Solid Phase Extraction Method Development Tips and Tricks

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Sources of Error Generated and Time Spent During a Typical Chromatographic Analysis

Sources of Error
- Contamination (4%)
- Sample Introduction (6%)
- Chromatography (7%)
- Integration (6%)
- Instrument (8%)
- Calibration (9%)
- Operator (19%)
- Columns (11%)

Time Spent
- Data Management (27%)
- Collection (6%)
- Analysis (6%)
- Sample Processing (61%)

(R.E. Majors, LC/GC Magazine, 2002)
Why not ‘dilute and shoot’?

Collected Sample \[\rightarrow\] Analyze

Current Sample = Unsuitable for further analysis!!!

Why?

**Too dilute** - analyte(s) not concentrated enough for quantitative detection

**Too dirty** - contains other sample matrix components that interfere with the analysis

**Too dangerous** - Contaminants can be ‘column killers’
Take the time to develop the method right the first time

Different vendors have slightly different sorbents.

This can affect selectivity and performance.

Optimization may be required for all new methods.
What Cartridge Do I Use?

• Know your matrix
• Know your compounds
  • pKa
  • log P
  • Chromatographic performance
### Cross Reference of Comparable Phases by Manufacturer

<table>
<thead>
<tr>
<th>If you are using...</th>
<th>Try this Agilent Sampler product...</th>
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<tbody>
<tr>
<td>Bond-Bot Plexa</td>
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**Silica and Other Sorbents**

<table>
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<th>Reversed Phase</th>
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<td>Bond-Bot C18</td>
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<td>Sampler Cyano</td>
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</table>
Analyte Assessment - Tetracycline

\[
pK_a = 3.3 / 7.7 / 9.7 \\
\log P = -1.3
\]

- = non-polar
- = polar
- = ionizable
### Phase Selection

<table>
<thead>
<tr>
<th>Solubility</th>
<th>Molecular Character</th>
<th>Organic Sample: MW &lt; 2000</th>
<th>Organic solvent-soluble</th>
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<tbody>
<tr>
<td>Water-soluble</td>
<td>Ionic</td>
<td>Soluble in polar solvent: MeOH (methanol), ACN (acetonitrile), THF (tetrahydrofuran)</td>
<td>Soluble in moderately polar solvent: Et OAc (Ethyl Acetate), CH₂Cl₂ (methylene chloride), Et₂O (diethyl ether)</td>
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<tr>
<td>Non-ionic (ion-paired)</td>
<td>Non-ionic</td>
<td>Soluble in non-polar solvent: C₅ (n-pentane), C₆ [n-hexane] and iC₈ (iso-octane)</td>
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</tbody>
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**Is your sample water soluble?**

<table>
<thead>
<tr>
<th>Phases: Polymeric</th>
<th>SCX Strong Cation Exchange</th>
<th>SAX Strong Anion Exchange</th>
<th>OPT, DVB, PS-DVB</th>
<th>OPT, DVB, PS-DVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phases: Si-based</td>
<td>Si-SCX, C₈/Si-SCX (mixed mode)</td>
<td>Si-SAX, Amino (WAX)</td>
<td>Cyano, Diol, Amino</td>
<td>Silica</td>
</tr>
<tr>
<td>Phases: C₁₈</td>
<td>C₁₈, C₁₈EC, C₈, C₂, Phenyl</td>
<td></td>
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<td>Silica</td>
</tr>
</tbody>
</table>

|----------------------|-------------------------------------------------------|

**EVIDEX** for drugs of abuse testing
Water Soluble Target Compounds in Organic Solvent Matrix- Tips

- No equilibration of cartridges required. Condition the cartridge with the same organic solvent as the sample.
- Acceptable non-polar loading and washing solvents are: hexane, chloroform, methyl-t-butyl ether.
- Acceptable polar elution solvents are: Tetrahydrofuran, ethyl acetate, isopropanol, acetonitrile and methanol as long as they are miscible with the loading/washing solvents.
### Phase Selection

**Organic Sample**  
MW < 2000

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**Is your sample soluble in organic?**
<table>
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<th>Soluble in moderately polar solvent: Et OAc (Ethyl Acetate), CH₂Cl₂ (methylene chloride), Et₂O (diethyl ether)</th>
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<tr>
<td>Polar</td>
<td>Moderately Polar</td>
<td>Non-polar</td>
</tr>
</tbody>
</table>

- **n-BPC Normal Bonded Phase Chromatography**
- **LSC Liquid-Solid Chromatography**
- **RPC Reversed Phase Chromatography**

**Yes, Soluble in organic solvent**

Are the target molecules soluble in polar, moderately polar or non-polar organic solvents?

If nonpolar, a reversed phase type sorbent will work best.

For methanol soluble compounds, frequently the reversed phase type sorbents will be effective, but greater selectivity can be obtained with a normal phase type sorbent.

As with water soluble compounds, the other sorbents are used for matrix-adsorption clean-up methods.
Organic Soluble Target Compounds in Aqueous Matrix- Tips

- Condition with methanol. Equilibrate with water, modify the pH up if target molecules are slightly basic, down if slightly acidic.

- Acceptable polar loading and washing solvents are:
  Water, buffered water to adjust the pH, water with small amount (1-10% methanol)

- Acceptable organic elution solvents are:
  Methanol, acetonitrile, ethyl acetate, acetone, isopropanol
Remember: Effect of pH on several bonded silica sorbents

- C2 – sorbent is stable between pH 5 and pH 8
- C18 – sorbent is stable between pH 2 and pH 8
- Amino – sorbent is stable between pH 5 and pH 7.8
- Polymeric mixed mode – sorbent is stable at all pH ‘s
All of Agilent’s polymer phases exhibit mixed-mode behavior. This characteristic results in the ability to retain target molecules over a wide range of pKa’s in any cartridge type. As a starting place for method development, the chart above can be used to identify the cartridge type for any application.

Strongly hydrophilic compounds, acids and bases should use SampliQ SCX or SAX.
What is Water Wettable? Applies to polymeric sorbents only.

- Highly reproducible recoveries wet or dry
  - Cartridges dried under vacuum for 10 minutes before the equilibration step
- RSD’s of the recoveries for each of the compounds (n=5) very low
- Compounds range from very polar, basic compounds to hydrophobic, neutral
Neutral, Hydrophobic, Non-Polar Target -- Aqueous Matrix

naphthalene

Tylenol

Dimetapp
SampliQ-OPT® Generic Method for 3mL Cartridge

1. Condition 3mL methanol

2. Equilibrate 3mL water
   - Recommended flow through cartridge: not faster than 1mL per minute
   - Dry <1 minute

3. Load 1mL prepared sample spiked with internal standard in water
   - Dry <1 minute

4. Wash 1mL 5% - 10% methanol in water
   - Dry 3 minutes

5. Elute 2mL methanol or 0.1% formic acid in methanol

6. Dry and reconstitute in mobile phase
Tips to optimize recovery on OPT

- Change the elution solvent (isopropanol, ethyl acetate or acetonitrile instead of methanol)
- Change the composition of the elution solvent (decrease and/or increase the % water in the elution solvent)
- Change the pH of the elution solvent (0.1% formic acid for enhancing neutral and base compound recovery on OPT)
- Add salt to the elution solvent (20mM ammonium acetate for OPT)
- Change the volume of solvent used (elution and wash solvents)
- Test fewer or more wash steps with different strength solvents
- Change the pH of the wash solvent (increase and decrease pH compared to current pH)
Acidic, basic, hydrophilic,

melamine

secobarbital

Aleve
Tips – Ionic Compounds in Aqueous or Organic Matrix

- Condition with methanol. Equilibrate with water, modify the pH down if target molecules are basic, up if acidic.
- Keep loading and wash flows slow (0.5 mL/min)
- Keep ionic strength low. <0.1M salt. May need to dilute sample.
**SampliQ-SCX® Generic Method Development Process**

1. **Condition 2% formic acid in methanol**
2. **Equilibrate 2% formic acid in water**
3. **Load prepared sample spiked with internal standard**
4. **Wash 2% formic acid in water**
5. **Interference removal: 2mL methanol**
6. **Elute with 5% NH4OH in methanol**
7. **Dry and reconstitute in mobile phase**

- Recommended flow through cartridge: not faster than 1mL per minute
- Dry <1 minute
- Dry 3 minutes
- Dry <1 minute

Methanol fraction may be collected if basic and neutral compound capture is desired.
**SampliQ-SAX** ® **Method Development Process for 3mL Cartridge**

1. **Condition 3mL methanol**
2. **Equilibrate 3mL water**
3. **Load 1mL prepared sample in phosphate saline buffer (pH 7) spiked with internal standard**
4. **Wash 1mL 50mM sodium acetate (pH7) in 5% methanol**
5. **Interference removal: 2mL methanol**
6. **Elute with 2mL, 2% formic acid in methanol**
7. **Dry and reconstitute in mobile phase**

**Recommended flow through cartridge:** not faster than 1mL per minute

**Dry:**
- <1 minute
- 2 minutes

Methanol fraction may be collected if basic and neutral compound capture is desired.
Method Development Tips – ion exchange

**Equilibration solvent**: methanol
   adjust pH to match conditioning solvent

**Conditioning solvent**: water
   pH adjusted to at least 2 units below the pKa of the target bases and 2 units above pKa of acids

**Loading solvent**: same as conditioning solvent

**Wash solvent**: organic modified conditioning solvent

**Elution solvent 1**: methanol

**Elution solvent 2**: methanol modified with:
   1) high ionic strength solution
   2) pH increased 2 units above the pKa of the target
Ion Exchange -

Remember:

With strong ion exchanger, the sorbent is charged at ALL pHs. Thus, in developing the SPE experiment, controlling the pH of the solution with the analyte in mind is all that is needed. (pH for retention below pKa of basic analyte and above for acidic analyte)

Ionic strength of the solution is critical. At high ionic strength (>0.1M) compounds will not retain regardless of pH. This is useful for elution because both pH and ionic strength can be adjusted to optimize release of analyte.

Compounds with multiple function groups require more optimization
Using Polymer SAX for Selective Fractionation of Neutral and Acidic Compounds

- For acidic and neutral compounds, two different polymer SAX cartridges show similar recoveries.

- The neutral compounds (secobarbital and nortriptyline) are isolated in a methanol eluent. The other compounds (all acids) are isolated in an acidic methanol eluent.
Method development - Summary

1. **Analyte assessment**
   1. Solubility
   2. pKa – single or multiple types of functional groups
   3. Chelating behavior
   4. Stability
   5. Protein binding

2. **Binding mechanism**
   1. Reversed phase (non-polar), normal phase (polar), anion exchange, cation exchange

3. **Phase selection**
   1. Frequently requires several cartridge tests

4. **Optimization**
   1. Frequently requires several wash experiments (increasing % organic)
   2. Frequently requires several elution experiments (decreasing % organic)
   3. Ruggedness tests in batch analysis of representative samples
Troubleshooting Sample Prep.

Problems areas:

1. Poor recovery
2. Poor reproducibility
3. Insufficiently clean
Poor Recovery, Poor Reproducibility

Flow-rate - speed kills

Applies to ALL sorbents

- Changes in sample: particulate, viscosity
- Changes in handling: filters
- Changes in SPE
Poor Recovery

Determine where the analyte is lost

A. Sample pretreatment
B. Load step and/or Wash
C. Not eluting
B. Load and Wash Losses

Problem: Target compounds are not retained effectively

Check: conditioning and equilibration: does the solvent standard recovery change?

Check: with silica cartridges is there any drying of the sorbent prior to loading?

Check: with ion exchange: does conditioning in 0.1% formic acid for SCX or 0.50mM ammonium acetate for SAX improve the recovery?

Check: does diluting the sample improve retention?

Check: Decrease the volume of wash

Check: Decrease the % organic in the wash

Check: Increase the amount of sorbent
C. Irreversible Binding to Sorbent

Problem: Target compounds are not eluting effectively

Check: has the solvent standard recovery changed?
Check: can the compound form complexes with salts, proteins?
Check: with silica cartridges is there any drying of the sorbent prior to loading?
Check: increase the ionic strength of the eluent to 100mM ammonium acetate
Check: increase the elution pH use 0.02% ammonium hydroxide in methanol
Check: decrease the elution pH with 0.1% formic acid in methanol
Check: increase the number of elution steps
Check: Change to a stronger elution solvent
Check: try a 2 minute ‘soak’ step
Check: try a less retentive sorbent
Solvent strength

Stronger elution solvents for normal phase

Water
Methanol
Acetonitrile
Isopropanol
Ethyl acetate
Tetrahydrofuran

Stronger elution solvents for reversed phase
2. Poor Reproducibility

**Analytical stability** – make sure the instrumentation is not contributing to the problem

**Matrix interferences**
- Ion suppression
- Protein binding

**Sample pre-treatment**

**Lot to lot variability of sorbent**
3. Insufficiently Clean

Symptoms of a sample which needs further clean-up are:

– poor reproducibility
– column failure (high backpressure, changing retention times)
– incorrect quantitation on QC samples
3. Insufficiently Clean

Fixes:

- ✓ try a different sorbent which uses the same retention mechanism (i.e. try silica C18 end capped instead of silica C18)
- ✓ change to a different retention mechanism sorbent
- ✓ change the wash solvent
- ✓ change the ionic strength (when using ion exchange sorbents)
- ✓ change the pH of the load and wash
- ✓ change the % organic of the load and wash
3. Insufficiently Clean

Fixes

✓ multiple wash steps
✓ add an acidified wash if protein binding is a problem
✓ use a low % organic followed by higher % organic if protein precipitation is a problem
✓ add a wash with a solvent that the sample is insoluble in but that may clean out interferences
✓ change the elution solvent
✓ using a weaker elution solvent
✓ filter the sample
Agilent SampliQ Quality Controls

- Rigorous particle size controls
- High surface coverage reduces the formation of fine
- Tri-functional bonded surfaces – results in a higher carbon load and fewer active silanol surface groups. (more consistent recoveries)
- Vacuum-packed cartridge bags
- Manufactured in Delaware along with Zorbax HPLC column manufacturing
- Certificate of Performance Shipped with each box of cartridges
- Material Safety Data Sheets (MSDS) available on-line
What’s New From Agilent

- Completely refreshed line of silica based sorbents
- Graphitized Carbon
- Mixed mode ion exchange (SCX and SAX)
- Mixed mode reverse phase (OPT)

www.agilent.com/chem/sampliq
Chloramphenicol in Honey
Using Agilent SampliQ OPT

broad spectrum bacteriostatic antibiotic
toxic side effects in humans
use of chloramphenicol in animal-derived foods is strictly regulated
European Union maximum residue limit (MRL) in food is 0.3 µg/kg
thiamphenicol and florfenicol are analogue compounds used to replace chloramphenicol
extremely effective for controlling infections in bee colonies

pKa 9.6 (basic)
Log P 1.01 (organic soluble)
Sample preparation– Liquid Liquid Extraction of Phenics in Honey

Accurately weigh 5g honey (±0.05g) in 50mL centrifuge tube

Spike 0.5mL of IS solution (50ng/mL of Chloramphenicol-D₅ in H₂O), vortex 1min for mixing.

Add 5mL of H₂O, vortex vigorously for 3min.

Add 5mL of Ethyl Acetate, then shake for 5min.

Centrifuge @ 3200rpm for 5min, transfer the upper organic layer to another tube.

Combine all of transferred organic layer (~ 14mL), blow down with N₂ flow @ 50°C

Reconstitute into 5mL of H₂O, vortex for 3min and sonicate for 2min

Ready for SPE
Sample clean-up – SampliQ OPT Solid Phase Extraction

1. **Condition 3mL methanol**

2. **Equilibrate 5mL Milli-Q H₂O (2.5mL x2)**

3. **Load 5mL extract (from previous sample preparation, 2.5mL x2), have sample pass through cartridge slowly with gravity.**

4. **Rinse the sample tubes and wash cartridge with 5mL x2 water**

5. **Apply full vacuum for 3min, dry the needle tip, put the collection tubes below**

6. **Elute with 5mL 2:8 Ethyl Acetate/MeOH (2.5mL x2)**

7. **Blow down @ 50°C, reconstitute into 0.5mL of 20:80 AcN/H₂O. Vortex 3min for mixing, then sonicate 2min.**

8. **Centrifuge @ 3200rpm for 2min, transfer to a 2mL autosampler vial for injection**
Chromatograms of 0.2 ng/g fortified honey extract.

- MRM (355.8 → 185.0) with Florefenicol at 4.307 min.
- MRM (354.0 → 184.9) with Thiamphenicol at 3.227 min.
- MRM (325.9 → 156.8) with Chloramphenicol-D₅ at 5.096 min.
- MRM (320.9 → 152.0) with Chloramphenicol at 5.079 min.
## LC/MS/MS Results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spike Level ng/g</th>
<th>% recovery</th>
<th>% RSD n=6</th>
<th>MRL ng/g</th>
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<tr>
<td>Chloramphenicol</td>
<td>0.1</td>
<td>96.4</td>
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<td>Florfenicol</td>
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<td>5990-3364EN</td>
<td>Determination of Penicillins in Meat by High Performance Liquid Chromatography (HPLC/UV) and HPLC/MS/MS</td>
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<td>5990-3235EN</td>
<td>Determination of Benzimidazole Fungicides in Apple Juice by SampliQ Polymer SCX Solid-Phase Extraction with High-Performance Liquid Chromatography</td>
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<tr>
<td>5990-3365EN</td>
<td>Determination of Melamine Residue in Milk Powder and Egg Using Agilent SampliQ Polymer SCX Solid Phase Extraction and the Agilent 1200 Series HPLC/UV</td>
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Questions??

www.agilent.com/chem/SampliQ