Denaturing size exclusion chromatography-MS for characterisation of AAV and assessment of CQAs

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Introduction

AAV consists of a ssDNA encapsulated by a protein capsid which is made of three viral proteins different (VP1, VP2 & VP3) normally 1:1:10 ratio with different properties.

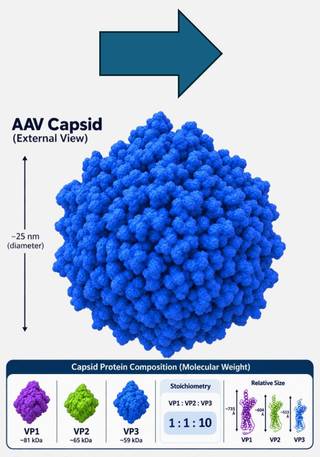


Figure 1: Showing the structure Viral proteins and the structure 3D structure of AAV Capsid Structure

ICH Q6B guidelines requires consistent PTMs and stoichiometry throughout manufacturing process because they could affect the transduction of the transgene.

Current method for intact mass analysis (RP-LCMS) provides poor resolution between VP1 and VP2 and shows a lack of sensitivity. This prevents analysis of important features of viral proteins.

Tiambeng et al, 2025 shows that denaturing size exclusion chromatography can successfully characterise AAV viral proteins.

Method

Run Parameters	
Method Of Separation	Uses organic solvents/acid to degrade substances online. The Size exclusion chromatography column contains different sized pores causing substances to elute in descending magnitude separating based on hydrodynamic volume (size).
Mobile Phase	25% ACN, 1% Formic acid, 5mM Ammonium Formate
Machine	Agilent 1290 HPLC system and Q-TOF 6545XT
Column	XBridge Protein BEH SEC Column, 450Å, 3.5 µm, 7.8 mm X 300mm
Elution method	Isocratic elution

Table 1: The run parameters used for Denaturing Size exclusion chromatography-MS

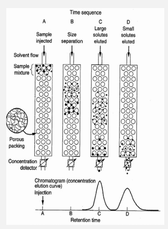


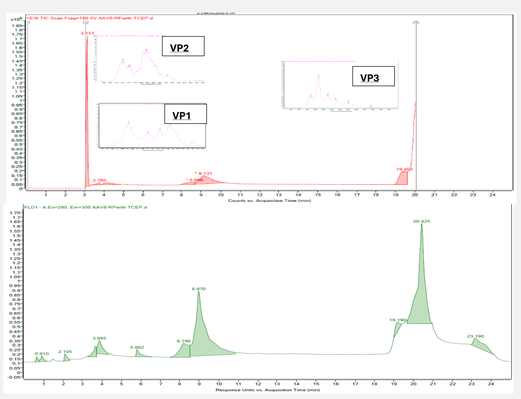
Figure 3: The separation of substances by SEC



Figure 4: Agilent LC-MS and Q-TOF [6]

Comparison to previous methods

Current Method



dSEC

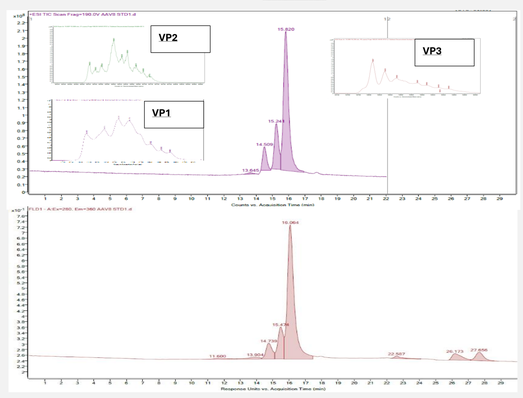


Figure 5: FLD and ESI of an AAV8 sample obtained using ZORBAX RRHD 300Å StableBond C18, 2.1 x 100 mm, 1.8 µm with a mobile phase of [A] 0.1% FA, [B]: 95% IPA, 4.8%ACN, 0.1%DFA, 0.1% FA

Figure 6: FLD and ESI-TIC of AAV8 Sample obtained using dSEC.

- The dSEC method shows greater sensitivity for AAV viral proteins and produces ~450 times greater signal than the current method.
- Results show dSEC produces greater resolution between VP1 and VP2 and less production of artefacts compared to RP-LCMS method.
- Neither produced baseline resolved results but purity can be established using the dSEC FLD.
- The protein deconvolution showed mixed results with dSEC identifying more peaks in VP1 yet less resolution of peaks, whilst VP2 produces greater resolution and identifies more peaks when using dSEC.
- VP3 deconvolution has a greater resolution in the current method shown through the identification of the base peak.

Characterisation of CE-SDS Peak

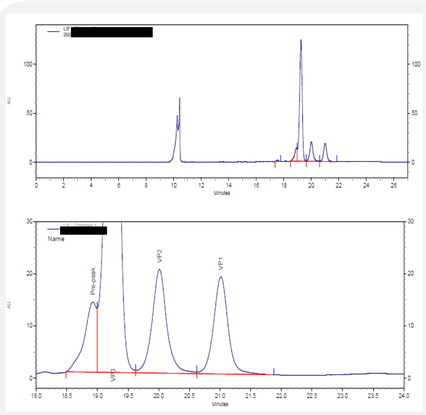


Figure 9: Capillary Electrophoresis-SDS results of an AAV2 sample

- CE-SDS data shows that a pre-peak arises prior to the VP3 peak that is likely to be induced by the temperature and the reducing agent causing a cleavage of VP3.
- By preparing the same product with a reducing agent and running through dSEC we can establish the molecular weight of the substance and hence the sequence of VP3 cleaved in AAV2
- Established the molecular/base peak has an average molecular weight of 45825.05 Da
- This suggests the N-terminus of the Viral protein undergoes cleavage leaving 409 amino acids in the sequence
- The breakage of the Asn124-Asp125 is theorised to be from spontaneous deamidation influenced by the pH and the steric hinderance of the VP3 chain

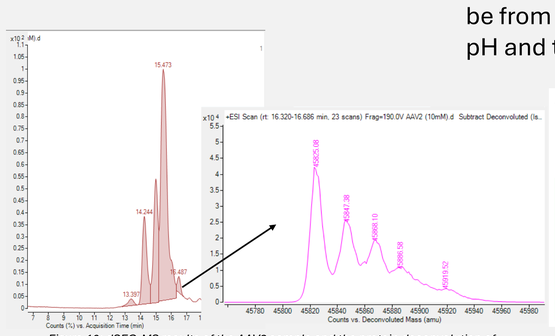


Figure 10: dSEC-MS results of the AAV2 sample and the protein deconvolution of the VP3 clip

AAV2 VP3 - (VP3 Fragment highlighted in Green)
N terminus –
MATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGD
RVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYST
PWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFK
LFNIQVKEVTQN**DGTTTIANNLTSTVQVFTDSEYQLPYVLG**
SAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFY
CLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRL
MNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQ
SRNWLPGPCYRQQRVSKTSADNNNSEYSWTGATKYHL
NGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSE
KTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNR
QAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDG
HFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKF
ASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNK
SVNVDFTVDTNGVYSEPRPIGTRYLTRNL – C-terminus

Figure 11: AAV2 VP3 protein sequence with the VP3-clip region highlighted in green



Figure 12: AAV2 VP3-Clip region protein structure interpreted by AlphaFold

Limit of Quantification and Linearity

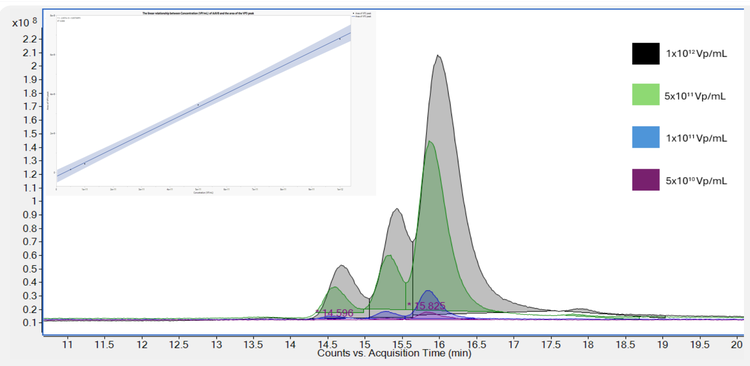


Figure 2: Stacked Total ion chromatogram of the same AAV8 STD at different concentration ($1x10^{12}$, $5x10^{11}$,$1x10^{11}$, $5x10^{10}$) along with a graph of the concentration (Vp/mL) against VP3 peak area showing the linearity of the method

- The linearity of the method shows that it can be used on the quantification of viral proteins of AAV shown through the R squared value of 0.999 and equation Y=(-2.007x10^{8}) + 0.007049x.
- Despite good linearity from 1x10^{12} to 5x10^{10} Vp/mL the LOQ was established based on signal to noise ratio of the VP1 (the smallest peak) being 10 times the noise of the chromatogram
- This meant the limit of quantification was established at 1x10^{11}Vp/mL.
- This method has also been shown to be relatively consistent as an AAV8 Standard obtained mean VP3 peak area of 1.93x10^{9} with a CV% of 11.37% (n=13).

dSEC-MS results across multiple serotypes

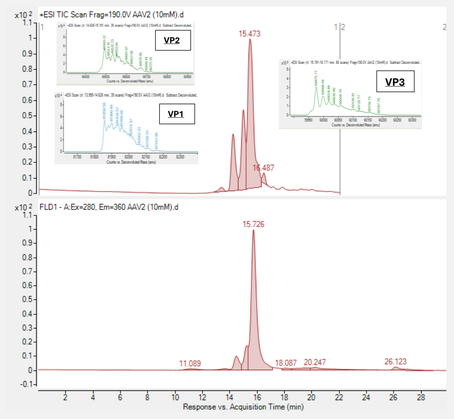
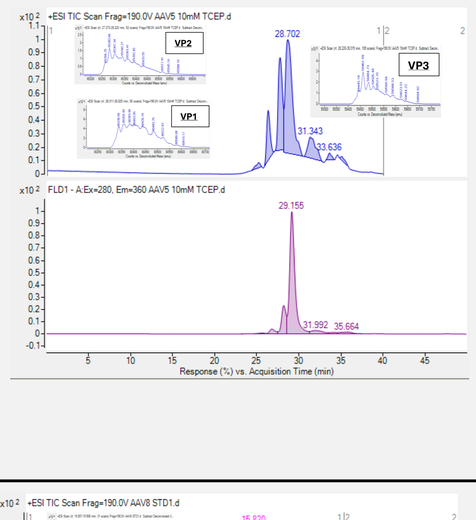
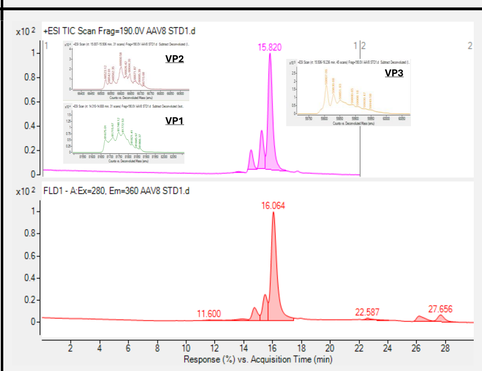
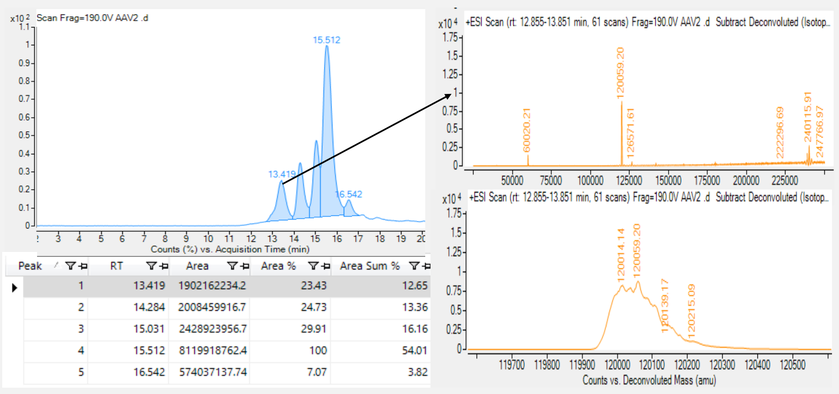
Serotype	Chromatograms and Protein deconvolution	Notes
AAV2		• In both the FLD and ESI there is good separation of VP1 and VP2. However, there is poorer separation of VP2 and VP3; this is expected due to the smaller 6.5 kDa difference in size. • In the protein deconvolution, post-translational modifications can be seen in VP3 and VP2. For VP1 this is less clear due to the lack of prominent peaks. All three can be used to confirm the mass of viral proteins and establish capsid serotypes. • This method requires TCEP to reduce artefacts.
AAV5		• AAV5 requires a slower flow rate of 0.25 mL/min compared with AAV2 and AAV8, which both use 0.45 mL/min. This allows proteins to diffuse into the bead pores more effectively, increasing separation, but also results in a longer run time of 55 minutes. • One reason this is required is that AAV5 has an average melting temperature 17°C higher than AAV8. • The ESI suggests that VP2 abundance is close to VP3 levels, but this is corrected in the FLD. Separation of viral proteins is visible but does not achieve the desired resolution value of 1. • Despite using TCEP, artefacts are also produced • The protein deconvolution for all AAV serotypes consists of broad peaks arising from the low flow rate, but it can still establish capsid identity.
AAV8		• AAV8 did not require TCEP in sample preparation, as no artefacts were produced and the viral proteins separated stably in dSEC. • The VP3 deconvolution program clearly marks the molecular ion peak in the mass spectrum, while the VP2 deconvolution marks the base peak. • VP1 deconvolution across all serotypes appears consistently incoherent, but in AAV8 the most abundant mass can still be observed in the deconvolution.

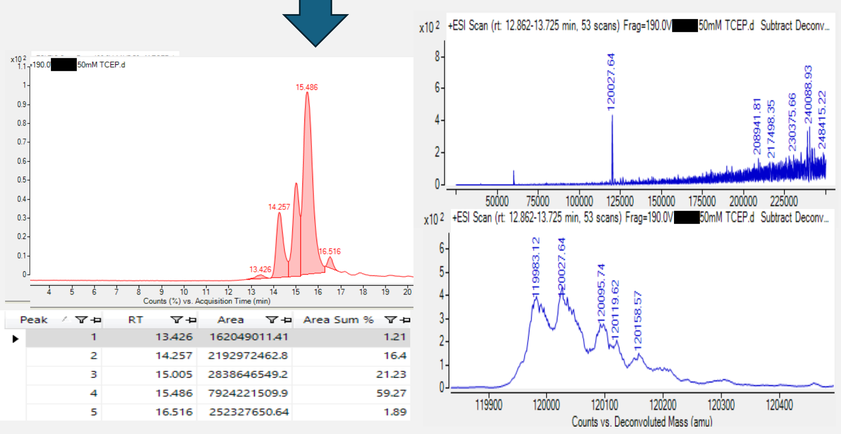
Table 2: Denaturing Size exclusion chromatography-MS results with protein deconvolution across multiple serotypes with FLD and ESI

Characterisation of VP3 dimerisation



Peak	RT	Area	Area %	Area Sum %
1	13.419	1902162234.2	23.43	12.65
2	14.284	2008459916.7	24.73	13.36
3	15.031	2428923956.7	29.91	16.16
4	15.512	8119918762.4	100	54.01
5	16.542	574037137.74	7.07	3.82

Figure 7: Untreated AAV2 dSEC results with the protein deconvolution of the Dimer pre-peak accompanied by a table showing the area of each peak



Peak	RT	Area	Area Sum %
1	13.426	162049011.41	1.21
2	14.257	2192972462.8	16.4
3	15.005	2838646549.2	21.23
4	15.486	7924221509.9	59.27
5	16.516	252327650.64	1.89

Figure 8: AAV2 dSEC results treated with 50mM of TCEP, and the protein deconvolution of the Dimer pre-peak accompanied by a table showing the area of each peak

- An artefact visible in the AAV2 and AAV5 ESI-TIC and FLD chromatograms were prominent peaks.
- Eluting prior to VP1 suggests that this peak consists of a molecular weight greater than 81KDa.
- The protein deconvolution mass spectrometry confirmed this through showing the mass to be 120059.20 Da in AAV2.
- For AAV2 there appears to be another peak in the deconvolution of 60020.21 Da, which is +45 Da adduct species of VP3.
- This suggests the PTM induces the dimerisation of VP3 through the strengthening of disulfide bonds
- This was then confirmed through sample preparation with TCEP (cleaves disulfide bonds through nucleophilic attack) and led to the significant decrease in the dimer peak area of AAV2.
- A possible PTM in this region is the formation of formic acid deprotonated molecular ions creating London dispersion forces and hydrogen bonds which can create a highly oxidative environment supporting the formation of disulfide bonds
- Unfortunately, the exact residue interacting with the formic acid deprotonated molecular ion is unable to be established. Yet, it is likely to be a positively charged amino acid such as His, Arg and Lys.

Conclusion

Findings

- Developed method to assess purity, post-translational modifications and quantify AAV Samples and compared to previously established methods and improved resolution of VP1 and VP2.
- Limit of quantification established to be 1x10^{14} VP/mL to 1x10^{11} VP/mL a much larger range than RPLC-MS
- Characterised artefacts from CE-SDS assay arising from N-terminal cleavage
- Gained knowledge in AAV Viral proteins disulfide bond formation

Future work

- Improve separation to baseline by purchasing a column with smaller pores (200Å> x <450Å)
- Further optimisation of mass spectrometry parameters to improve resolution of protein deconvolution profile
- Using UV detection to allow method to be able to establish empty: full ratio of AAV samples
- Explore possibility in implementing for other top-down analyses
- Trial on chimeras on AAV to ensure serotype agnosticism

Reference

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