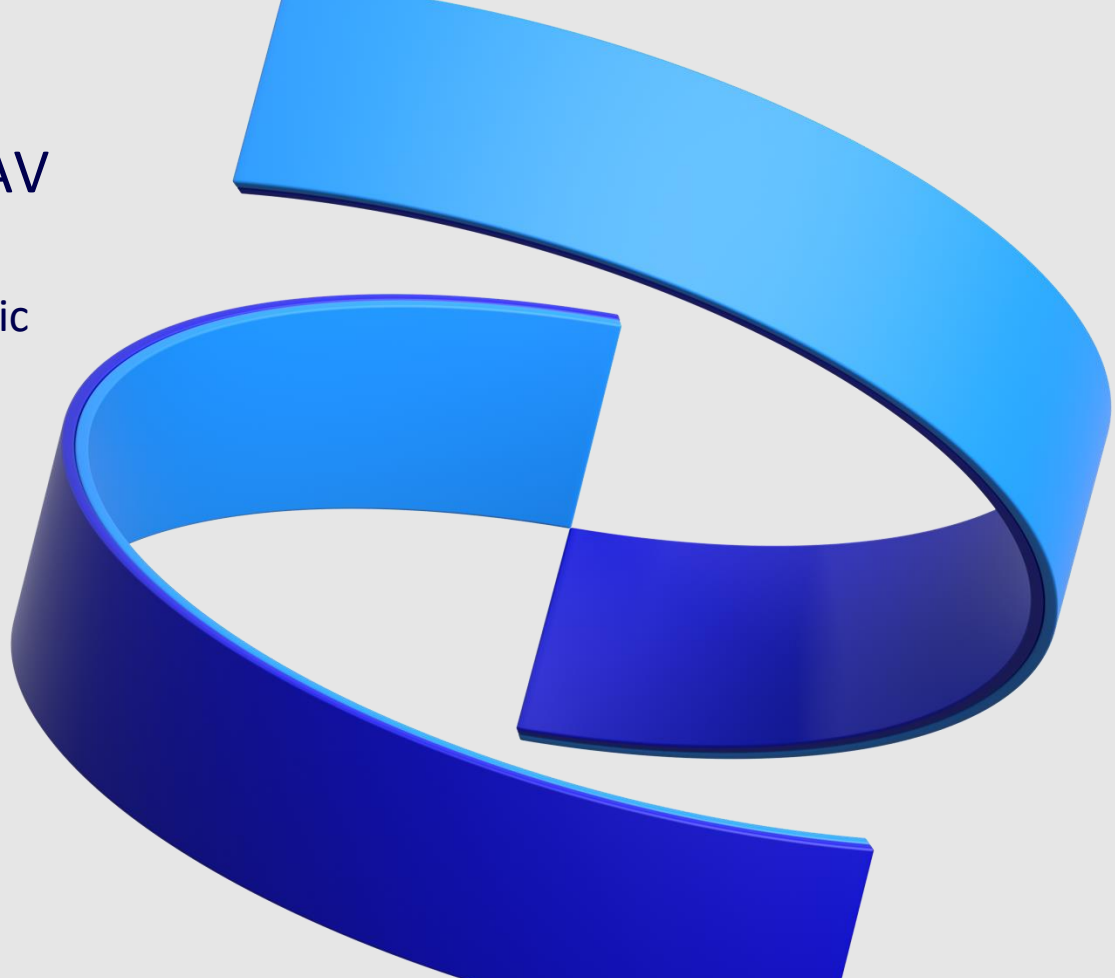
Unlocking Analytics for AAV Gene Therapy Programs:

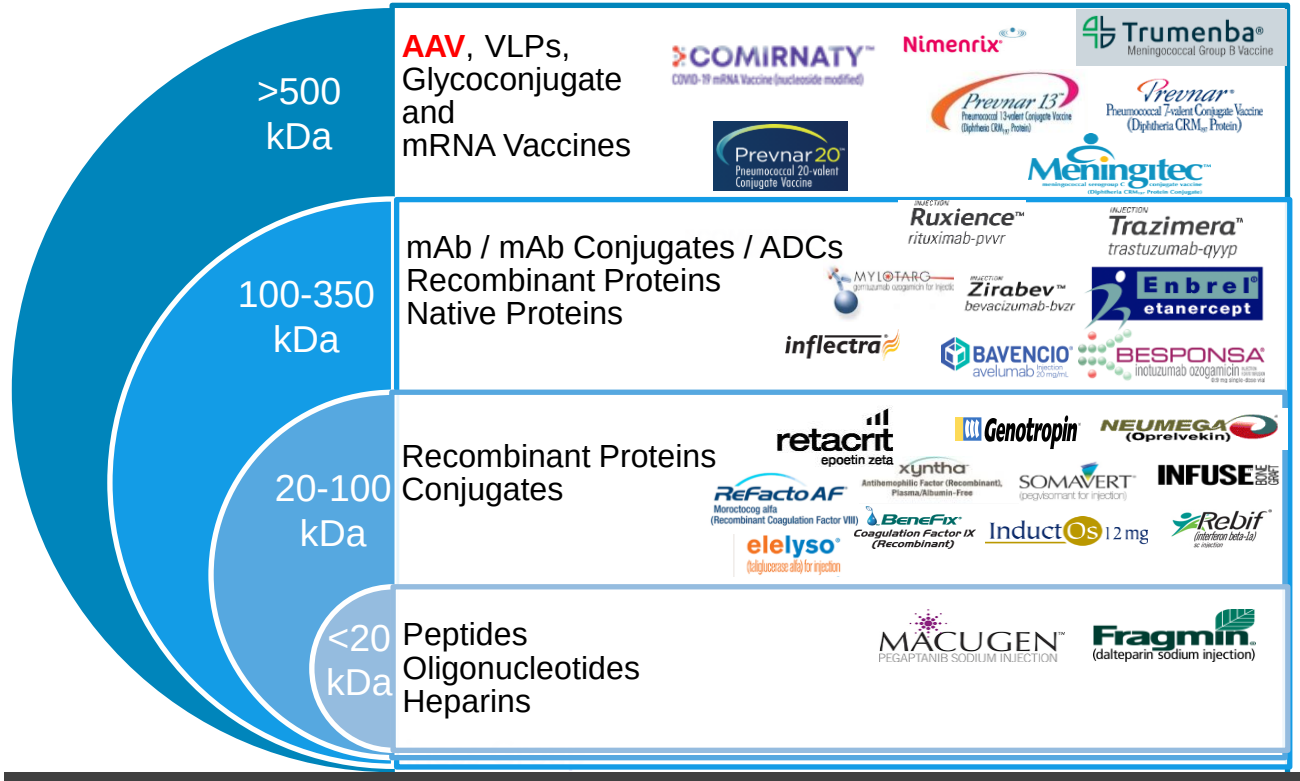
Leveraging Standard Biotherapeutic Strategies to Transform New Modalities

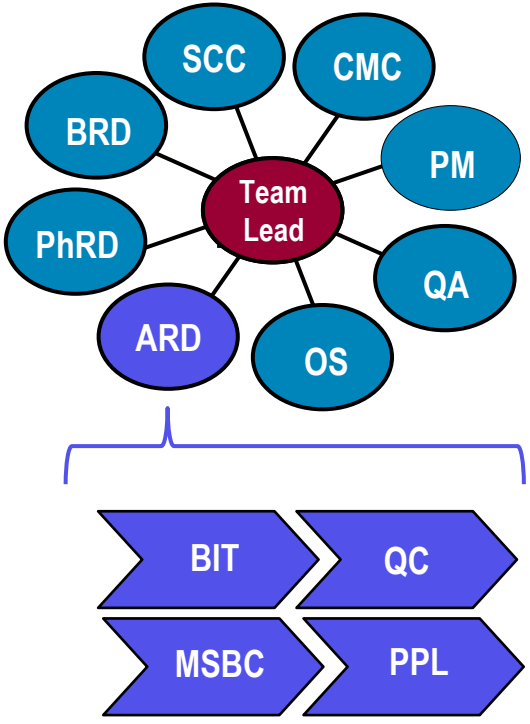


Thomas Powers, Pfizer
Senior Principal Scientist
BioTherapeutics Pharmaceutical Sciences
Analytical Research & Development

CASSS MS Presentation 27Sept2022

Overviewing Pfizer's Extensive Experience in Biotherapeutics



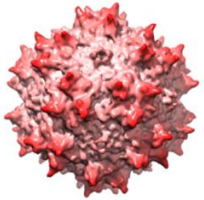


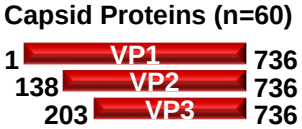
- Pfizer has diverse experience across biotherapeutic modalities
- Experience from traditional modalities has guided the characterization approach for GTx programs

Overview of MS Applications for AAV Programs in Pfizer

- Pfizer's implementation strategy of MS has been adapted from standard biotherapeutics.
- AAV workhorse assays include LC-MS/MS peptide map, MAM (LC-MS), capsid protein analysis (RP-HPLC-MS) and intact capsid analysis (CDMS)
- MS has been a pivotal for AAV programs
  - Product understanding (PQAs/CQAs)
  - Bioprocess and formulation support
  - Routine testing and stability studies
  - Support development of physiochemical methods
  - Support understanding of particle content
  - Aid in product investigations

Overview of the AAV Capsid





Capsid Information

~ 20 nm in size
~ 3.5 MDa in mass
Estimated 1:1:10 capsid protein ratio

Genome Information

~ 4.7 kb
~ 1.5 MDa in mass

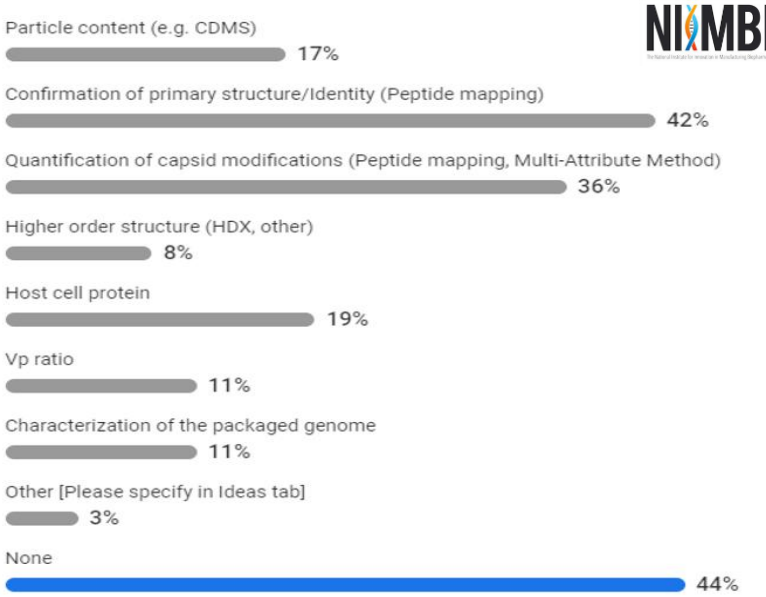
Application of MS for AAV GTx Programs

Overview of Mass Spec Utilization Rates in Traditional BTx BLAs (2017)

MS attribute	% of MS BLAs	MS attribute (< 10%)
Amino acid sequence analysis	97.5	Sequence variants (amino acid substitutions)
Molecular mass	92.4	Covalent dimers
Disulfide bonds	77.2	Methionine/cysteine formylation
Glycosylation	70.9	Phosphorylation
Sequence variants (C-term)	64.6	Truncation
Sequence variants (N-term)	64.6	Acetylation
Deamidation	58.2	Aggregation
Oxidation	57.0	Folding/HOS
Size variants	27.8	Host cell proteins (HCPs)
Free thiols	25.3	Partial reduction
Glycation	22.8	PEGylation
Charge variants	19.0	Translucent particles
Other impurities	17.7	Zinc
Proteolysis/fragmentation	13.9	Glutathionylation
Succinimidation	12.7	Methylation
Isomerization	10.1	Norleucine incorporation
Other	10.1	Phosphogluconylation

Rogstad, Sarah et al. "A Retrospective Evaluation of the Use of Mass Spectrometry in FDA Biologics License Applications" *Journal of the American Society for Mass Spectrometry* vol. 28,5 (2017)

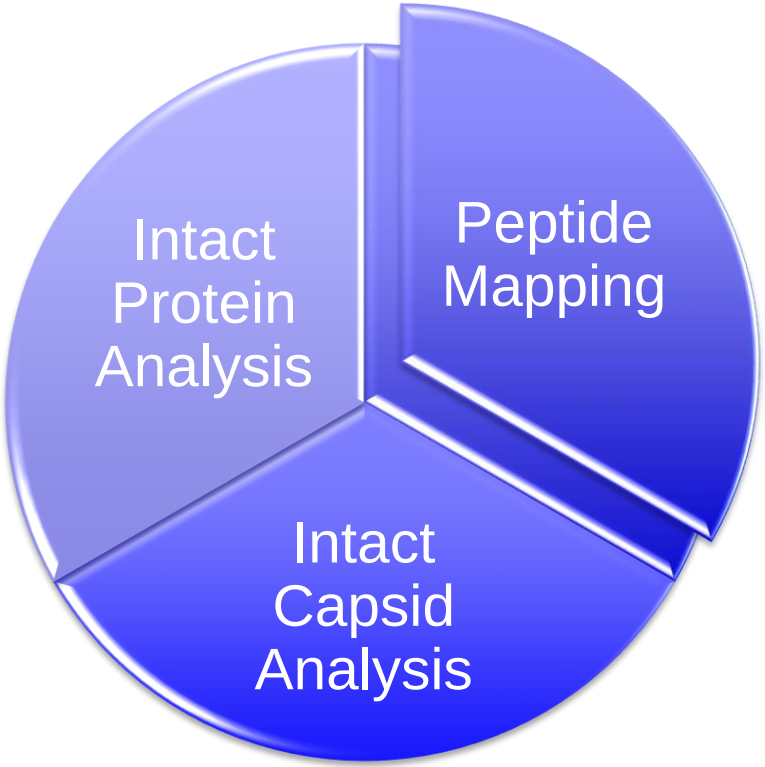
Overview of Mass Spec Utilization Rates in AAV Gene Therapy (2022)

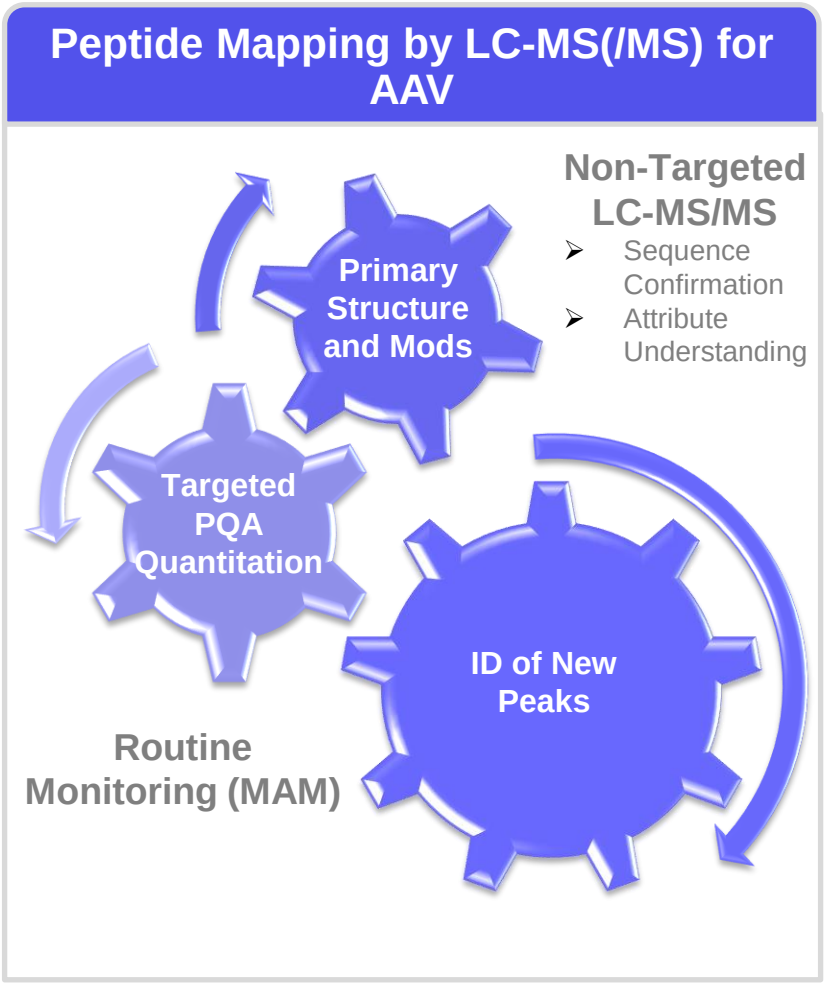


NIIMBL AAV Analytics Workshop hosted in April of 2022

- MS utilization rates for AAV programs are lower compared to traditional biotherapeutics

MS Applications for AAV:





Mass Spec Enables Superior Attribute Understanding:

Linking Capsid Modifications to Potency Data

Accelerated Temperature → **0W** → **1W** → **2W** → **4W** → **8W** → **12W**

Most Sensitive Attributes

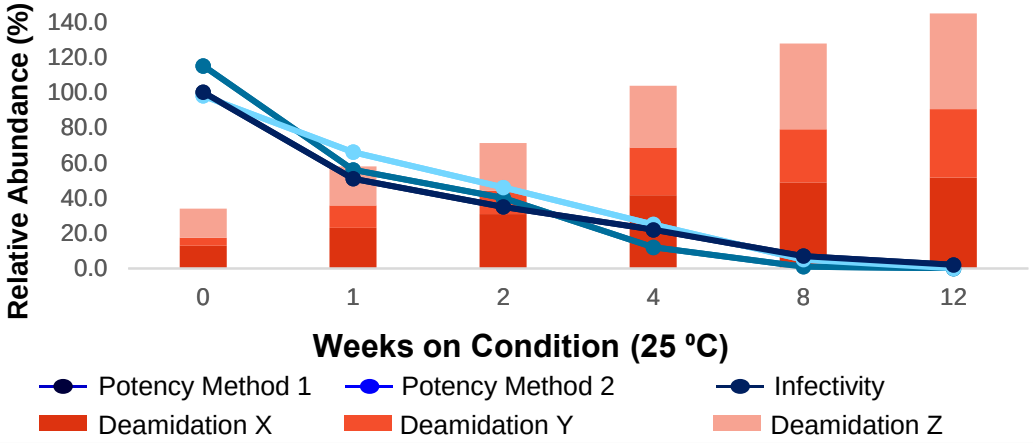
- ✓ Potency (Activity)
- ✓ Infectivity

No Changes Observed

- ➢ Titer (genome, capsid)
- ➢ Aggregation
- ➢ Purity

Investigation needed to understand what is driving potency decrease

Linking Bioassays and Attributes



Capsid modifications are inversely correlated with bioassay results

In-depth characterization coupled to sensitive bioassays identified a possible cause of the potency drop

Mass Spec Enables Superior Attribute Understanding:

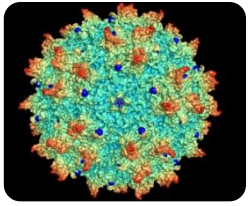
Mutants Confirm Hypothesis from Mass Spec Data

Single Base Mutations Mimic Deamidation

Site X

Site Y

Site Z



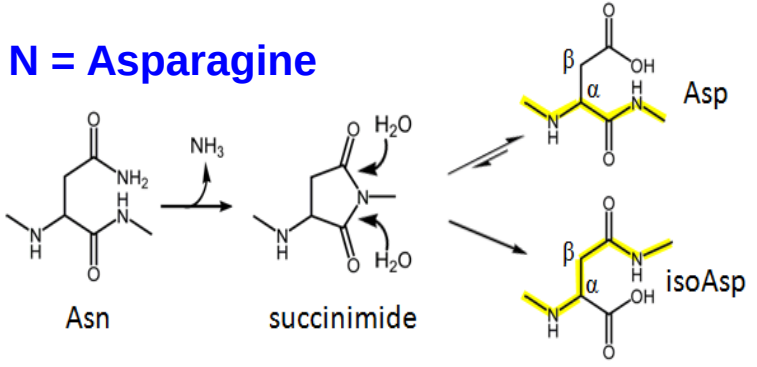
Individual N to D mutations mimic 100% deamidated capsid

Model of Site Z in the context of the AAV capsid

Process of Chemical Deamidation

D = Aspartic Acid

N = Asparagine



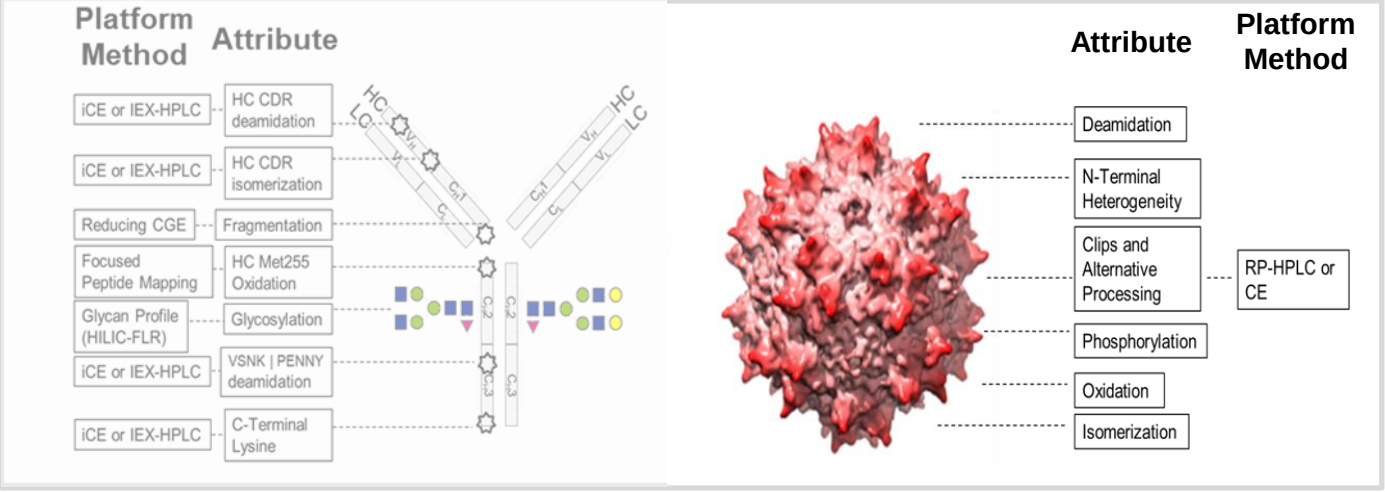
% Relative Potency

N->D Mutants at Specified Sites

Mutations mimicking deamidation impact % relative potency

Multi-Attribute Method Enables Unprecedented Access to Data

Contrasting Platform Methods for AAV vs. Standard BTx



Application of MAM

- **Peptide Map Method – Easy and Reproducible Digest**
- **Targeted Hotspots**
- **Automated Data Processing**

- The Multi-attribute Method (MAM) can simultaneously detect and quantitate product quality attributes.
- MAM can result in efficiencies for method development and analytical testing
- Method can be platformed and impact early product and process development
- Samples supported include: Batch MFG, stability and forced deg, LPQ/PPQ, comparability, formulation support, etc.

Implementation of MAM – Current and Future Efforts within Pfizer

Advances Enabling MAM Success (AAV and BTx)

Automated Preparation → Demonstrated Method Performance (Qual/Val)

Generic Report → Data Access – LIMS Compatibility

Data Trending: Spotfire

Implementation of High-Throughput Automation Enables Routine MAM Support

MABS
2021, VOL. 13, NO. 1
https://doi.org/10.1080/19420862.2021.1978131

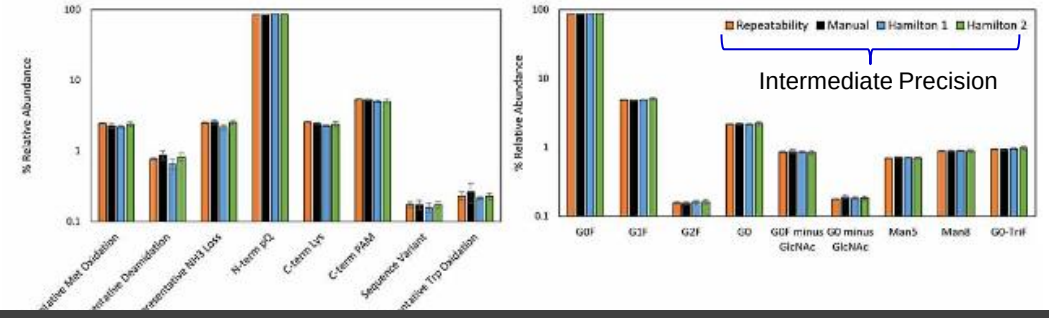
Taylor & Francis
Taylor & Francis Group

Open access

Enhancing the multi-attribute method through an automated and high-throughput sample preparation

Pongkwan Sitasuwan[a,†], Thomas W. Powers[b,†], Tiffany Medwid[b], Yuting Huang[b], Bradley Bare[b], and L. Andrew Lee[a]

[a] Integrated Micro-Chromatography Systems, Inc, Irmo, SC, USA [b] Pfizer, Inc, Chesterfield, MO, USA

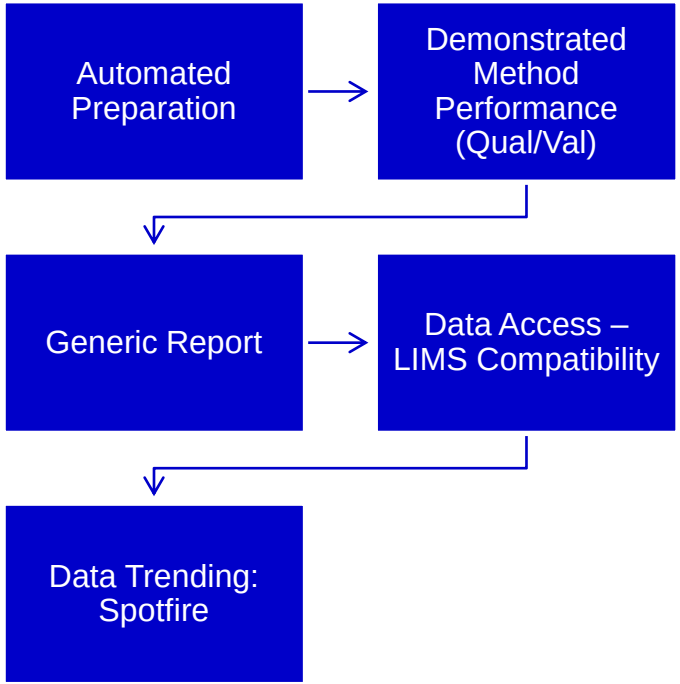


Automation performance mirrors manual sample preparation but at greater efficiency

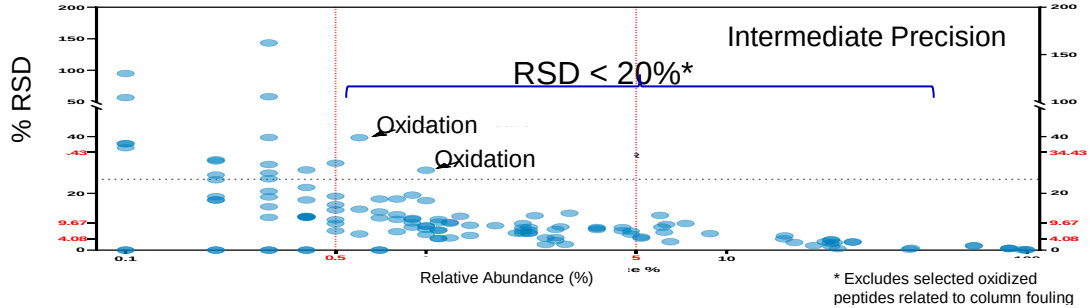
Pfizer

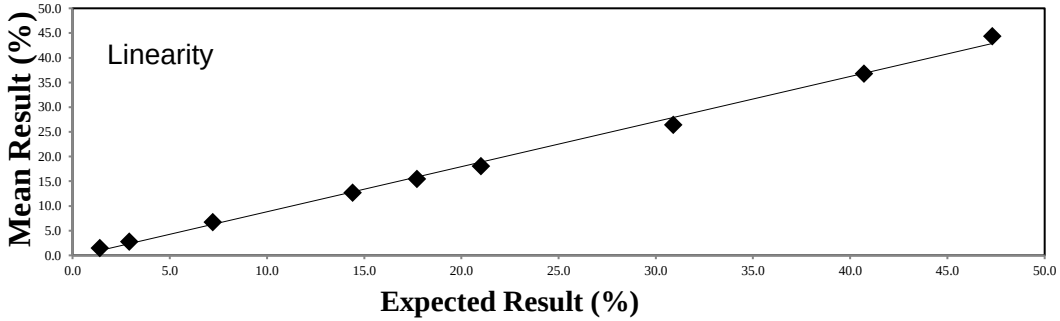
Implementation of MAM – Current and Future Efforts within Pfizer

Advances Enabling MAM Success (AAV and BTx)



Evaluating MAM Method Performance

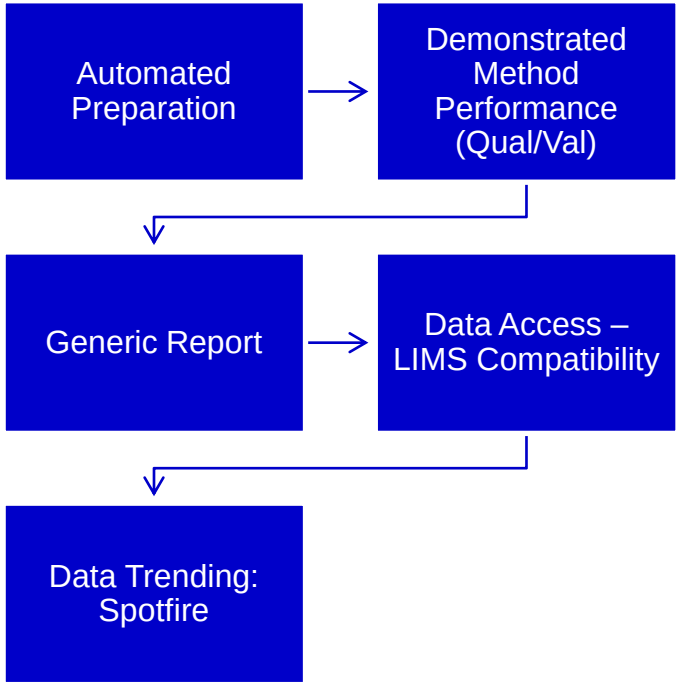




- Method evaluated for specificity, system precision, linearity/range, LOQ, sample stability, accuracy, and robustness

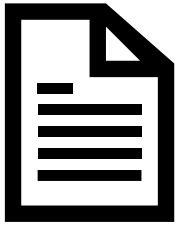
Implementation of MAM – Current and Future Efforts within Pfizer

Advances Enabling MAM Success (AAV and BTx)

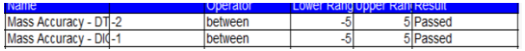


Enhancing MAM Quality and Access

Universal Report – No changes needed across methods!

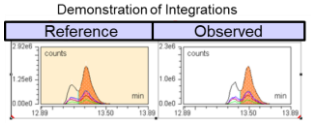


Criteria (SST, AC) Specified in Processing Method



Name		Operator	Lower Rang	Upper Rang	Result
Mass Accuracy - DT	-2	between	-5	5	Passed
Mass Accuracy - DI	-1	between	-5	5	Passed

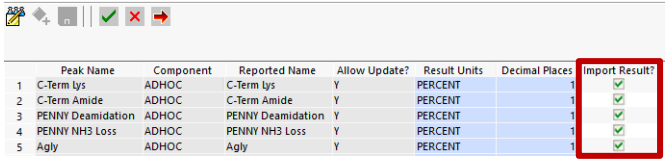
Automatic Integration Visualization



Assessment of Composite Scoring Criteria

Automatic Export

Exported Data compatible with LIMS

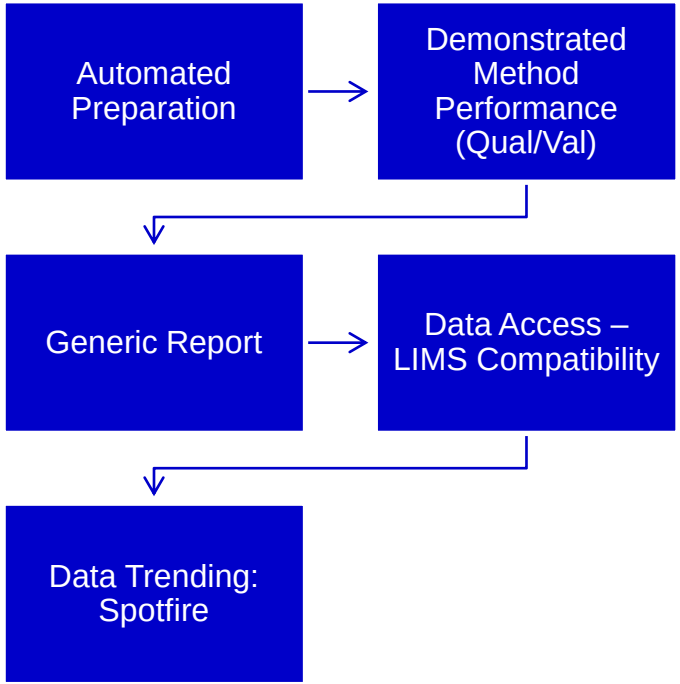


	Peak Name	Component	Reported Name	Allow Update?	Result Units	Decimal Places	Import Result?
1	C-Term Lys	ADHOC	C-Term Lys	Y	PERCENT	1	☑
2	C-Term Amide	ADHOC	C-Term Amide	Y	PERCENT	1	☑
3	PENNY Deamidation	ADHOC	PENNY Deamidation	Y	PERCENT	1	☑
4	PENNY NH3 Loss	ADHOC	PENNY NH3 Loss	Y	PERCENT	1	☑
5	Agly	ADHOC	Agly	Y	PERCENT	1	☑

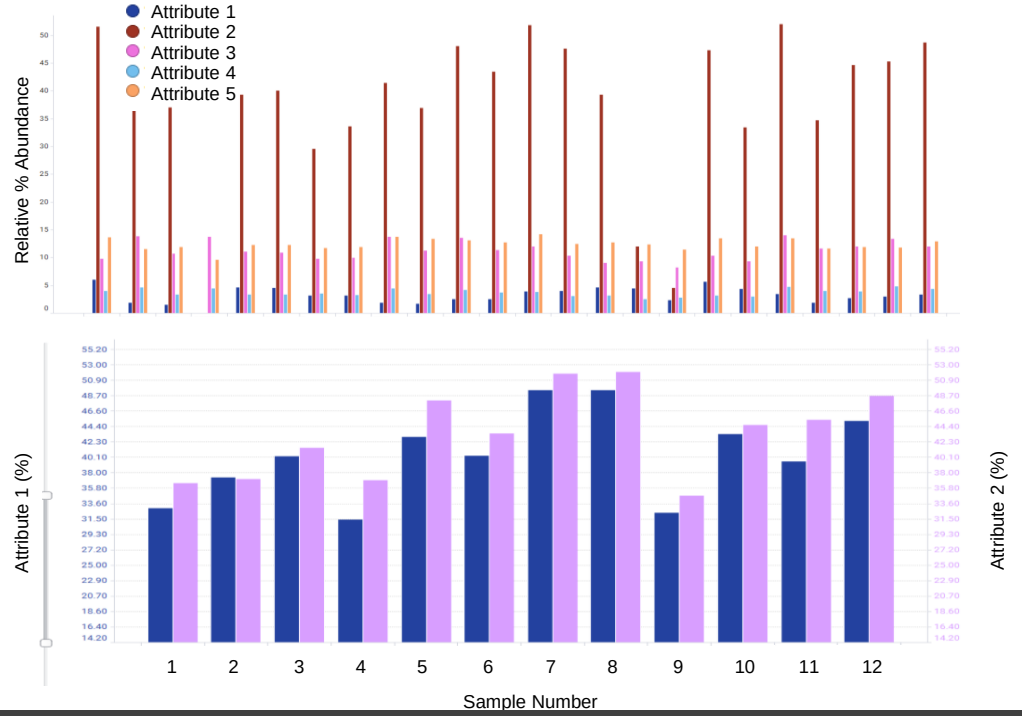
- Enhancing MAM Implementation and Data Documentation Practices are Key Enablers to the Technology

Implementation of MAM – Current and Future Efforts within Pfizer

Advances Enabling MAM Success (AAV and BTx)

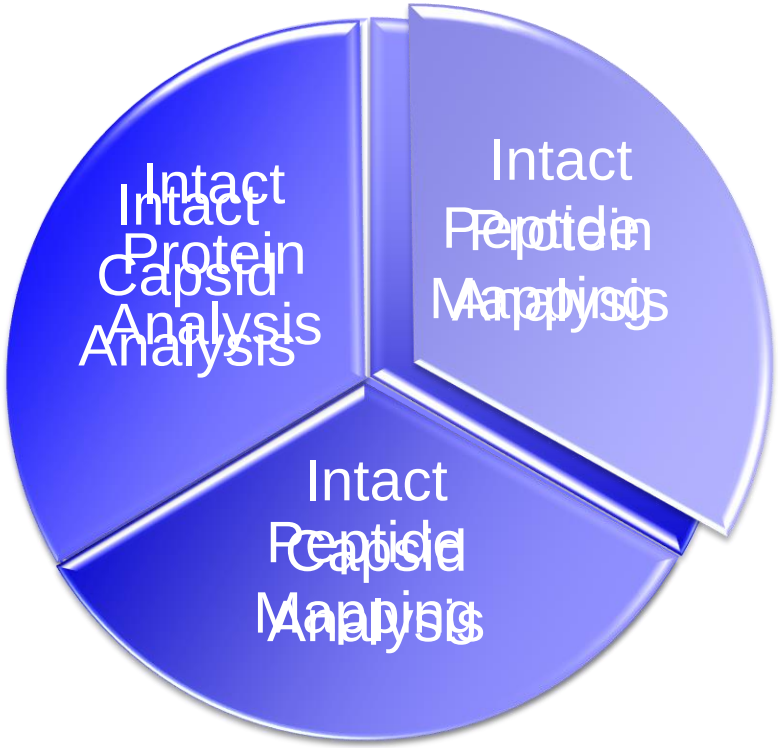


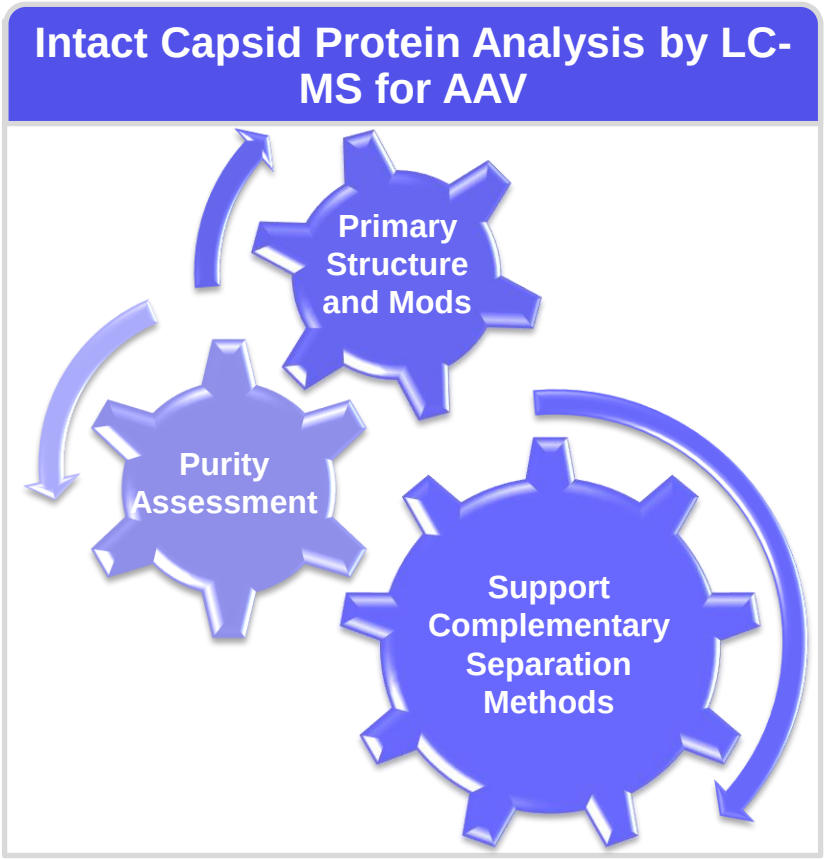
Enhancing MAM Quality and Access



- Spotfire Improves Access and Trending of Data

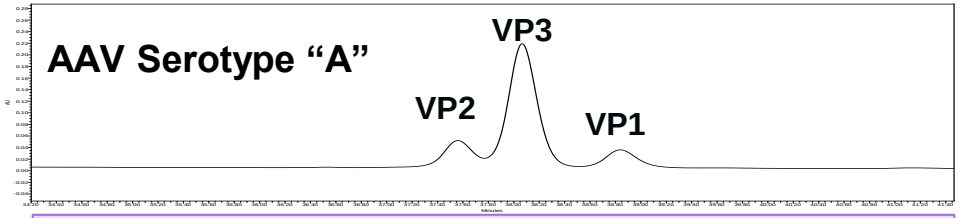
MS Applications for AAV:



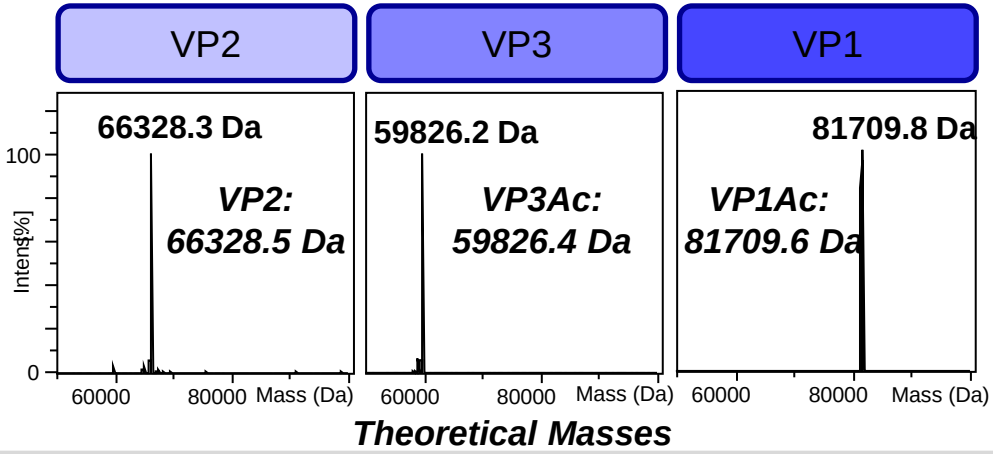


Capsid Protein Analysis Supports Product Quality Understanding

Characterizing AAV Capsid Proteins by LC-MS

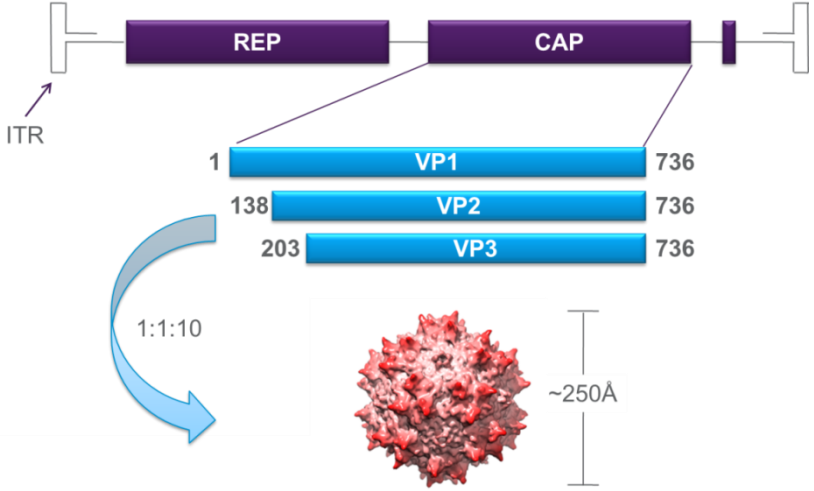


Deconvoluted Mass Spectra



Theoretical Masses

Understanding Capsid Heterogeneity



- Approach can support VP ratio
- Multiple modifications can be identified: N-term heterogeneity, phosphorylation, oxidation, deamidation, clips and alternative processing sites, etc.

Mass Spec Supports Development of Quality Analytics:

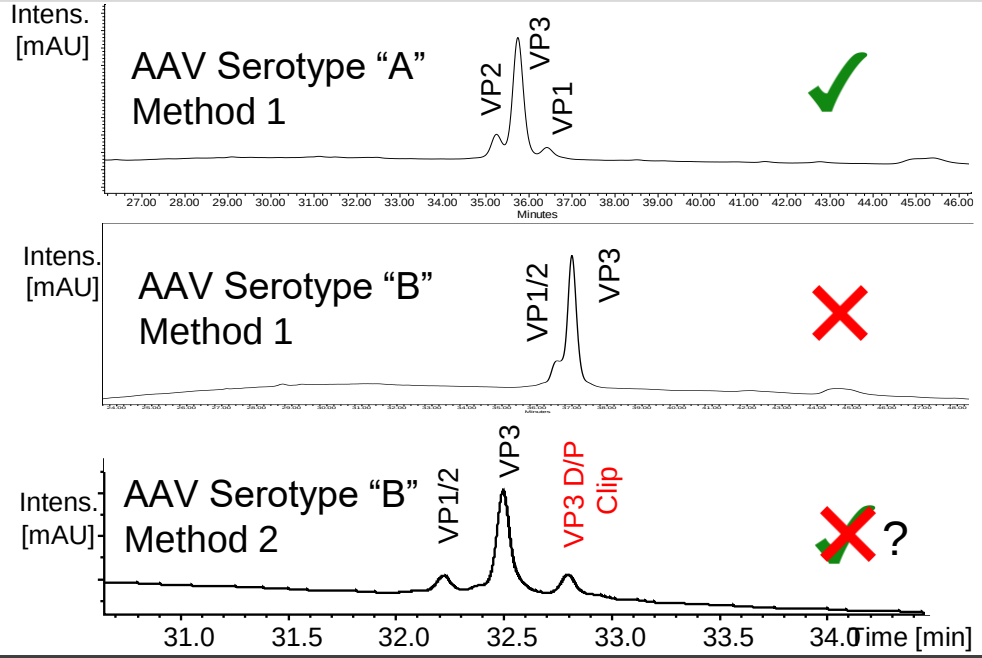
Advances Enabling MAM Success (AAV and BTx)

Supporting Method Development → ID of Unknowns

↓

Next Generation Methods

Understanding Capsid Heterogeneity



- Incorporating MS into method development ensures that peaks are understood before optimizing methods around them

Mass Spec Supports Identification of Unknowns

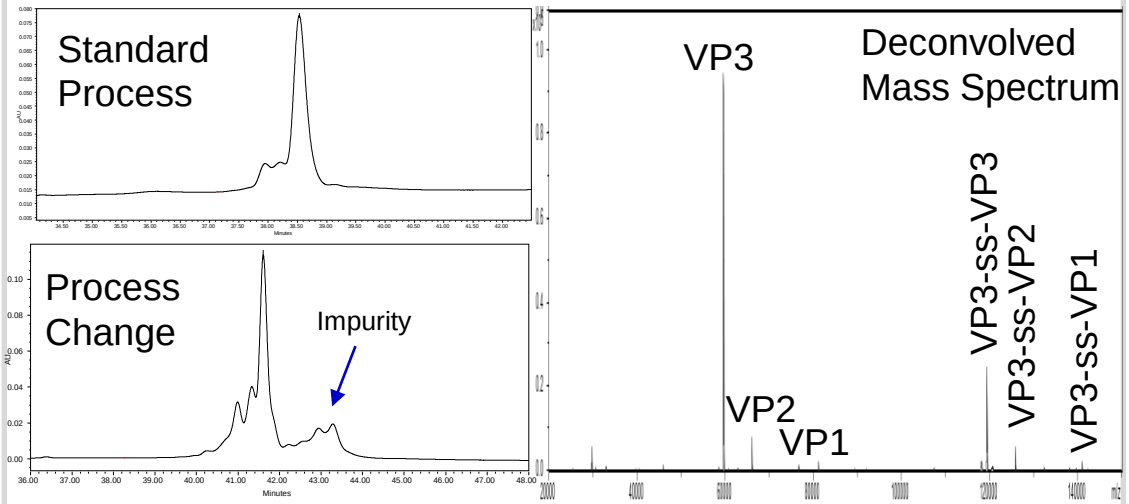
Advances Enabling MAM Success (AAV and BTx)

Supporting Method Development → ID of Unknowns

↓

Next Generation Methods

Understanding Capsid Heterogeneity



- As unknown impurities appear, having a method that can directly couple to the MS is valuable
- RP-HPLC/MS has also been used to support impurities that were observed by CGE purity assessments

Mass Spec Supports Next Generation Separation Methods

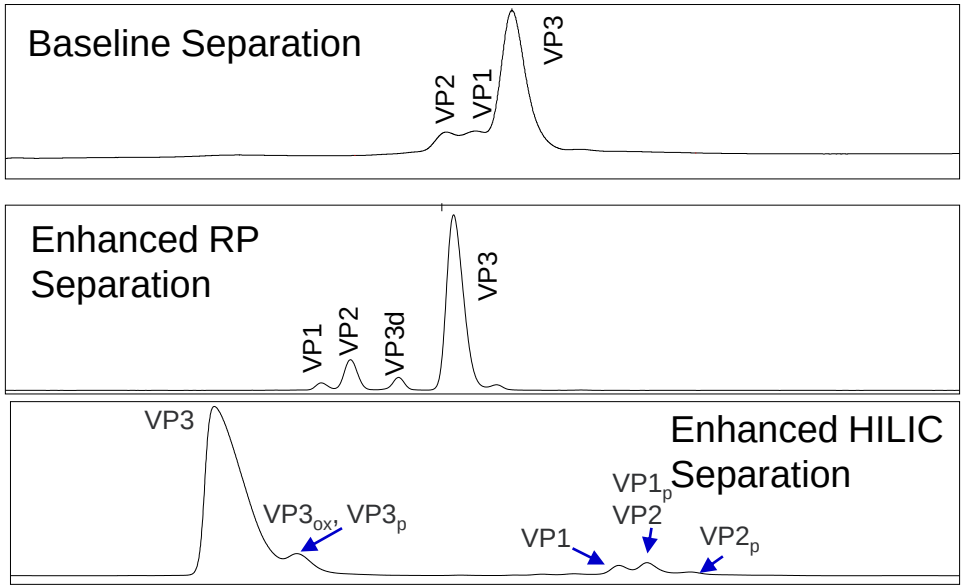
Advances Enabling MAM Success (AAV and BTx)

Supporting Method Development → ID of Unknowns

↓

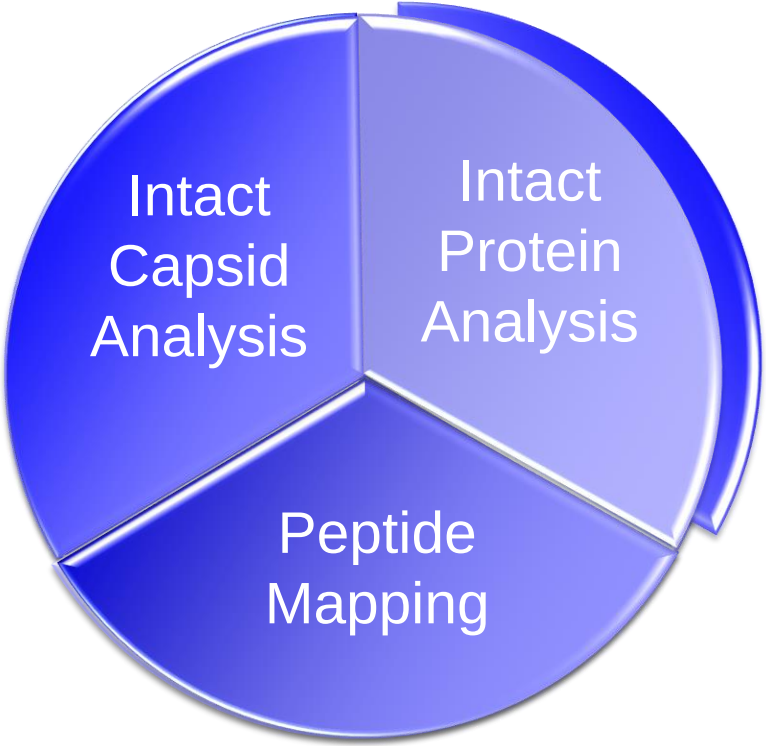
Next Generation Methods

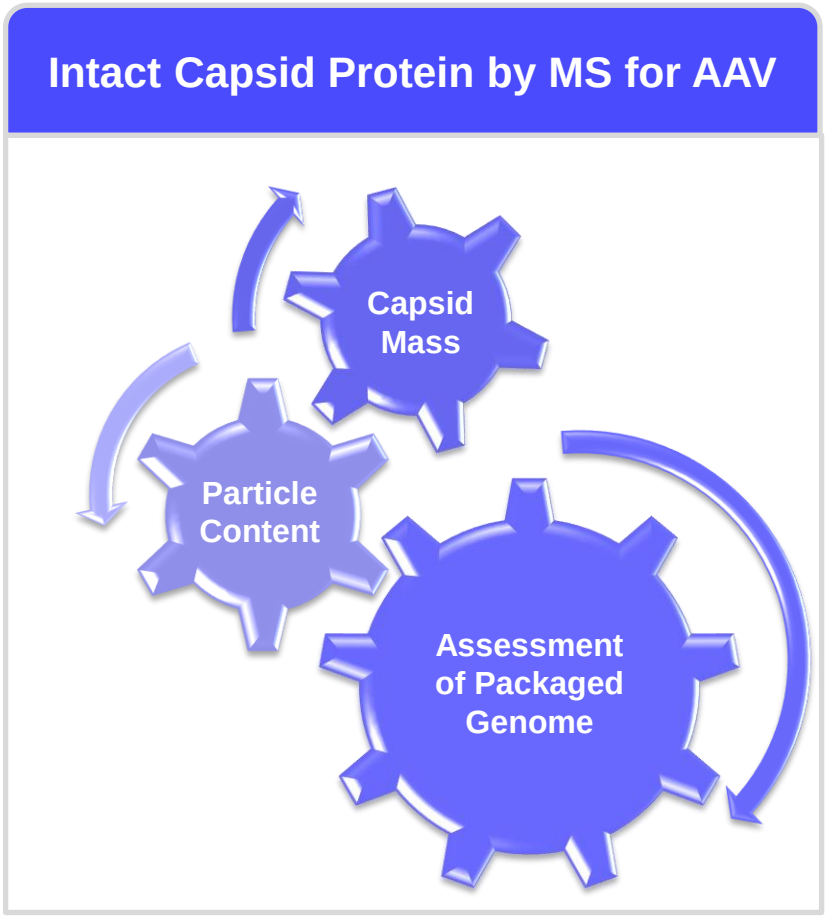
Understanding Capsid Heterogeneity



- More selective HPLC methods are being evaluated that have utility in separating PQAs. Coupling MS to the separation enables understanding to support method development

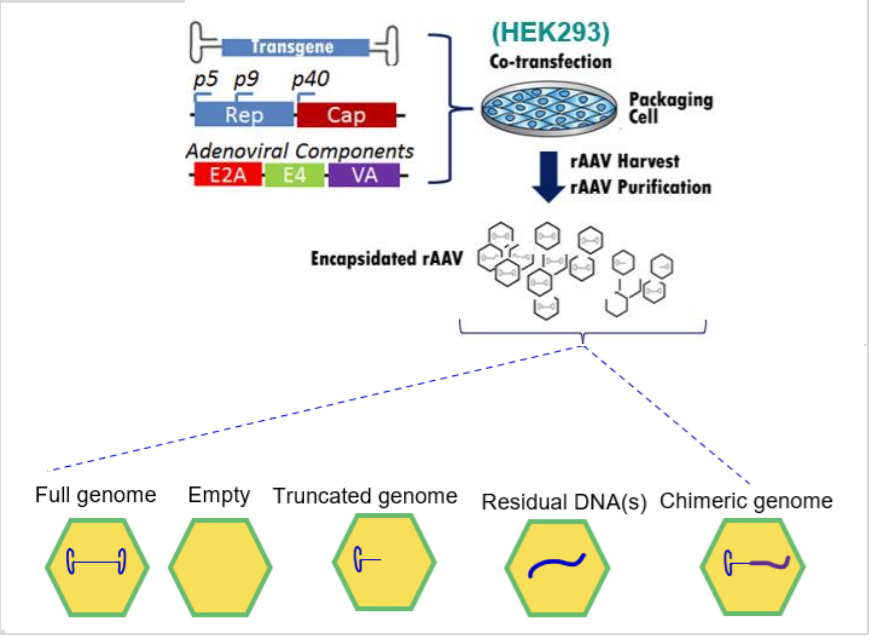
MS Applications for AAV:



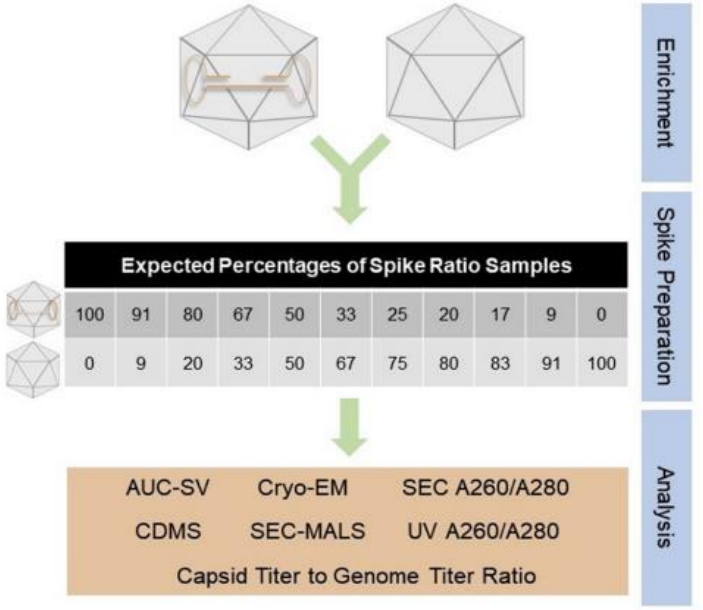


Overview of “Particle Content” in the AAV GTx Space:

Possible Impurities in AAV Materials



Pfizer Survey of Particle Content Methods



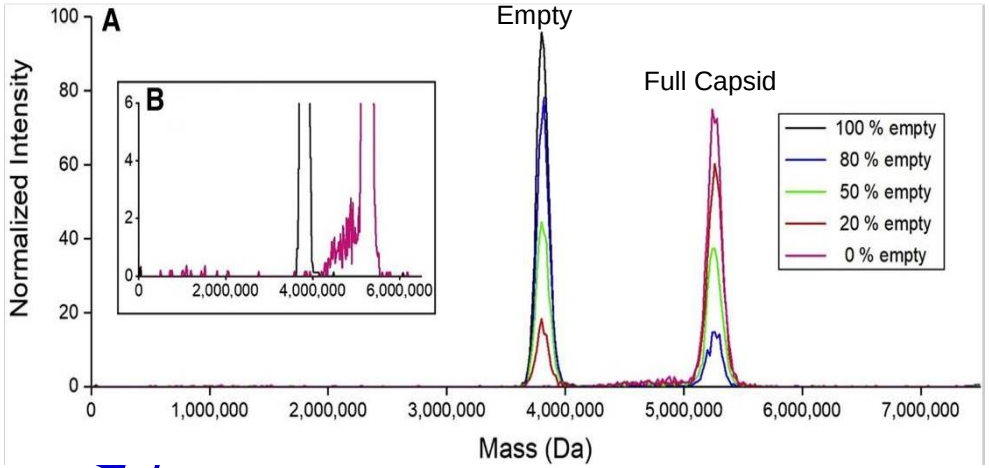
- Cellular, Tissue and Gene Therapies Advisory Committee (CTGTAC – Sept 2021) highlighted enhanced scrutiny on AAV impurities and a concern around the level of empty/partially-filled capsids
- Committee recommended additional characterization techniques for AAV empty/full capsids

CDMS has Promise for Assessing Particle Content

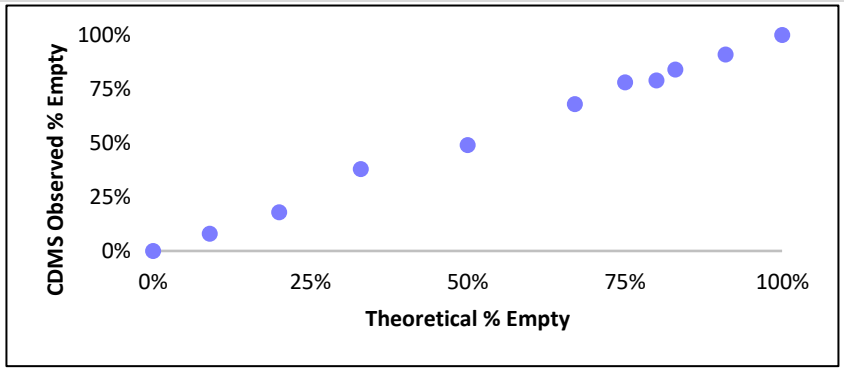
Comparison of analytical techniques to quantitate the capsid content of adeno-associated viral vectors

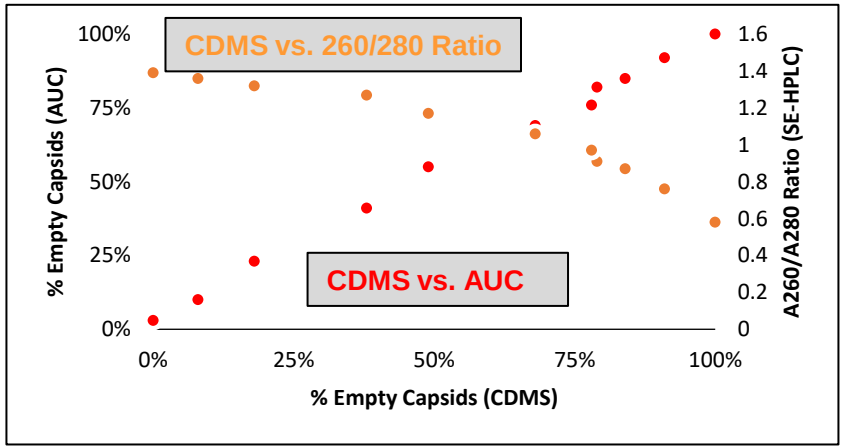
Amanda K. Werle,[1] Thomas W. Powers,[1] James F. Zobel,[1] Caitlin N. Wappelhorst,[1] Martin F. Jarrold,[2] Nicholas A. Lyktey,[2,3] Courtney D.K. Sloan,[1] Andrew J. Wolf,[1] Sharee Adams-Hall,[1] Phoebe Baldus,[1] and Herbert A. Runnels[1]

[1]Pfizer Inc., Analytical Research and Development, 875 Chesterfield Pkwy. West, Chesterfield, MO 63017, USA; [2]Indiana University, Chemistry Department, Bloomington, IN 47405, USA; [3]Gravity Diagnostics, Covington, KY 41011, USA



Evaluating CDMS Performance

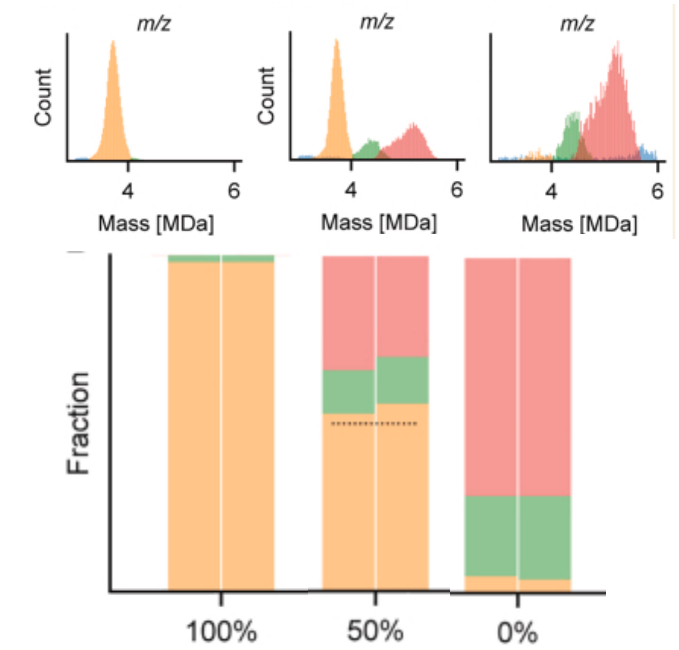




*Work in conjunction with the Jarrold Lab at Indiana University

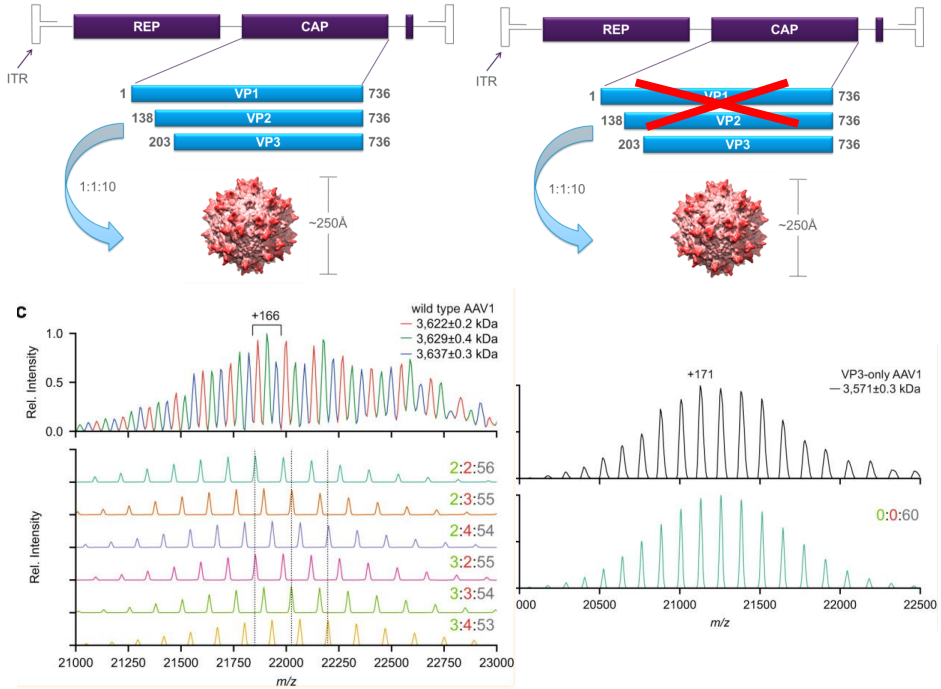
MS Reveals Additional Information on Capsid Composition

Particle Content Evaluation on Alternate System



Wörner TP, Snijder J, Friese O, Powers T, Heck AJR. Assessment of genome packaging in AAVs using Orbitrap-based charge-detection mass spectrometry. Mol Ther Methods Clin Dev. 2021 Nov 29;24:40-47.

UHMR-MS Documents Unique Capsid Heterogeneity

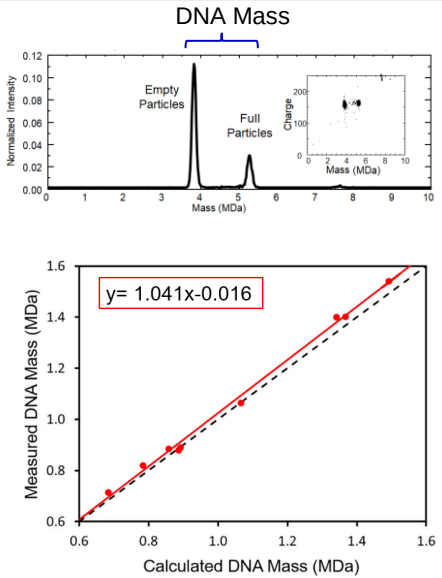


Wörner, T. P., Bennett, A., Habka, S., Snijder, J., Friese, O., Powers, T., Agbandje-McKenna, M., & Heck, A. (2021). Adeno-associated virus capsid assembly is divergent and stochastic. Nature communications, 12(1), 1642.

*Work in conjunction with the Heck Lab at Utrecht University

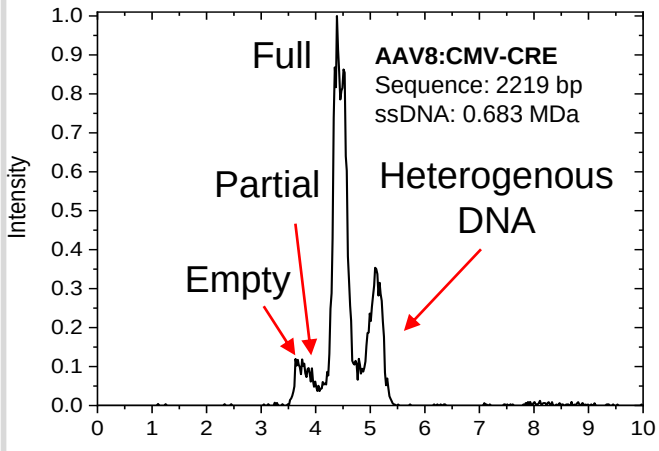
MS Can Support Characterization of the Packaged Genome

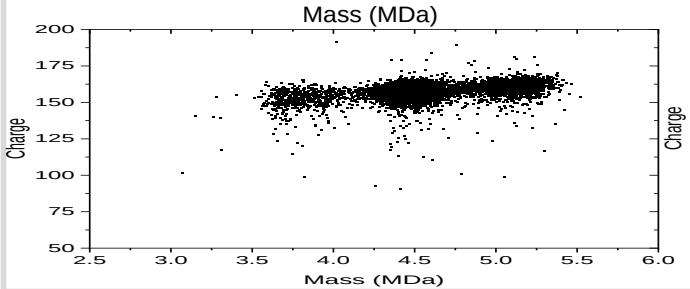
Assessment of AAV Capsids with Various Genome Sizes

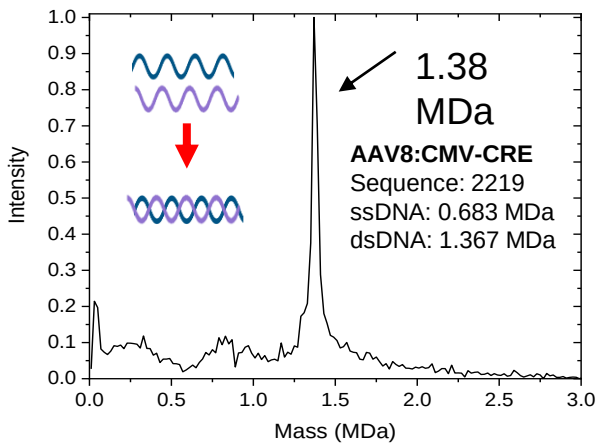


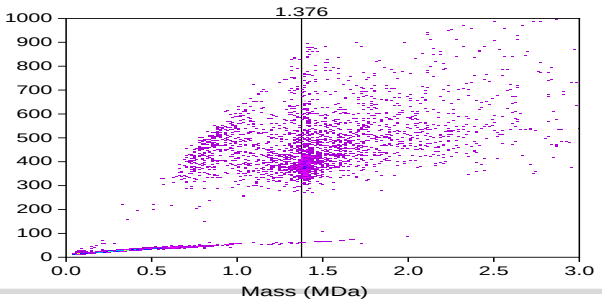
Barnes, L. F., Draper, B. E., Chen, Y. T., Powers, T. W., & Jarrold, M. F. (2021). Quantitative analysis of genome packaging in recombinant AAV vectors by charge detection mass spectrometry. *Molecular therapy. Methods & clinical development*, *23*, 87–97.

CDMS Supports Characterization of the Packaged Genome: Assessing the Assembled Capsid and Extracted Genome









*Work in conjunction with the Jarrold Lab at Indiana University

Conclusions

Mass spec remains a unique and key assay for AAV products. Novel applications continue to offer significant potential for GTx development.

- At Pfizer, mass spectrometry is an important tool in AAV analytical toolbox, where utilization ranges from characterization testing to impurity identification to routine testing support.
- Mass spectrometry has enabled superior product understanding by identifying stability indicating attributes, degradation pathways, and unique capsid and protein heterogeneity
- Mass spectrometry has aided in the development of novel, non-MS separation methods, including RP-HPLC and HILIC-HPLC
- Mass spectrometry can provide confirmation of results from orthogonal and complementary methods, including AUC for particle content

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Abstract

Mass spectrometry is a crucial component of the analytical toolbox used to support traditional biotherapeutics. While utilization rates of mass spectrometry for gene therapy programs may not match the utilization rates for traditional biotherapeutics, the technology offers unique insights into AAV gene therapy programs. The current presentation aims to outline several orthogonal mass spectrometry-based approaches that can be used to look wholistically at the AAV product, from bottom-up analysis to the assessment of intact AAV capsids. By leveraging the array of mass spectrometry-based methods for AAV characterization, mass spectrometry can be used to support a wide range of analytical studies. To date, mass spectrometry has been successfully implemented to better understand critical quality attributes (CQAs), support investigations, support routine manufacturing testing, aid in method development, and provide supportive data to complementary technologies.

While the application of mass spectrometry to AAV is still relatively new, the characterization of AAV products has been enabled by cutting edge movements in the field of mass spectrometry, including innovations in the multi-attribute method (MAM), charge detection mass spectrometry (CDMS), and ultra-high mass range (UHMR) systems. Importantly mass spectrometry has been useful in identifying new quality attributes and support methods in place that monitor already established CQAs. The current presentation details cutting edge research that has been implemented to AAV gene therapy programs.