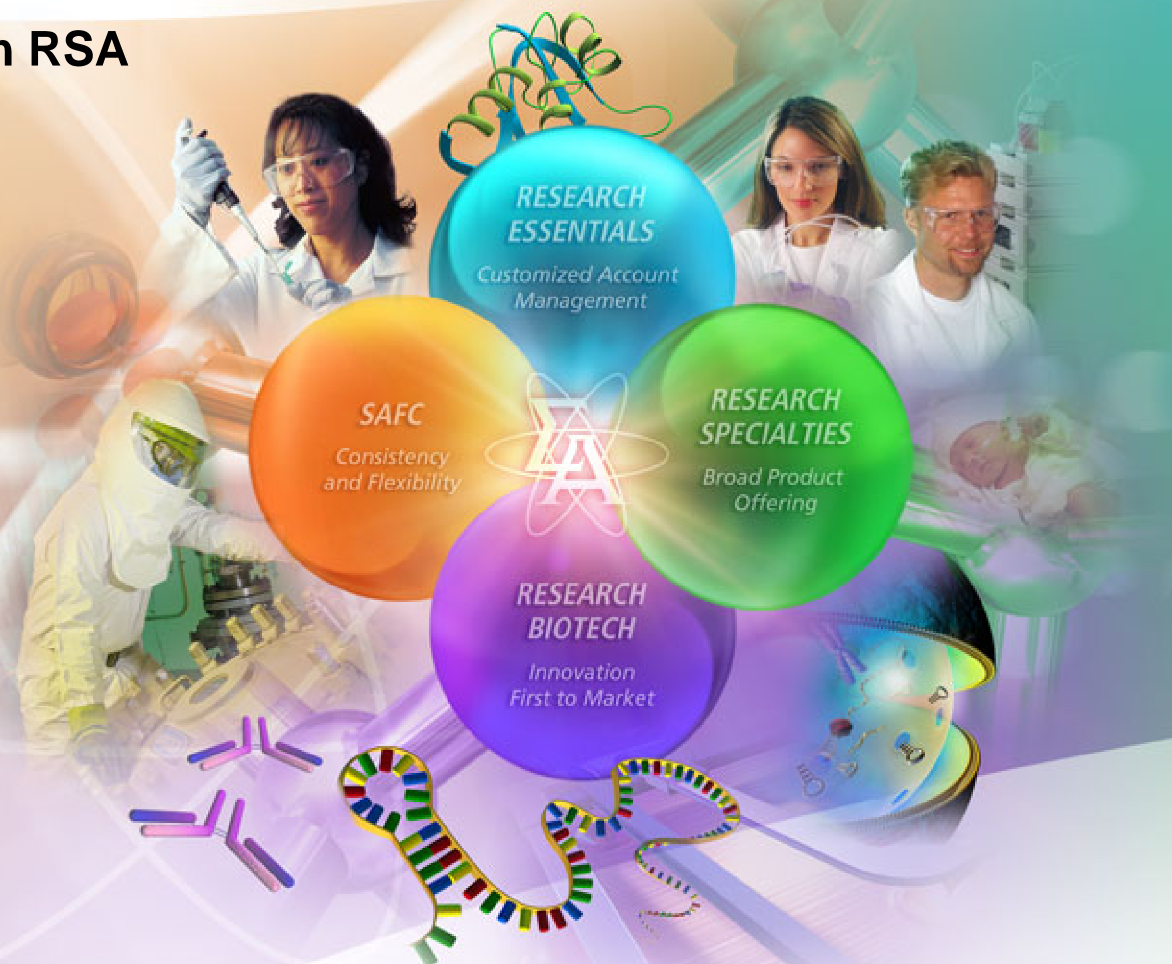
Basics of SPE
Technology & Mechanisms

Pieter Grobler,

Sigma-Aldrich RSA



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Agenda

- The Importance of Sample Prep
- Overview of SPE Technology
- SPE Strategies
- Understanding Retention Mechanisms

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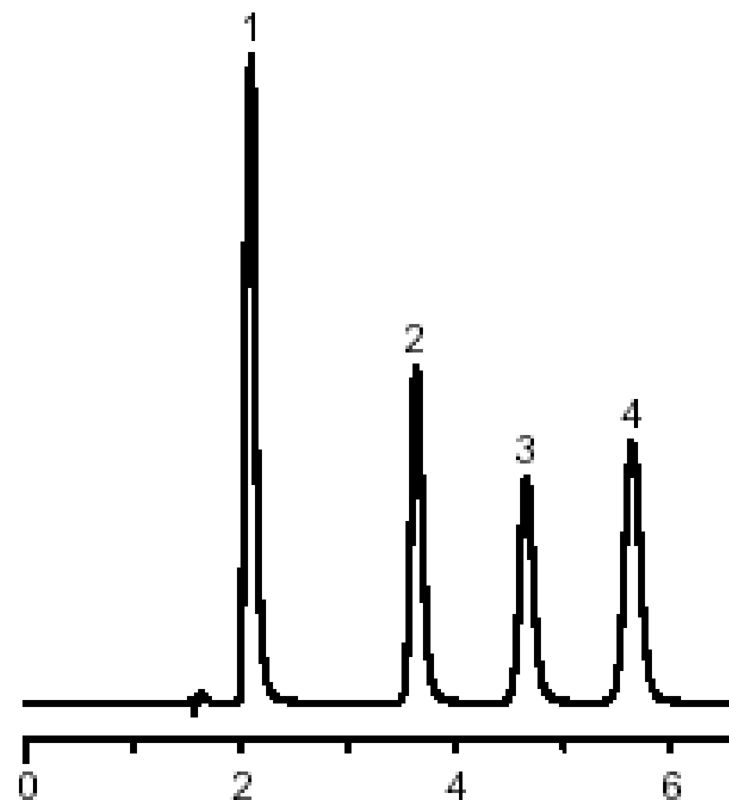
Analytical Chromatography Heaven

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

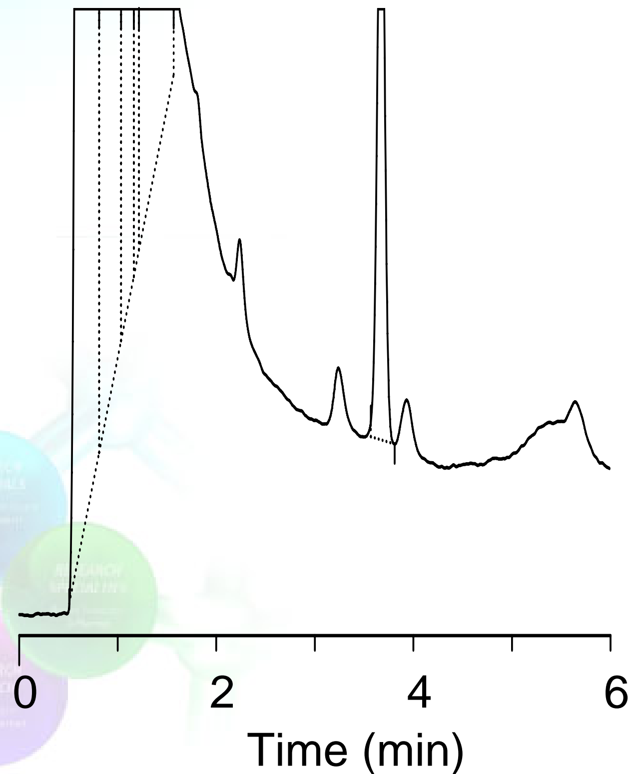


The Importance of Sample Preparation

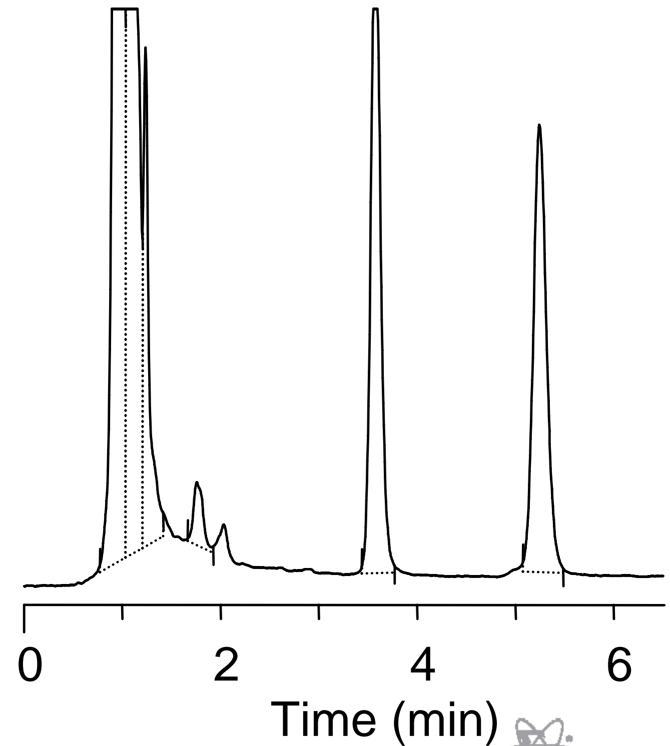
Real World & Real Samples

The Importance of Sample Preparation

Urine Sample without SPE



Urine Sample with SPE



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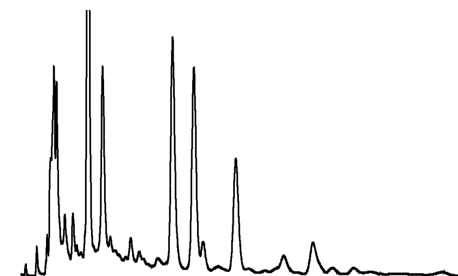
Why is sample preparation required?

Collected Sample

GC, HPLC, or LC-MS/MS Analysis



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

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

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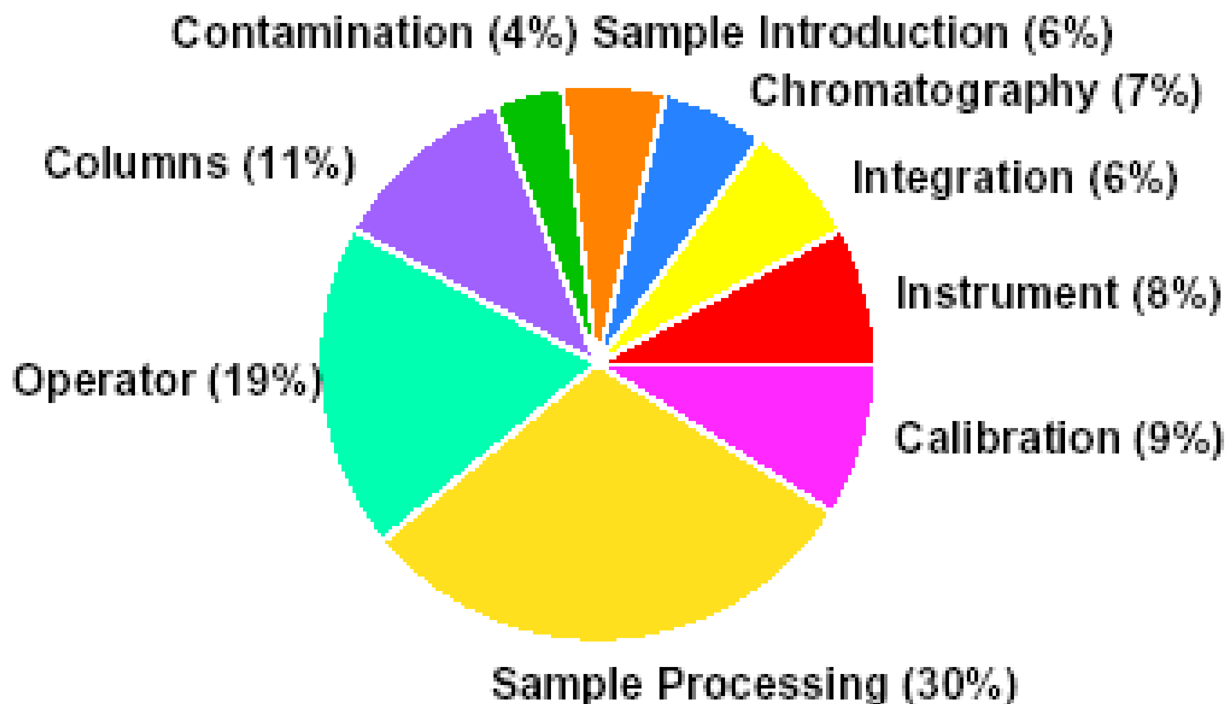
Sources of Chromatographic Errors



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

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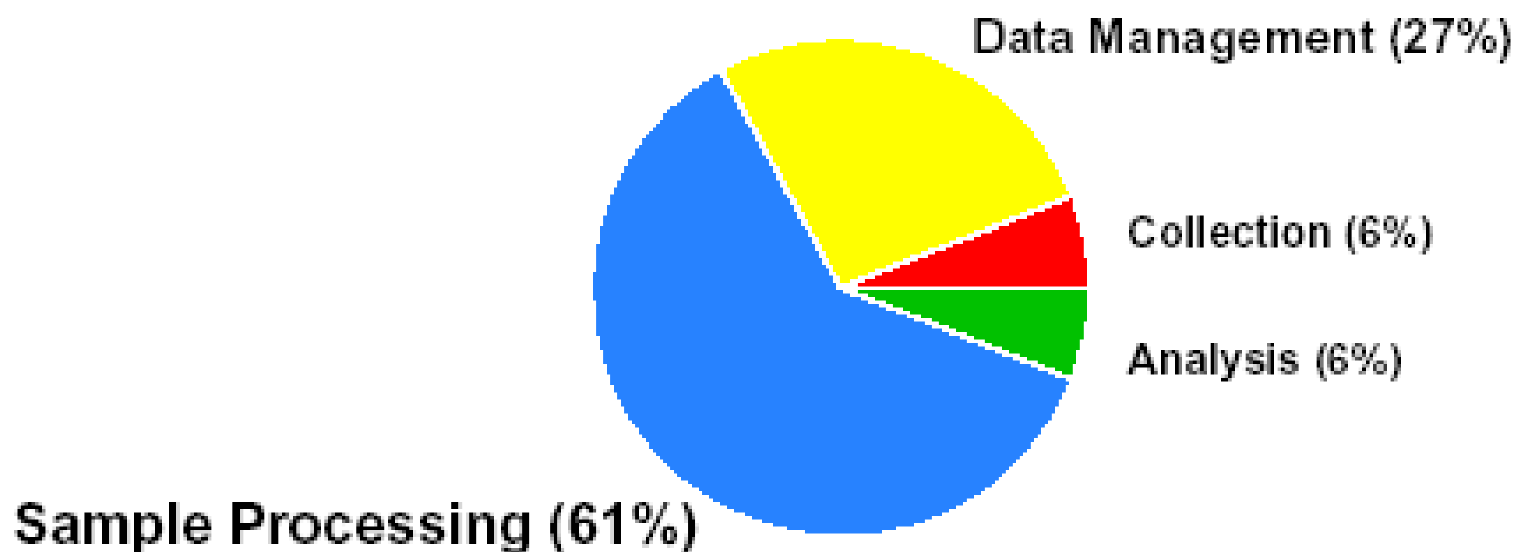
Time Spend on Analytical Process



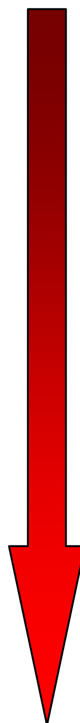
(R.E. Majors, LC/GC Magazine, 1991, 1997, 2002)

Many Tools/Technology for Sample Prep

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

Simpler

Generic Methodology

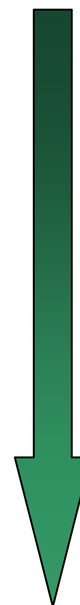


More Complicated

Requires Method Dev

Less Selective

Minimal Sample Cleanup & Concentration



Greater Selectivity

Optimal Sample Cleanup & Concentration

Separatory Funnels/LLE = Old Technology

- Works for many samples
- Large solvent consumption
  - Disposal of solvent
- Vigorous shaking/mixing
- Waiting for layers to separate
- Phase emulsions
- Longer Rotovap Times
- Separatory funnel is spacey equipment
  - (sample throughput, Automatisation?)

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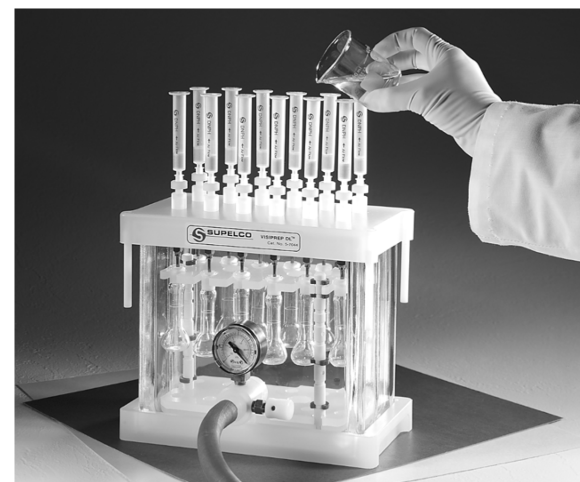
Purpose of Solid Phase Extraction (SPE)

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.



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Overview of Solid Phase Extraction (SPE)

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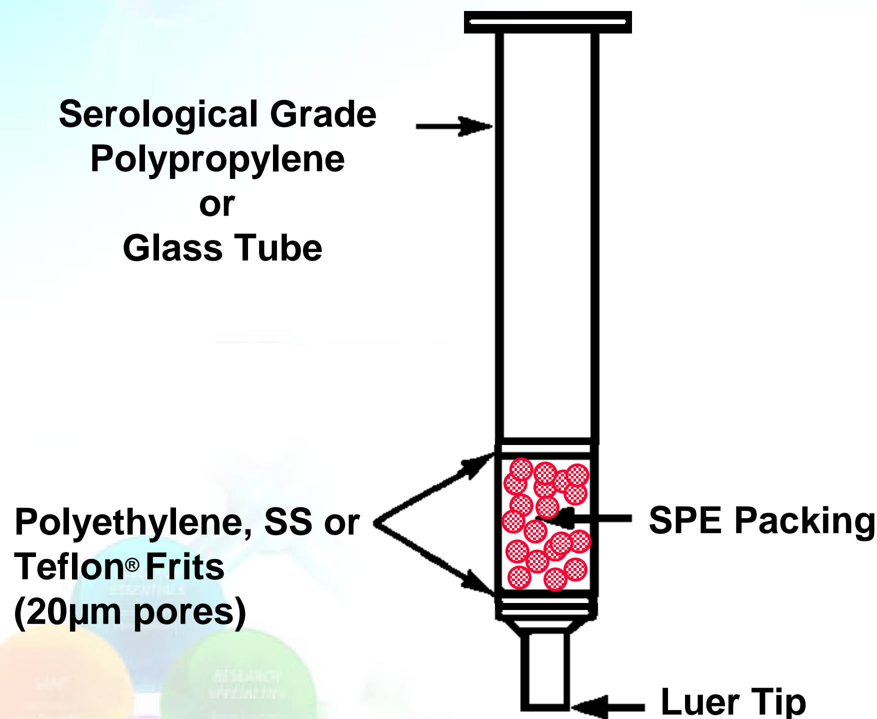
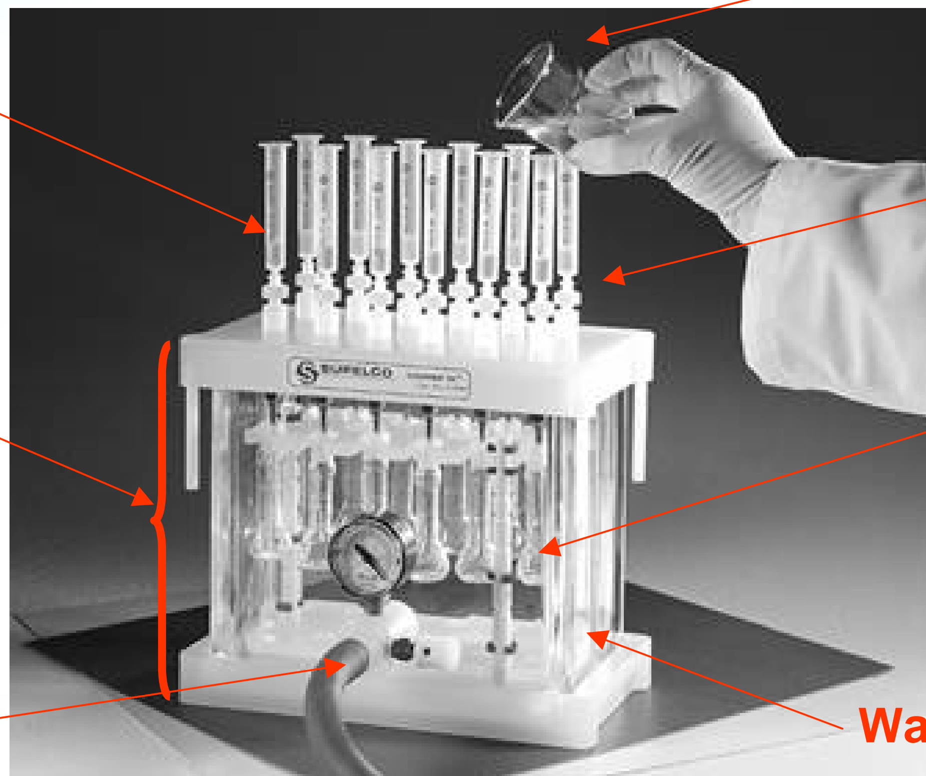
Basic SPE Concept



- 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 (>50µm) vs. HPLC (≤ 5µm)
  - SPE particle size distribution much broader than HPLC
- Use it only once

SPE Vacuum Manifold



Sample introduction

SPE tubes

Indiv. Port Valves

Vacuum manifold

Sample collection tubes (volumetric flasks)

Vacuum line and gauge

Waste reservoir

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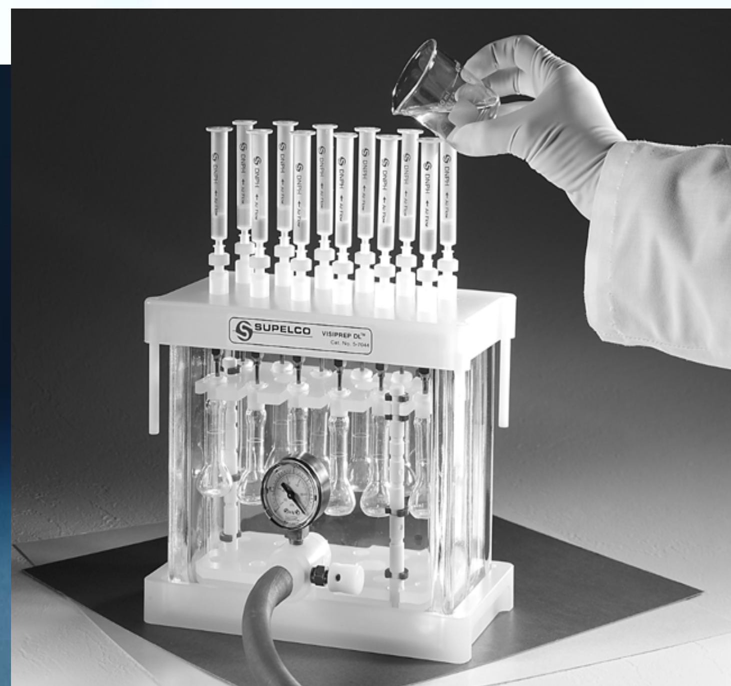
SPE Tube Device Processing Equipment



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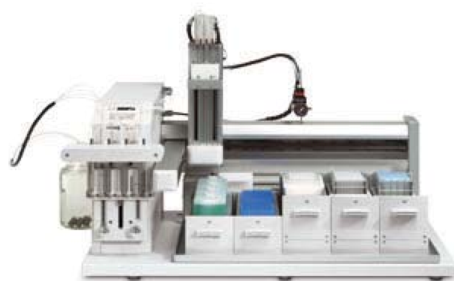
Most Common SPE Robots for Automated SPE

Zymark RapidTrace System

TomTec Quadra System



Gilson SPE 215 System

Code 802 & 803 "Tab-less" 1 & 3mL racks

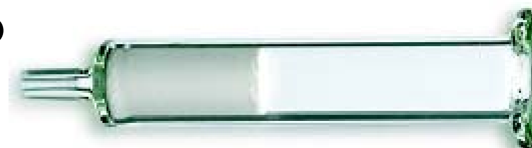
Types of SPE Tubes/Cartridges

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



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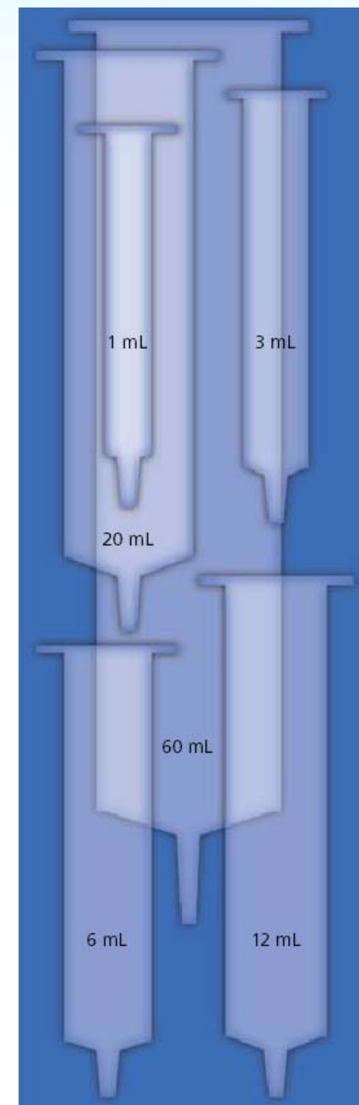
SPE Bed Weight/Tube Size Selection

- Smaller tube dimensions (1 mL) contain smaller bed weights.
  - reduced elution volumes which can be beneficial
- 3 mL SPE tubes are most common size
- 6 mL SPE tubes when one or more steps require volumes greater than 3 mL.
- 12, 20, and 60 mL tubes contain larger bed weights allow to use SPE as a prep purification or modified LPLC/Flash technique.

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

* This value depends on the analyte and sample matrix. As a rule of thumb, the bed capacity can be estimated with ~5% of the bed weight.



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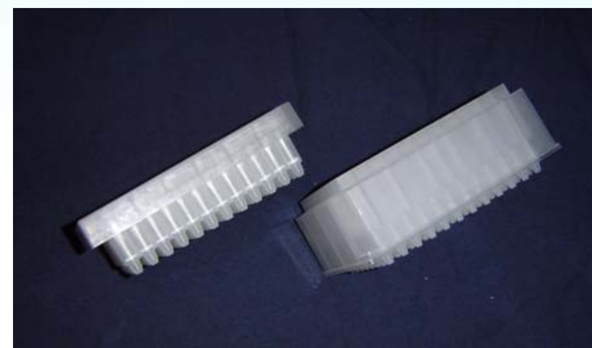
Common SPE Hardware

Funnels

Büchner format ideal for large sample volumes

Tubes

Glass or plastic, tubes are the most common SPE format



96-well plates

SPE Disks

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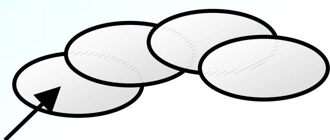
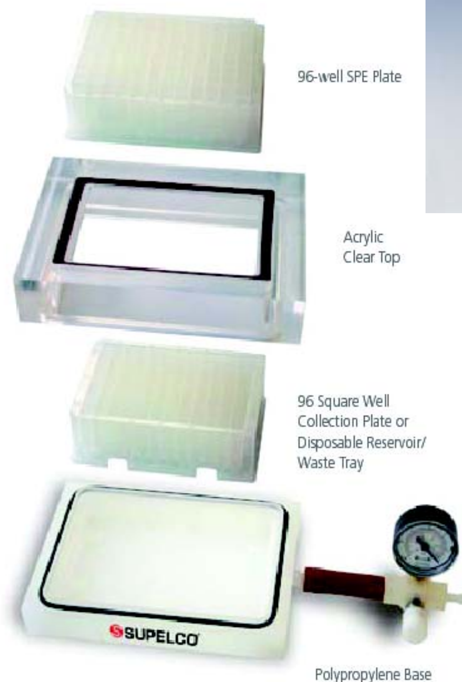
Disk & 96-Well Plates Manifold

ENVI-Disk™



SPE packing embedded in glass fiber matrix



96-well plates

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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 (really?)

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

Three different SPE Strategies

Which one to choose depends on the goal of the extraction.

1. **Bind & Elute Strategy**
   - Most common
   - Bind: Analytes bind to tube, unwanted matrix comp. 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 (Form of Bind Elute)**
   - Retain and sequentially elute different classes of compounds by modifying eluant pH or % organic

General Steps of an SPE Procedure (Bind & Elute)

1. Sample Pre-treatment
2. Conditioning & Equilibration
3. Sample Load
4. Washing
5. Elution
6. Evaporation

1) Sample Pre-treatment:

Dependent on analyte, sample matrix, and nature of retention chemistry; involves pH adjustment, centrifugation, filtration, dilution, buffer addition, etc..

2a) Conditioning:

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

2b) Equilibration:

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

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General Steps of an SPE Procedure (Bind & Elute)

3) **Sample Load:**

Introduction of the sample = analytes of interest are bound/ extracted onto the phase/ sorbent.

4) **Washing:**

Selectively remove unwanted interferences co-extracted with the analyte without prematurely eluting analytes of interest.

5) **Elution:**

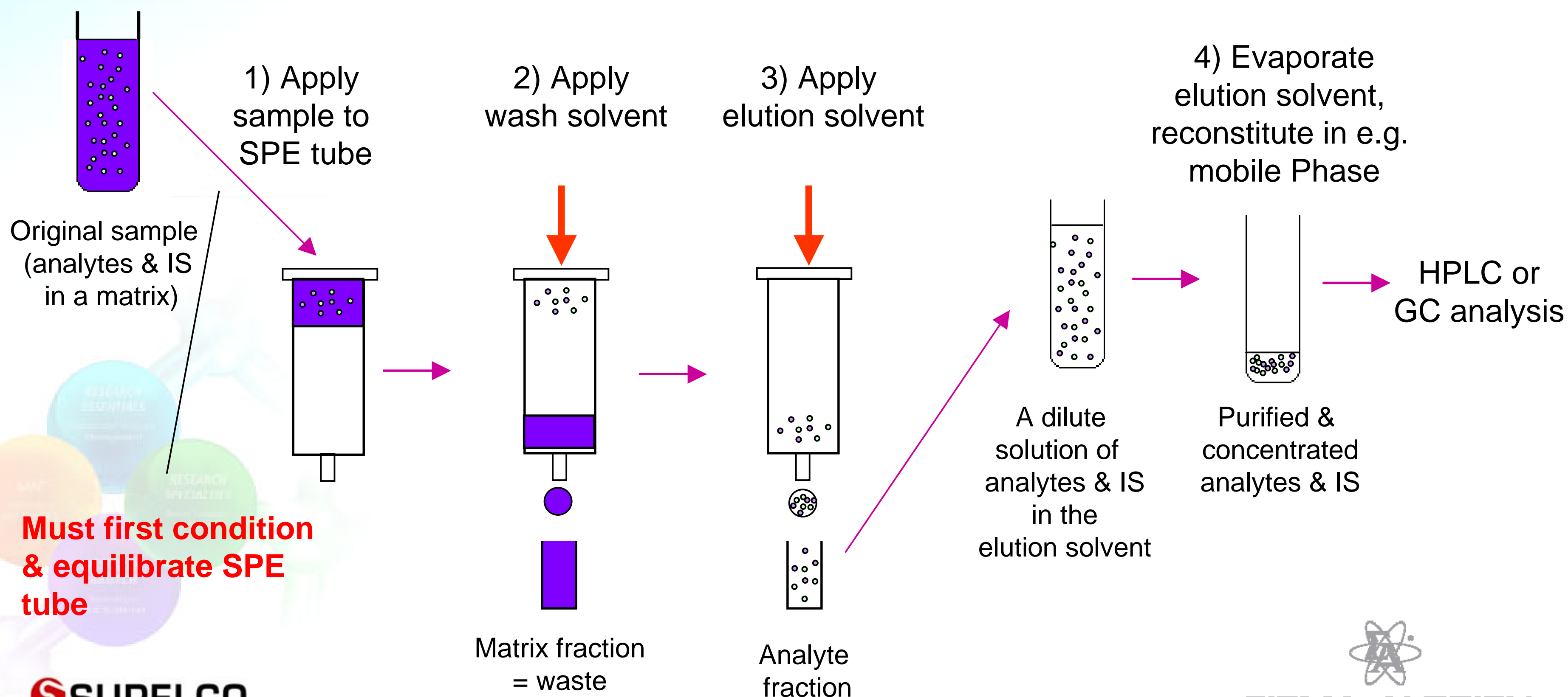
Removing analytes of interest with a solvent that overcomes the primary and secondary retention interactions b/w sorbent and analytes of interest.

6) **Evaporation**

of eluent/ reconstitution with mobile phase (optional).

Bind-elute strategy diagram

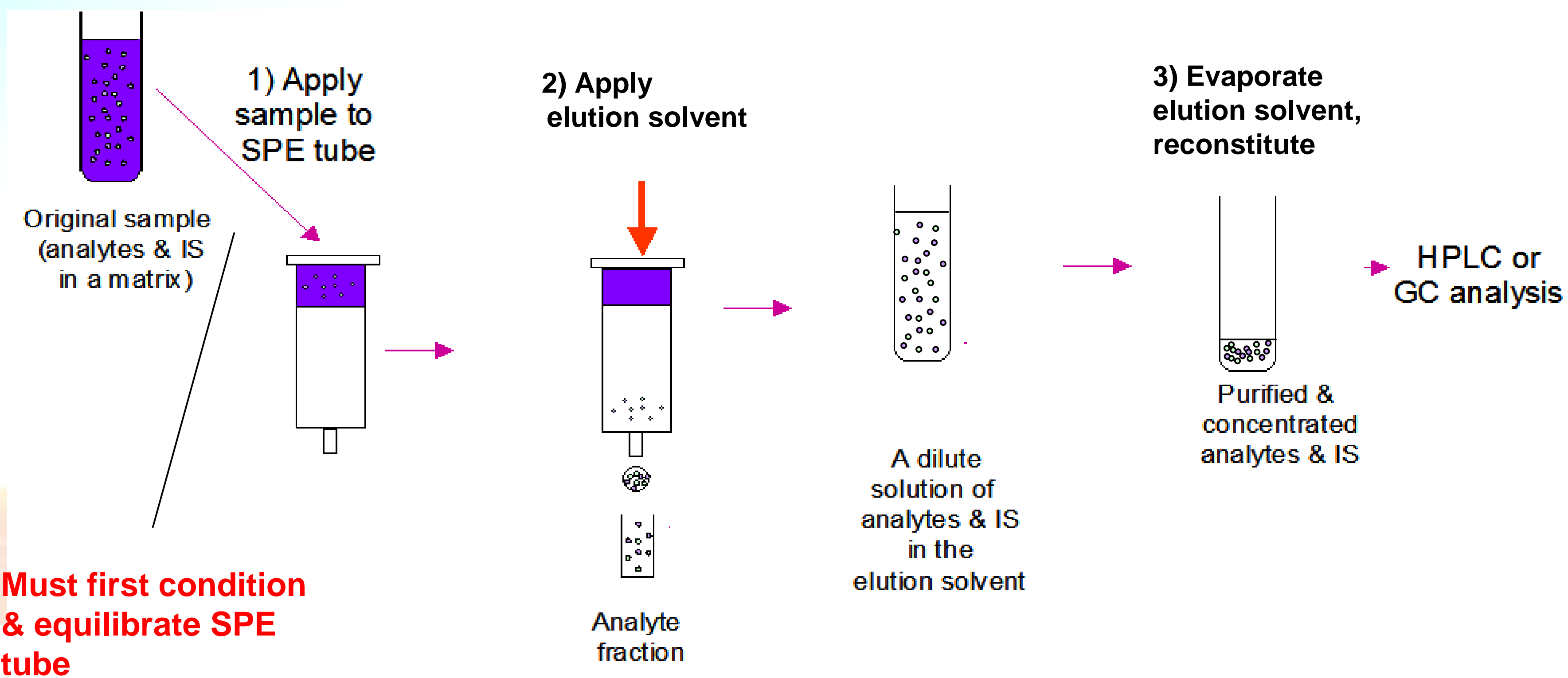
(Filtered) sample with internal standard (IS) → Analytes of interest in suitable matrix



Must first condition & equilibrate SPE tube

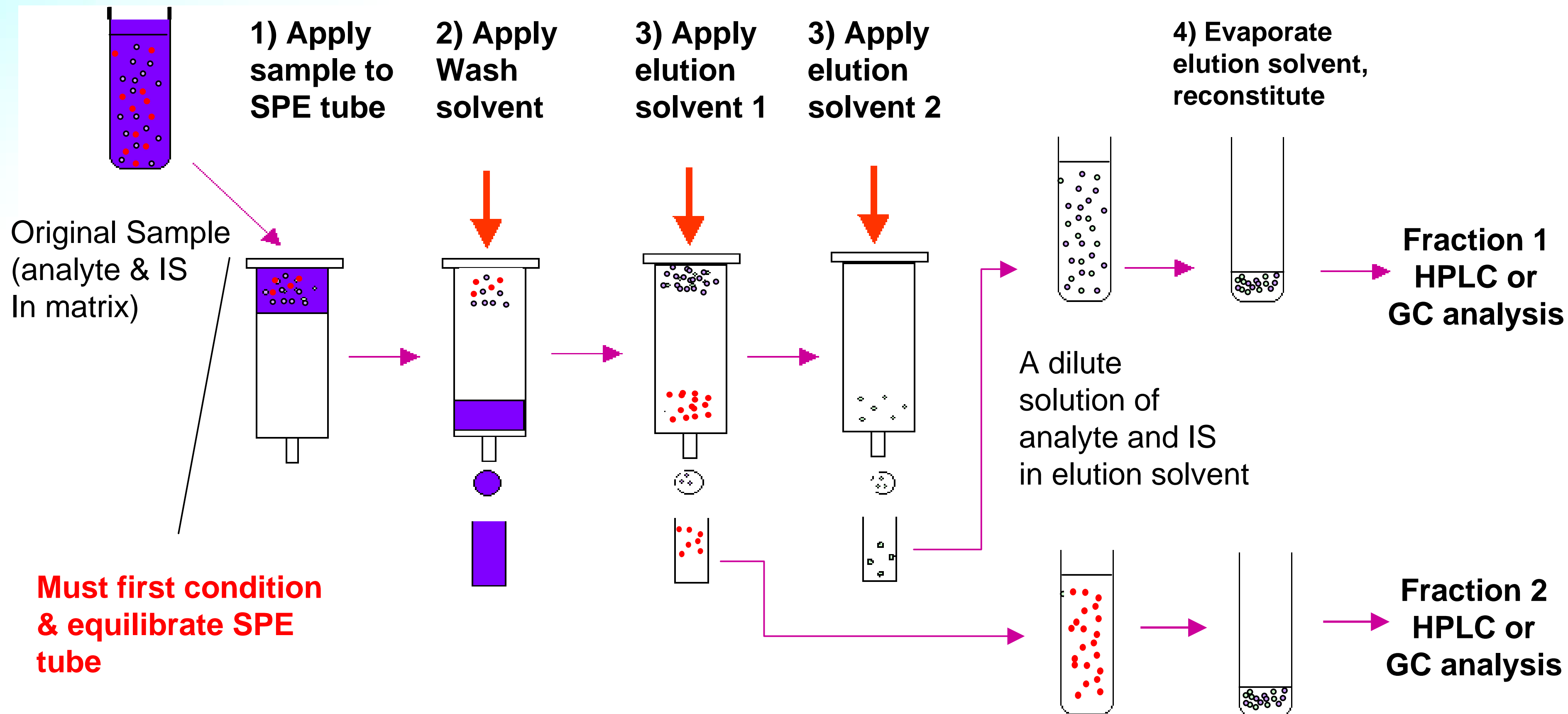
Interference removal strategy diagram

Sample with Internal Standard in Matrix → Matrix adsorbed → Analytes & IS pass



Fraction strategy diagram

Form of Bind and Elute Strategy with multiple elution steps



Understanding Retention Mechanisms

Reversed-Phase SPE

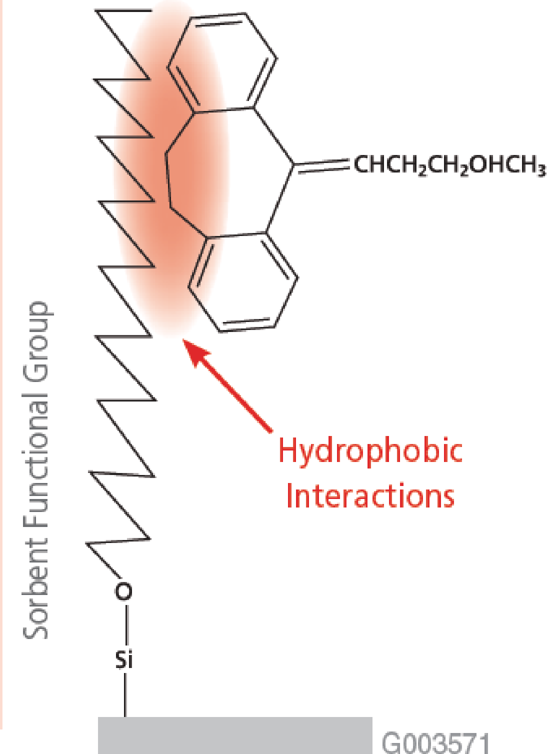
Retention Mechanism: **Non-polar or hydrophobic interactions**
- Van der Waals or dispersion forces

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
- Aqueous extracts of tissues and solids



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)

Example RP SPE Protocol

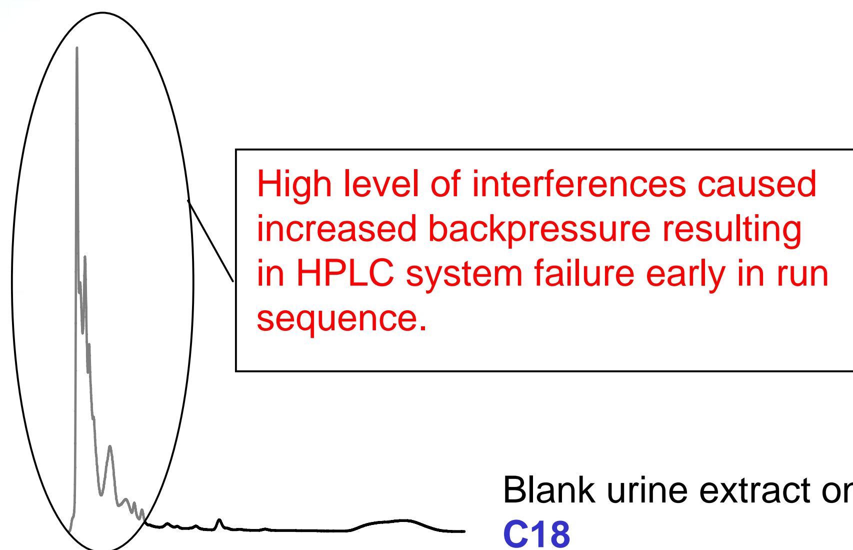
4. **Wash sorbent** (elutes co-retained interferences)
   - Critical for improving selectivity
   - 5-20% MeOH common
   - Dilute MeOH in buffer used during sample load

5. **Elute analytes of interest**
   - MeOH or MeCN most common
   - pH manipulation can improve recovery (adjust pH opposite to load conditions)

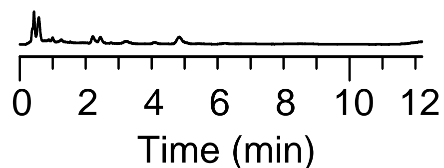
6. **Evaporate/reconstitute as necessary**

C18 vs. C8 vs. Ph vs. CN

More polar RP sorbents (e.g. CN, Ph) can offer better selectivity



Blank urine extract on conventional **C18**



Blank urine extract on **DSC-CN**

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C18 vs. C8 vs. Ph vs. CN

- More polar RP sorbents
  - can offer better selectivity
  - Often allow for weaker & smaller elution volumes
  - Greater risk of premature analyte elution during wash step
    - Requires weaker wash solvents
  - Less risk of sorbent over drying

- More non-polar RP sorbents
  - Have broader analyte retention range
  - Greater risk insufficient clean-up
  - Allows for stronger wash solvents
  - May require increased elution volume

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
  - Only critical with C18 & only critical in first conditioning step
  - Phase just needs to be moist during sample addition
  - All other steps non-critical
- 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

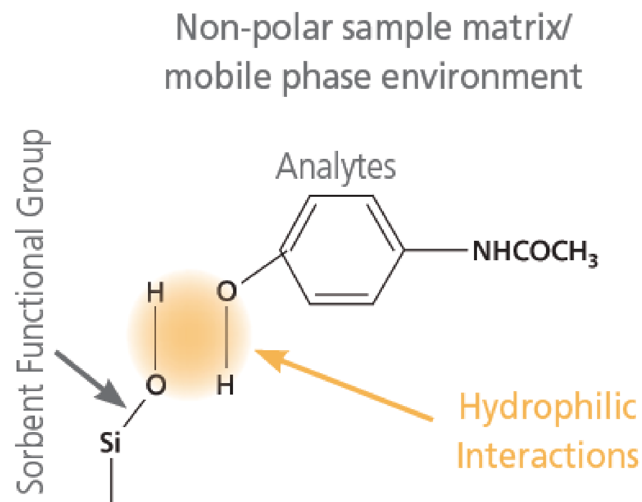
Retention Mechanism: **Polar Interactions**
- Hydrogen bonding, pi-pi, dipole-dipole, and induced dipole-dipole

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:
- Cleanup of organic extracts of soils and sludge
- Fractionation of petroleum hydrocarbons
- PCBs in transformer oil
- Isolation of compounds in cosmetics



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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 Mg-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 not be in MeCN or MeOH**

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Example NP SPE Protocol

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**

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Common Normal Phase Solvents

Solvent	Elutropic (e°) or elution strength on silica	
Hexane	0.00	Promotes Normal-Phase Retention
Isooctane	0.00	
Carbon tetrachloride	0.14	
Toluene	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	

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Ion-Exchange SPE

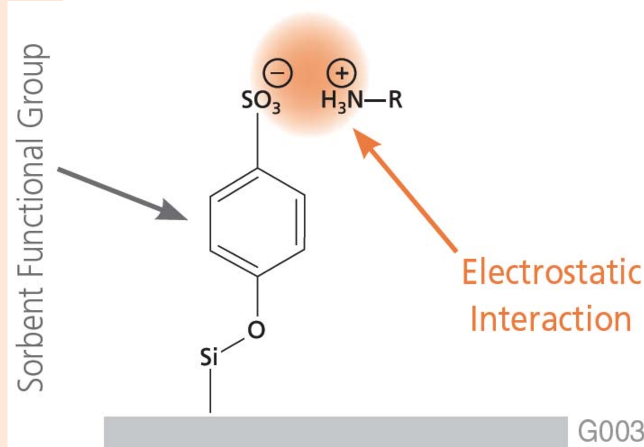
Retention Mechanism: Electrostatic attraction of charged functional groups of the analyte(s) to oppositely charged functional groups on the sorbent. Combination of reversed-phase and ion-exchange for mixed-mode

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
- Cleanup of synthetic reactions
- Organic acids from urine
- Herbicides in soil



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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)

Example IOX SPE Protocol

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^-

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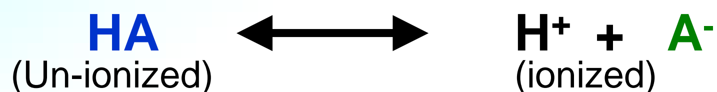
The Critical Role of pH in SPE

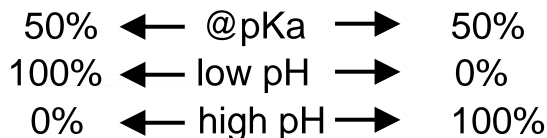
Neutral State (Blue) = promotes hydrophobic (RP) interaction

Ionized State (Green) = promotes electrostatic (IOX) interaction

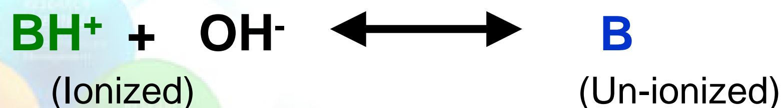
Ionization of Acidic & Basic Molecules-

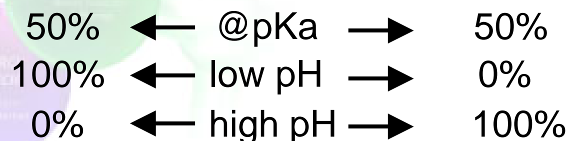
Acids (e.g., carboxylic acids): (e.g., R-COOH ⇔ $R\text{-}COO^-$)





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)**

SPE Phase Selection

Your Sample Matrix is:

- **Aqueous** (biological fluids, water, aqueous extracts of tissues, etc.)
- **Organic** (organic extracts of tissues, hexane, dichloromethane, etc.)

Recommended Retention Mechanisms:

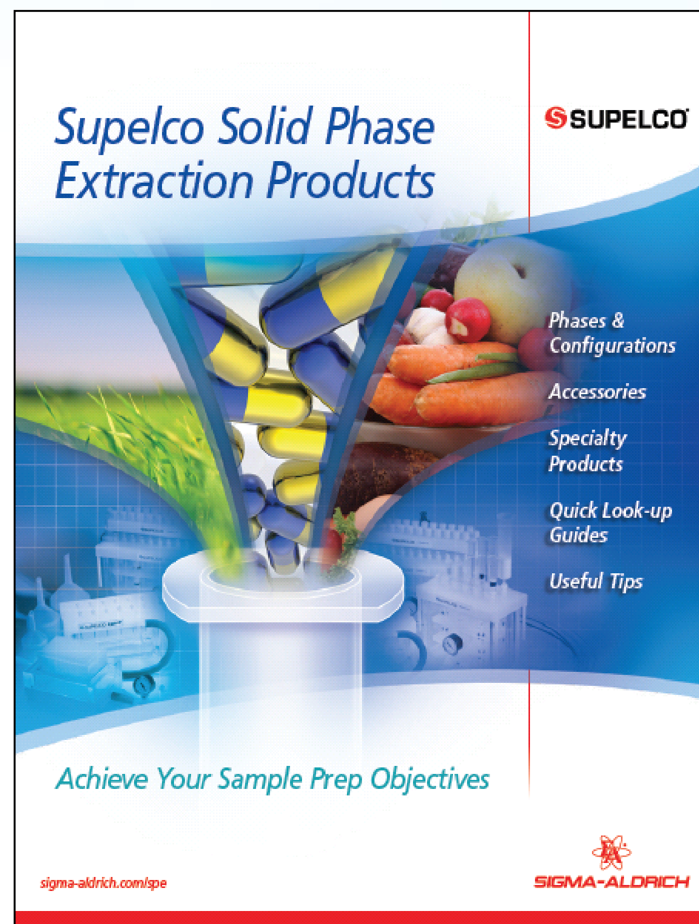
- **Reversed-Phase** See page 27 for more details
- **Ion-Exchange** See page 28 for more details
- **Normal-Phase** See page 29 for more details

Analyte Characteristics:	Moderately polar to non-polar compounds		Weak cations/ anions		Strong cations/ anions		Polar to moderately polar compounds	
Application:	**Pharma.**	**Environ.**	**Pharma.**	**Environ.**	**Pharma.**	**Environ.**	**Pharma.**	**Environ.**
Recommended SPE Phases:	DSC-18 DSC-18Lt DSC-8 DSC-Ph DSC-CN DPA-6S	ENVI™-18 ENVI-8 ENVI-Chrom P LC-18 LC-8 LC-Ph LC-CN	DSC-SAX DSC-SCX	LC-SAX LC-SCX	DSC-WCX DSC-NH_2	LC-WCX LC-NH_2 PSA	DSC-Si DSC-CN DSC-Diol DSC-NH_2	ENVI-Florisil® LC-Alumina LC-Florisil LC-Si LC-NH_2 LC-Diol LC-CN PSA

New SPE Brochure 2007

- T402150 (FEB)
- 28 pages

- Complete list of SPE products and accessories



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