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# Influence of Chromatographic Parameters on Empty/Full AAV Separation

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## 1. Introduction

Monoliths are commonly used for separation of empty and full AAV capsids on analytical and preparative scale. PrimaS® approach for AAV separation employs an ascending pH gradient, which can be sensitive towards small changes in chromatographic parameters. In present work 200 µL testing units extracted from large CIMmultus PrimaS® monolithic columns, were employed for the adjustment of critical chromatographic parameters.

## 2. Experimental Approach

Method development was conducted using a 0.2 mL PrimaS®, unit, tested with AAV 2/8 serotype sample. For evaluation of chromatographic parameters retention times and elution pH values of empty and full capsids were obtained. Tryptophan was added to the sample as a non-binding tracer. PATfix® HPLC system was used for chromatographic experiments.

Detectors:
- pH (and conductivity)
- Fluorescence (Ex/Em: 280/348 nm)

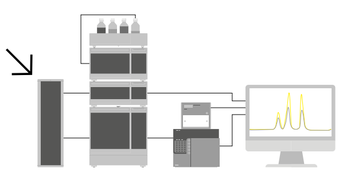
Buffer composition (pH gradient):
- Buffer A: 10 mM BTP, 10 mM TRIS, 2 mM $MgCl_2$, 1% sorbitol, 0.1% poloxamer 188, pH 8.00
- Buffer B: 10 mM BTP, 10 mM TRIS, 10 mM NaCl, 2 mM $MgCl_2$, 1% sorbitol, 0.1% poloxamer 188, pH 9.50
- Gradient: 5.0 min gradient from 100% buffer A to 100% buffer B
- CIP and column conditioning: 10 CV 0.1 M NaOH and 1 M NaCl; 30 CV 0.1 M acetic acid and 1 M NaCl, pH 5; 20CV $dH_2O$; 20 CV Buffer A; 20 CV Buffer B; 40 CV Buffer A

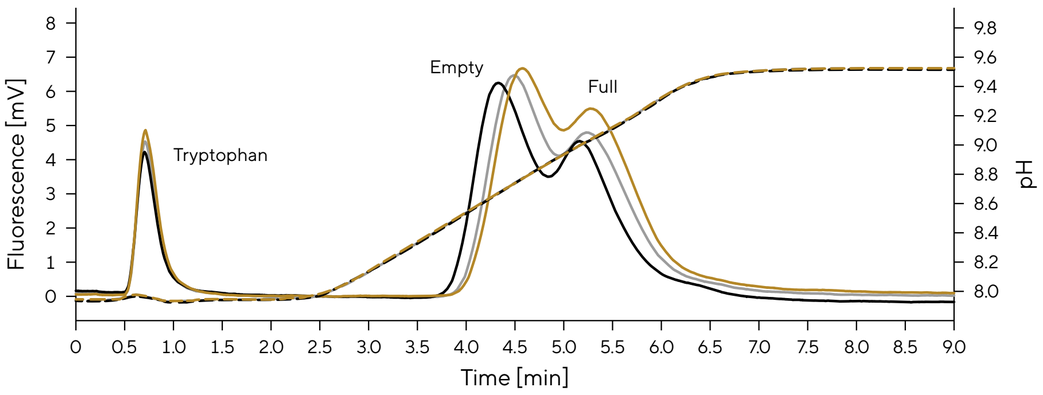
## 3. Results

### 3.1 Impact of Temperature Variation on Empty/Full Separation

**Figure 1:** *Chromatograms of Empty/Full AAV Separation Performed 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 |



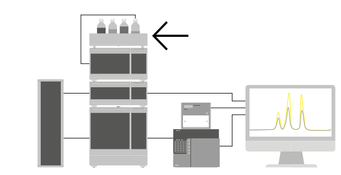


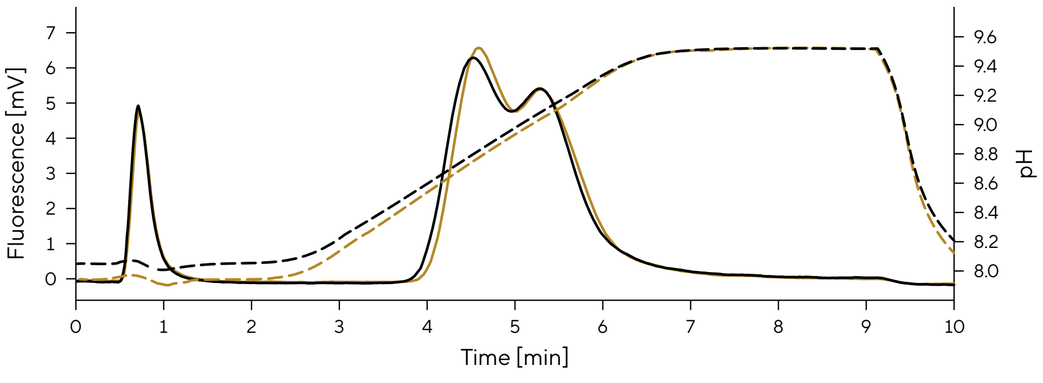

*Note.* An increase in temperature significantly improves the resolution between empty and full capsids but also affects retention times. Use of a **thermostat is essential** to provide reproducible column performance.

### 3.2 Buffer Preparation Affects Empty/Full Separation

**Figure 2:** *Chromatograms of Empty/Full AAV Separation With Buffer A pH 8.00 ±0.05.*

| | Buffer A pH | $t_{empty}$ (min) | R (/) |
|---|---|---|---|
| | 7.95 | 4.59 | 0.55 |
| | 8.05 | 4.52 | 0.58 |



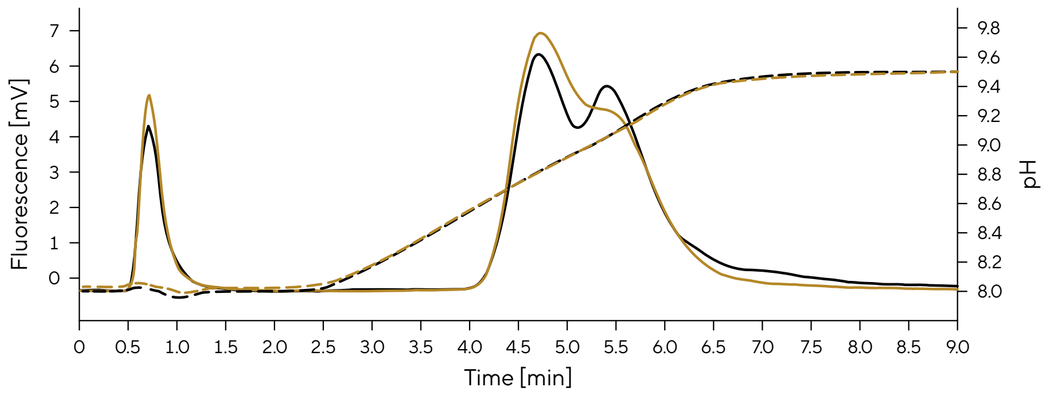



*Note.* It is critical to prepare buffers with **1% precision in pH value** for reproducible results. This was achieved by ajdusting the pH with **exact mass of standardized 1 M HCl.**

### 3.3 Influence of Column Conditioning on Empty/Full Separation

**Figure 3:** *Chromatograms of Empty/Full AAV Separation With Different Conditioning.*

| | Neutralization after CIP | $t_{empty}$ (min) | R (/) |
|---|---|---|---|
| | with | 4.71 | 0.58 |
| | without | 4.74 | 0.38 |

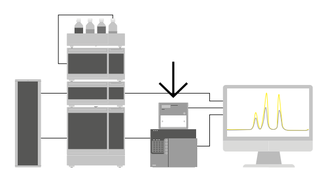


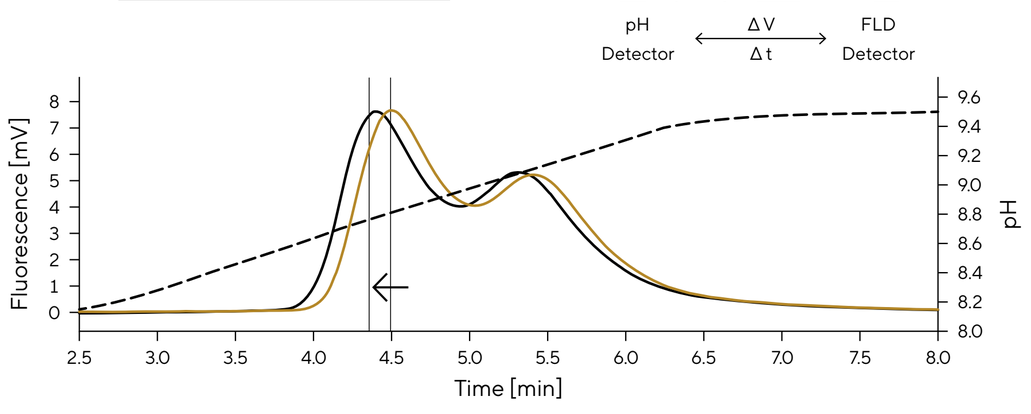

*Note.* Resolution between peaks dramatically decreases if the column is not conditioned in correct manner. Column needs to be neutralized with **sodium acetate and washed with deionized water** after CIP is performed. This step is crucial to get the optimal column effiency.

### 3.4 The Effect of Void Volume of the System

**Figure 4:** *Chromatograms of Empty/Full AAV Separation: Delay Based on dt(pH-FLD).*

| | dt (pH–FLD) | $t_{empty}$ (min) | R (/) |
|---|---|---|---|
| | with | 4.39 | 0.64 |
| | without | 4.49 | 0.64 |






*Note.* Void volume of chromatographic path plays an important role in interpretation and evaluation of chromatographic parameters which is even more significant when small columns are run on high void volume systems. Figure above illustrates the delay between the pH and fluorescence detectors – analyte passes pH detector first and then fluorescence. More accurate results are achieved if the **dt(pH-FLD) correction** is taken into account.

## 4. Conclusion

For reproducible empty/full AAV separation on PrimaS® it is important to:
- **use a thermostat,**
- **prepare standardized buffers** with 1% accuracy,
- **condition the column properly,**
- taking into account the **dt(pH-FLD) delay.**

Do Not Scale up the Method Before a Complete Control Over the Chromatographic Parameters.