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Analysis of Adeno-Associated Virus Quality Attributes with LC-FLD-(MS)

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Introduction

Adeno-associated viral vectors (AAVs) are promising delivery vehicles for the next generation of emerging gene therapies. AAVs consist of sixty copies of capsid protein assembled around a single-stranded DNA genome[1], and can be > 4MDa in molecular weight. Due to their size, complexity, and relatively low titers, AAV critical quality attributes (CQAs) can be challenging to measure accurately.

In this poster we demonstrate optimized workflows to address three important CQAs: (i) Aggregation, (ii) Full / Empty capsid ratio, and (iii) Capsid protein identity and relative abundance. Highly sensitive detection and quantitation was achieved by intrinsic fluorescence detection as well as high-resolution mass spectrometry where appropriate.

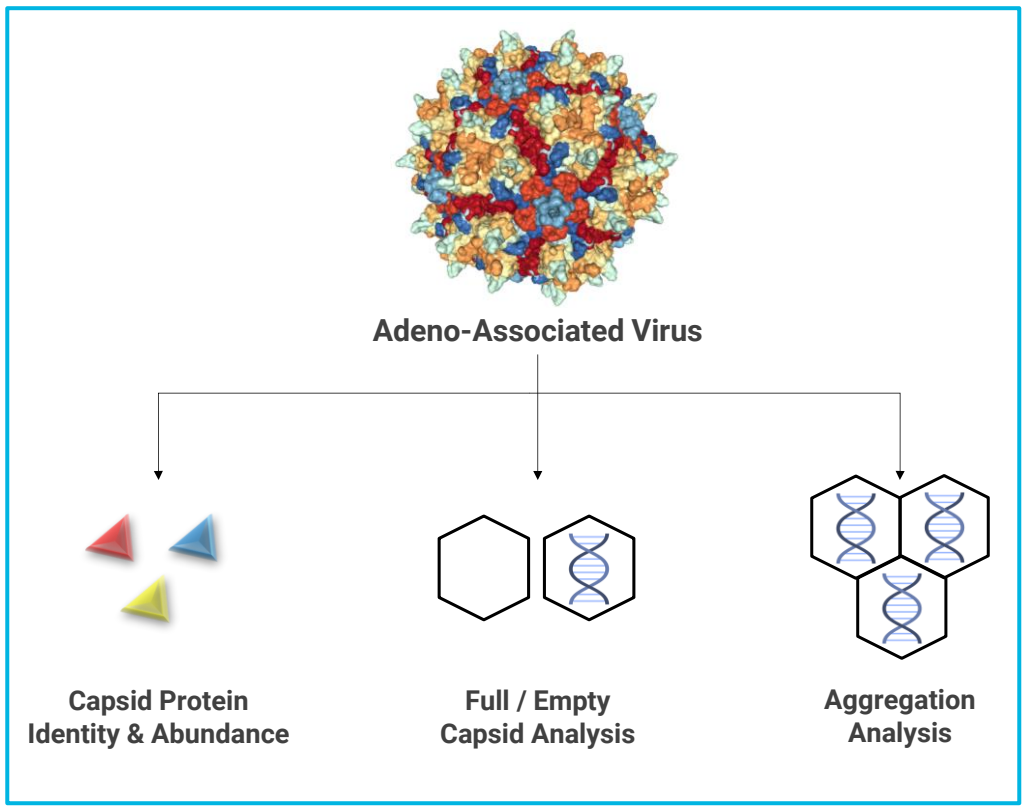

Figure 1. Critical quality attributes of AAV vectors.

Experimental

AAV serotypes 1, 2, 6, 7, 9, 7m8, DJ and rh10 were purchased from the Vector Core @ GIS (A*STAR, Singapore) or Vigene Biosciences. In all cases, capsid protein intrinsic fluorescence was detected using an Agilent 1260 Infinity II Fluorescence Detector set to Ex = 280nm, Em = 340nm.

Aggregation Analysis

AAV serotypes 2 and 9 were separated on an Agilent Bio SEC-5 column (4.6x300mm, 5µm, 1000Å) using a mobile phase consisting of 50mM phosphate + 400mM NaCl, pH 7.4, at a flow rate of 0.4ml/min. Stressed samples were generated by dilution into deionized water containing 0.4µg of dsDNA, followed by overnight incubation at 37°C.

Experimental

Full/Empty Ratio Analysis

AAV serotypes 1 and 6 were separated on an Agilent Bio SAX NP5 PK column (2.1x50mm, 5µm) using a mobile phase consisting of 70mM Bis-Tris Propane + 2mM $MgCl_2$. Elution was achieved using a tetramethylammonium chloride gradient ranging from 150 – 275mM over 25 mins at a flow rate of 0.1ml/min. Full- and empty-enriched standards were mixed at different ratios to assess the method's accuracy, linearity and inter-day reproducibility.

Capsid Protein ID and Stoichiometry

Gradient elution was carried out on Agilent Zorbax RRHD 300Å StableBond C3, Diphenyl or C18 columns (2.1x100mm, 1.8µm) using a mobile phase consisting of 80-90% IPA + 0-10% ACN + 9.8% water + 0.1% FA + 0.1% TFA at a temperature of 75-80°C. AAV samples were heat-denatured at 70°C in 3M GdHCl + 10mM DTT for 15 mins, then directly injected without further sample preparation.

Figure 2. Agilent 6545XT AdvanceBio LC/Q-TOF

Results and Discussion

Aggregation Analysis

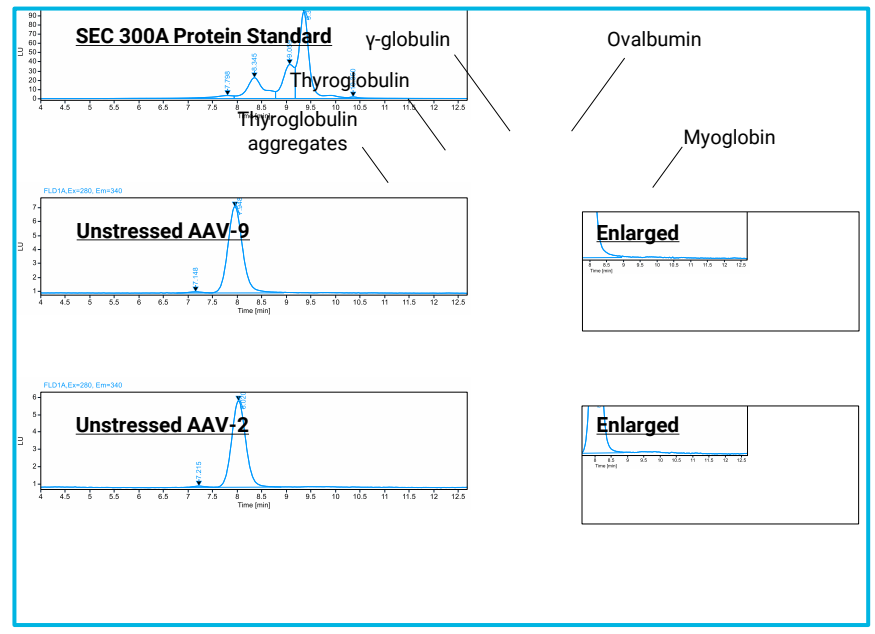

Figure 3. Unstressed AAV-2 and 9 aggregation analysis

Results and Discussion

Based on the hydrodynamic radius of monomeric Thryroglobulin (85.8Å), AAV monomers were expected to elute between monomeric and aggregated Thryroglobulin (Figure 3). Unstressed AAV-2 and 9 samples were of high purity, each containing 1.2 – 1.3% aggregates.

In contrast, stressed AAV-9 showed extensive both aggregation (7.5%) and fragmentation (8.8%). The total peak area of stressed samples was only ~60% that of unstressed samples, possibly indicating the formation of insoluble aggregates.

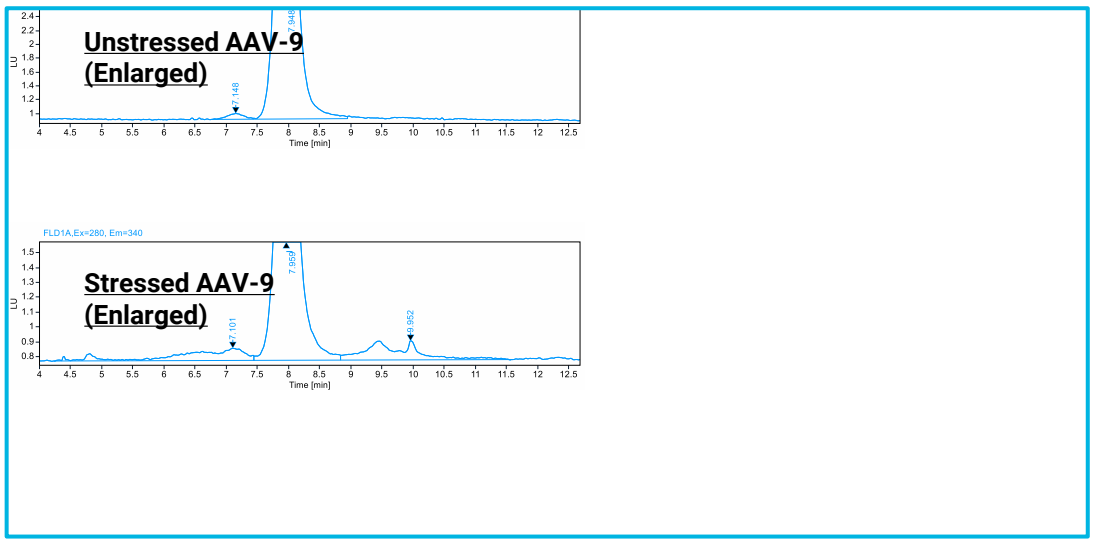

Figure 4. Stressed AAV-9 aggregates and fragments

Full/Empty Ratio Analysis

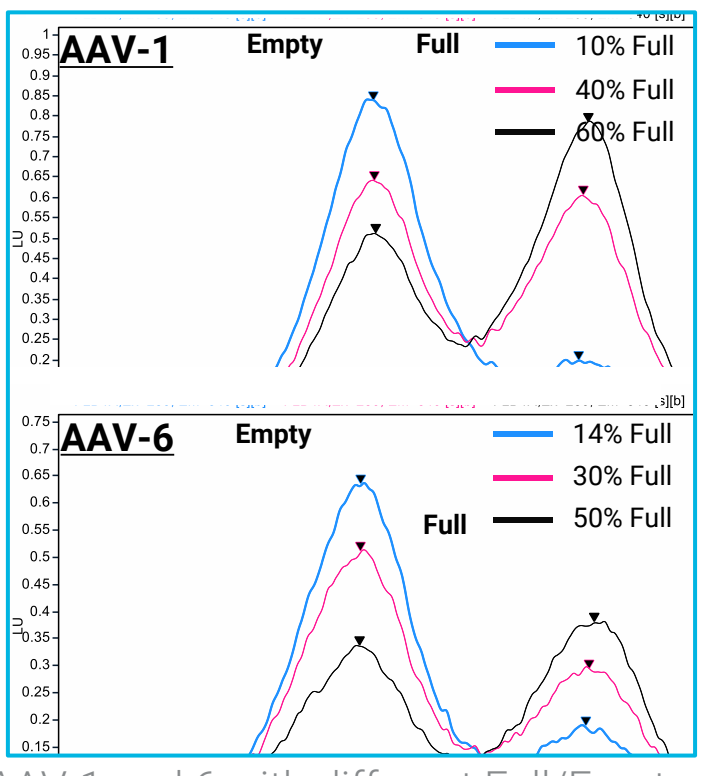

Figure 5. AAV-1 and 6 with different Full/Empty ratios

After method development on a 1290 Infinity II Bio LC equipped with quaternary Flexible Pump, the method was transferred to a binary High-Speed Pump to separate admixtures of AAV-1 and AAV-6 full and empty capsids at different ratios (Figure 5). Sample recovery was found to be negatively correlated with flow rate, hence a low flow rate of 0.1ml/min was maintained throughout[2].

As the Full and Empty peaks were not baseline separated, we found relative peak height to be superior to relative peak area for estimating Full/Empty capsid ratio.

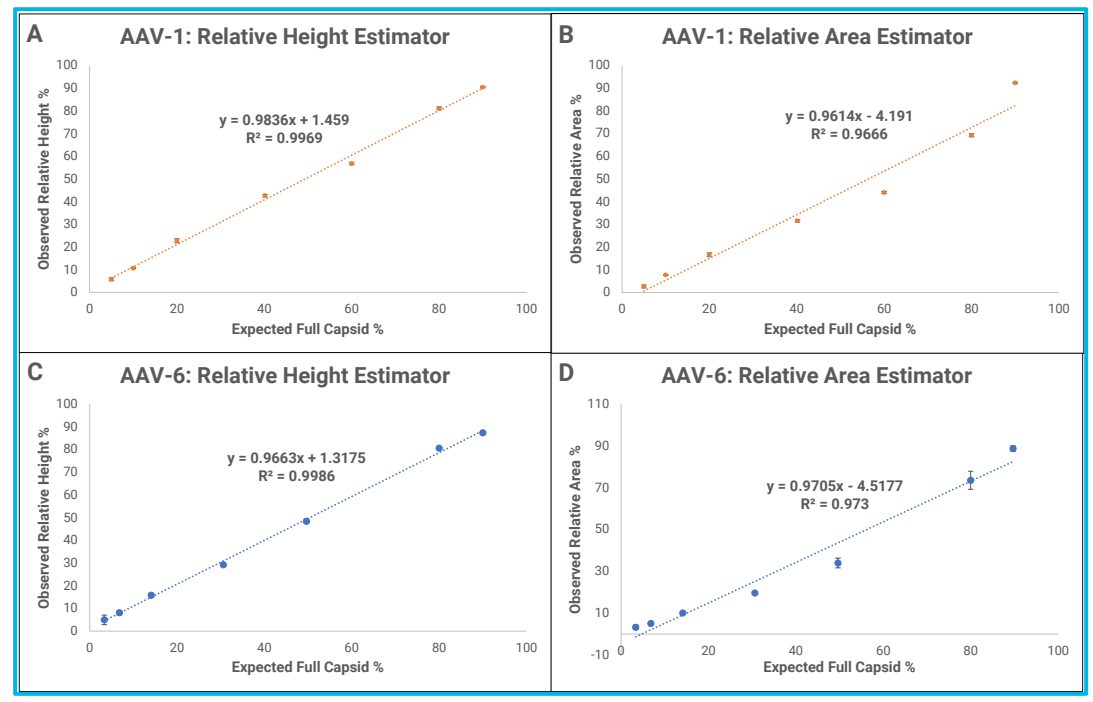

Figure 6. Relative peak height vs. area estimators

The relative standard deviation (RSD) of Full/Empty ratios were ≤10% for AAV-1 and ≤11.2% for AAV-6 over the range of 5 – 90% full capsids (Figure 7). This confirms the method's broad linear range and places the lower limit of quantitation for both serotypes at ≤5% full capsids if we assume an acceptable threshold RSD of 15%. Retention times for both full and empty capsids were also highly reproducible with RSD ≤2.4%.

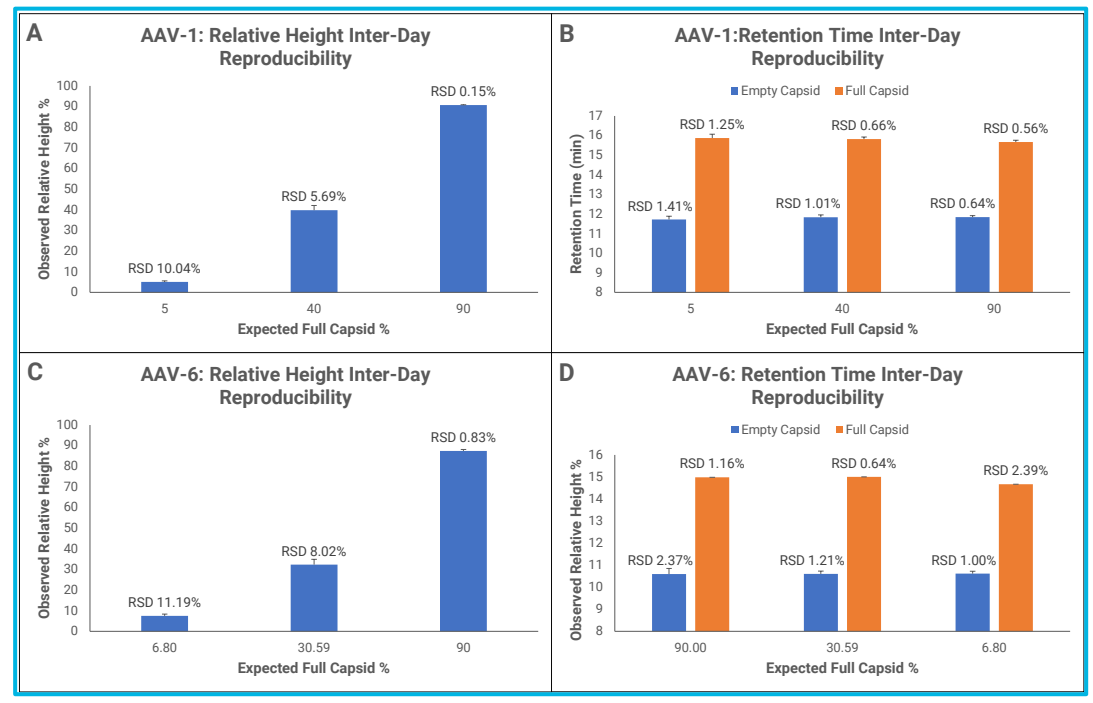

Figure 7. Inter-day reproducibility of Full/Empty ratios and retention times

Capsid Protein ID and Stoichiometry

AAV capsids contain three highly homologous proteins which differ only in their N-termini. This makes separation of the capsid proteins challenging, and indicates that measures to improve separation efficiency might be appropriate e.g. high temperature, strong eluents, and acidic modifiers to improve peak shapes.

For method development, denatured AAV-2 samples were separated on StableBond C3, Diphenyl and C18 columns, which have improved temperature and pH stability.

Results and Discussion

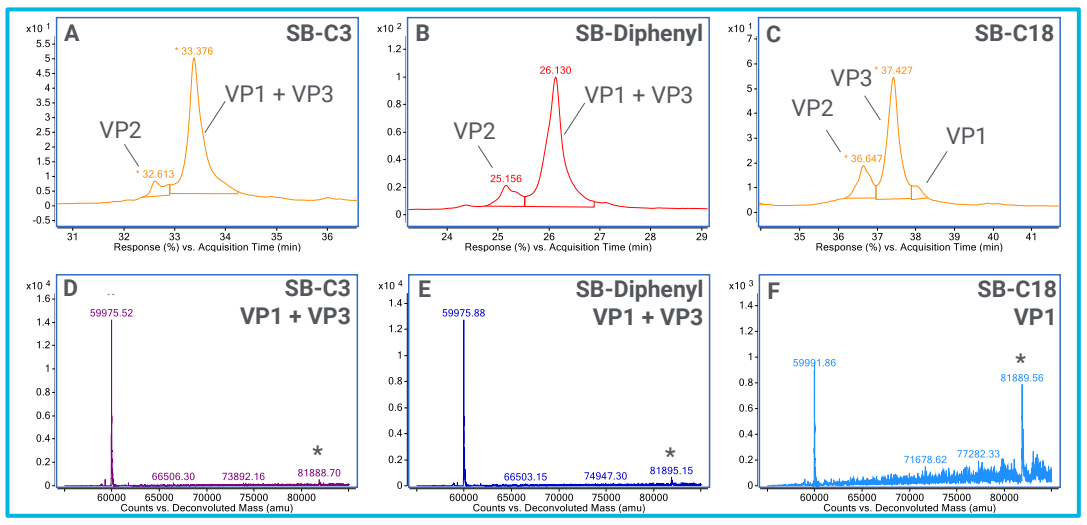

Figure 8. Separation of capsid proteins VP1 – 3 on C3, Diphenyl and C18 columns

Only C18 had the specificity needed to separate AAV-2 VP1 – 3. Figure 8D – F show the deconvoluted mass spectra of VP1 from each column, illustrating how the far more abundant VP3 protein (59974.70 Da) may interfere with and suppress the VP1 signal (asterisks), compromising mass accuracy and quantitation.

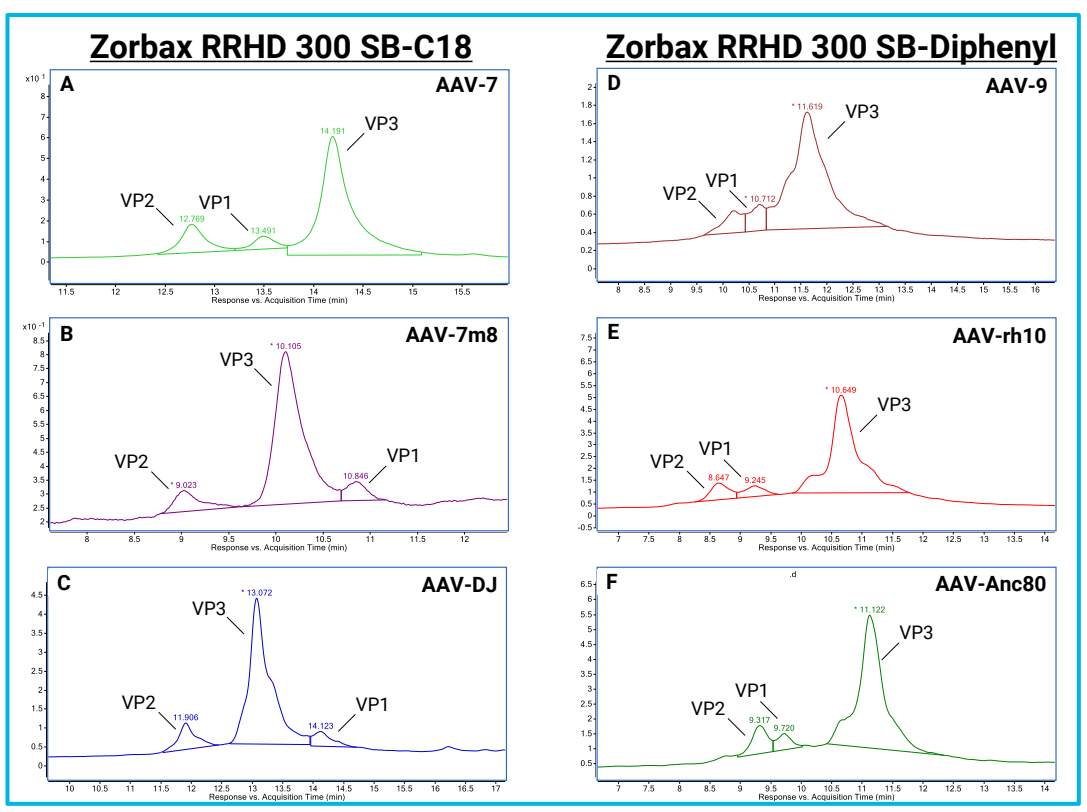

Figure 9. Separation of different AAV serotypes

Whilst AAV-7, 7m8 and DJ were also observed to separate well on C18 columns, other serotypes AAV-9, rh10 and Anc80 resolved only on Diphenyl columns.

Serotype	VP1	VP2	VP3
2	10.67	10.61	78.72
7	7.16	9.81	83.03
9	8.75	8.04	83.21
7m8	7.62	9.70	82.68
DJ	8.62	11.72	79.66
rh10	6.58	9.18	84.24
Anc80	5.64	10.62	83.74

Table 1. Capsid protein quantification (FLD%)

The relative quantities of each capsid protein (Table 1) as determined by fluorescence peak area generally conform to the expected VP1:2:3 stoichiometry[1] of 1:1:10. The abundance of VP1 appeared to be more variable than VP2 or 3 across the different samples tested. As VP1 is known to be a critical nuclear localization factor, this could be an important quality attribute accounting for differences in infectivity.

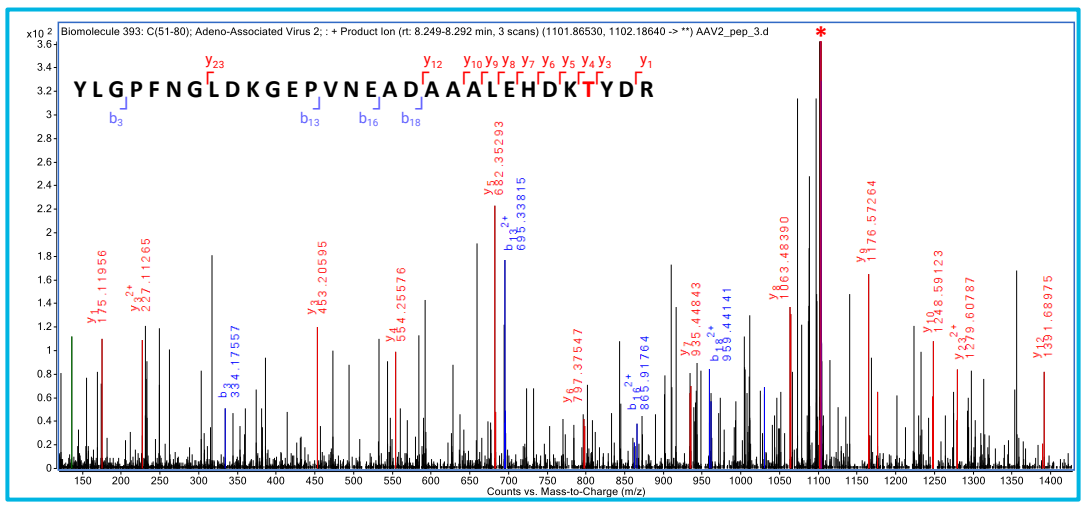

Figure 10. Confirmation of Ala → Tyr substitution on VP1

The LC-FLD-MS method proved useful in detecting an unanticipated Ala → Tyr mutation in AAV-2 VP1 based on the deconvoluted mass of the intact protein (theoretical = 81855.46 Da, observed = 81885.72 Da). This mutation was subsequently confirmed by MS/MS analysis of AAV-2 tryptic peptides (Figure 10).

Conclusions

Multiple AAV quality attributes were quantified by LC-FLD-(MS). At the whole-capsid level, reducing aggregation and optimizing Full/Empty capsid ratios are known to be critical for ensuring therapeutic efficacy. These attributes were efficiently addressed by size-exclusion and strong anion exchange chromatography with high accuracy, linearity and inter-day reproducibility.

At the level of capsid proteins, we found that VP1 – 3 could be separated on C18 or Diphenyl columns depending on the AAV serotype. This facilitated accurate capsid identification and permitted VP1:2:3 stoichiometry to be measured. Alterations to capsid protein primary sequences could be detected using MS1 and confirmed using peptide MS/MS.

References

[1]Backovic, A. et al. Capsid Protein Expression and Adeno-Associated Virus like Particles Assembly in Saccharomyces Cerevisiae. *Microb. Cell Fact* **2012**, 11, 124.

[2]Trilisky, E. I.; Lenhoff, A. M. Flow-Dependent Entrapment of Large Bioparticles in Porous Process Media. *Biotechnol. Bioeng.* **2009**, 104, 127–133.

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