Sample Preparation Techniques for Biological Matrices: Finding the right balance to achieve optimal results

Presenter:
Diana Wang
Applications Engineer
SPP-Support@agilent.com
Today’s Agenda

- Why sample prep
- 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

- Archiving
- Report Generation
- Information to customer

For Research Use Only. Not for use in Diagnostic procedures.
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>
</tr>
</tbody>
</table>
Striking the Right Balance in Sample Preparation

Just Right

Ideal

Realistic

Effort & Investment

Quality of Results
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 or samples

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 2nd secondary step
• Mechanical filtration for visible interference removal
  – Syringe filters
  – Syringeless filters
  – 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

**Advantages**

- Inorganic salts easily removed
- Short method development time
- Low cost
- Flexible for a variety of sample types
- Easy to perform

**Disadvantages**

- Labor-intensive
- Large volumes of organics
- Difficult to automate
- Variable results
- Expensive, clean glassware
- Emulsion formation

For Research Use Only. Not for use in Diagnostic procedures.
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
Solid-Supported LLE - 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
  – 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)

*For Research Use Only. Not for use in Diagnostic procedures.*
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”.

For Research Use Only. Not for use in Diagnostic procedures.
"Disappearing Matrix" – 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>Typical Elution Conditions (C18 column)</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>Short term effect (single injection)</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>Long term effect (multiple injections)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Decreased sensitivity, Increased variability</td>
<td>Decreased sensitivity, Increased variability</td>
</tr>
<tr>
<td>Likely long term causes</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>
</tr>
</tbody>
</table>
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

Nebulizer/Sprayer

Spray Shield/MS Inlet/Capillary
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

Particulates → Proteins → Lipids → Surfactants → Salts

Captiva Filtration
Captiva ND
Captiva ND Lipids
Chem Elut SLE
Bond Elut SPE
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
Solution: SLE Extraction of Beta-Blockers in Plasma

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.

↓

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

- Proteins Excluded
- Frequency of OH oligomers reduces
- Change in Polarity
- Polar and non-polar drugs bind in the SDVB hydrophobic end of the pore structure
- Polar drugs bind in hydrophobic end of pore structure
- Albumin and proteins of similar size will be excluded
- Lipids adsorb and do not elute under normal elution conditions
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

![Graph showing the comparison between Unfiltered Plasma extract and Centrifuged Plasma extract with respect to Column Backpressure (Bar) and Number of Injections of Unfiltered, Centrifuged and Filtered Human Plasma PPT Extract.](image)
Solution: Filtration Extends Column Lifetime

For Research Use Only. Not for use in Diagnostic procedures.
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

For Research Use Only. Not for use in Diagnostic procedures.
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

<table>
<thead>
<tr>
<th>Captiva ND Lipids</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<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 transfer injection plate for analysis.</td>
<td>0</td>
</tr>
</tbody>
</table>

Total time required for sample preparation **5**
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)
Challenge: Sample Logistics and Handling

Traditional Blood Collection, Transportation, and Storage

For Research Use Only. Not for use in Diagnostic procedures.
Solution: Agilent DMS Cards for Sample Collection and Storage

Dried Matrix Spot (DMS)

Spot. → Dry. → Send DMS cards in an envelope.
Dried Matrix Spot, DMS (Analysis)

1. Punch DMS cards.
2. Put punched spot in a centrifugation tube with organic solvent e.g. 80% MeOH.
3. Add internal standard.
5. Take supernatant for analysis.
6. Or you can take further to combine other sample prep techniques such as SPE, Captiva ND Lipids, etc.

For Research Use Only. Not for use in Diagnostic procedures.
Biological Sample Analysis

SUMMARY AND WRAP UP
Striking the Right Balance in Sample Preparation

![Graph showing the relationship between effort and investment and quality of results. The graph illustrates that there is an optimal balance (Just Right) between effort and investment, where the quality of results is maximized. There is a distinct point labeled 'Ideal' and another labeled 'Realistic' on the graph.]

For Research Use Only. Not for use in Diagnostic procedures.
Solution: Select an Agilent Sample Preparation Product Based on Interference Removal

Particulates → Proteins → Lipids → Surfactants → Salts

Captiva Filtration → Captiva ND → Captiva ND Lipids → Chem Elut SLE → Bond Elut SPE

For Research Use Only.
Not for use in Diagnostic procedures.
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

Reference Materials and Guides:
Agilent Sample Preparation Catalog:
www.chem.agilent.com/SPPCatalog

Agilent Sample Preparation Products
www.agilent.com/chem/sampleprep

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

* North America
Acknowledgements

Agilent applications chemists, product managers, and technical support colleagues who contributed to this presentation.
Questions?