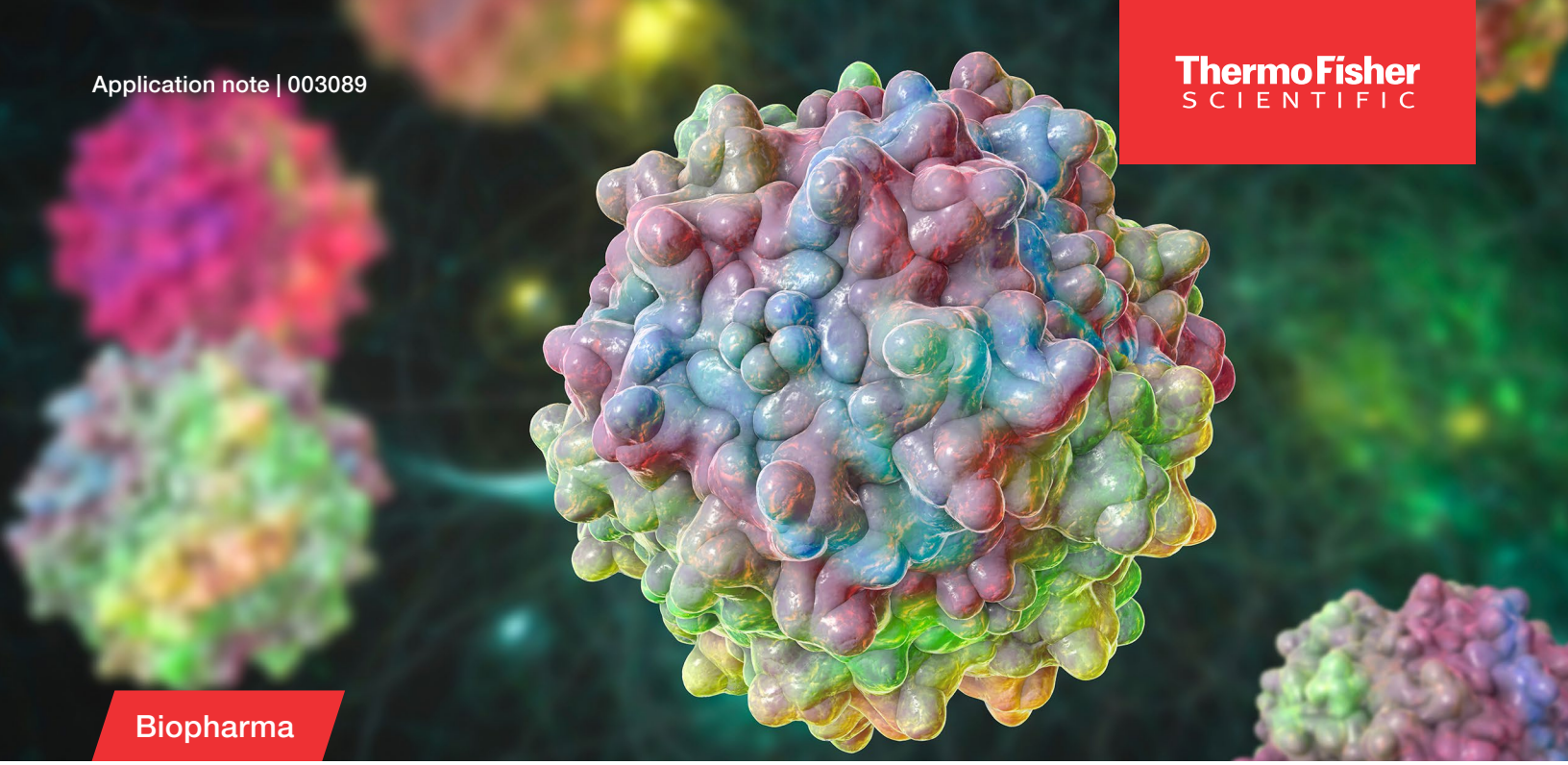

Application note | 003089

Biopharma

Size-exclusion chromatography of adeno-associated viruses with the SurePac Bio 550 SEC MDi column

Authors

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Keywords

Biopharmaceutical, size-exclusion liquid chromatography, SEC, adeno-associated virus, AAV, monomeric capsids, high molecular weight species, HMWS, 3 µm SurePac Bio 550 SEC MDi column, monodisperse

Key benefits

- High-resolution separation of monomeric AAV and aggregates by size-exclusion liquid chromatography within 4 minutes
- Utilizing UV and fluorescence detection for easy and conclusive confirmation of peak identities
- Resolution of aggregate and monomer peaks preserved even for injection of large volumes
- Consistent lot-to-lot and column-to-column performance

Goal

To demonstrate baseline separation of adeno-associated virus (AAV) monomeric capsids and high molecular weight species (HMWS) using a 4.6 × 150 mm size-exclusion liquid chromatography column packed with 3 µm monodisperse silica particles with a 550 Å pore size

Introduction

AAVs are small replication-defective, non-enveloped viruses that can be used as vectors for gene therapy. AAV vectors have emerged as pivotal tools in gene therapy, playing a transformative role in the delivery of therapeutic genes due to their low immunogenicity, safety, and long-term transient expression.[1] In AAV production, separating the monomeric form from aggregates is critical for optimal gene therapy outcomes.[2]

The success of AAV-based gene therapies critically depends on the purity and homogeneity of the AAV vector populations used in these applications. Therefore, monitoring AAV aggregation is important for their production and quality assurance. Size-exclusion liquid chromatography (SEC) has been the method of choice for the detection and quantification of AAV aggregates. SEC relies on the differential exclusion of molecules based on their size, making it particularly well-suited for separating AAV particles from aggregates. Chromatographic methods are advantageous, owing to their relatively straightforward method development and high sample throughput. They can also serve as orthogonal methods when combined with other commonly used techniques such as ELISA, cryogenic electron microscopy (cryo-EM), and AUC.[3]

The Thermo Scientific™ SurePac™ Bio 550 SEC MDi™ 3 µm column is ideal for the analysis of AAV monomeric capsids and HMWS. The monodispersed silica particles are covalently modified with a proprietary diol hydrophilic layer. This proprietary process brings an extremely low level of non-desired interaction sites. Compared to traditional polydisperse particles (right image, Figure 1), the consistent size distribution of the monodisperse particles (left image, Figure 1) not only facilitates precise control over media synthesis and column packing, but also significantly improves column-to-column and lot-to-lot reproducibility. The stationary phase is housed in state-of-the-art hydrophilic-coated stainless-steel hardware. The hydrophilic coating reduces secondary interactions, ensuring optimal performance during the initial injections. These column properties make the 3 µm SurePac Bio 550 SEC MDi column capable of analyzing AAV samples with high resolution and excellent reproducibility. In this application note, we provide practical examples of using the 3 µm SurePac Bio 550 SEC MDi column for the separation of AAV monomeric capsids and HMWS using UV and fluorescence (FLD) detection and multi-angle light scattering (MALS) detection.

Experimental

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Sodium phosphate monobasic (Sigma-Aldrich, P/N S8282)
- Sodium phosphate dibasic dodecahydrate (J.T. Baker, P/N 3822-01)
- Sodium chloride (Sigma-Aldrich, P/N S7653)
- Phosphate buffered saline (PBS) (Sigma-Aldrich, P/N P53683)
- 10x DPBS concentrate without calcium and magnesium salts, pH 7.4 (DPBS) (Teknova, P/N P0295)
- Poloxamer 188 non-ionic surfactant (100x), Gibco™ (P/N 24040032)
- AAV3-CMV-GFP, AAV5, 8 and 9-CMV-luciferase (Virovek, Hayward, CA)
- AAV5 sample used for SEC-MALs testing, generated in house
- 3 µm SurePac Bio 550 SEC MDi column (P/N 43903-15463)
- Thermo Scientific™ SureSTART™ 2 mL polypropylene screw top microvials (P/N 6ESV9-04PP)
- Thermo Scientific™ SureSTART™ 2 mL screw caps (P/N 6ASC9ST1)

Sample preparation

In the analysis of sample loading, AAV9 underwent a 10-fold dilution with 1x PBS, pH 7.4 buffer.

For all other tests, the AAV samples were used as received (2×10^{13} vg/mL)

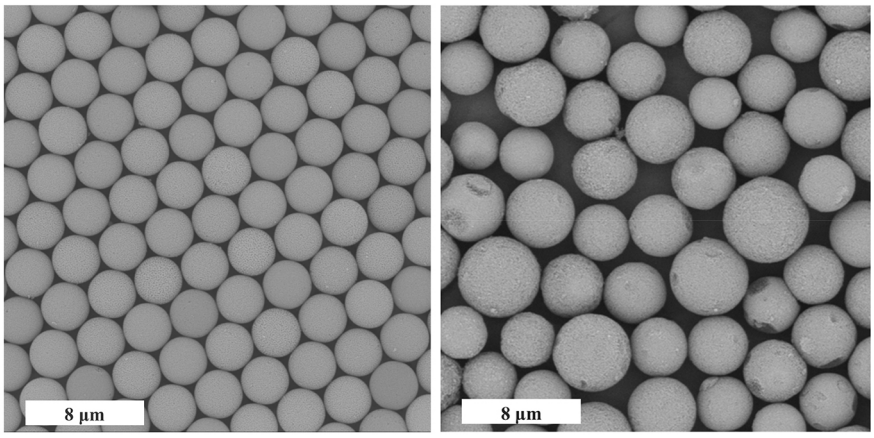

Figure 1. SEM image of 3 µm monodisperse silica particles (left) vs. 3 µm polydisperse particles (right)

Instrument

Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system, including:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump (P/N VF-P20-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A) with 25 µL (V = 50 µL) sample loop
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector (P/N VF-D40-A) with Thermo Scientific™ Vanquish™ Variable Wavelength Detector F Flow Cell (P/N 6077.0300)
- Thermo Scientific™ Vanquish™ Fluorescence Detector (P/N VF-D51-A) with Thermo Scientific™ Vanquish™ Fluorescence Detector F Flow Cell (P/N 6079.4230)
- Eppendorf ThermoMixer™ F1.5 (Eppendorf, P/N EP5384000020)

Below instruments are used for SEC-MALS analysis:

- Thermo Scientific™ UltiMate™ LPG-3400RS Rapid Separation Quaternary Pump (P/N 5040.0036)
- Thermo Scientific™ UltiMate™ WPS-3000TBRS Biocompatible Rapid Separation Well Plate Autosampler (P/N 5841.0020)
- Thermo Scientific™ UltiMate™ TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- Thermo Scientific™ Vanquish™ Diode Array Detectors and Multiple Wavelength Detector (P/N VH-D10-A)
- Wyatt Technology Optilab™ Detector (P/N WOP1-01)
- Wyatt Technology DAWN™ Light Scattering Detector for SEC-MALS (P/N WD3-02)

Data processing

Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System is used for data acquisition and analysis.

Wyatt Technology ASTRA™ 8.1.2 is used for SEC-MALS data acquisition and analysis.

Chromatographic conditions

Please refer to Table 1 for details on mobile phase compositions and method conditions—such as flow rate, column temperature, and injection volume—unless specified in the results and discussion section.

Table 1. Chromatographic conditions

Parameter	Setting
Column	SurePac Bio 550 SEC MDi (P/N 43903-154631)
Format	4.6 × 150 mm
Eluent	50 mM phosphate buffer and 300 mM NaCl, pH 6.5
Flow rate	0.3 mL/min
Run time	10 min
Injection volume (UV)	1 µL
Injection volume (FLD)	200 nL for AAV8, 400 nL for other AAVs
UV detection	260 nm and 280 nm
FLD detection	Ex 280 nm, Em 330 nm

Results and discussion

Within this section, we outline a straightforward approach for effectively separating AAV monomeric capsids and HMWS across four distinct AAV samples (AAV3, 5, 8, and 9). We delve into the nuanced impact of varying flow rates, ranging from 0.05 to 0.6 mL/min, on the separation dynamics of AAV. Additionally, we explore the consequential effects of sample loading on the refined separation of AAV monomer and HMWS. To provide a comprehensive perspective, we include a comparative analysis with columns from other vendors featuring a 2.5 µm particle size and 450 Å pore size, and a 5 µm particle size and 500 Å pore size. Concluding our exploration, we highlight the column's consistency by demonstrating its lot-to-lot reproducibility.

UV vs. fluorescence detection

The application of SEC in the analysis of various AAV serotypes stands as a critical endeavor in biopharmaceutical research. This section employs both ultraviolet (UV) and fluorescence (FLD) detection methods to comprehensively assess different AAV samples. UV detection at 280 nm and 260 nm proves instrumental in elucidating the capsid content of AAV particles, distinguishing between empty and full capsids, thereby confirming peak identity. This is because the absorbance at 280 nm primarily corresponds to the presence of proteins, which are abundant in the capsid, while the absorbance at 260 nm corresponds to nucleic acids, which are present only in full capsids. The differential absorbance at these wavelengths allows for clear differentiation between the two types of capsids. Simultaneously, the heightened sensitivity of fluorescence detection offers a robust alternative, allowing for substantial sample conservation as lower sample volumes can be employed without compromising signal strength. This integrated approach

not only advances the precision of AAV serotype analysis but also underscores the versatility of SEC in facilitating nuanced insights into viral vector characteristics for biopharmaceutical applications.

In Figure 2A, the distinction between the first and second peaks is discerned through the larger 260 nm absorbance relative to 280 nm. This disparity indicates that the first peak corresponds to HMWS, while the second peak represents the monomeric AAV3. The third peak, exhibiting greater 260 nm absorbance without FLD signal, is indicative of potential extracellular DNA. Analogously, Figure 2B can be interpreted with a parallel rationale, where the initial peaks denote HMWS and the second prominent peak signifies AAV5 monomer. The absence of FLD emission in the third peak suggests it may be extracellular DNA as well. Figures 2C and 2D correspond to AAV8 and AAV9, where the enlarged first peak showcases 260 nm absorbance surpassing 280 nm, characterizing it as aggregates. The subsequent second peak is identified as the AAV monomer, while the third peak is indicative of extracellular DNA. The consistent baseline separation of AAV monomers from HMWS across all four samples underscores the efficacy of the 550 Å pore size in the column. The sharpness of the monomer peak further attests to the high efficiency facilitated by the 3 µm monodispersed particles in the column.

SEC-MALS analysis

SEC coupled with MALS detectors has become the gold standard for simultaneous measurement of AAV titer, aggregate quantitation (including monomers, dimers, trimers, etc.), and aggregate molecular weight. This integrated approach is crucial in development and production settings, where rapid and accurate characterization of AAV samples is critical.

In this section, SEC-MALS analysis of AAV5 samples was conducted. Performance of the 4.6 × 150 mm, 3 µm SurePac Bio 550 SEC MDi column with a 4.6 × 300 mm, 5 µm, 500 Å column from a different vendor is compared in Figure 3. Remarkably, analysis time and sample usage were reduced by half or more using the SurePac column compared to the longer conventional column. Even with the reduced separation time, the resolution of the SurePac column is better for the dimer (resolution 1.72 versus 1.26) with improved definition of the trimer shoulder enabling more accurate mass characterization. The enhanced resolution and efficiency offer significant benefits in terms of throughput and mobile phase consumption due to reduced analysis time and reduced sample usage. By streamlining analysis time and sample consumption while uncovering previously unseen aggregates, the SurePac Bio 550 SEC MDi column presents a compelling solution for AAV characterization in both research and industrial applications.

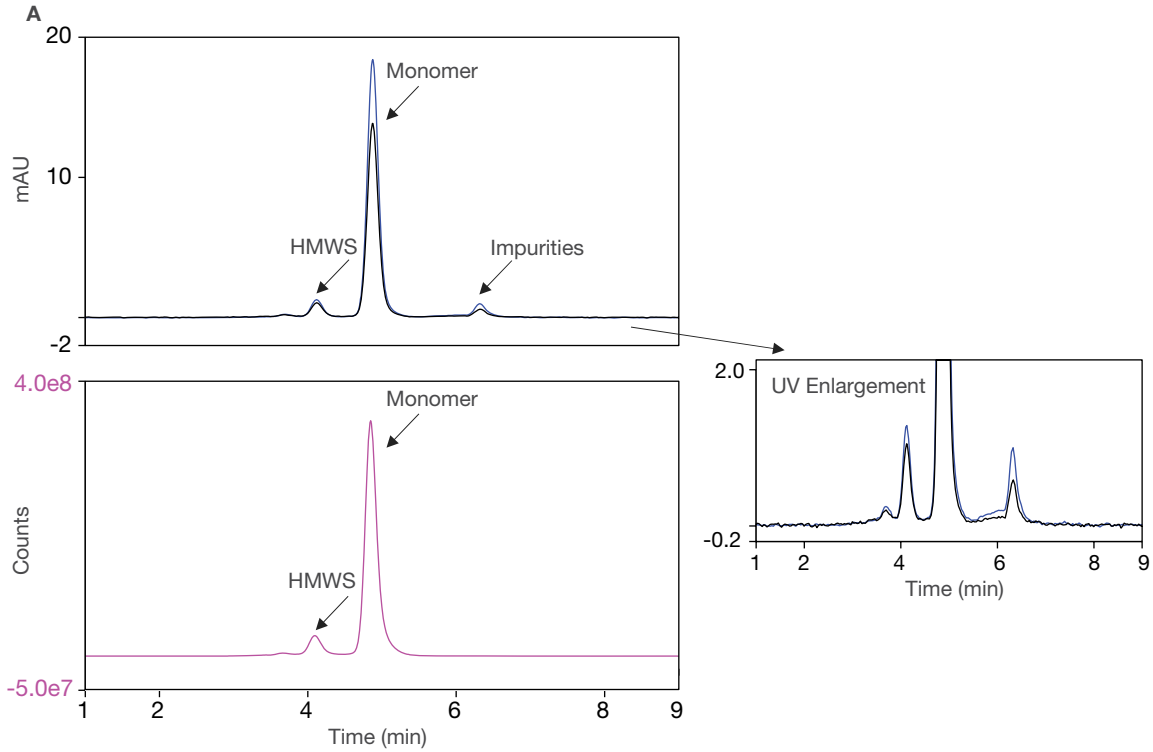

Figure 2A. Separation of different AAV3 serotypes using both UV and FLD detection. Black trace represents the UV 280 nm signal, blue trace represents the UV 260 nm signal, and pink trace represents the FLD signal.

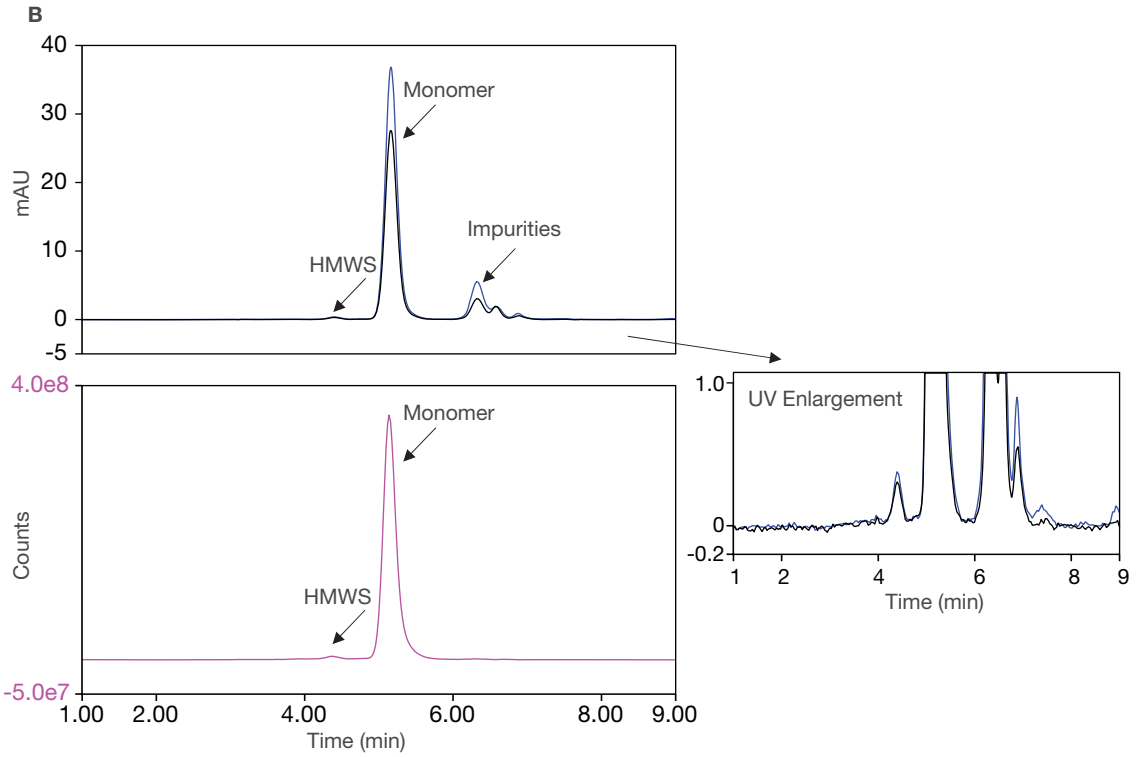

Figure 2B. Separation of different AAV5 serotypes using both UV and FLD detection. Black trace represents the UV 280 nm signal, blue trace represents the UV 260 nm signal, and pink trace represents the FLD signal.

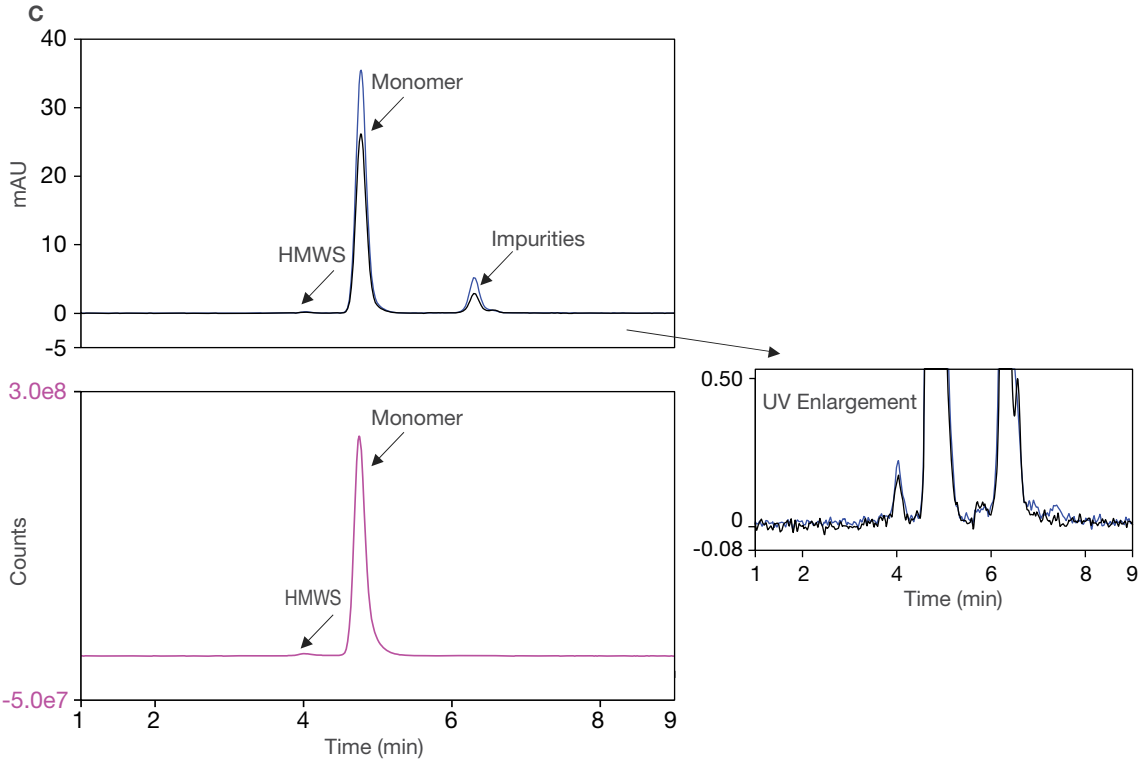

Figure 2C. Separation of different AAV8 serotypes using both UV and FLD detection. Black trace represents the UV 280 nm signal, blue trace represents the UV 260 nm signal, and pink trace represents the FLD signal.

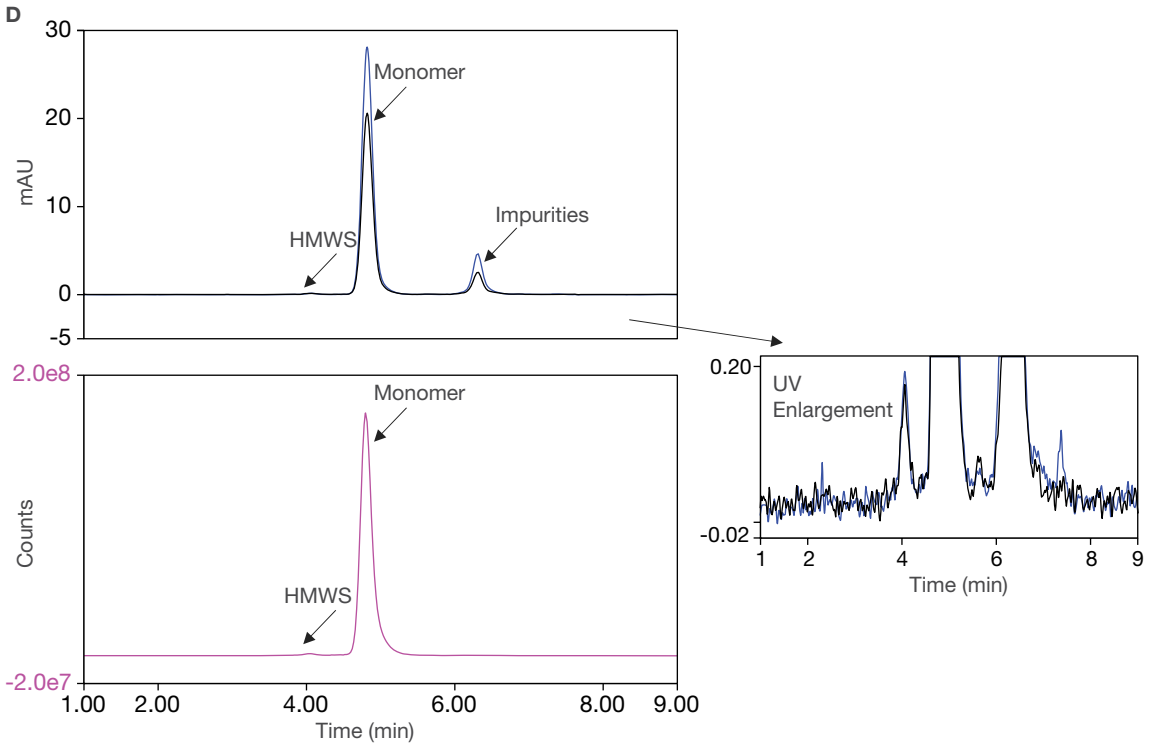

Figure 2D. Separation of different AAV9 serotypes using both UV and FLD detection. Black trace represents the UV 280 nm signal, blue trace represents the UV 260 nm signal, and pink trace represents the FLD signal.

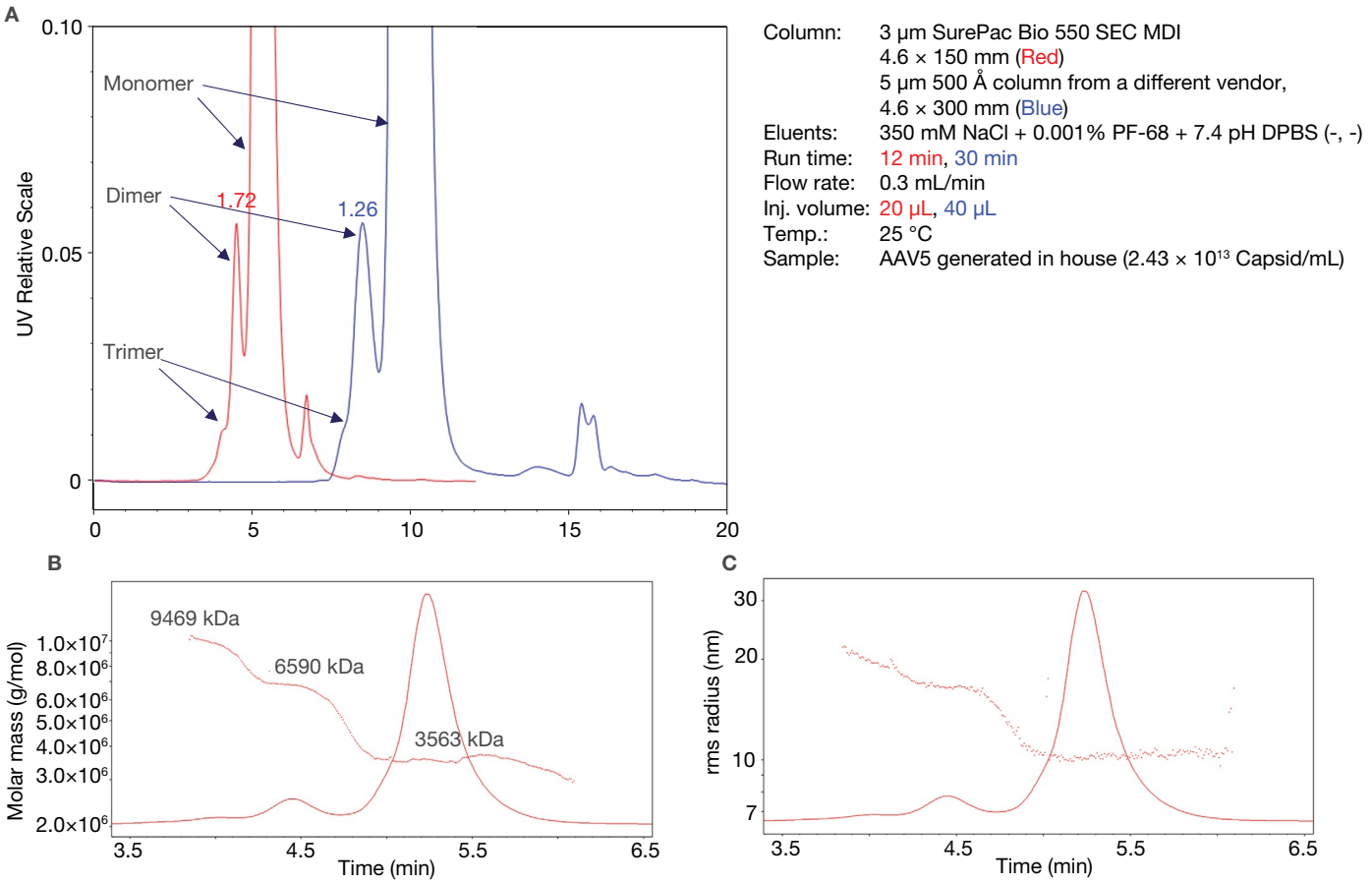

Figure 3. Separation of AAV5 generated in house using SEC-MALS with (A) 4.6 x 150 mm, 3 µm SurePac Bio 550 SEC MDi column (Red) and 4.6 × 300 mm, 5 µm, 500 Å column from a different vendor (Blue). Legend: resolution between monomer and dimer, (B) Molecular mass data using the 4.6 × 150 mm, 3 µm SurePac Bio 550 SEC MDi column, and (C) Rms radius data using the 4.6 × 150 mm, 3 µm SurePac Bio 550 SEC MDi column.

Flow rate effect on chromatography

The choice of flow rate plays a crucial role in SEC during the separation of AAV (Table 2). Figure 4 shows the chromatogram of AAV8 over a flow rate range of 0.05 to 0.6 mL/min. The flow rate significantly influences the dynamics of AAV separation. Optimal flow rates contribute to well-defined and efficient separation, ensuring clear distinctions between monomeric AAV and HMWS. Too high a flow rate might compromise resolution, while too low a rate could lead to extended analysis times. Thus, careful consideration and adjustment of the flow rate are essential to achieve optimal SEC performance in AAV separation studies.

Lower flow rates, such as 0.05 mL/min within the 45 min range (Figure 4A), yielded enhanced resolution (4.71) between the monomer peak and HMWS. This slower flow rate provided a longer analysis time, allowing for a more detailed characterization of HMWS composition. Conversely, higher flow rates, such as 0.6 mL/min (Figure 4B), demonstrated the potential for high-throughput AAV analysis. Despite the total run time within 4.0 minutes, the monomer remained baseline separated from HMWS, albeit with reduced resolution. This configuration is advantageous for rapid analyses where high throughput is prioritized.

Table 2. Separation of AAV8 using flow rates from 0.05 mL/min to 0.6 mL/min

Flow rate (mL/min)	Monomer RT (min)	HMWS RT (min)	Resolution
0.05	28.228	23.628	4.71
0.10	14.161	11.870	3.78
0.20	7.128	5.986	3.20
0.30	4.786	4.028	2.64
0.40	3.611	3.078	2.58
0.50	2.903	2.470	2.09
0.60	2.436	2.078	2.23

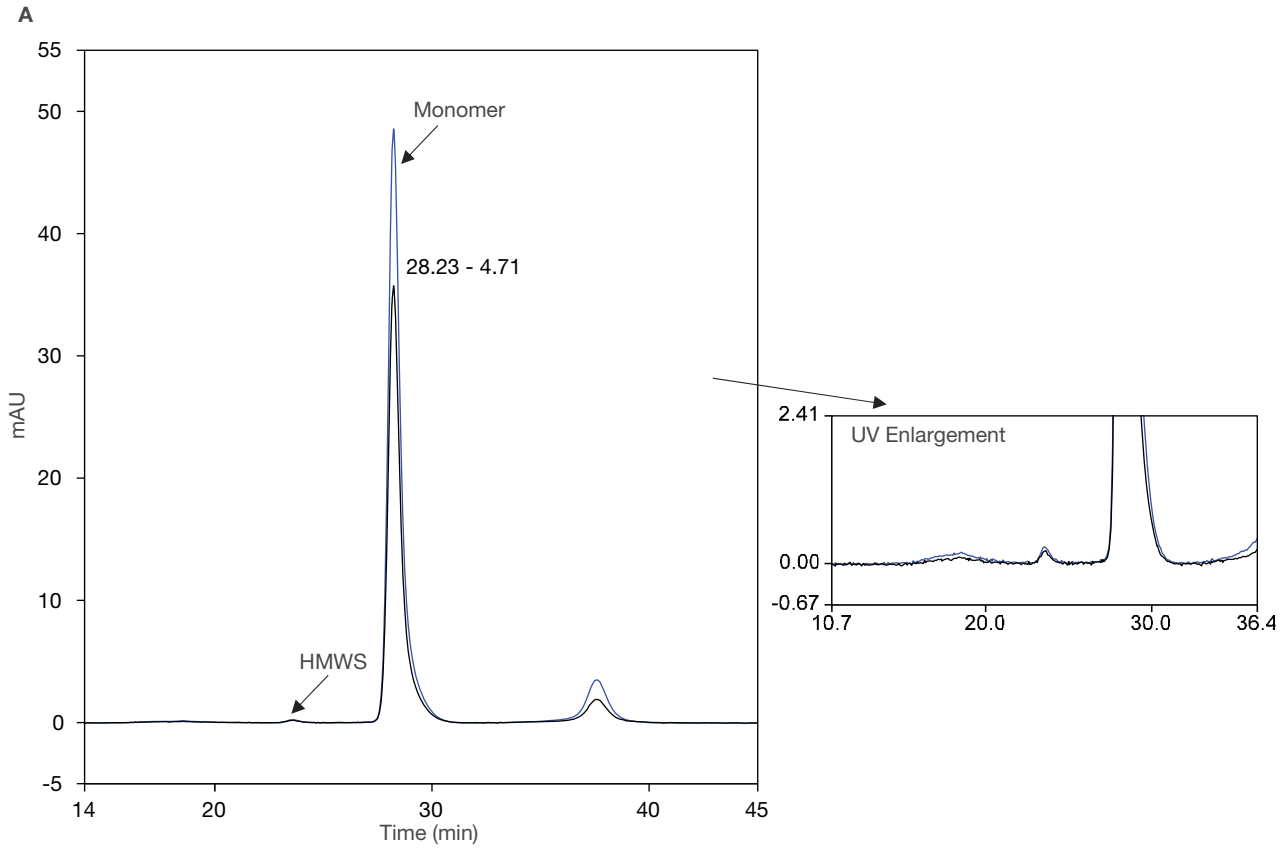

Figure 4A. Separation of AAV8 using flow rate of 0.05 mL/min. The black trace represents the UV 280 nm signal, and the blue trace represents the UV 260 nm signal. Legend: RT (min) – Resolution between monomer and HMWS.

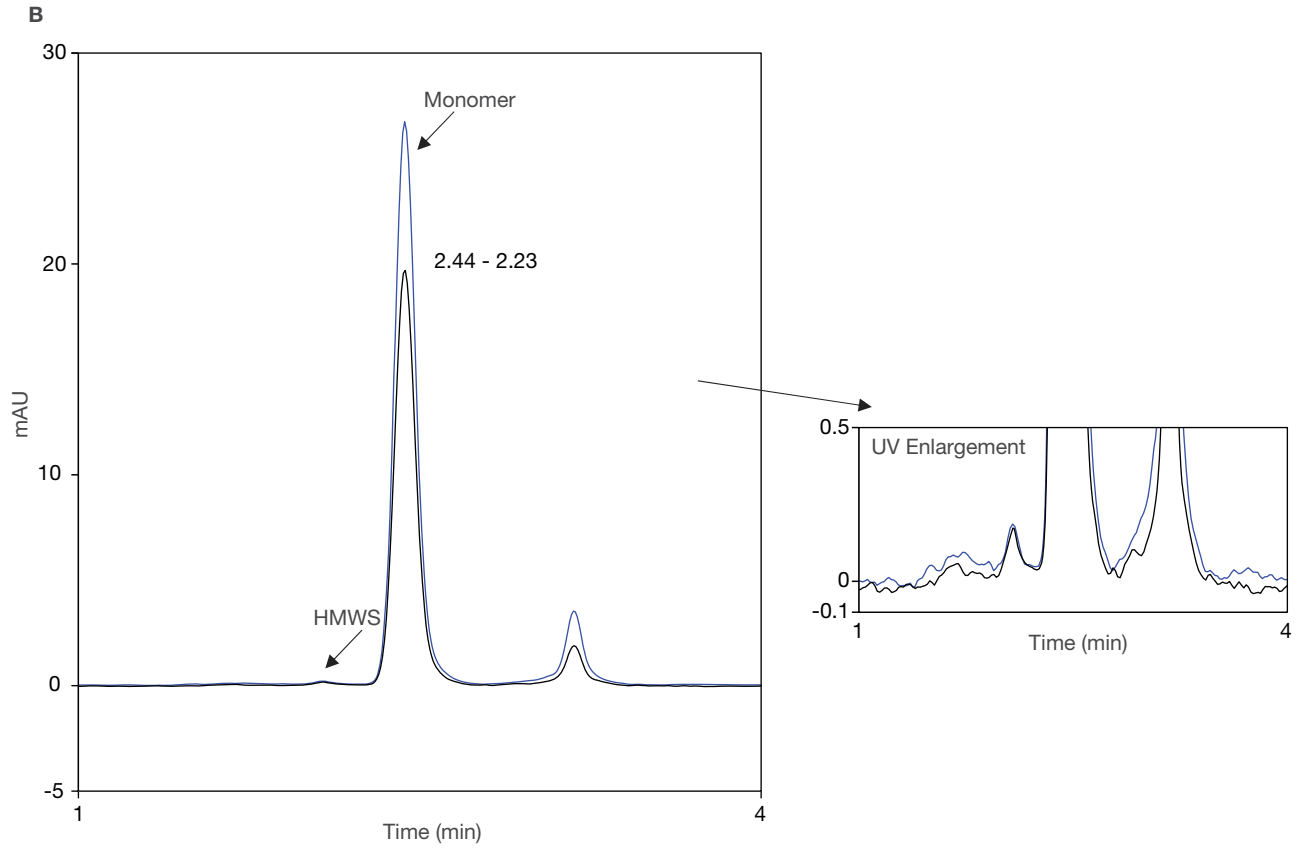

Figure 4B. Separation of AAV8 using flow rate of 0.6 mL/min. The black trace represents the UV 280 nm signal, and the blue trace represents the UV 260 nm signal. Legend: RT (min) – Resolution between monomer and HMWS.

The selection of the optimal flow rate should align with specific analytical objectives. Researchers seeking detailed insights into HMWS composition and enhanced resolution may prefer a slower flow rate for meticulous characterization. Meanwhile, those prioritizing high throughput can opt for a higher flow rate while still achieving baseline separation between the monomer and HMWS.

Sample loading analysis

The amount of sample loaded significantly impacts the separation of AAV. In this study, volume loading amounts ranging from 4 to 50 µL (conc. ~ 2×10^{12} vg/mL) are investigated. This parameter has a direct influence on the resolution and peak shape in the chromatogram. By systematically exploring various loading amounts, the study aims to identify the optimal range that ensures effective separation and precise identification of AAV components. The relationship between sample loading and sample volume is crucial in maintaining resolution and peak integrity in SEC. A large injection volume can lead to broader peak widths and a subsequent decrease in resolution, because of the contribution of the injection volume to the extra-column band dispersion. In Figure 5, baseline separation of HMWS from the monomer peak, is observed across all injection volumes ranging from 4 to 50 µL.

The relative peak area calculation reveals a consistent result, with the HMWS peak remaining constant at approximately 0.5%, and the AAV monomer peak consistently maintaining around 99.5% across the entire range of sample loading volumes. This constancy in relative peak area suggests that the quantification remains robust and unaffected by variations of volume and sample loading. These findings underscore the critical role of sample loading in sustaining optimal resolution and peak integrity in SEC AAV separation, emphasizing the 3 µm SurePac Bio 550 SEC MDi column's robust design and positioning it as an exemplary choice for accurate and reproducible AAV quantification in biopharmaceutical research and development.

We conducted loading experiments using increased volumes of diluted AAV8 samples. The injection volumes were as follows: 1 µL for undiluted AAV8, 10 µL for 10-fold diluted, 20 µL for 20-fold diluted, and 40 µL for 40-fold diluted AAV8 samples. Samples were diluted with 1x PBS buffer. These adjustments were made to maintain a consistent amount of AAV8 injected onto the column and evaluate uniquely the peak broadening due to the injection volume.

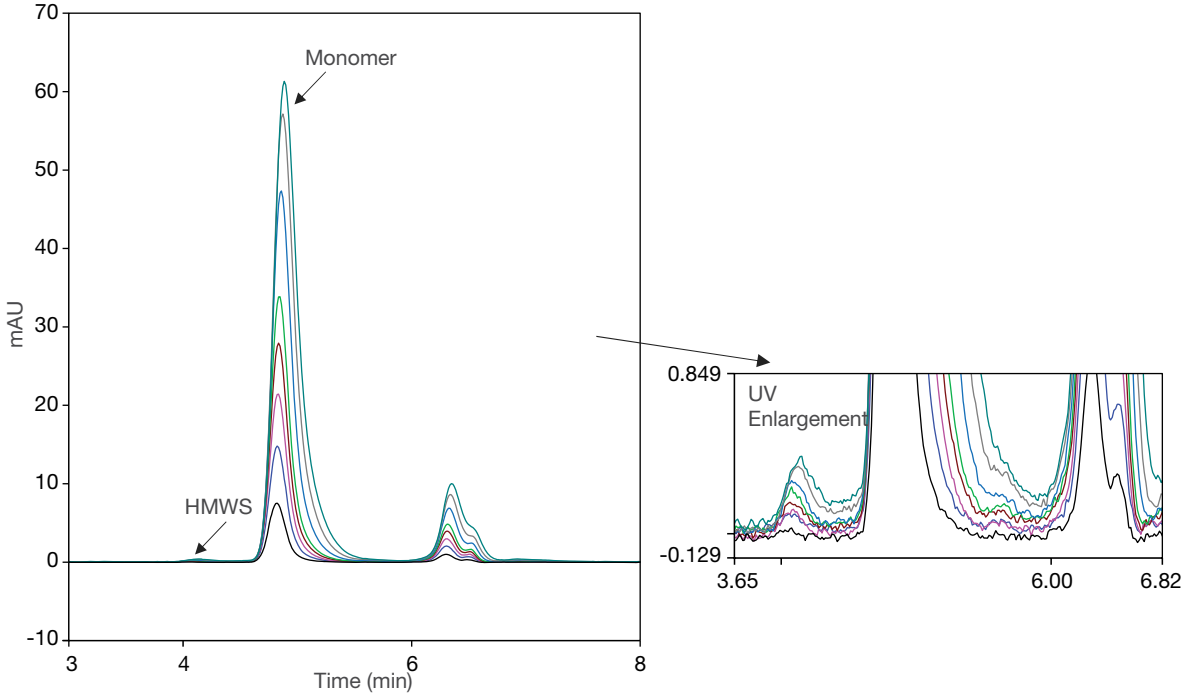

Figure 5. Separation of AAV9 sample with injection volume ranging from 4 to 50 µL

Figure 6 illustrates the overlay of chromatograms obtained from these four injection volumes. The slight difference in peak height may be attributed to errors introduced during sample dilution. Notably, despite the injection volume being 40 times that of the non-diluted sample, the increase in peak width at half height was only approximately 26%. This attribute of the SurePac column makes it particularly suitable for customers requiring large sample injection capacities. This attribute of the column is highly advantageous in applications where substantial sample injection volumes are often required like SEC-MALS.

Comparative analysis with another vendor column

In this section, we scrutinize the performance of the 3 µm SurePac Bio 550 SEC MDi column alongside another vendor column with 2.5 µm polydisperse particles and a 450 Å pore size on the analysis of AAV3, 5, 8, and 9 samples. The distinct advantages of the 3 µm SurePac Bio 550 SEC MDi column become evident, showcasing superior chromatographic capabilities (Figure 7). Notably, the employment of this column results in the generation of sharper peaks with enhanced resolution during the separation of the AAV monomer from HMWS. Noteworthy is the reduced backpressure (ca. 200 psi) observed with the utilization of the 3 µm SurePac Bio 550 SEC MDi column, underscoring its operational efficiency in AAV analysis compared to the competitor column. This detailed exploration not only sheds light on the specific merits of the 3 µm SurePac Bio 550 SEC MDi column in terms of finer resolution and enhanced peak sharpness but also emphasizes its overall operational efficiency of AAV sample analysis.

Column:	3 µm SurePac Bio 550 SEC MDi
Format:	4.6 × 150 mm
Eluents:	50 mM phosphate buffer and 300 mM NaCl. pH 6.5
Run time:	10 min
Flow rate:	0.3 mL/min
Inj. volume:	1 µL for undiluted (black) 10 µL for 10-fold undiluted (Blue) 20 µL for 20-fold undiluted (Pink) 40 µL for 40-fold undiluted (Brown)
Temp.:	30 °C
Detection:	UV, 280 nm
Sample:	AAV8-CMV-Luciferase (2×10^{13} vg/mL undiluted)

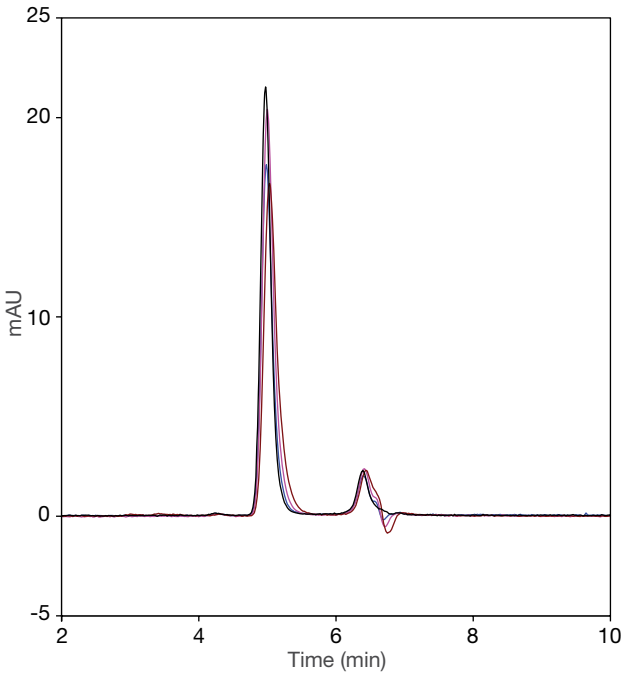

Figure 6. Separation of undiluted, 10-fold diluted, 20-fold diluted, and 40-fold diluted AAV8 sample. Sample is diluted with 1x PBS buffer.

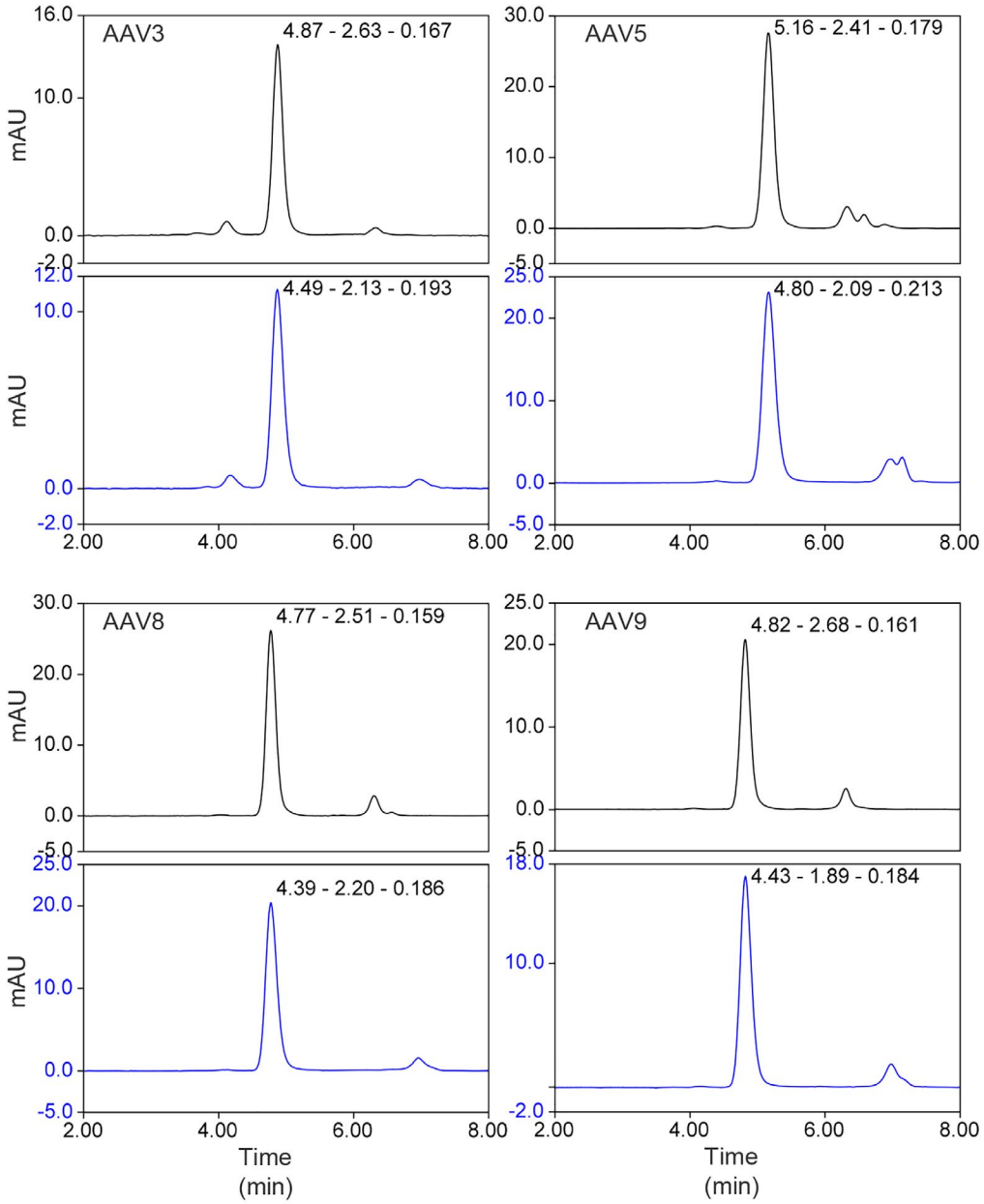

Figure 7. Chromatograms of using 3 µm SurePac Bio 550 SEC MDi column (black) and another vendor column with 2.5 µm particle size (blue) for AAV3, 5, 8, and 9 samples. Peak label: RT (min) – Resolution between monomer and HMWS – PWHH (peak width at half height).

Lot-to-lot reproducibility

Ensuring consistent and reproducible performance from one column lot to another is imperative for the reliability of chromatographic analyses. The utilization of the SurePac Bio MDi technology platform, characterized by the use of monodisperse particles and precision-controlled chemistry, plays a pivotal role in achieving this goal. Figure 8 illustrates the analysis of AAV3 using three distinct lots of media, each created from a unique lot of monodisperse silica particles. The observed robust performance in this analysis showcases excellent lot-to-lot reproducibility, providing chromatographers with a consistent and dependable separation profile. This noteworthy consistency not only enhances the reliability of analytical results but also underscores the effectiveness of the SurePac Bio MDi technology platform in ensuring uniform performance across different column lots, reinforcing its suitability for rigorous and reproducible chromatographic studies.

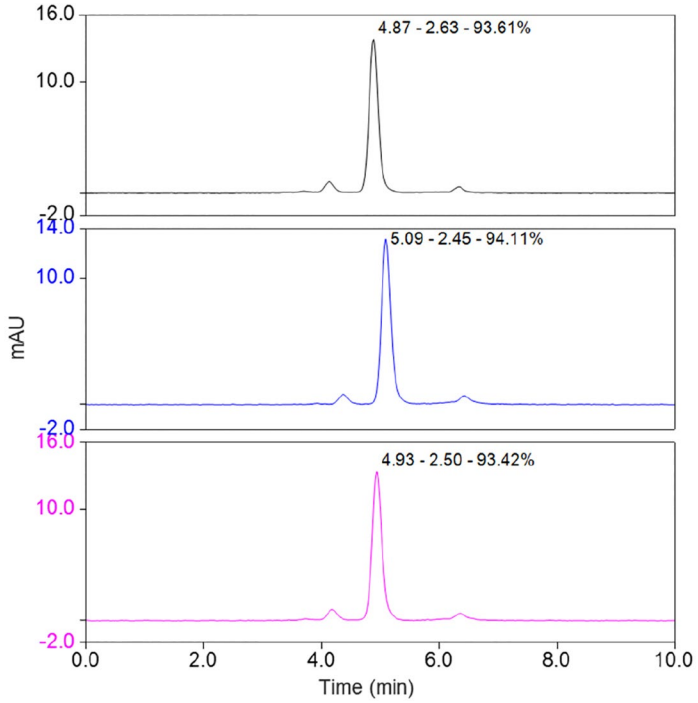

Figure 8. Chromatograms of AAV3 using three different lots of 3 µm SurePac Bio 550 SEC MDi columns using UV detection at 280 nm.
Peak label: RT (min) - Resolution between monomer and HMWS - %Area

Conclusions

- The SurePac Bio 550 SEC MDi column delivers baseline resolution of AAV monomer and aggregate peaks in less than 4 minutes.
- The SurePac Bio 550 SEC MDi column consistently delivers reliable separation of AAV across different lots.
- Careful optimization of flow rate, sample loading amounts, and buffer conditions is crucial for achieving accurate and consistent results in SEC AAV separation.
- The SurePac Bio 550 SEC MDi column outperforms other commercially available columns in AAV analysis, displaying advantages such as sharper peaks, improved resolution, and lower operational pressure.

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