Basics of SPE Technology & Mechanisms

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Agenda

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

Time (min)
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
Sources of Chromatographic Errors

- Contamination (4%)
- Sample Introduction (6%)
- Chromatography (7%)
- Columns (11%)
- Integration (6%)
- Instrument (8%)
- Operator (19%)
- Calibration (9%)
- Sample Processing (30%)

Time Spend on Analytical Process

Sample Processing (61%)

Data Management (27%)
Collection (6%)
Analysis (6%)

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

Less Selective
Minimal Sample Cleanup & Concentration

More Complicated
Requires Method Dev

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?)
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.
Overview of Solid Phase Extraction (SPE)
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

- SPE tubes
- Vacuum manifold
- Vacuum line and gauge
- Indiv. Port Valves
- Sample introduction
- Sample collection tubes (volumetric flasks)
- Waste reservoir
SPE Tube Device Processing Equipment
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

- **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
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.

<table>
<thead>
<tr>
<th>Bed Weight</th>
<th>Tube Volume</th>
<th>Minimum Elution Vol.</th>
<th>Bed Capacity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-100 mg</td>
<td>1 mL</td>
<td>100-200 μL</td>
<td>2.5-10 mg</td>
</tr>
<tr>
<td>500 mg</td>
<td>3 mL</td>
<td>1-3 mL</td>
<td>25-100 mg</td>
</tr>
<tr>
<td>0.5-1 g</td>
<td>6 mL</td>
<td>2-6 mL</td>
<td>25-100 mg</td>
</tr>
<tr>
<td>2 g</td>
<td>12 mL</td>
<td>10-20 mL</td>
<td>0.1-0.2 g</td>
</tr>
<tr>
<td>5 g</td>
<td>20 mL</td>
<td>20-40 mL</td>
<td>1.25-2.5 g</td>
</tr>
<tr>
<td>10 g</td>
<td>60 mL</td>
<td>40-100 mL</td>
<td>0.5-1 g</td>
</tr>
</tbody>
</table>

* 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.
Common SPE Hardware

- **Tubes**: Glass or plastic, tubes are the most common SPE format.
- **SPE Disks**: Ideal for large sample volumes.
- **Büchner format Funnel**: Ideal for large sample volumes.
- **96-well plates**
Disk & 96-Well Plates Manifold

ENVI-Disk™

SPE packing embedded in glass fiber matrix

96-well plates
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.
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

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

1) Apply sample to SPE tube
2) Apply wash solvent
3) Apply elution solvent
4) Evaporate elution solvent, reconstitute in e.g. mobile Phase

Original sample (analytes & IS in a matrix)

Must first condition & equilibrate SPE tube

Matrix fraction = waste
Analyte fraction

Purified & concentrated analytes & IS

A dilute solution of analytes & IS in the elution solvent

HPLC or GC analysis

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Interference removal strategy diagram

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

1) Apply sample to SPE tube
2) Apply elution solvent
3) Evaporate elution solvent, reconstitute

Must first condition & equilibrate SPE tube

Analyte fraction

A dilute solution of analytes & IS in the elution solvent

Purified & concentrated analytes & IS

HPLC or GC analysis
Fraction strategy diagram

Form of Bind and Elute Strategy with multiple elution steps

1) Apply sample to SPE tube
2) Apply Wash solvent
3) Apply elution solvent 1
3) Apply elution solvent 2
4) Evaporate elution solvent, reconstitute

Original Sample (analyte & IS in matrix)

Must first condition & equilibrate SPE tube

Fraction 1 HPLC or GC analysis

A dilute solution of analyte and IS in elution solvent

Fraction 2 HPLC or GC analysis
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

High level of interferences caused increased backpressure resulting in HPLC system failure early in run sequence.

Blank urine extract on conventional C18

Blank urine extract on DSC-CN
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
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, sonication, 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

SUPELCO
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
## Common Normal Phase Solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Elutropic (e°) or elution strength on silica</th>
<th>Promotes Normal-Phase Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Isooctane</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Tert-butyl methyl ether</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Methylene chloride (dichloromethane)</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>40% methanol in acetonitrile</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>20% methanol in diethyl ether</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>20% methanol in methylene chloride</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>&gt;0.73</td>
<td>Promotes Normal-Phase Elution</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>&gt;0.73</td>
<td></td>
</tr>
</tbody>
</table>
## 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
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+} > RN{H_3}^{2+} > NH_4^+ > Na^+ > H^+ > Li^+

**For Anion Exchangers:**
- Benzene Sulphonate > Citrate > HS{O_4}^- > NO_3^- > HS{O_3}^- > NO_2^- > Cl^- > HCO_3^- > HPO_4^- > Formate > Acetate > Propionate > F^- > OH^-
**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-COO⁻)

\[
\text{HA} \quad \text{(Un-ionized)} \quad \leftrightarrow \quad \text{H}^+ + \text{A}^- \quad \text{(ionized)}
\]

<table>
<thead>
<tr>
<th>pH State</th>
<th>pKa @</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pH</td>
<td>&lt;pKa</td>
<td>50%</td>
</tr>
<tr>
<td>High pH</td>
<td>&gt;pKa</td>
<td>100%</td>
</tr>
<tr>
<td>High pH</td>
<td>&lt;pKa</td>
<td>0%</td>
</tr>
<tr>
<td>Low pH</td>
<td>&gt;pKa</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Bases** (e.g., amines): (e.g., R-NH₃⁺ ↔ R-NH₂⁻)

\[
\text{BH}^+ + \text{OH}^- \quad \leftrightarrow \quad \text{B} \quad \text{(Un-ionized)}
\]

<table>
<thead>
<tr>
<th>pH State</th>
<th>pKa @</th>
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<tr>
<td>Low pH</td>
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<td>100%</td>
</tr>
</tbody>
</table>

**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.)

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**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

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**Anlyte Characteristics:**

**Moderately polar to non-polar compounds**

**Weak cations/anions**

**Strong cations/anions**

**Polar to moderately polar compounds**

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**Application:**

**Pharma.**

DSC-18
DSC-18Lt
DSC-8
DSC-Ph
DSC-CN
DPA-6S

**Environ.**

ENVI*-18
ENVI-8
ENVI-Chrom P
LC-18
LC-8
LC-Ph
LC-CN

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**Pharma.**

DSC-SAX
DSC-SCX

**Environ.**

LC-SAX
LC-SCX

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**Pharma.**

DSC-WCX
DSC-CN
DSC-Diol
DSC-NH₂

**Environ.**

LC-WCX
LC-NH₂
PSA

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**Pharma.**

DSC-Si
DSC-CN
DSC-Diol
DSC-NH₂

**Environ.**

ENVI-Florisil®
LC-Alumina
LC-Florisil
LC-Si
LC-NH₂
LC-Diol
LC-CN
PSA
New SPE Brochure 2007

- T402150 (FEB)
- 28 pages
- Complete list of SPE products and accessories