Transform Your Mindset for HPLC and UHPLC Method Development for Analyzing Proteins— Including Monoclonal Antibodies!

A discussion of wide-pore/core-shell technology for monoclonal antibodies, method development considerations with wide-pore/core-shell columns, size exclusion chromatography (SEC) using sub-2 µm particles, and method optimization for SEC.

Brian Rivera
Product Manager for Bioseparations
Phenomenex Inc.

Introduction

Analytical chemists have relied on high performance liquid chromatography (HPLC) columns such as Kinetex® Core-Shell and Luna® Omega Fully Porous Media for high-quality, high-efficiency, and robust reversed-phase analysis of small molecules. Recently, however, there has been a shift among pharmaceutical companies and contract research organizations (CROs) to move into the characterization of large biomolecules, including complex proteins such as monoclonal antibodies (mAbs). In reversed-phase separations for intact proteins and mAb fragments, the approach for both column selection and method development differs greatly from that of small-molecule separations. Applying a "small molecule" mindset to method development may hinder the chance of success; however, there may be analyses in which these tools are applicable.

Primer on Proteins and Protein Folding

Proteins are polypeptide chains with side functional groups that can be hydrophobic, polar, aromatic, or ionic. The side-chain functional groups form thermodynamic interactions within the protein and hold the protein together. They give the protein stability in the form of salt bridges, as well as hydrogen bonding and hydrophobic interactions (i.e., van der Waals forces).

Of these functional groups, the hydrophobic, non-polar groups must be consolidated away from water, which causes the protein to collapse from an extended coil into its globular structure. This final structure determines the protein's function, which gives reason for monitoring and identifying changes or post-translational modifications (PTMs). Any changes to these side chains could potentially be problematic to a protein therapeutic.

Changes in protein folding occur when pH, solvent composition, or temperature are altered. Specifically, heating the protein will expose the buried hydrophobic non-polar groups and allow them to interact with the alkyl stationary phase in HPLC separations similar to small-molecule reversed-phase separations. Unfolding proteins into their extended coil has the potential to affect retention time (see **Figure 1**).

Figure 1: Protein folding.
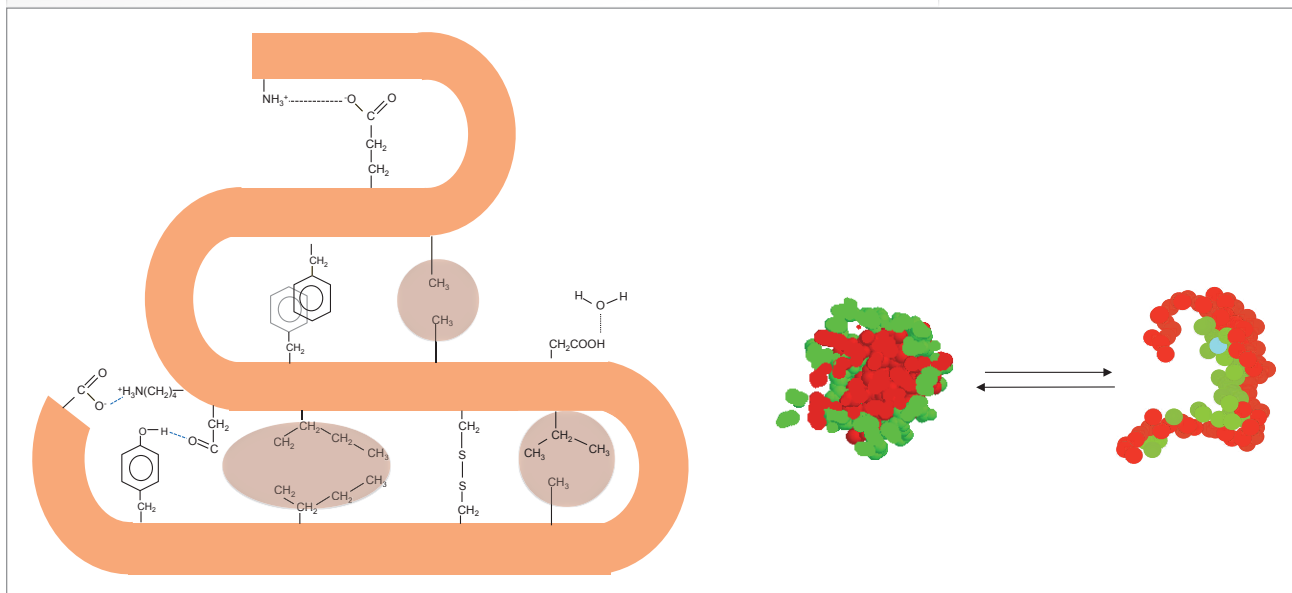
Identifying and Characterizing Therapeutic Proteins

Discussing monoclonal antibodies, immunoglobulin G (IgG) has a molecular weight of approximately 150 kilodaltons (kDa) and is composed of two different polypeptide chains. The protein is made up of about 1,320 amino acids total with two heavy chains (50 kDa) and two light chains (25 kDa). In IgG molecules, the two heavy chains are linked by disulphide bridges and each light chain is attached to one heavy chain, again by disulphide bridges.

Each heavy–light chain pairing creates an antigen binding site; therefore, each IgG molecule has two antigen binding sites that vary in binding specificity and affinity from antibody to antibody. The fragment crystallizable (F_c) region, which is composed of two heavy chain segments, cannot bind the antigen. The F_c region, however, is involved in the antibodies' effector functions such as complement fixation. The fragment antigen binding (F_{ab}) and the F_c regions can be cleaved by using site-specific proteases, such as immunoglobulin-degrading enzyme (IdeS) and papain.

By selectively cleaving an antibody with proteases and/or by using reducing agents, it is possible to engineer fragments with discrete characteristics for detection, separation, and characterization.

Antibody fragmentation methods are somewhat time consuming and require a significant amount of work to optimize the protease amount, the reaction's pH, and the reducing agent's type/amount.

Post-Translational Modifications (PTMs)

It may seem like a daunting task to characterize a biomolecule as large as 1,320 amino acids, but one can focus on certain hotspots for PTMs. PTMs are changes to a protein that happen after protein expression and can be monitored by reversed-phase separations. Examples of PTMs include methionines (particularly those found in the CH2 and CH3 regions, which can be prone to oxidation), aspartate isomerization, asparagine deamidation, N-terminal pyroglutamate formation, N-linked glycosylation, and C-terminal lysine clipping (see **Figure 2**). The F_{ab} region can also have a variety of PTMs; therefore, to properly characterize a protein, PTMs must be identified and criticality determined.

Method Development and Challenges

Although peptide mapping might be a comprehensive way to monitor and identify PTMs, a more streamlined route is to take a middle-down approach. This approach breaks up the antibody into more manageable fragments, thus simplifying PTM identification by reversed-phase analyses.

The most basic workflow for middle-down protein analysis is the reduction of the antibody using dithiotreitol (DTT), beta-mercaptoethanol, tris(2-carboxyethyl)phosphine (TCEP), or a reducing agent that can break up the protein into heavy and light chain fragments. Other site-specific proteases can be used to generate fragments as well, such as the branded FabRICATOR IdeS that generates antibody fragments by cleaving below the IgG's hinge region. This process generates F_{ab2} and $F_{c/2}$ fragments. This highly specific enzyme only cleaves at one site, thus preventing over-digestion. It can also be used as a complementary approach to N-glycan profiling as well as C-terminal clipping.

The more traditional route for generating F_{ab} and F_c fragments is using papain, which can be combined with another protease and reduction to achieve even smaller mAb fragments for better characterization.

Traditionally, columns packed with fully porous particles with wide pores of 300Å have been used in the reversed-phase

analysis of intact proteins; however, broad peak shapes and less-than-desirable separations occur. Poor protein recovery, especially with traditional alkyl stationary phases, has limited analytical options using stationary phases like C4s or butyls.

Phenomenex saw an opportunity in this area to develop a core-shell particle using silica sol-gel chemistries on the Kinetex® platform. By optimizing core-shell to solid-core ratios, a particle morphology was developed that was optimal for improved chromatography and better recovery.

However, one cannot simply make a "wider pore" Kinetex and expect the same improvements in chromatography. For example, a 0.73 solid-core to core-shell ratio could not be applied to proteins because their slower diffusion rate requires a smaller ratio. A rho of 0.89, for instance, affects the loadability, overall surface area, and retention because pore volumes are smaller.

To ensure efficient protein separations, the distance the analyte travels must be minimized. The Aeris™ WIDEPORE core-shell solution/platform for proteins was created with this in mind. It uses a 3.2 µm solid core with a 0.2 µm porous shell. This optimal particle morphology provides high efficiency for intact reversed-phase analysis and improves protein recovery due to low surface area. High temperature stability up to 90 degrees under acidic conditions can also be achieved with this technology, which is critically important in method development for proteins.

In addition, Aeris WIDEPORE is targeted for a different selectivity through the C4, XB-C18, and XB-C8 stationary phases. Selectivity between the three columns is illustrated in **Figure 3**.

Method Development Considerations Overview

Method development considerations using wide-pore core-shell particles include:

- **Mobile phase:** A standard 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile (ACN) are mainstays for LC-UV methods, while a standard 0.1% formic acid is used in LC–mass spectrometry methods. Chromatographers commonly use 10–50% isopropanol in mobile phase B. This may not be necessary for protein recovery, especially with Aeris WIDEPORE core-shell columns, which have a low surface area and less hydrophobic phase.

- **Gradient optimization:** If one is transferring a method from a fully porous 300Å method, it will likely be necessary to decrease the initial organic concentration approximately 5–10% below the usual reversed-phase methodologies because the Aeris WIDEPORE is less hydrophobic than a fully porous 300Å column. A good scout gradient for mAbs is about 25–40% mobile phase B. The initial and final percent B should be adjusted to increase the elution window. In addition, hot, fast, shallow gradients are required to prevent degradation (**Figure 4**). Gradients for proteins are influenced by how the folding affects retention as proteins are sensitive to changes in solvent compositions. Responses to these changes may not be linear or predictable and gradient times should be kept short.
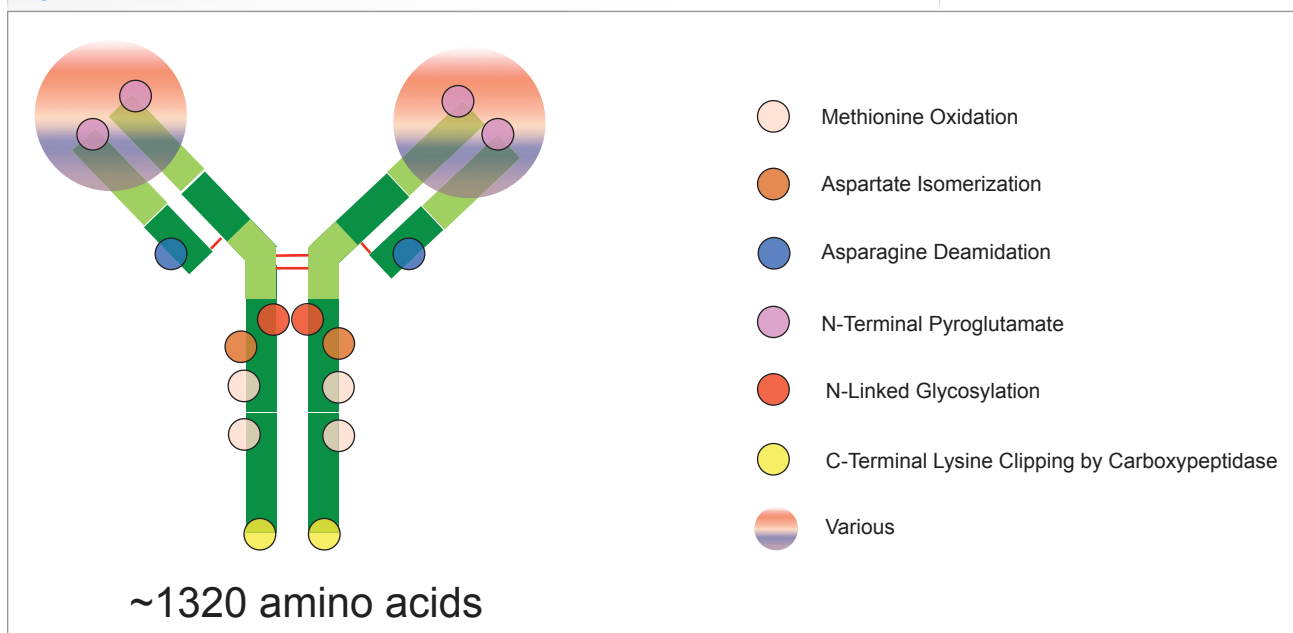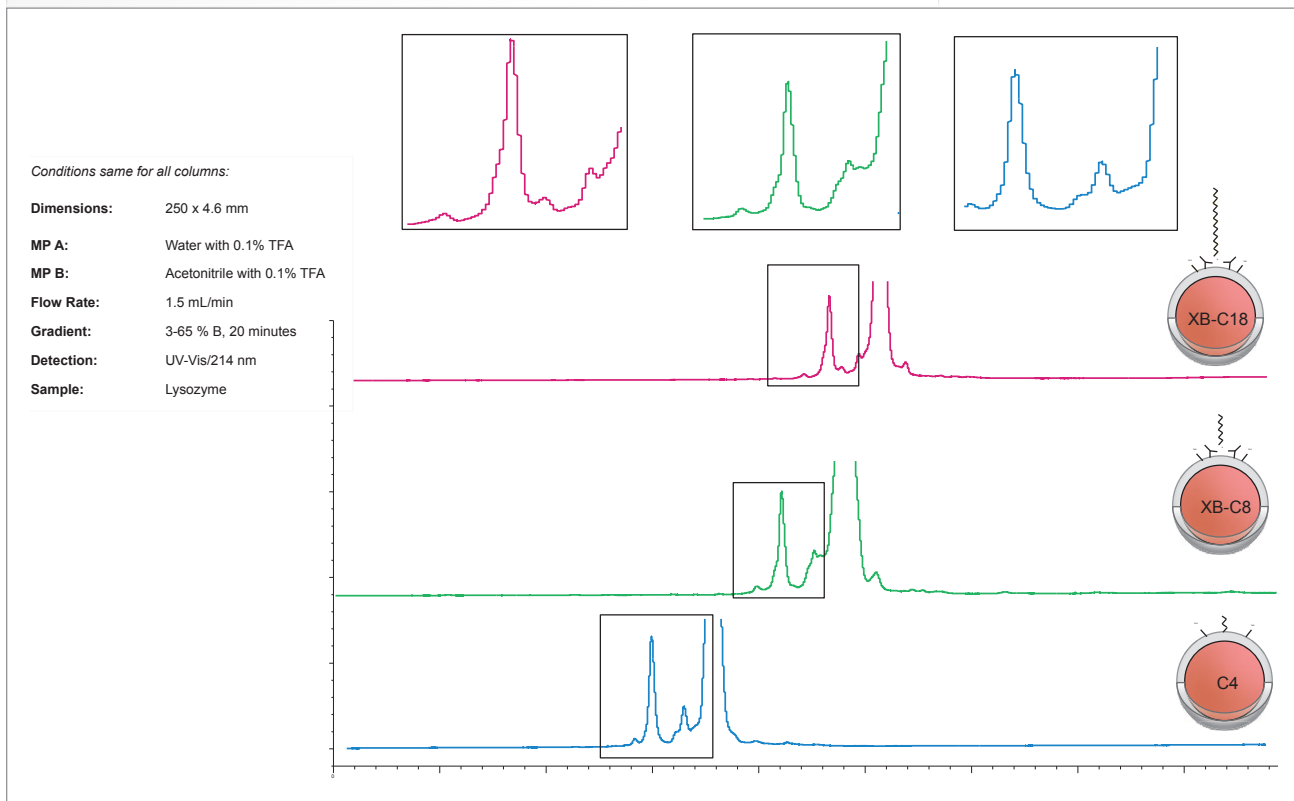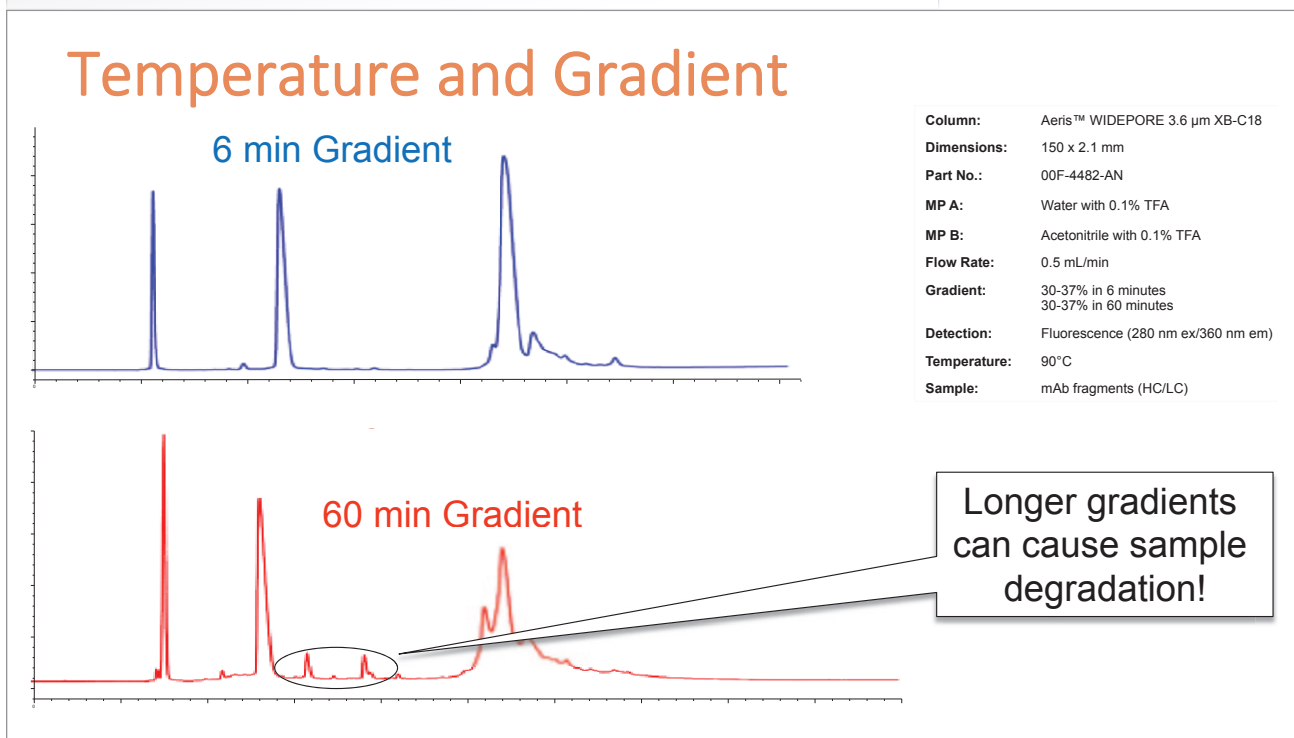

Figure 2: Post-translational modifications.

Methionine Oxidation

Aspartate Isomerization

Asparagine Deamidation

N-Terminal Pyroglutamate

N-Linked Glycosylation

C-Terminal Lysine Clipping by Carboxypeptidase

Various

~1320 amino acids

Figure 3: Selectivity of three columns.

Conditions same for all columns:

Dimensions:	250 x 4.6 mm
MP A:	Water with 0.1% TFA
MP B:	Acetonitrile with 0.1% TFA
Flow Rate:	1.5 mL/min
Gradient:	3-65 % B, 20 minutes
Detection:	UV-Vis/214 nm
Sample:	Lysozyme

Figure 4: Hot, fast, shallow gradients prevent degradation.

Temperature and Gradient

6 min Gradient

60 min Gradient

Longer gradients can cause sample degradation!

Column:	Aeris™ WIDEPORE 3.6 µm XB-C18
Dimensions:	150 x 2.1 mm
Part No.:	00F-4482-AN
MP A:	Water with 0.1% TFA
MP B:	Acetonitrile with 0.1% TFA
Flow Rate:	0.5 mL/min
Gradient:	30-37% in 6 minutes 30-37% in 60 minutes
Detection:	Fluorescence (280 nm ex/360 nm em)
Temperature:	90°C
Sample:	mAb fragments (HC/LC)

Source: Fekete et al, Analysis of recombinant monoclonal antibodies by RPLC: toward a generic method development approach. J Pharm Biomed Anal. 2012

- **Temperature:** Temperature is critical for good chromatography and protein recovery for monoclonal antibodies; running hot, as noted, is often a method requirement. The effect of temperature on selectivity of the F_c and F_{ab} fragments can be seen in **Figure 5**.

The two most critical method development parameters to optimize will be gradient to adjust the capacity factor (resolution) and temperature to adjust the selectivity in intact protein reversed-phase analyses.

Size-Exclusion Chromatography (SEC)

SEC involves separating analytes based upon their hydrodynamic volume in a solution. Gel permeation chromatography (GPC) is determining a molecular weight and distribution of analytes in a solution. It is often used to determine the polydispersity of a synthetic polymer in combination with RI and viscometers and light-scattering detection.

Aggregate analysis, sometimes referred to as gel filtration chromatography (GFC) due to its aqueous conditions, is an SEC technique used to fractionate relatively monodispersed macromolecules in their native undenatured state. This technique separates monomers from dimers and multimers and is a non-absorptive separation technique. The benefit of this method is that the protein remains in its native, undenatured state. This is important since denaturing methods like RP-HPLC would disrupt the interactions which stabilize the agglomerated dimers/multimers.
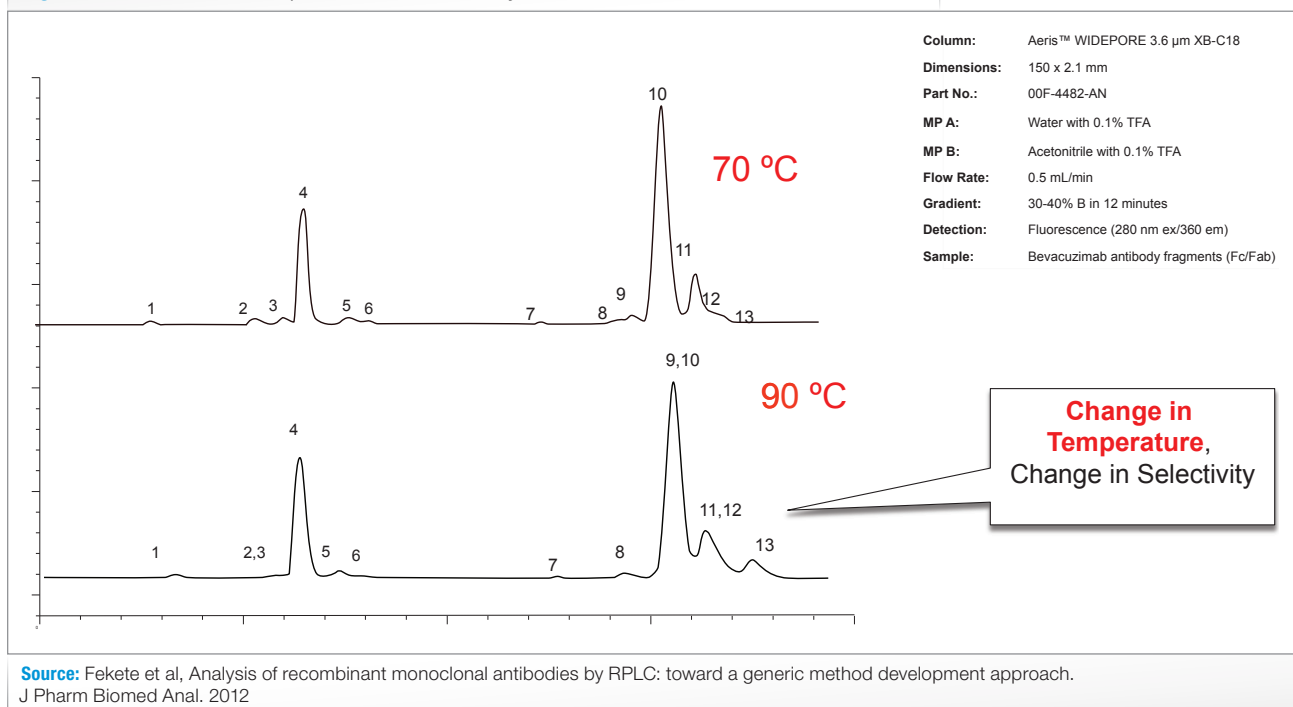
Silica-based SEC columns are packed with fully porous particles of a pore size distribution to accommodate the analytes of interest. The separation is based upon a hydrodynamic radius; the larger the analyte, the fewer the pores it can access, the lower the elution volume. The smaller the analyte, the more pores it can access, thus the higher the elution volume. SEC can then be used to separate large aggregates such as dimers from monomers, again, while keeping the protein in its native, folded state. The challenge in optimizing SEC methods is minimizing any interaction between the stationary phase and the protein.

Phenomenex recently released several sub-2 µm particles columns: Yarra™ 1.8 µm SEC-X150 and SEC-X300 columns (**Figure 6**). They vary slightly in exclusion ranges as the pore sizes are "nominal," and the molecular weights differ slightly because of variations in silica treatment.

SEC methods have relatively low efficiency and low-resolution separation; the slow diffusion rates of proteins results in more band broadening and higher linear velocities. Reducing the band broadening requires the packing of smaller particles (sub-2 µm particles). The result is an increase in the linear velocity along with improved analysis and reduced run times. However, when adjusting a method to a new column, dead volume and instrument differences should be considered due to the impact on plate count (efficiency) and performance.

Using a smaller particle provides higher efficiency, improved separation, and better characterization of protein

Figure 5: The effect of temperature on selectivity.

Column:	Aeris™ WIDEPORE 3.6 µm XB-C18
Dimensions:	150 x 2.1 mm
Part No.:	00F-4482-AN
MP A:	Water with 0.1% TFA
MP B:	Acetonitrile with 0.1% TFA
Flow Rate:	0.5 mL/min
Gradient:	30-40% B in 12 minutes
Detection:	Fluorescence (280 nm ex/360 em)
Sample:	Bevacuzimab antibody fragments (Fc/Fab)

Change in Temperature, Change in Selectivity

Source: Fekete et al, Analysis of recombinant monoclonal antibodies by RPLC: toward a generic method development approach. J Pharm Biomed Anal. 2012

aggregates. If a high extent of characterization is not required, then increasing throughput may be a higher priority. This can be accomplished with a shorter column, which has similar resolution but half the run time and lower back pressure with only a slight loss of resolution and efficiency on a 150 mm column versus a 300 mm column (see **Figure 7**). However, the longer column may increase accuracy, reproducibility, sensitivity, and robustness of the separation method.

SEC Method Development

Mobile phases and flow rates are critically important and under-utilized method development tools that should be used for a good, robust SEC method. Notably, high molecular weight species separate out much better using lower linear velocities. This technique ensures proper ingress and egress of the analytes through the pores.

In considering flow rate, changes are much more pronounced for larger molecular weight analytes. The system's back pressure must also be a consideration, as large

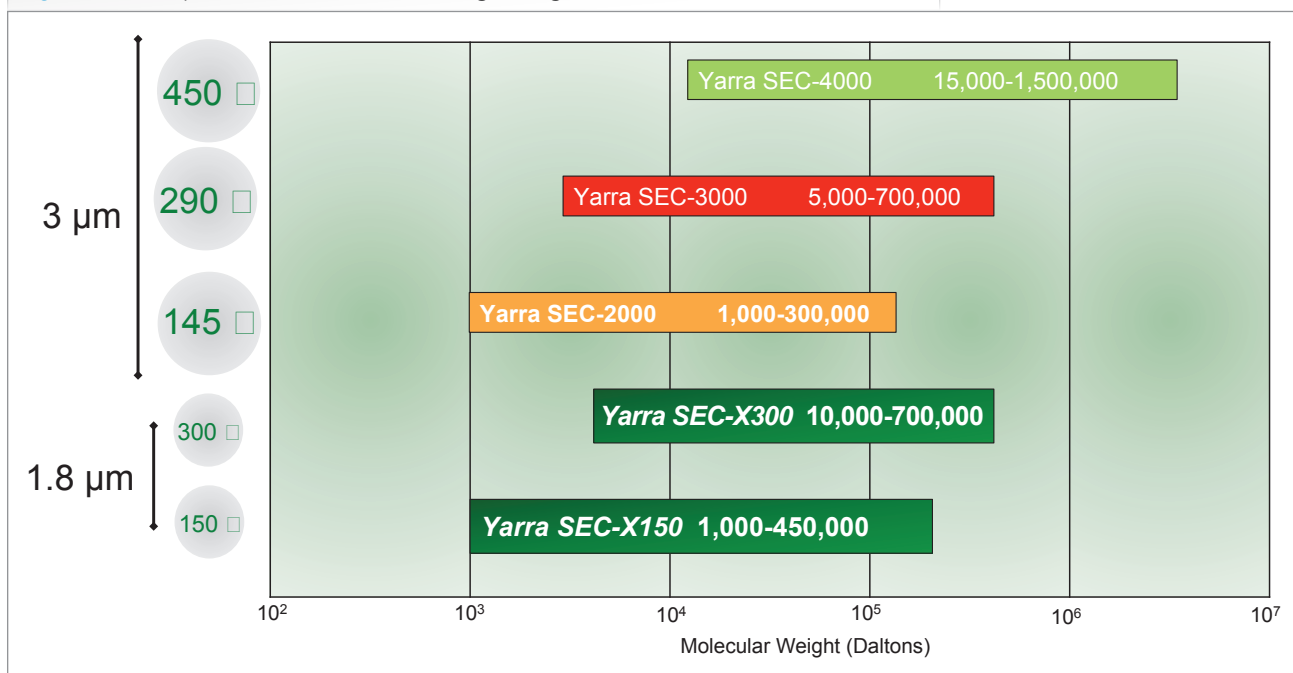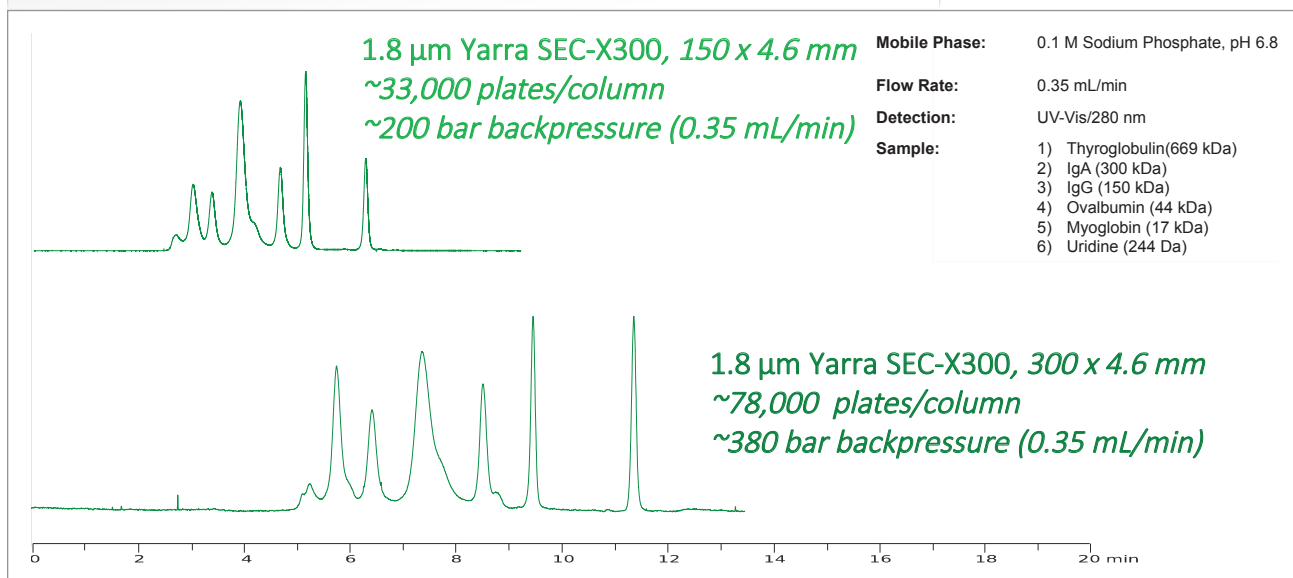**Figure 6:** Yarra™ pore size and molecular-weight range.

Figure 7: Comparison of column dimensions.

1.8 µm Yarra SEC-X300, *150 x 4.6 mm*
~33,000 plates/column
~200 bar backpressure (0.35 mL/min)

Mobile Phase:	0.1 M Sodium Phosphate, pH 6.8
Flow Rate:	0.35 mL/min
Detection:	UV-Vis/280 nm
Sample:	1) Thyroglobulin(669 kDa)
	2) IgA (300 kDa)
	3) IgG (150 kDa)
	4) Ovalbumin (44 kDa)
	5) Myoglobin (17 kDa)
	6) Uridine (244 Da)

1.8 µm Yarra SEC-X300, *300 x 4.6 mm*
~78,000 plates/column
~380 bar backpressure (0.35 mL/min)

molecules have a slower diffusion rate. Running at higher flow rates affect the large analyte's ability to ingress and egress at a pore resulting in a reduced ability to increase throughput.

In considering the mobile phases, the mobile phase composition in terms of buffer and salt concentration is critical. Salt is necessary to disrupt ionic interactions between the positively charged moieties of the protein and the acidic silanols that are inherent in any silica-based SEC column. Sodium phosphate versus potassium phosphate has a critical role in high molecular weight aggregate recovery with many analysts believing that potassium shields acidic silanols better than sodium. The salt and buffer concentrations stabilize the protein but can also cause it to be more hydrophobic, thus leading to secondary interactions. The result is broadening of peaks and poor protein recovery.

An additional consideration is that vendors prepare their columns under the different conditions. Different chemistries may be involved and differences in mobile phases and the amount of phosphate or sodium in the buffer will affect proteins in different ways.

Antibody drug conjugates (ADCs) are more challenging proteins to work with as they are hydrophobic and the peak shapes are typically very broad. The addition of organic solvent is often necessary and improvements in both peak shape and protein recovery are observed with incremental amounts of acetonitrile added (ACN). Other applications may use isopropanol, which is probably the most common organic modifier to minimize the hydrophobic interactions between the stationary phase and the hydrophobic ADCs. ACN is slightly advantageous in that it is less viscous in higher percentages than isopropanol or methanol. However, exceeding approximately 15% organic modifier may disrupt some of the interactions that are holding the aggregate together, which

may hinder a proper assessment of the amount of aggregates in a particular sample.

In addition to increased organic in the mobile phase, high back pressure and temperature under ultra-high performance liquid chromatography (UHPLC) conditions, while working with sub-2 µm particles, may lead to changes in measured aggregate. This is of concern because aggregate is what the method is intended to assess and is a trade off with the increased performance of UHPLC. Increased back pressure associated with sub-2 µm columns may create the propensity for the analytes of interest to aggregate on column. This is further exacerbated with temperatures that are intended to increase resolution or reduce viscosity and can result in differences in the percent aggregate reported. Additionally, changes in temperature will affect proteins differently as the extent of unfolding will be different.

Summary

Sub-2 µm columns can be used to improve resolution for demanding aggregate analysis methods and to improve throughput as well. The column is not the only part of the method, however, method development should definitely be a consideration for high performance and robust SEC methods. Factors method development should consider:

- Flow rate adjustment to improve separations between monomers and high molecular weight aggregates

- Mobile phase composition

- Differences in column preparation by different vendors

- Temperature adjustment, which may improve separation or reduce viscosity

- The potential for temperature and back pressure to result in column aggregation