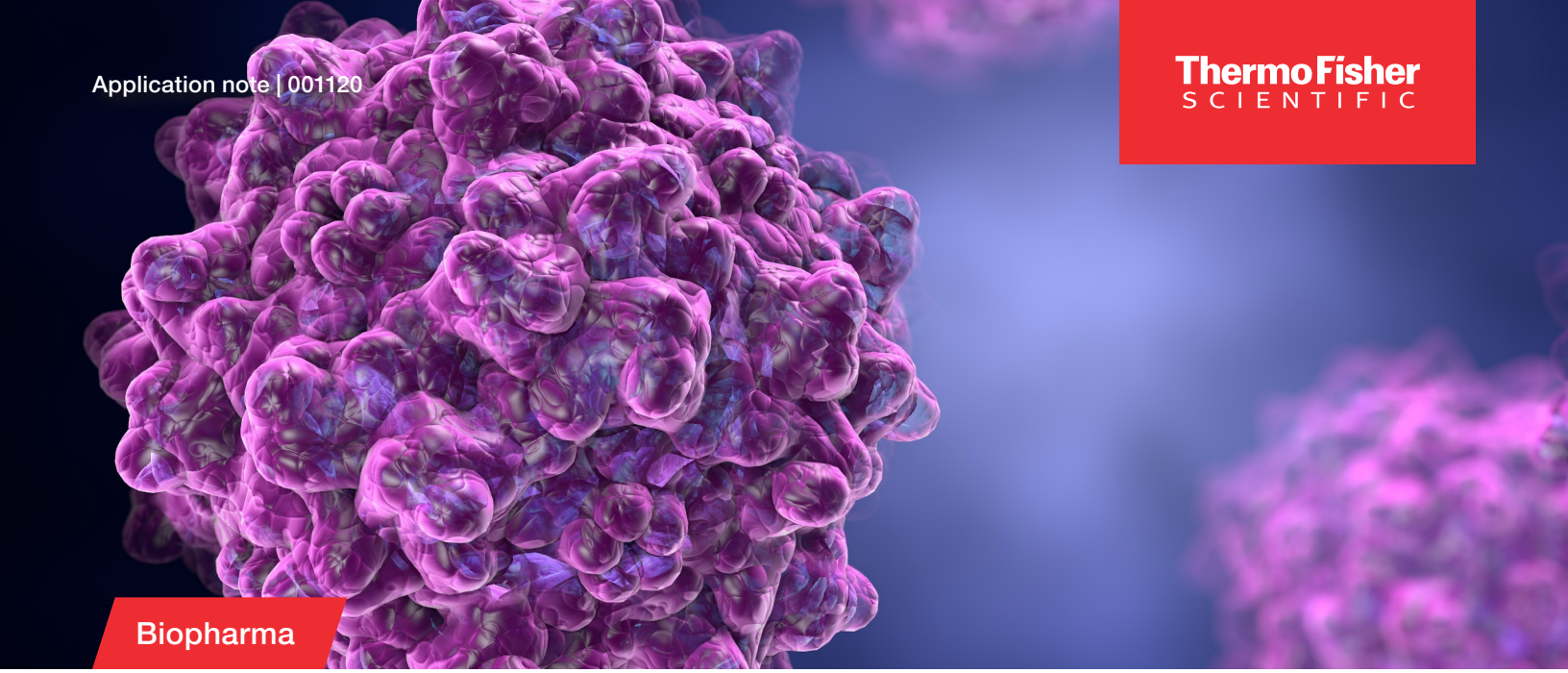

Application note | 001120

Biopharma

Assessing key attributes of adeno-associated viral proteins using HPLC-FLD-intact mass analysis

Authors

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Keywords

Adeno-associated virus (AAV), capsid protein, viral protein, post translational modification (PTM), high-resolution accurate mass (HRAM), Orbitrap Exploris MX mass detector, ultra-high performance liquid chromatography (UHPLC), reversed-phase liquid chromatography (RPLC), fluorescence detection (FLD)

Application benefits

- Rapid ratio assessment of adeno-associated virus (AAV) structural proteins VP1:VP2:VP3 through sensitive fluorescence detection
- Accurate intact mass measurement of AAV viral proteins and associate proteoforms with mass error < 5 ppm, ensuring rapid AAV serotype confirmation
- Sensitive detection and accurate intact mass measurement of low abundant AAV vial protein PTMs
- Generic method for monitoring the structure differences of AAV products, including ratios of VPs, identity of VPs and their associated PTMs/truncated forms across different batches and manufacturing processes

Goal

Develop a robust UHPLC-FLD-HRAM Thermo Scientific™ Orbitrap™ MS method for rapid characterization of AAV viral protein identities, relative ratios, and PTMs that are applicable to multiple serotypes

Introduction

Recombinant adeno-associated viral (rAAV) vectors have emerged as the leading gene delivery vehicles for gene therapy due to their high-efficiency transduction and safety. AAV viral proteins (VPs) are critical for viral infectivity and vector potency. The key attributes of AAV VPs, such as identity and relative ratio of VPs and their post-translational modifications (PTMs). PTMs need to be fully characterized and monitored during the viral vector development and manufacturing to ensure the safety, quality, and efficacy of AAV products.[1,2]

To address the analytical needs for supporting large-scale AAV product manufacturing, we developed a UHPLC-FLD-HRAM MS method for simultaneous relative expression ratio assessment of VP1, VP2, and VP3 using FLD and direct accurate intact mass measurement of the VPs, their associated proteoforms and truncated protein forms using an HRAM Orbitrap mass spectrometer. The market-leading resolution, mass accuracy, and sensitivity offered by Orbitrap technology enabled excellent spectral quality for accurate AAV viral protein intact mass analysis. The HPLC-FLD-HRAM MS method was applied to analyze multiple AAV serotype samples. The analytical results are reported here.

Experimental

Sample preparation

Two in-house AAV samples (AAV6, AAV9) expressed via transient transfection in HEK293 cells using the Gibco™ AAV-MAX Helper-Free AAV Production, and four commercially available AAV serotype samples (AAV1, AAV6, AAV8, and AAV9) were used for the HPLC-FLD-HRAM MS method development and evaluation. The AAV samples were buffer exchanged and concentrated into 80% H_2O/20% acetonitrile containing 5 mM TCEP and 0.1% formic acid using a 30K centrifugal filter (Amicon™ Ultra, 0.5 mL). The collected sample was incubated at room temperature for 2 hours. The estimated protein concentration per AAV sample was 0.2–0.3 µg/µL based on the AAV titer information provided by the manufacturers.

HPLC conditions

For all experiments, chromatographic separations were carried out using a C4 stationary phase column (1.0 × 100 mm, 300 Å, 1.7 µm) on the Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled with the Thermo Scientific™ Vanquish™ Fluorescence Detector F. The LC and gradient conditions used are listed in Table 1. The fluorescence detector (FLD) settings are shown in Table 2.

For all experiments, chromatographic separations were carried out using a Vanquish Horizon UHPLC system, consisting of the following modules:

- Thermo Scientific™ System Base Vanquish™ Horizon/Flex (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A)
- Thermo Scientific™ Vanquish™ Split Sampler FT (P/N VH-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment (P/N VH-C10-A)
- Vanquish Fluorescence Detector F (P/N VF-D50-A)

Table 1. LC conditions

Parameter	Value	
Solvents	A: 0.1% difluoroacetic acid in water B: 0.1% difluoroacetic acid in acetonitrile	
Flow rate (mL/min)	0.08	
Gradient	Time (min)	%B
	0	20
	1	32
	16	36
	20	80
	21.5	80
	22	20
	30	20
Column temperature (°C)	80	
Idle volume injector (µL)	130	
Mixer volume (µL)	25	
Injection loop volume (µL)	100	
Flow cell volume (µL)	2	
Capillary between column and FLD	Thermo Scientific™ nanoViper™ Fingertight Fittings, 75 µm × 550 mm (P/N 6041.5760)	
Capillary between FLD and MS	Thermo Scientific™ nanoViper™ Fingertight Fittings, 50 µm × 150 mm (P/N 6041.5124)	

Table 2. FLD settings

Parameter	Value
Excitation wavelength (nm)	280
Emission wavelength (nm)	350
Lamp mode	High power
Sensitivity	1
Data collection rate (Hz)	5

Mass spectrometry

The Thermo Scientific™ Orbitrap Exploris™ MX mass detector (P/N BRE725536) equipped with the Thermo Scientific™ BioPharma™ Option was used for MS data collection. The MS settings are shown in Table 3.

Table 3. ESI and MS settings

ESI source settings	
Sheath gas (a.u.)	25
Aux gas (a.u.)	8
Sweep gas (a.u.)	0
Spray voltage (+V)	3,400
Capillary temperature (°C)	320
Vaporizer temperature (°C)	100
MS conditions	**Intact**
Method type	Full MS
Scan range (*m/z*)	700–2,800
Application mode	Intact
Pressure mode	Low
Resolution	30,000 at *m/z* 200
RF lens (%)	150
AGC target value	75
Maximum inject time (ms)	100
Microscans	10
Source fragmentation (V)	25

Data analysis

Thermo Scientific™ BioPharma Finder™ software version 5.0 was used for the intact protein spectra deconvolution.

Results and discussion

The denatured samples were analyzed using the HPLC-FLD-intact MS method. The VP1, VP2, and VP3 of the multiple AAV serotype samples were efficiently separated with the above chromatography conditions. Additional viral protein proteoforms and truncated protein forms were also detected. The average mass of each viral protein and associated proteoforms and truncated proteins were determined through deconvolution of the intact mass raw data. The high resolution, high sensitivity, and high desolvation efficiency offered by the Orbitrap MS allowed great spectral quality and, hence, precise measurement of average mass of each viral protein and associated PTMs.

Figure 1 shows the deconvoluted spectra of AAV viral proteins. Excellent mass accuracy was observed with three viral proteins and the VP3 proteoform with deamidation. Exceptional spectral quality was observed not only for VP3 but also for the less abundant VP1, VP2, and VP3 with deamidation; all were measured with mass accuracy < 5 ppm. The average masses of detected additional truncated VP proteins are shown in Figure 2.

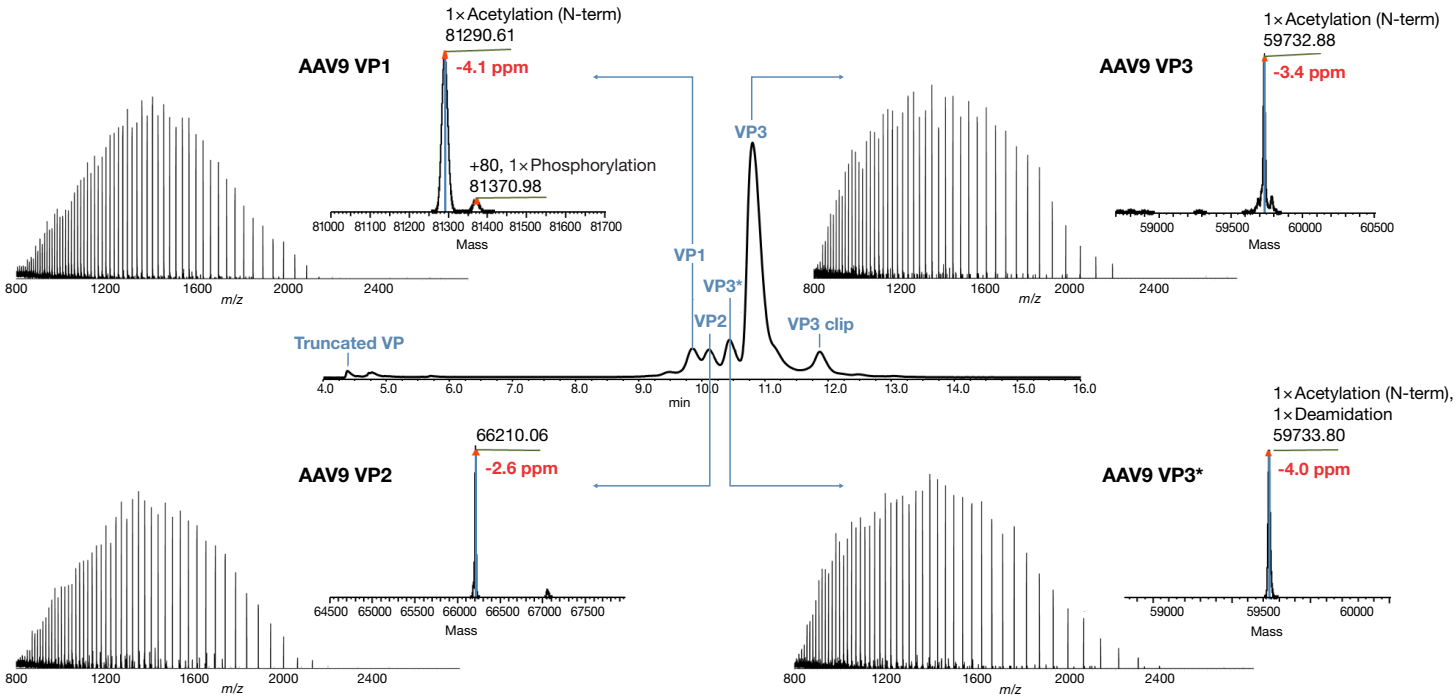

Figure 1. The Full MS raw spectra and their deconvoluted results for VP1, VP2, and VP3 proteoform from the internal manufactured AAV9 sample. The mass accuracy was less than 5 ppm per VP.

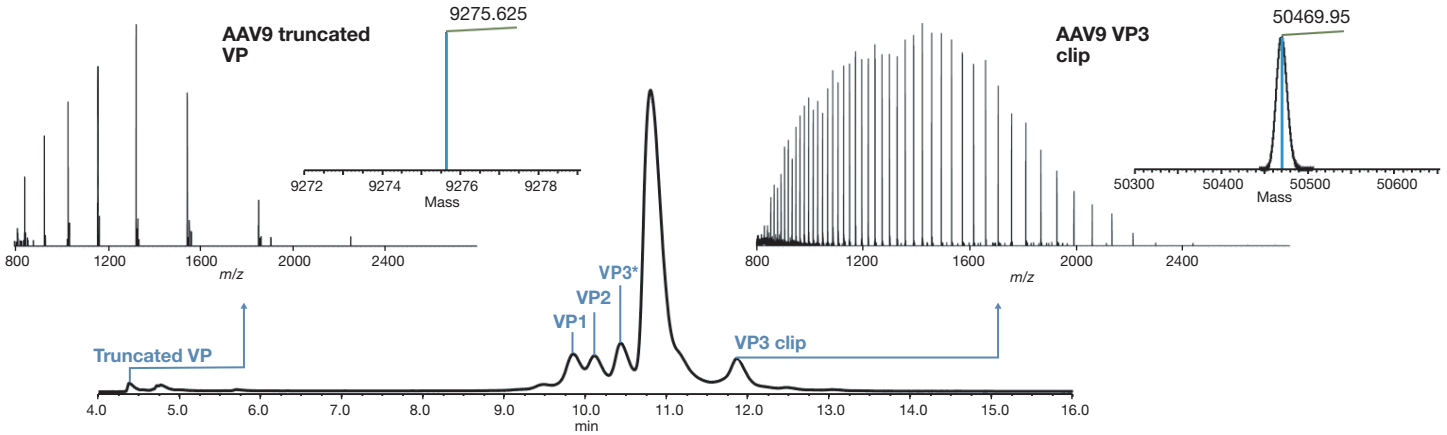

Figure 2. The Full MS raw spectra and their deconvoluted results for truncated VPs from the internal AAV9 sample

The combined FLD optical data and the accurate intact mass measurement data can be used for quick monitoring of the critical quality attributes of viral proteins, including the relative ratio of viral proteins and identify conformation of viral proteins, thus providing a powerful tool for monitoring the AAV product quality attributes across different batches, manufacturing processes, and different AAV serotypes. As an example, a quick comparison of the quality attributes for the two AAV9 samples is shown in Figure 3. The integrated FLD peak areas were used for relative quantitation of VPs, and the intact protein raw spectra were used for the average mass measurement of VPs and associated proteoforms. Although both AAV9 samples showed comparable separation profiles, the observed relative ratios of VP1:VP3 and VP2:VP3 were much lower for the commercially available AAV9 sample compared to the internal manufactured AAV9 sample. The observed average masses from the two truncated VP proteoform peaks are identical in both AAV9 samples, suggesting that they have the same identity.

The FLD elution profile and the accurate intact mass measurement data for other AAV serotype samples (AAV8, AAV6, and AAV1) are shown in Figures 4, 5, and 6, respectively. The accurate intact mass data (< 5 ppm) allowed rapid confirmation of AAV serotypes, rapid identification of PTMs on the viral proteins, and a quick estimation regarding the PTM abundance compared to the unmodified viral proteins. For our AAV samples, the AAV8 had the most abundant VP1 and VP2 with phosphorylation. AAV8 was also the only one that identified VP3 without and with acetylation. For the AAV6 and AAV1 samples, only VP3 with acetylation was identified.

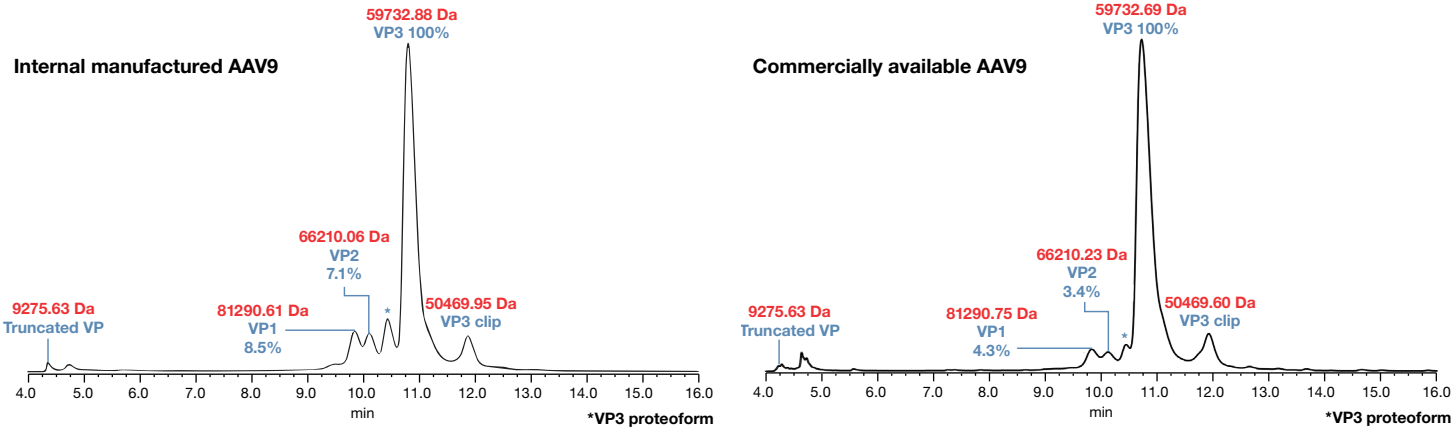

Figure 3. FLD elution profile with observed average mass results from the two AAV9 denatured capsid protein mixtures. The integrated FLD peak areas were used for relative quantitation of viral proteins. The relative abundances of the detected components were calculated against the integrated peak area of VP3, which were assigned as 100%. The average mass per detected VP and associated VP proteoform peak was determined through the deconvolution of the raw full MS spectra.

VP1				VP2				VP3			
AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)
2 (Ac)–738	81666.90	81667.31	-5.0	139–738	66518.50	66518.57	-1.1	204–736	59763.07	59763.07	0
2 (Ac)–738 +1×Phosphorylation	81747.32	81747.29	0.4	139–738 +1×Phosphorylation	66598.30	66598.55	-3.7	205 (Ac)–738	59804.82	59805.11	-4.9

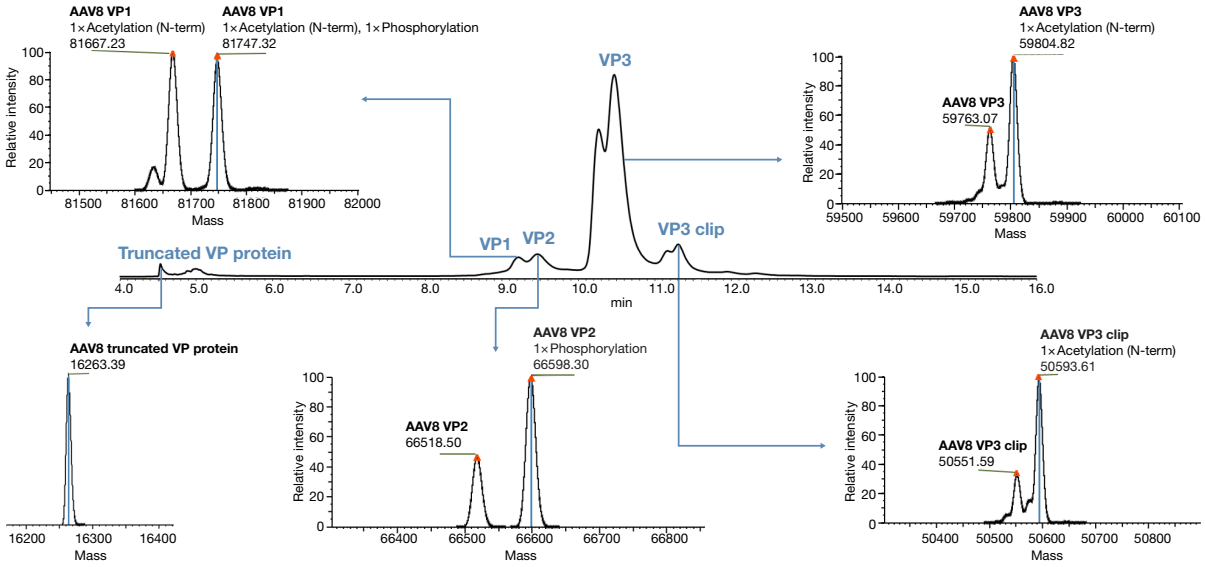

Figure 4. The FLD separation profile and intact mass deconvoluted results for the detected viral protein and associated truncated peaks from the AAV8 sample. The observed average masses for the viral proteins with and without PTMs are summarized in the table in the top of the figure. The mass accuracy was less than 5 ppm. Significant phosphorylation was observed with VP1 and VP2.

VP1				VP2				VP3			
AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)
2 (Ac)–736	81321.92	81322.21	-3.6	139–737	66095.12	66095.41	-4.4	204 (Ac)–736	59519.12	59519.13	-0.2
2 (Ac)–738 +1×Phosphorylation	81402.02	81402.19	-2.1								

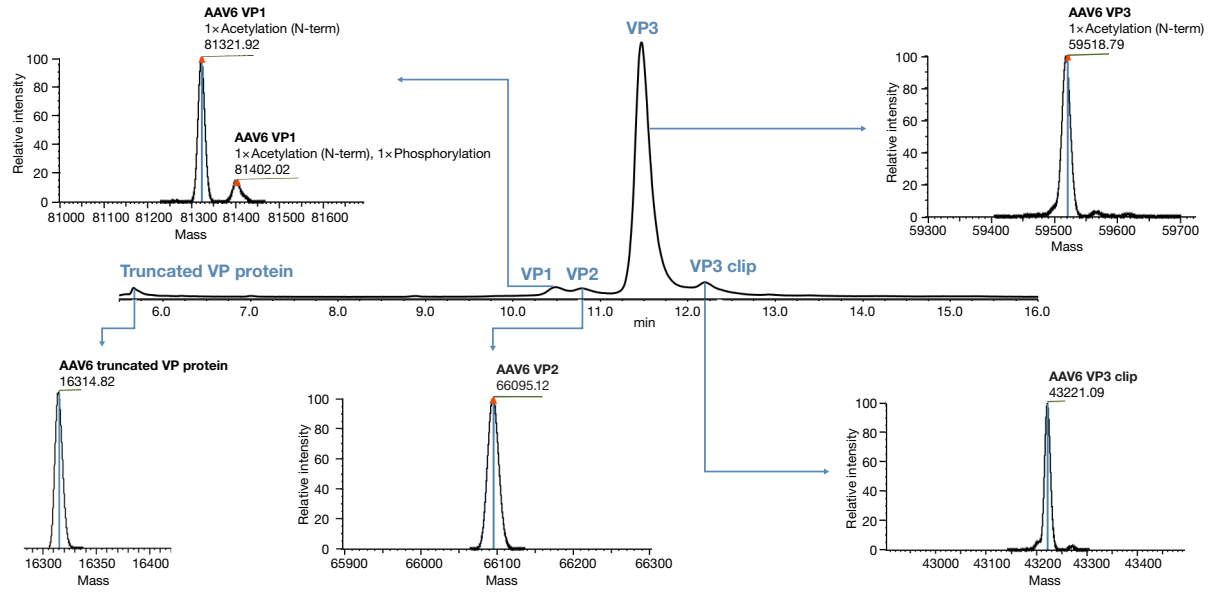

Figure 5. The FLD separation profile and intact mass deconvoluted results for the detected viral protein and associated truncated peaks from the AAV6 sample. The observed average masses for the viral proteins with and without PTMs are summarized in the table in the top of the figure. The mass accuracy was less than 5 ppm.

VP1				VP2				VP3			
AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)	AA sequence	Observed mass (Da)	Theoretical mass (Da)	Error (ppm)
2–736	81244.17	81244.05	1.5	139–736	66093.19	66093.30	-1.7	204 (Ac)–736	59516.95	59517.03	-1.3
2 (Ac)–736	81286.02	81286.09	-0.9	139–736 +1×Phosphorylation	66173.35	66173.28	1.1				
2 (Ac)–738 +1×Phosphorylation	81365.76	81366.07	-3.8								

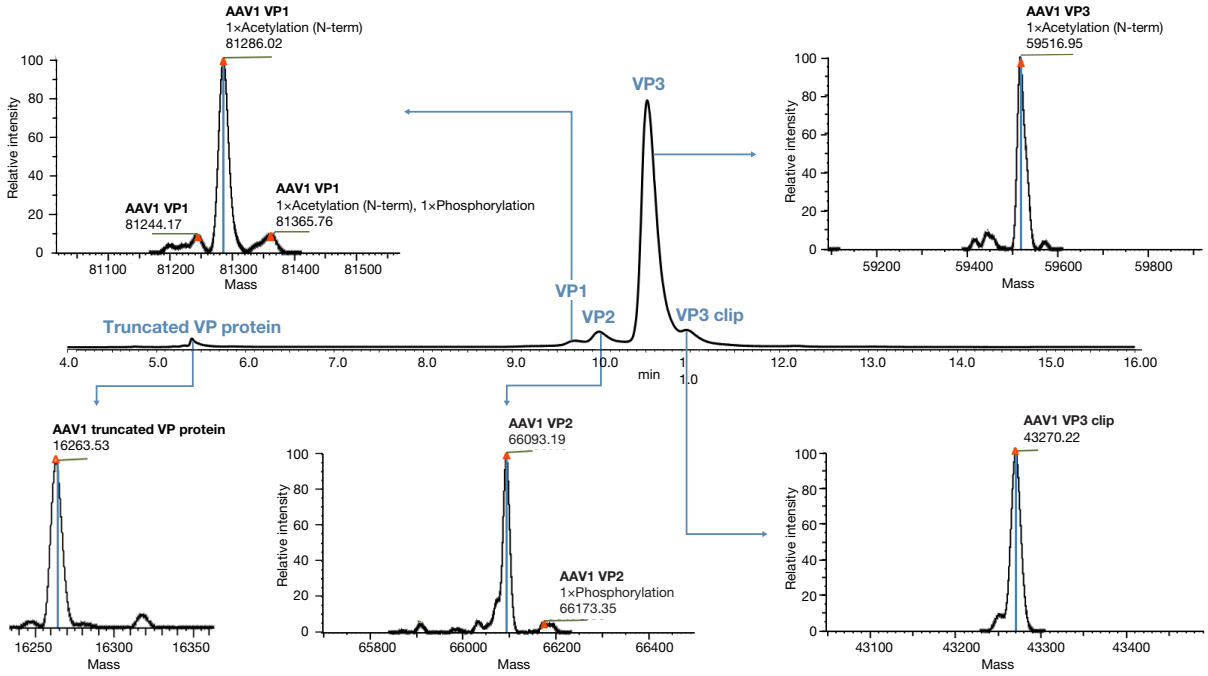

Figure 6. The FLD separation profile and intact mass deconvoluted results for the detected viral protein and associated truncated peaks from the AAV1 sample. The observed average masses for the viral proteins with and without PTMs are summarized in the table in the top of the figure. The mass accuracy was less than 5 ppm.

Conclusion

A robust UHPLC-FLD-HRAM Orbitrap Exploris MX method was developed for rapid characterization of viral protein identities, relative ratios, and PTMs across multiple AAV serotypes.

This method provides the following benefits:

- Rapid ratio assessment of AAV structural proteins VP1:VP2:VP3 through sensitive fluorescence detection
- Accurate intact mass measurement of AAV viral proteins and associate proteoforms with mass error < 5 ppm on an Orbitrap Exploris MX mass detector, ensuring rapid AAV serotype confirmation
- Sensitive detection and accurate mass measurement of low abundant AAV viral protein proteoforms
- Common platform for comparing the composition differences of AAV products, across different batches and manufacturing processes

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