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Refinement of HR Multi-Attribute Method from Sample Preparation to Data Analysis

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ABSTRACT

The Thermo Scientific™ HR Multi-Attribute Method (HR MAM)[1] takes advantage of the high resolution accurate mass (HRAM) capabilities of Orbitrap™ technology and a streamlined process of data analysis offered by BioPharma Finder™ Software and compliance-ready Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software for robust and consistent data acquisition, critical quality attribute (CQA) identification and quantitation, and new peak detection (NPD). Here we present our recent work on refining HR MAM workflow in terms of sample preparation, column assessment, and software feature enhancement.

INTRODUCTION

The multi-attribute method (MAM) was introduced in 2015[2] and has since gained increasing recognition and adoption among biopharmaceutical manufacturers and regulatory agencies[2-4]. MAM enables characterization and monitoring of a wide range of CQAs in a single HRAM-based liquid chromatography mass spectrometry (LC-MS) assay. Additionally, MAM allows for detection of known and unknown impurities – a procedure referred to as NPD. MAM can be implemented at different phases of biopharmaceutical drug development, from research and process development to quality control (QC). To improve the robustness of MAM, each of its steps – from sample preparation to data analysis – should be carefully optimized.

MATERIALS AND METHODS

Trypsin Digestion of NISTmAb

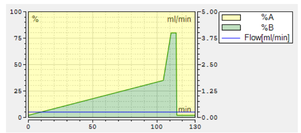
In-solution trypsin digestion on NISTmAb (National Institute of Standards and Technology, Gaithersburg, MD) was performed following the published protocol[5] and has previously been described in detail[1]. The SMART Digest of NISTmAb was performed using a Thermo Scientific™ Low pH SMART Digest™ Kit (Magnetic Bulk Resin Option; P/N 60109-101-MB-LPH) on a Thermo Scientific™ KingFisher™ Duo Prime Purification System (P/N 5400110) controlled by Thermo Scientific™ BindIt™ Software. Briefly, NISTmAb was mixed with low pH SMART Digest buffer and tris (2-carboxyethyl) phosphine (TCEP) in a 96-well plate. Then magnetic trypsin beads were transferred by magnetic heads into the sample, followed by simultaneous heat denaturation and digestion at 70 °C for 30-45 min. The digestion was terminated by removal of the trypsin beads. All the SMART Digest steps, with exception of initial manual mixing of sample and buffer, are automatically performed in the KingFisher Duo Prime system using a digestion protocol written in BindIt Software.

LC and MS Methods for NISTmAb Analysis

Peptide separations were performed using the LC gradient shown in Figure 1 on a Thermo Scientific™ Vanquish™ Horizon UHPLC system.

Figure 1. LC gradient for separation of NISTmAb peptides.

No	Time	Flow [ml/min]	%B	Curve
1	0.000		Run	
2	0.000	0.250	2.0	5
3	105.000	0.250	35.0	5
4	111.000	0.250	80.0	5
5	115.000	0.250	80.0	5
6	115.500	0.250	2.0	5
7	130.000	0.250	2.0	5
8	*New Row*			
9	130.000		Stop Run	



The performances of four commercially available Thermo Scientific columns (Table 1) were compared in terms of retention of hydrophilic peptides and separation of deamidated species.

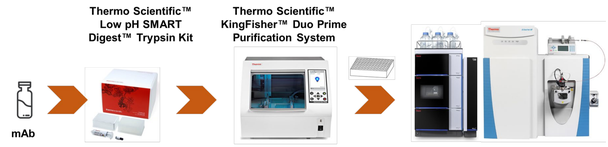
Table 1. Four commercially available Thermo Scientific™ columns used in this study

Column	Dimension (mm)	Particle size (µm)	Packing Material	Stationary Phase	P/N
Accucore™ Vanquish™ C18+	150 x 2.1	1.5	Spherical, Solid Core, Ultrapure Silica	C18	27101-152130
Acclaim™ Vanquish™ C18	250 x 2.1	2.2	Silica, Spherical Fully Porous Ultrapure	C18	074812-V
Hypersil GOLD™ Vanquish™ C18	200 x 2.1	1.9	Silica, Spherical Fully Porous Ultrapure	C18 Selectivity	25002-202130
Acclaim™ Vanquish™ PA II	250 x 2.1	2.2	Silica, Spherical Fully Porous Ultrapure	Polar Embedded	074814

The LC-MS data were acquired on a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer controlled by Chromeleon CDS (Version 7.2.10). The MS/MS data for peptide mapping were analyzed in BioPharma Finder Software Version 3.2 while full scan MS data were processed in Chromeleon CDS. A resolution setting of 140,000 (at *m/z* 200) was used to resolve overlapping peaks and to provide more accurate quantitation for deamidated peptides.

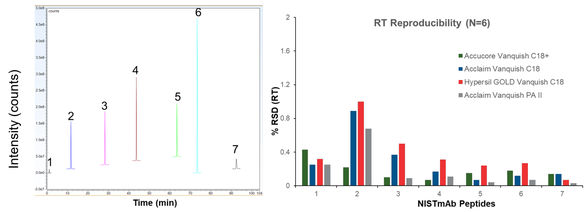
RESULTS

Figure 2. Low pH SMART Digest coupled with a KingFisher Duo Prime Purification System



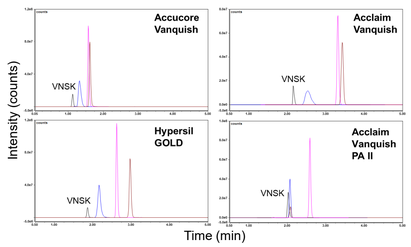
The easy-to-use low pH SMAR Digest coupled with KingFisher Duo Prime system offers automatic and consistent digestion with low artifacts due to minimal manual preparation required. The total process time of this approach is less than 1 hr, about 2-fold reduction in time compared to a 'rapid' in-solution protocol (~2 hr)[1,5]. The low pH SMART Digest and in-solution digestion protocols both produced 100% sequence coverage of NISTmAb (data not shown). The standard low pH SMART Digest protocol produced higher percentage of missed cleavages compared to the in-solution protocol. However, increasing the amount of trypsin beads can reduce the percentage of missed cleavages (data not shown). Full optimization of the low pH SMART Digest approach will be reported elsewhere.

Figure 3. RT reproducibility (%RSD) of four Thermo Scientific columns



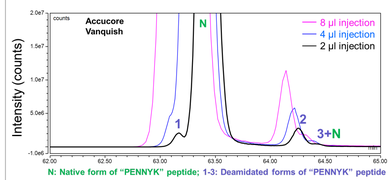
All four columns gave good RT reproducibility (%RSD < 2%) for the seven chosen peptides (1-7) across the whole RT range.

Figure 4. Retention of hydrophilic peptides



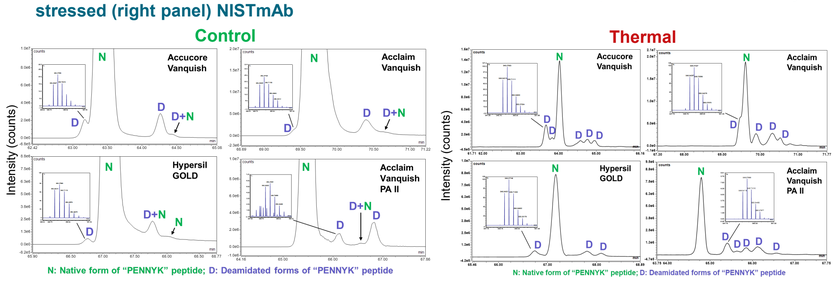
Different characteristics in retention of hydrophilic peptides were observed for the four columns. The Acclaim Vanquish and Hypersil GOLD columns showed good performance in retention of four early eluters including VNSK.

Figure 5. Effect on sample load on separation of deamidated peaks



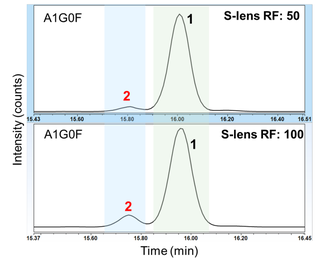
The increasing of sample load resulted in reduced separation of the deamidated peak 1 from its adjacent native form (N). By contrast, the separation of deamidated peaks 2 and 3 was not affected by the amount of sample injected.

Figure 6. Separation of 'PENNYK' deamidated species in the control (left panel) and thermally stressed (right panel) NISTmAb



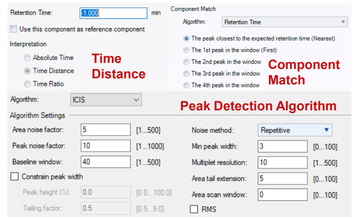
While the Accucore Vanquish, Acclaim Vanquish, and Hypersil GOLD columns produced deamidated peaks on both RT sides of the native forms, all the deamidate species were eluted later than the native form with the Acclaim Vanquish PA II column. The Accucore Vanquish and Hyperpsil GOLD columns provided good separation of deamidated species from the native form, although the Hypersil GOLD was unable to resolve some of the deamidated species.

Figure 7. Effect of S-Lens RF on glycopeptide EEQYN[A1G0F]STYR



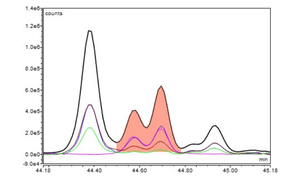
It is important to keep source condition "soft" (while maintaining good sensitivity) to avoid unintentional fragmentation (commonly called 'in-source fragmentation') of glycoforms. Figure 7 shows that the relative abundance of Peak 2 in the XIC of A1G0F increased from 3.3% to 9.0% by increasing S-lens RF from 50 to 100, indicating that Peak 2 was produced from unintentional fragmentation of larger glycoforms. It is essential for the chosen column to separate these species for accurate integration and quantitation of the native glycoform. Shown here is separation of two peaks by Hypersil GOLD. Similar result was obtained for Acclaim Vanquish (data not shown).

Figure 8. Chromeleon CDS features enable flexible and consistent peak detection and integration



Consistent peak detection and integration are key to reproducible CQA relative quantitation. This can be achieved in Chromeleon CDS by choosing the proper combination of the following settings, time distance (e.g. relative RT to a reference peptide), component match algorithm, peak detection algorithm (ICIS, Genesis, or Cobra), smoothing (Gaussian 5-7 points), and RT window.

Figure 9. Overcoming challenges for peak detection and integration



Shown here is a tricky example of consistently detecting and integrating two isomerization peaks in between deamidated peaks using the following settings:

- **RT window**: 0.50 min
- **Time distance**: -1.00 min (native form as the reference peak)
- **Component match algorithm**: Retention time with the Nearest option
- **Smoothing**: Gaussian 5 points
- **Peak detection algorithm**: ICIS with multiplet resolution = 10

CONCLUSIONS

As MAM sees increasing adoption throughout the biopharmaceutical industry, it has become clear that the ease of use, consistency, and robustness of the approach are critical to successful implementation of MAM from process and development to QC. In this poster, we describe a few factors to consider when developing and refining MAM workflow. The SMART Digest coupled with KingFisher Duo Prime system can be a viable approach to achieve automatic digestion with consistent result. In addition, we demonstrate the importance of choosing the right sample load and columns for retention and separation of CQAs. Lastly, we highlight Chromeleon CDS features for consistent detection and integration of CQAs, even in tricky scenarios.

REFERENCES

1. Liu, HC. *et al.* A high-resolution accurate mass multi-attribute method for critical quality attribute monitoring and new peak detection. Thermo Scientific™ Application Note 72916.
2. Rogers, R. *et al.* Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics. *MAbs*, **2015**, *7*, 881.
3. Rogers, R. *et al.* A view on the importance of "multi-attribute method" for measuring purity of biopharmaceuticals and improving overall control strategy. *The AAPS Journal*, **2018,** *20*, 7.
4. Rogstad, S. *et al.* Multi-attribute method for quality control of therapeutic proteins. *Anal. Chem.*, **2019**, *91*, 14170.
5. Ren, D. *et al.* An improved trypsin digestion method minimizes digestion-induced modifications on proteins. *Analytical Biochemistry*, **2009**, *392*, 12.

ACKNOWLEDGEMENTS

We would like to thank our customers and colleagues in the BioPharma and Product Marketing teams for collaboration and support.

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