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Purity and Identity Characterization of Adeno- Associated Virus Capsid Particles by Intact and Bottom- Up Based Liquid Chromatography-Mass Spectrometry Methods

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Introduction

Adeno-associated viruses (AAVs) are the main viral vectors for gene therapy 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. There are 11 known serotypes that transduce different cell types, allowing for increased selectivity for therapeutics. As AAVs continue to be explored as therapeutic delivery platforms, it is vital to ensure that all the critical quality attributes (CQAs) of the therapeutic product are maintained.

Characterizing viral capsid proteins yields several challenges. The protein shell is composed of three capsid proteins, VP1, VP2 and VP3, that assemble into a 3.9 megadalton structure in a ratio of 1:1:10 with 60 capsids per virion. In addition to the low molar ratios of VP1 and VP2, all three proteins have overlapping sequences at the C-terminus. Traditionally, SDS-PAGE is used to establish the molecular weight of the capsid proteins, however, this technique provides an approximate molecular weight and may not be able to distinguish between different serotypes. Mass spectrometry is a promising method to overcome these challenges and determine CQAs of the capsid proteins.

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. Trypsin and rAsp-N were purchased from Promega.

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, 20% H₂O and 80% acetonitrile with 0.1% formic acid (v/v). After the sample was collected, it was incubated at room temperature prior to injection. For peptide mapping, 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 workflow. All MS data was processed with Agilent MassHunter BioConfirm 10.0 software.



Figure 1: 6545XT AdvanceBio LC/Q-TOF

	Intact Analysis	Peptide Mapping Analysis
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
Injection Volume	20 µL	40 µL
Column Temperature	60°C	60°C

Table 1: Column and LC Conditions for Both Analyses

Intact Analysis of AAV8 on the 6545XT AdvanceBio LC/Q-TOF

The spectral clarity provided by the improved vacuum on the AdvanceBioLC/Q-TOF in combination with the large molecule SWARM autotune feature show all three viral capsid proteins with their post-translational modifications (PTMs) with high mass accuracy, under 10 ppm for all proteoforms. While it is not shown, VP1 and VP2 are chromatographically separated which can be challenging. While mass spectrometry can separate these proteins by mass, having chromatographic separation allows for less ion suppression of these two low abundant proteins.

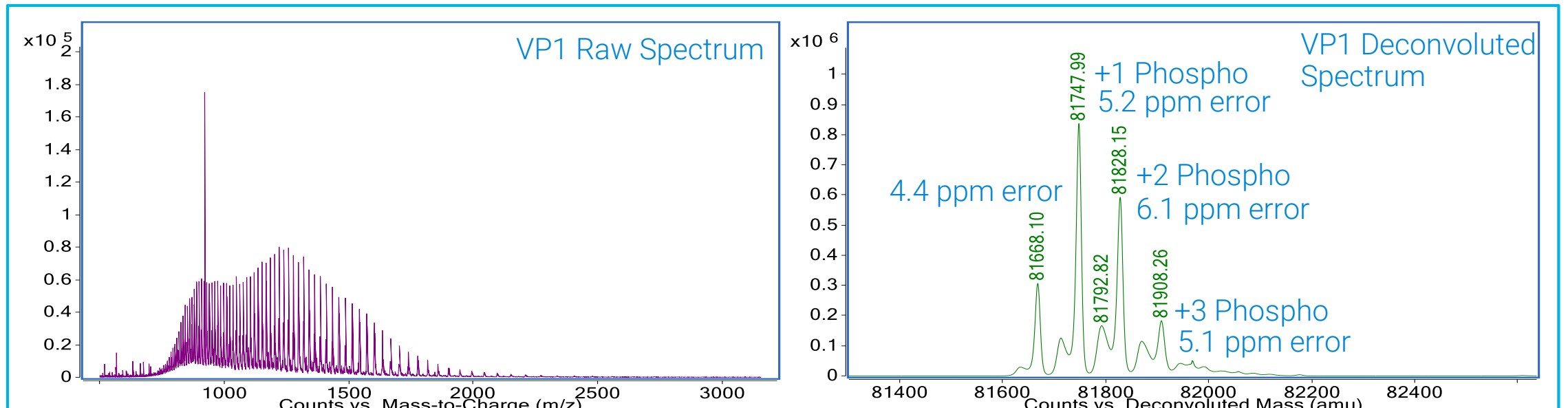


Figure 2: VP1 raw and deconvoluted mass spectra. Three phosphorylation sites were detected on VP1 with less than 10 ppm error. The accurate mass data confirmed that VP1 is missing its N-terminal amino acid residue and that the new N-terminus is acetylated.

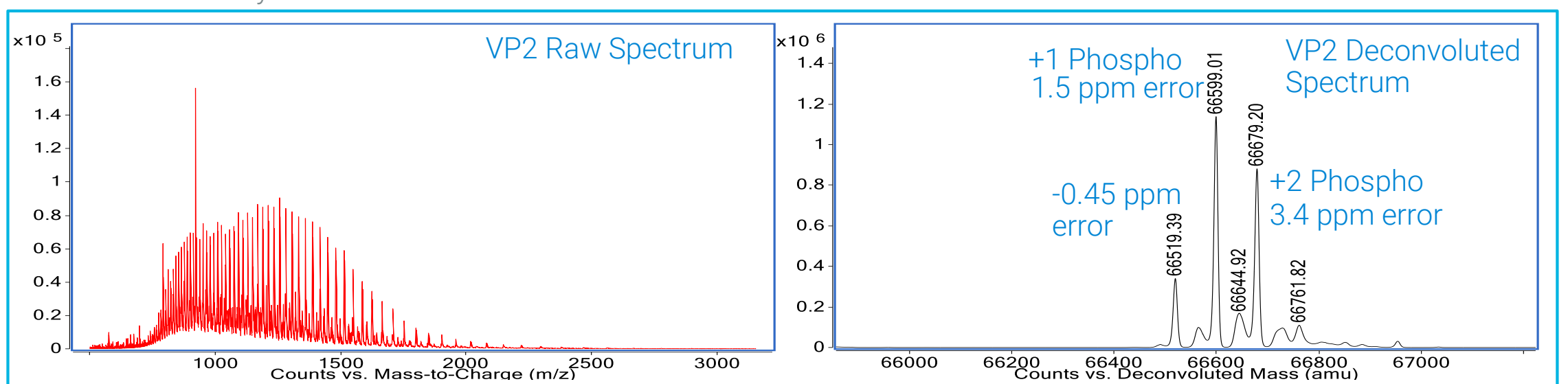


Figure 3: VP2 raw and deconvoluted mass spectra. The accurate mass data confirms at least two phosphorylation sites on VP2.

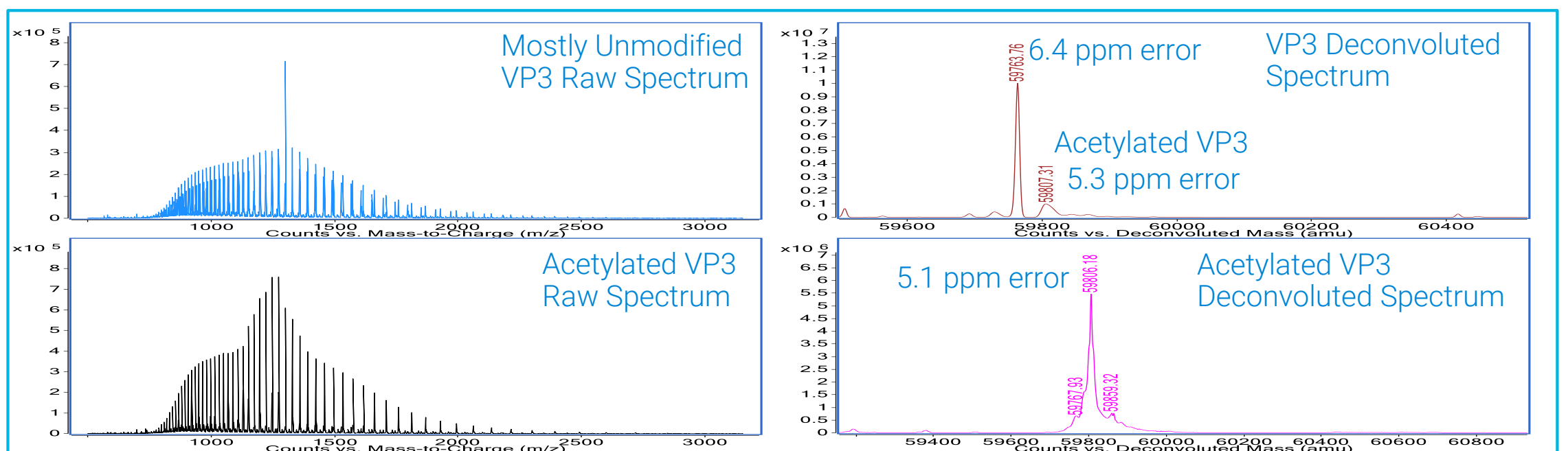


Figure 4: VP3 raw and deconvoluted mass spectra. The unmodified form of VP3 is mostly chromatographically separated from acetylated VP3. While VP1 was fully acetylated, about 70% of VP3 was acetylated.

Peptide Mapping of VP1, VP2, and VP3 on the 6545XT AdvanceBio Q-TOF.

Peptide mapping of biotherapeutics is an essential method to determine protein sequence and post-translational modifications, required by the ICH, FDA and other regulatory agencies. As of January 2020, the FDA recommends providing information regarding primary and secondary structure including PTMs for human gene therapy drug substances. Peptide mapping with the iterative MS/MS feature excludes peptides from all previous runs for isolation and fragmentation, allowing for selection and detection of low abundant peptides. In addition, BioConfirm 10.0 allows for multiple runs to be selected to provide a total sequence coverage. This feature is useful for combining results from iterative MS/MS runs as well as using multiple enzymes.

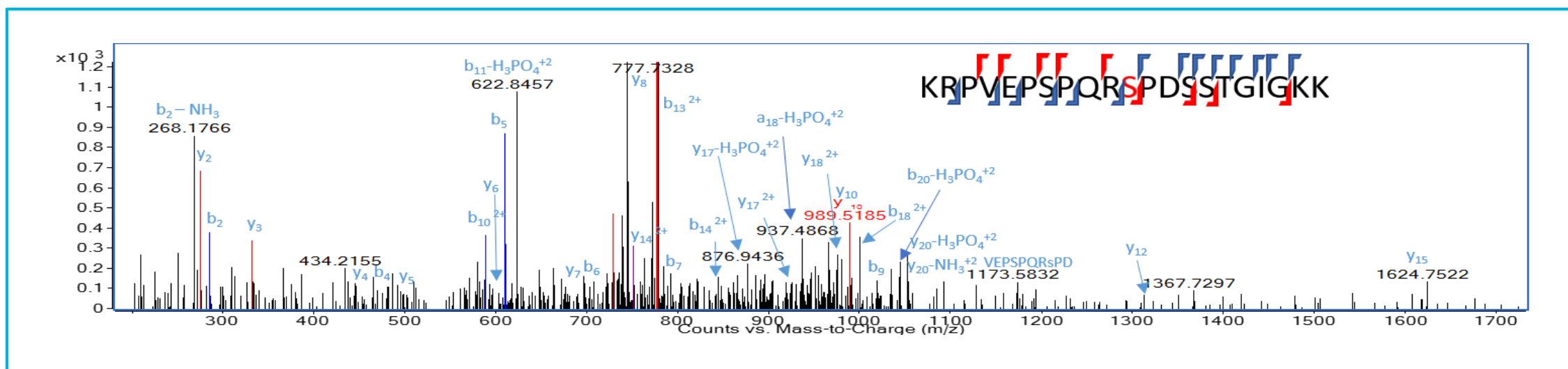


Figure 5. Example of MS/MS spectrum confirming site-specific phosphorylation of a serine residue.

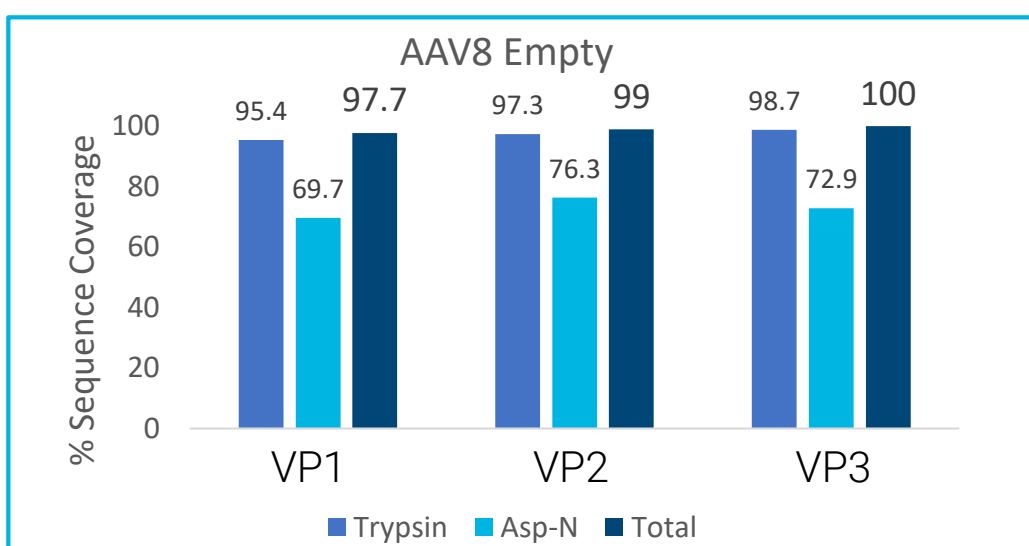


Figure 6. Peptide mapping results of each of the viral capsid proteins. Each protein has 100% or nearly 100% sequence coverage with MS/MS confirmation.

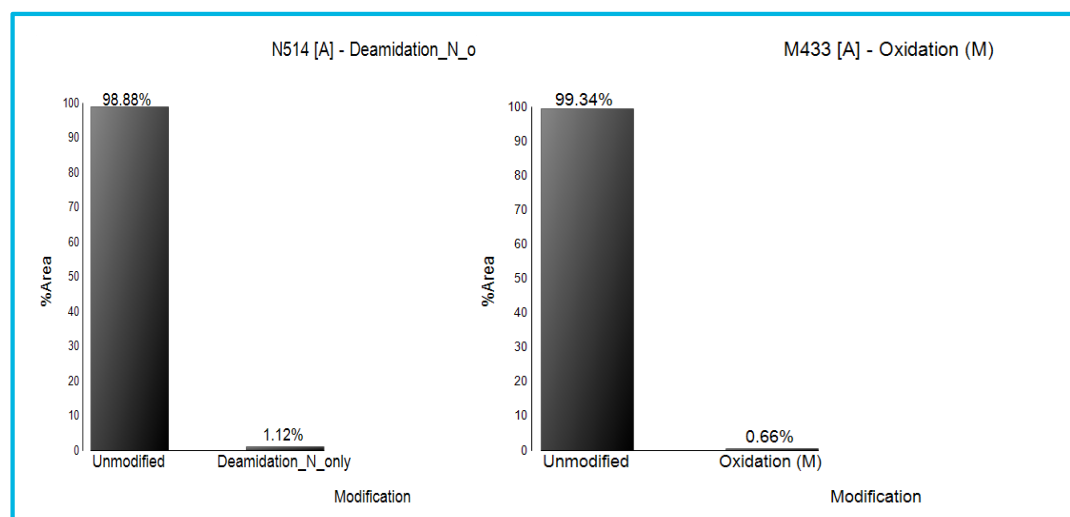


Figure 7. Examples of common PTMs. Low levels of deamidation and oxidation indicate this protein has not degraded.

Conclusions

- All three capsid proteins were chromatographically separated.
- The intact protein data has clear spectra and all proteoforms have less than 10 ppm error.
- The peptide mapping data gives between 97.7-100% sequence coverage with MS/MS confirmation.
- Site specific phosphorylation was localized.
- Relative quantitation of PTMs was performed.

References

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