Sample Preparation Techniques for Biological Matrices:

Finding the right balance to achieve optimal results

Presenter:

Golnar Javadi
Applications Engineer
SPP-Support@agilent.com

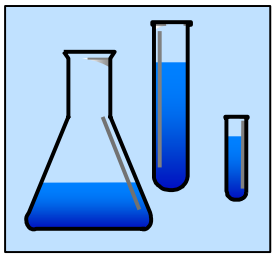
Today's Agenda

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

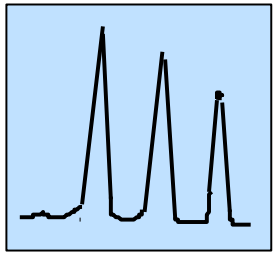
Biological Sample Analysis

THE IMPORTANCE OF SAMPLE PREPARATION

Sample Analysis Workflow Diagram in Biological Sample Testing



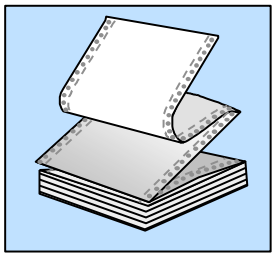
Sample Preparation



Sample Analysis

23.5 ng/mL
c-THC
✓

Data Handling

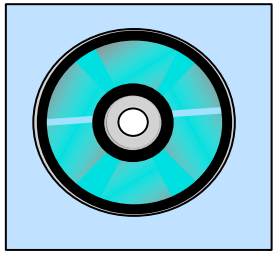


Report Generation

Information to customer

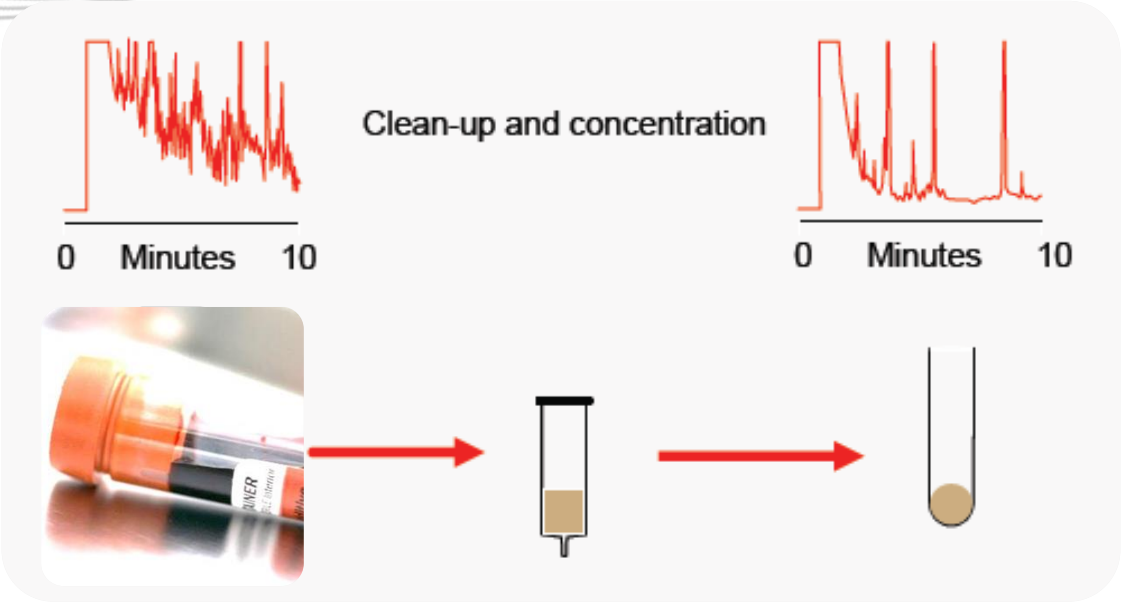
Sample Collection/ Sampling

Sample Transport



Archiving

Why is Sample Preparation Required?

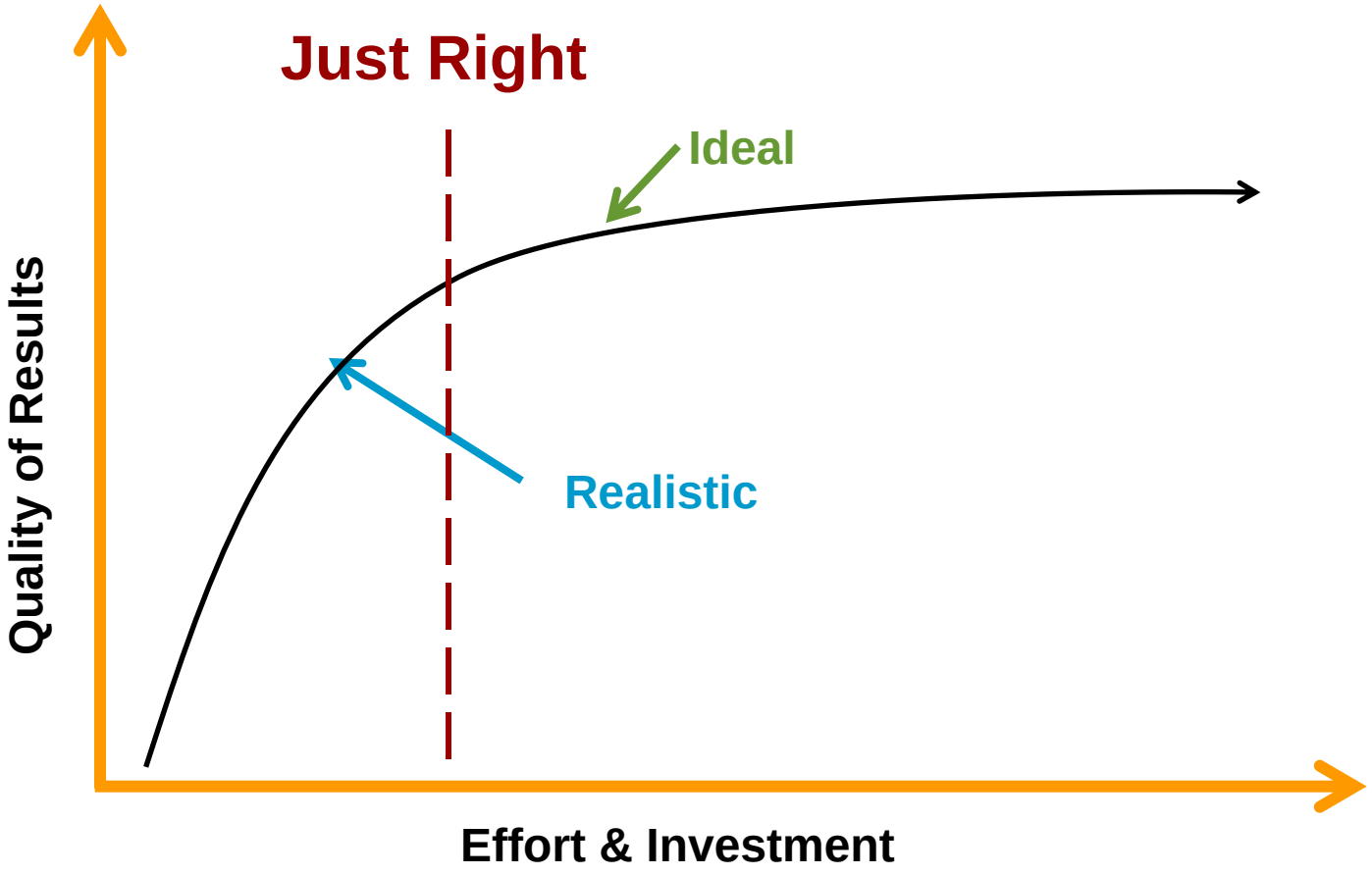


- **Concentration:** Target analyte(s) not concentrated enough for quantitative detection
- **Compatibility:** Sample is not compatible with or would be harmful to your chromatographic system
- **Cleanliness:** Sample matrix components will interfere with the analysis

Possible Effects of Biological Sample Components

Issue	Result
Poor peak shapes, co-elution, no resolution	Difficulty in identifying, quantifying components
Mechanical issues (particulates, blockages)	LC/GC column lifetime issues
Increased instrument downtime	Reduced productivity, increase in sample run time / cost
Interferences	Ion suppression in LC-mass spectrometry Peak integration issues
Overall lower sensitivity	Inability to meet detection limits

Striking the Right Balance in Sample Preparation



Biological Sample Analysis

SAMPLE PREPARATION OPTIONS

Sample Preparation Techniques For Today's Discussion

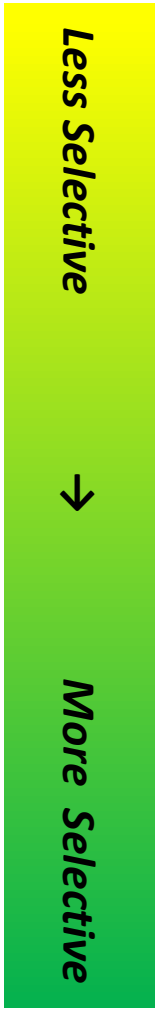
1. Filtration
   - Basic particulate removal from ALL kinds of samples
   - Useful when additional step of lipid content removal is needed
2. Liquid-Liquid Extraction
   - Straightforward sample preparation technique
   - Useful for in-house or commercial extraction
3. Solid supported liquid extraction (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

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

Solid Supported Liquid Extraction (SLE)

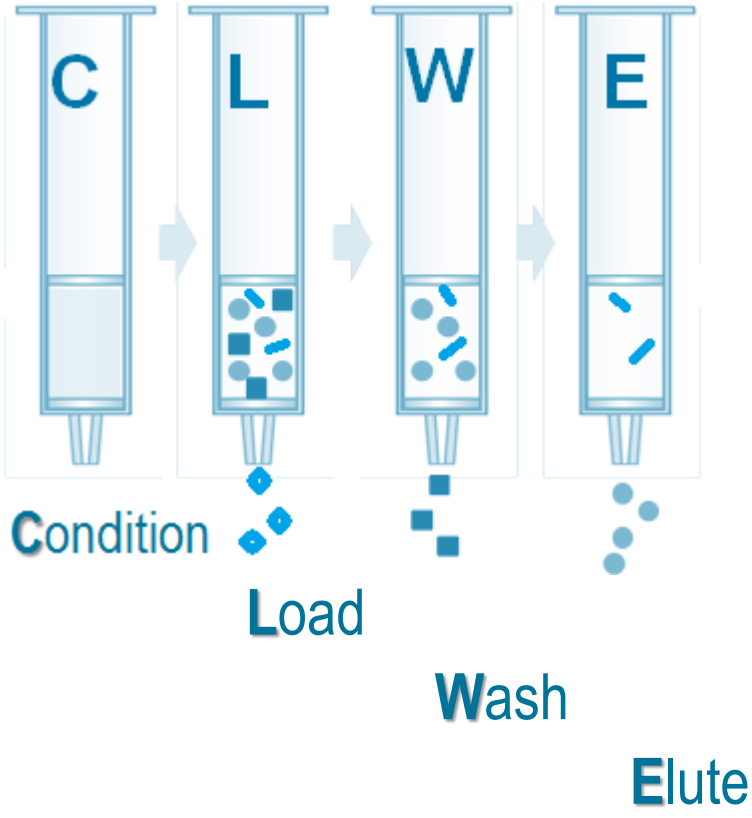
- Extraction mechanism: same as traditional liquid/liquid extraction (LLE)
- Simple, time-saving process
  - Apply aqueous sample to the solid bed
  - Extract with water-immiscible solvent (MTBE, dichloromethane, ethyl acetate)
  - Analyze extract or evaporate and reconstitute as needed
- Convert LLE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

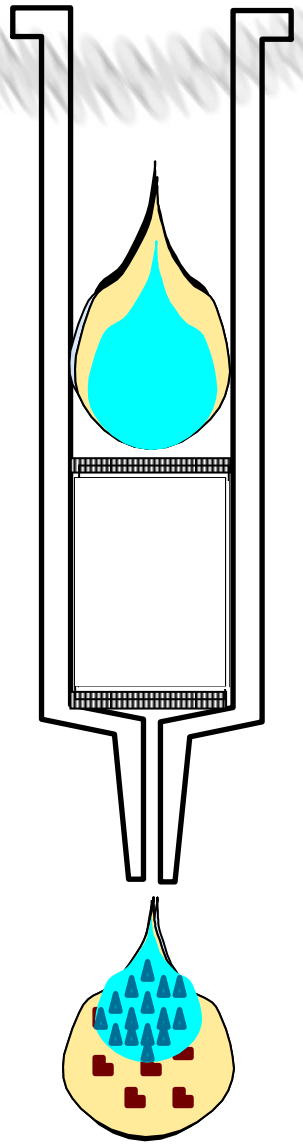
- Types of SPE
  - Reversed phase SPE
  - Cation exchange SPE
  - Anion exchange SPE
  - Polar SPE
  - Mixed mode SPE
  - Specialty SPE
- Capabilities
  - Very selective
  - Highly clean samples
  - Wide range of applicability
  - Automation friendly



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)**

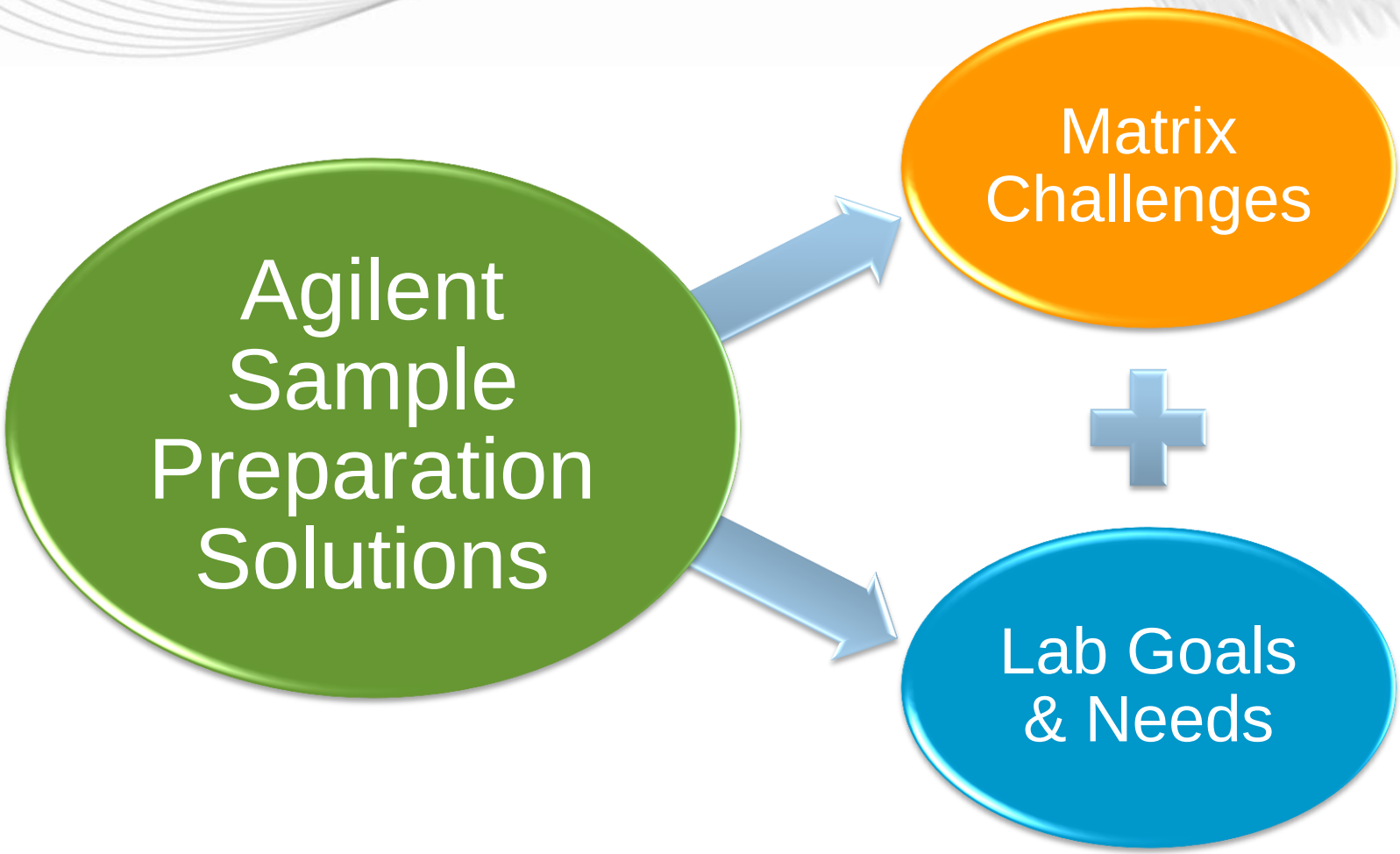
- Relatively clean and inexpensive

Filtration **(Agilent Captiva Syringe Filters, Tubes, Plates, and Vials)**

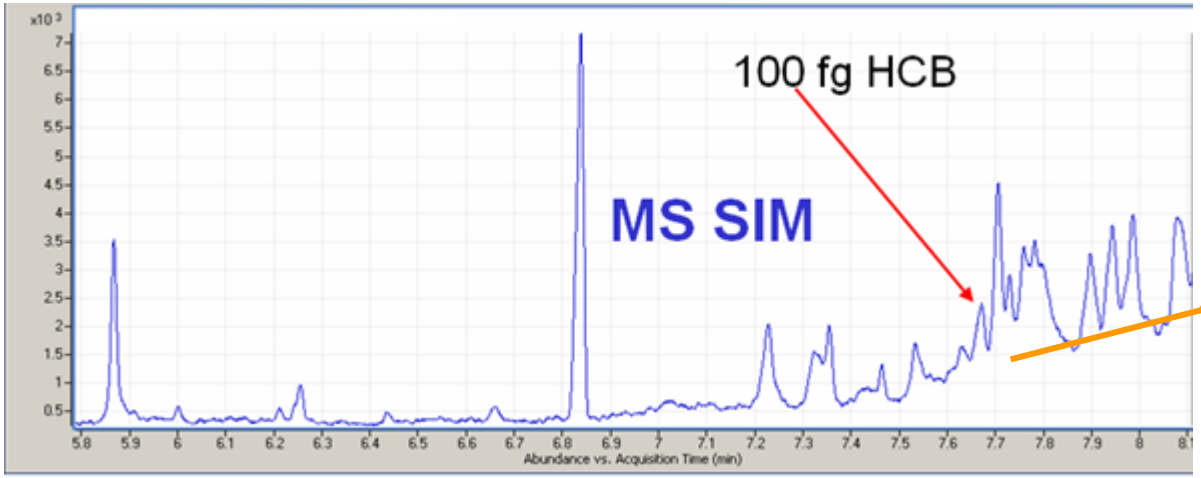
Dilute and shoot (guard columns or retention gaps)

Biological Sample Analysis

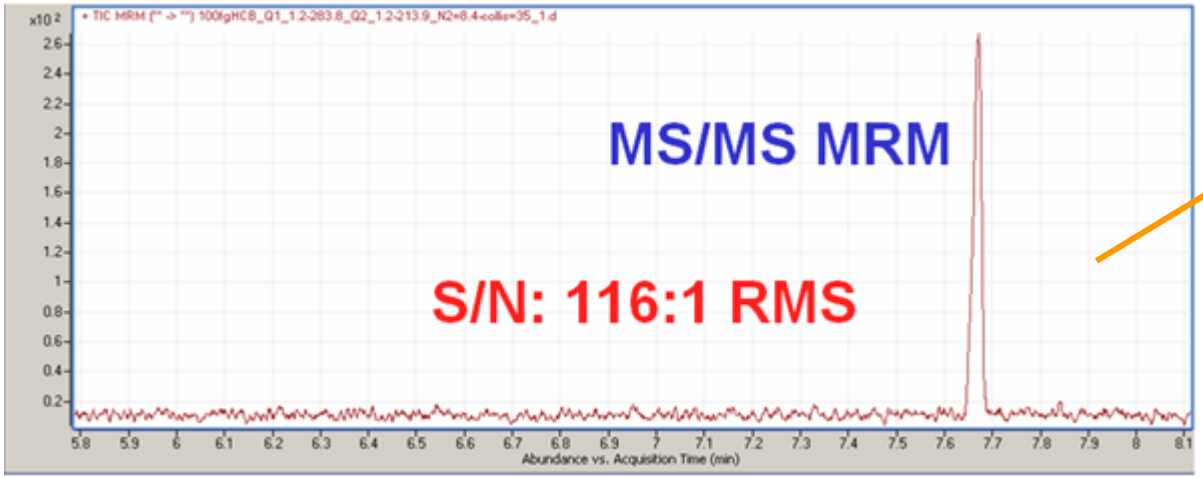
COMMON CHALLENGES IN BIOLOGICAL SAMPLE ANALYSIS


Agilent Sample Preparation Solutions
Matrix Challenges
Lab Goals & Needs

Tandem Mass Spectrometry and "The Case of the Disappearing Matrix"

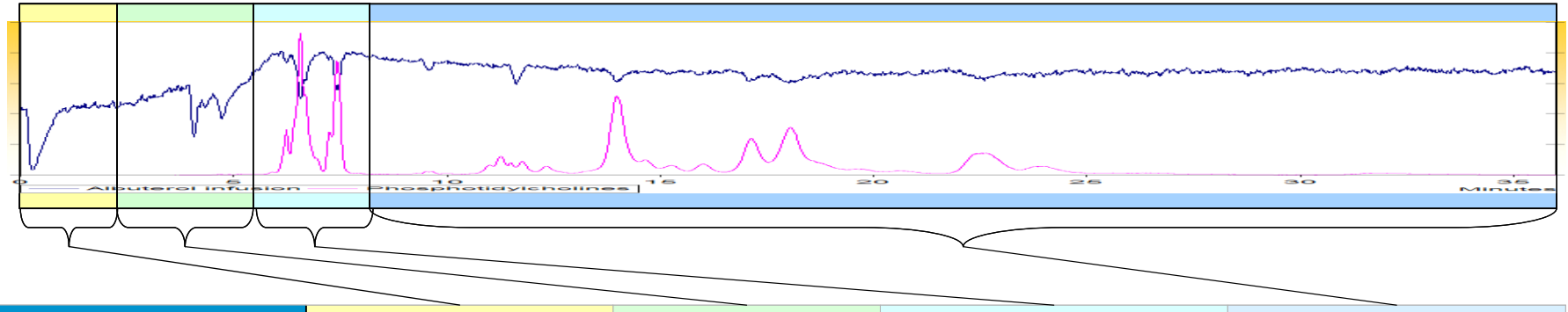


Selected ion monitoring looking at 100 fg HCB in matrix



MRM's better selectivity makes the matrix "disappear"

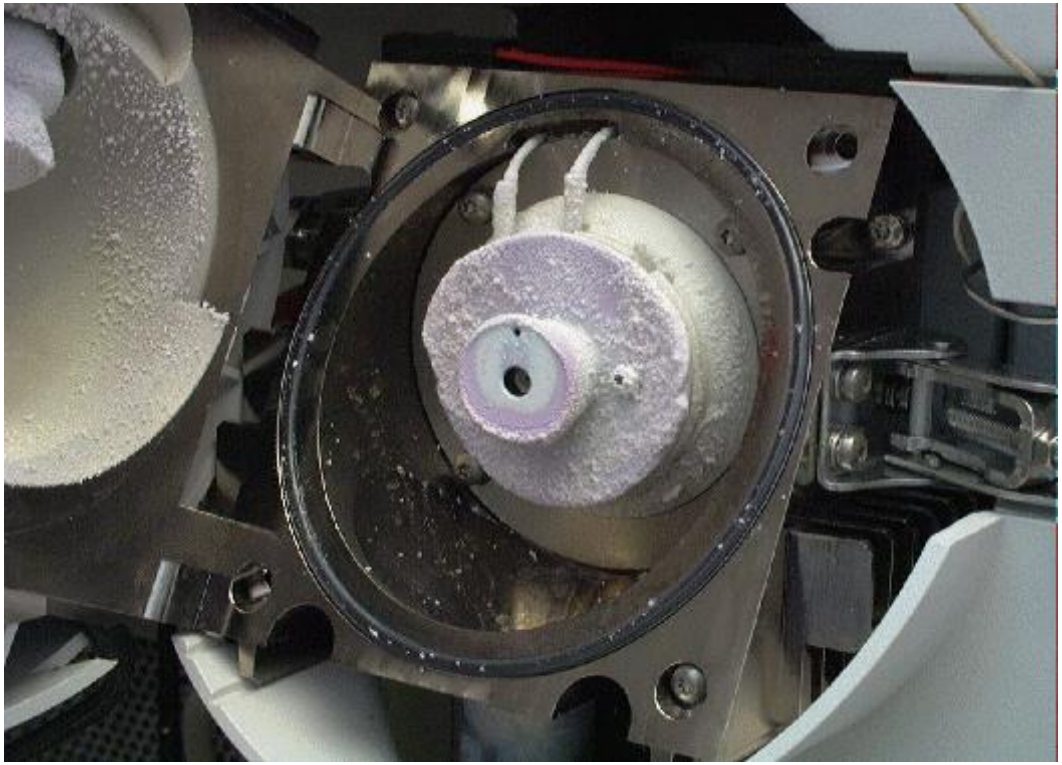
Ion Suppression: What Can Dirty Samples Do?



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

Challenge: Instrument Contamination

Example 1

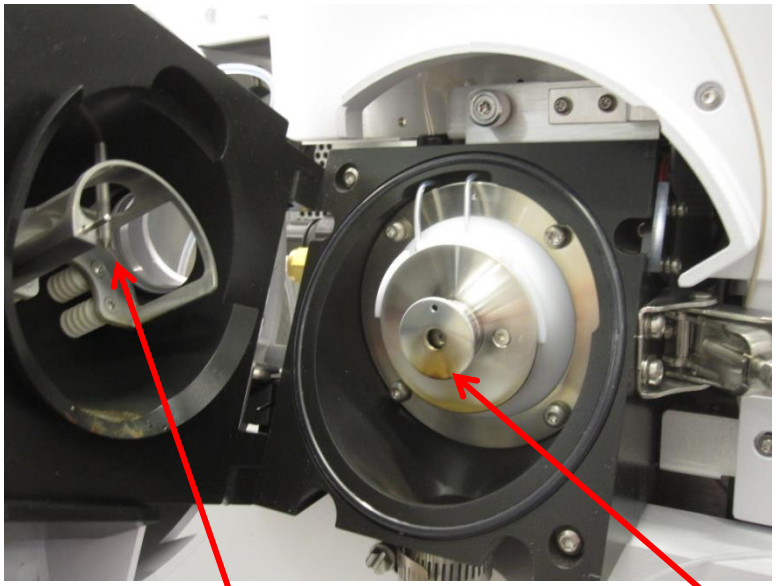


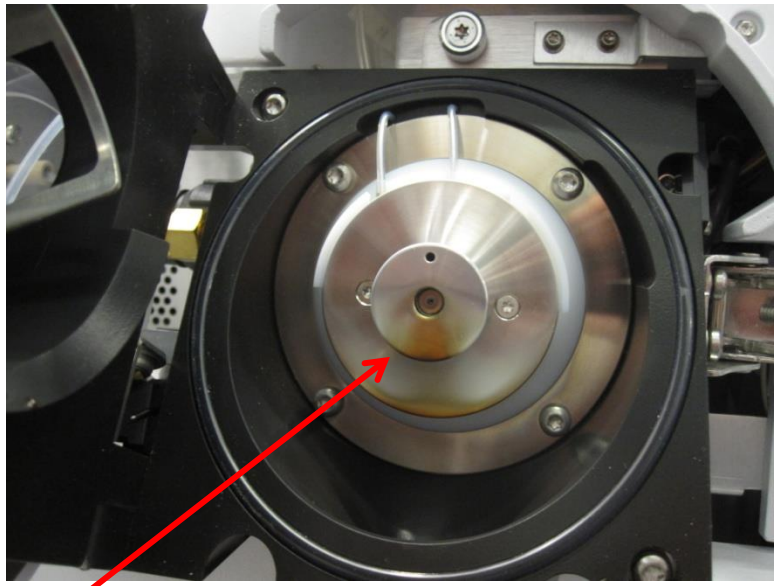
Salt build-up in LC-MS ion source from unextracted salts

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

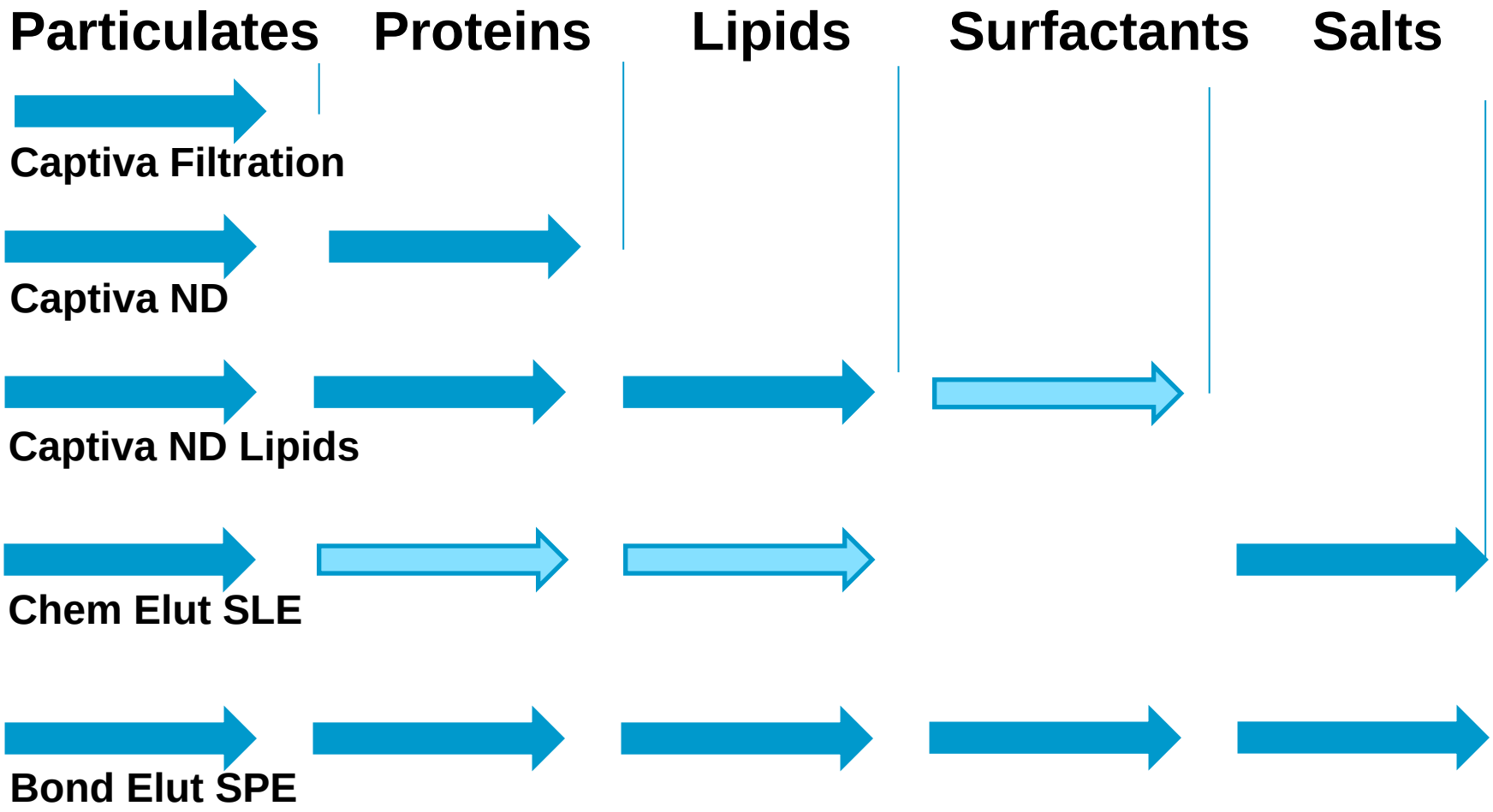
Example 3

GC System Component Contamination with Biological Samples

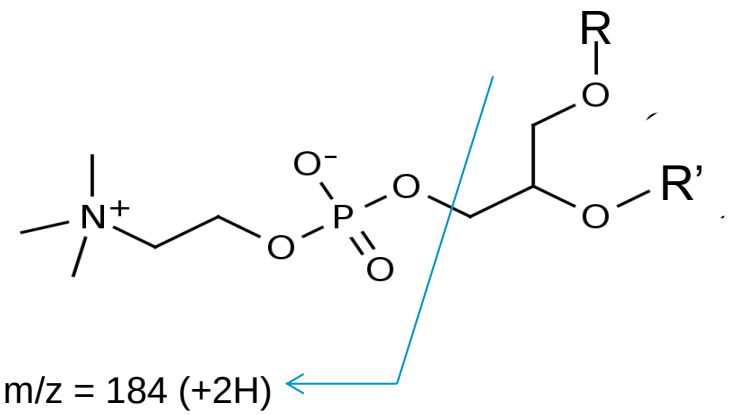
GC Inlet Liner

GC Inlet Seal

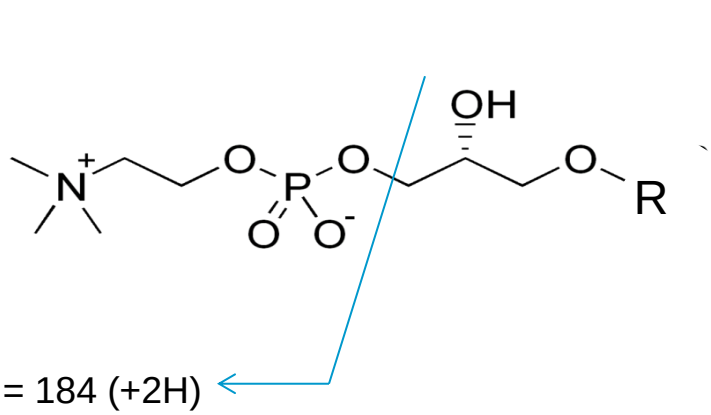
Solution: Select an Agilent Sample Preparation Product Based on Interference Removal



Lipids that Cause Interference and System Cleanliness Issues



phosphatidylcholine



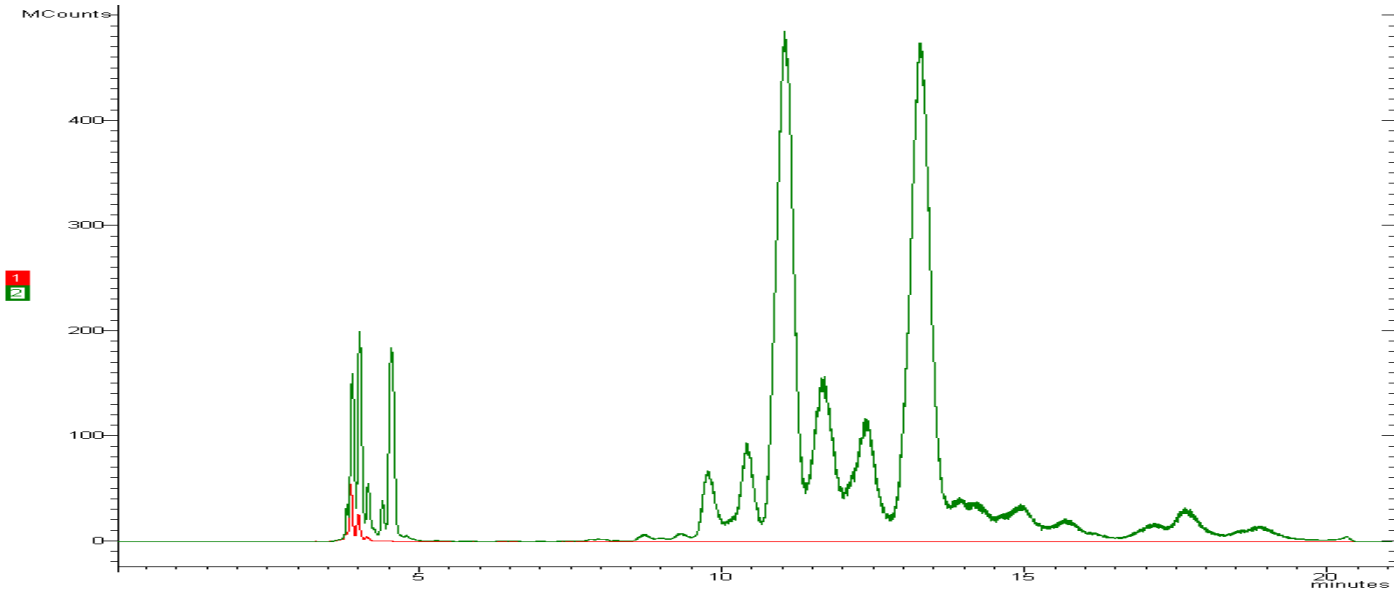
lysophosphatidylcholine

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. and collect the eluate

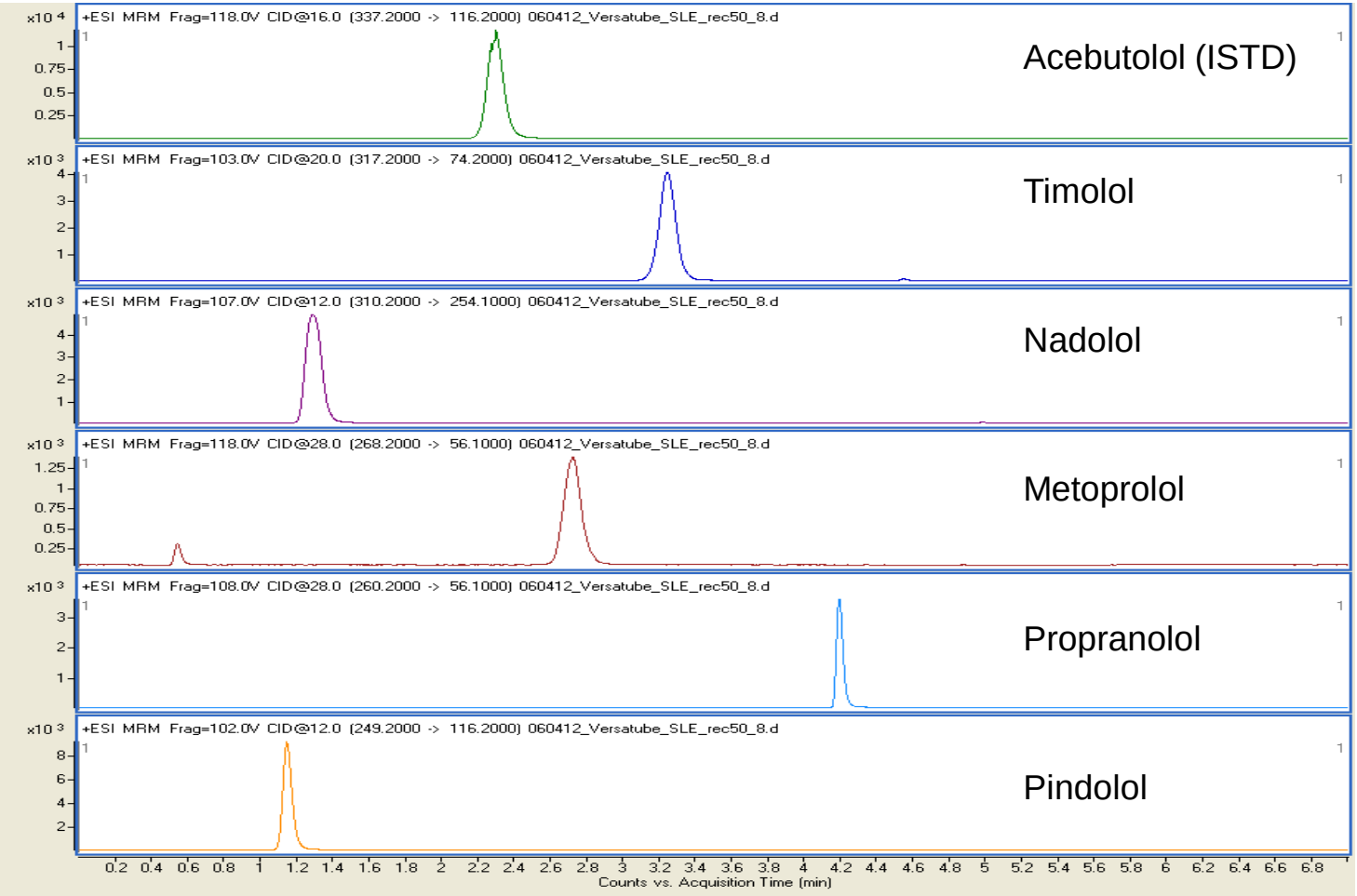
↓

After elution increase vacuum for 30 sec.

↓

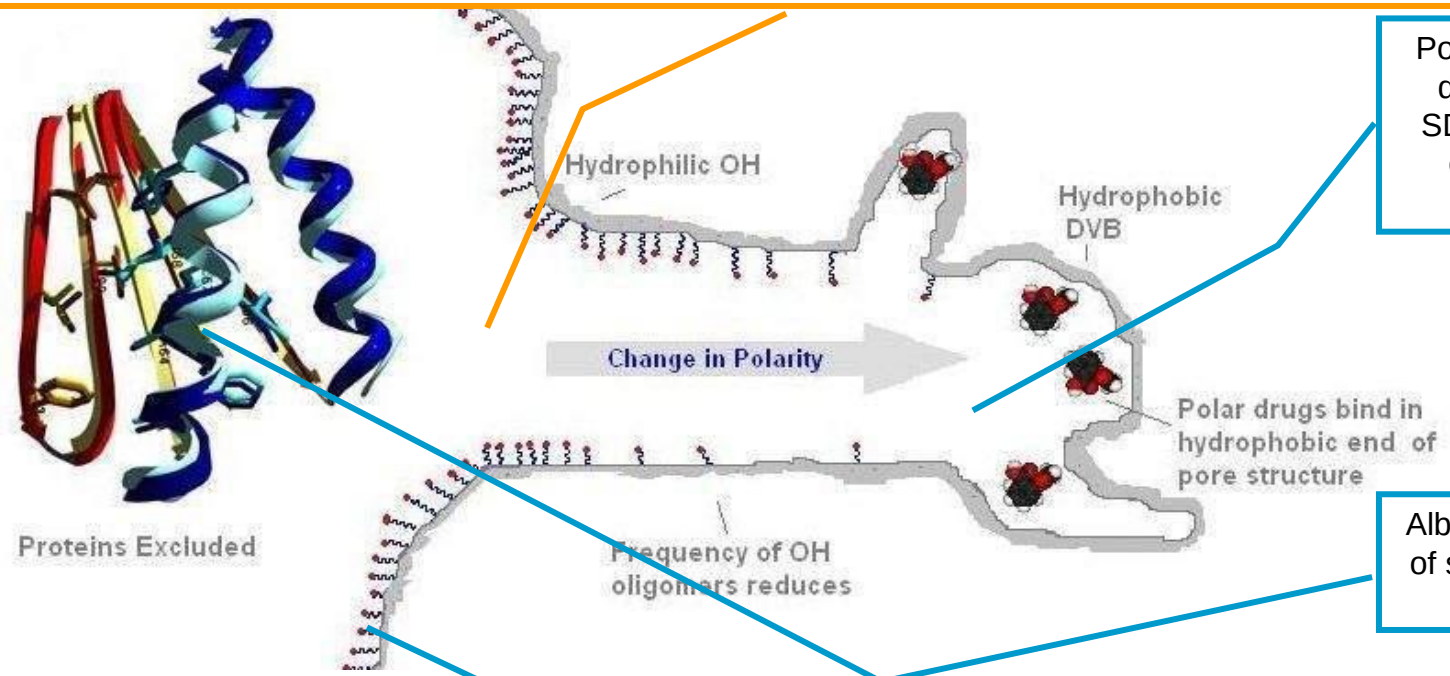
Evaporate and reconstitute in 0.15 mL of 30% MeOH for LC/MS/MS analysis.

Beta-Blockers by SLE – Chromatography



Solution: Remove Proteins & Lipids Using SPE

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



Polar and non-polar drugs bind in the SDVB hydrophobic end of the pore structure

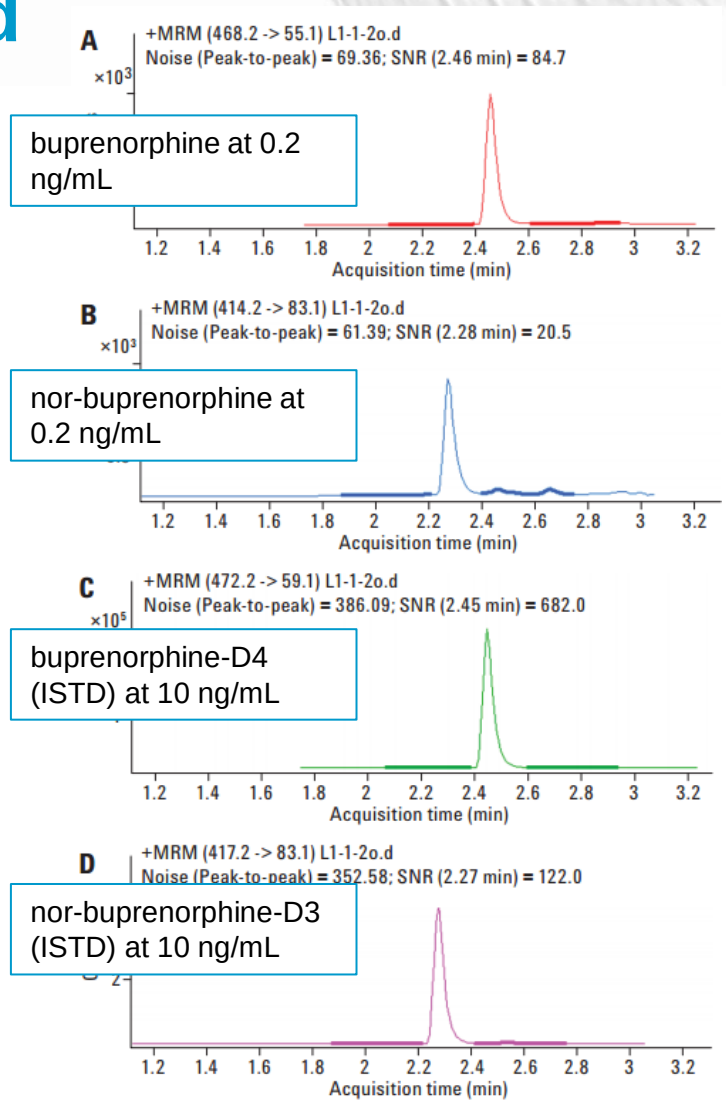
Albumin and proteins of similar size will be excluded

Lipids adsorb and do not elute under normal elution conditions

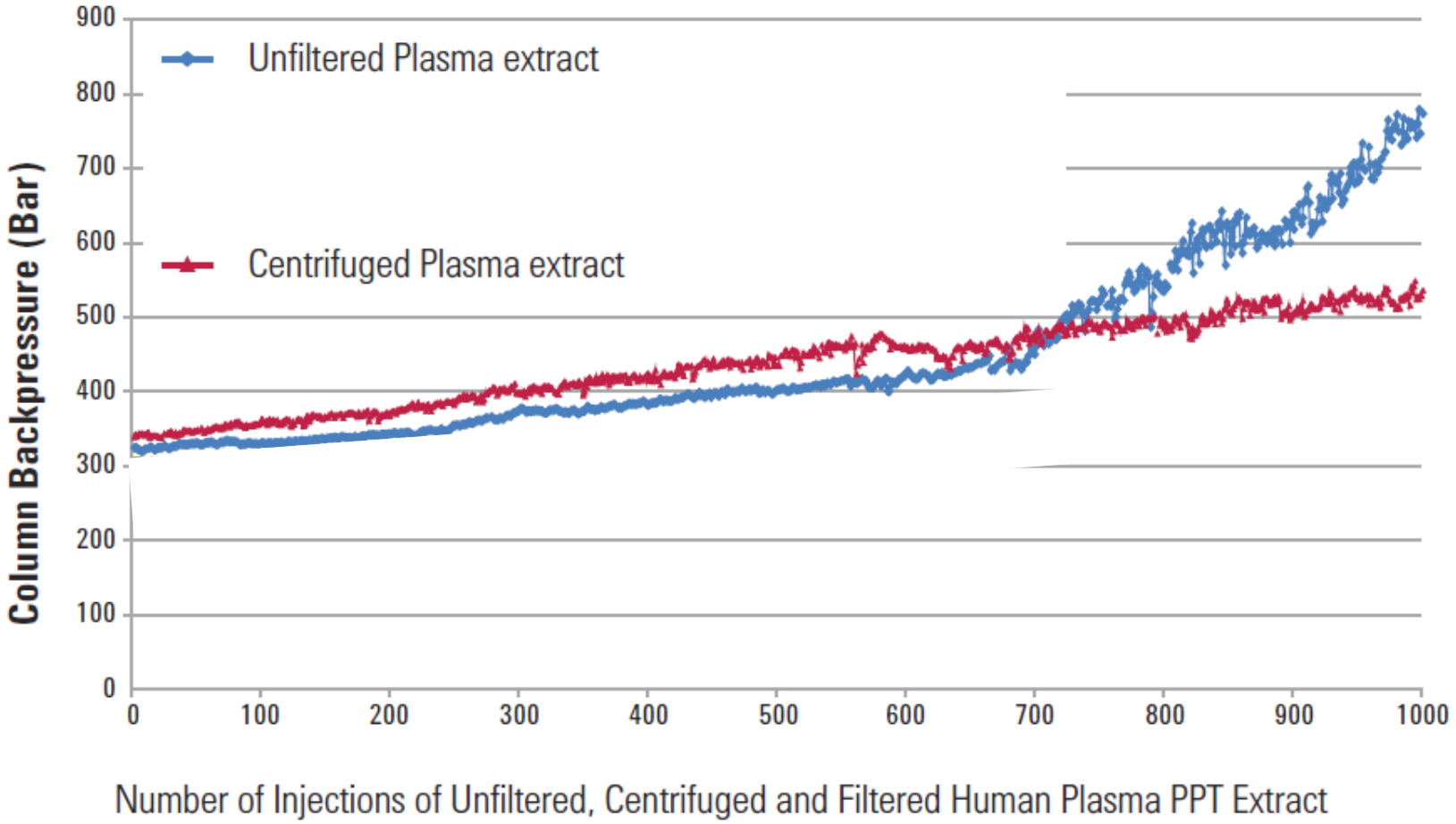
Solid Phase Extraction of Buprenorphine and nor-Buprenorphine in Whole Blood

SPE using Agilent Bond Elut Plexa PCX polymeric cation exchange

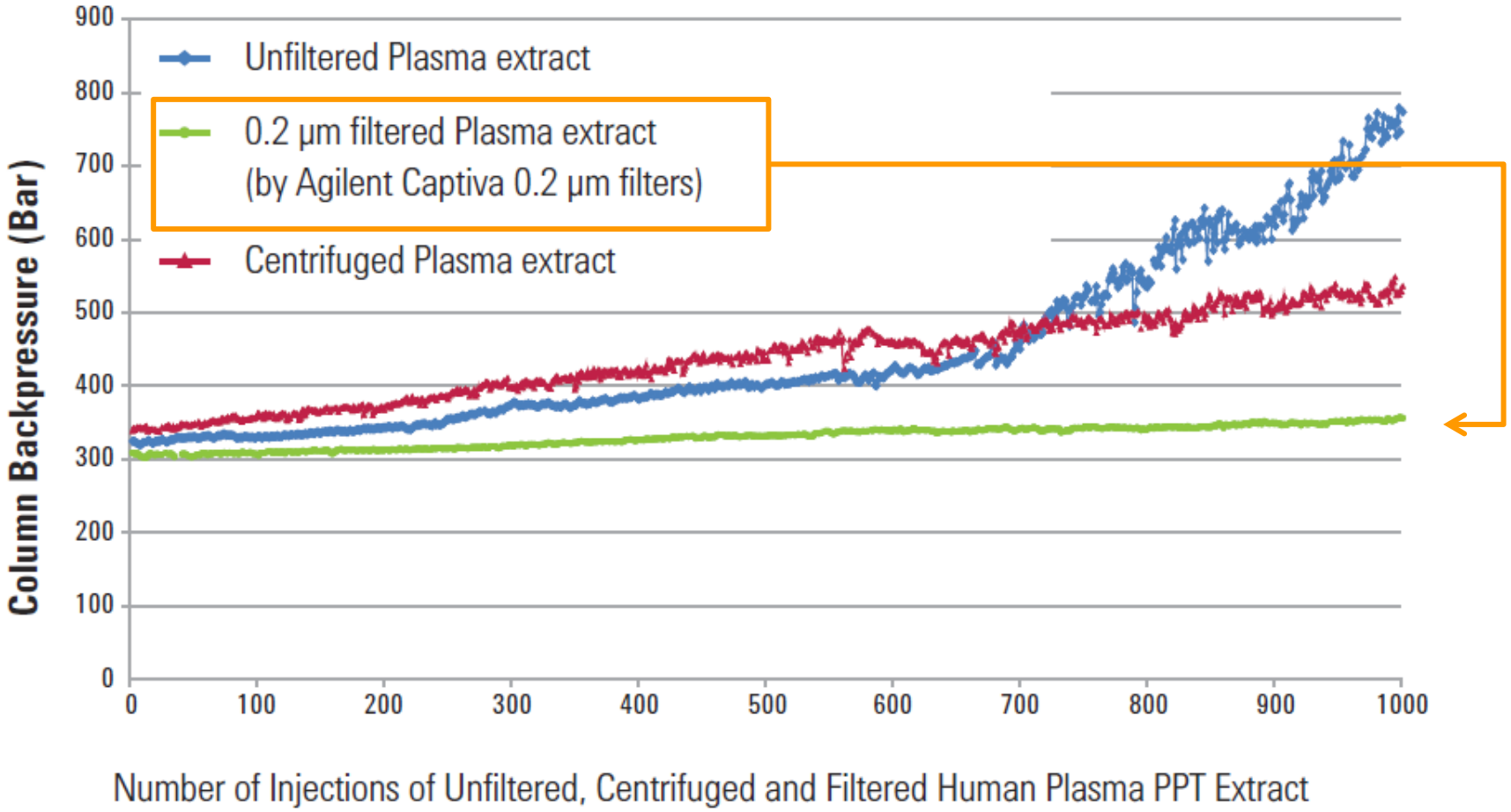
- Buprenorphine and nor-buprenorphine were efficiently extracted from whole blood
- Extraction method was simple and used only 0.5 mL of whole blood
- Mixed-mode cation exchange SPE ensures removal of matrix and high recoveries of targets
- Precise, reproducible extraction with low detection limits



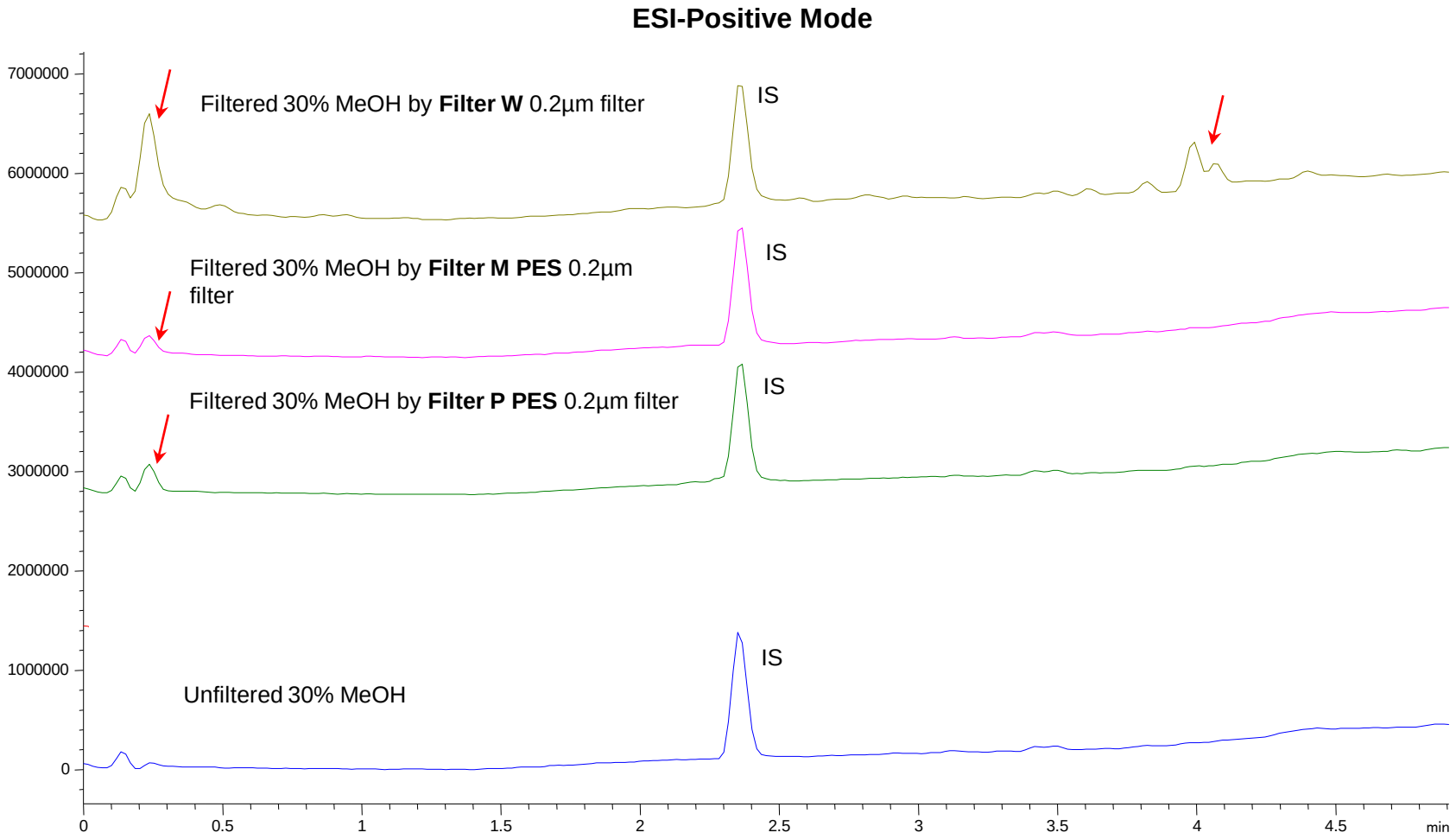
Challenge: Degradation of Sub-2-µm LC Column Performance Over Time



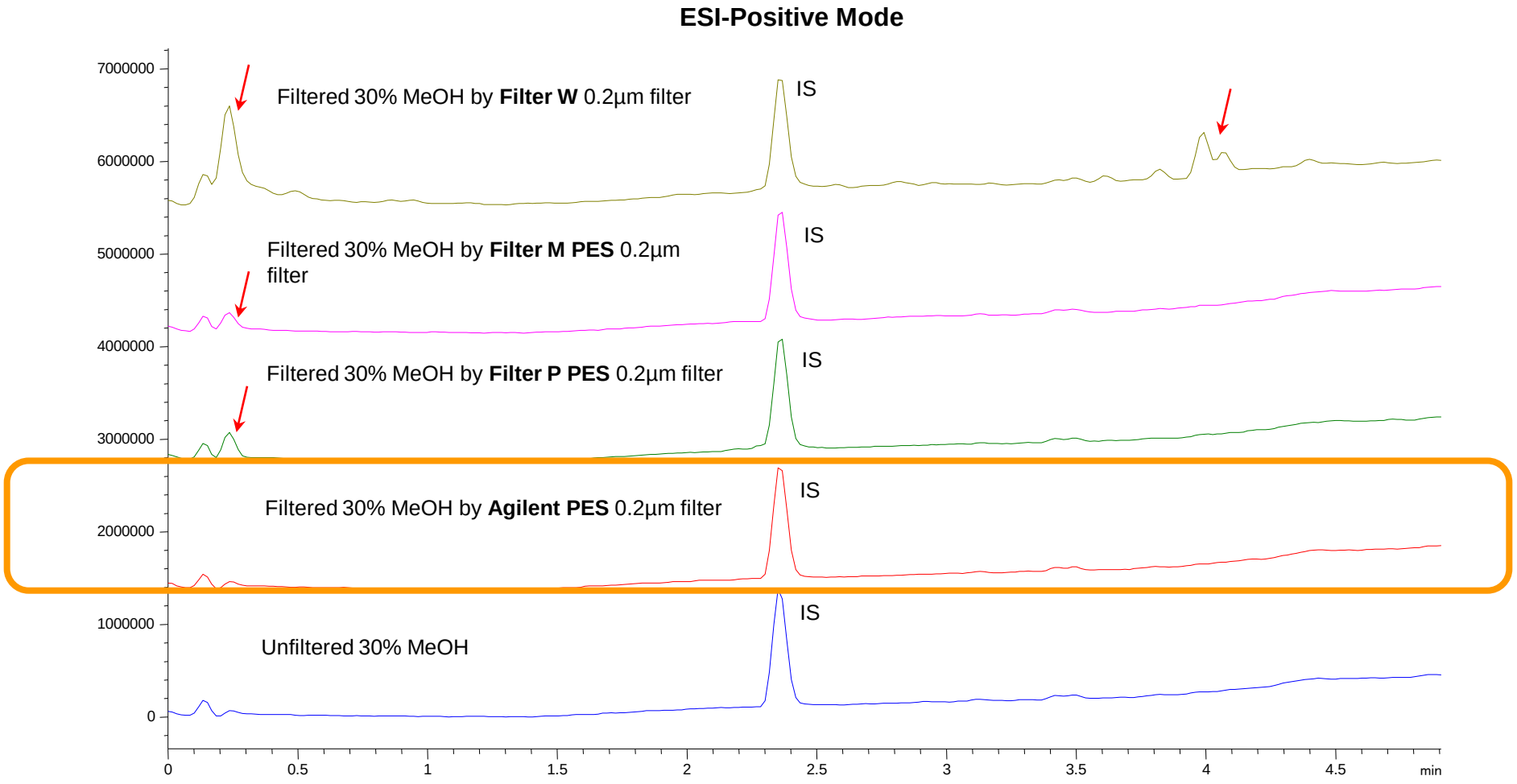
Solution: Filtration Extends Column Lifetime



Challenge: Unexplained Peaks in Sample



Solution: Use Certified Clean Filtration Products



Challenge: Sample Throughput and Productivity

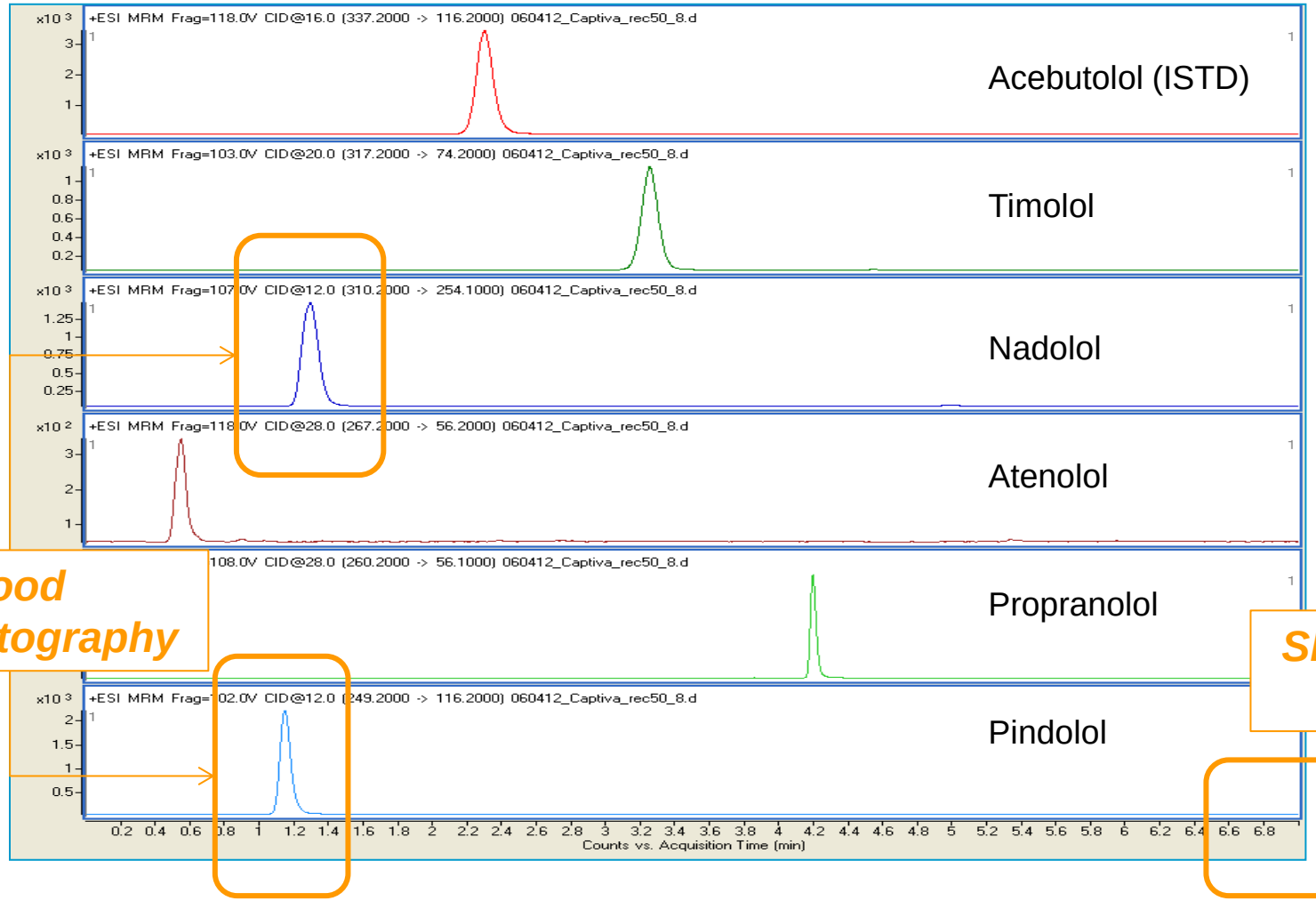
- 100s or 1000s of samples a day to be processed
- Consistency and simplicity to achieve high-quality results with few repeats
- Limit the operator-to-operator and day-to-day variation (ruggedness)
- Support for automation for increased throughput and unattended operation

Solution: Replace Conventional Protein Precipitation with Captiva ND Lipids

Captiva ND Lipids	Time (min)
Add 0.6 mL of MeOH and 0.2 mL of plasma sample to Captiva ND 96-well plate.	5
Mix each well with a pipette 5 times and apply vacuum for filtration.	
Directly collect filtrate into injection plate for analysis.	0
Total time required for sample preparation	5

This time comparison is based on the preparation of 96 samples.

Results: Captiva ND Lipids for Beta-blockers in Plasma



Good chromatography

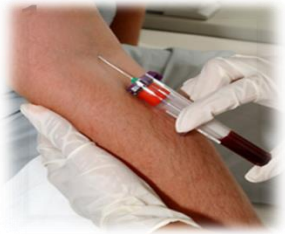
Short Run Time

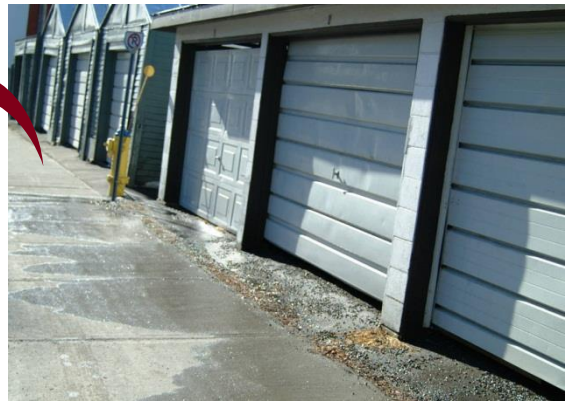
Agilent Captiva ND and Captiva ND Lipids Simplify Sample Prep

- 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

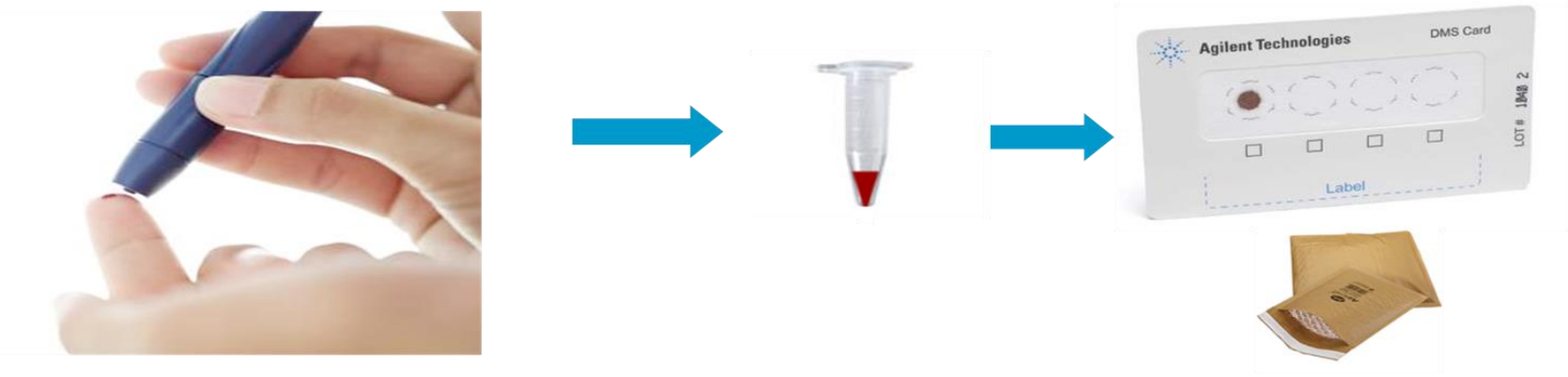
Traditional Blood Collection, Transportation, and Storage



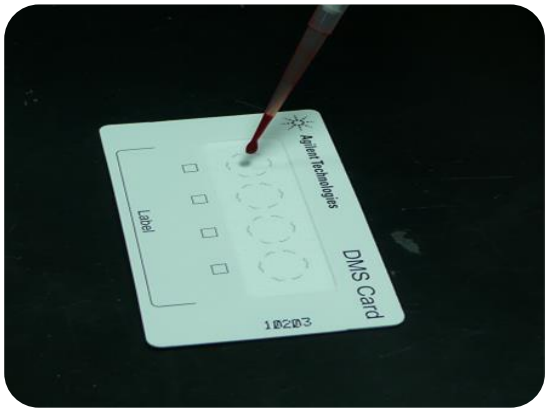


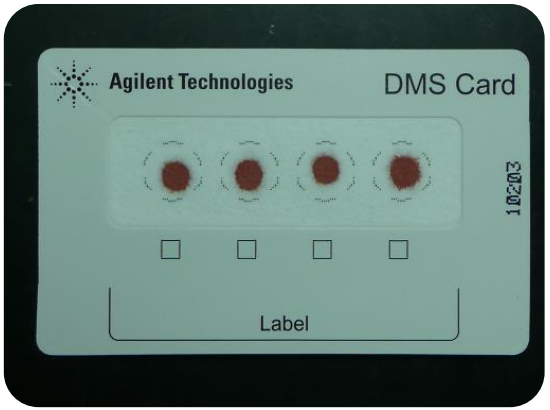
Solution: Agilent DMS Cards for Sample Collection and Storage

Dried Matrix Spot (DMS)



Spot. → Dry. → Send DMS cards in an envelope.





Dried Matrix Spot, DMS (Analysis)

Punch DMS cards.

↓

Put punched spot in a centrifugation tube with organic solvent e.g. 80% MeOH.

↓

Add internal standard.

↓

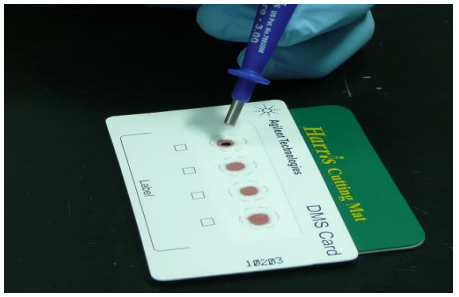
Centrifuge.

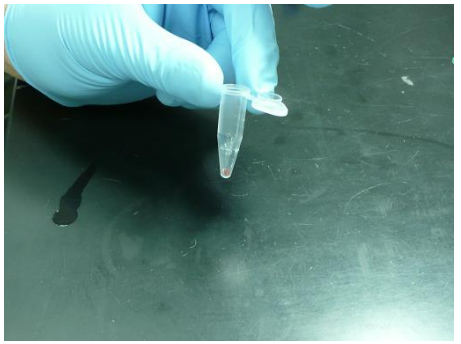
↓

Take supernatant for analysis.

↓

Or you can take further to combine other sample prep techniques such as SPE, Captiva ND Lipids, etc.

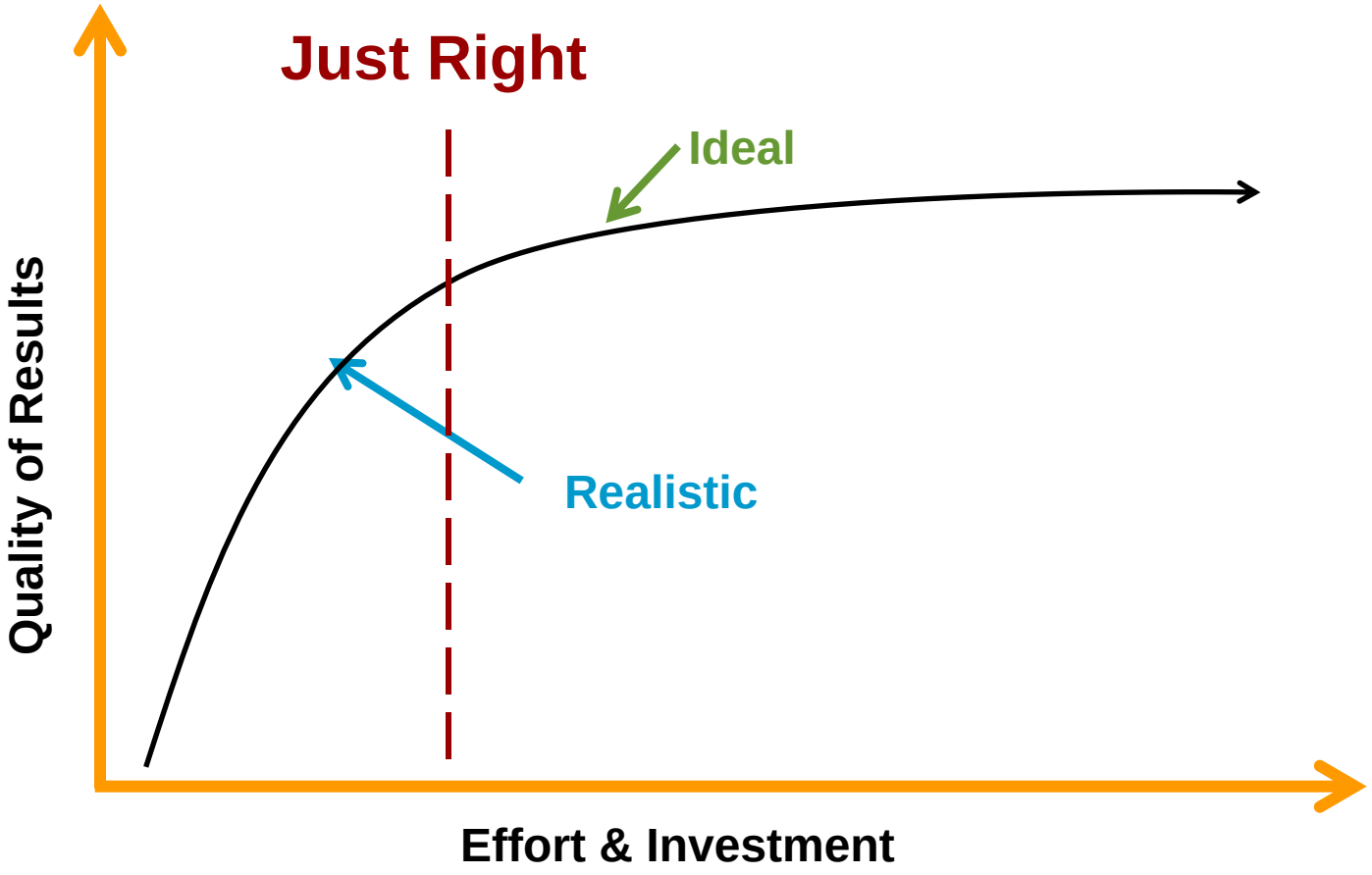




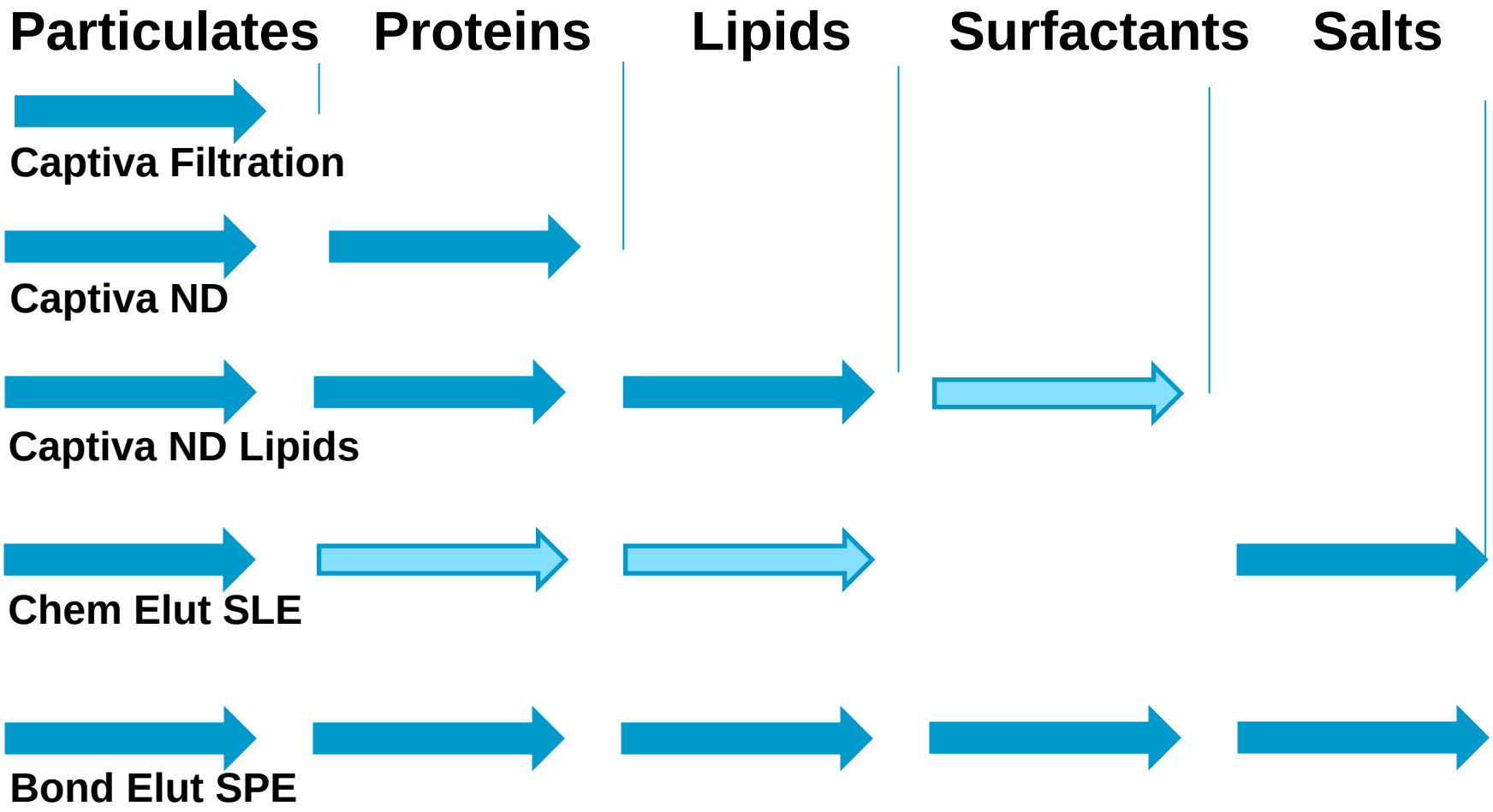
Biological Sample Analysis

SUMMARY AND WRAP UP

Striking the Right Balance in Sample Preparation



Solution: Select an Agilent Sample Preparation Product Based on Interference Removal



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:
http://www.chem.agilent.com/Library/brochures/Bond%20Elut%20Certify%20MethodsManual.pdf

Bond Elut Certify Video:

http://www.chem.agilent.com/en-US/products-services/Columns-Sample-Preparation/Sample-Preparation/Solid-Phase-Extraction-(SPE)/Bond-Elut-Certify/Pages/certifyvideo.aspx

Agilent QuEChERS Application Notebook:
http://www.chem.agilent.com/Library/brochures/5990-4977EN.pdf

Agilent Sample Preparation Catalog:

http://www.chem.agilent.com/Library/catalogs/Public/5991-1057EN%20Sample%20Prep%20Catalog.pdf

Agilent "Sample Preparation Fundamentals for Chromatography", a SPP primer handbook:

www.agilent.com/chem/SamplePrepBook

Agilent Sample Preparation portfolio brochure:

http://www.chem.agilent.com/Library/brochures/5991-2954EN_LR.pdf

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

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

Questions?