Relevant Parameters in Developing Protein Aggregation Methods Using Yarra™ GFC Columns

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A new high efficiency GFC column, Yarra, 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 sometimes require changes to operating parameters. Performing method development for protein aggregation analysis using next-generation Yarra GFC columns will be discussed.

Introduction

Being an isocratic method, gel filtration chromatography is assumed to be a simple method with little or no method development involved. On closer inspection, however, subtle changes in mobile phase and other parameters can have significant results on separation performance and accuracy of determining the aggregation state of a protein. Protein gel filtration columns are typically made by bonding a highly polar "diol-like" ligand to a porous silica matrix of a specific pore size. This polar "diol" coat on the silica is intended to minimize surface interactions between the silica and proteins, resulting in separations based on the size of a protein in solution as proteins are differentially excluded from the pores of the silica particle. One typically uses different pore-sized columns that provide maximum resolution of a specific molecular weight range based on the protein being separated. Often, two different pore-sized columns overlap in a molecular weight range, resulting in different selectivities based on the column being used. Sometimes a column at one edge of the overlap demonstrates better resolution in a protein specific manner. Since protein separations are typically looking at trying to quantitate non-covalent aggregates in solution, it is most common that a buffered aqueous mobile phase is used for GFC separations.

While coating GFC silica with a polar bonded phase greatly reduces the amount secondary interactions between proteins and the silica matrix, the reality is that some secondary interaction persists. The secondary interactions can be summed up into two categories: ionic interactions between acidic free silanol groups on the silica surface and basic residues of a protein, and hydrophobic interactions between the bonded phase ligand and hydrophobic pockets of a protein. Especially aggregated proteins tend to be more sensitive to hydrophobic and ionic interactions. Depending on the protein and column being used, different running conditions can be utilized to minimize such secondary interaction, providing for more accurate quantitation of low level impurities in a protein product. Examples will show how mobile phase composition and column selection can have a major impact on separations and factors to consider in developing a gel filtration method using the new Yarra brand of GFC columns.

Material and Methods

Standard proteins and mobile phase modifiers were purchased from Sigma Chemicals (St. Louis, MO, USA). Additional test proteins were purchased from R+D systems (Minneapolis, MN, USA). Mobile phases were obtained from EMD (San Diego, CA, USA). The instrument used for all separations was an Agilent® 1200 HPLC with an autosampler, column oven, and multi-wavelength detector. All separations used Yarra SEC-2000 and Yarra SEC-3000 columns (300 x 7.8 mm) obtained from Phenomenex (Torrance, CA, USA). Mobile phases with varying molarities of phosphate and sodium chloride were used (noted in each chromatogram).

Results and Discussion

Column Selection

Gel filtration chromatography separates proteins based on their size in solution and how much a particular protein can permeate into the pore of the bonded silica particle used in the column packing. Larger proteins will permeate a decreased distance into the porous particle versus smaller proteins which will permeate deeper into the particle. As a result, fully excluded aggregates and large proteins will elute first, followed by multimers, then the full-length monomer protein, and then protein fragments with salts and small molecules coming out last. Each specific Yarra column has an optimal molecular weight separation range which is based on the pore size of the porous media: Yarra SEC-4000 (500 Å), Yarra SEC-3000 (290 Å) and Yarra SEC-2000 (145 Å). **Figure 1** lists the suggested molecular weight range of each media based on the mobile phase conditions used. Note that there is significant molecular weight overlap between each individual phase. Depending on the separation goal of a specific GFC application, one or the other phase may be appropriate.

Molecular Weight (MW) Separation Ranges for Yarra

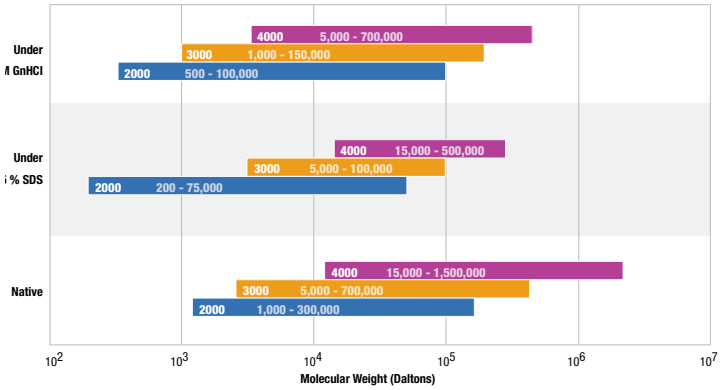


Figure 1.
Table of suggested separation ranges for particular Yarra SEC columns based on a specific class of mobile phase used for the separation (native or denaturing). Note that the three phases (SEC-2000, SEC-3000, and SEC-4000) all overlap under all conditions; in many cases more than one phase might be appropriate for a separation.

While the focus is more on buffer influences, comparing **Figure 2** and **Figure 3** demonstrates that in overlap region, application goals may influence column choice. The separation between Ig-G dimer and monomer (Rs 1,2) is slightly better on the Yarra SEC-3000 while resolution between Ig-G monomer and BSA (Rs 2,3) is slightly better on the Yarra SEC-2000. For any overlapping molecular weight region, both columns should be evaluated for that application.

Buffer Influence on GFC

While gel filtration columns are manufactured to minimize any secondary interactions between stationary phase and proteins of interest, both ionic and hydrophobic interactions do occur which can lead to peak tailing, loss of resolution, and poor recovery. Protein structure can be sensitive to changes in pH and osmolarity resulting in changes to net charge, solubility, and overall size based on the buffer conditions present in a protein solution. As a result, mobile phase can have a major impact on protein separations by GFC and is the main method for optimizing a particular separation. Mobile phase pH can have a significant effect on chromatography due to changes in the net polarity of a protein; even a change as small as 0.2 pH units can have an impact on a separation and should be investigated whenever practical. However, for most native GFC applications mobile phase pH will generally be between pH 6.5 to pH 7.5.

Figure 2a.
Ig-G and BSA on Yarra™ SEC-2000

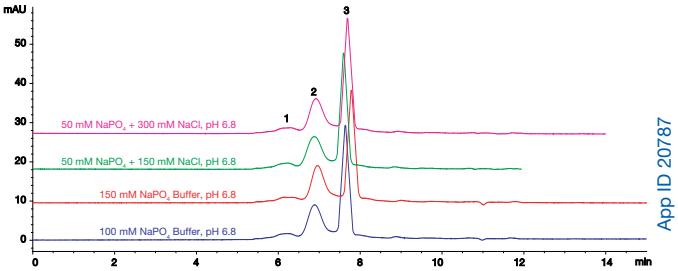
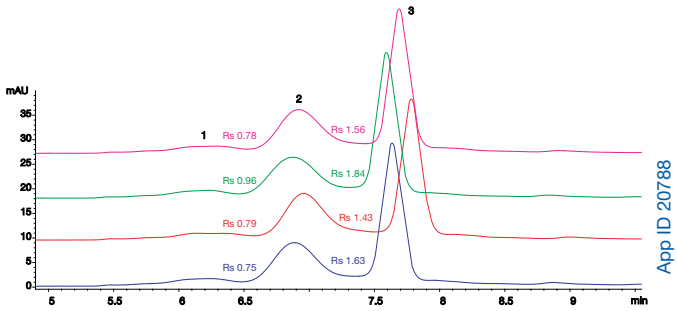


Figure 2b.
Ig-G and BSA on Yarra SEC-2000 (zoom in)



Conditions for both figures:

Column:	Yarra 3 µm SEC-2000
Dimension:	300 x 7.8 mm
Part No.:	00H-4512-KO
Mobile Phase:	(see figures)
Flow Rate:	1 mL/min
Detection:	UV @ 214 nm
Temp:	Ambient
Injection:	5 µL
Sample:	1. IgG dimer 300 kDa
	2. IgG (0.285 mg/mL) 150 kDa
	3. BSA (0.2 mg/mL) 66 kDa

Figure 2: An overlay of an Ig-G and BSA mixture run on a Yarra SEC-2000 column using different mobile phases. **Figure 2a** shows the full chromatogram and buffer conditions. **Figure 2b** is a zoom-in of the main components in the mixture. Note that resolution, peak shape and retention all change with mobile phase concentrations and that optimal resolution between Ig-G and BSA is obtained with moderate salt concentration on the Yarra SEC-2000 column demonstrating the utility of testing multiple mobile phase conditions

Buffer molarities and solution osmolarity can have the biggest impact on a protein separation because of its joint influence on both the protein and stationary phase. Some typical examples of this are shown in **Figure 2** and **Figure 3** for an Ig-G/ BSA mixture run on the Yarra SEC-2000 and Yarra SEC-3000, respectively. While there are multiple interactions occurring, increasing the buffer or salt concentration of a mobile phase influences the secondary interactions between analytes and the stationary phase.

Figure 3a.
Ig-G and BSA on Yarra SEC-3000

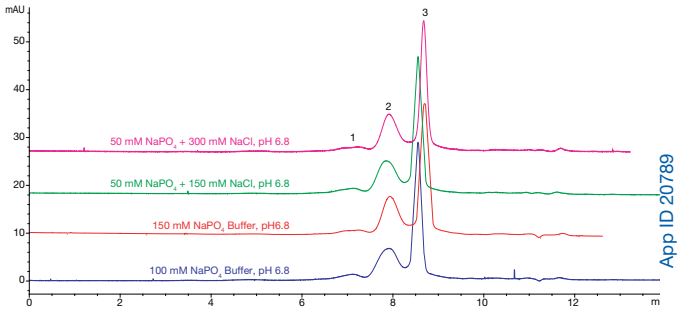
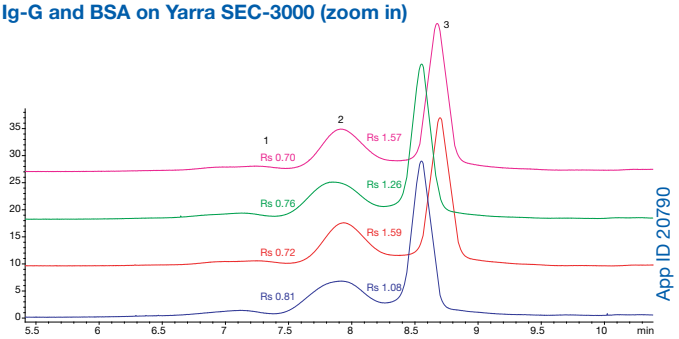


Figure 3b.
Ig-G and BSA on Yarra SEC-3000 (zoom in)



Conditions for both figures:

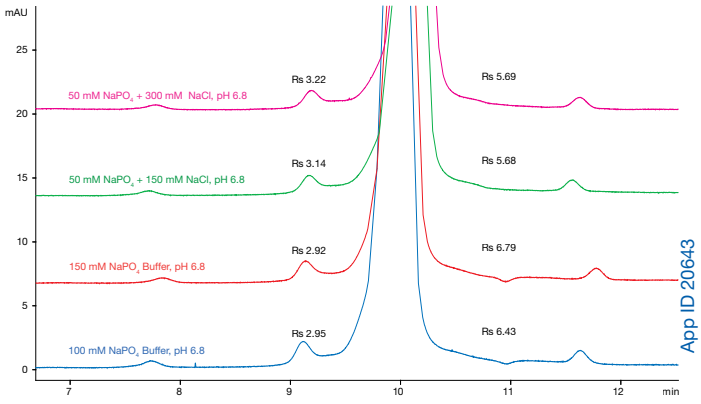
Column:	Yarra 3 µm SEC-3000
Dimension:	300 x 7.8 mm
Part No.:	00H-4513-KO
Mobile Phase:	(see figures)
Flow Rate:	1 mL/min
Detection:	UV @ 214 nm
Temp:	Ambient
Injection:	5 µL
Sample:	1. IgG dimer 300 kDa
	2. IgG (0.285 mg/mL) 150 kDa
	3. BSA (0.2 mg/mL) 66 kDa

Figure 3: An overlay of the same Ig-G and BSA mixture run on a Yarra SEC-3000 column using different mobile phases. **Figure 3a** shows the full chromatogram and buffer conditions. **Figure 3b** is a zoom-in of the main components in the mixture. Note that resolution, peak shape, and retention all change with mobile phase concentrations. Note that resolution between Ig-G and BSA is better for the Yarra SEC-2000 column while resolution between the monomer and dimer Ig-G peaks is better for the Yarra SEC-3000 column at low salt conditions.

APPLICATIONS

Increasing salt concentration of the mobile phase suppresses ionic interactions between the stationary phase and proteins which can result in better peak shape for basic proteins. However, increasing salt leads to increased hydrophobic interactions between the bonded stationary phase and hydrophobic protein resulting in increased anomalous retention, peak tailing, and reduced recovery. This is best shown in **Figure 4** where IGF-1, a small moderately hydrophobic protein, is injected on a Yarra™ SEC-2000. While recovery is not adversely affected, note that as salt increases in the mobile phase the peak shape and efficiency is adversely affected. Retention also increases slightly for the monomer that positively influences resolution between the dimer and monomer peak, but negatively influences resolution between the monomer peak and low molecular weight impurities. Thus, for specific protein aggregate applications investigating optimal buffer concentration is suggested. Good starting points for any method would be starting with phosphate (or tris) buffer between the 50-150 mM range and then investigating if additional salt (up to 300 mM NaCl) or organic (up to 10 % of either acetonitrile or methanol) improves a separation.

IGF-1 on Yarra SEC-2000



Column: Yarra 3 µm SEC-2000
Dimension: 300 x 7.8 mm
Part No.: 00H-4512-K0
Mobile Phase: (see figure)
Flow Rate: 1 mL/min
Detection: UV @ 214 nm
Temp: Ambient
Injection: 5 µL
Sample: IGF-1

Figure 4: An overlay of different mobile phase conditions for IGF-1 on Yarra SEC-2000. Note the different resolutions of particular critical components under the various salt concentrations. Low molecular weight resolution decreases with increasing salt while dimer/monomer resolution increases. Optimizing mobile phase is critical for any GFC separation.

Conclusion

Because GFC is an isocratic separation method based on differential pore exclusion, method development considerations are much different than what is typically used for other separation modes. Column selection is the main parameter considered in developing a GFC method where different pore size columns determine the molecular weight range of a separation. In the case of the Yarra brand of GFC columns, the Yarra SEC-2000 column is most appropriate for a lower molecular weight range of separations (1-150 kDa) and the Yarra SEC-4000 column is most appropriate for the largest molecular weight range of separations (300-1000 kDa). The Yarra SEC-3000 column is most appropriate for separations between the other two phases (between 50-500 kDa) with significant molecular weight overlap between the three phases. This overlap makes it highly advantageous to investigate two phases when overlap occurs to determine which column provides the optimal separation for a particular application.

The most overlooked area of method development for GFC phases is the mobile phase buffer used for a separation. Increasing or decreasing buffer and salt concentration can either increase or decrease secondary interactions between a protein and the stationary GFC phase. Increasing salt and buffer concentrations reduce ionic interactions possibly improving separation of basic and fairly polar proteins but come with a concomitant increase in hydrophobic interactions. Reducing salt concentrations reduces hydrophobic interactions but increases ionic interactions. As Yarra is very inert to compared to other GFC columns, optimal salt and buffer concentrations will generally be lower than other GFC methods. The key to any GFC separation is using the optimized phase and mobile phase conditions to achieve ones separation goals.

Ordering Information

Yarra™ 3 µm SEC Columns (mm)

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

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

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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