

Systematic SPE Method Development

A method development strategy leading to robust and reliable SPE methods



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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

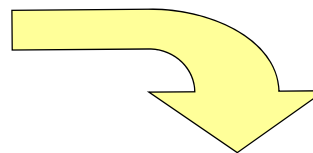
The Result:

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 **O**ptimized **S**olid phase extraction

or **S**electivity **P**rofiled **S**PE (**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”

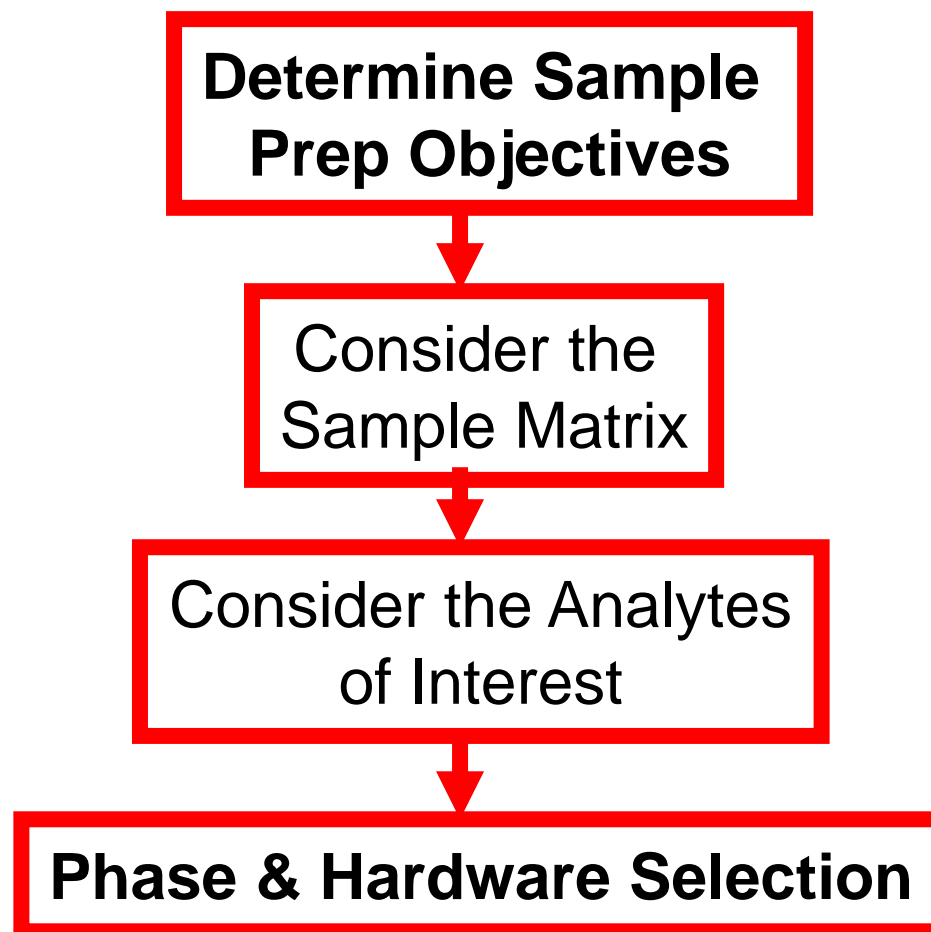
POS Idea:

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)

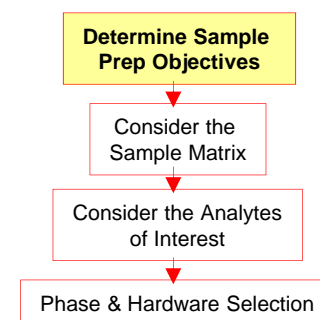
-> Greater Confidence and Efficiency

Profile Optimized SPE (POS) Method Development - Step1



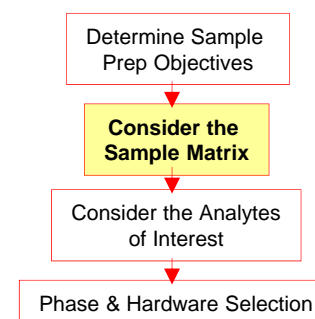
Determine Sample Prep Objectives

- 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

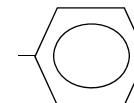
What functional groups may influence the analytes' solubility (Log P o/w), polarity, ionization state (pKa), etc.?

Hydrophilic Groups:

- Hydroxyl -OH
- Amino -NH₂
- Carboxyl -COOH
- Amido -CONH₂
- Guanidino -NH(C=NH)NH₃⁺
- 4° Amine -NR₃⁺
- Sulfate -SO₃⁻

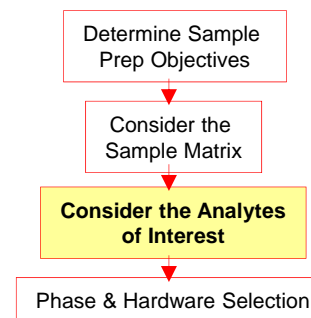
Hydrophobic Groups:

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



Neutral Groups:

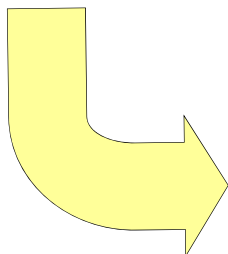
- Carbonyl -C=O
- Ether -O-R
- Nitrile -C=N



Phase & Hardware Selection

Summarizing the considerations

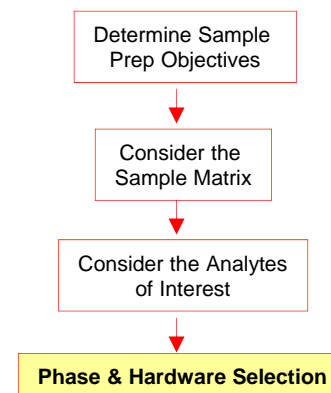
- *Sample Matrix*
- *Analyte of interest*



-> *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 0.25-1.0 $\mu\text{g}/\text{mL}$ Serum
- Post SPE **sample matrix** should be a buffered solvent **compatible with HPLC** mobile phase

Determine Sample Prep Objectives

Consider the Sample Matrix

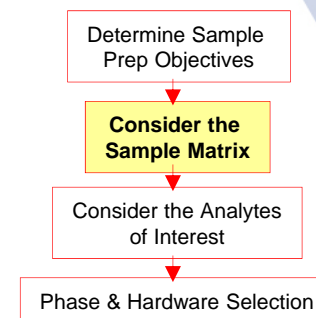
Consider the Analytes of Interest

Phase & Hardware Selection

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



POS Example: TCAs from Sheep Serum

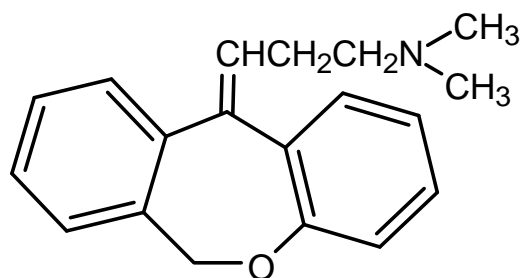
Determine Sample
Prep Objectives

Consider the
Sample Matrix

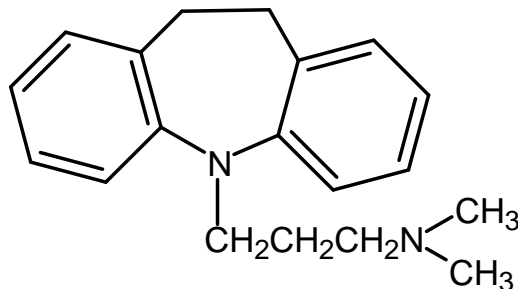
**Consider the Analytes
of Interest**

Phase & Hardware Selection

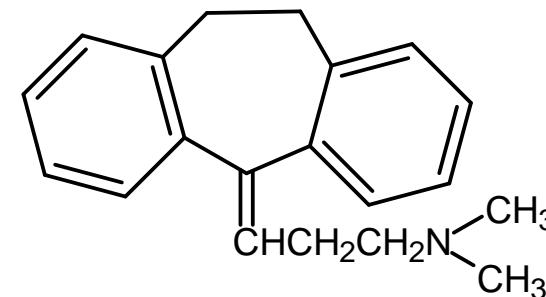
Consider the Analytes of Interest:



Doxepin



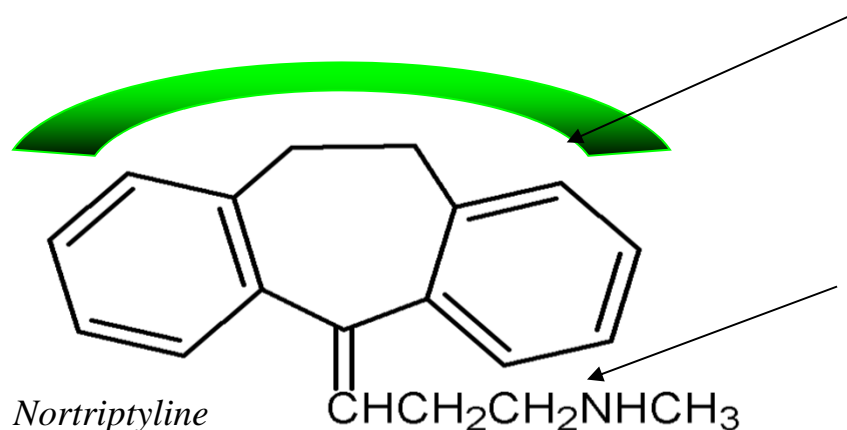
Imipramine



Amitrypytline

Tricyclic Antidepressants TCAs

POS Example: TCAs 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**:

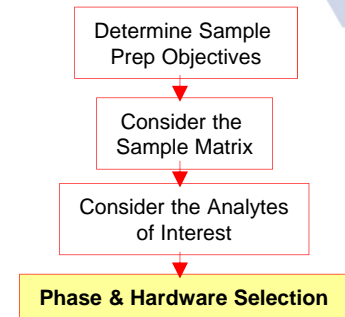
- At pH ≥ 11 , the 2° or 3° amine functional group should be **neutralized**.
- At pH ≤ 7 , the amine group should be **ionized**.

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

POS Example: TCAs from Sheep Serum

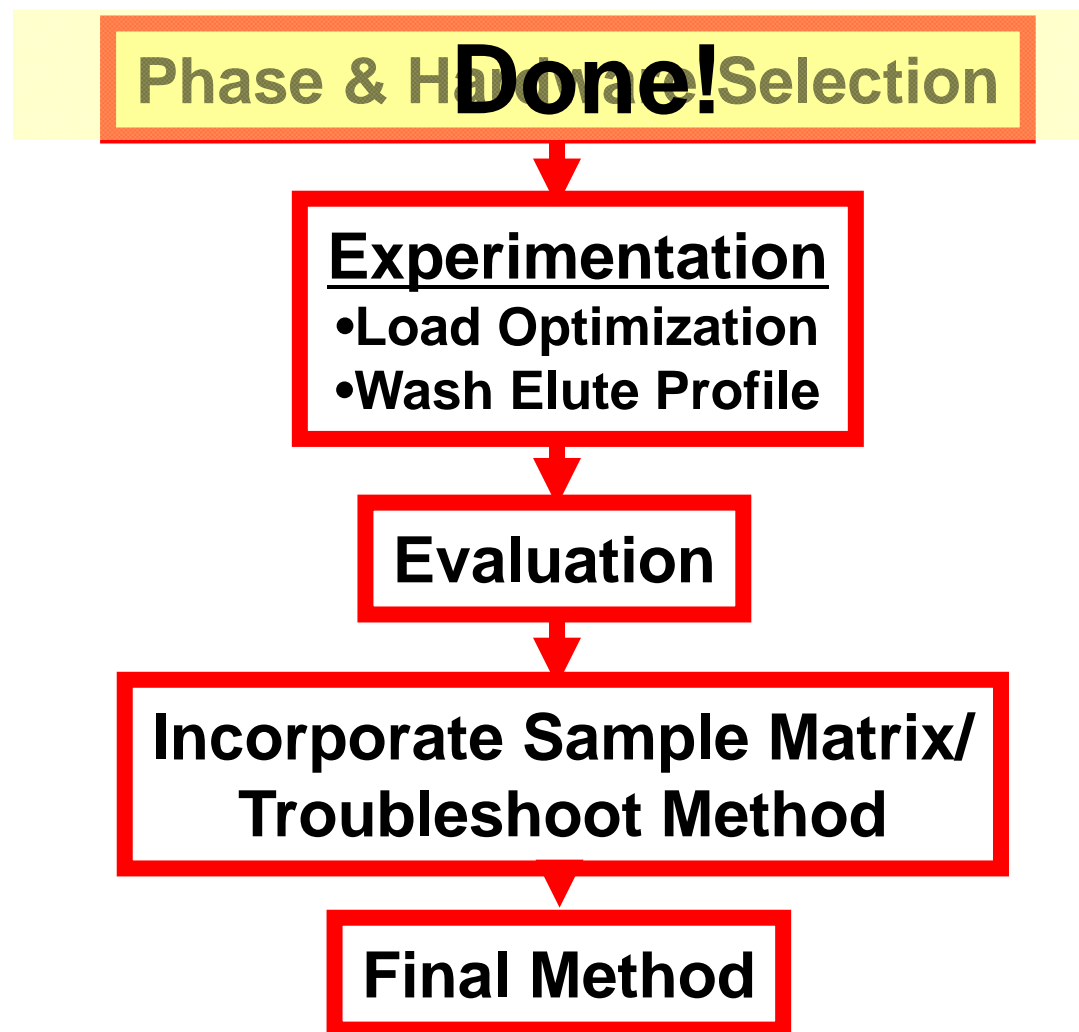
SPE Phase & Hardware Selection

- **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





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.

POS Example: TCAs from Sheep Serum

- Load Optimization

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

POS Example: TCAs from Sheep Serum

- Wash/Elute Profile

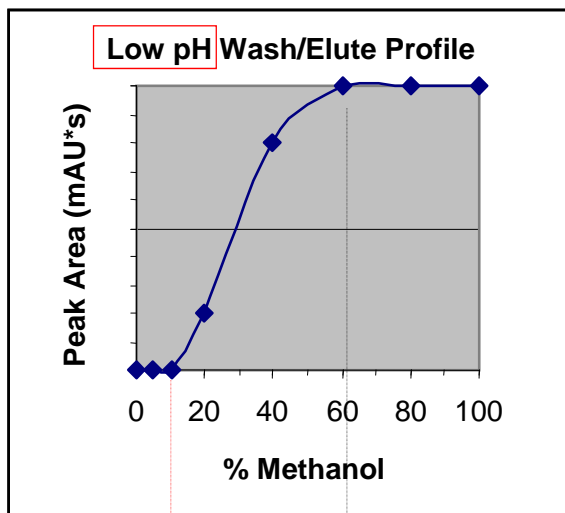
Determine analyte retention and elution patterns as a function of pH & %-Organic

1. Conditions DSC-18 wells with 1mL MeOH
2. Equilibrate DSC-18 wells with 1mL DI 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

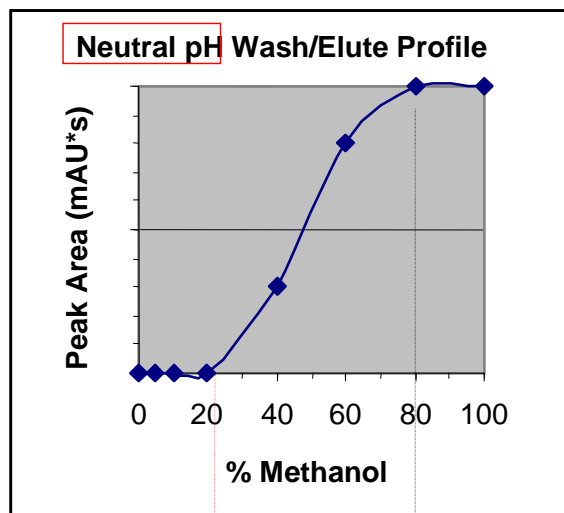
- Wash/Elute Profile

Evaluation



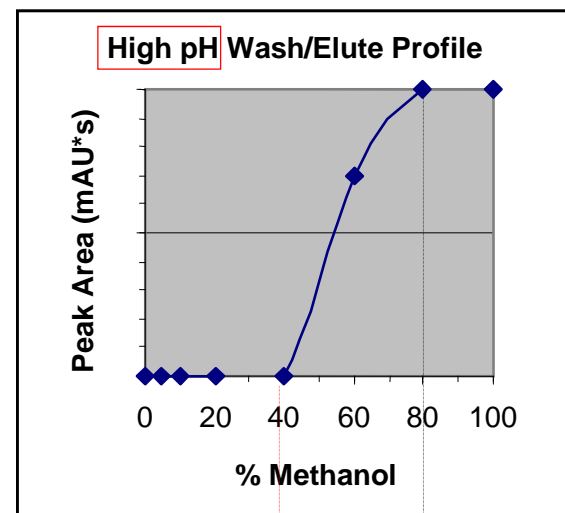
At **low pH**, complete elution occurs at 60% MeOH.

At **low pH**, retention limit is 10% MeOH.



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

At **neutral pH**, retention limit is 20% MeOH.



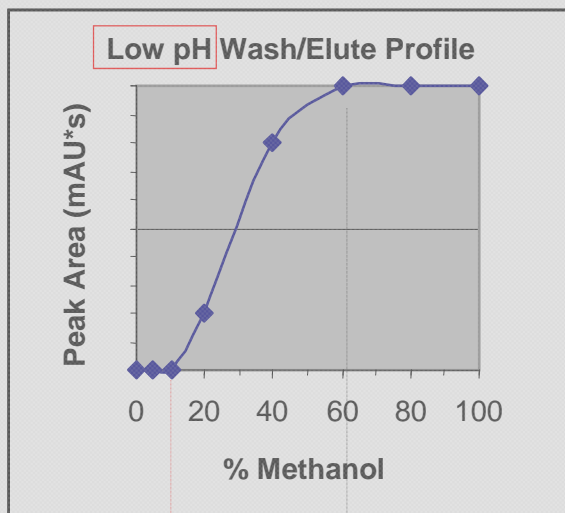
Under **basic pH**, complete elution occurs at 80% MeOH.

At **high pH**, retention limit is 40% MeOH.

POS Example: TCAs from Sheep Serum

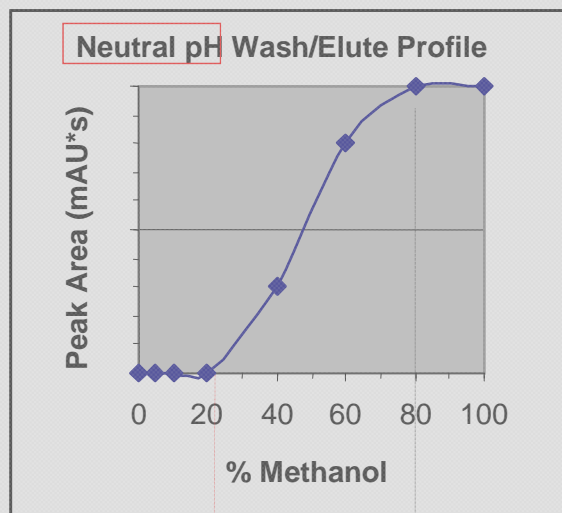
- Wash/Elute Profile

Evaluation



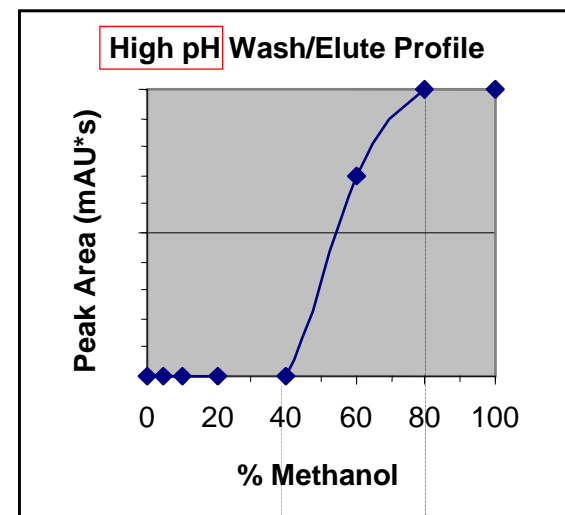
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At **neutral pH**, retention limit is 20% MeOH.



Under **basic pH**, complete elution occurs at 80% 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 it self



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



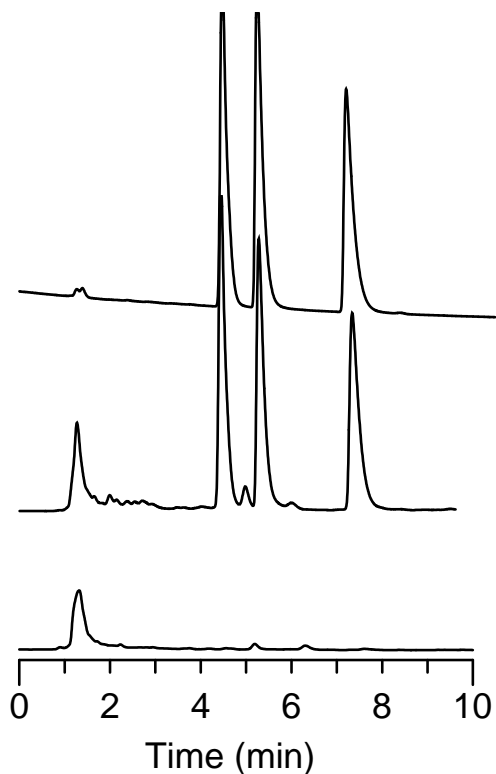
POS Method on DSC-18 Well Plate vs. Generic Method on Competitor Polymer Phase

Generic Method on Competitor Polymeric Phase (30mg/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 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



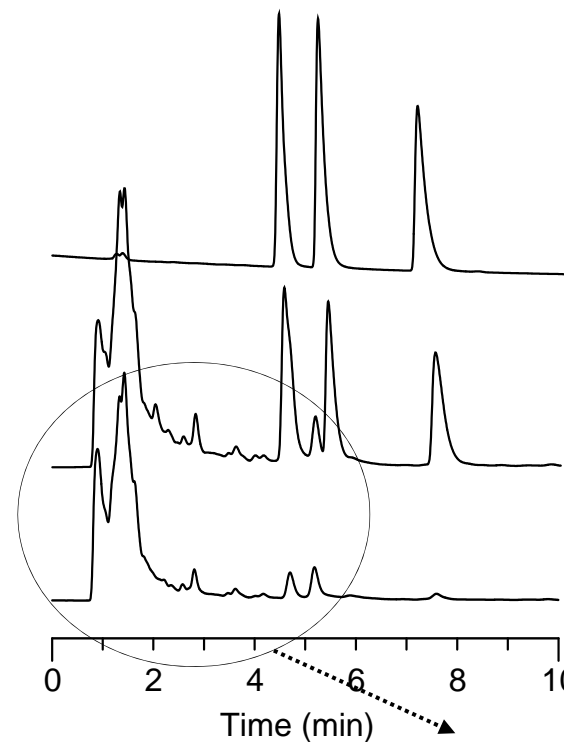
1. Doxepin
2. Imipramine
3. Amitriptyline

Ext. Stds

**1 µg/mL
Spiked serum**

Blank serum

Generic Method Using Competitor Polymeric Well plate



**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

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

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”

Bulletin 910

Guide to Solid Phase Extraction

| | |
|--|--------|
| Introduction | Page 1 |
| Phase Types Reversed phase packings Normal phase packings Ion exchange packings Adsorption packings | 2 |
| SPE Theory How compounds are retained by the sorbent Reversed phase SPE Normal phase SPE Ion exchange SPE Secondary interactions The role of pH in SPE | 3 |
| How to Use SPE Selecting the proper extraction scheme The five-step SPE method development process Sample pretreatment options - Liquid samples - Solid samples SPE hardware and accessories for processing samples | 6 |

Introduction
Solid phase extraction (SPE) is an increasingly used liquid/liquid extraction can be prevented, such as in breakable specialty glassware, and disposal of large yields quantitative extractions that are easy to perform. SPE is used most often to prepare liquid samples that are pre-extracted into solvents. SPE products exist in a wide variety of chemistries, adsorbents, and sizes.

SPE Method Development Process Overview

Determine Sample Prep Objectives:

- What level of recovery is required?
- What level of interference removal is required for analysis?
- Is concentration required for optimal analysis?
- In what solvent should the analyte(s) be in for optimal analysis?
- What resources are available for method development and routine analysis?

Consider Sample Characteristics (Matrix & Analytes):

- What is the sample matrix and is it more polar or non-polar?
- What is the sample volume?
- What interferences are endogenous to the sample?
- What functional groups may influence the analytes' solubility, polarity, ionization state (pKa), etc.?

Select retention mechanism, phase chemistry, bed weight and hardware configuration:

- What phase chemistry, bed weight and hardware configuration best meets the predefined sample prep objectives for the sample characteristics?

Develop Analytical Method- Load Optimization & Wash/Elution Profile:

Experimentation:

- Develop analysis method (LC-UV, GC-MS, etc.)
- Using standards and buffered/organically modified solutions, identify and determine the effects of key variables (pH, organic strength, etc.) that influence analyte retention and elution.

Evaluation:

- 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:

- Determine preliminary, optimized method and incorporate sample matrix.
- Make determinations of sample matrix effect on sample recovery, cleanliness, reproducibility, and analytical (LC or GC) resolution
- Refine optimum conditions to account for sample matrix effects

Validate Method

We are committed to the success of our Customers, Employees and Shareholders through leadership in Life Science, High Technology and Service.

SUPELCO

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Technical Report

Systematic SPE Method Development

Comparison of a systematically developed method using Discovery DSC-8 SPE 96-Well Plate vs. a generic method using conventional C18 for the extraction and HPLC analysis of diazepam and its three major metabolites from goat serum

Authors: An Trinh, Product Manager, Liquid Separations, Dave Bell, HPLC Applications Group, Supelco, Bellefonte, PA

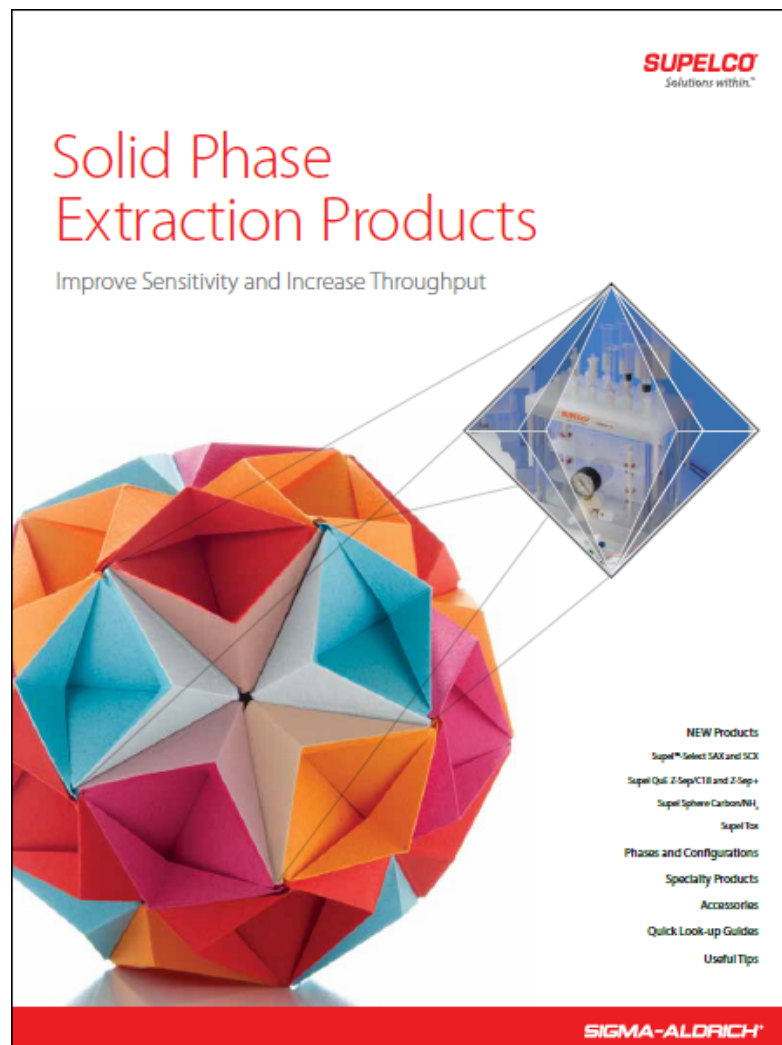
C18 has become the most commonly used phase chemistry for reversed-phase SPE due to its broad selectivity. However, there can be disadvantages to using C18 for some applications. Its higher hydrophobicity can lead to over retention of the analytes potentially leading to poor recovery/reproducibility from incomplete elution. For such applications, elution typically requires the use of stronger and/or larger volumes of solvent. The final eluate must then be evaporated and reconstituted with a solution suitable for LC-resolution and analysis. This prolongs and adds additional steps to the extraction procedure.

DSC-8 contains a monomerically bonded octyl chain with approximately half the carbon content of most C18 phases. Its less retentive nature allows for the rapid release of hydrophobic molecules using weaker eluents. Using the SPE method development approach illustrated in this report, a simple and highly selective extraction method using Discovery DSC-8 SPE 96-Well Plates was developed to recover diazepam and its three major metabolites from goat serum. When compared to a generic method using a conventional C18 phase, the systematic SPE method development approach provided a simpler method eliminating the final SPE eluate evaporation and reconstitution steps typical of most reversed-phase SPE procedures. Recoveries for the four compounds ranged from 90.0-99.9%, and RSDs were less than 3.5% for the 0.5µg/mL spike level tested.

SPE Brochure

- T402150 (FEB)
- 36 pages

- Complete list of SPE products and accessories



Thank You!!!



DISCOVERY & 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