Evaluation of Automated MAM Sample Preparation Utilizing SizeX IMCStips® on Hamilton Robotics

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ABSTRACT

Sample Preparation
- Denaturation
- Reduction
- Alkylation
- **Buffer exchange** or dilution
- Trypsin digest

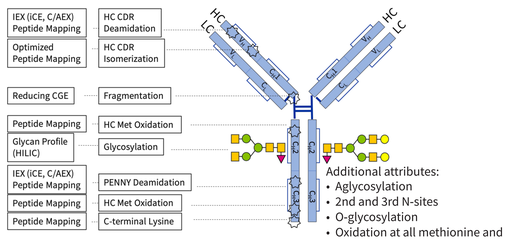
LC-MS or LC-MS/MS
- Sequence coverage
- Post-translational modifications
- Clip identification

Data Processing
- Relative CQA quantifications
- New peaks detection

INTRODUCTION

The Multi-Attribute Method (MAM) is an application of ultrahigh-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) for simultaneous detection, identification, quantitation and monitoring of molecular attributes of biotherapeutics. Critical Quality Attributes (CQAs) are identified based on the severity of harm to patient, which include efficacy (potency), pharmacokinetics and safety/immunogenicity. MAM is a single MS-based method used to monitor known CQAs as well as to detect new peaks, which are indicative of impurities ***(Figure 1)***.

Sample preparation for MAM often relies on a manual buffer exchange step to reduce the subsequent tryptic digestion time. Alternative to buffer exchange, denatured protein solution can be diluted and digested with trypsin overnight. Here we evaluate automated sample preparation utilizing SizeX IMCStips®, which are size exclusion chromatography pipette tips, on Hamilton STAR® liquid handling systems. The programmed workflow includes denaturation, reduction, alkylation, buffer exchange, trypsin digestion and trypsin quench. The automated program was evaluated by two independent laboratories. The evaluation compared automation to manual sample preparation from antibody stock concentrations ranging from 1 to 10 mg/mL. Denatured proteins with final concentrations ranging from 0.25 to 1 mg/mL were loaded on SizeX IMCStips. Precision data was also collected to determine consistency and robustness of automated program.



Additional attributes:
- Aglycosylation
- 2nd and 3rd N-sites
- O-glycosylation
- Oxidation at all methionine and tryptophan residues
- New peak detection

Figure 1. *Attributes of biotherapeutic monitored by MAM.*

MATERIALS AND METHODS

SizeX IMCStips® were provided by IMCS. For manual buffer exchange, Bio-Spin® 6 columns (Bio-Rad) were used. In brief, antibody stocks with concentrations ranging from 1 to 10 mg/mL were denatured, reduce and alkylated with proprietary protocols. The denatured protein with concentrations ranging from 0.25 to 1 mg/mL were then buffer exchanged using Bio-Spin column (manual) or SizeX IMCStips (automation). Protein volumes, concentrations and recoveries were measured. Next, desalted antibody was digested with trypsin to generate peptides for MAM analysis. Peptide samples were analyzed on Thermo Q Exactive plus and data was processed in BioPharma Finder. Known CQAs were quantified and new peptide peaks were screened.

REFERENCES

1. Roger RS, Nightlinger NS, Livingston B, Campbell P, Bailey R and A Balland (2015) Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics. *mAbs*, **7**, 881-890.
2. Roger RS, Abernathy M, Richardson DD, Rouse JC, Sperry JB, Swann P, Wypych J, Yu C, Zang L and R Deshpande (2017) A view on the importance of "Multi-Attribute Method" for measuring purity of biopharmaceuticals and improving overall control strategy. *The AAPS Journal*, **20**, 7.

RESULTS

LABORATORY ONE

The lowest concentration of antibody stock tested was 1 mg/mL. The maximum volume of antibody stock transferred was limited to 25% of total denaturation volume to prevent denaturation buffer dilution. The concentration of denatured antibody was 0.25 mg/mL prior to buffer exchange. Manual and automated sample preparations were compared as outlined in ***Figure 2.***

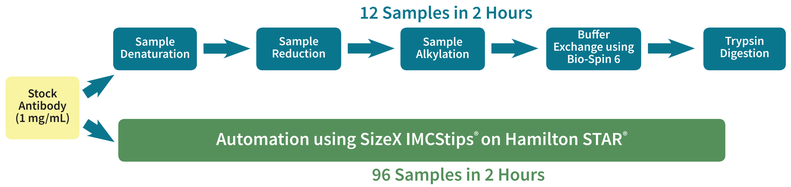


Figure 2. *Comparison of manual and automated MAM sample preparation using 1 mg/mL antibody stock.*

Digested samples were analyzed by LC-MS, and the total ion chromatogram (TIC) of each automated sample was very similar to that of manual sample ***(Figure 3)***. Biopharma Finder analysis did not identify any new peaks, indicating no contamination in automated samples. Glycosylation patterns of automated samples were the same as the manual sample ***(Figure 4)***. While glycation and deamidation percentages are comparable between automated and manual samples, oxidation percentages at four different sites were higher in automated samples compared to manual one ***(Figure 5)***. This suggests that light shielding during alkylation and methionine addition could improve automated MAM sample preparation.

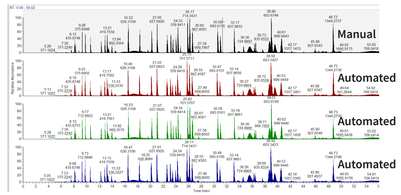


Figure 3*. Total ion chromatograms (TICs) of peptide samples from manual and automated methods using 1 mg/mL antibody stock.*

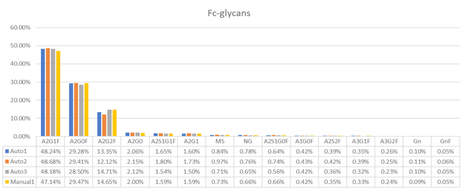


Figure 4. *Fc glycosylation patterns of automated samples compared to manual sample.*

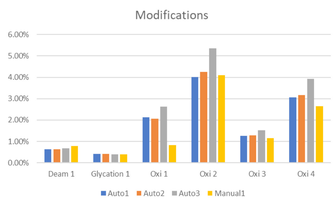


Figure 5. *Modifications observed in automated samples compared to manual sample; Deam: deamidation and Oxi: oxidation.*

LABORATORY TWO

Antibody recoveries were tested by a second laboratory using Bio-Spin 6 and two different SizeX IMCStips sizes ***(Figure 6)***. The highest recovery was measured based on NanoDrop measurements using $SizeX_{100}$ IMCStips, followed by Bio-Spin 6, and lowest recovery with $SizeX_{150}$ ***(Figure 7)***. Whether the sample loading was performed manually or using Hamilton liquid handling, the recoveries were within 4% RSD for $SizeX_{100}$ (n=4). The lower recovery from $SizeX_{150}$ is primarily due to a larger resin bed for increased sample loads, and further optimization of automation program was required to match the performance seen with $SizeX_{100}$.

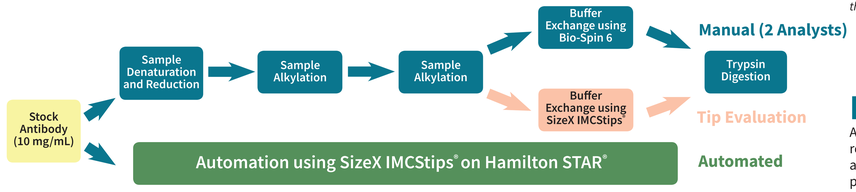


Figure 6. *Comparison of manual, tip evaluation, and automated MAM sample preparation using 10 mg/mL antibody stock.*

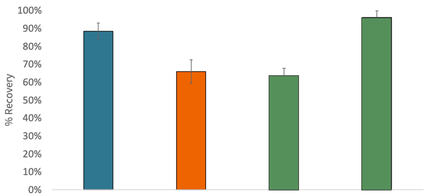


Figure 7. *Antibody recovery from manual, $SizeX_{150}$ tip evaluation, and automated MAM using $SizeX_{100}$ and $SizeX_{150}$ IMCStips preparations. n=4.*

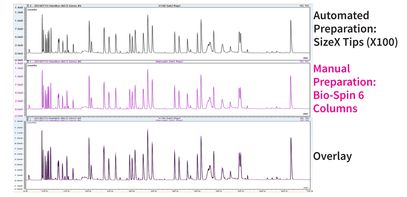


Figure 8. *Total ion chromatograms (TICs) of peptide samples from manual and automated $SizeX_{100}$ sample preparations using 10 mg/mL antibody stock.*

50 µg of each desalted antibody was digested with trypsin and peptide samples were analyzed by LC-MS. TICs of manual and automated samples were very similar as shown in ***Figure 8***. The modifications were found to be present at similar percentages when using SizeX automation compared to manual preparations ***(Figure 9)***. N-glycoforms were present at the same abundance between manual and automated sample preparations ***(Figure 9c)***.

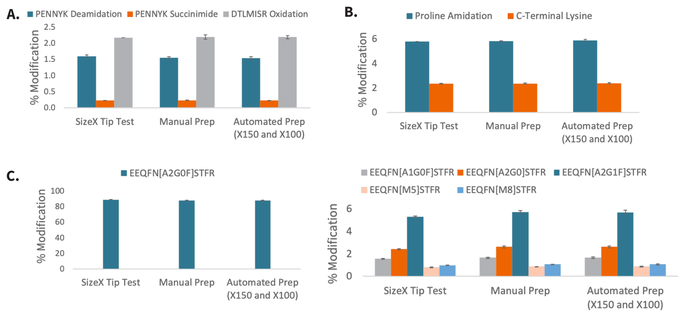


Figure 9. *Comparison of modification percentages of samples prepared manually with Bio-Spin columns, manually with the SizeX IMCStips for tip evaluation and automatically using Hamilton STAR with SizeX IMCStips. (a) deamidation, succinimide and methionine oxidation. (b) proline amidation and C-terminal lysine. (c) Fc glycosylation patterns, n = 4.*

Precision of automated sample preparation was evaluated by running the same protocol at three separate timepoints with four replicates at each timepoint. Modification percentages among the three timepoints were very similar ***(Figure 10)***. R Four analysts were used to compare the reproducibility of automated and manual preparations. While there was no significant difference in modification percentages between manual and automated preparations, automated sample preparations resulted in lower deviations as indicated by lower percentage RSD values in ***Figure 11***.

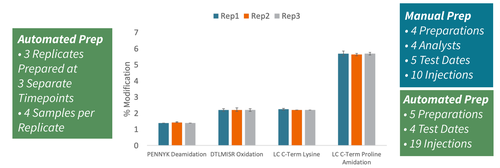


Figure 10. *Precision of automated MAM sample preparation performed at three different timepoints with 4 replicates at each timepoint.*

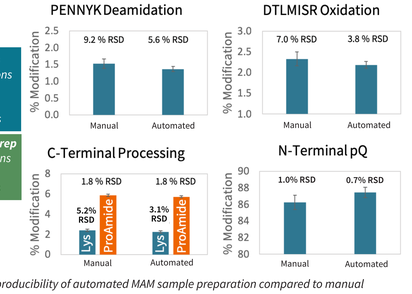


Figure 11. *Reproducibility of automated MAM sample preparation compared to manual preparation.*

DISCUSSION

Automated MAM sample preparation utilizing SizeX IMCStips on Hamilton STAR liquid handling showed comparable precision and improved reproducibility over manual preparation. Automating tedious and repetitive sample preparation is a promising improvement for obtaining accurate and reproducible data for monitoring CQAs of biotherapeutics. Furthermore, it could also serve as a platform to systematically optimize preparation conditions.

ACKNOWLEDGEMENT

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