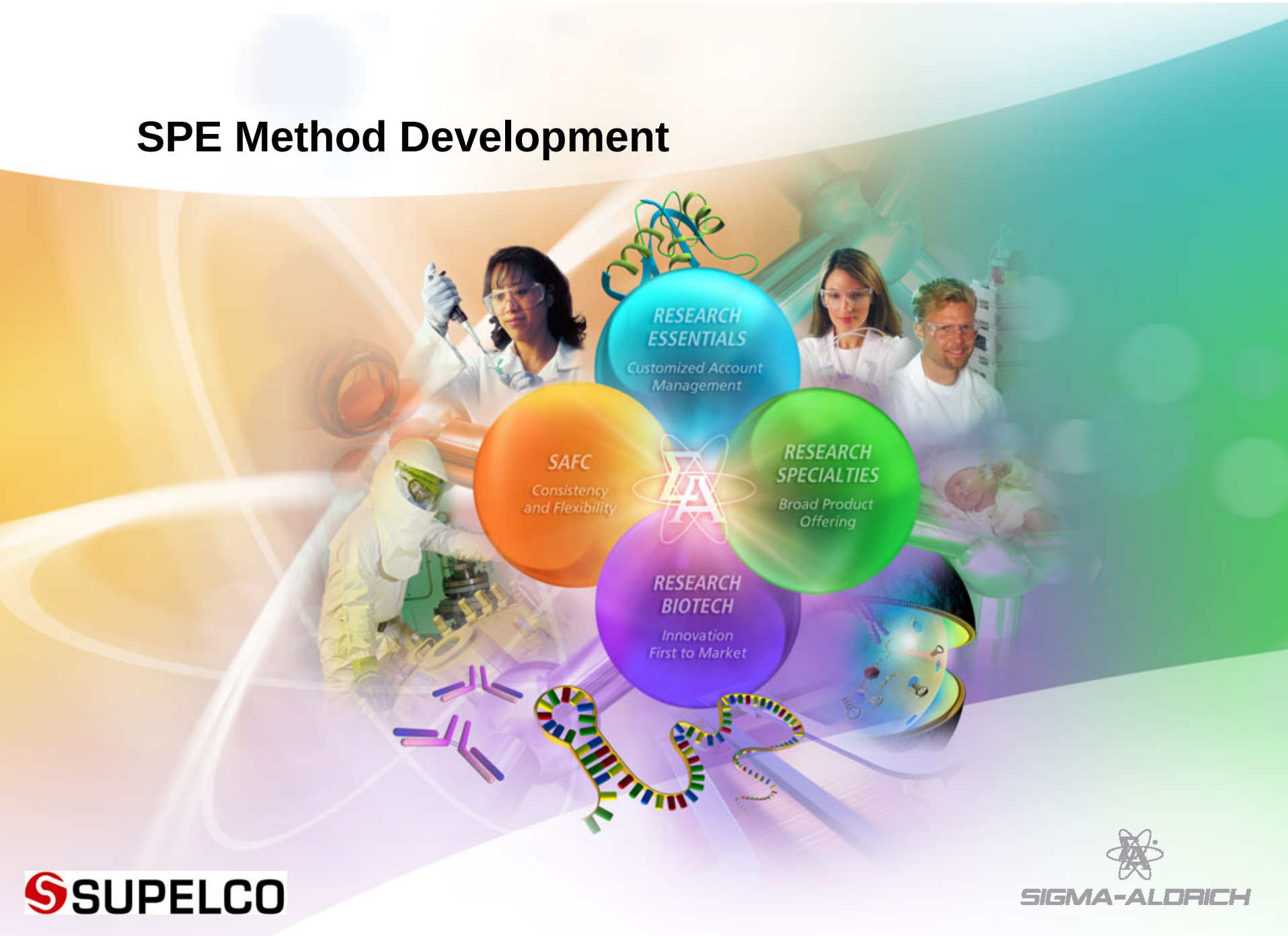
SPE Method Development



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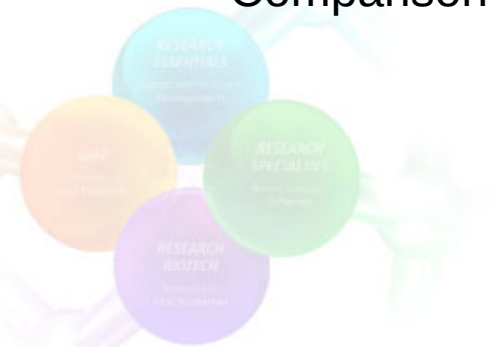
Presentation Outline

- SPE
  - Common Method Development Strategies
  - Problems with these
- Profile Optimized SPE (POS) Method Development
  - POS Example: Tricyclic Antidepressants from Sheep Serum
  - Comparison of POS Method vs. Generic Method



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SPE Basics - The Process

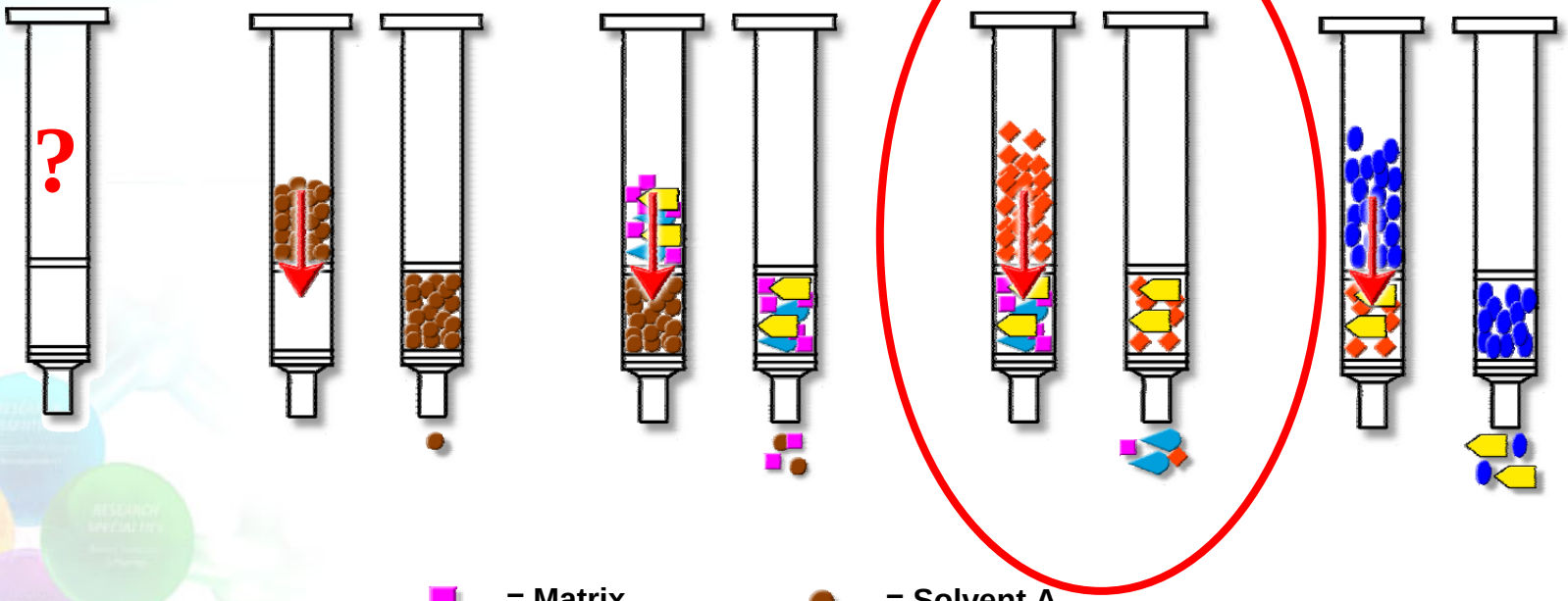
Step 1.
Select the Proper SPE Tube or Disk

Step 2.
Condition the Tube or Disk

Step 3.
Add the Sample

Step 4.
Wash the Packing

Step 5.
Elute the Compounds of Interest



= Matrix
= Impurity
= Compound of interest

= Solvent A
= Solvent B
= Solvent C

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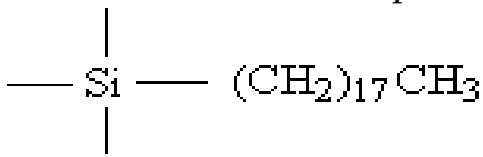
SPE Phases

Reversed Phase

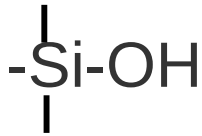
DSC-18, DSC-18Lt, DSC-8, DSC-Ph, DSC-CN, DPA-6S (Polyamide resin)

Examples



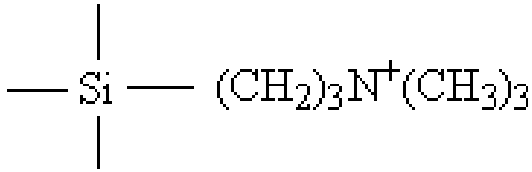
Normal Phase

DSC-Si, DSC-Diol, DSC-CN, DSC-NH_2



Ion Exchange

DSC-NH_2, DSC-SAX, DSC-WCX, DSC-SCX, PSA

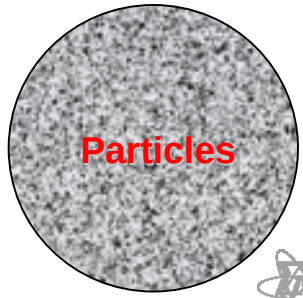


Mixed Mode

DSC MCAX (mixed mode C8 & SCX)

Adsorption

ENVI-Carb, ENVI-Florisil, ENV-Chrom P



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Basic Rules of Solid Phase Extraction

- Analyte must adsorb onto the SPE Sorbent
- There must be sufficient resident time for analyte
- Selectively remove 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 the **recovery** high enough?

Is the product/method yielding **reproducible** given good results?

Is the sample **clean** enough for analysis?

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How method development is often approached

Incorporate the sample matrix or real samples immediately and…

- Duplicate an existing or 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

Possible Problems

- Novel Analytes – Behavior different

- 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)

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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 to:

- **Select Hardware and Phase**
- **Understand the Analyte/Sorbent Interaction for optimal Conditions**
- **Systematically adjust 2 main Variables (organic strength & pH)**

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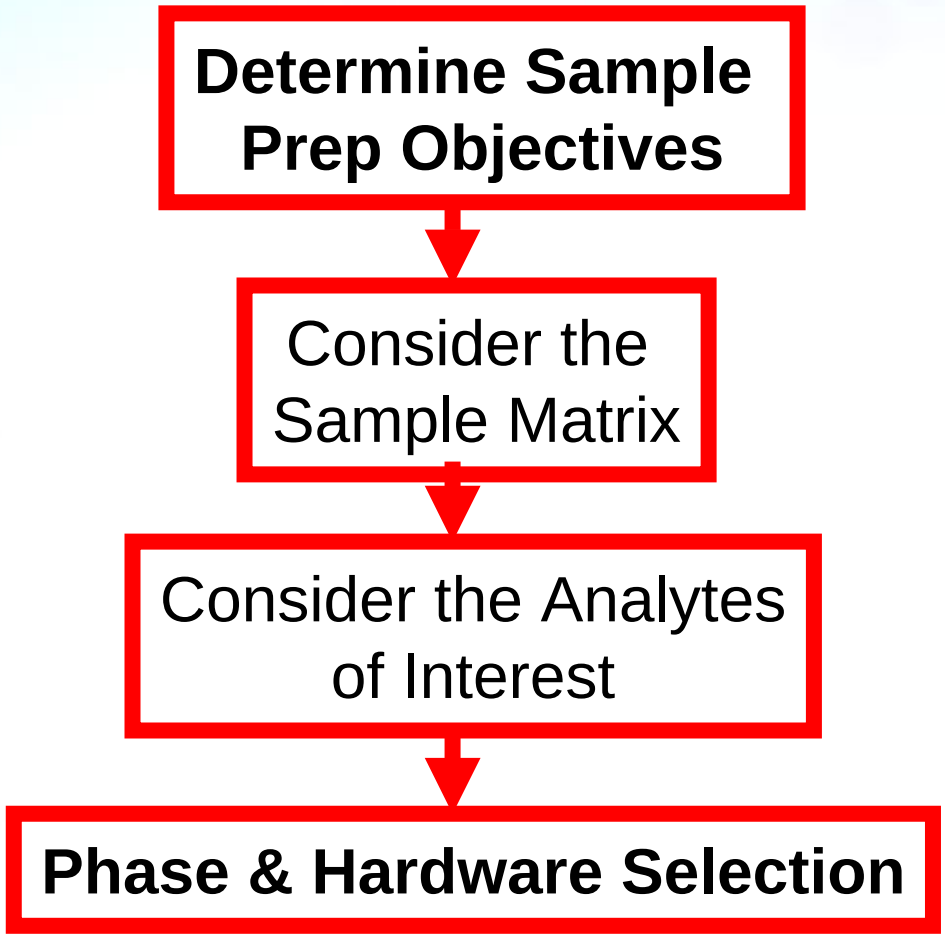
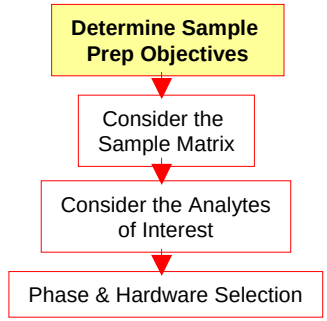
Profile Optimized SPE (POS) Method Development Step1



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.)?



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Consider the Sample Matrix

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

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

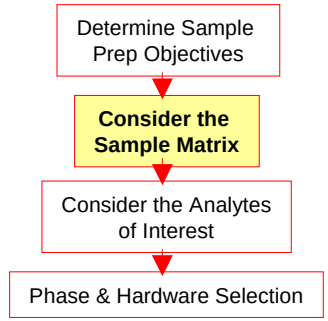
- **Is the sample matrix more polar or non-polar?**

  - **Serum, Plasma, Urine = Polar**

    → Reversed-Phase or Ion-Exchange

  - **Organic synthesis reactions or extractions = Non-Polar**

    → Normal-Phase



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Consider the analyte(s) of interest

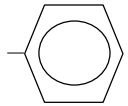
What functional groups may influence the analytes' solubility (Log P o/w), polarity, ionization state (pKa), etc.?

Hydrophilic Groups:

- **Hydroxyl** **-OH**
- **Amino** **$-NH_2$**
- **Carboxyl** **-COOH**
- **Amido** **$-CONH_2$**
- **Guanidino** **$-NH(C=NH)NH_3^+$**
- **4° Amine** **$-NR_3^+$**
- **Sulfate** **$-SO_3^-$**

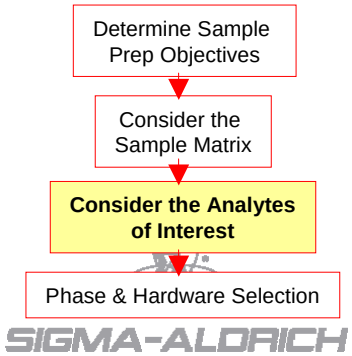
Hydrophobic Groups:

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



Neutral Groups:

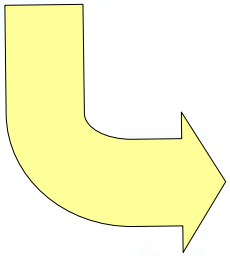
- **Carbonyl** **-C=O**
- **Ether** **-O-R**
- **Nitrile** **-C=N**



Phase & Hardware Selection

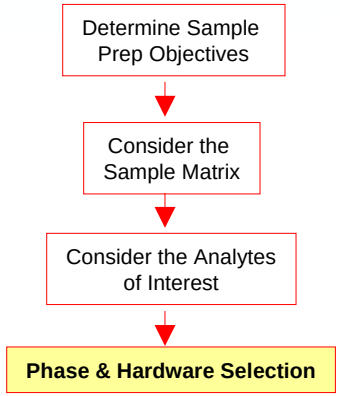
Summarizing:

- *Sample Matrix*
- *Analyte of inertest*



-> Choose most ideal

- Retention Mechanism,
- Phase Chemistry
- Hardware Configuration



Real Application Example of TCAs in Sheep Serum →

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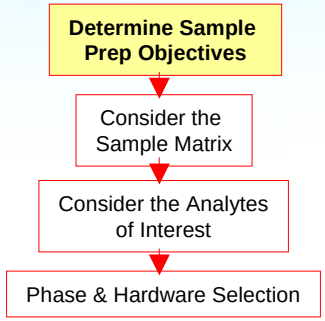
POS Example: Tricyclic Antidepressants (TCAs) from Sheep Serum



Determine Sample Prep Objectives:

- Develop a simple extraction procedure
- Achieve ≥ 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 limits of 0.25-1.0µg/ml Serum
- Post SPE sample matrix should be a buffered solvent compatible with HPLC mobile phase

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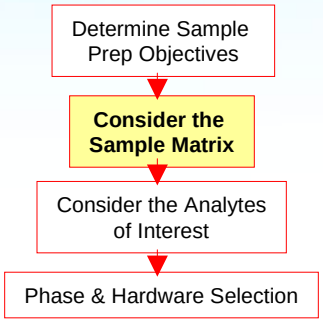
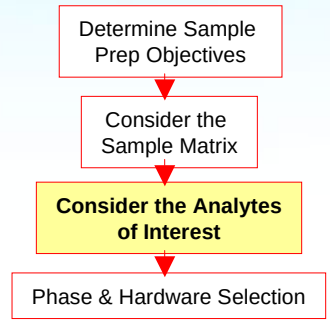
POS Example: TCAs from Sheep Serum



Consider the Sample Matrix:

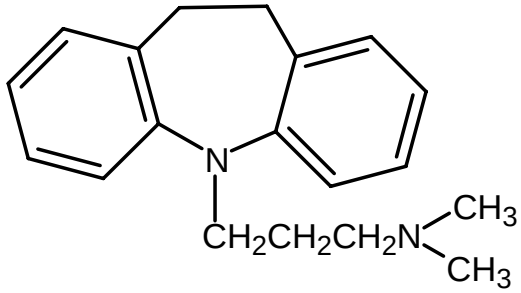
- Sample Volume 0.5 mL Sheep Serum
- Serum is the aqueous portion of blood = Polar
  - Platelets, corpuscles, and clotting factors have been removed
- Endogenous Interferences:
  - albumin, globulins, lipids, salts and carbohydrates

POS Example: TCAs from Sheep Serum

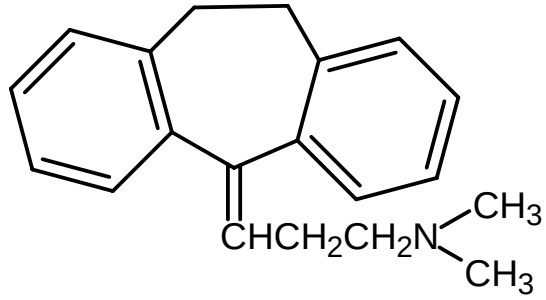


Consider the Analytes of Interest:

Doxepin



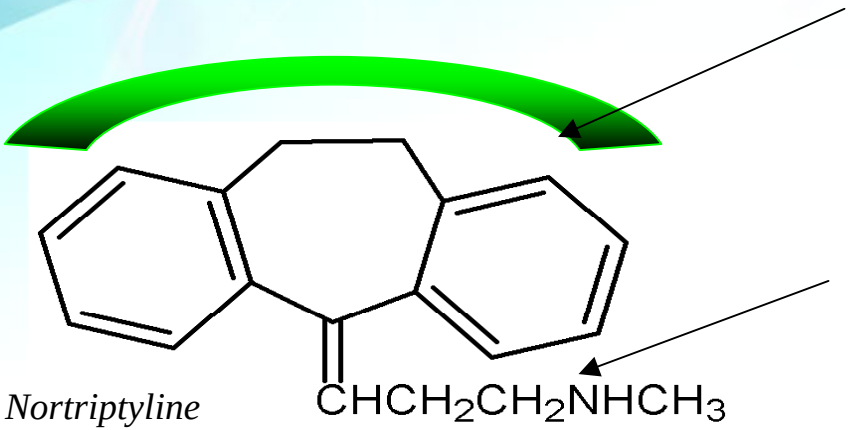
Imipramine



Amitrypityline

Tricyclic Antidepressants TCAs

POS Example: TCAs from Sheep Serum



Nortriptyline

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

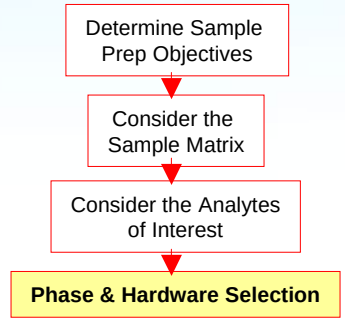
2° amine: basic functional group w/a pKa of ~9. Very useful for controlling analyte's ionization state:

- **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 eluants = Maximize Sample Clean-Up**

1st Choice = Discovery DSC-18 SPE-96 Well Plate

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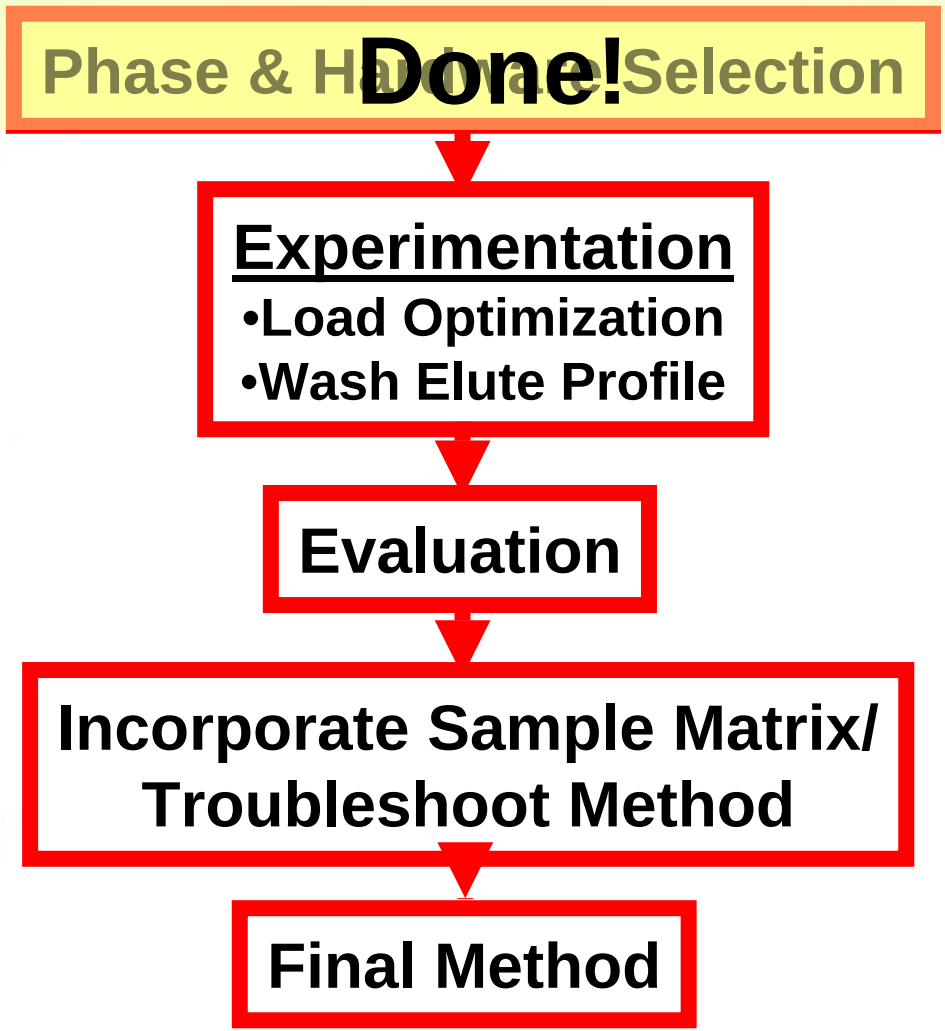
Profile Optimized SPE (POS) Method Development
Step 2



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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

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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 analyt´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 H_2O
3. Load 1mL 5µg/mL* standard test mix prepared at neutral (DI H_2O) and basic pH (2% NH_4OH).
4. Collect Load 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 load 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

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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 H_2O
3. Load 1mL 5µg/mL standard test mix prepared at basic pH (2% NH_4OH).
4. **Wash/Elute with 1mL of a test solvent ranging from 0-100% MeOH in 2% CH_3COOH (low pH), DI H_2O (neutral pH), and 2% NH_4OH (high pH)**
5. **Collect wash/elute eluate and analyze via HPLC-UV**

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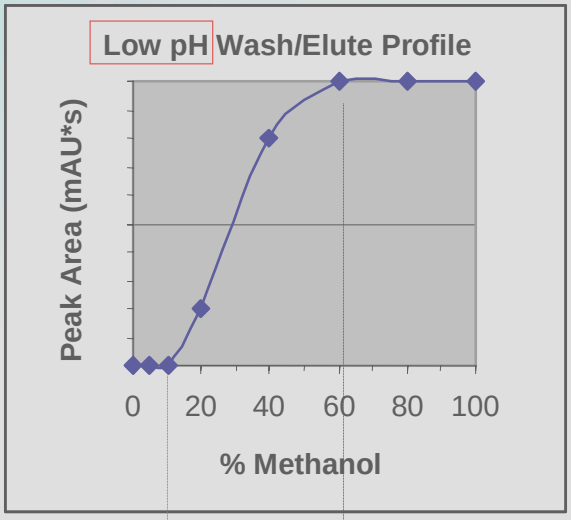
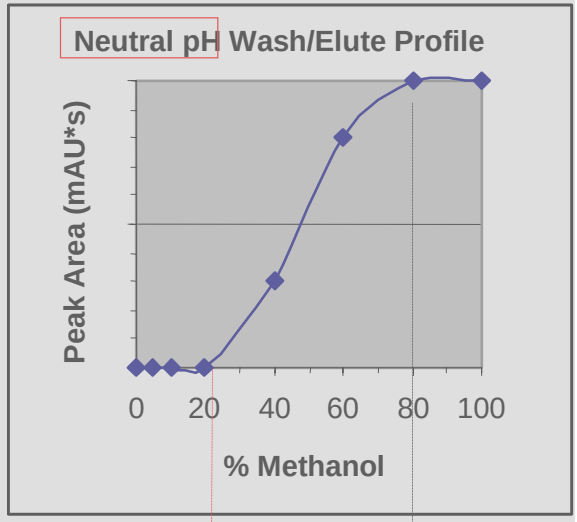
POS Example: TCAs from Sheep Serum
- Wash/Elute Profile

Evaluation



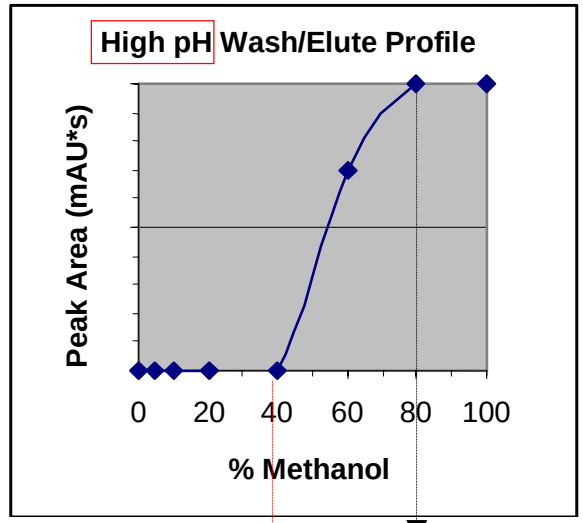
At low pH, complete elution occurs at 60% MeOH.

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.

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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 H_2O
2. Load 0.25-2.0µg/mL TCAs spiked in sheep serum diluted in 2% NH_4OH (1:1, v/v); n=3 for ea. concentration
3. Wash w/ 1mL *40% MeOH* in 2% NH_4OH
4. Elute w/ 1mL MeOH
5. Evaporate eluate with N-purge (30°C; ~10min.), and reconstitute in 300µL MP

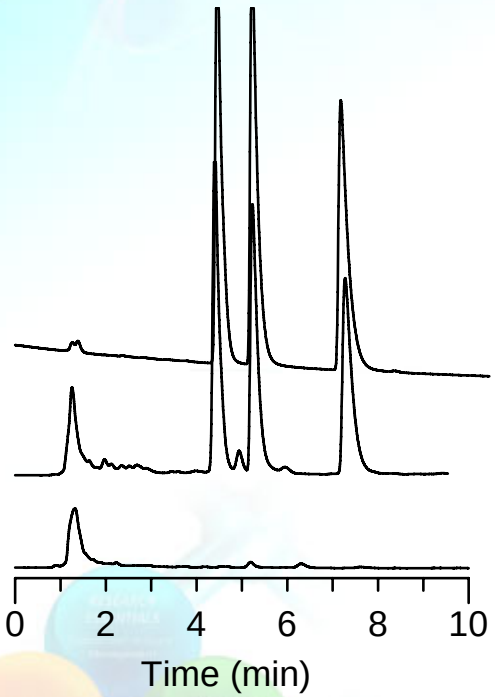
POS Method on DSC-18 Well Plate vs. Generic Method on Competitor Polymer Phase

Generic Method on Competitor Polymeric Phase (30mg/well):

1. Condition/Equilibrate w/ 1mL MeOH & 1mL DI H_2O
2. Load 0.25-2.0µg/mL TCAs spiked in sheep serum diluted in 2% NH_4OH (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

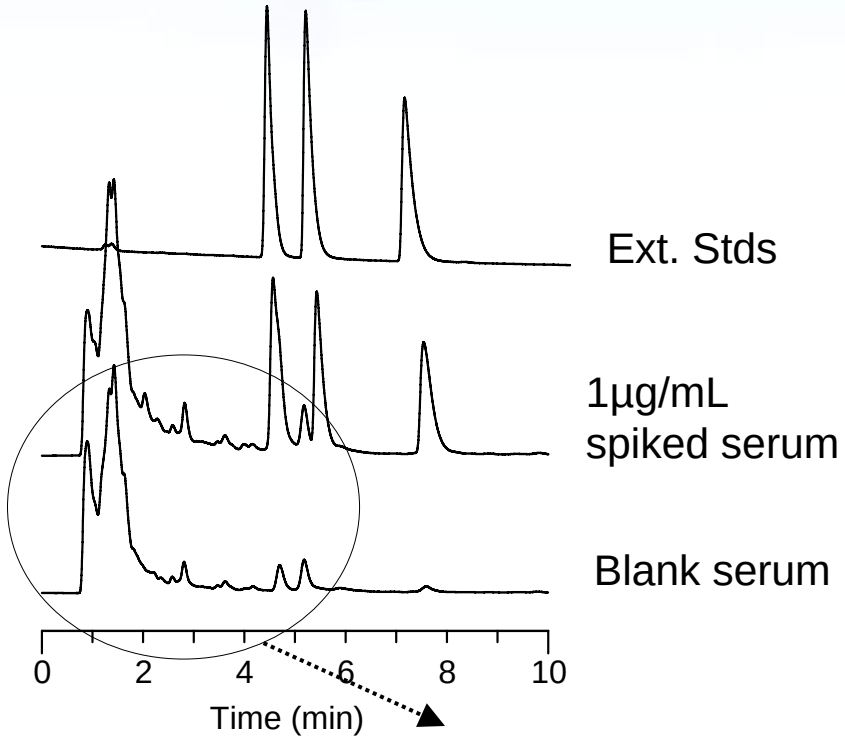
Results

POS Method Using DSC-18 SPE-96 Well plate



1. Doxepin
2. Imipramine
3. Amitryptyline

Generic Method Using Competitor Polyermic Well Plate



HPLC Method:

Column:	**Discovery C18**, 15cmx4.6mm, 5µm, & 2cm guard column & 0.5µm frit filter;
Mobile Phase:	MeCN: 25mM KH_2PO_4, pH 7 (45:55);
Flow Rate:	1.4mL/min; Temp: 30°C;
Det.:	UV, 254nm; **Inj:** 100µL

High Background; Misleading interfering responses

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

Phase and Hardware

- Pre-testing of the right hardware set-up and considering sample matrix and analytes of interest

→ strongest candidate for SPE method development

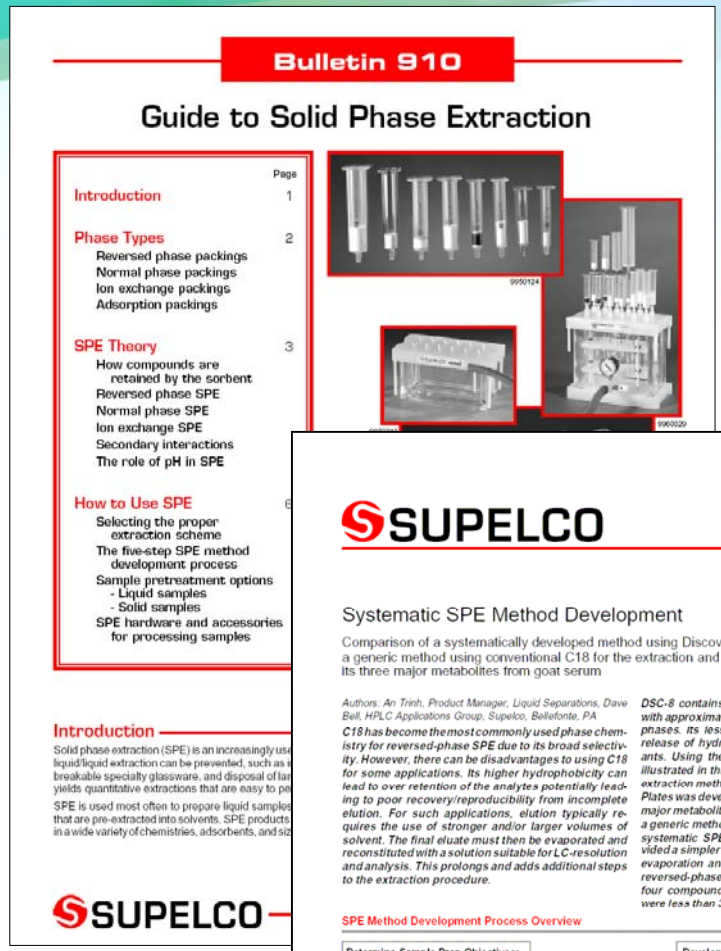
Parameters

- Use Standards and different influencing for testing the Analyte Retention and Elution,
- Strategically manipulate and make adjustments to the Extraction Method

SPE - Literature

Further
Method Development Aids

Bulletin 910
“Guide to Solid Phase Extraction”


Bulletin 910

Guide to Solid Phase Extraction

Introduction

Solid phase extraction (SPE) is an increasingly use
liquid/liquid extraction can be prevented, such as i
breakable specialty glassware, and disposal of lar
yields quantitative extractions that are easy to pe

SPE is used most often to prepare liquid samples
that are pre-extracted into solvents. SPE products
in a wide variety of chemistries, adsorbents, and siz

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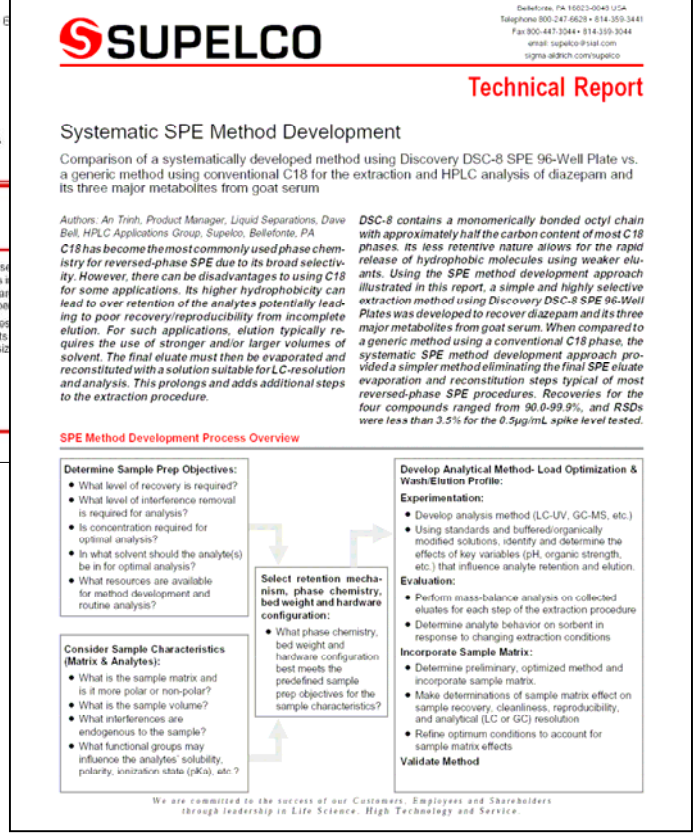
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Technical Report

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

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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 eluants. 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 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

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“Systematic SPE Method Development”