

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 - - - - H₂O

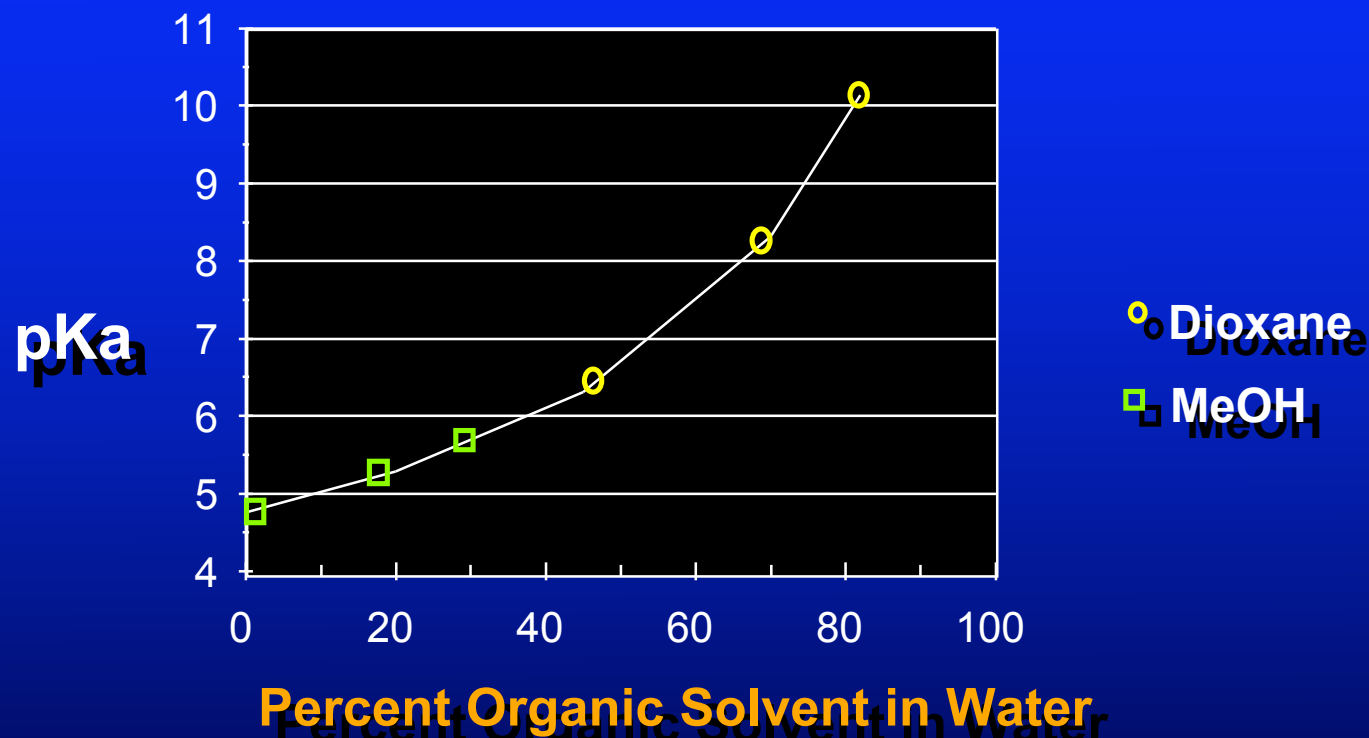
Hydrocarbon

H₂O - - - - - MeOH - - - - Hexane

Bonded silica

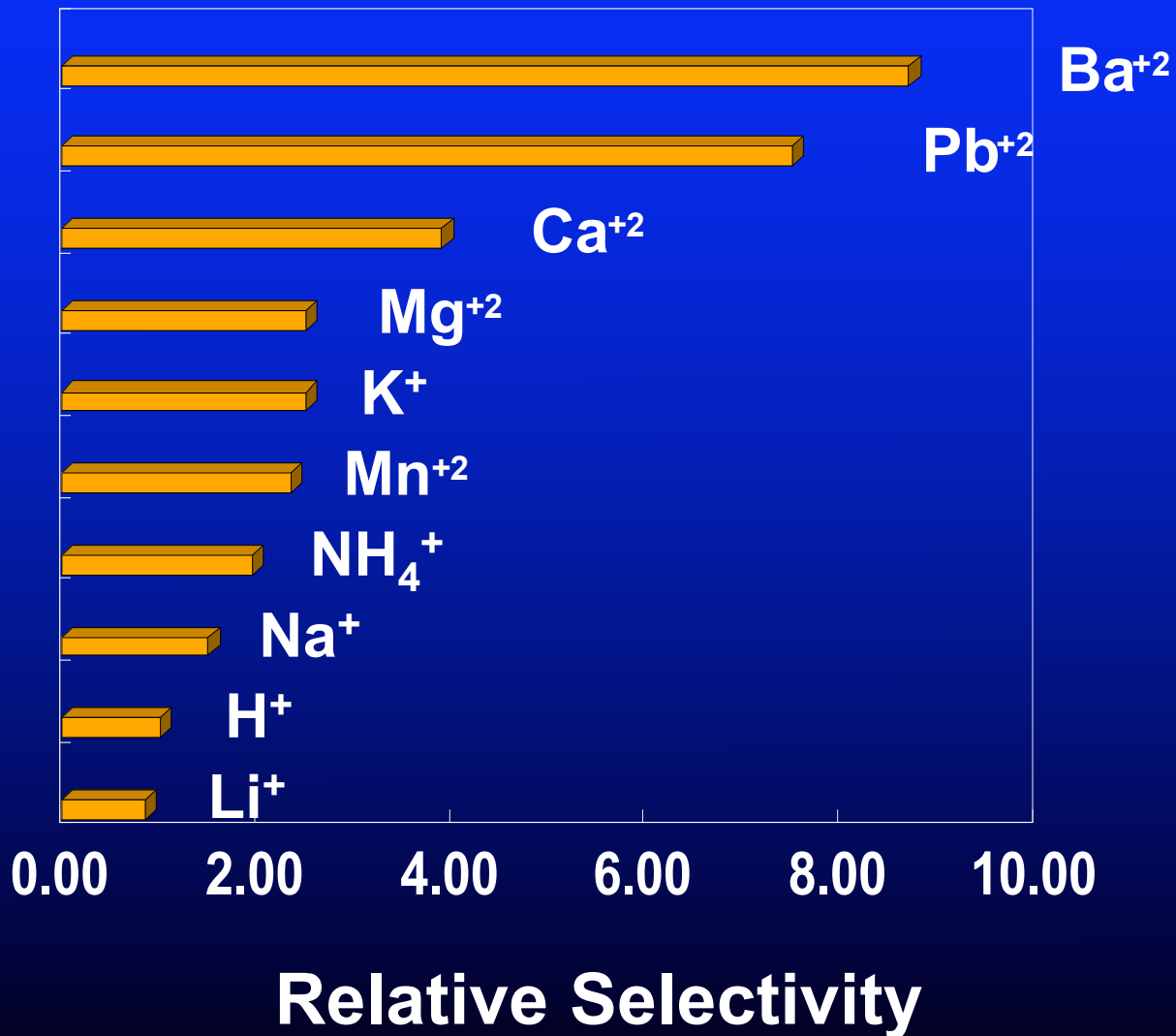
H₂O <<: MeOH - - - - H₂O: >> MeOH

Effect of Solvent on pKa of HOAc at 25 C

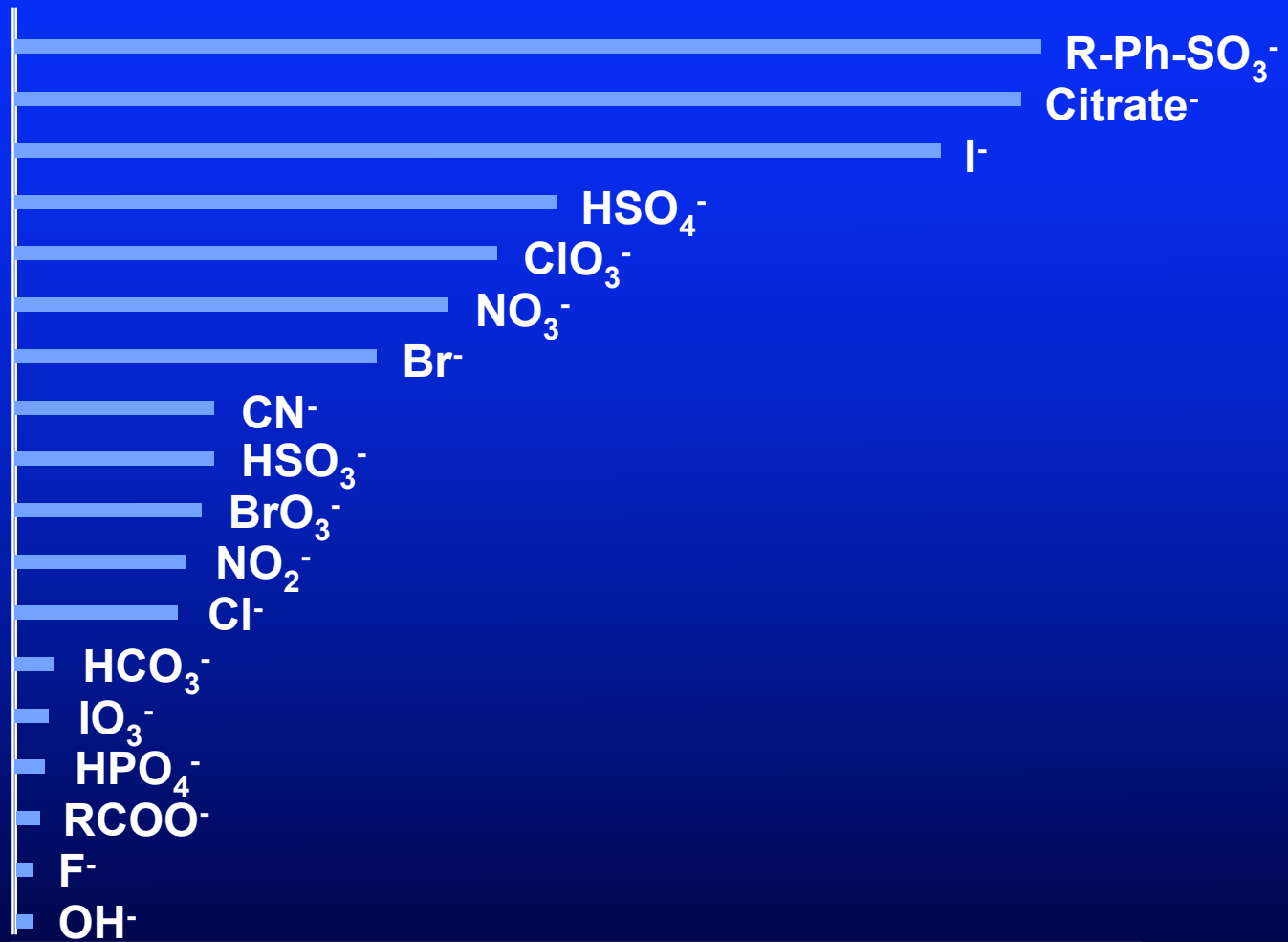


Reference: Hendrickson, Cram and Hammond,
"Organic Chemistry", 3rd Edition McGraw Hill p 303

SCX / PRS Counter-ion Selectivity



SAX Counter- ion Selectivity



Relative 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



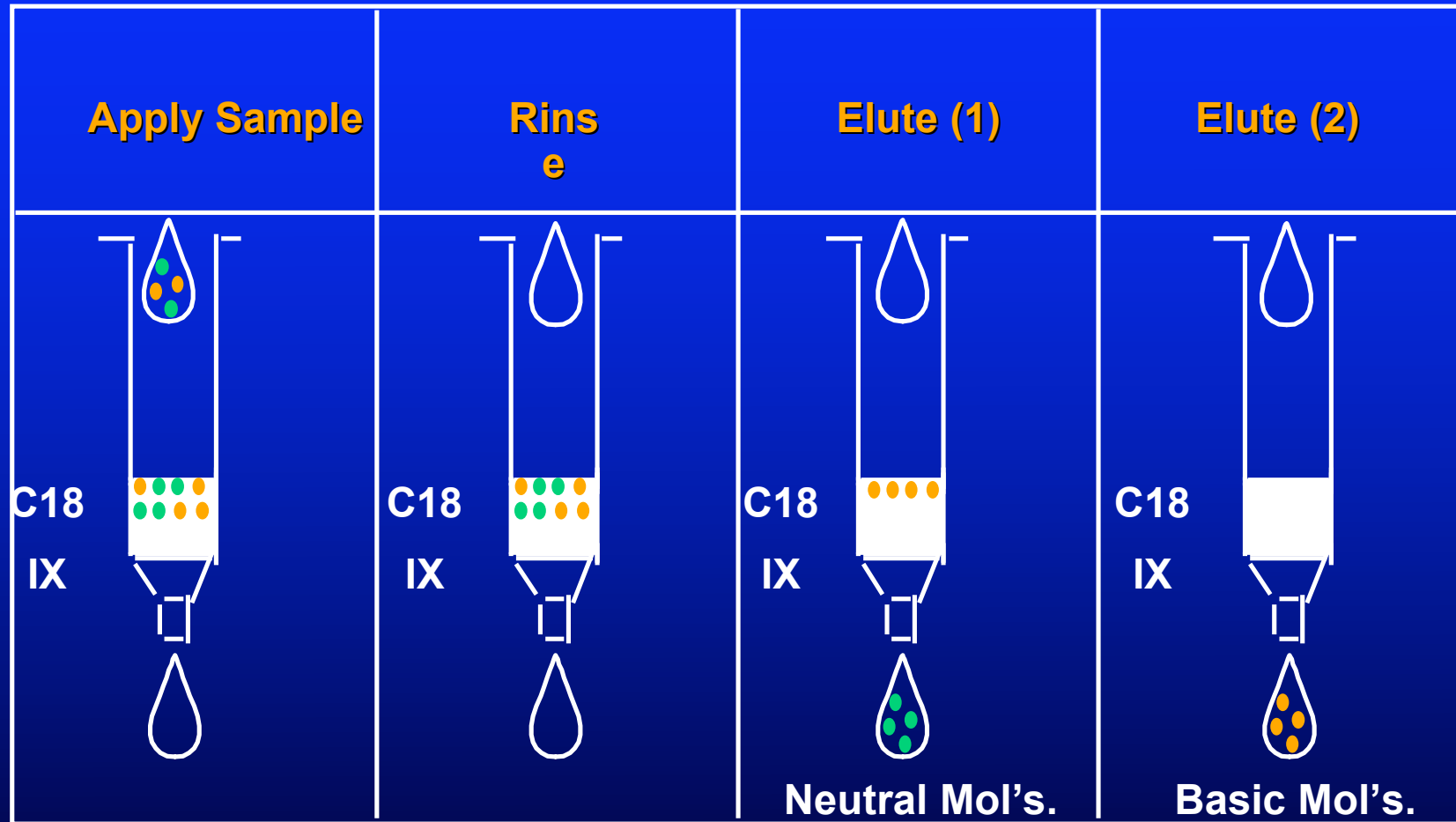
Contaminant Elution

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

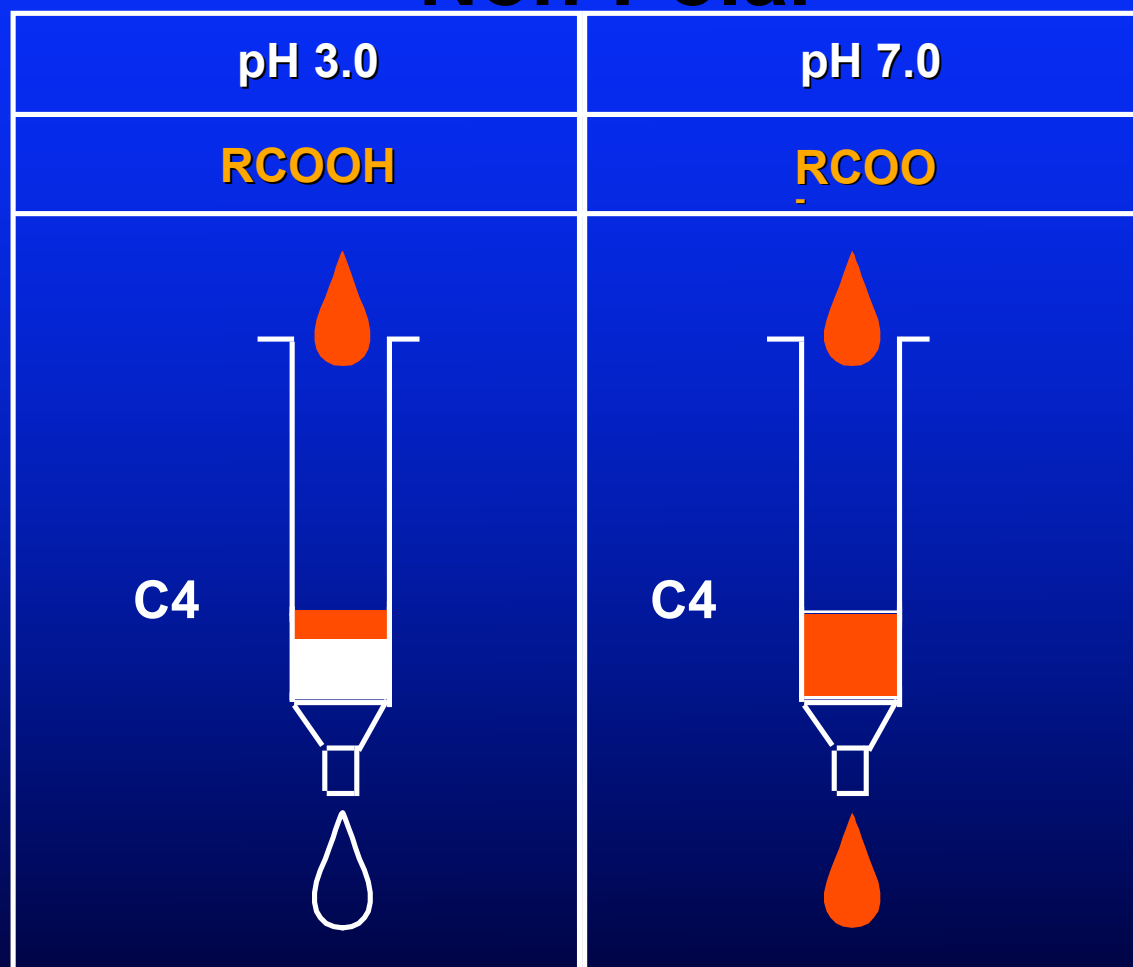
Why use Mixed-mode Columns?

- **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

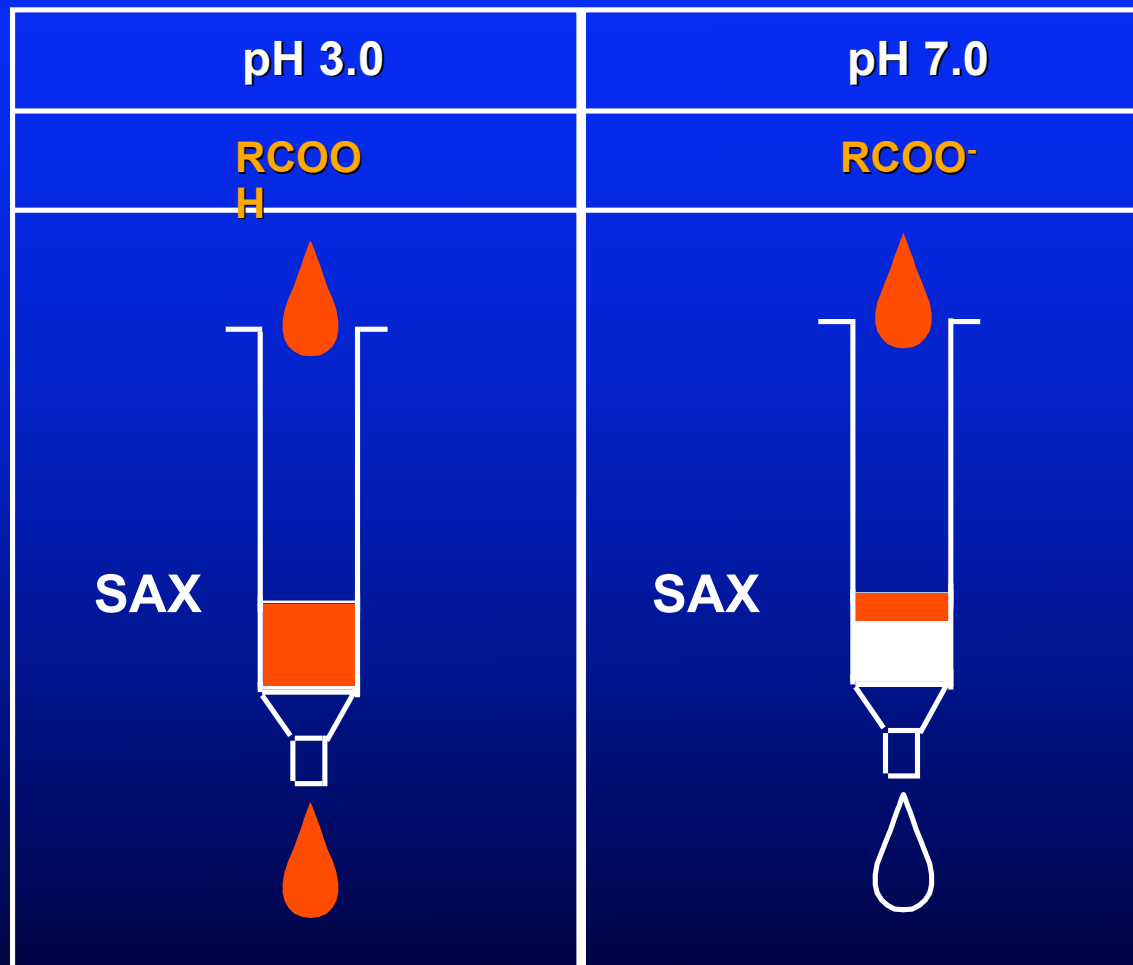


Influence of pH on Retention Non-Polar



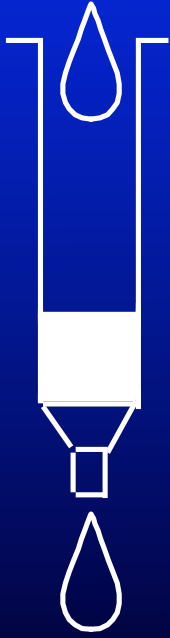


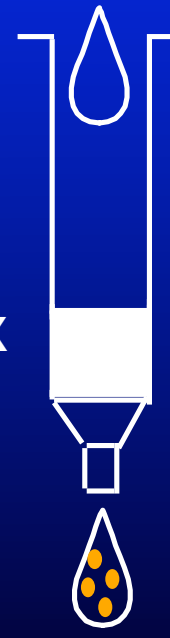
Adjust pH to Suppress Ionization
RCOOH pKa = 5.0

Influence of pH on Retention Ion Exchange



Adjust pH to Ensure Ionization

Anion Exchange

| Condition | Apply Sample | Interference Elution | Analyte Elution |
|---|---|---|--|
| <p>1. Methanol 2. Water or Buffer</p> | <p>2 pH Units Above Isolate pKa</p> | <p>Water or Buffer</p> | <p>2 pH Units Below pKa (500mM H₂SO₄)</p> |
| <p>SAX</p>  | <p>SAX</p>  | <p>SAX</p>  | <p>SAX</p>  <p>Homovanillic Acid</p> |

Rumsby, et. al.

Weak Anion Exchange (NH₂)

Analyte: Weak Anion, pKa 5.0

Sorbent: NH₂, pKa 9.8

Analyte: Strong Anion

Sorbent: NH₂, pKa 9.8

| | pH 3 | pH 7 | pH 11 |
|------------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Analyte: Weak Anion, pKa 5.0 | R-COOH | R-COO ⁻ | R-COO ⁻ |
| Sorbent: NH ₂ , pKa 9.8 | R-NH ₃ ⁺ | R-NH ₃ ⁺ | R-NH ₂ |
| Analyte: Strong Anion | R-SO ₃ ⁻ | R-SO ₃ ⁻ | R-SO ₃ ⁻ |
| Sorbent: NH ₂ , pKa 9.8 | R-NH ₃ ⁺ | R-NH ₃ ⁺ | R-NH ₂ |

Strong Anion Exchange (SAX)

Analyte: Weak Anion, pKa 5.0

Sorbent: SAX

Analyte: Strong Anion

Sorbent: SAX

| pH 3 | pH 7 | pH 11 |
|--------------------------------|--------------------------------|--------------------------------|
| R-COOH | R-COO ⁻ | R-COO ⁻ |
| R-NR ₃ ⁺ | R-NR ₃ ⁺ | R-NR ₃ ⁺ |
| R-SO ₃ ⁻ | R-SO ₃ ⁻ | R-SO ₃ ⁻ |
| R-NR ₃ ⁺ | R-NR ₃ ⁺ | R-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 <ul style="list-style-type: none">– Hydrogen-Bonding– Dipole-Dipole– π-π | 3-7 kcal/mole |
| • Non-Polar Interactions <ul style="list-style-type: none">– Van der Waals or dispersion | 1-2 kcal/mole |

Extraction Components

Matrix (Sample or Solvent)



Analyte(s)

Sorbent

Retention/Elution

Retention

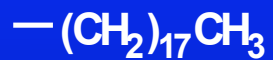
Elution

Analyte \leftrightarrow Sorbent Analyte \leftrightarrow Sorbent

Matrix \leftrightarrow Sorbent Matrix \leftrightarrow Sorbent

Analyte \leftrightarrow Matrix Analyte \leftrightarrow Matrix

Non-Polar Sorbents



Octadecyl



Octyl



Ethyl



Phenyl



Cyclohexyl



Cyanopropyl

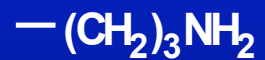
Polar Sorbents



Silica



Diol

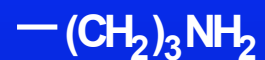


Aminopropyl

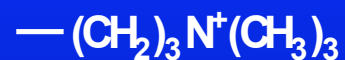


Cyanopropyl

Ion-Exchange Sorbents



Aminopropyl



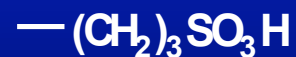
SAX (quaternary amine)



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:

$$V_r = V_m + KV_s$$

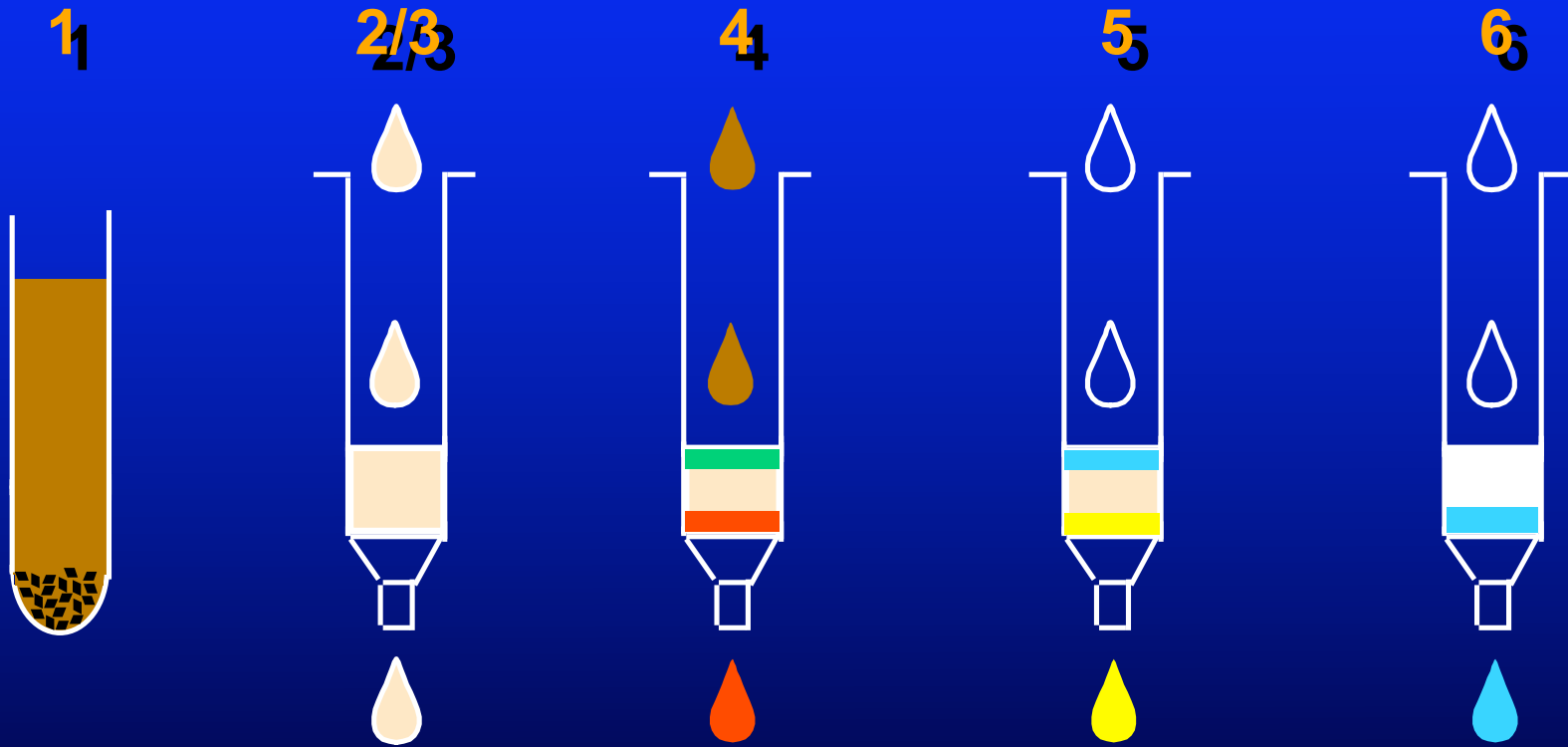
$$0.2 \ll K \ll 200$$

- Extraction: SPE Chromatography:

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

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

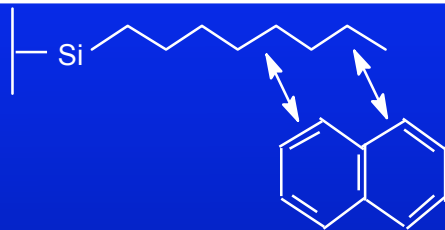

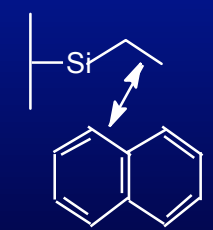
Six-Step SPE Procedure



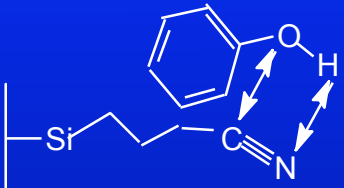
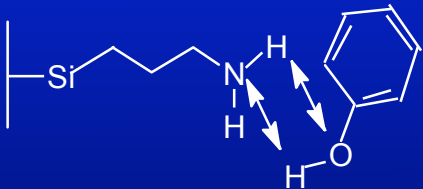
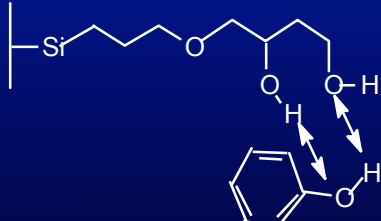
A Typical SPE Procedure Involves Six Steps

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- 6. Analyte elution**

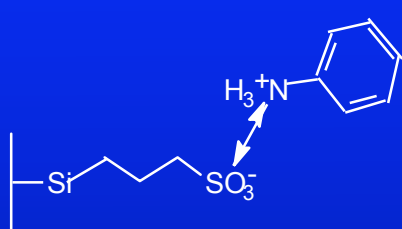
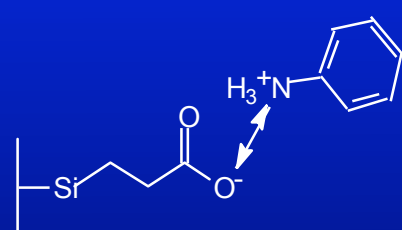
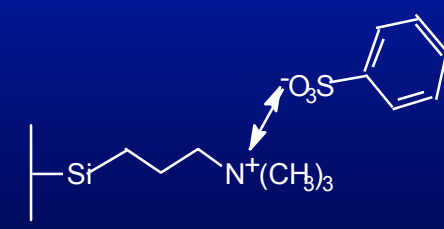
Non-Polar Interactions

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

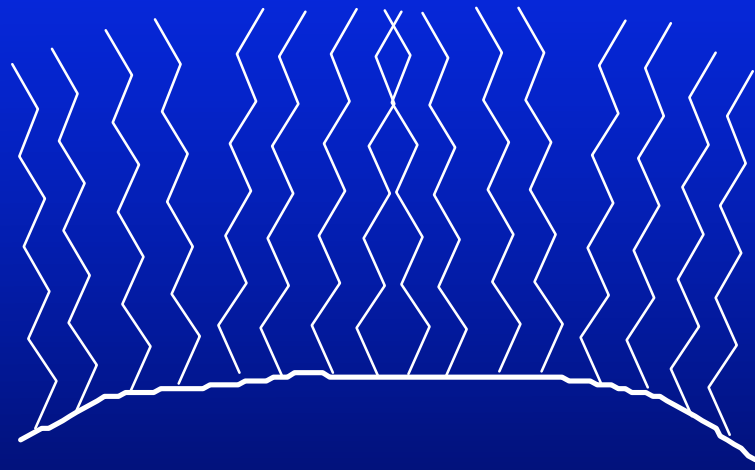
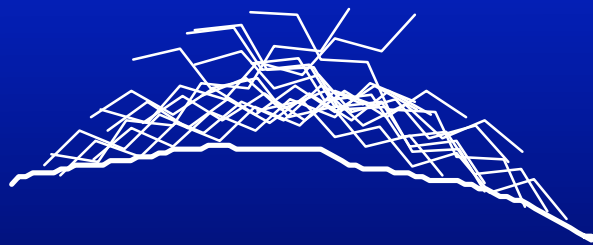
Polar Interactions

| | Sorbents | Interactions |
|-----------------------|---|-------------------------|
| C N |  <p>The diagram shows a silicon atom bonded to a propyl chain, which is further bonded to a cyano group (-C≡N). This cyano group is positioned near a phenol ring (benzene ring with an -OH group). Dashed arrows indicate dipole-dipole interactions between the carbon and nitrogen atoms of the cyano group and the oxygen and hydrogen atoms of the phenol group.</p> | Dipole/Dipole |
| NH₂ |  <p>The diagram shows a silicon atom bonded to a propyl chain, which is further bonded to a primary amine group (-NH₂). This amine group is positioned near a phenol ring. Dashed arrows indicate hydrogen bonding interactions between the hydrogen atoms of the amine group and the oxygen and hydrogen atoms of the phenol group.</p> | Hydrogen-Bonding |
| 2OH |  <p>The diagram shows a silicon atom bonded to a propyl chain, which is further bonded to an ether linkage (-O-), followed by a chain containing two hydroxyl groups (-OH). This dihydroxy chain is positioned near a phenol ring. Dashed arrows indicate hydrogen bonding interactions between the hydroxyl groups of the chain and the phenol group.</p> | Hydrogen-Bonding |

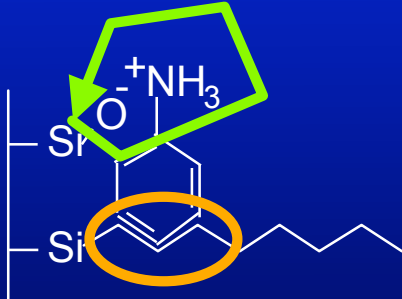


Ionic Interactions

| | Sorbents | Interactions |
|------------|---|----------------------|
| PRS |  <p>Chemical structure of PRS sorbent: A silane chain (Si) is bonded to a propyl chain, which is terminated by a sulfonate group (SO_3^-). This sulfonate group is shown interacting with a protonated benzylamine cation ($\text{H}_3^+\text{N}-\text{Ph}$).</p> | Electrostatic |
| CBA |  <p>Chemical structure of CBA sorbent: A silane chain (Si) is bonded to a propyl chain, which is terminated by a carboxylate group (COO^-). This carboxylate group is shown interacting with a protonated benzylamine cation ($\text{H}_3^+\text{N}-\text{Ph}$).</p> | Electrostatic |
| SAX |  <p>Chemical structure of SAX sorbent: A silane chain (Si) is bonded to a propyl chain, which is terminated by a trimethylammonium cation ($\text{N}^+(\text{CH}_3)_3$). This cation is shown interacting with a sulfonate anion ($\text{O}_3\text{S}-\text{Ph}$).</p> | Electrostatic |

Conditioning



Secondary Silanol Interactions on Non-Polar Phases

| Apply Sample | Rinse | Elute |
|---|--|--|
| <p data-bbox="317 643 688 688">Aqueous, pH 5-7</p>  | <p data-bbox="919 643 1178 688">Acetonitrile</p>  | <p data-bbox="1402 643 1892 688">0.5% HCL in Methanol</p>  |

Steps to be Optimized During Method Development

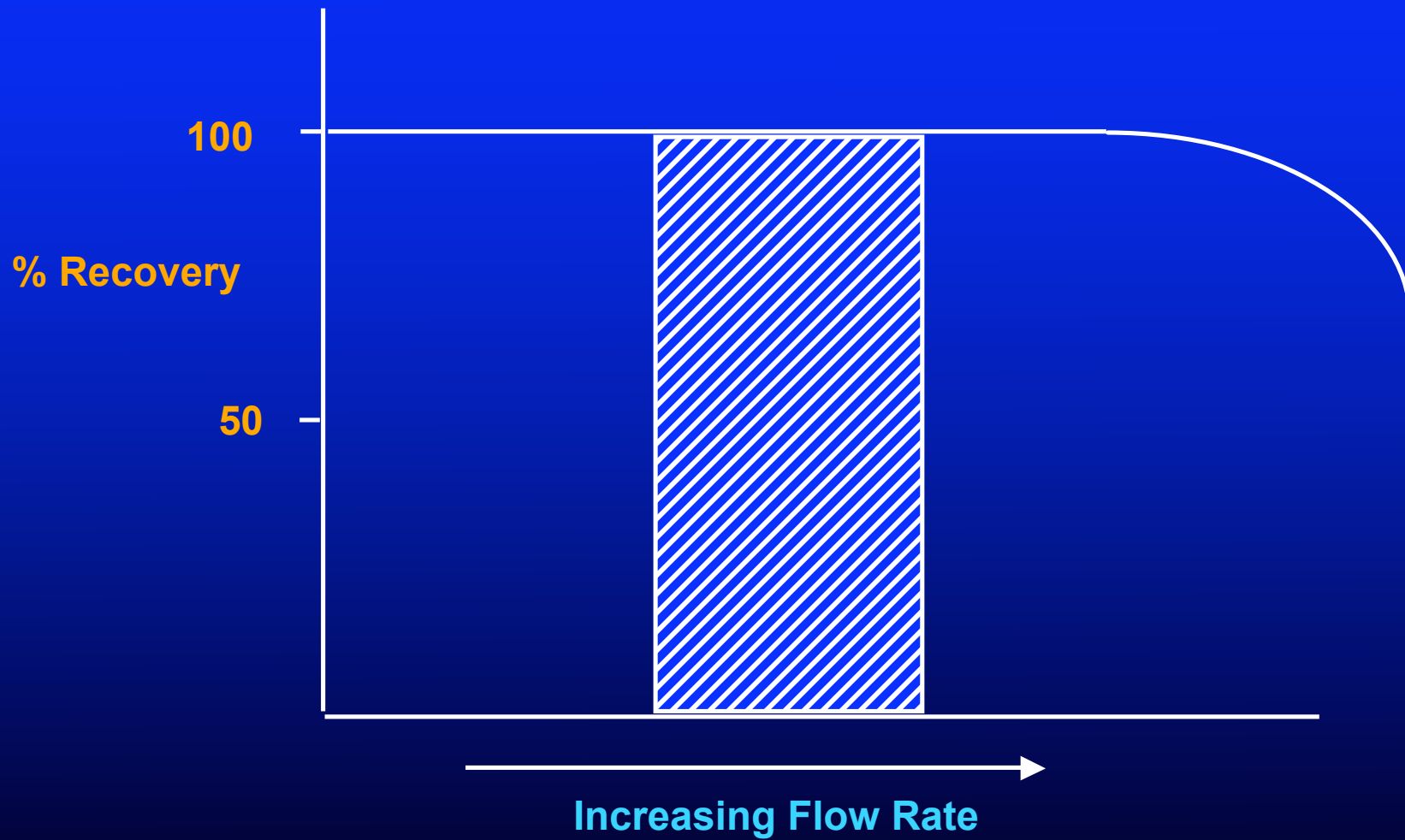
- Sorbent selection
- Sample preparation
- Column conditioning
- Sample loading rate
- Selection of interference elution solvent
- Interference elution solvent flow rate
- Selection of Isolate elution solvent
- Isolate elution solvent flow rate

Flow Rate Considerations

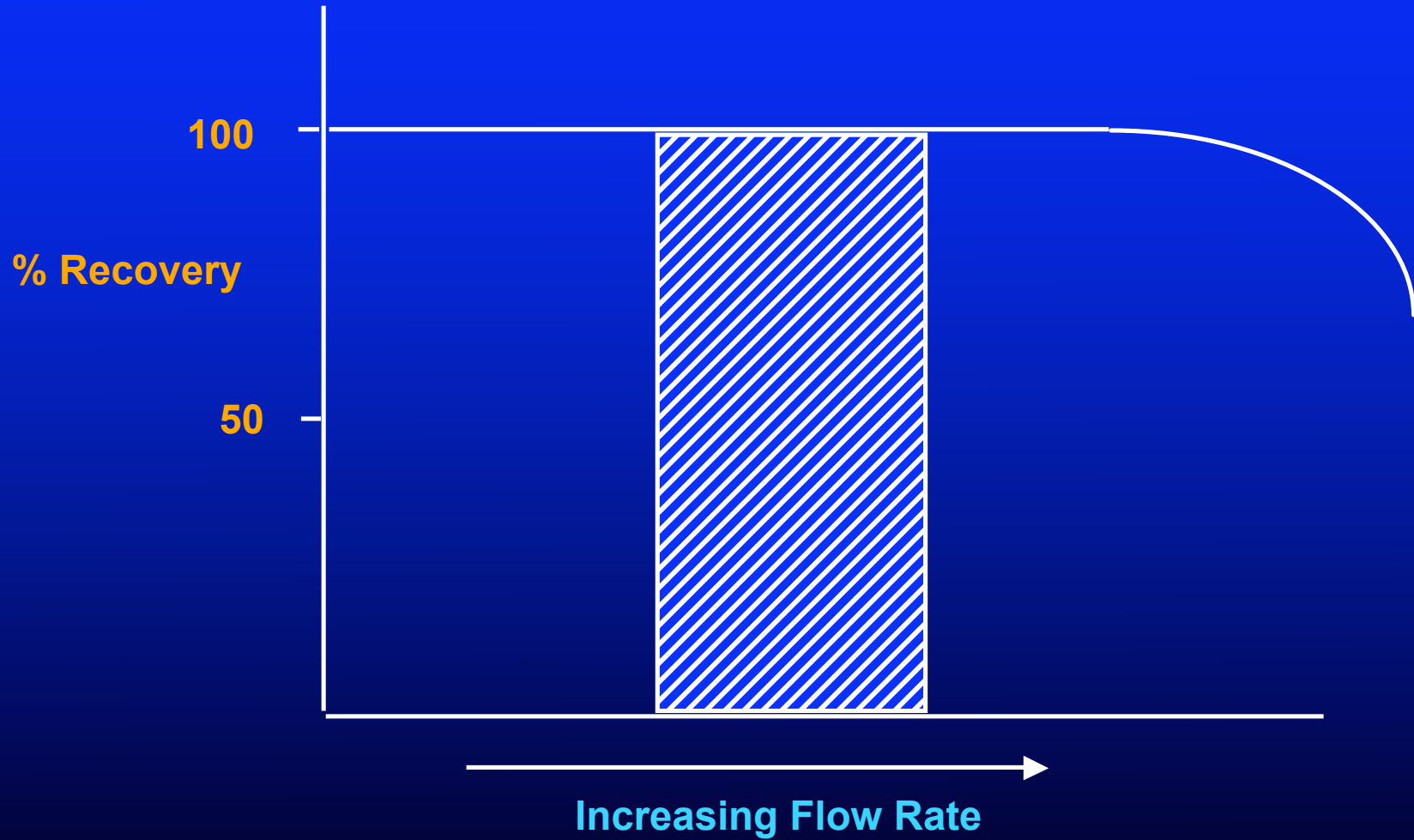
Optimize and Specify in Protocol for

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

Sample Loading Flow Rate vs. Extraction Efficiency

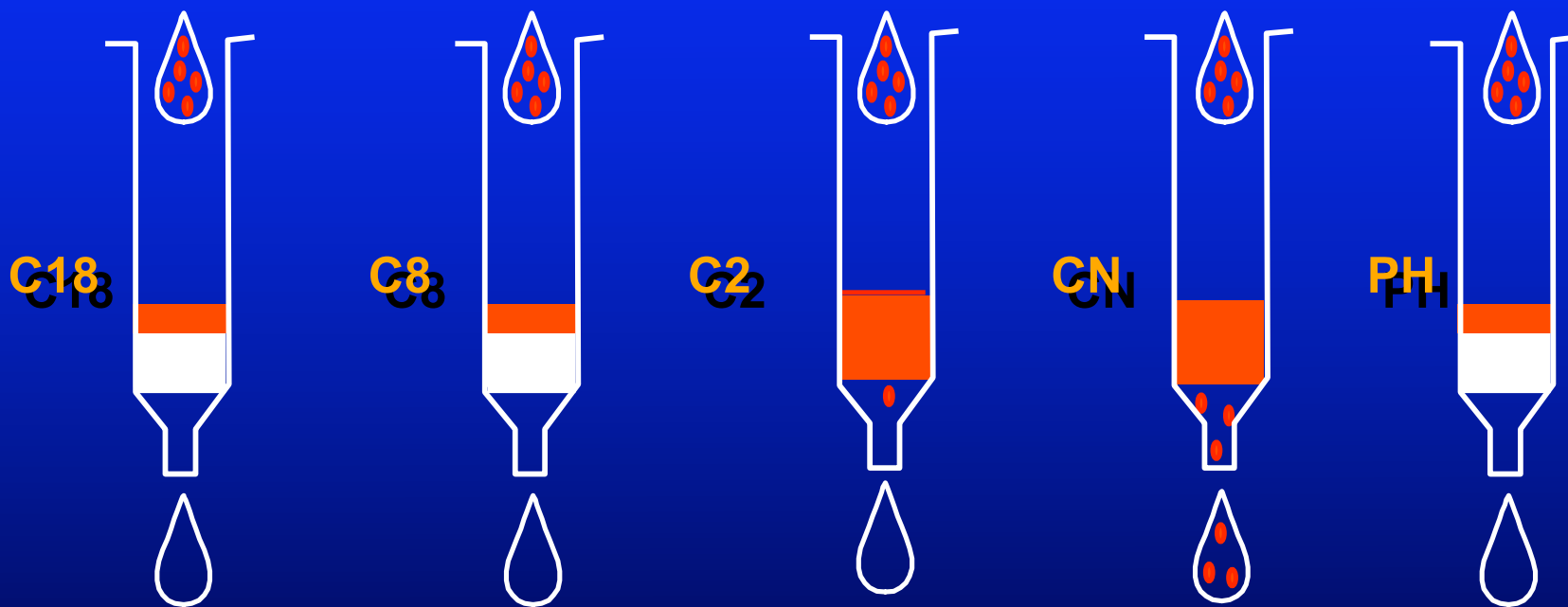


Elution Solvent Flow Rate vs. Extraction Efficiency

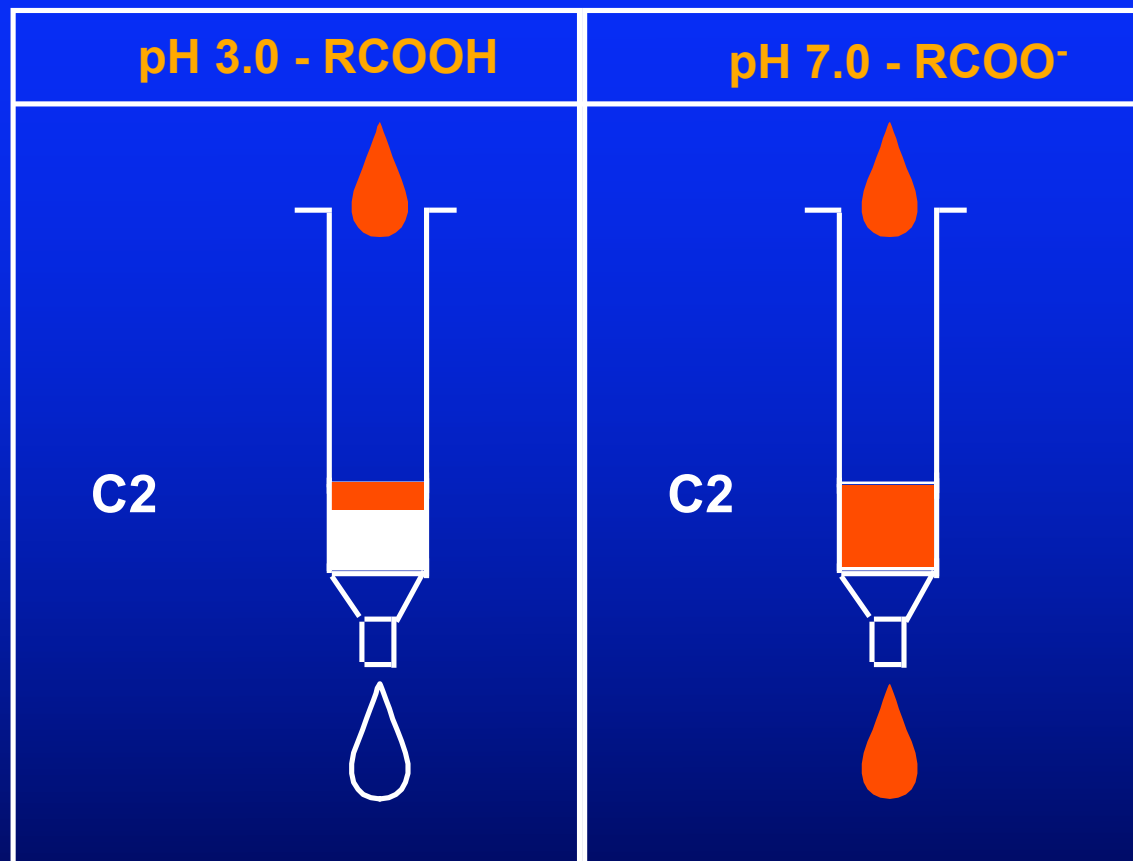


Sorbent Screening

Sample Application

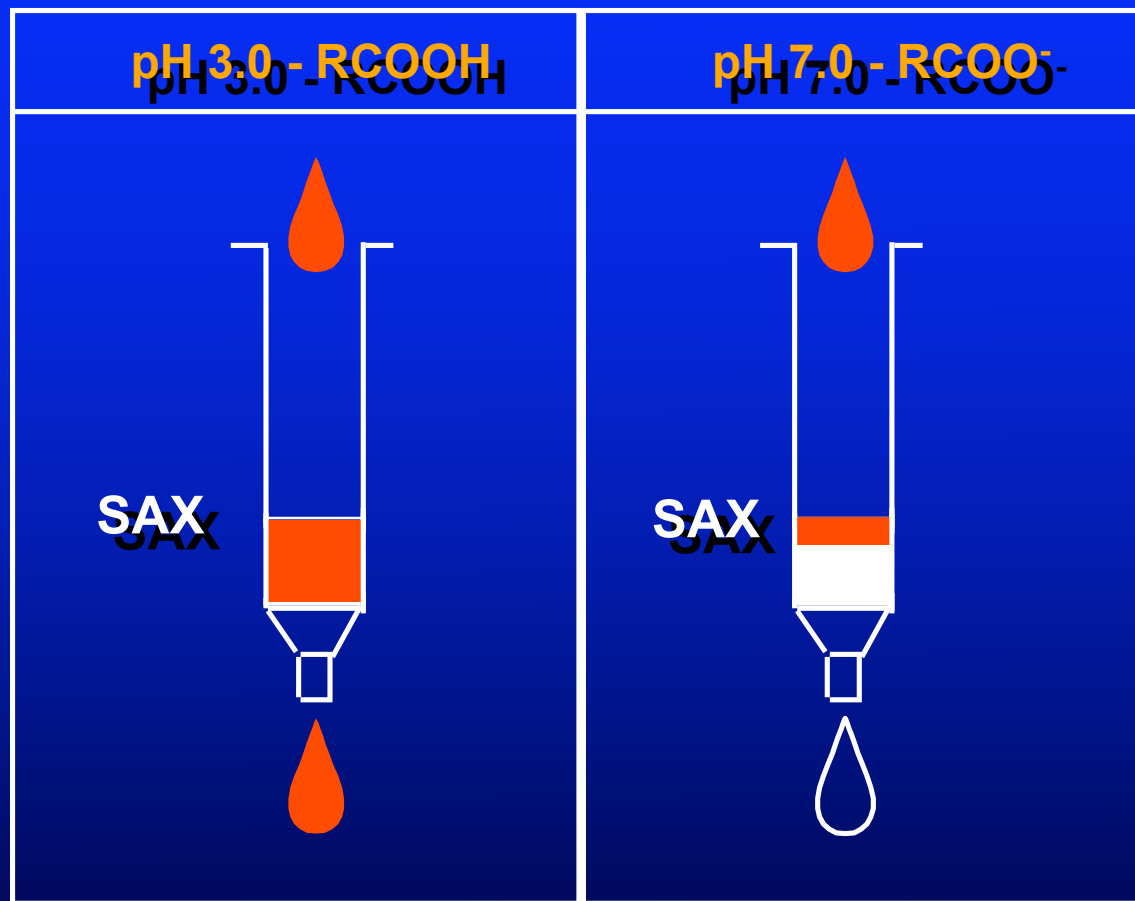


Influence of pH on Non-Polar Retention



Adjust pH to Suppress Ionization
RCOOH pKa = 5.0

Influence of pH on Ionic Retention



Adjust pH to Ensure Ionization

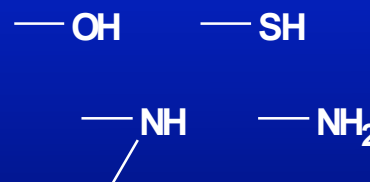
Analyte Characteristics

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

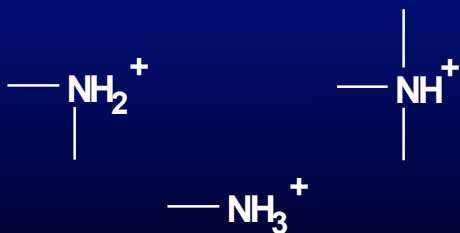
Hydrophobic



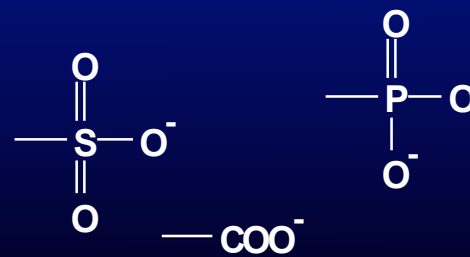
H-Bonding



Cationic

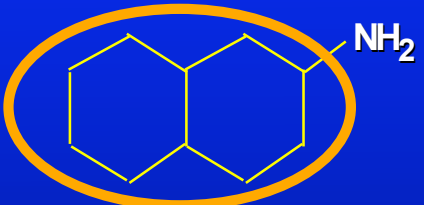




Anionic



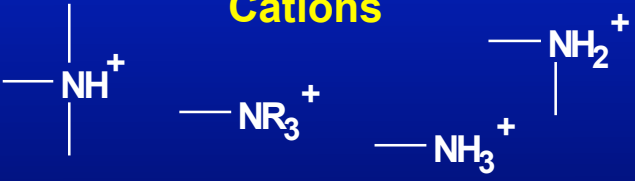
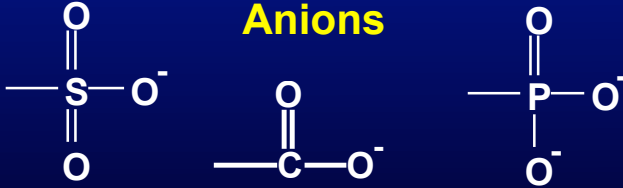


Mechanism Selection

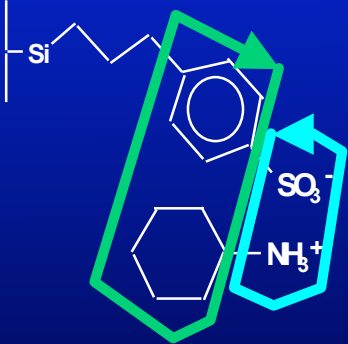
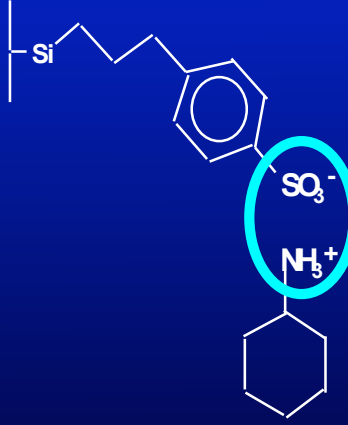


Analyte-Based

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

Mechanism Selection - Summary

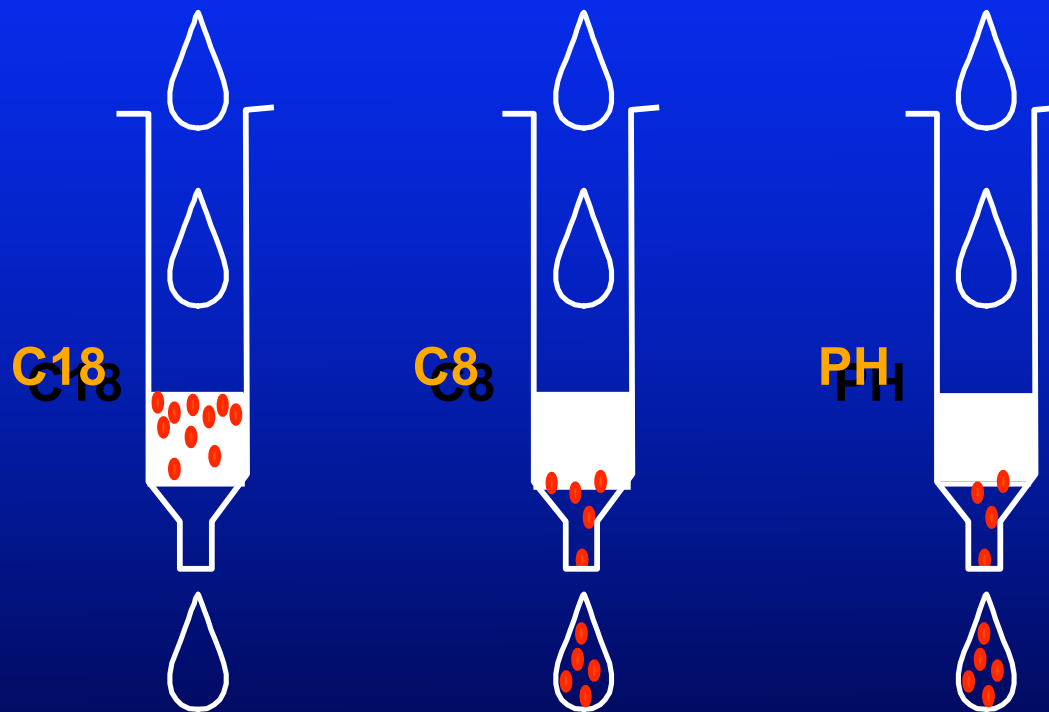
| Analyte | Matrix | Sorbent |
|---|--|--|
| <p>Hydrophobic</p>  | Aqueous | <p>Non-Polar</p> <p>C18 C8 PH CH C2 CN</p> |
| <p>H-Bonding</p>  | Aqueous or Non-Polar Solvent | <p>Polar</p> <p>SI NH2 2OH CN</p> |
| <p>Cations</p>  | <p>Aqueous (Low Ionic Strength)</p> <p>(High ionic strength)</p> | <p>Cation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

Secondary Interactions on Ion Exchange Phases

| Apply Sample | Rinse | Rinse | Elute |
|--|---|--|--|
| <p>Aqueous , pH 5-7</p> | <p>Acetonitrile</p> | <p>0.1M K_2HPO_4</p> | <p>Acetonitrile, 0.1M K_2HPO_4 (50:50)</p> |
|  |  |  |  |

Sorbent Screening

Analyte Elution



Validate for Ruggedness

- Vary Solvent Strength by $\pm 5\%$
- 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 | $-\text{NH}_3^+$ HO_2C^- | $-\text{NH}_3^+ \rightleftharpoons -\text{O}_2\text{C}^-$ | $-\text{NH}_2$ $-\text{O}_2\text{C}^-$ |
| Weak | Strong | $-\text{NH}_3^+ \rightleftharpoons -\text{O}_3\text{S}^-$ | $-\text{NH}_3^+ \rightleftharpoons -\text{O}_3\text{S}^-$ | $-\text{NH}_2$ $-\text{O}_3\text{S}^-$ |
| Strong | Weak | $-\text{NR}_3^+$ HO_2C^- | $-\text{NR}_3^+ \rightleftharpoons -\text{O}_2\text{C}^-$ | $-\text{NR}_3^+ \rightleftharpoons -\text{O}_2\text{C}^-$ |
| Strong | Strong | $-\text{NR}_3^+ \rightleftharpoons -\text{O}_3\text{S}^-$ | $-\text{NR}_3^+ \rightleftharpoons -\text{O}_3\text{S}^-$ | $-\text{NR}_3^+ \rightleftharpoons -\text{O}_3\text{S}^-$ |

Ion-Exchange Elution

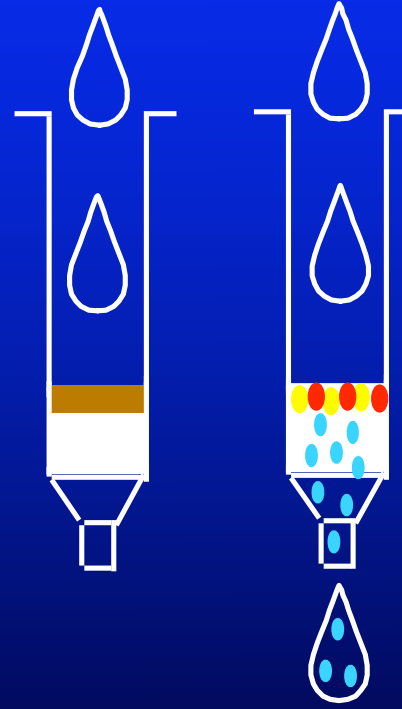
- **High Ionic Strength**
- **pH Change**
 - **Neutralize Sorbent**
 - **Neutralize Analyte**
- **High Selectivity Counter-ions**

Cleanup Techniques

Selective Rinse



Selective Elution

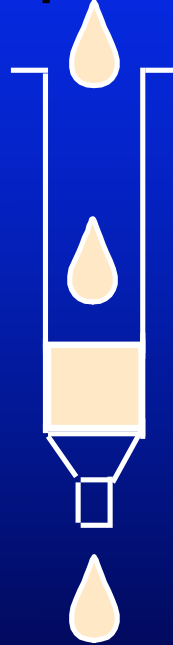


Typical SPE Procedure

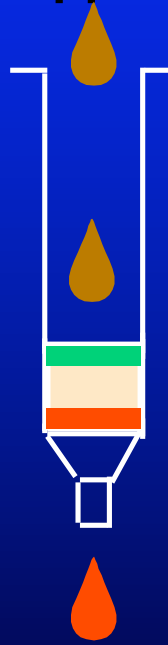
Column
Conditioning



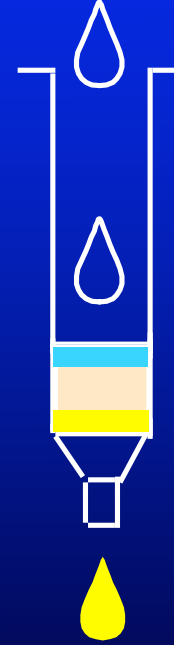
Column Pre-
Equilibration



Sample
Application



Interferenc
e Elution



Analyte
Elution

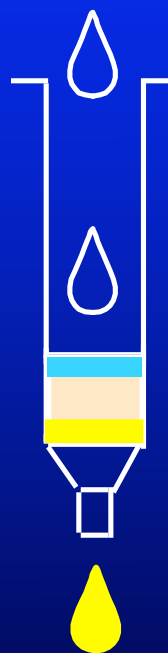


Typical SPE Procedure

Sample Application



Wash



Elution

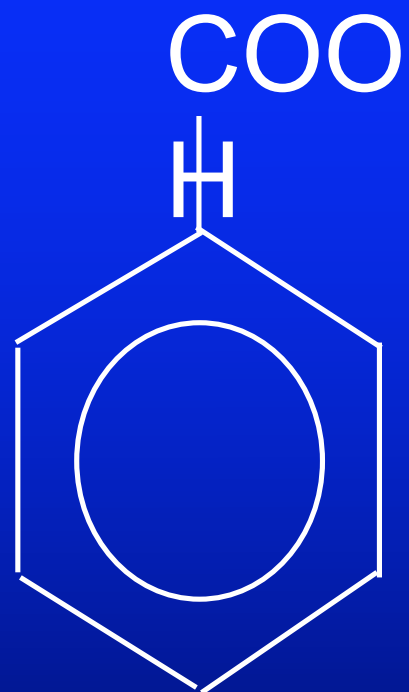


Cleanup Tips

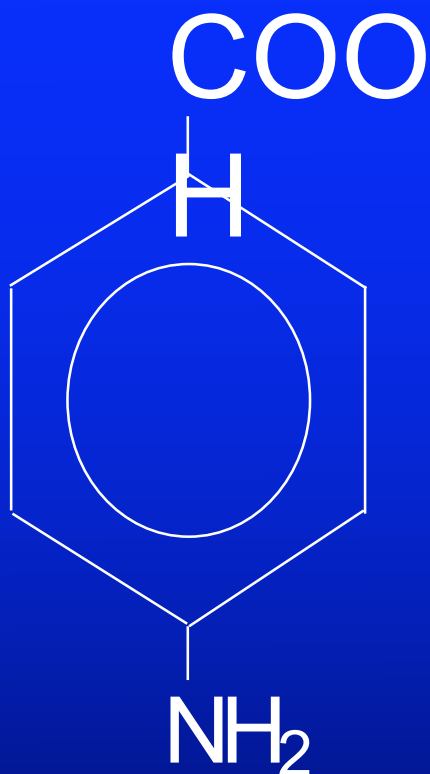
1. Non-retaining sorbents (from Sorbent Screening) are good cleanup sorbents
2. Non-eluting solvents (from Analyte Elution Screening) are good rinse solvents
3. Solvents intermediary between sample and elution solvent are good rinse solvents
4. Solvents in which the analyte(s) are insoluble are good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

Capacity & Elution Volume

- Capacity is $\sim 1-5\%$ of Sorbent Mass
- Minimum Elution Volume is $\sim 125 \mu\text{l}$ 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**