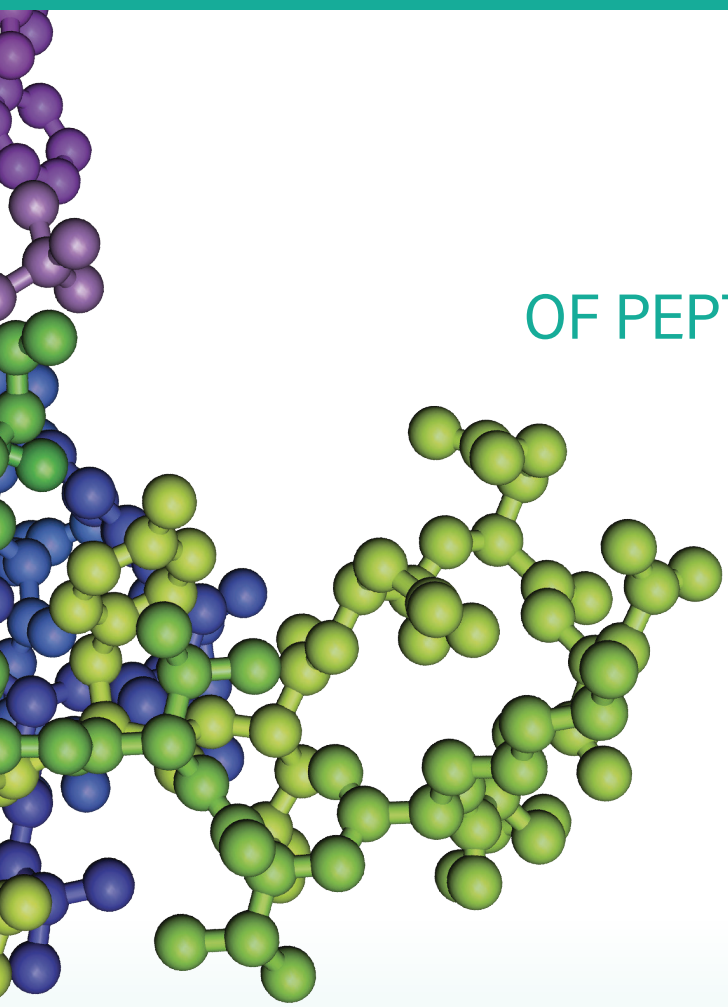
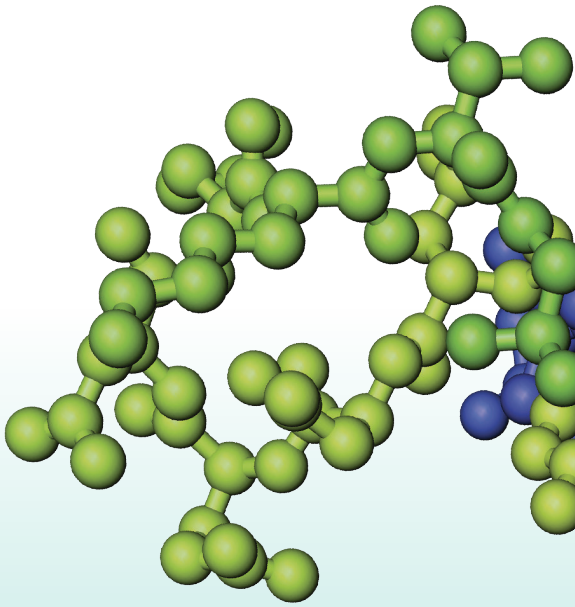
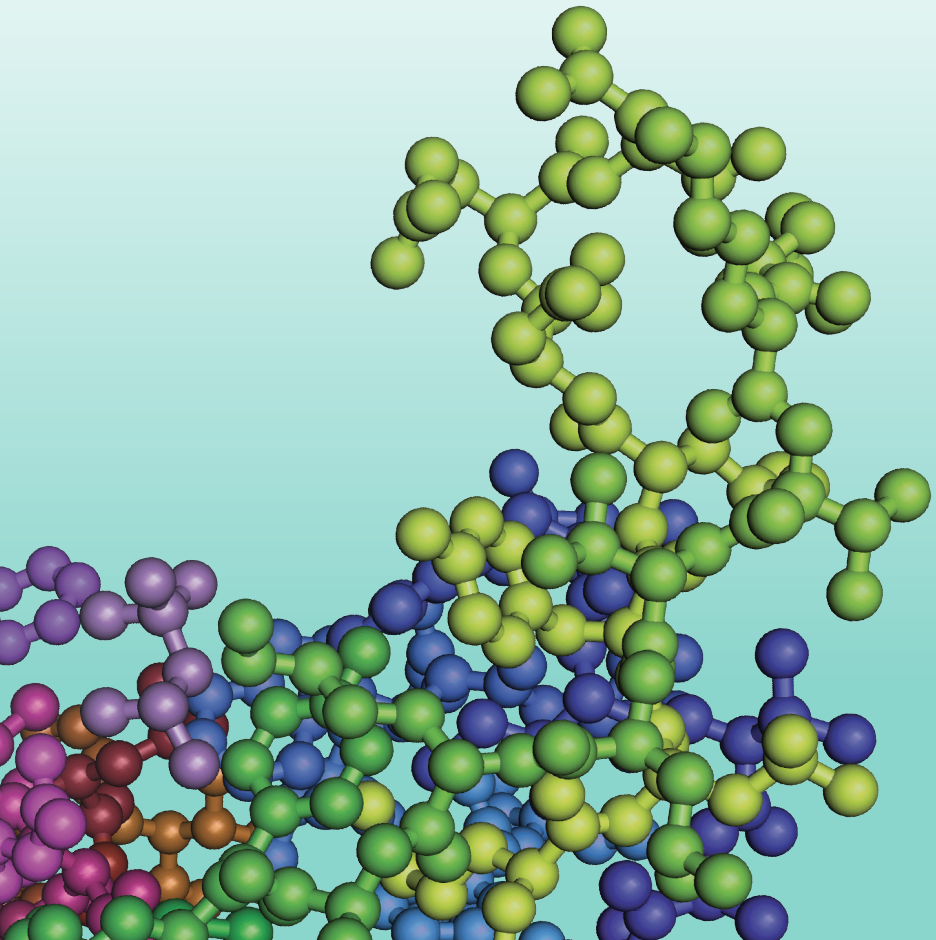
[APPLICATION NOTEBOOK]

SIZE-EXCLUSION CHROMATOGRAPHY (SEC) OF PEPTIDES, PROTEINS, AND AAVs







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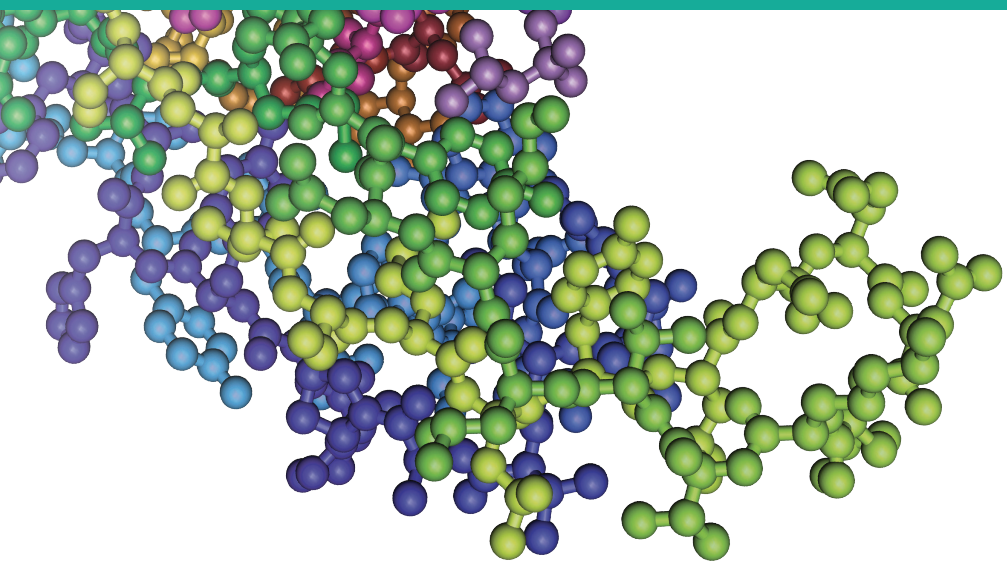
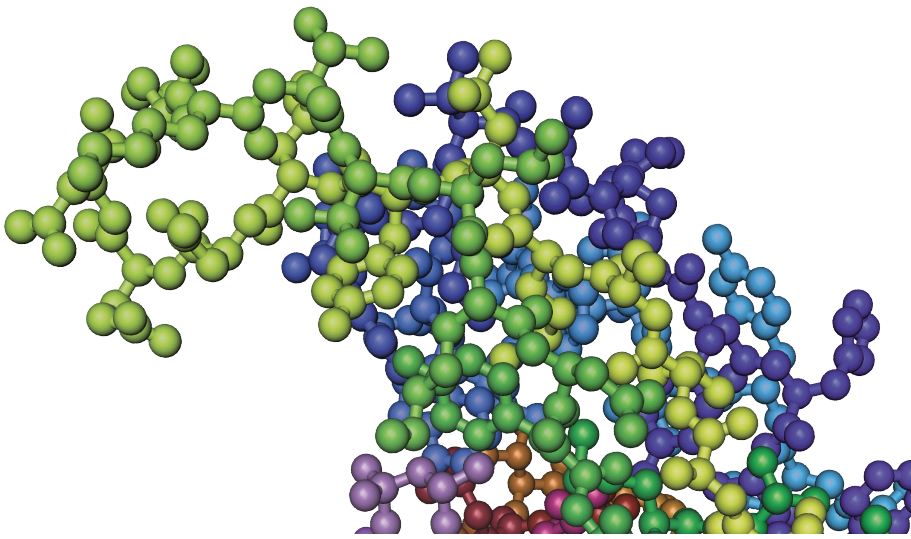


Introduction

Gel-permeation chromatography (GPC), size-exclusion chromatography (SEC), and gel-filtration chromatography (GFC) are commonly used terms to describe a liquid chromatographic technique that separates compounds based on their relative different size in solution, which loosely correlates with their molecular weight. For over 50 years, Waters™ has been the market leader in GPC- and SEC-based analyses, providing the highest quality columns , instrumentation, and applications support for both organic- and aqueous-based size separations.

SEC is the analytical "gold standard" for the separation and accurate quantitation of aggregates, monomers, and lower molecular weight fragments contained in biotherapeutic peptides and proteins, including monoclonal antibody (mAbs) drugs. There are more than 500 mAbs used as biotherapeutics, diagnostic reagents, or research tools, many of which require the use of SEC to help confirm their quality for the intended end use.

Waters has compiled the following references to help guide you through some common challenges and applications associated with SEC of peptides and proteins. The topics covered range from fundamentals of SEC to suggested best practices. We hope you find this information useful in your desire to obtain high-quality data from your SEC analyses.



Biographies

Robert Birdsall, Ph.D.

Robert is a Consultant Scientist with over 10 years of experience in the separation and analysis of biotherapeutics including antibodies, oligonucleotides, and glycoproteins. He works in the Scientific Operations' Biopharmaceutical Group of Waters Corporation with a focus on developing analytical solutions for the characterization, monitoring, and quantification of biotherapeutics using new separation technologies. Since obtaining his Ph.D. in Analytical Chemistry from Purdue University in 2013, Robert has authored multiple peer reviewed papers and patents in the application of new technology and methods for improving chromatographic performance in the separation of protein-based molecules.

Edouard S. P. Bouvier

Dr. Bouvier is a Principal Consultant in the Chemistry Technology Center at Waters Corporation, where he is responsible for identifying and assessing new technologies. For most of his career at Waters, Dr. Bouvier was an integral part of the Chemistry R&D Organization, where he was responsible for the research and development of several commercially successful products. He is an inventor on over thirty granted patents and author of over twenty papers. He was the primary inventor of a water-wettable, reversed-phase material for SPE, which was commercialized as Oasis HLB, and was a key member of the team developing the family of BEH SEC columns for peptide and protein characterizations. He managed a group which was responsible for performing the initial proof-of-concept work leading the commercialization of the ACQUITY product line. Dr. Bouvier has also been responsible for managing several external research collaborations.

Weibin Chen, Ph.D.

Weibin is the Director of Scientific Operations at Waters Corporation. He leads the development of analytical solutions to address the challenges in the biopharmaceutical industry by LC-MS and related technologies. Under his direction, the biopharmaceutical scientists develop new technologies and applications, demonstrate their usefulness, engage collaboration with partners, and disseminate the data, analysis and methodologies for use in the biopharmaceutical community. Weibin has a Ph.D. in Analytical Chemistry from Purdue University and published more than 40 peer-reviewed scientific papers/book chapters in biopharmaceutical analysis.

Paula Hong, Ph.D.

Paula is a Principal Consulting Scientist at Waters Corporation in the Systems Design and Development Application Laboratory. She received her Ph.D. in Inorganic Chemistry from the University of Pennsylvania, focusing on the reactivity of transition metal/silane complexes. Paula has over 15 years experience in the area of LC and LC-MS applications, including amino acid analysis, ion exchange, and peptide mapping. She began her career at Waters in the Chemistry Division focusing on novel column chemistries for biopharmaceutical applications, including studies on the first hybrid sub-2-µm SEC column. Her more recent work has focused on understanding the role of instrumentation characteristics on chromatographic separations. These studies have included method development for both reversed-phase and supercritical fluid applications as well as method transfer. Paula has authored over 25 journal articles, white papers, and application notes on a variety of chromatographic principles. Her more recent work has focused on the instrument attributes that affect method transfer and method lifecycle management.

Pamela Iraneta

Pamela joined Waters Corporation in 1987. Her interest in all forms of chromatography started while characterizing silica sols using Sedimentation Field Flow Fraction (SF^3) – a technique based on some of the same principles used in chromatographic separations. Once introduced to and mentored in liquid chromatographic separations by Uwe Neue, she became enamored with the complexities of chromatographic surfaces and the interplay of fluid dynamics, mass transfer, and thermodynamic equilibration that takes place as a separation is accomplished. Throughout her career at Waters, she has made significant contributions to the development of the Oasis family of solid-phase extraction products, and ACQUITY UPLC BEH, XBridge, XSelect, and CORTECS Column brands. Her present focus is in SEC research, troubleshooting, and development. She is a co-author on 26 peer-reviewed publications and an inventor on 14 patents. She graduated from the College of William and Mary with a Bachelor of Science in Chemistry and from Northeastern University with a Bachelor of Science in Electrical Engineering. She is a Consulting Scientist at Waters Corporation.

Stephan M. Koza, Ph.D.

Stephan has worked at Waters Corporation for seven years and leads an applications group that has a primary focus on the use of UPLC, HPLC, LC-MS, and sample preparation chemistries and columns for the analysis of biomolecules. Prior to joining Waters, he had nearly twenty years of experience with biopharmaceutical characterization and analytical method development.

Matthew Lauber, Ph.D.

Matthew is a Consulting Scientist within Research and Development for the Chemistry Technology Center at Waters Corporation, where he leads an evaluation team that focuses on developing new products and methods for the analysis of biomolecules. From his graduate studies in proteomics at Indiana University, he has for six years been applying his expertise in protein chemistry and LC-MS-based characterization methods toward the development and application of state-of-the-art reagents and separation chemistries. In this time, Matthew has aided the commercialization of numerous new technologies for analyzing biopharmaceuticals, including novel LC-MS N-glycan profiling, HILIC of intact proteins, high resolution peptide mapping, and improved protein reversed-phase separations. Among other things, his group is now pushing towards new capabilities for protein ion-exchange and size-exclusion chromatography.

Henry Shion

Henry is a Principal Scientist working in the Scientific Operation's Biopharmaceutical Group within Global Markets at Waters Corporation. He has more than 20 years of hands-on mass spectrometry (LC-MS, GC-MS, MALDI/MALDI imaging) experience in biotherapeutics and small molecule applications. He is currently focused on developing LC-MS (with reversed-phase, native, and HILIC separations) based analytical solutions for the characterization, monitoring, and quantification of biotherapeutics, such as monoclonal antibodies (mAbs), biosimilars, antibody-drug conjugates (ADCs), glycoproteins, and oligonucleotides. Henry authored/co-authored more than 20 peer reviewed papers/patents in various scientific subjects. He obtained his Ph.D. in Analytical Chemistry from Drexel University in 1997 and carried out a NIH-sponsored postdoc training at University of Illinois College of Pharmacy under Dr. Richard van Breemen before joining Waters as an Application Scientist in 1998.

Biographies

Thomas Walter, Ph.D.

Thomas received a Ph.D. in Chemistry from the University of Illinois at Urbana-Champaign, working on multinuclear solid-state NMR spectroscopy of heterogeneous catalysts. Joining the Chemistry R&D Group at Waters Corporation in 1987, he held positions of increasing responsibility, including serving as the Director of Chemistry R&D from 2000–2015. He is a coinventor of hybrid particle technology and an inventor on 11 issued patents. He has authored 30 publications on chromatographic materials, columns, and NMR spectroscopy. Thomas is currently a Corporate Fellow and manages a group focused on the chromatographic evaluation of new columns and sample prep devices for small molecule applications.

Bill Warren

Bill is a Principal Product Manager within Waters Chemical Technology Group at Waters Corporation. During his 14 years of his 34-year tenure, he functioned as a Senior Application Chemist focusing on protein as well as DNA/RNA purifications and analyses. For the past dozen years, he has been part of the Chemistry Marketing and Product Management Team that manages a comprehensive line of bioseparation offerings that included nano, capillary, analytical, and preparation columns as well as reagents and kits for DNA/RNA, amino acid, peptide, protein, and glycan applications. His current focus is on chemistry consumables for protein, DNA/RNA, and cell therapy related applications.

Kevin Wyndham, Ph.D.

Kevin is the Senior Director of Research and Development for the Chemistry Technology Center at Waters Corporation. He oversees innovation in new liquid chromatographic (HPLC, UHPLC) separation products that are used in the fields of healthcare, biopharmaceutical analysis, food safety, environmental analysis, and material science. He has over 25 years of experience in material science, hybrid organic/inorganic materials, and the design of new analytical separation products. His team's focus includes the preparation and surface modification of hybrid organic/inorganic materials for chromatography, including the introduction of BEH SEC particle technology. Kevin received a Bachelor's Degree in Chemistry from Boston College and a Ph.D. in Chemistry from the University of California, Irvine. He holds over twenty U.S. patents and has authored several publications in the areas of hybrid materials, porous chromatographic media, and separation science.

Hua Yang, Ph.D.

As a Principal Scientist, Hua has been working at Waters Corporation for 13 years. Originally from the Chemistry Research and Development Group, Hua did cutting-edge research in making advanced materials for analytical separation and sample preparation. In recent years, Hua joined the Biopharmaceutical Group in Scientific Operations to develop applications for bio-molecule separations such as ion-exchange and size-exclusion chromatography. She received her Ph.D. in Chemistry from the University of Pittsburgh and worked as a Postdoctoral Fellow in the University of California, San Francisco

Ying Qing Yu, Ph.D.

Dr. Ying Qing is a Senior Science Manager at Waters Corporation. She joined Waters in 2001, shortly after she received her Ph.D. from the Analytical Chemistry Department at Purdue University. She is a group leader in Scientific Operations at Waters. Her group's focus is on protein biotherapeutics characterization using UPLC/Q-Tof MS platform. The application areas her group focuses on are mAb characterizations, glycan profiling, multi-attribute method, AAV characterization, impurity analysis, and the hydrogen deuterium exchange mass spectrometry for protein higher order structure analysis.

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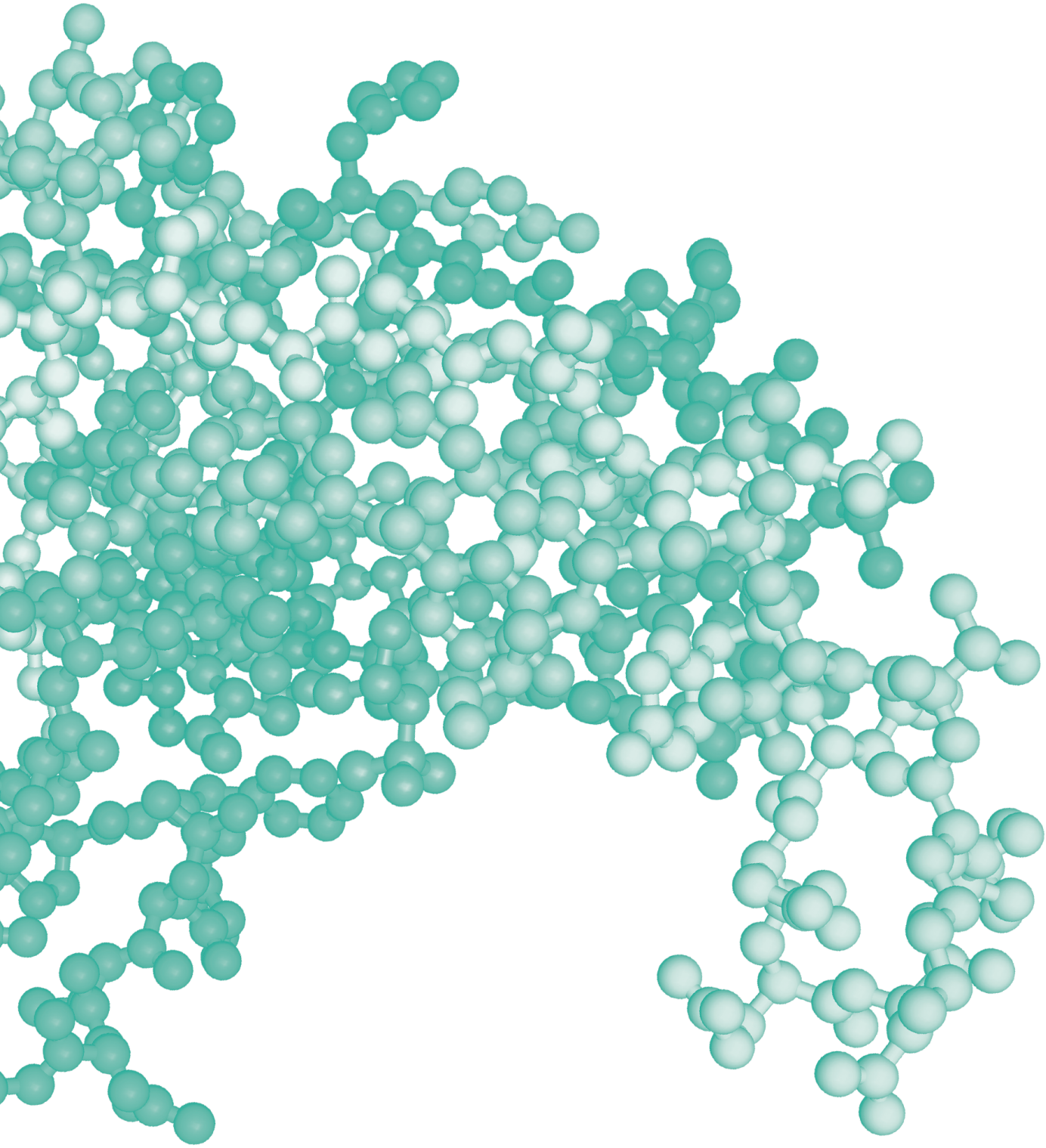

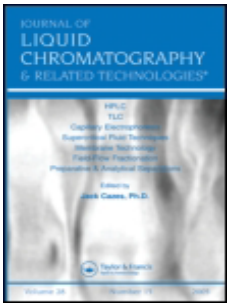

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A REVIEW
SIZE-EXCLUSION CHROMATOGRAPHY FOR THE ANALYSIS OF PROTEIN BIOTHERAPEUTICS AND THEIR AGGREGATES

Paula Hong, Stephan Koza, and Edouard S. P. Bouvier
Waters Corporation, Milford, MA, USA

□ *In recent years, the use and number of biotherapeutics has increased significantly. For these largely protein-based therapies, the quantitation of aggregates is of particular concern given their potential effect on efficacy and immunogenicity. This need has renewed interest in size-exclusion chromatography (SEC). In the following review we will outline the history and background of SEC for the analysis of proteins. We will also discuss the instrumentation for these analyses, including the use of different types of detectors. Method development for protein analysis by SEC will also be outlined, including the effect of mobile phase and column parameters (column length, pore size). We will also review some of the applications of this mode of separation that are of particular importance to protein biopharmaceutical development and highlight some considerations in their implementation.*

Keywords biomolecule, chromatography, monoclonal antibody, protein, size-exclusion chromatography (SEC)

INTRODUCTION

Given the complexity of protein and peptide-based parenteral therapies, a broad set of complementary techniques are required to monitor the critical quality attributes of intermediate drug substances and drug products.[1,2] As outlined in regulatory agency guidelines, one of these attributes is a quantitative assessment of the aggregation, including dimers and multimers, of the active protein. While numerous techniques have been developed to monitor protein aggregation, size-exclusion chromatography (SEC) has been predominantly favored for routine and validated analyses because of both its speed and reproducibility.[3–6] SEC is also an accurate method if confirmed with an orthogonal method, such as sedimentation velocity analytical ultracentrifguation (SV-AUC).[7–9] The intent of this review is to provide a summary of SEC, including background,

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theory, and applications with a primary focus on the analysis of peptide and protein aggregates.

Since the early introduction of biologic-based therapeutics, the presence of protein aggregates has been theorized to compromise safety and efficacy.[10] These concerns, which date to the 1980s, have led to routine analysis and quantitation of dimers, trimers, and higher order aggregates for a wide variety of biologic-based therapies, such as insulin,[3–6] recombinant human growth hormone (rGH),[11,12] and monoclonal antibodies.[8,13,14] Aggregate analyses are typically performed throughout the entire product lifecycle of biotherapies.[8] However, each stage of development may have different assay requirements including robustness, sensitivity, ease of use, and high throughput. These desired attributes have led to a wide variety of techniques for the analytical characterization of biotherapies based on the size of the biomolecules.[8] Commonly used techniques include SV-AUC,[15,16] asymmetric flow field flow fractionation (AF4),[16–18] multi-angle light scattering (**MALS**),[12,19,20] and SEC. While all of these techniques are frequently used, the dominant method continues to be SEC.[9]

HISTORY

The concept of size-based separations by chromatography was first speculated by Synge and Tiselius,[21] based on the observation that small molecules could be excluded from the small pores of zeolites as a function of their molecular size.[22] The term "molecular sieve," coined by J. W. McBain[23] to describe this property of zeolites, was subsequently used to describe the technique commonly known today as size-exclusion chromatography. Over the years, SEC has been known by a number of other names, such as exclusion chromatography,[24] steric-exclusion chromatography, restricted-diffusion chromatography,[25] liquid-exclusion chromatography,[26] gel-filtration chromatography, and gel-permeation chromatography. The first examples of size-based separations by liquid chromatography were noted by Wheaton and Bauman[27] in their work on ion-exclusion chromatography. They observed that various nonionic species could be separated on ion-exchangers by a size-based mechanism. Similarly, R. T. Clark[28] demonstrated the separation of sugar alcohols on a strong cation exchange resin.

Lindqvist and Storgårds[29] reported the first separation of biomolecules by a size-exclusion process, where they separated peptides from amino acids on a column packed with starch. Subsequently, Lathe and Ruthven[30,31] performed extensive characterizations on columns packed with potato or maize starch, which demonstrate very low adsorption of proteins. Using a column packed with maize starch, they were able to separate

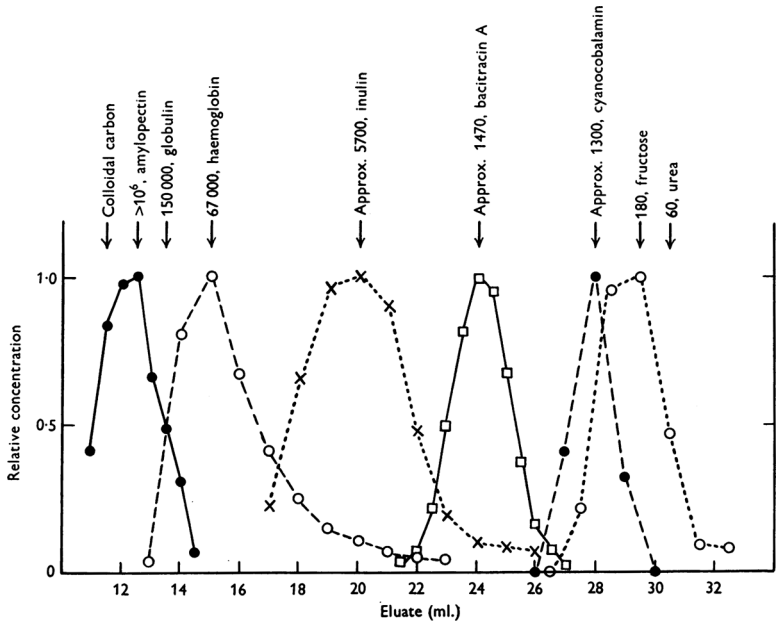

Figure 1 Separation of amylopectin, hemoglobin, inulin, bacitracin A, cyanocobalamin, and fructose on a column containing heat-swollen maize starch. Mobile phase: 25 mM borate, 25 mM potassium chloride, pH 8.5. Column bed dimensions: 16 mm diameter x ~16 cm. Flow rate: ~3 mL/hr. Reproduced from Reference[31] with permission from Portland Press Ltd.

a variety of compounds including proteins and peptides by the "molecular sieve" effect (see Figure 1).

However, the low mechanical strength of starch limited the speed of separations as it could not withstand high linear velocities before bed collapse. In addition, as a natural product, starch was relatively poorly defined. Shortly thereafter, dextrans crosslinked with epichlorohydrin were developed. These materials proved to be equally proficient at minimally interacting with proteins and additionally provided greater mechanical strength than starch.[32–35] Pharmacia commercialized these materials under the tradename Sephadex, and they became the standard media for size-based separation of proteins for many years. Sephadex was initially prepared as irregular particles and later synthesized as porous spheres.[36] By varying the degree of crosslinking, the inclusion or exclusion of the analytes from the pore network could be altered. The Sephadex gels were weakly acidic, showing some adsorption of basic analytes, with a binding capacity of about 10 μ-equivalent per g of dry gel.[37] By addition of salt to the eluent, ionic interactions could be minimized.

Other polymeric resins, such as agar and agarose,[38–40] polyacrylamide,[41–43] polyvinylethylcarbitol,[42] and polyvinylpyrrolidone[42] gels

were also used for size based separations. Polyacrylamide-based gels were commercialized by Bio-Rad under the trade name Bio-Gel.

Early on, it was realized that SEC materials follow the same chromatographic theory as adsorption chromatography. In one of the early papers describing Sephadex, Flodin[34] demonstrated the beneficial effect of reduced particle size on chromatographic performance. There has been a drive to further reduce particle size in order to achieve faster speed and greater chromatographic resolution. However, the soft polymeric resins compress under pressure and flow, which limits the extent that the particle size can be reduced for chromatographic applications.

In the 1970s, derivatized porous silica became the predominant chromatographic stationary phase media due to its superior mechanical strength, non-swelling nature and inertness over a fairly wide range of conditions. The utility of porous silica for SEC was explored, as the greater mechanical strength provided a means to further improve performance by reducing particle size. As a size-exclusion medium for proteins, it suffered from strong ionic interactions due to the acidic surface silanols. To mitigate these interactions, both surface modifications and mobile phase additives were employed. Surface modifiers include glyceropropylsilane[44] and N-acetylaminopropylsilane.[45] However, these functional groups are non-ideal as they exhibit significant hydrophobic interactions with proteins. The most commonly used surface modifier today is a diol[46,47] functional group, which has minimal hydrophobic interactions. However, even with high coverage, a significant concentration of surface silanols still remains.[48] To further diminish interactions with these residual silanols, high ionic strength mobile phases are typically required.

More recently, porous hybrid organic/inorganic particles[49] have been employed as the base particle for size-exclusion chromatography. The bridged ethyl hybrid (BEH) particles, surface modified with diol groups, provide a significant reduction in silanol activity, thus requiring lesser amounts of salt additives to minimize the ionic interactions with proteins.[50] In addition, the high mechanical strength of BEH particles enables a reduction in particle size to 1.7 μm, providing gains in chromatographic efficiency.

While most SEC columns are packed with porous particles, a couple of other types of sorbent configurations should be noted. Most packed beds of porous particles have an interstitial porosity of about 35–41%. In SEC, this interstitial porosity adds time to the analysis without benefitting the separation. Czok and Guiochon[51] utilized bundles of aligned porous fibers for size-exclusion chromatography. They were able to reduce the interstitial porosity to 15–18%, resulting in a significant increase in the intraparticle pore volume. However, the increased pore volume did not translate into improved resolution, as they could not prepare columns with good chromatographic efficiency, presumably because the aligned fibers restricted radial

dispersion in the column. Li et al.[52] prepared poly(ethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate) monoliths for biopolymer separations. These monoliths showed low protein binding in aqueous buffers, and chromatographic efficiency comparable to a packed bed of ca. 8 μm particles. The monolithic columns showed separation of peptides and proteins across a broad MW range of up to 670,000 Da, with most of the resolving power available for MW less than 66,000 Da.

THEORY

Thermodynamics

The free energy change of a chromatographic process can be described by,[53,54]

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = RT \ln k \tag{1}$$

where ΔG^0, ΔH^0, and ΔS^0 are the standard free energy, enthalpy, and entropy differences, respectivly; R is the gas constant: T is absolute temperature, and k is the partition coefficient. For most chromatographic modes of separation, the enthalpy of adsorption is the dominant contributor to the overall change in free energy. SEC is unique in that partitioning is driven entirely by entropic processes as there ideally is no adsorption, $\Delta H = 0$. Thus the previous equation becomes:

$$\ln K_D = -\Delta S^0 / R \tag{2}$$

where K_D is the thermodynamic retention factor in SEC. Thus, in SEC separations, temperature should have no impact on retention. In practice, temperature can indirectly impact retention to a small degree by altering the conformation of the proteins, as well as by affecting mobile phase viscosity and analyte diffusivity. Figure 2[50] shows an overlay of protein separations run at three different temperatures, demonstrating the minimal effects that temperature can have on retention.

The thermodynamic SEC retention factor is the fraction of intraparticle pore volume that is accessible to the analyte:

$$K_D = \frac{V_R - V_0}{V_i} \tag{3}$$

where V_R, V_0, and V_i are the respective retention volumes of the analyte of interest, the interstitial volume, and the intra-particle volume. K_D will range from a value of 0 where the analyte is fully excluded from the pores of the

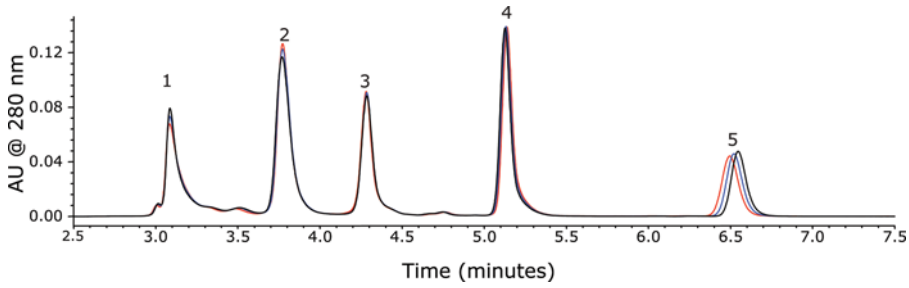

Figure 2 Separation of (1) thyroglobulin, (2) IgG, (3) BSA, (4) Myoglobin, and (5) Uracil on a Waters ACQUITY UPLC BEH200 SEC, 1.7 µ, 4.6 × 150 mm. Mobile phase: 100 mM sodium phosphate, pH 6.8. Flow rate: 0.3 mL/min. Temperature: 30°C (black), 40°C (blue), 50°C (red). Reproduced with permission from Waters Corporation, Milford, MA. (Color figure available online.)

stationary phase, to a value of 1 where the analyte fully accesses the intraparticle pores. By rearranging Eq. (3), one obtains:

$$V_R = K_D \cdot V_i + V_0 \tag{4}$$

Ideally, the separation of proteins and other compounds by SEC is based on the size (or more specifically the Stokes radii) of the analytes in solution. The size based separation, in principle, allows a calibration curve, derived from a set of known analytes, to be used to estimate the molecular weight of an unknown analyte.[54–58] Typical calibration curves are based on proteins or polymers of known molecular weight. By plotting log M vs. the retention volume, one typically obtains a third order polynomial, with a linear region which provides the highest resolution and molecular weight accuracy. For example, Figure 3 shows a typical calibration curve for various protein analytes. The linear range of the curve shows the molecular weight range that the column is suited for. In this example, the linear range is approximately 10 kDa∼500 kDa. By normalizing the x-axis to the volume of the column when empty, one can readily determine the interstitial volume fraction, intraparticle volume fraction, and stationary phase volume fraction. In this instance, the respective values are about 38%, 46%, and 16%, respectively.

As has been widely demonstrated and discussed by Yau and Kirkland among others,[57,59,60] the molecular weight range and slope of the calibration curve are highly dependent on the pore size of the packing matrix. Specifically, the pore size and/or geometry restricts access of molecules based on their Stokes radius. The largest proteins, which are excluded from the pores, elute first. Subsequent proteins elute in order of decreasing size.

As proteins vary in shape (e.g., globular, rod-like or flexible chains), their Stokes radii do not correlate exactly with molecular weight. The difficulty in obtaining accurate molecular weight information for proteins

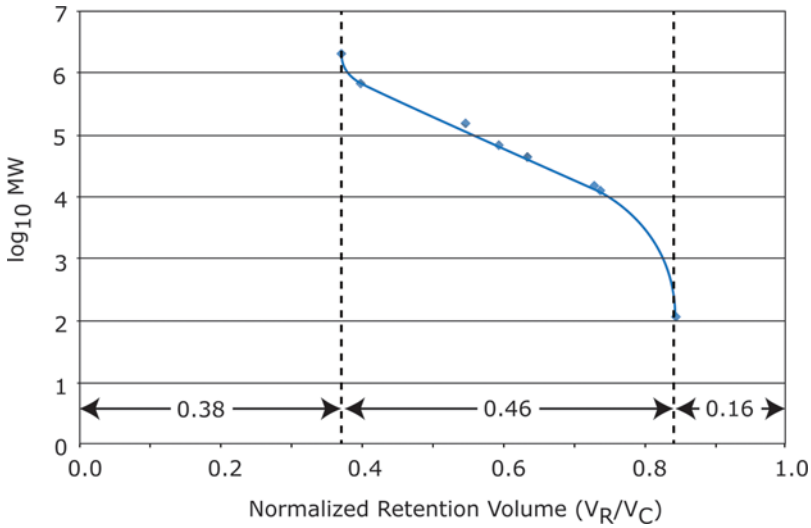

Figure 3 Typical SEC calibration curve. (Color figure available online.)

based on calibration curves has been well-studied.[58,61,62] Another source of error in the calibration curve is that nonideal adsorption may alter the retention volume.[63–65] However, some studies have successfully demonstrated the effectiveness of using a universal calibrant for proteins, primarily for protein collection. Guo et al.[55] described the use of pullan standards as a universal calibrant for molecular weight determination of heparin. However, mobile phase conditions affected the elution volume of the heparin, thereby affecting molecular weight accuracy. These studies required screening of the mobile phase to ensure minimal nonideal interactions which if not controlled could affect molecular weight determination.

The slope of the line in the linear portion of the calibration curve is a measure of the selectivity of the stationary phase, which can be defined by the relationship:

$$\log M = m \cdot K_D + b \tag{5}$$

where m and b are the respective slope and intercept of the line. As the pore size distribution of the particle narrows, the slope becomes shallower, which results in a greater selectivity to discriminate between analytes that are similar in size. Since K_D is bound between 0 and 1, this greater selectivity comes with a tradeoff in that it has less ability to separate analytes over a broad dynamic range. The point at which $K_D = 0.5$ is a measure of the particle's mean pore size. Ghrist et al.[66] define the term k" as the mass of solute

inside the particle divided by that outside the particle (which is similar, but not identical, to the retention factor k in adsorption chromatography):

$$k'' = K_D \frac{V_i}{V_0} \tag{6}$$

Substituting this into Eq. (5), one can see that the selectivity can be enhanced by decreasing V_0 or increasing V_i. These parameters can be altered by packing a column more densely, or by using particles with greater pore volume. However, the physical constraints to both of these approaches limit the extent to which selectivity can be altered in practice. The interstitial volume fraction of a randomly packed bed of particles cannot be easily reduced much beyond 35%, notwithstanding novel approaches such as using aligned fibers as noted earlier. Increasing particle pore volume comes at the expense of the skeletal volume, and so the maximum pore volume achievable depends on the mechanical stresses that the particle needs to be able to withstand.

Kinetics

If one uses the Van Deemter Equation to describe plate height H as a function of linear velocity u, diffusion coefficient D_m, and particle size d_p, one obtains the following relationship:

$$H = ad_p + bD_m/u + cud_p^2/D_m \approx ad_p + cud_p^2/D_m \tag{7}$$

Note that in the case of proteins, the "b" term is typically negligible compared to the other two terms due to the low diffusivity of the macromolecules.

Diffusion coefficients can be estimated for globular proteins using the following relationship:[67]

$$D_m = 8.34 \cdot 10^{-10} \cdot \frac{T}{\eta M^{1/3}} \tag{8}$$

where T is the absolute temperature in Kelvin, η is the mobile phase viscosity in Poise, and M is the molecular weight of the protein. Note that in the case of SEC, the molecular weight and resulting diffusion coefficients can vary considerably between analytes. In addition, with increasing size, analytes become excluded from the pores of the particles, and the intraparticle diffusion decreases, resulting in low c-terms for the highest MW analytes.[68]

As seen in Eq. (7), the plate height is a function of the particle size, with the last term dependent on d_p^2/D_m. Thus it would be expected that if particle size could be reduced, it would provide significant impact on

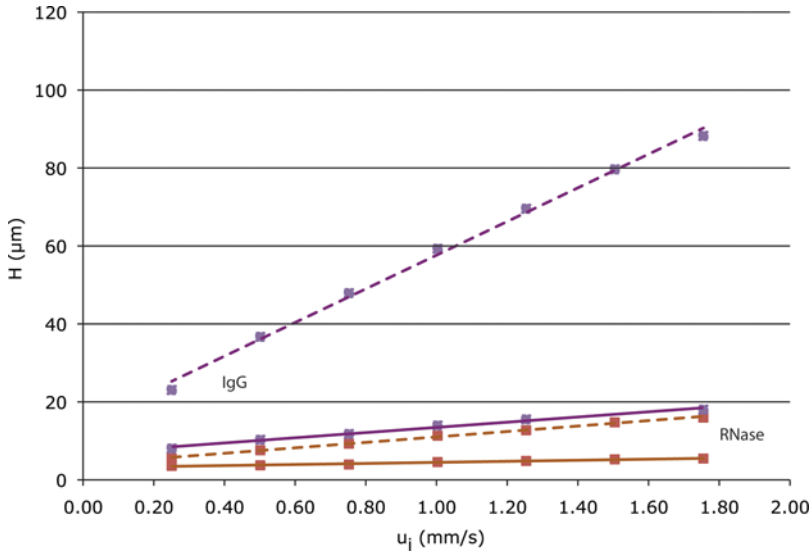

Figure 4 Effect of linear velocity on plate height for (a) ribonuclease A (red) and (b) a monoclonal antibody (blue) on two columns varying in particle size. The 4.6 × 150 mm columns were packed with either 1.7 micron (solid line) or 2.6 micron particles (dashed line). Pore size of stationary phase sorbent: 200 Å. Mobile phase consisted of 100 mM sodium phosphate, pH 6.8. Reproduced with permission from Waters Corporation, Milford, MA. (Color figure available online.)

chromatographic efficiency, particularly at high linear velocities. Figure 4 shows a plot of plate height at different linear velocities for two proteins, Ribonuclease A, and a monoclonal antibody (Ab), on columns packed with two different size particles, 1.7 µm and 2.6 µm. The results are generally in agreement with the theory. One sees that plate height increases linearly with flow rate. Also, the smaller particles provide improved efficiency, and the improvement is especially apparent at the high linear velocities.

Resolution

In chromatography, resolution between two analytes is typically defined as:

$$R_s = \frac{V_{R2} - V_{R1}}{4\sigma} = \frac{\Delta V_R}{4\sigma} \tag{9}$$

where the subscripts 1 and 2 denote the respective analyte, and σ is the mean standard deviation of the respective peak widths. The slope m in the linear region of the calibration curve as defined in Eqs. (3) and (5) is:

$$m = V_p \frac{\log M_1/M_2}{\Delta V_R} \tag{10}$$

Using Eq. (7) and the relationship:

$$N = \frac{V_R^2}{\sigma^2} = \frac{L}{H} \tag{11}$$

the Resolution equation can be rewritten as:

$$R_s = \frac{1}{4} \cdot \frac{V_p}{V_R} \cdot \frac{\Delta \log M}{m} \cdot \sqrt{\frac{L}{ad_p + cud_p^2/D_m}} \tag{12}$$

This equation shows the dependance resolution has on the pore volume, column length, and linear velocity.

INSTRUMENTATION CONSIDERATIONS

From the first analyses by Lathe and Ruthevin.[30,31] SEC separations of proteins have been performed under native conditions which preserve the biological activity of the macromolecule. Biocompatible chromatographic systems are most often used to minimize any metal-protein adducts or undesired protein interactions. Native conditions most often require physiological pH, high salt content, and 100% aqueous mobile phases, all of which can be problematic. The presence of high salt concentrations increases the potential of particulates in the mobile phases, thereby affecting system and column performance. Highly aqueous mobile phase can cause bacterial contamination within hours, particularly in the absence of bacteriocides or bacteriostats (e.g., sodium azide).[69]

In adsorption chromatography, separation typically occurs in a volume that is significantly greater than the volume of the chromatographic column. But in SEC, separation takes place in less than one column volume. As a peak migrates through a chromatographic column, its peak width increases, and the amount at which it increases depends on the retention factor or retention volume. Thus, in the case of SEC, where an analyte has a retention factor of zero, the amount that the peak broadens can be significantly less than other modes of chromatography. As a result, the impact of band broadening in SEC is of particular importance and has been the subject of a wide number of articles.[54,66,70–72]

Since the early introduction of SEC, instrumentation such as high performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC)[73,74] systems have sufficient pressure thresholds to accomodate silica-based SEC columns. However, these systems can have significant system dispersion because of the design and configuration. For example, Ghrist et al.[66] found in "non well-behaved" systems

SE-HPLC instrumentation can lead to increased band broadening (up to a 50% increase) as compared to expected values. Even in well-behaved systems, SE-HPLC can lead to significant band broadening. To minimize the impact that the instrument has on dispersion, large diameter SEC columns with inner diameters of greater than 7.5 mm are typically used.

As a result, HP-SEC instrumentation is typically optimized for these separations.[54,75–77] This is accomplished by the utilizing tubing with low inner diameters (0.005" or less) and minimizing the length of tubing. It is important that the chromatographic system contain no additional tubing and that any valves used in the chromatographic system have low volume connections. A number of studies, including Grznárová et al. have examined the effects of varying connector tubing lengths and diameters, injector tubing lengths and varying flow rates for macromolecules.[78] These studies reinforce previous work by Kirkland et al. that demonstrated the negative impact of extra system volume from injectors, guard columns, detectors and connectors on chromatographic resolution and accuracy.[79]

For the combination of SEC and light scattering detectors, which require the use of both a multi-angle light scattering detector as well as a concentration detector, extra column band broadening is of particular concern. A number of studies have looked at the effect of multiple detectors on the band broadening.[20,80] These studies have outlined varying influence of band broadening effects with multiple detectors. Some studies have found band broadening effects with dual detectors can have a significant impact on samples with higher polydispersities, affecting molar mass calculations. However, for non polydisperse samples, the volume shift for multiple detectors in SEC is minimal: molar mass averages are comparable and within 1% of actual values for most proteins, whether or not volume shift correction is applied.[81]

In 2004, the commercialization of reversed-phase LC columns with sub 2 micron particles provided the chromatographer with significantly improved resolving power, provided that the column was used on a low dispersion LC instrument.[82] It has only been recently that SEC columns with sub 2 μm particles have been developed which take advantage of the improved low dispersion instrumentation.[82] These instruments can provide lower system dispersion and improved resolution for SEC protein separations as compared to SEC-HPLC.

DETECTORS

For SEC analyses, UV continues to be the predominant mode of detection.[76,83] Near UV or longer wavelengths give greater response for aromatic amino acids, such as tryptophan, and are commonly used for

protein measurement.[84] Higher sensitivity provided by detection using far UV or low wavelengths (214 or 220 nm), where the amide peptide bond has a strong absorbance. Both wavelength ranges can be quantitatively inaccurate due to either scattering from particles at lower wavelengths or the presence of other chromophores absorbing at 280 nm. However, each wavelength range has its advantages: at lower wavelength, improved sensitivity allows for analysis of sample limited or low concentration proteins, while higher wavelengths provide a greater linear dynamic range.

The advantages of the two wavelength ranges can be combined by using dual wavelength detection, which has been proposed for purity profiling in SEC.[20,85] In this approach, the lower wavelength provides the sensitivity for the low abundant species, while the higher wavelength provides a higher linear range for the major species (i.e., the monomer). The wavelength ratio is experimentally determined for the major species. This factor is then used to calculate the percentage of aggregates and other impurities detected at the low wavelength which provides greater sensitivity. This type of approach, which has been demonstrated for monoclonal antibodies by Bond, can allow for lower levels of aggregates to be measured against a monomer et al.[20]

Some assays have also used fluorescence detectors for improved sensitivity and or selectivity.[17,86–88] Diress et al.[86] demonstrated the utility of fluorescence detection for improved sensitivity in cases where excipients may elute near or with the protein of interest. Gunturi et al.[87] also showed the sensitivity of fluorescence detection for recombinant human growth hormone, also in the presence of excipients. These studies confirm the applicability of fluorescence detectors to measure low level of aggregates.

Requirements for molecular weight confirmation have led to coupling of SEC with detectors that provide information on molecular weight. These include multi-light scattering (ALS) detectors which can determine the size and shape of proteins. The coupling of SEC and light scattering detectors has enabled better determination and/or confirmation of molecular weights.[12,19,89–91] SEC-MALS can provide information on the size, shape and concentration of the sample. Due to the dependence of MALS on concentration and the extinction coefficient of the protein, SEC-MALS must also be coupled to a separate detector for concentration determination. The most common detectors used for concentration determination in conjunction with MALS are refractive index (RI) and ultra-violet (UV) detection. Oliva et al.[12] compared the precision and accuracy of both SEC-MALS/UV-Vis and SEC-MALS/RI. The results of these methods were found to have a high degree of correlation, with the expected precision and accuracy for most proteins. SEC-MALS/UV-Vis was found to have slightly greater coefficients of variation (CV); however the values were within expected experimental error. Folta-Stogniew and Williams[19]

evaluated the precision and accuracy of SEC-MALS for a range of proteins from 12–480 kDa. While the median standard deviation was 2.3%, larger variation could be attributed to sample characteristics, such as dimer destabilization.[19] Since the response in MALS is proportional to the molecular weight of the protein, accuracy for lower molecular weight species can be influenced by sample load.

SEC-MALS has been shown to provide information not only proteins and their dimers but also high order aggregates. Ahrer evaluated aggregates for human IgG. This study showed that for those samples containing trace amounts of higher order aggregates, SEC-UV may not provide enough sensitivity. The same sample in MALS detector is significantly more sensitive to the high MW aggregates and enables their confirmation and analysis.[90]

Mass spectrometry is another method for obtaining molecular weight information. However, there are challenges to interfacing SEC with MS. As discussed extensively by García,[92] the greatest challenge is the incompatibility of mobile phases containing high concentrations of nonvolatile salts.[92] SEC mobile phases are typically nondenaturing aqueous solutions in the physiological pH range (6.5–8). These mobile phases lead to ion suppression and contamination of the mass spectrometer. The most suitable SEC-MS mobile phases provide non-denaturing conditions (ammonium formate and ammonium acetate) but not physiological conditions.[93]

In order to overcome this difficulty, SEC-MS methods have been developed using denaturing mobile phases containing organic solvents and ion-pairing reagents.[94–97] Lazar et al.[95] applied a similar principal for the analysis of immunoconjugates. In this case, covalently linked immunoconjugates were distinguished from the intact monoclonal antibody under SEC-MS conditions, providing utility that could not be achieved by either offline sample preparation or reversed phase desalting. Liu et al.[94] developed a similar approach for the analysis of reduced and alkylated monoclonal antibodies with acetonitrile and TFA in the mobile phase, thus allowing for rapid desalting (Figures 5b and 5c).[94,97]

METHOD OF OPTIMIZATION

Proteins are prone to interact with surface charged sites of chromatographic stationary phases.[9,63–65,98–100] These ionic interactions can result in adsorption of the protein,[74] shifts in retention time,[5] peak tailing or peak asymmetry,[6] or to changes in the three dimensional conformation of the protein.[63,101] As previously mentioned, chromatographic stationary phases and mobile phases have been used to mitigate nonideal interactions.

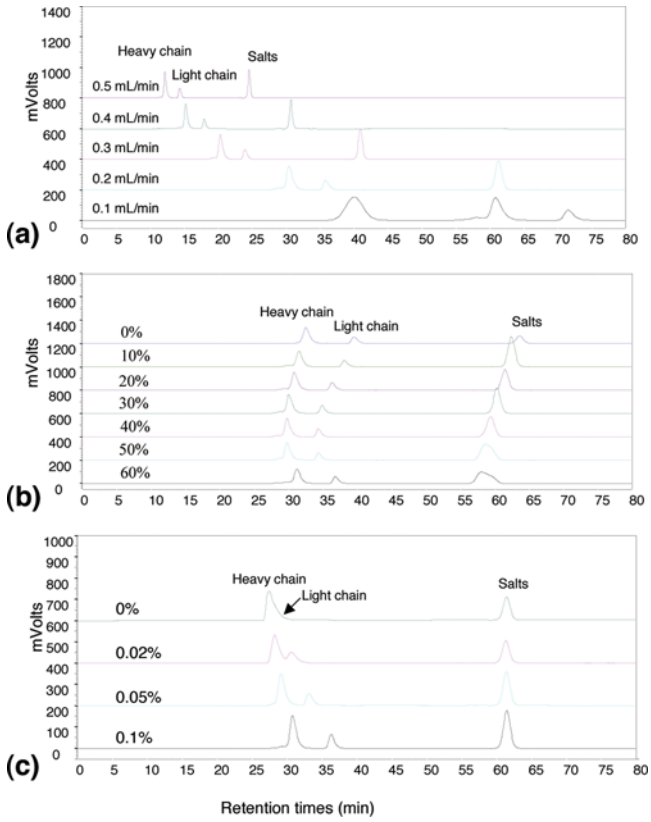

Figure 5 SEC chromatograms of antibody-A analyzed using TSKgel G3000SWxl column; (a) at various flow-rates of 0.5 mL/min, 0.4 mL/min, 0.3 mL/min, 0.2 mL/min, and 0.1 mL/min as labeled in the figure; (b) using mobile phase consisting of 0%, 10%, 20%, 30%, 40%, 50%, or 60% acetonitrile as labeled in the figure with 0.1% TFA, and 0.1% formic acid in Milli-Q water; (c) using mobile phase consisting of 20% acetonitrile with 0.1% formic acid and with 0%, 0.02%, 0.05%, or 0.1% TFA in Milli-Q water as labeled in the figure. Reprinted from Reference[93] with permission from Elsevier. (Color figure available online.)

Other factors can be used to manipulate SEC separations. Chromatographic conditions that can be evaluated include flow rate, column length, mass load, and volume load. Adjustment of these factors can impact resolution, analysis time, and/or sensitivity.[102]

Nonbinding interactions between the solute and the packing material are dominated by two types of chemical interactions: electrostatic interactions and hydrophobic interactions.[64] If the protein and the stationary phase surface are identically charged, "ion-exclusion" can result. In this case, the protein is prevented from entering into the pores of the particle, and thus elute faster than would be predicted. If the protein and the particle are oppositely charged, then adsorption of protein to the stationary phase surface may result from ion-exchange interactions, and results in

greater than expected retention. Hydrophobic effects can be produced from interaction of the solute with hydrophobic sites on the packing material and lead to increased retention.

Salt Concentration

A common approach to reducing electrostatic interactions in SEC involves increasing the ionic strength or salt concentration of the mobile phase.[63–65] This can reduce secondary interactions and improve peak symmetry, retention time, and quantitation. This approach has been recently demonstrated by Ricker and Sendoval[100] in which a number of monoclonal antibodies were analyzed at varying ionic strengths. While the results varied among the antibodies, some antibodies showed retention time shift and poor peak shape at low sodium chloride concentrations. Kamberi et al.[103] also examined the effects of electrostatic interactions on the recovery of aggregates of synthetic human parathyroid hormone. In this study, aggregate recovery was evaluated at different sodium chloride and acetonitrile concentrations. The addition of 100 mM sodium chloride was found to minimize electrostatic interactions and increase aggregate recovery.

Increasing the concentration of a counter ion in the mobile phase is a common approach to reducing electrostatic interactions. However, very high concentrations of these same ions can lead to an increase in hydrophobic or ion exclusion effects.[61] This interaction has been well-documented, particularly for peptides, and strongly hydrophobic proteins.[99,104]

Mobile Phase Modifiers

Numerous studies have evaluated the addition of of organic modifiers or other additives, such as arginine[11,105] to mitigate these secondary interactions. These additives are often used to aid in protein recovery. The reduced recovery of aggregates in SEC chromatography is an area of wide concern.[14,105] One strategy often used is the addition of arginine to the mobile phase to reduce secondary interactions.[11,101] Arginine acts as a binder to the analyte in solution, thus preventing it from interacting with the stationary phase. Arakawa analyzed the effect of arginine on protein aggregate quantitation and found an increase in aggregate recovery when arginine was added to the mobile phase.[101] Other studies have also found improvement in peak shape with the use of arginine as a mobile phase additive.[11] Methods using arginine in the mobile phase have been developed for both large biomolecules and small proteins, such as insulin.[4–6]

Mobile Phase pH

Mobile phase pH can also be manipulated to reduce secondary interactions.[63,65] Varying pH of the mobile phase can perturb the three dimensional conformation of the protein, resulting in changes in non-ideal interactions with the stationary phase. These interactions can be predicted based on the relationship between mobile phase pH and the isoelectric point of the protein. Golovchenko et al.[65] demonstrated that at low ionic strengths, ion exchange effects were observed at pH values below the pI of the protein, while ion-exclusion effects were observed at pH values above the isoelectric point of the protein.[65]

Flow Rate

Flow rate is one of the parameters available optimizing resolution.[2,100,106] As in many chromatographic separations of macromolecules, the optimum column efficiency is achieved at low linear velocities. The impact of flow on resolution can be seen from the discussions in the Theory section, and in particular Eq. (12). Engelhardt and Schön[106] demonstrated conditions for optimizing size-exclusion chromatography, including reduced flow rate. A study by Qian et al.[85] analyzed flow rates over a 10× (0.112–1.2 mL/min) range for the analysis of human serum albumin and interferon. Ricker and Sandoval[100] demonstrated the effect of flow rate on the SEC separation of a protein mixture: resolution improvement was observed for bovine serum albumin and ovalbumin with decreasing flow rate. Liu et al.[94] evaluated the effect of flow rate for the SEC separation of a reduced antibody under denaturing conditions (Figure 5a). For all of the examples, the improvements in resolution are accompanied by longer analysis times, broader peaks and lower sensitivity.

Sample Load

As in other chromatographic techniques, sample load, both volume and mass, affects SEC chromatographic resolution and sensitivity.[82,106,107] Ideally, proteins are separated based on size, limiting the resolution between analytes. In instances of column overloading, resolution between the analytes can deteriorate. Ideal volume loads correspond to sample volumes between 5–10% of the total column volume. If the sample volume increases beyond this range, resolution decreases. Oftentimes these higher volumes cause peak distortion (i.e., tailing). Ricker and Sandoval[100] demonstrated this phenomenon for a set of protein standards: injection volumes were tested over a 100-fold range (2–200 μL). Large injection volumes (> 100 μL) led to increased peak widths, resulting in decreased

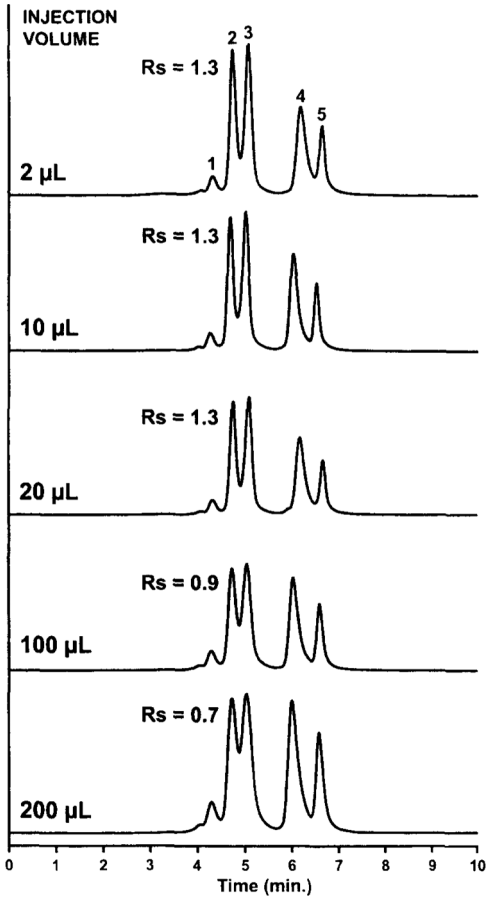

Figure 6 Effect of injection volume on separation efficiency in SEC. A 4-component protein mixture was separated on a Zorbax GF- 250 column (250 × 9.4 mm) using a mobile phase of 200 mM sodium phosphate, pH 7.0. The injection volume was varied from 2 to 200 µL] and ambient temperature was used. Detection, represented on the y-axis, was carried out at 230 nm. The flow rate was 2 ml/min. Resolution (Rs) between BSA and ovalbumin are shown. Peak Identities: 1 = BSA-dimer; 2 = BSA; 3 = ovalbumin; 4 = lysozyme and 5 = sodium azide. Reprinted from Reference[100] with permission from Elsevier.

resolution between the bovine serum albumin monomer and ovalbumin (Figure 6).[100]

Loss of recovery of monoclonal antibodies and their aggregates is a concern in SEC chromatography. Thus, for method development, the correlation between mass load and recovery is often analyzed. Gabrielson et al.[14] evaluated this phenomenon for unstressed and acidified monoclonal antibody formulation. In this study, loss of protein mass was observed for the acidified monomer at higher mass injection loads, while the unstressed sample showed no significant loss of protein mass. This loss

can be attributed to non-ideal interactions and illustrates the utility of analyzing varying mass loads in method development.

Column Dimensions

A common approach to method development in any chromatographic method also includes the effect of varying column length and inner diameter. Increasing column length provides a means of improving resolution in isocratic separations such as SEC.[100,108] From Eq. (12), one can see that resolution is proportional to $L^{1/2}$. While most SEC columns are 30 cm in length to provide optimum resolution, additional column length can be attained by linking multiple columns in series. Ricker and Sandoval[100] demonstrated this effect by linking two 4.6×250 mm columns for the analysis of BSA and ovalbumin. In this example improved resolution and higher column efficiencies were achieved, however, these were accompanied by increased time of analysis. Coupling of columns and/or increasing column length results in an increase in run time proportional to the additional column length.

As discussed earlier, increasing the inner diameter of SEC columns can significantly improve peak capacity and resolution by minimizing the system contribution to band broadening.[66,75,106] In cases where system dispersion is significant, 7.5 mm I.D. SEC columns may be required in order to maximize peak capacity.[109] However, with the introduction of newer low dispersion, instrumentation such as UHPLC, smaller ID columns (4.6 mm) can be used to achieve comparable resolution to SE-HPLC.

Particle Size

As discussed earlier, and shown in Eq. (12), efficiency in SEC is affected by the particle size of the chromatographic stationary phase. Evaluation of these smaller particles has shown the advantages in resolution as compared to larger particles.[106,110–112] Liu et al.[94] demonstrated these effects for the analysis of a reduced antibody: improved resolution and higher efficiencies were observed for the light and heavy chain using columns with smaller particle sizes (Table 1).[94]

Theoretical analysis of the optimum particle size has shown the benefits of 1–2 µm particle on SEC separations.[67] With the advent of sub-um SEC column packing materials, these resolution improvements can be realized. Diedrich evaluated columns of varying particle size for the analysis of monoclonal antibodies.[109] Improved resolution and higher efficiencies were observed for sub-2 µm SEC columns at higher flow rates resulting in shorter run times (Figure 7).

TABLE 1 Comparison of Columns: Effect of Particle Size on Efficiency and Resolution for a Reduced Antibody

[-17pt] Columns	Dimensions (m i.d. × mm length)	Particle size (μm)	Pore sizes (Å)	Theoretical Plates HC	Theoretical Plates LC	Resolution
TSKgel G3000SW	7.5 × 300	10	250	1980	3845	3
TSKgel G3000SWxl	7.8 × 300	5	250	5060	10674	4
Shodex KW-804	8.0 × 300	7	250	4952	8859	2
Protein-Pak 300SW	7.5 × 300	10	250	2078	4271	3
BioSuite 250	7.8 × 300	5	250	5149	9403	3

Conditions: 20% acetonitrile, 0.1% TFA, 0.1% formic acid at 0.2 mL/min. Reprinted from Reference [94] with permission from Elsevier.

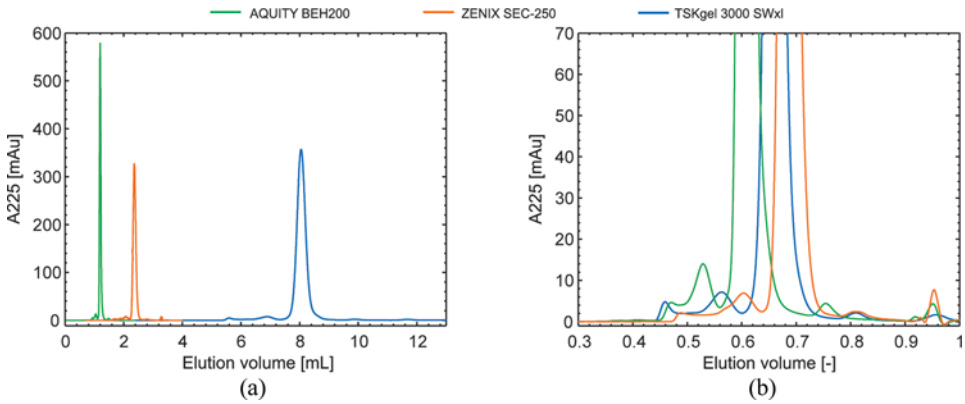

Figure 7 Effect of particle size (d_p). (A) Overlay of single injection chromatograms of a mAb sample (1.0 g/L) analyzed on AQCUITY BEH200(1.7 µm). Zenix SEC-250 (3 µm) and TSKgel 3000 SWxl (5 µm). (B) For comparability, elution volumes were normalized to column void volumes. Reprinted from Reference[109] with permission from Elsevier. (Color figure available online.)

APPLICATIONS

There have been numerous applications of SEC reported over the last few decades for a wide range of analytes. However, the scope of this section of the review will be the use of SEC in the field of biotherapeutic protein development and some of the considerations that may be important to addressed when using this separation mode for that purpose. The primary application for SEC in the biopharmaceutical industry is the routine monitoring of protein or modified protein (protein-drug conjugates, pegylated proteins, etc.) aggregation and quaternary structure.[113] Given the

sensitivity and reproducibility of the method, SEC may be considered as the standard method for monitoring protein aggregation,[114] and is included in the list of the typically used tests in the European Pharmacopoeia guidance document entitled "Technical Guide for the Elaboration of Monographs on Synthetic Peptides and Recombinant DNA Proteins," 1st Edition 2006.[115] In these regards, SEC can be used as an integral part of an analytical testing strategy designed to provide assurance of biopharmaceutical product safety and although this information is not included or has been redacted from the FDA Summary Basis for Approval for most products, the method is likely positioned as a registered test in the majority of the Chemistry, Manufacturing & Controls (CMC) sections of current regulatory filings.

SEC is often used as a tool to aid in manufacturing process and formulation development. As part of the manufacturing process development SEC can be used to guide cell-line selection. These data can be invaluable in not only selecting a cell-line that produces the lowest levels of aggregates, it can also discriminate between aggregate forms that may be more or less difficult to remove during downstream purification steps.[116] Another use for SEC during cell-line development is to ensure that the specific activity of the purified protein is not under-reported or in rare cases over-reported as the result of increased aggregation.[117,118]

SEC can also be used extensively to guide the development of the purification process for biopharmaceuticals. There have been vast numbers of biopharmaceutical new chemical entities (NCE) that are antibodies or antibody-like in recent years.[119] The primary purification workhorse for these molecules is Protein A or G affinity purification. Although this purification step can provide high log removal of conditioned media impurities as the first step in the purification process, a significant removal of aggregate impurities is not typically observed and often the elution conditions of the affinity purification step can potentially induce further aggregate formation.[120] As a result, further polishing steps including gel-filtration, ion-exchange, hydrophobic interaction, or hydroxyapatite chromatography are often required to control the levels of aggregate of the purified protein.[121–123] The use of SEC derived data to assist in the optimization of these methods is ideal due to the short run times and amenability of the method to most buffer systems. One disadvantage, however, is that since on-column analyte concentration is not possible the sample concentrations must be adequate to provide meaningfully results. To address this limitation alternative UV absorbance wavelengths or fluorescence can be used.[19,87]

Changes in the extent and forms of protein aggregation are primary concerns during product formulation development. The short run times and quantitative reproducibility make SEC an appropriate method for

stability monitoring. Stability protocols with multiple formulations, manufacturing batches, storage conditions, and time-points can generate large numbers of samples and the 15–20 min run-times achieved by HPLC columns and systems can provide good sample throughput. Recently, the availability of sub-2 µm particle size SEC columns and low dispersion UPLC systems have reduced these run-time by approximately two times or more, and through the use of sample interlacing routine analyses of under 2 min per sample have been reported.[109] A complication to the SEC analysis of a protein drug product can also be the coeleution of excipients such as the non-ionic surfactant polysorbate 80.[124] To address this issue, alternative UV absorbance wavelengths or the intrinsic fluorescence of the protein may be used to advantage.[86,87]

The limitations of SEC have been well documented.[15,16,125,126] During the development and application of an SEC method for the analysis of a biotherapeutic protein orthogonal techniques such as sedimentation velocity analytical ultracentrifugation (SV-AUC), asymmetrical flow field flow fractionation (AF4), and dynamic light scattering (DLS) may be needed to confirm that the SEC method is providing an accurate representation of the forms and level of aggregates in the protein sample.

In addition to providing assurance that an SEC method is providing an accurate assessment of the aggregate levels and forms present, the biochemical and biophysical characterization of the aggregate forms is also a valuable part of a thorough protein characterization study. During the initial phases of protein development, the characterization of the aggregate fraction may, for example, include host-cell protein analysis, bioactivity, posttranslational modifications, and mass analysis (MALLS or MS). Additionally, as the product moves through to commercialization this characterization may need to be repeated or enhanced depending on the extent of any changes in the manufacturing process as part of a comparability assessment to provide assurance that the nature of the aggregate forms present in the products produced by the two manufacturing processes are comparable.

Currently, with patent protections running their course, there has been a surge of interest in the area of biosimilars.[127–129] While no clear guidance with respect to SEC fraction characterization has been documented for biosimilars, a complete comparability package would likely contain a comparison of an appropriate combination of orthogonal analyses and aggregate peak characterization data for both the innovator and the biosimilar products.

CONCLUDING REMARKS

Since the introduction of the first recombinant insulin in 1982, nearly three decades after the first reported uses of SEC for protein analysis, SEC

has become the most widely applied method for the routine analysis of aggregation for biotherapeutic peptides and proteins. Additionally, SEC has been used extensively to guide manufacturing process and formulation development for these classes of biotherapeutics. The broad adoption of this method for these analyses can be attributed to its simplicity, reproducibility, sensitivity, and speed. More recently, dramatic improvements in resolution, sensitivity, and throughput provided by the use of smaller particle columns ($\leq$2 μm) on low dispersion UHPLC instrumentation have further enhanced the capabilities of SEC.[109]

A thorough understanding of the principles and practices of SEC is vital to developing robust, accurate, and precise methods. While SEC separations, in theory, are based solely on the size of the protein or peptide in solution, non-ideal interactions between these large molecules and the column packing materials are often encountered in routine practice. These interactions can deleteriously affect the retention time, peak shape, and recovery of the protein and should be minimized through method optimization. In addition, SEC method development should also include an appropriate evaluation of the chromatographic recovery of both the drug product and any aggregate forms present in the sample.

The analysis of protein and peptide aggregation as a critical quality attribute, will continue to be of importance into the future as a result of the steady introduction of novel protein and peptide based biotherapeutics into the clinic, at a rate of approximately 40 per year since 2007.[119] SEC, a nearly 50-year-old technique, has continued to evolve in order to meet the ever greater demands in terms of accuracy and sample throughput, is currently the primary means of routinely measuring protein aggregate levels. However, the use of SEC in this capacity should always be demonstrated to be appropriate for a specific biopharmaceutical sample.

LIST OF SYMBOLS

a, b, c	Constants in van Deemter Equation
D_m	Diffusion coefficient
d_p	Particle size
H	Plate height
k	Partition coefficient, retention factor
k''	Alternative retention factor (Equation 3.6)
K_D	Partition coefficient for SEC
m, b	Slope and intercept of SEC calibration curve in linear region
L	Column length
M	Molecular weight
N	Plate number
R	Gas constant

R_s	Resolution
T	Absolute Temperature
u	Linear velocity
V_i	Intraparticle volume
V_0	Interstitial volume
V_R	Retention volume
ΔG^0	Standard Gibbs free energy change
ΔH^0	Standard enthalpy change
ΔS^0	Standard entropy change
η	Viscosity
σ	Standard deviation of peak width

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Advances in size-exclusion separations of proteins and polymers by UHPLC

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ABSTRACT

The separation of molecular compounds based on their capacity to access the intra-particle pore volume of chromatographic media, which is dictated by the relative size in solution of those compounds, has been commonly known as size-exclusion chromatography (SEC) or gel-permeation chromatography (GPC). Conventionally, these two terms have been applied to the analysis of biomolecules and polymers, respectively. Over the more than half-a-century history of size-based separations, there has been a series of advancements, starting from the earliest soft-gel particles and culminating within the past few years in the use of sub-2-µm particles in ultra-high-performance liquid chromatography (UHPLC). The intent of this review is to provide a concise synopsis of the advancements of both chromatography columns and instrumentation for protein and polymer size-based separations. Also, this review presents brief summaries of the application of UHPLC technology for these classes of analytes.

Contents

1. Introduction

Size-exclusion chromatography (SEC) and gel-permeation chromatography (GPC) are two names for the same technique, the only difference being application area. SEC is predominately used to describe size-based separations of biomolecules, while GPC typically refers to separation of synthetic and natural polymers.

In this article, we discuss some of the more recent trends in the area of SEC separations. Historically, the technique was considered to be a low-resolution, time-consuming separation method. Indeed, the peak capacity for an SEC separation is substantially less than a gradient elution analysis. In SEC, the entire separation occurs within one column volume, while a gradient separation can be tens of column volumes, which lead to over an order of magnitude difference in peak capacity between the different separation modes. The materials traditionally used for SEC were limited in mechanical strength, thus precluding their use at higher flow rates. But, despite its limited peak capacity and lengthy separation time, SEC still plays an important role in separation and characterization of

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proteins and polymers. In this article, we discuss some of the new trends in SEC column and instrument design that are improving resolving power and enabling faster separations.

The predominant use of SEC for the analysis of biotherapeutic formulations has been in the measurement of the levels of reversible self-associated or aggregated (non-reversible) soluble high-molecular-weight (HMW) biomolecule forms that may impact the safety and the efficacy of a product. The level and the valency of soluble protein aggregation are critical quality attributes (CQAs) that require monitoring for monoclonal antibody (mAb) preparations intended for human use. Low-valency (e.g., dimer) HMW levels provide insight into process and product stability, as aggregation, which may occur throughout the manufacturing process from cell culture through final drug product formulation, may indicate partial denaturation or other perturbations of protein structure [1]. Also, the stability of the drug product, with respect to aggregation, must also be thoroughly understood. It is also critical to elucidate the distribution of high-valency, multimeric HMW forms in protein biotherapeutic preparations, since these multimeric forms have been reported to elicit an immune response aggressively by engaging an immunological pathway that is independent of T-cell involvement [2–4].

The use of SEC as the most common method for the quantitation of HMW levels in biotherapeutics is principally due to the sensitivity, the reproducibility, and the relatively high sample throughput of these analyses. However, one of the primary limitations of SEC is the potential of the method to not provide an accurate representation of the HMW forms present in a sample due to filtration or non-specific binding of the HMW forms by the column [5]. As a result, a crucial aspect of developing a reliable SEC method for the analysis of a biotherapeutic is confirmation of the separation observed by one or more orthogonal methods, such as sedimentation velocity analytical ultracentrifugation (SV-AUC), dynamic light scattering (DLS), or asymmetric flow field flow fractionation (afFFF) [6].

For the polymer industry, SEC provides critical information about the chemical composition and molar mass distribution, and how the molecule is constructed. This information provides data that can be correlated with some of the physical properties of a material, such as tensile strength, elasticity, and adhesion. The raw retention-time data generated from a chromatographic profile are transformed into a molecular-weight distribution. This is typically done by creating a calibration curve using standards of a range of known molecular weights (MWs). Narrow-dispersity polystyrene is most commonly used, and the calibration curve can be adjusted for the polymer composition of interest. This may require use of multiple detectors, such as ultraviolet (UV), refractive index (RI) and viscometry.

2. Stationary-phase development for SEC

The first demonstration of SEC was reported more than 60 years ago in 1953 by Wheaton and Bauman [7]. The broad application of this size-based separation for the isolation of biomolecules would begin six years later when Pharmacia brought to market spherical porous cross-linked dextran particles, under the trade name Sephadex [8–10], which is still commercially available. The size of the pore network of these particles depends on the degree of crosslinking, thereby modulating the optimal size range of biomolecules that can be separated. Other current gel-based particles were also produced in this era, including polyacrylamide-based gels [11,12]. These materials were commercialized by Bio-Rad under the trade name Bio-Gel.

The first SEC chromatographic media developed for hydrophobic polymers was by Moore of the Dow Chemical Company [13]. By cross linking with different amounts of divinylbenzene, porous gels could be synthesized with differing mean pore size. By packing the 44–75-µm particles into a 0.305 inch I.D. x 12 foot long tube, separations could be achieved in under 3 h. This was a significant improvement in time savings compared to the 3–4 weeks of extensive sample work-up required at the time [14]. Moore coined the term "gel-permeation chromatography'" to describe the technique of SEC specifically for polymer separations. Waters Associates licensed the technology from Dow, and commercialized the Styragel product line.

One of the key features that made Sephadex and Styragels widely used was their minimal interaction with proteins and organic polymers, respectively. However, both types of media were limited in mechanical performance. Their low operating pressure precluded their utility at high flow rates, or in configurations that utilized small particles. Since it was inherently a low-resolution technique, often two or three columns were connected together, resulting in run times of 30–60 min. Also, the polystyrene resins could shrink substantially and swell in different mobile phases, which meant that solvent switching could not be readily be performed without compromising the mechanical integrity of the packed bed. Manufacturers thus provided columns stored in several different solvents to remove the risk of adversely impacting column performance via solvent switching.

While it was well understood that reduced particle sizes would provide higher efficiency separations, it would not be until 1972 that a 10-µm porous silica particle would be brought to the market by Waters under the trade name of µPorasil [15]. The strength and rigidity of this particle enabled the creation of stable packed beds capable of operating at several hundred bars pressure, and able to withstand the shear stresses of high flow-rate mobile phases.

C18-modified silica became the workhorse tool for modern reversed-phase HPLC (RP-HPLC). Size-exclusion columns were also developed using porous silica, and typically optimized for this application by increasing the pore volume of the media. The surfaces required modification to minimize the strong ionic interactions between proteins and the acidic surface silanols of the silica by derivatizing with hydrophilic silanes [16–19]. Further reduction in interactions could be obtained by addition of mobile-phase additives [20]. Significant success was achieved with the use of a diol functional group. Even though acidic silanols remained, and could lead to ion-exchange adsorption of the charged proteins, the interaction could be substantially mitigated by utilizing high ionic strength mobile phases [19]. To this day, a diol phase remains as the most predominantly used silica-surface modifier for SEC of proteins.

In the case of polymers, a short-chain hydrocarbon silane was typically used for non-polar polymer separations, while unbonded silica proved effective for many hydrophilic polymers. However, while silica-based SEC columns became widely used for the characterization of proteins, cross-linked styrene is still widely used for polymer separations in non-aqueous media. One reason for this is the difficulty in effectively mitigating the ionic and hydrogen-bond interactions between silica and polymer analyte with compatible mobile-phase additives.

More recently, porous hybrid organic/inorganic particles [21] were developed and utilized for SEC. In 2010, Waters Corporation commercialized its first SEC column offerings with diol bonding, specifically for protein characterization. Subsequently, columns were commercialized with a trimethylsilyl (TMS) surface modification, or unbonded, for organic and aqueous separations, respectively [22,23]. One key advantage of these particles over silica is the significantly lower acidity of the hybrid silanols [24]. Fig. 1 shows the differences in silanol acidity for silica and bridged-ethyl hybrid (BEH) particles, both bonded and unbonded [24]. Acidity of the BEH-silica is seen to be substantially less than that of the silica. By surface-modifying the BEH particles with diol or trimethylsilyl (TMS) groups, silanol acidity could be further reduced.

One important consideration in the design of chromatographic media for SEC is the pore volume of the particle. In SEC, the differential size separation occurs almost entirely within the

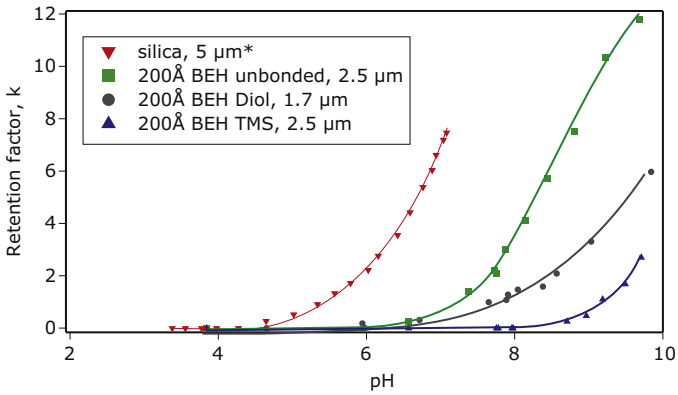

Fig. 1. Titration of silanol by plotting retention factor of nitrate ion as function of pH using method of Mendez et al. [25]. Mobile phase: 60% MeOH, 40% buffer (1 mM: sodium acetate, sodium phosphate, sodium carbonate, or sodium borate) Temp.: 30°C. Sample: 1.5 µL $LiNO_3$. Detection: Conductivity. *Data for silica (Waters Symmetry ®) adapted from Figure 3 in Mendez et al. [25], with permission from Elsevier. (Reproduced with permission from Waters Corporation).

intraparticle pores. Thus maximum separating power is achieved on particles with the greatest pore volume. However, this desire for high pore volume must be balanced against the mechanical strength requirements of the particle, as any increase in pore volume is at the expense of the solid structural component of the particle. Nonetheless, for the BEH particles, an increase of about 75% in pore volume was achieved while still maintaining the required mechanical rigidity for a 1.7-µm particle packed in a chromatographic bed and used at high pressures and shears [26,27]. These ultra-high-performance (or ultrahigh-pressure) liquid chromatography (UHPLC) columns provide significant gains in chromatographic efficiency when coupled with the appropriate UHPLC instrumentation.

Recently, monolith technology was demonstrated for SEC separations. For example, Li et al. [28] performed separations of protein mixtures in 30 min using a 23 cm x 150 µm capillary monolithic column comprised of poly(ethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate). Viktorova et al. [29,30] demonstrated the separation of up to 20×10^6 Da polystyrene on a monolithic divinylbenzene capillary column. One limitation of monolith technology is that the mesopore fraction of the column is typically substantially less than the intraparticle porosity of a bed packed with porous particles. This means that substantially longer column lengths are required for monoliths to achieve pore volumes similar to those of packed beds, resulting in longer separation times.

3. UHPLC instrument design for size-exclusion separations

The chromatographic efficiency of a peak that one observes is a result of both the column and the system. Ideally, one would like the system contribution to band spreading to be negligible compared to the band spread resulting from the chromatographic column. Modern UHPLC instrumentation is designed to add minimal dispersion to a chromatographic peak on a 2.1-mm ID column. This is typically the case for traditional adsorption modes of chromatography, such as RP, ion exchange, and normal phase. The process of adsorption within the column will broaden the peak, so demands on the system are reduced. The impact of retention on peak width is discussed in a related article in this issue [31], where the intrinsic peak variance is noted to be directly proportional to $(1 + k)^2$. UHPLC instruments were designed to add minimal system contributions to band broadening for retention factors greater than about 2. In the case of SEC, where there is no adsorption, the retention factor is zero, and the intrinsic peak width will be at a minimum. The peak variance obtained in SEC is thus seen to be almost an order of magnitude smaller than in adsorptive LC with a k of 2. Thus, to compensate for this, commercial size-exclusion UHPLC (SE-UHPLC) columns are provided with a 4.6-mm diameter in order increase the intrinsic peak variance, as this is proportional to the fourth power of the column diameter.

The extra-column dispersion of the injected sample can lead to significant losses of separation efficiency and undesired peak tailing [32–34]. These losses in efficiency can be introduced by unswept volumes in the autosampler, detector, and the tubing and end connections.

Another key attribute in instrument design is the compatibility of the system with mobile phases commonly used for SEC separations. For proteins, these are typically aqueous buffers with high salt concentrations. The chromatographic system used must be tolerant of the high-salt-concentration buffers used for these methods in addition to being biocompatible in order to minimize the formation of metal-protein adducts or protein-surface interactions. The wetted surfaces within chromatographic systems used for protein characterization are typically constructed of titanium, biocompatible polymers (e.g., PEEK) or biocompatible alloys (MP35N). For compatibility with polymer solvents, the system must be compatible with the broad range of non-aqueous solvents for dissolution and separation, often with aggressive/corrosive mobile -phase additives. These solvents must be delivered at pressures up to 1000 bar, without deleteriously affecting the flow delivery, seals and valves. Some of the solvents used for low-pressure GPC mobile phases may be limited due to their physical properties. For example, at room temperature, DMSO solidifies when subjected to pressures of about 500 bar. It is possible to use additives to depress the freezing point of DMSO, but this may induce adsorption or precipitation of the polymer of interest.

For polymer characterization, flow rate, precision and accuracy are critical to obtaining quality data. Because retention-time data are converted to MW, precision of the LC pump correlates directly with the precision of the molecular-weight distribution.

Recently, in 2013, Waters Corporation commercialized the Acquity APC® UHPLC, which was a system specifically designed for polymer separations. The isocratic system was designed to have low system dispersion. The materials contacting the fluidic components were chosen to be compatible with a wide range of mobile phases typically used for polymer characterization by SEC [23,35,36].

In SEC, a number of different detectors are used for characterization of polymers and biomolecules. For the analysis of proteins, peptides, and related compounds, UV absorbance detectors are most commonly used. A wavelength of approximately 280 nm provides good sensitivity for proteins and peptides that have amino acids tryptophan or tyrosine as part of their primary structure. However, disulfide bonds also absorb at this wavelength, and the molar extinction coefficient of this moiety is significantly lower than that of tryptophan or tyrosine [37]. The UV-absorbance band of the amide peptide bond (214–220 nm) can also be used and provides improved sensitivity over UV absorbance at 280 nm. However, this lower wavelength is more prone to baseline noise due to light scattering and may limit the use of some mobile-phase components. UV absorbance at 260 nm can be used to detect oligonucleotides separated by SEC. In the event that sample components interfere with protein detection by UV absorbance, the intrinsic fluorescence of these biomolecules can be used to advantage [38,39]. For the detection of polysaccharides, which have no chromophores, refractive-index (RI) detectors can be used [40]. Also, evaporating light-scattering detectors (ELSDs) have been commercially available for UHPLC use for several years.

In addition to using orthogonal methods, such as SV-AUC or DLS, to confirm the results observed by SEC indirectly, as previously noted, the direct characterization of the peaks separated by SEC is commonly performed using multi-angle light-scattering

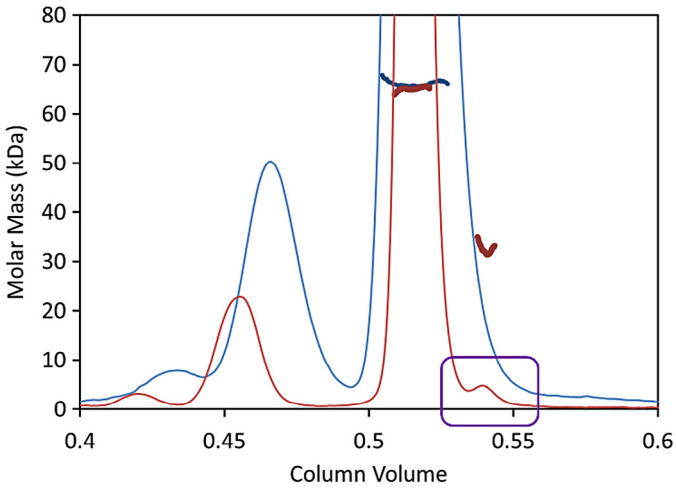

Fig. 2. Light-scattering data and measured molar mass for bovine serum albumin separated using UHPLC columns and instrumentation (red) and by standard HPLC columns and instrumentation (blue). Chromatographic conditions: Mobile phase 125 mM NaCl, 50 mM phosphate, pH 6.7; Temperature: 25°C. For UHPLC separation, detection was performed using a Wyatt μDAWN™ 660 nm, while HPLC separation was performed using a Wyatt miniDawn™ system. (Reproduced with permission from Wyatt Technologies).

(MALS) detectors. In conjunction with UV and/or RI detectors, absolute MW can be assigned [41,42]. More recently, low-dispersion RI detectors were commercialized in 2013 by both Waters and Wyatt [43,44]. In addition, Wyatt recently commercialized a low-dispersion MALS detector [45]. Fig. 2 shows overlays comparing the HPLC and UHPLC versions of the Wyatt MALS detector. The peak width is approximately 50% narrower on the UHPLC system, and is able to resolve a low-molecular-weight (LMW) constituent that could not be resolved using the HPLC detector.

Mass spectrometry (MS) detectors are increasingly being used for characterization of proteins and polymers [46]. However, there are particular challenges to coupling with SEC separations of proteins. Protein separations are typically performed using high concentrations of non-volatile salts, which can rapidly foul the MS source, and can also cause ion suppression [47]. SEC methods have been modified using denaturing mobile phases containing organic modifiers and with volatile buffers for use with MS detection [48–51]. For polymers, a distribution of charged species adds complexity to characterization of molar mass distribution. For this reason, matrix-assisted laser desorption/ionization (MALDI) is the most commonly used MS technique, as it generates primarily singly-charged species. However, MALDI is an off-line technique that requires deposition and evaporation of eluate onto a solid surface. Challenges remain in maintaining low dispersion from this process. As an alternative, Saucy et al. [52] have had success demonstrating the use of ^{210}Po as a means for charge reduction of electrosprayed polymers in aqueous media, but had less success with polymers in non-aqueous media. They had some success performing charge reduction for water-insoluble polymers when electrospraying in a solution of 5% trifluoroacetic acid in 1-methyl-2-pyrrolidone (NMP) [53].

Two-dimensional (2D) LC separations, which we discuss later in this article, often utilize NMR detection to obtain chemical composition information [54–56]. Reducing dispersion from transfer lines and the NMR flow cell presents challenges due to the distances needed to keep the LC instrument physically separated from the magnetic field.

4. Method development for biomolecule separations

Operationally, successful application of SEC for the analysis of biomolecules requires the consideration of two fundamental parameters. The first parameter is the use of an optimized mobile phase while the second is the extra-column dispersion of the chromatographic system. In order to achieve a separation primarily based on size or hydrodynamic radius of the analyte, the secondary interactions, both ionic and hydrophobic, between the biomolecule and the column must be eliminated or effectively minimized [57–59]. Not only can these interactions perturb the separation being attempted, resulting in observations of loss of protein recovery or deleterious changes in peak shape, they can also effectively alter protein secondary structure [60–62]. There are two principal types of ionic or electrostatic interactions that can affect SEC. The most readily noticeable of these is ionic adsorption, which occurs when the protein and chromatographic media have opposing charges and can result in low sample recoveries and peak tailing [63]. Less obvious is the phenomenon of "ion-exclusion", which can occur when the particles and the analyte have the same charge and will result in effectively excluding the analyte from the pores due to the ensuing repulsive forces. The chromatographic observation for this type of secondary interaction will be that the analyte will elute earlier than predicted based on its hydrodynamic radius.

Adjustments to the ionic strength and pH of the mobile phase are the primary means of reducing electrostatic interactions between the analyte and the SEC column [64,65]. While increasing the salt and/or buffer concentrations can minimize or eliminate undesired ionic interactions, there is also the possibility of introducing hydrophobic interactions with the diol ligands or other hydrophobic surfaces present in the column [65–68]. In these instances, using a more chaotropic anion, such as perchlorate, can be used to advantage [69]. Another approach to minimizing hydrophobic interactions is by adding an organic modifier, such as acetonitrile [70]. Another mobile-phase modifier that has been widely used to improve SEC protein and peptide separations is the basic amino acid arginine [62,71]. Arginine both stabilizes protein structure and prevents interactions between the protein and the column. While in the past there may have been concerns that arginine could be acting as a protein denaturant, as it has been observed to lower melting temperatures of proteins in solution, studies have shown otherwise [72]. As with other mobile-phase buffers, salts, and modifiers, it is important to use arginine of high purity in order to minimize chromatographic baseline noise to obtain optimal sensitivity. One of the limitations of arginine is that it absorbs and can therefore impair detection sensitivity at wavelengths below 220 nm.

5. SE-UHPLC applications

5.1. Biomolecules

There are numerous reported successful applications of size-exclusion HPLC (SE-HPLC) and many reviews and other publications have been devoted to this technology, some of which are in the References section of this review [20,69,73–76].

Certainly for the scientist who has initiated development of an SE-UHPLC method, much of the knowledge and many of the applications centered upon SE-HPLC can be directly applied to SE-UHPLC. By contrast, the number of applications reported for the use of SE-UHPLC is very limited, as this technology was only recently introduced (2010), and, currently, the only supplier of columns packed with sub-2-μm particles is Waters. However, commercially available SEC columns with 3-μm particles are available from Tosoh, Agilent, Phenomenex, Sepax, and Sigma-Aldrich. These columns provide some of the resolution, speed and sensitivity benefits relative to 1.7-μm particles compared to the classical SEC

columns with 5-µm and 10-µm particles. Both Waters and Thermo Scientific offer biocompatible UHPLC systems. As previously noted, UHPLC-compatible MALS and RI detectors are available from Wyatt.

The utility of SE-UHPLC separations has been realized in many areas of fundamental biochemistry research. In this capacity, these size separations have primarily been used to monitor the purity of laboratory-produced protein-related compounds [77–81]. In other examples, SE-UHPLC has been used as a purification step to purify cross-linked proteins in the study of cellular processes [82], and has also proved useful in protein-binding studies where differences in hydrodynamic radii between the reactant and the product can be used to advantage [83,84]. Proteomics is another area of research where the use of SE-UHPLC has been evaluated. Specifically, in the LC-MS mode, the utility of SE-UHPLC in a top-down proteomics strategy has been evaluated [85,86].

High-throughput and high-resolution separations, and the apparent molecular-weight range provided by SE-UHPLC have proved to be of significant value during the discovery and process-development activities associated with biotherapeutic proteins [87–91]. Also, SE-UHPLC has been successfully applied to the analysis of protein fragments [92], biotherapeutic leukocyte extracts [93], heparin [94], PEGylated proteins [95], and insulin and insulin variants [96,97]. An in-depth evaluation of the performance of SE-UHPLC was recently reported, and demonstrated that gains in sample throughput and the resolutions of high-efficiency separations can be achieved, when compared with SE-HPLC columns [98]. The authors also noted that the relative peak areas of the aggregate species of mAb panitumumab were observed to increase at higher temperatures and pressures, highlighting the importance of systematic method development and the confirmation of observed SEC profiles through the use of orthogonal methods [2].

In addition to these relatively traditional SEC applications, the characteristics of SE-UHPLC have been exploited in creative, novel methods. LC-MS separations under non-denaturing or native conditions have proved useful for the MS characterization of reduced mAbs, where the post-column addition of m-nitrobenzyl alcohol was used to improve electrospray ionization (ESI) and allow the MS identification of low-level species [99]. SEC LC-MS separations using direct ESI with a mobile phase of 25 mM ammonium acetate with 5% acetonitrile at a pH of 5.2 to evaluate the aggregation of a mixture of mAbs in stability studies were also reported [100].

Alternative separation strategies have been employed. The high sample-throughput solution using parallel interlaced SEC was reported as bringing the time of analysis for the aggregation levels of a mAb to below 2 min per sample [101]. An on-line 2D separation using an SE-UHPLC guard column (30 mm length) as a means of removing interfering small-molecule excipients in a sample prior to a mixed mode separation for the analysis of mAbs [102]. The reduced protein-column interactions and high efficiencies of the SE-UHPLC guard in comparison to SE-HPLC enabled the successful execution of this approach. The analysis of a mAb by a mixed-mode SEC and hydrophobic interaction liquid chromatography (HILIC) separation has also been reported [103]. In this example, the diol bonding and or the organosilica particle is being utilized as the ligand for HILIC interaction.

The high-efficiency separations provided by SE-UHPLC allow researchers to develop analytical SEC methods with greater resolution, improved sensitivity, and higher sample throughput than SE-HPLC methods. However, considerations of the performance of LC instrumentation and its implementation so as to minimize extra-column dispersion are critical in realizing the full potential of this technology.

5.2. Polymers

The first demonstration of the utility of UHPLC for polymer separations was in 2010 by Uliyanchenko et al. [104,105]. Using a 4.6 × 150 mm column packed with 1.7-µm 130-Å BEH C18, they were able to demonstrate separation of polystyrene standards with MW up to 50 kDa in less than 1 min. Separations were performed at a flow rate of 1.85 mL/min and an operating pressure of 660 bar. Columns were limited in pore volume, which reduced selectivity of the separation.

Janco et al. evaluated prototype columns packed with high pore -volume media for UHPLC separations by size exclusion [106]. They evaluated the impact of particle size on the polymer characterization. Using narrow-MW polymer standards with M_p of 11,600 g/mol, they compared the molar distribution on columns packed with 1.7-µm, 3.5-µm, 5-µm and 10-µm C18 particles. Fig. 3 shows the resulting chromatograms and molar mass. As particle size decreased, the calculated dispersity, Đ, defined as M_W/M_N, was found to become closer to the reported Đ value of 1.03.

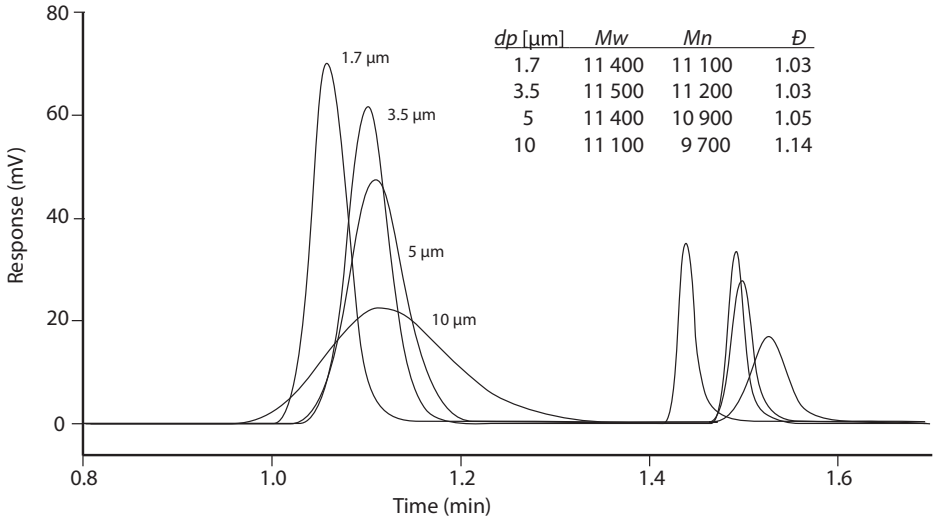

Fig. 3. Chromatograms of polystyrene standard (Mp 11 600 g/mol, Đ 1.03) obtained on XBridge® R C18 and Acquity C18 columns (4.6 × 150 mm) packed with different size particles: 1.7 µm, 3.5 µm, 5 µm and 10 µm. Mobile phase, THF; flow rate, 1 mL/min; detection, UV at 254 nm. {Reproduced from [106] under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License (CC BY-NC-ND)}.

The impact of surface chemistry on polymer characterization was explored by Bouvier et al. [24]. As an enthalpy-driven process, retention should not be affected by temperature to a great extent. While the hydrodynamic radius can be impacted by temperature, the relative retention change is minor compared to enthalpic adsorption. Bouvier et al. [24] looked at a limited number of polymers on columns packed with both an unmodified and trimethylsilyl-modified on 200-Å BEH particles. They found that in a tetrahydrofuran (THF) mobile phase, the non-polar polymers saw comparable retention time decreases of about 1–2% when run at 50°C compared to 30°C on unbonded and TMS-bonded phases. Similar retention-time changes were observed on a corresponding divinylbenzene (DVB) column. However, polyethylene glycol was substantially more retained on the unbonded BEH phase at the lower temperature, and poly(4-vinylphenol) and poly(2-vinylpyridine) did not elute on the columns packed with the unbonded phase. Retention of these analytes was not affected by temperature on the TMS-bonded column. This indicates that the available surface of the unbonded BEH columns is able to interact by ionic and/or hydrogen bonding with these polar analytes.

5.2.1. Oligomer separations

Synthetic oligomers are used for numerous applications: lubricants, plasticizers, coatings, and intermediate prepolymers. It is desirable to be able to separate and to resolve as many of the individual components of the oligomer from each other, as that enables better identification and quantitation of the oligomeric component of the polymer or prepolymer. The number of oligomeric SEC applications has grown by two orders of magnitude in the past 30 years [32].

One key driver in characterizing oligomers is legal requirements for pre-manufacture notification (PMN) and for export/import regulations. The US Environmental Protection Agency (EPA) has exempted some classes of polymers from PMN, if the oligomer content is below a certain threshold [107,108]. The (e)(1) exemption pertains to polymers with M_n 1000–10,000 g/mol. Oligomers with molar mass <500 g/mol and 1000 g/mol must be <10% (w/w) and 25% (w/w), respectively. The (e)(2) exemption pertains to polymers with M_n above 10,000 g/mol. Oligomers with molar mass <500 g/mol and 1000 g/mol must be <5% (w/w) and 2% (w/w), respectively.

Oligomer separations by SEC present difficult challenges to chromatographic column and instrument design. The limited peak capacity of an SEC system precludes resolving all of the individual constituents of the oligomer. As MW increases, the difference in retention time between a polymer of *n* units in length from one of *n+1* units in length decreases. Above MW of ~1000–2000 Da, no observable resolution can be achieved in SEC between an *n*-mer and an *(n+1)*-mer. In the past few years, columns packed with smaller 3-µm and 5-µm particles were utilized for oligomer separations, primarily to achieve gains in speed and resolution. For separation of non-aqueous oligomers, porous styrene/divinylbenzene particles were traditionally used, and can typically operate at pressures less than 70 bar and deliver efficiencies up to 110,000 plates/m. The recent introduction of UHPLC to polymer characterization demonstrated an improvement in the resolving power of oligomer separations in significantly shorter run times. The use of 1.7-µm BEH particles enables faster flow rates on UHPLC instruments that can operate at pressures of 1000 bar. Fig. 4 shows a separation of oligomer constituents of a 374-Da polystyrene standard that can be achieved in less than 2 min [109].

Fig. 5 shows the impact of flow rate on chromatographic efficiency. In the case of oligomers, in which components are individually resolved, chromatographic efficiencies are up to 230,000 plates/m [109]. In the case of higher MW polymers, in which individual components are not resolvable, the chromatographic efficiency appears to be substantially less. However, in this case, the dispersity of the polymer has the most significant contribution to the peak width.

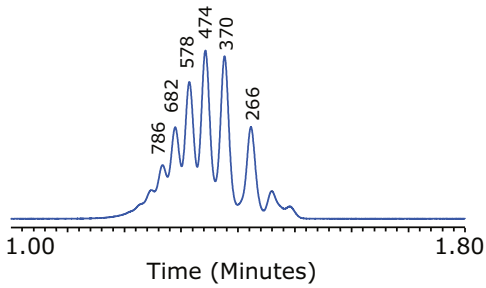

Fig. 4. Separation of polystyrene oligomers on a Waters APC 45, 4.6 × 150 mm, 1.7 µm in THF. Flow rate: 1.0 mL/min. (Reproduced with permission from Waters Corporation).

5.2.2. 2D separations

Complex polymers, such as blends and copolymers, present characterization challenges. They can contain distributions in MW and chemical composition that must be characterized. One such approach is to utilize comprehensive 2D separations (LC X LC), as discussed in a recent review article [56].

One common technique is to use LC under critical conditions (LCCC) as the first dimension [110]. In LCCC, conditions are chosen so that all constituents of the same composition elute at the same time, regardless of MW. Separations can be performed both off-line and on-line, but typically require several hours for complete analysis due to the time constraint of the second dimension, so the technique is impractical for routine use.

Recently, UHPLC-SEC was employed in the second dimension, with individual run times of less than 1 min, and total 2D separation occurring in 22 min [111]. This was demonstrated for the separation of polymethacrylate (PMMA) and polybutylmethacrylate (PBMA) copolymers. LCCC was employed in the first dimension, first to elute PMMA homopolymers, followed by an acetonitrile/THF gradient, providing a separation by chemical composition. SE-UHPLC was employed in on-line mode in the second dimension,

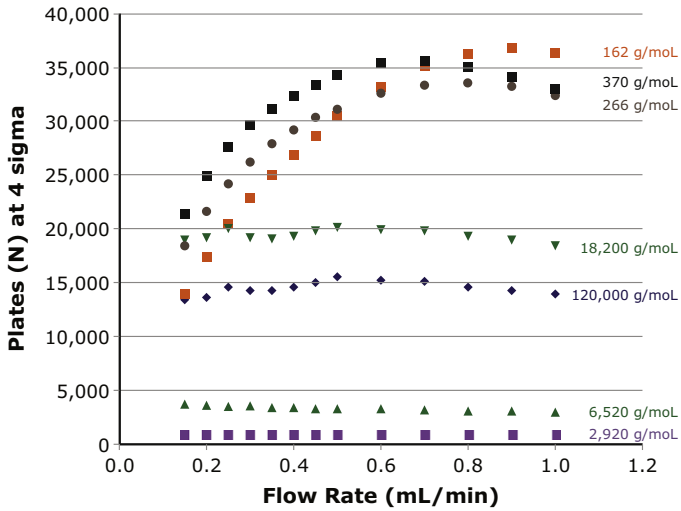

Fig. 5. Impact of flow rate on observed chromatographic efficiency for polystyrene standards. Column: Waters Acquity APC 45 XT, 4.6 × 150 mm; Mobile phase: THF; Temp: 25°C. (Reproduced with permission from Waters Corporation).

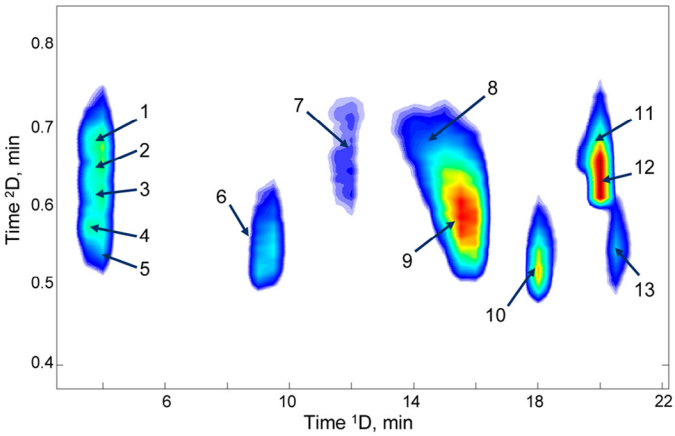

Fig. 6. Two-dimensional separation of PMMA and PBMA homopolymers and copolymers. First-dimension separation was performed on three Waters Acquity UPLC C18 columns connected in series, 2.1 mm x 250 mm total length. Gradient: 5 min at 15.5% THF in acetonitrile, followed by a 17-min linear gradient to 80% THF. Flow rate: 0.2 mL/min. Second dimension performed on an Acquity C18, 4.6 × 150 mm at a flow rate of 2 mL/min, in a THF mobile phase. {Reprinted with permission from [112], ©2012 American Chemical Society}.

providing the size-distribution information. Fig. 6 shows the results of the comprehensive separation.

Another approach, using conventional SE-HPLC as second dimension utilized high temperature to decrease mobile-phase viscosity and increase analyte diffusivity. This enabled faster separations with minimal degradation in chromatographic fidelity [111]. A 2D separation was performed, with second dimension runs of 1.6 min, for the analysis of polystyrene with different functional groups, and polystyrene-polyisoprene-polystyrene triblock copolymers.

One limitation in the use of multi-dimensional separations is the mobile-phase compatibility of the two techniques. When SEC is used in the second dimension, it is highly desirable for the sample diluent from the first dimension to be of sufficient strength for the analyte to be unretained on the stationery phase. Otherwise, adsorption during loading could impact the integrity of the peak and result in peak splitting [113]. Conversely, if adsorption chromatography is used for the second dimension, a weak solvent is needed for sample loading to concentrate the band. Peak spreading due to injection solvent can be mitigated by using smaller injection volumes. Alternatively, adding a make-up solvent and mixing tee could provide improvement, but at the expense of increasing the complexity of the system.

6. SE-UHPLC for HMW polymer characterization

HMW polymers are subject to shear stresses that can lead to deformation or shear [114]. As shear stresses are induced, the polymer can transition from a random coil to a stretched form. The extent of stretching can be characterized by the Deborah Number, a dimensionless number that represents the ratio of hydrodynamic forces to Brownian forces [115].

Both Uliyanchenko et al. [114] and Janco et al. [106] explored the effect of UHPLC on shear. Both groups found no shear-induced degradation of polymers up to 2–3 MDa. Slalom effects were observed for the HMW polymers, resulting in an increase in retention time. However, the slalom effects could be reduced by operating at lower linear velocities. Uliyanchenko found that shear-induced degradation could be induced for a 13-MDa polystyrene, but this could be avoided by operating at low linear velocities.

7. Benefits of UHPLC for size-based separations

SEC is an inherently a low-resolution technique, particularly when compared to other modes of chromatography. SEC separations are performed within one column volume, while isocratic and gradient elution chromatography use multiple column volumes to perform the separation. In the case of gradient separations, where band spread within the column is minimized, peak capacity can be more than an order of magnitude greater than in SEC [116,117]. However, SEC offers substantially improved selectivity over other separation modes when the primary characteristic being evaluated is size distribution. For example, determination of the extent of protein aggregation or the MW distribution of a polymer is most effectively provided by SEC.

The main utility of SEC is in the separation of large polymers and biopolymers, which have inherently low diffusivities. The resulting slow mass transfer of these analytes in and out of the stationary-phase pores limits the speed at which separations can take place.

Significant efforts have been made over the years to try to speed up or to increase the sample throughput of SEC separations [101,106,118–122]: by using higher flow rates, shorter columns, changing column aspect ratio, and performing staggered injections. However, the first three approaches result in decreased resolving power, while the last approach can add significant complexity to the chromatographic instrumentation.

The introduction of low-dispersion SE-UHPLC instrumentation and columns enables one to achieve faster separations without sacrificing resolution, by reducing particle size and column length, and maintaining the same L/d_p ratio. As discussed earlier, the success of this approach depends on using high pore-volume particles that have the requisite mechanical strength to maintain their integrity under high shear conditions.

Thus, speed is the primary benefit provided by SE-UPLC. By using a column packed with 1.7-μm particles instead of 5-μm particles, one can demonstrate that equivalent efficiency can be obtained in about one-ninth of the time. If one maintains the same L/d_p ratio, the approximate three-fold reduction in particle size enables a corresponding three-fold reduction in column length. In addition, the flow rate needs to be adjusted, since the optimum flow rate is inversely proportional to the particle size [123]. This combination of faster flow rate and shorter column length is what provides the nine-fold increase in sample throughput without sacrificing resolution.

In cases where even more resolution is needed, longer columns can be used, or multiple columns can be banked together to provide improved resolution without needing excessive run times. Since resolution is proportional to the square root of L/d_p, one would expect a 70% improvement in resolution for columns of equivalent length containing 1.7-μm *versus* 5-μm particles.

8. Conclusion

The benefits of enhanced chromatographic performance obtained with UHPLC were recently extended to separations by size exclusion, which has characteristics that place stringent demands on column and instrument design for UHPLC performance.

The dispersion requirements for SE-UHPLC are substantially more stringent than adsorption modes of chromatography, as the column contributions to band spread are at their smallest. In the past, column design suffered from several limitations:

- low-strength sorbents that could not operate at high pressures;
- swelling/shrinking when exposed to different mobile phases; and,
- adsorption to chromatographic media, particularly silica, which contained acidic silanols.

Recent advances in chromatographic column development have provided high-strength, high-pore-volume chromatographic media with low acidity. Surface modification has further reduced silanol acidity. Diol-bonded media have provided minimal interactions towards proteins using appropriate buffered aqueous mobile phases. Unbonded and TMS-bonded media can be used to perform effective size-based separations in aqueous and non-aqueous mobile phases, respectively.

While low-dispersion UHPLC UV and ELSD detectors have been available for the past decade, additional UHPLC-compatible detectors, such as RI and MALS, are beginning to be commercialized and can maintain the chromatographic integrity of these high-performance separations. MS detectors are successfully being used in conjunction with SE-UHPLC. By using volatile mobile phases, proteins have been effectively characterized with this powerful tool. We expect that SE-UHPLC separations of polymers will also benefit from MS and NMR detection, although challenges remain in interfacing these detectors with the separation to maintain low dispersion. Also, for MS, reducing charge distribution remains a challenge.

2D separations are expected to benefit greatly from SE-UHPLC. Chromatographic fidelity can be maintained for rapid SEC separations, and we expect the time required for comprehensive 2D separations to be reduced greatly from several hours to 30 min or less.

Even though the first commercial UHPLC columns for SEC were developed only four years ago, a number of protein-separation applications have already been developed, demonstrating the benefits of speed, resolution and sensitivity compared to conventional SE-HPLC. With the recent introduction of a system and columns for polymer characterization, the future also looks promising for characterization of these classes of analytes.

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A REVIEW OF WATERS HYBRID PARTICLE TECHNOLOGY.

Part 2. Ethylene-Bridged [BEH Technology] Hybrids and Their Use in Liquid Chromatography

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

A NEW LEVEL OF PERFORMANCE

First introduced in 1999 in XTerra™ Columns, Waters™ patented organic/inorganic Hybrid Particle Technology [HPT] surmounted significant limitations of silica-based reversed-phase packing materials, particularly their hydrolytic instability at high pH. In 2005, second-generation HPT, branded as BEH Technology™, embodied in new ACQUITY™ UPLC™ BEH Columns and in Waters XBridge™ HPLC Columns, marks a new milestone in chromatography.

BEH Technology columns demonstrate outstanding capability, especially for basic compounds, using traditional conditions. But now you have the freedom to broaden operation to a dramatically wider pH range of 1–12, typical of polymer packings, yet attain peak shape, efficiency, retention properties and high temperature stability equal to, or better than, that of the best silica-based reversed-phase columns.

With an order-of-magnitude improvement in high pH stability and a higher level of chromatographic performance, BEH Technology columns define the new benchmark for LC method development.

Problems with Silica

Before 1999, silica-based packings were predominant in reversed-phase HPLC columns because of their high chromatographic efficiency and excellent mechanical strength. They have continued to evolve via significant process improvements made in the last two decades. For example, almost all new column brands in the last 15 years have adopted the use of high purity, highly efficient, small-particle, spherical silica, which reduces peak tailing due to metal impurities.[1] Refinements in traditional approaches to bonding, e.g., using trifunctional[2] or sterically hindered monofunctional silanes, [3] have greatly increased the resistance to hydrolysis of silyl groups, attached to the surface via siloxane bonds, in low-pH [1-3] mobile phases. Embedding a polar functional group [e.g., a carbamate[4]] in a bonded alkyl chain was discovered to improve peak tailing factors for basic analytes as well as to impart unique selectivity for certain classes of important analytes such as phenols. Despite all these enhancements, one key chemical property of silica-based packings still limits their performance and operation: the hydrolytic instability of *silica*, especially above pH 8. In high-pH mobile phases, silica particles begin to hydrolyze, resulting in loss of efficiency and peak distortion due to voids in the packed bed.

Hybrid Particle Technology [HPT]

The XTerra family of reversed-phase HPLC columns featured the first commercially available hybrid organic/inorganic packing materials incorporating Waters patented Hybrid Particle Technology. They provided the first viable alternative to silica-based column technology.[5]

XTerra high purity methyl hybrid material is prepared in a highly reproducible process analogous to that used today to produce high-purity silica — with one important difference. It is formed from the mixed condensation of *two* high purity monomers (Figure 1): tetraethoxysilane [TEOS, the SiO_4 tetrahedral subunit precursor] *and* methyltriethoxysilane [MTEOS, an *organosilane* which adds carbon throughout the backbone].

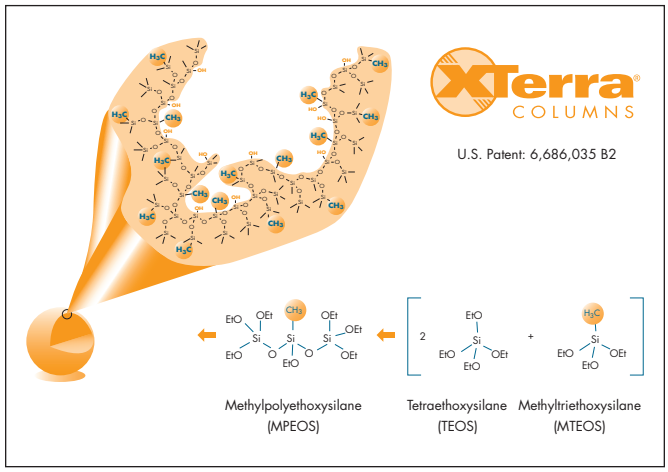


Figure 1: XTerra hybrid particle synthesis.

The XTerra substrate retains certain key advantages of silica: purity, mechanical strength, highly spherical shape, ability to tailor particle size, pore dimensions, surface area, and surface chemistry [using traditional bonding reagents *e.g.*, C_{18}-, C_8-, or phenyl-chlorosilanes].

While chromatographic evaluations of bonded-phase XTerra columns demonstrate performance (*e.g.*, efficiency, selectivity, and retentivity) comparable to that of silica-based reversed-phase materials, they also highlight key advantages:

- decreased tailing factors for basic analytes
- significant improvement in high-pH stability.[5,6]

Unlike other high-pH-stable packing materials [*e.g.*, organic polymers, graphitic carbon, alumina, titania, and zirconia], XTerra columns were the first commercially available option that allowed the use of conventional bonding reagents and convenient reversed-phase separation conditions *without* drawbacks such as unpredictable selectivity, decreased mechanical stability, bed swelling/shrinking, or inferior peak shape due to mass transfer issues [typical of polymers or organic coatings on inorganic phases] or secondary interactions [especially on highly active inorganic surfaces such as alumina, zirconia or titania].

There are several benefits of carrying out separations at high pH. Some compounds are not stable in acid and require a basic mobile phase. For many basic analytes, so predominant in the pharmacopoeia, using high-pH mobile phases provides a powerful tool for optimization of selectivity.[5] When the mobile-phase pH is two units above the pK_a of the base, it will be primarily in its neutral [unprotonated] form. In this state, peak tailing arising from interaction with silanols is minimized, retention is no longer strongly dependent on mobile-phase pH, and analytical methods become more rugged and reliable.[5b] Operating at a pH that ensures basic molecules are in their neutral form also dramatically increases the amount of sample that can be loaded before peak distortion occurs, a great benefit to preparative purification.[7]

Ongoing HPT Development

Waters is committed to improving separation technologies, and believes Hybrid Particle Technology is an important tool to achieve this goal. The impressive worldwide acceptance of the XTerra column brand validates the HPT approach to improving peak shape and extending the high pH stability of chromatographic sorbents.

Since the XTerra launch, Waters has continued its HPT research, exploring a variety of organofunctional silane precursors. Some of these new hybrid materials contained a terminal organofunctional group (*e.g.*, $RSiO_{1.5}$ where R = alkyl, aryl, vinyl); others had an internal bridging organofunctional group (*e.g.*, $O_{1.5}Si\text{-}R'\text{-}SiO_{1.5}$ where R' = alkylene, arylene). From our initial evaluations, the one particle formulation that stood out above the rest in terms of chromatographic performance was the ethylene-bridged hybrid.

We have since invested several years of research and development to ensure that we would bring to market this BEH Technology particle in an optimized form. Based upon crucial customer feedback, three goals for this second-generation hybrid particle were considered paramount:

- maximize efficiency
- further improve high-pH stability
- improve column ruggedness.

After four years of research, we introduced our second-generation BEH Technology hybrid particles.

BEH Technology Introduction

BEH Technology synthesis creates particles that ensure extreme column performance and long column lifetime under harsh operating conditions. In our development, using state-of-the-art techniques, we optimized each step of the production process, including particle synthesis, particle size distribution, and bondings, to achieve the desired properties reproducibly.[8] As shown in Figure 2, BEH Technology particles are prepared, like XTerra particles, from *two* high purity monomers: tetraethoxysilane [TEOS] and bis(triethoxysilyl)ethane [BTEE, which incorporates the pre-formed ethylene bridge].

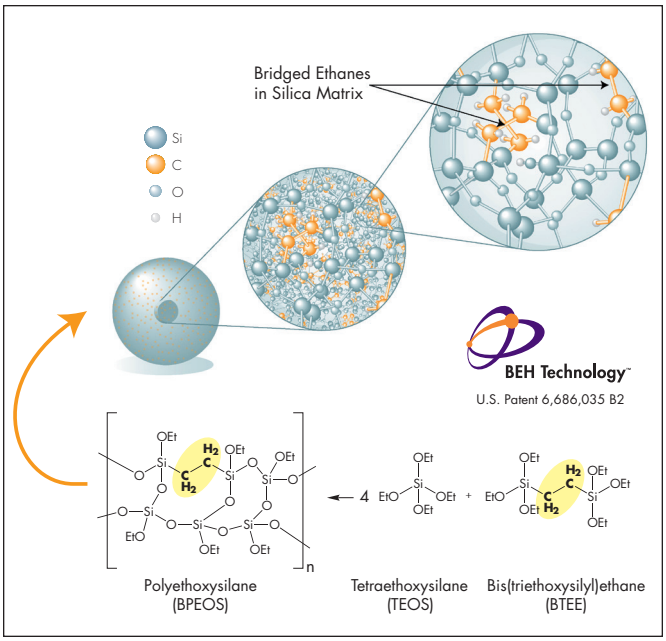


Figure 2: BEH Technology particle synthesis.

Optimal physicochemical characteristics [pore size distribution, surface area, particle size and strength] of the porous spheres are obtained using a 4:1 mole ratio of TEOS to BTEE. A sufficient population of accessible silanols remains to enable surface functionalization by traditional methods. By uniformly distributing an ethylene-bridged co-monomer throughout the backbone, the effective cross-linking of the second-generation hybrid particle is higher than that of the XTerra methyl-hybrid particle. This high degree of cross-linking ensures excellent mechanical strength. And, because the covalent Si–CH_2–CH_2–Si units are chemically stable and hydrophobic, their presence greatly improves hydrolytic stability.

Precise control over particle synthesis, coupled with newly developed processes for sizing and packing sub-2-µm particles, led us to create ACQUITY UPLC BEH C_{18} 1.7 µm Columns[9] which have enabled the holistically designed Waters ACQUITY Ultra Performance LC™ System.[10] We selected a particle size [1.7 µm] that would permit operation at the highest possible linear velocity for maximum column efficiency within our engineers' design constraints [15,000 psi] for state-of-the-art fluid delivery and sample injection.

At conventional HPLC pressures and particle sizes, advances in column performance provided by BEH Technology packings are equally dramatic.

XBridge HPLC Column Phases

The rational array of surface functionalities in the XBridge column family, shown in Figure 3, were carefully chosen to combine complementary selectivity with the highest efficiency and widest range of pH stability. XBridge C_{18}, C_8 and Phenyl packings are made using trifunctional silanes. A proprietary endcapping technique produces superior high- and low-pH stability and outstanding peak shape for basic analytes. XBridge Shield RP18 columns incorporate a patented monofunctional silane[4] whose embedded polar carbamate group imparts unique selectivity characteristics and superior peak shape for basic analytes.[4,11,12]

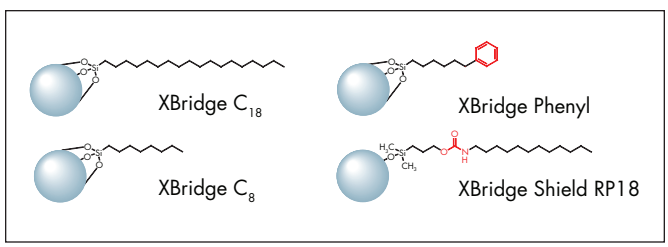


Figure 3: XBridge column surface function structural formulas.

XBridge Column Efficiency

The origins of band spreading, which decreases separation efficiency, are described by the van Deemter equation[13]:

$$h = a + b/\upsilon + c\upsilon$$

the reduced plate height, h, is a function of the reduced linear velocity, υ, [both normalized for particle size] and a, b, and c summarize the contributions of eddy diffusion, longitudinal diffusion, and the sum of stationary- and mobile-phase mass transfer terms, respectively.

Data for both silica and BEH Technology packings, fitted to the van Deemter equation as shown in Figure 4, demonstrate effective equivalence in efficiency. Furthermore, the c term for XBridge C_{18} is virtually identical to that for the two state-of-the-art C_{18} Silica columns, indicating that all these columns are comparable in mass transfer characteristics.

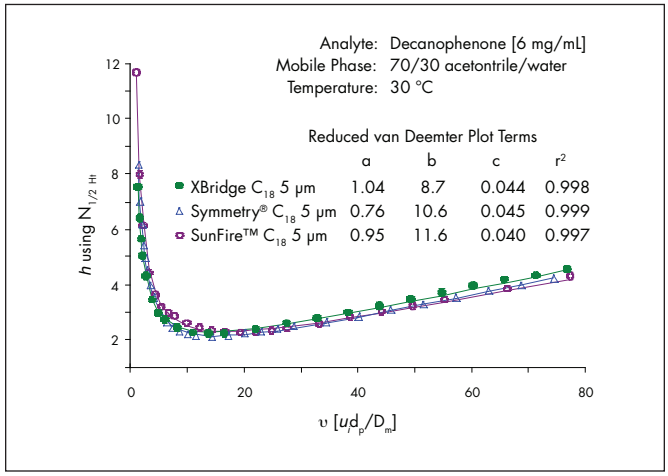


Figure 4: van Deemter curve comparison.

XBridge Peak Shape

Peak shape is an indicator of chromatographic column quality and performance.[1] An ideal peak is Gaussian, symmetrical about a vertical line drawn from the peak apex to its baseline. A horizontal chord, drawn at 5% of the peak height, from peak front to rear should be bisected by this axis of symmetry. The ratio of the total length of this bisected chord to twice the length of the front segment defines the U.S.P. Peak Tailing Factor [T_f]. T_f = 1.0 for a symmetrical peak; T_f > 1.0 for a tailed peak; T_f < 1.0 for a fronted peak.

When neutral analyte peaks are symmetrical, but a peak for a basic compound tails, interaction with silanols on the unbonded surface of a packing material via an ion-exchange or proton-transfer mechanism is implicated.[14] Propranolol and amitriptyline are basic

drugs commonly used to probe silanol activity on reversed-phase packings. With intermediate pH mobile phases, many silica-based C_{18} reversed-phase columns show high tailing factors for these bases.[1] For example, using the conditions shown in Figure 5, $T_f > 2.0$ is common for amitriptyline. In contrast, XBridge C_{18} columns display significantly reduced tailing factors for both propranolol ($T_f < 1.2$) and amitriptyline ($T_f < 1.4$). This we attribute to the Si–CH_2–CH_2–Si units that lower the population of surface silanol groups within both inaccessible and accessible pores, and that lower the acidity of surface silanols in hybrid particles ($pK_a > 8$) when compared with that typical of silica (pK_a 3.5 – 6.8).[15]

XBridge Column Selectivity

The availability of a variety of functional groups, each bonded to a common particle substrate, which exhibit a wide array of selectivity, is an important requirement for efficient HPLC method development. To compare the separation performance of the initial four XBridge column chemistries [C_{18}, C_8, Shield RP18 and Phenyl; see Figure 3], we used a well-documented isocratic method,[16] separating at pH 7.0 a set of seven acidic, neutral and basic analytes, as shown in Figure 5.

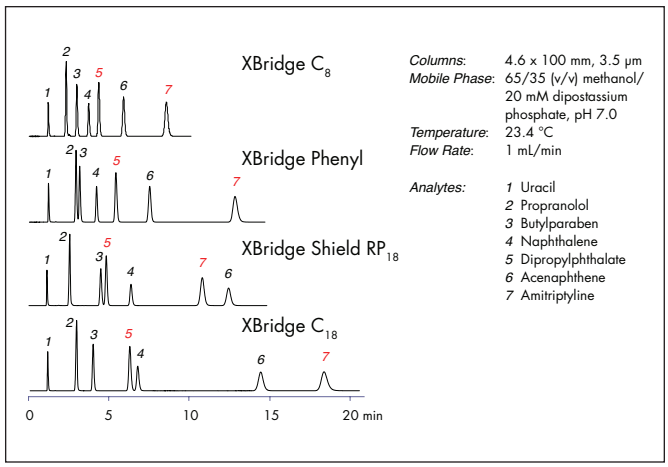
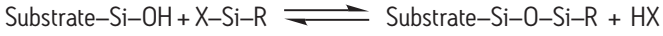


Figure 5: XBridge family selectivity comparison.

These test conditions are particularly suited to probe selectivity and retentivity differences between various column phases.[16c] Note the relative retention of naphthalene [peak 4] to dipropylphthalate [5], as well as of acenaphthene [6] to amitriptyline [7], in the four chromatograms. As expected, the most dramatic selectivity change is obtained by using the Shield RP18 phase while the highest retentivity is on the C_{18} phase. Compare also the selectivity of each phase for the aromatic neutral substrates, particularly dipropylphthalate [5].

XBridge Low-pH Stability

Both silica and hybrid substrates are stable in strong acids. The origin of low-pH instability for bonded phases is due to the often-overlooked *reversibility* of the reaction, devised by Speier,[17] commonly used to attach a silyl functional group to a surface silanol [Si–OH] via formation of a siloxane bond [Si–O–Si]:



Siloxane bond hydrolytic cleavage is catalyzed by both acids and bases. Many factors contribute to the rate of hydrolysis. A marked decrease in retention or deleterious change in selectivity signifies that the magnitude of surface function loss has ended the column's useful life.

To improve the low-pH stability of bonded phases, it is necessary to slow the rate of hydrolysis. We developed, evaluated, and adopted several approaches for XBridge C_{18}, C_8, and Phenyl packings. These include the use of well-characterized, highly monitored, proprietary procedures for trifunctional bonding and endcapping.

To compare the low-pH stability of XBridge C_{18} columns to that of a series of benchmark silica-based C_{18} columns, we used a previously developed procedure to accelerate column failure.[2] In this test, columns at 80 °C are equilibrated for one hour in an aqueous mobile phase [pH 1.0] containing 1% trifluoroacetic acid [TFA], then injected with a sample containing methyl- or ethylparaben to monitor retention loss. After each separation, the columns are flushed with 1% TFA in acetonitrile to remove any residual hydrolyzed bonded phase not previously eluted. Then, the whole cycle is repeated. Test data in Figure 6 illustrates the continuous loss of retention with increasing time of exposure to aqueous TFA.

As expected, bonded phases prepared using trifunctional silanes are much more stable at low pH than those using monofunctional silanes. A monofunctionally bonded benchmark silica column showed a large retention loss within 20 hours. In contrast, the trifunctionally bonded XBridge C_{18} column shows little retention loss in this accelerated test, with a lifetime equivalent to that of a sterically hindered C_{18}-silica-bonded phase.

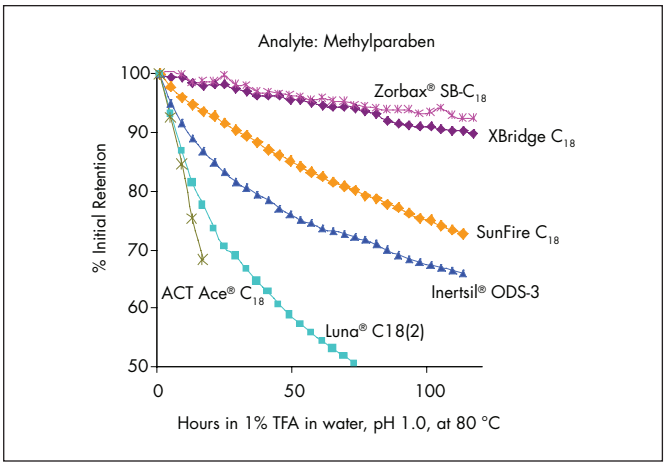


Figure 6: Accelerated low-pH stability tests of C_{18} phases.

The benefits of our bonding chemistry techniques are even more dramatic with smaller primary functional groups. Witness the comparison in Figure 7 of the low-pH stability test results for a series of Phenyl columns. XBridge Phenyl exceeds the stability of other silica-based phenyl-bonded phases under these conditions, including a benchmark silica column that relies solely on protection by a sterically hindered silane.

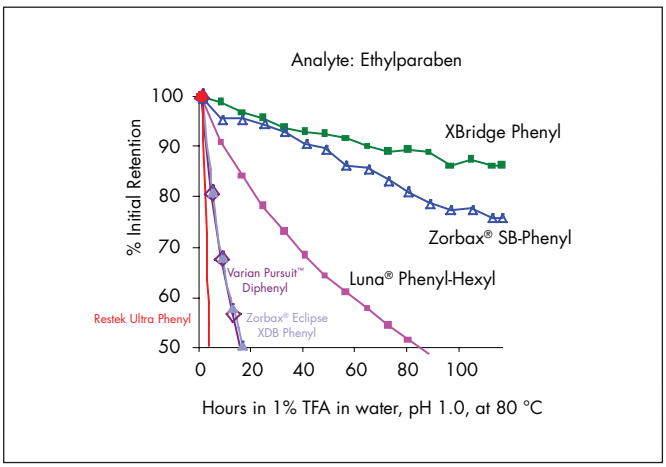


Figure 7: Accelerated low-pH stability tests of phenyl phases.

XBridge High-pH Stability

Silica is unstable in alkaline mobile phases. The failure mechanism generally accepted as predominant for a bonded-silica-based particle at elevated pH is nucleophilic attack by hydroxide ions on its structural siloxane bonds.[18] Once this process of silica dissolution proceeds to a critical point, the packed bed abruptly collapses, causing voids that result in a catastrophic loss of efficiency.

In contrast, our organic/inorganic hybrid substrates are more stable. We have previously attributed the improved high-pH stability of XTerra bonded phases to the protection by the organic component of the particle backbone.[5c,d] The hydrophobic methyl groups, present throughout the particle, not just on the accessible surface, are believed to shield the silica units. XBridge substrates also benefit from hydrophobic shielding, but have the further advantage that the structural ethylene bridges do not hydrolyze. Up to six siloxane bonds would need to be broken to free one ethylene-bridge unit from a particle. Thus, XBridge particles exhibit excellent resistance to high-pH solutions, even prior to bonding.[8] This fact, coupled with the excellent mechanical strength of the unbonded particle, explains the exceptional lifetime of XBridge columns in high-pH mobile phases.

To compare the high-pH stability of XBridge C_{18} columns to a series of benchmark silica-based C_{18} columns, we ran an accelerated stability test.[8] Columns at 50 °C are equilibrated with an aqueous triethylamine-containing [TEA] mobile phase buffered to pH 10. After periodic flushing with water and methanol, columns are tested for efficiency with the aromatic hydrocarbon analyte acenaphthene. As shown in Figure 8A, column efficiency remains nearly constant for a period of time, and then drops precipitously. In Figure 8B, a series of chromatograms, run at regular intervals during the high-pH lifetime study in Figure 8A , verify that 86% of the original XBridge column efficiency remains after 300 hours at pH 10 and elevated temperature, with little change in peak shape or retention time. XTerra MS C_{18} columns have about twice the lifetime of that for the most stable silica-based benchmark column. Amazingly, XBridge C_{18} column lifetime exceeds that of the best silica-based columns by an order of magnitude [1000%]!

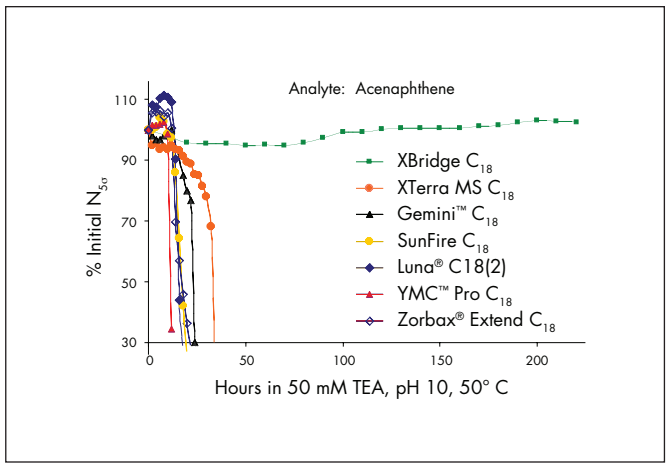


Figure 8A: Accelerated pH 10 stability test of C_{18} columns.

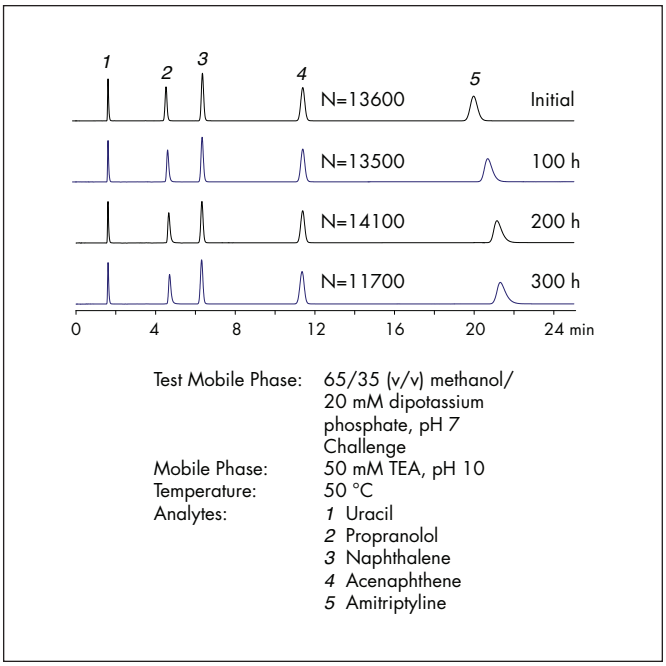


Figure 8B: XBridge Column efficiency maintained after 300 Hours at pH 10.

In order to explore the limits of high-pH stability, we devised a more severe test. We subjected the entire family of XBridge columns at 50 °C to a 0.02 N NaOH mobile phase at pH 12.3. As shown in Figure 9, the lifetimes of all XBridge columns ranged from 28 to 45 hours under conditions that cause silica-based columns to fail within 1 to 2 hours. Of particular note is the fact that the more hydrolytically resistant XBridge Phenyl, C_8, and C_{18} columns have nearly equivalent high-pH stability, in marked contrast to silica-based columns where the less sterically hindered, shorter-chain primary functional groups [phenyl, C_8] typically fail sooner.

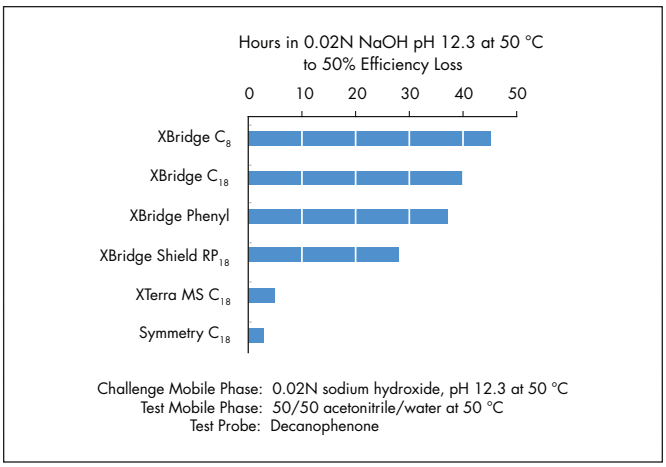


Figure 9: Exploring the limits of XBridge column lifetime at pH 12.3.

Conclusions

Chromatographic methods need to be developed rapidly, with confidence that the results will be both robust and reproducible. The convenience of a phase that has the chemical versatility to handle a spectrum of samples under a wide range of appropriate conditions, and the particle size range to accommodate straightforward scale up of sample loads when necessary, is most beneficial. The record-setting rapidity of worldwide acceptance of XTerra HPLC columns has validated the capability of Waters Hybrid Particle Technology to deliver such benefits.

Waters investment in, and ongoing commitment to, materials science has now created the second generation of HPT — BEH Technology particles. With their commercialization in ACQUITY UPLC BEH columns for the UltraPerformance Liquid Chromatography technique, followed by the XBridge family of HPLC columns, Waters has brought a higher level of stability and capability to the LC arena.

BEH Technology particles offer excellent peak shape and efficiency, especially for basic analytes, a rational array of chromatographic selectivity, and improvements in chemical stability at mobile-phase extremes, particularly dramatic at elevated pH.

Confidence, capability, capacity, convenience — key criteria for chromatographic method development — are now embodied in a new organic/inorganic hybrid particle platform.

XBridge Columns, in 2.5 µm, 3.5 µm, and 5 µm particle sizes, as C_{18}, C_8, Shield RP18, and Phenyl bonded phases, are being introduced at HPLC 2005. The ACQUITY UPLC 1.7µm BEH C_{18} columns were introduced at Pittcon 2004, the ACQUITY UPLC 1.7µm BEH C_8, Shield RP18 and Phenyl columns at Pittcon 2005.

— Kevin D. Wyndham, Thomas H. Walter, Pamela C. Iraneta, Uwe D. Neue, Patrick D. McDonald, Damian Morrison, Mark Baynham

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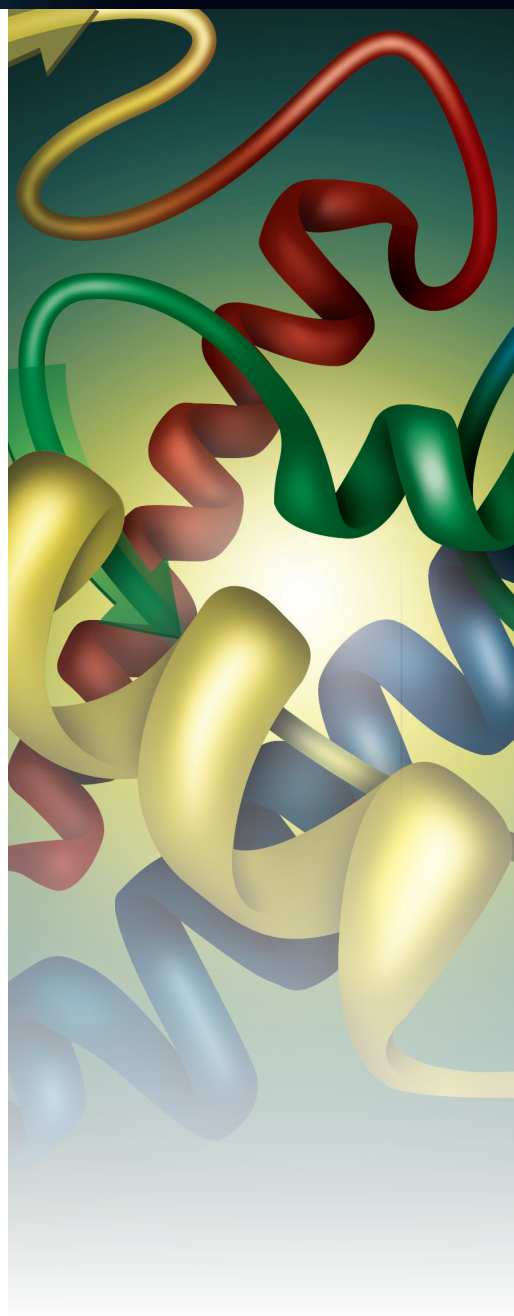
XBridge Protein BEH SEC Columns for HPLC-based Separations



Waters XBridge® Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns were developed for use on HPLC instrumentation and to complement our existing line of smaller particle sized, UPLC®-based SEC columns for size-exclusion chromatography (SEC) of proteins. These new HPLC SEC columns are based on the same Waters ethylene bridged hybrid (BEH)-based particle technology with stable diol-bonding to deliver superior performance compared to traditional, 100% silica-based SEC offerings. Consequently, chromatographers now have the ability to easily develop and/or transfer SEC methods based on laboratory instrumentation, required protein component resolution, and sample throughput requirements.

- HPLC-based SEC resolution of proteins from 10–1,500K Daltons with higher throughput capability
- Outstanding SEC column life
- Less non-desired, protein/column interactions than silica-based SEC columns
- Comprehensive testing to provide unmatched column consistency and increased confidence in validated methods
- Complement ACQUITY UPLC®-based SEC columns for seamless method transfer based on application needs

HPLC-BASED SEC COLUMNS FOR PROTEIN SEPARATIONS

Reliable, high resolving, SEC methods are routinely used in the discovery, development, and quality assessment of protein-based biotherapeutics. Waters XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns separate proteins from approximately to 10k to 1,500K Daltons. The diol-bonded XBridge Protein BEH SEC, 3.5 µm Columns are flow and pressure tolerant for increased sample throughput on HPLC systems compared to use of many traditional, silica-based SEC columns containing >5 µm particles.

Calibration Curves of Proteins and Peptides on XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns

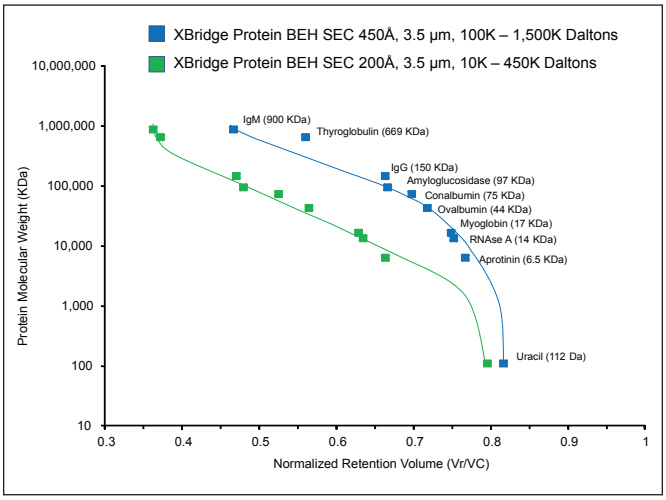


Figure 1. Calibration curves of various proteins, peptides, and uracil generated on the XBridge Protein BEH SEC 200Å (green) and 450Å (blue), 3.5 µm particle-size SEC Columns.

Separation of Protein and Peptide Standards on XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns

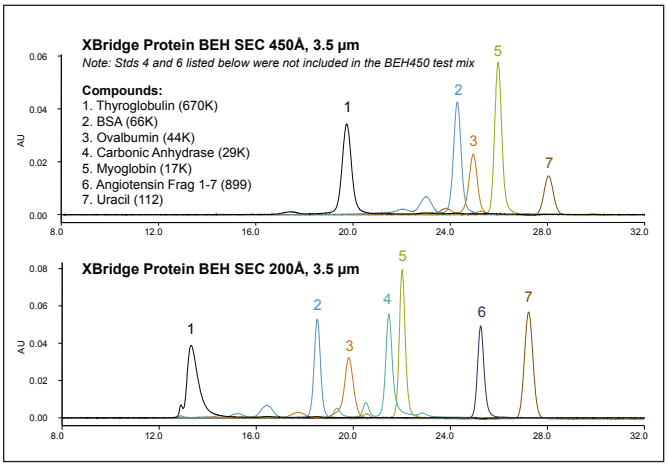


Figure 2. Comparative separations of curves of various proteins, peptides, and uracil on the XBridge Protein BEH SEC 450Å (top) and 200Å (bottom), 3.5 µm particle-size SEC Columns.

Higher Sample Throughput Using XBridge Protein BEH SEC 3.5 µm Columns

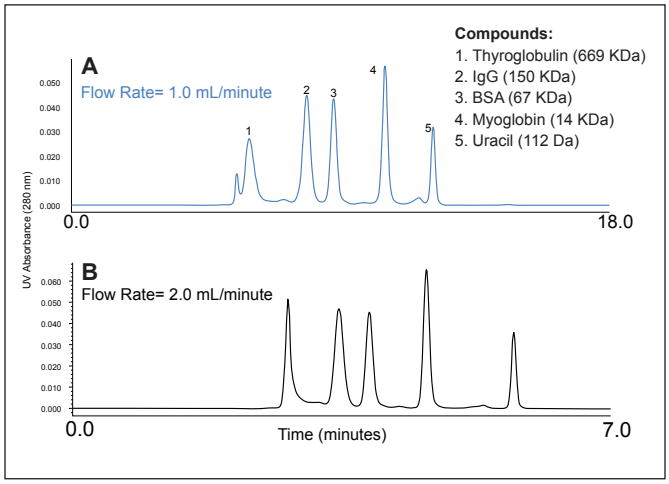


Figure 3. Comparative Separation of Waters BEH200 SEC Protein Standard Mixture (P/N: 186006518) on (A) competitor's 250Å, silica-based, 5 µm SEC column, flow rate 1.0 mL/minute and (B) XBridge Protein BEH SEC, 200Å, 3.5 µm Column, flow rate 2.0 mL/minute with 100 mM Sodium Phosphate Buffer, pH 6.8 mobile phase. Both column dimensions were 7.8 mm x 300 mm length and the same sample loads were injected. The time axis for the main chromatograms have been normalize. Peak identities for chromatograms A and B are: 1) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da).

Note: Comparable molecular weight standard profiles are observed, with the exception that the larger pore-size of the 250Å, 5 µm silica-based particles provide improved resolution of the thyroglobulin dimer peak (1.3 MDa) than what is observed on the 200Å, 3.5 µm BEH-based particle. Use of Waters XBridge Protein BEH SEC, 450Å, 3.5 µm is recommended for the analysis of proteins, such as thyroglobulin and its dimer, whose molecular weights exceed those recommended be analyzed on the XBridge Protein BEH SEC ,200Å, 3.5 µm Column.

OUTSTANDING SEC COLUMN LIFE WITH LESS NON-DESIRED INTERACTIONS USING BEH PARTICLE TECHNOLOGY

BEH Technology™ is well established for chromatography of various biological compounds with stability and performance attributes not found with many traditional, 100% silica-based particles (Anal Chem. 75 6781–6788 2003). The combination of the BEH base particle and innovative diol bonding process results in column stability, performance, and lifetime unheard of in traditional size-exclusion chromatographic columns. Also, compared to traditional silica-based SEC columns, less charged silanols exist on the diol-coated BEH particles contained in Waters XBridge Protein BEH SEC Columns which translates into less non-desirable ionic interactions between the protein and the SEC particle.

Waters XBridge Protein BEH SEC, 200Å, 3.5µm Column Performance Over 600 Injections

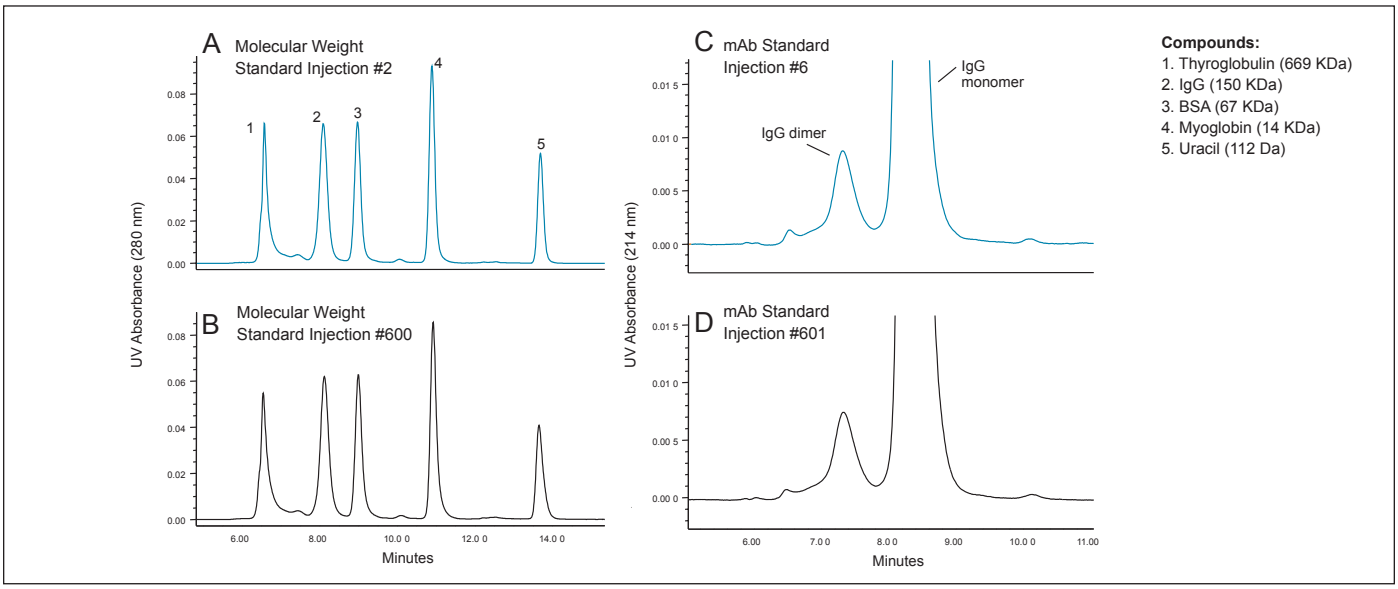


Figure 4. Column life time study using Waters BEH450 SEC Protein Standard (P/N 186006842) and Intact mAb Mass Check Standard (P/N 186006552, diluted to 1 mg/mL) on Waters XBridge Protein BEH SEC, 450Å, 3.5 µm, 7.8 x 300 mm Column. For the chromatograms of the mAb standard (C and D) the molecular weights of the IgG monomer and dimer are approximately 150 KDa and 300 KDa, respectively.

Effect of SEC Eluent Ionic Strength on the Analysis of the Basic Protein Lysozyme on 100% Silica vs. BEH SEC Particles

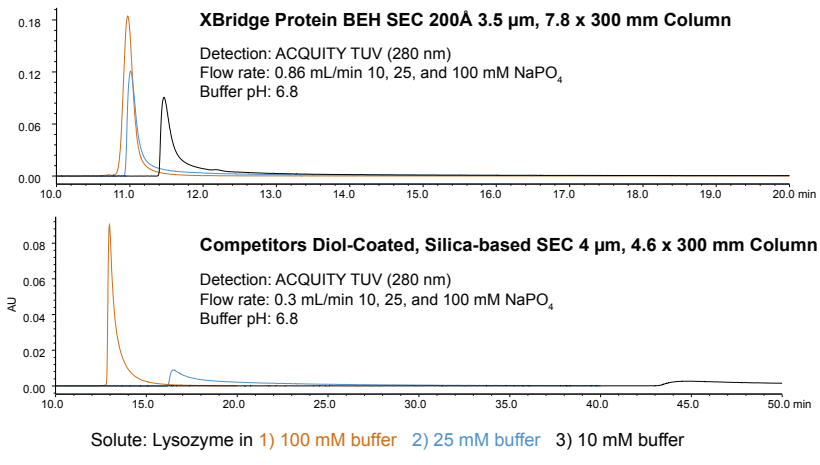


Figure 5. With many SEC columns containing silica-based, diol-bonded particles, undesirable secondary ionic interactions can occur between negatively-charged surfaces on the particle surface and basic proteins that can result in long retention times and excessive peak tailing. Traditionally, the solution frequently involves use of SEC eluents containing high concentrations of salt to minimize these ionic interactions. The unique BEH-diol particle surface on XBridge Protein BEH SEC, 200Å and 450Å Columns significantly reduces these secondary interactions, resulting in the ability to use less aggressive mobile phase salt concentrations.

STRINGENT MANUFACTURING QUALITY DELIVERS CONFIDENCE IN SEC GENERATED DATA

All Waters HPLC- and UPLC-based, BEH SEC particles are synthesized in state-of-the-art, ISO-certified manufacturing facilities from high quality raw materials, and are extensively QC tested throughout the synthetic process. In addition, each manufactured batch of XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm material is tested with relevant proteins to help ensure unmatched batch-to-batch consistency for supreme confidence in validated methods.

Batch-to-Batch and Column-to-Column Reproducibility on XBridge Protein BEH SEC Columns

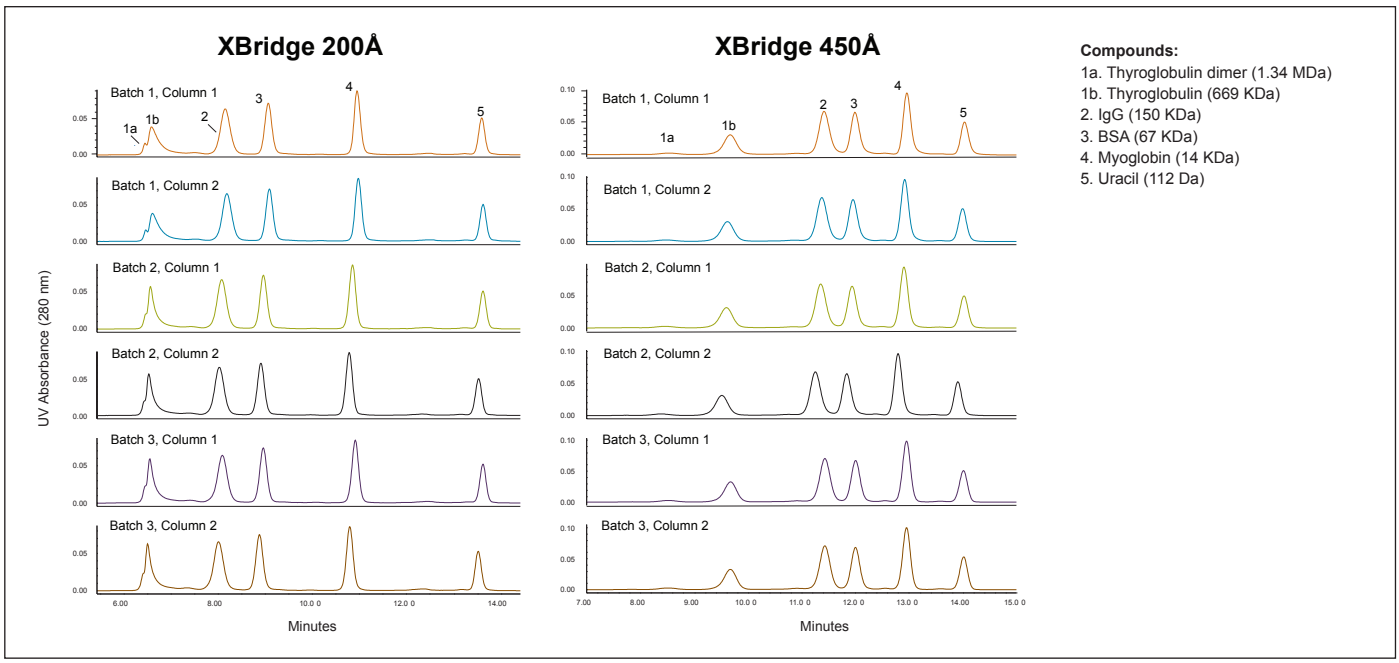
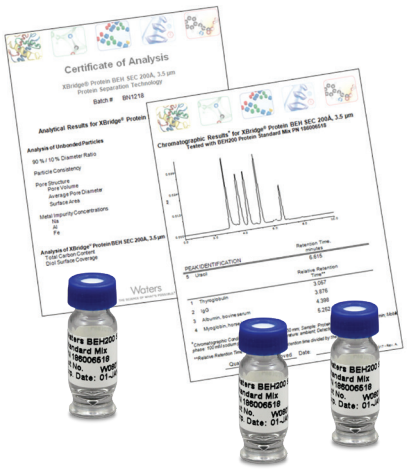


Figure 6. Separations of Waters BEH200 and BEH450 SEC Protein Standard Mixes on XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm, 7.8 mm x 300 mm Columns showing excellent manufacturing and column packing consistency. Two columns were packed from 3 different manufacturing batches of BEH-diol bonded particles to evaluate and confirm industry leading, column-to-column and batch-to-batch reproducibility.



BENCHMARKING, METHOD DEVELOPMENT, AND TROUBLESHOOTING: BEH200 OR BEH450 SEC PROTEIN STANDARDS

Each XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm Column is shipped with a lyophilized vial of the appropriate BEH SEC Protein Standard Mix. The same protein standard formulation is used by Waters manufacturing to ensure SEC Column batch-to-batch consistency. Consequently, chromatographers can now use these same materials to benchmark a new SEC column or troubleshoot issues that might arise in a validated method.

METHOD TRANSFER FOR PROTEIN SEC CHARACTERIZATION

In 2010, Waters introduced ACQUITY UPLC Protein BEH SEC Columns containing 1.7 µm particles designed for optimal performance on Waters low dispersion, UPLC instrumentation. For the first time, these columns delivered outstanding component resolution in less time compared to use of traditional SEC column containing 5–8 µm particles. Waters XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns can now effectively deliver comparable component resolution on HPLC platforms where high sample throughput is not a requirement.

Scalable Chromatography on Waters HPLC- vs. UPLC-based SEC Columns

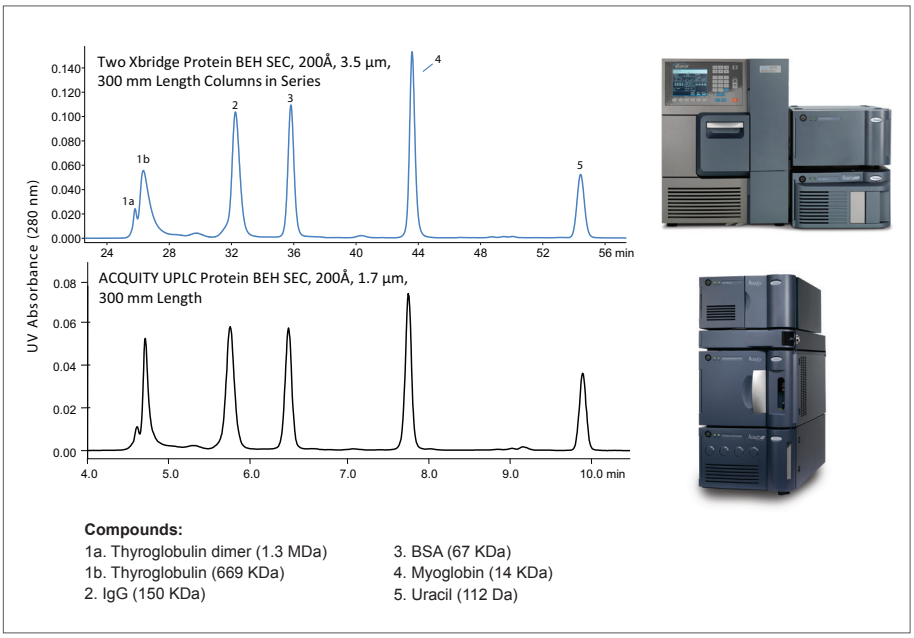


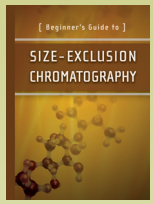
Figure 7. Separation of Waters BEH200 SEC Protein Standard (P/N 186006518) on two XBridge Protein BEH SEC 200Å, 3.5 µm, 7.8 x 300 mm Columns run in series using an Alliance® HPLC (top) and on a single ACQUITY UPLC Protein BEH SEC 200Å, 1.7 µm, 4.6 x 300 mm Column using an ACQUITY H-Class Bio UPLC (bottom). The flow rates were scaled based on particle diameter and column ID to 0.42 mL/minute for the two HPLC columns run in series and 0.3 mL/minute for the UPLC Column. Sample loads were also adjusted for column volume. Peak identities for chromatograms are: 1a) thyroglobulin dimer (1.3 MDa), 1b) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da).

A Review: Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and Their Aggregates

The 23-page, Journal of Liquid Chromatography Publication provides an excellent historical review and recent advancements involving the use of SEC for the analysis of proteins. It discusses various instrumentation considerations as well as method development strategies for the successful use of this important bioanalytical technique.

Literature Code:
720004595EN

Beginner's Guide to Size-Exclusion Chromatography



This 62-page paperback book details the principles and practice of using size-based separations for polymer characterization. It provides readers a straightforward introduction to traditional gel permeation chromatography (GPC) and includes clear and colorful diagrams to acquaint the reader with basic SEC concepts including instrument and detection considerations.

Literature Code:
715004398

ORDERING INFORMATION

Description	Configuration	Particle Size	Dimension	Part No.
XBridge Protein BEH SEC, 200Å Column with BEH200 SEC Protein Standard Mix	Guard Column	3.5 µm	7.8 x 30 mm	176003594
XBridge Protein BEH SEC, 200Å Column with BEH200 SEC Protein Standard Mix	Column	3.5 µm	7.8 x 150 mm	176003595
XBridge Protein BEH SEC, 200Å Column with BEH200 SEC Protein Standard Mix	Column	3.5 µm	7.8 x 300 mm	176003596
XBridge Protein BEH SEC, 450Å Column with BEH450 SEC Protein Standard Mix	Guard Column	3.5 µm	7.8 x 30 mm	176003597
XBridge Protein BEH SEC, 450Å Column with BEH450 SEC Protein Standard Mix	Column	3.5 µm	7.8 x 150 mm	176003598
XBridge Protein BEH SEC, 450Å Column with BEH450 SEC Protein Standard Mix	Column	3.5 µm	7.8 x 300 mm	176003599
Straight Connection Tubing and Fittings for XBridge Protein BEH SEC Column	—	—	—	WAT022681
U-Bend Connection Tubing and Fittings for XBridge Protein BEH SEC Column	—	—	—	WAT084080
BEH 200Å SEC Protein Standard Mix	—	—	—	186006518
BEH 450Å SEC Protein Standard Mix	—	—	—	186006842

ADDITIONAL INFORMATION

Description	Literature Code
Alliance System Brochure	720000370EN
A Review: Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and Their Aggregates	720004595EN
Beginner's Guide to Size-Exclusion Chromatography	715004398

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Impact of LC System Dispersion on the Size-Exclusion Chromatography Analysis of Monoclonal IgG Antibody Aggregates and Fragments: Selecting the Optimal Column for Your Method

Stephan M. Koza, Corey E. Reed, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- An educational and systematic demonstration of the impact of LC system dispersion on SEC-based mAb separations
- Guidance for selecting the optimal SEC column configuration based on the LC systems to be used and the analytical method requirements including resolution, sensitivity, reproducibility, and transferability
- A comparison of the SEC separation performance of the ACQUITY™ UPLC™ H-Class Bio (UPLC) and ACQUITY Arc™ Bio (UHPLC) Systems

WATERS SOLUTIONS

ACQUITY UPLC H-Class Bio System

ACQUITY Arc Bio System

ACQUITY UPLC Protein BEH SEC Columns

XBridge™ Protein BEH SEC Columns

KEYWORDS

Size exclusion, system dispersion, UPLC, UHPLC, HPLC, proteins, IgG, infliximab, trastuzumab, rituximab

INTRODUCTION

Historically, native size-exclusion chromatography (SEC) has been the most widely used methodology for the assessment of non-covalent protein aggregation (high molecular weight species [HMWS]) in recombinant protein based biotherapeutic products.[1] However, in recent years due to the improved capabilities of SEC columns and LC systems, there has also been a greater interest in using SEC for the non-denatured analysis of protein fragments (low molecular weight species [LMWS]) in these samples. Most notably, the analysis of IgG monoclonal antibody (mAb) fragments resulting from the hydrolytic degradation of the hinge region has been targeted.[2] In comparison to the more traditional separation of the dimer and higher molecular weight forms of HMWS (≥300 KDa) from the monomer (~150 KDa), the separation of LMWS fragments, a predominant form of which for a mAb is two-thirds the molecular weight of the mAb monomer (~100 KDa), can be more challenging. This is due to the LMWS and monomer being more similar in size (hydrodynamic radius) versus the size comparison between the monomer and HMWS protein forms. An additional difficulty is presented by the elution order of the proteins in which the low-abundance LMWS peak elutes as a trailing shoulder on the main (monomer) peak.

While the use of higher efficiency SEC columns with particle diameters of 2 µm and smaller has enabled improved efficiencies resulting in higher throughput analyses of HMWS and LMWS forms, due to column hardware and packing constraints, these high efficiency SEC particles have only been made available in columns with internal diameters of 4.6 mm and smaller. Whereas, 7.8 mm internal diameter (I.D.) columns are typically employed for 3 µm and larger SEC particle sizes when using an HPLC chromatographic system. While many HPLC systems are physically capable of operating at the flow rates and back pressures required for many of these smaller particle size 4.6 mm I.D. SEC columns, what is frequently not considered is the fact that extra-column dispersion in typical HPLC configurations is sufficiently large relative to the peak volumes generated by the sub-2-µm columns. The result of this is that the peak resolutions observed are significantly reduced.[3] Extra-column dispersion can be considered as the increase in the volume of an injected sample that occurs as it travel through the flow path of an LC system without a column in place.

The goal of this study was to systematically evaluate the impact that the SEC particle size, column length, and internal diameter, and LC system extra-column dispersion has on the SEC resolution of HMWS and LMWS impurities of a purified mAb noting that the basic illustrated principles are applicable to SEC of other protein classes. Additionally, the deleterious effect that system dispersion has on the lower limits of detection and the reliability of quantitative results for LMWS will also be demonstrated. In summary, recommendations for SEC column selections that are compatible with Waters LC systems and for the analysis of mAbs are provided.

EXPERIMENTAL

Sample description

The mAb samples of infliximab (Remicade®) and rituximab (Rituxan®) were used past expiry at the original concentration of ~21 mg/mL. Trastuzumab (Herceptin®) was used past expiry at a diluted (in water) concentration of 2.0 mg/mL.

Method conditions (unless noted otherwise)

LC conditions

Systems: ACQUITY UPLC H-Class Bio, unless otherwise noted

Detection: ACQUITY UPLC TUV Detector with 5 mm titanium flow cell

Wavelength: 280 nm, unless otherwise noted

Columns: ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 2.1 × 150 mm (p/n: 186008471)

ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 150 mm (p/n: 186005225)

ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 300 mm (p/n: 186005226)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 300 mm (p/n: 186009164)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 150 mm (p/n: 186009163)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 150 mm (p/n: 176004335)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 300 mm (p/n: 176004336)

XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 × 150 mm (p/n: 176003595)

XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 × 300 mm (p/n: 176003593)

BEH SEC, 200 Å, 3.5 µm, 4.5 × 300 mm (custom packed)

Column temp.: Ambient, ~22 °C

Sample temp.: 10 °C

Flow rates and injection volumes, unless otherwise noted:

Column dimension (mm I.D. × mm L)	Flow rate (mL/min)	Injection volume (µL)
4.6 × 150	0.350	1.0
4.6 × 300	0.350	2.0
7.8 × 150	1.000	5.8
7.8 × 300	1.000	10.0

Mobile phase A: 100 mM NaH_2PO_4

Mobile phase B: 100 mm Na_2PO_4

Mobile phase C: 1.00 M NaCl

Mobile phase D: H_2O

All 0.2 µm sterile filtered and Auto▪Blend Plus blended at 7.4% A, 12.6 % B, 35% C, and 45% D to yield 20 mm sodium phosphate, 350 mM NaCl, pH 6.8, unless otherwise noted

Sample vials: Polypropylene 12 × 32 mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 300 µL volume (p/n: 186002640)

Data management

Chromatography software: Empower™ 3

RESULTS AND DISCUSSION

MEASURING SYSTEM DISPERSION

One chromatography fundamental is that extra-column dispersion, or broadening of a chromatographic peak or band, that does not occur within the packed chromatographic bed of the column always has a deleterious effect on the resolution of a separation. In many protein and peptide gradient separations, such as reversed phase and ion exchange, the analyte binds strongly to the stationary phase under loading conditions and will re-concentrate at the head of the packed bed of the column until the gradient begins. As a result, for these gradient-based separations, the deleterious effect of pre-column band dispersion will be minimized or even eliminated to the point that the principal concern will be dispersion that occurs to the peaks once they are eluted from the column. Conversely, in ideal SEC separations, there is no binding that occurs between the protein sample and the packed SEC particle surface. The practical result of this is that for SEC separations pre-column dispersion will equally degrade the quality of a separation as compared to post-column dispersion. Consequently, evaluating the changes in an injected sample volume and profile that occur after it travels through an LC system without a column in place can be very instructive.

The measurement of extra-column dispersion (system band broadening) has been studied extensively and for the interested reader a far more thorough discussion of system dispersion and its measurement can be found in the companion publication to this application note (720006337EN). For this discussion the $5\sigma_{ec}$ dispersion volumes (based on peak width at 4.4% peak height) were determined as diagrammed in Figure 1. As can be seen in the dispersion profiles shown in Figure 2, the extra-column dispersion peaks are asymmetrical with a noticeable tailing or skewed profile, and by making a peak width measurement closer to the baseline more of the influence of peak tailing can potentially be accounted for. Therefore, using peak width at 4.4% peak height to determine 5σPeak values, while somewhat arbitrary, was selected due to it being the peak width at the lowest percentage of a peak height that is provided by most common chromatography data systems such as Waters Empower 3 and Agilent ChemStation. The varied extra-column dispersions shown in Figure 2 were created on an ACQUITY UPLC H-Class Bio System by adding sample loops at the inlet side of the column.

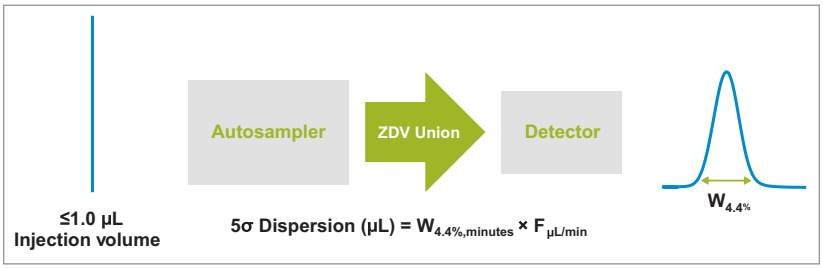

Figure 1. Measurements of extra-column dispersion were carried out using 3:7 water:acetonitrile as a mobile phase at a 0.3mL/min flow rate. The sample was 1 µL of 0.16 mg/mL caffeine in 1:9 water:acetonitrile. The UV absorbance was monitored at 273 nm at a sampling rate of 40 Hz.

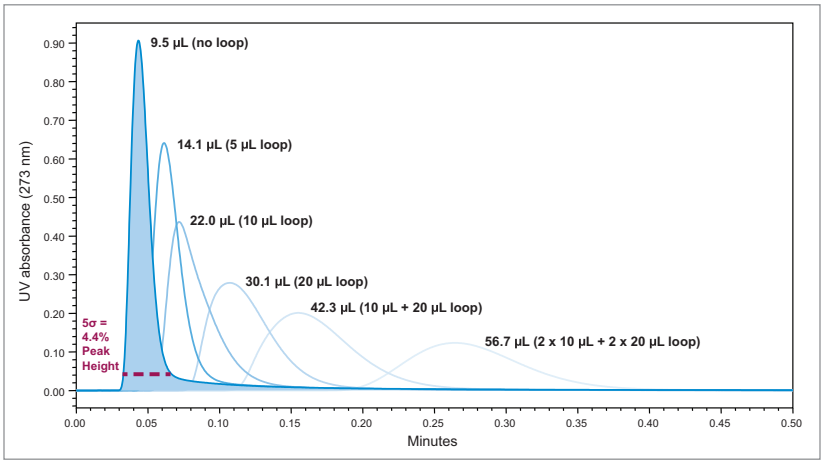

Figure 2. Shown are the measurements of 5-sigma extra-column dispersion volumes ($5\sigma_{ec}$) for this study based on peak width at 4.4% peak height. Experiments were carried out as described in the caption of Figure 1. Sample loops were connected pre-column to generate the larger dispersion volumes.

IMPACT OF SYSTEM DISPERSION ON SEPARATION AND QUANTIFICATION OF BIOTHERAPEUTIC IGG HMWS AND LMWS

Shown in Figure 3 is a well resolved SEC chromatogram of infliximab, a biotherapeutic chimeric human-mouse IgG monoclonal antibody. This sample was analyzed well past expiry and the HMWS, presumed to be predominantly dimer (~300 KDa), is present at a level of approximately 0.75% in the samples tested. The LMWS1 fragment is the result of one of the FAb domains of the antibody being hydrolytically cleaved from the protein at a site within the hinge region of the antibody and has a molecular weight of ~100 KDa while the LMWS2 fragment is a mixture of FAb and Fc domains (both ~50 KDa).[4] The free Fc domain being the result of both FAb arms being cleaved. Of these product related impurities, the most challenging to resolve is LMWS1 due to this form not being as proportionally different in size from the monomer fraction as the HMWS is from the IgG monomer, and by its elution position within the tailing segment of the far more abundant IgG monomer. A low abundance species that trails the main peak in a separation becomes more problematic to resolve and quantify since any increase in the tailing of the main peak will have a significant impact on the resolution and integrated area of that low abundance peak. To address the extent of this impact we evaluated several mixtures of the mAb that contained levels of LMWS1 ranging from approximately 0.4 to 4.1% peak area. These samples were evaluated on several different SEC columns using an LC system where the $5\sigma_{ec}$ volumes were purposely modified to be between 9.5 µL and 56.7 µL.

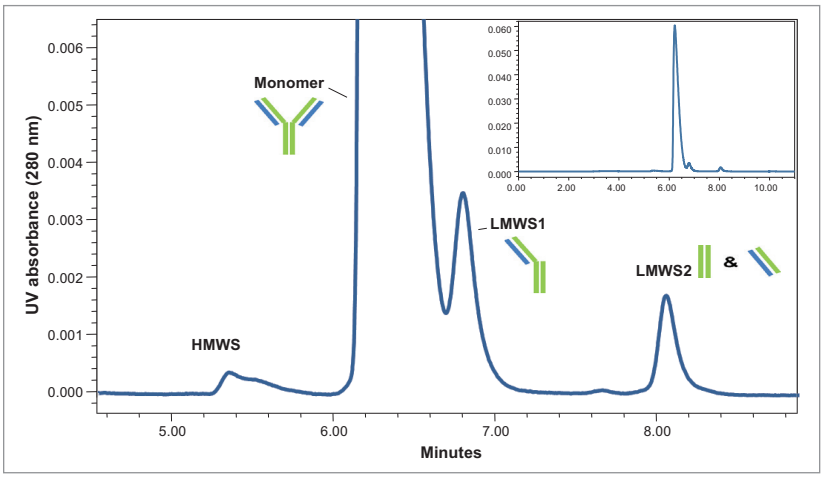

Figure 3. Sample chromatogram demonstrating the optimal separation obtained for a degraded infliximab sample using a 1.7 µm particle size SEC column with an internal diameter of 4.6 mm and length of 300 mm. The 5-sigma extra-column dispersion (σ_{ec}) of the LC system was 9.5 µL. Peak identifications are: high molecular weight (HMWS, ~300 KDa), mAB monomer (~150 KDa), 2/3 mAb fragment comprised of one Fab and one Fc domain (LMWS1, ~100 KDa), and co-eluting Fc and Fab domains (LMWS2, ~50 KDa). The measured peak area percent for HMWS in this sample is 0.75%, that of the LMWS1 fragment is a 4.1%, and that of the LMWS2 fragment is 1.7%.

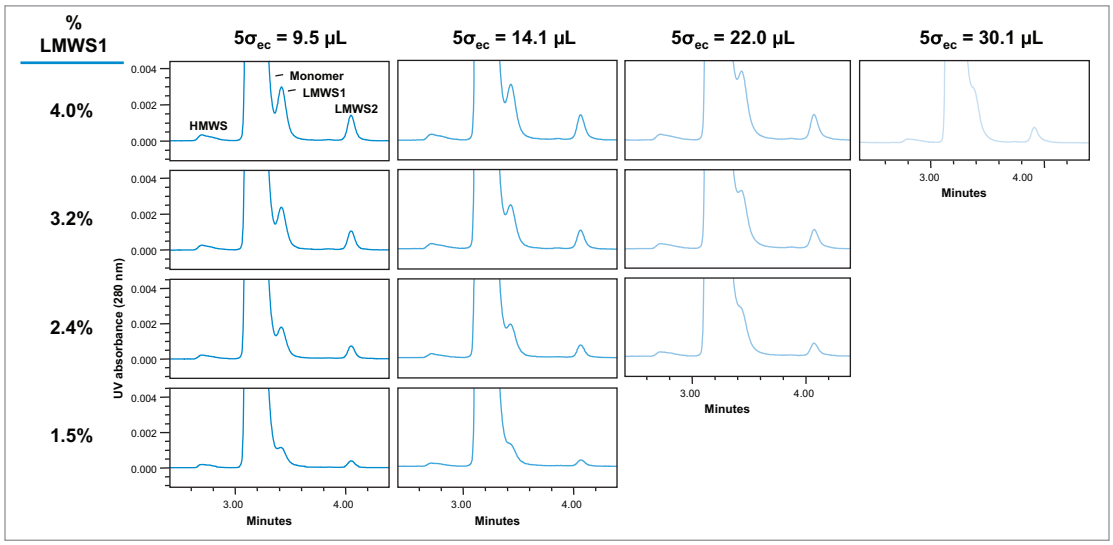

Figure 4. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σec) on the separation of LMW1 mAb fragment with 1.7 µm particle size SEC column with an internal diameter of 4.6 mm and length of 150 mm. The predicted peak area percents for the LMWS1 fragment in each of the samples evaluated at the σec conditions tested are shown in the left column and range from 4.1% to 1.5%. Peak identifications are provided in Figure 8.

Examples of the chromatographic results for the infliximab samples evaluated on the 4.6 mm I.D. (1.7 µm SEC particles), 150 mm and 300 mm length columns are shown in Figures 4 and 5, and those for the 7.8 mm I.D. (3.5 µm SEC particles), 300 mm length column are shown in Figure 6. The chromatograms for the 7.8 mm I.D. (3.5 µm), 300 mm column are not shown as the LMWS1 fragment was not resolved on this column. A summary of the quantitative results for HMWS determination using all four columns are presented in Figure 7. We observe that the resolutions between the HMWS and the mAb monomer, and the integrated HMWS percent peak areas for both 7.8 mm I.D. (3.5 µm) columns and for the 1.7 µm, 4.6 × 300 mm column is minimally impacted by increases in extra-column dispersion. This is due in part to the high degree of separation that is achieved between the two peaks (R_s>1.6), the large peak volumes generated by these three columns, and the order of elution such that peak tailing of the significantly more abundant monomer has less impact on resolution. Additionally, upon closer inspection of the chromatographic profiles, we observe that the HMWS peak is somewhat polydisperse and as a result, the resolution values will appear to be more consistent as they will be strongly dictated by the size distribution of self-associated forms eluting in the HMWS peak.

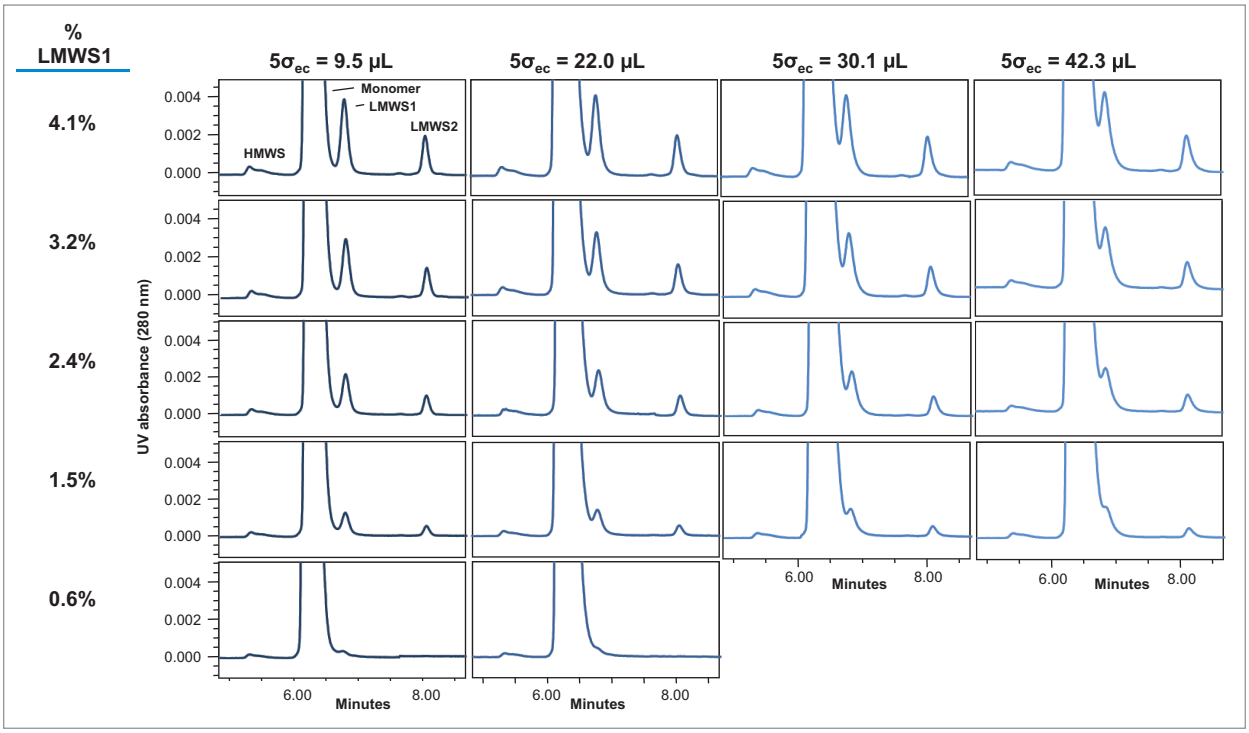

Figure 5. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σec) on the separation of LMWS1 mAb fragment with 1.7 µm particle size SEC column with an internal diameter of 4.6 mm and length of 300 mm. The predicted peak area percents for the LMWS1 fragment in each of the samples evaluated at the σec conditions tested are shown in the left column and range from 4.1% to 0.6%. Peak identifications are provided in Figure 8.

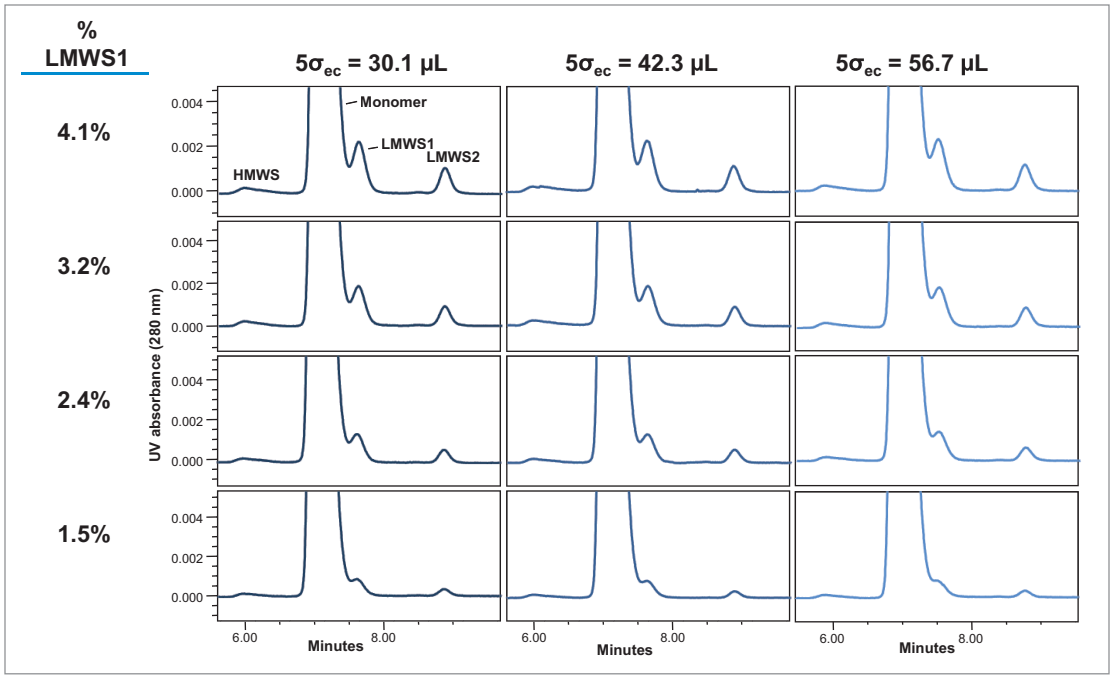

Figure 6. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σec) on the separation of LMWS1 mAb fragment with 3.5 µm particle size SEC column having an internal diameter of 7.8 mm and length of 300 mm. The predicted peak area percents for the LMWS1 fragment in each of the samples evaluated at the σec conditions tested are shown in the left column and range from 4.1% to 1.5%. Peak identifications are provided in Figure 8.

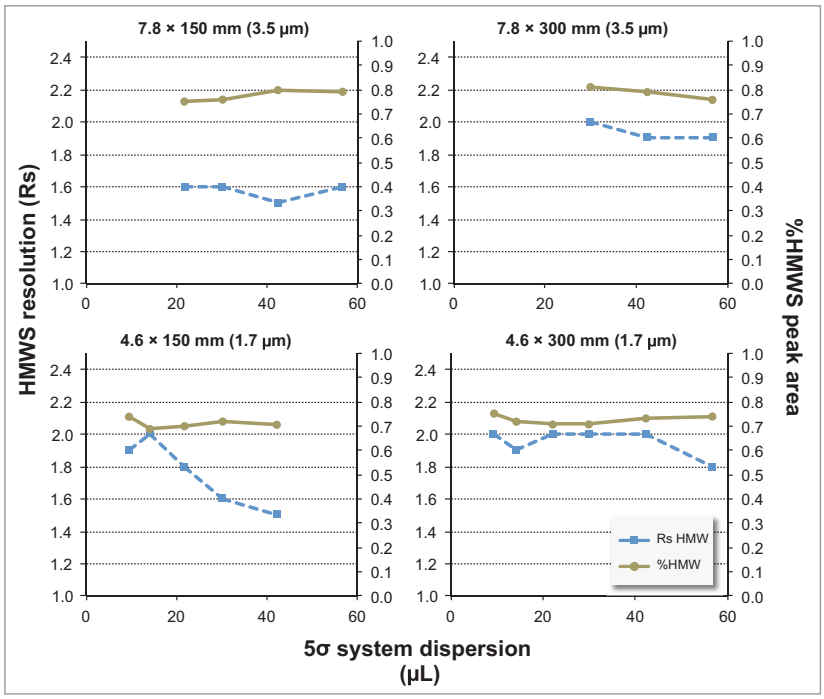

Figure 7. Shown are quantitative comparisons for the HMWS peak from the chromatograms shown in Figures 10 through 13. In addition, the results from the evaluation of HMW for the 3.5 µm particle size SEC column with an internal diameter of 4.6 mm and length of 150 mm are included. The USP resolution between the monomer and HMWS peak (dashed blue line with square markers) and the HMWS integrated percent peak areas (brown lines with round markers) are plotted against the system dispersions tested as described in the text. Sample injection volumes and flow rates were proportional to column internal diameter.

Despite this factor, we still observe a significant decrease in resolution for the 1.7 µm (4.6 × 150 mm) column as system dispersion increases. This is a result of the smaller peak volumes generated by this column (Figure 8). However, the integrated HMWS percent peak area is still consistent for this column. We see a similar trend when comparing the 300 mm columns although the 1.7 µm (4.6 × 300 mm) column is not outperformed until we operate at a $5\sigma_{ec}$ of greater than 40 µL. When we consider the IgG peak volumes based on 5σ peak widths generated by these columns (Figure 8), we can see why the longer length, larger internal diameter, and larger particle size columns are not as greatly impacted by extra-column dispersion. For example, in comparing the peak volumes generated by the 3.5 µm, 7.8 mm I.D. columns to those of the 4.6 mm I.D. (1.7 µm) columns, nearly a 4-fold increase in peak volume is observed. We also observe an approximate 50% increase in peak volume as column lengths are increased from 150 mm to 300 mm. These results indicate that for the SEC analysis of HMWS, system dispersion volumes will likely need to be minimized and controlled to derive a significant resolution benefit from 4.6 mm I.D. columns packed with 1.7 µm particles. In this example, $5\sigma_{ec}$ dispersion volumes should be lower than 15 µL for the 150 mm column, and below 40 µL for the 300 mm column.

We will next examine the effect that extra-column dispersion has on the separation of LMWS and mAb monomer. Based on the chromatographic results presented in Figures 4 through 6, a series of graphs are presented (Figure 9) showing the effect of extra-column dispersion on the peak-to-valley ratio between the monomer and the LMWS1 peaks (left axes) and percent areas of the LMWS1 peaks (right axes). The results for the 3.5 µm (7.8 × 150 mm) column have been omitted as the LMWS1 fragment was not resolved on that column.

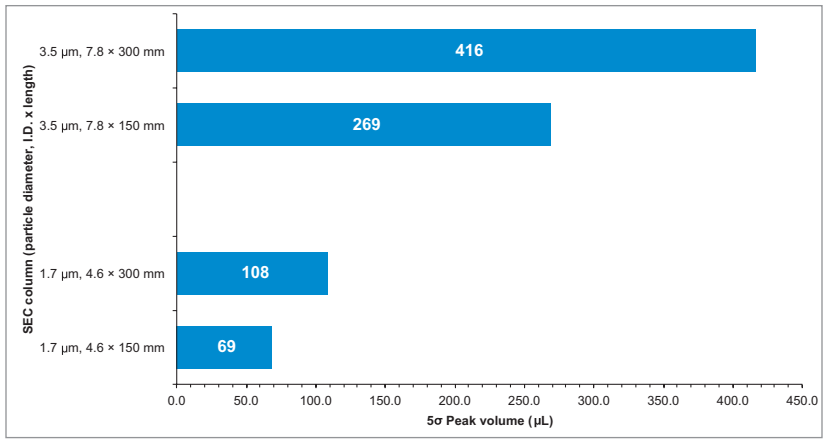

Figure 8. Shown are the estimated $5\sigma_{ec}$ peak volumes (based on peak width at 4.4% height) for an IgG peaks in the Waters BEH200 SEC Protein Standard Mix. Chromatograms are not shown. Peak volumes were corrected for 5-sigma system dispersion volumes.

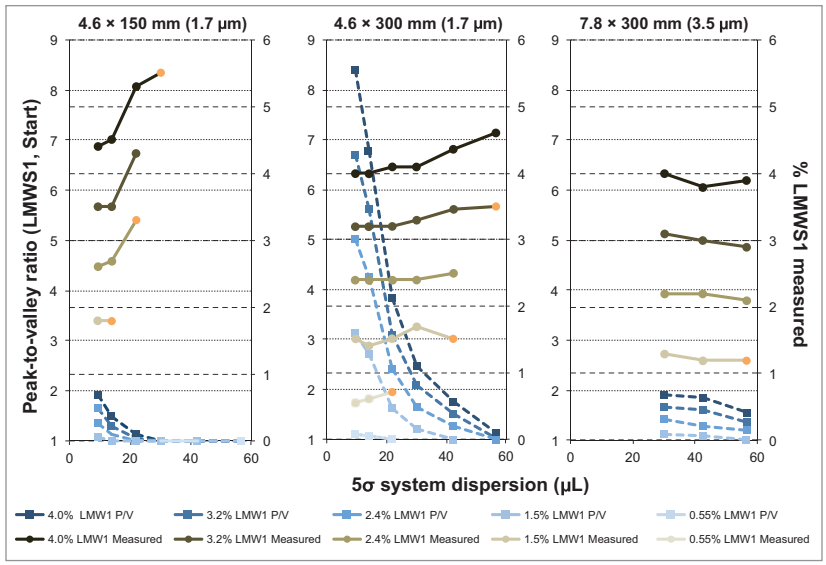

Figure 9. Quantitative comparisons for the determination of LMWS1 from the chromatograms shown in Figures 4 through 6. The peak-to-valley ratio for the LMWS1 mAB fragment (blue dashed lines with square markers) and the LMWS1 integrated percent peak areas (brown solid lines with round markers) are plotted against the system dispersions tested as described in the text. Orange circles indicate measured % LMWS1 data points where the P/V ratio for the LMWS1 peak was ≤1.01. Sample injection volumes and flow rates were proportional to column internal diameter.

In comparing the resolution (P/V) results between the two 1.7 µm, 4.6 mm I.D. columns, we can immediately see that the use of the 300 mm length column provides significantly greater resolution of the mAb monomer and LMWS1 peaks. This is primarily the result of approximately doubling the plate count or efficiency of the separation, however, as observed in Figure 8 the increased peak volume produced by the longer column also reduces the impact of extra-column volume. This greater resolution also provides for a lower limit of detection, which in this case will be arbitrarily defined as a P/V ratio greater than 1.01. Additionally, the 300 mm column can resolve the LMWS1 peak at larger $5\sigma_{ec}$ levels. When we compare the quantitative results (% LMWS1), we can see that throughout the range of $5\sigma_{ec}$ levels tested that the percent of the LMWS1 fragment is never consistent with respect to $5\sigma_{ec}$ for the 150 mm length column, while for the 300 mm length column the measured percent LMWS1 is more consistent although it does begin to deviate more significantly as $5\sigma_{ec}$ levels exceed 30 µL. It should be noted that the quantitative results for the more well resolved LMWS2 peak were consistent for these three column configurations and for the 3.5 µm (7.8 × 150 mm) column throughout the range of $5\sigma_{ec}$ volumes evaluated.

In comparing the performance of the 3.5 µm (7.8 × 300 mm) column to that of the 1.7 µm (4.6 × 300 mm) column, we observe that the P/V ratio was on average only 7 to 20% lower at $5\sigma_{ec}$ levels of 30 µL and that at $5\sigma_{ec}$ levels of 42 µL and greater the P/V ratios for the 3.5 µm column were reproducibly higher. However, unlike the 1.7 µm column, this larger column format produced more consistent quantitative results for LMWS1 as $5\sigma_{ec}$ increased above 22 µL. To summarize, these results for the SEC analysis of the LMWS1, the use of a 1.7 µm (4.6 × 150 mm) column is not recommended for a validated method due to the significant variation in quantification observed with changes in system dispersion. The 1.7 µm (4.6 × 300 mm) column produced the highest resolutions and reliable LMWS1 quantification when $5\sigma_{ec}$ levels of approximately 25 µL and lower are maintained. The 3.5 µm (7.8 × 300 mm) column produced significantly lower resolutions than what could be achieved using the 1.7 µm (4.6 × 300 mm) column on a low dispersion LC, however, the quantitative results were consistent with respect to system dispersion. In addition, for these mAb separations at $5\sigma_{ec}$ levels greater than 40 µL, the 3.5 µm (7.8 × 300 mm) column provided the highest resolution.

If more sensitive LMWS1 analysis is required, and when using an LC system that cannot take advantage of a 1.7 µm (4.6 × 300 mm) column, a reasonable option will be to operate two 3.5 µm (7.8 × 300 mm) columns in series resulting in a total column length of 600 mm. Although this will increase analysis time, the resultant method will be more reliable and easily transferrable. The use of multiple SEC columns in series is demonstrated by a comparison of the separations obtained for LMWS1 of trastuzumab on the 1.7 µm (4.6 × 300 mm) column, the 3.5 µm (7.8 × 300 mm) column, and on two 3.5 µm (7.8 × 300 mm) columns run in series (Figure 10). We did not rigorously transfer our method between these columns but instead elected to use the commonly used flow rates of 0.4 mL/min for the 1.7 µm (4.6 × 300 mm) column, and 1.0 mL/min for the 3.5 µm (7.8 × 300 mm) column. The 1.7 µm (4.6 × 300 mm) column was evaluated on both an ACQUITY UPLC H-Class Bio System ($5\sigma_{ec}$ = 20 µL) and on an ACQUITY Arc Bio System ($5\sigma_{ec}$ = 34 µL). We can see from these comparisons that the LMWS1 and the mAb monomer, which is estimated to be present at a ~0.3% relative abundance, are effectively separated on the 1.7 µm (4.6 × 300 mm) column when using the ACQUITY UPLC H-Class Bio System (P/V=1.32), whereas the higher dispersion of the ACQUITY Arc Bio System compromises the separation (P/V = 1.03) to a level that would likely result in unreliable quantification of LMWS1. The HMWS and LMWS2 are both adequately separated on both LC systems. When the method was run using a single 3.5 µm (7.8 × 300 mm) column run at a flow rate of 1.0 mL/min with the ACQUITY Arc Bio System, lower component resolution was observed in comparison to a 1.7 µm (4.6 × 300 mm) column run on the ACQUITY Arc Bio System, as would be predicted. However, when using two 3.5 µm (7.8 × 300 mm) columns in series at 1 mL/min the resolution (P/V = 2.1) was significantly improved over that observed for the 1.7 µm (4.6 × 300 mm) column configured to the ACQUITY UPLC H-Class Bio System (P/V = 1.32). This is because even with a $5\sigma_{ec}$ value of 20 µL, the ACQUITY UPLC H-Class Bio System degrades the resolution (P/V) of the separation between the monomer and LMWS1 from the theoretical maximum. We can observe this clearly based on the P/V ratios for the LMWS1 of infliximab on this column (See Figure 5). While this tandem column method requires an approximately two-fold increase in analysis time in addition to using more sample and mobile phase, it provides a reliable and sensitive method. Given the immense peak volumes generated (~550 µL), the separation efficiencies obtained are not predicted to be significantly impacted by $5\sigma_{ec}$ dispersions of even greater than 60 µL.

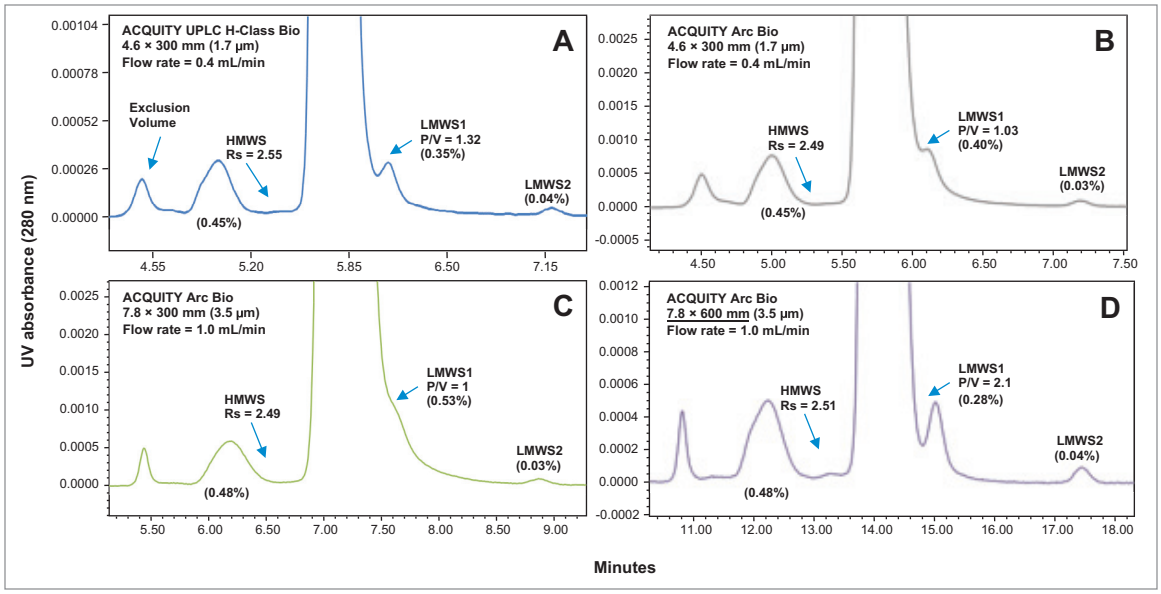

Figure 10. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σec) of an ACQUITY UPLC H-Class Bio System (30 cm column heater, $5\sigma_{ec}$ = 20 µL) and an ACQUITY Arc Bio System ($5\sigma_{ec}$ = 34 µL) on the SEC separation of HMWS, and the mAb fragments LMWS1 and LMWS2 for trastuzumab. The top two chromatograms (A and B) compare the results obtained using a 4.6 × 300 mm (1.7 µm) column on the ACQUITY UPLC H-Class Bio System (A) and the ACQUITY Arc Bio (B) at a flow rate of 0.4 mL/min. The bottom left chromatogram (C) was generated using a 7.8 × 300 mm (3.5 µm) column, while the bottom right chromatogram (D) was produced using two 7.8 × 300 mm (3.5 µm) columns in series, both at a flow rate of 1.0 mL/min. The mobile phase was 25 mM sodium phosphate, 400 mM NaCl, pH 7.2. Injection volumes were 5 µL (A and B), 15 µL (C) and 21 µL (D). The UV flow cell pathlength was 5 mm for the ACQUITY UPLC H-Class Bio and 10 mm for the ACQUITY Arc Bio.

2.5 µm BEH SEC COLUMNS FOR IGG HMWS AND LMWS ANALYSIS

Following the publication of the original version of this application note, Waters produced an intermediate 2.5 µm particle size series of BEH SEC columns. Based in part on some of the studies presented in this study, the goal of this product was to provide higher resolution separations versus the 3.5 µm particle size and resolutions approaching those that can be observed on 4.6 mm ID, 1.7 µm particle size, columns while using LC systems with 20 µL or larger $5\sigma_{ec}$ dispersion volumes. The data presented in this section represent an entirely different set of experiments as the samples used in the previous sections of this application note were no longer available. For additional information on the comparative performance of the 2.5 µm particle size BEH SEC columns, reference "High Resolution and High Throughput Size-Exclusion Chromatography Separations of IgG Antibody Aggregates and Fragments on UHPLC and HPLC Systems with 2.5 µm BEH Particles" (Waters Application Note, p/n: 720006522EN).

A comparison of the chromatograms produced using three 200 Å BEH SEC particles for the separation of rituximab, a chimeric (mouse/human) anti-CD20 IgG1 antibody, is shown in Figure 11. Only the 300 mm column lengths are compared in this study as it was already shown that the use of 1.7 µm (4.6 × 150 mm) columns significantly decreases the resolution and limit of quantification for the LMWS1 fragment. In Figure 11, the impact of system dispersion was evaluated on 1.7 µm (4.6 × 300 mm), 2.5 µm (4.6 × 300 mm), and 2.5 µm (7.8 × 300 mm) columns. The 3.5 µm (7.8 × 300 mm) column was only evaluated at a $5\sigma_{ec}$ system dispersion volume of 38.8 µL.

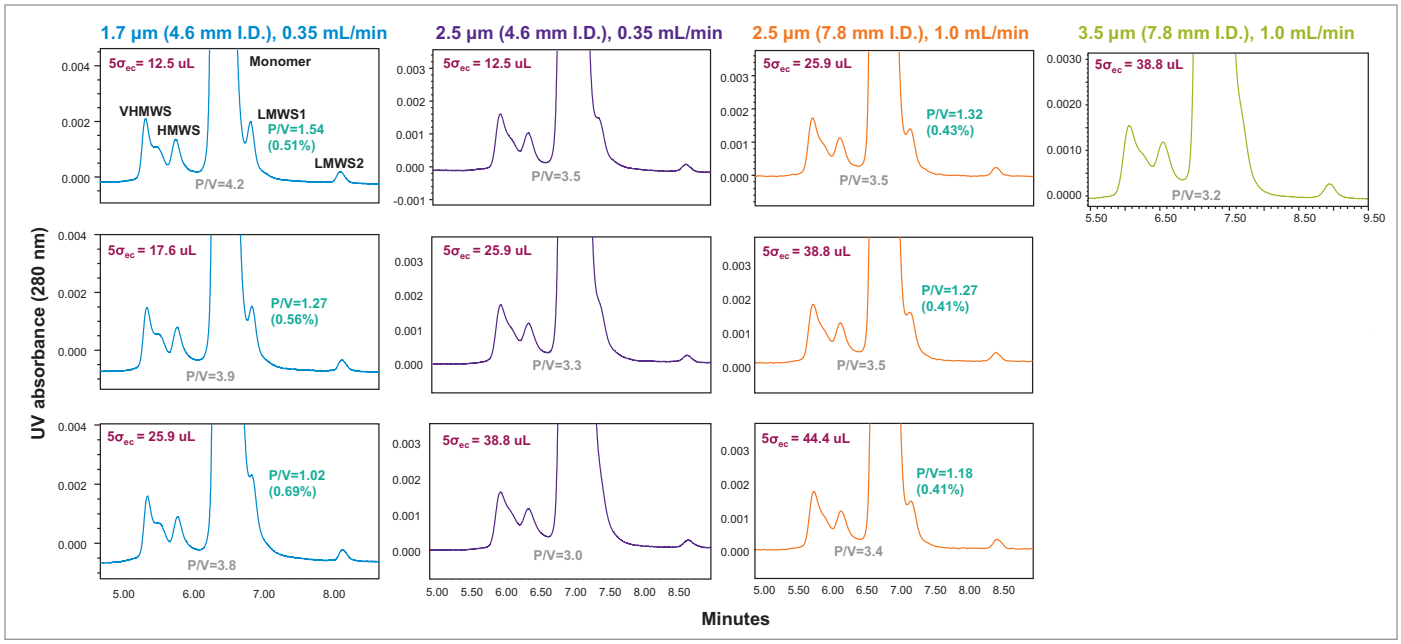

Figure 11. A comparison of the separation of rituximab on 200 Å pore size BEH SEC particles with diameters of 1.7 µm, 2.5 µm, and 3.5 µm. All columns were 300 mm in length, and sample loads and flow rates were proportional to the square of the column I.D. For the 1.7 µm and 2.5 µm (4.6 mm I.D.) columns, the flow rate was held constant, and system dispersion was increased. For the 2.5 µm and 3.5 µm (7.8 mm I.D.) columns, the system dispersion was constant, and flow rates were decreased. Mobile phase was 20 mm sodium phosphate, 400 mM NaCl, pH 7.2. Percent peak areas were determined by drop-baseline peak integration.

The first consideration is the critical pair separation between the HMWS and the monomer. HMWS is presumed to be predominantly a dimer (~300 KDa) that is present at a level of approximately 0.5% in the sample tested. By comparing the top row of chromatograms, it is observed that the HMWS-monomer separation improves (higher P/V values) as particle size is decreased when the columns are operated at equal linear velocities and on systems with appropriate dispersion volumes. Throughout the range of $5\sigma_{ec}$, system dispersion volumes show that the 1.7 µm particle size column provided better resolution than the 7.8 mm I.D. (2.5 µm or 3.5 µm) columns, although this advantage diminishes as system dispersion is increased. It also shows that for the 2.5 µm particles, the performance of the 4.6 mm I.D. column matches that of the 7.8 mm I.D. column at only the lowest dispersion volume tested ($5\sigma_{ec}$ = 12.5 µL); and, increases in system dispersion do not significantly alter the HMWS P/V values for the 7.8 mm I.D. (2.5 µm) column. Similar comparisons can be made for HMWS separations using 150 mm length columns. Although, as we would predict, the impact of increasing system dispersion is proportionally greater.

The other consideration is the separation of the IgG LMWS1 fragment on these columns when operated at equivalent linear velocities. As noted previously, this separation is further complicated by the elution position of the LMWS peak within the tailing segment of the far more abundant monomer and by the low abundance (~0.4%) of LMWS1 in the sample. The effect that system dispersion has on P/V ratio and percent peak area for 1.7 µm (4.6 × 300 mm) and 2.5 µm (7.8 × 300 mm) columns are also presented in Figure 12. For the 1.7 µm (4.6 × 300 mm) column, we observe a precipitous decrease in P/V as $5\sigma_{ec}$ system dispersion volume is increased from 12.5 µL to 25.9 µL. This loss in resolution also resulted in an increase in the integrated relative peak area of LMWS1 from 0.5% up to 0.7%, which is consistent with previous results (Figure 9).

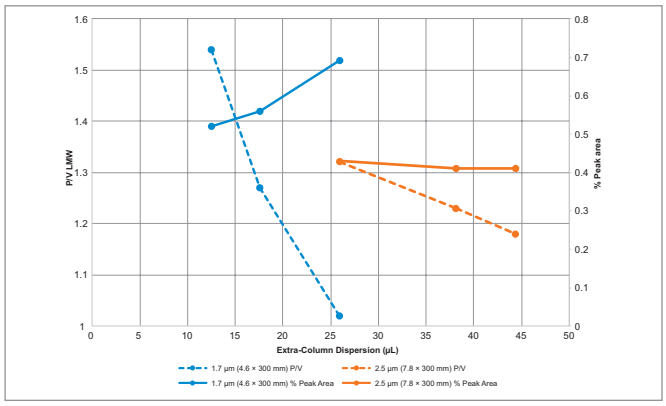

Figure 12. Shown are quantitative comparisons for the determination of LMWS1 from the chromatograms shown in Figure 11 for the 1.7 µm (4.6 × 300 mm) and 2.5 µm (7.8 × 300 mm) columns run at equivalent analysis times. The peak-to-valley ratio for the LMWS1 mAb fragment (dashed lines) and the LMWS1 integrated percent peak areas (solid lines) are plotted against the system dispersions tested (as described in the text). Sample injection volumes and flow rates were proportional to column internal diameter.

In comparison, the 2.5 µm (7.8 × 300 mm) column, while providing the same sample throughput, generated a comparable or greatly improved separation to that observed for the 1.7 µm column at a $5\sigma_{ec}$ system dispersion volume of 17.6 µL to 25.9 µL, performance that is more typical of UHPLC and some UPLC system configurations capable of using 30 cm columns. As further evidence of the impact that extra-column dispersion can have when using 4.6 mm I.D. SEC columns, it is also observed that there is a significant loss of LMWS1 resolution for the 4.6 mm I.D. (2.5 µm) column versus the 7.8 mm I.D. column. In addition, there is a modest decrease in LMWS1 resolution for the 2.5 µm (7.8 × 300 mm) column as $5\sigma_{ec}$ system dispersion volume is increased from 25.9 µL to 44.4 µL, however, this loss of resolution does not have a significant impact on the percent peak areas of LMWS1. As predicted, there is a significant increase in LMWS1 resolution for the 2.5 µm (7.8 × 300 mm) column versus the 3.5 µm (7.8 × 300 mm) column.

To match the LMWS1 resolution obtained on the 1.7 µm (4.6 × 300 mm) column ($5\sigma_{ec}$ = 12.5 µL) with the 2.5 µm and 3.5 µm particle size columns we need to either increase column length, as previously shown for the 3.5 µm (7.8 × 300 mm) column (Figure 10); or, decrease flow rate. As shown in Figure 13, the P/V achieved at a 0.75 mL/min flow rate when using the 2.5 µm (7.8 × 300 mm) column and 0.25 mL/min on the 3.5 µm (7.8 × 300 mm) column is comparable to that observed for the 1.7 µm (4.6 × 300 mm) column. This corresponds to a 33% longer analysis time for the 2.5 µm (7.8 × 300 mm) column and a 4-fold increase in analysis time for the 3.5 µm (7.8 × 300 mm) column.

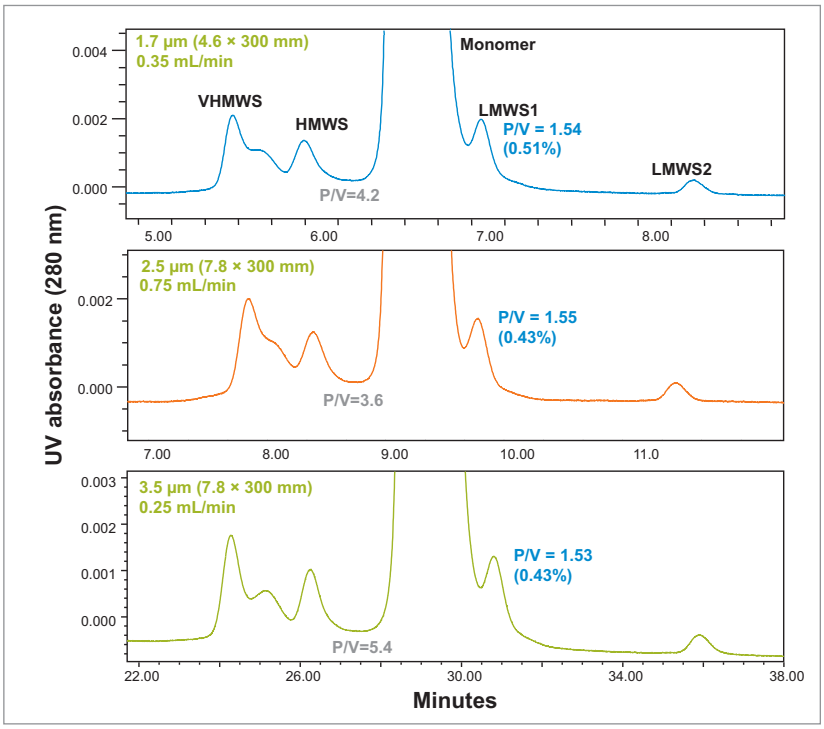

Figure 13. A comparison of the separation of rituximab on 200 Å pore size BEH SEC particles with diameters of 1.7 µm, 2.5 µm, and 3.5 µm. All columns were 300 mm in length and sample loads were proportional to the square of the column I.D. For the 2.5 µm and 3.5 µm columns, the flow rate was reduced to yield comparable resolution of the LMSW1 peak. System dispersion ($5\sigma_{ec}$) was 12.5 µL for the 1.7 µm (4.6 × 300 mm) column, and 38.8 µL for the 2.5 µm (7.8 × 300 mm) and 3.5 µm (7.8 × 300 mm) columns. Mobile phase was 20 mm sodium phosphate, 400 mM NaCl, pH 7.2. Further experimental details are provided in the text. Percent peak areas were determined by drop-baseline peak integration.

In summary, for the analysis of LMWS1 fragments by SEC, the use of a 1.7 µm (4.6 × 300 mm) column can provide shorter analysis times as compared to the 2.5 µm (7.8 × 300 mm) column provided that UPLC system dispersion is minimized and controlled. Alternatively, comparable HMWS and LMWS resolutions can be realized when using the 2.5 µm (7.8 × 300 mm) column at moderately lower linear velocities and increased analysis times with the added benefit of the methods being far less dependent on the system dispersion and run at lower pressures; thereby, allowing for the use of UHPLC and modern HPLC systems. The 2.5 µm particle size in a 7.8 × 300 mm column configuration will outperform the same particle in a 4.6 × 300 mm column configuration, and that performance increase improves as system dispersion increases. As a result, the 7.8 mm column I.D. is generally recommended unless there is a desire to limit sample or mobile phase volumes, and system dispersion will be controlled. In all cases, a 2.5 µm particle size column will outperform a 3.5 µm particle size column of the same length and I.D. The advantages of the 3.5 µm particle size will be an approximate 50% lower back pressure, enabling its use on some LC systems with low, upper pressure capabilities.

Table 1. SEC column recommendations based on LC system dispersion.

	Separation[1]	ACQUITY UPLC H-Class Bio (15 cm CH)[2] 5σ ≤ 12 µL UPLC	ACQUITY UPLC H-Class Bio[4] (30 cm CH)[2] 12 µL ≤ 5σ ≤ 25 µL UPLC/UHPLC	ACQUITY Arc Bio[4] (30 cm CH)[2] 25 µL ≤ 5σ ≤ 35 µL UHPLC	Alliance[4] (30 cm CH)[2] 35 µL ≤ 5σ ≤ 45 µL HPLC	Alliance[4] (30 cm CH)[2] 5σ ≥ 45 µL HPLC
300 mm Column length	HMWS	1.7>2.5>3.5	1.7[3]>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
	LMWS1	1.7>2.5>3.5	1.7[3]>$2.5_{7.8}$>3.5	$2.5_{7.8}$>1.7[3]	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
	LMWS2	1.7>2.5>3.5	1.7>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
150 mm Column length	HMWS	1.7>2.5>3.5	1.7[3]>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
	LMWS1	1.7[3]	Not recommended			
	LMWS2	1.7>2.5>3.5	1.7[3]>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5

[1] *Particle size (1.7 µm, 2.5 µm, and 3.5 µm) recommendations are provided in the order that is predicted to provide the most resolution for that separation for equivalent analysis times. The symbol (≈) indicates that performance advantage will depend on specific 5σ system dispersion of LC used and analytes. Analyses and LC configurations where only the 2.5 µm, 7.8 mm I.D. column is exclusively recommended are indicated by subscript (2.57.8). Where the 7.8 mm I.D. column is not specified, the 4.6 mm I.D. column may be considered, if reduced consumption of mobile phase or sample is desired; however, the separation on the 4.6 mm I.D. column may be of lower resolution due to LC system dispersion.*

[2] *CH: Column Heater Configuration. Note: 30 cm SEC columns will not fit into ACQUITY CH-A and comparably sized column heaters, but will fit in ACQUITY CH-30A and comparably sized column heaters.*

[3] *Control of system dispersion levels may be required to maintain resolution.*

[4] *Variations in LC configurations (detector, connectors, etc.) can cause variations in LC dispersion.*

CONCLUSIONS

We have attempted to systematically address the interplay between LC system extra-column dispersion with SEC particle size, column I.D., and column length with respect to the analyses of mAb HMWS as well as LMWS impurities. With these relationships in mind, we developed a set of general guidelines for matching Waters BEH SEC columns with three Waters LC systems most recommended for SEC separations (Table 1). In addition, these data suggest that an evaluation of extra-column dispersion might be an important variable to consider in robustness testing for some SEC methods. As a final note, if a developed method must be transferred for use on LC systems with unacceptably large extra-column dispersion, either decreasing the flow rate or increasing the column length of the method may effectively mitigate the impact of greater, extra-column dispersion without fundamentally altering the selectivity of the separation.

The reader is also referred to a companion publication to this application note "Evaluating the Impact of LC System Dispersion on the Size-Exclusion Chromatography Analysis of Proteins" (Waters Application Note, p/n: 720006337EN). This publication captures additional data and theoretical discussion on SEC and system dispersion, in addition to SEC method development advice.

Acknowledgments

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Evaluating the Impact of LC System Dispersion on the Size-Exclusion Chromatography Analysis of Proteins

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APPLICATION BENEFITS

- Understanding the measurement of LC system dispersion
- An educational and systematic demonstration of the impact of LC system dispersion on SEC-based protein separations
- Guidance for selecting the optimal SEC column configuration based on the LC systems to be used and the analytical method requirements including resolution, sensitivity, reproducibility, and transferability

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BEH200 SEC Protein Standard Mix

KEYWORDS

Size exclusion, system dispersion, UPLC, UHPLC, HPLC, proteins

INTRODUCTION

Size-exclusion chromatography (SEC) is the predominant method used for the assessment of non-covalent protein aggregation (high molecular weight species [HMWS]) in recombinant biotherapeutic protein and peptide products.[1] In the application note "Impact of LC System Dispersion on the Size-Exclusion Chromatography Analysis of Monoclonal IgG Antibody Aggregates and Fragments: Selecting the Optimal Column Configuration for Your Method" (p/n: 720006336EN), we presented an in-depth evaluation of the SEC analysis of monoclonal antibody (mAb) aggregates (HMWS) and fragments (LMWS), and the effect that extra-column dispersion has on that separation. This application note is a companion piece to that publication with the intent of providing a more detailed and generally applicable discussion of extra-column dispersion and SEC column selection. We have also included additional instructive information and data regarding the measurement of extra-column dispersion and the impact that extra-column dispersion has on the SEC separation of proteins. In doing so, we have also presented some of the same data and figures in both application notes in order to provide publications that can be read and referred to independently.

Extra-column dispersion can be considered as the increase in the volume of an injected sample that occurs as it travels through an LC system's flow-path without a column. Consequently, as the volume of this dispersion increases in proportion to the volume of the separated peaks, the resolution of the SEC separation will diminish.

A brief review of the history of SEC protein separations will help explain why LC system dispersion has had a greater impact on the quality of modern SEC separations. In 1977, the Tosoh Corporation (Japan) introduced a diol-bonded silica-based TSKgel SW series of SEC columns for the analysis of proteins which were packed with 10 to 13 µm particle sizes. The SW series of columns and the 5 to 8 µm particle size SWXL series of columns introduced by Tosoh 10 years later were typically packed in column hardware with internal diameters (I.D.) of 7.5 mm or greater and in 30 cm lengths. With these columns, the generated peak volumes were sufficiently large so that the measurable separation efficiencies were not significantly impacted by extra-column dispersion. While these TSKgel® SW and SWXL columns were the predominant column of choice for the analysis of biotherapeutic protein aggregation, the push toward higher sample throughput in recent years has resulted in the adoption of higher efficiency SEC columns with particle diameters of 2 µm and smaller. However, due to the use of shorter SEC column lengths made possible by use of these smaller and more efficient particles, column packing constraints have generally limited this hardware to internal diameters of 4.6 mm or smaller. Consequently, these smaller columns have significantly decreased packed bed volumes and higher efficiencies. This has resulted in a significant decrease in the peak volumes produced by these modern SEC columns such that the extra-column dispersion volume of a typical HPLC or UHPLC system is sufficiently large enough to cause a significant reduction in observed peak resolutions.[2]

This application note will detail the origins and measurement of extra-column dispersion and demonstrate the impact that extra-column dispersion has on the efficiency of SEC separations. We will conclude by summarizing useful considerations in the selection of an SEC particle size and column geometry for protein separations.

EXPERIMENTAL

Sample description

BEH200 SEC Protein Standard Mix (p/n: 186008476) was reconstituted in 500 µL of SEC mobile phase to yield the following:

Analyte	pI	MW
Thyroglobulin, 3 mg/mL	4.6	660,000
IgG, 2 mg/mL	6.7	150,000
BSA, 5 mg/mL	4.6	66,400
Myoglobin, 2 mg/mL	6.8, 7.2	17,000
Uracil, 0.1 mg/mL	N/A	112

The mAb sample of rituximab (Rituxan®) was used past expiry at an original concentration of ~21 mg/mL.

The mAb sample of trastuzumab (Herceptin®) was used past expiry at a diluted (in water) concentration of 2.0 mg/mL.

Intact mAb Mass Check Standard (p/n: 186006552) was reconstituted in 500 µL of SEC mobile phase to yield a nominal concentration of 2 mg/mL.

Method conditions (unless noted otherwise):

LC conditions

Systems:	ACQUITY UPLC H-Class Bio
Detection:	ACQUITY UPLC TUV detector with 5 mm titanium flow cell
Wavelength:	280 nm
Columns:	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 2.1 × 150 mm (p/n: 186008471)
	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 150 mm (p/n: 186005225)
	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 300 mm (p/n: 186005226)
	XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 300 mm (p/n: 186009164)
	XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 150 mm (p/n: 186009163)
	XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 150 mm (p/n: 176004335)
	XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 300 mm (p/n: 176004336)
	XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 × 150 mm (p/n: 176003595)
	XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 × 300 mm (p/n: 176003593)

Column temp.: Ambient, ~22 °C

Sample temp.: 10 °C

Flow rates and injection volumes unless, otherwise noted:

Column dimension (mm I.D.× mm L)	Flow rate (mL/min)	Injection volume (µL)
2.1 × 150	0.073	0.2
2.1 × 300 (two 150 mm, in series)	0.073	0.4
4.6 × 150	0.350	1.0
4.6 × 300	0.350	2.0
7.8 × 150	1.000	5.8
7.8 × 300	1.000	10.0

Mobile phase A: 100 mM NaH_2PO_4

Mobile phase B: 100 mm Na_2HPO_4

Mobile phase C: 1.00 M NaCl

Mobile phase D: H_2O

All 0.2 µm sterile filtered and Auto▪Blend Plus blended at 7.4% A, 12.6 % B, 35% C, and 45% D to yield 20 mm sodium phosphate, 350 mM NaCl, pH 6.8, unless otherwise noted.

Sample vials: Polypropylene 12 × 32 mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 300 µL Volume (p/n: 186002640)

Data management

Chromatography software: Empower™ 3

RESULTS AND DISCUSSION

UNDERSTANDING AND MEASURING SYSTEM DISPERSION

One of the fundamentals of chromatography is that extra-column or system dispersion, which is the broadening of a chromatographic peak or band that does not occur within the packed chromatographic bed of the column, always has a deleterious effect on the resolution of a separation. Extra-column dispersion can be visualized by the experiment diagrammed in Figure 1 where the peak volume resulting from a relatively small injection volume of analyte is determined without a column installed in the LC system. For this discussion, we will measure this extra-column dispersion peak width in units of time (min) at 4.4% of the peak and then multiply by the flow rate (µL/min) to generate the extra-column dispersion volume (µL). Since a normal Gaussian distribution is five standard deviations wide at 4.4% of its maximum height (approximately 99% of the peak area), we will analogously refer to this calculated extra-column dispersion volume as $5\sigma_{ec}$. Historically, chromatographic peak dispersion or band broadening volumes have often been represented as the unit σ value, which in this case would be obtained by dividing the 5σ value by 5 and still be expressed in units of µL. Dispersion is also often represented as a variance (σ^2), which is the square of the dispersion volume and has units of $\mu L.^2$ This nomenclature is analogous to the nomenclature used to describe statistical distributions where the square root of the variance is equal to the standard deviation of the distribution. Throughout this discussion we will most often refer to 5σ dispersion volumes which can be more easily visualized.

The approximate band broadening of a peak, σpeak, as it travels through an LC system and SEC column is shown in the following relationship where $\sigma_{Pre\text{-}column}$, σ_{Column}, and $\sigma_{Post\text{-}column}$, are the pre-column, on-column, and post-column dispersion volumes.

$$\sigma_{peak} = (\sigma^2_{Pre\text{-}Column} + \sigma^2_{Column}\,\sigma^2_{Post\text{-}Column})^{1/2} \quad \text{(Equation 1)}$$

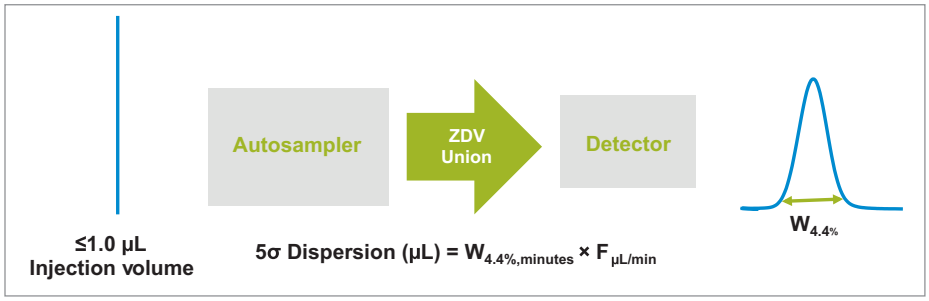

Figure 1. Measurements of extra-column dispersion were carried out using 3:7 water:acetonitrile as a mobile phase at a 0.3 mL/min flow rate. The sample was 1 µL of 0.16 mg/mL caffeine in 1:9 water:acetonitrile. The UV absorbance was monitored at 273 nm at a sampling rate of 40 Hz.

An important consideration within this relationship is $\sigma_{Pre\text{-}column}$, which occurs primarily in the injector and tubing connecting to the column inlet. The impact of $\sigma_{Pre\text{-}column}$ on σ_{Peak} is modulated by the retention factor (k') of the analyte.[3] At high k' values, where the analyte binds strongly to the stationary phase, the analyte will re-focus at the head of the packed bed of the column which will minimize and may even eliminate the deleterious effect of pre-column band dispersion. Examples of gradient based protein separations where analyte k' values can be sufficiently high during sample loading such that pre-column dispersion is not a concern include affinity (e.g., Protein A), ion-exchange, and reversed-phase separations. Conversely, in ideal SEC separations, as there is no partitioning occurring between the protein and the packed SEC particle surface, k' will be effectively zero. The practical result of this is that for SEC separations, pre-column dispersion will equally degrade the quality of a separation as compared to post-column dispersion, and as a result σ_{Peak} can be calculated by the simplified expression:

$$\sigma_{peak} = (\sigma^2_{ec} + \sigma^2_{Column})^{1/2} \qquad \text{(Equation 2)}$$

Where σ_{ec} is the total extra-column band broadening and is indeed the value that we determine by the experiment diagrammed in Figure 1. Another important relationship to note in either Equation 3 or Equation 4 is that the dispersion volumes are squared prior to being added and σpeak is then obtained by taking the square root of the summation. This relationship magnifies the impact that the larger σ value has in the relationship.

We will briefly consider the causes of extra-column dispersion. We can visualize the LC flow path to be a series of tubes and mismatched internal diameter connections of those tubes. The predominant contributions of extra-column dispersion can be understood based on the Taylor-Aris equation which models dispersion in a length of open capillary tubing.[4]

$$\sigma^2_{vol,tube} = \frac{\pi \cdot L \cdot r_c^4 \cdot F}{24 D_m} \qquad \text{(Equation 3)}$$

Where $\sigma^2_{vol,tube}$ is the contribution of a given tube to peak variance, L is tube length, r_c is tube internal radius, F is flow rate, and D_m is the diffusion coefficient of the analyte. Of note is that $\sigma^2_{vol,tube}$ is proportional to the 4th power of tubing radius, which underscores the importance of minimizing the internal diameter of connection tubing. In addition, as D_m decreases for larger molecular weight analytes, $\sigma^2_{vol,tube}$ will increase proportionally.

While dispersion in open tubing is typically the predominant contributor to band broadening, the presence of mismatched tube internal diameter connections is also important to consider.[4]

$$\tau^2_{chamber} = \frac{r^2_{c,1} - r^2_{c,2}}{2 D_m} \qquad \text{(Equation 4)}$$

Where $\tau^2_{chamber}$ is the peak variance that occurs at the mismatched interface, which is referred to as a diffusion chamber. Similarly to σ^2, τ^2 is represented in units of μL^2 and is added to peak volume in the same way. A mismatched fluidic interface can create an unswept volume within the LC flow path which leads to increased peak tailing as the analyte must diffuse back to the flow path, as a result, we can visualize the strong, inverse squared dependence that $\tau^2_{chamber}$ has on D_m.

The measurement of extra-column dispersion (system band broadening) has been studied extensively.[5] These measurements range from simply determining the peak widths to more involved mathematical approaches that include elaborate peak modeling and deconvolution algorithms.[6] The need for these advanced methods are the result of chromatographic and extra-column dispersion peak shapes that deviate significantly from a normal or Gaussian distribution in their nature. While these more complex approaches are certainly more accurate with regards to estimating the actual system band broadening and thereby elucidating the actual chromatographic performance of a column they are not very amenable to a routine analytical laboratory setting. As part of this study, we compared the more commonly used and easily obtained result for $5\sigma_{ec}$ dispersion volume as determined by direct peak width measurement at 4.4% of the peak height, and $5\sigma_{ec}$ as determined by the second moment of the peak.

Specifically, for this study, a zero-dead volume (ZDV) union was used in place of the SEC column (Figure 1) and the broadening of a small injection volume of caffeine was evaluated. In addition to the standard configuration, we also evaluated additional configurations in which sample loops or combinations of sample loops ranging from adding an additional 5 µL to 60 µL to the flow path to increase the system dispersion volume (Figure 2). The direct measurement of the $5\sigma_{ec}$ peak widths (peak width at 4.4% peak height) were then determined (Figure 1). The choice of using peak width at 4.4% peak height to directly determine $5\sigma_{Peak}$ values, while somewhat arbitrary, was selected due to it being the peak width value at the lowest percent of peak height that is reported in most common chromatography data systems such as Waters Empower 3 and Agilent ChemStation. For typical biotherapeutic protein analyses, the HMW and LMW peak heights are at 0.5% of the main peak height or lower. As stated previously, the assumption in using the direct peak width measurement approach is that the peak approximates a normal distribution. However, as can be seen in the dispersion profiles shown in Figure 2, the extra-column dispersion peaks are asymmetrical with a noticeable tailing or skewed profile, and making a peak width measurement closer to the baseline will potentially account for more of the influence of peak tailing.

For this same data set, the determination of σ_{ec} was also calculated as the square root of the second-moment (M_2) or variance of the peak. This approach is more appropriate for the determination of variance for a skewed distribution and is determined by the calculation:

$$M_2 = \frac{\int_{Peak\ Start}^{Peak\ End} (t - M_1)^2 h(t) dt}{M_0} = \sigma^2$$

(Equation 5)

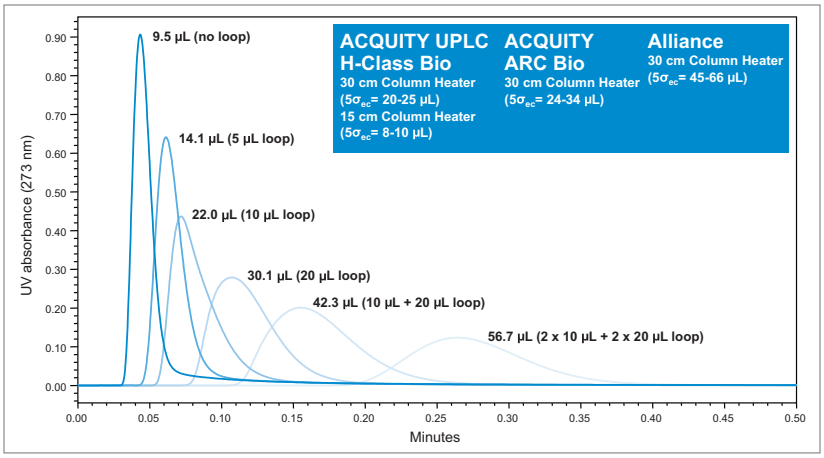

Figure 2. Measurements of 5-sigma extra-column dispersion volumes ($5\sigma_{ec}$) for this study based on peak width at 4.4% peak height and the expected range of values for Waters LC systems. Experiments were carried out as described in the caption of Figure 1. Sample loops were connected pre-column to generate the larger dispersion volumes.

Where M_0 and M_1 are zero and first moments of the peak, M_0 is calculated as the peak area and M_1 is the time at the geometric center or mean of the peak. For a normally distributed Gaussian peak, this is equivalent to retention time of the peak, but, for a tailing peak it will be located at a slightly later time. In this case, M_1 is then calculated as:

$$M_1 = \frac{\int_{Peak\ Start}^{Peak\ End} t\, h(t) dt}{M_0}$$

(Equation 6)

The most important relationship to note in the calculation of M_2 (Equation 5) is that the time difference between a point on the elution profile and M_1 is a square function. Hence, the calculation of the variance is more heavily weighted by values further from the mean even though their intensities are relatively low. Because of this dependency, the assignment of the end of the peak can significantly impact the determination of M_2. In order to provide a consistent, baseline end point, the M_2 values were calculated within the Empower 3 Software using Waters ApexTrack integration function with a percent touchdown value of 0.05% and a 5-point moving average smoothing function. The values of M_2 were then converted to σ_{ec} by multiplying the flow rate by the square root of M_2.

A correlation plot of the $5\sigma_{ec}$ values determined by the peak width and second moment calculations is presented in Figure 3. We can see that at low levels of extra-column dispersion, the peak width method under estimates σ_{ec} while the values at high levels of dispersion are more similar. The chromatographic inset shows an example of the system dispersion measurements. While this profile shows that more of the lower portion of the peak is incorporated into the M_2 determination of σ_{ec} versus the direct peak width method, it is also evident that the M_2 method baseline does not incorporate all of the peak tail.

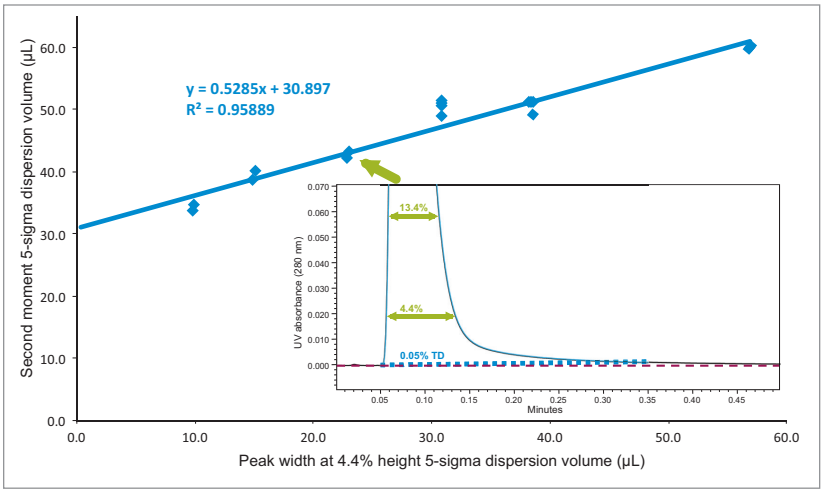

Figure 3. Shown is the correlation between $5\sigma_{ec}$ volumes based on the second moment peak variance and on the peak width at 4.4% peak height. The chromatogram for the 22.0 µL dispersion (width at 4.4% peak height) is shown in the inset. The green double arrows indicate where direct 4-sigma (13.4%) and 5-sigma (4.4%) peak volumes are determined. The blue dashed line indicates the location of the 0.05% touchdown (TD) baseline as determined by Empower 3. The red dashed line indicates the parallel zero baseline.

As a result, while this M_2 method provides a value for σ_{ec} that is perhaps closer to the true value, it is certainly an underestimation as well.[7] Although there is a noted bias between the peak width method and the M_2 method, there is a reasonable correlation between the two methods ($R^2 = 0.96$). It was also noted that the determinations of σ_{ec} using the M_2 method are significantly more variable than those obtained for the peak width method with a six times higher relative standard deviation on average. Therefore, we have opted to use the 4.4% peak width method for this study as it is adequate for the relative comparison of LC performance, and is more reproducible despite significantly underestimating the true system dispersion.[8] In addition, the direct 4.4% peak width method has the advantage of being more easily implemented in analytical laboratories using different chromatography data systems and where LC system performances can significantly vary.

It is important to recognize that we have arbitrarily defined the measurement of system dispersion to include a specific analyte and its concentration, as well as, a mobile phase composition and programmed running conditions. When comparing the dispersions of LC systems across an organization, these variables need to be consistent to provide meaningful results. However, in practice, using the SEC method mobile phase and a protein sample could be more convenient. To make this comparison, we measured a range of system dispersions using the caffeine standard and the Intact mAb Mass Check Standard reconstituted at a concentration of 2 mg/mL in the SEC mobile phase. Similarly, in order to minimize the refractive index difference between the sample buffer and the mobile phase, a reference material could be buffer exchanged into mobile phase. Or, if the concentration is high enough, and the buffer does not have any interfering chromophores, it may be possible to dilute the protein reference material directly into the mobile phase.

A comparison of the dispersion profiles for caffeine and IgG is shown in Figure 4. The peaks for the IgG tail were observed more significantly than those for caffeine, and the measured $5\sigma_{ec}$ volumes were greater. A correlation plot for the two methods (Figure 4) reveals that there is a reasonable correlation between the two methods with the values determined using IgG measuring approximately 33% higher based on the slope of the curve. This result is consistent with Equations 3 and 4, where dispersion has inverse relationships with the diffusion coefficient of the analyte. While we observe a significant bias between the two methods, these results demonstrate that using an appropriate protein sample can provide a useful and easy-to-implement relative evaluation of LC system dispersion.

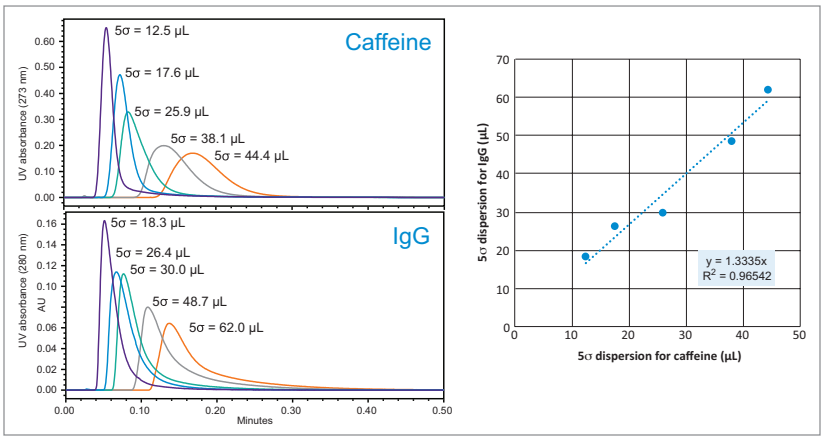

Figure 4. Shown is the comparison of system dispersion measurements at 5σ (width at 4.4% peak height) measured with a caffeine and an IgG standard. Experiments were carried out as described in the Figure 1 caption. Sample loops were connected pre-column to generate the larger dispersion volumes.

IMPACT OF SYSTEM DISPERSION ON SEC SEPARATION EFFICIENCY

It can be assumed that in the absence of extra-column dispersion, if two columns of the same length, but different internal diameters, are packed with the same particles and with the same plate count, then those columns will provide the same resolution when the same linear velocity, and a proportional sample load relative to the column volume, are maintained. Or more simply stated, resolution is independent of column I.D. Under these constraints, the peak widths observed for these two different size columns will be equivalent in the time domain, but the volume of a peak for the larger I.D. column will be proportionally larger due to its higher flow rate. The practical implications of these relationships are that as SEC peak volumes become smaller due to the use of smaller internal diameter columns, extra-column dispersion can have a greater deleterious impact on the net chromatographic result.

To assess the impact that σ_{ec} has on SEC separations as a function of particle size, column I.D., and column length, the SEC MW protein standard mix was injected on 2.1 mm and 4.6 mm I.D. columns packed with 1.7 µm particles, 4.6 mm and 7.8 mm I.D. columns were packed with 2.5 µm particles, and 7.8 mm I.D. columns were packed with 3.5 µm particles. Column lengths of 150 and 300 mm were tested, and, in all cases, the average pore diameter of the particles was 200 Å. The 300 mm bed length, 2.1 mm I.D. column was simulated by running two 2.1 × 150 mm length columns, in series. For these experiments, the linear velocities were held constant, therefore, for a given column length the analysis times are equivalent. In addition, sample loads were appropriately scaled based on column volume.

Representative chromatographic profiles for the 2.1 mm and 4.6 mm I.D. columns packed with 1.7 µm particles obtained over a range of system dispersions, are presented in Figure 5. These chromatograms were selected as they provide the most visually discernable changes. Quantitative measures of the USP plate counts (based on uracil) and the resolution between the two largest baseline resolved proteins, IgG and BSA, are presented in Figures 6 and 7, respectively. Additionally, to get a sense of the peak volumes produced by these SEC columns, we determined the estimated $5\sigma_{ec}$ peak volumes for the proteins IgG, BSA, and myoglobin based on the direct measurement of peak width at 4.4% peak height (Figure 8). To obtain a better estimate of the $5\sigma_{column}$ volumes, we have subtracted the contributed $5\sigma_{ec}$ from the measured protein peak widths ($5\sigma_{peak}$) using the relationship shown in Equation 1.

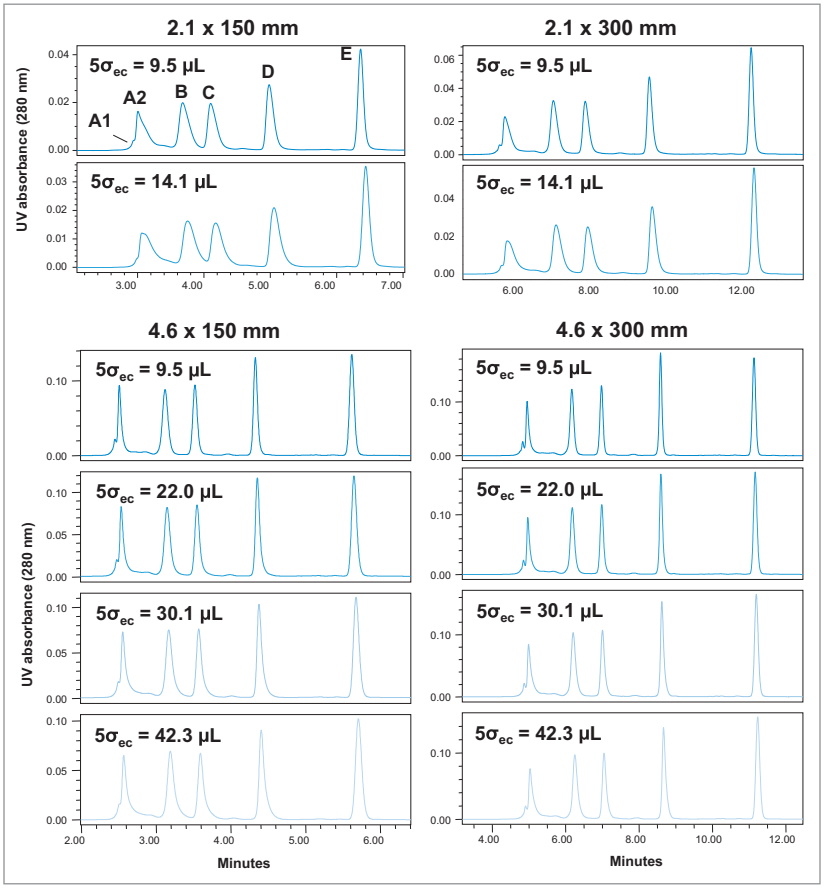

Figure 5. Impact of extra-column dispersion volume ($5\sigma_{ec}$) on the separation of MW standards with 1.7 µm particle size SEC with column internal diameters of 2.1 mm and 4.6 mm and lengths of 150 mm and 300 mm. Sample injection volumes and flow rates were proportional to column internal diameter. Peak identifications (shown in top left chromatogram) are: (A1) thyroglobulin dimer (1.32 MDa), (A2) thyroglobulin monomer (660 KDa), (B) IgG (150 KDa), (C) BSA (66 KDa), (D) myoglobin (17 KDa), and (E) uracil (112 Da).

It is visually obvious that the peak widths and resolutions obtained for the 2.1 × 150 mm and 2.1 × 300 mm columns are significantly degraded compared to those obtained on 4.6 mm I.D. columns of equivalent lengths and packed 1.7 µm particles (Figure 5). Indeed, the uracil derived plate counts (Figure 6) measured for even the longer 300 mm length, 2.1 mm I.D. columns ($5\sigma_{ec}$ = 9.5 µL) were 22% lower than those of the 4.6 mm I.D. column ($5\sigma_{ec}$ = 12.5 µL) and the resolution between IgG and BSA was 36% lower for the 2.1 mm I.D. column. These decreases in efficiency and resolution are a direct result of the $5\sigma_{peak}$ peak volumes for the 2.1 mm I.D. columns being only approximately 2 to 3 times greater than the $5\sigma_{ec}$ dispersion volume, while the $5\sigma_{peak}$ peak volumes for the 4.6 mm I.D. columns are 5 to 9 times greater than the $5\sigma_{ec}$ dispersion volume at which they were tested. Since the peak and dispersion volumes are squared before they are added together (Equation 2) this difference is even more significant.

The impact on system dispersion can also still be seen for larger sized columns, when comparing the results for the 4.6 mm and 7.8 mm I.D. columns packed with 2.5 µm particles. At a $5\sigma_{ec}$ of 25.9 µL, the plate counts were 23% and 12% lower, and the resolutions between IgG and BSA were measured to be 10% and 7% lower for the 4.6 mm I.D. columns at lengths of 150 mm and 300 mm, respectively. However, as $5\sigma_{ec}$ increases to 44.4 µL, which is a good performance for typical HPLC systems, we observe 38% and 24% decreases in the plate counts, and concomitant resolution decreases of 20% and 16%.

As an aside, it should be noted that the observed decreases in IgG and BSA resolution for the 2.1 mm I.D. (1.7 µm) column far exceeds the 12% decrease in peak resolution we would estimate based on the reduction in plate count (N).

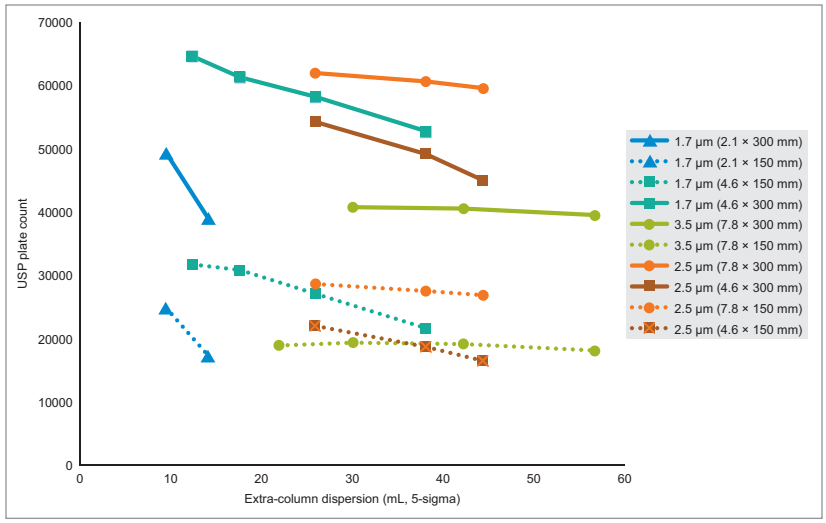

Figure 6. Shown are the measured USP plate counts determined based on the uracil peak plotted versus extra-column dispersion (σ_{ec}). Columns with 1.7 µm particle size are in blue hues, columns with 2.5 µm particle size are in orange or brown, and columns with 3.5 µm particle size are in green. Triangles, squares, and circles indicate 2.1, 4.6, and 7.8 mm I.D. columns, respectively. Sample injection volumes and flow rates were proportional to column internal diameter.

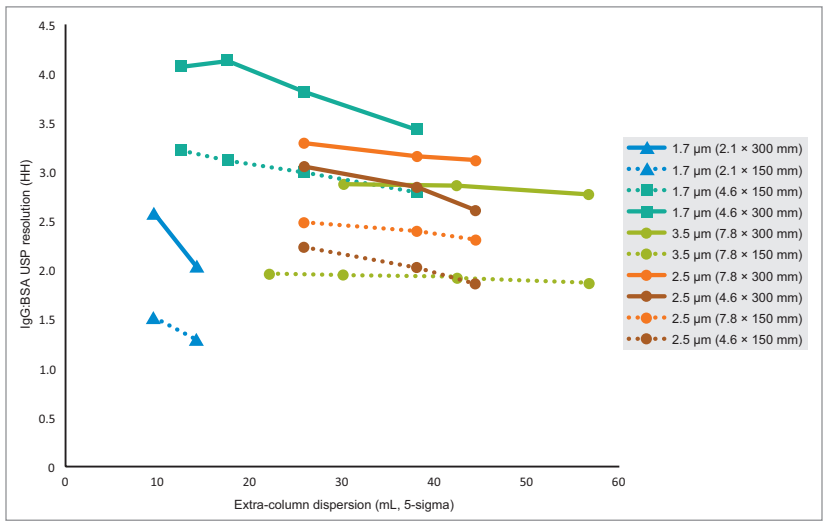

Figure 7. Shown are the measured USP resolution values between the primary IgG and BSA peaks. Columns with 1.7µm particle size are in blue hues, columns with 2.5 µm particle size are in orange or brown, and columns with 3.5 µm particle size are in green. Triangles, squares, and circles indicate 2.1, 4.6, and 7.8 mm I.D. columns, respectively. Sample injection volumes and flow rates were proportional to column internal diameter.

In comparison of the 4.6 mm I.D. (2.5 µm) and 7.8 mm I.D. (2.5 µm) columns, the plate count and resolution changes are more consistent with Equation 7. This discrepancy is a result of the extra-column dispersion volumes being larger for the protein standards (Equations 3 and 4) versus the uracil standard. In addition, the column derived $5\sigma_{peak}$ volumes for the protein standards are decreased for the 1.7 µm columns versus columns packed with 2.5 µm particles, as described by the Van Deemter equation.

$$Rs \propto \sqrt{N} \qquad \text{(Equation 7)}$$

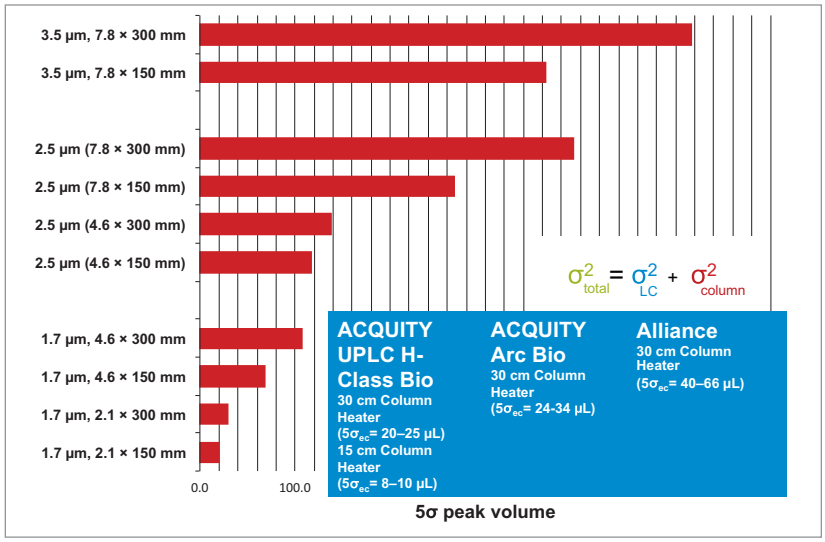

Figure 8. Shown are the estimated $5\sigma_{ec}$ peak volumes (based on peak width at 4.4% height) for the IgG, BSA, and myoglobin peaks in the column configurations evaluated. Peak volumes were corrected for 5-sigma system dispersion volume based on the relationship shown in the inserted equation (Equation 2 in the text). The expected range of values for Waters LC systems are also provided for comparison.

Where the theoretical plate height (H), which is inversely proportional to column efficiency (N), relates to mobile phase velocity (u), particle diameter (d_p), and the diffusion coefficient of the analyte (D_m). The "a" term reflects eddy diffusion and the "c" term represents mass transfer into the pores of the particle. For the SEC separation of proteins, the "b" term, or longitudinal diffusion term, can be ignored due to the small value of D_m. As a result, because dp is squared in the "c" term of the Van Deemter equation, the contribution that decreased values of D_m have toward increased plate heights is reduced for the 1.7 µm column versus the 2.5 µm column.

$$H = ad_p + \frac{bD_m}{u} + cud_p^2/D_m \approx ad_p + cud_p^2/D_m \qquad \text{(Equation 8)}$$

Practically, these results demonstrate that if two columns are packed with the same size particles, the larger I.D. column will result in a method with significantly improved efficiency (i.e., component resolution) when using LC systems where extra-column dispersion volume is substantial relative to analyte peak volumes. Additionally, the relative impact of extra-column dispersion will be greater for columns with shorter lengths and smaller particle sizes. The larger I.D. column will also provide greater analytical consistency and robustness as a method is transferred to various LC systems, and the method will also provide more sensitivity for low abundance analytes when the injection volumes are adjusted proportionally to column volume. The only advantages to be gained by using the smaller I.D. column packed with the same size particles will be the ability to use smaller volumes of sample and less total mobile phase; however, system dispersion may need to be more carefully controlled in order to not compromise separation quality.

SELECTING A COLUMN AND PARTICLE SIZE FOR AN SEC METHOD

The major considerations in selecting a column are interdependent, and also include the sample throughput needs and the performance capabilities of the LC systems being used for the method. Finally, both sample volume limitations and mobile phase use may also be considered. Understanding the extra-column dispersion and pressure limits of the LC systems to be employed, as has been demonstrated, is of paramount importance as these limits can restrict your choice of column geometries; but, more significantly, may limit the sample-throughput of your method by precluding the use of sub-2-µm columns which are only currently available with an I.D. of 4.6 mm or smaller. It should be noted that the impact of extra-column dispersion on the separation of a low-abundance, partially-resolved, antibody protein fragment (Figures 9A and 9B [LMWS1]) is greatly magnified relative to the changes observed for aggregate (HMW) protein peaks due to the tailing nature of system dispersion. The impact of extra-column dispersion on the SEC separation of monoclonal IgG antibodies is more thoroughly evaluated in a companion application note (p/n: 720006336EN).

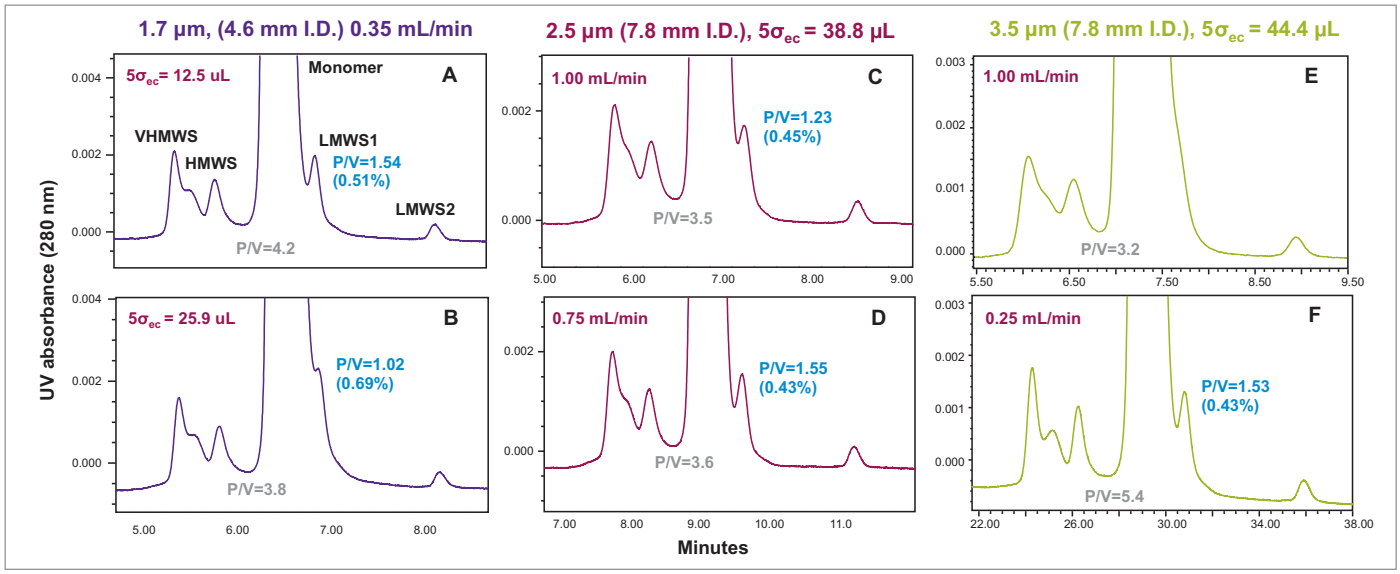

Figure 9. Zoomed view chromatograms demonstrating the impact of extra-column dispersion on the SEC separation of aggregates (VHMWS and HMWS, ≥300 KDa), and the mAb fragments LMWS1 (≥100 KDa) and LMWS2 (≥50 KDa). For rituximab (≥150 KDa), Frames A and B are the results obtained using a 4.6 × 300 mm (1.7 µm) column at a fixed flow rate while altering extra-column dispersion. Frames C and D, and frames E and F are the results obtained using a 7.8 × 300 mm (2.5 µm or 3.5 µm) column at fixed extra-column dispersion while altering flow rate. The separation conditions are as described in the text.

When used with low-dispersion UPLC systems ($5\sigma_{ec} \leq 25$ µL) 4.6 mm I.D. columns packed with 1.7 µm particles will always provide greater resolution than the same column size that is packed with 2.5 µm particles. However, in comparison to a larger 7.8 mm I.D. column of equivalent length and packed with 2.5 µm or larger particles, the performance advantage of the 4.6 mm I.D. (1.7 µm) columns may substantially decrease as system dispersion increases. This often can be the case in protein fragment separations, where it may be found that the use of a 7.8 mm I.D. (2.5 µm) column may provide a comparable or improved separation to that observed with a 4.6 mm I.D. (1.7 µm) column with little or no compromise in sample throughput (Figures 9A through 9D), depending on system dispersion levels. The larger column I.D. also provides the added benefits of a method that is more change tolerant in system dispersion as demonstrated by the better resolution of the LMWS1 peak observed using the 2.5 µm column at a $5\sigma_{ec}$ of 38.8 µL versus the resolution observed for the 1.7 µm column at a $5\sigma_{ec}$ of 25.9 µL (Figures 9B and 9C). In addition, the larger particle sized column operates at a lower pressure, and is more economical.

For high-dispersion LC systems, which for these studies could be defined as a $5\sigma_{ec}$ value of approximately 40 µL or more, columns with internal diameters of 7.8 mm are recommended. In all cases, the smaller 2.5 µm particle size will provide greater resolving power than the larger 3.5 µm particle size (Figures 9C and 9E). The advantage of the 3.5 µm particle sized columns, however, is lower cost and 50% lower operating pressures since column pressure is proportional to the square of particle diameter.

In the event that the larger particle size column does not provide the needed resolution, it may be necessary to either reduce flow rate as shown for the 2.5 µm and 3.5 µm columns in Figure 9. Another option is to increase column length. As demonstrated in Figure 10, by operating two 30 cm length, 3.5 µm particle sized columns in series at the same flow rate used for a single column, greater resolution of both the aggregate (fragment [LMW]) is achieved while analysis time is only doubled versus the 1.7 µm particle size column.

The final consideration to be discussed is when an SEC method is required for use with minimal sample volumes or reduced mobile phase consumption. This is most simply achieved by reducing the internal diameter of the column being used since injection volume and flow rate are proportional to the square of the column I.D. However, as we have seen, the impact of extra-column dispersion must be dutifully considered. Alternatively, decreasing flow rate and using a shorter column length will also reduce sample and mobile phase volumes. Decreasing flow rate not only provides greater efficiency from an SEC column, but it also decreases the extra-column dispersion of the separation. In taking this approach, either analysis time or resolution must be partially compromised.

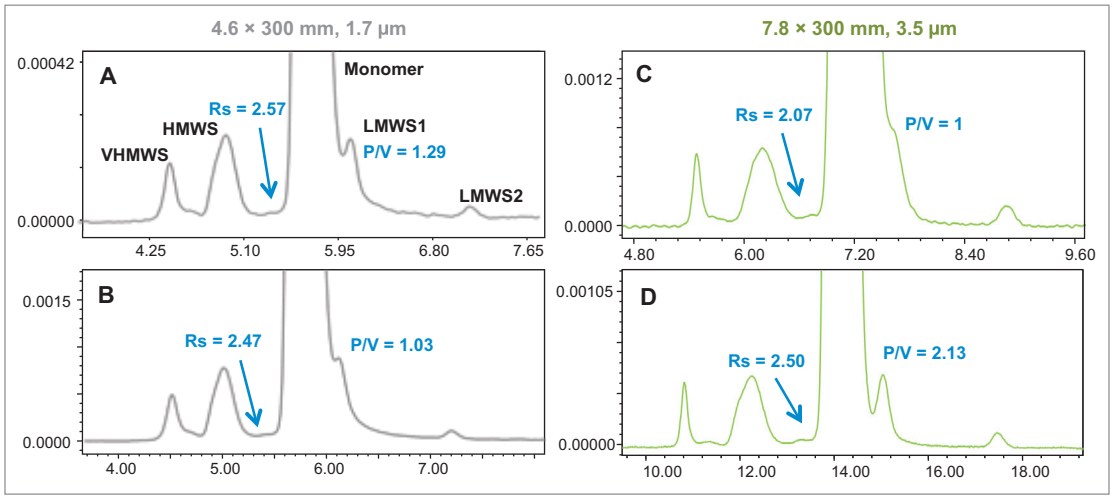

Figure 10. Shown are comparisons of 2.0 mg/mL trastuzumab (Herceptin) run with the ACQUITY UPLC BEH, 200 Å (1.7 µm, 4.6 × 300 mm) Column on an ACQUITY UPLC H-Class Bio System (Frame A, $5\sigma_{ec}$ = 20 µL) and an ACQUITY Arc Bio System (Frame B, $5\sigma_{ec}$ = 34 µL). The results for a single and tandem XBridge BEH 200 Å (3.5 µm, 7.8 × 300 mm) Columns on the ACQUITY Arc Bio System (Frames C and D) are also compared. The mobile phase was 25 mM phosphate and 400 mM NaCl at pH 7.2. Injections volumes were 5 µL for the 4.6 × 300 mm column, 15 µL for the 7.8 × 300 mm column, and 21.2 µL for the two 7.8 × 300 mm columns in series. The 4.6 mm I.D. column used a flow rate of 0.4 mL/min while the XBridge BEH 200 Å Column used a flow rate of 1.0 mL/min. Peak identifications are provided in Figure 9.

CONCLUSIONS

Selecting the most appropriate SEC particle size and hardware configuration for a column to be used in a protein SEC method will be influenced by the requirements of the separation and the performance capabilities of the LC systems to be used. In summary, these relationships, as illustrated in this application note, include:

- True SEC column efficiency (plate count) is independent of column I.D. when sample volume and mobile phase flow rate are scaled to the square of the column I.D.
- With particle size, column length, and packing efficiency being comparable, and flow rate and injection volume being scaled, a larger I.D. SEC column will provide better resolution than a smaller I.D. column due to the diminished impact of extra-column dispersion on the increased peak volumes generated by the larger I.D. column.

The separation efficiencies observed for 4.6 × 300 mm and shorter SEC columns are significantly more dependent on extra-column dispersion levels than 7.8 mm I.D. columns of equivalent length. As a result, while it is good practice to always include the determination of extra-column dispersion in LC system suitability testing, it is strongly encouraged for LC systems used to run SEC methods that employ 4.6 mm and smaller I.D. columns.

- SEC column efficiency is inversely proportional to particle size.
- SEC column efficiency is proportional to column length.
- SEC column efficiency is improved at lower flow rates.
- Decreased sample injection volume and mobile phase use can be achieved by using shorter length or narrower I.D. columns at lower flow rates.
  - Shorter columns will compromise either resolution or sample throughput.
  - Smaller I.D. columns may significantly compromise resolution if LC system dispersion is not considered and minimized.
  - Selecting a smaller I.D. column that also incorporates smaller sized particles will maximize resolution and sample throughput, however, operating pressure will also increase.

Acknowledgments

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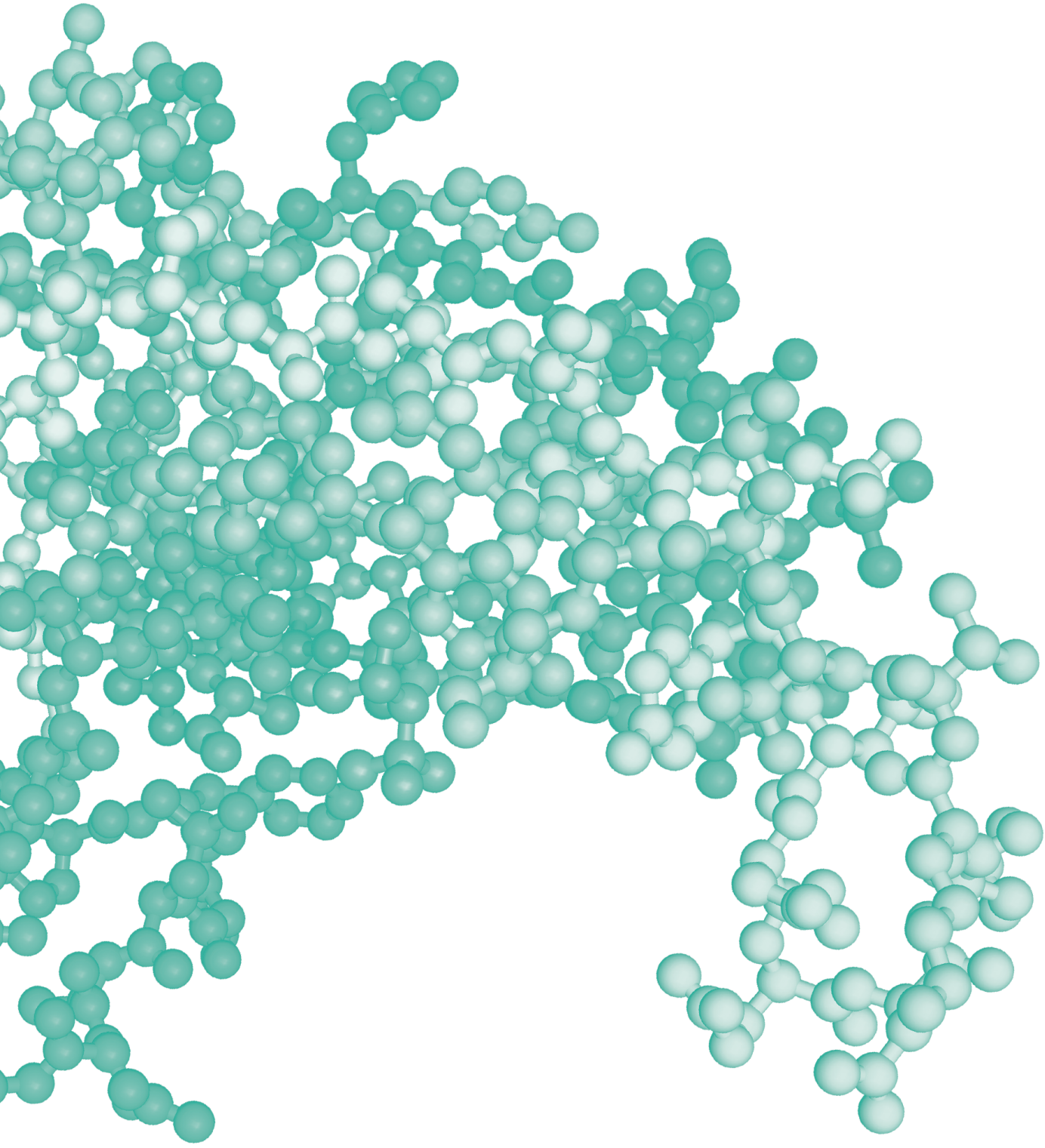
SEC Method Development and Transfer



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Method Development for Size-Exclusion Chromatography of Monoclonal Antibodies and Higher Order Aggregates

Paula Hong and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Robust analysis of mAb monomer and aggregates
- High throughput SEC separation
- Consistent purity profile
- Reproducible quantitation of higher order aggregates
- Easy SEC method development

WATERS SOLUTIONS

- ACQUITY UPLC® H-Class Bio System
- ACQUITY UPLC BEH200 SEC 1.7 µm Column
- Auto•Blend Plus™ Technology
- Empower® 2 Software

KEY WORDS

Size-exclusion chromatography, UPLC, monoclonal antibody, method development, aggregates

INTRODUCTION

Since the early introduction of biologic based therapeutics, the presence of protein aggregates can compromise safety and efficacy.[1] Given these factors, protein aggregates are typically monitored throughout the production of a biotherapeutic. While a variety of analytical techniques have been used to analyze soluble aggregates, the dominant technique continues to be size-exclusion chromatography (SEC).[2]

While SEC has been performed with silica-diol coated columns and HPLC instrumentation, the introduction of UPLC® or low dispersion systems in combination with sub-2-µm particles has allowed for improvements in these isocratic separations, including improved resolution, higher throughput and sensitivity.[3] However, as in any SEC method, a variety of parameters can be adjusted to improve resolution and method robustness. In the following application, we will investigate the impact of some of these parameters, including mobile-phase composition, flow rate and column length on a SEC separation. Evaluation of the separation will be based on a variety of criteria such as column calibration, resolution, and aggregate quantitation.

EXPERIMENTAL

Sample description

The protein standard (BioRad) containing bovine thyroglobulin (5 mg/mL), bovine γ –globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 mg/mL) and Vitamin B12 (0.5 mg/mL) in de-ionized water was analyzed. A murine monoclonal antibody, purified by Protein A affinity chromatography, was analyzed. The sample concentration was 10 mg/mL in 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3.

Samples were not controlled for inter-experiment conditions.

LC conditions

System:	ACQUITY UPLC H-Class Bio System with TUV and Titanium flow cell
Wavelength:	214 and 280 nm
Column:	ACQUITY UPLC BEH200 SEC 1.7 µm, 4.6 x 150 mm, PN: 186005225
Column temp.:	30 °C
Sample temp.:	4 °C
Injection volume:	2 µL (unless otherwise specified)
Flow rate:	0.4 mL/min (unless otherwise specified)
Mobile phase:	Prepared using Auto•Blend Plus technology
Final composition:	25 mM sodium phosphate, pH 6.8, 200 mM sodium chloride, (unless otherwise specified)

Data management

Software:	Empower 2

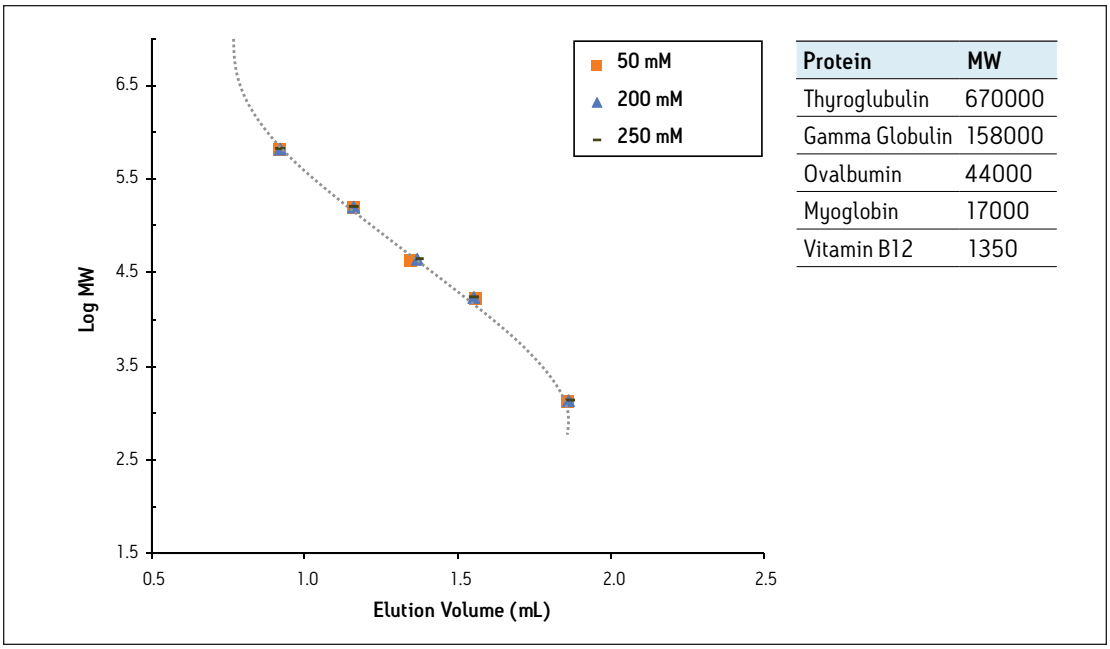
RESULTS AND DISCUSSION

A number of factors need to be evaluated in SEC method development. Ideally SEC separations are based on the size of the proteins in a solution. For this reason, size-exclusion chromatography of biomolecules is performed under aqueous, native conditions. However, the presence of mixed mode interactions can obscure size measurements.[4] More specifically, the charged sites on the packing material can interact with the proteins, resulting in an 'ion- exchange' effect. To determine the influence of these effects the mobile-phase conditions of the separation need to be evaluated. However, the conditions of the chromatographic separation can alter the protein structure and state. The concentration and identity of the salt and pH can affect the 3-D structure and the protein-protein interactions. For these reasons, evaluation of a SEC method must incorporate information of the biological activity of the biomolecule.

In the following discussion, we will outline considerations and parameters for developing a SEC method. While the SEC method development steps are illustrated on UP-SEC, the same principles apply to any HP-SEC separation. Methods will be evaluated based on peak shape, resolution, calibration accuracy, and quantitation. Optimization of the mobile-phase ionic strength and pH can easily be accomplished with a quaternary eluent management system in combination with software that can take advantage of this four eluent blending system.[5] This approach was used throughout the studies described.

Mobile-phase Ionic Strength

The ionic strength of the mobile-phase should be adjusted to minimize any secondary interactions between the packing material and proteins. To determine the effect of mobile-phase concentration on the calibration curve, a set of protein standards was analyzed at 50–250 mM sodium chloride. Sodium chloride was selected since it is the most common salt used in SEC separations. The buffer concentration (sodium phosphate) and pH were kept constant at 25 mM and pH 6.8, respectively. Over the concentration tested, the retention times for each protein were within 0.07 minutes with the greatest retention time variability observed for ovalbumin (Figure 1). These results indicate the calibration curves are not sensitive to salt concentration.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 1. Effect of sodium chloride on a SEC calibration curve.

Note: Calibration points deviate from a straight line because of protein shape in solution.

In addition to protein standards, the SEC separation of a murine monoclonal antibody (mAb) was evaluated at 50–250 mM sodium chloride (Figure 2). As is commonly observed with gel filtration packing materials,2 higher ionic-strength mobile phases lead to decreased peak tailing and narrower peaks for the mAb monomer. With increasing sodium chloride concentrations from 50–200 mM, the mAb peak height increases from 0.189–0.289. The USP tailing factor also decreases from 1.64 to 1.22. Changes are less pronounced as the ionic strength of the mobile phase is increased from 200 to 250 mM sodium chloride (USP Tailing = 1.20).

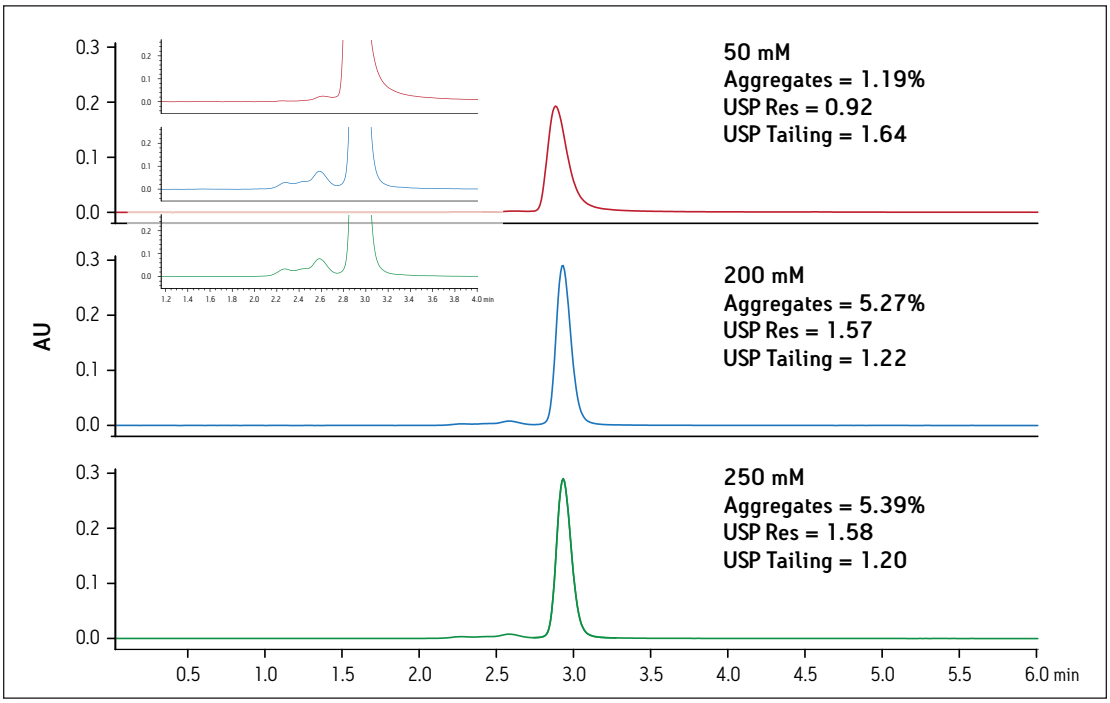


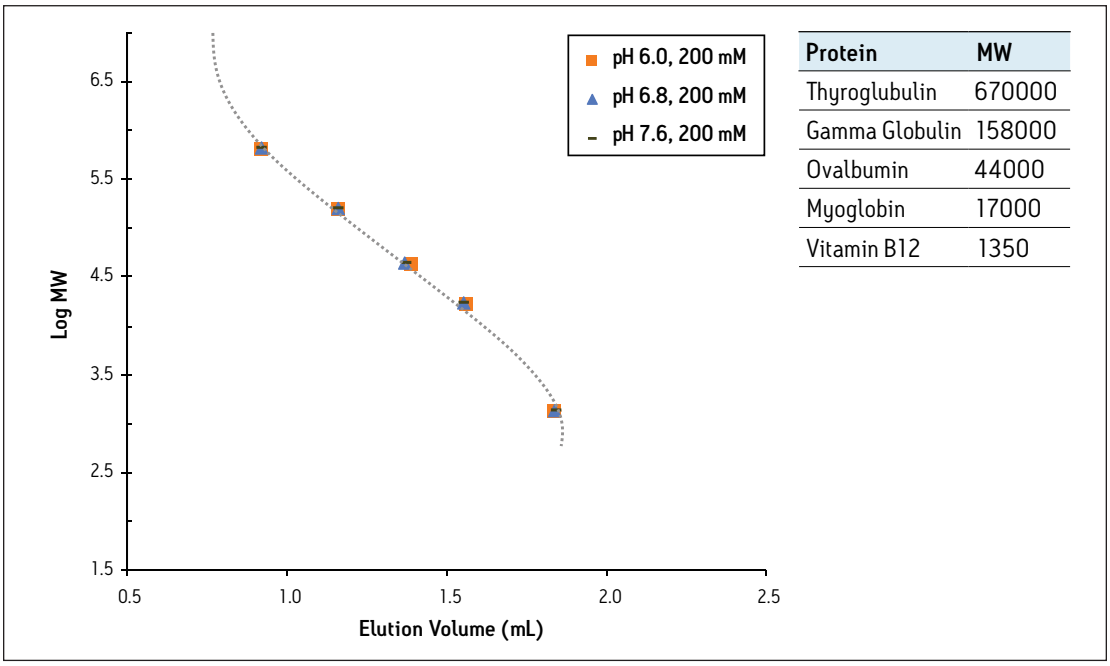
Figure 2. Effect of sodium chloride on the SEC separation of a murine mAb.

The effect of buffer ionic strength on the observed amount of aggregate was also analyzed. In the experiments previously described, increasing sodium chloride concentrations from 50–200 mM results in greater observed recovery of aggregates (see inset). The aggregate % area increased from 1.18% to 5.27%. However, at concentrations above 200 mM sodium chloride, aggregate quantitation did not change significantly. This suggests minimal secondary interactions above this concentration.

The variability in retention time and changes in peak shape indicate secondary interactions between the protein and the column packing material, as has been observed for the materials used to prepare SEC packings. These interactions, which can lead to increased retention and irregular peak shape, are easily minimized by increasing the ionic strength of the buffer.

Mobile-phase pH

Given the influence of pH on both secondary interactions and the structure of the protein, SEC method development should also evaluate pH and its influence, if any, on the separation and quantitation of the biomolecule. The BEH200 column was evaluated with the protein standard mix from pH 6.0–7.6. This analysis was performed to evaluate the effect of pH on the column calibration. The pH range was based on the buffering capacity range of the sodium phosphate buffer. The sodium chloride concentration was kept constant at 200 mM. The results show no significant shift in retention times were observed for the proteins. All of the retention times were within 0.02 minutes (Figure 3), suggesting pH has no significant affect on calibration under the conditions tested.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 3. Effect of mobile-phase pH on a SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

To test the effect of pH on a typical biotherapeutic, the mAb was analyzed under the same conditions (pH 6.0 to 7.6, 200 mM sodium chloride) (Figure 4). As the pH increases from 6.0 to 7.6, the mAb monomer peak height decreases and shifts to earlier retention time (Figure 4). However, the aggregate quantitation over the pH range from pH 6.0–7.6 was within 0.4% (5.7–5.3%), indicating mobile phase pH has no effect on the measured proportion.

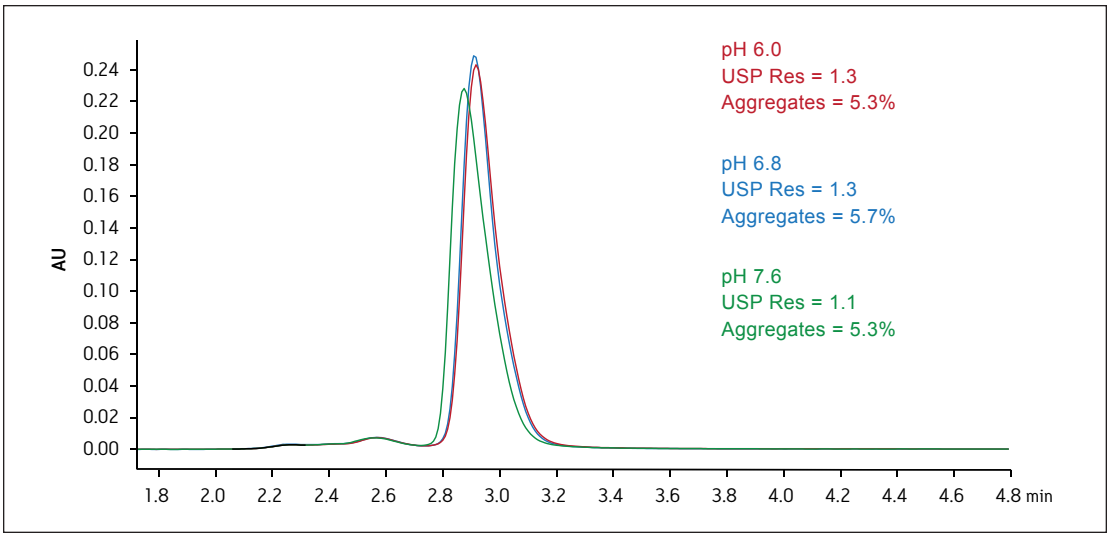


Figure 4. Effect of mobile-phase pH on a SEC separation of murine mAb. Mobile phase: 25 mM sodium phosphate, 200 mM sodium chloride pH 6.0–7.6.

The buffer pH can influence secondary interactions. In this case we observe changes for monomer elution profile but not for the dimer. This suggests a change in the hydrodynamic radius rather than a change in the secondary interactions.

Flow rate

Resolution in size based separations can be influenced by linear velocity. Although using lower flow rates results in longer run times, the increased resolution gives greater confidence in aggregate quantitation. In addition, the use of sub-2-µm particles for this application allows the use of shorter columns. Thus, the throughput achieved with UPLC-SEC is still greater than that of traditional HP-SEC.[3]

In order to test the reliability and robustness of the method, the effect of flow rate on the SEC separation of a mAb was analyzed. Triplicate injections of the mAb were analyzed at flow rates of 0.2 and 0.4 mL/min (Figure 5). Analysis of the separations shows no significant change in aggregate quantitation with flow rate. However, decreasing the flow rate did increase the monomer/dimer resolution by 15%. While the lower flow rates allow for increased resolution, higher flow rates allow for greater throughput and faster analyses times.

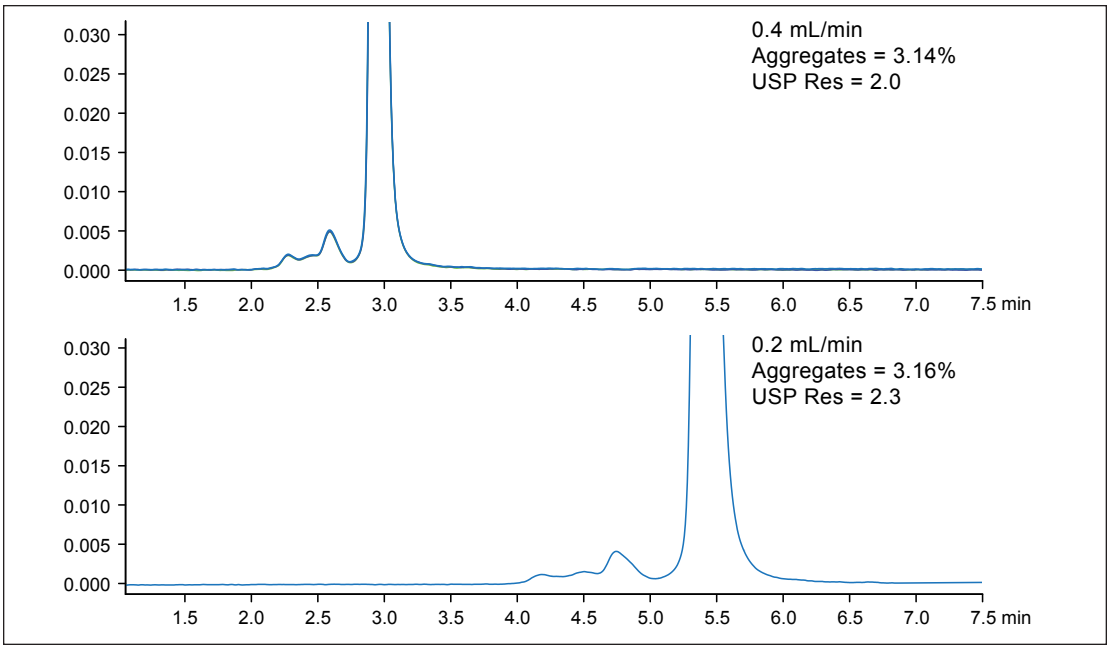
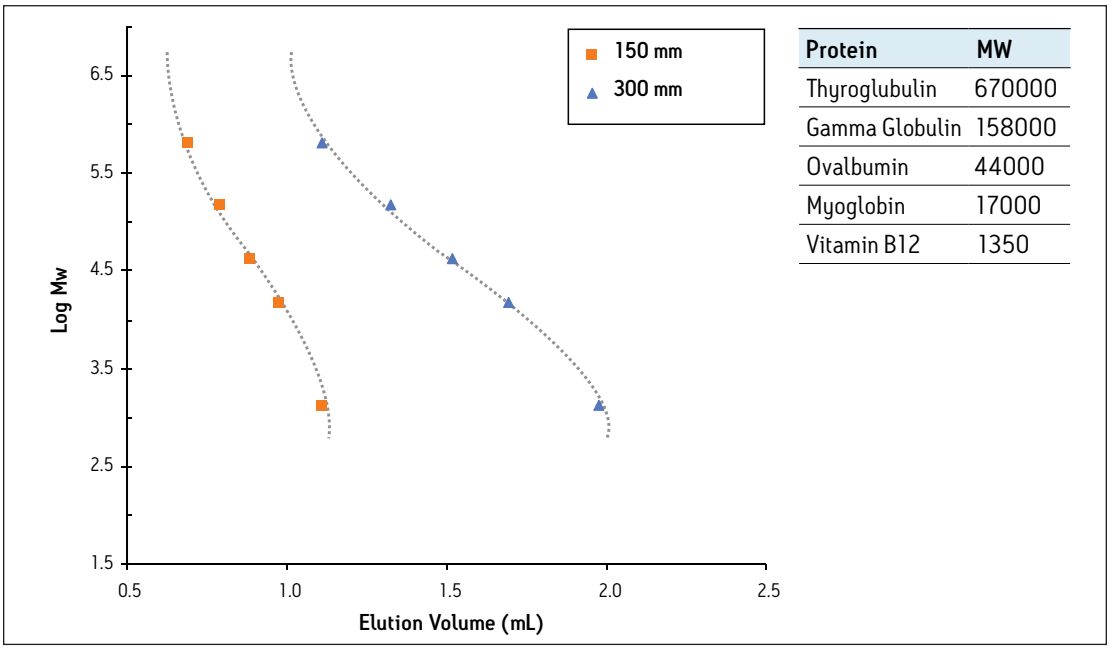


Figure 5. Effect of flow rate on a SEC separation of a murine mAb.

Column length

Improvements in SEC resolution can also be gained by increasing column length. SEC separations are based on diffusion into and out of the pores of the column's packing material. The larger proteins cannot access the pores and thus elute earlier. The smaller the protein, the longer the residence time within the pores, which results in longer retention times. These principles allow for greater resolution with longer column lengths.

To demonstrate these effects, a set of protein standards were run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Comparison of the calibration curves reveals a shallower slope for the 300 mm column as compared to the 150 mm, demonstrating the higher resolving power achievable on a longer column (Figure 6).



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 6. Effect of column length on SEC calibration curve.

Note: Calibration points deviate from a straight line because of protein shape in solution.

The effect of column length was also tested for the SEC separation of a murine mAb run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Under the same conditions, the longer column provided improved resolution for the monomer/dimer (2.07 to 2.80) (Figure 7) with comparable aggregate quantitation. The improved resolution is also apparent in the monomer peak tail, in which a small, lower molecular weight peak is partially resolved on the 300 mm but not on the 150 mm column. However, the improved resolution is accompanied by an increase in retention time (from 3.0 to 6.0 minutes).

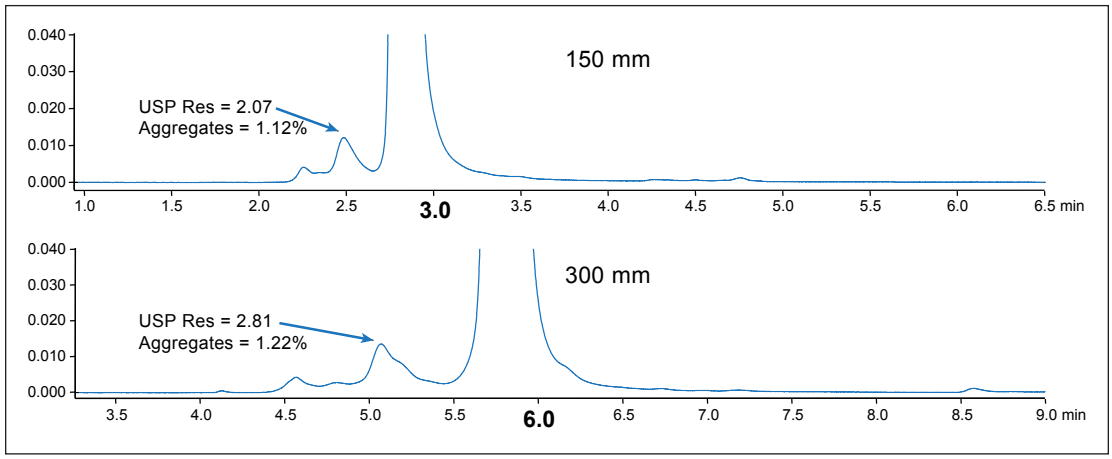


Figure 7. Effect of column length on a SEC separation of murine mAb.

These results indicate that column length can be a useful tool in method development. Depending on the method requirements, column length can be chosen to either provide improved resolution or higher throughput. For example, in a manufacturing environment a longer column allows for improved resolution. While in discovery or development, a shorter column allows for faster analysis time and high throughput.

CONCLUSIONS

Size-exclusion chromatography continues to be a standard technique for the analysis of monoclonal antibodies and their aggregates. However, as in any SEC method, a thorough evaluation needs to be performed to develop an optimum separation. While HP- SEC can be time consuming, the use of UP-SEC allows method optimization to be predicted in less time with a high level of efficiency and higher degree of confidence. In addition, the use of Auto•Blend Plus Technology makes it easier and less labor intensive to systematically examine the effects of mobile phase on protein structure and on secondary interactions.

As described, optimization should evaluate a number of conditions, including mobile phase (pH and ionic strength), flow rate, and column length. In addition – although not described in detail – injection volume, mass load and temperature can also affect SEC separations. Therefore, a suggested set of experiments should evaluate:

1. Ionic strength
2. pH
3. Column length
4. Flow rate
5. Other variables (mass load, injection volume, temperature, etc.)

These experiments should incorporate information on the biological activity of the protein. If factors affecting the proteins biological activity are limited, PBS is the recommended starting mobile phase.

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Advanced HPLC Size-Exclusion Chromatography for the Analysis of Macromolecular Proteins Using 3.5 µm Ethylene Bridged Hybrid (BEH) Particles

Stephan Koza, Susan Serpa, Hua Yang, Edouard Bouvier, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution of macromolecular proteins by SE-HPLC
- Outstanding column stability and reliable column-to-column reproducibility
- Both 200Å and 450Å pore sizes provide a broad protein size separation range
- 2-fold increased sample throughput with minimal compromise in resolution compared to traditional HPLC separation

WATERS SOLUTIONS

XBridge® Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns

Alliance® HPLC System

Auto•Blend Plus™ Technology

BEH200 and BEH450 SEC Protein Standard Mix

KEY WORDS

Size-Exclusion Chromatography, SEC, HPLC, proteins, SE-HPLC, Gel Filtration Chromatography, IgG, IgM

INTRODUCTION

In 2010 Waters first introduced a 200Å pore-size size-exclusion chromatography (SEC) based on UPLC® Technology.[1] These size-exclusion UPLC (SE-UPLC) columns consist of sub-2-µm diameter ethylene bridged hybrid (BEH) particles, which are more structurally and chemically stable than pure silica-based particles. It is the enhanced structural stability of these particle that has indeed enabled the advent of SE-UPLC. However, the small particle-size and narrow 4.6 mm internal diameter of SE-UPLC columns are not optimal for use with an HPLC system. As a result, Waters has introduced HPLC-compatible, 3.5 µm particle diameter and 7.8 mm internal diameter size-exclusion HPLC columns (SE-HPLC) based on the robust BEH chemistry. This provides laboratories with HPLC instrumentation a means to take advantage of the benefits provided by this unique particle technology including its capability to withstand higher back pressures as compared to silica-based SEC particles. This note will highlight the performance characteristics of both the 200Å and 450Å pore-size versions of these columns, designed for the separation of macromolecular proteins, with respect to resolution, column-to-column reproducibility, and column stability. Additionally, the distinct advantages in terms of resolution and sample-throughput that these sub-4-µm packing material offers over larger (5 and 8 µm) standard HPLC particle sizes for the separation of large proteins will also be shown.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures (Waters and Sigma-Aldrich). Sample concentrations were 1.0 mg/mL (nominal) unless noted otherwise.

Method conditions

LC conditions

LC system:	Alliance HPLC or ACQUITY UPLC® H-Class Bio System with 30 cm Column Heater
Detection:	Alliance HPLC TUV Detector ACQUITY UPLC TUV Detector with 5 mm titanium flow cell
Wavelength:	280 or 214 nm
Columns:	Waters XBridge Protein BEH SEC, 200Å, 3.5 µm, 7.8 x 150 mm (p/n 176003595) and 7.8 x 300 mm (p/n 176003596) XBridge Protein BEH SEC, 450Å, 3.5 µm, 7.8 x 150 mm (p/n 176003598) and 7.8 x 300 mm (p/n 176006599)

Comparator

Columns:	250Å, 5 µm, Silica-DIOL SEC, 7.8 x 300 mm Silica-DIOL SEC, 450Å, 8 µm, 7.8 x 300 mm
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	0.84 mL/min
Mobile phases:	25 mM sodium phosphate, 150 mm sodium chloride, pH 7.2 (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Standard:	BEH200 SEC Protein Standard Mix (p/n: 186006518) BEH450 SEC Protein Standard Mix (p/n: 186006842) Intact mAb Mass Check Standard (p/n: 186006552)
Sample vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with cap and preslit PTFE/Silicone Septa, 1 mL (p/n: 186000385DV)

Data management

Chromatography software:	Empower® Pro (v2 and v3)

RESULTS AND DISCUSSION

The benefits provided by BEH Technology™ when used in the manufacturing of size-exclusion UPLC (SE-UPLC) packing materials for the analysis of peptides and proteins; have been previously described.[2,3] However, the diameter of these UPLC particles precluded their use in column dimensions applicable to HPLC instrumentation. In order to take advantage of the chemical and structural capabilities of BEH particle technology for the SEC separation of proteins and other macromolecules on HPLC instrumentation 7.8 mm ID columns packed with 3.5 µm BEH particles with pore sizes of either 200Å or 450Å have been introduced. These two column types provide a broad molecular weight range of SE-HPLC separations to include biological macromolecules with large radii of hydration (Rh), ranging from approximately 10 KDa to nearly 2 MDa. As part of this evaluation, the separation efficiency advantages of this packing material with respect to larger particle-size (5 and 8 µm) HPLC packing materials, and the critical performance characteristics of column-to-column reproducibility and lifetime stability will be demonstrated. In addition, this note will define the protein size-separation range of these two columns.

Advantages of reduced BEH particle size

Due to the significantly higher extra-column dispersion volumes and lower pressure limits of HPLC systems relative to UPLC Systems the resolution benefits provided by UPLC size-exclusion particles have not been available to laboratories that currently use HPLC instrumentation. In an effort to provide optimal resolutions for the SE-HPLC separation of proteins, a series of columns have been introduced based on BEH particle technology. To demonstrate their performance, protein molecular weight standards and a monoclonal IgG standard were separated on 250Å pore-size silica-based SEC column (5 µm, 7.8 x 300 mm) and on a 200Å pore-size BEH-based SEC column (3.5 µm, 7.8 x 300 mm) using the same Alliance HPLC System and aqueous mobile phase conditions (Figure 1).

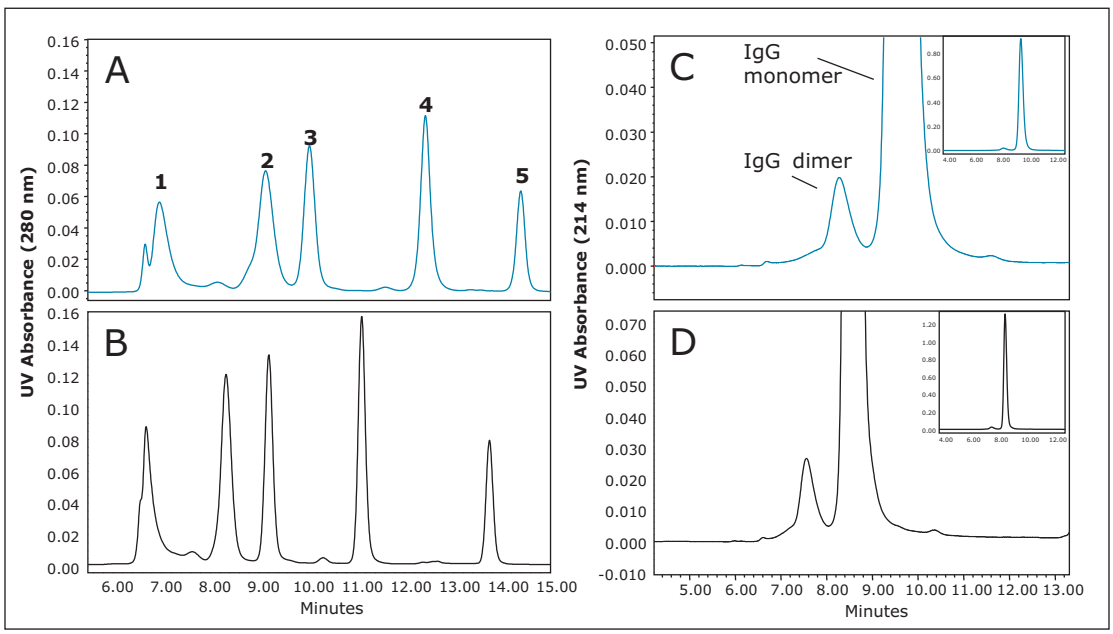

Figure 1. Shown is a comparison of separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 250Å, silica-based 5 µm (Frames A and C) and 200Å, BEH 3.5 µm (Frames B and D) SEC columns. Both columns were the same dimensions (7.8 x 300 mm) and separations were performed with the same flow rate (0.84 mL/minute) and the same sample loads. Peak identities for chromatograms A and B are: 1) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms C and D the molecular weights of the IgG monomer and dimer are approximately 150 KDa and 300 KDa, respectively.

The flow rates and injection volumes used were equivalent. Improved sensitivity and narrower peak widths were observed on the 3.5 µm packing material across the separation range of the molecular weight standards. USP resolution values (half-height measurement) calculated for the separation between the IgG monomer (MW=150 KDa) and dimer (MW=300 KDa) forms demonstrated an improvement of over 40% for the 3.5 µm particle over the resolution observed for the 5 µm particle size column. This improvement in resolution approaches the improvement that would be predicted by doubling the column length (Rs ∝ √L). Similar results comparing the chromatograms generated for the 450Å pore-size, silica-based, SEC column (8 µm, 7.8 x 300 mm) to the 450Å pore-size BEH-based SEC column (3.5 µm, (7.8 x 300 mm)) were observed (Figure 2). However, in this comparison the relative improvement observed for the separation between the IgG monomer and dimer is approximately 75%. This is due to the greater decrease in particle size between these two columns as compared to the smaller pore size 250Å silica-based and BEH 200Å particles. As a general observation, it should be noted from these data that the BEH 450Å SEC column provides an outstanding separation of both the dimeric and multimeric aggregate forms in this IgG sample.

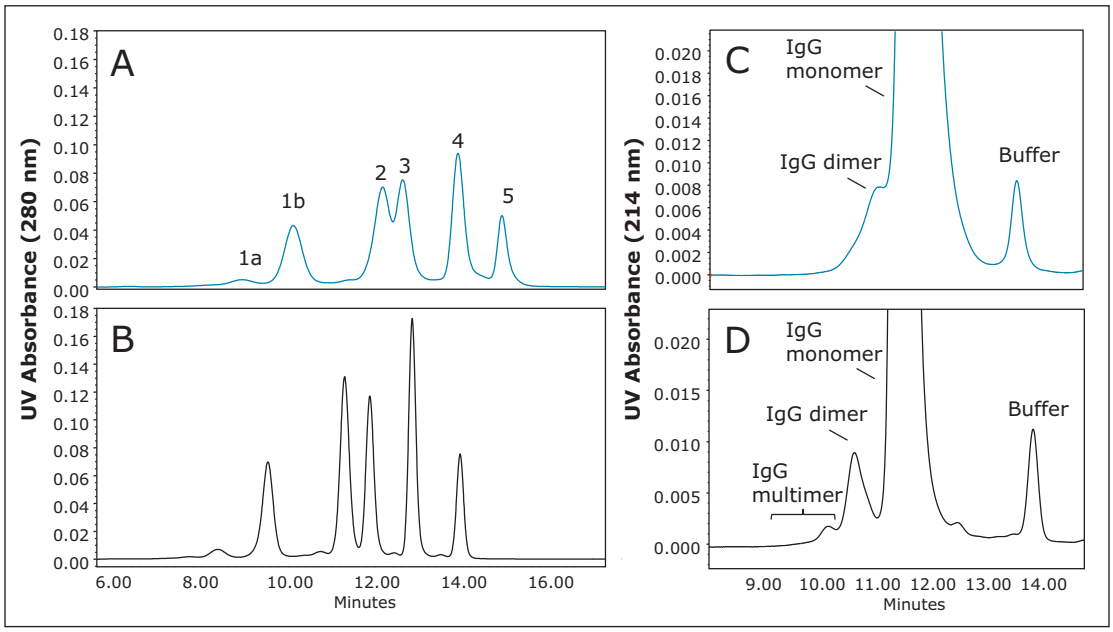

Figure 2. Shown is a comparison of separations of Waters BEH450 SEC Protein Standard Mix (p/n: 186006842) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 450Å, silica-based 5 µm (Frames A and C) and 450Å, BEH 3.5 µm (Frames B and D) SEC columns. Both columns were the same dimensions (7.8 x 300 mm) and separations were performed with the same flow rate (0.84 mL/minute) and with the same sample loads. Peak identities for chromatograms A and B are: 1a) thyroglobulin dimer (1.3 MDa), 1b) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms in frames C and D the molecular weights of the IgG monomer, dimer, and multimer are approximately 150 KDa ,300 KDa, and ≥450 KDa, respectively.

Advantages of BEH Particle Strength

BEH SEC particles have improved mechanical strength in comparison to silica-based particles. An opportunity presented to the analyst due to this characteristic is the ability to run at higher flow rates and pressures than can be tolerated by traditional SE-HPLC columns. By increasing the flow rate, the analysis time can be reduced proportionally in SEC, however, it should be noted that SEC resolution decreases as a function of flow rate. Taking these characteristics under consideration, if higher SE-HPLC sample throughput is an essential requirement the 3.5 µm BEH SE-HPLC can accommodate this demand. In this study a comparison (Figure 3) was made between a traditional 250Å, 5 µm silica based SE-HPLC column (7.8 x 300 mm). and a 3.5 µm BEH-based SE-HPLC column (7.8 x 300 mm). The 5 µm silica-based SE-HPLC column flow rate was set to 1.0 mL/minute (maximum flow rate: 1.2 mL/minute) and the 3.5 µm BEH SE-HPLC column was set to 2.0 mL/minute (maximum flow rate: 2.7 mL/minute). Comparable molecular weight standard profiles are observed, with the exception that the larger pore-size of the 250Å, 5 µm silica-based particle provides improved resolution of the thyroglobulin dimer peak (1.3 MDa) than what is observed on the 200Å, 3.5 µm BEH-based particle. While increasing the flow rate by a factor of two decreases the analysis time proportionally there will be a concomitant loss of resolution. As an example, the resolution observed between IgG and BSA was 2.5 on the 3.5 µm BEH based column as compared to 2.0 on the 250Å, 5 µm silica-based column (data not shown) at a flow rate of 1.0 mL/minute. However, at a flow rate of 2.0 mL/minute, the resolution on the 3.5 µm BEH-based column decreased approximately 25% to a resolution of 1.9.

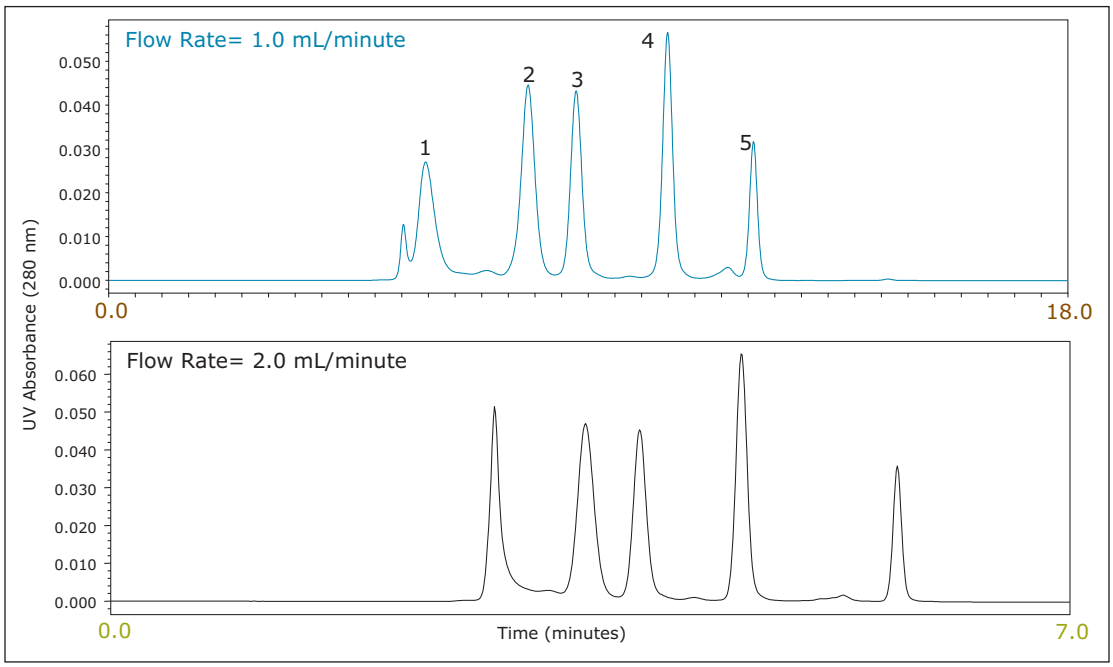

Figure 3. Shown is a comparison of separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) on 250Å, silica-based 5 µm SEC column, separated at 1.0 mL/minute (Frame A) and on a 200Å, BEH 3.5 µm (Frame B) SEC column separated at 2.0 mL/minute. Both columns were the same dimensions (7.8 x 300 mm) and the same sample loads were used. The time axis for the main chromatograms have been normalize, the actual times of the separations are provided in the inset. Peak identities for chromatograms A and B are: 1) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da).

Note: Comparable molecular weight standard profiles are observed, with the exception that the larger pore-size of the 250Å, 5 µm silica-based particles provide improved resolution of the thyroglobulin dimer peak (1.3 MDa) than what is observed on the 200Å, 3.5 µm BEH-based particle. Use of Waters XBridge Protein BEH SEC, 450Å, 3.5 µm is recommended for the analysis of proteins, such as thyroglobulin and its dimer, whose molecular weights exceed those recommended be analyzed on the XBridge Protein BEH SEC, 200Å, 3.5 µm Column.

XBridge Protein BEH SEC 200Å, and 450Å, 3.5 µm Columns: reproducibility and stability

Major concerns that an analyst has when selecting an SEC column for method development or use in a validated method are column-to-column and batch-to-batch reproducibility as well as obtained column lifetime when used in methods. Shown in Figure 4 is an overlay of the chromatograms for a series of molecular weight standards for both the 200Å and 450Å, 3.5 µm SEC columns in a (7.8 x 300 mm). These chromatograms demonstrate the reproducibility of 6 SEC columns packed from 3 different production lots of packing material. For these standards, and at a flow rate of 0.84 mL/minute, the retention time standard deviations for the 200Å pore size, SEC column ranged from a minimum of 0.037 minutes to 0.084 minutes with an average standard deviation of 0.064 minutes for all components labeled in Figure 1. For the 450Å pore size, SEC column the retention time standard deviations ranged from a minimum of 0.045 minutes to 0.068 minutes with an average standard deviation of 0.060 minutes.

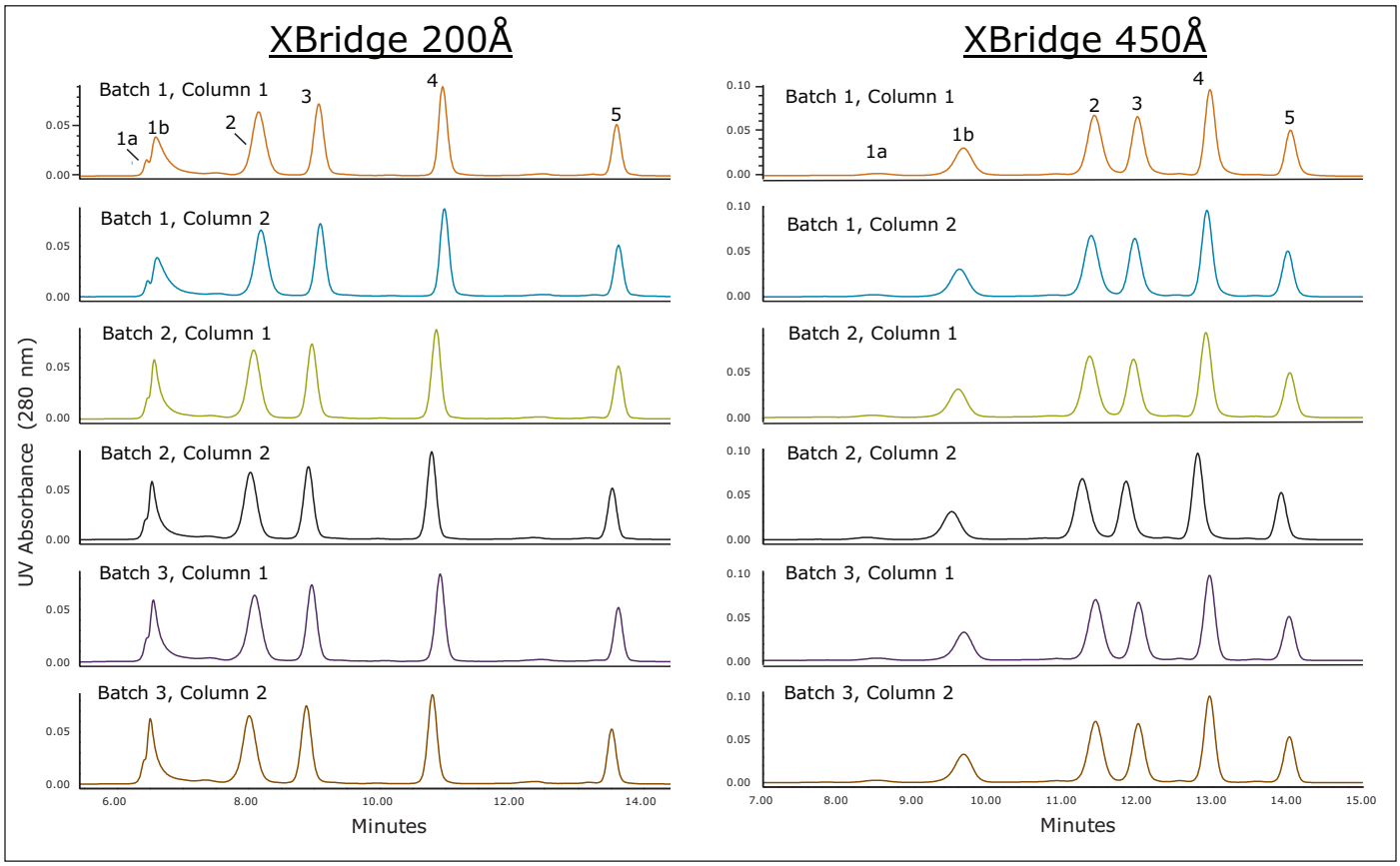

Figure 4. Shown are overlays of the separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) and BEH450 SEC Protein Standard Mix (p/n: 186006842) on 200Å and 450Å BEH 3.5 µm SEC columns. Two columns (7.8 x 300 mm) were packed from 3 individual manufacturing batch of particles to evaluate both column-to-column and batch-to-batch reproducibility. Peak identities are: 1a) thyroglobulin dimer (1.34 MDa), 1b) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). Separations were performed on an ACQUITY UPLC H-Class Bio System.

The stability of the 200Å and 450Å, 3.5 µm SEC columns (7.8 x 300 mm) was evaluated by injecting a series of standards over the course of over 600 total injections. Given that the stability of silica-based SEC columns can be deleteriously altered by mildly basic pH levels, the pH of the mobile phase was set to 7.2, equivalent to that of phosphate buffered saline (PBS) buffer. Shown in Figures 5 and 6 are comparisons of the profiles obtained for the molecular weight standards and the IgG standard from the start to the finish of the study for both columns.

The resolution between two of the critical peak pairs, IgG and BSA, and IgG Dimer and IgG monomer were determined for each column. Both columns demonstrated remarkable stability with only modest depreciation of the calculated resolutions as highlighted in the Figure caption. These data demonstrate that XBridge Protein BEH SEC columns containing 3.5 µm particles can provide the reproducibility and stability needed to develop reliable assays and run them routinely in a quality control environment.

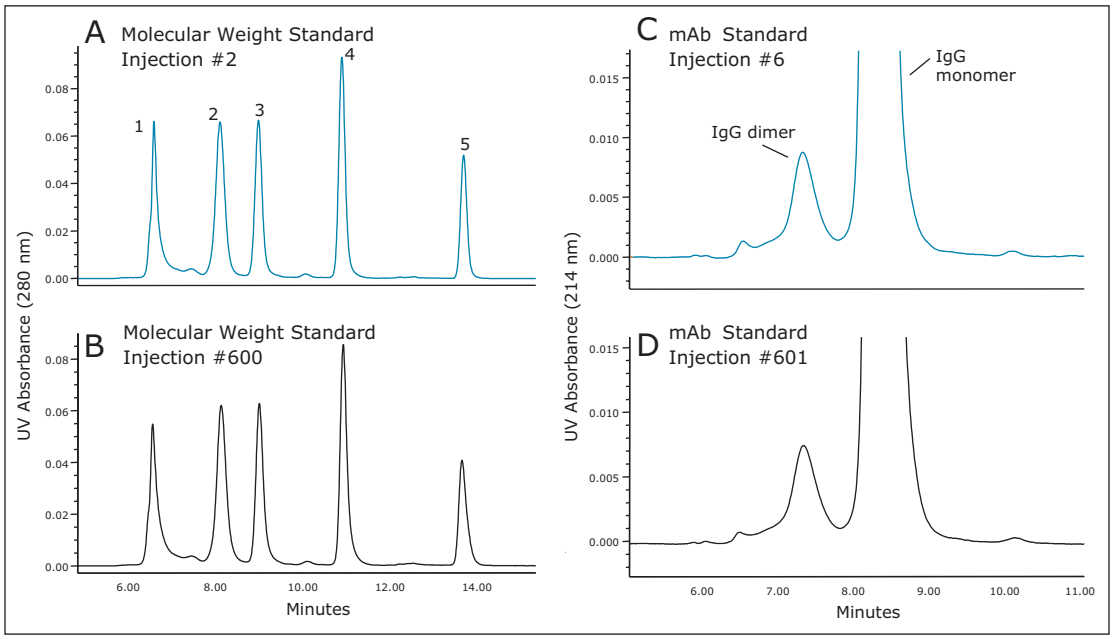

Figure 5. Shown are comparisons of column lifetime study separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 200Å BEH 3.5 µm SEC column (7.8 x 300 mm). Peak identities for chromatograms A and B are: 1) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms of the mAb standard the molecular weights of the IgG monomer and dimer are approximately 150 KDa and 300 KDa, respectively. Separations were performed on ACQUITY UPLC H-Class Bio System.

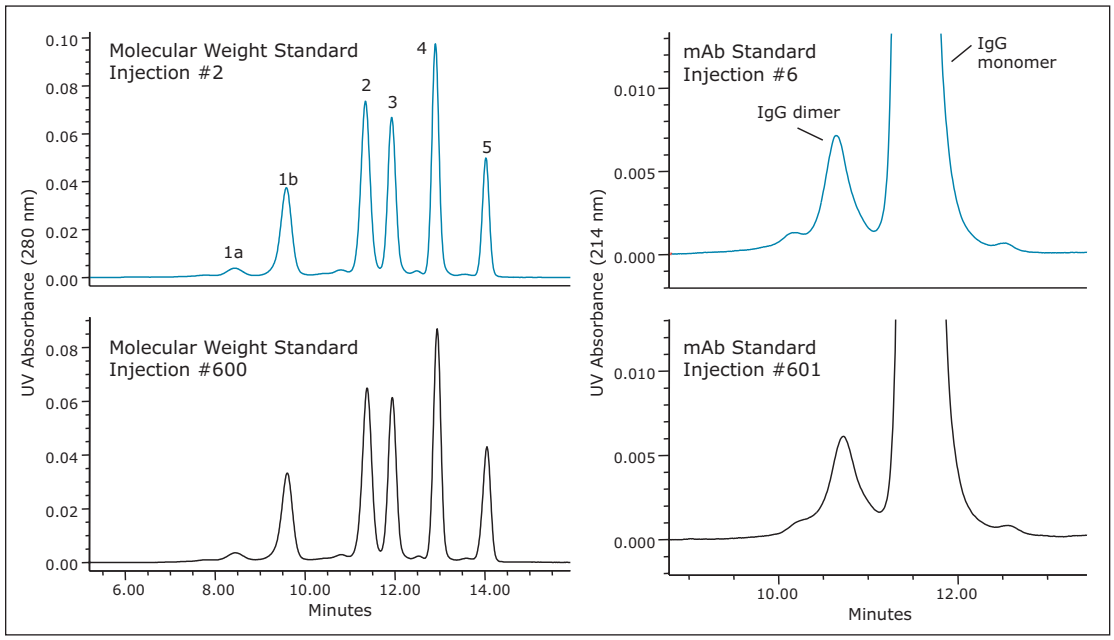

Figure 6. Shown are comparisons of column lifetime study separations of Waters BEH450 SEC Protein Standard Mix (p/n: 186006842) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 450Å BEH 3.5 µm SEC column (7.8 x 300 mm). Peak identities are: 1a) thyroglobulin dimer (1.34 MDa), 1b) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms of the mAb standard the molecular weights of the IgG monomer and dimer are approximately 150 KDa and 300 KDa, respectively. Separations were performed on an ACQUITY UPLC H-Class Bio System.

Molecular weight range

Comparisons were made between the XBridge Protein BEH SEC 450Å and 200Å, 3.5 µm columns for their ability to resolve a series of defined standards. The protein molecular weight calibration curves are shown in Figure 7. For proteins, the linear molecular weight range for the 200Å pore-size column is estimated to be from approximately 10 KDa to 450 KDa, whereas the 450Å pore-size column is estimated to be from approximately 50 KDa to over 1.3 MDa. This upper limit is based on the chromatographic separation observed (Figure 2) for thyroglobulin (669 KDa) and it dimer (1.3 MDa). The 450Å column separation of IgM pentamer (900 KDa) and IgM dipentamer (1.8 MDa) as shown in Figure 8 shows partial resolution between these two forms, which is indicative that the pore volume accessible to the dipentamer is limited, thereby demonstrating that 1.8 MDa is beyond the linear molecular weight range of this column and close to practical upper molecular weight limit for this column. This higher molecular weight range may be of use when analyzing multimeric protein aggregates or proteins conjugated to compounds that have relatively large radius of hydration values such as long chain polyethylene glycols or when running proteins under denaturing SEC conditions.

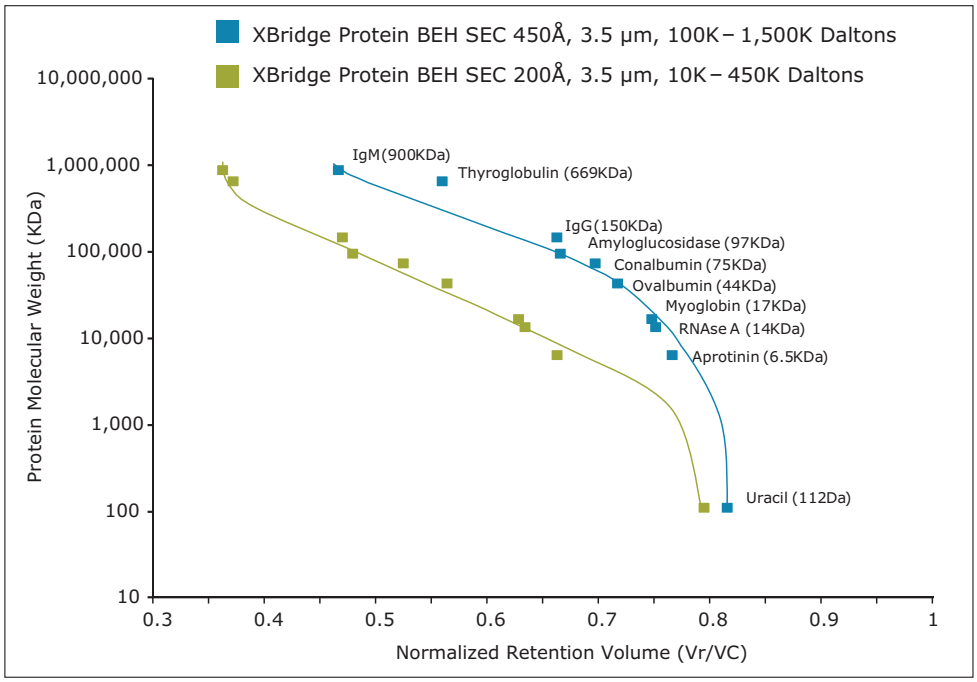

Figure 7. Shown are calibration curves of various proteins, peptides, and uracil generated for both the BEH 200Å and 450Å, 3.5 µm SEC columns.

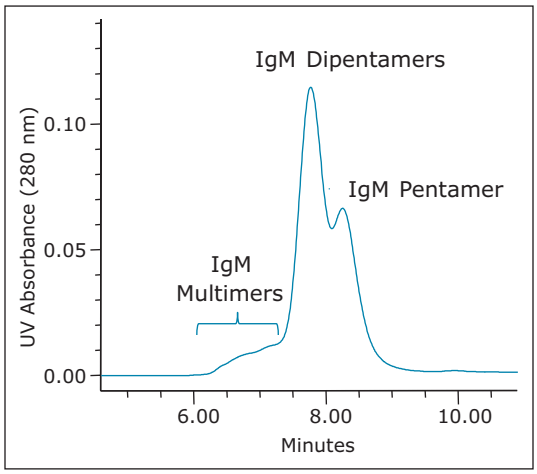

Figure 8. Shown is the separation of IgM pentamer, IgM dipentamer, and the multimeric forms of pentameric IgM separated on a BEH, 450Å, 3.5 µm SEC column. The molecular weight of the proteins are: IgM pentamer (900 KDa), IgM dipentamer (1.8 MDa), and IgM multimers (≥2.7 MDa). Separation was performed on an ACQUITY UPLC H-Class Bio System.

CONCLUSIONS

A reliable, high resolving, size-exclusion method is often an integral part of the quality assessment of a protein biopharmaceutical and also has a key role in the evaluation of protein samples in other areas of research. The introduction of HPLC-compatible, XBridge Protein BEH SEC 200Å and 450Å, columns containing 3.5 µm particles provide improved component resolution in LC-based SEC separations compared to use of traditional silica-based SEC columns containing 5 µm particles. In addition, higher throughput analyses are possible due to the structural strength of the BEH particle. This critical particle strength characteristic in combination with use of stable diol-bonded particles work to deliver outstanding column lifetimes. As part of the Waters' quality manufacturing guidelines, these columns are produced to rigorous tolerances and quality tested with relevant analytes. Although not presented within this report, these HPLC separations are also directly scalable to SE-UPLC separations using ACQUITY UPLC Protein BEH SEC Columns containing 1.7 µm or 2.5 µm diameter particles and narrower column internal diameters (4.6 mm I.D.) which can proved even greater resolution and sample-throughput when coupled with UPLC capable chromatographic systems.[4]

The XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns provide:

- HPLC-based SEC resolution of proteins from 10–1,500K Daltons with higher throughput capability
- Outstanding SEC column life
- Less non-desired, protein/column interactions than silica-based SEC columns
- Comprehensive testing to provide unmatched column consistency and increased confidence in validated methods
- Complement ACQUITY UPLC-based SEC columns for seamless method transfer based on application needs

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4. Stephan Koza, and Kenneth J. Fountain. Successful Transfer of Size-Exclusion Separations between HPLC and UPLC. Waters Corporation, Application Note, [serial on the Internet]. 2014; (720005193EN).

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Successful Transfer of Size-Exclusion Separations Between HPLC and UPLC

Stephan Koza, Susan Serpa, Edouard Bouvier, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Seamless scalability and transfer of protein SEC methods between UPLC and HPLC instrumentation
- Simplifying method transfer between laboratories with different instrumentation
- Enabling UPLC method scale-up for peak characterization
- 200Å and 450Å pore sizes provide a broad protein size separation range

WATERS SOLUTIONS

XBridge® Protein BEH SEC 200Å and 450Å, 3.5 µm Columns

ACQUITY UPLC® Protein BEH SEC, 200Å, 1.7 µm, and 450Å, 2.5 µm Columns

Alliance® HPLC System

ACQUITY UPLC H-Class Bio System

Empower® 3 Software

Waters BEH200 and BEH450 SEC Protein Standard Mixture and mAb Standard

KEY WORDS

Size-exclusion chromatography, UPLC, HPLC, method transfer, aggregates

INTRODUCTION

Over the last several years Waters has produced the highest resolving and highest sample throughput size-exclusion chromatography (SEC) columns available for protein and peptide analysis.[1,2] These size-exclusion UPLC® (SE-UPLC) columns consist of 1.7 µm diameter ethylene bridged hybrid (BEH) particles with 200Å or 125Å pore sizes, or a 2.5 µm diameter BEH particle with a 450Å pore size. Based on observations, SE-UPLC technology has been adopted in many biopharmaceutical analytical laboratories as an important tool to monitor protein aggregation during product development due to the high sample throughput that it provides. However, often times SE-UPLC methods cannot be transferred to other laboratories due to the lack of available UPLC instrumentation. Therefore, in an effort to provide the capability to transfer SEC methods between UPLC and HPLC instruments, Waters has introduced 3.5 µm particle diameter BEH-based size-exclusion HPLC (SE-HPLC) columns specifically for use on traditional HPLC instrumentation. These columns provide the analyst with the unique capability to use consistent particle chemistries that can be directly scaled between HPLC and UPLC instrumentation. This application note will highlight the considerations that must be made when transferring an SEC method between UPLC and HPLC columns and systems.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures (Waters® and Sigma-Aldrich®). Sample concentrations were 1.0 mg/mL (nominal) unless noted otherwise.

Method conditions

LC conditions

LC system:	Waters Alliance HPLC or ACQUITY UPLC H-Class Bio System with 30 cm Column Heater
Columns:	XBridge Protein BEH SEC, 200Å, 3.5 µm, 7.8 x 300 mm (p/n: 176003596); XBridge Protein BEH SEC, 450Å, 3.5 µm, 7.8 x 300 mm (p/n: 176003599); ACQUITY UPLC Protein BEH SEC, 200Å, 1.7 µm, 4.6 x 300 mm (p/n: 186005226); ACQUITY UPLC Protein BEH SEC, 450Å, 2.5 µm, 4.6 x 300 mm (p/n: 176002997)
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	0.84 mL/min
Mobile phases:	5 mM sodium phosphate, 150 mm sodium chloride, pH 7.2 (prepared using Auto•Blend Plus™ Technology or 5.5% A: 100 mM NaH_2PO_4, 14.5% B: 100 mM Na_2HPO_4, 15.0% C: NaCl, 65% D:H_2O)
Gradient:	Isocratic
Standard:	BEH200 SEC Protein Standard Mix (p/n: 186006518); BEH450 SEC Protein Standard Mix (p/n: 186006842); Intact mAb Mass Check Standard (p/n: 186006552)
Sample Vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and pre-slit PTFE/Silicone Septa, 1 mL (p/n: 186000385DV)
Detection:	Alliance HPLC TUV Detector; ACQUITY UPLC TUV Detector with 5 mm Titanium flow cell
Wavelength:	280 or 214 nm
Chromatography Software:	Waters Empower Pro (v2 and v3)

RESULTS AND DISCUSSION

LC system considerations

The performance of the LC system in an SEC separation can have a significant effect on the quality of the chromatographic resolution that can be achieved. Fundamentally, this can be represented by the equation highlighted in Figure 1, which has been adapted from Gritti and Guiochon.[3] From this equation, the total peak variance σ^2_{Total} can be derived from the sum of the peak variances that occur prior to the analyte reaching the column ($\sigma^2_{Pre\text{-}Column}$), on the column ($\sigma^2_{Column}$), and after the analyte elutes off of the column ($\sigma^2_{Post\text{-}Column}$). Using this relationship, it can be readily derived that as the peak variance contributions from extra-column dispersion ($\sigma^2_{Pre\text{-}Column}$ and $\sigma^2_{Post\text{-}Column}$) increase and become significant relative to the variance that occurs on the column (σ^2_{Column}), that the SEC separation efficiency will be measurably reduced. Another important concept illustrated by Figure 1 is the additive characteristic of both the pre-column and post-column variances in SEC. This is due to the absence of significant binding interactions between the analyte and the particle surface in SEC. Conversely, in other bind-and-elute based protein separations such as reversed-phase or ion-exchange chromatography, the contributions of $\sigma^2_{Pre\text{-}Column}$ become far less significant due to the strong binding-driven, analyte refocusing that occurs at the head of the column.

$$\sigma^2_{Total} = \sigma^2_{Pre\text{-}Column} + \sigma^2_{Column} + \sigma^2_{Post\text{-}Column}$$

Figure 1. Equation highlighting sources of peak dispersion in SEC. Dispersion in blue (pre-column and post-column) results in reduced resolution when levels are significant relative to dispersion in green (column). Consult text for further explanation.

The practical effect of these considerations is demonstrated in Figure 2, which shows a comparison of the separation of protein standards on two columns packed with the same batch of 3.5 µm diameter, 200Å pore-size BEH particles on an Alliance HPLC System. One of the columns was an experimental column with an internal diameter (I.D.) of 4.6 mm, and the other the commercially available 7.8 mm I.D. column. The flow rates were adjusted to provide equivalent linear velocities for each column. It is readily observed that the resolution for the 4.6 mm I.D. column is significantly lower than that observed for the 7.8 mm I.D. column on the same instrument. As an example, the resolution observed between IgG and BSA is 30% higher for the 7.8 mm I.D. column in comparison to the 4.6 mm I.D. column. To understand why the performance of the 7.8 mm I.D. is markedly better, we can reconsider the relationships of the equation presented in Figure 1. In this equation, the peak dispersion that occurs within the column (σ^2_{Column}) increases with column I.D., however, this is offset by a proportional increase in pore volume ,which increases separation efficiency, with the end result being that the resolution of an SEC column is fundamentally independent of column I.D.. Therefore, as the I.D. of an SEC column increases, the separation efficiency remains constant , assuming packing efficiencies are maintained, while σ^2_{Column} increases. This results in the contribution that $\sigma^2_{Pre\text{-}Column}$ and $\sigma^2_{Post\text{-}Column}$ have to σ^2_{Total} becoming less significant, which practically results in an improved chromatographic separation.

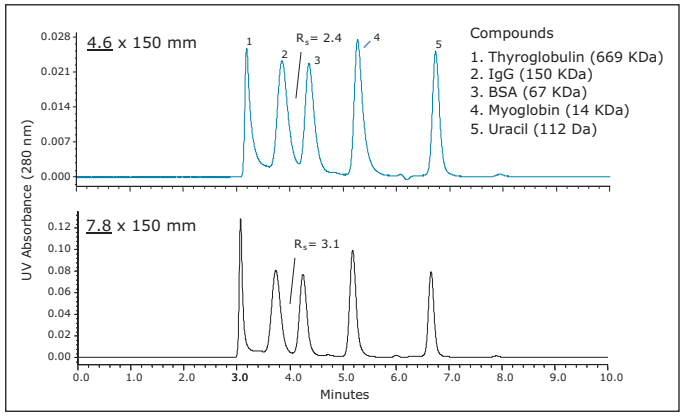

Figure 2. Shown is a comparison of separations of Waters BEH200 SEC Protein Standard Mix (p/n 186006518) link: on two XBridge Protein BEH SEC, 200Å, 3.5 µm Columns. The top frame is an experimental column with a 4.6 mm I.D. and the bottom frame is a commercially available column with a 7.8 mm I.D.. Both columns were run using an Alliance HPLC and the flow rates were scaled based on column I.D. to 0.3 mL/minute for the 4.6 mm I.D. column and 0.86 mL/minute for the 7.8 mm I.D. column. Samples loads were also adjusted for column volume.

Transfer of SEC methods between UPLC and HPLC columns

There are two primary considerations to be made when transferring an SEC method from one column to another. Most importantly the surface chemistry of the particles must be comparable. In addition to matching pore size, size-exclusion particles can potentially have both ionic and hydrophobic interactions with biomolecules, and the nature of these interactions must be comparable if the separations are going to be performed using the same mobile phase and temperature. Secondly, the separation must be appropriately scaled with respect to particle size. The first step in scaling relative to particle size is to match as best as possible the ratio between length and particle diameter for the two columns using Equation 1:

$$L_{HPLC} = \frac{L_{UPLC} \times d_{p,HPLC}}{d_{p,UPLC}}$$

Equation 1: Where: L_{HPLC} and L_{UPLC} are the lengths of the HPLC and UPLC columns (mm), and $d_{p,HPLC}$ and $d_{p,UPLC}$ are the particle diameters of the HPLC and UPLC columns (µm).

Following this, a flow rate can be calculated by running both columns at the same reduced linear velocity, which is proportional to the product of the linear velocity and the particle diameter for a given analyte. Since the flow rate is proportional to the product of the linear velocity and square of the column I.D., the correct scaled flow rate can be readily calculated using Equation 2:

$$F_{HPLC} = \frac{F_{UPLC} \times d_{p,UPLC} \times D^2_{HPLC}}{d_{p,HPLC} \times D^2_{UPLC}}$$

Equation 2: Where: F_{HPLC} and F_{UPLC} are the flow rates (mL/minute) of the HPLC and UPLC columns, and D_{HPLC} and D_{UPLC} are the internal diameters of the HPLC and UPLC columns (mm).

Finally, the injection volume can be scaled for column volume, which is proportional to the product of the square of the column and the column length. This can be represented by Equation 3:

$$V_{HPLC} = \frac{V_{UPLC} \times L_{HPLC} \times D^2_{HPLC}}{L_{UPLC} \times D^2_{UPLC}}$$

Equation 3: Where: V_{HPLC} and V_{UPLC} are the injection volumes of the HPLC and UPLC columns (µL).

We will first demonstrate the successful scaling between the ACQUITY UPLC Protein SEC 200Å, 1.7 µm, 4.6 X 300 mm column and the XBridge Protein SEC, 200Å, 3.5 µm, 7.8 mm I.D. HPLC column format. Given that there is an approximately 2-fold increase in particle size (based on Equation 1) the HPLC column length will need to be twice that of the UPLC Column (60 cm) to yield comparable resolution. This can be accomplished by connecting two 30 cm length HPLC columns in series. It should be noted that if a 15 cm, 1.7 µm particle size UPLC Column had been used, then the separation should be properly transferred to a single 30 cm length, 3.5 µm particle size HPLC column. Given the column I.D. and particle diameter values for each column, and using Equations 2 and 3, the flow rate should be 1.4 times greater and the injection volume should be 5.75 times greater for the HPLC analysis than for the UPLC analysis.

The results of this method scaling are shown in Figure 3 and Figure 4. Figure 3 is a comparison of the separation of the BEH200 SEC Protein Standard Mix, and Figure 4 is a comparison of the separation of the biotherapeutic monoclonal antibody infliximab. The UPLC separations were performed on an ACQUITY UPLC H-Class Bio System and the HPLC separations were performed on an Alliance HPLC System. The mobile phase used for both separations was a phosphate buffered saline (PBS) solution with low ionic strength and a slightly basic pH (25 mM sodium phosphate, 150 mm sodium chloride, pH 7.2). This buffer was selected due to its common use for SEC separations. Additionally, the pH and low ionic strength of this buffer will mask subtle variations in particle surface chemistry that can lead to undesired protein-particle interactions to a much lower extent as compared to higher ionic strength buffers.[4] In both comparisons, the time axis has been normalized. Both pairs of chromatograms show comparable profiles with the primary difference being the analysis time, which is four to five times lower for the UPLC separation. While the HPLC analysis time is significantly longer, it has the same selectivity (profile) as the UPLC separation, thus allowing methods to be developed more rapidly and used on UPLC and then subsequently transferred to HPLC when the receiving lab does not have UPLC instrumentation.

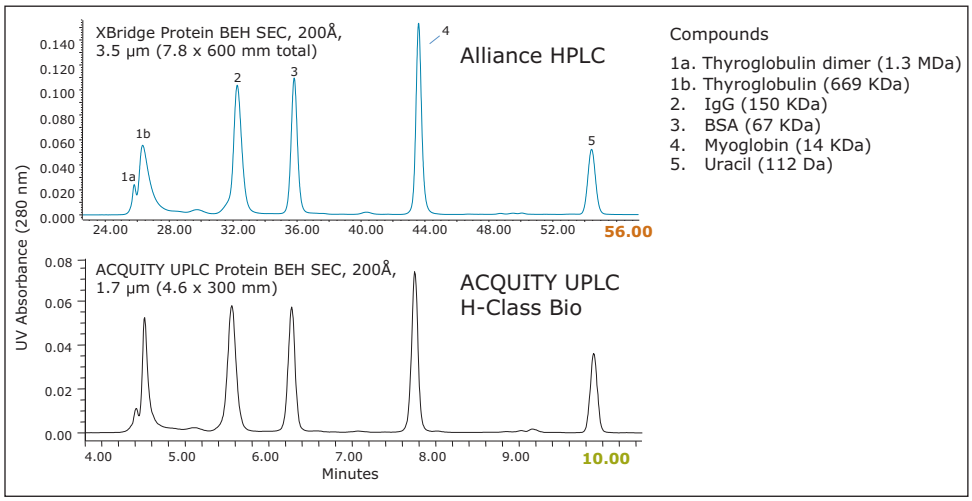

Figure 3. Shown is a comparison of separations of BEH200 SEC Protein Standard Mix (p/n 186006518) on two, XBridge Protein BEH SEC 200Å, 3.5 µm, 7.8 x 300 mm Columns run in series using an Alliance HPLC (top frame) and on an ACQUITY UPLC Protein BEH 200Å, 1.7 µm, 4.6 x 300 mm Column using an ACQUITY UPLC H-Class Bio (bottom frame). The flow rates were scaled based on particle diameter and column I.D. to 0.42 mL/minute for the two HPLC columns run in series and 0.3 mL/minute for the UPLC Column. Samples loads were also adjusted for column volume.

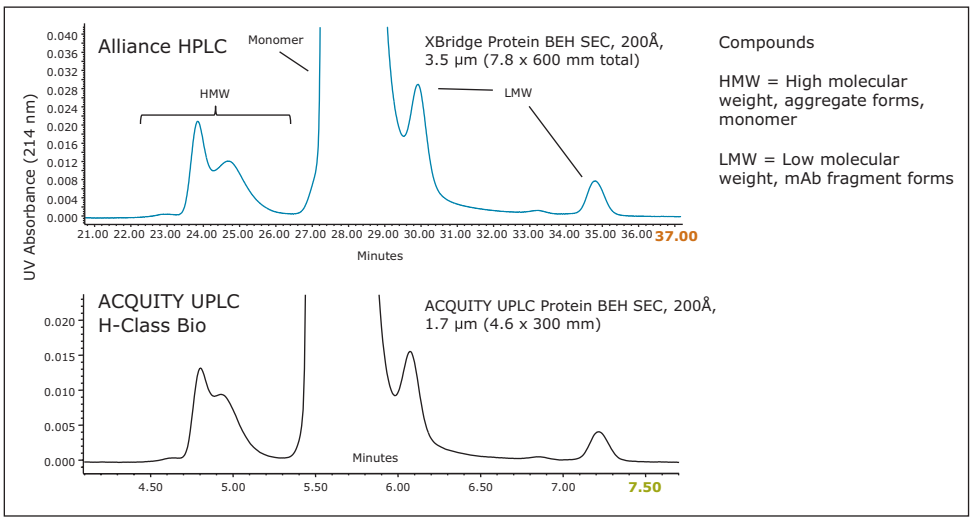

Figure 4. Shown is a comparison of separations of the biotherapeutic monoclonal antibody Infliximab on two, XBridge Protein BEH SEC 200Å, 3.5 µm columns (300 x 7.8 mm I.D.) run in series using an Alliance HPLC (top frame) and on an ACQUITY UPLC Protein BEH 200Å, 1.7 µm column (300 x 4.6 mm I.D.) using an ACQUITY H-Class Bio UPLC (bottom frame). The flow rates were approximately scaled based on particle diameter and column I.D. to 0.5 mL/minute for the two HPLC columns run in series and 0.4 mL/minute for the UPLC Column. Samples loads were also adjusted for column volume. Peak identities for chromatograms are: HMW (high molecular weight, aggregate forms), monomer, and LMW (low molecular weight, mAb fragment forms).

Next, we present the transfer between an ACQUITY UPLC Protein BEH SEC, 450Å, 2.5 µm, 4.6 x 300mm, column and the XBridge Protein BEH SEC, 450Å, 3.5 µm particle size, 7.8 mm I.D. HPLC column format. Using Equation 1, the length of the HPLC column should be 42 cm, which can be approximated by connecting a 30 cm and a 15 cm XBridge SEC, 3.5 µm Column in series. In the event that the method transfer is from a 15 cm length, 2.5 µm UPLC Column, the appropriate length HPLC column would be 21 cm, which is not an available configuration. In this case, a 30 cm HPLC column could be used. However, if matching reduced linear velocities were used, the resolution for the HPLC column should be approximately 20% greater, as the resolution difference is approximately proportional to the square root of compared column lengths.

Figure 5 and Figure 6 demonstrate the effectiveness of scaling from a 30 cm, 2.5 µm, 450Å, 4.6 mm I.D., SE-UPLC column to a total 45 cm length 3.5 µm particle size, 450Å, 7.8 mm I.D., HPLC column. Figure 5 is a comparison of the separation of the BEH450 SEC Protein Standard Mix, and Figure 6 shows the separations observed for IgM in its pentameric and dipentameric forms with molecular weights of approximately 900 KDa and 1.8 MDa, respectively. As in the previous example, the UPLC separations were performed on an ACQUITY UPLC H-Class Bio System and the HPLC separations were performed on an Alliance HPLC System, and in both comparisons, the time axis has been normalized. Both pairs of chromatograms show comparable profiles over the molecular weight range thereby demonstrating transferability between the UPLC and HPLC formats. In this example, UPLC is approximately 2-fold faster than HPLC.

Overall, these data demonstrate the seamless and effective method transfer from BEH-based, SE-UPLC columns operated on UPLC systems to SE-HPLC columns operated on HPLC systems. The chromatographic profiles observed underscore the chemical comparability of the particle surfaces, as well as their pore characteristics.

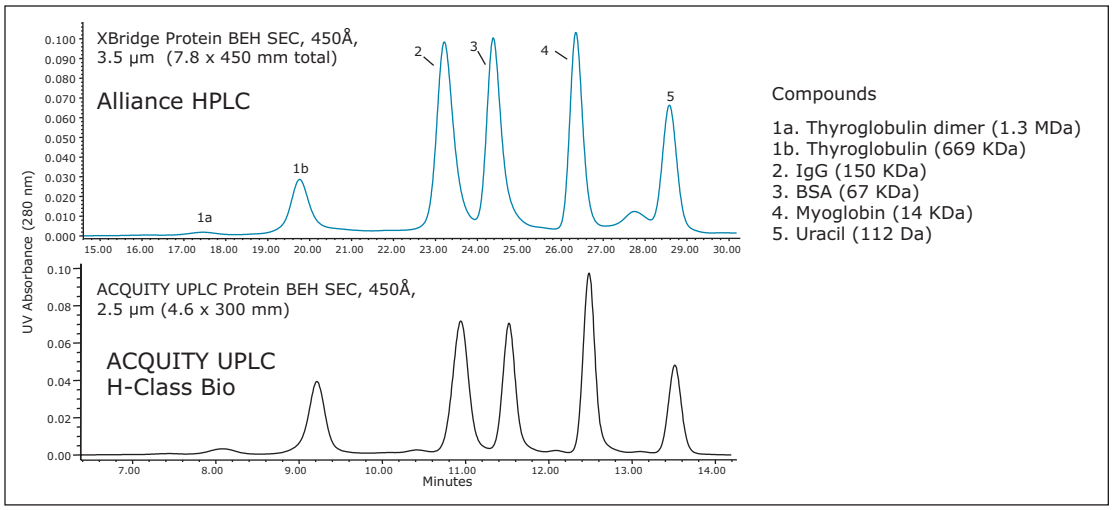

Figure 5. Shown is a comparison of separations of BEH450 SEC Protein Standard Mix (p/n 186006518) on two XBridge Protein BEH SEC, 450Å, 3.5 µm Columns (7.8 x 150 + 300 mm) run in series using an Alliance HPLC (top frame) and on an ACQUITY UPLC Protein BEH 450Å, 2.5 µm, 4.6 x 300 mm Column using an ACQUITY UPLC H-Class Bio (bottom frame). The flow rates were scaled based on particle diameter and column I.D. to 0.62 mL/minute for the two HPLC columns run in series and 0.3 mL/minute for the UPLC column. Samples loads were also adjusted for column volume.

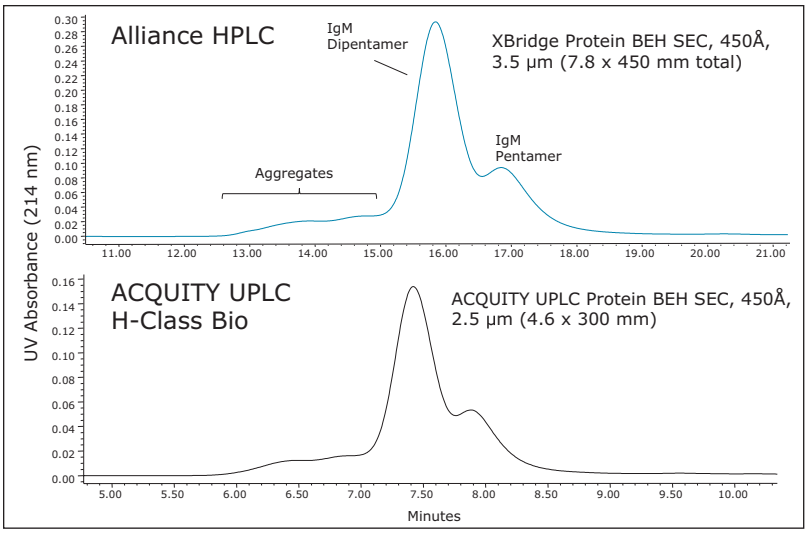

Figure 6. Shown is a comparison of separations of IgM (1 mg/mL) on two XBridge Protein BEH SEC 450Å, 3.5 µm, 7.8 x 150 + 300 mm Columns run in series using an Alliance HPLC System (top frame) and on an ACQUITY UPLC Protein BEH SEC, 450Å, 2.5 µm, 4.6 x 300 mm Column using an ACQUITY UPLC H-Class Bio (bottom frame). The flow rates were scaled based on particle diameter and column I.D. to 0.62 mL/minute for the two HPLC columns run in series and 0.3 mL/minute for the UPLC column. Samples loads were also adjusted for column volume.

CONCLUSIONS

Successful implementation of SE-UPLC separation technology requires high efficiency columns and low dispersion LC systems capable of operating at high back pressures. The advantage in doing so is the capability to dramatically improve sample throughput while decreasing sample requirements and mobile phase use. However, there are several situations where it is beneficial to be able to transfer between SE-UPLC and SE-HPLC separations, including instrumentation limitations in a method transfer, or in the event that a UPLC separation needs to be scaled up in order to facilitate the structural or functional characterization of low abundance species. However, in some cases, a direct method transfer using the same mobile phase and temperature conditions is not possible between SE-UPLC Columns packed with BEH-based particles and SE-HPLC columns packed with traditional silica-based particles due to the differences in the surface characteristics of the two particle types. These differences can necessitate the re-optimization of the method in order to get comparable results. Waters' recent development of HPLC compatible 200Å and 450Å, 3.5 µm particles based on the same diol-coated BEH-particle chemistry as that used in ACQUITY UPLC Protein BEH SEC Columns provides the capability to readily transfer between SE-UPLC and SE-HPLC for the first time. This application note details and provides examples of the methodology that can successfully employed for this method transfer.

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Transfer of an SEC Method for Monoclonal Antibody Analysis from HPLC to UHPLC Using the ACQUITY Arc System

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APPLICATION BENEFITS

- Emulate HPLC or UHPLC separations with Arc™ Multi-flow path™ technology
- Seamless SEC-HPLC method transfer from Agilent 1100 Series to the ACQUITY® Arc System
- Increased productivity by updating method conditions from HPLC to UHPLC

WATERS SOLUTIONS

ACQUITY Arc System

2489 UV/Visible (UV/Vis) Detector

XBridge® Protein Columns

Empower® 3 Chromatography Data Software (CDS)

KEY WORDS

Method transfer, ACQUITY Arc, SEC, monoclonal antibody

INTRODUCTION

Size exclusion chromatography (SEC) is a common technique used in the pharmaceutical industry for the analysis of biotherapeutics, including monoclonal antibodies. SEC is often used throughout the lifecycle of a drug product, from discovery through commercialization. Because analytical methods are commonly transferred to various laboratories within an organization, or to contract organizations throughout a product's lifetime, regulatory guidelines require that method equivalency be demonstrated between laboratories to ensure product quality and consistency. As with any assay used for release testing, it is important that the instrumentation used for analysis be robust and easy to deploy across laboratories. The ACQUITY Arc System is an LC platform designed to bridge the gap between HPLC and UPLC,® allowing users to seamlessly transfer methods across laboratories. Legacy HPLC methods can be easily replicated and UHPLC methods can be readily adopted with the use of Arc Multi-flow path technology.[1] This study uses a monoclonal antibody to assess SEC method transfer from an Agilent 1100 Series instrument to the ACQUITY Arc System. Peak area and retention time will be used as metrics to demonstrate equivalency between platforms, after which system repeatability of the ACQUITY Arc System will be evaluated. Finally, the HPLC method used to demonstrate method transfer will be updated to a UHPLC method to yield better resolution and a faster run time.

EXPERIMENTAL

LC conditions

LC systems:	ACQUITY Arc System with 2489 UV/Vis Detector, flow path 1 Agilent 1100 Series LC System with quaternary pump and DAD detector
Absorption wavelength:	280 nm
Sampling rate:	20 Hz
Column temp.:	30 °C
Mobile phase:	0.02 M sodium phosphate, 0.3 M sodium chloride, pH 6.8
Sample temp.:	5 °C
Injection volume:	30 µL

HPLC conditions

HPLC column:	Tosoh TSK gel G3000 SW_{XL} 250 Å, 5 µm, 7.8 mm x 300 mm
Flow rate:	0.5 mL/min
Method length:	35 min

UHPLC conditions

UHPLC column:	XBridge Protein BEH SEC, 200Å, 3.5 µm, 7.8 mm x 300 mm (p/n 176003596)
Flow rate:	0.714 mL/min
Method length:	24.5 min

Data management

Empower 3 CDS Software, SR2

RESULTS AND DISCUSSION

Legacy SEC-HPLC method shows equivalency and high level of reproducibility when transferred from an Agilent 1100 Series to the ACQUITY Arc System

A monoclonal antibody, rituximab, was prepared at 1 mg/mL in mobile phase and used to study HPLC method transfer from an Agilent 1100 Series instrument to the ACQUITY Arc System. The SEC method used was taken from the USP Medicines Compendium,[2] and although the compendium is now discontinued, the method is representative of a typical SEC analysis that would be used in the industry. To establish a benchmark chromatogram, rituximab was separated using a Tosoh SEC column on an Agilent 1100 Series instrument (Figure 1A). This same method was transferred to the ACQUITY Arc System and run using fluidic Path 1 under identical method conditions (Figure 1B). Visual inspection of the chromatograms shows a high degree of similarity. Evaluation of retention time and peak area percent, as reported in Table 1, confirms this agreement. The shift in retention time between instruments was approximately 0.2 minutes, but more importantly, peak area percent remained unchanged.

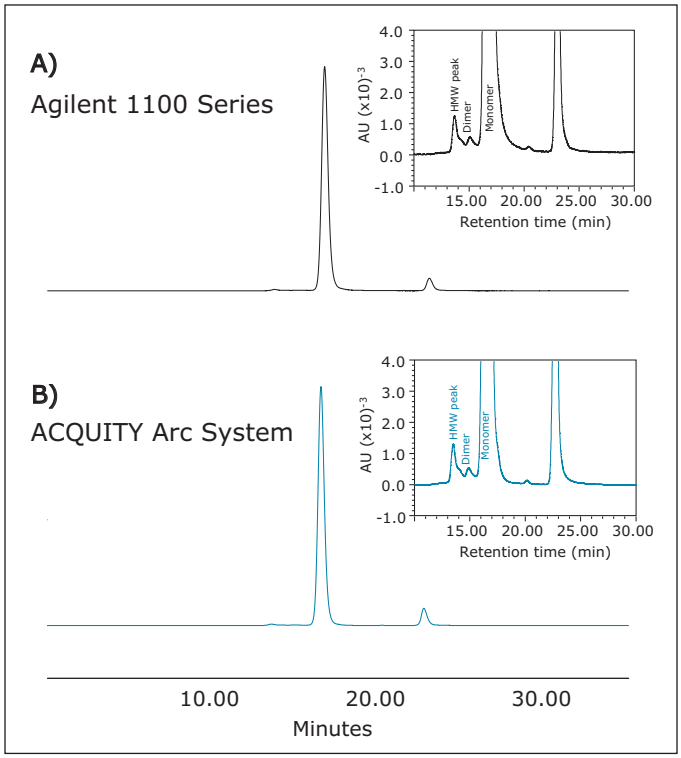

Figure 1. Comparison of SEC data acquired on an A) Agilent 1100 Series and B) ACQUITY Arc System. Inset shows separation of a dimer component and a higher molecular weight peak from the main monomer peak.

With industry standards demanding a high degree of product consistency and the need to meet suitability requirements, demonstrating instrument reproducibility is important. As shown in Figure 2, overlays of five injections on the ACQUITY Arc System are nearly indistinguishable when using the method parameters described above. Table 2 provides quantitative analysis of this data by reporting retention time, peak area percent, and resolution results for both systems.

Updating SEC-HPLC to SEC-UHPLC for improved resolution and quicker run time

The ACQUITY Arc System enables users to operate under both HPLC and UHPLC conditions on a single platform. To take advantage of this, the Tosoh SEC column was replaced with a XBridge Protein BEH SEC Column. Decreasing particle size requires that additional method parameters be scaled appropriately as well. Because column dimensions are the same, the new flow rate, F_2, is proportional to the ratio of particle diameter according to the following equation:

$$F_2 = F_1 \times \frac{d_2^2}{d_1^2} \times \frac{d_{p1}}{d_{p2}}$$

Where F_1 is the old flow rate, d_1 and d_2 are the old and new internal column diameters, and d_{p1} and d_{p2} are the old and new particle sizes. By using the equation above, a new flow rate of 0.714 mL/min was calculated. The adjusted flow rate can be used to determine the new run time, t_2, according to the following equation:

$$t_2 = t_1 \left(\frac{F_1}{F_2}\right)\left(\frac{d_2^2}{d_1^2}\right)\left(\frac{L_2}{L_1}\right)$$

Where t_1 is the old run time and L_1 and L_2 are the old and new column lengths. The adjusted run time was calculated to be 24.5 min. These new method conditions meet the guidelines for the USP's allowable adjustments,[3] which allows for the SEC method to be updated without requiring re-validation.

System	Retention time (min)			Peak area (%)	
	HMW peak	Dimer	Monomer	Higher order species	Monomer
Agilent 1100 Series	13.66	15.06	16.69	1.28	98.63
ACQUITY Arc System	13.49	14.87	16.47	1.29	98.65
Δ	0.17	0.19	0.22	0.01	-0.02

Table 1. Comparison of HPLC-SEC on an Agilent 1100 Series and the ACQUITY Arc System. Note that higher order species includes all peaks eluting earlier than the monomer, including the dimer and HMW peak identified in Figures 1 and 2. All results are average values from five injections.

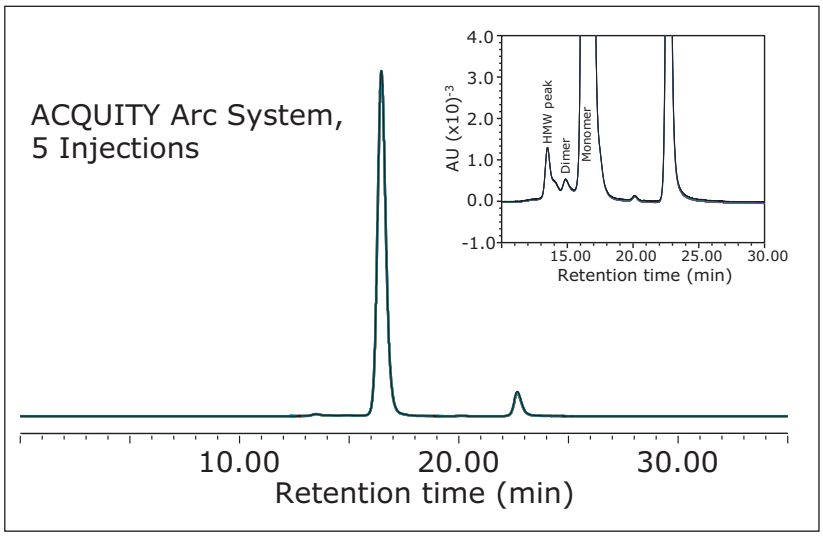

Figure 2. Overlay of SEC chromatograms acquired from five injections on the ACQUITY Arc System. Inset shows separation of a dimer component and a higher molecular weight peak from the main monomer peak.

System	Relative peak area (%)						Resolution (Dimer-Monomer)		
	Higher order species			Monomer					
	$\bar{x}$	σ	%RSD	$\bar{x}$	σ	%RSD	$\bar{x}$	σ	%RSD
Agilent 1100 Series	1.28	0.01	1.04	98.63	0.01	0.01	1.52	0.06	3.92
ACQUITY Arc System (HPLC)	1.29	0.01	1.04	98.65	0.01	0.01	1.54	0.03	1.64
ACQUITY Arc System (UHPLC)	1.29	0.02	1.49	98.63	0.02	0.02	1.75	0.02	1.26

Table 2. Quantitative comparison of HPLC-SEC on an Agilent 1100 Series instrument and the ACQUITY Arc System under both HPLC and UHPLC conditions. Note that higher order species includes all peaks eluting earlier than the monomer, including the dimer and HMW peak identified in Figures 1 and 2. All results are average values from five injections.

Rituximab was separated under UHPLC conditions and results were compared to those reported by HPLC. Improved resolution between the dimer and main peak are seen when comparing the HPLC data (Figure 3A) to the UHPLC data (Figure 3B). UHPLC conditions also led to sharper peaks with earlier elution times. Although UHPLC conditions showed improved resolution, relative peak area percentages of the higher order species and the monomer peak remained unchanged between the two methods. This indicates that there was minimal interaction between rituximab and the stationary phase, which is true of an ideal SEC separation. Table 2 contains data comparing UHPLC results to earlier reported HPLC results from both systems.

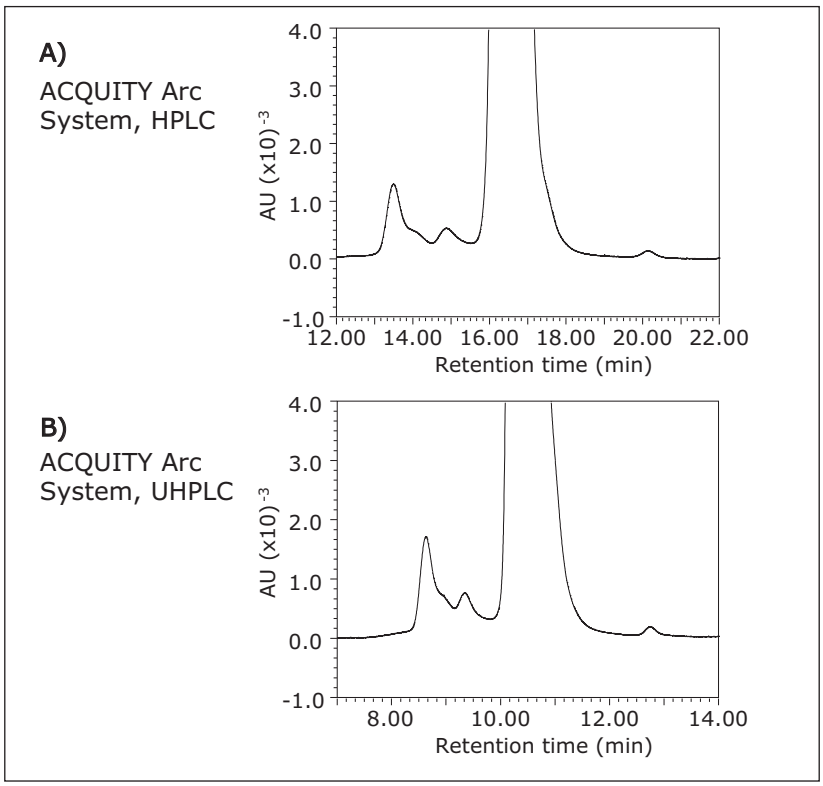

Figure 3. Comparison of SEC data acquired on an ACQUITY Arc System under A) HPLC conditions and B) UHPLC conditions. The UHPLC method results in more narrow peaks having better resolution and earlier elution times than the HPLC method.

CONCLUSIONS

The introduction of the ACQUITY Arc System recognizes the need to emulate legacy HPLC techniques, but also offers the advantage of adopting UHPLC technology if desired. An SEC method was successfully transferred from an Agilent 1100 Series HPLC to the ACQUITY Arc System without changing any method parameters. The ACQUITY Arc System demonstrated a high degree of reproducibility, which is important when verifying product consistency. Finally, the transition from SEC-HPLC to SEC-UHPLC showed improved resolution and a shorter run time while maintaining comparable peak area percentages to those obtained under HPLC conditions.

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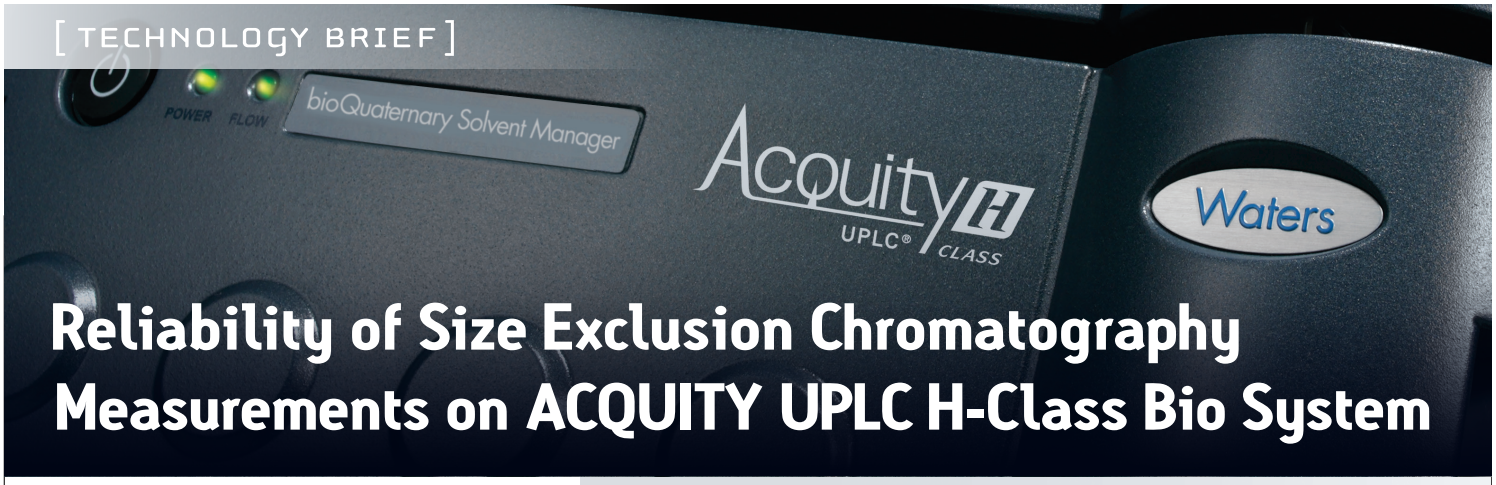

Reliability of Size Exclusion Chromatography Measurements on ACQUITY UPLC H-Class Bio System

GOAL

To demonstrate reliability of the quaternary-based ACQUITY UPLC® H-Class Bio System and the ACQUITY UPLC BEH200 SEC Column for the analysis of proteins by size exclusion chromatography (SEC).

BACKGROUND

The complete characterization and analysis of biopharmaceuticals includes the application of size exclusion chromatography (SEC) to measure protein aggregates and other size variants. Soluble protein aggregates, in particular, can contribute to immunogenicity; accurate analysis and quantitation of biotherapeutic protein aggregates is, therefore, often required.

Current HPLC/silica-based SEC methods can be time-consuming and unreliable. These uncertain results may be due to changes in retention time, peak shape, or spacing between peaks as well as irreproducibility between columns and changes in columns within a few runs.

With the introduction of the ACQUITY UPLC H-Class Bio System and sub-2-µm ACQUITY UPLC BEH200 SEC Column chemistry, SEC separations can be obtained reproducibly, reliably, and in shorter analysis time with minimal development. Methods can be easily developed with the system's quaternary solvent manager utilizing Auto•Blend Plus™ Technology. This new implementation of instrument control functions removes the need for buffer pH adjustment and reduces time spent in buffer preparation.

The ACQUITY UPLC H-Class Bio System along with an ACQUITY UPLC BEH200 SEC Column deliver reliable and reproducible SEC separations for biomolecules.

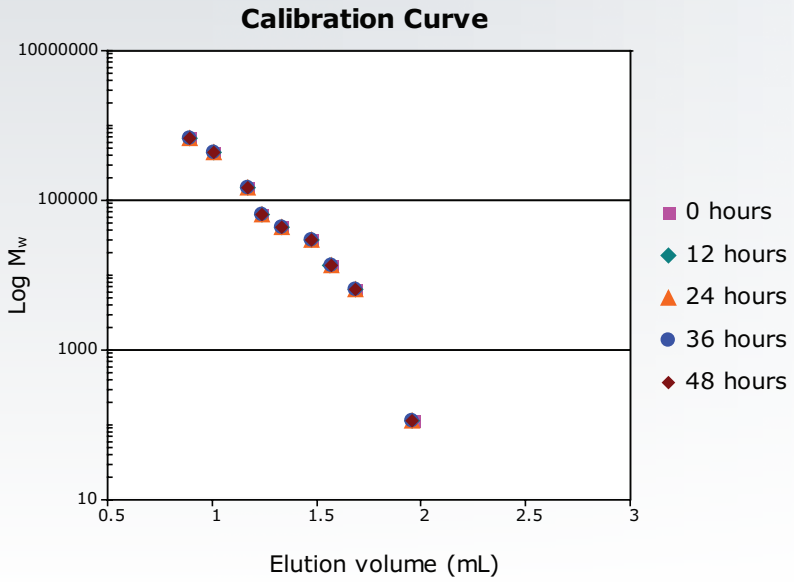

Figure 1. Protein calibration curve, ACQUITY UPLC BEH200 SEC 1.7-µm, 4.6 x 150 mm Column. Recommended molecular weight range is 10,000 to 450,000. Overlay of five calibration curves over 48 hours. Buffer: 20 mM Sodium Phosphate, 0.15 M NaCl, pH 6.8. Flow rate: 0.4 mL/min.

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The superior performance of this UPLC® SEC method relies on both the inert, low-dispersion system and the chemically-stable BEH column. The combination of these components allows users to obtain more accurate and reproducible results over a larger number of samples than is observed with current SEC methodologies.

THE SOLUTION

The SEC separation of biomolecules combines the ACQUITY UPLC H-Class Bio System with a 1.7-µm ACQUITY UPLC BEH SEC Column that provides the biochemist with a reliable separation. The low-dispersion, high-pressure system contains an inert flow path, that, when combined with four-solvent mixing and Auto•Blend Plus Technology, facilitates easy buffer preparation without pH adjustment.

The ACQUITY UPLC BEH200 SEC particle has an effective diol coating that provides a stable particle with minimal secondary interactions. The packing material is more resistant to chemical and mechanical degradation over time. These attributes combine to provide an SEC column stable more than 600 injections and requiring lower buffer concentrations than traditional silica-based columns.

In a series of experiments, protein standards and monoclonal antibody biotherapeutics were analyzed with UPLC-based SEC. Repeated analysis of the same sample was performed at regular intervals over a two-day period. Reproducibility of the calibration was tested by analysis of proteins standards over the molecular weight range of 10,000 to 450,000 Da.

The elution volume for each protein standard was found to be within 0.2% RSD. The calibration curve points do not fall on a perfect straight line because the elution volume reflects both size and shape of protein standard. The consistency of the calibration curve is, however, indicative of both the column life and instrument control of flow rate and injection volume.

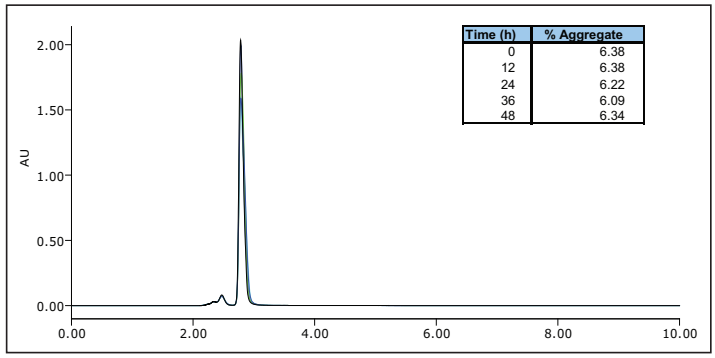

Figure 2. SEC separation of humanized IgG, 20 mg/mL. Injection of undiluted humanized IgG over 48 hours showed aggregate quantitation relative to the monomer of 6.09% to 6.38% with a RSD of 0.2%. Buffer: 20 mM Sodium Phosphate, 0.15 M NaCl, pH 6.8. Flow rate: 0.4 mL/min.

To test the reliability of quantitation, a humanized monoclonal antibody was analyzed. The sample shown was found to have an average aggregate quantitation of 6.82% ± 0.3% of the monomeric species over the time period. The reliability of this analysis is demonstrated by the reproducibility of this measurement. The SEC separations demonstrate the accuracy and reproducibility of UPLC SEC technology, which, in turn, ensures accurate identification and aggregate determination.

SUMMARY

The ACQUITY UPLC H-Class Bio System with an ACQUITY UPLC BEH200 SEC Column combine to provide reliable separations of proteins and their aggregates. As previously described, the analysis of both protein standards and monoclonal antibodies demonstrates the reliability of the calibration over a period of days. This reproducibility ensures accurate identification and quantitation of proteins and their aggregates, which can minimize analysis delays due to irreproducible results or incorrect peak identification. This, in turn, can increase throughput, thereby saving time and money. With the introduction of the ACQUITY UPLC H-Class Bio System and the new ACQUITY UPLC BEH200 SEC Column, reliable and reproducible SEC separations can be obtained for biomolecules.

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Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio System to Run SEC-HPLC and SEC-UPLC

Eoin F.J. Cosgrave and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Transfer size exclusion chromatography (SEC) applications from HPLC to UPLC®
- Future-proof laboratories with a flexible system that is capable of both HPLC and UPLC to perform aggregation assays in protein characterization

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

Biosuite™ SEC Column

ACQUITY UPLC Protein BEH SEC Column

BEH200 SEC Protein Standard Mix

Empower® 3 Chromatography Data Software

KEY WORDS

Size exclusion chromatography (SEC), HPLC, UPLC, method transfer, higher peak resolution, faster separation time, improved sensitivity, infliximab

INTRODUCTION

Aggregation represents a serious concern for companies manufacturing large-molecule therapeutics. Numerous assays aid in establishing the extent of aggregation for a given product, one of which is size exclusion chromatography (SEC). SEC is a straightforward assay requiring minimal sample preparation that exploits the size, or more specifically the hydrodynamic radius, of a given molecule as the mechanism of separation. SEC is unique from many other large-molecule chromatographic approaches in the sense that separation occurs under isocratic conditions. Because separation is influenced exclusively by an analyte's hydrodynamic radius and its ability to penetrate the pores of the stationary phase particle, there is no requirement for gradient conditions as adsorption is ideally non-existent and therefore does not influence migration times.

As observed with other large-molecule assays, significant benefits can be achieved in chromatographic quality when transitioning a method from HPLC to UPLC. The most obvious benefit is an increase in chromatographic resolution, driven principally by the increased chromatographic efficiency obtained through reduced column-particle sizes coupled with the use of low-dispersion instrumentation. This is particularly noticeable in SEC of large molecules as several higher-order aggregates can potentially be identified and quantified due to the increased resolution gained with SEC-UPLC. The added increase in sensitivity obtained through improved efficiency in SEC-UPLC also facilitates the identification of low abundant, higher-order aggregates that may have otherwise been undetected in SEC-HPLC.

Transferring SEC methods from HPLC to UPLC can be considered one of the more straightforward tasks as only a limited number of parameters need to be considered. With this in mind, there are significant opportunities for analysts to move legacy SEC-HPLC methods to UPLC technology.

To demonstrate the applicability of the ACQUITY UPLC H-Class Bio System for performing both SEC-HPLC and SEC-UPLC, we present here the transfer of an SEC-HPLC assay for monoclonal antibody aggregation from legacy HPLC instrumentation to the ACQUITY UPLC H-Class Bio. Following transfer of the HPLC method, we demonstrate a simplified approach to migrate the SEC-HPLC method to UPLC column chemistry.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLCH-Class Bio comprised of: ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (QSM)
	ACQUITY UPLC H-Class Bio Sample Manager (SM)
Extension loop:	100 µL (p/n 430002625)
Detector:	ACQUITY UPLC Tunable UV Detector with Ti flow cell
Absorption	Wavelength: 214 nm
Column temp.:	Ambient
Mobile phase:	20 mM Phosphate, 200 mM NaCl, pH 6.8
Sample:	Waters BEH200 SEC Protein Standard Mix (p/n 186006518)

HPLC method conditions

HPLC column:	Biosuite SEC Column, 250 Å, 10 µm, 7.5 mm x 300 mm (p/n 186002170)
Injection vol.:	20 µL
Flow rate:	0.400 mL min^{-1}
Method length:	35 min

UPLC method conditions

UPLC column:	ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 mm x 150 mm (P/N 18605225)
Injection vol.:	4 µL
Flow rate:	0.885 ml min^{-1}
Method length:	3 min

Results of this study illustrate that the ACQUITY UPLC H-Class Bio System is capable of producing highly similar SEC-HPLC data compared to legacy HPLC instrumentation. Moreover, improvement in chromatographic resolution is obtained by moving from HPLC to UPLC. A reduction in run time is also observed without compromise to monoclonal antibody aggregate quantification. The experiment also illustrates the flexibility of using the ACQUITY UPLC H-Class Bio System for both SEC-HPLC and SEC-UPLC and the benefits of moving to smaller particle sizes for SEC-based assays.

RESULTS AND DISCUSSION

Legacy SEC-HPLC methods prove highly comparable when run on the ACQUITY UPLC H-Class Bio System

To determine the ability of the ACQUITY UPLC H-Class Bio to perform legacy SEC-HPLC assays, we established a benchmark SEC-HPLC method using a quaternary pump HPLC instrument coupled with the BioSuite SEC 250 Å 10-µm Column (7.5 mm x 300 mm).

Two samples were used to evaluate the separation. The first sample was the Waters SEC200 protein standard mix, used to determine the total inclusion and exclusion volume of each column. The second sample was a therapeutic monoclonal antibody, infliximab, which was selected to measure aggregation, if any, and therefore a useful sample for evaluating the accuracy of method transfer between SEC run on the HPLC instrument and the ACQUITY UPLC H-Class Bio System. With respect to HPLC method parameters, separation in SEC is isocratic and, as such, the basic requirement of the method is that it run long enough to deliver a minimum of one column volume. For the selected HPLC column, a volume of approximately 13 mL was required and therefore a run time of 35 min was selected based on a delivered flow rate of 0.4 mL $min.^{-1}$ A standard mobile phase of 20 mM phosphate buffer, 200 mM NaCl at pH 6.8 was selected for use and initial analyses.

To first establish a benchmark chromatogram, the SEC200 protein mix standard was separated using the quaternary pump HPLC instrument with the SEC-HPLC column. Results of this separation are presented in Figure 1A. The SEC-HPLC column was then transferred to the ACQUITY UPLC H-Class Bio System without any changes to the method parameters. The same protein mix standard was separated, with results presented in Figure 1B. As can be clearly seen from these figures, very little difference is observed in the chromatography performed on each instrument. Details of the retention times and relative peak areas provided in Table 1 further indicate the accuracy of method transfer across analytical instruments, as evidenced by minimal differences between data recorded from each instrument.

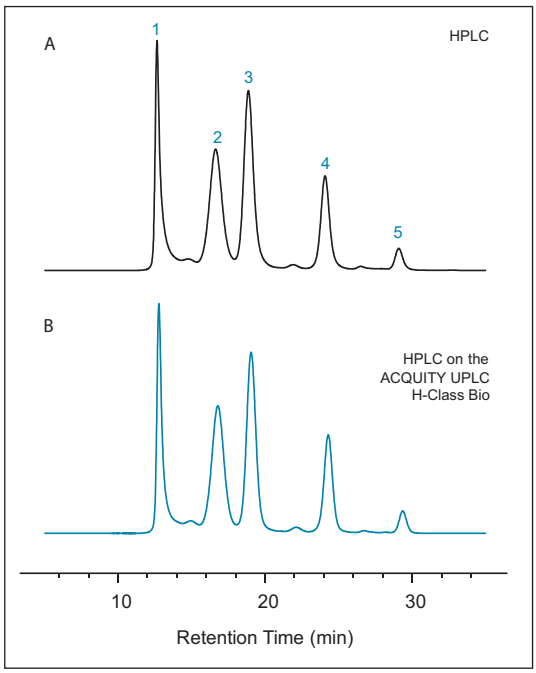

Figure 1. SEC transferred from HPLC to the ACQUITY UPLC H-Class Bio generates equivalent separation. (A) SEC on the HPLC instrument; (B) HPLC SEC on the H-Class Bio.

Peak	SEC Component	Retention Time (min)			Peak Area (%)			
		HPLC	H-Class	Δ	HPLC	H-Class	Δ	
1	Thyroglobulin	12.78	12.65	-0.13	23.24	23.79	-0.55	Figure 1.
2	IgG	16.78	16.70	-0.08	28.31	27.64	0.67	
3	BSA	19.04	18.82	-0.22	30.42	31.38	-0.96	
4	Myoglobin	24.28	24.16	-0.12	15.13	14.45	0.68	
5	Uracil	29.34	29.19	-0.15	2.90	2.74	0.16	
1	Mab Dimer	14.49	14.19	-0.30	0.48	0.47	0.01	Figure 2.
2	Mab Monomer	17.69	17.18	-0.51	99.52	99.53	0.01	

Table 1. Quantitative comparison of HPLC SEC run on a traditional HPLC versus the ACQUITY UPLC H-Class Bio. Retention time and peak area data represent the averaged data of triplicate analyses.

With conditions indicating a successful transfer of the SEC method to the ACQUITY UPLC H-Class Bio System, the monoclonal antibody infliximab was separated on both the quaternary pump HPLC instrument and the ACQUITY UPLC H-Class Bio System to determine the relative abundance of monomer and aggregate species within the sample. Similar to the protein standard results across instruments, very little difference was observed in chromatography (Figure 2) as well as relative peak areas in both the SEC200 protein standard mix and infliximab samples (Figure 3). Retention times, relative peak areas, and the resolution between the IgG monomer and dimer, also presented in Table 1, provide convincing evidence that SEC-HPLC assays can successfully be transferred to the ACQUITY UPLC H-Class Bio System without any compromise to the legacy analytical method criteria.

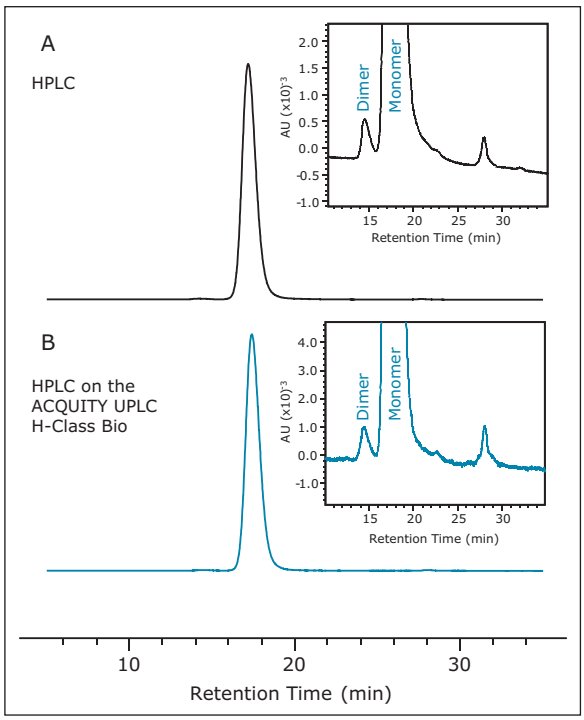

Figure 2. Separation of infliximab on SEC gives equivalent results between the HPLC instrument and the ACQUITY UPLC H-Class Bio. (A) SEC on the HPLC; (B) SEC on the H-Class Bio.

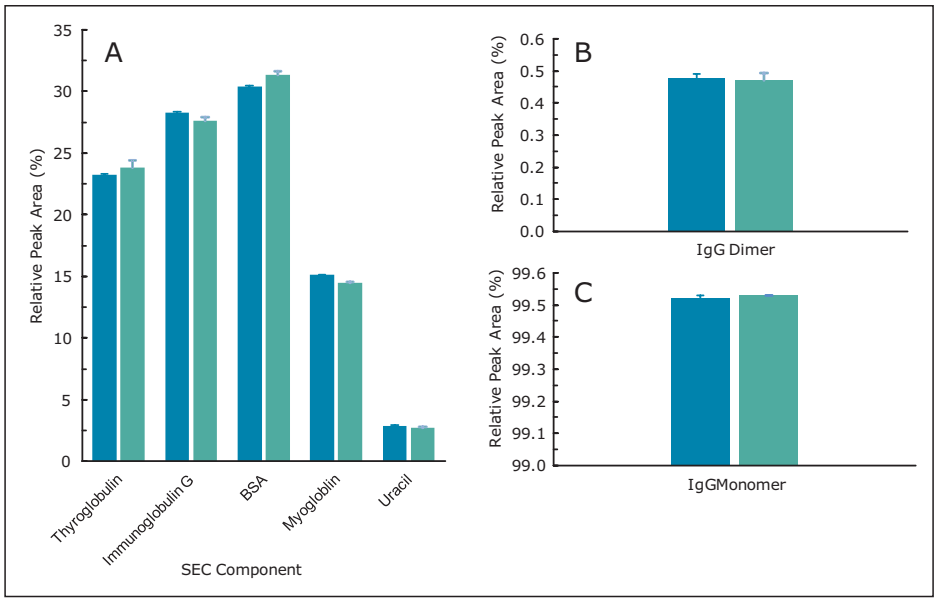

Figure 3. Evaluation of relative peak area between SEC performed on HPLC and the ACQUITY UPLC H-Class Bio System. In each figure, the dark blue columns represent values measured from the HPLC instrument while the light blue columns represent values measured from the ACQUITY UPLC H-Class Bio. (A) Peak areas measured from the SEC200 protein standard mix. (B) Measurement of the relative peak area of the infliximab dimer. (C) Measurement of the relative peak area of the infliximab monomer.

Migration from HPLC to UPLC improves resolution

Significant benefits can be obtained by transferring legacy SEC-HPLC methods to SEC-UPLC columns, which provide improved chromatographic resolution through reduced column particle size. To illustrate this benefit, the SEC-HPLC method used for method transfer between the quaternary pump HPLC and the ACQUITY UPLC H-Class Bio System was adapted to be run on SEC-UPLC using the Waters ACQUITY UPLC Protein BEH SEC Column (200 Å, 1.7 µm, 4.6 mm x 150 mm).

To demonstrate the improved resolution as a result of reduced particle size on SEC, we separated the SEC200 protein standard mix using the 10-µm Biosuite SEC Column and the 1.7-µm ACQUITY UPLC Protein SEC column and calculated the differences in chromatographic performance. Both HPLC and UPLC separations were performed using the Waters ACQUITY UPLC H-Class Bio System. To accommodate for differences in particle size and column dimensions between the HPLC and UPLC columns, the flow rate was adjusted based on the following formula:

$$F_2 = F_1 \left(\frac{d_2^2}{d_1^2} \right) X \frac{d_{p1}}{d_{p2}}$$

Where F refers to flow rate, d refers to column internal diameter, and dp refers to particle diameter. Based on the two columns used and an HPLC flow rate of 0.4 mL min,$^{-1}$ a new flow rate of 0.885 mL min^{-1} was calculated. A new run time of 3 minutes was also determined for the UPLC separation, given the reduced volume of the UPLC column and the increased flow rate.

To determine the improvement in UPLC-based SEC, the SEC200 protein standard mix was separated on the BEH SEC 200 Å 1.7-µm column using the updated run time and flow rate. Most noticeable from the separation was the significantly reduced run time from 35 min in HPLC to just 3 minutes in UPLC (Figure 4). This was not at the cost of chromatographic performance, where relative peak areas are equivalent between HPLC and UPLC (Figure 5A) but a significant improvement in resolution between all peak pairs is observed in UPLC (Figure 5B). Resolution between all peak pairs are tabulated for reference (Table 2).

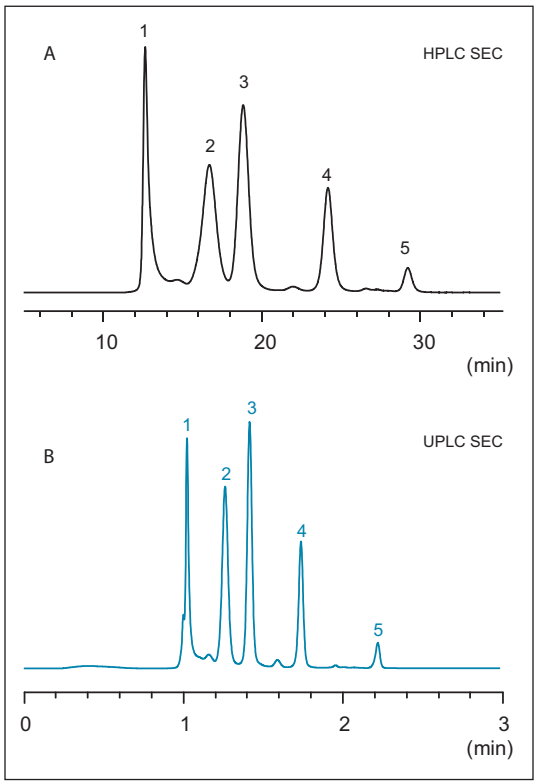

Figure 4. SEC transferred to UPLC generates higher peak resolution, faster separation time, and improved sensitivity. The HPLC SEC method was scaled for chromatography using the ACQUITY UPLC Protein BEH SEC, 200 Å column. A flow rate of 0.885 mL min^{-1} was performed over a 3-min time frame. Equivalent resolution was obtained. Each separation was performed in triplicate.

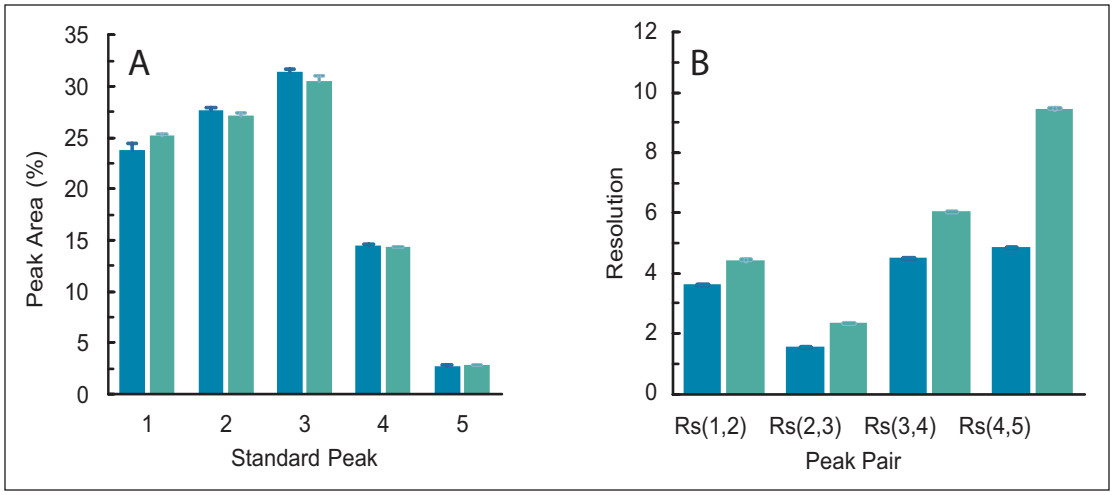

Figure 5. Comparative assessment of SEC-HPLC against SEC-UPLC. In each figure, dark blue columns refer to HPLC relative peak area measurements while light blue columns refer to UPLC measurements. (A) Measurement of the SEC200 protein standard mix where 1 represents thyroglobulin, 2 represents IgG, 3 refers to BSA, 4 represents myoglobin, and 5 represents uracil. (B) Measurement of the change in resolution between each peak pair of the SEC200 protein standard mix.

To determine what effect, if any, occurred on quantification in SEC, we separated the monoclonal antibody infliximab using the SEC-HPLC column and the SEC-UPLC column. In each SEC experiment, peaks corresponding to the monomer and dimer were integrated. As expected, a noticeable improvement in resolution was observed when separating infliximab in UPLC (Figure 6). Most importantly, the improvement in resolution did not affect the amount of aggregation quantified, where near identical relative peak areas for infliximab monomer and dimer were calculated on HPLC and UPLC. All chromatographic data has been reported in Table 2.

LC Mode	Peak	SEC component	Retention time			Peak area			Resolution		
			$\bar{x}$	σ	% RSD	$\bar{x}$	σ	% RSD	$\bar{x}$	σ	% RSD
HPLC	1	Thyroglobulin	12.65	0.001	0.008	23.79	0.638	2.683			
	2	IgG	16.64	0.002	0.010	27.64	0.260	0.940	3.63	0.003	0.072
	3	BSA	18.86	0.002	0.011	31.38	0.240	0.765	1.56	0.002	0.149
	4	Myoglobin	24.07	0.004	0.015	14.45	0.107	0.740	4.52	0.004	0.092
	5	Uracil	29.07	0.002	0.007	2.74	0.032	1.175	4.88	0.005	0.111
	1	Mab dimer	14.19	0.022	0.155	0.29	0.000	0.000			
	2	Mab monomer	17.18	0.001	0.006	99.71	0.000	0.000	1.91	0.014	0.733
UPLC®	1	Thyroglobulin	1.03	0.002	0.149	25.19	0.151	0.601			
	2	IgG	1.27	0.002	0.121	27.14	0.217	0.798	4.43	0.06	1.251
	3	BSA	1.42	0.001	0.081	30.52	0.482	1.580	2.36	0.02	0.757
	4	Myoglobin	1.75	0.001	0.066	14.30	0.084	0.586	6.06	0.00	0.038
	5	Uracil	2.23	0.002	0.078	2.85	0.032	1.127	9.43	0.04	0.452
	2	Mab dimer	1.10	0.002	0.188	0.48	0.006	1.57			
	3	Mab monomer	1.30	0.001	0.133	99.53	0.036	0.036	2.47	0.017	0.688

Table 2. Quantitative comparison of SEC-HPLC and SEC-UPLC performed on the ACQUITY UPLC H-Class Bio System. Retention time and peak area data represent the averaged data of triplicate analyses.

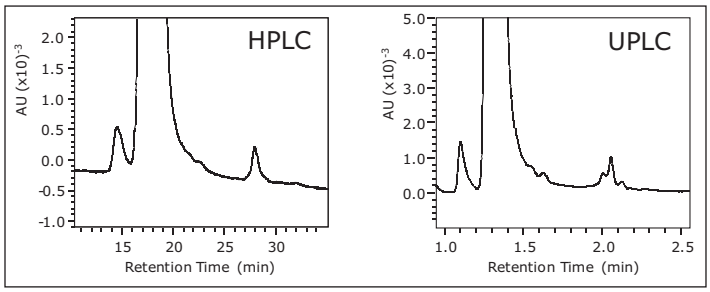

Figure 6. Improved resolution is observed when quantifying molecular species in UPLC. Comparison of peak resolution identified the presence of multiple species in UPLC that were unidentifiable in HPLC.

CONCLUSIONS

Size exclusion chromatography is a common method used for investigating the extent of aggregation in protein therapeutics. In this application note, we have demonstrated that legacy SEC-HPLC methods can be easily transferred to the ACQUITY UPLC H-Class Bio System without any modification to existing method details. With adjustments to flow rate and run time based on changes in particle size and column dimensions, SEC-UPLC can easily be performed with the same ACQUITY UPLC H-Class Bio System without any consequence to protein quantification requirements.

This permits QC labs to align LC technology and associated methods with development labs, while continuing to support legacy large-molecule assays currently deployed in the QC environment.

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Future-Proofing the Biopharmaceutical QC Lab: Benefits of Automating Mobile Phase Delivery to Improve pH Consistency in Size Exclusion Chromatography Methods

Eoin F.J. Cosgrave and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Using the ACQUITY UPLC® H-Class Bio System to perform size exclusion chromatography (SEC) in biopharmaceutical quality control (QC)
- Experimental approach for converting conventional mobile phase delivery to Auto•Blend Plus™ SEC assays

WATERS SOLUTIONS

ACQUITY UPLC H-Class Bio System

BioSuite™ SEC Column

ACQUITY UPLC Protein BEH SEC Column

BEH200 SEC protein standard mix

Empower® 3 Chromatography Data Software

KEY WORDS

Size exclusion chromatography, SEC, quality control, QC, AutoBlend Plus, automated mobile phase delivery, automated buffer management

INTRODUCTION

Large molecule separations that require buffered mobile phases represent a challenge in analytical labs due to the potential sensitivity of analytes to changes in pH and salt concentration. One such large molecule assay includes size exclusion chromatography (SEC), which is typically used to measure the extent of aggregation in protein-based therapies.

Mobile phases for SEC separations have historically been prepared by combining individual components of the mobile phase followed by adjustment to the desired pH using an appropriate acid or base. In this scenario, calibration of the pH meter and the associated accuracy of pH measurements can directly influence the final pH of the mobile phase, affecting the quality of the final separation. As a result, subtle changes in the preparation of mobile phase can lead to differences in chromatography in situations where pH differs between mobile phase preparations.

In this application note, we continue our discussion of using the ACQUITY UPLC H-Class Bio System for size exclusion chromatography[1] by demonstrating the benefits of Auto•Blend Plus Technology – which is included with all ACQUITY UPLC H-Class instruments – for consistent and reliable delivery of pH-dependent mobile phase for SEC-HPLC and SEC-UPLC.

Compared to manual approaches where mobile phase delivery is defined by percent composition of each solvent line, Auto•Blend Plus allows the user to define individual method steps based on the desired pH and salt concentration. This enables the analyst to explore an extensive list of method parameters in a single set of buffer preparations.

Auto•Blend Plus can be particularly advantageous in QC environments, where methods are expected to be accurate, precise, and robust. Variability in mobile phase preparation due to inconsistencies with pH can potentially lead to erroneous outcomes that can otherwise be controlled using automated chromatographic tools such as Auto•Blend Plus.

EXPERIMENTAL

LC conditions

ACQUITY UPLC H-Class Bio System, comprised of:

- ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (QSM)
- ACQUITY UPLC H-Class Bio Sample Manager (SM)
- ACQUITY UPLC Tunable UV Detector with Ti flow cell
- Extension loop: 100 µL (p/n 430002625)
- BioSuite SEC 10 µm, 250 Å Column, 7.5 mm x 300 mm (p/n 186002170)
- ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 x 150 mm (p/n 186005225)
- BEH200 SEC protein standard mix (p/n 186006518)

Column temp.:	Ambient
Seal wash:	10% acetonitrile in H_2O
Conventional mobile phase:	20 mM phosphate, 200 mM NaCl, pH 6.8

Auto•Blend Plus

Mobile phase A:	A: 100 mM NaH_2PO_4
Mobile phase B:	100 mM Na_2HPO_4
Mobile phase C:	1 M NaCl
Mobile phase D:	H_2O
Detection wavelength:	214 nm
Syringe purge:	H_2O
Syringe wash:	H_2O

HPLC conditions

Injection vol.:	20 µL
Flow rate:	0.400 mL min^{-1}
Method length:	35 min

UPLC conditions

Injection vol.:	4 µL
Flow rate:	0.885 mL min^{-1}
Method length:	3 min

In this application note, we demonstrate how a conventional SEC method is converted to an Auto•Blend Plus-enabled method using the ACQUITY UPLC H-Class Bio System. There is no disruption to mobile phase composition when performing this conversion: Auto•Blend Plus delivers identical chromatography to that obtained using mobile phase prepared and delivered in a conventional manner. The results presented in this application note show robust, precise, and reliable chromatography for both SEC-HPLC and SEC-UPLC, supporting the prospect of Auto•Blend Plus as a technology that can be successfully deployed in large molecule QC environments.

RESULTS AND DISCUSSION

Experimental design of conventional and Auto•Blend Plus assisted SEC

Preparation of aqueous, pH dependent mobile phases can be a cumbersome aspect for both method development experiments as well as high-throughput assay environments where mobile phase is used in high volume. In the latter case, each preparation of new mobile phase can be susceptible to variability due to differences in pH meter calibration and accuracy, pH adjustment of the mobile phase, and general differences in the way analysts prepare mobile phase.

To get around this inconsistency, control of mobile phase preparation can instead be accomplished using Auto•Blend Plus Technology. Solutions of appropriate acid, base, salt, and water can be prepared separately as concentrated stocks and mixed together using Auto•Blend Plus, which combines the necessary proportions of each solvent required for delivering a specified pH and salt concentration. This strategy is made possible by the ACQUITY UPLC H-Class System's Quaternary Solvent Manager, which can combine four separate solvents to form a desired mobile phase composition.

To evaluate the similarity between conventional SEC-HPLC and Auto•Blend Plus assisted SEC-HPLC, we created two sets of mobile phase for each SEC assay. For conventional SEC, the mobile phase consisting of 20 mM phosphate with 200 mM NaCl adjusted to pH 6.8 was prepared at the bench. For Auto•Blend Plus assisted SEC, four separate stock solvents of 100 mM NaH_2PO_4 buffer, 100 mM Na_2HPO_4 buffer, 1 M NaCl, and pure H_2O were prepared.

In each case, a BioSuite SEC 10-µm 250 Å Column (7.5 x 300 mm) was used for comparison. Two separate protein samples were used to evaluate the HPLC approaches. The first protein sample was a Waters® SEC200 protein standard mix consisting of five components intended for determining the total inclusion and exclusion volumes of SEC columns capable of separating proteins between approximately 10 kDa and 500 kDa. The second protein was the commercial monoclonal antibody, infliximab, previously shown to contain a minor amount of aggregate formation.[1]

For accurate delivery of a desired pH, an empirical table was generated that accounted for the effect of increasing salt concentration on mobile phase pH. Instrument methods for both conventional SEC-HPLC and Auto•Blend Plus SEC-HPLC were created using Empower 3 Software (Figure 1). For conventional SEC-HPLC, all relevant instrument details were outlined as depicted in Figure 1A. The Auto•Blend Plus SEC-HPLC method was created by selecting Auto•Blend Plus from the QSM option in the instrument method and itemizing the desired pH and salt concentration, as depicted in Figure 1B. Addition of empirical data was accessed by selecting Buffer System and then selecting the Empirical Data option on the right side of the new window (Figure 2). It is recommended that labs generate their own Auto•Blend Plus tables as suppliers of raw chemicals and standard operating procedures may yield different pH values than those listed in the figure.

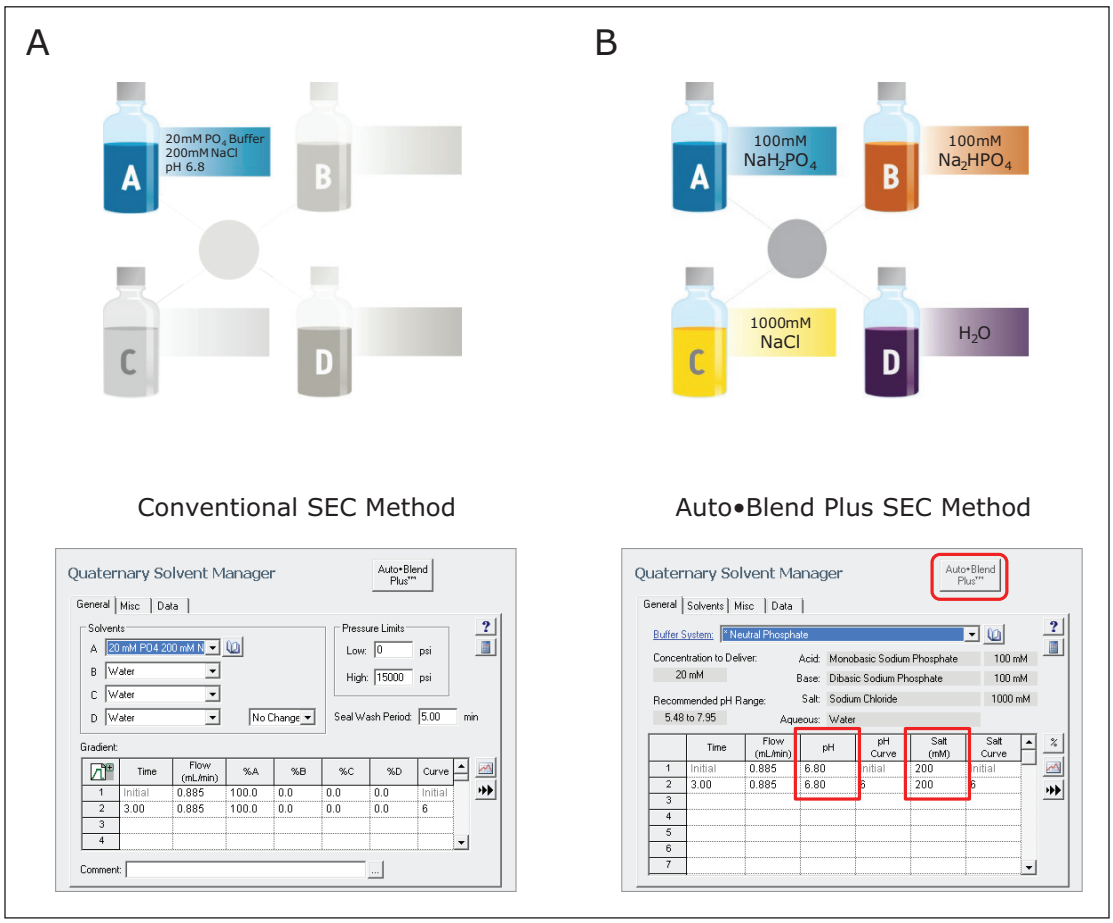

Figure 1. Conventional SEC and Auto•Blend Plus assisted SEC arrangements. Presented instrument method windows relate to the SEC-UPLC method. (A) Conventional SEC arrangement typically has a single prepared mobile phase on one solvent line, which is itemized in the instrument method as a 100% solvent A. (B) In Auto•Blend Plus SEC arrangements, mobile phases corresponding to acid (NaH_2PO_4), base (Na_2HPO_4), salt (NaCl), and water are configured on 4 solvent lines. The instrument method is modified to request the desired pH and salt composition rather than a percent mobile phase, as illustrated by the red boxed items. Similar windows exist for SEC-HPLC with appropriate changes to flow rate and method duration.

SEC-HPLC with Auto•Blend Plus generates identical results to conventional SEC-HPLC

To determine the comparability of Auto•Blend Plus for SEC-HPLC, a benchmark separation was first established using the conventional SEC-HPLC method with the BioSuite SEC 10-µm column. In the first instance, the SEC200 protein standard mix was chromatographically separated and all peaks were shown to elute within the method run as expected (Figure 3A). All relevant chromatographic data is recorded in Table 1. With migration times established for each protein standard component, the ACQUITY UPLC H-Class Bio was configured to run Auto•Blend Plus methods by exchanging the conventional SEC mobile phase arrangement (Figure 1A) with the Auto•Blend Plus mobile phase arrangement (Figure 1B). The same column and SEC200 protein standard mix were used. Each component of the standard was shown to exhibit near identical migration times when compared to the conventional SEC-HPLC method results (Figure 3B and Table 1). Relative peak areas associated with each component were also shown to be highly comparable, indicating the ability of Auto•Blend Plus to generate identical chromatography when compared to conventional HPLC.

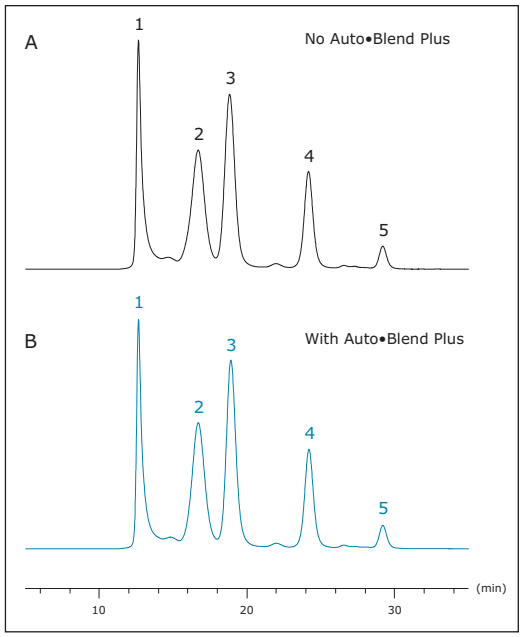

Figure 3. Auto•Blend Plus assisted SEC-HPLC generates equivalent chromatography to the conventional approach. In each chromatogram, 1 refers to thyroglobulin, 2 refers to IgG, 3 refers to BSA, 4 refers to myoglobin, and 5 refers to uracil. (A) SEC-HPLC using conventional mobile phase preparation; (B) SEC-HPLC using Auto•Blend Plus Technology for mobile phase delivery.

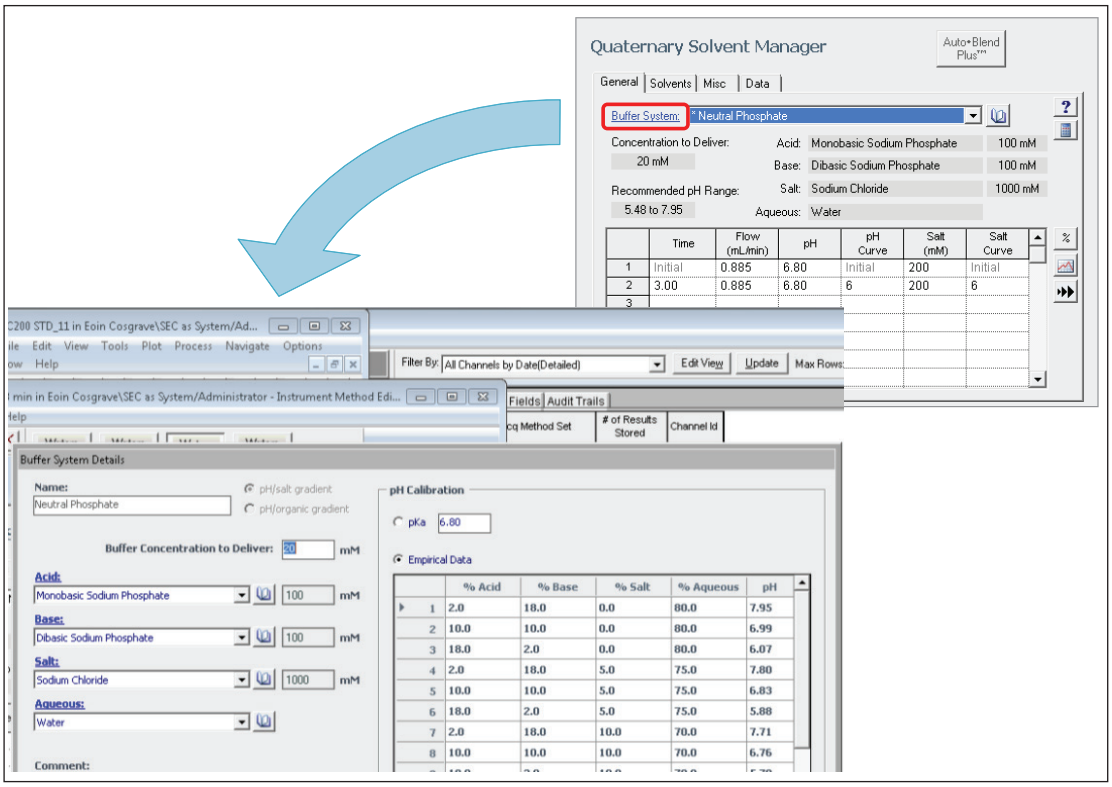

Figure 2. Recording empirical data in Auto•Blend Plus methods. Correcting pH due to salt concentration can be added to Auto•Blend Plus methods by first selecting the "Buffer System" in the QSM tab of the Empower instrument method window. In the new window, the option of "pKa" or "Empirical Data" is available. Selecting "Empirical Data" activates the table where pH values corresponding to the composition itemized in each row can be entered.

To investigate the comparison with a true commercial large molecule protein therapeutic, we used each SEC approach to measure the extent of aggregation in infliximab. As can be seen in Figure 4, the migration time for both the infliximab dimer and monomer were highly comparable, indicating Auto•Blend Plus as a suitable replacement for conventional mobile phase delivery.

SEC-UPLC with Auto•Blend Plus generates identical results to conventional mobile phase preparation

Moving from SEC-HPLC to SEC-UPLC offers a number of improvements to chromatography previously described.[1] In addition to increasing chromatographic resolution and sensitivity by moving to SEC-UPLC, method robustness can also be improved by incorporating Auto•Blend Plus into the instrument method. Transferring the SEC-HPLC method to SEC-UPLC results in an increase in flow rate from 0.4 mL min^{-1} to 0.885 mL min^{-1} with a corresponding reduction in run time from 35 min to just 3 min.

To determine if Auto•Blend Plus could generate comparable results as those observed with SEC-HPLC, we ran both the SEC200 protein mix standard and infliximab using either conventional SEC-UPLC or Auto•Blend Plus-assisted SEC-UPLC. An ACQUITY UPLC Protein BEH SEC 200 Å Column (1.7-µm, 4.6 x 150 mm) was used with the ACQUITY UPLC H-Class Bio System for the assay. Benchmark SEC-UPLC using the SEC200 protein standard mix was generated as illustrated in Figure 5A. Auto•Blend Plus-assisted SEC-UPLC was then run and compared to the conventional SEC-UPLC, with results indicating no difference in individual component migration times (Figure 5B and Table 1). The same comparison was performed using infliximab, where similar results were obtained (Figure 6 and Table 1).

Results of SEC-UPLC unequivocally illustrate that using Auto•Blend Plus Technology for SEC-UPLC can replace conventional SEC-UPLC with no impact on component migration time or relative peak area.

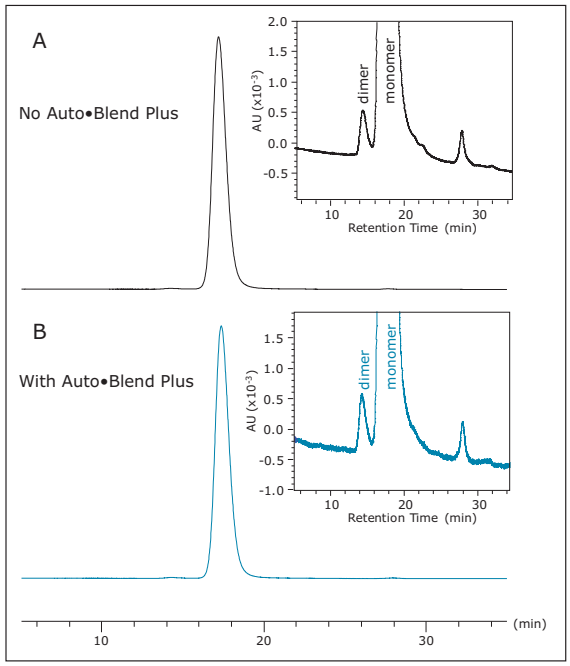

Figure 4. Auto•Blend Plus assisted SEC-HPLC of infliximab generates equivalent separation compared to conventional SEC-HPLC. (A) Infliximab separated using conventional SEC-HPLC; (B) Infliximab separated using Auto•Blend Plus assisted SEC-HPLC.

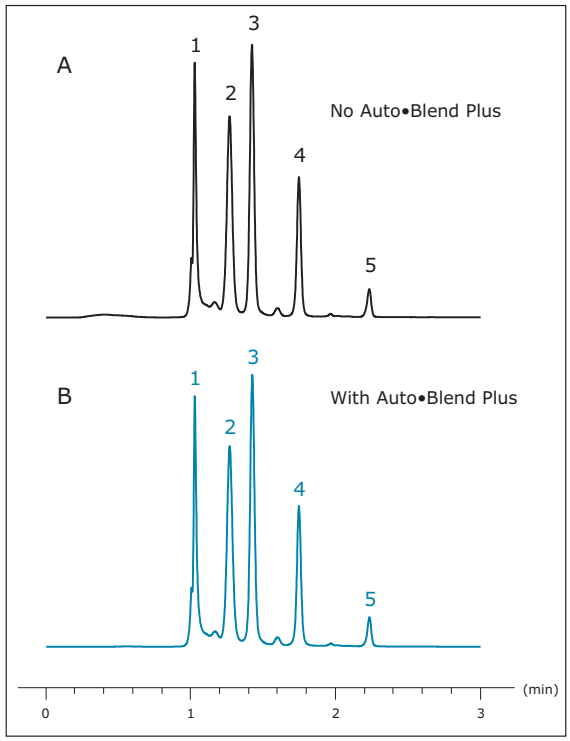

Figure 5. Auto•Blend Plus assisted SEC-UPLC generates equivalent chromatography to the conventional approach. In each chromatogram, 1 refers to thyroglobulin, 2 refers to IgG, 3 refers to BSA, 4 refers to myoglobin, and 5 refers to uracil. (A) SEC-UPLC using conventional mobile phase preparation; (B) SEC-UPLC using Auto•Blend Plus Technology for mobile phase delivery.

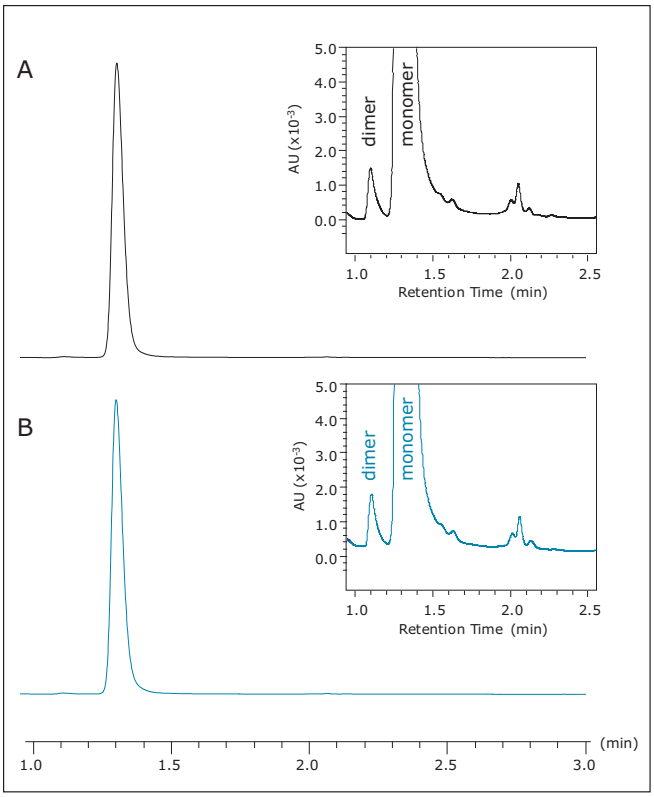

Figure 6. Auto•Blend Plus assisted SEC-UPLC of infliximab generates equivalent separation compared to conventional SEC-UPLC. (A) Infliximab separated using conventional SEC-UPLC; (B) Infliximab separated using Auto•Blend Plus assisted SEC-UPLC.

Peak	SEC component	Retention time (min)				Peak area (%)			
		Auto•Blend Plus				Auto•Blend Plus			
		-		+		-		+	
		$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
1	Thyroglobulin	12.65	0.001	12.66	0.002	23.79	0.638	23.64	0.015
2	IgG	16.64	0.002	16.70	0.002	27.64	0.260	27.83	0.012
3	BSA	18.86	0.002	18.91	0.005	31.38	0.240	31.15	0.021
4	Myoglobin	24.07	0.004	24.19	0.003	14.45	0.107	14.60	0.015
5	Uracil	29.07	0.002	29.19	0.002	2.74	0.032	2.78	0.012
1	Mab dimer	14.19	0.022	14.29	0.003	0.47	0.000	0.53	0.010
2	Mab monomer	17.18	0.001	17.53	0.004	99.53	0.000	99.47	0.010
1	Thyroglobulin	1.03	0.001	1.03	0.001	25.19	0.151	25.34	0.200
2	IgG	1.27	0.001	1.27	0.001	27.14	0.217	27.17	0.209
3	BSA	1.42	0.000	1.43	0.001	30.52	0.482	30.19	0.511
4	Myoglobin	1.7	0.001	1.75	0.002	14.30	0.084	14.43	0.087
5	Uracil	2.23	0.001	2.23	0.001	2.85	0.032	2.87	0.021
1	Mab dimer	1.14	0.000	1.11	0.001	0.47	0.030	0.46	0.020
2	Mab monomer	1.32	0.001	1.30	0.000	99.53	0.040	99.54	0.020

Table 1. Quantitative comparison conventional SEC versus Auto•Blend Plus assisted SEC Retention time and peak area data represent the averaged data of triplicate analyses.

CONCLUSION

Conventional SEC relies on the accurate preparation of pH dependent mobile phases where subtle variation in pH can lead to significant changes in chromatographic retention times. As a means for reducing variability in the preparation of buffered mobile phase, Auto•Blend Plus Technology available through the Waters ACQUITY UPLC H-Class Bio System can prepare buffered mobile phase across a range of pH and NaCl concentrations from 4 standard stock solvents. In this application note, we have demonstrated the steps required to convert conventional SEC methods to Auto•Blend Plus methods. The benefits of Auto•Blend Plus span both SEC-HPLC and SEC-UPLC, where equivalent chromatography can be achieved with a more robust and reproducible solvent delivery system for pH dependent mobile phases.

Reference

1. Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio to Run SEC HPLC and SEC UPLC. Waters Application Note. 2014: 720005057en.

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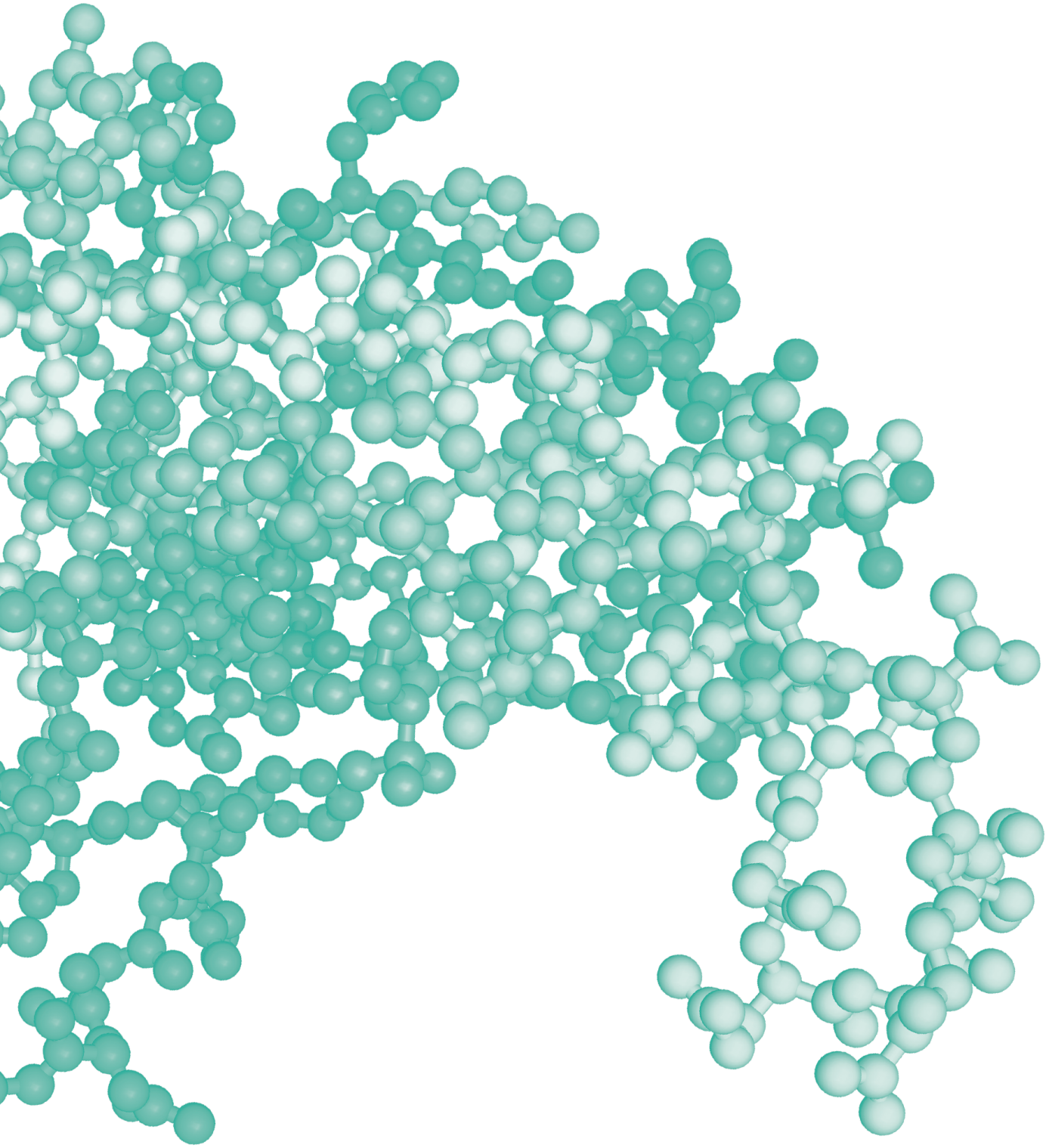
Application-Specific SEC Analysis



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Advances in Size-Exclusion Chromatography for the Analysis of Small Proteins and Peptides: Evaluation of Calibration Curves for Molecular Weight Estimation

Paula Hong, Stephan Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution and throughput of peptide separations and small proteins with SE-UPLC compared to SE-HPLC
- Optimized conditions for true size-based separations of peptides
- Organic mobile phase compatibility for reliable molecular weight estimation of hydrophobic peptides

WATERS SOLUTIONS

ACQUITY UPLC® BEH125 SEC, 1.7 µm Column

ACQUITY UPLC H-Class Bio System

Auto•Blend Plus™ Technology

KEY WORDS

Size-exclusion chromatography, SEC, peptides, proteins, SE-UPLC, Gel-Filtration Chromatography, calibration curves

INTRODUCTION

In 2010 over 60 therapeutic peptides were available in the US, Europe and/or Japan.[1] Recent trends indicate this number will only increase: the decline in development of small molecule pharmaceuticals, combined with improvements in peptide synthesis, have renewed interest in the research and development of peptide biotherapeutics a class of compounds that includes synthetic peptides such as vasopressin analogues and enfuvirtide.[2,3]

However, the complex nature of biotherapeutics requires a number of different analytical techniques for complete characterization, with each technique providing information on a different physical property of the biomolecule. One such technique, size-exclusion chromatography (SEC) can be used to provide molecular weight characterization for the both the biomolecule and any process related species.[2,3] In this chromatographic mode, apparent molecular weight, based on hydrodynamic radius, is determined by comparing the elution volume of the unknown biomolecule with the elution profile of a known set of standards. However, these results can only provide useful information if the separation is solely size-based and not influenced by non-ideal or secondary interactions.

We have previously described the benefits of Ultra Performance Liquid Chromatography (UPLC®) combined with 200 Å sub-2 µm SEC packing materials for the analysis of monoclonal antibodies; however, these packing materials are not ideal for small biomolecules (<80,000 Da).[4,5] In the following application, the impact of a 125 Å pore sub-2 µm packing material on the separation and resolution of small proteins and peptides will be demonstrated. We will also show the impact of both physical and chemical properties of SEC packing materials on SEC calibration curves used for molecular weight estimation.

EXPERIMENTAL

SAMPLE PREPARATION: All samples were prepared in 25 mM sodium phosphate, 150 mM sodium chloride pH 6.8 buffer. Proteins and peptides were purchased as individual standards (Sigma-Aldrich). Sample concentrations ranged from 1–5 mg/mL. All samples were tested as individual standards unless otherwise noted.

LC Conditions

LC System:	ACQUITY UPLC H-Class Bio System with Column Manager or 30 cm Column Heater
Detection:	TUV detector with 5 mm Titanium Flow Cell
Wavelength:	280 and 214 nm
Columns:	ACQUITY UPLC BEH125 SEC, 1.7 µm Column, 4.6 x 150 mm and 4.6 x 300 mm (Part Number: 186006505); ACQUITY UPLC BEH200 SEC, 1.7 µm Column, 4.6 x 150 mm (Part Number: 186005225); BioSuite™ 125 UHR, 4 µm Column, 4.6 x 300 mm (Part Number: 186002161)
Column Temp.:	30 °C
Sample Temp.:	10 °C
Injection Volume:	2–8 µL
Flow Rate:	0.4 mL/min
Mobile Phases:	25 mM sodium phosphate, 150 mm sodium chloride, pH 6.8, 25 mM sodium phosphate, 250 mm sodium chloride, pH 6.2 and 30% ACN, 0.1% TFA (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Vials:	Maximum Recovery Vials (Part Number: 186002802)

Data Management

Chromatography Software:	UNIFI™ v 1.5 Software

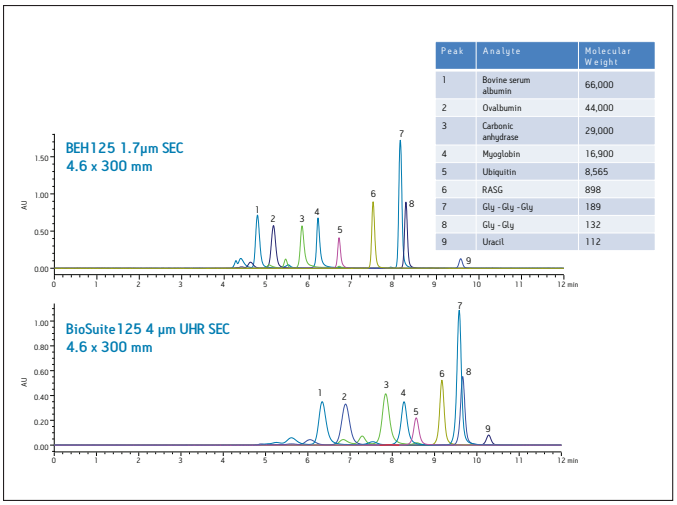
RESULTS AND DISCUSSION

Size-based separation calibration curves are based on known molecular weights of each protein as a function of elution volume or retention time. These curves, typically linear or third order polynomial, provide a means to get an approximate molecular weight of an unknown protein or peptide. While the linear portion of the calibration curve provides the highest resolution, non-linear behavior can also be observed since elution is dependent on the hydrodynamic radius of the molecule. While pore size is the predominant determining factor for the linear range of an SEC calibration curve, other factors include total pore volume of the column and pore size distribution.

Effect of Particle Size

The benefits of smaller particles for size-exclusion chromatography have been well documented demonstrating improvements in efficiency and resolution.[5] Until recently, most studies have evaluated packing materials consisting of particle sizes greater than 3 µm. The advent of sub-2 µm SEC column packing materials allows for further improvements in resolution and efficiency.

A set of proteins and peptides were analyzed on both a UPLC-based BEH SEC column (1.7 µm) and an HPLC-based silica SEC column (4 µm) using the same ACQUITY UPLC H-Class Bio System (Figure 1) and aqueous mobile phase conditions (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8). The elution volume of the peptides and proteins was lower for the ACQUITY UPLC BEH125 SEC, 1.7 µm column as compared to the HPLC-based silica column. In addition, improved sensitivity and narrower peak widths were observed on the sub-2 µm packing material. USP resolutions for the main constituents were also calculated for both the UPLC-based BEH SEC (1.7 µm) column and the HPLC-based silica SEC (4 µm) column (Figure 2). While resolution in SEC with respect to the particle used is primarily a function of pore size and pore volume, the particle size of the separation medium also affects the ability to resolve closely related, molecular weight species. As shown in Figure 2, the calculated USP resolutions for peaks 2–8 in the test mixture showed resolution gains from 24–200% as compared to the 4 µm SEC column. As predicted, the greatest improvements in resolution on the 125 Å SEC-UPLC column were obtained in the molecular weight range less than 20,000 Da.



Peak	Analyte	Molecular Weight
1	Bovine serum albumin	66,000
2	Ovalbumin	44,000
3	Carbonic anhydrase	29,000
4	Myoglobin	16,900
5	Ubiquitin	8,565
6	RASG	898
7	Gly - Gly - Gly	189
8	Gly - Gly	132
9	Uracil	112

Figure 1. Effect of particle size on SEC separation of proteins and peptides.

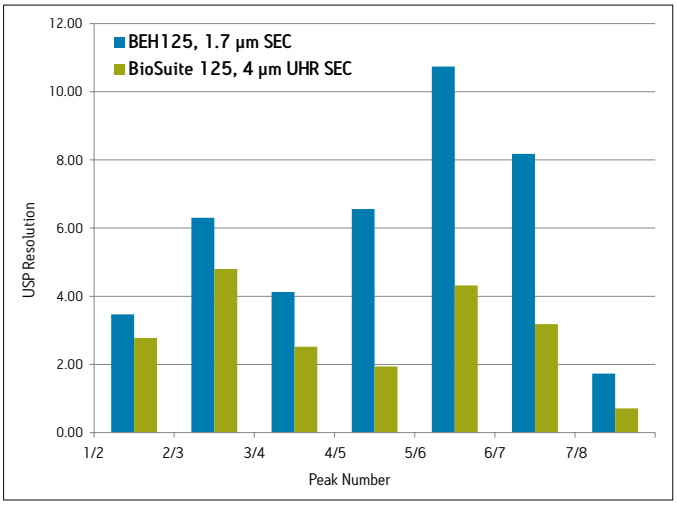
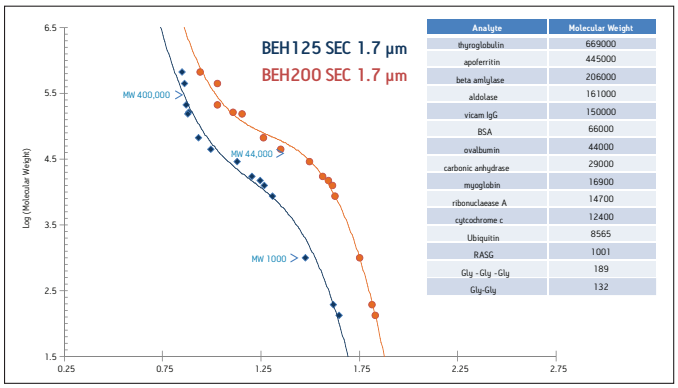


Figure 2: USP resolution of protein and peptides in Figure 4. USP Resolution was calculated by $2(t_{R2}- t_{R2})/(w_2+w_1)$, where t_R = retention time and w = peak width at 50% peak height.

Effect of Pore Size

For the analysis of small proteins and peptides, SEC packing materials typically contain pores of with a diameter <200 Å. These pore diameters have been shown to provide optimum resolution for solutes with less than 100,000 molecular weight. To evaluate the effect of pore size, a set of proteins and peptides were analyzed on both the 125 and 200 Å BEH sub-2 µm SEC columns under aqueous conditions (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8).

The calibration curve for each column was also evaluated to verify the effect of pore size on the molecular weight range (Figure 3). As described above pore size has a significant impact on the linear portion of an SEC calibration curve. Comparison of the 125 and 200 Å BEH sub-2 µm SEC columns illustrates this phenomenon. The calibration curve for the ACQUITY UPLC BEH200 SEC, 1.7 µm column showed greatest linearity and highest resolution, in the molecular weight range of 400,000 to 44,000 Da. Likewise, ACQUITY UPLC BEH125 SEC, 1.7 µm column provided highest resolution from 44,000 to 1,000 Da, the molecular weight range of most peptide biotherapeutics. As expected, pore size of the packing material had a significant impact on the useable molecular weight range of the column. The 200 Å packing material produced a separation with highest resolution over the molecular weight of 1,000,000 to approximately 44,000 Da, while the separation on the 125 Å packing material had greatest resolution from 44,000 to approximately 1,000 Da.



Analyte	Molecular Weight
thyroglobulin	669000
apoferritin	445000
beta amlylase	206000
aldolase	161000
vicam IgG	150000
BSA	66000
ovalbumin	44000
carbonic anhydrase	29000
myoglobin	16900
ribonuclaease A	14700
cytcochrome c	12400
Ubiquitin	8565
RASG	1001
Gly -Gly -Gly	189
Gly-Gly	132

Figure 3: Calibration curves: Effect of pore size. ACQUITY UPLC BEH125 SEC, 1.7 µm and ACQUITY UPLC BEH200 SEC, 1.7 µm columns, 4.6 x 150 mm comparison.

Effect of Mobile Phase Composition

SEC separations based on the hydrodynamic radius of the biomolecule rely on minimal adsorption between the analyte and packing material. These secondary interactions can be due to a number of different mechanisms including ionic interactions between the solute and the free silanols of the packing material or hydrophobic interactions between the solute and the hydrophobic sites on the packing material. While ion-exchange effects can be minimized by the addition of buffers and salts and/or pH adjustments of the mobile phase, hydrophobic effects are commonly minimized by the addition of organic solvents or other additives. Given these considerations, careful evaluation of mobile phase conditions must be conducted to ensure a predominantly size-based separation for peptides

As described above, the ACQUITY UPLC BEH125 SEC, 1.7 µm column provided improved component resolution in molecular weight range less than 20,000. To explore the SEC separations within a defined molecular weight range, a series of peptides less than 9,000 Da were analyzed under aqueous conditions. Method development experiments evaluated the effect of mobile phase pH and salt concentration. The results showed minimal effect of salt concentrations (150–350 mM) and mobile phase pH (6.2–7.4) on retention time (data not shown). All of the aqueous mobile phases resulted in later than expected elution for most small peptides and proteins (<17,000 Da) as well as elution order that did not correspond to published molecular weight values. For example, bradykinin fragment 1–7 (MW 757) eluted before greater molecular weight peptides such as angiotensin I (MW 1296) and bradykinin (MW 1,060). Figure 4a. These results also suggest the non-ideal interactions of the tested peptides with the media is not solely due to an "ion-exchange" mechanism since increasing salt concentration had no significant impact on retention time.

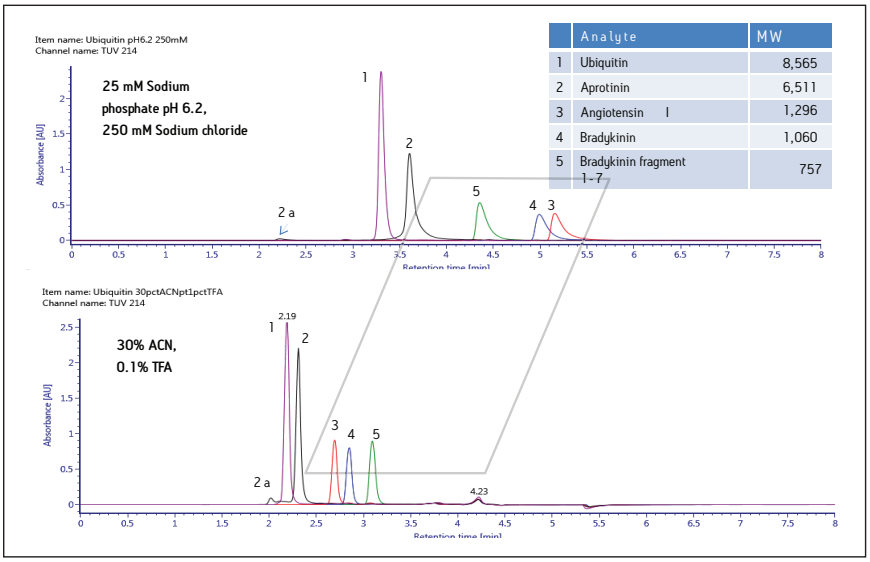
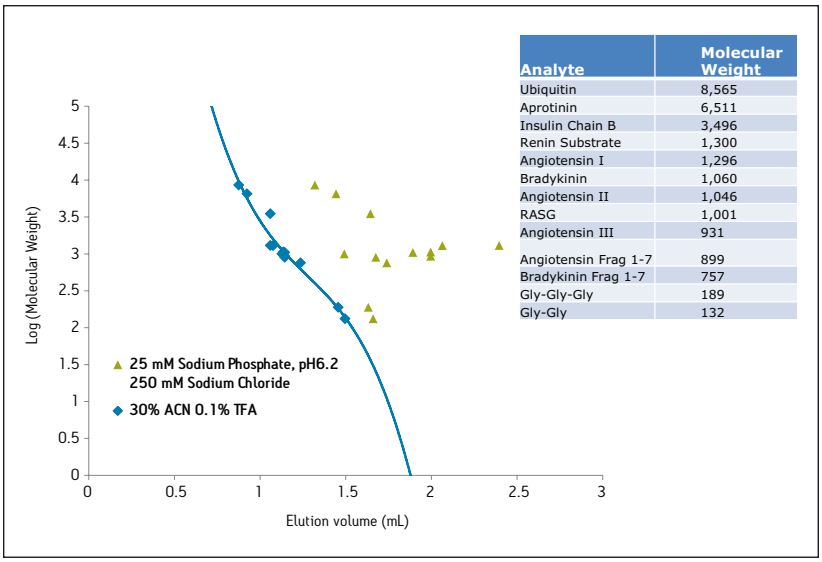


Figure 4. Effect of mobile phase on SEC separation of peptides.

In order to optimize the SEC separation for these peptides, evaluation of mobile phase was conducted. Mobile phases commonly used for SEC analysis of biotherapeutic peptides are denaturing and often contain organic solvents, acids and denaturants/charge additives such as arginine. These mobile phases minimize non-ideal (hydrophobic and/or ionic) interactions and thus are often needed to obtain a size-based separation for some peptides.[6] Additionally, these mobile phases can also affect retentivity by changing the structural conformation of the peptides. Under native conditions peptides may form stable secondary structures, while in the presence of denaturants these same polypeptides form random coil structures. These confirmation changes can increase the hydrodynamic radius of the biomolecule resulting in changes in elution volume.

The ACQUITY UPLC BEH125 SEC, 1.7 µm column was tested under similar conditions with organic/ion-pairing mobile phases (Figure 4). Acetonitrile was used to minimize hydrophobic interactions and trifluoroacetic acid was used as an ion pairing reagent to reduce "ion-exchange" or charge-charge interactions. As expected, this mobile phase (30% acetonitrile and 0.1 % trifluoroacetic acid [TFA]) produced earlier retention times and more symmetrical peak shapes for the peptides analyzed. Furthermore, in contrast to the SEC separation of peptides under 100% aqueous mobile phases conditions, the use of organic and ion-pairing mobile phases resulted in the expected elution order for bradykinin fragment 1–7, angiotensin I and bradykinin, based on their molecular weights (Figure 4b). These elution order changes could be due to reduction of secondary interactions and/or changes in the confirmation and hydrodynamic radii of the peptides.

Comparison of the SEC calibration curves more clearly illustrates the effect of mobile phase formulation on the SEC separation of small biomolecules (Figure 5). Under aqueous condition (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8), the elution order of the peptides appears random. However, the use of acetonitrile and TFA in the mobile phase produced a 3rd order polynomial calibration curve, as predicted in size-exclusion chromatography. This allows for reliable molecular weight estimation based on the linear region of the calibration curve. For example, the high molecular weight species of aprotinin (peak 2a) was calculated to be within 11% (or 14,370 Da) of the expected molecular weight (13,022 Da). This same estimation could not be performed under aqueous conditions because of the non-linearity of the calibration curve.



Analyte	Molecular Weight
Ubiquitin	8,565
Aprotinin	6,511
Insulin Chain B	3,496
Renin Substrate	1,300
Angiotensin I	1,296
Bradykinin	1,060
Angiotensin II	1,046
RASG	1,001
Angiotensin III	931
Angiotensin Frag 1-7	899
Bradykinin Frag 1-7	757
Gly-Gly-Gly	189
Gly-Gly	132

Figure 5. Effect of mobile phase on calibration curve of BEH125 SEC, 1.7 µm column.

CONCLUSIONS

Size-exclusion chromatography has been the preferred method for the analyses of biomolecules based on size. By combining 125 Å sub-2 µm packing materials with a low dispersion ACQUITY UPLC H-Class System, improved resolution and high-throughput of SE-UPLC can be realized for small biomolecule separations. However, secondary interactions may need to be minimized in the development of a size-based separation for reliable molecular weight estimation.

The ACQUITY UPLC BEH125 SEC, 1.7 µm column combined with the ACQUITY UPLC H-Class Bio System provides:

- Improved resolution and higher throughput as compared to traditional SE-HPLC packing materials
- Improved resolution for 80,000–1,000 Da as compared to larger pore sized packing materials
- Compatibility with denaturing mobile phase used to reduce secondary interactions between peptides and packing materials for molecular weight characterization and impurity testing in the production of peptide biotherapeutics.

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Advances in Size Exclusion Chromatography for the Analysis of Macromolecular Proteins

Stephan Koza and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution of macromolecular proteins by SE-UPLC®
- Outstanding column stability and reliable column-to-column reproducibility
- Increased size separation range when the new 450Å BEH450 SEC, 2.5 µm Column is used in series with the Waters® ACQUITY UPLC® BEH200, 1.7 µm, SEC Column

WATERS SOLUTIONS

ACQUITY UPLC BEH450 SEC, 2.5 µm Column

ACQUITY UPLC H-Class Bio System

Auto•Blend Plus™ Technology

BEH450 SEC Protein Standard Mix

KEY WORDS

Size-exclusion chromatography, SEC, peptides, proteins, SE-UPLC, gel filtration chromatography, calibration curves, macromolecules, IgM

INTRODUCTION

The separation of macromolecular proteins by size-exclusion chromatography has been an area of significant interest since the introduction of cross-linked dextran based soft-gels by Porath and Flodin in 1959.[1] This mode of separation was further improved by the introduction of particles comprised of cross-linked polystyrene (µ-Styragel™) and ultimately porous-silica particles. With that perspective, the newly introduced sub-3-µm 450Å BEH SE-UPLC particle presented in this note represents the latest step in the technological evolution of the size-based separation of biological macromolecules. This note will highlight the performance characteristics of this column with respect to column-to-column reproducibility and column stability. Additionally, the distinct advantages that sub-3-µm packing material offers over a larger (8 µm) particle size for the separation of large proteins will also be shown. Finally, we will demonstrate where the size-separation range of this 450Å pore-size SE-UPLC Column complements that of the 200Å ACQUITY® BEH200 SEC Column and how the two columns can be used together to increase the molecular weight range of a protein separation.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures (Waters and Sigma-Aldrich). Sample concentrations were 1.0 mg/mL (nominal) unless noted otherwise.

Method conditions

(unless noted otherwise)

LC conditions

System:	Waters ACQUITY UPLC H-Class Bio System with 30-cm Column Heater
Detection:	ACQUITY UPLC TUV with 5-mm titanium flow cell Wyatt miniDAWN TREOS light scattering detector
Wavelength:	280 or 214 nm
Columns:	Waters ACQUITY UPLC BEH450 SEC Column, 450Å, 2.5 µm, 4.6 x 150 mm (p/n 176002996) and 4.6 x 300 mm (p/n 176002997) Waters ACQUITY UPLC BEH200 SEC Column, 200Å, 1.7 µm, 4.6 x 150 mm (p/n 186005225) and 4.6 x 300 mm (p/n 186005226)
HPLC Column:	450Å, 8 µm, 7.8 x 300 mm
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.35 mL/min
Mobile phases:	25 mM sodium phosphate, 250 mm sodium chloride, pH 6.8 (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Standard:	BEH450 SEC Protein Standard Mix (p/n 186006842)
Sample vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 1 mL (p/n 186000385DV)

Data management

Chromatography software:	Waters Empower® Pro (*v*2, FR 5) Waters UNIFI® (v1.6) Wyatt Astra (v5.3.4.16)

RESULTS AND DISCUSSION

The benefits of UltraPerformance Liquid Chromatography (UPLC) combined with 125Å and 200Å pore-size, sub-2-µm size-exclusion UPLC (SE-UPLC) packing materials for the analysis of peptides and proteins have been previously described.[2,3] The newly introduced BEH 450Å pore size, sub-3-µm packing material, designed to expand the molecular weight range of SE-UPLC separations to include biological macromolecules with large radii of hydration (Rh), such as IgM and multimeric self-associated proteins, will be evaluated. As part of this evaluation, the critical performance characteristics of column-to-column reproducibility, lifetime stability, and the separation efficiency advantages of this packing material with respect to larger particle-size (8 µm) HPLC packing materials will be demonstrated. In addition, this note will demonstrate where the size-separation range of this 450Å pore-size SE-UPLC Column complements that of the 200Å ACQUITY BEH200 SEC Column and how the two columns can be used together to increase the molecular weight range of a protein separation.

ACQUITY UPLC BEH450 Column reproducibility and stability

In addition to analyte resolution and sensitivity, the two major concerns that an analyst has when selecting an SEC column for method development are column-to-column reproducibility and column lifetime. An overlay of the chromatograms for a series of molecular weight standards is shown in Figure 1. These chromatograms demonstrate the reproducibility of five 300-mm length ACQUITY BEH450 SEC Columns packed from three different production lots of packing material. For these standards at a flow rate of 0.35 mL/min, the retention time standard deviation ranged from a minimum of 0.005 min for the earliest eluting component (IgM pentadimer) to 0.022 min for the total permeation component (uracil) with an average standard deviation of 0.017 min for all components labeled in Figure 1.

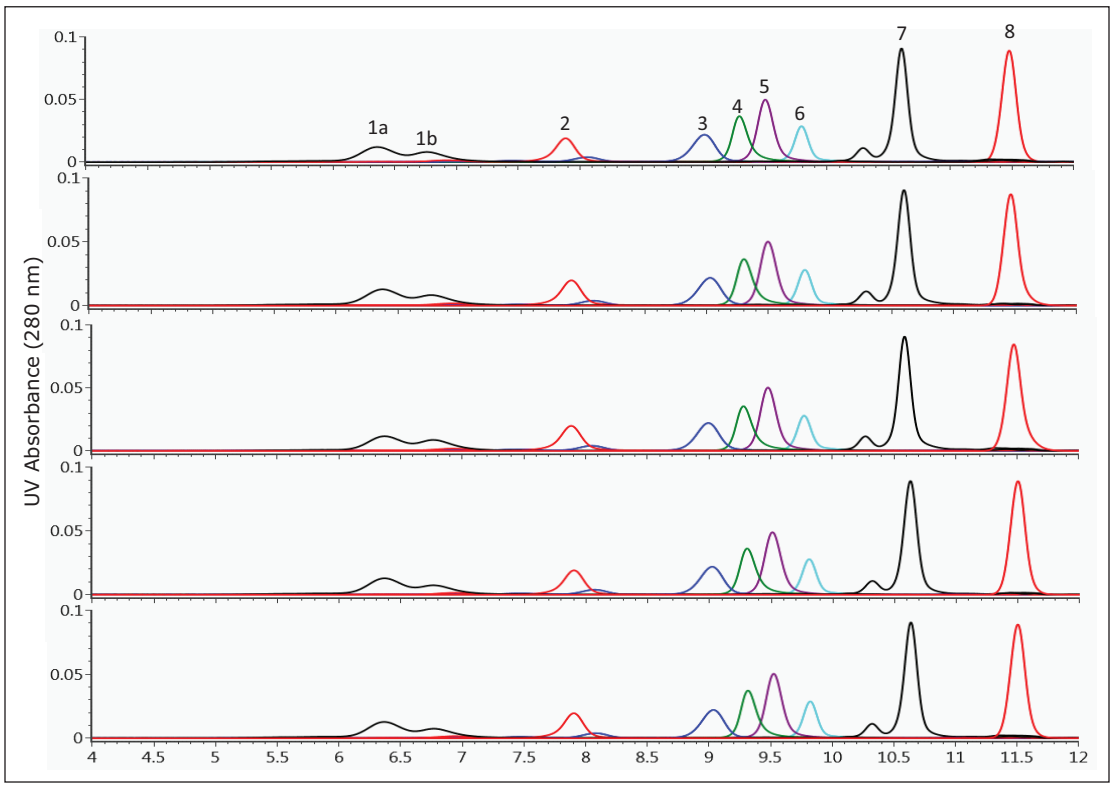

Figure 1. Column-to-column reproducibility of the 300 mm ACQUITY UPLC BEH450 SEC Column. The chromatograms for five columns packed from three different production batches are shown. Compounds: 1a. IgM Dipentamer (1.8MDa), 1b. IgM Pentamer (900 Kda), 2. Thyroglobulin (667 KDa), 3. Apoferritin (443KDa), 4. β-Amylase (200 Kda), 5. IgG (150 KDa), 6. BSA (66 KDa), 7. Myoglobin (17 KDa), and 8. Uracil (112 Da).

The stability of the ACQUITY BEH450 SEC Column (300 m) was evaluated by injecting a series of standards over the course of over 800 total injections. The retention time stability for the largest protein evaluated in this study (thyroglobulin), which is used to probe the chemical stability of the particle surface, is shown in Figure 2. The peak asymmetry of the uracil standard is also shown in Figure 2. This compound, which elutes in the total permeation volume, is used to test the mechanical integrity of the column. These data demonstrate that the ACQUITY BEH450 SEC Column can provide the reproducibility and stability needed to develop reliable assays for use in a quality control environment.

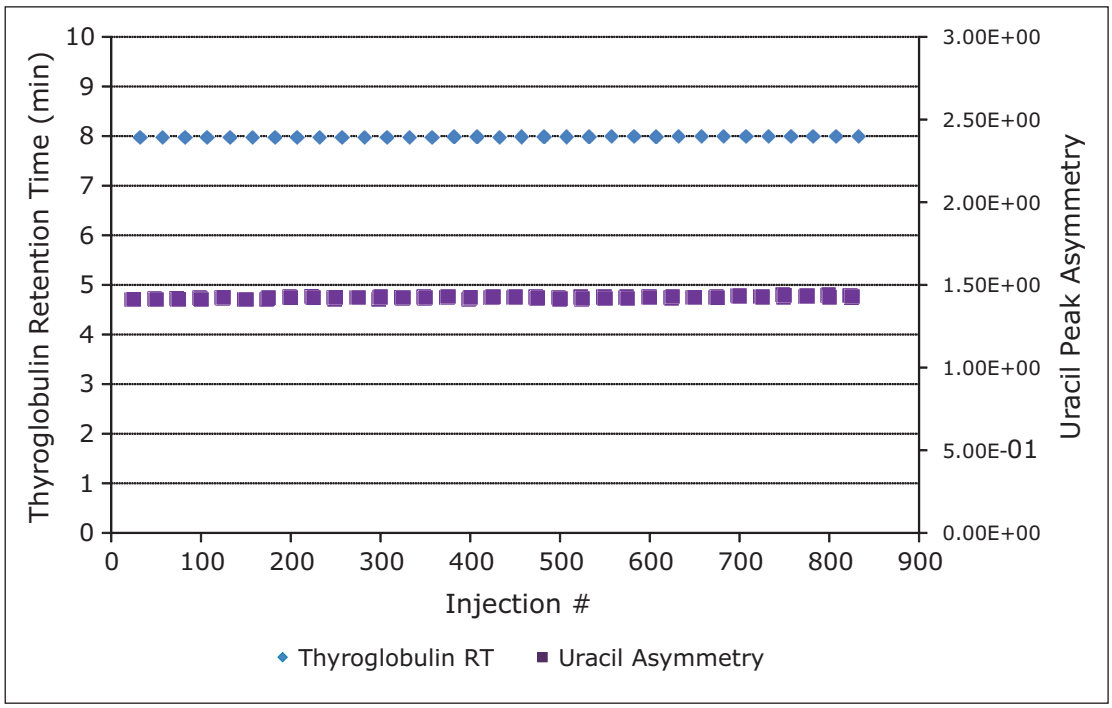

Figure 2. Performance stability of the 300 mm ACQUITY UPLC BEH450 SEC Column over 800 injections. The retention time of thyroglobulin and the asymmetry of the uracil standard (at 4.4% peak height) are shown.

Effect of particle size

The benefits of smaller particles for size-exclusion chromatography have been well documented with improvements in efficiency and resolution.[4] A set of proteins was analyzed individually on a 450Å pore-size UPLC-based BEH SEC column (2.5 µm) and a 450Å pore-size HPLC-based silica SEC column (8 µm) using the same ACQUITY UPLC H-Class Bio System and aqueous mobile phase conditions, shown in Figure 3. The flow rates and injection volumes used were proportional to the size of the column tested. Improved sensitivity and narrower peak widths were observed on the 2.5 µm packing material across the separation range. USP resolution values (half-height measurement) calculated for the separation between the apoferritin monomer (MW=443 KDa) and dimer (MW=886 KDa) forms, shown in Figure 4, were base-line resolved with a resolution of 2.49. This resolution is 1.8 times greater than that observed using the 8 µm particle-sized column (R_s=1.42).

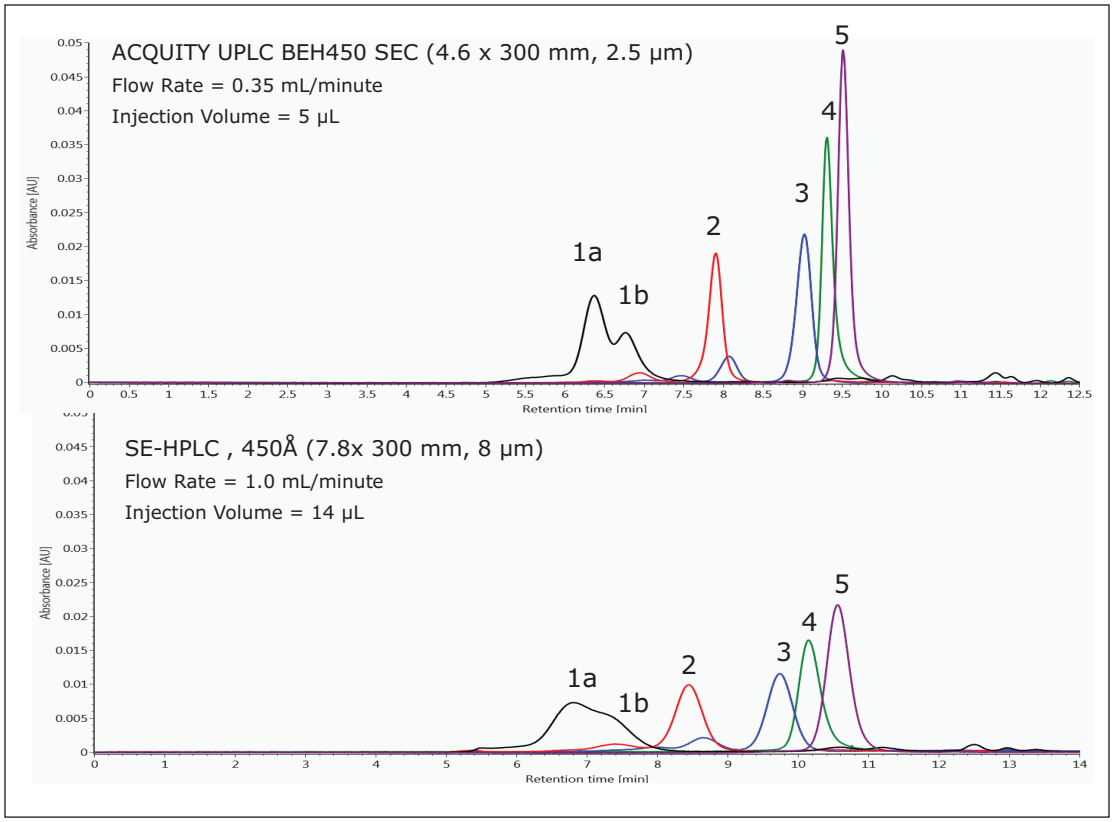

Figure 3. Comparison of the ACQUITY UPLC BEH450 SEC Column (300-mm length) to a Biosuite 450 HR Column (300-mm length). Compounds: 1a. IgM Dipentamer (1.8MDa), 1b. IgM Pentamer (900 Kda), 2. Thyroglobulin (667 KDa), 3. Apoferritin (443KDa), 4. β-Amylase (200 KDa), 5. IgG (150 KDa). Sample injection volumes and flow rate were normalized for column geometry. UV absorbance (280 nm) shown at same scale for both columns. The identities of the peaks observed for the IgM sample were confirmed by SEC-MALLS analysis.

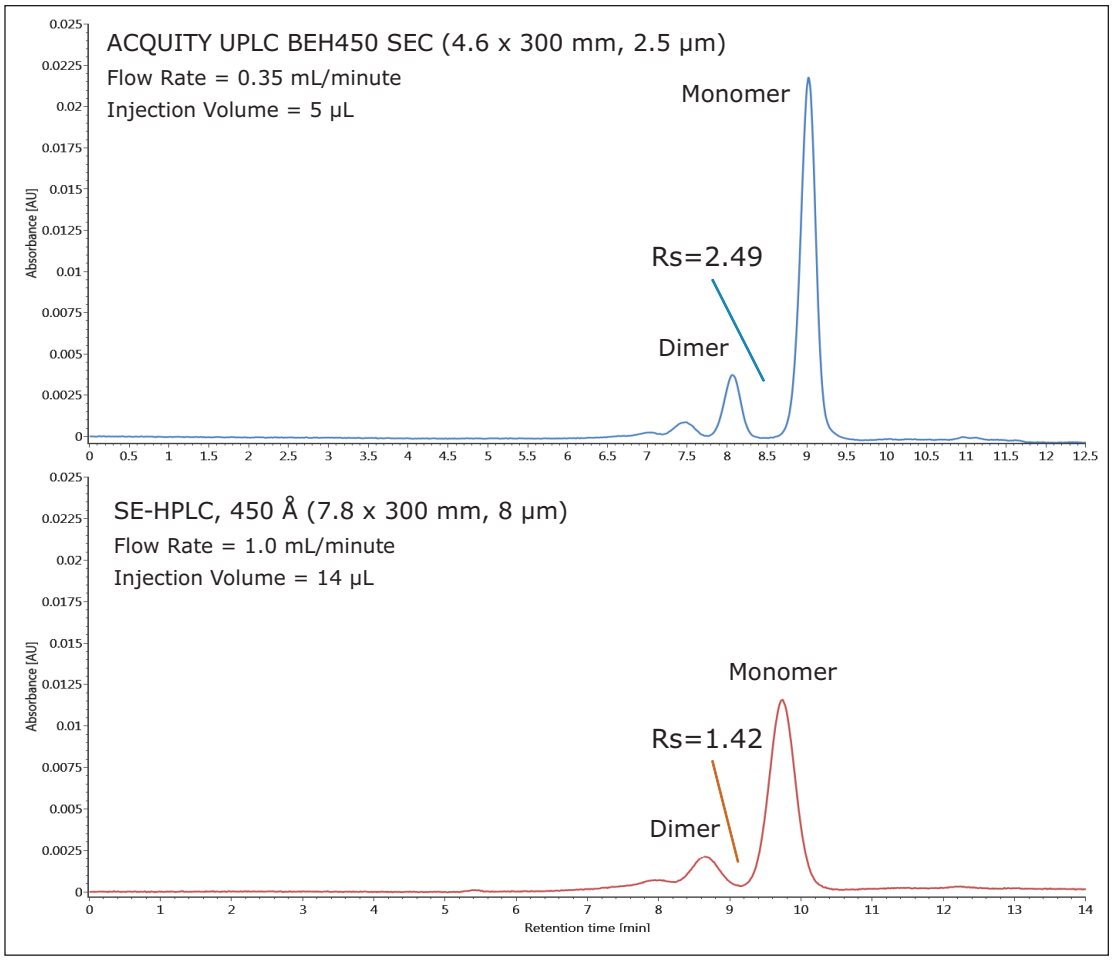

Figure 4. Comparison of the ACQUITY UPLC BEH450 SEC Column (300-mm length) to a Biosuite 450 HR Column (300-mm length) for the separation of apoferritin (443 KDa). Sample injection volumes and flow rate were normalized for column geometry. UV absorbance (280 nm) shown at same scale for both columns.

Expanding molecular weight range: combination with ACQUITY BEH200 SEC (200Å)

Comparisons were made between the 450Å and 200Å pore-size particles for the separation of proteins. Additionally, both columns used in the series were evaluated. The comparison of the separation achieved on the 200Å and 450Å SE-UPLC columns (300-mm length) and the 150-mm length version of both columns connected in series (BEH200 followed by BEH450) for the Waters BEH200 SEC Protein Standard Mix (p/n 186006518) is shown in Figure 5. For this standard mix, it is clear that the resolution of the separation for the standard components with molecular weights up to that of the IgG standard (150 KDa) is higher with the 200Å column as noted by the separation between IgG and BSA (66 KDa). For higher molecular weight components like thyroglobulin (667 KDa) and its dimeric form (1340 KDa or IgM pentamer (900 KDa) and IgM dipentamer (1800 KDa), however, the 450Å can provide improved separations, as shown in Figure 4. This higher molecular weight range may be of use when analyzing multimeric protein aggregates or proteins conjugated to compounds that have relatively large Rh values, such as long chain polyethylene glycols.

A third option available to the analyst in need of analyzing a sample containing both low molecular weight and multimeric high molecular weight forms in a sample, is to use the two columns in series. As the back pressure generated by the 200Å column is greater than that of the 450Å column due to the smaller particle size of 200Å column, the 200Å column was placed first in the series for this study. The result of this two-column configuration is shown in the center panel of Figure 5. By using both columns, the functional upper molecular weight range of the separation is increased as noted by the improved separation of thyroglobulin and its dimer compared to that same separation using the 200Å column alone. Additionally, for the lower molecular weight forms, there is an improvement in resolution compared to the use of the 450Å column alone, as evidenced by the improved separation between IgG and BSA. These observations can be confirmed by evaluating the elution volumes of these components in the calibration curves generated from these data, as shown in Figure 6. The calibration curve for the ACQUITY UPLC BEH200 SEC Column showed greater linearity, and elution volume differences up to at least the IgG (150 KDa) standard compared to the ACQUITY UPLC BEH450 SEC Column. Conversely, using the same criteria, the BEH450 column is better suited for proteins with a molecular weight of near that of thyroglobulin (667 KDa) and greater. The BEH200 and BEH450 columns in series produced an intermediate calibration curve with the broadest pseudo-linear range. The compromise of this two-column configuration is that the resolutions achieved for proteins within the optimal range of each individual column are diminished, however.

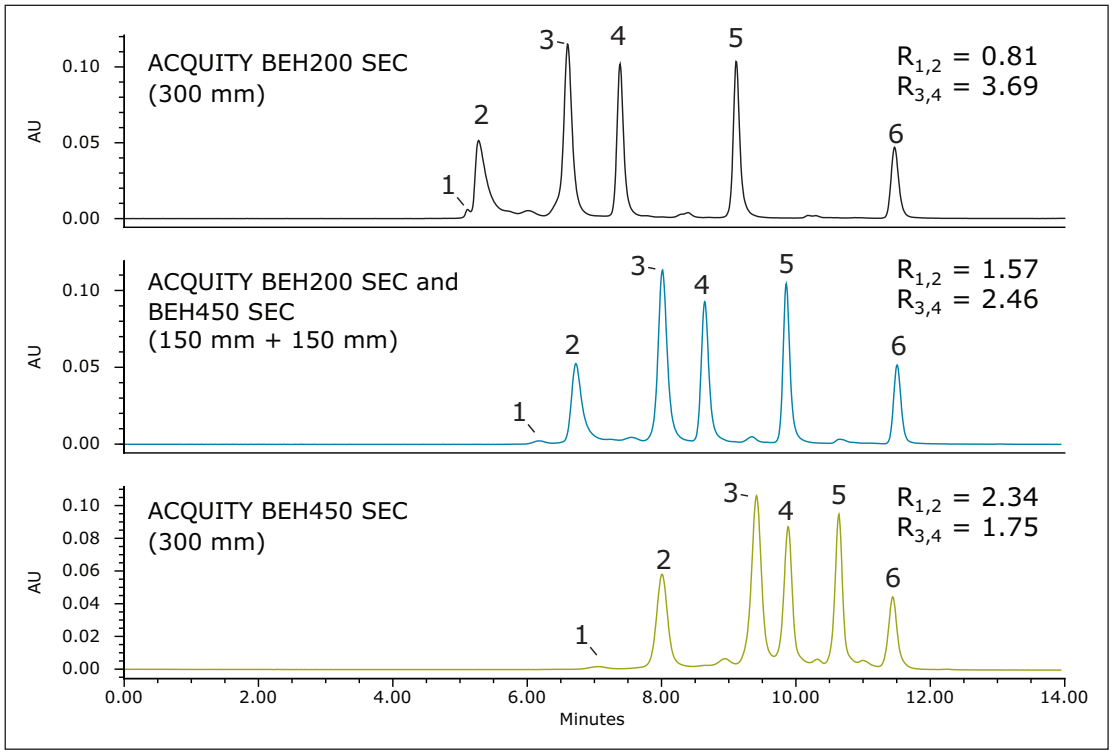

Figure 5. Comparison of the ACQUITY UPLC BEH450 SEC Column to the ACQUITY UPLC BEH200 SEC Column (both 300-mm length). The center panel was generated using both a BEH200 and BEH450 column in series. Compounds: 1. Thyroglobulin dimer (1,340 KDa), 2. Thyroglobulin (667 KDa), 3. IgG (150 KDa), 4. BSA (66 KDa), 5. Myoglobin (17 KDa), 6. Uracil (112 Da). Resolution values were calculated based on peak width at half maximum.

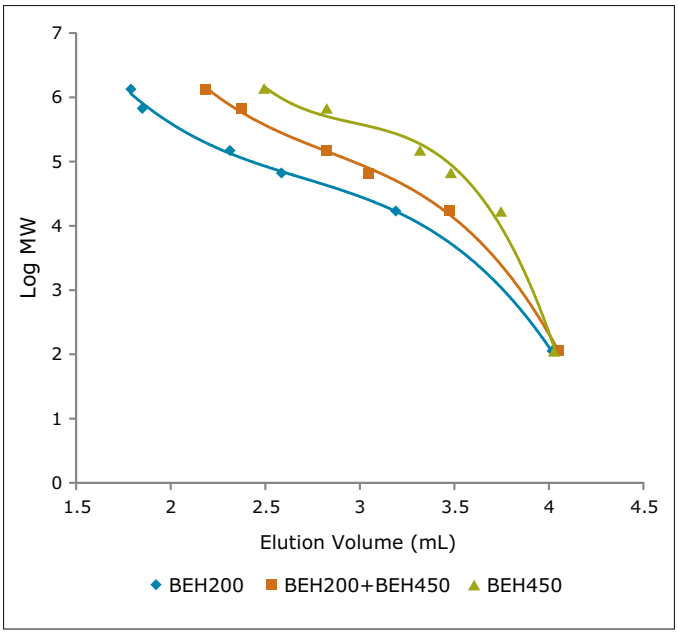

Figure 6. Comparison of the ACQUITY UPLC BEH450 SEC and ACQUITY UPLC BEH200 SEC Column calibration curves. Retention times of compounds tested in Figure 5 are represented.

CONCLUSIONS

Size-exclusion chromatography has been the preferred method for the analyses of proteins based on size. By combining 450Å sub-3-µm packing materials with a low dispersion ACQUITY UPLC H-Class System, separations with improved resolution and high-throughput of SE-UPLC can be realized for macromolecular proteins and highly aggregated proteins with molecular weights of up to approximately 2000 KDa. Additionally, the use of the BEH450 and BEH200 columns in series can provide a broader molecular weight range than can be obtained by using the columns individually.

The ACQUITY UPLC BEH450, 2.5 µm SEC Column in combination with the ACQUITY UPLC H-Class Bio System provides the following benefits:

- Outstanding column-to-column reproducibility and stability
- Nearly a two-fold increase in resolution compared to traditional SE-HPLC packing materials
- Improved resolution for large proteins and aggregates with molecular weights above the linear separation range of the smaller pore-size SE-UPLC columns
- Expanded molecular weight range when used in series with a UPLC BEH200, 1.7 µm SEC Column

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The Analysis of Multimeric Monoclonal Antibody Aggregates by Size-Exclusion UPLC

Stephan Koza, Matthew Lauber, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- ACQUITY UPLC® SEC Technology delivers increased high molecular weight protein (up to 1.8 MDa) component resolution compared to traditional, HPLC-based SEC separations.
- Size separation range increased with the BEH450 SE-UPLC® Column used in series with the BEH200 SE-UPLC Column for the analysis of dimer through multivalent mAb aggregates by SE-UPLC.

WATERS SOLUTIONS

ACQUITY UPLC BEH450 SEC, 2.5 µm Column

ACQUITY UPLC BEH200 SEC, 1.7 µm Column

ACQUITY UPLC H-Class Bio System

BEH450 SEC Protein Standard Mix

Auto•Blend Plus™ Technology

Empower® 3 Chromatography Data Software

KEY WORDS

Size-exclusion chromatography, SEC, monoclonal antibodies, mAb, proteins, SE-UPLC, gel-filtration chromatography, macromolecules, IgM, multimers

INTRODUCTION

Monoclonal antibodies (mAb) have become one of the predominant protein classes in the biotherapeutic landscape. Both the level and valency of soluble protein aggregation are critical quality attributes (CQA) that require monitoring for mAb preparations intended for human use. Protein aggregation, which may occur throughout the manufacturing process from cell culture through drug product shelf-life, may be indicative of partial denaturation or other perturbations of protein structure which can deleteriously effect the safety and efficacy of the protein biotherapeutic.[1] While it is important to quantitatively assess low valency (*e.g.* dimer) aggregate levels as a measure of process and product stability, as well as product safety, it is also critical to elucidate the distribution of high valency multimeric soluble aggregate forms in protein biotherapeutic preparations. These multimeric aggregate forms may be more effective in eliciting an immune response, due to their ability to trigger an immunological pathway independent of T-cell involvement.[2]

The recently introduced BEH 450Å pore size, sub-3-µm packing material has been designed to expand the molecular weight range of size-exclusion UPLC (SE-UPLC) separations to include biological macromolecules with large radii of hydration (R_h), such as IgM and multimeric self-associated proteins.[3] In this study, a 450Å pore sub-3-µm packing material (BEH450) was evaluated for the analysis of an mAb. The data demonstrate the advantages of a UPLC-based, size-exclusion separation compared to an HPLC-based, size-exclusion analysis for the separation of macromolecular protein complexes. In addition, data are presented showing the benefits of combined ACQUITY UPLC BEH SEC columns of 200Å and 450Å pores for the analysis of an mAb sample that contains high valency multimeric mAb aggregates.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures. The IgG_1 mAb sample was biotherapeutic trastuzumab that was analyzed past expiry. Sample concentrations were 1.0 mg/mL (nominal) unless otherwise noted.

Method conditions (unless otherwise noted)

LC conditions

System:	ACQUITY UPLC H-Class Bio with 30-cm Column Heater
Detection:	Waters® ACQUITY UPLC TUV Detector with 5-mm Titanium flow cell Wyatt miniDAWN TREOS light scattering detector
Wavelength:	280 or 214 nm
Columns:	ACQUITY UPLC PrST SEC, 450Å, 2.5 µm, 4.6 x 150 mm (p/n 176002996) and 4.6 x 300 mm (p/n 176002997) ACQUITY UPLC PrST SEC, 200Å, 1.7 µm, 4.6 x 150 mm (p/n 186005225) and 4.6 x 300 mm (p/n 186005226) HPLC column: Silica-based, diol bonded 450Å, 8 µm, 7.8 x 300 mm
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.35 mL/min
Mobile phases:	5 mM sodium phosphate, 250 mM sodium chloride, pH 6.8 (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Standard:	BEH450 SEC Protein Standard Mix (p/n 186006842)
Sample vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 1 mL (p/n 186000385DV)

Data management

Waters Empower® 3 Software

Waters UNIFI® Information System

Wyatt Astra Software

Cross-linking experimental detail

Covalent high molecular weight IgG aggregates were prepared using the Waters Intact mAb Standard (p/n 186006552), and the lysine-specific cross-linking agent, BS3 (Pierce, Rockford, IL). Reactions were performed with the antibody at a final concentration of 10 mg/mL and reagent-to-protein molar ratio of approximately 5:1 for 30 minutes.

RESULTS AND DISCUSSION

Comparison of SE-UPLC and SE-HPLC of IgM and IgM dimer

The BEH450 UPLC Column was compared to a silica-based 8-µm particle size HPLC column for the separation of IgM, a pentameric immunoglobulin with a molecular weight of 900 KDa, and the di-pentamer form of IgM with a molecular weight of 1.8 MDa (Figure 1). The sample loads and flow rates were adjusted for the column geometries used, and both analyses were performed on the same ACQUITY UPLC H-Class Bio System. The BEH450 column produced significantly better separation between the di-pentamer and pentamer forms, and improved sensitivity with peak height greater than 50% compared to the HPLC column. This remarkable improvement in separation efficiency is principally due to decreased particle size. Additionally, it can be observed that the molecular weight range of the BEH450 column extends above that of di-pentamer based on the observation of multimeric dimer forms eluting earliest in the chromatogram.

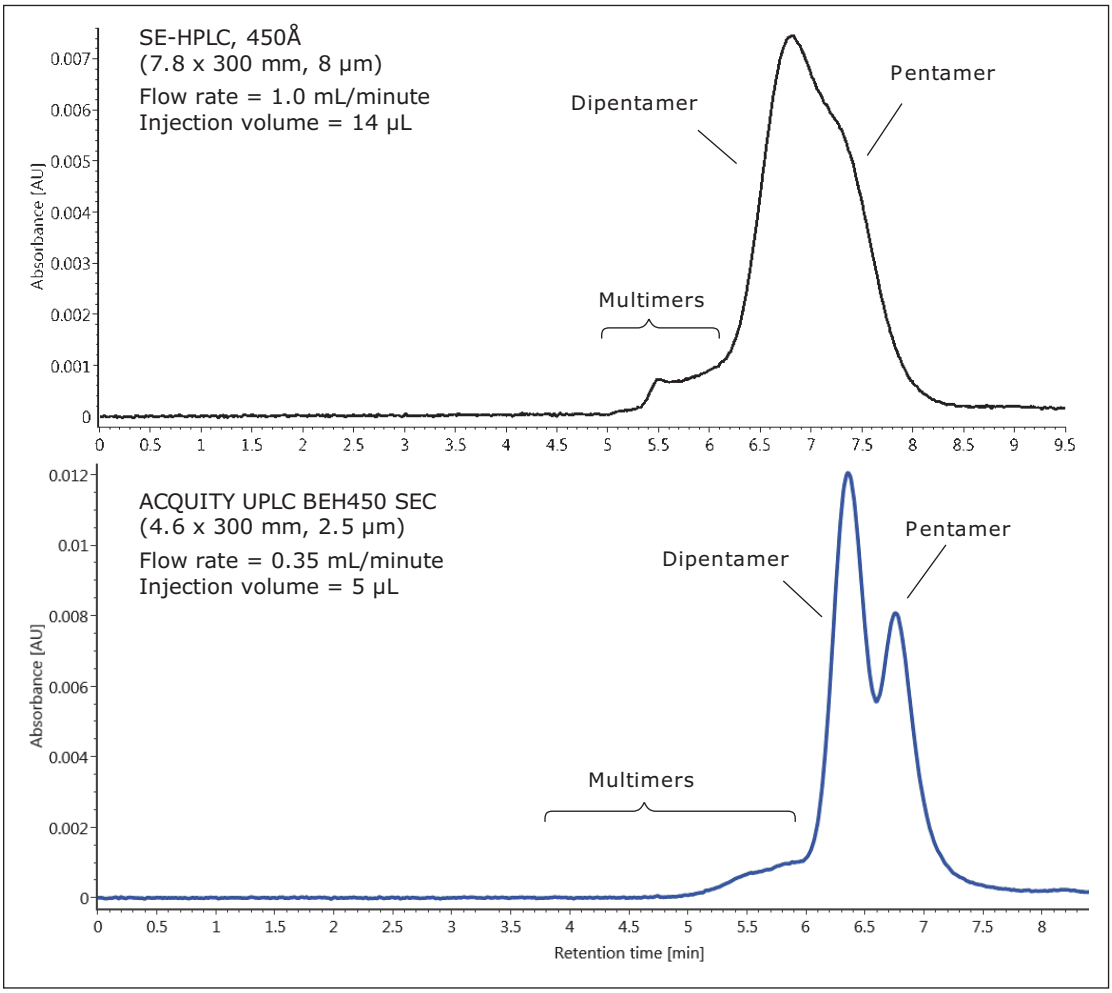

Figure 1. Comparison of an HPLC silica-based, 8-µm particle-size, 450Å SEC column (300-mm length) to an ACQUITY UPLC BEH450 SEC Column (300-mm length) for the separation of IgM pentamer (900 Kda) and IgM dipentamer (1.8 MDa). Sample injection volumes and flow rates were normalized for column geometry. The identities of the peaks were confirmed by SEC-MALS analysis.

Expanding the molecular weight range of mAb aggregation analysis

The outstanding efficiency provided by the BEH450 column for the separation of proteins above the upper molecular weight range of the BEH200 (approximately 450 KDa) suggests that using the two columns in series can provide some advantages for SE-UPLC separations over a broad molecular weight range. A comparison of the separation achieved on the BEH200 and BEH450 columns alone (each 300 mm in length) and the 150-mm length version of both columns connected in series (BEH200 followed by BEH450) for the Waters BEH200 SEC Protein Standard Mix (p/n 186006518) is shown in Figure 2. As the back pressure generated by the 1.7-µm particle size BEH200 column is greater than that of the 2.6-µm particle size BEH450 column, the BEH200 column was placed first in the series for this study. The result of this two-column configuration is shown in the center panel of Figure 2. By using both columns in series, the functional upper molecular weight range of the separation is increased as noted by the improved separation of thyroglobulin and its dimer compared to the separation using the 200Å column alone. Additionally, for the lower molecular weight forms, there is an improvement in resolution compared to the use of the 450Å column alone, as proven by the improved separation between IgG and BSA.

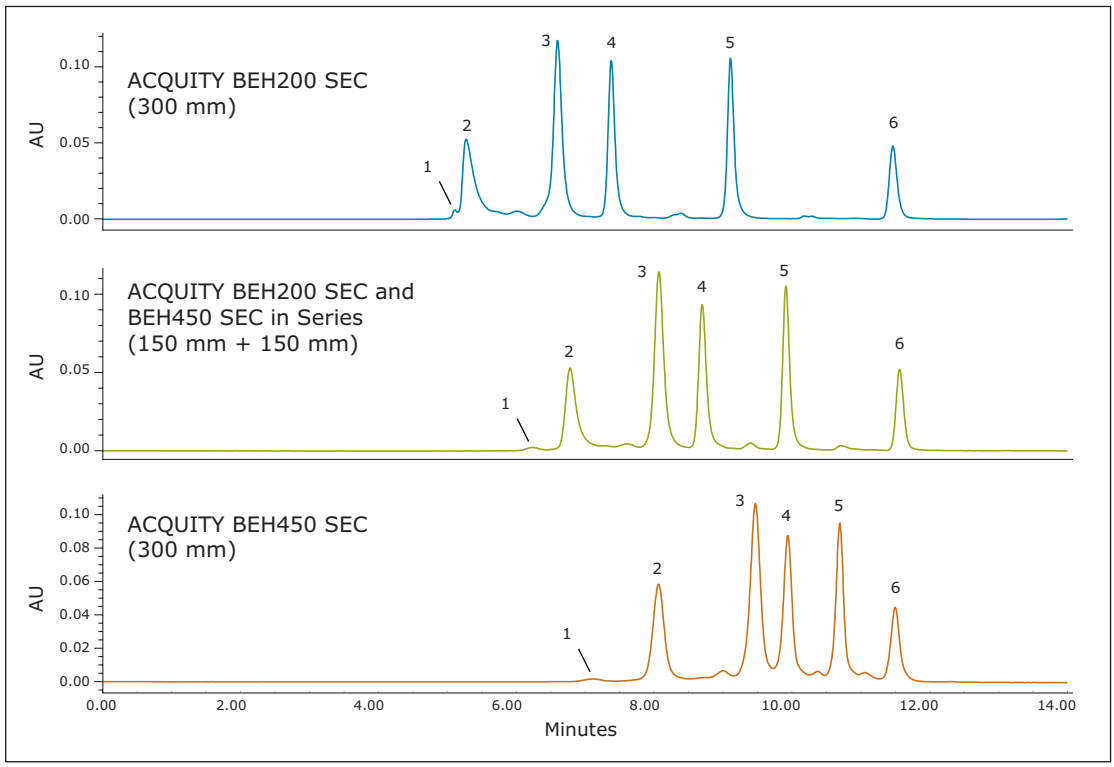

Figure 2. Comparison of the ACQUITY UPLC BEH450 SEC Column to the ACQUITY UPLC BEH200 SEC Column (300-mm lengths). The center panel was generated using both a BEH200 and BEH450 column in series. Columns were connected using Waters part number 186006613. Compounds included the following: 1. Thyroglobulin dimer (1340 KDa), 2. Thyroglobulin (667 KDa), 3. IgG (150 KDa), 4. BSA (66 KDa), 5. Myoglobin (17 KDa), and 6. Uracil (112 Da).

The use of two SEC columns of different pore size can provide separations over a broader molecular weight range. One example of such a separation is the multivalent aggregate, trimer, dimer, and monomeric forms of an mAb. To demonstrate this, a sample of IgG (p/n 186006552) was then cross-linked to generate covalent dimeric and multimeric forms in order to generate a stable sample with an abundant level of mAb multimeric species. This sample was then used to define the molecular weight range of aggregated mAb species that can be separated by the BEH200 and BEH450 columns. The cross-linking chemistry produced high levels of multimeric species that were easily characterized by multi-angle laser light scattering (MALS) measurements; however, the polydispersity of the peaks increased due to the nature of the cross-linker reaction, also resulting in non-crossed linked additions of the reagent to the proteins. These chromatograms, shown in Figure 3, along with the peak assignments based on the MALS data, demonstrate the advantages of using the BEH200 and BEH450 columns in series. For the separation using only the BEH200 column, excellent resolution is obtained between the monomer and dimer forms. However, when compared to the separation observed on the BEH450 column, the distribution of aggregate forms larger than trimer elute near the total exclusion volume of the BEH200 column. By using the two columns in series (middle chromatogram in Figure 3), the distribution of higher aggregate forms can be observed while better resolution between the monomer and dimer is achieved compared to using the BEH450 column alone.

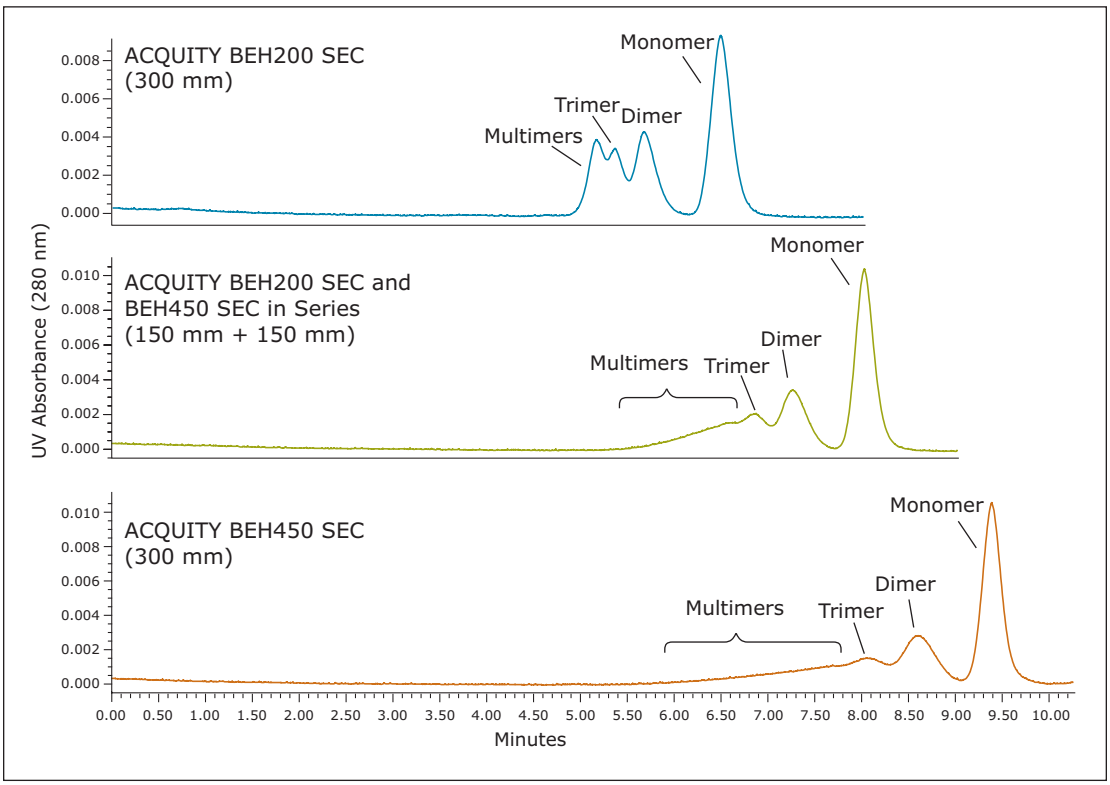

Figure 3. Comparison of the ACQUITY UPLC BEH450 SEC Column to the ACQUITY UPLC BEH200 SEC Column (300-mm lengths), and both a BEH200 and BEH450 column (150-mm lengths) in series for the separation of covalently cross-linked antibody sample. Columns were connected using Waters part number 186006613. The identities of the peaks were confirmed by SEC-MALS analysis.

Application of the BEH200 and BEH450 columns in series for the SE-UPLC analysis of a biotherapeutic IgG_1 mAb (trastuzumab) was then investigated. In order to generate a more relevant sample for this study, the trastuzumab sample was subjected to a series of freeze-thaw events to increase the levels of non-covalent aggregates in the sample. The aggregate levels were then evaluated using the BEH200 column (300-mm length) or the BEH200 and BEH450 columns in series (each 150-mm length). These results (Figure 4) show that the use of the two columns in series provides a separation in which the distribution of multimeric aggregate forms can be observed along with an improved separation between the dimeric and trimeric aggregate species when compared to the BEH200 column alone. Conversely, use of the BEH200 column alone provides a better separation of the mAb fragments that result from cleavage in the hinge region of the mAb.[4]

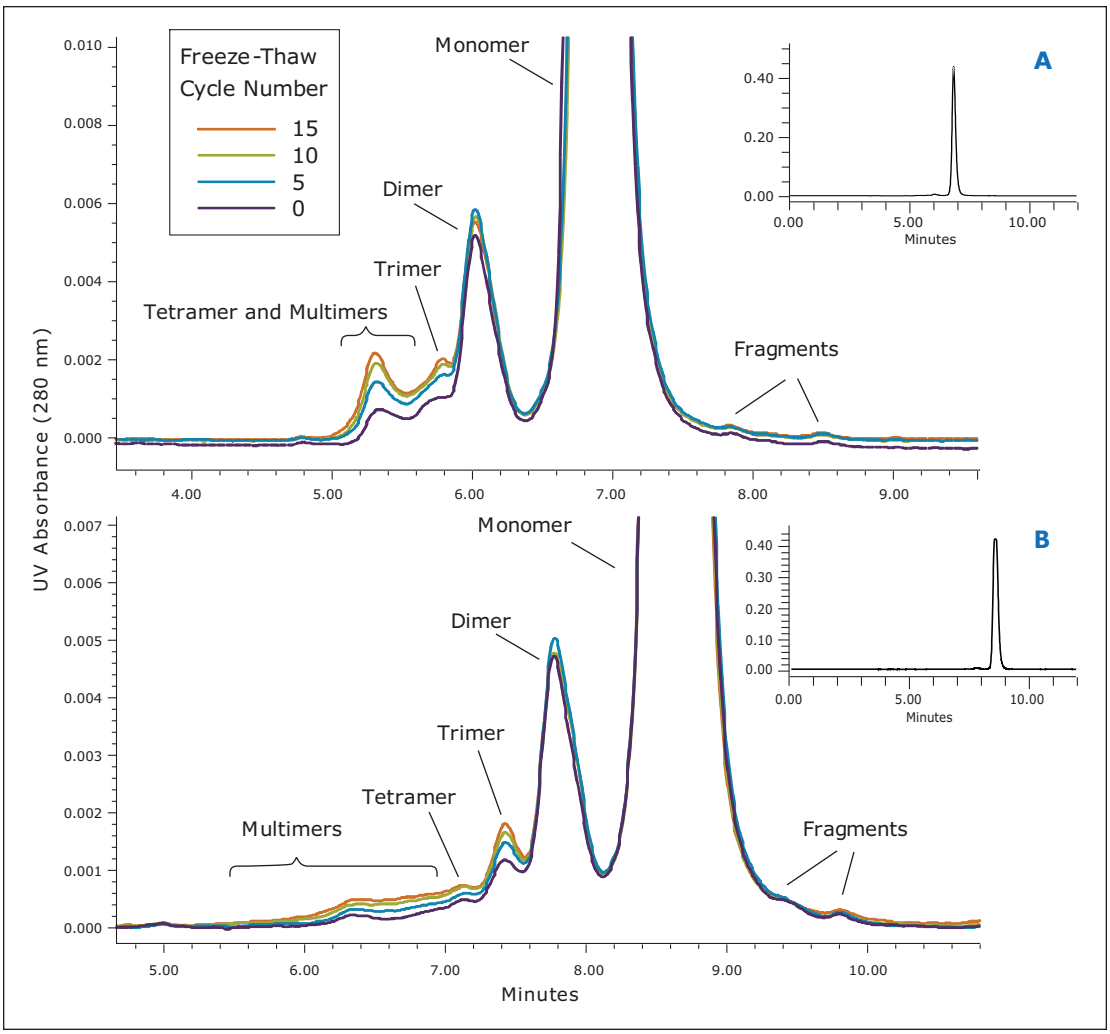

Figure 4. Comparison of a 300-mm length BEH200 column (A) versus a BEH200 and a BEH450 column (150-mm each) in series (B) for the separation of trastuzumab (IgG1) aggregation generated by increasing freeze-thaw cycles (0, 5, 10, and 15). Low-level peak identities are predicted based on elution position and previous monoclonal antibody assignments from Figure 3.

By evaluating the change in this profile over the course of the freeze-thaw study, it can be visually observed for both column configurations that the overall level of soluble trimer and multimeric aggregate is increasing. Both column configurations also provided comparable results for the determination of the relative levels of dimer, as well as the pooled trimer and multimer aggregate (Figure 5). This quantitative comparison required pooling the trimer and multimeric peak areas, as the resolution between the trimer and larger multimeric forms using the BEH200 column alone did not allow for accurate integration. However, the use of the BEH200 and BEH450 columns in series provides a significant benefit for this application in that the trimer and tetramer forms are more resolved compared to use of the BEH200 column alone. Additionally, the distribution of aggregate forms greater in valency than trimer and tetramer can be monitored better. These results are consistent with those presented previously (Figure 2), which demonstrate that the upper molecular weight range for the BEH200 column for a globular protein is approximately that of thyroglobulin (667 KDa), nearly the molecular weight of IgG tetramer (600 KDa). By comparison, the upper molecular weight range for the BEH450 column is approximately that of IgM dipentamer (1.8 MDa) which is the molecular weight of an IgG 12-mer. This additional information provided by the larger pore-size BEH450 column may be beneficial in characterizing a biotherapeutic protein, since in addition to the level of protein aggregation, the valency of that aggregation may potentially alter immunogenicity.

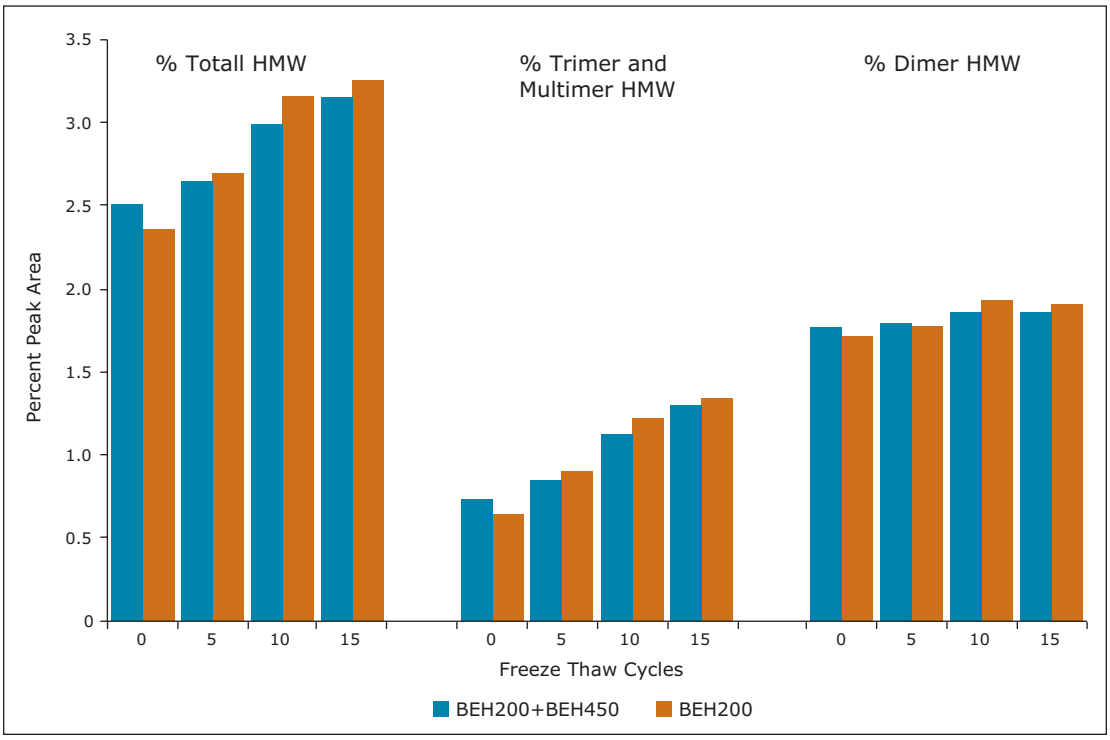

Figure 5. Comparison of relative peak areas observed in trastuzumab (IgG_1) as a result of aggregation generated by freeze-thaw cycles (0, 5, 10, and 15). Integrated results were determined from chromatograms presented in Figure 4.

CONCLUSIONS

Both the levels and the nature of soluble aggregates are important CQA for biotherapeutic protein preparations. The introduction of the BEH450 SEC column provides an extended upper molecular weight range for SE-UPLC analyses, and significantly improves resolution compared to a 450Å pore-size HPLC-based size-exclusion column. The use of the BEH450 SE-UPLC Column in series with the 200Å pore-size BEH200 column provides an expanded molecular weight range that can be used for the analysis of both dimeric and multimeric aggregates of an mAb, while taking advantage of the sensitivity, resolution, and throughput of SE-UPLC.

The ACQUITY UPLC BEH450, 2.5 µm SEC Column in combination with the ACQUITY UPLC BEH200, 1.7 µm SEC Column, and the ACQUITY UPLC H-Class Bio System provide the following benefits:

- Greater resolution and sample throughput compared to traditional SE-HPLC packing materials
- An extended useful SE-UPLC molecular weight range (approximately 10 KDa to 1800 KDa)
- Ability to observe the distribution of multivalent mAb aggregate forms while maintaining excellent resolution between the dimeric aggregate and monomeric mAb species

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Simultaneous Determination of Molecular Size, Concentration, and Impurity Composition of Biotherapeutics with SEC and the Biopharmaceutical Platform Solution with UNIFI

Robert Birdsall, Thomas E. Wheat, Henry Shion, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Efficient and integrated workflow for running batched samples to maximize analytical information content of biotherapeutics.
- Increased productivity through the automation of an analytical workflow including data acquisition, processing, and reporting.
- Increased productivity through automated assessment of protein aggregates.

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector with 5-mm titanium flow cell

Auto•Blend Plus™ Technology

ACQUITY UPLC Protein BEH SEC Column, 200Å, 1.7-µm

KEY WORDS

Size exclusion chromatography (SEC), monoclonal antibody (mAb), bioseparation, method development, quantification

INTRODUCTION

Size exclusion chromatography (SEC) is often used to assess the size distribution of molecular species for therapeutic proteins in a solution (*e.g.* protein clips, aggregates, etc.). The non-denaturing buffers commonly employed in SEC allow for the characterization of proteins in their native state. In addition to measuring molecular size, peak areas from SEC can be readily used in the relative and absolute quantitation of biological samples for increased productivity. As such, this technique has been particularly useful in the biotechnology industry for detecting and quantifying protein aggregation of biotherapeutics.

Protein aggregation in biotherapeutics have been linked to potential loss of therapeutic efficacy as well as unwanted immunogenic responses.[1,2] Controlling factors that contribute to aggregate formation, for example, protein misfolding during expression stages,[3] protein denaturation during purification processes,[1] and high protein concentration during formulation,[4] has been an area of continuing interest in the pharmaceutical industry.

Increasing demand from regulatory bodies to provide detailed information about the quantity and nature of aggregates in biotherapeutics, combined with rising development costs and a demanding work environment, require cost-effective solutions that have minimum impact on productivity. Efficient workflows that seamlessly combine characterization and quantitation information for biotherapeutics are highly desirable.

EXPERIMENTAL

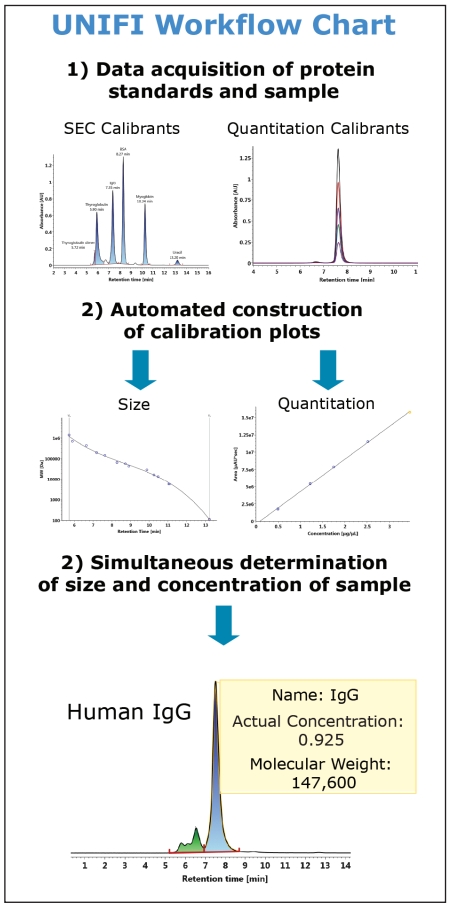
LC conditions

LC system:	ACQUITY UPLC H-Class System with Auto•Blend Plus Technology
Detector:	ACQUITY UPLC TUV
Absorption	Wavelength: 220 nm
Vials:	Total recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	ACQUITY UPLC Protein BEH SEC, 200Å, 1.7-µm, 4.6 x 150 mm (p/n 186005225)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	2 µL
Flow rate:	0.150 mL/min
Mobile phase A:	100 mM sodium phosphate monobasic monohydrate (NaH_2PO_4)
Mobile phase B:	100 mM sodium phosphate dibasic (Na_2HPO_4)
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Autoblend Plus Method:	Isocratic (150 mM NaCl in 20 mM phosphate buffer; pH 7.4)

Informatics for data collection and processing

UNIFI Scientific Information System, v1.6

The Waters Biopharmaceutical Platform solution with UNIFI is developed to streamline the analytical workflow to increase the productivity in the characterization of biotherapeutic samples. The ability to perform SEC using conditions that minimally perturb aggregate composition make it ideal in the assessment and communication of multiple attributes of biotherapeutics such as size, aggregate composition, and concentration. Through the use of calibrated standards, tools within the UNIFI Scientific Information System can simultaneously determine the molecular size (apparent molecular weight) and the concentration of chromatographically resolved species in a biotherapeutic sample in the same analysis.



The objective of this application note is to demonstrate the ability to determine molecular weight and amount of the constituents of an antibody sample using UNIFI informatics. A purified antibody from human serum was used as a model protein to test the application.

Protocol

A Waters ACQUITY UPLC Protein BEH SEC Column was conditioned as outlined by the manufacturer. Waters BEH200 SEC protein standards (p/n 186006518) and BEH125 SEC protein standards (p/n 186006519) were prepared in 1 mL and 0.2 mL of 18 MΩ water, respectively. Apoferritin (p/n A3660), β-amylase (p/n A8781), carbonic anhydrase (p/n C7025), insulin (p/n I0516), sodium phosphate monobasic monohydrate (p/n S3522), sodium phosphate dibasic (p/n S5136), and sodium chloride (S5886) were purchased from Sigma Aldrich. The Waters Glycoworks control standard (p/n 186007033; purified human IgG) was used as an "unknown" and prepared at a concentration of 1 µg/µL as per the labeled amount using 18 MΩ water. Apoferritin, β-amylase, carbonic anhydrase, and insulin were prepared at concentrations of 10 µg/µL, 2.9 µg/µL, 1.5 µg/µL, and 5.0 µg/µL, respectively. The Waters mAb mass check standard (p/n 186006552) used for quantification was prepared at concentrations of 0.49 µg/µL, 1.22 µg/µL, 1.74 µg/µL, 2.51 µg/µL, and 3.46 µg/µL in 18 MΩ water.

RESULTS AND DISCUSSION

Integrated informatics tools for the construction of calibration plots

Size exclusion chromatography is often employed by the pharmaceutical industry for the assessment of aggregate content in biotherapeutic samples. In principle, the elution time of a protein in a SEC separation is determined by how much of the intra-particle pore volume is accessible to the protein.[6] In practice, this separation mechanism prescribes that protein species will elute in order of decreasing hydrodynamic radius. This chromatographic behavior is illustrated by the lower left panel of Figure 1, where Waters SEC protein standards (calibrants) are separated using an ACQUITY UPLC Protein BEH SEC Column (200Å, 1.7-µm, 4.6 x 150 mm).

Using the known molecular weight of the calibrants defined in the component manager as shown in the top panel of Figure 1, the built-in UNIFI informatics tool automatically constructs a size calibration plot (log MW *vs.* RT) for the standards as shown in the lower right panel of Figure 1. Proteins used in this optimized size calibration plot include thyroglobulin dimer, thyroglobulin, apoferritin, β-amylase, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin, ribonuclease A, insulin, and uracil. The calibration plot can be constructed using the logarithmic scale of MW of protein standards as y-axis plotted against either the elution time or the elution volume (x-axis). The data is automatically fitted with a linear or a higher-order polynomial equation to acquire a calibration curve as shown in Figure 1.

Figure 1. Waters BEH200 SEC protein standards (p/n 186006518) are separated (bottom left panel) using an ACQUITY UPLC Protein BEH SEC Column (p/n186005225). A separation calibration plot of log MW versus retention time (lower right panel) is automatically constructed using UNIFI informatics tools from a set of defined protein standards (top panel).

For disparate measurements such as apparent molecular weight (elution time vs. molecular weight) and concentration (area vs. amount), individual assays that generate targeted data sets are usually required. UNIFI informatics allows for the incorporation of multiple calibration plots that can be applied to a single data stream for the measurement of such dissimilar attributes as size and concentration.

Figure 2 illustrates how UNIFI constructs a concentration calibration curve in the same analysis as the apparent molecular weight measurement using a Waters mAb mass check standard as a calibrant for proof of principle. Using the mAb standard, 200 µL of 18MΩ water was added to the 1 mg of lyophilized protein to generate a stock calibrant solution with a concentration of 5.00 µg/µL. From the stock calibrant, five standard samples were prepared at concentrations of 0.493 µg/µL, 1.22 µg/µL, 1.74 µg/µL, 2.51 µg/µL, and 3.46 µg/µL. Three replicates of the five standard samples were performed in a serial fashion with a constant volume (2 µL) injected on an ACQUITY UPLC Protein BEH SEC Column (200Å, 1.7-µm, 4.6 x 150 mm). Using the defined concentrations as indicated by their concentration level in the component summary window of Figure 2, UNIFI automatically constructs the concentration calibration plot as shown in the bottom right panel of Figure 2.

The ability to automate the construction of multiple calibration plots and apply them in a single data stream to discern uniquely disparate critical quality attributes makes the Waters Biopharmaceutical Platform Solution with UNIFI a preferred system for increasing productivity during the method development process.

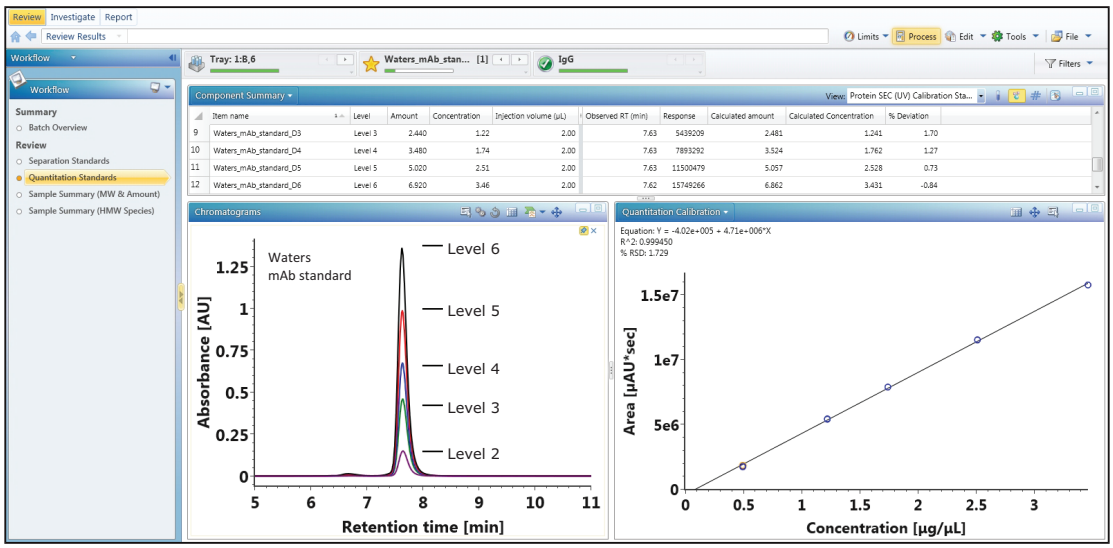


Figure 2. The Waters mAb mass check standard was injected at five different concentration levels (lower left panel). Using the supplied concentrations as indicated by their level in the component summary panel, UNFI informatics automatically constructs a calibration plot of peak area versus sample concentration (bottom right panel).

Simultaneous determination of apparent molecular weight and concentration of purified human IgG from a single injection

The experimental results for the calibration plots show that as an integrated platform, UNIFI is fully capable of determining the apparent molecular weight and concentration of biotherapeutics analyzed in the same sample set as the protein standards. A purified human IgG sample (p/n 186007033) was analyzed using the method to demonstrate the capability of the platform to generate such information for an "unknown" sample. The lyophilized sample was reconstituted in 100 µL of 18 MΩ water to a concentration of 1 µg/µL. Three replicate injections of the sample were analyzed within the same sample set of the calibration standards as shown in the bottom left pane of Figure 3. For each run, 2 µL of the sample was injected.

At the end of the analysis workflow, UNIFI automatically reports the calculated concentration (µg/µL), amount (µg), and molecular weight (Da) of the parent peak or monomer peak of the human IgG sample as shown in the upper component summary pane of Figure 3. Using the data from the component summary pane, the mean concentration and apparent molecular weight of the human IgG parent peak were calculated to be 0.93 µg/µL ± 0.01 µg/µL and 147,600 Da ± 100 Da, respectively. The ability to automatically determine apparent molecular weight and concentration of a sample within a single injection confirms that Waters Biopharmaceutical Platform with UNIFI is an integrated solution for increasing productivity and maximizing characterization content for the analysis of biotherapeutics.

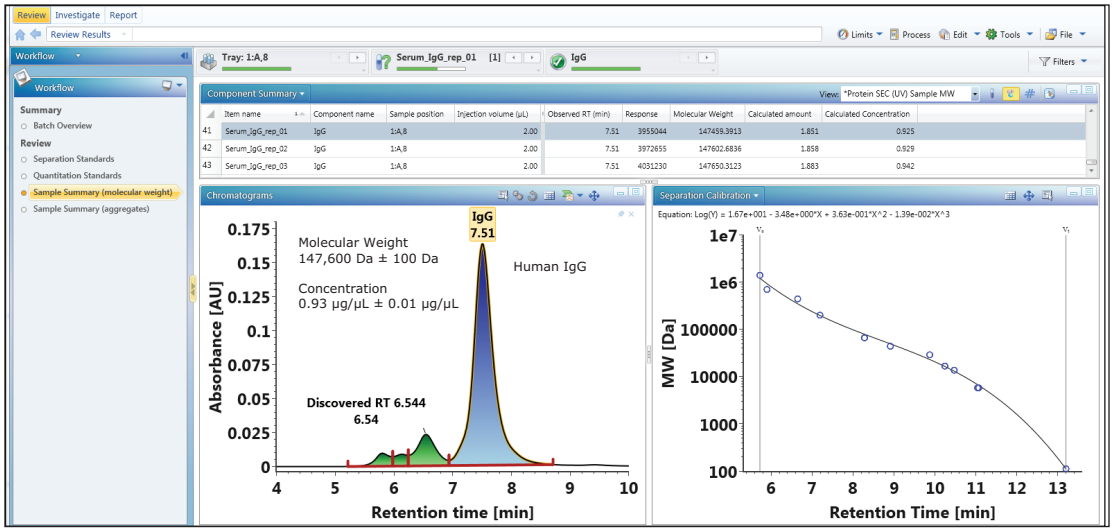
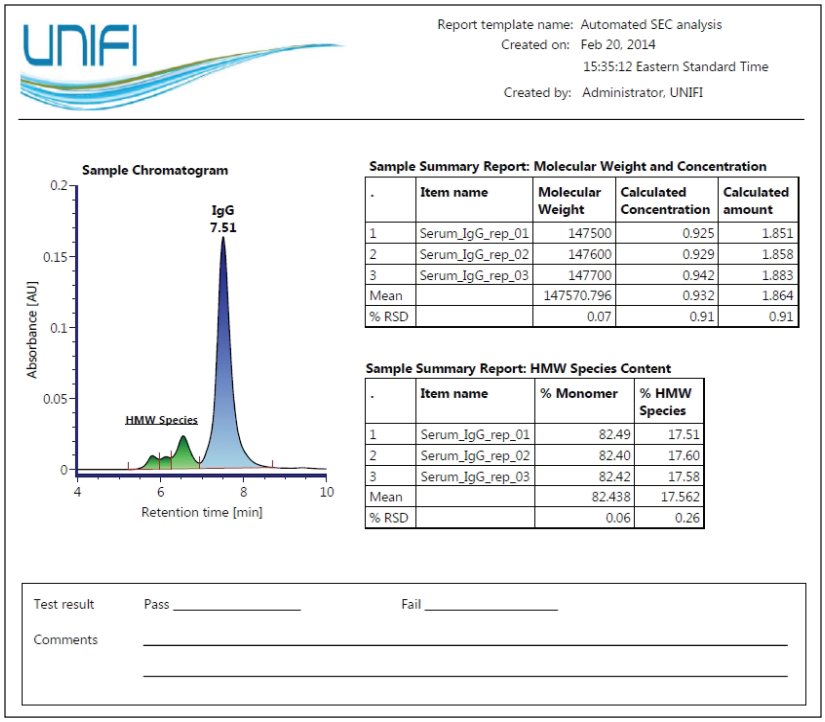


Figure 3. UNIFI reports the concentration, amount, and molecular weight (top pane) of a human IgG parent peak (lower left pane) using the calibration plots constructed from reference standards (lower right pane).

Bioinformatics tools for automated reporting of SEC characterization of biotherapeutics



Report template name: Automated SEC analysis
Created on: Feb 20, 2014
15:35:12 Eastern Standard Time
Created by: Administrator, UNIFI

Sample Chromatogram

Sample Summary Report: Molecular Weight and Concentration

.	Item name	Molecular Weight	Calculated Concentration	Calculated amount
1	Serum_IgG_rep_01	147500	0.925	1.851
2	Serum_IgG_rep_02	147600	0.929	1.858
3	Serum_IgG_rep_03	147700	0.942	1.883
Mean		147570.796	0.932	1.864
% RSD		0.07	0.91	0.91

Sample Summary Report: HMW Species Content

.	Item name	% Monomer	% HMW Species
1	Serum_IgG_rep_01	82.49	17.51
2	Serum_IgG_rep_02	82.40	17.60
3	Serum_IgG_rep_03	82.42	17.58
Mean		82.438	17.562
% RSD		0.06	0.26

Test result Pass _____ Fail _____

Comments _____

Figure 4. A report template example created by UNIFI. The calculated molecular weight concentration, amount, as well as the relative higher molecular weight species content is shown in the report for the IgG sample.

UNIFI integrates strong reporting functionalities with the ability to generate meaningful analytical measurements to form a seamless informatics workflow. These informatics tools allow for custom reports to be automatically generated for the efficient communication and cataloging of analytical results. Report templates can be readily constructed and customized for assessment of analysis results.

Figure 4 is an example of a report template designed for SEC characterization of biotherapeutics such as monoclonal antibodies. Using the results for the purified IgG sample from Figure 3, a summary report on the apparent molecular weight, concentration, and relative amount of the parent peak (monomer) and corresponding statistical evaluation (*e.g.* mean and % R.S.D) is generated after data acquisition and processing. In addition, pertinent biotherapeutic information on the level of relative aggregation in each sample is also assessed and reported as a percentage of monomer and the percentage of higher molecular weight (HMW) species (collectively).

The flexibility to design custom report templates based on analysis needs makes the Biopharmaceutical Platform Solution with UNIFI a powerful integrated system for the acquisition, processing, and reporting of analysis results.

CONCLUSIONS

Assessing and controlling aggregate content in therapeutic proteins is a critical component in the manufacturing process. Increasing sample complexity coupled with the rising research and development costs highlight the need for more efficient analytical methods that are readily deployable in therapeutic protein characterization. Waters' Biopharmaceutical Platform Solution with UNIFI offers answers to these challenging problems.

The efficient built-in workflow aided by the UNIFI's data acquisition, processing, and reporting capabilities enable simultaneous determination and reporting of the apparent molecular weight, concentration, and aggregate composition of biotherapeutic proteins. This process, which is fully automated, makes the Biopharmaceutical Platform Solution with UNIFI ideal for increasing productivity through efficient method deployment for biotherapeutic characterization.

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PEGylated Protein Analysis by Size-Exclusion and Reversed-Phase UPLC

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APPLICATION BENEFITS

- Two UPLC® alternatives, SE-UPLC and RP-UPLC, are presented for the analysis of PEG-Protein conjugate, non-PEGylated protein, and free active PEG levels in PEGylated protein preparations.
- SE-UPLC provides a rapid and high resolution separation of an unmodified protein from its PEGylated form. Successful application of SE-UPLC for this analysis can be predicted based on theoretical calculations of the hydrodynamic viscosity radii of the analytes.
- Separation of PEG-Protein conjugate, non-PEGylated protein, and free aPEG based on their differences in their hydrophobicities is provided by RP-UPLC for this application.

WATERS SOLUTIONS

ACQUITY UPLC® Protein Separation Technology (PrST) Columns

ACQUITY UPLC H-Class Bio System with ACQUITY UPLC TUV and ACQUITY UPLC ELSD

KEY WORDS

Size-exclusion chromatography, SEC, monoclonal, proteins, SE-UPLC, Gel filtration chromatography, polyethylene glycol, PEG, PEGylated protein, reversed-phase chromatography, RP-UPLC

INTRODUCTION

The first PEGylated biotherapeutic, pegademase, which is a bioconjugate of the bovine derived enzyme adenosine deaminase and 5 KDa molecular weight (MW) polyethylene glycol (PEG), was introduced in 1990. Pegademase is used for the treatment of individuals with severe combined immunodeficiency disease (SCID). As of 2012, there were ten approved PEGylated bioconjugates on the market and other candidates in clinical studies.[1] Among other benefits, PEGylation can improve the pharmacokinetics and stability of a biotherapeutic. Interestingly, however, it has been reported that approximately 25% of the normal healthy population has a titer of antibodies against PEG which may be a result of the prevalent use of these compounds in personal care products. The development of anti-PEG antibodies has also been observed in the clinic for PEG conjugates.[2, 3] Since both the efficacy and potentially the safety of PEGylated bioconjugates can depend on the extent of their PEGylation it is a critical quality attribute that should be monitored.

PEGylated proteins can be separated by a number of different methods including ion-exchange (IEC), size-exclusion (SEC), and reversed-phase (RPC) chromatography.[4] For this application, the separation of three species, a 50 KDa molecular weight protein, a 40 KDa activated-PEG (aPEG) and the conjugate, were evaluated using UPLC configurations of both SEC (SE-UPLC) and RPC (RP-UPLC), as these methods can be readily developed to be compatible with an evaporative light scattering detector (ELSD). While the use of SEC for this type of analysis has been reported,[5] the extent of success for the SEC mode of separation for this application type will ultimately be dependent upon the hydrodynamic viscosity radii of the three components as well as their polydispersity. Alternatively, the success of a RPC separation for this application is dependent on the differences in the hydrophobicities of the three components.

EXPERIMENTAL

Sample description

All samples were provided by a collaborator and stated concentrations are nominal values.

Method conditions

(unless noted otherwise):

LC Conditions

LC System:	ACQUITY UPLC H-Class Bio System with 30 cm Column Heater
Detection:	ACQUITY UPLC TUV Detector with 5mm Titanium flow cell Settings: 280 nm, 1 Hz sampling rate ACQUITY® ELSD Detector
Settings:	Gain = 500, Data Rate = 20 pps, Time Cont. = Fast, Gas Pressure = 40.0 psi, Nebulizer Heating at 10% Power Level, Drift Tube Temp. 50 °C
Columns:	Waters ACQUITY UPLC PrST SEC Column, 450Å, 2.5 µm, 4.6 x 150 mm (p/n: 176002996) Waters ACQUITY UPLC PrST SEC Column, 200Å, 1.7 µm, 4.6 x 150 mm (p/n: 186005225) Waters ACQUITY UPLC PrST C_4 Column, 300Å, 1.7 µm, 2.1 x 50 mm (p/n: 186004495)
Column Temp.:	SEC=40 °C; RPC=90 °C
Sample Temp.:	10 °C
Injection Volume:	SEC = 10 µL; C_4 = 5 µL (unless otherwise specified)
Flow Rate:	SEC = 0.4 mL/min, C_4 = 0.5 mL/min
Mobile Phases:	SEC = 200 mM ammonium formate, 5% ACN; C_4 = Water (A)/ACN(B), 0.1% (v/v) TFA
Gradient:	SEC=Isocratic C_4=Gradient

Time	%A	%B
Initial	95	5
1	95	5
16	5	95
17	5	95
20	95	5
25	95	5

Sample Vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/ Silicone Septa, 1 mL (p/n: 186000385DV)

Data Management

Chromatography Software:	Waters Empower® Pro (v2, FR 5)

RESULTS AND DISCUSSION

SE-UPLC

The use of both BEH200 (200Å pore-size) and BEH450 (450Å pore-size) SE-UPLC columns (150 mm lengths) in series was selected for this evaluation due to the extended MW weight range that this combination of columns can provide.[6] Proprietary samples were obtained from a collaborator and consisted of a 50 KDa molecular weight protein, a 40 KDa aPEG and the PEG-Protein conjugate. A volatile mobile phase comprised of 200 mM ammonium formate and 5% (v/v) acetonitrile was selected for these analyses. This buffer composition provided optimal separation of the active-PEG and conjugate critical pair and this volatile buffer could also be used if an evaporative light scattering detector (ELSD), which would provide improved sensitivity for the aPEG component in contrast to UV absorbance, was to be used. The 40 KDa aPEG used in this study has a broad and weak UV absorbance with a maximum at approximately 300 nm; therefore, for this study the UV absorbance at 280 nm provided adequate sensitivity for the high aPEG sample loads that were evaluated. The full-scale chromatograms of the conjugate, the 50 KDa protein, and the 40 KDa activated PEG are shown in Figure 1. Additionally, shown in Figure 2 is an overlay of the chromatograms of the aPEG and conjugate. Based on the chromatograms of these three samples, the SEC method provides useful resolution between the conjugated and the unconjugated protein, however, the separation between the conjugate and the aPEG is clearly not adequate for quantitation of a low level aPEG species in the presence of the predominant conjugate species.

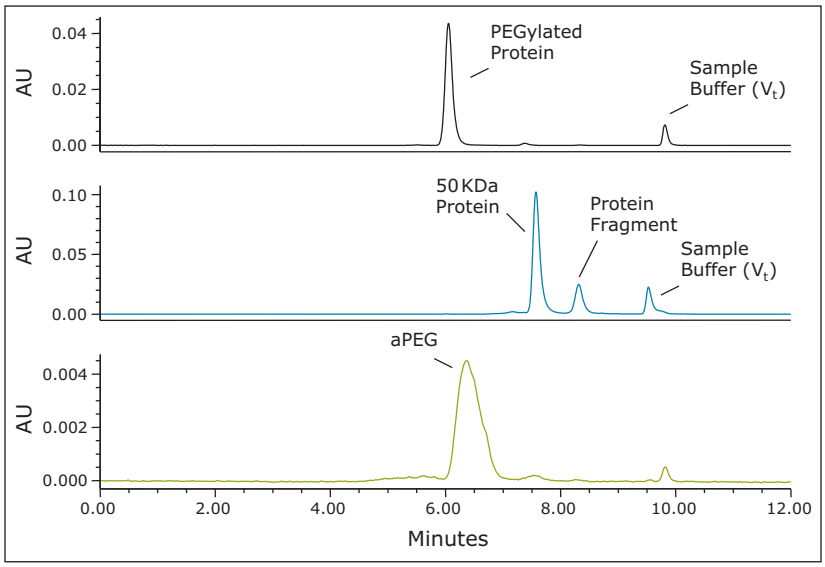

Figure 1. SE-UPLC UV traces (280 nm) for the 50 KDa PEGylated protein (black), the 50 KDa protein (blue), and the activated 40 KDa PEG (green).

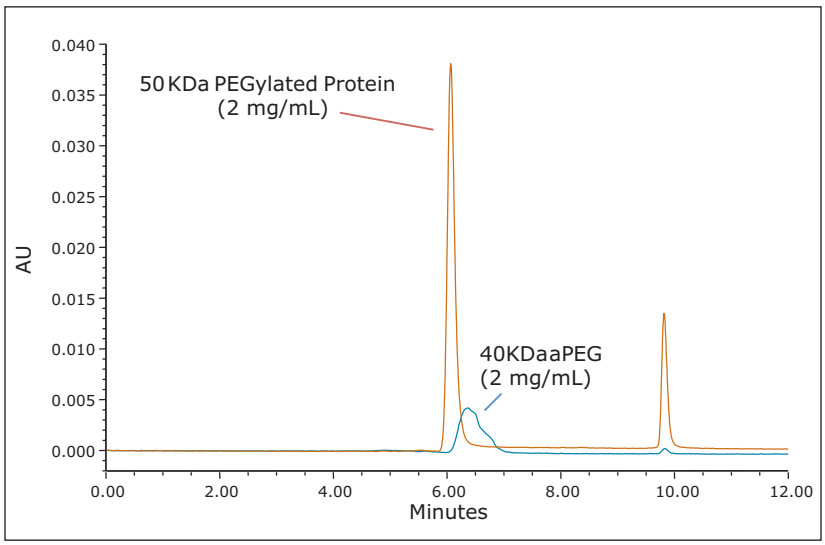

Figure 2. Overlaid SE-UPLC UV traces (280 nm) for the 50 KDa PEGylated protein (brown) and the activated PEG (blue).

These results demonstrate that achieving an SEC-based separation for the quantitation of PEGylated protein and the free aPEG forms may not be achievable in some cases. This can be due to a number of factors including the polydispersity of the aPEG, which will broaden its elution profile as well as that of the PEG-protein conjugate. Additionally, the nature of the interaction between the bound PEG and the surface of the protein may greatly limit the utility of a size-based separation. Ultimately, the critical factors that dictate the success of an SEC separation are the hydrodynamic viscosity radii (R_h) distributions of the aPEG, protein, and the conjugate. These R_h values can be empirically approximated using the relationships proposed in the work of Fee and Van Alstine.[7,8] Based on these relationships the R_h of PEG is typically much greater than that of a protein at a given molecular weight. Typically the ratio of the R_h values for two components should be approximately 1.26 or greater, or the inverse which is 0.79 or smaller, in order to resolve those components by SEC.[8] For globular proteins, this corresponds to a 2-fold increase in MW ($R_h \propto MW^{1/3}$). Using these theoretical relationships, it is it is clear to see that to develop a size-based separation that can resolve the non-PEGylated protein, the aPEG, and the conjugate from a mixture will be challenging. Shown in Table 1 are the predicted R_h ratios for various combinations of MW for these three components. Based on these predicted values covering a broad range of protein and PEG MW, there are only three combinations of components that would be predicted to have all three components resolve by SEC. The prediction for the 50 KDa protein and 40 KDa aPEG used in this study confirms what was observed experimentally where adequate resolution was achieved between the protein and the both the aPEG and the conjugate. However, the R_h ratio between the conjugate and the aPEG is well below 1.26 (1.07), which is in agreement with the insufficient resolution observed between those two species.

Protein MW (Da)	PEG MW (Da)	$R_{h,PEG}/R_{h,pro}$	$R_{h,pro+PEG}/R_{h,pro}$	$R_{h,pro+PEG}/R_{h,PEG}$
25000	5000	0.93	1.42	1.53
50000	5000	0.74	1.32	1.78
100000	5000	0.59	1.24	2.11
150000	5000	0.51	1.20	2.35
25000	10000	1.37	1.71	1.25
50000	10000	1.09	1.52	1.40
100000	10000	0.86	1.38	1.60
150000	10000	0.75	1.32	1.75
25000	20000	2.02	2.22	1.10
50000	20000	1.61	1.89	1.18
100000	20000	1.27	1.64	1.29
150000	20000	1.11	1.54	1.38
25000	40000	2.98	3.08	1.03
50000	40000	2.37	2.52	1.07
100000	40000	1.88	2.10	1.12
150000	40000	1.64	1.91	1.17

Table 1. Predicted ratios of the hydrodynamic viscosity radii for several PEG ($R_{h,PEG}$), proteins ($R_{h,pro}$), and their conjugate forms ($R_{h,pro+PEG}$). Ratio values of 1.26 or greater and 0.79 or less (green) indicate that adequate analytical separation between those species by SEC is predicted. R_h values between 0.79 and 1.26 (blue) predict that analytical resolution of the two compounds is not expected. The MW values of the protein and aPEG are highlighted in green for combinations for which resolution of all three components is predicted.

It should be noted that the predicted R_h ratios contained in Table 1 are approximations and that the possibility of successfully separating different species lessens as their R_h ratio approaches a value of 1.0. However, successful SEC separations could be obtained for species with borderline R_h ratios and such analyses may warrant experimental investigation. It is also worth noting that in cases where resolution of only two of the three components is required, such as in applications designed to quantitate the levels of the non-PEGylated protein and PEGylated protein a useful SEC separation is predicted (Column $R_{h,pro+PEG}$ / $R_{h,pro}$ in Table 1) for all but the largest proteins with the lowest MW 5 KDa PEGylation.

RP-UPLC

As an alternative to SEC, and with the understanding that PEGylation may likely have a profound effect on protein hydrophobicity, RP-UPLC using a C_4-bonded stationary phase was evaluated for the separation of the non-PEGylated protein, aPEG, and conjugate mixture. For this analysis, an ELSD was used in series after the UV detector to aid in the characterization of the observed peaks and to provide greater sensitivity for the unreacted PEG. A column temperature of 90 °C was selected for this separation to maximize sample recovery and peak shape quality. An overlay of the chromatograms obtained for the three components is presented in Figure 3. Under these conditions, the selectivity and peak widths obtained resulted in excellent resolution between the three analytes. Shown in Figure 4 is an overlay of the ELSD and TUV (A280) traces for the conjugated sample. In the ELSD trace (black), a low level (nominally 5%) aPEG peak is observed as well a low level unmodified protein peak (nominally 3.4%). The values determined by ELSD are relative as the response is not linear and is dependent on the nature of the analyte and the mobile phase composition. As a result, the level of unmodified protein based on the measured A280 peak areas is significantly higher (13.1%). However, the low level of free aPEG in the sample was below the limit of detection by UV absorbance. Consequently, the use of both detectors in series is essential in order to effectively monitor the levels of all three components in a single analysis.

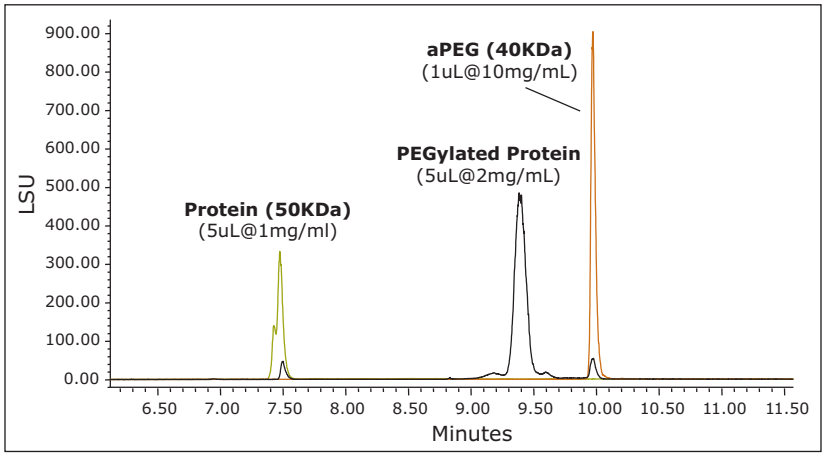

Figure 3. Overlay of the C_4 RP-UPLC ELSD traces for the 50 KDa PEGylated protein (black), the 50 KDa protein (green), and the activated 40KDa PEG (brown).

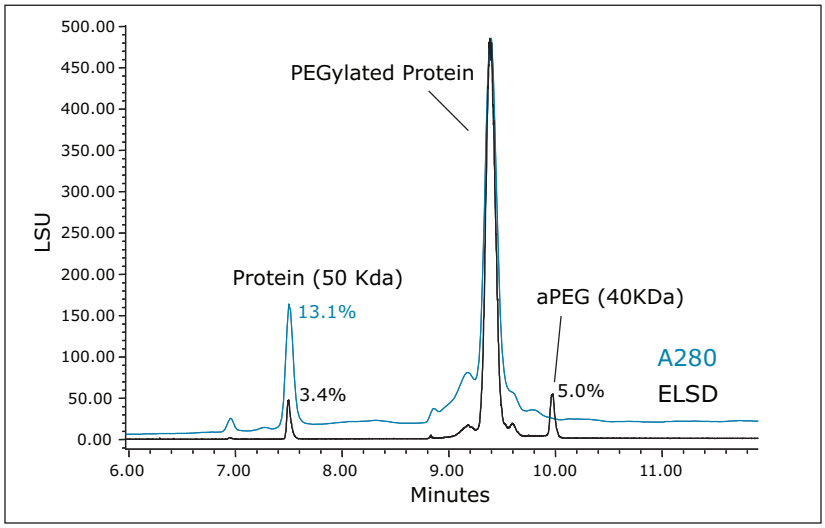

Figure 4. Normalized overlay of the ELSD (black) and 280 nm UV absorbance (blue) chromatograms for the 50 KDa PEGylated protein separated using a RP-UPLC BEH300 C_4 column.

CONCLUSIONS

SE-UPLC can provide rapid analysis of the products and unreacted components of a protein PEGylation reaction if the R_h values for the non-PEGylated protein, aPEG, and conjugate are sufficiently different. Based on predictions of the R_h values for combinations of protein and PEG molecular weights, in many circumstances SE-UPLC cannot provide the necessary analytical separation of all three components. This was indeed the case for this application where the model correctly predicted that the 40 KDa PEG and PEGylated 50 KDa protein R_h values were not significantly different to enable their separation by SE-UPLC. However, in many instances, SE-UPLC can be used to separate the modified and unmodified protein components of the sample, particularly for samples where large MW PEG (20 and 40 KDa) are being used.

By comparison, for this specific application it was found that all the three components were well separated based on differences in their hydrophobicities using a 300Å BEH column at high temperature (90 °C). Additionally, the use both a UV and an ELSD detector in series may be used to for their quantitation.

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Ultimate Resolution HPLC-Based Size Exclusion Chromatography for the Analysis of Small Proteins and Peptides Using 3.5 µm Ethylene Bridged Hybrid (BEH) Particles

Susan Serpa, Hua Yang, Edouard S. P. Bouvier, and Stephan Koza
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Outstanding column stability and reliable column-to-column reproducibility with SEC columns containing BEH-based particles for increased confidence in validated methods.
- Improved resolution of small proteins and peptides by SE-HPLC compared to larger particle size (5 µm) standard HPLC particle-size columns.
- 125Å pore size provides an effective size separation range for peptides and small proteins.

WATERS SOLUTIONS

XBridge® Protein BEH SEC, 125Å, 3.5 µm Column

Alliance® HPLC System

ACQUITY UPLC® H-Class Bio UPLC and ACQUITY UPLC Systems

BEH125 SEC Protein Standard Mix

KEY WORDS

Size-exclusion chromatography, SEC, HPLC, UPLC, proteins, SE-HPLC, SE-UPLC, gel filtration chromatography

INTRODUCTION

Waters currently provides the only sub-2-µm particle size size-exclusion chromatography (SEC) column with a pore-size (125Å) suitable for the analysis of small proteins and peptides.[1] This UPLC® Technology size-exclusion (SE-UPLC) column consists of 1.7 µm diameter ethylene bridged hybrid (BEH) particles, which are mechanically strong and more chemically stable than pure silica-based particles. However, the small particle-size and narrow 4.6 mm internal diameter of SE-UPLC columns are not optimal for use with an HPLC system due to the significantly higher extra-column dispersion and lower pressure limits of HPLC systems relative to UPLC systems. As a result, Waters has recently developed a 125Å pore-size, 3.5 µm particle-size BEH SEC column specifically for use on classic HPLC instrumentation. This provides laboratories with HPLC instrumentation a means to take advantage of the benefits provided by BEH particle technology. This application note will cover the performance characteristics of this column, designed for the separation of small proteins and peptides, with respect to UPLC method transfer, column-to-column reproducibility, and column stability. Additionally, the performance advantages that this 3.5 µm packing material offers over larger (5 µm), standard SE-HPLC particles for both non-denaturing and denaturing separations will be highlighted.

EXPERIMENTAL

Method conditions

(unless noted otherwise)

LC conditions

LC systems:	Alliance HPLC, ACQUITY UPLC H-Class Bio System, or ACQUITY UPLC
Detection:	Alliance HPLC TUV Detector, ACQUITY UPLC TUV Detector with 5 mm titanium flow cell
Wavelength:	280 or 214 nm
Columns:	XBridge Protein BEH SEC, 125Å, 3.5 µm, 7.8 x 300 mm (p/n: 176003596) and ACQUITY UPLC Protein BEH SEC, 125Å, 1.7 µm, 4.6 mm x 300 mm (p/n: 186006506)
Comparator columns:	Silica-diol SEC, 125Å, 5 µm, 7.8 x 300 mm
Column temp.:	Ambient
Sample temp.:	10 °C
Injection vol.:	10 µL (unless otherwise noted)
Flow rate:	0.84 mL/min (unless noted otherwise)
Mobile phases:	25 mM sodium phosphate, 150 mm sodium chloride, pH 7.2 (prepared using Auto•Blend Plus™ Technology)
Mobile phase A:	100 mM NaH_2PO_4, 14.5%
Mobile phase B:	100 mM Na_2HPO_4, 15.0%
Mobile phase C:	1.0 M NaCl, 65%
Mobile phase D:	Water or 30% (v/v) acetonitrile, 0.1% (v/v) TFA
Gradient:	Isocratic
Standard:	BEH125 SEC Protein Standard Mix (p/n: 186006842)
Sample Vials:	Deactivated Clear Glass 12 x 32mm Screw Neck Total Recovery Vial, with Cap and preslit PTFE/Silicone Septa, 1 mL (p/n: 186000385DV)
Chromatography software:	Empower® Pro (v2 and v3)

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins and peptides were purchased as individual standards or as mixtures (Waters® and Sigma-Aldrich®). Sample concentrations were 1.0 mg/mL (nominal) unless noted otherwise.

RESULTS AND DISCUSSION

The significant sample throughput and performance benefits provided by BEH particle technology when used in the manufacturing of size-exclusion UPLC (SE-UPLC) columns for the analysis of peptides and proteins have been previously described.[2,3] However, these advantages cannot be fully realized when using HPLC instrumentation due to the peak dispersion introduced by these systems. In order to take advantage of the chemical and structural capabilities of BEH particle technology for the SEC separation of small proteins and peptides on HPLC instrumentation, 7.8 mm I.D. columns packed with 3.5 µm BEH particles with a pore size of 125Å has been recently introduced. This column provides an optimal molecular weight range of SE-HPLC separations to include protein and peptide with radii of hydration (R_h), that translates to a molecular weight range from <1 KDa to approximately 80 KDa. As part of this evaluation, the separation efficiency advantages of this packing material with respect to larger particle-size (5 µm) HPLC-based packing materials, and the critical performance characteristics of column-to-column reproducibility and column stability (i.e. lifetime) will be demonstrated.

Enhanced SE-HPLC resolution using 3.5 µm BEH particles

To demonstrate their performance, a small protein (myoglobin, MW 17 KDa) and a series of peptides were separated on a silica-based SEC, 125Å, 5 µm, 7.8 x 300 mm column and on a BEH SEC, 125Å, 3.5 µm, 7.8 x 300 mm column using the same Alliance HPLC System and either aqueous or organic mobile phase conditions (Figures 1 and 2). Equivalent flow rates and injection volumes were used for both comparisons. Improved sensitivity and narrower peak widths were observed on the 3.5 µm packing material in comparison to the 5 µm particle size materials. The BEH SEC column has improved mechanical properties that allow operation at higher flow rates and pressures than can be tolerated by traditional SE-HPLC columns.

An example of how these capabilities can be used to advantage is shown in Figure 2 (bottom frame). For this analysis two BEH SEC, 125Å, 300 mm columns were operated in series (3.5 µm, 7.8 x 600 mm total length) and by increasing the flow rate two-fold a significant increase in resolution can be observed while maintaining equivalent analysis times. Another advantage that can be observed by employing BEH particles for the SEC separation of peptides is a reduced level of secondary interactions for certain peptides (Figure 2) such as ubiquitin and aprotinin in comparison to the secondary interactions observed for the silica-based SEC column. These secondary interactions are likely the result of silanol activity given the charge characteristics of these two peptides. Aprotinin is a very basic protein (pI = 10.5) and ubiquitin presents a highly basic surface charge with multiple lysines that are involved in the protein-ubiquitin interactions referred to as ubiquination.

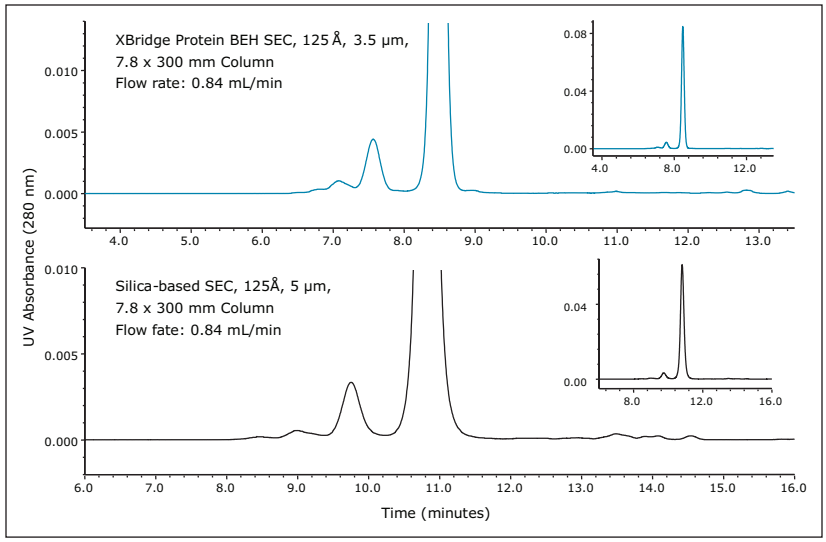

Figure 1. Shown is a comparison of the separation of myoglobin (17 KDa) on an XBridge Protein BEH SEC, 3.5 µm Column (top frame) and a traditional silica-based SEC, 5 µm column (bottom frame). Columns were operated under equivalent aqueous conditions as outlined in the text.

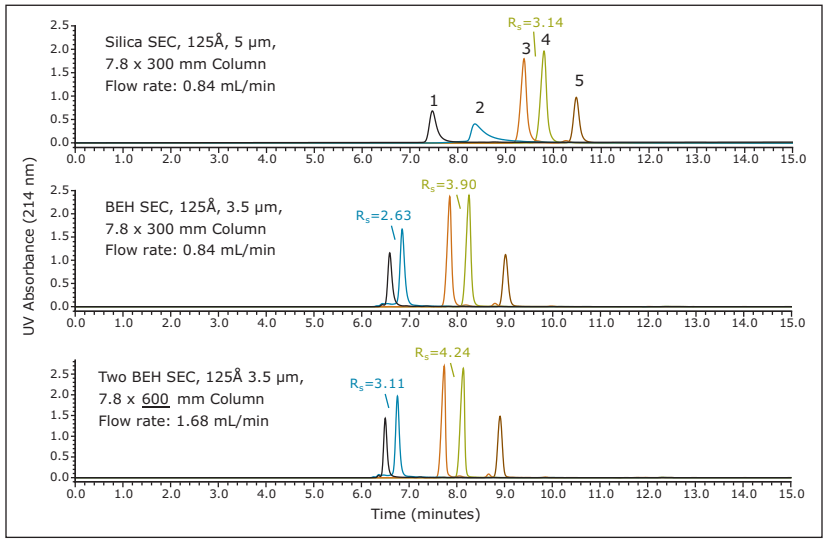

Figure 2. Shown is a comparison of the separation of several peptides on an XBridge Protein BEH, 3.5 µm SEC Column (middle frame) and a traditional silica-based, 5 µm SEC column (top frame), and two XBridge Protein BEH SEC, 3.5 µm Columns run in series while doubling flow-rate resulting in equivalent analysis times (bottom frame). Mobile phase used was 30% ACN, 0.1% TFA. Peak identities: 1. Ubiquitin (8565 Da), 2. Aprotinin (6511 Da), 3. Angiotensin I (1296 Da), 4. Bradykinin (1060 Da), 5. DLWQK (689 Da).

Exceptional column stability and reliable column-to-column reproducibility

Major concerns for method development are batch-to-batch and column-to-column reproducibility, as well as packed column stability. Shown in Figure 3 is an overlay of the chromatograms for a series of molecular weight standards separated on three 125Å, 3.5 µm, 7.8 x 300 mm columns. These chromatograms demonstrate the reproducibility of these SEC columns packed from 3 different production lots of packing material. At a flow rate of 0.42 mL/minute, the retention time standard deviations for the 125Å pore size columns ranged from a minimum of 0.06 minutes to 0.27 minutes with an average standard deviation of 0.17 minutes for all labeled components. The average retention time reproducibility relative to the retention time of uracil (total permeation volume) was 0.99% RSD.

The stability of the BEH SEC, 125Å, 3.5 µm, 7.8 x 300 mm column can be demonstrated by evaluating the results for a protein standard over the course of over 600 total injections. Given that the stability of silica-based SEC columns can be deleteriously altered by mildly basic pH levels, the pH of the mobile phase was set to 7.2, equivalent to that of phosphate buffered saline (PBS) buffer. Shown in Figure 4 is a comparison of the profiles obtained for the myoglobin standard from the start to the finish of the study for both the BEH SEC, 125Å, 3.5 µm column and a traditional pure silica-based, 125Å, 3.5 µm column. The resolution between the critical myoglobin monomer and dimer peaks were determined for each column. The BEH 125Å, 3.5 µm column demonstrated remarkable stability with no significant depreciation of resolution. These data demonstrate that XBridge Protein BEH SEC, 3.5 µm Columns can provide the reproducibility and stability needed to develop reliable assays and run them routinely in a quality control environment.

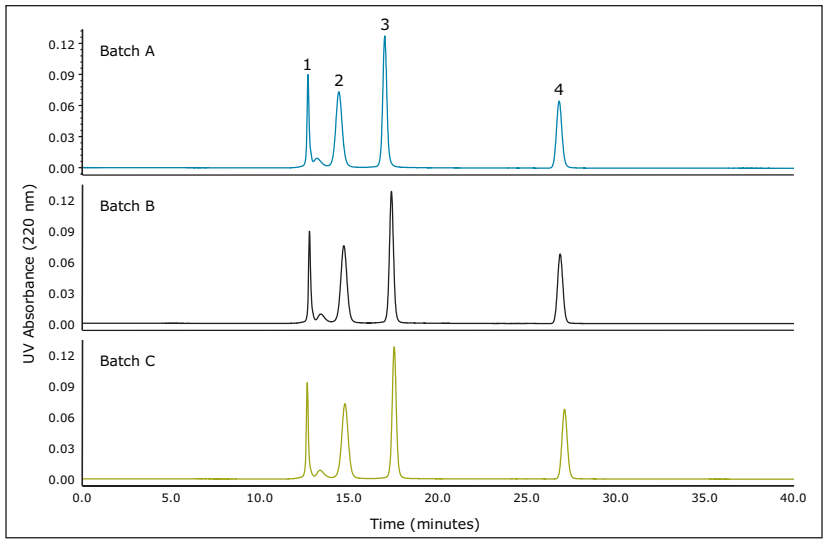

Figure 3. Shown is a comparison of the separation of BEH125 SEC Protein Standard Mix (p/n 186006842) on three XBridge Protein BEH SEC, 3.5 µm Columns representing three separate batches of manufactured particles. Mobile phase used was 100 mM sodium phosphate, pH 6.8 and flow rate was 0.42 mL/minute. Peak identities are: 1. Thyroglobulin, 2. Ovalbumin, 3. Ribonuclease, 4. Uracil.

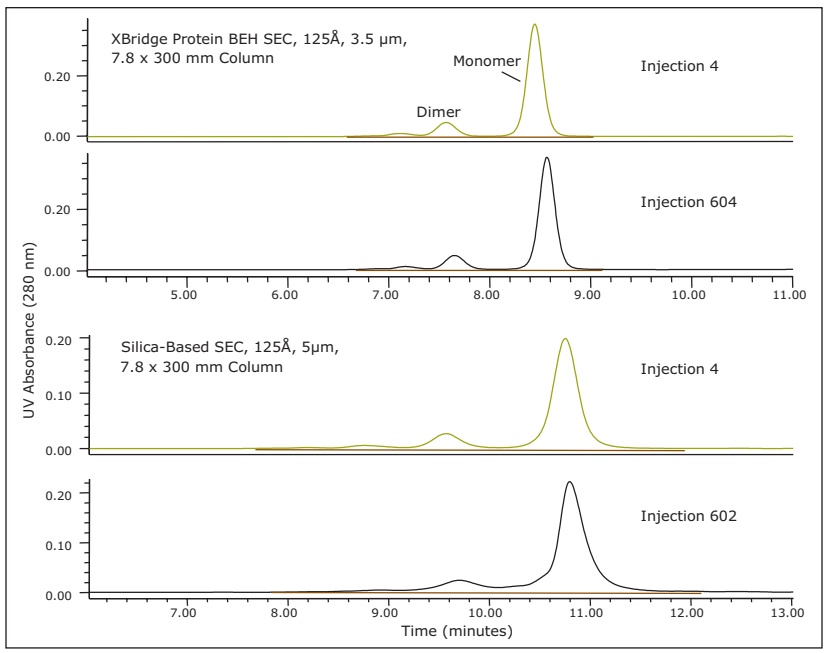

Figure 4. Shown is a lifetime (>600 injections) comparison of the separation of myoglobin (17 KDa) on an XBridge Protein BEH SEC, 3.5 µm Column (top frame) and a traditional silica-based SEC, 5 µm column (bottom frame). Columns were operated under equivalent aqueous conditions as outlined in the text.

125Å pore-size SEC HPLC and UPLC method transferability

Successful method transfers between UPLC® and HPLC platforms for both 200Å and 450Å pore-size SEC columns have been described previously.[3] The transferability of an SEC method between UPLC and HPLC platforms will be primarily impacted by the comparability of the pore-size and surface chemistry of the particles. For example, in comparing the peptide separations on the BEH and silica-based SEC columns (Figure 2) it is clear that under equivalent conditions that the secondary interactions observed for the peptides ubiquitin and aprotinin are not equivalent between the two particle surfaces. An exercise was undertaken to demonstrate the method transfer between an XBridge Protein BEH SEC, 125Å, 3.5 µm Column and an ACQUITY UPLC Protein BEH SEC, 125Å, 1.7 µm Column. Based on general chromatographic scaling principles it was determined that the column length for the HPLC column would need to be twice that of the UPLC column in order to maintain a comparable column length to particle diameter ratio (L/dp) and the reduced linear velocity would need to be half that of the UPLC Column. The result of this method transfer is shown in Figure 5. The observed profiles are comparable between the two separations when the time-axis is normalized, however, it should be noted that the analysis time for the 3.5 µm HPLC separation using a 7.8 mm I.D. column, takes 4 times longer than the separation on the 1.7 µm UPLC separation using a 4.6 mm I.D. column and uses nearly 6 times more mobile phase. By contrast, to appropriately scale a 1.7 µm, 4.6 mm SEC separation to a 5 µm, 7.8 mm HPLC column would require the use of three times the column length and the run-time would be approximately nine times longer.

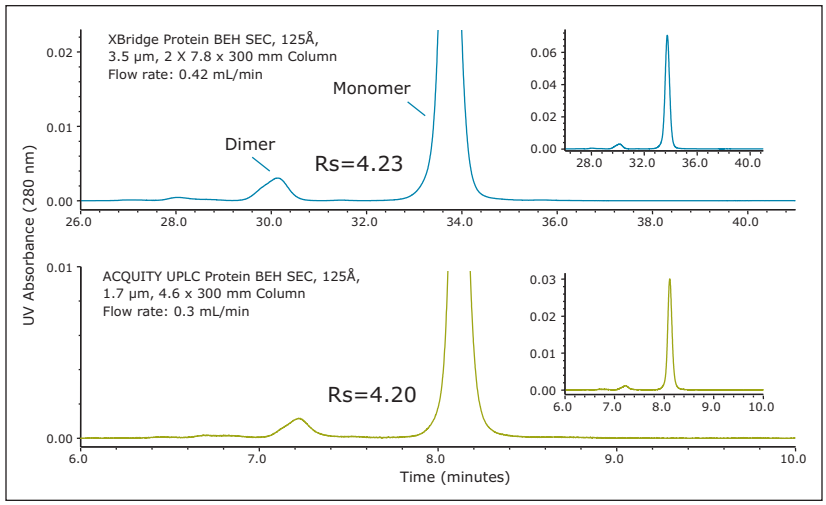

Figure 5. Shown is a comparison of the separation of myoglobin (17 KDa) on two XBridge Protein BEH SEC, 3.5 µm Columns (600 mm total bed length) run in series (top frame) and using an ACQUITY UPLC BEH SEC, 1.7 µm, 300 mm Column (bottom frame). Resolution is reported for the separation between dimer and monomer.

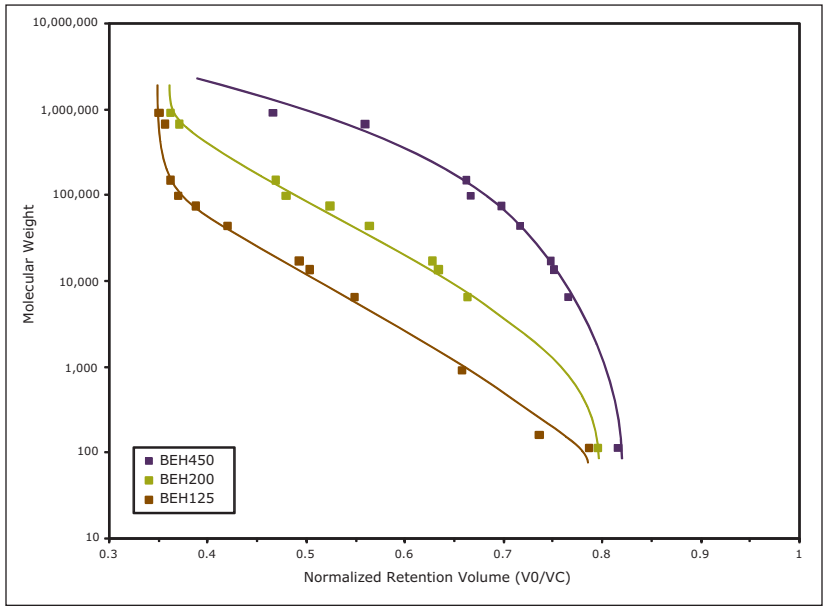

Figure 6. Shown are calibration curves of various proteins, peptides, and uracil generated the 125Å, 200Å, and 450Å Protein BEH SEC 3.5 µm particle-size columns.

Three pore sizes, 200Å and 450Å, for extended protein molecular weight range

Comparisons were made between the 125Å, 200Å, and 450Å pore-size particles for the separation of proteins and peptides. The protein molecular weight calibration curves are shown in Figure 6. The linear molecular weight range for the 125Å pore-size column is estimated to be from approximately <1 KDa to 80 KDa, the 200Å pore-size ranges from 10 KDa to 450 KDa, and the 450Å pore-size column is estimated to be from approximately 50 KDa to over 1.3 MDa.

CONCLUSIONS

The introduction of BEH SEC, 3.5 µm, HPLC-compatible columns with a pore-size of 125Å provides improved resolution SE-HPLC separations in comparison to traditional 5 µm silica-based particles. This characteristic, in combination with the chemical stability of BEH Technology,™ provides outstanding column lifetimes. As part of the Waters® Protein Separation Family of columns, these columns are manufactured to rigorous tolerances and quality tested with relevant analytes. These HPLC separations are also directly scalable to SE-UPLC separations using 1.7 µm diameter BEH technology particles and narrower column internal diameters (4.6 mm I.D.), which have even greater resolution and sample-throughput when coupled with UPLC-capable chromatographic systems.

The Waters XBridge Protein BEH SEC, 125Å, 3.5 µm Column provides:

- Increased resolution compared to the use of columns packed with traditional 5 µm, SE-HPLC particles.
- Reduced secondary interactions compared to the use of traditional silica-based particle SE-HPLC columns.
- Particle batches are QC tested with protein mixture that is supplied with the column.
- Outstanding batch-to-batch and column-to-column reproducibility.
- BEH particles deliver improved column stability in comparison to silica-based particle under basic pH conditions.
- Seamless SEC method transferability between HPLC platforms to UPLC using Waters ACQUITY UPLC Protein BEH SEC, 125Å, 1.7 µm Column.

References

1. Paula Hong, Stephan Koza, and Kenneth J. Fountain, Advances in Size-Exclusion Chromatography for the Analysis of Small Proteins and Peptides: Evaluation of Calibration Curves for Molecular Weight Estimation. Waters Corporation, Application Note 720004412EN, 2012.
2. Stephan Koza, Susan Serpa, Hua Yang, Edouard Bouvier, and Kenneth J. Fountain, Advanced HPLC Size-Exclusion Chromatography for the Analysis of Macromolecular Proteins Using 3.5 µm Ethylene Bridged Hybrid (BEH) Particles. Waters Corporation, Application Note 720005202EN, 2015.
3. Stephan Koza and Kenneth J. Fountain. Successful Transfer of Size Exclusion Separations between HPLC and UPLC. Waters Corporation, Application Note 720005214EN, 2014.

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High Resolution Size-Exclusion Chromatography Separations of mAb Aggregates, Monomers, and Fragments Using BioResolve SEC mAb Columns on UPLC, UHPLC, and HPLC Chromatography Systems

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- BioResolve SEC mAb Columns are individually performance tested using the Waters™ mAb Size Variant Standard to help ensure out-of-the-box performance for mAb analyses
- High resolution separations can be achieved on UPLC, UHPLC, and HPLC systems using appropriately selected I.D. and length columns
- The implementation of individual SEC column performance testing with the Waters mAb Size Variant Standard helps increase the confidence in obtaining the needed resolution for reliable mAb size variant quantification

WATERS SOLUTIONS

ACQUITY™ Arc™ Bio System

ACQUITY UPLC H-Class PLUS Bio System

Alliance™ HPLC System

BioResolve™ SEC mAb Column

ACQUITY UPLC Tunable UV Detector

Empower™ 3 Chromatography Data Software

KEYWORDS

Size exclusion chromatography (SEC), monoclonal antibodies, mAb aggregates, monomers, fragments, mAb size variant standard

INTRODUCTION

The historical importance of aqueous size-exclusion chromatography (SEC) is well established for the relative quantification of protein aggregates and self-associated forms (high molecular weight species, HMWS) to ensure the efficacy and safety of recombinant protein-based biotherapeutic products.[1] Recently, interest in quantifying monoclonal antibody (mAb) fragments (low molecular weight species, LMWS) in non-denaturing SEC has increased. The cause of the fragmentation appears not to be enzymatically or host cell protein driven, but rather a kinetic metal ion induced hydrolytic cleavage in the upper heavy chain hinge region resulting in the generation of Fab fragment (~50 kDa), Fc with a single Fab domain (Fab/c, ~100 kDa), and low levels of Fc fragment when both Fab domains are hydrolyzed.[2]

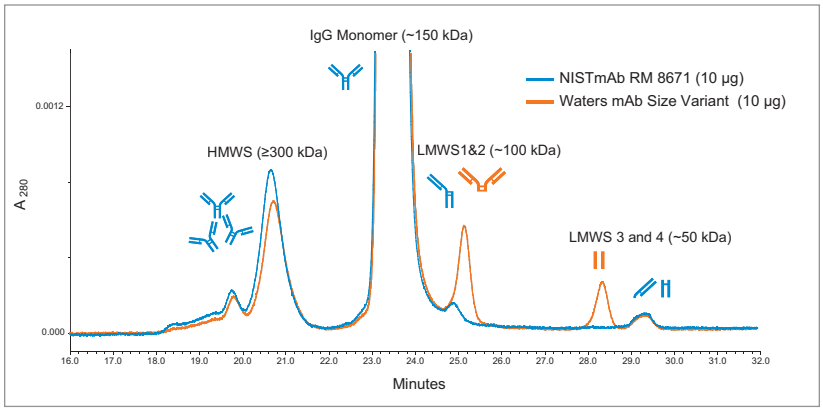

Figure 1. Separation of mAb aggregates, monomers, and fragments of NISTmAb RM 8671 and Waters mAb Size Variant Standard using a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column. LMWS: $F(ab')_2$ and $(Fc/2)_2$ IdeS fragments (orange); Fab/c, Fab, Fc hydrolytic degradation fragments (blue). Conditions: Ambient temperature and 0.3 mL/min.

The separation of HMWS (>300 kDa) from the main mAb monomer (~150 kDa) generally presents much less of a challenge compared to the separation of the Fab/c fragment, which is closer in size to the monomer and elutes at the tail of the monomer, and is typically present at low abundance (Figure 1). However, LC system improvements, higher efficiency SEC columns, and a better understanding of how LC system dispersion affects component resolution have increased the ability to obtain reliable separations of these different mAb forms.[3]

Waters BioResolve SEC mAb Columns are performance tested using a mAb Size Variant Standard on a low dispersion LC system to show their capabilities. The observed chromatographic efficiency of a separation is affected by each of the components in the chromatographic system: the injector, tubing, column, and detector. In this application note, a series of chromatograms are shown for a variety of LC systems demonstrating the relationship between column dimensions (internal diameter [I.D.] and length) and system dispersion on the observed chromatographic efficiency of a separation. The 7.8 mm I.D. × 300 mm length columns provided high resolution separations and accurate quantification of aggregates, monomers, and fragments, independent of modern LC system dispersions.

EXPERIMENTAL

System dispersion was measured using 0.16 mg/mL caffeine diluted in 50/50 acetonitrile/water (UPLC Absorbance Test Solutions Kit, p/n: 700002642, Solution 7). The mobile phase was 50/50 acetonitrile/water (v/v) and the flow rate was 0.5 mL/min. A zero dead volume union (p/n: 700002636) was used in place of the column. After a 10-min equilibration time, three blank mobile phase injections were followed by five consecutive 0.5 µL injections of caffeine using a run time of 1–2 min. The caffeine peak widths at 4.4% (5-sigma peak width) were averaged. The average was multiplied by 500 µL/min to obtain the reported 5-sigma extra-column dispersion ($5\sigma_{ec}$) volumes. The dispersion data on the three systems (four configurations) are reported in Table 1.

LC Systems	System Volume (µL)	5-Sigma W at 4.4% (µL)	USP Tailing
1) ACQUITY UPLC H-Class Bio with CH-A	16.8	10	1.25
2) ACQUITY UPLC H-Class Bio with CH-30A	22.6	13	1.33
3) ACQUITY Arc Bio with 30-cm CH	43.4	30	1.37
4) Alliance HPLC with CH	61.6	49	1.66

Table 1. System dispersion characteristics for LC systems.

Sample description

Waters mAb Size Variant Standard (p/n: 186009284) contains 160 µg of stabilized and lyophilized NISTmAb RM8671 which has been supplemented with 2 µg of nonreduced IdeS digested NISTmAb fragments $(F(ab')_2$ and $(Fc/2)_2)$. The lyophylized contents of each vial was solublizes using 70 µL of MilliQ water.

Method conditions

LC conditions

Systems:	ACQUITY UPLC H-Class Bio, 5-Sigma system dispersion = 10 µL, 3 µL	Vials:	Max Recovery Sample vials (p/n: 186000327C)
	ACQUITY Arc Bio, 5-Sigma system dispersion = 30 µL	Column(s):	BioResolve SEC mAb, 200 Å, 2.5 µm, 4.6 × 150 mm (p/n: 176004592*) 4.6 × 300 mm (p/n: 176004593*) 7.8 × 150 mm (p/n: 176004594*) 7.8 × 300 mm (p/n: 176004595*) *Includes column and one complimentary vial of mAb Size Variant Standard
	Alliance HPLC, 5-Sigma system dispersion = 49 µL		
Detectors:	Tunable Ultraviolet (TUV) with a 5 mm Ti Flow Cell for ACQUITY UPLC H-Class Bio, 2489 UV/Vis with 10 mm Bio Inert Flow Cell for the ACQUITY Arc Bio and Alliance	Column temp.:	35 °C active preheaters CH-A (ACQUITY UPLC H-Class), CH-30A (ACQUITY UPLC H-Class), and convection heaters 30-cm CH (ACQUITY Arc), CH (Alliance)
Detection:	280 nm, 10 Hz, fast filter		

Sample temp.:	8 °C
Sample:	2.28 mg/mL Waters mAb Size Variant Standard
Injection volume:	Varies: 1.8, 3.5, 5, or 10 µL depending on column configuration (I.D. and length)
Flow rate:	0.200 mL/min (for 4.6 mm I.D.)/ 0.575 mL/min (for 7.8 mm I.D.)
Seal wash:	10% HPLC-grade methanol/ 90% 18.2 MΩ water v/v (Seal wash interval set to 0.5 min)
Sample manager washes:	18.2 MΩ water
Mobile phase A:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Mobile phase B and C:	18.2 MΩ water
Mobile phase D:	10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM potassium chloride
Syringe draw rate:	30 µL/min
Needle placement:	1.0 mm
Air gaps:	None
Data channels:	ACQUITY TUV ChA 280 nm; System pressure, room temperature
Mobile phase A:	Prepare by mixing 2.66 g of anhydrous dibasic sodium phosphate, 4.36 g of monobasic potassium phosphate mono hydrate, and 14.91 g of potassium chloride per L of water followed by filtration using sterile 0.2 µm nylon filter units (filtered mobile phase pH 6.9)
Chromatography software:	Empower 3 FR 3.0

RESULTS AND DISCUSSION

SEC is a unique and challenging form of chromatography. Under ideal conditions (method development required), proteins migrate through the column with minimal-to-no interaction with the stationary phase. No retention, only diffusion driven separations achieved by the size hinderance provided by the specifically designed pore sizes in the packing's porous structure. As a result, the amount of band spreading that occurs to the analyte zone as it travels from the injector to the detector will have a significant impact on SEC resolutions. An in-depth evaluation of the effect of system dispersion on the SEC analysis of mAb aggregates (HMWS) and fragments (LMWS), and the effect that extra-column dispersion has on that separation can be found in previously published application notes.[4,5]

MEASURING SYSTEM DISPERSION

When conducting band spreading experiments to evaluate system dispersion, it is important to measure the band broadening at a peak width of 4.4% of its height when SEC is our intended analysis. Many of the impurities that we intend to separate and quantify have peak heights well below 4.4% of the peak height of the main peak (Figure 2). The effect of LC system dispersion on the 5-sigma efficiency and USP tailing factor for the main mAb peak plays a key role in achieving resolution for the ~100 kDa fragment. There is benefit in documenting these parameters during method development since these data are potential indicators of column failure or a system dispersion problem. Consequently, the data from this analysis can be incorporated in standard procedures to help ensure the column and system are fit-for-purpose prior to the analysis of valuable samples.

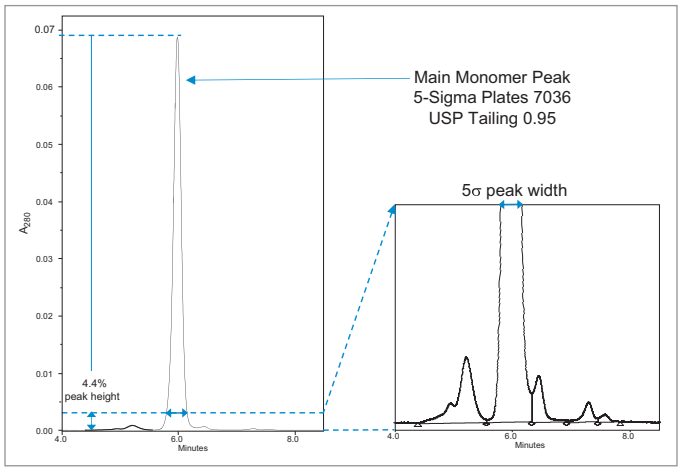

Figure 2. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 4.6 × 150 mm Column using an ACQUITY UPLC H-Class Bio System. Conditions provided in the experimental section.

The Waters mAb Size Variant Standard was used to systematically investigate the effect of the various system dispersions on the chromatographic performance of each of the four columns listed below. The same columns were evaluated on each system. Columns were checked before and after all evaluations and found to maintain their performance. The individual quality report chromatogram, that is provided in the BioResolve SEC mAb Column box, is essentially the same as that generated on the ACQUITY UPLC H-Class Bio System, in the examples below.

In these experiments, four BioResolve SEC mAb Columns (4.6 × 150 mm, 4.6 × 300 mm, 7.8 × 150 mm, and 7.8 × 300 mm) are used on three different relatively modern LC Systems:

- The lowest dispersion (UPLC) system is the ACQUITY UPLC H-Class Bio having a dispersion of 10 µL $5\sigma_{ec}$ for the 150 mm length column heater (CH-A) and 13 µL 5/sec for the 300 mm length column heater (CH-30A): both having active pre-heating.
- The intermediate dispersion (UHPLC) system is the ACQUITY Arc Bio having a dispersion of 30 µL $5\sigma_{ec}$. For all columns, the forced air convection 30-cm column heater (30-cm CH) was used.
- The largest dispersion (HPLC) system is the Alliance having a dispersion of 49 µL $5\sigma_{ec}$. For all columns, the forced air convection column heater box was used.

The chromatographic results of these experiments for the 7.8 mm I.D. columns are shown in Figure 3, and those for the 4.6 mm I.D. column are shown in Figure 4.

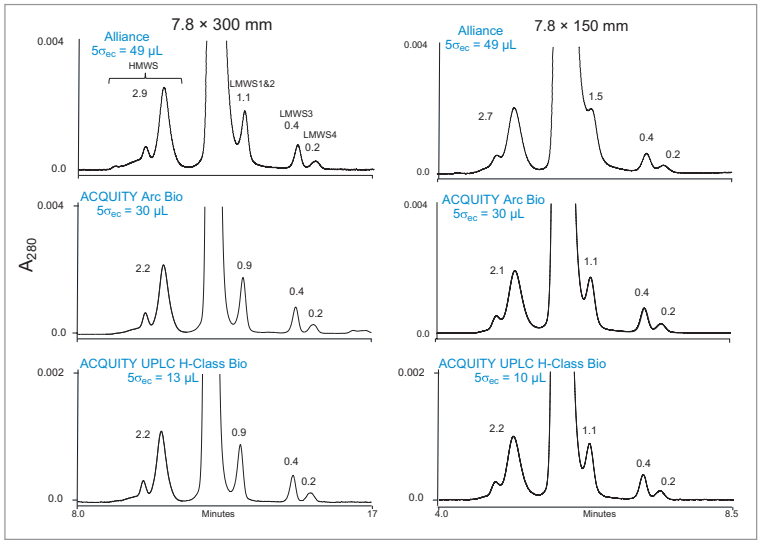

Figure 3. Separation of Waters mAb Size Variant Standard on BioResolve SEC mAb, 7.8 × 300 mm and 7.8 × 150 mm Columns on LC systems with 49 µL (Alliance), 30 µL (ACQUITY Arc), and 10 or 13 µL (ACQUITY UPLC H-Class) system dispersions. Percent areas are reported for each chromatogram. Conditions provided in the experimental section.

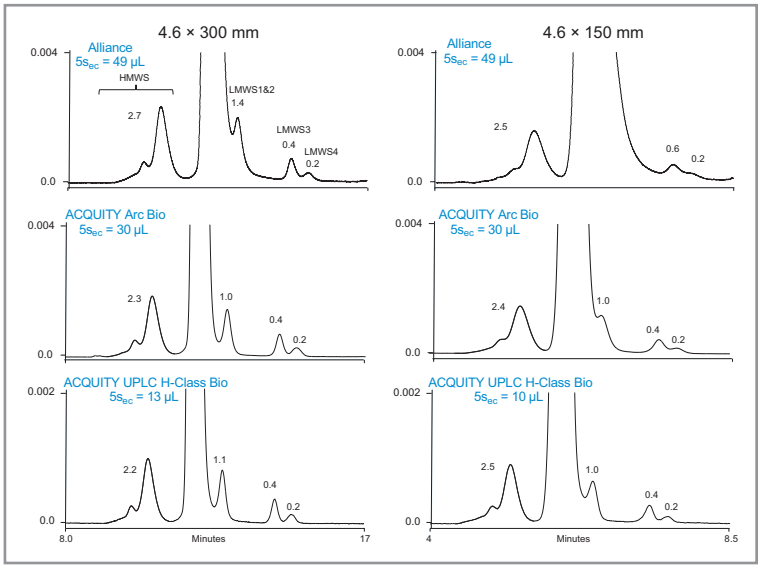

Figure 4. Separation of Waters mAb Size Variant Standard on BioResolve SEC mAb, 4.6 × 300 mm and 4.6 × 150 mm Columns on LC systems with 49 µL (Alliance), 30 µL (ACQUITY Arc), and 10 or 13 µL (ACQUITY UPLC H-Class) system dispersions. Percent areas are reported for each chromatogram. Conditions provided in the experimental section.

Before reviewing the chromatographic results, it is important to point out the various components in the Waters mAb Size Variant Standard. The percent composition of the lower molecular weight species in commercial mAb drug products are generally very low and variable among product batches, making the utility of such samples as standards undesirable. To address this shortcoming, Waters created a mAb Size Variant Standard. Each vial of the standard contains 160 µg of National Institute of Standards and Technology (NIST) mAb Reference Material (RM) 8671 that has been supplemented with 2 µg of the IdeS (FabRICATOR®) digested NISTmAb. Figure 5 elucidates the source of the various components in the mAb Size Variant Standard. As seen in the chromatogram (Figure 5b) the ~100 kDa Fab/c fragment endogenous to the NISTmAb elutes slightly earlier than the IdeS generated ~100 kDa $F(ab')_2$ fragment. Since the order of elution is taken as a difference in hydrodynamic volume, Fab/c will be more difficult to separate from the monomer as compared to $F(ab')_2$. The BioResolve SEC mAb, 7.8 × 300 mm Column was used at a lower flow rate (0.3 mL/min) in order to achieve the resolution shown for Fab/c in the NISTmAb RM 8671. More details can be found in the mAb Size Variant Standard care and use manual (p/n: 720006811EN). More information on NISTmAb RM 8671 can be found at nist.gov.[6]

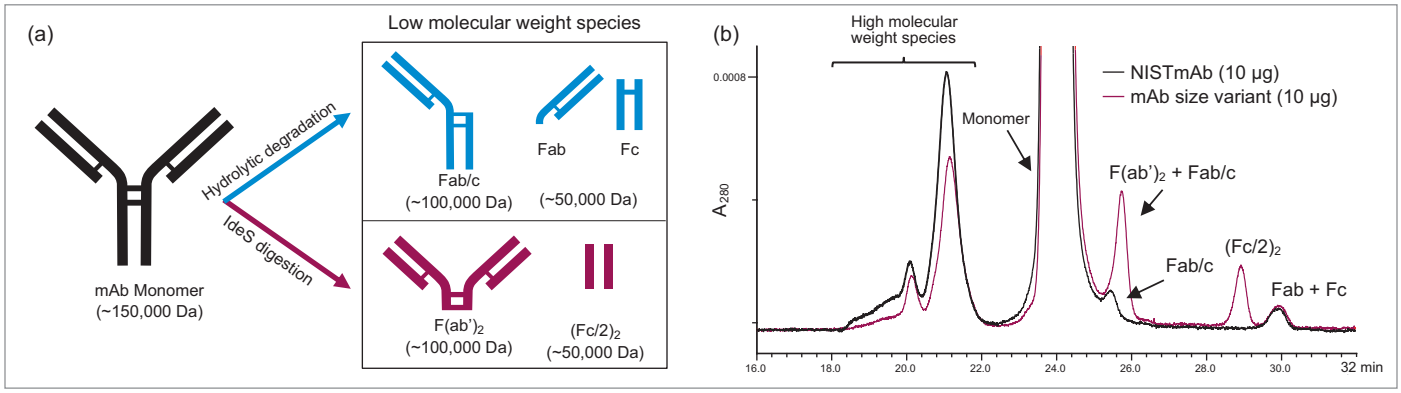

Figure 5. (a) and (b) mAb graphic illustrating the difference between the intact monomer and fragments found in NISTmAb and mAb Size Variant Standard. A representative A280 SEC chromatogram of NISTmAb (black trace) and modified mAb Size Variant Standard (red trace) cropped to show both high and low molecular weight species, in addition to the monomer. Due to similarity in hydrodynamic volume, $F(ab')_2$ and Fab/c are not resolved. Data were collected with a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column with absorbance measured at 280 nm using a flow rate of 0.3 mL/min at ambient temperature.

As shown in Figure 3, the separation of the mAb Size Variant Standard on 7.8 mm I.D. columns, we see that the chromatograms display very similar separation performance for the HMWS, main, and fragment peaks on the 7.8 × 300 mm column run on the HPLC, UHPLC, and UPLC systems. The shorter 7.8 × 150 mm column provides, as predicted, lower resolution separations, but also shows a visible degree of performance loss for LMWS1&2 on the HPLC system. HMWS are adequately separated on both 7.8 mm I.D. columns on the three LC systems.

Although the 7.8 × 150 mm column in Figure 3 provides what appears to be adequate resolution for LMWS1&2 on the UPLC system, it is important to understand that most of the LMWS1&2 peak area is due to the IdeS supplemented fragment, $F(ab')_2$. As previously mentioned, this fragment appears to be slightly smaller (therefore slightly better resolved) than the naturally occurring Fab/c fragment (Figure 5).

The effect of system dispersion on the 4.6 mm I.D. columns in Figure 4 presents a different picture than that for the 7.8 mm I.D. columns discussed above. The LMWS1&2 are separated with the 4.6 × 300 mm column on all three systems with a noticeable loss of resolution observed as system dispersion increases. This trend is even more abrupt for the 150 mm column length. No significant change in HMWS resolution is observed for the 4.6 × 300 mm column on the different LC systems, however, a slight loss of HMWS resolution is observed for the 150 mm column length, as system dispersion increases.

RESOLUTION OF AGGREGATES

The trends visually observed in the chromatograms of Figures 3 and 4 were quantified using the Empower system suitability parameters. For aggregate resolution, the USP resolution at half height (HH) was used to assess the quality of the separations between the main monomer peak and the dimer across the three systems for these columns. The frequently proclaimed value of 1.5 for baseline resolution is not relevant for most real-world chromatographic separations. A criterion adopted for acceptable resolution for real world samples is 1.75–2.0.7 This shift to higher values is due to effects of peak asymmetries and the dissimilar areas encountered in SEC separations.[7] In Figure 6, for the dimer-main peak resolution values we observe that for all, but the 4.6 × 150 mm column, the resolution remains essentially the same across the three LC systems. The 26% loss in resolution for the 4.6 × 150 mm column across the three LC systems remains above acceptable levels for many aggregate analyses.

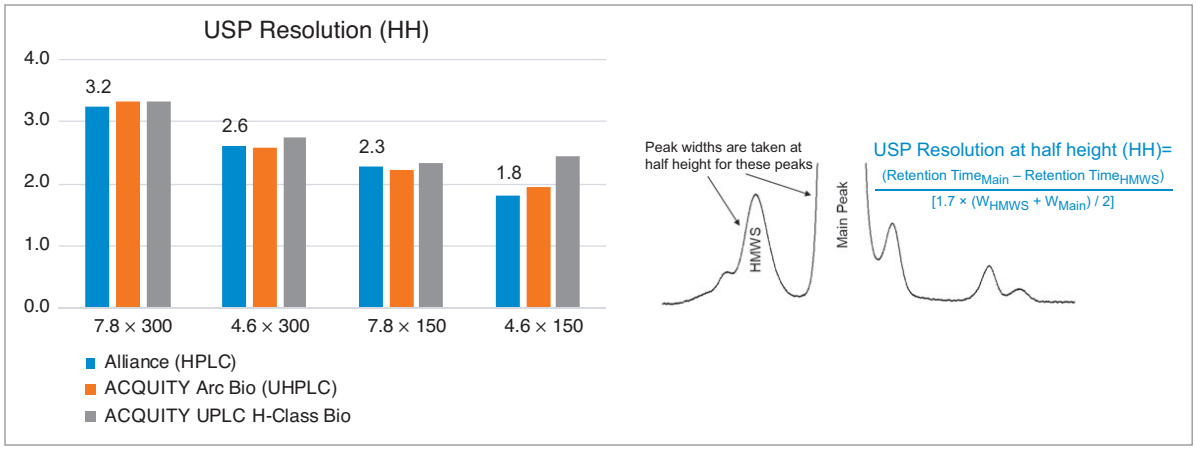

Figure 6. Dimer-Main peak USP resolution (HH) data for the four-column set across the three LC systems. Resolution equation shown. Conditions provided in the experimental section.

RESOLUTION OF FRAGMENTS

In order to quantitatively measure and compare the resolutions for LMWS1&2, another type of resolution metric is needed, the peak-to-valley ratio (p/v). The p/v parameter, illustrated in Figure 7, is typically used to quantify the quality of a separation between peaks that have disparate areas and are too closely eluting (partially coeluting) to obtain a resolution value from the traditionally used resolution equations. The value of p/v is used to allow quantitative assessment of very difficult separations such as those encountered for fragment analysis. An example, of the use of the p/v > 2.0 criterion for quantification of closely eluting peaks in SEC, can be found in the USP Pharmacopoeia Monograph for Insulin.[8] The USP Insulin monograph calls for an end p/v > 2.0 when quantifying the relative amount of high molecular weight protein (HMWP) in the insulin sample.[8] Because USP resolution (HH) values were not available for all the column configuration/system combinations in this study, the start p/v parameter was used for these comparisons.

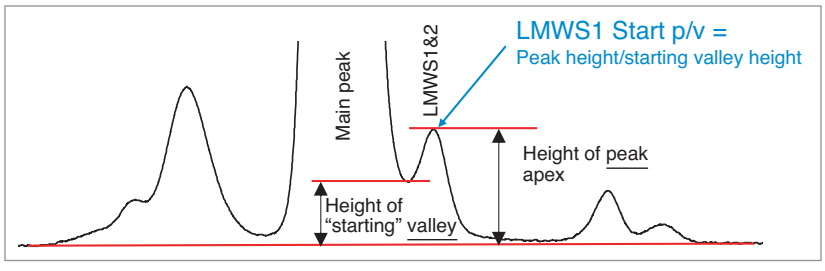

Figure 7. Chromatographic example and calculation for LMWS1&2 start p/v.

In Figure 8, the p/v resolution values are maintained consistently across the UPLC and UHPLC systems for all the configurations. Generally, p/v ratios are less reproducible as they increase above three (data not shown). As predicted, the resolutions on the 300 mm length columns are higher than on the 150 mm length columns. The 4.6 × 150 mm column was not included in this figure because it failed to resolve LMWS1&2 on the Alliance, and on the other systems the p/v was 1.6 and lower. As p/v resolutions increase above 1, the % areas for LMWS1&2 gradually decrease until they start to level off as they approach p/v > 2. This trend is visible for all the columns on the Alliance.

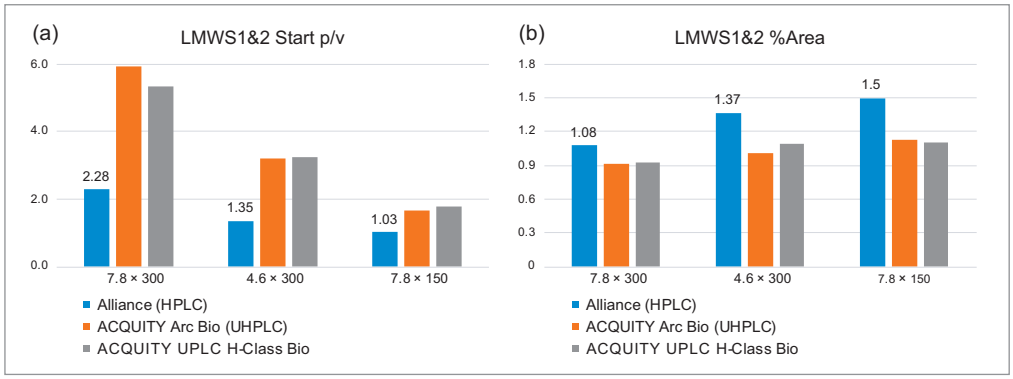

Figure 8. (a) LMWS1&2 start p/v and (b) LMWS1&2 % areas for the three columns used across three systems. Conditions provided in the experimental section.

MAIN PEAK

Central to all SEC mAb chromatograms is the main peak. It is apparent from the chromatograms in Figures 3 and 4 that the efficiencies for the main peak are degraded by system dispersion. As separations lose efficiency, analyte peak heights decrease. Not often discussed is the effect of a system's dispersion on the sensitivity of the analysis due to this decrease in peak height which directly impacts the signal-to-noise ratio of the impurities that we are intending to quantify in these separations.

In Figure 9a, the correlation coefficient squared (R^2) values between system dispersion volume and 5-sigma plates show increasingly stronger correlations as column volume decreases. R^2 values represent the percent of the variation in plates that can be explained by the difference in system dispersion volumes; meaning that 84–100% of the decrease in main peak 5-sigma plates can be explained by the increasing system dispersion volumes. The slopes of the linear correlations are steeper on the 4.6 mm I.D. columns compared to those on the 7.8 mm I.D. columns, indicating a greater impact of dispersion volume on the 4.6 mm I.D. columns. It is also noticeable that the efficiency even on the 4.6 × 300 mm column was not as high as that obtained on the 7.8 × 300 mm, suggesting that system dispersion is limiting its performance even on the UPLC system.

The dispersion volume and its tailing factor are not independent variables. However, comparing the R^2 values between the dispersion peak volumes (Figure 9a) and tailing factors (Figure 9b) to main peak plates suggests that it is the system dispersion tailing factor that plays a stronger role in influencing changes in the main peak plates on the 7.8 mm I.D. columns than on the 4.6 mm I.D. columns. It is not known at this time how universally applicable these observations are to all systems but does suggest that reducing tailing for the dispersion peak may lead to even better performance for 7.8 mm I.D. configurations.

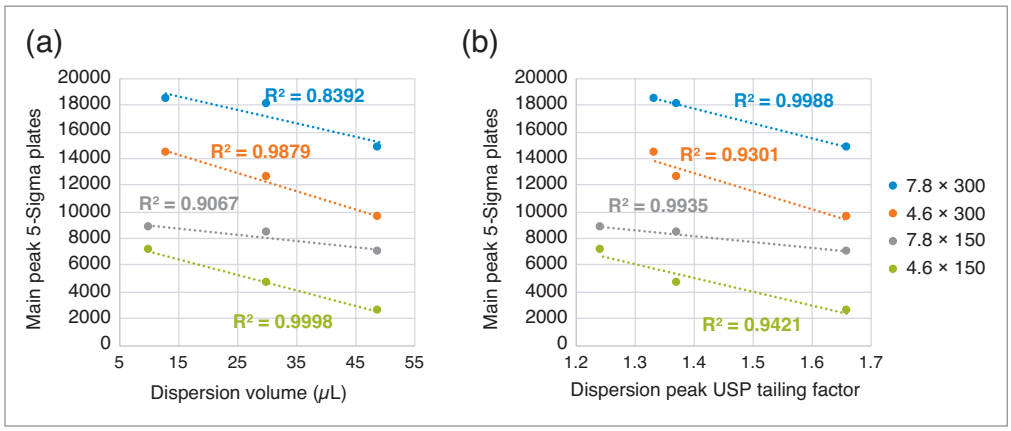

Figure 9. Effect of system dispersion on main peak 5-sigma plates; (a) Effect of dispersion volume; (b) Effect of dispersion peak USP tailing factor. Conditions provided in the experimental section.

Focusing on the 300 mm length columns, it was observed that the LMWS1&2 p/v was most strongly influenced by the tailing factors of both the main peak and the dispersion peak. The p/v values were still found to be influenced by the main peak plates (R^2 values of 0.88 for 4.6 mm I.D. and 0.95 for 7.8 mm I.D.) and system dispersion volume (R^2 values of 0.80 for 4.6 mm I.D. and 0.64 for 7.8 mm I.D.), just not as strongly as with those described in Figure 10. Since the tailing factor for the main peak is also strongly correlated with the tail factor for the dispersion peak (Figure 9b), minimization of tailing in the dispersion peak should be investigated as a means of improving the resolution for fragment analyses.

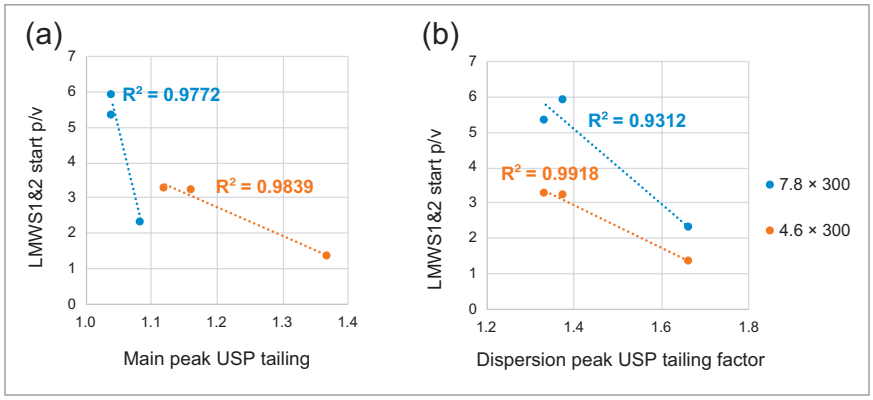

Figure 10. Factors influencing LMWS1&2 p/v: (a) Main peak USP tailing factor; (b) Dispersion peak USP tailing factor for 300 mm length columns across the three LC systems. Conditions provided in the experimental section.

CONCLUSION

Waters BioResolve SEC mAb Columns can provide high resolution and reproducible separations of mAb HMWS, monomers, and LMWS. The 7.8 mm column I.D. configurations provide better chromatographic performance than the 4.6 mm I.D. columns on HPLC, UHPLC, and UPLC systems, and are therefore the most recommended configurations when high resolution separations, particularly those requiring analysis of partially resolved LMWS1 (Fab/c). The 4.6 mm I.D. columns can be nearly as effective as the 7.8 mm I.D. columns on UPLC systems and for the analysis of HMWS on UHPLCs and in some cases HPLCs. The advantages offered by the 4.6 mm I.D. columns are reduced sample demands and mobile phase consumption, along with being more economical.

In these experiments, the LC systems used were not modified to further reduce dispersion from that of their typically configured components. For example, it is well known that reducing the length and I.D. of the connection tubing may further reduce dispersion.[9] The "out-of-the-box" performance of BioResolve SEC mAb Columns is tested by Waters for each column using the Waters mAb Size Variant Standard on low dispersion LC systems and chromatograms should appear similar to those presented on the ACQUITY UPLC H-Class System in Figures 3 and 4. Representative quality testing results for four different batches of BioResolve SEC mAb are presented in Figure 11 and demonstrate a high degree of reproducibility.

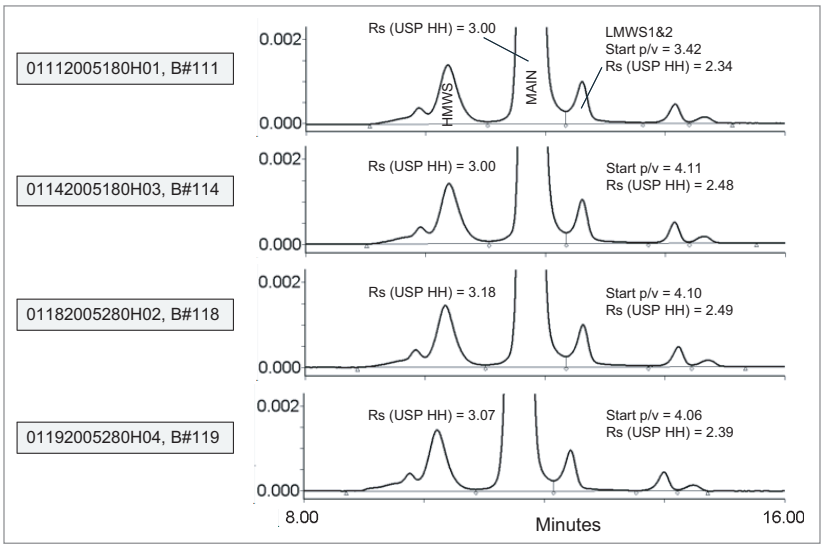

Figure 11. Resolution comparison using the Waters mAb Size Variant Standard on four different BioResolve SEC mAb batches in 7.8 × 300 mm columns run on the same ACQUITY UPLC H-Class Bio System. Conditions provided in the experimental section.

Successful implementation of SEC in the research, development, and manufacture of mAb biotherapeutics is dependent on understanding and selecting a column that is compatible with the dispersion levels of the utilized LC system(s) to help ensure the generation of reliable data especially in a validated method. Development of separation conditions that provide robust separations while minimizing secondary interactions with both the column and LC system flow path are critical to the success of any SEC method. Finally, it is important to consider establishing and using a reliable mAb-based reference sample to help ensure column, LC system, mobile phase, and method readiness prior to the analysis of desired mAbs.

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BioResolve SEC mAb Guard Columns for Production Process and Formulation Development Samples

Stephan M. Koza and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Enabling high sample throughput SE-HPLC analysis of protein samples with the potential to precipitate
- Demonstrated increased SE-HPLC column tolerance against resolution loss due to the injection of protein precipitates versus UP-SEC

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class PLUS System

ACQUITY UPLC Tunable UV Detector

BioResolve™ SEC mAb Columns

Empower™ 3 Chromatography Data Software

KEYWORDS

High molecular weight species, HMWS, size exclusion, SEC, UP-SEC

INTRODUCTION

Protein self-associated forms, or high molecular weight species (HMWS) are routinely assessed as a critical quality attribute (CQA) in biotherapeutic protein preparations as they can impact both the safety and efficacy of treatment.[1] SEC remains one of the predominant methods for the assessment of protein self-association due to the reliability and sample throughput of the method.[2] Although, it is critical that the veracity of the SEC measurement be confirmed by complementary methods such as analytical ultracentrifugation (AUC).[3] There has also been an increase in the measurement of protein fragment levels, or low molecular weight species (LMWS) by SEC, particularly for monoclonal antibody based therapeutics.[4] The increased demand for greater sample throughput has necessitated the use of more efficient, smaller particle size (sub-2-µm) ultra-performance SEC (UP-SEC) columns. UP-SEC columns are also packed in smaller 4.6 mm inner diameter (I.D.) columns that require LC systems with significantly lower dispersion versus larger particle sizes packed in 7.8 mm I.D. high-performance SEC columns (SE-HPLC).[5]

In addition to being less tolerant of LC system dispersion, UP-SEC columns are also more demanding of guard column performance in order to maintain optimal resolution. As a result, high efficiency guard columns are needed for UP-SEC. A previous publication showed the ability to obtain monoclonal antibody resolutions using a 2.5 µm particle size, 7.8 mm I.D. SE-HPLC column that were comparable to those obtained on an UP-SEC column with only a modest increase in analysis time.[6] For the same reasons that a 7.8 mm I.D. SE-HPLC column can be effectively used on LC systems with greater dispersion, the demands on SE-HPLC guard column efficiency are lower. Additionally, due to the larger particle size of the HP-SEC column, we observed an increased tolerance for samples with suspended protein precipitates versus a column with a 1.7 µm particle size as would be predicted. These attributes should certainly be considered when the protein samples to be tested may have uncertain levels of particulates.

The effectiveness of Waters™ BioResolve SEC mAb, 200 Å, 2.5 µm Guard (p/n: 186009443) to protect a 7.8 × 300 mm BioResolve SEC mAb Column (p/n: 186009441) from the injection of suspended protein precipitates was evaluated. In addition, a comparison of the susceptibility to the injection of precipitated protein was made between the BioResolve SEC mAb, 2.5 µm Guard and the ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm Guard (p/n: 186005793). Precipitated protein was generated by stressing the BEH200 SEC Protein Standard Mix (p/n: 186006518, reconstituted in 500 µL of water) at 80 °C on an orbital shaker set at 800 RPM for 30 minutes. The performance of the separation was evaluated using an expired sample of Erbitux (cetuximab) as provided in a liquid formulation at 2 mg/mL.

The impact that the BioResolve SEC mAb Guard has on the separation of cetuximab was evaluated (Figure 1). The experimental details are provided in the caption of Figure 1. We observe a predicted increase in retention time and minimal change in the quality of the separation. The resolutions (USP) for HMWS1 were equivalent and a slight decrease in the peak-to-valley ratio (P/V) of LMWS1 with the guard in place was observed. Given that LMWS1 is present at a 0.5% level, the modest decrease in P/V is likely the result of the increase in the extent of low-level tailing due to dispersion effects.[7]

Next, a series of 14 µL injections of the stressed protein standard were made onto the column with the guard in place, and the separation of cetuximab was evaluated after every 20 injections (Figure 2). Over the course of 40 stressed protein standard injections we observed a steady decrease in the resolution of HMWS1. Additionally, we observed some changes in the chromatographic profile of the HMWS1 and HMWS2. It was noted that the percent peak areas of the HMWS2 and HMWS1 changed throughout these studies which is predominately the result of the high levels and instability of these self-associated forms. The high-levels of HMWS2 and HMWS1 are the result of freeze-thaw cycles, which are not recommended when this product is to be used clinically. As a result, these values are not reported.

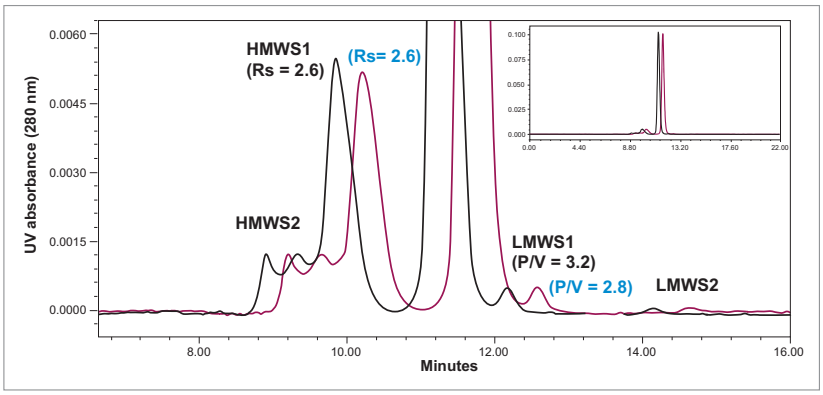

Figure 1. Shown is an overlay of the SEC separation of a 2 mg/mL cetuximab sample (14 µL injection volume) using a 2.5 µm particle size 7.8 × 300 mm BEH SEC column with a BioResolve SEC mAB Guard (blue) and without the guard (black). HMWS1 resolution (Rs) and LMWS1 P/V and percent peak areas are presented. The flow rate was 0.575 mL/minute and the mobile phase was 50 mM sodium phosphate, 200 mM KCl, pH 7.0. Analysis was performed on an ACQUITY UPLC H-Class Bio using Empower 3.

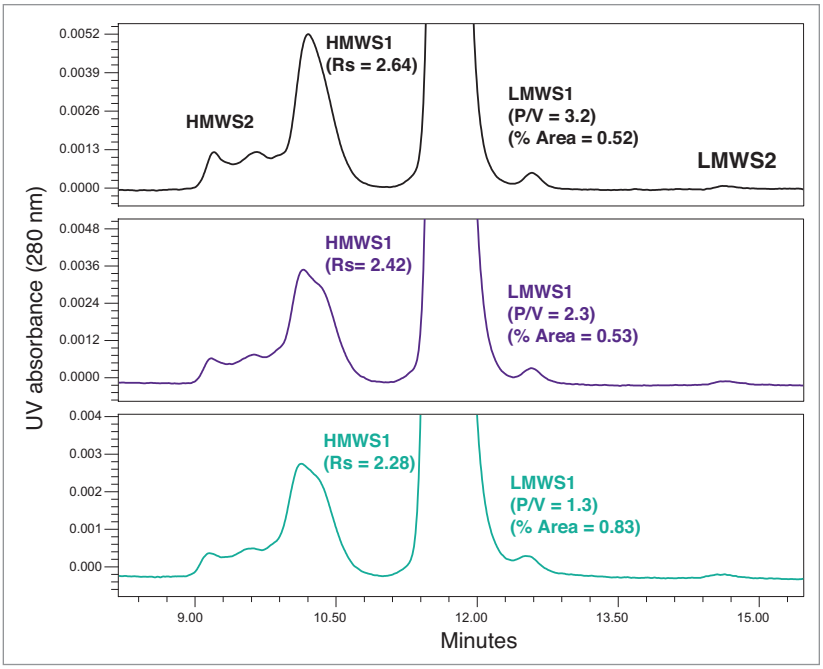

Figure 2. Shown are the SEC separations of cetuximab with analytical SEC column and BioResolve Guard initially (black) and then following 20 (blue) and 40 (green) 14 µL injections of a stressed SEC protein standard. Additional experimental condition are presented in the caption of Figure 1.

A decrease in the P/V of LMWS1 is also observed and more significantly, a 60% increase in the percent peak area of LMWS1 was measured. This is likely the result of increased low-level tailing of the monomer peak. This is important to note as the level of LMWS1 may be considered as a CQA for mAb-based therapeutics.

The fouled guard was then replaced, and the separation of cetuximab was reevaluated (Figure 3). As shown, the chromatographic performance was improved substantially. While the resolution of HMWS1 and the P/V of LMWS1 were not fully restored to their initial levels, most of the previous performance was recovered, and the significant increase observed in the percent peak area of LMWS1 was reversed. These data indicate that the BioResolve SEC mAb Guard can effectively extend the life of the analytical column when analyzing samples containing precipitated proteins.

As a final study, the rate of fouling of the BioResolve SEC mAb, 2.5 µm, 4.6 × 30 mm Guard was compared to that of the equivalently sized ACQUITY UPLC Protein BEH SEC, 1.7 µm Guard while using the same 2.5 µm particle size, 7.8 × 300 mm analytical column. For this study, smaller injections (5 µL) of the stressed protein standard were made on the 1.7 µm particle size guard, since it is intended to be used with a 4.6 mm I.D. analytical column, and the separation of cetuximab was evaluated after every 10 injections (Figure 4). We observed the rate of loss of initial performance was comparable for the resolution of HMWS1 and that of the P/V of LMWS1 appeared to be greater over the initial 20 injections of the precipitated protein sample. Taking into account that the two guards were of the same I.D. and length, and the injection volumes were almost three times larger for the 2.5 µm particle size guard, these results clearly indicate that a 2.5 µm particle size SEC column is significantly less prone to fouling by precipitated proteins versus an SEC column packed with 1.7 µm particles. This is predicted since a larger particle size would result in reduced filtration of these particulates. It would also be predicted that the impact that a fouled guard would have on these separations would be greater on a 4.6 mm I.D. versus a 7.8 mm I.D. analytical column due to the larger peak volumes generated by the latter.

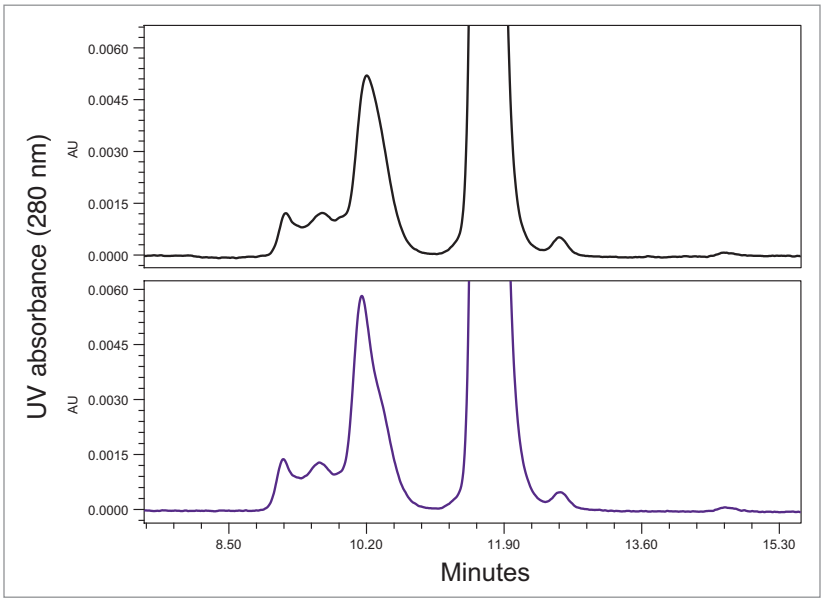

Figure 3. Shown are the SEC separations of cetuximab with analytical SEC column and BioResolve Guard initially (black) and replacement guard (blue) following 40 injections of a stressed SEC protein standard. Additional experimental condition are presented in the caption of Figure 1.

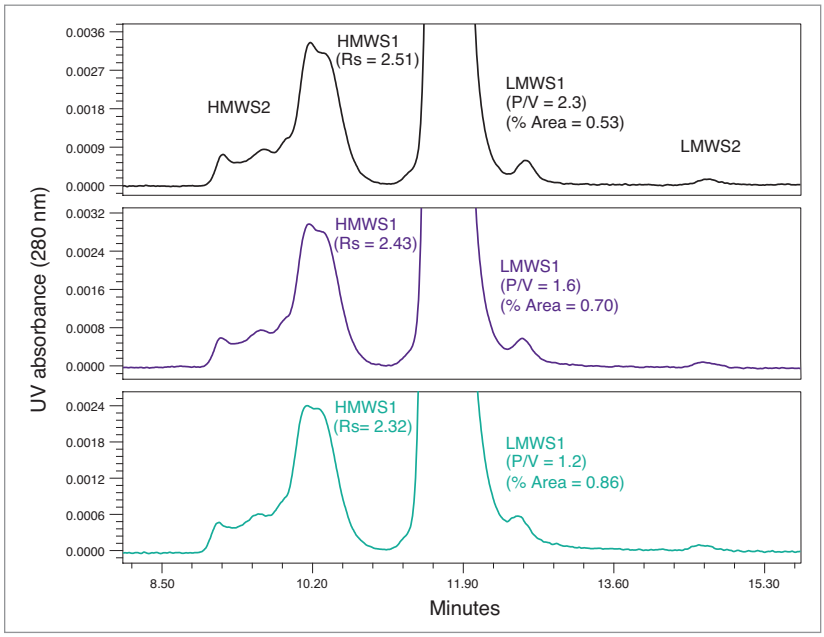

Figure 4. Shown are the SEC separations of cetuximab with analytical SEC column and ACQUITY UPLC SEC 200 Å Guard initially (black) and then following 10 (blue) and 20 (green) 5 µL injections of a stressed SEC protein standard. Additional experimental condition are presented in the caption of Figure 1.

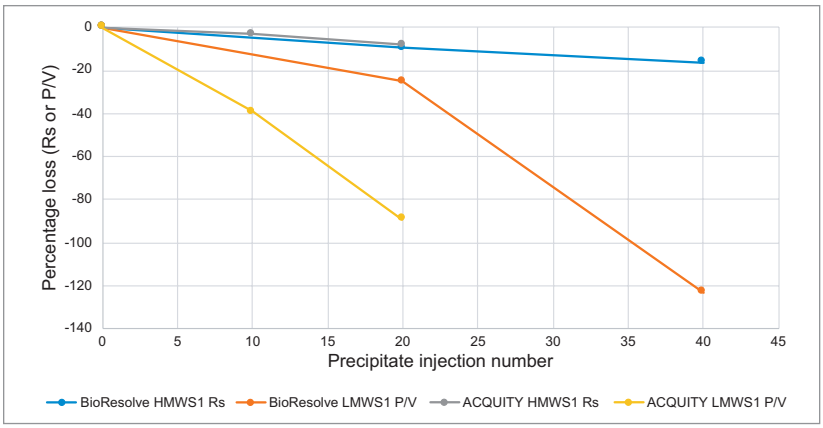

Figure 5. Shown are the comparisons of the trends for the resolution of HMWS1 and P/V of LMWS for the SEC separations of cetuximab with analytical SEC column with a BioResolve Guard or an ACQUITY UPLC SEC 200 Å Guard. Data are derived from the chromatograms presented in Figures 2 and 4.

CONCLUSION

These results demonstrate that the use of a BioResolve SEC mAB Guard (p/n: 186009443) to protect a BioResolve SEC mAb Column can be beneficial when analyzing both HMWS and LMWS in process development samples that may contain varying levels of precipitated protein.

It is also shown that 2.5 µm particle size HP-SEC columns are significantly more resistant to performance loss when injected with precipitated proteins versus an UP-SEC column packed with 1.7 µm particles.

As a final note, while the use of a guard column can be beneficial, sample preparation to remove particulates via filtration or more preferably centrifugation to minimize potential speciation should also be considered whenever possible.

Acknowledgements

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High Resolution and High Throughput Size-Exclusion Chromatography Separations of IgG Antibody Aggregates and Fragments on UHPLC and HPLC Systems with 2.5 µm BEH Particles

Stephan M. Koza and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A performance comparison of Waters™ Protein BEH SEC, 200 Å (2.5 µm, 1.7 µm, and 3.5 µm) Columns on UPLC™, UHPLC, HPLC LC systems
- Performance comparisons of Waters Protein BEH SEC, 200 Å, 2.5 µm Columns and current competitor sub-3-µm columns
- Guidance for selecting the optimal SEC column configuration based on the LC systems to be used for analysis

WATERS SOLUTIONS

ACQUITY™ UPLC Protein BEH SEC, 200 Å, 1.7 µm Columns

XBridge™ Protein BEH SEC, 200 Å, 2.5 µm Columns

XBridge Protein BEH SEC, 200 Å, 3.5 µm Columns

ACQUITY UPLC H-Class Bio System

KEYWORDS

Size exclusion, system dispersion, UPLC, UHPLC, HPLC, proteins, IgG, rituximab (Rituxan)

INTRODUCTION

Size-exclusion chromatography (SEC) has been the method of choice for the routine assessment of protein aggregation (high molecular weight species [HMWS]) for most recombinant protein-based biotherapeutic products.[1] Additionally, the use of SEC for the non-denatured analysis of protein fragments (low molecular weight species [LMWS]) in these samples has also been successfully applied.[2] A predominant form of which for many mAb biotherapeutics is two-thirds the molecular weight (~100 kDa) of the mAb monomer (~150 kDa) and is the result of proteolytic cleavage of a Fab domain (50 kDa) at the IgG hinge region of an IgG.[2] We will refer to this fragment as LMWS1. Additionally, a lower abundance LMWS peak is also observed eluting later than the LMWS1 fragment. This peak, referred to as LMWS2, is a mixture of cleaved Fab and Fc domains, where the Fc domains are the result of the cleavage of both Fab domains. Due to the LMWS1 and monomer being more similar in size (hydrodynamic radius) and eluting on the tail-end of the significantly larger monomer peak, the separation and reproducible quantification of this LMW form is typically more challenging than it is for the dimer HMWS protein form (~300 kDa), which elutes prior to the monomer, and for the LMWS2 fragments which are baseline resolved. While this study will focus on the mAb SEC separation as an example, the general principals demonstrated here may also be applied to other protein biotherapeutics as well.

While it has been demonstrated that the use of multiple SEC columns in series can be used to provide the efficiencies needed to reliably separate the 100 kDa mAB LMW fragment, this separation has generally been performed using higher efficiency SEC columns, with particle diameters of 2 µm and smaller, to enable higher throughput analyses.[3] However, as these columns are typically manufactured with internal diameters (I.D.s) of 4.6 mm and smaller, their use is limited for this application on HPLC and even some UHPLC chromatography systems with their somewhat larger system dispersion volumes, as compared to UHPLC systems.[4] As a result, BEH SEC, 200 Å, 2.5 µm columns packed in larger formatted 7.8 mm I.D. hardware were developed to effectively bridge the performance gap between the Waters Protein BEH SEC, 200 Å, 1.7 µm and 3.5 µm Columns, and provided more robust and easily transferred analyses with less dependency on the extra-column dispersion of the LC systems being used.

Throughout this discussion we will refer to $5\sigma_{ec}$ system dispersion volume as a primary measurement of LC system performance. Briefly, $5\sigma_{ec}$ system dispersion volume, also referred to as extra-column dispersion, is the volume of mobile phase that an injected sample slug (1 µL or less) will occupy after it has traveled through an LC system without a column in place as measured from the beginning to the end of the peak at a height of 4.4% of the peak maximum.[5] More detailed discussion of LC system dispersion, and the effect that it has on SEC separations, can be found in Waters Application Notes (p/n's: 720006336EN and 720006337EN).

The goal of this study was to demonstrate the performance of the Waters XBridge Protein BEH SEC, 200 Å, 2.5 µm Columns, which are provided in both 7.8 mm and 4.6 mm I.D. Comparisons will be provided for Waters SEC 1.7 µm and 3.5 µm particle size columns and current sub-3-µm competitor columns. In summary, recommendations are provided for column selections that are compatible with Waters LC instrumentation.

EXPERIMENTAL

Sample description

The mAb sample of rituximab (Rituxan®) was used past expiry at original concentration of ~21 mg/mL.

BEH200 SEC Protein Standard Mix (p/n: 186006518)

Method conditions (unless noted otherwise)

LC conditions

Systems: ACQUITY UPLC H-Class Bio

Detection: ACQUITY UPLC TUV with 5 mm titanium flow cell

Wavelength: 280 nm

Columns: ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 2.1 × 150 mm (p/n: 186008471)

ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 150 mm (p/n: 186005225)

ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 300 mm (p/n: 186005226)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 150 mm and BEH200 Protein Standard (p/n: 176004335)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 300 mm and BEH200 Protein Standard (p/n: 176004336)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 150 mm and BEH200 Protein Standard (p/n: 176004326)

XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 300 mm and BEH200 Protein Standard (p/n: 176004327)

XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 × 300 mm and BEH200 Protein Standard (p/n: 176003596)

Column temp.: Ambient, ~22 °C

Sample temp.: 10 °C

Flow rates and injection volumes, unless otherwise noted:

Column dimension (mm)	Flow rate (mL/min)	Injection volume (µL)
4.6 × 150	0.350	1.0
4.6 × 300	0.350	2.0
7.8 × 150	1.000	5.8
7.8 × 300	1.000	10.0

Mobile phase A: 100 mM NaH_2PO_4

Mobile phase B: 100 mm Na_2HPO_4

Mobile phase C: 1.00 M NaCl

Mobile phase D: H_2O (all 0.2 µm sterile filtered) and Auto•Blend Plus blended at 3.4% A, 16.6% B, 40% C, and 40% D to yield 20 mm sodium phosphate, 400 mM NaCl, pH 7.2, unless otherwise noted

Sample vials: Polypropylene 12 × 32 mm Screw Neck with Cap and PTFE/silicone Septum, 300 µL Volume (p/n: 186002640)

Chromatography software: Empower™ 3

RESULTS AND DISCUSSION

GENERAL PERFORMANCE COMPARISON OF 1.7 µm, 2.5 µm, AND 3.5 µm BEH SEC, 200 Å COLUMNS

The general performance of the BEH SEC 200 Å, 2.5 µm column was compared to that of the 1.7 µm and 3.5 µm particle size columns for the separation of the BEH200 SEC Protein Standard Mix. Sample chromatographic profiles presented in Figure 1 were performed on 300 mm length columns for all three particle sizes and the linear velocities (cm/min), and ultimately analysis times, were equivalent in this comparison. The 1.7 µm column I.D. was 4.6 mm, and 2.5 µm and 3.5 µm column I.D.s were 7.8 mm. The $5\sigma_{ec}$ dispersion volume of the LC system used for the 1.7 µm column was 17.6 µL, which is within the middle of the dispersion volume range expected for a UPLC system, and the $5\sigma_{ec}$ dispersion volume of the LC system used for the 2.5 µm and 3.5 µm columns was 38.1 µL, which is within the upper dispersion volume range expected for a typical UHPLC system or within the lower dispersion volume range expected for a typical HPLC system.

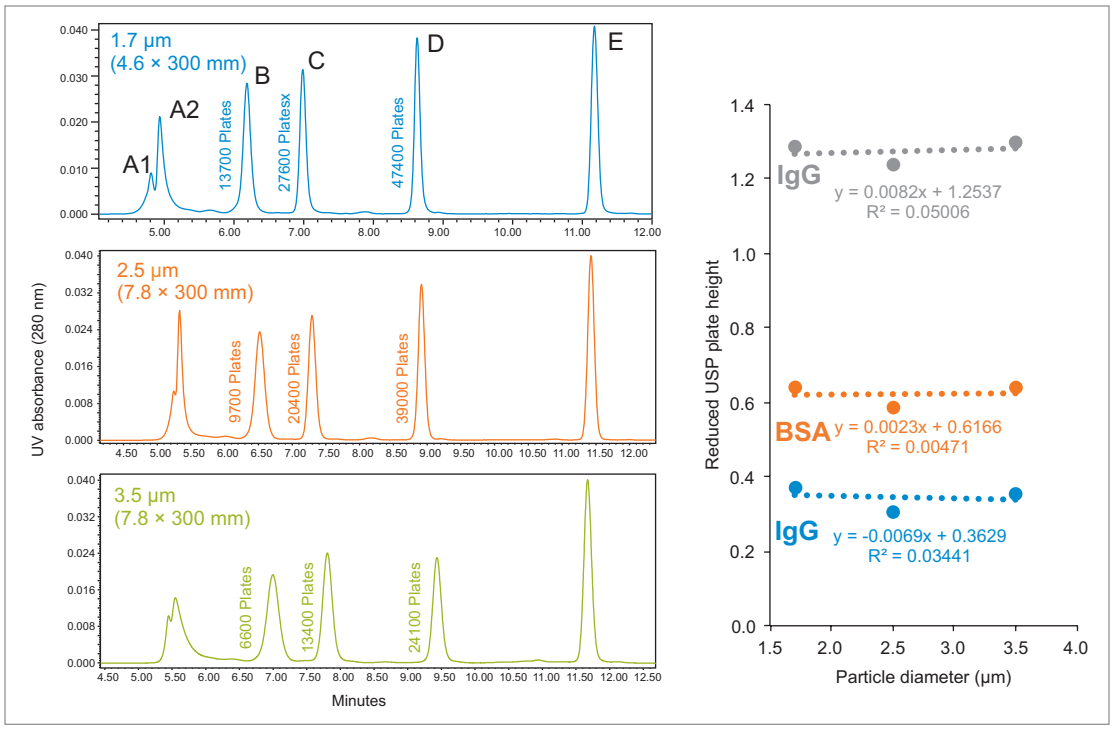

Figure 1. Shown is a comparison of the separation of standard proteins on 200 Å pore size BEH SEC particles with diameters of 1.7 µm, 2.5 µm, and 3.5 µm. Sample loads and flow rates were proportional to column I.D. The 5σec LC system dispersion volume was 17.6 µL for the 1.7 µm column and 38.8 µL for the 2.5 µm and 3.5 µm columns. Peak identifications (shown in top left chromatogram) are: thyroglobulin dimer (A1, 1.32 kDa), thyroglobulin monomer (A2, 660 kDa), IgG (B, 150 kDa), BSA (C, 66 kDa), myoglobin (D, 17 kDa), and uracil (E, 112 kDa). Mobile phase was 20 mM sodium phosphate, 400 mM NaCl, pH 7.2. Plate counts for specified peaks are based on USP tangent method and reduced plate count is determined by dividing the plate height (column length divided by plate count) by the particle diameter.

In comparing the profiles, it is readily observed that the peak widths are narrower, and resolutions improved as the particle size decreased, as would be predicted since peak width is proportional to the inverse of particle diameter. The USP plate counts determined for the immunoglobulin G (IgG), bovine serum albumin (BSA), and myoglobin protein standard are also provided alongside their respective peaks. These plate counts confirm that the column efficiency increases as particle size is decreased. It is also noted that the determined plate counts decrease as the protein size increases. This is consistent with the predicted proportional relationship between separation efficiency and the diffusion coefficient of the analyte.[5] To further quantitatively assess this performance difference, we compared the reduced plate heights for the protein standards (Figure 1). Reduced plate height is a dimensionless quantity determined by dividing the plate height (column length divided by plate count) by the particle diameter where both values are expressed in the same units of length. Comparable reduced plate heights were measured on all three columns, which demonstrates that the column efficiency is reasonably proportional to the BEH particle diameters, as would be predicted. Practically, these results show that greater resolution for an SEC separation can be achieved without an increase in analysis time by using smaller diameter particles.

ANALYSIS OF HMWS AND LMWS IMPURITIES IN mAb PREPARATIONS WITH 1.7 µm, 2.5 µm, AND 3.5 µm BEH SEC, 200 Å COLUMNS

Next, we compare the performance of the three 200 Å BEH SEC particle sizes in the separation of the recombinant rituximab, a chimeric (mouse/human) anti-CD20 IgG1 antibody (Figure 2). It was previously demonstrated that the use of 1.7 µm, 4.6 × 150 mm columns significantly decreases the resolution and limit of quantification for the LMWS1 fragment (100 kDa) of an IgG and also required tight control of system dispersion volumes in order to provide reproducible results in comparison to the use of a 4.6 × 300 mm column.[3] Therefore, we will only compare the 300 mm column lengths in this study. In Figure 2, the two sets of chromatograms produced by 4.6 × 300 mm columns containing either 1.7 µm or 2.5 µm diameter particles were run at a constant flow rate of 0.35 mL/min and the $5\sigma_{ec}$ system dispersion volume was altered. For the 7.8 × 300 mm columns packed with 2.5 µm and 3.5 µm diameter particles, the $5\sigma_{ec}$ system dispersion volume was set to 38.8 µL, while the flow rate was lowered as noted to provide more resolution. The quality of the separation will be assessed using peak-to-valley (P/V) ratio, which is calculated by dividing the height from the baseline of the smaller peak by the height of the valley between the critical pair.

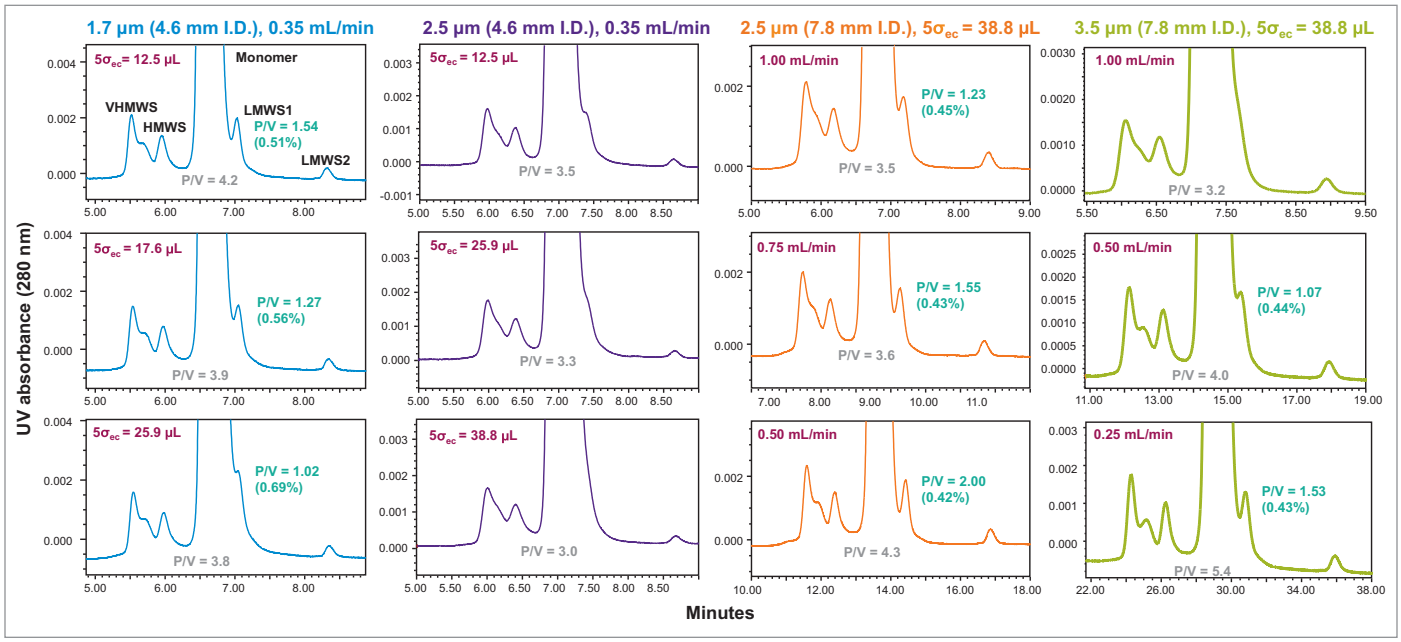

Figure 2. A comparison of the separation of rituximab on 200 Å pore size BEH SEC particles with diameters of 1.7 µm, 2.5 µm, and 3.5 µm. All columns were 300 mm in length, and sample loads and flow rates were proportional to the square of the column I.D. For the 1.7 µm and 2.5 µm, 4.6 mm I.D. columns the flow rate was held constant and system dispersion was increased; and for the 2.5 µm and 3. 5 µm, 7.8 mm I.D. columns, the system dispersion is constant and flow rates were decreased. Further experimental details are provided in the text. Percent peak areas were determined by drop-baseline peak integration.

We will first consider the critical pair separation between the HMWS and the monomer. HMWS is presumed to be predominantly a dimer (~300 kDa) that is present at a level of approximately 0.5% in the sample tested. We observe by comparing the top row of chromatograms that the HMWS-monomer separation improves (higher P/V values) as particle size is decreased when the columns are operated at equal linear velocities and on systems with appropriate dispersion volumes. Throughout, the range of $5\sigma_{ec}$ system dispersion volumes showed the 1.7 µm particle size column provided better resolution than the 7.8 mm I.D. (2.5 µm or 3.5 µm) columns, although this advantage diminishes as system dispersion is increased. We also see for the 2.5 µm particles that the performance of the 4.6 mm I.D. column matches that of the 7.8 mm I.D. column at only the lowest dispersion volume tested ($5\sigma_{ec}$ = 12.5 µL). Changes in system dispersion did not significantly alter the HMWS P/V values for the 7.8 mm I.D., 2.5 µm particle size column (data not shown).

By decreasing the flow rate for the 2.5 µm and 3.5 µm columns with 7.8 mm I.D., the HMWS P/V value can be improved to match and even exceed that of the 1.7 µm column, however, this will result in longer analysis times. Similar observations were also made for the separation of the VHMWS peak which is comprised of multimeric aggregate forms (data no shown). For both the 4.6 mm I.D. and 7.8 mm I.D. columns, variations in $5\sigma_{ec}$ system dispersion volumes or flow rate did not significantly alter the integrated percent peak areas of the VHMWS or the HMWS with the highest relative deviations of 4.9% and 6.7% being observed for VHMWS and HMWS during the flow rate study on the 3.5 µm, 7.8 × 300 mm column.

We will now consider the separation of the IgG LMWS1 fragment (Figure 2) which has a molecular weight of approximately 100 kDa. It is challenging to reproducibly separate and quantify LMWS1 due to this form not being as different in size from the monomer versus the size difference between HMWS and monomer. This separation is further complicated by the elution position of the LMWS peak within the tailing segment of the far more abundant monomer and by the very low abundance of LMWS1 in the sample being evaluated (~0.4%) in this study.[3] Of note is the effect that system dispersion can have on this resolution. This is clearly observed for the 1.7 µm (4.6 × 300 mm) column where we observe a precipitous decrease in P/V measured between LMWS1 and monomer as $5\sigma_{ec}$ system dispersion volume was increased 12.5 µL to 25.9 µL, where the latter volume is commonly observed in UHPLC and even some UPLC system configurations. This loss in resolution also resulted in an increase in the integrated relative peak area of LMWS1 from 0.5% up to 0.7%. In comparison, the LMWS1 separation provided by the 2.5 µm (7.8 × 300 mm) column when run at a flow rate of 0.75 mL/min, which results in a 33% increase in run time, was comparable to that observed for the 1.7 µm column ($5\sigma_{ec}$c = 12.5 µL). More practically, when run at the same linear velocity the 2.5 µm (7.8 × 300 mm) particle size column produced a comparable or greatly improved separation to that observed for the 1.7 µm column when used on an LC at a $5\sigma_{ec}$ system dispersion volume of 17.6 µL to 25.9 µL, performance that is more typical of UHPLC and UPLC systems capable of using 30 cm columns. As further evidence of the impact that extra-column dispersion can have when using 4.6 mm I.D. SEC columns, we also observe a significant loss of LMWS1 resolution for the 4.6 mm I.D., 2.5 µm particle size column versus the 7.8 mm I.D. column.

When we compare the LMWS1 separations produced by the 2.5 µm and 3.5 µm particle size columns, we observe that the comparable P/V values can be achieved using the 3.5 µm particle size, albeit at a considerably lower linear velocity. As an example, the P/V achieved at a 0.75 mL/min flow rate when using the 2.5 µm column is comparable to that observed at a flow rate of 0.25 mL/min on the 3.5 µm column. This corresponds to a sample throughput of approximately three times greater for the 2.5 µm column.

The robustness for the measurement of the LMWS1 fragment relative abundance is greatly improved for both larger format (7.8 × 300 mm) columns in comparison to that observed for the 1.7 µm particle size column (4.6 × 300 mm). Regardless of the resolution achieved, we observe a remarkably consistent LMWS1 percent peak area ranging between 0.42% and 0.45% for the two 7.8 mm I.D. columns. Additionally, in a separate experiment, the LMWS1 percent peak area was consistent for the 2.5 µm particle size (7.8 × 300 mm) column ranging from 0.41% to 0.43% as $5\sigma_{ec}$ system dispersion volume was increased from 25.9 µL to 44.4 µL (data not shown). Throughout this study the percent peak area for the 50 kDa LMWS2 fragments was consistent for all columns (data not shown). This is a result of this peak being fully baseline resolved under the conditions tested.

In summary, for the analysis of LMWS1 fragments by SEC, the use of a 1.7 µm particle size (4.6 × 300 mm) column can provide improved resolution and reliable results with the same analysis time as compared to the 2.5 µm particle size (7.8 × 300 mm) column provided that UPLC system dispersion is minimized and controlled. Alternatively, comparable HMWS and LMWS resolutions can be realized when using the 2.5 µm particle size (7.8 × 300 mm) column at moderately lower linear velocities and increased analysis times with the added benefit of the methods being far less dependent on the system dispersion and running at lower pressures. Thereby, allowing for the use of UHPLC and modern HPLC systems.

The 2.5 µm particle size in a 7.8 × 300 mm column configuration will outperform the same particle in a 4.6 × 300 mm column configuration and that performance increase improves as system dispersion increases. As a result, the 7.8 mm column I.D. is generally recommended unless there is a desire to limit sample or mobile phase volumes, and system dispersion will be controlled. In all cases, a 2.5 µm particle size column will outperform a 3.5 µm particle size column of the same length and I.D. The advantages of the 3.5 µm particle size will be an approximate 50% lower back pressure, enabling its use on some LC systems with low upper pressure capabilities.

HIGH-THROUGHPUT ANALYSIS OF HMWS

We receive numerous requests from customers who are interested in deploying high-throughput SEC analysis during process and formulation development for a biotherapeutic protein. Greatly reducing SEC analysis time has also provided the possibility of deploying SEC and SEC with multi-angle light scattering detection (SEC-MALS) for on-line monitoring of a manufacturing process step.[6] Given the utility of these applications we compared the performance of the 1.7 µm and 2.5 µm particles with a column length of 150 mm and at equivalent linear velocities. The 3.5 µm particle size was not considered due to the benefits smaller particles provide for high-throughput SEC analysis.

The impact of system dispersion on the separation of both VHMWS and HMWS for the 1.7 µm particle size (4.6 mm I.D.) and 2.5 µm particle size (4.6 mm and 7.8 mm I.D.) columns is shown in Figure 3. Here we observe that the 1.7 µm column provides superior separation of the VHMWS and HMWS peaks throughout the range of system dispersions evaluated. However, for the separation of HMWS and monomer, we observe that as $5\sigma_{ec}$ exceeds 25 µL that the performance advantage of the 1.7 µm particle size is substantially diminished. The greater impact of system dispersion on the separation of HMWS and monomer is a direct result of the significantly larger monomer peak size and the limited separation observed between those two peaks. These results indicate that for partially resolved HMWS separations the LC system dispersion should be minimized and controlled to derive the full benefits of the 1.7 µm particle size (4.6 × 300 mm) column.

Comparison of the performance of the 2.5 µm particle size in 4.6 mm and 7.8 mm I.D. columns (Figure 3) shows that the 7.8 mm I.D. significantly outperforms the 4.6 mm I.D. column for the separation of HMWS and monomer, and to a lesser extent the separation of VHMWS and HMWS. This is a result in that UHPLC and HPLC system dispersion levels have a much greater deleterious effect on separation quality for the smaller I.D. column.

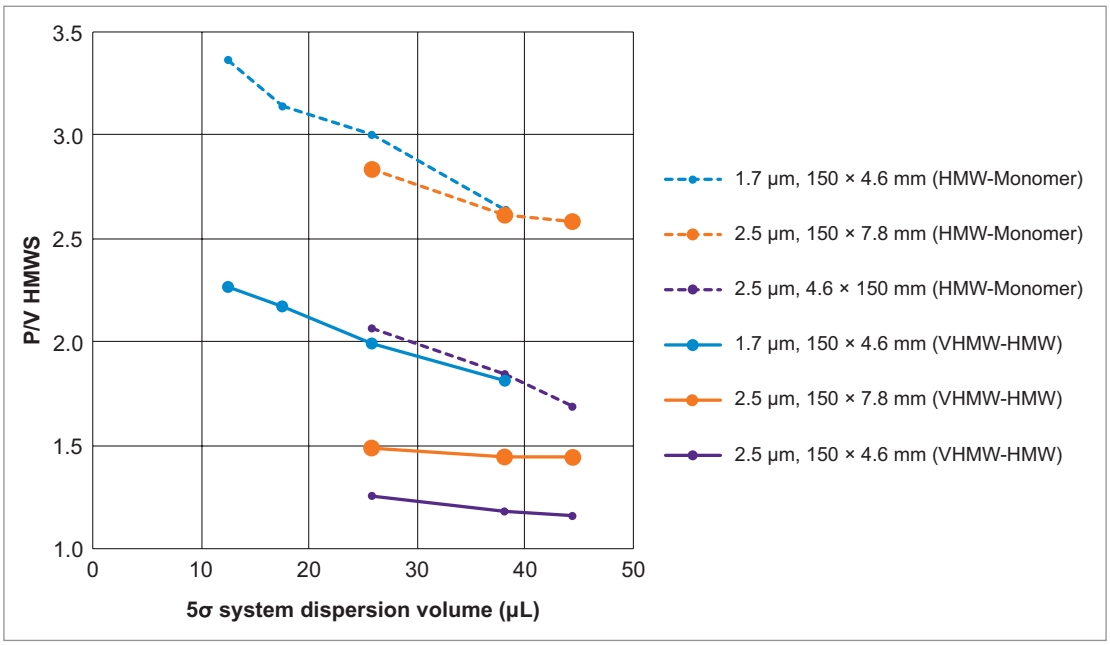

Figure 3. Plotted is a comparison of the peak-to-valley (P/V) measurements at the start (VHMW-HMW) and end (HMW-Monomer) of the HMWS peak as a function of system dispersion for the separation of rituximab on 200 Å pore size BEH SEC particles with diameters of 1.7 µm, 2.5 µm, and 3.5 µm. Linear velocities were constant for all columns as sample loads and flow rates were proportional to the square of the column I.D.

We next compare the performance of the 1.7 µm particle size (4.6 × 150 mm) and 2.5 µm particle size (7.8 × 150 mm) columns at increased flow rates (Figure 4). The flow rate studies were carried out at a $5\sigma_{ec}$ volume of 17.6 µL (UPLC performance) for the 1.7 µm particle size column and a $5\sigma_{ec}$ volume of 38.8 µL (UHPLC/HPLC performance) for the 2.5 µm particle size column. In comparing the HMWS and monomer separation, we observe that as the linear velocity is increased, the P/V value decreases to a lesser extent for the 1.7 µm particle size column. This behavior is a consistent chromatographic theory based on the van Deemter relationship for SEC.[1]

Based on these results, a 1.7 µm particle size (4.6 × 150 mm) column, when used in combination with a low dispersion UPLC system, will provide the higher resolutions and potentially higher sample throughput versus the 2.5 µm particle size (7.8 × 150 mm) column. However, if UHPLC and HPLC systems with larger dispersions are used, the use of the 2.5 µm particle size (7.8 × 150 mm) column can provide comparable separations with an approximate 50% to 75% increase in analysis time depending on flow rate.

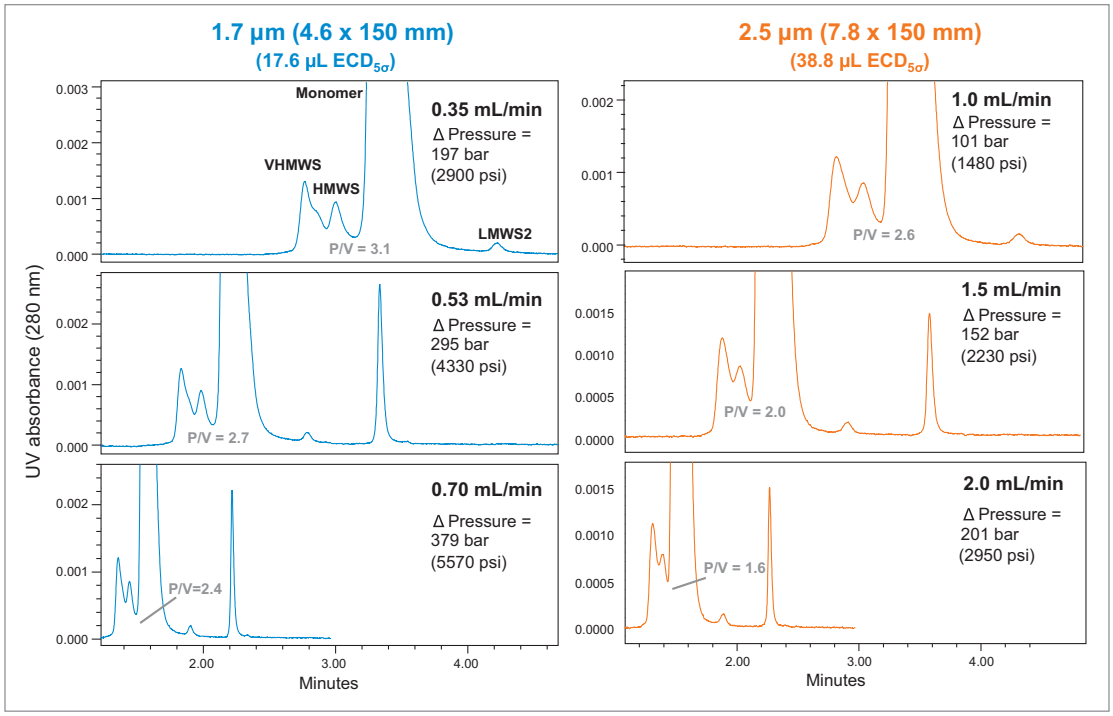

Figure 4. Shown is a comparison of the separation of rituximab aggregates VHMWS and HMWS as a function of flow rate on 200 Å pore size BEH SEC particles with diameters of 1.7 µm and 2.5 µm. Both columns were 150 mm in length, and sample loads and flow rates were proportional to the square of the column I.D. The reported Δ pressure values reflect the pressure drop across the column only. Further experimental details are provided in the text.

COMPARISON OF COMMERCIAL SUB-3-µm SEC COLUMNS

SEC columns with particle sizes between 2 µm and 3 µm provide a significant amount of efficiency for separations that will be performed on UHPLC and HPLC instrumentation. In evaluating the performance of the XBridge Protein BEH SEC, 200 Å, 2.5 µm Column with two other commercially available columns, a physiologically relevant 20 mM phosphate buffered saline (PBS) mobile phase with a pH of 7.2 was employed for the analysis. However, for the analysis of rituximab the NaCl concentration was increased to 400 mM for the XBridge Column and 500 mM for the comparison columns to minimize the increased ionic secondary interactions resulting from operating at a pH above neutral. The XBridge Column and the Competitor A, 300 Å, 2.7 µm column had dimensions of 7.8 × 300 mm and were tested at a $5\sigma_{ec}$ volumes of 38.8 µL while the Competitor B, 250 Å, 2.0 µm column had dimensions of 4.6 × 300 mm and was tested at a $5\sigma_{ec}$ volume of 25.9 µL that is typical of UHPLC systems. All three columns were operated at equivalent linear velocities and the separations were quantitatively assessed based on the plate counts observed for the protein standards. Thyroglobulin monomer plate count is not included as this larger protein eluted near the excluded volume for the XBridge and Competitor B columns, which would have resulted in artificially high plate counts versus the Competitor A column.

A chromatographic comparison and the plate counts determined for the protein standards are shown in Figure 5. We observe that the XBridge Column produces significantly higher plate counts for the IgG, BSA, and myoglobin standards versus the comparison columns, consistent with those peaks being narrower and of greater height. We also observe the impact of pore size where the larger 300 Å pore diameter of the Competitor A column provides clearly improved separation of thyroglobulin and its dimer form versus the other two columns. In contrast, due to their smaller pore diameters, higher resolutions (based on peak width at half-height) between the IgG and BSA standards were observed for the XBridge Column ($Rs_{(HH)}$ = 3.3) and Competitor B column ($Rs_{(HH)}$ = 2.9) versus the Competitor A column ($Rs_{(HH)}$ = 2.6).

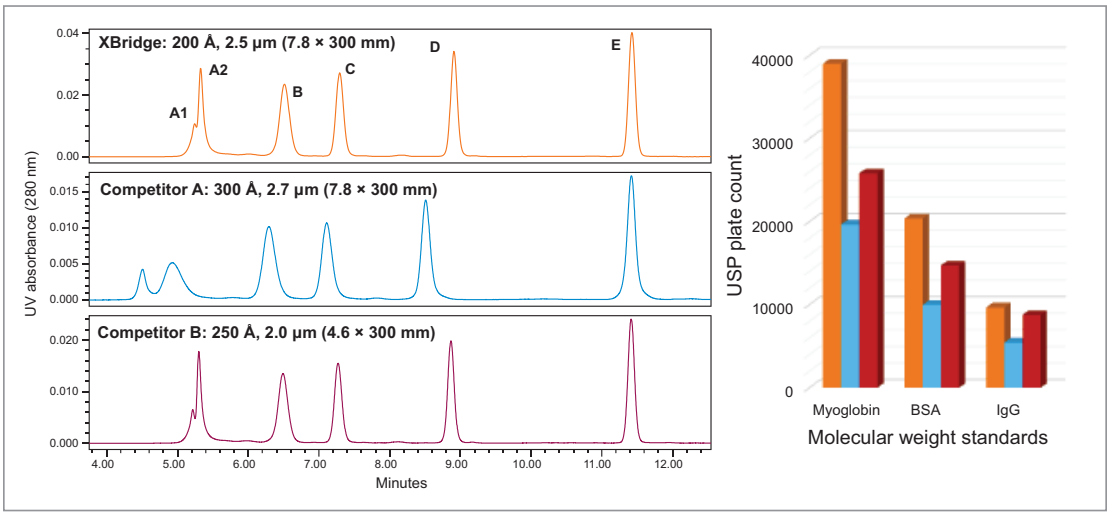

Figure 5. A comparison of the separation of standard proteins on three commercially available sub-3-µm particle size SEC columns. Sample loads and flow rates were proportional to column I.D. The $5\sigma_{ec}$ LC system dispersion volume was 25.9 µL for the Competitor B column and 38.8 µL for the XBridge and Competitor A columns. Mobile phase was 20 mM sodium phosphate, 400 mM NaCl, pH 7.2 for the XBridge Column and it was the same for Competitor A and B columns except for the NaCl concentration, which was increased to 500 mM. Plate counts for specified peaks are based on USP tangent method and reduced plate count is determined by dividing the plate height (column length divided by plate count) by the particle diameter. Peak identifications (shown in top chromatogram) are: thyroglobulin dimer (A1, 1.32 kDa), thyroglobulin monomer (A2, 660 kDa), IgG (B, 150 kDa), BSA (C, 66 kDa), myoglobin (D, 17 kDa), and uracil (E, 112 kDa).

The performance of the three columns for the analysis of rituximab was also evaluated and the chromatograms are presented in Figure 6. In this comparison, we observe comparable separation (P/V) between the HMWS and monomer peaks. Similar differences to those observed for the thyroglobin protein standard are seen for the multimeric VHMWS aggregate, where the larger pore size of the Competitor A column results the inclusion of the majority of the VHMWS aggregates versus the XBridge and Competitor B columns where a significant portion of the VHMWS aggregates are mostly excluded from the pores as indicated by the sharper peak profile of VHMWS on those two columns. Lastly, when we compare the separation for the LMWS1 fragment (100 kDa) we achieve a useful separation on only the XBridge Column. The ability to realize this separation on the XBridge Column is due to its more optimal 200 Å pore diameter and greater efficiency.

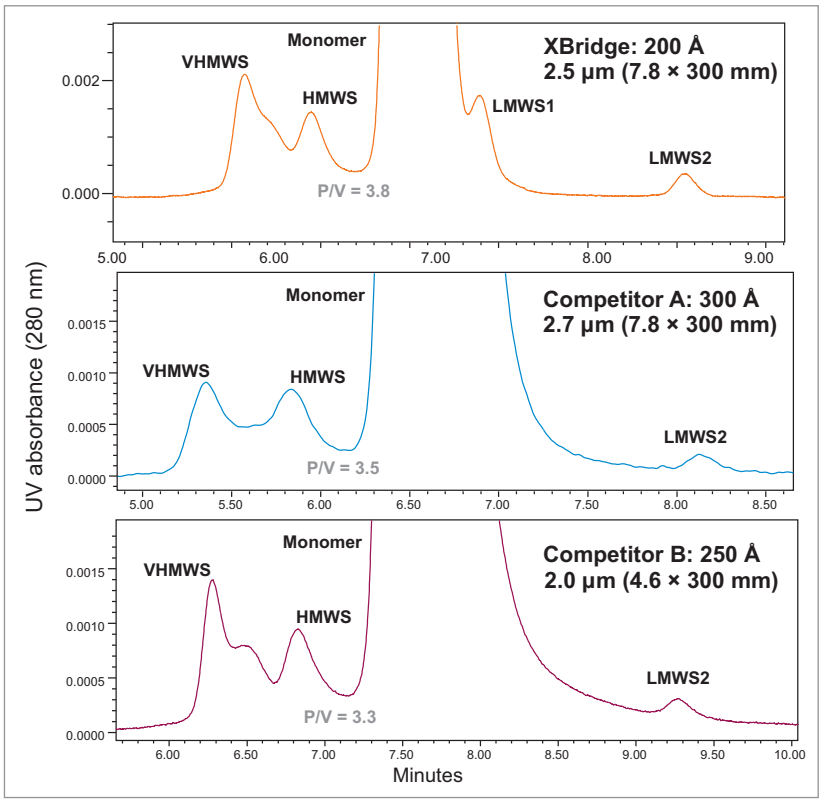

Figure 6. A comparison of the separation of rituximab on three commercially available sub-3-µm particle size SEC columns. Sample loads and flow rates were proportional to column I.D. The $5\sigma_{ec}$ LC system dispersion volume was 25.9 µL for the Competitor B column and 38.8 µL for the XBridge and Competitor A columns. Mobile phase was 20 mM sodium phosphate, 400 mM NaCl, pH 7.2 for the XBridge Column and it was the same for the Competitor A and B columns except for the NaCl concentration, which was increased to 500 mM.

These results demonstrate that the performance of the XBridge Protein BEH SEC, 200 Å, 2.5 µm Column compares favorably with other commercially available SEC columns packed with particle sizes ranging between 2 µm and 3 µm. The XBridge Column produced superior resolution for the 100 kDa IgG fragment (LMWS1) and comparable separation of HMWS and monomer. In addition, in contrast to the Competitor B, 2.0 µm particle size column that is only produced with a 4.6 mm I.D., the 7.8 mm I.D. configuration of the Competitor A and XBridge 2.5 µm Columns can provide this performance on most HPLC system configurations.

CONCLUSIONS

The XBridge Protein BEH SEC, 200 Å, 2.5 µm Columns can provide HMWS and LMWS resolutions equal to those that can be achieved on ACQUITY UPLC Protein BEH SEC, 1.7 µm Columns, however, use of the XBridge Column will result in moderately increased analysis times. The XBridge Column, however, when used in a 7.8 mm I.D. configuration, will provide performance that is far less dependent on the system dispersion and will generate lower back pressures to the extent that both UHPLC and HPLC systems can be employed. Whereas, minimization and control of system dispersion and the ability to operate at significantly higher pressures is required to gain the benefits provided by the more efficient ACQUITY UPLC SEC, 1.7 µm, 4.6 mm I.D. Column.

In addition, as would be predicted, XBridge 2.5 µm Columns provide improved efficiencies in comparison to equivalently sized XBridge 3.5 µm Columns, however, the back pressures generated by the smaller particle size will be greater. The XBridge Protein BEH SEC, 200 Å, 2.5 µm Column also compares favorably with other commercially available SEC columns packed with particle sizes ranging between 2 µm and 3 µm, making this column a worthwhile candidate for new method development, particularly when the resultant method will be run on different LC systems. However, as we have observed in this study, the optimal ionic strength mobile phase for SEC methods on the XBridge Column comprised of organo-silica hybrid particles will be lower than what is required for typical silica-particle-based SEC columns.

To aid in the selection between 1.7 µm, 2.5 µm, and 3.5 µm particle size BEH SEC columns we have summarized key considerations in Table 1. While this study focused on the 200 Å pore size column and the separation of a IgG and its HMW and LMW impurities, this generic guide can also be applied to other proteins as well. Also, the guidance, with regards to column diameter in this table, can be applied to the use of BEH and other SEC columns with other pore and particle sizes.

Table 1. BEH SEC Column Selection Guide.

Particle Size	Considerations
1.7 µm	Can provide the highest separation efficiencies at equivalent analysis times or equivalent separation efficiencies with the shortest analysis times
	Column efficiency advantages increase over larger particles as flow rate is increased to reduce analysis time
	Produces the highest column backpressures
	Typically provided in columns with I.D.s of 4.6 mm or less and thus may likely require LC systems with well-controlled and low dispersions (ACQUITY UPLC H-Class or ACQUITY UPLC I-Class performance) optimized to provide highest efficiencies and reproducible quantitative results for some analytes
2.5 µm	Can provide separation efficiencies equivalent to 1.7 µm particle size on all UPLC, UHPLC, and HPLC systems with a less than two-fold increase in analysis times when used in 7.8 mm I.D. column configurations
	Quantitative results for some analytes will be more independent of LC system dispersion when used in 7.8 mm I.D. column configuration versus 1.7 µm particle size
	Provides the highest separation efficiencies for UHPLC (ACQUITY Arc™ and ACQUITY Arc™ Bio) and HPLC (Alliance™) systems when used in 7.8 mm I.D. column configurations
	Can be used in 4.6 mm I.D. column configurations to conserve sample or mobile phase, however, LC system dispersion may have more impact on results
	Provides more economical analysis versus 1.7 µm particle size
3.5 µm	Produces lower backpressures versus 2.5 µm particle for better compatibility with some HPLC and FPLC systems
	Provides more economical analysis versus 2.5 µm particle size

Acknowledgments

The authors would like to thank Pamela Iraneta and William Warren for their insights and perspectives of the SEC analysis of proteins.

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Size-Exclusion Chromatography Analysis of Adeno-Associated Virus (AAV) Preparations Using a 450 Å Diol-Bonded BEH Column and Fluorescence Detection

Stephan M. Koza and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Optimization of SEC based separations for several AAV serotypes
- Resolution of low valency multimeric and dimeric AAV HMWS and AAV LMWS
- Low AAV sample volume and concentration requirements using intrinsic fluorescence detection to improve the signal-to-noise ratios

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class Bio System with ACQUITY FLR Detector

XBridge™ Protein BEH SEC Columns

BEH450 SEC Protein Standard Mix

Empower™ Software with Auto•Blend™ Plus Technology

KEYWORDS

Size exclusion, adeno-associated virus (AAV), fluorescence detector (FLR)

INTRODUCTION

As the development of gene therapy products accelerates, the need to develop sound and efficient analytical strategies to help guide the development of manufacturing processes and evaluate the quality of clinical adeno-associated virus (AAV) vectors has become more important. Among other critical quality attributes, the levels of potential AAV aggregates represented as high molecular weight species (HMWS) and AAV fragments represented as low molecular weight species (LMWS) may also require monitoring.[1] Here we present optimized size-exclusion chromatography (SEC) methods that can separate soluble AAV self-associated forms and fragments under non-denaturing conditions for several CMV-GFP control AAV serotypes including AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9.[2]

ACQUITY UPLC H-Class Bio System with ACQUITY FLR Detector and XBridge Protein BEH SEC Columns.

SAMPLE DESCRIPTION

Bulk AAV8-Null and AAV8-GFP control samples were supplied by BioReliance (Rockville, MD). The assorted AAV serotype samples were purchased from Vigene Biosciences (Rockville, MD) and were formulated in PBS. BEH450 SEC Protein Standard Mix resuspended in 500 µL PBS containing 10 µg/mL of L-tryptophan (Sigma).

Method conditions

(unless noted otherwise)

LC conditions

Systems:	ACQUITY UPLC H-Class Bio
Detection:	ACQUITY UPLC with FLR Detector (analytical flow cell), unless otherwise noted. Wavelengths: excitation at 280 nm and emission at 350 nm Wyatt microDAWN MALS detector (Santa Barbara, CA) Waters ACQUITY RI Detector
Column:	XBridge Protein BEH SEC, 450 Å, 3.5 µm, 7.8 mm × 300 mm with included BEH450 SEC Protein Standard Mix (p/n: 176003599)
Column temp.:	25 °C
Sample temp.:	6 °C
Mobile phase:	A: 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 6.6 (HCl); B: 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 500 mM KCl, pH 6.6 (HCl); All 0.1 µm sterile filtered, unless otherwise noted
Flow rate:	0.6 mL/min, unless otherwise noted
Sample vials:	Polypropylene 12 × 32 mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 300 µL Volume (p/n: 186002640)

Data management

Chromatography software:	Empower 3

RESULTS AND DISCUSSION

In developing an SEC separation for AAV, it is important to recognize the upper analyte size limitation of SEC, which is below the sub-visible range with a detectable upper particle size diameter limit of approximately 100 nm. As aggregate size exceeds 100 nm, it may be disrupted under SEC conditions or trapped by the frits or packed bed of the column. Therefore, the sizing of entities larger than 100 nm will typically fall in the realm of complementary methods such as dynamic light scattering and nanoparticle tracking analysis (NTA), among others.[3,4] It should also be noted that significant increases in the levels of subvisible aggregate forms may not be concomitant with an increase in lower valency aggregates such as dimer and trimer observed by SEC profiles.

With these considerations, the SEC separation of AAV monomer, dimer, and lower valency multimers in addition to low molecular weight forms was evaluated on a 450 Å (45 nm) average pore-size BEH diol-bonded SEC column. An SEC particle with an average pore size of 450 Å would not generally be considered adequate for the SEC separation of an analyte with a protein molecular weight of nearly 4000 KDa and a ssDNA molecular weight of approximately 1500 KDa. However, due to the compact structure of the AAV with a diameter of 25 nm, it was predicted that this high efficiency SEC particle with a large pore volume could provide the needed separation with the larger than average size pores providing most of the accessible pore volume for the HMWS. Additionally, while initial method development was undertaken on a 4.6 mm I.D. column packed with 2.5 µm diameter particles, a larger 3.5 µm particle size, 7.8 mm I.D. column was selected for the final method to minimize potential sample sieving effects.

Intrinsic protein fluorescence detection was also employed to provide maximum sensitivity given the low concentrations of many AAV formulated samples and the small injection volumes desired for SEC due to the low product yields of the manufacturing processes being developed. Intrinsic protein fluorescence also has an added advantage that the response factor is not as greatly impacted by changes in the DNA content of the AAV in comparison to UV absorbance.[5]

Method development initially used an AAV8-Null control sample due to availability. A null control sample is an AAV capsid that does not contain DNA. The separation observed using a phosphate buffered saline mobile phase (PBS, 10 mM sodium phosphate with 150 mM NaCl) was evaluated using a Wyatt microDAWN Multi-Angle Light Scattering detector (MALS) and an ACQUITY RI (refractive index) Detector (Figure 1). The SEC-MALS data confirmed that separation between the dimeric and monomeric AAV forms was observed. Additionally, putative multimeric forms preceding dimer were also observed by FLR but could not be assigned molecular weights by MALS due to their low abundance.

A single-factor optimization scheme for the mobile phase starting with PBS was employed to maximize aggregate recovery and decrease peak tailing of the monomeric capsid. Wherein, the evaluation of pH while holding ionic strength constant (150 mM NaCl), followed by evaluating ionic strength at the observed optimal pH was accomplished using Auto•Blend Plus Technology.[6] Finally, salt types (NaCl, KCl, and sodium perchlorate) and other additives (arginine and isopropanol) were evaluated. Ultimately, a mobile phase consisting of 20 mM sodium phosphate, pH 6.6, with 150 mM KCl was found to provide a functional separation for the AAV8-Null control sample. This single-factor optimization approach was opted for in lieu of a more rigorous full-factorial method development to conserve sample. However, it should be noted that using a full-factorial optimization is generally preferred to determine optimal method conditions since the use of a single-factor optimization scheme may result in settling on a local optimum. In deploying this method for the analysis of DNA containing AAV serotypes (CMV-GFP), it was observed that the optimal concentration of KCl required to maximize the recovery of dimer and multimer varied by serotype. The finalized method conditions and chromatographic profiles for serotypes AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9 are presented in Figure 2.

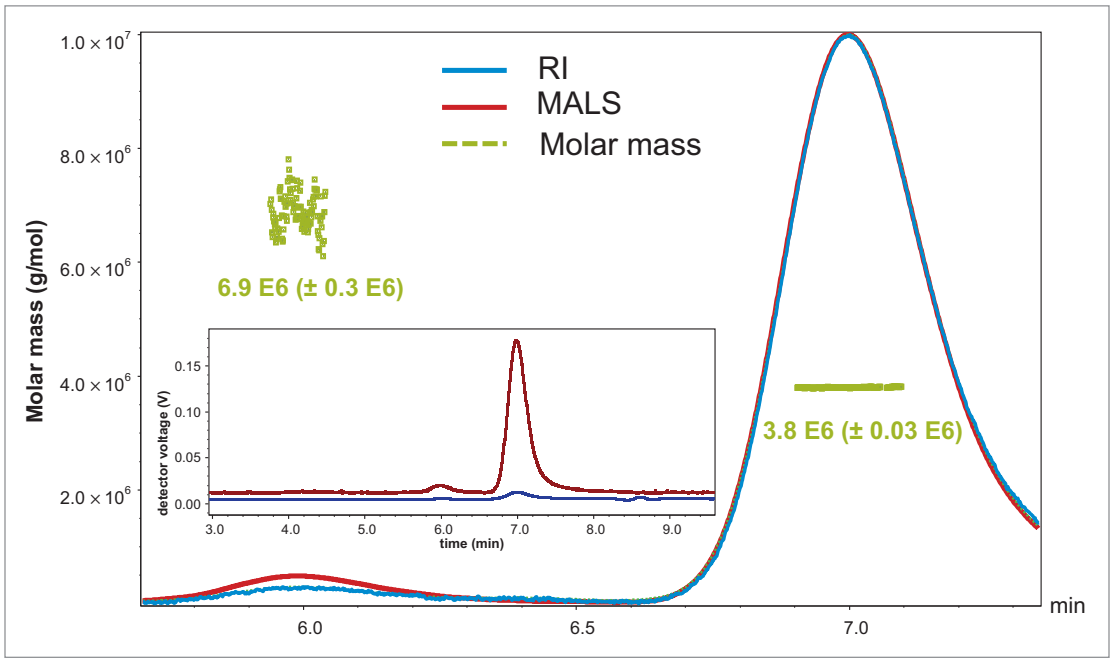

Figure 1. SEC-MALS of AAV8-Null sample (~1 × 10^12 capsids/mL) using refractive index (RI) for concentration measurement are shown. The MALS (red) and RI (blue) signals are normalized and the average and distribution of determined molar masses (green) were determined using Wyatt Astra (v. 7.3.1.9) based on a dn/dc of 0.185 and using a "sphere" model for the icosahedral AAV. Additional experimental details are provided in the text.

We observe that the peak shapes for both the dimer HMWS and monomeric AAV forms are symmetrical and return to baseline appropriately. In addition, detectable levels of HMWS with retention times that are consistent with multimeric AAV forms were observed for several of the serotypes. Significant amounts of LMW forms were only observed in the AAV9 serotype and to a much lower extent in AAV6.

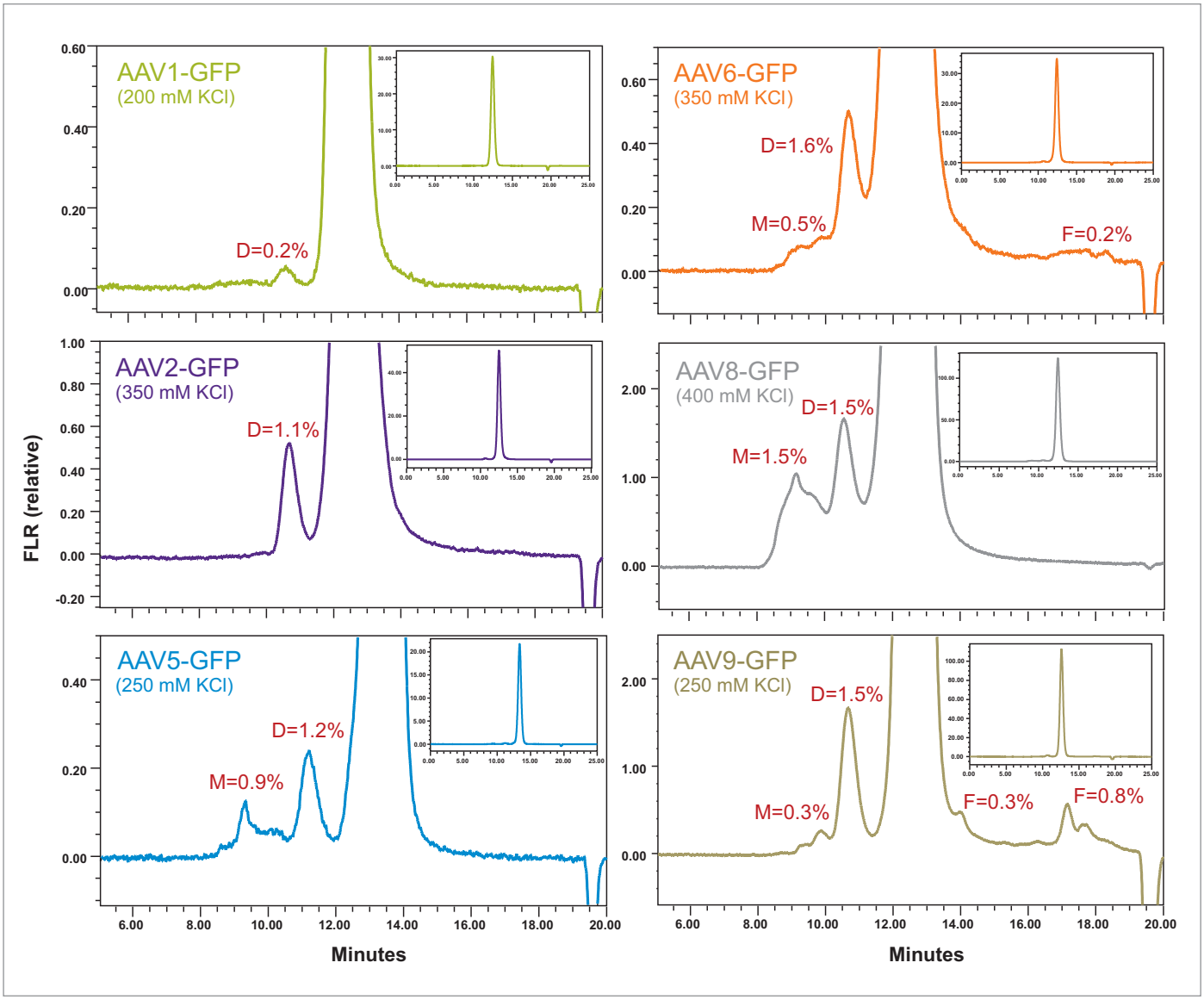

Figure 2. Shown are the SEC separations of a series of AAV serotype control samples containing ssDNA coding for green fluorescent protein (GFP). The peak percentages for dimer (D), multimer (M), and fragments (F) are provided. The chromatogram baselines are zoomed approximately 50x versus the full-scale chromatogram shown in the inset. Additional experimental details are provided in the text. Sample concentrations were approximately 1×10^{12} to 5×10^{12} capsids/mL.

As previously noted, intrinsic protein fluorescence detection was used for this method to enhance the sensitivity for low abundance high molecular weight and low molecular weight forms. However, when evaluating the SEC protein standard mix, the small molecule total permeation volume marker (uracil) is not observed due to low quantum yield. Being able to track the total permeation volume of an SEC column is valuable in SEC method development since in this case any FLR signal persisting after the total permeation volume is indicative of protein that is being excessively retained on the column. Therefore, it is recommended that L-tryptophan at 10 µg/mL be added to the standard to mark the total permeation volume since this amino acid is the primary fluorophore of the protein that is detected when using intrinsic protein fluorescence (Figure 3).

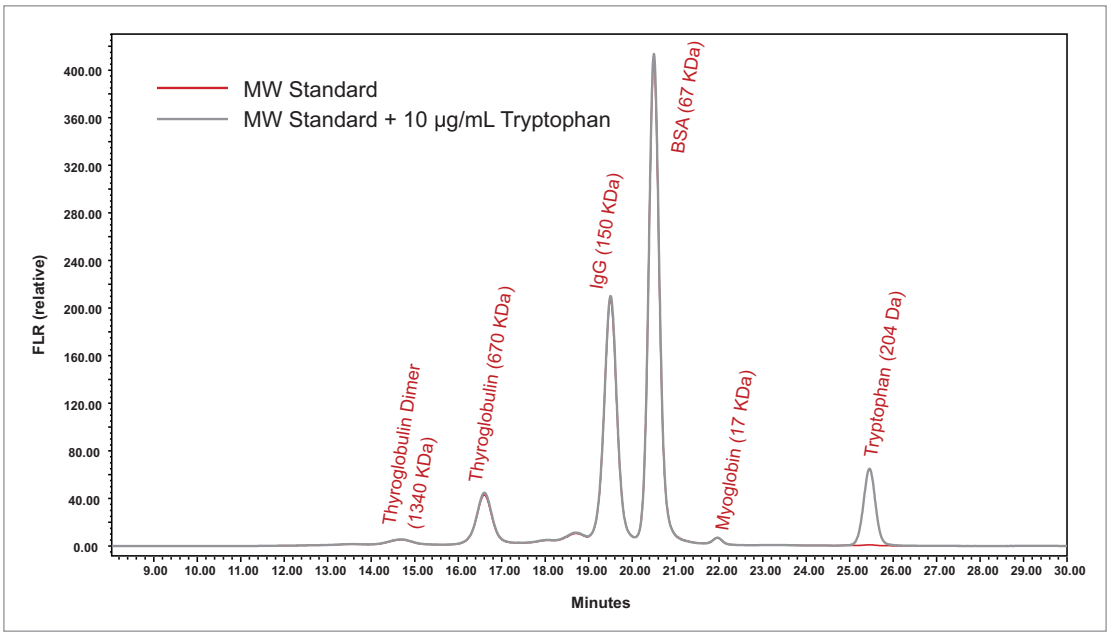

Figure 3. Shown are SEC separations of the Waters BEH450 SEC Protein Standard Mix. The standard was reconstituted with 500 µL of PBS containing 10 µg/mL L-tryptophan and one was injected on column at a flow rate of 0.5 mL/min. Additional experimental details are provided in the text.

CONCLUSIONS

A BEH SEC column with an average pore size of 450 Å and a particle diameter of 3.5 µm was demonstrated to be effective in the separation of AAV monomers from their HMW dimers, lower valency multimers, and LMW fragments. The minimal amount of ionic strength (KCl) required for optimal peak shape and recovery varied by serotype and these levels are reported for serotypes AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9. While a 450 Å average pore size may have been considered less than ideal for this separation, the results demonstrate that the high pore volume and efficiencies of these particles enable an effective separation. It is proposed that the AAV HMWS separation is driven primarily through the distribution of pore sizes larger than 450 Å. Additionally, intrinsic protein fluorescence was used to extend the sensitivity of the method for the AAV samples tested.

Acknowledgments

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Rapid AAV Concentration Determination Using Size-Exclusion Chromatography with Fluorescence and UV Dual Detection

Stephan M. Koza, Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Rapid and high throughput AAV capsid concentration determination
- Measurements down to 2×10^{11} capsids/mL or lower
- Approximation of AAV ssDNA E/F ratio

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class PLUS Bio System

ACQUITY UPLC Protein BEH SEC Guard Columns

ACQUITY UPLC FLR Detector

ACQUITY UPLC PDA Detector

Empower™ Chromatography Data System

KEYWORDS

Size-exclusion chromatography, adeno-associated virus, AAV

INTRODUCTION

Determining the concentration of capsid in a preparation of adeno-associated virus (AAV) designed for gene therapy treatments is essential in both process development and product quality analyses. Currently ELISA methods are typically used for capsid quantification. These methods are quite reliable, however they may take several hours to generate results, require well characterized reagents, and generally have lower precision (~20% CV at limits of quantification) than chromatographic methods.[1,2] Methods have also been introduced that use the differential ultraviolet absorbance (UV) of the capsid proteins and ssDNA of the AAV under denaturing conditions.[3] This approach is generally amenable to high throughput testing with minimal analysis times but may require significant sample volumes (100 µL) and will be susceptible to the presence of interfering chromophores. Additionally, the use of non-denaturing size-exclusion chromatography (SEC) with UV absorbance detection has also been reported (SEC-UV).[4] While linear calibration curves are demonstrated for this approach, the method would be limited to samples with little variation in ssDNA content.

In a more recent publication, the relative quantification of AAV capsid that does not contain ssDNA (empty) was evaluated using anion-exchange chromatography with intrinsic protein fluorescence (FLR) detection.[5] In this study, it was shown that the relative change of intrinsic protein fluorescence intensity for empty capsid versus those AAV that contain the requisite ssDNA was several fold lower than was observed for UV absorbance at 280 nm (A280) or 260 nm (A260). This potentially makes FLR better suited than A280 for AAV quantification since errors in the estimate of ssDNA content of the capsid in the sample will have significantly less impact on the final concentration determination. Other advantages of FLR include greater selectivity for the protein component versus other sample components including surfactants and DNA, and enhanced sensitivity for low concentration samples.

Two challenges presented by AAV as an analyte relative to UV absorbance and FLR optical detection methods are light scattering effects due to AAV particle size and the impact that can have on the UV absorbance and FLR quantum yields. As a result, the empirical UV absorbance and FLR response for intact AAV will be most effectively determined when other solution components in the AAV sample remain constant.

In addition, the level of particulates in the samples with sizes approaching or exceeding that of the UV wavelengths being employed should be minimized. In traditional optical methods this is generally accomplished using 0.2 µm and smaller filters to further purify the sample prior to spectral analysis.[6]

Here we present a rapid SEC method (two minutes) with FLR detection for the determination of AAV capsid concentration (Cp/mL). An SEC approach provides a consistent solution in which optical measurement of an analyte is undertaken and may provide additional removal of interfering components from the sample. In this method, the FLR signal response may be corrected for ssDNA content of the capsid in the sample as determined by a separate analysis such as anion-exchange chromatography (720006825EN). Alternatively, as demonstrated in this study the ssDNA content may be estimated from the ratio of UV absorbance based on peak areas at 260 nm and 280 nm (A260/A280) measured by a UV/Vis PDA Detector in tandem with and positioned prior to the FLR Detector (SEC-UV-FLR).

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EXPERIMENTAL

Sample description

AAV8 capsids without ssDNA (AAV8-Empty) or with Green Fluorescent Protein genes (AAV8-CMV-GFP) were injected directly onto the SEC column. The concentration of the AAV8-Null and AAV8-CMV-GFP samples were estimated to be approximately 1.67×10^{12} and 2.48×10^{12} capsid/mL (Cp/mL), respectively.

System:	ACQUITY UPLC H-Class PLUS Bio
Detection:	Fluorescence detector: excitation: 280 nm, emission: 350 nm; (10 points/sec) and ACQUITY UPLC PDA Detector with 5 mm titanium flow cell, 280 nm and 260 nm; (10 points/sec)
Vials:	Polypropylene 12 × 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)
Guard column:	ACQUITY UPLC Protein BEH SEC, 125 Å, 1.7 µm, 4.6 × 30 mm (p/n: 186006504)
Column temp.:	25 °C
Sample temp.:	10 °C
Injection volume:	1.0 µL
Flow rate:	0.20 mL/min
Mobile phase:	10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 6.6 (HCl), 200 mM KCl, pH 6.6 (HCl), 0.1 µm sterile filtered

Data management

Chromatography software:	Empower 3

RESULTS AND DISCUSSION

This study aimed to develop a chromatographic method that would allow for the rapid determination of the capsid content of an AAV sample with minimal sample preparation, limited sample consumption, and a practical lower limit of quantification. The SEC-UV-FLR method presented here employs a separation on a Protein BEH SEC Guard Column. This short bed-length column (30 mm) is packed with 1.7 µm diameter diol-bonded BEH particles with a 125 Å average pore size. An SEC column is used to buffer exchange the AAV capsid into the mobile phase, and by selecting a short column that excludes AAV from most of the pore volume, the dispersion of the AAV is minimized. However, if the AAV samples also contain significant amounts of interferences (e.g., proteins, DNA) that could co-elute with AAV then a longer column with a larger pore size may be required. Both a UV absorbance detector (UV) and fluorescence detector (FLR) were used in series. The FLR was positioned after the UV to accommodate the lower back pressure limits of the FLR.

The concentration of the AAV8-Null (without ssDNA) control sample (referred to hereafter as AAV-Empty) was assigned as 1.67×10^{12} capsid/mL (Cp/mL) based on ELISA measurement as reported by the supplier and the measured DNA-containing capsid levels were approximately 0.5% as determined by electron microscopy and 1.5% as determined by charge-detection mass spectrometry (CDMS). The concentration of the AAV8-CMV-GFP sample (AAV-Full) was estimated as 2.48×10^{12} Cp/mL based on the relative peak areas of a CDMS spectrum using a 50:50 equal volume mixture of the AAV-Empty and AAV-Full samples. The AAV-Full sample contained 2.2% empty capsids based on CDMS analysis. The AAV-Full sample was serially diluted using the AAV-Empty sample to generate a set of mixtures with the mole fractions of AAV-Full (X_{Full}) and capsid concentrations shown in Table 1. The predicted X_{Full} values of the mixtures also compared favorably to those observed by CDMS with a correlation of 0.996 and a slope of 1.008 for a linear fit forced through the origin (data not shown). It should be noted, however, that while the use of these relative concentration values is adequate to demonstrate the principles of this methodology a more rigorous assessment of the capsid concentrations of the AAV-Empty and AAV-Full standards should be considered when greater method accuracy is required.

Dilution (Full:Empty)	Predicted X_{Full}	Capsid concentration (Cp/mL)	Relative capsid concentration
100:0	0.9788	2.40E+12	1.00
87.5:12.5	0.8916	2.31E+12	0.963
75:25	0.7972	2.22E+12	0.925
50:50	0.5832	2.04E+12	0.850
25:75	0.327	1.85E+12	0.771
0:100	0.0148	1.67E+12	0.696

Table 1. AAV8 serial dilutions (AAV-Full:AAV-Empty), X_{Full} values and sample concentrations (Cp/mL).

The SEC FLR and SEC-UV at 260 nm and 280 nm (SEC-A260 and SEC-A280) peak areas were determined in duplicate for the set of AAV-Full and AAV-Empty sample serial dilutions. In the overlays of representative chromatograms (Figure 1), we observe that the change in response for the FLR signal is significantly lower than the changes observed in the UV absorbances as X_{Full} of AAV8 is varied from 1 to 0. This difference is predominately the result of the strong UV absorbance of DNA within the full capsid at 260 nm. The SEC-FLR and SEC-UV peak areas of the mixtures were then normalized for concentration by dividing the peak areas by their respective relative concentrations (Table 1). The normalized peak areas were then plotted against X_{Full} (Figure 2). Based on the fitted linear equations presented in Figure 2 the peak areas predicted for X_{Full} values of 1 and 0 ($Area_{X_{Full}=1}$ *and* $Area_{X_{Full}=0}$) were extrapolated and AAV8-Full to AAV8-Empty response factors ($R_{F/E}$) of 6.81 for A260 ($R_{F/E,260}$), 2.98 for A280 ($R_{F/E,280}$), and 0.875 for FLR ($R_{F/E,FLR}$) were calculated using Equation 1 below. The values of $R_{F/E,260}$, $R_{F/E,280}$, and $R_{F/E,FLR}$ are expected to vary with the composition of the ssDNA and to a lesser extent with AAV serotype.

Equation 1.

$$R_{F/E} = \frac{Area_{X_{Full}=1}}{Area_{X_{Full}=0}}$$

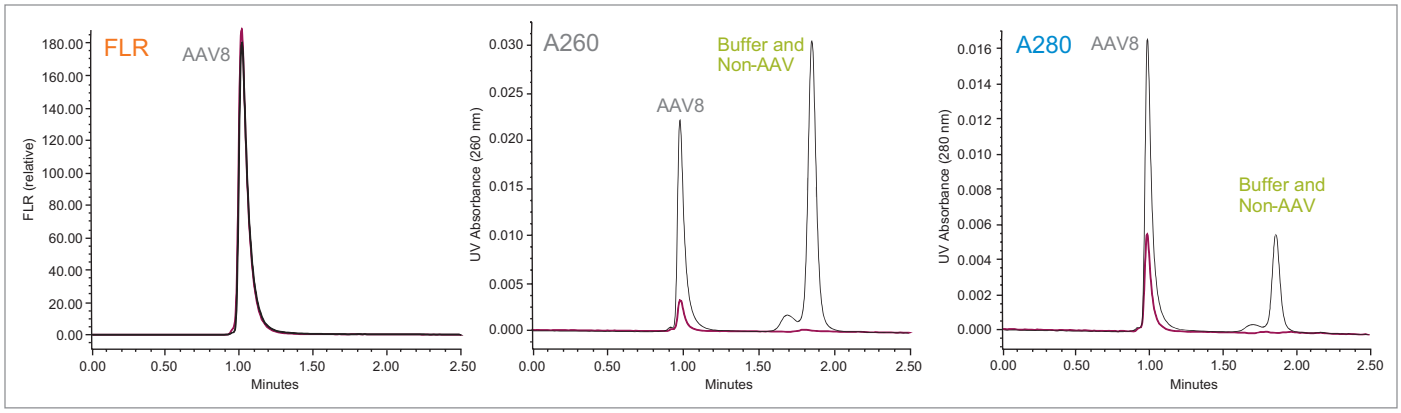

Figure 1. A comparison of the SEC fluorescence and UV absorbance responses for a series of AAV8 samples in which the mole fraction of AAV8-Full ranges from approximately 1.0 (black) to 0.0 (red). Experimental procedures provided in text.

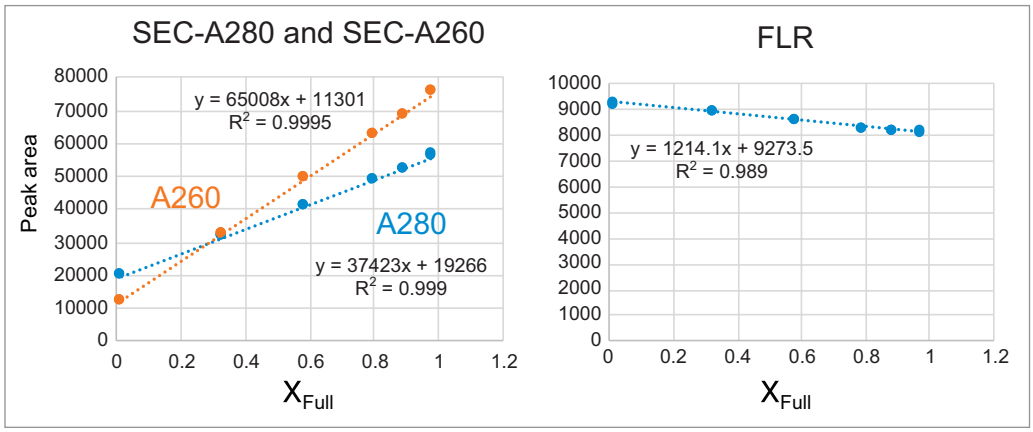

Figure 2. Shown are the changes in the SEC-UV (280 nm and 260 nm) and SEC-FLR peak areas for a series of AAV8 samples (n = 2) where the mole fraction of AAV8-Full (X_{Full}) ranges from approximately 1.0 to 0.0. Peak areas are normalized to sample concentration (Cp/mL). Experimental procedures provided in text.

In routine use, $Area_{X_{Full}}$=1 and $Area_{X_{Full}}$=0 may be directly calculated using the peak areas of the AAV-Full and AAV-Empty control samples for which both Cp/mL and X_{Full} have been determined. Additionally, since $R_{F/E,}$FLR has a value closer to 1 versus $R_{F/E,}$280 and $R_{F/E,}$260, a correction for FLR response may not be necessary for SEC-FLR capsid concentration estimates in which X_{Full} of the samples does not vary significantly or less precise capsid concentration determinations are acceptable.

In order to apply $R_{F/E}$, we will need to know X_{Full} of the sample ($X_{Full, Sample}$). While $X_{Full, Sample}$ may be determined through methods such as electron microscopy or anion exchange chromatography,[5] it may be possible to use the ratio of the SEC-A260 and SEC-A280 peak areas ($Area_{A260}/Area_{A280}$) to approximate X_{Full} for the sample being evaluated in an SEC-UV-FLR or SEC-UV experiment. The UV absorbance-based estimate of $X_{Full, Sample}$ is reliant on the standards used and it is important to ensure measurements are made under well controlled experimental conditions since the absorbance values can deviate significantly with changes to the buffer composition.

In this study, a photodiode array UV-Vis detector (PDA) was used in series with the FLR Detector. FLR Detector flow cells are typically less tolerant of higher pressures and should generally be positioned after the UV detector. The advantages of determining the A260/A280 UV absorbance ratio in this manner is that additional sample is not required, and any potentially interfering chromophores or fluorophores in the samples may be separated from the AAV capsid on the SEC column if they are different in size. A PDA Detector was preferred over a dual-wavelength tunable UV-Vis detector (TUV) for this SEC-UV-FLR method due to its significantly higher sampling rate given the peak widths observed (~12 seconds). If a longer SEC column is used or the flow rate is reduced a TUV Detector could be used provided that approximately 40 or more points are collected across the peak.

The relationship between the observed peak area ratio of the sample mixtures and X_{Full} for AAV8 shows a non-linear response curve (Figure 3). The predicted peak area ratios for the sample mixtures are depicted by the dashed line in Figure 3 and can be calculated using the relationship:

Equation 2.

$$\frac{Area_{A260,Sample}}{Area_{A280,Sample}} = \frac{X_{Empty,Sample} * Area_{A260,X_{Full}=0} + X_{Full,Sample} * Area_{A260,X_{Full}=1}}{X_{Empty,Sample} * Area_{A280,X_{Full}=0} + X_{Full,Sample} * Area_{A280,X_{Full}=1}}$$

Where $X_{Empty, Sample}$ and $X_{Full, Sample}$ are the mole fractions of AAV-Empty and AAV-Full in the sample. $Area_{A260, X_{Full}=0}$, $Area_{A260, X_{Full}=1}$, $Area_{A260, X_{Full}=0}$, and $Area_{A260, X_{Full}=1}$ are extrapolated from the fitted linear equation for SEC-A280 and SEC-A260 presented in Figure 2, as previously described.

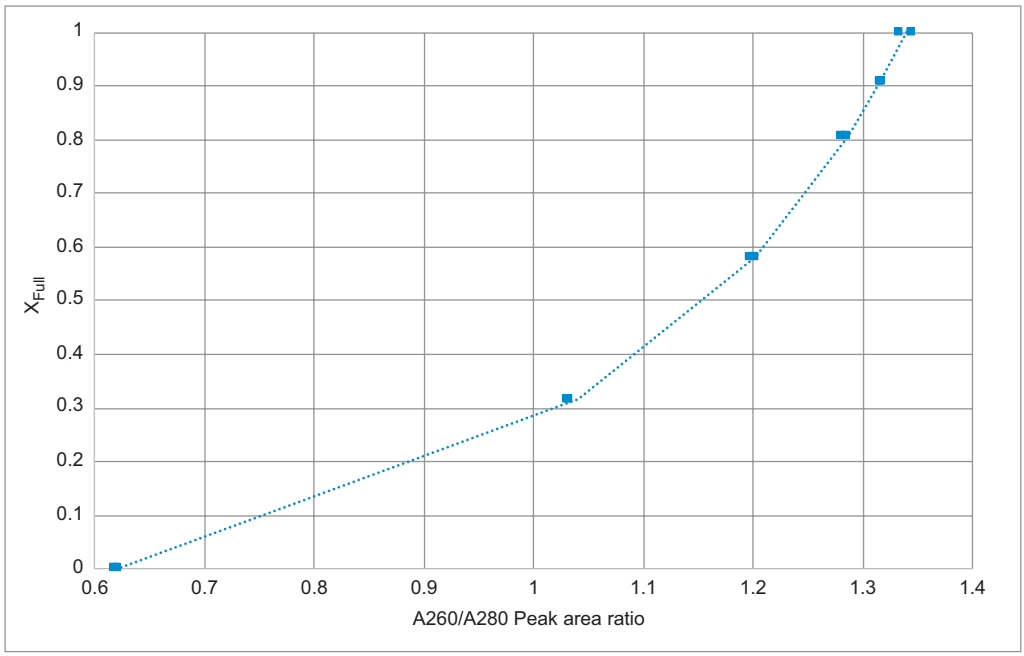

Figure 3. Shown are the change in the SEC 260 nm and 280 nm UV absorbance peak area ratios (n = 2) for a series of AAV8 samples in which the mole fraction of AAV8-Full (X_{Full}) ranges from approximately 1.0 to 0.0. The dashed line highlights the predicted A260/A280 ratios. Experimental procedures provided in text.

To determine X_{Full} of a sample using the SEC-UV peak area ratio measured for the test sample ($Area_{A260, Sample}/Area_{A280, Sample}$) the relationship in Equation 2 may be rearranged to Equation 3. For those interested in mathematic detail, Equation 3 in its reduced form is an asymptotic non-linear regression between X_{Full}, Sample and ($Area_{A260, Sample}/Area_{A280, Sample}$) of the form $y = a/x + bx + c$.

Equation 3.

$$X_{Full,Sample} = \frac{Area_{A260,X_{Full}=0} - \left(\frac{Area_{A260,Sample}}{Area_{A280,Sample}}\right)}{Area_{A260,X_{Full}=0} - Area_{A260,X_{Full}=1} - \left(\frac{Area_{A260,\ Sample}}{Area_{A280,\ Sample}}\right) * Area_{A280,X_{Full}=0} + \left(\frac{Area_{A260,Sample}}{Area_{A280,Sample}}\right) * Area_{A280,X_{Full}=1}}$$

Equation 3 is independent of the concentration of the AAV samples being tested. Moreover, once $Area_{A260}$, $X_{Full} = 0$, $Area_{A260}$, $X_{Full} = 1$, $Area_{A260}$, $X_{Full} = 0$, and $Area_{A260}$, $X_{Full} = 1$ are determined on an LC system they may be considered as constants in further measurements. The resulting values of X_{Full}, Sample, which were calculated from the measured values of $Area_{A260, Sample}/Area_{A280, Sample}$ and Equation 3, correlated strongly with the predicted values ($R^2 = 0.9995$, slope = 0.991, intercept = 0.000, correlation plot not shown). As an additional assessment of the methodology, the measured values were also found to correlate well with those obtained by CDMS (Figure 4).

These results demonstrate that the precision and accuracy of this method for the measurement of X_{Full}, Sample are acceptable for the estimation of FLR and UV response factors and may even be adequate for estimates of X_{Full}, Sample for process intermediate samples with relatively high abundances of empty capsid. However, due to greater slope of the response curve as X_{Full} approaches one (Figure 3) this method will likely deliver less precision and accuracy for the determination of X_{Full}, Sample in high purity samples.

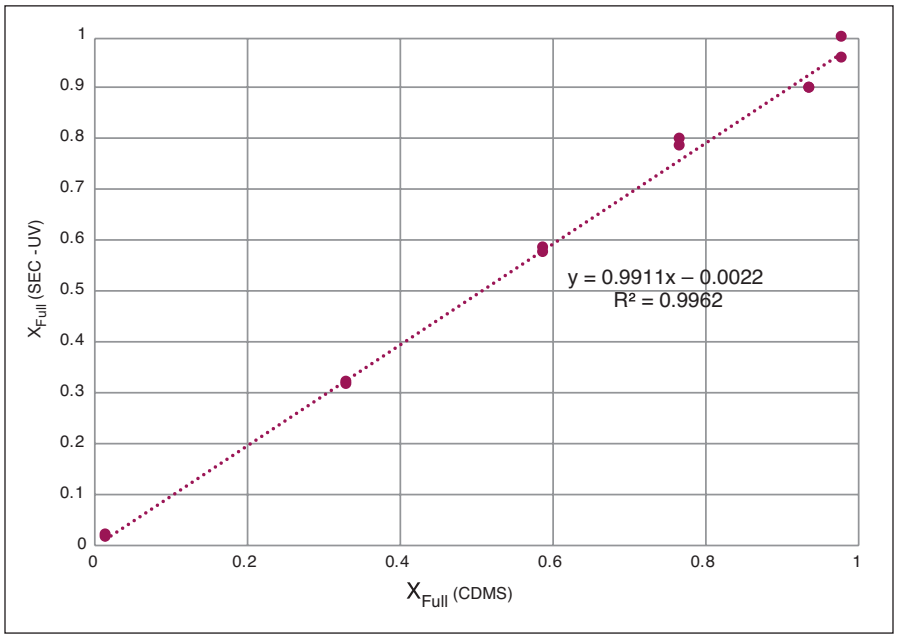

Figure 4. The correlation for the determination of the mole fraction of AAV8-Full (X_{Full}) as determined by SEC 260 nm and 280 nm UV absorbance peak area ratios (n = 2) and CDMS for a series of AAV8 samples. Experimental procedures provided in text.

Once X_{Full}, Sample is determined it can then be used along with RF/E (Equation 1) to normalize the SEC-FLR or SEC-UV peak area of a sample or standard ($Area_{Sample, Norm}$) to account for the difference in response between an empty and full capsid. This peak area normalization will also be similarly applied to the concentration calibration standards. In this example, we have arbitrarily elected to normalize peak area to the value predicted if $X_{Full, Sample}$ were 1. To derive the normalization equation we can define the test sample (or standard) peak area ($Area_{Sample, Meas}$) as a combination of the maximum peak area contributions of the full and empty capsids, $Area_{Sample, XFull} = 1$ and $Area_{Sample, XFull} = 0$, multiplied by their respective mole fractions.

Equation 4.

$$Area_{Sample,Meas} = Area_{Sample,X_{Full}=1} * X_{Full,Sample} + Area_{X_{Full,Sample}=0} * X_{Empty,Sample}$$

By substituting in Equation 1 and the relationship $X_{Full,sample} + X_{Empty,sample} = 1$, Equation 4 can be rearranged to yield Equation 5, which can be used to normalize the response of a sample or standard for its measured value of X_{Full}.

Equation 5.

$$Area_{Sample,Norm} = \frac{Area_{Sample,Meas}}{\left(X_{Full,Sample} + \frac{(1 - X_{Full,Sample})}{R_{F/E}}\right)}$$

As an initial test of the methodology described above, the data set from the samples described in Table 1 were evaluated. In this case, the A280 UV absorbance and FLR peak area data for the dilution series of samples were evaluated without normalization for the known concentration differences with the intent of determining the capability of this method to predict the Cp/mL values of the serially diluted samples. For this study the two undiluted samples with X_{Full} values of 0.0148 and 0.9788 and respective concentrations of 1.67×10^{12} Cp/mL and 2.48 Cp/mL (Table 1) were used to apply response factor corrections using Equations 3 and 5, and to define the concentration calibration curves based on SEC-A280 and SEC-FLR.

The determined sample concentrations were then compared to the predicted values (Figure 5). The correlation was greater, and the slope of the curve was closer to one for the SEC-FLR data results versus those observed for SEC-A280. Additionally, the variance between the duplicate measurements of concentration was lower for the SEC-FLR results (0.67% average difference) versus the SEC-A280 concentration measurements (1.27% average difference) despite the variance being lower for the actual SEC-A280 peak areas (0.43% average difference) versus the SEC-FLR peak areas (0.63% average difference). Since the same values of X_{Full} are used in the SEC-FLR and SEC-A280 calculations, the more precise analytical results observed using SEC-FLR are predominantly due to the value of $R_{F/E}$, FLR being closer to one (0.898) than the value of $R_{F/E, A280}$ (2.98) since a response factor closer to one minimizes the impact of the variance in the determination of X_{Full}. The additional impact of the greater variance in the determination of X_{Full} for samples with low levels of empty capsid content, as noted previously, can also be observed for these results.

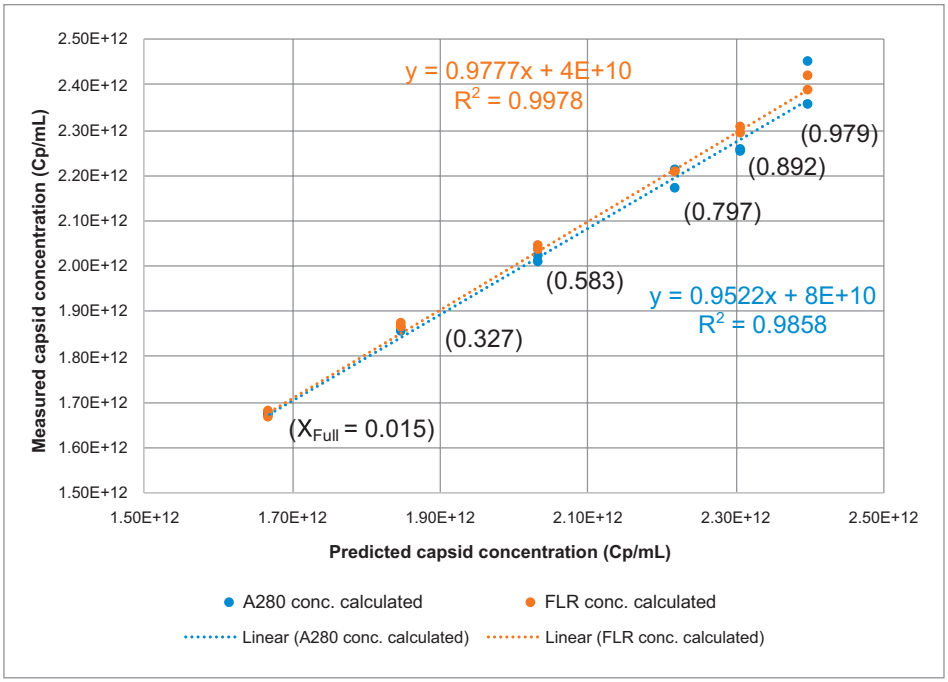

Figure 5. Comparison of the SEC-FLR-UV and SEC-UV based determinations of capsid concentrations. In both cases the determined concentration is corrected by a response factor based on X_{Full} calculated from on-line UV A260 and A280 measurements as described in the text. The capsid concentration of capsid ranges from approximately 1.7 × 10^{12} Cp/mL to 2.4 x 10^{12} Cp/mL and the X_{Full} values (shown in parentheses) of the samples ranged from approximately 0 to 1, respectively. Experimental procedures provided in text.

To evaluate an extended concentration response curve a sample with an X_{Full} value of approximately 0.55 and an estimated concentration of 2.07 × 10^{12} Cp/mL was serially diluted to yield samples of 1.04 x 10^{12}, 5.19 × 10^{11}, and 2.59 × 10^{11} Cp/mL. These samples were analyzed on an LC system with both an FLR and UV detector in line (SEC-UV-FLR). A comparison of the UV absorbance (260 nm and 280 nm) and FLR chromatograms for the lowest concentration sample (2.59 x 10^{11} Cp/mL) is presented in Figure 6. Here we observe that adequate signal is observed for all three optical channels with the FLR Detector having significantly higher signal-to-noise (~10x) versus the UV absorbance channels indicating that the quantitative limits may likely be lower for FLR.

Figure 7 shows the FLR response curves for the SEC-FLR and SEC-A280 peak areas that have been normalized by their respective response factors ($R_{E/F, FLR}$ and $R_{E/F, A280}$). While we expect the diluted samples to have equivalent values of X_{Full}, the correction was applied to interrogate the reproducibility of the SEC-UV-FLR and SEC-UV methods. A useful linear fit is observed for the SEC-FLR and SEC-A280 calibration curves with correlation coefficients of 0.9985 and 0.9989, respectively. These results indicate that when the levels of empty capsid in AAV samples are higher and more consistent, the SEC-A280 method, with applied X_{Full} response correction, may provide comparable analytical performance in comparison to the SEC-FLR method.

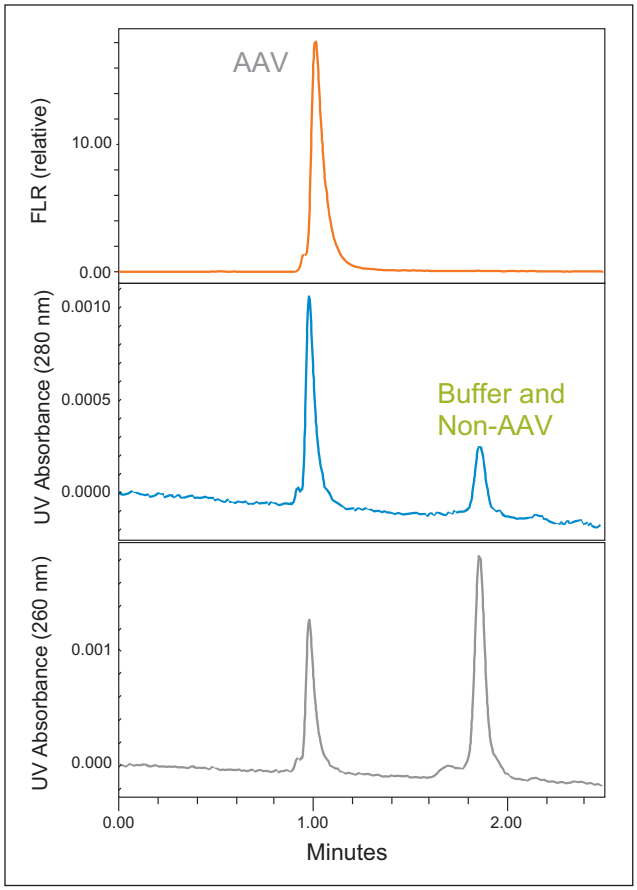

Figure 6. Comparison of the SEC-UV absorbance responses for a series of AAV8 samples with an approximate capsid concentration of 2.59 × 10^{11} Cp/mL. Experimental procedures provided in text.

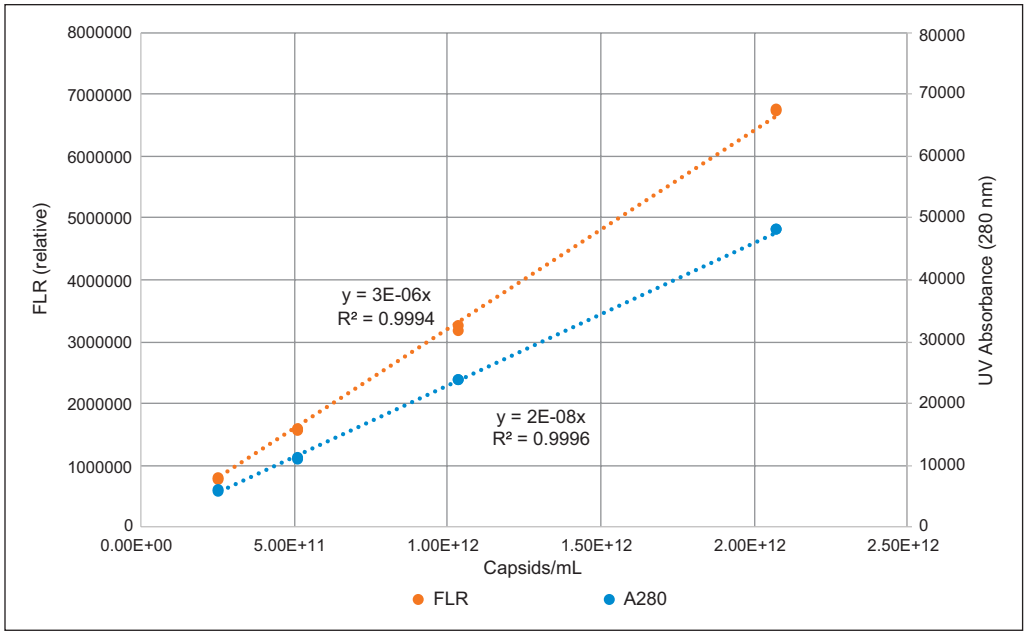

Figure 7. The change in the SEC-FLR-UV and SEC-UV peak area corrected by a response factor based on X_{Full} calculated from on-line UV A260 and A280 measurements as described in the text. The concentration of capsid ranges from approximately 2 × 10^{11} Cp/mL to 1.6 × 10^{12} Cp/mL. Experimental procedures provided in text.

CONCLUSIONS

The use of an efficiently packed SEC guard column as an online buffer exchange device prior to intrinsic protein fluorescence detection was demonstrated to be effective for the rapid determination of capsid content (Cp/mL) in AAV8 samples from 2.6×10^{11} Cp/mL to 2.0×10^{12} Cp/mL. The proposed method uses 1 µL of sample per replicate with minimum sample preparation. However, for samples with higher levels of visible or sub-visible particulates a centrifugation step may be advisable.

An FLR Detector monitoring intrinsic protein fluorescence (tryptophan) offers benefits versus UV absorbance. These benefits include the Empty/Full FLR response factor for AAV8 that is closer to 1 in comparison to the response factors observed for UV absorbance at 280 nm ($R_{F/E, A280} = 2.98$) and 260 nm ($R_{F/E, A260} = 6.81$). Therefore, FLR detection is less dependent on the precise and accurate determination of the mole fraction of DNA containing capsid (X_{Full}). Additionally, intrinsic protein fluorescence has approximately 10-fold higher S/N and is a more selective protein detection method that is not sensitive to free DNA or RNA in the sample.

The level of DNA containing capsid, X_{Full}, can be determined by an additional analysis (e.g., anion exchange chromatography) or empirically estimated from the ratio of SEC-A280 and SEC-A260 peak areas using a UV/VIS PDA detector positioned prior to the FLR Detector (SEC-UV-FLR), as shown. Also, while not demonstrated in this work, a TUV detector may be used instead of the UV/VIS PDA detector if an SEC method producing larger peak widths is used.

Purified samples were used in demonstrating the general principal of this method. However, when analyzing samples that contain macromolecular interfering fluorophores (e.g., proteins) that co-elute with AAV it may be necessary to use a larger pore size SEC particle along with a longer SEC column or slower flow rate to gain better resolution. Also, when employing SEC-A260 and SEC-A280 peak areas for the determination of X_{Full}, interfering chromophores (e.g., proteins, RNA, or DNA) may also need to be separated from the AAV if those interferences are at high enough levels to significantly impact the determination of capsid ssDNA content. Also, while not demonstrated here, larger diameter (7.8 mm I.D.) columns packed with larger size particles (2.5 µm or 3.5 µm) can be deployed for HPLC systems. Larger particle size columns will be less readily fouled with sample particulates, although proportionally higher sample amounts (3 µL) will be required to gain similar sensitivity and lower sample throughputs may result.

Acknowledgement

The authors would like to thank Benjamin Draper of Megadalton Solutions for providing the CDMS analyses for this study.

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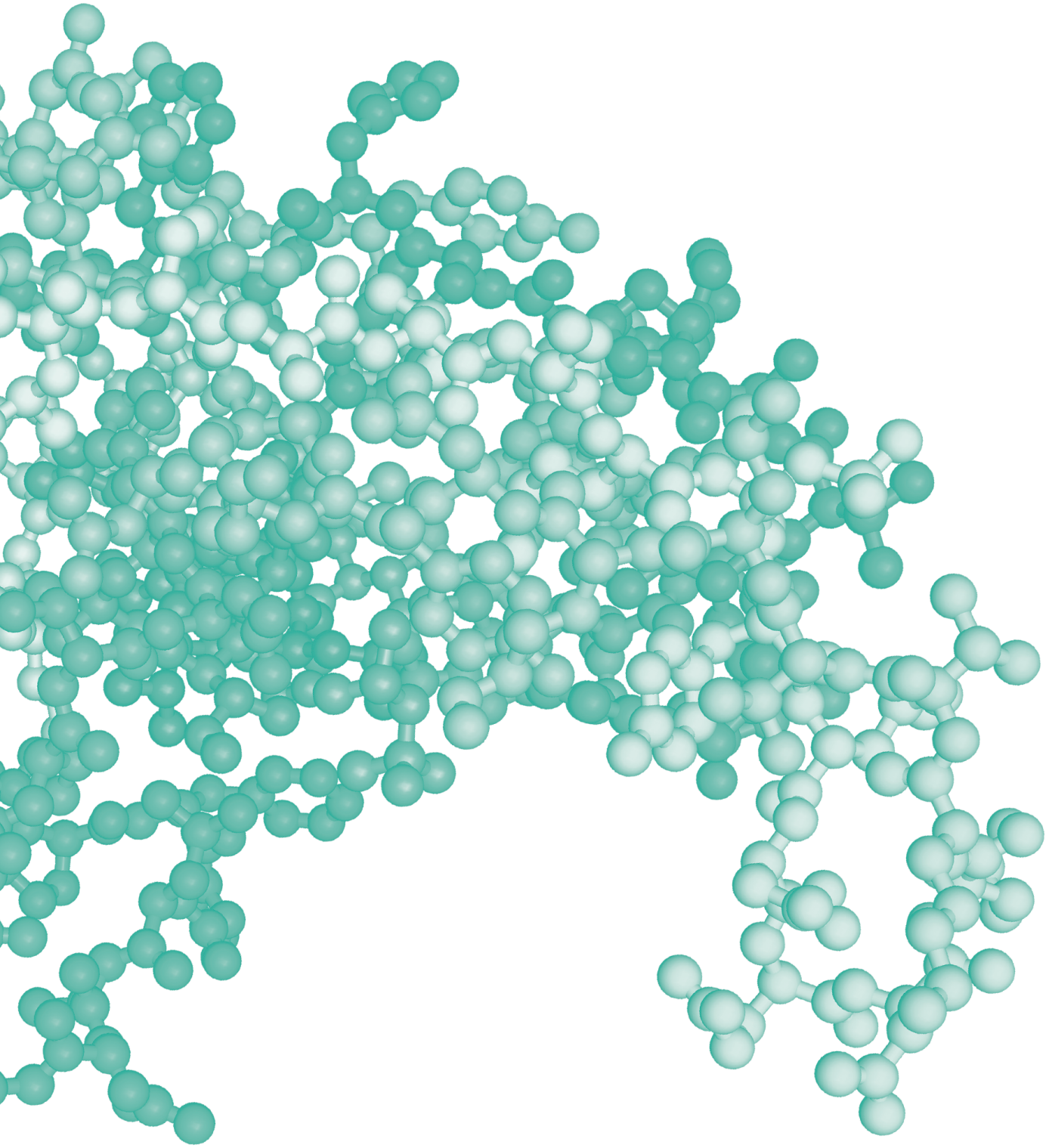

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Improving SEC-MALS Data Quality with Ethylene Bridged Hybrid HPLC Size-Exclusion Columns

Stephan M. Koza and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Substantially improved performance of a Waters™ XBridge™ Protein BEH SEC Column versus an industry standard silica-based SE-HPLC column in an SEC-MALS experiment
- Significantly reduced column conditioning requirements prior to use in SEC-MALS experiments
- Improved resolution of monoclonal antibody fragments in an SE-HPLC separation

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class System

Waters XBridge Protein BEH SEC Column

Waters BEH200 SEC Protein Standard Mix

KEYWORDS

Size-exclusion, SEC-MALS, HPLC, proteins, IgG, adalimumab (Humira®)

INTRODUCTION

The assessment of protein aggregation is an essential part of any testing plan for recombinant protein based biotherapeutic products. Protein aggregation or self-association can impact both the efficacy and the safety profile of these therapies. Specifically, the primary safety concern is the increased risk of an immunogenic response due to the presence of multivalent epitopes.[1] Size-exclusion high-performance liquid chromatography (SE-HPLC) is most commonly relied on for monitoring protein aggregation in drug substance and drug product samples. SEC using a UV absorbance detector provides reproducible results for these analyses that can be relied upon once the veracity of the results are demonstrated with complementary analyses such as analytical ultracentrifugation (AUC) and dynamic light scattering (DLS). Additionally, the online molecular weight characterization of peaks observed in SEC can be facilitated through the use of multi-angle light scattering detectors (SEC-MALS).[2] SEC-MALS has also been reported as a common method in biosimilarity assessments,[3] and in the analysis of heparin molecular weights as a qualified method.[4]

The goal of this application was to evaluate and compare the performance of an industry standard diol-bonded silica-based SE-HPLC column to a diol-bonded ethylene bridge hybrid (BEH) organo-silica based column. The quality of data obtained, and ultimately the accuracy of molecular weight assignments, from an SEC-MALS experiment is greatly impacted by both particulates within the sample, the mobile phase and particulates that may originate from the column itself. These particulates or fines may either be "released" as a bolus from the column during a pressure transient that occurs during the injection of the sample or gradually shed from the column throughout the course of the separation. In the former case, a large light scattering peak may be observed very early in the chromatogram while in the latter case the baseline noise of the light scattering channels throughout the chromatogram may have higher noise levels.

EXPERIMENTAL

Sample description

BEH200 SEC Protein Standard Mix was reconstituted in 500 µL of SEC mobile phase to yield the following:

Analyte	pI	MW
Thyroglobulin, 3 mg/mL	4.6	660,000
IgG, 2 mg/mL	6.7	150,000
BSA, 5 mg/mL	4.6	66,400
Myoglobin, 2 mg/mL	6.8, 7.2	17,000
Uracil, 0.1 mg/mL	N/A	112

A mAb sample of adalimumab (Humira) was used past expiry at a diluted concentration of 1.0 mg/mL.

Method conditions (unless noted otherwise):

LC conditions

System:	ACQUITY UPLC H-Class Bio
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell
Wavelength:	280 nm
Columns:	XBridge Protein BEH SEC, 200Å, 3.5 µm, 7.8 × 300 mm (p/n: 186003596) and BEH200 Protein Standard Mix, (p/n: 186006518); Diol-bonded silica-based SEC, 250Å, 5 µm, 7.8 × 300 mm
Column temp.:	25 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	1.0 mL/min
Mobile phase:	Fisher phosphate buffered saline prepared 2x to a final concentration of 20 mM phosphate, 5.4 mM KCl, and 274 mM NaCl, pH 7.4, 0.2 µm sterile filtered
Sample vials:	Polypropylene 12 × 32 mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 300 µL volume (p/n: 186002640)
MALS detector:	Wyatt miniDAWN TREOS

Data management

Chromatography software:	Empower™ 3
MALS software:	Wyatt Astra 7

RESULTS AND DISCUSSION

The extent of column conditioning required prior to performing an SEC-MALS experiment was evaluated for each of the columns using the same mobile phase and on the same LC-MALS system. Each column was installed and washed at 1 mL/minute for one hour with mobile phase. The system back pressure for silica-based SEC column was 193 bar (2800 psi) with a pressure drop across the column of approximately 41 bar (600 psi) and for the XBridge SEC Column the pressures were 228 bar (3300 psi) and 76 bar (1100 psi). Following conditioning, a series of four 10 µL injections of the mobile phase was performed, followed by analysis of the protein standard mix and the adalimumab sample. Following the protein samples, three additional mobile phase blanks were injected in order to re-evaluate the baseline noise. A comparison of the entire baseline for Channel 1 of the miniDAWN TREOS (43.6° scattering angle) for the two columns is shown in Figure 1. Channel 1 was selected for these comparisons as it exhibits the highest noise. These results show that the peak induced by the pressure transient during the injection of the sample is significantly greater for the silica-based SEC column and while it is observed to gradually decrease upon subsequent injections it is still present at a height of ~4 mV for the fourth blank. In comparison, the initial blank run with the XBridge BEH SEC Column is observed with a peak height of ~0.1 mV which is reduced to ~0.02 mV upon the fourth blank injection.

A comparison of the third blank injection chromatograms produced by the two columns after sample analysis is also shown in Figure 1. For the silica-based SEC column the pressure transient induced peak is still detected at a height of ~1 mV while this peak is no longer observed for the XBridge BEH SEC Column.

In addition to the pressure transient induced baseline disturbance, another SEC-MALS method suitability concern is the noise level of the baseline. This measurement was determined for the first two minutes of the fourth blank analysis (Figure 2). In this comparison the noise level of the baseline observed for the silica-based SEC column was approximately four times greater than that of the XBridge BEH SEC Column. This decrease in baseline noise increases the sensitivity of the SEC-MALS data for lower molecular weight proteins and for lower abundance proteins and protein aggregates. This is demonstrated in the analysis of myoglobin (17 kDa) in the standard protein mix (Figure 3) and in the analysis of the high molecular weight species (HMWS) of adalimumab (Figure 4).

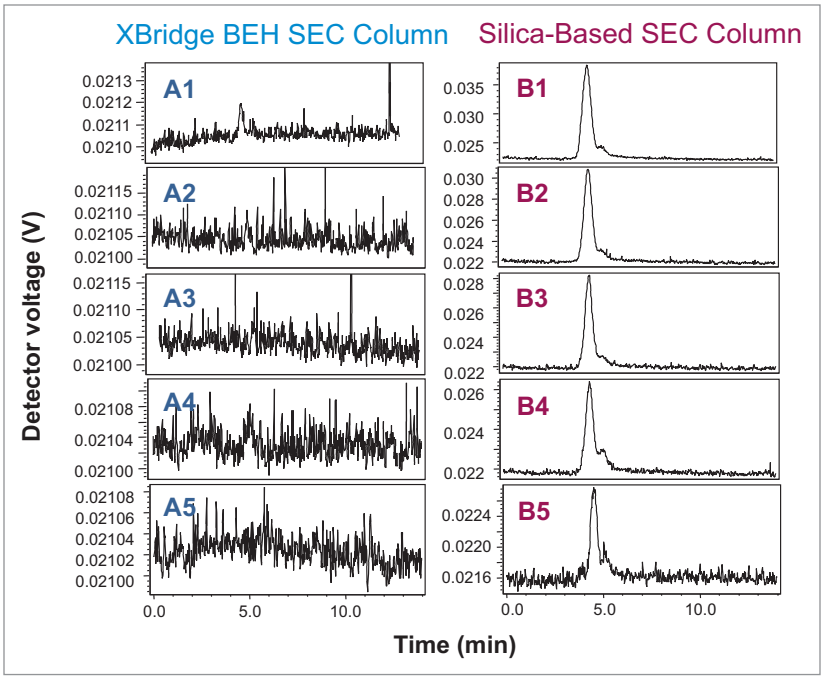

Figure 1. SEC-MALS baseline noise profiles for Channel 1 (43.6°) of a Wyatt miniDAWN TREOS MALS detector for the columns evaluated. Profiles A1 through A4 and B1 through B4 represent the first four blank injections on a new column after one hour of conditioning. Profiles A5 and B5 represent the third blank after a series of six samples.

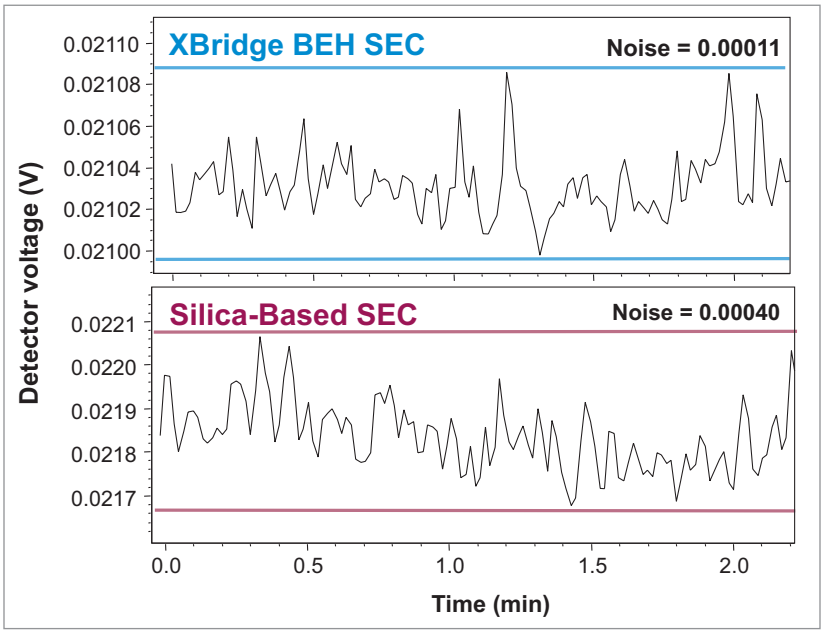

Figure 2. Zoomed-in view of the SEC-MALS baseline noise profiles for Channel 1 (43.6°) of a Wyatt miniDAWN TREOS MALS detector from the fourth blank run on a previously unused column.

Comparing the chromatograms obtained for the standard protein mix (Figure 3) shows several differences in both the overall chromatographic separation quality and in the MALS signal. Under the conditions tested and using uracil as a low molecular weight marker (Peak E) the calculated USP plate count was 45,000 for the XBridge BEH SEC Column and 32,000 for the silica-based SEC column. This 41% improvement is consistent with the 43% increase predicted based on 3.5 µm and 5 µm particle sizes of the respective columns. Chromatographically the XBridge BEH SEC Column provides greater resolution (Rs = 2.62) between the IgG monomer (Peak B2) and BSA (Peak C) peaks than what is observed for the silica-based SEC column (Rs = 1.89). Additionally, comparisons of the peak-to-valley (P/V) measurements between the IgG HMW (Peak B1) and the IgG monomer (Peak B2) are also modestly improved for the XBridge BEH SEC Column (P/V = 1.99) versus the silica-based SEC column (P/V = 1.84). However, the P/V measurements between the IgG HMW (Peak B1, ~300 kDa) and the thyroglobulin monomer (Peak A2, ~660 kDa) were equivalent for the XBridge BEH SEC Column (P/V = 1.20) and silica-based SEC column (P/V = 1.19). This is the result of the 250Å pore size of the silica-based SEC column being more optimal for proteins in this size range than the 200Å pore size of the XBridge BEH SEC Column. In comparing the MALS data of the standard protein mix for the two columns, we will compare the signal-to-noise (S/N) measurements for the lowest molecular weight protein in the standard mix, myoglobin (17 kDa) which produces that lowest MALS signal intensity per unit mass. The lower baseline noise levels observed for the XBridge BEH SEC Column directly translate into improved S/N levels for this low molecular weight protein (S/N = 25) in contrast to the silica-based SEC column (S/N = 9). This improved S/N may provide more accurate and reproducible molecular weight assignments.

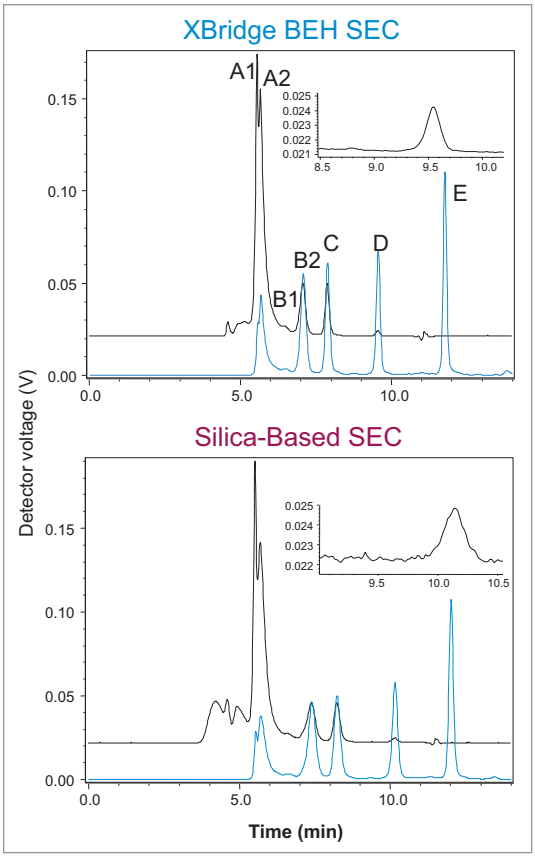

Figure 3. SEC-MALS UV (280 nm, blue) and Channel 1 (43.6°, black) MALS chromatograms for the protein standard mix tested on both columns. The peak identities are: (A1) thyroglobulin dimer 1.32 kDa, (A2) thyroglobulin 660 kDa, (B1) IgG dimer 300 kDa, (B2) IgG 150 kDa, (C) BSA 67 kDa, (D) myoglobin 17 kDa, (E) uracil 112 kDa. Shown in the insert are zoomed views of the MALS myoglobin peak.

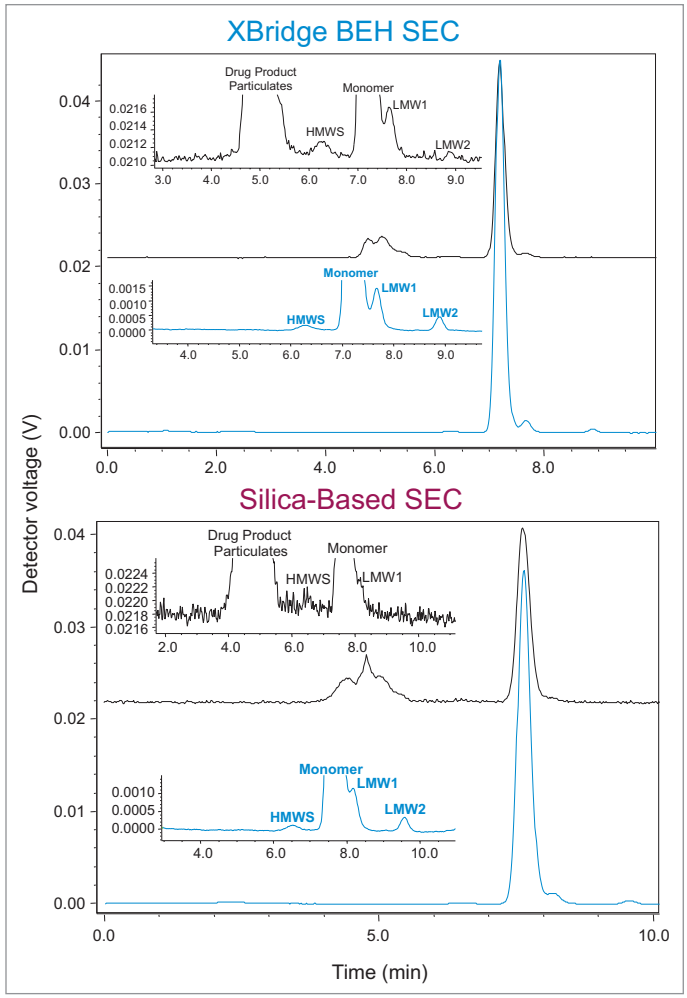

Figure 4. SEC-MALS UV (280 nm, blue) and Channel 1 (43.6°, black) MALS chromatograms for the adalimumab at 1 mg/mL. The peak identities are described in the text. Shown in the insert are zoomed views of the MALS channel.

In a final comparison we will evaluate the chromatograms and MALS data observed for a 1 mg/mL sample of monoclonal antibody (mAb) adalimumab (Humira) shown in Figure 4. Chromatographically the XBridge provides significantly greater resolution of the LMW1 species. This fragment is the result of the hydrolytic cleavage of one of the antigen binding fragment (FAb) arms of the mAb to yield a fragment with a molecular weight of ~100 kDa that consists of one FAb and Fc fragment. The P/V ratio measured for the LMW1 species was nearly two times greater for the XBridge BEH SEC Column (P/V = 2.04) versus the silica-based SEC column where this peak was marginally separated (P/V = 1.11). For the HMW species observed, the USP resolution observed for the XBridge BEH SEC Column (2.14) was slightly higher than that observed for the silica-based SEC column (2.05). Noted in the MALS data for both columns is a large peak eluting before the HMW peak. This peak is not observed in the UV trace and is assumed to be predominantly silicone oil and other protein and non-protein related particles in the drug product.[5] In comparing the MALS data, it is visually clear that the S/N for this separation is superior on the XBridge BEH SEC Column (S/N ~3) in comparison to the silica-based SEC column when we focus on the low abundance (~0.6%) HMW species. This low level signal allowed for the reasonable average molecular weight assignment of this peak as a mAb dimer (Figure 5) at 270 kDa ±5% while the average molecular weight for the monomer was determined as 140 kDa ±0.4%. Molecular weight assignments were made based on a reported UV extinction coefficient of 1.39 mg mL^{-1} cm^{-1} (US Patent 20070292442 A1) and a dn/dc of 0.1815 mL $gm.^{-1}$ A molecular weight assignment could not be made for the HMW peak with the silica-based SEC column experiment.

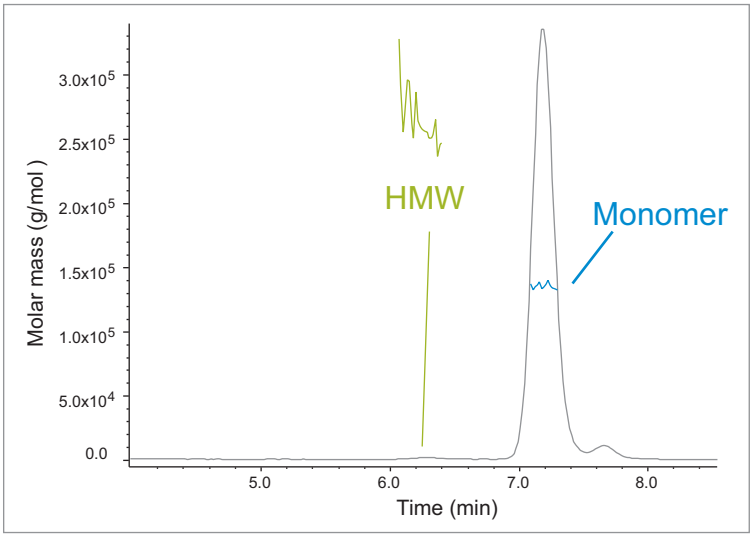

Figure 5. Molecular weight assignments for the HMW (green) and monomer peaks (blue) observed on the XBridge BEH SEC Column for the adalimumab at 1 mg/mL. The UV profile (280 nm) is shown in gray.

CONCLUSIONS

The HPLC compatible XBridge Protein BEH SEC Column with a pore size of 200Å and a particle size of 3.5 µm offers significant improvements in both the quality of MALS data and chromatographic resolution and that can be obtained during the SEC-MALS analysis of small proteins and mAbs in comparison to a standard silica-based SEC column with a pore size of 250Å and a 5 µm particle size. The BEH SEC column was observed to be conditioned much more rapidly; and following the conditioning, the level of both continuous and sample-injection pressure pulse related baseline noise was dramatically lower for the XBridge BEH SEC Column. These performance attributes can be beneficial allowing for the molecular weight determination of low abundance protein species and in the reliability of SEC-MALS data for protein species that elute near the baseline disturbance. The higher efficiency of the XBridge BEH SEC Column also afforded significant improvements in chromatographic resolution of protein species up to a molecular weight of approximately 660 kDa. The impact of this improved efficiency was readily observed in the profiles of the molecular weight standards BSA (66 kDa) and myoglobin (17 kDa). In addition, the XBridge BEH SEC Column provided greater resolution among the protein fragments, monomer, and dimer HMW species present in the mAb drug product sample.

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Analysis of Proteins by Size-Exclusion Chromatography Coupled with Mass Spectrometry Under Non-Denaturing Conditions

Paula Hong, Stephan Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution and sensitivity with SE-UPLC as compared to traditional SE-HPLC
- Non-denaturing SEC method for MS identification of unknown biotherapeutic components
- Exact molecular weight confirmation of intact biomolecules
- BEH particles provide columns with reduced secondary interactions that allow for mobile phases with reduced salt concentrations
- SEC column with minimal MS column bleed provides improved sensitivity

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC BEH200 SEC, 1.7 µm Column

Xevo® G2 Q-Tof Mass Spectrometer

MassLynx® Software

KEY WORDS

SE-UPLC, SEC-MS, QuanTof, monoclonal antibodies, biotherapeutics

INTRODUCTION

Ultra performance size-exclusion chromatography (SE-UPLC) provides a high throughput, robust method for separation of biomolecules based on size in solution.[1] SE-UPLC is typically performed under non-denaturing conditions, which are intended to preserve the state of self-association of the biomolecule, with a UV detector for quantification. Molecular weight estimates based on this technique require the use of an appropriate set of molecular weight standards for calibration. Other methods capable of providing molecular weight information under non-denaturing conditions include on-line multi-angle light scattering (MALS) and off-line analytical ultracentrifugation (AUC), both of which do not rely on molecular weight standards. These low resolution techniques cannot always resolve minor differences in molecular weight due to post-translational modifications or degradation. The combination of SEC using non-denaturing mobile phase and mass spectrometry (MS) provides accurate on-line mass determination for biomolecular species observed by SE-UPLC, however, the form of the non-covalent self-associated species is not provided by this method.

In this application, we describe SEC-MS under non-denaturing conditions. While a similar application has been evaluated for SE-HPLC,[2] UPLC® Technology in combination with sub-2-µm SEC column packing and a time-of-flight mass spectrometer, Xevo G2 Q-Tof, allows for direct analysis with improved chromatographic resolution and sensitivity. The resulting separations are comparable in retention time to those obtained using typical SEC mobile phases that are not MS compatible. By combining these conditions with a Xevo G2 Q-Tof, SE-UPLC-MS analysis can be used as an effective complementary characterization method to low-resolution, non-denaturing mass determination methods such as MALS or AUC, and low-resolution, denaturing size separations such as capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) to confirm the identification of biomolecular species observed by size-exclusion chromatography.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System with PDA detector
Flow cell:	Titanium 5 mm (part number 205000613)
Wavelength:	280 nm
Column:	ACQUITY UPLC BEH200, SEC 1.7 µm, 4.6 x 300 mm (part number 186005226)
Column temp.:	30 °C
Sample temp.:	4 °C
Injection volume:	2 µL
Flow rate:	0.15 mL/min or 0.2 mL/min
Mobile phase:	100 mM ammonium formate and 25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8
Additive:	Acetonitrile, 0.8% formic acid, at 0.2 mL/min
External pump:	Waters 515 HPLC pump
Vials:	LCMS Certified Max Recovery vials (part number 600000755CV)

MS conditions

MS system:	Xevo G2 QTof
Ionization mode:	ESI+
Analyzer mode:	Sensitivity
Acquisition range:	500-5000
Capillary voltage:	3.00 kV
Cone voltage:	40.0 V
Source temp.:	150 °C
Desolvation temp.:	450 °C
Cone gas flow:	0.0 L/Hr
Desolvation gas flow:	800.0 L/Hr
Calibration:	NaI 2 µg/µL from 1000-4000 *m/z*

Data management

MassLynx Software

MaxEnt™1 Software

Sample description

The protein standard (obtained from Bio-Rad) containing bovine thyroglobulin (5 mg/mL), bovine γ–globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 µg/µL) and Vitamin B_{12} (0.5 µg/µL) in deionized water was analyzed. Horse heart myoglobin (Sigma) was prepared at 2 mg/mL in deionized water. A recombinant humanized monoclonal antibody, trastuzumab, was analyzed past expiry undiluted (21 µg/µL).

RESULTS AND DISCUSSION

The analysis of proteins by size exclusion chromatography (SEC) is typically performed under non-denaturing conditions which preserve the three dimensional structure and can be correlated with biological activity of the protein. Common mobile phases are 100% aqueous in a physiological pH range (6-8) and typically require non-volatile buffers and salts such as sodium phosphate and sodium chloride.[3] In order to obtain MS characterization of sample fractions separated under these conditions, the most common solution is to desalt the sample prior to analysis; however, this approach can result in sample speciation and can be cumbersome.

Another strategy is to perform SEC under denaturing conditions, so that species are efficiently ionized for detection by MS.[4, 5] These methods typically require the use of mobile phases containing acetonitrile, formic acid and trifluoroacetic acid (TFA) for direct coupling of SEC to MS. While TFA does cause ion suppression in MS, it is required to minimize secondary interactions between the column packing material and the biomolecule. This application provides a useful tool for desalting of a sample without the need for column re-equilibration and has been used for the analysis of reduced and alkylated monoclonal antibodies as well as other smaller proteins.[5, 6] This method does not typically preserve the self-associated state of the protein.

An alternative approach to SEC-MS has been the use of aqueous mobile phases that are MS compatible such as ammonium formate and ammonium acetate at low concentrations (<100 mM). While these mobile phases may not completely preserve the native structures for biomolecules,[3] they have been found to provide MS sensitivity while best preserving protein self-association and size-based chromatographic separation.

METHOD DEVELOPMENT

The ACQUITY UPLC BEH200 SEC, 1.7 µm Column was evaluated at varying ammonium formate concentrations (5-200 mM) for resolution and MS sensitivity. Initial screening by UV evaluated the effect of salt concentration on both peak shape and resolution. A protein standard (Bio-Rad Laboratories) was used for the analysis. At low ammonium formate concentrations (<100 mM), secondary interactions result in poor peak shape and increased tailing for most of the proteins compared to phosphate buffers. These interactions can be due to either an “ion-exchange” or ”ion-exclusion” effect between the free silanols on the packing material and the biomolecules.[7] While peak shape and resolution improved at higher ammonium formate concentrations, ion suppression in the ESI process was also observed with lower intensity counts. The final mobile-phase conditions were selected to balance resolution and ion suppression. At 100 mM ammonium formate, no tailing significant was observed and the MS signal was adequate for peak identification.

Comparison of the UV chromatograms with 100 mM ammonium formate and PBS (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8) mobile phases show similar retention and peak shape (Figure 1). For this example, ammonium formate provides an adequate SEC separation. However, not all biomoelcules exhibit the same degree of secondary interactions. In instances in which there are greater secondary interactions, the ammonium formate concentration can be altered to improve peak shape.

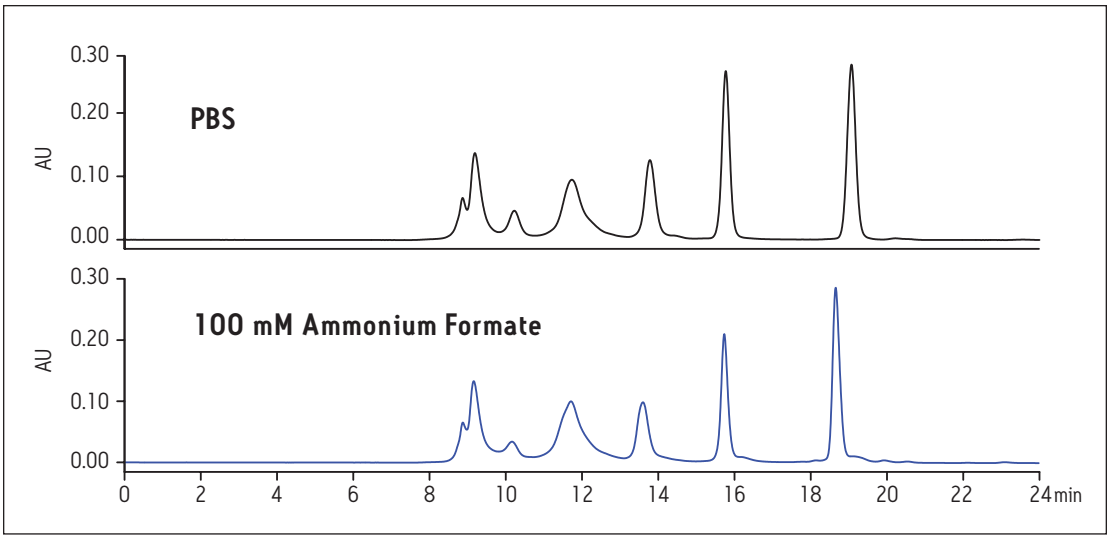
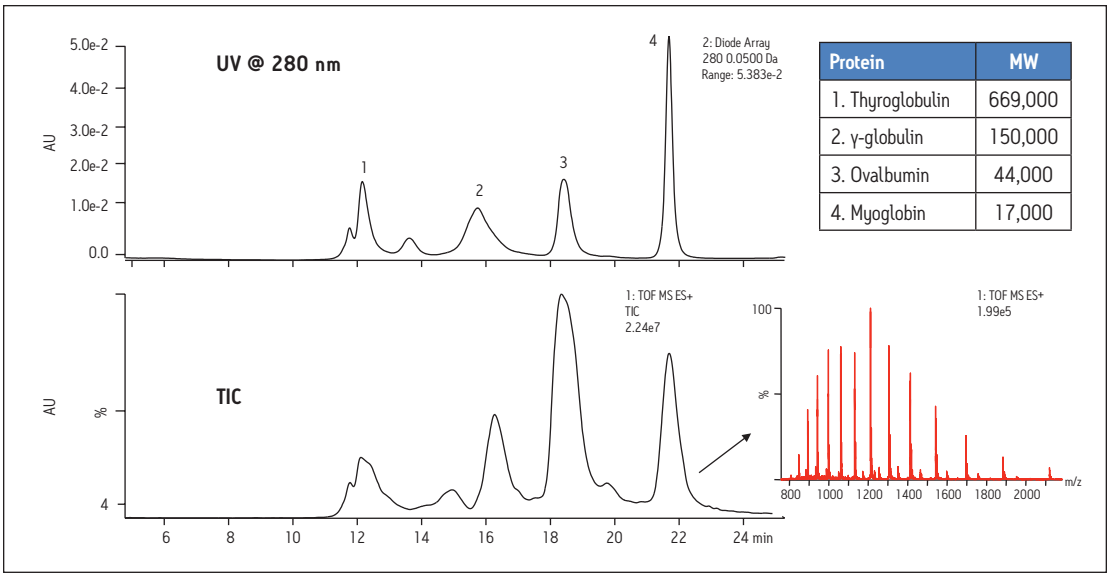


Figure 1. Influence of mobile-phase composition on the SEC separation of a protein standard.

As described above, ammonium formate was selected because of its volatility and MS compatibility. Since the use of non-denaturing mobile phases such as ammonium formate can reduce MS signal by a factor of 10 or greater,[8] a denaturing modifier (formic acid in acetonitrile) was added to the eluent post-column. The post-detector tubing and external pump were connected with a tee just prior to the MS inlet valve. Differences in resolution between the UV and TIC were minimal (Figure 2). As expected, there were significant differences in relative peak area ratios of the proteins in the TIC and UV chromatograms due to differential ionization efficiencies of the protein species. In these experiments the ESI-MS TIC was used solely for identification purposes, and the UV traces for quantification, where relevant.



Protein	MW
1. Thyroglobulin	669,000
2. γ-globulin	150,000
3. Ovalbumin	44,000
4. Myoglobin	17,000

Figure 2. SEC-UV-MS analysis of a protein standard.

Analysis of myoglobin aggregates

The ACQUITY UPLC BEH200 SEC, 1.7 µm Column provides adequate resolution and MS sensitivity of the myoglobin size variants, including the monomer (peak 1), dimer (peak 2) and higher order aggregates (peak 3) (Figure 3). The ESI mass spectrum of the myoglobin monomer and dimer show multiple charged ion signals (Figure 4). The spectrum for the monomer reveals multiple-charge states from *m/z* approximately 800 to 2000 corresponding to charge states from $[M+8H]^{+8}$ to $[M+21H]^{+21}$. The deconvoluted spectrum of the monomer mass spectrum confirms the intact mass of myoglobin at 16,951. The MS signal for the dimer is a factor of 10 weaker than that of the monomer. The ESI mass spectrum of the dimer shows multiple charge states from $[M+20H]^{+20}$ to $[M+40H]^{+40}$. The deconvoluted spectrum shows the presence of both myoglobin monomer and the dimer (*m/z* 16,951 and 33,886).

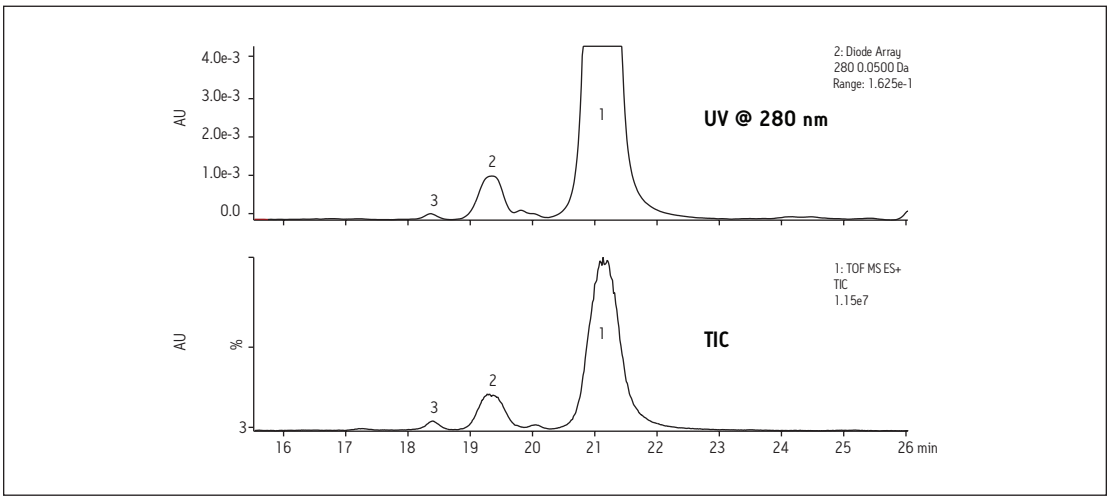


Figure 3. SEC-UV-MS anslysis of myoglobin monomer and aggregates.

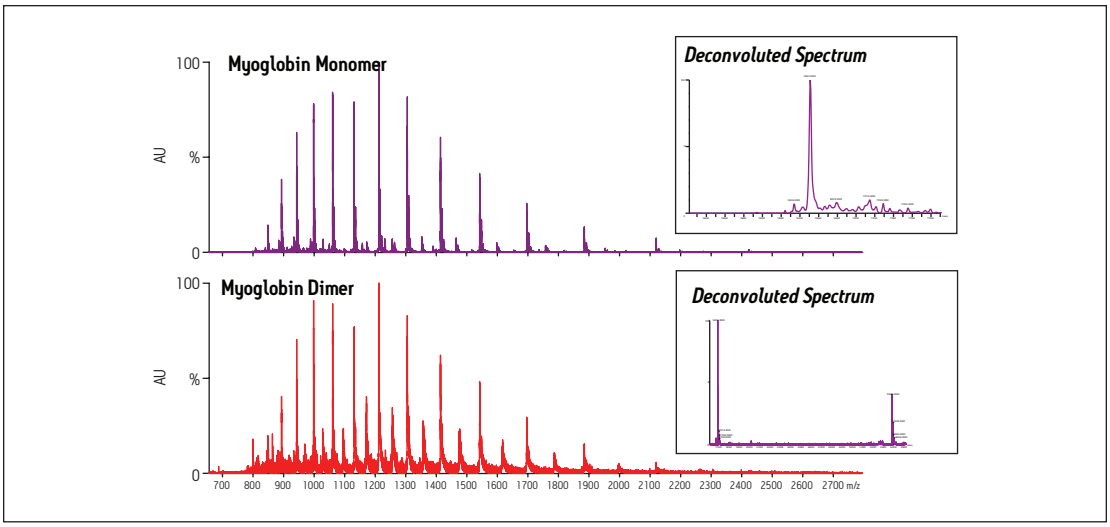
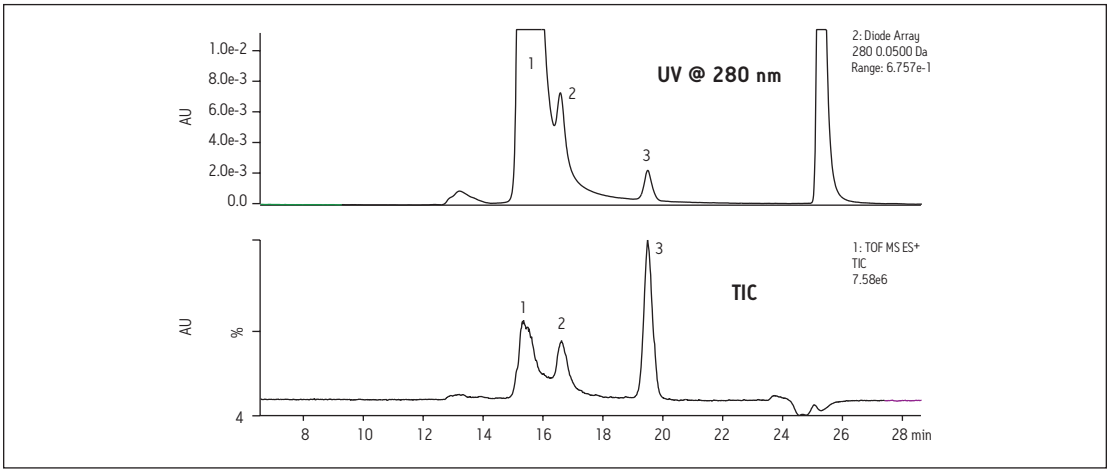


Figure 4. ESI mass spectrum and deconvoluted spectrum (inset) of myoglobin monomer and dimmer.

The simultaneous presence of monomer and dimer in the deconvoluted spectrum may be due to a variety of factors including dissociation of the non-covalent dimer in source, and/or presence of additional size variants. As described above, an acidic organic modifier is required post-column to provide adequate ionization of the proteins. These sample conditions can cause the proteins to denature, thus disrupting protein-protein interactions including non-covalent interactions.[2] An additional factor may be due to the presence of misfolded forms of myoglobin. While separation of the myoglobin monomer and dimer is achieved, a minor peak is present between the two peaks, possibly due to misfolded proteins or other size variants. These forms may be one factor for the appearance of the monomer mass in the deconvoluted spectrum of the dimer. Nevertheless, the presence of only myoglobin monomer and dimer indicates that the aggregation is primarily related to self-association of myoglobin.

Identification of unknown components in a biotherapeutic

An intact monoclonal antibody biotherapeutic, which was past expiry, was analyzed by SEC (Figure 5) using MS-friendly, non-denaturing conditions. In the UV chromatogram, not only are the mAb aggregate and monomer observed, but a low molecular weight (LMW) peak eluting after the intact mAb is partially resolved as well. In addition to these peaks, the UV chromatogram reveals two other LMW species.



The ESI-mass spectrum of the monoclonal antibody (1) shows charge-states from $[M+34H]^{+34}$ to $[M+70H]^{+70}$ (Figure 6). The sensitivity of the method is illustrated by the high TIC satellite peaks of the $[M+39H]^{+39}$ and $[M+40H]^{+40}$ charge-states of the monomer . The deconvoluted spectrum of the monomer peak confirms the presence of the major glycosylated forms of the intact antibody with values corresponding to previously published results.[9] The exact masses can be assigned to G0F/G0F (148,058 *m/z*), G0F/G1F (148,219 *m/z*) and (G01F)2 or G0F/G2F (142,379 *m/z*).

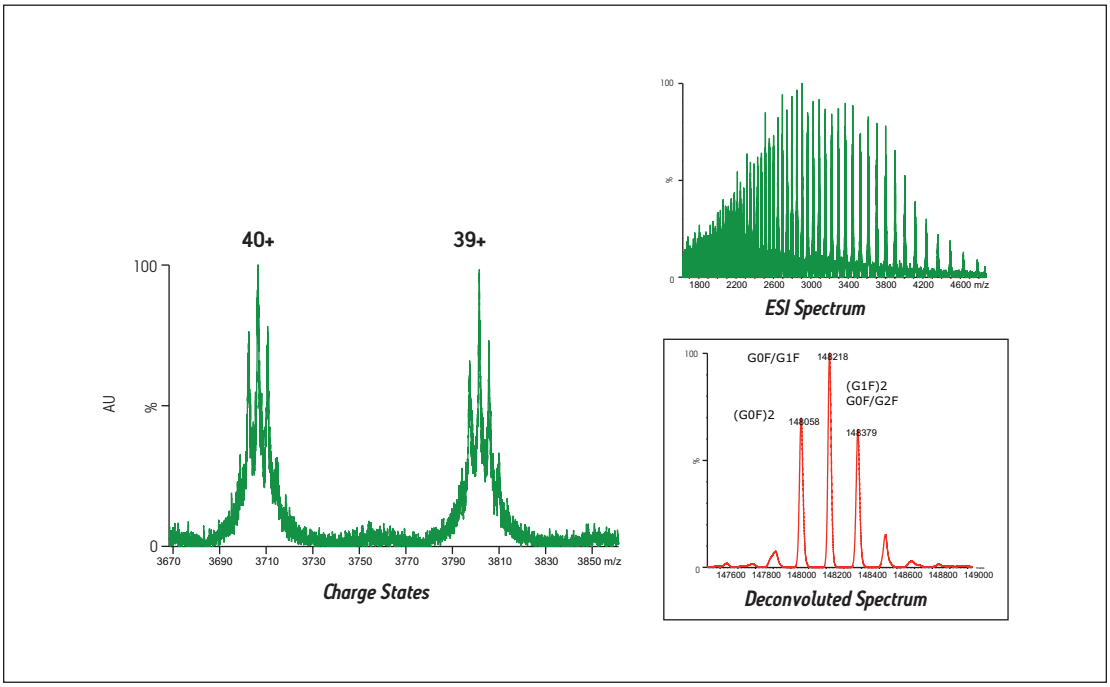


Figure 6. ESI mass spectrum of an intact monoclonal antibody. Deconvoluted spectrum (inset) shows intact mAb as well as glycosylated forms.

The LMW peak (peak 3) eluting at 19 minutes also provides an adequate MS signal for molecular weight confirmation. Analysis of the ESI spectrum shows the presence of two different charge envelopes from 1100-2400 *m/z* (Figure 7). This is evident in the magnified view in which the satellite peaks for both sets of charge-states are resolved. The deconvoluted spectrum shows multiple peaks (Figure 8 inset), with 47,269 *m/z* (F1) and 47,636 *m/z* (F2) having the highest intensities. These intact masses correspond to the two prominent multiply charged ion states in the ESI mass spectrum: the charge states from $[M+19H]^{+19}$ to $[M+31H]^{+31}$ are shown in the zoomed spectrum. Based on the sequence of the protein, the main peaks in the deconvoluted spectrum can be assigned to Fab fragments resulting from hydrolytic cleavage of the heavy chain: the mass of F1 (47,270 *m/z*) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Asp^{224} while the mass of F2 (47,637 *m/z*) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Thr^{228}.

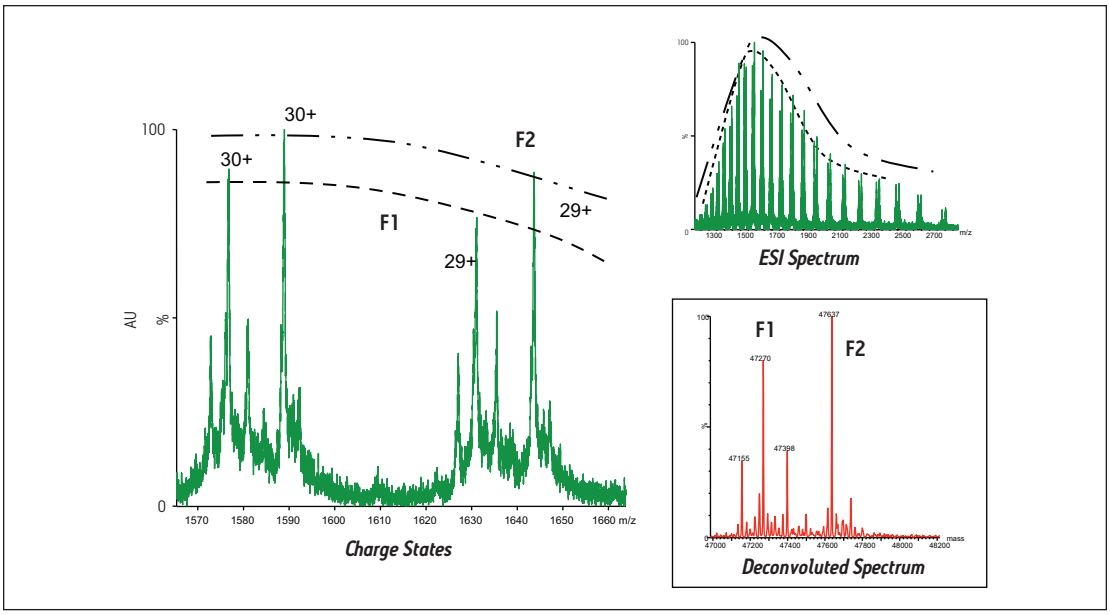


Figure 7. ESI mass spectrum of low molecular weight species (peak 3) in a recombinant humanized monoclonal antibody.

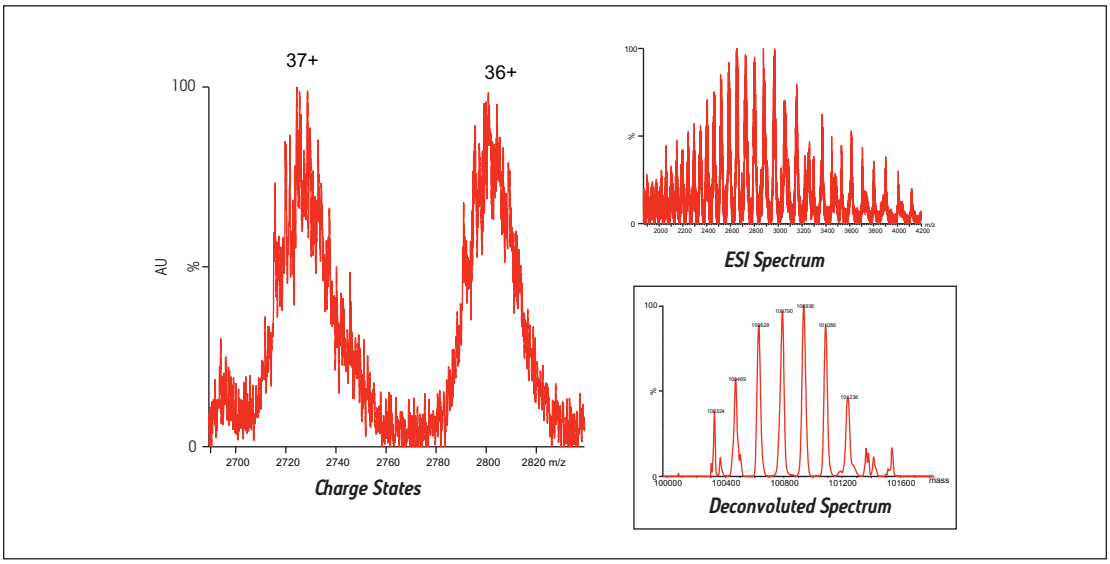


Figure 8. ESI mass spectrum of fragment (peak 2) in a recombinant humanized monoclonal antibody.

CONCLUSIONS

Size exclusion chromatography under non-denaturing conditions is a standard method for testing biomolecules and their aggregates. MALS and AUC are established detectors but cannot provide exact mass for unknown species with a sufficient accuracy. The presence of an unexpected peak requires further investigation and/or confirmation of molecular weight, and SE-UPLC-MS under aqueous, non-denaturing conditions can provide valuable information that would more rapidly solve an organization's issues with characterization or quality.

While SEC-MS does not typically preserve protein self association, it can assist in identification. The analysis of myoglobin illustrates the utility of an SEC-MS approach by confirming that the HMW forms observed in the myoglobin sample are related to the protein. The SEC-MS analysis of a humanized monoclonal antibody under non-denaturing conditions provides exact masses for LMW antibody fragments. By efficiently combining the ACQUITY UPLC BEH200 SEC, 1.7 µm Column and the benchtop Xevo G2 Q-Tof with an extended *m/z* range, the intact antibody and its associated fragments can be identified, providing a rapid method for exact molecular weight determination of intact biomolecules.

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[TECHNOLOGY BRIEF]

A Generic On-Line UPLC-SEC/UV/MS Method for the Analysis of Reduced Monoclonal Antibodies

GOAL

To demonstrate the capabilities of Waters' integrated UPLC-SEC/UV/MS system for fast and routine characterization of reduced monoclonal antibodies.

BACKGROUND

The pipeline for biotherapeutics is growing rapidly as pharmaceutical organizations shift their focus from small molecule drugs to biotherapeutic drugs. The efficient characterization of antibody drugs is increasingly important to both regulatory agencies and pharmaceutical companies to ensure the safety and efficacy of biotherapeutic products.

Although the selectivity of antibodies varies appreciably, the overall structures of antibodies are highly conserved within an antibody class. The ability to analyze the same class of antibodies using a generic method is highly desirable for improving the efficiency of analyses in the pharmaceutical industry.

Reversed-phase (RP) chromatography coupled with mass spectrometry is frequently used for the analysis of reduced antibodies. RP chromatography separates proteins based on their hydrophobicity and is a popular technique for rapid, intact mass analysis. Nonetheless, proteins with different sizes may show similar hydrophobicity and are therefore difficult to separate by RP. High temperature is often used in RP chromatography to achieve better peak shape and to minimize carryover. This, however, raises questions on whether high temperature introduces changes to the protein structure.

This on-line UPLC®-SEC/MS method, accomplished by utilizing a sub-2-µm ACQUITY UPLC® BEH SEC Column, the ACQUITY UPLC System, and the SYNAPT® G2, offers a powerful solution for antibody characterization.

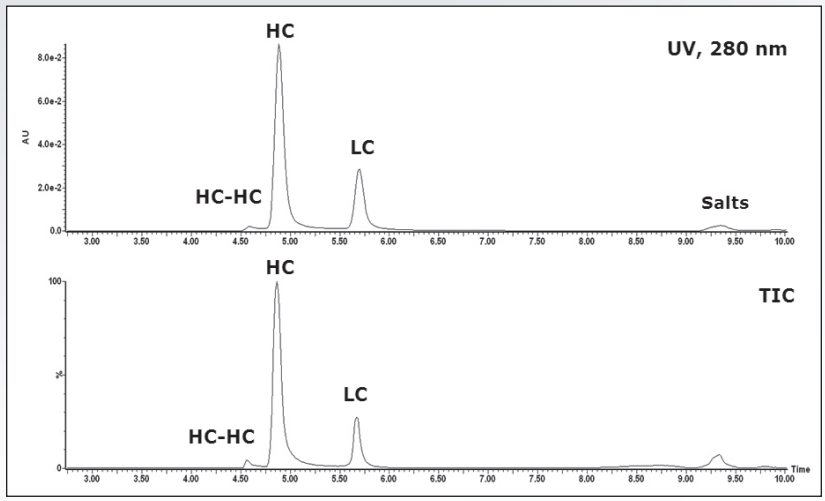

Figure 1. UPLC-SEC/UV/MS chromatogram of a reduced humanized mAb. This method is able to resolve light chain, heavy chain, and heavy chain-heavy chain clip, providing a generic way for antibody screening.

Size exclusion chromatography (SEC) separates proteins predominantly based on their size differences using an isocratic elution rather than hydrophobicity and avoids the use of high column temperature and gradient optimization. SEC is widely used in the biopharmaceutical industry to detect antibody aggregates and fragments.

However, traditional SEC mobile phases are incompatible with electrospray ionization MS, and historically optical detection methods with lower specificity have been used. Lower specificity presents a problem for organizations wishing to characterize biotherapeutics according to the well-characterized biotherapeutic product model as adopted by regulators around the world.

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This work describes an efficient on-line UPLC-SEC/MS method for the direct mass analysis of reduced humanized monoclonal antibodies (mAb), and demonstrates that rapid, high-resolution SEC separations in combination with high-performance SYNAPT G2 MS provides an efficient, generic method for routine antibody characterization.

THE SOLUTION

The successful coupling of SEC to ESI-MS was achieved using an MS-friendly mobile phase. The SEC separation was achieved using an ACQUITY UPLC BEH200 SEC Column (1.7-µm, 4.6 x 300 mm) with an ACQUITY UPLC System using isocratic elution with a flow rate of 0.46 mL/min. The mobile phase contains 30% acetonitrile, 0.1% TFA, and 0.1% FA in Milli-Q water. The flow passed through a TUV detector operated at 280 nm wavelength and then directed to the SYNAPT G2 MS.

The use of sub-2-µm UPLC BEH column packing materials along with the low-dispersion, high-pressure ACQUITY UPLC System resulted in significant improvements in size-based separation as displayed in Figure 1. The 10-minute SEC run completely resolved the earlier eluting glycosylated heavy chain (HC) from the later eluting light chain (LC), a separation not achievable in such a limited timeframe with existing techniques.

In addition, the glycosylated HC-HC fragment/clip was well separated from the heavy chain. In contrast to RP columns, the Waters UPLC BEH SEC column showed no memory effect. Consequently, no blank runs were needed between sample runs.

Figure 2 (top) depicts the summed raw mass spectrum (inset) and the resulting MaxEnt1 deconvoluted mass spectrum of the glycosylated heavy chain. The major peaks correspond to the heavy chain containing the core fucosylated glycan (G0F), a minor nonfucosylated form (G0), and core glycan variants extending by one or two terminal galactose residues (G1F, G2F). Below, it also shows the summed raw mass spectrum (inset) and the MaxEnt1 deconvoluted spectrum of the light chain.

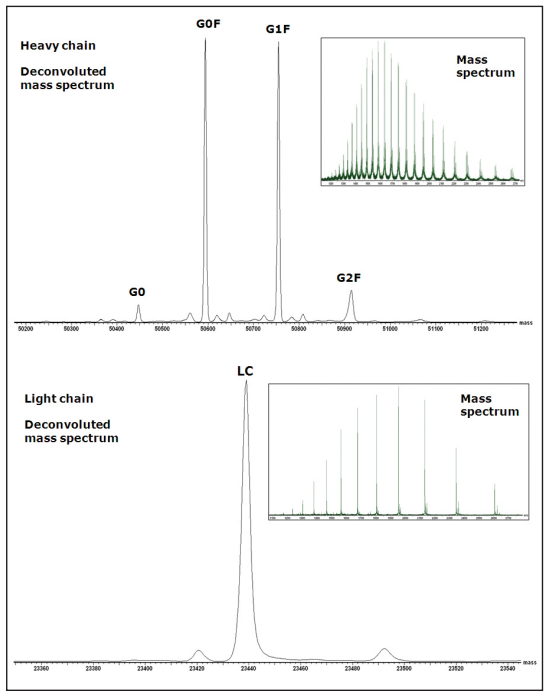

Figure 2. Combined mass spectrum (inset) and MaxEnt1 deconvoluted mass spectrum of the heavy chain (top). Combined mass spectrum (inset) and MaxEnt1 deconvoluted mass spectrum of the light chain (bottom).

SUMMARY

The optimized UPLC-SEC/UV/MS method enables the direct pairing of size exclusion chromatography with a mass spectrometer to measure the molecular weights of reduced mAbs. This well-established separation technique is now linked to mass spectrometric detection for those needing to characterize their biotherapeutics and satisfy regulators that they have sufficiently understood their biotherapeutic products.

The performance of the UPLC system and SEC column allows scientists to resolve chain, heavy chain, and heavy chain-heavy chain clip of an antibody without employing high column temperature. This optimized SEC method with separation based on size differences provides a complement to RP chromatography, and, coupled with MS, offers a powerful, routine, and generic solution for antibody characterization.

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Analysis of Antibody Drug Conjugates (ADCs) by Native Mass Spectrometry on the BioAccord System

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Waters Corporation, Milford, MA, USA

GOAL

To demonstrate the performance of the BioAccord™ System for the analysis of antibody drug conjugates (ADCs) under native conditions.

BACKGROUND

Drug-to-Antibody Ratio (DAR) is a critical quality attribute (CQA) for ADCs because it directly affects their therapeutic efficacy and pharmacokinetics. Determination (and monitoring) of DAR is essential across the ADC development process and within commercial manufacturing operations.

Native electrospray mass spectrometry (native MS) has emerged as a powerful tool in the analysis of covalent complex therapeutic proteins and non-covalent protein complexes. Under native MS conditions, proteins are subject to electrospray ionization using a non-denaturing MS-friendly buffer system. These conditions for LC-MS analysis enable many proteins to remain in their folded states that demonstrate characteristically low charge states, requiring sensitivity over a broader and higher mass to charge (*m/z*) range than that for the analysis of the denatured proteins. Native MS

Drug-to-Antibody Ratio (DAR) determination of Lys and Cys conjugated ADCs was accomplished using analytical scale size exclusion chromatography (SEC) and the BioAccord System for native LC-MS analysis.

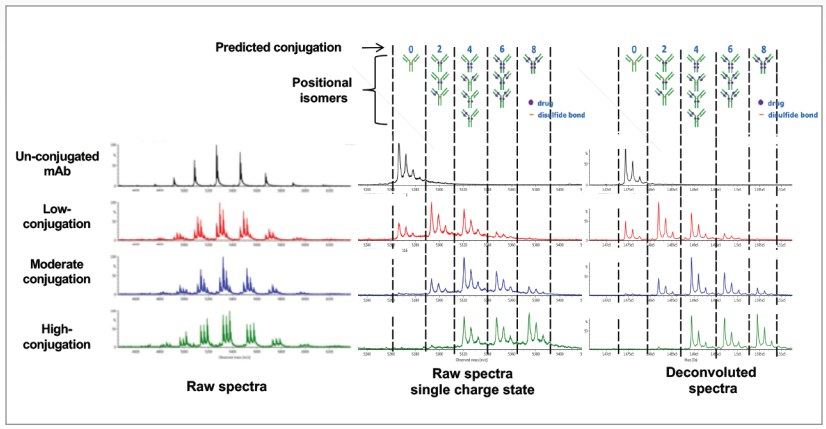

Figure 1. The combined raw spectra from multiple charge state envelope (left), the zoomed-in region (single charge state) of the combined raw spectra (center), and the deconvoluted spectra (right) of the reference materials (mAb), the low, moderate, and high conjugation level cysteine-conjugated ADC samples without deglycosylation treatment from the BioAccord System native LC(SEC)-MS analysis. Drug distribution was compared for three different cysteine-conjugated ADC samples with increasing drug load.

Cysteine-conjugated ADCs drug loading distribution and DAR

	Low				Moderate				High			
	HIC	QTof1	QTof2	Tof	HIC	QTof1	QTof2	Tof	HIC	QTof1	QTof2	Tof
ADC 2	0.81	0.74	0.64	0.68	0.38	0.41	0.35	0.36	0.07	0.09	0.05	0.05
ADC 4	1.14	1.17	1.37	1.36	1.67	1.57	1.81	1.82	1.23	1.11	1.19	1.15
ADC 6	0.75	0.60	0.64	0.65	1.61	1.45	1.51	1.47	1.72	1.72	1.86	1.85
ADC 8	0.12	0.21	0.05	0.10	0.78	0.97	0.70	0.75	2.95	3.05	2.98	2.96
DAR	2.83	2.72	2.70	2.79	4.44	4.40	4.37	4.40	5.97	5.97	6.07	6.01

QTof 1	deglycosylated samples, run on Xevo G2-S in 2014
QTof 2	non-deglycosylated samples, run on Vion in 2017
Tof	non-deglycosylated samples, run on BioAccord in 2018

Table 1. Total average DARs and drug distribution comparison amongst the HIC (UV) and the three native SEC-MS experiments exhibit agreement across all three drug loading levels. The results indicated that DAR measurements can be measured consistently using orthogonal approaches (HIC vs MS), or across different QTof or Tof MS systems (Xevo™ G2-S, Vion™ IMS QTof MS, and the BioAccord System). With its streamlined workflow for automated data acquisition, processing, and reporting of DAR calculated results, the BioAccord System proved effective for native LC(SEC)-MS analysis of ADCs to determine lot to lot, batch to batch comparability.

faces several unique challenges including the need for extensive sample clean-up before analysis if infusion MS is attempted, and greater operator skill to produce and interpret experimental results. Previously, we have made efforts to simplify the acquisition of native MS data by coupling inline SEC with existing MS technologies[1,2] to facilitate the sample desalting and buffer exchange for the study of the population of cysteine-conjugated ADCs.

In this study, we employ the BioAccord System to improve accessibility to an analytical solution for native MS analysis of both cysteine-conjugated and lysine-conjugated ADCs. The BioAccord System is a small footprint, high performance bench top system that was designed and developed with simplified user interface as well as automatic system setup and self-diagnostic capabilities.

THE SOLUTION

The BioAccord System is physically comprised of an ACQUITY™ UPLC™ I-Class PLUS System configured with an optical detector (TUV or FLR) coupled in-line to an ACQUITY RDa™ Detector (compact oa-TOF MS). The system is operated under a UNIFI Scientific Information System that enables streamlined workflow solutions for regulated and non-regulated laboratories with the combination of automated data acquisition, processing, and reporting, including automating the DAR calculation for ADC characterization.

Cysteine conjugated ADC analysis

Native mass spectrometry of cysteine-conjugated ADCs analysis requires non-denaturing conditions to maintain the non-covalently linked ADC molecules intact to determine DAR values and the drug loading distribution for the ADC samples. In this study, the BioAccord System was directly coupled with an analytical scale SEC column (ACQUITY UPLC Protein BEH SEC Column, 200Å, 1.7 µm, 2.1 mm x 150 mm, p/n=186008471) with isocratic elution (50 mM ammonium acetate (NH_4OAc) over a 10-minute run).

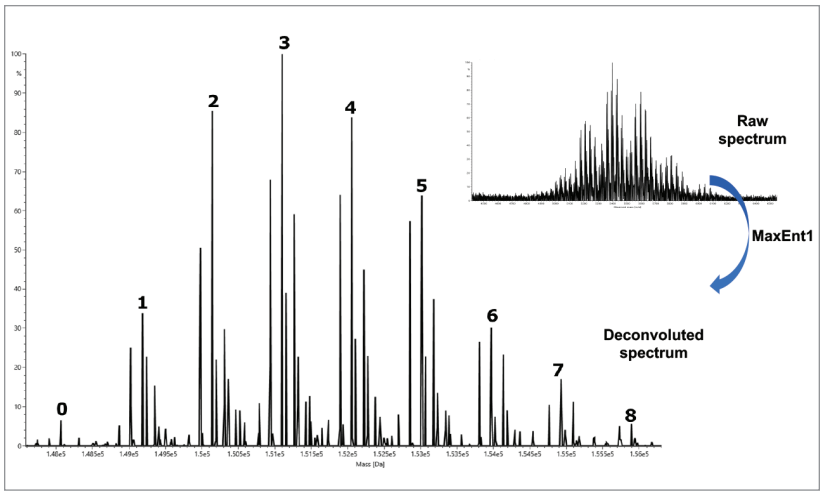

Figure 2. Raw and deconvoluted spectrum of LC(SEC)-MS analysis of Lysine conjugated ADC, Kadcyla (Trastuzumab Entansine (T-DM1)), by BioAccord System. The number labeled on the deconvoluted spectrum represents the detected number of drugs that are conjugated to the protein Trastuzumab in the intact level. The calculated average DAR is 3.46, vs. the published DAR of 3.50.[3] The experiment was conducted without deglycosylation of the ADC drug.

Under these SEC conditions the ADC subunits maintain a quazi-native state, maintaining quaternary structural interaction, and producing a surface area much smaller than that of its denatured, unfolded forms found with the acidic-organic mobile phases typical of reversed-phase separations. Proteins under native electrospray mass spectrometry conditions will carry fewer charges than in reversed-phase conditions, and the smaller charge envelope appears at a higher *m/z* range in the resulting mass spectra. As shown in Figure 1, the extended mass range (up to *m/z* 7,000) of the BioAccord System meets the need of high *m/z* measurement and demonstrates the detection of complex non-deglycosylated cysteine-conjugated ADCs. These results are consistent with spectra from the ADC sample previously analyzed on other high-resolution MS systems,[1,2] with comparable DAR values obtained between the BioAccord and the previous QTof MS systems (Figure 1 and Table 1).

The combined raw spectral charge state envelope (Figure 1, left), a zoomed-in single charge state (center), and the deconvoluted spectra (right) are shown for the naked antibody mAb, and three levels (low, moderate, and high) of conjugated ADCs on the BioAccord System. The glycosylation pattern displayed in the naked mAb spectrum is repeated for each conjugation form (0, 2, 4, 6, and 8) across all three levels of conjugated samples. The combined integrated peak areas of each of the glycoforms from the deconvoluted spectra were used for automated calculation of the total average DAR, and the drug loading distribution, within the UNIFI data processing workflows, as previously described in detail.[1]

Lysine conjugated ADC data

The raw and deconvoluted spectra of LC(SEC)-MS analysis of lysine-conjugated ADC Kadcyla (Trastuzamab Entansine (T-DM1)) were obtained without the need of deglycosylation of the sample (Figure 2). The detailed benefits of using native MS approach for covalent lysine-conjugated ADC analysis were described previously.[2] The number of conjugated drugs detected is labeled on the deconvoluted spectrum peaks. The DAR value calculated by UNIFI was 3.46, which is in good agreement with DAR of 3.50 reported by the drug manufacturer.[3] Again, results were comparable to a previous study using a Vion IMS QTof system.[2]

SUMMARY

In this tech brief, we have demonstrated that the BioAccord System is well suited for routine LC(SEC)-native MS analysis of both cysteine and lysine conjugated ADCs. The average DARs and drug loading distribution results were comparable to that generated from Hydrophobic Interaction Chromatography (HIC) separation (with TUV detection), and results from previous generations of QTof MS systems. Simplified system operation and configurable compliance features will enable the BioAccord System to be readily adopted by scientists with less MS experience, which will allow organizations to more readily deploy the mass spectrometry in supporting ADC across development and manufacturing processes. The capabilities demonstrated in this tech brief and other published applications using the BioAccord System[4-8] show the breadth of the BioAccord System for supporting routine analysis of biotherapeutic product quality attributes.

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Analytical Scale Native SEC-MS for Antibody-Drug Conjugates (ADCs) Characterization

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- An improved native mass spectrometry with analytical scale Size Exclusion Chromatography (SEC) method is developed for characterization of cysteine-conjugated and lysine-conjugated ADCs.
- This improved method offers increased sensitivity, robustness and simplified sample preparation process for DAR and drug load distribution of cysteine-conjugated and lysine-conjugated ADCs
- UNIFI™ Scientific Information System automates the DAR and drug load distribution calculations and is well suited for streamlined critical quality attributes (CQAs) such as ADC DARs characterization

WATERS SOLUTIONS

Vion™ IMS QTof Mass Spectrometer

ACQUITY™ UPLC™ H-Class Bio System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

UPLC Protein BEH SEC Column

UNIFI Scientific Information System

KEYWORDS

Native SEC-MS, Vion IMS QTof Mass Spectrometer, QTof, biotherapeutics, ADC, cysteine-conjugated, lysine-conjugated, ACQUITY UPLC H-Class Bio System, UNIFI Scientific Information System, intact mass analysis, DAR, drug loading distribution, isocratic, ammonium acetate

INTRODUCTION

Native mass spectrometry (MS) is widely used in academic and industrial labs for a variety of applications, such as protein folding,[1] protein-ligand, protein-protein interactions,[2] protein complex architecture,[3] small protein aggregation,[4] antibodies/antibody derivatives,[5,6] and antibody drug conjugates (ADCs).[7,8] Most of these native MS applications use static infusion from glass nano flow capillaries following extensive sample clean-up. The notable drawbacks for the infusion approach are the need of extensive sample clean-up before the analysis, as well as the need of highly skilled scientists to produce interpretable data. The effort of applying online native approaches, however, is still far from practical and routine. To address the challenges in performing native MS analysis, we developed an analytical scale native SEC-MS method that can be adopted for cysteine- and lysine-conjugated ADC Drug-to-Antibody Ratio (DAR) measurements.

Figure 1. The ACQUITY UPLC H-Class Bio System (right) coupled to a bench top Vion IMS QTof Mass Spectrometer (left) controlled by a compliance-ready and workflow-driven software, UNIFI Scientific Information System, presents a single platform for robust native SEC-MS characterization of biotherapeutics (such as ADCs) with streamlined data acquisition, data processing, and reporting workflow.

Cysteine-conjugated ADCs use the intra chain thiol groups to conjugate with small cytotoxic drug molecules. This type of modification transforms the active mAbs from covalently linked tetramers to non-covalently linked complexes. If exposed to standard reversed-phase liquid chromatography (RPLC) mobile phases (e.g. organic solvent or acid), these non-covalently linked complexes will dissociate to smaller subunits. Therefore, native mass spectrometry with non-denaturing condition is used to keep the protein in its near-native state. The purpose of the analysis is to characterize DAR and drug loading distribution.

RPLC-MS is widely used for Lysine-conjugated ADC characterization. However, deglycosylation treatment is usually needed to reduce the MS spectrum complexity for DAR calculations; as well as the link-only species formed in a two-step conjugation process. Compare to RP-MS, native SEC-MS extends the charge envelope to higher *m/z* mass window, therefore, it could improve mass spectrum quality for lysine-conjugated ADCs without the need for deglycosylation using PNGase F.

In this study, we describe a streamlined native SEC-MS methodology using an analytical scale SEC column (p/n: 186008471) for ADCs analysis. The Waters™ Vion IMS QTof Mass Spectrometer, coupled with an ACQUITY UPLC H-Class Bio System and an ACQUITY UPLC Tunable Ultraviolet (TUV) Detector is used in the study. The workflow includes automated data acquisition, processing, and reporting. The SEC-MS results are compared to hydrophilic interaction chromatography (HIC) analysis,[9] the goal is to demonstrate the benefit of using analytical scale SEC-MS for routine ADC DAR characterization.

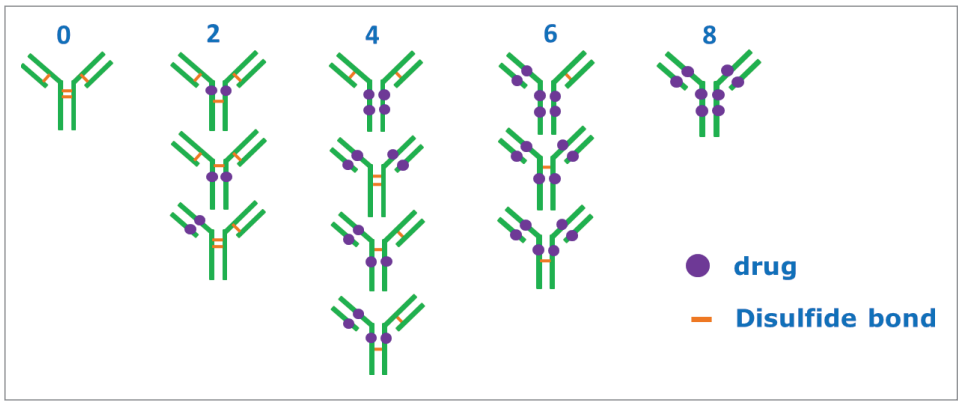

Figure 2. Cysteine-conjugated ADC molecules held together by non-covalent interactions between the light and heavy chains. The non-denaturing conditions are needed to preserve the non-covalent interactions and bonding. Possible combinations of ADC conjugation are shown in Figure 2.

EXPERIMENTAL

Reagents, solvents, and sample preparation

All of the ADCs used in this study were obtained from external collaborations. Samples were stored at -80 °C in the original buffer and were thawed and diluted to 2 µg/µL concentration with 50 mM ammonium acetate (NH_4OAc) in H_2O before SEC-MS analysis. Lockmass compound [Glu1]-Fibrinopeptide B Standard (p/n: 700004729) was diluted to 320 fmol/µL in 50/50 H_2O/ACN with 0.1% Formic Acid (FA) and was used during the acquisition.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detector:	ACQUITY UPLC TUV, absorption wavelength: 280 nm
Column:	ACQUITY UPLC Protein BEH SEC 200Å, 1.7 µm, 2.1 mm × 150 mm (p/n: 186008471)
Column temp.:	25 °C
Sample temp.:	5 °C
Injection volume:	2 µL
Mobile phase:	50 mM ammonium acetate (NH_4OAc) in H_2O
Gradient:	Isocratic at 0.065 mL/min with total run time of 10 min

Gradient table:

Time (min)	Flow rate (mL/min)	50 mM NH_4OAc
Initial	0.065	100
10	0.065	100

Total run time = 10 min

MS conditions

MS system:	Vion IMS QTof
Acquisition range:	*m/z* 500–8,000 Da
Mode:	ESI+
Capillary voltage:	2.00 kV
Cone voltage:	140 V
Source offset:	80 V
Source temp.:	125 °C
Desolvation temp.:	250 °C
Desolvation gas low:	600 L/h
Lock mass:	[Glu1]-Fibrinopeptide B at 320 fmol/µL in 50/50 H_2O/ACN, 0.1% FA

Software for data acquisition and processing

UNIFI Scientific Information System 1.8.2

Vion IMS QTof driver pack 2.0

Software used for data collection and processing was UNIFI Version 1.8.2, which is configured using an intact protein analysis type that defines the automated processing for DAR calculation, please see reference [9] for detailed description of the automatic DAR calculation steps in UNIFI.

RESULTS AND DISCUSSION

Native mass spectrometry (MS) is widely used in academic and industrial labs for a variety of applications. In this method, protein samples are usually introduced to the MS via static infusion from glass nanoflow capillaries. Some of the benefits of this approach are high desolvation and ionization efficiency of the protein molecules due to the very low volume of aqueous mobile phase and lower temperature of the MS source conditions that are required. However, there are notable drawbacks associated with this approach, such as the need for extensive sample clean-up before the analysis, as well as the need for highly skilled scientists to produce interpretable data. In addition, the static infusion would not be very useful for complex samples without pre-separation. In a previous study,[9] in an attempt to address some of the challenges in performing native MS analysis, we applied online native SEC-MS approach, using a Waters ACQUITY UPLC Protein BEH SEC Column (p/n: 186005225) (with an ACQUITY UPLC H-Class Bio QSM and TUV coupled to a Xevo™ G2-S MS System) to analyze a set of cysteine-conjugated ADC samples after deglycosylation treatment. The experiment results show very good agreement with the data from an orthogonal method using Hydrophobic Interaction Chromatography (HIC) for the average DARs and drug load distributions. In this current study, we employed a Waters ACQUITY UPLC Protein BEH SEC Column (p/n: 186008471) with an ACQUITY UPLC H-Class Bio QSM and TUV coupled to a Vion IMS QTof MS System to analyze the same set of cysteine-conjugated ADC samples, however, without deglycosylation sample treatment. The smaller diameter of the column (2.1 mm vs. 4.6 mm) enabled lower LC flow rate, while maintained a higher and stable LC system back pressure to retain high peak retention reproducibility form run to run. Lower flow rate also meant that less harsh MS source conditions can be applied to obtain high desolvation and ionization efficiency, therefore increasing the MS sensitivities. In general, a 5× increase in sensitivity was observed when switching from 4.6 mm to 2.1 mm I.D. column. In addition, the improved performance of the Vion IMS QTof MS System[10] with the new QuanTof 2 technology and enhanced TOF analyzer vacuum system resulted in better glycoforms mass resolution at the raw spectra level as shown in Figure 3. The advanced MS system facilitated the ability to analyze these ADC samples without deglycosylation treatment, therefore improved the sample analysis throughput.

Figure 3 shows the TUV and TIC chromatograms from a typical native SEC-MS experiment. The protein eluted at around 4.5 minutes, and the inorganic salts (buffers, etc.) in the injected sample eluted later at about 6.5 minutes. The total run time for the isocratic gradient is about 10 minutes. Raw MS spectrum can be generated by combining the MS scans of the TIC peak at 4.5 minutes.

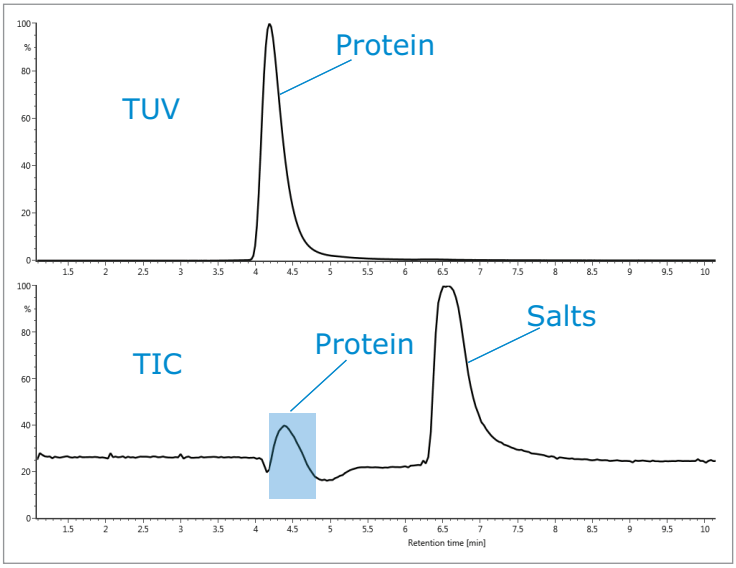

Figure 3. The TUV and TIC chromatograms from a typical native SEC-MS experiment.

Figure 4 shows the combined raw spectra multiple charge states envelope (left), the zoomed-in region (single charge state) of the combined raw spectra (center), and the deconvoluted spectra (right) of the reference materials (mAb), the low, moderate, and high conjugation level of cysteine-conjugated ADC samples without deglycosylation treatment from native SEC-MS analysis. The glycosylation pattern displayed in the reference mAb spectrum is repeated with good consistency in each conjugation form (0, 2, 4, 6, and 8) across all three levels of conjugated samples.

The integrated peak areas of each of the glycoform peaks from the deconvoluted spectra were used for automatic total average DAR and the drug loading distribution calculation within UNIFI as described in detail in reference [9].

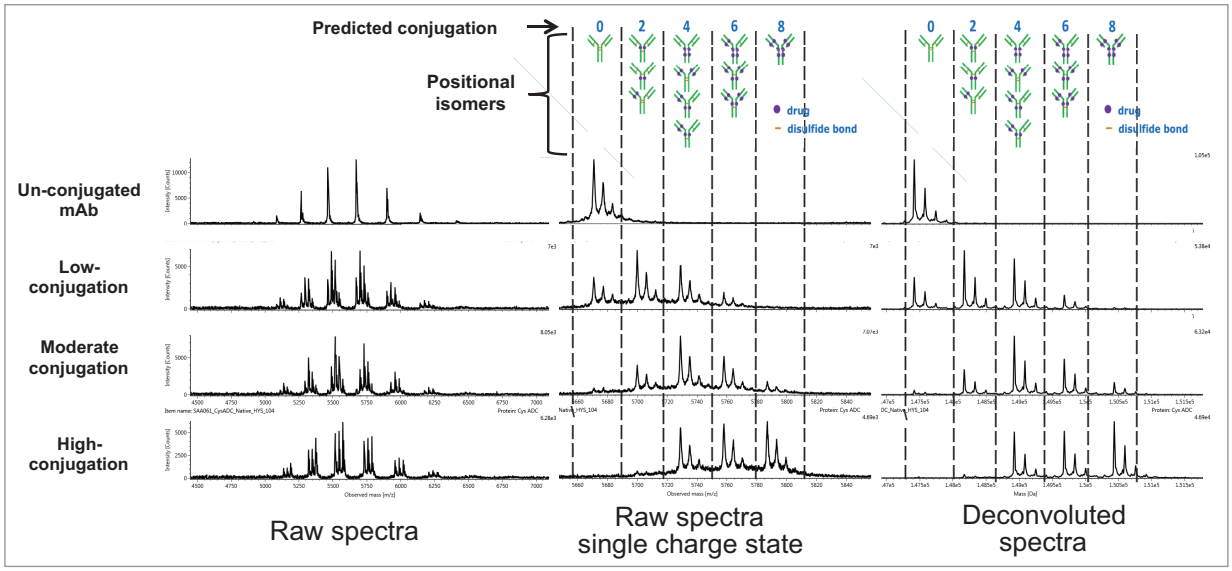

Figure 4. The combined raw spectra from multiple charge state envelope (left), the zoomed-in region (single charge state) of the combined raw spectra (center) and the deconvoluted spectra (right) of the reference materials (mAb), the low, moderate, and high conjugation level cysteine-conjugated ADC samples without deglycosylation treatment from native SEC-MS. Drug distribution was compared for three different cysteine-conjugated ADC samples with increasing drug load.

Table 1 compares the calculated DAR and drug load distribution for three batches of ADCs among the HIC using data collected from 3 years apart, the original native SEC-MS analysis using Xevo G2-S QTof System[9] and the new native SEC-MS analysis with Vion IMS QTof MS. HIC data was also generated in 2014. The 2014 SEC-MS data was collected after the ADC was treated with PNGase F. The experiment results from the HIC methods shown good agreement among the methods for both the individual DARs as well as the total average DARs for all three drug loading levels. For example, the individual DARs with six drug payload for the three samples from HIC were 0.75, 1.61, and 1.72; for the 2014 native SEC-MS method they were 0.60, 1.45, and 1.72; and for the 2017 native LC-MS method they were 0.64, 1.51, and 1.86 respectively. The total DAR values from the HIC method were 2.83, 4.44, and 5.97 (low, moderate, and high); for the 2014 native SEC-MS method they are 2.72, 4.40, and 5.97, and for the 2017 native SEC-MS method, they were 2.70, 4.37, and 6.07 respectively. The results from this study indicated that the measured DARs for this set of cysteine-conjugated ADC samples were consistent from orthogonal approaches (HIC vs. MS) and from different QTof MS systems (Xevo G2-S vs. Vion) and sample preparations (with and without deglycosylation). The results therefore also validated the improved method with increased sensitivity and robustness, as well as simplied sample preparation. This could be very benefitial for lot-to-lot, batch-to-batch comparison studies.

Cysteine-conjugated ADCs drug loading distribution and DAR									
	Low			Mod			High		
	HIC	QTof 1	QTof 2	HIC	QTof 1	QTof 2	HIC	QTof 1	QTof 2
ADC 2	0.81	0.74	0.64	0.38	0.41	0.35	0.07	0.09	0.05
ADC 4	1.14	1.17	1.37	1.67	1.57	1.81	1.23	1.11	1.19
ADC 6	0.75	0.60	0.64	1.61	1.45	1.51	1.72	1.72	1.86
ADC 8	0.12	0.21	0.05	0.78	0.97	0.70	2.95	3.05	2.98
DAR	2.83	2.72	2.70	4.44	4.40	4.37	5.97	5.97	6.07

QTof 1 *sample deglycosylated, run in 2014.* QTof 2 *sample non-deglycosylated, run in 2017.*

Table 1. Total average DARs and drug distribution comparison amongst the HIC, the original and the new improved native SEC-MS experiments shown very good agreement of the three methods for all three drug loading levels.

Figure 5 shows the raw and deconvoluted spectra of SEC-MS analysis of lysine-conjugated ADC Kadcyla (Trastuzamab Entansine (T-DM1)) without deglycosylation. The number on top of the deconvoluted spectrum peaks represents the detected number of drugs that are conjugated to the mAb, trastuzumab, in the intact level. The automatically calculated DAR in UNIFI is 3.56 compared to the reported DAR 0f 3.50 from the manufacture Genentech.[11] For lysine-conjugated ADCs, characterization has been successfully performed using standard reversed-phased (denaturing) LC separation/desalting conditions. However, pre-analysis sample treatment of deglycosylation is usually required in order to reduce the sample complexity, such as removing glycosylation to improve the sample heterogeneity, so that we can identify and quantify the distribution of different numbers of conjugated drugs, as well as the link-only species formed in the 2-steps conjugation process with less ambiguities. Compared to reversed-phased conditions, native SEC-MS raw spectrum shifts the charge envelope to higher *m/z* mass window separation of multiple charge species envelope, therefore, has the potential benefit of better resolving complexity of the lysine-conjugated ADCs without deglycosylation as demonstrated from the SEC-MS analysis of Kadcyla in here.

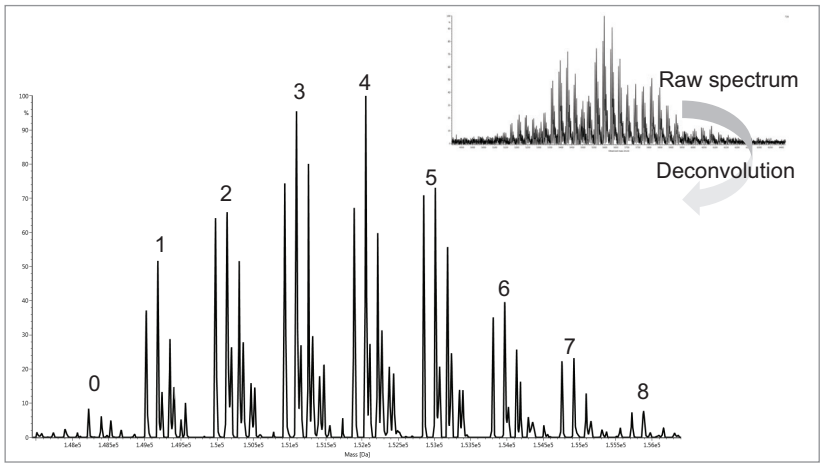

Figure 5. Raw and deconvoluted spectrum of SEC-MS analysis of Lysine conjugated ADC, Kadcyla (trastuzumab Entansine (T-DM1),) without deglycosylation. The number on top of the deconvoluted spectrum peaks represents the detected number of drugs that are conjugated to the protein Trastuzumab in the intact level. The calculated average DAR is 3.56, vs. the theoretical DAR of 3.50 [11].

CONCLUSIONS

We demonstrate the use of an analytical scale native SEC-MS analytical platform to examine both cysteine and lysine conjugated intact ADCs. Average DARs and drug loading distributions from the native SEC-MS analysis were compared with the values generated from HIC separation. We also compared the DARs value from two different QTof systems. Excellent agreement in DAR values was observed from HIC and SEC-MS studies. The current SEC-MS method has the benefit of increased sensitivity and robustness (higher system back pressure to ensure the peak retention reproducibility) and simplified sample preparation process (no need for sample deglycosylation). We believe that this native SEC-MS platform method adds benefit to 1) DAR and drug distribution calculations; 2) evaluation of new constructs and drug linker technologies; and 3) characterization of research molecules.

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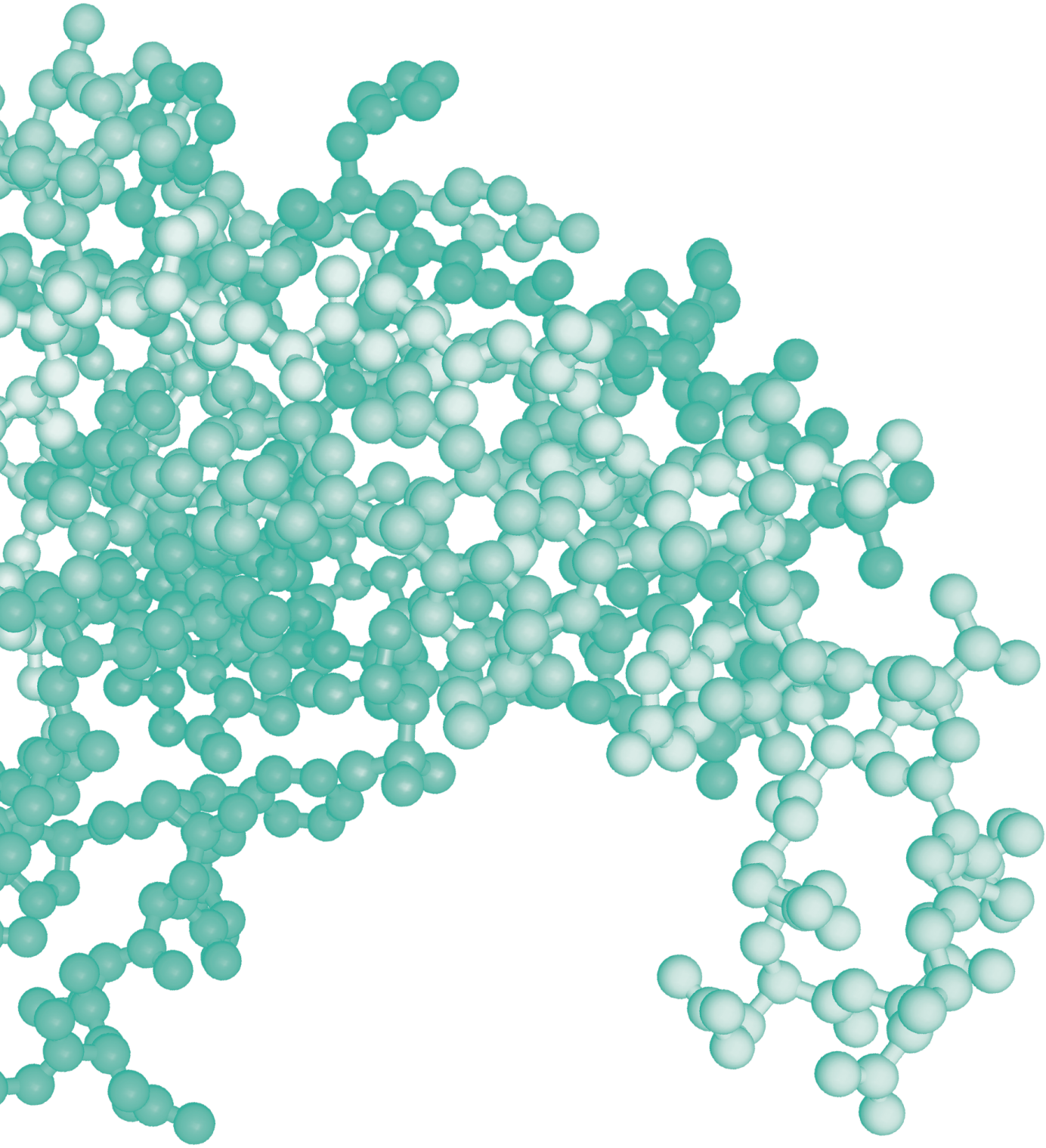
Best Practices for SEC Analysis



[TECHNOLOGY BRIEF]

Improving the Lifetime of UPLC Size-Exclusion Chromatography Columns using Short Guard Columns

GOAL

To demonstrate the improved long-term stability of the ACQUITY UPLC® BEH200 SEC, 1.7 µm column with the use of a guard column.

BACKGROUND

Size-exclusion chromatography (SEC) is commonly used to measure aggregates and other size variants in protein-based therapeutics. Of particular interest are soluble aggregates, which are thought to play a role in immunogenicity. Studies have found a variety of chemical and physical conditions can affect the amount of aggregates in biotherapeutics. To minimize these effects, excipients or stabilizers are typically added to protein-based therapeutics. Additives can decrease long-term column stability in SEC, resulting in inaccurate quantitation due to changes in retention, peak shape or spacing between peaks. While a variety of cleaning protocols may be used to try to restore the column, this approach can be time consuming and ultimately ineffective. As a last resort, column replacement is often required, resulting in higher costs and further delays in analysis time. With the introduction of the ACQUITY UPLC BEH200 SEC, 1.7 µm guard column, long-term stability for the SEC column can be increased, thereby saving time and money.

THE SOLUTION

The UPLC® SEC separation for biomolecules combines the ACQUITY UPLC H-Class Bio System with a sub-2 µm BEH SEC column. While the ACQUITY UPLC BEH200 SEC packing material is resistant to chemical and mechanical degradation for hundreds of injections, various sample components may adversely affect the column. With the use of a guard column in combination with the BEH200 SEC column, the column stability can be extended for hundreds of injections in the presence of these excipients.

In this set of experiments, protein standards, uracil and a murine monoclonal antibody (mAb) were analyzed on both a BEH200 SEC, 1.7 µm, 4.6 x 150 mm column alone and a BEH200 SEC, 1.7 µm, 4.6 x 150 mm column equipped with a guard column with the same packing material (4.6 x 30 mm). The mAb was diluted in a representative biotherapuetic formulation containing polysorbate 80, sucrose and sodium phosphate. The samples were analyzed on a single BEH200 SEC column over the course of 500 injections and on a BEH200 SEC column equipped with a guard column for over 900 injections. The guard column was replaced approximately every 200 injections. Evaluation of the mAb monomer efficiency on the BEH200 SEC column alone shows a decrease in plate count (from 5000 to 3000) after 550 injections. The same sample tested on the column and the guard combination shows no significant change in mAb monomer efficiency over 900+ injections (Figure 1). Any drops in monomer efficiency were restored after replacement of the guard column.

Before replacement of each guard column, the BEH200 SEC, 1.7 µm column was tested alone to check column efficiency. Comparison of this test to the initial column testing demonstrates the long-term stability achievable with a guard column (Figure 2). The mAb separation on the column alone shows a decrease in peak height and an increase in peak tailing over the course of 480 injections. Furthermore, the mAb monomer/dimer USP resolution decreases 26%. In contrast, the separation on the column protected by a guard shows no significant change in

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peak height or peak tailing over the course of 900 injections. The mAb monomer/dimer USP resolution remains relatively unchanged, demonstrating the effectiveness of the guard column.

SUMMARY

The combination of the BEH200 SEC analytical column and guard column allow for a stable analysis and quantitation of a monoclonal antibody in the presence of excipients. While the presence of excipients or other matrix components may adversely affect a SEC separation over time, the regular replacement of the guard column preserves the performance of the analytical column for over 900 injections. This allows the biochemist to reduce costs and minimize down time, thereby resulting in more stable analyses for monoclonal antibodies and their soluble aggregates.

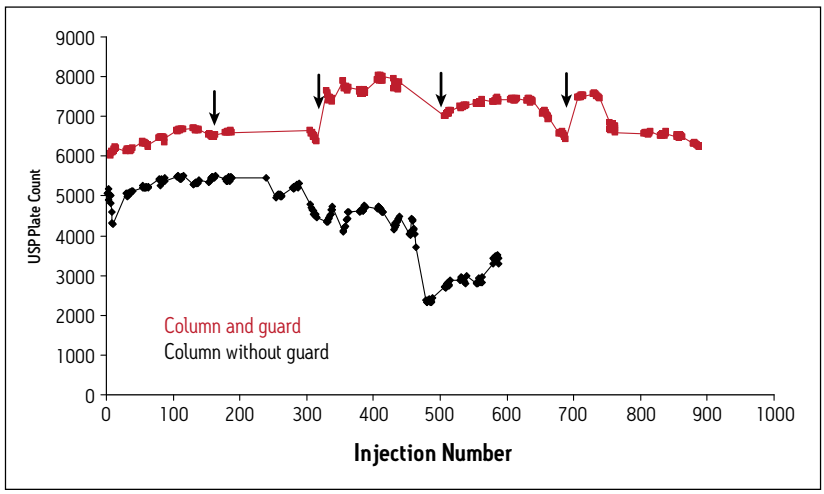
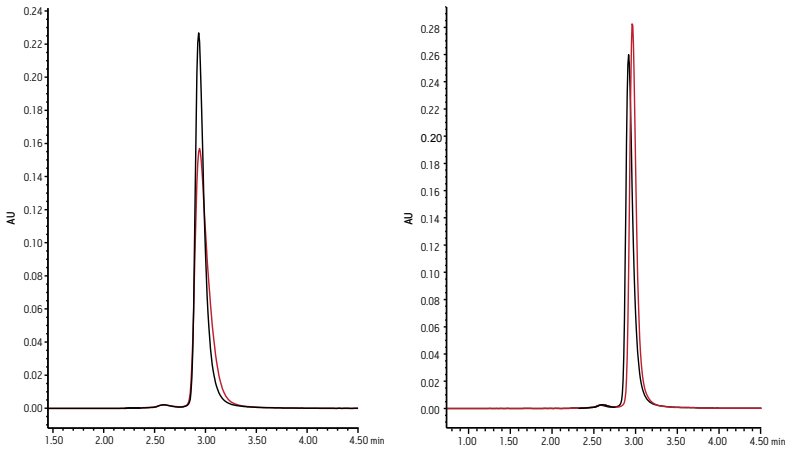


Figure 1. Effect of using a 30 mm guard column on column efficiency. The arrows indicate where the guard column was changed.



No Guards Over Lifetime

Injection	USP Res	USP Tailing
6	1.84	1.70
488	1.35	1.80

Guards Replaced Every 200 Injections

Injection	USP Res	USP Tailing
2	1.75	1.63
902	1.79	1.45

Figure 2. Effect of a 30 mm guard column on mAb separation performance. Chromatograms of mAb on BEH200 SEC column before and after multiple injections of a formulated mAb sample. Note: Chromatograms at right were run on column without guard to confirm column efficiency, i.e. column check. Conditions: 0.025 M sodium phosphate pH 6.8, 0.15 M sodium chloride, 0.4 mL/min at 30 °C.

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Best Practices for Achieving Optimal Separations and Long Column Lifetimes in UPLC SEC of Proteins

Pamela Iraneta, Matthew Lauber, Susan Rzewuski, Bill Warren, Stephan Koza and Tom Walter
Waters Corporation, Milford, MA

INTRODUCTION

Size-exclusion chromatography (SEC) is a technique commonly used throughout the development and commercialization of biotherapeutic proteins, such as monoclonal antibodies (mAbs). Ideally, these proteins and their molecular weight variants are separated based solely on their relative size in solution. ACQUITY™ UPLC™ Protein BEH SEC, 200 Å, 1.7 µm Columns are frequently used for the relative quantification of mAb monomers and associated high and low molecular weight species in less than 8 minutes.

Several factors contribute to the generation of high quality and reproducible UPLC-based SEC separations of proteins. Included are the selection of an appropriately configured LC system, and the proper attachment of the column to the LC system. Particular attention will be given to practices for avoiding microbial contamination of the mobile phase and particulates from the samples.

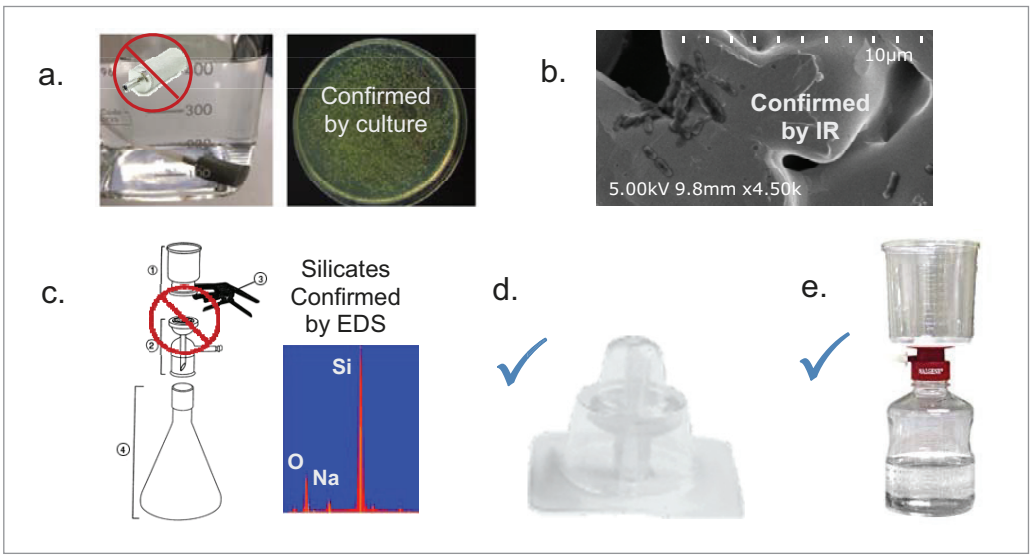


Figure 1. Problems and Recommendations: a. Metal sinkers harbor microbes; b. Scanning electron micrograph of bacteria on column frit; c. Silica-based supports can add silicates and apparatus is not sterile; d. Low-bind sterile 0.22µm syringe filters; e. Sterile Nalgene 0.2µm nylon filter unit.

METHODS

System: ACQUITY UPLC H-Class Bio

- ACQUITY UPLC TUV with 5 mm path titanium flow cell (p/n: 205000611)
- Column Heater (CH-A pn 186015042) with MP35N active pre-heater (p/n: 20500756) set to 30 °C
- Sample Manager with an MP35N 15 µL Flow-Through Needle (SM-FTN)
- bioQuaternary Solvent Manager (bioQSM)

Column: ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 150 mm and BEH200 SEC Protein Standard Mix (p/n: 176003904)

Mobile phases:

- 0.1 M sodium phosphate 0.1M NaCl pH 7.0 filtered w/ Nalgene 0.2 µm nylon filter unit in sterile bag
- Milli-Q water using a point of use Millipak® Express 40, 0.22 µm filter
- Wash solvent and seal wash: 10% acetonitrile/90% water
- Purge Solvent: 10% methanol/90% water

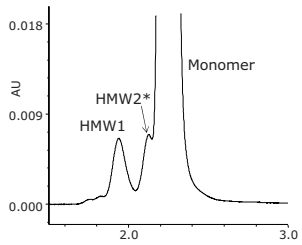
Samples:

- Protein Mix (mg/mL): thyroglobulin (1.36), IgG (0.9), bovine serum albumin (2.3), myoglobin (0.9), uracil (0.05) reconstituted with 1.1 mL water
- Panitumumab at 10 mg/mL – diluted from 20 mg/mL (lot 1069811) with water
- Fully humanized monoclonal IgG2 antibody specific to the epidermal growth factor receptor
- Uracil at 0.05 mg/mL in phosphate mobile phase

Flow rate: 0.5 mL/min with a 5 minute run time

UV detection wavelength: 280 nm

Injection volume: 3.5 µL of Vectibix was injected (6 µL for uracil and protein mix)



Average ± stdev during *1000 injection* study:
1) Uracil JP Plate: 35,824±775
2) Monomer JP Plate Count: 7,890±190
3) HMW2 end p/v: 1.10±0.03
4) % Areas: 1.56±0.02 for HMW1, 0.97±0.02 for HMW2
All RSDs ≤2.4%

ACQUITY UPLC BEH SEC separation of a biotherapeutic monoclonal antibody (mAb).

Performing lifetime studies is not easy to do. We endured power outages and supply chain issues during the winter of 2016. However, we were one step ahead of the game. Mobile phase sinkers (Fig. 1a) were not in use in the lab for many years, given our prior knowledge of their source of microbial contamination.[1] This eliminated a major source of microbial contamination. Early in these studies we experienced problems with microbial (Fig. 1b) and silicate column contamination when using silica-based support filtrations apparatus (Fig. 1c). These issues were resolved when we filtered our mobile phases using prepackaged sterile 0.2 µm Nylon, Nalgene filter units (Fig. 1e).

We started our studies using a mixture of lyophilized proteins (Fig. 2a). Initially, the protein mix was reconstituted with 1.1 mL of water but was not filtered or centrifuged before use. Very short lifetimes (≤340 injections) were obtained on two different columns from different lots.

The mechanical stability was checked by performing injections of uracil with every 10th as the protein mix. This column reached 600 injections with no efficiency loss when the study was stopped. Another column was then tested using a filtered (Fig. 1d) protein mix sample and it reached 695 injections when it suddenly lost 75% efficiency (Fig. 2b). It was clear that sample filtration extended column lifetime.

For our next column lifetime study we chose a formulated IgG_2, panitumumab (Vectibix), because of its challenging HMWS peak shape. The HMW2 peak was reported to be potentially a trimer of light chain subunits by Guillarme et al.[2] Its resolution was monitored using p/v ratio. A formulated IgG solution was also chosen because it was expected to be free of particulates unlike the protein mix used in the scouting experiments.

It is important to point out that even formulated protein solutions do not remain particulate free. Mishandling (agitation, stirring, vortexing, exposure to multiple freeze-thaw cycles or heating) can lead to the formation of insoluble protein aggregates of visible and sub-visible size.[3] In one paper the authors demonstrated that centrifugation for 10 min at 9300g, removes insoluble material and that this process did not affect SEC data for the soluble aggregates in the protein species distribution.[4]

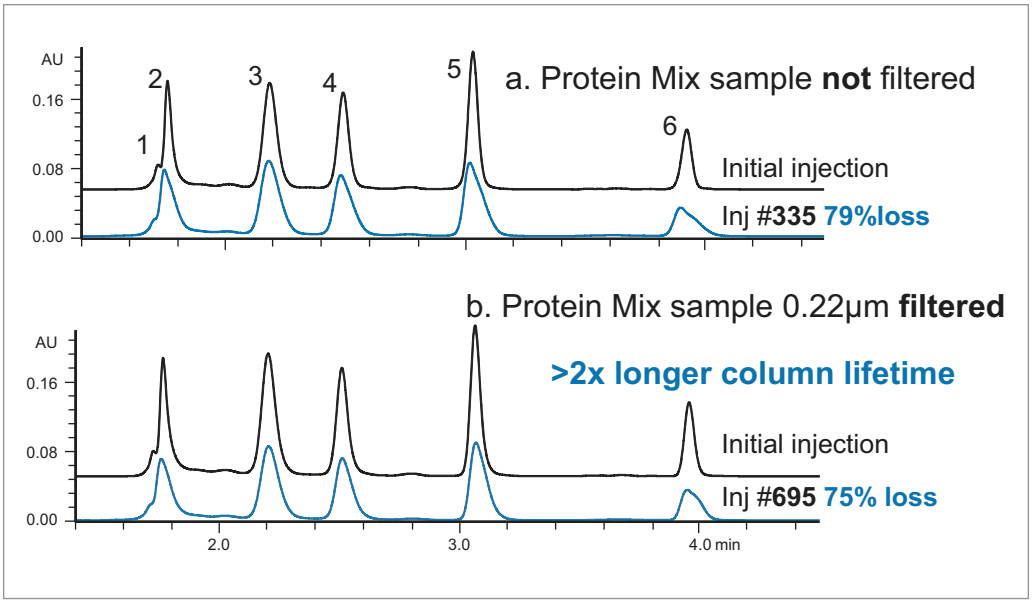


Figure 2. Protein Mix 1) thyroglobulin dimer, 2) thyroglobulin, 3) IgG, 4) BSA, 5) myoglobin and 6) uracil (JP Plate loss).

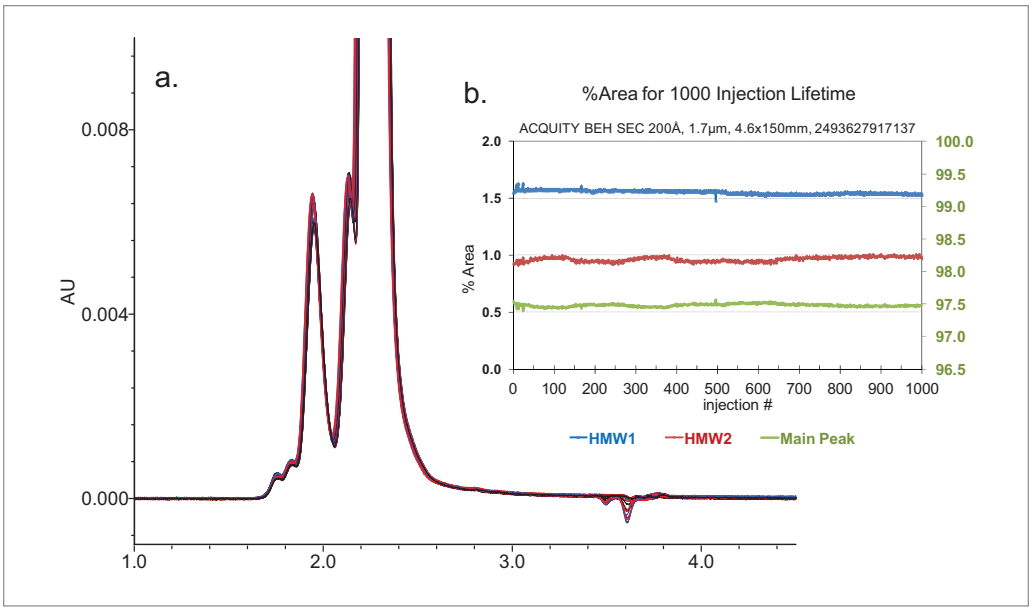


Figure 3. 1000th injection lifetime on a BEH200 SEC, 1.7 µm, 4.6 x 150 mm column: a. Overlay of every 20th injection for panitumumab (10 mg/mL); b. Relative %area data for 1000 injections

To generate high quality UPLC-based SEC separations on sub-2-µm packed columns, an LC system with very low dispersion is required. To take advantage of the speed and resolution capabilities of sub-2-µm packed columns, one must use of smaller diameter (4.6 mm I.D. vs. 7.8 mm I.D.) columns to mitigate detrimental radial thermal gradients that form due to viscous frictional heating of the mobile phase as it is forced through the much smaller channels in sub-2-µm packed columns.[5, 6, 7] The variance of our ACQUITY UPLC H-Class System used in these studies was 2.8 μL^2 (8.3 µL width at 4.4%).

The need for particulate free mobile phases and samples is not new to those familiar to the art of ultra-high pressure LC. Use of 0.2 µm filtered mobile phases for UPLC has been standard practice since its introduction in 2005. *Particulate free mobile phases and samples are required for long column lifetimes.*

In addition to particulate free, STERILE filtration, for mobile phases containing organic solvents (in HILIC and RP LC) or very high molar salts (in IEX and HIC) is often not rigorously practiced due to the reduced risk of microbial contamination in these solutions. SEC mobile phases are often ideally suited to nurture the growth of microbes — particularly with the addition of a little iron (Fig 1a) contamination. *Sterility is required for reproducible peak shape and column lifetime.*

Under appreciated is the fact that iron is an essential element for microbial growth as well as for all other living organisms.[8,9] The metal sparge/sinkers in our mobile phase bottles have, at best, 10 µm filtering capabilities. At worst, supply our systems with trace metals and microbial contaminants. *PLEASE remove sinkers from your SEC systems.*

As one of the common column lifetime failure modes, especially in early spring and through the summer months, *use 70% isopropanol (IPA) or 70% ethanol to effectively kill bacteria in your system.*[10] This "cleaning" is best done through the Waters Console, selecting System > to Control > to the Prime solvents... function. To avoid accidental precipitation of salt, flush lines with water before initiating the 70% IPA prime function for a 6 min duration per lines. The use of the prime function, at 4 mL/min through the selected lines, vigorously purges and helps clean the system.

For details on cleaning protocols and other useful tips and tricks see the SEC Optimization Guide (p/n: 720006067EN).

CONCLUSION

1. An inert LOW DISPERSION (<5.8 μL^2) LC system is required for the acquisition of high quality sub-2-µm SEC.
2. REMOVE sinkers from ALL solvent lines and do NOT replace them.
3. CLEAN and STERILIZE (70% isopropanol) LC system as outlined in the SEC optimization guide.
4. Prepare FRESH SEC eluents and STERILE filter using 0.2 µm Nylon Nalgene units.
5. Do NOT "top off" SEC mobile phases.
6. FILTER or CENTRIFUGE samples to remove insoluble particulates that will shorten column lifetime if they remain in injected samples.

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Best Practices for Maintaining Column Performance in Size-Exclusion Chromatography during Long-Term Storage

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APPLICATION BENEFITS

- Data confirms the non-corrosive nature of the 10% acetonitrile/25 mM sodium phosphate pH 7, 100 mM potassium chloride on 316 stainless-steel column frits used in Waters BEH-based SEC columns
- Results support the use of 10% acetonitrile in 25 mM sodium phosphate pH 7, 100 mM potassium chloride over 20% methanol/80% water for long-term column storage
- Data provides insights into factors influencing SEC column performance after storage

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class PLUS Bio System

BioResolve™ SEC mAb Columns

Empower™ Chromatography Data System

ACQUITY UPLC Tunable UV Detector

KEYWORDS

Size exclusion, monoclonal antibodes, mAbs

INTRODUCTION

It is well known that the mobile phases used for the characterization of native peptides and proteins in SEC are capable of growing microorganisms which can "infect" columns and lead to degradation of a column's resolving power. Furthermore, it is known that these columns infected with microorganisms produce contaminated fractions. What might not be appreciated is the fact that the mobile phases used for the characterization of native peptides and proteins are well within the pH and salt ranges that enhance bacterial growth.[1,2,3]

Most SEC column manufacturers use sodium azide as the bacteriostatic agent for the shipping and storage of SEC columns. Concentrations between 0.02–0.05% are typically used with the highest recommended concentration of 0.1%. Sodium azide at a concentration of 0.05% has been found to be effective as a bactericide for many gram-negative bacterial.[4] Gram-positive bacteria are more resistant to sodium azide and have been found to grow in media containing 1% sodium azide.[5] The content of the cited papers and others[6,7] indicate that there is no silver bullet for preventing microbial growth that would be suitable under SEC conditions. Due to a bacteria's capacity for rapid change via horizontal DNA transfers (plasmids), bacteria find ways to adapt to stressors. As one of the earliest examples of this, just four years after the scaled-up production of penicillin (1947), the first strains of penicillin resistant *Staphylococcus aureus* were found. The best recourse against damage to SEC columns due to microbial "infection" is prevention. Unlike other modes of column fouling, microbial growth can continue even when the stored column is not in use if it does not contain a bacteriostatic or cidal reagent.

At the time of this writing, Waters Corporation declines/prohibits the use of sodium azide in any of its manufacturing facilities because of the risks associated with its use. Sodium azide is a highly water soluble, inorganic salt that is very acutely toxic, RTECS #VY8050000.[8] Even small amounts, if swallowed, can be fatal[9] and no known antidotes have been found.[10] Mixing sodium azide with acid produces the highly toxic hydrazoic gas and contact with copper, lead, brass, or solder in plumbing systems can lead to the formation and accumulation of explosive metal azides.[11]

For these reasons we have chosen to use 20% methanol/80% water or 10% acetonitrile (ACN) in 25 mM sodium phosphate ($Na\text{-}PO_4$) pH 7, 100 mM potassium chloride (KCl) as the bacteriostatic solutions for the elimination/minimization of microbial growth during SEC column storage.

Based on our historical use of these solutions[12] we believe they are as effective as sodium azide in protecting SEC columns from microbial growth. Searches for organic solvent-tolerant (OST) bacteria, useful for remediation purposes, indicate that OST bacteria are difficult to find. The successful identification of an acetonitrile tolerant strain found that 10% ACN killed the bacteria when in its initial growth phase.[6]

The goal of SEC column storage protocols is to maintain existing chromatographic performance of a column during short term and/or long-term intervals of inactivity. Common issues encountered after storage are changes in protein retention times, poor recovery of HMWS, and/or loss of the resolving power for the column. The focus of this investigation is to provide experimental support for the choice of our new shipping/storage solvent: 10% acetonitrile (ACN) in 25 mM Na-PO_4 pH 7, 100 mM KCl.

Changes in protein retention times are typically minor for SEC columns in buffers with pH <7. Other common issues associated with column storage, although not desirable, can typically be mitigated with injections of protein samples such as Waters™ BEH200 SEC Protein Standard Mix (p/n: 186006518) to re-condition or "re-passivate" the column. The most significant factor contributing to the catastrophic failure of a column during storage is microbial growth. This failure mode is not directly addressed in this application note, but based on historical use in our laboratories, low concentrations of organic solvents effectively mitigate the risk of microbial growth in SEC columns free of gross contamination. The goal of this application note is to review the impact of the long-term storage solvents on the performance of BioResolve SEC mAb Columns after storage periods of one to four months.

Before proceeding to the long-term storage data, the following provides general guidance for maintaining column performance during short-term storage:

- Storage under refrigeration (never frozen) can only be successful if column end plugs are tightly installed to prevent solvent/buffer evaporation which can cause the precipitation of buffers and/or salts. In addition, the introduction of oxygen has been linked to enhancing the growth of microbes.[4] Addition of a bacteriostatic agent to the storage solvent is highly recommended even in a refrigerator at 4 °C. Refer to your column's instruction manual for guidance on the best short-term storage solvent.
- Although many, if not most, size-exclusion chromatography (SEC) sample analyses begin with purging the SEC column with high purity sterile water (which remains a good operating step) the amount of water used should be limited to less than 10 column volumes (CV). Extensive purging (200 CV) with 100% Milli-Q water can result in poor recovery for the high molecular weight species (HMWS). See Figure 1.
- Never store a SEC column on the LC system, under zero flow conditions, in pH 5–8 buffers without the use of a bacteriostatic agent. A single colony forming unit (CFU) can easily multiply into millions provided conditions can support the growth when a column is stored.[2] Maintaining a low flow on the column is preferable to stopping the flow both for the column and the system,[13] but only if the mobile phase and system remains microbe free.
- Bacteria are known to produce metabolic products that enhance their ability to survive and/or grow.[2,4] Studies in which bacteria have been "washed" prior to subsequent inoculation have shown that survival is less likely in hostile environments.[2,4] Maintaining a low flow rate for short periods of time while the column is not actively in use on the system may minimize the possibility of a bacterium entering a rapid growth phase. This is useful only if the system and mobile phase remains microbe free and is preferable to the short-term storage of the column under zero flow conditions without a bacteriostatic agent added to storage solvent.

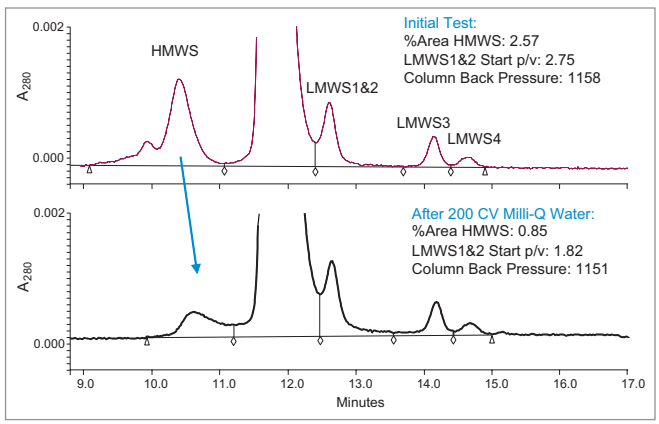

Figure 1. Effect of extensive purging with Milli-Q water on recovery of HMWS and resolution for LMWS1&2 on BioResolve SEC mAb, 200 Å, 2.5 µm, 4.6 × 300 mm Column. One column volume (CV) for the 4.6 × 300 mm column is equal to 5 mL. Sample: Waters mAb Size Variant Standard. Conditions given in the experimental section.

EXPERIMENTAL

Sample description

Waters mAb Size Variant Standard (p/n: 186009284) contains 160 µg of stabilized and lyophilized NISTmAb RM8671 which has been supplemented with 2 µg of non-reduced IdeS (Fabricator) digested NISTmAb fragments ($F(ab')_2$ and $(Fc/2)_2$). The lyophilized contents of each vial were solubilized using 70 µL of Milli-Q water. More information on the Waters mAb Size Variant Standard can be found on waters.com, search for 720006811EN.

LC conditions

Systems:	ACQUITY UPLC H-Class Bio
Detectors:	Tunable Ultraviolet (TUV) with a 5 mm Ti Flow Cell for ACQUITY UPLC H-Class Bio
Detection:	280 nm, 10 Hz, fast filter
Vials:	Max Recovery Sample Vials (p/n: 186000327C)
Column(s):	BioResolve SEC mAb, 200 Å, 2.5 µm, 4.6 × 150 mm (p/n: 176004592*) *Includes column and one complimentary vial of mAb Size Variant Standard
Column temp.:	35 °C Active preheater CH-A (H-Class)
Sample temp.:	8 °C
Sample:	2.28 mg/mL Waters mAb Size Variant Standard
Injection volume:	1.8 µL
Flow rate:	0.200 mL/min
Seal wash:	10% HPLC-grade methanol/90% 18.2 MΩ water v/v (seal wash interval set to 0.5 min)
Sample manager washes:	18.2 MΩ water
Mobile phase A:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Mobile phase B and C:	18.2 MΩ water
Mobile phase D:	10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM potassium chloride
Syringe draw rate:	30 µL/min
Needle placement:	1.0 mm
Air gaps:	None
Data channels:	System pressure, room temperature
Mobile phase A:	Prepare by mixing 2.66 g of anhydrous dibasic sodium phosphate, 4.36 g of monobasic potassium phosphate mono hydrate, and 14.91 g of potassium chloride per L of water followed by filtration using sterile 0.2 µm nylon filter units (filtered mobile phase pH 6.9)
Chromatography software:	Empower 3, FR 3.0

RESULTS AND DISCUSSION

There is very little literature comparing the effects of various column storage solvents on the performance of SEC columns. Only one paper could be found which compared 100% methanol, 10% methanol, and 0.001% sodium azide in water as storage solvents.[14] The 100% methanol solvent was found to remove peptides that had previously been used to condition the examined SEC columns.[14] Support for the use of buffers containing sodium azide and low organic/water solvent solutions can be found in most SEC column manufacturer's instruction manuals. Unique to the shipping/storage solvent recommended for BioResolve SEC mAb Columns is the addition of a buffer plus salt to a 10% acetonitrile solution.

The present study explores the effects of 10% acetonitrile in water or in combinations of buffers with and without salt. Concerns over the use of chloride-containing storage solvents are addressed by several corrosion studies. Comparisons are made to the previously recommended storage/shipping solvent of 20% methanol in water. The five different shipping/storage solvents compared for use with BioResolve SEC mAb, 200 Å, 2.5 µm, 4.6 × 150 mm Columns are:

A: 10% acetonitrile/90% 25 mM Na-PO_4, pH 7.0 with 100 mM KCl

B: 10% acetonitrile/90% 2.5 mM Na-PO_4, pH 7.0 with 10 mM KCl

C: 10% acetonitrile/90% 20 mM Na-PO_4, pH 6.8

D: 10% acetonitrile/90% Milli-Q water

E: 20% methanol/80% Milli-Q water

In this study, a total of 15 columns were packed and tested using a 50 mM Na-PO_4, pH 7.0, 200 mM KCl mobile phase with the Waters mAb Size Variant Standard (p/n: 186009284). After the initial testing of all 15 columns, sets of three columns were flushed with 10 column volumes (CV) into each of the above five storage solvents. After the initial testing of the 15 columns, one column from each storage solvent group was retested after one, two, and four months of storage at room temperature.

The Waters mAb Size Variant Standard is supplied with a certificate of analysis for each prepared standard lot. It is comprised of the NISTmAb Reference Material (RM) 8671 (a humanized monoclonal antibody) and non-reduced IdeS digested NISTmAb fragments LMWS2 (~100,000 Da) and LMWS3 (~50,000 Da), two mAb fragments with similar molecular weights as the IdeS fragments are endogenous to NIST RM 8671, LMWS1, and LMWS4, respectively. An example chromatogram of the Waters mAb Size Variant Standard and the NIST RM 8671 is shown in Figure 2. For more information, see 720006811EN.

All the investigated storage solvents maintained equivalently unchanged column performance for up to one month. Most of the Waters Empower Chromatography Data System parameters monitored during the four-month study did not show significant changes. Minor changes in retention time for the main peak were observed for all solvent groups (<0.2 min). All solvents showed a slight increase in retention which remained steady after two months. These slight changes would have likely to of occurred in a shorter period with routine column use.

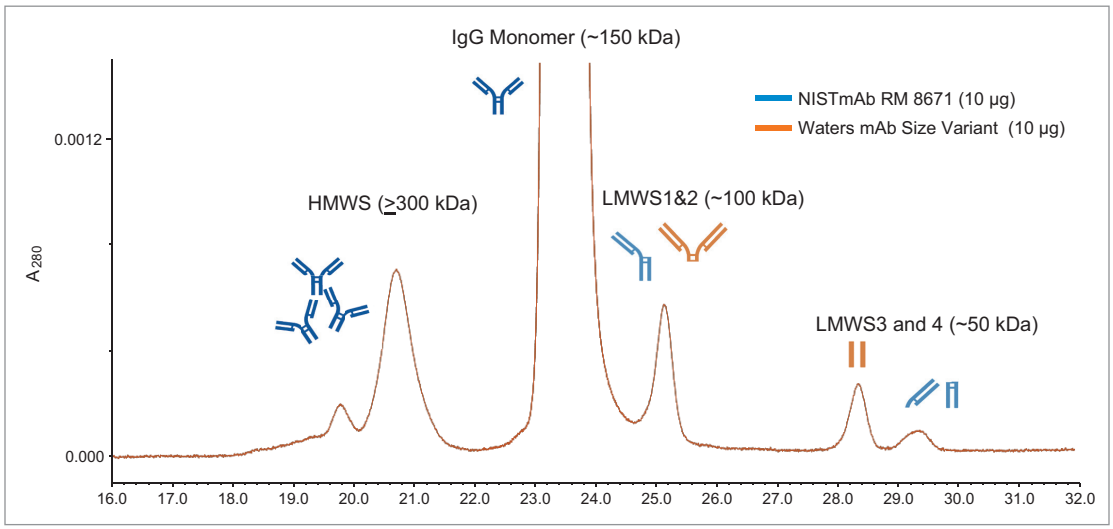

Figure 2. Separation of mAb aggregates, monomers, and fragments of NISTmAb RM 8671 and Waters mAb Size Variant Standard using Waters BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column. LMWS: $F(ab')_2$ and $(Fc/2)_2$ IdeS fragments (orange); Fab/c, Fab, Fc hydrolytic degradation fragments (blue). Conditions: Ambient temperature and 0.3 mL/min.

HIGH MOLECULAR WEIGHT SPECIES

The Empower chromatographic parameters that showed significant changes are shown in Figure 3. The % areas for the HMWS continued to decline (Figure 3a) after one month of storage in solvents that did not contain buffer and/or salt. The dimer-main peak resolution (USP Resolution at Half-Height, USP Res. HH) also continued to decrease over time in these buffer/salt free solvents. Of interest is the contrast illustrated using 10% acetonitrile in water versus the 10% acetonitrile with buffer and salt (Figure 4). The 10% acetonitrile in water showed the largest loss of the HMWS at four months. This is consistent with a previous study (data not shown) that found purging a column with larger amounts (>10 CV) of 20% methanol/80% water showed a similar effect. We did not select methanol as the antimicrobial agent for the BioResolve SEC mAb Column storage solvent due to the impact of unknown low-level impurities in methanol that in the past negatively impacted column performance.

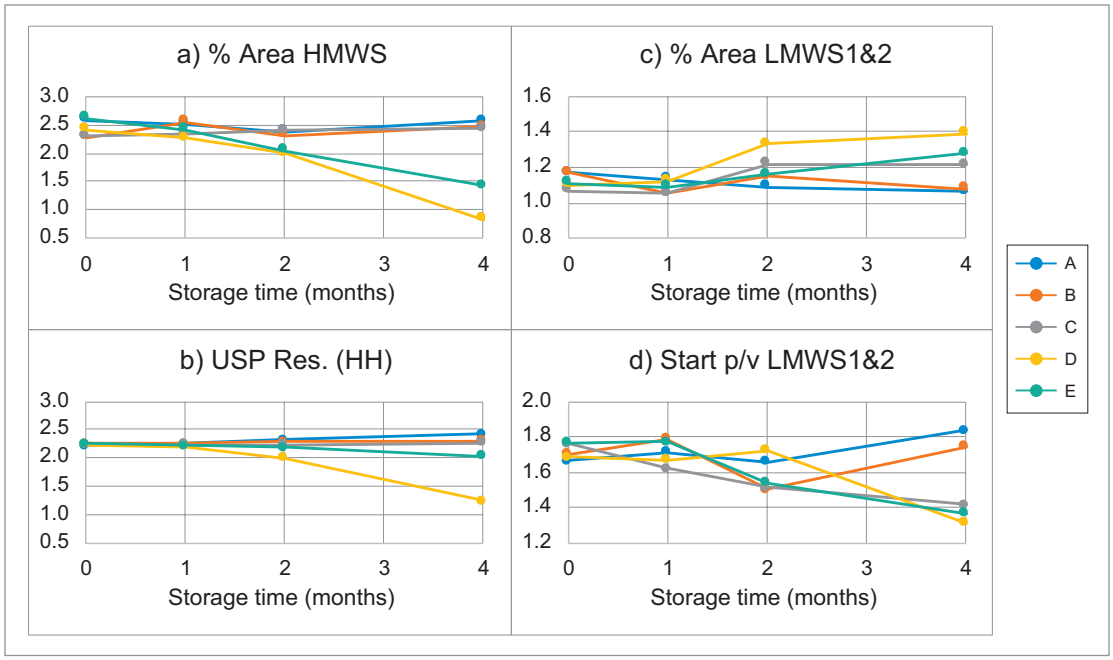

Figure 3. Effect of selected storage solvents on BioResolve SEC mAb, 4.6 × 150 mm Column performance using Waters mAb Size Variant Standard: a) %HMWS, b) USP resolution at half height, c) %LMWS1&2, and d) Start p/v for LMWS1&2. Conditions given in the experimental section.

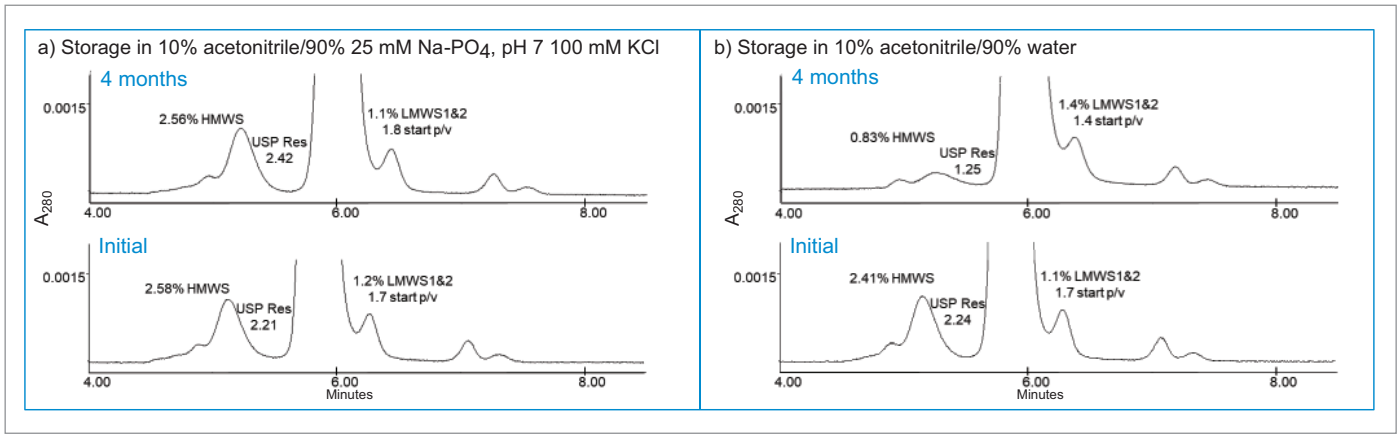

Figure 4. Chromatographic comparison of Waters mAb Size Variant Standard on BioResolve SEC mAb, 200 Å, 2.5 µm, 4.6 × 150 mm Columns initial and after four months storage in a) 10% acetonitrile/90% 25 mM Na-PO_4 pH 7, 100 mM KCl and b) 10% acetonitrile/90% water. Conditions given in the experimental section.

LOW MOLECULAR WEIGHT SPECIES

Figures 3c and 3d show the impact of the storage solvent on the % area for LMWS1&2 and its resolution from the main peak. The LMWS1&2 % area increases as a result of decreasing resolution from the main peak. It has been observed that as resolution decreases the % area for LMWS1&2 increases due to the increasing addition of main peak area. This resolution was monitored using the Empower parameter: start peak to valley heights (start p/v) for LMWS1&2 because the more typically used resolution parameter (USP Res. HH) could not be calculated. Again, the storage solvents that did not contain buffer plus salt did not maintain the column's initial start p/v after a storage period of about two months.

CORROSION STUDIES

Stainless steel (SS) is a specialized group of steel alloys designed to resist corrosion. All steel components in the fluid path of Waters SEC columns are austenitic 316 SS. This steel is designed to be corrosion resistant. Corrosion of stainless steel in aqueous solutions is a function of pH, halide (chloride) and/or sulfide concentration, and temperature. The corrosion rate of SS is enhanced by lower pHs, increased chloride concentrations, and higher temperatures. Working with the chloride salt containing buffers used in SEC brings up concerns of rusting for SS parts. Rust (iron oxide) forms as an oxidation product of iron. Iron combines with water and oxygen to form the insoluble reddish-brown ferric hydroxide oxide.

We are all familiar with the risk of rust formation in LC systems as well as columns with the use hydrochloric acid (HCl). HCl is frequently used to facilitate aggressive ranking of various types of steel for pitting corrosion. Its aggressive nature is blamed on the combined effects of the acidic environment as well as the chloride ion. The corrosion situation is quite different when the pH is in the range used during SEC (pH 5–8) as is the case for all five of the storage solvents tested. This is thanks to metal oxide/hydroxide layers that are maintained on the metal surface, protecting it from attack. These layers are more readily dissolved under acidic conditions.

Based on extensive experience, the most frequent site for rust formation in columns is the frits. This is due to the higher surface area present compared the column tube wall in addition to the higher oxygen levels at the column inlets and outlets. Other sources of contamination such as microbes, catalysts, or other metals can also accelerate corrosion. In order to check for evidence of frit rusting, all the columns were opened and examined for rust after four months storage in the different storage solvents. None of the storage solutions caused rusting during the four months of storage at ambient temperatures.

Although ASTM G48 is one of the most common standard tests used to rank various metals for corrosion, it relies on conditions (the very acidic ferric chloride solution) that are dissimilar from those required to monitor corrosion under SEC conditions. To assess rusting under our test conditions, frit "soaking" experiments were conducted using:

1. 10% acetonitrile/90% 25 mM Na-PO_4, pH 7.0 with 100 mM KCl
2. 10% acetonitrile/90% 2.5 mM Na-PO_4, pH 7.0 with 10 mM KCl
3. 10% acetonitrile/90% 0.25 mM Na-PO_4, pH 7.0 with 1 mM KCl
4. 10% acetonitrile/90% Milli-Q water

In this second experiment, 10 mL of each of the above solutions was added to 20 mL scintillation vials. Each solution was set up in duplicate with four, 0.2 µm 316 SS frits. The capped vials were sonicated for 5 minutes then aged at ambient temperature or 60 °C. The vials were checked under a microscope periodically for seven weeks. During this time period there was no evidence of rust formation even at 60 °C. After the seven weeks, all the vials were maintained at ambient temperature for nine months and still no signs of rust formation were detected.

In the third experiment, ASTM B895 was used. The ASTM B895 test is specifically designed to test porous stainless-steel samples. It consists of soaking porous stainless steel (SS) in 5% NaCl (855 mM) until corrosion occurs. The test was made more aggressive by soaking the frits in an oven at 60 °C. Our SS frits were tested along with other porous materials. After seven weeks submerged in the 5% NaCl the other materials showed a significant amount of rust while our SS frits remained rust-free.

CONCLUSIONS

The choice of acetonitrile as the co-solvent in the shipping/storage solvent was based at least in part on its cell toxicity profile as shown by its efficacy in killing an extremophile bacterial strain at 10% acetonitrile.[6] Only storage solvents that contained buffer plus salt maintained the initial performance of the BioResolve SEC mAb Columns. The storage solution that performed the best was the 10% acetonitrile/90% 25 mM sodium phosphate pH 7, 100 mM KCl. The more dilute version of this buffer gave very similar performance suggesting that there is range of buffers plus salt that can be useful as storage solvents. It is noteworthy to mention that the column containing only buffer in 10% acetonitrile (no salt) maintained its performance for the HMWS but showed degradation in the main peak/LMWS1&2 resolution. In contrast, the 10% acetonitrile in water showed a 66% loss in HMWS % area, with a 44% decrease in the USP resolution at half height between the dimer and monomer, and a 26% increase in the LMWS1&2 % area that resulted from a 22% decrease in the start p/v resolution. The 20% MeOH/80% water solvent showed similar changes to those of the 10% ACN/90% water but to a lesser degree.

Corrosion testing of the column frits further confirmed the non-corrosive nature of the selected storage solvent. This study suggests that the use of a buffer plus salt environment helps maintain the initial column performance after long-term storage with the use of 10% acetonitrile as the bacteriostatic agent.

Acknowledgements

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Comparison of Sub-3-µm HP-SEC Columns for the Analysis of IgG Antibody Aggregates (HMWS) and Fragments (LMWS)

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APPLICATION BENEFITS

- Improved performance using the Waters™ BioResolve™ SEC mAb Column for mAb (cetuximab) fragment analysis while providing comparable aggregate analysis in comparison with several commercially available SEC columns.
- A column (7.8 × 300 mm) and particle size (2.5 µm) with previously demonstrated compatibility with HPLC, UHPLC, and UPLC™ systems.
- Improved efficiency and peak tailing performance versus commercially available SEC, sub-3-µm, 7.8 × 300 columns for improved separations of mAbs and other comparable-sized therapeutic proteins.

WATERS SOLUTIONS

BioResolve SEC mAb Columns

Empower™ 3 Chromatography Data Software

KEYWORDS

Size-exclusion chromatography, mAbs, HMW, LMWS, cetuximab

INTRODUCTION

Size-exclusion chromatography (SEC) has been the method of choice for the routine assessment of protein aggregation (high molecular weight species, HMWS) and has also been used for the non-denatured analysis of protein fragments (low molecular weight species, LMWS).[1] LMWS for many mAb biotherapeutics are the result of proteolytic cleavage at the IgG hinge region resulting in an Fab-Fc LMWS (LMWS1, ~100 kDa) and Fab and Fc domains (LMWS2, ~50 kDa).[2]

While multiple HPLC SEC (HP-SEC) columns in series or reduced linear velocity can be used to provide the efficiencies needed to reliably separate LMWS1 from the mAb monomer, this separation has generally been performed using higher efficiency UPLC-SEC (UP-SEC) columns with particle diameters of 2 µm and smaller to enable reasonable analytical throughput.[3] While SEC columns packed with sub-2-µm particles can provide the highest sample throughputs for the analysis of HMWS, these columns are typically manufactured with internal diameters (I.D.) of 4.6 mm, and as a result when used for the analysis of LMWS1, UPLC systems with exceedingly low and well-controlled dispersion volumes must be employed in order to obtain consistent separations and reproducible relative LMWS1 peak areas.[3,4]

As a result, an HP-SEC column with a 200 Å pore diameter and 2.5 µm BEH particles packed in larger format 7.8 mm I.D. column hardware (XBridge Protein BEH SEC, 200 Å, 2.5 µm Column, p/n: 186009164) was developed to effectively bridge the performance gap between the UPLC-SEC columns. This column provides for more robust and easily transferred analyses with less dependency on the extra-column dispersion of the LC systems being used while increasing analysis time by 50% or less.[5] This general purpose column has since been reoptimized with respect to column packing to specifically improve upon the separation of IgG mAb monomer and LMWS1 to produce the BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column (p/n: 186009441).

The goal of this study was to demonstrate the performance of the BioResolve SEC mAb, 7.8 × 300 mm Column in comparison to three commercial SEC, sub-3-µm, 7.8 × 300 mm columns. The performance fundamentals of efficiency and peak shape, and the separation of the HMWS and LMWS of a mAb were compared. Almost invariably, SEC vendor column comparisons are made using the same mobile phase that a vendor has optimized for their column to evaluate other manufacturer's columns. To avoid the potential bias that this approach presents, we identified a therapeutic mAb (cetuximab) for which several column vendors have published their SEC separation method details (i.e., mobile phase composition) for either the same column tested in this study or for a column that appears to have the same SEC particle chemistry with either a change in particle size or column hardware.

RESULTS AND DISCUSSION

The Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) was used for the comparison of the BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column and the three commercial SEC, sub-3-µm, 7.8 × 300 mm columns. A visual comparison of the respective chromatograms and the separation conditions used for each column are presented in Figure 1. We observed that all four columns had comparable pore volumes (within 10%, data not shown) based on the difference between the elution volumes of thyroglobulin multimer (T3) and uracil (U). However, it was noted that the relative elution volumes of the individual protein standards varied. We observed the most similarities between the BioResolve Column and Column Y, while the separations on Column X and Column Z appeared relatively similar. These differences related predominantly to the average pore diameter of the packed particles and are most clearly observed in the separations between thyroglobulin monomer (T1) and the HMWS of thyroglobulin (T2 and T3) that are more resolved on Column X and Column Z due to their larger pore diameters. Conversely, we observed more separation between IgG (I) and BSA (B) for the BioResolve Column and Column Y due to their smaller pore diameters. We will later see how these pore diameter differences are manifested in the mAb separations.

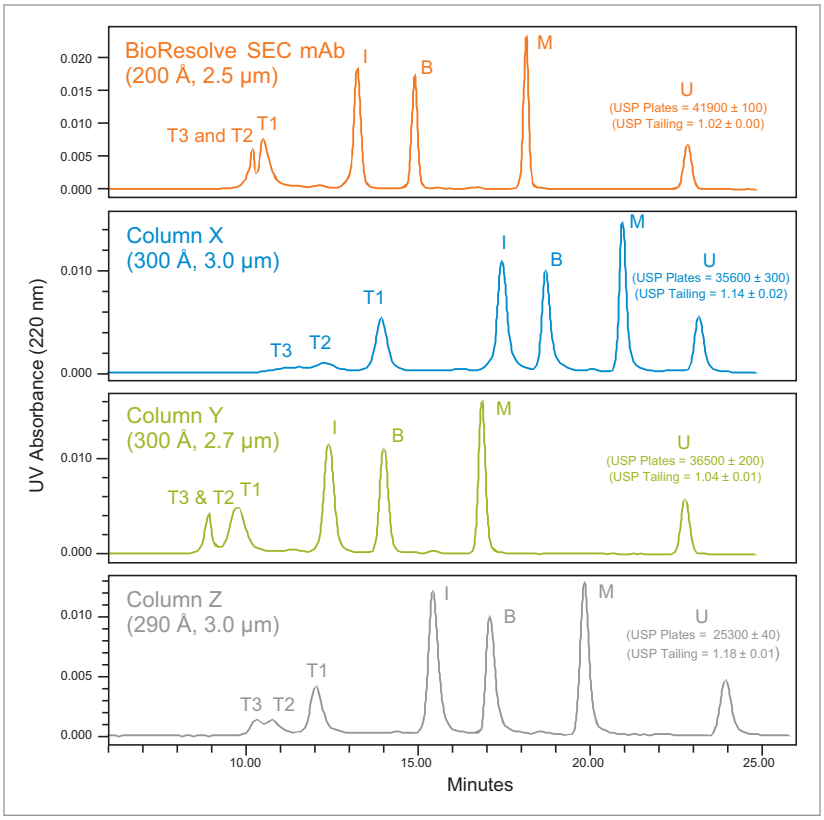


Figure 1. Shown are the separations of Waters BEH200 SEC Protein Standard Mix (injection volume 3 µL) on a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column, and three equally sized commercial SEC columns, containing sub-3-µm particles. Peak identifications are: thyroglobulin multimer (T3, ≥1.98 MDa), thyroglobulin dimer (T2, 1.32 MDa), thyroglobulin monomer (T1, 660 KDa), IgG (I, 150 KDa), BSA (B, 66 KDa), myoglobin (M, 17 KDa), and uracil (U, 112 Da). USP plate counts for uracil are based on tangent method, and USP tailing is measured at 5% peak height (n = 2). Mobile phases were: 50 mM sodium phosphate pH 7.0, 200 mM potassium chloride for the BioResolve Column; 200 mM sodium phosphate, pH 6.7 (NaOH) for Column X; 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 (NaOH) for Column Y; and 50 mM potassium phosphate, 250 mM potassium chloride, pH 6.8 (KOH) for Column Z. Flow rate was 0.50 mL/min, at-column temperature was 25° C. Analysis was performed in duplicate on an Empower 3 controlled ACQUITY™ UPLC H-Class Bio System with a 5σ dispersion volume of 14 µL. Value uncertainties represent the range.

As one measure of relative SEC column performance, we compared separation efficiencies using the tangential USP plate count (N) method for uracil (U), which is the total included SEC marker. The measured plate count was higher for the BioResolve Column as was predicted due to that column having the smallest sized particles of the SEC columns tested. If plate height (H = L/N, where L is column length) is normalized for particle size (reduced plate height, h = H/dp, where dp is particle diameter) we saw that Column X (h = 2.81) and Column Y (h = 3.04) were more comparable to the BioResolve Column (h = 2.87) indicating that Column X and Column Y are also packed efficiently.

We then looked at USP tailing as a measure of column packing quality. As USP tailing approached a value of 1.0, the peak was more symmetrical. USP tailing is a measure of peak symmetry at 5% peak height. Here we saw that the BioResolve Column and Column Y produce relatively symmetrical peaks, while Column X and Column Z produced peaks that tail slightly more. For the SEC separation of proteins, columns packed to tail slightly will result in improved separation of HMWS and monomer while degrading the separation of LMWS, and columns with more symmetrical peaks will provide a more consistent separation of both HMWS and LWMS.

The full-scale and expanded-view chromatograms for cetuximab are presented in Figures 2 and 3. In the full-scale chromatograms, we observe the same general trend in retention time as observed for the protein standards with sharp and relatively symmetrical peak shapes for the mAb monomer on all four columns. In the expanded view (Figure 3), we observe that the chromatographic profiles are well behaved with the baseline returning to its origin before the elution of LMWS2 for the BioResolve Column and Column Y, and immediately following LMWS2 for Column X and Column Z. This indicates that the mobile phases used for each column are reasonably appropriate.

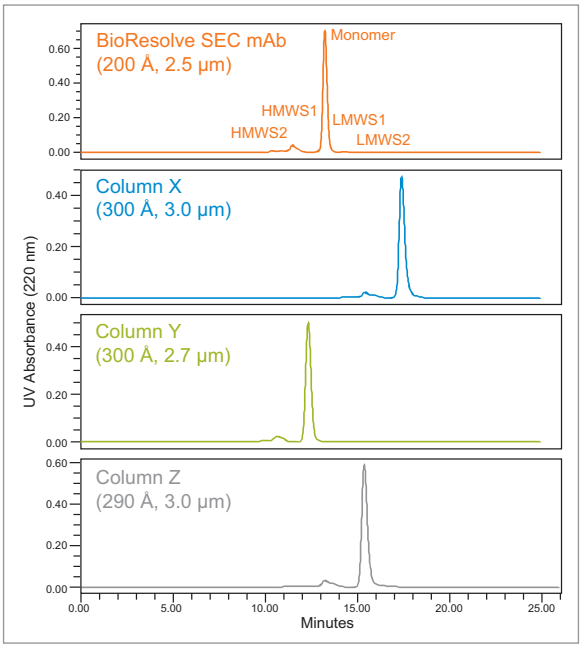
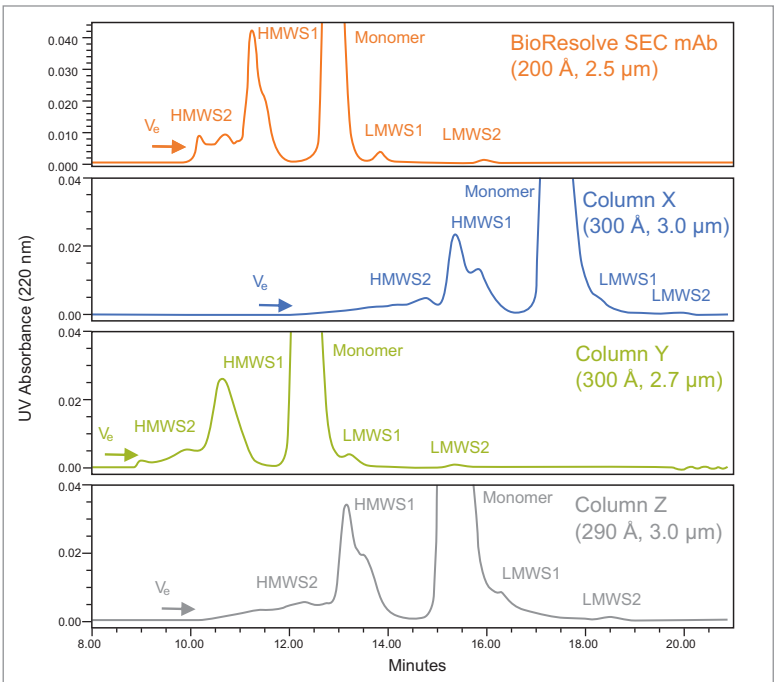


Figure 2. Shown are the full-scale separations of stressed Erbitux (cetuximab) drug product samples at a concentration of 2 mg/mL (injection volume 10 µL) using a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column, and three equally sized commercial SEC columns containing sub-3-µm particles. Peak identifications are provided in the text, and additional experimental conditions are provided in Figure 1.



Column	HMWS2 % Area	HMWS1 % Area	HMWS1 Rs	LMWS1 % Area	LMWS1 P/V	LMWS2 % Area
BioResolve	3.04 ± 0.02	8.97 ± 0.02	3.36 ± 0.02	0.48 ± 0.01	4.98 ± 0.11	0.11 ± 0.00
Column X	2.92 ± 0.13	7.96 ± 0.04	2.27 ± 0.01	ND	1.00 ± 0.00	0.15 ± 0.01
Column Y	2.04 ± 0.00	8.45 ± 0.01	2.26 ± 0.00	0.81 ± 0.01	1.24 ± 0.01	0.11 ± 0.00
Column Z	3.18 ± 0.04	8.22 ± 0.02	2.27 ± 0.00	ND	1.00 ± 0.00	0.17 ± 0.02

Figure 3. Shown are the expanded-scale separations of stressed Erbitux (cetuximab) drug product samples presented in Figure 2, and relevant tabulated percent area and separation quality results. Peak identifications are provided in the text. V_e represents the excluded volume of the column. Resolution (Rs) is determined based on USP half-height method. Peak-to-valley ratio (P/V) is based on LMWS1 peak height divided by the Monomer-LMWS1 valley height. HMWS1 resolution was determined in relation to the monomer. ND indicates that the value could not be determined. Samples were analyzed in duplicate, and listed uncertainties represent the range of the values.

We observe comparable separation between HMWS1 and the monomer for the four columns. While the USP half-height resolution for HMWS1 was 26–50% greater for the BioResolve Column, this value is confounded by the increased separation of a second HMWS1 size variant by the larger pore diameter columns. We also observe that the multimeric HMWS2 forms had an increased degree of separation on Column X and Column Z, consistent with the results observed for thyroglobulin in the SEC Protein Standard. The relative amounts of HMWS1 and HMWS2 were found to be variable, which is likely the result of sample instability following several freeze-thaw cycles, which is not recommended for this liquid formulation drug product. While they are provided (Figure 3), the relative amounts of HMWS1 and HMWS2 were not deemed to be critical for this comparison.

When considering the separation of the 50 kDa LMWS2 fragment, we observe that all four columns provide this separation. The percent peak area of this fragment is exceedingly low (~0.1%), and as a result, UV absorbance was monitored at 220 nm to improve signal-to-noise. The BioResolve Column and Column Y produced baseline resolution of LMWS2 and identical percent peak areas (0.11%). Column X and Column Y produced low-level tailing of the monomer peak resulting in partial resolution and ultimately artificially higher integrated HMWS2 percent peak areas.

For the separation of monomer and LMWS1, a discernable valley was only observed on the BioResolve Column and Column Y. The extent of this separation, as measured by the peak-to-valley ratio (P/V) for LMWS1, is significantly greater for the BioResolve SEC mAb Column with a P/V of 4.98 as compared to 1.24 for Column Y. This is likely the result of a more optimal pore diameter, increased plates, and decreased low level peak tailing for the BioResolve Column. The marginal resolution observed on Column Y also resulted in an artificially increased LMWS1 percent peak area (0.81%) versus the BioResolve Column (0.48%). As the abundance of LMWS1 decreases, it becomes more challenging to separate from the monomer and reliably quantify due to low-level tailing of the predominant monomer peak that can be caused by either the column or the LC system. Although not evaluated in this study, all four of the tested SEC columns appear to partially separate LMWS1 from monomer. As a result, reliable quantification may possibly be obtained, if the relative abundance of LMSW1 were higher in the sample. Otherwise, the separation efficiency would need to be improved by increasing column length or decreasing flow rate for these columns.

CONCLUSION

Currently, there are several commercially available SEC columns containing sub-3-µm particles and a 7.8 × 300 mm column geometry that can be effectively used for the analysis of HMWS and LMWS product-related impurities in mAb samples. These modern sub-3-µm SEC columns deliver significantly greater separation efficiencies than the previous generation of SEC columns containing 5 to 8 µm particles. Also, while 4.6 mm I.D. SEC columns containing sub-2-µm particles provide the greatest sample throughput for the separation of HMWS and LMWS, the reproducible analysis of LMWS1 can only be realized on extremely low dispersion UHPLC and UPLC systems. In contrast, an optimized modern 7.8 mm I.D., sub-3-µm SEC column can be used effectively on a wide range of HPLC system platforms while resulting in only a 33% or lower decrease in sample throughput.

The Waters BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column provided comparable separations of mAb HMWS1 (dimer) and monomer when compared to the commercially available and appropriately evaluated SEC columns. All of the evaluated SEC columns provided adequate and reliable separation between mAb HMWS1 and HMWS2 (multimer) with the smaller average pore diameter of the BioResolve SEC mAb Column and SEC Column Y generating less separation versus Column X and Column Z (which have greater average pore diameters).

For fragment analysis, the smaller average pore diameter of the BioResolve Column and Column Y provided comparable baseline separation of LMWS2 (50 kDa) and a discernable valley between LMWS1 (100 kDa) and mAb monomer. The BioResolve Column provided more separation of LMWS1 with an average P/V of 4.98 versus a P/V of 1.24 for Column Y. The reduced resolution of LMWS1 on Column Y and LMWS2 on Column X and Column Z resulted in the integrated relative areas of these impurities being artificially high in comparison to the values observed on the BioResolve Column.

The high efficiency and symmetrical peak shape of the BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 × 300 mm Column can provide high resolution separations of HMWS and LMWS for mAb-based therapeutics and similarly sized proteins on HPLC, UHPLC, and UPLC platforms.

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