



Optimized liquid chromatography mass spectrometry methods for intact protein analysis and peptide mapping of adeno-associated virus capsid proteins

Recombinant adeno-associated viruses (AAV) have emerged as one of the preferred platforms for gene therapy strategies and vaccine development.

Introduction

Due to their extremely large molecular sizes, structural complexity, and heterogeneity, the characterization and analysis of adeno-associated viruses is challenging. The confirmation of the AAV capsid proteins' identity, ratio and heterogeneity profile are required quality attributes to support the development of therapies with AAV vectors. The timsTOF Pro has previously been shown to be well suited for this purpose by Oyama et al. Here, we present a rapid and robust UHPLC-MS method for intact capsid protein analysis and peptide mapping of different AAV serotypes.

Keywords:
Adeno-associated Virus,
maXis II, BioPharma Compass,
Intact Protein, Peptide
Mapping

Materials and Methods

Sample preparation: rAAV samples (serotype 2, 5, 6 and 8) were purchased from Virovek (Virovek Inc, Hayward, CA, USA) and Vigene Biosciences (now Charles River Laboratories, Rockville, MD, USA). Each vial contained $\sim 1 \times 10^{13}$ vector genomes per milliliter.

For intact analysis, the samples were treated with 4% acetic acid for 5 minutes to dissociate the viral proteins and directly analyzed by LC-MS.

For peptide mapping, the sample was denatured by RapiGest™ (Waters, Milford, MA, USA), reduced with 2.5 mM dithiothreitol, and alkylated using 5 mM Iodoacetamide, followed by enzymatic digestion (2 hr, 37°C) using trypsin and chymotrypsin (Promega, Madison, WI, USA).

UHPLC system: LC separation was carried out using an Elute UHPLC system (Bruker Scientific LLC, Billerica, MA, USA).

Mass Spectrometry: Data was acquired on a maXis II UHR QTOF (Bruker Scientific LLC, Billerica, MA, USA).

Chromatography

1. Intact analysis:

The separation of the intact viral proteins was performed using an ACQUITY UPLC protein BEH C4 column (2.1 x 50 mm, 1.7 μ m, 300 Å, Waters, Milford, MA) held at 70°C using a 30-minute gradient.

Mobile phase A (aqueous phase): LC-MS grade water with 0.1% DFA

Mobile phase B (organic phase): LC-MS grade acetonitrile with 0.1% DFA

Time [min]	Flow [mL/min]	% A	% B
0.00	0.20	80.0	20.0
1.00	0.20	68.0	32.0
14.00	0.20	64.0	36.0
18.00	0.20	20.0	80.0
21.00	0.20	20.0	80.0
22.00	0.20	80.0	20.0
30.00	0.20	80.0	20.0

2. Peptide Mapping:

Tryptic peptides were separated using an ACQUITY Premier C18 column (2.1x100 mm, 1.7 μ m, 130 Å, Waters, Milford, MA) held at 50°C using a 70-minute gradient.

Mobile phase A (aqueous phase): LC-MS grade water with 0.1% FA

Mobile phase B (organic phase): LC-MS grade acetonitrile with 0.1% FA

Time [min]	Flow [mL/min]	% A	% B
0.00	0.20	99.0	1.0
3.00	0.20	99.0	1.0
18.00	0.20	85.0	15.0
48.00	0.20	70.0	30.0
51.00	0.20	45.0	55.0
65.00	0.20	5.0	95.0
67.00	0.20	5.0	95.0
70.00	0.20	99.0	1.0
85.00	0.20	99.0	1.0

maXis II ETD HRS-MS

1. Intact protein:

Intact protein data was acquired in full scan MS mode from 300 – 3000 m/z with a scan rate of 1 Hz.

Apollo ESI source				Transfer	
Nebulizer	Dry gas	Dry temp	End plate offset	Funnel 1 RF	400 Vpp
1.2 bar	8 L/min	220°C	500 V	isCID	0
Capillary	Scan range	Scan mode	Spectra rate	Multipole RF	400 Vpp
4500 V	600-3000 m/z	MS	1 Hz	Quadrupole ion energy	3 eV
				Quadrupole low mass	600 m/z
				Collision energy	8 eV
				Collision RF	2500 Vpp
				Transfer time	80 μ s
				Pre-pulse storage	10 μ s

2. Peptide Mapping:

Peptide mapping experiments were carried out in DDA mode with variable MS/MS acquisition rate (2-12 Hz). The data was processed with DataAnalysis® and BioPharma Compass® 2021 for data visualization and automated deconvolution (Bruker Scientific LLC, Billerica, MA, USA).

Apollo ESI source				Stepping at Basic Mode			MS/MS only		
Nebulizer	Dry gas	Dry temp	End plate offset	Time (%)	Collision RF (Vpp)	Transfer time (μs)	Time (%)	Collision energy (%)	
1.5 bar	8 L/min	180°C	500 V	1	35	150	1	50	100
Capillary Scan range Scan mode Spectra rate				2	65	1500	2	50	100
4500 V	50-2000 <i>m/z</i>	Auto MS/MS	2 Hz						

Transfer		MS/MS parameter - Auto MS/MS	
Funnel 1 RF	400 Vpp	Cycle time	2.5 sec
isCID	0	MS spectra rate	2 Hz
Multipole RF	400 Vpp	Threshold (Active exclusion)	1000 counts; Exclude after 1 spectra; Release after 0.1 min; Reconsider precursor if Current intens./Previous intens.: 3.0
Quadrupole ion energy	4 eV	MS/MS spectra acquisition (Dynamic)	Max. rate: 12 Hz, Min. rate: 2 Hz Target intensity: 50,000 cts
Quadrupole low mass	322 <i>m/z</i>		
Collision energy	8 Ev		
Pre-pulse storage	7 μs		

Data processing

1. Intact protein:

The raw intact LC-MS data were processed in DataAnalysis® using Maximum Entropy deconvolution.

2. Peptide mapping:

The raw LC-MS/MS data were processed in BioPharma Compass® using the Peptide mapping (autoMS/MS) workflow method.

Results and discussion

1. Intact analysis:

- Intact viral proteins of AAV 2, 5, 6, and 8 were well separated by RPLC on a C4 column with high chromatographic resolution values of 1.5 - 3 (Figure 1).

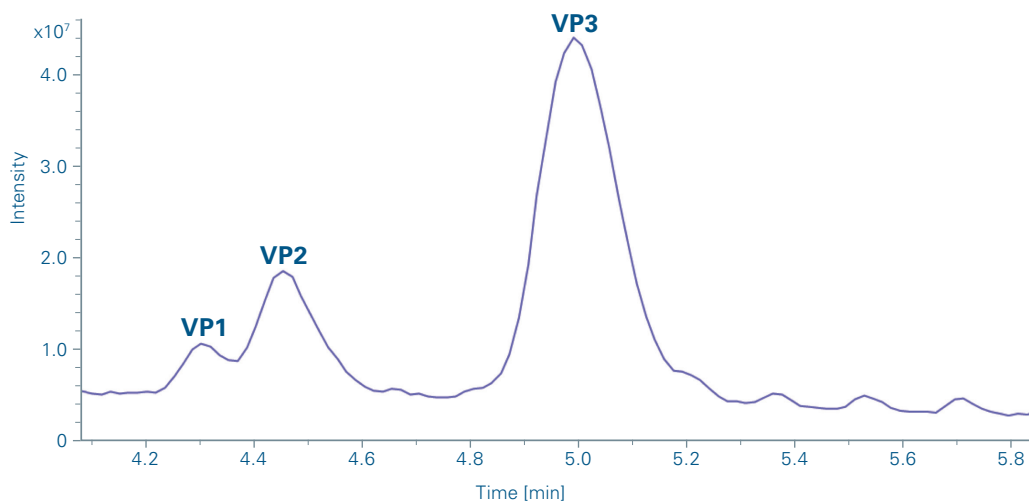


Figure 1
Separation of AAV2 viral proteins in less than 10 minutes on the C4 columns.

Serotypes	AAV8		AAV6		AAV2		AAV5		
	Mass/VP	Observed (Da)	Delta mass (ppm)	Observed (Da)	Delta mass (ppm)	Observed (Da)	Delta mass (ppm)	Observed (Da)	Delta mass (ppm)
VP1		81,668	12	81,320	5	81,856	20	81,336	30
VP2		66,518	15	66,096	32	66,488	30	65,283	11
VP3		59,805	33	59,519	30	59,974	15	59,463	33

Table 1

Observed mass and delta mass of the AAV serotypes on maXis II ETD.

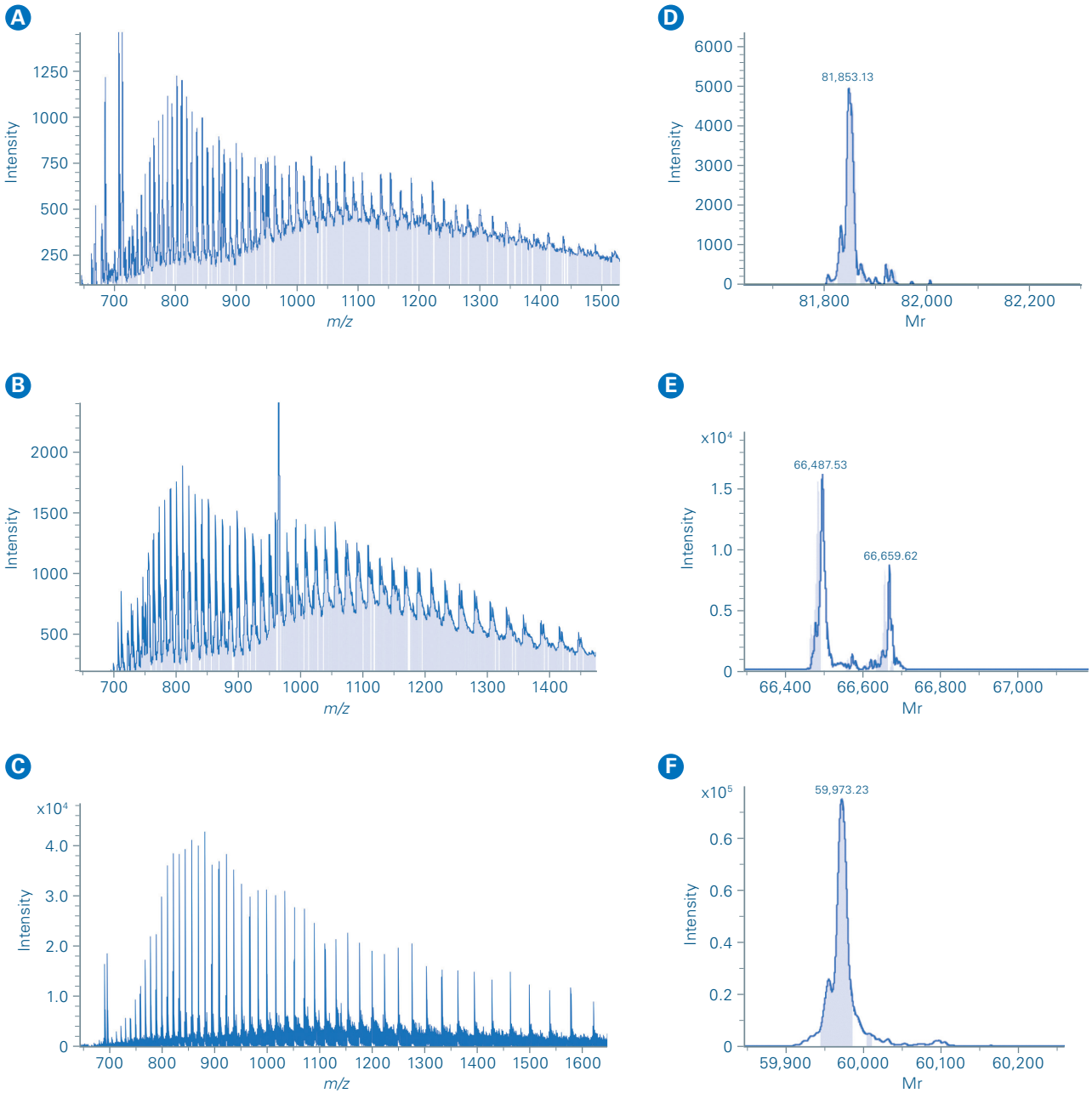


Figure 2

Raw mass spectra of the viral proteins for AAV2.

(A) Viral protein 1, (B) Viral protein 2, and (C) Viral protein 3. The deconvoluted spectra of the AAV2 (D) Viral protein 1, (E) Viral protein 2, and (F) Viral protein 3.

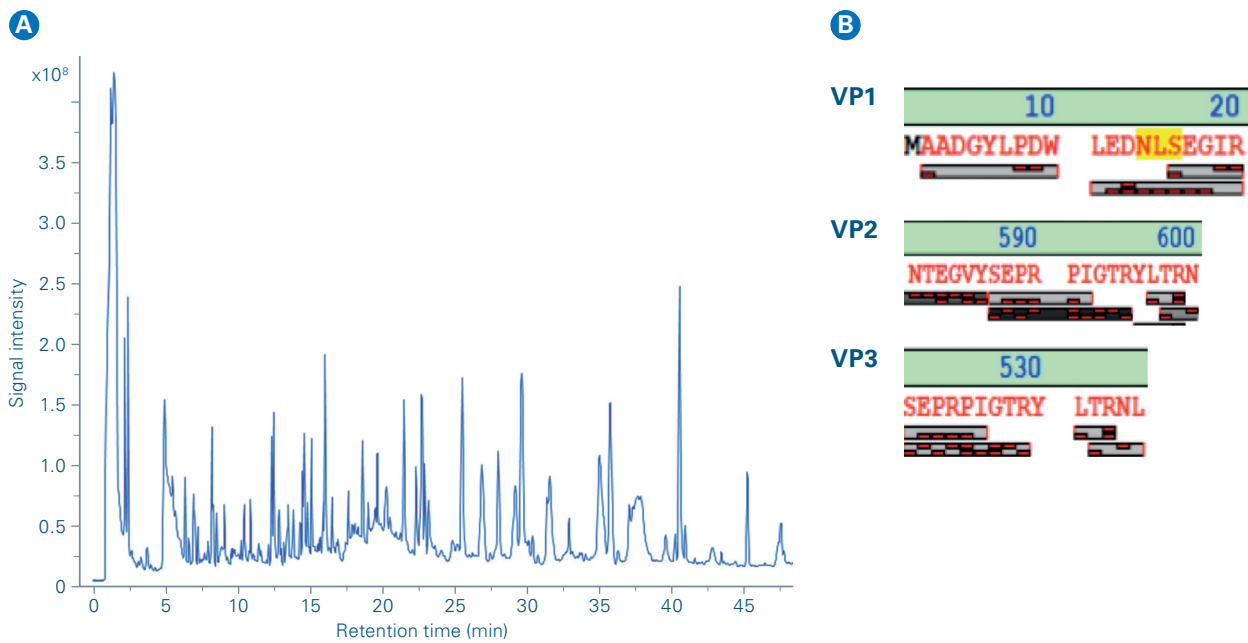


Figure 3

A AAV8 peptides are well resolved on the C18 column and show high sequence coverage. **B** The full sequence coverage of the unique peptide sequence of the VP1 (1-20 aa), VP2 (585-601 aa), and the VP3 common region (525,536).

- High mass accuracy data were obtained for all VP proteins from different serotypes with a good correlation between the theoretical and observed mass (Table 1).
- Accurate mass determination of the VP proteins allowed the assignment of potential post-translational modifications such as phosphorylation (Figure 2).
- Peptide mapping of the AAV serotypes was successfully achieved in 60 minutes by reversed-phase LC-MS/MS with a capsid protein sequence coverage of 94% or higher (Figure 3).
- Chymotrypsin and trypsin were used in combination for digestion. As there are limited Arg and Lys residues on the AAV proteins, a combination of the two enzymes gave ~15% more coverage than using only trypsin.
- Several PTMs were identified including deamidation, oxidation, phosphorylation, and acetylation on VP3 of both serotypes AAV8 and AAV6.

Serotypes	AAV8	AAV6
Coverage/Viral protein	Coverage (%)	Coverage (%)
VP1	92.2	90.2
VP2	95.5	91.5
VP3	96.2	95.2

Table 1

Peptide coverage of the serotypes AAV6 and AAV8. An average of 94% peptide sequence coverage is seen for the 3 viral proteins.

Conclusion

- The high data quality from the Bruker maXis II allowed the development of a robust platform for the intact protein analysis of AAV viral capsid proteins and the identification of modifications such as phosphorylation.
- Intact viral proteins (VP1, VP2, VP3) of AAV serotypes 2, 5, 6, 8 were well-separated on a reverse phase C4 column in less than 10 minutes with accurate mass determination for all VP proteins.
- A robust LC-MS/MS peptide mapping workflow was developed using chymotrypsin and trypsin.
- Sequence coverage of 94% or higher was achieved for the 3 viral proteins as well as a complete sequence coverage of the unique peptides for VP1, VP2, and the common region for VP3.
- Several post-translational modifications including deamidation, oxidation, phosphorylation, and acetylation were identified on VP3 of the serotype AAV8 and AAV6.

References

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