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 <u>immediately</u> 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

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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-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
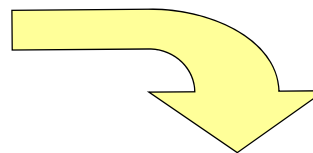
Solid Phase Extraction

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?

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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?

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How to solve the Problems?

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

- <u>Systematic approach</u>

  $\Rightarrow$ **POS**

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

  or **S**electivity **P**rofiled 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 w/ Standards to

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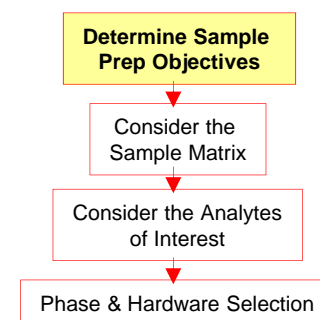- Systematically adjust 2 main Variables (organic strength & pH)

-> Greater Confidence and Efficiency

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Profile Optimized SPE (POS) Method Development - Step1

Determine Sample Prep Objectives

↓

Consider the Sample Matrix

↓

Consider the Analytes of Interest

↓

Phase & Hardware Selection

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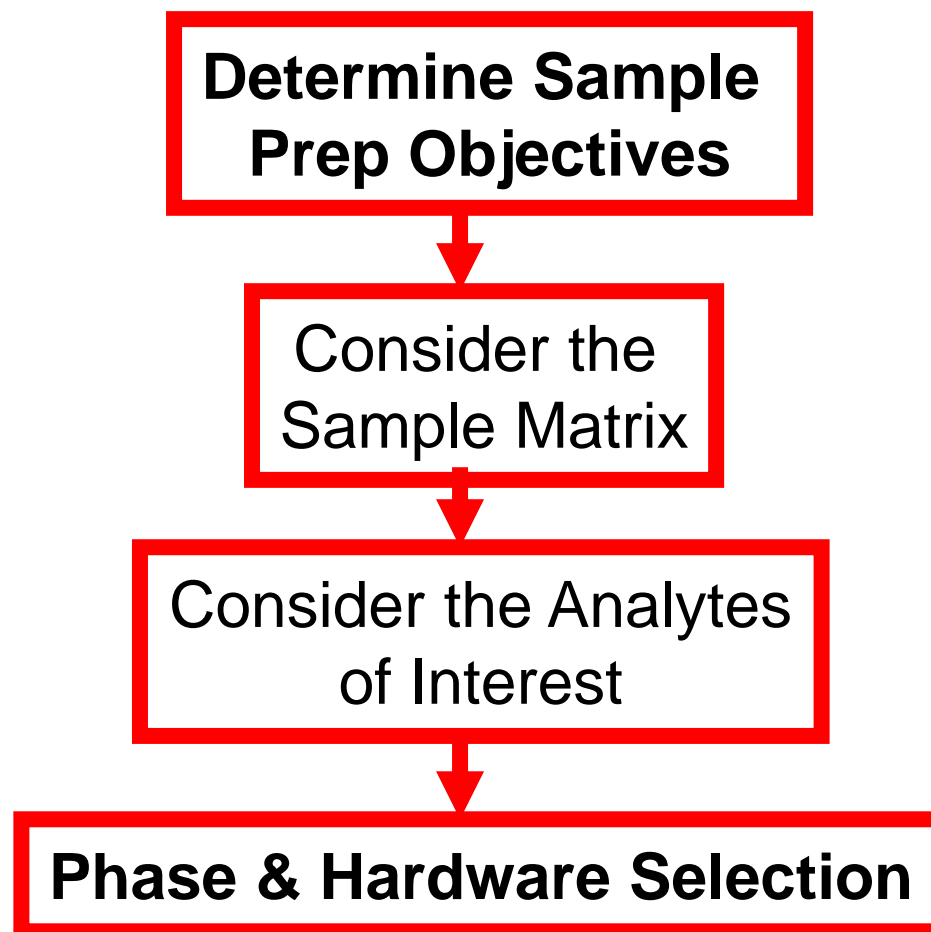
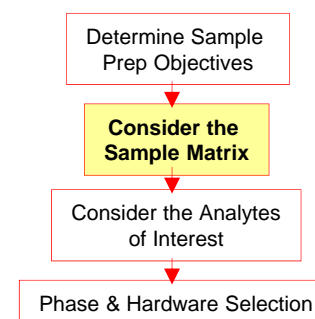# Determine Sample Prep Objectives

- 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.)?

Determine Sample Prep Objectives

↓

Consider the Sample Matrix

↓

Consider the Analytes of Interest

↓

Phase & Hardware Selection

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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**

Determine Sample Prep Objectives

Consider the Sample Matrix

Consider the Analytes of Interest

Phase & Hardware Selection

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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
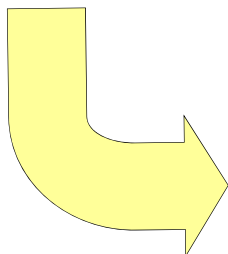- Carbon-Halogen   -C-Cl
- Olefin   -C=C
- Aromatic
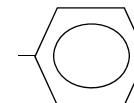
Neutral Groups:
- Carbonyl   -C=O
- Ether   -O-R
- Nitrile   -C=N

Determine Sample Prep Objectives

Consider the Sample Matrix

Consider the Analytes of Interest

Phase & Hardware Selection

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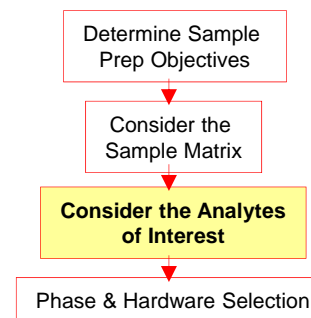
Phase & Hardware Selection

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!*
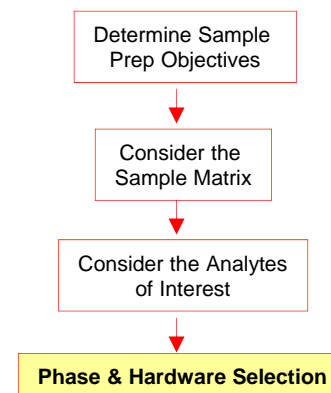
Here's an example: TCAs from Serum

Determine Sample
Prep Objectives

Consider the
Sample Matrix

Consider the Analytes
of Interest

Phase & Hardware Selection
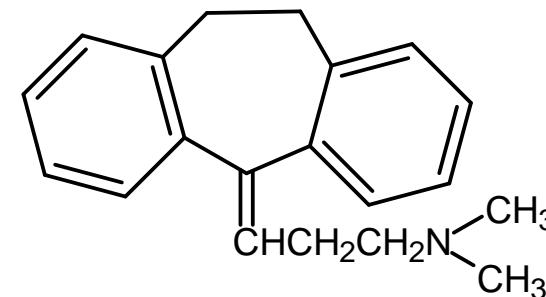
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POS Example: Tricyclic Antidepressants (TCAs) from Sheep Serum

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

Determine Sample Prep Objectives

↓

Consider the Sample Matrix

↓

Consider the Analytes of Interest

↓

Phase & Hardware Selection

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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
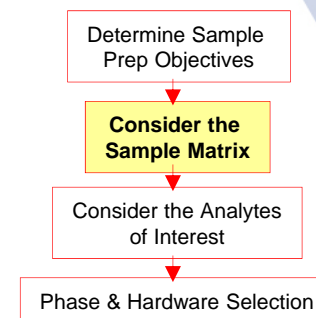
Determine Sample
Prep Objectives

↓

**Consider the
Sample Matrix**

↓

Consider the Analytes
of Interest

↓

Phase & Hardware Selection

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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:
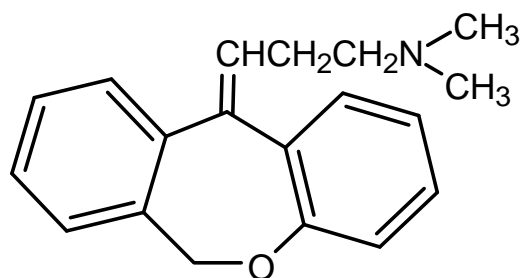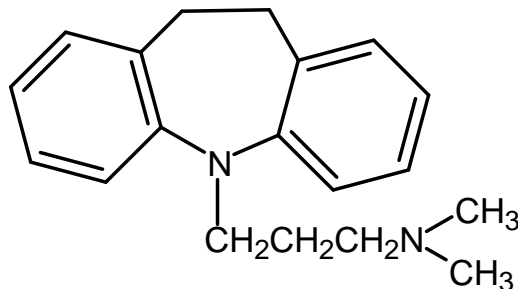
Doxepin　　　　　**Imipramine**　　　　**Amitrypityline**

Tricyclic Antidepressants TCAs

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POS Example: TCAs from Sheep Serum
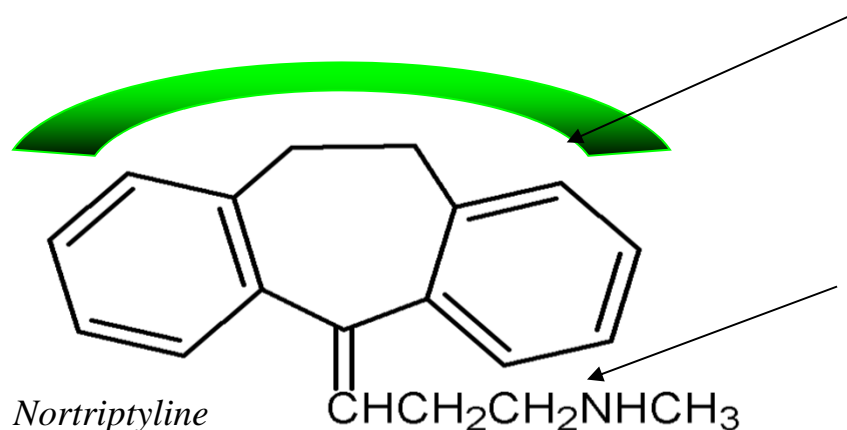
Dibenzocycloheptene skeleton = excellent hydrophobic foot print for potential reversed-phase interaction.

Nortriptyline

CHCH₂CH₂NHCH₃

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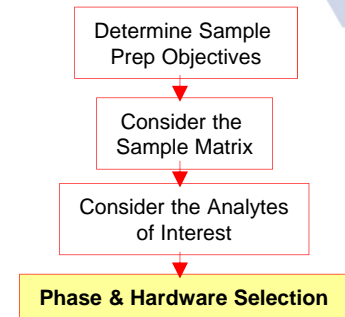
1st Choice = Discovery DSC-18 SPE-96 Well Plate

Determine Sample
Prep Objectives

Consider the
Sample Matrix

Consider the Analytes
of Interest

Phase & Hardware Selection

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Profile Optimized SPE (POS)
Method Development Step 2

Phase & Hardware Selection

Done!

Experimentation
- Load Optimization
- Wash Elute Profile

Evaluation

Incorporate Sample Matrix/ Troubleshoot Method

Final Method

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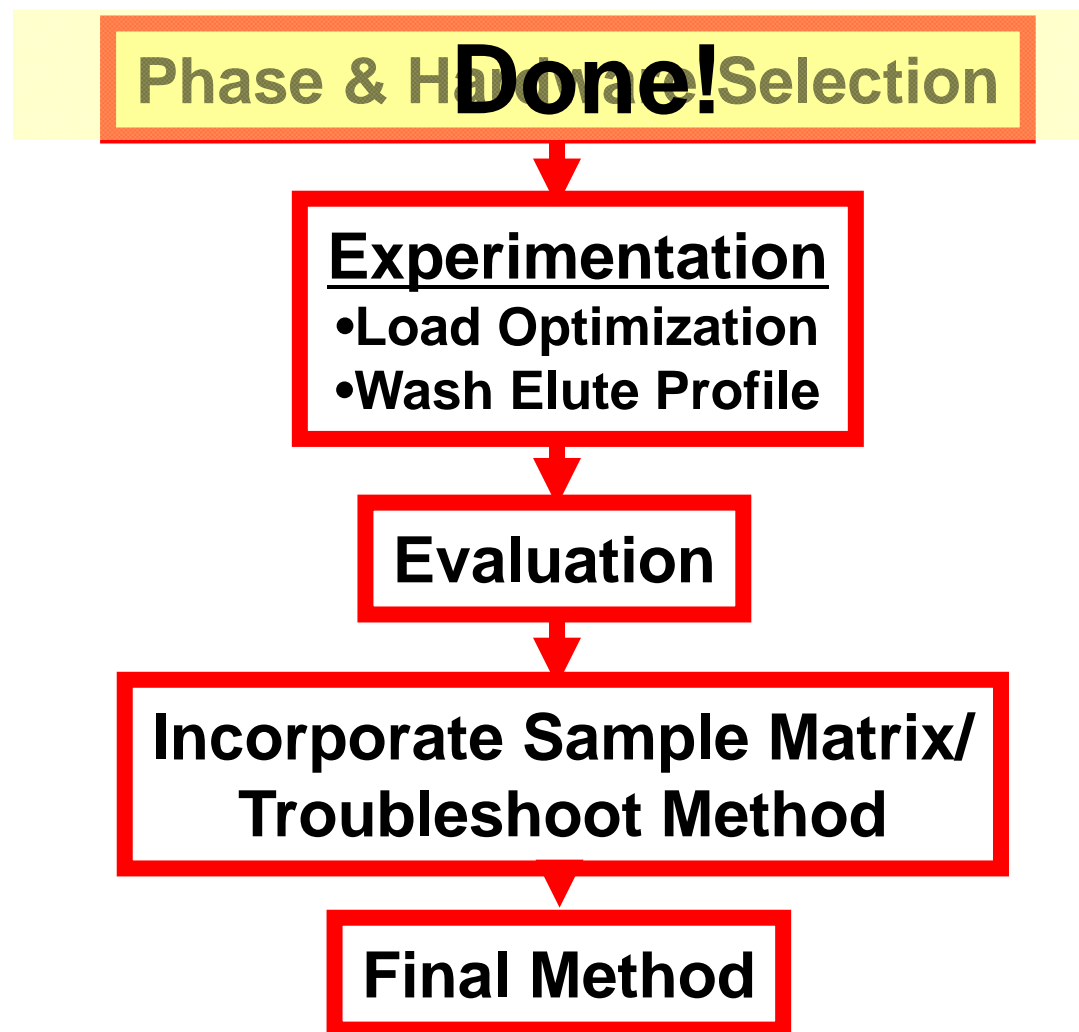
Experimentation, Evaluation, Incorporate Sample Matrix & Troubleshoot Method

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 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

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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 (1% NH_4OH).

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.

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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

$\Rightarrow$ 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 (1% 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

Low pH Wash/Elute Profile

Peak Area (mAU*s) vs % Methanol

Neutral pH Wash/Elute Profile

Peak Area (mAU*s) vs % Methanol

High pH Wash/Elute Profile

Peak Area (mAU*s) vs % Methanol

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.

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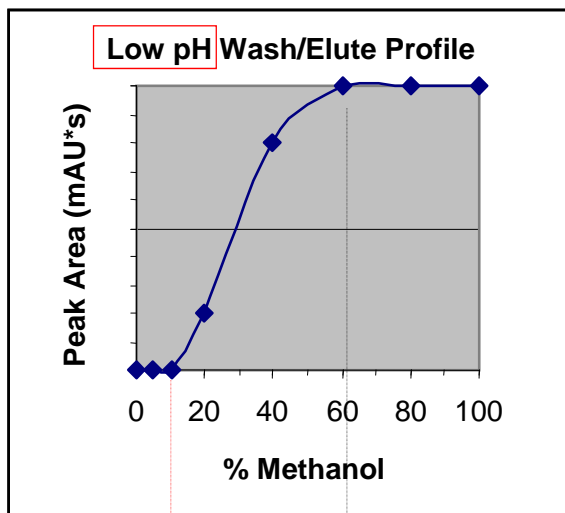
POS Example: TCAs from Sheep Serum
- Wash/Elute Profile

Evaluation

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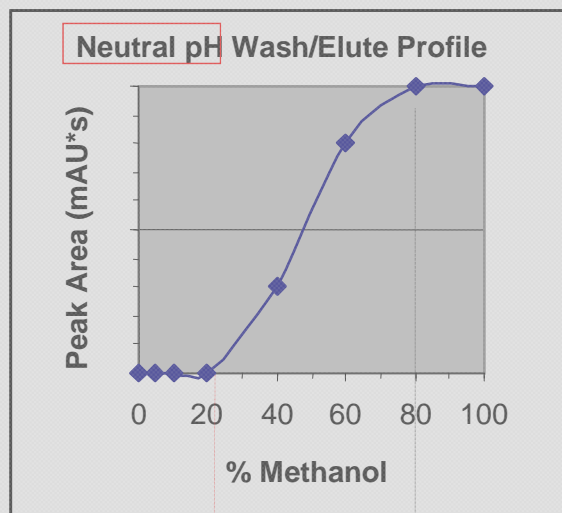
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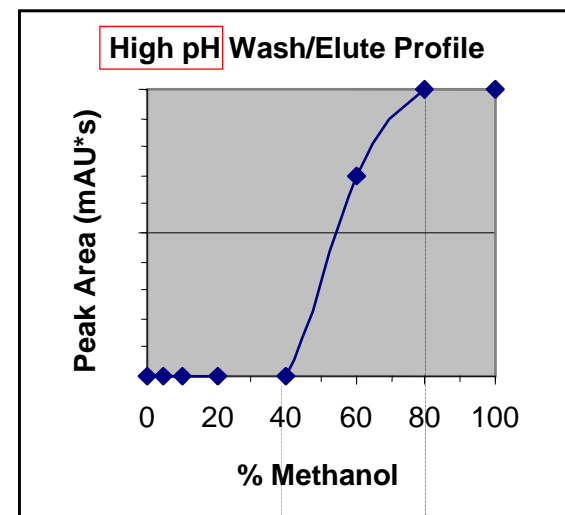
Rule of Thumb

"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

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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

* 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

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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

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Results

POS Method Using
DSC-18 SPE-96 Well plate

Generic Method Using
Competitor Polymeric Well plate
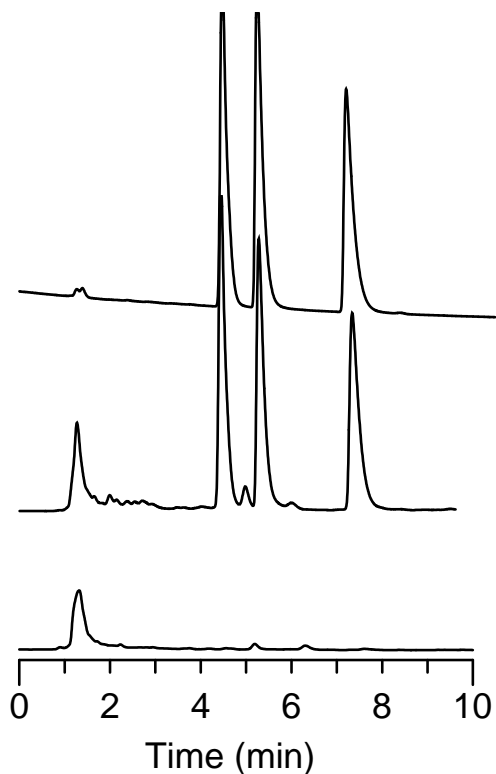
1. Doxepin
2. Imipramine
3. Amitryptyline

Ext. Stds

**1µg/mL
Spiked serum**

Blank serum

Time (min)

Time (min)
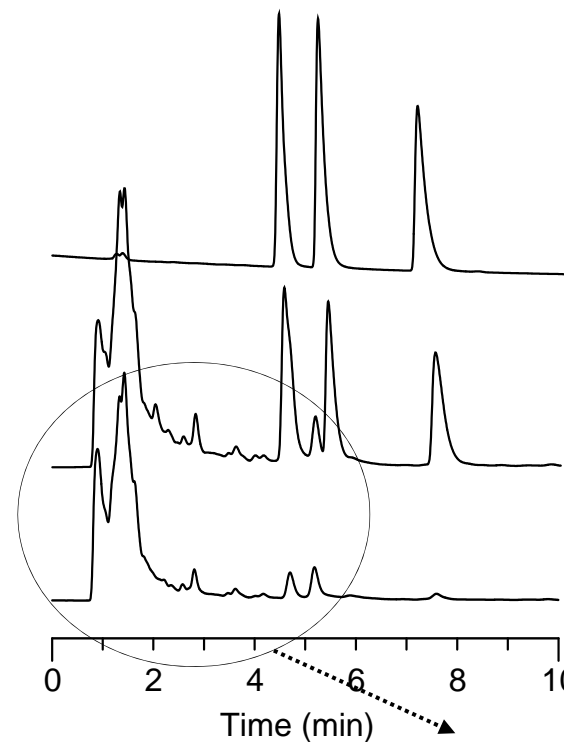
**High Background;
Misleading interfering
responses**

<u>**HPLC Method:**</u>
Column: **Discovery C18**, 15cmx4.6mm, 5µm, & guard column & 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

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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%

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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

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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

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SPE - Literature

Further
Method Development Aids

Bulletin 910
"Guide to Solid Phase Extraction"

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

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SPE Brochure

- T402150 (FEB)
- 36 pages

- Complete list of SPE products and accessories

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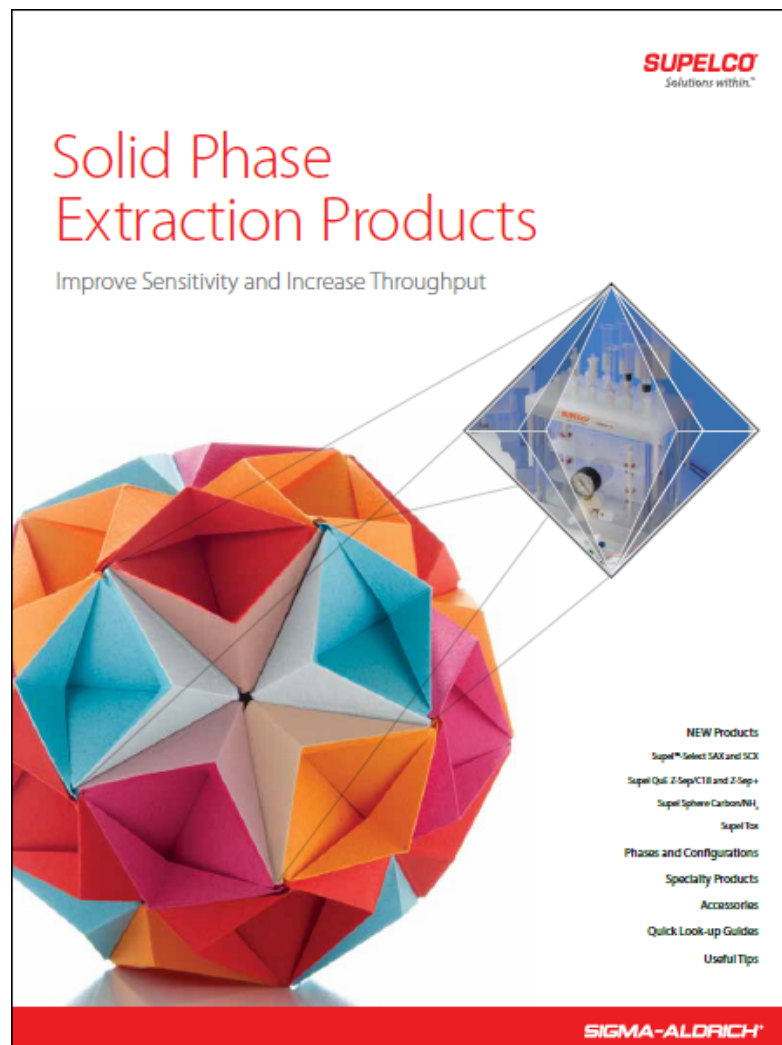
Thank You!!!

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-NH$_2$
- **IE:**   DSC-NH$_2$, 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, Na$_2$SO$_4$/Florisil
- **MIP**   SupelMIPs - Highly selective SPE
- **HybridSPE®**   Phospholipid removal from Plasma & Serum Samples

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