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Application Note

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Roadmap Towards Robust Chromatographic Separation: AAV Capsid Separation Case Studies on CIM® PrimaS (HR) Monoliths

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Abstract

This application note thoroughly describes how small deviations in various chromatographic parameters can influence the separation of empty and full AAV (E/F AAV) capsids. Understanding how to ensure method reproducibility, is a key when dealing with delicate chromatographic separations. High control of parameters affecting the chromatographic method is essential to achieve consistent and reliable purification results. Even slight deviations in these parameters can result in changed outcomes of precise chromatography operations – examples being shifts in retention time, elution conductivity and reduced peak resolution.

Introduction

CIM® PrimaS (HR) monolith is a multimodal ligand, that exploits a combination of anion exchange and hydrogen bonding to achieve unique selectivity.

The main difference in chromatography performance between PrimaS® and PrimaS® HR (High Reproducibility) is that the HR line ensures consistent elution profiles across all column sizes or batches. In terms of resolution, impurity reduction, dynamic binding capacity (DBC), recovery, and other metrics, both PrimaS and PrimaS HR perform similarly. The differences between them arise from different release criteria, leading to different reproducibility performance. When using PrimaS HR, AAVs consistently elute under the same conditions, whereas with PrimaS, AAVs may elute across a broader range of conditions.

Although the following experiments focus on PrimaS HR, deviations in various chromatographic parameters have the same influence on both PrimaS and PrimaS HR columns.

One of PrimaS (HR) applications is separation of empty (E) and full (F) AAV capsids using an ascending linear pH gradient, thoroughly described in this publication.

Influence of critical chromatographic parameters on E/F AAV separation was demonstrated using a 0.2 mL specimen monolithic column extracted from large CIMmultus HR monoliths, tested with an internal AAV2/8 standard sample. Specimen is a small non-GMP unit, extracted from the same batch of bulk monolith as its parental column. Specimen from CIMmultus PrimaS HR matches chromatographic properties of its parental column, therefore specimen can be used to control lot-to-lot variabilities of its parental columns, as described in the following publication. Retention times of empty AAV capsids and resolution between empty and full capsids were used as output parameters for the evaluation of chromatographic reproducibility.

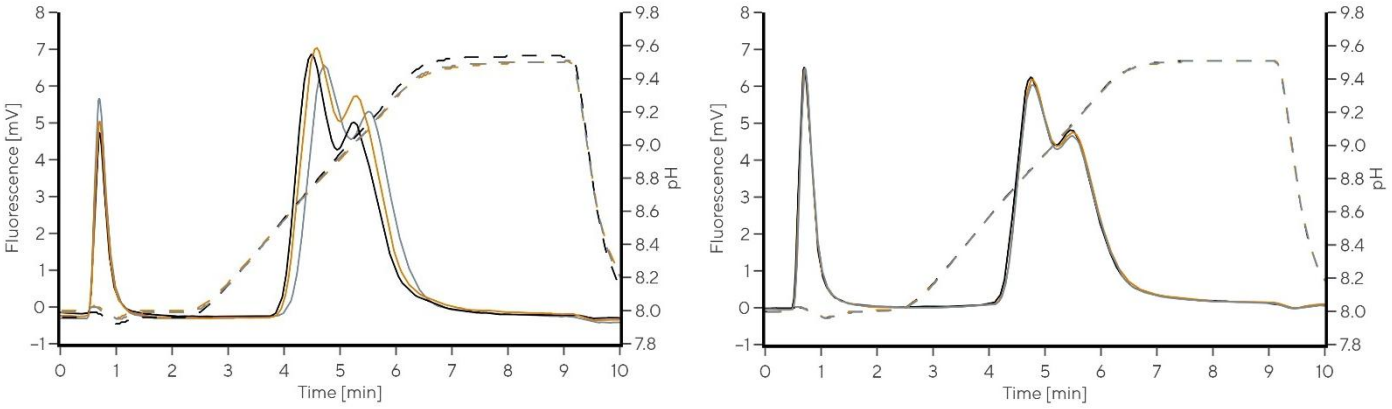
NOTICE: Specimen unit is only available for HR line of products.

The retention time of empty AAV2/8 capsids was selected as the parameter for column release, since retention time is much more reliable and stable to pH signal (see Notice below). The retention time in our method directly correlates with the elution pH and it was determined that 0.1 pH unit difference corresponds to a time interval of 0.27 minutes. Please note that this finding is based on internal testing and is not included in this document.

NOTICE: The monitored pH signal from the LC pH detector has a precision within a ±0.2 pH unit range and accuracy within a ±0.15 pH unit range. This level of accuracy is insufficient for distinguishing differences in CIM PrimaS (HR) batches when used for E/F AAV empty/full AAV separation.

Consistency in chromatographic process performance can be influenced by various factors, including variability in upstream sample, chromatographic conditions, amount of sample loaded, systems used, temperature, buffer pH, column batch-to-batch and scale-to-scale variations, and other additional factors. These factors can lead to inconsistent AAV capsid separations, as it is shown on the left side of Figure 1, where the variability of the same tested specimen is evident despite using normal good laboratory practice (GLP) requirements. This document guides users of CIM® columns to enhance the control of their chromatographic process, transitioning from the scenario on the left side of Figure 1 to the robust chromatographic process shown on the right side of Figure 1.

Figure 1: *Chromatograms of E/F AAV separation performed on the same specimen PrimaS HR column. Left: Overlay of chromatograms showing non-reproducible testing conditions, where retention times for empty AAV capsids shifts between the days for 0.22 min. Right: Control of main chromatographic parameters improves test reproducibility (retention times for empty AAV capsids shifts between the days for 0.07 min).*



NOTICE: Chromatographic effects, showed with CIM PrimaS HR columns in this document can be also applicable to other anion exchange based CIM columns (e.g. CIM QA, CIM QA HR, CIM PrimaT, CIM DEAE) and modalities (e.g., lentivirus purification etc.).

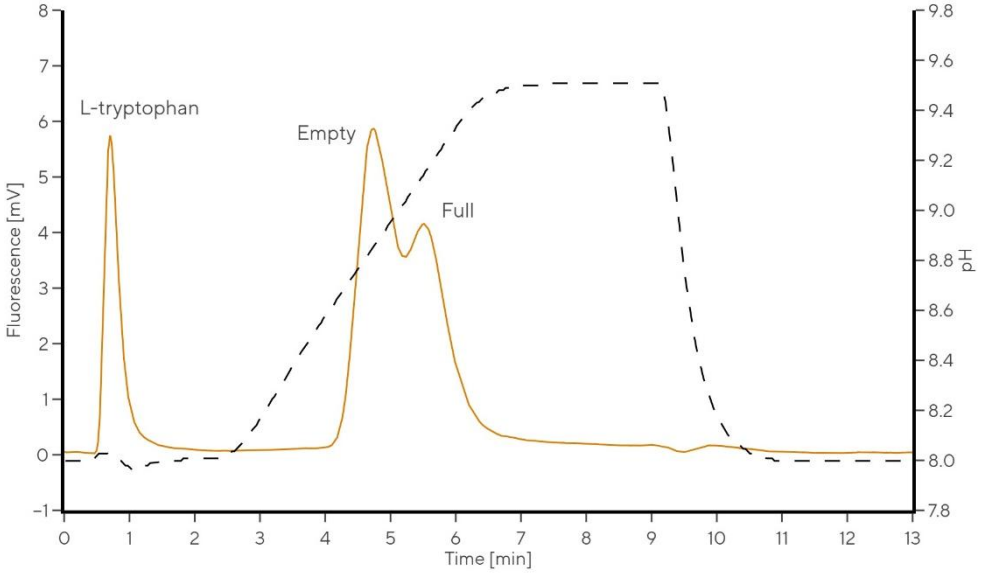
Methodology

Table 1: *Chromatographic method used for the study.*

Column	Specimen PrimaS HR, 0.2 mL, 2 µm channels size			
Chromatography system and configuration	▪ PATfix® biochromatography system. Details of the system configuration is described in Specimen QA HR Method Guide ▪ Autosampler temperature 8 °C ▪ Column thermostat temperature 23 °C ▪ pH detector ▪ Fluorescence (FLD) detector (Detector RF-20A, Shimadzu)			
Sample injection	100 µL of standard sample containing AAV2/8 and L-tryptophan (Sartorius BIA Separations)			
Mobile phases (MP)	**MPA:** 10 mM BTP + 10 mM Tris + 2 mM MgCl2 + 1 % D-sorbitol + 0.1 % poloxamer 188, pH 8.00 ± 0.05 **MPB:** 10 mM BTP + 10 mM TRIS + 2 mM MgCl2 + 10 mM NaCl + 1 % D-sorbitol + 0.1 % poloxamer 188, pH 9.50 ± 0.05 **Cleaning in place (CIP) solution:** 0.1 M NaOH + 2 M NaCl **Column neutralization buffer:** 100 mM CH3COOH, 1 M NaCl, pH 5.0			
Column preparation procedure	10 column volume (CV) ddH20 10 CV CIP 30 CV column neutralization buffer. 15 min incubation 30 CV ddH20 20 CV MPA at 23°C (column thermostat) 20 CV MPB at 23°C (column thermostat) 40 CV MPA at 23°C (column thermostat) Flow rate: 1 mL/min (5 CV/min).			
Chromatographic method	**Time [min]**	**Mobile phase A [%]**	**Mobile phase B [%]**	**Flow rate (mL/min)**
	0	100	0	1.0
	1	100	0	1.0
	5	0	100	1.0
	8.20	0	100	1.0
	8.22	100	0	1.0
	13	100	0	1.0

A typical chromatogram for AAV2/8 empty and full separation employing ascending pH gradient on 0.2 mL specimen from CIMmultus PrimaS HR column is shown in Figure 2.

Figure 2: *Reference chromatogram of E/F AAV separation on 0.2 mL PrimaS HR specimen.*



Results

We have identified the following chromatographic parameters as crucial for reliable separation of AAV 2/8 empty/full capsids:

- Buffer preparation
- Column neutralization/equilibration
- Temperature
- System void volume; time delay between detectors
- System comparison; binary analytical vs preparative
- Capillaries

Below are a few examples demonstrating how variations in chromatographic (method) parameters influence the separation of empty and full AAV capsids. These examples highlight the need for caution and precision to achieve successful and reproducible results.

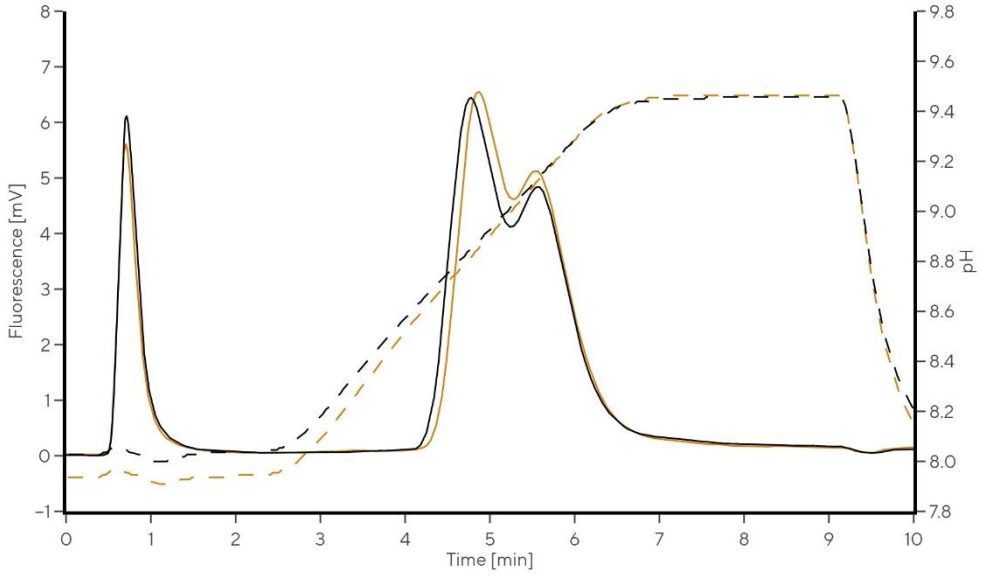
NOTICE: We have only focused to the parameters, where a substantial effect on the AAV separation reproducibility using CIMmultus PrimaS HR was observed. For other chromatography columns, the crucial parameters could be different.

Buffer Preparation

Figure 3 shows an overlay of two chromatograms, where the only variable was the difference in MPA preparation. The most reproducible and precise buffer preparation was achieved when pH of the buffer was adjusted by adding a fixed amount of standardized 1 M HCl. Such buffer preparation procedure allows us to prepare buffers within ± 0.05 pH consistency.

Using the procedure above, we have prepared two different MPA buffers, differing in the amount of added standardized HCl. One buffer contained 2 % w/w more 1 M HCl, which resulted in a 0.05 lower pH. The results from Figure 3 illustrate that such a small change led to a 0.1-minute shift in AAV elution. To ensure highly reproducible results, it is crucial to prepare buffers within not more than ± 0.05 pH difference.

Figure 3: *Chromatograms of E/F AAV separation on PrimaS HR specimen column using buffer A at pH 8.00 (black) and buffer B at pH 7.95 (yellow).*



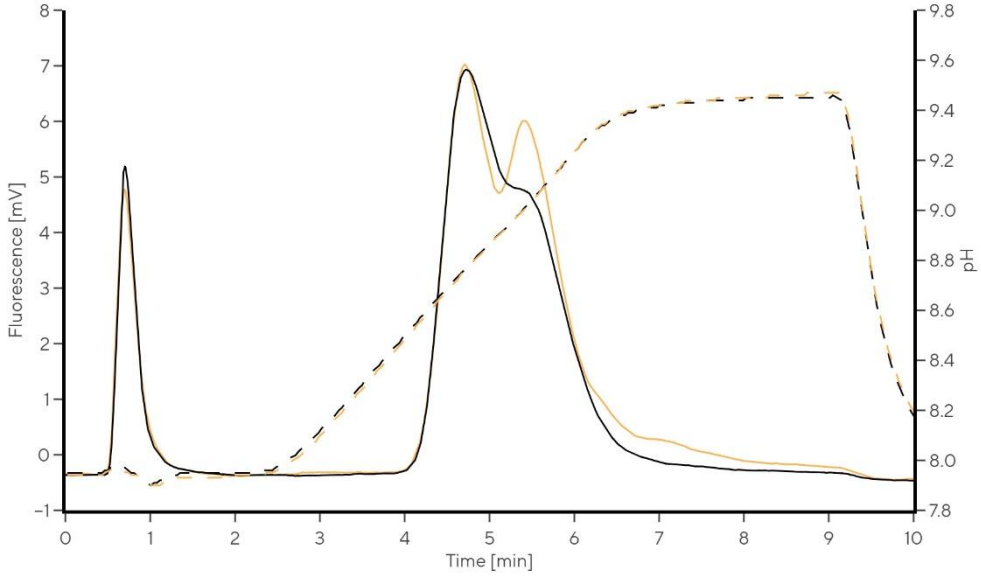
Buffer A	pH	t_{empty} [min]	R [/]
	7.95	4.87	0.57
	8.00	4.77	0.62

Our suggestion in buffer preparation is to titrate the buffer with the exact mass of standardized 1 M HCl and avoid buffer pH adjustment using a pH meter. Additionally, it is necessary to prepare fresh buffers daily. Aging the buffers for more than 2 days can affect the separation reproducibility and lead to results falling outside of the acceptance criteria. More details about buffer preparation practice at Sartorius BIA Separations can be obtained at help.bia@sartorius.com.

Column Neutralization | Equilibration

The effect of insufficient column neutralization after NaOH treatment is demonstrated in Figure 4. Standard neutralization/equilibration procedure after NaOH cleaning requests column neutralization with 100 mM CH3COOH, 1 M NaCl, pH 5.0 and water (yellow chromatogram). Inappropriate neutralization/equilibration step leads to loss of resolution (black chromatogram).

Figure 4: *Chromatograms of E/F AAV separation on PrimaS HR specimen column that underwent different neutralization/equilibration procedures).*



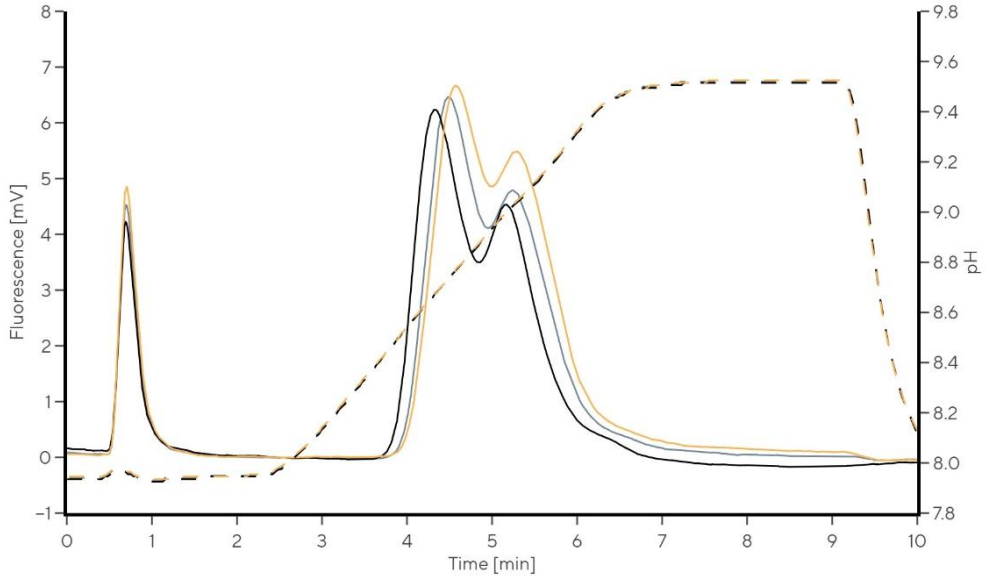
After CIP Washed With	t_{empty} [min]	R [/]
Buffer+dH_2O	4.71	0.58
dH_2O	4.74	0.38

NOTICE: Equilibration, optimized for PrimaS (HR) columns, cannot be directly translated to other surface chemistries. Proper equilibration protocol for each specific CIM column can be found in Instructions for Use.

Temperature Effect

E/F AAV separation at three different temperatures was tested to assess the impact of temperature on chromatographic elution (Figure 5).

Figure 5: *Chromatograms of E/F AAV separation on PrimaS HR specimen column performed in column thermostat at three different temperatures - 23°C, 26°C, and 30°C.*



	T [°C]	t_{empty} [min]	R [/]
	23	4.57	0.54
	26	4.49	0.57
	30	4.33	0.66

An increase in temperature improves the resolution between empty and full AAV capsids but also affects retention times (elution pH) over the limits of measurement uncertainty. The change of operating temperature from 23°C to 30°C shifts the elution pH for 0.24 min (corresponding to 0.09 pH units). When transferring linear gradient elution to step gradient elution without taking into account the temperature effect, this could lead to a significant purity decrease or recovery loss of full AAV capsids. Therefore, using a thermostat is one of the solutions enabling consistent and reproducible column performance for this specific application.

System Void Volume: Delay Between Detectors and Detector Response Time

The effect of void volume of the chromatographic path should be considered in the interpretation and evaluation of chromatographic parameters. This is particularly important when small volume columns are used on preparative systems with substantial system void volumes.

The time delay between in-series connected detectors, like the pH and fluorescence detectors, is an important factor, especially in short chromatographic methods. Since the analyte first passes through the pH detector and then through the fluorescence detector, this results in a time delay between the pH and fluorescence readings. More accurate results are achieved if the time delay (dt) between the pH and fluorescence detectors correction is considered – dt (pH-FLD).

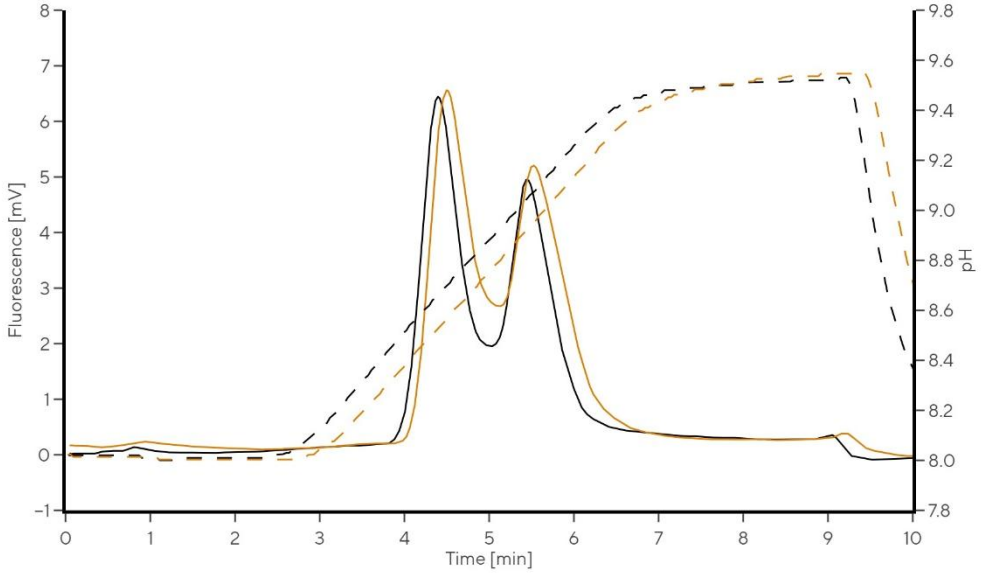
NOTICE: For precise determination of the dt (pH-FLD) correction, detailed instructions are provided in Specimen QA HR Method Guide.

The second hidden problem, when analysing data from two in-series connected detectors, is different response time of each detector. For example – pH probe could have longer response time than fluorescence, what shifts the peak detection. When the flow rate is increased with the same set of detectors, this shift changes, which blurs the comparison of chromatograms at different flow rates.

System Void Volume: Capillary Inner Diameter

Different capillary inner diameter (ID) has an impact on the retention time and resolution of the elution peaks (Figure 6).

Figure 6: *Chromatograms of E/F AAV separation on PrimaS HR specimen column – the effect of changing the capillary diameter. Resolution using capillaries with 0.25 mm ID is 1.17, while resolution using capillaries with 0.5 mm is 0.91.*



Capillary ID [mm]	t_{empty} [min]	R [/]
0.50	4.50	0.91
0.25	4.40	1.17

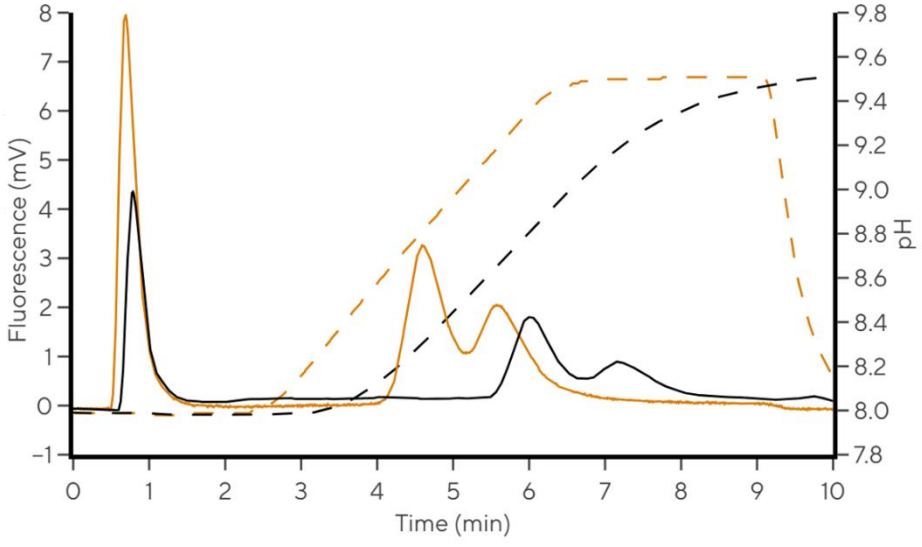
When using capillaries with ID 0.25 mm, the resolution between empty and full capsids increased by 20-25% compared to analysis using capillaries with ID 0.5 mm. The retention time shifts to the right simultaneously, but this change could be normalized with conductivity line.

System Void Volume: Comparison of Analytical vs. Preparative LC System

Variations in void volumes of the chromatographic systems are applicable across all chromatographic equipment; not only does capillary diameter affect outcomes, but also factors such as capillary length, void volume between chromatography skids of varying sizes, mixing chambers, and bubble traps can increase the void volume of the entire system and impact chromatographic resolution.

Different chromatographic systems can generate different chromatographic profiles, especially when comparing analytical vs. preparative chromatographic systems. In experiment depicted in Figure 7, the same method was employed on two different chromatographic systems – PATfix biochromatography system and ÄKTA Pure 150 M, as an example of an FPLC system

Figure 7: *Chromatograms of E/F AAV separation on PrimaS HR specimen column with two different chromatographic systems.*



	LC System	t_{empty} [min]	R [/]
	Analytical - PATfix	4.59	0.82
	Preparative - ACTA	6.02	0.87

Delay in elution of AAV sample was observed when method was run on the preparative system. This delay mainly corresponds to larger mixing chamber used on the preparative system (1.6 mL), whereas a smaller 0.25 mL mixing chamber was employed on the analytical system.

During scale-up of chromatographic methods, it is crucial to maintain a consistent ratio between the system's void volume and the column volume. Removal of unnecessary void volumes, such as bubble traps, across all systems should be considered. An increase in the ratio of void volume vs. chromatographic column volume during scale-up or scale-down may require adjustments, such as extending lengths of chromatographic steps to ensure reproducible E/F AAV results.

Other Parameters Influencing the Chromatographic Separation

The examples above highlight just a few of the parameters that can significantly impact the separation of empty and full AAV capsids. However, for highly reproducible results, variations in the following parameters could become equally important:

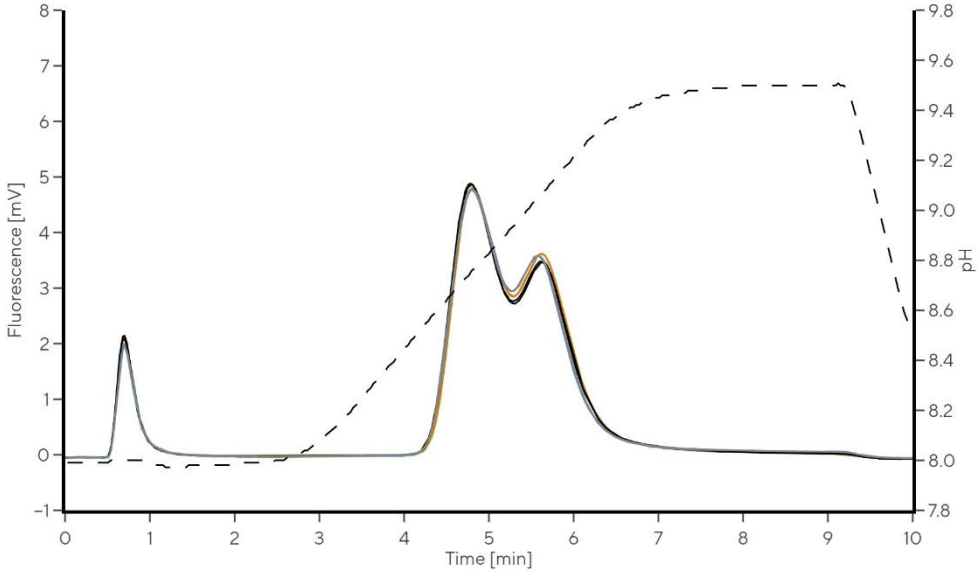
- post-column pressure,
- buffer degassing,
- detector calibration,
- amount of injected sample,
- sample stability,
- AAV sample sedimentation.

Inconsistencies between purification runs can arise if any of these factors are not reproducible. It is crucial to carefully consider each factor and assess all relevant parameters that could impact your target separation, as inconsistencies often result from multiple contributing factors.

Instead of Conclusions

By taking serious and cautious control over chromatographic parameters, day-to-day reproducibility can be improved from the scenario shown in Figure 1 (left side) to the level demonstrated in Figure 8.

Figure 8: *Standard AAV sample was analysed on the same PrimaS HR specimen column during the three-day period, each day two consecutive injections. Buffers were prepared each day separately.*



Day-to-day Reproducibility	t_{empty} [min]	R [/]
Day 1, Rep 1	4.81	0.66
Day 1, Rep 2	4.81	0.66
Day 2, Rep 1	4.79	0.67
Day 2, Rep 2	4.78	0.67
Day 3, Rep 1	4.81	0.65
Day 3, Rep 2	4.80	0.64

Retention times of the empty AAV capsids are between 4.78 and 4.81 min (0.01 pH unit difference), which corresponds to RSD of 0.2%. This represents a significant improvement in method robustness, enabling excellent and reliable control over the downstream purification process.

Ordering Information

Cat No.	Product Name
BIA-311.5119-2	CIMmultus PrimaS® HR 1 mL Monolithic Column (2 µm channels)
BIA-414.5119-2	CIMmultus PrimaS® HR 4 mL Monolithic Column (2 µm channels)
BIA-411.5119-2	CIMmultus PrimaS® HR 8 mL Monolithic Column (2 µm channels)
BIA-914.5119-2	CIMmultus PrimaS® HR 40 mL cGMP Compliant Monolithic Column (2 µm channels)
BIA-911.5119-2	CIMmultus PrimaS® HR 80 mL cGMP Compliant Monolithic Column (2 µm channels)
BIA-924.5119-2	CIMmultus PrimaS® HR 400 mL cGMP Compliant Monolithic Column (2 µm channels)
BIA-921.5119-2	CIMmultus PrimaS® HR 800 mL cGMP Compliant Monolithic Column (2 µm channels)
BIA-934.5119-2	CIMmultus PrimaS® HR 4000 mL cGMP Compliant Monolithic Column (2 µm channels)
BIA-931.5119-2	CIMmultus PrimaS® HR 8000 mL cGMP Compliant Monolithic Column (2 µm channels)
311.5118-2	CIMmultus PrimaS® 1 mL Monolithic Column (2 µm channels)
414.5118-2	CIMmultus PrimaS® 4 mL Monolithic Column (2 µm channels)
411.5118-2	CIMmultus PrimaS® 8 mL Monolithic Column (2 µm channels)
614.5118-2	CIMmultus PrimaS® 40 mL Monolithic Column (2 µm channels)
611.5118-2	CIMmultus PrimaS® 80 mL Monolithic Column (2 µm channels
814.5118-2	CIMmultus PrimaS® 400 mL Monolithic Column (2 µm channels)
811.5118-2	CIMmultus PrimaS® 800 mL Monolithic Column (2 µm channels)
BIA-1014.5118-2	CIMmultus PrimaS® 4000 mL Monolithic Column (2 µm channels)
901.5118-2	CIMmultus PrimaS® 8 mL cGMP Compliant Monolithic Column (2 µm channels)
914.5118-2	CIMmultus PrimaS® 40 mL cGMP Compliant Monolithic Column (2 µm channels)
911.5118-2	CIMmultus PrimaS® 80 mL cGMP Compliant Monolithic Column (2 µm channels)
924.5118-2	CIMmultus PrimaS® 400 mL cGMP Compliant Monolithic Column (2 µm channels)
921.5118-2	CIMmultus PrimaS® 800 mL cGMP Compliant Monolithic Column (2 µm channels)
934.5118-2	CIMmultus PrimaS® 4000 mL cGMP Compliant Monolithic Column (2 µm channels)
931.5118-2	CIMmultus PrimaS® 8000 mL cGMP Compliant Monolithic Column (2 µm channels)

References

Publication

Miklavčič, R., Simčič, T., Rotar, S., Komel, P., Žigon, R., Pavlovič, D., Bergoč, I., Ipavec, D., Simčič Zuljan, A., Žnidaršič, A., Kukanja, D., Vidič, J., Štrancar, A. and Černigoj, U. (2025), Development and Validation of AAV Capsids Separation on Specimen Columns for Reproducibility Evaluation of Large-Scale Chromatographic Monoliths. J Sep Sci., 48: e70114. https://doi.org/10.1002/jssc.70114

Gagnon P, Goričar B, Drmota Prebil S, Jug H, Leskovec M, Štrancar A. Separation of empty and full adeno-associated virus capsids from a weak anion exchanger by elution with an ascending pH gradient at low ionic strength. *BioProcess J*, 2021; 20. https://doi.org/10.12665/J20OA.Gagnon

Method guide

Specimen QA HR Method Guide | Sartorius BIA Separations

Additional Literature

Library Resources for AAV

https://www.biaseparations.com/library/aav/

Library Resources for CIM HR Line

https://www.biaseparations.com/library/cim-monolith-qa-hr-line/

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