WHITE PAPER

Method Transferability and Robustness for Modern Size Exclusion Chromatography Methods

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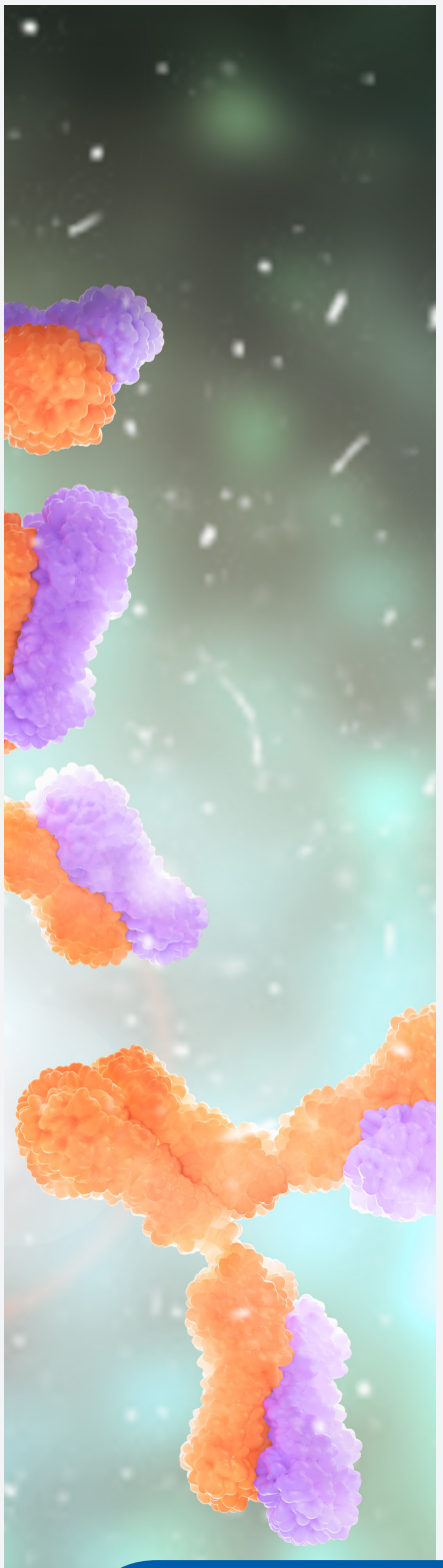


Introduction

Size exclusion chromatography (SEC) is a non-adsorptive chromatographic modality where large molecules, such as proteins or polymers separate based upon hydrodynamic radius. Larger molecules which cannot permeate all the pores in the chromatographic media are partially excluded, thus elute earlier, wherein smaller molecules which can permeate more pores, elute later. In this technical note, we investigate which method parameters and variables to consider for developing a robust and transferable method.

Background

SEC is a primary method used for aggregate analysis of protein therapeutics, supporting the entire product life cycle.[1] Advancements in HPLC columns have led to drastic improvements in data quality for aggregate analysis. For example, if method parameters such as mobile phase and column selection are optimized, the SEC method can separate both mAb high molecular weight aggregate (HMW), as well as low molecular weight (LMW) fragments.[2] This is particularly the case with the advent of sub-3 µm ultra-high performance SEC columns, which can improve not only resolution of size variants but sensitivity and throughput as well.[3]

Although SEC is used throughout the development of a biotherapeutic, because of its relative simplicity and method transferability, it is commonly the preferred analytical method for downstream stability studies and quality control for drug substance and product. As defined in the ICH Q2B guidelines, method robustness is the purposeful variation of method parameters to assess their impact on the data obtained. With SEC, percent purity of monomer is the primary target method attribute. However, other considerations - such as monomer peak shape, resolution of monomer from dimer and/or fragment, and retention time of monomer, should all be considered.

Unfortunately, often the only purposeful variation for assessing method robustness for an SEC method are column-to-column differences. These columns may or may not be from the same silica bonded batch. However, batch-to-batch issues aside, since the intent for size exclusion is to minimize non-SEC interactions, method attributes related to mobile phase - namely, ionic strength and pH - are even more critical. Other method parameters, such as flow rate, might also be interesting to look at though this parameter might be more critical for improving separation, which is indirectly associated with method robustness.

In this white paper, we demonstrate impact of particle size and column length on method transferability, as well as variation of mobile phase composition to assess method robustness using a commonly used standard for method benchmarking, the National Institute of Standards and Technology (NIST) RM 8671 Monoclonal Antibody (mAb).

Materials and Methods

NIST mAb and all reagents were purchased from MilliporeSigma® (St. Louis, MO, USA). SEC analysis was performed either on an Agilent® 1260 HPLC system or a Waters® H-Class Bio, UPLC® system with UV-detection, at 280 nm. Data analysis was performed using ChemStation® and Empower™ software. Biozen™ dSEC-2 columns (Phenomenex) were used for all separations. NIST mAb samples were diluted with 1X PBS to 10 mg/mL. Injections of 10 µL were performed.

Results and Discussion

Batch-to-Batch Variation

The most common robustness parameter to assess is column-to-column performance and reproducibility. This straightforward assessment is easy and practical to implement. However, batch differences can heavily influence method performance, as pore volume and bonded silica

will vary slightly. An HPLC column is often considered to be a consumable, and the same column obviously cannot be used to support the biotherapeutic life cycle due to the passage of time and changes in column performance.

The most important method performance parameter to assess for different column batches is peak areas for aggregate, as this is the quality attribute that is ultimately the goal for a SEC method applied to mAbs. Resolution of monomer and aggregate should be considered and investigated. **Figure 1** shows an overlay of 3 different batches of columns packed with 1.8 µm 200 Å silica-based media for NIST mAb. **Table 1** shows low % CVs for retention time and percent areas for HMW aggregate and monomer. However, low molecular weight fragment is less consistent. Batch 1 seems to partially separate fragment 1 as a post-peak, but fragment 1 is not separated in the other two batches. No batches are able to resolve the post-peak without manual integration, which may not be reliable for routine testing. As such, if fragment separation was more critical, one would consider optimization of the method to improve the separation of the post-peak. There are several approaches to improvement of the separation, including decreasing flow rate, optimizing mobile phase, running columns in series, or selecting a column packed with smaller particle sizes. However, if the intent for the method is simply aggregate assessment, the method as shown demonstrates very good reproducibility.

Figure 1. Overlay of SEC chromatograms, each generated from a different vendor lot, for NIST mAb. Column dimensions are 7.8 mm ID by 300 mm length, packed with 3 µm 200 Å pore size media. Separation of monomer and aggregate is reproducible, however, fragment 1 is not resolved from the main peak, and fragment 2 shows slight variation in peak area.

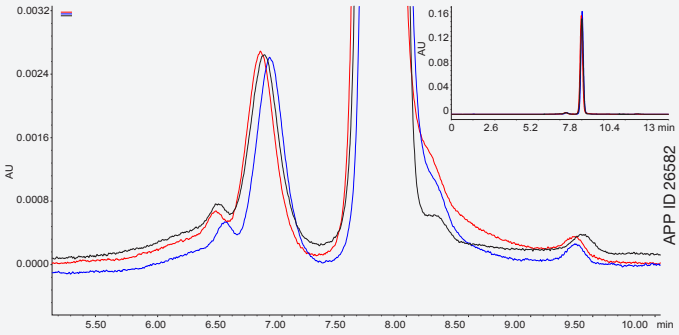


Table 1.
Chromatographic Results for NIST mAb Analyses using Different SEC Column Batches

	Peak	Retention Time (min)	Percent Area	USP Resolution
Batch 1		6.826	1.91	
Batch 2	HMW	6.900	1.94	
Batch 3		6.859	1.82	
	%CV	0.5%	3.3%	
Batch 1		7.771	97.86	2.65
Batch 2	Monomer	7.822	97.84	2.60
Batch 3		7.806	97.77	2.05
	%CV	0.3%	0.0%	1.9%
Batch 1		11.066	0.23	11.35
Batch 2	Fragment 2	11.033	0.22	11.69
Batch 3		11.184	0.41	10.25
	%CV	0.7%	37.3%	6.8%

Particle Size and System Variation

If the analytical method is to be run at multiple sites, for the sake of transferability, particle size should be taken into consideration. Larger particle formats tend to have more favorable frit configurations for the rigors of routine testing. **Figure 2** is an example of NIST mAb run on a 3 µm, 200 Å column, 4.6 mm ID by 300 mm length, showing resolution of 2.2 for monomer and high molecular weight aggregate, and resolution of fragment 1. The system used was a Waters® UPLC® H-Class Bio, a relatively low dispersion system (<400 µL system dwell volume per the manufacturer). These results exceed what typically would be acceptable. Further, using the 4.6 mm ID column would require less mobile phase and sample than a larger ID column, should sample amount be limited.

However, some sites running an analytical method may lack a low dispersion UHPLC. Because system dwell volume contributes greatly to band broadening with SEC methods, it may be more practical to run a 7.8 mm ID column. The larger ID column will be less effected by system volume outside the column, whether that is related to flow-cell or PEEK tubing used within the HPLC system. **Figure 3** shows an example of the same lot of NIST mAb run on a 3 µm with the same packing, but with a 7.8 mm ID column. Flow rate was scaled appropriately to 1 mL/min to ensure linear velocities remained consistent. This was on an Agilent® 1100 system; although the HPLC was upgraded with a microflow cell (2 µL), system dwell volume is significantly more than the UHPLC system used in the previous example. Even with this suboptimal system, resolution of monomer and aggregate was 2.4 for the 7.8 mm ID, superior to what was run on the UHPLC system. As such, the 7.8 mm ID is the preferred choice to accommodate system variance among different sites, and should be considered the most practical choice as it will likely yield an acceptable separation regardless of the system used.

Figure 2. NIST mAb SEC profile. Column dimensions were 4.6 mm ID, 300 mm length, and packed with 3 µm 200 Å pore size media Mobile phase used was 2X PBS (50 mM Sodium Phosphate, 300 mM Sodium Chloride, pH 6.8). Method flow rate was 0.35 mL/min.

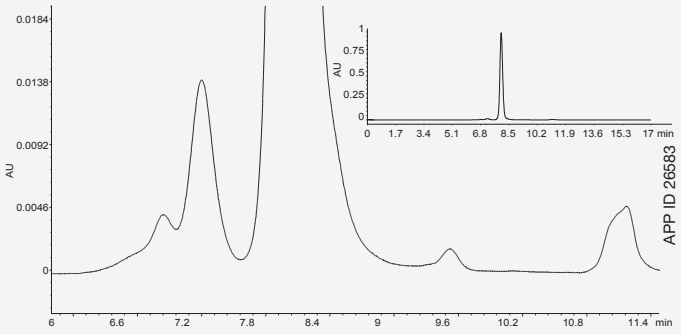
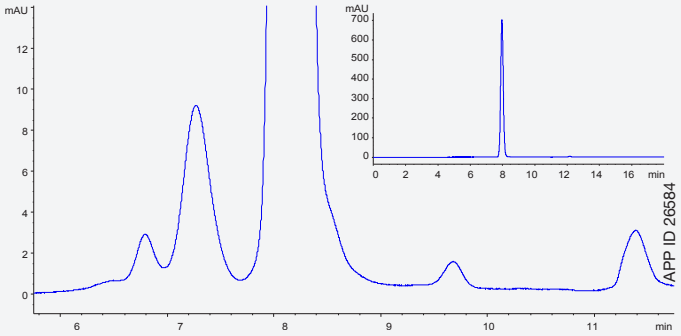


Figure 3. SEC profile for NIST mAb using a column packed with 3 µm 200 Å particles. Column dimensions were 7.8 mm ID by 300 mm length. Mobile phase identical to **Figure 2** with linear velocity scaled to ID (i.e. 1mL/min).

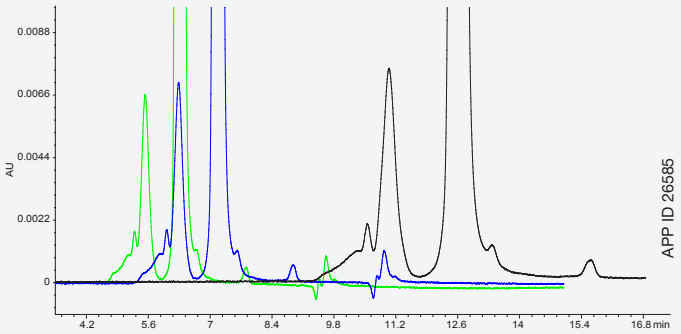


Impact of Flow rate and Effect on Method Robustness

Flow rate is a common method parameter to include as part of the design of experiment for any analytical method development and robustness study. Typically, flow rate is investigated for reversed phase methods wherein optimal linear velocity must be maintained to ensure a good separation. However, SEC is a unique separation modality in that lower flowrates often yield improvements in separation, and resolution can improve simply by running at a lower linear velocity. Consequently, it might be more prudent to implement a method at a relatively low flowrate for downstream methods, as throughput needs are not quite as high.

Figure 4 demonstrates the utility of method improvements by running at a lower flow rate. Flow of 0.4 mL/min yielded resolution of 2.97 for monomer and HMW, whereas 0.2 mL/min yielded resolution of 3.42. Although the method will effectively take twice as long, because resolution is often a requirement for system suitability, the superior result with lower flow rate allows a wider margin of error before method failure. As such, if one is prioritizing performance and robustness over throughput, lower flow rate is one of the easiest method parameters to implement.

Figure 4. Chromatographic overlay of SEC method for NIST mAb at different flow rate. Columns were 4.6 mm ID, 300 mm length, and packed with 1.8 µm 200 Å particles. The lowest flow rate (0.2 mL/min-black trace) yields monomer and HMW resolution when compared to faster linear velocities (0.35 mL/min - blue trace, 0.4 mL/min - green trace). Fragment, including post-peak separation of fragment, also improved at 0.2 mL/min.



Mobile Phase Assessment for Robustness

As previously mentioned, SEC is non-adsorptive, so minimizing secondary, non-SEC interactions is critical to method reproducibility and overall robustness. Although it is prudent to evaluate different batches of media, depending on the mobile phase composition and physicochemical properties of the analyte, non-SEC interactions may be more pronounced. For example, kosmotropic phosphate buffers are ubiquitous in SEC methods, as they are protein stabilizing and minimize intermolecular interactions. However, kosmotropes may expose hydrophobic moieties of the protein, causing interactions with the stationary phase; even a propanediol commonly used in SEC might still interact with the protein if the phosphate concentration is sufficiently high and the protein is close enough to its isoelectric point.

As such, an assessment of mobile phase composition is a critical component to any experimental design to assess method robustness. We present a reference SEC method used for a United States Pharmacopeia (USP) monograph.[4] Note that particle sizes implemented (1.8 µm) were outside the allowable USP adjustments. Using the USP monograph mobile phase as a starting point, the following experimental design was run, varying phosphate, chloride, cosolute and pH, to assess the impact on resolution and peak area recoveries, as defined by a percentage of purity. Note that with arginine as the co-solvent, phosphate concentration remained the same (0.2M) while arginine varied in molarity at similar concentrations to what one would use with a chloride salt. **Table 2** summarizes the experimental design. **Figures 5-9** show representative chromatograms obtained when varying the buffer components as summarized in **Table 2**. Additionally, **Table 3** summarizes the chromatographic results.

Table 2.
Experimental Design for Mobile Phase Robustness Assessment

Method Parameter	Ranges
Potassium Phosphate Concentration	50, 100, 200 mM
Potassium Chloride Concentration	100, 200, 250 mM
Cosolute (arginine) Concentration	100, 200, 250 mM
Mobile Phase pH	6.2, 6.8, 7.2

Figure 5. NIST mAb SEC Profile, running the USP monograph method mobile phase (200 mM Potassium Phosphate, 250 mM KCl, pH 6.2). Column used was packed with 1.8 µm 200 Å particles.

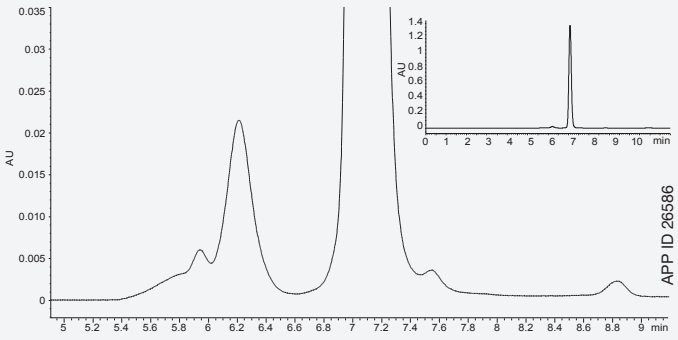


Figure 6. Overlay of NIST mAb SEC chromatograms varying potassium phosphate conditions: 50 mM - black trace, 100 mM - blue trace, 200 mM - green trace.

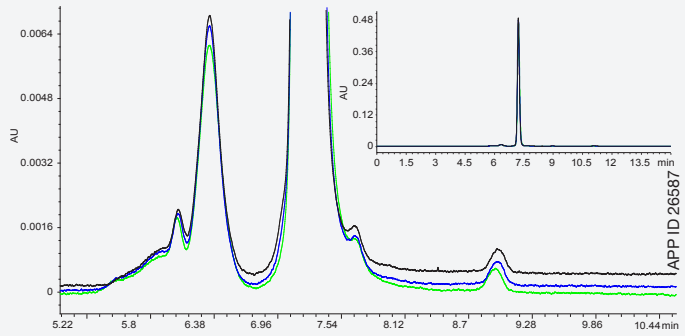


Figure 7. Overlay of NIST mAb SEC chromatograms varying potassium chloride conditions: 100 mM KC l- black trace, 200 mM KCl - blue trace, 250 mM KCl - green trace.

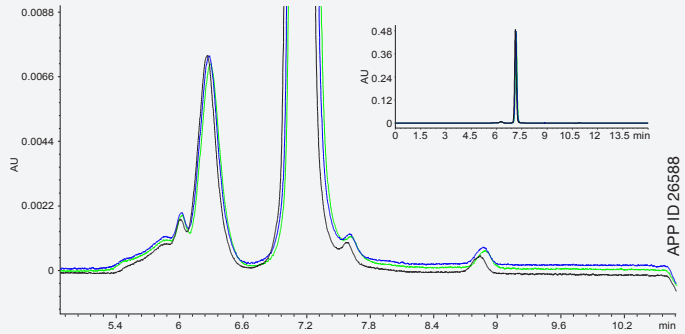


Figure 8. Overlay of NIST mAb SEC chromatograms varying mobile phase pH: 6.2 - black trace, 6.8 - blue trace, 7.4 - green trace.

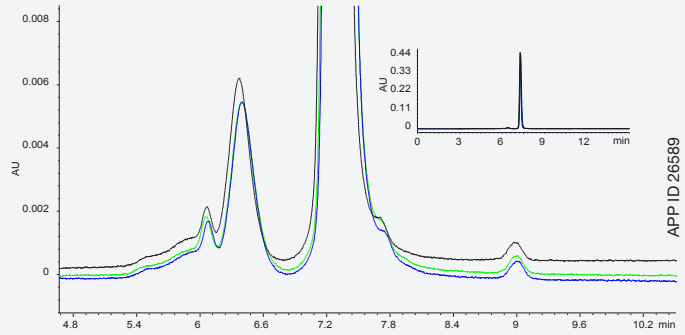
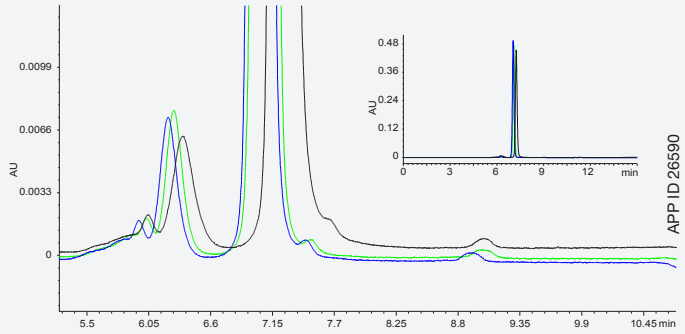


Figure 9. Overlay of NIST mAb SEC chromatograms when varying arginine (as an alternative to varied chloride salt): 100 mM - black trace, 200 mM - blue trace, 250 mM - green trace.



As observed with overlays when phosphate, potassium chloride, and even pH (**Figures 6**-8) is modulated, there is a negligible change in separation, as retention time and peak shapes remain consistent. The only appreciable difference is with arginine running as low as 100 mM (**Figure 9**). Even then, the resulting retention time shift is still within an acceptable range, % CV is still less than 2%. Resolution and percent purity of monomer also show acceptable results, with less than 5% and 1% CVs, respectively.

Table 3.
Summary of Chromatographic Results as Influenced by Mobile Phase Component Concentrations

Mobile Phase	Conditions	Monomer Retention Time	Resolution 1,2	% Purity Monomer
Variable Potassium Phosphate, 250 mM KCl, pH 6.2	50	7.214	3.01	96.81
	100	7.219	3.02	96.4
	200	7.224	2.91	96.47
200 mM Potassium Phosphate, variable KCl, pH 6.2	100	7.165	3.15	97.06
	200	7.147	3.12	96.42
	250	7.117	3.19	96.55
200 mM Potassium Phosphate, 250 mM KCl, variable pH	6.2	7.264	2.96	97.32
	6.8	7.298	2.88	97.13
	7.2	7.287	2.82	97.23
200 mM Potassium Phosphate, variable Arginine, pH 6.2	100	7.235	3.02	97.55
	200	7.037	3.26	96.91
	250	7.078	3.25	95.32
	%CV	1.15%	4.76%	0.61%

Column Bed Stability and Ruggedness During Routine Testing

The primary failure mode for size exclusion columns is voiding, wherein the column packed bed compresses and collapses. Ultimately, this yields a source of significant extra column volume, peak broadening and tailing. Column voids can occur because of spikes in backpressure, switching mobile phase resulting in changes in solvent viscosity (e.g. storage solvent containing 20% methanol) and suboptimal method/sequence start up and shut down, wherein method flow rate starts or stops suddenly. Either way, the column becomes unusable upon voiding and must be replaced. As such, column lifetime must be considered for any lab that might be performing routine testing, as column voids can occur within the sequence and invalidate data generated within a sequence. This also delays testing, which can be further exacerbated if column availability is an issue.

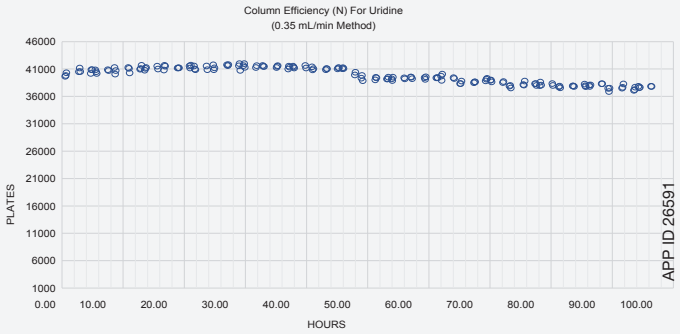
To demonstrate column method robustness against column voiding during routine testing, a 4.6 mm ID by 150 mm length column packed with sub-2 µm, 200 Å media was run with a viscous mobile phase containing 10% isopropanol. To further stress the column packed bed, flow rate was modulated between 0.35 mL/min and 0.45 mL/min, the latter of which resulted in a backpressure of ~320 bar. Finally, flow was intermittently stopped for 8 hours to replicate the system start up and shut down which can also result in chromatographic bed collapse. A total of >100 hours of run time, or 226 injections, were performed. Column

failure is defined as a drop of 25% efficiency for uridine, a small molecule which elutes in the total permeation volume. A summary of the sequence is indicated below (**Table 4**). Column efficiency remained acceptable at the end of the sequence (**Figure 10**).

Table 4.
Summary of SEC Column Bed Stability Sequence

Mobile Phase	Flow rate (mL/min)	Method Run Time (min)	Number of Injections
0.1 M Phosphate Buffer, pH 6.8 + 10% IPA	0.35	6.5	4
0.1 M Phosphate Buffer, pH 6.8 + 10% IPA	0.45	6.5	4
0.1 M Phosphate Buffer, pH 6.8 + 10% IPA	0.35	6.5	3
0.1 M Phosphate Buffer, pH 6.8 + 10% IPA	0.35	90	1
N/A (no system flow)	0	480	0
		Sequence repeated until 100 hours of run time was exceeded	

Figure 10. Column efficiency for uridine over hours of run time. Sequence as shown in **Table 4**. Only a 3% drop in efficiency is observed over the course of the sequence.

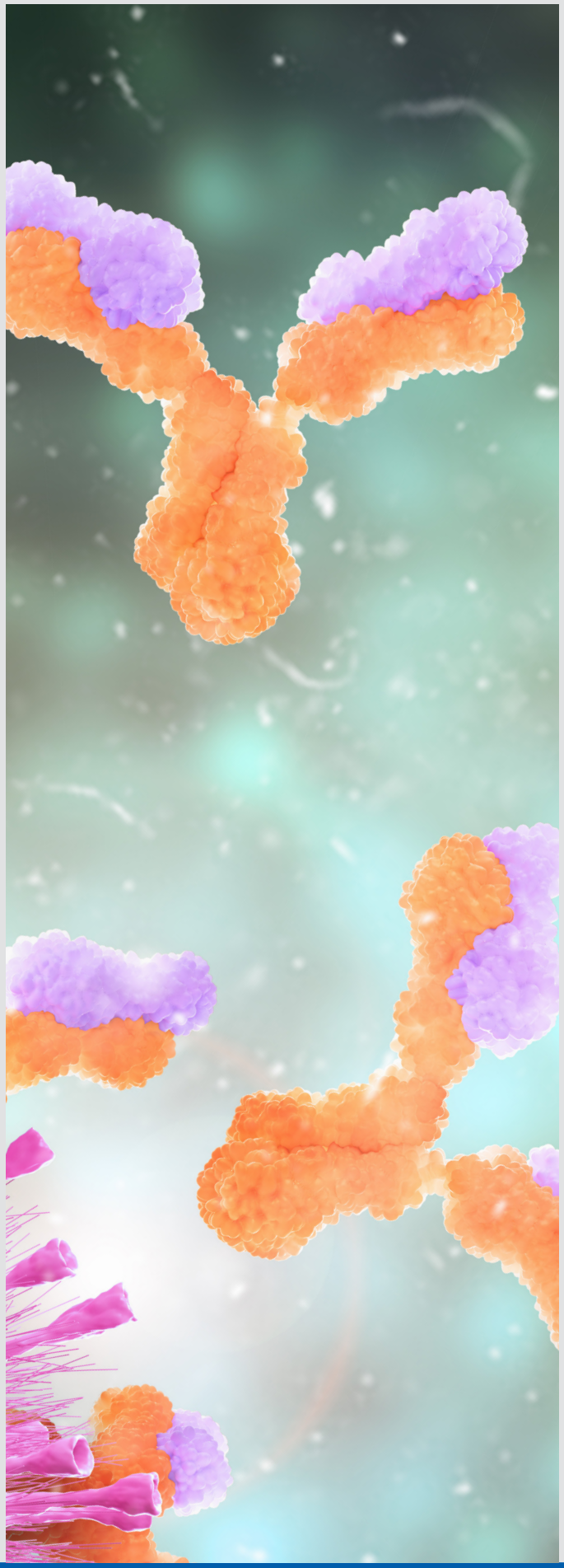


Conclusion

In this white paper, we provided guidelines for ensuring a robust and transferable method for SEC, specifically for mAbs. Methods should be practical regarding column dimensions and particle sizes used, with 7.8 mm ID by 300 mm length columns lending benefits for transferability irrespective of the LC system used. Flow rate should be run as low as practically possible, as throughput for SEC is typically not the primary rate limiting factor, and lower flow rates often confer improvements in chromatography. Mobile phase should be investigated and varied to understand the potential impact of differences in surface activity and inertness of the analyte to the stationary phase. Finally, ensuring column robustness in a routine environment should be taken into consideration, as column failures due to poor packing density can lead to misreporting of data and delays in projects.

There are some limitations in this study, in that NIST mAb was the only analyte evaluated. NIST was used as it is recognized as the common surrogate standard for method development. However, as the standard is an IgG1k mAb, it is not representative of other mAb isotypes or engineered mAbs which deviate significantly in physicochemical properties, so each analyte should be evaluated individually.

Finally, although limited batch-to-batch data was presented, it is prudent to evaluate multiple columns from multiple batches. Our recommendation is to evaluate at least one column from three different manufacturing batches. However, during the method lifecycle, it is prudent to investigate all columns evaluated for inter- and intra-batch variation to fully assess performance.



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