APPLICATIONS

Better Gel Filtration: A New Look at Method Development Parameters Using Yarra™ GFC Columns

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An affordable, high efficiency Yarra GFC column was recently introduced and is significantly more efficient than other GFC columns on the market. In addition to higher efficiency, Yarra columns demonstrate significantly higher inertness to ionic interactions versus other GFC columns; however, such chemical characteristics require optimization to operating parameters. In particular, salt and buffer concentration can have a significant impact on secondary interactions which can influence GFC separations. Performing method development through mobile phase composition using next-generation Yarra GFC columns will be discussed.

Introduction

Gel filtration chromatography (GFC) is a technique focused on separating proteins based on their size in solution (which directly correlates to their molecular weight). This makes GFC particularly useful for quantitating protein aggregation state as well as post translational modifications that impact solution structure.[1] Despite being a seemingly simple isocratic separation method there are several critical parameters in optimizing separation methods for specific proteins. Buffer and salt concentrations of the mobile phase can have a significant impact on the retention and resolution of many proteins (especially basic and hydrophobic proteins) and optimization strategies for GFC will be discussed.[2]

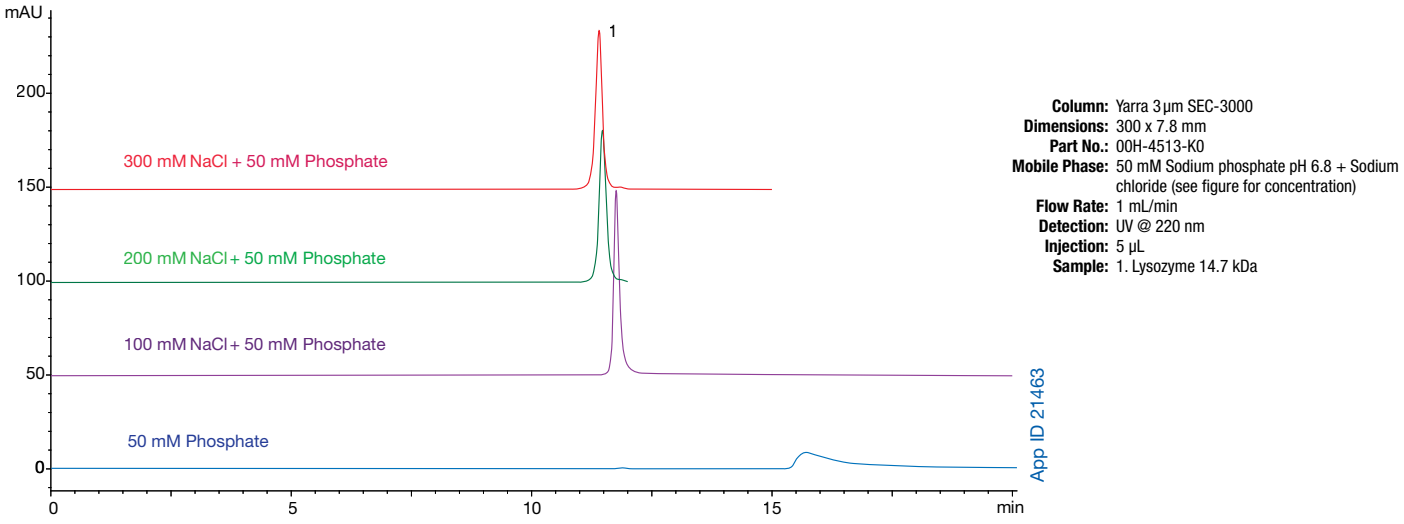
Materials and Methods

GFC standards using various proteins and void markers (thyroglobin, IgA, IgG, ovalbumin, myoglobin, lysozyme, and uridine; Sigma Chemical, St. Louis, MO, USA) were used to evaluate secondary interactions of the stationary phase as well as determine molecular weight linearity. Isocratic HPLC runs were performed on an Agilent® 1100 with autosampler, UV detector, and ChemStation™ software (Agilent Technologies, Palo Alto, CA, USA). HPLC columns used for GFC studies were Yarra 3 µm SEC-2000 and Yarra 3 µm SEC-3000 (300 x 7.8 mm dimension; Phenomenex, Torrance, CA, USA). Columns were run at ambient temperature and at a flow rate of 1.0 mL/min unless otherwise noted. Mobile phase buffer composition is listed in specific figures.

Results and Discussion

Efforts were undertaken to show influences that mobile phase can have on GFC separations in order to give researchers a better understanding on method development actions to take in developing methods. Although GFC bonded stationary phases are designed to minimize interactions between negatively-charged silanol groups on the silica surface and basic proteins, some interaction does occur and that can be minimized by increasing salt concentration in the mobile phase thus reducing ionic interactions. An excellent example of this is shown in **Figure 1** where lysozyme (a basic protein) is injected on a Yarra SEC-3000 column under different mobile phase concentrations. Note that as the salt concentration in the mobile phase increases, the retention time of lyzosyme decreases to the appropriate retention for a protein of its molecular weight. In addition, both recovery and peak shape improves as salt concentration of the mobile phase increase. Such results would suggest that increasing salt concentration is the key for all GFC separations. Unfortunately that is not the case as ionic interactions are only one of the interactions that occur during GFC that can impact performance.

Figure 1.
Yarra SEC-3000: Lysozyme pH 6.8

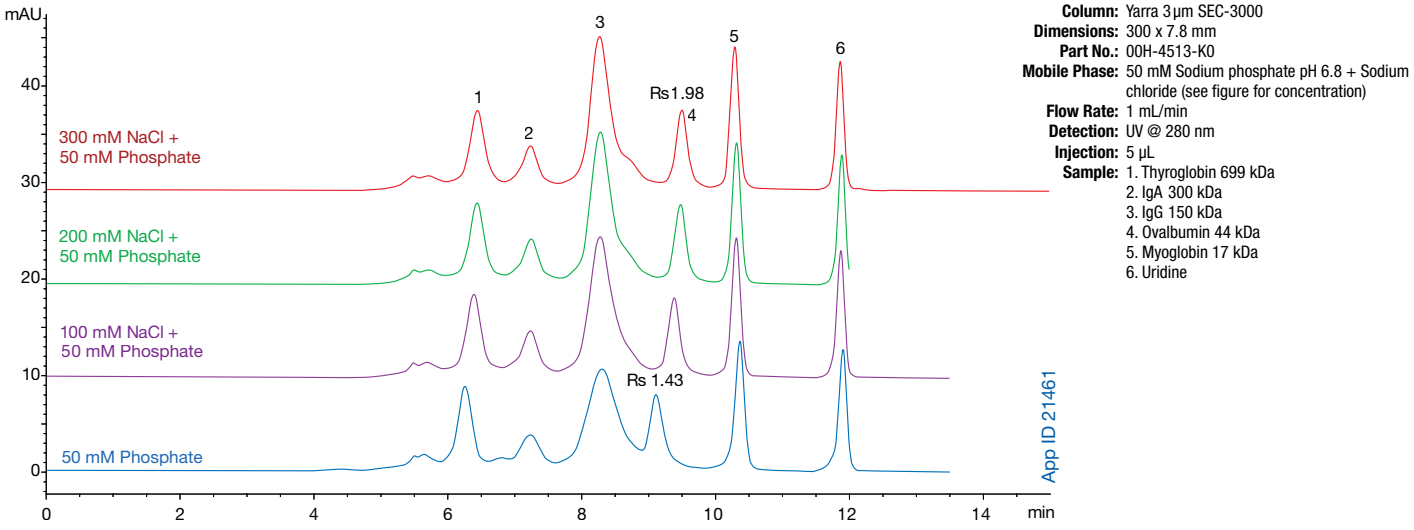


An overlay of lysozyme run on a Yarra SEC-3000 column using different mobile phase conditions. Lysozyme was injected using increasing salt (sodium chloride) in the mobile phase to demonstrate its influence on basic proteins like lysozyme. Note that the retention decreases, peak shape improves, and recovery increases as ionic interactions between the stationary phase and analytes are disrupted by increasing salt in the mobile phase.

Bonded phases used for gel filtration chromatography typically have some diol functionality. The diol ligand both covers the silica surface as well as displays a polar functionality that "mimics" water. However, besides GFC, diol phases are sometimes used as very weak hydrophobic interaction media; thus one would expect that at increased salt concentrations in the mobile phase one would start seeing hydrophobic interactions occurring between the bonded phase and hydrophobic proteins. To test this hypothesis, a mixture of standard proteins was run on a Yarra™ SEC-3000 column and salt concentration was increased. **Figure 2** compares the retention of ovalbumin (a moderately hydrophobic protein) to other proteins as the salt concentration in the mobile phase increases. One can see ovalbumin increase in retention to a molecular weight anomalous to the expected molecular weight. Note that most of the other proteins only change minimally in retention time versus ovalbumin. This difference can be better visualized when one makes a plot of retention time versus the log of the molecular weight for the proteins in the mixture. The log plot in **Figure 3** for this experiment shows that only ovalbumin moves substantially with changes in mobile phase concentrations of salt suggesting that for hydrophobic proteins too much salt can be detrimental to separation. Further, it suggests that method development for GFC revolves around finding the optimal mobile phase to minimize ionic and hydrophobic interactions.

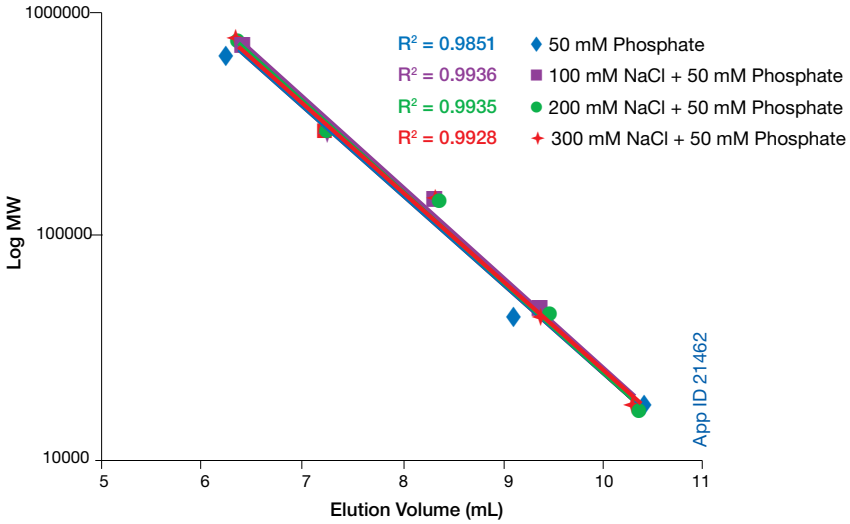
To determine if this observation is specific to the GFC phase used in these experiments and mobile phase buffer salts, the experiment was repeated using a different pore size GFC column (Yarra SEC-2000) and utilizing sodium sulfate as the mobile phase salt. When ones looks at the results in **Figure 4**, one can see a similar result for ovalbumin as in previous figures with retention time increasing as salt concentration increases. Another particular observation unique for the Yarra SEC-2000 column revolves around the thyroglobulin/aggregate peak early in the chromatogram; under low salt conditions, thyroglobulin appears to be fully excluded, yet with other mobile phase conditions some resolution is obtained. It is possible that a similar hydrophobic effect is being observed with the increased retention of the thyroglobulin peak leading to some resolution between it and a high molecular weight aggregate peak. Other proteins in the mixture do shift slightly, but not as profound as observed with ovalbumin or thyroglobulin. These results suggest that salt can influence the hydrophobic retention of some proteins on GFC phases in general but not specific to a particular salt or column phase.

Figure 2.
Yarra SEC-3000 pH 6.8



An overlay of a standard protein mixture (thyroglobulin, IgA, IgG, ovalbumin, myoglobin, and uridine) run on a Yarra SEC-3000 column with increasing salt concentration (mobile phase concentration listed with each chromatogram). Note that ovalbumin increases retention as salt increases. Increasing salt concentration increases hydrophobic interactions between the bonded diol phase and some protein analytes.

Figure 3.
Protein Mixture Calibration Curve on Yarra SEC-3000



A log molecular weight plot for the protein standards shown in **Figure 2** under different mobile phase conditions. Most proteins demonstrate limited retention change except for ovalbumin which shifts to greater retention with increased salt.

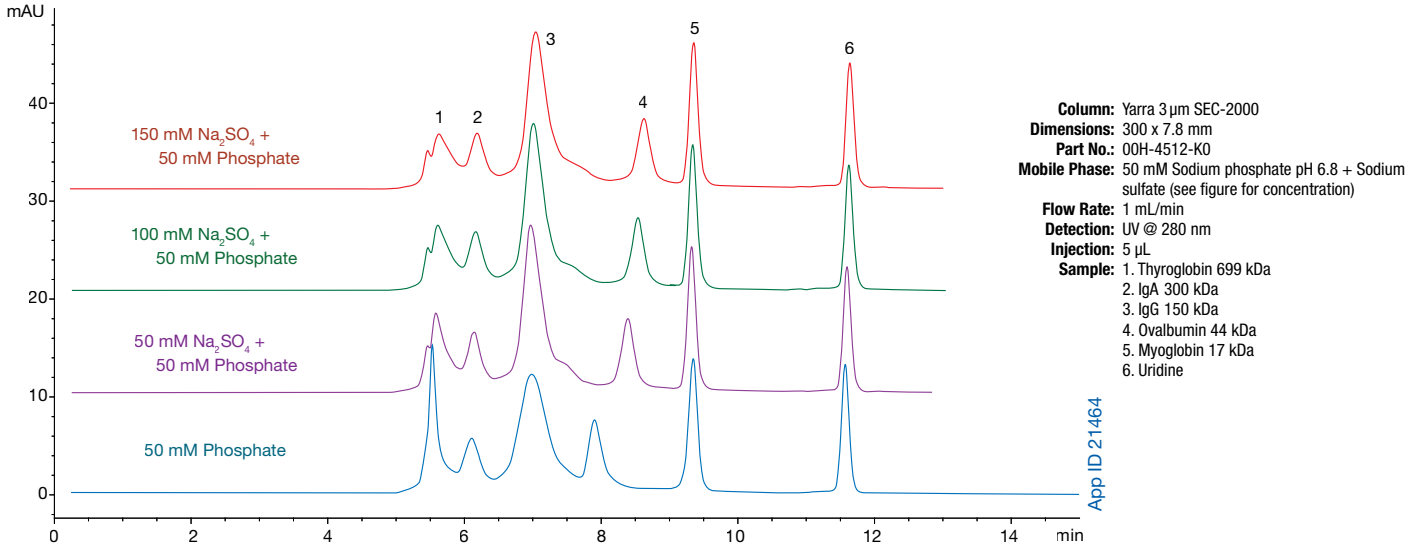
Conclusion

Gel filtration chromatography is an isocratic method for separating proteins based on their size in solution, but secondary interactions between the stationary phase and protein analytes make it far from simple. Mobile phase composition plays a significant role in the separation of protein by influencing proteins in solution as well as the ionic and hydrophobic interactions that the stationary phase may impact on the separation. While not studied in this paper, mobile phase pH can influence the net charge of a protein as well as the surface silanols on the silica media. The use of buffer (and non-buffer) salts in the mobile phase can dramatically influence the retention, peak shape, and recovery of proteins by modulating ionic secondary interactions. While increasing salt concentrtion reduces ionic interaction, it also increases hydrophobic interactions making method development for GFC separation a balance between the two interactions. The key to optimizing any GFC method is investigating a range of salt concentrations and compositions to achieve the desired separation of the proteins or other biomolecules of interest.

References

1. J. Engelsman; P. Garidel; R. Smulders; H. Koll; B. Smith; S. Bassarab; A.Seidl; O. Hainzl; W. Jiskoot; *Pharm Res* (2011) 28:920–933
2. T. Arakawa; D. Ejima; T. Li; J. Philo; *Journal of Pharmaceutical Sciences* V99 (4) pg 1674–1692 (2010

Figure 4.
Yarra SEC-2000 pH 6.8



Overlay of the standard protein mixture on a Yarra SEC-2000 column. In this figure, the standard used in **Figure 2** was injected on a smaller pore size GFC column (Yarra SEC-2000) using a different salt (sodium sulfate). Similar retention shifts for ovalbumin were observed indicating that this effect is not salt or column dependent.

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Ordering Information

Yarra™ 3 µm SEC Columns (mm)

	Narrow Bore	Analytical	Analytical	SecurityGuard™ Cartridges (mm)
Phases	**300 x 4.6**	**150 x 7.8**	**300 x 7.8**	**4 x 3.0***
				/10 pk
Yarra 3 µm SEC-2000	00H-4512-E0	00F-4512-K0	00H-4512-K0	AJ0-4487
Yarra 3 µm SEC-3000	00H-4513-E0	00F-4513-K0	00H-4513-K0	AJ0-4488
Yarra 3 µm SEC-4000	00H-4514-E0	–	00H-4514-K0	AJ0-4489
				for ID 4.6 - 7.8 mm

*SecurityGuard Analytical cartridges require holder, Part No.: KJ0-4282

SecurityGuard Cartridge Holder Kit

Part No.	Description
KJ0-4282	for 4.6 to 7.8 mm ID column

guarantee

If Yarra analytical columns do not provide at least an equivalent or better separation as compared to competing column with similar dimension, phase, and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

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