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

Differentiator	SPE	PPT	LLE	Online
Charged Analyte Recovery	1	2	4	3
Neutral Analyte Recovery	2	4	1	2
Highly Polar Analyte Recovery	1	2	4	3
Thermally Labile Analyte Recovery	3	2	4	1
Generic Protocol	2	1	4	2
Assay Ruggedness	3	4	1	2
Matrix effects	2	4	1	2
Selectivity	2*	4	1	3
Sample Concentration	2	4	1	3
96-well plate prep	1	3	4	2
Automatable	2	3	4	1
Simplicity	3	1	2	4
Method development speed	4	1	3	2
Cost (cheapest)	4	1	2	3
Sample Type Variance	3	2	1	4
Prepartion time (fastest)	4	2	3	1
Sample Volume (limited)	3	2	3	1
Extract direct injection	3	2	4	1
* = ion exchange SPE mode	Rank ordered 1=best, 4=worst			

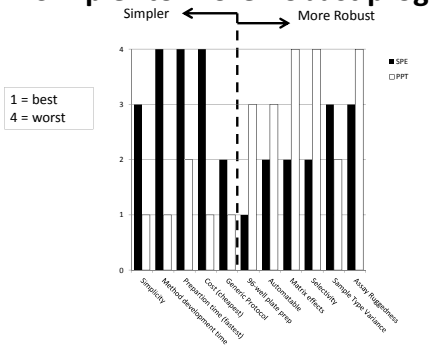
*table from Russell Grant, Ph.D. – MSACL Quant. MS Development & Validation short course, with permission

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. – MSACL Quant. MS Development & Validation short course, with permission

Simpler to More Robust progression



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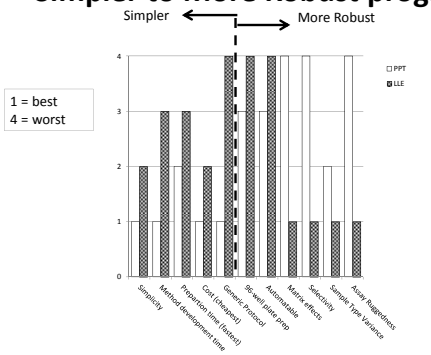


But dilution &/or protein crash works just fine in my lab…….

- **Workload**
- **Test menu/instrument**
- **Criteria used for data review & robustness?**
- **How to quantify instrument down time & troubleshooting time (technical & service support)?**
- **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 MeOH/ZnSO4 + Acetonitrile w 1% formic acid had better linearity, long term precision and was "cleaner" [less cleaning of MSMS] than Acetonitrile alone)

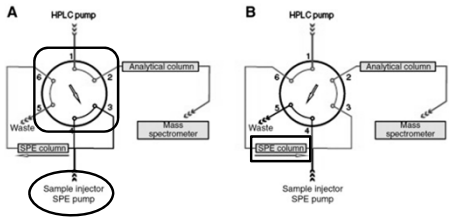
* Take Russell Grant's Quant MS Development & Validation short course – MSACL, March 2014!!

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 10 repeats/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.?)

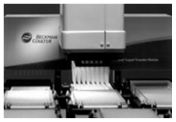
Automation

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

- <u>Online extraction</u>
  - 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
- <u>Automated Liquid Handling (ALH)</u> – 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

Parameter	Online	ALH
CLS Hands on time/sample	Less	More
LC sophistication needed	More	Less
Capitol Cost (online vendor complete solution)	More~	Less
Capitol Cost (adding online home brew to existing LC)	Less~	More
Flexibility (options for different types of extraction)	Less	More

Considerations for Online Extraction- ROI & support

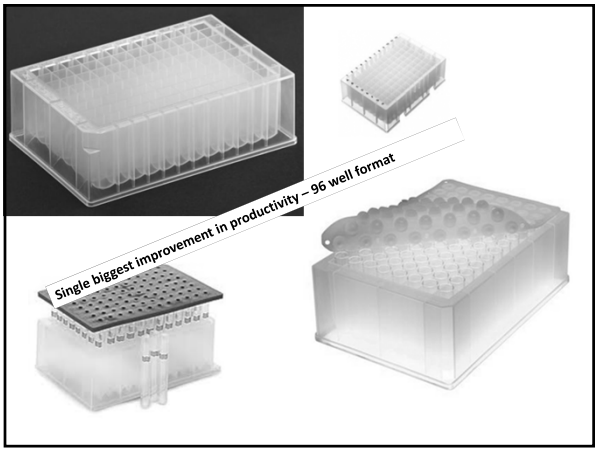
- Do you have a single, high volume assay or multiple, lower volume assays ?
- In-house troubleshooting, repair?
- Realistic throughput estimate - extraction vs LC time, parallel or serial?
- Vendor support for method development?

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** (>$ = >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)**?**

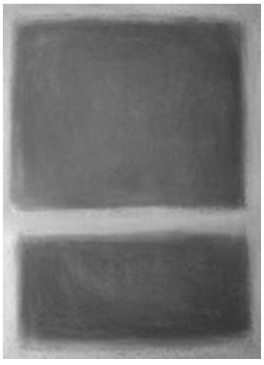
Considerations for Automated Liquid Handling – Analytical Issues

- **Minimum and maximum pipettable volumes** (and with what precision)**?**
- **Pipetting of difficult liquids precisely & w/o damaging components?** (hexane, dichoromethane, 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

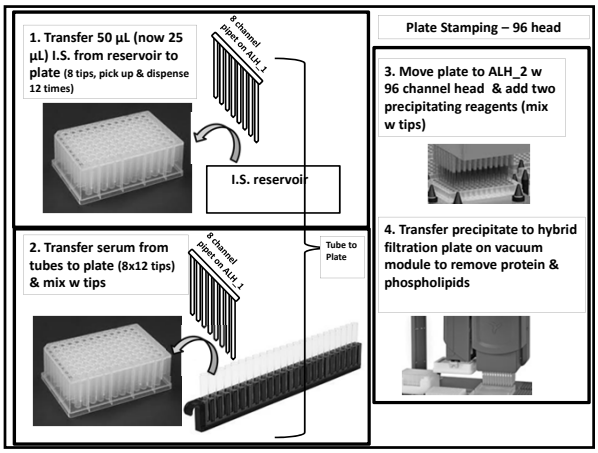
Development History

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



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

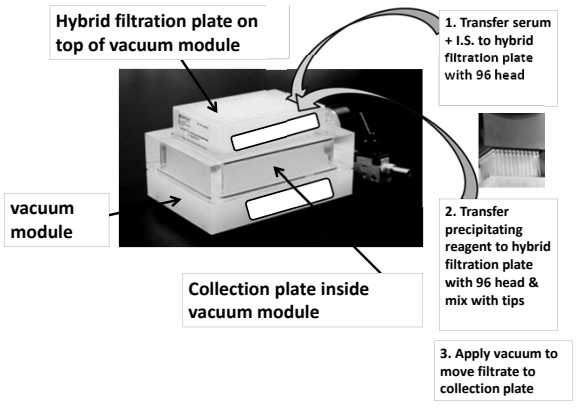
– Precipitate serum + I.S. in a hybrid (protein & phospholipid removal) filtration plate – transfer filtrate to collection plate (vacuum)

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

?

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?

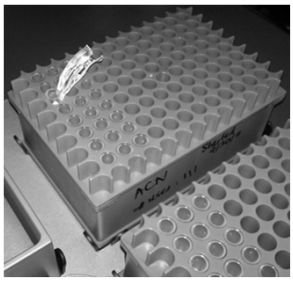
Solution - crash, wait, then "sip" & transfer

- 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