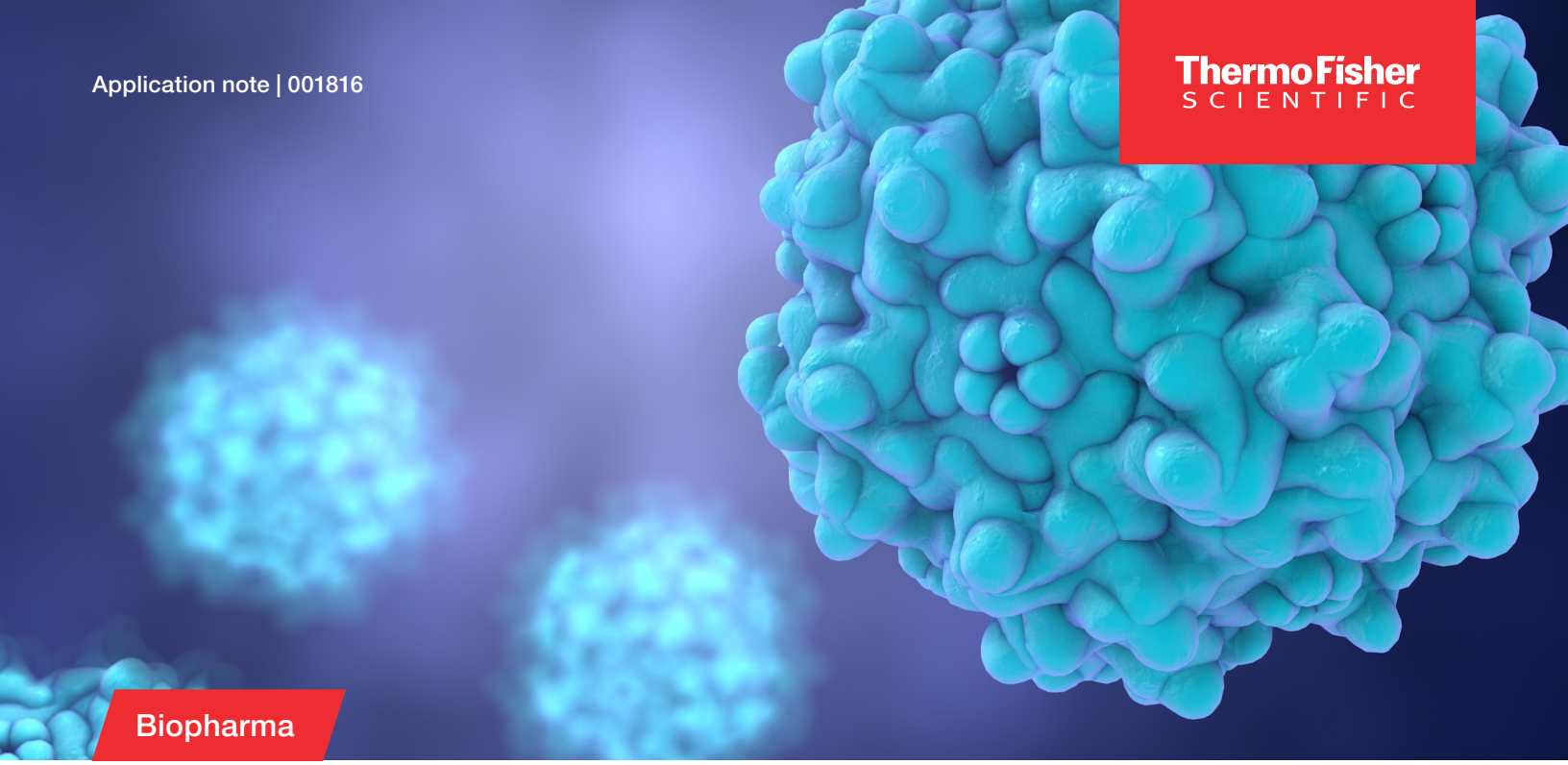
Application note | 001816



Biopharma

Salt gradient separation and analysis of adeno-associated virus samples using a 3 µm monodisperse strong anion exchange chromatography column

Authors

Ke Ma, Shane Bechler

Thermo Fisher Scientific, Sunnyvale, CA

Keywords

Biopharma, strong anion exchange, SAX, adeno-associated virus, AAV, empty capsid, full capsid, salt gradient, gene therapy, ProPac 3R SAX 3µm column, monodisperse, high performance liquid chromatography

Application benefits

- High-resolution separation of empty and full AAV capsids by anion exchange chromatography
- Easy and straightforward method development
- Consistent lot-to-lot performance

Goal

To demonstrate salt gradient method development and optimization for adeno-associated virus (AAV) empty and full capsid separation and characterization using a 2 × 50 mm strong anion exchange chromatography column packed with a 3 µm monodisperse SAX resin

Introduction

Adeno-associated viruses (AAV) are small replication-defective, non-enveloped viruses that can be used as vectors for gene therapy. AAVs are one of the most commonly used viral vectors for delivering genes due to their low immunogenicity, safety, and long-term transient expression.[1] Quantitation of the empty and full capsids of a given AAV therapeutic are important for understanding their quality and potential efficacy. Analytical ultra-centrifugation (AUC) using cesium chloride is a classic technique for separating and characterizing empty and full capsids; however, the process is time intensive and

often laborious. In contrast, liquid chromatography techniques enable straight-forward method development for separating and quantifying empty and full capsids peaks in addition to other impurities that may be present in the sample.

Anion exchange (AEX) chromatography is commonly used to characterize AAV particles as both the empty and full capsids typically have a negative surface charge. As such, AEX chromatography can be leveraged to separate and accurately quantify the AAV empty-to-full capsid ratio.[2-4] Strong anion exchange (SAX) chromatography is particularly suited for this application as the functional quaternary amine AEX group of the SAX phase is always charged. This enables adsorption and separation of AAV particles using a salt gradient with a buffer pH sufficiently greater than the AAV isoelectric point (pI). The empty capsid typically has a higher pI (~0.4 pH units on average) than the full capsid, which may be due in part to the full capsid being loaded with negatively charged DNA.[3] Conformational differences in the full capsid relative to the empty capsid likely contribute to the empty/full separation as DNA-loaded particles may have an increased number of accessible surface charges on the capsid. The resolution of the empty and full capsids due to the accessible surface pI difference is generally sufficient to separate and quantify the empty-to-full AAV ratio and characterize AAV heterogeneity. 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), AUC, etc.[4]

The Thermo Scientific™ ProPac™ 3R SAX column is well-suited for the analysis of empty and full AAV capsids. The 3 µm, non-porous, monodisperse resin is coated with a hydrophilic layer and quaternary ammonium groups to provide exceptionally high resolving power. Compared to traditional polydisperse particles (right image, Figure 1), the monodisperse particles have a consistent size distribution (left image, Figure 1) resulting in improved column packing and lot-to-lot reproducibility. The thin, hydrophilic layer grafted to the particle core and precisely controlled quaternary amine chemistry reduce secondary interactions between the stationary phase and sample to minimize band broadening. The quaternary ammonium functionality grafted to the hydrophilic layer introduces permanently charged cationic sites to provide the strong anion exchange character required for promoting sample binding. The reproducible resin chemistry and manufacturing processes minimize column variability as a concern in method development and data analysis. The PEEK (polyether ether ketone) column hardware has well-established bioinert properties to minimize nonspecific adsorption of protein samples compared to metal-based hardware.[5] These column properties make the ProPac 3R SAX column capable of analyzing complex samples with high resolution and excellent reproducibility. In this application note, we provide practical examples of method design using the ProPac 3R SAX column for the separation of AAV empty and full capsids using a linear salt gradient and a linear salt gradient with an isocratic hold on the ProPac 3R SAX column.

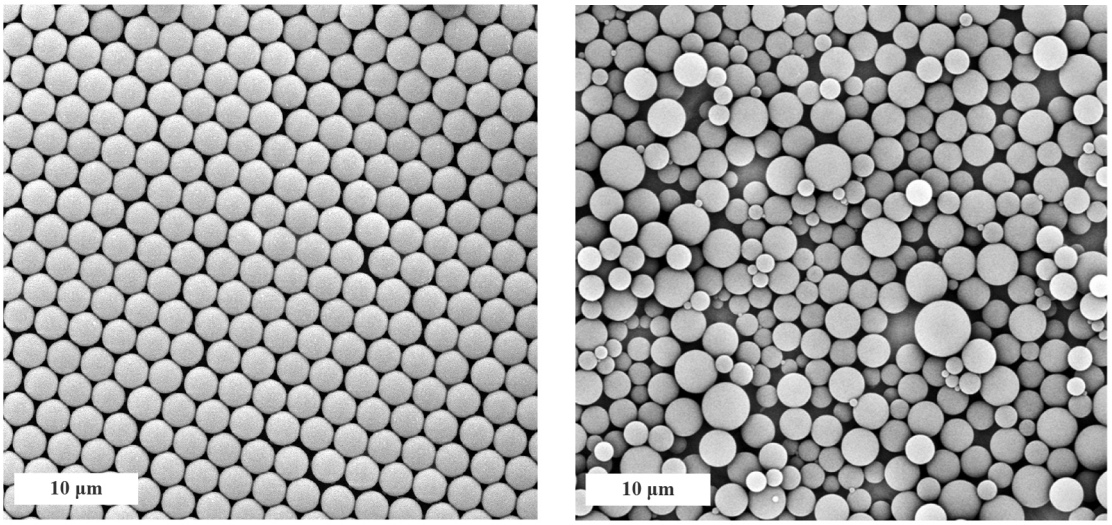


Figure 1. SEM image of 3 µm monodisperse particles (left) vs. 3 µm polydisperse particles (right). White scale bars are 10 µm in length.

Experimental

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Tetramethylammonium chloride (Sigma-Aldrich, P/N T19526)
- BIS-TRIS propane (Sigma-Aldrich, P/N B4679)
- 5N Hydrochloric acid (J.T.Baker, P/N 5618-02)
- AAV 1, 6, 8 Empty (Virovek, Hayward, CA)
- AAV 1, 6, 8-CMV-GFP (Virovek, Hayward, CA)
- 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

- AAV 1, 6, and 8 empty and full samples were used as received (2×10^{13} vg/mL).
- For linear salt gradient methods, AAV empty and full samples were mixed with a 1:10 ratio.
- For linear salt gradient with isocratic hold methods, AAV6 full sample was used directly without further modification.

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™ Fluorescence Detector (P/N VF-D51-A) with Thermo Scientific Vanquish™ Fluorescence Detector F Flow Cell (P/N 6079.4230)

Column

- ProPac 3R SAX, 3 µm, 2 × 50 mm (P/N 43203-052068)

For mobile phase compositions and gradient conditions including flow rate, column temperature, and injection volume, reference the text and figures in the results and discussion section. Excitation at 280 nm and emission at 330 nm were used for detection of all samples.

Data processing

The Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System (CDS) was used for data acquisition and analysis.

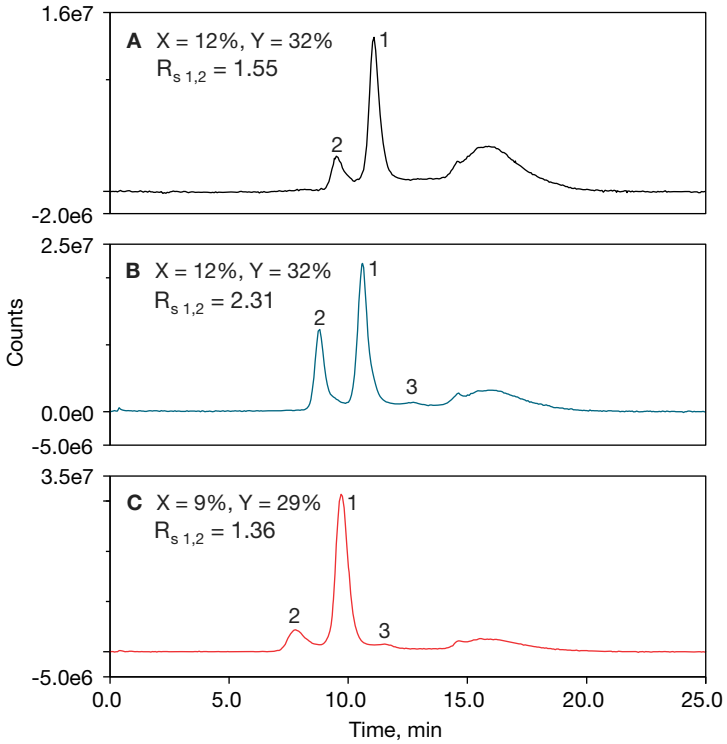
Results and discussion

In this section, we demonstrate a straightforward approach for the separation of empty and full capsids and associated impurities for three AAV samples (AAV1, AAV6, and AAV8). First, we evaluate the separation of empty and full capsids using a simple linear gradient. Second, we show the development of a method to optimize the separation of these components for AAV6 by incorporating an isocratic hold during the linear gradient. These basic approaches can be extended to other AAV samples to create methods for the rapid analysis of empty/full capsids and other sample impurities.

Because AAV analysis are often sample limited, a 2 × 50 mm column and a fluorescence detector (FLD) were used for these separations. The 2 mm column i.d. was chosen for the increased detection sensitivity provided by running at a low flow rate when using low sample mass loading. The high capacity of the 3 µm ProPac 3R SAX media enables the use of a short 50 mm column format without sacrificing separation performance. The FLD detector was chosen over a variable wavelength detector due to the greater sensitivity of fluorescence-based detection techniques when using limited sample quantities.

Linear salt gradient results

Figure 2 shows the analysis of AAV1, AAV6, and AAV8 samples using a simple linear salt gradient. Each AAV sample was created by mixing stock empty and full capsid samples in a 1:10 ratio. AAV1 and AAV6 mixed samples were analyzed using a linear salt gradient from 12% to 32% mobile phase B (MPB) over 15 minutes. The AAV8 mixed sample was analyzed using a gradient from 9% to 29% MPB over 15 min. Previous analyses of only the empty and full capsid samples were used to determine the elution time of each peak in the chromatograms (data not shown). For each of the AAV samples tested, empty (peak 2) and full (peak 1) capsid peaks were baseline resolved using the linear gradient indicating that a simple linear salt gradient is often sufficient for separating these peaks for the purposes of quantitation. For AAV6 and AAV8 samples, an impurity peak (peak 3) was observed to elute after the full capsid peak. For the AAV separations, the relative peak areas for empty capsids (peak 2) compared to full capsids (peak 1) are visually greater than 10% as expected based on the 1:10 mixing of empty:full standards. This is particularly obvious for AAV6 and may be due in part to the accuracy of sample concentrations from the supplier. However, we note that the fluorescence signal response for empty and full capsids is not equivalent, which will contribute to the differences in relative peak areas measured.[6] Calibration curves for both empty and full capsids would be needed to accurately measure the exact amounts of each capsid. For simplicity in this Application Note, we report the relative peak areas for empty and full capsids in subsequent analyses.



Column:	ProPac 3R SAX, 3 µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium chloride
Mobile phase C:	200 mM BIS-TRIS propane, pH 9.0

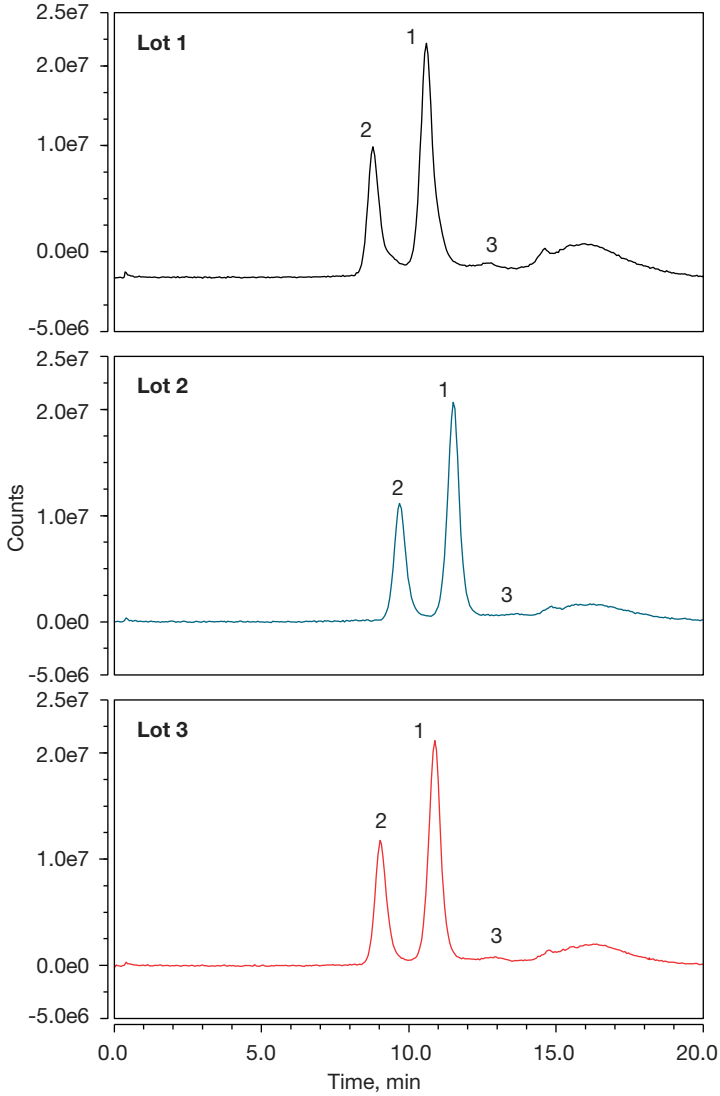
Gradient:

Time (min)	%A	%B	%C
0.0	90-X	X	10
1.0	90-X	X	10
11.0	90-Y	Y	10
11.1	0	90	10
13.0	0	90	10
13.1	90-X	X	10
25.0	90-X	X	10

Temperature:	20 °C
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	Concentration: 2 X 10^{13} vg/mL A: AAV1, Empty:Full 1:10 B: AAV6, Empty:Full 1:10 C: AAV8, Empty:Full 1:10
Peaks:	1. Full capsid 2. Empty capsid 3. Impurity

Figure 2. Linear salt gradient separation of full capsid AAV samples spiked with empty capsid to give a 1: 10 Empty:Full ratio: (A): AAV1 sample, (B): AAV6 sample, and (C): AAV8 sample

Using the method for spiked AAV6 sample analysis in Figure 2, we evaluated the lot-to-lot reproducibility for three different lots of ProPac 3R SAX media using the linear gradient as shown in Figure 3. Good lot-to-lot reproducibility with baseline separation for the spiked AAV6 sample is observed for ProPac 3R SAX columns.



Column:	ProPac 3R SAX, 3µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium chloride
Mobile phase C:	200 mM BIS-TRIS propane, pH 9.0

Gradient:

Time (min)	%A	%B	%C
0.0	78	12	10
1.0	78	12	10
11.0	58	32	10
11.1	0	90	10
13.0	0	90	10
13.1	78	12	10
25.0	78	12	10

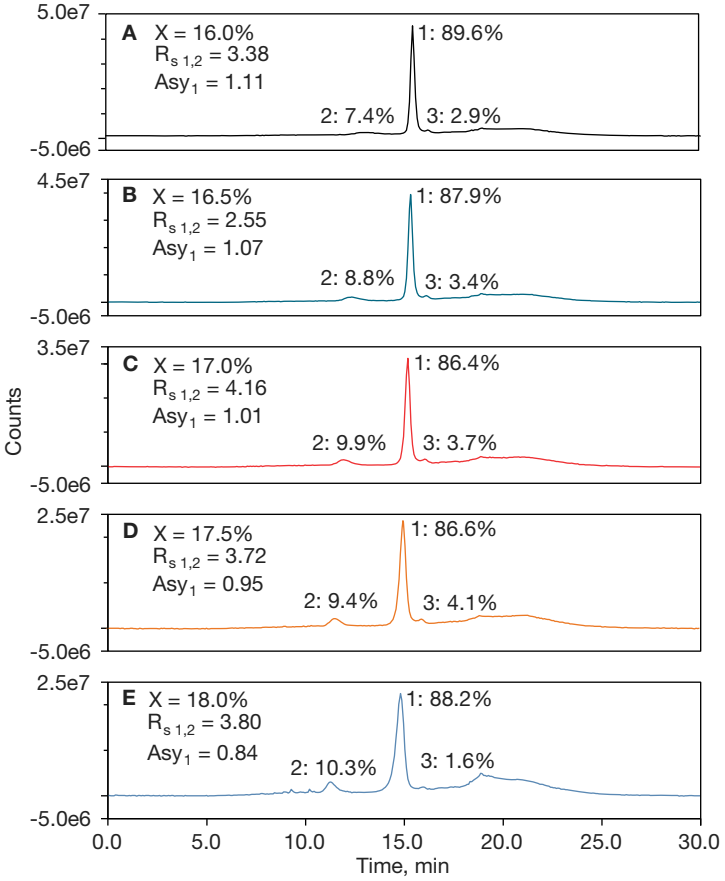
Temperature:	20 °C
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	Concentration: 2 X 10^{13} vg/mL AAV6, Empty:Full 1:10
Peaks:	1. Full capsid 2. Empty capsid 3. Impurity

Figure 3. Separation of spiked AAV6 sample on 3 different lots of ProPac 3R SAX media using a linear salt gradient

Linear salt gradient with isocratic hold method development

The simple linear salt gradient method is straightforward to implement and provides good separation for each of the AAV samples evaluated; however, further improvements to the separation between the AAV empty and full capsid may be required for more difficult to separate samples to either increase resolution or improve detection sensitivity. In this section, we demonstrate a method to improve the separation of AAV6 using a linear gradient by incorporating an isocratic hold midway through the method to increase the separation of the empty capsid from the full capsid. The isocratic hold is used to elute the empty capsid at a constant salt concentration that is insufficient to promote elution of the full capsid, which remains bound to the column stationary phase. After elution of the empty capsid, a linear gradient of increasing mobile phase B concentration is performed to elute the bound full capsid, resulting in significantly improved resolution between the AAV empty and full capsid.[3,4]

Figure 4 shows the results of different %MPB from 16.0% to 18.0% for the isocratic elution stage. Baseline separation of empty and full capsid are achieved for all the choices of isocratic %MPB tested; however, differences are observed in the quality of the separation. As the initial %MPB increases, the separation between empty and full capsid peaks improves as the empty capsid peak elutes earlier in the chromatogram. At lower %MPB (16.0% and 16.5%) for isocratic elution, the empty capsid peak is broad, and the signal strength is weak as the elution strength does not promote fast elution of the empty capsid. At higher %MPB (17.5% and 18.5%), the empty capsid elutes with increased signal strength and peak sharpness; however, the elution strength is such that the full capsid begins to elute isocratically as evidenced by fronting of peak 1. Based on the above analysis, using isocratic elution at 17.0% MPB is considered for further optimization of this method since the empty and full capsid are baseline separated, and the full capsid peak asymmetry is close to 1 (Asy_1 = 1.01).



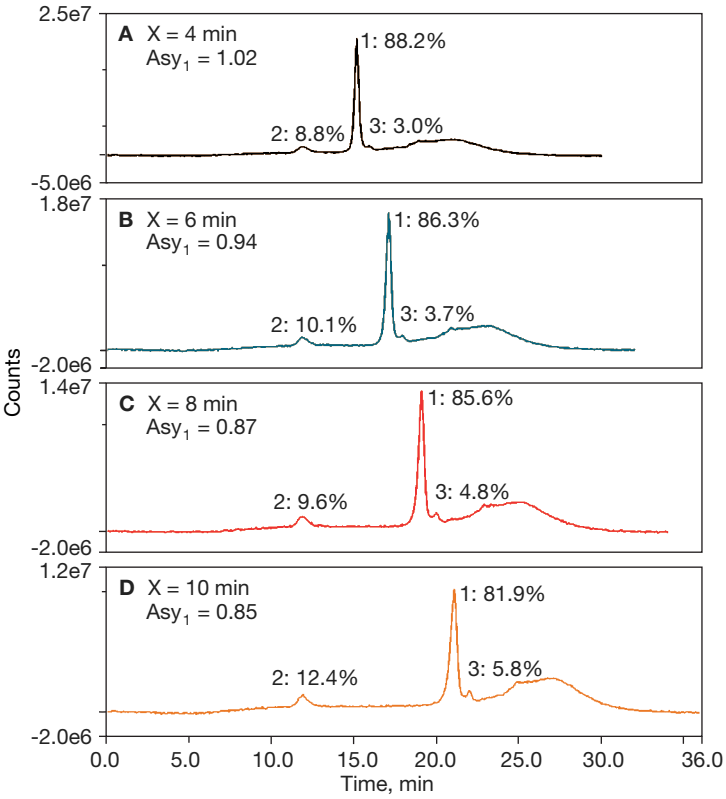
Column:	ProPac 3R SAX, 3µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium chloride
Mobile phase C:	200 mM BIS-TRIS propane, pH 9.0

Gradient:

Time (min)	%A	%B	%C
0.0	88	2	10
1.0	88	2	10
6.0	90-X	X	10
10.0	90-X	X	10
15.0	40	50	10
15.1	0	90	10
18.0	0	90	10
18.1	88	2	10
30.0	88	2	10

Temperature:	20 °C
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	AAV6 (2 X 10^{13} vg/mL)
Peaks:	1. Full capsid 2. Empty capsid 3. Impurity

Figure 4. Salt gradient separation of AAV6 separation using an isocratic hold at different %MPB: (A): 16.0%, (B): 16.5%, (C): 17.0%, (D): 17.5%, and (E): 18.0%



Column:	ProPac 3R SAX, 3µm
Format:	2 × 50 mm
Mobile phase A:	Water
Mobile phase B:	1 M Tetramethylammonium chloride
Mobile phase C:	200 mM BIS-TRIS propane, pH 9.0

Gradient:

Time (min)	%A	%B	%C
0.0	88	2	10
1.0	88	2	10
6.0	73	17	10
6.0+X	73	17	10
11.0+X	40	50	10
11.1+X	0	90	10
14.0+X	0	90	10
14.1+X	88	2	10
26.0+X	88	2	10

Temperature:	20 °C
Flow rate:	0.2 mL/min
Inj. volume:	0.2 µL
Detection:	FLD (Ex: 280 nm, Em: 330 nm)
Sample:	AAV6 (2 X 10^{13} vg/mL)
Peaks:	1. Full capsid 2. Empty capsid 3. Impurity

Figure 5. AAV6 analysis using a linear salt gradient with isocratic hold with different isocratic stage holding times: (A): 4 min, (B): 6 min, (C): 8 min, and (D): 10 min

Once the ionic strength (%MPB) at the isocratic stage is determined, the isocratic stage holding time can be further tuned to improve the separation between empty and full capsid. Figure 5 shows the method used in Figure 4 with an isocratic hold at 17% MBP with holds ranging in time from 4 min to 10 min. This shows separation of the empty (peak 2) and full (peak 1) capsid peaks due to the later elution of the full capsid peak with increasing isocratic holding time. However, as the isocratic stage holding time prolongs, there is a decrease in the intensity and relative peak area of the full capsid peak due to peak broadening. The full capsid peak also fronts more as asymmetry decreases from 1.02 to 0.85. The above evidence indicates that the full capsid also elutes slowly during the 17% MPB isocratic stage, which may lead to inaccurate quantitation of empty and full capsid if holding time is too long.[3] In the absence of additional information about the exact empty/full composition of the sample, we would recommend using 17% MPB for isocratic elution for 4 min as shown in Figure 5A. The user can employ orthogonal methods such as AUC or cryo-EM[4] in combination with AAV standards to determine which isocratic hold time method will provide the most accurate characterization of the empty/full ratio. These more time-consuming methods, however, would facilitate choosing the proper chromatography method, which could be used on a routine basis for fast and accurate characterization of AAV samples.

Conclusion

- The ProPac 3R SAX 3 µm column provides excellent separation of AAV empty and full capsids and other impurities using both a linear salt gradient and linear salt gradient with isocratic hold. The unique column design provides high resolution, robust performance, and lot-to-lot reproducibility needed for AAV analysis.
- Design of a linear salt gradient with an isocratic hold at an appropriate isocratic elution salt concentration and holding time can provide optimized methods for the separation and quantitation of AAV empty and full capsid.

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