Considerations for Sample Prep and Method Development

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• Nothing to disclose

Learning Objectives

After this presentation you should be able to:
1. List advantages and disadvantages of the common sample preparation techniques used for clinical mass spectrometry (MS)
2. Create a due diligence plan to select sample prep automation for clinical MS
3. Describe pitfalls and solutions encountered when using automated liquid handlers for clinical MS sample preparation
**Common Sample Prep Options**

1. Dilution (Urine)
2. Protein Crash or Ultrafiltration (Serum) / Phospholipid removal (PPT)
3. Liquid-Liquid extraction (LLE) & Supported Liquid Extraction (SLE)
4. Solid Phase extraction (SPE) – offline & online
5. Other less common options, not listed

**Comparing Sample Prep Techniques**

<table>
<thead>
<tr>
<th>Differentiator</th>
<th>SPE</th>
<th>PPT</th>
<th>LLE</th>
<th>On-line</th>
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</thead>
<tbody>
<tr>
<td>Complete Phage Recovery</td>
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<tr>
<td>Intrinsic Analyte Recovery</td>
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<td>4</td>
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<td>Targets Unknown Analyte Recovery</td>
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<td>Reverse phase</td>
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<td>Simplicity</td>
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<td>Calibration</td>
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<td>Linear Range (ng/mL)</td>
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<td>3</td>
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<td>Precision (ranging)</td>
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<tr>
<td>System Original</td>
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<td>4</td>
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<tr>
<td>Accessory equipment</td>
<td>5</td>
<td>4</td>
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<td>3</td>
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</table>

*Table from Russell Grant, Ph.D. – MSAF Quant. MS Development & Validation short course, with permission

**What Extraction mode to use?**

* Scientific approach*:
  - Nature of the molecule is 1st decision point (charged, neutral, highly polar, pKa)
  - 2nd decision point - concentrate or dilute to achieve desired LLOQ & robustness?

* Compromise with reality approach:
  - Expertise/FTEs available for development & production (comfort zones)?
  - Accessory equipment & capitol $ available to “get cleaner,” scale up, automate?

* Russell Grant, Ph.D. – MSAF Quant. MS Development & Validation short course, with permission
But dilution &/or protein crash works just fine in my lab…….

• All dilution & PPT protocols are not equal – the details matter.

• Workload

• Test menu/instrument

• Criteria used for data review & robustness?

• How to quantify instrument down time & troubleshooting time (technical & service)

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• All dilution & PPT protocols are not equal – the details matter.
Qualifiers – the details* matter

• **SLE** (supported liquid extraction) easier to automate than **LLE** → similar robustness [but sample volume limitations – able to concentrate x4]

• **C18 SPE** << selective than mixed mode SPE (C18 + cation/anion exchange) [need a charged analyte]

• **PPT** combined with phospholipid removal plates can be very robust [but precipitation in the plate doesn’t always work]

• All **PPT** protocols are not the same [two-stage H2O/MeOH + Acetonitrile w/1% formic acid had better linearity, long term precision and was “cleaner” [less cleaning of MSMS] than Acetonitrile alone]

* Take Russell Grant’s Quanit MS Development & Validation short course – MSACL, March 2014!!

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Lessons learned the hard way

• Do qualitative (post-column infusion with injection of multiple patient extracts) AND quantitative (no matrix & pre- & post-extraction spikes with matrix) testing for ion suppression [getting pre- & post-spikes correct, accurate & precise – NOT SO EASY!]

• Challenge with numerous patient samples EARLY in development [avoid the sample prep that is beautiful with calibrators and QC but, oops, doesn’t work with patient samples]

• Don’t ignore outliers [1 failure in 10 samples translates to 1 repeat/day with a batch of 100 and 250 repeats/day with batches of 2,500! How will you resolve MRM ratio failures, I.S. low recovery failures, interfering peaks, etc.??]

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Automation

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Online Extraction - concept

Automated Liquid Handling
- 1, 2, 4, 8 and/or 96 pipetting channels
- Washable/reusable vs disposable tips
- Accessories for extraction (shaker, heater, cooler, vacuum module, evaporator)
- 96 well plates and tubes
- Barcoding & interfacing capabilities

Choices for Sample Prep Automation
- Online extraction
  - Vendor “complete” solutions
    - Thermo Scientific/Cohesive Technologies - TLX Turbo-Flow online SPE extraction
    - Spark-Holland/Symbiosis – dual channel cartridge online SPE
    - Gerstel MPS Workstation with in-tip dispersive SPE
  - User-developed online SPE
    - Most LC-MSMS vendors have packages/software - extraction pump(s), switching valves, installation, application notes
- Automated Liquid Handling (ALH) – many vendors & options, useful also for tube to plate with online or off-the-deck extraction (e.g. positive pressure SPE)
Comparing Online vs ALH (Automated Liquid Handling) if you can only have one

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Online</th>
<th>ALH</th>
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</thead>
<tbody>
<tr>
<td>Hands on time/sample</td>
<td>Less</td>
<td>More</td>
</tr>
<tr>
<td>LC sophistication needed</td>
<td>More</td>
<td>Less</td>
</tr>
<tr>
<td>Capital Cost (online vendor complete solution)</td>
<td>More~</td>
<td>Less</td>
</tr>
<tr>
<td>Capital Cost (adding online home brew)</td>
<td>Less~</td>
<td>More</td>
</tr>
<tr>
<td>Flexibility (options for different types of extraction)</td>
<td>Less</td>
<td>More</td>
</tr>
</tbody>
</table>

Considerations for Online Extraction- ROI & support

- Do you have a single, high volume assay or multiple, lower volume assays?
- In-house troubleshooting, repair?
- Realistic throughput estimate - extraction vs LC time, parallel or serial?
- Vendor support for method development?

Considerations for Online Extraction- Analytical Issues

- Limited sample volume (entire aspirated sample to analytical column)?
- Analyte thermally labile (no dry down needed)?
- # of solvents, solvent types for wash & regeneration of SPE column (solvent select valves, miscibility, CARRYOVER)?
- Elute (strong solvent) from the extraction column and retain downstream on the analytical column (weaker solvent) - how to
Considerations for Online Extraction - Process

- How to do LC gradient development, SST, validate recovery & ion suppression (can you inject with bypass of the extraction column)?
- Sample and internal standard still have to be mixed before the sample is put into the LC-MS/MS (glucuronide/sulfate hydrolysis?)
- Evaluate cycle times of extraction & LC run with care
  - Are two extraction lines "multiplexed" to one LC?
  - Are more than one extraction + LCs lines "multiplexed" to one MSMS?
  - Will the LC be waiting for the extraction or the extraction waiting for the LC?
- Compatible with all MSMS vendors (customers in production)?

Considerations for Automated Liquid Handling – ROI & Support

- Disposable tips vs Washable (consumable cost vs carryover risk, less throughput)
- # of pipettor channels (4-6 = throughput, 8 channel for tube to plate transfer, 96 channel for "plate stamping")?
- Barcode reading (plates & tubes) & aliquot/primary tube automated feed (barcode heights, formats?)
- Deck space (# of SBS footprint positions – tips, plates, shaker, vacuum, etc.)
- Redundancy (service support)?
- Application support (programming)?
Considerations for Automated Liquid Handling – Analytical Issues

- Minimum and maximum pipettable volumes (and with what precision)?
- Pipetting of difficult liquids precisely & w/o damaging components? (hexane, dichloromethane, acids, bases)
- Liquid level sense & clot detection?
- Reagent delivery channel(s) - how many solvents?
- Hard to effectively MIX a column of liquid(s) in a tall, narrow, plastic well

Considerations for Automated Liquid Handling – Process

- # of plates/tubes/tips that can be stored on the deck (stackable)?
- Off deck labware storage with auto-transfer to the deck?
- Moving plates around the deck (gripper)?
- Span capability (X, Y, Z independence of pipettor channels – for different tube sizes, racks, plates)?
- File In/Out capability (interfacing to LIS/middleware)?

Automated Liquid Handling – Case Histories
Thanks to my colleagues who did the work for liquid handling cases

- Julia Drees, PhD, Scientific Director – TPMG Kaiser Regional Laboratories, NCal
- Tony DaSilva, MS, Automation/IT Applications Engineer - TPMG Kaiser Regional Laboratories, NCal
- Bret Martin – Applications Programmer, Hamilton Scientific

Case 1 - To touch off or not to touch off – that is the question

Task
- Pipette serum and internal standard (I.S.) to a 96-well plate for a 25-OH Vitamin D assay by LC-MSMS.
- Desired intra-assay precision (within plate) = <5% CV

Constraints
- Sample + I.S. volume must = 75 µL
- 50 µL of serum needed for LLOQ – so I.S. must be 25 µL
- Conical bottom, 1 mL, polypropylene plate

Development History

Original Protocol:
- 50 µL I.S. dispensed into plate
- 50 µL serum added, mix with tips
- Intra-assay precision 4-6%

Revised Protocol:
- 2 precipitation reagents instead of 1 (more robust)
- Total volume had to stay the same
- I.S. volume must be decreased to 25 µL
1. Transfer 50 µL (now 25 µL) I.S. from reservoir to plate (4 tips, pick up & dispense 3 times)

2. Transfer serum from tubes to plate (8x12 tips) & mix w tips

3. Move plate to AlH 2 w 96 channel head & add two precipitating reagents (mix w tips)

4. Transfer precipitate to hybrid filtration plate on vacuum module to remove protein & phospholipids

Problem - %CV↑ to 6-9% when I.S. volume↓ from 50 µL to 25 µL

- Re-measure bottom of plate dimensions w calipers & adjust height of dispense – not fixed
- Lower height of dispense (with only 25 µL not touching off on bottom of plate?) – not fixed
- Slow speed of dispense – not fixed
- Pause after dispense – not fixed
- Blow out of residual volume in tips – not fixed

Solution

- Dispense serum 1st
- Dispense I.S. 2nd - touch off to serum (liquid) instead of questionable touch off to (dry) plate
- Intra-assay % CV decreased to 2-4%
- Trade offs
  - touch off to serum requires new tip each well
  - increased tip cost (from 8 to 96 tips)
  - tube to plate time increased 15-27 min
- Lesson learned – small changes can make a big difference in liquid handling precision
Case 2 – Missing filtrates

Task
– Precipitate serum + I.S. in a hybrid (protein & phospholipid removal) filtration plate – transfer filtrate to collection plate (vacuum)

Constraints
– Maintain (sample + I.S.) volume to precipitating reagent volume at 1:3
– Total volume in hybrid filtration plate ≤ 450 μL
– Don’t move plates off deck during the process to maintain viable work flow for 25 plates/day

Problem
• First few plates look good
• Then for a few wells in every plate – no filtrate transferred to collection plate
• All other wells in those plates have expected recovery

Hybrid filtration plate on top of vacuum module

1. Transfer serum + I.S. to hybrid filtration plate with 96 head

2. Transfer precipitating reagent to hybrid filtration plate with 96 head & mix with tips

3. Apply vacuum to move filtrate to collection plate

Collection plate inside vacuum module
Investigation

• Call vendor and complain – onsite support
• Change of plate lot? - not fixed
• Breakthrough of filtration membrane in surrounding wells ➞ inadequate vacuum for problem wells? - unable to prove or disprove
• Clogged membrane in problem wells – why OK before & not now?

Eureka!

• Frozen aliquots of pooled serum in use ➔ switch to fresh patient samples
• Vendor validated with frozen rat serum
• Hypothesis
  – frozen serum precipitate ≠ fresh serum precipitate
  – difference in size of particulate clogging filter?

Solution - crash, wait, then “sip” & transfer

• Crash in plain, conical bottom, 1 mL, plate INSTEAD of crashing INSIDE hybrid filtration plate
• Wait 5 min for heavier clumps to settle
• “Sip” upper layer of milky white precipitate (avoid heavy clumps at bottom) with 96 head and transfer to hybrid filtration plate (apply vacuum)
• Plate could stay on deck – no off deck centrifugation required
• Recovery was acceptable
Lessons learned

- Give the low-tech solution a try
- Extraction automation is about surface chemistry:
  - characteristics of different plastics
  - surface tension, flow & density of liquids
  - micro/macro-architecture of particulates, media, filters
- Positive pressure is more reliable than vacuum
- Mixing can be a challenge
- Pay attention to the millimeters (teaching the robot)
- Don’t give up & be creative – the details matter!

Case 3 – static cling of tips to pipet head (tips not shucked)

Solution

- Purchase tips without plastic film wrapping of individual tip boxes
- Only re-use reagent tips a few times (static builds up with re-use)
- Static seemed to vary with humidity
- Anti-static devices were not too helpful
In conclusion...

- Attention to trivial detail & persistence was the key to success
- Automation made possible extraction & LC-MSMS batch submission of 2,500 samples/day by two CLS in 4-5 hrs (LC-MSMS ran for 20 hrs)
- Long term (18 mos) precision across 3 instruments (6 streams) - 4-6% CV

Thanks for your attention