Automated multi-attribute method sample preparation using high-throughput buffer exchange tips

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Rationale: The multi-attribute method (MAM) has become a valuable mass spectrometry (MS)-based tool that can identify and quantify the site-specific product attributes and purity information for biologics such as monoclonal antibodies (mAbs) and fusion molecules in recent years. As we expand the use of the MAM at various stages of drug development, it is critical to enhance the sample preparation throughput without additional chemical modifications and variability.

Methods: In this study, a fully automated MAM sample preparation protocol is presented, where rapid desalting in less than 15 minutes is achieved using miniaturized size-exclusion chromatography columns in pipette tips on an automated liquid handler. The peptide samples were analyzed using an electrospray ionization (ESI) orbitrap mass spectrometer coupled to an ultra-high-performance liquid chromatography (UHPLC) system with a dual column switching system.

Results: No significant change was observed in product attributes and their quantities compared with manual, low-artifact sample preparation. The sample recovery using the buffer exchange tips was greatly enhanced over the manual spin cartridges while still demonstrating excellent reproducibility for a wide variety of starting sample concentrations. Unlike a plate desalting system, the individual columns provide flexibility in the number of samples prepared at a time and sample locations within plates.

Conclusions: This automated protocol enables the preparation of up to 96 samples with less “at-bench” time than the manual preparation of a smaller batch of samples, thereby greatly facilitating support of process development and the use of the MAM in quality control.
several conventional electrophoretic and chromatographic methods used in a quality control (QC) environment. Since then, the method has been utilized in all stages of drug development, from product characterization and process development to dispositioning clinical materials. The first application of the MAM in the current Good Manufacturing Practice (cGMP) environment was reported in 2019, replacing cation-exchange chromatography (CEX) or capillary isoelectric focusing (cIEF) for charge variants analysis; hydrophilic interaction liquid chromatography (HILIC) glycan mapping for glycosylation species analysis; and reduced capillary electrophoresis sodium dodecyl sulfate (rCE-SDS) for other modifications such as clipping. While the conventional methods provide attribute information at the molecule level, the MAM provides site-specific information at the amino acid residue level. This detailed information is significant in cases where there are multiple glycan sites as well as when one needs to differentiate the critical quality attributes (CQAs) from other product quality attributes (PQAs). As regulatory agencies now expect a quality by design (QbD) approach to ensure product safety, developing a quality target profile and identifying site-specific CQAs has become essential. Because of these key unique benefits, the MAM has established its place as a critical tool in product characterization, sequence confirmation, process and formulation development, and stability testing in addition to becoming a platform method in QC.

The first step in the MAM is sample preparation, which generally involves denaturation, disulfide bond reduction, free cysteine alklylation, and enzymatic digestion of the biotherapeutics. In the second step, the resulting peptides are separated by reversed-phase chromatography and analyzed with a high-resolution mass spectrometer directly coupled to the liquid chromatographic system. In the last step, the raw data generated from the liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis are used to identify the product quality attributes, and, depending on the stages of development, LC/MS or LC/MS/MS data are used for quantitative data analysis on targeted product attributes (monitoring) and nontargeted impurities (new peak detection).

Sample preparation is a critical component of the MAM as it is a foundation for robust and reproducible data across the samples analyzed over extended periods of time. Optimizing the time required in each step as well as the reagent pH and concentrations minimizes the probability of artefactual chemical modifications during sample preparation. In particular, enzymatic digestion of biotherapeutics generally requires incubation at an elevated temperature and pH, which can induce chemical modifications such as deamidation and aspartic acid isomerization. Therefore, rapid yet effective digestion is prioritized and can be achieved by removing the denaturant and the reducing and alkylating agents. Guanidine hydrochloride (GuHCl) is a common denaturant – depending on the molecules and enzyme used for digestion, it is possible to dilute GuHCl before digestion. However, GuHCl is a known trypsin inhibitor and acts by forming a hydrogen bond/salt bridge within the active region of trypsin. It is difficult to dilute sufficiently for complete digestion in shorter incubation periods when the initial sample concentrations and quantities are limited. Dilution of GuHCl also lowers the concentration of target proteins for digestion, affecting digestion kinetics or requiring longer incubation times. Alternatively, GuHCl can be chromatographically removed using a size-exclusion column for more effective digestions, which allows most monoclonal antibodies (mAbs) to be fully digested in 30 min.

Manual sample preparation using size-exclusion spin cartridges can produce results with excellent reproducibility with minimal chemical modifications. However, it is not suited for a large set of samples, is amenable to human errors, and creates challenges when implementing identical processes across multiple locations. In addition, sample recovery from size-exclusion spin columns can vary greatly depending on the initial sample concentrations. In this study, we developed and evaluated a fully automated MAM sample preparation protocol using buffer exchange pipette tips on an automated liquid-handling system. Two proprietary IgG1 antibodies were used in this study: mAb 1 and 2. Sample recoveries and peptide chromatograms were compared between manual and automated preparations using mAb 1. Attribute quantification was compared using mAb 2, which has a glycosylation site in the light chain (LC) in addition to the traditional Fc N-glycosylation site. After digested samples had been thawed, they were transferred to autosampler vials stored at 4°C and analyzed within 24 h to minimize the effects from the waiting periods in the autosampler.

Unlike a plate-based buffer exchange system, pipette-based buffer exchange provides flexibility in the sample number and 96-well plate sample locations to be performed. Furthermore, the buffer exchange process is completely integrated into the liquid handler without needing a centrifuge. The system allows up to 96 samples with varying sample concentrations to be processed at a time from the initial denaturation to vialing for LC/MS analysis with significantly reduced abench time. The sample recovery after buffer exchange improved with excellent reproducibility for a wide variety of starting concentrations. Following both automated and manual sample preparations, the post-translational modifications (PTMs) were identified, and their levels were compared between the two modes of sample preparations.

2 | MATERIALS AND METHODS

2.1 | Materials

Two IgG1 antibodies were produced using stable CHO-K1 cell lines and purified using standard protein A and polishing chromatography. Both mAb 1 and mAb 2 were formulated in 20 mM acetate with 9% sucrose at pH 5.2 and stored at < −70°C until use.

2.2 | Chemicals and reagents

The materials used for the reduction, alklylation, and trypsin digestion are the following: Tris buffer (pH 7.5 and pH 8.4) was from Teknova.
(Hollister, CA, USA); guanidine hydrochloride (GuHCl) and trifluoroacetic acid (TFA) solutions were from Thermo Scientific (Waltham, MA, USA); hydrochloric acid (HCl), dithiothreitol (DTT), and sodium iodoacetate (IAA) were from Sigma (St Louis, MO, USA). Glacial acetic acid, sodium acetate, and sodium hydroxide were from J.T. Baker (Phillipsburg, NJ, USA). Lyophilized trypsin was from Thermo Scientific Pierce (Waltham, MA, USA). L-Methionine was from Sigma.

2.3 | Methods

### 2.3.1 Manual sample preparation

Antibody stocks with concentrations ranging from 1 to 11 mg/mL were denatured and reduced at room temperature (RT) using a solution of >5 M GuHCl in 100 mM Tris, pH 8.3, with 10 mM DTT for 30 min followed by alkylation with 20 mM IAA for 25 min in the dark. Micro Bio-Spin® P-6 columns (Bio-Rad, Hercules, CA, USA) were prepared by removing the storage buffer, adding 50 mM Tris pH 7.9 and removing the solvents by centrifugation at 1000 g for 30 s three times. The denatured proteins were concentrated by absorption at 280 nm manually using a Nanodrop™ 2000 (Thermo Scientific). Trypsin was added to 20 μg of reduced-alkylated antibody at a 1:10 enzyme/substrate ratio, and the mixture was incubated at 37°C for 60 min. After digestion, 2.5% TFA was added at a 1:10 ratio to quench the digestion. The sample concentration was calculated, and the digested samples were stored at < −70°C.

### 2.3.2 Automated sample preparation

Protein LoBind® 96-well plates (Eppendorf, Hamburg, Germany), SizeX100 IMCSTips (IMCS, Irmo, SC), a Hamilton Microlab® STAR™ liquid-handling system and accessories (reagent reservoirs and syringes, Hamilton, Burlington, VT, USA) were used in the automated method. The program calculated the amount and corresponding volumes of samples, buffers, and reagents needed for the automated method. In brief, the program populated the denaturation plate with denaturing buffer and antibody sample. DTT was then added, followed by mixing and incubation for 30 min at RT. IAA was then added and mixed and allowed to incubate at RT for an additional 25 min. The resulting reduced-alkylated antibody samples with concentrations ranging from 0.25 to 1 mg/mL were then desalted using SizeX500. The buffer exchange process was completed in less than 15 min, which includes three washes to equilibrate SizeX with buffer, followed by sample loading and its subsequent elution after chaser addition. Trypsin was added to 50 μg of reduced-alkylated antibody at a 1:10 enzyme/substrate ratio, and the mixture was incubated at 37°C for 60 min. Then 2.5% TFA was added at a 1:10 ratio to quench the digestion. Protein volumes, concentrations, and recoveries were measured. A280 measurements of the remaining sample (after transfer for trypsin digest) were manually completed using the Nanodrop™ 2000. The sample concentration was calculated, and the digested samples were prepared for analysis or stored at < −70°C.

### 2.3.3 Addition of free methionine

Free L-methionine was added to GuHCl denaturing buffer and 50 mM trypsin digestion buffer at 1, 3 and 5 mM concentrations. The digestion and buffer exchange protocols were identical to the ones without methionine addition.

### 2.3.4 Reversed-phase liquid chromatography

A dual column Vanquish™ Flex Binary UPLC system was used (ThermoFisher). The system allowed columns to be switched after sample analysis so that the column wash and reconditioning were done while the next sample was injected and analyzed. Mobile phase A contained 0.1% formic acid (Millipore, Suprapur®) in water and mobile phase B contained 0.1% formic acid in ACN. The following LC conditions were used to separate the peptides: flow rate at 0.3 mL/min, column temperature at 50°C, and the autosampler at 4°C. For each analysis, a nominal load of 2 μg of the digest, based on final sample concentration, was injected onto a Zorbax C18 300-SB column (300 Å pore size, 1.8 mm particle size, 150 mm length; Agilent). The gradient started at 2% B until 5 min, then increased to 10% in 1 min. Next, the gradient was ramped up from 10% B to 35% B in 44 min. Next, the % B was increased to 60% in 5 min. At 55 min, the % B was dropped to 2% for 5 min. The total analysis time was 60 min. After column switching, the previous column was washed with saw tooth gradients (2% B to 90% B and back down) until minute 45. From minute 45 to 60, 2% B was used to recondition the column.
2.3.5 | Mass spectrometry

A Q-Exactive HF orbitrap mass spectrometer (Thermo Scientific) coupled to the HPLC system was used for MS and MS/MS analysis. The MS capillary temperature was maintained at 250°C. For the top 5 precursor ions, the MS spectra were acquired at mass resolution of 120,000 with an automatic gain control (AGC) target of 3E6, maximum ion time of 200 ms, and a scan range of 300 to 1800 m/z between 5 and 55 min run time. MS/MS spectra were acquired at mass resolution of 15,000 with an AGC target of 1E5, maximum ion time of 250 ms, with an isolation window of 3.0 m/z. The dynamic exclusion was set to 10 s.

2.3.6 | Data analysis

Biopharma Finder version 3.2 (Thermo Scientific) was used for peptide and attribute identification using the default variable modifications and the CHO glycan library. A static modification was set for cysteines carboxymethylation and unspecified modifications were allowed between 58 and 162. After peptide and attribute identification, the monitoring of attributes was done using Expressionist Refiner MS (Genedata). New peak detection was done using Biopharma Finder with MS area ratios set for ≥5 or ≤0.2.

3 | RESULTS

3.1 | Automated liquid-handling system setup

The setup on the automated liquid-handling system (ALH) was based on the manual sample preparation protocol with some adjustments such as the use of a heater/shaker block for the automated method versus a water bath in the manual method. The reagent placements, plastic consumables, and hardware configurations were designed to reduce carryover and minimize movements (Figure 1). Full details of the automation protocol are provided in section 2.

The program allows two options for the sample source: a 96-well PCR plate or a single tube in the multiflex carrier. The samples are denatured in a 96-well plate where subsequent reduction and alkylation occurs (Figure 1, Denaturation Plate, tracks 5–10) with a lid to minimize light exposure. Automated buffer exchange processes the samples from the denaturation plate and elutes them into the elution plate (Figure 1, Elution Plate, tracks 5–10). The samples are then transferred to the trypsin digest plate (Figure 1, Trypsin Digest Plate, tracks 12–18) to be incubated with trypsin on the heater/shaker module (Figure 1, Hamilton Heater Shaker [Trypsin Digest], tracks 30–35) with a lid to minimize evaporation. Once the trypsin digest plate has been removed from the heater, TFA is added and the plate is ready for further analysis, transfer to autosampler vials, or storage at −70°C until use.
Representative peptide chromatograms of mAb 1 (manual vs. automated) are shown in Figure 2. The chromatograms from automated and manual preparations were nearly superimposable, and we did not find any new peaks with a threshold set at 5-fold difference with the minimum signal intensity of 1E5 for all charge states using BioPharma Finder software program from the initial test sets of samples prepared in triplicate.

3.2 | Effect of protein concentration on recovery

We evaluated the sample recovery and variability by measuring the concentrations of samples before and after buffer exchange for both the manual and the automated protocol. Sample concentrations of 1, 2, 5 and 9 mg/mL of mAb 1 in triplicate were used for evaluation. The results are summarized in Figure 3A. The sample recovery for manual preparation increased with increasing sample concentration, ranging from 29.2% at 1 mg/mL to 61.5% at 9 mg/mL with a maximum relative standard deviation (RSD) value of 6.9% at 9 mg/mL. On the other hand, the relative % recovery after buffer exchange using SizeX IMCStips was consistently higher at all concentrations with the lowest observed recovery of 86.4% at 1 mg/mL with a maximum RSD of 2.5% at 5 mg/mL.

The sample recovery from SizeX IMCStips was also evaluated for mAb 1 samples at 11 mg/mL (n = 72) and mAb 2 samples at 10 mg/mL (n = 25) (Figures 3B and 3C). The average sample recoveries were 88.0% for mAb 1 with RSD of 5.3% and 94.3% for mAb 2 with RSD of 10.4%.

3.3 | Evaluation of edge effects

While all reagents and sample conditions tested were the same throughout the experiments, there were a few notable differences between the manual and automated sample preparations, namely sample heating and mixing strategies. With the Hamilton STAR, a heating block equipped with a PCR plate adapter was used during trypsin digestion while a water bath was used for manual preparations. When using a heating block it is possible that the temperature of the wells at the edge of the sample plate differ slightly from the inside wells. Because of this difference in heating methods, we evaluated if the edge effects during automated preparations impacted results. Six edge and six inside samples were placed in a plate as shown in Figure 4, and two plates were prepared on separate days to provide 12 edge and 12 inside samples. The starting mAb 2 sample concentration was 10 mg/mL for both automated and manual preparations done in triplicate.
The deamidation and succinimide formation levels over 0.3% for mAb 2 are reported in Figures 5A–5C. For asparagine positions 2 and 3 on peptide B in Table 1, the average total succinimide and the deamidation levels are reported. There are three asparagine residues on this peptide, and two of them were deamidated to form aspartic acid or iso-aspartic acid leading to four chromatographic peaks.

**FIGURE 4** The plate layout for edge effect evaluation. Water was placed between wells to mimic a full plate. The samples were incubated at 37°C using a heating plate within the Hamilton STAR.

**FIGURE 5** A. The average succinimide and deamidation levels at asparagine positions 1, 2 and 3 are graphed for edge and inside samples and compared with the average values from manual preparation. B. The average methionine and tryptophan oxidation levels at methionine position positions 1, 2 and tryptophan position 1 are graphed for edge and inside samples and compared with the average values from manual preparation. C. The average unprocessed C-term lysine levels. Error bars show ±1 S.D.
theoretically: however, we observed coelution of peaks. Since two coeluting peaks belong to the deamidation products of asparagine positions 2 and 3, the sum of all deamidation values on this peptide are reported. The sum of succinimide levels for positions 2/3 are reported for simplicity. The succinimide levels ranged from 1.93 to 2.1% and 1.87 to 2.04% for position 1 in peptide A and positions 2/3 in peptide B, respectively. The total deamidation levels for positions 2/3 were all below 1% ranging from 0.58 to 0.62%. The asparagine positions 2/3 are located in the Fc region of this molecule and the most vulnerable to deamidation in this molecule,19 but we saw very low levels of modification. All RSD values were less than 10% within plates. Overall, there was no significant difference in succinimide and deamidation levels at all asparagine positions between edge and inside samples, and their values were comparable to manually prepared samples. Iso-aspartic acid formation is another post-translational modification that can be influenced by varying temperatures. No aspartic acid residues showed isomerization levels over 0.1%.

Both methionine and tryptophan oxidation levels were measured and compared with the values from manual preparations (Figures 5D–5F). The average oxidation levels ranged from 1.29 to 1.61% for methionine position 1 in peptide C and from 0.78 to 1.07 for methionine position 2 in peptide D. Tryptophan oxidation was observed at position 1 in peptide D. All preparations were below 1%. Again, all RSD values were less than 10% within plates, and all oxidation levels were comparable to or lower than manual preparations. All tryptophan double oxidation levels were less than 0.5% (not shown).

Figures 5G and 5H show the unprocessed C-term lysine and non-cyclized N-term glutamine, respectively. The levels of both unprocessed lysine and N-term glutamine were consistent within plates as well as with values from manual preparations. RSD values were all within 10%.

The mAb 2 molecule has two N-glycosylation sites: one in the light chain (LC) and the other in the heavy chain (HC) fragment.

### Table 1: Peptides monitored for post-translational modifications

| Peptide | Modification positions |
|---------|------------------------|
| A       | XXXXXXXXXXXXX-N1-XX    |
| B       | XXXXXXXXXXXXX-N2-XXXX-N3-NXX |
| C       | XXX-M1-XXX             |
| D       | XXX-W1-XXXXXXXX-M2-XXX |

### Figure 6

A. The average light chain (LC) glycosylation levels for the top 10 glycans. B. The average Fc glycosylation levels for the top 6 glycans. Error bars show ±1 S.D.

### Figure 7

Selected attribute levels with varying initial sample concentrations. Error bars show ±1 S.D.
crystallizable (Fc)-region. The LC top 10 and HC top 6 glycan levels are shown in Figures 6A and 6B, respectively, with excellent reproducibility within plates as well as with manual preparations.

3.4 | Varying starting sample concentrations

We evaluated the effects of varying starting concentrations on the modification levels for both manual and automated sample preparations in triplicates. We saw no change in the levels of succinimide, deamidation and aspartic acid isomerization. The only change we observed was methionine oxidation levels as shown in Figure 7. At both methionine positions 1 and 2, oxidation levels were slightly increased at lower concentrations for both automated and manual sample preparations, but the changes in oxidation levels were larger for manually prepared samples.

3.5 | Methionine addition during sample preparation

One of the common remedies used to suppress methionine oxidation is addition of free methionine to sample preparation reagents or in some cases HPLC solvents. We evaluated the oxidation levels after addition of methionine to the final concentrations of 0 (control), 1, 3 and 5 mM in GuHCl denaturing buffer and 50 mM trypsin digestion buffer in triplicates using mAb 2 at 10 mg/mL initial concentration. The oxidation levels at methionine positions 1 and 2 are summarized in Figure 8. The manual preparation appears more susceptible to day-to-day differences in preparation conditions as the oxidation levels without free methionine addition at protein concentration of 10 mg/mL differed more from the set described in section 3.4. Overall, we observed reduction in oxidation levels even at 1 mM concentration for both manual and automated preparations. Though the oxidation levels continued to be reduced as the methionine concentrations increased to 3 and 5 mM, the changes were minimal.

4 | DISCUSSION

We have observed exponential growth in the market share and the production of biologics in the last decade along with the technological advancement in the analytics. As part of growing needs for more cost-effective, comprehensive, and site-specific characterization of biotherapeutics, the MS-based MAM has become widely used as part of cell line, process, and formulation development as well as in QC. Sample preparation is a critical aspect of the MAM as it should add very little artifacts in order to correctly characterize the attributes. Buffer exchange after denaturation can reduce the digestion time significantly while providing fully digested peptides with little additional artifacts; however, incorporating the buffer exchange step in the automated MAM sample preparation has not been fully explored and comprehensively compared with the manual preparation. In this study, we developed and evaluated a fully automated, low-artifact MAM sample preparation protocol using buffer exchange with a Hamilton liquid-handling system.

The customizable scripts for the liquid-handling system seamlessly executed all the digestion steps reducing the at-bench time significantly. The entire automated sample digestion time was similar to manual digestion, and up to 96 individual samples could be prepared in parallel, even with varying sample concentrations. Because SizeX IMCStips are individual tips instead of a plate, the number of samples and well locations can vary for each preparation without wasting the unused columns. After centrifuging to remove the storage buffer, all exchange steps take minutes to complete with excellent recovery and reproducibility for a wide range of mAb concentrations. In our study, the sample recovery ranged from 85 to 95% for all sample types and concentration tested with RSD values less than 10%. Because of the reproducibility exhibited by the buffer exchange process, we eliminated the concentration measurement step using A280 after buffer exchange to further simplify the procedure. For all the experiments, the yield was estimated as 85% to transfer 50 μg of protein sample before trypsin digestion. The transferring volumes are automatically calculated for each well from

![Oxidation at M Position 1](image.png)

![Oxidation at M Position 2](image.png)

**FIGURE 8** Methionine oxidation levels at positions 1 and 2. Error bars show ±1 S.D
the initial sample concentrations eliminating the need to manually adjust the sample volume for each sample. After sample digestion, the quenched peptide samples can be transferred to autosampler vials using a Hamilton system. The samples can be analyzed immediately or quenched peptide samples can be transferred to autosampler vials to adjust the sample volume for each sample. After sample digestion, the total ion current chromatograms were nearly superimposable, and the modification levels showed no significant difference between the two preparation methods. Deamidation can be particularly sensitive to the incubation pH, temperature, and duration,\textsuperscript{20–22} but the levels were all under 1\% even for the most vulnerable asparagine positions 2/3 in the Fc region combined.

The trypsin digestion using the plate heater showed no well location dependent variabilities or edge effects in modification levels indicating no temperature gradient in the plate due to the heating strategies or cooling. We observed very little tryptophan oxidation in both preparation methods, which can be influenced by the light exposure. We did not expect glycan, and N and C-terminal modifications to vary between the two preparation methods unless there is a significant change in the digestion efficiency. Indeed, we saw nearly identical results with excellent reproducibility for such modifications.

We also evaluated the effects of starting mAb concentrations on the product attributes. The only modification that showed concentration-dependent changes in levels was methionine oxidation. The samples with the lowest starting mAb concentration showed the highest methionine oxidation levels at two methionine positions. The differences in the oxidation levels were even larger for the manually prepared samples. This difference may be because mAb concentrations are significantly lower after the buffer exchange step and the following sample digestion process due to lower recovery at the buffer exchange step. We also evaluated the overall effect of free methionine in denaturing and the digestion solution on oxidation levels. There was significant reduction in oxidation levels even at 1 mM for both preparation methods while reduction in methionine oxidation was minimal, from 1 to 5 mM. Based on these results, we recommend addition of free methionine in sample preparation solutions at concentrations over 1 mM.

Overall, we conclude that the automated sample preparation provides comparable relative PQA values to manual preparation with excellent reproducibility, improved recoveries across different concentrations, more streamlined walk away solution and higher throughput capability. The seamless sample preparation without intervention from the start to finish makes the MAM more suitable for all stages of drug development and production.

**PEER REVIEW**

The peer review history for this article is available at https://publons.com/publon/10.1002/rcm.9222.

**DATA AVAILABILITY STATEMENT**

Research data are not shared due to proprietary nature of the antibodies used for analysis.

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**REFERENCES**

1. Grilo AL, Mantalaris A. The increasingly human and profitable monoclonal antibody market. *Trends Biotechnol.* 2019;37(1):9-16. https://doi.org/10.1016/j.tibtech.2018.05.014

2. Rogers RS, Nightlinger NS, Livingston B, Campbell P, Bailey R, Ballard A. Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics. *MAbs.* 2015;7(5):881-890. https://doi.org/10.1080/19420862.2015.1069454

3. Rogers RS, Abernathy M, Richardson DD, et al. A view on the importance of “multi-attribute method” for measuring purity of biopharmaceuticals and improving overall control strategy. *AAPS J.* 2018;20(1):7. https://doi.org/10.1208/s12248-017-0168-3

4. Ren D. Advancing mass spectrometry technology in cGMP environments. *Trends Biotechnol.* 2020;38(10):1051-1053. https://doi.org/10.1016/j.tibtech.2020.06.007

5. Luciani F, Galluzzo S, Gaggioli A, et al. Implementing quality by design for biotech products: Are regulators on track? *MAbs.* 2015;7(3):451-455. https://doi.org/10.1080/19420862.2015.1023058

6. Aksu B, Paradkar A, De Matas M, Özer Ö, Güneri T, York P. Quality by design approach: Application of artificial intelligence techniques of tablets manufactured by direct compression. *AAPS PharmSciTech.* 2012;13(4):1138-1146. https://doi.org/10.1208/s12249-012-9836-x

7. Aksu B, De Beer T, Folestad S, et al. Strategic funding priorities in the pharmaceutical sciences allied to quality by design (QbD) and process analytical technology (PAT). *Eur J Pharm Sci.* 2012;47(2):402-405. https://doi.org/10.1016/j.ejps.2012.06.009

8. Yu LX, Amidon G, Khan MA, et al. Understanding pharmaceutical quality by design. *AAPS J.* 2014;16(4):771-783. https://doi.org/10.1208/s12248-014-9598-3

9. Alt N, Zhang TY, Motchnik P, et al. Determination of critical quality attributes for monoclonal antibodies using quality by design principles. *Biotechnological Sciences.* 2016;44(5):291-305. https://doi.org/10.1016/j.bios.2016.06.005

10. Von Kreudenstein TS, Escobar-Cabrera E, Lario PI, et al. Improving biophysical properties of a bispecific antibody scaffold to aid developability: Quality by molecular design. *MAbs.* 2013;5(5):646-654. https://doi.org/10.4161/mabs.25632

11. Rathore AS. Quality by design (QbD)-based process development for purification of a biotherapeutic. *Trends Biotechnol.* 2016;34(5):358-370. https://doi.org/10.1016/j.tibtech.2016.01.003

12. Rogstad S, Faustino A, Ruth A, Keire D, Boyne M, Park J. A retrospective evaluation of the use of mass spectrometry in FDA biologics license applications. *J Am Soc Mass Spectrom.* 2017;28(5):786-794. https://doi.org/10.1007/s13361-016-1531-9

13. Rogstad S, Yan H, Wang X, et al. Multi-attribute method for quality control of therapeutic proteins. *Anal Chem.* 2019;91(22):14170-14177. https://doi.org/10.1021/acs.analchem.9b03808

14. Ren D, Pipes GD, Liu D, et al. An improved trypsin digestion method minimizes digestion-induced modifications on proteins. *Anal Biochem.* 2009;392(1):12-21. https://doi.org/10.1016/j.ab.2009.05.018

15. Tajiri-Tsukada M, Hashii N, Ishii-Watabe A. Establishment of a highly precise multi-attribute method for the characterization and quality control of therapeutic monoclonal antibodies. *Biotechnology and Bioengineering.* 2020;11(1):984-1000. https://doi.org/10.1002/bit.26979.
16. Mangel WF, Singer PT, Cyr DM, et al. Structure of an acyl-enzyme intermediate during catalysis: (Guanidinobenzoyl)trypsin. Biochemistry. 1990;29(36):8351-8357. https://doi.org/10.1021/bi00488a022

17. Turk D, Stürzebecher J, Bode W. Geometry of binding of the Nα-Nitosylated piperidides of m-amidino-, p-amidino- and p-guanidino phenylalanine to thrombin and trypsin. X-ray crystal structures of their trypsin complexes and modeling of their thrombin complexes. FEBS Lett. 1991;287(1–2):133-138. https://doi.org/10.1016/0014-5793(91)80033-Y

18. Song YE, Dubois H, Hoffmann M, et al. Automated mass spectrometry multi-attribute method analyses for process development and characterization of mAbs. J Chromatogr B Analyt Technol Biomed Life Sci. 2021;1166:122540. https://doi.org/10.1016/j.jchromb.2021.122540

19. Yan Q, Huang M, Lewis MJ, Hu P. Structure based prediction of asparagine deamidation propensity in monoclonal antibodies. MAbs. 2018;10(6):901-912. https://doi.org/10.1080/19420862.2018.1478646

20. Bobaly B, D’Atri V, Goyon A, et al. Protocols for the analytical characterization of therapeutic monoclonal antibodies. II – Enzymatic and chemical sample preparation. J Chromatogr B Analyt Technol Biomed Life Sci. 2017;1060:325-335. https://doi.org/10.1016/j.jchromb.2017.06.036

21. Xu W, Jimenez RB, Mowery R, et al. A quadrupole Dalton-based multi-attribute method for product characterization, process development, and quality control of therapeutic proteins. MAbs. 2017;9(7):1186-1196. https://doi.org/10.1080/19420862.2017.1364326

22. Mouchahoir T, Schiel JE. Development of an LC-MS/MS peptide mapping protocol for the NISTmAb. Anal Bioanal Chem. 2018;410(8):2111-2126. https://doi.org/10.1007/s00216-018-0848-6

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