Systematic SPE Method Development
A method development strategy leading to robust and reliable SPE methods

Frank Michel, Klaus Buckendahl,
SIGMA-ALDRICH Chemie GmbH
Eschenstraße 5, 82024 Taufkirchen,
Germany
How method development is often approached

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
Basic Rules of Solid Phase Extraction

• Analyte must adsorb onto the SPE Sorbent

• There must be **sufficient resident time** for analyte-sorbent interaction to occur

• Must be able to **selectively remove** endogenous sample interferences from the analyte

• Analyte must be able to be **removed from the sorbent**
Solid Phase Extraction

The Result:

Sample is in a simpler matrix
Sample is semi-purified
Sample is trace enriched
Sample is chromatography friendly

Major Concerns:

Is recovery high enough?
Is the product/method yielding reproducible results?
Is the sample clean enough for analysis?
Possible Problems

- Dealing with novel Analytes - different Behavior
- 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? Different SPE phase?
How to solve the Problems?

• By almost randomly “Try and Error”
  - might lead to Time consuming Troubleshooting
  - might be less less robust

• Systematic approach

⇒ POS
  Profile Optimized Solid phase extraction

or Selectivity Profiled SPE (SPS)
What is POS all about?
→ Adjust Selectivity

“Selectivity -
the ability of the sorbent and extraction method to discriminate between the analyte(s) of interest and endogenous interferences within the sample matrix”

POS Idea:
2-3 Experiments w/ Standards to
- Select Hardware and Phase
- Understand the Analyte/Sorbent Interaction for optimal Conditions
- Systematically adjust 2 main Variables (organic strength & pH)

→ Greater Confidence and Efficiency
Profile Optimized SPE (POS) Method Development - Step 1

1. Determine Sample Prep Objectives
2. Consider the Sample Matrix
3. Consider the Analytes of Interest
4. Phase & Hardware Selection
Determine Sample Prep Objectives

• What level of interference removal is required for the analysis?

• What solvent should the analyte(s) be in for optimal analysis?

• Is concentration required for optimal sensitivity?

• What resources are available to invest towards method development and routine analysis (time, personnel, instrument availability, etc.)?
Consider the Sample Matrix

- **What is the sample volume?** -> Hardware?
  - Configuration of SPE (Tube, Filter, 96-Well plates)

- **What are the endogenous sample interferences?**

- **Is the sample matrix more polar or non-polar?**
  - Serum, Plasma, Urine = Polar
    → Reversed-Phase or Ion-Exchange
  - Organic synthesis reactions or extractions = Non-Polar
    → Normal-Phase
Consider the analyte(s) of interest

What functional groups may influence the analytes’ solubility (Log P o/w), polarity, ionization state (pKa), etc.?

**Hydrophilic Groups:**
- Hydroxyl \(-\text{OH}\)
- Amino \(-\text{NH}_2\)
- Carboxyl \(-\text{COOH}\)
- Amido \(-\text{CONH}_2\)
- Guanidino \(-\text{NH(C=NH)NH}_3^+\)
- 4° Amine \(-\text{NR}_3^+\)
- Sulfate \(-\text{SO}_3^-\)

**Hydrophobic Groups:**
- Carbon-Carbon \(-\text{C-C}\)
- Carbon-Hydrogen \(-\text{C-H}\)
- Carbon-Halogen \(-\text{C-Cl}\)
- Olefin \(-\text{C=C}\)
- Aromatic

**Neutral Groups:**
- Carbonyl \(-\text{C=O}\)
- Ether \(-\text{O-R}\)
- Nitrile \(-\text{C=N}\)

© 2009 Sigma-Aldrich Co. All rights reserved.
Phase & Hardware Selection

Summarizing the considerations

• **Sample Matrix**
• **Analyte of interest**

-> choose most ideal
• Retention **Mechanism**,
• Phase **Chemistry**
• Hardware **Configuration**

for achieving the pre-determined sample prep objectives!

Here’s an example: **TCAs from Serum**
POS Example: Tricyclic Antidepressants (TCAs) from Sheep Serum

Determine Sample Prep Objectives:

• Develop a simple extraction procedure

• Achieve $\geq 85\%$ Recovery & Excellent Reproducibility for HPLC-UV Quantitation

• Endogenous serum interferences should be substantially removed
  - Simplifies HPLC resolution, prolongs Column Life, & Minimizes misleading background responses

• Achieve detection/quantitation limits of 0.25-1.0µg/mL Serum

• Post SPE sample matrix should be a buffered solvent compatible with HPLC mobile phase
POS Example: TCAs from Sheep Serum

Consider the Sample Matrix:

- **Sample Volume** 0.5 mL Sheep Serum
- Serum is the aqueous portion of blood = **Polar**
  - Platelets, corpuscles, and clotting factors have been removed
- **Endogenous Interferences**:
  - albumin, globulins, lipids, salts and carbohydrates
Consider the Analytes of Interest:

Doxepin  Imipramine  Amitryptyline

Tricyclic Antidepressants TCAs
The pH has influence & can be used for retention control as different ionic forms retain differently on a given sorbent.

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

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

- At pH $\geq 11$, the 2° or 3° amine functional group should be neutralized.
- At pH $\leq 7$, the amine group should be ionized.

POS Example: TCAs from Sheep Serum

Nortriptyline

CHCH$_2$CH$_2$NHCH$_3$
POS Example: TCAs from Sheep Serum
SPE Phase & Hardware Selection

• Sample volume = 0.5mL
  - 96-well plate or 1mL SPE tubes

• Smaller bed weights (25-100mg)
  - Smaller elution volumes = higher Analyte Concentrations

• Aqueous sample matrix + hydrophobic character of TCAs
  - Excellent candidate for Reversed-Phase SPE
  - C18 will ensure optimal retention for the potential use of stronger wash eluents
    = Maximize Sample Clean-Up

1st Choice = Discovery DSC-18 SPE-96 Well Plate
Profile Optimized SPE (POS)
Method Development Step 2

Phase & Hardware Selection

Experimentation
• Load Optimization
• Wash Elute Profile

Evaluation

Incorporate Sample Matrix/
Troubleshoot Method

Final Method

Done!
Experimentation, Evaluation, Incorporate Sample Matrix & Troubleshoot Method

Experimentation

• Develop **Analytical Method** (LC, GC, etc.)
• **Using standards** and buffered/organically modified solutions, identify and test key variable parameters (pH, organic strength, etc.)

Evaluation of Selectivity

• **Perform mass-balance analysis** on collected eluates for each step of the extraction procedure
• **Determine analyte behavior** on sorbent in response to changing extraction conditions →

Incorporate Sample matrix/ Troubleshoot

• **Define method** and incorporate sample matrix
• **Make determinations of recovery, matrix effect, cleanliness, and LC/GC resolution**
How to control Selectivity?

**Organic Strength**-
Higher (and/or stronger) organic content will cause less analyte retention via *reversed-phase* mechanism.

RP = aqueous loading and wash with low organic

**pH**-
Adjusting the pH of the MP +/- 2 pH units relative to the analyte’s pKa will make the molecule fully charged or fully ionized.

In RP SPE, charged molecules will not adsorb whereas un-charged molecules will more likely adsorb.
POS Example: TCAs from Sheep Serum
- Load Optimization

Ensure retention of the analytes of interest

1. Conditions DSC-18 wells with 1mL MeOH
2. Equilibrate DSC-18 wells with 1mL DI H₂O
3. Load 1mL 5µg/mL* standard test mix prepared at neutral (DI H₂O) and basic pH (1% NH₄OH).
4. Collect Eluate and analyze via HPLC-UV

*Note: Load concentration was increased (Method request was 0.25-1.0µg/mL) to provide adequate signal response for detecting small analyte breakthrough percentages. Also note that acidic load conditions were avoided.
Load Optimization Evaluation:
A lack of analyte presence in the 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
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 H₂O
3. Load 1mL 5µg/mL standard test mix prepared at basic pH (1% NH₄OH).
4. Wash/Elute with 1mL of a test solvent ranging from 0-100% MeOH in 2% CH₃COOH (low pH), DI H₂O (neutral pH), and 2% NH₄OH (high pH)
5. Collect wash/elute eluate and analyze via HPLC-UV
POS Example: TCAs 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.

At high pH, retention limit is 40% MeOH.
POS Example: TCAs 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 high pH, retention limit is 10% MeOH.
At neutral pH, retention limit is 20% MeOH.
At high pH, retention limit is 40% MeOH.
POS Example: TCAs from Sheep Serum
Incorporate Sample Matrix/Troubleshoot Method

Rule of Thumb

“For many applications, recovery values observed for the real-matrix based solutions will parallel values obtained with standard solutions”

• Profiling major parameters affecting Analyte Retention/Elution
  - e.g. Major matrix components, Viscosity, Particles, Stability of Analyte in the Matrix and the Matrix itself
POS Example: TCAs from Sheep Serum

POS-Method on DSC-18 SPE-96 Well Plate (100mg/well):
1. Condition/Equilibrate w/ 1mL MeOH & 1mL DI H₂O
2. Load 0.25-2.0µg/mL TCAs spiked in sheep serum diluted in 2% NH₄OH (1:1, v/v);
   n=3 for ea. concentration
3. Wash w/ 1mL 40% MeOH in 2% NH₄OH
4. Elute w/ 1mL MeOH*
5. Evaporate eluate with N-purge (30°C; ~10min.), and reconstitute in 300µL MP

* Although a 60% acidified may have been a potential elution eluant, in order to maintain sensitivity limits,
  further experimentation would be required to determine minimum elution volume
POS Method on DSC-18 Well Plate vs. Generic Method on Competitor Polymer Phase

1. Condition/Equilibrate w/ 1mL MeOH & 1mL DI H₂O
2. Load 0.25-2.0µg/mL TCAs spiked in sheep serum diluted in 2% NH₄OH (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 Mobile Phase
Results

POS Method Using DSC-18 SPE-96 Well plate

Generic Method Using Competitor Polymeric Well plate

1. Doxepin
2. Imipramine
3. Amitryptyline

Ext. Stds

1µg/mL Spiked serum

Blank serum

High Background; Misleading interfering responses

HPLC Method:
Column: Discovery C18, 15cmx4.6mm, 5µm, & guard column & frit filter;
Mobile Phase: MeCN: 25mM KH₂PO₄, pH 7 (45:55);
Flow Rate: 1.4mL/min; Temp: 30° C; Det.: UV, 254nm; Inj: 100µL
## Results

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

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

Cleaner Extracts:
• POS Method on DSC-C18 vs. Generic Method on a competitor polymeric phase shows cleaner extracts

Translates to
• Lower Background -> Increased Sensitivity
• No misleading overlapping Responses from Interferences
• Longer Column Life
• Simpler and shorter chromatographic Analysis
• More accurate Results
Summary – Systematic Method Development SPE

Phase and Hardware

- Pre-determining of sample prep objectives and carefully considering sample matrix and analytes of interest

→ strongest candidate for SPE method development

Parameters

- Use Standards and testing key variables influencing Analyte Retention and Elution,
- Strategically manipulate and make quick adjustments to the Extraction Method

→ Meet the Sample Prep Objectives
SPE - Literature

Further Method Development Aids

Bulletin 910
"Guide to Solid Phase Extraction"

Technical Report T403039 (FOP)
"Systematic SPE Method Development"

© 2009 Sigma-Aldrich Co. All rights reserved.
SPE Brochure

• T402150 (FEB)
• 36 pages

• Complete list of SPE products and accessories
Thank You!!!

& Supelclean ENVI SPE Products

- for reliable & easy Sample Clean Up and Concentration -

• RP: DSC-18, DSC-18lt, DSC-8, DSC-Ph, DSC-CN, DPA-6S
  Supel-Select HLB
• NP: DSC-Si, DSC-Diol, DSC-CN, DSC-NH₂
• IE: DSC-NH₂, DSC-SAX, DSC-WCX, DSC-SCX
• Mixed Mode: DSC-MCAX (C8 & SCX)
• Adsorption: ENVI-Carb, ENVI-ChromP, ENVI-Florisil
• Special Ag-Ion, PSA, ENVI-carbon/PSA, Na₂SO₄/Florisil
• MIP SupelMIPs - Highly selective SPE
• HybridSPE® Phospholipid removal from Plasma & Serum Samples