Assessing the Repeatability and Quality of an Automated Peptide Mapping Workflow for use with Monoclonal Antibodies

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1. Introduction/Background

Peptide mapping is a well-established method used in the biopharmaceutical industry to characterize biotherapeutic proteins at the peptide level. Protein samples are enzymatically digested into small peptides, which are separated using high-performance liquid chromatography and coupled with a mass spectrometer to identify the peptides. Resulting data are used to confirm the primary amino acid sequence on a peptide level and characterize post-translational modifications to specific amino acid sites in the starting sample.

Peptide mapping sample preparations are complex, multi-step processes involving denaturation, reduction, alkylation, buffer-exchange, and enzymatic digestion, that are tedious and labor-intensive, making them difficult to scale and especially vulnerable to preparation related variability. To address these challenges, we sought out automation options such as liquid-handling robots. Ultimately, we selected the Waters Andrew+™ Robot for peptide mapping applications because of its ability to perform solid-phase extraction and heating in a single continuous workflow. Another feature taken into consideration was the high accessibility of the Andrew+ Robot's operating software, OneLab™. The tools of the Andrew+ Robot allowed adaptation of the manual sample preparation to an automated method by shifting the preparation to a plate-based method, along with other adjustments.

Evaluation was conducted with an IgG1 sample, with the main assessment criteria involving the Andrew+ Robot's ease of use and its ability to produce repeatable and quality results.

2. Method

Experimental design:

IgG1 sample was prepared in triplicate using the Waters Andrew+ Liquid Handling Robot. The automated workflow was adapted directly from the Waters PeptideWorks™ method and executed using OneLab Software. High-performance liquid chromatography was performed using an Agilent 1290 Infinity II series LC system. Mass data was collected with an Agilent QTOF 6545 XT and analyzed with Protein Metrics Byos software (v5.5). UV chromatograms and post-translational modifications levels of these triplicate preparations were used to assess repeatability. To assess quality, the automated method was directly compared to the results of an established method (prepared manually) for the same sample. The extent of digestion was used as the quality measure and thus the levels of nonspecific and missed cleavages were compared between the two methods.

Established manual method:

Denaturation, reduction, alkylation:
1. Guanidine-HCl
2. TCEP
3. Iodoacetamide

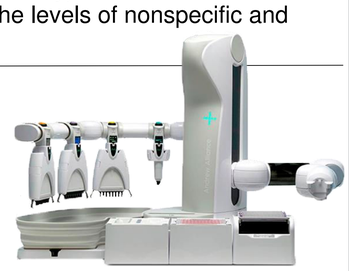
Buffer exchange
- Desalted using Zeba spin columns
- Centrifugal driven
- 7 kDa MWCO

Digestion:
- Samples digested using Promega Lys-C/trypsin mix
- 16-hour (overnight) digestion at 37°C

Digested protein sample ready for LC/MS analysis

Automated method:

Blue text: Adjustments to the manual method.



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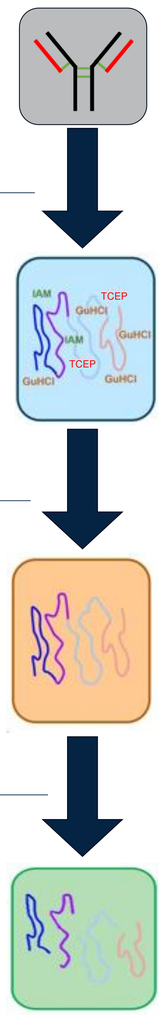
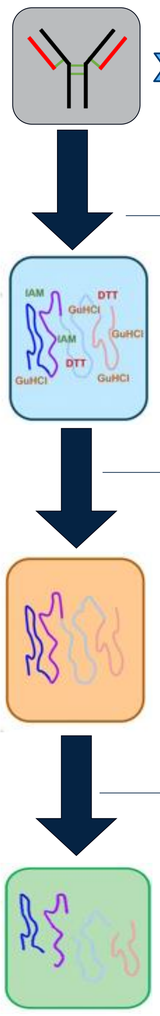
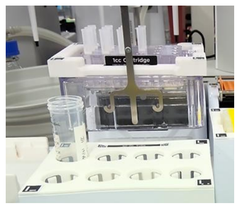




Denaturation, reduction, alkylation:
1. Guanidine-HCl
2. DTT
3. Iodoacetamide

Buffer exchange
- Desalted using Sep-Pak™ SEC Cartridges
- Gravity driven
- 5 kDa MWCO

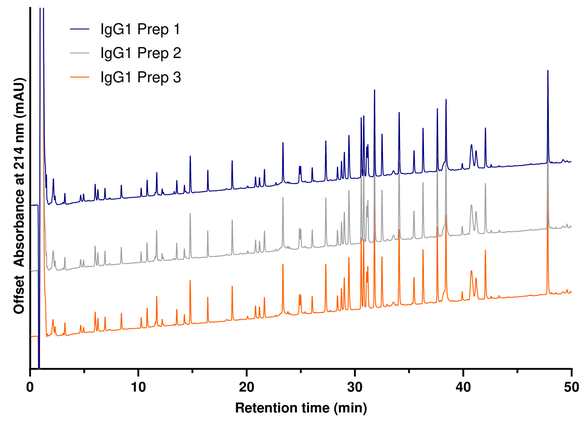


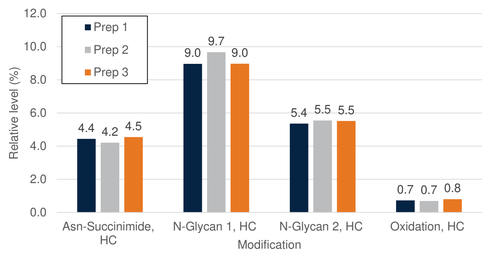
Digestion:
- Samples digested using Waters RapiZyme™ (trypsin)
- 30-minute digestion at 37°C with Peltier+™ Connected Device

Digested protein sample ready for LC/MS analysis

3. Results

Reproducibility: Preparation replicates prepared by the Andrew+ Robot were highly repeatable.

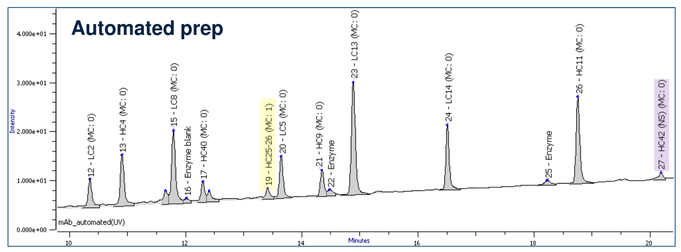


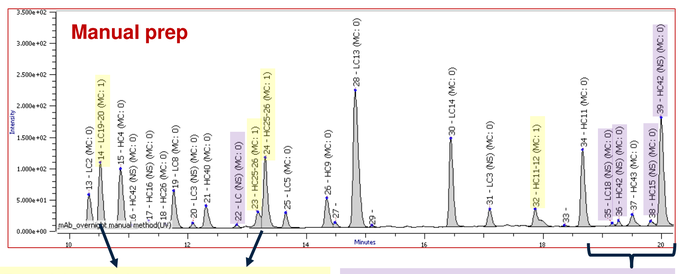


↑ Post-translational modification levels were repeatable across replicates.

←UV 214 nm chromatograms of replicates had identical profiles.

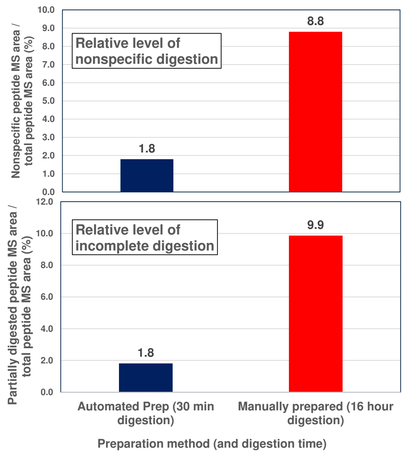
Quality: The automated method had better extent of digestion with lower levels of nonspecific and missed cleavages over the established, manual method.



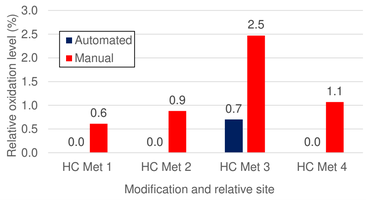


Peptides with missed tryptic cleavages (MC), are highlighted in yellow. Elevated levels of this type of cleavage indicates under digestion.

Nonspecific cleavages (NS), cleavages outside the target sites of trypsin (Lys, Arg) are highlighted in purple. Elevated levels of this type of cleavage indicates over digestion by the enzyme.



Preparation induced oxidation levels were lower in the automated method over the manual method likely due to the shorter digestion time.



4. Conclusions

- The Andrew+ Robot and the PeptideWorks procedure produced repeatable peptide mapping results, including a consistent chromatographic profile and post-translational modifications.
- Advantages of the automated method:
  - The automated Waters PeptideWorks method resulted in more complete, specific digestion than the established manual method.
  - Preparation-to-preparation variability is minimal.
  - Limited preparation-induced modifications.
  - The OneLab Software interface is user-friendly, allowing for simple protocol creation without the need for coding.
  - Potentially high efficiency gain for medium to large samples sets (>10 samples).
- Limitations of the automated method:
  - A 2.5x dilution of the sample occurs during buffer exchange with the Sep-Pak SEC Cartridges.
  - Sample plates are exposed without capping, posing a risk of evaporation, air-oxidation, and contamination. *Note: The shortened digestion was implemented to minimize this effect.*
  - Low efficiency gains for small sample sets due to the need for analyst intervention at specific points, such as opening and closing reagent containers and capping sample trays.

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