Sample Prep for Chromatography

Sorbents, Devices and Techniques to Improve Sensitivity, Specificity and Throughput

2010 Innovation Seminar Series
Supelco, Div. of Sigma-Aldrich
Time Spend on the Analytical Process

- Sample processing: 61%
- Data management: 27%
- Collection: 6%
- Analysis: 6%

Real World & Real Samples

Ideal situation

Typical starting situation

Sample prep tools
Overview of Presentation

General SPE theory

Featured sample prep devices & techniques

1. Hybrid SPE particles
   - Improved sensitivity for LC-MS in bioanalysis
2. Molecularly-imprinted particles
   - Analyte/matrix-specific sorbents
3. Solid phase microextraction fibers
   - Solventless sample prep

For each device: What it is, how it works, examples
Goals of Sample Preparation

Remove matrix
Increase concentration of analyte
Exchange solvent
Other considerations:
  • Automation
  • Exhaustive vs. equilibrium
Surface chemistry
  • Affects k and alpha

\[ R_s = \frac{\sqrt{N}}{4} \cdot \frac{k}{k+1} \cdot \frac{\alpha-1}{\alpha} \]
Basic SPE Concept

- Cartridge
- Frits
- Packing material
### Three SPE Strategies

#### Bind-Elute Strategy
- **Bind:** Analytes bind to sorbent, unwanted matrix components are washed off
- **Elute:** Change eluant
- **Analytes are concentrated via evaporation prior to analysis**

#### Interference Removal Strategy
- **Bind** all unwanted matrix components and allow analytes to pass through during the sample loading stage

#### Fractionation Strategy
- **Retain** and sequentially elute different classes of compounds by modifying eluant pH or % organic
**Bind-Elute Strategy Diagram**

1) **Apply sample to SPE cart.**
   - Original sample (analytes & IS in a matrix)

2) **Apply wash solvent**
   - Matrix fraction = waste

3) **Apply elution solvent**
   - Analyte fraction
   - A dilute solution of analytes & IS in the elution solvent

4) **Evaporate elution solvent, reconstitute**
   - Purified & concentrated analytes & IS
   - HPLC or GC analysis

**Bind-Elute sorbent types:**
- DSC-C18, Supel-Select HLB, SupelMIP, ENVI-Carb Plus, PS/DVB, DSC-MCAX, Envi-ChromP
Interference Removal Strategy Diagram

1) Apply sample to SPE cart.

Original sample (analytes & IS in a matrix)

Interference Removal sorbent types: HybridSPE, QuEChERS, PSA, Envi-Carb, Dual Layer

2) Apply elution solvent

Analyte fraction

A dilute solution of analytes & IS in the elution solvent

3) Evaporate elution solvent, reconstitute

Purified & concentrated analytes & IS

HPLC or GC analysis
Fractionation Strategy Diagram

1) Apply sample to SPE cart.
2) Apply wash solvent
3) Apply elution solvent 1
3) Apply elution solvent 2

Original Sample (analyte & IS in matrix)

Fractionation sorbent types: DSC-MCAX, Ag-Ion, DSC-SCX, DSC-SAX

A dilute solution of analyte and IS in elution solvent

Fraction 1 HPLC or GC analysis

Fraction 2 HPLC or GC analysis
Sample Prep Devices

Hybrid SPE particles
- Improved sensitivity for LC-MS in bioanalysis

Molecularly-imprinted particles
- Analyte/matrix-specific sorbents

Solid phase microextraction fibers
- Solventless sample prep

Strategy: Interference removal
Goal: Remove matrix
HybridSPE™-Precipitation (HybridSPE-PPT)

96-well SPE plates and cartridges
Zirconia-coated silica particles

Features:
- Selective removal of phospholipid interferences and precipitated proteins
- Simple 2-3 step procedure

Benefits
- Improved LC-MS sensitivity (reduced matrix effect)
- Enhanced column lifetime
- Gradients not needed to clean column
Monitoring Phospholipid Contamination

- PLs major component of cell membranes
- Polar head group, non-polar tail
- Largest subclass (phosphatidylcholine) monitored using m/z 184 or m/z 104 fragment ions
- Used as a marker for ion-suppression risk assessment during LC-MS/MS
- Determine selectivity effectiveness of sample prep technique

polar head group

non-polar “tail”
Problem: Protein and Phospholipid Accumulation on HPLC Column

- Standard protein ppt technique
- Reduces performance
- Increases backpressure
- Unpredictable carry-over & elution in future injections
- Gradients needed to clean column

HPLC column: Sub-2um C18, 5 cm x 2.1 mm I.D.
Solution: Phospholipids Selectively Removed using HybridSPE-PPT Technology

- The Zr atom on the particle acts as a Lewis acid
- The phosphate groups on the phospholipids are strong Lewis bases and complex with the zirconium atoms
- Analytes are eluted free of phospholipids
HybridSPE-PPT Method (96-Well Format)

Precipitate proteins in well
- 100 µL plasma/serum
- 300 µL 1% formic acid in acetonitrile
- Add I.S. as necessary

Mix

Apply vacuum

Resulting filtrate/eluate is free of proteins and phospholipids, ready for LC-MS
Improved Situation: No Protein or Phospholipid Accumulation Using HybridSPE-PPT

Consistent column performance
No increase in backpressure
Eliminates carry-over & elution in future injections
Extends column lifetime
Gradients are not needed to clean column

Monitoring PLs at 184 m/z

Inj. #1, 1920 psi

Inj. #20, 1925 psi

No change in back-pressure and baseline
Improved Through-put with HybridSPE-PPT

Elimination of need for gradient clean-up improves sample throughput

Gradient, 20 min.
~ 70 inj./day

Isocratic, 2 min.
~ 700 inj./day
Overlay of HybridSPE-Small Volume and Protein Precipitation Samples

Methadone and metabolites from plasma
Sample was extracted using HybridSPE-PPT or standard PPT
High concentration (1200 ng/mL), still shows suppression with standard ppt method

Column: Ascentis Express RP-Amide 10 cm X 2.1, mm I.D., 2.7um; ESI+ detection
Comparison of response HybridSPE-PPT vs. standard protein ppt

Calibration: Methadone Plasma Extracts

- Methadone STD
- Plasma HybridSPE
- PPT
HybridSPE™ – Precipitation Technology (HybridSPE-PPT)

- Simplicity of protein precipitation and selectivity of SPE
- Nearly complete depletion of phospholipids and precipitated proteins
- 2-3 step generic procedure
- 96-well and cartridge dimensions
  - 50 mg/2 mL per well
  - 15 mg/0.8 mL per well
- Compatible with automation
- No need for gradients to clean HPLC column
Sample Prep Devices

Hybrid SPE particles
  • Improved sensitivity for LC-MS in bioanalysis

Molecularly-imprinted particles
  • Analyte/matrix-specific sorbents

Solid phase microextraction fibers
  • Solventless sample prep

Strategy: Bind-elute
Goal: Remove matrix
High-Specificity SPE (SupelMIPs)

96-well SPE plates and cartridges
Molecularly imprinted polymer particles
Developed by MIP Technologies, Lund, Sweden

Features:
• Very selective extraction
• Predefined protocols: no method development

Benefits:
• Permits more rigorous washing to remove matrix
• Analysis at extremely low concentrations (ppb, ppt)
The Molecular Imprinting Process

**Molecularly imprinted polymers (MIPs)** are polymers that have been prepared by polymerizing either pre-formed or self-assembled monomer-template complexes together with a cross-linking monomer. After removal of the template molecule, a polymer with binding sites for the template is obtained.
The MIP Binding Site

Graphical representation of the MIP binding site, which contains a cavity of the right size and attractive molecular features that can bind to the target molecule(s).
Overview of a Typical SupelMIP SPE Procedure

Very simple methods. Full protocols are included with each MIP product. Protocols may require optimization depending on the sample matrix.
SupelMIP Chloramphenicol: Analysis in Honey

Chloramphenicol is an antibiotic that is monitored in honey.

Background from honey sample cleaned by SupelMIP-SPE and LLE for chloramphenicol analysis.

Comparison of matrix effect (ion suppression) between different clean-up methods for honey. Samples were spiked with CAP prior to analysis.
SupelMIP Products

- **PAHs** in edible oils
- **Nitroimidazoles** in milk, eggs and other foods
- Nonsteroidal anti-inflammatory drugs (**NSAIDS**) in wastewater and other matrices
- **Fluoroquinolones** in bovine kidney, honey and milk
- **Amphetamines** and related compounds in urine
- **Chloramphenicol** in plasma, urine, milk, honey and shrimp
- **NNAL** - nitroso compound in urine
- **TSNAs** - tobacco specific nitrosamines in urine and tobacco
- **β-agonists** and **β-blockers** in tissue, urine and wastewater
- **Clenbuterol** in urine
- **Triazines** in water
- **Riboflavin** in milk

**In development:**

- **Nicotine** and **Cotinine** in gum, urine
- **Aminoglycosides** in cell culture broth, honey, kidney
- **Crystal violet** in fish tissue
- **Malachite green** in fish tissue
Detailed MIP Protocols

- Optimized for analyte and matrix
- Eliminates method development time

Protocol for Extraction of Fluoroquinolones from Bovine Kidney:

Sample Pre-treatment:
Dissolve milk in an equal amount of 10 mL ammonium acetate, pH 5. Centrifuge for 5 min at 10,000 rpm. Adjust supernatant to pH 7 as necessary with ammonium hydroxide and acetic acid.

Condition/equilibratce cartridge with:
1 mL methanol
2 mL ultra pure water

Load sample:
Apply a maximum of 2 mL sample

Wash:
5 mL ultra pure water
1 mL acetic acid
1 mL 1% acetic acid in ultra pure water
1 mL 0.5% acetic acid in acetic acid (v/v)
1 mL 3% ammonia in ultra pure water

Analyte elution:
Elute 10 mL with 1 mL 2% ammonia in methanol (v/v)

Evaporate the elution solvent to dryness at a maximum temp. of 35 °C under gentle nitrogen. Redissolve in 150 μL 50% acetic acid in 0.1% formic acid prior to analysis. Filter through a 0.45 μm filter if necessary.
Sample Prep Devices

Hybrid SPE particles
- Improved sensitivity for LC-MS in bioanalysis

Molecularly-imprinted particles
- Analyte/matrix-specific sorbents

Solid phase microextraction fibers
- Solventless sample prep, plus...

Strategy: Bind-elute
Goal: Analyte concentration
Solid Phase Microextraction (SPME)

Holder assemblies (manual, autosampler, robots)
Coated fibers (adsorbent and absorbent)
Janusz Pawliszyn, Univ. of Waterloo; unique and proprietary to Supelco
Economical enrichment technique mainly for trace analysis
Initially for GC analysis, now new fibers for LC

Features:
- Very limited or no use of solvents
- All types of samples & matrixes
- Direct immersion or headspace
- Designs for manual, autosamplers and robots

Benefits:
- Economical
- Highly consistent, quantifiable results
- Portable (field use) and reusable
- Reduces lab animal sacrifice
Enlargement of the SPME fiber coating

Enlargement of analyte conc. in fiber and sample

SPME Fiber Coating: The Business End

Not an exhaustive extraction technique

An equilibrium is set up between analytes dissolved in the sample (solution or gas phase) and in the liquid coating on the fiber.

The fiber coating consists of:
- Matrix/binder (e.g. PDMS)
- Particles (e.g. C18, carbons, DVB)
PDMS-DVB Fiber SEM

Cross section of the PDMS-DVB fiber. The center is a fused silica core, surrounded by a Stableflex core. The 3-5µm DVB particles are suspended in PDMS and layered over the cores. 275x magnification.

Photomicrograph of SPME fiber provided by Prof. Dan Armstrong, U. Texas Arlington
3000X magnification of the Carboxen PDMS coating. The 3-5µm Carboxen particles are suspended in PDMS.

Photomicrograph of SPME fiber provided by Prof. Dan Armstrong, U. Texas Arlington
Distribution Constant

Concentration of analyte in stationary phase compared to concentration of analyte in solution:

\[ K = \frac{n_s}{V_1 C_2^\circ} \]

- \( K \): Distribution constant
- \( n_s \): Moles of analyte in stationary phase
- \( V_1 \): Volume of stationary phase
- \( C_2^\circ \): Final analyte concentration in sample
Adsorption Mechanism for SPME

Rapid uptake onto fiber
“Dials” to Turn in SPME Methods

**Device**
- Type of coating (polarity)
- Coating thickness

**Sample**
- Headspace vs. direct immersion extraction
- Ionic strength, pH, polarity of sample solution
- Stirring (sample) & agitation (fiber)
- Extraction time
- Extraction temperature

**Instr.**
- Inlet liner volume (GC)
SPME Extraction of Odor-Causing Compounds in Water at 2 ppt (GC/MS)

1. 2-Isopropyl-3-methoxypyrazine (IPMP)
2. 2-Isobutyl-3-methoxypyrazine (IBMP)
3. 2- Methylisoborneol (MIB)
4. 2,4,6-Trichloroanisole (I.S. 8ppt)
5. (±) Geosmin

Sample: 25mL water containing odors at 2 ppt 25% NaCl 40mL vial
Fiber: 2cm DVB/Carboxen™/PDMS
Extraction: heated headspace, 30 min, 65°C, rapid stirring
Desorption: 3 min, 250°C, splitter closed
Column: Equity-5, 30m x 0.25mm x 0.25µm film
Oven: 60°C (1 min) to 250°C at 8°C/min
Det.: quadrupole MS, selected ion mode
Quantitative SPME

Linearity of Odor-Causing Compounds from Water at ppt Levels (SPME-GC/MS)

- IPMP $r^2=0.9900$, $y_{int}=+0.015$
- IBMP $r^2=0.9959$, $y_{int}=+0.028$
- MIB $r^2=0.9983$, $y_{int}=0.021$
- Geosmin $r^2=0.9988$, $y_{int}=-0.071$

part per trillion
Peppermint Oil in Chocolate Cookie Bar

1. Solvent
2. Internal standard
3. cis-Menthone
4. trans-Menthone
5. Menthol

SPME Fiber: 100µm PDMS
Sample: 4g peppermint cookie bar
Extraction: headspace, 1 min, 45°C
Desorption: 5 min at 250°C
Column: PTE™-5, 30m x 0.25mm ID, 0.25µm film
Detector: FID, 250°C
Injector: Splitless (3 min), 250°C
Residual Solvents in Commercial Ibuprofen

**Brand “A”**
- 1. Acetaldehyde
- 2. Ethanol
- 3. Acetonitrile
- 4. Acetone
- 5. 2-Propanol
- 6. 2-Methylpentane
- 7. 3-Methylpentane
- 8. Hexane
- 9. Ethyl acetate
- 10. 2,2-Dimethylpentane
- 11. 2,4-Dimethylpentane
- 12. Methylcyclopentane

**Brand “B”**
- 1. Acetaldehyde
- 2. Ethanol
- 3. Acetonitrile
- 4. Acetone
- 5. 2-Propanol
- 6. 2-Methylpentane
- 7. 3-Methylpentane
- 8. Hexane
- 9. Ethyl acetate
- 10. 2,2-Dimethylpentane
- 11. 2,4-Dimethylpentane
- 12. Methylcyclopentane
New SPME Research Focus: LC Sample Prep

Single use biocompatible fiber probes for *in vivo* analysis
Inert to sample matrix
Comprise C18-silica in a special binder
Solvent-stable coatings ideal for:
- Difficult matrixes (plasma, tissue)
- Non-volatile analytes
- Living systems (e.g. animals, plants, cell culture)
- Multiple data points per sample
- Reducing lab animal sacrifice

For laboratory use only
Comparison of SPME *in-vivo* PK Study of Carbamazepine from Mice Whole Blood to Extracts of Plasma Removed from Mice

![Graph showing the comparison of SPME *in-vivo* PK Study of Carbamazepine from Mice Whole Blood to Extracts of Plasma Removed from Mice. The graph plots CBZ Concentration (ng/mL) against Time (min). The data points for 'SPME 1 mouse' are represented by red squares with error bars, and the data points for 'Terminal blood draw Plasma from 3 mice per data point (18 mice total)' are represented by black triangles with error bars.](image)

Slide Courtesy of Ines de Lannoy, NoAb BioDiscoveries
SPME in Bioanalysis

Protein Precipitation Sample, MRM
184/104 for Phospholipids

propranolol and 4HP-metabolite
SPME Sample, MRM 184/104 for Phospholipids
Forensics
Food & Beverage
Flavor & Fragrance
Environmental
Biotech
Pharmaceutical

SPME Technical Literature

Explosives

Using SPME, it is possible to detect explosives, compounds from an explosive sample, and their associated organic compounds, such as explosives, with a single sample. Investigators at theMiami-Dade Police Crime Laboratory in Miami, FL, USA and at theDepartment of Chemistry at Florida International UniversityMiami, used SPME for the analysis of high explosives from solid samples and solid samples.2 They claim their technique, involving solidphase microextraction (SPME), is more effective than stripping the fiber from the sample and extracting the explosive. They claim the technique is more effective than using a sample and extracting the explosive. The technique was used to detect explosives at concentrations less than 50 ng per sample. (See Figure A.)

Figure A. Explosives Sampled Without Solvent

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Sample Prep Innovations

• **HybridSPE™-PPT** for reducing matrix effects
• **SupelMIP™** high-specificity sample prep devices
• **SPME**, including biocompatible fibers
Other Supelco Sample Prep Devices

General Purpose
- Polymer SPE
- Flash chromatography
- Carbonaceous adsorbents

Food/Food Safety
- Dispersive SPE (QuEChERS) (pesticides)
- DSC-SCX (melamine)
- Silver ion SPE (FAMEs)
- Dual-layer SPE (pesticides)

Biochromatography
- Packed pipette tips

Synthetic reaction mixtures
- Mixed-mode SPE (DSC-MCAX)

Environmental
- Radiello® Passive-Diffusive air sampling products
- BPE/DNPH for simultaneous measurement of ozone and carbonyls
- Supelclean Sulfoxide (PCBs in oil)
- Supelpak 2 (purified XAD-2)
- Dioxin Prep System
- Mercury sampling tubes
- Deactivated Thermal Desorption Tubes
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Supelco and Fluka R&D Teams
Our customers worldwide