Sample Preparation Techniques for Biological Matrices: 
Finding the right balance to achieve optimal results
Today’s Agenda

- Why sample preparation?
- Overview of sample preparation options
- Common challenges in bioanalysis applications
- Sample preparation solutions to these challenges
- Summary, Questions and Wrap Up
Biological Sample Analysis

THE IMPORTANCE OF SAMPLE PREPARATION
Sample Analysis Workflow Diagram in Biological Sample Testing

Sample Collection/Sampling → Sample Preparation → Sample Analysis → Data Handling

- Sample Analysis: 23.5 ng/mL c-THC

Archiving → Information to Customer → Report Generation

Sample Transport
Why is Sample Preparation Required?

- **Concentration**: Target analyte(s) not concentrated enough for quantitative detection
- **Compatibility**: Sample is not compatible with or would be harmful to your chromatographic system
- **Cleanliness**: Sample matrix components will interfere with the analysis
## Possible Effects of Biological Sample Components

<table>
<thead>
<tr>
<th>Issue</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor peak shapes, co-elution, no resolution</td>
<td>Difficulty in identifying, quantifying components</td>
</tr>
<tr>
<td>Mechanical issues (particulates, blockages)</td>
<td>LC/GC column lifetime issues</td>
</tr>
<tr>
<td>Increased instrument downtime</td>
<td>Reduced productivity, increase in sample run time / cost</td>
</tr>
<tr>
<td>Interferences</td>
<td>Ion suppression in LC-mass spectrometry</td>
</tr>
<tr>
<td></td>
<td>Peak integration issues</td>
</tr>
<tr>
<td>Overall lower sensitivity</td>
<td>Inability to meet detection limits</td>
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</table>
Striking the Right Balance in Sample Preparation

![Graph showing the relationship between Effort & Investment and Quality of Results, with points labeled Ideal, Realistic, and Just Right.](image-url)
Biological Sample Analysis

SAMPLE PREPARATION OPTIONS
Sample Preparation Techniques For Today’s Discussion

1. Filtration
   - Basic particulate removal from ALL kinds of samples
   - Useful when additional step of lipid content removal is needed

2. Liquid-Liquid Extraction
   - Straightforward sample preparation technique
   - Useful for in-house or commercial extraction

3. Solid supported liquid extraction (SLE)
   - Increased productivity using liquid/liquid extraction principle and the concept of automation
   - Ideal for aqueous sample

4. Solid phase extraction (SPE)
   - Ultra-clean sample preparation for analysis when high selectivity and sensitivity are required
Sample Prep Options: An Overview

- Direct injection
- Dilute & Shoot
- Filtration
- Liquid/liquid extraction (LLE, SLE)
- QuEChERS
- Solid phase extraction (SPE)
- MIPS and Immunoaffinity Columns
Dilution (Dilute & Shoot)

Simple sample dilution

Advantages

• Fast and easy
• High throughput

Limitations

• Interferences are not removed
• Concentration is reduced
• Instrument and column contamination
• Matrix interferences – ion suppression or poor peak shapes
Filtration

- Basic sample prep method for ALL kinds of samples
- Can be the 1st choice of sample prep or a secondary step
- Mechanical filtration for visible interference removal
  - Syringe filters
  - Syringeless filters (filter vials)
  - Agilent Captiva (cartridge and 96-well plate formats)
  - Agilent Captiva ND (cartridge and 96-well plate formats)

- Mechanical filtration + extraction by sorbent for lipid removal
  - Agilent Captiva ND Lipids (cartridge and 96-well plate formats)
## Sample Preparation: Liquid-Liquid Extraction

<table>
<thead>
<tr>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic salts easily removed</td>
<td>Labor-intensive</td>
</tr>
<tr>
<td>Short method development time</td>
<td>Large volumes of organics</td>
</tr>
<tr>
<td>Low cost</td>
<td>Difficult to automate</td>
</tr>
<tr>
<td>Flexible for a variety of sample types</td>
<td>Variable results</td>
</tr>
<tr>
<td>Easy to perform</td>
<td>Expensive, clean glassware</td>
</tr>
<tr>
<td></td>
<td>Emulsion formation</td>
</tr>
</tbody>
</table>
Solid Supported Liquid Extraction (SLE)

- Extraction mechanism: same as traditional liquid/liquid extraction (LLE)
- Simple, time-saving process
  - Apply aqueous sample to the solid bed
  - Extract with water-immiscible solvent (MTBE, dichloromethane, ethyl acetate)
  - Analyze extract or evaporate and reconstitute as needed
- Convert LLE methods to SLE to save time and money, and increase throughput
SLE- Benefits

• No emulsions → easier extractions
• No special glassware → lower cost per sample
• Less time, minimal method development → faster implementation
• Reduced technique dependence → better ruggedness
• Increased reproducibility → better results
• Automatable → enables batch processing
Solid Phase Extraction (SPE)

- Types of SPE
  - Reversed phase SPE
  - Cation exchange SPE
  - Anion exchange SPE
  - Polar SPE
  - Mixed mode SPE
  - Specialty SPE

- Capabilities
  - Very selective
  - Highly clean samples
  - Wide range of applicability
  - Automation friendly
A Typical SPE Sequence

• Condition the cartridge (Step 1)

• Apply sample (e.g. food extract, water, plasma) (Step 2)
  - Some compounds “retain”

• First wash of the cartridge, interference removal (Step 3a)

• Second wash of the cartridge, additional interference removal (Step 3b)

• Apply a different liquid to “elute” (Step 4)

✓ The extract is cleaner, in a different liquid, and typically more concentrated

Some sorbent technologies let you reduce the number of steps for easier, faster extractions
Why Choose SPE?

- Flexible - match a broad spectrum of sample and target compound types to different sorbents and forms
- Wide array of formats and sorbents for lower detection limits and longer instrument uptime from cleaner extracts
- Increase sample throughput with automation-friendly formats
- Easy adoption of methods due to high number of publications and applications
- Get the right answer the first time with highest accuracy and confidence
- Best balance of sample cleanliness, accuracy of results, and cost-per-sample
How do Sample Prep Options Compare?

Solid Phase Extraction  *(Agilent Bond Elut)*
- Often very clean, allows for trace analysis, built in concentration
- Potential for the most selectivity (and hence cleanliness)

Liquid/Solid Extraction  *(Agilent Chem Elut, Agilent QuEChERS)*
- Relatively clean and inexpensive

Filtration  *(Agilent Captiva Syringe Filters, Tubes, Plates, and Vials)*
- Dilute and shoot (guard columns or retention gaps)
COMMON CHALLENGES IN BIOLOGICAL SAMPLE ANALYSIS
Agilent Sample Preparation Solutions

Matrix Challenges +

Lab Goals & Needs
Tandem Mass Spectrometry and “The Case of the Disappearing Matrix”

Selected ion monitoring looking at 100 fg HCB in matrix

MRM’s better selectivity makes the matrix “disappear”
Ion Suppression: What Can Dirty Samples Do?

<table>
<thead>
<tr>
<th>Interference type</th>
<th>Salt/Polar ionics</th>
<th>Proteins/ Peptides</th>
<th>Lyso-phosphatidylcholines</th>
<th>Lipids and other hydrophobics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Typical Elution Conditions (C18 column)</strong></td>
<td>At or near void with &lt; 20% organic</td>
<td>10’s of column volumes at 40% - 70% organic</td>
<td>10’s of column volumes at 70% - 90% organic</td>
<td>10’s to 100’s of column volumes at &gt; 90% organic</td>
</tr>
<tr>
<td><strong>Short term effect (single injection)</strong></td>
<td>Significant ion-suppression</td>
<td>Significant ion-suppression</td>
<td>Significant ion-suppression</td>
<td>Some ion suppression, however, usually retained on LC column</td>
</tr>
<tr>
<td><strong>Long term effect (multiple injections)</strong></td>
<td>Unknown</td>
<td>Unknown</td>
<td>Decreased sensitivity, Increased variability</td>
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</tr>
<tr>
<td><strong>Likely long term causes</strong></td>
<td>Ion source contamination</td>
<td>Ion source contamination</td>
<td>Ion source contamination, Some column build-up</td>
<td>Ion source contamination, Column build-up</td>
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Challenge: Instrument Contamination

Example 1

Salt build-up in LC-MS ion source from unextracted salts
Challenge: Instrument Contamination

Example 2

Orthogonal ESI Ion Source Condition after 3000x Urine Dilute/shoot Injections

Spray Shield/MS Inlet/Capillary

Nebulizer/Sprayer
Challenge: Instrument Contamination

Example 3

GC System Component Contamination with Biological Samples

GC Inlet Liner

GC Inlet Seal
**Solution: Select an Agilent Sample Preparation Product Based on Interference Removal**

<table>
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<tr>
<th>Particulates</th>
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<th>Lipids</th>
<th>Surfactants</th>
<th>Salts</th>
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Lipids that Cause Interference and System Cleanliness Issues

Two major lipid interferences that exist during bioanalysis by LC/MS/MS. By soft ionization conditions, these two phospholipids can generate the common fragment, 184 m/z. Removal of ion suppressing lipids can be verified by monitoring 184→184 m/z transition.
Solution: Targeted Lipid Removal

Green = ppt only
Red = lipid-stripped ppt with Captiva ND Lipids

MS Transition 184 → 184
Plasma spiked with beta blockers was diluted by 2% ammonia 1:1.

Load 0.3 mL of spiked & diluted plasma to VersaPlate.

Apply slight vacuum to initiate flow. When sample is soaked below the top frit stop vacuum.

Wait for 5 min for aqueous sample adsorption.

Elute with 2 X 0.9 mL of EtOAc. Apply vacuum to have 1 – 2 drops per sec. and collect the eluate

After elution increase vacuum for 30 sec.

Evaporate and reconstitute in 0.15 mL of 30% MeOH for LC/MS/MS analysis.
Beta-Blockers by SLE – Chromatography

- Acebutolol (ISTD)
- Timolol
- Nadolol
- Metoprolol
- Propranolol
- Pindolol
Solution: Remove Proteins & Lipids Using SPE

Exploit the chemistry and physical properties of Agilent Bond Elut Plexa SPE sorbent

- Albumin and proteins of similar size will be excluded.
- Lipids adsorb and do not elute under normal elution conditions.
- Polar and non-polar drugs bind in the SDVB hydrophobic end of the pore structure.
Solid Phase Extraction of Buprenorphine and nor-Buprenorphine in Whole Blood

SPE using Agilent Bond Elut Plexa PCX polymeric cation exchange

- Buprenorphine and nor-buprenorphine were efficiently extracted from whole blood
- Extraction method was simple and used only 0.5 mL of whole blood
- Mixed-mode cation exchange SPE ensures removal of matrix and high recoveries of targets
- Precise, reproducible extraction with low detection limits
Challenge: Degradation of Sub-2-µm LC Column Performance Over Time

- Unfiltered Plasma extract
- Centrifuged Plasma extract

Number of Injections of Unfiltered, Centrifuged and Filtered Human Plasma PPT Extract
Solution: Filtration Extends Column Lifetime

![Graph showing column backpressure with different plasma extracts and filtration options.]

- Unfiltered Plasma extract
- 0.2 μm filtered Plasma extract (by Agilent Captiva 0.2 μm filters)
- Centrifuged Plasma extract

Number of Injections of Unfiltered, Centrifuged and Filtered Human Plasma PPT Extract
Challenge: Unexplained Peaks in Sample

**ESI-Positive Mode**

- Filtered 30% MeOH by **Filter W** 0.2µm filter
- Filtered 30% MeOH by **Filter M** PES 0.2µm filter
- Filtered 30% MeOH by **Filter P** PES 0.2µm filter
- Unfiltered 30% MeOH
Solution: Use Certified Clean Filtration Products

ESI-Positive Mode

- Filtered 30% MeOH by **Filter W 0.2µm filter**
- Filtered 30% MeOH by **Filter M PES 0.2µm filter**
- Filtered 30% MeOH by **Filter P PES 0.2µm filter**
- Filtered 30% MeOH by **Agilent PES 0.2µm filter**
- Unfiltered 30% MeOH
Challenge: Sample Throughput and Productivity

- 100s or 1000s of samples a day to be processed
- Consistency and simplicity to achieve high-quality results with few repeats
- Limit the operator-to-operator and day-to-day variation (ruggedness)
- Support for automation for increased throughput and unattended operation
Solution: Replace Conventional Protein Precipitation with Captiva ND Lipids

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<th>Time (min)</th>
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<td>Add 0.6 mL of MeOH and 0.2 mL of plasma sample to Captiva ND 96-well plate.</td>
<td>5</td>
</tr>
<tr>
<td>Mix each well with a pipette 5 times and apply vacuum for filtration.</td>
<td></td>
</tr>
<tr>
<td>Directly collect filtrate into injection plate for analysis.</td>
<td>0</td>
</tr>
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This time comparison is based on the preparation of 96 samples.
Results: Captiva ND Lipids for Beta-blockers in Plasma

Acebutolol (ISTD)

Timolol

Nadolol

Atenolol

Propranolol

Pindolol

Good chromatography

Short Run Time
Agilent Captiva ND and Captiva ND Lipids Simplify Sample Prep

- Extended column lifetime especially for UHPLC system with sub-2 µm columns
- Designed for high throughput via automation (cartridges or 96-well plate formats)
- Reduced instrument downtime/maintenance/repair
- Fast! No conditioning, evaporation, and reconstitution steps are required. No analyte loss during washing and evaporation steps.
- Reduces ion suppression caused by lipids (Captiva ND Lipids)
Biological Sample Analysis

SUMMARY AND WRAP UP
Striking the Right Balance in Sample Preparation

![Graph showing the relationship between effort and investment vs. quality of results. The curve peaks at the point labeled "Just Right," with "Ideal" and "Realistic" markers.](image)
Solution: Select an Agilent Sample Preparation Product Based on Interference Removal

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Summary and Wrap-Up

- Biological sample analysis presents multiple challenges, both from the matrix and from the laboratory needs and goals
- Sample preparation is an investment that can help solve those challenges and achieve your analytical goals
- Matching the sample preparation approach to the challenge ensures that you are employing the right tool for the task

*Agilent’s sample preparation options and expertise are ideally suited for bioanalysis challenges*
Additional Resources and Application Support

Bond Elut Certify Methods Manual:

Bond Elut Certify Video:

Agilent QuEChERS Application Notebook:

Agilent Sample Preparation Catalog:

Agilent “Sample Preparation Fundamentals for Chromatography”, a SPP primer handbook:
www.agilent.com/chem/SamplePrepBook

Agilent Sample Preparation portfolio brochure:

Agilent Sample Preparation Technical and Application Support Contact Information*:
  Phone: 800-227-9770, Options 3, 3, 3
  Email: spp-support@agilent.com
* North America
Acknowledgements

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Questions?