Definition of SPE

"Separation or removal of an analyte or analytes from a mixture of compounds by selective partitioning of the compounds between a solid phase (sorbent) and a liquid phase (solvent)."

Definition of a Robust SPE Procedure

"A robust SPE procedure is one that can provide sample extracts of adequate purity and high reproducible extraction efficiencies when performed by any analyst on any sample of the matrix type for which the procedure was optimized."

A Typical SPE Procedure Involves Six Steps

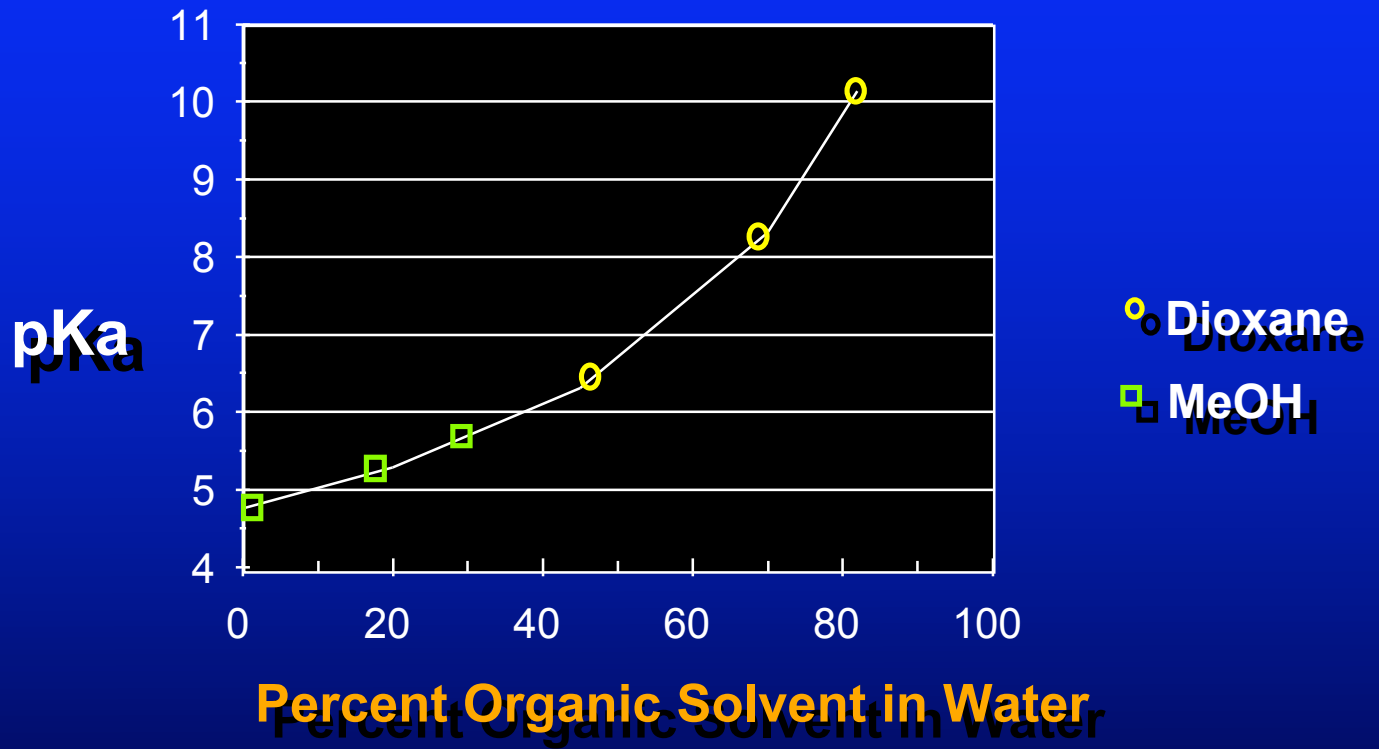
1. Sample pre-treatment
2. Column solvation
3. Column equilibration
4. Sample application
5. Interference elution
6. Analyte elution

Solvent Strength

(Chemical Strength is always relative)

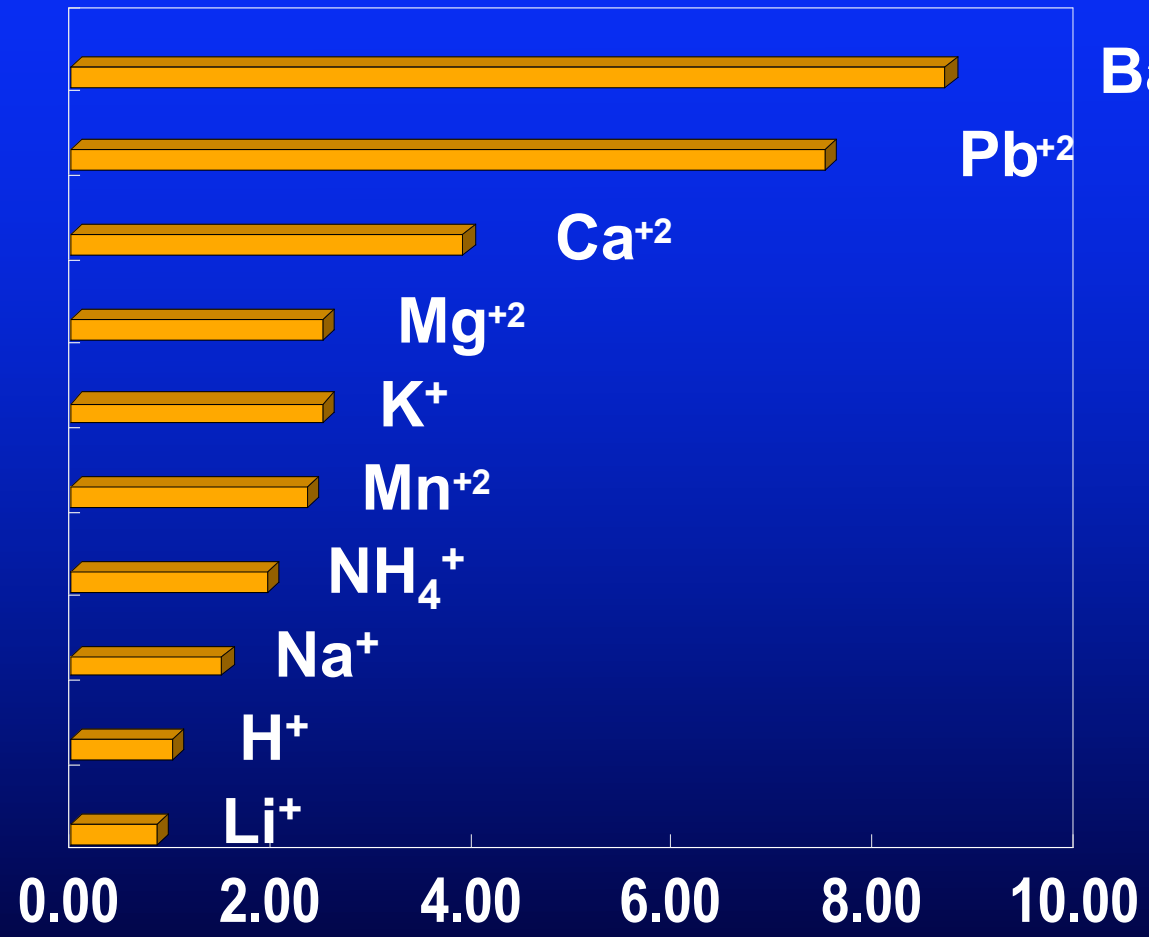
SORBENT	INCREASING STRENGTH
Silica	Hexane - - - MeOH - - - H_2O
Hydrocarbon	H_2O - - - - - - MeOH - - - Hexane
Bonded silica	H_2O << MeOH - - - H_2O >> MeOH

Effect of Solvent on pKa of HOAc at 25 C

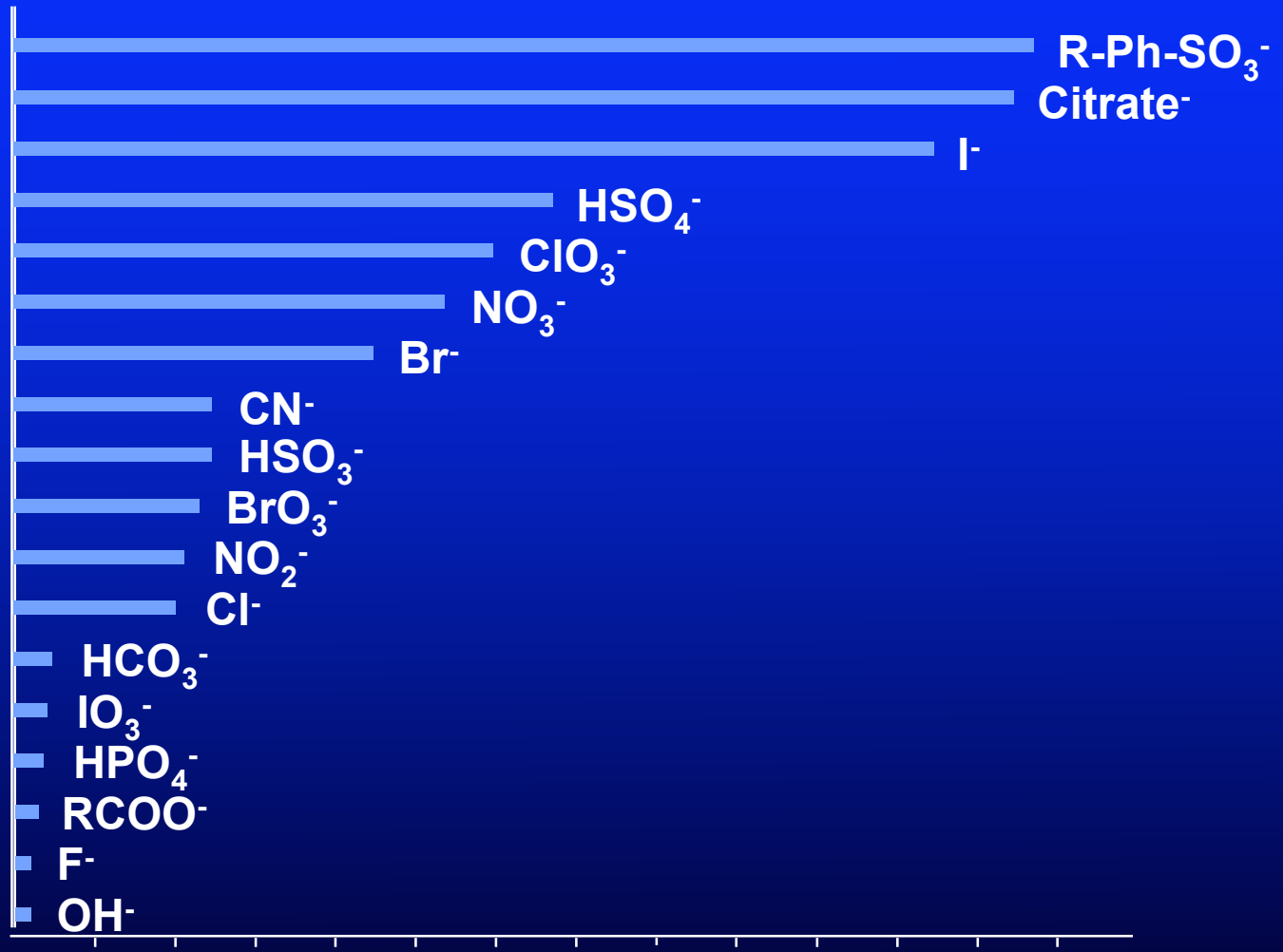


Reference: Hendrickson, Cram and Hammond, "Organic Chemistry", 3rd Edition McGraw Hill p 303

SCX / PRS Counter-ion Selectivity



SAX Counter- ion Selectivity



Steps to be Optimized During Method Development

- Sorbent selection
- Sample preparation
- Column conditioning
- Sample loading rate
- Selection of interference elution solvent
- Interference elution solvent flow rate
- Selection of Isolate elution solvent
- Isolate elution solvent flow rate

SPE Column Conditioning

- Solvation of the Sorbent
- Purification of the SPE Column / Sorbent

Comparison of 0.1 ml and 0.4 ml SPE Columns

0.1 ml

0.4 ml

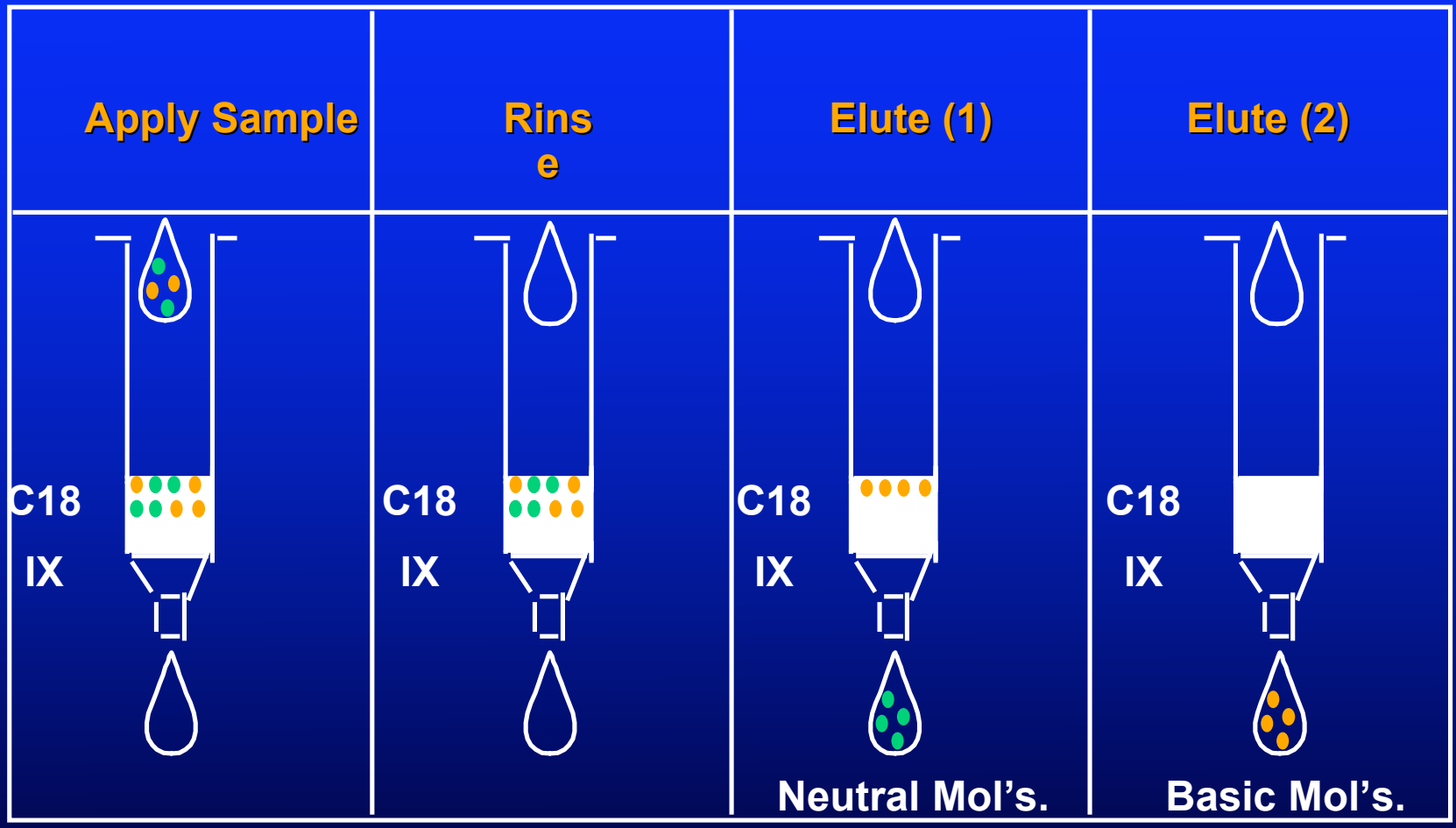
Contaminant Elution

- Analyte-Insoluble Solvents
- Selective Mixtures
- Maintain Retention Conditions
- Verify no Analyte Bleed
- Proper Flow Rate

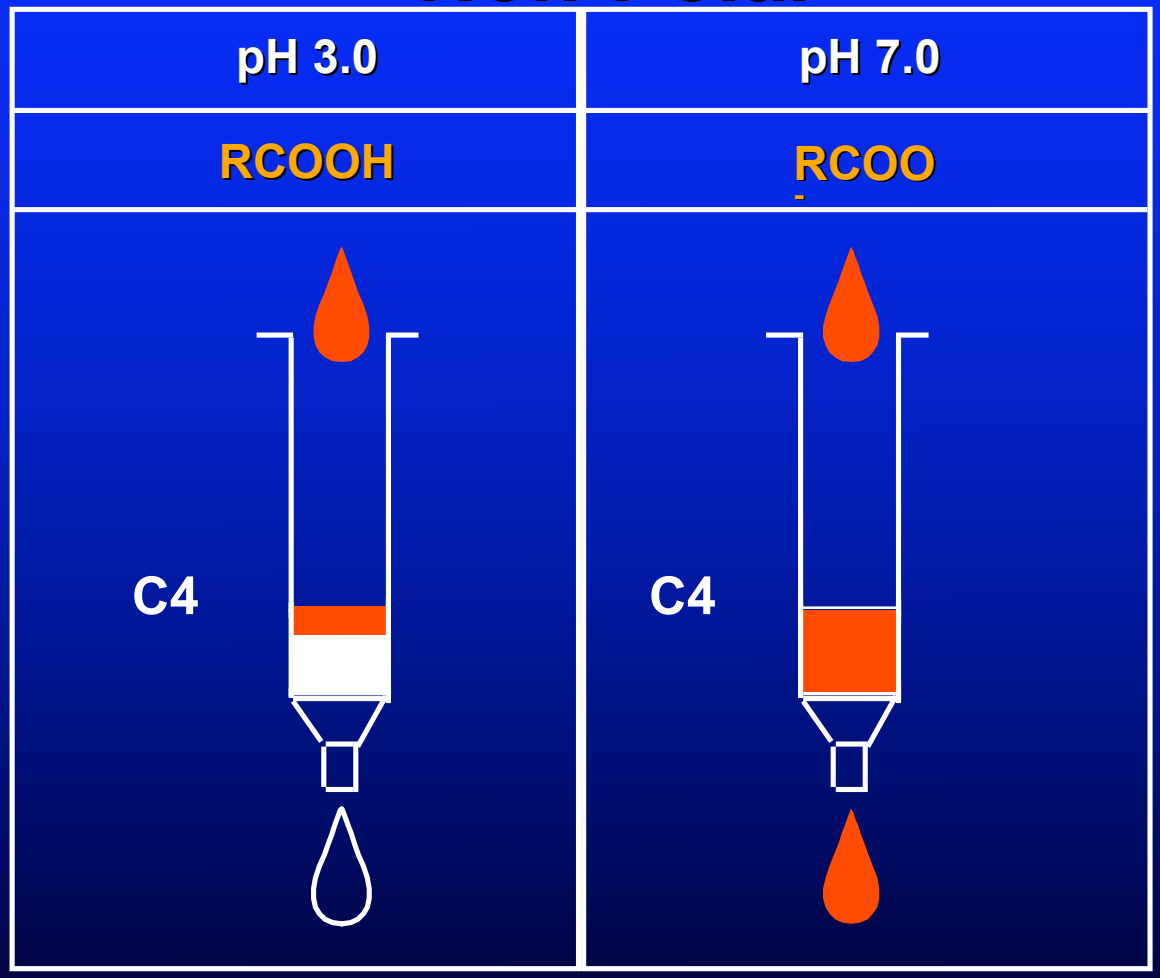
Why use Mixed-mode Columns?

- More robust procedures
  (Less matrix dependent vs. 100% ion exchange)
- Columns designed to provide multiple interaction
  (ion exchange and polar partitioning capacity optimized for some PTM bio-applications)
- Cleaner extract possible
- Ability to fractionate complex mixtures
- Methods for biomolecules or from biomatrices

Mixed-Mode Extraction



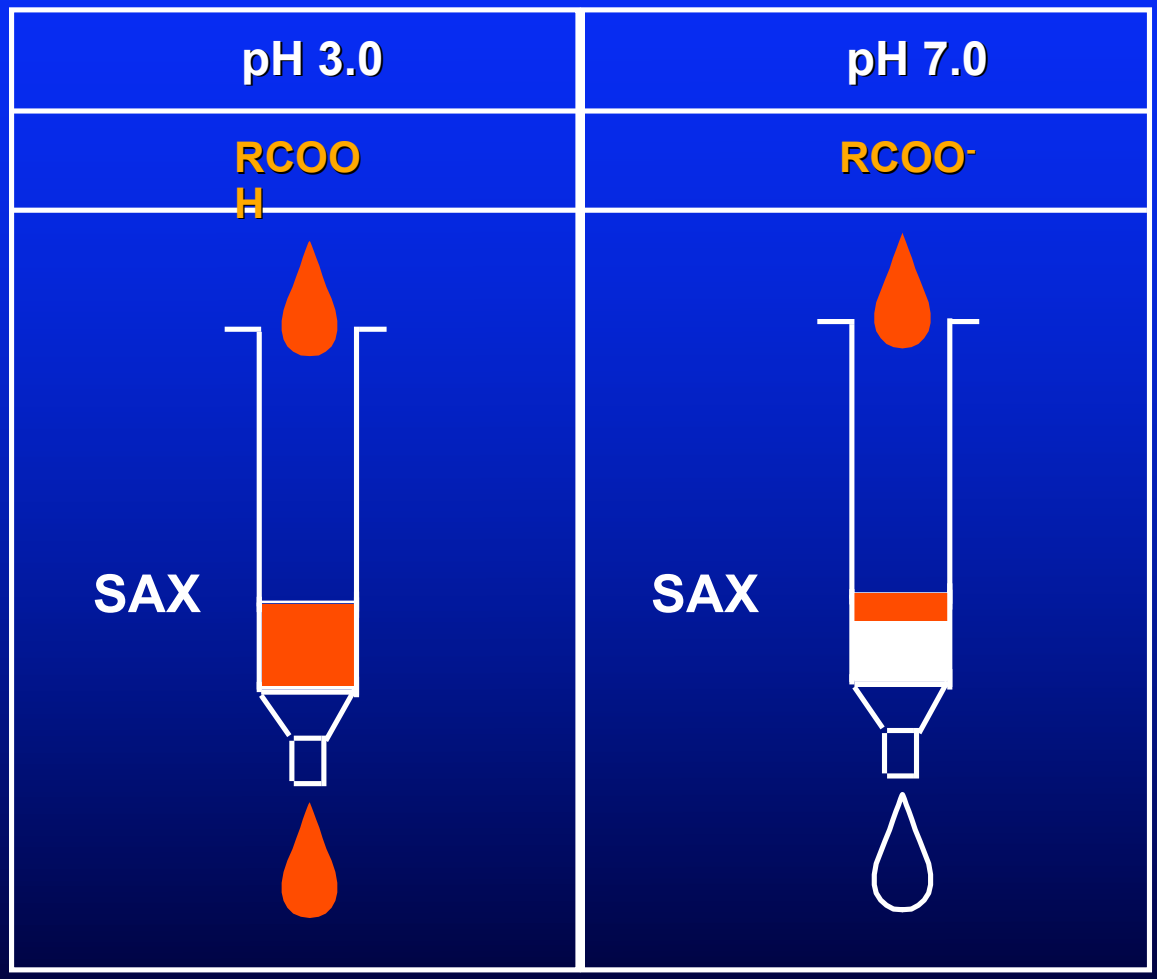
Influence of pH on Retention
Non-Polar



Adjust pH to Suppress Ionization

RCOOH pKa = 5.0

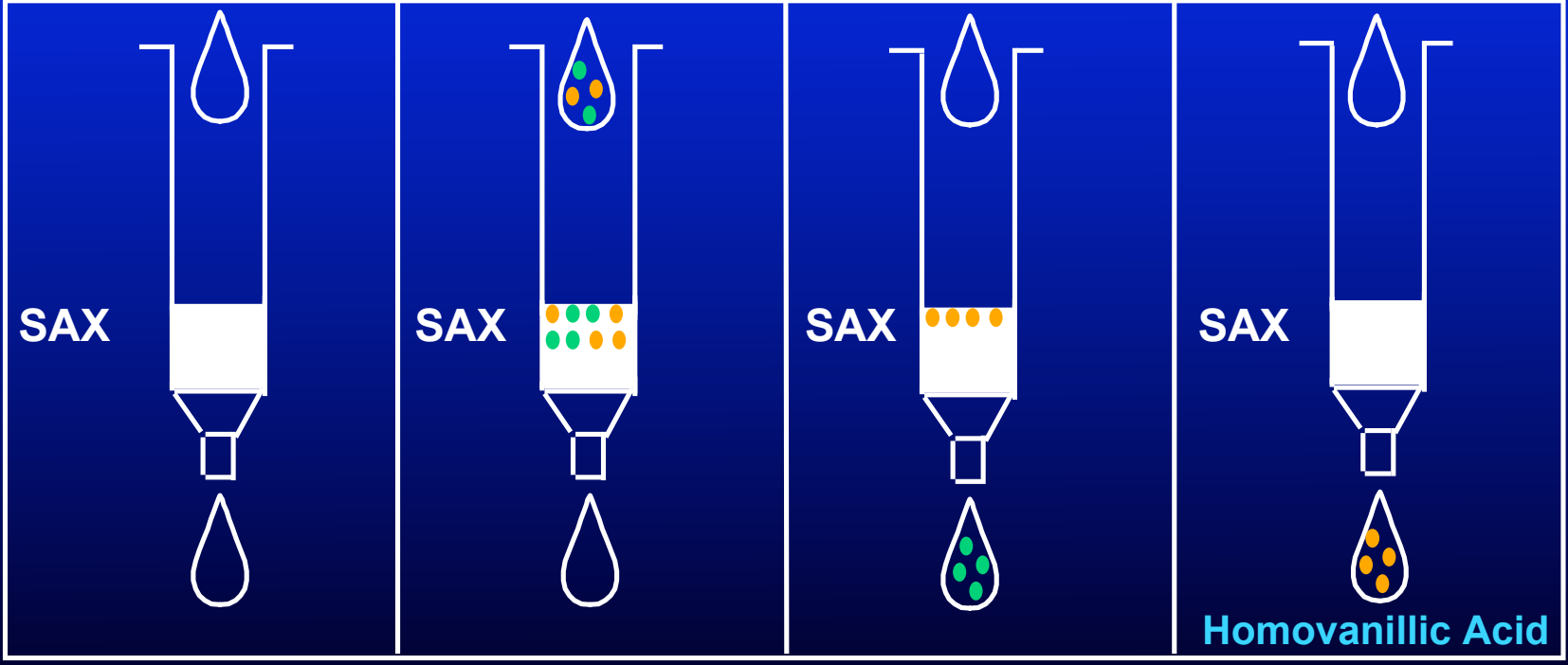
Influence of pH on Retention
Ion Exchange



Adjust pH to Ensure Ionization

Anion Exchange

Condition	Apply Sample	Interference Elution	Analyte Elution
1. Methanol 2. Water or Buffer	2 pH Units Above Isolate pKa	Water or Buffer	2 pH Units Below pKa (500mM H_2SO_4)



Rumsby, et. al.

Weak Anion Exchange (NH2)

	pH 3	pH 7	pH 11
Analyte: Weak Anion, pKa 5.0	R-COOH	$R\text{-}COO^-$	$R\text{-}COO^-$
Sorbent: NH2, pKa 9.8	$R\text{-}NH_3^+$	$R\text{-}NH_3^+$	$R\text{-}NH_2$
Analyte: Strong Anion	$R\text{-}SO_3^-$	$R\text{-}SO_3^-$	$R\text{-}SO_3^-$
Sorbent: NH2, pKa 9.8	$R\text{-}NH_3^+$	$R\text{-}NH_3^+$	$R\text{-}NH_2$

Strong Anion Exchange (SAX)

	pH 3	pH 7	pH 11
Analyte: Weak Anion, pKa 5.0	R-COOH	$R\text{-}COO^-$	$R\text{-}COO^-$
Sorbent: SAX	$R\text{-}NR_3^+$	$R\text{-}NR_3^+$	$R\text{-}NR_3^+$
Analyte: Strong Anion	$R\text{-}SO_3^-$	$R\text{-}SO_3^-$	$R\text{-}SO_3^-$
Sorbent: SAX	$R\text{-}NR_3^+$	$R\text{-}NR_3^+$	$R\text{-}NR_3^+$

L/L Extraction

- Two Extraction Solvents per Step
- Solvents must be Immiscible
- Solubility Differentiation (Non-Selective)
- Emulsions
- Large Solvent Volumes
- Extract Concentration Required
- Serial Process

Solid Phase Extraction

- Sorbent Phase and Solvent Phase
- Inherent “Immiscibility”
- Functional Group Differentiation
- No Emulsions
- Small Solvent Volumes
- Inherent Concentration
- Batch Process

Interference Elution Step

- Analyte-Insoluble Solvents
- Selective Mixtures
- Maintain Retention Conditions
  (pH control can be important)
- Verify no Analyte Bleed
- Optimize Flow Rate

Extraction Steps

- Sample Pretreatment
- Column Conditioning
- Column Equilibration
- Sample Application to Column
- Interference Elution
- Analyte Elution

Column Conditioning

- Hygienic Cleaning
- Solvation of the Sorbent

SPE Column Conditioning

- **Non-Polar Sorbents**
  MeOH, MeCN, THF
- **Bonded Polar Sorbents (HILIC)**
  MeOH, MeCN, THF then 5-10% water /w buffer
- **Unbonded Polar Sorbents**
  Non-Polar Solvents, Hexane, Ethyl Acetate,
  (Same solvent as the sample matrix)
- **Ion-Exchange Sorbents**
  MeOH, MeCN, THF, then water /w buffer ions

Column Equilibration

- Remove Excess Solvation Solvent
- Normalize Sorbent to Sample Condition
- Promote Optimum Retention
  - Solvent Composition
  - Ionic Strength
  - pH
- Ion-Exchange
  - Optimize Choice of Counter-ion
  - Optimize pH for ionization of sorbent

Sample Pretreatment

Optimize Sample for Analyte Retention

- Proper Dilution/Ionic Strength
- Correct pH
- Analytes Free in Solution
- Remove Particulates

Sample Application

Proper Flow Rate

Analyte Elution

- Elution solvent must overcome both PRIMARY and SECONDARY interactions
- 100% Elution in 2-10 Bed Volumes
- Use Selective Solvents/Mixtures
- Smaller elution volume ➡ More conc. extract
- Optimize Flow Rate

Flow Rate Considerations

Optimize and Specify in Protocol for

- Column Equilibration
  (Counter-ion Exchange)
- Sample Loading
- Interference Elution
- Analyte Elution

Molecular Interactions

- Covalent Interactions — 100-300 kcal/mole
- Ionic Interactions — 50-75 kcal/mole
- Polar Interactions — 3-7 kcal/mole
  - Hydrogen-Bonding
  - Dipole-Dipole
  - π-π
- Non-Polar Interactions — 1-2 kcal/mole
  - Van der Waals or dispersion

Extraction Components

Matrix (Sample or Solvent)

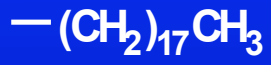
Analyte(s)

Sorbent

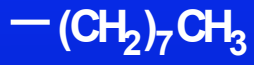
Retention/Elution

Retention	Elution
Analyte ⇔ Sorbent	Analyte ⇔ Sorbent
Matrix ⇔ Sorbent	Matrix ⇔ Sorbent
Analyte ⇔ Matrix	Analyte ⇔ Matrix

Non-Polar Sorbents



Octadecyl



Octyl

Ethyl

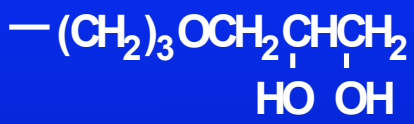
Phenyl

Cyclohexyl

Cyanopropyl

Polar Sorbents

Silica

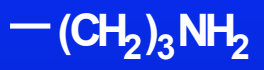


Diol

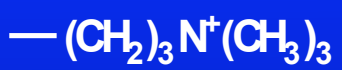
Aminopropyl

Cyanopropyl

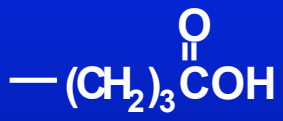
Ion-Exchange Sorbents



Aminopropyl

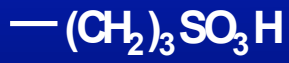


SAX (quaternary amine)



CBA (carboxylic acid)

SCX (benzenesulfonic acid)



PRS (propylsulfonic acid)

Barriers to SPE Use

- More complex than liquid/liquid extraction
- More choices than liquid/liquid extraction
- Less user familiarity
- Longer method development cycle

HPLC vs. SPE

- **Elution: Column Chromatography:**

$$V_r = V_m + KV_s$$

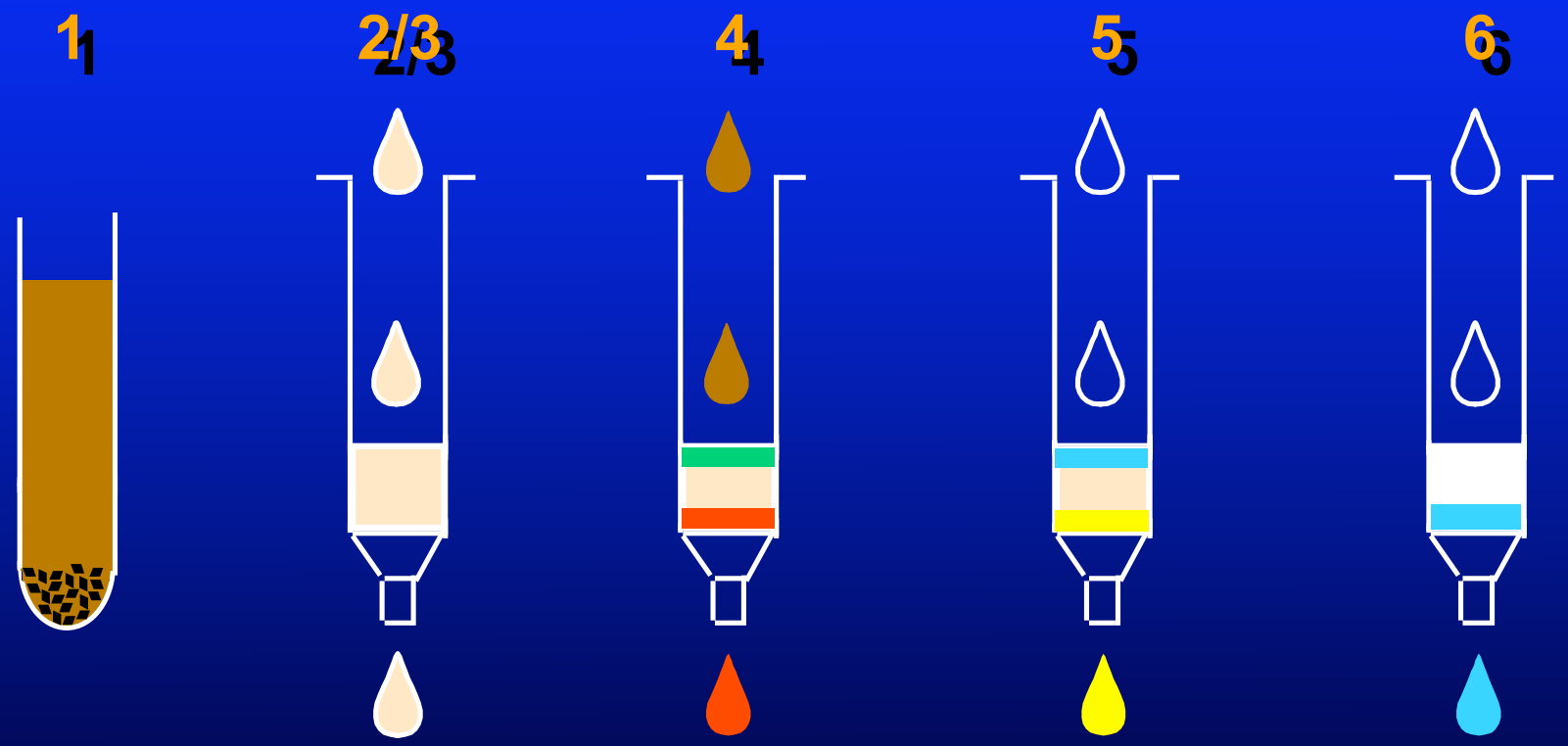
$$0.2 << K << 200$$

- **Extraction: SPE Chromatography:**

$$K = \frac{[stat]}{[matrix]} > 1000 \longrightarrow \text{Retention}$$

$$K = \frac{[stat]}{[eluent]} < 0.001 \longrightarrow \text{Elution}$$

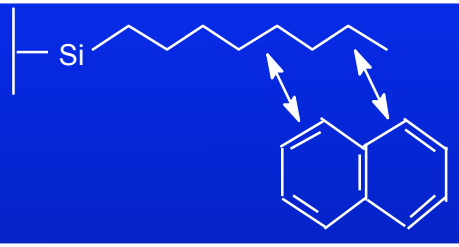
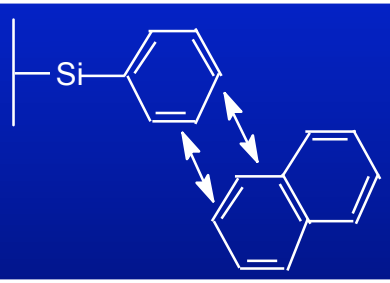
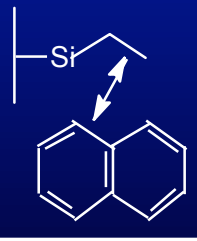
Six-Step SPE Procedure



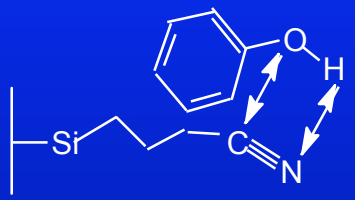
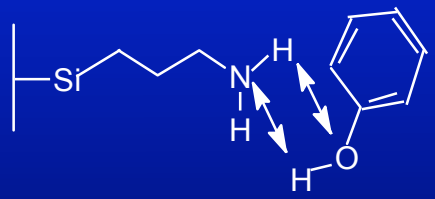
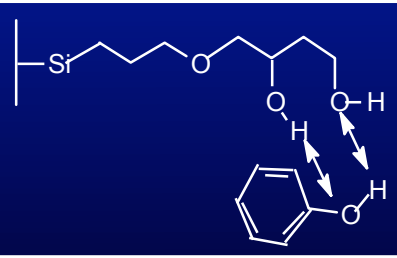
A Typical SPE Procedure Involves Six Steps

1. Sample pre-treatment
2. Column solvation
3. Column equilibration
4. Sample application
5. Interference elution
6. Analyte elution

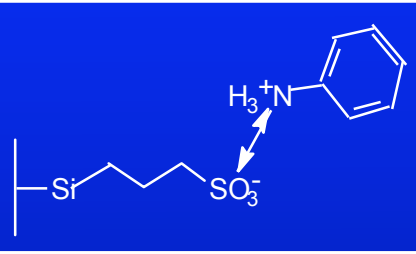
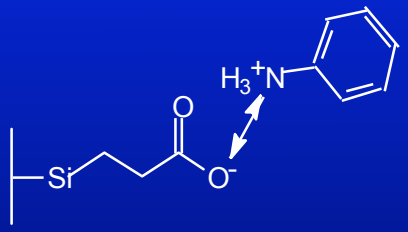
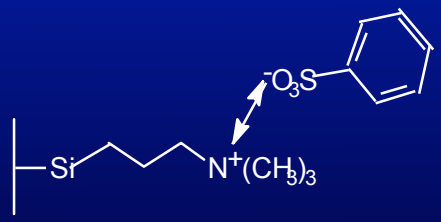
Non-Polar Interactions

Sorbents		Interactions
C8		van der Waals
P H		van der Waals
C2		van der Waals

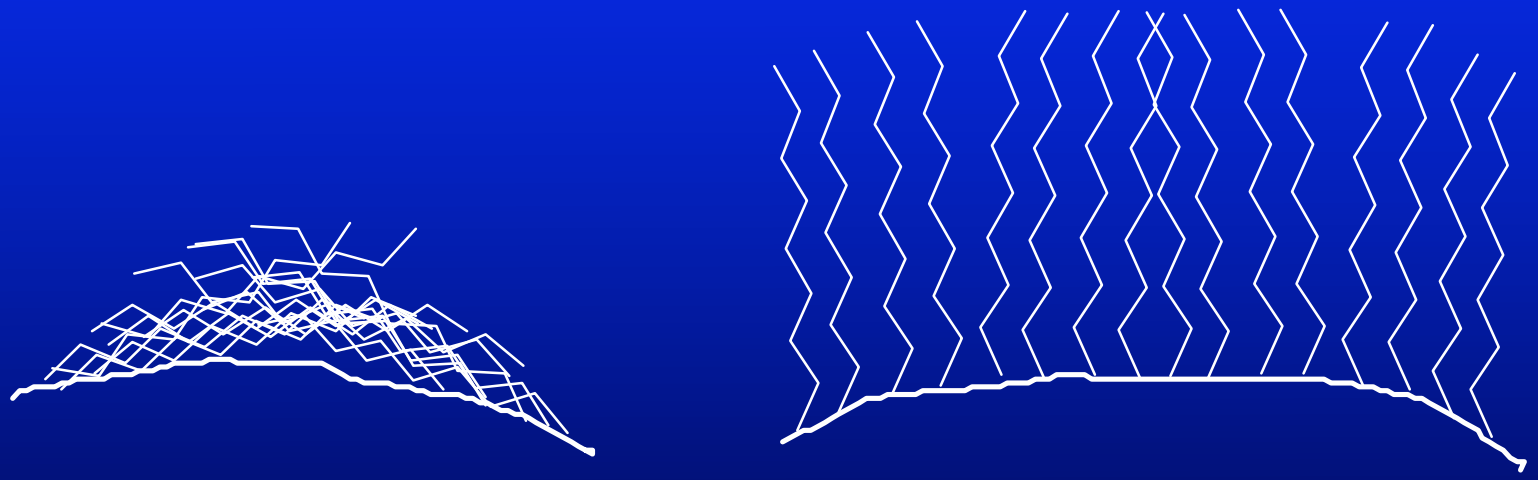
Polar Interactions

Sorbents		Interactions
C N		Dipole/Dipole
NH2		Hydrogen-Bonding
2OH		Hydrogen-Bonding

Ionic Interactions

Sorbents		Interactions
PRS		Electrostatic
CBA		Electrostatic
SAX		Electrostatic

Conditioning



Secondary Silanol Interactions on Non-Polar Phases

Apply Sample	Rinse	Elute
Aqueous, pH 5-7	Acetonitrile	0.5% HCL in Methanol
		

Steps to be Optimized During Method Development

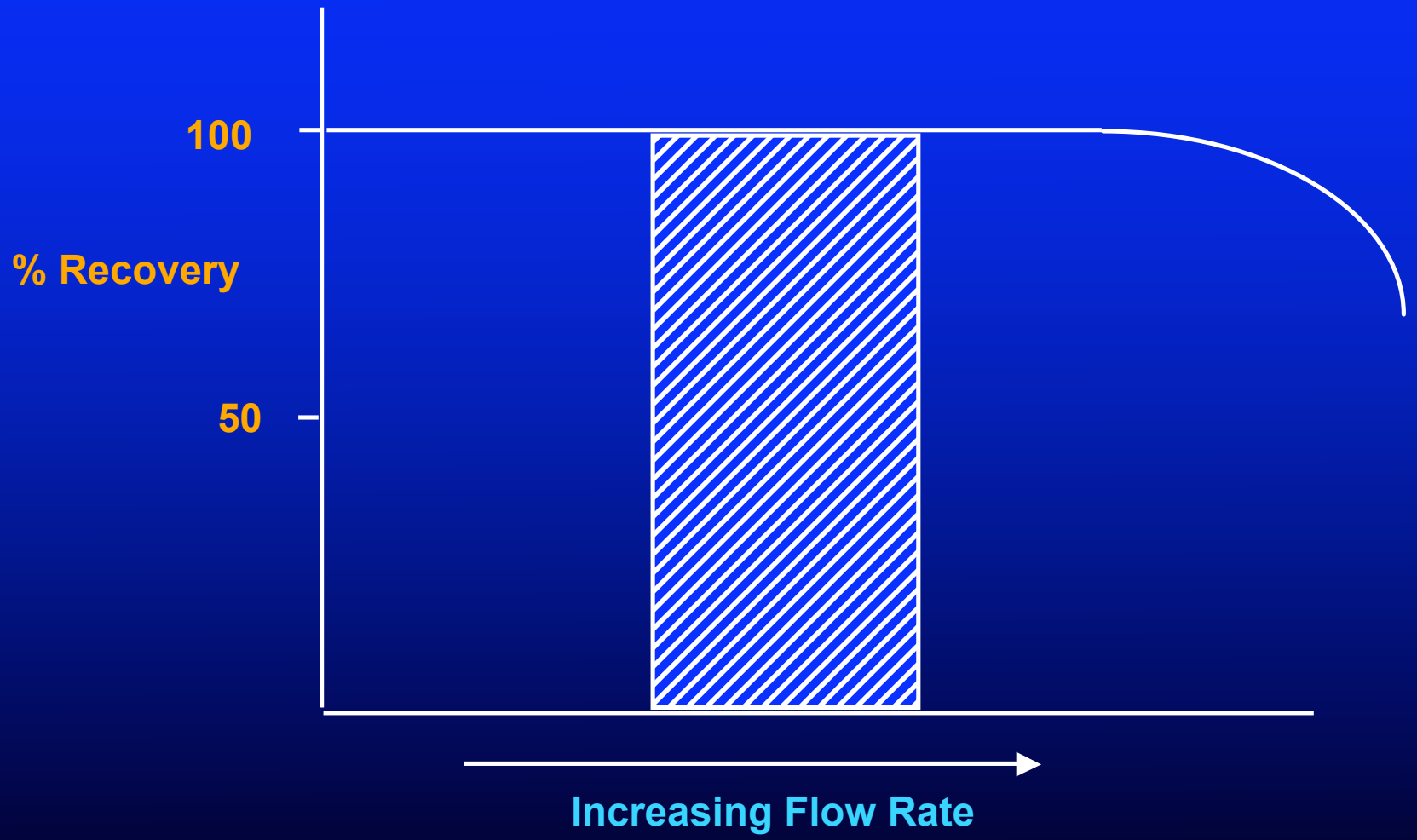
- Sorbent selection
- Sample preparation
- Column conditioning
- Sample loading rate
- Selection of interference elution solvent
- Interference elution solvent flow rate
- Selection of Isolate elution solvent
- Isolate elution solvent flow rate

Flow Rate Considerations

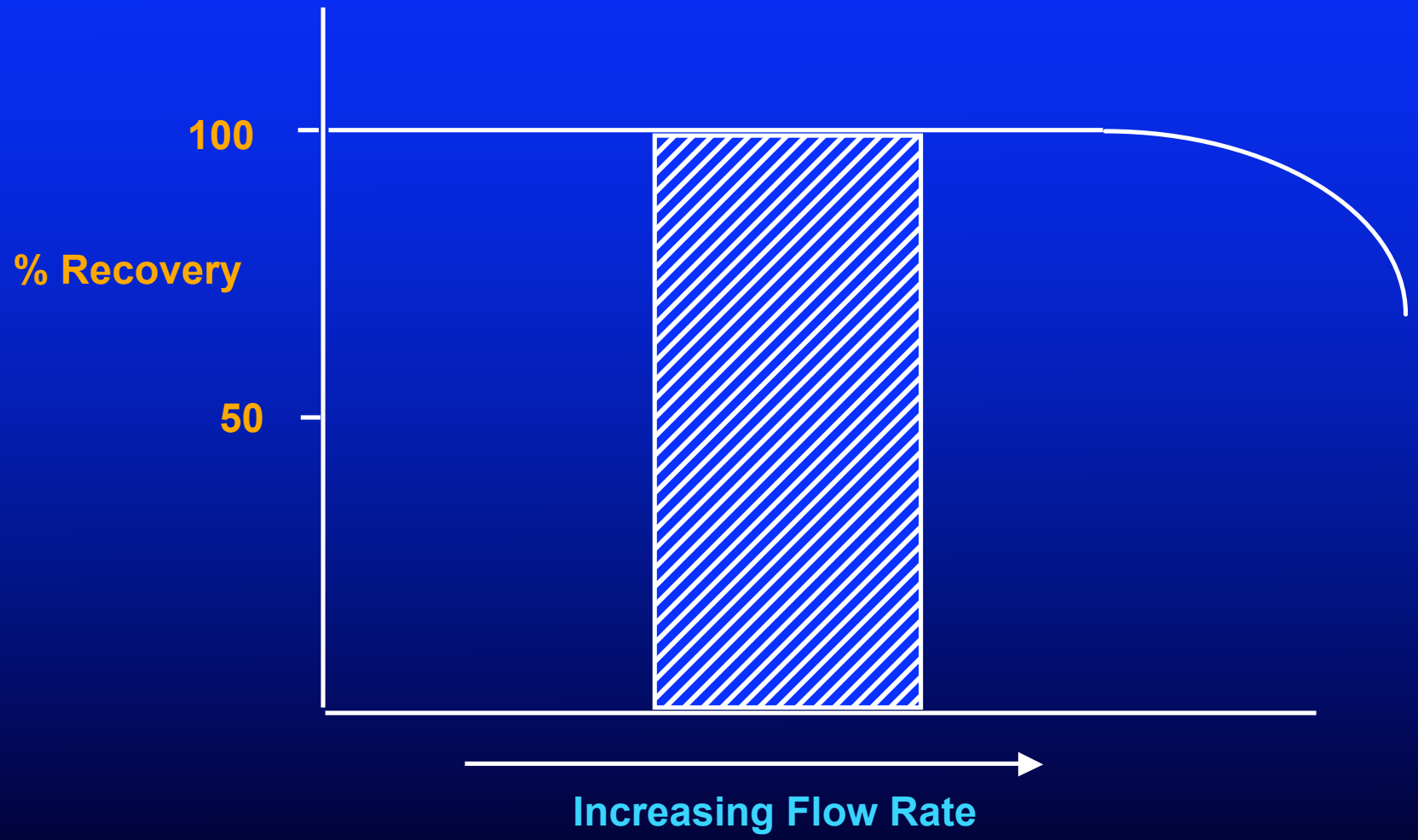
Optimize and Specify in Protocol for

- Column Equilibration
- Counter-ion Exchange
- Sample Loading
- Interference Elution
- Analyte Elution

Sample Loading Flow Rate vs. Extraction Efficiency

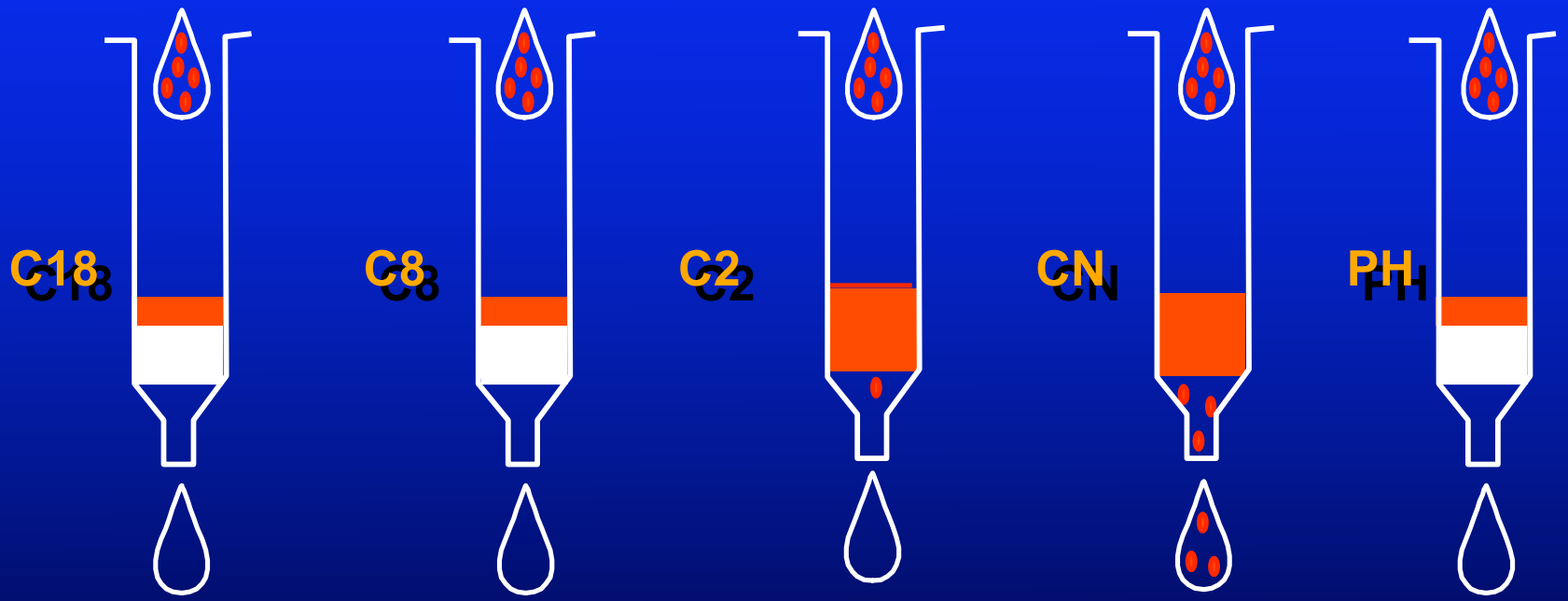


Elution Solvent Flow Rate vs. Extraction Efficiency

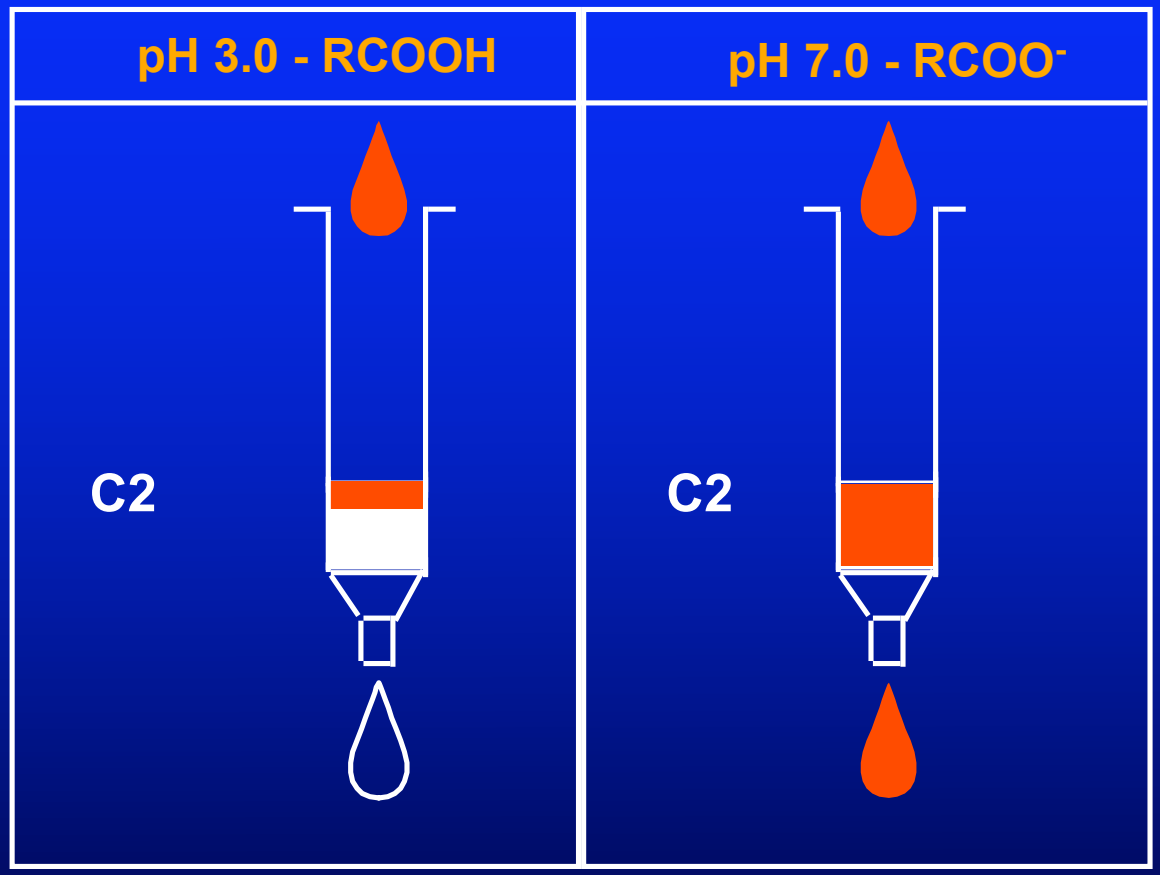


Sorbent Screening

Sample Application



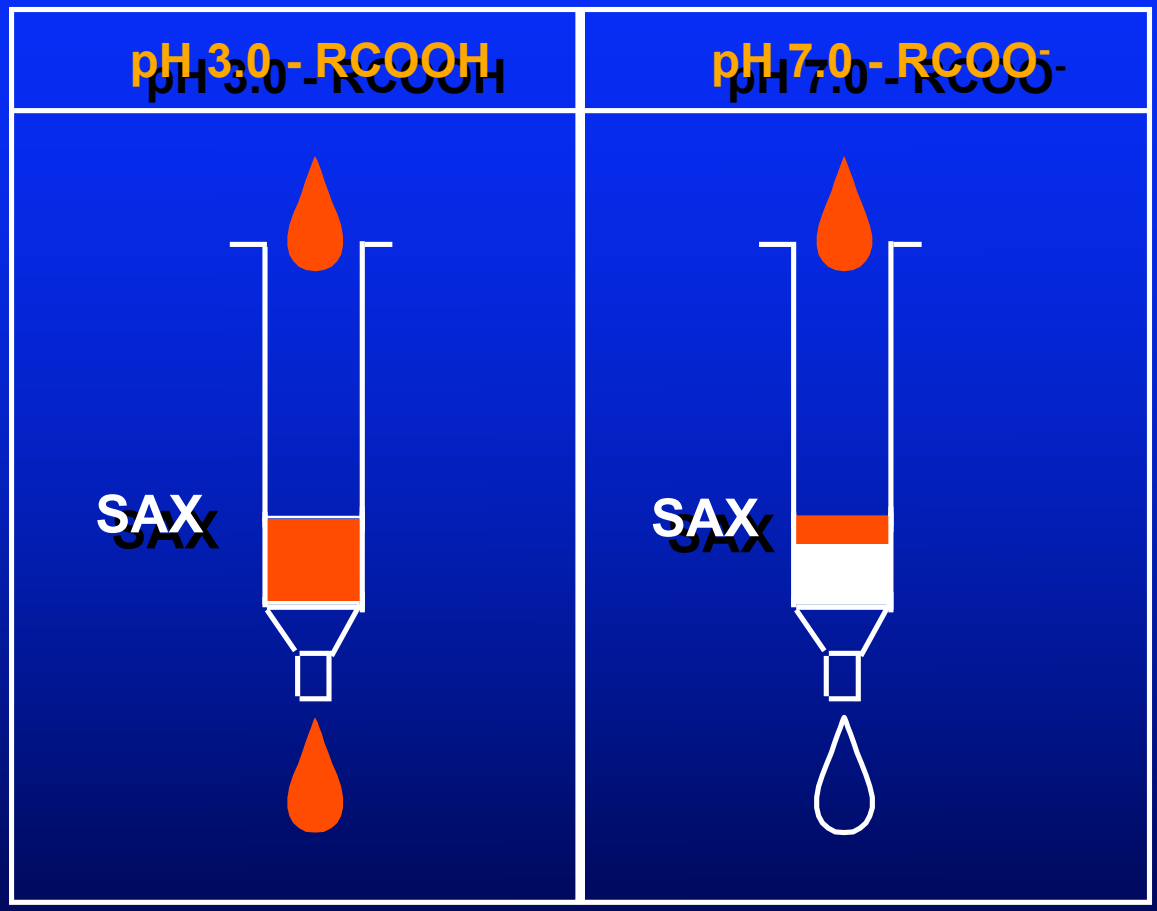
Influence of pH on Non-Polar Retention



Adjust pH to Suppress Ionization

RCOOH pKa = 5.0

Influence of pH on Ionic Retention

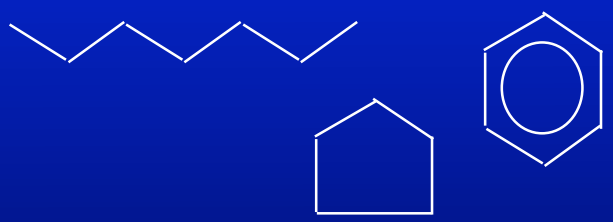


Adjust pH to Ensure Ionization

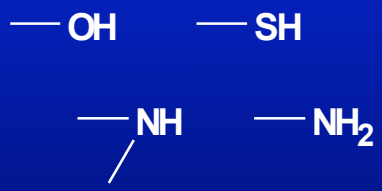
Analyte Characteristics

- Functional Groups
- Solubilities
- pK_a Values
- Stability Characteristics
- Chromatographic Behavior

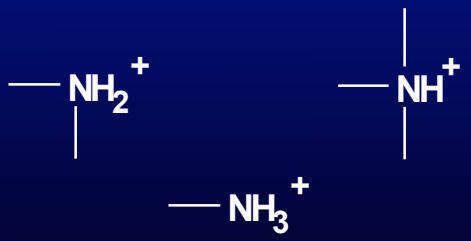
Hydrophobic



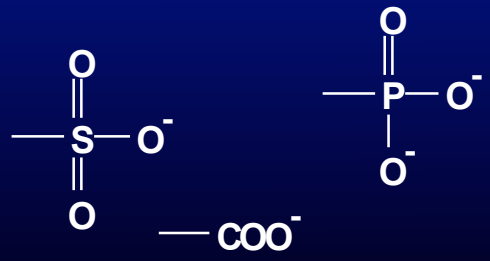
H-Bonding



Cationic

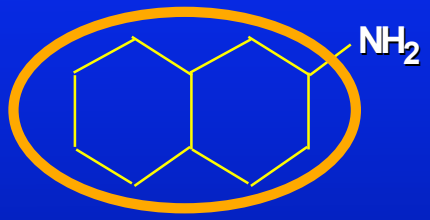


Anionic

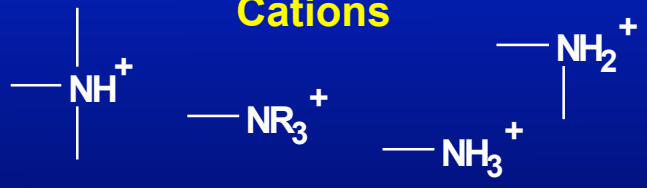
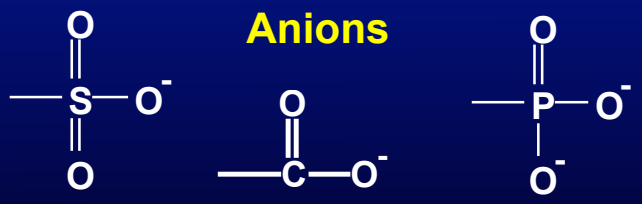


Mechanism Selection

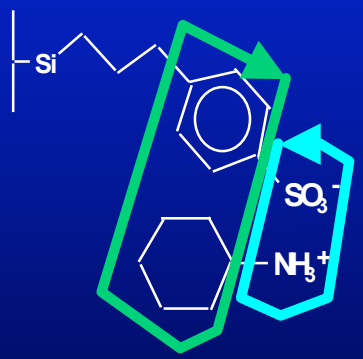
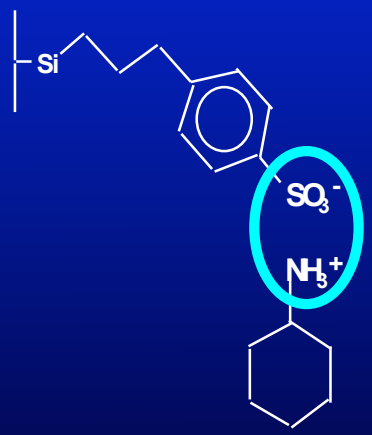
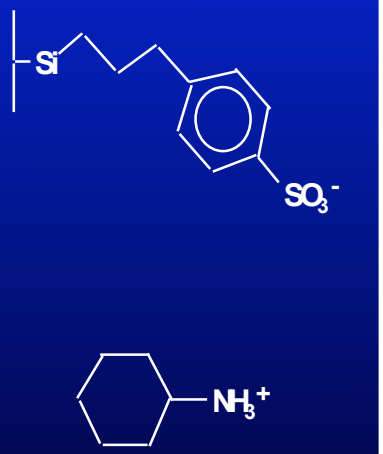
Analyte-Based

Functionality	Analyte	Mechanism
Hydrophobic		Non-Polar
H-Bonding		Polar
Ionic		Ion-Exchange

Mechanism Selection - Summary

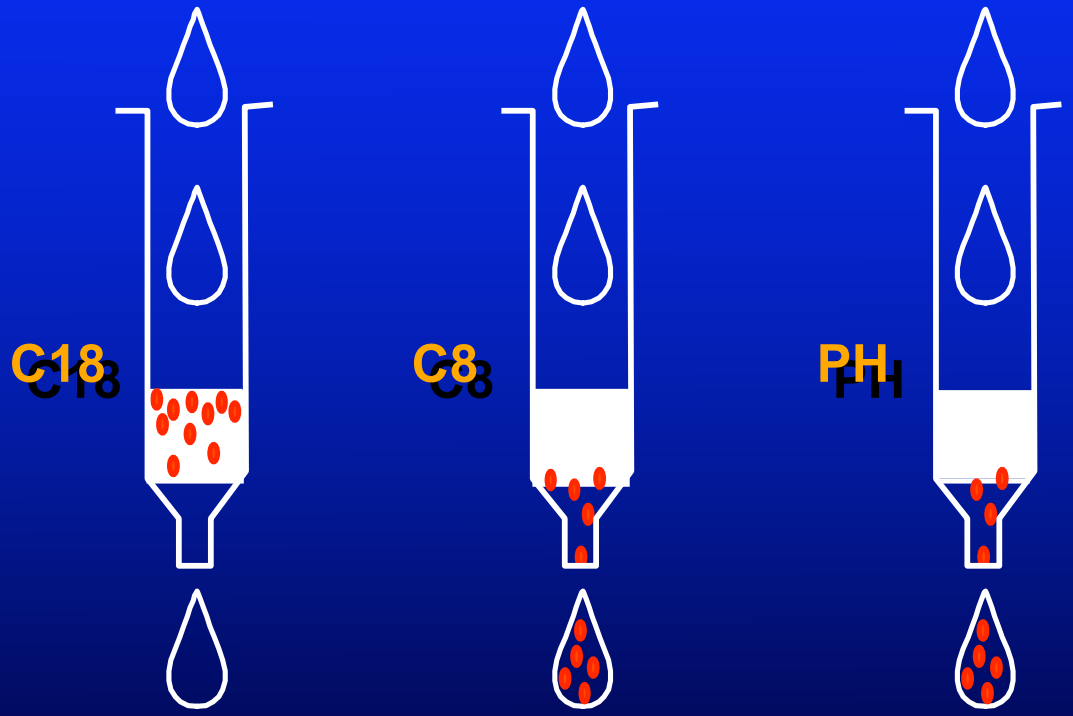
Analyte	Matrix	Sorbent
Hydrophobic 	Aqueous	Non-Polar C18 C8 PH CH C2 CN
H-Bonding 	Aqueous or Non-Polar Solvent	Polar SI NH2 2OH CN
Cations 	Aqueous (Low Ionic Strength) (High ionic strength)	Cation Exchange CBA PRS SCX (ENV+ C18 C8)
Anions 	Aqueous (Low Ionic Strength) (High ionic strength)	Anion Exchange SAX NH2 (ENV+ C18 C8)

Secondary Interactions on Ion Exchange Phases

Apply Sample	Rinse	Rinse	Elute
Aqueous , pH 5-7	Acetonitrile	0.1M K_2HPO_4	Acetonitrile, 0.1M K_2HPO_4 (50:50)
			

Sorbent Screening

Analyte Elution



Validate for Ruggedness

- Vary Solvent Strength by ± 5%
- Vary pH of Ionic Steps
- Change Sample Volumes
- Change Flow Rates
- Verify Linearity
- Check Multiple Sorbent Lots

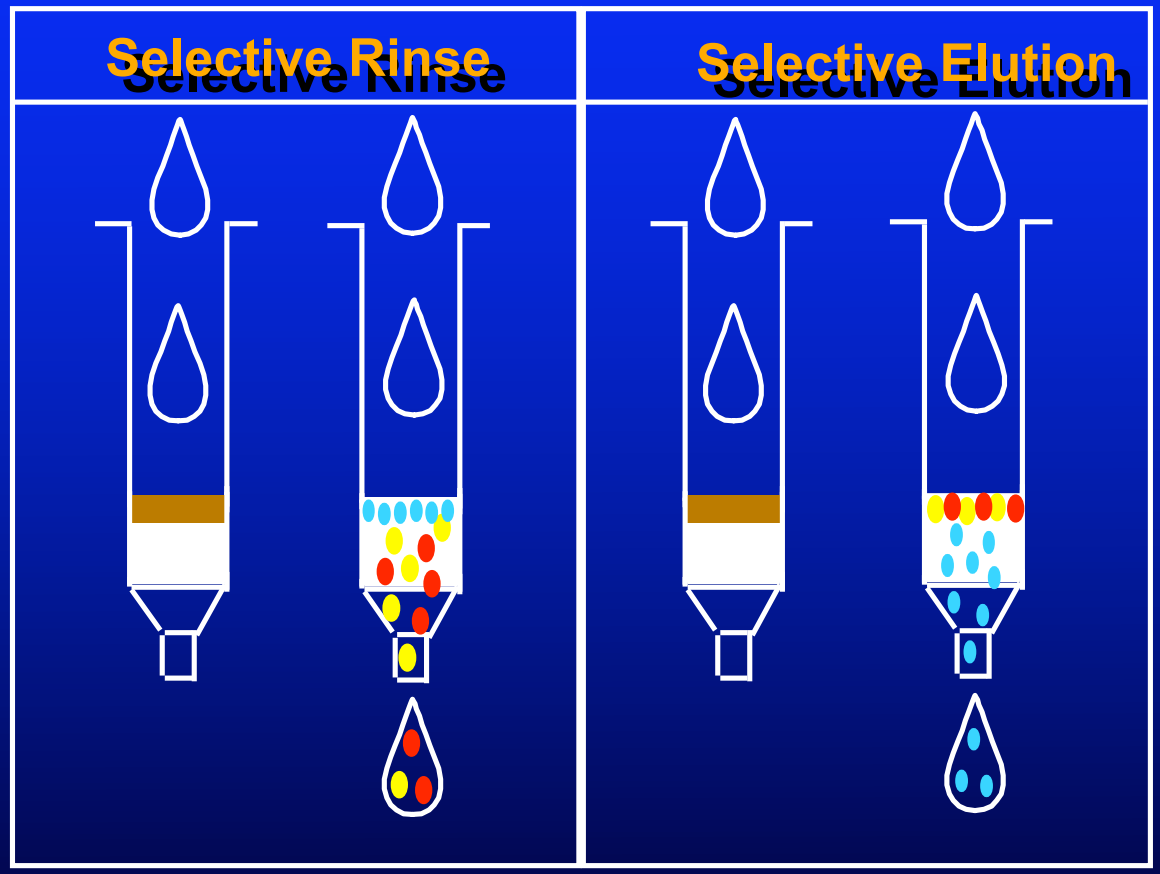
Ionic Combinations

Cation	Anion	pH = 3.0	pH = 7.0	pH = 11.0
Weak	Weak	$-NH_3^+$ HO_2C-	$-NH_3^+ \rightleftharpoons {}^-O_2C-$	$-NH_2$ $^-O_2C-$
Weak	Strong	$-NH_3^+ \rightleftharpoons {}^-O_3S-$	$-NH_3^+ \rightleftharpoons {}^-O_3S-$	$-NH_2$ $^-O_3S-$
Strong	Weak	$-NR_3^+$ HO_2C-	$-NR_3^+ \rightleftharpoons {}^-O_2C-$	$-NR_3^+ \rightleftharpoons {}^-O_2C-$
Strong	Strong	$-NR_3^+ \rightleftharpoons {}^-O_3S-$	$-NR_3^+ \rightleftharpoons {}^-O_3S-$	$-NR_3^+ \rightleftharpoons {}^-O_3S-$

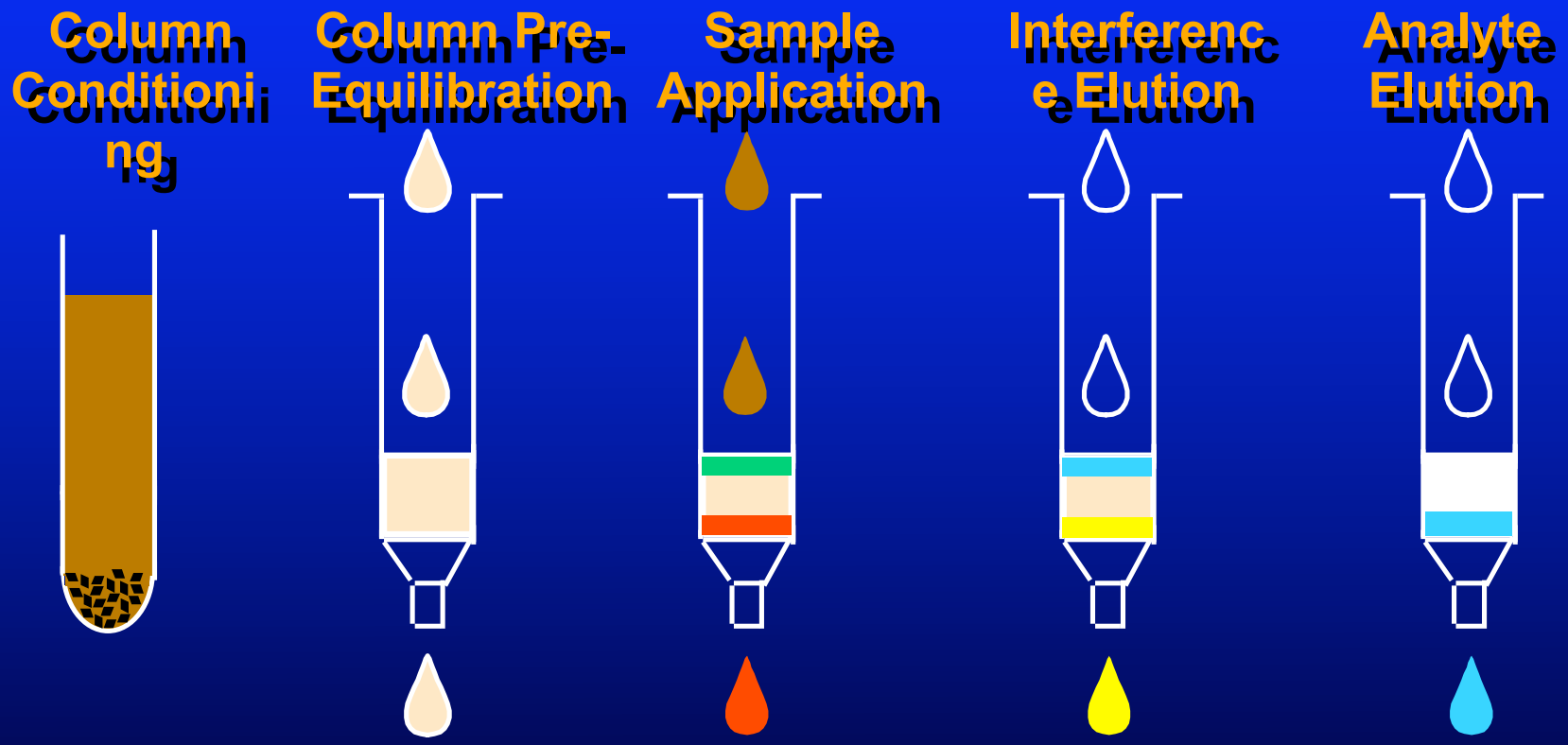
Ion-Exchange Elution

- High Ionic Strength
- pH Change
  - Neutralize Sorbent
  - Neutralize Analyte
- High Selectivity Counter-ions

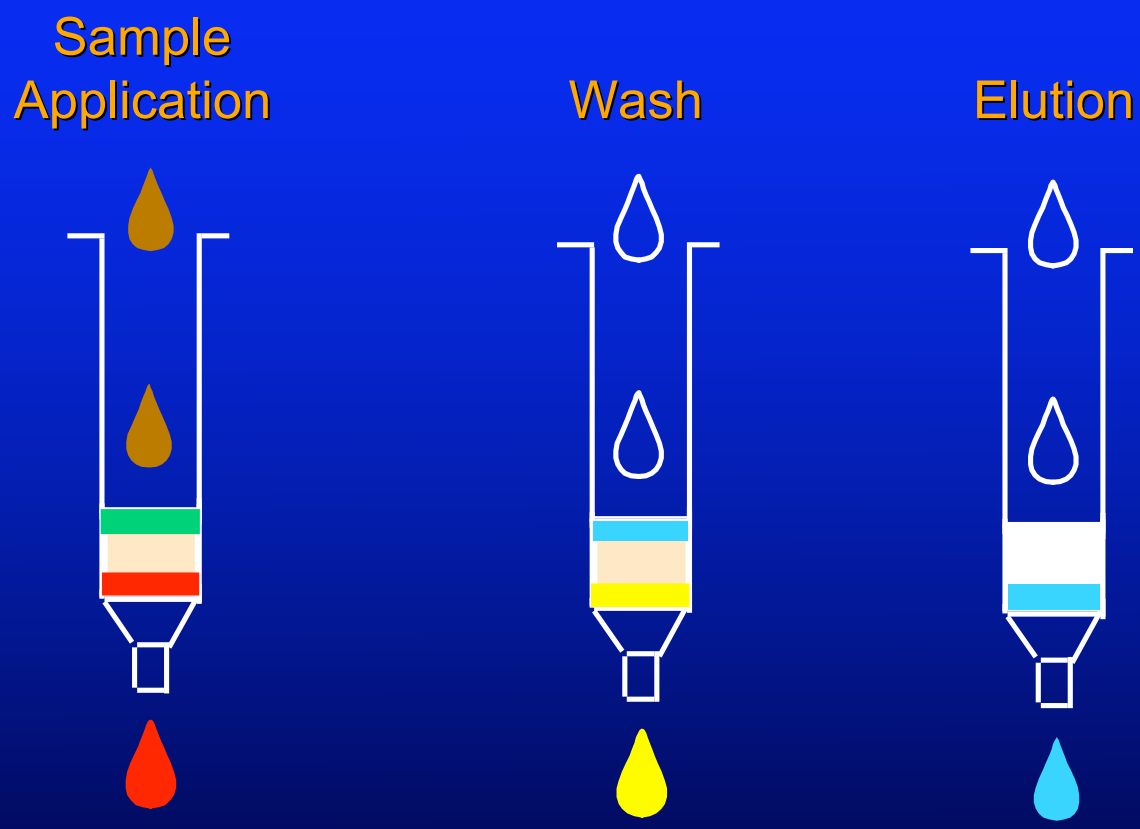
Cleanup Techniques



Typical SPE Procedure



Typical SPE Procedure

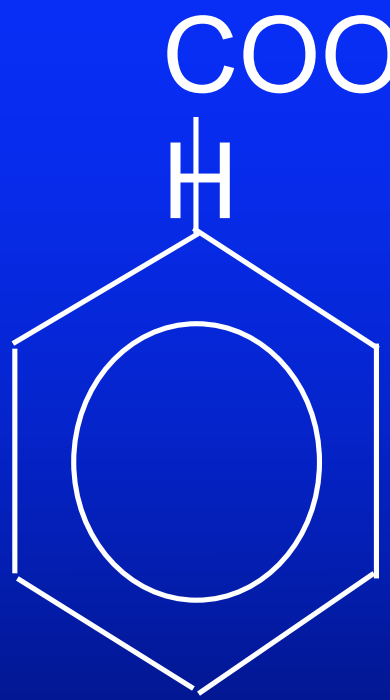


Cleanup Tips

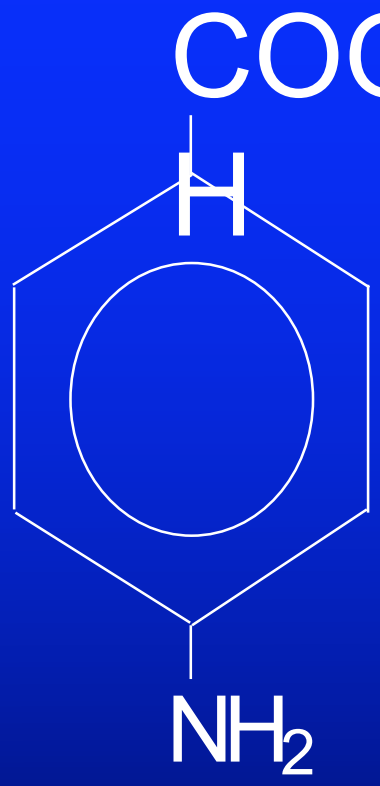
1. Non-retaining sorbents (from Sorbent Screening) are good cleanup sorbents
2. Non-eluting solvents (from Analyte Elution Screening) are good rinse solvents
3. Solvents intermediary between sample and elution solvent are good rinse solvents
4. Solvents in which the analyte(s) are insoluble are good rinse solvents

Capacity & Elution Volume

- Capacity is ~ 1 - 5% of Sorbent Mass
- Minimum Elution Volume is ~ 125 µl per 100 mg Sorbent Mass for Spin Columns
- "Effective" Capacity is increased through Selective Extractions
- If large sorbent mass is required initially, first elution may be reconcentrated on smaller bed



Benzoic acid, pKa 4.80



***p*-Aminobenzoic Acid**

pKa's 4.65 , 4.80

Automation of SPE

- Routine Analysis
- Method Development

A TYPICAL SPE PROCEDURE:

Automation of the six steps

Step One: SAMPLE PRETREATMENT

- pH
- Ionic strength
- Redox
- Wetting / protein binding
- Filtering
- Mixing

A TYPICAL SPE PROCEDURE:
Automation of the six steps

Step Two: COLUMN SOLVATION

- Variable volumes
- Variable flow rates
- Multiple solvents

A TYPICAL SPE PROCEDURE:
Automation of the six steps

Step Three: COLUMN EQUILIBRATION

- Multiple solvents
- Variable volumes
- Variable flows

A TYPICAL SPE PROCEDURE:
Automation of the six steps

Step Four: SAMPLE APPLICATION

- Variable volumes
- Variable flow rate
- Multiple collection options

A TYPICAL SPE PROCEDURE: Automation of the six steps

Step Five: INTERFERENCE ELUTION

- Multiple solvents
- Variable volumes
- Variable flow rates
- Multiple collection options
- Drying options
  - Volume
  - Flow

A TYPICAL SPE PROCEDURE: Automation of the six steps

Step Six: ANALYTE ELUTION

- Multiple solvents
- Variable volumes
- Variable flow rates