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REVIEW

2016 ASMS Fall Workshop: Sample Preparation for the Real-World Analysis of Samples by Mass Spectrometry

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The ASMS Workshop on Sample Preparation for the Real-World Analysis of Samples by Mass Spectrometry: The Common Denominator for Quality Data, sponsored and co-organized by the American Society for Mass Spectrometry, was held November 3 and 4, 2016, in Baltimore, MD at the Sheraton Inner Harbor Inn. The topic was specifically focused on a wide variety of strategies for the preparation of samples ranging from biological samples to foods and environmental matrices. The Workshop was co-organized by Jack Henion (Q2 Solutions) and Russell Grant (LabCorp) along with very helpful organizational assistance from Mr. Brent Watson from the ASMS office. The workshop brought together 10 invited speakers from the US pharmaceutical industry, government agencies, clinical diagnostic laboratories, and private startup companies, along with over 150 participants from a wide variety of laboratories from around the world. Since poster presentations are not generally part of an ASMS workshop, the venue consisted only of invited speakers plus two presentations each from the respective co-organizers and one clinical diagnostic/academic invitee. Digital PDF copies of each lecture were provided on-line or via thumb drive to each participant so they could follow the slides presented by the speakers. The oral presentations progressed systematically from introductory overviews of commonly employed sample methods to those that are in development now and may be important parts of future strategies.

The ASMS workshops are by design of a tutorial nature. The invited speakers were instructed to 'teach' the methods and workflow of their title topics rather than present their latest research. The material presented was to be of a practical nature with 'tips and tricks' that the audience could retain and employ as appropriate when they returned to their laboratories. The oral presentations were confined to a maximum of 50 min to allow adequate time for questions and discussion after each lecture. In general, this worked out well as many positive comments were received from the attendees so that the Q&A sessions were very informative, with additional input not only from the speakers but from the audience.

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The workshop opened with overview comments by Jack Henion, which addressed the general opinion of most that while sample preparation may not be considered to be fun, the value and importance of appropriate sample preparation is often critical to generate quality data from mass spectrometric techniques. The attendees were presented with the list below of common frustrations about sample preparation:

- Why don't LC/MS results from biological extracts work as well as analytical standards?
- Why do I have to do sample preparation?
- Isn't there a generic procedure I can use for all samples?
- Why do I need chromatography if mass spectrometry is so great?
- If I extract larger volumes of sample won't I have better sensitivity?
- Blood and urine sample prep is easy; try fish, cannabis plants, cabbage, etc.
- I must run thousands of samples per month; I cannot do exhaustive sample prep on all these!
- If only sample preparation could be fully automated.

The participants were advised that each of these points would be addressed in the presentations. The first presentation was given by Russell Grant on the topic of solution-based extraction techniques. The various extraction modes were overviewed along with the many 'Gotchas', including adsorptive losses to container surfaces, sample to extraction volume ratios and matrix suppression by phospholipids, etc. Although protein precipitation is a favorite approach because of its simplicity and facile automation, the pitfalls of this and related steps involved were detailed and summarized. With a focus on liquid–liquid extraction (LLE), the relatively cleaner extracts were contrasted with limitations for automation as well as many options for optimal solvent mixtures and back-washing strategies, together with subsequent steps needed to ready the sample for 96-well plate-based LC-MS/MS analysis.

The next lecture was presented by Erin Chambers of Waters on the subject of solid-phase extraction (SPE). Erin gave an excellent, systematic presentation on the benefits and

challenges of SPE techniques with a focus on small drug molecules, but with some reference to the even more challenging sample preparation aspects of larger biomolecules. She covered the relatively new phospholipid removal (PLR) 96-well plates along with solid-supported liquid extraction plates (SSLE) in addition to modern solid-phase extraction (SPE) plates. The importance of pretreatment of different sample matrices (like the pH of urine) and the importance of bed-to-sample aspect ratio were highlighted in Erin's presentation. Each step of an SPE sample preparation procedure can be important for the ultimate selectivity and recovery of the desired analytes, and these were highlighted in this presentation.

The next presentation was given by Professor Andy Hoofnagle of the University of Washington on the topic of affinity purification for sample preparation. The basics of this approach were nicely described, followed by the relative analytical merits of polyclonal versus monoclonal antibodies for selective isolation of targeted compounds from complex sample matrices. The merits and challenges of using effective antibodies coupled with various pre- and post-digestion strategies of larger molecules were described. A comparison of antibody enrichment with SPE and other techniques along with an indication of the amount of enrichment possible (up to 20,000-fold with a monoclonal anti-peptide antibody) were presented. Magnetic beads and other solid supports were described along with robotics such as the KingFisher. It was pointed out that antibodies are not always perfect in that the degree of selectivity with, for example, vitamin D, can be less than desired (using chemiluminescent measurement); however, the cross-reactivity of commercial antibodies enabled analysis of the vitamin D metabolites family with selectivity provided by the analytical horsepower of LC-MS/MS techniques.

The next lecture was titled Sample Preparation for Small Molecule Drug and Metabolite Bioanalysis, presented by Jack Henion. The focus of this talk was based on the quantitative determination of drugs and their metabolites in biological samples within a regulated bioanalysis GLP laboratory. Key issues are selectivity, sensitivity, linear dynamic range, signal-to-noise, and a host of other necessary credible analytical factors in order to obtain reliable, accurate, and precise bioanalytical data. The importance of removing the very high levels of endogenous components from a biological sample, not losing the targeted analyte(s) because of poor recovery, adsorptive losses, etc., and the importance of a stable isotope internal standard were covered. Also of importance is to obtain the same quantitative value for a sample on another day underscores what is now called incurred sample reanalysis (ISR) and the factors that affect this. Several real-world case studies were presented to demonstrate the points covered.

Paul West from Stemina Biomarker Discovery gave a presentation on an overview of sample preparation for untargeted metabolomics. An overview of metabolomics with respect to genomics, transcriptomics, and proteomics was given to provide a comparison with each. The goal is a comparative analysis of the small molecule metabolites found in biological samples. This is particularly challenging given the large number of such molecules, their sometimes very low concentrations, and the often minor changes in their concentrations as a result of a biochemical process. Paul differentiated between discovery and targeted metabolomics, the latter being somewhat more straightforward albeit not trivial. The importance of sample preparation in this work was highlighted since that process can potentially discriminate against possibly relevant molecules in the sample.

Sample preparation for quantitative protein LC/MS was presented by Hendrik Neubert of Pfizer. Hendrik contrasted the relative merits of quantifying intact proteins (top-down) versus enzymatic digestion and analysis using surrogate peptides (bottom-up) and concluded that at least for now the latter is preferred. Many of the important details for achieving optimal enzymatic digestion of proteins as well as the important systematic workflow components that affect a successful sample preparation strategy were reviewed and discussed. Elegant examples of coupled enrichment strategies (protein and peptide immunoaffinity) using on-line column switching technologies were described. Key examples of multiplexed measurement were demonstrated through rational selection of antibodies to specific epitopes of protein isoforms (proform and mature cytokines) followed by peptide immunoaffinity enrichment and subsequent quantitative analysis using LC-MS/MS.

Clinical biomarkers and the associated optimal sample preparation strategies were presented by Andy Hoofnagle. Andy started out by asking what is the difference between clinical sample prep and validation. A key difference is the demands placed upon a clinical lab with essentially a continuous production phase (the same test can be run for years), performance specification (precision, linearity, accuracy, QC, etc.), and reference ranges versus cut-off and the generalized trend of immediate data turnaround (batch size trending to 1). Other important topics relevant to sample preparation and its importance in the clinical laboratory included harmonization, proficiency testing, and standardization. The audience was left with a good appreciation of the rigor and challenges faced by today's clinical laboratory.

The first day ended with audience participation (with encouragement provided by liquid and solid refreshments) in a game proposed by Russell Grant called 'Battleships'. A series of 'methods' that contained steps recognized by an experienced sample preparation analytical chemist to be suboptimal or very poor choices for various reasons were presented and the audience was tasked to find these poor steps and explain why they would contribute to a poor bioanalytical method. With 'direct liquid introduction' and a variety of crazy ideas shown in these methods, the Battleship game went on for a full hour. The two admirals (chairs) suggested that the exercise end in a tie, and live on to battle another day.

On the second day, sample preparation for intact tissue analysis was discussed by Jeremy Norris of the Vanderbilt University School Medicine. The content of this presentation differed considerably from the prior topics. The measurement of amounts and importantly localization of analytes in solid tissue samples was the key content with this lecture. MALDI

imaging and a surprisingly significant degree of sample preparation were described. Handling of the sample is key and this differs dramatically from how one treats a biological fluid. Since imaging involves depicting the spatial location of drugs, proteins, and metabolites, maintenance of spatial and molecular integrity is very important (and much more complicated than initially thought). The process of sampling, freezing, microtoming (10-μm thick slices), matrix deposition, and minimizing temperature effects were discussed, together with the relevant steps required to control not merely the localization of analytes but the morphology of the original tissue.

In contrast to MALDI imaging of intact tissues, Chad Briscoe of PRA Health Sciences described the sample preparation approaches employed in so-called 'disruptive' tissue analysis; the selected tissue is homogenized using both chemical and mechanical means followed by more conventional sample preparation techniques. Chad opened his talk by commenting that this approach to tissue analysis is quite difficult based on the different mechanical strengths of tissues (bone versus muscle versus fat, and subgroups thereof). Additionally, as with intact tissue analysis, considerations of temperature control through mechanical mixing was elaborated upon with relevant experiments to assess and control the sample preparation process. The additional complexities of calibrating assays (matrix selection, preparation as homogenate, use of surrogate analytes, and matrix equivalency studies) were described in detail for quantifying analytes in tissue sample in support of regulated studies.

Steven Lehotay from the USDA ARS program then presented yet another set of sample preparation challenges that arise when dealing with food and environmental samples. The current most important and successful sample preparation approach for these samples is called QUECHERS (Quick, Easy, Cheap, Effective, Rugged, and Safe). This technique, which essentially involves a 'salting out' of miscible solvents to enable immiscibility and further tailoring of solvent chemistry to force the extractables into one solvent layer, is the method of choice for many sample preparation venues. Particular attention was given to the process of using sample preparation as a removal technique for unwanted analytes versus selectively extracting the analyte of choice. In addition, Steve described dispersive solid-phase extraction (addition of solid phase media to liquid samples with centrifugation) as an additive step for QUECHERS. Finally, the utility of solid phase microextraction (SPME) was discussed as an alternative sample preparation tool.

Clever alternatives to modern sample preparation was the title of a presentation by Kevin Bateman (Merck). Kevin continues to innovate and explore new techniques that can provide improved throughput and performance relative to current processes. One of the most impressive things he showed was the standard curve preparation of 96-well plate of standards and QCs via ink jet printing technology. An entire calibration curve was prepared in less than 30 s! Kevin also highlighted the trend towards micro-sampling and microfluidics. Kevin referenced Don Cooper's (Waters) work regarding random access LC/MS analysis, which is a novel way to perform quantitative analysis without analyzing a separate set of calibrators. This is done by adding the internal calibrators directly to the sample such that a calibration curve is created within each individual sample. It will remain to be seen whether this kind of an approach will be accepted by the regulators, but it certainly appears to be scientifically credible.

The next presentation was given by Jack Henion on the topic of micro sample collection, preparation, and analysis. This presentation focused on dried blood spots (DBS) and dried plasma spots (DPS). The first examples described essentially miniaturizing whole blood collection from standard phlebotomy in vacutainer tubes to lancet sampling and specimen collection into glass capillaries. Volumes up to 75 μL of blood are collected and the capillary centrifuged to separate the plasma, yielding approximately 30 μL of plasma for subsequent conventional sample preparation and analysis by the usual approach. Alternatively, work reported by Walter Korfmacher was discussed using similar capillary blood collection without plasma separation. Instead, the capillary is placed into a small vial with aqueous acetonitrile and mixed on a vortex mixer to produce aqueous diluted blood. This sample is frozen, stored, and later subjected to sample prep and analysis. Finally, automated analysis of DBS and DPS cards was shown using the DBS-A autosampler from Spark Holland.

The last presentation was given by Russell Grant on the topic of automating workflows for sample preparation. On-line sample preparation using column switching techniques and turbulent flow chromatography was described. This approach can allow direct injection of urine or plasma onto the turbulent flow column wherein larger molecules pass through the column and essentially allow separation of smaller drugs and metabolites to be resolved by chromatofocusing. A variety of commercially available robotic systems was shown, but it was stressed that the implementation (methods design and setup), training (liquid classes, deck layout, key assay steps, and personnel), and efficient use of these systems can require considerable time and investment. There are plenty of reasons for automation in the laboratory, but it is sometimes easier said than done. Several references were made to the differences between humans doing the lab work versus a robot. An important difference is humans can 'think'; robots do only what they are told. If we humans forget to tell the robot a particular step or to communicate when an error occurs (clots, bubbles, etc.), errors may ensue. Specific experiments were described to assess the fundamental differences (timing, tips, carry-over) when considering the modifications that an automated workflow contains when compared with manual techniques.

In summary, the ASMS post-workshop survey suggested that the attendees enjoyed the program and left with an appreciation of the importance of sample preparation for LC/MS analyses as well as many new ideas that they can use in their own laboratories. Some attendees suggested the workshop should be repeated and that short courses or other tutorial formats should be provided in the future.

Photo courtesy of Brent Watson (ASMS office)