In-depth Characterization of Adeno-Associated Viruses (AAVs) using Microchip CE-MS

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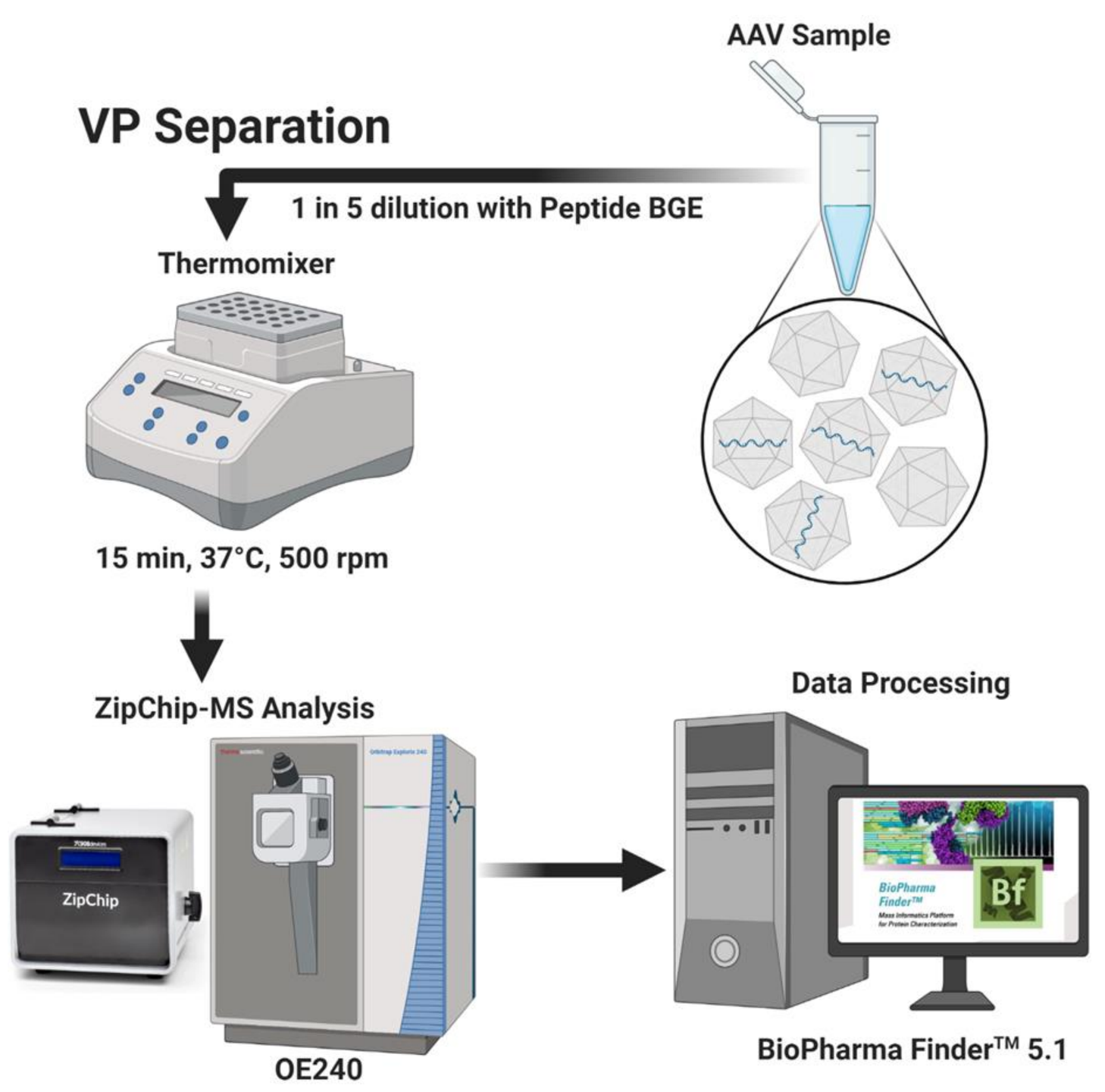
Introduction

- Adeno-associated viruses (AAVs) are promising vectors for gene therapy products due to their low immunogenicity.
- These icosahedral, 60-mer capsids are composed of the three viral proteins (VPs) VP1, VP2, and VP3, in an approximate 1:1:10 ratio, respectively.
- Characterization of AAV viral capsid proteins (VPs) and their post translational modifications (PTMs) is critical to ensure product quality and safety.
- Commonly utilized liquid chromatography–mass spectrometry (LC-MS) characterization methods are often time consuming, with analysis alone often taking 30 minutes to >2 hours, depending on the desired characterization application.
- AAV sample is often limited, so minimizing the sample required for analysis is crucial.
- Microchip capillary electrophoresis coupled with MS (CE-MS) addresses such concerns by providing rapid characterization capabilities with minimal sample. It has previously proven capable of performing peptide mapping on AAVs in under 10 minutes [1] and intact analysis of capsid VPs in under 5 minutes [2].
- Here we demonstrate the utilization of microchip CE-MS for VP characterization and peptide mapping for rapid in-depth AAV characterization. Performed across a spectrum of AAV serotypes, we illustrate how this platform quickly provides crucial information related to the characteristics of AAVs including the presence of VP proteoforms and PTMs.

Viral Capsid Protein (VP) Analysis

Methodology



Acquisition	Settings
ZipChip	Field Strength: 500 V/cm Injection Volume: 5.5nL
Global Settings	Application Mode: Intact protein, Pressure Mode: Low Pressure, Expected Peak Width: 10 Default Charge State: 35
MS1	15k Res, m/z 740-2000, RF Lens 125%, Normalized AGC 50%, max IT 200 ms, Collision Energy: 35V

Samples: AAV6, AAV8, and AAV9 (2E13vp/mL, Virovek)

Sample Preparation: 5µL of AAV is diluted with 20µL of Peptide Background Electrolyte [Peptide BGE; Part of ZipChip Peptides Kit (908 Devices)] and incubated for 15 minutes, at 37°C, on a Thermomixer, shaking at 500 rpm. Sample is then loaded directly into well of a ZipChip chip for analysis.

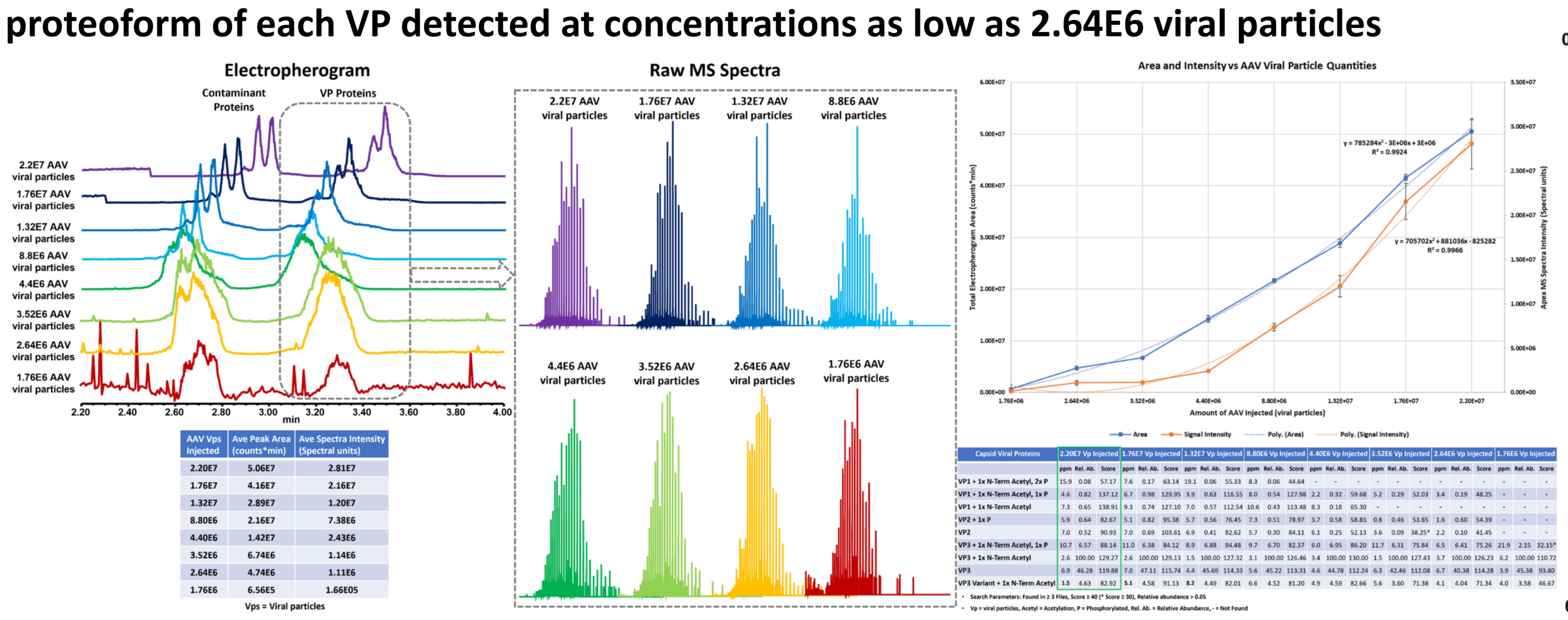
Sample Analysis: Separation is performed using a ZipChip™ Device (908 Devices Inc.) coupled to an Orbitrap Exploris™ 240 MS (Thermo Scientific). A standard (HR) chip (908 Devices Inc.) primed with Peptide BGE containing 4% DMSO is used for analysis. Each run is 5 minutes, and 5 injections were performed for each sample.

Software: MS Instrument control and data acquisition was performed using Chromeleon CDS 7.3.1. Data analysis is performed using BioPharma Finder (BPF) 5.1.

Figure 1: **Workflow for AAV VP Separation using microchip CE-MS**

Limit of Detection (LOD) Analysis

Figure 2: **LOD testing of AAVs for microchip CE-MS using Empty AAV8. All Identified VP proteoforms were detected at concentrations ≥8.80E6 viral particles, with at least one proteoform of each VP detected at concentrations as low as 2.64E6 viral particles**



Viral Capsid Protein (VP) Analysis Continued

Proteoform Identification

- Rapid AAV VP analysis of Full AAV8 using the microchip CE-MS.
- 5-minute runs enable increased replicate injections without sacrificing reproducibility or sensitivity (Figure 3 and Figure 4A)
- High sensitivity enables detection of low abundant proteoforms using BPF 5.1
- (Figure 4B and 4C).

Figure 3: **Illustration of reproducibility of VP analysis performed using microchip CE-MS**

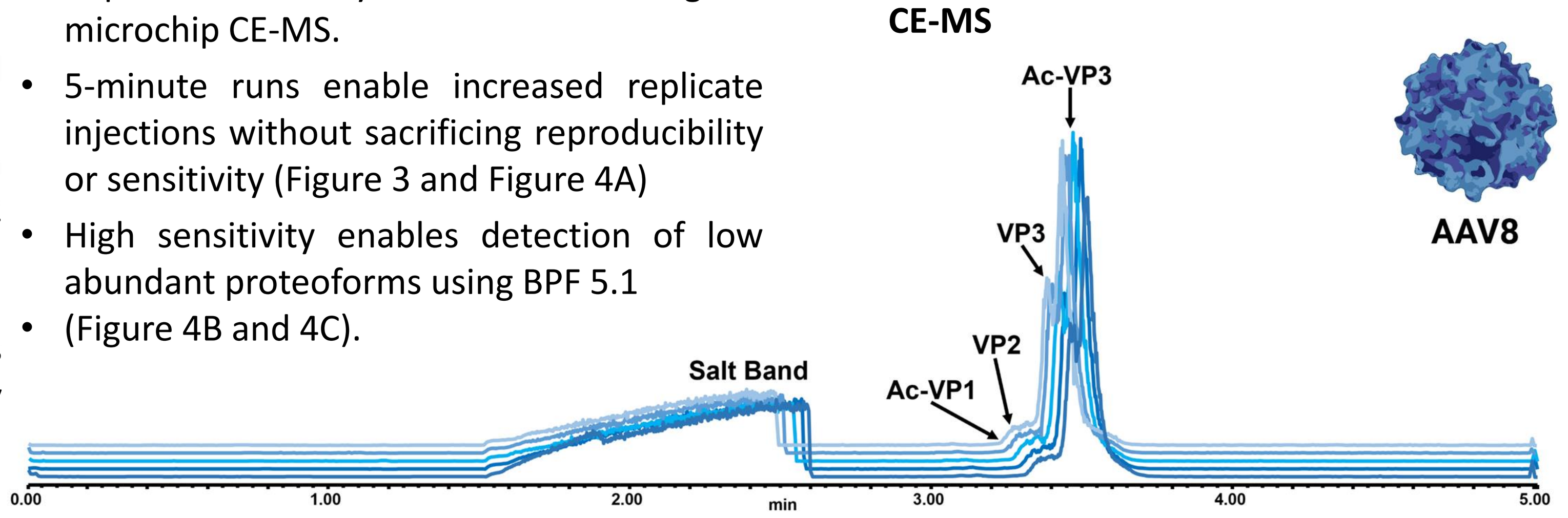
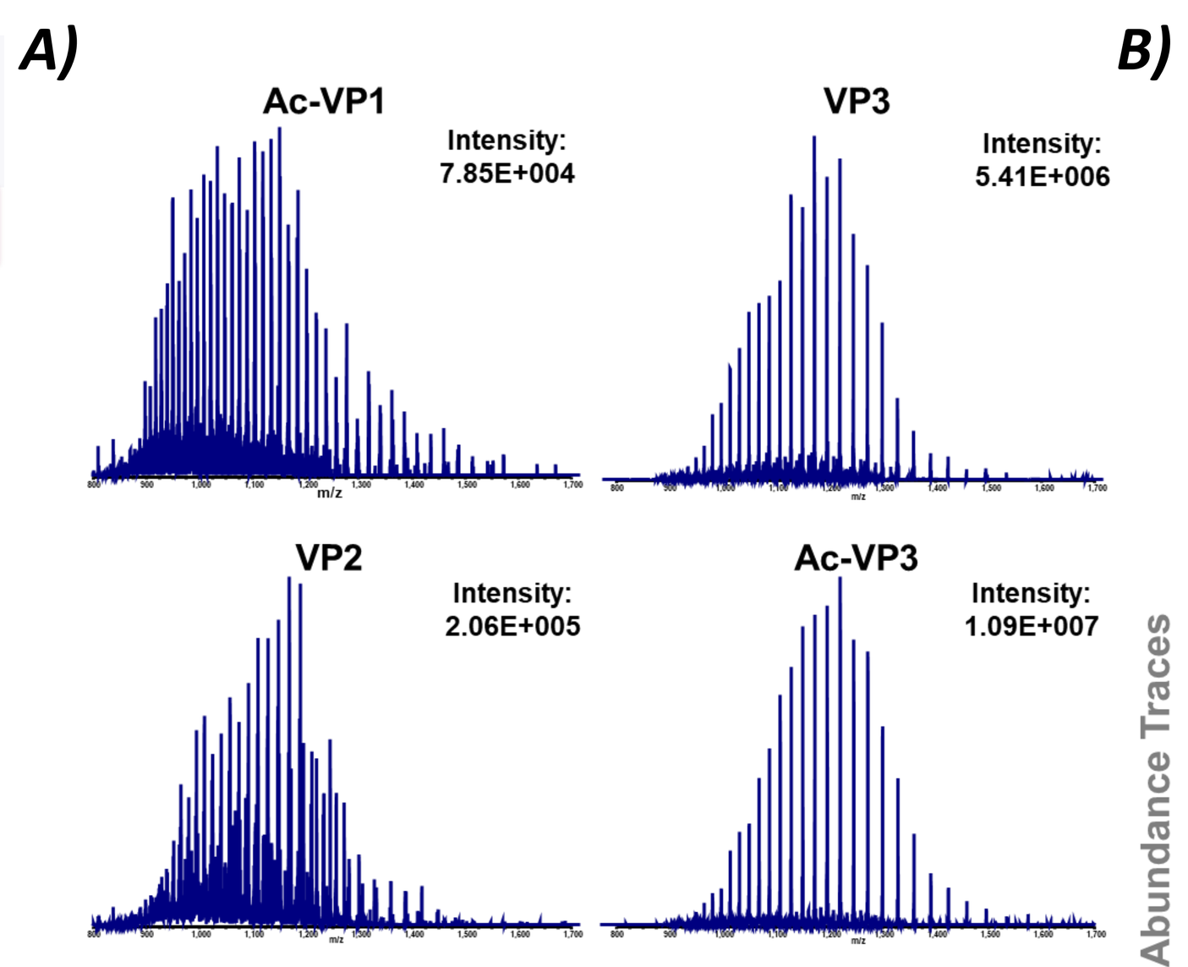
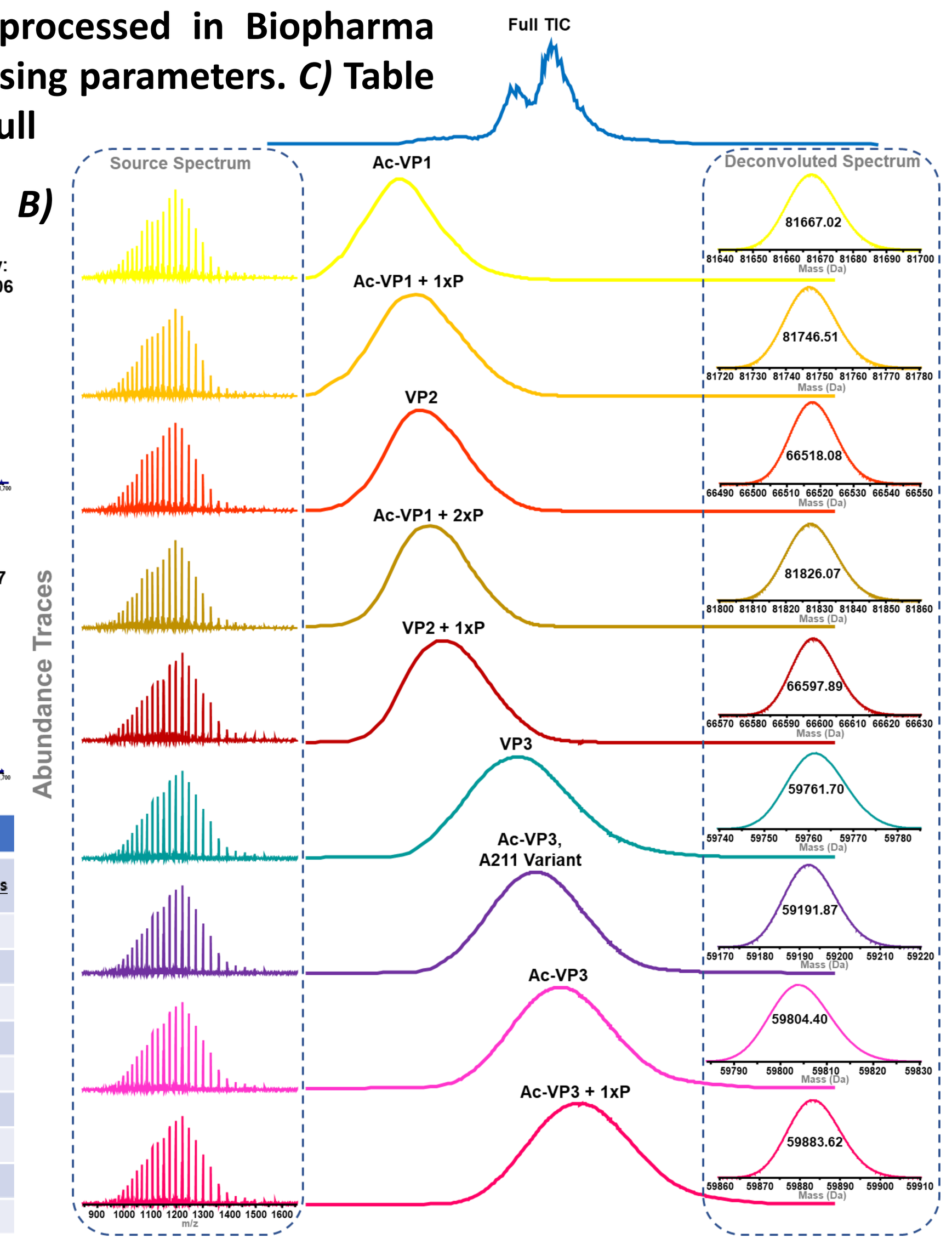


Figure 4: **Data processing of AAV VPs illustrated using AAV8 Full. *A)* Raw MS Spectra of VPs. *B)* Data processed in Biopharma Finder 5.1 using sliding windows processing parameters. *C)* Table of Identified VP proteoforms for AAV8 Full**





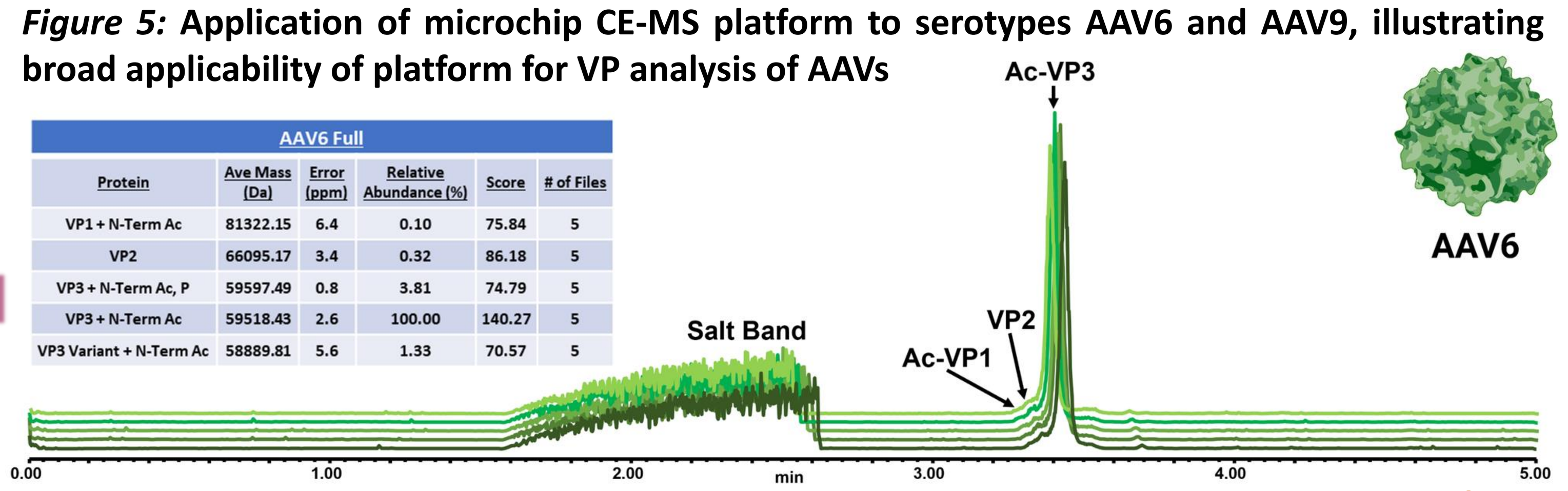
C)

AAV8 Full					
Protein	**Ave Mass (Da)**	**Error (ppm)**	**Relative Abundance (%)**	**Score**	**# of Files**
VP1 + N-Term Ac, 2xP	81826.07	7.4	0.08	40.90	5
VP1 + N-Term Ac, P	81746.51	2.2	0.41	119.09	5
VP1 + N-Term Ac	81667.02	3.8	0.21	97.69	4
VP2 + P	66597.89	2.8	0.89	112.65	5
VP2	66518.08	0.2	0.38	91.04	5
VP3 + N-Term Ac, P	59883.62	17.4	6.85	86.24	5
VP3 + N-Term Ac	59804.40	4.7	100.00	127.80	5
VP3	59761.70	15.8	45.28	113.27	5
VP3 Variant + N-Term Ac	59191.87	1.8	4.94	88.77	5

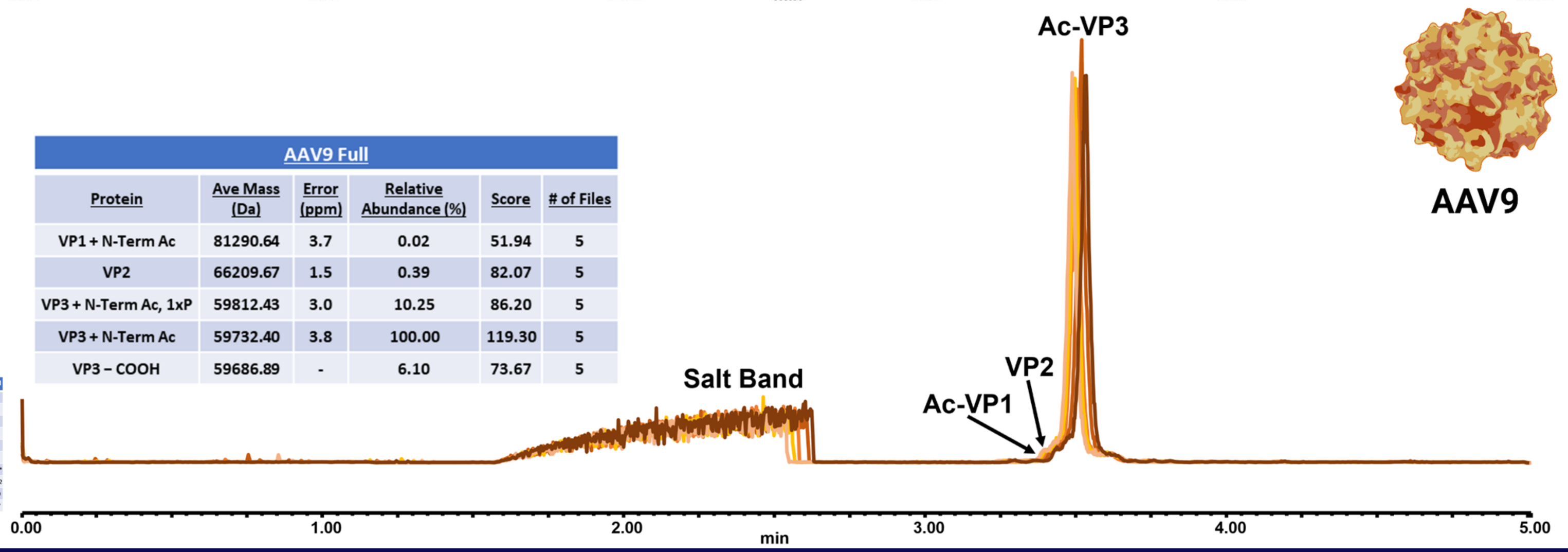
Multiple Serotype Application

Figure 5: **Application of microchip CE-MS platform to serotypes AAV6 and AAV9, illustrating broad applicability of platform for VP analysis of AAVs**

AAV6 Full					
Protein	**Ave Mass (Da)**	**Error (ppm)**	**Relative Abundance (%)**	**Score**	**# of Files**
VP1 + N-Term Ac	81322.15	6.4	0.10	75.84	5
VP2	66095.17	3.4	0.32	86.18	5
VP3 + N-Term Ac, P	59597.49	0.8	3.81	74.79	5
VP3 + N-Term Ac	59518.43	2.6	100.00	140.27	5
VP3 Variant + N-Term Ac	58889.81	5.6	1.33	70.57	5



AAV9 Full					
Protein	**Ave Mass (Da)**	**Error (ppm)**	**Relative Abundance (%)**	**Score**	**# of Files**
VP1 + N-Term Ac	81290.64	3.7	0.02	51.94	5
VP2	66209.67	1.5	0.39	82.07	5
VP3 + N-Term Ac, 1xP	59812.43	3.0	10.25	86.20	5
VP3 + N-Term Ac	59732.40	3.8	100.00	119.30	5
VP3 – COOH	59686.89	-	6.10	73.67	5



Peptide Mapping

Methodology

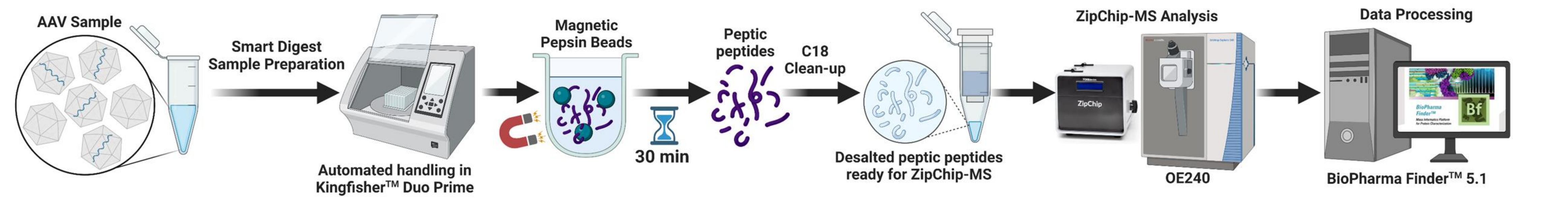


Figure 6: **Workflow for AAV Peptide mapping using microchip CE-MS**

Peptide Mapping Acquisition Settings	
ZipChip	Field Strength: 500 V/cm, Injection Volume: 8.0nL
Global Settings	Application Mode: Peptides, Pressure Mode: Standard, Expected Peak Width: 15, Default Charge State: 2
MS1	Polarity: Positive, 30K Res, m/z 200-1800, RF Lens: 50%, Normalized AGC: 100%, Microscans: 1, Max IT: 200 ms
MS2	Isolation window (m/z): 2, NCE: 28, 15K Res, Ref Lens: 70%, AGC 100%, Microscans: 1 Max IT: 50 ms
	DDA: Cycle Time, Scan Event: 1, Dynamic Exclusion: 1s Mass Tolerance: 10ppm, Charge State: 2-8, Intensity Threshold: 5.0e4

Samples: Same as for VP Separation

Sample Preparation: 10 µg of AAV (determined using NanoOrange protein assay) was digested SMART Digest pepsin magnetic beds on a Kingfisher™ DuoPrime. Desalting is performed using a C18 spin column and then reduced to dryness in a speed-vac.

Sample Analysis: Separation is performed using a ZipChip™ Device coupled to an Orbitrap Exploris™ 240 MS. Sample is reconstituted in 10uL of Peptide BGE and loaded directly into the well of an HR chip primed with Peptide BGE for analysis. Each run is 20 minutes, and 2 injections were performed for each sample.

Software: Same as for VP Separation

Sequence Coverage and PTM Identification

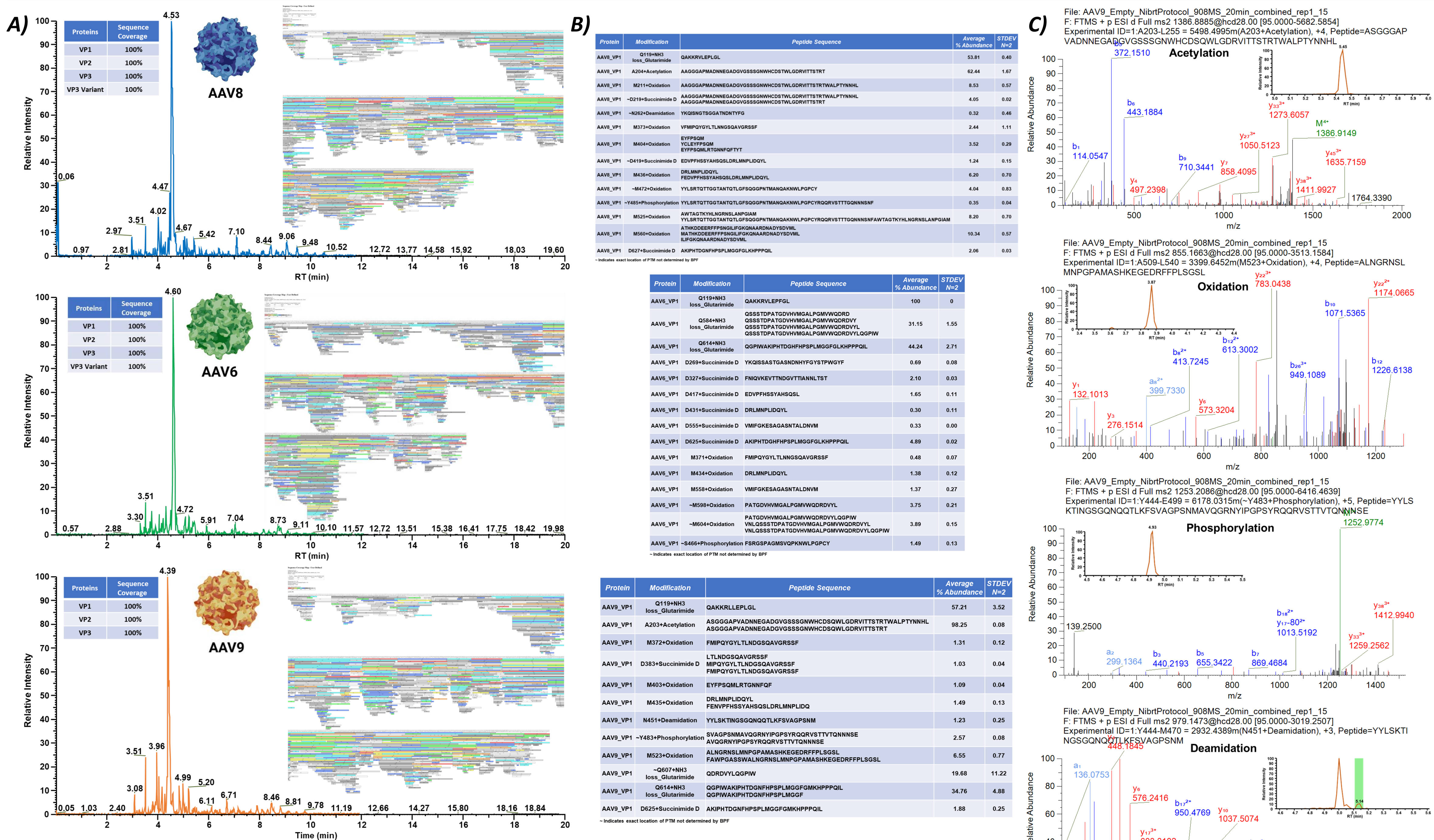


Figure 7: **Microchip CE-MS separation for peptide mapping and identification of PTMs. *A)* CE-MS base peak electropherograms of AAV serotypes illustrating high sequence coverage. *B) Tables of i*dentified PTMs for each serotype. *C)* Select identified PTMs and their MS² Spectra.**

Conclusions & Future Work

- Rapid microchip CE-MS can be utilized for the in-depth characterization of AAVs including viral capsid protein analysis and peptide mapping.
- VP separation is performed in as little as 5 minutes while detecting multiple proteoforms of each viral capsid protein.
- Peptide mapping provides high sequence coverage of all VP proteins in under 20 minutes and can characterize PTMs.
- Application demonstrated with serotypes AAV6, AAV8, and AAV9 to show the broad applicability of this platform across AAV serotypes.
- With characterization performed at greatly increased speeds compared to standard LC-MS analysis, the microchip CE-MS ZipChip platform can provide an attractive alternative for laboratories looking for high throughput AAV characterization.
- **Future work:** Investigate VP ratios; Improve detection of PTMs on N-terminus of VP1; Apply microchip CE-MS to empty/full AAV intact capsid analysis

[1] Kulkarni et al. ThP360, ASMS 2022
[2] Zhang, Y.; Wang, Y.; Sosic, Z.; Zhang, L.; Bergelson, S.; Zhang,W. *Anal. Biochem.* **2018**, *555*, 22.

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