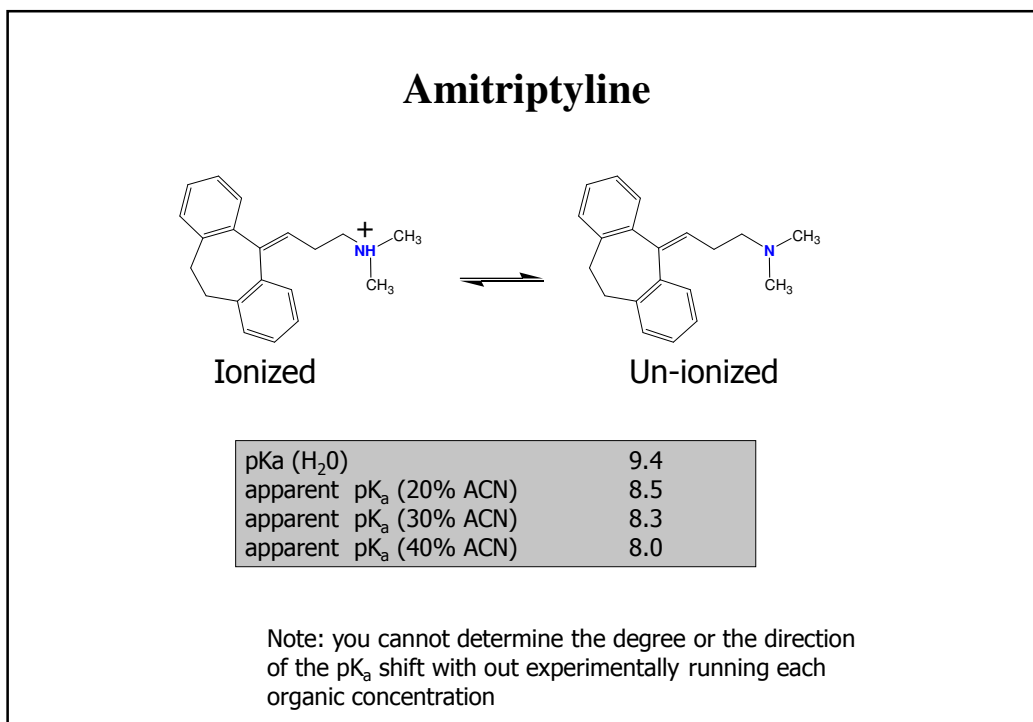
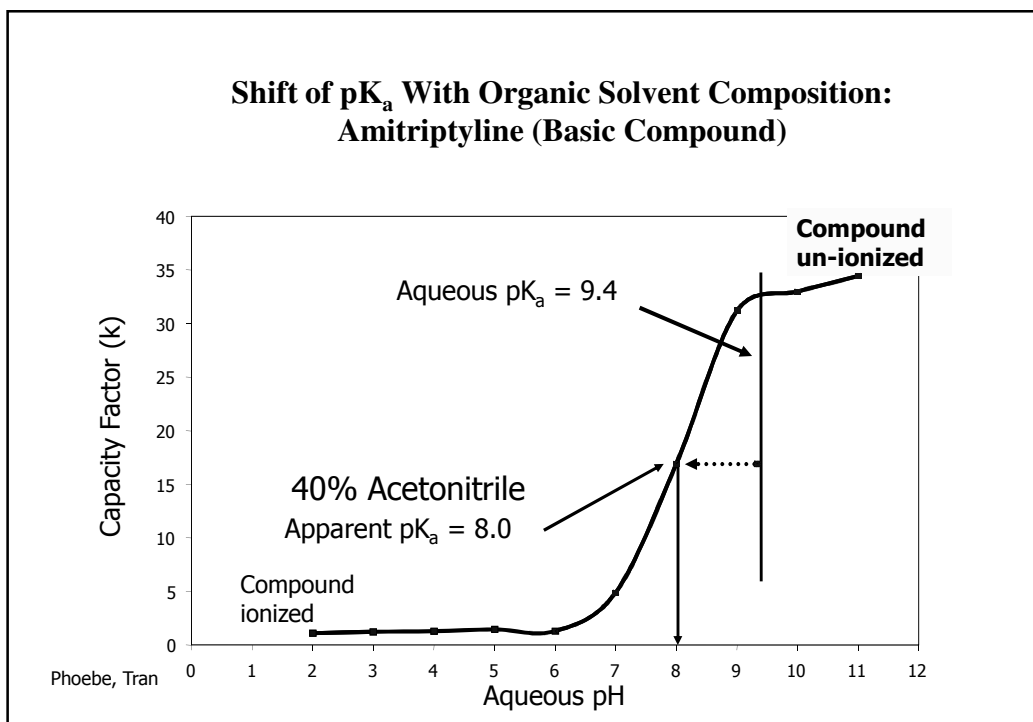
Acid	Aqueous pK_a	pK_a in 50% methanol
Ammonium	9.24	8.76

Appendix: Methanol References 2 and 3

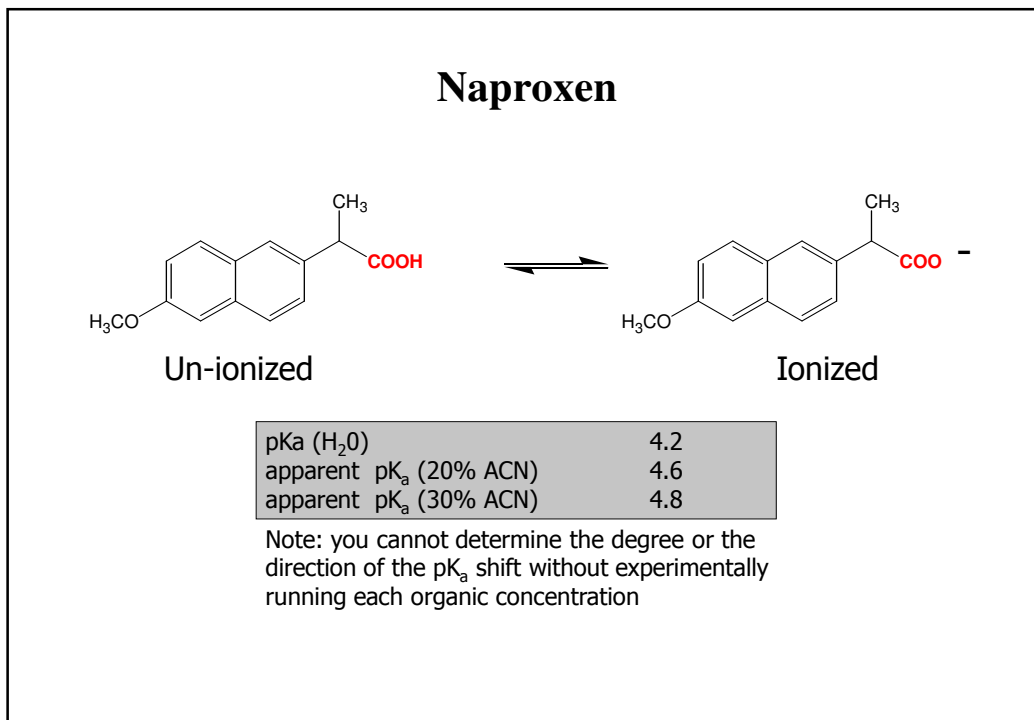
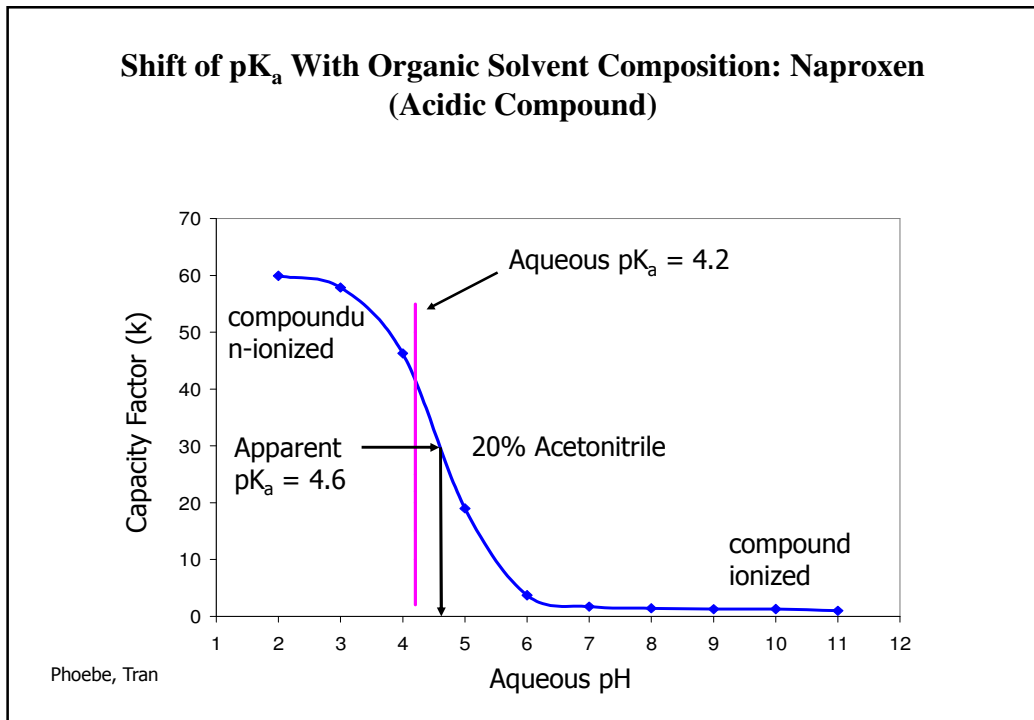
Impact of Organic Concentration on the pK_a of the Analyte

- In general:
 - Basic Compounds: pK_a will decrease with the addition of an organic solvent
 - Acidic Compounds: pK_a will increase with the addition of an organic solvent
- The specific change in pK_a will be compound dependent

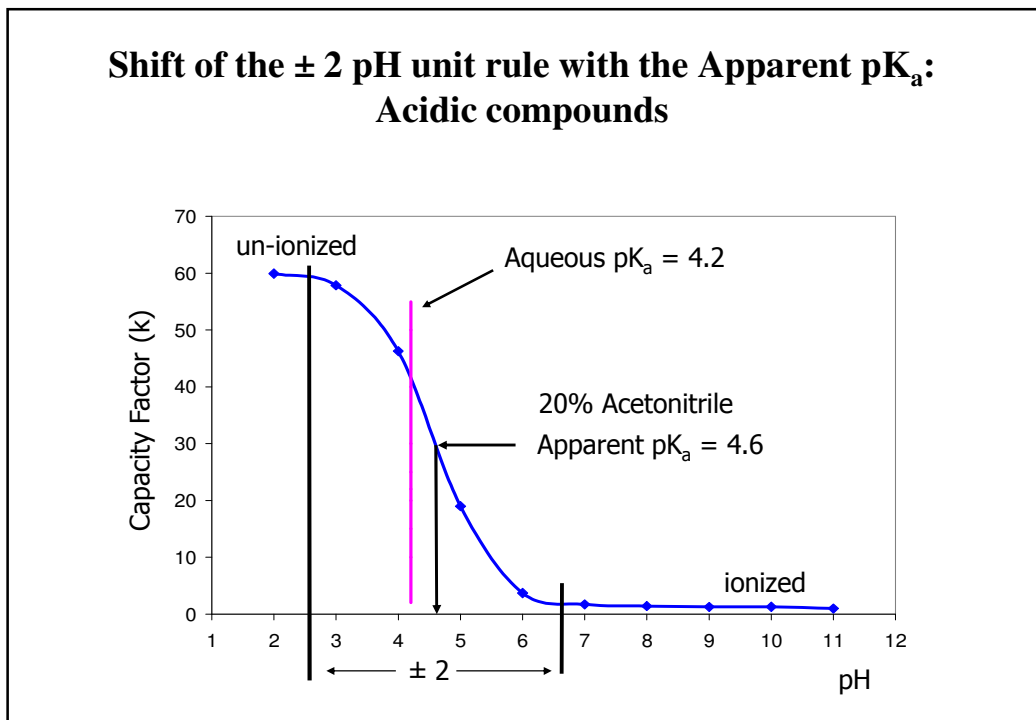
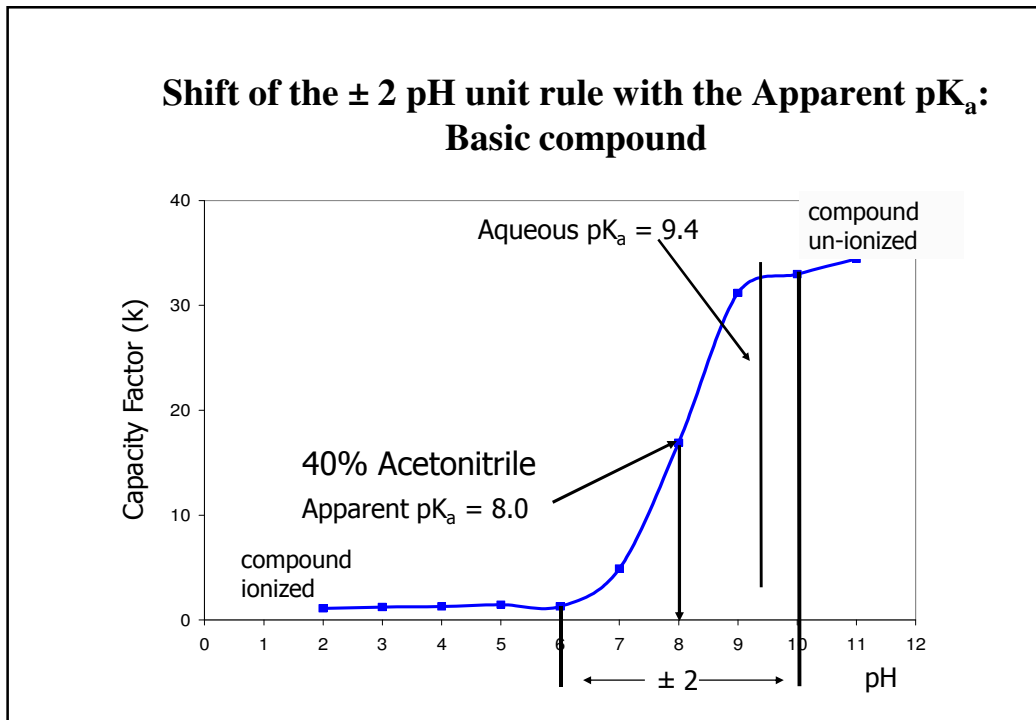
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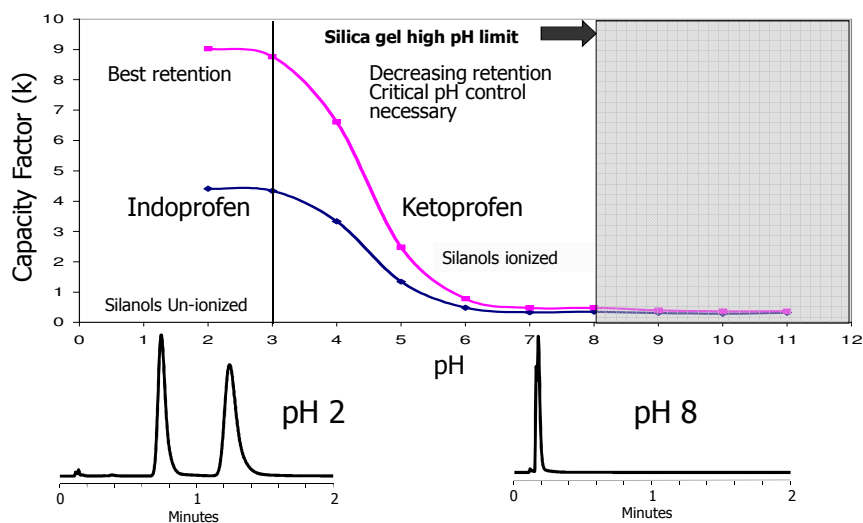
Reversed Phase Chromatography: Mobile phase Considerations



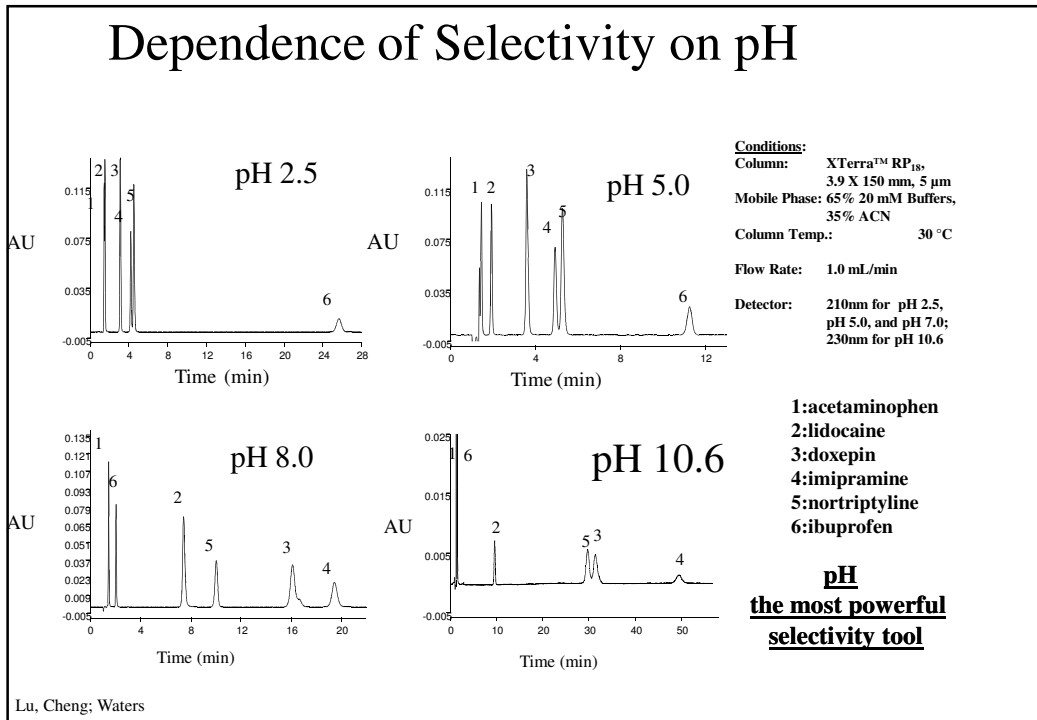
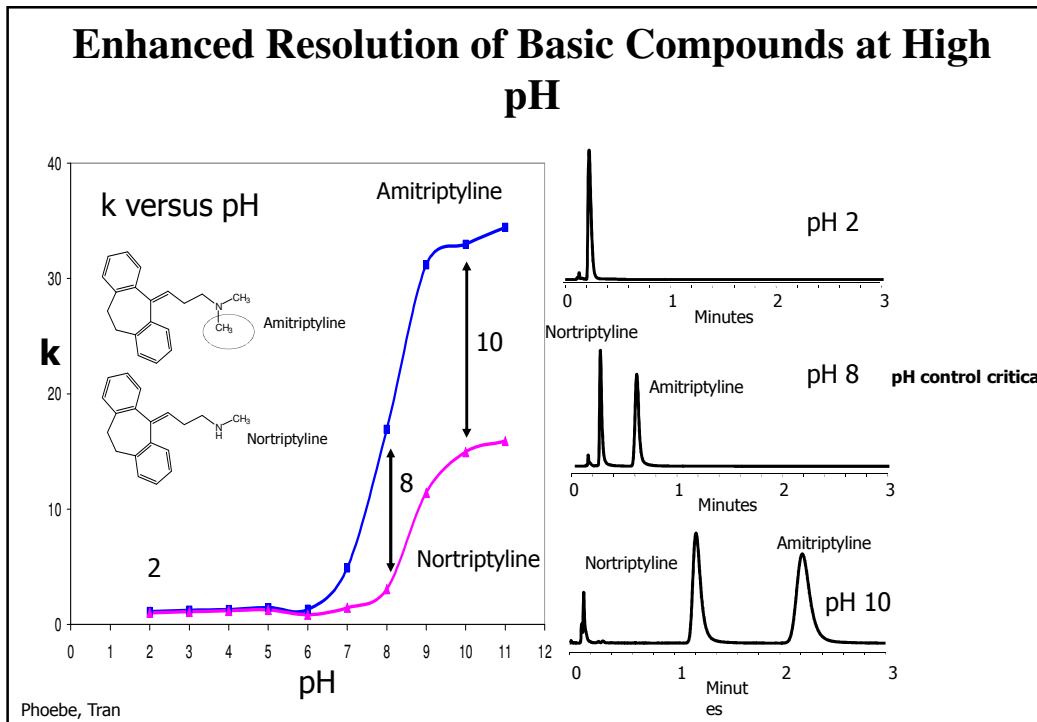
General Retention and Mobile Phase pH Rules

- Retention of ionizable analytes is most affected within the ± 2 pH unit envelope surrounding the pK_a
- Strong selectivity changes are observed at pH's close to the pK_a
 - Requires tight control of mobile phase pH for reproducible chromatographic retention
- Most stable chromatographic retention times observed outside of the ± 2 pH unit envelope
- Remember always measure pH before adding the organic solvent

Resolution of Two Acidic Compounds at Different Mobile Phase pH's

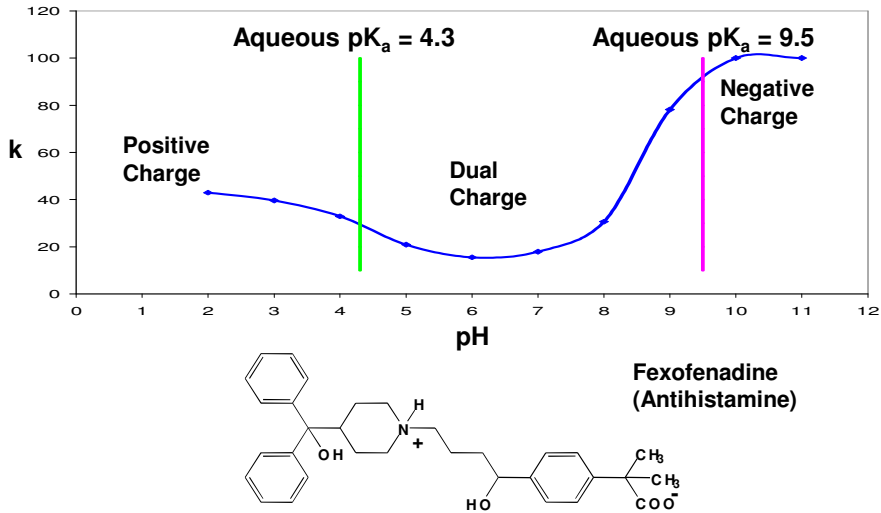


Reversed Phase Chromatography: Mobile phase Considerations

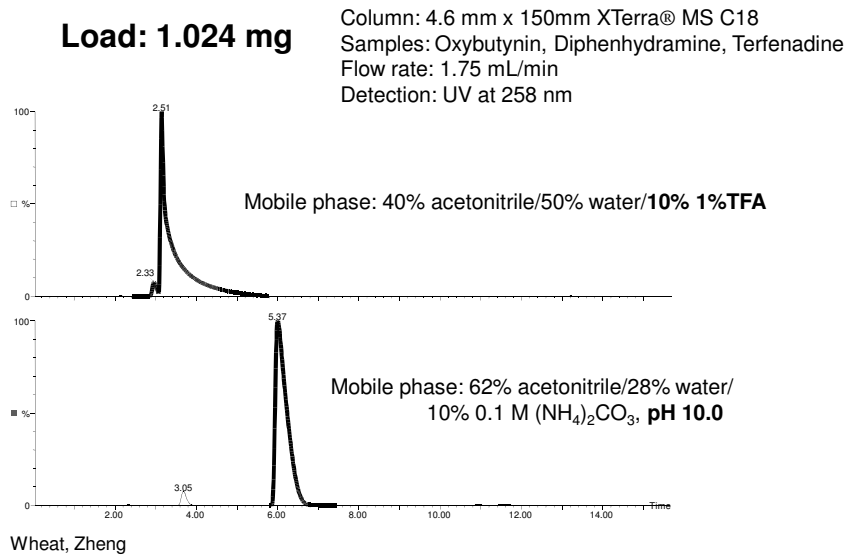


Reversed Phase Chromatography: Mobile phase Considerations

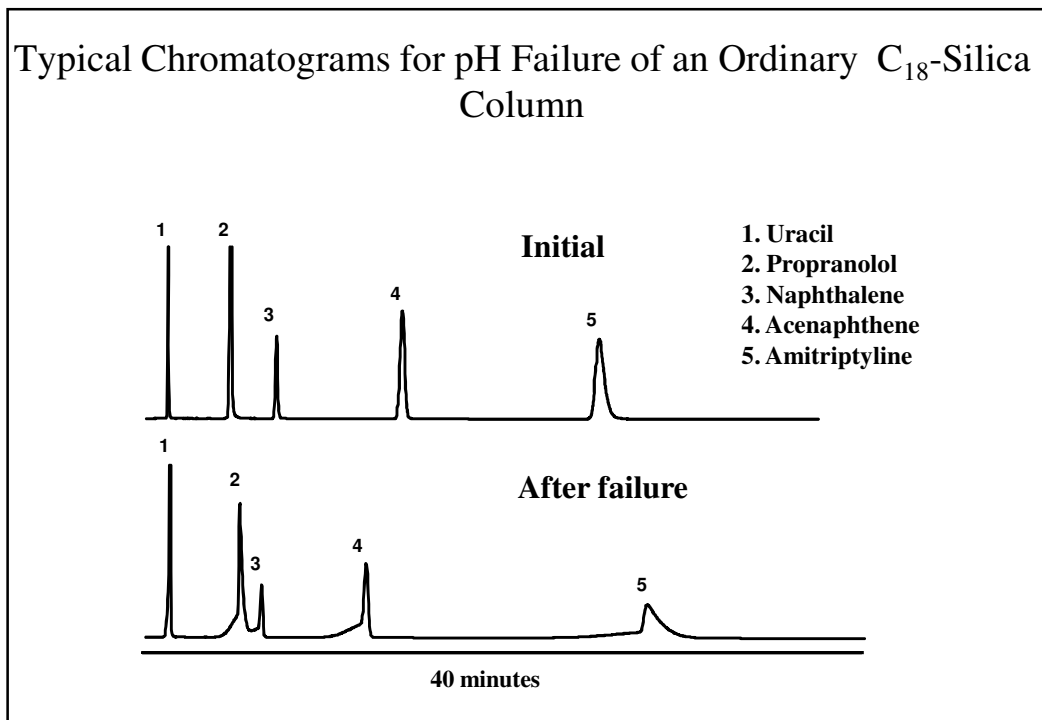
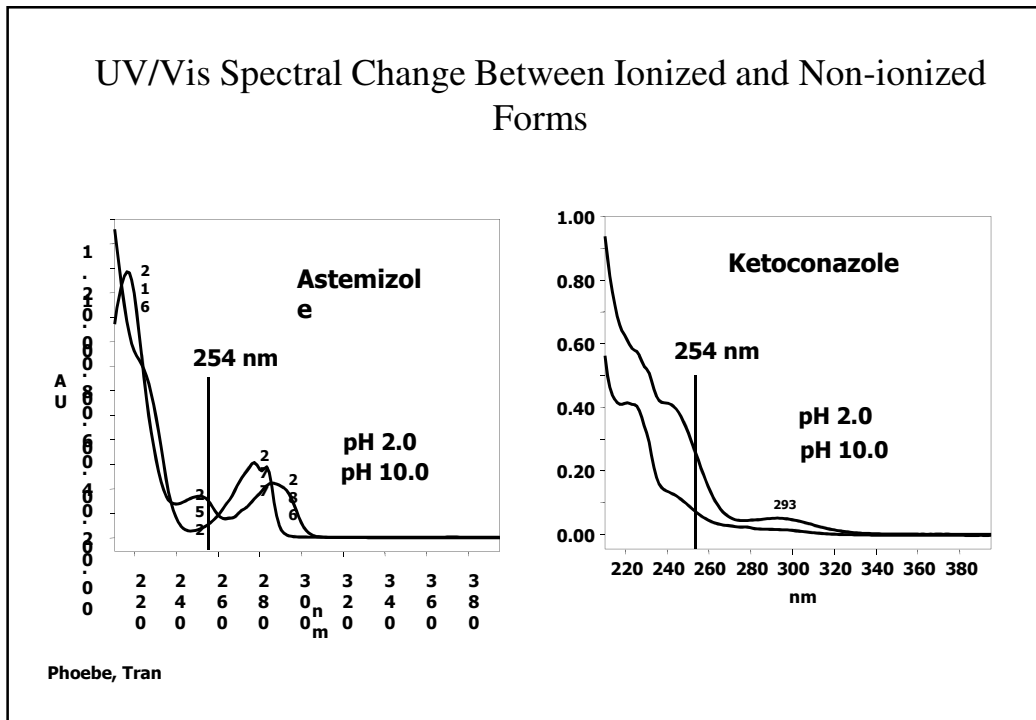
Impact of pH on the Retention of a Zwitterionic Compound



pH Dependent Loadability: Equal Load



Reversed Phase Chromatography: Mobile phase Considerations



**MOBILE PHASE
Parameters that Affect Separation**

- * **TYPE OF MODIFIER (MeOH, ACN)**
- * **MOBILE PHASE COMPOSITION (% modifier)**
- * **pH**
- * **TYPE OF BUFFER (phosphate, acetate)**
- * **IONIC STRENGTH (Salts, buffer concentration)**
- * **ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)**

Types of Buffers Used in Reversed Phase Mode

Commonly Used Buffers for Reversed Phase HPLC

Buffer	pKa	Buffer Range	UV Cutoff (nm)
Phosphate	2.1	1.1 – 3.1	200
	7.2	6.2 – 8.2	
	12.3	11.3 – 13.3	
Formic acid*	3.8	2.8 – 4.8	210
Acetic acid*	4.8	3.8 – 5.8	210
Citrate	3.1	2.1 – 4.1	230
	4.7	3.7 – 5.7	
	5.4	4.4 – 6.4	
Tris	8.3	7.3 – 9.3	205
Triethylamine*	11.0	10.0 – 12.0	200
Pyrrolidine	11.3	10.3 – 12.3	200

* Volatile buffers

**Recommended Buffers for pH's 2-7
(Silica and Hybrid Packing Materials)**

Additive or Buffer	pK _a	pH range (± 1 pH unit)	Volatile or Non-Volatile	Recommended Concentration Ranges and Common Counter-Ions
TFA	0.3		Volatile	(0.02-0.1%)
Acetic Acid	4.76		Volatile	(0.1-1.0%)
Formic Acid	3.75		Volatile	(0.1-1.0%)
Acetate	4.76	3.76 – 5.76	Volatile/Non-volatile	(1-10mM) NH ₄ , Na, K
Formate	3.75	2.75 – 4.75	Volatile/Non-volatile	(1-10mM) NH ₄ , Na, K
Phosphate	2.15	1.15 – 3.15	Non-volatile	
	7.20	6.20 – 8.20	Non-volatile	Not for pH's >7.0 (reduce the temperature & conc. for longer column lifetime)

**Non-Recommended Buffers for
pH's >7**

Buffer	pK _a	pH range (± 1 pH unit)	Volatile or Non-Volatile	Effect if buffer used (Dissolution)
Phosphate	12.3	11.3 – 13.3	Non-Volatile	Short Column Lifetime
Borate	9.2	8.2 – 10.2	Non-Volatile	Short Column Lifetime

Not recommended for either hybrid (XTerra) or silica gel based columns.

Reversed Phase Chromatography: Mobile phase Considerations

Recommended Buffers at pH's >7 (Hybrid & Polymeric HPLC Packings)

Buffer	pK _a	pH range (± 1 pH unit)	Volatile or Non-Volatile	Recommended Concentration Ranges
4-Methyl-Morpholine	~8.4	7.4 – 9.4	Volatile	10mM
Ammonia	9.2	8.2 – 10.2	Volatile	<10 mM and <30° C
Bicarbonate	10.3	9.3– 11.3	Volatile	5mM (do not use carbonate)
1-Methyl-Piperidine	10.3	9.3 – 11.3	Volatile	10mM
Triethylamine	10.7	9.7 – 11.7	Volatile	(0.1-1%) also DEA
Pyrrolidine	11.3	10.3 – 12.3	Volatile	10mM

Recommended Organic Buffers for pH's 2-7

Additive or Buffer	pK _a	pH range (± 1 pH unit)	Volatile or Non-Volatile	Recommended for use with Extended pH Packings
TFA	0.3		Volatile	Yes (0.02-0.1%)
Acetic Acid	4.76		Volatile	Yes (0.1-1.0%)
Formic Acid	3.75		Volatile	Yes (0.1-1.0%)
Acetate	4.76	3.76 – 5.76	Volatile/Non- volatile	Yes (1-10mM) NH ₄ , Na, K
Formate	3.75	2.75 – 4.75	Volatile/Non- volatile	Yes (1-10mM) NH ₄ , Na, K
Phosphate	2.15	1.15 – 3.15	Non-volatile	Yes
	7.20	6.20 – 8.20	Non-volatile	No for pH's >7.0 (lower the temperature the longer the column lifetime)

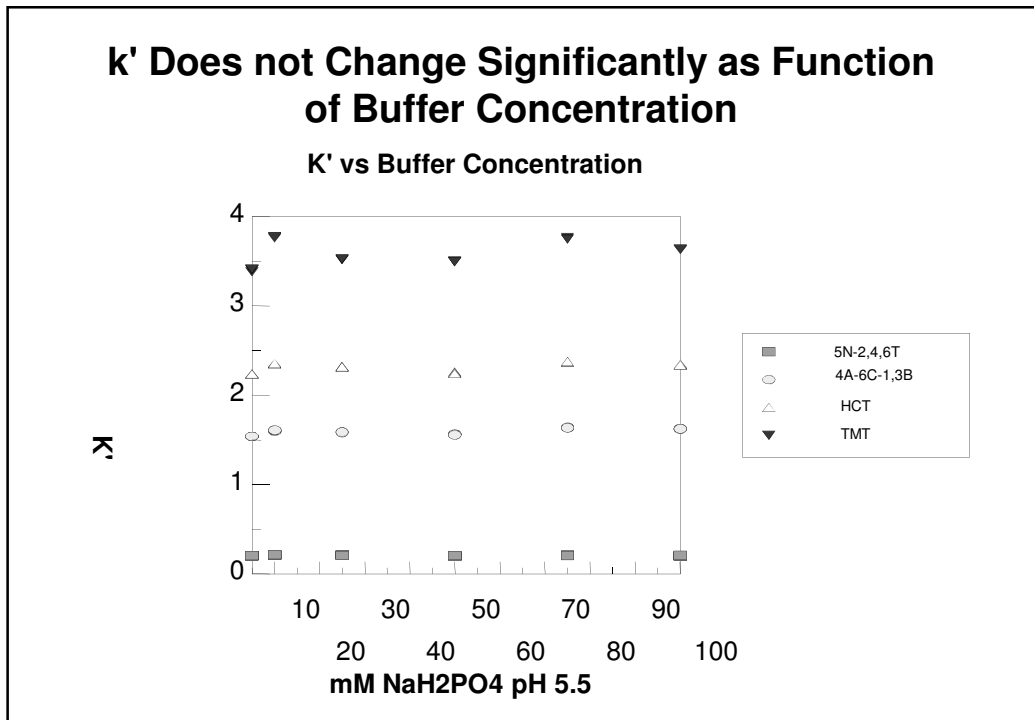
Reversed Phase Chromatography: Mobile phase Considerations

Some Considerations in the Selection of Mobile Phase Buffers for Reversed Phase HPLC

- Phosphate is more soluble in CH₃OH/water than in CH₃CN/ water or THF/water.
- NH₄⁺ > K⁺ > Na⁺ soluble in organic/water mobile phases .
- TFA and TEA degrade with time and increase their UV absorbance. Mobile phases containing these buffers should be made fresh often.
- Citrate buffers attack stainless steel. When using these buffers, be sure to flush them out of the system as soon as you complete your assay.
- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and damage chromatographic performance. These mobile phases should be made fresh daily and pre-column filters should be used to protect columns.
- Using boiled water to prepare buffered mobile phase and storing it under refrigeration will help reduce the problem of microbial growth.
- At pH greater than 7, phosphate buffers accelerate the dissolution of silica and severely shorten the lifetime of silica-based HPLC columns. Organic buffers should be used at pH higher than 8.

MOBILE PHASE Parameters that Affect Separation

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- * **MOBILE PHASE COMPOSITION (% modifier)**
- * **pH**
- * **TYPE OF BUFFER (phosphate, acetate)**
- * **IONIC STRENGTH (Salts, buffer concentration)**
- * **ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)**



Types of Buffers and Recommended Ionic Strength

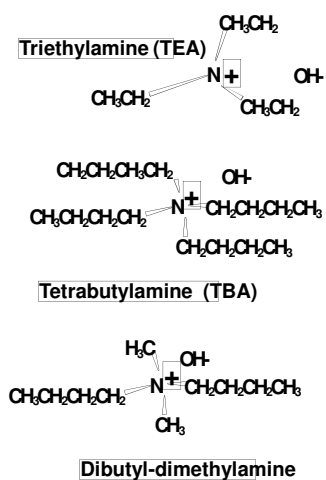
- **pH 10: Borate**
 - 20 mM H₃BO₃
- **pH 7: Phosphate**
 - 20 mM K₂HPO₄
- **pH 4-5: Acetate**
 - 10 mM CH₃COONH₄
 - 100 mM CH₃COOH
- **pH 2-3.5: Phosphate**
 - 20 mM H₃PO₄ - KH₂PO₄

MOBILE PHASE Parameters that Affect Separation

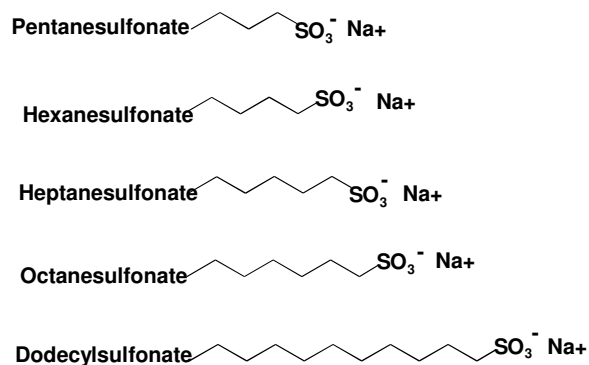
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Ion Pair Reagent

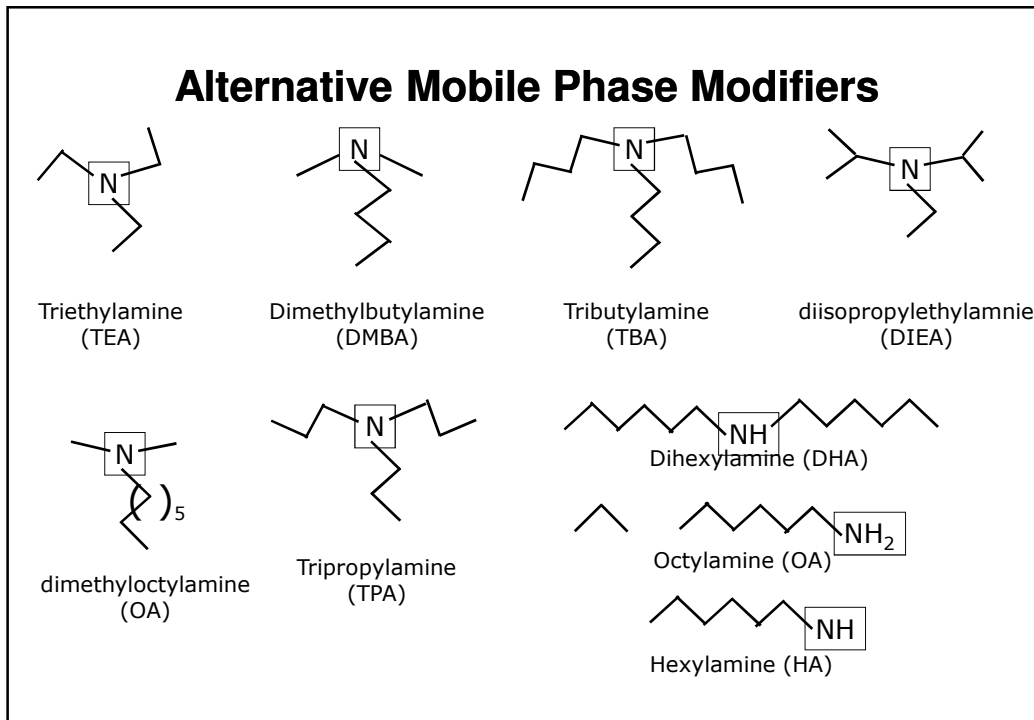
Alkylamines



Alkylsulfonates



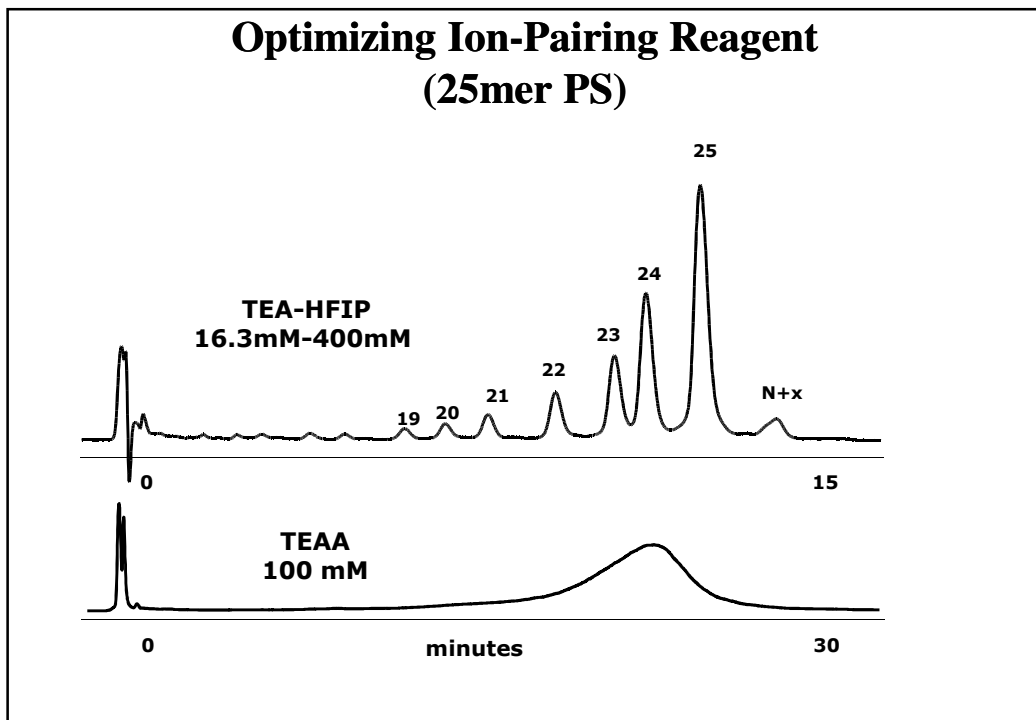
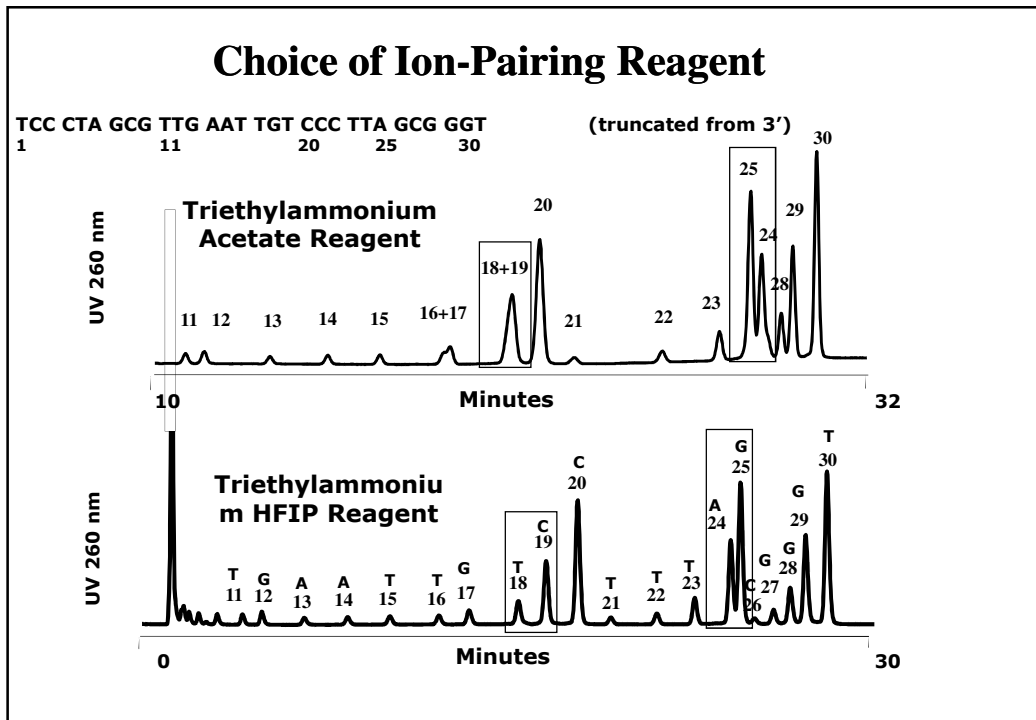
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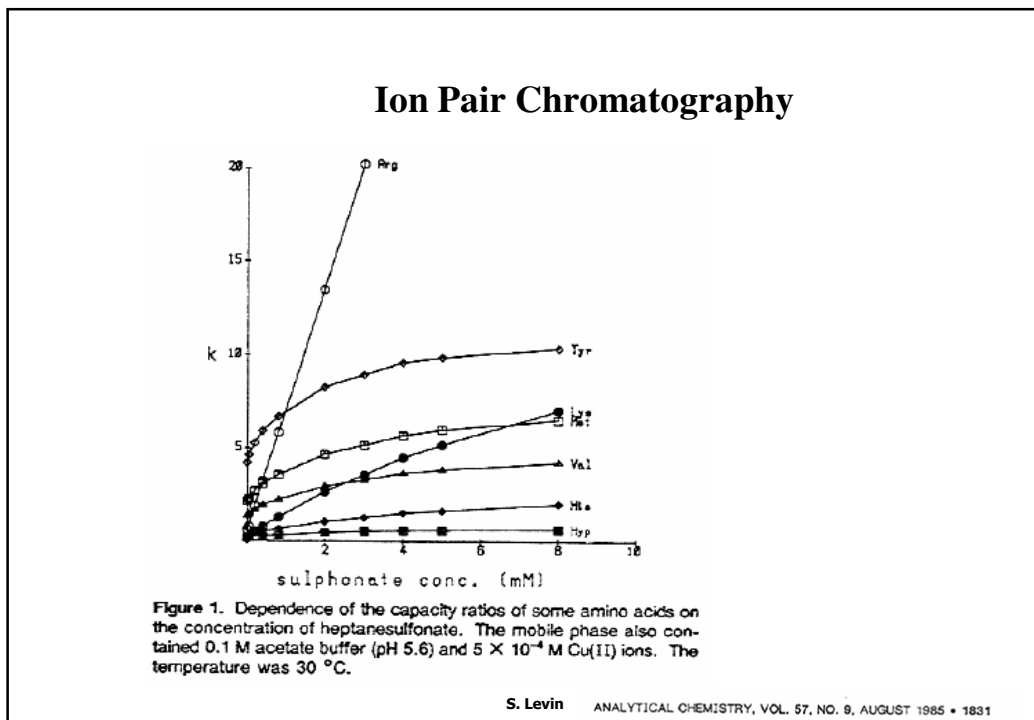
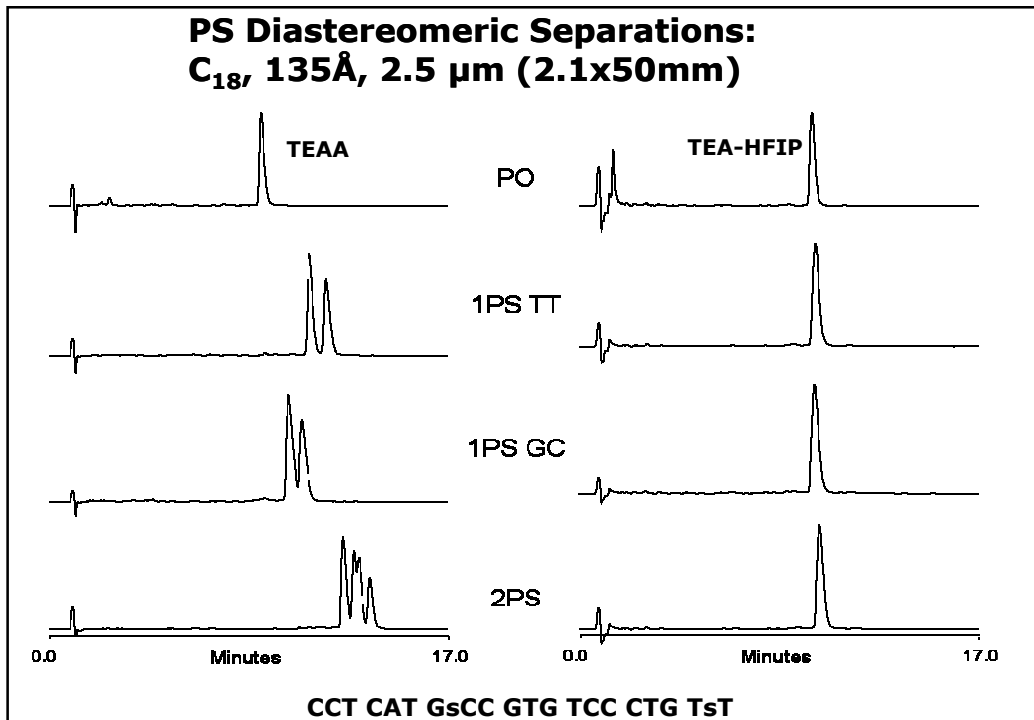


Choice of Ion-Pairing Reagent

<u>Ion-Pairing Agent</u>	<u>Acid</u>	<u>Abbreviation</u>
Triethylamine	acetic acid	TEAA
Triethylamine	bicarbonic acid	TEAB
Dimetylbutylamine	acetic acid	DMBAA
Tetrabutylamine	acetic acid	TBAA
Triethylamine	hexafluoroisopropanol	TEA-HFIP

Reversed Phase Chromatography: Mobile phase Considerations





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Dependence on Chain Length of Ion-Pair Reagent

S. Levin ANALYTICAL CHEMISTRY, VOL. 57, NO. 9, AUGUST 1985

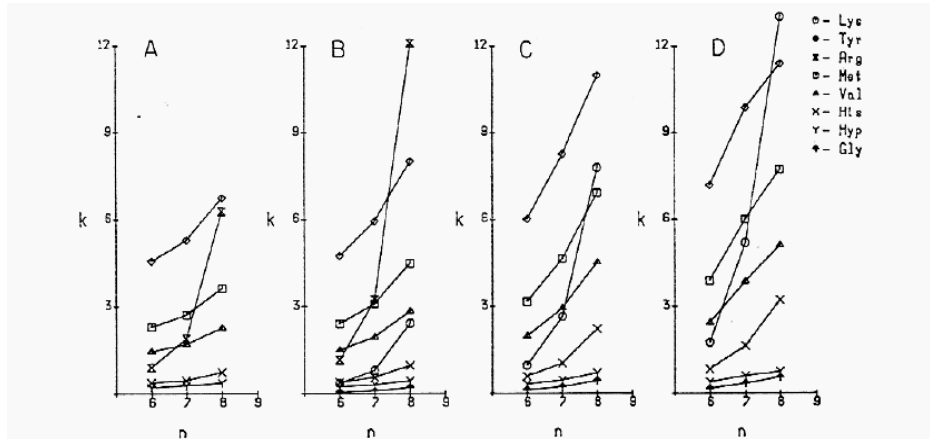
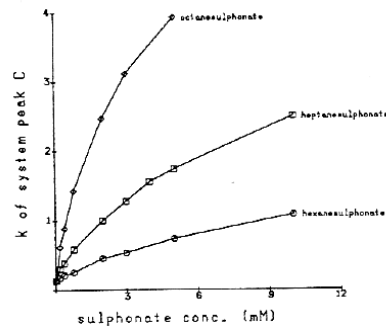
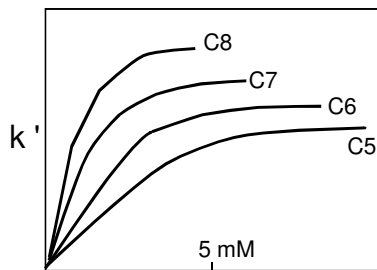


Figure 2. Dependence of the capacity ratios of some amino acids on the side-chain length of the alkylsulfonates. Concentrations of the ion-pair reagents are (a) 0.2 mM, (b) 0.4 mM, (c) 2 mM, and (d) 5 mM. All other conditions are as given in Figure 1.

Concentration of Ion-Pair Reagent in the Mobile Phase

The larger the alkyl, the longer are retention times

The larger alkyls saturate the stationary phase at lower concentrations



Conc. of Ion Pair Reagent in the Mobile Phase

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