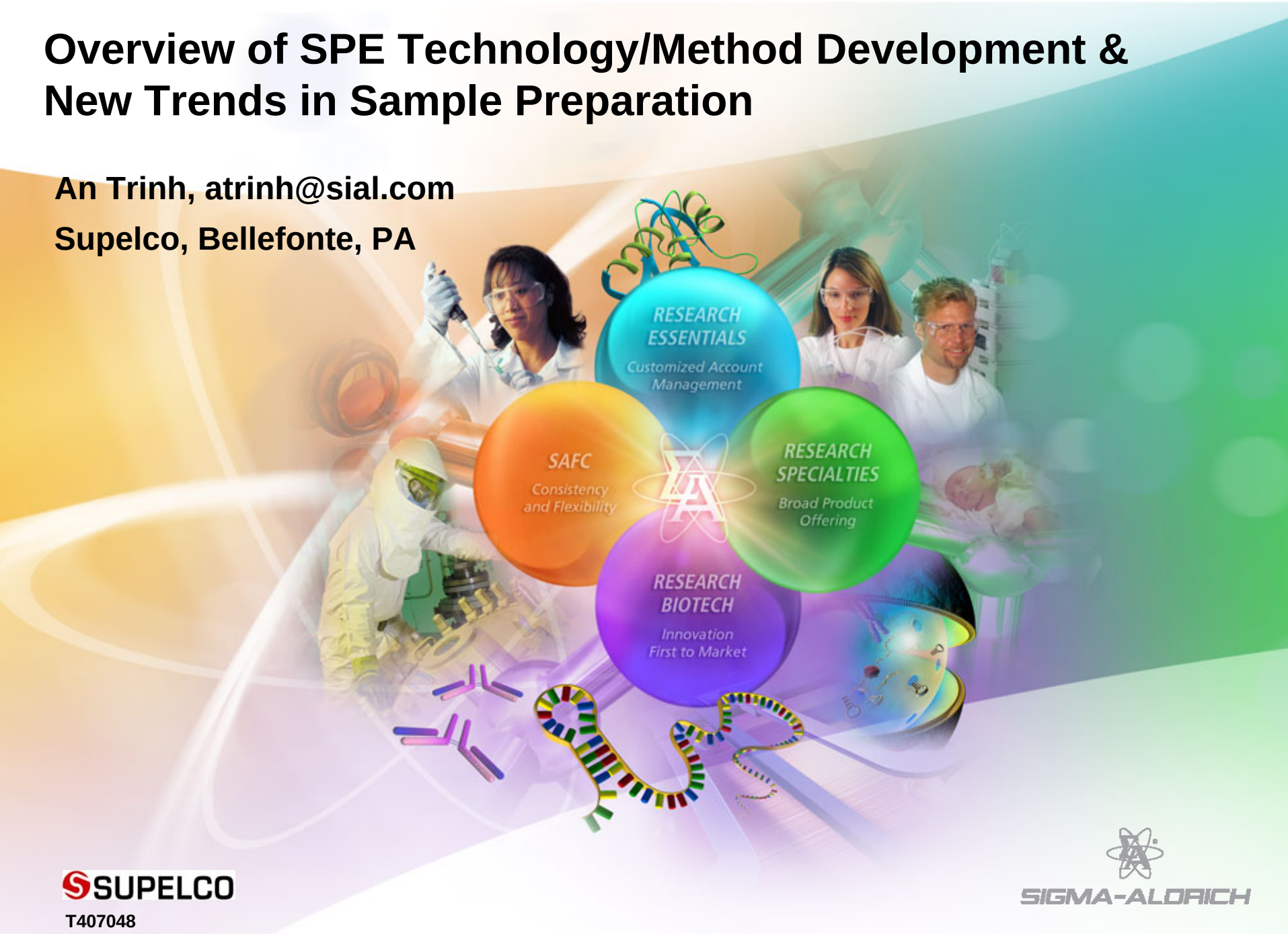
Overview of SPE Technology/Method Development & New Trends in Sample Preparation

An Trinh, atrinh@sial.com

Supelco, Bellefonte, PA



SUPELCO

T407048

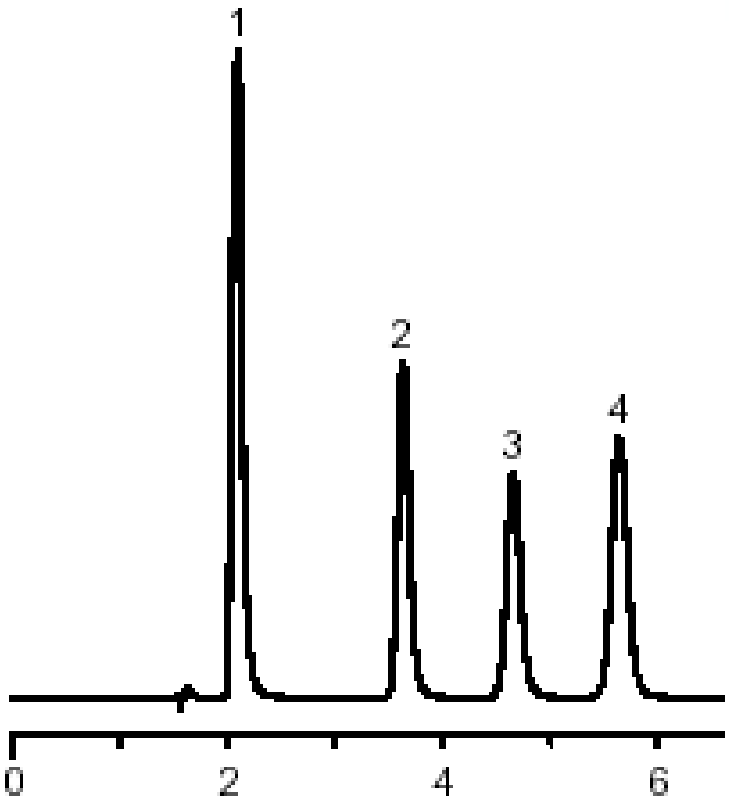
SIGMA-ALDRICH

Agenda

- The Importance of Sample Prep
- Overview of SPE Technology
- Understanding Retention Mechanisms
- A Systematic Approach to SPE MD
- New Developments in Sample Prep

Analytical Chromatography Heaven

- Short run times
- Baseline resolution
- Symmetric peak shape
- Good S/N ratio
- No misleading peaks
- High precision/accuracy



SUPELCO

SIGMA-ALDRICH

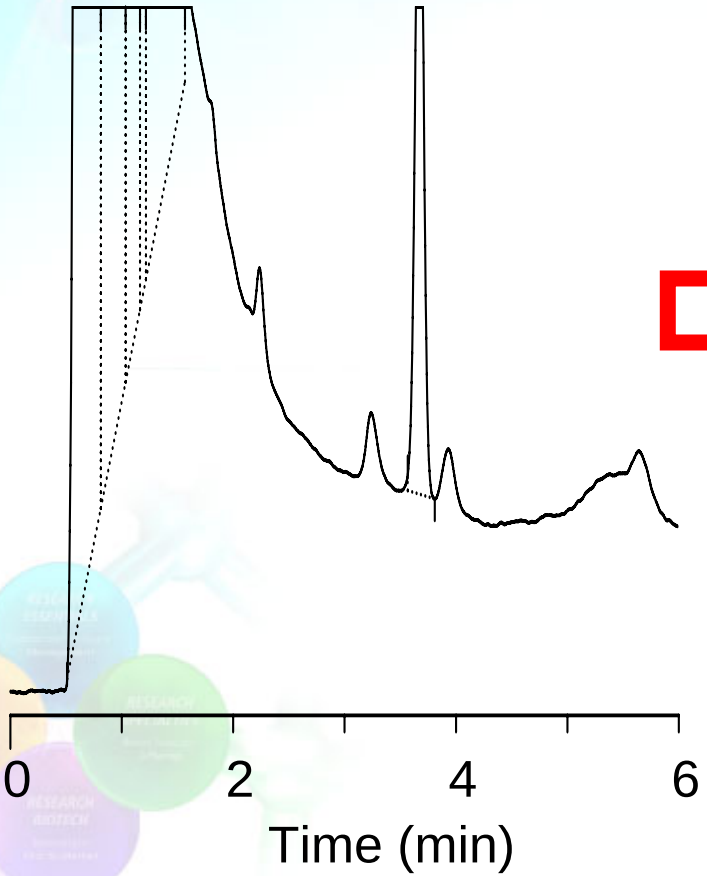
The Importance of Sample Preparation

SUPELCO

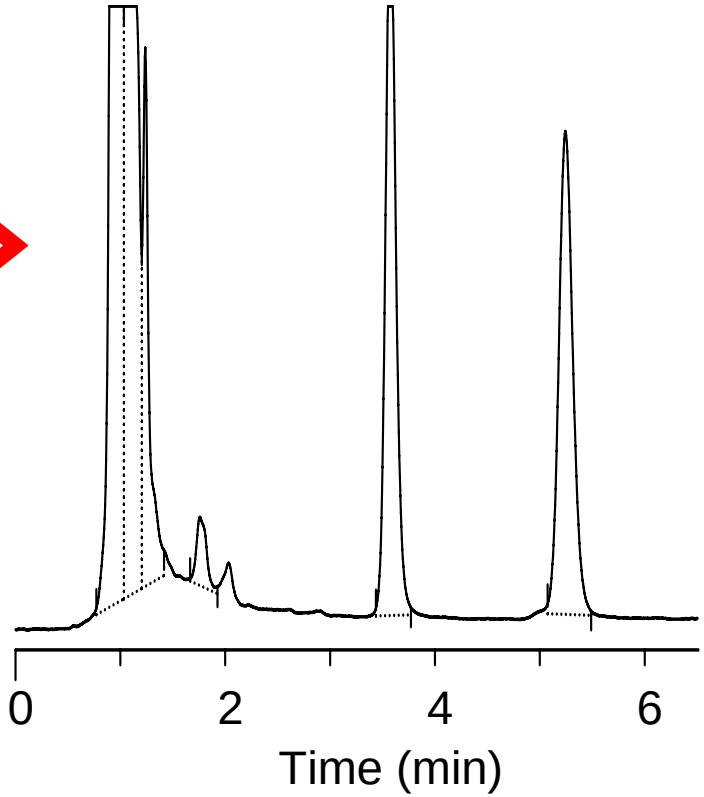
SIGMA-ALDRICH

Real World & Real Samples

Urine Sample without SPE



Urine Sample with SPE



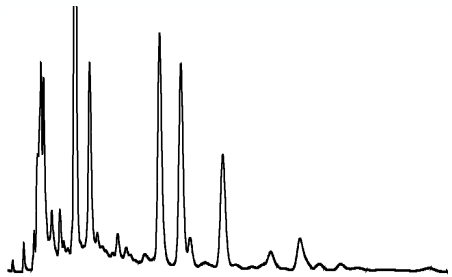
SUPELCO

SIGMA-ALDRICH

Why is sample preparation required?

Collected Sample

GC, HPLC, or LC-MS/MS Analysis



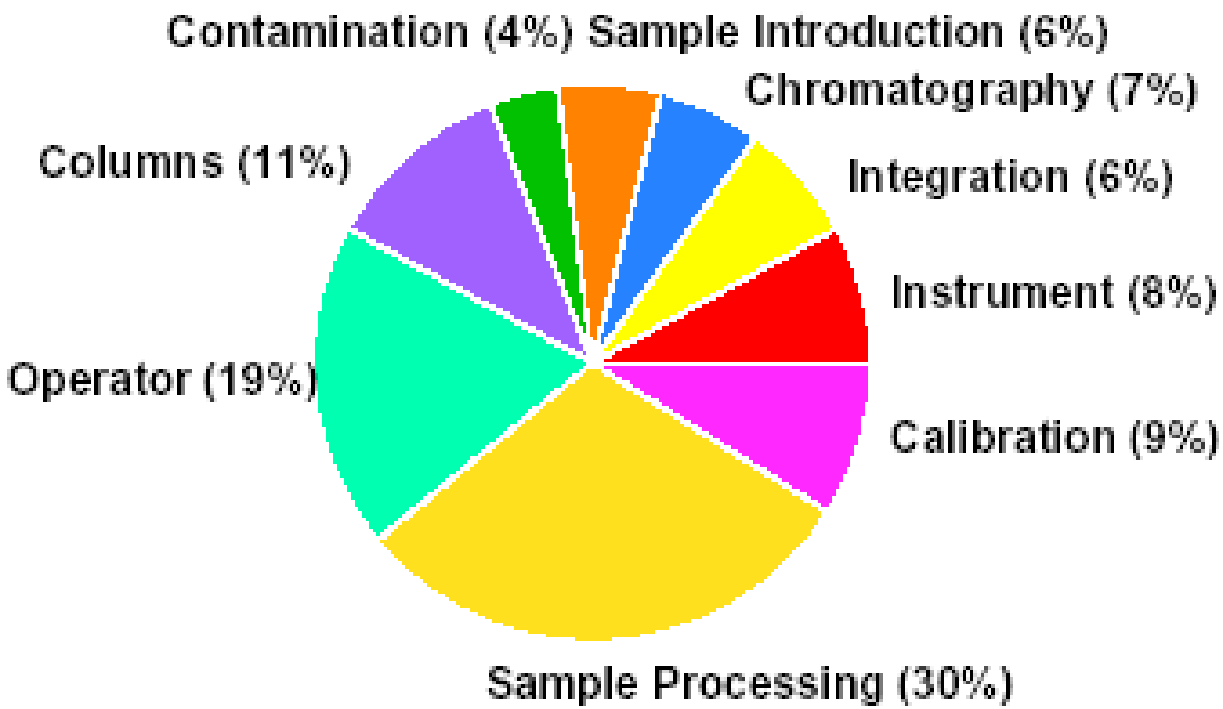
Current Sample = Unsuitable for further analysis!!!… Why?

- **Too dilute**- analyte(s) not concentrated enough for quantitative detection
- **Too dirty**- contains other sample matrix components that interfere with the analysis
- Present **sample matrix not compatible** with or harmful to the chromatographic column/system

SUPELCO

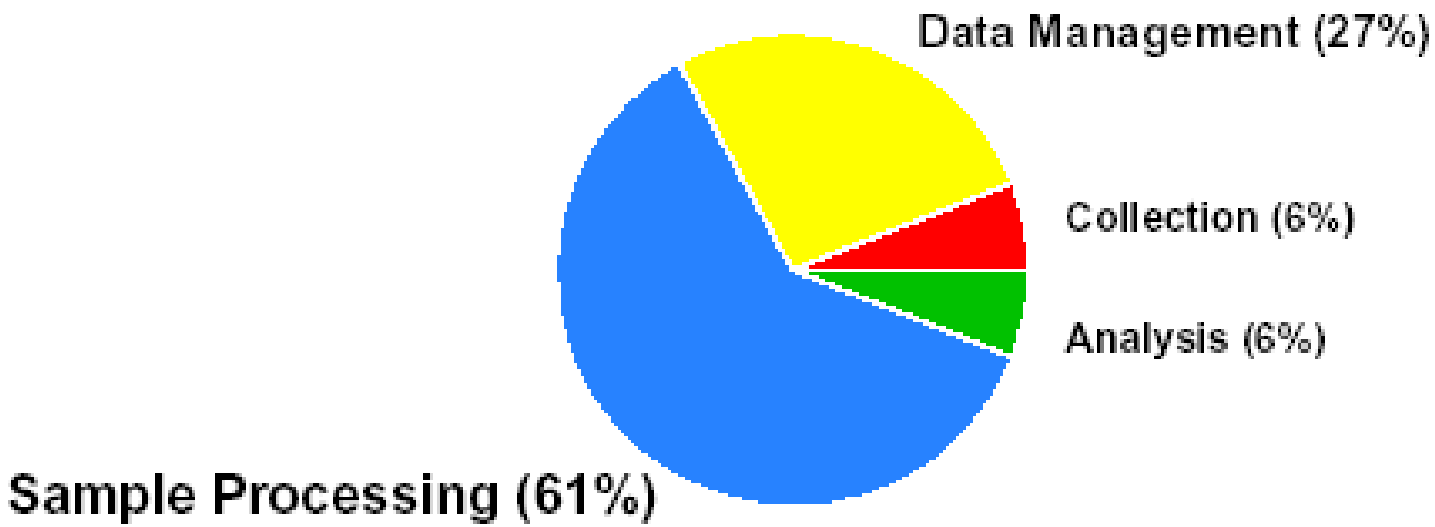
SIGMA-ALDRICH

Sources of Chromatographic Errors



(R.E. Majors, LC/GC Magazine, 1991, 1997, 2002)

Time Spend on Analytical Process



(R.E. Majors, LC/GC Magazine, 1991, 1997, 2002)

Why is sample prep especially important in bioanalysis?

- Due to many types of sample matrices encountered:
  - Plasma- proteins, lipids, and other endogenous macromolecules
  - Urine- contains uric acid and many nitrogenous base products
  - Serum
  - Bile
  - Tissue Homogenates
  - Perfusates
  - Saliva
  - Seminal Fluid
  - Caco-2 buffer
  - Others

Many Tools/Technology for Sample Prep

Simpler; Generic Methodology

- Dilute and Shoot
- Filtration]
- Protein Precipitation
- Equilibrium dialysis/ ultrafiltration
- Liquid Liquid Extraction
- Solid Phase Microextraction (SPME)
- Solid Phase Extraction (off-line and on-line)
- Turbulent Flow Chromatography
- Monolithic Chromatography
- Immunoaffinity

Less Selective

Minimal Sample Cleanup & Concentration

Greater Selectivity

Optimal Sample Cleanup & Concentration

More Complicated; Requires Method Dev

SUPELCO

SIGMA-ALDRICH

How to choose the right sample prep technology?

- Should depend on three specific criteria:
  - **Requirements of the assay**
  - **Time allowed to run sample prep method**
  - **Possible investment towards method development time**
- Example:
  - **Late Discovery/Early Development**
    - **Requires rapid sample turn around**
    - **Higher limits of quantitation**
    - **Very little method development time (1-2 days)**
    - **Protein Precipitation may be ideal choice**
  - **Development (pre-clinical and clinical)**
    - **Drugs more potent and dosed at lower levels**
    - **Requires ultra-sensitivity, great selectivity and rugged method development**
    - **Greater method development time (3-5 days)**
    - **SPE is more ideal choice**

Separatory Funnels/LLE = Old Technology

- Large solvent consumption
- Vigorous shaking/mixing
- Waiting for layers to separate
- Phase emulsions

SUPELCO

SIGMA-ALDRICH

More Common Sample Prep Tools/Technology

Protein Precipitation:

- Advantages
  - Requires little to no method development (universal)
  - Amenable to automation
  - Very simple (2-3 steps), and relatively inexpensive
- Disadvantages
  - Sample dilution effect => requires concentration which is time consuming due to aqueous portion of sample
  - Poor removal of matrix interferences => stress on analytical system and increased ion-suppression resulting in poor reproducibility, accuracy and sensitivity

SPE Advantages & Disadvantages

Disadvantages

- ***Perceived*** difficulty to master its usage (method development)
  - Wide range of chemistries, many choices for manipulating solvent and pH conditions make it difficult to grasp
- More steps and MD time required
- Greater cost per sample

Advantages

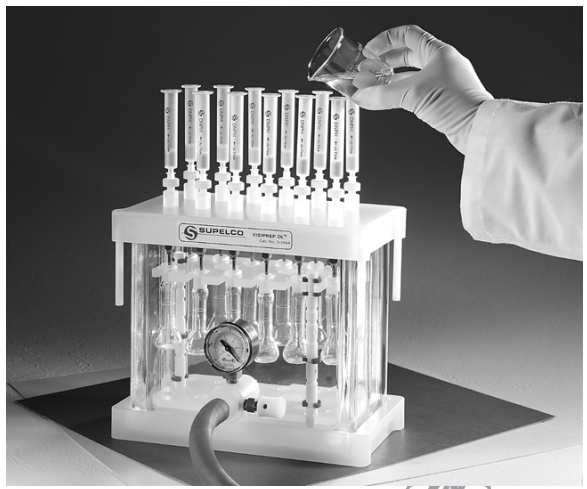
- Greater selectivity- paramount importance (e.g., bioanalysis (pg/mL))
- Wide variety of sample matrices
- High recoveries & good reproducibility
- Amenable to automation
- Low solvent volumes

Purpose of Solid Phase Extraction (SPE)

Prior to the actual analysis, SPE is most commonly used to…

1. Clean Up - Strip the analyte(s) away from endogenous interferences.
2. Concentrate analytes(s) for better sensitivity.
3. Exchange sample environments for better chromatography

   -e.g., analytes in serum => analytes in mobile phase.



SUPELCO

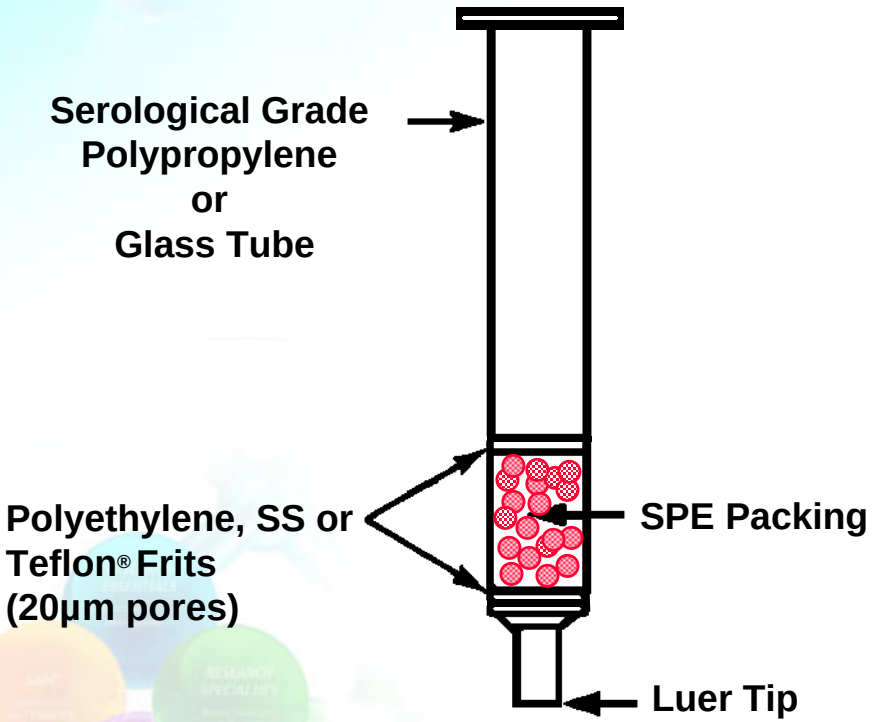
SIGMA-ALDRICH

Overview of Solid Phase Extraction (SPE)

SUPELCO

SIGMA-ALDRICH

Basic SPE Concept



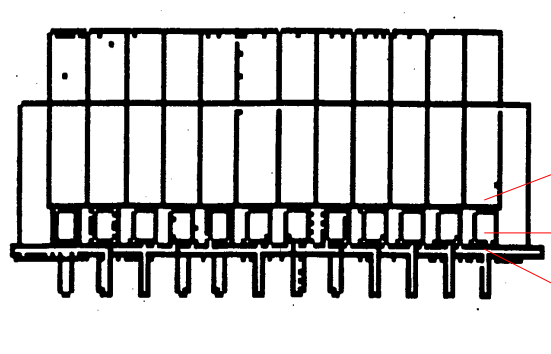
- Another form of chromatography
- Hardware = plastic (polypropylene) or glass
- Sorbent held in place by two PE frits
- Packing material is very similar to HPLC
  - Often irregular shape vs. spherical (HPLC)
  - Much larger particle size (>50um) vs. HPLC (</= 5um)
  - SPE particle size distribution much broader than HPLC
- Use it only once

High Throughput Solid Phase Extraction

Discovery SPE 96-Well Plates

Plate Description:

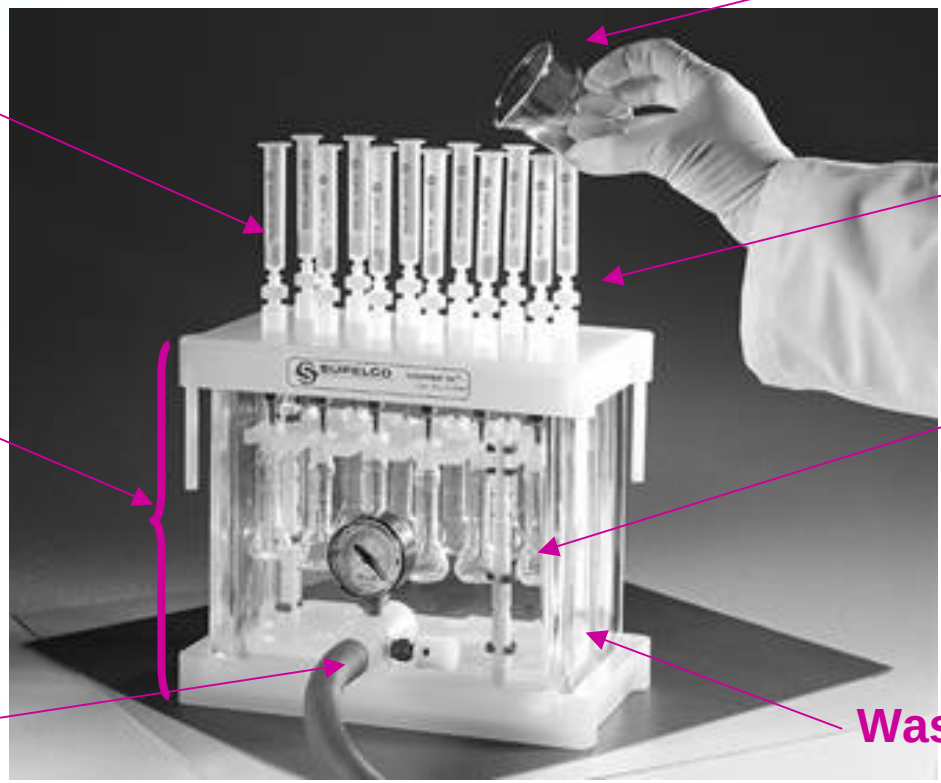
- **Square well extraction plate, 2.0-2.25 ml capacity, polypropylene.**
- **Available for all Discovery SPE phases**
- **Bed weight = 25, 50, or 100mg/well**
- **Compatible with most robotic systems and automated sample processing systems: TomTec Quadra, Gilson SPE 215, etc.**



SUPELCO

SIGMA-ALDRICH

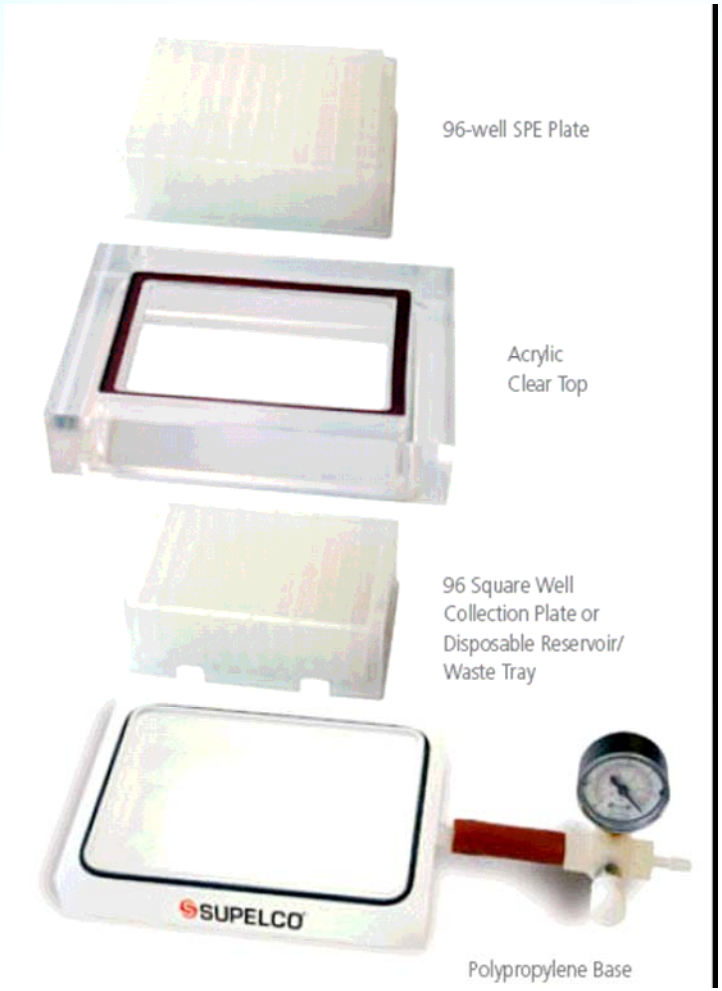
SPE Vacuum Manifold



SUPELCO

SIGMA-ALDRICH

SPE Vacuum Manifold (cont.)



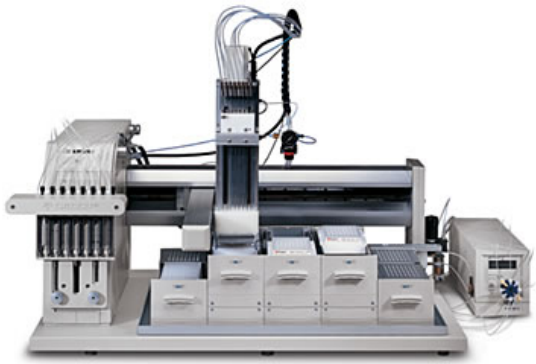
SUPELCO

SIGMA-ALDRICH

Most Common SPE Robots for Automated SPE

Zymark RapidTrace System

TomTec Quadra System



Gilson SPE 215 System

Code 802 & 803 “Tab-less” 1 & 3mL racks

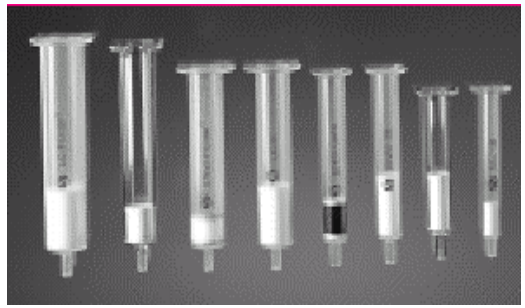
SUPELCO

SIGMA-ALDRICH

Types of SPE Tubes/Cartridges

SPE tubes are available in two materials:

- **Polypropylene (serological grade)**
  - Most common
  - Suitable for most SPE applications
  - Inexpensive



An assortment of Supelco SPE tubes. Second tubes in from either side are glass.

- **Glass (serological grade)**
  - Greater solvent resistance than plastic
  - No phthalates or plasticizers to leach into sample
  - Can be heated
  - More expensive than plastic
  - Common in environmental analysis

SUPELCO

SIGMA-ALDRICH

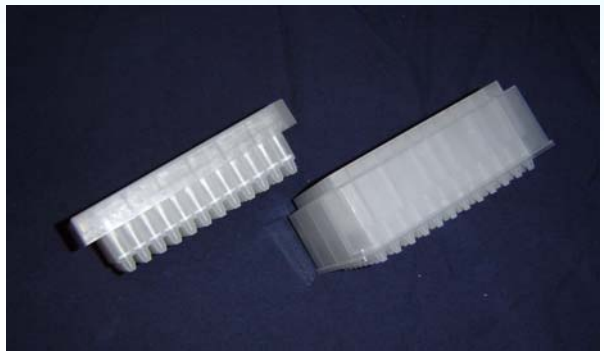
Common SPE Hardware

Funnels

Büchner format ideal for large sample volumes

Tubes

Glass or plastic, tubes are the most common SPE format



96-well plates

SPE Disks

SUPELCO

SIGMA-ALDRICH

Basic SPE Steps & Approaches

SUPELCO

SIGMA-ALDRICH

General Steps of an SPE Procedure

1) **Sample Pre-treatment:** Dependent on compound of interest, sample matrix, and nature of retention chemistry; involves pH adjustment, centrifugation, filtration, dilution, buffer addition, etc..

2) **Conditioning:** Solvent is passed through the SPE material to ***wet*** the bonded functional groups => ensures consistent interaction.

3) **Equilibration:** Sorbent/ phase is treated with a solution that is similar (in polarity, pH, etc.) to the sample matrix => maximizes retention.

4) **Sample Load:** Introduction of the sample = analytes of interest are bound/ extracted onto the phase/ sorbent.

5) **Washing:** Selectively remove unwanted interferences co-extracted with the analyte without prematurely eluting analytes of interest.

6) **Elution:** Removing analytes of interest with a solvent that overcomes the primary and secondary retention interactions b/w sorbent and analytes of interest.

7) **Evaporation** of eluent/ reconstitution with mobile phase (optional).

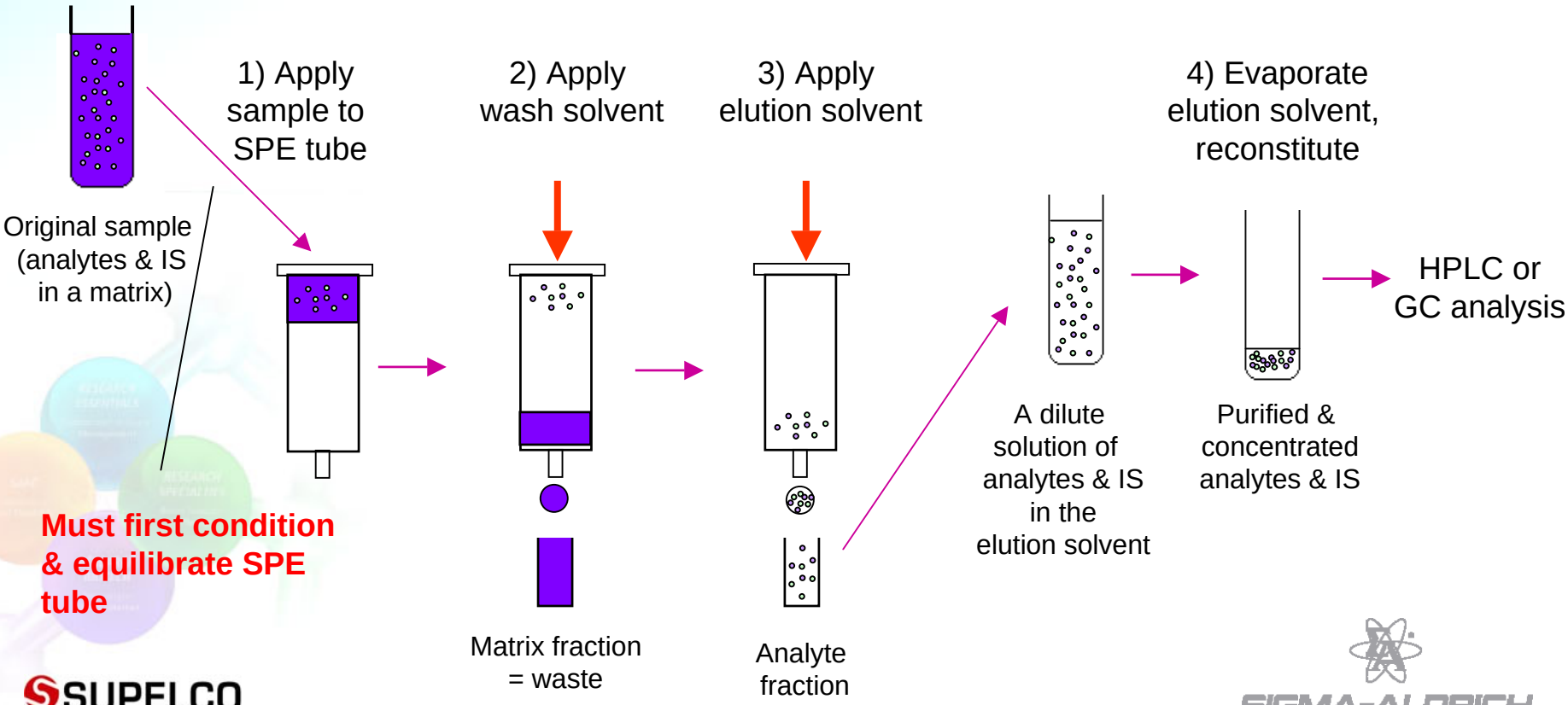
Three different SPE Strategies

There are 3 different elution strategies in SPE. Which one to choose depends on the goal of the extraction.

1. **Bind-Elute Strategy**
   - Most common
   - Bind: Analytes bind to tube, unwanted matrix components are washed off
   - Elute: Eluant changed to remove analytes from tube
   - Analytes are concentrated via evaporation prior to HPLC or GC analysis
2. **Interference Removal Strategy**
   - Bind all unwanted matrix components and allow analytes to pass through during the sample loading stage
   - Like chemical filtration
3. **Fractionation Strategy**
   - Retain and sequentially elute different classes of compounds by modifying eluant pH or % organic

Bind-elute strategy diagram

Shown is a step-by-step bind-elute SPE extraction, beginning with a filtered sample containing analytes and internal standard (IS) in a matrix and ending with purified and concentrated analytes and internal standard ready for HPLC or GC analysis.

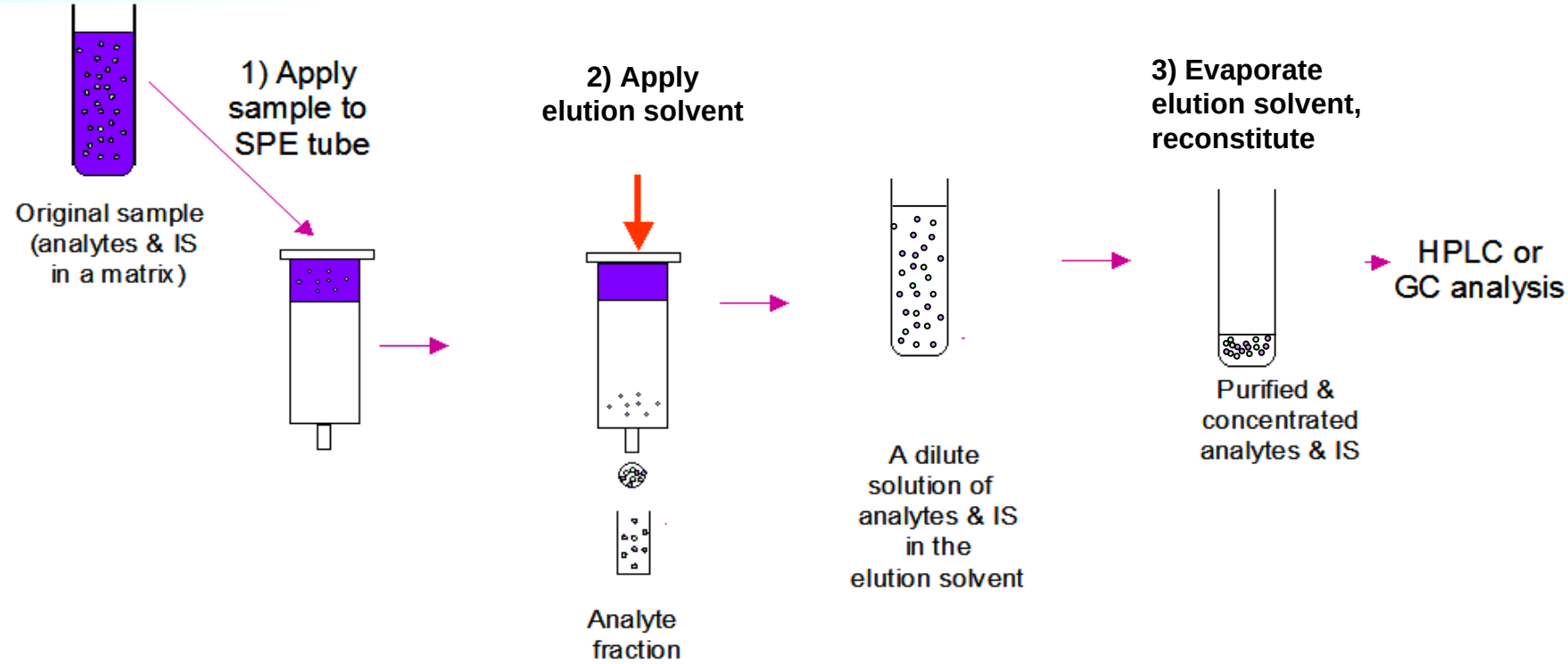


SUPELCO

SIGMA-ALDRICH

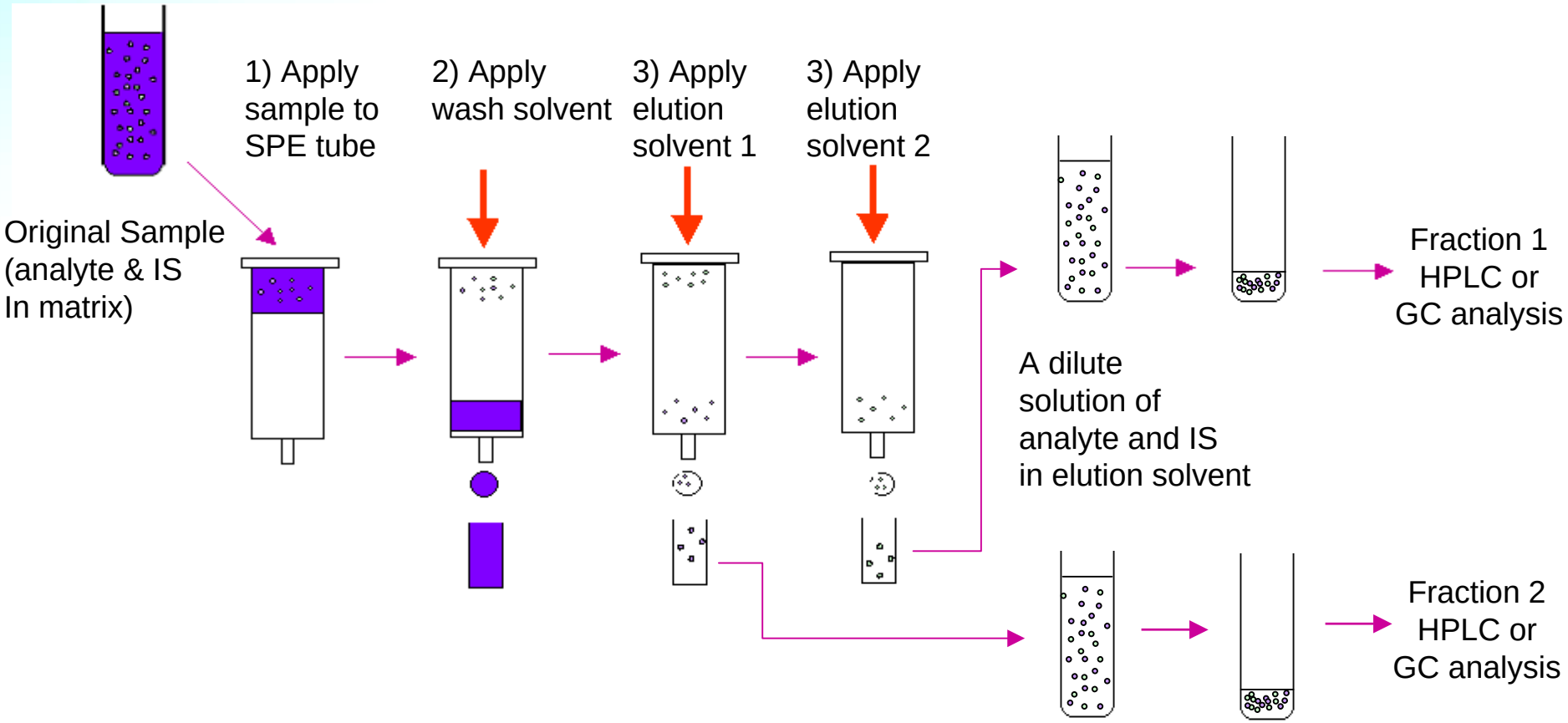
Interference removal strategy diagram

Shown is a step-by-step bind-elute SPE extraction, beginning with a filtered sample containing analytes and internal standard (IS) in a matrix and ending with purified and concentrated analytes and internal standard ready for HPLC or GC analysis.



Fraction strategy diagram

Shown is a step-by-step bind-elute SPE extraction, beginning with a filtered sample containing analytes and internal standard (IS) in a matrix and ending with purified and concentrated analytes and internal standard ready for HPLC or GC analysis.



Understanding Retention Mechanisms

SUPELCO

SIGMA-ALDRICH

Reversed-Phase SPE

General Guidelines

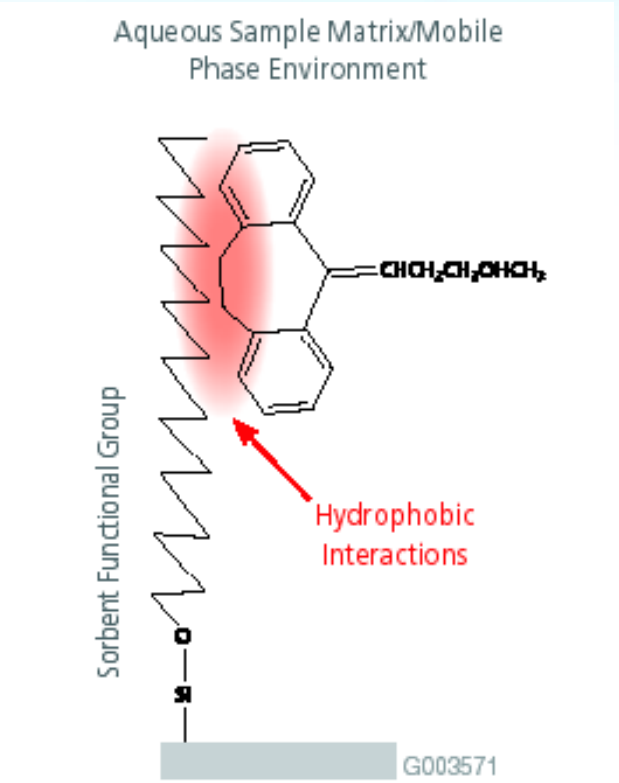
Retention Mechanism: **Non-polar or hydrophobic interactions**
- Van der Waals or dispersion forces

Sample Matrix: **Aqueous samples**
- Biological fluids (serum, plasma, urine)
- Aqueous extracts of tissues
- Environmental water samples
- Wine, beer and other aqueous samples

Analyte Characteristics: **Analytes exhibiting non-polar functionalities**
- Most organic analytes
- Alkyl, aromatic, alicyclic functional groups

Elution Scheme: **Disrupt reversed-phase interaction with solvent or solvent mixtures of adequate non-polar character**
- Methanol, acetonitrile, dichloromethane
- Buffer/solvent mixtures

Common Applications:
- Drugs and metabolites in biological fluids
- Environmental pollutants in water
- Aueous extracts of tissues and solids



Reversed-phase SPE is considered the least selective retention mechanism when compared to normal-phase or ion-exchange SPE. In other words, it may be difficult for a reversed-phase method or bonded-chemistry to differentiate between molecules that are structurally similar. However, because reversed-phase will retain most molecules with any hydrophobic character, it is very useful for extracting analytes that are very diverse in structure within the same sample.

SUPELCO

SIGMA-ALDRICH

Example RP SPE Protocol

1. **Sample Pre-Treatment**
   - Dilute samples 1:1 with buffer (10mM ammonium acetate)
   - pH manipulation important for ionizable analytes
   - Filter or centrifuge out particulates
2. **Condition & Equilibrate**
   - Condition with 1-2 tube volumes MeOH or MeCN
   - Equilibrate with 1-2 tube volumes buffer
3. **Load sample** (consistent rate; 1-2 drops per second)
4. **Wash sorbent** (elutes co-retained interferences)
   - Critical for improving selectivity
   - 5-20% MeOH common
   - Dilute MeOH in buffer used during sample load
5. **Elute analytes of interest**
   - MeOH or MeCN most common
   - pH manipulation can improve recovery (adjust pH opposite to load conditions)
6. **Evaporate/reconstitute as necessary**

Useful RP SPE Tips

- Drugs in biological fluids risk drug-protein binding effect
  - Disrupt during sample pre-treatment using 40uL 2% disodium EDTA or 2% formic acid per 100uL plasma
- Sorbent over drying only a concern during first conditioning step
- If eluate evaporation necessary, dry SPE tube with vacuum for 10-15 min. prior to elution to remove residual moisture
- Pass DCM through SPE before conditioning to remove SPE tube impurities for highly sensitive analyses
- Reduce bed weight to minimize elution volume
- Increase bed weight to retain more polar compounds

Normal-Phase SPE

General Guidelines

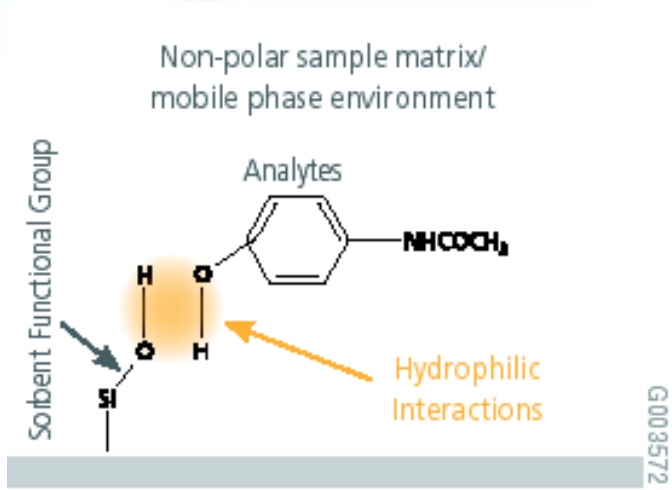
Retention Mechanism: **Polar Interactions**
- Hydrogen bonding, pi-pi, dipole-dipole, and induced dipole-dipole

Sample Matrix: **Non-polar samples**
- Organic extracts of solids
- Very non-polar solvents
- Fatty oils, hydrocarbons

Analyte Characteristics: **Analytes exhibiting polar functionalities**
- Hydroxyl groups, carbonyls, amines, double bonds
- Hetero atoms (O, N, S, P)
- Functional groups with resonance properties

Elution Scheme: **Polar interactions disrupted with a more polar solvent or solution**
- Acetonitrile, methanol, isopropanol
- Combinations of buffer/solvent or solvent/solvent mixtures

Common Applications:
- Clean-up of organic extracts of soils and sludge
- Fractionation of petroleum hydrocarbons
- PCBs in transformer oil
- Isolation of compounds in cosmetics



In order for polar retention to occur between the sorbent and the sample, the analyte must be introduced to the SPE device in a non-polar sample or mobile phase environment. Therefore, typical sample matrices that can be employed in normal-phase SPE include hydrocarbon or fatty oils diluted in an organic solvent, hexane, isoocatane, chlorinated solvents, THF, diethyl ether, and ethyl acetate.

Most organic analytes exhibit some polar functionalities that can be exploited for normal-phase separation. Because many molecules exhibit polar functionality, each interaction can provide different levels of selectivity offering highly selective separations of compounds very similar in structure.

Example NP SPE Protocol

1. **Sample Pre-Treatment**
   - Liq samples extracted/diluted with non-polar solvent (e.g. hexane, DCM)
   - Solid samples (soil, sediment, etc.) extracted (soxhlet, sonnication, etc.) with non-polar solvent, and concentrated
   - Dry solvent extract with Na-sulfate or Ma-sulfate
     - Residual moisture can greatly affect analyte retention
2. **Condition & Equilibrate** w/ 1-2 tube volumes non-polar solvent
3. **Load sample** (consistent rate; 1-2 drops per second)
   - Sample should <u>not</u> be in MeCN or MeOH
4. **Wash sorbent** (elutes co-retained interferences)
   - Use a more polar solvent, but not so polar as to elute analytes of interest
   - Fractionation common in NP SPE
5. **Elute analytes of interest with polar solvent**
   - MeOH, MeCN, Acetone, IPA are common
6. **Evaporate/reconstitute as necessary**

Common NP Solvents

Solvent	Elutropic (e°) or elution strength on silica	
Hexane	0.00	Promotes Normal-Phase Retention
Isooctane	0.00	
Carbon tetrachloride	0.14	
Tolulene	0.22	
Benzene	0.27	
Tert-butyl methyl ether	0.29	
Chloroform	0.31	
Methylene Chloride (dichloromethane)	0.32	
Diethyl ether	0.29	
Ethyl Acetate	0.43	
Tetrahydrofuran	0.35	
Acetone	0.45	
Acetonitrile	0.50	
40% methanol in acetonitrile	0.67	
20% methanol in diethyl ether	0.65	
20% methanol in methylene chloride	0.63	
Isopropanol	0.63	
Methanol	0.73	
Water	>0.73	Promotes Normal-Phase Elution
Acetic Acid	>0.73	

SUPELCO

SIGMA-ALDRICH

Ion-Exchange SPE

General Guidelines

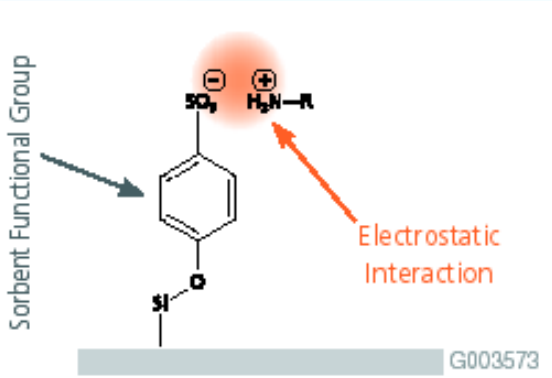
Retention Mechanism: Electrostatic attraction of charged functional groups of the analyte(s) to oppositely charged functional groups on the sorbent. Combination of reversed-phase and ion-exchange for mixed-mode

Sample Matrix: Aqueous or organic samples of low salt concentration (< 0.1M)
- Biological fluids
- Solution phase synthesis reactions

Analyte Characteristics:
- Use cation-exchange for isolating basic compounds: primary, secondary, tertiary, and quarternary amines
- Use anion-exchange for isolating acidic compounds: carboxylic acids, sulphonic acids, and phosphates

Elution Scheme: Electrostatic interactions disrupted via:
- pH modification to neutralize compound and/or sorbent functional groups
- Increase salt concentration (> 1M); or use a more selective counter-ion to compete for ion-exchange binding sites

Common Applications:
- Drugs of abuse and pharmaceutical compounds in biological fluids
- Fatty acids removal in food/agricultural samples
- Clean-up of synthetic reactions
- Organic acids from urine
- Herbicides in soil



In order for electrostatic retention to occur, both analyte and sorbent functional groups must be in their ionized form. This is done through strict pH control of the sample matrix. For basic analytes, the pH should be adjusted to at least 2 pH units below the molecule's pKa. For acidic analytes, the pH should be adjusted to at least 2 pH units above the molecule's pKa.

To elute, the opposite is true. By adjusing the pH of the eluant to at least two pH units above or below the analytes' and/or sorbent's pKa, one can effectively neutralize one or both functional groups disrupting the electrostatic interaction allowing for elution to occur.

Note: Because the kinetic exchange processes between sample and sorbent functional groups are considerably slower for ion-exchange than for normal- and reversed-phase, flow rates should be drop wise (~1 drop/second). One may also need to increase elution and wash volumes allowing for sufficient residence time for the mobile phase and stationary phase to interact.

Example IOX SPE Protocol

1. **Sample Pre-Treatment:**
   - Basic compounds: dilute w/ 10-25mM buffer (e.g., potassium phosphate, ammonium acetate), pH 3-6
   - Acidic compounds: dilute with 10-25mM buffer (e.g. acetate), pH 7-9
   - BOTH sorbent functional group & analyte most be ionized
2. **Condition & Equilibrate**
   - Condition with 1-2 tube volumes MeOH or MeCN
   - Equilibrate with 1-2 tube volumes buffer (used during sample pre-treatment
3. **Load sample** (consistent rate; 1-2 drops per second)
4. **Wash sorbent** (elutes co-retained interferences)
   - Wash interferences with buffer
   - Wash with 100% MeOH to remove hydrophobic interferences
5. **Elute analytes of interest**
   - Adjust pH opposite to load conditions (e.g. 2-5% ammon hydroxide for basic compounds)
   - May require organic modifier (50-100% MeOH)
6. **Evaporate/reconstitute as necessary**

Useful IOX SPE Tips

- IOX kinetics slower than RP & NP => reduce flow rate
- Strong vs. weak ion-exchangers
  - Strong = sorbent functional group always ionized regardless of pH
  - Weak = sorbent functional group has controllable pKa; commonly used for extracting strong analytes
- Counter-Ion Selectivity in IOX

For Cation Exchangers:

- $Ca^{2+} > Mg^{2+} > K^{+} > Mn^{2+} > RNH_3^{2+} > NH_4^{+} > Na^{+} > H^{+} > Li^{+}$

For Anion Exchangers:

- Benzene Sulphonate > Citrate > $HSO_4^{-} > NO_3^{-} > HSO_3^{-} > NO_2^{-} > Cl^{-} > HCO_3^{-} > HPO_4^{-}$ > Formate > Acetate > Propionate > $F^{-} > OH^{-}$

SUPELCO

SIGMA-ALDRICH

SPE Method Development – SPE Selection & The Role of pH

SUPELCO

SIGMA-ALDRICH

Critical Questions to Ask

- What are requirements and goals to method development?
- What is known about the sample (sample matrix, analyte Log Po/w, pKa(s), functional groups)?
- What investment in MD time can be made?
- Any known information from previous work with similar analytes?

Best practices for method development

- Historically MD = hit or miss experiments in which many random variables evaluated at the same time
- Results in user not knowing why a set of conditions worked or what type of leeway can be associated with operator variation, changes in pH, etc.
- Problems can easily arise during method transfer.
- Best method developers isolate one variable at a time, and use 96-well technology to evaluate multiple variables in parallel

Key to Successful SPE

- Choose the appropriate SPE phase by understanding the sample matrix and identifying analyte(s) functional groups that influence its solubility, polarity, etc..
- Understand how the analyte(s) behaves on the sorbent in response to changing extraction conditions.
- Manipulate these conditions to meet the defined sample prep objectives

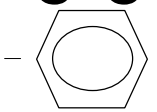
Consider the analyte(s) of interest

What functional groups may influence the analytes' solubility, polarity, ionization state (pKa), etc.?

Hydrophilic Groups:

•Hydroxyl	-OH
•Amino	$-NH_2$
•Carboxyl	-COOH
•Amido	$-CONH_2$
•Guanidino	$-NH(C=NH)NH_3^+$
•4° Amine	$-NR_3^+$
•Sulfate	$-SO_3^-$

Hydrophobic Groups:

•Carbon-Carbon	-C-C
•Carbon-Hydrogen	-C-H
•Carbon-Halogen	-C-Cl
•Olefin	-C=C
•Aromatic	

Neutral Groups:

•Carbonyl	-C=O
•Ether	-O-R
•Nitrile	-C≡N

SUPELCO

SIGMA-ALDRICH

Retention Mechanism Quick Look-Up

Reversed-Phase

Compounds:
- small molecules
- moderately polar to non-polar

Sample matrix:
- Aqueous samples!!!
- e.g., biological fluids (urine, plasma, serum), water, buffer

Ion-Exchange

Compounds:
- Charged/ ionizable compounds

Sample matrix:
- Aqueous or organic samples
- Low salt concentration

Normal-Phase

Compounds:
- Polar to moderately non-polar compounds

Sample matrix:
- Organic samples/extracts e.g., Hexane, ethyl acetate, dichloromethane

DPA-6S - Polyamide phase (nylon)- good for working with plant extracts (chlorophyl, tannins, humic acid); Good for extracting phenolic compounds (multiple –OH groups) from aqueous samples

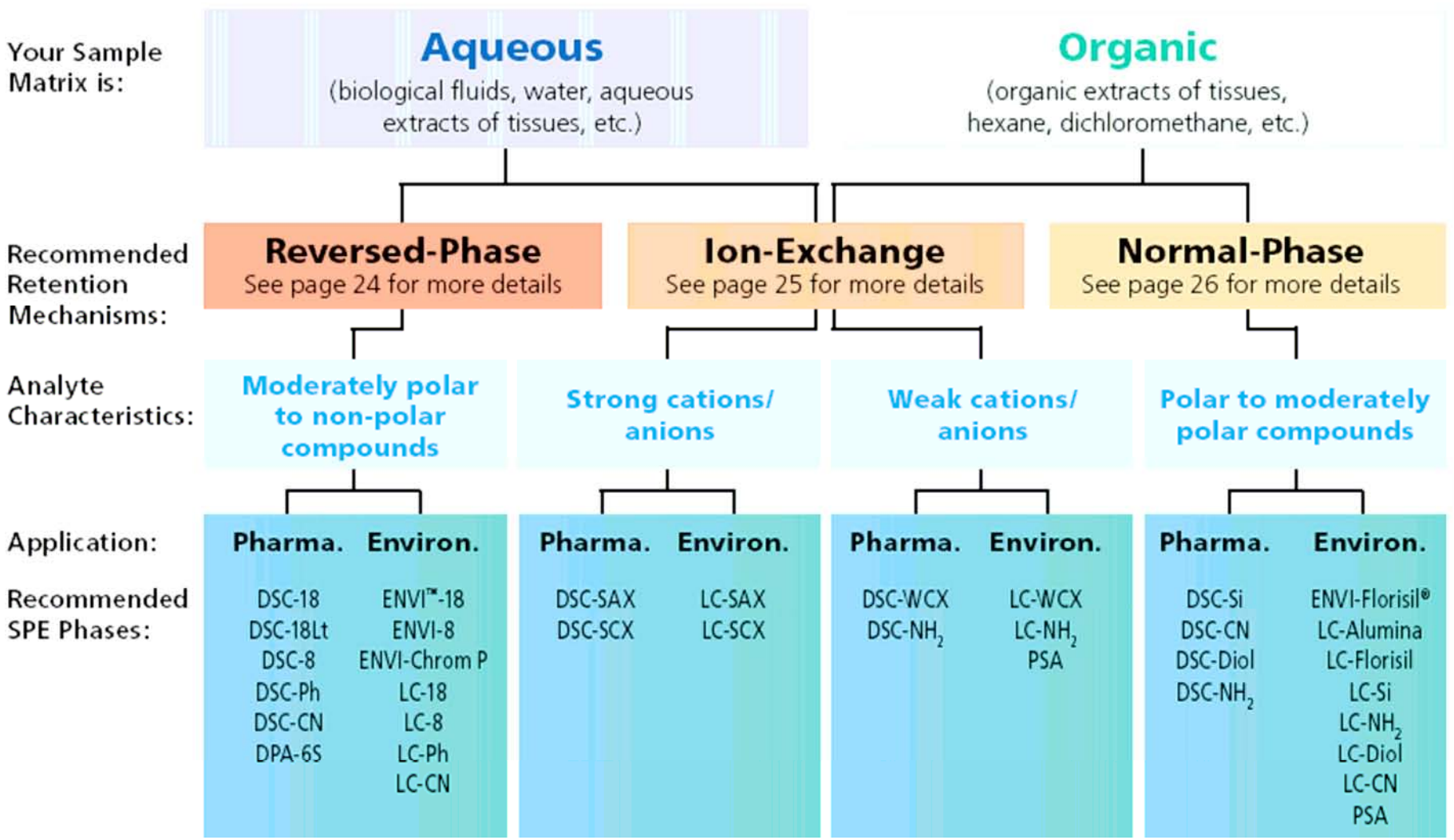
ENVI-Carb- Graphitized carbon excellent for extracting polar compounds from aqueous samples

DSC-MCAX- mixed-cation phase for superior selectivity for extracting basic compounds from biological fluids

SUPELCO

SIGMA-ALDRICH

SPE Phase Selection



Choosing the Appropriate Phase Chemistry

Reversed-Phase:

- C18 (18%C)
  - Less risk of silanol activity for predictable extractions
  - Broad affinity for a wide range of compounds
  - Potential use of stronger wash solvents
  - Greater risk of co-retention of matrix interferences
  - Extract many analytes with generic methodology
- CN (Cyanopropyl; 6%C)
  - Weaker affinity to compounds
  - May retain compounds more selectivity than C18
  - Weaker wash solvents are required
  - Could yield weaker elution solvents
  - Could elute with smaller elution volumes
  - Increased risk of silanol activity (may not be bad though)
- C8 (9%C)
  - retains compounds with log Po/w ≥ 1

Choosing the Appropriate Phase Chemistry (cont.)

Ion-Exchange:

- SCX & SAX (strong ion exchange)
  - Can be very selective
  - Elution typically done via pH manipulation to neutralize analytes
  - Always some mixed-mode properties (requires a combination of pH adjustment and organic strength to elute compounds)

- WCX & NH_2 (weak ion exchange)
  - Used for extracting strong bases and acids where elution cannot be done through pH manipulation of analytes
  - Instead pH adjustment used to neutralize sorbent functional groups

Choosing the Appropriate Phase Chemistry (cont.)

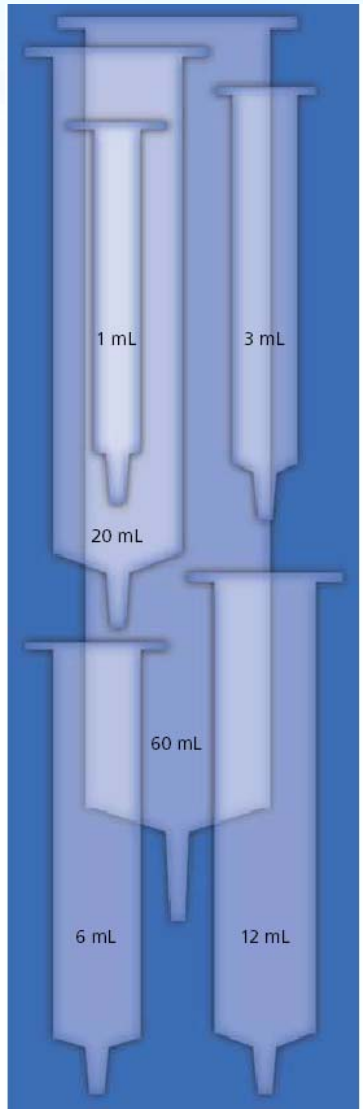
Mixed-Mode SPE:

- Dual mechanisms of attraction
  - Reversed-phase + ion-exchange = broad affinity for a wide range of compounds
  - Many compounds contain ionizable functional groups
  - Combination of hydrophobic and strong electrostatic interactions allows researcher to use vigorous wash steps
  - Results in more selective extractions

SPE Bed Weight/Tube Size Selection

Bed Weight	Tube Volume	Minimum Elution Vol.	Bed Capacity
50-100 mg	1 mL	100-200 µL	2.5-10 mg
500 mg	3 mL	1-3 mL	25-100 mg
0.5-1 g	6 mL	2-6 mL	25-100 mg
2 g 5 g 10 g	12 mL 20 mL 60 mL	10-20 mL 20-40 mL 40-100 mL	0.1-0.2 g 1.25-2.5 g 0.5-1 g

- Smaller tube dimensions (1 mL) contain smaller bed weights. Smaller bed weights allow for reduced elution volumes which can be beneficial for sensitive analyses, and when further processing is required (e.g., evaporation).
- 3 mL SPE tubes are the most common size dimension
- 6 mL SPE tubes should be used when one or more steps in the SPE process require volumes greater than 3 mL. 6 mL tubes also contain larger bed weights (up to 1g) which offers greater capacity, and can be beneficial when extracting difficult to retain compounds.
- 12, 20, and 60 mL tubes contain larger bed weights and head space volume which offer greater capacity. This allows researchers to use SPE as a purification or modified LPLC/Flash technique.

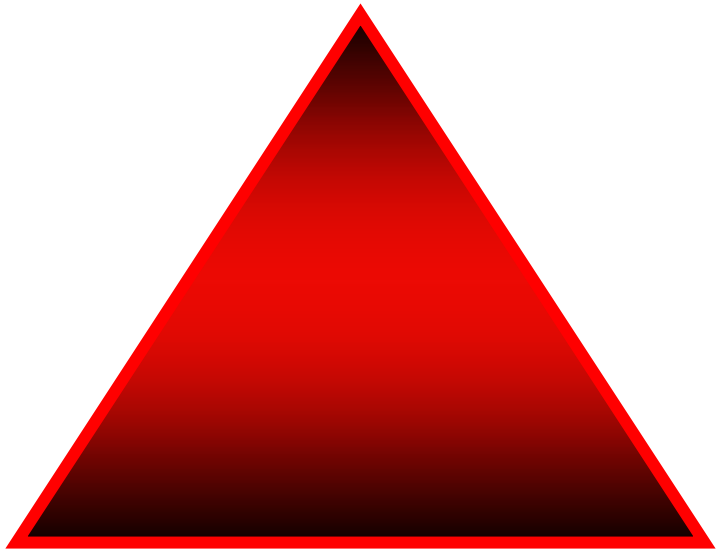


SUPELCO

SIGMA-ALDRICH

SPE is form of Chromatography!

Analyte(s) of Interest



Solid Phase Chemistry

Mobile Phase Environment

SUPELCO

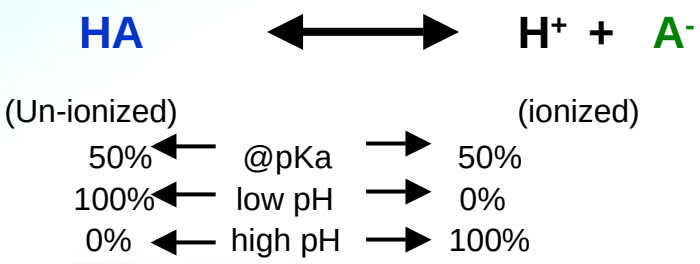
SIGMA-ALDRICH

The Critical Role of pH in SPE

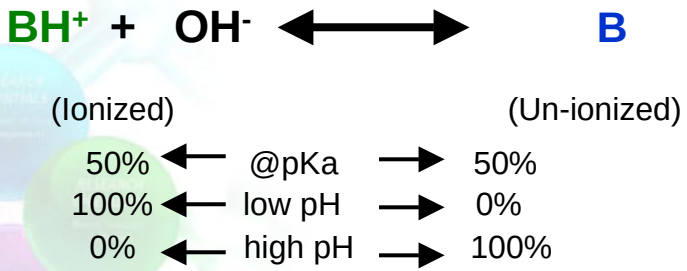
Neutral State (Blue) = promotes hydrophobic (RP) interaction
Ionized State (Green) = promotes electrostatic (IOX) interaction

Ionization of Acidic & Basic Molecules

Acids (e.g., carboxylic acids): (e.g., R-COOH ⇔ $R\text{-}COO^-$)



Bases (e.g., amines): (e.g., $R\text{-}NH_3^+$ ⇔ $R\text{-}NH_2$)



pKa of most acids (e.g. –COOH) is 3-5

- **Presence of halogen atom near a carboxy group strengthens acid effect (electron sink)**
- **e.g., acetic acid (pKa 4.75), monochloro acetic acid (pKa 2.85), dichloroacetic acid (pKa 1.48)**

pKa of most amines is 8-11

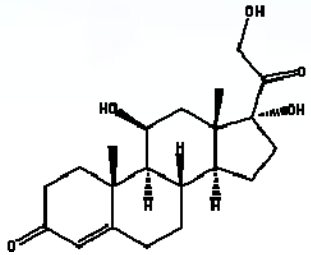
- **Aromatic (electron sink) amines have a lower pKa than aliphatic amines**
- **e.g., Aromatic amines- aniline (pKa 4.6), pyridine (pKa 5.2); Aliphatic amines- (pKa 9.7), dimethylamine (pKa 10.7)**

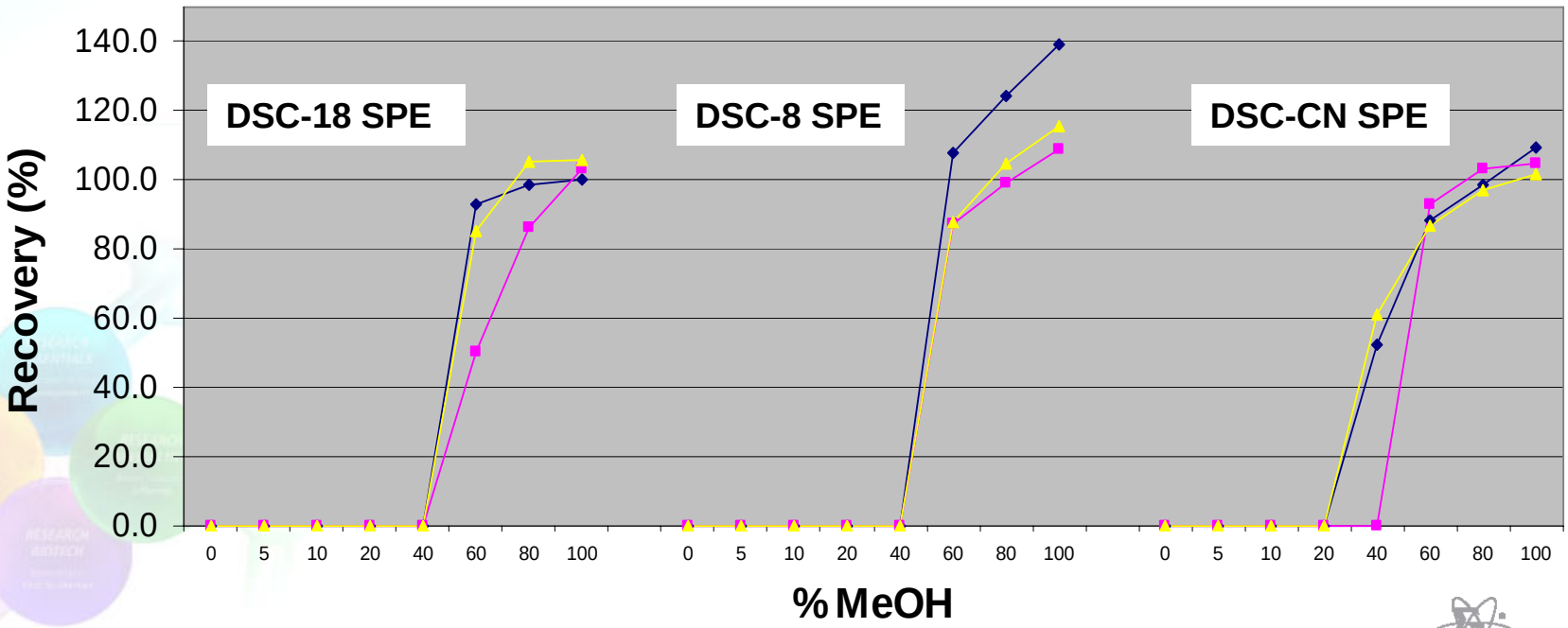
SUPELCO

SIGMA-ALDRICH

Wash/Elute Profile- Neutral Compounds

Hydrocortisone



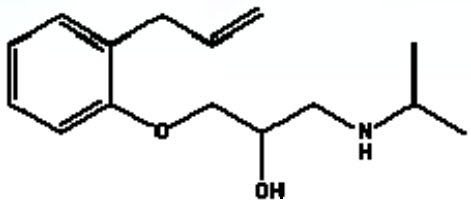


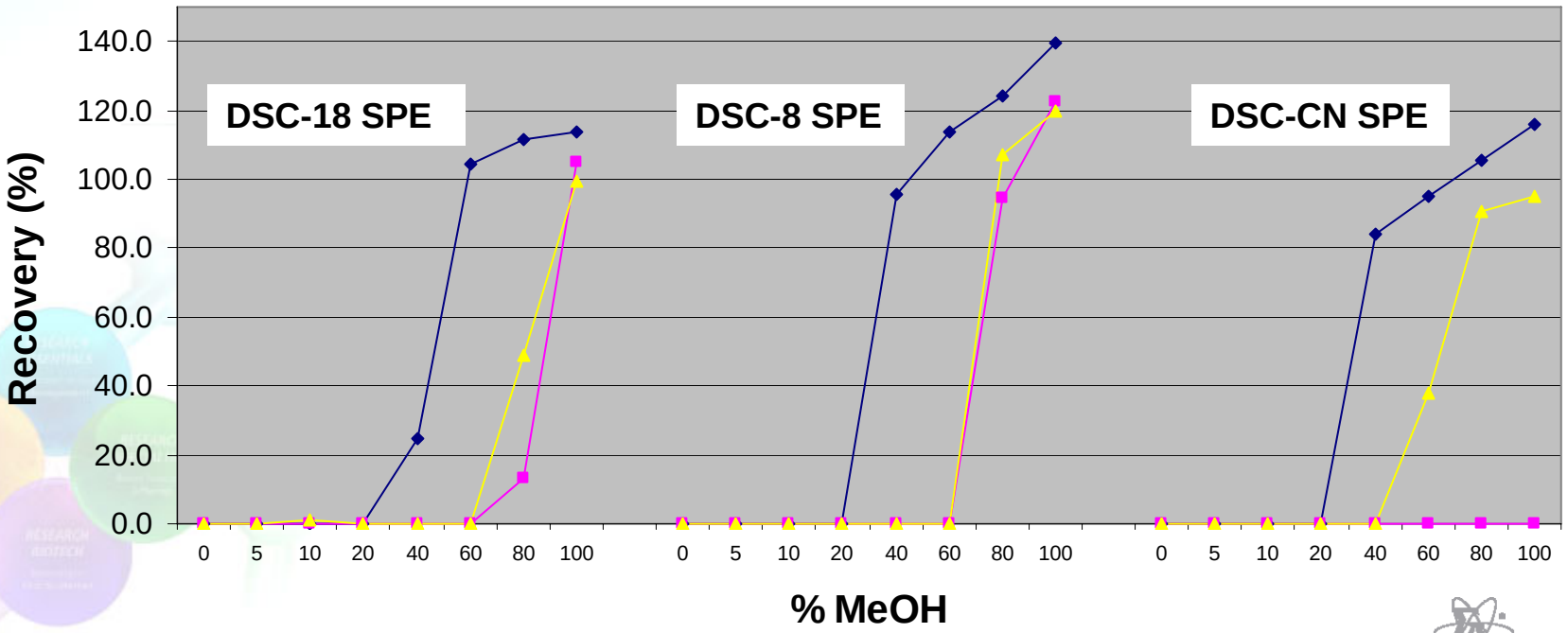
SUPELCO

SIGMA-ALDRICH

Wash/Elute Profile- Basic Compounds

Alprenolol



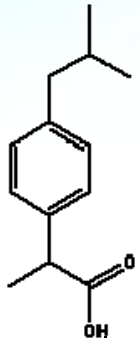


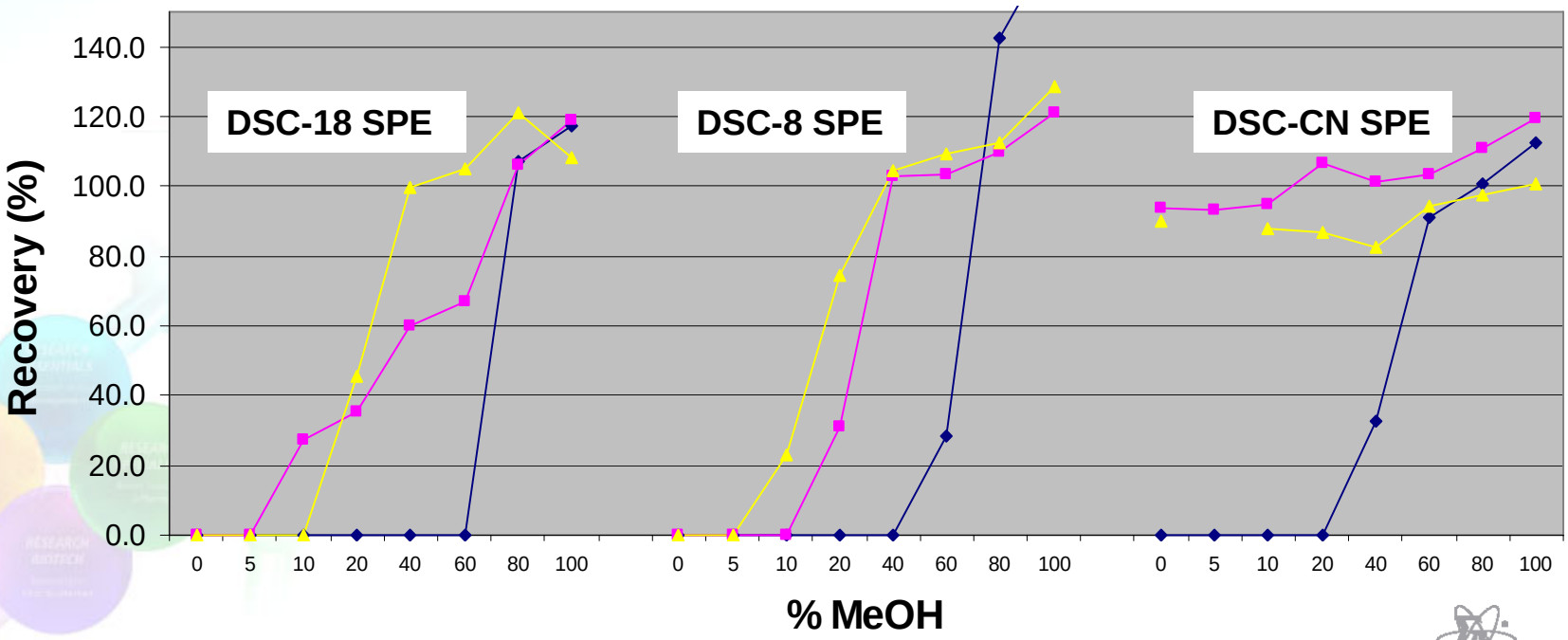
SUPELCO

SIGMA-ALDRICH

Wash/Elute Profile- Acidic Compounds

Ibuprofen





SUPELCO

SIGMA-ALDRICH

A Systematic Approach to SPE Method Development – Case study examples in pharma bioanalysis

SUPELCO

SIGMA-ALDRICH

Specific Challenges for Bioanalyses

- Determine analyte concentrations in biological fluids
  - Data used to understand time course of drug action and pharmacokinetics of an in-vivo system
  - Data used to support epidemiological studies
  - Requires efficient/adequate sample preparation, good chromatographic separation, and sensitive detection technique.
- Difficulties in analyzing biological samples
  - Many sample matrices encountered (e.g. plasma, urine, tissues, etc.)
  - Difficult Sample Matrices => Selectivity is Key
  - Must separate drugs, metabolites, and/or other small molecules from endogenous matrix interferences

How are most SPE methods developed?

Incorporate the sample matrix or real samples immediately and…

- Choose a very generic or robust method
- Duplicate an existing/similar application from a previous method
- Copy an existing application from an SPE vendor or literature reference
- Go to the local SPE “guru” for help

The Problem with these approaches…

More often, investigator will have more questions than answers

→ Leads to a Non-Systematic Approach to method development and optimization & Variable MD Time

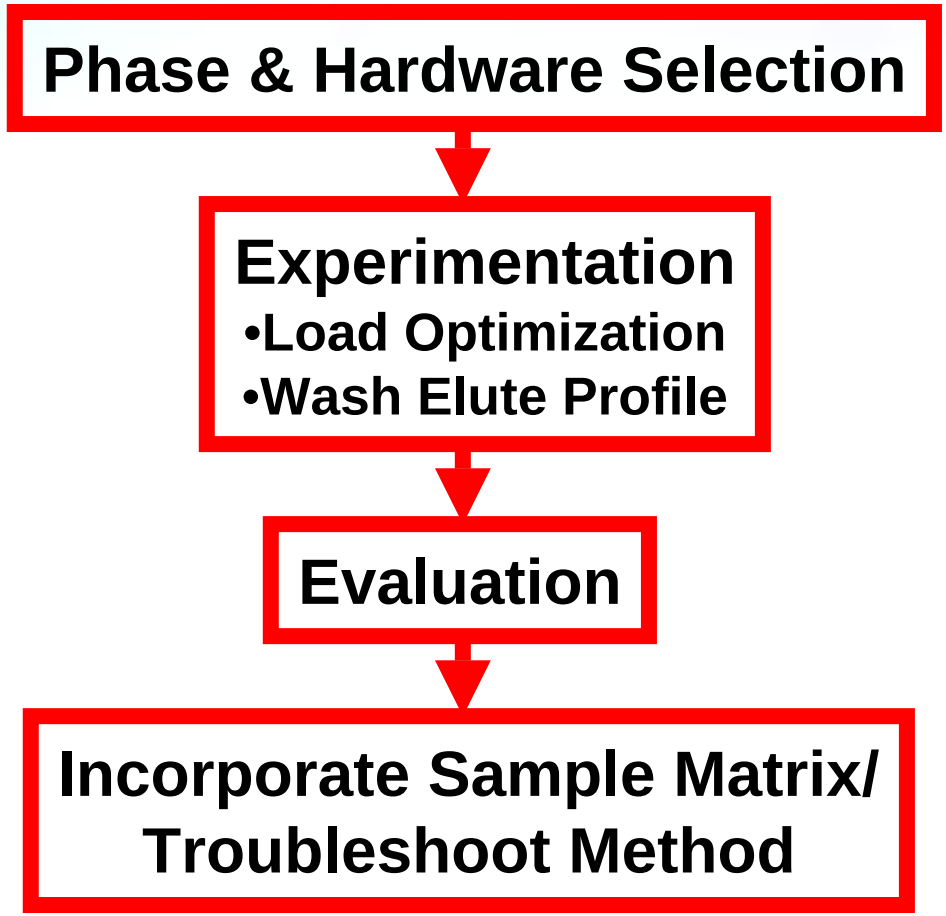
Example of Problems:

- **Dealing with novel analytes**
- **Poor Recovery. Is it due to…**
  - **Poor retention?**
  - **Pre-mature elution?**
  - **Over retention?**
- **Poor Reproducibility. Typically caused by one or more inadequate steps. Which one?**
- **Insufficient clean-up. Stronger wash solvent? Maybe a different SPE phase?**

SUPELCO

SIGMA-ALDRICH

Systematic SPE Method Development



SUPELCO

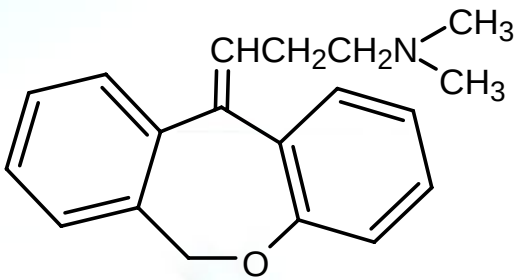
SIGMA-ALDRICH

What is Systematic SPE MD all about?

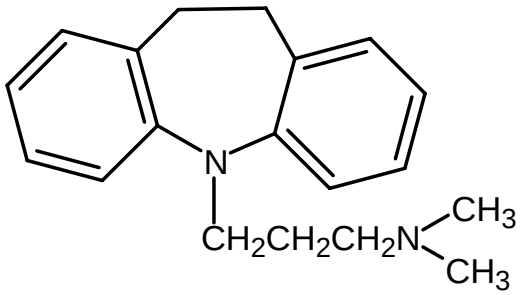
- **Maximizing Selectivity**
  - The ability of the sorbent and extraction method to discriminate between the analyte(s) of interest and endogenous interferences within the sample matrix
- **By employing two or three expts. using standard solutions w/o sample matrix**
  - systematically adjust the two main variables that control selectivity (pH & organic strength).
- **Benefits**
  - understand how the analytes interact with the sorbent under specific conditions
  - allows for a systematic approach to finding the optimal sample prep conditions with greater efficiency and confidence.

Example: Tricyclic Antidepressants from Sheep Serum

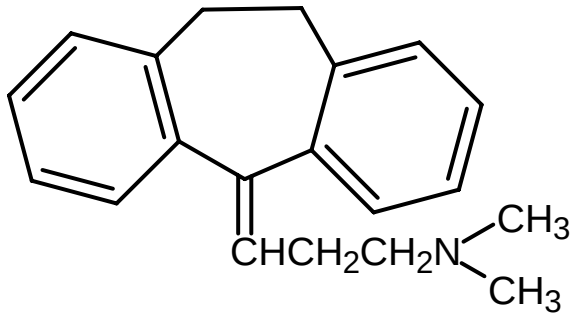
Consider the Analytes of Interest:



Doxepin



Imipramine

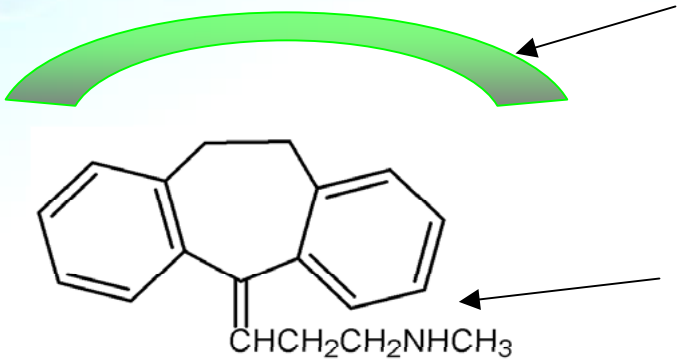


Amitrypityline

SUPELCO

SIGMA-ALDRICH

Example: Tricyclic Antidepressants from Sheep Serum



Dibenzocycloheptene skeleton = excellent hydrophobic foot print for potential reversed-phase interaction.

2° amine: basic functional group w/ a pKa of ~9. Very useful for controlling analyte's ionization state .

- Different ionic forms retain differently on a given sorbent.
- pH manipulation can control retention and selectivity on a given sorbent.
  - At pH ≥ 11, the 2° or 3° amine functional group should be neutralized.
  - At pH ≤ 7, the amine group should be ionized.

Example: Tricyclic Antidepressants from Sheep Serum

Load Optimization- Ensures retention of the analytes of interest

1. Conditions DSC-18 wells with 1mL MeOH
2. Equilibrate DSC-18 wells with 1mL DI H_2O
3. Load 1mL 5µg/mL standard test mix prepared at neutral (DI H_2O) and basic pH (1% NH_4OH).
4. Collect Load eluate and analyze via HPLC-UV

Note: load concentration was increased to provide adequate signal response for detecting small analyte breakthrough percentages. Also note that acidic load conditions were avoided.

Example: Tricyclic Antidepressants from Sheep Serum

Load Optimization Evaluation:

- A lack of analyte presence in the load eluate was found for both pH conditions

  → Indicates adequate retention for both neutral and basic load conditions

- Basic pH was chosen to ensure maximum retention for the three basic analytes.
- Stronger retention permits the potential use of stronger wash solvents increasing overall sample clean-up

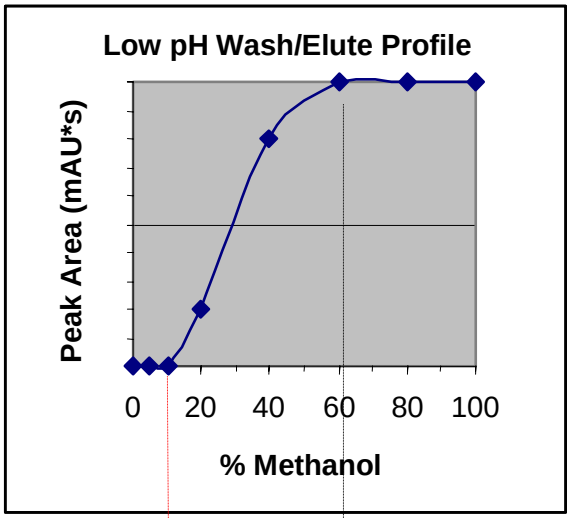
Example: Tricyclic Antidepressants from Sheep Serum

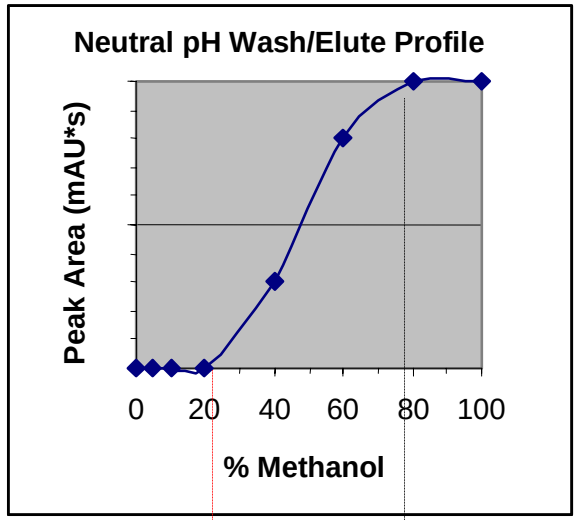
Wash/Elute Profile- Determine analyte retention and elution patterns as a function of pH & % Organic

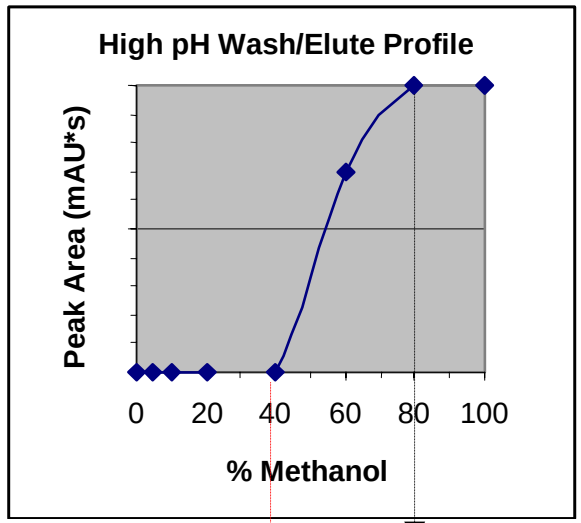
1. Conditions DSC-18 wells with 1mL MeOH
2. Equilibrate DSC-18 wells with 1mL DI H_2O
3. Load 1mL 5µg/mL standard test mix prepared at basic pH (1% NH_4OH).
4. Wash/Elute with 1mL of a test solvent ranging from 0-100% MeOH in 2% NH_4OH (high pH), DI H_2O, and 2% CH_3COOH (low pH)
5. Collect wash/elute eluate and analyze via HPLC-UV

Example: Tricyclic Antidepressants from Sheep Serum

Wash/Elute Profile Evaluation-







At low pH, complete elution occurs at 60% MeOH.

At neutral pH, complete elution occurs at 80% MeOH.

Under basic pH, complete elution occurs at 80% MeOH.

At low pH, retention limit is 10% MeOH.

At neutral pH, retention limit is 20% MeOH.

Under high pH, retention limit is 40% MeOH.

Example: Tricyclic Antidepressants from Sheep Serum

- Incorporate Sample Matrix/Troubleshoot Method-
  - Obtain specific guidelines for defining, optimizing, and troubleshooting the extraction method
  - For most applications, recovery values observed for real-matrix based solutions will parallel values obtained with standard solutions

Systematic Method on DSC-18 Well Plate vs. Generic Method on Polymer Phase

Systematically Developed Method on DSC-18 SPE-96 Well Plate (100mg/well)

1. **Condition/Equilibrate w/ 1mL MeOH & 1mL DI H_2O**
2. **Load 0.25-2.0µg/mL TCAs spiked in sheep serum diluted in 2% NH_4OH (1:1, v/v); n=3 for ea. concentration**
3. **Wash w/ 1mL 40% MeOH in 2% NH_4OH**
4. **Elute w/ 1mL MeOH**
5. **Evaporate eluate with N-purge (30°C; ~10min.), and reconstitute in 300µL MP**

Note: 60% acidified MeOH may have been a potential elution eluant

Generic Method on Competitor Polymeric Phase (30mg/well)

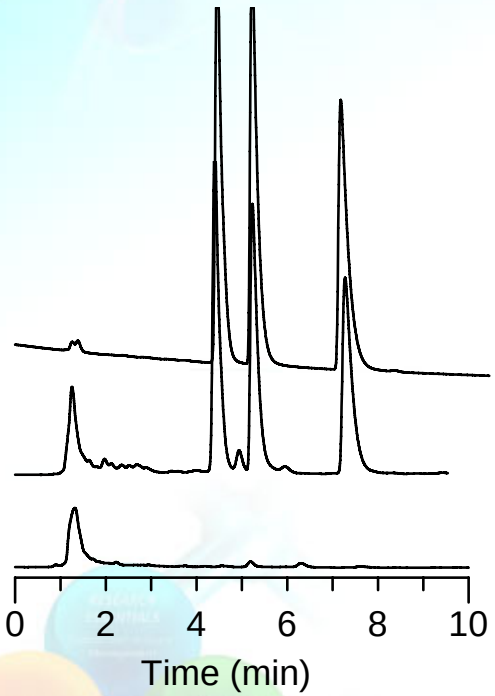
1. **Condition/Equilibrate w/ 1mL MeOH & 1mL DI H_2O**
2. **Load 0.25-2.0µg/mL TCAs spiked in sheep serum diluted in 2% NH_4OH (1:1, v/v); n=3 for ea. concentration**
3. **Wash w/ 1mL 5% MeOH**
4. **Elute w/ 1mL MeOH**
5. **Evaporate eluate with N-purge (30°C; ~10min.), and reconstitute in 300µL MP**

SUPELCO

SIGMA-ALDRICH

Results

POS Method Using DSC-18 SPE-96 Well plate



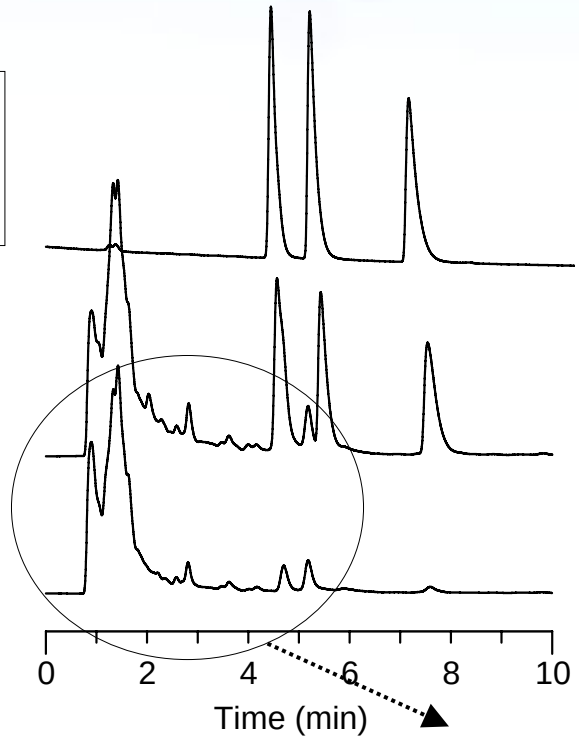
Ext. Stds

1µg/mL Spiked serum

Blank serum

1. Doxepin
2. Imipramine
3. Amitryptyline

Generic Method Using Competitor Polymeric Well Plate



Ext. Stds

1µg/mL spiked serum

Blank serum

HPLC Method:

Column: **Discovery C18**, 15cmx4.6mm, 5µm, & 2cm guard column & 0.5µm frit filter;

Mobile Phase: MeCN: 25mM KH_2PO_4, pH 7 (45:55);

Flow Rate: 1.4mL/min; Temp: 30°C;

Det.: UV, 254nm; **Inj:** 100µL

High Background; Misleading interfering responses

SUPELCO

SIGMA-ALDRICH

Results

Efficiency of Absolute Recovery ofTricyclic Antidepressants on POS Method Using Discovery DSC-18 SPE Vs. Generic Method Using Competitor Polymer Phase

Compound	Concentration	%Recovery ± RSD (n=3) on Discovery DSC-18	%Recovery ± RSD (n=3) on Competitor Polymer Phase
1. Doxepin	1.0µg/mL	90.8 ± 1.2%	108.8 ± 8.2%
	0.5µg/mL	91.1 ± 1.6%	127.6 ± 13.5%
	0.25µg/mL	89.2 ± 2.2%	167.8 ± 3.2%
2. Impipramine	1.0µg/mL	95.5 ± 2.5%	88.4 ± 5.6%
	0.5µg/mL	97.7 ± 0.6%	98.2± 14.7%
	0.25µg/mL	97.8 ± 3.7%	93.1± 0.3%
3. Amitryptyline	1.0µg/mL	91.0 ± 2.0%	92.4 ± 5.1%
	0.5µg/mL	87.4 ± 1.4%	104.9 ± 12.6%
	0.25µg/mL	89.5 ± 3.5%	133.5 ± 1.4%

Example: Tricyclic Antidepressants from Sheep Serum

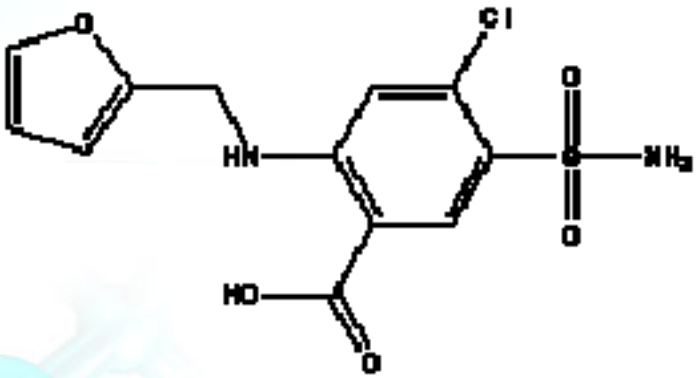
Summary

- **In this study, through systematic SPE method development we were able to:**

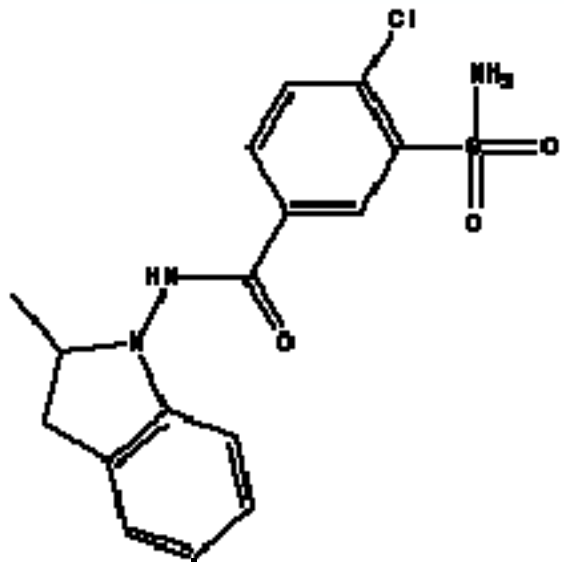
1. optimize the wash solvent to maximize sample clean-up resulting in minimal background and more accurate results
   - Determination of 40% MeOH in 2%NH_4OH as wash solvent
   - 60% MeOH in 2% CH_3COOH
2. achieve high and reproducible recoveries at the spike levels tested (> 90% recovery, $\leq$ 4% RSD).
3. High background observed on generic method on polymeric SPE well plate

Example: Furosemide from Horse Serum

Consider the Analytes of Interest:



Furosemide



Indapamide (I.S.)

SUPELCO

SIGMA-ALDRICH

Example: Furosemide from Horse Serum

Load Optimization

SPE: **Discovery DSC-18 SPE 96-well, 50mg/well**

1. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
2. Load 1mL standards containing 5.0µg/mL furosemide and indapamide in 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)
3. Collect load flow-through eluate & analyze for compound break through via HPLC-UV

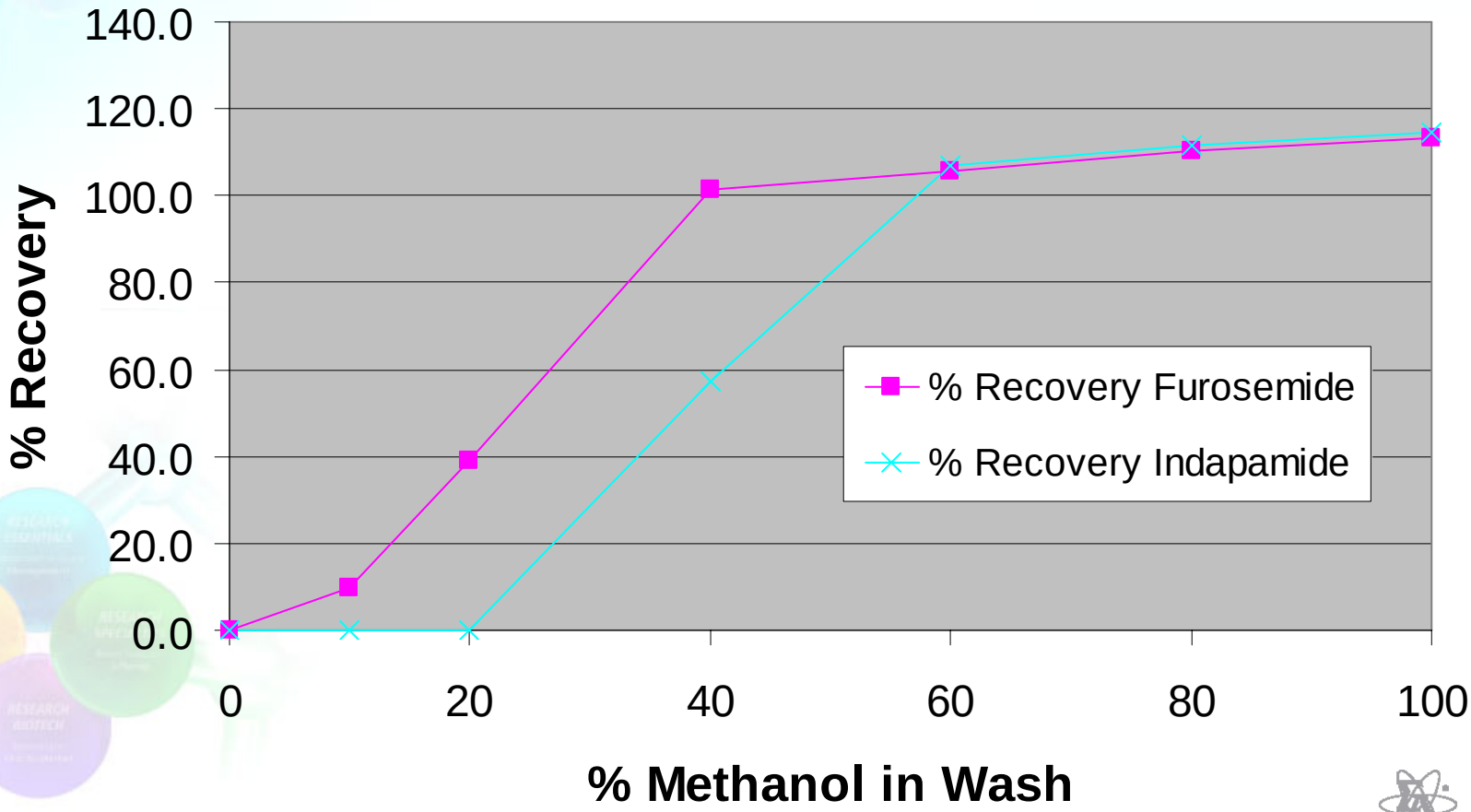
Wash/Elute Profile

1. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
2. Load 1mL standards containing 5.0µg/mL furosemide and indapamide in 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)
3. Wash/elute with 1mL test solvents ranging from 0-100% methanol
4. Collect wash/elute eluate & analyze for compound elution via HPLC-UV

SUPELCO

SIGMA-ALDRICH

Wash/Elute Profile for Furosemide & Indapamide (I.S.) on Discovery DSC-18 SPE



SUPELCO

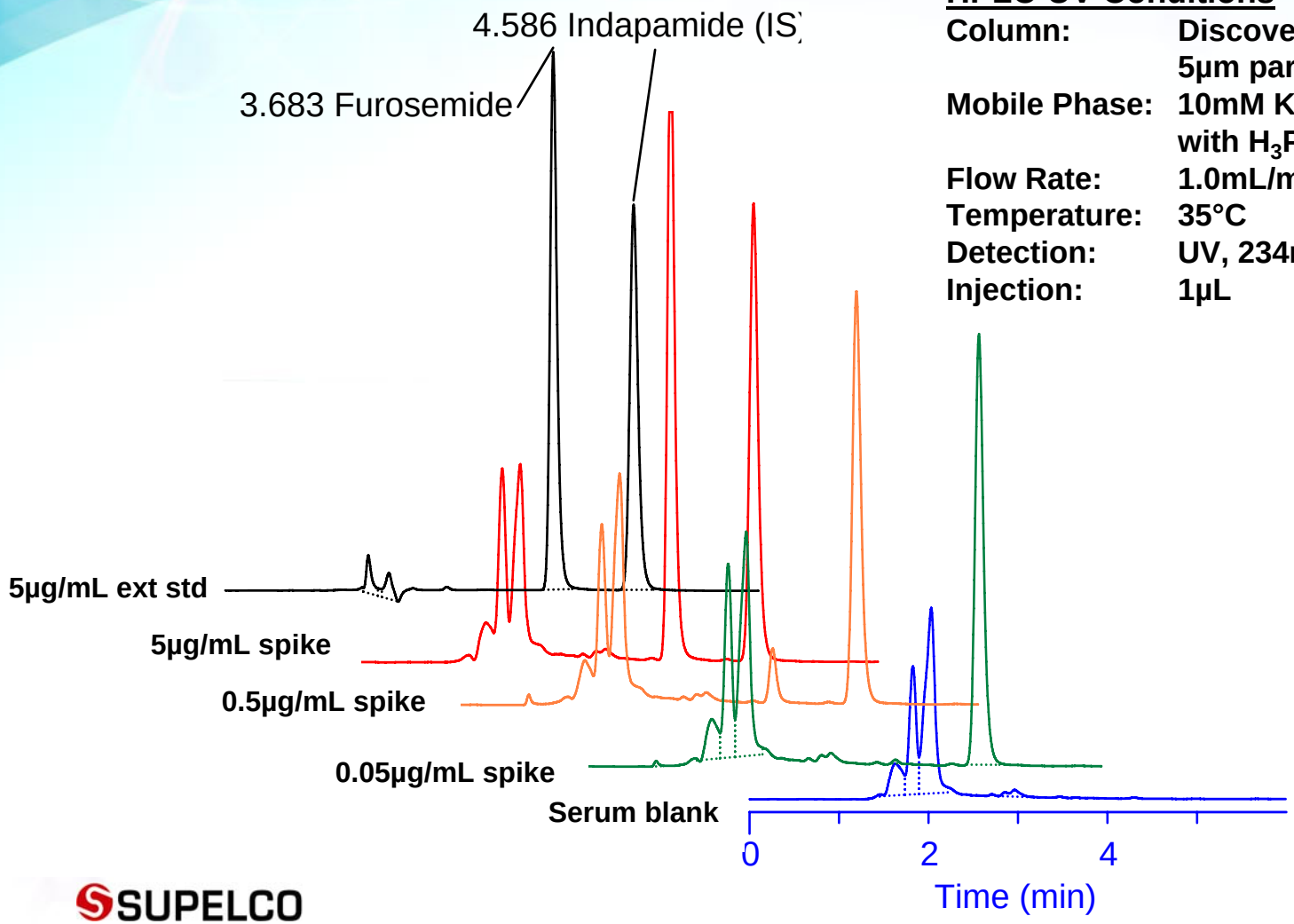
SIGMA-ALDRICH

Systematically Developed SPE Method For Furosemide & Indapamide from Serum

SPE: Discovery DSC-18 SPE, 50mg/1mL

1. Condition with 1mL methanol
2. Equilibrate with 1mL 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)
3. Load 1mL sample
4. Wash with 1mL 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)
5. Elute with 1mL 60% methanol in DI H_2O
6. Directly analyze eluate (no evaporation/reconstitution) via HPLC-UV.
7. Determine relative recovery and RSD against working calibration standards not subjected to SPE sample preparation.

Example Chromatograms of Extracts Generated from the Systematically Developed Method on Discovery DSC-18 SPE



HPLC-UV Conditions

Column:	**Discovery C18, 15cm x 4.6mm ID, 5µm particles**
Mobile Phase:	**10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4):MeCN (60:40)**
Flow Rate:	**1.0mL/min**
Temperature:	**35°C**
Detection:	**UV, 234nm**
Injection:	**1µL**

SUPELCO

SIGMA-ALDRICH

Relative Recovery of Furosemide from Horse Serum Using Discovery DSC-18 SPE

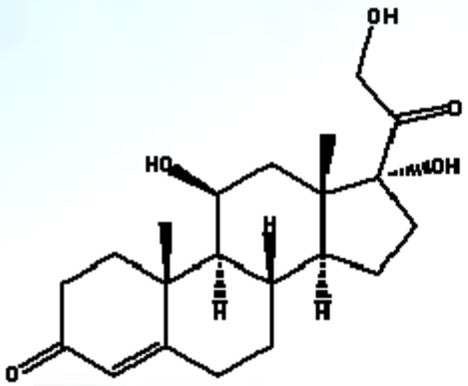
Sample	Furosemide Spike Concentration (µg/mL)	Avg. Response Factor	% Recovery ± RSD (n=3)
A	10.00	2.307	99.3 ± 3.1
B	5.00	1.168	100.8 ± 1.4
C	0.50	0.107	97.4 ± 2.8
D	0.10	0.065	120.7 ± 1.3
E	0.05	0.009	132.8 ± 8.3

Example: Furosemide from Horse Serum

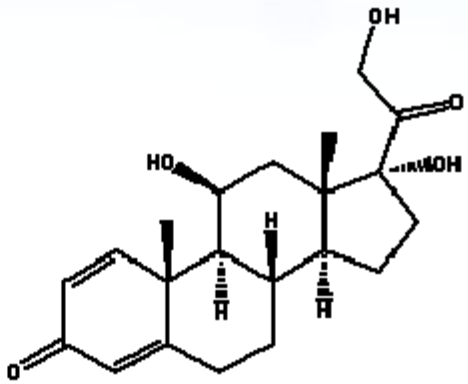
Summary

- Furosemide not strongly retained on C18 SPE under neutral conditions
- Wash solvents stronger than DI water caused compound elution
- Selectivity improved by eluting with weaker elution solvent (e.g. 60% MeOH)
- Weaker elution solvent = direct injection of the final eluate.
- Average relative recovery and RSD = 100.2 ± 3.4%.
- Note that the procedure is quantitative down to 0.5µg/mL serum.
- Below this level, reasonable precision is evident; but, accuracy suffers.
- Primarily to the detection limitations of UV absorbance for furosemide. Flourescence detection or mass spectrometry is likely to provide increased sensitivity.
- Decreasing SPE elution volume may also be a viable choice for improving sensitivity.

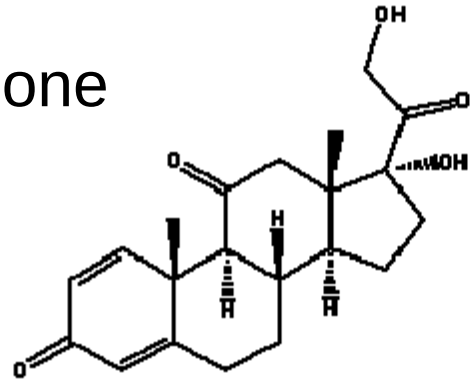
Example: Corticosteroids from Urine



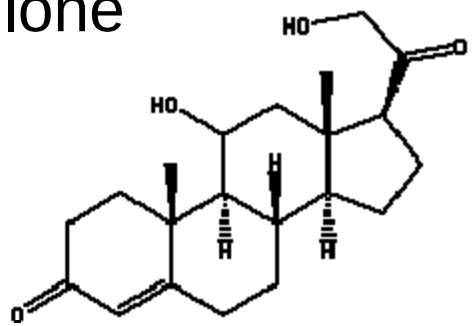
Hydrocortisone



Prednisilone



Predinisone



Corticosterone

Example: Corticosteroids from Urine

Load Optimization

SPE: Conventional C18 96-well SPE (100mg/well)

Discovery DSC-CN SPE 96-well (100mg/well)

1. Condition & equilibrate SPE wells (C18 & CN) with 1mL methanol & DI H_2O
2. Load 1mL standards containing 5.0µg/mL of each of the four corticosteroids in DI H_2O
3. Collect load flow-through eluate & analyze for compound break through via HPLC-UV

Wash/Elute Profile

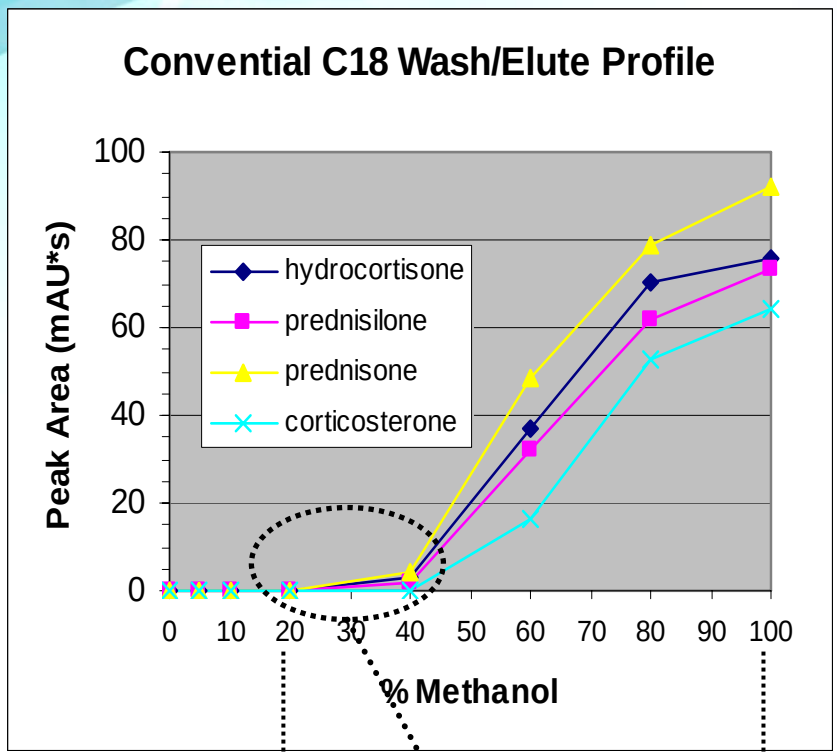
1. Condition & equilibrate SPE wells (C18 & CN) with 1mL methanol & DI H_2O
2. Load 1mL standards containing 5.0µg/mL of each of the four corticosteroids in DI H_2O
3. Wash/elute with 1mL test solvents ranging from 0-100% methanol
4. Collect wash/elute eluate & analyze for compound elution via HPLC-UV

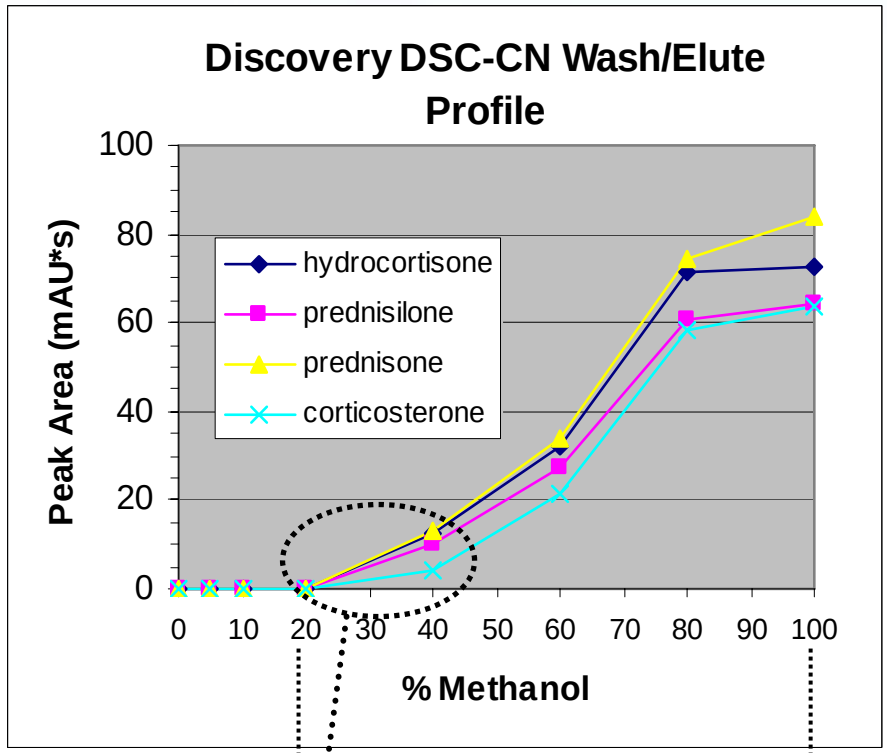
Example: Corticosteroids from Urine

HPLC-UV Conditions

Column:	Discovery HS F5, 5cm x 4.6mm ID, 3µm particles
Mobile Phase:	Methanol:DI H_2O (40:60)
Flow Rate:	1.5mL/min
Temperature:	35°C
Detection:	UV, 240nm
Injection:	5µL

Wash/Elute Profile for Corticosteroids on C18 & CN SPE





For C18, Retention Limit is 20% MeOH

For C18, Full elution at 100% MeOH

For CN, Retention Limit is 20% MeOH

For CN, Full elution at 100% MeOH

Increased hydrophobic retention observed on C18 relative to CN

Systematically Developed SPE Method For Steroids From Urine

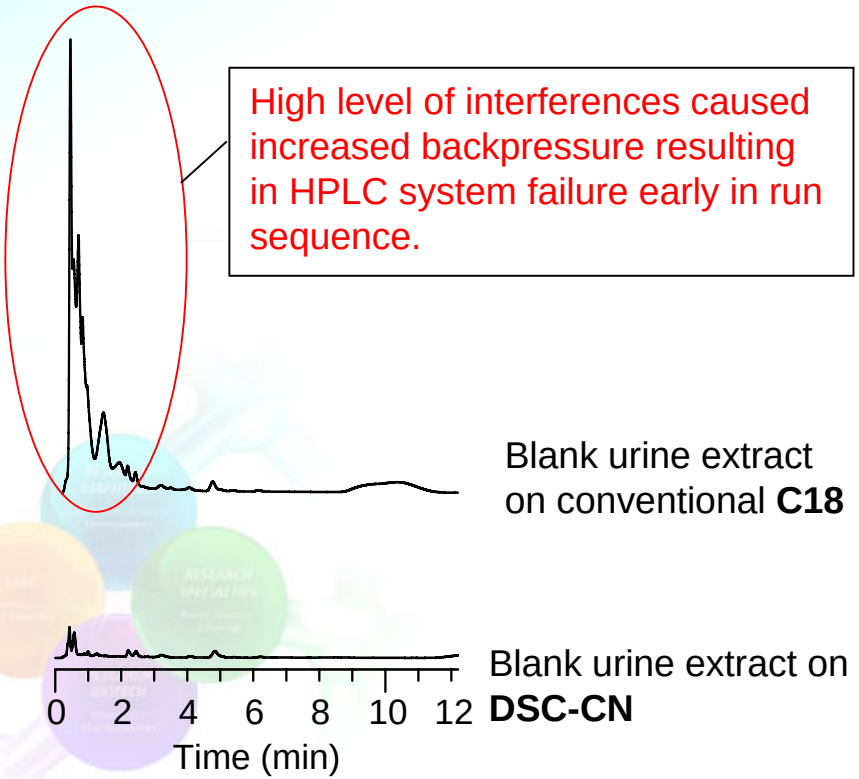
SPE: Conventional C18 96-well SPE (100mg/well)

Discovery DSC-CN SPE 96-well (100mg/well)

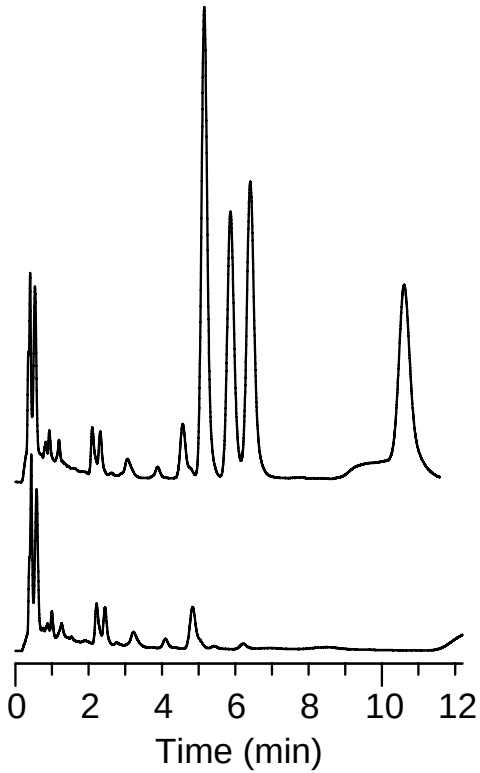
1. Condition & equilibrate with 1mL methanol and 1mL DI H_2O
2. Load 0.5 & 1.0µg/mL corticosteroids spiked in human urine diluted in DI H_2O (1:1, v/v); n=3
3. Wash with 1mL 20% methanol
4. Elute with 1mL 100% methanol
5. Evaporate eluate with nitrogen purge (30°C; ~10 min), and reconstitute in 200µL HPLC mobile phase

Chromatograms of Blank & Spiked Urine Extracts Generated on C18 & CN SPE

Blank urine extracts on C18 & CN SPE



Blank & spiked urine extracts on CN SPE



SUPELCO

SIGMA-ALDRICH

Recovery of Steroidal Compounds from Urine on Discovery DSC-CN SPE

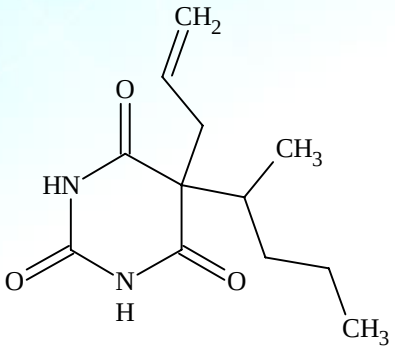
	% Absolute Recovery ± RSD (n=3)	
Compound	0.5ug/mL spike level	1.0ug/mL spike level
1. Hydrocortisone	123.3 ± 1.4%	95.9 ± 1.7%
2. Prednisilone	107.2 ± 1.1%	91.9 ± 1.1%
3. Prednisone	103.2 ± 1.0%	88.4 ± 1.8%
4. Corticosterone	102.0 ± 1.2%	93.1 ± 5.6%

Example: Corticosteroids from Urine

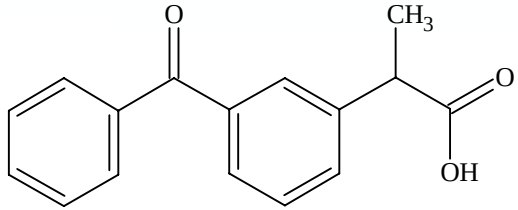
Summary

- Under identical SPE protocols, C18 SPE eluate carried a yellow tint => lead to system failure due to high back pressure
- Stronger wash solvents required; but stronger wash solvents will lead to premature analyte elution
- In contrast, improved selectivity was observed on DSC-CN
- Chromatograms were free of interfering components
- On DSC-CN SPE , avg. absolute recovery and RSD was 100.6 ± 1.9%.
- Recovery values for C18 SPE were not obtained due to HPLC system failure caused by insufficient sample clean-up.

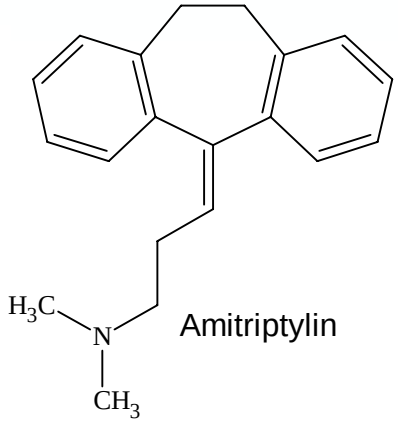
Example: Five pharmaceutical compounds from human urine



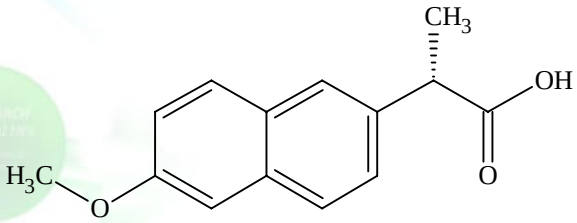
Secobarbital



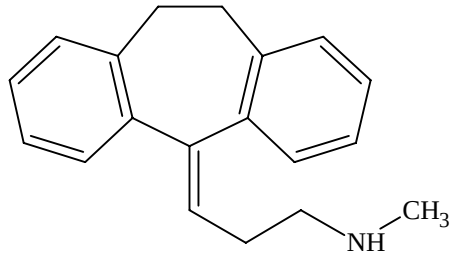
Ketoprofen



Amitriptylin



Naproxen



Nortriptyline

SUPELCO

SIGMA-ALDRICH

Table 1. Fractionation Protocol Used for Discovery DSC-MCAX SPE, 1g/6mL[1]

Condition:

6mL methanol
6mL 10mM acetic acid, pH 3

Load:

1mL spiked urine sample Urine samples diluted 1:1 with 10mM potassium phosphate, pH 3 prior to loading

- Low sample pH neutralizes all acidic compounds inducing reversed-phase retention
- Low sample pH ionizes basic compounds inducing ionic-exchange retention

Wash:

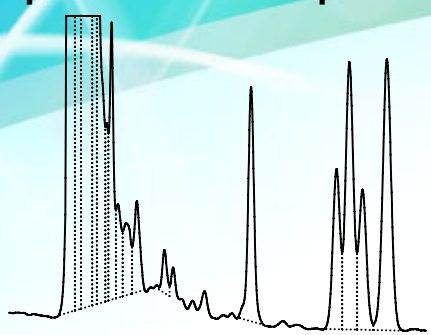
Wash with 6mL 10mM acetic acid, pH 3The low pH aqueous wash solvent removes all non-basic hydrophilic interferences, and also locks basic compounds onto the sorbent reinforcing both ionic and reversed-phase retention.

Elute:

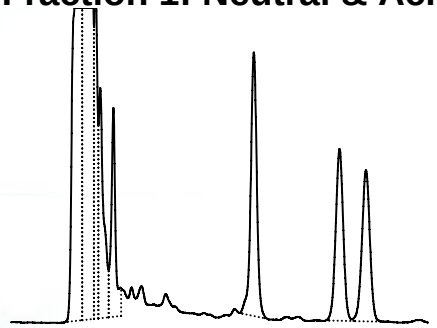
Elute basic compounds with 6mL 1%TEA in methanol or 5% ammonium hydroxide in methanolThe increase in elution pH neutralizes basic compounds disrupting ionic interactions between compound and sorbent. Favorable hydrophobic interactions disrupted via the presence of methanol

Eluate EvaporationEluate fractions were evaporated to dryness at 37°C under N_2-purge, and reconstituted with 1mL mobile phase.

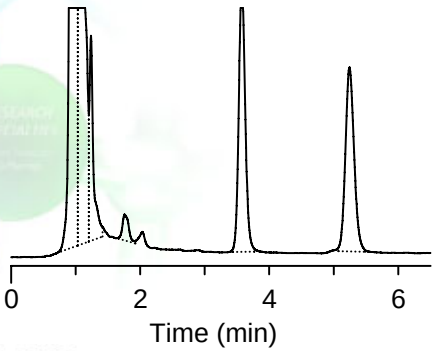
Spiked Urine Samples before DSC-MCAX SPE



Fraction 1: Neutral & Acidic Compounds



Fraction 2: Basic Compounds



SPE Tube: **Discovery DSC-MCAX**, 1g/6mL
HPLC Col.: Discovery C8,
15cm x 4.6mm, 5µm particles

Mobile Phase 1 (Pre-SPE & Fraction 1):
0.1% TFA:MeOH (50:50)
Mobile Phase 2 (Fraction 2):
10mM ammonium acetate:MeOH
(45:55)

Flow Rate: 2mL/min.
Temp.: Ambient
Det.: 214nm, UV
Inj. Vol.: 10µL

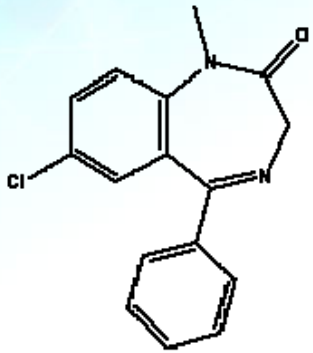
SUPELCO

SIGMA-ALDRICH

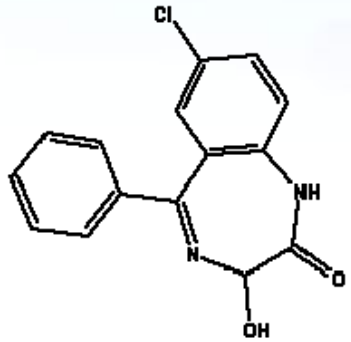
Example: Five pharmaceutical compounds from human urine using DSC-MCAX

Compound Description		% Recovery ± RSD (n=3)
Neutral	1. Secobarbital (10µg/mL spike)	105.8 ± 2.1%
Acidic	2. Ketoprofen (5.0µg/mL spike)	101.7 ± 1.3%
Acidic	3. Naproxen (2.5µg/mL spike)	101.5 ± 0.8%
Basic	4. Notriptyline (5.0µg/mL spike)	100.3 ± 0.5%
Basic	5. Amitriptyline (5.0µg/mL spike)	103.3 ± 1.7%

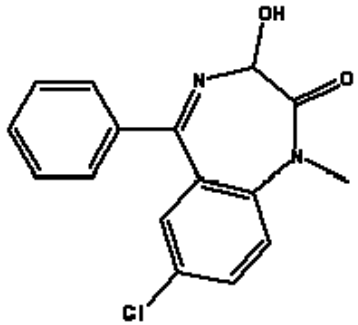
Example: Diazepam/metabolites from porcine serum



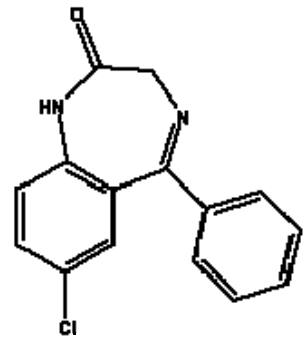
diazepam



oxazepam



temazepam



desmethyl diazepam

SUPELCO

SIGMA-ALDRICH

Example: Diazepam/metabolites from porcine serum

Load Optimization

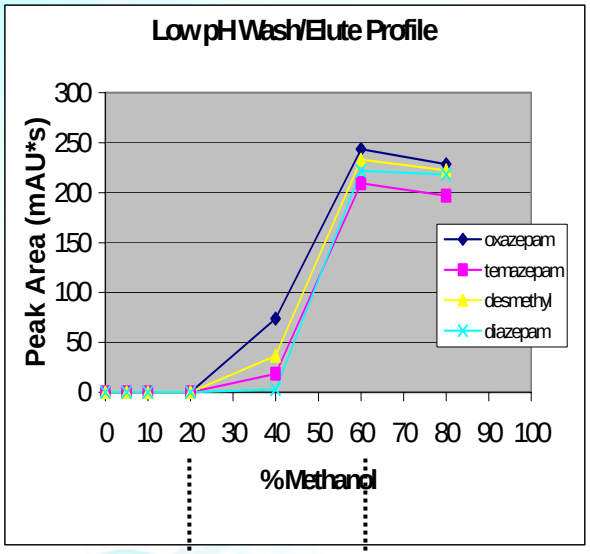
SPE: Discovery DSC-8 SPE 96-well (100mg/well)

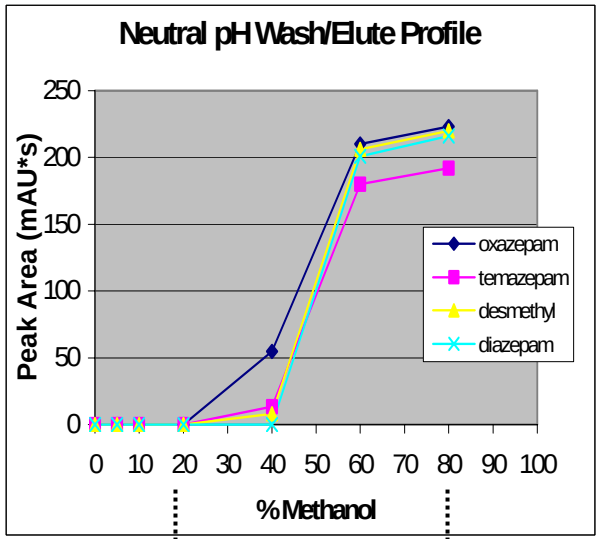
1. Prepare standards containing 2.5µg/mL diazepam and metabolites in neutral (10mM ammonium formate, pH 7.1), and basic (1% NH_4OH) solutions
2. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
3. Load 1mL of each standard test mix (neutral and high pH)
4. Collect load eluate & analyze for compound break through via HPLC-UV

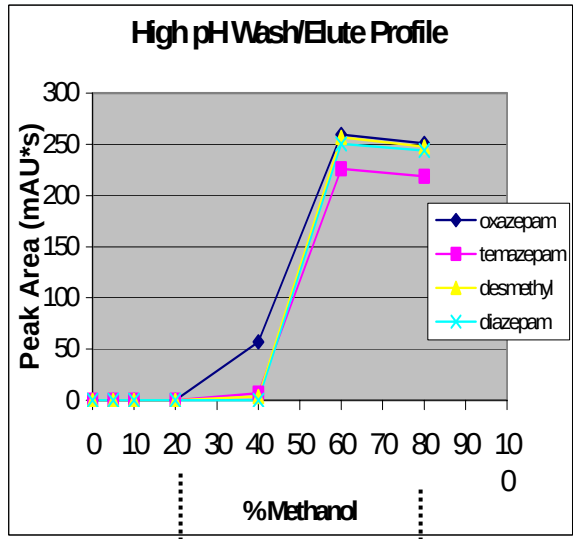
Wash/Elute Profile

1. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
2. Load 1mL standards containing 2.5µg/mL diazepam in 25mM ammonium formate, pH 7.1
3. Wash/elute respective wells with 1mL test solvents ranging from 0-100% methanol in 1% NH_4OH, pH 11 (high pH), 10mM ammonium formate, pH 7.1 (neutral pH), and 10mM ammonium formate, pH 2.7 (low pH)
4. Collect wash/elute eluate & analyze for compound elution via HPLC-UV

Wash/Elute Profile for Diazepam & Metabolites on Discovery DSC-8 SPE







At low pH, complete elution occurs at 60% methanol

At neutral pH, complete elution occurs at 80% methanol

At high pH, complete elution occurs at 80% methanol

At low pH, retention limit is 20% methanol

At neutral pH, retention limit is 20% methanol

At high pH, retention limit is 20% methanol

SUPELCO

SIGMA-ALDRICH

SPE Methods Employed for Extracting Diazepam and Metabolites from Serum

Systematically Developed Method on C8

SPE: **Discovery DSC-8 SPE 96-well Plate (100mg/well)**

1. Condition & equilibrate each well with 1mL methanol & DI H_2O
2. Load 1mL, 0.5µg/mL diazepam and metabolites spiked in goat serum diluted in 10mM ammonium formate, pH 7.1 (1:1; v/v)
3. Wash with 1mL 20% methanol in 1% NH_4OH, pH 11
4. Elute with 1mL 60% methanol in 25mM ammonium formate, pH 2.75

Generic Method on C18

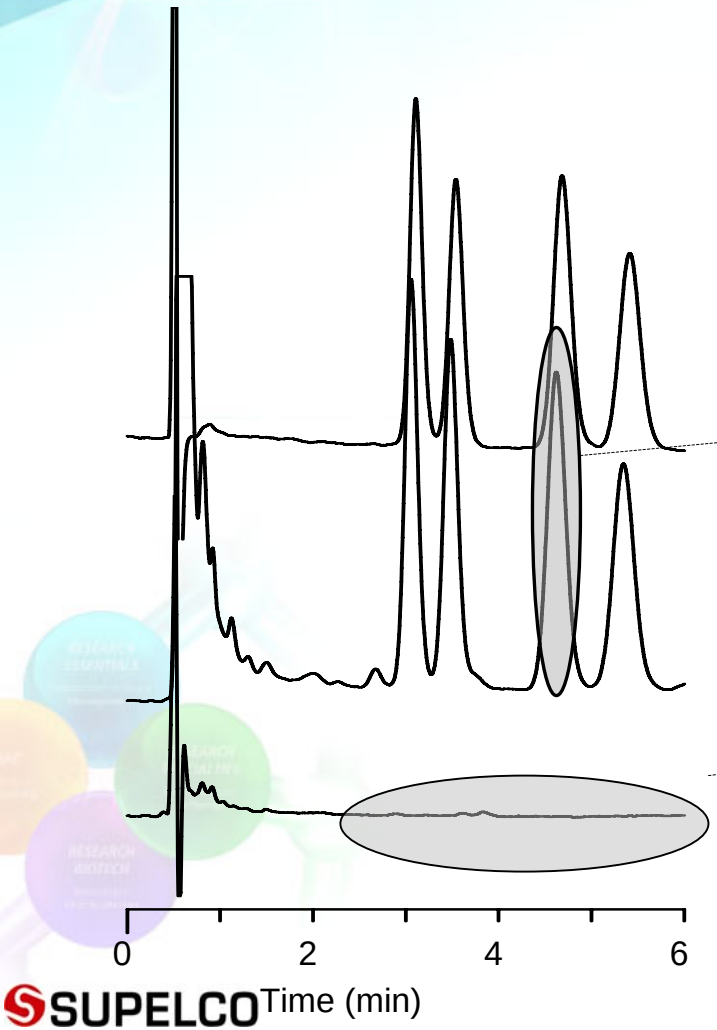
SPE: **Conventional C18 SPE 96-well Plate (100mg/well)**

1. Condition & equilibrate each well with 1mL methanol & DI H_2O
2. Load 1mL, 0.5µg/mL diazepam and metabolites spiked in goat serum diluted in 10mM ammonium formate, pH 7.1 (1:1; v/v)
3. Wash with 1mL 5% methanol
4. Elute with 1mL methanol
5. Evaporate eluate with nitrogen purge (30°C; ~15 min); and reconstitute with 200µL HPLC mobile phase

Example Chromatograms of Blank & Spiked Serum Extracts Generated on C8 SPE

HPLC-UV Conditions

Column:	Discovery C18, 5cm x 4.6mm ID, 5µm particles
Mobile Phase:	Methanol:10mM ammonium acetate, pH 4.5 (45:55)
Flow Rate:	1.5mL/min
Temperature:	35°C
Detection:	UV, 240nm
Injection:	25µL



Ext. Stds

Excellent Peak Shape

0.5µg/mL spiked serum

Blank serum

Low background/minimal interferences for optimal sensitivity and resolution at reduced run times

SUPELCO

SIGMA-ALDRICH

Absolute Recovery of 0.5µg/mL Diazepam & Metabolites on Systematically Developed Method Using C8 vs. Generic Method on C18

Compound	%Recovery ± RSD (n=3)	
	Developed Method on Discovery DSC-8	Generic Method On Conventional C18
1. Oxazepam	94.7 ± 1.2%	82.8 ± 4.0%
2. Temazepam	99.9 ± 1.1%	89.1 ± 4.0%
3. Nordiazepam	94.2 ± 1.8%	82.4 ± 5.0%
4. Diazepam	90.0 ± 3.4%	68.5 ± 9.1%

Example: Diazepam/metabolites from porcine serum

Summary

- **Weaker eluent (60% methanol in low pH buffer) was determined for DSC-8 allowing for direct analysis of the SPE eluate.**
- **Provided good selectivity signified by chromatograms with low background (Figure C).**
  - **allowed for minimal run times (6 min) resulting in faster and more accurate results.**
- **Average absolute recovery and RSD on C8 via the developed method was 94.7 ± 1.9%.**
- **In contrast, the generic method on C18 yielded an average absolute recovery and RSD of 80.7 ± 5.3% (Table 3).**

Important SPE Tips

- Drug Protein Binding Effects - must be disrupted during sample pre-treatment:
  - 40µL 2% disodium EDTA per 100µL mouse plasma
  - 40µL 2% formic acid per 100µL mouse plasma
  - Other possible reagents (per 100µL matrix): 40µL 2% TCA, 40µL 2% acetic acid, 40µL 2% TFA, 40µL 2% phosphoric acid, or 200µL MeCN (protein ppt.).
- Sorbent over drying
  - Only critical with C18 & only critical in first conditioning step
  - Phase just needs to be moist during sample addition
  - All other steps non-critical

Important SPE Tips

Wash Step

- Water wash step alone does not provide a clean eluate; Need some sort of organic modifier
- For IOX SPE, MeOH or MeCN may be used as a wash solvent

Sorbent Drying prior to elution

- Important to dry sorbent prior to elution, otherwise, subsequent eluate evaporation will take a real long time.

Compound volatility during evaporation

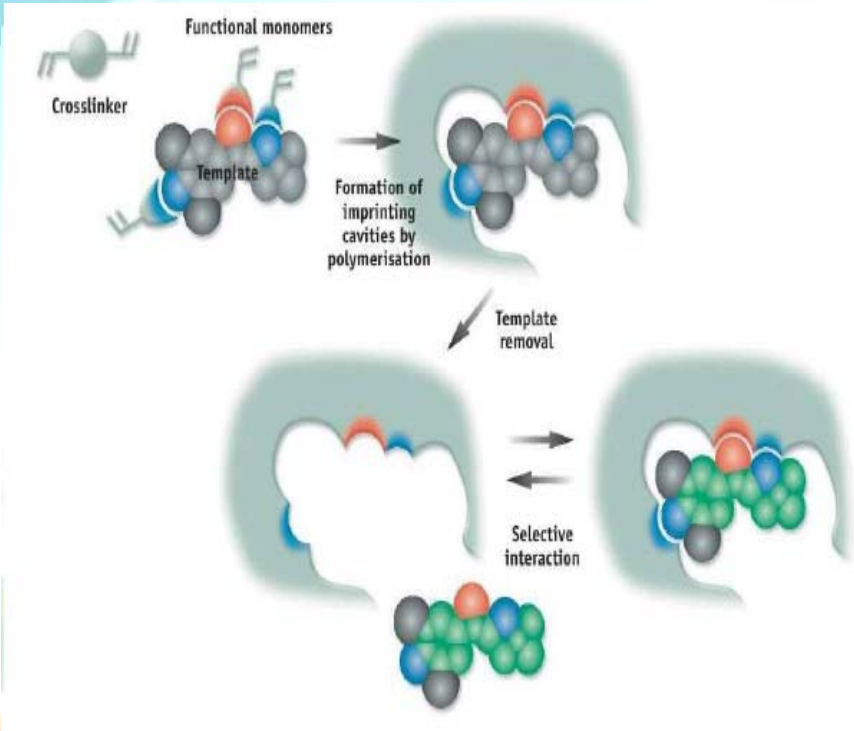
- Lower heat during evaporation, or Use a keeper solvent (e.g. dodecane)

NEW Developments in Sample Prep

SUPELCO

SIGMA-ALDRICH

SupelMIP (Molecular Imprinted Polymers) SPE

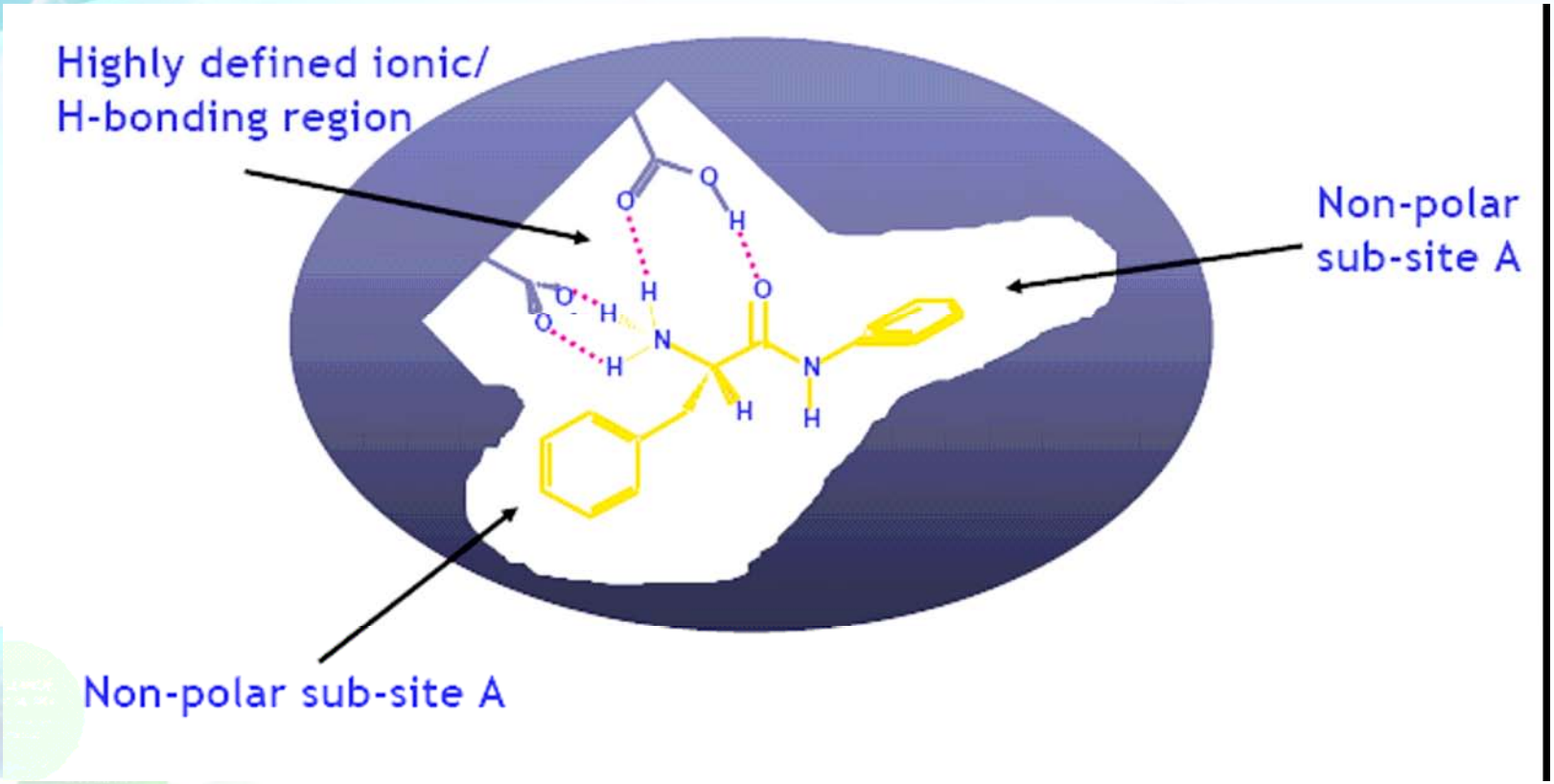


- Highly cross-linked polymer prep'd in presence of template molecule (analyte of interest or analog of analyte of interest)
- Functional monomers interact with template non-covalently prior to or during polymerization
- After polymerization using a cross linker, the template is removed through exhaustive wash steps.
- Leaves specific cavities or imprints in the polymer that are chemically and sterically complementary to the template.
- Benefit = High selectivity => lower LLOQs when extracting analyte of interest from difficult sample matrices.

SUPELCO

SIGMA-ALDRICH

SupelMIP Binding Site



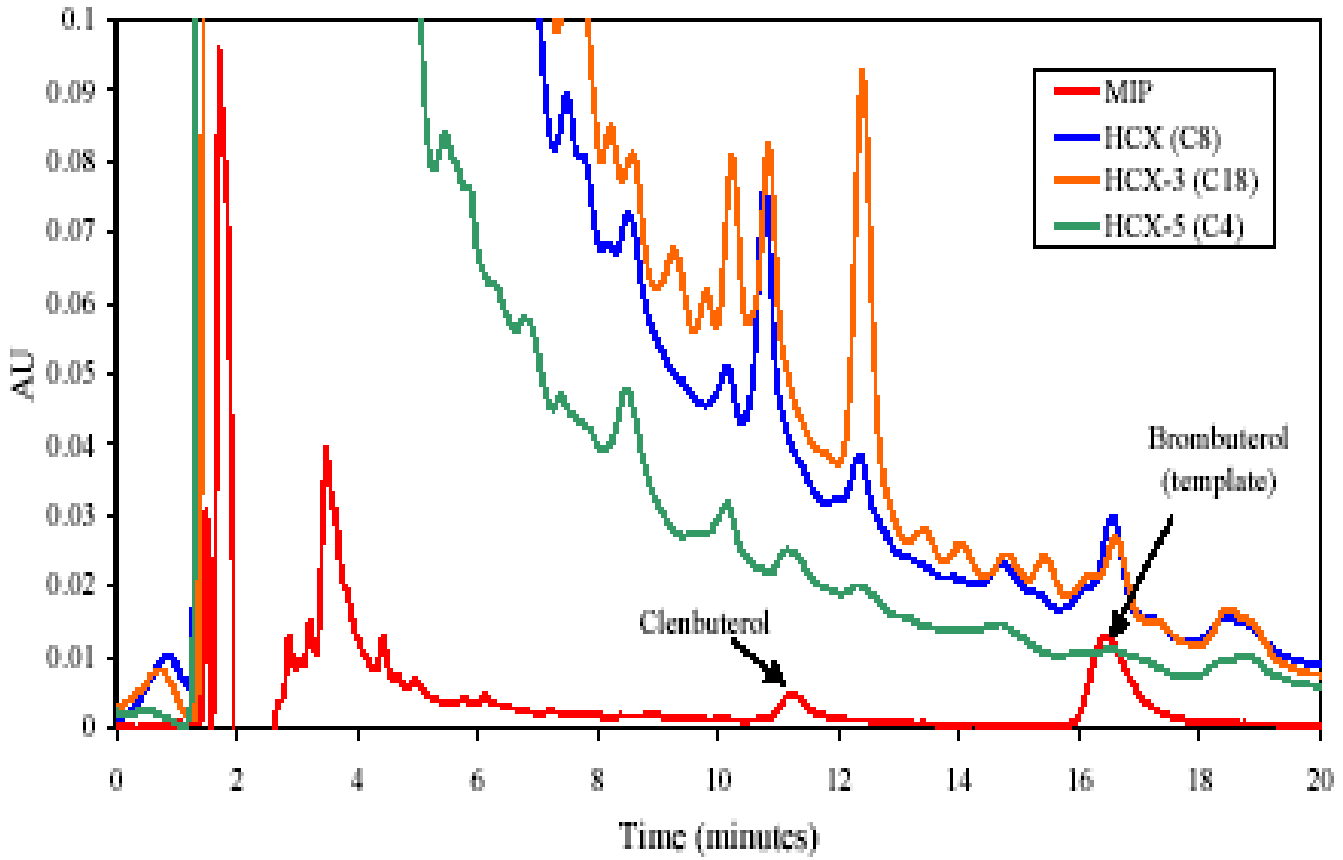
SUPELCO

SIGMA-ALDRICH

Extraction of Clenbuterol/Beta-Agonists

- Used as therapeutic drugs
- Banned substance group world-wide
- Illegally used as growth promoter
- Maximum residue limits at trace levels (0.1 and 0.3 ppb) * e.g.EU Council regulation ECC No. 2377/90
- Conventional methods based on mixed-phase SPE. NOT enough selectivity

Clenbuterol from Urine – SupelMIP vs. Mixed-Mode



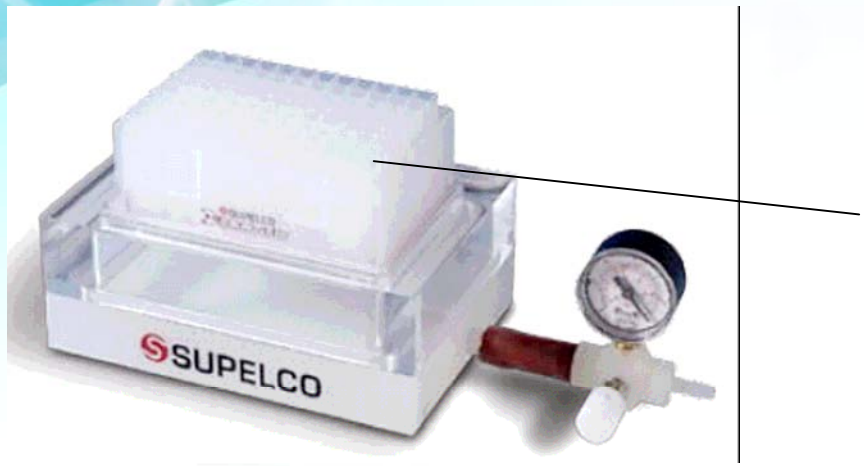
SUPELCO

SIGMA-ALDRICH

Application Examples using MIP SPE

- Beta-agonists from urine and tissue samples
- Riboflavin (vitamin B2) from aqueous samples (milk)
- Triazine herbicides from water, soil, and food products
- Chloramphenicol from biological matrices
- Beta Blockers from water and biological samples
- NNAL (tobacco specific nitrosamines) and nicotine from nictonine gum and biological samples

96-well SPE Method Development Plates



	1	2	3	4	5	6	7	8	9	10	11	12
A	Discovery® DSC-PS/DVB (polystyrene divinyl benzene) [1]											
B	Discovery DSC-18 (tC18) [1]											
C	Discovery DSC-8 (C8) [1]											
D	Discovery DSC-CN (cyanopropyl) [1]											
E	Discovery DSC-MCAX (mixed-mode cation exchange) [2]											
F	Discovery DSC-WCX (weak cation exchange) [2]											
G	Discovery DSC-SAX (strong anion exchange) [3]											
H	Discovery DSC-NH_2 (aminopropyl weak anion exchange) [3]											

[1] Reversed-phase; [2] Cation-exchange; [3] Anion-exchange

Discovery 96-well SPE MD Plate

- Courtesy of Biotage

SUPELCO

SIGMA-ALDRICH

96-well MD Plate Generic Protocols

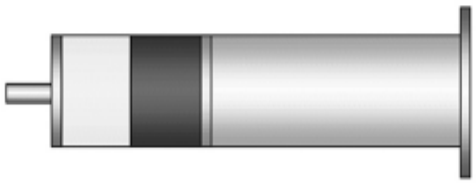
SPE Step:	[1] Reversed-phase	[2] Cation-exchange	[3] Anion-exchange
1. Sample Pre-Treatment	Dilute biological sample 1:1 with 10-50 mM buffer (phosphate, ammonium acetate, or ammonium formate) at 2 pH units above analytes' pKa for basic analytes, or 2 pH units below pKa for acidic analytes.	Dilute biological sample 1:1 with 10-50 mM buffer (phosphate, ammonium acetate, or ammonium formate), pH 3 for basic analytes.	Dilute biological sample 1:1 with 10-50 mM buffer (phosphate, ammonium acetate, or ammonium formate), pH 10 for acidic analytes.
2. Condition / Equilibrate	Condition with methanol. Equilibrate with DI water or buffer used in sample pre-treatment.	Condition with methanol. Equilibrate with DI water or buffer used in sample pre-treatment.	Condition with methanol. Equilibrate with DI water or buffer used in sample pre-treatment.
3. Sample Load	Load pre-treated sample from step 1.	Load pre-treated sample from step 1.	Load pre-treated sample from step 1.
4. Wash	Wash off co-retained interferences with 5-20% methanol diluted in DI water or buffer used in sample pre-treatment.	Wash off co-retained interferences with low pH buffer used in sample pre-treatment, followed by 1M acetic acid and 100% methanol.	Wash off co-retained interferences with high pH buffer used in sample pre-treatment, followed by 100% methanol.
5. Elution	Elute with methanol or acetonitrile. pH modification may be necessary to facilitate elution. Use 2% acetic acid in methanol or acetonitirile for basic analytes; or 2% ammonium hydroxide in methanol or acetonitrile for acidic analytes.	Elute basic analytes with 2-5% ammonium hydroxide in methanol or acetontirile.	Elute acidic analytes with 2-5% acetic acid in methanol or acetonitrile.
6. Evaporate / Reconstitute	Evaporate SPE eluate, and reconstitute with analytical mobile phase		

SUPELCO

SIGMA-ALDRICH

Dual-Layer SPE Products for Multi-Residue Pesticide Analysis in Foods

Supelclean ENVI-Carb-II/PSA SPE Products



Retention Mechanism: Reversed-phase and anion-exchange
Sample Matrix Compatibility: Organic or aqueous solutions

- Dual layer SPE tube that contains both Supelclean ENVI-Carb-II (upper layer) and PSA (lower layer) SPE sorbents (separated by PE frit)
- Developed to offer superior clean up when conducting multi-residue pesticide analysis from food (e.g. agricultural products, meats, etc.).
- ENVI-carb-II has a strong affinity towards planar molecules, and can isolate/remove pigments (e.g., chlorophyll and carotinoids) and sterols commonly present in foods and natural products
- Supelclean PSA is a polymerically bonded, ethylenediamine-N-propyl phase that contains both primary and secondary amines
- Supelclean PSA has a strong affinity and high capacity for fatty acids, organic acids, and some polar pigments and sugars
- Tested for superior cleanliness using GC-FID and GC-MS

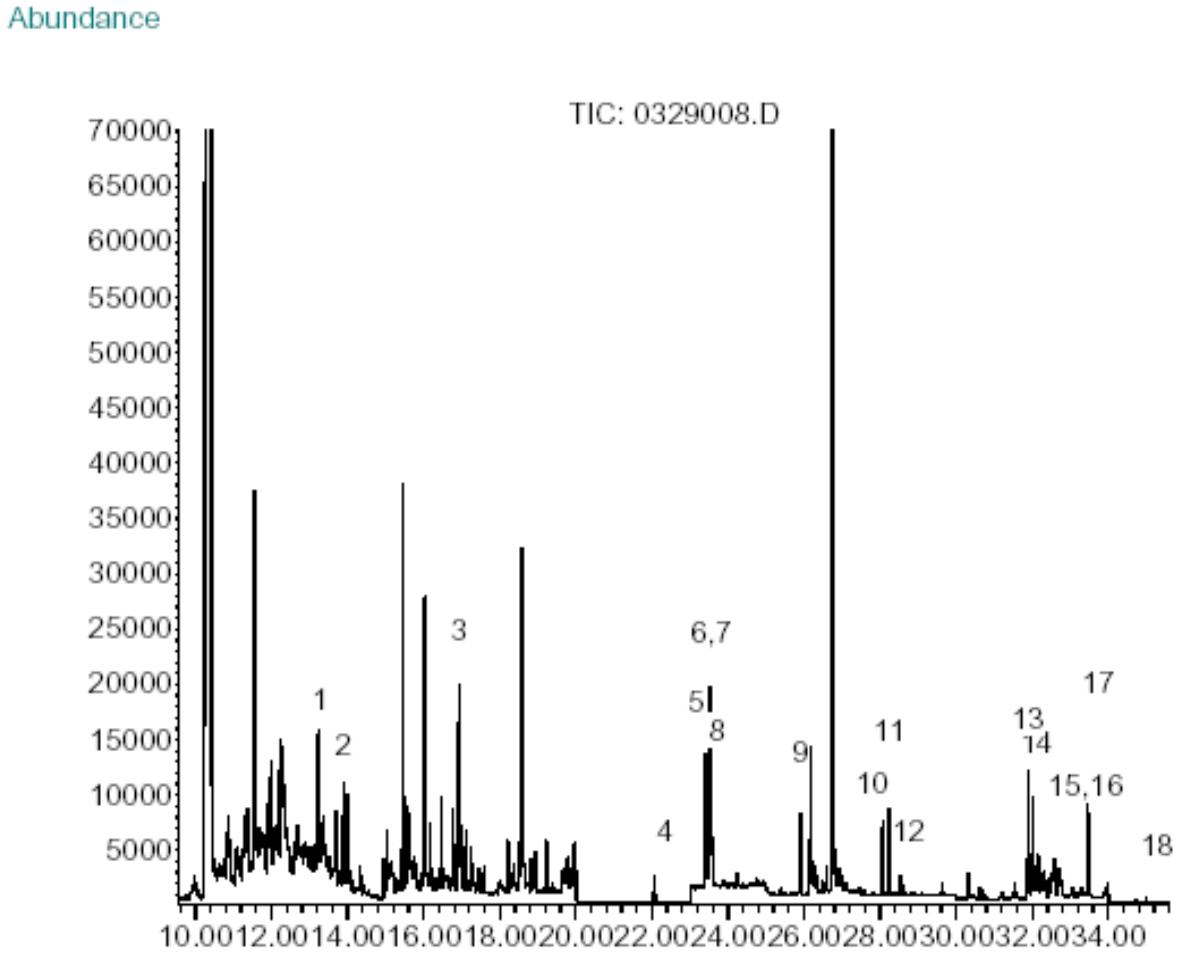
Pre-SPE Extraction:

1. For solid samples with less than 2% fat content (e.g., chopped vegetables/fruits), combine every 10 g homogenized food product with 20 mL acetonitrile; vortex/shake 1 min. For liquid samples (e.g. milk) combine every 10 mL food product with 20 mL acetonitrile; vortex/shake 1 min.
2. Add 2-4 g sodium chloride for every 20 mL acetonitrile used for extraction; Add I.S. as necessary
3. Centrifuge or filter to remove particulate matter
4. Transfer acetonitrile layer to a separate vessel
5. Dry acetonitrile layer over anhydrous sodium sulfate or magnesium sulfate
6. Evaporate acetonitrile extract and reconstitute as necessary to achieve a final acetonitrile extract volume of 1 mL

SPE:

7. Condition multi-Layer Supelclean ENVI-Carb-II/PSA SPE cartridge with 5mL acetonitrile:toluene (3:1)
8. Load acetonitrile extract from step 7
9. Elute weakly retained pesticides with 20mL acetonitrile:toluene (3:1)
10. Evaporate acetonitrile:toluene eluate (3:1); and reconstitute with acetone:hexane (1:1)

Representative GC-MS C-gram for Pesticides in Orange

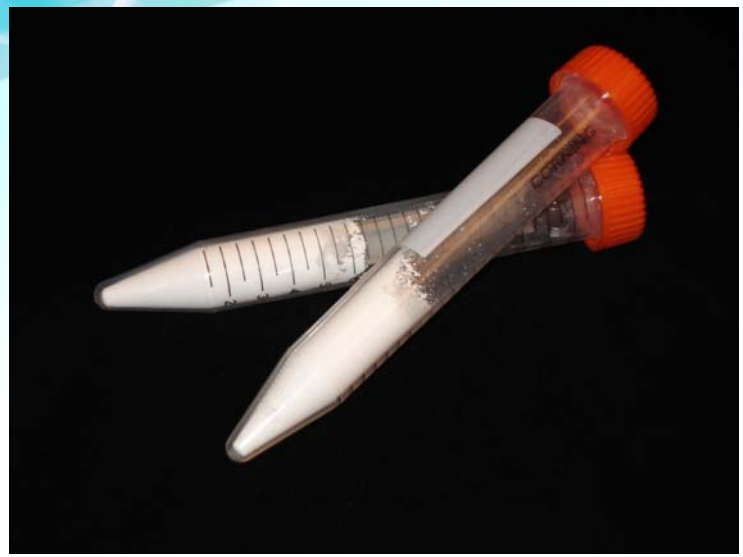


1. Methamidophos
2. Dichlorvos
3. Acephate
4. Quintozene
5. Methyl parathion
6. Carbaryl
7. Methyl chloropyriphos
8. Vinclozolin
9. Procymidone
10. Chlorothiophos
11. Tetrasul
12. Endosulfan sulfate
13. Acrinathrin
14. Bitertanol
15. cis-Permethrin
16. trans-Permethrin
17. Cypermethrin isomers
18. Deltamethrin

SUPELCO

SIGMA-ALDRICH

Dispersive SPE Products for Multi-Residue Pesticide Analysis in Foods



Dispersive SPE Products for the "QuEChERS" Method

1. Food initially extracted with aq miscible solvent (e.g. MeCN)
2. High amounts of salts (NaCl, Mg-sulfate) and buffering agents added to induce phase separation and stabilize acid/base labile pesticides
3. Shake/centrifuge. Isolate aliquot of sup for SPE clean-up.
4. SPE done with bulk sorbents and salts; not SPE tubes.

Supelco carries a line of centrifuge tubes containing pre-determined salts and sorbents to support most common method configurations used today.

SUPELCO

SIGMA-ALDRICH

Dispersive SPE Product Line

Description	Qty.	Cat. No.
Dispersive SPE (dSPE) Products, packed in 15 mL centrifuge tube		
Citrate Extraction Tube 4 g magnesium sulfate (Cat. No. 63135) 1 g sodium chloride (Cat. No. 71379) 0.5 g sodium citrate dibasic sesquihydrate (Cat. No. 71635) 1 g sodium citrate tribasic dihydrate (Cat. No. 32320)	50	**55227-U**
Mg_2SO_4 Extraction Tube 6 g magnesium sulfate (Cat. No. 63135) 1.5 g sodium acetate (Cat. No. 24,124-5)	50	**55234-U**
PSA SPE CleanUp Tube 1 900 mg magnesium sulfate (Cat. No. 63135) 150 mg Supelclean PSA (Cat. No. 52738-U)	50	**55228-U**
PSA/C18 SPE CleanUp Tube 1 900 mg magnesium sulfate (Cat. No. 63135) 150 mg Supelclean PSA (Cat. No. 52738-U) 150 mg Discovery DSC-18 (Cat. No. 52600-U)	50	**55229-U**
PSA/ENVI-Carb SPE CleanUp Tube 1 900 mg magnesium sulfate (Cat. No. 63135) 150 mg Supelclean PSA (Cat. No. 52738-U) 15 mg Supelclean ENVI-Carb (Cat. No. 57210-U)	50	**55230-U**
PSA/ENVI-Carb SPE CleanUp Tube 2 900 mg magnesium sulfate (Cat. No. 63135) 150 mg Supelclean PSA (Cat. No. 52738-U) 45 mg Supelclean ENVI-Carb (Cat. No. 57210-U)	50	**55233-U**

Discovery Ag-ION SPE

Discovery Ag-Ion (silver-ion) SPE

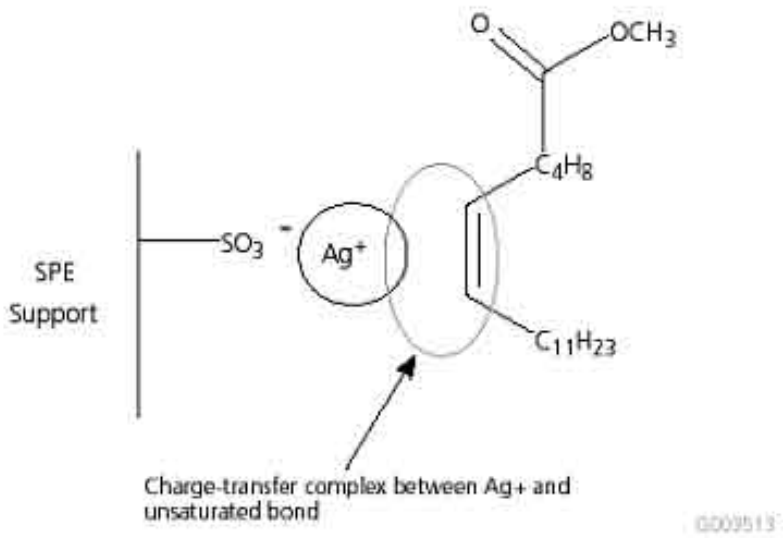
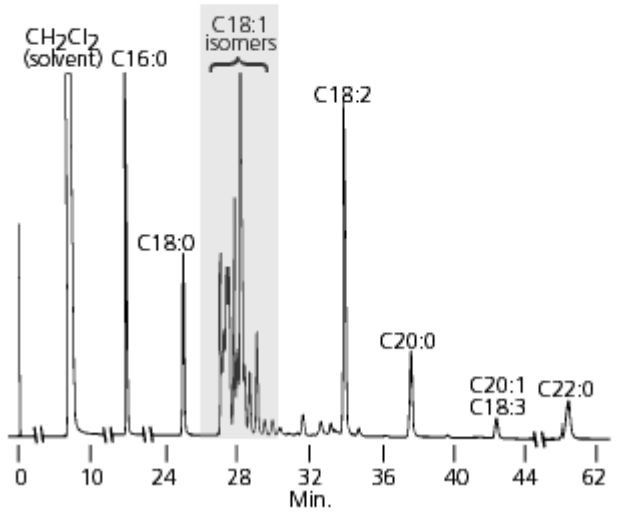


Figure 1. Difficult Separation of C18:1 Fatty Acid Isomers

column: SP-2560, 100 m x 0.25 mm I.D., 0.20 µm (24056)
oven: 175 °C
det.: FID, 200 °C
carrier gas: helium, 20 cm/sec. at 175 °C
injection: 1 µL of positional *cis*/*trans* standard (5.0 mg/mL FAME isomers in methylene chloride), split 100:1, 200 °C



SUPELCO

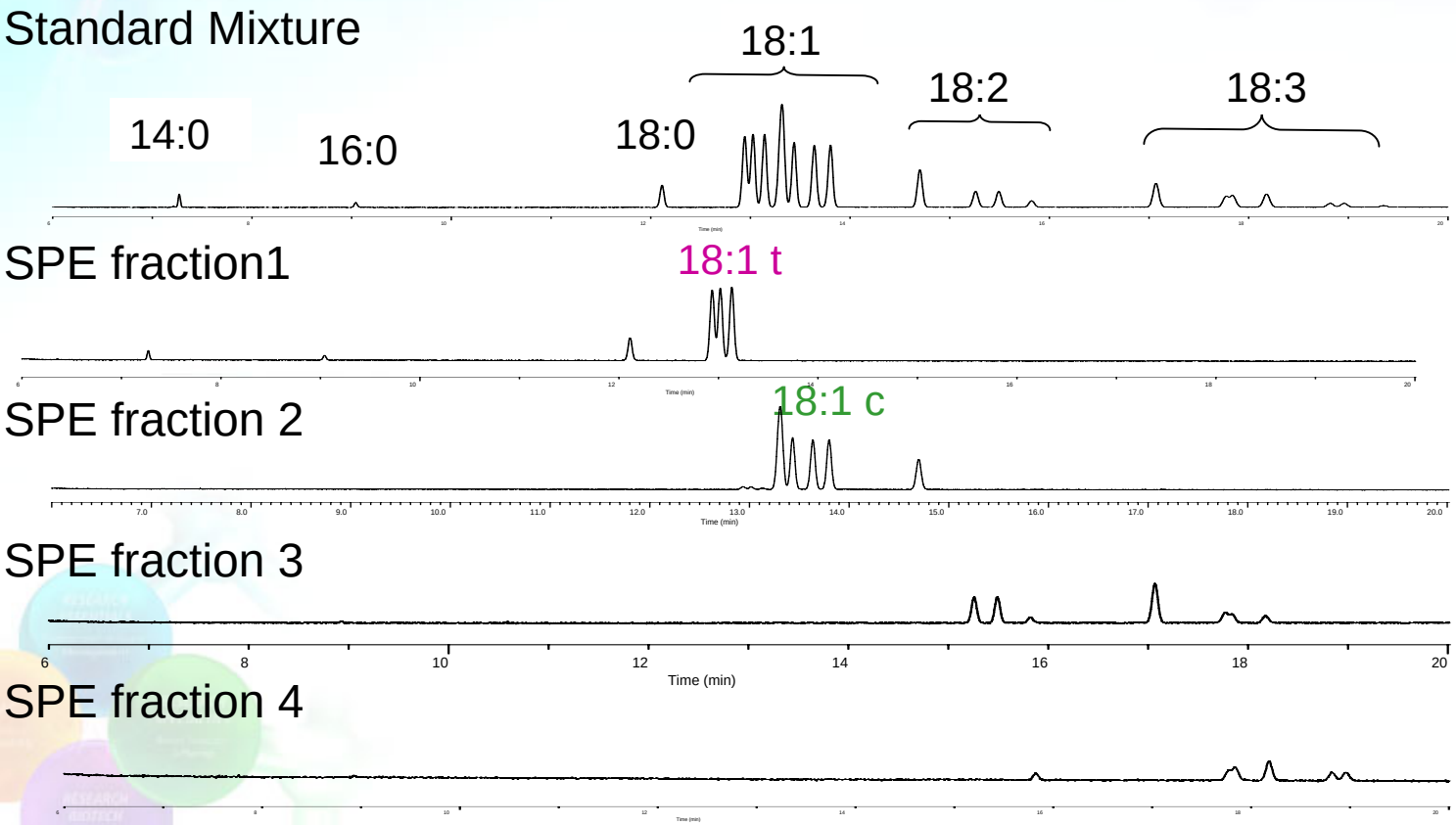
SIGMA-ALDRICH

Discovery Ag-ION Protocol

SPE Step	Description	Comments
1. Condition 2. Equilibrate	Condition SPE with 4 mL acetone. Equilibrate cartridge with 4 mL hexane	Moisture adsorbed on to the SPE phase can affect normal-phase performance. Acetone conditioning removes any residual moisture from the SPE phase.
3. Sample Load	Load 1 mL of 1 mg/mL FAMEs in hexane derived from sample extraction.	Discovery Ag-Ion 750 mg cartridges have a maximum capacity of 1 mg FAMEs. Exceeding the capacity will reduce resolution efficiency of the cartridge.
4. Fraction 1	Elute fraction 1 with 6 mL hexane:acetone 96:4	Fraction 1 will target: • Saturated fatty acids • Trans monoenes • Cis/cis and trans/trans conjugated linoleic acids (CLAs)
5. Fraction 2	Elute fraction 2 with 4 mL hexane:acetone 90:10	Fraction 2 will target: • Cis monoenes • Trans/trans dienes • Cis/trans and trans/cis CLAs
6. Fraction 3	Elute fraction 3 with 4 mL acetone	Fraction 3 will target: • Cis/cis dienes • Other dienes • Most trienes
7. Evaporation/Reconstitution	Evaporate all fractions at 40 °C under N_2 sparge. Reconstitute in 1 mL hexane prior to GC analysis.	

Fractionation of the standard FAME mixture

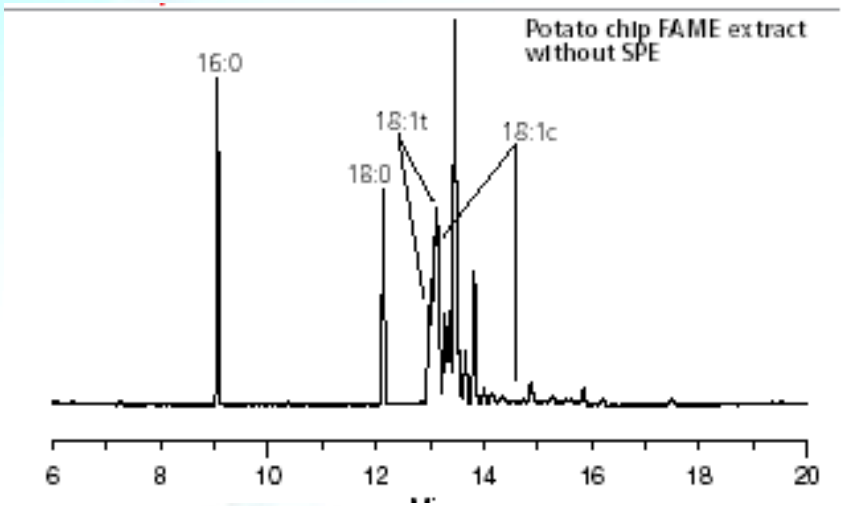
Standard sample, total FAMEs at 1 mg/ml

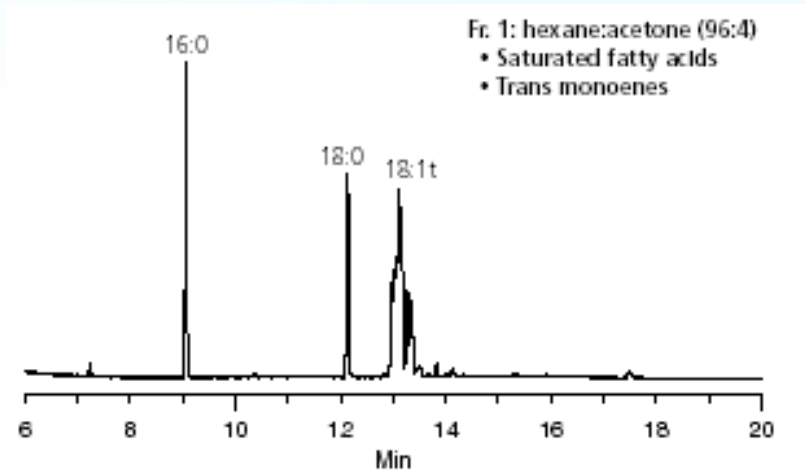


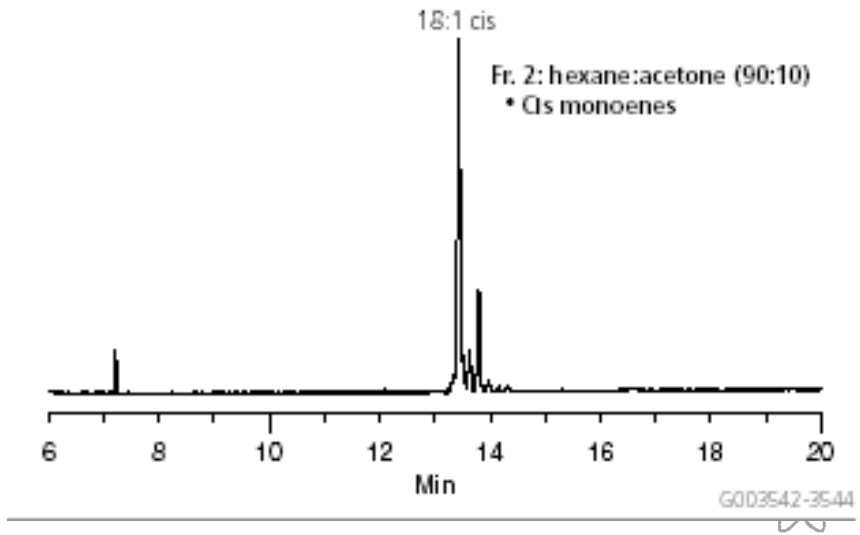
SUPELCO

SIGMA-ALDRICH

Cis/Trans Fractionation of FAMEs using Discovery Ag-ION SPE



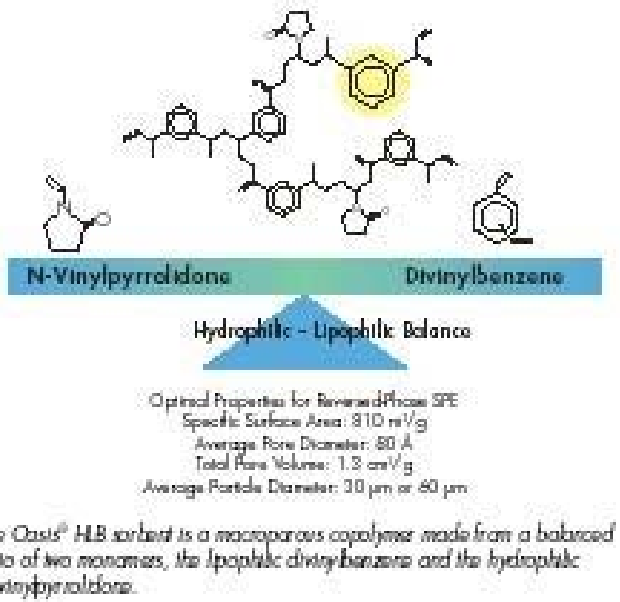




SUPELCO

SIGMA-ALDRICH

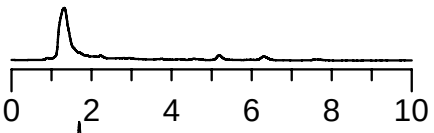
Hydrophilic Polymer SPE



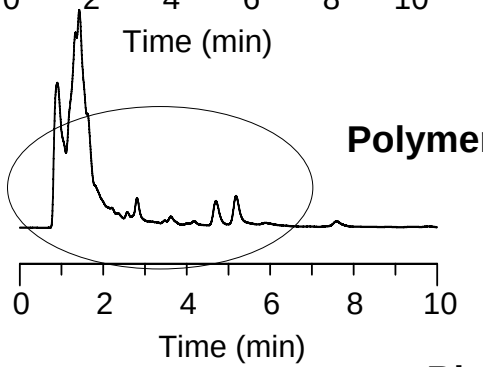
- Courtesy of Waters

- **Water wettable**
- **Broad affinity for a wide range of compounds**
- **Generic methodology = high recoveries**
- **Drawback = Possible poor selectivity**

C18



Polymer SPE



Blank serum

Similar Hydrophilic Polymers/Co-polymers

- **Varian Focus – amide functionalized PS/DVB**
- **Phenomenex Strata X**
- **Biotage/Argonaut/IST Evolute**
- **3M Universal Resin**

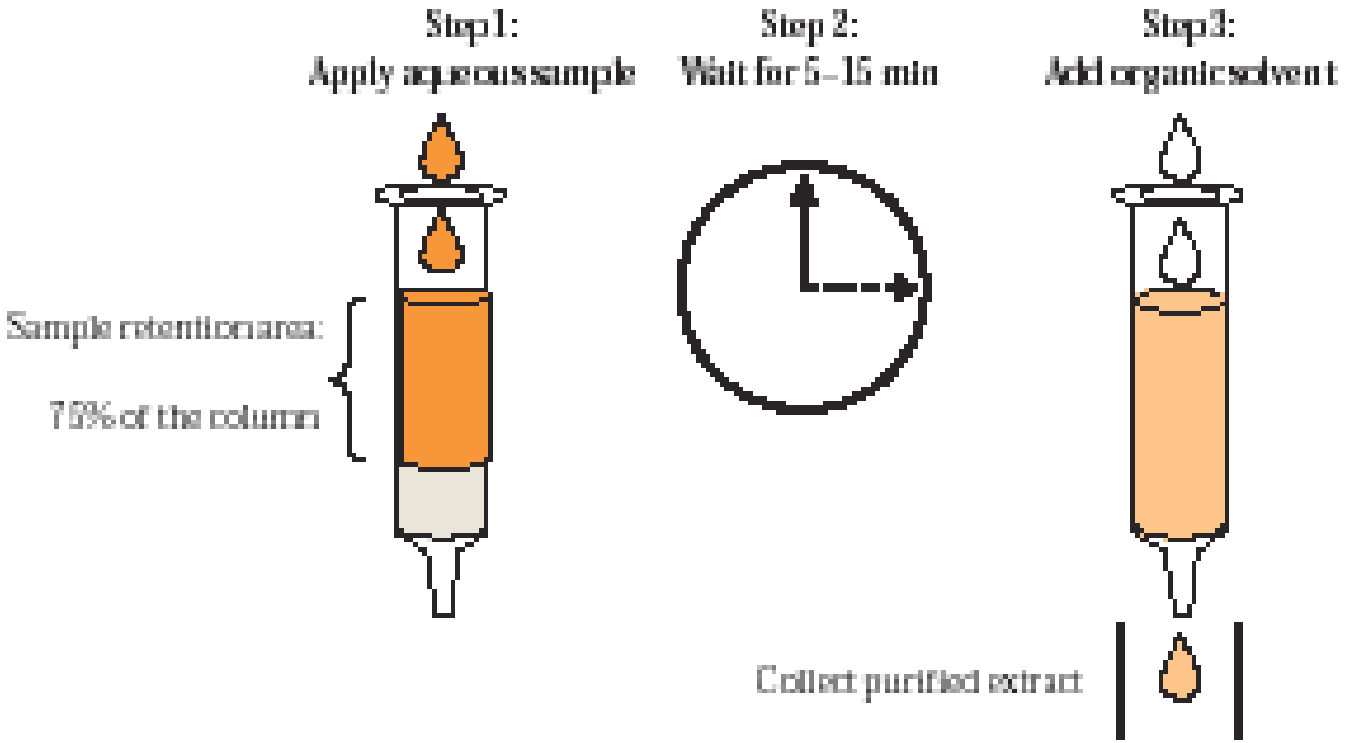
High Background; Misleading interfering responses

SUPELCO

SIGMA-ALDRICH

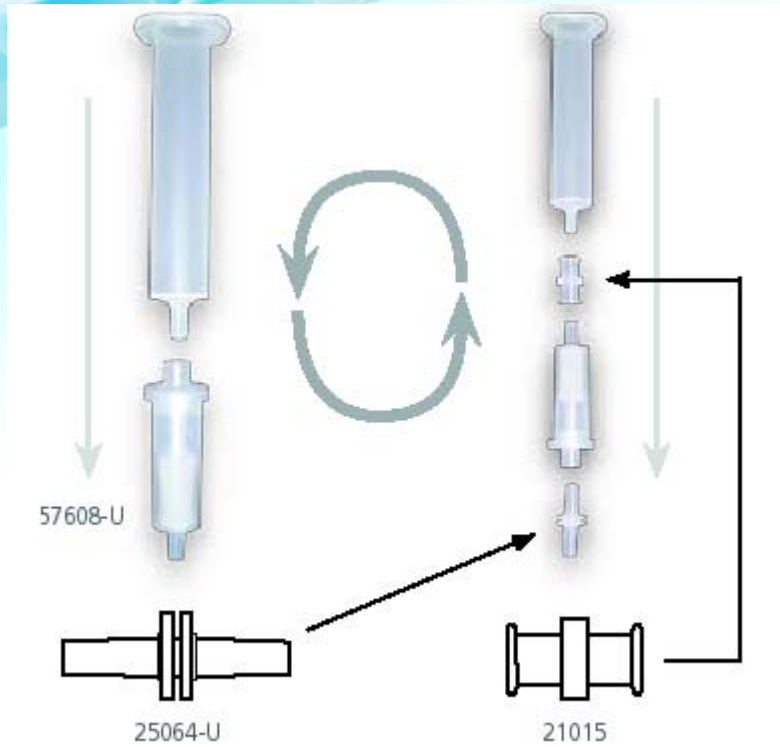
New or Unique SPE Phase Chemistries (cont.)

Surface Driven LLE or Supported LE



- Courtesy of Biotage/Argnoaut/IST

Alternative SPE Formats



Reversible SPE Tubes

Rezorian SPE Cartridges

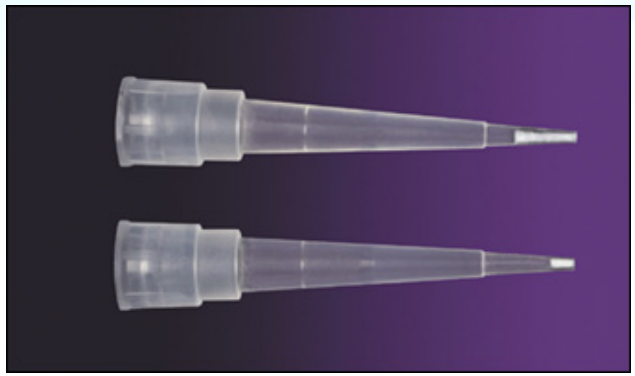
SUPELCO

SIGMA-ALDRICH

Alt. SPE Formats (cont.)

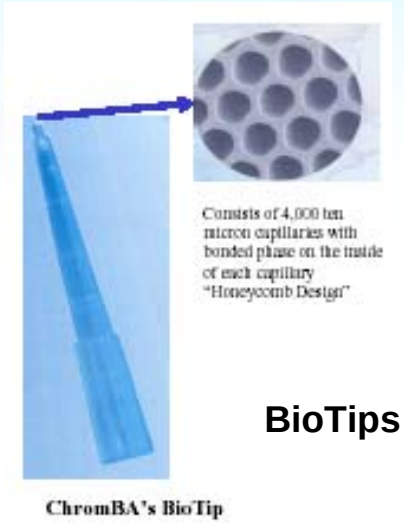
ZipTip Pipette Tips

- Courtesy of Millipore



Omix Tips

- Courtesy of Varian, Inc.



BioTips

- Courtesy of ChromBA

- Originally designed for proteomics applications
  - Peptide desalting/detergent removal
  - Small volume applications (< 10uL applictation)
- Expanding to drugs/metabolites in biological fluids
- Bi-directional flow; amenable to some x-y-z robotic liquid handlers
- MiniTips™ also available through Sigma-Aldrich (developed by Supelco)

SUPELCO

SIGMA-ALDRICH

New or Unique SPE Phase Chemistries (cont.)

Supelclean ENVI-Carb SPE Products

Graphitized Non-Porous Carbon

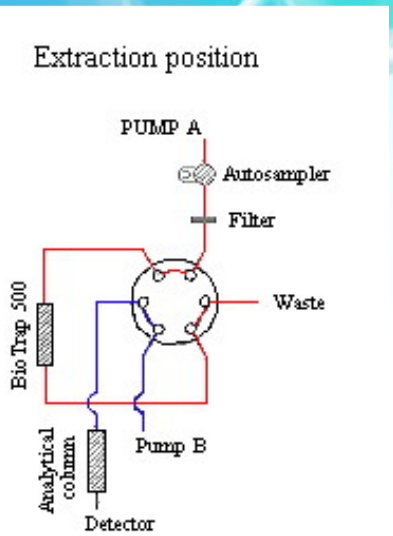
Retention Mechanism: Reversed-phase
Sample Matrix Compatibility: Aqueous solutions (drinking, ground, waste water)

- Surface area: 100 m^2/g, Particle size: 100-400 mesh
- Extreme affinity for organic polar and non-polar compounds from both non-polar and polar matrices when used under reversed-phase conditions
- Carbon surface comprised of hexagonal ring structures, interconnected and layered into graphitic sheets
- Non-porous nature of the carbon phase allows for rapid processing, adsorption does not require analyte dispersion into solid phase pores
- Independent investigators have found ENVI-Carb extremely useful for the rapid sample preparation of over 200 pesticides from various matrices including ground water, fruits, and vegetables

SPE Disk Technology

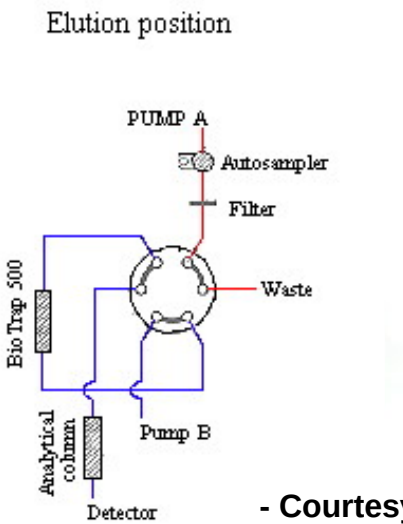
- Courtesy of 3M

Alt. SPE Formats (cont.)



Online SPE (Prospekt)

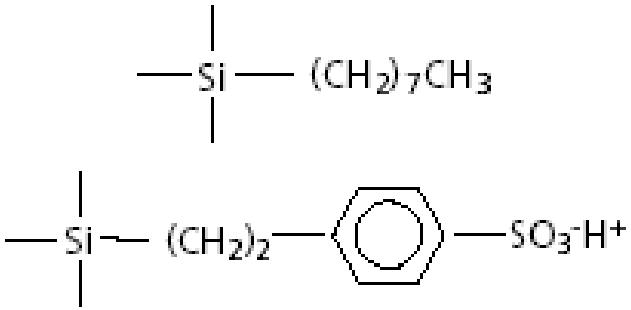
- Courtesy of Varian & Spark



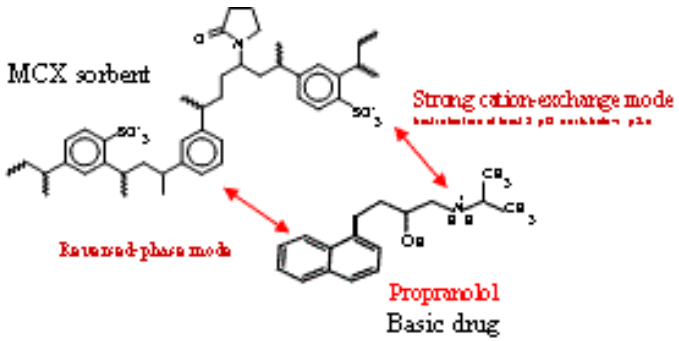
- **Spark provides dedicated instrument for online SPE called Prospekt**
- **Online SPE also possible with switching valve capabilities (hardware = guard column packed with SPE)**
- **Becoming more popular in drugs/metabolites in biological fluids**

- Courtesy of ChromTech

New or Unique SPE Phase Chemistries – Mixed-Mode SPE



Discovery DSC-MCAX SPE



- Courtesy of Waters

Condition with methanol *& equilibrate with pH 3-6 buffer*

Load Sample @ *low pH 3-6*

***Wash 1*: low pH 3-6 buffer**

***Wash 2*: 100% methanol**

Elute with *basified (high pH)methanol*

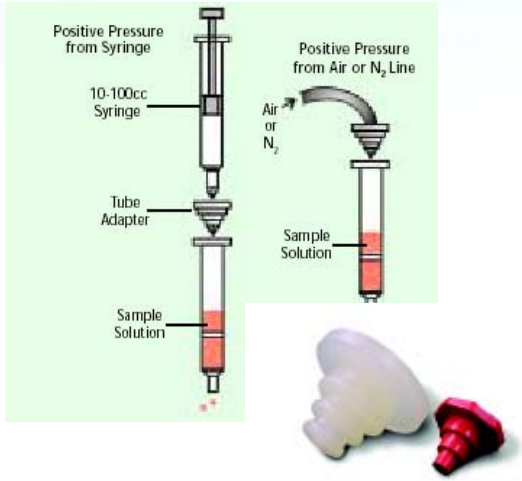
SIGMA-ALDRICH

Useful SPE Accessories

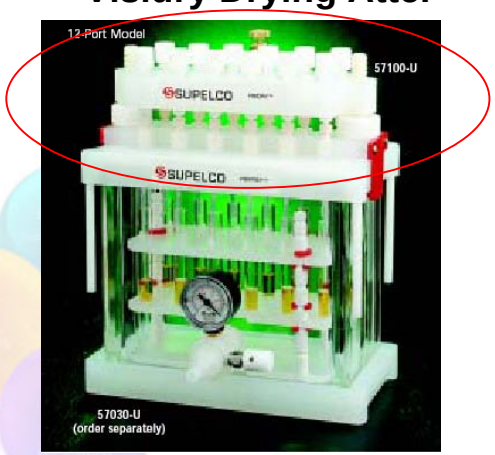
Visiprep DL (Disposable Liner)

Visiprep Lg. Vol. Sampler

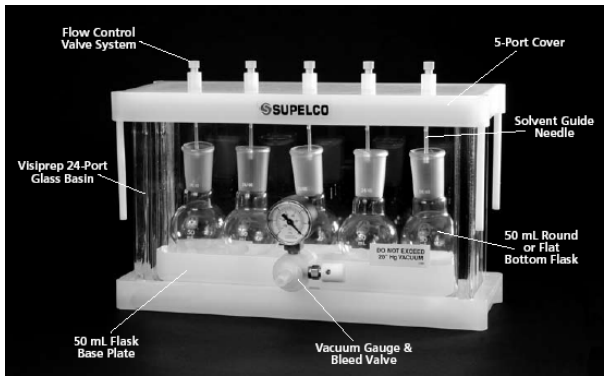
SPE Tube Adapter



Visidry Drying Attc.



Visidry 5-Port Flask Manifold



Zymark Turbovap

- Courtesy of Zymark

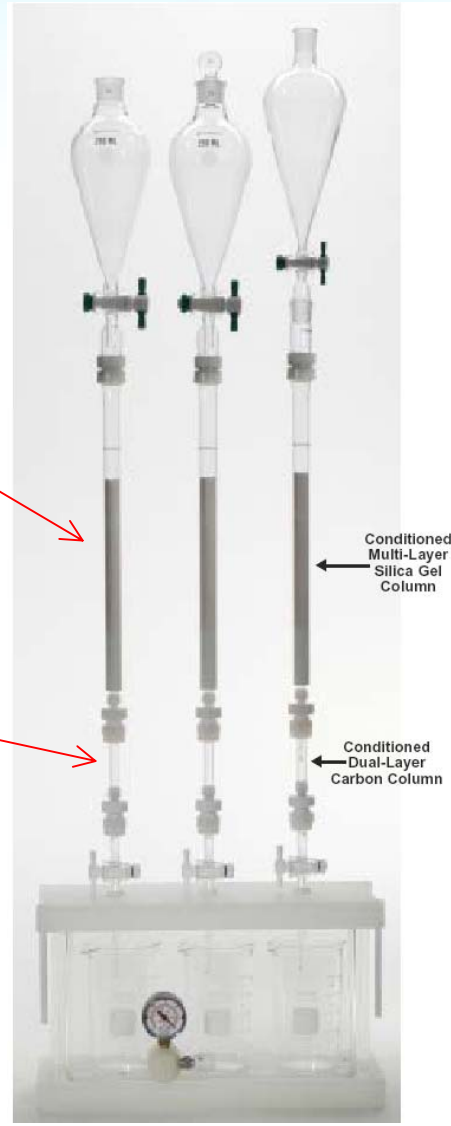
SUPELCO

SIGMA-ALDRICH

Dioxin Prep System

Multi Layer Silica Tube:

7-layers of acid and base treated silica optimized to remove unsaturated hydrocarbons, phthalates, organochlorides, pigments, PAHs, lipids, proteins, phenols, sulfur, pesticides, etc.



- **Dual Layer Carbon reversible tube**: Isolates and concentrates coplanar PCBs, dioxins, furans

alternatively

- NEW! Florisil reversible tube isolates PCBs from dioxins/furans

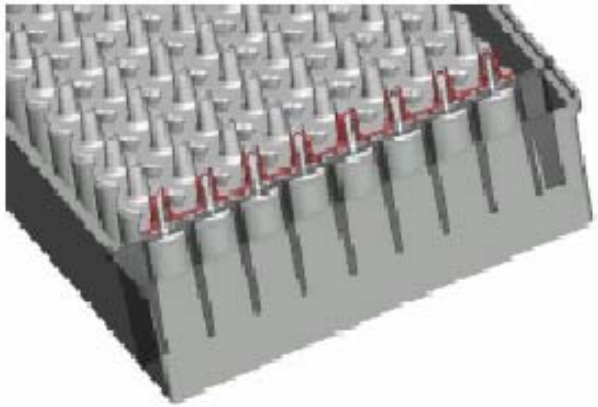
SUPELCO

SIGMA-ALDRICH

Alternative Sample Prep Techniques (cont.)

Protein Ppt. Plates

Waters Sirocco Plate



Specially designed vented closures
prevent solvent flow until vacuum is
applied.

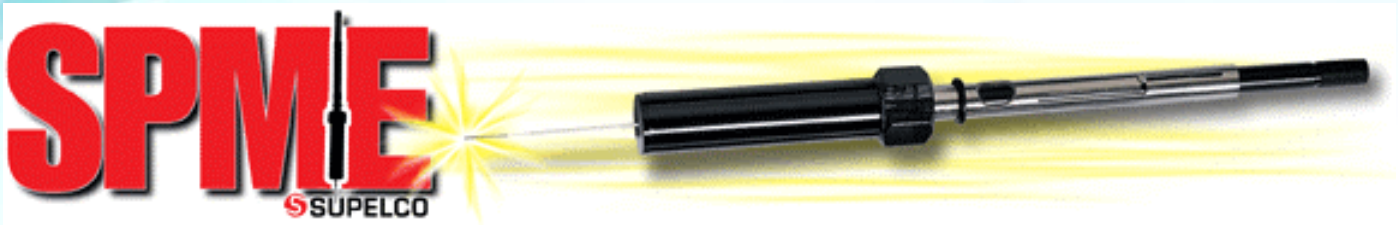
Orachem Protein PPT Plate

Hydrophobic graded filters to prevent leaking during mixing and incubation.

SUPELCO

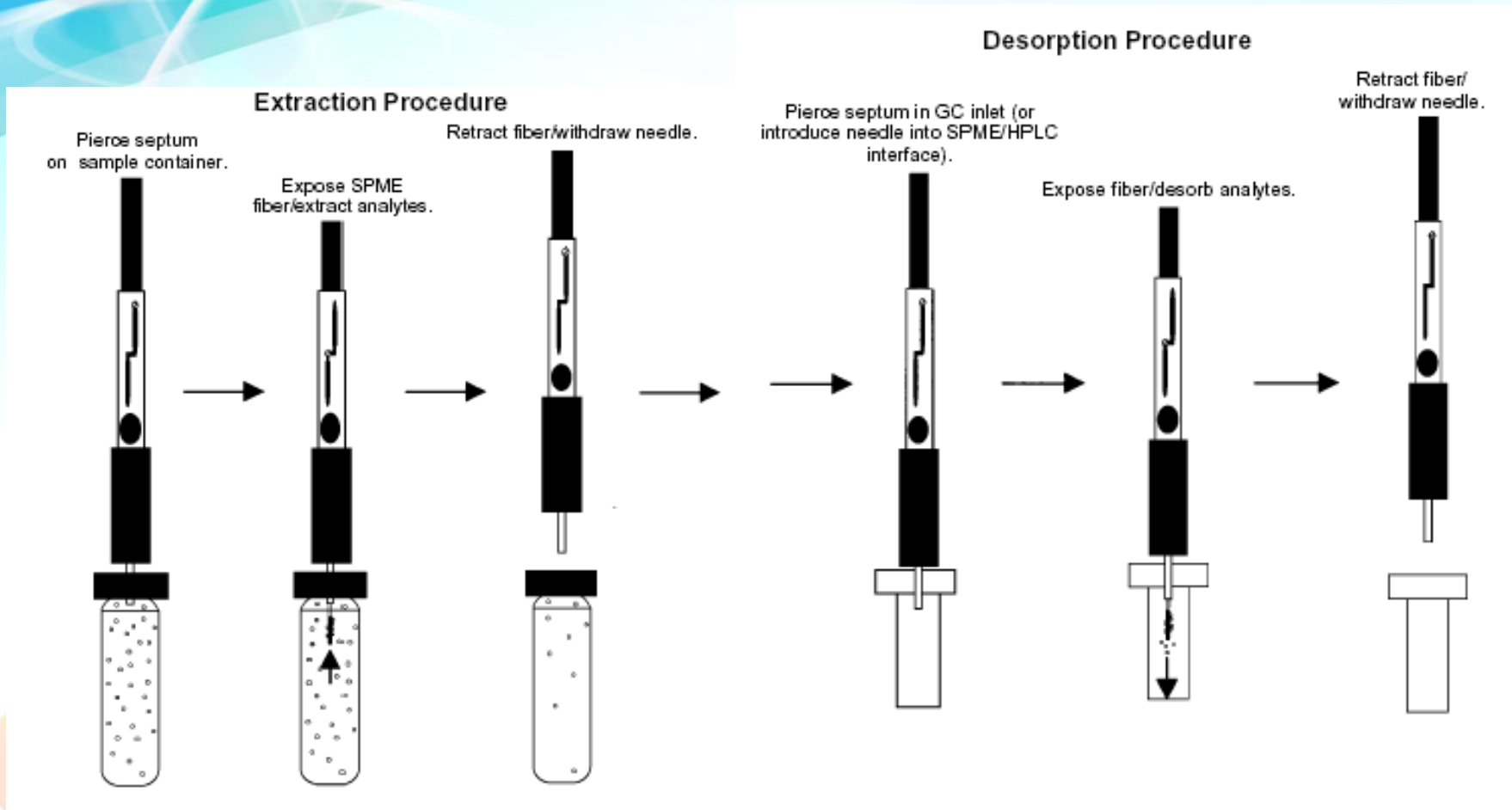
SIGMA-ALDRICH

Alternative Sample Prep Techniques



- Fiber coated with a liquid polymer, sorbent or both
- Fast, solvent-less, and reusable
- Amenable to quantitation with proper technique and calibration standards
- Fiber chosen on basis of polarity, MW, and volatility
- Amenable to GC, LC, and automation

Alternative Sample Prep Techniques (cont.)



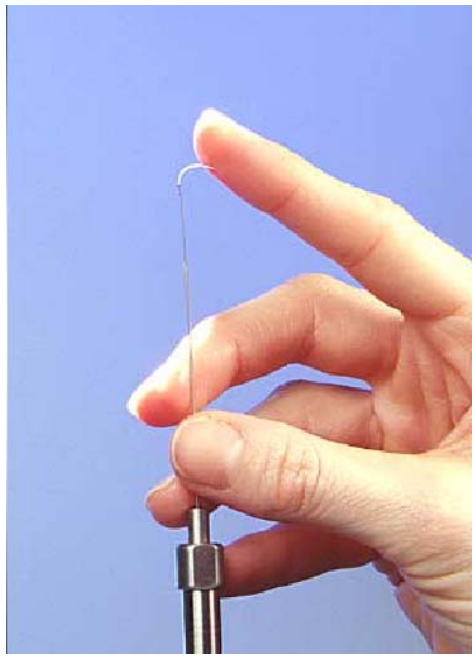
SUPELCO

SIGMA-ALDRICH

Alternative Sample Prep Techniques (cont.)

Fused Silica →

StableFlex



→

SPME All Metal

For More information (SPME CD, FAQs, Literature, etc.):

http://www.sigmaaldrich.com/Brands/Supelco_Home/Spotlights/SPME_central.html

SUPELCO

SIGMA-ALDRICH

Alternative Sample Prep Techniques (cont.)

GERSTEL Twister – Stir Bar Sorptive Extraction (SBSE)

The PDMS coated GERSTEL Twister is stirred in the sample for several minutes. The analytes of interest come in contact with the PDMS phase and are extracted.

Without additional sample preparation, the Twister is placed in a GERSTEL TDS 2 ThermoDesorption System. Here the analytes are thermally desorbed, focussed in the inlet, and transferred to the GC capillary column.

SUPELCO

SIGMA-ALDRICH

References

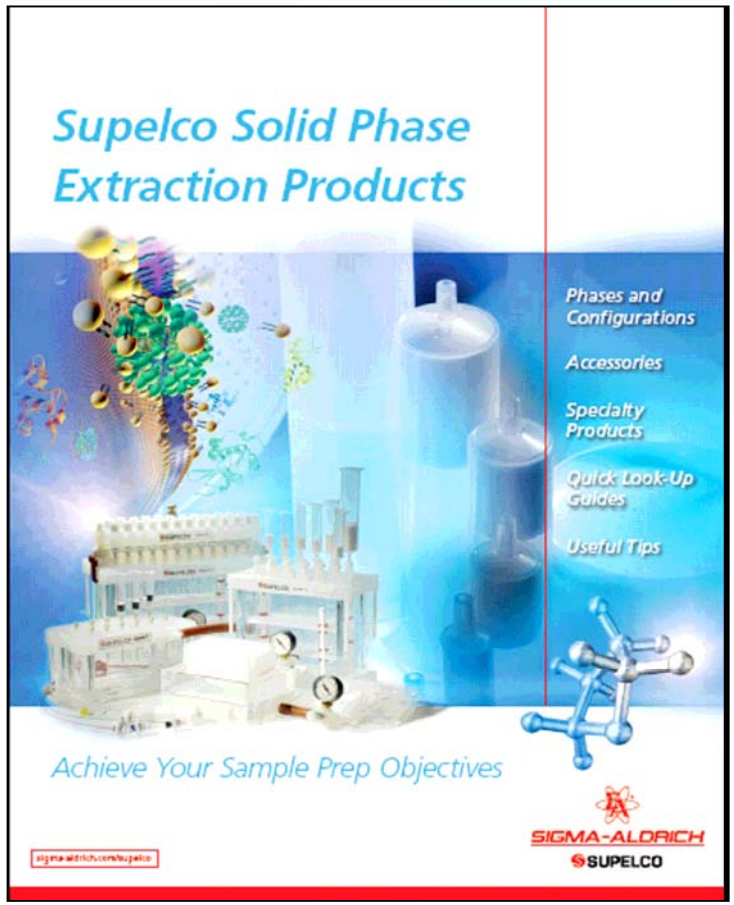
- R.E. Majors, New Sample Prep Technologies for the Sample Prep Industry, 2006, GMP Training Systems
- D.A. Wells, High Throughput Bioanalytical Sample Preparation, Elsevier, Amsterdam, The Netherlands (2003)
- N.J.K. Simpson, Ed., Solid-Phase Extraction: Principles, Techniques, Applications, Marcel Dekker, NY (2000)
- J.S. Fritz, Analytical Solid-Phase Extraction, John Wiley & Sons, NY (1999)
- H. Wiltshire, In: R.F. Venn, Ed., Principles and Practice of Bioanalysis, Taylor & Francis, London (2000)
- N.J.K. Simpson, Ed., Solid-Phase Extraction: Principles, Techniques, Applications, Marcel Dekker, NY (2000)
- J.S. Fritz, Analytical Solid-Phase Extraction, John Wiley & Sons, NY (1999)

Acknowledgments for the vendors that provided product information: Waters, Varian, Biotage, Gerstel, Orochem, Gilson, Zymark, Gerstel, Fondazione Salvatore Maugeri, Pfizer, Chromsys, LC/GC, 3M, MIP Technologies

New SPE Brochure 2006

- T402150 (FEB)
- 28 pages

- Complete list of SPE products and accessories



SUPELCO

SIGMA-ALDRICH