SPE Method Development for Pharmaceutical Bioanalysis

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Agenda

- **Specific challenges for bionalytical sample prep**
- **Available tools/technology for bioanalytical sample prep**
- **Understanding SPE Theory and Practice**
- **SPE Method Development Approaches**
  - Generic Approach
  - Selective Approach
  - Systematic Approach

Specific Challenges for Bioanalyses

- **Develop bioanalytical methods that selectively separate drugs/metabolites from endogenous matrix interferendces**
- **Must determine drug concentrations in biological fluids**
  - Data used to understand time course of drug action and pharmacokinetics of an in-vivo system
  - Requires efficient/adequate sample preparation, good chromatographic separation, and sensitive detection technique.
- **Although MS is very sensitive and specific, the importance of a well chosen column and method conditions cannot be overlooked.**

Why is sample preparation required?

Collected Sample HPLC, or LC-MS/MS Analysis

Current Sample = Unsuitable for further analysis!!!… Why?

- **Too dirty- contains other sample matrix components that interfere with the analysis**
- **Too dilute- analyte(s) not concentrated enough for quantitative detection**
- **Present sample matrix not compatible with or harmful to the chromatographic column/system**

Why is sample prep especially important in bioanalysis?

- **Due to many types of sample matrices encountered:**
  - Plasma- proteins, lipids, and other endogenous macromolecules
  - Urine- contains uric acid and many nitrogenous base products
  - Serum
  - Bile
  - Tissue Homogenates
  - Perfusates
  - Saliva
  - Seminal Fluid
  - Caco-2 buffer
  - Others

Primary Sample Prep Objectives

1. **Remove unwanted sample matrix components**
2. **Concentrate analytes to meet detection limits**
3. **Solvent exchange- chromatographic compatibility**
4. **Isolate analytes of interest (esp. if LC resolving power is insufficient)**
5. **Reduce backpressure and LC system fouling**

Many Tools/Technology for Sample Prep

- **Dilute and Shoot**
- **Protein Precipitation**
- **Filtration**
- **Equilibrium dialysis/ultrafiltration**
- **Liquid Liquid Extraction**
- **Solid Phase Extraction (off-line and on-line)**
- **Turbulent Flow Chromatography**
- **Monolithic Chromatography**
- **Immunoaffinity**

How to choose the right sample prep technology?

- **Should depend on three specific criteria:**
  - Requirements of the assay
  - Time allowed to run sample prep method
  - Possible investment towards method development time
- **Example:**
  - Late Discovery/Early Development
    - **Requires rapid sample turn around**
    - **Higher limits of quantitation**
    - **Very little method development time (1-2 days)**
    - **Protein Precipitation may be ideal choice**
  - Development (pre-clinical and clinical)
    - **Drugs more potent and dosed at lower levels**
    - **Requires ultra-sensitivity, great selectivity and rugged method development**
    - **SPE is more ideal choice**

More Common Sample Prep Tools/Technology

Protein Precipitation:

- **Sample matrix combine with precipitating agent (MeCN) 1:3 or 1:4 (v/v) => vortexing/mixing => filtration or centrifugatioin => analyze filtrate or supernatant**
- **Advantages**
  - Requires little to no method development (universal)
  - Amenable to automation
  - Very simple (2-3 steps), and relatively inexpensive
- **Disadvantages**
  - Sample dilution effect => requires concentration which is time consuming due to aqueous portion of sample
  - Poor removal of matrix interferences => stress on analytical system and increased ion-suppression resulting in poor reproducibility, accuracy and sensitivity

More Common Sample Prep Tools/Technology

Liquid-Liquid Extraction:

- **Separates analytes from interferences in sample matrix by partitioning analytes between two immiscible solvents**
- **Advantages**
  - Widely amenable to many applications and can achieve good selectivity for the target analytes
  - Becoming more amenable to automation via liquid handling stations
  - Relatively inexpensive
- **Disadvantages**
  - Can be labor intensive with several disjointed vortex mix and centrifugation steps required
  - Organic solvents are volatile and dangerous
  - Phase emulsions

SPE Basics

SPE Advantages & Disadvantages

Disadvantages

- Perceived difficulty to master its usage (method development)
  - Wide range of chemistries, many choices for manipulating solvent and pH conditions make it difficult to grasp
- More steps and MD time required
- Greater cost per sample

Advantages

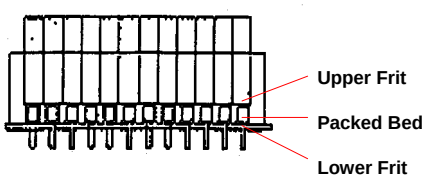
- Greater selectivity- paramount importance in bioanalysis (pg/mL)
- Wide variety of sample matrices
- High recoveries & good reproducibility
- Amenable to automation
- Low solvent volumes and amenable to automation

High Throughput Solid Phase Extraction

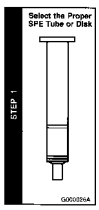
Discovery SPE 96-Well Plates

Plate Description:

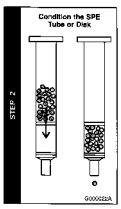
- Square well extraction plate, 2.0-2.25 ml capacity, polypropylene.
- Available for all Discovery SPE phases
- Bed weight = 25, 50, or 100mg/well
- Reduced height for larger volumes without leak prone extensions.
- Standard dimensions common to most if not all square well designs.
- Compatible with most robotic systems and automated sample processing systems: TomTec Quadra, Gilson SPE 215, etc.



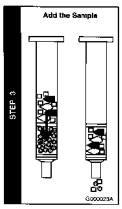
The SPE Process: A form of digital chromatography



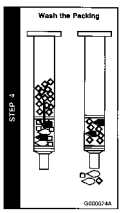
Phase & Hardware Selection



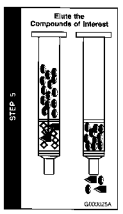
Condition/ Equilibrate



Load Sample



Wash Weakly Retained Co-Extracted Impurities



Elute Analytes

General Steps of an SPE Procedure

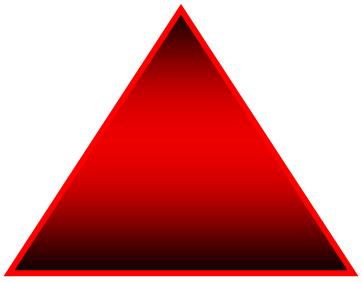
1) **Sample Pre-treatment:** Dependent on compound of interest, sample matrix, and nature of retention chemistry; involves pH adjustment, centrifugation, filtration, dilution, buffer addition, etc..
2) **Conditioning:** Solvent is passed through the SPE material to ***wet*** the bonded functional groups => ensures consistent interaction.
3) **Equilibration:** Sorbent/ phase is treated with a solution that is similar (in polarity, pH, etc.) to the sample matrix => maximizes retention.
4) **Sample Load:** Introduction of the sample = analytes of interest are bound/ extracted onto the phase/ sorbent; unretained components
5) **Washing:** Selectively remove unwanted interferences co-extracted with the analyte without prematurely eluting analytes of interest.
6) **Elution:** Removing analytes of interest with a solvent that overcomes the primary and secondary retention interactions b/w sorbent and analytes of interest.
7) **Evaporation** of eluent/reconstitution with mobile phase (optional).

Rules of SPE

- Analyte must adsorb onto the SPE sorbent
- There must be sufficient resident time for analyte sorbent interaction to occur
- Endogenous sample interferences must be selectively separated from the analyte
- Analyte must be able to be removed from the sorbent

Interactions in SPE (mirrors LC)

Analyte(s) of Interest



Solid Phase Chemistry

Mobile Phase Environment

Reversed-Phase SPE Rules:

Retention mechanism:	**Non-Polar or Hydrophobic Interactions**
Sample Matrix:	**Aqueous Samples** ▪ Biological fluids ▪ Environ water samples
Analyte Characteristics:	**Analytes exhibit non-polar functionalities** ▪ Most organic analytes ▪ Alkyl, aromatic, alicyclic functional groups
Elution Scheme:	**Hydrophobic interaction disrupted with more hydrophobic solution or solvent** ▪ MeOH, MeCN, DCM, etc. ▪ Combinations of water or buffer/solvent mixtures

Ion-Exchange SPE Rules:

Retention mechanism:	**Electrostatic Interaction** ▪ Sorbent and analyte functional groups must be oppositely charged
Sample Matrix:	**Non-Polar or Polar Samples of low salt concentration (<0.1M)**
Analyte Characteristics:	**Cation Exchange for Basic compounds** ▪ E.g., all amines **Anion Exchange for acidic compounds** ▪ E.g., carboxylic acids, sulphonic acids, phosphates
Elution Scheme:	**Disrupt electrostatic interaction via one of two ways:** ▪ Modification of pH to neutralize analyte or sorbent functional groups ▪ Increase salt concentration (> 0.1M) ▪ Use counter-ion of greater selectivity for sorbent than annalyte

The Critical Role of pH in SPE

Neutral State (Blue) = promotes hydrophobic (RP) interaction
Ionized State (Green) = promotes electrostatic (IOX) interaction

Ionization of Acidic & Basic Molecules-

Acids (e.g., carboxylic acids):

$HA \leftrightarrow H^+ + A^-$ (e.g., R-COOH ⇔ $R\text{-}COO^-$)

HA (Un-ionized) ⇔ H^+ + A^- (ionized)

50% ← @pKa → 50%
100% ← low pH → 0%
0% ← high pH → 100%

Bases (e.g., amines):

$BH^+ + OH^- \leftrightarrow B$ (e.g., $R\text{-}NH_3^+$ ⇔ $R\text{-}NH_2$)

BH^+ + OH^- (Ionized) ⇔ B (Un-ionized)

50% ← @pKa → 50%
100% ← low pH → 0%
0% ← high pH → 100%

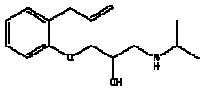
Prediction of Analytes' pKa

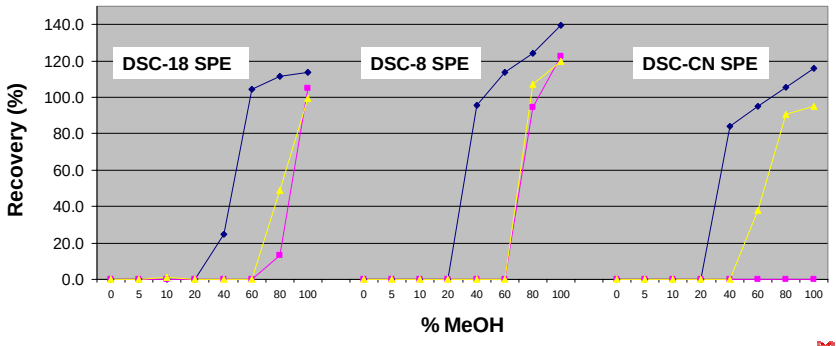
Often, the pKa for a drug is not known

- **pKa of most amines is 8.0 – 11.0**
  - Aromatic (electron sink) amines generally have a lower pKa than aliphatic amines.
  - E.g.- Aromatic amines- aniline (pKa 4.6), pyridine (pKa 5.2); Aliphatic amines- trimethylamine (pKa 9.7), dimethylamine (pKa 10.7)
- **pKa of most acids (e.g. –COOH) is 3.0-5.0**
  - Presence of a halogen atom near a carboxy group strengths the acid effect (electron sink)
  - E.g.- acetic acid (pKa 4.75), monochloraceticacid (pKa 2.85), dichloroacetic acid (pKa 1.48)

Wash/Elute Profile- Basic Compounds

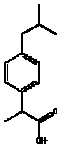
Alprenolol

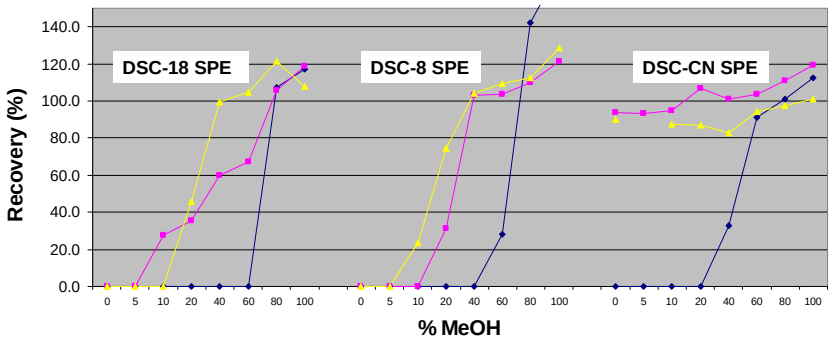




Wash/Elute Profile- Acidic Compounds

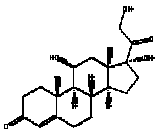
Ibuprofen

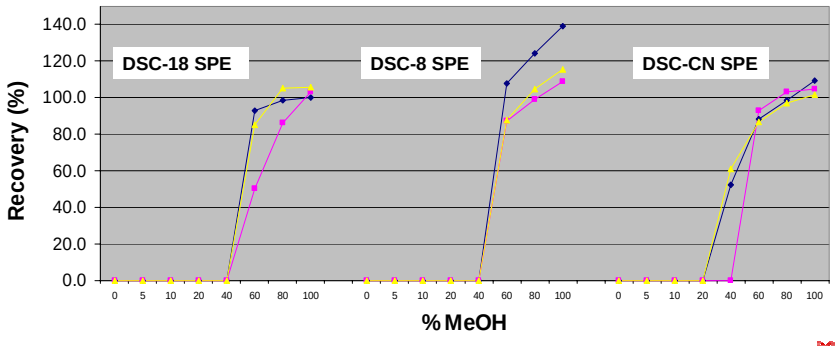




Wash/Elute Profile- Neutral Compounds

Hydrocortisone





SPE Method Development

Best practices for method development

- Historically MD = hit or miss experiments in which many random variables evaluated at the same time
- Results in user not knowing why a set of conditions worked or what type of leeway can be associated with operator variation, changes in pH, etc.
- As a result, problems can easily arise during method transfer.

- Best method developers isolate one variable at a time, and use 96-well technology to evaluate multiple variables in parallel

Key to Successful SPE

- Choose the appropriate SPE phase by understanding the sample matrix and identifying analyte(s) functional groups that influence its solubility, polarity, etc..
- Understand how the analyte(s) behaves on the sorbent in response to changing extraction conditions.
- Manipulate these conditions to meet the defined sample prep objectives

Consider the analyte(s) of interest

What functional groups may influence the analytes' solubility, 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

Choosing the Appropriate Phase Chemistry

Reversed-Phase:

- **C18 (18%C)**
  - **Less risk of silanol activity for predictable extractions**
  - **Broad affinity for a wide range of compounds**
  - **Potential use of stronger wash solvents**
  - **Greater risk of co-retention of matrix interferences**
  - **Extract many analytes with generic methodology**
- **Cyanopropyl (CN; 6%C)**
  - **Weaker affinity to compounds**
  - **May retain compounds more selectivity than C18**
  - **Weaker wash solvents are required**
  - **Could yield weaker elution solvents**
  - **Could elute with smaller elution volumes**
  - **Increased risk of silanol activity (may not be bad though)**
- **C8 (9%C)- retains compounds with log Po/w ≥ 1**

Choosing the Appropriate Phase Chemistry (cont.)

Ion-Exchange:

- **SCX & SAX (strong ion exchange)**
  - Can be very selective
  - Elution typically done via pH manipulation to neutralize analytes
  - Always some mixed-mode properties (requires a combination of pH adjustment and organic strength to elute compounds)
- **WCX & NH_2 (weak ion exchange)**
  - Used for extracting strong bases and acids where elution cannot be done through pH manipulation of analytes
  - Instead pH adjustment used to neutralize sorbent functional groups
- **In general though, standard ion-exchange is not commonly used for bioanalysis**
  - Ionic strength in biological fluids are high and fluctuates greatly
  - Higher salt concentrations found in the SPE eluate not desirable for LC/MS

Choosing the Appropriate Phase Chemistry (cont.)

Mixed-Mode SPE:

- **Dual mechanisms of attraction**
  - Reversed-phase + ion-exchange = broad affinity for a wide range of compounds
  - Most pharma compounds contain one or more amine groups
  - Therefore, mixed-cation phases are of great utility in bioanalysis
  - Combination of hydrophobic and strong electrostatic interactions allows researcher to use vigorous wash steps
  - Results in more selective extractions

Choosing the Appropriate Bed Weight

- **Smaller Bed Weights allow for smaller elution volumes**
  - 25mg/well = 75-400µL
  - 50mg/well = 200-800µL
  - 100mg/well = 300-1000 µL
- **Smaller elution volumes = less evaporation time; dilute and shoot methods**
- **Note that there's a greater risk of channeling with smaller bed weights**
- **Also greater risk of phase over-drying with smaller bed weights**

Three Main Approaches to SPE MD for Pharma Bioanalyses

- **Generic Approach to SPE Method Development**
  - Single sorbent universal method
  - Multiple sorbent universal method
- **Selective Approach to Method Development (Dave Wells Approach)**
- **Supelco's Systematic SPE Method Development Approach**

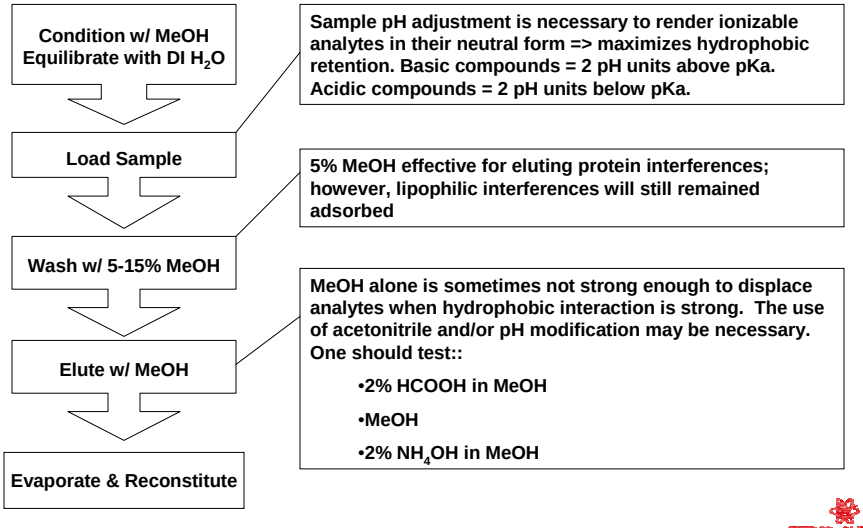
Critical Questions Prior to Lab Work

- **What are requirements and goals to method development?**
- **What is known about the sample (sample matrix, analyte Log Po/w, pKa(s), functional groups)?**
- **What investment in MD time can be made?**
- **Any known information from previous work with similar analytes?**

Generic Method Development Approach

- Involves a generic or universal set of conditions which are expected to adsorb and desorb a range of analyte polarities involved (e.g. parent drug + metabolites)
- Can be used with C18 or C8 SPE
- Development process = running method at three concentrations with multiple replications and assaying for recovery and performance (precision and matrix effect)
- Most commonly used when little time is available for method development (1-2 days).

Generic Extraction Conditions on C18 or C8 SPE



Example generic method development template on C18 or C8 96-well SPE

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample Blank; Low pH Elute			Spike Level 1; Low pH Elute			Spike Level 2; Low pH Elute			Spike Level 3; Low pH Elute		
B	Sample Blank; Neutral pH			Spike Level 1; Neutral pH			Spike Level 2; Neutral pH			Spike Level 3; Neutral pH Elute		
C	Sample Blank; High pH Elute			Spike Level 1; High pH Elute			Spike Level 2; High pH Elute			Spike Level 3; High pH Elute		
D												
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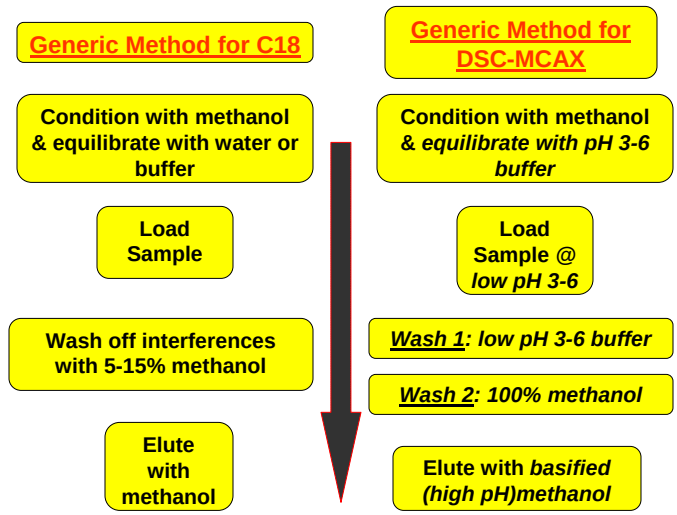
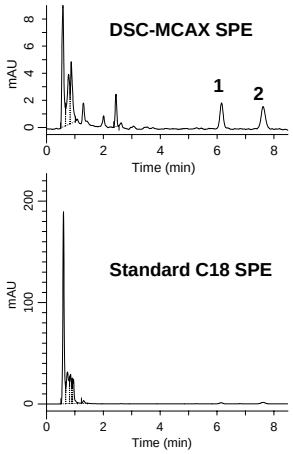
G												
H												

Generic conditions on Mixed-Cation SPE



Comparative Extraction of Amphetamines Using DSC-MCAX SPE and Standard C18 SPE



Comments:

- **Note the Y-axis scale difference between DSC-MCAX and C18 SPE**
- **DSC-MCAX SPE offered a maximum background height of ~9mAU. In contrast, standard C18 background levels were ~20 x's greater than DSC-MCAX SPE**
- **Also, on DSC-MCAX, absolute recovery averaged at 100.3 and 101.7% for amphetamine and methylamphetamine, respectively.**
- **On standard C18, absolute recovery avaraged at 48 and 79% for the two compounds.**

1. **Amphetamine**
2. **Methylamphetamine**

SPE: Discovery DSC-MCAX, 100mg/3mL; Standard C18, 100mg/3mL; **HPLC:** Discovery HS F5 15cm x 4.6mm, 5µm; **Mobile Phase:** 10mM ammonium acetate, pH 4.5:MeCN (35:65); **Flow Rate:** 2mL/min; **Temp.:** 40ºC; **Det.:** 210nm, UV; **Inj. Vol.:** 10µL

Accessing Method Performance

- **Evaluating matrix effect**
  - **Can lead to decreased reproducibility and accuracy**
  - **Failure to reach desired LOQ**
  - **Must compare blank sample spiked post extraction against external standard in buffer (no sample prep) to determine matrix influence after sample prep**
  - **Matrix effect (% ion-suppression) = [Response of spiked matrix blank] / [Response of unextracted standard]**
- **Determination of Recovery**
  - **% Recovery = [Response of pre-extracted spike] / [Response of post extracted spike] x 100**

Selective Method Development Approach

- **In one test using 96-well SPE, the goal is to obtain as much information as possible about the affinity of analytes in matrix for a particular sorbent relative to changes in pH environment.**
- **Goal is to determine the most selective method possible.**
- **With more application based data determined early in MD, the easier it is to troubleshoot and the more reliable the method is as it progresses through development.**

Selective Method Development Approach

1. **Determine optimal load elution pH conditions**
   - Load at three pH conditions (neutral, high, and low)
   - Evaluate elution with MeOH and MecN (neat, high, and low pH conditions)
   - Results in 18 combinations (3 load pH by 6 elution conditios)
2. **Select one or two best load/elute combinations & perform wash solvent optimization**
   - Determine max percent organic modifier (0 – 40% MeOH or MeCN) before compound elution occurs
3. **Select best load/elution/wash conditions and optimize elution volume conditions vs. bed weight**

Important Tips-

Drug Protein Binding Effects:

- Must be disrupted during sample pre-treatment:
  - 40µL 2% disodium EDTA per 100µL mouse plasma
  - 40µL 2% formic acid per 100µL mouse plasma
  - Other possible reagents (per 100µL matrix): 40µL 2% TCA, 40µL 2% acetic acid, 40µL 2% TFA, 40µL 2% phosphoric acid, or 200µL MeCN (protein ppt.).

Sorbent over drying-

- Only critical with C18 & only criticial in first conditioning step
- Phase just needs to be moist during sample addition
- All other steps non-critical

Wash Step-

- Water wash step alone does not provide a clean eluate; Need some sort of organic modifier

Sorbent Drying prior to elution-

- Important to dry sorbent prior to elution, otherwise, subsequent eluate evaporation will take a real long time.

Compound volatility during evaporation

- Lower heat during evaporation, or Use a keeper solvent (e.g. dodecane)

Systematic SPE Method Development

How are most SPE methods developed?

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 "gooroo" for help**

The Problem with these approaches

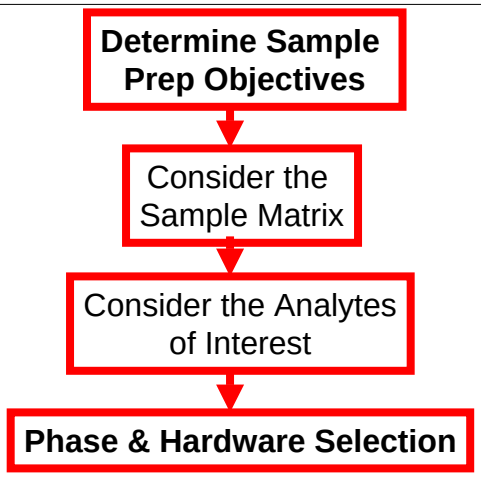
"With some experience, they might meet their sample prep objectives on the first pass; however, more often than not, the investigator will have more questions than answers."

Leads to a Non-Systematic Approach to method development and optimization & Variable MD Time

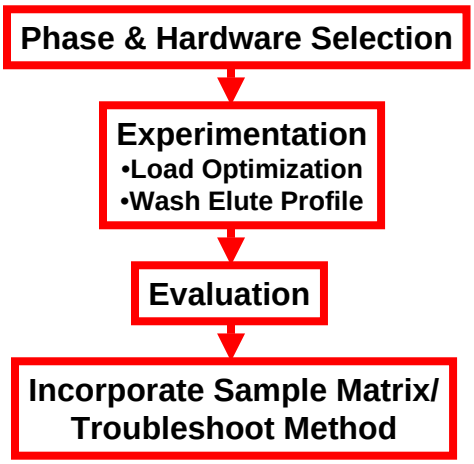
Examples of Problems

- **Dealing with novel analytes**
- **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? Maybe a different SPE phase?**

Systematic SPE Method Development



Systematic SPE Method Development



Experimentation, Evaluation, Incorporate Sample Matrix & Troubleshoot Method

Experimentation	• Develop Analytical Method (LC-MS) • Using standards and buffered/organically modified solutions, identify and test key variable parameters (pH, organic strength, etc.)
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/ Troubleshoot	• Define method and incorporate sample matrix • Make determinations of recovery, matrix effect, cleanliness, and LC/GC resolution

What is Systematic SPE MD all about?

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

By employing two or three expts. using standard solutions w/o sample matrix, the researcher can systematically adjust the two main variables that control selectivity (pH & organic strength).

By understanding how the analytes interact with the sorbent under specific conditions, it allows for a systematic approach to finding the optimal sample prep conditions with greater efficiency and confidence.

Example: Tricyclic Antidepressants 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/quantitation limits of 0.25-1.0µg/mL Serum**
- **Post SPE sample matrix should be a buffered solvent compatible with HPLC mobile phase**

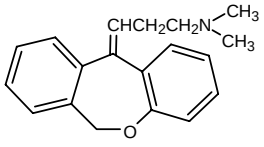
Example: Tricyclic Antidepressants from Sheep Serum

Consider the Sample Matrix:

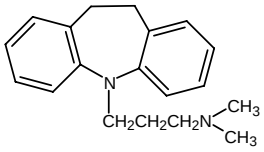
- **0.5 mL Sheep Serum = Polar**
- **Serum is the aqueous portion of blood**
  - Platelets, corpuscles, and clotting factors have been removed
- **Endogenous interferences:**
  - albumin, globulins, lipids, salts and carbohydrates

Example: Tricyclic Antidepressants from Sheep Serum

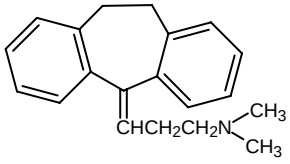
Consider the Analytes of Interest:



Doxepin

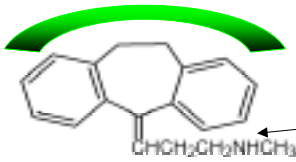


Imipramine



Amitrypityline

Example: Tricyclic Antidepressants 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 .

Different ionic forms retain differently on a given sorbent. pH manipulation plays a critical in controlling retention and selectivity on a given sorbent.

- **At pH >/=11, the 2° or 3° amine functional group should be neutralized. At pH </=7, the amine group should be ionized.**

Example: Tricyclic Antidepressants 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 = ensure optimal retention for the potential use of stronger wash eluants ⇨ Maximize Sample Clean-Up**

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

Example: Tricyclic Antidepressants from Sheep Serum

<u>Load Optimization</u>- Ensures 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 Load eluate and analyze via HPLC-UV**

Note: load concentration was increased to provide adequate signal response for detecting small analyte breakthrough percentages. Also note that acidic load conditions were avoided**.**

Example: Tricyclic Antidepressants from Sheep Serum

<u>Load Optimization Evaluation</u>:

- **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**

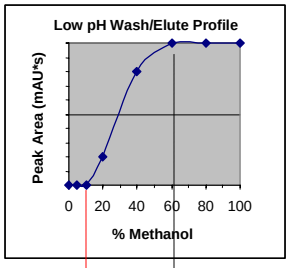
Example: Tricyclic Antidepressants from Sheep Serum

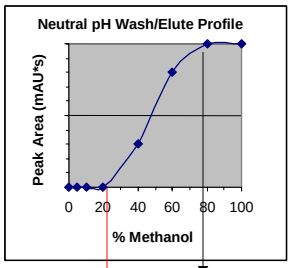
<u>Wash/Elute Profile</u>- Determine analyte retention and elution patterns as a function of pH & % Organic

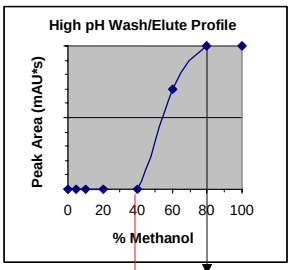
1. **Conditions DSC-18 wells with 1mL MeOH**
2. **Equilibrate DSC-18 wells with 1mL DI H_2O**
3. **Load 1mL 5µg/mL standard test mix prepared at basic pH (1% NH_4OH).**
4. **Wash/Elute with 1mL of a test solvent ranging from 0-100% MeOH in 2% NH_4OH (high pH), DI H_2O, and 2% CH_3COOH (low pH)**
5. **Collect wash/elute eluate and analyze via HPLC-UV**

Example: Tricyclic Antidepressants 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.

Under high pH, retention limit is 40% MeOH.

Example: Tricyclic Antidepressants from Sheep Serum

Wash/Elute Profile Evaluation-

- **At low pH, basic analytes predominately in their ionic form**
- **TCAs amine functional groups counteracted hydrophobic interaction b/w analyte and C18 alkyl groups ➔ Full elution observed at 60% MeOH**
- **Equates to more selective elution**

- **At high pH, basic analytes predominately in their neutral form**
- **This promotes stronger hydrophobic interaction ➔ 40% MeOH can be used as potential wash solvent**
- **Equates to a more selective wash step**

Example: Tricyclic Antidepressants from Sheep Serum

Incorporate Sample Matrix/Troubleshoot Method-

- **Profiling major parameters affecting analyte retention/elution = Obtain specific guidelines for defining, optimizing, and troubleshooting the extraction method**
- **For most applications, recovery values observed for real-matrix based solutions will parallel values obtained with standard solutions**

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

Systematically Developed 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

Note: Although a 60% acidified MeOH may have been a potential elution eluant

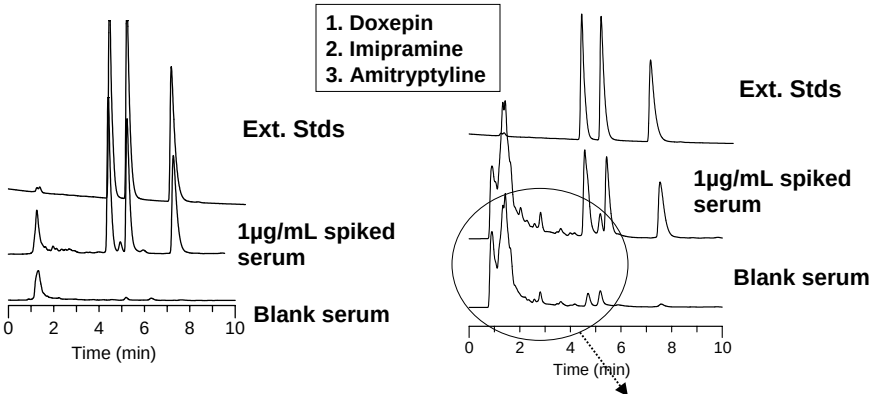
Generic Method on Competitor Polymeric Phase (30mg/well)

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 MP

Results-

SPS Method Using DSC-18 SPE-96 Well Plate

Generic Method Using Competitor Polyermic Well Plate



Discovery C18, 15cmx4.6mm, 5µm, preceeded by 2cm guard column & 0.5µm filter frit;
MP: 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

Efficiency of Absolute Recovery of TCAs on Sys SPE 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%

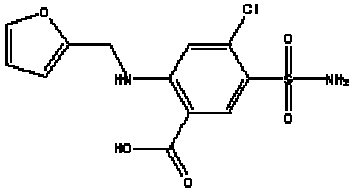
Example: Tricyclic Antidepressants from Sheep Serum

Summary

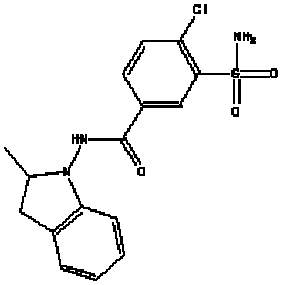
- **In this study, through systematic SPE method development we were able to:**
  1. optimize the wash solvent to maximize sample clean-up resulting in minimal background and more accurate results
     - Determination of 40% MeOH in 2%NH_4OH as wash solvent
     - 60% MeOH in 2% CH_3COOH
  2. achieve high and reproducible recoveries at the spike levels tested (> 90% recovery, ≤ 4% RSD).
  3. High background observed on generic method on polymeric SPE well plate

Example: Furosemide from Horse Serum

Consider the Analytes of Interest:



Furosemide



Indapamide (I.S.)

Example: Furosemide from Horse Serum

Load Optimization

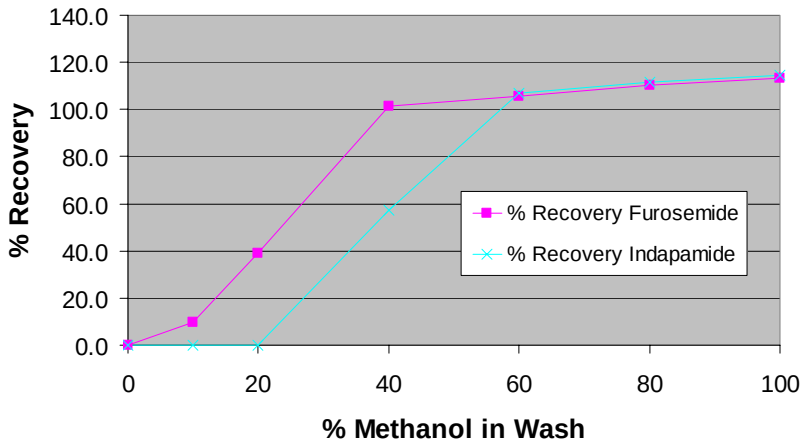
SPE: Discovery DSC-18 SPE 96-well, 50mg/well

1. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
2. Load 1mL standards containing 5.0µg/mL furosemide and indapamide in 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)
3. Collect load flow-through eluate & analyze for compound break through via HPLC-UV

Wash/Elute Profile

1. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
2. Load 1mL standards containing 5.0µg/mL furosemide and indapamide in 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)
3. Wash/elute with 1mL test solvents ranging from 0-100% methanol
4. Collect wash/elute eluate & analyze for compound elution via HPLC-UV

Wash/Elute Profile for Furosemide & Indapamide (I.S.) on Discovery DSC-18 SPE



Evaluation- Furosemide from Horse Serum

Load Optimization

- A lack of analyte presence in the load flow-through eluate during sample load indicated adequate retention under low pH conditions

Wash/Elute Profile

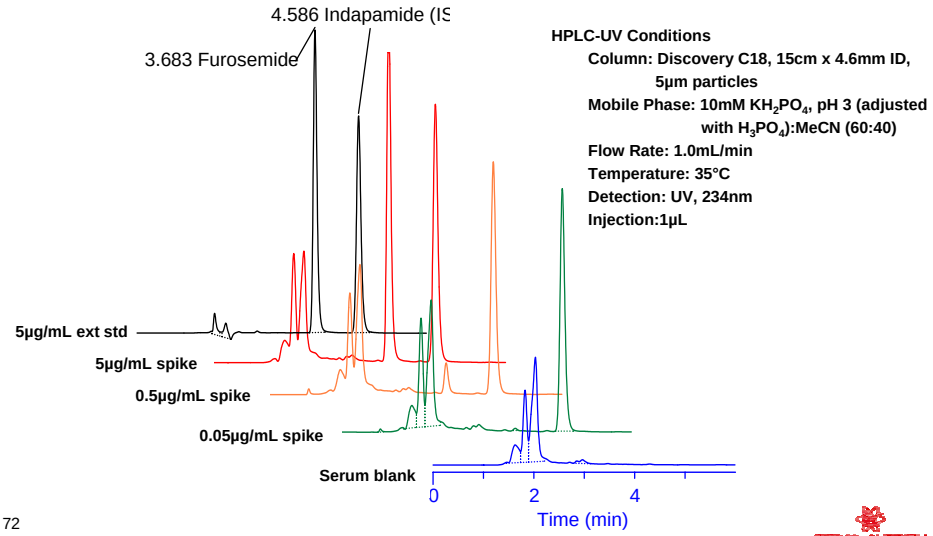
- Under neutral conditions, furosemide is not strongly retained on DSC-18 SPE
- Furosemide begins elution between 0-10% methanol
- Stronger wash solvents would result in premature elution potentially leading to poor recovery
- Indapamide is more strongly retained than furosemide
- A wash strength of 10-20% methanol is possible before indapamide break through occurs
- A minimum solvent strength of 60% methanol is required to fully elute both compounds from DSC-18 SPE

Systematically Developed SPE Method For Furosemide & Indapamide from Serum

SPE: Discovery DSC-18 SPE, 50mg/1mL

1. **Condition with 1mL methanol**
2. **Equilibrate with 1mL 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)**
3. **Load 1mL sample- See Sample Info**
4. **Wash with 1mL 10mM KH_2PO_4, pH 3 (adjusted with H_3PO_4)**
5. **Elute with 1mL 60% methanol in DI H_2O**
6. **Directly analyze eluate (no evaporation/reconstitution) via HPLC-UV.**
7. **Determine relative recovery and RSD against working calibration standards not subjected to SPE sample preparation.**

Example Chromatograms of Extracts Generated from the Systematically Developed Method on Discovery DSC-18 SPE



Relative Recovery of Furosemide from Horse Serum Using Discovery DSC-18 SPE

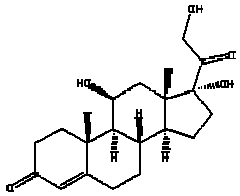
Sample	Furosemide Spike Concentration (µg/mL)	Avg. Response Factor	% Recovery ± RSD (n=3)
A	10.00	2.307	99.3 ± 3.1
B	5.00	1.168	100.8 ± 1.4
C	0.50	0.107	97.4 ± 2.8
D	0.10	0.065	120.7 ± 1.3
E	0.05	0.009	132.8 ± 8.3

Example: Furosemide from Horse Serum

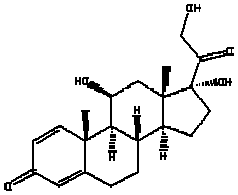
Summary

- Furosemide not strongly retained on C18 SPE under neutral conditions
- Wash solvents stronger than DI water caused compound elution
- Selectivity improved by eluting with weaker elution solvent (e.g. 60% MeOH)
- By using a weaker elution solvent, direct injection of the final eluate was possible resulting in less processing steps and reduced processing time.
- Average relative recovery and RSD for the five concentrations were 100.2 ± 3.4%.
- Note that the procedure is quantitative down to 0.5µg/mL serum.
- Below this level, reasonable precision is evident; but, accuracy suffers.
- This is mainly due to the detection limitations of UV absorbance for furosemide. Flourescence detection or mass spectrometry is likely to provide increased sensitivity.
- Decreasing SPE elution volume may also be a viable choice for improving sensitivity.

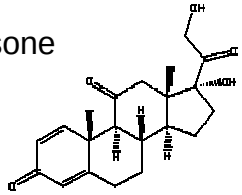
Example: Corticosteroids from Urine



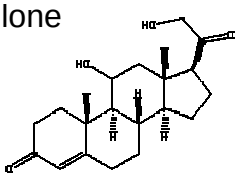
Hydrocortisone



Prednisilone



Predinisone



Corticosterone

Example: Corticosteroids from Urine

Load Optimization

SPE: **Conventional C18 96-well SPE (100mg/well)**
Discovery DSC-CN SPE 96-well (100mg/well)

1. **Condition & equilibrate SPE wells (C18 & CN) with 1mL methanol & DI H_2O**
2. **Load 1mL standards containing 5.0µg/mL of each of the four corticosteroids in DI H_2O**
3. **Collect load flow-through eluate & analyze for compound break through via HPLC-UV**

Wash/Elute Profile

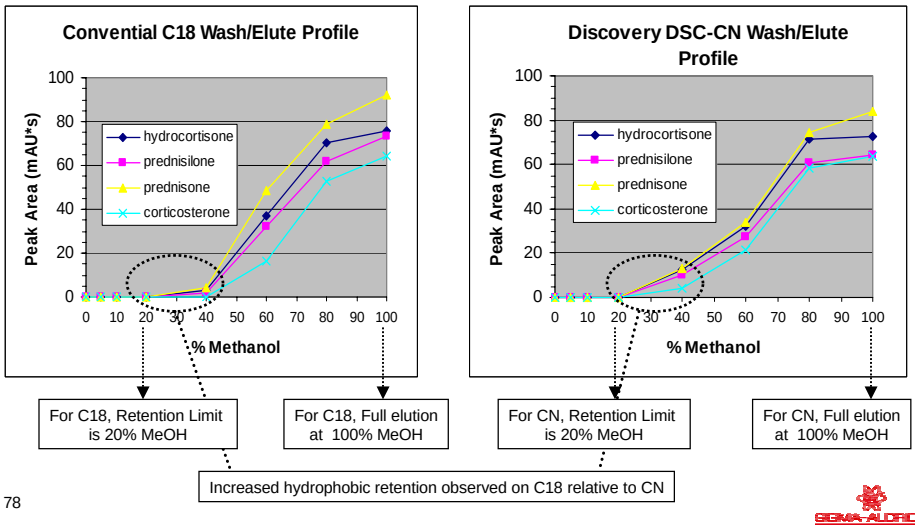
1. **Condition & equilibrate SPE wells (C18 & CN) with 1mL methanol & DI H_2O**
2. **Load 1mL standards containing 5.0µg/mL of each of the four corticosteroids in DI H_2O**
3. **Wash/elute with 1mL test solvents ranging from 0-100% methanol**
4. **Collect wash/elute eluate & analyze for compound elution via HPLC-UV**

Example: Corticosteroids from Urine

HPLC-UV Conditions

Column:	**Discovery HS F5, 5cm x 4.6mm ID, 3µm particles**
Mobile Phase:	**Methanol:DI H_2O (40:60)**
Flow Rate:	**1.5mL/min**
Temperature:	**35°C**
Detection:	**UV, 240nm**
Injection:	**5µL**

Wash/Elute Profile for Corticosteroids on C18 & CN SPE



Evaluation: Corticosteroids from Urine

Load Optimization

- A lack of analyte presence in the load flow-through eluate for both phase chemistries indicated adequate retention was observed for both C18 & CN SPE phases under neutral aqueous conditions

Wash/Elute Profile (Figure C)

- Steroidal compounds behaved similarly on both C18 and CN SPE
- Stronger retention observed on C18 phase. Particularly evident in 20-40% methanol range of wash/elute profile
- Up to 20% methanol can be used as a potential wash solvent on both phases before premature analyte elution occurs
- 100% methanol is required to completely elute the analytes of interest from both C18 & CN SPE

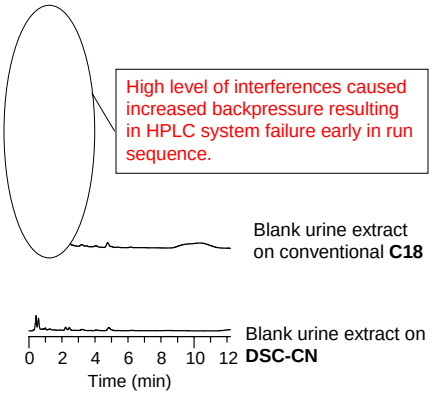
Systematically Developed SPE Method For Steroids From Urine

SPE: Conventional C18 96-well SPE (100mg/well)
Discovery DSC-CN SPE 96-well (100mg/well)

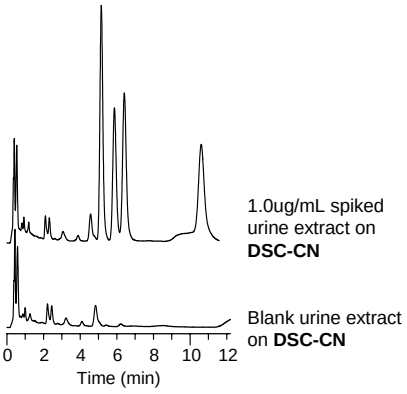
1. Condition & equilibrate with 1mL methanol and 1mL DI H_2O
2. Load 0.5 & 1.0µg/mL corticosteroids spiked in human urine diluted in DI H_2O (1:1, v/v); n=3
3. Wash with 1mL 20% methanol
4. Elute with 1mL 100% methanol
5. Evaporate eluate with nitrogen purge (30°C; ~10 min), and reconstitute in 200µL HPLC mobile phase

Chromatograms of Blank & Spiked Urine Extracts Generated on C18 & CN SPE

Blank urine extracts on C18 & CN SPE



Blank & spiked urine extracts on CN SPE



Recovery of Steroidal Compounds from Urine on Discovery DSC-CN SPE

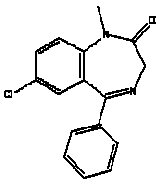
	% Absolute Recovery ± RSD (n=3)	
Compound	0.5ug/mL spike level	1.0ug/mL spike level
1. Hydrocortisone	123.3 ± 1.4%	95.9 ± 1.7%
2. Prednisilone	107.2 ± 1.1%	91.9 ± 1.1%
3. Prednisone	103.2 ± 1.0%	88.4 ± 1.8%
4. Corticosterone	102.0 ± 1.2%	93.1 ± 5.6%

Example: Corticosteroids from Urine

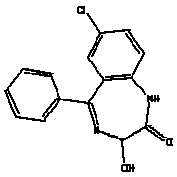
Summary

- **Under identical SPE protocols, C18 SPE eluate carried a yellow tint => lead to system failure due to high back pressure**
- **Stronger wash solvents required; but stronger wash solvents will lead to premature analyte elution**
- **In contrast, improved selectivity was observed on DSC-CN**
- **Chromatograms were free of interfering components that can result in column fouling, high background, and misleading peak responses**
- **On DSC-CN SPE , avg. absolute recovery and RSD for the four corticosteroids at the two spike levels tested was 100.6 ± 1.9%.**
- **Recovery values for C18 SPE were not obtained due to HPLC system failure caused by insufficient sample clean-up.**

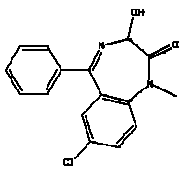
Example: Diazepam/metabolites from porcine serum



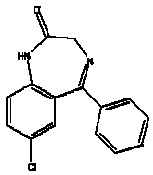
diazepam



oxazepam



temazepam



desmethyl diazepam

Example: Diazepam/metabolites from porcine serum

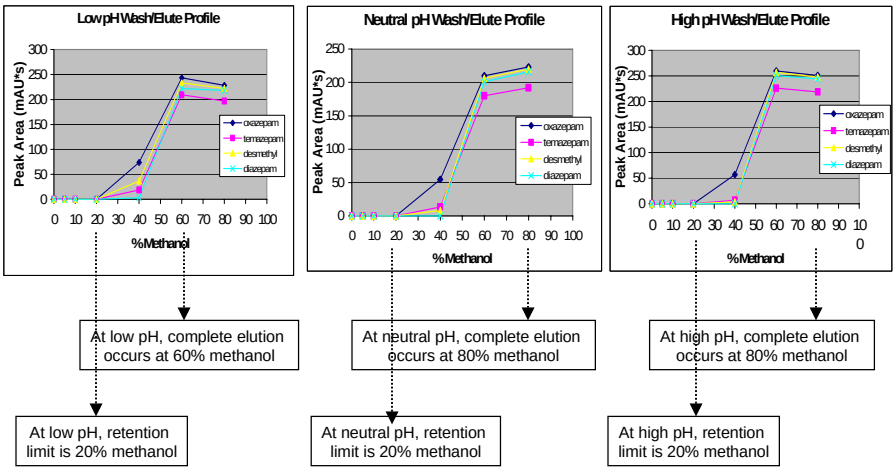
Load Optimization **SPE: Discovery DSC-8 SPE 96-well (100mg/well)**

1. Prepare standards containing 2.5µg/mL diazepam and metabolites in neutral (10mM ammonium formate, pH 7.1), and basic (1% NH_4OH) solutions
2. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
3. Load 1mL of each standard test mix (neutral and high pH)
4. Collect load eluate & analyze for compound break through via HPLC-UV

Wash/Elute Profile

1. Condition & equilibrate SPE wells with 1mL methanol & DI H_2O
2. Load 1mL standards containing 2.5µg/mL diazepam in 25mM ammonium formate, pH 7.1
3. Wash/elute respective wells with 1mL test solvents ranging from 0-100% methanol in 1% NH_4OH, pH 11 (high pH), 10mM ammonium formate, pH 7.1 (neutral pH), and 10mM ammonium formate, pH 2.7 (low pH)
4. Collect wash/elute eluate & analyze for compound elution via HPLC-UV

Figure B. Wash/Elute Profile for Diazepam & Metabolites on Discovery DSC-8 SPE



Evaluation- Diazepam/metabolites from Serum

Load Optimization

- A lack of analyte presence in the load flow-through eluate was observed at both pH conditions indicating adequate retention for both neutral and basic load conditions. Either load conditions would have been adequate. Neutral pH load conditions was chosen for this application.

Wash/Elute Profile (Figure B)

- Diazepam (most hydrophobic of four analogs) can withstand elution at 40% methanol and each of the pH conditions tested. Oxazepam (the most polar) elutes the most at 40% methanol
- At low pH, the basic analytes are in their ionic form. As a result, the analytes' amine functional groups counteract the hydrophobic interaction between the sorbent alkyl chains and analytes' hydrophobic moieties. This allowed for easier and potentially more selective elution using weaker eluents.
- Up to 20% methanol can be employed as potential wash solvents at the three pH levels tested. Under low pH conditions, the analytes are in their ionic form and full recovery can be achieved at 60% methanol.

SPE Methods Employed for Extracting Diazepam and Metabolites from Serum

Systematically Developed Method on C8

SPE: Discovery DSC-8 SPE 96-well Plate (100mg/well)

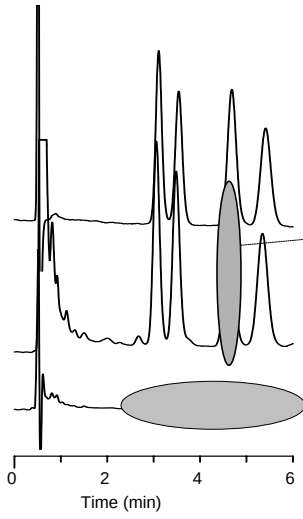
1. **Condition & equilibrate each well with 1mL methanol & DI H_2O**
2. **Load 1mL, 0.5µg/mL diazepam and metabolites spiked in goat serum diluted in 10mM ammonium formate, pH 7.1 (1:1; v/v)**
3. **Wash with 1mL 20% methanol in 25mM ammonium formate, pH 2.75**
4. **Elute with 1mL 60% methanol in 25mM ammonium formate, pH 2.75**

Generic Method on C18

SPE: Conventional C18 SPE 96-well Plate (100mg/well)

1. **Condition & equilibrate each well with 1mL methanol & DI H_2O**
2. **Load 1mL, 0.5µg/mL diazepam and metabolites spiked in goat serum diluted in 10mM ammonium formate, pH 7.1 (1:1; v/v)**
3. **Wash with 1mL 5% methanol**
4. **Elute with 1mL methanol**
5. **Evaporate eluate with nitrogen purge (30°C; ~15 min); and reconstitute with 200µL HPLC mobile phase**

Example Chromatograms of Blank & Spiked Serum Extracts Generated on C8 SPE



HPLC-UV Conditions

Column:	Discovery C18, 5cm x 4.6mm ID, 5µm particles
Mobile Phase:	Methanol:10mM ammonium acetate, pH 4.5 (45:55)
Flow Rate:	1.5mL/min
Temperature:	35°C
Detection:	UV, 240nm
Injection:	25µL

Ext. Stds

Excellent Peak Shape

0.5µg/mL spiked serum

Blank serum

Low background/minimal interferences for optimal sensitivity and resolution at reduced run times

Absolute Recovery of 0.5µg/mL Diazepam & Metabolites on Systematically Developed Method Using C8 vs. Generic Method on C18

Compound	%Recovery ± RSD (n=3)	
	Developed Method on Discovery DSC-8	Generic Method On Conventional C18
1. Oxazepam	94.7 ± 1.2%	82.8 ± 4.0%
2. Temazepam	99.9 ± 1.1%	89.1 ± 4.0%
3. Nordiazepam	94.2 ± 1.8%	82.4 ± 5.0%
4. Diazepam	90.0 ± 3.4%	68.5 ± 9.1%

Example: Diazepam/metabolites from porcine serum

Summary

- Through a systematic method development approach, a weaker eluent (60% methanol in low pH buffer) was determined allowing for direct analysis of the SPE eluate. As a result, processing time was reduced when compared to most generic reversed-phase methods that require a final eluate evaporation/reconstitution prior to analysis.
- The systematically developed C8 method provided good selectivity signified by chromatograms with low background (Figure C). The clean extracts also allowed for minimal run times (6 min) resulting in faster and more accurate results.
- Average absolute recovery and RSD for the four compounds on C8 via the developed method was 94.7 ± 1.9%. In contrast, the generic method on C18 yielded an average absolute recovery and RSD of 80.7 ± 5.3% (Table 3).

Conclusion

- **Many sample prep challenges in pharmaceutical bioanalysis**
- **Many tools available for sample preparation**
- **SPE is the most selective sample prep tool; but many negative connotations associated with method development**
- **Proposed three general approaches to SPE Method Development**
  - Generic Approach, Selective Approach, and Systematic Approach
- **Systematic SPE Method Development**
  - Excellent quantitative precision and accuracy
  - Improved recovery and sensitivity
  - Improved method ruggedness
  - Application specific knowledge for more effective method transfer and troubleshooting

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