Supplementary Materials

1. Supplementary Methods

1.1. Recovery Determination Using Cation-Exchange Chromatography (CEX)

In addition to anion-exchange–based separations, strong cation-exchange (CEX) chromatography was employed as an analytical tool to assess total AAV8 capsid recovery across downstream processing (DSP). Samples were diluted with the appropriate mobile phase A (MPA; Table S3F) and analyzed using a CIMac SO3 monolithic column (1.3 µm channel size, 0.1 mL column volume).

The chromatographic method consisted of sample loading followed by an isocratic hold at 100% MPA for 3 min (30 column volumes – CVs) to ensure complete binding. A linear gradient from 100% MPA to 100% MPB was applied over 3 min (30 CVs), followed by a 2 min (20 CVs) hold at 100% MPB. A cleaning-in-place (CIP) step using 100% MPC was subsequently performed (2 min, 20 CVs), after which column regeneration, equilibration, and conditioning were carried out according to standard operating procedures. All analyses were conducted at a constant flow rate of 1 mL/min, with multiple MPA blank injections performed before sample analysis to establish a stable detector baseline. To minimize carryover, DSP samples were injected in order of decreasing purity.

2. Supplementary Results and Discussion

2.1. Additional Method Optimization on AEX

To avoid extensive DEAE method optimization, which is beyond the scope of this study, we adopted an approach adapted from a previous report [9], in which longer-chain counter-anion organic salts in the presence of magnesium were shown to enhance E–F separation on QA monoliths. As an equivalent 3-C-chain magnesium salt (magnesium propionate) was not available, we titrated our buffers with propionic acid rather than the standard hydrochloric acid to adjust the pH from approximately 10.0 to 8.0. Following this strategy, we observed a modest improvement in separation when using buffers titrated with propionic acid (Figure S1B). Based on these results, we concluded our optimization efforts regarding salt or organic solvent screening.

The most common methods for E–F separation are based on salt-gradient elution, which remains the primary elution mechanism to this day [9,39]. Encouraged by the strong influence of pH on DEAE (Figure 1), we evaluated whether a pH gradient alone could further enhance

the E–F separation on DEAE. The rationale was to modulate the charge of AAV8 capsids by exceeding their isoelectric point (pI) and shifting toward alkaline pH, thereby neutralizing the net charge on the CIMac DEAE column and promoting capsid elution (Figure 2).

For the initial pH-gradient experiment, we selected a loading pH of 6.0, near the pI of AAV. During titration of a weak AEX such as DEAE under a pH gradient, changes in proton concentration cause the buffer pH to vary. These pH changes depend on the buffer composition and the column's titration behavior, so the pH shifts as the buffer flows through the column. In contrast to weak AEX, strong AEX, such as QA, is not affected by these pH changes [64]. Buffer compositions were therefore adopted from a recent study on a multimodal weak AEX – CIMac Swiper [40] to ensure a smooth pH gradient, which is particularly critical when working with a weak AEX, where the buffer compositions and the column's titration curves result in changes in pH as the buffer passes through the column [64].

However, using a pH gradient alone (Figure S2) did not yield improved separation than that achieved with a salt gradient (Figure S1). The E–F resolution obtained was only about 1.00, indicating that a pH gradient is not suitable for enhanced AAV8 elution from a weak AEX. From this pH screening, it is further evident that acidic and neutral pH conditions are not favourable for improved E–F separation on DEAE, as the best results were obtained at a loading pH of 8 in both salt and pH-gradient experiments (Figure S1 and Figure S2). This finding that an alkaline pH is needed to improve E–F separation is consistent with our initial hypothesis and with the results reported in [9] for QA HR columns. A similar trend may be expected for cation-exchange (CEX) columns, where improved separation would likely occur at mildly elevated pH rather than at strongly acidic conditions.

2.2. Assessment of AAV8 Capsid Recovery and Empty–Full Separation Using Cation Exchange Chromatography

Cation-exchange (CEX) chromatography is mainly used in AAV downstream processing (DSP) as a capture step for enriching total capsid populations and is generally not expected to provide effective empty–full (E–F) capsid separation [9,31,32,65,66]. According to these studies, strong CEX materials, such as CIMac SO3, are also commonly used in the analytical workflows to discriminate total AAV capsids from process-related impurities, particularly when coupled with multi-angle light scattering (MALS) detection. Compared with commonly used UV detectors (260 nm for nucleic acid–related impurities and 280 nm for protein-related

impurities) and fluorescence detectors for protein-related impurities, multi-angle light scattering (MALS) provides enhanced detection selectivity by distinguishing larger particles, such as AAV, from smaller species, such as proteins [9,31,32]. As illustrated in Figure S3, only MALS (LS monitored at 90° angle) clearly resolved the AAV peak from other co-eluting nucleic acid- (UV 260nm) and protein-related (UV280 nm, FLD) impurities. Moreover, the LS chromatographic signal of the initial harvest sample largely overlaps with that of the purified DSP sample, exhibiting minimal or no peak shoulders compared to the UV or fluorescence signals, in agreement with previous studies [9,31,32]. Under acidic (pH 3.5) analytical conditions (see Table S3F), AAV capsids—independent of serotype or genome content—typically elute as a single, well-defined peak (Figure S3), consistent with previous reports [31,66]. This behavior enables robust relative estimation of total AAV recovery across the DSP by combining chromatographic MALS peak areas with sample volume and dilution factors. Relative recovery values obtained using CEX–MALS were compared with orthogonal techniques, including digital PCR (dPCR) and ELISA, as summarized in Table S2 (more detailed conditions are written in section 1. Supplementary Methods). Compared with these conventional assays, CEX-based recovery determination provides a rapid, high-throughput alternative for monitoring overall process performance.

Overall and DSP step recoveries were calculated by directly incorporating the MALS signal area (specifically, the integrated peak area of light scattering (LS) monitored at 90° angle) into Equation 1. No extinction coefficient was applied, as this approach is based on relative quantification by comparing the signal area of each in-process step to that of the initial load step. The baseline was defined with the injection of a mobile phase A (blank run).

Equation (1): Relative recovery estimation during DSP. Annotations: Ri—recovery of individual DSP sample (i) based on its initial harvest sample (Load); Vprep—total volume of AAV sample [mL]; D—dilution factor; A—area of AAV LS 90°signal peak [mVs].

$$Ri\ [\%] = \frac{(Vprep\ x\ D\ x\ A)i}{(Vprep\ x\ D\ x\ A)Load}$$

However, beyond theestablished analytical role of CEX chromatography, we explored whether partial E–F separation could be achieved directly on a strong CEX column, despite prior evidence suggesting that such separation is unlikely. This exploratory assessment was motivated by the broader mechanistic hypothesis developed throughout this study, namely that pH-dependent modulation of capsid surface charge governs separation behavior across ion-

exchange modalities. However, consistent with observations on AEX, lower pH conditions are expected to adversely affect E–F resolution, as previously reported [67].

AAV8 samples were therefore loaded onto the CIMac SO3 column under the mildest feasible acidic to near-neutral conditions (pH 6.5, see Table S3D). At lower pH values (such as e.g., pH 3.5 as commonly used in capture steps), SO3 typically produces a single, symmetric AAV peak (Figure S3). However, operating at such low pH values has been reported to compromise capsid integrity and infectivity [68,69]. Moreover, exposure to acidic conditions can promote capsid dissociation, aggregation, or dimer formation, as recently reported [67].

Although a slight peak asymmetry was observed under these conditions (Figure S4), no distinct E–F separation was achieved. Increasing the loading pH further would likely have resulted in non-robust binding and partial elution in the flow-through, as elution already occurred near the onset of the salt gradient. Consequently, no further E–F separation studies were pursued on the CIMac SO3 column.

Taken together, these results confirm that acidic and near-neutral pH conditions provide little to no E–F resolution on CEX media, consistent with trends observed for AEX systems, and further emphasize the central role of capsid charge modulation in governing chromatographic separation behavior.

3. Supplementary Figures and Tables

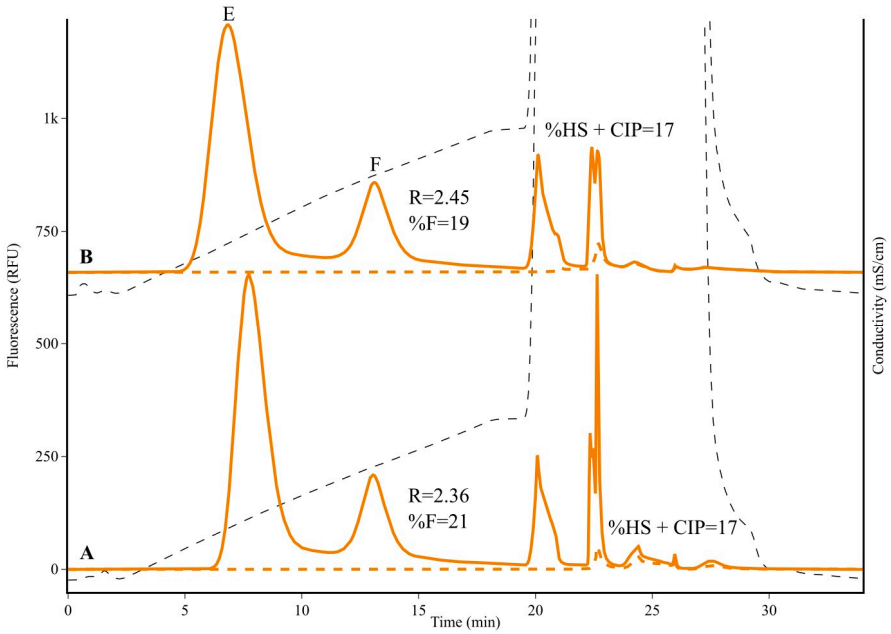


Figure S1. Minor method optimization on DEAE using magnesium acetate gradients: (A) 2–25 mM magnesium acetate gradient (pH 8.0, HCl-titrated); (B) 2–25 mM magnesium acetate gradient (pH 8.0, propionic acid–titrated).

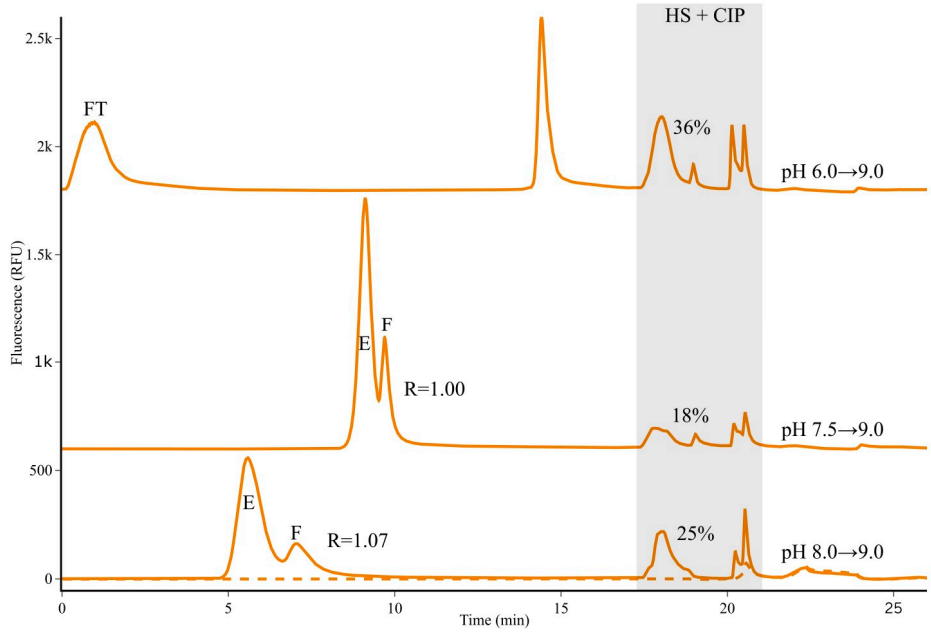


Figure S2. Empty–full (E–F) capsid separation on DEAE using pH-gradient elution. FT = flow-through.

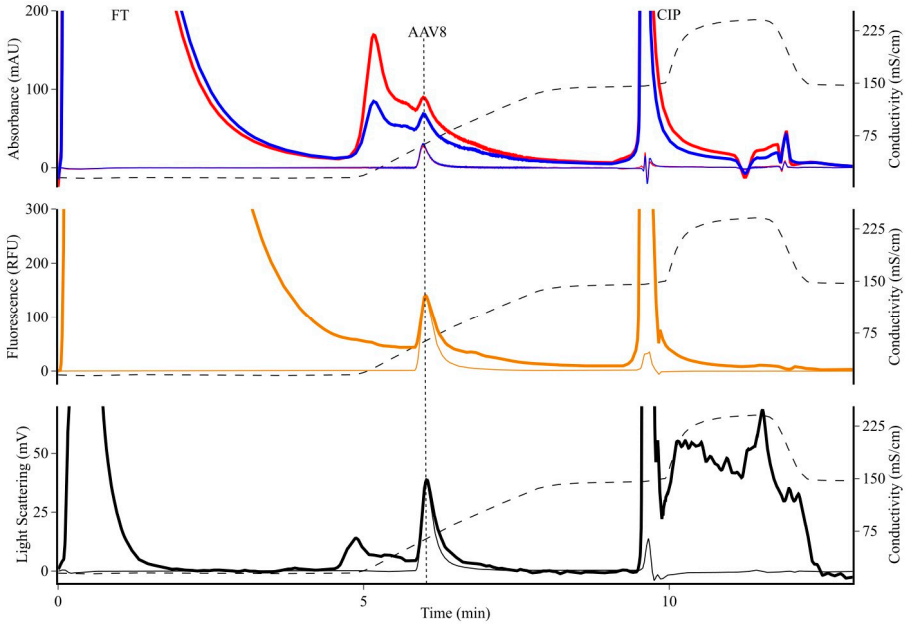


Figure S3. Capture behavior of AAV8 capsids on a strong cation-exchange (CEX) monolithic column (SO3). Annotations: red—UV 260 nm; blue—UV 280 nm; orange—tryptophan fluorescence; black—light scattering (90°); black dashed—conductivity. FT = flow-through; CIP = cleaning-in-place. The bold chromatographic trace corresponds to the initial harvest sample, whereas the lighter trace represents the pre-purified AAV sample.

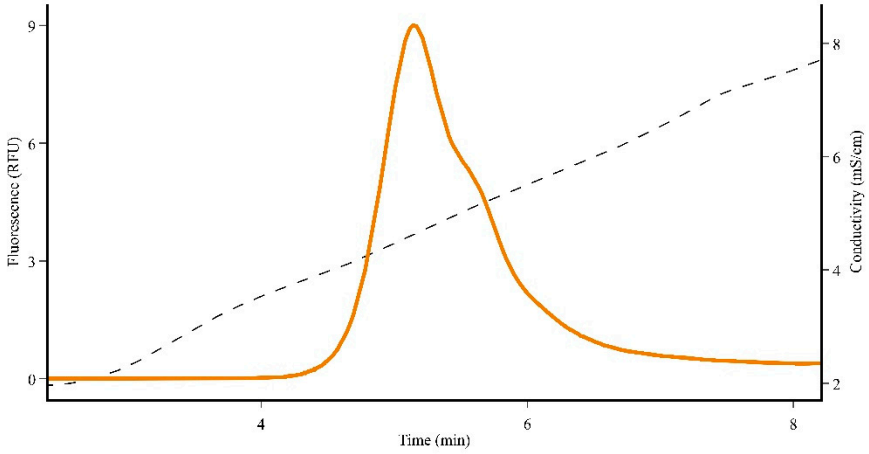


Figure S4. Attempted empty–full (E–F) separation of AAV8 capsids on a strong CEX (SO3) monolithic column. Annotations: red—UV 260 nm; blue—UV 280 nm; orange—tryptophan fluorescence; black—light scattering (90°); black dashed—conductivity.

Table S1. Composition of commonly used in-house downstream process buffers evaluated for AAV8 sample dilution and in-line buffer exchange chromatographic analysis.

Buffer	Composition
Formulation Buffer	20 mM Tris + 2 mM $MgCl_2$ + 150 mM NaCl + 0.01% Poloxamer; pH 7.0
SEC Buffer	10 mM K_2HPO_4 + 200 mM KCl + 0.05% Poloxamer; pH 7.4

QA HR Buffer (adopted from [9])	20 mM Tris + 2.5% EtOH + 2 mM $MgAc_2$ + 1% sorbitol; pH 8.5
QA HR Buffer (adopted from [9]; higher pH)	20 mM Tris + 2.5% EtOH + 2 mM $MgAc_2$ + 1% sorbitol; pH 9.5
SO3 Buffer	50 mM Na-citrate + 1% sorbitol + 0.01% poloxamer, pH 3.50
DGUC Buffer	100 mM Tris, 3 M CsCl, 0.1 % Poloxamer, pH 7.4

Table S2. Recovery assessment of AAV8 capsids using PATfix CEX and orthogonal analytical methods. *dPCR is not suitable for recovery determination after QA loading due to the selective enrichment of empty and full capsids.

Sample	Recovery [%]		
	PATfix CEX (MALS)	dPCR	ELISA
Clarified harvest	87	109	132
TFF Retentate	116	121	157
SO3 Load	78	78	133
SO3 Eluate	52	52	43
QA Load	49	50	88
QA Empty	24	3*	25
QA Full	26	43*	23

Table S3. Summary of working buffers used in chromatographic experiments.

A

Column	Composition
DEAE; pH 6.0	MPA: 20 mM MES + 5 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 6.0 MPB: 20 mM MES + 50 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 6.0 MPC: 20 mM MES + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 6.0
DEAE; pH 6.5	MPA: 20 mM MES + 5 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 6.5 MPB: 20 mM MES + 50 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 6.5 MPC: 20 mM MES + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 6.5
DEAE; pH 7.0	MPA: 20 mM TRIS + 5 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 7.0 MPB: 20 mM TRIS + 50 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 7.0 MPC: 20 mM MES + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 7.0
DEAE; pH 7.5	MPA: 20 mM TRIS + 5 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 7.5 MPB: 20 mM TRIS + 50 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 7.5 MPC: 20 mM MES + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 7.5
DEAE; pH 8.0	MPA: 20 mM TRIS + 5 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.0 MPB: 20 mM TRIS + 50 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.0 MPC: 20 mM MES + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 8.0
DEAE, QA; pH 8.5	MPA: 20 mM TRIS + 5 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.5 MPB: 20 mM TRIS + 50 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.5 MPC: 20 mM MES + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 8.5

B

Column	Composition
DEAE; pH 8.0 (titration with hydrochloric or propionic acid)	MPA: 20 mM TRIS + 2 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.0 MPB: 20 mM TRIS + 25 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.0 MPC: 20 mM TRIS + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 8.0

C

Column	Composition
DEAE; pH 6.0 → 9.0	MPA: 2 mM $MgAc_2$ + 10 mM MES + 10mM HEPES + 15mM BTP + 1% Sorbitol + 2.5% EtOH; pH 6.0 MPB: 2mM $MgAc_2$ + 10mM MES + 10mM HEPES + 15mM BTP + 1% Sorbitol + 2.5% EtOH; pH 9.0 MPC: 0.5 M NaAc; pH 5.5 MPD: 0.1 M NaOH + 2 M NaCl
DEAE; pH 7.5 → 9.0	MPA: 2mM $MgAc_2$ + 10mM MES + 10mM HEPES + 15mM BTP + 1% Sorbitol + 2.5% EtOH; pH 7.5 MPB: 2mM $MgAc_2$ + 10mM MES + 10mM HEPES + 15mM BTP + 1% Sorbitol + 2.5% EtOH; pH 9.0 MPC: 0.5 M NaAc; pH 5.5 MPD: 0.1 M NaOH + 2 M NaCl
DEAE; pH 8.0 → 9.0	MPA: 2 mM $MgAc_2$ + 20 mM TRIS + 1% Sorbitol + 2.5% EtOH; pH 8.0 MPB: 2 mM $MgAc_2$ + 20 mM TRIS + 1% Sorbitol + 2.5% EtOH; pH 9.0 MPC: 0.5 M NaAc; pH 5.5 MPD: 0.1 M NaOH + 2 M NaCl

D

Column	Composition
SO3; pH 6.5	MPA: 25 mM MES + 1% Sorbitol + 2.5% EtOH, pH 6.5 MPB: 25 mM MES + 150 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 6.5 MPC: 25 mM MES + 2 M KAc + 1% Sorbitol + 2.5% EtOH, pH 6.5

E

Column	Composition
SEC	MPA: 20 mM TRIS + 2 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.5
QA	MPA: 20 mM TRIS + 2 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.5 MPB: 20 mM TRIS + 50 mM $MgAc_2$ + 1% Sorbitol + 2.5% EtOH, pH 8.5 MPC: 0.5 M NaAc; pH 5.5 MPD: 0.1 M NaOH + 2 M NaCl

F

Column	Composition
SO3	MPA: 50 mM Na-citrate + 1% Sorbitol + 0.01% Poloxamer 188, pH 3.5 MPB: 150 mM Na-citrate + 1% Sorbitol + 0.01% Poloxamer 188 + 2 M NaCl, pH 3.5 MPC: 1 M NaOH + 2 M NaCl