# Electronic Supplementary Information (ESI)

## Mass spectrometry friendly pH-gradient anion exchange chromatography for the separation of full and empty Adeno-Associated Virus (AAV) capsids

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## Experimental

### Chemicals and Regents

Ammonium bicarbonate (LC-MS grade), ammonium acetate (99.999%, trace metal basis), acetic acid (LC-MS grade) and ammonium hydroxide (1M solution) was obtained from Sigma-Aldrich (Wicklow, Ireland). Formic acid (LC-MS grade) and ultrapure water (MS grade) was obtained from Fisher Scientific (Dublin, Ireland). Micro Bio-Spin™ 6 columns were obtained from Bio-Rad (Naas, Ireland). Research grade Sf9 derived standards of AAV 5, 6 and 8 full (CMV-GFP) and empty material was obtained from Virovek (Heyward, CA, USA) at a concentration of 2 E 13 vg/ml.

### Sample preparation

Once received, AAV samples in 1X PBS containing 0.001% pluronic F-68 were stored in a -80°C freezer until analysis Samples were thawed at room temperature for 5 min and injected as received. Injection amounts were 2e10 viral particles per condition per run (1µl in case of full and empty capsid samples, 2 µL in case of the 1:1 mixture). For direct infusion experiments the AAV5 F/E sample was buffer exchanged to 100 mM ammonium acetate.

### Liquid chromatography

Liquid chromatography was conducted on a Vanquish Flex UHPLC system, equipped with fluorescence detection (Thermo Scientific, Germering, Germany). The separation column was a ProPac 3R SAX column with dimensions of 2.1 x 50 mm, 3µm particle size obtained from Thermo Fisher Scientific (Sunnyvale, CA, USA). Mobile phase A was 20 mM ammonium bicarbonate and 20 mM ammonium hydroxide, pH 10.3. Mobile phase B was 15 mM formic acid and 30 mM acetic acid, pH 2.6. The final gradient was as follows: 20% B for 0.5 minutes, from 20 to 70% B over 20 minutes which was followed by 2.5 minutes at 100% B before decreasing the amount of B to 20% for 12 minutes to ensure adequate column equilibration. The total run time was 35 minutes. The column oven temperature was held at either 40 ˚C in case of AAV5 and 8 and 60 ˚C for AAV6, depending on the AAV analyzed. The flow rate was 0.2 mL/min and fluorescence detection was performed at excitation and emission wavelengths of 280 and 348 nm, respectively.

**Liquid chromatography-mass spectrometry**

For mass spectrometric detection the LC system was interfaced to a Q Exactive™ Ultra High Mass Range (UHMR) mass spectrometer (Thermo Scientific, Bremen, Germany) which was equipped with an ExD cell from e-MSion (Corvallis, OR, USA) which was tuned for transmission of AAVs. The spray voltage was set to 3.8 kV, the capillary temperature was 250 ˚C, sheath and auxiliary gasses were set to 20 and 10 arbitrary units, respectively. The probe heater temperature was 150 ˚C and the s-lens RF level was 200. The IST desolvation was set to -100 V, the number of microscans was set to 5, the resolution setting was 12,500 at m/z 400. The maximum IT was 100 ms, the scan range was 16,000 to 50,000 m/z. Detector m/z optimization and ion transfer target were set to high m/z, respectively, the trapping gas pressure was set to 2.0 corresponding to a UHV pressure of 4.6e-10 mbar, the trapping gas used was Sulfur hexafluoride.

**Static nano-ESI-based CDMS**

Experiments were based on the same MS system as is described in the Liquid chromatography-mass spectrometry section employing the Thermo Scientific Direct Mass Technology™ (DMT) mode. Ion transfer and detector m/z optimization modes as well as the trapping gas type and pressure were the same as were used for native MS experiments. The resolution setting applied was 50,000 at m/z 400, the injection time was set to 100 ms. All other acquisition parameters were tuned to obtain information rich single ion spectra of an AAV sample. Acquisition was performed for 30 minutes.

**Data analysis**

Data analysis for illustrative purposes was performed either in Chromeleon CDS, version 7.3.1 or in Xcalibur 4.2.47. MS chromatograms in Xcalibur underwent 5-point boxcar smoothing. Chromatographic quality metrics were either extracted from Chromeleon CDS or manually calculated. Resolution was calculated according to the formula: $1.18 * ((t_{R2} - t_{R1}) / (w_{1/2}2 + w_{1/2}1))$ with $t_R$ being the respective retention time and $w_{1/2}$ being the width at half height of each peak, respectively. Peak capacity was calculated according to the formula: $1 + t_g / w_b avg$ with $t_g$ being the gradient time and $w_b avg$ being the average peak width at the base. Results represent the average of triplicate measurements. Results shown for standard deviations of retention time and peak area also represent the average of triplicate measurements. Analysis of CDMS data was performed in STORIboard 1.0 (Proteinaceous, Evanston, Il, USA) using a preestablished calibration curve with charges of up to +80

which was extrapolated to cover a region of up to 240 charges. The set of processing parameters was optimized for AAV characterization, no filters were applied.

**Table S1** Proportions of full and empty capsids of 1:1 full/empty mixtures of AAV5, 6 and 8. Values represent the average of triplicate measurements.

| Species | Relative abundance (%) | F:E ratio |
|---|---|---|
| AAV5 full | 38.2 | 0.61 |
| AAV5 empty | 61.8 | |
| AAV6 full | 22.8 | 0.30 |
| AAV6 empty | 77.2 | |
| AAV8 full | 55.7 | 1.26 |
| AAV8 empty | 44.3 | |

**Table S2** Calculated relative standard deviation of retention time and peak area based on full/empty mixtures. Values were calculated based on three replicate runs, respectively.

| Sample/peak | σ RT (%) | σ area (%) |
|---|---|---|
| AAV5 full | 0.015 | 2.21 |
| AAV5 empty | 0.012 | 1.62 |
| AAV6 full | 0.019 | 4.79 |
| AAV6 empty | 0.022 | 1.50 |
| AAV8 full | 0.054 | 1.19 |
| AAV8 empty | 0.042 | 2.09 |

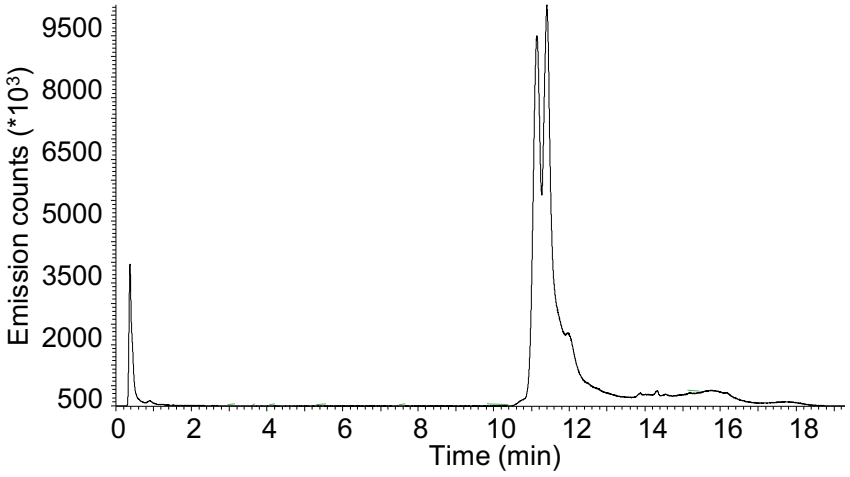


**Fig. S1** Separation of 1:1 AAV8 F:E mixture during gradient optimization with starting conditions of 30% mobile phase B. A clear injection peak is visible at minute 0.4, indicating insufficient sample binding.

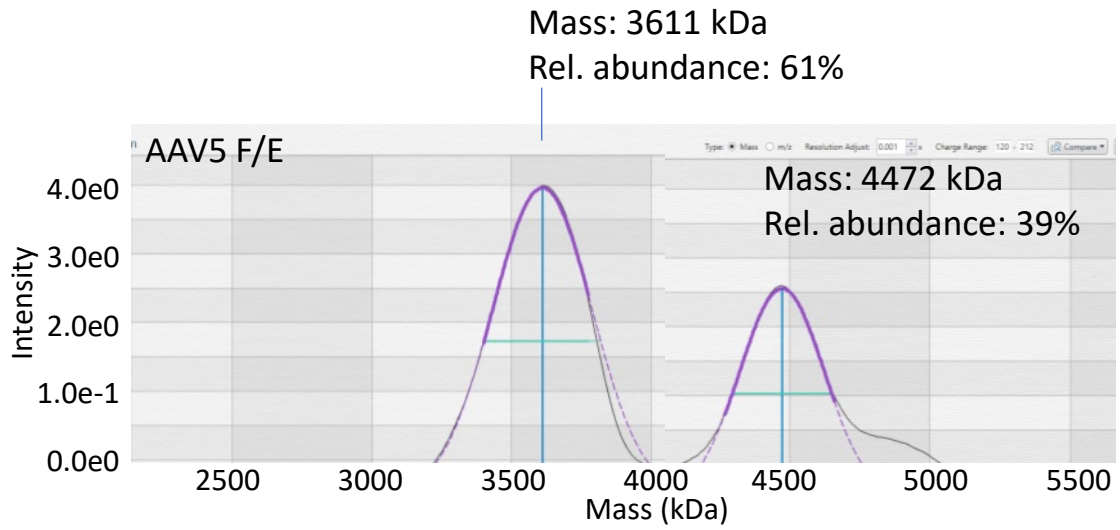


**Fig. S2** True mass spectrum of an AAV5 1:1 mixture obtained by CDMS. The masses obtained for full and empty peaks correspond well to theoretical masses with mass deviations of 2.4 and 0.6% for full and empty species, respectively (assuming a theoretical capsid mass of 3699 kDa and a transgene size of 0.8 kDa). Relative abundances of full and empty species align well with results from AEX shown in Table S1.

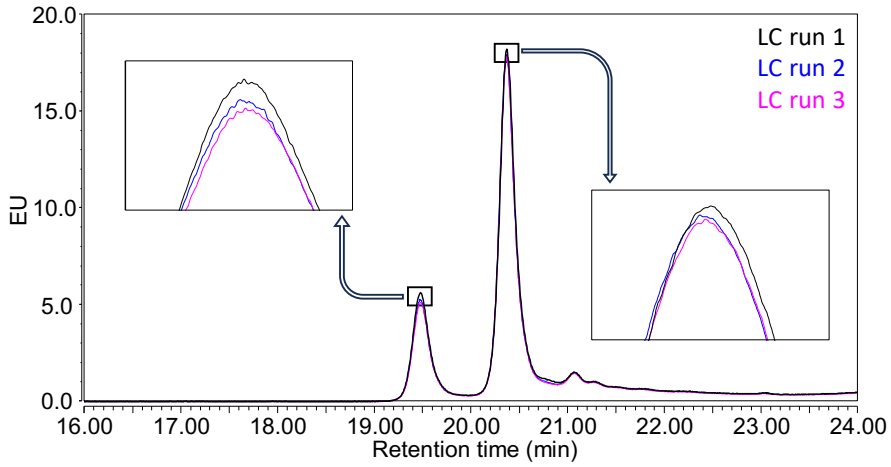


**Fig. S3** Overlay of replicate chromatograms of the full/empty mixture of AAV6. Peak tips are shown magnified to demonstrate a decrease in signal response over time.