Application Note

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Analysis of Full/Empty Capsid Ratios in Adeno-Associated Virus 1 and 6 Serotypes Using Biocompatible Liquid Chromatography

Excellent linearity and reproducibility using the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX strong anion exchange columns

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Abstract

Ion-exchange chromatography is an invaluable tool both for the purification of adeno-associated viruses (AAV) and analysis of their quality attributes. This study demonstrates the determination of the full/empty capsid ratio in AAV-1 and AAV-6 samples using the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX strong anion exchange columns. The method showed excellent linearity and reproducibility, establishing the 1290 Infinity II Bio LC as an advanced liquid chromatography system with industry-leading performance.

Introduction

During production of recombinant AAVs, a significant proportion of viral capsids contain no genetic payload and are therefore called "empty" capsids.[1] Empty capsids have been shown to lower the gene transduction efficacy of AAV preparations[2], and are therefore considered by regulatory authorities to be a process-related impurity that must be characterized.[3]

In this study, the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX column was used to determine the full/empty capsid ratio in purified AAV-1 and AAV-6 samples. The 1290 Infinity II Bio LC has a biocompatible flow path. When paired with Agilent nonporous poly(styrene divinylbenzene) (PS-DVB) Bio SAX strong anion exchange PEEK columns, the LC ensures the integrity of biomolecules by minimizing unwanted surface interactions, making it ideal for large molecule applications.

Experimental

AAV reference standards were bought from Vigene Biosciences as full- and empty-enriched samples. All chemicals were bought from Sigma-Aldrich, unless otherwise stated.

Instrumentation

Two configurations of the 1290 Infinity II Bio LC were used in this study (Figure 1). The LC was equipped with either a quaternary Flexible Pump, or binary High-Speed Pump. The Flexible Pump accommodates four solvent lines at once, reducing the number of different mobile phases that must be prepared during method development. The High-Speed Pump combines the highest efficiency mixing with the lowest delay volume providing excellent levels of sensitivity and reproducibility. Both configurations are resistant to corrosion from high salt concentrations and can withstand high backpressures of up to 1300 bar. An in-line Agilent 1260 Infinity II Fluorescence Detector was used to achieve the high sensitivity required for analysis of the relatively dilute AAV samples.

Figure 1. Agilent 1290 Infinity II Bio LC.

Chromatography

An Agilent Bio Sax NP5 column in PEEK hardware (2.1 × 50 mm, 5 µm, part number 5190-2472) was used to separate full and empty AAV capsids. Salt gradient elution was performed using the mobile phase and gradient conditions shown in Tables 1 to 3.

For the Flexible Pump method, Agilent Buffer Advisor Software was used to determine the percentages of each mobile phase component required to attain 70 mM *bis-tris* propane, pH 9.0, during the analysis. The AAV-1 and AAV-6 standards were diluted in 50 mM Tris-HCl, pH 7.4, before analysis, either individually as empty- or full-enriched, or as mixtures. Data acquisition and analysis was controlled by Agilent OpenLab CDS software using the integration settings shown in Table 4.

Table 1. Composition of mobile phases.

	Flexible Pump	High-Speed Pump
Type Of Pump	Quaternary	Binary
Mobile Phase Composition	A: De-ionized water B: 1 M tetramethylammonium chloride C: 0.3 M HCl D: 0.2 M *bis-tris* propane A to D contain 2 mM $MgCl_2$	A: 70 mM *bis-tris* propane, pH 9.0 B: 70 mM *bis-tris* propane + 1 M tetramethylammonium chloride, pH 9.0 A and B contain 2 mM $MgCl_2$

Table 2. Flexible Pump gradient conditions.

Time	%A	%B	%C	%D	Flow Rate (mL/min)
0	36.2	15	13.8	35	0.1
40	15.8	35	14.2	35	0.1
40.1	0.6	50	14.4	35	0.3
43	0.6	50	14.4	35	0.3

Table 3. High-Speed Pump gradient conditions.

Time	%A	%B	Flow Rate (mL/min)
0	85	15	0.1
25	72.5	27.5	0.1
25.1	0	100	0.3
28	0	100	0.3

Table 4. Agilent OpenLab CDS 2.3 software integration settings.

Parameter	Value
Slope Sensitivity	0.02
Peak Width	2
Area Reject	0.05
Height Reject	0.05
Shoulders Mode	Tangential
Tangent Skim Mode	Straight
Tail Peak/Skim Height Ratio	0.05
Front Peak/Skim Height Ratio	0.05
Skim Valley Ratio	20
Baseline Correction Mode	Classical
Peak-to-Valley Ratio	500
Blank Subtraction	Yes

Results and discussion

The relative response factors of AAV-1 and AAV-6 were first estimated by injecting identical numbers of capsids from either full- or empty-enriched samples. Full capsids were observed to be brighter than empty capsids with an apparent response ratio relative to empty capsids of approximately 1.2 for both serotypes. This value is in good agreement with the published response ratio of 1.3 for AAV-6.2.[4]

As shown in Figure 2, separations of full and empty capsids in the mixed samples were achieved with acceptable resolutions (R = 1.1 for AAV-1, and R = 1.3 for AAV-6) using the Flexible Pump. Sample recovery was found to be negatively correlated with flow rate (data not shown), which was consistent with observations reported in the literature.[5] Therefore, the flow rate for this method was maintained at a low value of 0.1 mL/min during the analytical gradient.

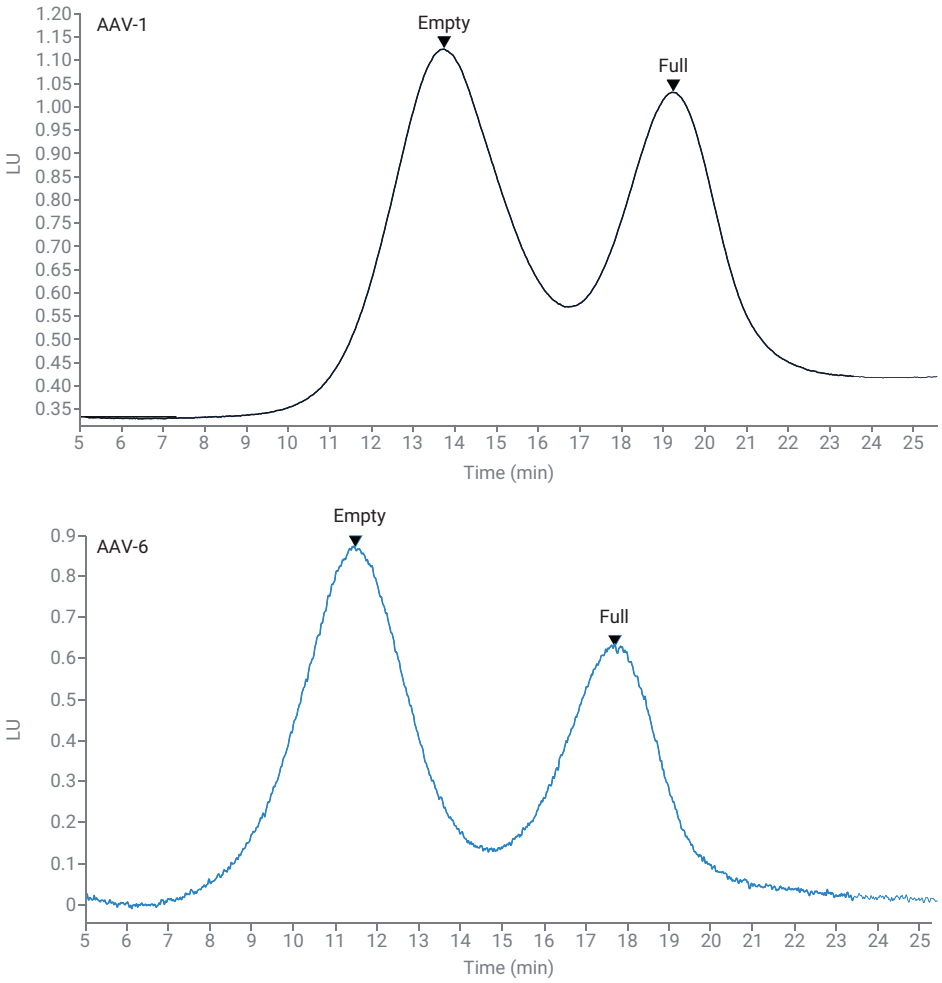

Figure 2. Separation of mixtures of full and empty capsids using the Agilent quaternary Flexible Pump.

The method was transferred to a 1290 Infinity II Bio LC equipped with a binary High-Speed Pump. Admixtures of full and empty capsids at different ratios were injected repeatedly over three days to assess the accuracy, linearity, and reproducibility of the assay. As shown in the overlaid chromatograms in Figure 3, the retention times and peak shapes of full and empty capsid peaks were reproducible over a wide range of capsid ratios.

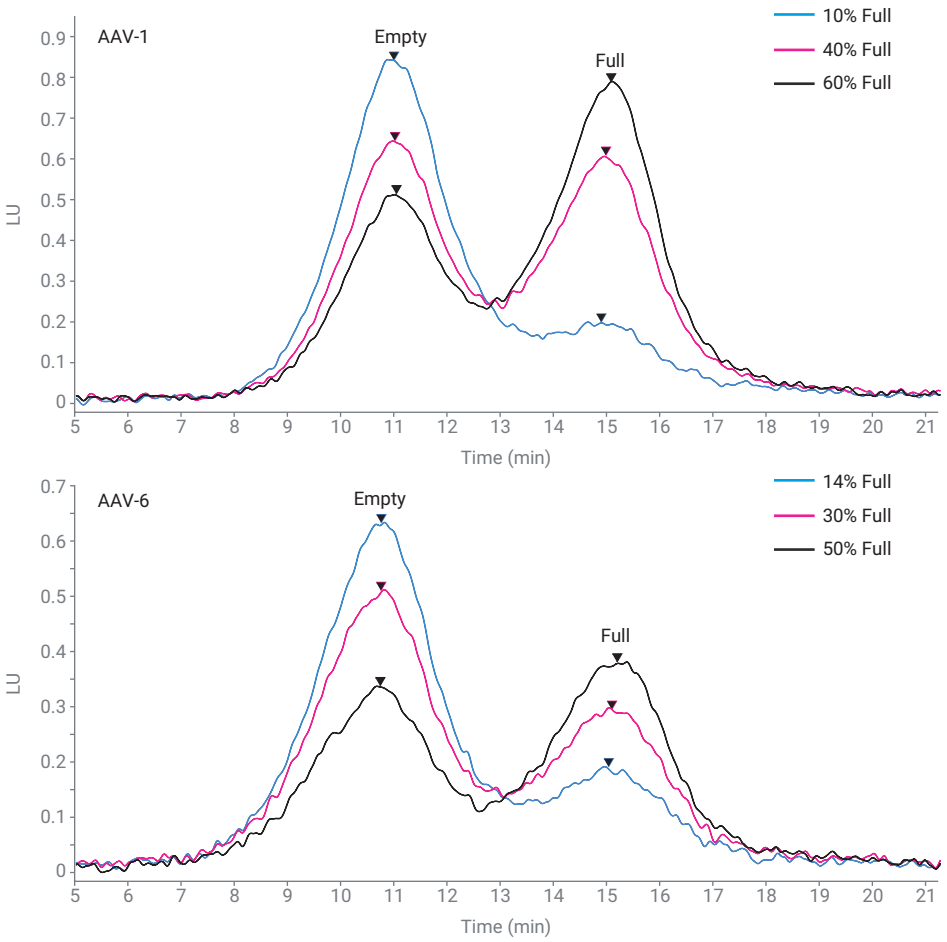

Figure 3. Separation of AAV-1 and AAV-6 samples with different full/empty capsid ratios using the Agilent binary High-Speed Pump.

As previously demonstrated[6], relative peak heights were found to provide more accurate and robust estimates of the full/empty capsid ratio compared to relative peak areas. This finding was because the peaks possessed some degree of tailing so were not fully resolved. Figure 4 illustrates the differences in linearity and accuracy for both AAV-1 and AAV-6 using either relative peak heights or peak areas as estimators. Linear trendlines for relative peak heights were closer to the origin and had higher R^2 values.

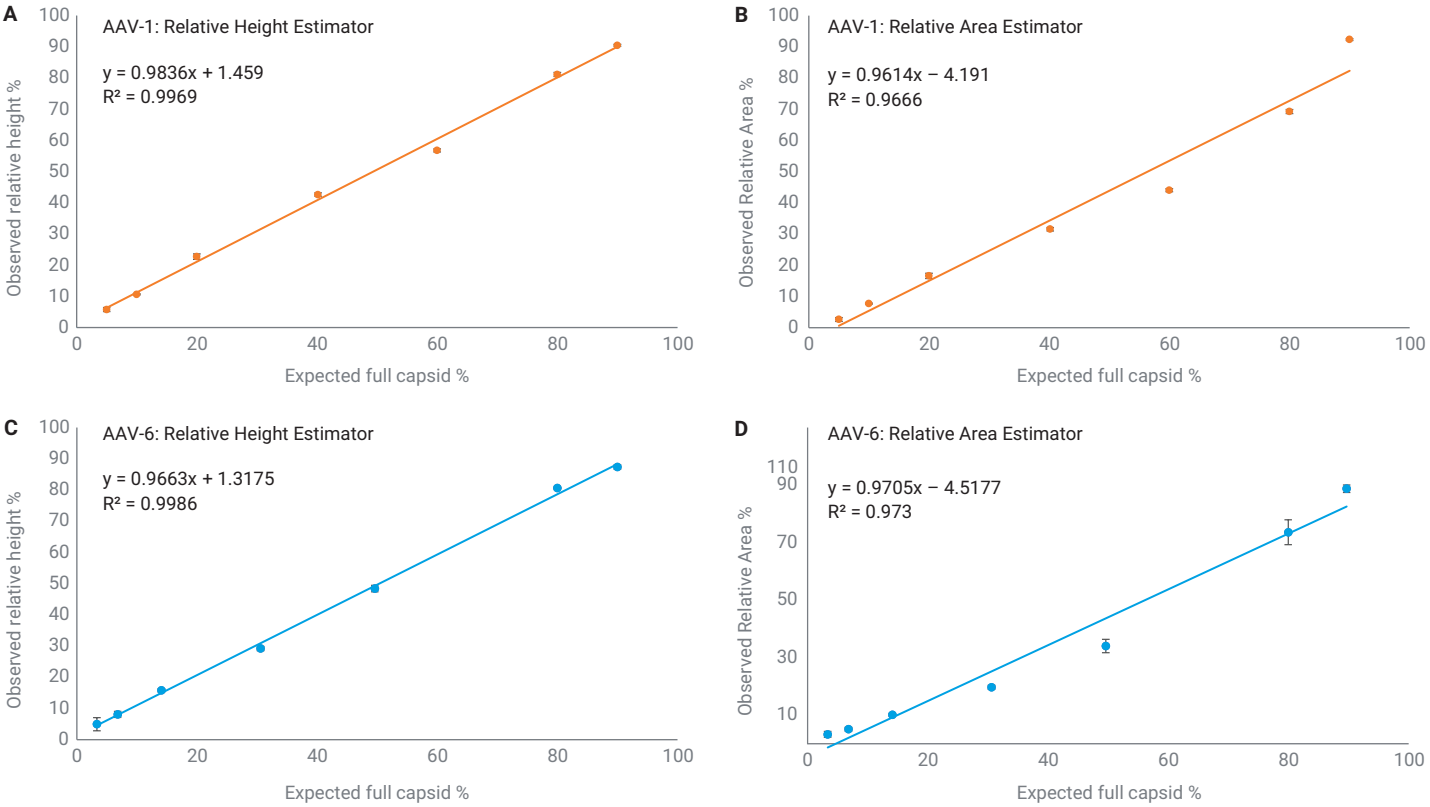

Figure 4. Comparison of relative peak heights and relative peak areas as estimators of full/empty capsid ratio. Relative peak height (A and C) provides superior accuracy and linearity compared to relative peak area (B and D).

Figure 5 shows the method's interday reproducibility. The relative standard deviations (RSD) of full/empty capsid ratios were ≤10.1% for AAV-1 and ≤11.2% for AAV-6 for samples containing between ~5 to 90% full capsids. The results show that the method has a broad linear range. The lower limit of quantitation for both serotypes were ≤5% full capsids, assuming an acceptable RSD of under 15%. The retention times for both full and empty capsid peaks were also highly reproducible, with RSDs of ≤2.4% for both serotypes for all tested samples.

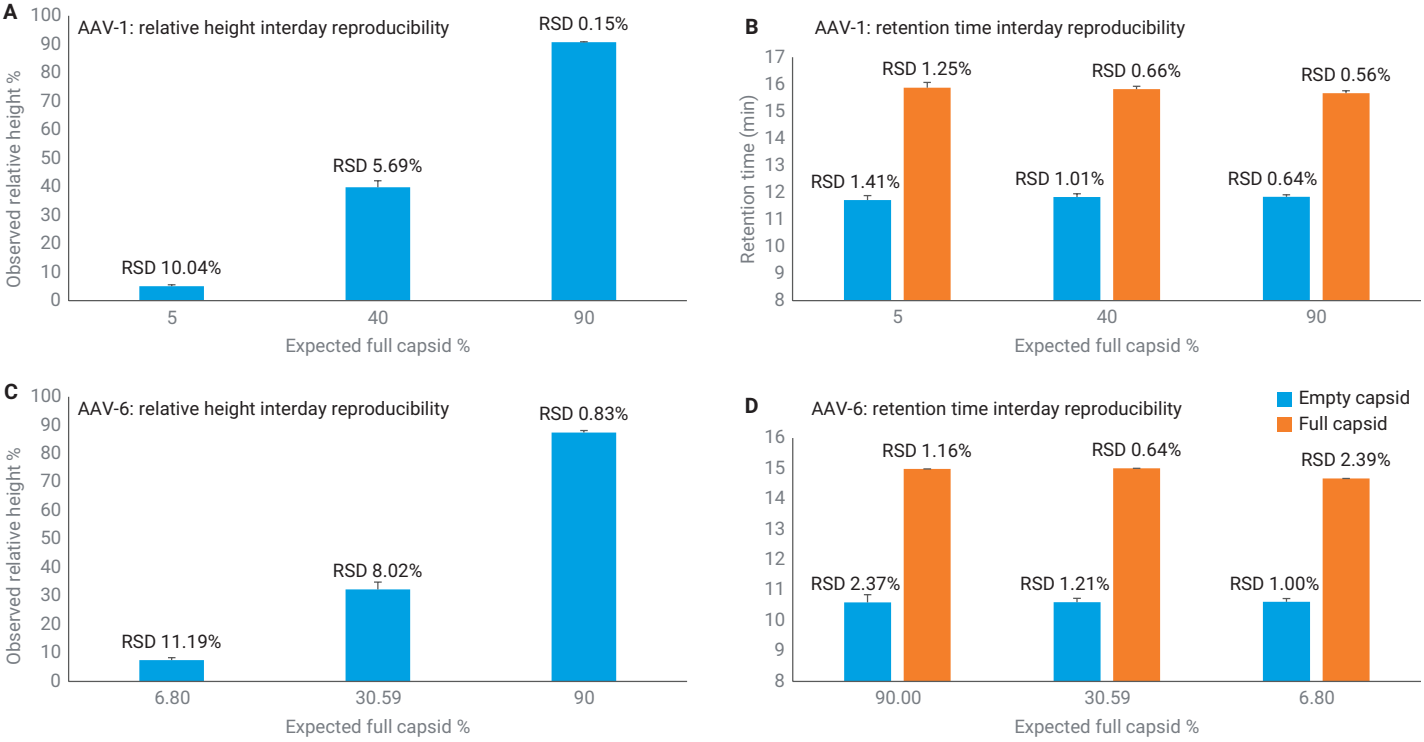

Figure 5. Interday reproducibility of relative height estimators and peak retention times.

Conclusion

This study shows that accurate and reproducible full/empty capsid ratios can be measured for AAV-1 and AAV-6 serotypes using the Agilent 1290 Infinity II Bio LC fitted with Agilent Bio SAX strong anion exchange columns.

References

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