Biopharma Analysis Guide

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Supelco®
Analytical Products

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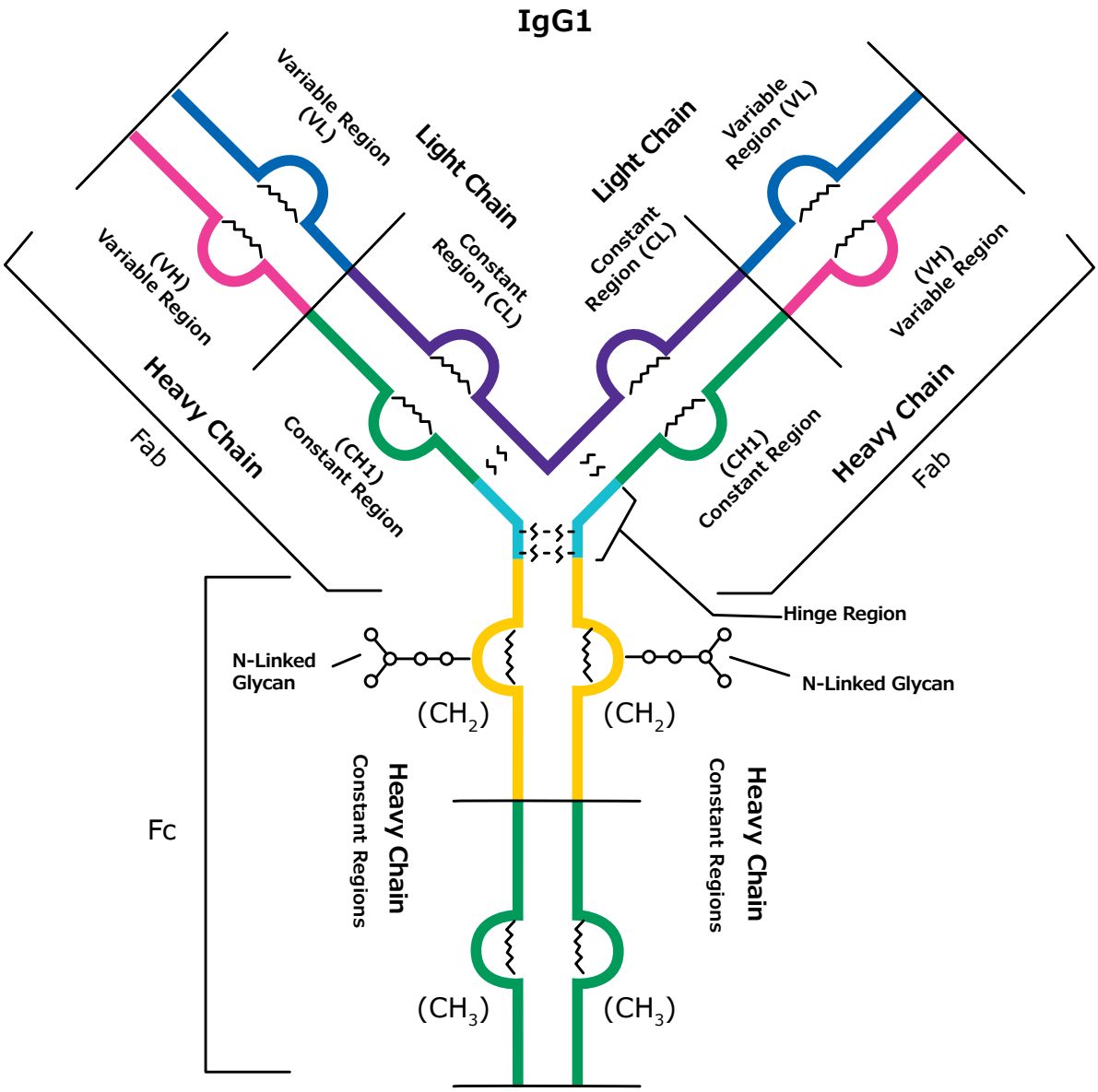
Introduction

The importance of therapeutic antibodies

Antibodies play a critical role in drug therapies, offering a wide range of benefits in the field of medicine. These proteins, produced by the immune system in response to foreign substances, have the remarkable ability to recognize and bind to specific targets. In drug therapies, antibodies can be harnessed for various purposes. These biomolecules have the ability to directly neutralize pathogens, preventing their entry into cells and halting infection. Additionally, antibodies can mark abnormal cells, such as cancer cells, for destruction by the immune system, enhancing the body's natural defense mechanisms. Moreover, antibodies can be engineered and modified in the laboratory to enhance their therapeutic properties, such as increasing their potency or extending their half-life in the body. With their specificity, versatility, and ability to interact with the immune system, antibodies have become invaluable tools in drug development and treatment, paving the way for innovative and targeted therapies that improve patient outcomes and quality of life. Several of the block buster drugs today are monoclonal antibodies.



Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs) are a class of innovative therapeutic agents that combine the specificity of monoclonal antibodies with the potency of cytotoxic drugs. ADCs are designed to deliver a toxic payload directly to target cells, increasing the selectivity and efficacy of the treatment, while minimizing damage to healthy cells. The structure of an ADC typically consists of three components: a monoclonal antibody that recognizes a specific target on the surface of a cell, a linker that connects the antibody to the cytotoxic drug, and the cytotoxic drug itself. Once the ADC binds to the target cell, it is internalized and the linker is cleaved, releasing the cytotoxic drug directly into the cell, where it exerts its effects. In the treatment of cancer, this targeted approach enables a higher concentration of a drug to accumulate in the tumor, improving efficacy and reducing systemic side effects compared to conventional chemotherapy. ADCs have shown promising results in the treatment of various cancers, providing a new avenue for precision medicine and targeted therapies in oncology.

Bi-Specific Antibodies

A bispecific antibody is a type of antibody engineered to simultaneously bind to two different target molecules. This unique feature allows the antibodies to bridge different cells or molecules, facilitating specific interactions and modulating immune responses. Bispecific antibodies are designed to have one binding site that recognizes a target molecule on one cell or tissue and another binding site that recognizes a different target on a separate cell or tissue. This dual targeting capability opens a range of therapeutic possibilities. Bispecific antibodies can redirect immune cells to attack cancer cells, enhance the specificity of drug delivery, or modulate signaling pathways involved in immune regulation. By bringing different components together, these antibodies offer a novel approach to treat conditions such as cancer, autoimmune disorders, and infectious diseases. These innovative molecules hold great potential for personalized medicine and have sparked considerable interest in the field of biotechnology and drug development.

Overview of the Analytical Workflow

Sample Prep

Purification & Depletion

Recombinant/Fusion Tag Protein Purification

ProteoExtract® Depletion

Seppro® Protein Depletion

Inhibitor Cocktails

cOmplete™ Protease Inhibitors

MS-SAFE Protease and Phosphatase Inhibitor

Protein Concentration

Amicon® Filters

Stirred Cell

Filtration & SPE

Millex® Syringe Filters

Supelclean™ LC-4 Side Pore SPE

ZipTip® Pipette Tips

Lysis Reagents

CelLytic™ Reagents

ProteoPrep® Kits

Digestion & Labeling

Proteases

SOLu-Trypsin

Trypsin

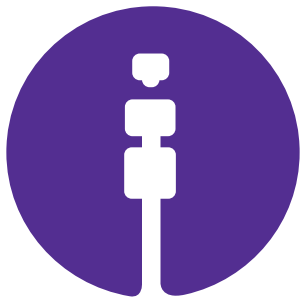
Other Proteases

Isotopically Labeled Amino Acids

Chromatographic Separation

Buffers

Solvents



Columns for Biopolymers

Ion Pair Reagents

Analysis

Products for Protein Mass Spectrometry

Amino Acid, Protein & Peptide Standards

USP Biologics

peptide Mapping applications

Peptide Mapping

Peptide mapping is a vital technique in the field of biochemistry and pharmaceutical research, playing a crucial role in the characterization and quality control of proteins. The process involves the identification and sequencing of peptides within a protein, providing valuable information about its primary structure, post-translational modifications, and overall integrity. Peptide mapping is essential for ensuring the safety, efficacy, and consistency of protein-based drugs, as any alterations or variations in the peptide sequence can significantly impact the protein's function and therapeutic properties. By thoroughly analyzing the peptide map, researchers can gain insights into protein structure-function relationships and detect any modifications or impurities that may arise during production or storage.

Typically, peptide mapping is performed through a combination of techniques, including enzymatic digestion, high-performance liquid chromatography (HPLC), and mass spectrometry (MS). The process begins with the enzymatic cleavage of the protein using specific proteases, such as trypsin or chymotrypsin, which break it down into smaller peptides. HPLC separates the peptides based on their hydrophobicity, size, and charge, allowing for the identification and quantification of each peptide in the sample. Finally, mass spectrometry is employed to determine the exact molecular weight and sequence of the peptides, providing accurate information about the protein's primary structure and any modifications present.

Peptide mapping is a critical tool for protein analysis and quality control in various industries, including biopharmaceuticals, biotechnology, and academic research. The technique enables researchers to assess the structural integrity of proteins, identify modifications, and monitor the consistency of protein-based drugs. By ensuring the accuracy and stability of protein products, peptide mapping contributes to the development of safe and effective therapies, as well as the advancement of scientific knowledge in the field of protein biochemistry.

Workflow for Peptide Mapping

An Optimized Protocol for Peptide Mapping of Therapeutic Monoclonal Antibodies with Minimum Deamidation and Oxidation Artifacts

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Abstract

Post-translation modifications (PTM), such as oxidation and deamidation, can have serious consequences on therapeutic monoclonal antibodies. Peptide mapping is a widely used method for the identification of site-specific PTMs, but typical protein digestion workflows often end up introducing significant amounts of artifacts. Hence, to obtain an accurate assessment of the modifications, it is critical to reduce the artifacts that occur during sample preparation steps. This study used NISTmAb as a model monoclonal antibody to demonstrate an optimized peptide mapping protocol resulting in minimal artificial asparagine deamidation and methionine oxidation. The protocol utilizes shorter incubation times and an improved digestion buffer, allowing for complete sample preparation in less than six hours.

Introduction

The development, production, and storage of therapeutic mAbs must be monitored for post-translational modifications (PTMs), to assure consistent quality and safety. PTMs such as deamidation and oxidation are known to influence the efficacy, safety, and stability of therapeutic monoclonal antibodies (mAb).[1,2] Deamidation of asparagine (ASN or D) and the oxidation of methionine (Met or M) are major chemical degradation pathways for protein therapeutics and have been studied extensively.[3,4,5] Asparagine residues can form a succinimide intermediate that subsequently hydrolyzes into isoaspartic or aspartic acid (**Figure 1A**).[6,7] Whereas, hydroxyl radicals can oxidize methionine residues to form methionine sulfoxide (**Figure 1B**).[8,9]

LC-MS based peptide mapping is the method of choice for measuring the relative abundance of PTMs. The sample preparation prior to the LC-MS analysis involves three steps of denaturation/reduction, alkylation, and digestion. The digestion of different mAbs produces different peptide fragments having a wide range of sizes — from single amino acids to longer polypeptides. Since these peptides vary widely in their hydrophobicity, reversed-phase (C18) is the preferred mode of chromatography for peptide mapping.

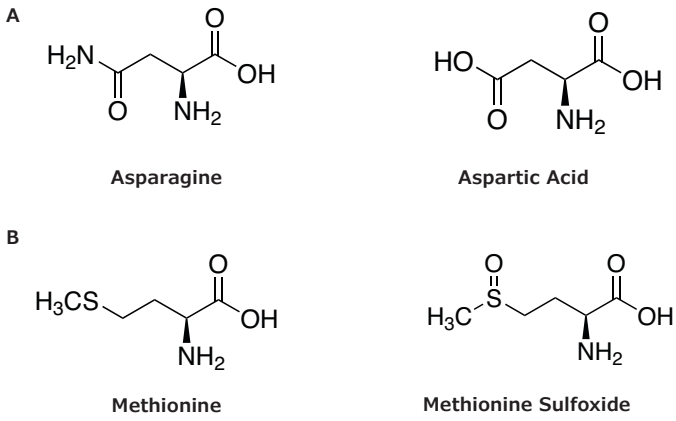

Figure 1. (A) Asparagine residues can undergo deamidation to form aspartic acid, and **(B)** methionine can undergo oxidation to form methionine sulfoxide.

The conventional trypsin digestion of monoclonal antibodies is lengthy, involving an overnight digestion step. The conditions and reagents used in this step are known to induce artifactual deamidation and oxidation of the mAb sample, leading to inaccurate measurement of PTMs.[10] The first part of this paper compares methionine and asparagine deamidation between conventional trypsin digestion and an optimized digestion protocol that takes less than six hours to complete. The second part compares the optimized protocol with the protocol published by NIST. All LC-MS analyses were carried out using C18 columns with superficially porous particles (BIOshell™ A160 Peptide C18).

Experimental

Sample: NIST Monoclonal Antibody Reference Material 8671 (NISTmAb)

Digestion: **Figure 2** outlines the digestion protocols.

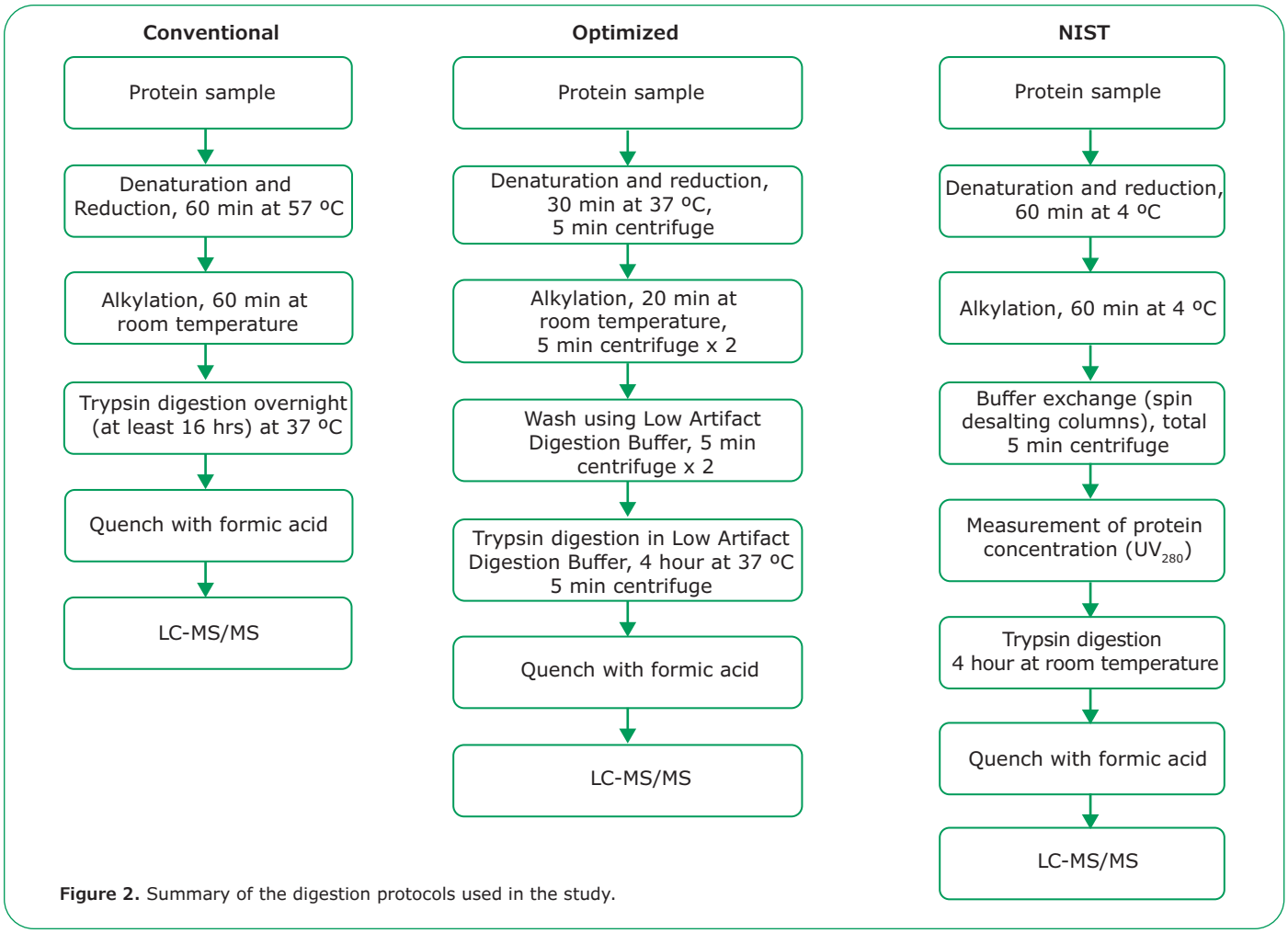

Figure 2. Summary of the digestion protocols used in the study.

The conventional protocol uses sodium deoxycholate (60 mg) in methanol (1 mL) as the denaturation solution. 20 mM TCEP (tris(2-carboxyethyl)phosphine) and the denaturation solution were mixed in 1:1 (v/v), to which 20 µL of dried down sample was added and incubated at 57 ºC for one hour. The sample was brought back to room temperature and centrifuged at 14,000 x g per gram for 30 seconds. This was followed by the alkylation step using 5 µL of 200 mM iodoacetamide (in 50 mM ammonium bicarbonate) and subsequent incubation for one hour in the dark at room temperature. Digestion was carried out by adding enough trypsin solution (trypsin in 50 mM ammonium bicarbonate) to have an enzyme:protein ratio of 1:20 and incubating at 37 ºC overnight (at least 16 hours) on a thermo-shaker. 2 µL neat formic acid was used to quench the digestion.

Detailed procedure for the optimized protocol is described in the technical bulletin for Low Artifact Digestion Buffer.[11] A NIST[12] paper describes the protocol provided by NIST. The reagents used in each protocol are shown in **Table 1**.

Table 1. Reagents used in the protocols of the study.

Reagent	Conventional	Optimized	NIST
Denaturing solution/buffer	Sodium deoxycholate	Urea	Guanidine HCl
Reduction	TCEP	TCEP	DTT
Alkylation	Iodoacetamide	Iodoacetamide	Iodoacetamide
Digestion buffer	Ammonium bicarbonate	Low Artifact Digestion Buffer	Urea
Trypsin	SOLu-Trypsin	SOLu-Trypsin	Recombinant, proteomics grade, expressed in Pichia pastoris

LC-MS Conditions HPLC:

HPLC Conditions	
Instrument:	Waters™ Acquity UPLC
Column:	BIOshell™ A160 Peptide C18, 15 cm x 1.0 mm, 2.7 µm particles (**67099-U**), two columns in series
Mobile phase	[A] 0.1% formic acid in water [B] 0.1% formic acid in acetonitrile
Gradient:	**Time (min) / %B** 0 / 1 120 / 35 121 / 97 136 / 97 137 / 1 162 / 1
Flow rate:	80 µL/min
Column temp.:	room temperature
Injection:	10 µL (3-4 µg mAb digest)

Mass Spectrometry:

MS Conditions	
Instrument:	Thermo QE Plus
Polarity:	Positive
Spray voltage:	4.0 kV
Capillary temp:	320 ºC
Sheath gas:	10
Aux gas:	5
S-Lens:	50 V
m/z range:	300-4000

Data analysis

The raw MS files were subjected to BioPharma Finder™ 3.0 (Thermo Fisher Scientific) for peptide mapping. The peptide identifications were performed by searching the processed data against the NISTmAb sequence-based accurate mass of a full mass scan and assignments of product ions in MS/MS spectra. The data was filtered to report only the peptides with a mass tolerance of ±10 ppm. The % deamidation and oxidation were calculated by BioPharma Finder™ software using the mapping tab. Also, the result was manually checked by creating the extracted ion chromatograms (XICs) for unmodified and modified peptide within 10 ppm mass error. Equations 1 and 2 were used to calculate the % modification (oxidation, deamidation) and % missed cleavage (% MC), respectively.

Equation 1:

$$\% \text{ Modification} = \frac{\text{area under the peak of XIC of modified peptide}}{\text{area under the peak of XIC of modified peptide + area under the peak of XIC of un-modified peptide}} \times 100$$

Equation 2:

$$\% \text{ MC} = \frac{\text{area under the peak of XIC of MC peptide}}{\text{area under the peak of XIC of standard peptide + area under the peak of XIC of MC peptide}} \times 100$$

Results and Discussions

Peptide mapping using LC-MS has become a routine analysis in the development and manufacture of therapeutic mAbs. Traditional sample preparation procedures used prior to LC-MS are often cumbersome. These procedures generally involve chemical denaturation, reduction and alkylation, buffer exchange, and overnight protease digestion of the protein sample at elevated pH and temperature. Asparagine deamidation and methionine oxidation take place during these various steps, the extent of which depends on the conditions such as reagents used, ionic strength, temperature, pH, incubation time, digestion buffer, and presence of trace metals (in the case of methionine oxidation).[7] A simpler, shorter method with minimal artifacts is certainly desired to obtain accurate endogenous levels of deamidation and oxidation.

Figure 3A is the base peak chromatogram of tryptic digested NISTmAb, showing examples of typical tryptic peptides used to measure the levels of Met (M) oxidation and Asn (D) deamination in this work. **Figure 3B** is the extracted ion chromatogram and MS spectrum of the peptide DTLMISR (t_R = 51 min) and the peptide with an oxidized methionine residue (position HC:M255), t_R = 44 min. The oxidation of methionine rendered the molecule less hydrophobic, thus less retentive on the BIOshell™ A160 Peptide C18 column. **Figure 3C** is the XIC and MS spectrum of the peptide GFYPSDIAVEWESNGQPENNYK (t_R 88.90 min) and the deamidated peptide (position HC:N387). The deamidated forms (isoASP and ASP) elute before and after the unmodified peak at ~87.91 and 91.92 min, respectively.

Optimized versus Conventional Protocol

The optimized and conventional protocols use the same reduction (TCEP) and alkylation (iodoacetamide) reagents, but they differ in the denaturing solution used, incubation times, and temperature (see **Figure 2** and **Table 1**). The conventional protocol has much longer incubation times and uses higher temperature for the denaturation/reduction step. The digestion step happened overnight with the conventional protocol at a higher pH of 8.5

With the optimized protocol, digestion took only four hours. The digestion buffer used was specifically developed to minimize deamidation and oxidation during the digestion step without sacrificing the digestion efficiency. The buffer was formulated at an optimal pH and contained a proprietary antioxidant.

In both protocols, the protease used was SOLu-Trypsin, a proprietary formulation of recombinant Trypsin (porcine sequence expressed in *Pichia pastori*) and stable in solution when refrigerated.

The deamidation levels between the protocols were extremely different at the two sites (**Figure 4A**). The biggest difference was observed at site HC:N387, where deamidation was 41.1% for the conventional

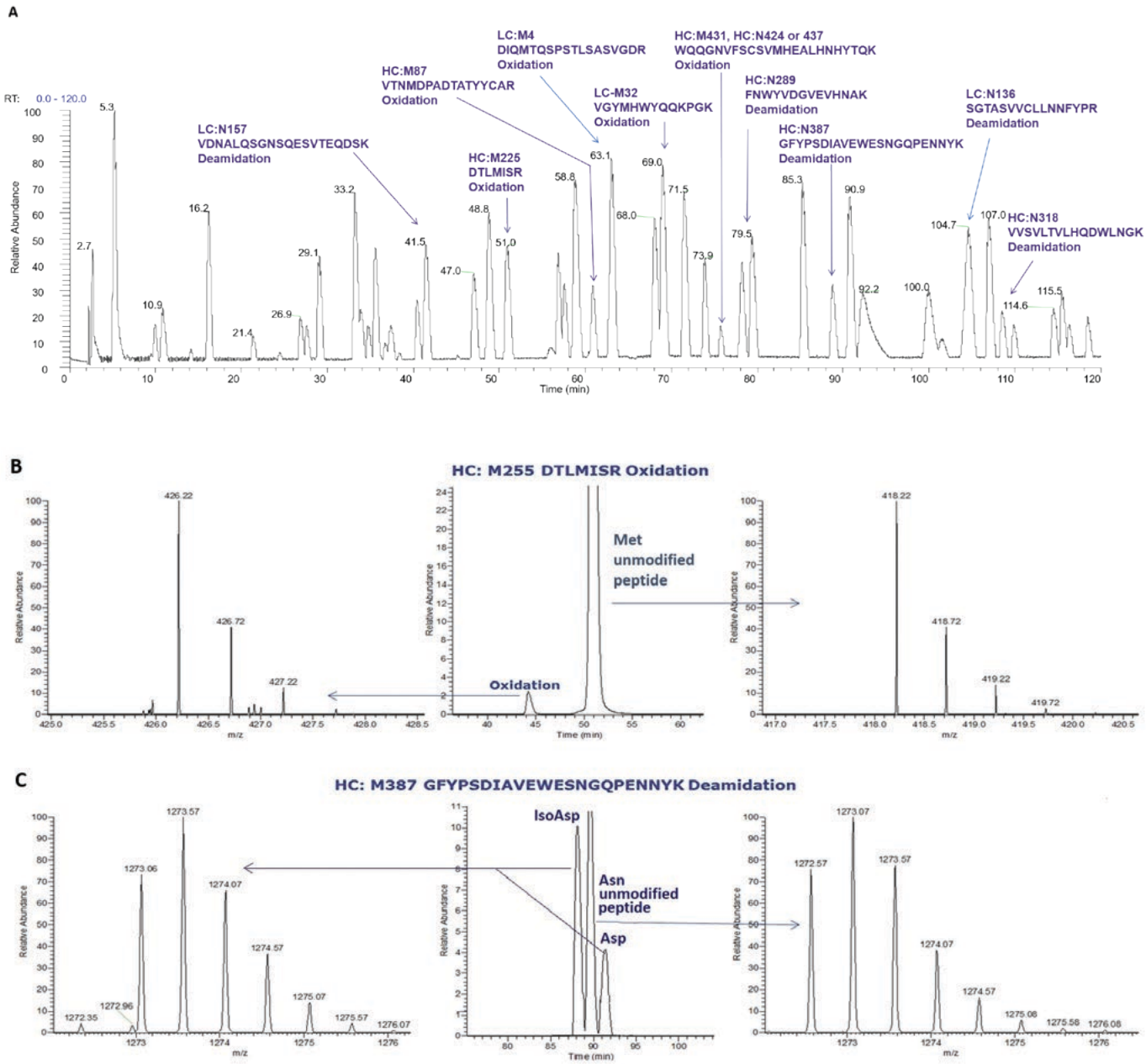

Figure 3. Analysis of tryptic digested NISTmAb (A) Base peak chromatogram of tryptic digested NISTmAb. Labeled peptides are examples of typical tryptic peptides used for determining Met oxidation and Asn deamination levels. (B) Extracted ion chromatogram and spectra of unmodified DTLMISR peptide and the peptide with oxidized Met. (C) Extracted ion chromatogram and spectra of unmodified GFYPSDIAVEWESNGQPENNYK peptide and the peptide with deamidated Asn.

protocol and 0.6% for the optimized one. At site HC:N318, a 21.2% deamidation was observed for the conventional protocol and none was observed for the optimized protocol. These results are not surprising. It is well known that the incubation times of protein samples in the denaturing/reduction and alkylation steps, and to a larger extent, the length of digestion, are directly proportional to the levels of artificial modification.[13] It has been reported that deamidation artifacts are reduced at lower temperatures;[14] in the conventional protocol, the denaturation/reduction step was carried out at an elevated temperature (57 ºC).

The difference in % oxidation was not as high as observed for deamidation. At site LC:M32, the conventional protocol had 2.9% higher oxidation than the optimized protocol, and it was 4.2% higher at site HC:M255 (**Figure 4B**).

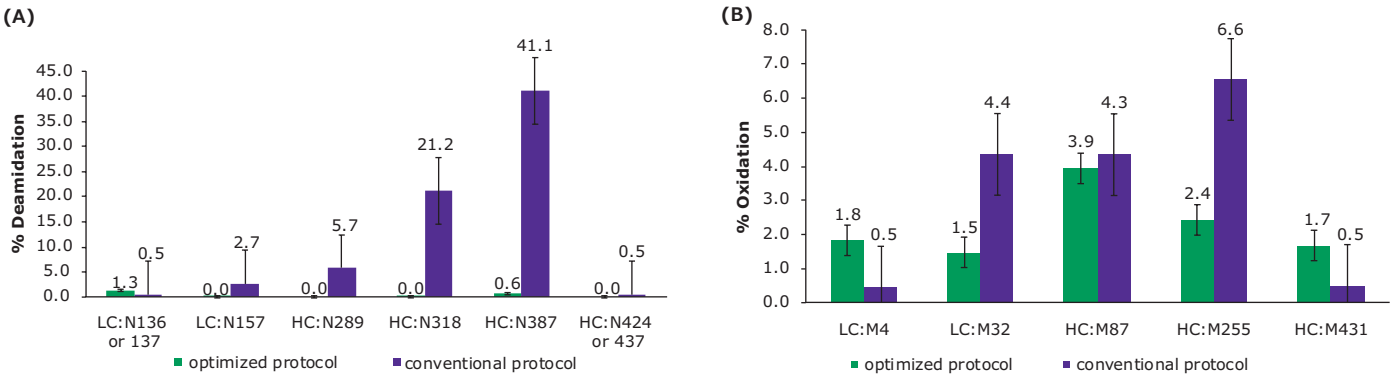

Figure 4. Levels of (A) Asn deamidation and (B) Met oxidation of NISTmAb tryptic peptides using the conventional and optimized protocols.

Optimized Protocol versus NIST Protocol

In 2018, NIST published a paper wherein they described the development of a tryptic digestion protocol used for peptide mapping. Their study focused on parameters such as buffer concentration, digestion time and temperature, and the source and type of trypsin used.[12] (See **Figure 2** for the outline of the protocol and **Table 1** for the reagents used.)

The denaturation/reduction and alkylation steps were carried out at a very conservative temperature (4 ºC) with incubation times much longer than the one for the optimized protocol. In addition, the protocol required a buffer exchange step (into the urea containing digestion buffer) before the tryptic digestion. Overall, the NIST protocol requires more time for reagent and sample preparation compared to the optimized protocol.

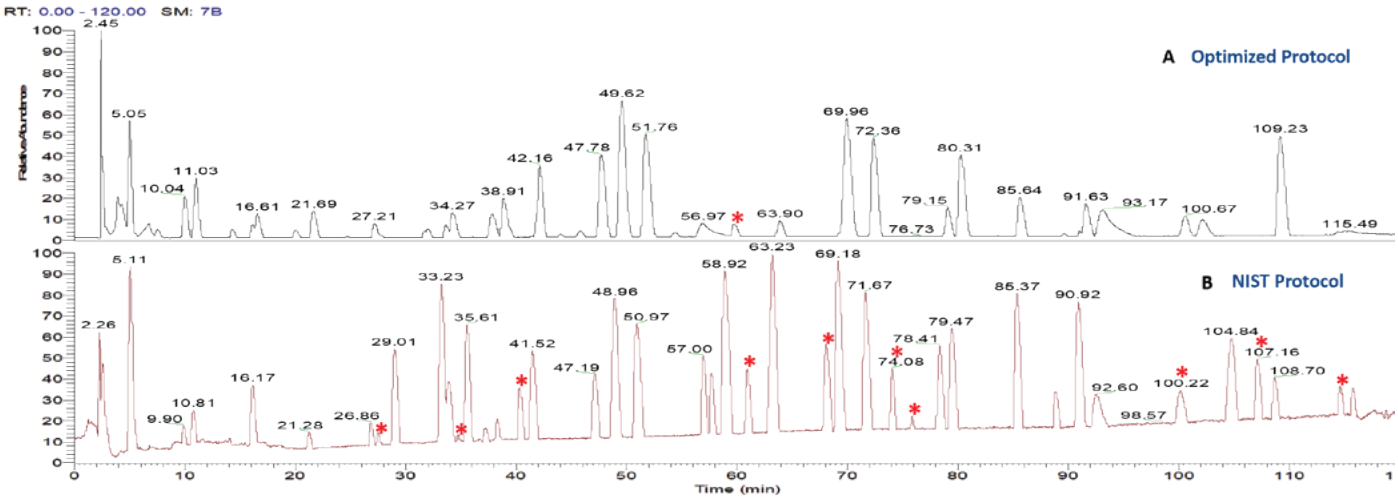

Figure 5. Comparison of base peak chromatograms of tryptic digested NISTmAb using the (A) optimized and (B) NIST protocols. Missed cleavage peptides are labeled with a red asterisk (*).

Figure 5 compares the base peak chromatograms of the digested NISTmAbs using the optimized protocol and the NIST protocol. The profile for the optimized digestion protocol is less complex. The chromatogram from the NIST protocol exhibited many extra peaks which were identified as missed cleavage peptides. The average percent missed cleavage was 16% for the optimized protocol and 35% for the NIST protocol (**Figure 6**).

In terms of Met oxidation and Asn deamidation, the performance of the two protocols is similar. The level of Met oxidation for both the methods is <5% and for deamidation <1.7% (**Figure 7**).

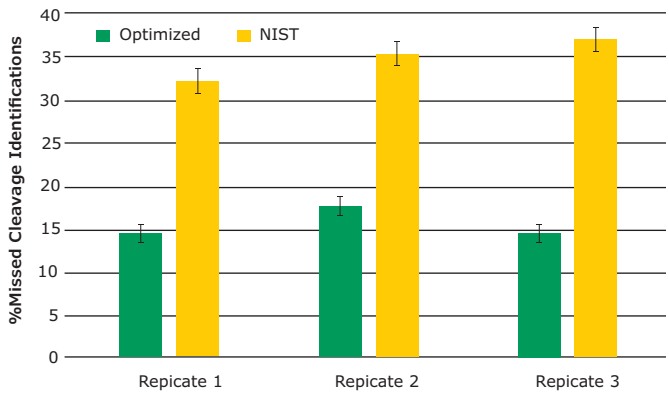

Figure 6. Average percent missed cleavage for the optimized and NIST protocols.

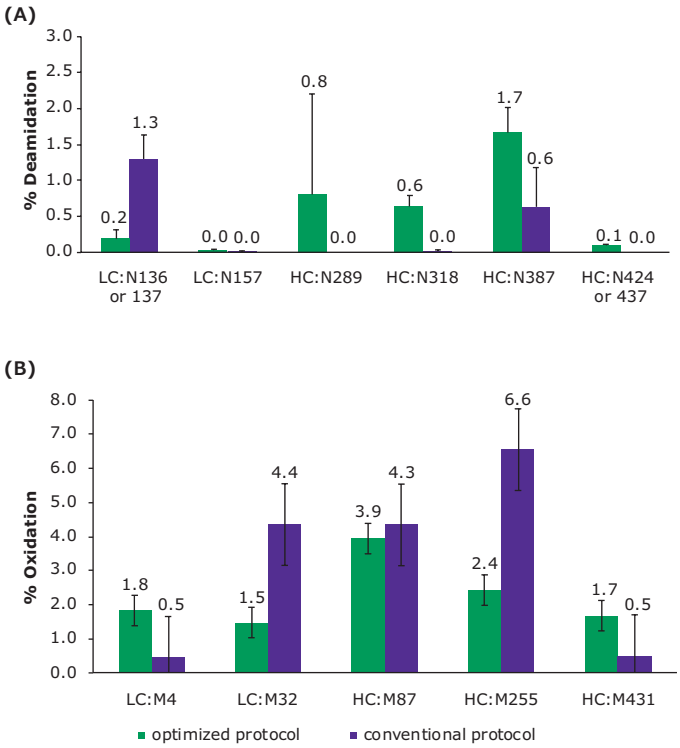

Figure 7. Levels of (A) Asn deamidation and (B) Met oxidation of NISTmAb tryptic peptides using optimized protocol and NIST protocol.

It is important to note that the HPLC conditions are also critical for the study. To enhance resolution and sequence coverage, two BIOshell™ A160 Peptide C18 15 cm x 1.0 mm columns arranged in series were used to provide for a total effective column length of 30 cm. Hydrophilic peptides with less than five amino acids such as VDK, TISK, EYK that typically elute in flow-through, could be retained on the longer column and be subsequently analyzed by mass spectrometry. In addition, the BIOshell™ columns are composed of superficially porous particles (SPPs) containing a solid, nonporous silica core with a porous silica outer layer, providing higher separation efficiency. This particle attribute results in a narrower peak width and improved resolution of the peptide analytes. Together with the optimized gradient conditions shown in the experimental section, a good separation of the unmodified and modified peptides was achieved. This result is well illustrated in **Figure 3B**, where the unmodified DTLMISR peptide and the peptide with oxidized Met were well resolved, allowing for accurate quantitation of each species.

Conclusions

The optimized protocol gave significantly lower levels of Asn deamidation compared to the conventional protocol, particularly at two sites, HC:N387 (over 40% lower) and HC:N318 (over 20% lower). The levels of oxidation (<5%) and deamidation (1.5 %) were comparable with the NIST protocol. In addition, more missed cleavage peptides were observed with the NIST protocol (35%) compared to the optimized protocol (16%). The optimized protocol also offers the advantage of allowing complete digestion in less than 6 hours, with minimal deamidation and oxidation artifacts. The use of two BIOshell™ A160 Peptide C18 (15 cm) columns in series allowed the successful separation of peptides in the tryptic digestion.

Featured Products

Description	Cat.No.
HPLC	
BIOshell™ A160 Peptide C18, 15cm x 1.0 mm, 2.7 µm	**67099-U**
Acetonitrile with 0.1% (v/v) Formic acid, hypergrade for LC-MS LiChrosolv®	**1.59002**
Water with 0.1% (v/v) Formic acid, hypergrade for LC-MS LiChrosolv®	**1.59013**
Formic acid 98% - 100%, for LC-MS LiChropur™	**5.33002**
Standards, Reagents, and Accessories	
NISTmAb, Humanized IgG1k Monoclonal Antibody	**NIST8671**
Low-Artifact Digestion Buffer	**EMS0011**
SOLu-Trypsin	**EMS0004**
Microcon® 30kDa Centrifugal Filter Unit with Ultracel® 30 membrane	**MRCF0R030**

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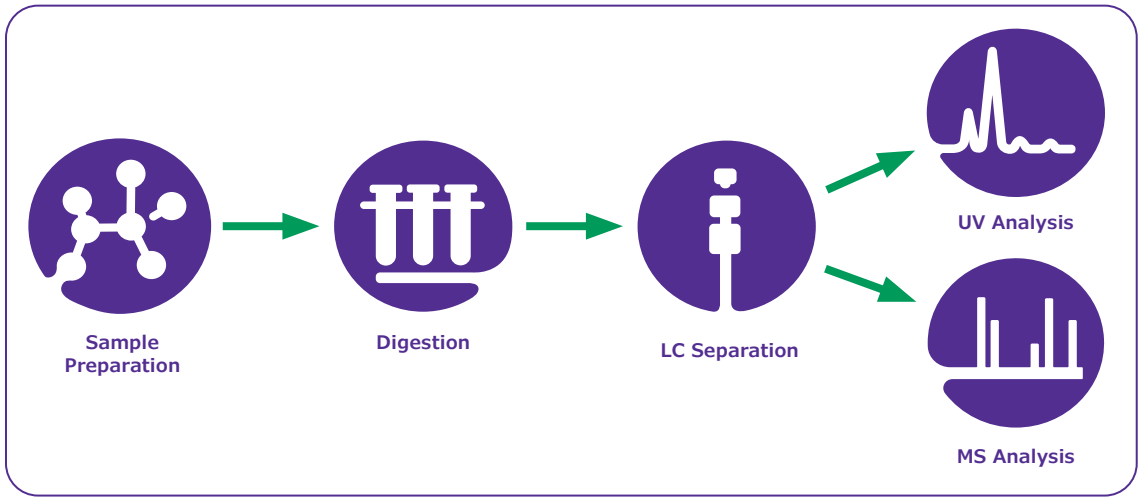
Teriparatide Peptide Mapping Analysis by LC-UV-Mass Spectrometry

Workflow for Teriparatide Peptide Mapping



A complete workflow by LC-UV-MS has been developed for teriparatide peptide mapping with 100% sequence coverage. The workflow offers the following:

- Step-by-step instructions for enzyme digestion of teriparatide
- Chromatography conditions on five Supelco® columns to separate teriparatide digestion fragments
- Identification of teriparatide digestion fragments with high accuracy on high resolution, accurate mass QTOF mass spectrometer

Introduction

Peptide mapping is a widely used analytical technique to examine primary structures and post-translational modifications (PTMs) of biopharmaceuticals.[1, 2] The general workflow is a bottom-up methodology including enzyme digestion followed by separation of the resulting peptide fragments and analysis via ultraviolet (UV) and/or mass spectrometry (MS).[1] The major use of the peptide mapping technique is to confirm the primary structure and thereby the identity of the analyte by the amino acid sequence. In the quality control (QC) environment, identity is confirmed by peptide mapping and comparing the sample map to a reference standard map in terms of the peak retention time, peak height and resolution.[1] A difference between the sample and reference standard may indicate a change in, or degradation of, the drug substance/product.[1] When coupled with mass spectrometry, the mass difference can be used to identify the type of degradation or modification (e.g. oxidation, deamidation and glycosylation of biomolecules).[1, 2]

Teriparatide is a recombinant form of parathyroid hormone (PTH) used in the treatment of osteoporosis. It consists of the first 34 amino acids (N-terminal) which is the bioactive portion of the hormone, also called rhPTH (1-34).[3] It is a single-chain linear polypeptide without any post-translational modifications. This molecule, with the active sequence of endogenous PTH, promotes the remodeling of bone through binding and activation of the PTH-R1 receptor.[4] The originator product of Forteo® from Eli Lilly was approved by US Food and Drug Administration (FDA) in November 2002 and European Medicines Agency (EMA) in June 2003. Since the patents on Forteo® expired in August 2019, biosimilars of teriparatide have been approved or are under development.

In this application note, a complete workflow for teriparatide peptide mapping by LC-UV-MS was developed on an Agilent 6545 XT AdvanceBio Q-TOF system. USP teriparatide standard and teriparatide sample were digested by Endoproteinase Glu-C according to the USP monograph method conditions. The resulting fragments were separated on multiple Supelco® columns including BIOshell™ A160 Peptide C18 (150 mm × 2.1 mm, 2.7 µm), Chromolith® RP-18e (100 mm × 4.6 mm), Chromolith® High Resolution RP-18e (100 mm × 2.0 mm), Ascentis® Express Phenyl-Hexyl (100 mm × 3.0 mm, 2.7 µm), and Ascentis® Express F5 (100 mm × 3.0 mm, 2.7 µm).

General Procedures

Reagent Preparation

20 mM Sodium Phosphate Buffer

A solution of 20 mM of sodium phosphate buffer is prepared by combining sodium phosphate monobasic and sodium phosphate dibasic heptahydrate together in water to reach the final concentration of 0.52 mg/mL sodium phosphate monobasic and 4.34 mg/mL sodium phosphate dibasic heptahydrate. [E.g., dissolve 0.104 g of sodium phosphate monobasic and 0.868 g of sodium phosphate dibasic heptahydrate in 200 mL of HPLC grade water.]

Enzyme Solution

Add 400 µL of HPLC grade water to a vial containing 100 units of Endoproteinase Glu-C, from Staphylococcus aureus V8, and mix well. This solution is stable for 72 h when stored in the refrigerator (2-8 °C).

Mobile Phase A: 0.1% TFA in water

Add 1 mL of trifluoroacetic acid (TFA) into 1000 mL of water. Mix well.

Mobile Phase B: 0.1% TFA in acetonitrile: water (60:40, v: v)

Mix 1 mL of trifluoroacetic acid (TFA) to 600 mL of acetonitrile and 400 mL of water. Mix well.

Sample Preparation

Teriparatide (USP) Standard Preparation

- Bring the USP teriparatide reference standard vial to room temperature.
- Open the USP teriparatide vial which typically contains about 1 mg of teriparatide (free base). Based on the exact amount in COA, add corresponding amount of 20 mM of sodium phosphate buffer using a positive displacement pipette to generate a 1.5 mg/mL of teriparatide reference standard (RS) solution without further weighing.
- Mix USP teriparatide RS solution with the enzyme solution at a teriparatide to protease ratio of 10:1. Mix well.
- Incubate the USP teriparatide RS and enzyme at 37 °C for 18-24h in a heat block to keep the temperature constant.
- Quench the digestion by adding mobile phase A (0.1% TFA in water) to reach a final teriparatide concentration of approximately 0.25 mg/mL. This solution is stable for 72 hours when stored at 2-8 °C.
- Transfer this solution to an autosampler vial for LC-UV-MS analysis. Or transfer a portion of this solution to an autosampler vial and store the remaining amount in a freezer for up to 1 month for future analysis.

Teriparatide Sample Preparation

- Bring the vial containing about 1 mg of lyophilized teriparatide to room temperature.
- Add 20 mM of sodium phosphate buffer using a positive displacement pipette and mix well to reach a teriparatide concentration of 1.5 mg/mL.
- Mix this solution with enzyme solution to achieve a teriparatide to protease ratio of 10:1. Mix well.
- Incubate teriparatide solution with enzyme at 37 °C for 18-24h in a heat block to keep the temperature constant.
- Quench the digestion by adding mobile phase A (0.1% TFA in water) to reach a final teriparatide concentration of approximately 0.25 mg/mL. This solution is stable for 72 hours when stored at 2-8 °C.
- Transfer this solution to an autosampler vial for LC-UV-MS analysis. Or transfer a portion of this solution in to an autosampler vial and then store the remaining amount in a freezer for up to 1 month for future analysis.

Blank Preparation

- In a 1.5 mL microcentrifuge tube, combine 20 mM of sodium phosphate buffer with enzyme solution in the same portions used for the Standard solution and Sample solution. Mix well.
- Cap the vial and incubate at 37 °C for 18-24 hours, in a heat block to keep the temperature constant.
- Quench the digestion by adding the same volume of mobile phase A (0.1% TFA in water) as the Standard solution and Sample solution and mix well.
- Transfer this solution to an autosampler vial for LC-UV-MS analysis.

LC-UV-MS System Setup and Data Analysis

The essential settings of the LC-UV-MS chromatography system applied in the analysis of teriparatide peptide mapping are listed in **Table 1** and **Table 2** below.

HPLC Parameters	
HPLC system	Agilent 1290 Infinity II
Software	Agilent MassHunter 10.0
Column	BIOshell™ A160 Peptide, C18, 150 x 2.1 mm, 2.7 µm Chromolith® RP-18e, 100 x 4.6 mm Chromolith® High Resolution RP-18e, 100 x 2.0 mm Ascentis® Express Phenyl-Hexyl 100 x 3.0 mm, 2.7 µm Ascentis® Express F5, 100 x 3 mm, 2.7 µm
Column temp	40 °C
Autosampler temp	5 °C
Injection volume	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	0.1% TFA in Acetonitrile: water (60:40, v: v)
UV Detector	214 nm

Table 1. HPLC-UV general system settings.

Mass Spec. Settings	
Mass Spectrometer	Agilent 6545 XT AdvanceBio Q-TOF
Software	Agilent MassHunter 10.0
Ion source	Dual ESI
Polarity	Positive
Gas temp	320 °C
Drying gas	8 L/h
Nebulizer	35 psi
Fragmentor	175 V
Skimmer	65 V
VCap	3500 V
Mass range	100-1700 m/z

Table 2. QTOF Mass Spectrometer settings.

Data Analysis

Data analysis for LC-UV and LC-MS was processed through Agilent MassHunter Qualitative Analysis 10.0 software. Digested teriparatide fragments were identified by comparing the *m/z* value from the extracted MS spectra to the ExPASY[6] teriparatide Glu-C digestion fragment database. The LC-UV separation of the teriparatide fragments was compared with USP teriparatide standard as well as with USP teriparatide standard certificate of analysis.[5]

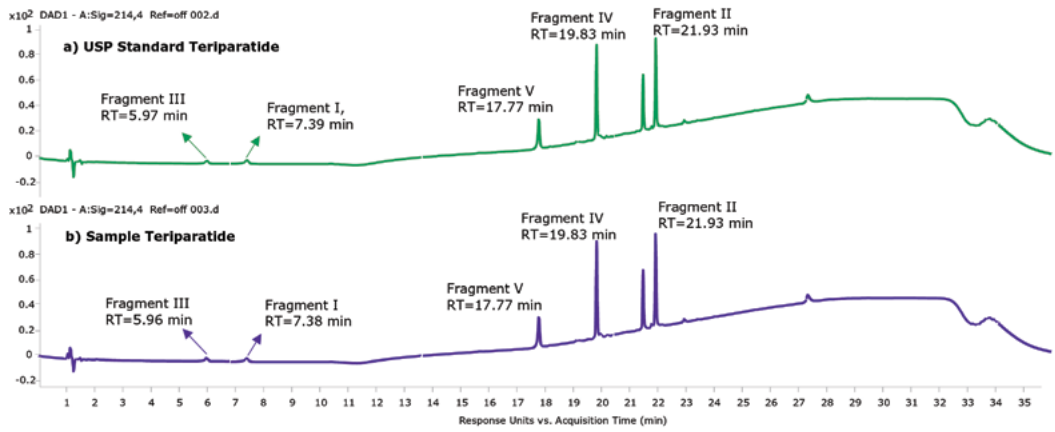
Results and Discussion

For each run of the digested teriparatide sample, a USP teriparatide standard digest was injected as a system suitability check to evaluate column and system performance. The LC-UV requirements for the system suitability sample were that resolution between fragment III and I is no less than (NLT) 1.5 and tailing factor for fragment IV is no more than (NMT) 2.3. All five digestion fragments found should match the chromatogram provided with USP certification. The retention time ratio between the USP teriparatide standard and the teriparatide sample should be within the range of 1.00±0.03.

Here, we included a second dimension of detection, by mass spectrometry, to confirm the digestion resulting fragments. One hundred percent sequence coverage was achieved with this digestion protocol. All five Supelco® columns evaluated here showed adequate separation of teriparatide fragments.

BIOshell™ A160 Peptide, C18, 150 x 2.1 mm, 2.7 µm Column

Digested teriparatide USP standard, sample, and blank were analyzed on Agilent 6545 XT AdvanceBio QTOF LC-UV-MS system with a BIOshell™ A160 Peptide, C18 column (150 mm x 2.1 mm, 2.7 µm). USP teriparatide standard was run as a system suitability sample before injection of the teriparatide sample. The HPLC gradient is shown in **Figure 1** along with LC-UV chromatograms for USP teriparatide standard and sample solution. All five fragments were identified by comparing to the USP teriparatide certificate. Resolution between fragment III and I is 8.4 which meets the USP monograph criteria of NLT 1.5.[5] Tailing factor for fragment IV is 1.1 which meets the USP monograph[4] criteria of NMT 2.3 as shown in **Table 2.** The retention time ratio between USP standard and the sample were all within the criteria 1.00 ± 0.03 **(Table 2).** One unknown peak, shown in USP teriparatide certificate as well, eluted in front of Fragment II with retention time at 21.48 min was used for Fragment II peak resolution calculation in **Table 2.**



Time (min)	Mobile Phase A	Mobile Phase B
0.01	99%	1%
8.00	99%	1%
10.00	90%	10%
15.00	80%	20%
26.00	20%	80%
30.00	20%	80%
31.00	99%	1%
36.00	99%	1%
36.00	Stop	

Figure 1. LC-UV chromatograms of teriparatide fragments resulting from Glu-C digestion on BIOshell™ A160 Peptide, C18, 150 mm x 2.1 mm, 2.7 µm. a) USP teriparatide standard, b) teriparatide sample. Flow rate: 0.3 mL/min, column temperature: 40 °C.

Peak ID	RT (min)	Area	Height	Symmetry	Width	Plates	Resolution	Tailing
Fragment III	5.97	13.16	2.14	0.69	0.096	22395	NA	1.2
Fragment I	7.39	17.27	2.53	0.81	0.108	27611	8.4	1.1
Fragment V	17.77	110.78	23.13	1.4	0.07	376655	70.6	0.9
Fragment IV	19.83	257.53	74.02	0.88	0.055	968726	20.9	1.1
Unknown I	21.48	144.74	44.22	0.75	0.05	1094124	20.4	1.2
Fragment II	21.93	234.8	70.79	0.88	0.051	1279074	5.6	1.1

Table 2. Result table for USP teriparatide standard (system suitability injection) on BIOshell™ A160 Peptide, C18 column.

A second dimension identification of the five fragments was conducted by extracting the mass spectra to match with ExPASY PeptideMass database for teriparatide Glu-C digestion.[6] The *m/z* values of each fragment for both the USP standard and sample are shown in **Figure 2a** and **Figure 2b** listed following the elution order which proves the 100% sequence coverage of teriparatide from Glu-C digestion. The agreement further confirms that the teriparatide sample matches the USP standard.

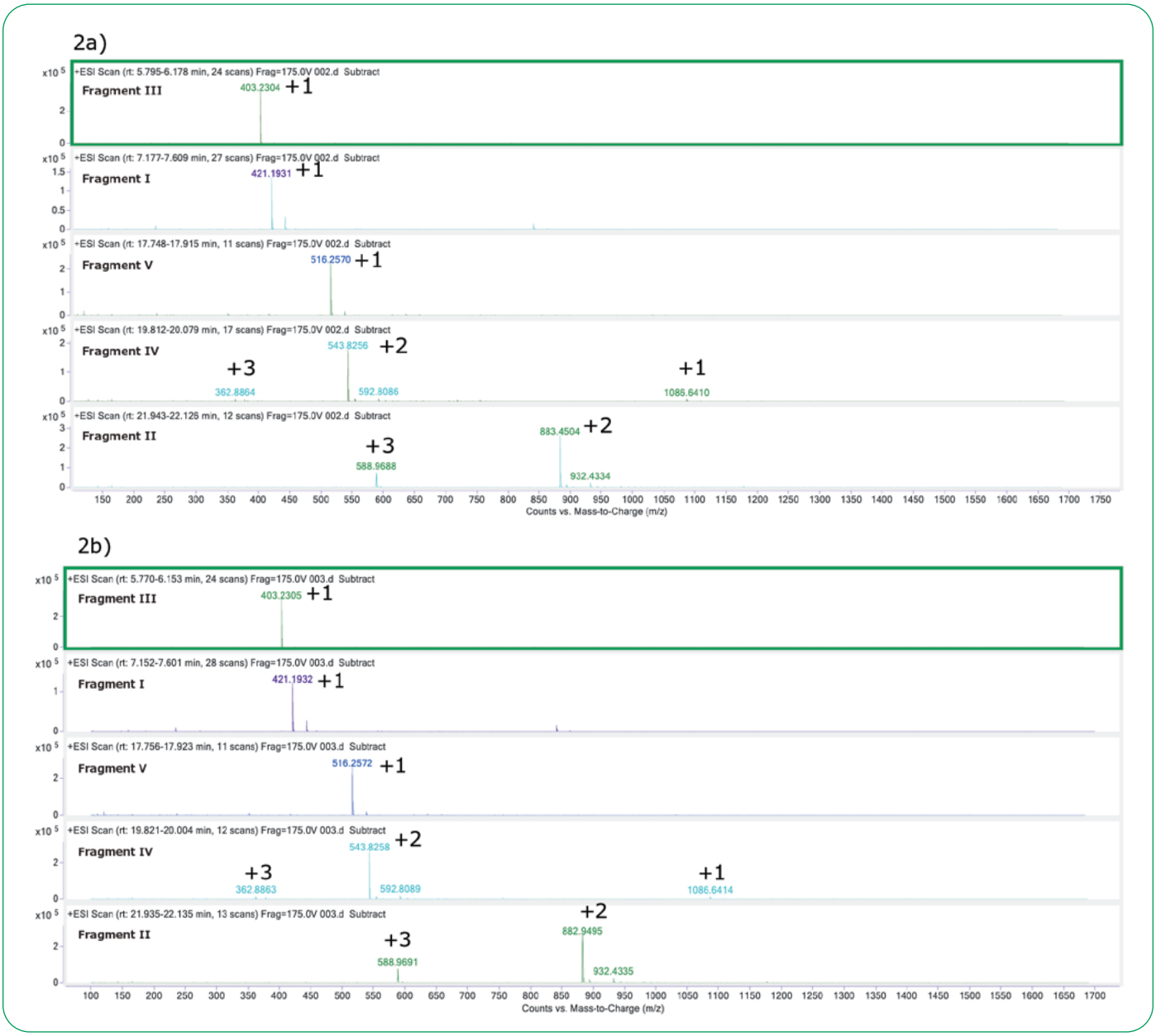


Figure 2. Mass spectra for the five peptides from teriparatide Glu-C digestion on BIOshell™ A160 Peptide, C18, 150 x 2.1 mm, 2.7 µm column, a) teriparatide USP standard, b) teriparatide sample.

Table 3 shows the mass accuracy of the five measured/deconvoluted fragments from Glu-C digestion compared to the theoretical calculated value from ExPASY PeptideMass.[6] The deconvoluted mass for all five fragments from both the USP teriparatide and the sample teriparatide is very consistent to the theoretical monoisotopic mass with mass errors less than 2 ppm.

Teriparatide Glu-C digested fragments	Position	Peptide sequence	Calculated Mass (Da)	USP teriparatide		Sample teriparatide	
				Measured Mass (Da)*	Mass Error (ppm)	Measured Mass (Da)	Mass Error (ppm)
Fragment II	5-19	IQLMHNLGKHLNSME	1763.8811	1763.8835	1.36	1763.8845	1.93
Fragment IV	23-30	WLRKKLQD	1085.6345	1085.6369	2.21	1085.6365	1.84
Fragment V	31-34	VHNF	515.2492	515.2498	1.16	515.2499	1.36
Fragment I	1-4	SVSE	420.1856	420.1858	0.48	420.1859	0.71
Fragment III	20-22	RVE	402.2227	402.2232	1.24	402.2232	1.24

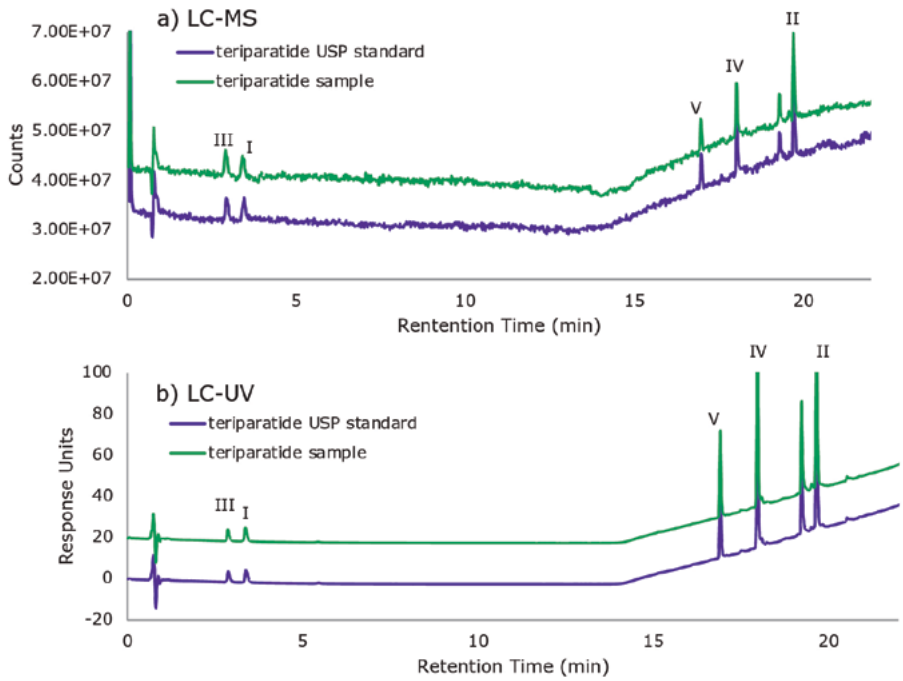
Table 3. Mass accuracy of the five fragments from the QTOF high resolution accurate mass.

*Measured mass is the deconvoluted neutral mass without any charge.

Chromolith® High Resolution RP-18 endcapped, 100 x 2.0 mm Column

Teriparatide peptide mapping was done on a Chromolith® High Resolution RP-18 endcapped column (100 x 2.0 mm). The same mobile phases were used as on the BIOshell™ column above but with an optimized gradient as shown in **Figure 3**. Resolution between fragment III and fragment I in system suitability sample is 3.9 and the tailing factor for fragment IV is 2.1 **(Table 4)**, which both meet USP monograph acceptance criteria. The retention time ratio between the USP standard vs teriparatide sample is in the range of 0.99-1.00 **(Table 4)** which is within the USP criteria of 1.00 ± 0.03.

The LC-MS trace of the five fragments confirms the elution order and the fragment sequence is consistent with the theoretical value from ExPASY database (data not shown).



Time (min)	Mobile Phase A	Mobile Phase B
0.01	98%	2%
12.00	98%	2%
20.00	45%	55%
25.00	0%	100%
29.00	0%	100%
30.00	98%	2%
35.00	98%	2%
35.00	Stop	

Figure 3. Chromatogram of teriparatide Glu-C digestion fragments separation on Chromolith® High Resolution 18-e, 100 x 2.0 mm, a) LC-MS, b) LC-UV. Flow rate: 0.3 mL/min, column temperature: 40 °C.

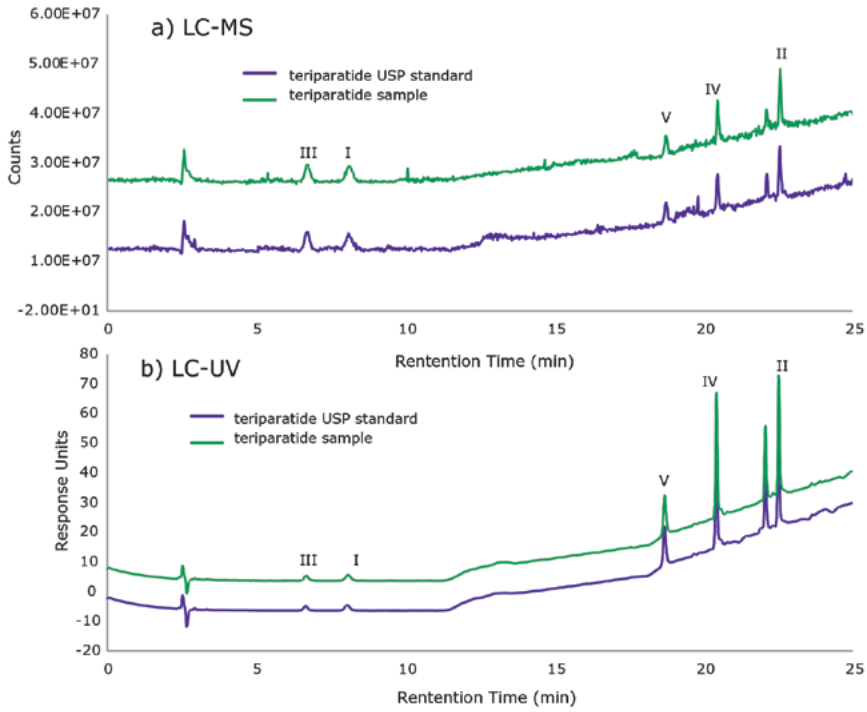
Peak	RT (min)	Area	Height	Symmetry	Width	Plates	Resolution	Tailing	RT ratio
Fragment III	2.878	25.36	5.15	0.69	0.075	8530	NA	1.3	0.99
Fragment I	3.387	35.11	6.52	0.71	0.083	9830	3.9	1.2	0.99
Fragment V	16.921	127.65	40.38	0.75	0.049	694598	124.3	1.3	1.00
Fragment IV	17.975	295.12	82.57	0.67	0.056	819985	13.1	2.1	1.00
Unknown I	19.243	154.03	41.82	0.6	0.055	846007	15.6	1.7	1.00
Unknown II	19.528	6.42	1.98	0.88	0.052	1001269	3.5	1.1	1.00
Fragment II	19.665	276.96	78.43	0.75	0.053	956370	1.7	1.3	1.00

Table 4. LC-UV result data table for USP teriparatide standard system suitability injection on Chromolith® High Resolution 18-e, 100 x 2.0 mm column.

Chromolith® RP-18 endcapped, 100 mm × 4.6 mm Column

Teriparatide peptide mapping was also done on a Chromolith® RP-18 endcapped column (100 x 4.6 mm). The same mobile phases were used as before but with the gradient further optimized as shown in **Figure 4**. In system suitability sample, resolution between fragment III and fragment I is 4.8 and the tailing factor for fragment IV is 1.7 (**Table 5**) meeting USP monograph acceptance criteria. The retention time ratio between the USP standard vs teriparatide sample is rounded to 1.00 (**Table 5**) showing the retention consistency between USP standard and sample teriparatide. Column performance was not as good as the Chromolith® High Resolution RP-18 100 mm x 2.0 mm endcapped column and further optimization maybe required.

LC-MS extracted spectra of the five fragments confirms the elution order, and the observed *m/z* values are consistent with theoretical values from ExPASY (data not shown here).



Time (min)	Mobile Phase A	Mobile Phase B
0.01	98%	2%
8.00	98%	2%
10.00	90%	10%
15.00	80%	20%
26.00	20%	80%
30.00	20%	80%
31.00	98%	2%
36.00	98%	2%
36.00	Stop	

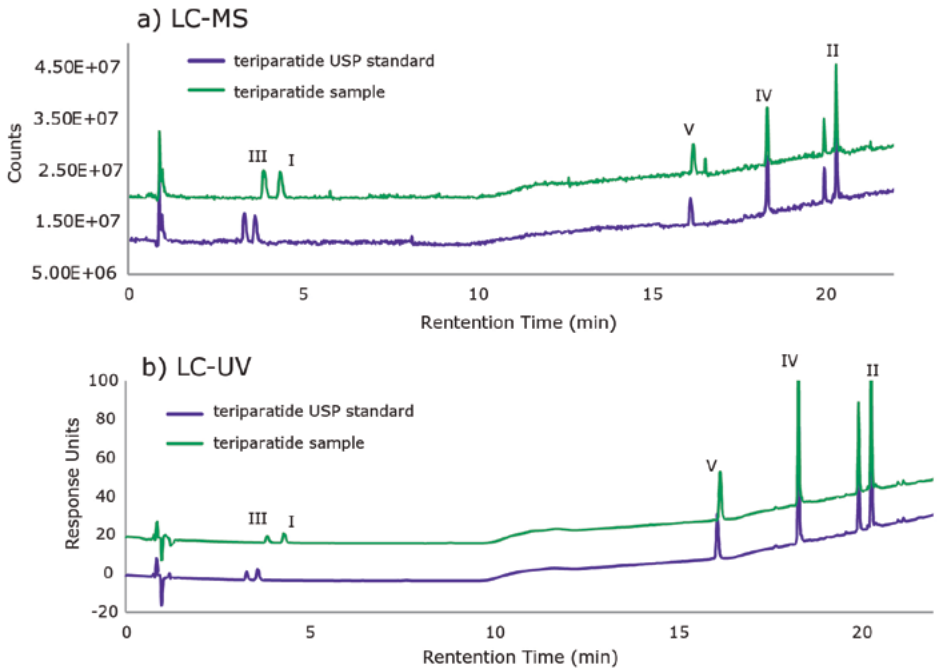
Figure 4. Chromatogram of teriparatide Glu-C digestion fragments separation on Chromolith® 18-e 100 x 4.6 mm, a) LC-MS, b) LC-UV. Flow rate: 0.6 mL/min, column temperature: 40 °C.

Peak	RT (min)	Area	Height	Symmetry	Width	Plates	Resolution	Tailing	RT ratio
Fragment III	6.656	15.91	1.54	0.88	0.158	10171	NA	1.1	1.00
Fragment I	8.055	21.76	1.81	0.89	0.184	10465	4.8	1.1	1.00
Fragment V	18.698	103.37	13.41	0.94	0.114	186835	43.6	1	1.00
Fragment IV	20.433	216.7	40.85	0.69	0.078	530435	12.2	1.7	1.00
Unknown I	22.082	146.02	24.57	0.91	0.085	624120	14.7	0.7	1.00
Fragment II	22.517	171.86	38.05	0.82	0.069	718526	4	1.2	1.00

Table 5. LC-UV result data table for USP teriparatide standard system suitability injection on Chromolith® 18-e 100 x 4.6 mm column.

Ascentis® Express Phenyl-Hexyl, 100 × 3.0 mm, 2.7 µm Column

Teriparatide peptide mapping was done on an Ascentis® Express Phenyl-Hexyl column (100 x 3.0 mm, 2.7 µm). The same mobile phases, 0.1% TFA in water and 0.1% TFA in acetonitrile/water (60:40, v: v) were used as above but with further gradient optimization. The system suitability injection of USP teriparatide standard yielded resolution between fragment III and fragment I of 2.6 and a tailing factor for fragment IV of 1.4 (**Table 6**), meeting USP monograph acceptance criteria. The retention time ratio for fragment V, IV, and II between USP standard and teriparatide sample all rounded to 1.00 (**Table 6**). Fragment III and I retention ratios, between USP standard and teriparatide sample, were 1.17 and 1.20, which were out of the USP monograph criteria. This may have been caused by a system equilibration issue since these two fragments are very polar (**Figure 5**). However, LC-MS spectra were used to confirm the identity of the five fragments and *m/z* values are consistent with theoretical values from ExPASY (data not shown). Therefore, Ascentis® Express Phenyl-Hexyl is capable of the analysis of teriparatide peptide mapping with adequate resolution and peak shapes.



Time (min)	Mobile Phase A	Mobile Phase B
0.01	99%	1%
8.00	99%	1%
10.00	90%	10%
15.00	80%	20%
26.00	20%	80%
30.00	20%	80%
31.00	99%	1%
36.00	99%	1%
36.00	Stop	

Figure 5. Chromatogram of teriparatide Glu-C digestion fragments separation on Ascentis® Express Phenyl-Hexyl column (100 x 3.0 mm, 2.7 µm). a) LC-MS, b) LC-UV. Flow rate: 0.5 mL/min, column temperature: 40 °C.

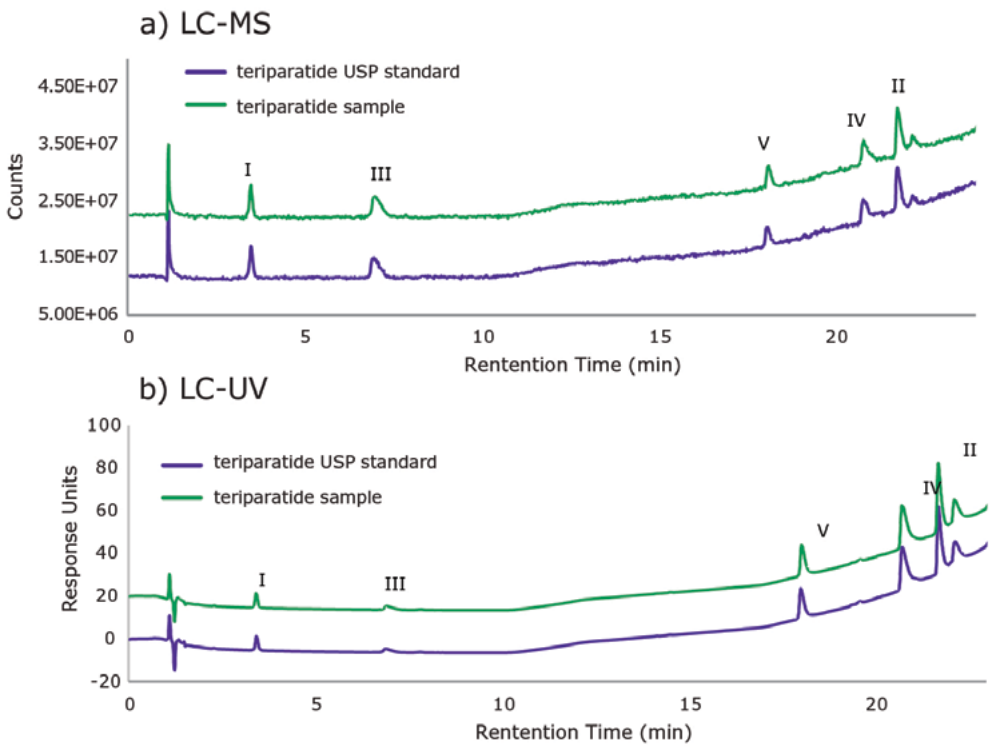
Peak	RT (min)	Area	Height	Symmetry	Width	Plates	Resolution	Tailing	RT ratio
Fragment III	3.296	20.4	4.46	0.82	0.071	13232	NA	1.2	1.17
Fragment I	3.602	28.18	5.87	0.83	0.074	15138	2.6	1.1	1.20
Fragment V	16.111	103.35	23.57	0.73	0.069	361151	111.5	1.3	1.00
Fragment IV	18.322	237.38	73.07	0.86	0.050	863831	23.8	1.4	1.00
Unknown I	19.962	133.64	44.03	0.86	0.047	1102834	21.2	1.1	1.00
Fragment II	20.308	213.84	69.20	0.86	0.048	1103408	4.5	1.1	1.00

Table 6. LC-UV Result data table for USP teriparatide standard system suitability injection on Ascentis® Express Phenyl-Hexyl column.

Ascentis® Express F5, 100 × 3.0 mm, 2.7 µm Column

Teriparatide enzyme digests were evaluated on an Ascentis® Express F5 column (100 x 3.0 mm, 2.7 µm) as well. Again, the same mobile phases were used as above with the gradient further optimized. System suitability injection of USP teriparatide standard generated peak resolution between fragment I and fragment III was 13.9 **(Table 7)** meeting USP monograph acceptance criteria well. The tailing factor for fragment IV is 2.6, which is slightly higher than the USP monograph criteria of 2.3 **(Table 7)**. On this column, the elution orders between fragment III, fragment I, the unknown peak, and fragment II are different from the columns used above. Peak identification was confirmed by MS results shown in **Figure 7**. Spectra for each of the fragment peaks was extracted and the observed *m/z* compared to the ExPASY database for teriparatide Glu-C digestion. Comparing the USP teriparatide standard and the sample teriparatide, the retention time ratios for all five fragments all rounded to 1.00 (**Figure 6** and **Table 7)**.

The Ascentis® Express F5 column provided adequate resolution for fragment III and I which is the most challenging separation in teriparatide peptide mapping. Fragment elution order on Ascentis® Express F5 is different from the C18 columns and the Phenyl-Hexyl column which indicates Ascentis® Express F5 would provide alternative selectivity for teriparatide peptide mapping work. Peak shapes on the Ascentis® Express F5 were not as good as with the other columns under the conditions tested. Optimization of chromatographic conditions to improve the peak shape may prove beneficial.



Time (min)	Mobile Phase A	Mobile Phase B
0.01	99%	1%
8.00	99%	1%
10.00	90%	10%
15.00	80%	20%
26.00	20%	80%
30.00	20%	80%
31.00	99%	1%
36.00	99%	1%
36.00	Stop	

Figure 6. Chromatogram of teriparatide Glu-C digestion fragments on Ascentis® Express F5 column (100 x 3.0 mm, 2.7 µm), a) LC-MS, b) LC-UV. Flow rate: 0.4 mL/min, column temperature: 40 °C.

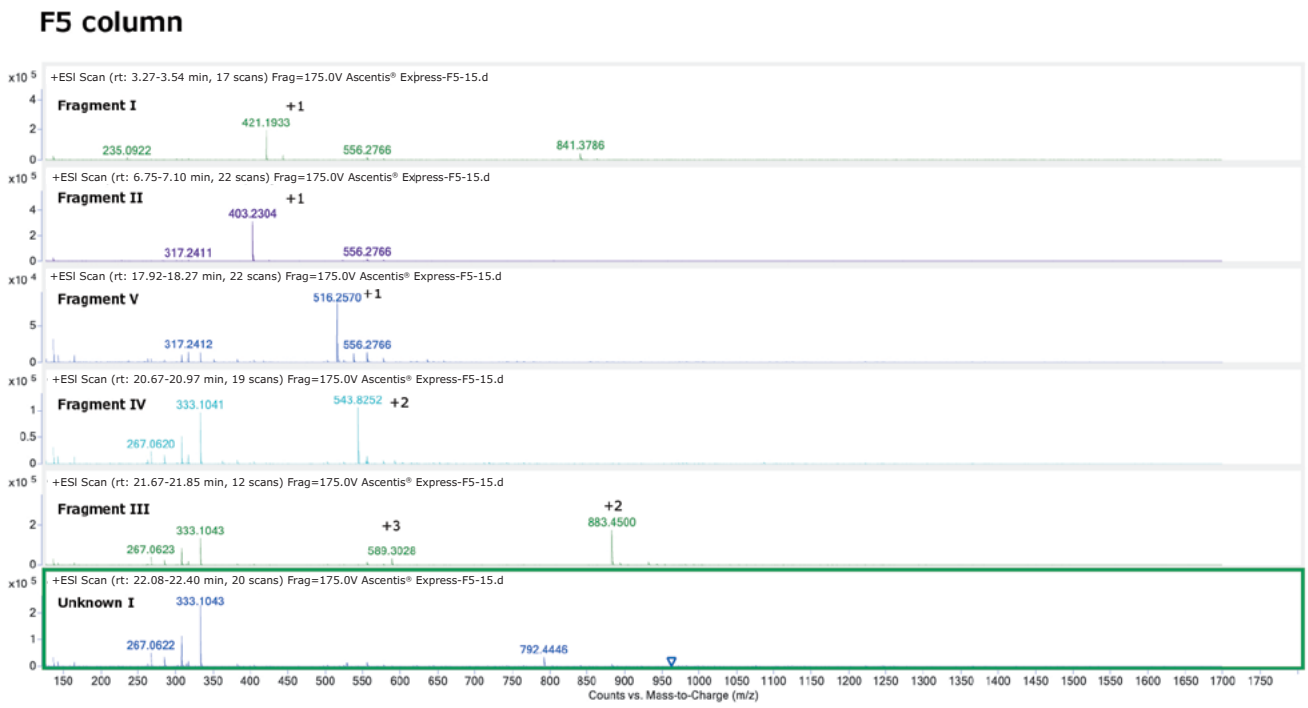


Figure 7. Mass spectra for the five fragment peptides from Glu-C digestion of teriparatide sample on Ascentis® Express F5.

Peak	RT (min)	Area	Height	Symmetry	Width	Plates	Resolution	Tailing	RT ratio
Fragment I	3.427	35.52	6.88	0.77	0.078	12524	NA	1.1	1.00
Fragment III	6.882	23.07	1.69	0.32	0.192	5409	13.9	1.8	1.00
Fragment V	18.035	119.04	13.89	0.39	0.130	107957	37.6	1.8	1.00
Fragment IV	20.757	226.80	19.47	0.25	0.170	72367	10.3	2.6	1.00
Fragment II	21.726	244.57	31.25	0.28	0.113	183852	3.8	2.4	1.00
Unknown I	22.169	138.09	10.99	0.64	0.185	89051	1.8	1.5	1.00

Table 7. LC-UV Result data table for USP teriparatide standard system suitability injection on Ascentis® Express F5 column.

Conclusions

A complete LC-UV-MS workflow has been developed for peptide mapping of teriparatide. This work illustrates the utility of orthogonal column selectivity and LC-UV-MS in peptide mapping analyses, as a means of confirming product identity and sequence coverage. Such approaches maybe useful in development of biosimilars and in monitoring lot to lot variations in manufacturing of peptide and protein drugs. The current USP compendial methods is LC-UV based. Here we show both the LC-UV and LCMS data to identify the peptide fragments detected in the LC-UV using HRMS. Modifications to column dimensions and gradients in compendial methods may require additional validation work. The workflow reported herein offers the following:

- System suitability testing with a USP teriparatide standard
- A step-by-step teriparatide digestion protocol using Endoproteinase Glu-C
- 100% sequence coverage
- Five options for LC column separation of teriparatide digestion resulting fragments
- Complete list of all reagents, consumables, columns, and related products

USP teriparatide standard served as a system suitability sample to evaluate the teriparatide digestion. A detailed enzyme digestion procedure, to achieve 100% sequence coverage, is provided herein. Five different columns, listed in the HPLC column product list, have been evaluated based on USP monograph LC-UV criteria, as well as on mass spectrometry to confirm the peptide fragments identification. BIOshell™ A160 Peptide C18, Chromolith® RP-18e, Chromolith® High Resolution RP-18e, and Ascentis® Express Phenyl-Hexyl columns all showed excellent separation of the five digest fragments, with sufficient resolution and tailing factors to pass USP criteria. The Ascentis® Express F5 showed different elution orders for fragment III and fragment I, the unknown peak and fragment II, compared with the first four columns, indicating that it could be used as an alternative for different selectivity.

References

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6. ExPASY PeptideMass. **https://web.expasy.org/peptide_mass/** Gasteiger E., Gattiker A., Hoogland C., Ivanyi I., Appel R.D., Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31:3784-3788(2003)

Featured Products

Description	Cat. No.
HPLC columns	
BIOshell™ A160 Peptide C18, 2.7 µm particle size, L × I.D. 15 cm × 2.1 mm	**66905-U**
Chromolith® RP-18e, L × I.D. 100 mm × 4.6 mm	**1.02129**
Chromolith® High Resolution RP-18e, L × I.D. 100 mm × 2.0 mm	**1.52322**
Ascentis® Express Phenyl-Hexyl, 2.7 µm particle size, L × I.D. 10 cm × 3.0 mm	**53345-U**
Ascentis® Express F5, 2.7 µm particle size, L × I.D. 10 cm × 3.0 mm	**53578-U**
Chemicals & reagents	
Teriparatide (USP)	**1643962**
Endoproteinase Glu-C from Staphylococcus aureus V8	**P2922-100UN**
Water, HPLC-Grade	**270733**
Acetonitrile, HPLC -Grade	**900667**
Sodium phosphate monobasic	**52074**
Sodium phosphate, dibasic, heptahydrate	**S9390**
Sodium hydroxide solution (50% in H2O)	**415413-100ML**
Phosphoric acid	**49685**
Trifluoroacetic acid (TFA)	**302031**
Instruments & consumables	
Eppendorf ThermoMixer® F1.5	**EP5384000012**
Vials, amber glass, volume 2 mL	**27344**
Pipette 0.5-10 µL	**EP4924000223**
Pipette 10-100 µL	**EP4861000716-1EA**
Pipette 100-1000 µL	**EP4924000282**
Pipette tips 0.1-20 µL box	**Z640204**
Pipette tips 2-200 µL box	**Z640220**
Pipette tips 50-1000 µL box	**Z640247**

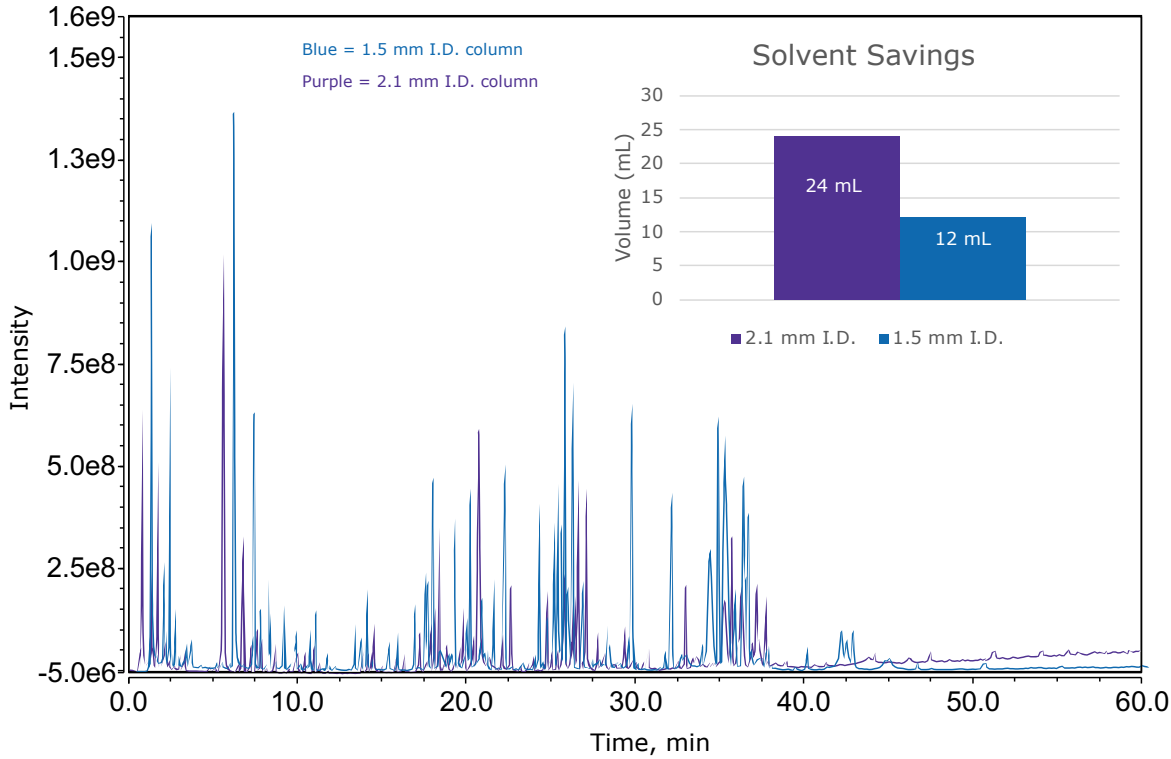
Related Products

Description	Cat. No.
Teriparatide, certified reference material, pharmaceutical secondary standard	**PHR8659-1MG**

UHPLC-MS Bottom-Up Analysis of Trastuzumab on a BIOshell™A160 Peptide C18 Column

Introduction

Bottom-up analysis (also called peptide mapping) is a routine assay performed by analysts in the biopharmaceutical industry as determining the primary structure of a biotherapeutic is a critical quality attribute (CQA). Narrow I.D. columns with 15 cm lengths are typically employed for this analysis in order to achieve high resolution and sensitivity. However, peptide mapping methods require a long run time and, therefore, utilize larger volumes of solvent than shorter methods. This requirement leads to higher costs of the method in terms of higher volumes of solvent used as well as additional expense in removing the solvent from the laboratory. This application note demonstrates the use of a new, 1.5 mm I.D. column in reducing solvent consumption for peptide mapping assays without a compromise in method performance.



Conditions

Column:	BIOshell™ A160 Peptide C18, 15 cm x 2.1 or 1.5 mm I.D., 2.7 µm
Mobile phase:	[A] Water (0.1% (v/v) DFA); [B] Acetonitrile (0.1% (v/v) DFA)
Gradient:	2 – 50% B in 60 min
Flow rate:	0.2 mL/min (1.5 mm I.D.) or 0.4 mL/min (2.1 mm I.D.)
Column temp.:	60 °C
Detector:	MSD, ESI-(+)
Injection:	2.0 µL
Sample:	Trastuzumab tryptic digest, 1.25 mg/mL, 1.5 M Guanidine hydrochloride, 0.5% (v/v) formic acid

MS Conditions

Spray voltage:	3.8 kV
Capillary temp:	320 °C
Sheath gas:	35
Aux gas:	10
RF lens:	50

Conclusion

This application note described the use of a new 1.5 mm I.D. column to reduce solvent consumption in peptide mapping workflows without a compromise in efficiency. As noted, 50% less solvent was consumed, as compared to a 2.1 mm I.D. column, using the 1.5 mm I.D. column as the optimum flow rate for this column is 0.2 mL/min. This observation translates to only 12 mL of solvent being used in this assay versus 24 mL using a 2.1 mm I.D. column. By using less solvent, the cost per sample is reduced as well as the cost of waste disposal, making this a truly “green” method. Finally, sensitivity, in general, was improved using the 1.5 mm I.D. column versus the 2.1 mm I.D. column, enabling more accurate quantitation of signature peptides as well as the detection of post-translational modifications.

Featured Products

Product Part Number	Description
66922-U	BIOshell™ A160 Peptide C18, 15 cm x 1.5 mm I.D., 2.7 µm UHPLC Column
66905-U	BIOshell™ A160 Peptide C18, 15 cm x 2.1 mm I.D., 2.7 µm UHPLC Column
900682	Water, for UHPLC, suitable for MS
900667	Acetonitrile, for UHPLC, suitable for MS
G4505	Guanidine hydrochloride, ≥99% (titration), organic base and chaeotropic agent
00922	Difluoroacetic acid, for LC-MS, LiChropur™
00940	Formic acid, for LC-MS LiChropur™, 97.5-98.5% (T)

Related Products

Product Part Number	Description
MSQC22	SILu™Lite SigmaMAb™ Trastuzumab Monoclonal Antibody
EMS0004	SOLu-Trypsin

Protein Fingerprinting of a Viral Vector, AAV5

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Introduction

The culture and use of adeno-associated virus (AAV) as a gene delivery device has seen much interest in recent years as a strategy for delivering targeted gene therapies towards muscle, nerve, liver, and eye disorders. Different serotypes of AAV target specific tissues and genetic engineering is also being used to create hybrid types with altered tissue specificity. In-depth characterization of the viral capsid proteins, as well as the genomic content, is essential to verify critical quality attributes of these particles. Both the amino acid sequence as well as post-translational modifications (PTMs) that are found on the viral capsid proteins can have an impact on the tissue tropism, efficacy, and immunogenicity of AAV.[1] PTMs that have been identified on AAV include phosphorylation, SUMOylation, ubiquitination, acetylation, methylation, and glycosylation.[2]

The USP released draft guidelines[3] for the analytical characterization of viral vectors in 2022 to provide method starting points for determination of critical quality attributes. A variety of tests are described to characterize identity, purity, concentration, and potency, among other traits. For determination of capsid identity, starting methods are provided that use Western blotting, reverse-phase HPLC with UV detection, and HPLC with MS detection. The latter approach describes the use of both intact mass analysis of the capsid proteins, as well as amino acid sequence analysis using peptide mapping. Here we describe our own work to develop methods for protein fingerprinting of AAV serotype 5 using both intact mass analysis and peptide mapping. Several post-translational modifications of the viral proteins VP1, VP2 and VP3 were identified.

Experimental Methods

A system suitability mix, MSRT1, was prepared according to the instructions on the data sheet but with a final acetonitrile concentration of 1.6%. The injection volume was 10 µL. This solution is a mix of 14 isotopically labelled peptides injected prior to injection of samples to verify instrument performance.

AAV Production

AAV5 subtype (Q9YIJ1) was produced in HEK293 non-T VirusExpress® 293 AAV Production Cells (**cat. no. VP002**) using EX-CELL® CD HEK293 Viral Vector Medium (**cat.no. 14385C**). Three transfection plasmids carrying: 1. Replication and capsid genes; 2. certain adenovirus genes on a helper plasmid and 3. a plasmid with the gene of interest, in this case green fluorescence protein (GFP), were used. After three days of transfection, full AAV capsids, as well as product related impurities such as empty capsids, and capsids filled with host DNA fragments or plasmid backbones, were produced. The HEK cells were then lysed with detergent, treated with benzonase/magnesium, to digest all unused plasmid DNA and host nucleic acids, and finally treated with 0.5 M sodium chloride. The clarified lysate was affinity purified on an FPLC system (ÄKTA) using commercially available resin, from host cell proteins and digested nucleic acids. This process resulted in viral particle (vp) concentrations between 4E+12 and 2E+13 vp/mL as determined by ddPCR.

Intact Mass Analysis

AAV particles were treated with 10% acetic acid for 15 minutes to dissociate capsid proteins before centrifugation at 12,000 rpm for five minutes. Approximately 0.6 µg of total capsid protein was injected for each run.

Chromatography was performed on BIOshell™ A400 Protein C4, 3.4 µm particle, and BIOshell™ IgG 1000 Å C4, 2.7 µm particle columns, with a competitor column, mentioned in the draft guideline, for comparison. All had dimensions of 100 x 2.1 mm. Mass spectrometry was conducted on a Waters™ Xevo® G2-S QTof in positive ion mode. Chromatography conditions and instrument parameters for the applied LC-UV-MS method were as shown in **Tables 1** and **2** below.

Table 1. HPLC conditions for intact mass analysis

LC Conditions			
Instrument:	ACQUITY™ Premier UPLC		
Columns:	BIOshell™ A400 Protein C4, 100 x 2.1 mm, 3.4 µm (**66825-U**); BIOshell™ IgG 1000 Å C4, 100 x 2.1 mm, 2.7 µm (**63288-U**)		
Mobile phase:	[A] Water containing 5% (v/v) acetonitrile with 0.1% TFA; [B] Acetonitrile (0.1% v/v TFA)		
Gradient:	**Time (min)**	**A (%)**	**B (%)**
	0.0	80	20
	1.0	68	32
	16.0	64	36
	20.0	20	80
	21.5	20	80
	22.0	80	20
	30.0	80	20
Flow rate:	0.2 mL/min		
Column temp.:	80 °C		
Detector:	UV @ 280 nm and mass spectrometry (**Table 2**)		
Injection:	5 µL		
Sample:	As described in text		

Table 2. MS Conditions used for intact mass analysis

Mass Spectrometer Conditions	
Instrument:	Waters™ Xevo® G2-S QTof
Polarity:	Positive ion
Capillary (kV):	3.0 kV
Sampling cone:	120 V
Source offset:	120 V
Source temperature:	125 °C
m/z range:	500-4000

Peptide Mapping

Digestion of purified AAV particles was conducted with both trypsin and, separately, chymotrypsin, using a Low-Artifact Digestion Buffer (**cat.no. EMS0011**) and a filter-assisted, sample preparation protocol. For details on this protocol, please see the online application note.[4]

Both a Supelco® BIOshell™ A160 Peptide C18, 2.7 µm particle column and an identical BIOshell™ column with 2 µm particles were used with gradient conditions outlined in the USP draft guidelines.[3] Both columns were 150 x 2.1 mm in dimension. For comparison, a competitor C18 column, mentioned in the draft guideline, was evaluated using the same conditions (**Table 3**) and column dimensions.

Mass spectrometry was conducted on a Thermo™ QE Plus in positive ion mode using a scan range of 200 to 2000 m/z and data dependent MS2 of the top ten ions (**Table 4**).

Table 3. HPLC conditions used for peptide mapping

LC Conditions			
Instrument:	ACQUITY™ UPLC		
Columns:	BIOshell™ A160 Peptide C18, 2.7 µm (**66905-U**); BIOshell™ A160 Peptide C18, 2 µm (**67243-U**); Competitor C18 130 Å, 2.5 µm FPP column All 150 x 2.1 mm		
Mobile phase:	[A] 0.1% Formic acid in water; [B] 0.1% Formic acid in acetonitrile		
Gradient:	**Time (min)**	**A (%)**	**B (%)**
	0.0	97	3
	0.5	97	3
	50.0	45	55
	50.1	10	90
	55.0	10	90
	55.1	97	3
	75.0	97	3
Flow rate:	0.25 mL/min		
Column temp.:	40 °C		
Pressure:	2890 psi (2.7 µm column); 5725 psi (2 µm column); 3555 psi (2.5 µm column) at start of run.		
Detector:	MS (**Table 4**)		
Injection:	5 µL		
Sample:	As described in text		

Table 4. Mass spectrometer conditions used for peptide mapping

MS Conditions	
Instrument:	Thermo™ QE Plus
Polarity:	Positive ion
Spray voltage:	3.5 kV
Capillary temperature:	320 °C
Sheath gas:	10
Aux gas:	5
S-Lens	50 V
m/z range:	200-2000
ddMS2:	Top 10

Results

Protein Fingerprinting

The draft USP guidelines provided a starting point for MS characterization of viral vectors by both intact capsid fingerprinting and by peptide mapping. The draft guideline also describes an additional method, using UV detection and a 2-hour chromatographic run, for determination of capsid stoichiometry. While we did not replicate that method, we did use UV detection in conjunction with mass spectrometry of the intact viral capsids over the shorter 30-minute run. Integration of the UV detected peaks was then used to evaluate capsid stoichiometry. The combined LC-UV-MS analysis provided a convenient one-method assessment of fingerprint and stoichiometry in a shorter run time.

Intact Mass Analysis of VP1, VP2, and VP3

The comparison of columns for separation of the intact capsid proteins is shown in **Figure 1**. Both the BIOshell™ A400 C4 and BIOshell™ IgG 1000 Å C4 columns provided good retention of the proteins using the gradient described in the draft guideline. Separation of VP1 from VP2 and VP3 was also achieved. The BIOshell™ A400 C4 column also provided partial separation of a VP3-clip from VP3. The competitor column gave faster elution with the same conditions but did not show a distinct peak for VP1. Deconvolution of the peaks observed on the A400 C4 column resulted in the mass determinations shown in **Figure 2**.

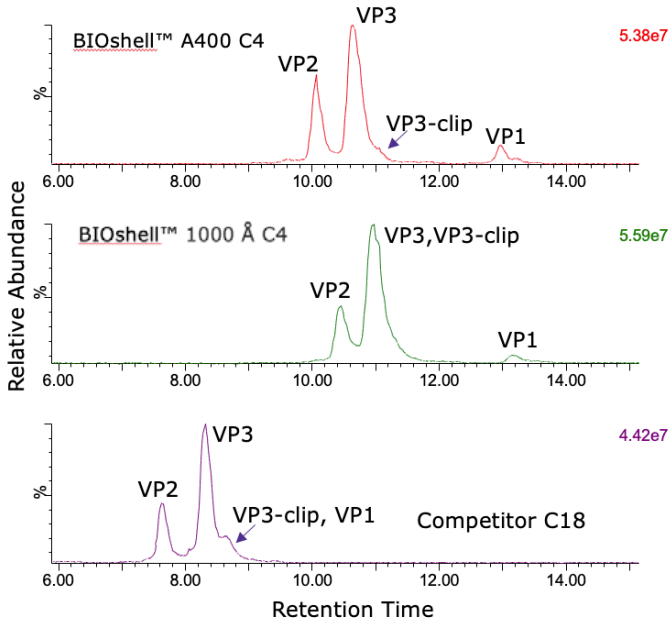


Figure 1. Total ion current (TIC) profile comparison of AAV5 capsid protein retention and separation on three columns evaluated.

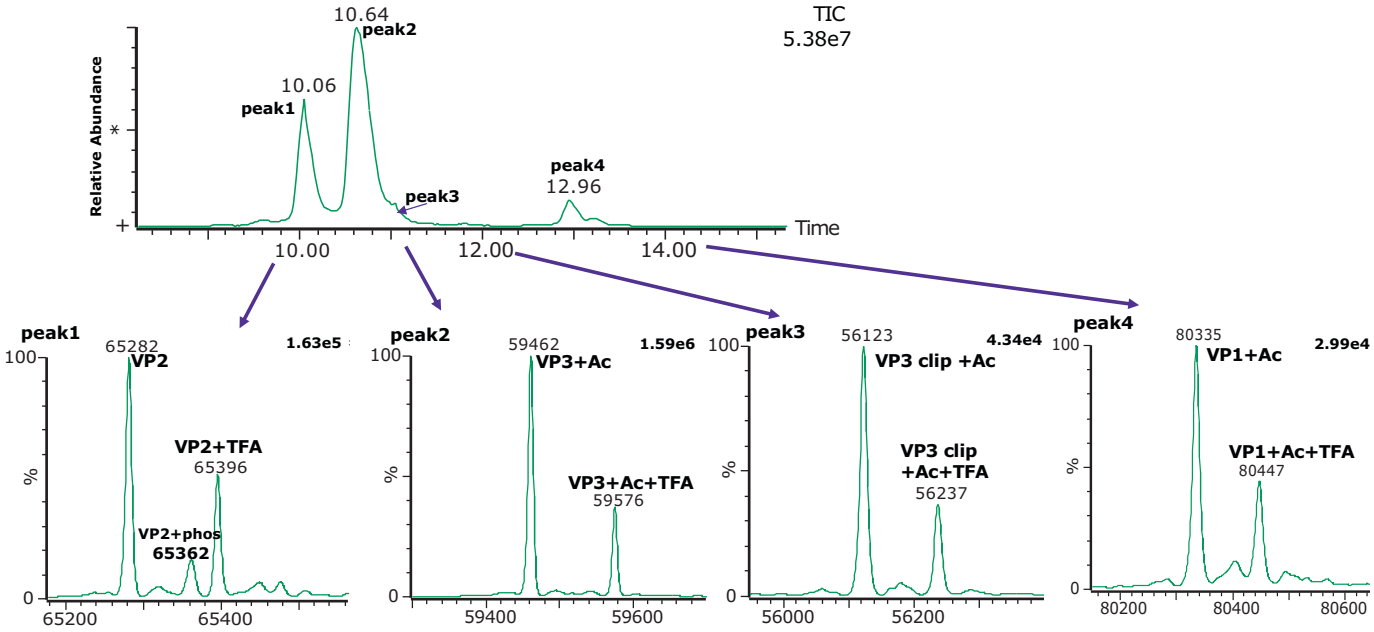


Figure 2. Deconvoluted spectra from each of 4 dominant peaks observed on the BIOshell™ A400 Protein C4 column.

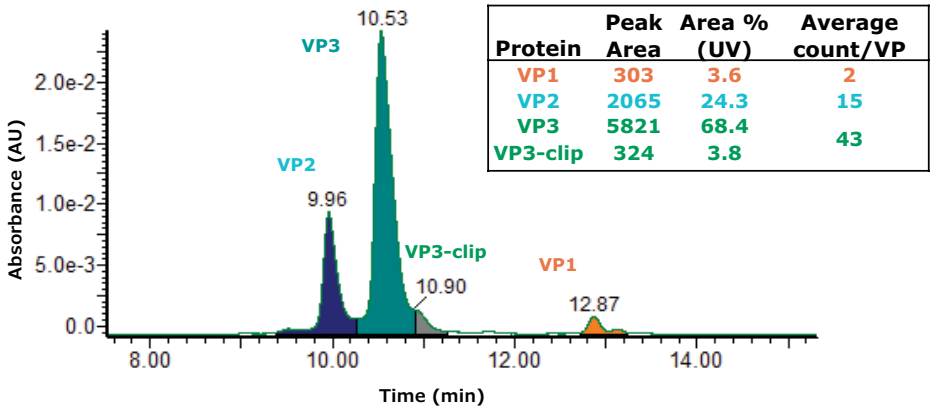
Both VP1 and VP3 were observed to be highly acetylated while VP2 showed a degree of phosphorylation. The presence of 0.1% TFA in the mobile phases was found to result in a substantial amount of TFA adduction on each of the proteins. Interestingly, other AAV serotypes (AAV2, AAV8) we have examined have not shown this same degree of TFA adduction. Replacing the TFA with 0.1% formic acid resulted in a loss of chromatographic separation of the three capsid proteins although the TFA adduction was eliminated (data not shown). The use of difluoroacetic acid (DFA) could be evaluated as a compromise between chromatographic separation and MS sensitivity.

A clipped form of VP3 was also observed, corresponding to the cleavage of the aspartic acid-proline(695) bond. This bond is known to be particularly labile to hydrolysis under acidic conditions and elevated temperature, and similar clip proteoforms have been reported by others.[5,6] **Table 5** shows the close agreement of the observed masses and the theoretical masses for the capsid proteins.

Table 5. Observed masses and the theoretical masses for the capsid proteins

Viral Protein	AA seq	Modification	Theoretical mass (Da)	Observed mass (Da)	Mass error (%)
VP1	2-724	N-term Acetylation	80336	80335	0.0012
VP2	138-724		65283	65282	0.0015
VP3	194-724	N-term Acetylation	59463	59462	0.0017
VP3-clip	194-694	N-term Acetylation	56125	56123	0.0036

Using the same conditions shown in **Table 1**, but with UV detection, a measurement of the relative abundance of the four main peaks was made, as shown in **Figure 3**.



Protein	Peak Area	Area % (UV)	Average count/VP
VP1	303	3.6	2
VP2	2065	24.3	15
VP3	5821	68.4	43
VP3-clip	324	3.8	

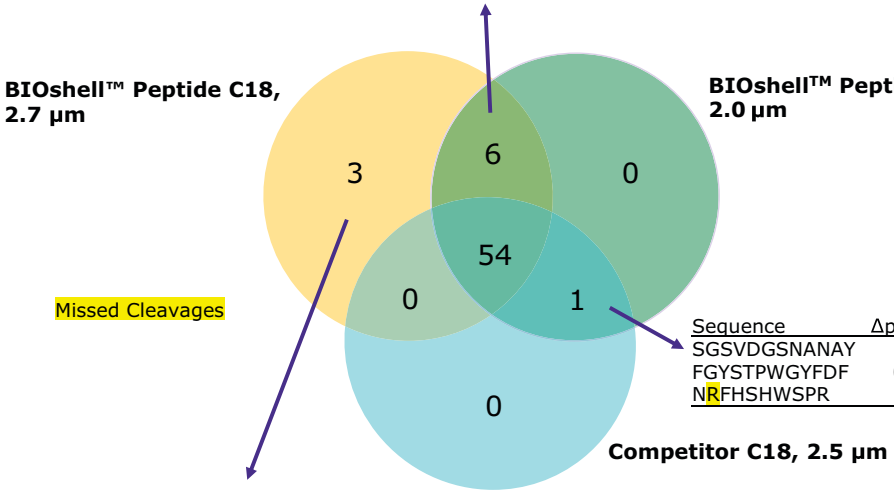
Figure 3. Stoichiometry determination using UV (280 nm) detection on the BIOshell™ A400 Protein C4 column.

While the stoichiometry of AAV capsids is sometimes suggested to have a specific ratio of capsid proteins, such as 1:1:10, Wörner et al.[7] nicely describe the process of capsid formation as being a stochastic sampling of capsid proteins available in the cell pool. AAV capsids are then made up of a highly heterogenous composition of three capsid proteins so that measured stoichiometry represents only an average composition. Combining both the UV and MS detection in the same run is an added convenience in evaluation of samples.

Peptide Mapping

For the comparison here, we used the column and conditions suggested in the draft USP guidelines; a competitor column and gradient conditions shown in **Tables 3** and **4**, above. This gradient is delivered over a 50-minute period. On both the competitor and BIOshell™ columns, good sequence coverage was obtained using tryptic digestion but with slightly better sequence coverage on the BIOshell™ columns due to retention of several small, early eluting peptides including VVTK, ADEVAR, GEPVNR, SLRVK, RIDDHFPKR (**Figure 4**). Importantly, the N-terminal sequence of VP2, (APTGK) was also identified on these columns, in addition to the N-terminal sequences of VP1 and VP3 as shown in **Figure 5**.

	Sequence	Δppm	RT	MS Area	Protein	start	end
Polar/Early eluters	VVTK	-1.84	1.89	4.24E+06	VP1	229	232
	ADEVAR	-0.65	3.05	3.35E+05	VP1	67	72
	GEPVNR	-0.45	3.22	2.44E+05	VP1	61	66
	SLRVK	-1.07	4.11	1.88E+05	VP1	301	305
	RIDDHFPKR	-0.77	7.98	1.99E+05	VP1	143	151
VP2 N-terminus	APTGK	1.18	1.58	8.11E+04	VP2	138	142



Sequence	Δppm	RT	MS Area	Protein	start	end
SGSVDGSNANAY FGYSTPWGYFDF NRFHSHWSPR	0	28	4.46E+06	VP1	252	285

Sequence	Δppm	RT	MS Area	Protein	start	end
STRTWVLPSYNNHQYREIK	0.36	15.3	1.02E+05	VP1	233	251
STRTWVLPSYNNHQYR	0.39	15.5	1.03E+05	VP1	233	248
DVYLQGPIWAKIPETGAHFH PSPAMGGFGLK	0.94	24.9	6.95E+04	VP1	600	630

Figure 4. Venn diagram showing the number of peptides identified using each of the three columns evaluated.

One large section of 60 amino acids was not covered by tryptic digestion alone so a separate digestion was performed with chymotrypsin to increase overall sequence coverage. Similar multi-enzyme digestion of AAV capsids has been shown useful by others.[8] Using the two enzymes, separately, coverage was increased to 100% on the BIOshell™ columns.

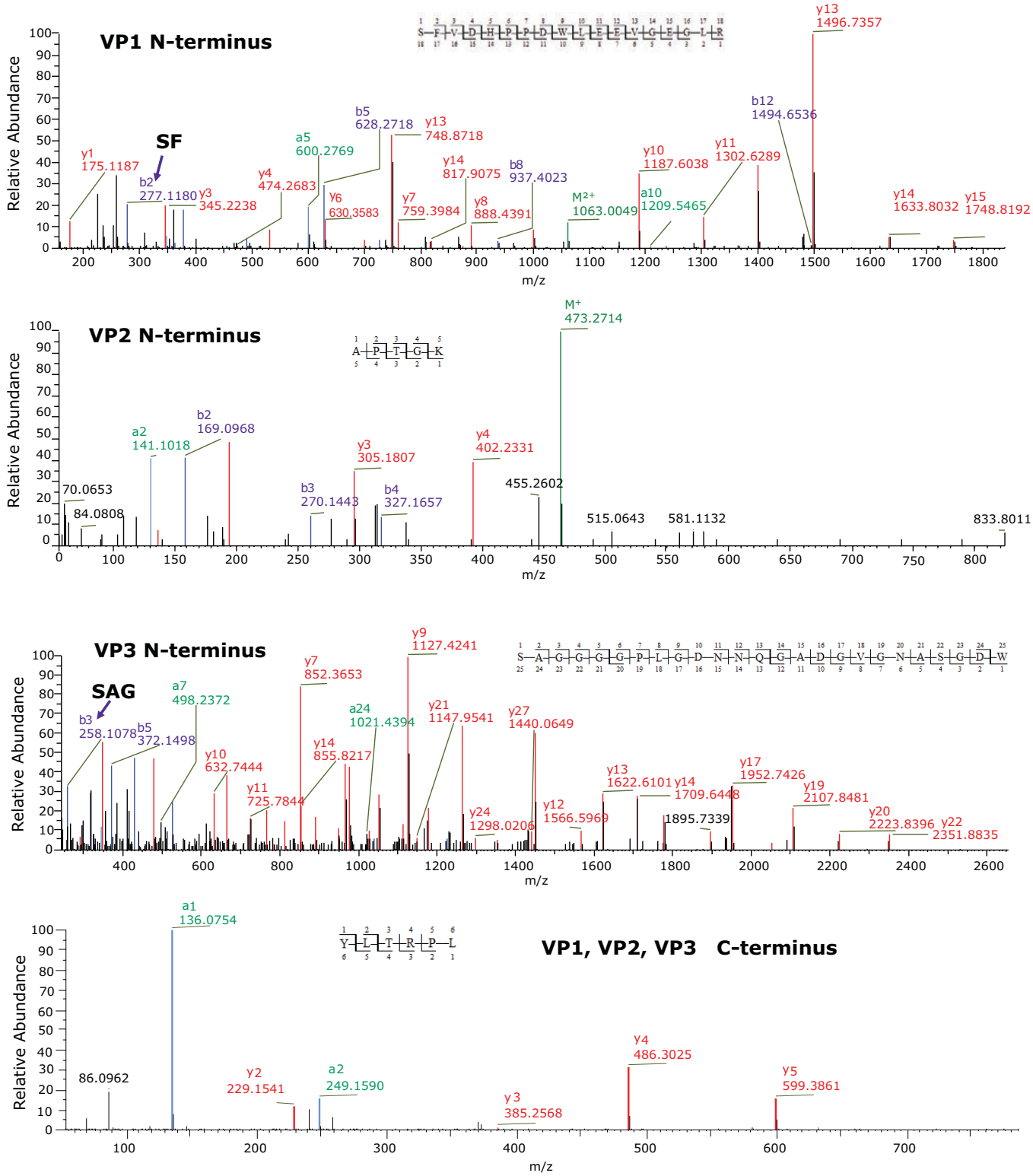


Figure 5. Product ion spectra providing sequence coverage of VP1, VP2, and VP3 N-termini as well as C-terminus of each.

A summary of the PTMs identified, along with their percent abundance on each of the three columns is shown in **Table 6.**

Table 6. Post-translational modifications and their abundance determined

Protein	Residue #	Modification	Peptide Sequence	% Abundance Competitor C18	% Abundance BIOshell™ Peptide C18 2 µm	% Abundance BIOshell™ Peptide C18 2.7 µm
VP1	1	S1+Acetylation	SFVDHPPDWLEEVGEGLR	100	100	100
VP3	1	S1+Acetylation	SAGGGGPLGDNNQGADGV GNASGDWHCDSTWMGDR	100	100	100
VP1	55	N55+Deamidation	GLVLPGYNYLGPGNGLDR	28.2	30.2	25.2
VP1,2,3	468	M468+Oxidation	NWFPGPMGR	0.53	0.56	0.67
VP1,2,3	474	W474+Oxidation	TQGWNLGSGVNR	0.21	0.21	0.20
VP1,2,3	568	M568+Oxidation	VAYNVGGQMATNNQSSTTA PATGTYNLQEIVPGSVWMER	0.73	0.76	0.59
VP1,2,3	623	M623+Oxidation	IPETGAHFHPSPAMGGFGLK HPPPMMLIK; IPETGAHFHPSPAMGGFGLK	0.28	1.24	0.34
VP1,2,3	530	S530+Phosphorylation	IFNSQPANPGTTATY	5.70	5.80	5.90
VP1,2,3	~648	~S648+Phosphorylation	MLIKNTPVPGNITSF; IKNTPVPGNITSF	2.12	2.70	2.54
VP2	1-41	Phosphorylation	APTGKRIDDHFPKRKKARTEE DSKPSTSSDAEAGPSGSQQL	9.55	5.50	9.51
VP1,2	155-227	Phosphorylation	TEEDSKPSTSSDAEAGPSGSQ QLQIPAQPASSLGADTMSAG GGGPLGDNNQGADGVGNA SGDWHCDSTWMGDR	7.68	11.59	9.10

Both VP1 and VP3 were found to be 100% acetylated while at least four sites of phosphorylation were identified. Acetylation of the N-terminus of AAV capsids appears to be highly conserved across serotypes.[2] In addition to the deamination site, four sites of oxidation were observed with all being less than 1% abundance of the unoxidized forms. Overall, the agreement in the abundances of the PTMs between the columns was very good.

Conclusions

Several column comparisons were shown to demonstrate uses of the BIOshell™ line of columns for characterizing viral vectors, in this case AAV serotype 5. Conditions outlined in the USP draft guideline were used for both intact mass fingerprinting of viral capsids and for peptide mapping experiments, but we suggest that further improvements in chromatography might be made with additional gradient optimization.

The BIOshell™ columns, particularly the A400 Protein C4 column, have proven to be effective in separating the three capsid proteins of AAV5 for intact mass analysis and stoichiometry evaluation. In addition, the A400 column provides partial separation of the VP3-clip from VP3. The competitor column also shows partial separation of VP3-clip from VP3 but with coelution of VP1.

The Peptide C18 columns, in both the 2.7 and 2.0 µm particle sizes proved to be useful in retaining short, polar peptides to provide slightly improved sequence coverage over the competitor column. Retention of the N-terminus of VP2 was only provided by the BIOshell™ columns.

The USP draft conditions for mobile phase and gradient conditions to both approaches to characterizing capsids were used here, but we suggest these conditions might benefit from further optimization with other AAV serotypes or specific PTMs.

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Featured Products

Product	Cat. No.
HPLC	
BIOshell™ A160 Peptide C18, 15 cm x 2.1 mm I.D., 2.7 µm	**66905-U**
BIOshell™ A160 Peptide C18, 15 cm x 2.1 mm I.D., 2.0 µm	**67243-U**
BIOshell™ A400 Protein C4, 10 cm x 2.1 mm I.D., 3.4 µm	**66825-U**
BIOshell™ IgG 1000 Å C4, 10 cm x 2.1 mm I.D., 2.7 µm	**63288-U**
Acetonitrile, LiChrosolv® hypergrade for LC-MS	**1.00029**
Formic acid, for LC-MS LiChropur™	**533002**
Trifluoroacetic acid, eluent additive for LC-MS, LiChropur™	**80457**
Difluoroacetic acid, for LC-MS LiChropur™	**00922**
Water, LiChrosolv® for LC-MS	**1.15333**
Samples and System Suitability Reagents	
MS RT Calibration Mix, Proteomics Retention Time Standard for LC-MS	**MSRT1**
Sample Preparation	
Acetic acid, for LC-MS LiChropur™	**5.33001**
Chymotrypsin Sequencing Grade, 4x25 µg	**11418467001**
Iodoacetamide (IAM), Single use vial of 56 mg	**A3221**
Low Artifact Digestion Buffer	**EMS0011**
SOLu-Trypsin, recombinant, expressed in *Pichia pastoris*, Proteomics Grade, liquid	**EMS0004**
Tris(2-carboxyethyl)phosphine (TCEP), 0.5 M, pH 7.0(aqueous solution; pH was adjusted with ammonium hydroxide)	**646547**
Microcon®-30kDa Centrifugal Filter Unit with Ultracel®-30 membrane	**MRCF0R030**

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intact/middle up analysis

Intact and Middle-Up Analysis

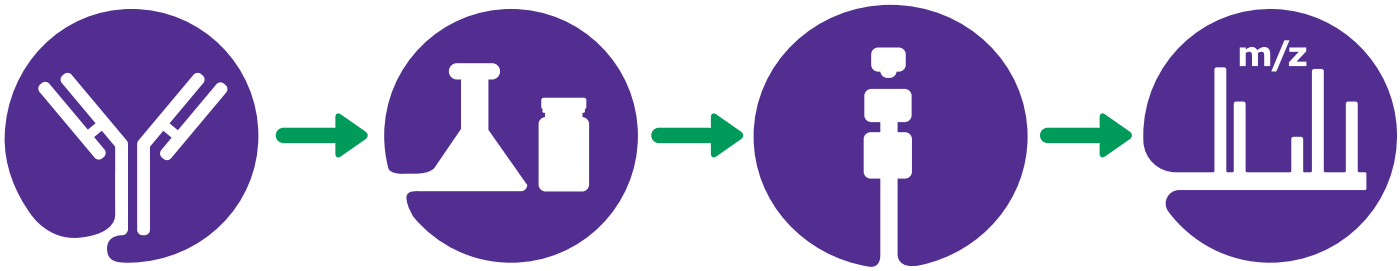
Intact antibody analysis and middle-up analysis are valuable techniques in the field of biopharmaceutical research, providing crucial insights into the structural characterization and quality control of therapeutic antibodies. Intact antibody analysis focuses on determining the intact mass and evaluating the overall integrity of antibodies, while middle-up analysis involves the characterization of subunits or fragments of the antibody. These analyses are vital for ensuring the safety, efficacy, and consistency of antibody-based drugs, as any alterations or variations in the structure can impact their functionality and therapeutic properties. By employing these techniques, researchers can gain a comprehensive understanding of the antibody's structure and detect any modifications or impurities that may arise during production or storage.

Performing intact antibody analysis typically involves a combination of techniques, including liquid chromatography (LC) and mass spectrometry (MS). The process begins with the separation of the intact antibody from any impurities in the sample using LC. The separated antibody is then introduced into the mass spectrometer, where it is ionized and analyzed to determine the intact mass. By comparing the measured mass with the theoretical mass of the antibody, researchers can assess the presence of any modifications or variations in the structure.

Middle-up analysis, on the other hand, focuses on the characterization of antibody subunits or fragments. This analysis involves the digestion of the antibody using specific proteases, such as IdeS, which cleave it into smaller fragments while preserving structural information. The resulting fragments are then separated and analyzed using LC-MS, allowing for the identification and quantification of the subunits. Middle-up analysis provides insights into the structural heterogeneity, post-translational modifications, and subunit interactions within the antibody, enabling researchers to evaluate its quality and functionality.

Intact antibody analysis and middle-up analysis play vital roles in the structural characterization and quality control of therapeutic antibodies. These techniques provide valuable information about the intact mass, subunit composition, and modifications of the antibody, ensuring the safety, efficacy, and consistency of antibody-based drugs. By employing advanced analytical methods such as liquid chromatography and mass spectrometry, researchers can accurately assess the structural integrity of antibodies and monitor any modifications that may impact their therapeutic properties.

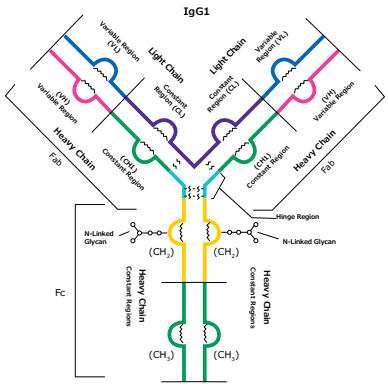
Workflow for Middle-up Analysis

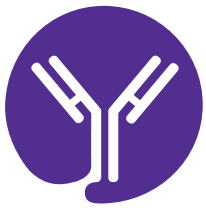


Analysis of Therapeutic Monoclonal Antibody Trastuzumab using BIOshell™ A400 Protein C4 Column

Fast and high-resolution analysis of intact monoclonal antibodies (mAbs)

Sundaram Palaniswamy, Segment Lead, Pharma QC; Aditya Pratihast, Application Scientist





Abstract

Although the majority of small molecules analysed by reversed phase have a mass below 1500 Da, there is a growing need to improve the performance of HPLC columns for the separation of therapeutic proteins and protein drug conjugates. This application note demonstrates a fast and reproducible reversed phase method with high-resolution for the analysis of intact therapeutic monoclonal antibody, trastuzumab. Separation and quantification were achieved using a BIOshell™ A400 Protein C4 column in less than 5 minutes, and more importantly, the optimised method was able to monitor degradation compounds created by heat stress studies.

Introduction

Over the past few years, monoclonal antibodies (mAbs) have become the best-selling drugs in the pharmaceutical market, and in 2018, eight of the top 10 best-selling drugs worldwide were biologics. The global therapeutic monoclonal antibody market was valued at approximately $115 billion in 2018 growing up to $300 billion by 2025. And although as of December 2019, 79 therapeutic mAbs have been approved by the US FDA for sales worldwide, there is a significant potential for the number to increase.[1] HPLC is a well-established method for the analysis of intact mAbs by Size Exclusion and Ion Exchange chromatography. However, technological advancements in the field of Reversed Phase (RP) have made them promising tools for the analysis on intact proteins.[2] Intact mAbs are yet analyzed with limited success using wide pore, fully porous particles due to their large molecular size leading to slow mass transfer and long analysis times. Superficially porous particles have overcome these challenges, but have lower loadability and still a rather limited offering of different stationary phases. Moreover, highly resolving core-shell columns easily separate intact mAbs quickly and with high efficiency.

Here, we have demonstrated the suitability of the BIOshell™ A400 Protein C4 column for a fast and high-resolution separation of intact trastuzumab using RP-HPLC. Retention time and area precision of the method were excellent, demonstrating the suitability of the column. Further we also showcase quantification and robustness that is highly suitable for biopharmaceutical QC applications.

Experimental

Equipment and Sample

The study was performed on a Shimadzu LC-2010CHT HPLC System. The therapeutic trastuzumab was purchased from a local pharmacy.

Methods

Chromatographic parameters for intact trastuzumab using a BIOshell™ A400 Protein C4 column are shown in Table 1.

Table 1. Chromatographic parameters used for RP HPLC analysis of trastuzumab

LC Parameters	
Column:	BIOshell™ A400 Protein C4, 100 x 2.1 mm I.D., 3.4 µm (**66825-U**)
Mobile phase:	[A] Water + 0.1% TFA; [B] Acetonitrile + 0.09% TFA
Gradient program:	**Time** / **%A** / **%B** 0 / 95 / 5 1 / 95 / 5 2 / 80 / 20 6 / 50 / 50 8 / 5 / 95 8.1 / 95 / 5
Post time:	2 minutes
Flow rate:	1 mL/min
Autosampler temp.:	5 °C
Column temp.:	80 °C
Detector:	UV 280 nm, 20 Hz
Injection volume:	10 µL
Sample:	1 mg/mL trastuzumab (1:10 dilution of formulation in mobile phase A)

Linearity, Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The calibration curve was constructed with nine standard concentrations of trastuzumab from 1 to 25 µg/mL. The mAb concentration that provided a signal-to-noise ratio (S/N) > 3 was considered as LOD and S/N > 10 was considered as LOQ.

Forced Degradation Studies

We compared the chromatographic profiles of native and heat-stressed trastuzumab for monitoring degraded products. For the forced degradation studies, 1 mg/mL of trastuzumab was exposed to 10 ppm hydrogen peroxide (H_2O_2) followed by heating at 80 °C for 60 min. An aliquot of 10 µL was used for RP HPLC analysis.

Results and Discussion

Intact Trastuzumab Analysis

For the HPLC analysis, a BIOshell™ A400 Protein C4, 3.4 µm HPLC column with core-shell particles and 400 Å pore size delivered reproducible, fast and high-resolution separation of intact trastuzumab, making it suitable for biopharma development and QC applications. **Figure 1** demonstrates excellent peak shape and overlays of six replicates in less than 5 minutes under the chromatographic conditions.

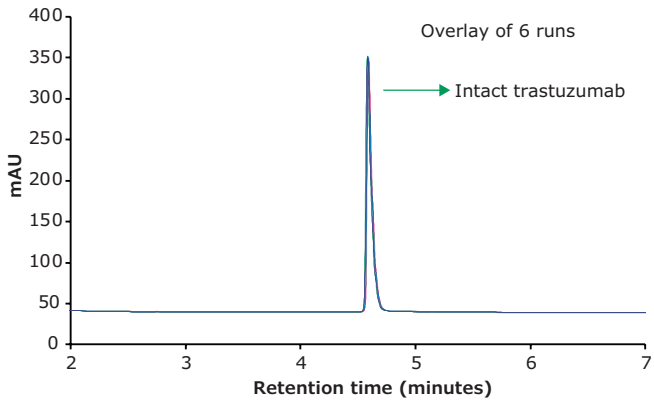


Figure 1. RP-HPLC analysis of trastuzumab on a BIOshell™ A400 Protein C4, 100 x 2.1 mm, 3.4 µm HPLC column.

Precision of Retention Time and Area

Table 2 shows the average Retention Time (RT) and Area RSDs from six replicates of trastuzumab injections. The Retention Time and Peak Area RSDs were less than 0.1% and 0.29 %, respectively, which demonstrates excellent reproducibility of the method and, thus, the precision of the method.

Table 2. Retention time and peak area precision (n = 6) for trastuzumab (1 mg/mL)

	Mean	RSD (%)
Retention Time (min)	4.58	0.1
Peak Area	987268	0.29

Limit of Detection and Limit of Quantitation

The LOD and LOQ were 0.125 µg/mL and 0.25 µg/mL, respectively, for trastuzumab, indicating that the method was sensitive. Observed LOD and LOQ values of trastuzumab are reported in **Table 3**. Representative chromatograms on same scale for 2 calibration runs & blank are shown overlayed in **Figure 2**.

Table 3. LOD, LOQ, and mean area and retention time (n = 3)

	Concentration (µg/mL)	Mean Area (n=3)	Retention Time (min)
LOD	0.125	9562	4.58
LOQ	0.25	21977	4.58

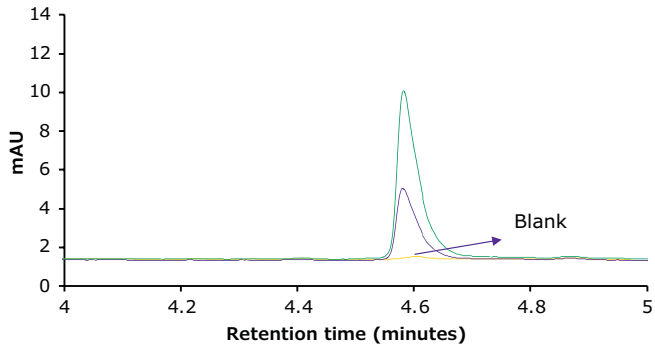


Figure 2. Overlay of representative chromatograms on same scale for 2 calibration runs & blank.

Linearity

Linearity curves for trastuzumab were constructed from 1 µg/mL up to 25 µg/mL in this study using area response and concentration of trastuzumab. The average peak areas are listed in Table 4. The linearity curve for trastuzumab is shown in **Figure 3**.

Table 4. Summary of linearity range (n = 3) for trastuzumab

Trastuzumab Conc. (µg/mL)	Average Area
1	95,961
2	194,821
4	394,886
6	593,986
8	791,940
10	984,370
15	1,480,051
20	1,940,216
25	2,447,554

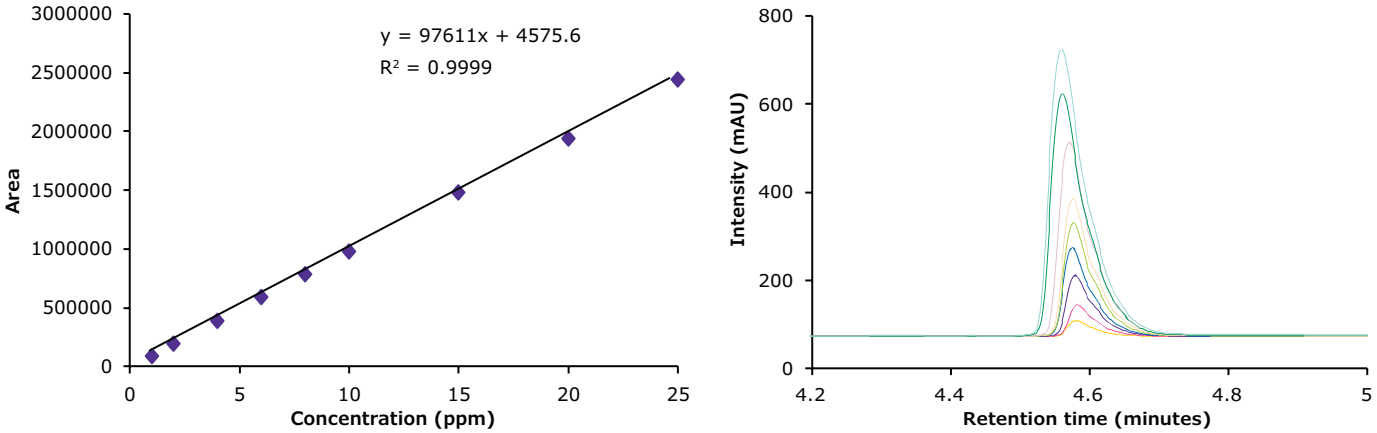


Figure 3. Linearity curve with nine standard concentrations of trastuzumab ranging from 1 to 25 µg/mL showing excellent coefficient values. Also shown are chromatogram overlays for the linearity ranges.

Trastuzumab Degradation Studies

We compared the intact and stressed trastuzumab using RP-HPLC to evaluate if this method is stability indicating. Any deviations in peak RT or Area as a result of stress were considered degradation products.

Figure 4 compares the RP-HPLC profile of unstressed and heat stressed trastuzumab. The profiles indicate that the BIOshell™ A400 Protein C4, HPLC column was able to distinguish between unstressed and stressed trastuzumab based on the peak shape and area.

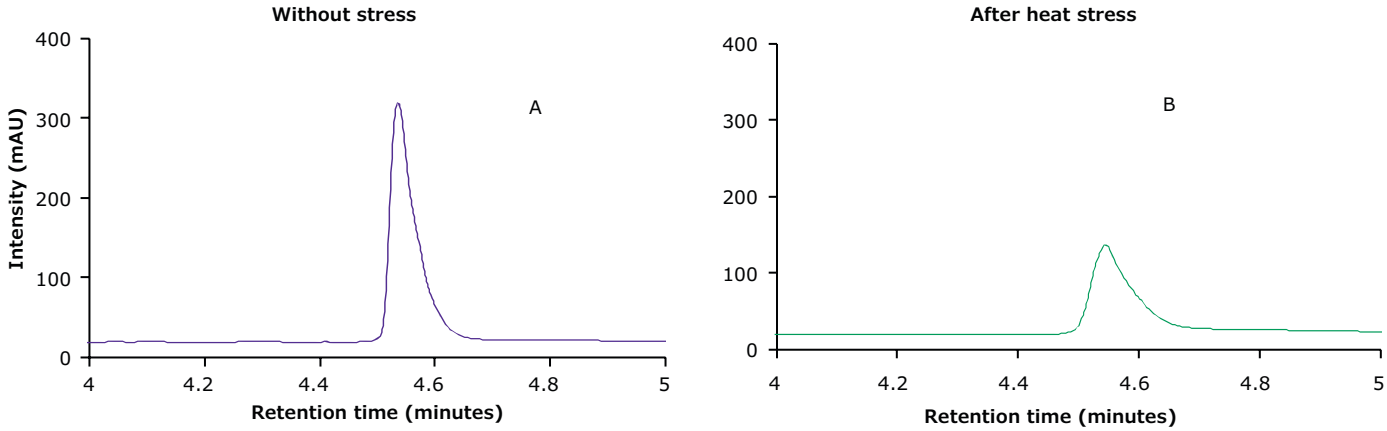


Figure 4. BIOshell™ A400 Protein C4, 100 x 2.1 mm, 3.4 µm RP-HPLC profiles of unstressed (A) and heat stressed trastuzumab sample (B)

Conclusion

Analysis of intact mAbs provides a first level of interrogation of size, post translational modification and heterogeneity. RP-HPLC analysis of mAbs requires large pore sizes, a hydrophobic stationary phase and appropriate chromatographic methods. In this application note a simple LC-UV method for the analysis of intact trastuzumab was showcased. Using a BIOshell™ A400 Protein C4 column, a high resolution and rapid separation of intact trastuzumab was developed. Area and RT precision of the method were excellent and showed the reliability of the method. The calibration curves with nine standard concentrations of trastuzumab had excellent coefficient of linearity values displaying that the method was quantitative and accurate. The LOD and LOQ for trastuzumab were found to be 0.125 µg/mL and 0.25 µg/mL, respectively, indicating the method was sensitive. In addition, heat stressed studies demonstrated that the BIOshell™ A400 Protein C4 column was able to monitor degraded mAbs and the method could be used for stability studies.

References

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Featured Products

Description	Cat. No.
BIOshell™ A400 Protein C4, 100 x 2.1 mm I.D., 3.4 µm	**66825-U**
LiChrosolv® Acetonitrile Isocratic Grade	**1.14291**
Trifluoroacetic acid HPLC grade	**302031**
Water for chromatography LiChrosolv®	**1.15333**
SILu™Lite SigmaMAb™ Trastuzumab Monoclonal Antibody	**MSQC22**

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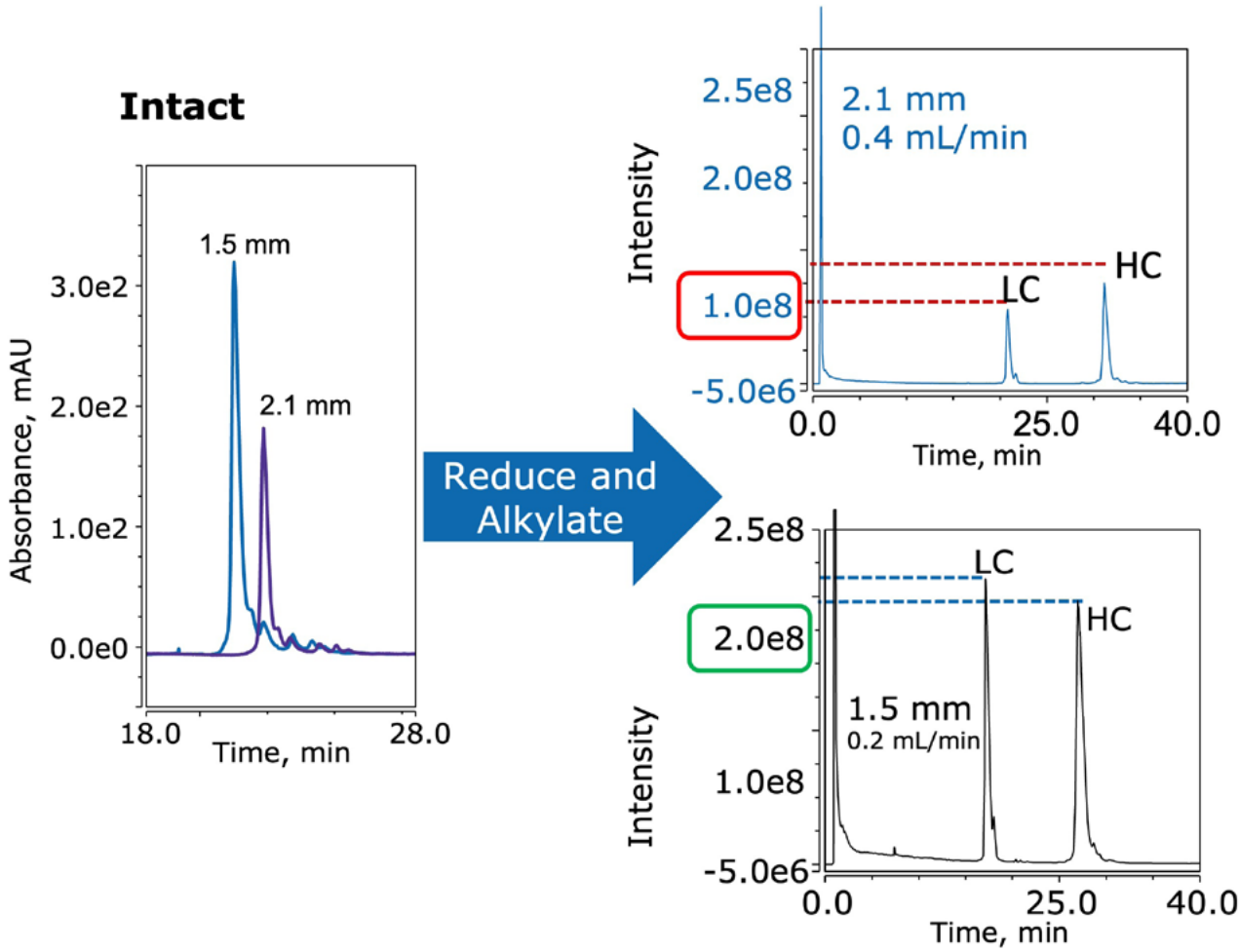
UHPLC-MS Middle-Up and Intact Analysis of Trastuzumab on a BIOshell™ IgG 1000 Å Diphenyl Column

Introduction

Sensitivity and recovery are two desirable traits in any method, especially in methods for characterizing monoclonal antibodies (mAbs). Having a method that can provide sharp, well-resolved peaks with sufficient area counts allows the analyst to better quantitate the analyte of interest and determine if any degradation products are in the sample. This application note demonstrates the advantage of using a smaller I.D. column (1.5 mm) versus a conventional 2.1 mm I.D. column. In the intact analysis of trastuzumab, double the area for the mAb was obtained on the 1.5 mm I.D. column as compared to the 2.1 mm I.D. column. In addition, in the reduced and alkylated assay, the area counts and sensitivity were improved significantly (2.7-fold increase in area count for the light chain (LC) and 2.3-fold increase in area count for the heavy chain (HC)) when using a 1.5 mm I.D. column as compared to a 2.1 mm I.D. column.



Conditions

Column:	BIOshell™ IgG 1000 Å Diphenyl, 15 cm x 2.1 or 1.5 mm I.D., 2.7 µm
Mobile phase:	[A] Water (0.1% (v/v) DFA); [B] 50:50 Acetonitrile (0.1% (v/v) DFA): n-Propanol (0.1% (v/v) DFA)
Gradient:	27 – 36% B in 40 min
Flow rate:	As indicated
Column temp.:	60 °C
Detector:	MSD, ESI-(+)
Injection:	3.0 µL
Sample:	Trastuzumab, 1.0 mg/mL, 100 mM Ammonium bicarbonate

Conclusion

This application note describes the use of a 1.5 mm I.D. column in analyzing both intact and reduced trastuzumab. Improvements in both recovery and sensitivity were observed with the use of a 1.5 mm I.D. column over a conventional 2.1 mm I.D. column.

No special equipment or modifications to existing UHPLC systems were needed to achieve the benefits of the 1.5 mm I.D. column, which allows for any analyst to reap the benefits of this new geometry.

Materials

Product Part Number	Description
577450-U	BIOshell™ IgG 1000 Å Diphenyl, 15 cm x 1.5 mm I.D., 2.7 µm
577421-U	BIOshell™ IgG 1000 Å Diphenyl, 15 cm x 2.1 mm I.D., 2.7 µm
900682	Water, for UHPLC, suitable for MS
900667	Acetonitrile, for UHPLC, suitable for MS
1.01024	1-Propanol, for liquid chromatography, LiChrosolv®
00922	Difluoroacetic acid, for LC-MS, LiChropur™
09830	Ammonium bicarbonate, BioUltra

Charge Variant Analysis (CVA) with Strong Cation Exchange and MS Compatible Buffers

Geoffrey Rule, Principal Scientist, MilliporeSigma
Nivesh K. Mittal, Product Manager, HPLC, Shimadzu Scientific Instruments

Introduction

Protein therapeutics require thorough characterization to determine if all critical quality attributes (CQAs) meet specifications. This process includes the determination of various charge isoforms (variants) of the protein. Protein charge can vary based on amino acid composition, as well as from post-translational modifications (PTMs), such as deamidation, lysine clipping, N-terminal pyroglutamate formation, and glycosylation. A measure of protein charge is the isoelectric point, or pI, the pH at which the protein has an overall neutral charge. If pH is above the pI, then the protein will have a net negative charge. If below the pI, then a net positive charge will exist, and the protein can be retained on a cation exchange column.

Charge variant analysis (CVA) is often performed using cation exchange chromatography on either a weak, carboxylic acid-based resin, or a strong, sulfonic acid-based phase. In some cases, a gradient of non-volatile salts is used to affect the chromatographic separation of charge variants. With a gradient increase in cation concentration, the protein will be displaced from the resin and migrate down the column. In other cases, a mix of non-volatile organic buffers may be utilized to create a well-controlled pH gradient. In this case, the protein will elute through the column when the net charge equals zero. Neither of these approaches can be used with electrospray ionization mass spectrometry since these mobile phases are not compatible with electrospray sources.

The use of electrospray ionization with high resolution, high mass accuracy mass spectrometry, is one of the most powerful approaches to characterizing whole proteins, especially when combined with a chromatographic separation. Intact mass analysis of protein in “native” form refers to analysis of non-digested protein in its natural, biological conformation. This technique is important to get an accurate picture of charge variants of the protein as they exist in biological conditions. Under denaturing conditions, such as in organic solvent or strongly acidic conditions, a protein is no longer in its native conformation and amino acids in the interior of the protein become exposed to the solvent conditions present. This method can alter the charge and conformation of the protein so that the biological state is no longer represented in the analysis. Native MS provides spectra at a higher mass, lower charge state, yielding broader separation of peaks in the spectral charge envelope, and may improve mass accuracy.[1] One other benefit of cation exchange-native MS, with a high-resolution instrument, is that one can discern the modifications creating the variants.[2]

Here, we report on the use of a polymeric cation exchange column to separate charge variants of two monoclonal antibodies using an LC-MS compatible, multi-modal gradient separation of ammonium acetate and pH.

Experimental Conditions

In this application note we use MS compatible buffers to separate mAb charge variants on an SCX column but with UV detection only (**Table 1**). The Proteomix® column used is a polymeric, nonporous particle with a sulfonic acid bonded phase.

Table 1. HPLC-UV conditions

LC Conditions			
Instrument:	Shimadzu Nexera XS Inert UHPLC		
Column:	Proteomix® SCX-NP5, 50 x 2.1 mm I.D., 5 µm (**Z777156**)		
Mobile phase:	[A] 50 mM Ammonium acetate, pH 5.5 (acetic acid) followed by addition of 2% acetonitrile; [B] 200 mM Ammonium acetate, pH 8.5 (ammonium hydroxide) followed by addition of 2% acetonitrile		
Gradient:	**Time (min)**	**A (%)**	**B (%)**
	0.00	75	25
	1.00	45	55
	21.00	5	95
	22.00	75	25
	28.00	75	25
Flow rate:	0.1 mL/min		
Column temp:	30° C		
Detector:	UV @ 280 nm		
Injection:	1 µL		
Sample(s):	NISTmab solution: 10 mg/mL, in 12.5 mM L-histidine, 12.5 mM L-histidine HCl (pH 6.0) in water Infliximab solution: 10 mg/mL, in 12.5 mM histidine buffer		

Results and Discussion

When doing charge variant analysis by pH gradient, others have shown that the buffering capacity of the column itself can influence the gradient.[3] Consequently, to minimize the influence of column buffering capacity, it was found beneficial to use shorter columns with larger particle sizes. Additionally, we have seen in the literature, as well as had personal communications, suggesting that metals in the HPLC system and column hardware can have a negative impact on separations of proteins by adsorbing to certain groups in the protein. For this application, we used an LC system designed to be free of wetted metal components in the sample flow path.

Heterogeneity of protein isoforms gives rise to both acidic and basic variants. These impurities are typically related to the most abundant form, or “main peak”, so that one has early eluting acidic forms and later eluting basic forms (**Figure 1**). As the gradient proceeds, proteins elute from the column when they reach their isoelectric point, or pI.

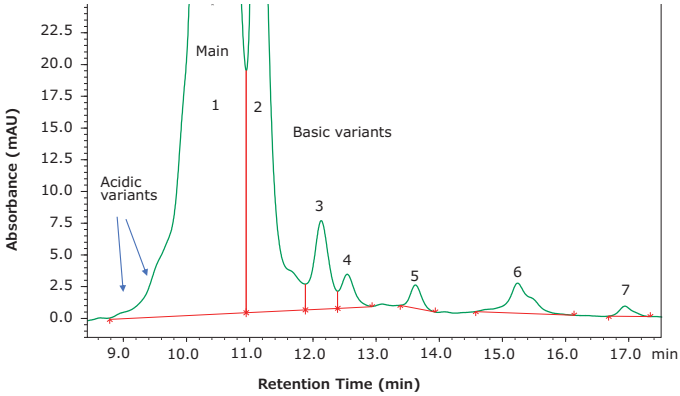


Figure 1. Separation of several variant forms of the monoclonal antibody NISTMab.

Reproducibility

We wanted to evaluate the reproducibility of our charge variant separation over several days and using the same mobile phase preparation. The mobile phase was prepared on day 1 and after performing a series of four injections the mobile phases were left on the instrument at room temperature. On days 2 and 3, the same series of injections were made. Afterwards peaks in the region of interest were integrated and used to evaluate system reproducibility.

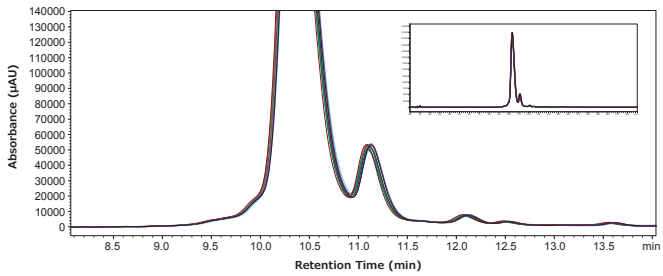


Figure 2. Overlay of 12 chromatograms analyzed over three days, four injections per day and using the same mobile phase preparation.

As shown in **Figure 2**, the 12 injections overlaid very well indicating stability of the column and mobile phase used. Analysis of the seven most abundant components of NISTmAb were used to generate the statistics in **Table 2**. The precision calculations of percent abundance for each component are seen to be excellent and increase only when evaluating very minor components. Retention time stability is also seen to be excellent, while recovery (based on peak area) was found to be very good with no significant drift observed over the course of the injections.

Table 2. Integration Statistics for seven charge variant peaks of NISTmAb across 12 injections over three days.

	Abundance			Retention Time			Peak Area		
	Avg (%)	Std Dev	CV (%)	Avg (min)	Std Dev	CV (%)	Avg Area (mAU*min)	Std Dev	CV (%)
Peak 1	86.325	0.092	0.11	10.344	0.0155	0.15	7707134	173062	2.25
Peak 2	10.902	0.089	0.81	11.111	0.0166	0.15	973336	21474	2.21
Peak 3	1.326	0.013	1.00	12.100	0.0223	0.18	118333	2358	1.99
Peak 4	0.437	0.017	3.95	12.508	0.0245	0.20	39037	1177	3.02
Peak 5	0.258	0.020	7.86	13.582	0.0293	0.22	23052	1704	7.39
Peak 6	0.620	0.047	7.59	15.201	0.0363	0.24	55417	5116	9.23
Peak 7	0.131	0.006	4.63	16.899	0.0420	0.25	11723	753	6.42

In addition to our evaluation of NISTmAb, we used the same conditions and mobile phases to evaluate a separation of infliximab. This material is available as a certified reference material (CRM) at 10 mg/mL in aqueous histidine buffer. **Figure 3** shows the separation achieved with infliximab using the same column and mobile phases but with a slightly modified gradient (shown in figure inset).

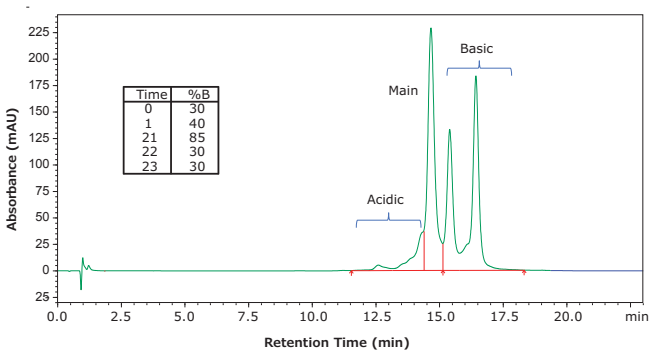


Figure 3. Separation of infliximab charge variants using gradient conditions shown in the inset and with a flow rate of 80 µL/min. All other conditions the same as reported above. Integration of the peaks yielded 8% acidic variants (combined), 40% main peak, and 51% basic variants.

Conclusion

The Proteomix® SCX-NP5 column shows very good performance in separating several charge variants of NISTmAb and infliximab. While mass spectrometric detection was not used here, we show the separation of charge variants using MS compatible mobile phases (ammonium acetate buffers), as demonstrated by the literature cited below, on a metal-free UHPLC system. These mobile phases were found to provide reproducible chromatography over at least three days.

References

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Featured Products

Description	Cat. No.
HPLC	
Proteomix® SCX-NP5 (2.1 mm x 50) mm, 5 µm	**Z777156**
Acetonitrile, LiChrosolv® hypergrade for LC-MS	**1.00029**
Water, LiChrosolv® for LC-MS	**1.15333**
Ammonium acetate, LiChropur™ for LC-MS	**73594**
Ammonium hydroxide, OmniTrace® Ultra *	**AX1308**
Acetic acid, LiChropur™ for LC-MS	**5.33001**
Samples and System Suitability Reagents	
NISTmAb, Humanized IgG1$_k$ Monoclonal Antibody	**NIST8671**
Infliximab (Remicade) solution, certified reference material, Cerilliant®, 0.25 mL	**I-042**

*Only available in North America, alternative is LiChropur™ product **cat.no. 5.43830**

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Protocol for Purification, Optional Reduction, and SEC-MS Analysis of a Monoclonal Antibody

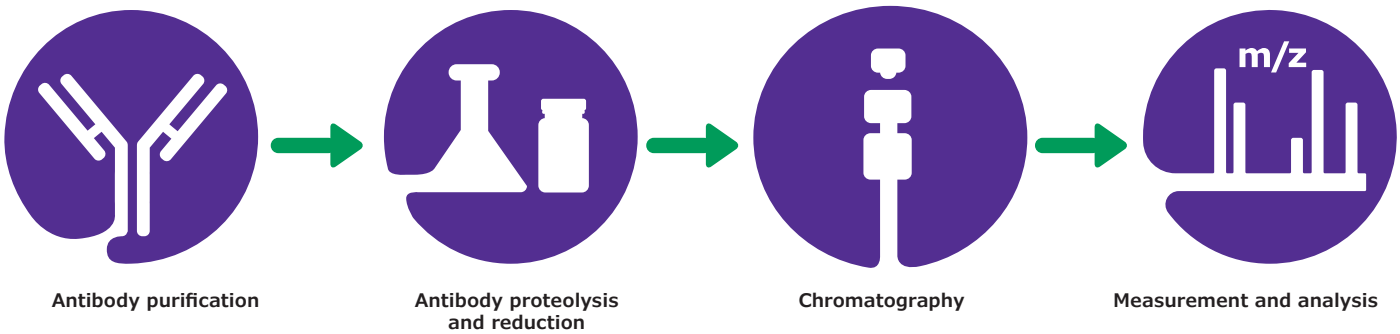
Stephan Altmaier[1], Pegah Jalili[2], Uma Sreenivasan[3], Kevin Ray[2]
[1]Darmstadt, Germany, [2]St.Louis, MO, USA, [3]Round Rock, TX, USA

Workflow For Intact And Middle-Up Mass Analysis Of Adalimumab

A complete SEC-MS workflow has been developed to simplify intact mass analysis of both non-reduced and reduced monoclonal antibodies (mAbs).

In detail, it includes:

- Antibody purification using immobilized protein A
- Antibody reduction procedure (optional)
- Mass spectrometer calibration
- System suitability test utilizing a recombinant human monoclonal antibody reference
- One generic SEC-MS method suitable for sample separation and analysis of both non-reduced and reduced monoclonal antibodies



Characterization of Monoclonal Antibodies and the Role of SEC-MS in Intact and Subunit Mass Analysis

Monoclonal antibodies (or immunoglobulins - IgGs) are large glycoproteins with a molecular weight of approximately 150 kDa (150,000 g/Mol). They are composed of two identical light chains (LC, molecular weight ca. 25 kDa each) and two identical heavy chains (HC, molecular weight ca. 50 kDa each) linked through covalent inter- and intra-chain disulfide bonds. They are utilized for the treatment of various types of cancer, and other diseases such as multiple sclerosis, Alzheimer's disease, and migraine.

Careful and thorough characterization of therapeutic mAbs is essential for ensuring drug safety and efficacy. mAbs are typically manufactured in mammalian host cell lines in bioreactors, generating a large number of heterogeneous drug molecules. It is important to establish critical quality attributes (CQAs) for each mAb and demonstrating that production batches are within acceptable limits are requirements for both innovator and biosimilar therapeutics.[1,2]

In many cases, the characterization of an antibody-based drug is performed using a specific chromatographic technique (e.g., size exclusion, reversed phase or hydrophilic interaction liquid chromatography, respectively – SEC, RP or HILIC)[3,4] coupled with mass spectrometry (MS). This combination allows for different types of analyses to be carried out, e.g., accurate mass measurement of the intact mAb and subunits, peptide mapping, and the determination of post-translation modifications such as glycosylation, oxidation, and deamidation.

Several techniques are applied to simplify antibody analysis by either fragmentation or removal of glycans. The latter can be performed by treatment with PNGase F, whereas proteolysis with IdeS[5] or reduction of inter-chain disulfide bonds with reducing agents, such as dithiothreitol, result in the formation of different antibody fragments with masses of 25 – 50 kDa. Various combinations of these techniques can be applied. For analysis of mAbs in cell culture supernatants, these may be combined with a preceding affinity purification step.[6] These approaches are referred to as intact mass and middle-up analysis methods.[7] The former term relates to the measurement of the mass of an intact mAb without controlled dissociation being performed. Such an experiment reveals information about stoichiometry, proteoforms, and modifications. Middle up experiments include mass measurement

after cleaving mAbs into several large fragments/ subunits via chemical reduction or proteolytic digestion. An example of this approach is the analysis of mAbs light and heavy chains, providing insight into posttranslational modifications of the individual chains. **Figure 1** provides an overview of antibody sample preparation and various digestion options prior to intact mass analysis.

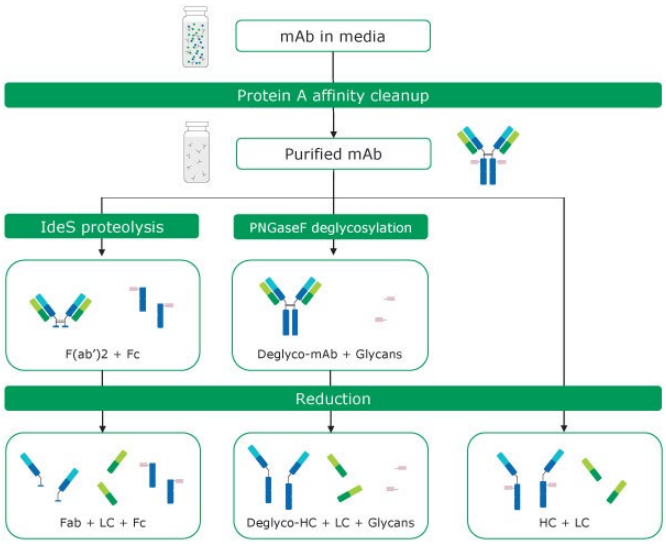


Figure 1. Antibody sample preparation

This report describes the application of both non-reducing and reducing SEC-MS workflows for the generation of intact mass and middle-up data of adalimumab. It includes the correlation of de-charged masses of respective heavy and light chains with calculated theoretical intact masses.

In all experiments, a recombinant human antibody standard, SILu™ Lite SigmaMAb™ (#MSQC4), was utilized as a reference and assay control sample. Throughout the text this is referred to simply as SigmaMAb™ although several different SigmaMAb™ standards are also available commercially.

General Procedures - Sample and Reference Preparation and System Setup for Antibody Purification and SEC-MS

Antibody Purification Procedure

The target antibody purification was performed on cell culture supernatants using immobilized protein A resin in a 96-well format. The suggested minimum working mAb titer is 100 µg/mL.

All procedures were conducted using both reference and assay control samples of SigmaMAb™ with a molecular mass of ~150 kDa. The reference sample consisted of the pure antibody reconstituted in water; it is used for the system suitability tests and instrument check. The assay control sample contained the antibody spiked into or delivered as a mixture with cell culture media or spent media (cell broth including nutrients etc.); it goes through the entire workflow and functions as a control sample.

In detail, the high-throughput purification of mAbs from cell culture media using protein A resin was performed as follows:

1. System/Workflow suitability
   As part of the workflow suitability, an assay control of SigmaMAb™ in media was purified along with the samples. Reference sample (SigmaMAb™) is prepared as follows: [SA1]
   - Reconstitute each vial of MSQC4 in 1.0 mL water to obtain a solution with an antibody concentration of 1 mg/mL.
   - Prepare assay control (spent media sample) by spiking SigmaMAb™ in EX-CELL® CHOZN® Platform Medium, or equivalent, to obtain a final concentration of 100-500 µg/mL.
2. Preparation of equilibration and elution buffers
   - Prepare equilibration buffer (20 mM citrate, 150 mM NaCl, pH 7) by dissolving 5.82 g trisodium citrate dihydrate, 0.04 g citric acid, and 8.77 g sodium chloride in 1 L water. Adjust pH of resulting solution to 7 using 1 M NaOH or HCl as needed; subsequently filter solution using a 0.2 µm filter.
   - Prepare elution buffer (25 mM citrate, pH 3) by dissolving 4.8 g citric acid in 1 L water. Adjust pH of resulting solution to 3 using 1 M NaOH or HCl as needed.
3. Clarify samples
   Centrifuge samples in tubes at maximum speed for five minutes and samples in plates at maximum speed for 60 minutes.
4. Protein A loading
   - Add or remove water from top portion of settled protein A slurry to obtain a 50% protein A suspension.
   - Mix slurry by constant pipette action and gentle shaking of reagent reservoir.
   - Use a multichannel pipette to deliver 200 µL of protein A slurry to each well of a 96-well filter plate. Place protein A filter plate on a vacuum manifold. Catch any flow-through from the filter plate by placing the filter plate on top of a used collection plate.
5. Protein A equilibration
   - Add 200 µL of equilibration buffer to each well of protein A and apply vacuum to void wells of buffer.
   - Repeat both steps twice.

6. Washing bound mAb
    - Place protein A filter plate on vacuum manifold with a waste collection plate inserted and the film cover removed.
    - Apply vacuum to void wells of buffer media and transfer the filter plate onto a waste collection plate.
    - Add 200 µL of equilibration buffer to wells and centrifuge plates at 3700 rpm for five minutes (this step helps in clearing the sample film on sides of filter plate wells).
    - Add 200 µL of equilibration buffer to wells and apply vacuum to remove buffer.
    - Repeat once more for a total of three washes.
7. Eluting bound mAb

- Place protein A filter plate on a new collection plate and secure with a rubber band.
- Add 100 µL of elution buffer to each well, incubate filter plate on orbital shaker at 170 rpm for five minutes.
- Centrifuge plates at 3700 rpm for five minutes.
- Repeat addition of elution buffer, incubation on orbital shaker, and centrifugation for a total of three elution steps (300 µL of total elution volume). Typical antibody recovery using this procedure is 60%.

Antibody Reduction Procedure

Disulfide (S-S) bond reduction was performed as follows:

1. Prepare a 1 M dithiothreitol (DTT) solution by dissolving 154.25 mg DTT in 1 mL water.
2. Prepare a 1 M ammonium bicarbonate (ABC) solution by dissolving 79.06 mg ABC in 1 mL water.
3. Combine equal volumes of 1 M ABC and 1 M DTT to prepare the reduction solution.
4. Transfer aliquots of 50 µL of each sample, system suitability reference, and control to autosampler vials.
5. Reduce by addition of 5 µL 0.5 M ABC/0.5 M DTT solution.
6. Incubate for one hour at room temperature or 30 min at 37 °C.

Note: Reduction is performed under non-denaturing conditions, where the inter-chain disulfide bonds (which are more susceptible to reduction) will break and produce the light and heavy chains, while the intra-chain disulfide bonds within each individual domain remain intact.

Alternatively, reduction of samples can be performed by using this protocol:

1. Prepare a 100 mM solution of tris(2-carboxyethyl) phosphine (TCEP) in 6 M aqueous guanidine hydrochloride by dissolving 2.87 g of TCEP and 57.32 g of guanidine hydrochloride in 100 mL of water (if less solution is needed, scale down accordingly).
2. Combine 30 µL of the resulting solution with 10 µL of sample.
3. Incubate for two hours at 37 °C.

Note: Reduction performed under denaturing conditions, where both the inter-chain and intra-chain disulfide bonds (which are more susceptible to reduction) will break.

Instrument Calibration

The Waters™ QToF Xevo® G2XS mass spectrometer was calibrated in a mass range of 500 – 6000 m/z with a 20 µL/min infusion of 0.4 mg/mL of cesium iodide in water. Alternatively, calibration can be performed with a 20 µL/min infusion of 0.4 mg/mL of polyalanine in water, prior to running the samples.

System Suitability

To evaluate the performance of the entire workflow, an assay control (SigmaMAb™ in media) was prepared and analyzed along with the samples. SigmaMAb™ reference was also tested to ensure system suitability (see section above).

In addition, reduced SigmaMAb™ antibody reference (formulated at 2 mg/mL and further diluted to 1 mg/mL) was analyzed alongside the samples to determine system suitability of the SEC-MS platform.

SEC-MS System Setup and MS Data Analysis

SEC-MS System Setup

The essential settings of the UHPLC-PDA chromatography system and the qToF mass spectrometer applied in the analysis of both reduced and non-reduced antibodies are listed in **Tables 1** and **2** below.

Instrument	Waters™ H-Class Acquity UPLC Chromatography System
Software:	MassLynx® 4.1
Column:	Tosoh TSK Gel SW3000XL, 300 x 2.0 mm, 4 µm
Column temp:	Ambient
Autosampler temp:	8 °C
Mobile phase:	Acetonitrile/water 30/70 (v/v) + 0.1% TFA
Gradient:	Isocratic
Flow:	0.1 mL/min
Loop volume:	20 µL
Injection method:	Partial loop or full loop
Injection volume:	20 µL
Run time:	10 min
Photodiode array:	280 nm
Flow divert:	6.7 - 9.9 min

Table 1. UHPLC-PDA settings.

Instrument	Waters™ QToF Xevo® G2X2 Mass Spectrometer
Software:	MassLynx® 4.1
Capillary (V):	3,500
Sample cone (V):	45
Extraction cone (V):	3
Ion guide (V):	3
Desolvation temp (°C):	100
Source temp (°C):	300
Scan range (Da):	400 – 4,000
Desolvation gas (L/h):	40
Cone gas (L/h):	600
Collision energy (V):	5
Pusher (V):	930
RF setting:	Autoprofile

Table 2. qToF-MS settings.

MS Data Analysis

Data were processed using the MaxEnt1 module within the MassLynx® 4.1 software to generate and analyze deconvoluted (zero charged) mass spectra. In general, a summed spectrum was created from the corresponding total ion chromatogram (TIC) of the eluted intact mAb, heavy chain (HC), or light chain (LC). The summed m/z spectrum was then processed by the MaxEnt1 algorithm. Detailed parameters are listed in **Table 3.**

For glycoform analysis, data were processed using UNIFI software from Waters™. Glycoforms were matched by the software and HC glycoform glycans are listed in the respective section below. Glycoform relative abundance data was tabulated based on peak intensities of the coeluting glycoform species. A deconvolution filter setting employing a base peak intensity of 2% was utilized to preclude noise incorporation, and an output resolution setting of 5 Da was used.

Non-Reduced Intact Mass	
m/z range	Full spectral range
Damage model	Gaussian, FWHM 3 Da
Resolution (Da/channel)	2
Mass range (Da)	140,000 - 160,000
Minimum intensity ratios, L and R (%)	33
Iterations	30
Background subtract post MaxEnt1	25th order, 5%, 0.01% tolerance
Center spectrum	Peak width half-height 20. Centroid top 30%, height
Reduced – Heavy Chain	
m/z range	1,200 - 3,000
Damage model	Gaussian, FWHM 0.5 Da
Resolution (Da/channel)	1
Mass range (Da)	40,000 - 60,000
Minimum intensity ratios, L and R (%)	33
Iterations	12
Reduced – Light Chain	
m/z range	1,000 - 2,600
Damage model	Gaussian, FWHM 0.5 Da
Resolution (Da/channel)	1
Mass range (Da)	20,000 - 25,000
Minimum intensity ratios, L and R (%)	33
Iterations	12

Table 3. Deconvolution parameters.

Adalimumab SEC-MS Intact and Middle Up Analysis Results

Intact Mass Analysis of Non-Reduced Adalimumab

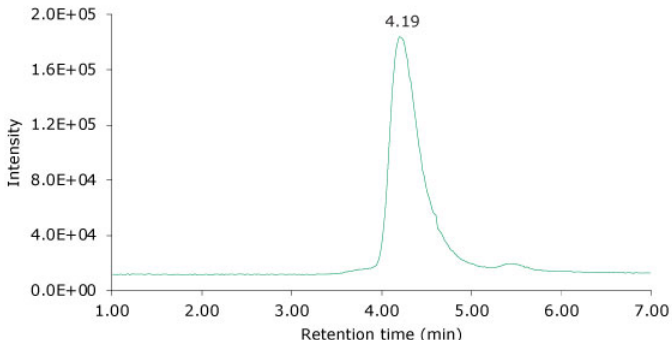
The analysis objective was to perform non-reduced SEC-MS intact mass analysis on all submitted samples to verify the molecular weight of adalimumab.

Protein A purification of samples of adalimumab and SigmaMAb™ antibody control was performed as described in the previous section. Intact SigmaMAb™ was used to determine system suitability. All mAb samples were solubilized in 100 µL H2O to obtain a final concentration of 1 mg/mL. Subsequently, samples were analyzed in their non-reduced form via SEC-MS.

System Suitability Test Results

SigmaMAb™ reference sample (10 µL) was injected on the SEC-MS system. **Figure 2** illustrates the photodiode array (280 nm) and TIC (total ion current) traces of the non-reduced antibody, while **Figure 3** displays the deconvoluted mass spectrum of the SigmaMAb™ reference. The observed intact mAb glycoforms matched the common glycoform masses of MSQC4, as listed in **Table 4**. The measured discrepancies between the observed masses and the theoretical values for four glycoforms were within 0.005% mass error or less.

2a)



2b)

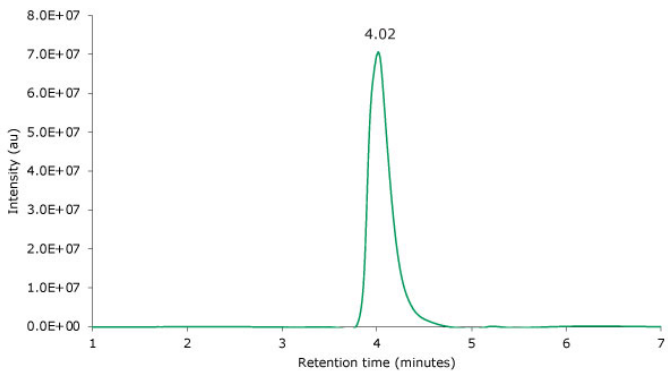


Figure 2. Photodiode array (280 nm, **2a**) and TIC traces (**2b**) of non-reduced SigmaMAb™ reference.

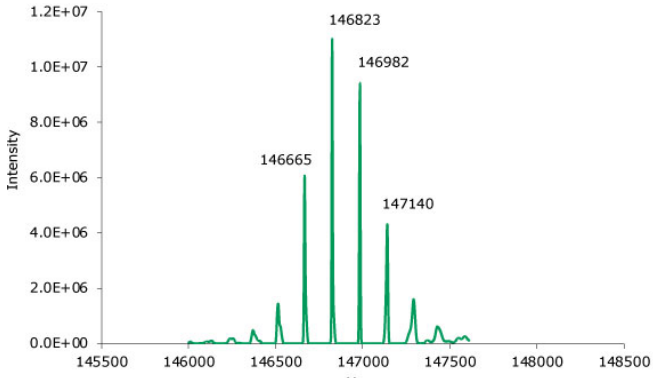


Figure 3. Deconvoluted mass spectrum of non-reduced SigmaMAb™ reference.

Table 4. Observed and theoretical masses of non-reduced SigmaMAb™ reference glycoforms.

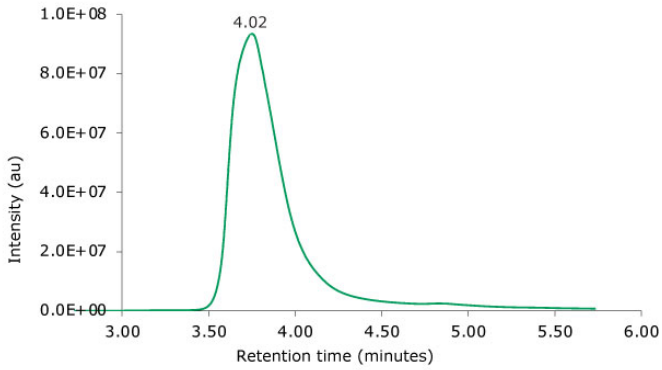
Species	Molecular Formula	Theoretical Mass (Da)*	Observed Mass (Da)
Heavy Chain/ G0FG0F	$C_{6486}H_{10048}N_{1716}O_{2070}S_{46}$	146658	146665
Heavy Chain/ G0FG1F	$C_{6492}H_{10058}N_{1716}O_{2075}S_{46}$	146821	146823
Heavy Chain/ G1FG1F	$C_{6498}H_{10068}N_{1716}O_{2080}S_{46}$	146983	146982
Heavy Chain/ G1FG2F	$C_{6504}H_{10078}N_{1716}O_{2085}S_{46}$	147145	147140

G0F: GlcNAc2Man3GlcNAc2Fuc
G1F: GalGlcNAc2Man3GlcNAc2Fuc
G2F: Gal2GlcNAc2Man3GlcNAc2Fuc
*Masses based on NIST Physical Reference Data

Non-Reduced Sample Results

The monoclonal antibody samples were analyzed in their non-reduced form using SEC-MS. The corresponding photodiode array (280 nm) traces, TICs, MS spectra, and the deconvoluted MS spectra of adalimumab are shown in **Figures 4** and **5**. The observed masses of the non-reduced mAb correlate well with the calculated theoretical masses for all submitted samples, as shown in **Table 5**, and the observed mass error was found to be 0.010% or less.

4a)



4b)

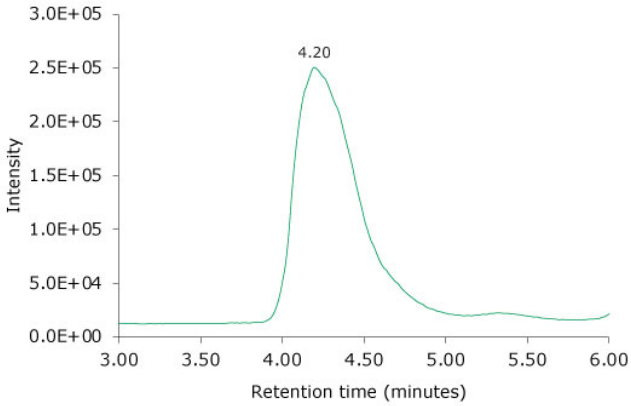


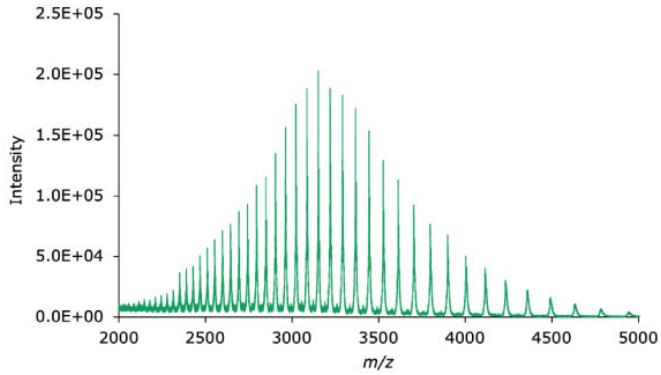
Figure 4. Photodiode array (280 nm, **4a**) and TIC traces (**4b**) of non-reduced adalimumab.

Species	Molecular Formula	Theoretical Mass (Da)*	Observed Mass (Da)
Heavy Chain/ G0FG0F	$C_{6267}H_{10040}N_{1898}O_{1906}S_{15}$	148080	148082
Heavy Chain/ G0FG1F	$C_{6273}H_{10050}N_{1898}O_{1911}S_{15}$	148242	148244
Heavy Chain/ G1FG1F	$C_{6279}H_{10060}N_{1898}O_{1916}S_{15}$	148405	148398
Heavy Chain/ G1FG2F	$C_{6285}H_{10070}N_{1898}O_{1921}S_{15}$	148567	148552

Table 5. Calculated and experimental masses of non-reduced adalimumab.

G0F: GlcNAc2Man3GlcNAc2Fuc
G1F: GalGlcNAc2Man3GlcNAc2Fuc
G2F: Gal2GlcNAc2Man3GlcNAc2Fuc
*Masses based on NIST Physical Reference Data

5a)



5b)

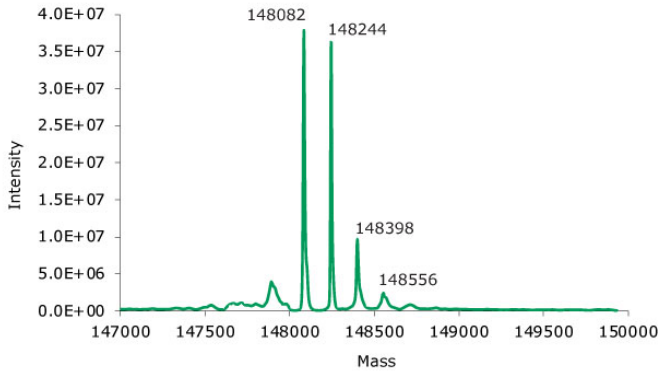


Figure 5. MS data for non-reduced adalimumab. **5a**: summed spectrum; **5b**: deconvoluted spectrum.

Intact Mass Analysis of Reduced Adalimumab

The objective of the intact mass analysis was to verify the molecular weight of adalimumab light and heavy chains by applying an SEC-MS middle-up approach.

Protein A purification of samples of cell culture supernatants containing expressed adalimumab and SigmaMAb™ assay control was performed as described in the previous section. Reduced MSQC4 reference was used to determine system suitability. All mAb samples were solubilized in 100 µL H2O for a final concentration of 1 mg/mL. Subsequently, samples were analyzed in their reduced form via SEC-MS.

System Suitability Test Results

10 µL of a reduced SigmaMAb™ reference sample was injected on the SEC-MS system. The observed heavy chain mAb glycoforms matched the expected glycoform masses of SigmaMAb™, as listed in **Table 6**. Discrepancies between the observed masses and the theoretical values for all three glycoforms were within 0.003% mass error or less. **Figure 6** illustrates the UV chromatogram (280 nm) trace of the SigmaMAb™ reference, while **Figure 7** shows the summed and deconvoluted mass spectra of the reduced SigmaMAb™ reference light and heavy chain glycoforms.

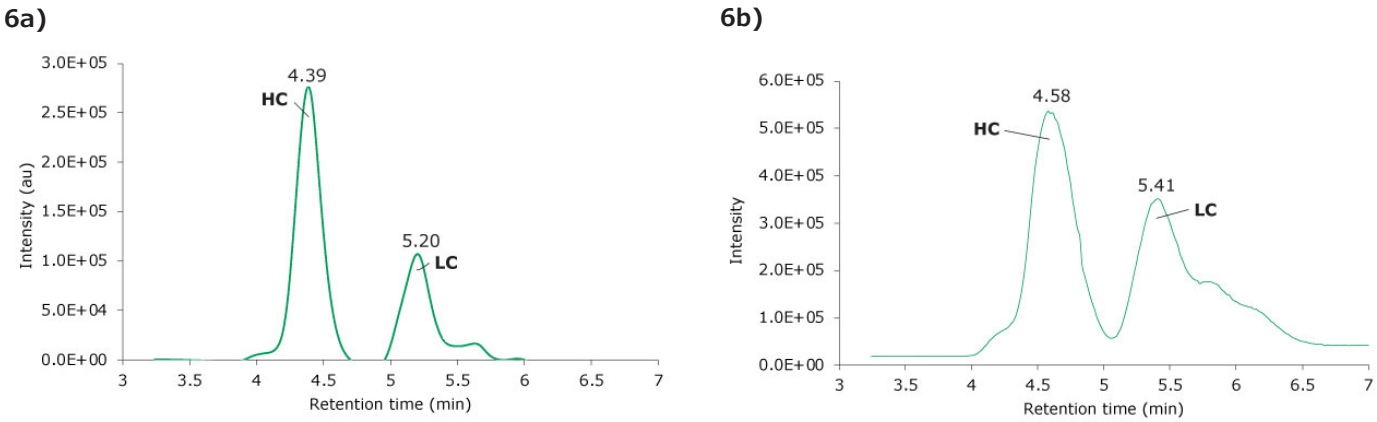


Figure 6. Photodiode array (280 nm, **6a**) and TIC trace (**6b**) of reduced SigmaMAb™ reference.

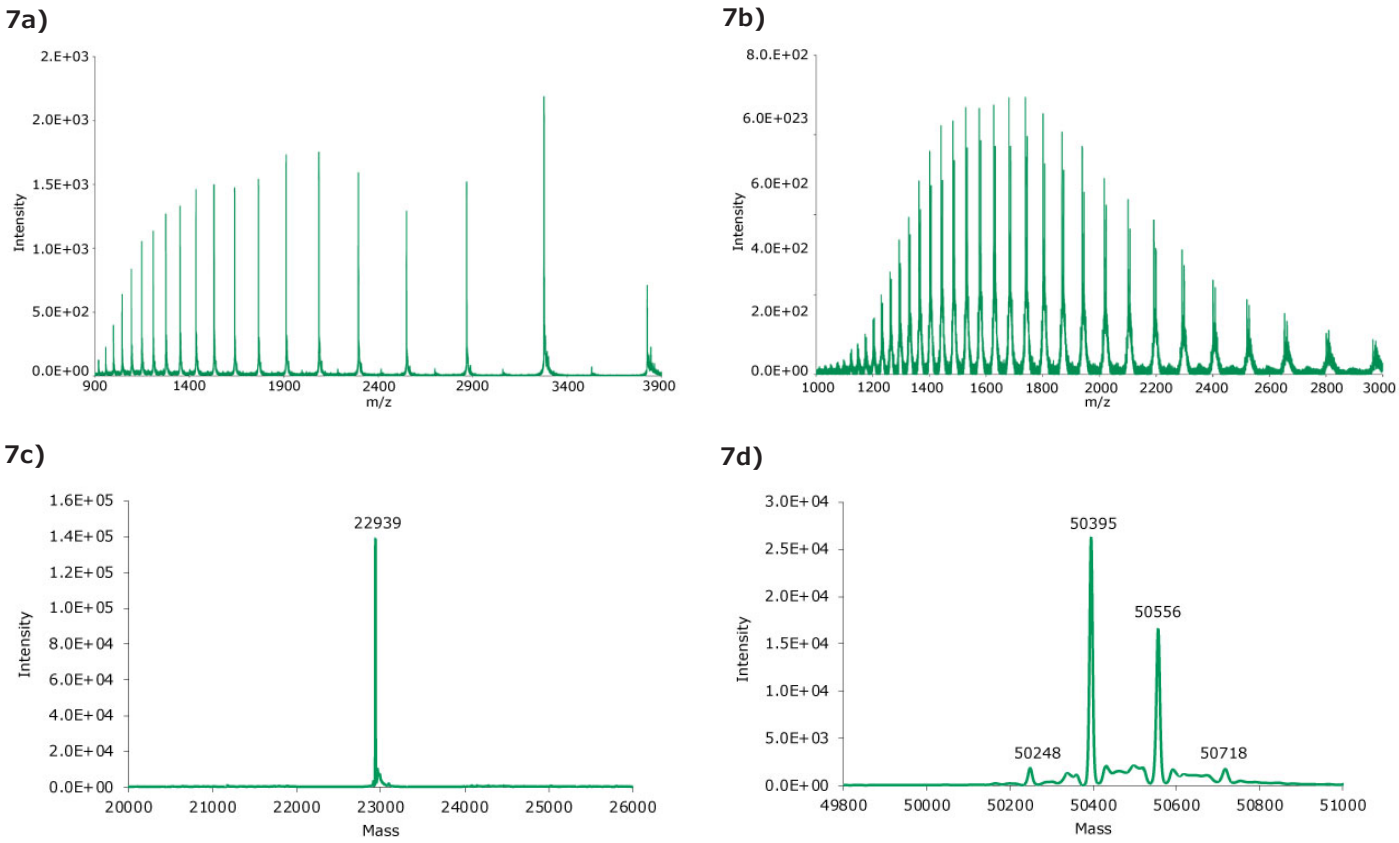


Figure 7. Summed (**7a and 7b**) and deconvoluted (**7c and 7d**) mass spectra of the light and heavy chains (left and right, respectively) of reduced SigmaMAb™ reference.

Species	Molecular Formula	Theoretical Mass (Da) Fully Reduced	Intra Disulfide Bonds	Theoretical Mass (Da) Partially Reduced
Light chain	$C_{1006}H_{1555}N_{267}O_{333}S_7$	22,942.2	2 (-4 Da)	22,938.2
Heavy Chain/ G0F	$C_{2237}H_{3485}N_{591}O_{702}S_{16}$	50,403.2	4 (-8 Da)	50,395.2
Heavy Chain/ G1F	$C_{2243}H_{3495}N_{591}O_{707}S_{16}$	50,565.3	4 (-8 Da)	50,557.3
Heavy Chain/ G2F	$C_{2249}H_{3505}N_{591}O_{712}S_{16}$	50,727.5	4 (-8 Da)	50,719.5

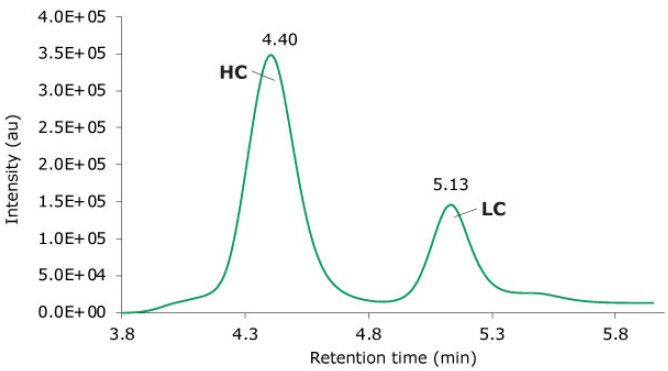
Table 6. Observed and theoretical masses of reduced SigmaMAb™ reference light chain and heavy chain glycoforms and (theoretical mass was calculated based on NIST Physical Reference Data).

G0F: GlcNAc2Man3GlcNAc2Fuc
G1F: GalGlcNAc2Man3GlcNAc2Fuc
G2F: Gal2GlcNAc2Man3GlcNAc2Fuc
*Masses based on NIST Physical Reference Data

Reduced Sample Results

The reduced adalimumab samples were analyzed using SEC-MS. The corresponding photodiode array (280 nm) traces, TICs, MS spectra, and the summed and deconvoluted MS spectra of the samples are shown in individual **Figures 8** and **9**. The measured masses of reduced forms correlated well with the calculated masses as shown in **Table 7** (mass error 0.005% or less). Deconvoluted masses of reduced light and heavy chains correlate well with the expected calculated masses for all submitted samples.

8a)



8b)

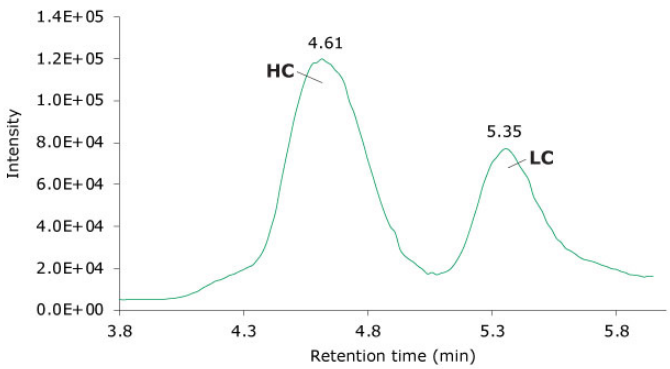
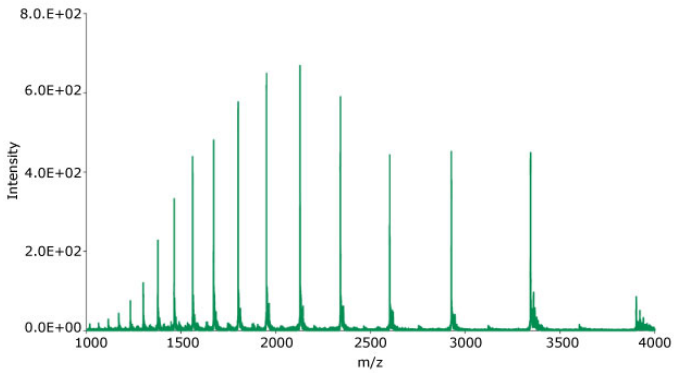
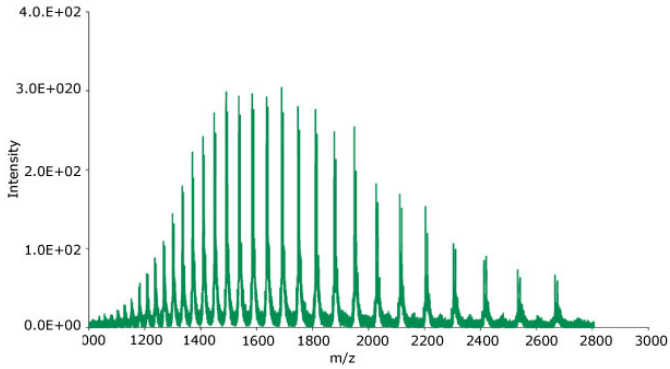


Figure 8. Photodiode array (280 nm, **8a**) and TIC trace (**8b**) of reduced adalimumab.

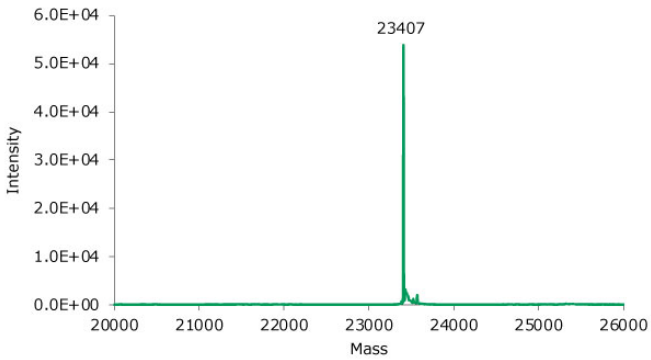
9a)



9b)



9c)



9d)

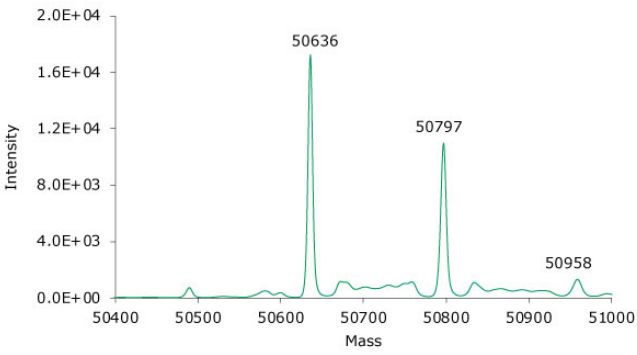


Figure 9. Summed (**9a and 9b**) and deconvoluted (**9c and 9d**) mass spectra of the light and heavy chains (left and right, respectively) of reduced adalimumab.

Conclusion

A workflow for the SEC-MS intact and middle-up mass analysis of reduced and non-reduced monoclonal antibodies was developed, using adalimumab as a model mAb and SILu™Lite SigmaMAb™ as a reference and assay control sample.

The workflow was comprised of an antibody purification process using immobilized Protein A, an optional mAb reduction procedure, a mass spectrometer calibration method, and a system suitability test utilizing a recombinant human monoclonal antibody reference. In addition, a generic SEC method suitable for sample separation and analysis of both reduced and nonreduced mAbs was established.

Results for non-reduced SigmaMAb™ reference revealed measured discrepancies between the observed masses and the theoretical values for four glycoforms of 0.005% mass error or less. For adalimumab, the measured masses of the non-reduced mAb showed a strong agreement with the expected calculated masses for all submitted samples, and the observed mass error was observed to be 0.010% or less.

Analysis of reduced SigmaMAb™ reference sample revealed that the observed heavy chain mAb glycoforms matched the expected glycoform masses of the antibody. The discrepancies between the observed and the theoretical values for three glycoforms were all within 0.003% mass error or less. Similarly, the measured masses of reduced adalimumab correlated well with the calculated masses (mass error 0.005% or less).

The experimental data demonstrated that the workflow can be used for either reduced or non-reduced monoclonal antibody sample analysis, with accurate results allowing for an unambiguous identification of various glycoforms.

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Species	Molecular Formula	Theoretical Mass (Da) Fully Reduced*	Intra Disulfide Bonds	Theoretical Mass (Da) Partially Reduced*
Light chain	$C_{1027}H_{1610}N_{282}O_{332}S_6$	23411.9	2 (-4 Da)	23407.9
Heavy chain/ G0F	$C_{2247}H_{3484}N_{586}O_{716}S_{15}$	50644.3	4 (-8 Da)	50636.3
Heavy chain/ G1F	$C_{2253}H_{3494}N_{586}O_{721}S_{15}$	50806.5	4 (-8 Da)	50798.5
Heavy chain/ G2F	$C_{2259}H_{3504}N_{586}O_{726}S_{15}$	50968.6	4 (-8 Da)	50960.6

Table 7. Calculated and experimental masses of reduced adalimumab light chain and heavy chain glycoforms (theoretical mass was calculated based on NIST Physical Reference Data).

G0F: GlcNAc2Man3GlcNAc2Fuc
G1F: GalGlcNAc2Man3GlcNAc2Fuc
G2F: Gal2GlcNAc2Man3GlcNAc2Fuc
*Masses based on NIST Physical Reference Data

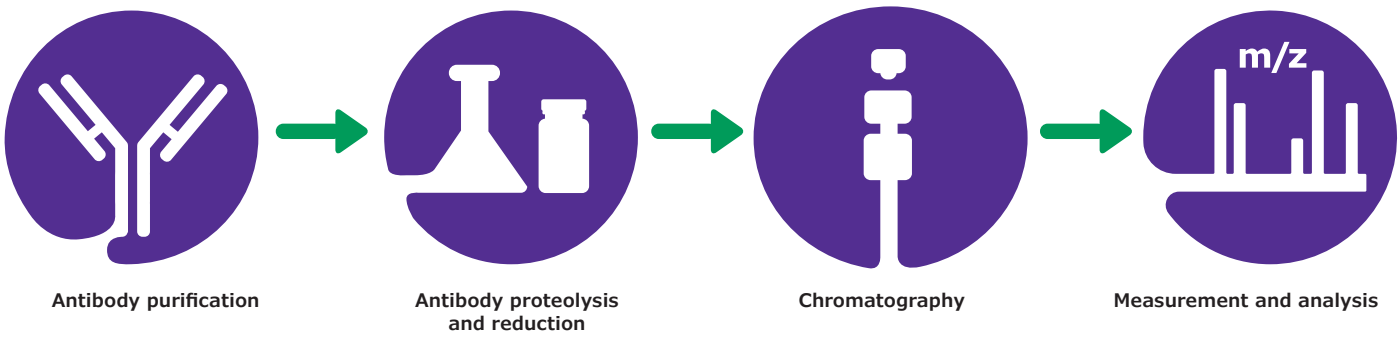
Related Products

Product No.	Description
MSQC4	**SILu™Lite SigmaMAb™ Universal Antibody Standard human**
821485	**TSKgel® SuperSW3000 HPLC Column** phase diol, L × I.D. 30 cm × 2 mm, 4 µm particle size
1.03728	**Water** for UHPLC-MS LiChrosolv®
1.03725	**Acetonitrile** for UHPLC-MS LiChrosolv®
80457	**Trifluoroacetic acid** eluent additive for LC-MS, LiChropur™, ≥99.0% (GC)
68957	**Tris(2-carboxyethyl)phosphine hydrochloride** BioUltra, suitable for electrophoresis, SDS-PAGE tested
75259	**Tris(2-carboxyethyl)phosphine hydrochloride** BioUltra, ≥98% (NMR)
SRE0066	**Guanidine hydrochloride solution, 6M** Reagent designed and manufactured under cGMP controls suitable for use in an IVD application
50933	**Guanidine hydrochloride** BioUltra, for molecular biology, ≥99.5% (AT)
09830	**Ammonium bicarbonate** BioUltra, ≥99.5% (T)
P3476	**Protein A–Agarose Fast Flow** 50%, aqueous suspension
S1804	**Trisodium citrate dihydrate** meets USP testing specifications
251275	**Citric acid** ACS reagent, ≥99.5%
1.09137	**Sodium hydroxide solution** c(NaOH) = 1 mol/l (1 N), Titripur®, reag. Ph. Eur., reag. USP
1.09057	**Hydrochloric acid solution** c(HCl) = 1 mol/l (1 N), Titripur®, reag. Ph. Eur., reag. USP
S9888	**Sodium chloride** ACS reagent, ≥99.0%
D5545	**DL-Dithiothreitol** BioXtra, ≥99.0% (titration)
21004	**Cesium iodide** analytical standard, suitable for mass spectrometry (MS)
P9003	**Poly-DL-alanine** mol wt 1,000-5,000
MSRLN04	**MultiScreen® Solvinert 96 Well Filter Plate** Clear, Sterile, Hydrophilic Polytetrafluoroethylene (PTFE), 0.45 µm pore size membrane, 0.5mL volume, Polyolefin copolymer device, 50 plates

Middle-up Mass Analysis of Protease Digested Cetuximab

Protocol for purification, proteolysis, reduction, and UHPLC-MS analysis of unlabeled and labeled monoclonal antibodies

Workflow for Middle-up Mass Analysis of Protease Digested Cetuximab



A complete reversed phase UHPLC-MS workflow has been developed to simplify middle-up mass analysis of an immunoglobulin G antibody. The protocol describes the analysis of proteolyzed and reduced samples. This type of antibody fragment analysis is a much faster assay than peptide mapping for establishing the presence or absence of antibody fragment modifications.

In detail, the workflow includes:

- Antibody purification using immobilized protein A
- Antibody proteolysis utilizing IdeS enzyme
- Antibody reduction procedure
- Mass spectrometer calibration
- UV spectrophotometric mAb quantification method
- System suitability test utilizing an intact protein LC-MS standard
- RP-UHPLC-MS method for separation and analysis of unlabeled and labeled samples

Introduction to Monoclonal Antibody Sample Preparation and Analysis

Monoclonal antibodies (mAbs or immunoglobulins - IgGs) are large glycoproteins with a molecular weight of approximately 150 kDa (150,000 g/Mol). They are composed of two identical light chains (LC, molecular weight ca. 25 kDa each) and two identical heavy chains (HC, molecular weight ca. 50 kDa each) linked through covalent inter- and intra-chain disulfide bonds. They are utilized for the treatment of various types of cancer, and other diseases such as multiple sclerosis, Alzheimer's disease, and migraine.

Careful and thorough characterization of therapeutic mAbs is essential for ensuring drug safety and efficacy. MAbs are typically manufactured in mammalian host cell lines in bioreactors, generating a large number of heterogeneous drug molecules. Establishing a number of critical quality attributes (CQAs) for each mAb and demonstrating that production batches are within acceptable limits are requirements for both innovator and biosimilar therapeutics.[1,2]

In many cases, the characterization of an antibody-based drug is performed using a specific chromatographic technique (e.g., size exclusion, reversed phase or hydrophilic interaction liquid chromatography, respectively – SEC, RP or HILIC) [3,4] coupled with mass spectrometry (MS). This combination allows for different types of analyses to be carried out, e.g., accurate mass measurement of the intact mAb and subunits, peptide mapping, and the determination of post-translation modifications such as glycosylation, oxidation, and deamidation.

Several techniques are applied to simplify antibody analysis by either fragmentation or removal of glycans. The latter can be performed by treatment with PNGase F, whereas proteolysis with IdeS[5] or reduction of inter-chain disulfide bonds with reducing agents, such as dithiothreitol, result in the formation of different antibody fragments with masses of 25 – 50 kDa. Various combinations of these techniques can be applied. For analysis of mAbs in cell culture supernatants, these may be combined with a preceding affinity purification step.[6] These approaches are referred to as intact mass and middle-up analysis methods.[7] The former term relates to the measurement of the mass of an intact mAb without controlled dissociation being performed. Such an experiment reveals information about stoichiometry, proteoforms, and modifications. Middle-up experiments include mass measurement after cleaving mAbs into several large fragments/subunits via chemical reduction or proteolytic digestion. An example of this approach is the analysis of mAbs light and heavy chains, providing insight into amino acid and post-translational modifications of the individual chains. **Figure 1** shows an overview of antibody sample preparation and various digestion options prior to intact mass analysis.

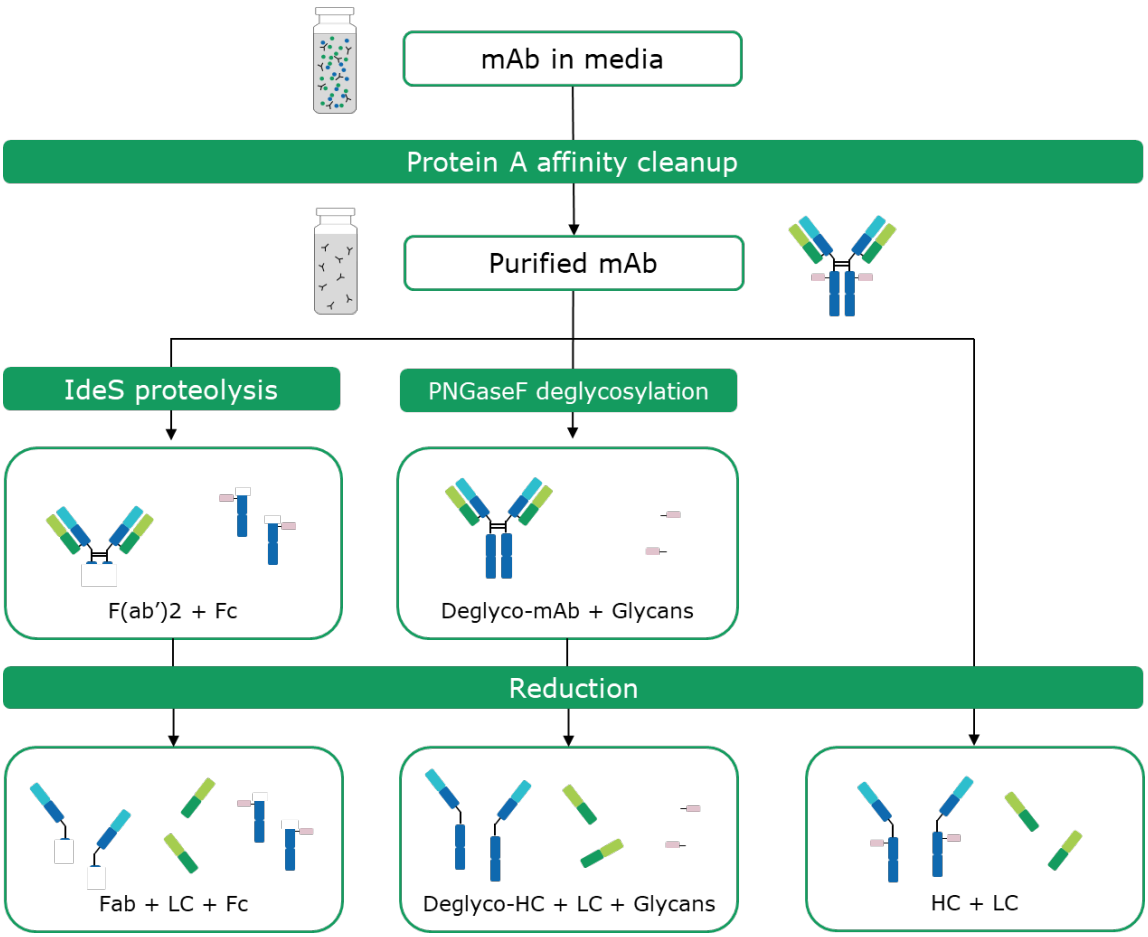


Figure 1. Antibody sample preparation by protein A affinity cleanup and chemical and proteolytic digestion options: Proteolysis with IdeS (formation of Fc and Fab fragments); PNGase F treatment (deglycosylation); chemical reduction (release of heavy and light chains). A combination of proteolysis and chemical reduction is also possible.

This report describes a middle-up approach for mass analysis of cetuximab IgG1 antibody samples. After protein A purification, each sample was subjected to IdeS digestion, reduced, and analyzed by reversed phase UHPLC-MS in order to determine the Fc, LC, and Fab masses of unlabelled and labelled antibody samples and reference.

The recombinant human monoclonal antibody SILu™Lite SigmaMAb™ (#MSQC4) in media was utilized as a purification assay control and pre-purified SILu™ Lite SigmaMAb™ reference served as a digestion control. System suitability control was performed using SigmaProt™ Intact Protein LC-MS Standard (#MSRT2 containing a set of nine proteins: ribonuclease B, insulin, lysozyme, transferrin, bovine serum albumin, trypsin inhibitor, β-lactoglobulin A, carbonic anhydrase, lactate dehydrogenase).

General Procedures – Antibody Preparation and System Setup for Reversed Phase LC-MS Analysis

Antibody Purification Procedure

The target antibody purification was performed on cell culture supernatants using immobilized protein A resin in a 96-well format. The suggested minimum working mAb titer is 100 µg/mL.

All procedures were also conducted using a cetuximab and a SigmaMAb™ reference with a molecular mass of ~150 kDa. The assay control sample contains the antibody spiked into or delivered as a mixture with cell culture media or spent media (cell broth including nutrients etc.); it goes through the entire workflow and serves as a control sample within the protein A purification process (and not as an SST for SEC-MS).

In detail, the high-throughput purification of mAbs from cell culture media using protein A resin was performed as follows:

1. System/Workflow suitability

As part of the workflow suitability, an assay control of SigmaMAb™ in media was purified along with the samples.

a) Reconstitute each vial of MSQC4 in 1.0 mL water to obtain a solution with an antibody concentration of 1 mg/mL.

b) Prepare assay control (spent media sample) by spiking SigmaMAb™ in EX-CELL® CHOZN® Platform Medium, or equivalent, to obtain a final concentration of 100-500 µg/mL.

2. Preparation of equilibration and elution buffers

a) Prepare equilibration buffer (20 mM citrate, 150 mM NaCl, pH 7) by dissolving 5.82 g trisodium citrate dihydrate, 0.04 g citric acid, and 8.77 g sodium chloride in 1 L water. Adjust pH of resulting solution to 7 using 1 M NaOH or HCl as needed; subsequently filter solution using a 0.2 µm filter.

b) Prepare elution buffer (25 mM citrate, pH 3) by dissolving 4.8 g citric acid in 1 L water. Adjust pH of resulting solution to 3 using 1 M NaOH or HCl as needed.

3. Clarify samples

Centrifuge samples in tubes at maximum speed for five minutes and samples in plates at maximum speed for 60 minutes.

4. Protein A loading

a) Add or remove water from top portion of settled protein A slurry to obtain a 50% protein A suspension.

b) Mix slurry by constant pipette action and gentle shaking of reagent reservoir.

c) Use a multichannel pipette to deliver 200 µL of protein A slurry to each well of a 96-well filter plate. Place protein A filter plate on a vacuum manifold. Catch any flow-through from the filter plate by placing the filter plate on top of a used collection plate.

5. Protein A equilibration

a) Add 200 µL of equilibration buffer to each well of protein A and apply vacuum to void the wells of buffer.

b) Repeat both steps twice.

6. mAb binding

a) Remove 750 µL of solution of sample and control without disturbing the pellet and load plate.

b) Cover the plate with film and secure filter and collection plates with a rubber band.

c) Incubate on an orbital shaker at 170 rpm for 30 minutes.

7. Washing bound mAb

a) Place protein A filter plate on vacuum manifold with a waste collection plate inserted and the film cover removed.

b) Apply vacuum to void wells of buffer media and transfer the filter plate onto a waste collection plate.

c) Add 200 µL of equilibration buffer wells and centrifuge plates at 3700 rpm for five minutes (this step helps in clearing the sample film on sides of filter plate wells).

d) Add 200 µL of equilibration buffer to wells and apply vacuum to remove buffer.

e) Repeat once more for a total of three washes.

8. Eluting bound mAb

a) Place protein A filter plate on a new collection plate and secure with a rubber band.

b) Add 100 µL of elution buffer to each well, incubate filter plate an orbital shaker at 170 rpm for five minutes.

c) Centrifuge plates at 3700 rpm for five minutes.

d) Repeat addition of elution buffer, incubation on orbital shaker, and centrifugation for a total of three elution steps (300 µL of total elution volume).

Typical antibody recovery using this procedure is 60%.

Antibody Proteolysis

The protein A purified or pre-purified antibody is proteolyzed and reduced into Fab, Fc, and LC components. The immunoglobulin-degrading enzyme of Streptococcus pyogenes (IdeS, Genovis brand name FabRICATOR®) proteolyzes IgG1 at the heavy chain sequence PAPELLGGP, between adjacent glycines; this gives a F(ab')2 and two Fc fragments. The F(ab')2 fragment, prior to reduction, contains all inter-molecular disulfide bonds of IgG. After reduction, three Fab fragment types with a molecular mass of approximately 25 kDa each are observed.

Additionally, pre-purified SigmaMAb™ reference is used as a digestion control to test if the IdeS workflow/ treatment worked. SigmaProt™ Intact Prot Protein LC-MS Standard is used as a system suitability control.

The antibody proteolysis utilizing IdeS was performed as follows:

1. pH adjustment buffer: Add 932 µL water to 67.6 µL NH_4OH 28-30% solution to prepare a 1 M ammonium hydroxide solution.
2. Add 5 µL of pH adjustment buffer to 40 µL of the cetuximab sample and SigmaMAb™ reference (1 mg/mL).

   Note: pH of the sample should be ≥ 7.
3. Add 0.6 µL of IdeS (67 units/µL) to pH adjusted sample.
4. Incubate at 37 °C for 30 minutes.

Antibody Reduction Procedure

Partial disulfide (S-S) bond reduction was performed as follows:

1. Prepare a 1 M dithiothreitol (DTT) solution by dissolving 154.25 mg DTT in 1 mL water.
2. Prepare a 1 M ammonium bicarbonate (ABC) solution by dissolving 79.06 mg ABC in 1 mL water.
3. Combine equal volumes of 1 M ABC and 1 M DTT to prepare the reduction solution.
4. Transfer aliquots of 50 µL of each sample, system suitability reference, and control to autosampler vials.
5. Reduce by addition of 5 µL 0.5 M ABC/0.5 M DTT solution.
6. Incubate for one hour at room temperature or 30 min at 37 °C.

Note: Partial reduction is performed under non-denaturing conditions, where the inter-chain disulfide bonds (which are more susceptible to reduction) will break and produce the light and heavy chains, while the intra-chain disulfide bonds within each individual domain remain intact.

Alternatively, complete reduction of samples can be performed by using this protocol:

1. Prepare a 100 mM solution of tris(2-carboxyethyl) phosphine (TCEP) in 6 M aqueous guanidine hydrochloride by dissolving 2.87 g of TCEP and 57.32 g of guanidine hydrochloride in 100 mL of water (if less solution is needed, scale down accordingly).
2. Combine 30 µL of the resulting solution with 10 µL of sample.
3. Incubate for two hours at 37 °C.

Note: Complete reduction is performed under denaturing conditions, where both the inter-chain and intra-chain disulfide bonds will break.

Calibration

The Waters™ Xevo® G2S mass spectrometer was calibrated with a 20 µL/min infusion of 0.4 mg/mL of polyalanine and a lock mass of Glu-Fib was used. Alternatively, calibration can be performed with a 20 µL/min infusion of 0.4 mg/mL of cesium iodide prior to running the samples.

System Suitability

To evaluate performance of the entire workflow, an assay control (SigmaMAb™ in media) was prepared and analyzed along with the samples. SigmaProt™ Intact Prot Protein LC-MS Standard was also tested to ensure system suitability.

UHPLC-MS System Setup and Data Analysis

1. UHPLC-MS System Setup

The essential settings of the UHPLC-PDA chromatography system and the qToF mass spectrometer applied in the analysis of both reduced and non-reduced antibodies are listed in **Tables 1** and **2** below.

Table 1. RP-UHPLC-PDA settings.

Instrument	Waters™ H-Class Acquity UPLC Chromatography System
Software:	MassLynx® 4.1
Column:	BIOshell™ A400 Protein C4 15 cm x 1.0 mm, 3.4 µm
Column temp:	Ambient
Autosampler temp:	10 °C
Mobile phase:	(A) 0.1% TFA in water (B) 0.1% TFA in ACN
Gradient:	20-46% B in 13.0 min, 0.5 min to 80% B, 2 min at 80% B, 0.5 min to 20% B, 7 min at 20% B
Flow:	70 µL/min
Loop volume:	20 µL
Injection method:	Partial loop or full loop
Injection volume:	10 µL
Run time:	23 min
Photodiode array:	280 nm
Divert valve:	0 – 3.5 min

Table 2. qToF-MS settings.

Instrument	Waters™ Xevo® G2S Mass Spectrometer
Software:	MassLynx® 4.1
Capillary (V):	3,500
Sample cone (V):	120
Ion energy (V):	1
Desolvation temp (°C):	300
Source temp (°C):	100
Scan range (Da):	300 - 5000
Desolvation gas (L/h):	600
Cone gas (L/h):	0
Collision energy (V):	5
Pusher (V):	1900
RF setting:	Automatic

2. MS Data Analysis

Data were processed using Waters™ MassLynx® 4.1 software to generate and analyze deconvoluted (zero charged) mass spectra. In general, a summed spectrum was created from the corresponding total ion chromatogram (TIC) of the eluting mAb species. The summed m/z spectrum was then processed by the MaxEnt1 algorithm and converted to a decharged (deconvoluted) mass spectrum. Detailed parameters are listed in **Table 3**.

For glycoform analysis, data were processed using UNIFI software from Waters™ Glycoforms were matched by the software and HC glycoform glycans are listed in the respective section below. Glycoform relative abundance data were tabulated based on peak intensities of the co-eluting glycoform species. A deconvolution filter setting employing a base peak intensity of 2% was used to preclude noise incorporation, and an output resolution setting of 5 Da was used.

Table 3. Deconvolution parameters.

MassLynx® deconvolution parameters	
Output mass	MassLynx® 4.1
Ranges	20,000 – 30,000
Resolution	1 Da/channel
Damage model	
Uniform Gaussian FWHM	1 Da
Minimum intensity ratios	
Left	33.0%
Right	33.0%
Completion options	
Iterate to convergence	No
Maximum iterations	12
RF setting	Automatic

Protein Quantification Method – Determination of Protein Concentration

Protein quantification of cetuximab samples was performed by analysis of the UV absorbance of all samples at 280 nm (“A280 method”). The system applied was a Varian Cary® UV 50 Bio UV-Vis spectrophotometer. Protein A elution buffer was recorded as a blank prior to sample measurement. To meet system suitability requirements, NIST BSA with a known concentration of 1.04 mg/mL was subjected to analysis. The mAb sample was measured without additional dilution, using 80 µL eluted sample. In detail, the method was run as follows:

1. Sample preparation

a) Take sample vial out of freezer and leave at room temperature for min. 15 minutes.

b) Centrifuge at 14,000 g for 5 minutes using a bench-top centrifuge (collection of lyophilized product at bottom of vial).

c) Add 500 µL of 0.1% formic acid in water to the vial.

d) Gently invert and mix content in vial, min. 5 times.

e) Leave vial at room temperature for min. 15 minutes.

f) Gently invert and mix content in vial, min. 5 times prior to UV measurement.

2. UV instrument suitability

a) Measure UV absorbance using WAV-7 solid-state reference cell at wavelengths of 270 nm, 280 nm, 300 nm, 320 nm, and 340 nm. Expected absorbance readings are as follows:

Wavelength (nm)	Expected absorbance reading
270	1.0050
280	0.9060
300	0.8000
320	0.5120
340	0.4000

The observed absorbance reading should be within 5% of the expected absorbance reading.

b) Measure UV absorbance using H_2O (blank for BSA protein standard solution).

c) Measure UV absorbance at 280 nm and 320 nm of control, BSA protein standard (sourced from NIST).

– Calculate concentration of control (ε = 0.67 mL/mg), conc. should be within ± 0.1 mg/mL.

3. Sample UV measurement

a) Measure UV absorbance using 0.1% formic acid in water (blank for sample) at 280 nm and 320 nm.

b) Transfer adequate volume of solution (e.g., 80 µL) to UV cell and measure absorbance at 280 nm and 320 nm.

c) If difference between absorbance at 320 nm for blank and sample is < 0.1 skip to step f.

d) If difference between absorbance at 320 nm for blank and sample is > 0.1, centrifuge samples at 14,000 g for 5 minutes using a bench-top centrifuge.

e) Measure UV absorbance at 280 nm and 320 nm again.

f) Calculate corrected UV absorbance at 280 nm by subtracting blank at 280 nm.

g) Calculate recovered mAb amount in µg (ε = 1.4) using this formula:

$[mAb]=AU/\varepsilon$

AU = corrected absorbance reading at 280 nm

ε = 1.4 mL/mg

[mAb] = Antibody concentration (mg/mL)

[mAb]×500 (µL) = content amount (µg)

Results of RP-UHPLC-MS Analysis of Proteolyzed and Reduced Cetuximab

The analysis objective was to perform middle-up mass analysis of all submitted cetuximab samples. After protein A purification, each sample was subjected to IdeS digestion, reduction, and analysis with reversed phase UHPLC-MS for the determination of Fc, LC, and Fab fragment masses.

All media samples (see **Table 4**) were received and stored at -20 °C prior to protein A purification.

To meet system suitability requirements, NIST BSA with a known concentration of 1.04 mg/mL was subjected to an A280 analysis. Concentration of the NIST BSA was calculated to be 1.13 mg/mL, which is within the +/- 0.1 mg/mL system suitability requirements. A280 value, corrected A280 value, and the calculated concentration of the purified Cetuximab samples are listed below in **Table 4**.

Table 4. Denotations and properties of submitted samples.

Sample	A280 value	Corrected A280 value	Concentration (mg/mL)
Blank	0.3486	0	-
Cetuximab E2 Light	0.6761	0.3275	0.24
Cetuximab E2 Heavy	0.5875	0.2389	0.17

System Suitability Test Results

1. Cetuximab Reference

Cetuximab reference sample (10 µL) was injected on the Waters™ Xevo® G2S. **Figure 2** illustrates the TIC (total ion current) and photodiode array (280 nm) traces of the digested and reduced antibody, while **Figure 3** displays the charged and decharged mass spectra of the reference. The observed intact mAb glycoform masses matched the common glycoform masses of cetuximab, as listed in **Table 5** below. The measured discrepancies between the observed masses and the theoretical values for four glycoforms are all within 0.004% mass error or less.

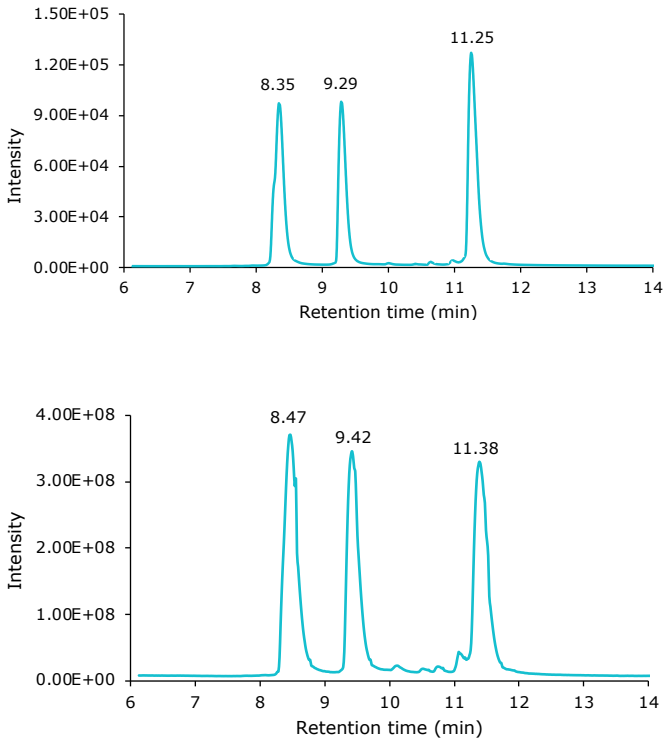


Figure 2. Cetuximab reference after proteolysis and reduction. Top: Photodiode array (280 nm) trace, Bottom: Total ion chromatogram (TIC).

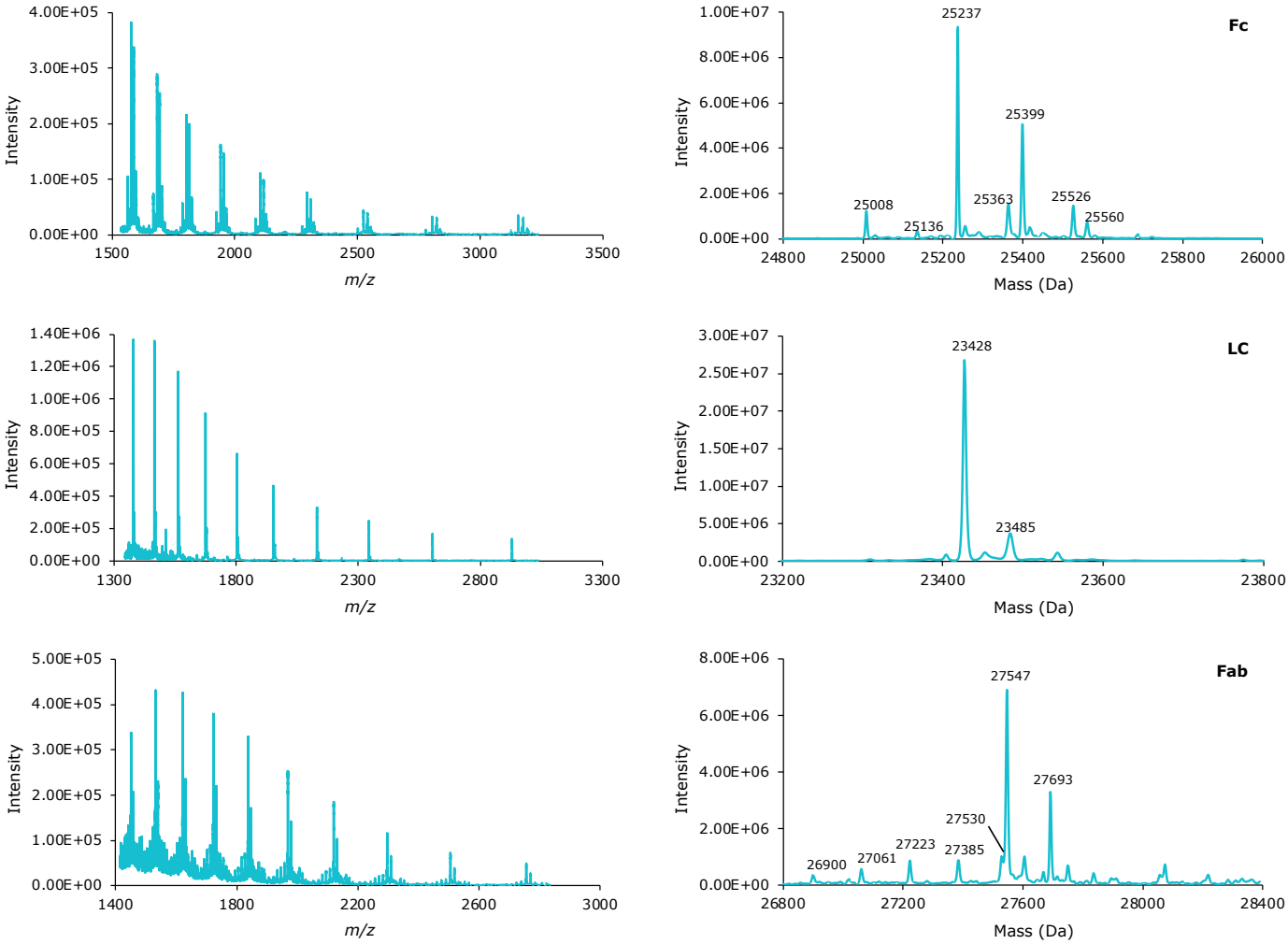


Figure 3. Cetuximab reference after proteolysis and reduction. Summed (left) and deconvoluted (right) mass spectra for Fc, LC, and Fab (top to bottom) regions.

Table 5. Observed peaks for IdeS-treated and reduced cetuximab reference.

Peak	Fragment glycoform	Composition	Calculated mass (Da)	Measured mass (Da)	% Error
Peak 1 (Fc)	Man5	$C_{1112}H_{1724}N_{284}O_{357}S_7$	25008	25008	0.000
	Man5 + Lys	$C_{1118}H_{1736}N_{286}O_{358}S_7$	25136	25136	0.000
	G0F	$C_{1122}H_{1740}N_{286}O_{361}S_7$	25236	25237	0.004
	G0F + Lys	$C_{1128}H_{1752}N_{288}O_{362}S_7$	25364	25363	0.004
	G1F	$C_{1128}H_{1750}N_{286}O_{366}S_7$	25398	25399	0.004
	G1F + Lys	$C_{1134}H_{1762}N_{288}O_{367}S_7$	25526	25526	0.000
	G2F	$C_{1134}H_{1760}N_{286}O_{371}S_7$	25560	25560	0.000
Peak 2 (LC)	NA	$C_{1025}H_{1599}N_{281}O_{338}S_5$	23427	23428	0.004
	LC + 57 Da*	$C_{1027}H_{1604}N_{283}O_{338}S_5$	23484	23485	0.004
Peak 3 (Fab)	G0F	$C_{1192}H_{1839}N_{301}O_{391}S_8$	26899	26900	0.004
	G1F	$C_{1198}H_{1849}N_{301}O_{396}S_8$	27061	27061	0.000
	G2F	$C_{1204}H_{1859}N_{301}O_{401}S_8$	27223	27223	0.000
	G3F	$C_{1210}H_{1869}N_{301}O_{406}S_8$	27385	27385	0.000
	G2FS'	$C_{1215}H_{1876}N_{302}O_{410}S_8$	27530	27530	0.000
	G4F	$C_{1216}H_{1879}N_{301}O_{411}S_8$	27547	27547	0.000
	G4F2	$C_{1222}H_{1889}N_{301}O_{415}S_8$	27693	27693	0.000

*The +57 Da mass shift correlates with one glycine

G0F: GlcNAc2Man3GlcNAc2Fuc

G1F: GalGlcNAc2Man3GlcNAc2Fuc

G2F: Gal2GlcNAc2Man3GlcNAc2Fuc

G3F: Gal3GlcNAc2Man3GlcNAc2Fuc

G2FS': NeuAcGal2GlcNAc2Man3GlcNAc2Fuc

G4F: Gal4GlcNAc2Man3GlcNAc2Fuc

G4F2: Gal4GlcNAc2Man3GlcNAc2Fuc2

2. SigmaMAb™ Reference

SigmaMAb™ reference sample (10 µL) was injected on the Waters™ Xevo® G2S. **Figure 4** illustrates the photodiode array (280 nm) and TIC traces of the digested and reduced antibody, while **Figure 5** displays the charged and decharged mass spectra of the reference. The observed masses are listed in **Table 6** below.

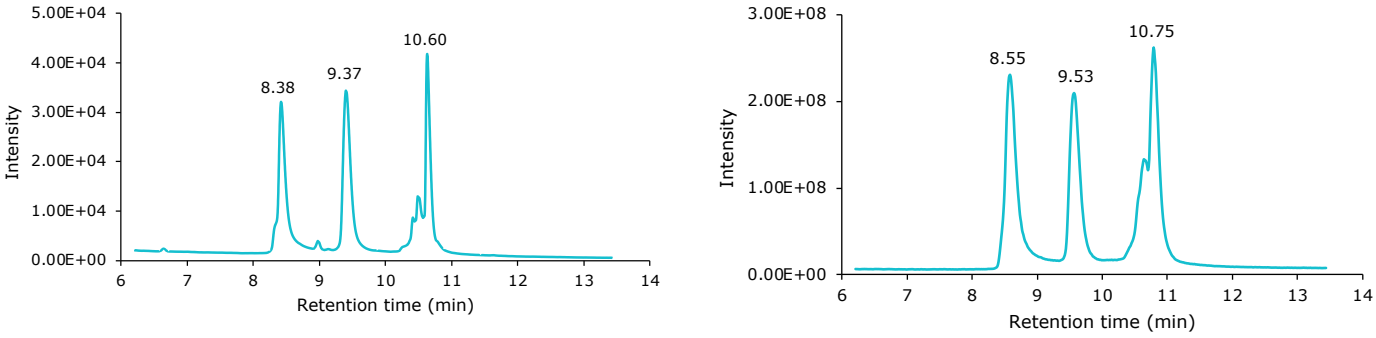


Figure 4. Photodiode array (280 nm, left) and TIC traces (right) of proteolyzed and reduced SigmaMAb™.

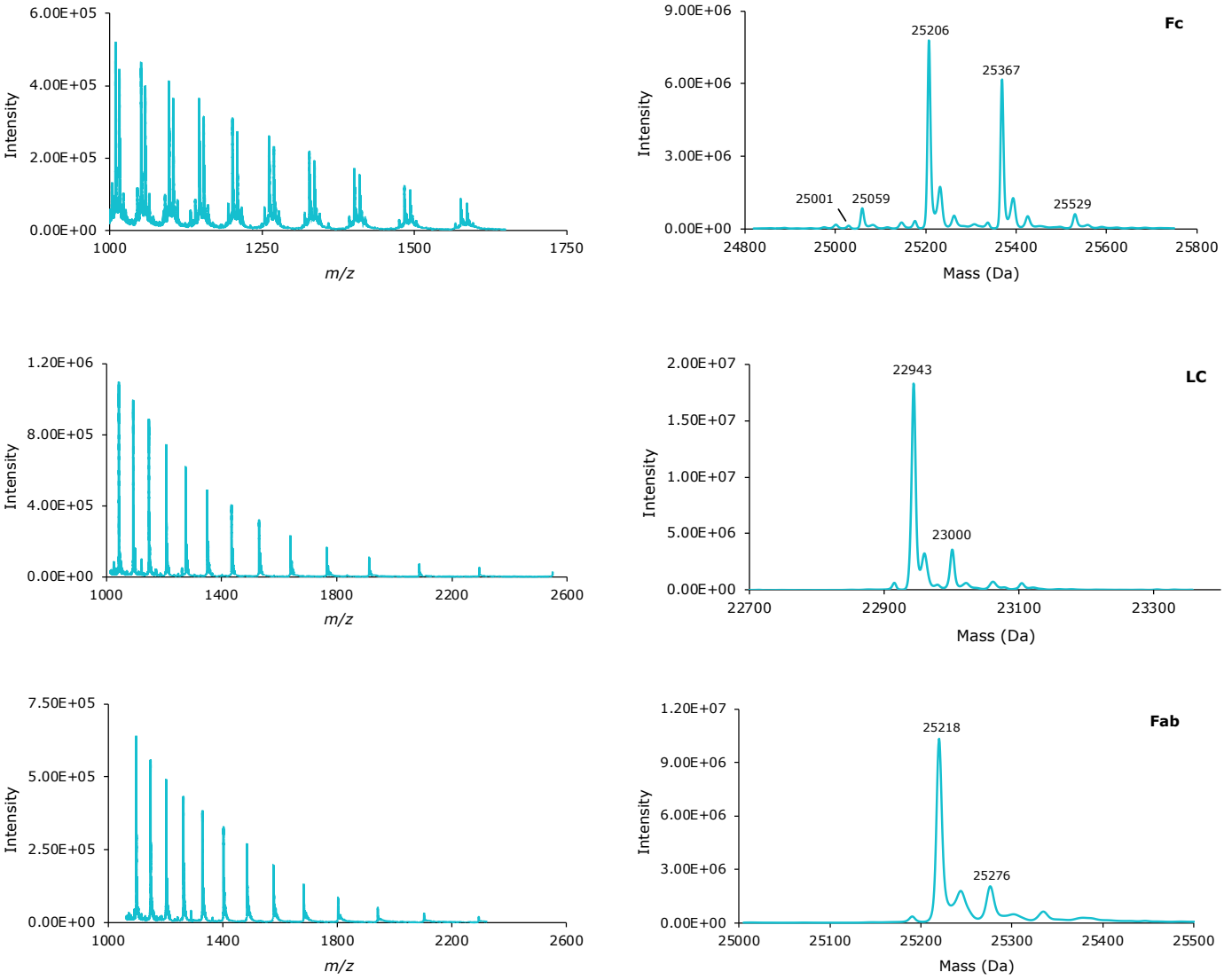


Figure 5. Proteolyzed and reduced SigmaMAb™. Summed (left) and deconvoluted (right) spectra for Fc, LC, and Fab (top to bottom) regions. Peaks 1, 2, and 3 correspond to Fc, LC, and Fab fragments.

Table 6. Observed peaks for IdeS-treated and reduced SigmaMAb™.

Peak	Fragment glycoform	Theoretical mass (Da)
Peak 1 (Fc)	Fc unmodified	23761
	G0F-N	25001
	G0	25059
	G0F*	25206
	G1F**	25367
	G2F	25529
Peak 2 (LC)	LC	22943
	LC + glycine	23000
Peak 3 (Fab)	Fab unmodified	25218
	Fab + glycine	25276

G0F-N: GlcNAc2Man3GlcNAcFuc
G0: GlcNAc2Man3GlcNAc2
G0F: GlcNAc2Man3GlcNAc2Fuc
G1F: GalGlcNAc2Man3GlcNAc2Fuc
G2F: Gal2GlcNAc2Man3GlcNAc2Fuc
*, **: G0F and G1F species are expected to be the most abundant

Cetuximab Sample Results

The submitted cetuximab E2 Light (unlabelled) and cetuximab E2 Heavy (labelled) samples were analyzed in a proteolyzed and reduced form using RP-UHPLC-MS.

1. Cetuximab E2 Light (unlabelled)

The TIC, photodiode array (280 nm) trace, and summed and deconvoluted MS spectra for the unlabeled sample cetuximab E2 Light are shown in individual **Figures 6** and **7**. Corresponding observed masses are displayed in **Table 7**. Minor amounts of oxidized species were observed and are not listed.

The IdeS-treated and reduced cetuximab E2 Light unlabeled fractions Fc, LC, and Fab match with the theoretical masses within an error of 0.004% or less. Comparison of the submitted samples to the reference revealed a difference in the glycosylation profile.

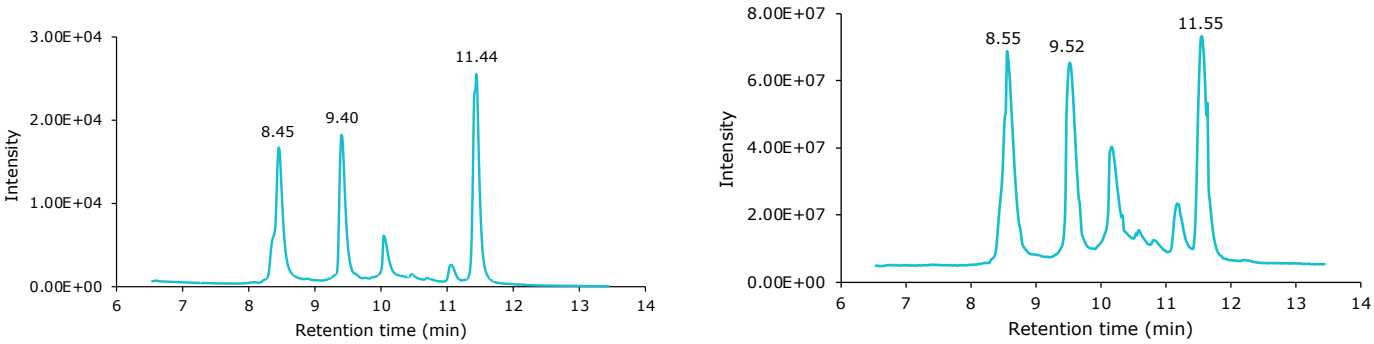
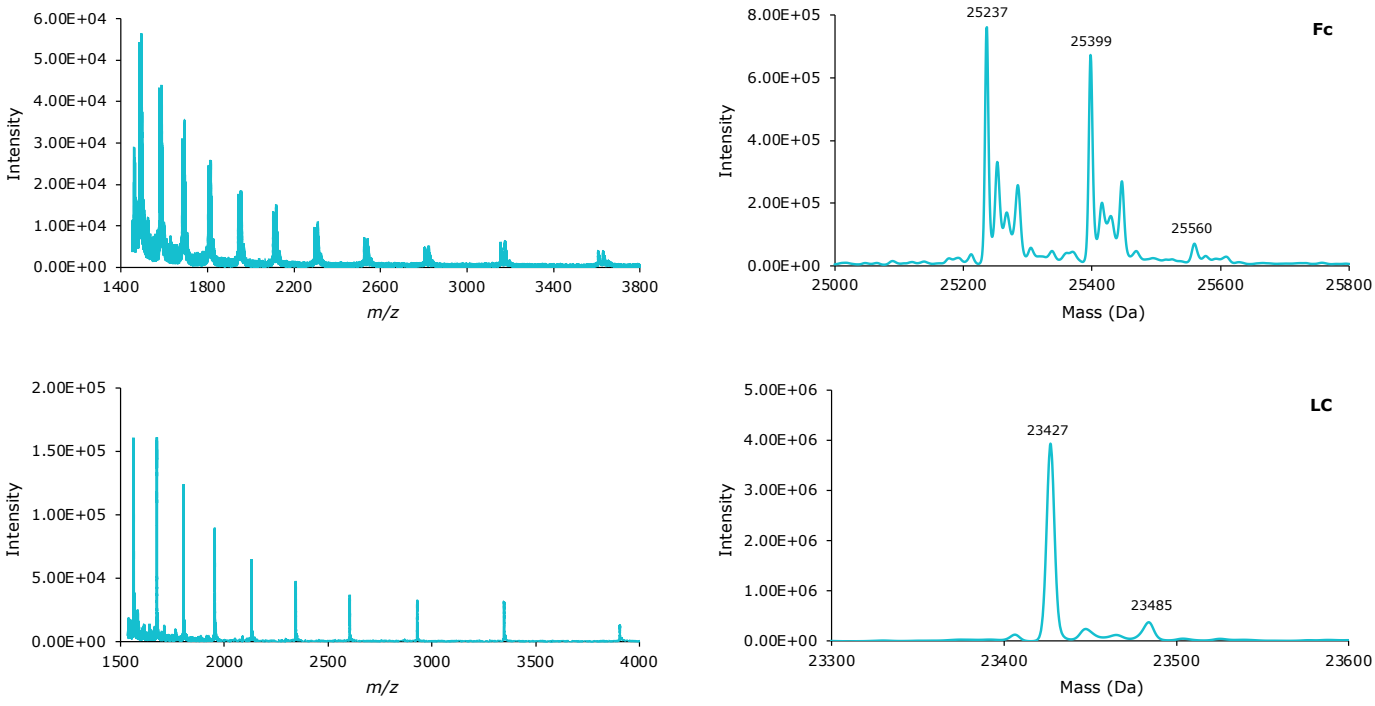


Figure 6. Cetuximab E2 Light unlabeled sample after proteolysis and reduction. Photodiode array (280 nm, left) and TIC traces (right).



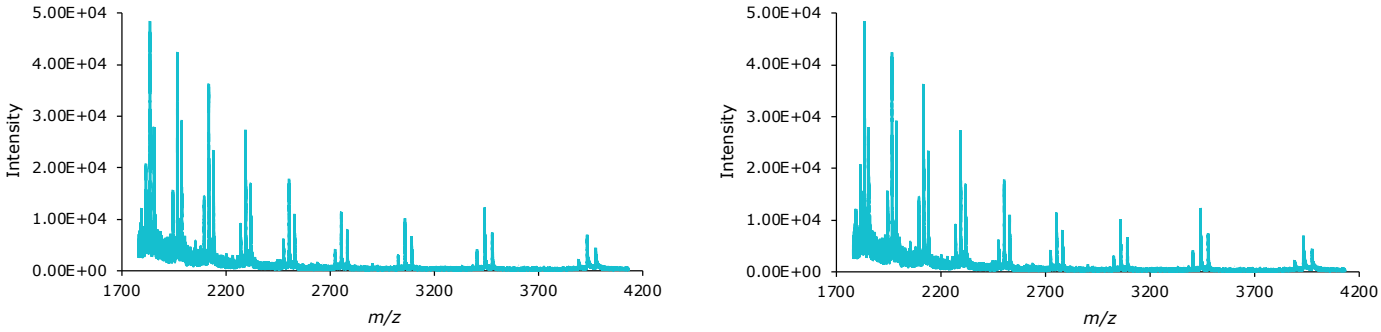


Figure 7. Cetuximab E2 Light unlabeled sample after proteolysis and reduction. Charged (left column) and decharged (right column) mass spectra for Fc, LC, and Fab (top to bottom) regions.

Table 7. Observed peaks for IdeS-treated and reduced Cetuximab E2 Light unlabeled sample..

Peak	Fragment glycoform	Composition	Calculated mass (Da)	Measured mass (Da)	% Error
Peak 1 (Fc)	G0F	$C_{1122}H_{1740}N_{286}O_{361}S_7$	25236	25237	0.004
	G1F	$C_{1128}H_{1750}N_{286}O_{366}S_7$	25398	25399	0.004
	G2F	$C_{1134}H_{1760}N_{286}O_{371}S_7$	25560	25560	0.000
Peak 2 (LC)	NA	$C_{1025}H_{1599}N_{281}O_{338}S_5$	23427	23427	0.000
	LC + 57 Da*	$C_{1027}H_{1604}N_{283}O_{338}S_5$	23484	23485	0.004
Peak 3 (Fab)	G2F	$C_{1204}H_{1859}N_{301}O_{401}S_8$	27223	27223	0.000
	G2FS	$C_{1215}H_{1876}N_{302}O_{409}S_8$	27514	27514	0.000
	G2FS2	$C_{1226}H_{1893}N_{303}O_{417}S_8$	27806	27805	0.004

*The +57 Da mass shift correlates with one glycine

G0F: GlcNAc2Man3GlcNAc2Fuc

G1F: GalGlcNAc2Man3GlcNAc2Fuc

G2F: Gal2GlcNAc2Man3GlcNAc2Fuc

G2FS: NeuAcGal2GlcNAc2Man3GlcNAc2Fuc

G2FS2: NeuAc2Gal2GlcNAc2Man3GlcNAc2Fuc

2. Cetuximab E2 Heavy (labelled)

The TIC, photodiode array (280 nm) trace, and summed and deconvoluted MS spectra for the labelled sample cetuximab E2 Heavy are shown in individual **Figures 8** and **9**. Corresponding observed masses are displayed in **Table 8**. This sample shows oxidized species that are also listed and that may have occurred during sample preparation.

The IdeS-treated and reduced cetuximab E2 Heavy labelled fractions Fc, LC, and Fab match with the theoretical masses within an error of approx. 0.01%. Comparison of the submitted samples to the reference revealed a different glycosylation profile.

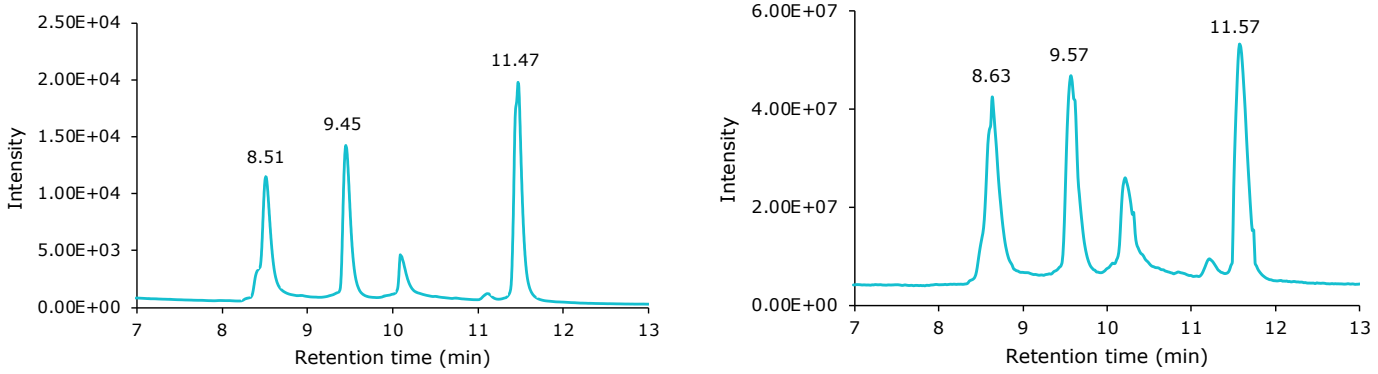


Figure 8. Cetuximab E2 Heavy labelled sample after proteolysis and reduction. Photodiode array (280 nm, left) and TIC traces (right).

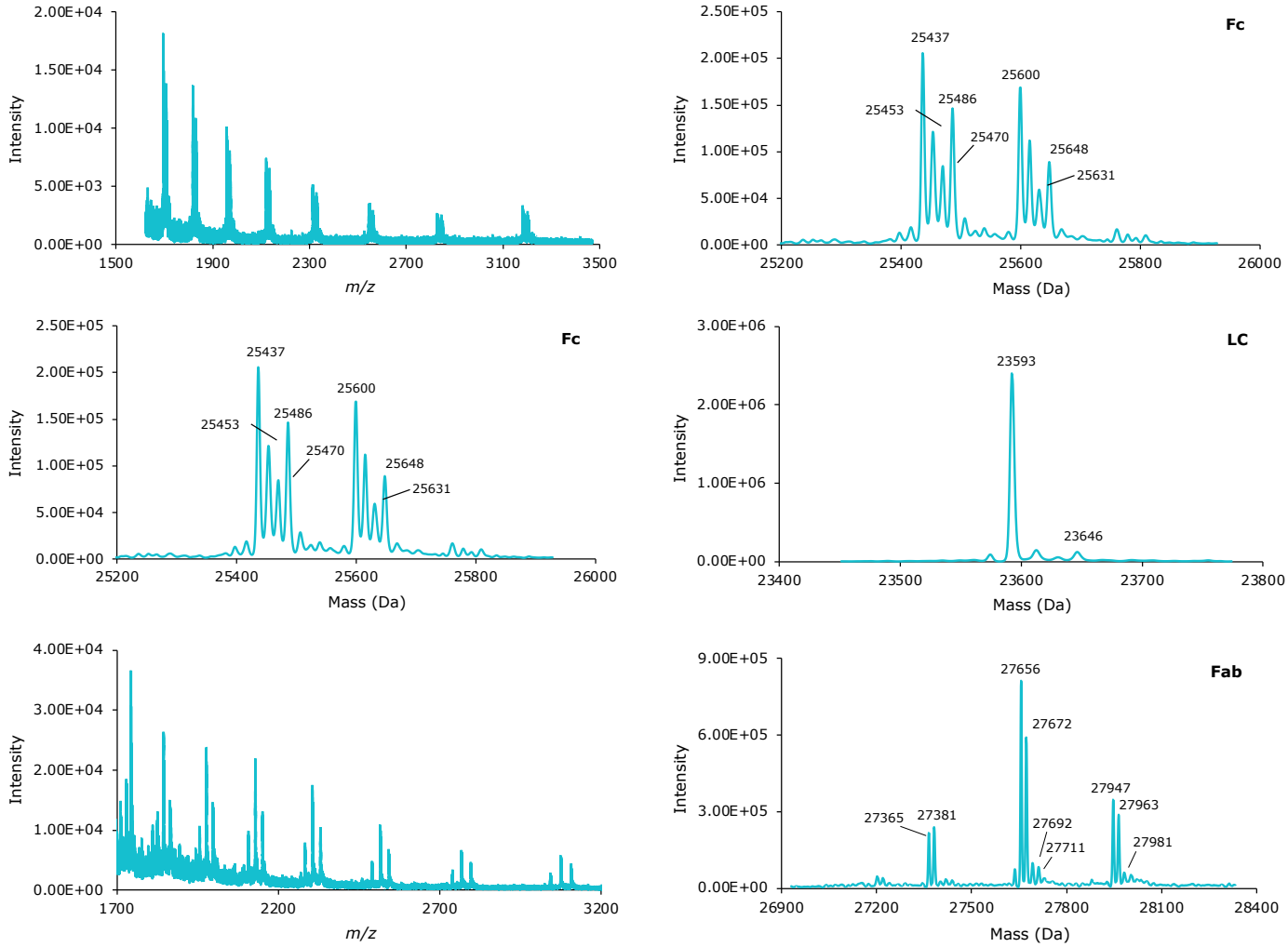


Figure 9. Cetuximab E2 Heavy labelled sample after proteolysis and reduction. Charged (left column) and decharged (right column) mass spectra for Fc, LC, and Fab (top to bottom) regions.

Table 8. Observed peaks for IdeS-treated and reduced Cetuximab E2 Heavy labelled sample.

Peak	Fragment glycoform	Composition	Calculated mass (Da)	Measured mass (Da)	% Error
Peak 1 (Fc)	G0F	$C_{1122}H_{1740}N_{286}O_{361}S_7$	25438	25437	0.004
	G0F+O	$C_{1122}H_{1740}N_{286}O_{362}S_7$	25450	25453	0.004
	G0F+2O	$C_{1122}H_{1740}N_{286}O_{363}S_7$	25469	25470	0.004
	G0F+3O	$C_{1122}H_{1740}N_{286}O_{364}S_7$	25485	25486	0.004
	G1F	$C_{1128}H_{1750}N_{286}O_{366}S_7$	25600	25600	0.000
	G1F+O	$C_{1128}H_{1750}N_{286}O_{367}S_7$	25616	25616	0.000
	G1F+2O	$C_{1128}H_{1750}N_{286}O_{368}S_7$	25632	25631	0.004
	G1F+3O	$C_{1128}H_{1750}N_{286}O_{369}S_7$	25647	25648	0.004
Peak 2 (LC)	NA	$C_{1025}H_{1599}N_{281}O_{338}S_5$	23594	23593	0.004
	LC + 57 Da*	$C_{1027}H_{1604}N_{283}O_{338}S_5$	23651	23646	0.020**
Peak 3 (Fab)	G2F	$C_{1204}H_{1859}N_{301}O_{401}S_8$	27366	27365	0.004
	G2F+O	$C_{1204}H_{1859}N_{301}O_{402}S_8$	27382	27381	0.004
	G2FS	$C_{1215}H_{1876}N_{302}O_{409}S_8$	27657	27656	0.004
	G2FS+O	$C_{1215}H_{1876}N_{302}O_{410}S_8$	27673	27672	0.004
	G2FS+2O	$C_{1215}H_{1876}N_{302}O_{411}S_8$	27689	27692	0.010
	G2FS+3O	$C_{1215}H_{1876}N_{302}O_{412}S_8$	27705	27711	0.020**
	G2FS2	$C_{1226}H_{1893}N_{303}O_{417}S_8$	27948	27947	0.004
	G2FS2+O	$C_{1226}H_{1893}N_{303}O_{418}S_8$	27964	27963	0.004
	G2FS2+2O	$C_{1226}H_{1893}N_{303}O_{419}S_8$	27980	27981	0.004

*The +57 Da mass shift agrees with one glycine

**The % error was slightly higher than 0.01%

E2 Heavy sample Peak 1 Fc G0F: 25236+ (((18K x 8.01) + (6R x 10.01)) x 99% = 25438

E2 Heavy sample Peak 2 LC: 23427+ (((11K x 8.01) + (8R x 10.01)) x 99% = 23594

E2 Heavy sample Peak 3 Fab G2F: 23423+ (((13KX8.01) + (4R X 10.01)) X 99% = 27366

G0F: GlcNAc2Man3GlcNAc2Fuc

G1F: GalGlcNAc2Man3GlcNAc2Fuc

G2F: Gal2GlcNAc2Man3GlcNAc2Fuc

G2FS: NeuAcGal2GlcNAc2Man3GlcNAc2Fuc

G2FS2: NeuAc2Gal2GlcNAc2Man3GlcNAc2Fuc

G4F2: Gal4GlcNAc2Man3GlcNAc2Fuc2

Conclusion of RP-UHPLC-MS Middle-up Mass Analysis of Proteolyzed and Reduced Monoclonal Antibodies

A workflow for the RP-UHPLC-MS middle-up mass analysis of proteolyzed and reduced monoclonal immunoglobulin G antibodies was developed, using unlabeled and labelled cetuximab as a model mAb and SILu™Lite SigmaMAb™ Universal Antibody Standard human as a reference and assay control sample for purification and digestion control, respectively. The workflow was comprised of an antibody purification process using immobilized protein A, a proteolysis step utilizing IdeS, a mAb reduction procedure, a mass spectrometer calibration method, and a system suitability test applying a recombinant human monoclonal antibody reference. In addition, a generic reversed phase UHPLC-MS method suitable for sample separation and analysis of proteolyzed and reduced mAbs was established. System suitability control was performed using an LC-MS standard comprised of nine proteins. Compared to peptide mapping, the advantage of this type of antibody fragment analysis is its much higher speed for establishing the presence or absence of antibody fragment modifications.

The experimental data demonstrated that the workflow can be used for middle-up mass analysis on cetuximab samples. Deconvoluted masses for unlabeled and labelled cetuximab and SigmaMAb™ fragments Fc, LC, and Fab were generated, and all demonstrate a strong correlation with the theoretical masses within an error of approximately 0.01% or less. A comparison of submitted cetuximab samples to the reference mAb revealed a difference in the glycosylation profile.

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Featured Products

Description	Cat. No.
Antibodies	
SILu™Lite SigmaMAb™ Universal Antibody Standard human	**MSQC4**
SILu™Lite SigmaMAb™ Cetuximab Monoclonal Antibody	**MSQC18**
SigmaProt™ Intact Protein LC-MS Standard	**MSRT2**
HPLC columns	
BIOshell™ A400 Protein C4 15 cm x 1.0 mm, 3.4 µm	**67045-U**
Solvents & reagents	
Ultrapure water from Milli-Q® IQ 7 series water purification system or LiChrosolv® UHPLC-MS grade water	**Milli-Q® IQ 7000 or 1.03728**
Acetonitrile for UHPLC-MS LiChrosolv®	**1.03725**
Trifluoroacetic acid eluent additive for LC-MS, LiChropur™	**80457**
Formic acid for LC-MS LiChropur™	**00940**
Guanidine hydrochloride solution 6M, manufactured under cGMP controls	**SRE0066**
Guanidine hydrochloride BioUltra	**50933**
Ammonium hydroxide solution 28.0-30.0% NH_3 basis	**221228**
Protein A–Agarose Fast Flow 50%, aqueous suspension	**P3476**
EX-CELL® CHOZN® platform medium	**24367C-1L**
Trisodium citrate dihydrate, meets USP testing specifications	**S1804**
Citric acid ACS reagent	**251275**
Sodium hydroxide solution 1 M	**1.09137**
Hydrochloric acid solution 1 M	**1.09057**
Sodium chloride ACS reagent	**S9888**
Dithiothreitol BioXtra	**D5545**
Tris(2-carboxyethyl)phosphine BioUltra	**68957 / 75259**
Ammonium bicarbonate BioUltra	**09830**
Cesium iodide analytical standard, suitable for mass spectrometry	**21004**
Poly-DL-alanine	**P9003**
Equipment & consumables	
Microcentrifuge tubes volume 0.6 mL	**T5149**
Autosampler vials volume 0.3 mL	**29661-U**
Stericup® Quick Release-GV Sterile vacuum filtration system	**S2GVU05RE**
PlatePrep 96-well vacuum manifold	**575650-U**
MultiScreen® Solvinert 96 well filter plate	**MSRLN0410**
Corning® Costar® reagent reservoirs	**CLS4870**
BRAND® 96-well deep well plate, stackable	**BR701346**
AlumaSeal® 96 film	**Z721549**
EZ-Pierce™ films	**Z721581**

Appendix

Sequence

FC

GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

LC

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSIN
SVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWK
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

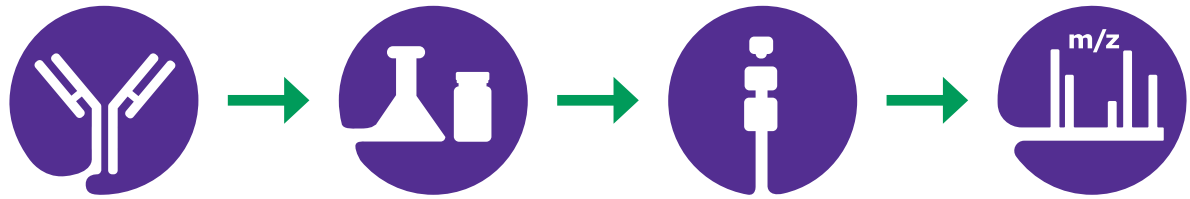
Fab

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNSKSQVF
FKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKT HTCPPCPAPELLG

N terminus modification: pyro-Glu

Workflow for High-Throughput Glycoprofiling of Rituximab

Protocol for purification, reduction, and SEC-MS glycoform analysis of a therapeutic monoclonal antibody



A complete SEC-MS workflow has been developed to enable rapid glycoprofiling of monoclonal antibodies in cell culture supernatants. In detail, it includes:

- Antibody purification using immobilized protein A
- Antibody reduction procedure
- Mass spectrometer calibration
- System suitability test utilizing a recombinant human monoclonal antibody reference
- SEC-MS method for sample separation and analysis

1. Introduction

Monoclonal antibodies (or immunoglobulins - IgGs) are large glycoproteins with a molecular weight of approximately 150 kDa (150,000 g/mol). They are composed of two light chains (LC, molecular weight ca. 25 kDa each) and two heavy chains (HC, molecular weight ca. 50 kDa each) linked through covalent inter-chain disulfide bonds. They are utilized for the treatment of various types of cancer, and other diseases such as multiple sclerosis, Alzheimer's disease, and migraine.

Careful and thorough characterization of therapeutic mAbs is essential for ensuring drug safety and efficacy. MAbs are typically manufactured in mammalian host cell lines in bioreactors, generating a large number of heterogeneous drug molecules. Establishing a number of critical quality attributes (CQAs) for each mAb and demonstrating that production batches are within acceptable limits are requirements for both innovator and biosimilar therapeutics.[1,2]

In many cases, the characterization of an antibody-based drug is performed using a specific chromatographic technique (e.g., size exclusion, reversed phase or hydrophilic interaction liquid chromatography, respectively – SEC, RP or HILIC)[3,4] coupled with mass spectrometry (MS). This combination allows for different types of analyses to be carried out, e.g., accurate mass measurement of the intact mAb and subunits, peptide mapping, and the determination of post-translation modifications such as glycosylation, oxidation, and deamidation.

Several techniques are applied to simplify antibody analysis by either fragmentation or removal of glycans. The latter can be performed by treatment with PNGase F, whereas proteolysis with IdeS[5] or reduction of inter-chain disulfide bonds with reducing agents, such as dithiothreitol, result in the formation of different antibody fragments with masses of 25 – 50 kDa. Various combinations of these techniques can be applied. For analysis of mAbs in cell culture supernatants, these may be combined with a preceding affinity purification step.[6] These approaches are referred to as intact mass and middle-up analysis methods.[7] The former term relates to the measurement of the mass of an intact mAb without controlled dissociation being performed. Such an experiment reveals information about stoichiometry, proteoforms, and modifications. Middle-up experiments include mass measurement after cleaving mAbs into several large fragments/subunits via chemical reduction or proteolytic digestion. An example of this approach is the analysis of mAb light and heavy chains, providing insight into post-translational modifications of the individual chains. **Figure 1** provides an overview of antibody sample preparation and various digestion options prior to middle-up mass analysis.

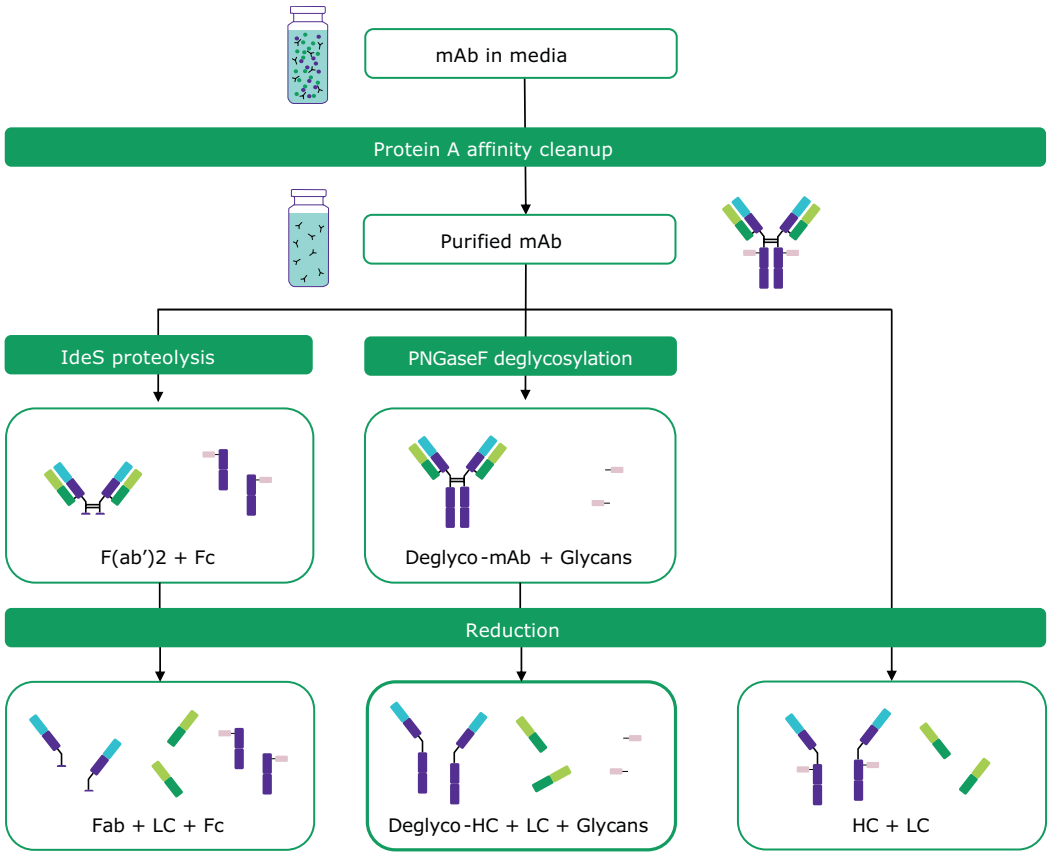


Figure 1. Antibody sample preparation by protein A affinity cleanup and chemical and proteolytic digestion options: Proteolysis with IdeS (formation of Fc and Fab fragments); PNGase F treatment (deglycosylation); chemical reduction (release of heavy and light chains). A combination of proteolysis and chemical reduction is also possible.

Glycosylation is one of the most common and important post translational modifications for mAbs. Glycans attached to antibodies play an important role in their pharmacokinetics, efficacy, and safety. Glycosylation involves the attachment of glycans at specific sites on a protein, most commonly at asparagine (Asn, N-linked) or serine/threonine (Ser/Thr, O-linked) amino acid residues. Both types of glycosylation are important for protein conformation, protein activity, protection from proteolytic degradation, and intracellular trafficking and secretion. Based on the large influence of glycosylation on protein function an accurate study and analysis of glycans is essential. N-glycan composition can be determined by the analysis of four different target structures: intact glycoproteins, glycopeptides, released glycans, and monosaccharide analysis.

This report describes the generation of heavy chain mass data for the rapid glycoprofiling of reduced rituximab recombinant monoclonal antibody samples expressed in 30 separate CHO cell clones along with an authentic rituximab reference material. Samples were purified using protein A resin, reduced, and analyzed by SEC-MS. Deconvoluted heavy chain spectra were generated, and glycoform relative distributions were determined. In all experiments, the recombinant human monoclonal antibody SILu™Lite SigmaMAb™ (#MSQC4) was utilized as a reference antibody.

2. Procedures for mAb Purification, Reduction, and SEC-MS Glycoform Analysis

The target antibody purification was performed on cell culture supernatants using immobilized protein A resin in a 96-well format. The suggested minimum working mAb titer is 100 µg/mL.

All procedures were conducted using both reference and assay control samples of SigmaMAb™ with a molecular mass of ~150 kDa. The reference sample consisted of the pure antibody reconstituted in water; it is used for the system suitability tests and instrument check. The assay control sample contained the antibody spiked into or delivered as a mixture with cell culture media or spent media (cell broth including nutrients etc.); it goes through the entire workflow and functions as a control sample.

2.1 mAb Sample Preparation and System Setup

In detail, the high-throughput purification of mAbs from cell culture media using protein A resin was performed as follows:

1. System/Workflow suitability

As part of the workflow suitability, an assay control of SigmaMAb™ in media was purified along with the samples.

Reference sample (SigmaMAb™) is prepared as follows:

c. Reconstitute each vial of MSQC4 in 1.0 mL water to obtain a solution with an antibody concentration of 1 mg/mL.

d. Prepare assay control (spent media sample) by spiking SigmaMAb™ in EX-CELL® CHOZN® Platform Medium, or equivalent, to obtain a final concentration of 100-500 µg/mL.

2. Preparation of equilibration and elution buffers

a. Prepare equilibration buffer (20 mM citrate, 150 mM NaCl, pH 7) by dissolving 5.82 g trisodium citrate dihydrate, 0.04 g citric acid, and 8.77 g sodium chloride in 1 L water. Adjust pH of resulting solution to 7 using 1 M NaOH or HCl as needed; subsequently filter solution using a 0.2 µm filter.

b. Prepare elution buffer (25 mM citrate, pH 3) by dissolving 4.8 g citric acid in 1 L water. Adjust pH of resulting solution to 3 using 1 M NaOH or HCl as needed.

3. Clarify samples

Centrifuge samples in tubes at maximum speed for five minutes and samples in plates at maximum speed for 60 minutes.

4. Protein A loading

a. Add or remove water from top portion of settled protein A slurry to obtain a 50% protein A suspension.

b. Mix slurry by constant pipette action and gentle shaking of reagent reservoir.

c. Use a multichannel pipette to deliver 200 µL of protein A slurry to each well of a 96-well filter plate. Place protein A filter plate on a vacuum manifold. Catch any flow-through from the filter plate by placing the filter plate on top of a used collection plate.

5. Protein A equilibration

a. Add 200 µL of equilibration buffer to each well of protein A and apply vacuum to void wells of buffer.

b. Repeat both steps twice.

6. mAb binding

a. Remove 750 µL of solution of sample and control, without disturbing the pellet, and load plate.

b. Cover the plate with film and secure filter and collection plates with a rubber band.

c. Incubate on an orbital shaker at 170 rpm for 30 minutes.

7. Washing bound mAb

a. Place protein A filter plate on vacuum manifold with a waste collection plate inserted and the film cover removed.

b. Apply vacuum to void wells of buffer media and transfer the filter plate onto a waste collection plate.

c. Add 200 µL of equilibration buffer to wells and centrifuge plates at 3700 rpm for five minutes (this step helps in clearing the sample film on sides of filter plate wells).

d. Add 200 µL of equilibration buffer to wells and apply vacuum to remove buffer.

e. Repeat once more for a total of three washes.

8. Eluting bound mAb

a. Place protein A filter plate on a new collection plate and secure with a rubber band.

b. Add 100 µL of elution buffer to each well, incubate filter plate on orbital shaker at 170 rpm for five minutes.

c. Centrifuge plates at 3700 rpm for five minutes.

d. Repeat addition of elution buffer, incubation on orbital shaker, and centrifugation for a total of three elution steps (300 µL of total elution volume).

Typical antibody recovery using this procedure is 60%.

2.2 Antibody Reduction Procedure

Partial disulfide (S-S) bond reduction was performed as follows:

1. Prepare a 1 M dithiothreitol (DTT) solution by dissolving 154.25 mg DTT in 1 mL water.
2. Prepare a 1 M ammonium bicarbonate (ABC) solution by dissolving 79.06 mg ABC in 1 mL water.
3. Combine equal volumes of 1 M ABC and 1 M DTT to prepare the reduction solution.
4. Transfer aliquots of 50 µL of each sample, system suitability reference, and control to autosampler vials.
5. Reduce by addition of 5 µL 0.5 M ABC/0.5 M DTT solution.
6. Incubate for one hour at room temperature or 30 min at 37 °C.

Note: Partial reduction is performed under non-denaturing conditions, where the inter-chain disulfide bonds (which are more susceptible to reduction) will break and produce the light and heavy chains, while the intra-chain disulfide bonds within each individual domain remain intact.

Alternatively, complete reduction of samples can be performed by using this protocol:

1. Prepare a 100 mM solution of tris(2-carboxyethyl) phosphine (TCEP) in 6 M aqueous guanidine hydrochloride by dissolving 2.87 g of TCEP and 57.32 g of guanidine hydrochloride in 100 mL of water (if less solution is needed, scale down accordingly).
2. Combine 30 µL of the resulting solution with 10 µL of sample.
3. Incubate for two hours at 37 °C.

Note: Complete reduction is performed under denaturing conditions, where both the inter-chain and intra-chain disulfide bonds will break.

2.3 Instrument Calibration

The Waters™ QToF Xevo® G2XS mass spectrometer was calibrated in a mass range of 500 – 6000 m/z with a 20 µL/min infusion of 0.4 mg/mL of cesium iodide in water. Alternatively, calibration can be performed with a 20 µL/min infusion of 0.4 mg/mL of polyalanine in water prior to running the samples.

2.4 System Suitability

To evaluate performance of the entire workflow, an assay control (SigmaMAb™ in media) was prepared and analyzed along with the samples. Reduced SigmaMAb reference was also tested to ensure system suitability (see section above).

2.5 SEC-MS System Setup and MS Data Analysis

2.5.1 SEC-MS system setup

The essential settings of the UHPLC-PDA chromatography system and the qToF mass spectrometer applied in the analysis of reduced antibodies are listed in **Tables 1** and **2** below.

Table 1. UHPLC-PDA settings.

Instrument	Waters™ H-Class Acquity UPLC Chromatography System
Software	MassLynx® 4.1
Column	Tosoh TSKgel® SW3000XL, 300 x 2.0 mm, 4 µm
Column temp	Ambient
Autosampler temp	8 °C
Mobile phase	Acetonitrile/water 30/70 (v/v) + 0.1% TFA
Gradient	Isocratic
Flow	0.1 mL/min
Loop volume	20 µL
Injection method	Partial loop or full loop
Injection volume	20 µL
Run time	10 min
Photodiode array	280 nm
Flow divert	6.7 - 9.9 min

Table 2. qToF-MS settings.

Instrument	Waters™ QToF Xevo® G2X2 Mass Spectrometer
Software	MassLynx® 4.1
Capillary (V)	3,500
Sample cone (V)	45
Extraction cone (V)	3
Ion guide (V)	3
Desolvation temp (°C)	100
Source temp (°C)	300
Scan range (Da)	400 – 4,000
Desolvation gas (L/h)	40
Cone gas (L/h)	600
Collision energy (V)	5
Pusher (V)	930
RF setting	Autoprofile

2.5.2 MS data analysis

Data were processed using the MaxEnt1 module within the MassLynx® 4.1 software to generate and analyze deconvoluted (zero charged) mass spectra. In general, a summed spectrum was created from the corresponding total ion chromatogram (TIC) of the eluted heavy chain (HC). The summed m/z spectrum was then processed by the MaxEnt1 algorithm; detailed parameters are listed in **Table 3**.

For glycoform analysis, data were processed using UNIFI software from Waters™. Glycoforms were matched by the software and HC glycoform glycans are listed in the respective section below. Glycoform relative abundance data were tabulated based on peak intensities of the co-eluting glycoform species. A deconvolution filter setting employing a base peak intensity of 2% was utilized to preclude noise incorporation, and an output resolution setting of 5 Da was used.

Table 3. Deconvolution parameters.

Heavy Chain	
m/z range	1,200 - 3,000
Damage model	Gaussian, FWHM 0.5 Da
Resolution (Da/channel)	1
Mass range (Da)	40,000 - 60,000
Minimum intensity ratios, L and R (%)	33
Iterations	12

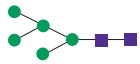
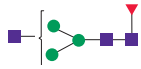
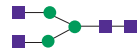
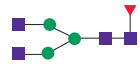
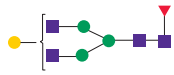
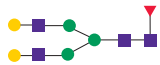
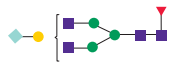
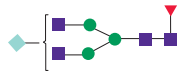
3. Results

The analysis objective was to perform rapid glycoprofiling of a set of reduced rituximab samples by SEC-MS. 30 cell culture supernatant samples from separate CHO clones expressing rituximab were received in 1.5 mL microcentrifuges tubes and stored at -20 °C prior to analysis. An authentic rituximab sample was also tested for comparison. In addition, SigmaMAb™ antibody reference and control samples were used to determine system suitability.

Protein A purification and reduction of rituximab samples and SigmaMAb™ antibody control was performed as described in the previous section. Purification was performed on 500 µL of each sample and 750 µL of SigmaMAb™ control. SigmaMAb™ reference underwent reduction only (no protein A purification) and was used to determine system suitability. Subsequently, samples were analyzed in their reduced form via SEC-MS.

Tables 4 and **5** list all glycans searched in this work and glycan constituent monosaccharides, respectively.

Table 4. Glycans searched as variable modifications.

Glycan	Monosaccaride Composition	Modification Mass	Structure*
Man5	Man_5 $GlcNAc_2$	1217.1	
G0F-N	Fuc_1 Man_3 $GlcNAc_3$	1242.1	
G0	Man_3 $GlcNAc_4$	1299.2	
G0F	Fuc_1 Man_3 $GlcNAc_4$	1445.3	
G1F	Fuc_1 Man_3 Gal_1 $GlcNAc_4$	1607.5	
G2F	Fuc_1 Man_3 Gal_2 $GlcNAc_4$	1769.6	
G1FS'	Fuc_1 Man_3 Gal_1 $GlcNAc_4$ Neu_5Gc_1	1914.7	
G2FS'	Fuc_1 Man_3 Gal_2 $GlcNAc_4$ Neu_5Gc_1	2076.9	

*Purple square: N-acetylglucosamine, green circle: mannose, red triangle: fucose, yellow circle: galactose, light blue diamond: N-glycolylneuraminic acid.

Table 5. Glycan constituent monosaccharides.

Glycan	Short Name	Residue Mass	Representation
N-Acetylglucosamine	GlcNAc	203.08	■
Mannose	Man	162.05	●
Galactose	Gal	162.05	●
Fucose	Fuc	146.06	▲
N-Glycolylneuraminic acid	Neu5Gc	325.27	◆

3.1 System Suitability Test Results

10 µL of a reduced SigmaMAb™ reference sample was injected on the SEC-PDA-MS system. **Figure 2** displays the photodiode array (280 nm) and TIC traces of the SigmaMAb™ reference, while **Figure 3** shows the summed and deconvoluted mass spectra of the reduced SigmaMAb™ reference heavy chain glycoforms. Obtained data demonstrated that the system and assay were suitable for glycoform mass analysis. The system reliably measured glycoform distributions throughout the acquisition queue, with control data falling within historical ranges for relative composition of G0F and G1F glycoforms and indicating proper calibration and sample reduction conditions. In more detail, the observed heavy chain mAb glycoform masses matched the expected masses of SigmaMAb™, as listed in **Table 6**. Discrepancies between the observed masses and the theoretical values for three glycoforms are all within 0.003% mass error or less.

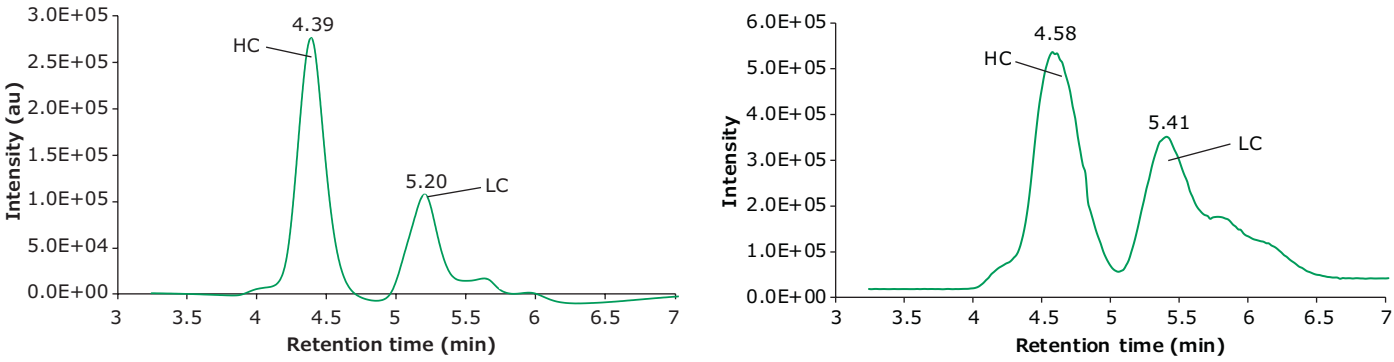


Figure 2. Photodiode array (280 nm, left) and TIC trace (right) of reduced SigmaMAb™ reference.

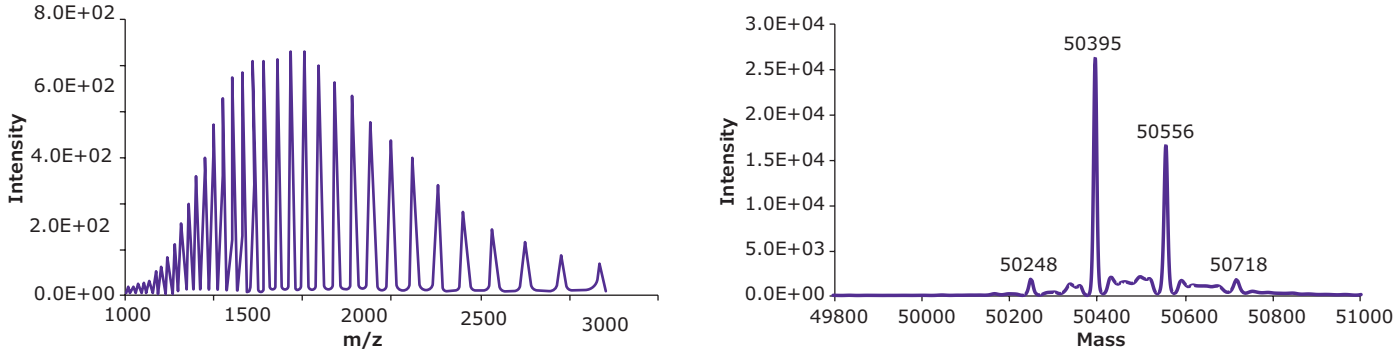


Figure 3. Summed (left) and deconvoluted (right) mass spectra of heavy chain of reduced SigmaMAb™ reference.

Table 6. Observed and theoretical masses of reduced SigmaMAb™ reference heavy chain glycoforms (theoretical mass was calculated based on NIST Physical Reference Data).

Species	Molecular Formula	Theoretical Mass (Da) Fully Reduced	Intra Disulfide Bonds	Theoretical Mass (Da) Partially Reduced	Observed Mass (Da)	Error (%)
Heavy Chain/G0F	C2237H3485N591O702S16	50,403.2	4 (-8 Da)	50,395.2	50395	+0.001
Heavy Chain/G1F	C2243H3495N591O707S16	50,565.3	4 (-8 Da)	50,557.3	50556	+0.002
Heavy Chain/G2F	C2249H3505N591O712S16	50,727.5	4 (-8 Da)	50,719.5	50718	-0.003

3.2 Sample Test Results

30 rituximab monoclonal antibody samples from separate CHO clones were analyzed for their glycoform composition by SEC-MS along with an authentic rituximab reference. **Figure 4** displays deconvoluted spectra of the glycoform composition of two exemplary antibody samples (clones 8 and 27). Typical protein adducts surrounding the major species are at low but appreciable levels; hence, care should be taken when

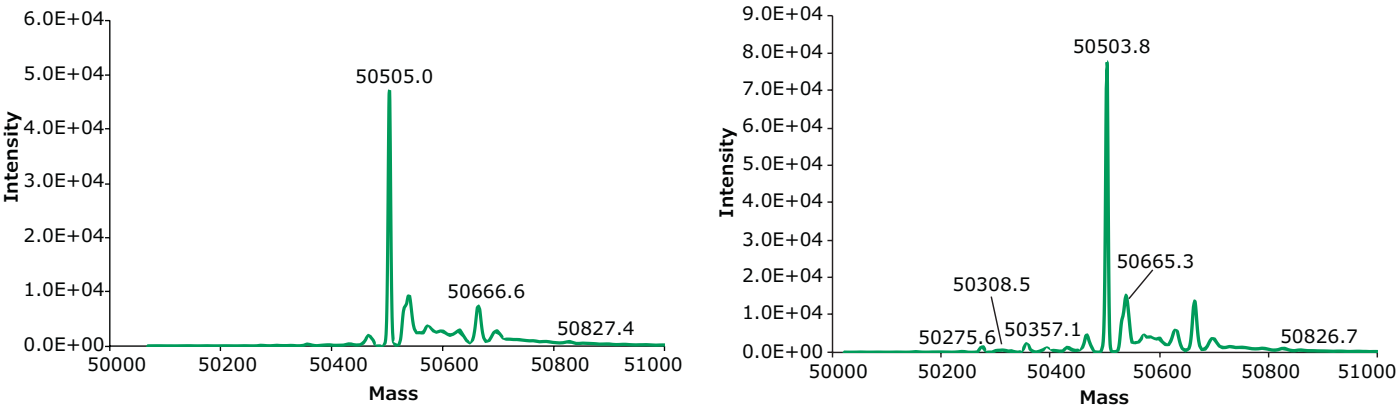


Figure 4. Exemplary deconvoluted SEC-MS spectra of rituximab from clones 8 (left) and 27 (right). The MS peak annotations correspond to glycoform glycans listed in **Tables 7** and **8**.

considering glycoforms with composition values less than 3%. **Tables 7** and **8** show observed and theoretical masses of the two reduced rituximab samples from clones 8 and 27. The mass errors between the observed masses and the theoretical values for the different glycoforms are in the range of 0.004 to 0.023%.

Table 8 summarizes the results for all 30 samples and authentic reference and **Table 9** provides an overview over the glycan composition of all 30 clones analyzed. G0F, G1F and G2F were the main glycans observed. In addition, a small fraction of the samples was also bearing G0, G0F-N and Man glycans.

Table 7. Observed and theoretical masses of reduced rituximab from clone 8 heavy chain glycoforms (expected mass was calculated based on NIST Physical Reference Data).

Component name	Observed mass (Da)	Expected mass (Da)	Mass error (mDa)	Mass error (ppm)	Error (%)
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G0F N [1/301]	50,505.0	50,514.0	-9,087.6	-179.9	-0.018
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G1F N [1/301]	50,666.6	50,676.2	-9,583.8	-189.2	-0.019
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G2F N [1/301]	50,827.4	50,838.3	-10,902.4	-214.5	-0.021

Table 8. Observed and theoretical masses of reduced rituximab from clone 27 heavy chain glycoforms (expected mass was calculated based on NIST Physical Reference Data).

Component name	Observed mass (Da)	Expected mass (Da)	Mass error (mDa)	Mass error (ppm)	Error (%)
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation Man5 N [1/301]	50,275.6	50,285.8	-10,230.4	-203.5	-0.020
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G0F-GlcNAc N [1/301]	50,308.5	50,310.9	-2,348.8	-46.7	-0.004
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G0 N [1/301]	50,357.1	50,367.9	-10,775.3	-214.0	-0.021
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G0F N [1/301]	50,503.8	50,514.0	-10,269.1	-203.3	-0.020
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G1F N [1/301]	50,665.3	50,676.2	-10,856.8	-214.3	-0.021
Rituximab Pyroglutamic Acid Q N-TERM [1/1], Glycosylation G2F N [1/301]	50,826.7	50,838.3	-11,597.5	-228.2	-0.023

Table 9. Percent sample composition (ND = not detected).

Sample	Man5	G0F-N	G0	G0F	G1F	G2F
Clone 1	ND	ND	ND	61.8	30.0	8.1
Clone 2	ND	ND	ND	59.7	31.7	8.6
Clone 3	ND	ND	ND	69.4	24.6	6.0
Clone 4	1.4	ND	ND	67.9	24.6	6.1
Clone 5	ND	ND	ND	67.8	26.0	6.2
Clone 6	ND	ND	ND	68.7	25.3	6.1
Clone 7	1.4	ND	1.3	66.8	24.3	6.2
Clone 8	1.5	ND	ND	68.3	23.9	6.3
Clone 9	ND	ND	ND	73.7	21.3	5.1
Clone 10	ND	ND	ND	73.9	21.3	4.9
Clone 11	ND	ND	ND	63.3	29.4	7.3
Clone 12	ND	ND	ND	60.6	31.5	7.9
Clone 13	ND	ND	ND	47.4	41.0	11.6
Clone 14	ND	ND	ND	46.5	41.1	12.4
Clone 15	ND	ND	ND	50.9	38.2	10.9
Clone 16	ND	ND	ND	50.2	38.4	11.4
Clone 17	ND	ND	ND	45.5	41.9	12.6
Clone 18	ND	ND	ND	47.0	41.0	12.0
Clone 19	ND	ND	1.8	65.3	25.7	7.1
Clone 20	ND	ND	1.7	64.2	26.6	7.5
Clone 21	ND	ND	2.2	75.0	19.3	3.6
Clone 22	ND	ND	2.1	75.1	19.1	3.7
Clone 23	ND	ND	2.7	71.0	22.3	4.0
Clone 24	ND	ND	2.6	70.2	23.1	4.1
Clone 25	ND	ND	3.6	74.5	18.9	3.0
Clone 26	ND	ND	3.3	73.6	19.6	3.6
Clone 27	3.1	1.8	4.0	67.1	20.2	3.8
Clone 28	3.0	1.8	3.9	67.6	19.9	3.8
Clone 29	ND	ND	4.7	59.7	29.9	5.7
Clone 30	ND	ND	4.7	60.0	29.8	5.4
Reference	1.6	0.9	0.7	38.0	45.0	13.7

4. Conclusion

The objective of this work was to develop a CHO clone screening workflow using a SEC-MS middle-up mass analysis of reduced rituximab recombinant monoclonal antibody samples, to generate protein mass data for the antibody heavy chains and to determine their glycoform compositions. Samples were purified from spent media, reduced, analyzed by SEC-MS and deconvoluted heavy chain spectra were generated for the determination of glycoform relative distributions.

The workflow was comprised of an antibody purification process using immobilized protein A, a mAb reduction procedure, a mass spectrometer calibration method and a system suitability test utilizing SILu™ Lite SigmaMAb™ Universal Antibody Standard human. In addition, a SEC method suitable for sample separation and analysis of reduced mAbs was utilized.

A total of 30 rituximab cell culture supernatants from separate CHO clones underwent a protein A purification and a reduction procedure and were then subjected to SEC-MS analysis, along with an authentic rituximab reference. In addition, SigmaMAb™ was utilized as a reference and was treated in the same way.

Experiments utilizing a reduced SigmaMAb™ reference sample revealed that the observed mAb heavy chain glycoform masses matched the expected masses of the antibody fragments. In this case, discrepancies between the observed and the theoretical values for three glycoforms are all within 0.003% mass error or less, proving the system and assay were suitable for middle-up mass analysis. Control data falling within historical ranges for relative composition of G0F and G1F glycoforms indicated proper calibration and sample reduction conditions.

Glycoform composition of rituximab from 30 CHO clones was determined by SEC-MS. Results reveal significant variation in the glycoprofiles of individual clones, with G0F being the most abundant glycan for all clones screened in this experiment. The most abundant glycan observed in the authentic reference rituximab was G1F.

All generated data display that the implemented workflow can be used for reduced monoclonal antibody sample glycoform analysis, with accurate results allowing for an unambiguous identification and relative quantitation of six different glycans.

5. References

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6. Featured Products

Description	Cat. No.
Antibodies	
SILu™ Lite SigmaMAb™ Universal Antibody Standard human	**MSQC4**
SILu™ Lite SigmaMAb™ Rituximab Monoclonal Antibody	**MSQC17**
HPLC columns	
TSKgel® SuperSW3000 HPLC Column phase diol, L × I.D. 30 cm × 2 mm, 4 µm particle size	**821485**
Solvents & reagents	
Ultrapure water from Milli-Q® IQ 7 series water purification system or LiChrosolv® UHPLC-MS grade water	**Milli-Q® IQ 7000 or 1.03728**
Acetonitrile for UHPLC-MS LiChrosolv®	**1.03725**
Trifluoroacetic acid eluent additive for LC-MS, LiChropur™	**80457**
Tris(2-carboxyethyl)phosphine BioUltra	**68957 / 75259**
Guanidine hydrochloride solution 6M, manufactured under cGMP controls	**SRE0066**
Guanidine hydrochloride BioUltra	**50933**
Ammonium bicarbonate BioUltra	**09830**
Protein A–Agarose Fast Flow 50%, aqueous suspension	**P3476**
EX-CELL® CHOZN® platform medium	**24367C-1L**
Trisodium citrate dihydrate, meets USP testing specifications	**S1804**
Citric acid ACS reagent	**251275**
Sodium chloride ACS reagent	**S9888**
Dithiothreitol BioXtra	**D5545**
Cesium iodide analytical standard, suitable for mass spectrometry	**21004**
Poly-DL-alanine	**P9003**
Consumables	
Microcentrifuge tubes volume 0.6 mL	**T5149**
Autosampler vials volume 0.3 mL	**29661-U**
Stericup® Quick Release-GV Sterile Vacuum Filtration System	**S2GVU05RE**
PlatePrep 96-well Vacuum Manifold	**575650-U**
MultiScreen® Solvinert 96 Well Filter Plate	**MSRLN0410**
Corning® Costar® reagent reservoirs	**CLS4870**
BRAND® 96-well deep well plate, stackable	**BR701346**
AlumaSeal® 96 film	**2721549**
EZ-Pierce™ films	**Z721581**

Middle-Up Analysis of Antibodies by Reversed-Phase Chromatography:

Comparison of Chromolith® WP 300 RP-18, 2 mm I.D., with other Columns

Benjamin Peters and Gisela Jung
HPLC R&D, Darmstadt, Germany

Introduction

Analysis of monoclonal antibodies (mAbs) is a difficult analytical challenge that requires a multi-pronged approach. Different modes of chromatography, in addition to different sample preparation strategies, need to be employed for complete characterization. In reversed-phase mode, three levels of analysis are typically performed: top-down (intact), bottom-up (fully digested peptide fragments), and middle-up (larger protein sub-units).

In middle-up analysis, a digestion procedure, such as using dithiothreitol (DTT) or IdeS (a protease) is employed to break the protein into larger fragments. DTT breaks a mAb down into a light chain (LC) fragment and a heavy chain (HC) fragment; IdeS breaks the mAb down into a fragment crystallizable (Fc) fragment and an antigen-binding fragment (Fab). These two approaches lead to a simpler mixture than a full tryptic digest which enables easier identification of variants.

The purpose of this application is to subject the SigmaMAb™ antibody reference standard and the NIST mAb reference standard to DTT and IdeS digestion and analyze each of the resulting digests by RPC. In addition, a series of columns will be employed to perform the analysis, and the differences in selectivity, efficiency, and peak shape will be examined.

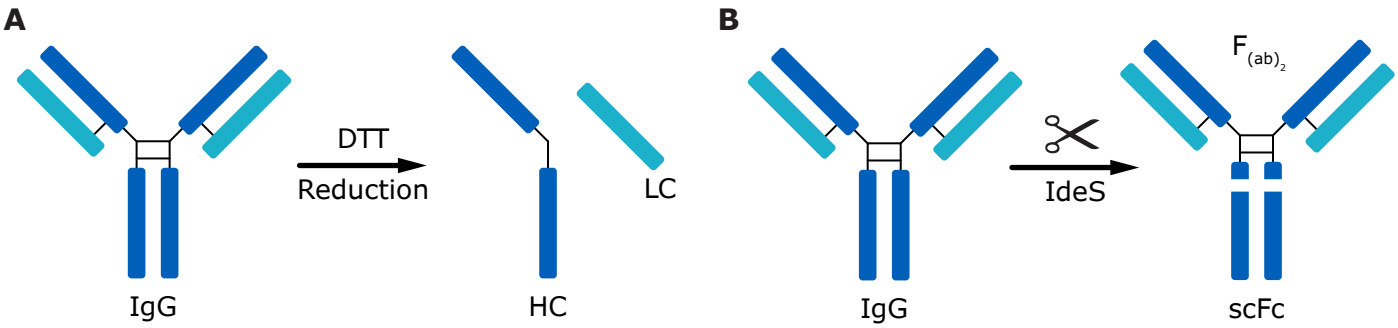
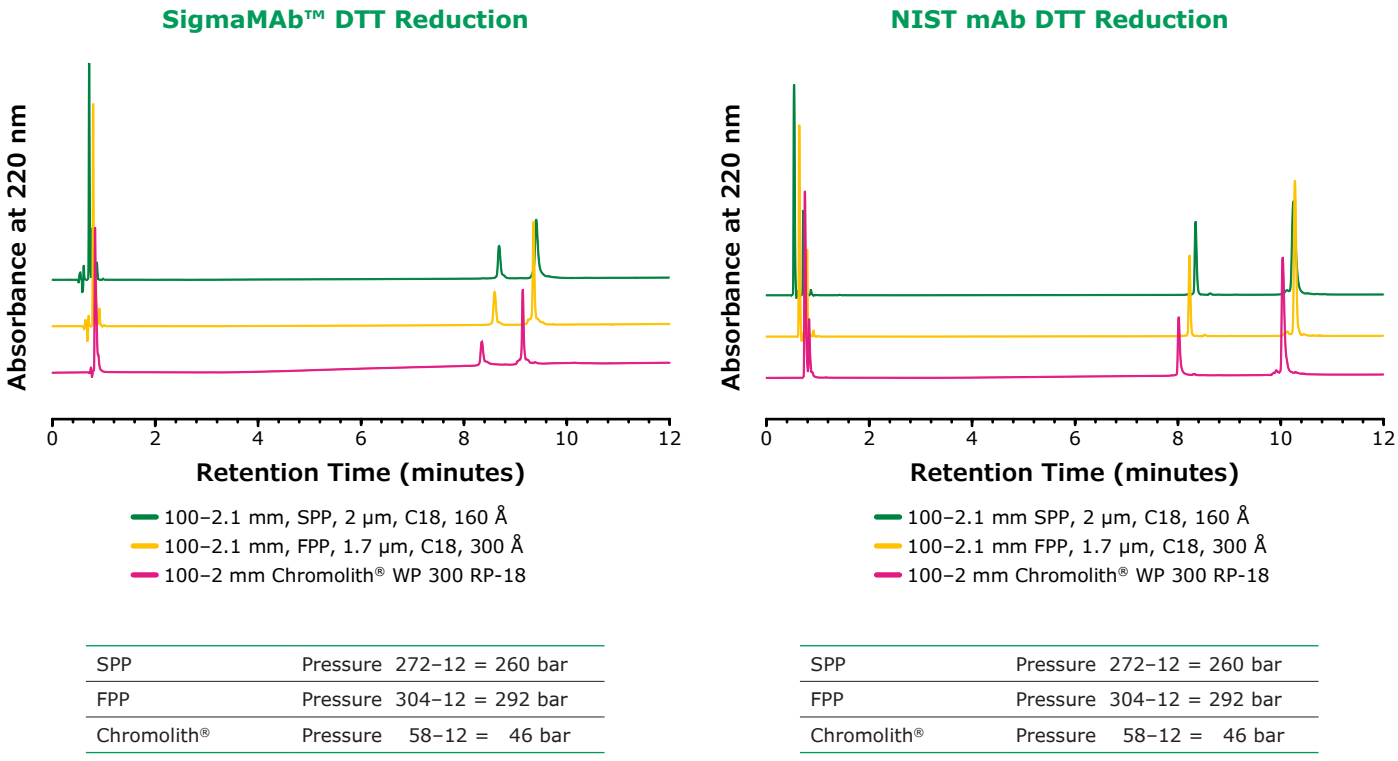


Figure 1: Digested products of an IgG antibody after being subjected to a) DTT and b) IdeS

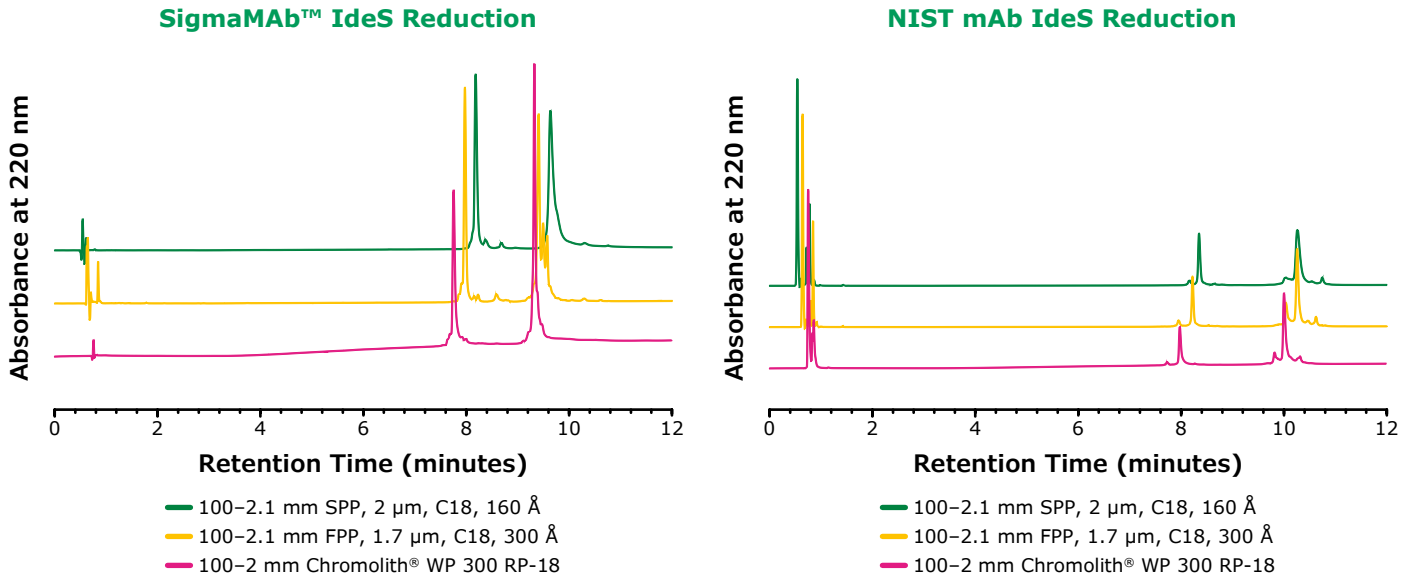
Experimental Conditions	
Column:	As indicated; 10 cm x 2.1 mm I.D., 2.0 µm (SPP), 1.7 µm (FPP)
Detection:	UV at 220 nm (analytical flow cell; 10 µL)
Mobile phase A:	Water (0.1% TFA v/v)
Mobile Phase B:	Acetonitrile (0.08% TFA v/v)
Gradient:	Time (min) / %B: 0 / 20; 1 / 20; 10 / 45
Sample:	Digested mAb, varied concentration, water (0.1% TFA v/v)
Sample Prep (DTT Method):	60 µL of 40 mM DTT solution was added to 40 µL mAb and allowed to incubate in a vial at 37 °C for 30 minutes.
Sample Prep (IdeS Method):	4 µL IdeS and 56 µL water were mixed with 40 µL mAb and allowed to incubate at 37 °C for 30 minutes.
Injection volume:	1.0 µL
Flow rate:	0.38 mL/min
Temperature:	Column: 80 °C Autosampler: 10 °C
Pressure drop:	As indicated



SPP	Pressure 272–12 = 260 bar
FPP	Pressure 304–12 = 292 bar
Chromolith®	Pressure 58–12 = 46 bar

SPP	Pressure 272–12 = 260 bar
FPP	Pressure 304–12 = 292 bar
Chromolith®	Pressure 58–12 = 46 bar

Figure 2: Comparison of separation performance between Chromolith® WP 300, a SPP, and a FPP-packed column analyzing DTT reduced SigmaMAb™ and NIST mAb. Chromolith® columns perform comparably to the UHPLC columns (SPP and FPP) but at a significantly lower backpressure.



SPP	Pressure 272–12 = 260 bar
FPP	Pressure 296–12 = 284 bar
Chromolith®	Pressure 62–12 = 50 bar

SPP	Pressure 268–12 = 256 bar
FPP	Pressure 296–12 = 284 bar
Chromolith®	Pressure 62–12 = 50 bar

Figure 3: Comparison of separation performance between Chromolith®, a SPP, and a FPP-packed column in analyzing IdeS reduced SigmaMAb™ and NIST mAb. Chromolith® columns are able to separate the Fab and Fc species with high resolution without generating artifacts due to higher pressure, as illustrated by extra peaks and peak broadening, as seen in the SigmaMAb™ example with the FPP and SPP columns, respectively.

Conclusion

The purpose of this study was to compare the performance of a series of columns in resolving medium-sized fragments of antibodies, after digestion, by RPC. Two different digestion protocols were employed in this study, DTT and IdeS. All three columns achieved excellent resolution between the two protein subunits. For the DTT digest, the Chromolith® WP 300, 2 mm I.D. column achieved equivalent UHPLC performance to the two, dedicated UHPLC columns, indicating that it has equivalent separating performance. As an added benefit, this performance was achieved at ~16% the pressure drop of the FPP column.

For the IdeS digestion, the Chromolith® WP 300, 2 mm I.D. column exhibited better separation performance than the SPP column, as is illustrated by the improved resolution of variants around the Fab peak in both SigmaMAb™ and NIST mAb. In what appears to be peak splitting occurring on the Fab peak of SigmaMAb™ on the FPP is actually a new variant generated from the high backpressure seen on the FPP. This artifact is mitigated, without a compromise in efficiency, on the Chromolith® WP 300, 2 mm I.D. column. Further research will examine the composition of this variant.

Featured Products

Product list	Cat. No
Chromolith® WP 300, 2 mm I.D., RP-18, 100–2 mm	**1.52370**
BIOshell™ A160 Peptide C18, 10 cm x 2.1 mm I.D., 2.0 µm	**67242-U**
Water for chromatography (LC-MS grade) LiChrosolv®	**1.15333**
Acetonitrile for UHPLC-MS LiChrosolv®	**1.03725**
Trifluoroacetic Acid (LC-MS grade) LiChrosolv®	**80457**
DL-Dithiothreitol (>98% HPLC)	**D0632**
SILu™Lite SigmaMAb™ Universal Antibody Standard human	**MSQC4**
NISTmAb, Humanized IgG1k Monoclonal Antibody	**NIST8671**

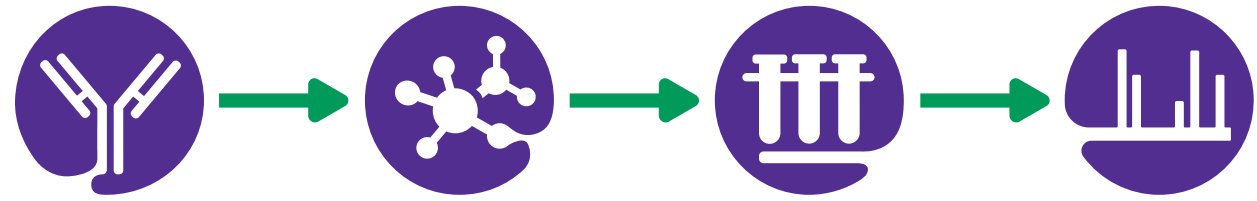
Released N-linked Glycan Analysis Workflow of Adalimumab

Step-by-step protocol for the procainamide labeled glycan profiling of a monoclonal antibody

Romana Rigger PhD[1], Pegah Jalili[2], Uma Sreenivasan PhD[3], Kevin Ray PhD[2]

[1]Buchs, Switzerland, [2]St.Louis, MO, USA, [3]Round Rock, TX, USA

Workflow for released N-glycan analysis



A complete workflow based on UHPLC-FLR-MS is developed to analyze the N-glycan profile. The workflow offers the following:

- Step-by-step instructions for sample preparation and analysis
- Procainamide labeling for increased detection sensitivity
- Separation using BIOshell™ Glycan HPLC column
- Low flow rate for reduced solvent consumption
- Compatibility with mass spectrometry

1. Introduction

Careful and thorough characterization of therapeutic mAbs is essential to ensuring drug safety and efficacy. Hence, establishing several critical quality attributes (CQAs) for each of the proteins and demonstrating that their production batches are within acceptable limits is necessary for both the innovator and biosimilar therapeutics. In this application note, we describe glycan analysis of the innovator mAb adalimumab (Humira®) as well as a recombinant mAb of the same sequence.

Monoclonal antibodies (mAbs) are target specific and have high efficacy and few side effects. Glycosylation is one of the most common and important post translational modifications for mAbs. Glycans attached to the antibodies play an important role in the pharmacokinetics, efficacy, and safety of therapeutic adalimumab. Glycosylation involves the attachment of glycans at specific sites on a protein, most commonly at asparagine (Asn, N-linked) or serine/threonine (Ser/Thr, O-linked) amino acid residues. There are two types of glycosylation— N-linked glycosylation and O-linked glycosylation, and both types are important for protein conformation, protein activity, providing protection from proteolytic degradation, and intracellular trafficking and secretion. N-glycan moieties also play a key role in the folding, processing, and secretion of proteins from the endoplasmic reticulum (ER) and the golgi apparatus. Based on the large influence of glycosylation on protein function, an accurate study and analysis of glycans is essential. Protein glycosylation is specifically mentioned in established technical guidelines, e.g., ICH Q5E and Q6B and FDA's published guidance for industry titled "Development of Therapeutic Protein Biosimilars".

There are four options to approach the N-glycan analysis: intact glycoproteins, glycopeptides, released glycans, or monosaccharides. This article focuses on the analysis of released N-glycans by UHPLC, combined with fluorescence (FLR) and mass spectrometric (MS) detection. The analysis of released N-glycans presented here is one of the most powerful and commonly used approaches for glycan composition analysis. Fluorescent derivatization increases the MS ionization efficiency and allows relative quantification of percent abundances of glycan species by fluorescence detection. Among fluorescent derivatization molecules, procainamide offers one of the best signals, surpassing the more traditional 2-AB and 2-AA labeling. Hydrophilic interaction liquid chromatography (HILIC) is a proven

technique for the separation and quantitation of glycans and has notable advantages over the other HPLC separation modes (e.g. reversed-phase, anion exchange). In this protocol, a BIOshell™ Glycan HPLC column is used to analyze adalimumab N-glycans—labeled with procainamide.

2. General procedure for released N-linked glycan analysis

2.1 Samples

Two samples are analyzed and compared using the following protocol:

- **Adalimumab Reference** (Humira®, from Abbvie, Inc., North Chicago, IL)
- **MSQC16** SILu™Lite SigmaMAb™ Adalimumab Monoclonal Antibody (equivalent, recombinant protein from us)

2.2 Reagent preparation

2.2.1 Buffers and Enzymes

- **8 M Guanidine HCl, prepared in water**
  1. Dissolve 7.6 g guanidine HCl in water and bring the final volume to 10 mL
  2. 0.2 mL/sample (152.85 mg guanidine HCl/sample) is required
- **50 mM Ammonium bicarbonate (ABC buffer), prepared in water**
  1. Dissolve 395 mg ABC in 100 mL water
  2. 2 mL/sample (7.91 mg ABC/sample) is required
- **1 unit/µL PNGase F**
  1. Dissolve in water; may be aliquoted and stored for 6 months or longer at -20 °C

2.2.2 System suitability reagents

The workflow, including glycan release, labeling, and SPE steps is tested on human IgG (hIgG). A sample of IgG is handled alongside other samples. Procainamide labeled dextran hydrolysate is used as an external standard for the analysis of glycans by HPLC. When analyzed on the BIOshell™ Glycan HPLC Column, the standard gives a characteristic ladder profile, from a monomeric glucose to approximately a 20-mer of glucose oligosaccharide. This ladder provides calibration reference points that can aid in identifying more complex glycans based upon retention characteristics.

- **IgG purified from human serum for workflow suitability**

  IgG purified from human serum (200 µg) is processed as a workflow suitability control.
  1. Prepare a 10 µg/µL solution in 8 M guanidine HCl and aliquot in 200 µg portions
  2. IgG is handled identically to the samples
  3. Store any unused portions at -20°C for later use

Adalimumab from different sources was analyzed. It is a recombinant human IgG1 monoclonal antibody (mAb), specific for human tumor necrosis factor (TNF). It has a molecular mass of about 150 kDa and is N-glycosylated on the Fc region.

- **Dextran hydrolysate, procainamide labeled for HPLC-FLR-MS system suitability**

Dextran hydrolysate is solubilized in 25% 75 mM ammonium formate / 75% acetonitrile (v/v), and procainamide labeled according to 2.4. A prelabelled procainamide dextran ladder is also commercially available (SMB01378).

2.3 N-Glycan release

Prior to the analysis, samples are reconstituted to a concentration of 1 mg/mL in water and 200 µg of each protein is used for N-glycan analysis.

2.3.1 Denaturation

1. Set heating block to 50 °C
2. Start with at least 100 µL protein at neutral pH
3. Add 200 µL 8 M guanidine HCl solution and quickly vortex to mix
4. Incubate at 50 °C for 30 min to denature (shaking optional)
5. Bring the temperature of the sample to RT

2.3.2 Buffer exchange to 50 mM ABC buffer

1. Transfer sample to a 30 kDa spin filter
2. Centrifuge at 14,000 x g for 15 min
3. Add 400 µL ABC buffer
4. Centrifuge at 14,000 x g for 30 min
5. Repeat the previous two steps once more, making sure all the solution has passed through the filter
6. Discard the flow through and place filters in new collection tubes

2.3.3 Enzymatic release of glycans

1. Set up heating block to 37 °C
2. Add 50 µL of 50 mM ABC buffer to each filter unit
3. Add 4 µL of 1 UN/µL PNGase F to each filter unit
4. Cap and seal centrifuge device with parafilm
5. Incubate at 37°C for 14-20 hours with shaking at 300 rpm

Note: The digestion time can be decreased to only 30 min using **PNGase Fast** (PN# **EMS0001-kit**) which produces a comparable result for most antibodies.

2.3.4 Recovery of glycans

1. Centrifuge at 1,000 x g for 10 sec to collect lid condensate
2. Add 40 µL ABC buffer
3. Centrifuge at 14,000 x g for 5 min
4. Add 100 µL ABC buffer
5. Centrifuge at 14,000 x g for 5 min
6. Repeat previous two steps once more
7. Transfer glycans from collection tube to 0.6 mL microcentrifuge tubes for labeling
8. Dry the glycans using speed vacuum

2.4 Procainamide labeling

Dried samples are labeled with procainamide in a one-pot reductive amination solution, purified by normal-phase SPE, and the resulting labeled product is then dried again. Glycans are solubilized in 50 µL of 25% 75 mM ammonium formate/75% acetonitrile (v/v) prior to UPLC-FLR-MS.

Note: All preparation and labeling must be performed in a fume hood except for weighing reagents. Prepare the incubation block by moving to the fume hood and set the temperature to 65 °C.

2.4.1 Procainamide labeling reagent

1. Weigh at least 1.8 mg sodium cyanoborohydride (NaBH3CN) per labeling reaction in a tube
    a. Tare a microcentrifuge tube
    b. Transfer NaBH3CN to the tube in the fume hood; a pencil eraser-head volume is usually sufficient
    c. Cap the tube and blow off any dust with N2 gas in the fume hood
    d. Weigh the tube
2. Weigh at least 2.033 times more procainamide hydrochloride, by mass, than NaBH3CN in a separate tube
3. Prepare 9.1 µL of a 70% dimethyl sulfoxide (DMSO)/30% acetic acid (AcOH) (v/v) solution per mg procainamide
4. Solubilize the procainamide with the 70% DMSO/30% AcOH (v/v) solution
5. Ensure the solution is homogenous by vortexing
6. Add 18.5 uL of solubilized procainamide per mg of NaBH3CN

Note: NaBH3CN will not completely solubilize; As exposure to strong acid releases cyanide gas, this step especially warrants working in the fume hood

7. Add 5 µL water per mg of NaBH3CN to completely dissolve NaBH3CN
8. Cap and mix by vortexing in the fume hood to fully solubilize NaBH3CN

2.4.2. Procainamide labeling of glycans

1. Add 40 µL labeling reagent per sample to reaction solution
2. Vortex for 1 min and briefly centrifuge
3. Place capped tube in incubator block and incubate at 65 °C for 3 hours

Note: Cover with foil to limit condensation on the lid and keep dark

2.5 SPE clean up

1. Prepare glycans for clean up by adding 200 µL of 70% ACN in water solution to the labeled glycans
2. Prepare Discovery® DPA-6S SPE tubes and vacuum manifold
    a. Place falcon tube under cartridge for waste collection
    b. Wash with 1 mL water, with minimum pressure gradient by vacuum manifold
    c. Equilibrate with 1 mL 99% ACN in water, with minimum pressure gradient by vacuum manifold
    d. Stop flow when meniscus completely enters top frit
3. Loading the samples
    a. Place microcentrifuge tube under cartridge for breakthrough collection
    b. Add full sample volume to cartridge bed
    c. Pass sample through bed by gravity
    d. When meniscus completely enters top frit, add 500 µL of 99% ACN
    e. Pass volume through by gravity, collecting in same tube
    f. Stop flow when meniscus completely enters top frit
    g. Place falcon tube under cartridge for waste collection
    h. Add breakthrough + 99% ACN to bed
    i. Pass volume through bed by gravity
    j. Stop flow when meniscus completely enters top frit
4. Wash
    a. Add 1 mL of 99% ACN, and pass the solution through cartridge with minimum pressure gradient by vacuum manifold
    b. Repeat the above step four more times

5. Elute
    a. Place new microcentrifuge tube under cartridge for purified glycan collection
    b. Add 200 µL of 20% ACN to bed
    c. Pass volume through bed by gravity
    d. When meniscus completely enters top frit, repeat the previous two steps once more
    e. After the collection drip has stopped, apply medium vacuum manifold pressure to evacuate all liquid from SPE to the collection tube (Total volume ~400 µL)
6. Dry glycans by SpeedVac™, 2-4 h
    a. Labeled glycans can be stored at -20 °C for at least 6 months

2.6 UHPLC-FLR-MS

2.6.1 Solubilize glycans

1. Dissolve the dry glycans in 50 µL of 75% ACN / 25% of 75 mM ammonium formate (v/v) pH 4.4 (adjusted with formic acid) and vortex for 2 min
2. Centrifuge at 16,000 x g for 2 min
3. Transfer 40 µL to autosampler vials

2.6.2 UHPLC-FLR-MS parameters

HPLC Parameters	
Instrument	Waters™ Acquity UPLC-Waters FLR fluorescence detector
Software	MassLynx® 4.1
Column	BIOshell™ Glycan 15 cm x 2.1 mm, 2.7 µm
	Equilibrate the column with 20 x column volume
Column temperature	58 °C
Column equilibration time	9 min
Sample Manager temperature	8° C
Mobile Phase	Mobile Phase A: 75 mM Ammonium formate pH 4.4
	Mobile Phase B: Acetonitrile
Gradient	Gradient: 75% B - 59% B in 75 min
Flow	0.3 mL/min
Injection method	Partial Loop
Injection volume	10 µL
Run time	110 minutes

MS Settings	
Instrument	Thermo Q Exactive™ Plus mass spectrometer
Source	IonMax
Ion source	ESI
DDA mode	One MS2 per MS, most abundant ion, any charge state
Data-dependent exclusion	3 s
Isolation width	5 Da
Normalized collision energy (%)	30
Capillary (V)	4000
Capillary temperature	200 °C
Sheath gas	10
Aux gas	12
Scan range (Da)	400-2000

2.6.3 Sample analysis

1. Run 1-2 blanks at the start of sample list
2. Analyse dextran ladder and hIgG samples first
3. After every 5 samples, run a blank followed by a dextran to update GU values
4. Run a blank after samples and before column flush
5. At completion of queue, flush column with water for 30 minutes followed by 80% ACN/20% water for 30 minutes and store

2.7 Data analysis

LC peaks are identified by their level of residue composition (**Table 1**) from the calculated glucose unit (GU) of their elution. GU levels are determined for each LC feature's retention time by interpolation to a 5th-order polynomial standard curve of the dextran hydrolysate ladder chromatogram (see **Figure 1**). Glycan assignment is done by comparing the GU values to a custom database of glycan GU values for the BIOshell™ column. The dextran ladder is analysed via UHPLC FLR-MS after every fifth sample to correct for any retention time shifts. For relative quantification, fluorescence peak areas are normalized to the sum of all identified glycan fluorescence peak areas. The limit of quantification (LOQ) is defined as 0.5% of the most abundant peak area. This allows for compositions less than 0.5% of the total peak area because composition is normalized to the sum of all peak areas. The peak area is calculated using Xcalibur™ Qual Browser. For general sample analysis, the Thermo Xcalibur™ Qual Browser software retrieves and records the base peak intensity for each sample.

Table 1: Glycan constituent monosaccharides.

Glycan/Modification	Short Name	Generic Name	Residue Mass	Representation
Procainamide Modification	-	-	219.17	-
N-Acetylglucosamine	GlcNAc	HexNAc	203.08	
Mannose	Man	Hex	162.05	
Galactose	Gal	Hex	162.05	
Fucose	Fuc	Fuc	146.06	
N-Acetylneuraminic Acid	Neu5Ac	Neu5Ac	291.10	

3. Results

3.1 System suitability results

The procainamide-labeled dextran hydrolysate and control acquisitions demonstrated that the UHPLC-FLR system and column were suitable to resolve and identify labeled oligosaccharides, as per the four system suitability requirements shown in **Table 2**. First the workflow, including glycan release, labeling, and SPE steps was tested with hIgG and was found to be suitable, with 14 common hIgG glycans detected—where a minimum of 10 hIgG glycan identifications are required. The second system suitability requirement was also met; peaks G1F (1,6) and G1F (1,3) were observed to be partially resolved in the fluorescence chromatogram (**Figure 1**). Although resolution between the G1F (1,6) and G1F (1,3) peaks was not complete, they could be visually differentiated upon magnification. The third system suitability requirement was met by the analysis of hIgG control. The relative abundances of a representative subset of these glycans gave an R2 correlation of 0.99 with the historical glycan profile. Finally, the slope created during analysis of the hIgG control was observed to be 0.99, which is within the slope requirement of 1.00 ± 0.07.

Table 2: Four system suitability requirements and results for hIgG.

Type	Criteria	Result
Qualitative	≥ 10 hIgG peaks detected	Yes
	G1F(1,6); G1F(1,3) Partially Resolved	Yes
Quantitative	Correlation Coefficient R2≥ 0.95	0.99
	Slope 1.00 ± 0.07	0.99

Fluorescence chromatograms of procainamide-labeled dextran hydrolysate and hIgG control.

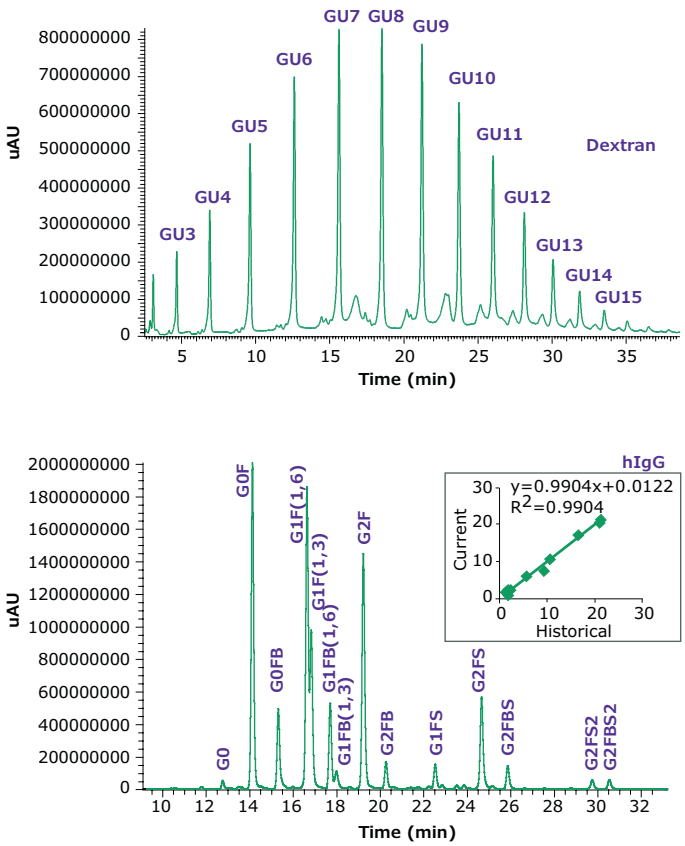


Figure 1: Fluorescence chromatograms of procainamide-labeled dextran hydrolysate and hIgG control. Top pane: annotations indicate the number of glucose units (GU) in each dextran hydrolysate-procainamide feature. Each feature of the control hIgG in the bottom pane was similarly identified. The inset shows the correlation of the glycan features' relative compositions with the historical values of previous hIgG data.

Fluorescence chromatograms of procainamide-labeled dextran hydrolysate and hIgG control showing the number of glucose units (GU) in each LC feature of the two, obtained in the released N-glycan analysis of adalimumab based on UHPLC-FLR-MS.

3.2 Adalimumab sample results

Sixteen glycan features for adalimumab samples were quantified. **Table 3** contains glycan compositions for all the samples. And **Figure 2** illustrates the fluorescence chromatograms for each sample type of adalimumab. Overall, the N-glycan profiles are broadly similar, but some differences exist in the observed relative compositions of some components. **Table 4** illustrates the structures of the observed glycans. The area under the peaks of glycans in the TIC chromatogram are summed and MS spectra were created to confirm the mass of glycans. **Figure 3** shows typical examples of MS spectra.

Table 3: N-Glycan % Composition.

Peak	Glycan	Adalimumab Reference (%)	MSQC16 (%)
1	G0-N	0.6	0.5
2	G0F-N	4.1	0.6
3	G0	0.9	3.1
4	Man5	5.3	1.0
5	G0F	65.0	44.4
6	G1F-N	1.7	-
7a	G1(1,6)	0.4	1.1
7b	G1(1,3)	0.4	0.7
8	Man6	1.8	-
9a	G1F(1,6)	12.1	29.6
9b	G1F(1,3)	5.1	10.0
10	G2F	2.7	7.3
11	G1FS(1,3)	-	0.5
12a	G2FS(1,6)	-	0.6
12b	G2FS(1,3)	-	0.4
13	G2FS2	-	0.4

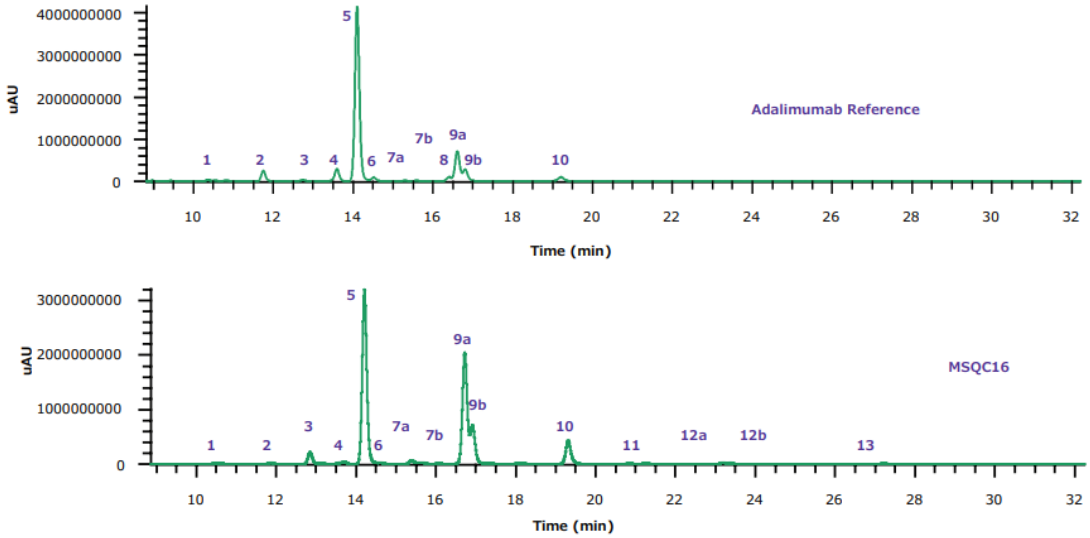


Figure 2: Fluorescence chromatograms of adalimumab samples with the numbered LC features obtained in the released N-glycan analysis of adalimumab based on UHPLC-FLR-MS.

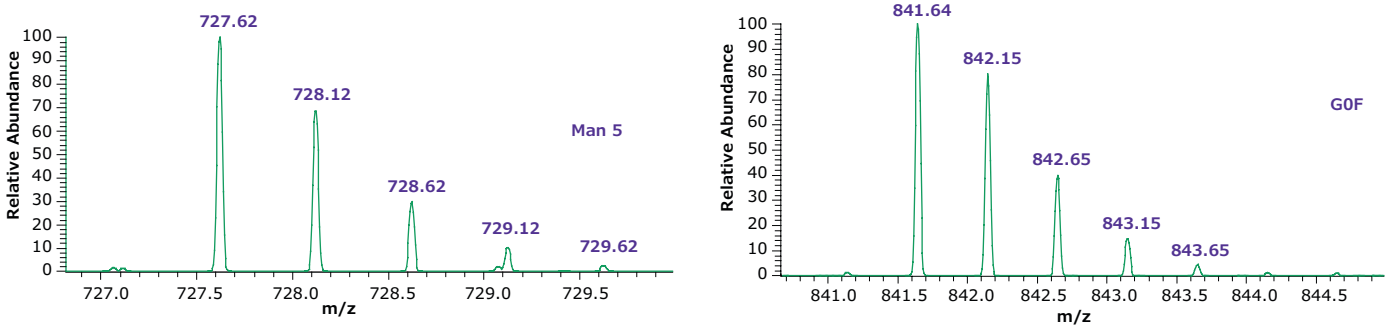


Figure 3: MS spectra of peak 4 (Man 5) and 5 (G0F) obtained on the fluorescence chromatograms of adalimumab samples in the released N-glycan analysis of adalimumab based on UHPLC-FLR-MS.

Table 4: N-Glycans Observed.

Peak	RT (min)	RT GU (min)	Theoretical Mass	Glycan	Monosaccaride Composition	Structure
1	10.46	5.29	1332.58	G0-N	Man3 GlcNAc3	
2	11.83	5.75	1478.64	G0F-N	Fuc1 Man3 GlcNAc3	
3	12.80	6.07	1535.66	G0	Man3 GlcNAc4	
4	13.66	6.35	1453.61	Man5	Man5 GlcNAc2	
5	14.17	6.51	1681.72	G0F	Fuc1 Man3 GlcNAc4	
6	14.56	6.65	1640.69	G1F-N	Fuc1 Man3 Gal1 GlcNAc3	
7a,	15.36	6.91	1697.71	G1	Man3 Gal1 GlcNAc4	
7b	15.62	6.99				
8	16.44	7.28	1615.66	Man6	Man6 GlcNAc2	
9a,	16.68	7.35	1843.77	G1F	Fuc1 Man3 Gal1 GlcNAc4	
9b	16.87	7.42				
10	19.27	8.26	2005.82	G2F	Fuc1 Man3 Gal2 GlcNAc4	
11	21.09	8.94	2134.9	G1FS	Fuc1 Man3 Gal1 GlcNAc4 Neu5Ac1	
12a,	23.23	9.78	2296.92	G2FS	Fuc1 Man3 Gal2 GlcNAc4 Neu5Ac1	
12b	23.47	9.88				
13	27.23	11.56	2588.01	G2FS2	Fuc1 Man3 Gal2 GlcNAc4 Neu5Ac2	

4. Conclusion

A complete UHPLC-FLR-MS workflow has been developed to simplify the analysis of N-linked glycans. This workflow offers the following:

- MS and Fluorescence compatibility
- System suitability testing using human IgG
- Rapid and reproducible N-Glycan release. The protocol provides detailed instructions for sample washing and denaturation
- Procainamide labeling ensuring high fluorescence intensity and ESI efficiency while showing comparable chromatographic separation compared to the other fluorescence labeling systems
- BIOshell™ HPLC column based UHPLC-FLR-MS analysis— suitable for the analysis of protein-linked glycans— and typical mobile phases used for hydrophilic interaction liquid chromatography (HILIC)
- Complete listing of all reagents, consumables, and related products.

A total of 16 glycan features were quantified for the mAb adalimumab. The glycan profile, including the qualitative and quantitative aspects, is comparable to the results found by other analytical laboratories.[1, 2]

Characterizing and monitoring the glycosylation pattern of therapeutic mAbs is required by regulatory authorities to ensure efficacy and safety of the drug.

This detailed protocol can be used for the analysis of N-linked glycans of mAbs and for complex and heterogenous glycoproteins.

References

1. Nayoung Lee, JongAh Joanne Lee, Hahymn Yang, Serim Baek, Soohwan Kim, Sooshin Kim, Tongkeun Lee, Dami Song & Gwangmin Park (2019) Evaluation of similar quality attribute characteristics in SB5 and reference product of adalimumab, mAbs, 11:1, 129-144, DOI: 10.1080/19420862.2018.1530920
2. Tebbey PW, Varga A, Naill M, Clewell J, Venema J. Consistency of quality attributes for the glycosylated monoclonal antibody Humira® (adalimumab). MAbs. 2015;7(5):805-811. doi:10.1080/19420862.2015. 1073429

Featured Products

Product	Cat. No.
Samples and System Suitability Reagents	
SILu™Lite SigmaMAb™ Adalimumab Monoclonal Antibody	**MSQC16**
Dextran from Leuconostoc mesenteroides	**31417**
IgG from human serum	**I4506**
2-AA Labelled dextran ladder	**SMB01376**
2-AB Labelled dextran	**SMB01377**
PC Labelled dextran ladder	**SMB01378**
Glycan Release	
Guanidine hydrochloride	**50933**
Ammonium bicarbonate	**09830**
PNGase F from Elizabethkingia meningoseptica	**F8435**
Microcon®-30kDa Centrifugal Filter Unit with Ultracel-30 membrane	**MRCF0R030**
Labeling	
Sodium cyanoborohydride	**156159**
Procainamide hydrochloride	**PHR1252**
Dimethyl sulfoxide	**D8418**
Acetic acid	**695092**
Cleanup	
Discovery® DPA-6S SPE Tube	**52624-U**
Acetonitrile	**1.00029**
Visiprep™ SPE Vacuum Manifold	**57044**
HPLC	
BIOshell™ Glycan HPLC Column, 15 cm x 2.1 mm, 2.7 µm	**50994-U**
Acetonitrile	**1.00029**
Ammonium formate	**70221**
Formic acid	**5.33002**
Water	
Ultrapure water from Milli-Q® system e.g. Milli-Q® IQ 7000	**ZIQ7000T0**
Water for UHPLC-MS LiChrosolv®	**1.03728**
Accessories	
Microcentrifuge tubes volume 0.6 mL	**T5149**
Autosampler vials volume 0.3 mL	**29661-U**

Supelco® products Sigma-Aldrich® products Milli-Q® products Millipore® products

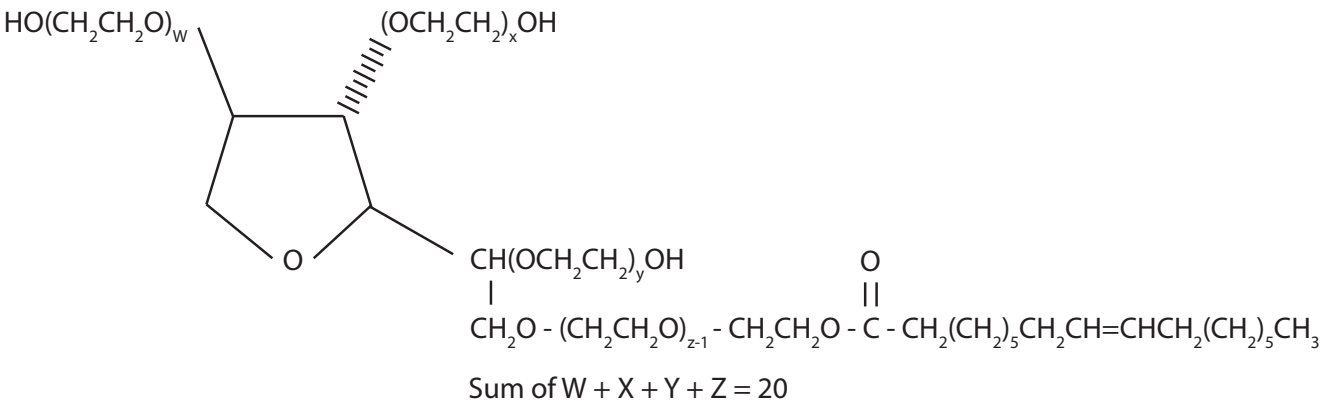
excipients & impurities analysis

Impurities

In the rapidly advancing field of biopharmaceutical modalities, including proteins, peptides, bispecific antibodies, and cell and gene therapies, it is essential to control various impurities that can arise during the manufacturing process. These impurities can be biological, such as host-cell proteins, DNA, viruses, aggregates, and variants of therapeutic proteins, or process-related impurities such as residual solvents, reagents, and excipients. There may also be product-related impurities, such as product variants, degradation products, and impurities resulting from manufacturing equipment, which are also monitored during the manufacturing process.

In addition to internal quality control measures, the biopharmaceutical industry follows guidelines provided by regulatory agencies worldwide to ensure that the impurities are controlled to acceptable levels during production. For example, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) provides guidelines such as ICH Q6B, which addresses the characterization and evaluation of impurities in biotechnology-derived products, and ICH Q11, which provides a framework for developing a description of the manufacturing process and the controls used to monitor the process. The US Food and Drug Administration (FDA) provides guidelines such as the Quality by Design (QbD) initiative, which emphasizes a systematic approach to product development and manufacturing,

HPLC and LC-MS have become indispensable tools in the development and manufacturing of biopharmaceutical modalities, ensuring their quality and purity. Proper use of HPLC and LC-MS techniques, in conjunction with regulatory guidelines, helps to ensure the safety and efficacy of biopharmaceutical modalities, allowing for successful drug development and regulatory approval.



Workflow for the Analysis of Polysorbate 80 in Erbitux® Formulation

Protocol for sample preparation and reversed phase HPLC-ELSD analysis of a nonionic surfactant in a monoclonal antibody formulation

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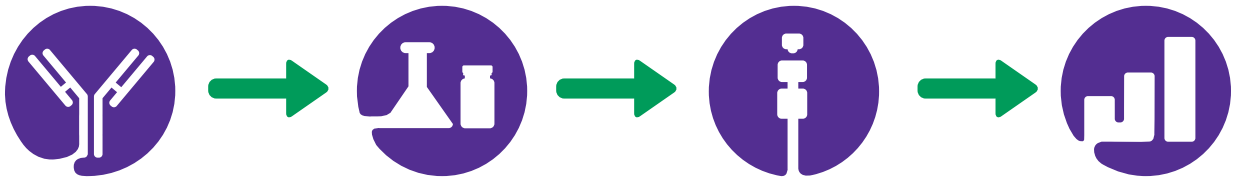
Workflow for the Analysis of Polysorbate 80 in a mAb Formulation



A complete reversed phase HPLC-ELSD workflow has been developed for the quantification of polysorbate 80 (Tween® 80) in antibody formulations.

In detail, it includes:

- Solid phase extraction (SPE) sample preparation procedure
- Preparation of calibration solutions
- Reversed phase HPLC-ELSD method for quantitative analysis of surfactant concentration

Introduction to Polysorbate 80 Analysis in mAb Formulations

Monoclonal antibodies [mAbs or immunoglobulins (IgGs)] are large glycoproteins with a molecular weight of approximately 150 kDa (150,000 g/Mol). They are composed of two identical light chains (LC, molecular weight ca. 25 kDa each) and two identical heavy chains (HC, molecular weight ca. 50 kDa each) linked through covalent inter- and intra-chain disulfide bonds. They are utilized for the treatment of various types of cancer, and other diseases such as multiple sclerosis, Alzheimer's disease, or migraine.

Careful and thorough characterization of therapeutic mAbs is essential for ensuring drug safety and efficacy. mAbs are typically manufactured in mammalian host cell lines in bioreactors, generating a large number of heterogeneous drug molecules.

Establishing a number of critical quality attributes (CQAs) for each mAb and demonstrating that production batches are within acceptable limits is a requirement for both innovator and biosimilar therapeutics.[1,2]

Polysorbate 80 (PS 80; commercial name: Tween® 80) is a nonionic surfactant that is utilized as a stabilizing excipient in protein therapeutics. PS 80 stabilizes proteins, prevents aggregation and nonspecific adsorption of primary and secondary antibodies to surfaces, reduces the rate of protein denaturation and increases the drug solubility and stability.[3,4] In order to ensure product quality, the accurate quantitation of PS 80 in the final drug product is crucial.

Figure 1 displays the chemical structure of polysorbate 80.

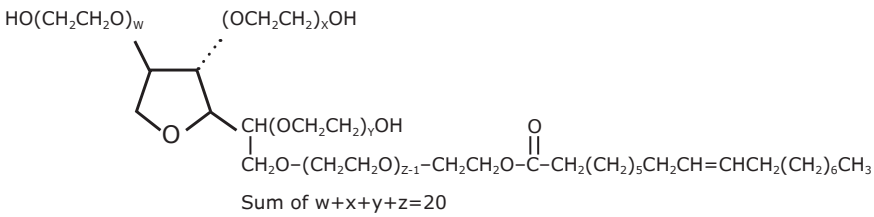


Figure 1. Chemical structure of polysorbate 80 (PS 80; commercial name: Tween® 80).

This report describes the application of reversed phase HPLC-ELSD (high performance liquid chromatography - evaporative light scattering detection) for the quantification of polysorbate 80 in an Erbitux® antibody drug formulation (Erbitux® is the trade name of the drug formulation using the monoclonal antibody cetuximab). Sample preparation is performed using solid phase extraction and a set of seven calibration solutions is prepared for system calibration.

General Procedures – Erbitux® Drug Product Sample Preparation, Polysorbate 80 Standard Preparation, System Setup and Calibration

The samples were received as Erbitux® drug product (DP, formulation of 5 mg/mL cetuximab, excipients: sodium chloride, glycine, polysorbate 80, citric acid monohydrate, sodium hydroxide, water) and were stored at 8 °C. Prior to sample preparation the samples were heated up to room temperature.

A set of two Erbitux® DP batches was analyzed in this work. The polysorbate 80 concentration of the samples was approximately 0.1 mg/mL. No dilution was required before loading onto the solid phase extraction (SPE) cartridge. All solvents applied during sample preparation were of gradient grade HPLC quality or higher.

Sample Preparation

The blank sample was represented by pure water and does not undergo the solid phase extraction process.

The Erbitux® DP samples were purified by SPE. In detail, sample preparation was executed as follows:

1. 4M Guanidinium hydrochloride solution

Dilute 80 mL guanidinium hydrochloride solution 6M with 40 mL water.

2. 10% Methanol

Dilute 10 mL methanol with 90 mL water to obtain a solution of 10% methanol in water.

3. Sample preparation – Solid phase extraction

- Position Supel™ Swift HLB SPE cartridge in Visiprep™ SPE Vacuum Manifold.
- Prime with 1 mL methanol.
- Condition with 1 mL water.
- Load 0.5 mL of an Erbitux® DP sample solution, add 0.5 mL water.
- Wash with 1 mL 4M guanidinium hydrochloride solution.
- Wash with 1 mL 10% methanol.
- Elute with 1 mL acetonitrile and collect eluent in 15 mL centrifuge tube.
- Repeat elution once and collect eluent in the same centrifuge tube.
- Evaporate acetonitrile in vacuum rotary evaporator at 40 °C for 25 minutes.
- Reconstitute sample with 250 µL water, vortex mix well and then transfer to HPLC glass vials.

Standard Preparation

The preparation of calibration standards was performed as follows:

4. PS 80 standard stock solution 1.2 mg/mL

For the preparation of PS 80 stock solution (c = 1.2 mg/mL) weigh approximately 60 mg PS 80 into a 50 mL volumetric flask and fill up to mark with water.

5. PS 80 calibration standards

Prepare dilution series according to **Table 1** to obtain a set of seven calibration standards.

Table 1. Polysorbate 80 calibration standards compositions. Final concentrations resulted from an initial weighed portion of PS 80 of 59.61 mg.

Calibration standard #	Standard stock solution (µL)	Water (µL)	Total volume (µL)	Final concentration (mg/mL)
1	20	980	1000	0.0238
2	40	960	1000	0.0477
3	80	920	1000	0.0954
4	140	860	1000	0.1669
5	200	800	1000	0.2384
6	260	740	1000	0.3099
7	320	680	1000	0.3814

RP-HPLC-ELSD System Setup and Data Analysis

RP-HPLC-ELSD System Setup

The essential settings of the Hitachi Chromaster chromatography system and the gradient conditions applied in the analysis of polysorbate 80 are listed in **Tables 2** and **3** below.

Table 2. HPLC-ELSD settings.

Instrument	Hitachi Chromaster
Software	Chromeleon™ 7.2.10
Column	Supelco® Ascentis® Express C18 5 µm 7.5 cm × 2.1 mm
Column temp	40 °C
Gradient	See Table 3
Flow	0.6 mL/min
Injection volume	50 µL
Run time	18 min
Detection	ELS

Table 3. HPLC-ELSD gradient conditions. A: Water, B: methanol, C: 2-propanol (all solvents LC-MS grade quality).

Minute	% A	% B	% C
0	95	5	0
2.5	95	5	0
5	10	20	70
10	0	10	90
10.5	95	5	0
18	95	5	0

Data Analysis

Data was processed with Chromeleon™ 7.2.10 software; due to application of a gradient profile and the necessity to summarize peak areas in the retention time range from approximately 6 to 9 minutes, the integration was executed manually. Integration of peaks outside the mentioned range was inhibited automatically. The calibration type applied was "Quad with offset".

Calibration data

A total of seven polysorbate 80 calibration standards was prepared. Quadratic regression revealed an excellent fit of the resulting calibration curve over the entire calibration range, with an R2 value of 0.9997 (see **Figure 2**). Experimental data obtained from the calibration experiments are listed in **Table 4. Figure 3** displays an overlay of the chromatograms obtained by the analysis of the seven calibration standards.

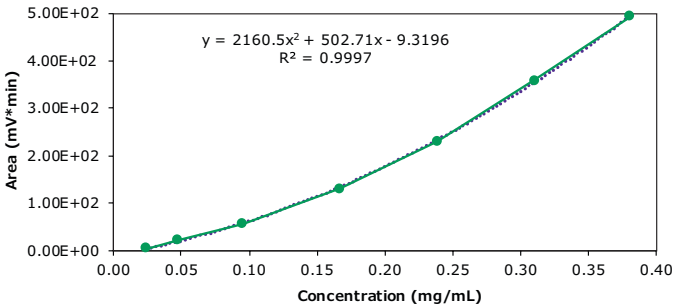


Figure 2. HPLC-ELSD calibration curve obtained by injection of polysorbate 80 calibration standards 1-7.

Table 4. Polysorbate 80 standard solution concentrations, peak areas (median of duplicates) and RSD (%).

Standard solution #	Concentration (mg/mL)	Peak area (mV*min)	RSD(%)
1	0.0238	3.39	0.40
2	0.0477	21.16	1.08
3	0.0954	58.02	0.84
4	0.1669	133.27	2.53
5	0.2384	230.70	3.86
6	0.3099	359.78	2.12
7	0.3814	494.15	2.05

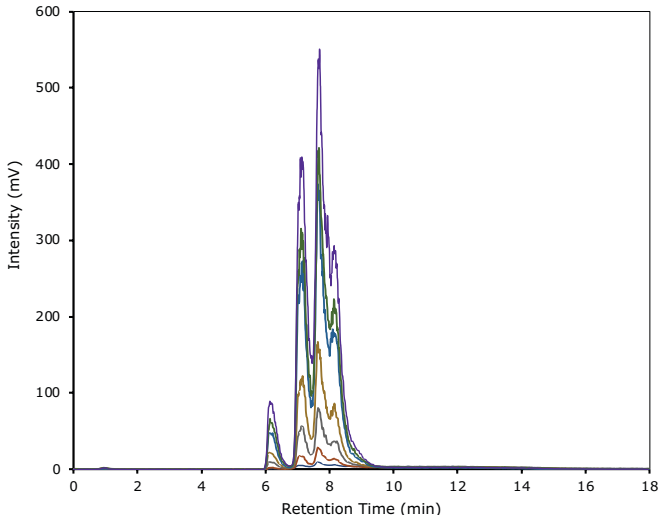


Figure 3. Overlay of chromatograms obtained by the HPLC-ELSD analysis of all seven PS 80 calibration standards.

Results

In this work, an Ascentis® Express C18 HPLC column was utilized for the HPLC-ELSD analysis of polysorbate 80 in two different batches of an Erbitux® antibody drug formulation. The HPLC column applied is comprised of reversed phase-modified, superficially porous silica particles and enables a fast, high-performance analysis.

Sample preparation by hydrophilc-lipophilic balanced solid phase extraction was shown to effectively separate PS 80 from major amounts of drug product excipients.

Duplicates of a total of five samples of each of the batches B4G and BM9 were analyzed to determine their polysorbate 80 content (see also **Table 5**). The corresponding ELSD traces of two representative samples of each batch are shown in **Figure 4**.

The analysis results revealed a PS 80 content of the samples of 0.12 and 0.14 mg/mL, which is in line with typical surfactant concentrations in antibody drug formulations. The calibration curve displayed an excellent quadratic fit over the entire calibration range, with an R2 value of 0.9997, and the LOD for the HPLC-ELSD method was 0.0055 mg/mL.

Table 5. Results of the polysorbate 80 analysis of two Erbitux® DP batches. Concentrations are provided as the median of duplicates.

Batch / sample #	Concentration (mg/mL)	RSD (%)
B4G 1	0.1103	0.0062
B4G 2	0.1117	0.0026
B4G 3	0.1292	0.0031
B4G 4	0.1123	0.0255
B4G 5	0.1488	0.0133
BM9 1	0.1418	0.0002
BM9 2	0.1398	0.0105
BM9 3	0.1455	0.0007
BM9 4	0.1431	0.0233
BM9 5	0.1291	0.0087

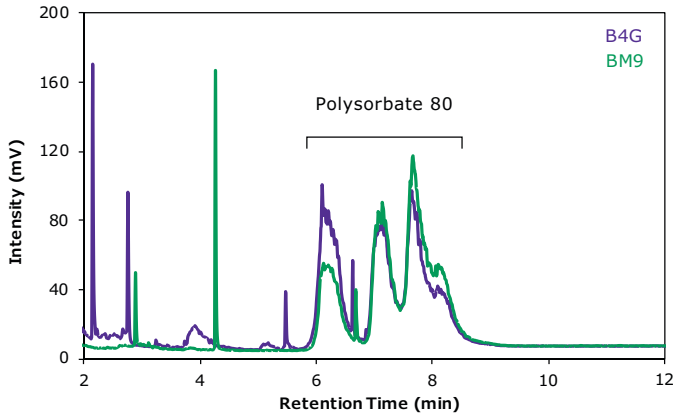


Figure 4. RP-HPLC-ELSD chromatogram of the Erbitux® antibody drug samples B4G (purple trace) and BM9 (green trace). Several polysorbate 80 peaks are visible in the range from approximately 6 to 9 minutes.

Conclusion

This report describes the entire workflow for the quantitative analysis of polysorbate 80 in two Erbitux® antibody drug formulations, using reversed phase HPLC-ELSD analysis. A Supelco® Ascentis® Express C18 HPLC column packed with superficially porous silica particles was applied for the separation of PS 80 and matrix compounds.

The workflow includes a sample purification process using solid phase extraction with HLB cartridges and subsequent analysis of the samples by reversed-phase HPLC-ELSD. HPLC system calibration data was obtained by the preparation and analysis of seven polysorbate 80 standard solutions and allows for a simple quantification of polysorbate 80 content in mAb samples.

The chromatographic method established is suitable for sample separation and analysis of PS 80, and can also be applied in the quantification of similar non-ionic surfactants

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1. K. Sandra, I. Vandenheede, P. Sandra, J. Chrom. A 1335, 81 – 103 (2014).
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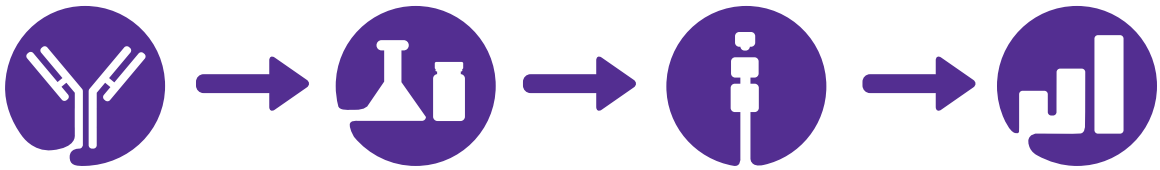
Product List

Description	Cat. No.
HPLC columns & sample preparation	
Supelco® Ascentis® Express C18 5 µm 7.5 cm × 2.1 mm	**50511-U**
Supel™ Swift HLB SPE tubes 30 mg (bed), volume 1 mL	**57493-U**
Solvents & reagents	
Polysorbate 80, certified reference material, pharmaceutical secondary standard	**PHR3790-5X1G**
Ultrapure water from Milli-Q® water purification system or bottled water	**ZIQ7005TOC or 1.15333**
Methanol gradient grade for liquid chromatography LiChrosolv®	**1.06007**
Methanol hypergrade for LC-MS LiChrosolv®	**1.06035**
2-Propanol gradient grade for liquid chromatography LiChrosolv®	**1.01040**
2-Propanol hypergrade for LC-MS LiChrosolv®	**1.02781**
Acetonitrile gradient grade for liquid chromatography LiChrosolv®	**1.00030**
Guanidine hydrochloride solution 6M, manufactured under cGMP controls	**SRE0066**
TWEEN® 80 BioXtra	**P8074-100ML**
Equipment & Consumables	
Visiprep™ SPE Vacuum Manifold, standard, 12-port model Supelco®	**57030-U**
Vacuum centrifuge Eppendorf Concentrator Plus	**EP5305000100-1EA**
Corning® 15 mL centrifuge tubes	**CLS430053-500EA**
Heidolph rotary evaporator Laborota 4003	**Z619094**
Snap Seal™ HPLC vials	**29141-U**

Workflow for Antibody Aggregate and Fragment Analysis

Protocol for heat stressing and SEC-UV analysis of monoclonal antibody aggregates and fragments

Workflow for Antibody Aggregate and Fragment Quantification



A complete SEC-UV workflow has been developed to enable quantitative analysis of antibody fragments and aggregates. In detail, it includes:

- Procedure for heat stressing of antibodies
- System suitability test utilizing a gel filtration standard
- SEC-UV method for sample separation and analysis

Introduction to Therapeutic mAb Characterization

Monoclonal antibodies (mAbs or immunoglobulins - IgGs) are large glycoproteins with a molecular weight of approximately 150 kDa (150,000 g/mol). These biomolecules are composed of two light chains (LC, molecular weight ca. 25 kDa each) and two heavy chains (HC, molecular weight ca. 50 kDa each) linked through covalent inter- and intra-chain disulfide bonds. IgGs are utilized for the treatment of various types of cancer and other diseases such as multiple sclerosis, Alzheimer's disease, and migraine.

Careful and thorough characterization of therapeutic mAbs is essential for ensuring drug safety and efficacy. MAbs are typically manufactured in mammalian host cell lines in bioreactors, generating many heterogeneous drug molecules. Establishing a number of critical quality attributes (CQAs) for each mAb and demonstrating that production batches are within acceptable limits are requirements for both innovator and biosimilar therapeutics.[1,2]

In many cases, the characterization of an antibody-based drug is performed using a specific chromatographic technique (e.g., size exclusion, reversed phase or hydrophilic interaction liquid chromatography, respectively – SEC, RP or HILIC)[3,4] coupled with UV or mass spectrometry (MS) detection. This combination allows for different types of analyses to be carried out, e.g., accurate mass measurement of aggregates,[5] the intact mAb and subunits, fragment analysis, peptide mapping, and the determination of post-translational modifications such as glycosylation, oxidation, and deamidation. SEC is a frequently utilized method for the determination of mAb fragments and aggregates.

Monoclonal antibody formulations can undergo various degradation processes during production, formulation, transport and storage, leading to the formation of protein aggregates and fragments - impurities that are CQAs and that must stay within specific limits. Typically, the total amount of impurities (high and low molecular weight - HMW/LMW) is required to be below 5% and no individual peak area should exceed 1-2%. Usually, the concentration of aggregates in a mAb formulation is higher than the fragment concentration and ranges from 1-5%. Specific limits are validated based on holistic characterization data or on stability trends.

During aggregation, two or more mAb monomers form a stable complex, a process that is difficult to reverse. This aggregation can be induced by electrostatic interactions, for example, leading to self-association of mAbs in concentrated formulations. Another pathway for aggregate formation is misfolding or (partial) unfolding of antibodies creating hydrophobic regions which subsequently bind to each other. Chemical reactions such as disulfide bonding of thiol groups can also form mAb aggregates. Depending on the process, reversible and irreversible covalent and non-covalent species of different sizes can be observed.

Factors influencing aggregation can include temperature, pH value, shear stress during shaking or stirring, or exposure to light or hydrophobic surfaces.[6]

Fragmentation of mAbs can be described as the chemically or enzymatically induced disruption of a covalent protein bond.[7] In the same manner as aggregation, fragmentation is a CQA that needs to be monitored.

An inactivation of a mAb by irreversible fragmentation reactions is most frequently caused by temperature, pH or mAb concentration or the presence of enzymes, metals, or radicals.

The presence of aggregates or fragments in antibody formulations is considered a paramount issue, since they can cause effects such as decreased or increased activity, increased cytotoxicity and immunogenicity and decreased solubility of the drug.[8] More specifically, side effects such as headache, shivers, anaphylactic reactions or renal failure have been observed[9] as well as the development of an immune response by patients. In such a situation, the effectiveness of a drug is strongly reduced.[10] Hence, it is essential to characterize mAb aggregate and fragment formation during drug formulation and to establish a suitable quality control process for these therapeutics.

The focus of this application note is to conduct forced temperature stress studies on a monoclonal antibody and to separate and quantify resulting aggregates and fragments as well as monomers by utilizing SEC-UV analysis.

General Procedures – Eluent and Sample Preparation and System Setup for SEC-UV Analysis of Unstressed and Heat-Stressed Antibodies

Eluent and Sample Preparation

Eluent Preparation

Add 38.1 g potassium di-hydrogen phosphate (KH_2PO_4), 21.1 g of di-potassium hydrogen phosphate (K_2HPO_4) and 37.3 g of potassium chloride (KCl) to a 2 L volumetric flask containing about 1.8 L water and mix thoroughly. Check pH (6.2 ± 0.1) and adjust, if needed, with 85% phosphoric acid (H_3PO_4) or 47% potassium hydroxide (KOH) under stirring. Fill up to 2 L with water and filter solution using a 0.2 µm bottle-top filter. Solution can be stored at 23 ± 5 °C for two weeks.

Sample Preparation

In this work, a research antibody was utilized as a model mAb and is referenced as “antibody”. The antibody sample was obtained as a formulation in 10 mM histidine (c = 21.6 mg/mL, pH 5.5).

Blank sample

Utilize undiluted eluent as a blank sample.

Gel filtration standard solution (system suitability)

Reconstitute one vial of a gel filtration standard (GFS; 1,350–670,000 Da; contains thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, vitamin B12) with 0.5 mL water. Dilute 480 µL of reconstituted solution by adding 9.12 mL water. Dispense resulting solution in 200 µL aliquots in amber vials. Avoid direct light exposure of the vials (GFS contains vitamin B12 which is light sensitive). Store at -80 °C ± 10 °C and use within three years.

Unstressed antibody sample solution

Dilute 93 µL of antibody sample solution with 907 µL eluent in amber vial to yield a final sample concentration of 2 mg/mL.

Heat-stressed antibody sample solution

Stress 1 mL of antibody sample solution for 6 hours at 60 °C utilizing an Eppendorf Thermomixer (or equivalent). Dilute 93 µL of resulting sample solution with 907 µL eluent in amber vial to yield a final sample concentration of 2 mg/mL.

SEC-UV System Setup and Data Analysis

System Setup

The essential settings of the SEC-UV chromatography system applied in the analysis of antibody aggregates and fragments are listed in **Table 1** below.

Table 1. SEC-UV system settings.

Instrument	ThermoScientific™ Vanquish™
Software	Chromeleon™ Version 7.2 SR4
Column	TSKgel® UP-SW3000 HPLC Column, phase diol, L × I.D. 30 cm × 4.6 mm I.D., 2 µm
Column temp	25 °C
Autosampler temp	5 °C ± 3 °C
Mobile phase	0.2 M Potassium phosphate + 0.25 M potassium chloride in water, pH 6.2±0.1
Gradient	Isocratic
Flow	0.35 mL/min
Injection volume	10 µL
Run time	15 min
UV	214 nm
Data collection rate	10.0 Hz
Response time	0.5 s

A typical sample injection scheme applied was as follows:

Sample	Number of injections
Blank	1
GFS	5*
Blank	1
Reference material (unstressed sample)	3
Blank	1
Sample 1 injection 1	1
Sample 1 injection 2	1
Sample 2 injection 1	1
Sample 2 injection 2	1
Blank 1	1
Reference Material	3

* Use 15 injections of GFS in case of new columns

Data Analysis

For quantitative analysis of aggregates, monomers and fragments, all sample peaks that were not present in the blank were integrated; both automatic and manual integration approaches were applied.

Results of SEC-UV Analysis of Unstressed and Heat-Stressed Antibodies

The analysis objective was to perform fragment and aggregate analysis of a research antibody by SEC-UV.

The antibody sample was received as a formulation in 10 mM histidine and used without further treatment. Both unstressed as well as heat-stressed antibody samples were tested. In addition, a gel filtration standard was utilized to determine system suitability.

System Suitability Test Results

A volume of 10 µL of the gel filtration standard was injected on the SEC-UV system. The obtained chromatographic result is displayed in **Figure 1**. The chromatogram shows five peaks for thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B12. In addition, thyroglobulin aggregate and fragment peaks are visible. Depending on the analytical needs, critical parameters such as the resolution or the plate count for specific peaks can be predefined (“acceptance criteria”).

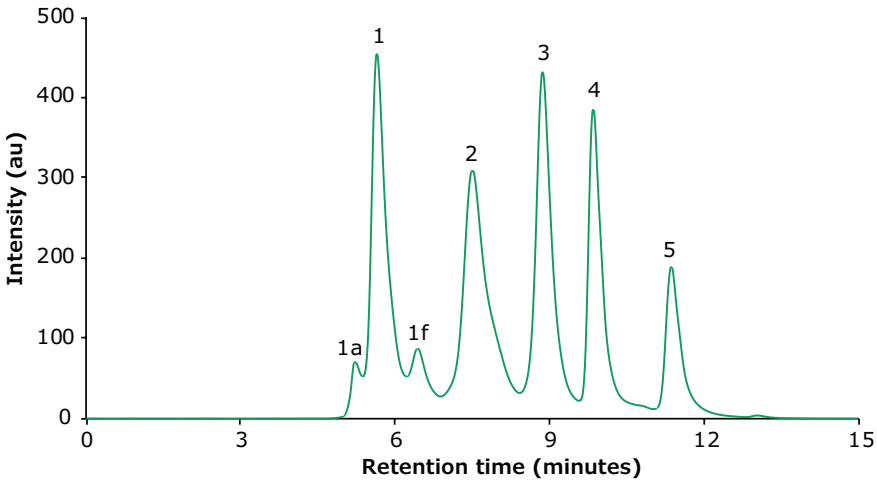


Figure 1. Chromatogram obtained by a gel filtration standard analysis utilizing SEC-UV. 1 Thyroglobulin, 2 bovine γ-globulin, 3 chicken ovalbumin, 4 equine myoglobin, 5 vitamin B12. 1a and 1f are thyroglobulin aggregate and fragment peaks, respectively.

Sample Test Results – Unstressed Antibody Sample

The SEC-UV chromatogram of an overlay of six injections of 10 µL of an unstressed antibody sample is displayed in **Figure 2**. The monomer antibody elutes as the main peak at 7.40 minutes; minor amounts of antibody fragments and aggregates elute between 9.19 to 14.62 minutes and at 6.33 minutes, respectively.

The relative peak areas of six consecutive runs of an unstressed mAb sample are listed in **Table 2**. Median peak area for the monomer was determined at 92.378%, with the standard deviation (SD) being as low as 0.320. Respective values for HMW and LMW compounds were 0.138% (SD 0.004) and 7.485% (SD 0.321), respectively.

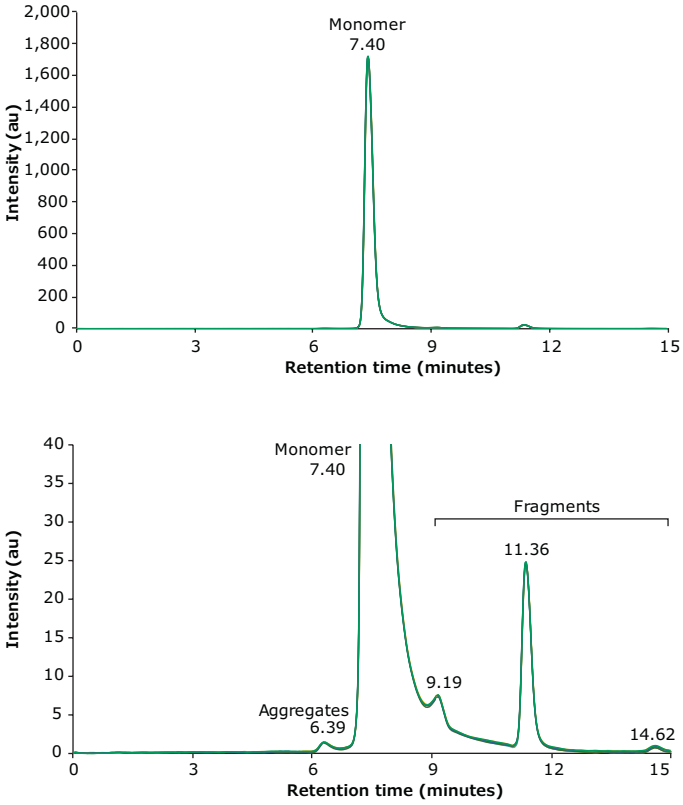


Figure 2. Overlay of SEC-UV traces of six consecutive runs of an unstressed mAb sample (top) and zoom-in (bottom).

Table 2. Relative peak areas of SEC-UV traces of six consecutive runs of an unstressed mAb sample.

Injection #	1	2	3	4	5	6	Median	Standard
Peak	Peak area [%]							deviation
HMW	0.14	0.14	0.13	0.14	0.14	0.14	0.138	0.004
Monomer	92.29	92.74	92.34	91.78	92.72	92.40	92.378	0.320
LMW	7.58	7.12	7.53	8.08	7.14	7.46	7.485	0.321

Sample Test Results – Heat-Stressed Antibody Samples

Heat-stressing of a research antibody sample was performed for 6 hours at 60 °C. 10 µL of the resulting sample was then injected on the SEC-UV system. **Figure 3** displays an overlay of six injections of the stressed antibody. The monomer antibody elutes as the main peak at 7.40 minutes; several antibody fragment peaks are visible between 8 to 15 minutes, and aggregates elute between 4.5 to 7 minutes.

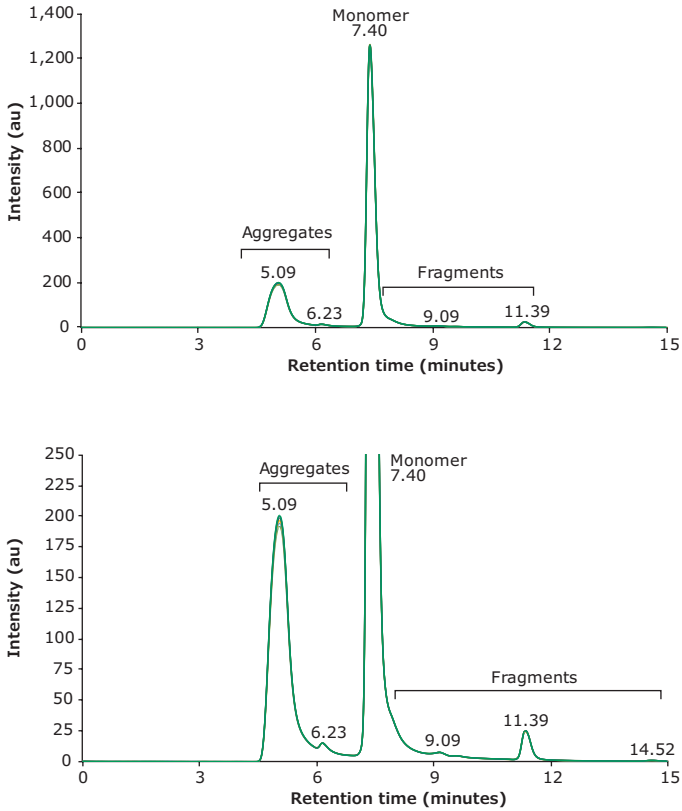


Figure 3. Overlay of SEC-UV traces of six consecutive runs of a mAb sample heat-stressed for 6 h at 60 °C (top) and zoom-in (bottom).

Table 3 shows the relative peak areas of six consecutive runs of the temperature-stressed mAb sample. Median peak area for the monomer, aggregates and fragments was 26.345%, 66.292% and 7.367%, respectively, and the corresponding standard deviations were 0.299, 0.312 and 0.033. Comparison of these results with the composition of the unstressed mAb sample revealed a negligible influence of temperature stress on the number of fragments detected. In contrast, monomer content dropped by approximately 26% and HMW content increased by the same amount.

Table 3. Relative peak areas of SEC-UV traces of six consecutive runs of a heat-stressed mAb sample.

Injection #	1	2	3	4	5	6	Median	Standard
Peak	Peak area [%]							deviation
HMW	25.86	26.10	26.33	26.41	26.64	26.73	26.345	0.299
Monomer	66.80	66.55	66.33	66.17	66.02	65.88	66.292	0.312
LMW	7.35	7.35	7.34	7.43	7.34	7.39	7.367	0.033

Conclusion of SEC-UV Analysis of Antibody Aggregates, Monomers and Fragments

The objective of this work was to develop a workflow for the quantification of antibody aggregates, monomers, and fragments by SEC-UV.

The workflow was comprised of a system suitability test utilizing a gel filtration standard and a procedure for heat stressing of antibodies. In addition, a SEC-UV method suitable for sample separation and analysis of mAb monomers, fragments and aggregates was established.

The research antibody analyzed in this work underwent temperature stressing at 60 °C for 6 h. A comparison of SEC-UV chromatograms of unstressed and stressed antibody samples revealed a strong influence of temperature on the formation of several mAb aggregates. The unstressed antibody sample contained the monomer as the main component (peak area 92.4%), 7.5% of various fragment molecules and 0.1% of aggregate species.

Heat-stressing of the antibody sample was utilized to cause accelerated change of sample composition. The applied process led to a strong shift in sample composition: While monomer concentration dropped to 66.3% (peak area) and the monomer remained the predominant species, the aggregate fraction portion increased to 26.4% and the amount of fragments remained almost constant and was calculated at 7.4%.

The generated data displays that the implemented workflow can be used for the quantitative analysis of unstressed and temperature stressed antibody aggregates, monomers, and fragments by SEC-UV.

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Product List

Description	Cat. No.
HPLC columns	
TSKgel® UP-SW3000 HPLC column, phase diol, L × I.D. 30 cm × 4.6 mm, 2 µm particle size	**80023448**
Solvents & reagents	
Ultrapure water from Milli-Q® IQ 7 series water purification system or LiChrosolv® UHPLC-MS grade water	**Milli-Q® IQ 7000 or 1.03728**
Methanol for analysis	**1.06009**
Potassium di-hydrogen phosphate	**1.04873**
di-Potassium hydrogen phosphate	**1.05101**
Potassium chloride	**1.04936**
Phosphoric acid 85%	**49685**
Potassium hydroxide solution 47%	**1.05545**
Instruments & consumables	
Eppendorf ThermoMixer® F1.5	**EP5384000012**
Mettler Toledo SevenCompact™ pH meter S210	**MT30130863**
Vials, amber glass, volume 2 mL	**27344**
Bottle-top filter unit, pore size 0.2 µm	**Z377422**
Pipette 0.5-10 µL	**EP4924000223**
Pipette 10-100 µL	**EP4861000716-1EA**
Pipette 100-1000 µL	**EP4924000282**
Pipette 500-5000 µL	**EP4924000304**
Pipette tips 0,1-20 µL box	**Z640204**
Pipette tips 2-200 µL box	**Z640220**
Pipette tips 50-1000 µL box	**Z640247**
Pipette tips 100-5000 µL box	**Z640271**

oligonucleotide analysis

Oligonucleotides

Therapeutic oligonucleotides, including antisense oligonucleotides, aptamers, and short interfering RNAs (siRNA), have emerged as a promising class of drugs with the potential to treat a wide variety of diseases, including cancer, genetic disorders, and viral infections. However, the development and manufacturing of oligonucleotide-based therapeutics require strict quality control measures to ensure their efficacy, safety, and regulatory compliance.

In this regard, HPLC and LC-MS techniques play a critical role in the analysis and characterization of oligonucleotide-based therapeutics. These techniques enable the detection, quantification, and identification of impurities, degradation products, and variants of the oligonucleotide, thereby ensuring the purity, consistency, and stability of the drug product. HPLC and LC-MS have become essential tools in the development and manufacturing of oligonucleotide-based therapeutics, enabling accurate and precise quality control and facilitating regulatory approval.

Analysis of Oligonucleotides by SEC-MALS

Presented application data courtesy of Tosoh Bioscience

Anders Fridström, Analytical Scientific Liaison Manager

Abstract

The importance of oligonucleotides in the generation of new pharmaceuctical therapies has been increasing in recent years with 10 FDA approved therapies in 2020.[1] This emerging field of therapies often requires improved or alternative analytical methods to accelerate development and assure the safety of the drug. This article describes the ability of ultra-high performance size exclusion chromatography to distinguish N and N-1 oligonucleotide species.

Introduction

In recent years, several oligonucleotide drugs for gene silencing, such as short interfering RNA (siRNA) and antisense oligonucleotides (ASOs) have been approved and microRNA (miRNA) and aptamers are being developed as therapeutic platforms. The promising CRISPR-Cas system also requires a specific RNA moiety - guiding RNA - to recruit and direct the Cas nuclease activity.

Therapeutic oligonucleotides are produced through a synthetic, solid-phase chemical synthesis. Despite improvements in oligonucleotide synthesis, and despite the most ardent post-synthesis clean-up, there is always some heterogeneity with regards to oligonucleotide distribution. Monitoring of impurities in this distribution is a fundamental aspect of process and quality control. This fundamental assessment is typically done by capillary gel electrophoresis (CGE) or anion exchange chromatography. Here, we present the ability of size exclusion chromatography (SEC) to discriminate oligonucleotides differing by one base in length. The 2 µm silica-based stationary phase, TSKgel® UP-SW2000, with a pore size of 125 Å, was used in combination with UHPLC and UHPLC-MALS systems.[2]

Analysis of Oligonucleotides by SEC[2]

TSKgel® UP-SW2000 is a recently developed silica-based 2 µm, 125 Å pore size SEC column designed for the separation of small proteins, peptides, and oligonucleotides. The column can be used both in HPLC and UHPLC systems and is ideally suited for method transfer from conventional silica-based size exclusion columns to UHPLC technology. Two, 30 cm TSKgel® UP-SW2000 columns in series were used to analyze a mixture of two oligonucleotides differing by only one base.

Materials and Method

Columns:	TSKgel® UP-SW2000, 2 x 300 x 4.6 mm I.D., 2 µm (**823514**)
Mobile phase:	[A] 50 mM phosphate buffer, pH 6.7, [B] 300 mM sodium chloride, 0.03% W/V sodium azide
Flow rate:	0.2 mL/min
Detection:	UV, 260 nm
Injection:	10 µL
Sample:	19-mer (5'-AATTCATCGGTTCAGAGAC-3') & 20-mer(5'-GAATTCATCGGTTCAGAGAC-3')

Results

Figure 1 demonstrates that UP-SW2000 can be used to separate a 20-mer and its N-1 19-mer.

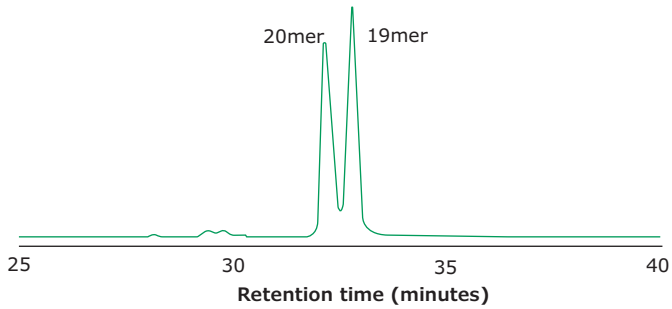

Figure 1. Separation of N and N-1 Oligonucleotides.

SEC-MALS Analysis of Oligonucleotides[2]

Crude and purified oligonucleotide samples were analyzed by SEC-MALS using $LenS_3$® multi-angle light scattering detector.

Materials and Method

Instrument:	Thermo Fisher Dionex™ Ultimate 3000 UHPLC system with LenS$_3$® MALS
Column:	TSKgel® UP-SW2000 300 x 4.6 mm I.D., 2 µm (**823514**)
Mobile phase:	0.5 M Sodium chloride, 0.1 M EDTA, pH 7.5; 0.1 M sodium sulfate; 0.03% w/v sodium azide in 0.1 M phosphate buffer
Flow rate:	0.3 mL/min
Column temp:	Room temperature
Detection:	UV, 260 nm
Injection vol.:	10 µL
Sample:	20 Bases custom oligonucleotide with MW= 6141 Da (purified sample 0.3 mg/mL; crude sample 1 mg/mL)

Results

Figure 2 shows the comparison of chromatograms of the crude and purified oligonucleotide samples and **Figure 3** shows the molecular weight distribution of the unpurified 20-mer. The molecular weight trace clearly indicates the presence of higher and lower molecular weight impurities.

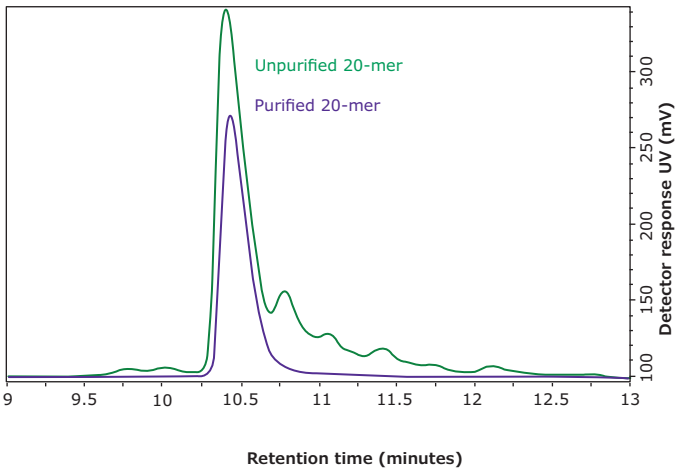

Figure 2. Overlay of unpurified and purified 20-mer UV chromatograms.

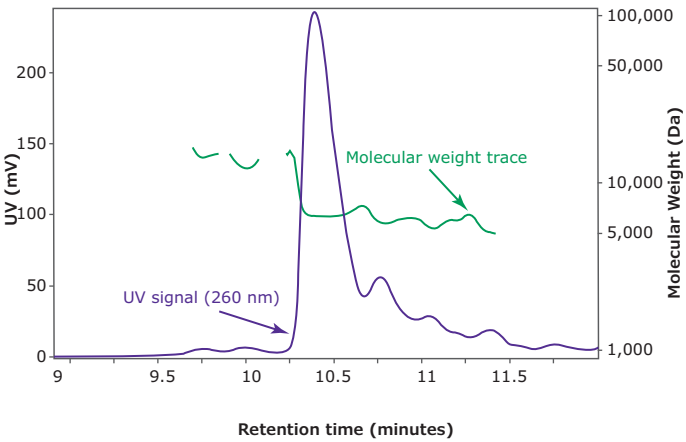

Figure 3. Molecular weight distribution (green) of the unpurified 20-mer.

The peak analysis (**Figure 4** and **Table 1**) allows a molecular weight profiling of the product and the impurities. The MALS analysis of the purified sample (**Figure 5**) proves the high purity of the 20-mer oligonucleotide. The good reproducibility of retention time and calculated molecular weight of the purified 20-mer is shown in **Table 2** (triplicate injection).

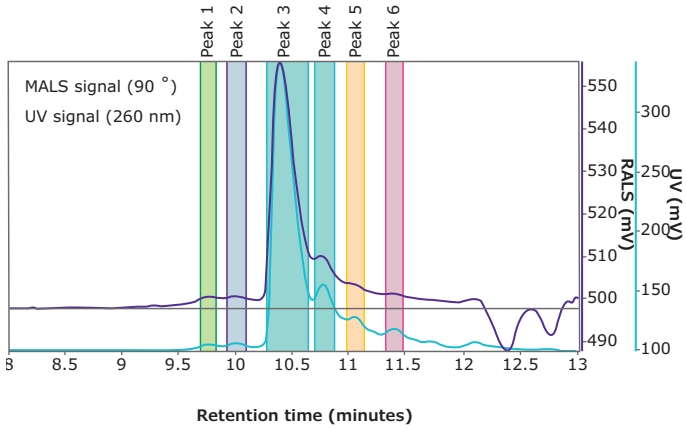

Figure 4. Peak analysis of the unpurified 20-mer.

Table 1. Molecular weight profiling

Peak	Retention time	% RSD	MW (Da)	% RSD
1	9.774	0.1%	13,599	2.1%
2	10.012	0.0%	11,550	1.9%
3	10.398	0.1%	6,398	0.7%
4	10.776	0.1%	5,751	1.5%
5	11.053	0.1%	5,177	2.3%
6	11.422	0.2%	4,446	5.5%

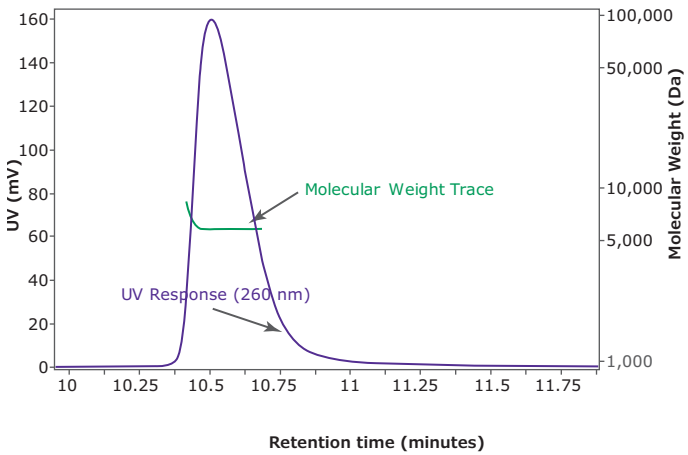

Figure 5. Molecular Weight distribution (green) of the purified 20-mer.

Table 2. Reproducibility of retention time

Injection	Retention time (Min)	MW (Da)
1	10.431	6.066
2	10.443	6.023
3	10.445	6.038
Average	10.440	6.042
% RSD	0.1%	0.3%

Conclusion

TSKgel® UP-SW2000 is a size exclusion column designed for UHPLC analysis of biomolecules having molecular weight of 1 to 150 kDa. The separation range is ideally suited to analyze small proteins or peptides and their aggregates.

This study shows that this column can also be used to analyze oligonucleotides by (U)HPLC. Multi-angle light scattering detection delivers additional information on the molecular weight of the oligonucleotide and any impurities present in the sample.

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Featured Product

Description	Cat. No.
TSKgel® UP-SW2000, phase diol, 300 × 4.6 mm, 2 µm	**823514**

Related Products

Description	Cat. No.
EDTA disodium salt suitable for HPLC, LiChropur™, 99.0-101.0% (KT)	**79884**
Potassium dihydrogen phosphate anhydrous for HPLC LiChropur™	**5.43841**
Sodium azide, purum p.a., ≥99.0% (T)	**71290**
Sodium chloride for HPLC LiChropur™	**5.43832**
di-Sodium hydrogen phosphate anhydrous for HPLC LiChropur™	**5.43838**
Sodium sulfate suitable for HPLC LiChropur™, 99.0-101.0% (T)	**80948**
Sodium azide, purum p.a., ≥99.0% (T)	**71290**

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1. Roberts, T.C.; Langer R.; Wood, J. A. Advances in oligonucleotide Drug Delivery. Nature Reviews, Drug Discovery, volume 19, 673–694 (2020) https://www.nature.com/articles/s41573-020-0075-7
2. Tosoh Application note, Analysis of Oligonucleotides by SEC-MALS https://www.separations.eu.tosohbioscience.com/OpenPDF.aspx?path=~/File%20Library/TBG/Products%20Download/Application%20Note/an0008a-g.pdf

Analysis of Oligonucleotide Standard 6 Mix by Liquid Chromatography-UV

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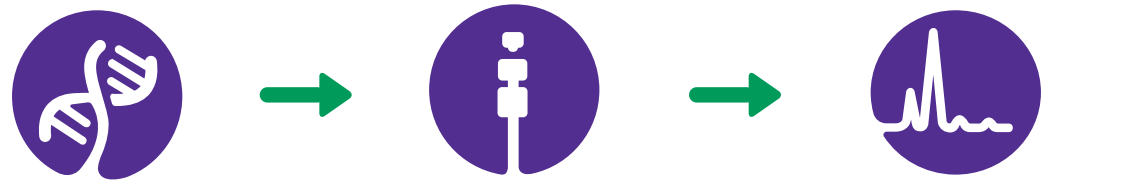


Introduction

With the COVID-19 pandemic, oligonucleotides (Oligos) have proven their importance in diagnostic and therapeutic applications. Currently, 11 oligonucleotide drugs crossing many disease areas have been approved by the FDA.[1, 2] Obstacles preventing quicker development of oligonucleotide therapeutics include the challenges of unfavorable absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies for many clinical trials.[2] Some strategies have been developed to tackle the challenges, such as chemical modification to improve drug delivery.

Synthetic oligonucleotides are typically small, single- or double-stranded modified nucleic acids.[2] There are many established techniques to analyze and characterize oligonucleotides, including capillary gel electrophoresis (CGE), ion exchange chromatography (IEX), and ion pair reversed phase liquid chromatography (IP-RPLC). Generally, liquid chromatography of Oligos is very challenging due to the similarity of oligonucleotide structures, very polar characteristics, presence of truncated and/or modified Oligos, ease of self-association into a variety of conformations, and affinity for metal surfaces.[1,2] This application describes the separation of an internally produced oligonucleotide standard (Oligo Standard 6) mix, which includes six oligonucleotides, on Supelco® Chromolith® RP-18e columns.

General Procedures

Oligo Standard 6 is an internal (in-house) system suitability mix for HPLC-UV evaluation of oligonucleotide separations. The standard contains six components with molecular weights of 3588.3 Da (Oligo 1), 4157.93 Da (Oligo 2), 7580.83 Da (Oligo 3), 10014.35 Da (Oligo 4), 6116.97 Da (Oligo 5), and 4395.8 Da (Oligo 6) following their elution order on Chromolith® RP-18e columns tested here.

Reagent Preparation

50 mM of Triethylammonium Acetate (TEAA)

To prepare 1 L of 50 mM TEAA, 50 mL of TEAA (commercial 1 M solution) was added into 950 mL of HPLC grade water and mixed well.

20 mM of Triethylammonium Acetate (TEAA)

To prepare 1 L of 20 mM TEAA, 20 mL of TEAA (commercial 1 M solution) was added into 980 mL of HPLC grade water and mixed well.

5 mM of Triethylammonium Acetate (TEAA)

To prepare 1 L of 5 mM TEAA, 5 mL of TEAA (commercial 1 M solution) was added into 995 mL of HPLC grade water and mixed well.

Sample Preparation

5 µM of Oligo Standard 6 sample

1 mL of HPLC grade water was added into the sample vial which contains 5 nmol each of the six Oligo components and mixed well.

HPLC-UV System Setup and Data Analysis

Essential settings of the HPLC-UV chromatography system for analysis of Oligo Standard 6 are listed in **Table 1** below.

Table 1. HPLC-UV general system settings.

Instrument Setup	
HPLC system	Agilent 1260 Infinity II
Software	Agilent ChemStation
Column	Chromolith® RP-18e, 100 x 4.6 mm; Chromolith® High Resolution RP-18e, 100 x 2.0 mm/50 x 2.0 mm
Column temp.	25 °C; 40 °C
Autosampler temp.	5 °C
Mobile phase A	5–50 mM TEAA
Mobile phase B	Acetonitrile
Flow	0.4–3 mL/min
Injection volume	5 µL
Run time	12 min
Detector	UV; 260 nm

Results and Discussion

With the linkage of phosphate groups, oligonucleotides tend to stick to metal surfaces present in stainless steel column hardware and the LC system, resulting in reduced sensitivity and inaccurate quantitation. Researchers have made a variety of efforts to mitigate this adsorption inside instrumentation, such as treatment of the system with EDTA, high pH mobile phase, or utilizing bio-inert HPLC system components.[3] Conventional HPLC columns are typically packed in metal columns, exposing the metal surfaces with positive charge which can adsorb acidic molecules, such as oligonucleotides containing phosphate groups. Chromolith® HPLC columns are made of highly porous monolithic rods of silica, with an innovative bimodal pore structure and packed in metal-free PEEK (polyetheretherketone) columns, which make it a good candidate for oligonucleotide analysis.

Chromolith® RP-18e, 100 x 4.6 mm Column

Flow Rate Test

To improve separation efficiencies, the particle size of packing material is usually reduced. Currently, conventional HPLC columns contain 5, 3, 2, and even sub 2 µm silica particles.[4] However, the smaller particle size will cause higher back pressure affecting the assay throughput, robustness, and column lifetime. The optimal solution is to use a column that offers faster throughput without the risk of high back pressure. Since Chromolith® is not packed with silica particles, but a single rod of high-purity, polymeric silica gel, the unique construction enables highly efficient separations at accelerated speeds, which is ideal for high throughput analysis.[4]

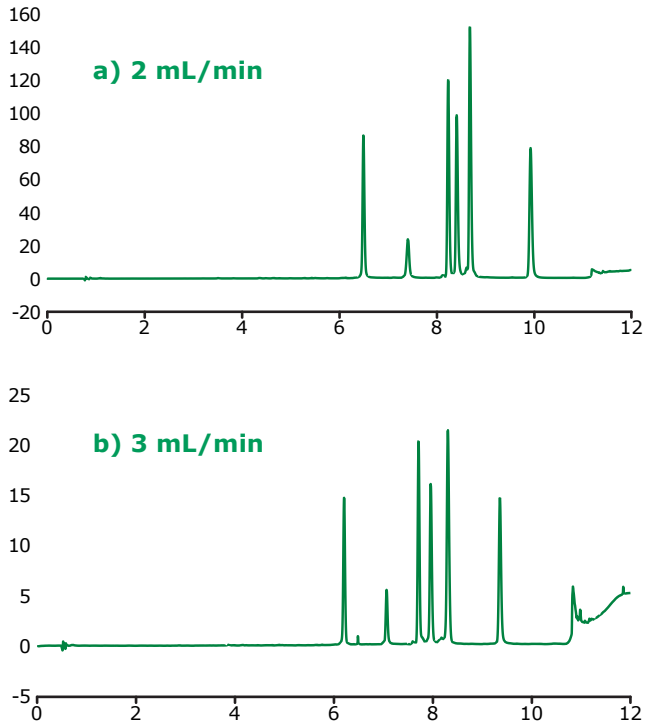
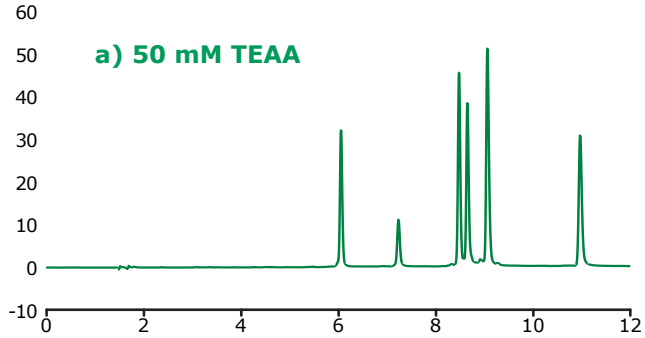


Figure 1. Oligo Standard 6 separation on Chromolith® RP-18e, 100 x 4.6 mm column at flow rates: a) 2 mL/min; b) 3 mL/min with a gradient of 5% B to 15 % B in 10 minutes. Mobile phase A: 50 mM TEAA in water; Mobile phase B: acetonitrile. Note: injection volume for 2 mL/min is 20 µL and 5 µL for 3 mL/min.

Figure 1 shows the separation of Oligo Standard 6 on a Chromolith® RP-18e column under flow rates of 2 mL/min and 3 mL/min with only 25 pmol on column injection for each oligonucleotide. 50 mM of TEAA was used as mobile phase A and acetonitrile as mobile phase B with a gradient of 5% B ramping to 15% B in 10 minutes. The typical back pressure at 2 mL/min and 3 mL/min is 30–50 bar which is beneficial for high throughput assays.

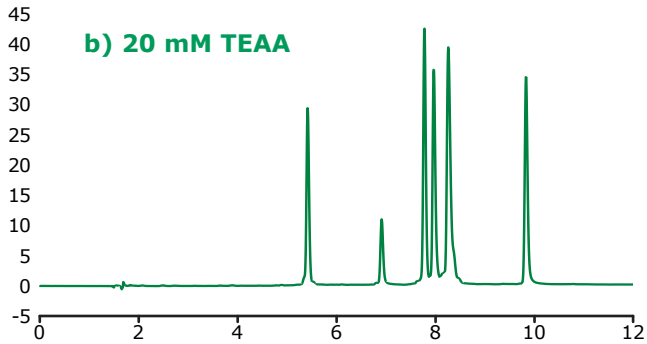
Ion-Pairing Additive Concentration Test

In the qualitative and quantitative analysis of oligonucleotide impurities, ion-pair reversed phase liquid chromatography has been the dominant technique. The ion-pairing reagents added in mobile phase are typically several alkylammonium salts which are adsorbed on the column sorbent with the positive charges exposed to interact with the negatively charged oligonucleotides. Triethylammonium acetate (TEAA) is one of the commonly used ion-paring reagents in LC-UV analysis of oligonucleotides. Optimizing ion-pairing additive concentration is important to achieve efficient separation while minimizing cost from additive consumption. In this work, optimization of TEAA concentration was conducted.



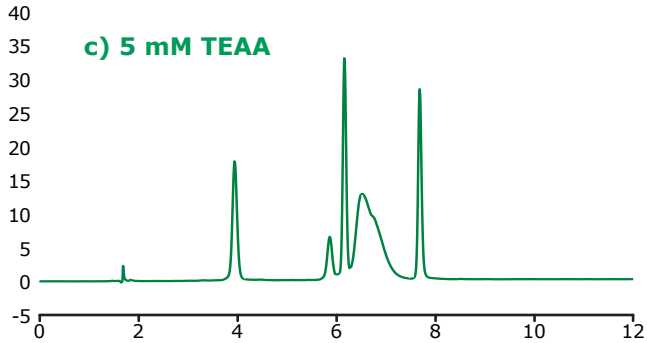
50 mM TEAA

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	6.051	113.191	32.336	
Oligo 2	7.232	45.290	11.023	12.549
Oligo 3	8.476	153.993	45.496	13.167
Oligo 4	8.647	136.388	38.111	1.938
Oligo 5	9.058	205.826	50.765	4.293
Oligo 6	10.964	142.741	30.822	17.499



20 mM TEAA

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	5.418	110.992	29.261	
Oligo 2	6.916	44.280	10.811	15.225
Oligo 3	7.780	158.234	42.434	8.820
Oligo 4	7.969	145.570	35.631	1.917
Oligo 5	8.263	215.256	39.189	2.522
Oligo 6	9.835	144.133	34.367	13.334



5 mM TEAA

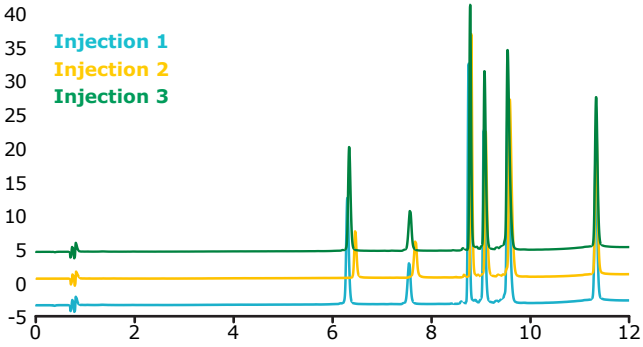
Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	3.942	121.392	17.759	
Oligo 2	5.865	40.805	5.964	10.811
Oligo 3	6.163	157.698	32.526	1.925
Oligo 4/5	6.517	368.327	12.332	0.749
Oligo 6	7.685	143.739	28.318	2.462

Figure 2. Oligo Standard 6 separation on Chromolith® RP-18e, 100 x 4.6 mm column with different TEAA concentration in mobile phase A: a) 50 mM TEAA; b) 20 mM TEAA; and c) 5 mM TEAA. Resolution is calculated between each two adjacent peaks.

Figure 2 shows the different concentrations of TEAA tested in mobile phase A with acetonitrile as mobile phase B in the separation. Five microliters of Oligo Standard 6 sample was injected on a Chromolith® RP-18e, 100 x 4.6 mm column at a flow rate of 1 mL/min with a gradient of 8% B to 15% B in 10 minutes for each test. With 50 mM of TEAA in mobile phase A, the oligonucleotides were well separated with the retention time as indicated in **Figure 2**. When the TEAA concentration was lowered to 20 mM, Oligo 1 to 6 eluted in the same order but with less retention on column. With the exception of Oligos 1 and 2, the resolution between each peak pair is seen to be lower as well. When TEAA concentration was further lowered to 5 mM, Oligos 4 and 5 were not separated which indicates the ion-pairing strength is not high enough to separate these two oligonucleotides. Comparing the peak heights of the six Oligos under the three different TEAA concentrations, 50 mM TEAA produced the highest peak height as shown in the table in **Figure 2**. Therefore, the ion-pairing additive concentration needs to be optimized based on the characteristics of the oligonucleotides.

Chromolith® High Resolution RP-18e Column

The Chromolith® High Resolution column possesses 1.15 µm macropores compared with 2 µm on the standard Chromolith® column. This modification results in higher separation efficiency and better peak shape. Although this creates higher back pressure, it is still less than half that of any particulate column of similar efficiency.[4]

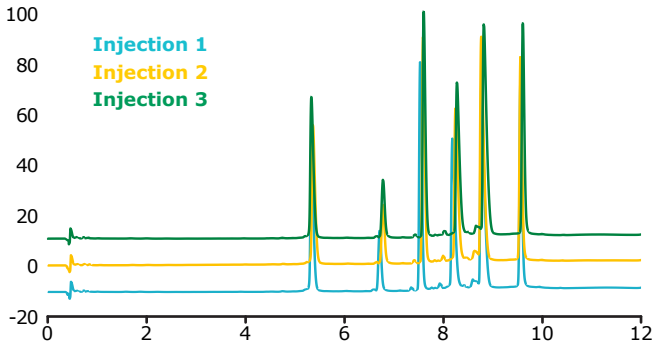


Chromolith® High Resolution RP-18e, 100 x 2.0 mm—3 µL injection

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	6.337	102.148	27.621	
Oligo 2	7.568	50.707	10.807	11.691
Oligo 3	8.781	167.619	65.201	13.279
Oligo 4	9.072	150.772	47.522	3.972
Oligo 5	9.539	228.983	53.013	4.936
Oligo 6	11.327	159.908	39.979	17.292

Figure 3. Oligo Standard 6 separation on Chromolith® High Resolution RP-18e, 100 x 2.0 mm column. Mobile phase A: 50 mM TEAA in water, Mobile phase B: acetonitrile; gradient: 8% B to 15% B in 10 minutes at flow rate of 0.4 mL/min, column temperature: 40 °C.

Here, 3 µL of Oligo Standard 6 sample was injected onto the Chromolith® High Resolution RP-18e, 100 x 2.0 mm column at 0.4 mL/min with a gradient of 8% B to 15% B in 10 minutes. **Figure 3** is an overlay of three injections showing consistent retention and response. 50 mM TEAA concentration was used as mobile phase A and acetonitrile was mobile phase B. Resolution between Oligo 4 and 5 is 4.936. A shorter column of Chromolith® High Resolution RP-18e, 50 x 2 mm was compared with the same conditions with 5 µL of injection volume used in **Figure 3**. As shown in **Figure 4**, on a 50 x 2 mm column, all six oligonucleotides were eluted within 10 minutes with the resolution between Oligo 4 and 5 of 3.921. Thus, Chromolith® HR RP-18e column is capable of oligonucleotide analysis using LC-MS compatible flow rates.



Chromolith® High Resolution RP-18e, 50 x 2.0 mm —5 µL injection

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	5.343	261.641	56.765	
Oligo 2	6.794	106.792	23.310	12.458
Oligo 3	7.618	357.882	90.331	7.641
Oligo 4	8.292	316.582	61.032	5.746
Oligo 5	8.836	479.91	84.329	3.921
Oligo 6	9.625	307.511	84.625	6.631

Figure 4. Oligo Standard 6 separation on Chromolith® High Resolution RP-18e, 50 x 2.0 mm column. Mobile phase A: 50 mM TEAA in water, Mobile phase B: acetonitrile; gradient: 8% B to 15% B in 10 minutes at flow rate of 0.4 mL/min, column temperature: 40 °C.

Conclusion

In this application note, the separation of Oligo Standard 6, an internally created HPLC-UV system suitability mix, was demonstrated on Chromolith® and Chromolith® High Resolution RP-18e columns. Flow rates up to 3 mL/min were evaluated on a Chromolith® column with excellent separation of the six Oligos indicating that it is ideal for high throughput assays. The results of the ion-pairing reagent optimization experiments indicate that 50 mM TEAA provides the best separation and sensitivity for Oligo Standard 6. Separation of Oligo Standard 6 on Chromolith® High Resolution column with flow rate of 0.4 mL/min produced better resolution of Oligo 4 and 5 compared to 3 mL/min method on Chromolith® column, with resolution (USP) of 3.9 vs 1.9. This result demonstrates that Chromolith® High Resolution column is suitable for oligonucleotide analysis by LC-MS with mass spectrometer favorable flow rates tested here. In addition, the polymeric column housing can be used as part of a metal free, or bio-inert HPLC system.

Acknowledgement

The authors would like to thank Pierre Potier for providing the Standard 6 oligonucleotide mix and for technical support.

References

1. Roberts, T.C.; Langer, R.; Wood, J. A. Advances in oligonucleotide drug delivery. Nature Reviews, Drug Discovery, volume 19, 673–694 (2020).
2. Hammond, S.M.; Aartsma-Rus, A.; Alves, S. et al. Delivery of oligonucleotide-based therapeutics: challenges and opportunities. EMBO Molecular Medicine 13: e13243 (2021).
3. Gilar M.; DeLano M.; Gritti F. Mitigation of analyte loss on metal surfaces in liquid chromatography. J. Chrom. A 1650 (2021) 462247
4. Chromolith® HPLC columns brochure-pb6401, Race through separations with revolutionary technology.

Product List

Description	Cat. No.
HPLC columns	
Chromolith® HPLC column RP-18e, L × I.D. 100 mm × 4.6 mm	**1.02129.0001**
Chromolith® HPLC column HR RP-18e, L × I.D. 100 mm × 2.0 mm	**1.52322.0001**
Chromolith® HPLC column HR RP-18e, L × I.D. 50 mm × 2.0 mm	**1.52321.0001**
Chemicals & reagents	
Triethylammonium Acetate, 1 M Solution	**90358**
Water, HPLC-Grade	**270733**
Acetonitrile, HPLC-Grade	**900667**
Instruments & consumables	
Eppendorf ThermoMixer® F1.5	**EP5384000012**
Vials, amber glass, volume 2 mL	**27344**
Pipette 0.5–10 µL	**EP4924000223**
Pipette 10–100 µL	**EP4861000716-1EA**
Pipette 100–1000 µL	**EP4924000282**
Pipette tips 0.1–20 µL box	**Z640204**
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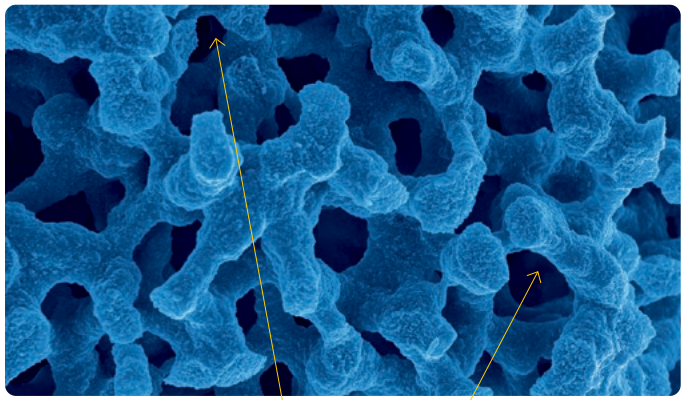
Learn more about Chromolith® columns by clicking **here**



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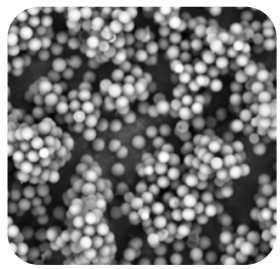

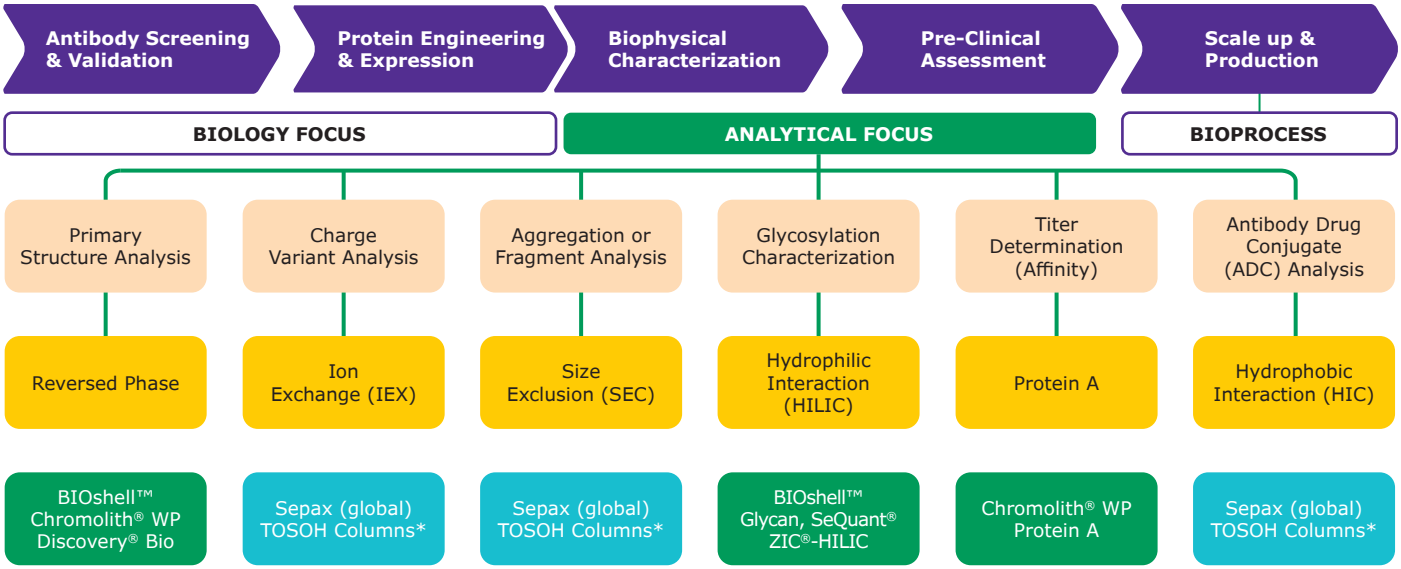

* Tosoh Bioscience columns are available in select countries.

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Featuring comparison of heat-stressed vs non-heat stressed antibodies; SEC-UV analysis on a TSKgel® UP-SW3000 HPLC Column

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