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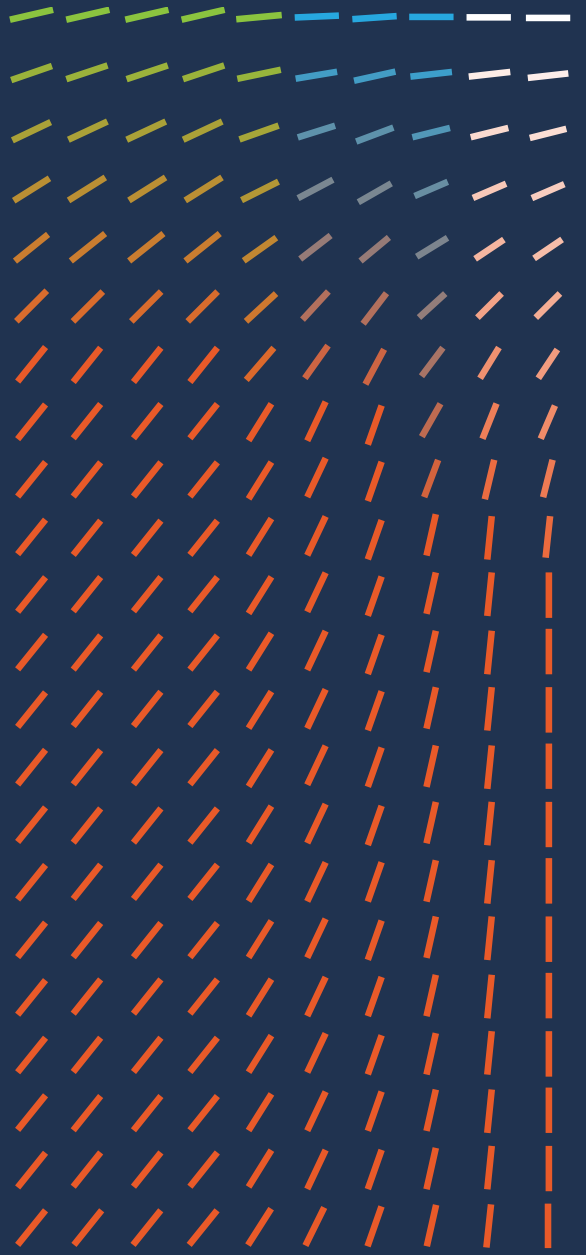
Complex Characterization Challenges and Analytical Solutions for Development of AAV Products

Chen Li, Director, Analytic Sciences

Yang Shen, Associate Director, Analytic Sciences

26 January 2023



Agenda

1. LC-UV-MS Based Methods for Characterization of Capsid Proteins and Monitoring Vector Product Attributes
2. Empty/Partial/Full Capsid Separation by Anion-Exchange Chromatography (AEX)
3. Complexities of Analyzing Surfactants in AAV Products

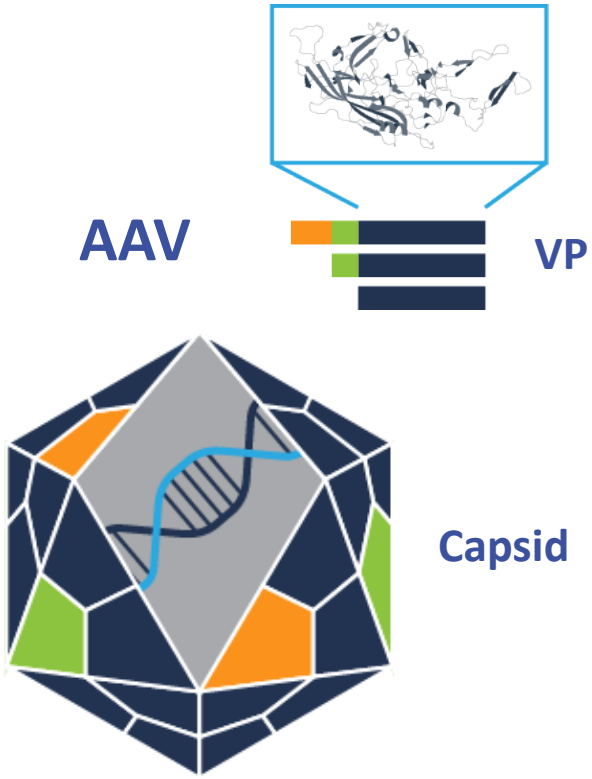
Analytical Characterization of AAV Gene Therapy Products

Capsid Proteins Characterization

- VP Ratio (LC-UV-MS, CE, SDS-PAGE)
- VP Identity (LC-MS, UV/FLD-HPLC, Western)
- PTMs/Degradants (LC-MS)
  - N-terminal acetylation
  - Phosphorylation
  - Methylation
  - Deamidation, oxidation
- N-terminal Sequences (LC-MS)
- Splice Variants (LC-MS)

Particle Characterization

- Full vs Empty/Partial (AUC, HPLC, SEC-MALLS)
- Aggregation (AUC, DSF, AF4)
- Particle Size & Counts (DLS, MFI, MALLS)



Purity

- HCP Profiling (ELISA, LC-MS)
- Residual Host Cell DNA (qPCR, NGS, CE, HPLC)
- Process Impurities & Residuals (HPLC, LC-MS, NGS)

Nucleotide Characterization

- Identity (LC-MS, q/ddPCR, NGS)
  - polyA heterogeneity (mRNA)
  - % Cap (mRNA)
- PTMs (LC-MS)
  - Cap methylation (mRNA)
  - Lipid conjugation (LNPs)
- Size / Conformer (HPLC, CE, Agarose Gel)

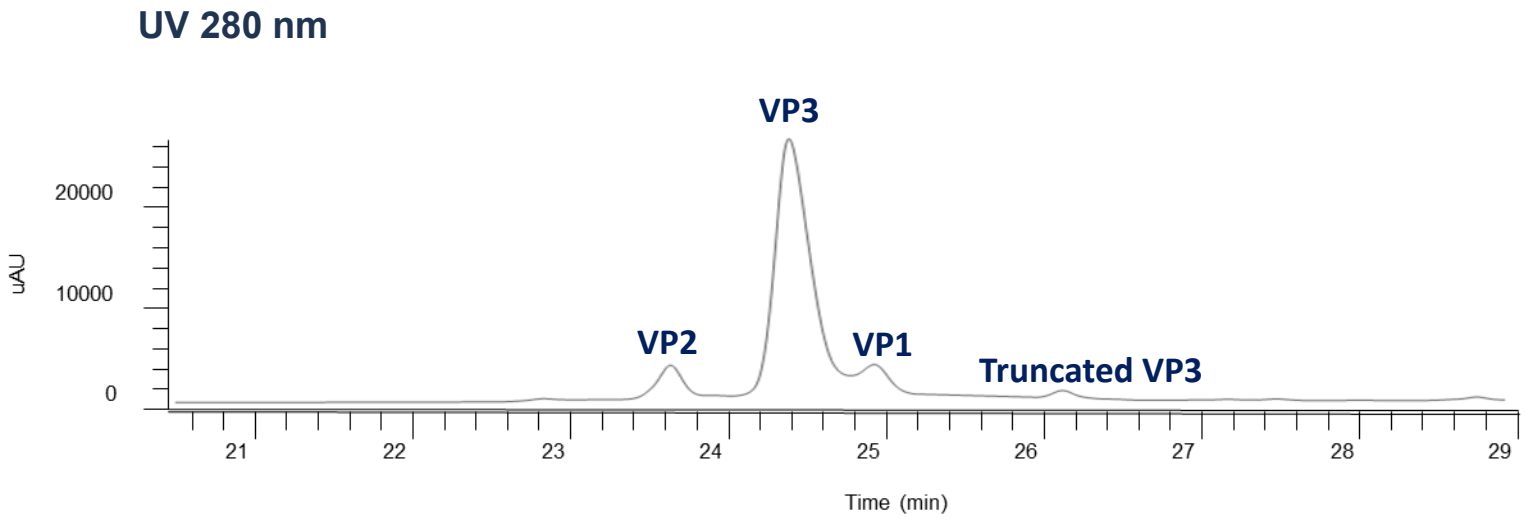
Potency/Activity

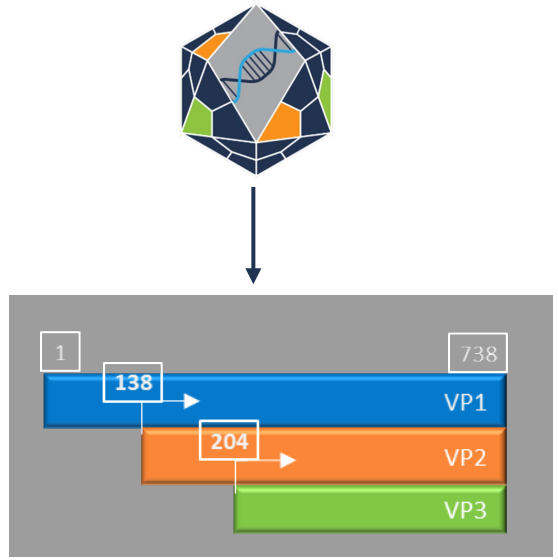
- Vector Genome Titer (qPCR)
- Infectivity / Infectious Titer
- Post-administration Product Monitoring
  - Expressed Protein: LC-MS, Western, ELISA
  - Nucleotide: mRNA, insertion sites

LC-UV-MS Based Methods for Characterization of Capsid Proteins and Monitoring Vector Product Attributes

Intact Mass Approach

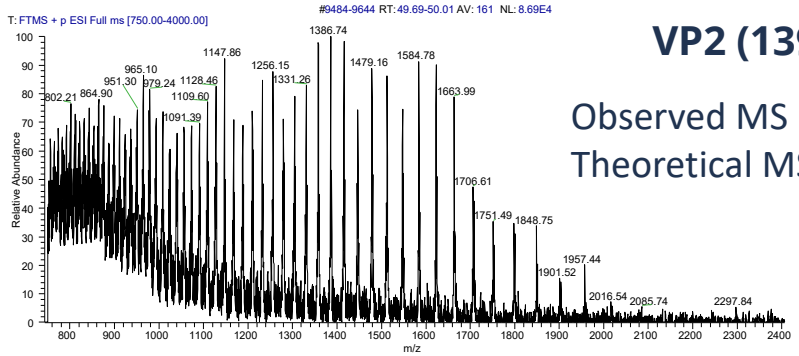
LC-UV-MS Method for Analysis of Intact Viral Proteins (UV Portion)





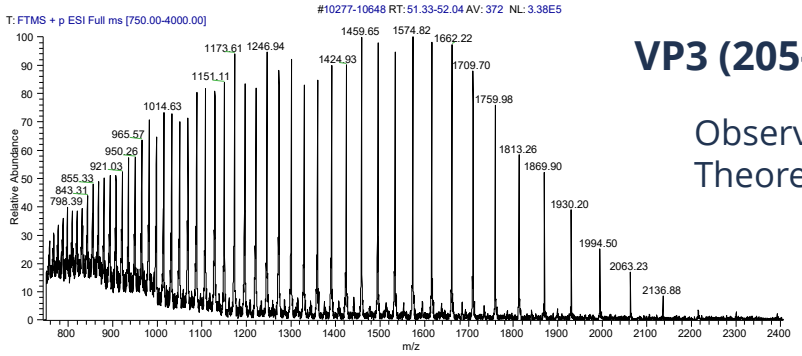
- Separation of intact viral proteins, along with related truncation variant, accomplished by a RPLC-UV-MS method.
- Determine relative ratio of the 3 major VPs and relative level of the truncation variant from the UV trace.

LC-UV-MS Method for Analysis of Intact Viral Proteins (MS Portion)



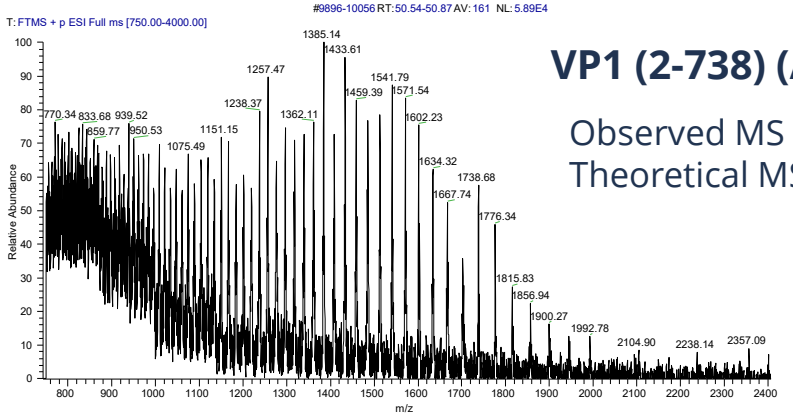
VP2 (139-738)

Observed MS = 66518.7
Theoretical MS = 66518.1



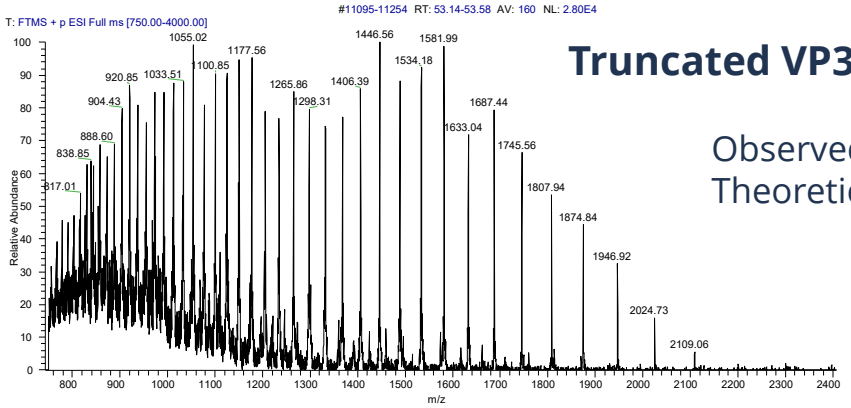
VP3 (205-738) (Acetylated)

Observed MS = 59804.7
Theoretical MS = 59804.7



VP1 (2-738) (Acetylated)

Observed MS = 81666.9
Theoretical MS = 81666.7



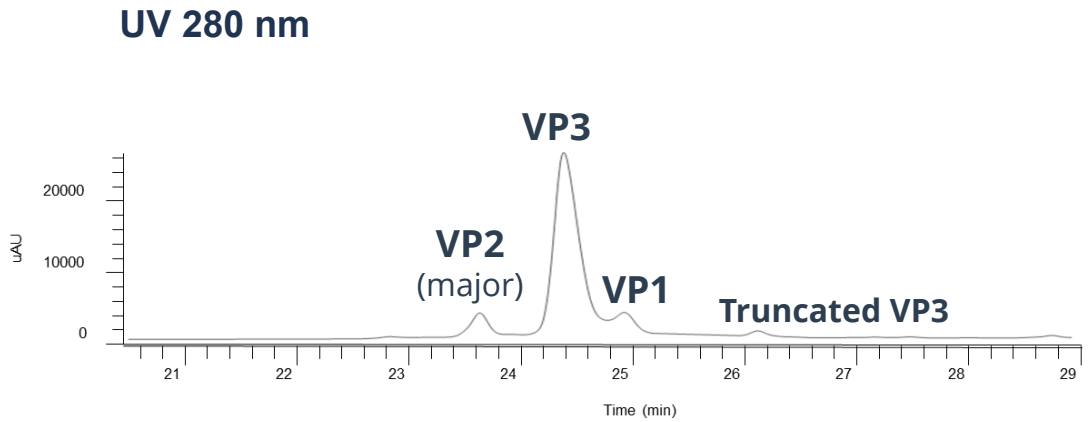
Truncated VP3 (205-659) (Acetylated)

Observed MS = 50592.8
Theoretical MS = 50593.9

- **Identities of the 3 major VPs with truncation and PTM (oxidation) variants determined by MS.**

Ratio of VPs by LC-UV-MS

Peak	Ref Sample
1 (VP2)	10.8%
2 (VP3)	75.9%
3 (VP1)	9.8%
4 (VP3 truncated form)	3.5%
Ratio of 3 VP	**1.2 : 1.2 : 10**



VP1 % = Peak3 %

VP2 % = Peak1 % (major portion 90%)

VP3 % = Peak2 % + Peak1 % (oxidized VP3 10%) + Peak 4 %

Ratio of 3 VP determined by corresponding %

For example, VP1:VP2:VP3 = 9.8% (Peak3) : 9.7% (Peak1 x 0.9) : 80.5% (Peak2 + Peak1 x 0.1 + Peak 4)

= 1.2: 1.2 : 10.0

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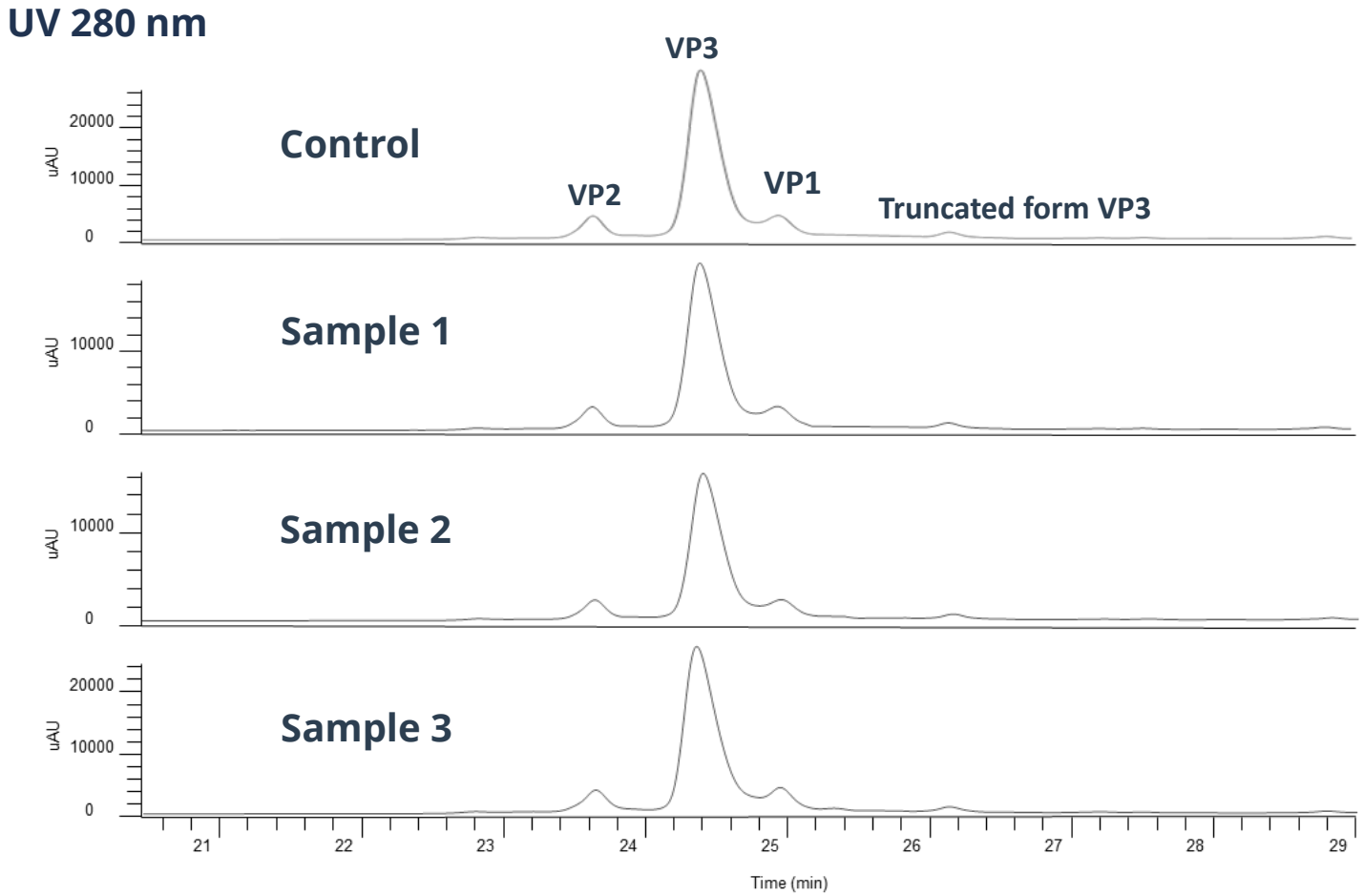
Intact Method LC-UV-MS (N=4 Samples)

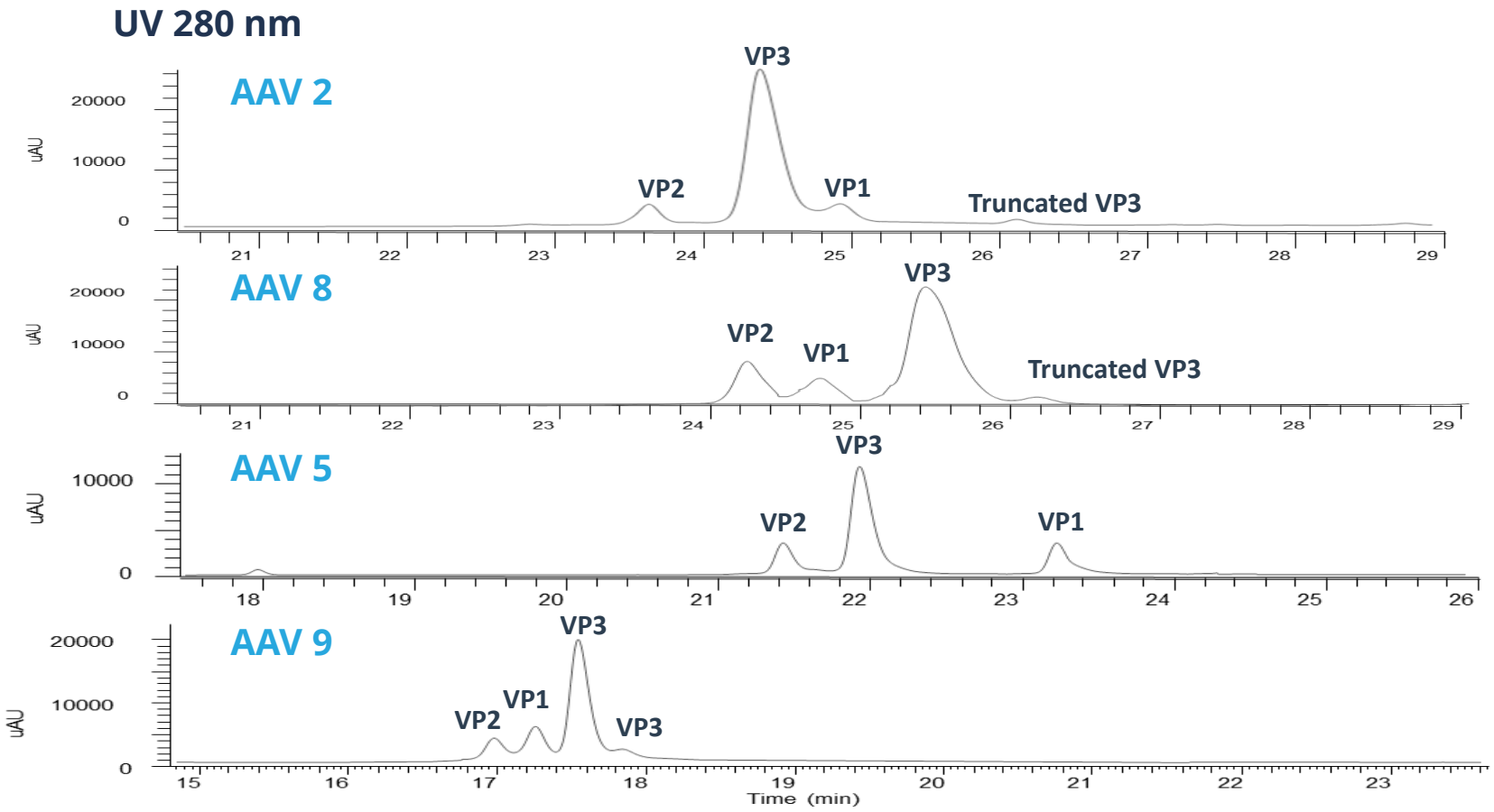


- **Each Peak can be identified/confirmed by MS.**

VPs Ratios (N=4 Samples)

Peak	Control	Sample 1	Sample 2	Sample 3
1 (VP2)	10.8%	11.3%	10.9%	11.2%
2 (VP3)	75.9%	76.1%	76.7%	76.4%
3 (VP1)	9.8%	9.7%	9.4%	9.6%
4 (Truncated VP3)	3.5%	2.9%	3.0%	2.8%
Ratio of 3 VP	**1.2 : 1.2 : 10**	**1.2 : 1.3 : 10**	**1.2 : 1.2 : 10**	**1.2 : 1.3 : 10**

Intact Method LC-UV-MS of Different AAV Serotypes



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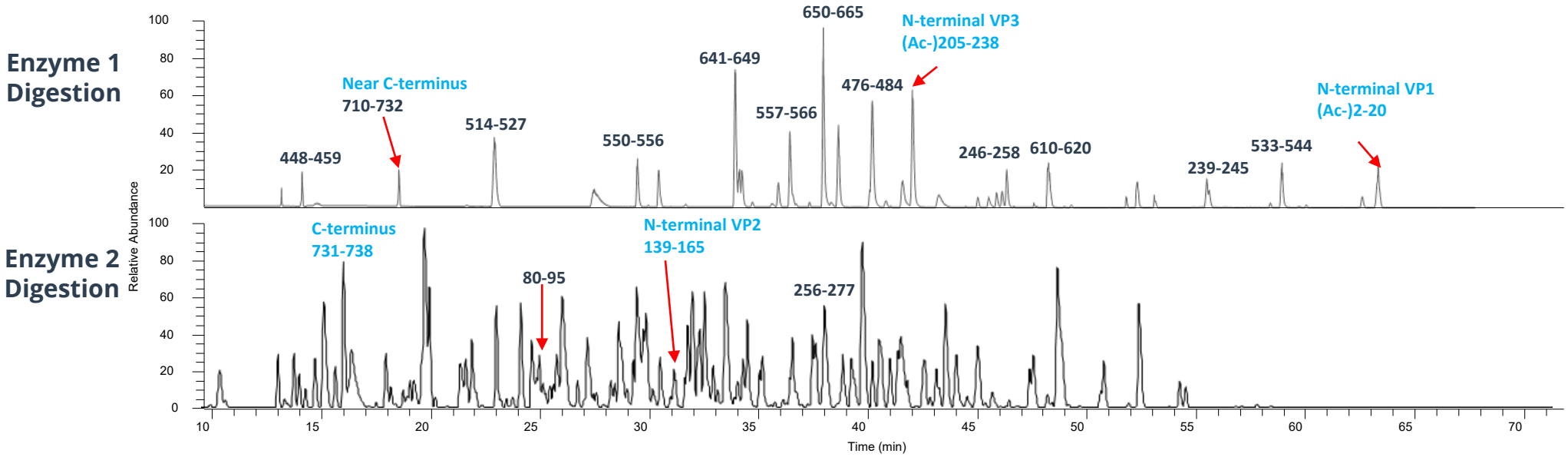
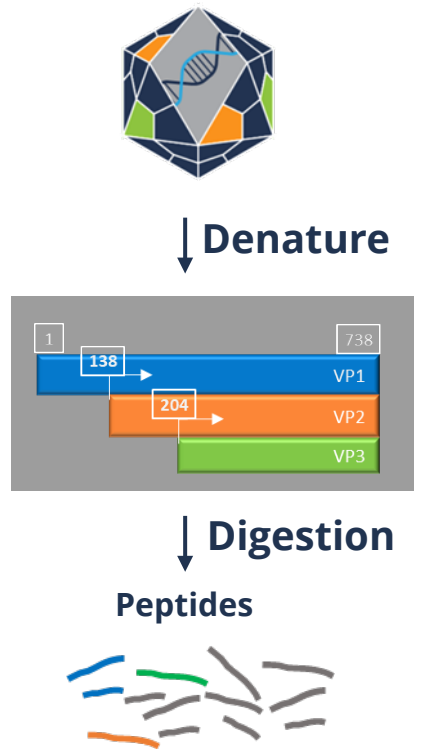
LC-UV-MS Based Methods for Characterization of Capsid Proteins and Monitoring Vector Product Attributes

Peptide Mapping Approach

LC-MS Peptide Mapping





- Sequence coverage (100% by two Enzyme digestion, >95% each one).
- Enzyme 2 covers the sequences not identified in Enzyme 1.
- Identify the expected N/C-termini (matches with Intact MW).
- Sample preparation under different conditions (e.g., low pH) to avoid of artifactual PTMs.

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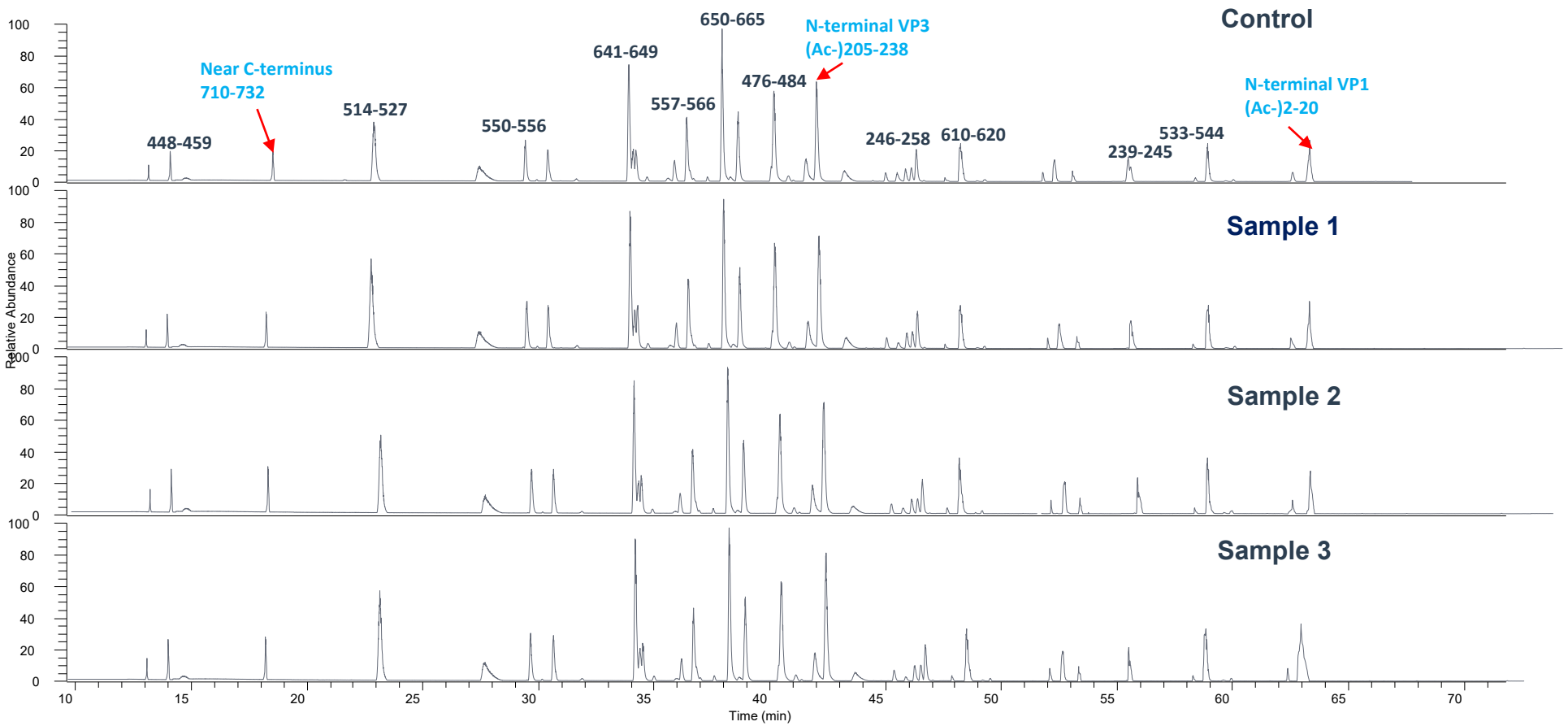
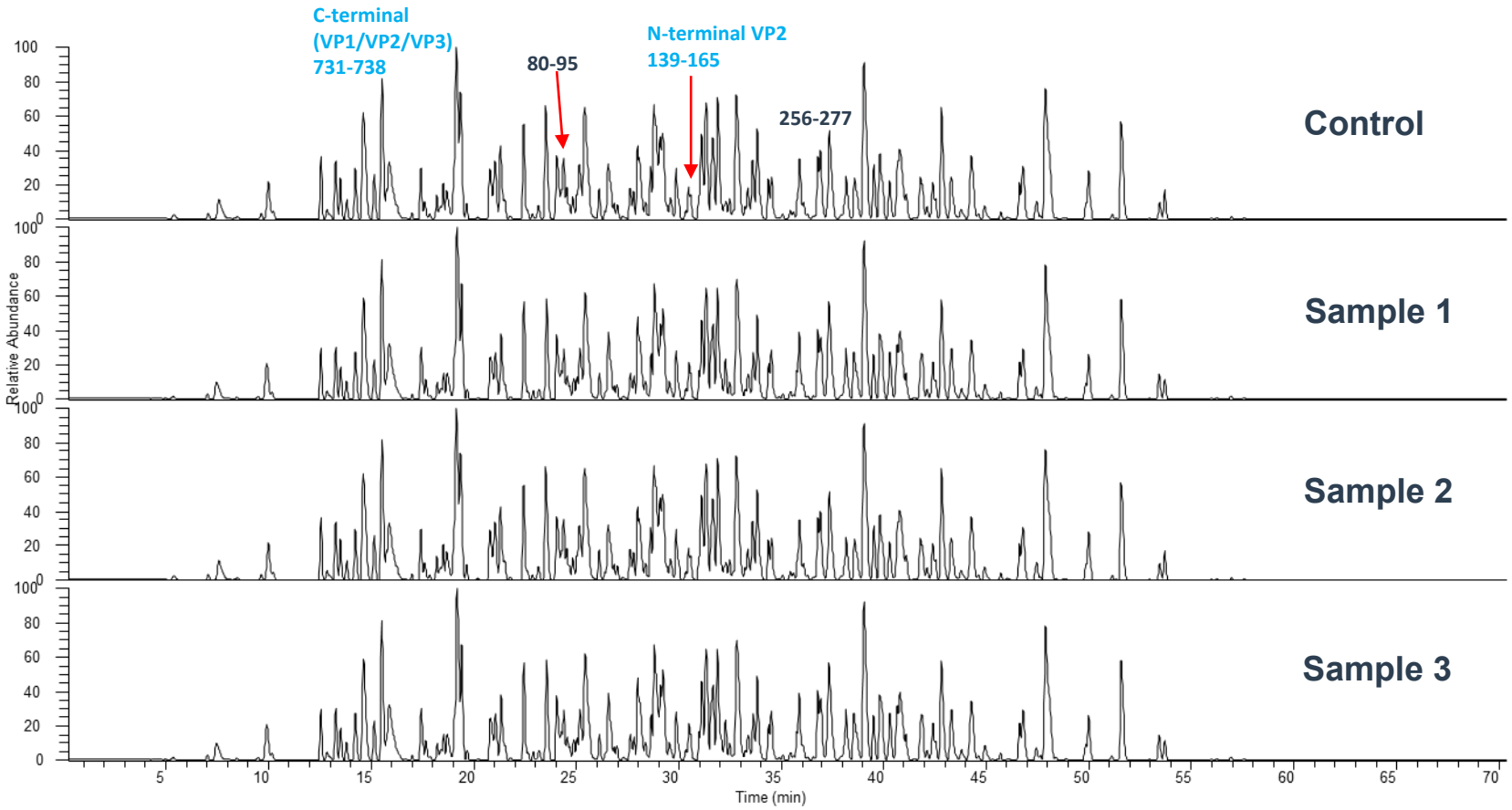
Comparative LC-MS Peptide Mapping (Enzyme 1 Digestion)



- **Similar chromatograms and sequence coverage (>95% for each sample).**

Comparative LC-MS Peptide Mapping (Enzyme 2 Digestion)



- Similar chromatograms and sequence coverage (>95% for each sample).

Summary of VP Identifications (Example: N-terminal Portion)

Region	Theoretical MW (mono)	Observed MW (mono)	Mass Error (ppm)	RT (min)	Notes
Ac-2-20	1791.850	1791.851	1	63.7	VP1 N-term
26-36	1660.810	1660.811	1	32.8	
37-29	1300.750	1300.752	2	31.0	
30-41	1360.237	1360.237	0	32.0	
42-48	1471.694	1471.695	1	19.3	
49-59	2388.232	2388.232	0	33.0	
59-65	1386.824	1386.823	-1	26.2	
66-85	2016.602	2016.603	0	22.4	
80-95	2342.476	2342.478	1	25.1	By Enzyme 2
88-123	4275.148	4275.148	0	54.1	
124-144	3495.057	3495.059	1	35.7	
139-165	3915.493	3915.493	0	37.4	VP2 N-term, by Enzyme 2
145-161	2463.279	2463.281	1	16.5	
162-184	2277.402	2277.405	1	27.0	
185-194	1758.258	1758.261	2	26.8	
195-238	4259.150	4259.145	-2	29.3	
Ac-205-238	3100.025	3100.026	0	42.5	VP3 N-term

- **The identity of each viral protein may be confirmed.**

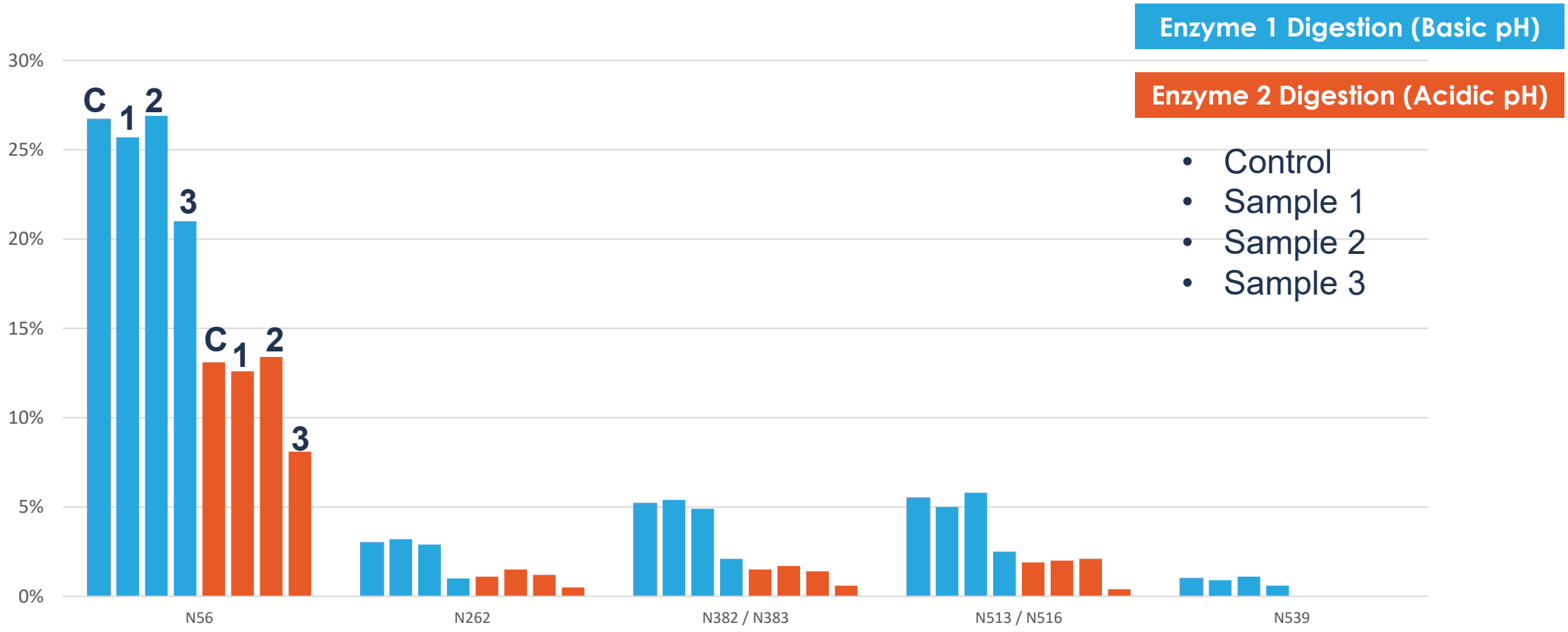
PTMs (N/C-termini)

% Acetylation

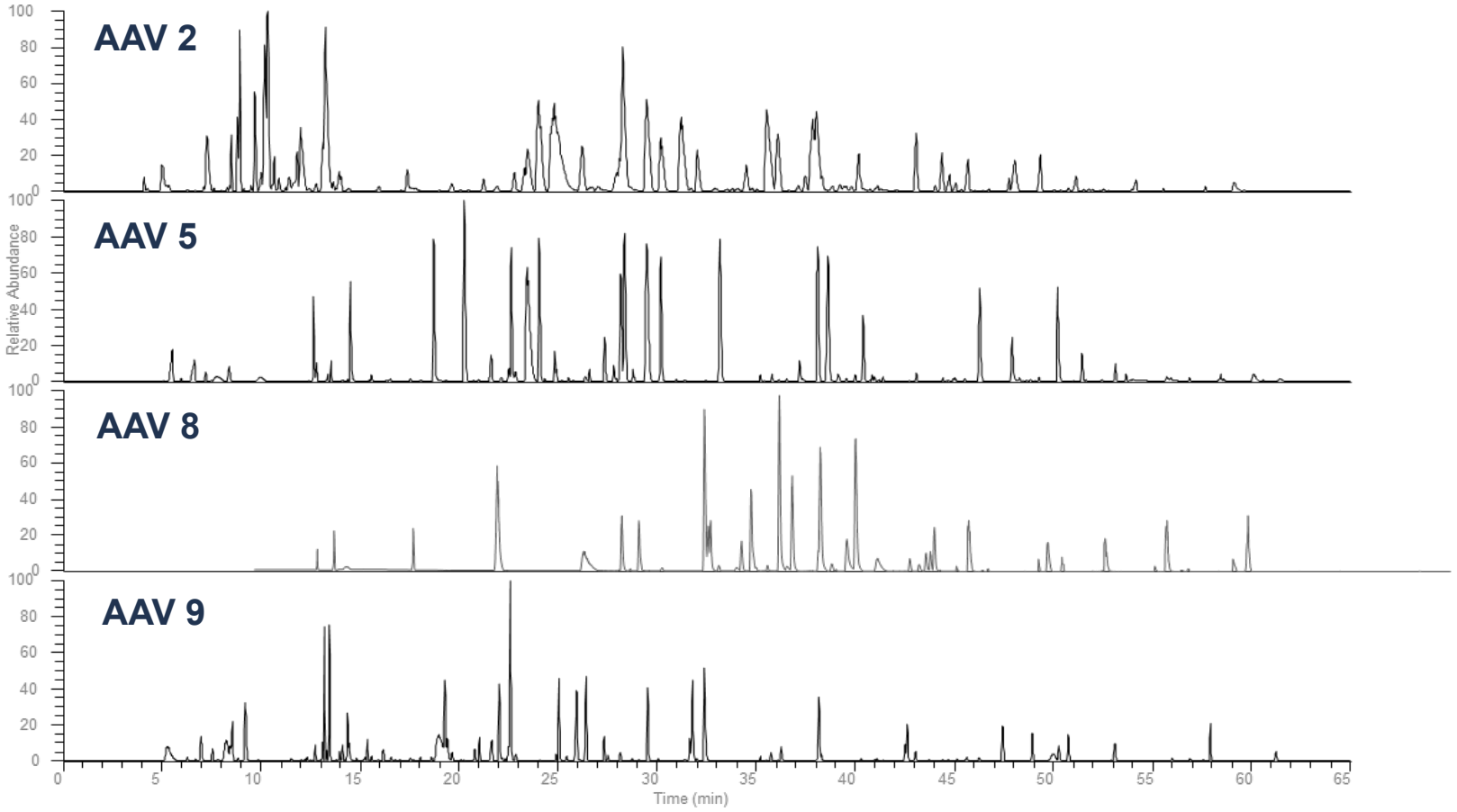
Region	Position	Site	Sequence	Control	Sample 1	Sample 2	Sample 3
VP1	2-20	2	XXXXXXXXXXXXXXXXXX	100	100	100	100
VP3	205-238	205	XXXXXXXXXXXXXXXXXXXXXXXXXX	99.2	99.5	99.4	99.6

- **Similar results obtained as with Intact MS analysis.**

PTMs (Deamidation)



Peptide Mapping (Different AAV Serotypes)



- **Peptide Mapping LC-UV (MS) can be further developed for ID test method.**

Summary of LC-UV-MS Based Methods for Characterization of Capsid Proteins and Monitoring Vector Product Attributes

- Intact masses and major PTMs for VPs
- VPs ratio monitoring from batch to batch
- 100% sequence coverage for each lot and PTMs monitoring
- Methods apply to main AAV serotypes

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Empty/Partial/Full Capsid Separation of Challenging AAV Samples by Anion-Exchange Chromatography (AEX)

AAVs Are Complex Products: Particle, Proteins and DNA

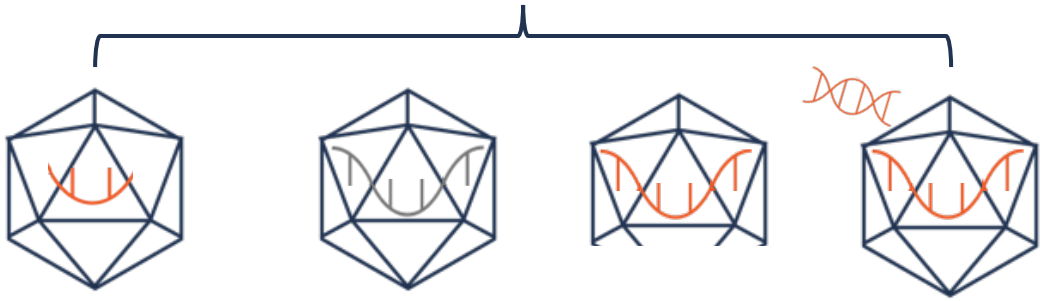
Capsid

Empty

~3.8 MDa (~25 nm)

Full

Partial/Overloaded



Truncated/partial transgene

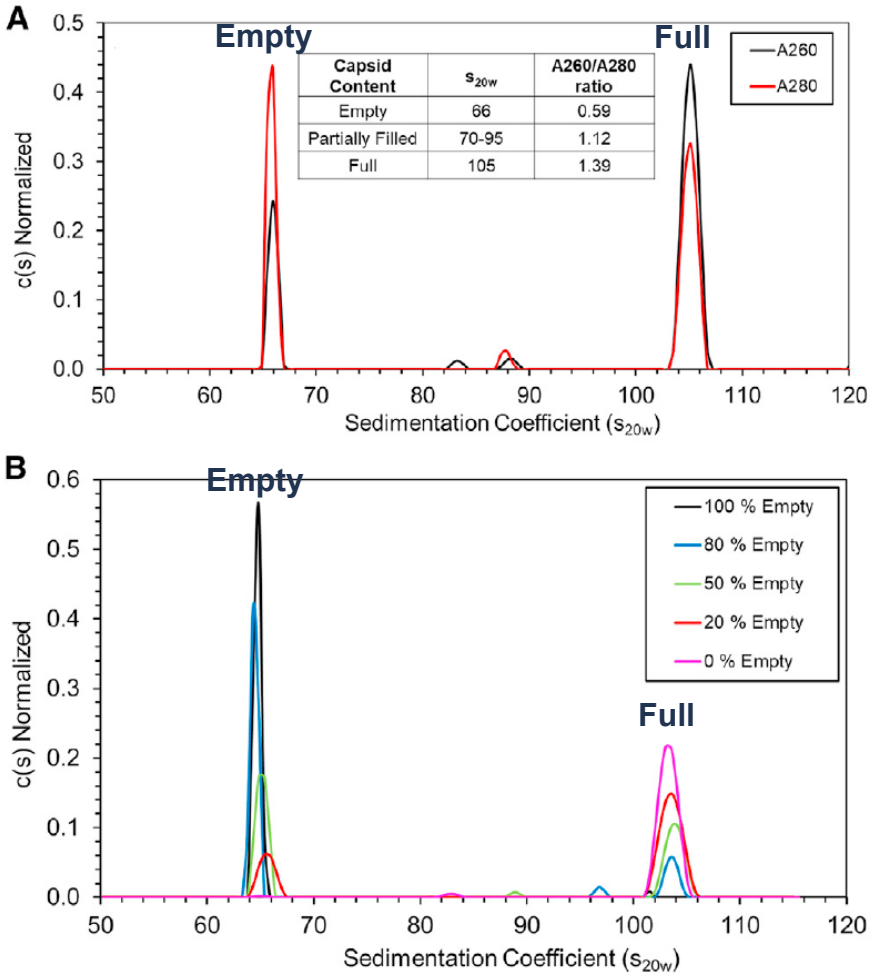
Contaminant DNA/protein

Incomplete capsid or alternative VP ratios

Capsid with DNA attached to surface

AUC – The Gold Standard for Empty/Partial/Full Capsid Analysis

- Excellent resolution of Empty (66 S), Partial (~80 S), and Full (105 S) Capsids
- A260/280 ratios support Empty and Full Capsid identification
- AUC serves as an excellent tool for orthogonal support of Empty/Partial/Full analytical method development
- Not validated



Werle, Amanda K., et al. "Comparison of analytical techniques to quantitate the capsid content of adeno-associated viral vectors." *Molecular Therapy-Methods & Clinical Development* 23 (2021): 254-262.

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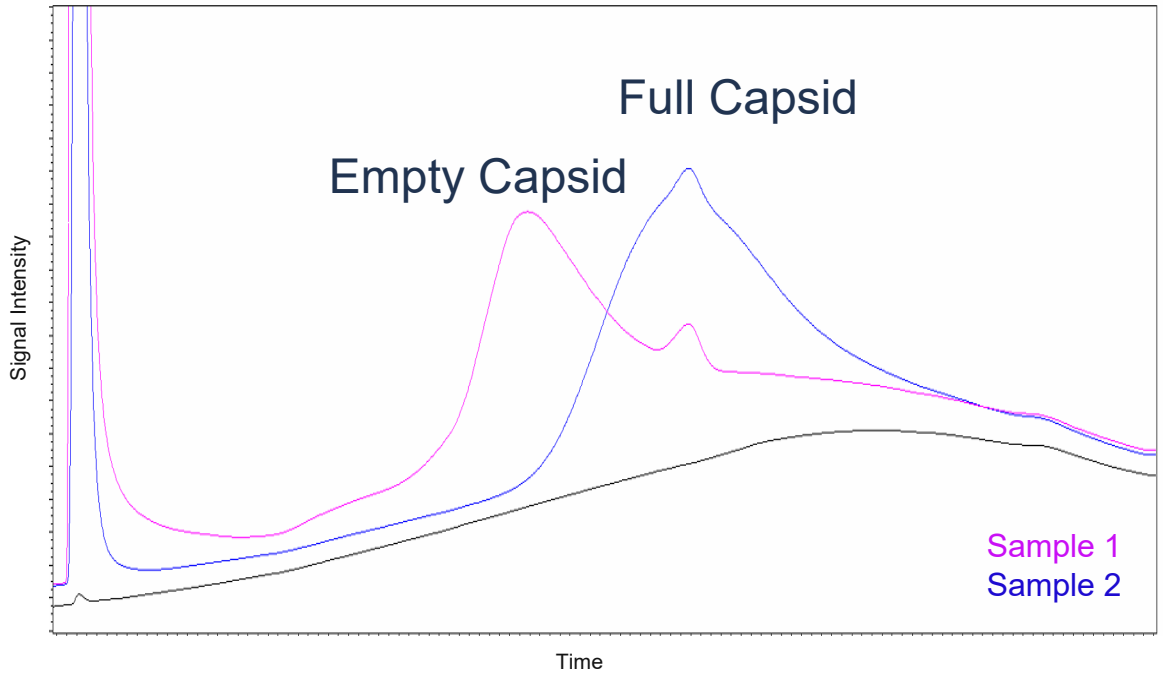
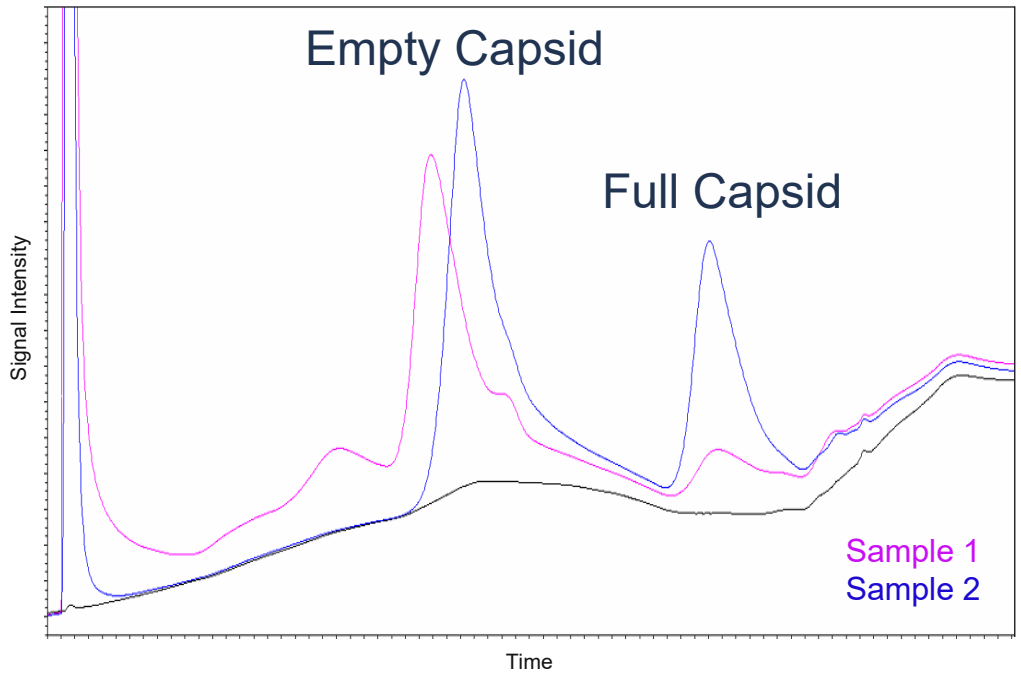
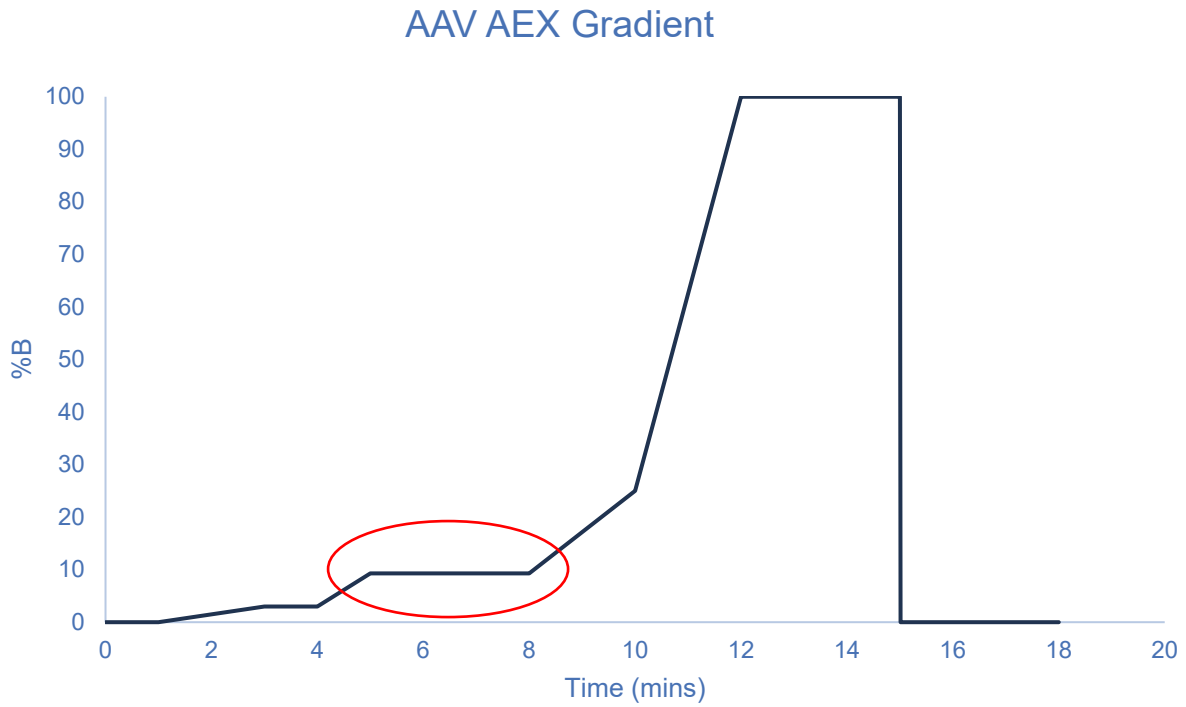
HPLC AEX-based AAV Empty/Full Capsid Analysis





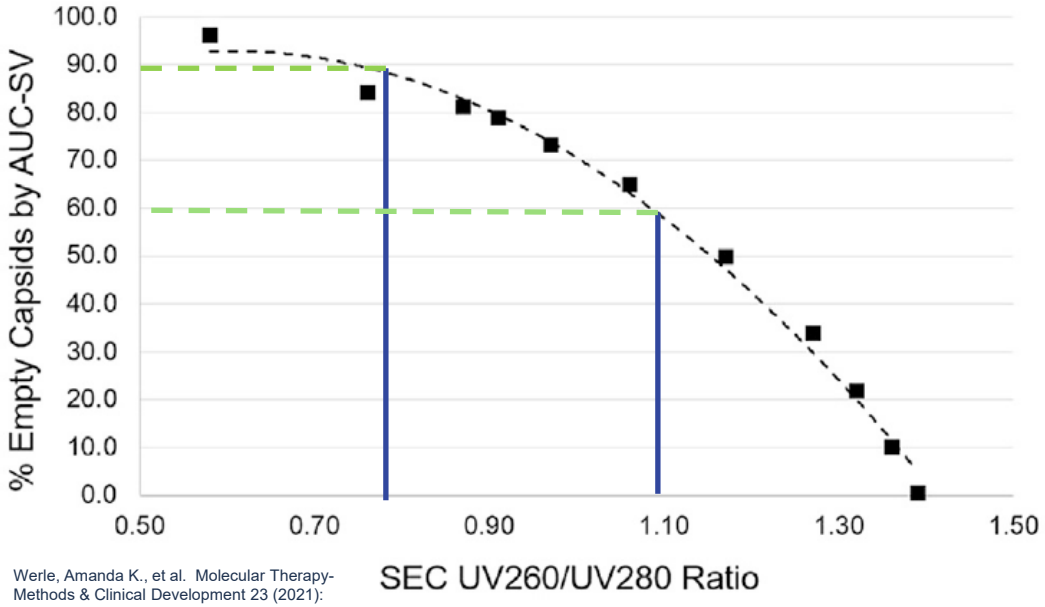
- **Different AAV products have unique properties and different methods are needed to achieve good separation**
  - **Column choice**
  - **Gradient profile (slope(s), plateau use and timing)**
  - **Mobile phase compositions can vastly differ in terms of what leads to well-resolved and identifiable peaks (complex solutions/mixtures, inclusion of unusual additives)**

Adding a Plateau into the Gradient Can Yield Improved Empty/Full Separation



- **With some products, the addition of a plateau allows for efficient separation of Empty and Full Capsids. The length of the plateau can vary as can the position within the gradient to accomplish good separation of Empty and Full Capsids.**

A260/A280 Ratios Confirm Empty/Full Ratios Determined by AEX



Werle, Amanda K., et al. Molecular Therapy-Methods & Clinical Development 23 (2021): 254-262.

- **As an orthogonal measure of Capsid composition, both AAV samples were concentrated and scanned on Shimadzu UV-1900 Spectrophotometer. A260/A280 ratios were also determined via AEX and HIC.**
- **Excellent consistency with respect to % Empty Capsid was observed between the various measurements.**

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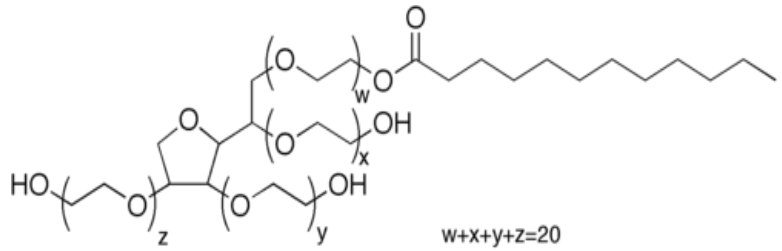
Complexities of Analyzing Surfactants in AAV Products

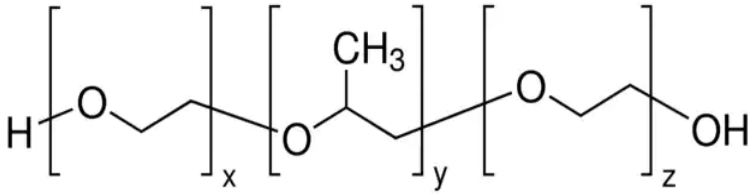
- An overview on surfactants used in gene therapy products and specific analytical challenges
- Approach to surfactant quantitation in AAV products by LC-CAD
- Characterization of surfactant species by LC-QDA

Popular Surfactants Used in AAV Product Manufacturing

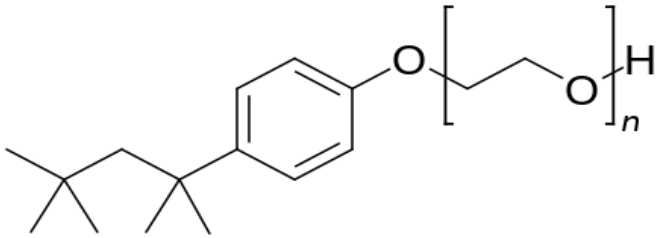
Non-ionic



Polysorbate 20

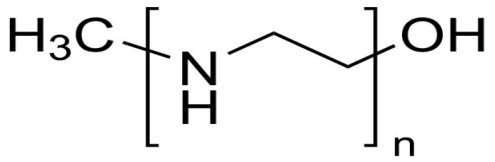


Poloxamer 188 (P188; Pluronic F68)



Triton X-100

Cationic



Polyethyleneimine (PEI)

- Surfactants are a diverse class of polymers with distinct chemistry, structure and charge
- Their heterogeneity complicates structure confirmation and quantification

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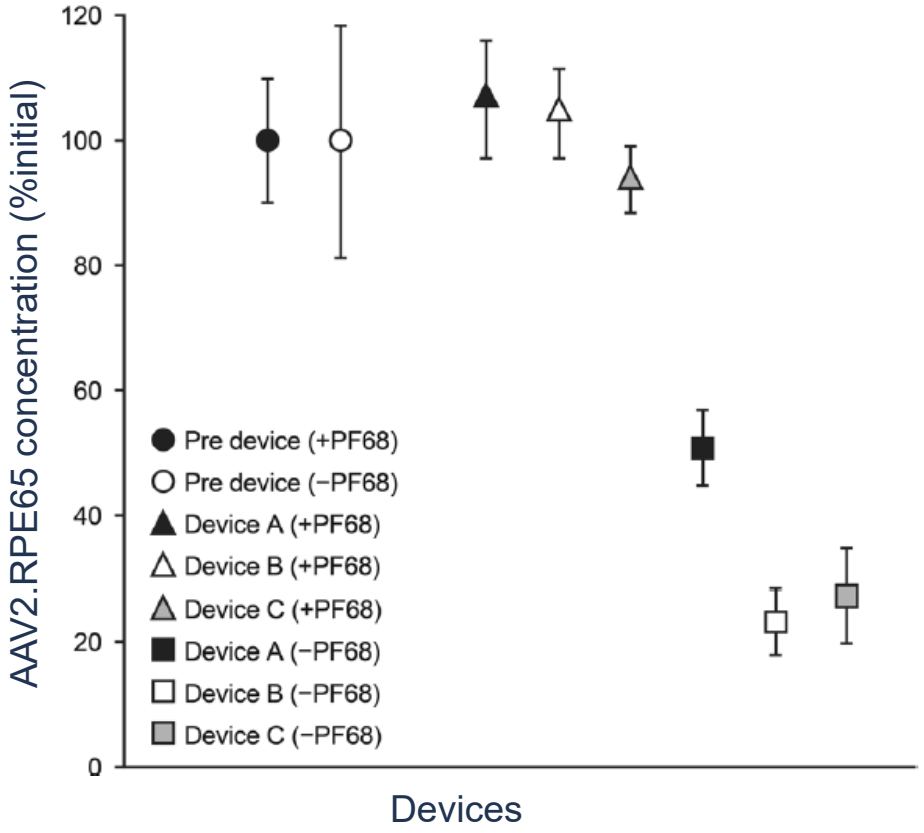
Surfactants Utilization in AAV: Process Reagents and Excipients

Function and Potential Risks

Surfactant	Function	Potential Risks
Polysorbate 20/80	Cell Lysis Reagent Formulation Excipient	Susceptible to chemical degradation Formation of visible and subvisible particles
Poloxamer 188	Formulation Excipient	Susceptible to chemical degradation Formation of visible and subvisible particles
Triton X-100	Cell Lysis Reagent	Acute oral toxicity, eye damage, skin irritation
Polyethyleneimine	Plasmid Transfection Reagent	Cytotoxicity

- Surfactants are used as process reagents in manufacturing and as excipients in the final formulation of AAV DS and DP
- There are different analytical requirements that apply to surfactants as process-related impurities or formulation excipients

Importance of Surfactants as Excipients in AAV Products



- Compatibility studies were undertaken to evaluate AAV2 in different delivery devices.
- Significant losses of AAV2 (up to 80%) were observed in formulations without surfactant in different delivery devices.
- Surfactants, such as Pluronic F68 at low concentrations (0.001%), can prevent this loss and ensure product delivery to the patient.

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Gerard A. Rodrigues, Pharm Res (2019) 36: 29

Regulatory Expectations of Excipients and Process Impurities in Gene Therapy Products

"Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials" from EMA

870 **S.3.2. Impurities**

871 During the production of an ATIMP, variable amounts of impurities, product- and process-related, may
872 be introduced into the active substance. Any reagents known to have clinical impact in humans should
873 be analysed in the active substance (or in individual components if otherwise not possible) and
874 acceptance criteria should be set. The specification limits should be justified by levels detected in
875 batches used for toxicological and/or clinical studies.

885 Process related impurities (e.g. media residues, growth factors, host cell proteins, host cell DNA,
886 column leachables) and product related impurities (e.g. cell types not linked to the therapeutic effect,
887 cell fragments or non-viable cells, precursors, degradation products, aggregates) should be kept to the
888 minimum or a risk assessment provided. Based on the risks identified, consideration should be given to
889 the maximum amount for the highest clinical dose and an estimation of the clearance should be
890 provided. In case only qualitative data are provided for certain impurities, this should be justified.

1279 Upper limits, taking safety considerations into account, should be set for the impurities. For the
1280 impurities not covered by the active substance specification, upper limits should be set, taking safety
1281 considerations into account.

1306 The final product should be tested for residual manufacturing reagents with known or potential
1307 toxicities and the test procedure described. Limits need to be included in the specifications, unless
1308 otherwise justified

Challenges and Approaches for Surfactant Analysis in AAV Products

- Surfactants are often associated with capsid proteins and oligonucleotides
  - Requirement to optimize procedures for surfactant release prior to analysis
- Chemical nature of surfactants means small differences in sample handling can cause significant change in quantitative results
  - Importance of specifying sample handling procedure and evaluating recovery at all stages
- Surfactants contain multiple species including a complex variety of potential degradants
  - Utilization of reversed-phase or mixed-mode chromatography for separation
  - For quantification, gradients can be modified to either quantify total surfactant level or separate species for monitoring individual constituents
- Surfactants are generally UV-transparent
  - Use of special detectors including CAD, ELSD or QDa
- Surfactants are ‘sticky’ leading to carryover and complicating quantification
  - Implementation of column cleaning steps into LC method or sequence and careful consideration of contact surfaces

Instrumentation and Techniques for Quantitation and Characterization of Surfactants in AAV Products

LC - CAD

LC - QDa

LC - ELSD

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Approach to Surfactant Quantitation in AAV Products by LC-CAD

Charge Aerosol Detector (CAD)

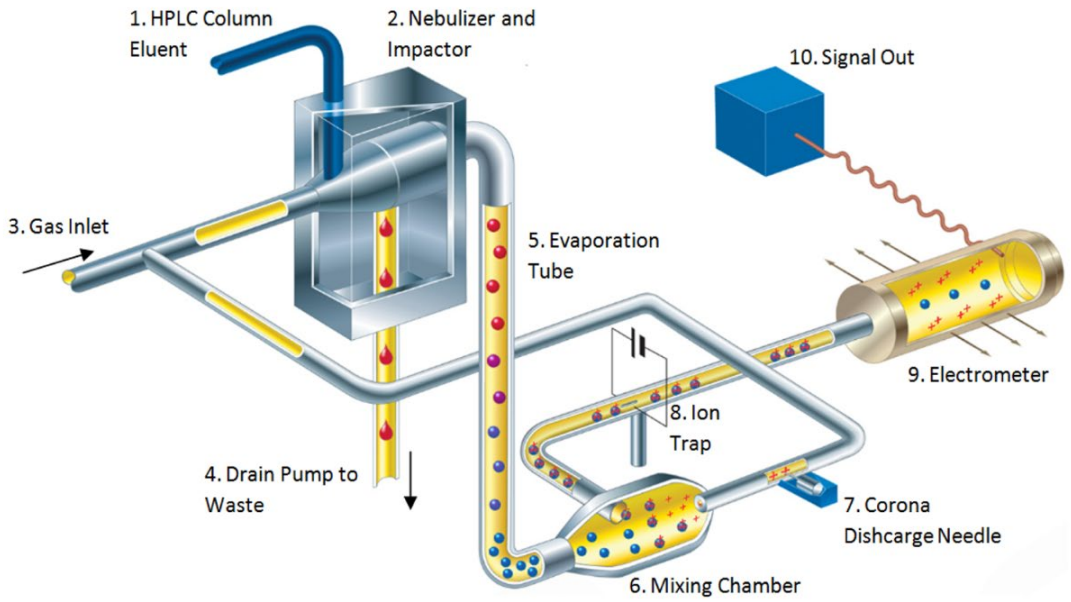
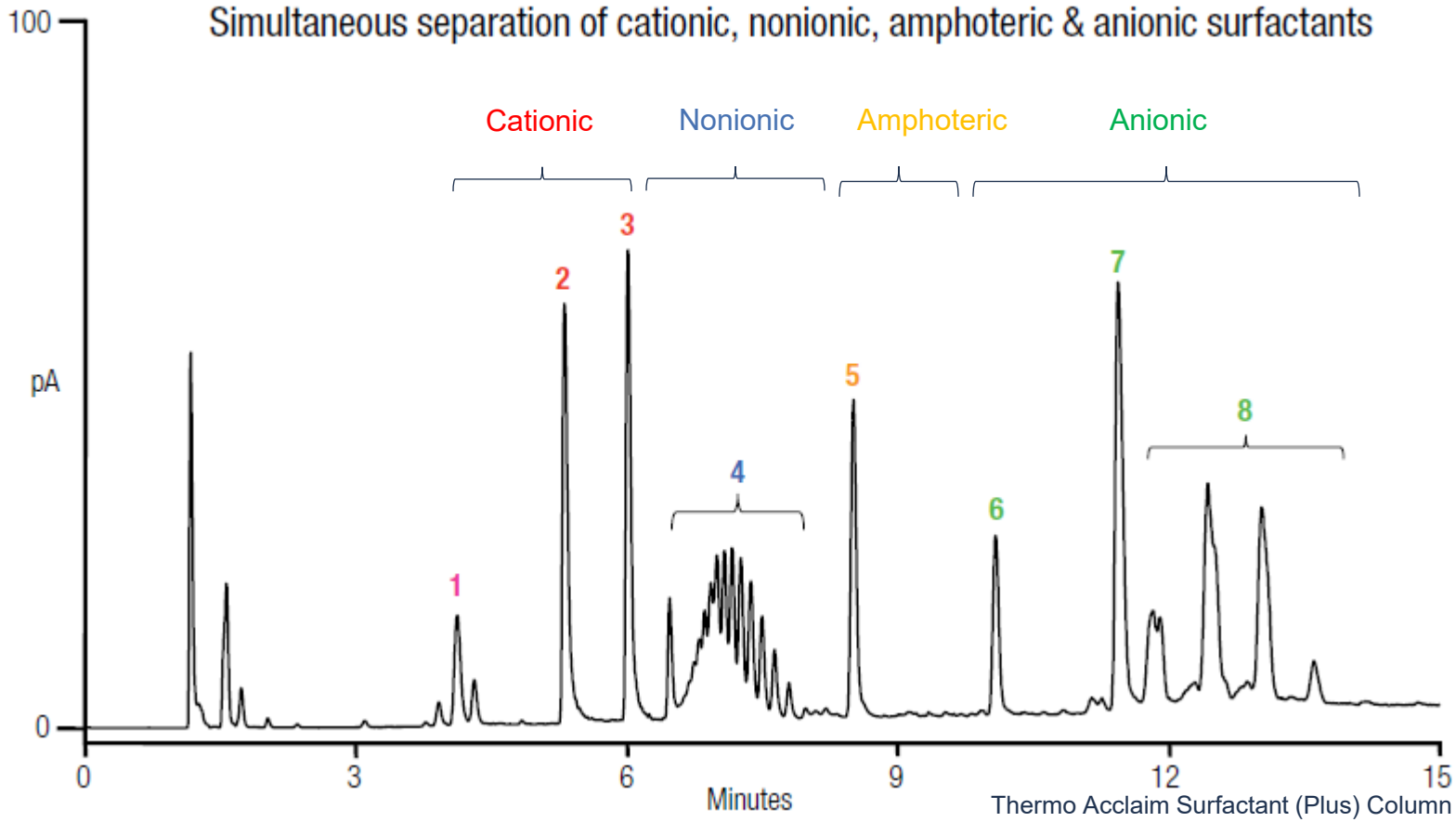


Image Curtesy of Thermo Scientific

- Nebulize HPLC eluent into aerosol, then dry small aerosol droplets into particles in evaporation tube.
- Mix particles with a stream of charged nitrogen gas in the mixing chamber to form charged particles
- Charged particles pass through the ion trap, then generate responses based on the charge detected by a sensitive electrometer.

- Fundamentally a particle detector
- Provide almost universal response independent of the physical or chemical properties of the analyte

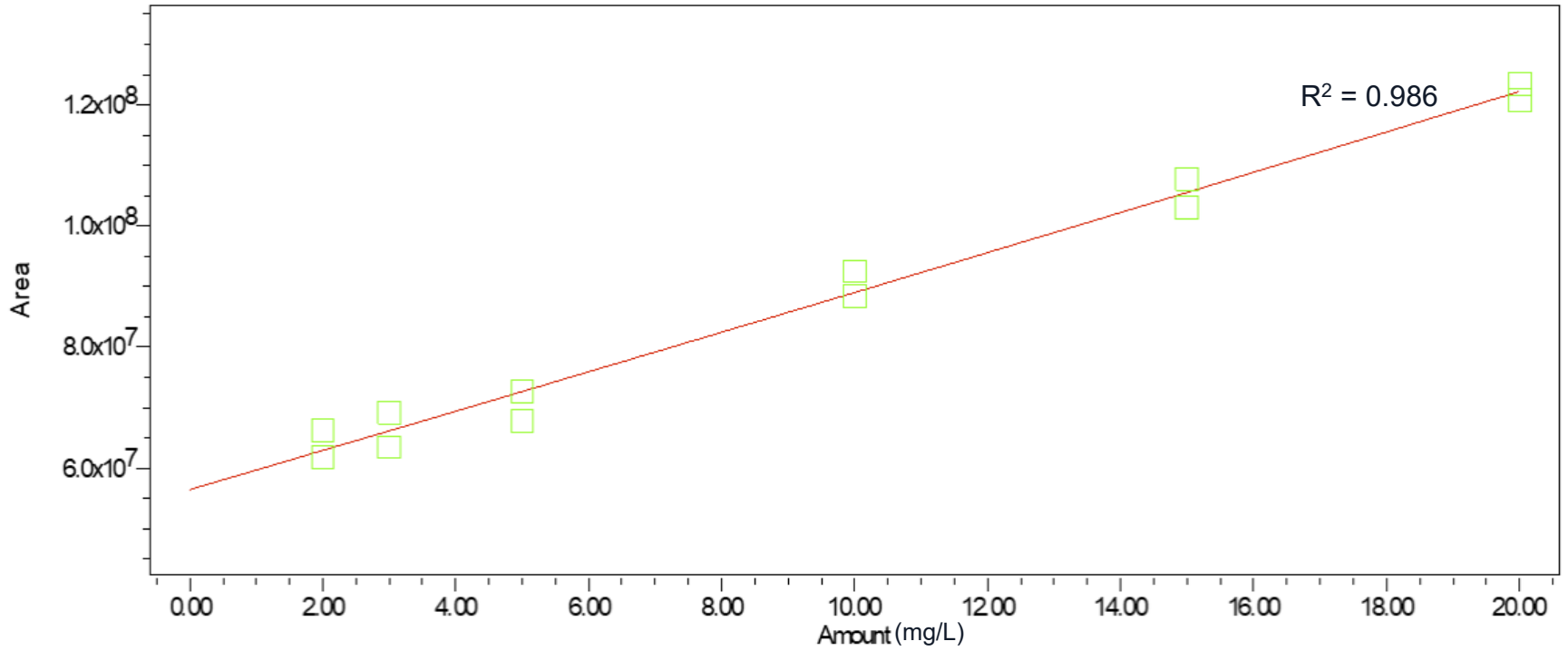
Mixed-mode Chromatography Column for Surfactant Analysis



1. Sodium Xylene Sulfonate
2. Laurylpyridinium Chloride
3. Lauryldimethylbenzyl ammonium chloride
4. Triton X-100
5. Cetyl Betaine
6. Sodium Decyl Sulfate
7. Sodium Dodecyl Sulfate
8. Sodium Dodecylbenzene Sulfonate

- Mixed-mode chromatography column is a powerful tool for surfactant separation

Quantification of Surfactant 1 in AAV Formulation A by LC-CAD



- Mixed Mode analysis was used to analyze Surfactant 1 in the presence of two other surfactants
- LC-CAD achieved excellent linearity (R^2 = 0.986) and repeatability of quantification of Surfactant 1 standards down to 10 mg/L.
- Levels of Surfactant 1 in AAV products were below the limit of detection of this method

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Characterization of Surfactant Species by LC-QDa

Single Quadrupole (QDa) MS Analysis of Analytes

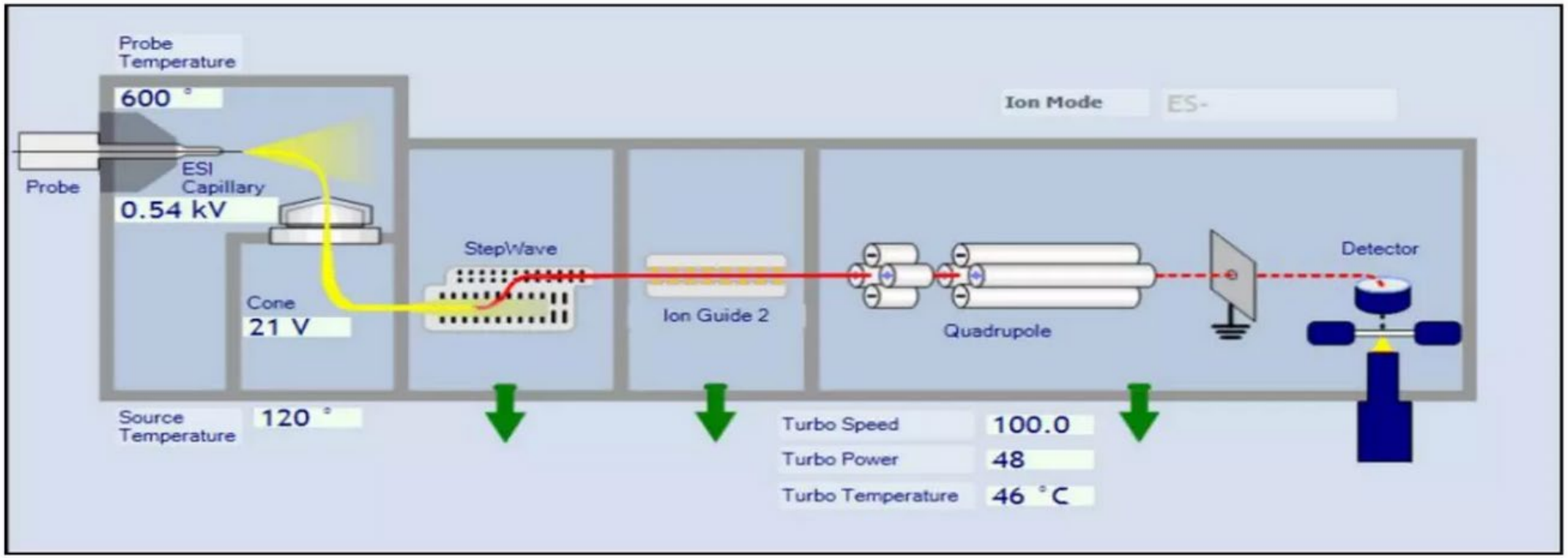
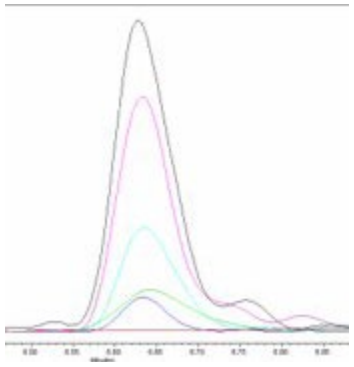


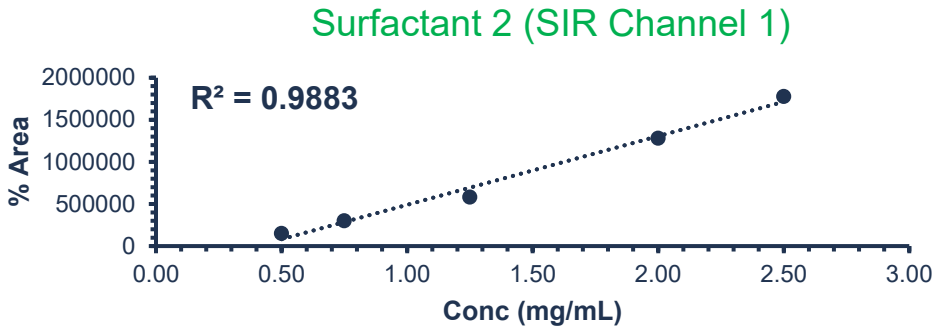
Image Curtesy of Waters

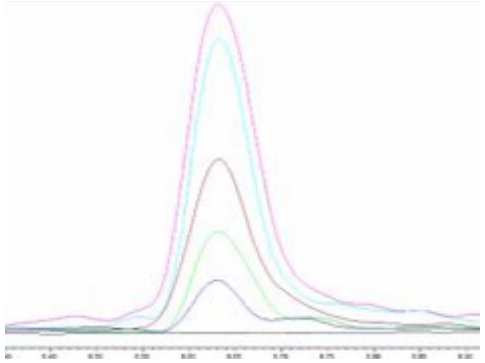
- Can perform Full Scan (MS mode) and Single Ion Recording (SIR Mode)
- Lower LOQ than CAD detector
- Suitable for surfactant quantitation and characterization
- Plug and play MS

Targeted MS for Surfactant Analysis

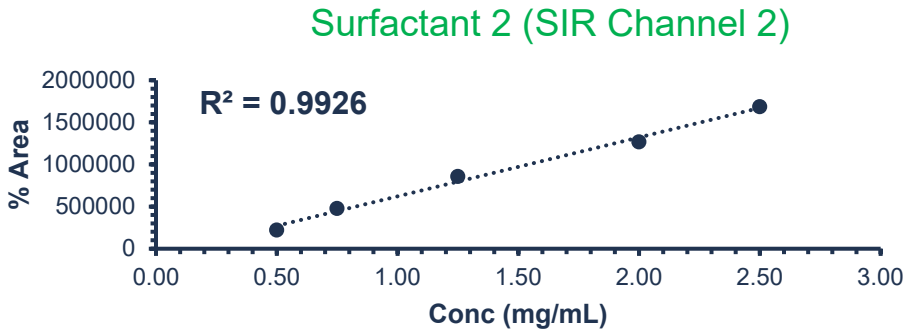


Targeted MS
(Selected Ion Monitoring)





Targeted MS
(Selected Ion Monitoring)



- MS can be utilized to study specific ions within a surfactant utilizing selected ion monitoring (selected SIR channel on QDa detector)
- Excellent linearity (R^2 ≥0.99) of Surfactant 2 standards was observed using both selected ions
- If an AAV sample contains multiple surfactants, selected ion monitoring by MS can improve specificity and S/N values

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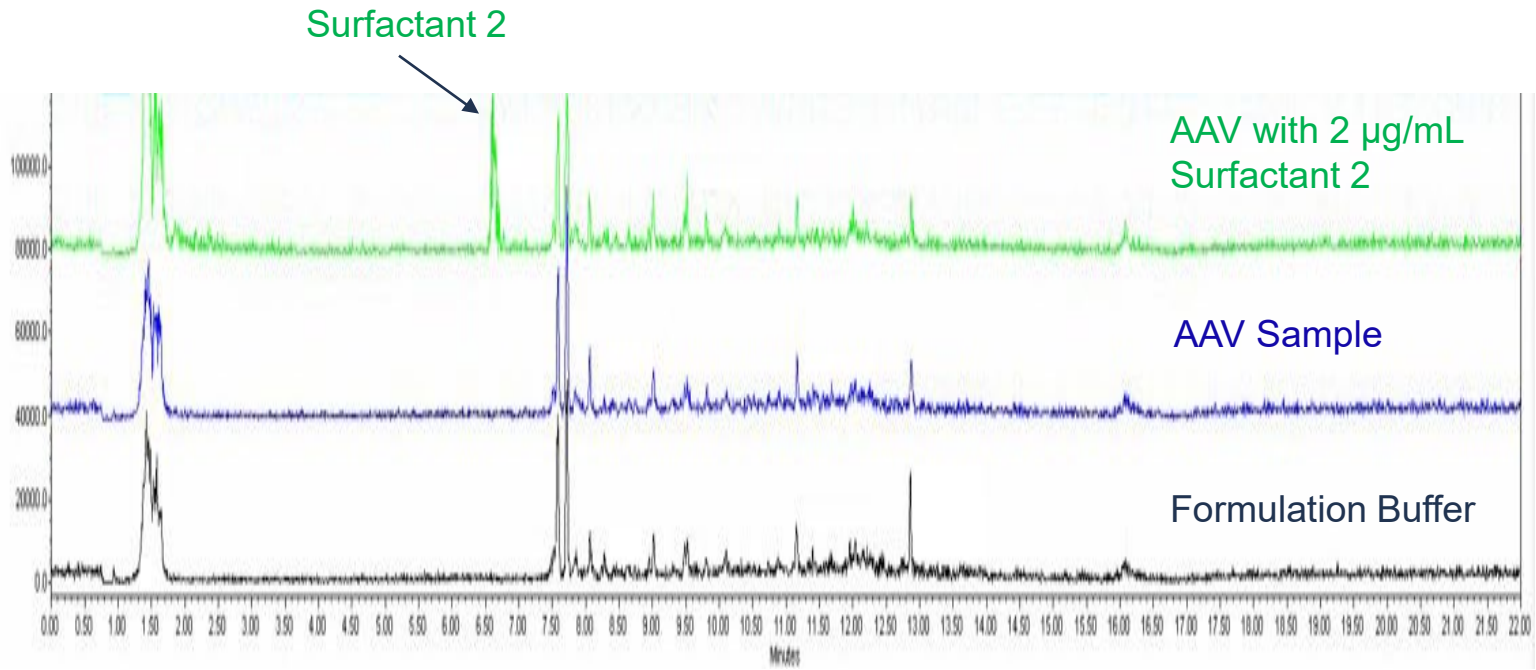
Targeted MS for Surfactant Analysis: Process Residual Clearance



- Analysis of Surfactant 2, a process-related impurity in an AAV product

- Targeted MS was used to demonstrate clearance of Surfactant 2 in this AAV sample
- The level was below the detection limit of 2 µg/mL established for the assay

Summary

- Analysis of surfactants in AAV products is important both for monitoring formulation excipients and process-related residuals and impurities
- Based on the nature of surfactants, sample preparation need to be optimized to release surfactants before analysis
- Mixed-mode chromatography can be used if multiple surfactants need to be monitored simultaneously
- Both CAD detector and MS detectors can provide accurate quantitation of UV transparent surfactants
- Surfactant monitoring has become expected in the release panel by regulators

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Thank You

ProtaGene.com
contact@protagene.com

Europe
ProtaGene GmbH
Inselwiesenstraße 10
74076 Heilbronn
Germany
Phone: +49 7131 74504-0

North America
ProtaGene US, Inc
4 Burlington Woods Drive
Burlington, MA 01803
USA
Phone: +1 857-829-3200

Europe
ProtaGene CGT GmbH
Im Neuenheimer Feld 582
D-69120 Heidelberg
Germany
Phone: +49 6221 42790-0

Europe
ProtaGene GmbH
Otto-Hahn-Straße 15
44227 Dortmund
Germany
Phone: +49 231 9742-6100