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Comprehensive Adeno-Associated Virus Critical Quality Attribute Analysis with Liquid Chromatography-Mass Spectrometry

Wendi A. Hale[1], Michelle English[2], Lawrie Veale[2], St John Skilton[2], Christopher M. Colangelo[1]

[1]Agilent, Lexington, MA
[2]Protein Metrics, Cupertino, CA

Introduction

Adeno-associated viruses (AAVs) are a crucial tool for development and delivery of cutting-edge gene therapies and have been successful in treating inherited retinal diseases and spinal muscular atrophy. AAV is composed of an icosahedral protein shell with a single-stranded genome of approximately 4.7 kb. The intact AAVs act as a vehicle to protect and deliver oligonucleotide therapeutics.

As AAVs grow in popularity as therapeutic delivery platforms it is essential that all critical quality attributes (CQAs) of the therapeutic product are maintained. CQAs such as post-translational modifications and truncations are routinely monitored by mass spectrometry. Other CQAs such as host cell protein analysis and sequence variant analysis have traditionally been monitored with techniques such as ELISA and next-generation sequencing tools. However, identification and relative quantitation of these CQAs are shifting to being monitored by mass spectrometry as mass spectrometry hardware and software become more powerful and improve in sensitivity and speed..

We developed liquid chromatography mass spectrometry (LC-MS) methods for AAV characterization for host cell protein analysis (HCP) and sequence variant analysis (SVA). In addition, orthogonal methods of subunit and peptide mapping were compared directly to capture discrepancies.

Experimental

Materials:

AAV8 was produced by Lake Pharma (Worcester, MA). Molecular weight cutoff filters and (tris(2-carboxyethyl)phosphine) (TCEP) were purchased from Millipore Sigma (St. Louis, MO). Trypsin and rAsp-N were purchased from Promega (Madison, WI).

Sample Preparation:

For intact analysis, AAVs underwent a buffer exchange three times at 10,000 g with a 10 kDa molecular weight filter. The buffer contained 5 mM TCEP, 80% H_2O and 20% acetonitrile with 0.1% formic acid (v/v). After the sample was collected, it was incubated at room temperature prior to injection. For HCP analysis and SVA, the AAVs underwent denaturation, reduction, alkylation and digestion. Enzymes utilized in this experiment were trypsin and rAsp-N.

Experimental

LC/MS Analysis:

LC/MS analysis was performed on a 1290 Infinity II LC coupled to a 6545XT AdvanceBio LC/Q-TOF system with a dual Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with the large molecule SWARM autotune feature was used for intact analysis. The instrument was further calibrated and operated in standard mass mode. The iterative MS/MS feature was used for the peptide mapping, HCP, and SV workflow. All MS data was processed with Protein Metrics Byos Software (Cupertino, CA). The SVA Validator algorithm was applied to the SV workflow.

Figure 1: 6545XT AdvanceBio LC/Q-TOF

	Intact Analysis	Peptide Mapping/HCP/ SVA
Column	Zorbax Diphenyl RRHD 300Å, 2.1 x 150 mm. 1.8 µm	AdvanceBio Peptide Mapping, 2.1 x 150 mm. 2.7 µm
Flow Rate	0.4 mL/min	0.4 mL/min
Column Temperature	60°C	60°C

Table 1: Column and LC Conditions for both analyses.

Reconstruction Data

Comparing orthogonal methods such as intact analysis and peptide mapping is challenging to analyze. Byos has a package that takes peptide mapping data and reconstructs it into a theoretical intact spectrum. This spectrum is overlaid with the experimental intact spectrum and provides a direct comparison of the two data sets.

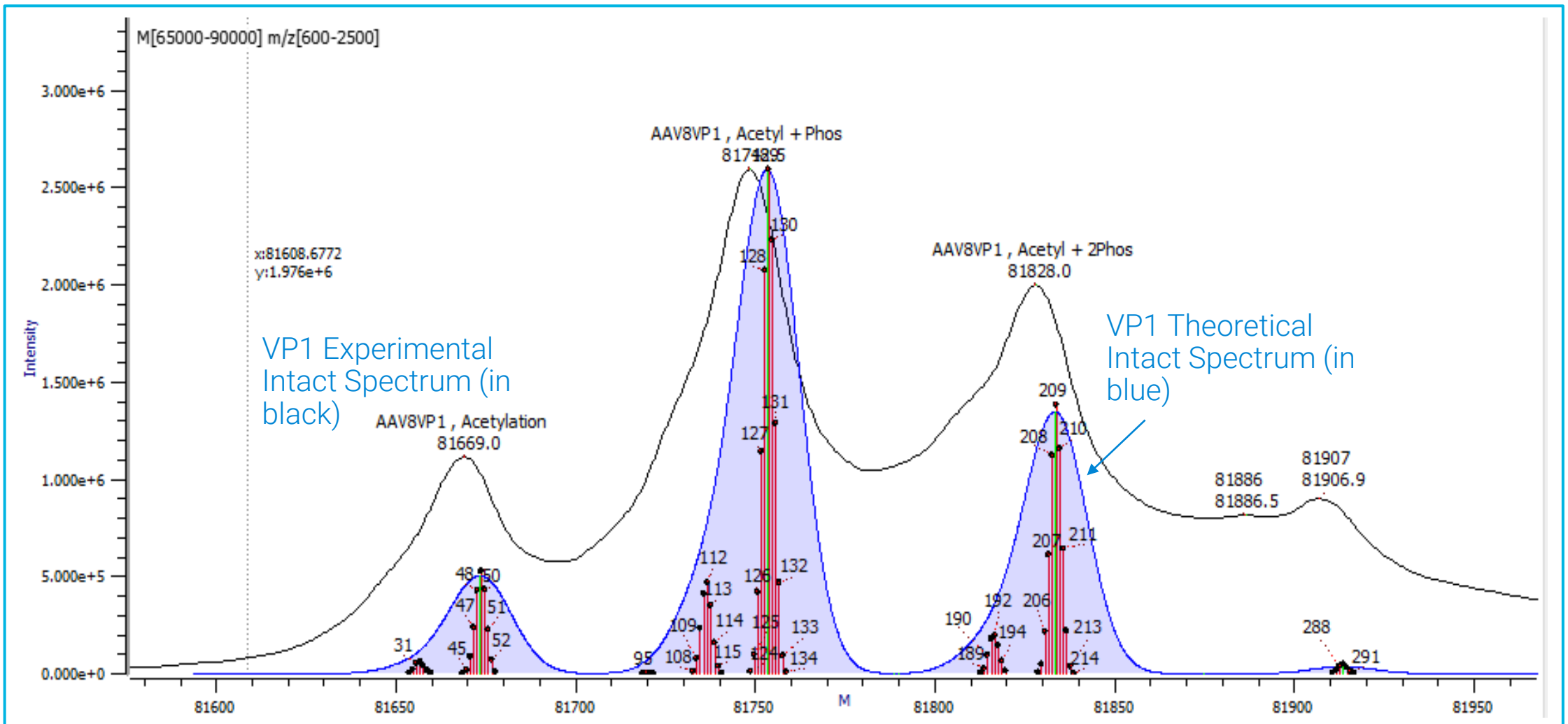

Figure 2: Reconstruction Data of VP1. The red lines represent relative quant of modifications on specific amino acids. The red lines are used to draw the theoretical blue intact spectrum. Shoulder peaks in the experimental spectrum (outlined in black) are a result of multiple low abundant and low mass PTMs, which is revealed in the reconstruction data.

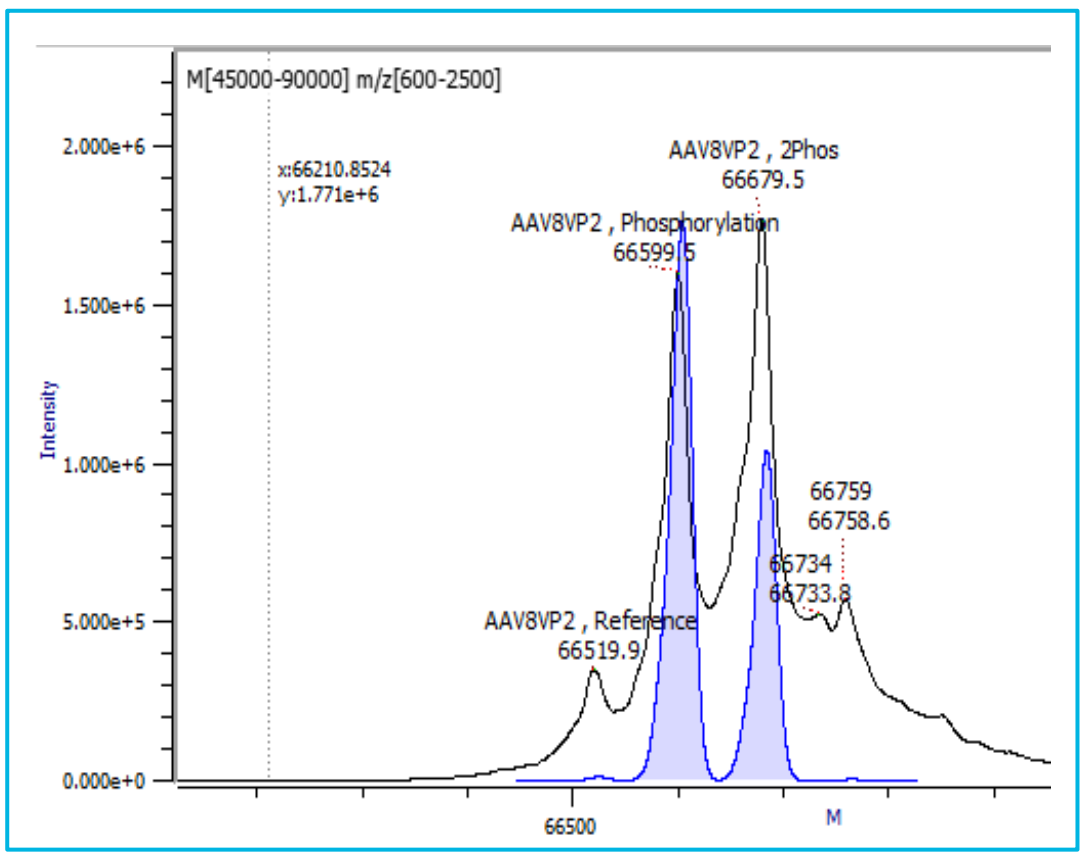

Figure 3: Reconstruction Data of VP2. The reconstruction data underestimates the second and third phosphorylation in VP1 (Figure 2) and VP2.

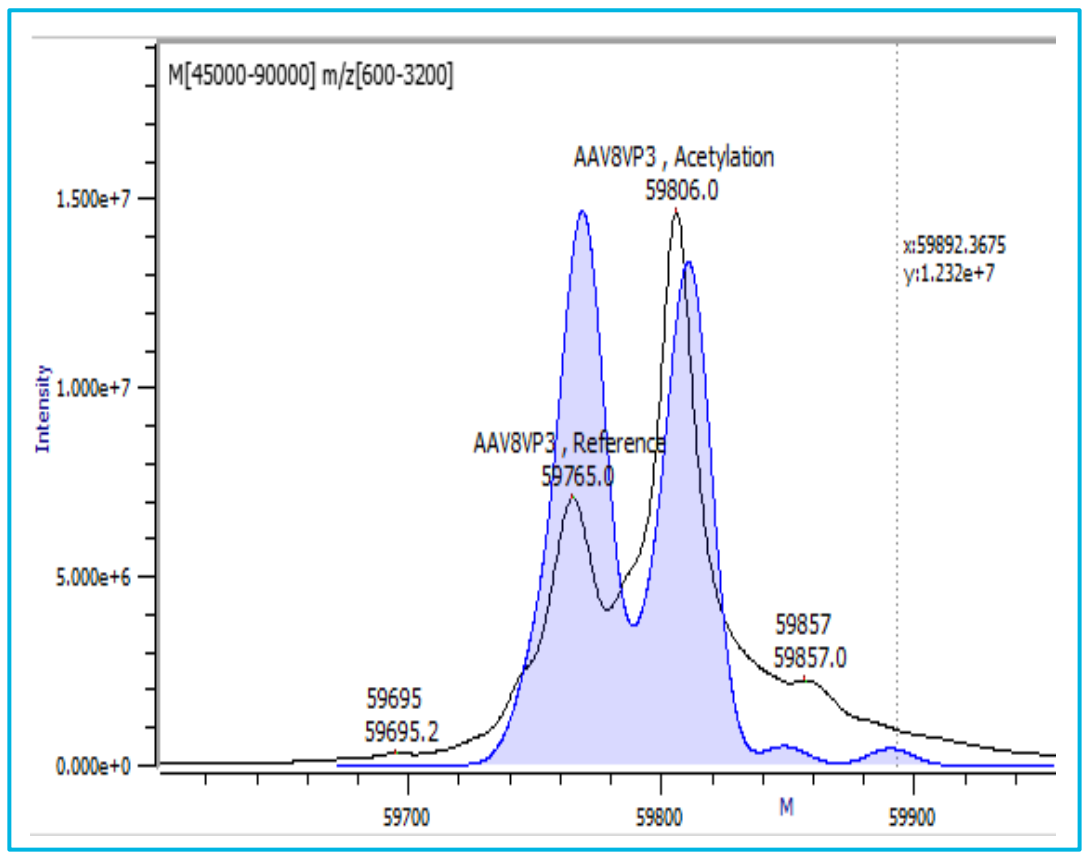

Figure 4: Reconstruction Data of VP3. The non-acetylated data is overstated in the peptide mapping data. This may be due to a clip as the clip mapping data was included in the reconstruction.

Results and Discussion

Host Cell Protein Analysis

HCP analysis is commonly performed with ELISA. ELISA, while easy to use, is not specific and cannot identify individual proteins. HCP identification by LC/MS/MS is becoming more popular with traditional biotherapeutic analysis, but it is not frequently used as an HCP assay with AAVs.

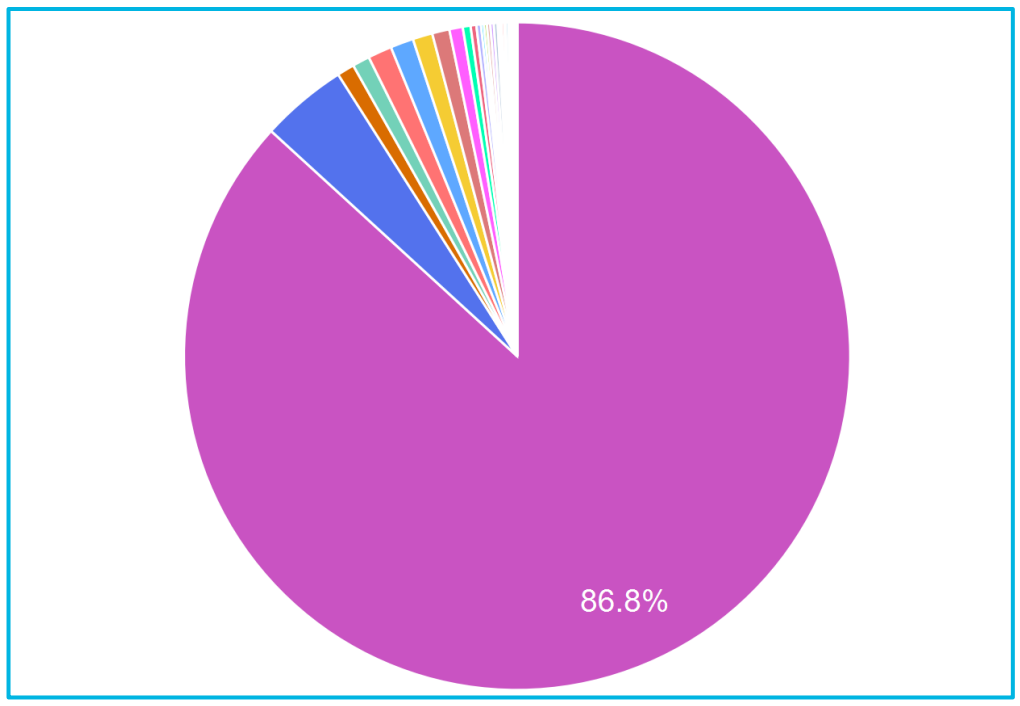

Figure 5: The top three Extracted Ion Chromatograms (EICs) of each protein were added together for relative quantitation. As expected, AAV8 is overwhelmingly abundant compared to protein impurities.

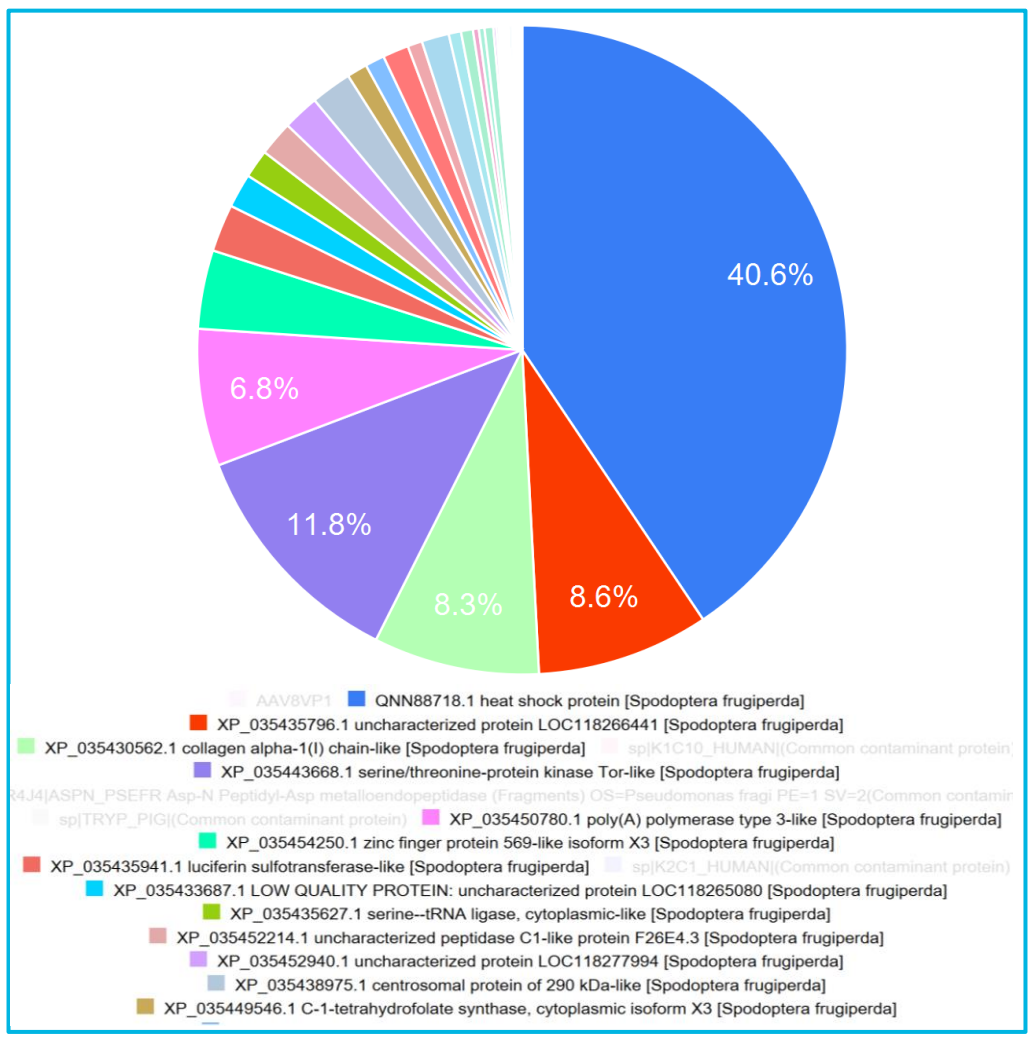

Figure 6: Top 3 EIC of all HCPs (AAV8 removed). Only one protein is more abundant than artefact proteins. The remaining proteins include a few uncharacterized proteins and a number of enzymes.

Sequence Variant Analysis

Similarly to HCP analysis, SVA by LC/MS/MS is becoming routine for traditional biotherapeutics, but less common for AAV analysis.

Variant	XIC Ratio%	Var. Pos. in VP1
Gly->Asp	0.41	545
Ile->Met	0.03	648
Met->Xle	0.57	404
Met->Xle	0.48	560
Ser->Asn	4.47	557
Ser->Asn	2.60	670

Table 2: List of sequence variants and their relative abundance.

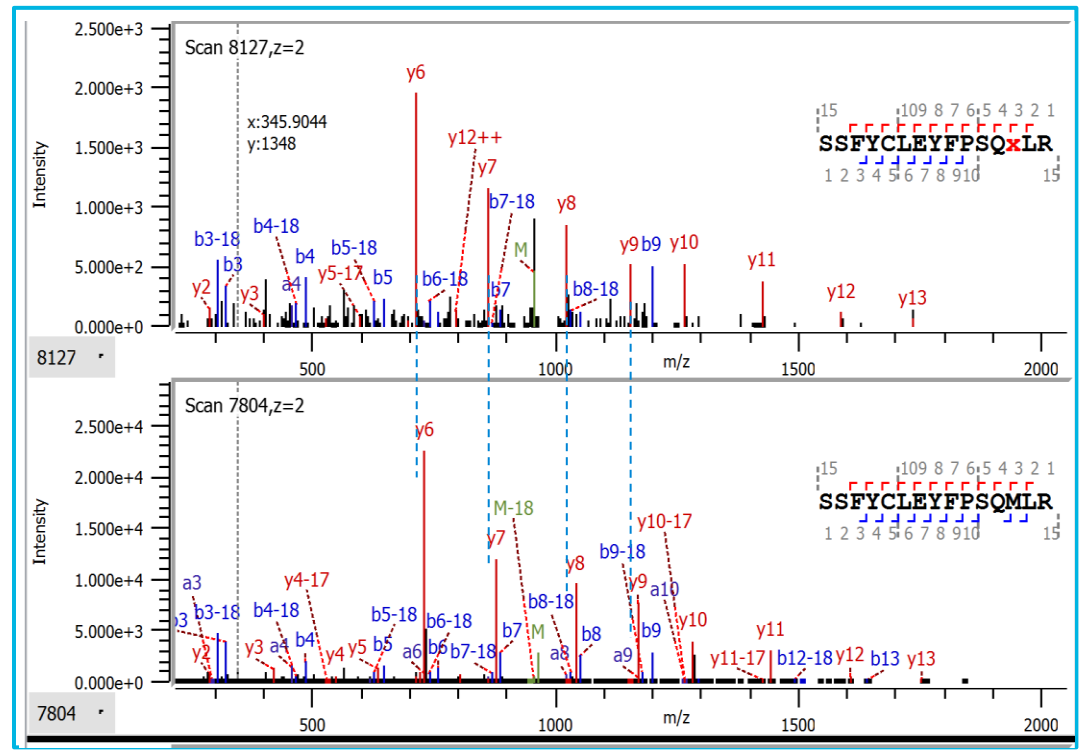

Figure 7: MS/MS confirmation of M to I/L variant. Dotted blue lines added to clarify differences in y-ions.

Conclusions

- Reconstruction data revealed low abundance and low mass PTMs contributed to peak shoulders, confirming intact data.
- Several proteins from the host cell were identified and relatively quantified.
- Several sequence variants were identified and confirmed with MS/MS.

References

[1]Huang, Y. et al. J. Pharm. Biomed. Anal. 2021. 200, 114069.
[2] Valliere-Douglass, J. et al. *PDA J Pharm Sci and Tech* 2019. 73, 622-634.

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