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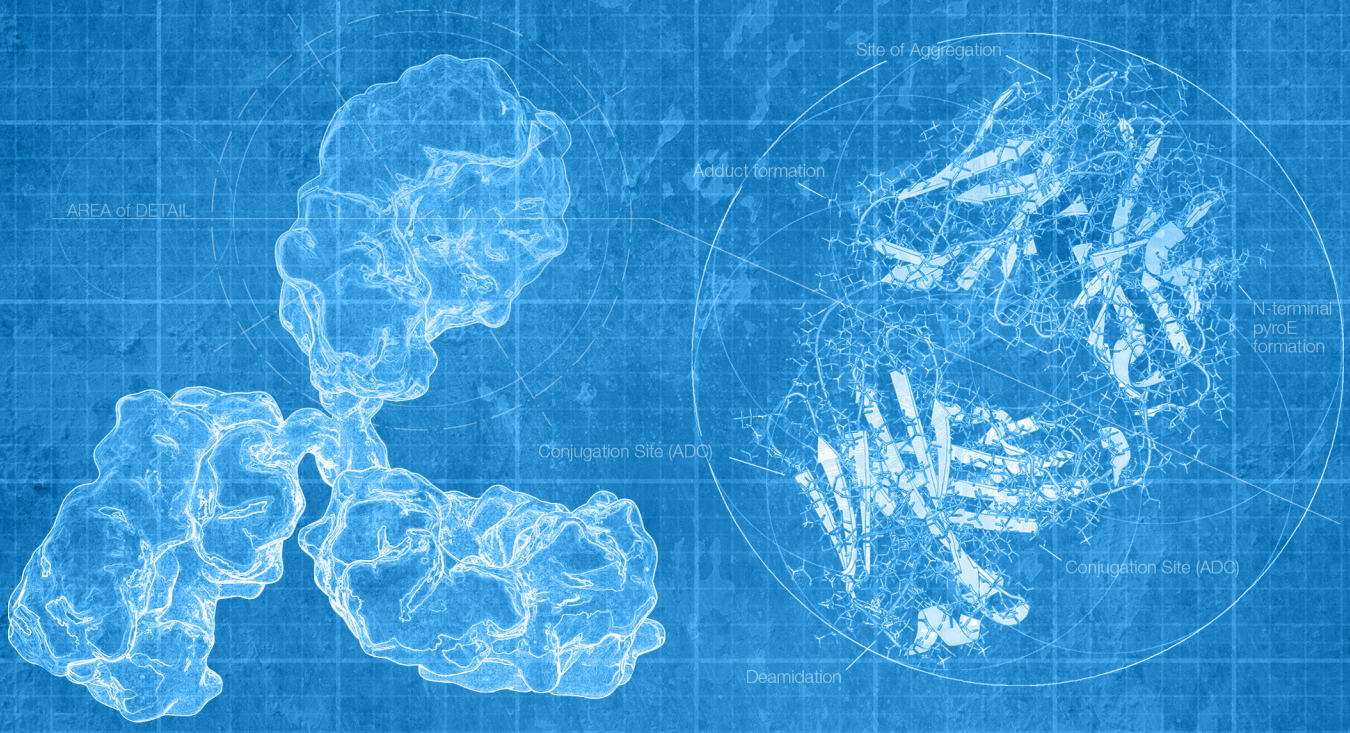

Immunoglobulin protein | ca. 150,000 Daltons | participates in the immune reaction as the antibody for a specific antigen | There are five main types: IgA, IgD, IgE, IgG, and IgM

Humanized IgG antibody fragment (Fab) | 50,000 Daltons | VH, CH1 and VL, CL regions, linked by an intramolecular disulfide bond.

STRUCTURAL INSIGHTS

BioPharma Applications Compendium

- Full Biotherapeutic Characterization
- Intact Protein Analysis
- Glycan Analysis
- Hydrogen Deuterium Exchange
- Peptide Mapping
- Antibody Drug Conjugates Analysis
- Bispecific Antibody Analysis
- Oligonucleotide Analysis

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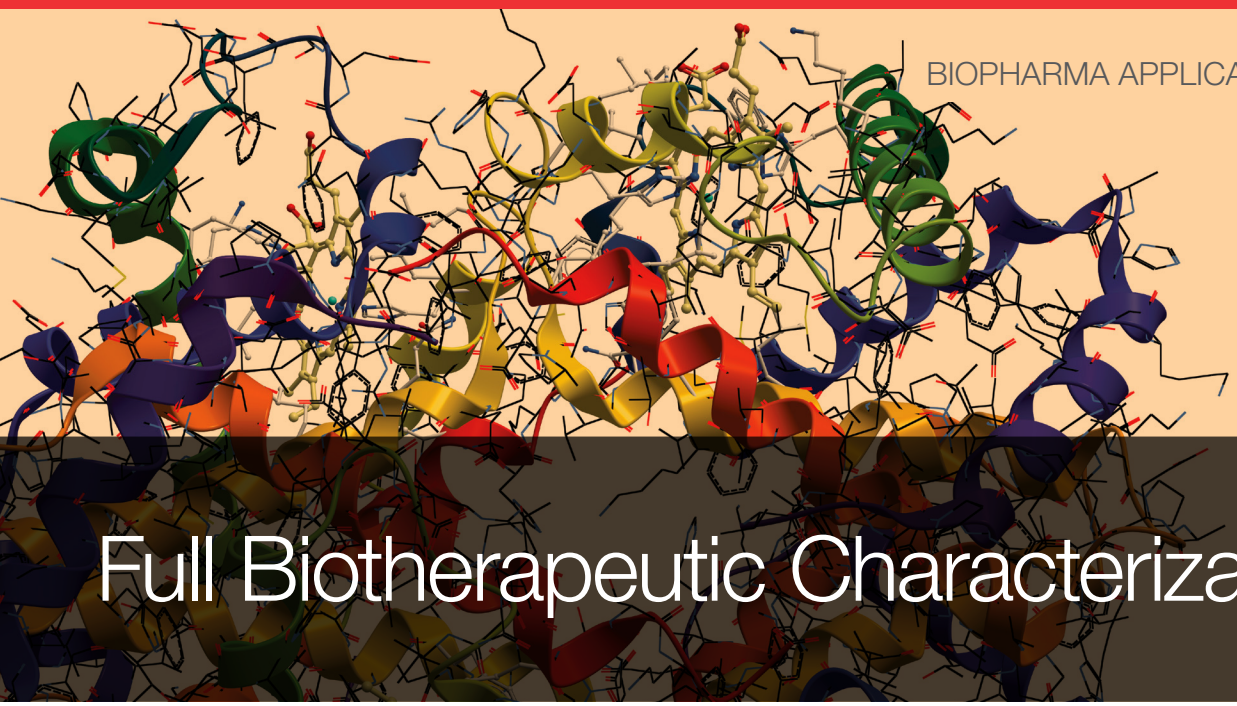
BIOPHARMA APPLICATION COMPENDIUM



Full Biotherapeutic Characterization

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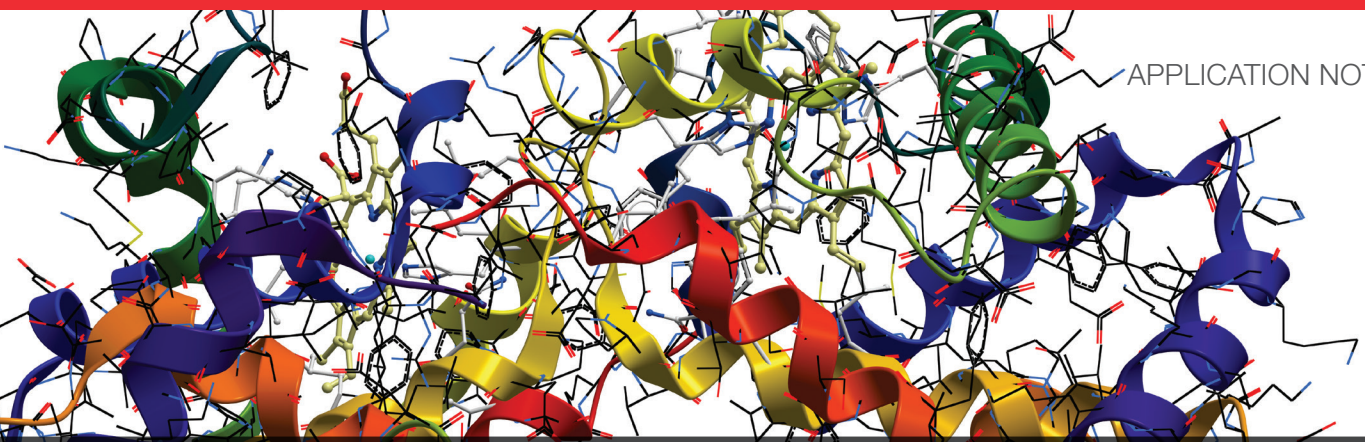

APPLICATION NOTE 21688

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

Authors

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Keywords

NIBRT, Biopharmaceutical, QA/QC, Critical quality attribute, Peptide mapping, Somatotropin, MAbPac RP, Acclaim 300 SEC, Acclaim VANQUISH C18, Magnetic SMART Digest, KingFisher Purification System, Vanquish Flex UHPLC, Q Exactive Plus MS

Application benefits

- Native and denatured intact protein analysis and peptide mapping on a single platform
- The entire workflow for the peptide mapping assay, including the digestion step, was completed in a little over one hour with minimal effort from the user
- The accurate and reproducible confirmation of the sequence with 100% sequence coverage, verification of the correct disulfide bond linkages, and quantification of several post-translational modifications
- Associated ease-of-use through automation

Goal

To demonstrate the applicability of a single Thermo Scientific™ LC-MS platform for extensive characterization of biotherapeutic proteins, by peptide mapping and intact protein analysis on the recombinant protein somatotropin.

Introduction

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biological production by living cells, there are many attributes that need to be analyzed to guarantee their safety and efficacy. This can involve multiple analytical

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techniques based on several different instrument platforms. There is an industry desire to simplify the processes, produce multi-attribute methodologies, and increase reproducibility between laboratories.[1] Here we use a single instrument platform and software with multiple characterization workflows that generate data for multiple quality controlled attributes.

Peptide mapping is one of the most important assays in the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and as a check for several post-translational modifications. High-resolution mass spectrometry is coupled to chromatography for peak identification and quantitation. Many QC methods use solely UV detection after the peak identities have been confirmed to simplify the method for a QC environment. However, UV-only data limits the attributes that can be measured and does not give absolute positive identification of the peptides. Here we show the possibility to use a simple, easy-to-implement LC-MS method with evidence of the benefits of such a system over UV-only detection.

Trypsin is the most commonly used proteolytic digestion enzyme due to its high specificity. However, many trypsin-based protocols and kits that have been developed for proteolytic digestion of proteins are labor intensive, prone to manual errors and may also introduce post-translational modifications during digestion.[2,3] Reproducible digestion is imperative for peptide mapping sample preparation, yet often leads to difficulty during method transfer. The digestion method used here is simplified and improved using immobilized heat stable trypsin.[4,5]

Intact protein analysis confirms that product with the correct molecular weight has been expressed and is an important characterization step for biotherapeutic proteins. High-resolution, accurate-mass (HRAM) Orbitrap mass spectrometry has been shown to be essential for this technique.[6] The same instrument platform, incorporating an easily automated change of columns and separation gradient, was used for the peptide mapping analysis. The intact molecular weight analysis was performed under native and denaturing conditions at high resolution to give isotopically resolved mass spectra.

Somatotropin is a small recombinant biotherapeutic protein used here as a model protein to describe the use of a new improved single instrument platform for extensive protein characterization analysis.

It is essential to detect, characterize, and quantify any undesirable modifications and confirm the correct product identity of recombinant proteins. In this application note, we demonstrate that typical protocols used for this type of characterization can be simplified and made more reproducible with new workflows performed on the same system. Peptide mapping is the most common analytical method employed for this purpose and delivers a wealth of information from correct amino acid sequence to the presence, location, and quantification of several post-translational modifications. Multiple quality attributes can be defined by peptide mapping analysis. Although a widely accepted and powerful technique, the digestion protocols for sample preparation are labor intensive and prone to manual errors and unwanted modifications. This can affect the quality of the analytical data and creates a source of irreproducibility. Incomplete digestion may render the accurate quantification of modifications impossible, however, a small amount of missed cleavage may enable 100% sequence coverage. A careful balance of digestion completion needs to be maintained in a very reproducible manner.

The Thermo Scientific™ SMART Digest™ kit was used for the sample preparation for peptide mapping analysis. This protocol greatly simplifies the digestion process and increases reproducibility. Intact protein analysis is a complimentary technique used to ensure the correct molecular weight of the protein biotherapeutic. Both these techniques can be performed on the same analytical platform with no change in the eluents used for chromatography. The Thermo Scientific™ BioPharma Finder™ software combines the identification and quantitation tools for peptide mapping with the deconvolution software used for intact protein analysis. Use of the described workflows on a single platform for this extensive characterization easily facilitates implementation in the laboratory.

Experimental

A Thermo Scientific™ Vanquish™ Flex UHPLC system connected to a Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer equipped with the BioPharma option was the LC-MS platform used for the analysis. The same system was utilized for the intact protein analysis, which was performed under both native and denaturing conditions with the data for all techniques analyzed using the both native and denaturing conditions. The data for all techniques was analyzed using BioPharma Finder 2.0 software.

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Ammonium acetate LC-MS grade (P/N A11450)
- Fisher Scientific TCEP Tris [2-carboxyethyl]phosphine (P/N 20490)
- SMART Digest Kit (P/N 60109-101)
- Fisher Scientific LC-MS grade water (P/N W/011217)
- LC-MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ formic acid LC-MS grade (P/N 28905)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ MAbPac™ RP column, 4 µm, 2.1 × 100 mm (P/N 088647)
- Thermo Scientific™ Acclaim™ SEC-300 column, 5µm, 4.6 x 300 mm (P/N 079723)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Sample pre-treatment

Somatotropin dry stock was made to a final concentration of 5 mg/mL in water with gentle swirling to aid in solubilization.

Digestion for peptide mapping

The sample was submitted to the SMART Digest protocol: 250 µg of somatotropin in 50 µL formulation buffer (5 mg/mL) was diluted to 200 µL with SMART Digest buffer. This was incubated for 5 and 15 minutes at 70 °C and 1400 rpm in an Eppendorf ThermoMixer®. The immobilized trypsin beads were then removed by spinning down in a micro-centrifuge for 5 minutes at 1,000g. The sample was gently removed and the supernatant carefully aspirated from the beads. The sample was split in half. One aliquot was reduced by adding DTT to a final concentration of 5 mM and incubated at 25 °C for 30 min. The other half remained untreated and was used for the detection of disulfide-bridged peptides and disulfide bond analysis.

Separation conditions

Instrumentation

Thermo Scientific Vanquish Flex Quaternary UHPLC system equipped with:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Mobile phase

Eluent A:	Water + 0.1% formic acid
Eluent B:	80% acetonitrile + 0.1% formic acid
Eluent C:	50 mM ammonium acetate
Flow rate:	0.3 mL/min
Column temperature:	70 °C
UV:	214 nm
Run conditions:	Tables 1, 2, and 3

Table 1. Gradient for peptide mapping with the Acclaim VANQUISH C18 column.

Retention time (min)	Flow (mL/min)	%B
0.0	0.3	4.0
30.0	0.3	70.0
31.0	0.3	100.0
34.0	0.3	100.0
35.0	0.3	4.0
60.0	0.3	4.0

Table 2. Gradient for intact protein analysis under denaturing conditions with the MAbPac RP column.

Retention time (min)	Flow (mL/min)	%B
0.0	0.3	10
0.1	0.3	10
10.0	0.3	55
10.1	0.3	90
11.0	0.3	90
11.1	0.3	10
15.0	0.3	10

Table 3. Run conditions for native intact protein analysis with the Acclaim SEC-300 column.

Flow (mL/min)	%C
0.25	100

MS conditions

Table 4. Q Exactive BioPharma MS parameter settings.

Parameter	Peptide mapping	Intact native/ denaturing conditions
Source probe	HESI II	HESI II
Polarity	Positive	Positive
Spray voltage	4.0 kV	4.0 kV
Vaporizer temp.	300 °C	320 °C/300 °C
Sheath gas flow rate [arb. units]	45	25/25
Auxiliary gas flow rate [arb. units]	12	10/5
Capillary temp.	320 °C	275 °C
Resolution (Full MS/MS^2)	60k/15k	120k/240k
Top-N MS^2	5	n.a.
S-lens RF level	50	80
Max inject time (Full MS/MS^2)	100 ms/200 ms	150 ms

Data processing

BioPharma Finder 2.0 software was used for analysis of all data acquired on the peptide and protein level. For deconvolution of isotopically resolved mass spectra of the intact proteins under native and denaturing conditions, the Xtract algorithm was used with a signal-to-noise threshold of 2, a fit factor of 80%, and a remainder of 25%.

For peptide mapping, searches were performed using a single-entry protein FASTA database with oxidation and deamidation set as variable modifications, 5 ppm mass accuracy, and a confidence level of 0.8 for MS/MS spectra.

Results and discussion

The sequence for somatotropin outlined in Figure 1 shows the expected cleavage positions for trypsin in red and the position for the disulfide bond linkages in yellow. This protein represents a good model system for peptide mapping and intact protein analysis with 18 peptides expected of varying size and two disulfide bond linkages (20 peptides when reduced). There are also several sites available for possible post-translational modifications by deamidation, isomerization, and oxidation.

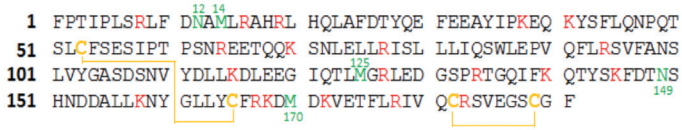

Figure 1. Amino acid sequence of somatotropin with cysteine residues involved in disulfide bonds marked in yellow and asparagines and methionine residues prone for oxidation and deamination marked in green.

Peptide Mapping

The samples obtained after performing digestion with and without subsequent reduction were subjected to LC-MS analysis under the conditions outlined above. The base peak chromatograms obtained for the reduced and unreduced samples are shown in Figure 2.

The chromatogram of the non-reduced somatotropin digest sample shows two extra peaks with retention times of 7.96 min and 16.50 min labelled with a black star in the upper panel of Figure 2. These correspond to the two disulfide-linked peptides present in the native somatotropin. The SMART Digest protocol does not use upfront reduction or alkylation as the proteins are digested in heat denaturing conditions at 70 °C. Thus, the disulfide linked peptides still maintain the covalent

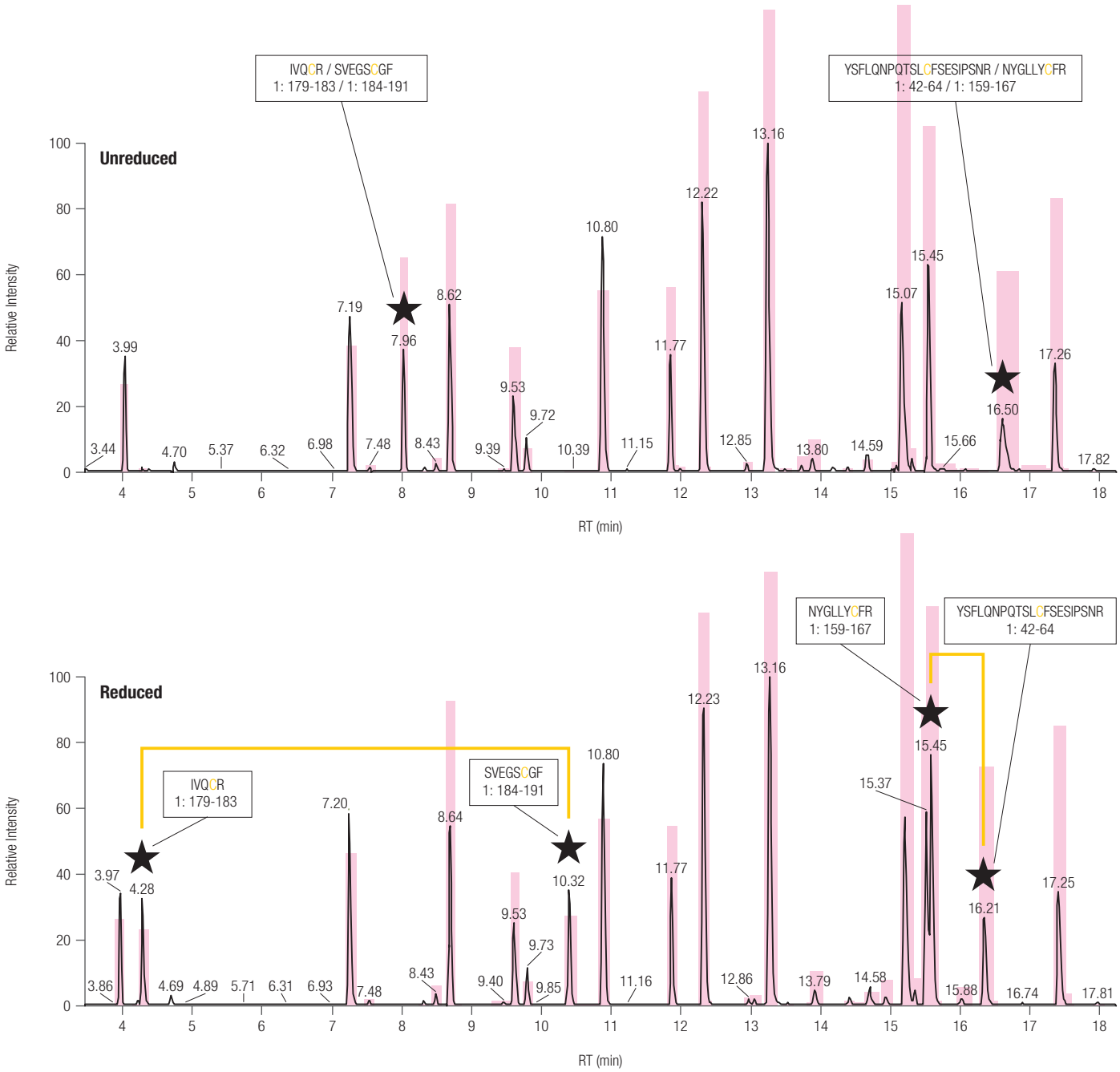

Figure 2. Base peak chromatograms obtained for the unreduced (top) and reduced (bottom) somatotropin trypsin digest. Cysteine containing peptides are labelled with black stars indicating four disconnected peptides in the reduced sample and two peptides in the unreduced sample bearing disulfide bonds.

linkage following the digestion procedure. This linkage can be broken by reduction with DTT at the peptide level following digestion and the effects are visualized in the bottom panel of Figure 2. The two peaks representing the disulfide-linked peptides in the chromatogram of the non-reduced sample disappear with the subsequent appearance of four additional peptides that are not initially observed in the non-reduced sample. The released peptides from the broken disulfide bonds are marked in the lower panel with stars. The peptides at retention times 4.28 min and 10.32 min relate to one disulfide linkage, and the peptides at retention times 15.45 min and 16.21 min relate to the other. The direct confirmation of the disulfide bond linkages is given by the BioPharma Finder software, which can identify the presence of the linked peptides in the non-reduced digestion by accurate parent ion mass alone. Further verification of the di-peptide can be achieved based on MS^2 spectra. This is an advantage of the described workflow, as an effective digestion of the unreduced protein can prove difficult to achieve with some proteins using standard in-solution digestion protocols.

The disulfide bond assignments in Figure 3 of the chromatogram are marked with a star for clarity. Unlike the other peptides that have a peak label indicating the position of the peptide in the sequence, the peak label for the disulfide-linked peptides show sequence positions for both peptides. The first to elute contains the tryptic peptides at positions 179-183 linked to the peptide at position 184-191. The second has 159-167 and 42-64 linked together by a disulfide bond. This is also shown in the sequence coverage map highlighted with yellow and red boxes, respectively. The yellow boxes indicate the peptides in the first eluting disulfide-linked peptide. The retention times indicate that they are eluting at the same retention time of 8.0 min due to the covalent linkage connecting them together. The last eluting disulfide-linked peptides are marked with a red box showing the identical elution time of 16.5 min. BioPharma Finder has this disulfide bond assignment method as a default choice from the menu. The results were obtained without any further manipulation of the

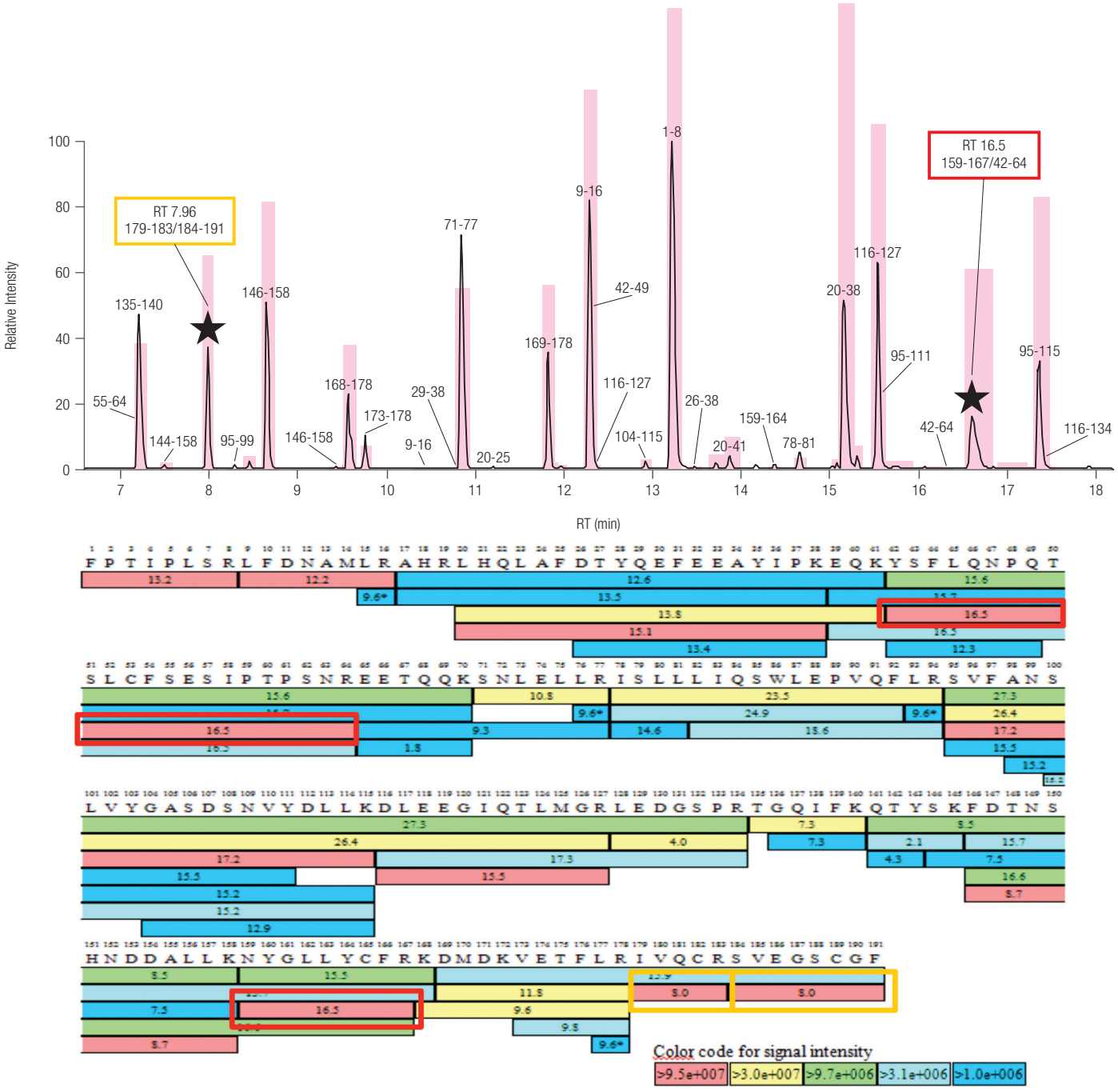

Figure 3. Assignment of peaks in the chromatogram representing peptides with intact disulfide bonds and location of the peptides involved in disulfide bonds in the protein sequence highlighted with boxes in red for the peptide aa 159-167/42-64 and with boxes in yellow for the linked aa peptides 179-183/184-191.

disulfide bond default method. Using the standard built-in methods sequence coverage of 100% was achieved for the peptide map of somatotropin without reduction of the digested peptides.

Proteins	Number of MS peaks	MS peak area	Sequence coverage	Abundance (mol)
Somatotropin	457	90.9%	100.0%	100.0%
Unidentified	14	9.1%		

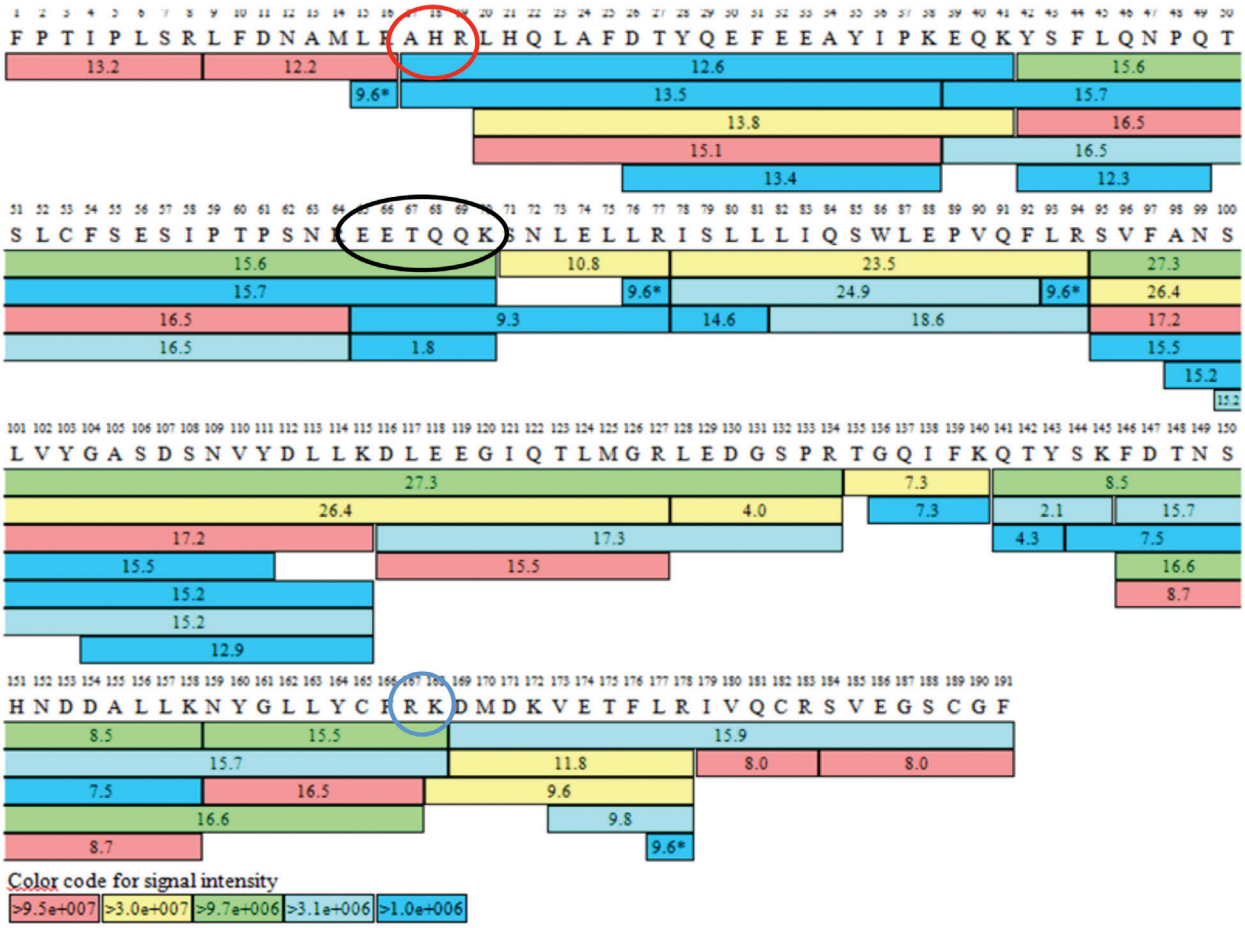

Figure 4. Sequence coverage map for somatotropin showing 100% coverage. Full MS and MS/MS data were used. The bars underneath the sequence represent peptides with colors indicating the precursor intensities and the numbers in the bars representing the retention time.

A small amount of a missed cleavage product is required to see a very hydrophilic peptide EETQQK, which is circled in black in Figure 4. The full cleavage product is difficult to see without a high sensitivity MS system and impossible with UV detection only, as it does not ionize well and elutes in the column void at a retention time of 1.8 min (Figure 4). Another short peptide is circled in red with the sequence AHR, which is also identified by a very low abundant missed cleavage product. The full cleavage product elutes very early in the column void at 1.3 min but does not ionize well at all (Figure 4). If the digestion time is increased to 60 minutes, full digestion occurs at this site and the low level missed cleavage product used in the confirmation of the AHR peptide sequence is lost (data not shown). Somatotropin also has two trypsin cleavage sites adjacent to each other at positions R167 and K168. This is circled in blue in Figure 4 with the two resulting abundant peptides eluting at 9.6 min and 11.8 min (yellow bars). Both peptides contain an unusually abundant missed cleavage site at position K172, a position just after the adjacent R167 and K168 sites and in front of a methionine at M170, which is susceptible to oxidation (Table 5). This missed cleavage is possibly due to the acidic nature of the glutamate and

aspartate residues near the cleavage site. The other trypsin cleavage sites throughout the somatotropin sequence are cleaved perfectly using the SMART Digest kit, showing high abundant peptides that are easily identified with BioPharma Finder software and can be converted to a method using UV absorption only for detection.

An additional benefit of the LCMS methodology is the identification of post-translational modifications. There are several sites in the sequence that could be prone to deamidation, oxidation, and isomerization of aspartic acid. These can be identified in BioPharma Finder software by simply adding the modifications that are interesting from a built-in list to the sequence manager. In this case, we looked for all the above modifications in the somatotropin sample. Results are indicated in Table 5 for a 5 and 15 minute trypsin digest using the SMART Digest kit.

Table 5. Post-translational modifications identified and quantified from the somatotropin SMART Digest samples after 5 min and 15 min digestion time.

Position	Sequence	Retention time [mod./unmod.]	Modification	% (5 min digest)	% (15 min digest)
N149	FDTNSHNDDALLK	9.43 / 8.68	Deamidation	1.05	1.64
M170	DMDKVETFLR	9.58 / 10.31	Oxidation	0.59	0.60
M14	LFDNAMLR	10.41 / 12.28	Oxidation	1.52	1.34
M125	DLEEGIQTLMGR	12.40 / 15.48	Oxidation	0.18	0.21
D130	LEDGSPR	3.95 / 4.06	Isomerization	0.18	0.55

There is one deamidation site, three oxidation sites, and one isomerization site identified, all at very low levels. The low levels alone would make these modifications difficult to impossible to quantify by UV only, especially in the presence of significant numbers of high abundant peptides. In addition, the isomerization site modified peptide did not separate well from the unmodified form at the peptide level with the short reversed-phase gradient chromatography used in this example. This would make their identification and quantification impossible by UV detection only with the chromatographic conditions applied. The mass differences for isomerization and deamidation modifications are also very small, which makes the use of high-resolution mass spectrometry the correct choice for confident results.

The results in Table 5 show a small increase in the levels of deamidation and isomerization modifications with time, and this has been noticed previously.[2,4] This increase is still relatively small over the digestion period with the SMART Digest Kit even if digestion times are increased to 60 minutes.[4] Optimization of the time of digestion should be considered, however, to minimize any digestion-induced modifications. For somatotropin, a 15 minute digestion seems optimal, providing a good balance between complete digestion suitable also for quantification while keeping induced modifications to a minimum.

In addition, a robust and complete digestion process is critical for the identification of modifications using high-resolution, accurate-mass (HRAM) mass spectrometry. If complete digestion is not achieved then missed cleavages around the modification site will lead to more than one peptide that contains the same modification eluting at different positions, making accurate quantification difficult or impossible. Table 6 shows the effects of a short digestion time of 5 minutes, creating a small level of an additional missed cleavage peptide for the deamidation at N149. A digestion site in front of the peptide has not been cleaved, creating an additional longer peptide with five additional amino acids, QTYSK, at the front of the sequence. In this example, the SMART Digest procedure still gives a credible result as the missed cleavage product is only present in relatively small amounts. This is made more important as the percentage levels of the deamidation product in the missed cleavage peptides are shown here to be different. An incomplete digestion would lead to several peptide products containing the same modification at different levels with the modified and unmodified peptides for deamidation all eluting at different retention times. This applies considerable pressure on obtaining an efficient reliable digestion for the assay. With less predictable and difficult to use digestion procedures this will cause a problem with accurate reproducible quantification.

Table 6. Quantification result of the deamidation level of asparagine at position 149 obtained from a sample after 5 min digestion time.
Two peptides are shown, one of which includes a missed cleavage site.

Position	Sequence	Retention time	Modification	%
Native	FDTNSHNDDALLK	8.69	None	
N149	FDTNSHNDDALLK	9.43	Deamidation	1.05
Native	QTYSKFDTNSHNDDALLK	8.48	None	
N149	QTYSKFDTNSHNDDALLK	9.11	Deamidation	1.69
			Average	0.99

In this series of experiments, the procedure using the SMART Digest kit was found to be extremely reproducible and efficient. Optimization is simple to obtain a complete digest that gives a small enough number of missed cleavages to help with sequence coverage but not enough not to interfere with the accurate determination of modifications.

The use of HRAM MS can be daunting to implement in the standard QC environment where HPLC systems with UV or fluorescence detection are the standard instruments and there is often little experience in high-end mass spectrometry. In this setting, a simple high-resolution MS-only instrument can be implemented in much the same way as another detector for the HPLC system. This adds a detector with minimal functional settings to give an additional mass trace in the chromatogram running under the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software. The additional online mass analysis enables all the advantages of quantification of some post-translational modifications as well as positive identification of the peptide sequence as demonstrated above. Figure 5 shows a peptide map of somatotropin with identification of the peptides using the intact peptide mass as measured in the MS1 scan only. Confident identification can be done in the Chromeleon CDS software, combining accurate mass and retention time with this simple system configuration. In the example in Figure 5, sequence coverage of 100% was achieved with standard settings in BioPharma Finder software. The peptides produced from the workflow give high abundant (red and yellow shading), perfectly cleaved tryptic peptides making identification easy. Missed cleavage peptides are in very low abundance (blue shading) and do not interfere with the quantitative analysis of the modifications.

Proteins	Number of MS peaks	MS peak area	Sequence coverage	Abundance (mol)
Somatotropin	205	89.6%	100.0%	100.0%
Unidentified	4	10.4%		

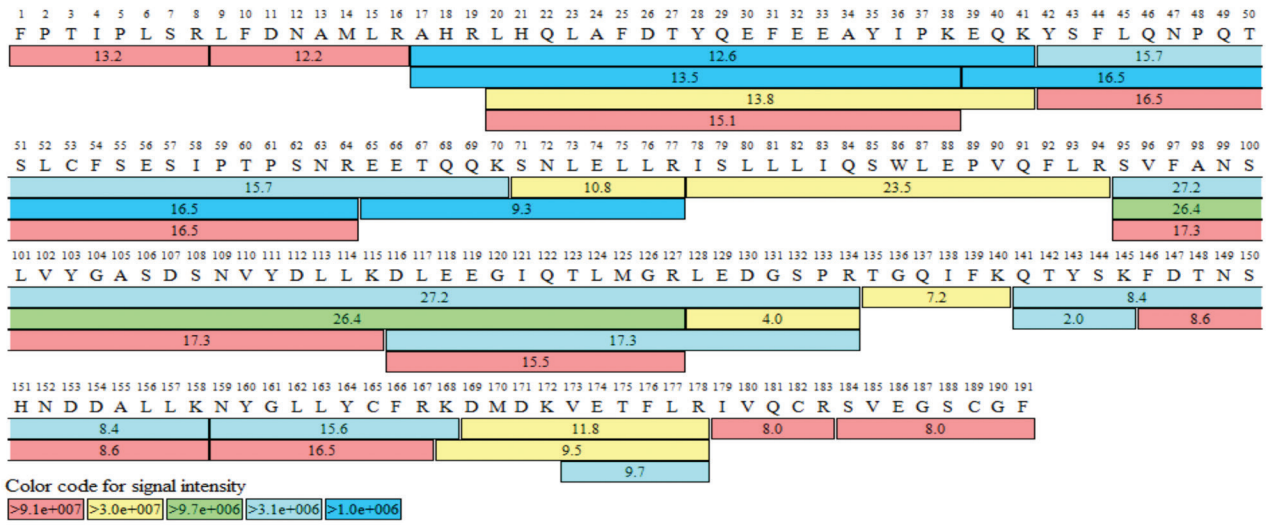

Figure 5. Sequence coverage map of somatotropin of identified peptides based on MS full scan data only.

The same system configuration can be used for denatured intact protein analysis as for the peptide mapping analysis, with a simple column change that may be performed automatically with a column change valve. The column of choice for intact mass analysis is the MAbPac RP column, a polymeric column with large pore size that exhibits high resolution of intact proteins with very little to no carryover. The eluents are the same for both analyses, with a slightly different gradient in the chosen intact analysis chromatography method. The example in Figure 6 shows the intact analysis of somatotropin with the deconvoluted monoisotopic mass at 22,111.0920 Da. The mass difference of ~4 Daltons corresponds to the four missing hydrogens lost from the cysteine residues involved in the two disulfide bonds, which were still intact in this sample. The theoretical mass with the disulfide bonds intact is 22,111.0409 Da, which is with 2.31 ppm deviation in very close agreement with the experimental value. The excellent resolution provided by the Orbitrap mass analyzer of this relatively small protein can easily be isotopically resolved at a resolution setting of 120,000. The charge envelope obtained for somatotropin analyzed under denaturing conditions spans charge states from 10 to 20 with the most abundant charge state detected at *m/z* 1,476.

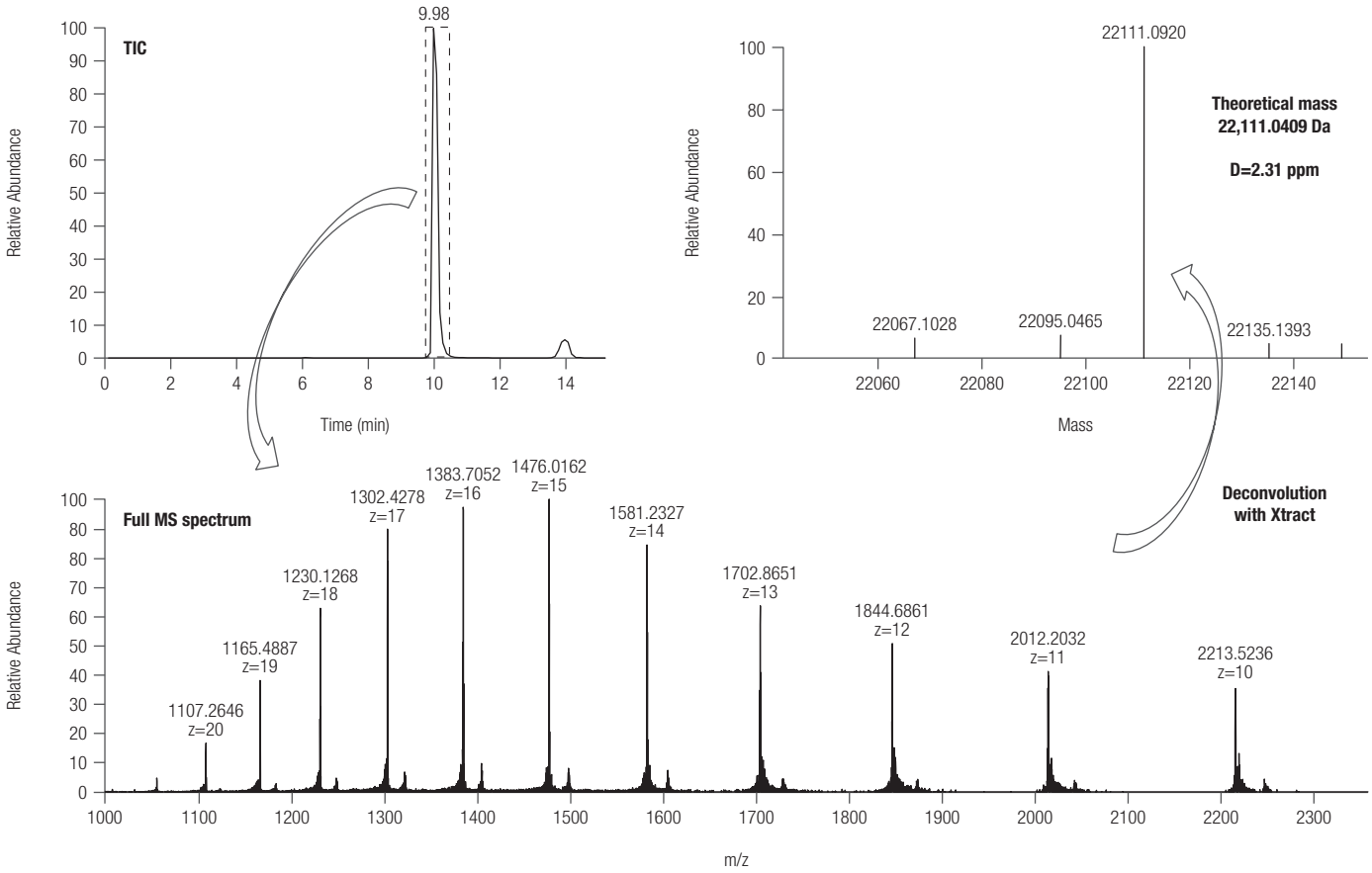

Figure 6. Mass analysis of intact somatotropin under denaturing conditions showing the total ion chromatogram, the Full MS spectrum, and the mass obtained after deconvolution with Xtract.

The mass of the native protein can also be characterized using this instrument platform. The analysis in the native form can give additional information that is not available after the protein is denatured. Binding characteristics in the native form and any higher-order structural variations can be studied with this approach. As the protein will be in its native folded state, the charges available on the surface of the protein are much lower in number than in the fully unfolded, denatured state, resulting in a reduced charge distribution on the protein and so the mass-to-charge ratio elevates to a higher *m/z* range. The reduced charge distribution can help with spectral resolution between modifications that could overlap with different variants from different charge states. For this analysis, an Acclaim SEC-300 column was utilized to introduce the intact protein to the MS, while maintaining low salt concentration. This is a polymeric size-exclusion chromatography (SEC) column that shows very little secondary interaction with proteins even at low salt eluent conditions. This was preferred over a silica-

based SEC column as it requires less salt in the eluent system to maintain excellent chromatography under size-exclusion conditions. The low salt concentration is beneficial to the mass spectrometry system for reduction of the background signal and greatly reduces the signal suppression inherent with salt gradients, resulting in overall increased sensitivity in the MS.

Figure 7 shows the base peak chromatogram, the full MS spectrum with a zoom into the most abundant charge state at *m/z* 2459.2446, and the deconvoluted spectrum for somatotropin acquired under native conditions. The distribution shows a smaller number and a shift to lower charge states due to the native form of the protein that is amenable to accepting fewer charges when compared to the analysis under denaturing conditions. The monoisotopic mass was calculated at 22,111.0754 Da, (1.56 ppm mass deviation) also in excellent agreement to the expected mass for somatotropin with the disulfide bonds intact.

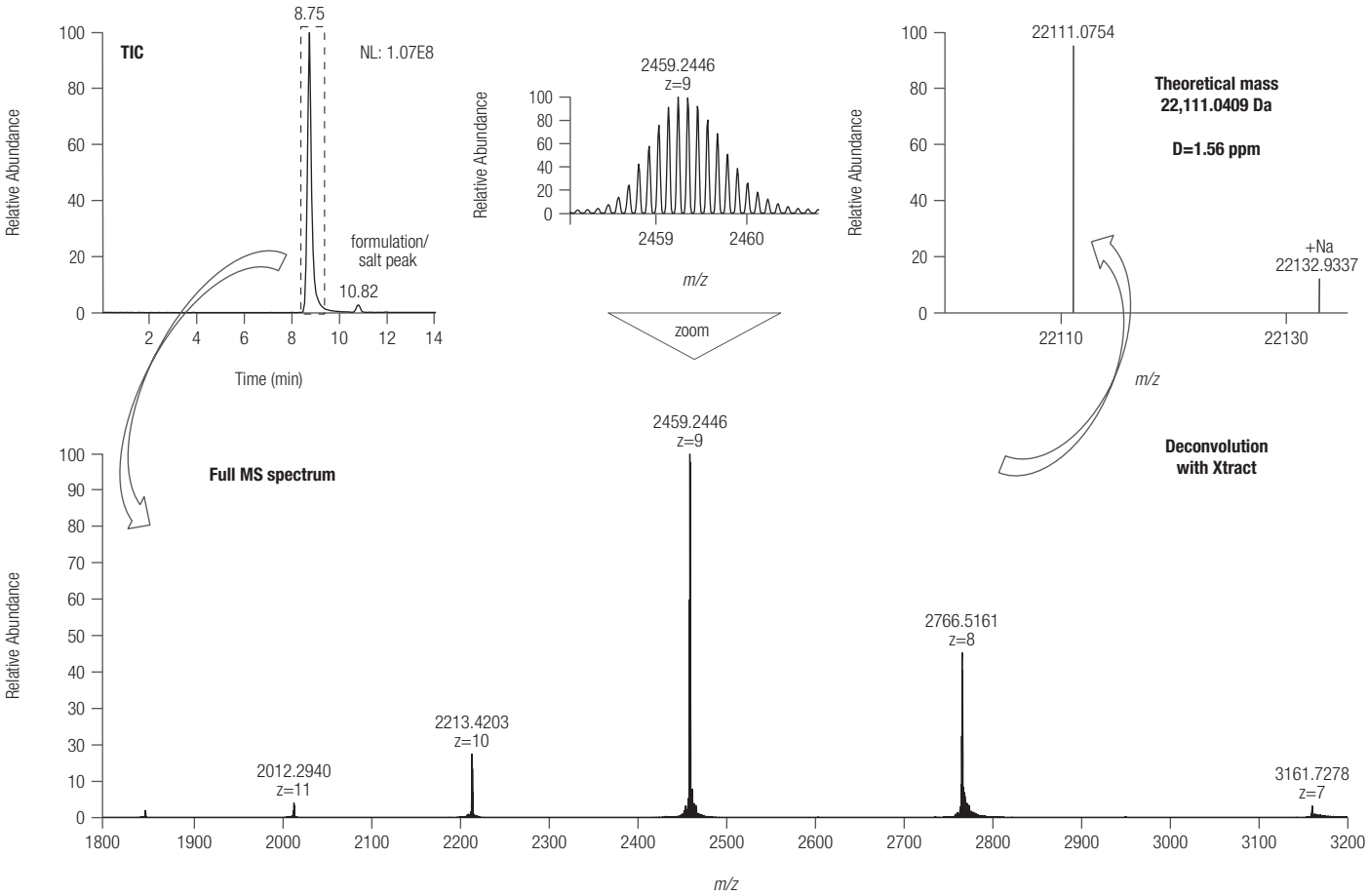

Figure 7. Analysis of intact somatotropin under native conditions showing the total ion chromatogram, the Full MS spectrum with a zoom into the most abundant charge state, and the mass obtained after deconvolution with Xtract.

Conclusions

An extensive characterization of the biotherapeutic protein somatotropin was successfully achieved using a single instrument platform. The peptide mapping workflow was simplified with excellent and reproducible data generated using the SMART Digest workflow, which showed consistent and complete digestion and good specificity for cleavage at the correct trypsin cleavage sites. This produced a simple peptide map that was easy to interpret using the BioPharma Finder software. The digestion efficiency and reproducibility was shown to be an important factor for the combination of total sequence coverage and accurate determination of posttranslational modifications. The information from the peptide mapping experiment included the following:

- Confirmation of the sequence with 100% sequence coverage
- Verification of the correct disulfide bond linkages
- Quantification of several post-translational modifications

The accuracy and reproducibility of the procedure using the SMART Digest kit allows the user to simply modify the digestion by time to allow the production of small amounts of missed cleavage products that may be required for complete sequence coverage and ensure correct conditions for complete digestion to allow accurate measurement of post-translational modifications. This is very difficult to achieve reproducibly with other digestion techniques.

The entire workflow for the peptide mapping assay, including the digestion step, could be accomplished in a little over one hour with minimal effort from the user. This assay could be implemented in a routine environment with little prior knowledge or experience in mass spectrometry using Chromeleon software to control the entire LC-MS system with the acquisition of Full MS data only.

In addition to the information from peptide mapping experiments, the verification of the correct monoisotopic mass of the intact protein based on isotopically resolved mass spectra can be done using the same system configuration. This can be achieved by analyzing the protein in the native or denatured state.

The described instrument platform and workflows show the possibilities for routine use to measure several critical quality attributes in routine characterization of biotherapeutics. The system configuration of UHPLC/MS with BioPharma Finder software has proven to be a simple, versatile, and powerful platform for the analysis of somatotropin, which could be extended to any biotherapeutic protein product.

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Acknowledgement

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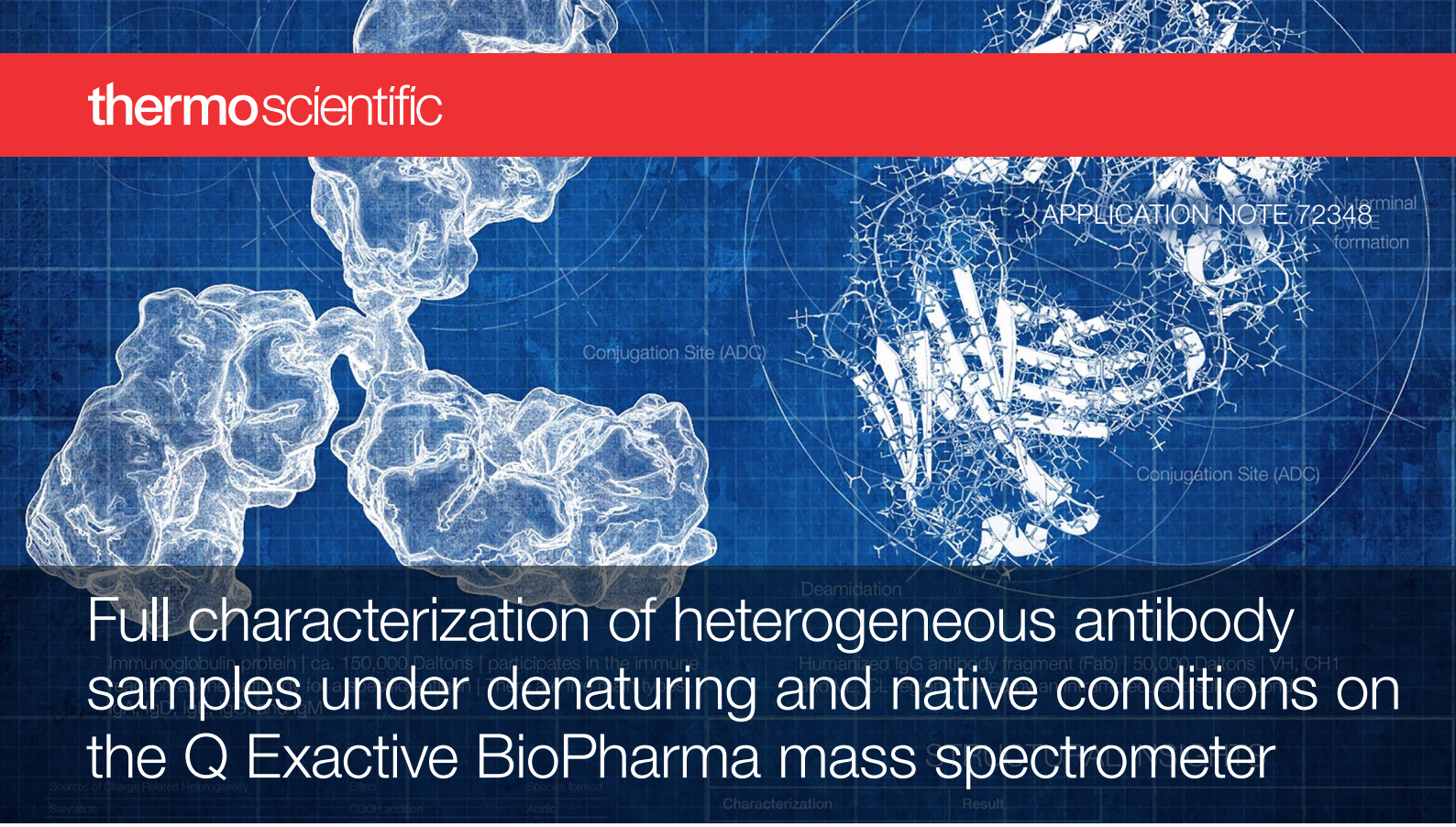

Full characterization of heterogeneous antibody samples under denaturing and native conditions on the Q Exactive BioPharma mass spectrometer

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Keywords
Q Exactive BioPharma, antibody characterization, HMR Mode, Protein Mode, native conditions

Goal

To demonstrate the capabilities of the Thermo Scientific™ Q Exactive™ BioPharma mass spectrometry platform, particularly the High Mass Range (HMR) Mode for the characterization of antibody samples. The three different operational modes of the BioPharma option are described and application data are presented for the major application workflows.

Introduction

MS analysis of antibodies at the protein and peptide levels is critical during development and production of biopharmaceuticals. The compositions of current generation therapeutic proteins are often complex due to their heterogeneity caused by various modifications that are relevant for their efficacy. Intact proteins analyzed by ESI-MS are detected in higher charge states that also provide more complexity in mass spectra.

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Analysis of proteins in native or native-like conditions with zero or minimal organic solvents and neutral or weakly acidic pH can allow proteins to preserve non-covalent interactions and retain high degrees of folding. This effect has analytical benefits: greater protein folding leads to reduced charge states, increased mass separation, and increased signal at higher *m/z*. This strategy has been utilized for the analysis of antibodies and antibody drug conjugates present in highly complex mixtures of different antibody/drug combinations.[1] Requirements for performing native MS on antibody samples include scanning towards 8,000 *m/z* and increased transmission optimization for large compounds. This feature has so far only been available on the Thermo Scientific™ Exactive™ Plus EMR mass spectrometer. Here are shown the results obtained after successful implementation of the HMR Mode as part of the BioPharma Option now available on both the Thermo Scientific™ Q Exactive™ Plus and Q Exactive HF™ mass spectrometers aimed at adding the capability to perform native MS analysis with mass detection up to 8,000 *m/z* without compromising performance of normal operation modes. These enhanced capabilities are necessary for the analysis of antibody samples on the intact level under native conditions requiring the detection of masses beyond the standard mass range of up to 6,000 *m/z*.

The BioPharma Option adds superior denatured and native MS intact mass analysis and subunit top/middle-down analysis capabilities to the most powerful benchtop peptide mapping instruments available. The BioPharma Option offers distinct operational modes that have been optimized for the top three protein characterization workflows:

- Intact mass analysis under native and denaturing conditions with the new High Mass Range Mode
- Subunit and top/middle-down analysis with Protein Mode
- Peptide mapping with Standard Mode

For the Q Exactive Plus mass spectrometer, the BioPharma Option includes: Standard Mode, Protein Mode, Enhanced Resolution Mode with resolution up to 280,000 @ *m/z* 200, and the High Mass Range Mode with extended mass range up to *m/z* 8,000. For the Q Exactive HF mass spectrometer, the BioPharma Option includes: Standard Mode, Protein Mode, and High Mass Range Mode with extended mass range up to *m/z* 8,000.

The increase of the upper mass range on the mass spectrometers was achieved by implementing instrument control software changes. The analysis of molecules across the full mass range including the detection of proteins under native conditions required the use of optimized parameter settings to ensure efficient desolvation in the front region of the instrument, efficient transfer via multipoles, efficient trapping in the C-trap/HCD region, and sensitive injection and detection in the Orbitrap™ mass analyzer. Critical parameters include the optimization of in-source fragmentation that strongly influences the support of the desolvation process. Also, for transmission efficiency, specific voltages have been evaluated and optimized to ensure robust and sensitive performance in the higher mass range when performing analyses under native conditions, experiments that have not been possible so far on this type of mass spectrometer. Additionally, the standard calibration routine previously used was modified and adapted to ensure high mass accuracy across the full mass range. The addition of the BioPharma Option to the Q Exactive Plus and Q Exactive HF mass spectrometers does not compromise the performance of the instruments in any way but rather extends it. That is, small molecule applications always run in Standard Mode can be performed with the same level of sensitivity as on instruments not equipped with the add-on option.

The profiles of three monoclonal antibodies, trastuzumab, bevacizumab, and infliximab, have been analyzed on the intact protein level under denaturing and native conditions as well as on the peptide level upon digestion with the Thermo Scientific™ SMART™ Digest kit using the three workflows laid out in Figure 1.

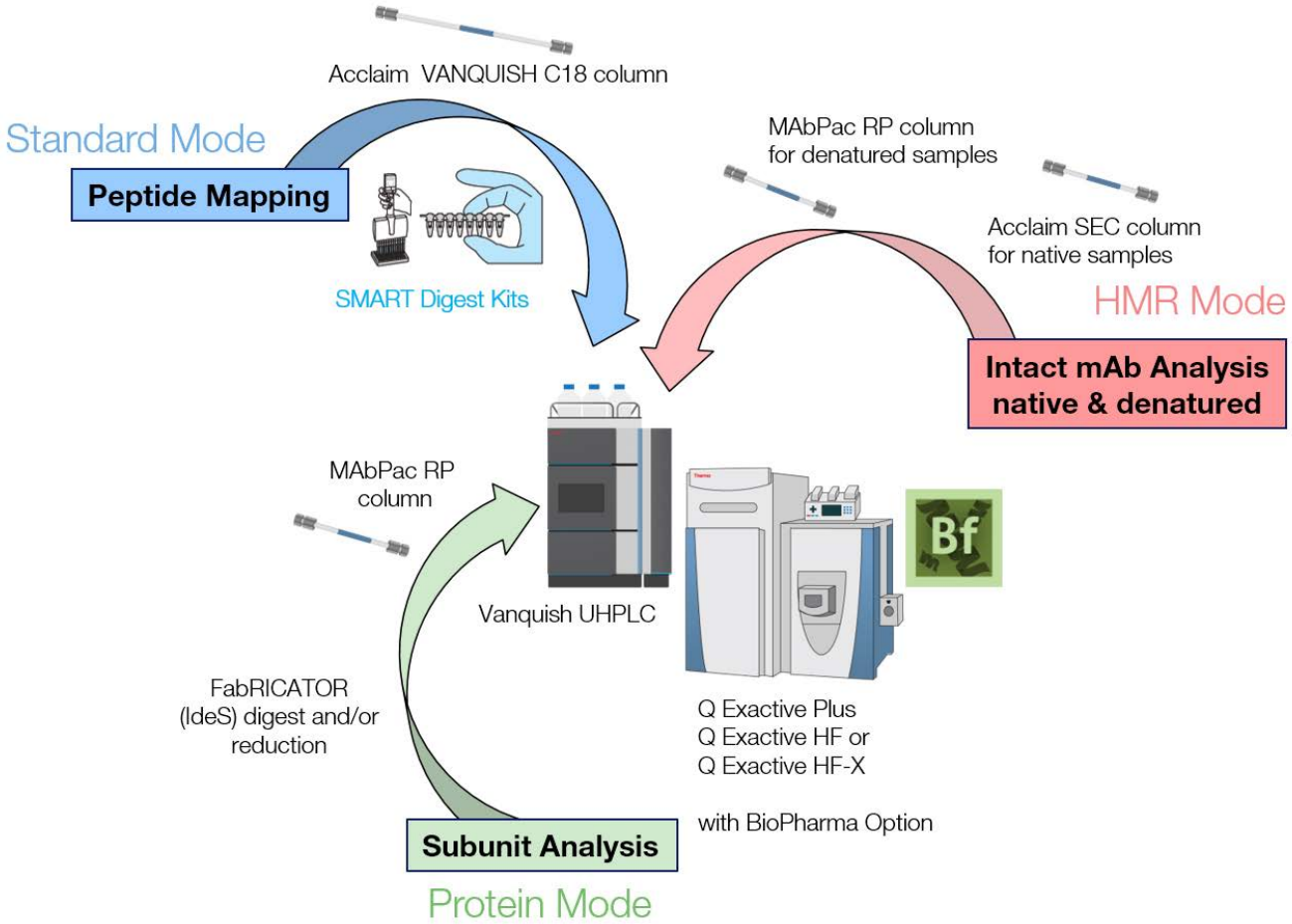


Figure 1. The Q Exactive Plus and Q Exactive HF mass spectrometers equipped with the BioPharma Option provide three different modes to cover the three major workflows in BioPharma.

The data collected on the different types of samples and presented in this study demonstrate the successful analysis after implementation of the HMR Mode, successful desolvation, and optimized critical hardware operation settings. This makes the instrument an ideal platform to cover the three major workflows in the BioPharma Option: intact mass analysis under denaturing and under native conditions in HMR Mode, subunit analysis (reduced mAb and/or IdeS digested mAb) on the MS^1 and MS^2 level in Protein Mode, and lastly peptide mapping in Standard Mode.

Experimental

Sample Preparation

The three commercially available monoclonal antibodies trastuzumab (tradename Herceptin™), infliximab (tradename Remicade™) and bevacizumab (tradename Avastin™) obtained in manufacturer's formulation buffer were used for all experiments.

Intact mAb analysis

For intact mass analysis under native conditions using size exclusion chromatography (SEC)-LC-MS, the antibodies were injected without any further dilution. For LC-MS analysis under denaturing conditions, antibody samples were diluted in 0.1% formic acid. For direct infusion experiments applying denaturing conditions, samples were desalted via Bio-Rad™ P6 desalting columns and diluted to achieve 50% acetonitrile/0.1% formic acid in the solvent.

Subunit analysis

For subunit analysis samples were reduced in 4 M guanidine hydrochloride (GdHCl)/50 mM tris(2-carboxyethyl)phosphine (TCEP) and incubated at 57 °C for 45 min. For LC, Fc/2, Fd', $F(ab')_2$ subunit analysis, samples were first digested with FabRICATOR™ (Genovis) enzyme according to the manufacturer's protocol to obtain the Fc/2 and $F(ab')_2$ subunits and in a subsequent step reduction was performed using 4 M GdHCl/50 mM TCEP and incubated at 57 °C for 45 min. FabRICATOR is also commonly known as IdeS (immunoglobulin-degrading enzyme from *Streptococcus pyogenes*), an engineered recombinant protease overexpressed in *Echerichia coli.* The protease cleaves specifically below the hinge region to yield $F(ab')_2$ and Fc/2 subunits (Figure 2).

Peptide analysis

For peptide analysis, 100 µg total protein per antibody sample was diluted to a volume of 50 µL and combined with 150 µL of SMART Digest buffer. The resulting 200 µL of sample were added to one vial of the SMART Digest kit (P/N 60109-101) containing immobilized, heat-stable trypsin. The proteolytic digestion was carried out at 70 °C and at 1,400 rpm shaking for 60 min. After completed digestion, the sample was separated from the beads and transferred into a fresh vial followed by reduction with 10 mM dithiothreitol (DTT) for 45 min at 57 °C. For disulfide bridge analysis, the sample obtained after the digest was split in half and only one portion was reduced, whereas the other remained unreduced.

Chromatography

All experiments were performed using Thermo Scientific™ Vanquish™ Horizon or Vanquish™ Flex Quarternary UHPLC systems.

For intact mass analysis under native conditions, proteins were desalted online using size exclusion chromatography (SEC) on a Thermo Scientific™ Acclaim™ SEC-300 4.6 × 300 mm column (5 µm particle size, P/N 079723) and isocratic elution with 50 mM ammonium acetate.

For reversed-phase chromatography of intact mAbs and mAb subunits under denaturing conditions, the Thermo Scientific™ MAbPac™ RP 50 mm × 2.1 mm column was used (P/N 088648) with a gradient of solvent A consisting of water/0.1% formic acid and solvent B consisting of acetonitrile/0.1%formic acid.

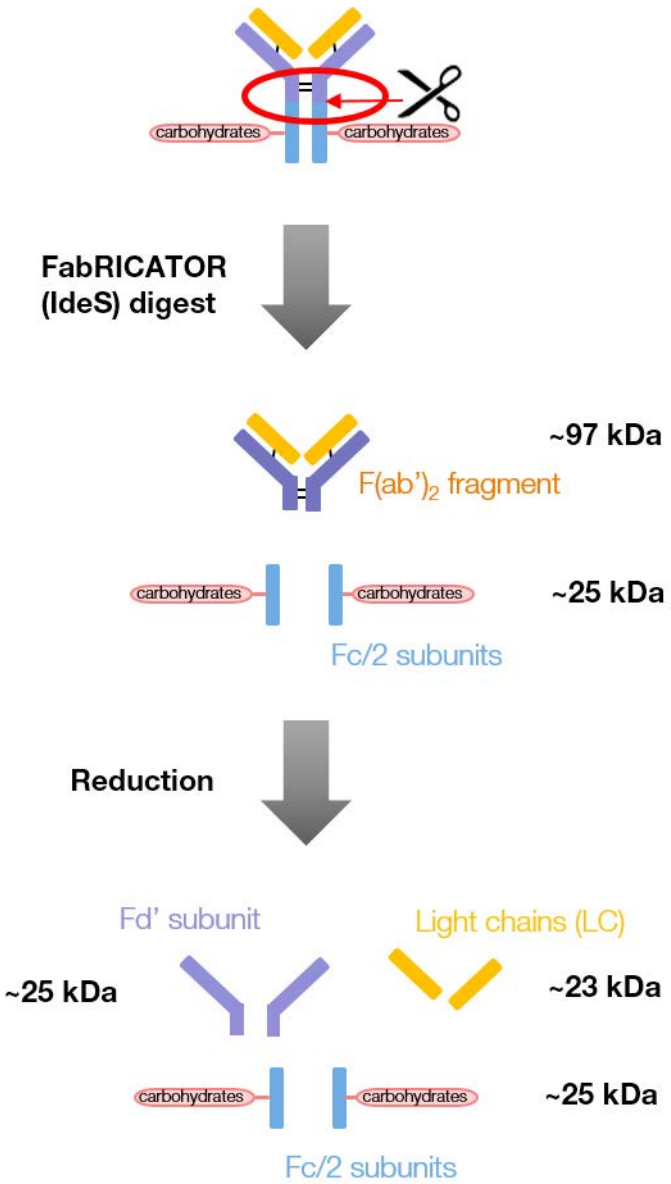


Figure 2. Schematic displaying the subunits obtained after FabRICATOR (IdeS) digest, which are the $F(ab')_2$ fragment and the Fc/2 subunits resulting after reduction in the Fd', LC, and Fc/2 subunits.

Peptide mapping experiments were performed on a Thermo Scientific™ Acclaim™ VANQUISH™ C18 2.1 × 250 mm reversed-phase column with 2.2 µm (120 Å) particles (P/N 074812), run with a gradient of solvent A consisting of water/0.1% formic acid and solvent B consisting of acetonitrile/0.1% formic acid.

All LC gradients used for the different analyses are summarized in Table 1.

Table 1. Overview of LC conditions: columns, flow rates, solvents, and gradients used for MS analysis of A) intact antibodies under native conditions, B) intact antibodies under denaturing conditions, C) light and heavy chain subunit analysis, D) mAb subunit analysis upon IdeS digest, with reduction and without reduction, and E) peptide mapping.

Table 1A. Intact Native		
Column:	SEC Acclaim-300 4.6 × 300 mm	
Column Temp.:	25 °C	
Heating Mode:	Still air	
Flow Rate:	300 µL/min	
Solvent A:	Water	
Solvent B:	100 mM ammonium acetate	
	Time [min]	*%B*
Isocratic:	0.0	50
	10.0	50

Table 1B. Intact Denatured		
Column:	MabPac RP 2.1 × 50 mm	
Column Temp.:	70 °C	
Heating Mode:	Still air	
Flow Rate:	250 µL/min	
Solvent A:	Water/0.1% formic acid	
Solvent B:	ACN/0.1% formic acid	
	Time [min]	*%B*
	1.0	25
	9.0	35
	10.0	80
	11.0	80
	12.0	25
	20.0	25

Table 1C. Reduced mAb (LC & HC)		
Column:	MabPac RP 2.1 × 50 mm	
Column Temp.:	70 °C	
Heating Mode:	Still air	
Flow Rate:	250 µL/min	
Solvent A:	Water/0.1% formic acid	
Solvent B:	ACN/0.1% formic acid	
	Time [min]	*%B*
	0.0	25
	1.0	25
	13.0	32
	14.0	80
	16.0	80
	16.5	25
	25.0	25

Table 1D. mAb Subunit Analysis (IdeS Digest w/wo Reduction)		
Column:	MabPac RP 2.1 × 50 mm	
Column Temp.:	70 °C	
Heating Mode:	Still air	
Flow Rate:	250 µL/min	
Solvent A:	Water/0.1% formic acid	
Solvent B:	ACN/0.1% formic acid	
	Time [min]	*%B*
	0.0	25
	1.0	25
	7.0	35
	8.0	80
	9.0	80
	9.5	25
	15.0	25

Table 1E. Peptide Mapping			
Column:	MabPac RP 2.1 × 50 mm		
Column Temp.:	70 °C		
Heating Mode:	Still air		
Flow Rate:	250 µL/min		
Solvent A:	Water/0.1% formic acid		
Solvent B:	ACN/0.1% formic acid		
	20 min gradient	40 min gradeint	
	time [min]	*time [min]*	*%B*
	0	0.0	2
	22.0	40.0	40
	23.0	42.0	80
	25.0	45.0	80
	25.5	45.5	2
	40.0	60.0	2

Mass spectrometry

The mass spectrometers used for all experiments were commercially available Q Exactive Plus and Q Exactive HF mass spectrometers, each equipped with the BioPharma Option and controlled by Exactive Series Tune 2.8 software. The MS parameter settings are summarized in Table 2.

The top-down experiments of trastuzumab light and heavy chains were performed on the Q Exactive HF mass spectrometer with direct nanospray infusion. The mass range was set to 300–3,000 *m/z* and fragment ion spectra were acquired for 3–5 different light and heavy chain precursor ions at a resolution setting of 240,000 for the heavy chain (HC) and 120,000 for the light chain (LC). The AGC target was 5e6 for the HC and 3e6 for the LC. A maximum injection time of 500 ms was set for the HC and 200 ms for the LC, 10 µscans and an isolation width

Table 2. Parameter settings for all experiments described in this application note regarding source and method parameters. *Resolution settings listed apply to the Q Exactive Plus mass spectrometer and relate to 30,000, 60,000, and 120,000 or 240,000 on the Q Exactive HF mass spectrometer.

MS Conditions	Intact Native	Intact Denatured	Reduced mAb	Subunit Analysis	Peptide Mapping
Method type	Full MS	Full MS	Full MS [two segments]	Full MS	Full MS-ddTop5 HCD
Instrument mode / Trapping gas pressure setting	HMR Mode / 1.0	HMR Mode / 1.0	Protein Mode / 0.2	Protein Mode / 0.2	Standard Mode / -/-
Total run time	10 min	20 min	25 min [0-9.9/9.9-25min]	15 min	40 min
Scan range [*m/z*]	2,500–8,000	2,200–5,000	600–2,400	700–2,800	200–2,000
Resolution (Full MS/MS^2)*	35,000	35,000	140,000/17,500	140,000	70,000/17,500
AGC targt value (Full MS/MS^2)	3e6	3e6	3e6	3e6	3e6/1e5
Max inject time [Full MS/MS^2]	200 ms	200 ms	200 ms	200 ms	100 ms /200 ms
Isolation window (MS^2)	-	-	-	-	2Th
Microscans (Full MS/MS^2)	10	10	5 / 10	5	1/1
SID [eV]	130	80	-	-	-
NCE [%]	-	-	-	-	28
Intensity threshold	-	-	-	-	1e4
Dynamic exclusion	-	-	-	-	10 ms
Lock mass used for internal calibration	-	-	-	-	391.28429
Source settings					
Probe heater temperature [°C]	175	150	150	150	150
Source voltage [kV]	4.2	3.50	3.5	3.8	3.5
Capillary temperature [°C]	275	300	300	320	320
S-lens RF level	200	100	100	60	60
Sheath gas	20	25	20	25	20
Aux gas	5	5	5	10	8
Sweep gas	0	0	0	0	0

of 1.2 Th. Rolling averaging was used for acquisition of the heavy chain top-down fragment ion spectra. Normalized collision energies of 10%, 12%, 14%, 16%, and 18% were applied. Averaging of several hundred µscans per precursor was performed with collision energies in the range of 10–18%. Fragment ions from all spectra from the different precursors and different collision energies applied were combined for sequence matching using ProSight Lite software.

IdeS digested and reduced trastuzumab was acquired on a Q Exactive Plus mass spectrometer using a Full MS method with the mass range set to 600–2,400 *m/z*, an AGC target value of 3e6, a maximum injection time of 200 ms, a resolution setting of 140,000 and 5 µscans. For top-down fragmentation a method was used consisting of MS^2 scans using a fixed 200 Th wide isolation window with a center mass of 950 *m/z,* a resolution setting of 140,000, an AGC target value of 3e6, maximum injection time of 500 ms, 10 µscans and a fixed first mass of 300 *m/z*. Normalized collision energies of 10%, 12%, 14%, 16%, and 18% were used in separate, duplicate runs. On average 25 scans with 10 µscans per scan were acquired for each subunit at each of the five different collision energies in duplicate runs, resulting in a total of 2,500 µscans that were used for sequence matching.

Data analysis

Raw data files obtained from intact protein samples, mAb subunit, and peptide mapping samples were analyzed with Thermo Scientific™ BioPharma Finder™ 2.0 software. For top-down analysis data ProSight Lite software was used after spectral deconvolution with the Xtract algorithm.

Results and discussion

Standard Mode, Protein Mode, and HMR Mode

There are many factors that play a key role in the analysis of proteins, some of which relate to sample preparation (buffers, solvents, additives) while others relate to the mass spectrometer's source conditions as well as the physical environment inside the instrument.[2,3] The Q Exactive Plus and Q Exactive HF mass spectrometers (Figure 3a) have previously been introduced with the Protein Mode option, which was one of many advancements for intact protein analysis on the Orbitrap platform. For these two instruments, an automated HCD gas control was introduced by using an electronically controlled valve for nitrogen gas in the HCD cell for easier optimization of experimental conditions required for different types of analyses wished to run on a single platform.

In Standard Mode, pressure settings are factory-optimized and suitable for most analyses (e.g. any small molecules application as well as peptides) and ions are cooled in the C-trap (Figure 3b). The trapping gas pressure setting is 1, which corresponds to a high vacuum pressure delta (ΔHV) of 3.1e-5 mbar. The ΔHV is defined as the difference between HV with HCD gas on minus HV with HCD gas off.

In Protein Mode, the default trapping gas pressure setting is 0.2, which corresponds to a ΔHV that is 5× lower than in Standard Mode. Additionally, ions are transferred and cooled in the HCD cell and thus have a longer flight path (Figure 3c).

The combination of reduced C-trap and HCD cell gas pressures and trapping ions in the HCD cell prior to mass analysis extends the life time of protein ions, resulting in increased signal intensities of isotopically resolved species (Figure 3c).

For higher gas pressures, high charge states of the same protein decay faster than lower charge states. This is because center-of-mass collision energy is proportional to the charge state z.

$$K_{ce} = E*m/(M/z)$$

with *E:* ion energy inside the Orbitrap mass analyzer
m: mass of residual gas, nitrogen
M/z: the mass-to-charge ratio for a given charge state

This explains observations of charge envelope shifts on the *m/z* scale when comparing data acquired in different modes with different pressure regimes in the HCD cell and C-trap region.

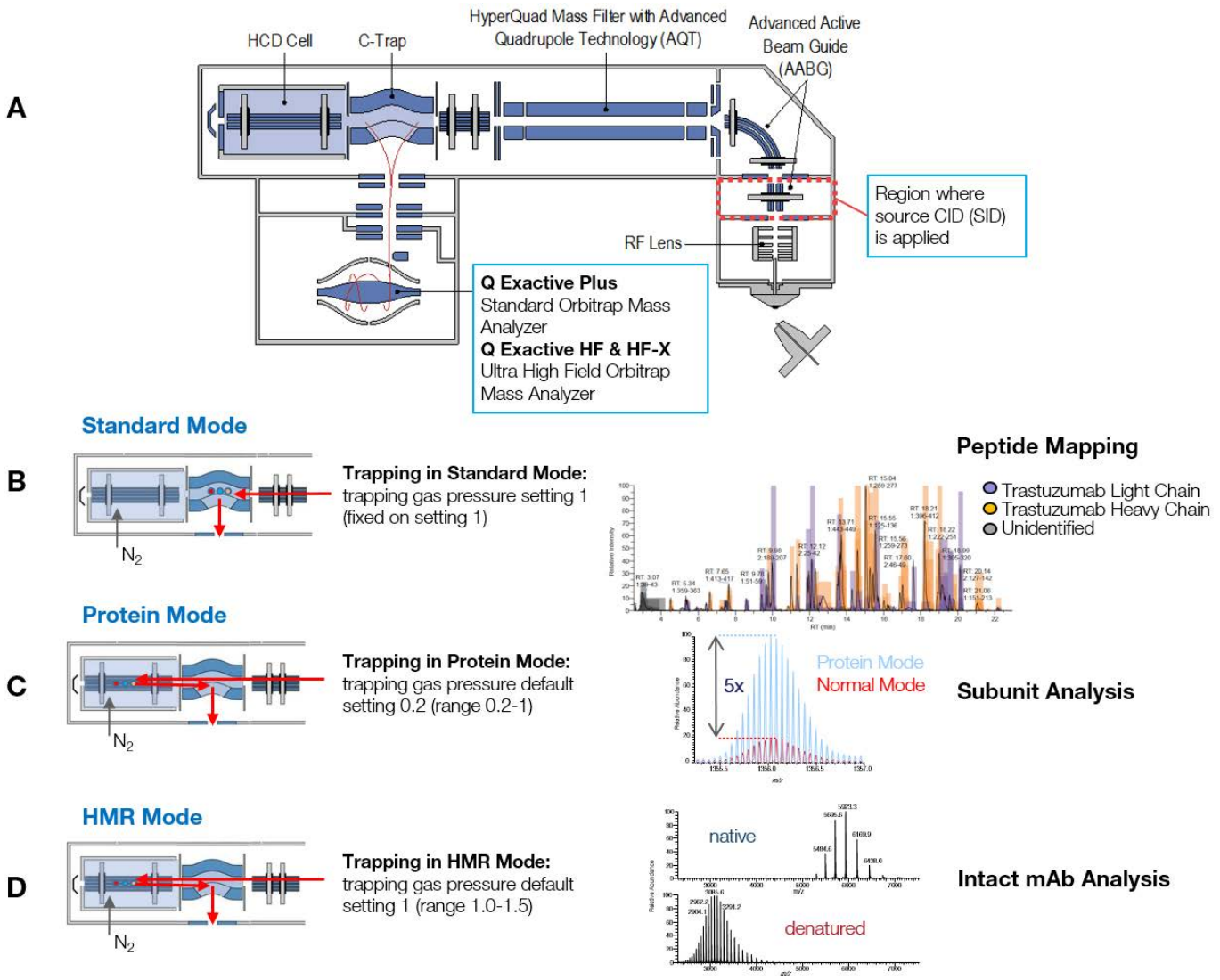


Figure 3. A) Schematic of the Q Exactive Plus and Q Exactive HF mass spectrometers and differences in the trapping path in the three different operating modes available: B) Standard Mode, C) Protein Mode and D) HMR Mode.

In HMR Mode, the default trapping gas pressure setting is 1 and can be slightly increased up to 1.5 for improved trapping of certain species such as protein complexes and heterogeneous large proteins (e.g. antibody drug conjugates). The trapping path in HMR Mode is the same as in Protein Mode with ion cooling taking place in the HCD cell. Additionally, mass detection up to *m/z* 8,000 is enabled compared to *m/z* 6,000 in the two other modes.

The trapping gas pressure in all modes is set and saved in the tune files. Since a method allows for segmentation using different tune files, different pressure settings can be used within one LC-MS run. In contrast, the mass range setting is set in the method and the method editor allows for several nodes with different experiment types using different mass ranges within one LC-MS run.

Intact mAb analysis under native and denaturing conditions

The analysis of intact antibodies under native and denaturing conditions requires different chromatographic conditions. Whereas some researchers refer to the native analysis of antibodies as the mass detection of the intact antibodies but under denaturing conditions, here native analysis is referred to as the analysis of an intact antibody under native conditions: near neutral pH, no acid or organic solvent involved.

Under denaturing conditions, the protein is exposed to acid and organic solvent and separated over a reversed phase column resulting in an envelope representing charge states from ~35 to 65, detected in a mass range from ~2,000 to 4,000 *m/z*. Under native conditions, the protein is kept in acqueous solution at near neutral pH only containing volatile salts such as ammonium acetate. These conditions preserve the protein's three dimensional structure, providing a smaller surface to accept protons during the ionization process. This results in an envelope representing fewer and lower charge states, typically ranging from 20 to less than 30, detected in the mass range between 5,000 and 7,000 *m/z* (Figure 5). The reduced number of charge states representing the ions detected in native conditions can contribute to improved sensitivity.

For the analysis of mAbs under native conditions, the parameter settings regarding in-source CID, probe heater, and capillary temperatures were found to have a significant impact on the declustering/desolvation efficiency and thus spectral quality.[5] Good starting conditions for optimization are provided in Table 2 of the Experimental section.

Under native conditions, a higher spatial resolution is obtained due to the detection at higher *m/z* as highlighted in the zoom of the most abundant charge states in Figure 4. The glycoform pattern, however, and the masses obtained after deconvolution match well with very good mass accuracies obtained. The expected theoretical average masses for the detected and most commonly observed glycoforms are listed in Table 3.

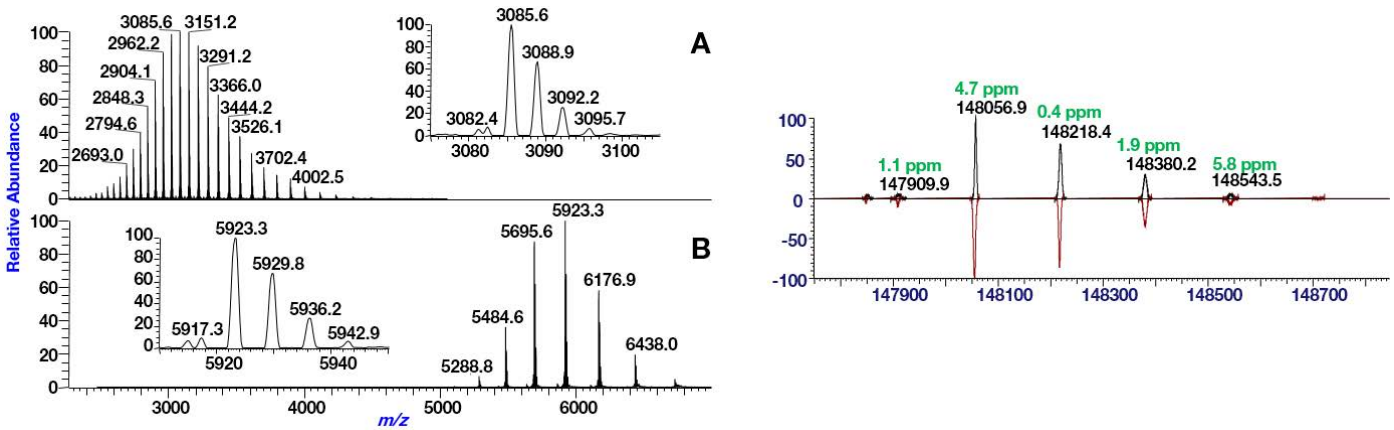


Figure 4. Full MS spectra acquired from intact trastuzumab under denaturing (A) and native conditions (B).

Table 3. Theoretical masses for trastuzumab glycoforms.

Glycoform	Average MW
Trastuzumab unglycosylated	145,165.5
Trastuzumab Man5/Man5	147,599.7
Trastuzumab G0/G0	147,796.2
Trastuzumab G0/G0F	147,910.1
Trastuzumab G0F/G0F	148,056.2
Trastuzumab G0F/G1F	148,218.3
Trastuzumab G0F/G2F or (G1F)2	148,380.5
Trastuzumab G1F/G2F	148,542.6
Trastuzumab G2F/G2F	148,704.8
Trastuzumab G1F/G2F SA	148,261.0
Trastuzumab G1F/G2F (SA)2	149,125.1
Trastuzumab G2F/G2F SA	148,996.0
Trastuzumab G2F/G2F (SA)2	149,287.3

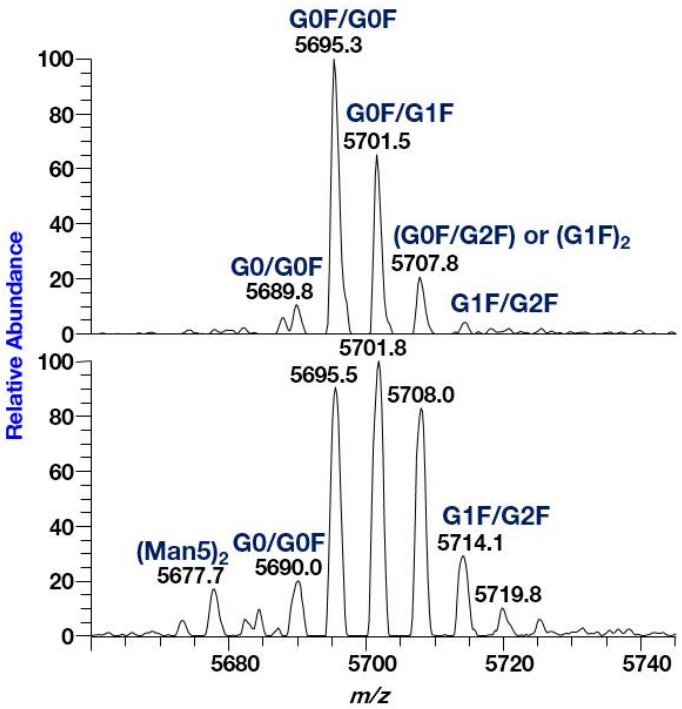


Figure 5. Observed glycoform patterns for two different lots of trastuzumab on the raw data level showing significantly different relative abundances.

The glycoform patterns of antibodies generally, but also of commercially available antibodies, show variations and differences can be detected when different production batches are compared. Two different batches of trastuzumab were analyzed, one that was obtained several years ago and one that was obtained in 2016. The observed glycoform patterns are different in the relative abundance and number of glycoforms as detected on the raw data level as well as after deconvolution (Figure 5). The respective pattern of each sample is consistent and reproducible across different instruments and platforms.

The three antibodies trastuzumab, infliximab, and bevacizumab were analyzed under native and denaturing conditions as single samples, as well as in a mixture (Figure 6a, c). The mixed sample provided the most complex pattern (also due to Lys-heterogeneity of infliximab, Figure 6e), which can be well resolved in both conditions (Figure 7b, d). However, under native conditions a higher spatial resolution is obtained due to the detection at higher *m/z* values.

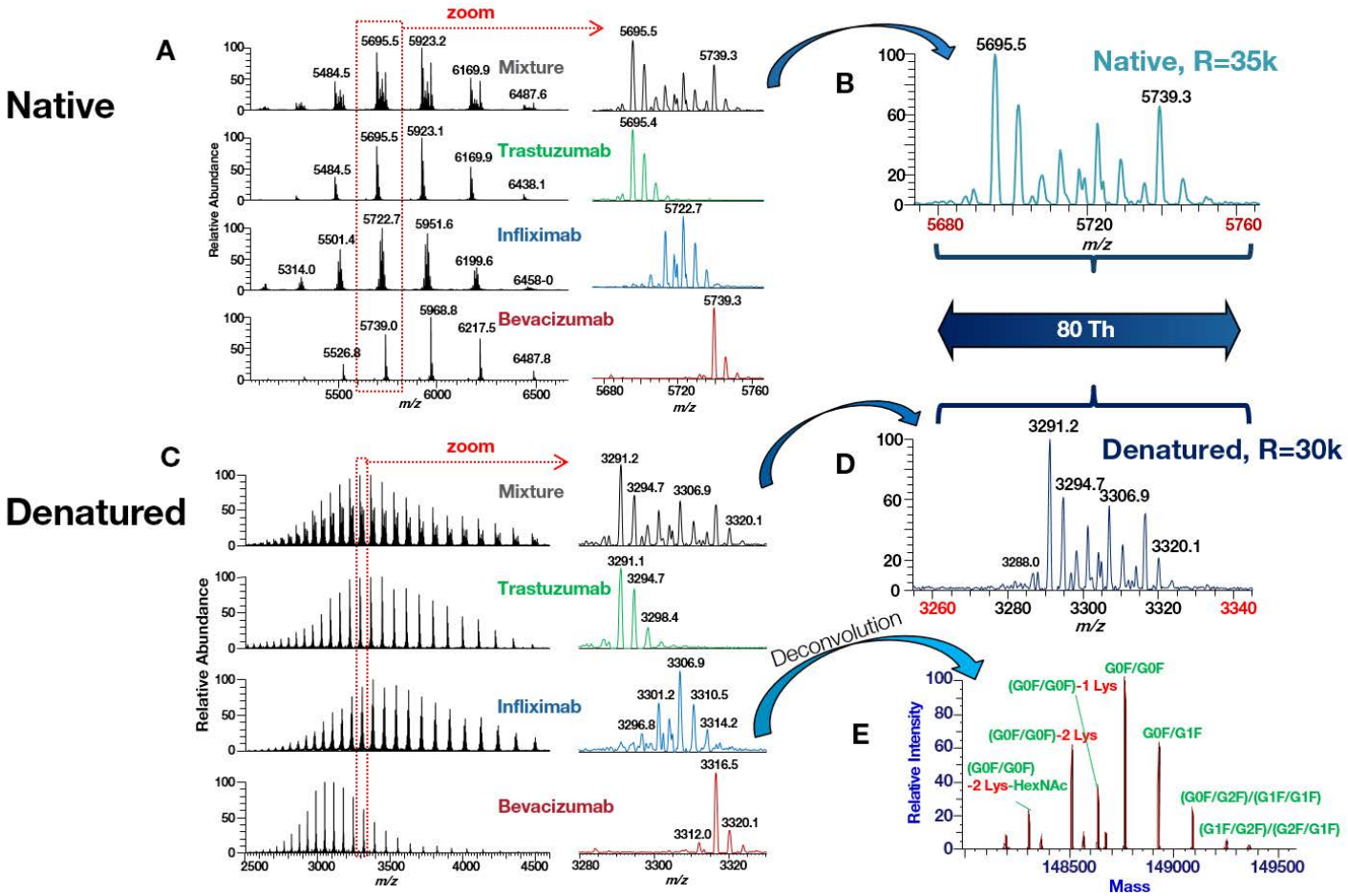


Figure 6. Analysis of the three mAbs trastuzumab, infliximab, and bevacizumab individually, as well as a mixture, under denaturing and native conditions.

To demonstrate the sensitivity of the instrument for intact mAb analysis under denaturing conditions, a dilution series of trastuzumab ranging from 100 pg to 1 µg total protein injected on column was performed as shown in Figure 7.

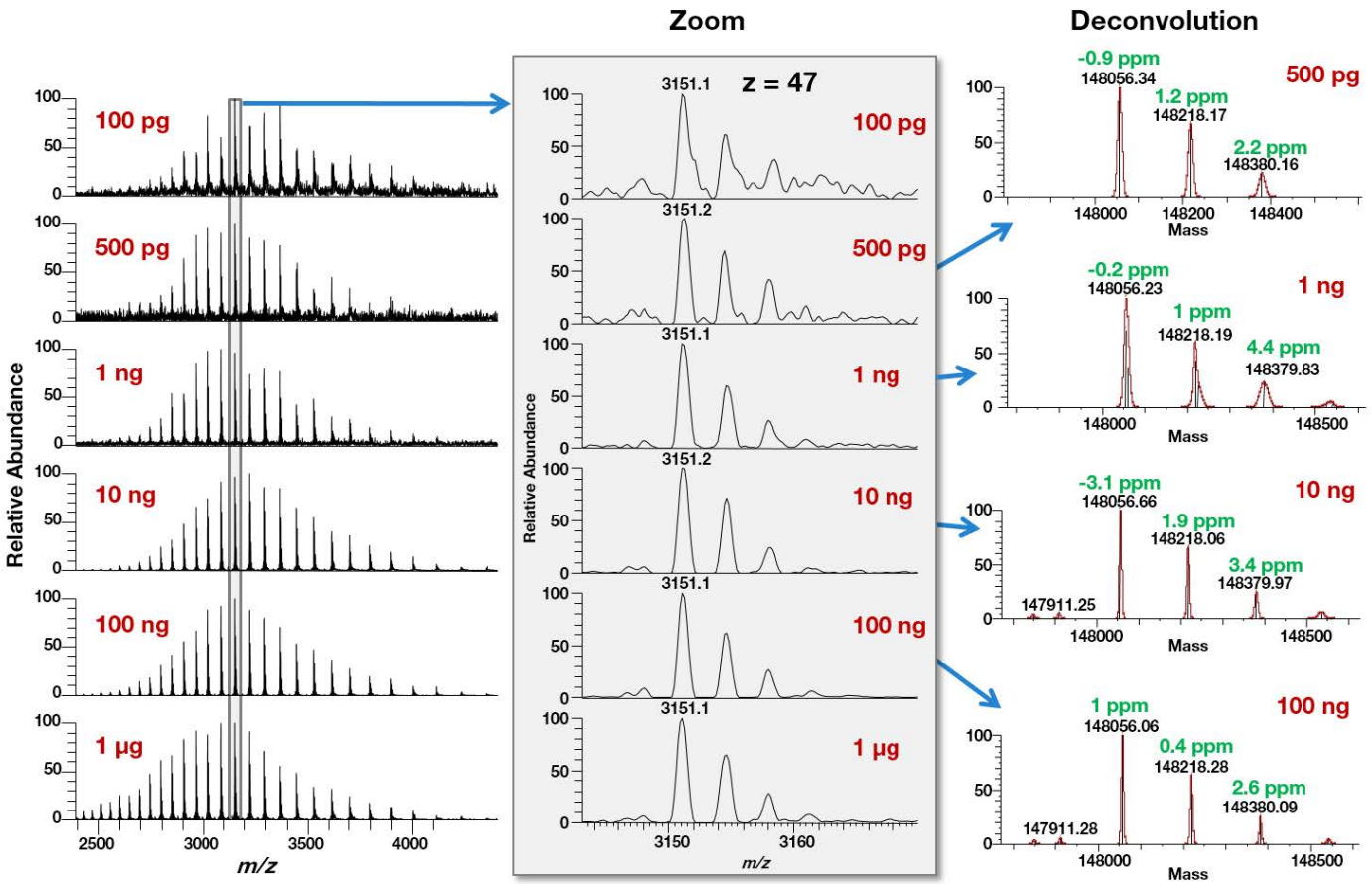


Figure 7. Serial dilution series of trastuzumab ranging from 100 pg to 1 µg total protein loaded on column.

mAb subunit analysis

The three antibodies trastuzumab, infliximab, and bevacizumab were analyzed after reduction as well as after FabRICATOR digest with and without reduction. Figure 8 shows the analysis of reduced trastuzumab as a representative for UHPLC separation of light and heavy chains typically obtained on the MAbPac RP column. The method setup comprised the full scan acquisition at high (light chain) and low (heavy chain) resolution settings to achieve intact molecular weight information in the first instance. In a separate direct infusion experiment acquiring SIM scan data of a single charge state of a single glycoform, isotopic baseline-resolved peaks were obtained also for the heavy chain. In a third experiment performing top-down analysis, MS/MS spectra for the light and the heavy chain were acquired and applied for sequence confirmation based on detected fragment ion masses matched to the expected subunit sequences.[6]

Another option to dissect an antibody in subunits is to perform a FabRICATOR digest using the immunoglobulin-degrading enzyme (IdeS) from *Streptococcus pyogenes,* an engineered recombinant protease overexpressed in *Echerichia coli.* The protease cleaves specifically below the hinge region to yield $F(ab')_2$ and Fc fragments (see Figure 2). Since the molecular weight of the $F(ab)_2$ fragment is too large (~90 kDa) to obtain isotopic resolution, and the Fc subunit with a MW of ~25 kDa can easily be isotopically resolved, a similar method as for the analysis of the reduced mAb was applied. In this method

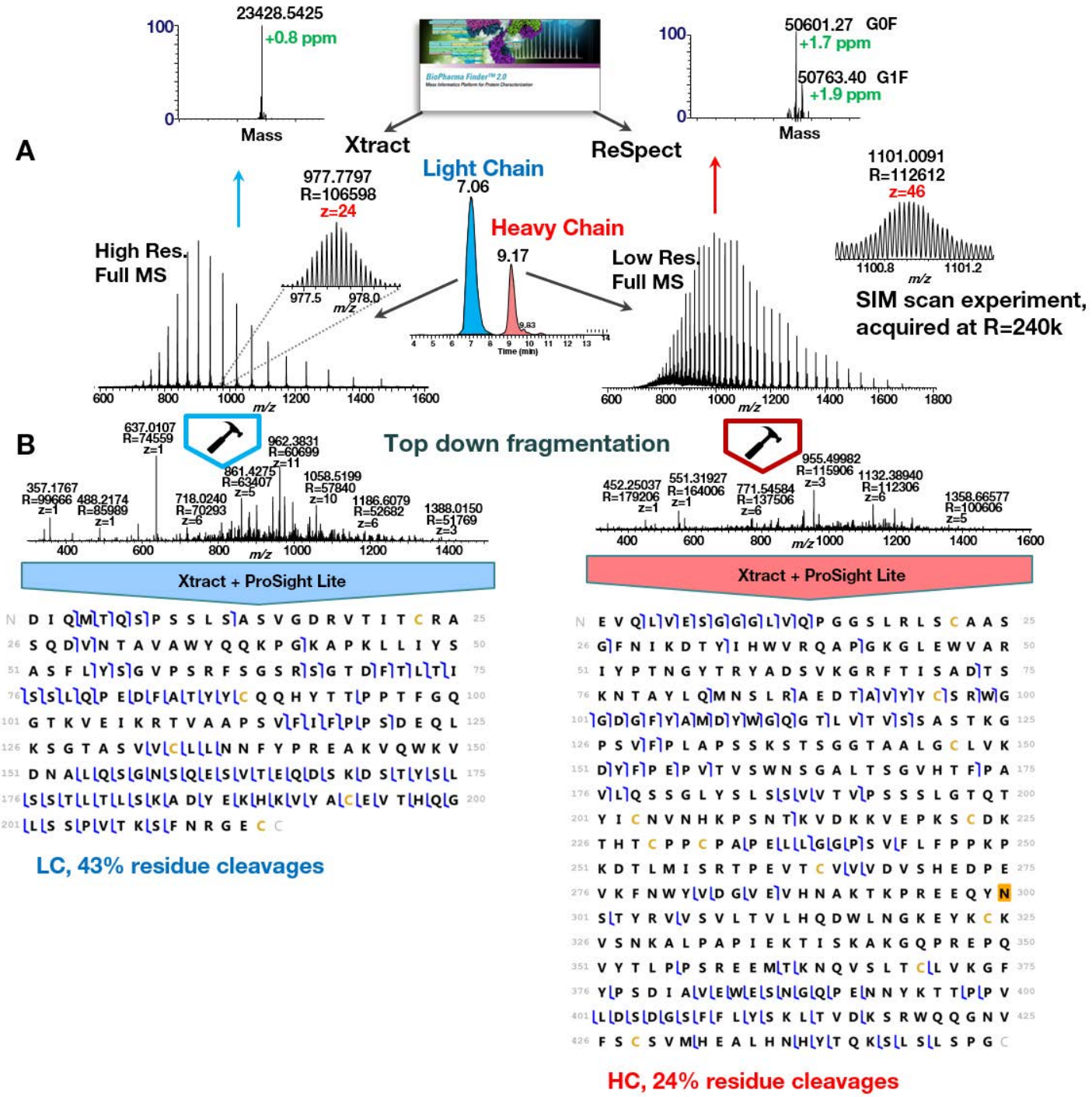


Figure 8. A) Detection of the separated light (LC) and heavy chains (HC) in Full MS as well as a SIM scan experiment of one charge state of one glycoform of the heavy chain providing a baseline resolved isotope pattern. B) Top-down subunit analysis of light and heavy chain of trastuzumab and assignment of detected fragment ions to the expected amino acid sequence.

the resolution is switched from high resolution (140k) for the detection of the Fc/2 subunit to lower resolution (35k) for the detection of the $F(ab)_2$ fragment (Figure 9). Due to the different resolution settings applied, the isotopically resolved spectra are deconvoluted with Xtract, whereas the unresolved spectra are deconvoluted with Respect. Both algorithms are implemented in the BioPharma Finder software. Both subunits are detected with excellent mass accuracies between 0.1 and 2.2 ppm. For theoretical masses please refer to Table 4.

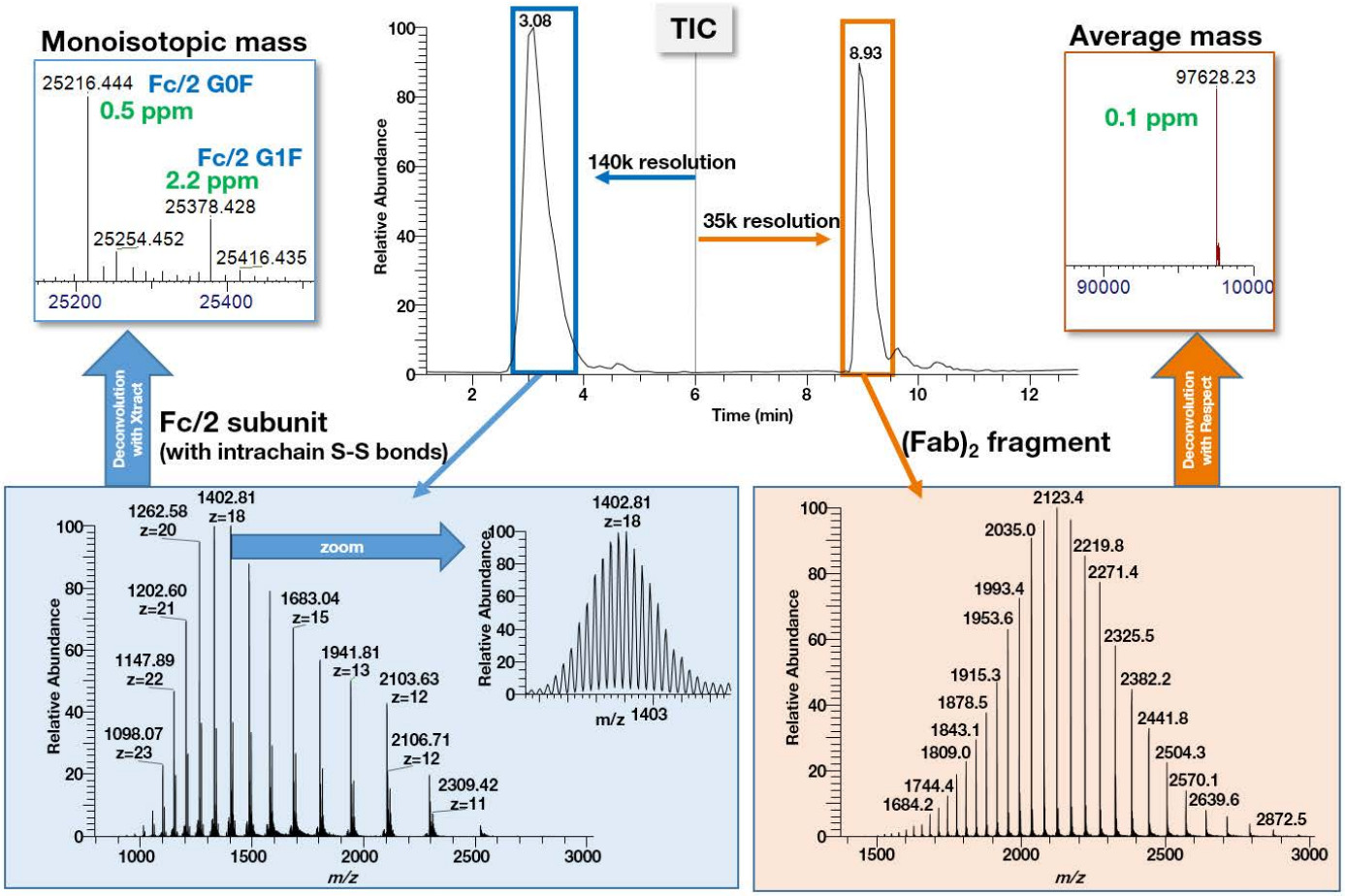


Figure 9. Total ion chromatogram and obtained MS spectra of trastuzumab after FabRICATOR digest without reduction resulting in the Fc/2 and F(ab')$_2$ regions with both subunits bearing intact disulfide bonds, resulting in observed molecular weights of ~25 kDa for the Fc/2 subunits and ~97 kDa for the F(ab')$_2$ fragment.

Table 4. Theoretical monoisotopic and average masses for the subunits of trastuzumab: light chain, Fc/2 region with terminal Lys truncation, reduced and unreduced, unglycosylated and with G0F and G1F glycoforms, Fd' region reduced and unreduced, the F(ab')$_2$ fragment and the heavy chain in the three glycoforms G0F, G1F and G2F.

	MW (Monoisotopic)	MW (Average)
Light chain, aa 1-213	23,428.52384	23,442.9
Fc/2 (-Lys) reduced	23,775.92951	23,790.7
Fc/2 (-Lys) G0F reduced	25,220.46338	25,236.0
Fc/2 (-Lys) G1F reduced	25,382.51621	25,398.2
Fc/2 (-Lys) unreduced	23,771.89821	23,786.7
Fc/2-(Lys) G0F unreduced	25,216.43208	25,232.0
Fc/2 (Lys) G1F - unreduced	25,378.48490	25,394.1
F(ab')$_2$ unreduced	97,567.89467	97,628.2
Fd' unreduced	25,363.48610	25,379.3
Fd' reduced	25,367.51740	25,383.3
Heavy chain G0F (- Lys, fully reduced)	50,569.97021	50,601.4
Heavy chain G1F (- Lys, fully reduced)	50,732.02304	50,763.5
Heavy chain G2F (- Lys, fully reduced)	50,894.07586	50,925.6

The $F(ab)_2$ fragment can be further separated into the light chain (LC) and Fd' subunit upon reduction. Figure 10 demonstrates the chromatographic separation of the three subunits Fc/2, LC, and Fd' obtained from trastuzumab, bevacizumab, and infliximab after FabRICATOR digest and reduction, nicely achieved in only 9 minutes based on superb separation capabilities of the MAbPac RP column. Infliximab shows a significantly lower degree of Lys-truncation than the other two mAbs, resulting in a doublet chromatographic peak (Fc/2 and Fc/2-Lys) and relates to the glycoform pattern obtained on the intact level (Figure 6).

Mass spectra were acquired at high resolution to obtain monoisotopic masses after deconvolution with Xtract. Mass accuracies for all species are below 2 ppm obtained with external calibration as depicted for trastuzumab.

In a separate experiment, a top-down analysis of the subunits as shown in Figure 10 was performed. Experiments were performed in Intact Protein Mode with a resolution setting of 140,000 to ensure resolving and detection of isotope patterns from highly charged and overlapping species. Figure 11 shows one example of an HCD spectrum obtained from the trastuzumab light chain. This MS/MS spectrum demonstrates the peak density across the mass spectrum and the well-resolved and nicely shaped isotope patterns of detected fragment ions in different charge states providing very good bond coverage as highlighted for all three subunits of trastuzumab (Figure 11).[6]

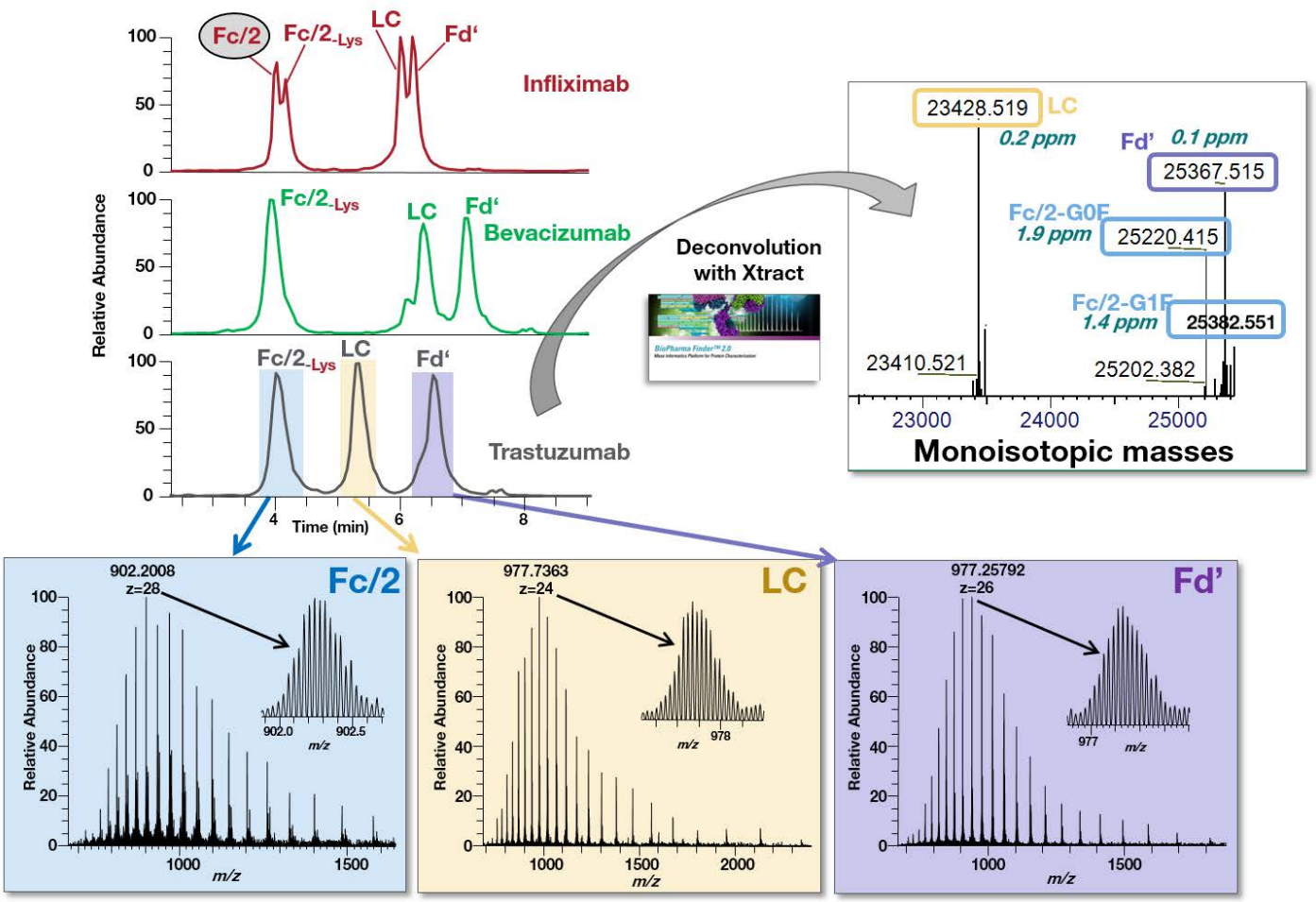
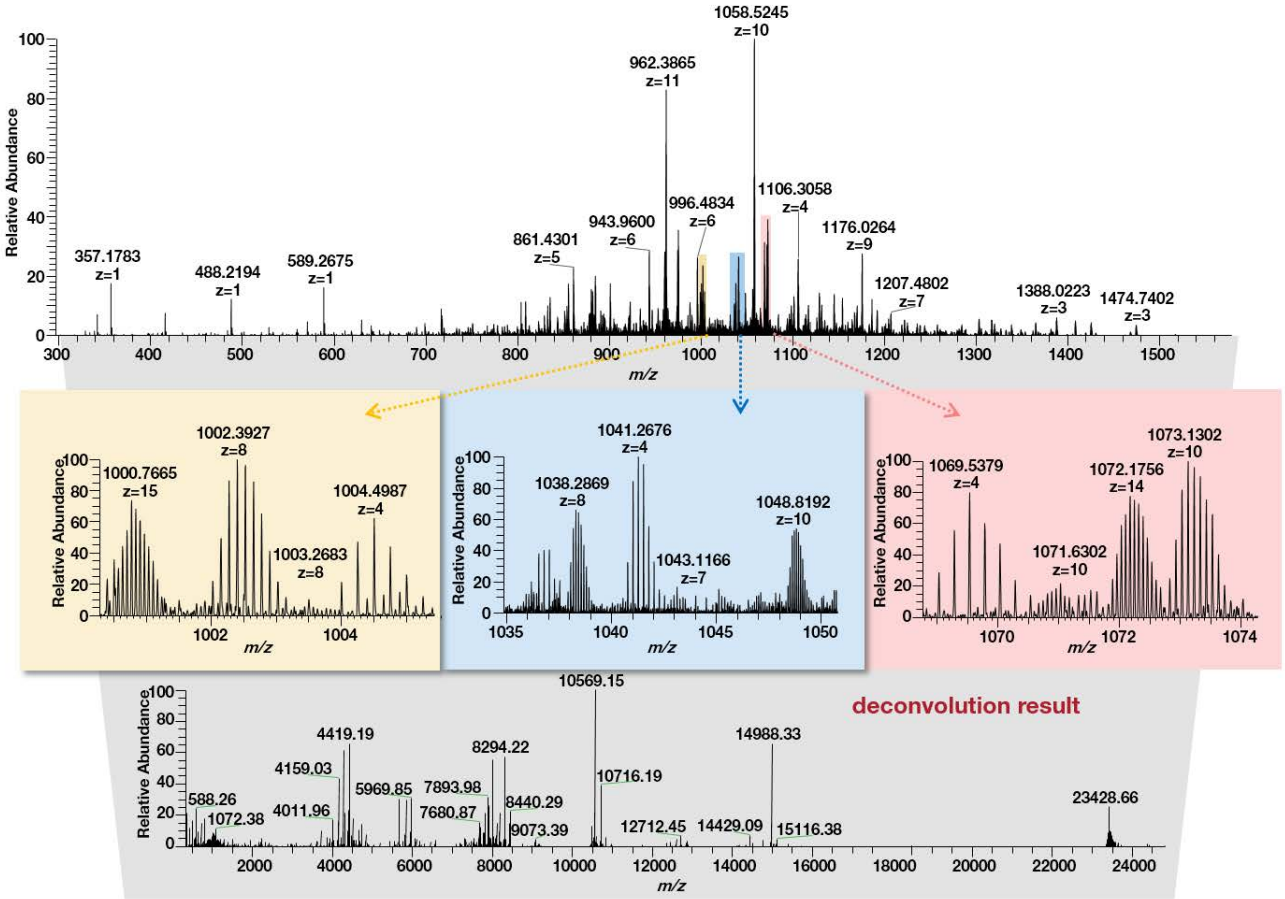


Figure 10. Total ion chromatograms of separated subunits obtained after FabRICATOR digest and reduction from trastuzumab, bevacizumab, and infliximab. Obtained Full MS mass spectra and baseline-resolved isotope patterns of the individual charge states, as well as the result after deconvolution, is showcased for trastuzumab.



Fc, 39% residue cleavages

N G P S|V|F|L|F|P P|K|P K D|T|L|M|I|S R T|P E|V|T|C 25
26|V|V|V|D V S|H E D|P E|V|K F N|W|Y|V|D G V E|V H N 50
51 A K T K P R E E Q Y **N** S T Y R V|V S V L T V L H Q 75
76 D W L N G K E Y K C K V S N K A L P A P I E K T I 100
101 S K A K G Q P R E P Q V Y T L P P S R E E M T K N 125
126 Q V|S|L T C L|V|K|G F|Y|P|S D|I|A|V|E|W|E|S|N|G|Q 150
151|P E|N N Y|K|T|T|P|P V L|D|S|D|G|S|F|F|L|Y|S K L|T 175
176 V|D|K S R W Q Q G N V|F|S C|S V|M|H E|A L H|N|H|Y 200
201|T|Q|K S L S L S P G C

Fd', 38% residue cleavages

N E V Q|L|V|E|S|G|G|G|L|V|Q|P G G S L R L S C|A|A S 25
26 G F N I K D T Y|I H W V R|Q|A P|G K|G L E W V A R 50
51 I Y P T N G Y T R Y A D S V K G R F T I S A D T|S 75
76 K N T A Y L|Q|M N S L R|A|E D T|A|V|Y|Y|C|S R|W|G 100
101|G|D G|F|Y|A|M|D|Y|W|G|Q G T|L V|T|V S S A S T K|G 125
126 P S V F|P L A|P S S K|S|T S G G T|A A L G C L V K 150
151 D|Y|F|P E|P V|T V|S|W N S G A L T S G V H T F|P A 175
176|V L Q|S|S G|L|L|Y|S L|S|S|V|V|T|V|P|S S|S L|G T|Q|T 200
201 Y|I C N V N H K|P|S N|T|K|V D|K K V E P K S C D|K 225
226 T H T C|P|P C|P A|P E|L L G C

LC, 49% residue cleavages

N D I Q|M|T|Q|S|P S S|L S|A S V G|D R V T I|T C R|A 25
26 S Q D|V|N T A V A W Y Q Q K P G K A|P K L L I Y|S 50
51 A S|F|L|Y|S|G V P S R F S G S R S|G T D F|T|L|T|I 75
76|S|S|L|Q|P E|D|F|A|T|Y|Y C Q Q H Y T T P P T|F|G Q 100
101 G T|K V E I K R T V A A P S|V|F|I|F|P|P S D E Q L 125
126 K S G T A|S V|V|C|L|L|N N F Y P R E A K V Q W K V 150
151 D N A L|Q|S|G|N|S|Q|E|S|V|T|E|Q|D|S|K|D|S|T|Y|S|L 175
176|S|S|T|L|T|L|S|K|A|D|Y E|K|H|K|V|Y|A|C|E|V T|H|Q|G 200
201|L|S|S|P|V|T|K|S|F|N|R G E C C

Figure 11. Top-down spectrum of the light chain of trastuzumab and bond coverage obtained for all three subunits: LC, Fd', and Fc/2.

Peptide mapping

The three antibodies trastuzumab, infliximab, and bevacizumab were analyzed on the peptide level after performing digestion with the SMART Digest Kit. Obtained base peak chromatograms are very similar but show distinct differences (Figure 12). All antibodies were identified with 100% sequence coverage when analyzed separately as well as in a mixture. Glycopeptides as well as common modifications such as low level oxidation and deamidation are confidently identified based on MS/MS spectra.

Figure 13 details the chromatogram obtained from the peptide mapping analysis of infliximab and the chromatographic peak shading provided by BioPharma Finder software after performing a peptide mapping data analysis based on the known amino acid sequence. This feature facilitates the optical evaluation of results obtained, in particular if the desired 100% sequence coverage is not obtained. A quick evaluation will provide missed peaks either due to a too broad elution profile requiring paramater optimization or resulting from deviations or errors in the provided amino acid sequence.

Figure 14 highlights the low degree of Lys-truncation of infliximab on the peptide level compared to trastuzumab and bevacizumab, confirming the results obtained on the intact and subunit levels. XICs were created for both versions of the peptide, with and without lysine truncation. For XICs, the singly and double charged precursor masses were considered with a ±5 ppm mass window. The relative intensities for the charge states of the peptide with and without C-terminal lysine are different. Since lysine is a basic amino acid strongly capturing a proton, the peptide containing the C-terminal lysine shows a more abundant doubly charged than singly charged ion. Comparing the relative intensities obtained for the two versions of the peptide shows for trastuzumab and bevacizumab only low levels of the lysine-containing peptides (0.15% and 0.2%) reflecting a high degree in lysine clipping. For infliximab, only low levels of lysine clipping are observed resulting in a ratio of ~1:3 clipped vs. unclipped.

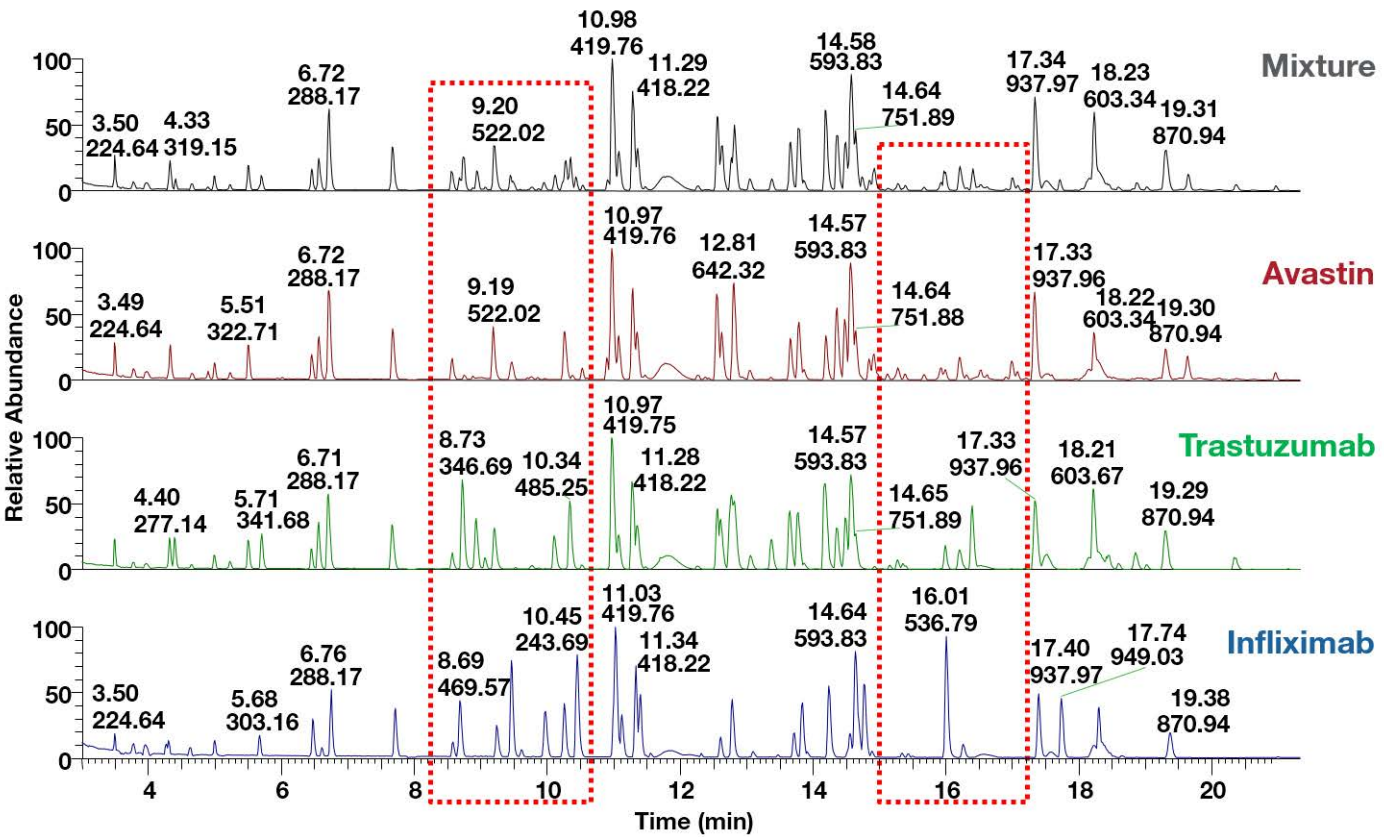


Figure 12. Base peak chromatograms obtained from individual digests of trastuzumab, bavacizumab, and infliximab as well as a mixture of all three. The basic peak pattern is very similar due to high similarity of the amino acid sequences amongst the three mAbs. Areas reflecting distinct differences are highlighted with the red boxes.

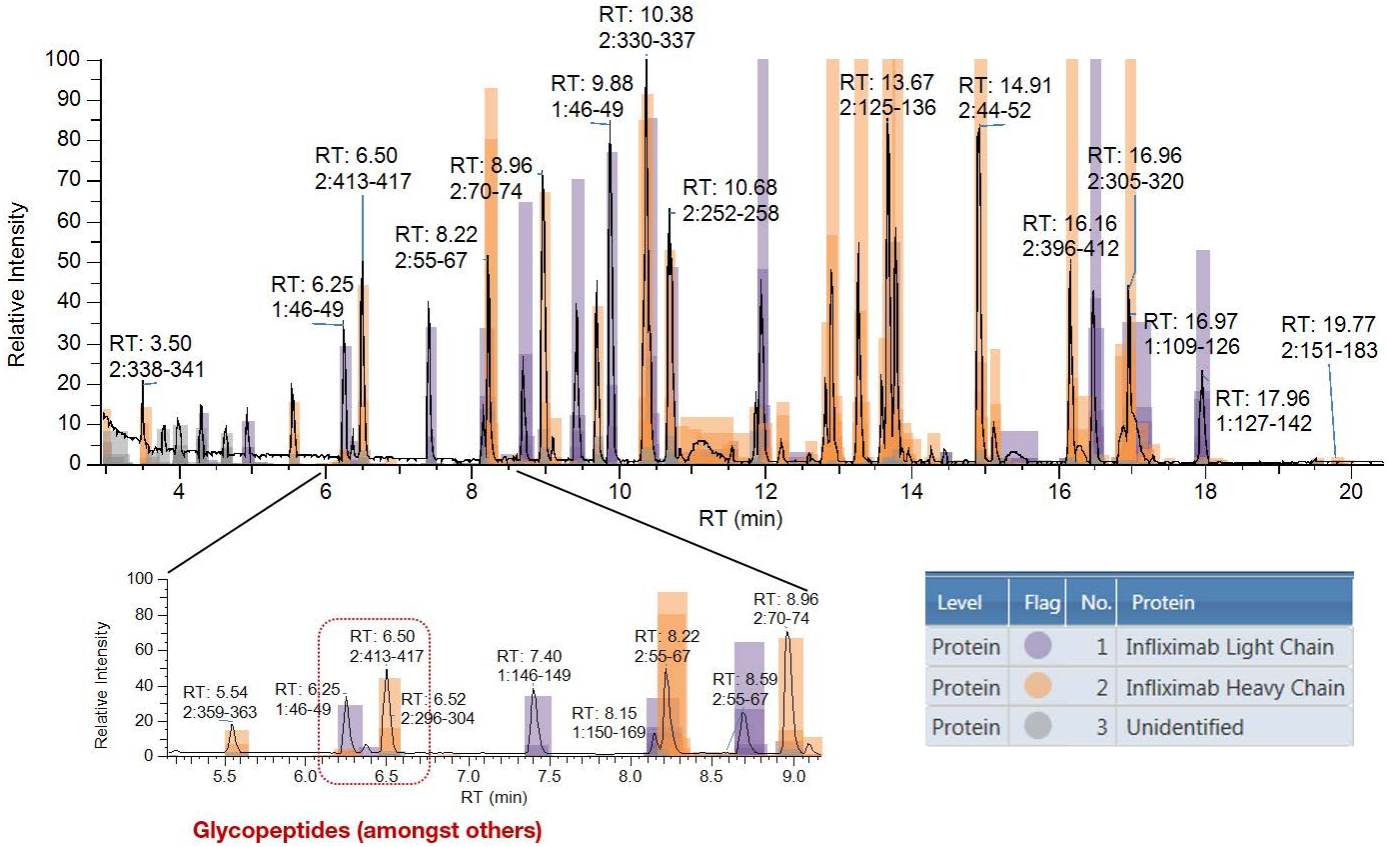


Figure 13. Peak shading provided by BioPharma Finder software based on peptides assigned to the light chain or heavy chain of infliximab. Some peaks remain unidentified, most commonly towards the onset or the end of the chromatogram.

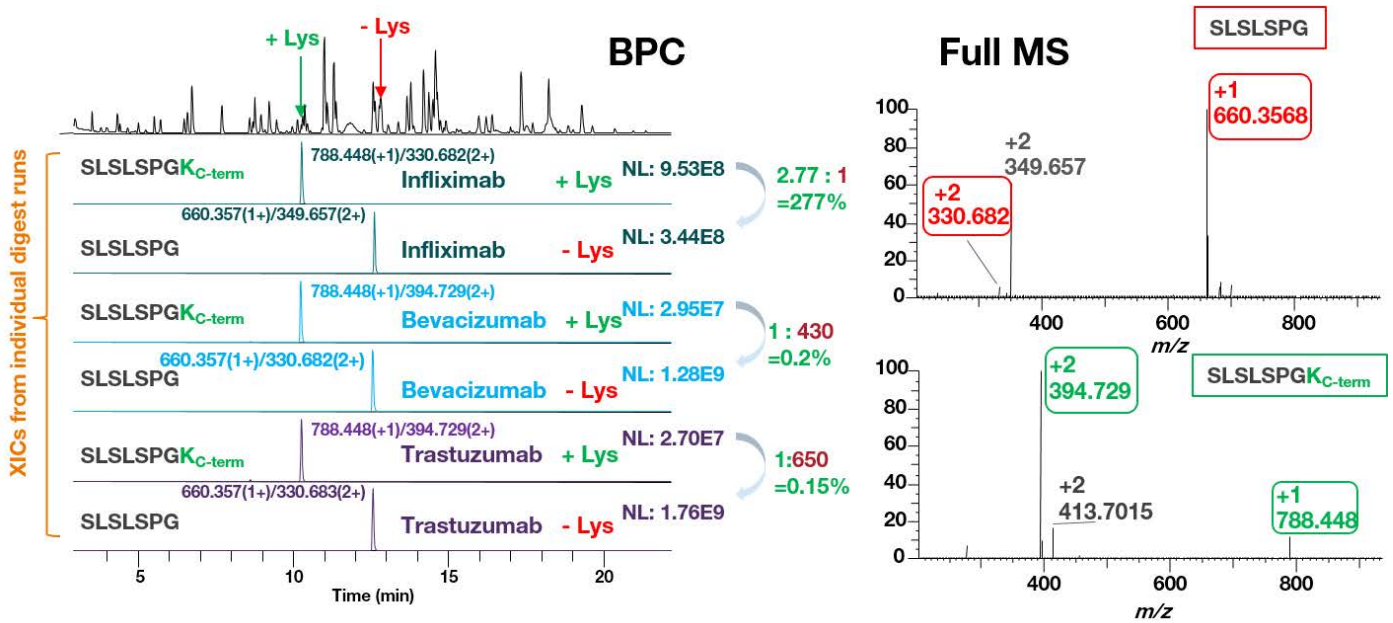
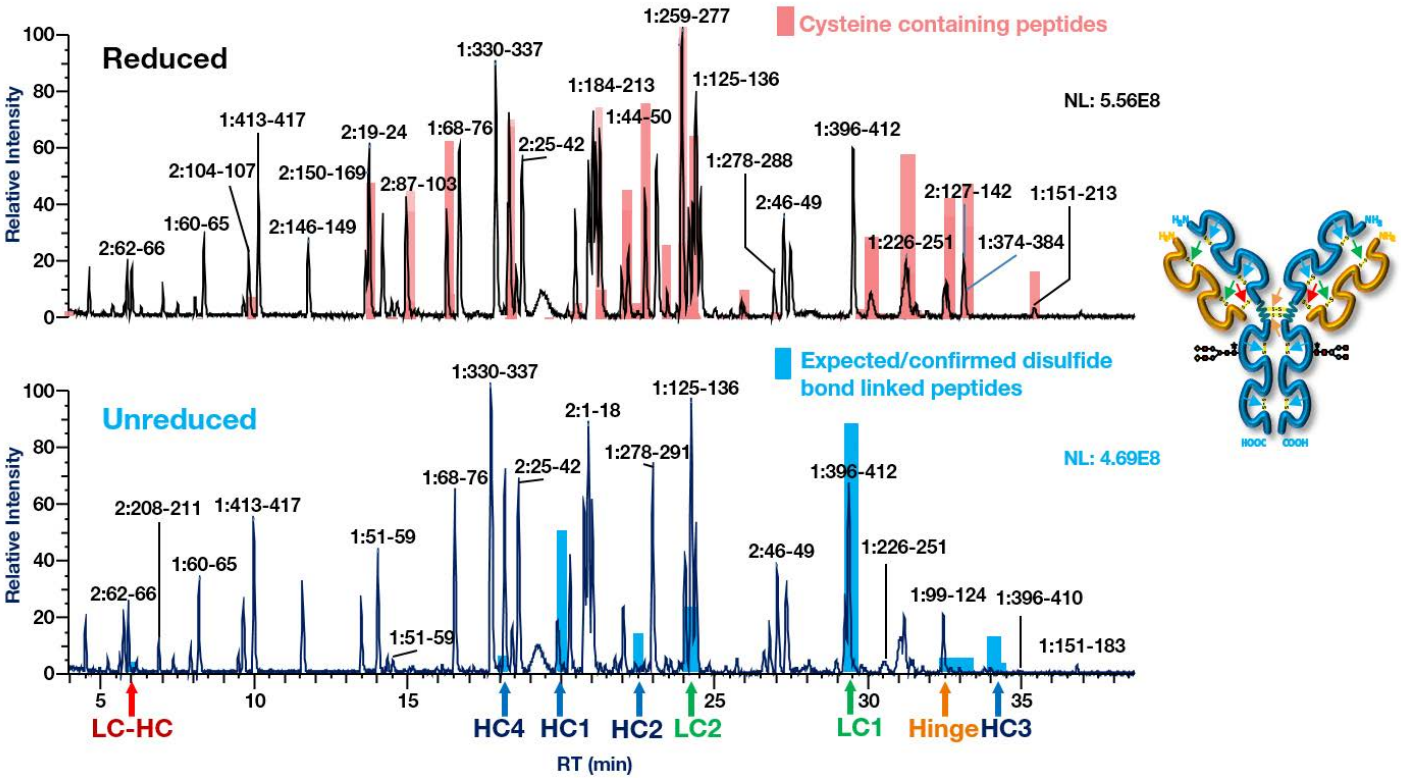


Figure 14. Base peak chromatogram (BPC) of infliximab and XICs for the C-terminal peptides of the heavy chain with and without lysine truncation for infliximab, bevacizumab, and trastuzumab. XICs were taken from the individual digests of the three mAb analyses. The Full MS spectra highlight the relative abundances of the singly and doubly charged peptides.

Disulfide bond mapping

Figure 15 highlights results from disulfide bond experiments of trastuzumab comparing a reduced vs. un-reduced sample. Differences in the base peak chromatograms are obvious by visual inspection, and using BioPharma Finder software they are identified as either free Cys-containing peptides (reduced sample) or of disulfide linked peptides (non-reduced sample) and provided as shaded peaks in the chromatogram. All expected intra- and interchain disulfide bonds were detected and confirmed with very good mass accuracies.



S-S Bond type		Peptide Sequence	Position	Δ ppm	RT
Intra-chain	LC 1	VTITCR/SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKR	C23-C88	1.42	29.28
	LC 2	SGTASVVCLLNNFYPR/VYACEVTHQGLSSPVTK	C134-C194	1.98	24.05
	HC 1	LSCAASGFNIK/AEDTAVYYCSR	C22-C96	1.54	19.94
	HC 2	NQVSLTCLVK/SRWQQGNVFSCSVMHEALHNHYTQK	C370-C428	-0.57	22.43
	HC3	STSGGTAALGCLVK/DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK	C147-C203	4.16	33.82
	HC4	CKVSNK/TPEVTCVVVDVSHEDPEVK	C324-C264	2.66	18.04
Inter-chain	LC-HC	SCDK/SFNRGEC	HC:C223-LC:C214	1.84	6.1
	Hinge region	THTCPPCPAPELLGGPSVFLFPPKPK/THTCPPCPAPELLGGPSVFLFPPKPK	C229-CC229/ C232-C232	2.33	32.43

Figure 15. Comparison of chromatograms obtained from a digested and reduced vs. digested and unreduced sample of trastuzumab. Color shading highlights cysteine-containing peptides. The table lists all expected intra- and interchain disulfide bridges that were identified and confirmed.

Figure 16 showcases one example of a light chain's intrachain disulfide bond marked yellow in the sequence window. The XIC of the precursor mass of the disulfide linked peptides shows the absence of this mass in the reduced sample. The precursor ion's isotope pattern detected in the non-reduced sample matches very well with the simulated pattern. Lastly, the fragment ion coverage map represents fragment ion assignment to both peptides involved based on the MS/MS spectrum obtained from the disulfide linked peptides, providing a high level of confidence in the correct identification.

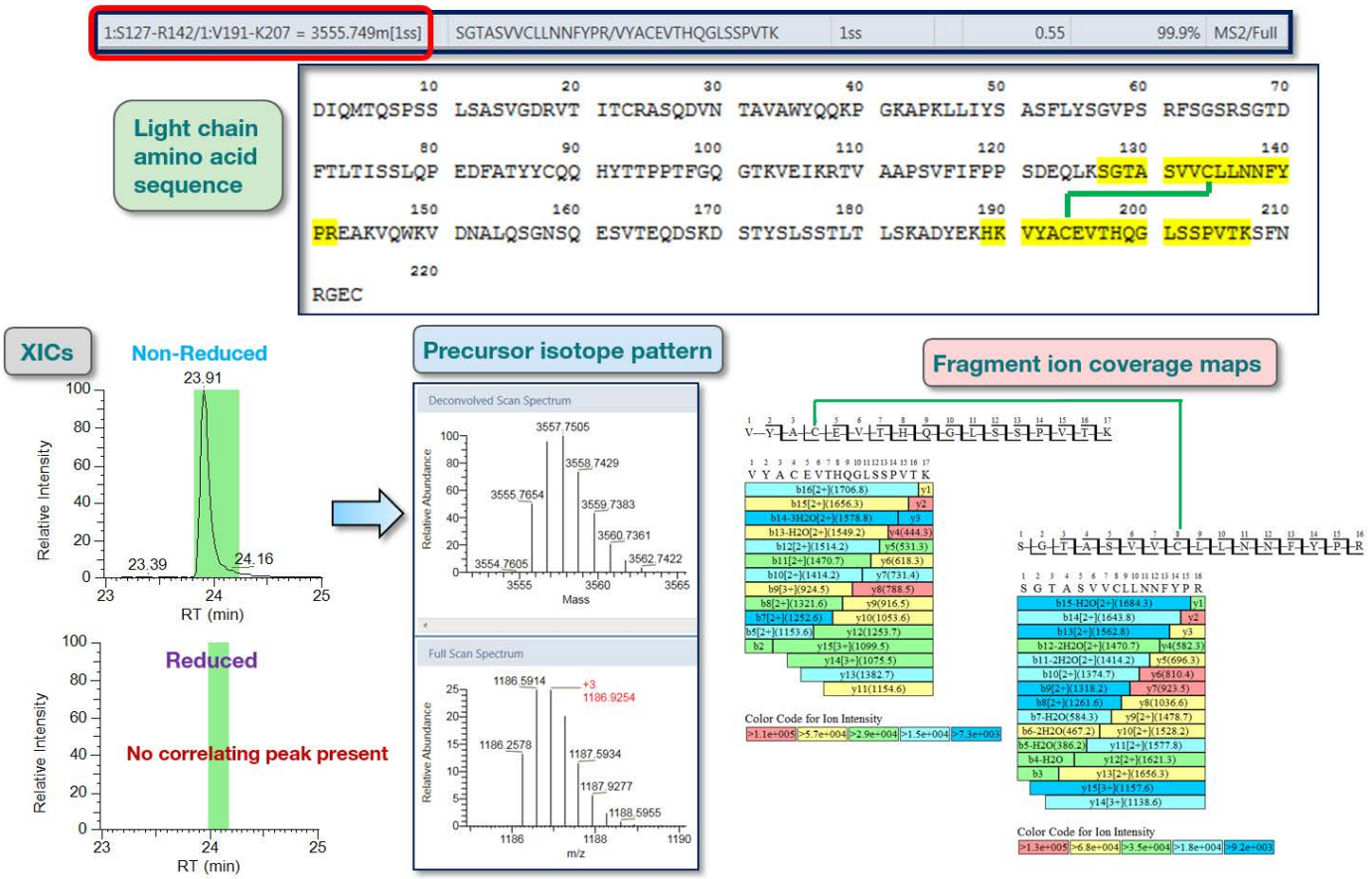


Figure 16. Example of an identified and confirmed intrachain disulfide linked peptide. The XIC based on the linked peptide mass shows the absence upon reduction of the sample. The detected isotope pattern of the un-reduced peptide matches very well with the theoretical isotope pattern. The fragment ion coverage maps provide assignment of fragment masses detected in the MS/MS spectrum resulting from fragmentation of both peptides involved.

Conclusions

- Here data obtained applying the three major workflows for characterization of biopharmaceuticals on one single instrument LC-MS platform is provided: 1) intact mass analysis, 2) subunit analysis, and 3) peptide mapping - aiming at confirming antibody sequences and disulfide bonds, elucidating modifications, and probing for scrambled disulfide bonds.
- The examples provided showcase the use of the three different modes included in the BioPharma Option: Standard Mode for peptide mapping analysis; Protein Mode for subunit and top-down analysis, and HMR Mode for intact mass analysis under denaturing and native conditions.
- The new High Mass Range Mode now also allows for analysis of antibodies and antibody drug conjugates under native conditions requiring a higher mass range up to *m/z* 8,000.
- Excellent mass accuracy, resolution, and sequence coverage were obtained at all stages of the workflows: intact molecular masses, masses of subunits, top-down fragments of subunits as well as peptides, providing very high confidence results.
- The SMART Digest kit provided an easy to use process with efficient and reproducible digestion of antibody samples in only 60 minutes.
- For infliximab, low lysine truncation was observed and confirmed at all molecular levels: on the intact mAb, the Fc/2 subunit, and the peptide level.

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Complete Characterization of a Cysteine-linked Antibody-Drug Conjugate Performed on a Hybrid Quadrupole-Orbitrap Mass Spectrometer with High Mass Range

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Poster Note 64802

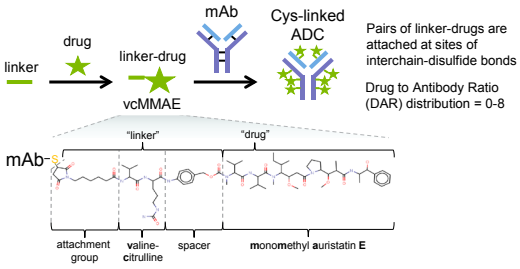
ABSTRACT

We have modified the instrument control software of a benchtop quadrupole-Orbitrap mass spectrometer to add native MS capability. In this study we demonstrate complete characterization of *Brentuximab vedotin*, a cysteine-linked ADC, which requires native MS conditions for intact analysis. We demonstrate preservation of non-covalent bonding of antibody subunits during electrospray ionization. HMR mode can be turned off for peptide mapping. We use trypsin peptide mapping approach with HCD fragmentation to achieve 99% coverage of the *Brentuximab vedotin* sequence using a single LC-MS analysis of a 90 min reverse phase gradient. Finally, we demonstrate that signature ions specific for HCD fragmentation of Brentuximab vedotin can be utilized to increase MS/MS assignment confidence.

INTRODUCTION

The complexity of modern therapeutic proteins, such as antibody-drug conjugates (ADCs), present a great analytical challenge which requires high resolution chromatography combined with high resolution mass spectrometry. Complementary MS approaches such as peptide mapping and intact mass analysis are needed for complete characterization of therapeutic proteins. Cysteine-linked ADCs present a unique challenge for characterization as proper intact analysis requires native MS conditions to preserve structurally-critical non-covalent binding between antibody chains. We have modified commercially-available Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF Orbitrap™ mass spectrometers to perform native LC-MS experiments. In the present study, we demonstrate this capability with intact analysis of *Brentuximab vedotin*, a cysteine-linked ADC **(Figure 1)**. Additionally, we have performed denaturing LC-MS and peptide mapping on these same instruments to generate complementary datasets for complete characterization.

Figure 1. Schematic for Constructing Cysteine-Linked ADC
Brentuximab vedotin is a cysteine-linked ADC which is constructed by modifying an antibody with vcMMAE, a preformed linker-drug comprised of a valine-cirtuline-based linker and a monomethyl auristatin E toxic drug. Saturatured (8 drugs) cys-linked ADCs are held intact with only non-covalent binding.



MATERIALS AND METHODS

Brentuximab vedotin was prepared for peptide mapping (reduction, alkylation, and trypsin digestion) or intact analysis (no treatment). For denaturing LC-MS intact analysis 1 µg of protein samples were separated using a 10 min gradient of 10-90% ACN in H_2O and 0.1% formic acid (Thermo MAb-Pac RP; flow rate 250 µL/min). For native LC-MS intact analysis 10 µg of sample was desalted online using size exclusion chromatography (Waters™ BEH SEC 4.6x150mm; 50 mM NH_4OAc isocratic elution, flow rate 300 µL/min) and directly presented to the mass spectrometer via electrospray ionization. Peptide mapping was performed using 2.5 µg of sample separated using a 90 min gradient of 2-90% ACN in H_2O and 0.1% formic acid (Acclaim RSLC 120 C18; flow rate 250 µL/min). Commercially-available Orbitrap mass spectrometers (Q Exactive HF and Q Exactive Plus) which were modified to include High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to m/z 8000. Native intact and denaturing MS data were acquired in HMR mode at setting of R=15k or 17.5k and deconvolved using the ReSpect™ algorithm and Sliding Window integration in Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software. Deconvolution species were identified automatically using the publicly-available FASTA sequence for Brentuximab vedotin, a mass tolerance of 50 ppm, and a static modification of Gln>Pyro-Glu for the heavy chain. Peptide mapping data were acquired by data dependent selection with R=60k or 70k for Full MS and R=15k or 17.5k for MS/MS. Peptide mapping data were searched using the MassAnalyzer algorithm in BioPharma Finder software with a tolerance of 5 ppm.

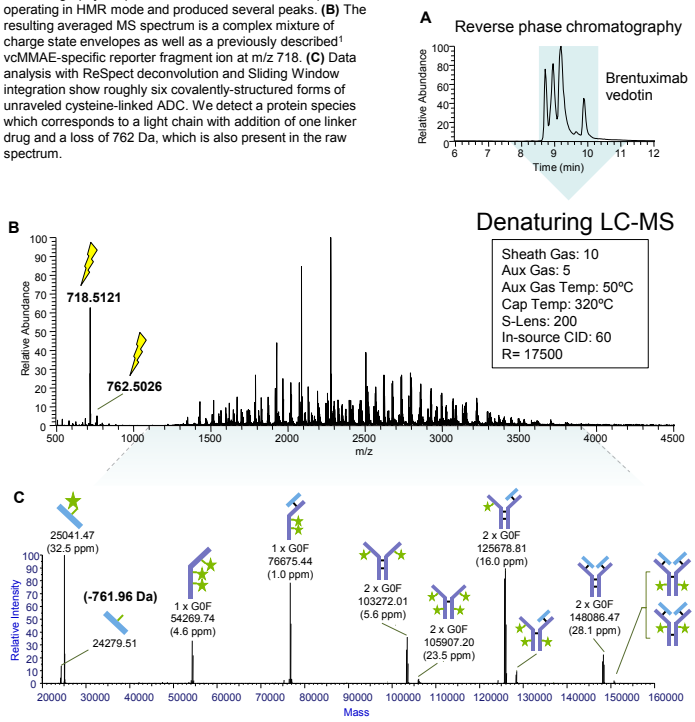
Figure 2. LC-MS Instrumentation for Complete ADC Characterization
All experiments were performed using a Vanquish UHPLC connected to a Exactive HF or Q Exactive Plus with High Mass Range (HMR) mode.

RESULTS

DENATURING LC-MS, CYSTEINE-LINKED ADC

Intact protein LC/MS analysis conventionally involves using mobile phases which are comprised of organic and acidic/basic pH, often suited specifically for reverse phase chromatography. This strategy can be useful for achieving high resolution protein separations. Conditions such as these, however, are not compatible for performing intact analysis on certain classes of compounds which require preservation of non-covalent bonds to maintain structural integrity, such as cysteine-linked ADCs. We demonstrate this phenomenon using the cysteine-linked ADC *Brentuximab vendotin*. Denaturing (reverse phase) LC/MS analysis of *Brentuximab vedotin* results in detection of roughly six unraveled forms **(Figure 3A-C)**. We observed a previously-reported[1] collisionally-induced m/z 718 fragment of the fragile vcMMAE linker-drug **(Figure 3B)**. Upon deconvolution we also observed a mass corresponding to light chain with addition of one vcMMAE and a loss of approximately 762 Da.

Figure 3. Denaturing LC-MS analysis
(A) Unmodified sample (1 ug) was analyzed by reverse phase chromatography coupled to a Q Exactive Plus Orbitrap MS operating in HMR mode and produced several peaks. **(B)** The resulting averaged MS spectrum is a complex mixture of charge state envelopes as well as a previously described[1] vcMMAE-specific reporter fragment ion at m/z 718. **(C)** Data analysis with ReSpect deconvolution and Sliding Window integration show roughly six covalently-structured forms of unraveled cysteine-linked ADC. We detect a protein species which corresponds to a light chain with addition of one linker drug and a loss of 762 Da, which is also present in the raw spectrum.



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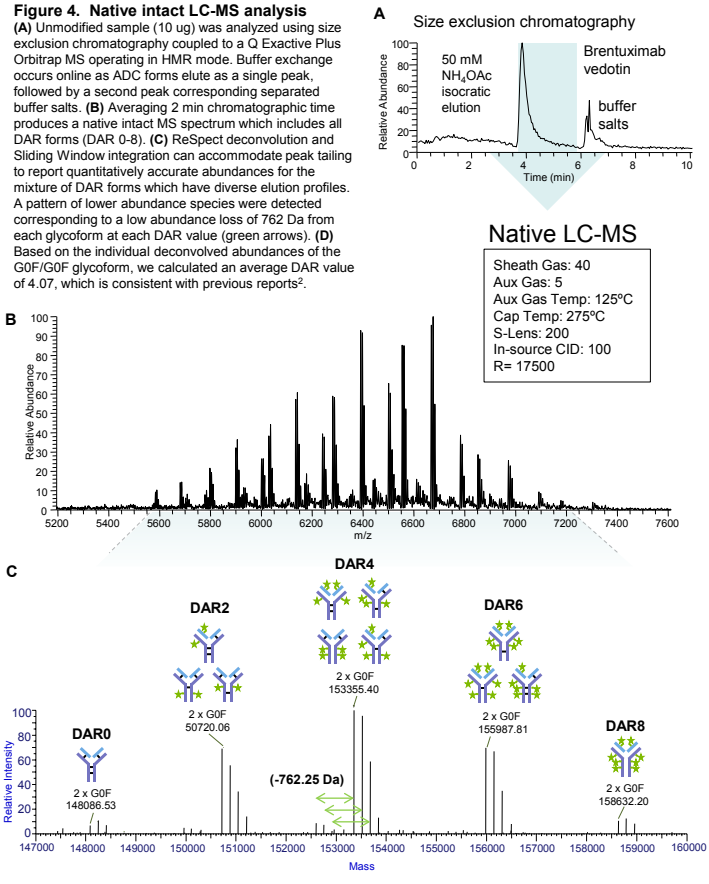
NATIVE INTACT LC-MS, CYSTEINE-LINKED ADC

Native MS intact protein analysis allows direct observation of molecules which rely on non-covalent interactions to preserve critical structural features, such as maintaining interchain associations which hold together cysteine-linked ADCs. The use of 100% aqueous physiological pH buffers in native MS analysis produces decreased charge states (increased m/z) and improves mass separation of heterogeneous mixtures. We performed native size exclusion LC-MS and observed 5 distinct species corresponding to intact *Brentuximab vedotin* with 0, 2, 4, 6, or 8 vcMMAE linker-drugs **(Figure 4)**. We measured an average drug-to-antibody ratio of 4.07, which is consistent with a previously published studies reporting 3.9-4.2 drugs per antibody[2] .

Figure 4. Native intact LC-MS analysis
(A) Unmodified sample (10 ug) was analyzed using size exclusion chromatography coupled to a Q Exactive Plus Orbitrap MS operating in HMR mode. Buffer exchange occurs online as ADC forms elute as a single peak, followed by a second peak corresponding separated buffer salts. **(B)** Averaging 2 min chromatographic time produces a native intact MS spectrum which includes all DAR forms (DAR 0-8). **(C)** ReSpect deconvolution and Sliding Window integration can accommodate peak tailing to report quantitatively accurate abundances for the mixture of DAR forms which have diverse elution profiles. A pattern of lower abundance species were detected corresponding to a low abundance loss of 762 Da from each glycoform at each DAR value (green arrows). **(D)** Based on the individual deconvolved abundances of the G0F/G0F glycoform, we calculated an average DAR value of 4.07, which is consistent with previous reports[2].



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G0F/G0F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	11.7	6.77
DAR2	23.1	69.23
DAR4	22.4	100.00
DAR6	40.5	69.75
DAR8	17.6	10.61

Average Drug-to-Antibody Ratio (DAR)
4.07

PEPTIDE MAPPING, CYSTEINE-LINKED ADC

A fundamental component of biotherapeutic protein characterization is peptide mapping. Whereas intact mass analysis aims to detect the abundances and distributions of mass deviation combinations, peptide mapping allows highly sensitive analysis of site-specific sequence features. The vcMMAE linker-drug on *Brentuximab vedotin* poses particular challenges when attempting to identify drug conjugation sites. We prepared a sample for peptide mapping using reduction and alkylation to block non-drug-conjugated cysteines, followed by trypsin digestion. In one 90 min LC-MS gradient we were able to achieve 99% sequence coverage for both light and heavy chains and detect peptides spanning all four drug conjugation sites. HCD fragmentation allowed detection of a peptide in the hinge region of the heavy chain that is differentially modified with 0-2 vcMMAE drugs. As a result efficient elution requires sustained delivery of high organic mobile phase.

Figure 5. Peptide Mapping of Cysteine-Linked ADC *Brentuxmab vedotin*
Reduced, alkylated, and trypsin-digested sample (2.5 ug) was separated using a 90 min gradient and eluted into a Q Exactive HF (equipped with HMR mode) operating in Standard mode. Using a mass accuracy cut off of 5 ppm, we achieved 99% sequence coverage of both light and heavy chains. We detected known glycopeptides and were able to detect MMAE-conjugated peptides at all four cysteines (red circles) which are normally involved in interchain disulfide pairs in naked antibodies. A trypsin peptide sequence at the hinge region of the heavy chain (red asterisk) was present in forms ranging from 0-2 vcMMAE conjugations. A missed cleavage peptide contained up to 3 conjugations.

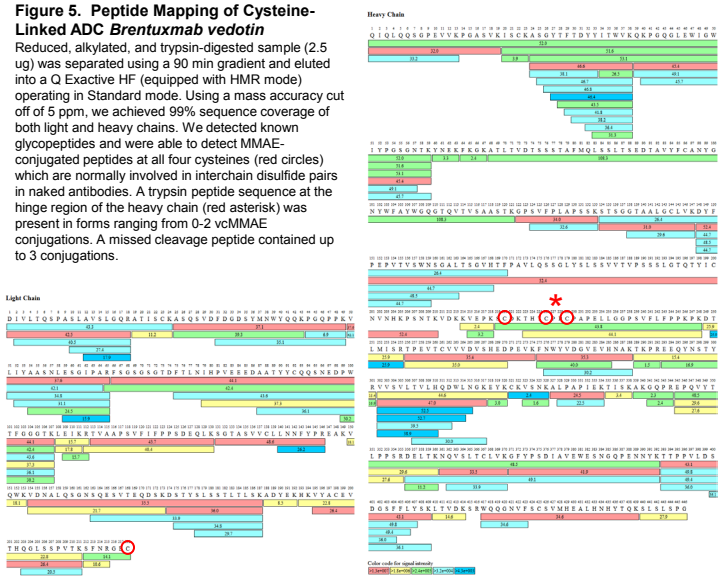


Figure 6. Hinge Region Peptide of Cysteine-linked ADC is Site of Multiple Conjugations
(A) Our data analysis in BioPharma Finder software resulted in detection of peptides which covered the hinge region of the heavy chain (red asterisk). A faithfully-trypsin-cleaved THT-KPK peptide was detected with 0-2 vcMMAE conjugations at cysteines (red circles) normally involved in interchain disulfide pairs. **(B)** Addition of vcMMAE to peptides dramatically increases hyphobicity which results in poor elution and increased retention time. MS/MS analysis of the **(C)** 1 linker-drug (both positional isomers) and **(D)** two linker-drug forms in BioPharma Finder allowed clear sequencing of y-ions in the hinge peptides, and thus facilitated automatic detection.

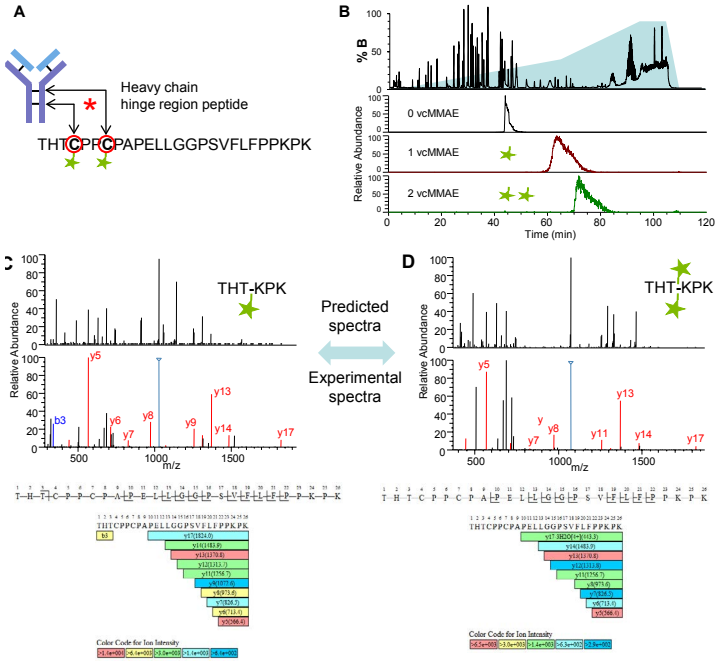
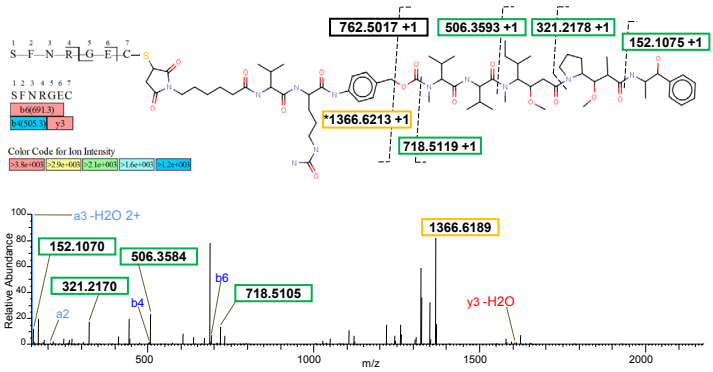


Figure 7. HCD Signature Fragment Ions for vcMMAE Linker-Drug
The light chain C-terminal peptide SFN-GEC is a conjugation site for vcMMAE. This modified peptide was automatically identified by BioPharma Finder (left side top panel). Further manual inspection produced additional fragment assignments for vcMMAE signature ions (right side top panel). Theoretical masses (top panel) were calculated manually and matched to experimental masses (bottom panel) within 5ppm (green boxes). A cleavage site for the loss of 762 Da is shown (black box; theoretical monoisotopic mass = 762.5017). We observed a high abundance ion at m/z 1366.6189 (orange box, asterisk) which corresponds to the peptide-retaining fragment pair of a 762 Da loss with an additional loss of 2 protons, presumably due to formation of a seven-membered aromatic ring.



CONCLUSIONS

•We have modified the control software in Q Exactive Plus and Q Exactive HF mass spectrometers to add native MS capability.

•Native LC/MS intact analysis of Brentuximab vedotin resulted in detection of intact ADC forms, DAR0-8. ReSpect deconvolution and Sliding Window integration showed an average DAR of 4.07, consistent with previous studies.

•Acquisition of MS/MS spectra with HCD fragmentation on Q Exactive Plus and Q Exactive HF Orbitrap mass spectrometers followed by data analysis with BioPharma Finder resulted in 99% sequence coverage from a single 90 min gradient using 5 ppm mass tolerance.

•Addition of vcMMAE linker drug dramatically increases peptide hydrophobicity and retention time.

•Signature HCD fragment ions of linker-drug may allow additional means for identifying drug-conjugated peptides.

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- Native intact mass analysis
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Separation of mAb Fragments on a High-Resolution HIC Column

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Key Words

Hydrophobic interaction chromatography, HIC, monoclonal antibody, mAb, papain digestion, Fab and Fc fragments, MAbPac HIC-20

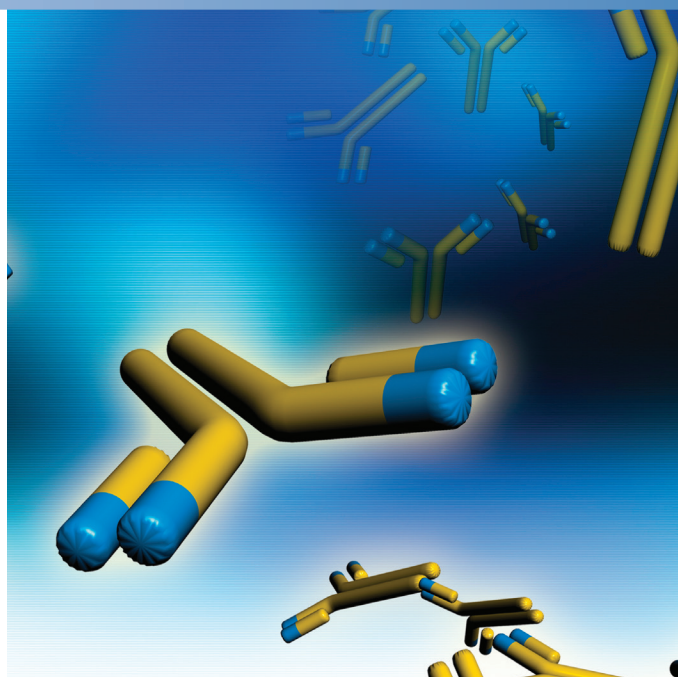
Goal

To demonstrate the separation of Fab and Fc fragments obtained by papain digestion by using a Thermo Scientific™ MAbPac™ HIC-20 column.

Introduction

Monoclonal antibodies (mAbs) are the most prominent and fastest growing class of therapeutic proteins. With their excellent biocompatibility and high specificity, mAbs have been shown to be effective against not only cancer and inflammatory diseases but also rare diseases such as Crohn's disease and paroxysmal nocturnal haemoglobinuria.[1] Recombinant mAbs can be highly heterogeneous due to various biochemical modifications such as sialylation, oxidation, deamidation, and C-terminal lysine truncation. Some of these modifications may reduce stability and efficacy of the drug. Therefore, it is critical to detect, characterize, and quantify impurities, as well as biochemical variants, during production of mAb therapeutics. A variety of HPLC methods are used to assess mAb heterogeneity by analyzing either the intact mAb or fragments after digestion.[2]

Separation of Fab and Fc fragments generated by papain digestion of mAbs is often employed to enhance the detection of some mAb variants. Papain cleaves an antibody into three fragments at the hinge region which produces two Fab fragments and one Fc fragment (Figure 1). Analysis of papain digested mAbs not only allows for the detection of variants that are not visible at the intact mAb level but also provides positional information on whether the modification is on the Fab or Fc fragment. Among various HPLC methods, hydrophobic interaction chromatography (HIC) often provides high-resolution separation of Fab and Fc fragments, thus is generally used for mAb variant analysis.[2-4]



HIC separates proteins in order of increasing hydrophobicity under non-denaturing conditions. Analytes bind to the weakly hydrophobic stationary phase in the presence of high salt concentration and elute off the column as the salt concentration decreases. Unlike reversed-phase liquid chromatography that typically denatures the protein, effectively destroying the native conformation, HIC typically preserves the native structure and bioactivity of the protein, which is useful for downstream functional analysis such as binding and cell-based potency assays. It also allows for the separation on the basis of conformational changes occurring in the native form. In addition, HIC typically provides separation with little or no carryover. Therefore, HIC is not only useful for separation of mAb variants but also valuable as a purification method for mAb products by biopharmaceutical companies.

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This application note describes the use of the new MAbPac HIC-20 column to separate Fab and Fc fragments of mAb therapeutics. The MAbPac HIC-20 column is a wide-pore (1000 Å), 5 µm silica-based HIC column well suited for the separation of high molecular weight mAb variants. The proprietary column chemistry provides high resolution, rugged stability, and desired selectivity for the analysis of mAb heterogeneity. In the examples shown here, high resolution separations of Fab and Fc fragments were achieved and the variants of these fragments were observed as well.

Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Isopropanol (Fisher Scientific P/N A461-4)
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, ≥98.0%)
- Ammonium sulfate [$(NH_4)_2SO_4$, ≥99.0%]
- Tris (hydroxymethyl) aminomethane (Fisher Scientific P/N T395-500)
- Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8\ 2H_2O$, ≥ 99%)
- L-cysteine (Fisher Scientific P/N AC173600250)
- Papain, lyophilized (≥10 units/mg protein)

Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Preparation of 2× papain digestion buffer

Dissolve 0.485 g of Tris, 0.060 g of EDTA, and 0.024 g of L-cysteine in 15 mL of DI water. Adjust the pH to 7.6 using HCl and bring the volume to 20 mL with DI water.

Papain digestion

Prepare a 2 mg/mL papain solution in DI water. Then mix the following in a microtube and incubate it for 4 h in a 37 °C water bath:

mAb solution (5 mg/mL)	80 µL
Papain (2 mg/mL)	4 µL
Water	16 µL
2× Papain digestion buffer	100 µL

Add 200 µL of mobile phase A before HPLC analysis. The final concentration of mAb is 1 mg/mL.

LC Separation

The LC separation conditions were as follows:

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS LC system equipped with:
	SR-3000 Solvent Rack (without degasser) (P/N 5035.9200)
	LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	VWD-3400RS Rapid Separation Variable Wavelength Detector equipped with a micro flow cel (P/N 5074.0010)
Columns	MAbPac HIC-20, 4.6 × 100 mm (P/N 088553)
	MAbPac Protein A, 4 × 35 mm (P/N 082539)
Mobile phase A	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0 Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) and 264.2 g of ammonium sulfate in 800 mL of DI water. Adjust the pH to 7.0 with 50% sodium hydroxide (NaOH) solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.
Mobile phase B	100 mM sodium phosphate, pH 7.0 Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) in 900 mL of DI water. Adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.

Gradient 1 (Standard gradient)

Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

Gradient 2 (Shallow gradient)

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Flow rate	1 mL/min
Run time	20 min
Temperature	30 °C
UV detector wavelength	280 nm

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

Two different therapeutic mAbs were digested with papain resulting in Fab and Fc fragments. The separations of these fragments were carried out using the MAbPac HIC-20 column, and the data was compared with that of intact mAbs. Initially, these samples were tested with 2 M ammonium sulfate as the starting mobile phase. For both mAb1 and mAb2, Fab and Fc fragments were well resolved in 20 minutes (Figures 2b and 3b). Separation was further optimized by simply reducing the starting ammonium sulfate concentration to 1.2 M (by adjusting the gradient) while maintaining the overall gradient time (Figures 4b and 5b). Addition of organic solvent did not further improve the resolution (data not shown).

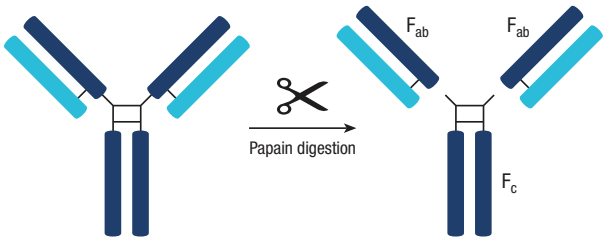

Figure 1. Schematic representation of papain digestion of monoclonal antibody

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 × 100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-5.0	100	0
	0.0	100	0
	1.0	100	0
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

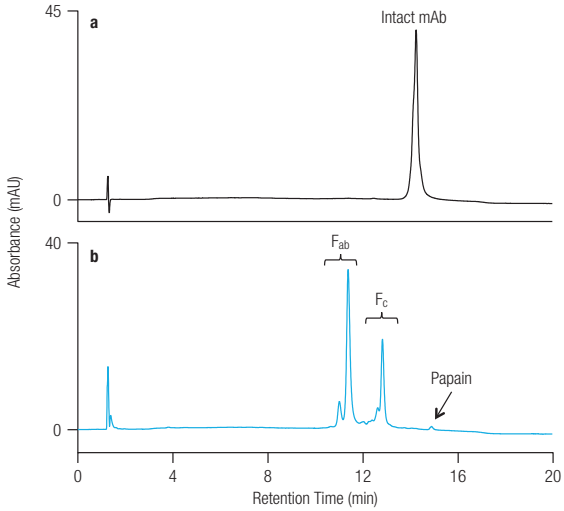

Figure 2. Separation of mAb1 and papain digested mAb1 using standard gradient

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 × 100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-5.0	100	0
	0.0	100	0
	1.0	100	0
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

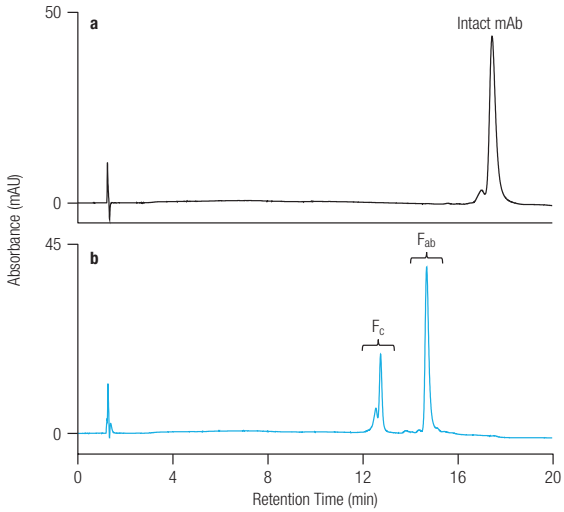

Figure 3. Separation of mAb2 and papain digested mAb2 using standard gradient

Since two Fab fragments and one Fc fragment are generated when a mAb is digested with papain, the peak assignment was done based on peak area. Further confirmation of the peak assignment was carried out using the MAbPac Protein A column (data not shown).

For mAb1, the Fab fragment is less hydrophobic than the Fc fragment and thus eluted first; for mAb2, the opposite trend is observed. Compared to corresponding intact mAbs (Figures 2a and 3), mAb fragments were more hydrophilic in both cases. The intact mAb1 chromatogram showed an asymmetric peak most likely due to the presence of unresolved variants (Figure 4a). After papain digestion, minor variant peaks in front of both Fab and Fc fragments were observed. The separation of these variant peaks was improved with a shallower gradient (Figure 4b). For mAb2, the intact mAb chromatogram shows a small peak in front of the main mAb peak (Figure 5a) and one variant peak is observed in front of the Fc fragment (Figure 5b).

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 × 100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-5.0	60	40
	0.0	60	40
	1.0	60	40
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

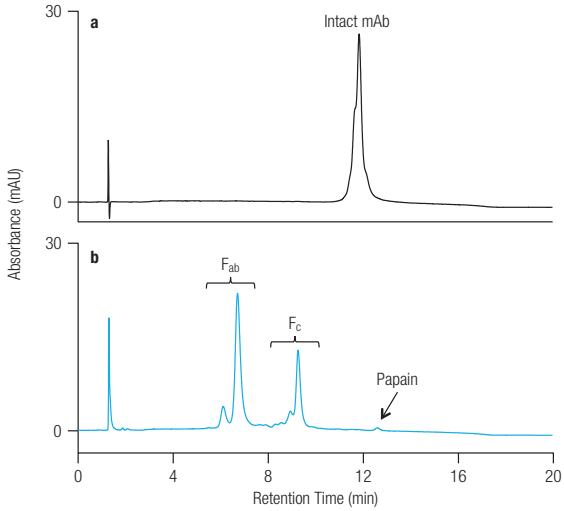

Figure 4. Separation of mAb1 and papain digested mAb1 using shallow gradient

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 × 100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-5.0	60	40
	0.0	60	40
	1.0	60	40
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	Intact mAb	5 µL	
	Papain digest	12 µL	
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	a. Intact mAb (2.5 mg/mL)		
	b. Papain digest (1 mg/mL)		

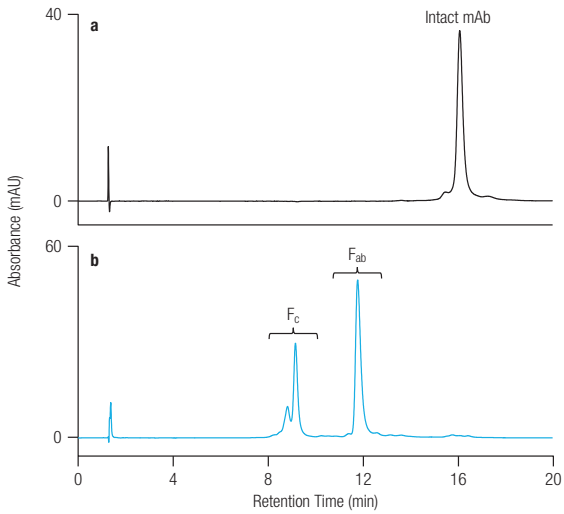

Figure 5. Separation of mAb2 and papain digested mAb2 using shallow gradient

Conclusion

- The MAbPac HIC-20 column provides the desired selectivity for separation of Fab and Fc fragments from the native mAb.
- The MAbPac HIC-20 column is a valuable tool for the analysis of mAb variants.

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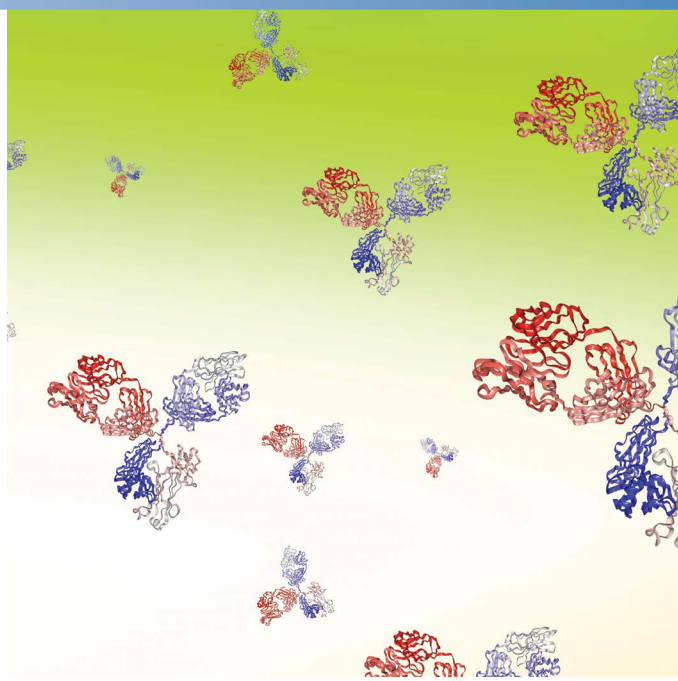
Fast Analysis of Therapeutic Monoclonal Antibody Fragments Using a Supermacroporous, Reversed-Phase Chromatography Column

Shanhua Lin, Nebojsa Avdalovic, and Xiaodong Liu
Thermo Fisher Scientific, Sunnyvale, CA

Application Note 21200

Key Words
MAbPac RP, rituximab, trastuzumab, infliximab, bevacizumab

Goal
To describe a high-resolution, high-speed separation of monoclonal antibody (mAb) fragments using a supermacroporous, reversed-phase column. Baseline separations of light chain (LC) and heavy chain (HC), crystallizable fragment (Fc) and antigen-binding fragment (Fab), single chain Fc (scFc) and $F(ab')_2$ were achieved for rituximab, trastuzumab, infliximab, and bevacizumab using a 10 minute gradient with water/acetonitrile/formic acid/trifluoroacetic acid mobile phases.



Introduction

A comprehensive characterization of mAb purity, aggregates, and variants is required for the final biopharmaceutical product approval and subsequent manufacturing process. We chose therapeutic mAbs (rituximab, trastuzumab, infliximab, and bevacizumab), produced mostly by mammalian cells, to study the reversed-phase chromatographic behavior of intact mAbs as well as of their fragments. These biological products are heterogeneous due to post-translational modifications.[1] Additional modifications, such as oxidation, could be introduced during the manufacturing process,[2] and the results of this analysis are also included.

There is a growing trend to obtain intact mass information as well as the glycan profile in the QC of mAbs using high-resolution mass spectrometers (MS). The most commonly employed LC-MS method is to analyze mAbs via reversed-phase liquid chromatography coupling with mass spectrometry detection. Further MS analysis of mAb fragments such as light chain (LC), heavy chain (HC), crystallizable fragment (Fc), and antigen binding fragment (Fab) can quickly reveal the location as well as nature of the modification.[3]

Here, we present a fast separation method for mAb fragments from rituximab, trastuzumab, infliximab, and bevacizumab using a novel supermacroporous, reversed-phase column. Baseline separations of LC and HC, Fc and Fab, single chain Fc (scFc) and $F(ab')_2$ fragments (two antigen-binding Fab portions linked together by disulfide bonds) were achieved in all cases using a 10 min gradient with water/acetonitrile/formic acid/trifluoroacetic acid mobile phase. The results demonstrate the general applicability of Thermo Scientific™ MAbPac™ RP column for mAb analysis.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Fisher Chemical™ Optima™ LC/MS acetonitrile (MeCN) [C_2H_3N; P/N A955-1]
- Fisher Chemical Optima LC/MS formic acid (FA) [CH_2O_2; P/N A117-1AMP]
- Fisher Chemical Optima LC/MS trifluoroacetic acid (TFA) [$C_2HF_3O_2$; P/N A116-50]
- Dithiothreitol (DTT) and papain, purchased from Sigma-Aldrich®
- FabRICATOR® (IdeS protease), purchased from Genovis
- Rituximab, trastuzumab, infliximab, and bevacizumab were from the original manufacturers

Sample Handling Equipment

Thermo Scientific™ Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Reduction

Reduction of inter-chain disulfides in a mAb (4 mg/mL) was achieved by incubation of mAb with 20 mM dithiothreitol (DTT) at 37 °C for 30 min.

Papain digestion

Digestion was carried out by incubating mAb (2 mg/mL) with papain (0.04 mg/ml) in 100 mM Tris-HCl, pH 7.6, 4 mM EDTA, and 5 mM cysteine buffer at 37 °C for 4 hours.

IdeS digestion

IdeS protease was added at 1 unit enzyme per 1 µg of mAb ratio. The digestion was carried out in 50 mM sodium phosphate, 150 mM NaCl (pH 6.6) buffer at 37 °C for 30 min.

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:		
	SRD-3400 Solvent Racks with Degasser (P/N 5035.9245)		
	HPG-3400RS Biocompatible Binary Rapid Separation Pump (P/N 5040.0046)		
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)		
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)		
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a semi-micro flow cell PEEK, 2.5 µL volume, 7 mm path length		
Column	MAbPac RP, 4 µm, 3.0 × 50 mm (P/N 088645)		
Mobile phase A	H_2O/FA/TFA (99.88:0.1:0.02 v/v/v)		
Mobile phase B	MeCN/H_2O/FA/TFA (90:9.88:0.1:0.02 v/v/v/v)		
Gradient	Time (min)	%A	%B
	0.0	80	20
	1.0	80	20
	11.0	55	45
	12.0	55	45
	14.0	80	20
	15.0	80	20
Flow rate	0.5 mL/min		
Column temperature	80 °C		
UV detector wavelength	280 nm		
Injection volume	5 µL		
Samples	Rituximab, 5 mg/mL; trastuzumab, 5 mg/mL; infliximab, 5 mg/mL; bevacizumab, 5 mg/mL		

Software

The Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System was used for data acquisition and analysis.

Results and Discussion

The MAbPac RP column is packed with supermacroporous polymer resin. Its large pore size (~ 1500 Å) is suitable for separation of large proteins, such as 150 kDa mAb and its 25, 50, and 100 kDa fragments. In addition, mAb analysis by reversed-phase liquid chromatography requires high temperatures such as 70 to 80 °C to reduce the strength of secondary interactions between mAb and the stationary phase.[4] The polymeric nature of the MAbPac RP column provides both pH and temperature stability, making it an ideal column for mAb analysis.

Monoclonal antibodies are heterogeneous (Figure 1). Comprehensive analysis of mAb post-translational modifications, such as deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine (Met) oxidation, and glycosylation, requires complete digestion of the mAbs and sequencing of all the peptides.

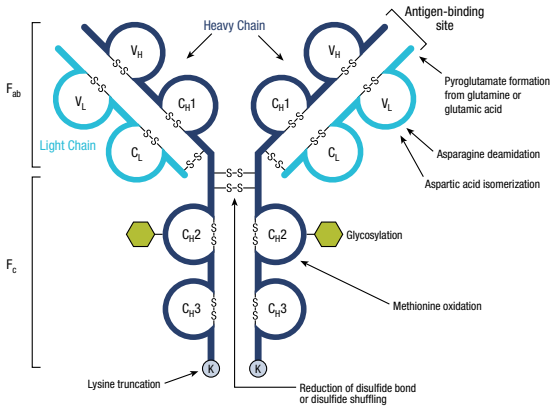


Figure 1. Structure of IgG and typical forms of heterogeneity.

However, peptide mapping can be time consuming. A simple and direct way to analyze mAb variants and locate the modification sites is to measure mAb fragments.[3] Light chains (LC) and heavy chains (HC) are generated by the reduction of mAbs with DTT (Figure 2a). Fc and Fab fragments are generated by papain digestion (Figure 2b). Single chain Fc (scFc) and F(ab')2 fragments are generated by IdeS digestion (Figure 2c).

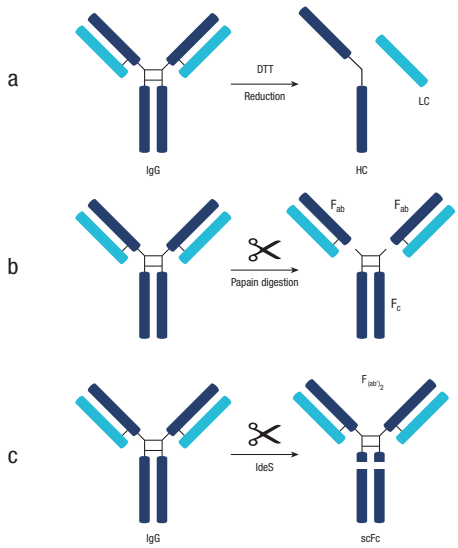
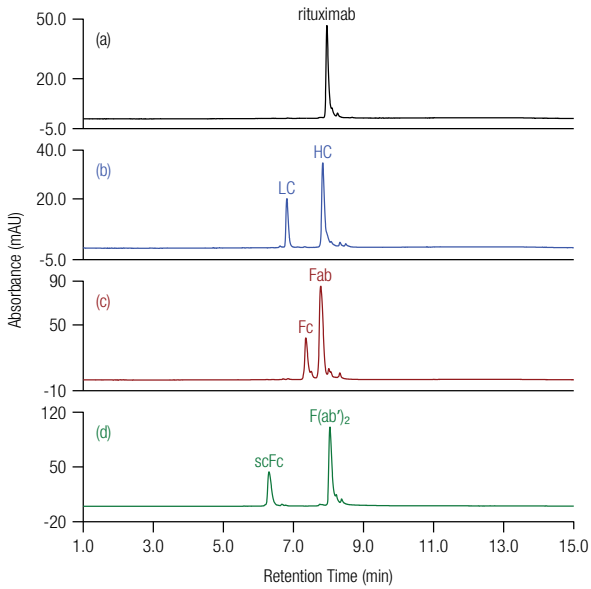


Figure 2. Monoclonal antibody fragments generation flow chart. (a) Reduction; (b) Papain digestion; (c) IdeS digestion.

Rituximab (Figure 3a), trastuzumab (Figure 4a), infliximab (Figure 5a), and bevacizumab (Figure 6a) were analyzed on an MAbPac RP column using a 10 minute gradient. In all cases, in addition to the main mAb peak, minor variant peaks eluting at a later retention time were observed. DTT reduction of mAb produces two copies of LC (25 kDa) and two copies of HC (50 kDa). The analysis of LC and HC of each mAb (Figures 3b, 4b, 5b, and 6b) showed two major peaks, plus small variant peaks eluting after HC. The peak area ratio between LC and HC is approximately 1:2. The degree of separation varied, with rituximab LC and HC giving the best resolution, and infliximab LC and HC the least.



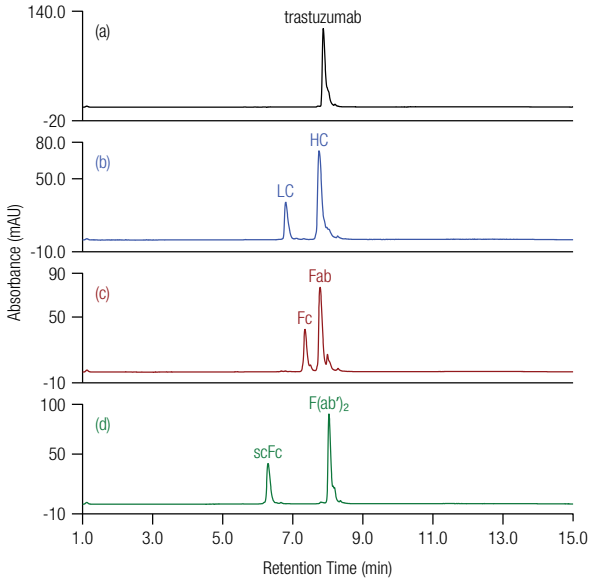
Column:	**MAbPac RP,** 4 µm
Format:	3 × 50 mm
Mobile Phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile Phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) rituximab (5 mg/mL) (b) rituximab + DTT (4 mg/mL) (c) rituximab + Papain (2 mg/mL) (d) rituximab + IdeS (2 mg/mL)

Figure 3. Analysis of rituximab and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and $F(ab')_2$ fragments.

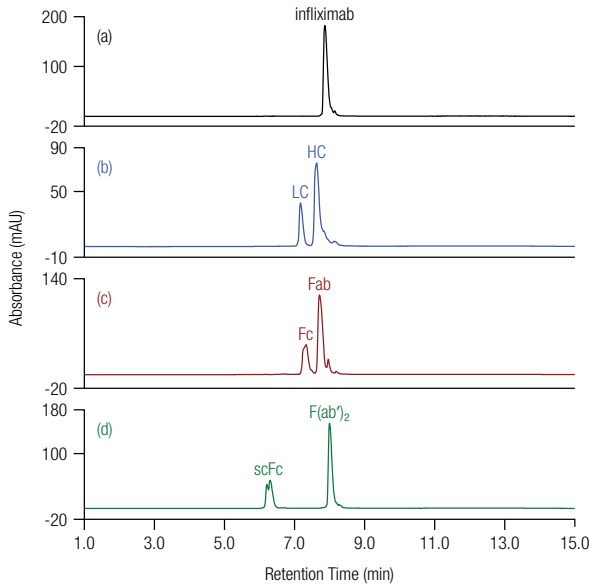
Papain digestion produces one copy of Fc (50 kDa) and two copies of Fab (50 kDa). The separation of Fc and Fab of each mAb (Figures 3c, 4c, 5c, and 6c) showed two major peaks, plus small variant peaks eluting after both Fc and Fab peaks. The peak area ratio between Fc and Fab is approximately 1:2. In the case of bevacizumab, we observed peaks co-eluting in the Fab region. Further MS analysis identified two polypeptide chains with the later eluting peak corresponding to the bevacizumab Fab, based on the molecular weight calculation. The earlier eluting peak has a smaller molecular weight comparing to the Fab (data not shown).



Column:	**MAbPac RP**, 4 µm
Format:	3 × 50 mm
Mobile Phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile Phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	Time (min) %A %B 0.0 80 20 1.0 80 20 11.0 55 45 12.0 55 45 14.0 80 20 15.0 80 20
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) trastuzumab (5 mg/mL) (b) trastuzumab + DTT (4 mg/mL) (c) trastuzumab + Papain (2 mg/mL) (d) trastuzumab + IdeS (2 mg/mL)

Figure 4. Analysis of trastuzumab and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and F(ab')$_2$ fragments.

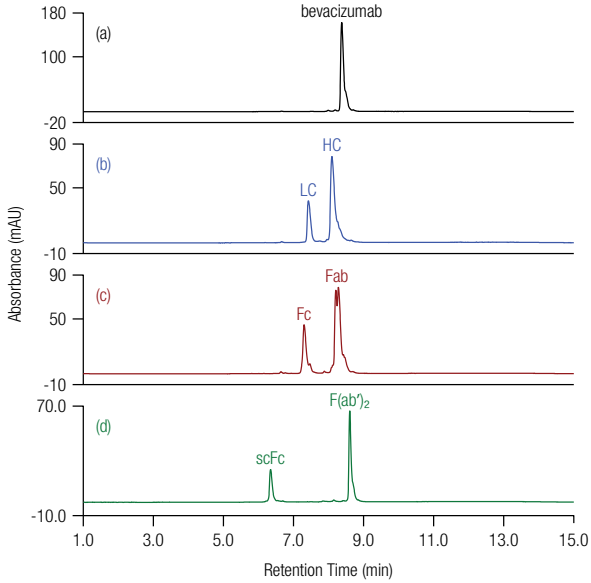
IdeS digestion produces two copies of scFc (25 kDa) and one copy of F(ab')$_2$ (100 kDa). The analysis of scFc and F(ab')$_2$ of each mAb (Figures 3d, 4d, 5d, and 6d) showed two major peaks, plus small variant peaks eluting after the F(ab')$_2$ peak. In the case of infliximab (Figure 5d), we observed two co-eluting scFc peaks. Further MS analysis revealed that the early eluting peak has one c-terminal lysine while the later eluting one does not have c-terminal lysine (data not shown). The chromatogram of infliximab Fc (Figure 5c) showed a broad peak. We hypothesize that this is due to co-eluting charge-variant species containing zero lysine, one C-terminal lysine, and two C-terminal lysines.



Column:	**MAbPac RP**, 4 µm
Format:	3 × 50 mm
Mobile Phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile Phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	Time (min) %A %B 0.0 80 20 1.0 80 20 11.0 55 45 12.0 55 45 14.0 80 20 15.0 80 20
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) infliximab (5 mg/mL) (b) infliximab + DTT (4 mg/mL) (c) infliximab + Papain (2 mg/mL) (d) infliximab + IdeS (2 mg/mL)

Figute 5. Analysis of infliximab and and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and F(ab')$_2$ fragments.

The mobile phase used in this study contained 0.1% FA and 0.02% TFA. The lower TFA content resulted in a more MS-compatible eluent. Alternative mobile phases containing higher concentrations of TFA (0.1%) would result in better separation of mAb fragments.



Column:	**MAbPac RP**, 4 µm
Format:	3 × 50 mm
Mobile Phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile Phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	Time (min) %A %B
	0.0 80 20
	1.0 80 20
	11.0 55 45
	12.0 55 45
	14.0 80 20
	15.0 80 20
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) bevacizumab (5 mg/mL)
	(b) bevacizumab + DTT (4 mg/mL)
	(c) bevacizumab + Papain (2 mg/mL)
	(d) bevacizumab + IdeS (2 mg/mL)

Figure 6. Analysis of bevacizumab and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and F(ab')$_2$ fragments

Conclusion

- The MAbPac RP column provides the ideal solution for separation of mAbs and related variants.
- The MAbPac RP column baseline separates reduction products: LC and HC.
- The MAbPac RP column baseline separates papain digest products: Fc and Fab.
- The MAbPac RP column baseline separates IdeS digest products: scFc and F(ab')$_2$.

References

1. Vlasak, J.; Ionescu, R. Heterogeneity of Monoclonal antibodies revealed by charge-sensitive methods, *Curr. Pharm*. Biotechnol. **2008**, *9*, 468.
2. Haverick, M.; Mengisen, S.; Shameem, M.; Ambrogelly, A. Separation of mAbs molecular variants by analytical hydrophobic interaction chromatography HPLC, *mAbs* **2014**, *6,* 852.
3. Stackhouse, N.; Miller, A. K.; Gadgil, H. S. A high-throughput UPLC method for the characterization of chemical modifications in monoclonal antibody molecules, *J. Pharm. Sci*. **2011**, *100*, 5115.
4. Fekete, S.; Rudaz, S.; Veuthey, J. L.; Guillarme, D. Impact of mobile phase temperature on recovery and stability of monoclonal antibodies using recent reversed-phase stationary phases., *J. Sep. Sci*. **2012**, *35*, 3113.

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LC-MS Analysis of Digested mAbs Using a Reversed-Phase Desalting Cartridge and High-Resolution, Accurate Mass Spectrometry (HRAMS)

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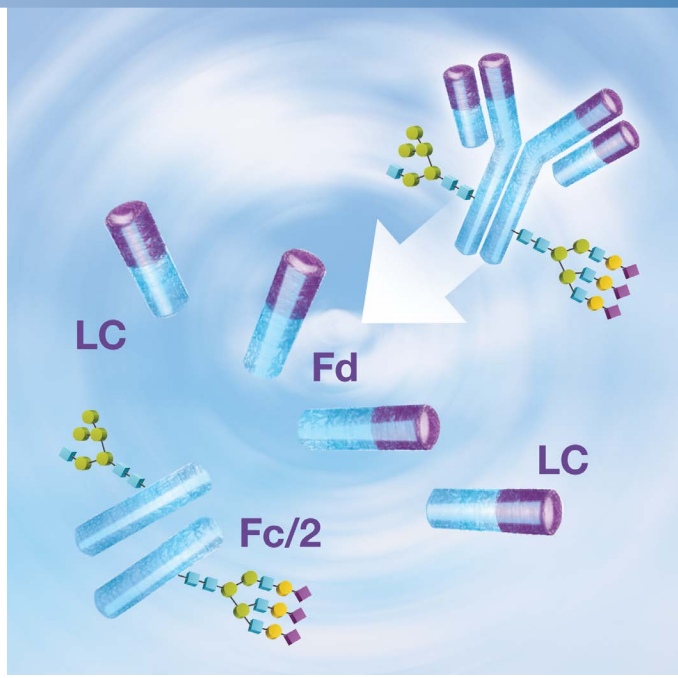
Application Note 21239

Key Words
Desalting, MSPac, monoclonal antibody, mAb, fragment, Fc, Lc, Fd', reversed phase, proteomics, mass spectrometry, biopharmaceutical, biomolecules, glycosylation, carry-over, intact protein

Goal
To describe a proof of concept analysis that demonstrates a simple, straightforward desalting method to optimize chromatographic separations for reduced analysis time, increased resolution, and improved MS detection of monoclonal antibody (mAb) fragments (Lc, Fc/2, and Fd') following digestion and reduction of a pharmaceutical mAb. Desalting and separation were conducted on a 2.1 × 10 mm Thermo Scientific™ MSPac™ DS-10 cartridge using conventional water/acetonitrile based eluents.

Introduction
The development and application of mAbs as biopharmaceutical therapeutics has grown rapidly in the last decade. This class of biomolecular drugs has proven effective for the treatment of a broad spectrum of diseases including cardiovascular, autoimmune disorders, and cancers.[1] While the fidelity of recombinant mAb production is generally very high, a range of biochemical modifications can occur during cellular production, mAb recovery, purification, and storage. These variations in structure can influence the safety and efficacy of the mAb, and it is therefore essential to fully characterize the nature and occurrence of structural variation and the subsequent effects on drug properties.[2]

Mass spectrometry (MS) is an essential tool in the characterization of mAbs, providing molecular weight determinations and structural information of intact as well as digested mAbs. Direct infusion of the sample into the MS is the simplest approach to characterize the sample. However, salts, detergents, and other matrix components can interfere with accurate MS characterization by decreasing sensitivity and resulting in instrument fouling, ultimately leading to downtime through extended instrument maintenance periods. Additionally, information regarding the types and locations of post-translational protein modifications can be difficult to determine without chromatography.



Reduction and/or digestion of mAbs to generate smaller protein fragments is a common analytical approach. Reducing the disulfide bonds breaks the mAb into smaller fragments. These are easier to characterize by MS than the intact mAb, therefore improving identification of structural changes across the mAb structure . Reducing the size of the proteins in the sample allows the acquisition of isotopically resolved charge states and the possibility of deconvolution to exact monoisotopic masses. Additionally, structural changes to the mAb can be isolated to a specific region (e.g., Fc, Lc, or Fd'). These sample preparation steps often utilize buffers and reagents rich in salts and other compounds that will interfere with MS detection. Liquid chromatography can be used to remove salts and other buffer components prior to MS detection, enabling complete characterization of all species. In some cases, users have applied short reversed-phase columns for the purpose of trapping their analyte while allowing salts to elute to waste.

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Figure 1 shows the resulting Fd', Lc, and Fc/2 fragments generated after digestion and disulfide bond reduction. Digestion occurs specifically under the hinge region of the mAb using the commercially available protease IdeS, while reduction of disulfide bonds is carried out using the common reduction agent TCEP (tris(2-carboxyethyl) phosphine). The resulting fragments generally range in size from 23–26 kDa and thus can be more easily characterized by MS with the possibility of acquiring isotopically resolved protein spectra. To improve the MS characterization of each individual fragment, however, the individual components need to be separated in order to generate a simplified mass spectrum.

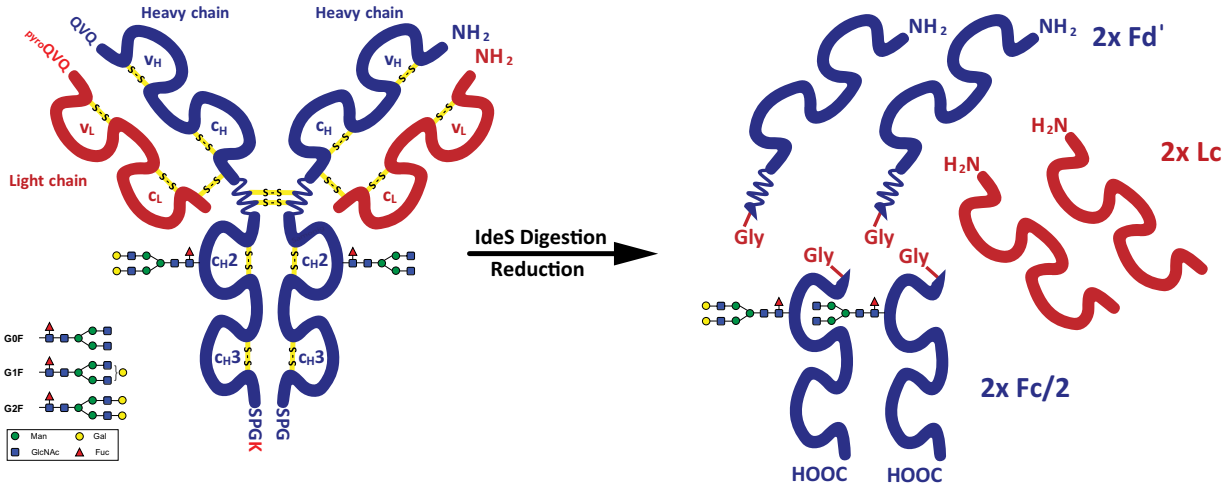


Figure 1. General structure and commonly observed post translational modifications (PTMs) on heavy and light chain of mAbs. IdeS digestion and reduction of humanized IgG1 class monoclonal antibody generates light chain (Lc), C-terminal heavy chain (Fc/2), and N-terminal heavy chain (Fd') antibody fragments.

Reversed-phase chromatography materials are well suited for protein desalting prior to LC-MS analysis; this step enables advanced protein characterization by providing clean, interference-free, mass spectra. Any viable chromatographic media for this purpose must therefore be robust and able to handle complex sample matrices without significant fouling. The MSPac DS-10 cartridge was developed for this purpose based on three beneficial attributes:

- The media retains proteins under aqueous conditions while salts and hydrophilic matrix components are eluted from the cartridge.
- The large pore structure of the supermacroporous resin allows the use of high flow rates at low temperatures with a low column backpressure.
- The lower hydrophobicity of the phenyl functionalized polymer results in reduced carryover between analyses comparatively to many alkyl functionalized particles.

In combination, these attributes allow fast loading of high sample masses to desalt proteins, therefore improving the quality of protein spectra. Additionally, despite the short length of the cartridge (10 mm), the high loading capacity and low dispersion properties of the stationary phase provide greater protein separation compared to larger particle cartridges that have reduced resolution.

In this work desalting, separation, and MS characterization of digested monoclonal antibody fragments of the pharmaceutical mAb rituximab (tradenames MabThera™ and Rituxan®) have been investigated. This demonstrated the ability to load and desalt up to 10 µg of protein with no detectable carryover using a 2.1 × 10 mm desalting cartridge. The Lc, Fc/2, and Fd' antibody fragments were partially separated and characterized with HRAMS. The methods here are broadly applicable to other mAbs and can be used to fully characterize the structure of mAbs and similar pharmaceutical compounds.

Experimental

Consumables

- MSPac DS-10 Desalting Cartridge, 2.1 × 10 mm, 2/pack (P/N 089170)
- Thermo Scientific™ Acclaim™ Guard Cartridge Holder (P/N 069580)
- Thermo Scientific™ National Mass Spec Certified 2 mL clear vial with blue bonded PTFE silicone cap (P/N MSCERT5000-34W)
- Fisher Scientific™ LC-MS grade water (P/N W/011217)
- Fisher Scientific LC-MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ LC-MS grade formic acid (P/N 28905)
- Molecular Probes™ Tris-(2-Carboxyethyl)phosphine, Hydrochloride (TCEP) (P/N T-2556)
- FabRICATOR® (IdeS protease), purchased from Genovis

Note: Unless stated otherwise, consumables are from Thermo Fisher Scientific.

Sample Pretreatment and Sample Preparation

Rituximab (10 mg/mL, 147 kDa) from Hoffmann-La Roche Ltd (Basel, Switzerland) in the formulation buffer:

- 0.7 mg/mL polysorbate 80
- 7.35 mg/mL sodium citrate dehydrate
- 9 mg/mL sodium chloride
- Sterile water adjusted to pH 6.5 using sodium hydroxide

IdeS protease was used to digest the rituximab mAb according to the manufacturer's protocol. Following sample digestion, the resulting protein fragments were reduced in 5 mM TCEP for 30 min at 60 °C.

Separation Conditions	
Instrumentation	Thermo Scientific™ Vanquish™ System Base (P/N VH-S01-A)
	Vanquish Binary Pump H (P/N VH-P10-A)
	Vanquish Split Sampler HT (P/N VH-A10-A)
	Vanquish Column Compartment H (P/N VH-C10-A)
	Vanquish Active Pre-heater (P/N 6732.0110)
	Vanquish Diode Array Detector (P/N VH-D10-A)
	LightPipe Flow Cell, 10 mm (P/N 6083.0100)
	Vanquish MS Connection Kit Vanquish (P/N 6720.0405)
	Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer

Gradient Conditions	
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	20:80 (v/v) water/acetonitrile + 0.1% formic acid
Flow rate	0.2 mL/min
Temperature	50 °C

Table 1. LC gradient conditions.

Time (min)	A	B
0	75	25
1	75	25
11	30	70
11.1	0	100
13	0	100
14	75	25
18	75	25

MS Conditions	
Source	HESI-II
Sheath gas pressure	40 psi
Auxiliary gas flow	10 arbitrary units
Capillary temperature	260 °C
S-lens RF voltage	50
Source voltage	3.5 kV
Full MS mass range	550–3000 *m/z*
Full MS parameters	
Resolution settings	240.000/15.000
Target value	3e6
Max injection time	200 ms
Microscans	3/10
SID	10 eV

Data Processing

The Thermo Scientific™ Dionex™ Chromeleon™ 7.2 SR2 Chromatography Data System and Thermo Scientific™ Protein Deconvolution™ 3.0 software were used for data acquisition and analysis.

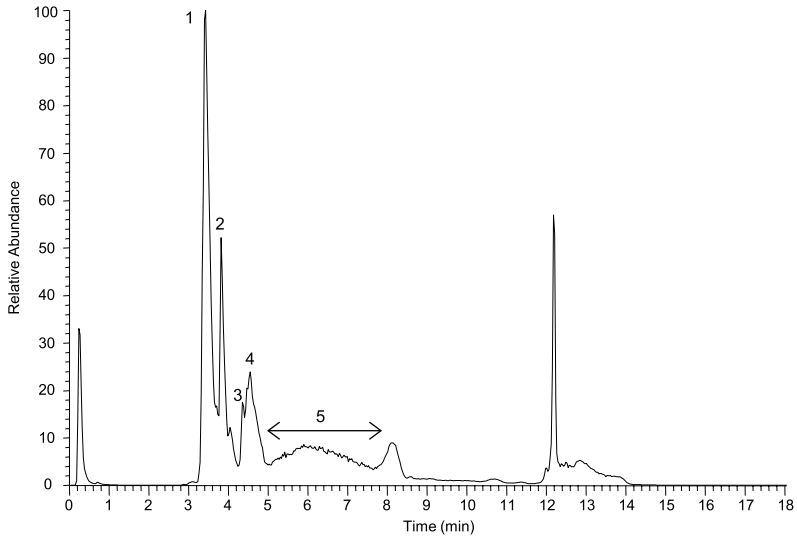
Results and Discussion

Effectiveness of Desalting

Figure 2 shows the separation and MS detection of the IdeS digested and TCEP reduced monoclonal antibody rituximab on a 2.1 × 10 mm MSPac DS-10 cartridge. This illustrates the benefits of the desalting protocol with multiple species being detected during the gradient separation.

The peak at 0.24 minutes shows the elution of salts and other sample matrix components that do not bind to the polymeric stationary phase and would normally interfere with MS detection. Typically, the MS diverter valve is used to send salts to waste during loading; however, here we show detection of the eluted sample salts to demonstrate their removal from the protein prior to MS detection.

Peaks 1, 2, and 3 (retention times of 3.49, 3.86, and 4.35 minutes, respectively) correspond to the Fc/2, Lc, and Fd' species. Peak 4 is the intact mAb, and the broad range of peaks in region 5 are the result of partially digested and/or reduced species (e.g., Hc, Hc+Lc, and 2 × Lc + 2 × Fd'). These results illustrate the desalting and separation capabilities of this short cartridge when handling samples composed of a range of ionic and organic matrix components and mAb fragments with similar molecular weights. In order to achieve resolution, the use of an analytical column is recommended. The Thermo Scientific™ MAbPac™ RP column is designed for this purpose and baseline resolution has been shown using the 3 × 50 mm format.[3]



Cartridge:	**MSPac DS-10** Desalting Cartridge
Format:	2.1 × 10 mm
Mobile Phase A:	Water + 0.1% formic acid
Mobile Phase B:	20/80 (v/v) water/acetonitrile = 0.1% formic acid
Flow Rate:	0.2 mL/min
Inj. Volume:	0.8 µL
Temp.:	50 °C
Detection:	MS; See methods for details
Gradient:	

Time (min)	A	B
0.0	75	25
1.0	75	25
11.0	30	70
11.1	0	100
13.0	0	100
14.0	75	25
18.0	75	25

Sample: Rituximab digested with IdeS and reduced with TCEP; 1 mg/mL

Peaks:
1. Fc/2 – C-terminal fragment of heavy chain with glycosated N301
2. Lc – Light chain
3. Fd' – N-terminal fragment of heavy chain
4. Intact mAb
5. Undigested and partially reduced species

Figure 2. Analysis of IdeS digested and reduced rituximab (0.8 µg total protein) on the MSPac DS -10 cartridge showing the separation of fragments, intact mAb, and partially digested and/or reduced mAb fragments.

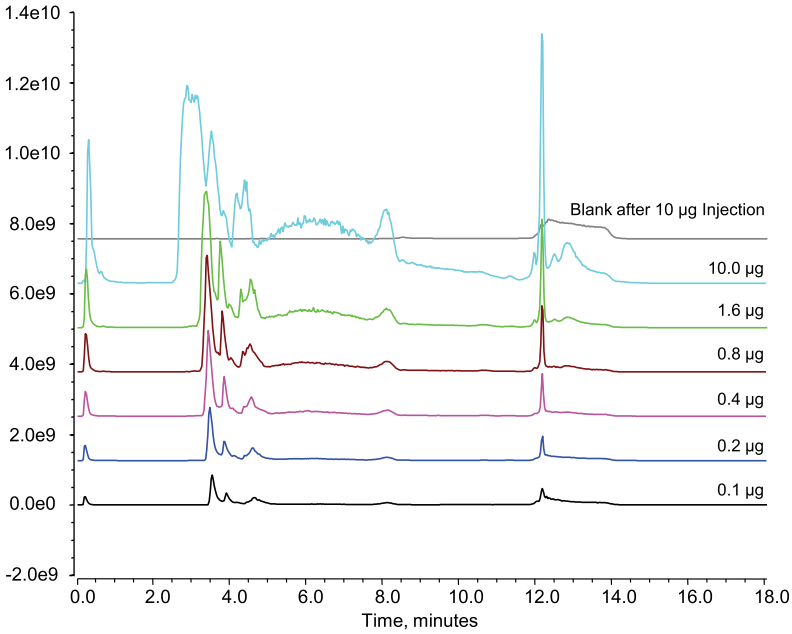
Protein Loading

The effective MS characterization of product-related impurities and low-abundant modifications often requires higher sample loading for sufficient signal strength to obtain clean protein mass spectra. For this reason, it is important to be able to load a sufficient amount of protein onto the cartridge. Figure 3 shows the spectra for increasing mass loading of the digested and reduced mAb ranging from 0.1–10 µg of total protein and the blank run after the 10 µg injection.

On increased loading, the signal strength increased for all components including salts and other matrix components observed to elute at ~0.25 minutes. This indicated effective desalting even at high mass loading amounts. Above 1.6 µg total protein loaded, the peak width at half height is observed to increase to the extent that sample component peaks overlap.

Carryover and Column Fouling

Following the 10 µg protein injection, no detectable carryover was observed illustrating the low fouling properties of the MSPac DS-10 solid phase. As is common in reversed-phase chromatography, hydrophobic sample artifacts are observed to elute as several peaks from 12 to 13 minutes during the step wash at 100% mobile phase B, resulting in a cleaned stationary phase. Together, these results demonstrate the ability to load high levels of mAb fragments onto the cartridge with effective desalting for improved MS signal and characterization with no detectable carryover between subsequent runs.



Cartridge:	**MSPac DS-10** Desalting Cartridge
Format:	2.1 × 10 mm
Mobile Phase A:	Water + 0.1% formic acid
Mobile Phase B:	20/80 (v/v) water/acetonitrile = 0.1% formic acid
Flow Rate:	0.2 mL/min
Inj. Volume:	0.1–10 µL
Temp.:	50 °C
Detection:	MS; See methods for details
Sample:	Rituximab digested with IdeS and reduced with TCEP; 1 mg/mL

Gradient:

Time (min)	A	B
0.0	75	25
1.0	75	25
11.0	30	70
11.1	0	100
13.0	0	100
14.0	75	25
18.0	75	25

Figure 3. Loading analysis (0.1–10 µg protein) of mAb fragments from IdeS digested and reduced rituximab on a 2.1 × 10 mm MSPac DS-10 cartridge and the blank run following the 10 µg injection.

Structural Identification

Figure 4 shows the mass spectra and deconvoluted mass spectra for the Fc/2, Lc, and Fd' fragments of the digested and reduced rituximab mAb for the chromatogram shown in Figure 2. The clean mass spectrum obtained for each component demonstrates the excellent desalting power of the MSPac DS-10 cartridge. The quality of the spectra allowed easy deconvolution to provide the measured molecular weight for each fragment including the glycosylated variants of the Fc/2 fragments. These results are summarized in Table 2 including the deviation from the theoretical monoisotopic molecular weight. The mass deviation of only -4.6 to -1.2 ppm for the molecular weight fragments demonstrates the detection power of the Q Exactive HF mass spectrometer.

In addition to these species, MS analysis and deconvolution of the MS spectra in region 5 (as shown in Figure 2) yielded the measured molecular weight of additional fragment variants and partially digested and/or reduced fragments. Effective desalting resulted in suitable mass spectra even in this region, which contains multiple protein species; this allowed deconvolution and determination of structural features for these species. These measurements are summarized in Table 3. Generally, observed mass deviations are directly related to the poorer spectrum quality for some of the fragments eluted in this complex region of the chromatogram. Inspection of the data in Table 3 shows that the measured molecular weight of the Hc (Fc/2 + Fd'), Hc + Lc, and 2 x Lc + 2 × Fd' fragments correspond to the calculated theoretical molecular weight of the individual fragments.

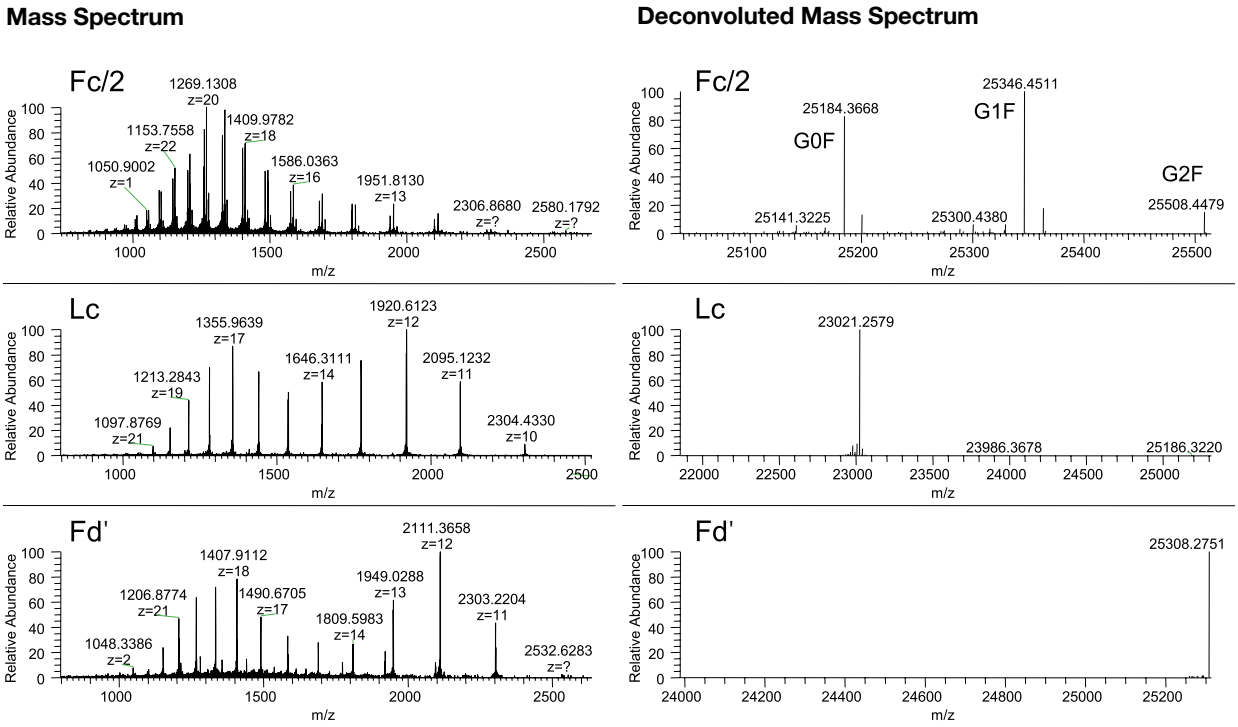


Figure 4. (Left) Mass spectra for Fc/2, Lc, and Fd' rituximab fragments and (right) the deconvoluted mass spectra showing the measured molecular weight of each fragment including glycosylated variants for the Fc/2 fragment.

Table 2. Theoretical monoisotopic MW, measured MW, and mass deviation for rituximab fragments and associated variants.

Fragment	Modifications	# C	# H	# N	# O	# S	MW (monoisotopic) [Da]	MW measured [Da]	Mass deviation [ppm]
Lc	N-terminal pyro Glutamic acid, 2 internal S-S bonds	1016	1570	272	328	6	23021.28593	23021.2579	-1.22
Fd'	N-terminal pyro Glutamic acid, 2 internal S-S bonds	1125	1724	292	354	10	25308.30854	25308.2751	-1.32
Fc/2	G0F glycan 2 internal S-S bonds	1122	1736	286	361	6	25184.46011	25184.3668	-3.71
Fc/2	G1F glycan 2 internal S-S bonds	1128	1746	286	366	6	25346.51294	25346.4511	-2.44
Fc/2	G2F glycan 2 internal S-S bonds	1134	1756	286	371	6	25508.56576	25508.4479	-4.62

Table 3. Measured MW for assumed fragment variants and partially digested and/or reduced rituximab fragments.

Fragment	MW measured [Da]	Theoretical MW [Da]
Lc	23036.352	23035.353
Fc/2+ G1F (2 internal S-S bonds)	25361.822	25362.019
Hc + G1F (4 internal S-S bonds)	50602.141	50668.124
Hc+ Lc + G1F (6 internal S-S bonds)	73680.188	73699.445
2 x Lc + 2 x Fd' (7 internal S-S bonds)[a]	96713.414	96712.9

[a] Partially reduced F(ab')2, with 7 unreduced S-S bonds

Conclusion

The analyses shown here for rituximab demonstrate a broadly applicable desalting method that can be used with a range of intact biomolecules and their associated fragments for the purposes of determining variations in structure and for proper clone selection for production in biopharmaceutical processes. Gradient conditions can be further optimized to develop a high-throughput platform for analyzing hundreds of samples in a short time frame.

- The MSPac DS-10 cartridge provides excellent separation of digested and reduced mAbs resulting in a clean protein spectrum for each fragment with minimum signal overlay.
- Using a reversed phase gradient and a high flow rate salts, adducts, and other sample matrix components are effectively removed from the mAb fragments resulting in clean MS spectra.
- The MSPac DS-10 cartridge provides these benefits without carryover enabling the user to reliably do successive injections of high protein loading amounts without requiring additional blank runs.
- The resolving power of the Q Exactive HF mass spectrometer is able to isotopically resolve mass spectra for all subunits enabling the calculation of the exact monoisotopic masses of each mAb fragment.

References

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2. Liu, H.; Gaza-Bulseco, G.; Faldu, D.; Chumsae, C.; Sun, J. Heterogeneity of Monoclonal Antibodies, *J. of Pharmaceutical Sciences*, **2008**, *97*, 2426.
3. Lin, S., Zhang, T., Wang., H., Josephs, J., and Liu, X., fast Analysis of Therapeutic Monoclonal Anitbodies, Fragments, and Oxidation Variants Using a Super-Macro Porous Reversed-Phase Column Coupled with an Orbitrap Mass Spectrometer, ASMS 2015, Thermo Scientific Poster Note PN64398.

Useful Links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:
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Fast Online Desalting of mAbs Using a Reversed-Phase Desalting Cartridge for LC-MS Analysis

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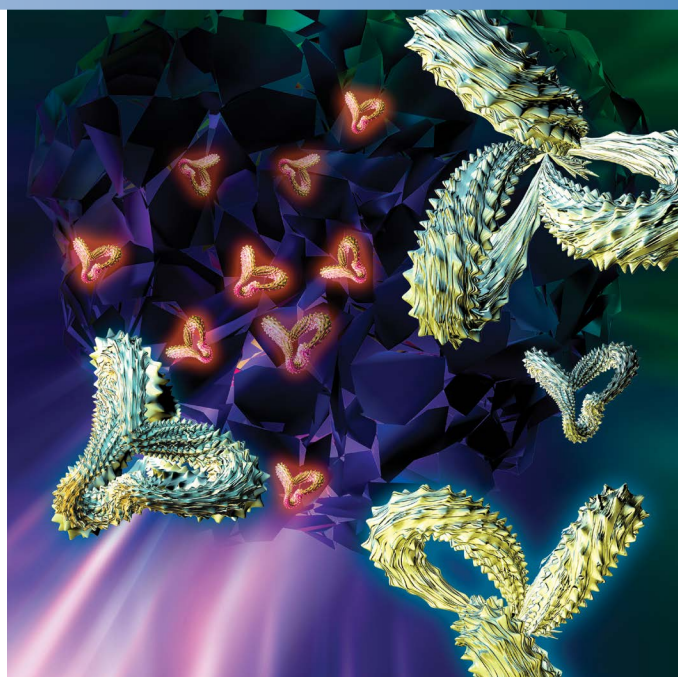
Application Note 21465

Key Words
Desalting, MSPac, monoclonal antibody, mAb, Vanquish, reversed phase, mass spectrometry, Q Exactive, biopharmaceutical, biomolecules, glycosylation, intact protein

Goal
To demonstrate a simple on-line desalting method for rapid, automated analysis of mAbs with LC-MS to obtain information-rich, high-quality spectra. The desalting method optimization was conducted on a 2.1 × 10 mm Thermo Scientific™ MSPac™ DS-10 cartridge using conventional water/acetonitrile-based separations on the Thermo Scientific™ Vanquish™ UHPLC system and MS detection using the Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer.

Introduction
Monoclonal antibodies (mAbs) as biopharmaceutical therapeutics have grown rapidly over the last decade. This class of drug is forecasted to be the primary treatment for a wide range of diseases including cardiovascular, autoimmune disorders, and cancers.[1] During drug development, biopharmaceutical companies typically use mass spectrometry (MS) to structurally characterize mAbs produced by hundreds of different monoclonal cell lines. The main objective is to determine monoclonal cell populations that produce the desired mAb with a high degree of fidelity and high activity for a specific antigen. To make the MS characterization process economical, high-throughput methods must be developed that enable rapid and inexpensive characterization of mAbs to select promising cell lines for further development.

Mass spectrometry is an essential tool in the characterization of mAbs, providing molecular weight determination and structural information of intact mAbs as shown in Figure 1. Direct infusion of the dissolved mAb into the MS is the simplest approach to characterize the proteins. However, the sample matrix of the recovered and purified mAb possesses a variety of salts, stabilizers, detergents, and other adduct forming ions. These can interfere with MS characterization by decreasing sensitivity and can result in instrument fouling, ultimately leading to downtime.



Liquid chromatography can be used to remove salts and other buffer components prior to MS detection. Reversed-phase chromatographic approaches are particularly well suited for protein desalting prior to MS analysis due to the technique's ability to retain proteins under aqueous conditions, allowing salts and other hydrophilic matrix components to elute to waste. However, careful column selection and method development is required for effective protein desalting using reversed phase methods.

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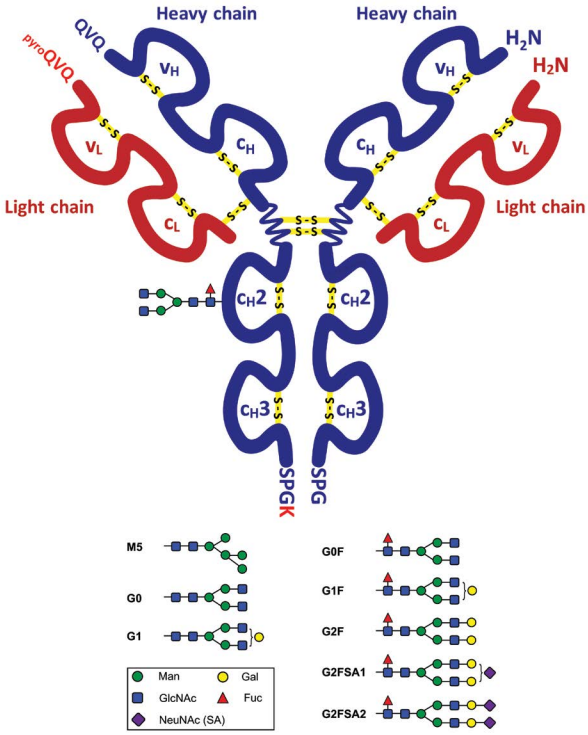

Figure 1. General structure, commonly observed post translational modifications (PTMs), and nomenclature of carbohydrate structures for intact mAbs.

For high-throughput applications, the chromatographic media must be robust and able to cope with complex sample matrices without significant impact on performance. The MSPac DS-10 cartridge was developed for this purpose based on the following benefits:

- The stationary phase retains proteins under aqueous conditions while salts and hydrophilic matrix components are eluted from the cartridge.
- The stationary phase is mechanically and chemically robust with the ability to handle rapid changes in flow rate and solvents commonly used for reverse phase applications.
- The large pore structure of the supermacroporous resin allows the use of high flow rates at low temperatures with a low column backpressure for improved desalting efficiency.
- The lower hydrophobicity of the phenyl functionalized polymeric phase results in reduced carryover between analyses relative to many alkyl functionalized particles.

In combination, these attributes complement high-throughput methods to allow rapid protein loading, desalting, and elution for MS analysis. The resulting clean mass spectra provide valuable information for the characterization of important mAb structural properties such as the glycoform profile.

In this work, the method development for desalting and MS characterization of the intact pharmaceutical monoclonal antibody rituximab has been investigated systematically for high-throughput purposes. This resulted in a fast chromatographic method for loading, desalting, and analyzing the intact mAb within 4 minutes using a 2.1 × 10 mm cartridge. The optimized method is broadly applicable to other mAbs and proteins and can be used to rapidly analyze mAbs and similar biopharmaceutical compounds.

Experimental

Consumables

- MSPac DS-10 Desalting Cartridge, 2.1 × 10 mm, 2/pack (P/N 089170)
- Thermo Scientific™ Acclaim™ Cartridge Holder (P/N 069580) (Figure 2)
- Thermo Scientific™ National Mass Spec Certified 2 mL clear vial with blue bonded PTFE silicone cap (P/N MSCERT5000-34W)
- Fisher Scientific™ LCMS grade water (P/N W/011217)
- Fisher Scientific™ LCMS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ LCMS grade formic acid (P/N 28905)

Figure 2. MSPac DS-10 Desalting Cartridge with Acclaim Cartridge Holder.

Sample Pretreatment and Sample Preparation

The sample used in the experiments is the commercially available monoclonal antibody rituximab (Hoffmann La Roche, Basel, Switzerland) supplied at a concentration of 10 mg/mL in a formulation buffer with the following composition:

- 0.7 mg/mL polysorbate 80
- 7.35 mg/mL sodium citrate dihydrate
- 9 mg/mL sodium chloride
- Sterile water adjusted to pH 6.5 using sodium hydroxide

The sample was diluted 1:1000 with water + 0.1% formic acid to give a final mAb concentration of 10 µg/mL.

- 0.7 µg/mL polysorbate 80
- 7.35 µg/mL sodium citrate dihydrate
- 9 µg/mL sodium chloride

LC Conditions	
Instrumentation	Vanquish UHPLC system consisting of:
	System Base (P/N VH-S01-A)
	Binary Pump H (P/N VH-P10-A)
	Split Sampler HT (P/N VH-A10-A)
	Column Compartment H (P/N VH-C10-A)
	Biocompatible 2-Position/6-Port Column Switching Valve (150 MPa) (P/N 6036.1560)
	Active Pre-Heater (P/N 6732.0110)
	Vanquish MS Connection Kit (P/N 6720.0405)

Separation Conditions	
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Water/acetonitrile (20:80 v/v) + 0.1% formic acid
Flow rate	See Table 1
Temperature	70 °C

Table 1. LC gradient conditions.

Gradient			
Time (min)	A	B	Flow Rate (mL/min)
0.0	70	30	0.5*
1.0	70	30	0.5*
1.01	70	30	0.2
3.0	0	100	0.2
3.2	0	100	0.2
3.3	0	100	1.0
3.5	0	100	1.0
3.6	70	30	1.0
4.0	70	30	0.5*

* Flow rate for loading is 0.5 mL/min unless noted otherwise in the text.

Table 2. MS divert valve configuration.

Valve Configuration		
Time (min)	Valve Configuration	Analysis Stage
0	To Waste	Sample loading and desalting
1.5	To MS	Elution to MS for analysis
3.1	To Waste	Cleaning and equilibration for loading next sample

MS Conditions	
Instrumentation	Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer
	The MS source and method parameters are shown in Table 2.

Table 3. Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer settings.

MS Source Parameters	Setting
Source	Ion Max source with HESI-II probe
Sheath gas pressure	35 psi
Auxiliary gas flow	10 arbitrary units
Vaporizer Temperature	260 °C
Capillary temperature	260 °C
S-lens RF level	80
Source voltage	3.5 kV
MS Method Parameters	
Method type	Full MS only
Full MS mass range	*m/z* 1800–5000
Resolution settings	17,500 (FWHM at *m/z* 200)
Target value	3e6
Max injection time	150 ms
Microscans	10
SID	20 eV

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Data System, version 7.2 SR3, and Thermo Scientific™ Protein Deconvolution™ software, version 4.0, were used in this work.

Results and Discussion

Separation Method for High-Throughput Desalting and MS Analysis

The objective of high-throughput analysis is characterization of the samples of interest with minimal total analysis time for each sample. LC-MS methods for high-throughput analysis must be carefully designed in order to optimize the chromatographic separation for accurate MS analysis. Flow rate, temperature, gradient composition, and valve control are chromatographic tools used to optimize the LC-MS method for MS characterization with minimal analysis time. Figure 3 shows a schematic of the gradient, flow rate, and valve positioning for the three different stages of high-throughput mAb analysis: 1) Sample loading and desalting, 2) Elution and MS analysis and 3) Cartridge wash and equilibration.

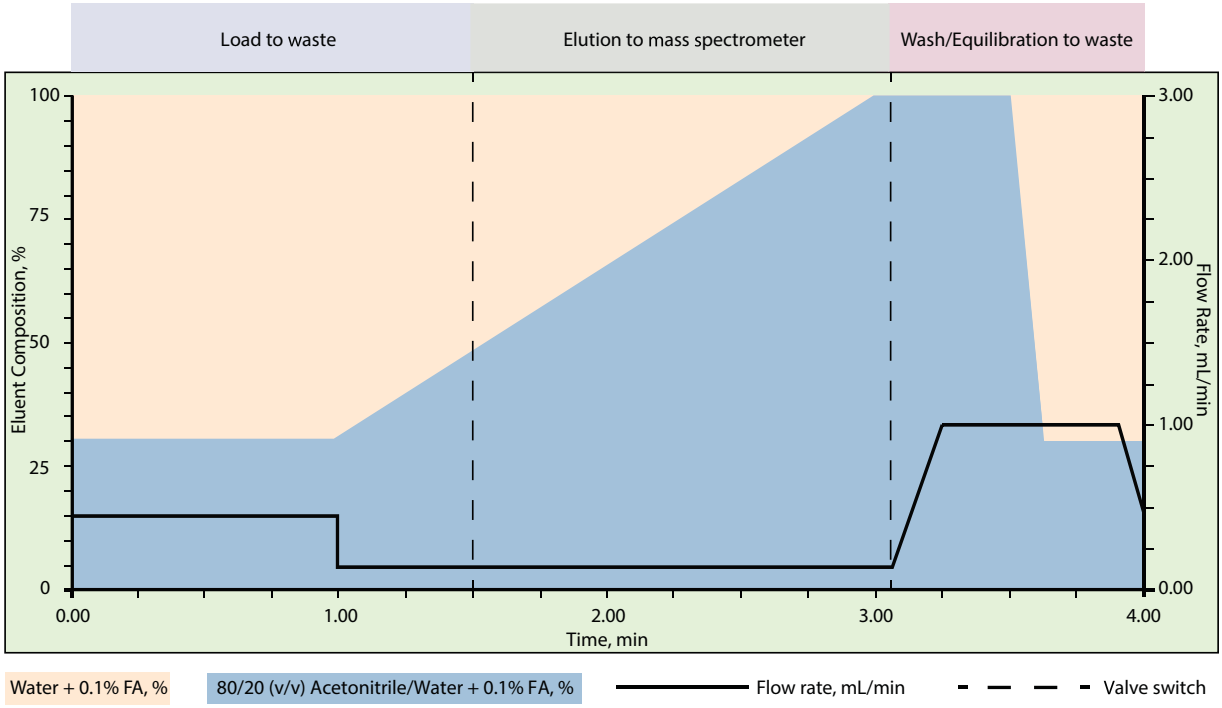

Figure 3. Schematic of the chromatographic program designed to load, desalt, and elute the mAbs for high-throughput MS characterization.

1) **Sample loading and desalting:** 0 to 1.5 minutes – Use of a high flow rate during loading minimizes the time required and maximizes the desalting efficiency. The MS divert valve sends the flow to waste to avoid contamination of the MS instrument. The mAb is loaded onto the cartridge within approximately 15 seconds but the loading step is extended to 1 minute to maximize the removal of salts and other hydrophilic sample artifacts resulting in a clean protein sample for MS analysis. The eluent composition is held constant at 30% B to enable mAb binding to the MSPac DS-10 solid phase. Prior to elution and MS analysis, the flow rate is decreased to 0.2 mL/min to be consistent with the optimized flow rate for the electrospray ionization (ESI) source.

2) **Elution and MS analysis:** 1.5 to 3.1 minutes – Prior to valve switching, a steep gradient from 30% to 100% B from 1 to 3 minutes elutes the mAb from the cartridge. At 1.5 minutes during the gradient, the divert valve directs flow to the MS for analysis of the eluting mAb.

3) **Cartridge wash and equilibration:** 3.1 to 4.0 minutes – At 3.1 minutes the divert valve directs flow to waste and at 3.2 minutes the flow rate is ramped to 1 mL/min over 0.1 minutes. The mobile phase composition is held at 100% B until 3.5 minutes to remove residual protein from the cartridge then the composition is decreased to 30% B over 0.1 minutes and held to equilibrate the cartridge solid phase for loading the next sample. The high flow rate reduces the total time required for cartridge washing and equilibration in this stage. From 3.9 to 4.0 minutes, the flow rate is decreased to 0.5 mL/min in preparation for the next sample loading.

During all stages, the cartridge temperature is held at a constant 70 °C. Elevated temperatures increase mAb recovery and also reduce the amount of carryover between analyses improving MS sample characterization.

Effect of Loading Flow Rate on MS Analysis

Prior to use of the optimized high-throughput method for mAb analysis, MS characterization of the mAb was evaluated at different protein loading flow rates to determine the optimal loading conditions. Figure 4 shows the chromatograms and mass spectra for rituximab at different loading flow rates. Neither the elution time nor signal intensity for the glycosylation variant profile showed any significant differences at the different flow rates. For these reasons, 500 µL/min was used for all subsequent loading steps since higher flow rates improve the desalting efficiency and the MS characterization is not adversely affected.

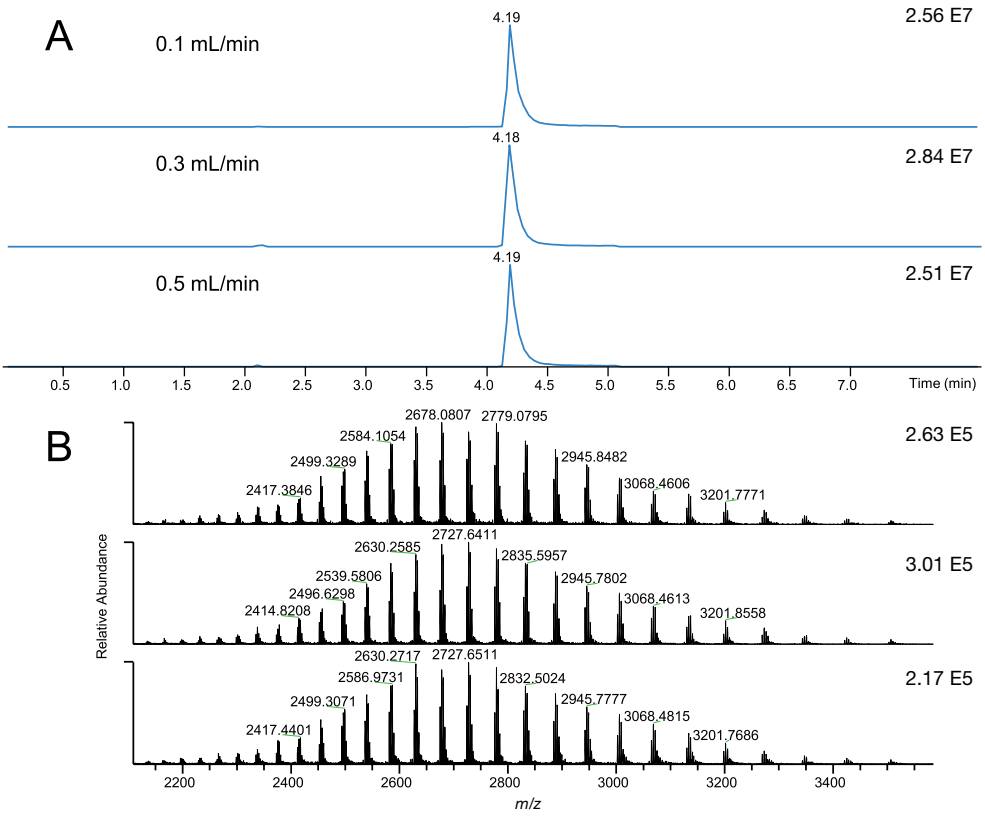

Figure 4. Effect of flow rate for sample loading obtained on (A) the total ion chromatograms and (B) the corresponding mass spectra.

Rapid mAb Desalting and MS Analysis

Figure 5 shows the desalting and MS characterization of 100 ng of the mAb rituximab using the optimized loading flow rate and an optimized SID (in-source collision induced dissociation) voltage of 20 eV. The enlarged region of the mass spectra of Figure 5B shows MS characterization that is free of any interference from salts or other sample artifacts. These results clearly show the desalting power of the MSPac DS-10 cartridge when using an optimized method for sample clean up prior to MS analysis. For comparison, Figure 5D shows desalting of a mAb on a competitor's column resulting in affected MS detection due to the presence of formulation buffer components. In this analysis, 2.5 ng of a mAb were separated on a 100 µm × 250 mm C4 column using a water/acetonitrile gradient at a flow rate of 2 µL/min and 70 °C. This general reversed-phase method resulted in the detection of a significant amount of salts and other adduct forming formulation buffer components in the mass spectra. The competitor's column spectra highlight the importance of selecting an appropriate reversed-phase product in combination with an optimized gradient for effective protein desalting.

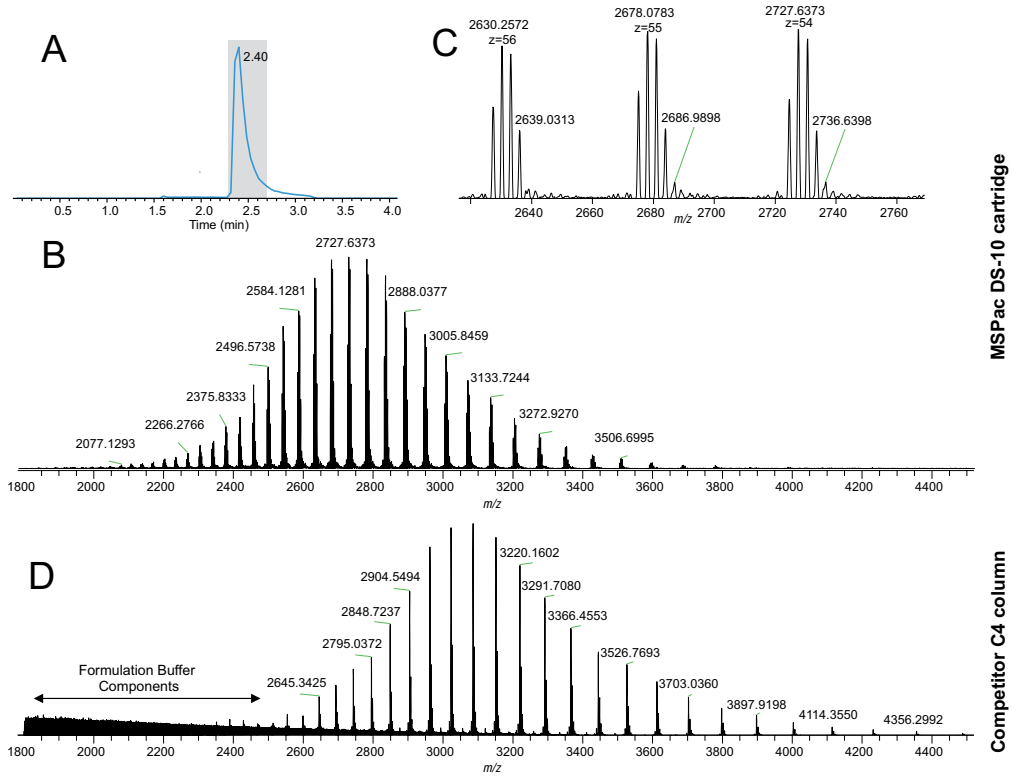

Figure 5. Rapid loading, desalting, and MS analysis of 100 ng of the mAb rituximab. (A) Total ion chromatogram, (B) average full mass spectrum of the mAb as detected in the gray region of the top panel, and (C) enlarged region of the mass spectrum shown in panel B displaying the three most abundant charge states typical for the mAb rituximab. The bottom panel (D) shows the presence of mAb formulation buffer components observed in the mass spectrum following reversed phase separation of a comparable mAb on a competitor's C4 chromatography column and is an indicator of ineffective desalting.

High-Throughput Analysis and Cartridge Ruggedness

Figure 6 shows the utility of the MSPac DS-10 cartridge for high-throughput desalting and MS analysis of 10 ng rituximab injections using the optimized method described above with consecutive sample injections (i.e., no blank runs were used). This data shows excellent reproducibility of spectrum quality up to 100 runs. The characterization for the relatively low sample amount highlights the sensitive detection power of the Q Exactive Plus MS.

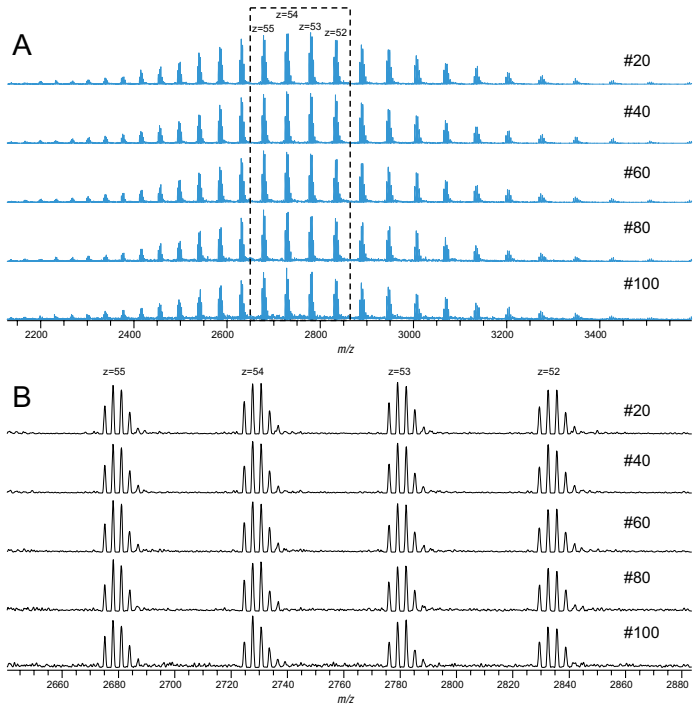

Figure 6. Mass spectrum quality over 100 runs (A) and enlarged view of charge states (B) for high-throughput loading, desalting, and MS analysis of 10 ng injections of rituximab. For a detailed comparison of glycoform detection, see Figure 7.

Characterization of the glycoform pattern for mAbs is a common practice during development of these biopharmaceutical therapeutics. Glycoform characterization is of particular relevance when screening a large number of clones for the quality and manufacturability of a mAb in cell culture. For this reason, it is important to be able to characterize the mAb glycoform pattern with a high confidence of detection for low abundance glycoforms using a fast, straightforward method. Figure 7 shows five examples of the deconvoluted spectra highlighting the glycoform pattern over 100 consecutive runs. Effective desalting with the MSPac DS-10 cartridge and the high resolving power of the Q Exactive mass spectrometer enable the accurate characterization and relative quantification of glycoforms over all 100 runs. The long lifetime of the cartridge paired with a high-throughput method enables the rapid analysis of a large sample set.

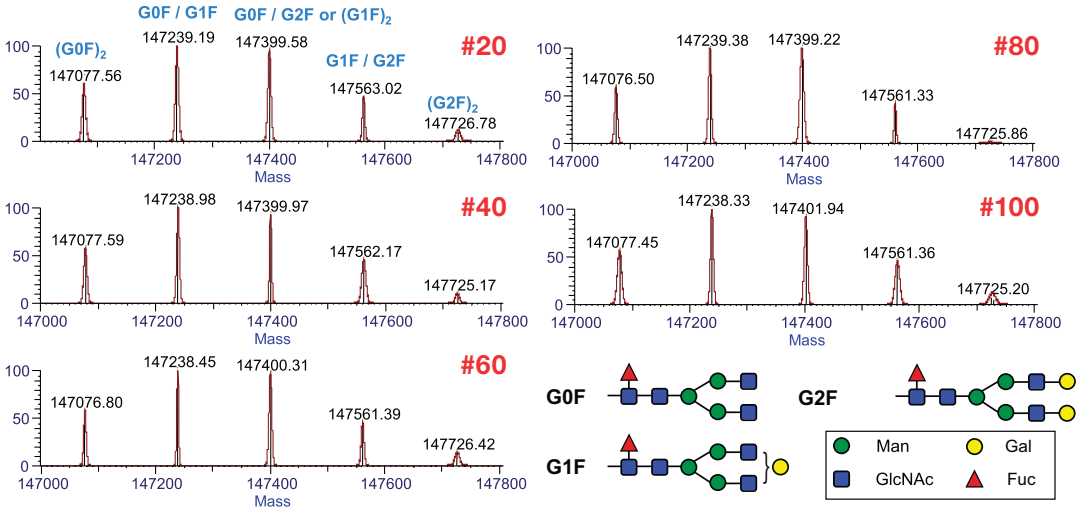

Figure 7. Comparison of deconvoluted mass spectra showing glycoform detection for runs 20, 40, 60, 80, and 100 of the high-throughput mAb analysis shown in Figure 6.

Analysis of High Mass Loading and Carryover

Higher mass loading levels are commonly used for detailed analysis of low abundance mAb glycoforms and other variants. For these analyses, sample carryover from run to run can interfere with characterization of variants in subsequent injections. Using the high-throughput method described above, Figure 8 shows the chromatogram for a 1000 ng injection of rituximab and the following two blank runs. As measured by peak area, the first blank run shows carryover of 1.48% (0.69% by signal intensity). In the second blank run, the carryover by peak area is 0.79%. These results indicate that only one blank run is required to sufficiently reduce carryover for MS analysis of structurally distinct mAbs when using this high loading amount. This in-depth characterization results in the detection of eight different glycans attached to the two heavy chains of the intact mAb, leading to a total of 13 different glycoforms.

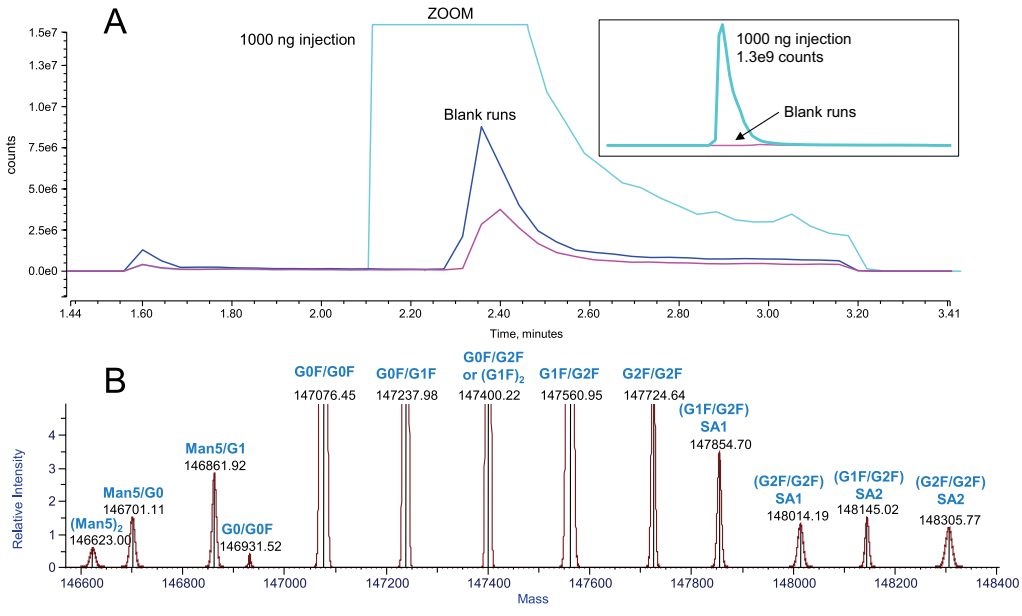

Figure 8. Total Ion Chromatogram (TIC) for analysis of a 1000 ng mAb injection and the following two blank runs showing carryover (A). The bottom panel (B) shows the the enlarged region of low abundance glycoforms for the 1000 ng injection.

Conclusion

This study summarizes the evaluation of the new MSPac DS-10 desalting cartridge and demonstrates the applicability for a fast, robust, and sensitive 4 min desalting method developed using rituximab as a representative for a large biomolecule.

- The MSPac DS-10 cartridge in combination with the Vanquish UHPLC system and the Q Exactive Plus mass spectrometer enables in-depth intact protein characterization of mAb glycoforms using fast desalting gradients of only 4 minutes.
- Using a reversed-phase gradient, the MSPac DS-10 cartridge provides excellent removal of salts, adducts, and other sample matrix components prior to MS analysis, resulting in clean and highly reproducible protein mass spectra for intact monoclonal antibodies.
- Sample loading and desalting of 1 µg of monoclonal antibody on the MSPac DS-10 cartridge enables the MS characterization of low abundance (< 1%) glycoforms.
- Sample carryover following a 1 µg rituximab injection and subsequent single blank run is less than 1%. With such low carryover, it is possible to analyze different protein samples within the same sequence.

References

1. Leavy, O. Therapeutic antibodies: past, present, and future, *Nature Reviews Immunology*, **2010**, *10*, 297.

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TECHNICAL NOTE

A pre-concentration and online solid phase extraction setup for the LC-MS analysis of therapeutic protein mixtures

Authors: Martin Samonig, Sabrina Patzelt, Martin Rühl, and Remco Swart
Thermo Fisher Scientific,
Germering, Germany

Key words

Pre-concentration, trap and elute, desalt, MSPac, MAbPac, monoclonal antibody, mAb, Vanquish, reversed phase, mass spectrometry, Q Exactive, biopharmaceutical, biomolecules, intact protein

Goal

Demonstrate a fully automated UHPLC setup applying simple and efficient online solid phase extraction method. Showcase assay performance by using a column cartridge for sample trapping and a Thermo Scientific™ MAbPac™ RP column for separation with water/acetonitrile-based gradients. Leverage the new technologies such as the Thermo Scientific™ Vanquish™ UHPLC platform in combination with the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole Orbitrap mass spectrometer.

Introduction

Solid-phase extraction (SPE) is a commonly used laboratory technique to isolate analytes of interest from complex matrices. Because this technique is typically performed manually, it may not satisfy productivity and automation requirements for all laboratories. When repeatability between different analysts shows too much variation or the sample is subject to contamination during the manual process, an automated sample cleanup is preferable.

In the online-SPE approach for the enrichment of low abundant compounds in liquid chromatography, the sample is pre-concentrated on a trap column prior to chromatographic separation. This technique can be applied for the sample cleanup of protein mixtures often containing high amounts of nonvolatile salts, which are present in various biopharma formulation buffers. The presence of such buffers may interfere with the operation of electrospray ion sources by suppressing ionization. In this technical note, the Vanquish UHPLC setup for fully automated pre-concentration and sample cleanup is described as well as the detection of intact proteins using the Q Exactive HF mass spectrometer.

Experimental

Consumables

- Analytical column: Thermo Scientific MAbPac RP column, 1 × 100 mm, 4 µm (P/N 302695)
- Trap column: Thermo Scientific™ MSPac™ DS-10 Desalting Cartridge, 2.1 × 10 mm, 2/pack (P/N 089170)
- Thermo Scientific™ Acclaim™ Cartridge Holder (P/N 069580)
- Fisher Scientific™ LC-MS grade water (P/N W/011217)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A/0638/17)
- Fisher Scientific™ Optima™ LC-MS grade trifluoroacetic acid (P/N 10125637)
- Thermo Scientific™ Pierce™ Formic acid LC-MS grade (P/N 28905)

Sample pretreatment and sample preparation

The sample used was the commercially available monoclonal antibody rituximab (Hoffmann La Roche™, Basel, Switzerland) supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.7 mg/mL polysorbate 80, 7.35 mg/mL sodium citrate dehydrate, 9 mg/mL sodium chloride, and sterile water adjusted to pH 6.5 using sodium hydroxide or hydrochloric acid.

Solutions of 1 mg/mL alpha-lactalbumin, cytochrome c, myoglobin, ribonuclease A (Sigma-Aldrich®, St. Louis, MO, USA) in 0.1% formic acid (FA) in water and rituximab in formulation buffer were diluted with 0.1% FA in water in the ratio of 1:1:1:1:2 (w/w) to a final protein concentration of 60 µg/mL. This protein mixture represents a sample including different sizes of proteins (12–148 kDa) with various post-translational modifications like glycosylation and acetylation.

LC conditions

Instrumentation

- Vanquish Flex UHPLC system consisting of:
  - System Base Vanquish Flex (P/N VF-S01-A)
  - Binary Pump F (P/N VF-P10-A-01)
  - Quaternary Pump F (P/N VF-P20-A)
  - Split Sampler FT (P/N VF-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Variable Wavelength Detector F (P/N VF-D40-A)
  - Ultra-Low Dispersion UV Monitor Flow Cell, 45 nL (P/N 6074.0285)
  - Vanquish MS Connection Kit (P/N 6720.0405)
- Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (P/N 0726041)

Figure 1 shows the Vanquish Flex UHPLC system chosen for this setup, consisting of a quaternary low-pressure gradient pump (LPG) used as the loading pump and a binary high-pressure gradient pump (HPG) used as the analytical pump. All required capillaries and additional parts for this setup are defined in Table 1. Separation conditions are shown in Table 2.

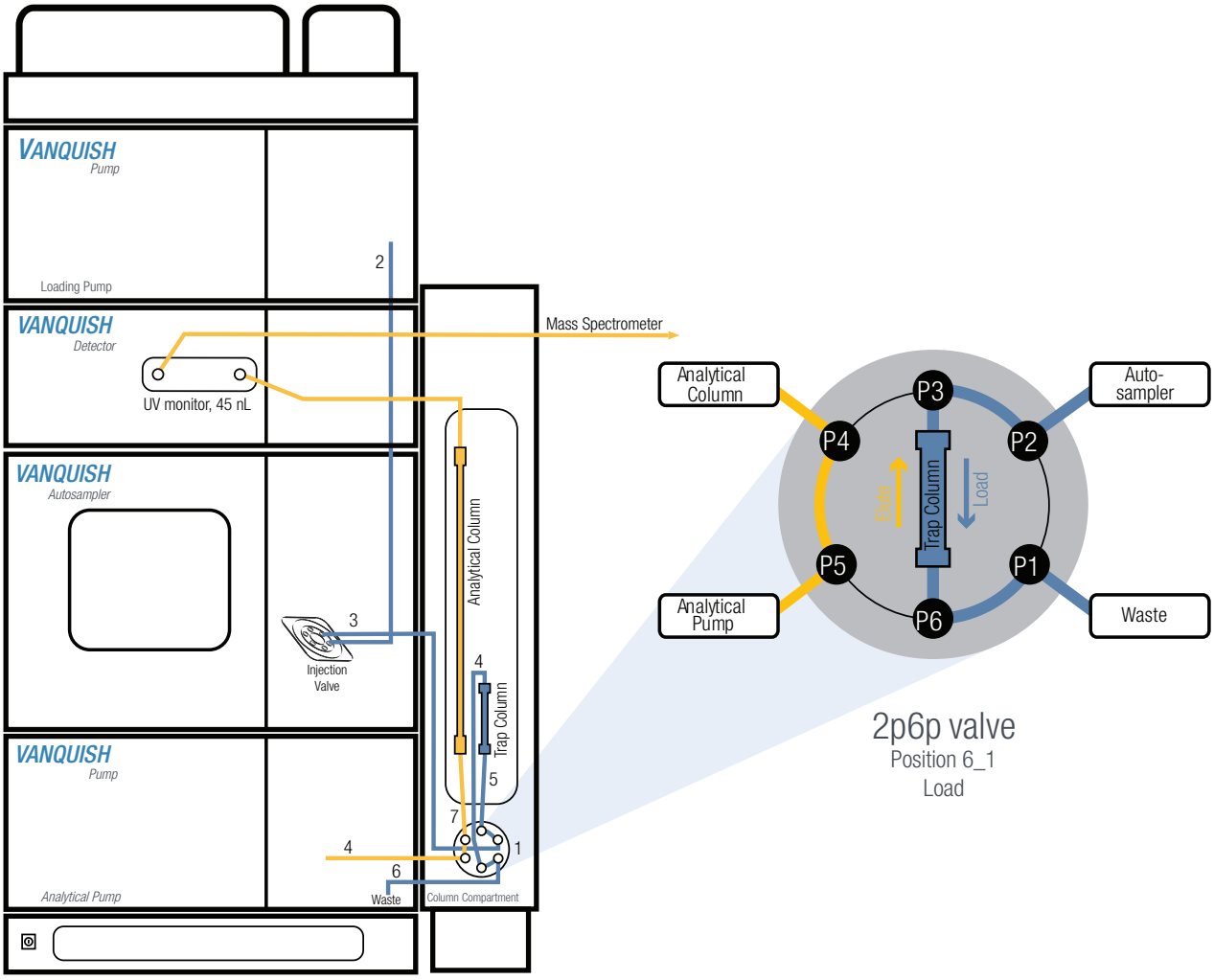

Figure 1. Vanquish Flex online SPE setup with the detailed 2-position/6-port (2p6p) valve configuration and recommended capillaries defined in Table 1.

Table 1. Additional parts needed for the online SPE setup.

#	Amount	Viper Capillary	P/N	2p6p Valve Port Assignment
1	1×	Biocompatible 2p6p column switching valve	6036.1560	
2	1×	Viper Capillary, MP35N, biocompatible, 0.1 × 650 mm	6042.2370	
3	1×	Viper Capillary, MP35N, biocompatible, 0.1 × 450 mm	6042.2350	Port 2 - Autosampler
4	2×	Viper Capillary, MP35N, biocompatible, 0.1 × 350 mm	6042.2340	Port 5 - Analytical pump; Port 6 - Trap column
5	1×	Viper Capillary, MP35N, biocompatible, 0.1 × 150 mm	6042.2320	Port 3 - Trap column
6	1×	Viper Capillary, MP35N, biocompatible, 0.5 × 350 mm	5083.2425	Port 1 - Waste
7	1×	Active Pre-heater, 0.1 × 380 mm[(a)]	6732.0110	Port 4 - Analytical column inlet

[a]Included in the System Base Vanquish Flex Ship Kit

Table 2. Separation conditions.

LC Parameters	Setting
Mobile phase A1	0.1% FA in water
Mobile phase B1	0.1% FA in water/ acetonitrile (10:90 v/v)
Mobile phase A2	0.1% TFA in water
Flow rate	See Table 3
Temperature	70 °C, forced air, unless noted otherwise in the text
Gradient	See Table 3

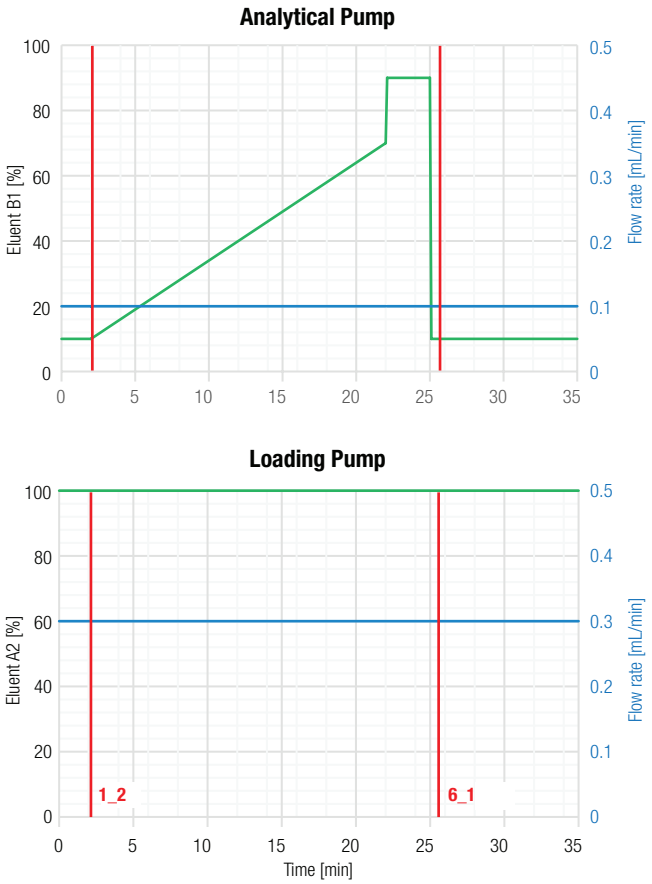

Figure 2. Valve configuration and LC gradient conditions for analytical pump / loading pump.

The lower switching valve of the Vanquish Thermostatted Column Compartment (VTCC) was used to switch between the sample loading and sample elution configuration (Figure 3). The loading pump was utilized to load the sample for two minutes on the trap column (MSPac DS-10 Desalting Cartridge) running an isocratic flow of 0.1% trifluoroacetic acid (TFA) in water at 300 µL/min. After the switching valve was switched to the elute position (Table 4), the trapping column was part of the analytical flow path and the HPG pump was delivering a 0.1% FA in water/acetonitrile gradient (Table 3) to elute the proteins from the trap column in back-flush mode and separate them on the MAbPac RP analytical column. The column outlet was coupled to the variable wavelength detector (VWD) and to the Q Exactive HF mass spectrometer in series. At 25.1 minutes, the switching valve was again switched to position 6_1 to allow the trap column to be equilibrated with the isocratic flow of the loading pump and to be ready for the next injection. If a setup with forward-flush is required to use the trap column also as a guard column, the position of the two capillaries of Port 4 and Port 5 on the switching valve (Figure 1) have to be swapped.

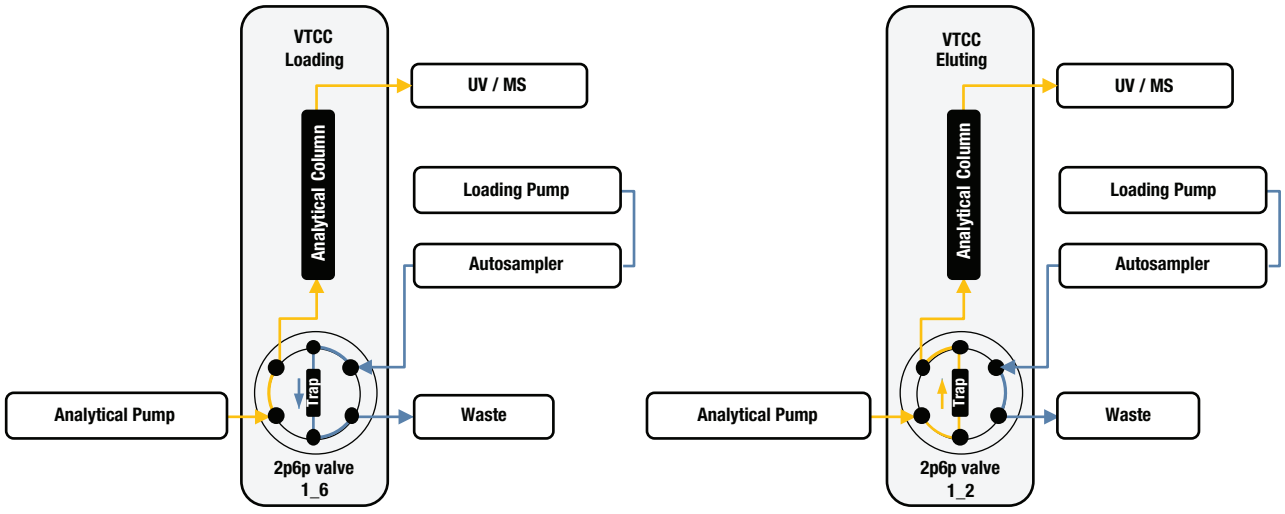

Figure 3. Valve configuration for sample trapping and sample elution/separation.

Table 3. LC gradient conditions.

Analytical Pump			
Time [min]	**A1 [%]**	**B1 [%]**	**Flow Rate [mL/min]**
0.0	90	10	0.1
2.0	90	10	0.1
22.0	30	70	0.1
22.1	10	90	0.1
25.0	10	90	0.1
25.1	90	10	0.1
35.0	90	10	0.1
Loading Pump			
Time [min]	**A2 [%]**		**Flow Rate [mL/min]**
isocratic	100		0.3

Table 4. Configuration of the lower switching valve.

Time [min]	Valve Configuration	Analysis Stage
0.0	6_1	Loading
2.0	1_2	Elute
25.1	6_1	Equilibration

MS conditions

Instrumentation

Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with MS source and method parameters shown in Table 5.

Data processing

The data were acquired exclusively with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR4, and Thermo Scientific™ BioPharma Finder™ software, version 2.0 was used for data analysis.

Table 5. Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer settings.

MS Source Parameters	Setting
Source	Ion Max source with HESI-II probe
Sheath gas pressure	25 psi
Auxiliary gas flow	10 arbitrary units
Vaporizer temperature	150 °C
Capillary temperature	280 °C
S-lens RF level	60
Source voltage	3.5 kV
MS Method Parameters	**Setting**
Method type	Full MS only
Full MS mass range	500–2800 / 1800–5000 *m/z*
Resolution settings	120k / 15k (FWHM at *m/z* 200)
Target value	3e6
Max injection time	200 ms
Microscans	1 / 10
SID	10 / 20 eV

Results and discussion

It has been shown that the column temperature is a critical parameter for the chromatographic separation of proteins with reversed phase chromatography[1] and thus needs to be optimized during the method development. At a column temperature of 50 °C or lower, the peak shape of mAbs is very poor, resulting from an extended elution time. However, an elevated column temperature of 70 °C results in improved peak shapes and peak widths.[2] Secondary interactions with the stationary phase provide one explanation for this phenomenon.[3] This effect also influences the trapping efficiency of proteins as shown in

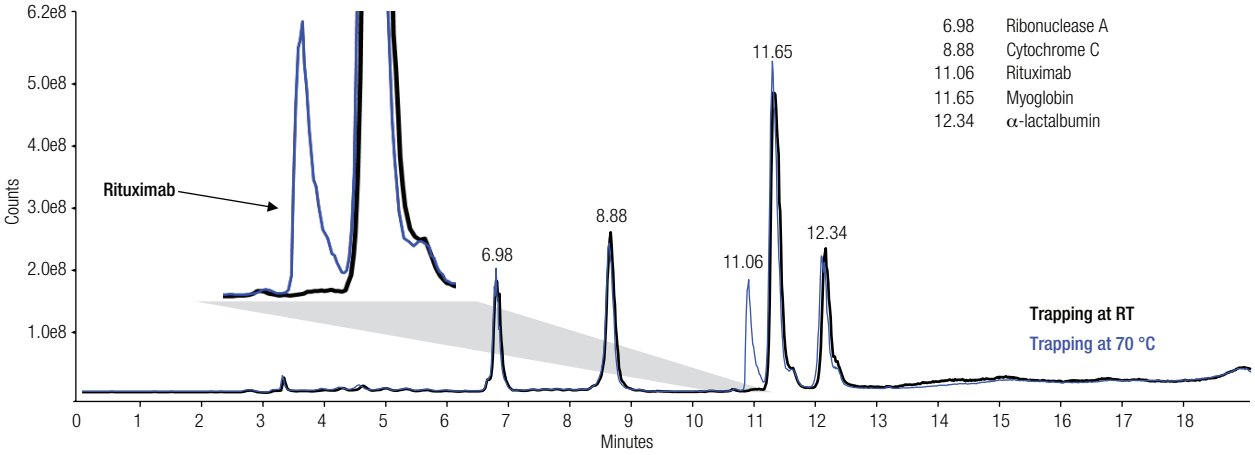

Figure 4. Analysis of a five protein mixture trapped on a MSPac DS-10 cartridge at 70 °C (blue trace) and at room temperature (black trace). Proteins were separated on a MAbPac RP column at 70 °C, showing the result for ribonuclease A, cytochrome C, rituximab, myoglobin, and α-lactalbumin A (total protein concentration: 420 ng).

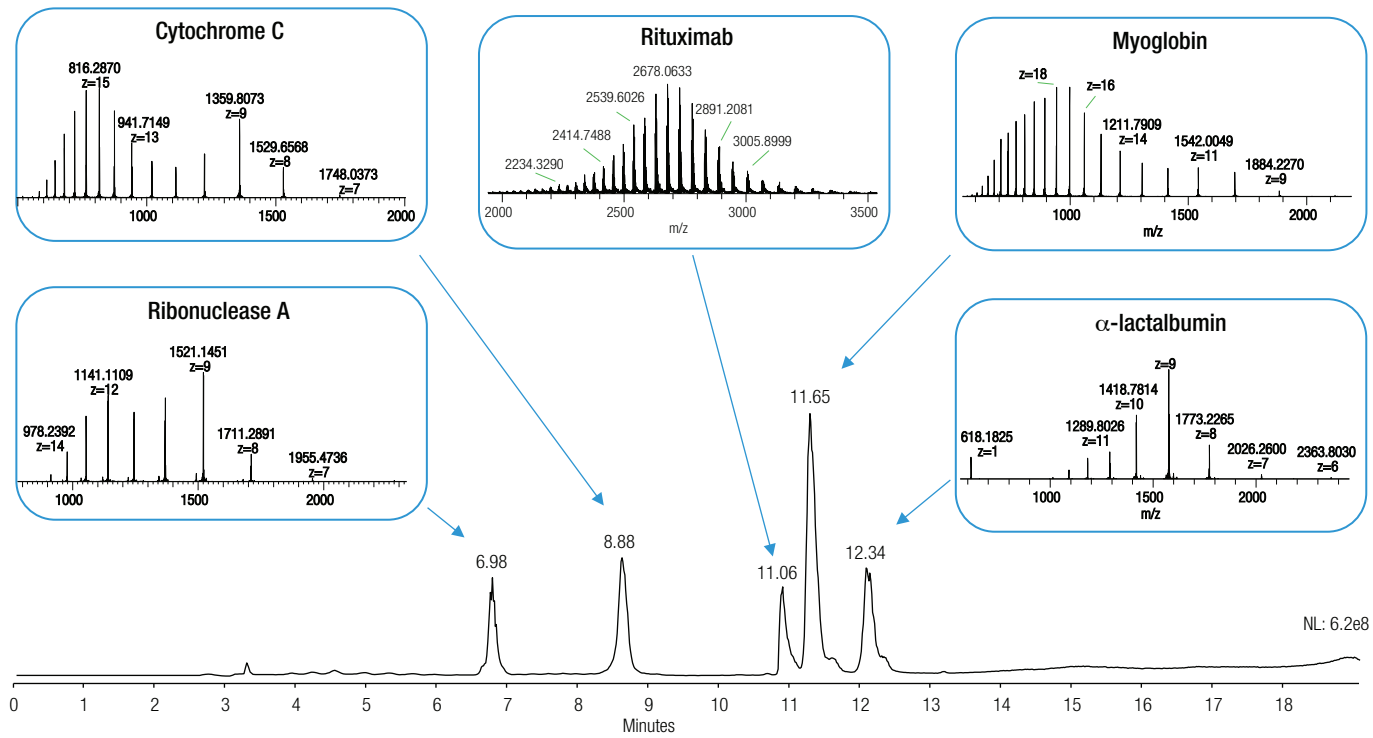

Figure 5. Analysis of a five protein mixture showing the separation and representative mass spectra for ribonuclease A, cytochrome C, rituximab, myoglobin, and α-lactalbumin A (total protein concentration, 600 ng) trapped on a MSPac DS-10 cartridge and separated on a MAbPac RP column.

Figure 4. Trapping and separating the proteins at 70 °C results in a baseline-resolved chromatogram for all five proteins. Changing the trapping temperature to room temperature and keeping the analytical column at 70 °C results in a loss of the rituximab peak (#3 -RT 11.06 min). The supressed elution of the mAb from the trapping column at room temperature causes this missing rituximab peak in the chromatogram and leads to a slightly increased baseline between 11 and 16 minutes. The trap column temperature has a major influence on the loading efficiency of monoclonal antibodies, but a less pronounced effect for smaller proteins (10–20 kDa). This example outlines the importance of elevated temperatures for protein trapping as well as protein separations.

Using the described online SPE setup for protein samples allows a very fast and simple way to efficiently clean up, enrich, and separate proteins for LC-MS analysis. Figure 5 shows a representative total ion current (TIC) chromatogram for the baseline separation of the five protein mixture. The sample was loaded to the trap column and the actual separation started at 2.0 minutes. The peak at 3.2 minutes

shows the eluting TFA, retained to the trap column during the loading step, and other very polar compounds eluting from the analytical column. All proteins of the mixture could be separated and the corresponding averaged mass spectra are shown in Figure 5. The rituximab spectrum consists of 15 averaged scans and all other spectra are an average of 87 scans. The mass spectrum for rituximab was acquired with a resolution of 15,000 and 10 microscans. For all other protein spectra, a resolution setting of 120,000 and 1 microscan were used to acquire isotopically resolved spectra for the determination of the monoisotopic mass.

Using the binary HPG pump for the chromatographic separation enables very fast separations, due to the very low gradient delay volume of this pump. Despite the larger gradient delay volume the quaternary LPG pump, it is sufficient for loading under isocratic conditions and can be used in this setup also for very fast separations.

The intact mass of the five proteins in the mixture and the four most abundant glycoforms of rituximab are obtained after the deconvolution of the full MS mass spectra with the BioPharma Finder software. Theoretical and measured masses are shown in Table 6 with the individual mass deviation for each protein/isoform. The calculated mass deviation for all proteins is below 5 ppm and demonstrates the remarkable mass accuracy of the Q Exactive mass spectrometer.

Table 6. Theoretical monoisotopic / average molecular weights (MW), measured MW, and mass deviation for the proteins and associated variants.

Protein	UniProt Accession Number	Theoretical MW (*monoisotopic*/ average) [Da]	Peak # - Retention Time [min]	Measured MW (*monoisotopic*/ average) [Da]	Mass Deviation [ppm]
Ribonuclease A (bovine)	**P61823**	*13673.260*	Peak 1 – 6.98 min	*13673.236*	**1.76**
Cytochrome C (bovine)	**P62894**	*12222.200*	Peak 2 - 8.88 min	*12222.180*	**1.64**
Rituximab + 2 G0F glycans	–	**147074.60**	Peak 3 -11.06 min	**147074.63**	**-0.14**
Rituximab + G0F/G1F glycans	–	**147236.74**	Peak 3 -11.06 min	**147237.31**	**-3.81**
Rituximab + G0F/G2F or (G1F)2 glycans	–	**147398.89**	Peak 3 -11.06 min	**147398.17**	**4.89**
Rituximab + G1F/G2F glycans	–	**147561.03**	Peak 3 -11.06 min	**147560.97**	**0.41**
Myoglobin (horse)	**P68082**	*16940.960*	Peak 4 – 11.65 min	*16940.956*	**0.24**
α-lactalbumin A (bovine)	**P00711**	*14168.747*	Peak 5 – 12.34 min	*14168.728*	**1.36**

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Conclusion

The method shown here for the separation of a five protein mixture demonstrates the applicable online SPE setup using Vanquish UHPLC systems for biopharma samples of medium complexity. The setup can be used for fully automated sample cleanup and enables direct injection of untreated samples. For the analysis of low abundant compounds, the setup can also be used for analyte enrichment, with the possibility of high volume injections. In comparison to time- and labor-intensive manual offline SPE, this automated method is faster and less prone to errors. The LC-MS system with single point Chromeleon CDS control fulfills GMP/GLP requirements and is a turn-key solution for fully integrated and automated sample handling.

Acknowledgements

The authors would like to thank Shanhua Lin and Shane Bechler for providing the MAbPac RP and MSPac columns used in this study.

References

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TN72161-EN 0217S

Application Note 1133

SEC-MS with Volatile Buffers for Characterization of Biopharmaceuticals

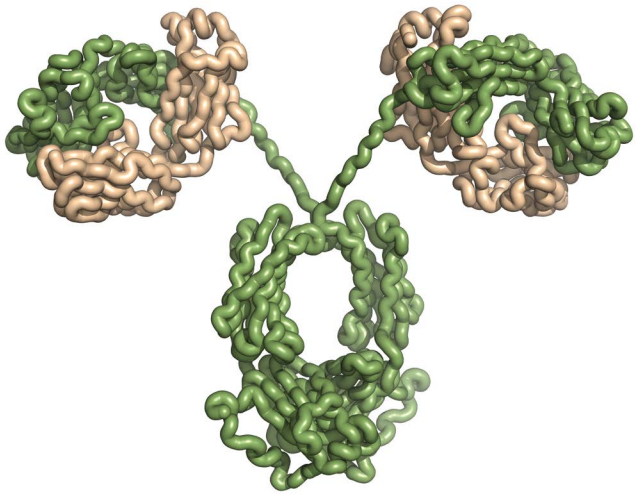
Martin Samonig, Remco Swart
Thermo Fisher Scientific, Germering, Germany

Key Words
Monoclonal Antibodies, MAbPac SEC-1, Biocompatible UHPLC, Biotherapeutics Characterization, Biopharma

Goal
Prove the suitability of the biocompatible Thermo Scientific™ Vanquish™ Flex system for size exclusion chromatography separations with mass spectrometry compatible volatile buffers.

Introduction
Size exclusion chromatography (SEC) is a routine method for the characterization of biopharmaceuticals. The conventional SEC setup uses UV detection, due to high salt concentrations in commonly used buffers. In this study, the separation of proteins by denaturing SEC with direct coupling to a mass spectrometer (MS) was investigated. A mobile phase containing acetonitrile, trifluoroacetic acid, and formic acid was used, which also allowed a direct molecular weight measurement by mass spectrometry.

The denaturing SEC method with a separation based on size differences provides an alternative to the commonly used (ion pair) reversed-phase chromatography (IP-RPC). Especially for large proteins, IP-RPC peaks often have significant tailing, due to secondary interactions with the column. This effect can be reduced with elevated column temperatures (>70 °C), but can be problematic for temperature-sensitive proteins. SEC can be used at room temperature with satisfying peak shape also for large proteins. Trifluoroacetic acid (TFA) is a common ion-pairing reagent that is often used to increase the separation efficiency for proteins in reversed-phase separations.[1] In SEC buffers, the addition of TFA reduces the protein-protein and protein-column interactions and is used instead of salts to avoid these negative effects.[2] However, it has been known that TFA suppresses the mass spectrometry signal; therefore, a very low TFA concentration of 0.05% was used.



Experimental
System evaluation was performed using the HPLC protein standard mixture (Sigma-Aldrich Chemie GmbH). The commercially available monoclonal antibodies rituximab (F. Hoffmann-La Roche, Ltd) and denosumab (Amgen) were used for this study, and disulfide bonds were reduced by incubation for 30 minutes at 60 °C with 5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) to separate light and heavy chain.

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Equipment

Vanquish Flex UHPLC system consisting of:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer

Experimental Conditions - HPLC	
Column	Thermo Scientific™ MAbPac™ SEC-1, 5 µm, 300 Å (4 x 300 mm (P/N 074696)
Mobile Phase	A: 0.1% FA and 0.05% TFA in 3/7 acetonitrile/water (v/v) , (P/N acetonitrile TS-51101)
Gradient	Isocratic
Flow Rate	0.2 mL/min
Temperature	25 °C
Injection Volume	1 µL
Detection	214 nm Data Collection Rate: 10 Hz Response Time 0.4 s
Flow Cell	10 mm LightPipe

Experimental Conditions - MS	
Source	HESI-II
Sheath Gas Pressure	35 psi
Auxiliary Gas Flow	10 arbitrary units
Capillary Temperature	250 °C
S-lens RF Voltage	60
Source Voltage	3.5 kV
Full MS Parameters	
Full MS Mass Range	1500–5000 / 400–3000 *m/z*
Resolution Settings	15.000 / 240.000
Target Value	3e6
Max Injection Time	200 ms
Microscans	10 / 1
SID	10–100 eV

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.2 SR3 was used for data acquisition, and the data analysis was performed using Thermo Scientific™ ProteinDeconvolution™ software version 3.0.

Results and Discussion

The mixture of four standard proteins (cytochrome c, myoglobin, ribonuclease A, and transferrin) was used to evaluate the setup. The separation and corresponding mass spectrum for each protein is shown in Figure 1. The measured mass after deconvolution has a mass deviation to the calculated theoretical mass of 0.75–1.23 ppm and shows the outstanding mass accuracy of the Q Exactive mass spectrometer.

The concentration of the additive TFA is a critical parameter in this method and has to be optimized for protein separations; it varies between 0.01 and 0.2%. The quaternary pump of the Vanquish Flex system enables a straightforward mobile phase optimization using one channel containing an adequate concentration of TFA. All common therapeutic mAb products are dissolved in formulation buffers with MS-incompatible components like polysorbate 80 and salts to stabilize the protein. The established SEC-MS setup is able to separate the small buffer components from the protein, can be used as a desalting step prior to mass detection and provides an alternative to IP-RPC separations. (Figure 2A and 2C).

The isocratic separation allows fast separations, due to the absence of an equilibration step and the low carryover of SEC separations. This method can also be used to separate the intact mAb from the detached chains for product related impurity characterizations, due to a retention time difference of 0.7 minutes from the intact mAb to the next eluting peak of the heavy chain. The disulfide bridges of the mAb were reduced with TCEP to separate light (23 kDa) and heavy chain (50 kDa). The two chains were baseline separated and the measured protein spectra are shown in Figure 2B and 2D.

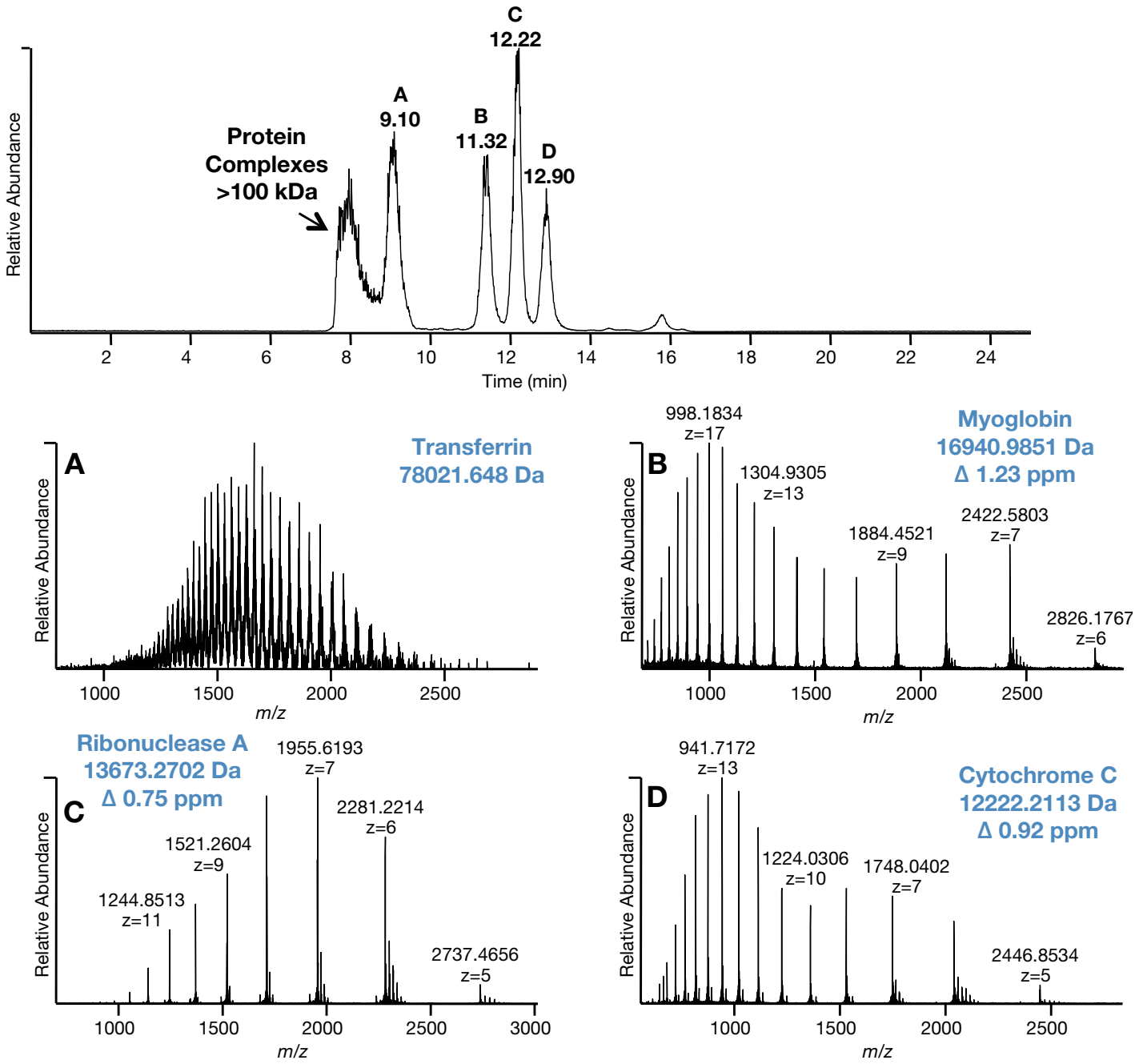

Figure 1. Separation of a mixture of the four standard proteins and the measured mass spectra for cytochrome c (1D), myoglobin (1B), ribonuclease A, (1C) and transferrin (1A). The protein complexes at RT = 8 min can be minimized with increased TFA concentrations (~ 0.1%), with the drawback of decreased signal intensities of the protein spectra.

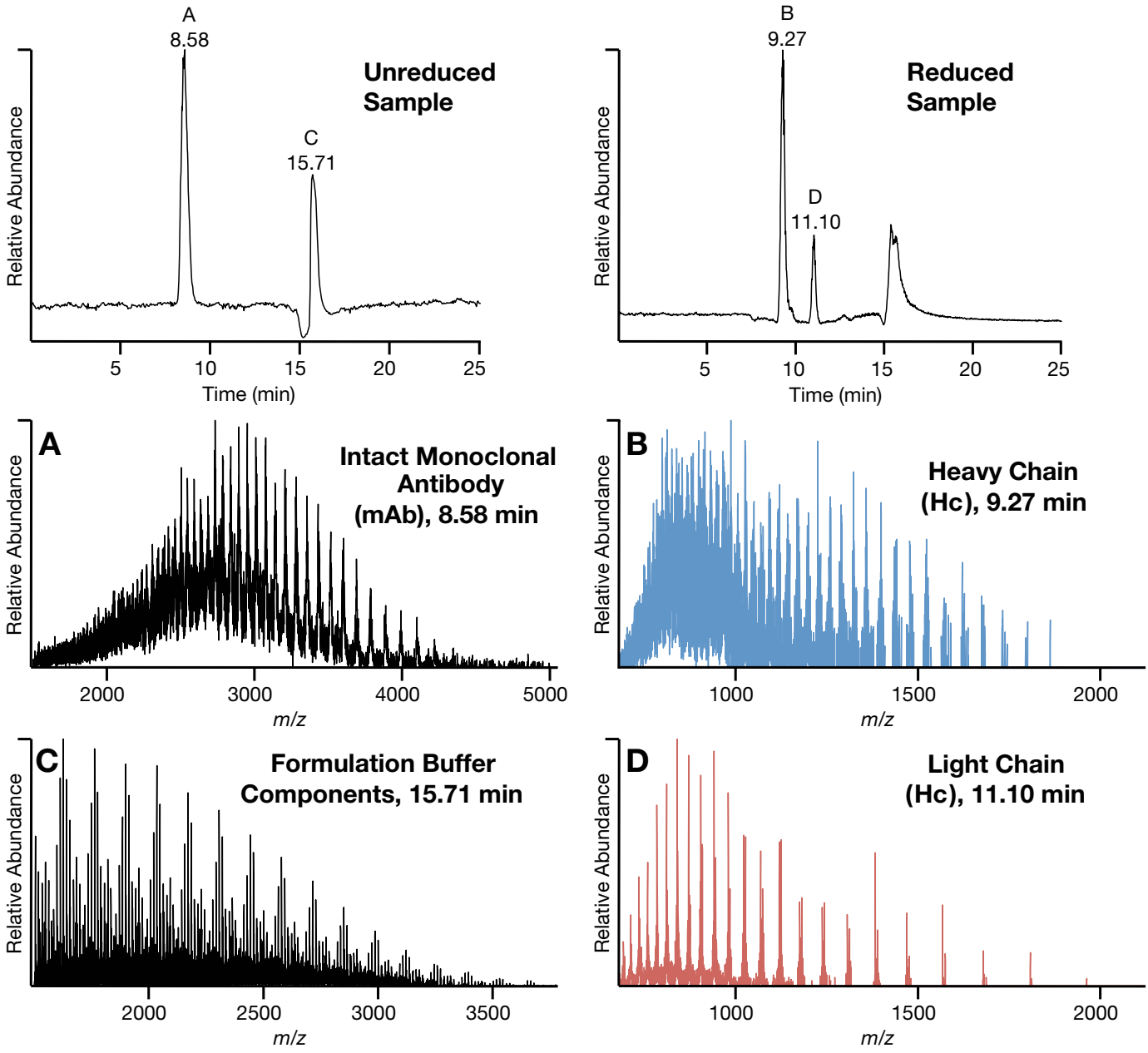

Figure 2. Separation of the unreduced and reduced monoclonal antibody rituximab.

Conclusion

The use of TFA and FA as additives for volatile SEC buffers enables the possibility for MS detection and delivers sufficient separation efficacy to characterize biopharmaceuticals. The biocompatible flow path of the Vanquish Flex system and the solvent flexibility of the quaternary pump delivers the ideal system for SEC-MS analysis and even establishes the possibility of method scouting.

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Application Note 21069

Separation of Monoclonal Antibody (mAb) Oxidation Variants on a High-Resolution HIC Column

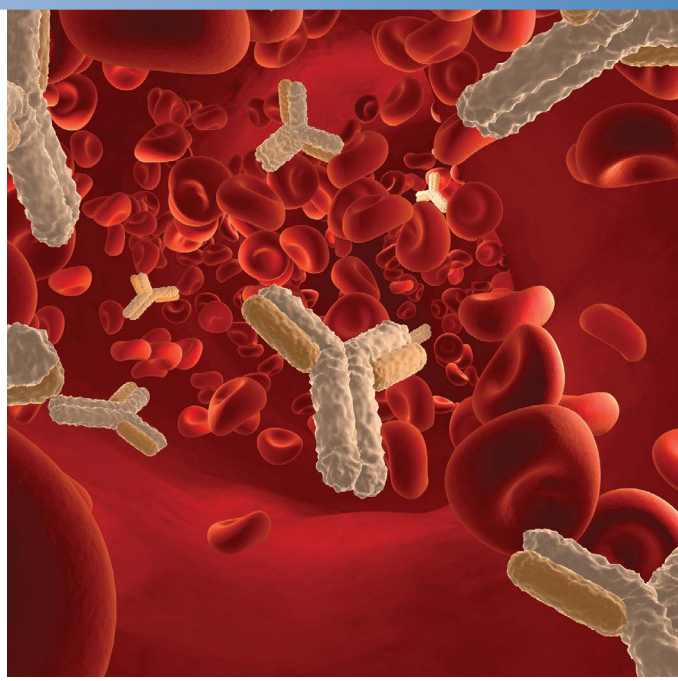
Julia Baek and Xiaodong Liu, Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words

Hydrophobic interaction chromatography, HIC, monoclonal antibody, mAb, oxidation, MAbPac HIC-20

Goal

To demonstrate the separation of oxidized mAb variants from their native form using Thermo Scientific™ MAbPac™ HIC-20 columns.



Introduction

Monoclonal antibody (mAb) drugs are a rapidly growing class of biotherapeutics that target various diseases including autoimmune disorders, cardiovascular diseases, infectious diseases, and cancers.[1] Recombinant mAbs are subject to a variety of biochemical modifications during processing, delivery, and storage. Some of these modifications have been shown to affect the safety and efficacy of mAb therapeutics, increasing the importance of analytical methods to detect mAb variants. Among these modifications, oxidation of exposed amino acid residues such as tryptophan (Trp) and methionine (Met) is a major concern in therapeutic mAb stability studies. A number of researchers have reported oxidation of mAbs and the adverse effect on product shelf life and bioactivity.[2] Oxidation of amino acid residues on a mAb can alter the hydrophobic nature of the mAb by either the increase in polarity of the oxidized form or the resulting conformational change. Therefore, hydrophobicity-based HPLC methods such as reversed-phase liquid chromatography (RPLC) and hydrophobic interaction liquid chromatography (HIC) are often used to characterize oxidized mAb products.[2]

Several recent studies have indicated that the Thermo Scientific™ ProPac™ HIC-10 column provided desired selectivity for oxidized mAbs, as an excellent alternative to RPLC.[3] HIC is a method that separates proteins, including monoclonal antibodies based on molecular hydrophobicity. The HIC mobile phase usually consists of a salting-out agent, which at high concentration retains the protein by increasing hydrophobic interaction between the protein and the stationary phase. Bound proteins are eluted by decreasing the salt concentration. In contrast to RPLC, HIC mobile phases typically contain little or no organic solvent at physiological pH levels, which allows the protein to preserve its native structure. Thus, conformational changes in the native form of the protein may be analyzed using HIC.[4]

The MAbPac HIC-20 column is a high-resolution, silica-based HIC column designed for the separation of mAbs and mAb variants. Its unique proprietary column chemistry provides high resolution, rugged stability, and desired selectivity for the analysis of mAbs and related variants. Here we describe the separation of oxidized variants of two mAbs on MAbPac HIC-20 columns.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Isopropanol (Fisher Scientific P/N A461-4)
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, ≥98.0%)
- Ammonium sulfate ($(NH_4)_2SO_4$, ≥99.0%)
- Hydrogen peroxide (H_2O_2) (Fisher Scientific P/N H325-500)
- 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Fisher Scientific P/N AC40156-0250)
- Sodium chloride (NaCl ≥99.5%)
- Sodium acetate trihydrate (Fisher Scientific P/N 50-877-058)

Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Two therapeutic mAbs were provided by a customer.

Preparation of 2× oxidation buffer

Dissolve 2.014 g of 360 mM sodium chloride and 0.136 g of 10 mM sodium acetate in 90 mL of DI water. Adjust the pH to 5.0 with 10 N HCl. Bring the final volume to 100 mL with DI water and filter the solution with a 0.22 µm filter.

Preparation of H_2O_2 oxidized mAb

Dilute the mAb solution (5 mg/mL) in half with the 2× oxidation buffer. Then add H_2O_2 to a final concentration of 0.01% (v/v) and incubate the sample for 24 h at room temperature. Dilute the oxidized mAb solution in half with mobile phase A (2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0) before analysis. The final oxidized mAb concentration is 1.25 mg/mL.

Preparation AAPH oxidized mAb

Dilute the mAb solution (5 mg/mL) in half with the 2× oxidation buffer. Then add AAPH to a final concentration of 1 mM and incubate the sample for 24 h at 40 °C. Dilute the oxidized mAb solution in half with mobile phase A (2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0) before analysis. The final oxidized mAb concentration is 1.25 mg/mL.

LC Separation

The LC separation conditions were as follows:

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS LC system equipped with:
	SR-3000 Solvent Rack (without degasser) (P/N 5035.9200)
	LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a micro flow cell
Column(s)	MAbPac HIC-20, 4.6 × 100 mm (P/N 088553)
	MAbPac HIC-20, 4.6 × 250 mm (P/N 088554)
Mobile phase A	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0 Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) and 264.2 g of ammonium sulfate in 800 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.
Mobile phase B	100 mM sodium phosphate, pH 7.0 Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$) in 900 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.
Gradient	Specified in Figures 1–4
Flow rate	1.0 or 0.5 mL/min
Temperature	30 °C
UV detector wavelength:	280 nm

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

A recent study on protein oxidation showed that hydrogen peroxide (H_2O_2) and t-butyl hydroperoxide (t-BHP) primarily oxidize Met residues, while 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and H_2O_2 + Fe(II) oxidize both Met and Trp residues. AAPH was reported to be more efficient in oxidizing Trp on a model protein.[5] In this study, H_2O_2 and AAPH were used as oxidants to induce Met and Trp oxidation on mAb samples. Using the standard HIC mobile phase condition, the MAbPac HIC-20 was able to differentiate oxidized mAb variants from the untreated mAb (Figure 1). Oxidation of the mAb with H_2O_2 resulted in two peaks with lower retention times, presumably by introducing conformational changes. Over time, the intensity of peak 1 continued to increase as the intensity of peak 2 decreased (data not shown). It is most likely that peak 1 has more residues oxidized than peak 2. As the oxidation continues, concentration of the variant with more sites oxidized increases.

For the AAPH oxidized mAb sample, a broader peak with two non-resolved shoulders where H_2O_2 oxidized variants (peaks 1 and 2) elute was observed. We speculate that AAPH oxidized both Met and Trp residues. The oxidation of a more buried Trp can lead to a more dramatic structural change that may cause the mAb to partially unfold. Partially unfolded mAb is more likely to have more conformational variations and result in a broader peak. This phenomenon has been reported previously with another mAb sample on ProPac HIC-10 column.[3] To optimize the resolution of the chromatograms, conditions using a lower starting salt concentration and addition of organic solvent into both mobile phases were investigated. Both approaches reduced the retention time. However, reducing the starting ammonium sulfate concentration to 1 M improved the resolution of the two oxidized mAb variants (Figure 2), while addition of isopropanol in both mobile phases reduced the resolution (data not shown). Mobile phases containing sodium acetate pH 5.2 buffer were also examined, but no improvement on the separation of the mAb and its oxidized variants was exhibited (data not shown).

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 × 100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-5.0	100	0
	0.0	100	0
	1.0	100	0
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	10 µL		
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	mAb (1.25 mg/mL)		
	H_2O_2 oxidized mAb (1.25 mg/mL)		
	AAPH oxidized mAb (1.25 mg/mL)		

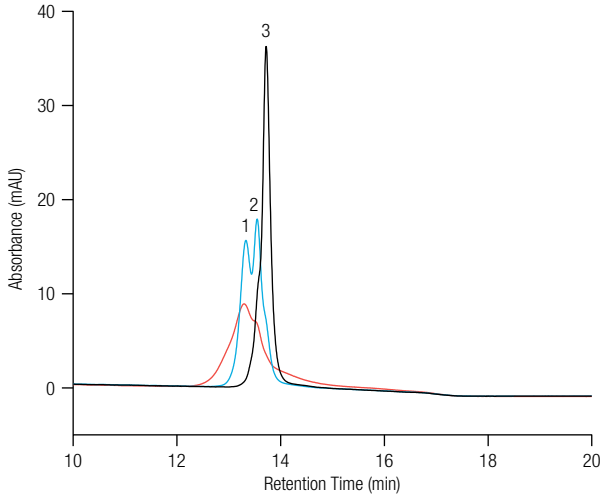

Figure 1. Separation of oxidized mAb1 using standard condition

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 × 100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-5.0	50	50
	0.0	50	50
	1.0	50	50
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	10 µL		
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	mAb (1.25 mg/mL)		
	H_2O_2 oxidized mAb (1.25 mg/mL)		
	AAPH oxidized mAb (1.25 mg/mL)		

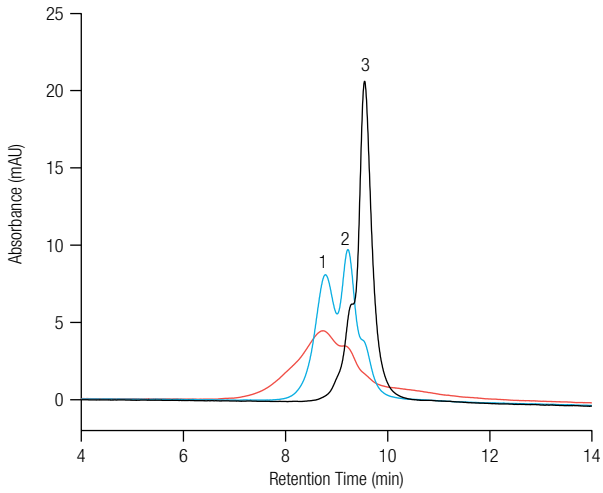

Figure 2. Separation of oxidized mAb1 using lower starting salt concentration

To further improve the resolution, a 4.6 × 250 mm column was employed. Using a flow rate of 0.5 mL/min and a starting ammonium sulfate concentration of 1 M, highest resolution was achieved with a relatively short analysis time (Figure 3). In this chromatogram, two oxidized species are also clearly observed from the untreated mAb chromatogram.

Using a similar condition another oxidized mAb sample was analyzed (Figure 4). Similar to the first mAb, H_2O_2 oxidized mAb2 has two distinct variant peaks and the AAPH oxidized sample has a broad peak with two non-resolved shoulders where the H_2O_2 oxidized peaks (peak 1 and 2) elute. This mAb sample appears to have two small oxidation variants as well as two hydrophobic variants (peak 4 and 5) on the right side of the main peak (peak 3).

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 × 250 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-6.0	50	50
	0.0	50	50
	2.0	50	50
	30.0	0	100
	35.0	0	100
Flow Rate:	0.5 mL/min		
Inj. Volume:	20 µL		
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	mAb (1.25 mg/mL)		
	H_2O_2 oxidized mAb (1.25 mg/mL)		
	AAPH oxidized mAb (1.25 mg/mL)		

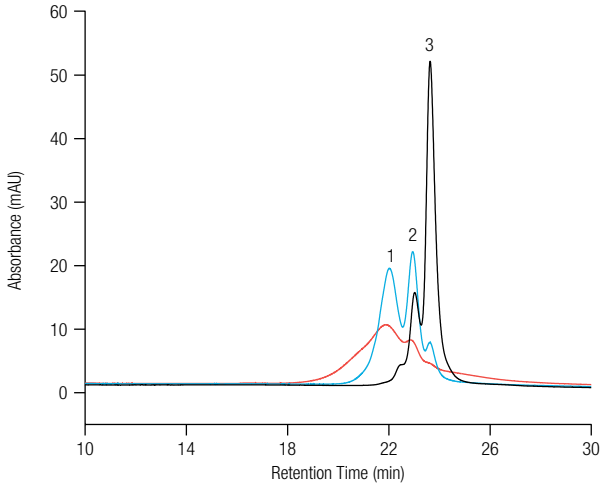

Figure 3. Separation of oxidized mAb1 on a 4.6 × 250 mm column

Column:	**MAbPac HIC-20**, 5 µm		
Format:	4.6 ×250 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-6.0	40	60
	0.0	40	60
	2.0	40	60
	30.0	0	100
	35.0	0	100
Flow Rate:	0.5 mL/min		
Inj. Volume:	20 µL		
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	mAb (1.25 mg/mL)		
	H_2O_2 oxidized mAb (1.25 mg/mL)		
	AAPH oxidized mAb (1.25 mg/mL)		

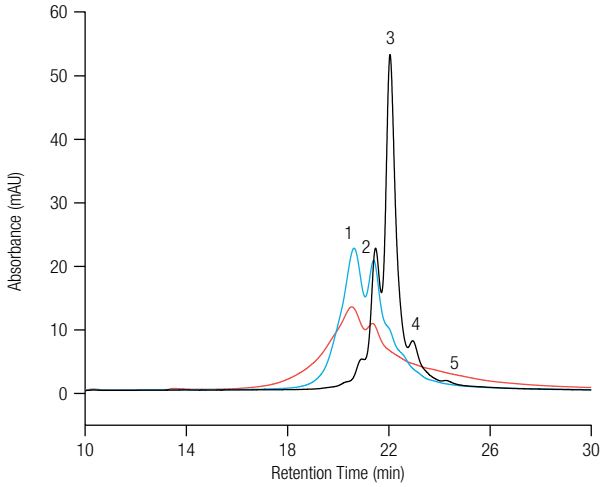

Figure 4. Separation of oxidized mAb2 on a 4.6 × 250 mm column

Conclusion

- The MAbPac HIC-20 column is a high-resolution HIC column that allows the separation of oxidized mAb variants from its native form.
- Using the MAbPac HIC-20 column, separation of oxidized mAb variants can be achieved without further sample processing.
- Best separation was achieved with 0.5 mL/min and 1.0–1.2 M starting salt concentration on a 4.6 × 250 mm MAbPac HIC-20 column.

References

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AN21069-EN 0616S

Reversed-Phase Separation of Intact Therapeutic Antibodies Using the Vanquish Flex UHPLC System

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Application Note 1131

Key Words
Biotherapeutics, Biosimilars, Intact Proteins, Stability, mAbs

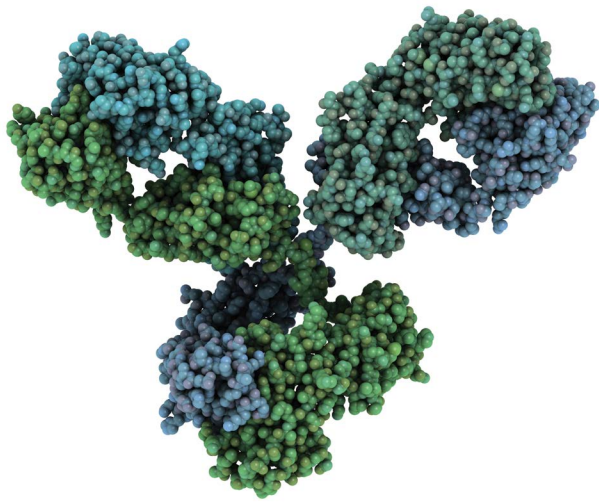
Goal

Provide examples of reversed-phase chromatography of monoclonal antibodies with the Thermo Scientific™ Vanquish™ Flex UHPLC System and Thermo Scientific™ MAbPac™ RP column.

Introduction

In biopharmaceutical early analytical development, characterization of monoclonal antibodies is required to support process development. Separation techniques such as hydrophobic interaction chromatography, size-exclusion chromatography, ion-exchange chromatography, and reversed-phase chromatography are routinely applied to profile the therapeutic protein during this stage of development. Reversed-phase chromatography can be run with mass spectrometry (MS)-compatible mobile phase, hence the method can be easily transferred to MS characterization laboratories when required.

Besides providing separation of impurities based on hydrophobicity, reversed-phase chromatography is an excellent tool for protein quantitation of main compound and minor variants. Reversed-phase separation of intact proteins is typically run at high temperatures to improve peak shape and recovery of proteins. Thus, high-resolution columns, packed with temperature-stable material are required. In addition, the method should be sufficiently fast, in order to allow the processing of a large number of samples in a reasonable time. An initial stability evaluation of the new biological entities has to be provided by early development laboratories. The analytical methods for the early stability assessment need to be able to indicate, and approximately quantify, sample degradation.



The MAbPac RP column is dedicated to separations of intact proteins. It is based on supermacroporous 4 µm polymer particles with exceptional thermal stability. The Vanquish Flex UHPLC system offers column thermostatting up to 120 °C and features a low-dispersion active pre-column eluent heater. This device actively regulates the thermal balance between the mobile phase and the stationary phase. The accurate temperature control allows avoiding loss of efficiency due to temperature mismatch between the column and the incoming solvent.

In this work, the MAbPac-RP column was operated with the Vanquish Flex UHPLC system for the reversed-phase chromatography of several intact therapeutic antibodies. To assess the suitability for stability studies, the chromatograms of a reference and a stressed mAb were compared.

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Experimental

Instrumentation

Vanquish Flex UHPLC system, equipped with:

- System Base (P/N VH-S01-A)
- Quarternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A) with Active Pre-heater VH-C1 (P/N 6732.0110) and Post-column Cooler 1 µL VH-C1 (P/N 6732.0510)
- Diode Array Detector HL (P/N VH-D10-A) equipped with LightPipe™ Standard Flowcell (P/N 6083.0100)

Chromatographic Conditions

Column:	MAbPac RP (2.1 x 50 mm) (P/N 088648)	
Mobile phase A:	0.1:100 TFA/water (v/v)	
Mobile phase B:	0.1:90:10 TFA/acetonitrile/water (v/v/v)	
Flow rate:	300 µL/min	
Column compartment temperature settings:	Column compartment: Active pre-heater: Post column cooler:	80 °C Forced air mode 80 °C 50 °C
Detector settings:	Detection wavelength: Data acquisition rate: Response time:	280 nm 10 Hz 0.4 s

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2

Results and Discussion

Four commercial antibodies were eluted with a 10 minute linear gradient. The linear increase of acetonitrile in the mobile phase was (9%)/min, in the case of trastuzumab (Figure 1), and (3.6%)/min for cetuximab (Figure 4). In all cases, the elution of the intact antibodies resulted in very sharp peaks. The peak width at half height spanned from 1.6 seconds to 3.1 seconds for the steepest and the shallowest gradient, respectively. Peak symmetry was excellent for all mAbs, as it can be observed in Figures 1–4.

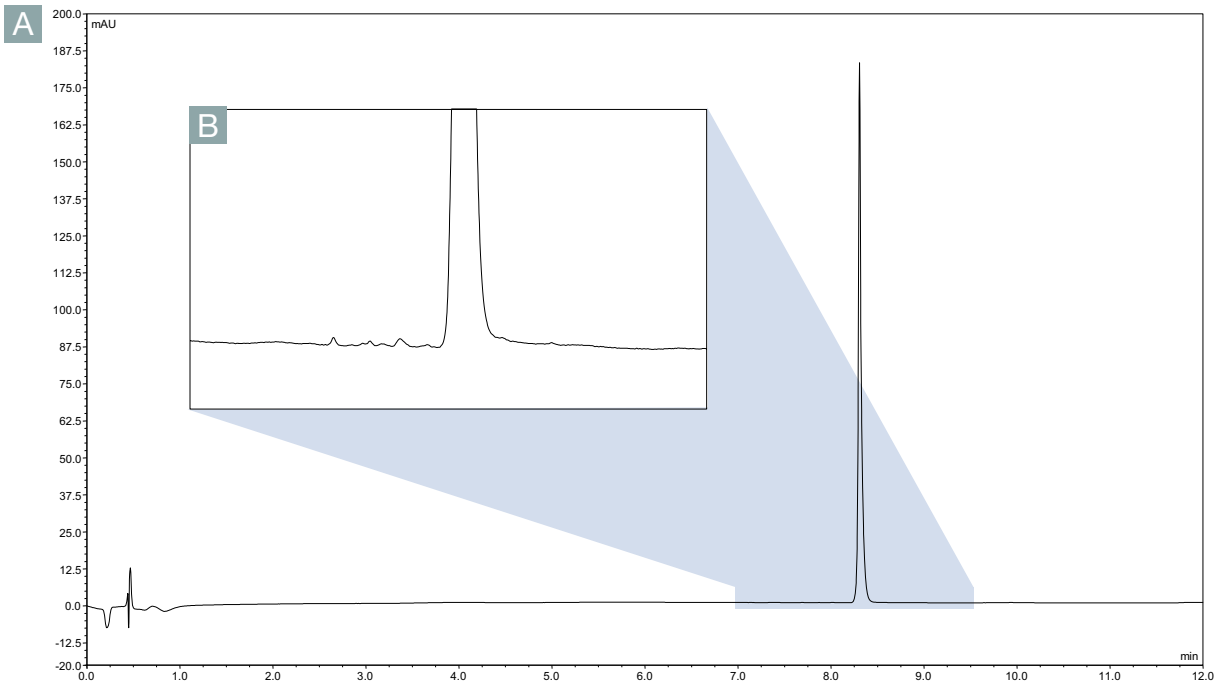

Figure 1. Injection of 4.2 µg of trastuzumab. Gradient 0–100% B in 10 minutes. Full view (a) and enlarged view (b).

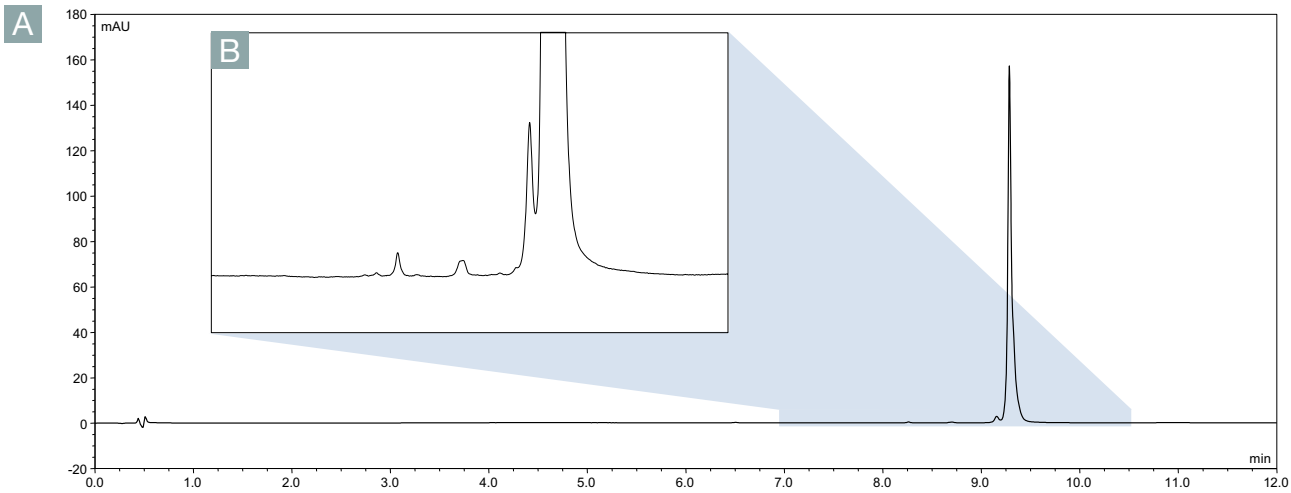

Figure 2. Injection of 25 µg of bevacizumab. Gradient 10–60% B in 10 minutes. Full view (a) and enlarged view (b).

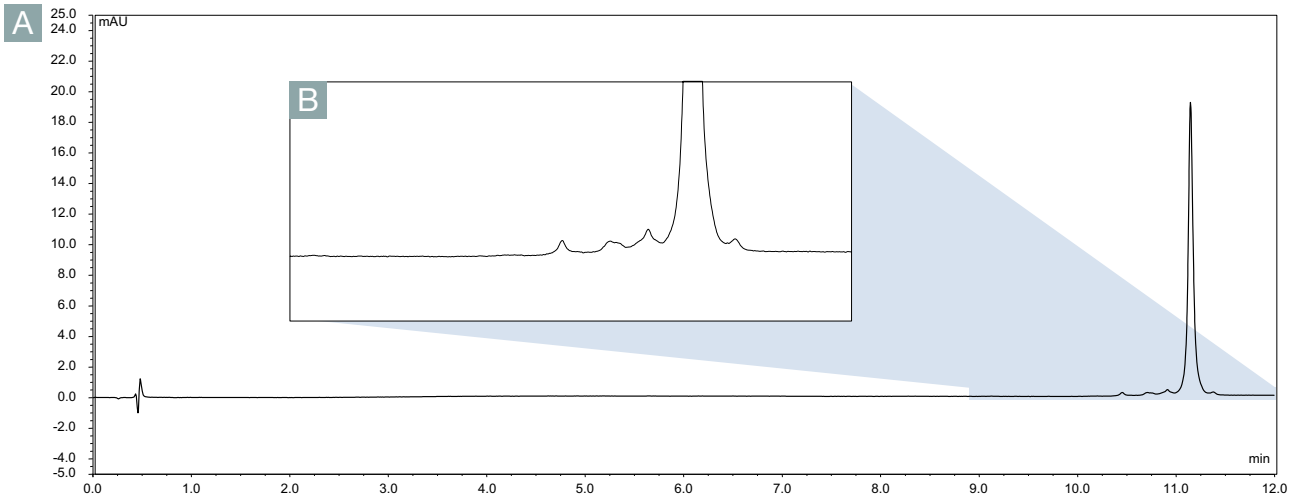

Figure 3. Injection of 1.25 µg of cetuximab. Gradient 20–45% B in 10 minutes. Full view (a) and enlarged view (b).

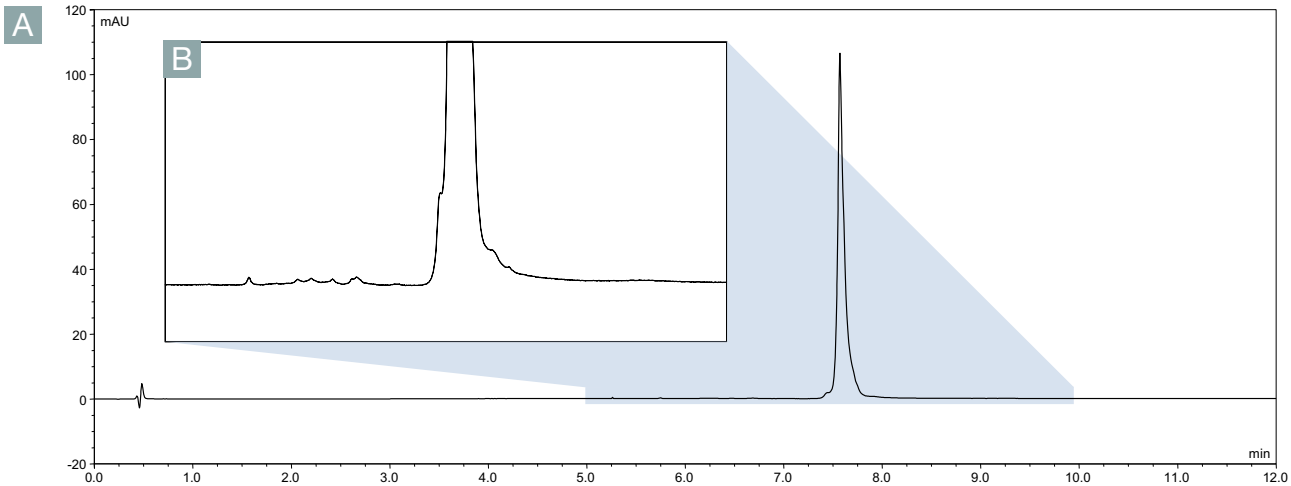

Figure 4. Injection of 1 µg of rituximab. Gradient 22–60% B in 10 minutes. Full view (a) and enlarged view (b).

Detailed views of the intact antibodies chromatograms revealed that the MAbPac RP column provides selectivity to separate minor variants, even with relatively simple and fast gradient programs. This feature can be exploited in cases such as preliminary stability studies. In Figure 5, the chromatogram of a reference antibody is compared to a stressed one. The sample was donated by a customer and the stressing conditions were not disclosed. Here, the effects of stress-related degradation of an antibody are observed by running a simple 10 minute gradient from 0 to 100% B. The increased relative area of the impurities eluting before the main peak of the stressed mAb, confirmed the degradation of the sample. Additionally the degradation/denaturation of the sample can be estimated by the increased width of the main peak. The width of the main peak at half height was 2.0 seconds for the reference, and 3.3 seconds for the stressed sample. This effect is likely caused by close-eluting species present in the stressed antibody but not in the reference one.

Conclusion

Reversed-phase chromatography is a powerful and convenient tool for the characterization of intact antibodies. With the extended thermostatting temperature range of the Vanquish Flex system and the new MAbPac RP column, very fast and efficient separations are achieved. Chromatograms with sharp symmetrical peaks are obtained that can be used to assess antibody purity in a very straightforward way.

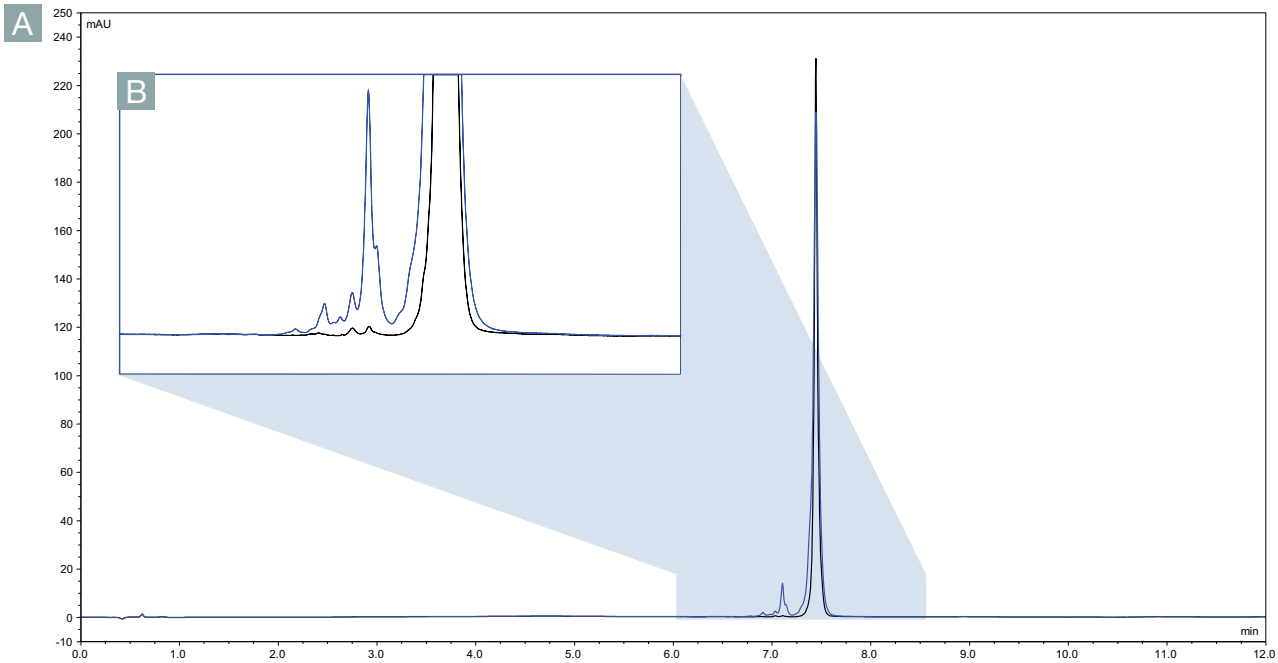

Figure 5. Comparison between a reference (black trace) and a stressed antibody (blue trace). Gradient: 0–100% B in 10 minutes. Full view (a) and enlarged view (b).

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High Temperature, High Throughput Reversed Phase Separation of Intact Monoclonal Antibodies

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Thermo Fisher Scientific, Germering, Germany

Poster Note 71695

Overview

Purpose: Demonstrate reversed phase chromatography of intact IgG with a novel UHPLC system and a novel dedicated column

Methods: In this work, the Thermo Scientific™ MAbPac™ RP column was operated with the Thermo Scientific™ Vanquish™ Flex UHPLC system. To assess the suitability of the approach for stability studies, the separation of a reference and a stressed mAb chromatogram is compared.

Results: The novel reversed phase column for intact protein allowed fast and efficient elution of monoclonal antibodies with a generic gradient. The suitability of the method for stability-indicating studies was demonstrated by comparing the profile of a reference and stressed antibody

Introduction

In the biopharmaceutical early analytical development, characterization of monoclonal antibody is required to support process development. Reversed Phase Chromatography (RPC) is routinely applied to profile the therapeutic protein during this stage of development. RPC can be run with MS-compatible mobile phase, hence the method can be easily transferred to the MS characterization laboratories when required. RPC is an excellent tool for protein quantitation of main compound and minor variants. RPC of intact proteins is typically run at high temperature to improve peak shape and recovery. Thus, high resolution columns, packed with temperature-stable material are required. The method should be sufficiently fast, in order to allow the processing of a large number of samples in a reasonable time.

A typical example of early-stage analytical development is the early stability evaluation of the new biological entities. The analytical methods for the stability assessment need to be able to indicate, and approximately quantify, sample degradation.

Methods

Sample Preparation

All antibody samples were dissolved in aqueous buffers. The concentration of the samples were the following: trastuzumab 21 mg/mL; bevacizumab 25 mg/mL; cetuximab 5 mg/mL; rituximab 1 mg/mL. The antibody referred as mAb-C (reference and stressed) was donated by a customer; the original solution was diluted 10 times.

Liquid Chromatography (or more generically Separations)

Chromatography systems:1) Vanquish Flex UHPLC System equipped with low pressure mixing quaternary pump. 2) Thermo Scientific Vanquish UHPLC system equipped with high pressure mixing binary pump

Mobile Phases: **A**- 0.1/100 = TFA/water (v/v); **B**- 0.1/9/1 TFA/acetonitrile/water (v/v/v)

Flow rate= 0.3 mL/min

LightPipe™ DAD detector setting: wavelength: 280 nm, Data Acquisition Rate: 10Hz, Response Time: 0.4sec

Thermostatted Column Compartment: still air mode at temperature 70-120 °C; active pre-column heater set at same temperature as column compartment. Post-colum cooler at 50 °C

Column: MAbPac-RP 4 µm, 2.1 × 50 mm

Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2

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Results

Reversed Phase of intact antibodies

Five intact antibodies were eluted with a generic gradient 0→100 %B in 10 minutes at 80 °C Sharp peaks were obtained for all samples (Figure 1). Active mobile phase pre-column heating prevented extra band broadening effects related to thermal mismatch between column and in-coming mobile phase.

The generic gradient is suitable to identify and quantify degradation products in early stability studies without the need to optimize for each antibody. Figure 2 shows the overlay between a reference and a stressed sample. The main peak is broader for the stressed protein, and degradation products are detected in front of the main peak. By using the same gradient and directly comparing the results, the 12 minute assay allows a fast confirmation of antibody purity.

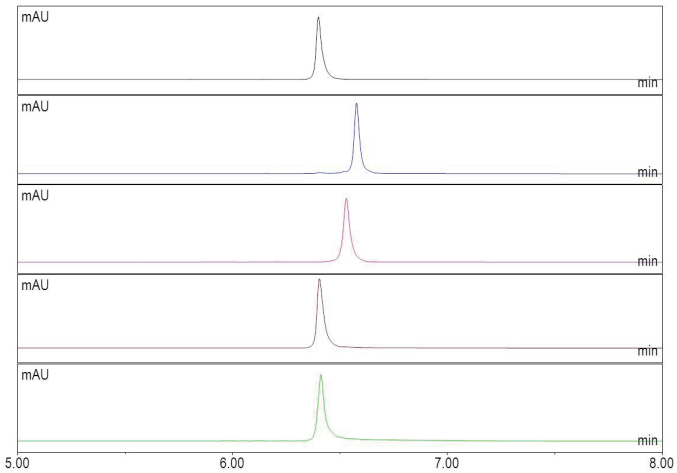

FIGURE 1. Elution of 5 intact mAb with generic gradient 0→ 100 %B in 10 minutes., at 80 °C From top to bottom panel: trastuzumab (0.2 µL), cetuximab (1 µL), mAb-C (0.25 µL), rituximab (1 µL), infliximab (1 µL).

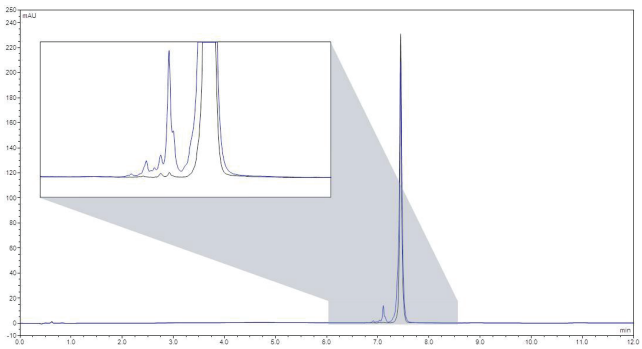

FIGURE 2. Comparison between the reference (black trace) and stressed (blue trace) antibody mAb-C. Gradient 0-→ 100 %B in 10 minutes, 80 °C. Degradation products elute in front of the main peak.

Influence of Column Temperature

High temperature column thermostatting is used for reversed phase of intact proteins. Normally temperature above 60 °C are the rule, with the range 70-90 °C as the preferred option.. One of the reasons temperatures above 90 °C are not common, is that the majority of silica-based columns lack the required thermal stability. In fact many columns are stable up to 60 °C. The other reason is to avoid the risk of thermally degrading the protein.

The exceptional thermal stability of the MabPac RP column, combined with the thermostatting capabilities of the Vanquish UHPLC system allowed to experiment with temperatures normally not accessible. Even though sharp peaks were obtained at 80 °C, in some cases we observed that higher temperatures were required to obtain satisfactory peak shape and recovery. Figure 3 for instance, shows the comparison between the same IgG sample eluted at 80 and 120 °C.

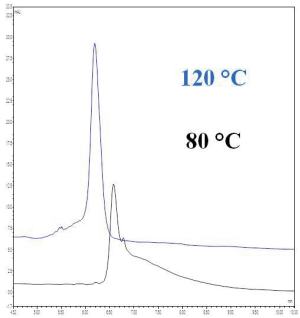

FIGURE 3. Injection of 1 µL rabbit IgG. At 80 °C the peak is affected by large tailing whereas at 120 °C the tailing has disappeared. Peak area was increased with temperature.

The chromatographic behavior of two antibodies eluted at different temperatures was studied in detail. Height and width of the main peak, and the sum area of main peak and impurities were evaluated. Peak width should decrease with temperature for pure peaks; increase of peak width at higher temperature indicates an increase of protein heterogeneity that may indicate that a degradation process is occurring. Peak area is an indicator of the recovery. Peak height is influenced both by degradation and recovery.

In Figure 4 and 5, these parameters recorded at 70,90 and 100 °C for Cetuximab and mAb-C are observed. The main peak shape is in all cases excellent, however small but important variations of peak parameters can be observed.. Firstly, the peak height, hence the sensitivity of the method, decreased with temperature for both antibodies.

Cetuximab shows a slight increase of peak width from 70 to 100 C. Recovery estimated by the sum area of all peaks improve from 70 to 90 °C, but drops at 100 °C. The behavior of mAb-C at different temperatures was more homogeneous, and the parameters relative variation less pronounced. Unlike cetuximab, peak width decrease from 70 to 90 °C. Ramping up of the width at 100 °C indicates likely degradation at this temperature.

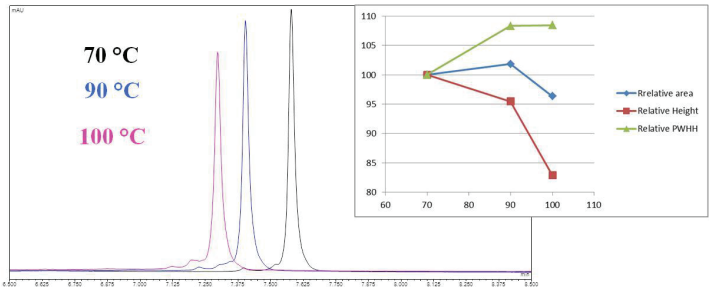

FIGURE 3. Injections of 0.25 µL cetuximab at different temperatures.

The graph shows some peak parameters of the chromatograms at different temperature: area (sum of main peak and impurities), height of the main peak, and Peak Width at Half Height of the main. Values are expressed as relative to those at 70 °C

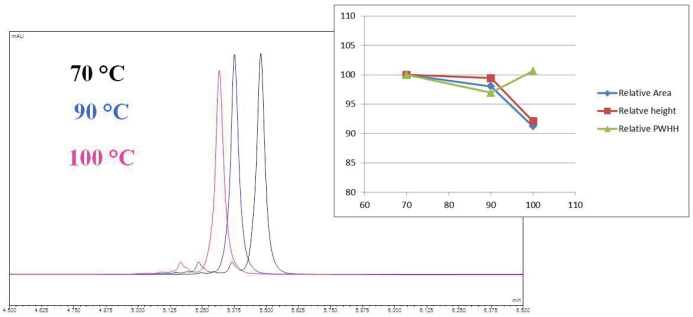

FIGURE 4. Injections of 0.25 µL mAb-C at different temperatures.

The graph shows some peak parameters of the chromatograms at different temperature: area (sum of main peak and impurities), height of the main peak, and Peak Width at Half Height of the main. Values are expressed as relative to those at 70 °C

High throughput separation of intact antibodies from degradation products can be obtained by running 5 minutes gradient (Figure 6). For high-throughput separation the Vanquish UHPLC system with binary high-pressure mixing pump is preferred, because of the low gradient delay volume (? µL)

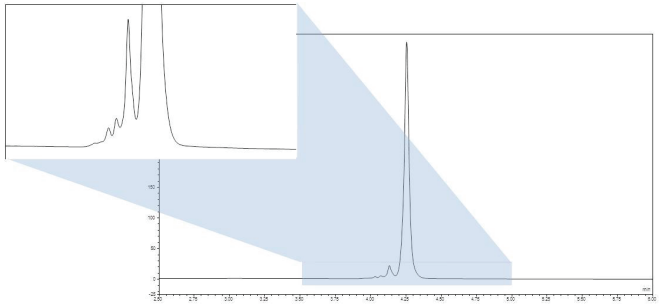

FIGURE 6. High-throughput separation of intact mAb-C and degradation products. Injection volume 0.25 µL. Gradient 0→ 100 %B in 5 minutes at 80 °C

Conclusion

- All tested mAb were eluted at 80 °C as sharp peaks with the same general purpose gradient
- Satisfactory peak shape for the rabbit IgG required column thermostatting at 120 °C
- Significant effects of temperature in the range 70-100 °C are sample-dependent
- The MabPac RP column with the Vanquish Flex system allows for rapid assessment of sample integrity in stability-indicating assays

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Application Note 20946

A Fast and Robust Linear pH Gradient Separation Platform for Monoclonal Antibody (mAb) Charge Variant Analysis

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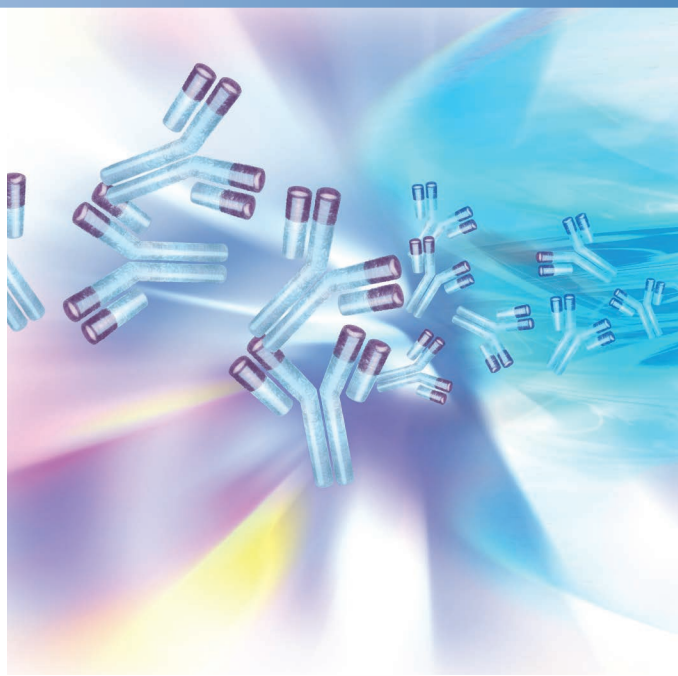
Key Words

CX-1 pH Gradient Buffer Kit, MAbPac SCX-10, mAb charge variant analysis

Abstract

This application note describes a fast, 10 min cycle time separation of mAb charge variants using a linear pH gradient separation method. A gradient from pH 5.6 to pH 10.2 was generated over time by running a linear pump gradient from 100% Thermo Scientific™ CX-1 pH Gradient Buffer A (pH 5.6) to 100% CX-1 pH Gradient Buffer B (pH 10.2). Linear UV response with up to 300 µg of protein loading was achieved. The elution pH values of mAbs are in linear relationship with their corresponding pI values.



Introduction

Recombinant monoclonal antibodies (mAbs) can be highly heterogeneous due to modifications such as sialylation, deamidation, and C-terminal lysine truncation. Salt gradient cation exchange chromatography has been used with some success in characterizing mAb charge variants; however, significant effort is often required to tailor the salt gradient method for an individual mAb. In the fast-paced drug development environment, a fast and robust platform method is desirable to accommodate the majority of the mAb analyses.

Thermo Fisher Scientific recently introduced cation-exchange pH gradient buffers that enable the fast and robust generic platform method requirements [1]. This buffer system consists of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. A linear pH gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% buffer A to 100% buffer B.

Using mAbs with a variety of isoelectric points (pI values) from 7 to 10, we have demonstrated that the linear pH gradient method separates charge variants consistently. In addition, the loading capacity on a Thermo Scientific™ MAbPac™ SCX-10 column when running pH gradient was investigated by injecting increasing amount of protein standards. The study showed that there was a linear signal response up to 300 µg of protein loading. Furthermore, a fast separation of charge variants was achieved in a 10 min cycle time using a 2 mL/min flow rate on a MAbPac SCX-10, 5 µm, 4 × 50 mm column.

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Experimental Details

Consumables		Part Number
Standard proteins were purchased from a reputable supplier. Monoclonal antibodies were a gift from a biotechnology company.		
Columns:	MAbPac SCX-10, 10 µm, 4 × 250 mm	074625
	MAbPac SCX-10, 5 µm, 4 × 50 mm	078656
Buffers:	CX-1 pH Gradient Buffer A (pH 5.6), 125 mL	083273
	CX-1 pH Gradient Buffer B (pH 10.2), 125 mL	083275

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System equipped with SRD-3600 Membrane Degasser, DGP-3600RS Biocompatible Rapid Separation Pump, TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves, WPS-3000TBRS Biocompatible Rapid Separation Thermostated Autosampler, VWD-3400RS UV Detector equipped with a Micro Flow Cell, and PCM-3000 pH and Conductivity Monitor.

Eluents

Eluent A and B were prepared by simply diluting the corresponding CX-1 pH Gradient Buffer 10 fold using deionized water. Proteins and mAb were dissolved in deionized water.

Gradients

The linear pH gradient was generated by running a linear pump gradient from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). For pH gradient analysis carried out on the MAbPac SCX-10, 10 µm, 4 × 250 mm columns, the gradient method in Table 1 was used. For pH gradient analysis carried out on the MAbPac SCX-10, 5 µm, 4 × 50 mm columns, the gradient method in Table 2 or Table 3 was used. All methods cover the pH range from pH 5.6 to pH 10.2.

Data Processing

Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8

Results

pH Gradient Loading Capacity

The separation of proteins with different pI values by pH gradient was demonstrated in Figure 1 using the gradient specified in Table 1. The protein mixture contained lentil lectin with three isoforms [lectin-1 (pI 7.8), lectin-2 (pI 8.0), lectin-3 (pI 8.2)], trypsinogen (pI 8.7), ribonuclease A (pI 9.3), and cytochrome C (pI 10.2). The concentrations of these proteins were 3 mg/mL, 2 mg/mL, 3 mg/mL, and 2 mg/mL, respectively. Figure 1 shows the multiple UV traces of chromatograms recorded from a series of injections of the protein mixture at 2 µL, 4 µL, 8 µL, 16 µL, 32 µL, 50 µL, and 100 µL. The peak area and peak width at half height (PWHH) were plotted against the sample loading in Figures 2a, 2b, 2c, and 2d for lentil lectin-1, trypsinogen, ribonuclease A, and cytochrome C, respectively. Trypsinogen, ribonuclease A, and cytochrome C exhibited a linear response up 100 µL of sample loading, while lentil lectin-1 showed linear response up to 50 µL of sample loading. The separation of these proteins was maintained throughout the range of 20 µg to 1,000 µg total protein loading.

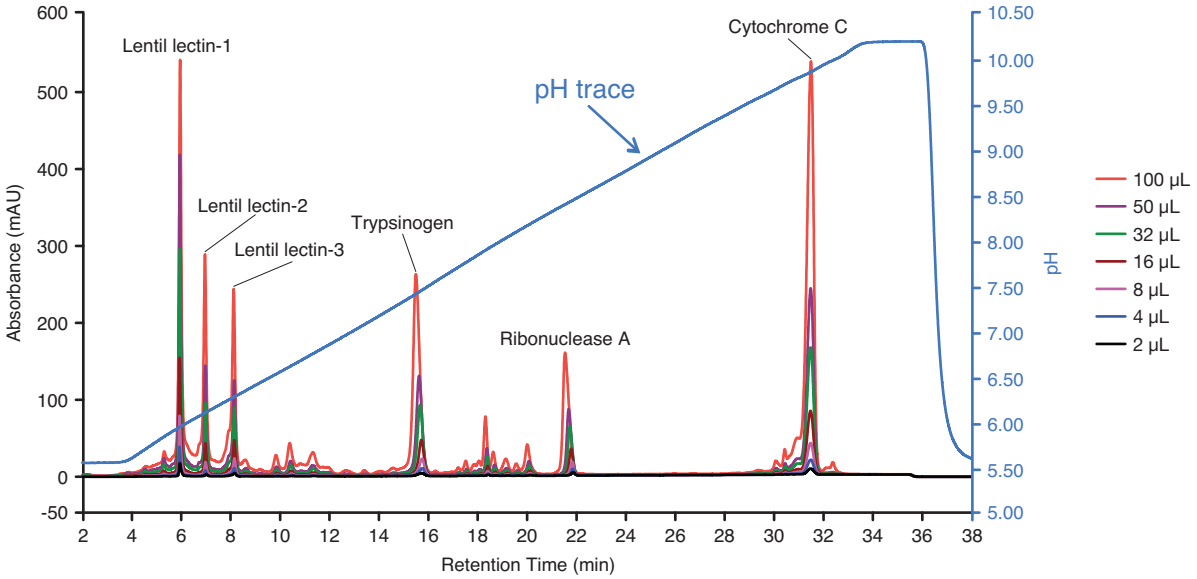

Figure 1: Chromatogram of a protein mixture separated using a 30 min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Protein names are labelled for each peak. The protein concentrations in the sample mixture were: lentil lectin, 3 mg/mL; trypsinogen, 2 mg/mL; ribonuclease A, 3 mg/mL; cytochrome C, 2 mg/mL. Increasing amounts of samples were loaded onto the column using the following injection volumes: 2 µL (black), 4 µL (blue), 8 µL (pink), 16 µL (brown), 32 µL (green), 50 µL (purple), and 100 µL (red). The pH trace is blue.

Time (minutes)	Flow rate (mL/min)	% A	% B
0–1	1	100	0
1–31	1	100–0	0–100
31–34	1	0	100
34–40	1	100	0

Table 1: A 30 min linear gradient method used with the MAbPac SCX-10, 10 µm, 4 × 250 mm, cation exchange column. Total run time is 40 min. The linear pH range covers from pH 5.6 to pH 10.2.

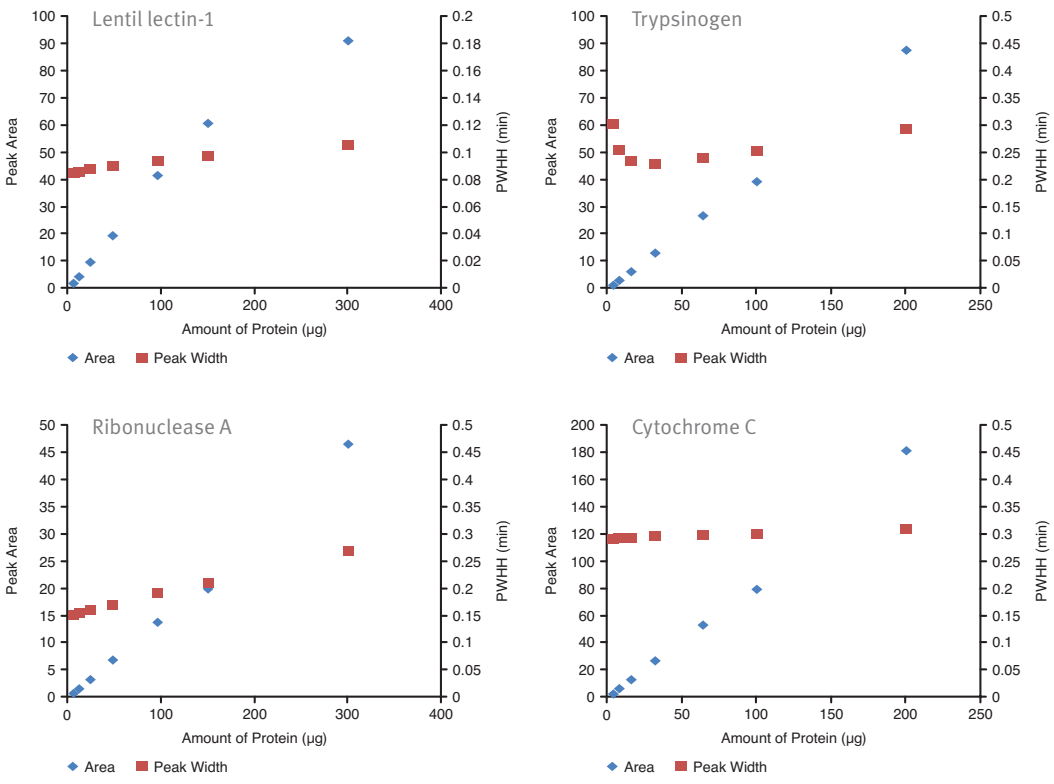

Figure 2: Plots of peak area and PWHH versus sample loading. Peak area is plotted on the primary Y-axis and the PWHH is plotted on the secondary Y-axis.

Linear Correlation of Elution pH Versus Protein pI Value

An analysis was performed to show that there is a correlation between the elution pH versus the corresponding pI values of the protein components. Figure 3 compares the measured pH values for six protein component peaks in Figure 1 as a function of the corresponding pI values. The measured pH values for the six protein component peaks exhibited a strong linear correlation to the literature based pI values, with an R^2 value of 0.9929. Since mAb molecules within the IgG1 class share most of the protein sequence, we expect to see a more linear correlation of the mAb elution pH versus their pI. Figure 4 shows pH gradient separation of charge variants from six mAbs with pI values at 7.2, 7.6, 7.8, 8.3, 9.0, and 10.0. Figure 5 is a plot of the elution pH of each mAb major variant versus the mAb pI value. The linear fit yielded an R^2 value of 0.9988. Thus, after a calibration procedure, this example supports the fact that linear regression coupled with the gradient method described here can be used to estimate the pI of a mAb based on the peak retention time and elution pH.

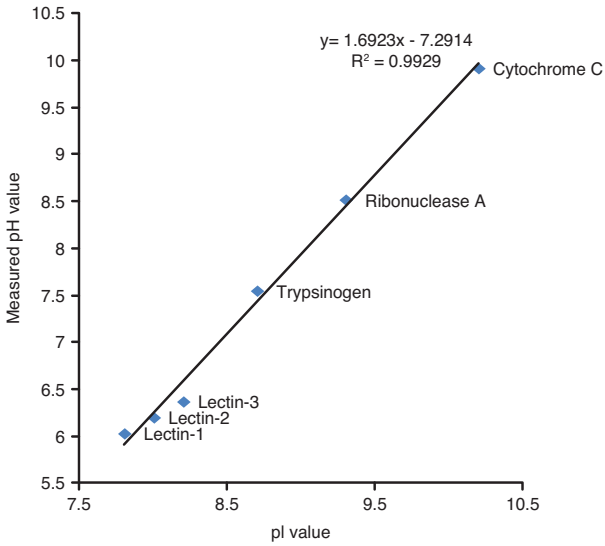

Figure 3: A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.

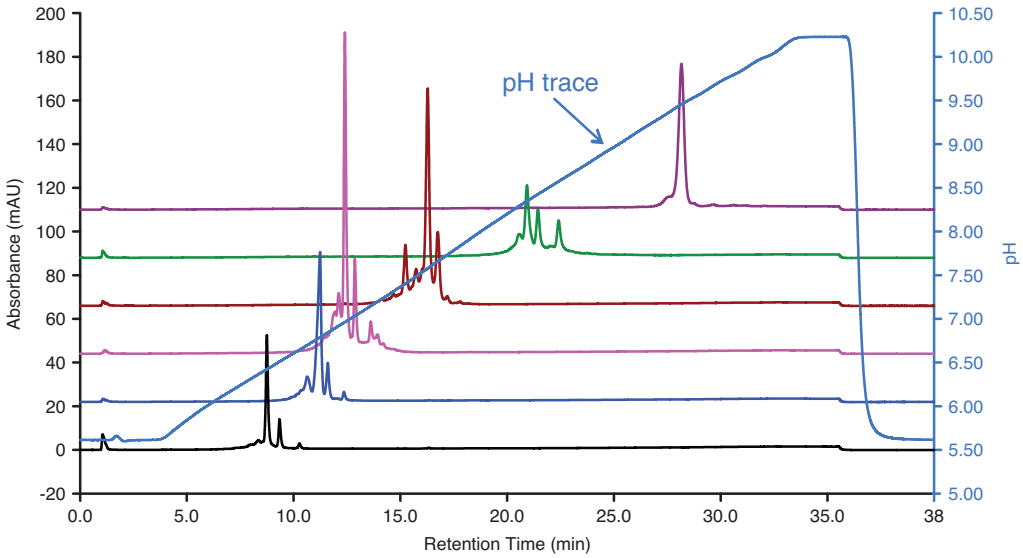

Figure 4: Chromatogram of six mAbs separated using a 30 min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. The UV traces are correspond to the mAbs with the following pI: 7.2 (black), 7.6 (blue), 7.8 (pink), 8.3 (brown), 9.0 (green), and 10.0 (purple). The pH trace is blue.

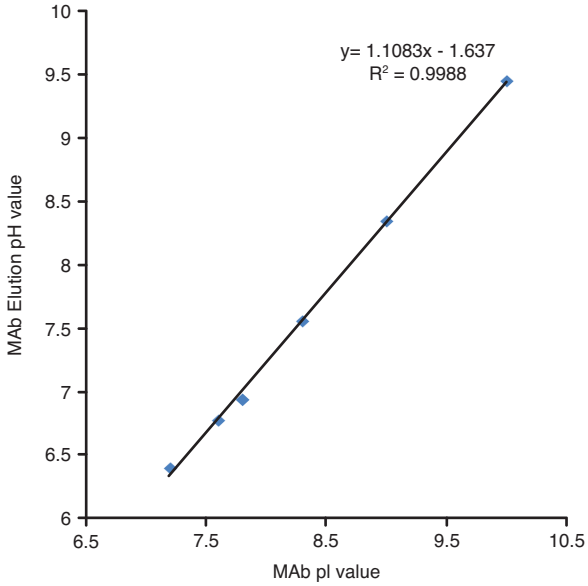

Figure 5: A graph plotting the elution pH values for six mAbs as a function of the corresponding pI values. The measured pH values of the major variant from all six mAbs were exported from the same experiment shown in Figure 4.

Fast pH Gradient Separation

The pH gradient method is fast and rugged. Figure 6 shows pH gradient runs on a MAbPac SCX-10, 5 µm, 4 × 50 mm column. With the gradient specified in Table 2 and a 1 mL/min flow rate, mAb variants separation was achieved within 15 min with a total run time of 20 min (Figure 6). Using the gradient specified in Table 3 and a 2 mL/min flow rate, mAb variants separation was achieved within 7.5 min with a total run time of 10 min (Figure 7). In both cases, the linearity of the pH gradient was maintained. Once the elution pH of the unknown mAb was determined in the scouting run, a bespoke method was set up with increasing % of eluent A as starting point and decreasing % of eluent B as ending point.

Time (minutes)	Flow rate (mL/min)	% A	% B
0–1	1	100	0
1–16	1	100–0	0–100
16–17	1	0	100
17–20	1	100	0

Table 2: A 15 min linear gradient method used with the MAbPac SCX-10, 5 µm, 4 × 50 mm, cation exchange column. Total run time is 20 min. The linear pH range covers from pH 5.6 to pH 10.2.

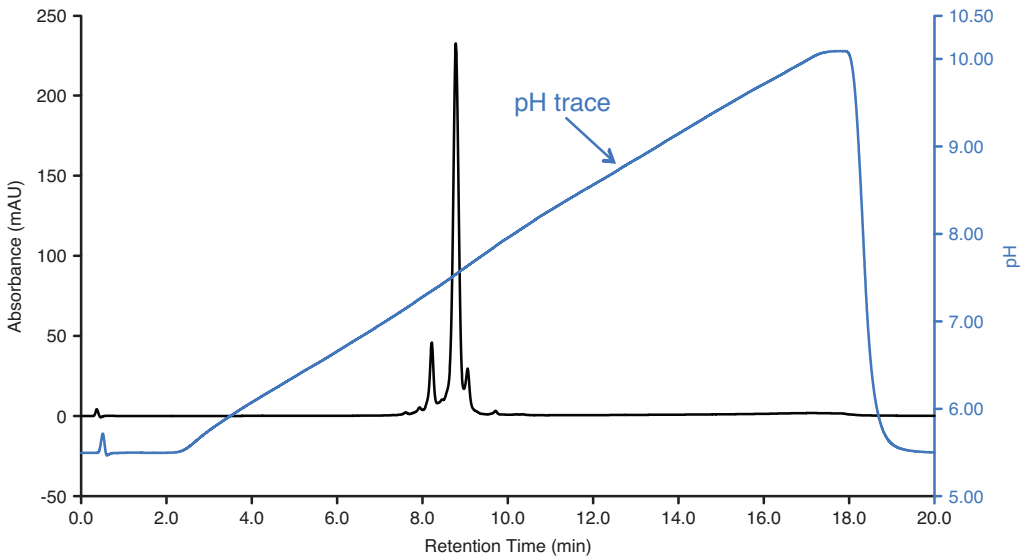

Figure 6: Fast mAb variant separation using pH gradient on a MAbPac SCX-10, 5 µm, 4 × 50 mm column with a 15 min gradient method. The mAb sample concentration was 5 mg/mL.

Time (minutes)	Flow rate (mL/min)	% A	% B
0–0.5	2	100	0
0.5–8	2	100–0	0–100
8–8.5	2	0	100
8.5–10	2	100	0

Table 3. A 7.5 min linear gradient method used with the MAbPac SCX-10, 5 µm, 4 × 50 mm, cation exchange column. Total run time is 10 min. The linear pH range covers from pH 5.6 to pH 10.2.

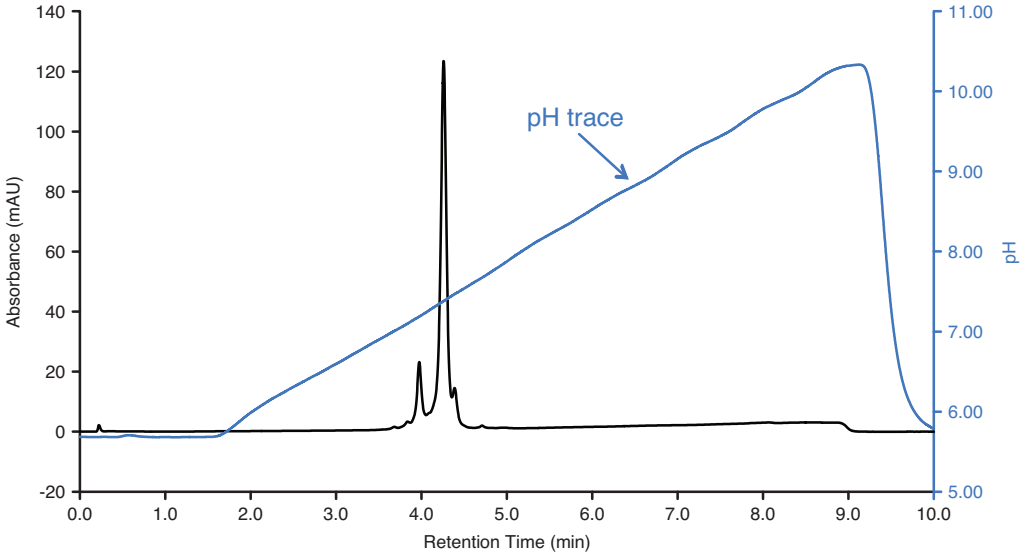

Figure 7: Fast mAb variant separation using pH gradient on a MAbPac SCX-10, 5 µm, 4 × 50 mm column with a 7.5 min gradient method. The mAb sample concentration was 5 mg/mL.

Conclusion

Using the pH gradient method:

- Linear signal response was achieved with up to 300 µg protein loading, such as Ribonuclease A.
- Elution pH values of mAbs exhibited a linear relationship with their corresponding pI values.
- Fast separation of mAb charge variants was achieved within a 10 min cycle time.

Reference

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Application Note 20997

High-Resolution Separation of Intact Monoclonal Antibody Isoforms

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Key Words
Protein heterogeneity, cation exchange, glycosylation, glycoforms, MAbPac SCX-10 column, CX-1 pH gradient buffer

Abstract
To develop a high-resolution, fast separation of intact protein sialylation isoforms using the Thermo Scientific™ MAbPac™ SCX-10 column and CX-1 pH gradient buffer on a Thermo Scientific™ Dionex™ UltiMate™ 3000 Dual Biocompatible Analytical LC system and an UltiMate 3000 BioRS LC system.

Introduction
Glycosylated proteins–including erythropoietins, monoclonal antibodies (mAbs), and various hormones–constitute a large portion of major approved therapeutic protein drugs. Sialic acid, usually attached at terminal positions of glycan molecules, is important to many biological processes such as cell recognition and migration. Sialic acids also have significant effects on the properties of therapeutic proteins, especially on their circulation half-life. For example, the circulation half-life of sialylated rhEPO is 5.6 h, whereas that of nonsialylated rhEPO is 1.4 min [1]. Thus, monitoring protein glycosylation, including sialylation, is important for both glycoprotein characterization and quality control purposes. Recently, an application note from Thermo Fisher Scientific showed good resolution of mAb sialylation isoforms by pH gradient ion-exchange chromatography using Thermo Scientific™ ProPac™ SCX-10 columns and self-prepared mobile phases [2]. The work described here shows a fast, reproducible, high-resolution, pH gradient ion-exchange chromatography approach for the separation of monoclonal antibody sialylation isoforms using the MAbPac SCX-10 column and CX-1 pH gradient buffer.

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Experimental Details

Consumables	Part Number
Deionized (DI) water, 18.2 MΩ-cm resistivity, generated from the Thermo Scientific™ Barnstead™ GenPure™ Pro UV-TOC Water Purification System	50131948
CX-1 pH gradient buffer A (pH 5.6), 250 mL, Thermo Scientific	085346
CX-1 pH gradient buffer B (pH 10.2), 250 mL, Thermo Scientific	085348
Neuraminidase, Fisher Scientific™	P0720S

Sample Handling Equipment
UltiMate 3000 Dual Biocompatible Analytical LC system*, including:
DGP-3600BM Biocompatible Dual-Gradient Micro Pump
WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
TCC-3000SD Thermostatted Column Compartment
DAD-3000 Diode Array Detector with 13 µL flow cell
UltiMate 3000 BioRS system, including:
LPG-3400RS Quaternary Rapid Separation Pump
WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted
TCC-3000RS - UltiMate 3000 Rapid Separation Thermostatted Column Compartment
DAD-3000RS - UltiMate 3000 Rapid Separation Diode Array Detector
Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version

* This application was successfully evaluated on the two UltiMate systems listed.

Preparation of Mobile Phases	
Mobile Phase A	10-fold dilution of CX-1 pH gradient buffer A (pH 5.6) with DI water
Mobile Phase B	10-fold dilution of CX-1 pH gradient buffer B (pH 10.2) with DI water

Sample Preparation

The mAb sample [5 mg/mL] was kindly provided by Shanghai National Engineering Research Center of Antibody Medicine Co., Ltd. (Shanghai, China)

Neuraminidase Digestion Procedure
This procedure follows vendor recommended reaction conditions with minor modifications. Combine 4 µL (equal to 20 µg) of mAb, 31 µL water, and 5 µL of 10X G1 Reaction Buffer to make a 40 µL total reaction volume. Add 10 µL neuraminidase and incubate at 37 °C for 1 h.

Separation Conditions		Part Number
Columns:	MAbPac SCX-10, 10 µm, 4 × 250 mm MAbPac SCX-10, 5 µm, 4 × 150 mm	074625 085198
Mobile Phase:	A: 10-fold dilute CX-1 pH gradient buffer A (pH 5.6) B: 10-fold dilute CX-1 pH gradient buffer B (pH 10.2)	
Gradient:	For MAbPac SCX-10 column (10 µm): 0–30 min: 10–35% B	
	For MAbPac SCX-10 column (5 µm): 0–12 min: 18–40% B	
Flow Rate:	1.0 mL/min for the columns with 10 µm particle size 0.8 mL/min for the columns with 5 µm particle size	
Injection Volume:	50 µL for the columns with 10 µm particle size 25 µL for the columns with 5 µm particle size	
Column Temperature:	30 °C	
Detection:	UV, absorbance at 280 nm	

Results

Separation of a Highly Sialylated mAb on a MAbPac SCX-10 Column

A new generation of ion exchange column, the MAbPac SCX-10 column, has been shown to provide better resolution of mAb charge variants compared to earlier generation columns [3]. Figure 1 shows that when using the CX-1 pH gradient buffer, the MAbPac SCX-10 column resolved 19 mAb peaks, many of which are likely sialylation isoforms with this highly sialylated mAb. The MAbPac SCX-10 column not only provided better resolution between major peaks compared to the ProPac SCX-10 column, but also resolved more minor peaks. The MAbPac SCX-10 column resolved some small peaks that are not observed in the ProPac SCX-10 column chromatogram [2]. Under these optimized conditions (column, mobile phase, and narrow pH range), the pH gradient ion-exchange chromatogram is impressive in its resolution of the large number of isoforms present in this mAb, many of which are putative sialylation isoforms (glycoforms).

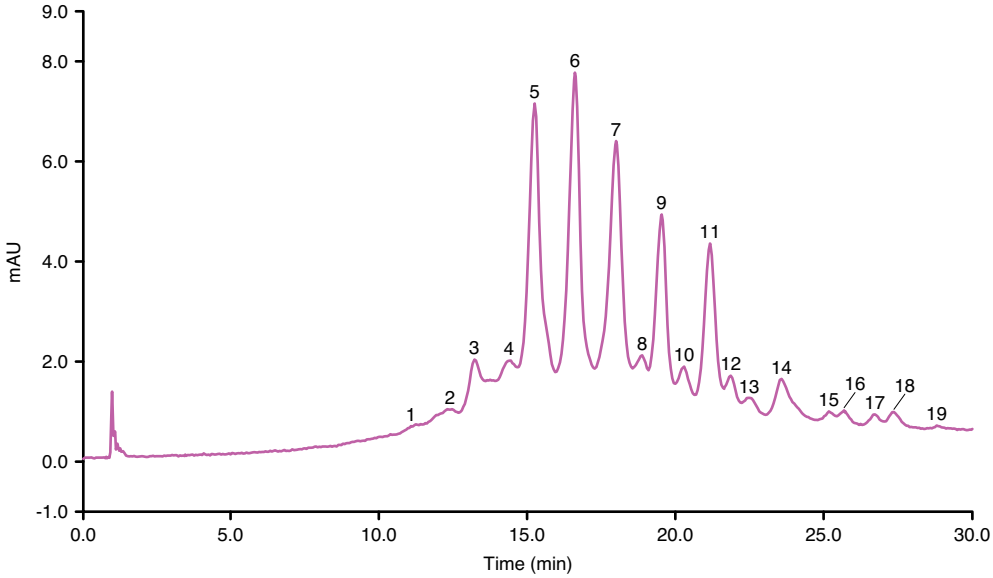

Figure 1: Chromatogram of mAb charge variants separated using the CX-1 pH gradient buffer solutions and a MAbPac SCX-10 column, 4 × 250 mm, 10 µm

Characterizing mAb Charge Variants

The pattern of evenly distributed mAb charge variants is similar to sialylation profiling by capillary electrophoresis [4]. To investigate whether the mAb charge variants separated on the MAbPac SCX-10 column are due at least in part to sialylation, mAb was desialylated with neuraminidase. Figure 2 shows the mAb sample with and without neuraminidase treatment. The chromatogram without neuraminidase treatment in Figure 2a has ten times less sample than the chromatogram in Figure 2b, so the minor peaks are not observed. After neuraminidase treatment, peaks 1–3 completely disappeared and peak 4 decreased, while peaks 5 and 6 increased. This strongly suggests that neuraminidase treatment has removed the sialic acids on the glycans resulting in a neutral glycan pattern on the mAb and increasing some of the peaks, which are presumably the original neutral glycan variant peaks. There are also several new peaks such as a, b, and c that appear. This can also be explained by desialylation of other charged variants that the pH gradient has separated, reducing the negative charge and so giving longer retention time on the SCX chromatogram. These new isoform peaks are more easily seen now due to the individual isoform originally being split into several different sialylation forms at low levels that have now added together as a single neutral glycan variant at a higher amount.

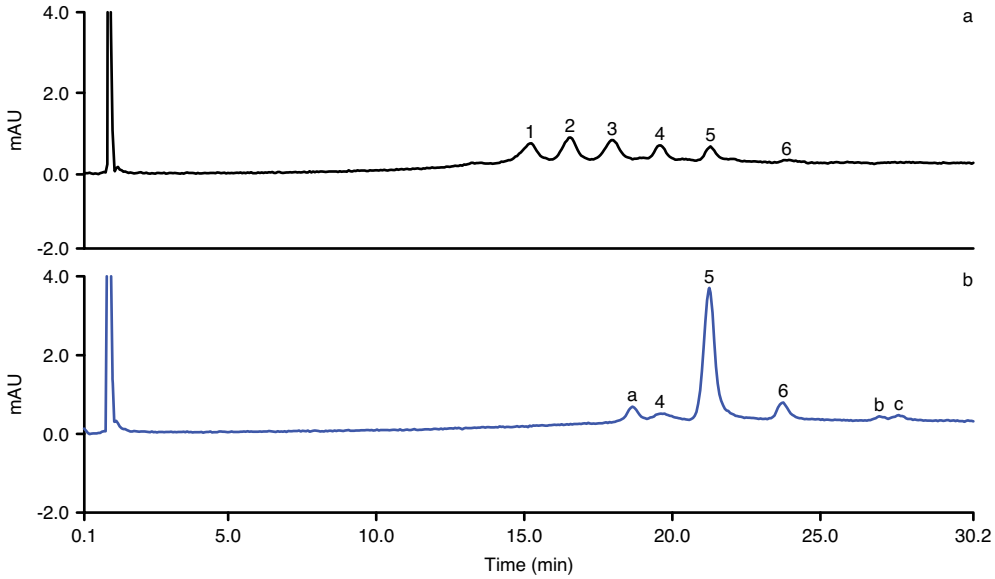

Figure 2: Chromatograms of a mAb (a) without neuraminidase treatment, and (b) with neuraminidase treatment. The chromatogram was generated under the same conditions as in Figure 1.

Comparison of MAbPac SCX-10 Column Separations using Different Particle Sizes

All the above chromatograms were obtained with 10 µm particle size columns in a 4 × 250 mm format. Although the pH gradient ion-exchange chromatograms achieved remarkable resolution, 50 min run times are too long when a fast analysis is needed. To reduce analysis time, a 5 µm particle size column, 4 × 150 mm was evaluated. As shown in Figure 3, the 5 µm column resolved 14 peaks in 11 min (total run time of 16 min). Peaks 6, 8, 10, and 11 can be identified in the chromatogram, but with compromised resolution.

The 5 µm column showed good reproducibility. Overlaid chromatograms of mAb sialylation variants obtained by five consecutive injections are shown in Figure 4. RSDs of main peak (peak 3, 4, 5, 7 and 9) retention times are less than 0.2%, and the RSDs of these peak areas are less than 1.5%.

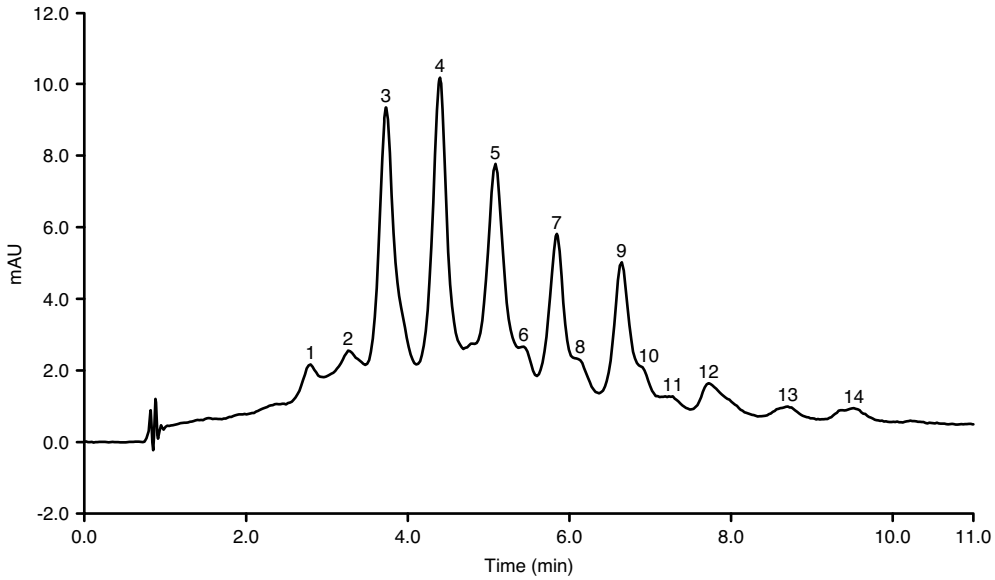

Figure 3: Chromatogram of mAb sialylation isomers separated using the CX-1 pH gradient buffer solutions and a MAbPac SCX-10 column with 5 µm particle size

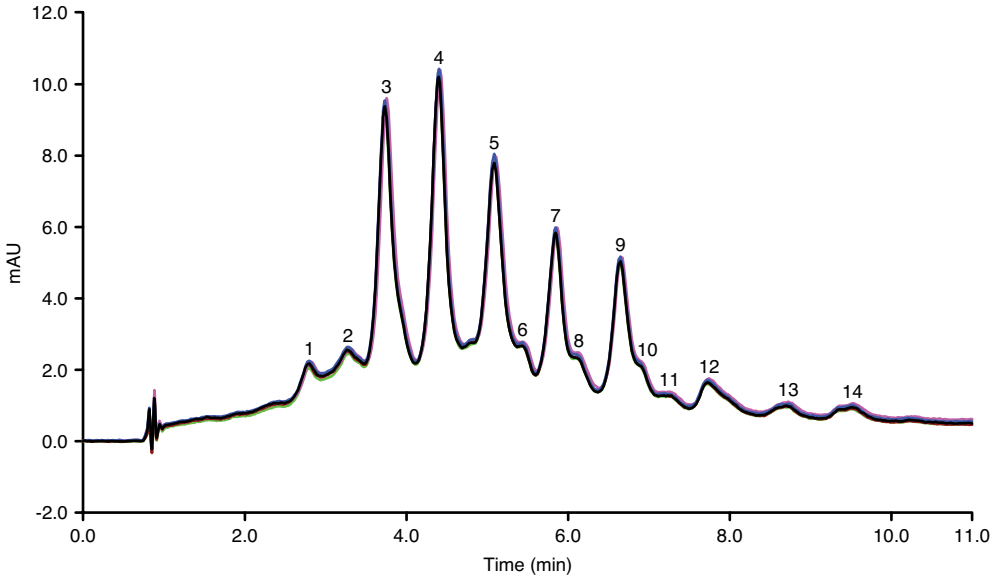

Figure 4: Overlay of chromatograms of five consecutive injections of mAb sialylation isomers separated on a 5 µm MAbPac SCX-10 column

Conclusion

This application note shows that pH gradient-based strong cation-exchange chromatography on a MAbPac SCX-10 column can provide excellent resolution for mAb charged variants including different glycan isoforms of sialylated mAbs. The CX-1 pH gradient buffer approach provides high resolution, reproducibility, and convenience. Fast separation could be achieved by using short columns with 5 µm particles and an UltiMate BioRS HPLC.

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Application Note 21092

High-Resolution Charge Variant Analysis for Top-Selling Monoclonal Antibody Therapeutics Using a Linear pH Gradient Separation Platform

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Key Words

Rituxan, rituximab, Herceptin, trastuzumab, Humira, adalimumab, Avastin, bevacizumab, CX-1 pH Gradient Buffer Kit, MAbPac SCX-10, mAb charge variant analysis

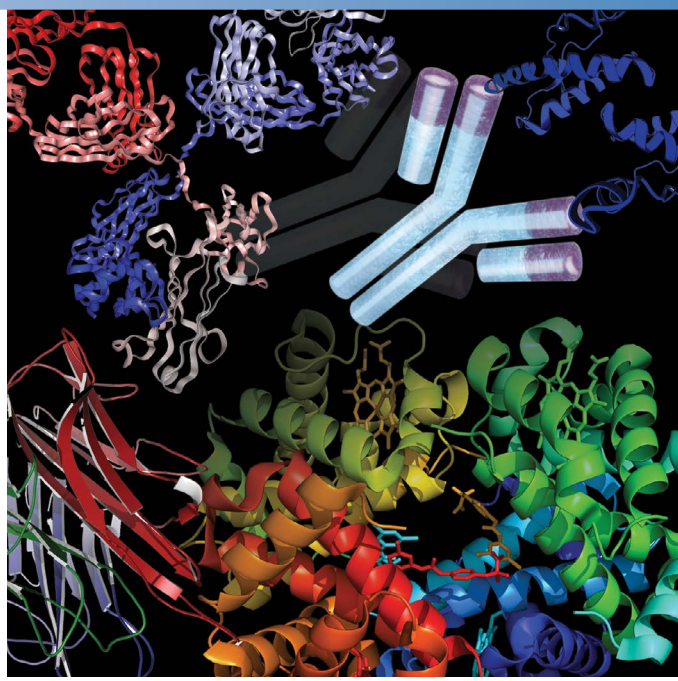
Goal

To develop a high-resolution charge variant analysis for Rituxan® (rituximab), Herceptin® (trastuzumab), Humira® (adalimumab), and Avastin® (bevacizumab) using a linear pH gradient separation method.

Introduction

Monoclonal antibody (mAb) therapeutics is a quickly growing market. In 2013, of the top ten bestselling pharmaceutical products, five were monoclonal antibodies (Humira, Remicade, Rituxan, Herceptin, and Avastin). Humira sales revenue alone exceeded $11 billion.[1] With the patent protection for these blockbuster drugs expiring in the next few years, many companies are entering the development of biogenerics, also known as the biosimilars. In order to demonstrate the safety and efficacy of the biosimilars and gain approval of the regulatory agency, it is essential to detect, characterize, and quantify impurities as well as structural variants of these biosimilars. Some structural variants, such as charge variants, have been demonstrated as critical quality attributes (CQAs).

Charge variants of mAbs are due to modifications such as sialylation, deamidation, and C-terminal lysine truncation. Traditionally, salt gradient cation-exchange chromatography has been used with some success in characterizing mAb charge variants.[2] However, significant effort is often required to tailor the salt gradient method for each individual mAb. In the fast-paced drug development environment, a quick and robust platform method is desirable to accommodate the majority of the mAb analyses. Thermo Fisher Scientific recently introduced cation-exchange pH gradient buffers that meet these platform method requirements.[3] The buffer system consists of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. A linear pH gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% buffer A to 100% buffer B.



In this study, the charge variants of Rituxan (rituximab), Herceptin (trastuzumab), Humira (adalimumab), and Avastin (bevacizumab) are analyzed on a Thermo Scientific™ MAbPac™ SCX-10 column with a linear pH gradient separation method. The linear gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% Thermo Scientific™ CX-1 pH Gradient Buffer A (pH 5.6) to 100% CX-1 pH Gradient Buffer B (pH 10.2). The results demonstrate the general applicability of the pH gradient method on monoclonal antibody charge variant analysis. The data also show that the pH gradient method delivers higher-resolution power than the traditional salt method. The methods described here can be widely used in the development of the biosimilars of these top-selling mAbs.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- MES hydrate [≥99.5%]
- Sodium chloride [NaCl, ≥99.5%]
- Rituxan, Herceptin, Humira, and Avastin were a gift from a biotechnology company.

Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

Columns and Buffers

- MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)
- CX-1 pH Gradient Buffer A (pH 5.6), 125 mL (P/N 083273)
- CX-1 pH Gradient Buffer B (pH 10.2), 125 mL (P/N 083275)

LC Separation

The LC separation conditions were as follows:

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:
	SRD-3400 Solvent Racks with degasser (P/N 5035.9245)
	HPG-3400RS Biocompatible Binary Rapid Separation Pump (P/N 5040.0046)
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a semi-micro flow cell PEEK, 2.5 µL volume, 7 mm path length, PCM-3000 pH and Conductivity Monitor (P/N 6082.2005)
pH gradient mobile phase A	1X CX-1 pH Gradient Buffer A Dilute CX-1 pH Gradient Buffer A 10-fold using deionized water.
pH gradient mobile phase B	1X CX-1 pH Gradient Buffer B Dilute CX-1 pH Gradient Buffer B 10-fold using deionized water.
Salt gradient mobile phase A	20 mM MES (pH 5.6) + 60 mM NaCl Add 3.9 g of MES (Sigma) to 900 mL of deionized water. Adjust the pH with NaOH to 5.6 and add 17.53 g of NaCl, adjust volume to 1 L and filter through a 0.22 µm filter before use.
Salt gradient mobile phase B	20 mM MES (pH 5.6) + 300 mM NaCl Add 3.9 g of MES (Sigma) to 900 mL of deionized water. Adjust the pH with NaOH to 5.6 and add 58.44 g of NaCl, adjust volume to 1 L and filter through a 0.22 µm filter before use.

Full pH gradient pH 5.6 to pH 10.2

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	100
34.0	0	100
34.1	100	0
45.0	100	0

Half pH gradient pH 5.6 to pH 7.9

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	50
34.0	0	50
34.1	100	0
45.0	100	0

Full salt gradient 60 mM NaCl to 300 mM NaCl

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	100
34.0	0	100
34.1	100	0
45.0	100	0

Half salt gradient 60 mM NaCl to 180 mM NaCl

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	50
34.0	0	50
34.1	100	0
45.0	100	0

Flow rate	1 mL/min
Run time	40 min
Temperature	30 °C
UV detector wavelength	280 nm
Injection volume	5 µL
Samples	Rituximab, 5 mg/mL; Trastuzumab, 5 mg/mL; Adalimumab, 5 mg/mL; Bevacizumab, 1 mg/mL

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

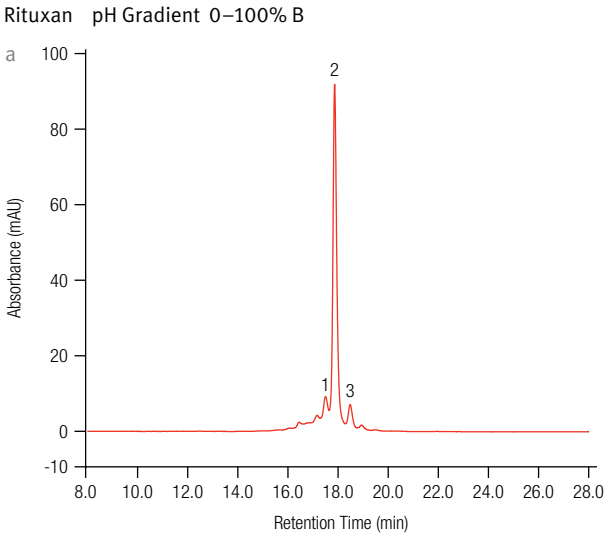
The CX-1 pH gradient buffer kit generates a linear pH gradient when a linear pump gradient is run from 100% CX-1 buffer A to 100% buffer B. This pH gradient method serves as a platform method for the mAb charge variant analysis, covering the pH range from 5.6 to 10.2. Most of the therapeutic mAbs have pI values falling within this pH range. Rituxiamab (Figure 1a), trastuzumab (Figure 3a), adalimumab (Figure 5a), and bevacizumab (Figure 7a) were analyzed on a MAbPac SCX-10 column using the full pH gradient method. Satisfactory separations of multiple variants were observed with all four samples. After the initial survey runs of the full pH gradient, the subsequent runs were aimed at improving resolution by decreasing the pH range and gradient slope. The fact that the pH gradient was linear made the method optimization simple. Rituximab (Figure 1b), trastuzumab (Figure3b), adalimumab (Figure 5b), and bevacizumab (Figure 7b) were analyzed using a shallower pH gradient with half the pH range. The variants were identified as peaks 1, 2, 3 in the chromatograms. Improved separations of the variants were observed in all cases.

Traditionally, the salt gradient method has been used for mAb charge variants analysis. The salt gradient method development usually requires screening at different pH values using different buffers. In addition, the minimum salt concentration required to elute the mAb off the cation exchange column must be individually determined. For comparison and speed, the same initial conditions and buffers (20 mM MES and 60 mM NaCl at pH 5.6) were used for all the samples in this study. Rituxiamab (Figures 2a and 2b), trastuzumab (Figures 4a and 4b), adalimumab (Figures 6a and 6b), and bevacizumab (Figures 8a and 8b) were each analyzed by two salt gradient methods: one with steeper gradient slope and the other one with shallower gradient slope.

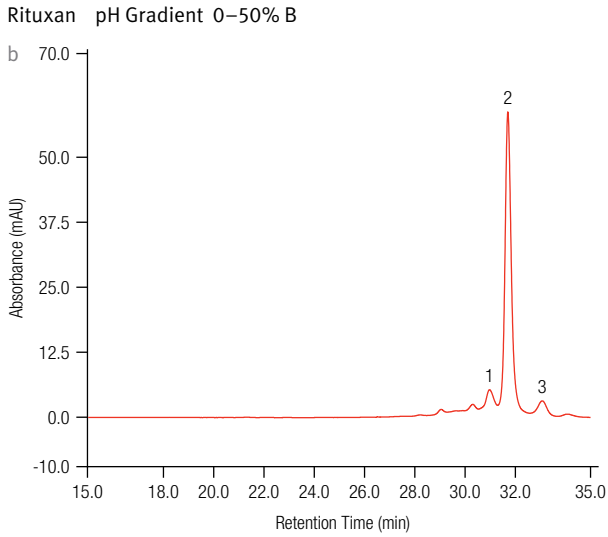
The separation profiles obtained by the pH and salt gradient methods were similar for the same molecule. In order to simplify the comparison, the acidic variant adjacent to the major variant was labeled as peak 1, the major variant was labeled as peak 2, and the basic variant adjacent to the major variant was labeled as peak 3 for each chromatogram (Figures 1–8). In the case of the trastuzumab salt gradient chromatogram, the minor acidic variant was very close to the major peak and could be not be determined, but this was resolved by the pH gradient. Table 1 lists the retention time of peak 1 (RT1), peak 2 (RT2), and peak 3 (RT3) and the difference between RT1 and RT2 (ΔRT1-2), as well as RT2 and RT3 (ΔRT2-3). In the case of rituximab, trastuzumab, and bevacizumab, it is clear that the ΔRTs between variants were greater when using the pH gradient profile. In the case of adalimumab, the ΔRTs were similar between the pH gradient profile and the salt gradient profile.

Table 1. Retention time of mAb charge variants analyzed by linear pH gradient and salt gradient methods.

	Method	Gradient	RT1 (min)	RT2 (min)	RT3 (min)	ΔRT2 (min)	ΔRT3 (min)
Rituxan/rituximab	pH	full	17.497	17.86	18.477	0.363	0.617
		half	30.98	31.71	33.077	0.73	1.367
	salt	full	12.927	13.11	13.504	0.183	0.394
		half	22.44	22.783	23.567	0.343	0.784
Herceptin/trastuzumab	pH	full	15.257	15.744	16.207	0.487	0.463
		half	27.404	27.914	28.818	0.51	0.904
	salt	full	n.a.	12.204	12.477	n.a.	0.273
		half	n.a.	21.494	21.844	n.a.	0.35
Humira/adalimumab	pH	full	16.063	16.26	16.593	0.197	0.333
		half	28.834	29.244	29.907	0.41	0.663
	salt	full	15.354	15.56	15.9	0.206	0.34
		half	26.877	27.267	27.947	0.39	0.68
Avastin/bevacizumab	pH	full	11.537	11.824	12.264	0.287	0.44
		half	19.977	20.553	21.433	0.576	0.88
	salt	full	13.494	13.724	14.11	0.23	0.386
		half	23.36	23.817	24.587	0.457	0.77

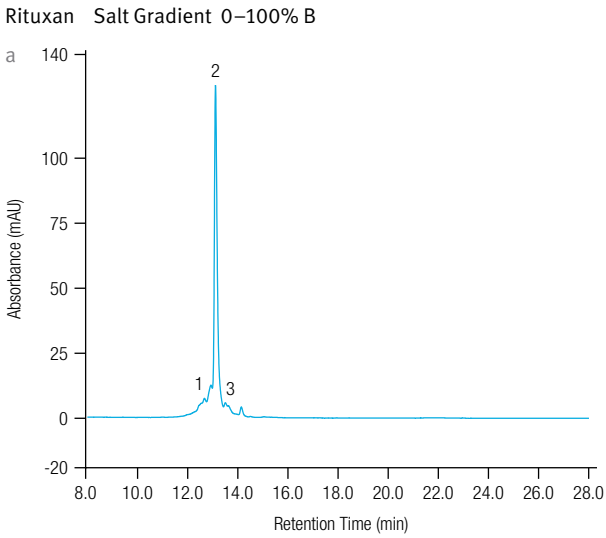


Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)

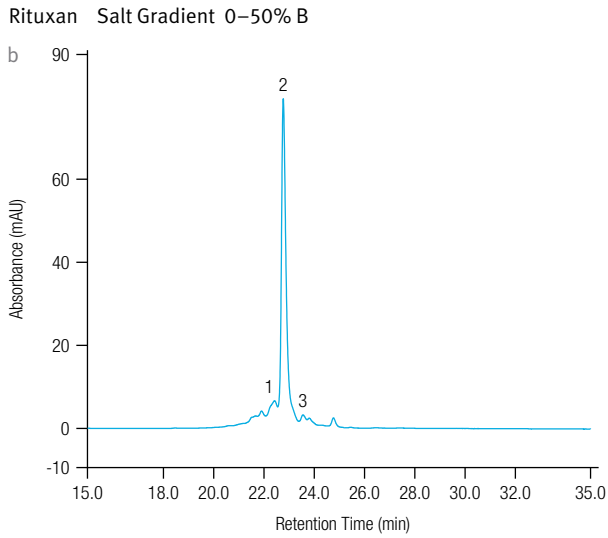


Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)

Figure 1. Rituxan/rituximab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.



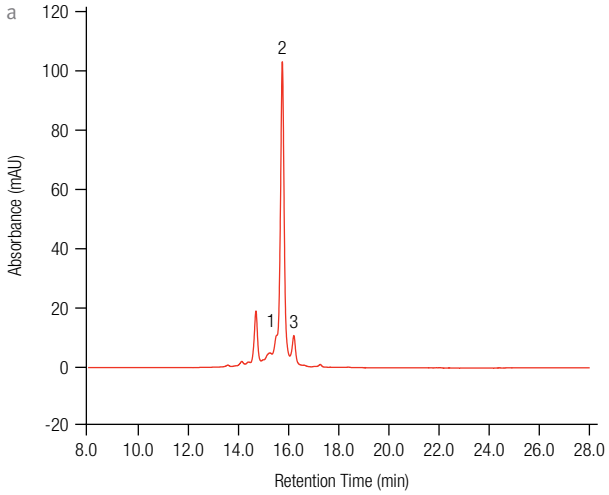
Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)

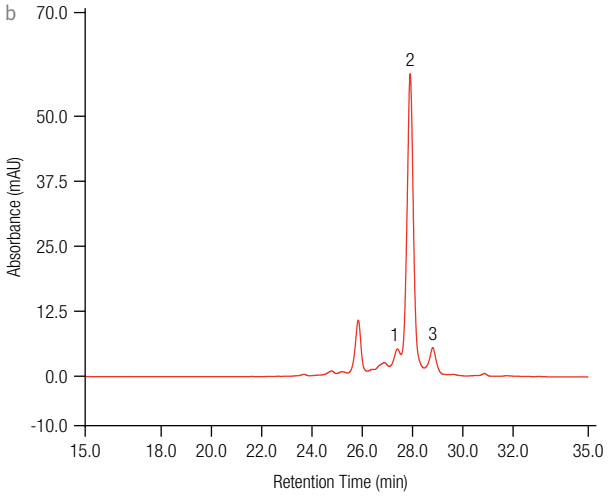
Figure 2. Rituxan/rituximab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Herceptin pH Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

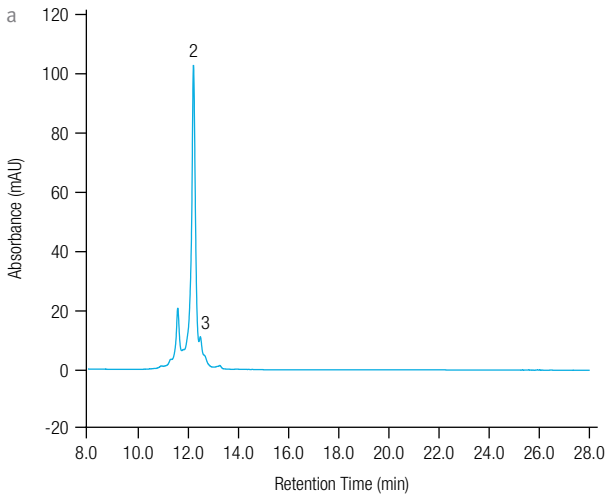
Herceptin pH Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

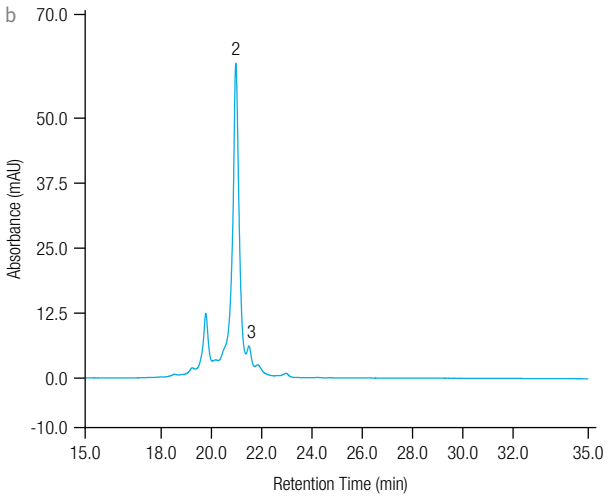
Figure 3. Herceptin/trastuzumab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.

Herceptin Salt Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

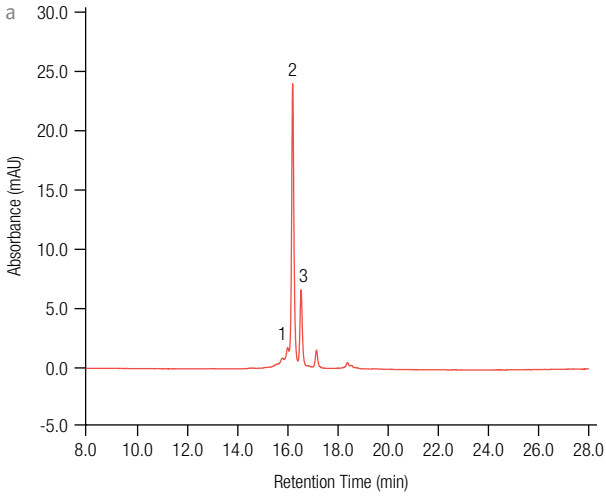
Herceptin Salt Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

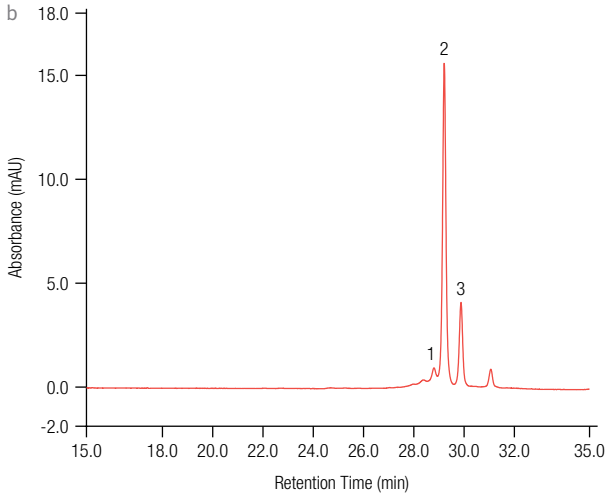
Figure 4. Herceptin/trastuzumab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Humira pH Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

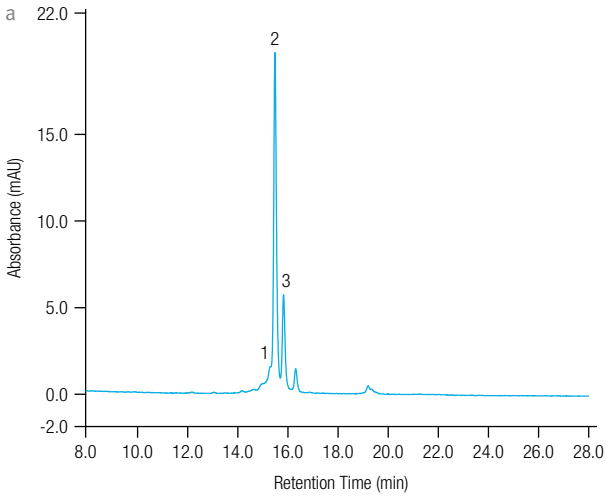
Humira pH Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

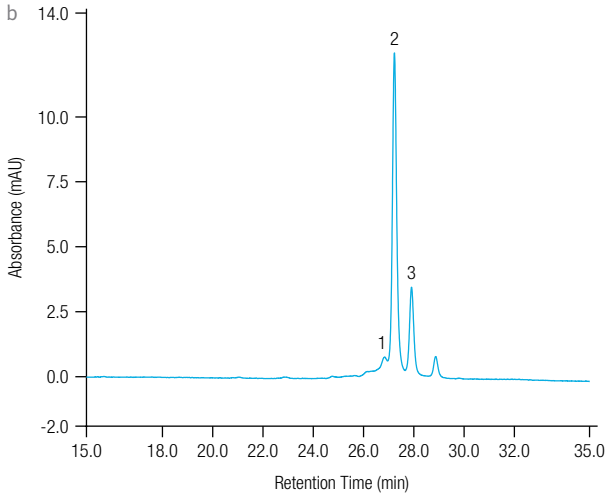
Figure 5. Himira/adalimumab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.

Humira Salt Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

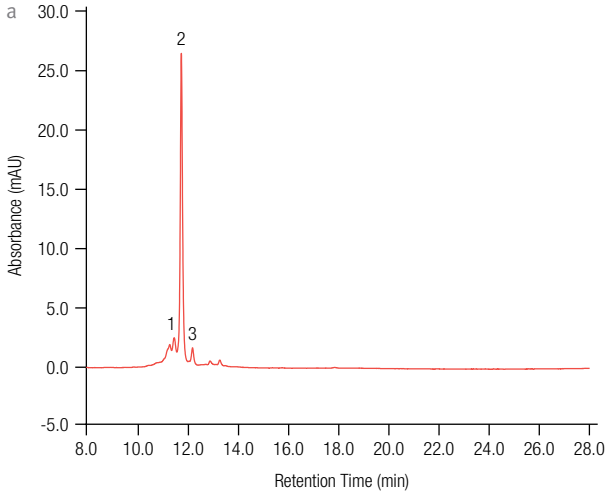
Humira Salt Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

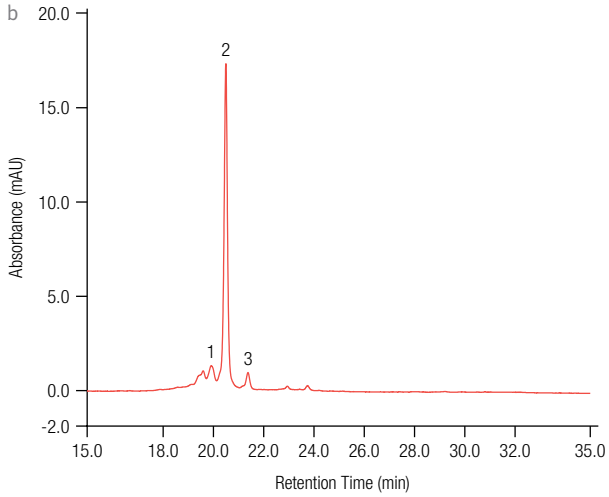
Figure 6. Himira/adalimumab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Avastin pH Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab (1 mg/mL)

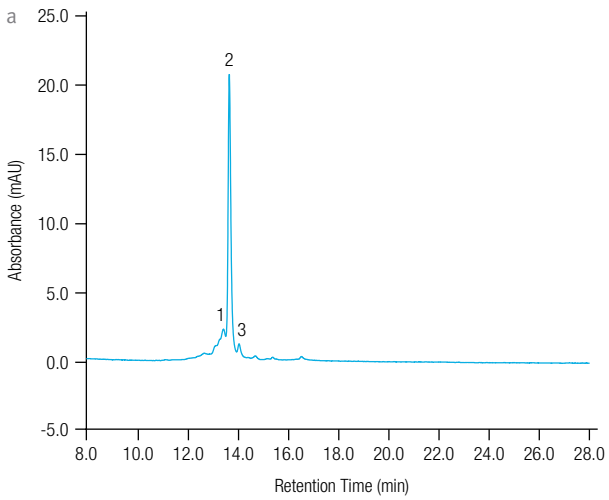
Avastin pH Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab (1 mg/mL)

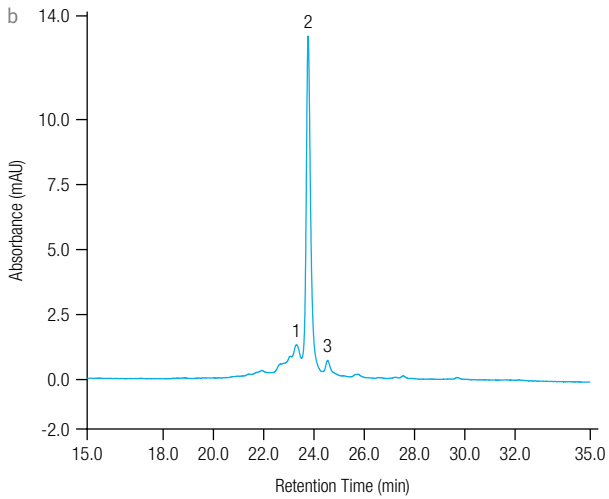
Figure 7. Avastin/bevacizumab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.

Avastin Salt Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab(1 mg/mL)

Avastin Salt Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 0% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab (1 mg/mL)

Figure 8. Avastin/bevacizumab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Conclusion

The linear pH gradient method is a platform method for mAb charge variant analysis. It can be easily optimized to improve separation and delivers better charge variant separation than the salt gradient method.

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Development of Ultra-fast pH-Gradient Ion Exchange Chromatography for the Separation of Monoclonal Antibody Charge Variants

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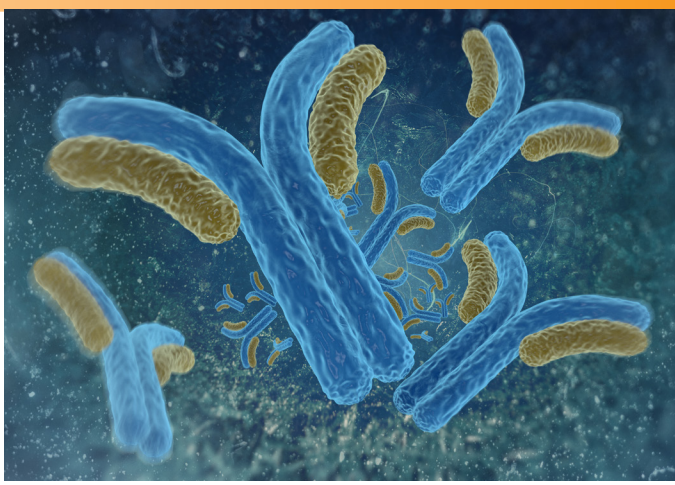
Technical Note 160

Key Words
Critical Quality Attributes, Biotherapeutics, Intact Proteins, Vanquish UHPLC, MAbPac SCX-10 RS Column



Introduction

The pre-formulated buffers for pH gradient, introduced by Thermo Fisher Scientific, have greatly simplified the development of ion exchange chromatography (IEX) of monoclonal antibodies (mAbs). Three features make this simplification possible. The first feature is that the buffers can cover a pH range from 5.6 to 10.2; this pH window enables the characterization of mAbs with a wide range of isoelectric points, providing a global pH gradient ion exchange screening method that will accommodate the majority of therapeutic mAbs. The second feature is that, if a linear solvent gradient is programmed in the pump, the actual pH gradient produced in the column will be linear as well. Although this sounds trivial, scientists involved in pH gradient studies know how difficult it is to develop buffer formulations capable of fulfilling this requirement. The advantage of a genuine linear pH gradient is that the method can be confidently fine-tuned merely by narrowing down the pH range around the mAb and its variants, thus allowing for the adjustment of the gradient time according the resolution requirements. The third feature is the mobile phase preparation: the pre-formulated pH buffers only needs to be diluted by a factor of 10 in deionized water, and the mobile phase is ready to use. Compared to a salt gradient ion exchange chromatography method, where no generic screening can be easily designed, and where method development goes through the rather tedious preparation of several buffers at different pH values, the time and effort invested in method development are substantially reduced.

High resolution pH gradient separations are obtained with 30 minute gradients and relatively long columns, such as the Thermo Scientific™ MAbPac™ SCX-10 column, 10 µm, 4 × 250 mm.[1] Fast separation of mAb variants were demonstrated by using the MAbPac SCX-10 column, 5 µm, 4 × 50 mm with no significant loss in resolution[2] on a Thermo Scientific™ UltiMate™ 3000 BioRS system. In this case, the pH gradient from 5.6 to 10.2 was completed in 7.5 minutes, and the resolution between variants was still satisfactory, despite the short analysis time.

In this work, we show how to push the throughput of pH gradient IEX even further. To achieve this, a MAbPac SCX-10 RS column, 5 µm, 2.1 × 50 mm was operated on a new Thermo Scientific™ Vanquish™ UHPLC system. The Vanquish UHPLC is a fully biocompatible system, suitable for the analysis of intact proteins. The combination of low gradient delay volume and high precision gradient formation makes it the ideal system for high throughput analysis with gradient elution. Here the system was used with the default configuration and a total system gradient delay volume of 175 µL. Fast charge variant separations of 5 mAbs are shown.

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Goal

Provide high throughput pH gradient separations of mAb charge variants

Experimental

Vanquish UHPLC, consisting of:

- Vanquish System Base (P/N VH-S01-A)
- Binary Pump H with Default Mixer (P/N VH-P10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)

Chromatographic Conditions	
Column:	MAbPac SCX-10 RS, 5 µm, 2.1 × 50 mm (P/N 082675)
Buffers:	Thermo Scientific CX-1 pH-Gradient buffer A (pH 5.6) 125 mL (P/N 083273) CX-1 pH-Gradient buffer B (pH 10.2) 125 mL (P/N 083275)
Mobile Phase A:	CX-1 pH-Gradient buffer A (pH 5.6) diluted 10x in deionized water
Mobile Phase B:	CX-1 pH-Gradient buffer B (pH 10.2) diluted 10x in deionized water
Column Compartment Temperature	30 °C, forced air
Detector and Conditions	
Detector:	LightPipe™ 10 mm Standard Flowcell (P/N 6083.0100)
Detection Wavelength:	280 nm
Data Acquisition Range:	5 Hz (for flow rate ≤ 0.5 mL/min) and 50 Hz (for flow rate ≥ 1.0 mL/min)
Response Time:	2 s (for flow rates ≤ 0.5 mL/min) and 0.1 s (for flow rates ≥ 1.0 mL/min)
Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS)

Samples

mAb Name	Concentration (mg/mL)	Injection Volume (µL)
Bevacizumab	25	1
Cetuximab	5	4
Infliximab	10	4
Trastuzumab	21	2
mAb A	21	2

Results and Discussion

One of the benefits of using CX1 buffers for the pH-gradient is the simplified method development and optimization. This is due to the fact that the pump running a linear solvent gradient will result in a linear pH gradient in the column. This is not the case for most of home-made buffer formulations, which would produce a non-linear pH gradient in response to a linear programmed gradient. A non-linear pH gradient makes method optimization difficult due the uncertainty of the actual effects of any changes in the programmed gradient.

It is recommended to perform a generic screening from pH 5.6 to 10.2 when the pH at which a given mAb elutes is not known. In this work, the generic screening was run in 10 minutes at 0.45 mL/min. As it can be seen in Figure 1 in some cases, satisfactory separation of the charge variants was achieved during the first run. This was the case for cetuximab and infliximab: several charge variants could be resolved with sufficient resolution.

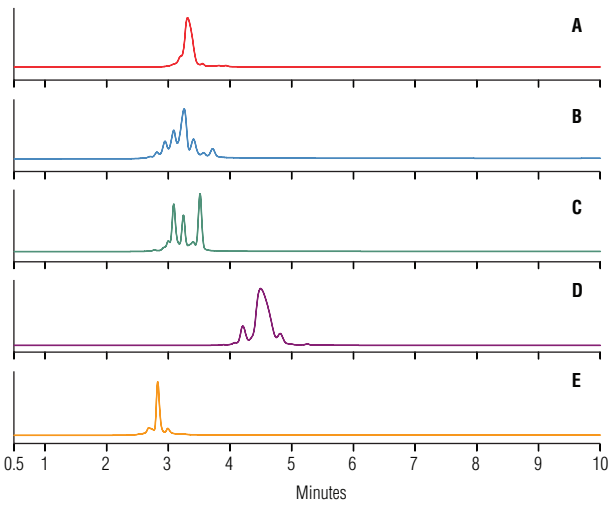

Figure 1. Separation of 5 mAbs with a generic 0→100 %B in 10 min at 0.45 mL/min (method #1 Table 1). Samples are as follows: A) bevacizumab, B) cetuximab, C) infliximab, D) trastuzumab, E) mAb A.

Table 1. Description conditions used for the separation of charge variants of the different monoclonal antibodies.

Method #	Figure	Gradient Range (%B)	Gradient pH Range	Gradient Time (min)	Flow Rate (mL/min)	Gradient Slope* (%B/mL)
1	1	0–100	5.6–10.2	10.0	0.45	22.2
2	2A	20–40	6.5–7.4	5.0	0.50	8.0
3	2B	23–35	6.7–7.2	2.5	1.00	4.8
4	3A	10–35	6.1–7.2	5.0	0.50	10.0
5	3B	10–35	6.1–7.2	2.5	1.00	10.0
6	4A	20–40	6.5–7.4	5.0	0.50	8.0
7	4B	18–27	6.4–6.8	0.8	1.20	9.4
8	5A	35–60	7.2–8.4	5.0	0.50	10.0
9	5B	33–45	7.2–7.7	2.5	1.00	4.8
10	6B	5–30	5.8–7.0	2.5	1.00	10.0

*Slope based on gradient volume

After the generic screening, the method development efforts aimed at decreasing the analysis cycle time and at the same time improving resolution. To achieve this goal, two parameters were modified, namely the pH range and the gradient slope. Here we used gradient slope based on gradient volume, i.e. $\Delta(\%B)/V_G$, where %B is the amount of B eluent and V_G is the volume of mobile phase delivered by the pump during the gradient. A narrower pH window allowed for a reduced run-time, whereas a shallower gradient slope provided better resolution.

The gradient slope of the generic screening between pH 5.6 and 10.2 was 22.2 (%B)/mL; the improved and faster analysis were obtained with gradient slopes of 8 (%B)/mL and 10 (%B)/mL. Details of the conditions are listed in Table 1. Figures 2a, 3a, 4a, and 5a, show the results obtained by this approach. The number of resolved variants was larger than in the initial screening. Gradient time was 5 minutes, hence half of the time of the initial screening.

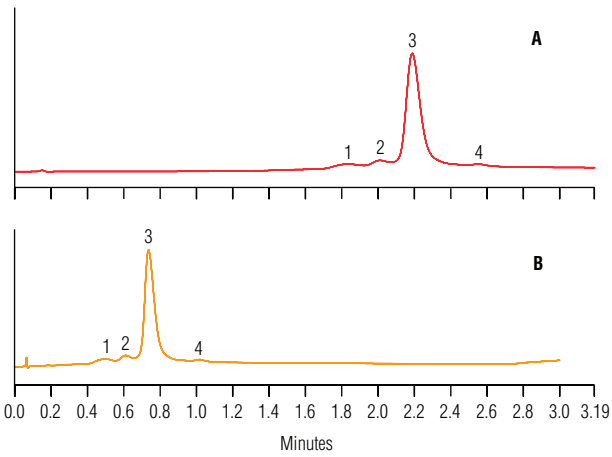

Figure 2. Separation of bevacizumab with different gradient conditions. Chromatogram A: method #2 Table 1; chromatogram B: method#3 Table 1.

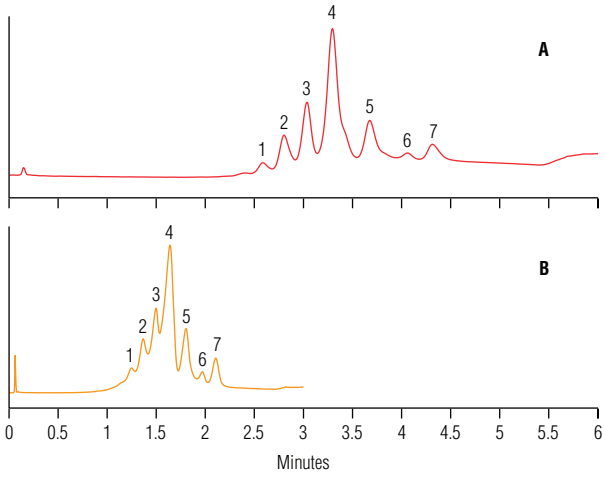

Figure 3. Separation of cetuximab with different gradient conditions. Chromatogram A: method #4 Table 1; chromatogram B: method#5 Table 1.

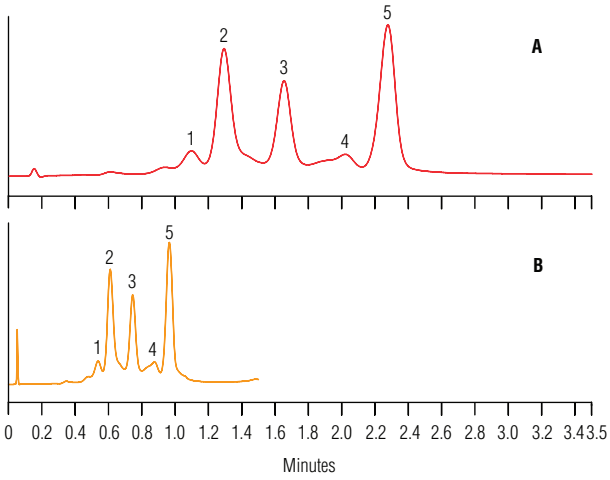

Figure 4. Separation of inflixumab with different gradient conditions. Chromatogram A: method #6 Table 1; chromatogram B: method#7 Table 1.

The following step was used to develop fast analysis cycles compatible with high throughput. With this approach we aimed for a 2.5 minute gradient time or lower, and total analysis time of less than 4 minutes, including column re-equilibration. Data are shown in Figures 2b, 3b, 4b, 5b, and 6b. The purpose was to develop a method suitable for high-throughput that can run at least 300 samples a day. High flow rate was used for this purpose. A high flow rate allows running short gradients with relatively shallow gradient slopes; the shallow slope is required to preserve selectivity between charge variants. Additionally, column equilibration, which is directly dependent on the volume of mobile phase flowing through the column, is reached quicker. MAbPac SCX-10 RS column is pressure rated up to 7000 psi (~ 480 bar), therefore it can be operated at high linear flow rate. In this work, we used flow rates up to 1.2 mL/min.

The chromatographic pattern between methods at moderate and high flow rate was preserved. The separation capabilities of different methods were compared based on the resolution between charge variants. Since in several instances peak pairs were overlapping, and it was not always possible to measure peak width at half height or at the baseline, here we used resolution based on statistical moments. Resolution was calculated directly by Chromeleon 7.2 CDS according to the formula:

$$R = \frac{(t_R^2 - t_R^1)}{2 * (\sqrt{\mu_2^2} + \sqrt{\mu_2^1})}$$

where t_R^2 and t_R^1 are the retention times of the more and less retained peak respectively, and μ_2^2 and μ_2^1 are the related second moment.

In some cases, the ultra-fast separation approach was accompanied by some resolution loss. This is the case of trastuzumab, where average resolution loss was ~ 3%. In the case of the complex variants pattern of cetuximab, the average resolution loss at high flow rate was ~ 13%. Infliximab resolution decreased by ~ 11%, however separation of the 5 main charge variants and 2 minor ones was achieved in 1 minute. This impressive result was obtained by running the column at 1.2 mL/min with a 0.8 min gradient time. In the case of bevacizumab, the ultra-fast separation approach even yielded to ~ 4% improved resolution. The better resolving power can be explained by a slightly narrower pH range and a shallower gradient slope.

mAb A was analyzed only with the generic screening and high flow rate (1 mL/min) method. The high-throughput method provided 19% better resolution on average of 5 charge variants.

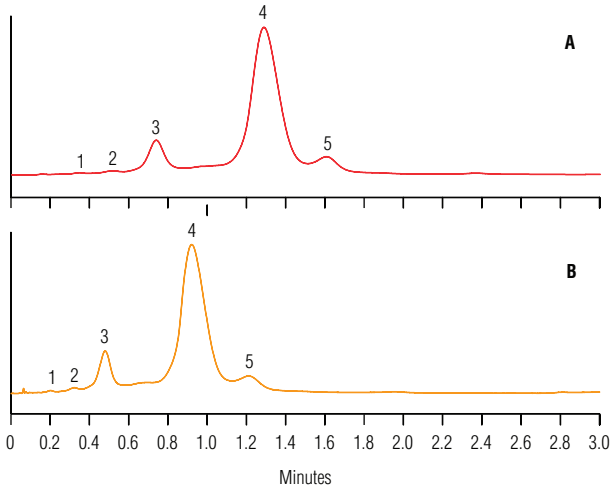

Figure 5. Separation of trastuzumab with different gradient conditions. Chromatogram A: method #8 Table 1; chromatogram B: method#9 Table 1.

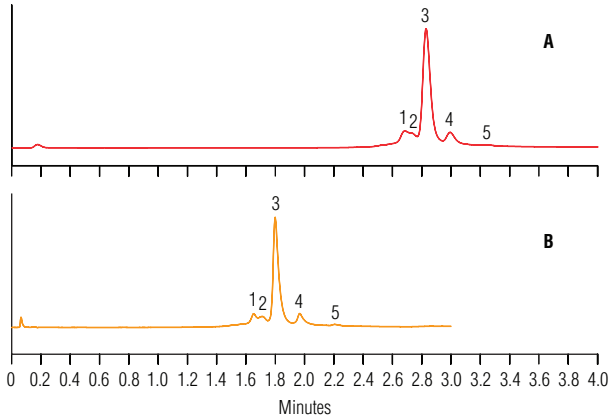

Figure 6. Separation of mAb A with different gradient conditions. Chromatogram A: method #1 Table 1; chromatogram B: method#10 Table 1.

Table 2. Overview of the resolution between charge variants at different conditions. Resolution was calculated by Chromeleon CDS using statistical moments.

Statistical Resolution Charge Variants							
mAb	Gradient Time (min)	Peaks 1–2	Peaks 2–3	Peaks 3–4	Peaks 4–5	Peaks 5–6	Peaks 6–7
Bevacizumab	5	0.94	0.87	1.81	—	—	—
	2.5	1.00	0.89	1.89	—	—	—
Cetuximab	5	0.79	1.14	1.00	1.13	1.28	0.98
	2.5	0.53	1.05	0.94	0.93	1.20	0.91
Infliximab	5	0.68	1.56	1.33	0.87	—	—
	0.8	0.59	1.40	1.30	0.70	—	—
Trastuzumab	5	1.25	1.14	1.70	0.87	—	—
	2.5	1.27	1.11	1.63	0.79	—	—
mAb A	10	0.14	1.31	0.94	1.26	—	—
	2.5	0.32	0.96	1.22	2.02	—	—

Conclusion

The ultra-fast charged variant separations described here are achieved because of several advances in chromatography techniques. The mechanism of pH gradient chromatography lends itself to the use of shorter, faster columns. The availability of high pressure rated small particle size ion exchange columns are a perfect match to pH gradient methodology. The commercial buffer formulations used here form a linear gradient which allows intelligent optimization of the methods. Finally, there is the use of the new Vanquish UHPLC system which has extremely low delay volumes, high precision gradient formation and a totally inert flow path.

Ultra-fast separation that requires total analysis cycle in the order of 2 minutes, including column re-equilibration and injection time, enables users to run more than 1,400 samples during 48 hours continuous operations. To allow unattended tasks with such large amount of samples, the Vanquish UHPLC system can be extended with the Vanquish Charger Module. This can host up to 9000 samples in a thermostatted environment, and transfer them to the Vanquish Autosampler.

References

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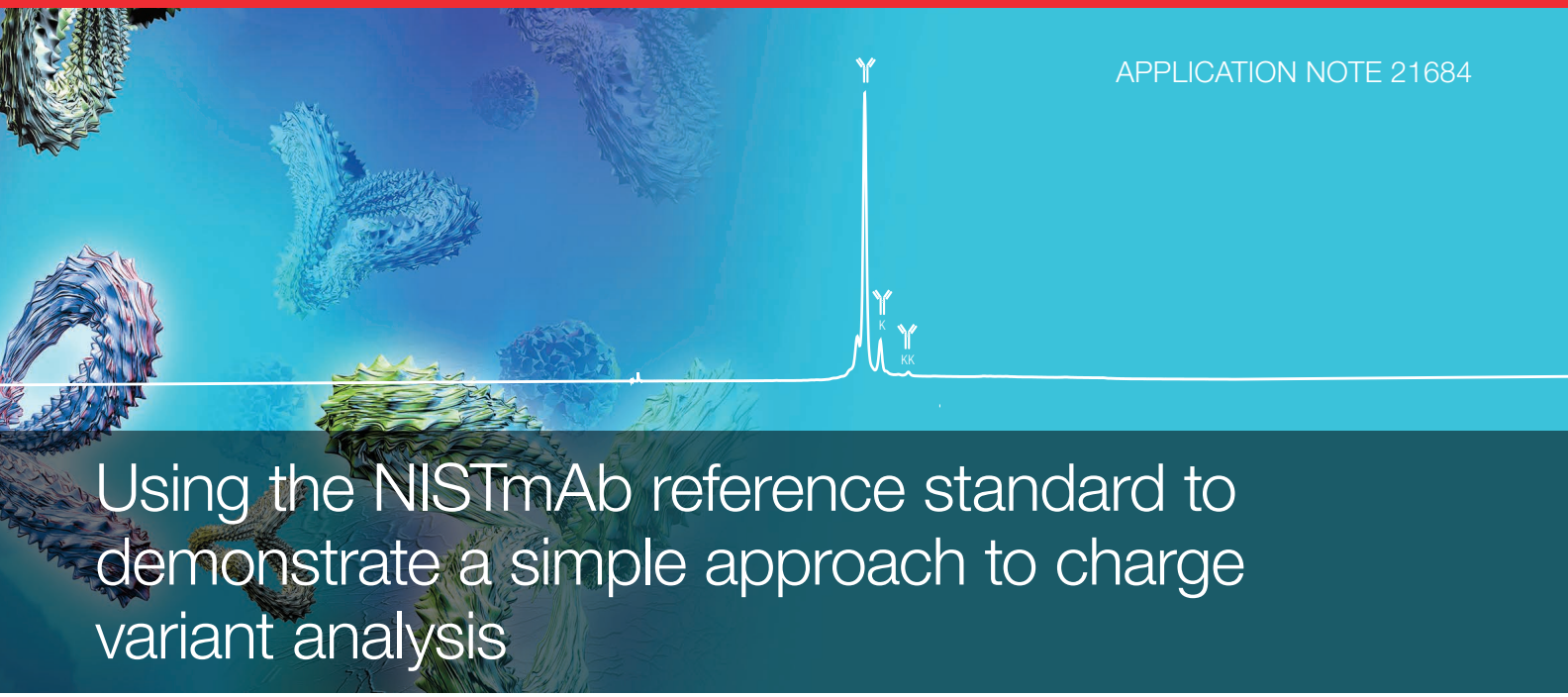

Using the NISTmAb reference standard to demonstrate a simple approach to charge variant analysis

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Keywords

NIBRT, Biopharmaceutical, Bioproduction, QA/QC, Biotherapeutic, IgG, Monoclonal antibody (mAb), Critical quality attribute, Intact Protein Analysis, NISTmAb, Reference Material 8671, Humanized IgG1K, Ion Exchange Chromatography, MAbPac SCX-10, ProPac WCX-10, pH Gradient Buffer, CX-1 pH Buffer, Vanquish Flex UHPLC

Application Benefit

- Integrated solution for superior charge variant separation and characterization
- Rapid, easy-to-optimize and highly reproducible method development

Goal

To demonstrate the effectiveness of a simple pH gradient/ion-exchange chromatography workflow approach to the characterization of charge variants in monoclonal antibodies using the NISTmAb reference standard as a model analyte. To show the assay is simple, reproducible, easily optimized, and resolves variants more effectively than previously published salt gradients.[1]

Introduction

The characterization of monoclonal antibodies (mAbs) during biopharmaceutical development involves the identification, monitoring, and analysis of charge variants. Antibodies can exhibit changes in charge heterogeneity during production and purification caused by amino acid substitutions, glycosylation, and other post-translational or chemical modifications. Not only can these changes impact stability and activity, they can also cause adverse immunological reactions. Identification of charge

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variants in development, and their monitoring throughout manufacturing is therefore critical.[2,3]

Ion-exchange chromatography (IEX) is widely used for the characterization of therapeutic proteins[4,5,6] and can be considered a powerful reference technique for the qualitative and quantitative evaluation of charge heterogeneity. IEX separates charge variants by differential interactions with a charged support.

Numerous variants are commonly observed when mAbs are analysed by charged-based separation techniques. These variants are generally referred to as acidic or basic species, compared with the main isoform, and are defined based on their retention times relative to the main peak. Acidic species are variants with lower pI that elute before the main peak by cation exchange (CEX), and basic species are variants with higher pI that elute after the main peak by CEX. Deamidation of asparagine residues and sialic acid content have been widely reported to contribute to the formation of acidic species.[7] Other modifications have also been shown to result in the generation of acidic species such as the non-classical disulfide linkages or high mannose oligosaccharides content. So far, basic species can be fully explained by known modifications including N-terminal glutamine, *N*-terminal leader sequences, C-terminal lysine, C-terminal amidation, or succinimide.[8]

State-of-the-art and emerging analytical and biophysical methodologies provide very detailed process and product information; however, their accuracy, precision, robustness, and suitability are also of critical importance. The NIST monoclonal antibody IgG1κ (NISTmAb) is intended to provide a well-characterized, longitudinally available test material that is expected to greatly facilitate analytical development applications associated with the characterization of originator and follow-on biologics for the foreseeable future.[9]

The NISTmAb reference material is a recombinant humanized IgG1κ expressed in murine suspension culture, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process related impurities. It is an approximately 150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds. The molecule has a high abundance of *N*-terminal pyroglutamination, C-terminal lysine clipping, and glycosylation of the heavy chain sub-units. The protein also has low abundance post-translational modifications including methionine oxidation, deamidation, and glycation.[9]

This application note presents the benefits of using pH gradient elution for separation of charge isoforms of NISTmAb reference material 8671 (Lot No. 14HB-D-002) using the combination of the Thermo Scientific™ MAbPac™ SCX-10 strong cation exchange (SCX) column and the Thermo Scientific™ CX-1 pH Gradient Buffers on a Thermo Scientific™ Vanquish™ Flex UHPLC for optimum performance. Additionally, NISTmAb charge variants were characterized following the chromatographic conditions described in the recently published book '*State-of-the art and emerging technologies for therapeutic mAb characterization. Volume 2*', which serves as both a foundational body of NISTmAb product knowledge as well as an evaluation of its suitability as an industry-appropriate reference material (RM), containing representative methods and associated data for the NISTmAb of which extent and quality is comparable to that in a Biologics Licence Application (BLA).[1]

Experimental

Chemicals and reagents

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- CX-1 pH Gradient Buffer A, pH 5.6 (P/N 085346)
- CX-1 pH Gradient Buffer B, pH 10.2 (P/N 085348)
- Thermo Scientific™ Chromacol™ Ultra High Recovery vials (P/N 1.2-UHRSV)
- Thermo Scientific™ 9mm Open Top Short Screw AVCS Caps and Septa (P/N 9-SCK(B)-ST1X)
- Carboxypeptidase B (150 units/mg; Roche® Diagnostic P/N 10103233001)
- Fisher Scientific™ Sodium phosphate monobasic monohydrate (P/N BP330-500)
- Sodium phosphate dibasic heptahydrate (P/N BP331-500)
- Sodium chloride (P/N S/3160/60)

Equipment

Vanquish Flex Quaternary UHPLC system, including:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump (P/N VF-P20-A)

- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A) with 25 µL (V=50 µL) sample loop
- Diode Array Detector HL (P/N VH-D10-A) with Thermo Scientific™ LightPipe™ 10 mm Standard Flow Cell (P/N 6083.0100)

MAbPac SCX-10 column, 10 µm, 4.0 × 250 mm (P/N 074625)

Thermo Scientific™ ProPac™ WCX-10 column, 10 µm, 4.0 × 250 mm (P/N 054993)

Buffers preparation for MAbPac SCX analysis

- Pump Eluent A: Dilute CX-1 buffer A pH 5.6 ten times with DI water.
- Pump Eluent B: Dilute CX-1 buffer B pH 10.2 ten times with DI water.

Buffers preparation for ProPac WCX analysis

- Pump Eluent A (20 mM phosphate buffer, pH 6.7): Dissolve 1.2 g of NaH_2PO_4 in approximately 450 mL of DI water. Add 13.449 g NaCl. Titrate to pH 6.7 with monovalent strong base or acid base as needed. Make up to 500 mL with DI water.
- Pump Eluent B (20 mM phosphate buffer, pH 6.7, 0.5 M NaCl): Dissolve 1.2 g of NaH_2PO_4 in approximately 450 mL of DI water. Add 13.449 g NaCl. Titrate to pH 6.7 with monovalent strong base or acid base as needed. Make up to 500 mL with DI water.

Sample preparation

Samples were injected directly at 10 mg/mL in sample buffer (12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl (pH 6.0). To verify which peaks correspond to C terminal lysine content, samples were digested with carboxypeptidase B (Roche Diagnostic) by incubation at 37 °C for 2 hours at 500 rpm.

UHPLC-UV charge variants analysis using MAbPac SCX-10 column

Charge variants were separated by strong cation-exchange chromatography on a Vanquish Flex Quaternary UHPLC system using an optimized linear gradient of 30–80% eluent B (CX-1 Buffer B pH 10.2) at 1 mL/min in 30 min on a MAbPac SCX-10 column, 10 µm, 4.0 × 250 mm at 30 ºC. The detection wavelength was 280 nm. NISTmAb samples were injected in 5 µL sample buffer.

UHPLC-UV charge variants analysis using ProPac WCX-10 column

Charge variants were separated by weak cation-exchange chromatography[1] on a Vanquish Flex Quaternary UHPLC system at 1 mL/min in 40 min. Eluent A was 20 mM phosphate buffer, pH 6.7, and eluent B was 20 mM phosphate buffer, pH 6.7, with 0.5 M NaCl. The column temperature was 35 ºC. The column was held at an initial condition of 3% eluent B for 3 minutes, followed by a linear gradient to 23% eluent B over 30 minutes. The column was held at 23% eluent B for 0.1 minute, ramped up to 99% eluent B over 0.5 minutes, held for 3 minutes, ramped back down to the initial conditions over 0.5 minutes, and then equilibrated at the initial conditions for 3 minutes. The detection wavelength was 280 nm. Injection volume for the NISTmAb samples, dissolved in sample buffer, was 5 µL.

Data processing and software

Chromatographic software:	Thermo Scientific™ Chromeleon™ CDS 7.2 SR4

Results and discussion

NISTmAb charge variants were characterized using a MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and a Vanquish Flex system equipped with a quaternary pump and DAD detection.

A pH gradient mode was used for mAb variants separation where the ionic strength of the mobile phase was kept low and constant, while the pH was varied to generate a linear gradient. This buffer system consisted of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. A linear pH gradient from pH 5.6 to 10.2 was generated over the time by running a linear pump gradient from 100 % eluent A to 100 % eluent B (Figure 1a). To optimize the NISTmAb variants separation, a shallower gradient was run (Figure 1b). Figure 1 demonstrates the MAbPac SCX-10 10 µm column providing fast, monoclonal antibody variant analysis using pH-based gradient. Higher resolution was achieved with a shallower gradient of 30% to 80% buffer B (Figure 1b) showing nine variant forms, with the main isoform assigned for peak 5. It illustrates good resolution of C-terminal lysine truncation variants (Figure 1b: peaks 5, 6, and 9), and other acidic and basic variants.

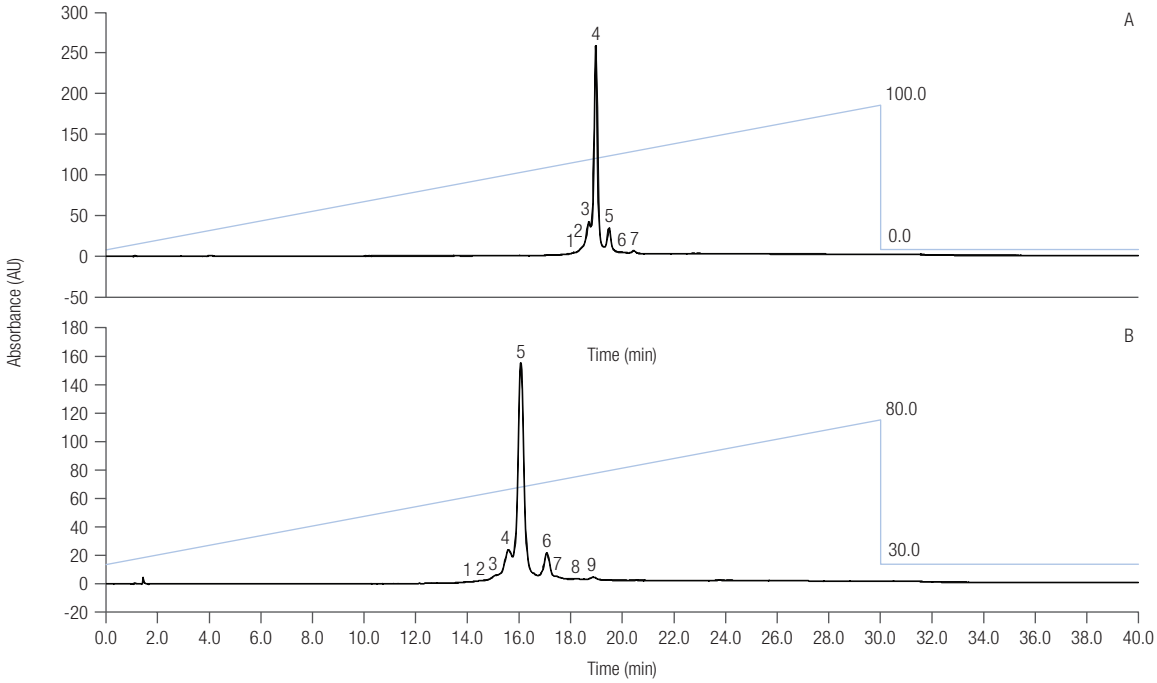

Figure 1. Chromatographic separation of NISTmAb (RM8671) charge variants using pH gradient (A) 0–100% over 30 min and (B) 30–80% B over 30 min, on a MAbPac SCX-10, 10 µm, 4 × 250 mm column.

Retention time precision (expressed as relative standard deviation, RSD) is essential in charge variants analysis for peak identification. Retention time precision was measured for repeated injections of the NISTmAb reference sample (Figure 2). Nine peaks were evaluated. The retention time precision for these peaks is shown in Table 1. The retention time precision for this separation was ≤ 0.25% for all peaks observed.

Table 1. Retention time (RT) precision (n=8) for charge variants peaks from NISTmAb sample RM 8671) shown in Figure 2.

Charge Variant Peak	1	2	3	4	5	6	7	8	9
Minimum RT (min.)	14.17	14.66	15.16	15.58	16.06	17.06	17.44	18.22	18.87
Maximum RT (min.)	14.23	14.78	15.28	15.60	16.08	17.08	17.47	18.25	18.90
Average RT (min.)	14.21	14.71	15.23	15.59	16.07	17.08	17.45	18.24	18.88
RT precision (% RSD)	0.19%	0.25%	0.22%	0.05%	0.05%	0.04%	0.06%	0.07%	0.06%

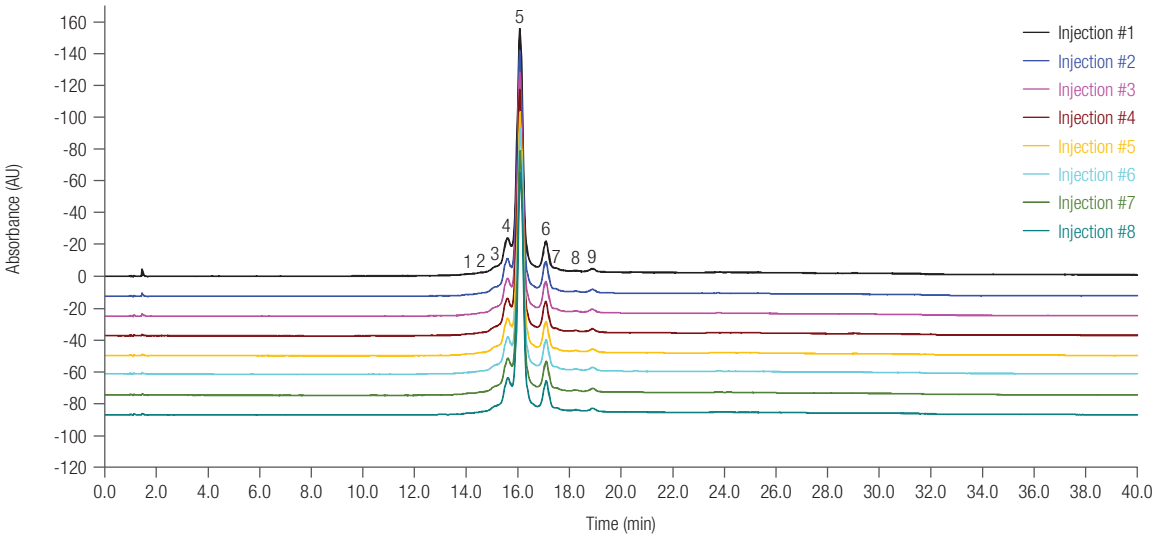

Figure 2. Repeated injections of NISTmAb sample (RM 8671) on a MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH-based gradient.

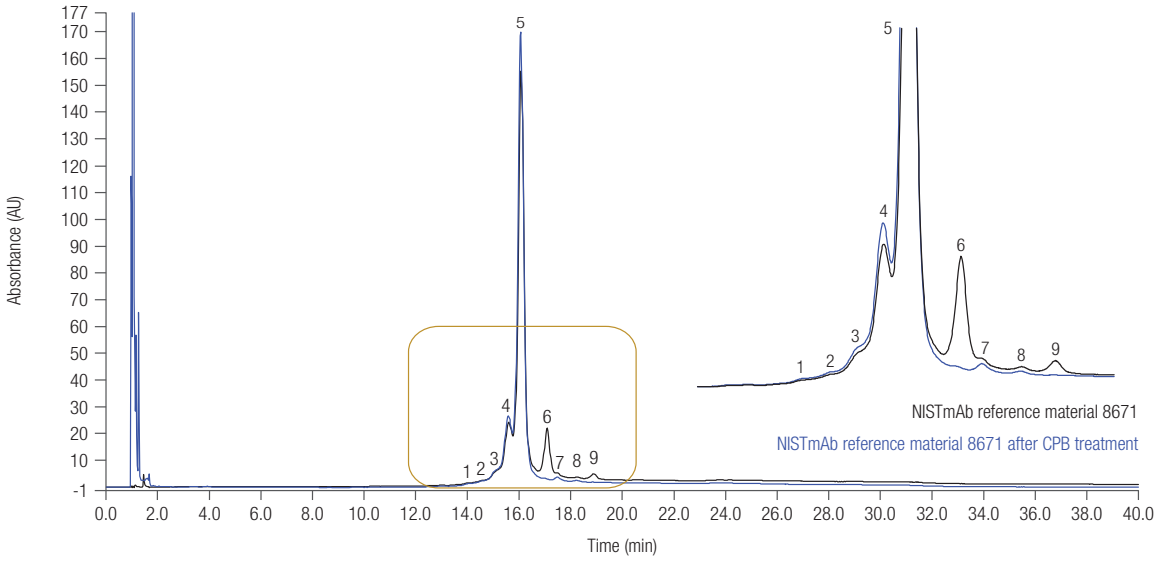

Figure 3. Characterization of NISTmAb (RM 8671) C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH gradient mode.

With the aim to verify heavy chain C-terminal lysine content, NISTmAb was treated with carboxypeptidase B (CPB), an exopeptidase that specifically cleaves C terminal lysine residues (Figure 3). This resulted in the absence of peaks 6 and 9 (containing 1 and 2 terminal lysine residues, respectively, on their heavy chains). The decreased peak areas in peaks 6 and 9 are accompanied by a corresponding increase in area of peaks 4 and 5 where no lysine is present (Figure 3, blue trace).

Figure 3 illustrates that three major peaks (black trace: peaks 5, 6, and 9) are due to variations in C-terminal content, after the treatment with CPB only one major peak remains (blue trace: peak 5).

Additionally, NISTmAb charge variants were characterized using a ProPac WCX-10 column as described in detailed in the NISTmAb biopharmaceutical characterization book chapter published by Michels, DA et al.[1] Figure 4 illustrates the comparison of charge variant profiles obtained with the ProPac WCX-10 column using a gradient of phosphate buffer (black trace) and the MAbPac SCX-10 column using a pH gradient mode (blue trace). The MAbPac SCX-10 column provided better resolution and the identification of nine variant forms compared to the results obtained with the ProPac WCX-10 column where only seven variant forms could be detected.

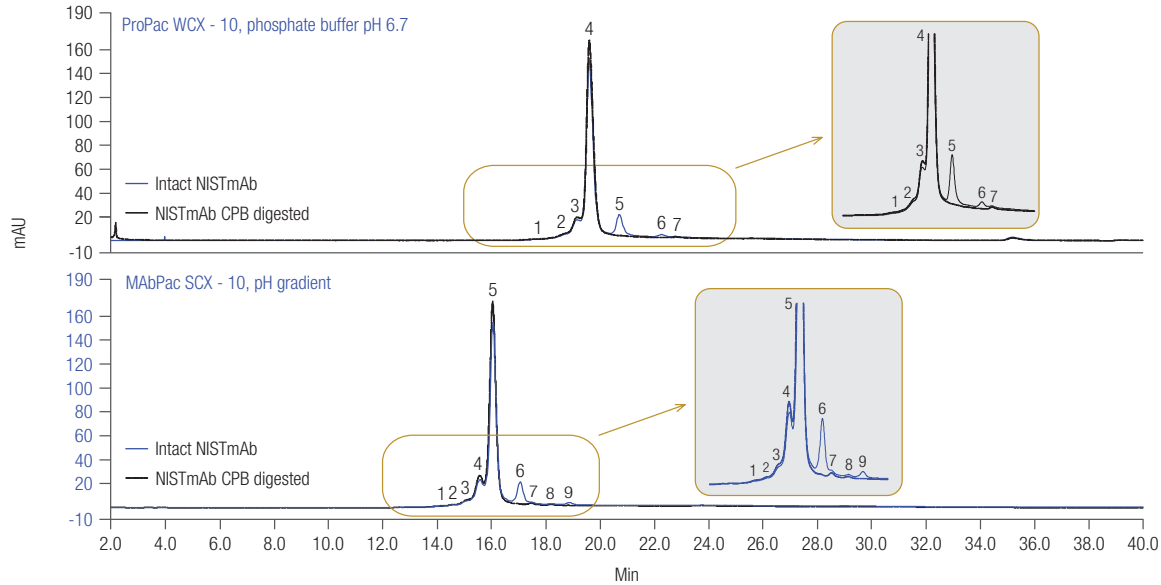

Figure 4. Characterization of NISTmAb (RM 8671) C-terminal lysine truncation variants using a ProPac WCX-10 column (4.0 × 250 mm, 10 µm) and a phosphate buffer gradient (black trace), and a MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH gradient mode (blue trace).

Conclusions

- pH-based gradients can be used for high resolution separation of monoclonal antibody charge variants. The CX-1 buffers are easy to prepare, highly reproducible, and provide a linear pH gradient, which minimize the issues of manually prepared buffers and online mixing variability. The CX-1 pH gradient buffers meet the fast and robust platform method requirements and the gradients can easily be altered to improve resolution.

- The availability of a commercial IgG1k reference material is intended to provide a widely available test product that is not associated with product-specific intellectual property concerns. This material will be useful for the assessment of current and emerging analytical technology and will establish a more robust framework for method quantification.

- The Vanquish Flex platform equipped with the Vanquish quaternary pump and Vanquish DAD detector is a powerful tool for charge variant analysis. Hence, this system can be effectively used for qualitative and quantitative evaluation of charge heterogeneity of NISTmAb reference material facilitating process development for novel mAbs and biosimilar products.

- The MAbPac SCX-10 column is complementary to the ProPac WCX-10 column for monoclonal antibody variant analysis, offering an alternative selectivity and providing higher resolution and efficiency for variant analysis of mAbs samples.

- The MAbPac SCX-10 column provides excellent separation for charge variants from NISTmAb reference material 8671, with excellent retention time precision.

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APPLICATION NOTE

No. 21601

A universal chromatography method for aggregate analysis of monoclonal antibodies

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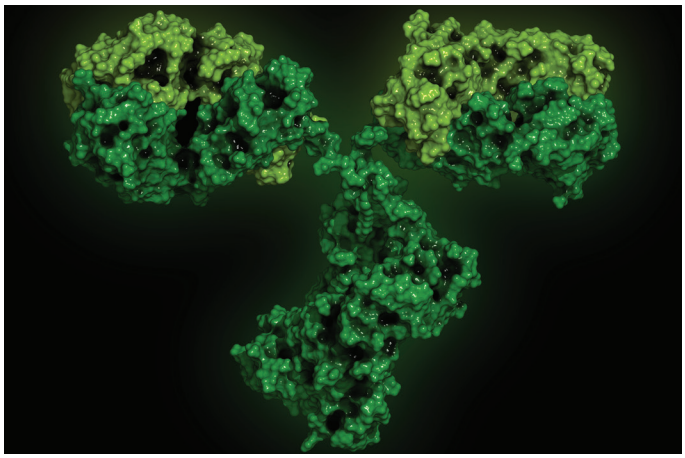
Key words
MAbPac SEC-1, monoclonal antibody, mAb, aggregation analysis, biotherapeutics, size-exclusion chromatography

Goal

Analysis of protein aggregation of five important biotherapeutic monoclonal antibodies (mAbs) by size-exclusion chromatography, showing the universal applicability of the Thermo Scientific™ MAbPac™ SEC-1 column for aggregate analysis of mAbs.

The aim of this study was threefold:

- **Produce a single globally applicable SEC chromatography method for the five mAb samples**
- **Show peak symmetry (implying limited secondary interaction with the column)**
- **Maintain the required monomer and dimer resolution**



Introduction

The biopharmaceutical industry continues to develop mAb-based biotherapeutics in increasing numbers. Due to the complexity of these biotherapeutics, there are several key quality attributes (CQAs) that need to be measured and controlled to guarantee their safety and efficacy. The presence of aggregates in a formulated drug product must be assessed to avoid potential issues with immunogenicity.

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Aggregates are typically dimers, trimers, or larger order structures of antibody molecules. They are formed at the following stages:

- Product expression during fermentation
- Product purification in downstream processing of the drug substance
- Storage or mishandling of the drug prior to patient administration.

Protein aggregation has been implicated as the cause of adverse immunological reactions that result in serious safety and efficacy issues. Aggregation must be monitored throughout the production process and during storage of the formulated biotherapeutic. MAb fragments that are smaller than the expected molecular weight elute after the parent peak and can also be determined.

Size-exclusion chromatography (SEC) is the standard method for this important analysis, but the compounds can show non-specific binding to the columns, which leads to retention time shifts, peak tailing, or even complete loss of protein peaks.[1,2] The MAbPac SEC-1 column is silica based with a proprietary, covalently bonded diol hydrophilic layer to prevent secondary interactions. Even so, the mobile phase eluents usually contain high salt concentrations to prevent ionic interactions, which can lead to corrosion of metal components. For this reason, an inert UHPLC system is recommended.[3] The MAbPac SEC-1 column separates by size and the pore size for this column (300 Å) was chosen to give a good separation in the molecular weight range of the monomer and dimers of a typical 150 kDa mAb. This column therefore serves as a good, broadly applicable column for mAb aggregate analysis.

Five important biotherapeutic mAbs (bevacizumab, cetuximab, infliximab, rituximab, and trastuzumab) were selected to investigate column performance. The monoclonal antibodies chosen are structurally diverse to investigate secondary interactions over a wide range of the physicochemical space. They cover a pI range between 7.6 and 8.7 and have widely different glycosylation patterns from very simple (bevacizumab) to highly complex (cetuximab). The MAbPac SEC-1 column and Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system were applied to the aggregate analysis of five important biotherapeutic mAbs using a common high salt buffer mobile phase at pH 6.8.

Experimental

Consumables

- Fisher Scientific™ HPLC grade water (P/N 10449380)
- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific Sodium phosphate dibasic anhydrous (P/N 10440481)
- Fisher Scientific Sodium phosphate monobasic anhydrous (P/N 10751135)
- Fisher Scientific Sodium chloride (P/N 11964051)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)
- Virtuoso 9 mm Wide Opening SureStop Screw Thread Vial Convenience Kit (P/N 60180-VT405)

Sample pre-treatment

Samples were reconstituted in water for injection with gentle swirling to aid in mAb solubilization as directed from the manufacturer's product insert information.

The following formulated drug products were injected directly:

- Bevacizumab 25 mg/mL
- Cetuximab 5 mg/mL
- Infliximab 10 mg/mL
- Rituximab 10 mg/mL
- Trastuzumab 21 mg/mL

Separation conditions

Instrumentation

Vanquish Flex Quaternary UHPLC system, standard configuration, equipped with:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)

Column: MAbPac SEC-1, 7.8 × 300 mm (P/N 088460)

Mobile phase

Composition:	0.2 M sodium chloride in 100 mM phosphate buffer pH 6.8
Flow rate:	0.3 mL/min
Column temperature:	25 °C
Injection volume:	1 µL
UV:	214 nm

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System software, version 7.2 SR4, was used for data acquisition and analysis.

Results and discussion

The separation profiles obtained from each mAb sample are represented in Figures 1A to 1E and normalized in an overlay in Figure 2.

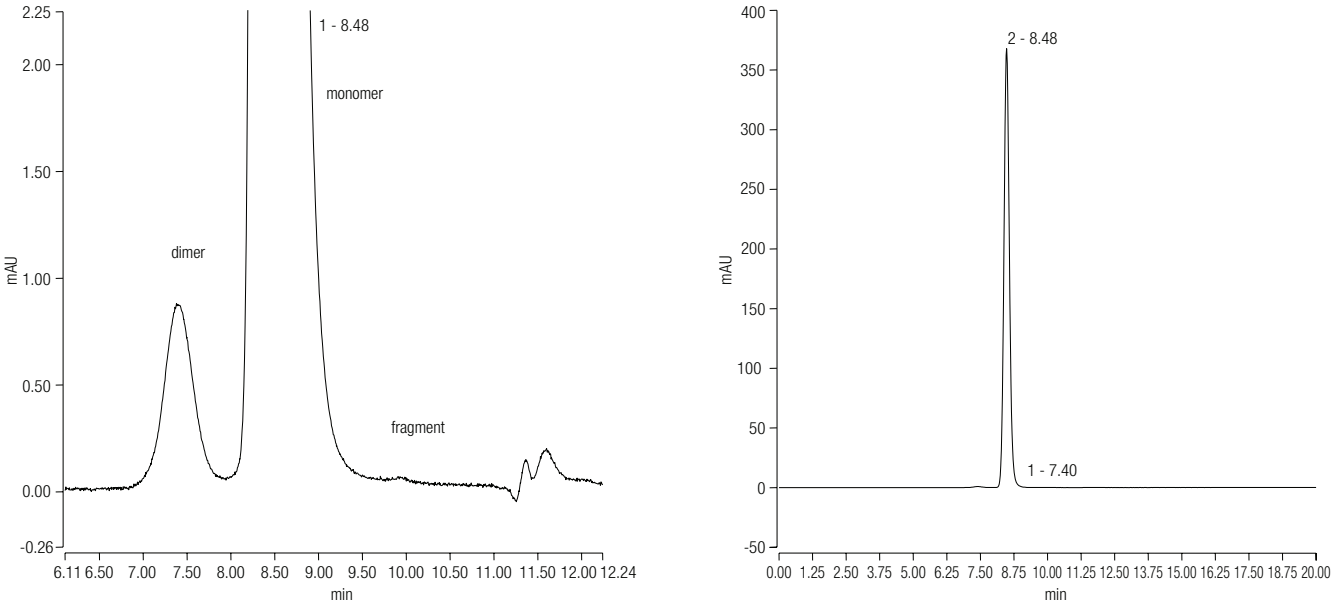

Figure 1A. Trastuzumab SEC separation, expanded view (left), full range chromatogram (right).

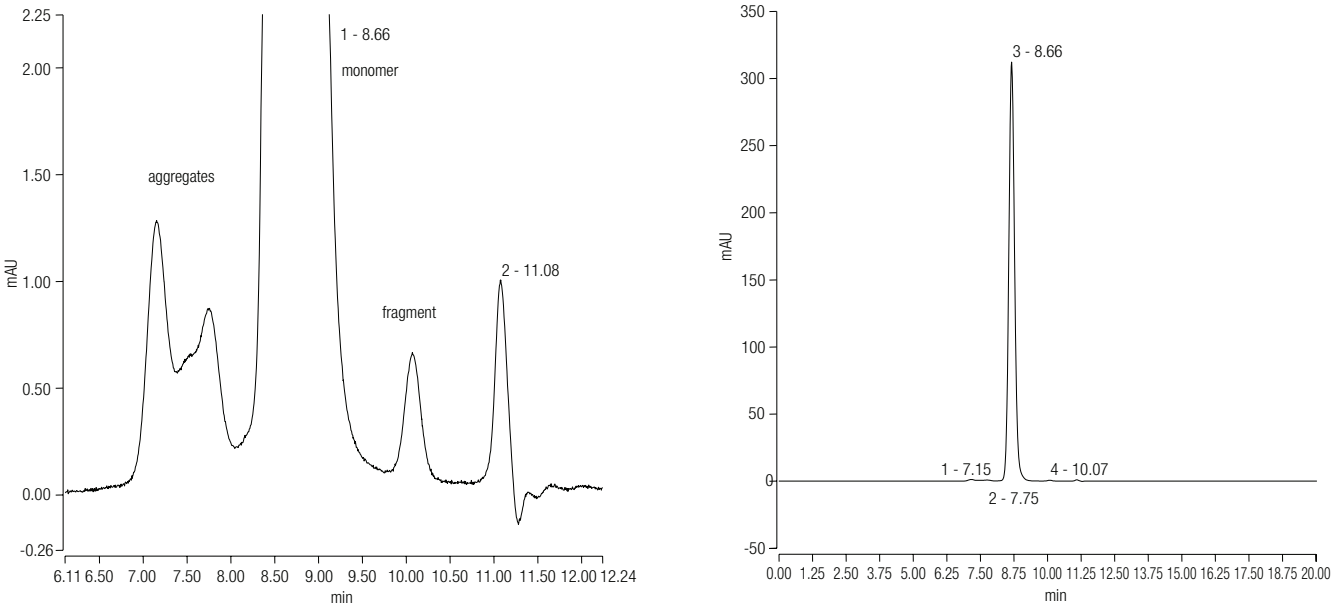

Figure 1B. Rituximab SEC separation, expanded view (left), full range chromatogram (right).

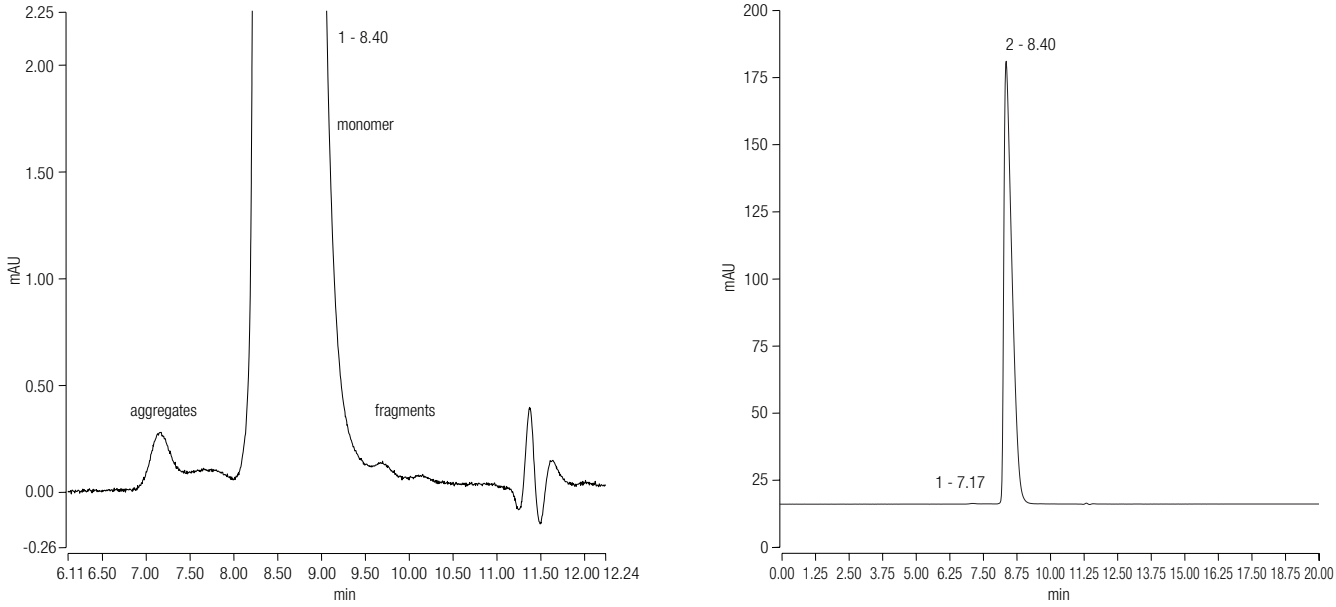

Figure 1C. Infliximab SEC separation, expanded view (left), full range chromatogram (right).

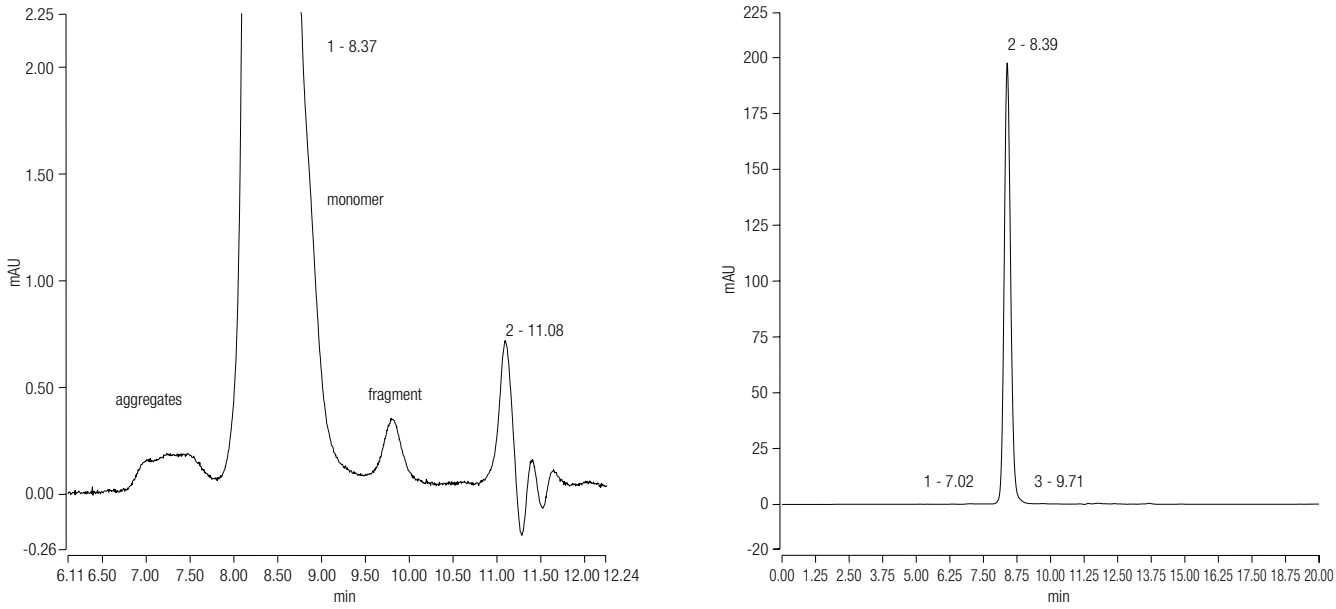

Figure 1D. Cetuximab SEC separation, expanded view (left), full range chromatogram (right).

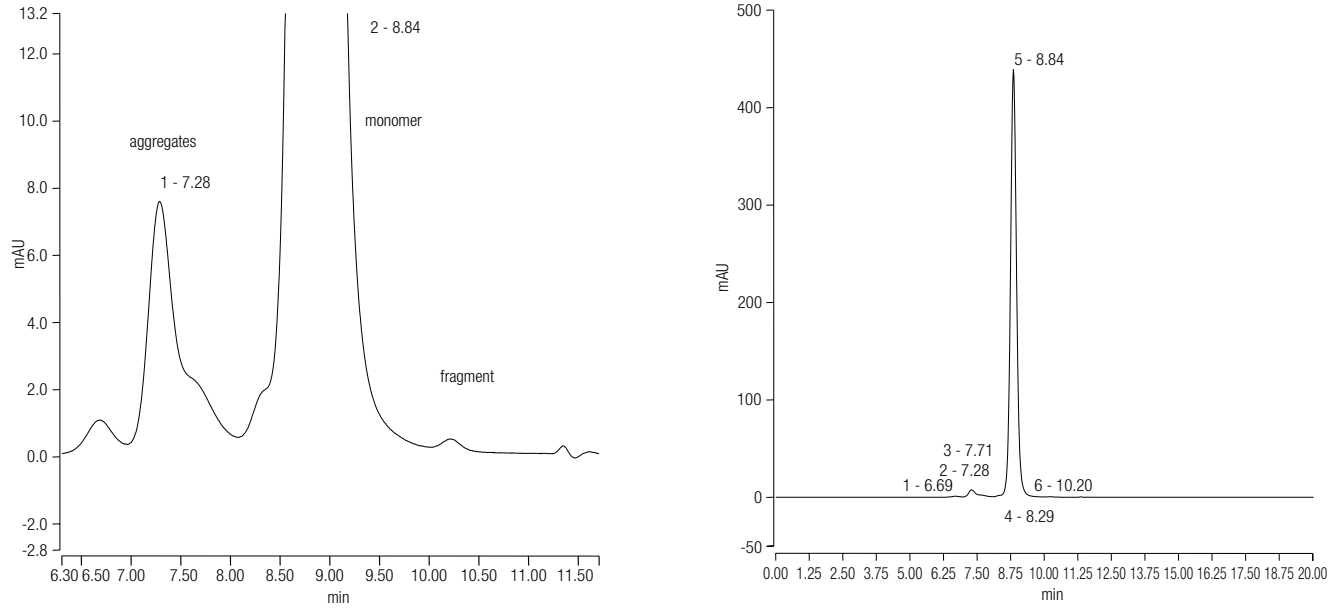

Figure 1E. Bevacizumab SEC separation, expanded view (left), full range chromatogram (right).

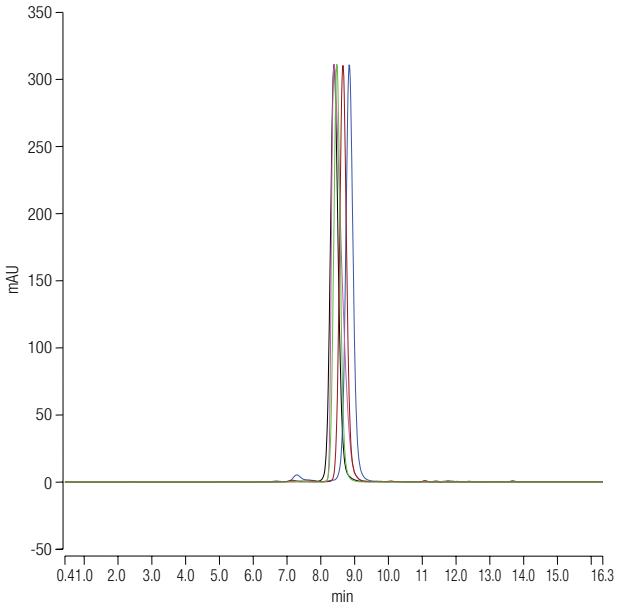

Figure 2. All 5 mAb samples overlaid to show relative retention time and peak shape (cetuximab, infliximab, trastuzumab, rituximab, bevacizumab).

Each pharmaceutically available mAb drug product shows a different aggregation pattern and different levels of aggregation (Figures 1A to 1E). Table 1 shows the percentage of aggregates in each mAb. Trastuzumab has a clear dimer peak that is well resolved from the monomer. Cetuximab and infliximab show the lowest level of aggregation and lowest fragment peaks. In contrast, bevacizumab exhibits a higher level of aggregation with a complex aggregation pattern, but has a low level of fragments.

Table 1. Percentage of aggregates in each mAb.

mAb Sample	% Aggregates
Trastuzumab	0.39
Rituximab	0.92
Infliximab	0.10
Cetuximab	0.29
Bevacizumab	2.99

In terms of performance, despite structural differences in the mAb samples, the MAbPac SEC-1 column gives resolution of the aggregates and fragments from the main monomer peak for all the samples tested. This resolution between monomer and dimer peaks was high enough to allow a good determination of the percentage aggregation. This was best demonstrated with trastuzumab (Figure 1A) where the calculated resolution was 2.21, which compares favorably with other SEC methods.

Figure 2 shows the overlaid chromatograms for the five mAbs. Molecular weights for these proteins are all relatively close to each other, which is reflected by their similar retention times. Given the retention times and the symmetrical peaks observed (Table 2), this demonstrates a lack of secondary interactions with the column packing material and hardware. Infliximab exhibits the worst asymmetry, which does not improve upon addition of solvent.[4]

In general, the relationship between retention time and mass is conserved with the largest mAb, cetuximab, eluting first and the smaller mAbs eluting in reverse molecular weight order as would be expected in SEC. The only anomaly is bevacizumab, which elutes last but is not the smallest protein. This may be a result of protein folding or differences in the N-glycans structures present on this molecule relative to the other mAbs studied. These subtle effects are magnified due to the narrow molecular weight range of the analytes.

The results demonstrate the general applicability of the MAbPac SEC-1 column for aggregate analysis for a range of monoclonal antibodies. The data also shows that this SEC column delivers high-resolution power for the separation of monomers and dimers, allowing easy quantitative analysis of any aggregates present in the samples. The method described here can be applied to the characterization of these top-selling mAbs and mAbs in general.

Table 2. Retention time comparison of mAbs and associated molecular weights.

mAb Sample	Molecular Weight (kD)	Retention Time (min)	Asymmetry
Rituximab	145	8.66	1.10
Trastuzumab	148	8.48	1.10
Bevacizumab	149	8.84	1.07
Infliximab	149	8.40	2.20
Cetuximab	152	8.37	1.13

Conclusions

Non-specific interactions with the column resin that occur during SEC analysis have been shown to be eliminated when using the MAbPac SEC-1 column, and the column exhibits the required resolution for aggregate analysis. Using the MAbPac SEC-1 column allows a single globally applicable SEC chromatography method for biotherapeutic monoclonal antibodies.

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4. Thermo Fisher Scientific Application Note AN21602: The Importance of Correct UHPLC Instrument Setup for Protein Aggregate Analysis by Size-Exclusion Chromatography, **2016**.

APPLICATION NOTE

No. 21602

The importance of correct UHPLC instrument setup for protein aggregate analysis by size-exclusion chromatography

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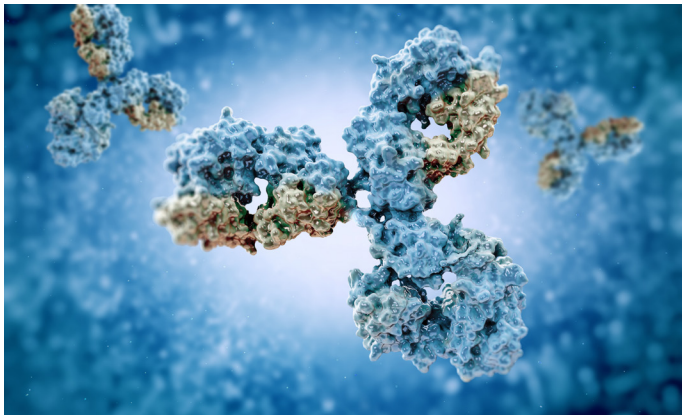
Key words
MAbPac SEC-1, monoclonal antibody, mAb, protein aggregation analysis, biotherapeutics, pre-column dispersion

Goal

Show the applicability of Thermo Scientific™ MabPac™ SEC-1 columns for monoclonal antibody aggregate analysis using the Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system.



Introduction

The industry-standard size-exclusion chromatography (SEC) column dimension for protein aggregate analysis is 7.8 mm internal diameter (i.d.), running at 1.0 mL/min. This is usually carried out using older HPLC systems, which perform well enough with these relatively high flow rates. The introduction of UHPLC, with much lower dispersion, has allowed the use of lower flow rate columns and higher resolution stationary phases. It is commonly believed that smaller dimension SEC columns are difficult to pack and this accounts for the reduction in performance seen with these columns compared to higher flow rate 7.8 mm i.d. columns. A more likely cause for apparent reduced performance is that the UHPLC system used for the comparison does not have the correct tubing or low dispersion flow path required to maintain peak integrity, an essential factor when using SEC at lower flowrates.[1,2]

A Vanquish Flex Quaternary UHPLC system was applied for the SEC analysis. This is a low dispersion, inert UHPLC, which can be used successfully for this type of application. For this analysis, pre-column dispersion was intentionally introduced to show the effects of dispersion in front of the column at different flow rates.

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The separation was performed on MabPac SEC-1 columns of differing internal diameters and flow rates.

The Vanquish UHPLC system was used to show the applicability of the MAbPac SEC-1 column for monoclonal antibody aggregate analysis. The MAbPac SEC-1 column is silica based and has been covalently modified with a proprietary diol hydrophilic layer to prevent secondary interactions which can hinder the chromatography of certain proteins.[2,3] SEC is one of the few chromatography methods that exhibits no 'on-column' focusing. Due to this, the pre-column dispersion on the system used is extremely important, especially at reduced flow rates on smaller i.d columns, as there will be no focusing of broad peak volumes at the head of the column. In adsorption chromatography, even under isocratic elution conditions, one would expect some focusing of the injection volume at the head of the column. In SEC, the volume in which the sample is presented to the column will only get larger in volume as it moves through the column. Therefore, many columns do not attain their expected resolution when using older HPLC systems with inherent dispersion on-injection. The effect of pre-column dispersion has been the subject of several reviews and can easily lead to up to 50% increase in peak widths on dispersive HPLC systems. The low dispersion Vanquish UHPLC system was applied for the SEC analysis to control and study the effects of dispersion. The separation was performed on MabPac SEC-1 columns with a commonly used high salt buffer at pH 6.8.

Experimental

Consumables

- Fisher Scientific™ HPLC grade water (P/N 10449380)
- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific Sodium phosphate dibasic anhydrous (P/N 10440481)
- Fisher Scientific Sodium phosphate monobasic anhydrous (P/N 10751135)
- Fisher Scientific Sodium chloride (P/N 11964051)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)
- Virtuoso 9 mm Wide Opening SureStop Screw Thread Vial Convenience Kit (P/N 60180-VT405)

Sample preparation

Bevacizumab was diluted to a 25 mg/mL solution with mobile phase containing 0, 5, 10, or 20% solvent as appropriate (see Figure 4).

Separation conditions

Instrumentation

Vanquish Flex Quaternary UHPLC system equipped with:

- System Base Vanquish Flex (VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)

Columns:	MAbPac SEC-1, 7.8 x 300 mm (P/N 088460) MAbPac SEC-1, 4 x 300 mm (P/N 074696)

SEC buffer

Mobile phase:	0.2 M NaCl in 100 mM phosphate buffer pH 6.8
Flow rate:	1.0 mL/min for 7.8 mm i.d. column and 0.3 mL/min for 4 mm i.d. column
Column temperature:	30 °C
Injection volume:	1 µL, unless stated
UV:	214 nm

Data processing

The Thermo Scientific™ Chromeleon™ 7.2 SR2 Chromatography Data System was used for data acquisition and analysis.

Results and discussion

Pre-column dispersion

Columns of 4.0 and 7.8 mm i.d. were used to test the effects of pre-column dispersion. The optimal flow of these columns is 0.3 and 1.0 mL/min, respectively. The flow rate used has a profound effect on the setup of the system. Lower flow rates were much more prone to the effects of any pre-column dispersion. At the higher flow rate of 1.0 mL/min using the 7.8 mm i.d. column, there was no discernible difference when the pre-column tubing was changed from the standard 100 µm i.d. tubing to 75 µm. Peak width, asymmetry, and resolution were all the same in both analyses, as can be seen in the overlay in Figure 1. This column was flowing at 1.0 mL/min, which generated 30 bar backpressure on the pre-column 100 µm tubing alone. The backpressure generated by the 75 µm tubing was 90 bar, but the change showed no improvement, indicating the chromatography was already optimum with the standard configuration on the system.

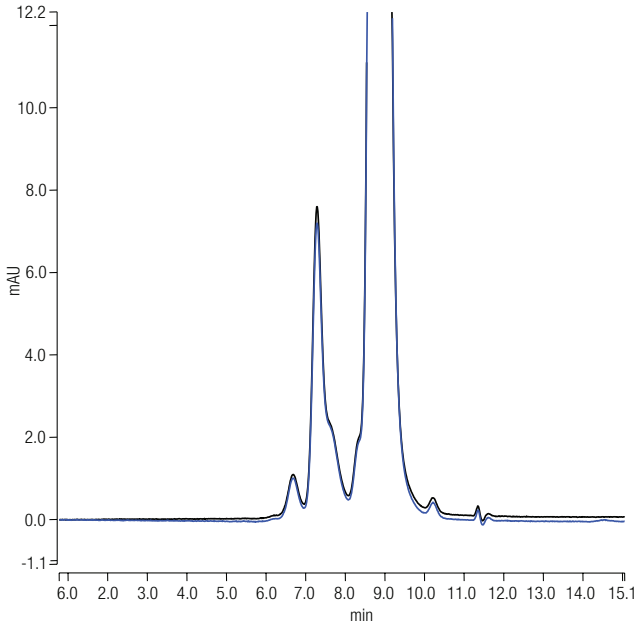
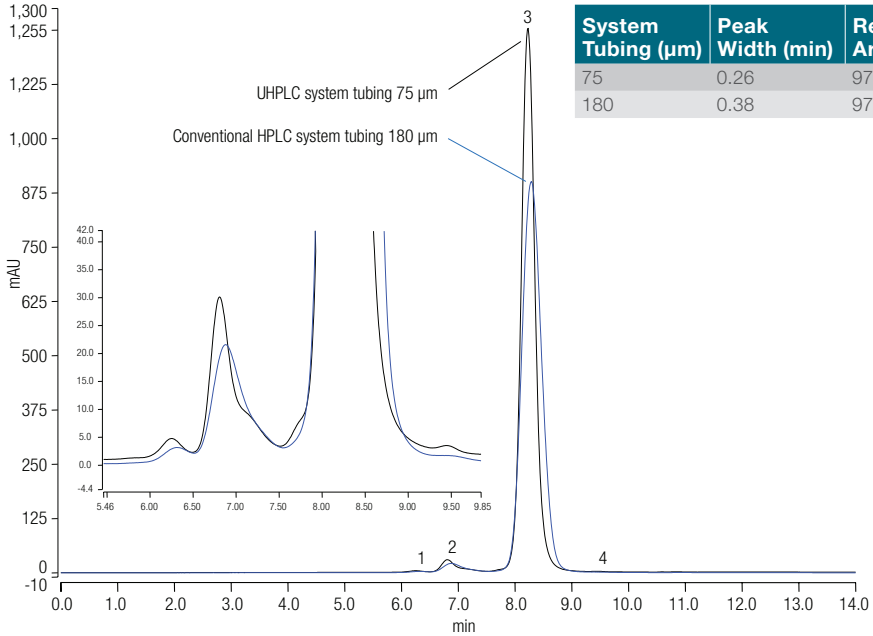


Figure 1. Overlay of bevacizumab SEC analysis with 100 µm and 75 µm i.d. tubing placed between the injection valve and the front of the column. Column i.d. was 7.8 mm.

The change when using the 4 mm id. column at 0.3 mL/min is quite dramatic. To mimic the configuration of a standard HPLC system, 180 µm tubing was used as well as 100 µm and 75 µm. These configurations are commonly used in SEC separations. In the overlay shown in Figure 2 there is a marked reduction in performance using 180 µm tubing in front of the column. This is what can be expected using a standard HPLC system at this flow rate on 4 mm i.d. SEC columns. This effect is compounded by the addition of the dispersion in the injection valves of older HPLC systems.

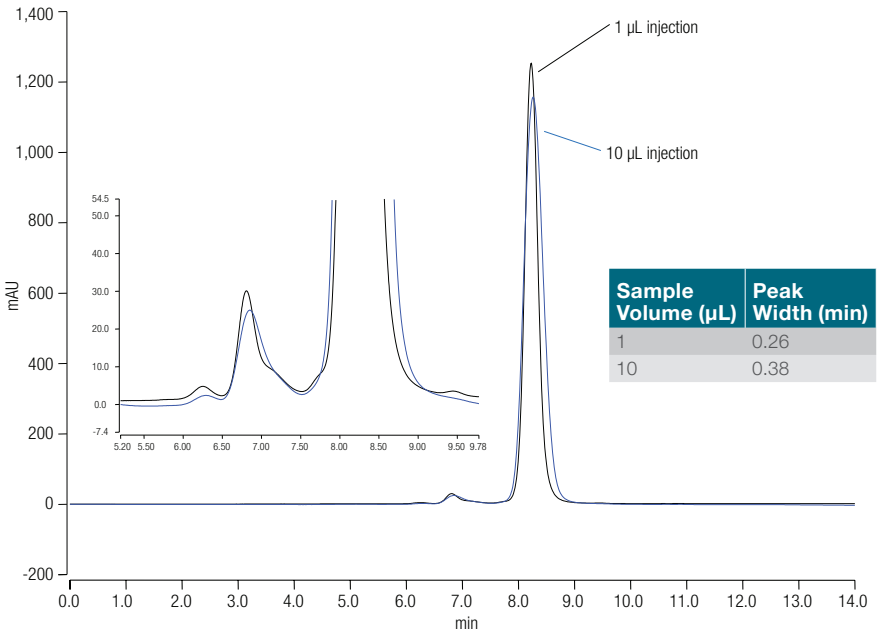


System Tubing (µm)	Peak Width (min)	Relative Area (%)
75	0.26	97.48
180	0.38	97.77

Figure 2. Overlay of bevacizumab using 180 µm and 75 µm i.d. tubing in front of the column. Column i.d. was 4.0 mm.

The relative area of the main monomer peak remains consistent. However, the peak shows marked dispersion with an increase in peak width at half height from 0.26 to 0.38 min and reduced sensitivity due to peak height reduction. There is also a loss of resolution and definition in the aggregate peaks, which leads to difficulty in identification of the aggregate forms. Peak dispersion and broader peak shape also influence the accuracy of integration with lower abundant variants. The 100 µm tubing showed some signs of dispersion (not shown for clarity). However, reducing the tubing inner diameter to 50 µm showed no further improvement over the 75 µm tubing, indicating that the optimum tubing to eliminate peak dispersion was 75 µm.

The volume of sample injected also plays an important part in peak dispersion in SEC, as it is essentially a form of inherent dispersion in the method. First, 1 µL of bevacizumab was injected onto the 4 mm i.d. column at a flow rate of 300 µL/min. The next injection was a 10× dilution using the same amount of sample in a 10 µL injection. The dispersion is evident in the increase in peak width and the loss in resolution and definition of the smaller aggregate peaks (Figure 3). There is a shoulder in front of the main peak that disappears along with the resolution of a second dimer peak. This confirms the importance of keeping injection volume as low as possible for SEC. Maintaining a high concentration of sample allows less volume to be injected onto the column and therefore lowers dispersion.

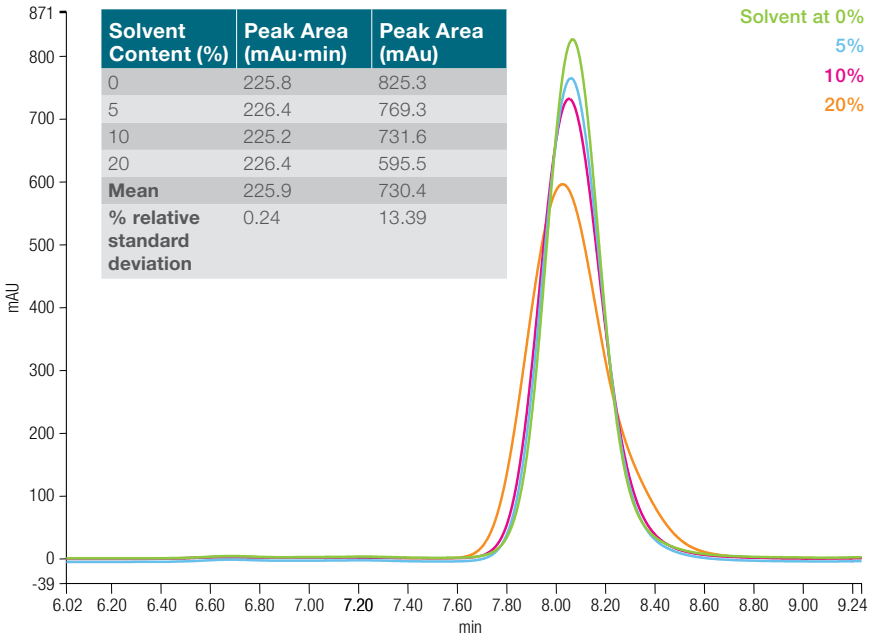


Sample Volume (µL)	Peak Width (min)
1	0.26
10	0.38

Figure 3. Effect of injection volume on peak dispersion with 4 mm SEC columns.

Solvent addition

There are reports of the use of solvents to improve peak shape on SEC columns that show secondary hydrophobic interactions. The use of any solvent with native protein samples can lead to unfolding of the protein, and the severity of the effect will be protein dependent. Proteins fold in a way that keeps the hydrophobic amino acids in the center of the folded protein with the hydrophilic amino acids on the outside in contact with the hydrophilic environment. The use of solvent changes the properties of the surrounding matrix and affects the structural folding of the protein. The MAbPac SEC-1 column has a proprietary hydrophilic boundary layer to eliminate secondary interactions with the silica resin. The effects of solvent addition can be seen in Figure 4.



Solvent Content (%)	Peak Area (mAu·min)	Peak Area (mAu)
0	225.8	825.3
5	226.4	769.3
10	225.2	731.6
20	226.4	595.5
Mean	225.9	730.4
% relative standard deviation	0.24	13.39

Figure 4. Inclusion of solvent in the SEC buffer conditions.

The peak retention time and asymmetry do not change with the addition of solvent, in this case acetonitrile, to the mobile phase. This would be expected if there were no secondary hydrophobic interactions between the column and the protein sample. However, even at 5% solvent, the peak height reduces and the peak width increases. This is possibly the effects of partial unfolding of the protein and a loss of structural stability. The shape and folded size of the protein has a greater distribution and creates a wider peak on size exclusion analysis. At 20% solvent, the effect is quite dramatic and the peak shape is compromised. This result has been confirmed with different mAb samples [not shown]. The level of the effect varies with the protein but all show some peak disruption with solvent. The addition of solvent is not required or recommended as peak asymmetry is acceptable without it.

Conclusions

SEC is prone to the effects of pre-column dispersion. Effects can be minimized by using higher flow rates on wider bore column formats. After the flow rates are reduced to 300 µL/min, or column formats are less than 4 mm i.d., the use of low dispersion UHPLC systems becomes essential for optimum performance. These systems may even need further optimization to include the use of 75 µm i.d. transfer tubing from the injection valve to the column.

Issues from non-specific interactions with the column resin during SEC analysis appear to have been eliminated with the measures taken to produce the MAbPac SEC-1 column. It proves to have more than satisfactory resolution for protein aggregate analysis and, in particular, for monoclonal antibodies. The use of solvent is not required to improve peak shape.

The addition of solvent to improve peak shape on columns that exhibit hydrophobic secondary interactions may help reduce the unwanted interaction but thought must be given to the additional effect of the solvent on the protein itself.

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POSTER NOTE 72229

Optimizing protein aggregate analysis by size exclusion chromatography

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Keywords

MAbPac SEC-1, monoclonal antibody, mAb, aggregation analysis, biotherapeutics, size-exclusion chromatography, pre-column dispersion

ABSTRACT

Purpose: Monoclonal antibody (mAb)-based therapeutics are structurally complex. A number of critical quality attributes (CQAs) must be monitored during development, bioproduction and drug product manufacture to meet standards for clinical use. Protein aggregation can occur during manufacture and storage and could seriously affect product safety and efficacy. Robust analytical methods capable of quantifying the extent of protein aggregation are essential to maintain product performance and patient safety. Size exclusion chromatography (SEC) is commonly used for this purpose, but without proper system optimization and conditions, problems that affect analytical performance can arise. In this study a universal SEC method for monitoring and quantifying the aggregation of mAbs was developed and an optimized ultra high performance liquid chromatography (UHPLC) system setup for aggregate analysis by SEC was determined.

Methods: MAb samples (bevacizumab, cetuximab, infliximab, rituximab and trastuzumab) were analysed on a bio-inert quaternary UHPLC system with a diode-array detector (DAD). The column employed was a 7.8 × 300 mm silica - based column with a proprietary, covalently bonded diol hydrophilic layer. System optimization was conducted using the mAb bevacizumab analysed on both 7.8 and 4 mm i.d. variants of the silica-based column. Dispersion effects were studied through variation of pre-column tubing internal diameter (i.d.) and the addition of solvent to the mobile phase.

Results: The mAbs studied did not display any non-specific interactions with the stationary phase of the selected column, allowing a single, globally applicable SEC chromatography method to be developed. It was determined that poor peak resolution associated with pre-column dispersion at low flow rates can be minimized through the use of pre-column transfer tubing of narrower i.d. and smaller injection volumes.

INTRODUCTION

MAbs are a dominant class of protein biotherapeutics which have achieved outstanding success in treating many life-threatening and chronic diseases. Over 20 mAb targeted therapy products reached ‘blockbuster’ status in 2015 (1). With many commercially successful biologic patents now expired, or nearing expiry over the next few years, there exists a great opportunity for mAb-based biosimilars to enter the global market. MAbs are known to form aggregates in the course of product expression during fermentation, product purification in downstream processing, or in storage or through mishandling of the product prior to patient administration.

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Aggregation of mAb monomers to dimers, trimers and other higher order structures is undesirable for two key reasons:

1. Aggregates may cause a decrease in product efficiency by lowering the effective concentration of the product.
2. Aggregation can expose normally unexposed epitopes leading to increased immunogenicity.

In order to demonstrate the safety and efficacy of the mAb and gain regulatory approval, it is essential to monitor the formation of aggregation products throughout the production process.

SEC is the standard method for protein aggregate analysis. The technique involves passing molecules through a column containing porous polymer or silica beads. The choice of pore size is related to the size of the molecule to be separated. For the separation of mAbs and their aggregates this is around 300 Å. Molecules are separated based on their hydrodynamic volume. Smaller molecules can penetrate fully into the pores of the stationary phase, while larger molecules cannot get totally inside the porous bead and therefore have less distance to travel and so elute through the column more quickly.

One requirement of the technique is that the analyte does not interact with the surface of the stationary phase. Ideally, differences in elution time are based solely on a protein's hydrodynamic volume, rather than its chemical or electrostatic interactions with the stationary phase.

However, mAbs are structurally diverse and can exhibit unwanted secondary interactions with residual groups on the column during analysis, affecting analytical data quality. Non-specific hydrophobic binding of proteins to the columns can, for example, lead to retention time shifts, peak tailing, or even a complete loss of protein peaks (2,3). A general SEC method applicable to a wide range of mAbs is therefore highly sought after.

MATERIALS AND METHODS

Sample Preparation

Universal MAb Aggregate SEC Method Development:

Samples were reconstituted in water for injection with gentle swirling to aid in mAb solubilization as directed from the manufacturer's product insert information. The following formulated drug products were injected directly:

- Bevacizumab 25 mg/mL
- Cetuximab 5 mg/mL
- Infliximab 10 mg/mL
- Rituximab 10 mg/mL
- Trastuzumab 21 mg/mL

Optimized Instrument Setup Determination:

Bevacizumab was diluted 1:1 with mobile phase (see Table 1).

Columns

Thermo Scientific™ MAbPac™ SEC-1, 5 µm, 4.0 × 300 mm (P/N 074696)
Thermo Scientific™ MAbPac™ SEC-1, 5 µm, 7.8 × 300 mm (P/N 088460)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system equipped with:

- System Base Vanquish Flex (VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)

Test Methods

Pre-Column Dispersion

180 µm ,100 µm, 75 µm, and 50 µm i.d. tubing was fitted pre-column to test dispersion.

Solvent Addition

0.2 M sodium chloride (NaCl) in 100 mM phosphate buffer, pH 6.8 containing 0, 5, 10 and 20 % (v/v) ACN was used as mobile phase to test the effect of solvent on peak shape and retention time (see Figure 6).

Chromatography Conditions

Table 1. Chromatography conditions applied when developing the universal mAb method and testing the various SEC conditions.

Test	Mobile Phase	Flow Rate (mL/min)	Column Temperature (° C)	Injection Volume (µL)	UV Wavelength (nm)
Universal Method – 7.8 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	1.0	25	1	214
Dispersion Test – 7.8 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	1.0	30	1	214
Dispersion Test – 4 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	0.3	30	1	214
Injection Volume Test – 4 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	0.3	30	1 & 10	214
Solvent Addition Test - 4 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8 containing 0, 5, 10 or 20 % (v/v) ACN	0.3	30	1	214

Data Analysis

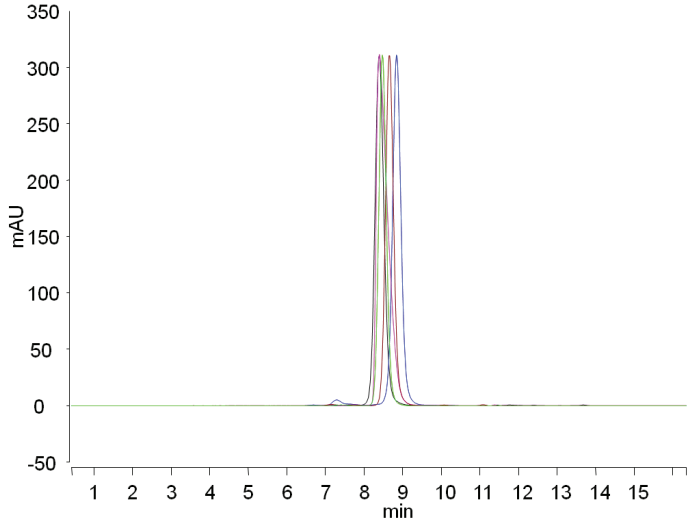
Thermo Scientific™ Chromeleon™ 7.2 SR2 Chromatography Data System was used for data acquisition and analysis.

RESULTS

Universal MAb Aggregate SEC Method Development

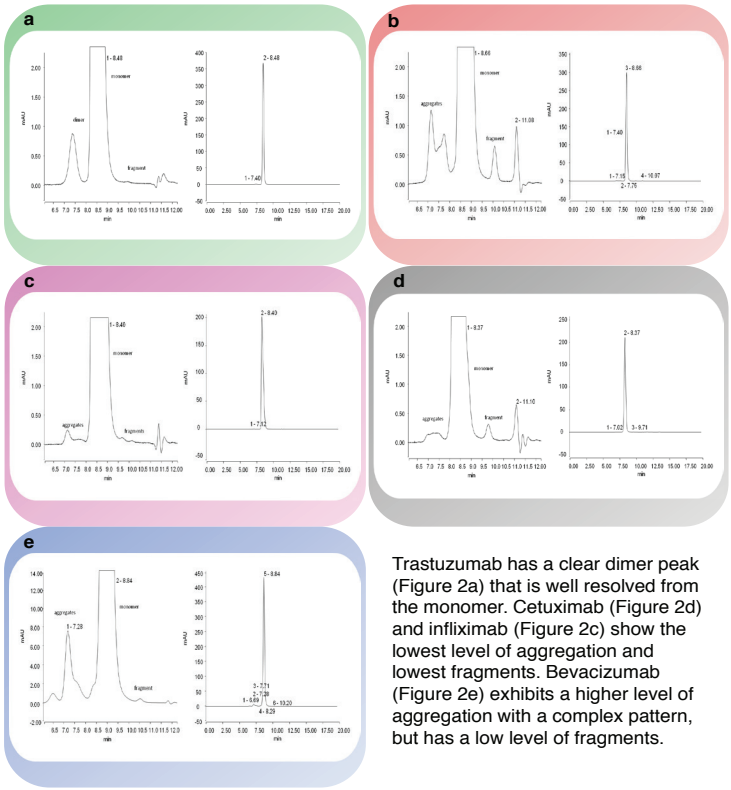
Using the MAbPac SEC-1 column, suitable resolution of aggregates and fragments from the monomer was achieved for all five mAbs (Figure 1), permitting determination of the percentage aggregation in each case (Table 2).

Figure 1. All 5 mAb samples overlaid showing relative retention time and peak shape (traztuzumab, rituximab, infliximab, cetuximab, bevicuzumab).



Cetuximab and infliximab showed the lowest levels of aggregation and fragmentation. Bevacizumab exhibited a higher level of aggregation and a complex aggregation pattern, but had a lower level of fragmentation (Figure 2).

Figure 2. SEC mAb separations. Expanded view (left) and full range chromatogram (right). Traztuzumab (a), rituximab (b), infliximab (c), cetuximab (d), bevicuzumab (e).



Trastuzumab has a clear dimer peak (Figure 2a) that is well resolved from the monomer. Cetuximab (Figure 2d) and infliximab (Figure 2c) show the lowest level of aggregation and lowest fragments. Bevacizumab (Figure 2e) exhibits a higher level of aggregation with a complex pattern, but has a low level of fragments.

Given the narrow range of molecular weights of the proteins studied, the similar retention times obtained for the samples, shown in Table 2, indicates a lack of secondary interactions with the column. This conclusion is further supported by the good symmetry of the chromatogram peaks.

Table 2. Retention time comparison of mAbs, associated molecular weights, asymmetry and percentage of aggregates in each mAb.

mAb Sample	Molecular Weight (kDa)	Retention Time (min)	Asymmetry	% Aggregates
Rituximab	145	8.66	1.10	0.92
Trastusumab	148	8.48	1.10	0.39
Bevacizumab	149	8.84	1.07	2.99
Infliximab	149	8.40	2.20	0.10
Cetuximab	152	8.37	1.13	0.29

Optimized Instrument Setup Determination

SEC is one of the few chromatography methods that exhibits no on-column focusing. Therefore, careful control of pre-column dispersion is essential to achieve optimum separation results, especially at reduced flow rates on smaller i.d. columns as broad peak volumes are not focused at the column head. To investigate the effect of pre-column dispersion on analytical performance, pre-column tubing of various diameters were used in combination with 4 and 7.8 mm i.d. columns for the analysis of bevacizumab.

Using the 7.8 mm i.d. column at the higher flow rate of 1.0 mL/min, there was essentially no difference in the produced chromatograms when the pre-column tubing was changed from the standard i.d. of 100 µm to 75 µm. Peak width, asymmetry and resolution were the same in both analytical runs, as shown in Figure 3.

Figure 3. Overlay of bevacizumab SEC analysis with 100 µm and 75 µm i.d. tubing placed between injection valve and column. Column i.d. was 7.8 mm.

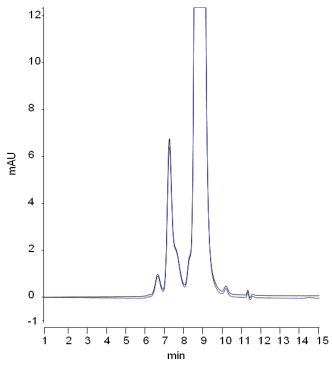
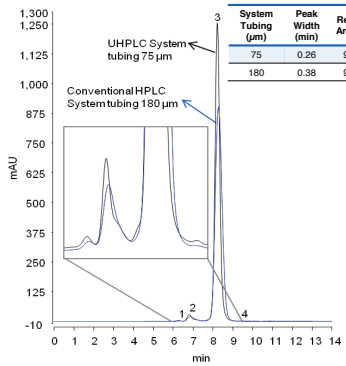


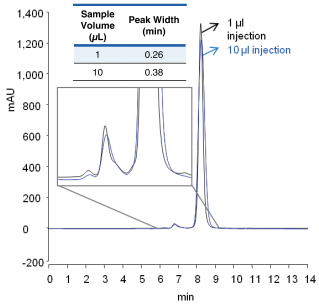
Figure 4. Overlay of bevacizumab using 180 µm and 75 µm i.d. tubing in front of the column. Column i.d. was 4.0 mm.



System Tubing (µm)	Peak Width (min)	Relative Area (%)
75	0.26	97.48
180	0.38	97.77

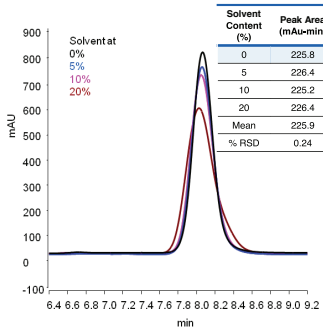
Using the 4 mm i.d. column at the lower flow rate of 0.3 mL/min, pre-column dispersion had a significant impact on analytical performance. Using 180 µm i.d. tubing markedly reduced peak shape quality (Figure 4), this effect would be compounded by the dispersion associated with the injection valves of older HPLC systems. Reduced tubing i.d. provided a significant improvement in peak resolution, the optimum tubing diameter was determined to be 75 µm.

Figure 5. Effect of injection volume on peak dispersion with 4 mm i.d. SEC column.



Sample Volume (µL)	Peak Width (min)
1	0.26
10	0.38

Figure 6. Inclusion of solvent in the SEC buffer conditions.



Solvent Content (%)	Peak Area (mAu·min)	Peak Area (mAu)
0	225.8	825.3
5	226.4	769.3
10	225.2	731.6
20	226.4	595.5
Mean	225.9	730.4
% RSD	0.24	13.39

The effect of injection volume on analytical performance was also studied (Figure 5). The 10 µL injection volume resulted in greater peak width and a loss in resolution and definition of the smaller aggregate peak.

The use of solvent changes the properties of the surrounding matrix and affects the structural folding of the protein. Peak retention time and asymmetry do not change with the addition of solvent (Figure 6). This would be expected if there were no secondary hydrophobic interactions between column and protein. At 5 % solvent, the peak height reduces and the peak width increases. At 20 % solvent peak shape is compromised (Figure 6).

CONCLUSIONS

- Robust and accurate protein aggregation analysis was achieved for five structurally diverse mAbs using a SEC method that employed a MAbPac SEC-1 silica-based column.
- The column's hydrophilic diol layer eliminates non-specific protein–column interactions.
- Poor peak resolution, associated with pre-column dispersion at lower flow rates, could be minimized through the use of pre-column transfer tubing of narrower i.d. (75 μm) and smaller injection volumes (1 μL).
- The addition of solvent is not required or recommended as peak asymmetry is acceptable without it.
- These findings highlight the importance of correct instrument set-up when using UHPLC systems, operating at lower flow rates.
- Through correct instrument optimization and the appropriate choice of chromatography columns, protein aggregation analysis by SEC can be a useful technique to ensure the safety and efficacy of mAb-based biotherapeutic products.

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Enabling Mass Spectrometric Analysis of Intact Proteins in Native Conditions on A Hybrid Quadrupole-Orbitrap Mass Spectrometer

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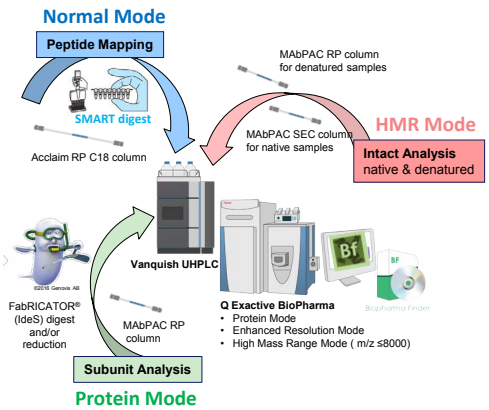
Poster Note 64804

INTRODUCTION

Analysis of proteins in native-like conditions free of organic solvents can allow proteins to preserve non-covalent interactions and retain high degrees of folding. This effect has analytical benefits: greater protein folding leads to reduced charge states leading to increased mass separation and increased signal at higher m/z. This strategy has been utilized for analysis of antibodies and antibody drug conjugates present in highly complex mixtures of different antibody/drug combinations [1]. Requirements for performing native MS on antibody samples include scanning towards 8000 m/z and increased transmission optimization for large compounds. Such features are not compatible with current commercially available quadrupole-Orbitrap instruments. Here we show results obtained after successful implementation of modifications aimed at adding the capability to perform native MS analysis without compromising performance of normal operation modes.

The analysis of intact proteins under native conditions is more challenging than under denaturing conditions since the buffers used don't contain any organic solvents. Performing electrospray from aqueous buffer solutions produces larger solvent droplets size and desolvation is less efficient. Moreover, for large proteins such as intact antibodies the required mass range for analysis under native conditions requires a mass range of more than 6000 m/z due to a smaller number of accepted charges. The increase of the upper mass range on the mass spectrometer was achieved by implementing instrument control software changes. The analysis of molecules across the full mass range including the detection of proteins under native conditions required the use of optimized parameter settings to ensure efficient desolvation in the front region of the instrument, the efficient transfer via multipoles, efficient trapping in the C-trap/HCD region and the sensitive injection and detection in the Orbitrap mass analyzer. Critical parameters are the optimization of in-source fragmentation that strongly influences the support of the desolvation process. Also, for the transmission efficiency specific voltages have been evaluated and optimized to ensure robust and sensitive performance in the higher mass range when performing analyses under native conditions, experiments that have not been possible so far on this type of mass spectrometer. Also, the standard calibration routine previously used was modified and adapted to ensure high mass accuracy across the full mass range.
With the data collected on different types of samples and presented in this study we demonstrate the successful analysis after implementation of the High Mass Range (HMR) mode, successful desolvation and optimized critical hardware operation settings. This makes the instrument an ideal platform to cover the three major workflows in BioPharma: intact mass analysis under denaturing and under native conditions in HMR mode, subunit analysis (reduced mAb and or IdeS digested mAb) in protein mode and peptide mapping in standard mode (see Figure 1).

Figure 1: Operating modes for the three major BioPharma workflows: Normal Mode, Protein Mode and HMR mode



MATERIALS AND METHODS

Samples:
Samples used in this study are ammonium hexafluorophosphate (AHFP, Fisher Scientific, part number A0368370), and Trastuzumab (tradename Herceptin, Roche, UK).

Chromatography:
A Thermo Scientific™ Vanquish™ UHPLC system was used for all LC/MS experiments. For native analysis, 50 mM ammonium acetate buffer (99.99%, Sigma Aldrich) was used. Reversed phase chromatography was performed with water/0.1% formic acid and acetonitrile/0.1% formic acid on a Thermo Scientific™ MAbPac™ RP 2.1x50 mm column.

Mass Spectrometry:
Mass spectrometers used in this study are the Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF systems with BioPharma Option. The instruments were operated under Tune 2.8 instrument control software in HMR mode, in which the RF applied to the C-trap was increased from 2,400 V p-p to 2,900 Vp-p for better trapping of the high m/z ions. Also, to ensure better capture of high-*m/z* ions in the Orbitrap analyzer, the initial central electrode voltage was adjusted from −3.7 kV to −3.4 kV, while the setting during detection remained unchanged (−5 kV). The S-lens RF level was allowed to be increased to a setting of 200 in HMR mode and set to that level for all experiments shown here.

Data Analysis:
Data analysis was performed with Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software.

RESULTS

There are many factors that play a key role in the analysis of proteins, some of which relate to sample preparation (buffers, solvents, additives) while others relate to the mass spectrometer's source conditions as well as the physical environment inside the instrument [2,3]. The Q Exactive Plus and Q Exactive HF mass spectrometers (Figure 2A) have previously been introduced with the Protein Mode option, which was one of many advancements for intact protein analysis on the Orbitrap platform. For these two instruments an automated HCD gas control was introduced by using an electronically controlled valve for nitrogen gas in the HCD cell for easier optimization of experimental conditions required for different types of analyses wished to run on a single platform.
In Normal Mode pressure settings are factory-optimized, suitable for most analyses and ions are cooled in the C-trap (Fig. 2B). The trapping gas pressure setting is 1 which corresponds to a high vacuum pressure delta (ΔHV) of ~3.1 e-5mbar. The ΔHV is defined as the difference between HV with HCD gas on minus HV with HCD gas off.
In Protein Mode the default trapping gas pressure setting is 0.2 and that corresponds to a ΔHV which is 5x lower than in Normal Mode. Additionally, ions are transferred and cooled in the HCD cell and thus have a longer flight path (Fig. 2C).
The combination of reduced C-trap and HCD cell gas pressures, and trapping ions in the HCD cell prior to mass analysis extends the life time of protein ions resulting in increased signal intensities of isotopically resolved species (Fig. 2E).

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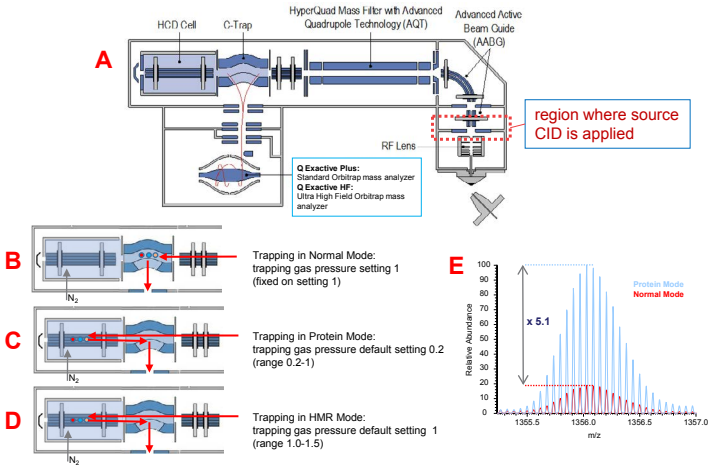
Figure 2. A) Schematic of the Q Exactive Plus/HF mass spectrometers and differences in the trapping path in the three different operating modes available: B) Normal Mode, C) Protein Mode and D) HMR Mode. E) Illustration of improvement in signal intensity for +17 charge state of a mAb light chain comparing Protein Mode and Normal Mode.



For higher gas pressures, high charge states of the same protein decay faster than lower charge states. This is because center-of-mass collision energy

$$K_{ce}=E*m/(M/z)$$

with
M/z: the mass-to-charge ratio for a given charge state
m: mass of residual gas, nitrogen
E: ion energy inside the Orbitrap

resulting in: **K_{ce} is proportional to the charge state z.**

This explains observations of charge envelope shifts on the m/z scale when comparing data acquired in different modes with different pressure regimes in the HCD cell and C-trap region, as shown in one example in Figure 6.

Here we have investigated and implemented the new High Mass Range (HMR) Mode that is especially required for the analysis of proteins under native conditions when samples are kept in aqueous buffers with no organic solvents involved at near neutral pH.

For HMR mode the default trapping gas pressure setting is 1 but it can be slightly increased up to 1.5 for even improved trapping of certain species such as protein complexes and heterogeneous large proteins (e.g. antibody drug conjugates). The trapping path in HMR mode is the same as in Protein Mode with ion cooling taking place in the HCD cell. And also, mass detection is enabled ranging up to m/z 8000 compared to m/z 6000 in the two other modes.

The trapping gas pressure in all modes is set and saved in the tune files and since a method allows for segmentation using different tune files different pressure settings can be used within one LC-MS run. In contrast the mass range setting is set in the method and the method editor allows for several nodes with different experiment types using different mass ranges within one LC-MS run.

Figure 3. Full MS spectra of ammonium hexafluorophosphate direct infusion experiment in Normal and HMR modes.

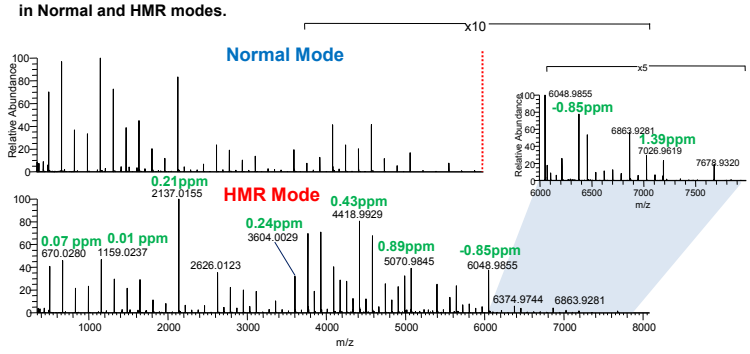


Figure 4. HMR Mode spectral mass accuracy test using ammonium hexafluorophosphate. The displayed test results in rms = 0.5 ppm.

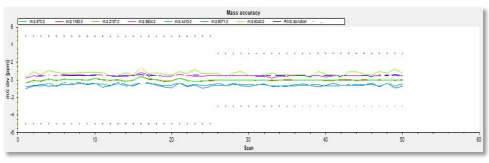


Table 1. Theoretical and measured masses (Figure 3) of ammonium hexafluorophosphate for calibration of HMR mode

theoretical m/z	experimental m/z	Δ Mass (ppm)
670.02805	670.0280	0.07
1159.02371	1159.0237	0.01
2137.01504	2137.0155	-0.21
3604.00204	3604.0029	-0.24
4418.99481	4418.9929	0.43
5070.98903	5070.9845	0.89
6048.98036	6048.9855	-0.85
7026.97170	7026.9619	1.39

hexafluorophosphate (AHFP) in direct infusion mode. Figure 3 displays the spectra obtained in Normal versus HMR mode with excellent mass accuracy (Table 1) even for masses detected with low abundance at m/z higher than 6000, also shown in the mass accuracy test in Figure 4.

Figure 5 displays the spectra obtained for intact Trastuzumab analyzed in native and denaturing conditions resulting in different charge envelopes inherent to the solvent and pH conditions (aqueous, near neutral pH for native; organic solvent content and low pH for denaturing). Both types of spectra result in nearly identical results after spectra deconvolution (Fig. 5C).

Figure 5. Trastuzumab analyzed under native (A) and denaturing (B) conditions resulting in highly similar deconvolution results (C).

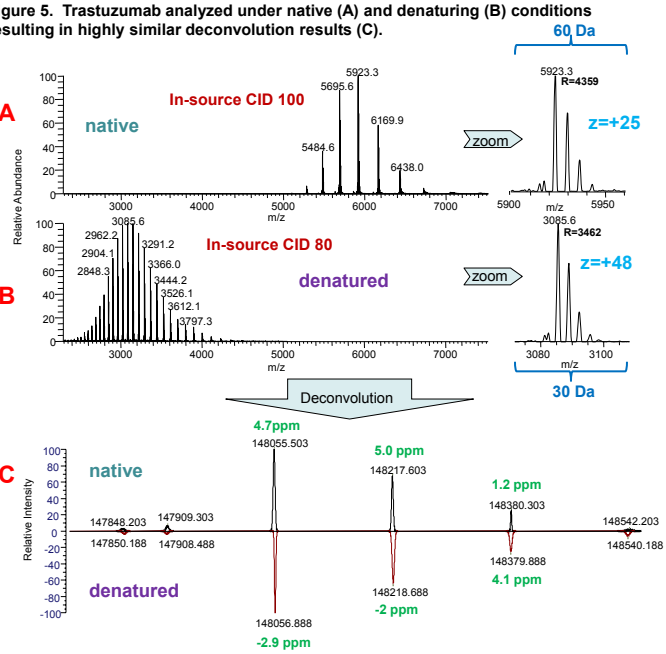
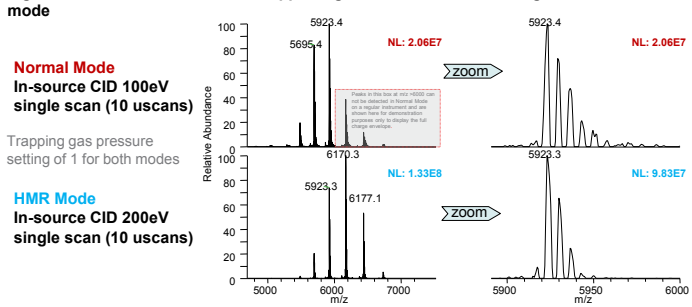
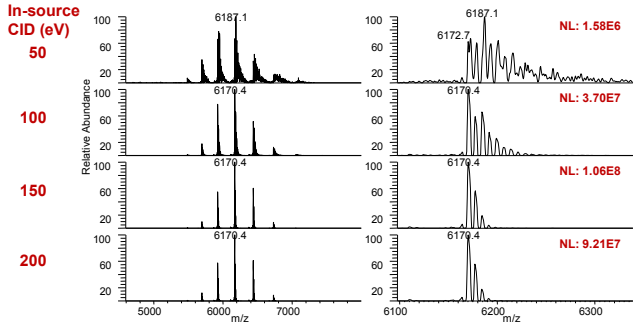


Figure 6. Effect of in-source CID supporting desolvation/ declustering in Normal vs. HMR mode



Figures 6 and 7 depict the influence and importance of source fragmentation supporting desolvation and declustering required to obtain the correct pattern of glycoforms. The data in these two figures were acquired in nanospray mode, requiring significantly higher in-source CID settings for native analyses. Due to neither gases available in the nanospray source nor a heated probe, the desolvation process needs to be supported with source fragmentation taking place inside the mass spectrometer, a potential step implemented between the S-lens and the Q00 (Figure 2A).

Figure 7. Intact Trastuzumab analysis under native conditions with increasing in-source CID settings highlighting the importance of this parameter on desolvation/ declustering.



Further parameters that were found critical in optimizing source conditions for native analysis using size exclusion chromatography (SEC) were the capillary temperature (also referred to as transfer tube) and the probe heater temperature (also referred to as Aux heater temperature). Figure 8 shows one example of two different temperature settings resulting in differences in charge state distribution.

Figure 8. Intact Trastuzumab analysis under native conditions with different probe heater and capillary temperatures.

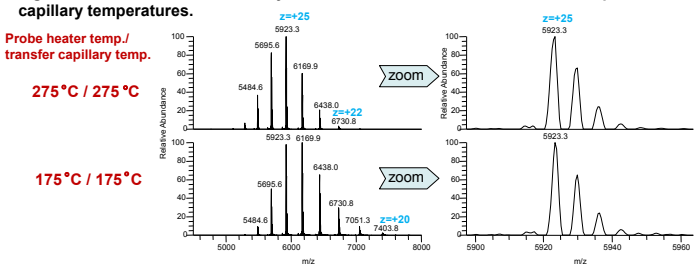


Figure 9. Intact Trastuzumab SEC-MS analysis under native conditions with different resolution settings.

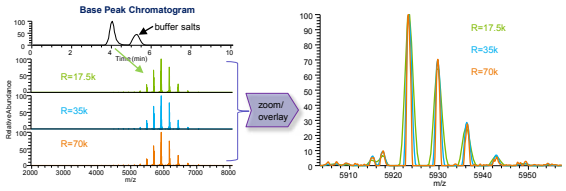
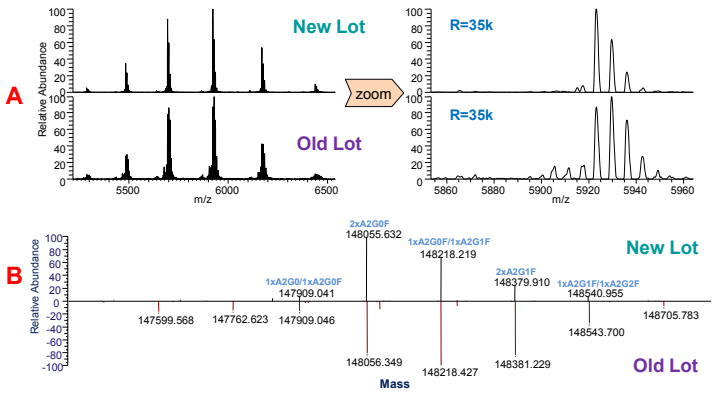


Figure 9 shows the SEC-LC/MS analysis of intact Trastuzumab under native conditions acquired on the Q Exactive Plus in HMR mode at different resolution settings of 17.5k, 35k and 70k. Increasing the resolution setting in this case is not aiming at achieving isotopic resolution but allowing to resolve possible sodium and potassium adducts [1] arising e.g. from salts and/or formulation buffer and thus significantly improving mass accuracy after deconvolution.
Figure 10 displays glycoform pattern variability from two different lots of Trastuzumab analyzed with SEC-LC/MS, which is not desired but has been observed and reported previously. The possibility for such a variation is inherent to the production process of biopharmaceuticals and shows the capabilities of mass spectrometry to easily pick up these variations since the analyses of glycoform patterns on the intact antibody level are very reproducible and reliable, considering the use of consistent optimized parameter settings for all experiments in a study.

Figure 10. Comparison of two different lots of Trastuzumab showing significant variation in the pattern of the glycoforms, a known issue in production of biopharmaceuticals.



CONCLUSIONS

- We have successfully implemented the High Mass Range Mode on the Q Exactive Plus and Q Exactive HF mass spectrometers allowing for mass detection up to m/z 8000.
- This new operating mode extends the instrument's capabilities to cover all three major workflows for BioPharma characterization.
- Desolvation/declustering conditions, ions transfer and trapping have been optimized to allow for improved sensitivity in HMR mode for resolution settings as high as 70k.
- Critical parameters for online LC-MS analyses under native conditions are capillary and probe heater temperatures as key factors in supporting desolvation/declustering.

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[2] Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. Science. 1989;246(4926):64-71.
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Poster Note 64404

The Sliding Window Algorithm for the Analysis of LC/MS Intact Protein Data

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Overview

Purpose: Identification of large molecules such as intact proteins in LC/MS is complicated by the difficulty of identifying the relevant peaks in the chromatography. In general, peaks associated with large molecules will have complicated profiles, ill-defined start and stop times, and often overlap with other components in complex protein samples. In this study, we discuss a novel "sliding window" approach that eliminates the need to identify chromatographic peaks and takes advantage of power of deconvolution algorithm to identify components directly.

Methods: The Sliding Window Algorithm averages spectra over a succession of windows in retention time, deconvolves each average spectrum, then merges similar masses from consecutive deconvolutions to identify components. This new algorithm has several advantages over conventional approaches: 1) it avoids the problems involved in trying to identify peaks associated with large molecules, 2) it can identify and characterize components that coelute at overlapping retention time ranges, 3) it produces a meaningful elution profile for each components it identifies, 4) it can reduce the rate of false positives, 5) in many cases it can also increase sensitivity.

Results: We apply the Sliding Window Algorithm to three representative data sets: a protein mixture, an antibody data set, and an ADC data set. The algorithm identifies components and their associated elution profiles.

Introduction

The Sliding Window Scheme is an alternative to the conventional approach to identifying components in LC/MS data. Rather than try to identify chromatographic peaks, then detect components associated with those peaks, it averages spectra over a succession of sliding windows in retention time, deconvolves each averaged spectrum, and merges similar masses to identify components. This algorithm is incorporated into Thermo Scientific™ Protein Deconvolution™ 4.0 Software.

Methods

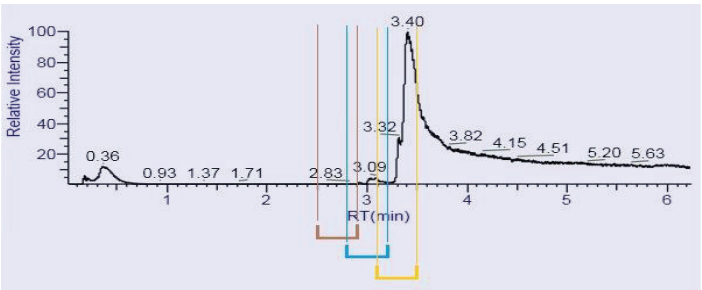
General Approach

The Sliding Window Algorithm involves two steps: the sliding window step and the mass merge step. These are described below.

The Sliding Window Step

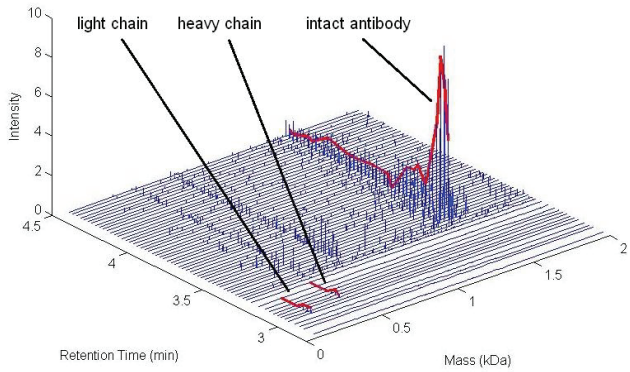
The sliding window step applies a sliding window in retention time to generate a succession of time-averaged spectra. The sequence of sliding windows is determined by four parameters: start time, stop time, width, and offset. This is illustrated in Figure 1, which shows a succession of three retention time windows starting at 2.5 min, with a width of 0.4 min and an offset of 75%, stopping at 3.5 min.

FIGURE 1. A succession of three windows in retention time, used to generate time-averaged spectra



Each average spectrum is then deconvoluted using the appropriate deconvolution scheme – Xtract for isotopically resolved and ReSpect™ for isotopically unresolved spectra – to generate a list of 'component peaks' for the successive retention time windows. The sliding window step produces a list of 'component peaks' for individual retention times. Figure 2 shows a plot of component intensities vs mass and retention time for a typical succession of sliding windows

FIGURE 2. component intensities vs mass and retention time for the example in Figure 1.



Valid components, such as the light chain, heavy chain, and intact antibody, occur at similar mass values, causing them to 'line-up' in the plot. Noise signals appear as randomly distributed peaks with no significant correlation in mass or retention time..

The Mass Merge Step

The mass merge step applies a sliding window in mass to the component peaks produced by the sliding window step and merges them to produce a list of 'merged components'. This sliding window in mass can be implemented as a window of constant width or as a minimum separation between components. The first approach will produce a list of components with a fixed width in mass, some of which could plausibly be merged. This approach is used for results from isotopically resolved spectra, for which masses are extremely well-determined The second approach produce a list of components that are separated by more than some minimum difference in mass. This will guarantee that related components peaks are merged, but can also incorporate unfortunately-placed noise peaks (see the discussion of Figure 6). This approach second is used for isotopically unresolved data.

The Sliding Window Algorithm returns a list of components and an 'abundance trace' for each component. Unlike XICs, which can incorporate unrelated parts of the original signal that might happen to share some m/z values with the primary component, the abundance trace is the actual elution profile associated with that component. This is illustrated in the following examples.

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Results

The Sliding window algorithm was applied to three representative data sets – a protein mixture, the antibody data set shown in Figures 1 and 2, and ADC data -- to evaluate its effectiveness. The results are discussed below.

Protein Mixture Data

The protein mixture consisted of 9 proteins. Some of these involved isotopically resolved spectra and will be ignored for the purposes of this particular.. Seven component groups associated with isotopically unresolved spectra eluted between a retention time of 10 and 17 minutes. These are listed in Table 1.

Table 1. 7 Component groups from the 9 protein mixture that eluted between a retention time of 10 and 17 minutes

ID	Mass Range (Da)	Retention Time Range
1	14,300-14,300	10.3-10.6 min
2	18,300-18,700	13.1-13.4 min
3	19,900-20,100	12.5-12.7 min
4	28,900-29,100	14.6-14.9 min
5	36,100-36,200	13.9-14.2 min
6	66,400-66,600	11.7-12.1 min
7	79,200-80.200	10.9-11.2 min

The Sliding Window Algorithm was applied in conjunction with ReSpect™ to the data for this retention time range. Figure 3 shows the resulting deconvoluted spectrum. The algorithm identified all 7 component groups in Table 1. Each component group is associated with a well-defined cluster of peaks in Figure 3.

FIGURE 3. Deconvoluted spectrum generated by the Sliding Window Algorithm for a protein mixture.

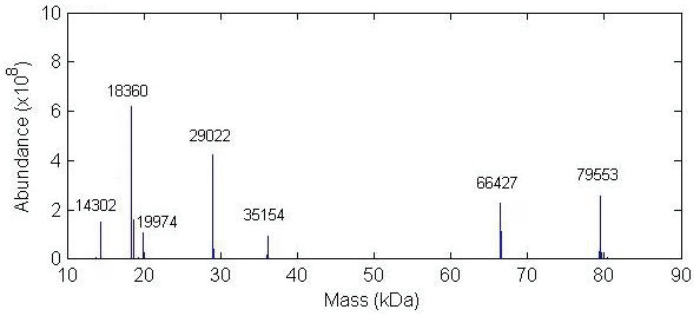
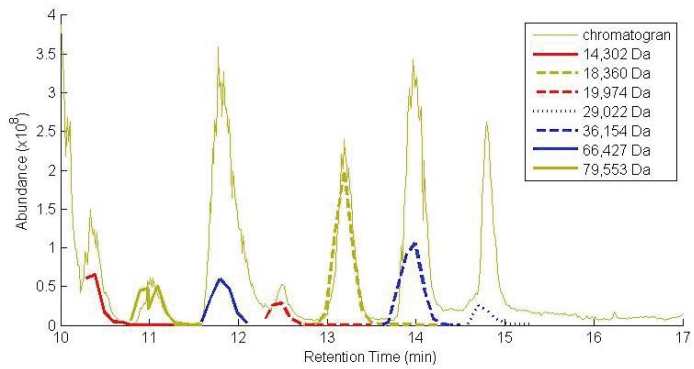


Figure 4 shows the original chromatogram and elution profiles generated by the Sliding Window algorithm for the most abundant modification for the components at 14,302, 18,360, 19,974, 29,022 , 36,154, 66,427, and 79,553 Da.

FIGURE 4. Chromatogram and elution profiles of 7 components identified by the Sliding Window Algorithm for a protein mixture



The algorithm identified retention times and generated elution profiles even for time periods such as the one between of 10.7 and 11 min when two components coeluted.

Antibody data

The antibody data set consisted of an intact antibody and associated modifications in the vicinity of 151 kDa along with component groups associated with a light chain, a heavy chain, and a combination of light and heavy chains. These are listed in Table 2

Table 2. 4 Component groups in the antibody sample

ID	Mass Range (Da)	Retention Time Range	Comments
1	23,400-25,200	2.9-3.2 min	Light chain
2	47,100-47,400	3.0-3.1 min	Heavy chain
3	135,000-135,400	3.2-3.3 min	Light + 2 Heavy
4	150,100-152,800	3.2-6.2 min	Intact antibody

Figure 5 shows the deconvoluted spectrum generated by the Sliding Window Algorithm in conjunction with ReSpect™. Due to the more challenging nature of this data set, it is not as clean as Figure 3, and it includes some false positives that the current version of the algorithm was unable to exclude, but it shows well-defined clusters of peaks corresponding to all four of the component groups listed in Table 2.

FIGURE 5. Deconvoluted spectrum generated by the Sliding Window Algorithm for an antibody sample.

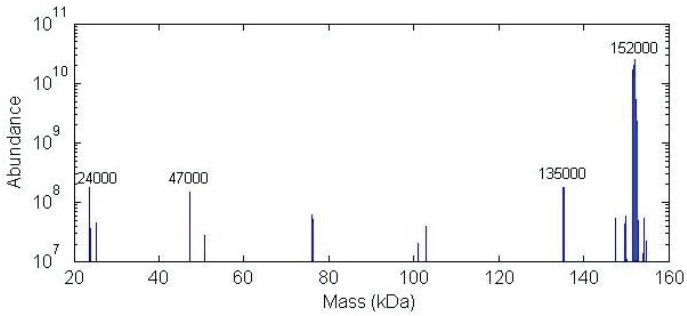
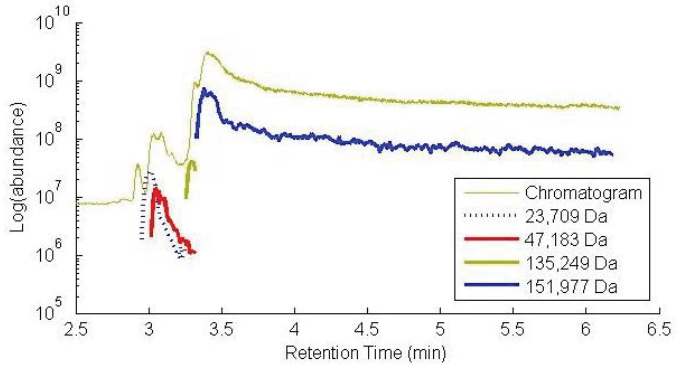


Figure 6 shows the original chromatogram and elution profiles generated by the Sliding Window for the most abundant modification of each of the 4 component groups listed in Table 2: at 27,709, 47,183, 135,249, and 151,977 Da. Like the deconvoluted spectrum, these profiles are not as clean as the results for the protein mixture in Figure 4, but they successfully resolve the two coeluting components at 23,709 and 47,183. They also resolve the component associated with the shoulder in the chromatogram at 3.2 min that can be difficult to detect using conventional techniques

FIGURE 6. Chromatogram and elution profiles of 4 components identified by the Sliding Window Algorithm for IgG_source_cid



ADC Data

The ADC data consisted of the antibody plus 8 components groups associated with ADCs. These are listed in Table 3.

Table 3. 9 Component groups in the ADC sample

ID	Mass (Da)	Retention Time Range
0	145,168	8.45-8.60 min
1	146,125	8.45-8.65 min
2	147.082	8.55-8.75 min
3	148,038	8.55-8.80 min
4	148,996	8.55-8.80 min
5	149,953	8.55-8.60 min
6	150,911	8.55-8.80 min
7	151,865	8.65-8.85 min
8	152,826	8.70-8.90 min

The top panel of Figure 7 shows the deconvoluted spectrum generated by the Sliding Window Algorithm for this data set. The bottom panel shows results from a conventional deconvolution for the same time range. The Sliding Window Algorithm performed significantly better, identifying ADCs at 151,866 and 151,827 Da that the conventional failed to detect.

FIGURE 7. Comparison between deconvoluted spectra generated by the Sliding Window Algorithm and conventional methods for an antibody sample.

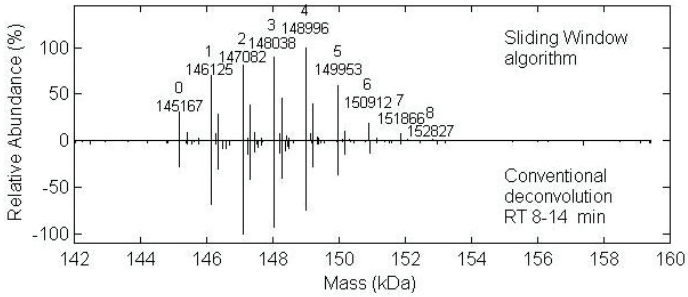
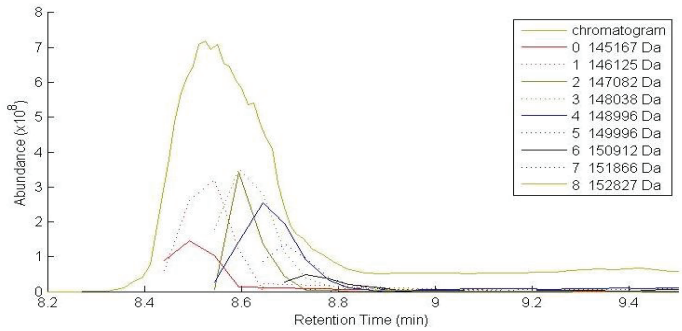


Figure 8 shows the original chromatogram and elution profiles generated by the Sliding Window Algorithm. The algorithm is able to distinguish between the 9 different components and generate unique profiles in a way that would be difficult using conventional techniques.

FIGURE 8. Chromatogram and elution profiles of 9 components identified by the Sliding Window Algorithm for an ADC sample



Conclusions

The Sliding Window Algorithm is a novel approach to identifying components in LC/MS data. This new algorithm has several advantages over the conventional approach:

- it avoids all the problems involved in trying to identify the complicated and ill-defined chromatographic peaks associated with large molecules,
- it can identify and characterize components that coelute at overlapping retention time ranges,
- it produces a meaningful elution profile for each components it identifies,
- it can dramatically reduce the rate of false positives.
- In many cases, it can increase sensitivity.

The Sliding Window Algorithm has been incorporated into Protein Deconvolution 4.0 software. It can be used to significantly improve the results of a charge state deconvolution. In the future, we will explore enhancements to this algorithm, such as improved noise peak rejection and the addition of a fitness measure to reduce incidence of false positives.

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POSTER NOTE

No. 64888

Versatile data processing software leverages Orbitrap data for intact and sub-unit mass analysis of protein biotherapeutics

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ABSTRACT

Intact protein analysis workflows benefit greatly from Orbitrap measurements, both in low resolution (average mass) spectra and high resolution (monoisotopic mass) spectra. A redesigned set of intact protein analysis tools for BioPharma Finder ™ 2.0 software allows new analytical capabilities for sub-unit and intact mAb mass analysis workflows.

INTRODUCTION

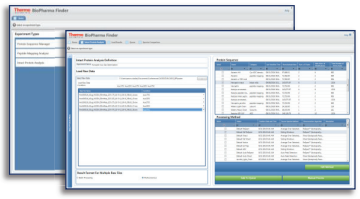
The increasing requirements in characterizing complex biotherapeutics for safety and efficacy place ever-increasing demands on analytical technologies and scientists. Therefore, high quality raw data must be converted into meaningful information. Data processing software is the key interface between high resolution accurate mass data and product knowledge. Intact and sub-unit mass analyses, routine assays in the biopharmaceutical industry, are important because these assays provide critical quality attributes (CQAs) and are usually among the first steps of biotherapeutic characterization. Here, we present three different examples on how data processing software can provide insight not only on product CQAs for antibody drug conjugates and monoclonal antibodies but also on how this software platform can be used to optimize raw data quality.

MATERIALS AND METHODS

The intact protein analysis side of BioPharma Finder software was redesigned to incorporate new functions such as automatic average drug-to-antibody ratio (DAR) and multiconsensus analysis (Figure 1). Raw files of Trastuzumab Emtansine were acquired under native conditions without removing the N-glycans. After ReSpect ™ deconvolution, masses corresponding to the combination of different DAR values and glycoforms were identified and a global average DAR of 3.65 was automatically calculated. The software offers the flexibility for users to calculate the average DAR ratio for specific glycoforms. Several raw files were acquired by varying the source settings. Raw files were processed in BioPharma Finder 2.0 using the multiconsensus option. A master table was created automatically and the mass accuracy and intensity of components can be compared between conditions. This table allows the user to quickly find the optimum source settings. Finally, a commercially available murine mAb test standard was stressed in an ammonium bicarbonate buffer (pH7.8) for either zero or four days at an elevated 37ºC temperature. Each sample was split in two. One half was subjected to trypsin proteolysis for peptide mapping purposes. The other was denatured and reduced for subunit analysis. All raw files obtained following HRAM LC-MS were processed using the sliding window feature combined with the Xtract ™ deconvolution algorithm. We found that high resolution accurate mass data showed deamidation at the sub-unit level, with approximately 1 ppm mass accuracy for both unmodified and deamidated species. Reconstructed chromatograms of the deconvolved components also showed a slight shift in the elution profile for deamidated vs. unmodified light chain subunits.

RESULTS

Figure 1. Intact Protein Analysis in BioPharma Finder 2.0 software



BioPharma Finder 2.0 software was redesigned and it now incorporates new functions such automatic average drug-to-antibody ratio (DAR) and multi consensus analysis.

Multiconsensus raw file comparisons

Five raw files were processed using the multiconsensus option to optimize the auxiliary gas temperature. The auxiliary gas temp was varied from 175ºC to 375ºC in individual SEC-MS runs (300 µL/min, 50 mM $NH_4CH_3CO_2$ (Figure 2). By looking at the raw data, the auxiliary gas at 375°C is probably too high but to differentiate the other conditions, the processed data will be have to be reviewed carefully.

Figure 2. Multiconsensus results table allows simultaneous visualization of both raw and deconvolution data. (A) Chromatogram, (B) raw spectrum. and (C) detail of m/z range of charge 24+ for different source auxiliary gas temperatures**.**

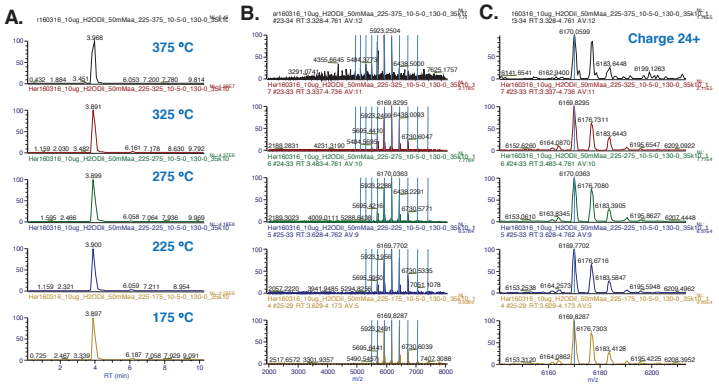


Figure 3. ReSpect score comparison of known glycoforms allows optimization.

Relative ReSpect score was calculated by dividing each ReSpect score by the average of all runs (each temperature)**.** The overall best auxiliary gas temperature is around 325°C.

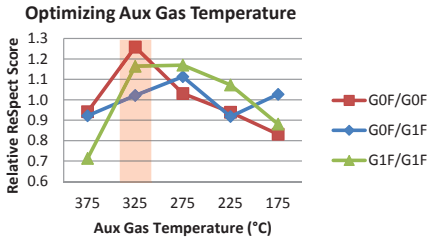
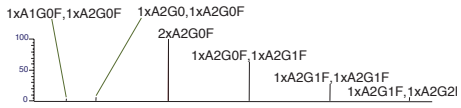


Figure 4. ReSpect deconvolution results of optimized native SEC-MS intact mAb analysis (A) Deconvolution spectrum. (B) Table of deconvolution results.

A.



B.

Glycoform	Mass Measured (Da)	Mass Accuracy (ppm)	Relative Abundance (% Intensity)
1xA1G0,1xA2G0F	147708.93	11.7	0.59
1xA2G0,1xA2G0F	147912.60	14.9	5.41
2xA2G0F	148057.19	4.5	100.00
1xA2G0F,1xA2G1F	148219.37	4.7	72.03
1xA2G1F,1xA2G1F	148381.74	6.2	23.36
1xA2G1F,1xA2G2F	148543.29	2.2	4.92

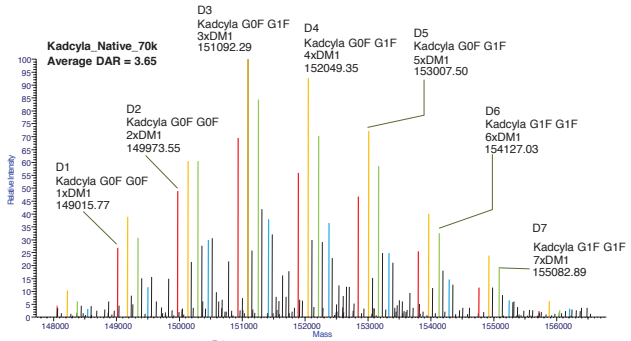
Automatic drug-to-antibody ratio calculation

After ReSpect deconvolution using the Sliding Window algorithm, the resulting masses were searched against the Trastuzumab amino acid sequence with the different glycoforms as fixed modification and the linker-drug mass (+957.53) as a variable modification. When a component is highlighted (ex: G0F/G1F +2 linker-drugs) in the master table, an abundance trace is created on the chromatogram window and data used for deconvolution are identified with blue bars in the raw spectrum. A blue bar is also present in the deconvoluted spectrum to mark the selected component. Average DAR value is calculated automatically and the software offers the flexibility for users to calculate the average DAR ratio for specific glycoforms.

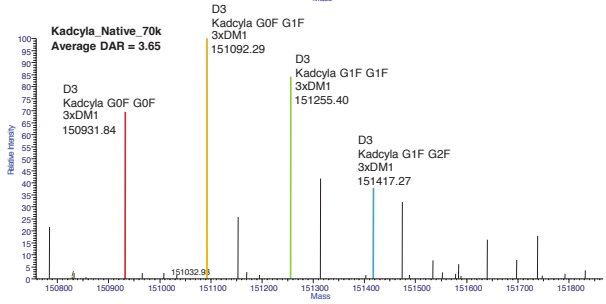
Figure 5. Data Vizualization in BioPharma Finder 2.0. (A) Comparison of raw data and deconvolution results. (B) Setting refercence modification for DAR measurement. (C) DAR measurement results panel.

Figure 6. Automated DAR calculation allows use of multiple reference masses to generate high confidence. (A) Full spectrum of deconvoluted masses of Trastuzumab Emtansine show a broad distribution of linker-drug additions. (B) A detail of D3 forms shows 4 abundant glycoforms. (C) Average DAR value calculated using 4 glycoforms after processing a raw file of Trastuzumab Emtansine is in agreement with previous measurements **(Ref 1)**.

A.



B.



C.

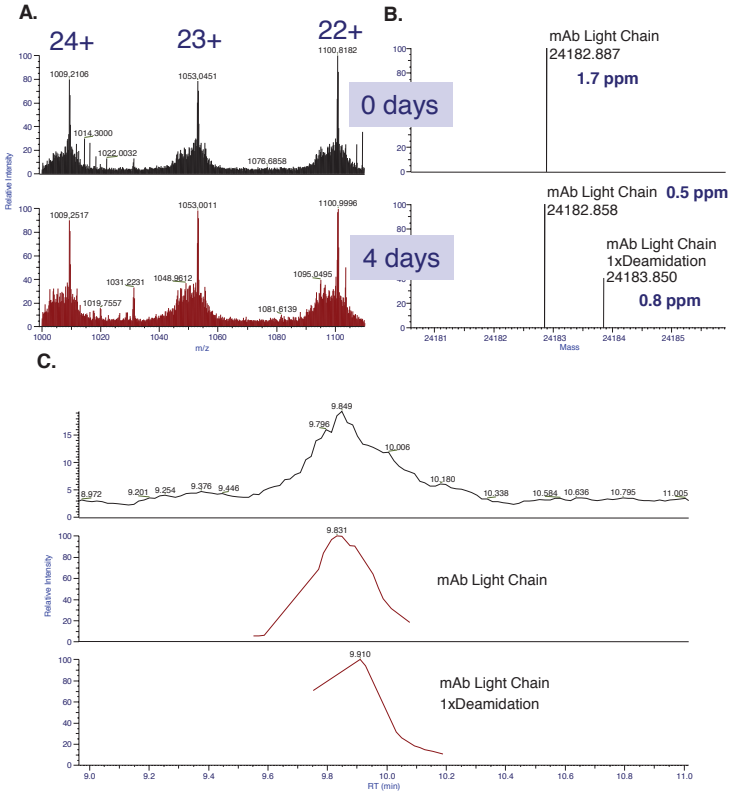
Glycoform	G0F/G0F	G0F/G1F	G1F/G1F	G1F/G2F	Top 4 avg
DAR	3.58	3.71	3.64	3.65	3.65

True intact analysis using a native MS approach allows measurement of DAR ratio without need for deglycosylation. This approach offers a faster, more direct route to analysis, in which we remove the possibility of artifacts due to sample preparation. Also with one LC-MS run, four independent average DAR values are calculated, increasing the confidence level of the average DAR value.

Sub-unit deamidation analysis using Sliding Window deconvolution

Deamidation can be measured using isotopic resolution of proteins or protein sub-units **(Ref 2)**. We incubated a monoclonal antibody sample in alkaline conditions for 4 days and analyzed the sample using a shallow reverse phase gradient (23-30% ACN in 10 min) and a selected ion monitoring (SIM) MS method (R=280,000). Xtract deconvolution showed that sample treatment resulted in observation of a new mass corresponding to deamidation of light chain. These data are easily visualized in multiconsensus, where the 0 and 4 day time points can be compared.

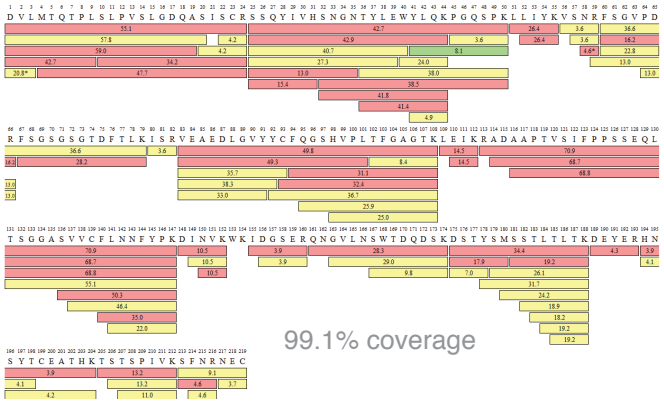
Figure 7. Xtract deconvolution of deamidated mAb Light Chain.
(A) Data were collected using a SIM method which acquired data on three charge states of the mAb light chain. (B) Xtract deconvolution using the Sliding Window feature detected a new species corresponding to deamidation of light chain at ~40% intensity. (C) Abundance traces of the deconvolved components showed a slightly shifted elution profile of the deamidated species.



To confirm our sub-unit analysis results we performed a trypsin peptide mapping analysis of the mAb light chain. Triplicate analysis of each sample type resulted in coverage of 99.1% of the sequence. We found 2 sites of deamidation; N33 and N162. Deamidation was present at a low level (~1%) at site N33 and in moderate amounts (~23%) at site N162 in the untreated sample (Figure 8). After 4 days of treatment deamidation levels increased a total of approximately 50% (~5% at N33 and 68% at N162). As we did not measure any deamidation in the untreated sub-unit analysis we suspect that our trypsin digestion protocol is responsible for the moderate amounts of deamidation present at site N162 in the peptide mapping results.

Figure 8. Peptide mapping confirmation of subunit-level deamidation results

A.



B.

	0 days			4 days		
Light Chain deamidation site	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
N33	0.80%	0.92%	0.84%	6.22%	5.44%	5.20%
N162	23.23%	23.02%	23.74%	67.65%	68.25%	68.79%

CONCLUSIONS

- BioPharma Finder 2.0 allows new analytical capabilities for intact protein analysis workflows
- Multiconsensus view allows cross-comparison of deconvolution results from multiple raw files
- Automatic DAR calculation allows use of multiple reference species to confidently measure ADCs
- Sliding Window algorithm produces abundance trace of deconvolved components which helps sensitive determination of new species.

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2. Zabrouskov V, Han X, Welker E, Zhai H, Lin C, van Wijk KJ, Scheraga HA, McLafferty FW. Stepwise deamidation of ribonuclease A at five sites determined by top down mass spectrometry. Biochemistry. 2006 Jan 24;45(3):987-92.

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PN64888-EN 11/16S

Poster Note 72101

New Integrated Informatics Solution for Protein Biotherapeutics Characterization

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Overview

Purpose: Integrated informatics solution for protein biotherapeutics characterization. Two software packages Thermo Scientific™ Protein Deconvolution and Thermo Scientific™ PepFinder™ software are integrated into one software platform.

Methods: Intact and sub-unit mass analysis and peptide mapping were performed to characterize trastuzumab. The new Thermo Scientific™ BioPharma Finder™ software was used for data processing.

Results: One integrated software solution was used for full characterization of trastuzumab. Intact protein mass was determined and all major glycoforms were identified using the intact protein analysis (Protein Deconvolution). Sub-unit analysis used a novel sliding window algorithm which improves peak detection in complex mixtures, to generate deconvoluted masses for the Fc/2, Fd and light chain molecules. A simulated stress study is automatically processed using the peptide mapping workflow (PepFinder software) where expected modifications are identified and a relative amount is determined automatically. New visualization for data mining and data interpretation enable both expert and beginner users to be successful with this new software while providing more confident results.

Introduction

Increasing requirements to fully characterize complex protein biotherapeutics for safety and efficacy place analytical scientists under pressure. In spite of this, the discovery and development of protein biotherapeutics continues to thrive and demands faster and better tools. Here we present a new, powerful software that can leverage chromatographic separations and High Resolution Accurate Mass (HRAM) analysis for the characterization of biotherapeutics.

Methods

Sample: Trastuzumab was used for both intact analysis and tryptic peptide mapping analysis.

LC: Thermo Scientific™ Vanquish™ UHPLC system

Column: Thermo Scientific™ MAbPac™ RP, (50 mm * 2.1 mm; 4 mm)

Thermo Scientific™ Accucore™, C18 (100mm * 2.1 mm; 1.7 mm)

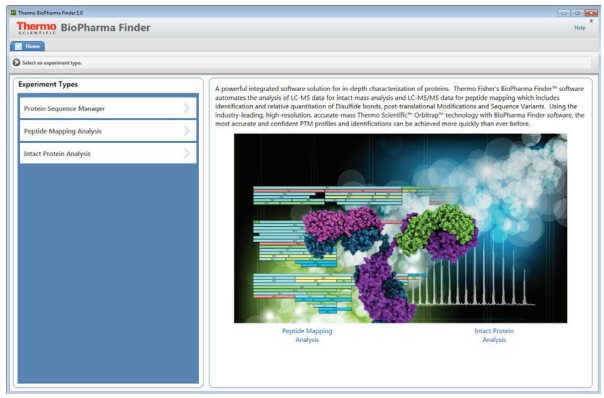
Mass Spectrometry: Thermo Scientific™ Orbitrap Fusion™ Tribrid™ MS

Data Analysis: Raw files were processed with Thermo Scientific™ BioPharma Finder software.

Results

BioPharma Finder software allows users to organize and store protein sequences with the Protein Sequence Manager and then the user can attach the sequence to the intact and peptide mapping methods (Figure 1). This simple interface allows users to easily navigate through the software seamlessly from one workflow to another.

FIGURE 1. BioPharma Finder software homepage



For the intact protein and sub-unit analyses, Trastuzumab was analyzed using the Orbitrap Fusion MS at 17K and 120K resolutions respectively. The deconvolution processing method used the ReSpect™ algorithm for the intact protein and Xtract™ algorithm was used for sub-unit study. MS scans were processed as “static” for the intact or “sliding window” for the sub-unit. All major glycoforms were identified and annotated. Average masses, sum intensities, matched delta masses and identification as well as abundances are reported in an exportable table. Sub-unit raw file was processed automatically using the sliding window tool eliminating the need to define manually the time range for each chromatographic peak.

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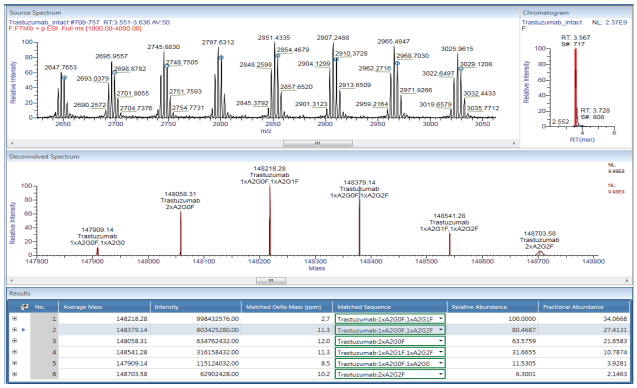
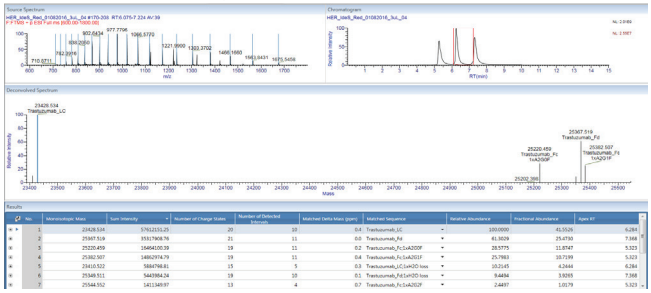
FIGURE 2. Intact mass analysis of Trastuzumab.



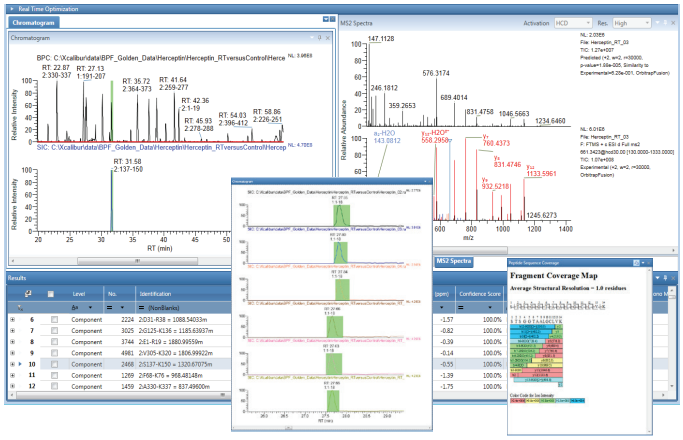
Displayed in the software is the source mass spectrum used for deconvolution with interactive graphics for manual interrogation of the results, chromatogram for reviewing elution profile, interactive deconvoluted spectrum and a results table which contains all of the relevant information.

FIGURE 3. Sub-unit analysis of Trastuzumab.



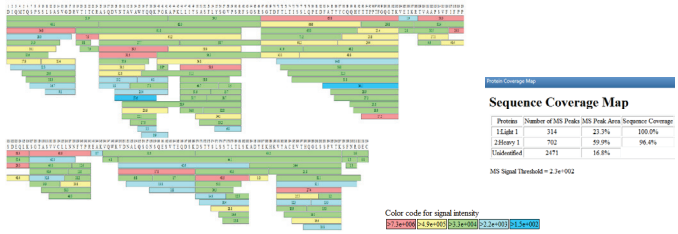
The width and the overlap between consecutive sliding window boxes for deconvolution is user defined. During processing the box will move across the chromatogram providing real time visualization.
All of the sub-units were identified with a delta mass error below 1ppm.

FIGURE 4. Peptide Mapping analysis of Trastuzumab. Main process & review page in BioPharma Finder software with interactive plots and tables for user friendly data mining.



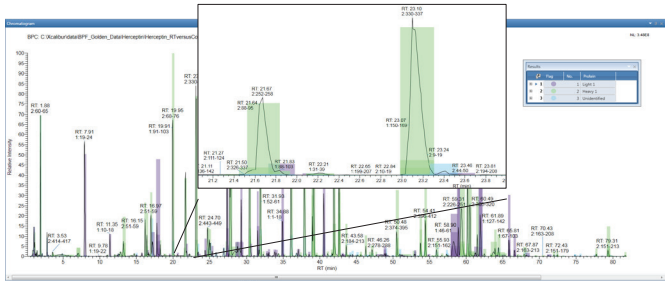
A “stress” sample was generated by storing at room temperature for 48 hrs a tryptic digest sample. For peptide mapping analysis, raw files were divided in two groups, control and stress. Data were acquired using an Orbitrap Fusion mass spectrometer. Data processing was performed using the peptide mapping workflow in BioPharma Finder software. The core algorithms for peptide mapping are from the PepFinder software. However, BioPharma Finder software has an updated user interface which provides a significantly improved user experience for data mining and data interpretation. Figure 4 shows the process & review page of the peptide mapping workflow. The main page has an interactive table, chromatogram plotting capability with 6 different types of plots and the ability to stack chromatograms from multiple raw files allowing the user to view peptides across samples. All of the plots and tables can be floated on the screen or moved to a second monitor for custom layout.

FIGURE 5. Sequence coverage map.



The sequence coverage map is automatically generated and provides a visual display of the depth of identification that is achieved in the peptide mapping workflow. Each peptide is colored based on abundance and a summary report is generated for each data file.

FIGURE 6. Novel chromatographic shading plot.



A new feature in BioPharma Finder software peptide mapping workflow is shown in Figure 6. The basic peak chromatogram is shaded using different colors based on the protein identification. The purple are peptides from the light chain, green are from heavy chain and blue are unidentified. This interactive plot provides the user an image where they can quickly see non-identified peaks, which peptides are the most abundant and displays co-eluting peptides.

FIGURE 7. Modification table for the oxidation on W420 of the heavy chain.

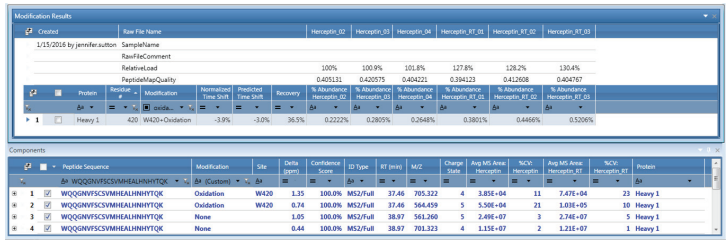


Figure 7 is a dispay of the new and improved modification summary report which was part of PepFinder software. This report is now an interactive report in which the user can select a specific modification on the top table and see the components used in the abundance calculation. Normalized time shift, a new feature, is the comparison of the modified and non-modified peptides retention times. The normalized time shift can be compared to the predicted time shift providing an extra level of confidence for the identification.

Conclusions

BioPharma Finder software provides:

- Confident deconvoluted molecule weight of proteins in denaturing and native conditions.
- Extra confidence in peptide identification by using a novel MS/MS predictive algorithm.
- Quantification of modifications
- Characterization of disulfide linkages
- Low level impurities - sequence variants identification
- Sequence alteration – stress samples, level of deamidation or oxidation

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BIOPHARMA APPLICATION COMPENDIUM

Glycan Analysis

- Sample preparation
- Monosaccharide and sialic acid determination
- Structural characterization

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Analysis of Human IgG Glycans on a Solid Core Amide HILIC Stationary Phase

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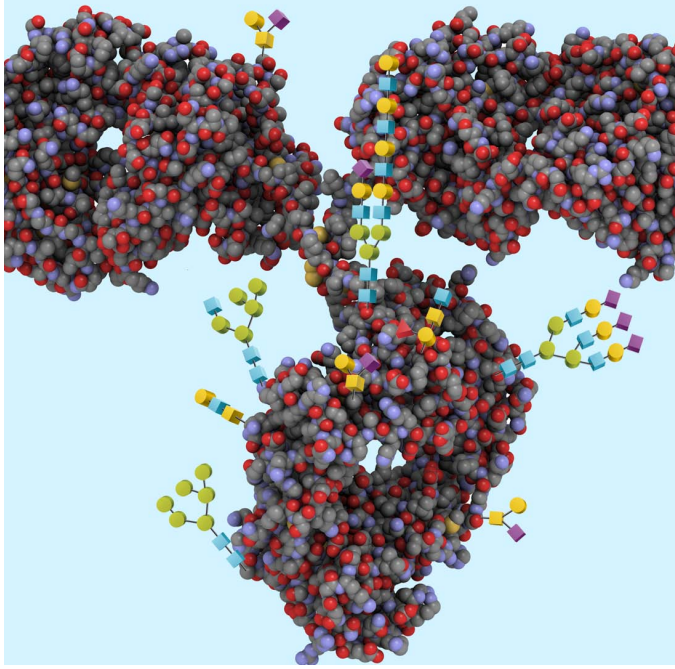
Key Words
Accucore 150-Amide-HILIC, Core Enhanced Technology, solid core, amide HILIC, glycans, human IgG, HILIC

Abstract
This application note demonstrates the analysis of human IgG glycans labeled with a fluorescent tag (2-aminobenzamide) by hydrophilic interaction liquid chromatography (HILIC). The separation is carried out with a Thermo Scientific™ Accucore™ 150-Amide-HILIC 150 Å pore diameter solid core HPLC column. The method displays excellent separation at backpressures compatible with conventional HPLC systems.

Introduction
Glycans are oligosaccharides and polysaccharides found on proteins and cell surfaces. These entities play fundamental roles in cellular function by creating a fingerprint tag for the protein they are bound to, which in turn affects cellular activity. Glycans are often key biomarkers for disease states such as cancer. Due to the branching of the chains and post-translational modifications, their structures are very complex. Minor changes in glycan structure can result in dramatic differences in cell function.

It is crucial when analyzing glycans to be able to efficiently separate all isomeric and branching variants present within the sample to achieve maximum structural elucidation. The polarity of the fragments, however, often presents a challenge to chromatographic retention and separation. Smaller fragments are often too polar to be retained by conventional reversed phase methods, and the absence of ionizable groups on many glycans (such as the IgG glycans analyzed here) reduces the usefulness of ion exchange chromatography. For glycans that contain ionizable groups, a mixed mode HILIC/anion exchange column like the Thermo Scientific™ GlycanPac™ AXH-1 column demonstrates a complementary solution for the analysis of charged glycans [1]. Hydrophilic interaction liquid chromatography (HILIC) features increased retention of polar species and has been shown to give good retention of oligosaccharides.

Antibodies have rapidly become a target for bio-pharmaceutical research; accurate structural elucidation is required to control their functioning for the development



of bio-therapeutic drugs. In this application note the performance of an Accucore 150-Amide-HILIC HPLC column for the chromatographic separation of human IgG glycans labeled with a fluorescent tag (2-aminobenzamide) is demonstrated.

Accucore HPLC columns use Core Enhanced Technology™ to facilitate fast and highly efficient separations. The 2.6 µm diameter particles are not totally porous, but instead have a solid core and a porous outer layer. The optimized phase bonding creates a series of high coverage, robust phases. The tightly controlled 2.6 µm diameter of Accucore particles results in much lower backpressures than typically seen with sub-2 µm materials. The Accucore 150-Amide-HILIC HPLC phase is designed for the separation of hydrophilic bio-molecules. Hydrophilic interaction liquid chromatography (HILIC) features a partitioning mechanism from an aqueous layer created by water molecules adsorbed on the media surface. Polar analytes are therefore retained in the water

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layer. Additionally, the amide bonded phase on the Accucore 150-Amide-HILIC HPLC column interacts with hydroxyl groups in the analytes via hydrogen bonding, and the larger pore diameter optimizes performance for larger bio-molecules.

Experimental Details

Consumables	Part Number
Fisher Scientific™ HPLC grade water	W/0106/17
Fisher Scientific HPLC grade acetonitrile	A/0627/17
Fisher Chemical™ ammonium formate	A/8050-15
Fisher Scientific™ Optima™ grade formic acid	A117-50

Vials and Closures

Thermo Scientific™ Chromacol™ 9 mm screw thread vial 200 µL, Fused insert-GOLD grade glass(02-FISVG) to be used in conjunction with Thermo Scientific Chromacol 9 mm open top short screw cap 6 mm hole (9-SC(B)-ST1). The high purity glass used for these vials results in extremely low concentration of active sites, therefore minimizing adsorption of basic or highly polar analytes that would otherwise interact with conventional glass surfaces.

Separation Conditions			Part Number
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC HPLC System equipped with a Thermo Scientific Dionex FLD fluorescence detector		
Column:	Accucore 150-Amide-HILIC, 2.6 µm, 100 × 2.1 mm		16726-102130
Mobile phase A:	Acetonitrile		
Mobile phase B:	50 mM ammonium formate pH 4.4 (prepared from LS-N-BUFFX40, Ludger Ltd)		
Gradient:	Time (min)	% B	
	0	20	
	26	40	
	27	50	
Flow rate:	1 mL/min		
Column temperature:	60 °C		
Backpressure:	300 bar		
Injection details:	5 µL in water, 50 µL loop		
Injection wash solvent:	Acetonitrile / water (78:22 v/v)		
Excitation wavelength:	330 nm		
Emission wavelength:	420 nm		

Sample Preparation

2AB labeled IgG glycans (CAB-IgG-01, Ludger Ltd) were diluted to approximately 2 nmol/mL concentration in HPLC grade water.

Data Processing

Software:	Thermo Scientific™ Chromeleon™ 7 software

Results

A sample of human IgG glycans was analyzed on an Accucore 150-Amide-HILIC HPLC column. The chromatography is shown in Figure 1 with the details of the labeled peaks contained in Table 1. Symmetrical peak shape and excellent separation of the glycans are observed.

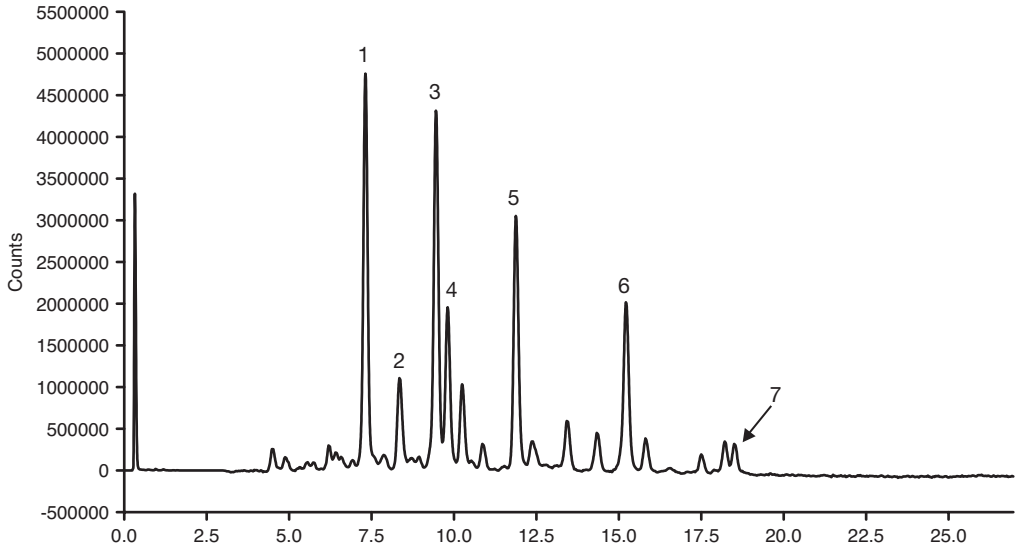

Figure 1: Chromatographic separation of human IgG glycans using an Accucore 150-Amide-HILIC HPLC column

Peak Number	Glycan
1	FA2
2	FA2B
3	FA2G1 (6 arm)
4	FA2G1 (3 arm)
5	FA2G2
6	FA2G2S1
7	FA2G2S2

Table 1: Peak identification for human IgG glycan labeled peaks

Conclusion

- The analysis of 2AB labeled human IgG glycans has been achieved on an Accucore 150-Amide-HILIC HPLC column. The analysis is simple and robust, leading to the separation and detection of 7 individual glycan peaks.
- The solid core technology allows for a highly efficient separation with a system backpressure compatible with conventional HPLC systems (300 bar at gradient apex).
- The Accucore 150-Amide-HILIC column efficiently retains and separates hydrophilic biomolecules.

Reference

[1] Thermo Scientific GlycanPac AXH-1 Product Specification: http://www.dionex.com/en-us/webdocs/114170-PS-GlycanPac-AXH1-Column-PS20695_E.pdf

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Separation of 2AB-Labeled *N*-Linked Glycans from Bovine Fetuin on a Novel Ultra High Resolution Mixed-Mode Column

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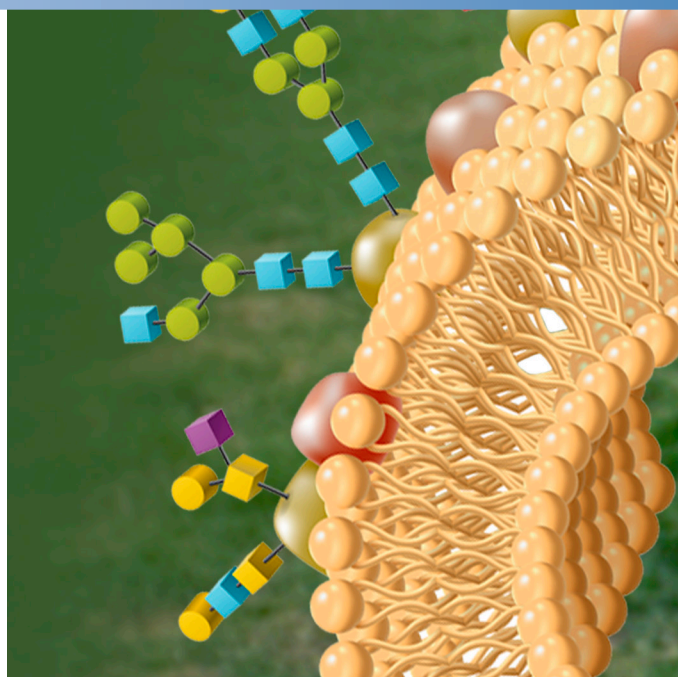
Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words
GlycanPac AXR-1, charge based separation, isomeric separation, mixed-mode chromatography, 2AB-labeled *N*-linked glycans, bovine fetuin, glycan analysis

Abstract
This application note demonstrates the separation of 2AB-labeled *N*-linked glycans released from proteins (bovine fetuin) by reversed phase (RP) / weak anion exchange (WAX) mixed-mode chromatography. The separation is carried out with both 1.9 µm and 3 µm Thermo Scientific™ GlycanPac™ AXR-1 columns using fluorescence detection. The method exhibits an excellent separation based on charge, isomeric structure and size.



Introduction
Glycans are involved in a wide range of biological and physiological processes including cell and pathogen recognition, regulatory functions, cellular communication, gene expression, cellular immunity, growth and development. The functions of proteins are often dependent on the structure and types of their attached oligosaccharides. *N*-linked glycans are commonly investigated as important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficacy are altered by glycosylation. Understanding, measuring and controlling glycosylation in glycoprotein- based drugs, glycoprotein products and biosimilars is increasingly important. However, glycan structures are highly diverse, complex and heterogeneous due to branch and linkage isomers that make comprehensive glycan characterization challenging [1].

Various HPLC separation modes have been developed for glycan analysis, including normal phase (or hydrophilic interaction, HILIC), ion-exchange and reversed-phase [2,3]. Because glycans are very hydrophilic (polar) a common separation mode utilizes amide HILIC columns; as exemplified by the Thermo Scientific™ Accucore™ 150-Amide-HILIC column [4], that resolves glycans based on hydrogen bonding, resulting in size and composition-based separations. Amide HILIC columns are particularly useful for the separation of 2AB-labeled *N*-linked glycans released from antibodies, for example MAbs, in which the majority of glycans harbor no charge. However, Amide HILIC amide columns do not provide adequate separations when glycans harbor 2 or more charge states (e.g., neutral and mono- or di-sialylated *N*-linked glycans) because glycan isoforms with different charge states are intermingled in the separation envelope.

Recently we developed a mixed-mode column (Thermo Scientific™ GlycanPac™ AXH-1) with both weak anion-exchange (WAX) and hydrophilic interaction (HILIC) properties [5] which separates *N*-linked glycans based on charge, polarity, and size. The GlycanPac AXH-1 provides improvements to the amide HILIC phases due to its charge-based separation. This column enjoys broad applicability for qualitative, quantitative and structural analysis of both labeled (2AB- and 2AA-) and native *N*-linked glycans from proteins using fluorescence and/or mass spectrometry (MS) detection [6]. Here we describe the new GlycanPac AXR-1 mixed-mode column that further improves separations by resolving glycans into different charge groups, and also separates glycans within

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each charge group based on isomerization and size, producing substantially increased resolution of *N*-linked glycans.

The GlycanPac AXR-1 column is based on novel mixed-mode column chemistry, combining the retention mechanisms of both WAX and reversed phase (RP) properties for optimal selectivity and resolution. The WAX functionality provides retention and selectivity for negatively charged glycans, while the reversed phase mode facilitates the separation of glycans of the same charge according to their isomeric structure, and size. As a result the GlycanPac AXR-1 column provides industry-leading resolution of charged 2AB-labeled *N*-linked glycans. The GlycanPac AXR-1 column is designed for, and tested with, LC-fluorescence detection and LC-MS applications using volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) with, or without acetonitrile. The substrate of the GlycanPac AXR-1 column is a modified high-purity spherical silica. The column is available in both 1.9 µm particle size for UHPLC and 3.0 µm particle size for HPLC applications.

Experimental Details

Consumables	Part Number
Deionized water, 18.2 MΩ-cm	
Fisher Scientific HPLC grade acetonitrile	AC610010040
Fisher Scientific LC-MS grade formic acid	A117-50
Fisher Scientific ammonium formate	AC401152500
Unlabeled non-reduced glycans are released from glycoproteins with PNGase F enzyme (New England BioLabs, P0705L). The released glycans are conjugated with the 2-amino benzamide (2-AB) label group with slight modification from the reported procedure of Bigge et. al. [7]	
Fisher Scientific 2-Amino benzamide	AC 10490-1000
Fetiun *N*-linked glycan library, labeled with 2AB	Prozyme GKSB-002

Buffer Preparation

Ammonium formate (100 mM, pH 4.4): Dissolve 6.35 ± 0.05 g ammonium formate and 0.70 ± 0.05g formic acid in 999.6 g of D.I. water. Confirm pH to 4.4 using a pH meter. Mix this solution well and filter through a 0.2 µm pore filter.

Sample Preparation

Dissolve 2AB-labeled *N*-linked glycans from fetuin or individually labeled standards (approximately 5000 pmol each) in 100 µL D.I. water in a 250 µL autosampler vial. Samples are ready for injection. Inject 1–5 µL.

Note: store the standard at -20 oC

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC Analytical LC system consisting of DGP-3600RS pump, TCC-3000RS thermal compartment, WPS-3000TRS autosampler, FLD3400RS fluorescence detector (with Dual-PMT) and a biocompatible 2 µL micro flow cell (6078.4330)

Separation Conditions for Figure 1

Column:	GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm)
Mobile Phase:	A: D.I. water
	B: Ammonium formate (100 mM, pH 4.4)
Flow rate:	400 µL/min
Initial pressure:	~300 bar with new column
Column temperature:	30 °C
Sample amount:	100 pmoles
Fluorescence detector:	λ_{Ex} = 320 nm & λ_{Em} = 420 nm

Separation Conditions for Figure 2	
Column:	GlycanPac AXR-1 (1.9 µm, 250 × 2.1 mm)
Mobile Phase:	A: Acetonitrile
	B. D.I. water
	C: Ammonium formate (100 mM, pH 4.4)
Flow rate:	400 µL/min
Initial pressure:	~500 bar with new column
Column temperature:	30 °C
Samples amount:	100 pmoles
Fluorescence detector:	λ_{Ex} = 320 nm & λ_{Em} = 420 nm

Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results

The GlycanPac AXR-1 column is designed for high-resolution separation of charged and neutral glycans present in glycoproteins, glycolipids and glycopolymers. It is noted that the GlycanPac AXR-1 works exceptionally well for charged *N*-linked glycan species.

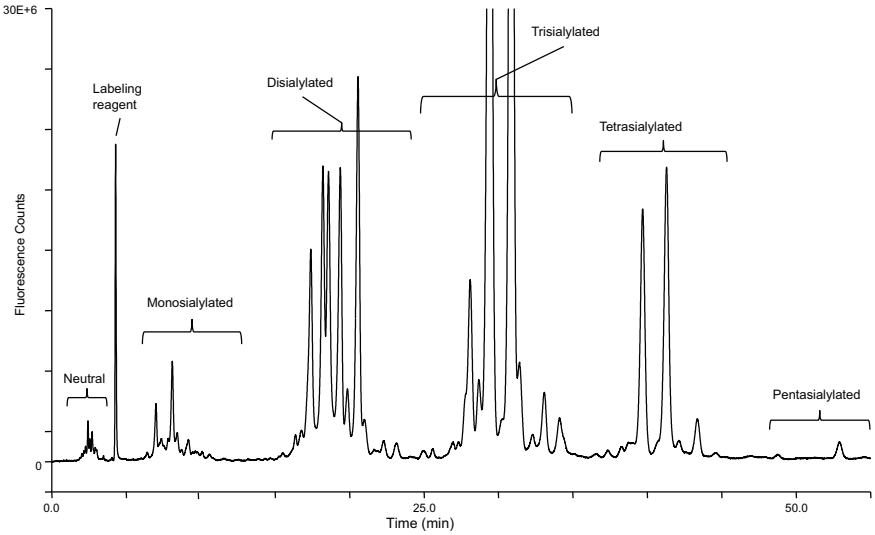

Figure 1: Separation of 2AB labeled *N*-linked glycans from bovine fetuin by charge, size, polarity and isomeric structure using the GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm) column and a binary gradient

Time (min)	%A	%B
-10	93	7
0	93	7
80	0	100
84	0	100

Table 1: Binary gradient for GlycanPac AXR-1 150 × 2.1 mm column (1.9 µm)

Figure 1 shows the separation of 2AB-labeled *N*-linked glycans from bovine fetuin using a GlycanPac AXR-1 (1.9 µm; 150 × 2.1 mm) column with a binary gradient (Figure 1, Table 1). The glycan elution profile consists of a series of peaks grouped into several clusters with neutral glycans eluting first, near the void, followed by monosialylated, disialylated, trisialylated, tetrasialylated and finally pentasialylated species. Peaks in each cluster represent the glycans of the same charge, grouped by ion exchange interaction. Within each cluster glycans with the same charge are further separated according to their isomeric structures and size by reversed phase interaction. *N*-linked glycan structures present in each peak were identified using LC-MS/MS (data not shown). Using the binary gradient the 150 × 2.1 mm GlycanPac AXR-1 (1.9 µm) resolved more than 70 components from the 2AB-labeled bovine fetuin *N*-linked glycans.

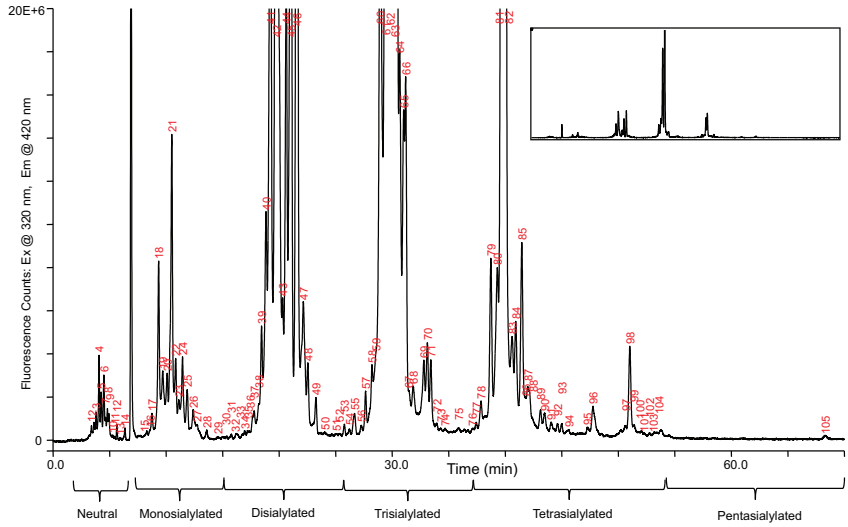

Figure 2: Improvements to resolution of fetuin 2AB-labeled *N*-linked glycans on a longer, 250 × 2.1 mm GlycanPac AXR-1 column. Inset is full Chromatogram

Time (min)	%A	%B	%C
-10	0	93	7
0	0	93	7
90	25	0	75
90.5	0	0	100

Table 2: Ternary gradient for 250 × 2.1 mm GlycanPac AXR-1 column (1.9 µm)

The effect of column length and a ternary gradient program (Table 2) are shown in Figure 2, where the 250 × 2.1 mm GlycanPac AXR-1 revealed at least 105 glycan-containing peaks in less than 70 minutes. In the 250 mm column, acetonitrile starts at 0% and increases over 90 minutes to 25 %. The longer column also results in more efficient peaks, thus increasing detection sensitivity for glycans.

Conclusion

- The GlycanPac AXR-1 is designed to provide high- resolution separations for biologically important complex glycans based on charge, isomeric structure and size.
- The GlycanPac AXR-1 column provides exceptional selectivity and resolution for 2AB-labeled *N*-linked glycans from bovine fetuin.
- The GlycanPac AXR-1 accomodates injection of fully aqueous samples as native or 2AB-derivatized *N*-linked glycans.

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Separation of 2AB Labeled N-Glycans from Bovine Fetuin on a Novel Mixed-Mode Stationary Phase http://www.separatedbyexperience.com/documents/AN-LC-GlycanPac-N-Glycans-Bovine-Fetuin-AN20754_E.pdf

Structural Analysis of Native N-Glycans Released from Proteins Using a Novel Mixed-Mode Column and a Hybrid Quadrupole-Orbitrap Mass Spectrometer http://www.separatedbyexperience.com/documents/AN-LC-GlycanPac-Native_N-Glycans-Bovine-Fetuin-AN20827_E.pdf

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Application Note 20910

Separation of 2AA-Labeled *N*-Linked Glycans from Glycoproteins on a High Resolution Mixed-Mode Column

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Key Words

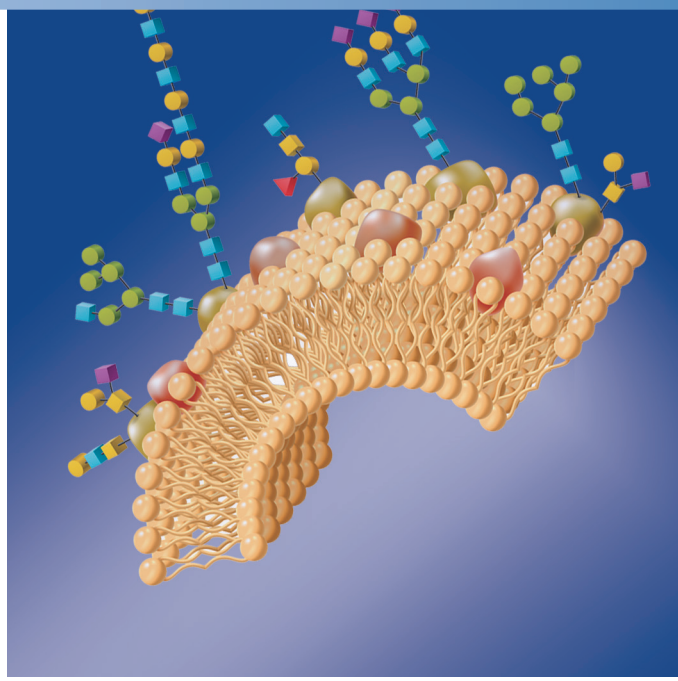
GlycanPac AXR-1, mixed-mode chromatography, *N*-linked glycans, glycoproteins, reversed-phase, anion-exchange, 2AA-labeled *N*-linked glycans, bovine fetuin, isomeric separation, charge based separation, glycan analysis

Abstract

This application note demonstrates the separation of 2AA-labeled *N*-linked glycans released from bovine fetuin using a reversed-phase / weak anion-exchange mixed-mode column (Thermo Scientific™ GlycanPac™ AXR-1, 1.9 µm, 150 × 2.1 mm) with fluorescence detection. The method exhibits excellent separation of glycans based on charge, isomeric structure and size.

Introduction

Glycosylation is a common post-translational modification (PTM) on proteins, and is often found on therapeutic proteins [1]. In fact, most of the protein pharmaceutical candidates in preclinical and clinical development are glycosylated. The efficacy of recombinant protein based drugs (e.g. erythropoietin [EPO] and Follicle-Stimulating Hormone [FSH]) is often dependent on the structure and the types of glycans attached to the protein [2]. Further, protein glycosylation is a prime source of therapeutic protein heterogeneity with respect to both structure and function, and variation in glycosylation is one of the main factors in product batch-to-batch variation. This affects product stability *in vivo* and significantly influences biological activity, pharmacokinetics, and clearance, as well as immunogenicity [2,3]. Glycan structures are diverse, complex and heterogeneous. Variation in glycosylation can be attributed to several factors including the type of cell in which the glycoprotein is produced as well as processes involved in cell culture, purification, formulation, and storage. Thus, understanding the structure of glycans in proteins provides detailed information necessary for control of reproducible production during development and manufacturing of clinically useful proteins. Structural characterizations of glycans, including monosaccharide composition, linkage and branch isomerism, charge, and size variations are essential for bio-therapeutics and bio-pharmaceutical projects [3].



Various HPLC separation modes have been developed for glycan analyses, including normal phase or hydrophilic interaction (HILIC), ion-exchange, and reversed-phase (RP) [4]. Because glycans are very hydrophilic (polar), a common separation mode employs amide-HILIC columns; as exemplified by the Thermo Scientific™ Accucore™ 150-Amide-HILIC column, that resolves glycans by hydrogen-bonding, producing a size and composition-based separation. Amide-HILIC columns are particularly useful for the separation of 2-aminobenzamide-(2AB) labeled *N*-linked glycans released from antibodies, for example MAbs, in which the majority of glycans harbor no charge. However, amide-HILIC columns do not provide adequate separations where glycans that harbor 2 or more charge states are present (e.g., neutral and sialylated glycans) because glycan isoforms with different charge states co-elute in the separation envelope.

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Recently we developed a mixed-mode column (GlycanPac AXH-1) with both weak anion-exchange (WAX) and hydrophilic interaction (HILIC) properties [5, 6] which separates *N*-linked glycans based on charge, polarity, and size. The GlycanPac AXH-1 improves characterization of charge states (sialylation) compared to the amide-HILIC phases. The GlycanPac AXH-1 separations support broad applicability for qualitative, quantitative and structural analysis of both labeled (2AB and 2AA) and unlabeled *N*-linked glycans from proteins using fluorescence and/or mass spectrometry (MS) detection [5]. This is particularly useful for antibodies (including human IgG).

Here we describe the new GlycanPac AXR-1 mixed-mode column that further improves separations by resolving glycans into different charge groups, and separating glycans within each charge group based on isomerization and size. This substantially increases resolution of complex *N*-linked glycan structures, and helps differentiate isomeric structures not resolved by other approaches. This application note demonstrates the separation of 2AA-labeled *N*-linked glycans from bovine fetuin on the GlycanPac AXR-1 column using both binary and ternary gradient eluent systems.

Experimental Details

Consumables	Part Number
Deionized (D.I.) water, 18.2 MΩ-cm resistivity	
Fisher Scientific acetonitrile HPLC grade	AC610010040
Fisher Scientific LC-MS grade formic acid	A117-50
Fisher Scientific ammonium formate (≥99 %)	AC-401152500
Thermo Scientific Premium 2 mL vial convenience kit	60180-600
PNGase F, New England BioLabs	P0705L
Bovine fetuin	
Fisher Scientific glacial acetic acid	AA36289AP

Buffer Preparation

Ammonium formate (100 mM, pH 4.4): Dissolve 6.35 ± 0.05 g of ammonium formate and 0.70 ± 0.05 g of formic acid in 999.6 g of D.I. water. Mix the eluent well and filter through a 0.2 µm pore filter.

Sample Preparation

Dissolve 2AA-labeled *N*-linked glycans from bovine fetuin or individual labeled standards (approximately 5000 pmol each) in 100 µL D.I. water in a 250 µL autosampler vial, Thermo Scientific 055428.

Inject 1–5 µL to introduce 100 pMol per injection.

Note: store the standard at -20 oC

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system, including pump: LPG-3400RS, thermal compartment, TCC-3000RS, split-loop well plate auto sampler: WPS-3000TRS, fluorescence detector with Dual-PMT: FLD3400RS, flow cell: 2 µL micro flow cell: 6078.4330

Separation Conditions	
Column:	Thermo Scientific™ GlycanPac™ AXR-1 column (1.9 µm, 150 × 2.1 mm)
Mobile Phase:	A: Acetonitrile
	B: D.I. Water
	C: Ammonium formate (100 mM, pH =4.4)
Flow rate:	400 µL/min
Column temperature:	30 °C
Injection volume:	1–5 µL
Sample Amount:	100 pmoles
Samples:	2AA-labeled *N*-linked glycans from bovine fetuin
Fluorescence detector:	λ_{Ex} = 320 nm & λ_{Em} = 420 nm

Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results

The GlycanPac AXR-1 column is used for qualitative and structural characterization of neutral and charged glycans present in glycoproteins. For this Application Note the *N*-linked glycans were released from bovine fetuin by PNGase-F treatment and labeled using a modification of the procedure detailed by Bigge et.al, [7].

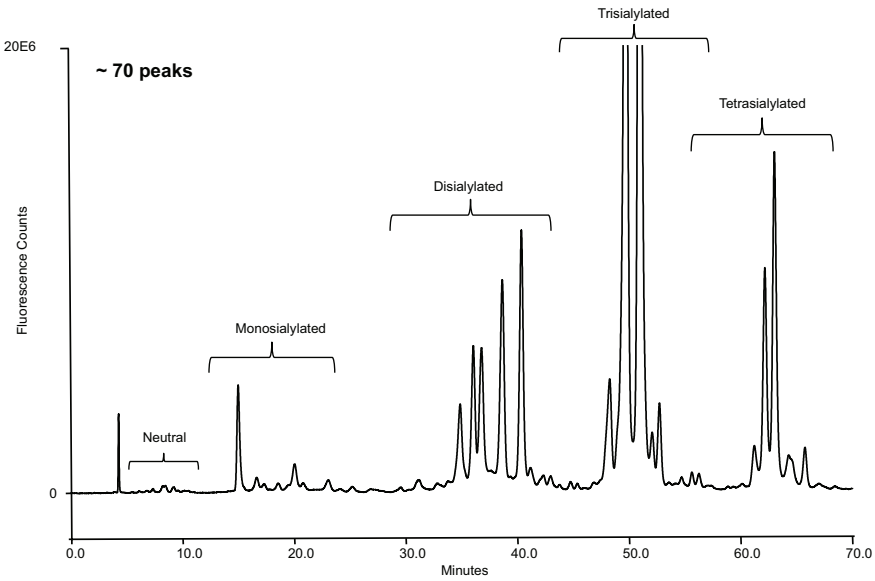

Figure 1: Separation of 2AA-labeled *N*-linked glycans from bovine fetuin by charge, isomerism and size using a ternary gradient with late introduction of acetonitrile on a GlycanPac AXR-1 column

Time (min)	%A	%B	%C
-10	0	85	15
0	0	85	15
1	0	85	15
25	0	70	30
70	10	40	50

Table 1: Ternary gradient conditions for Figure 1

Figure 1 shows the separation of neutral and acidic 2AA-labeled *N*-linked glycans from bovine fetuin using a GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm) column with a ternary gradient. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, followed by monosialylated, disialylated, trisialylated and tetrasialylated species. Analytes in each cluster represent glycans of the same charge. Within each cluster, glycans having the same charge are further separated according to their isomeric structure, sized and polarity by reversed-phase mechanisms. Note: The gradient was completed before elution of the few penta-sialylated glycans known to be present in bovine fetuin.

As shown in Figure 1, 2AA-labeled neutral glycans elute between 4 and 12 min, monosialylated glycans elute between 12 and 27 min, disialylated glycans elute between 27 and 45 min, trisialylated glycans elute between 45 and 58 min, and tetrasialylated glycans elute between 58 and 70 min. More than 70 peaks are identified in less than 70 minutes in this chromatogram of 2AA-labeled *N*-linked glycans from bovine fetuin.

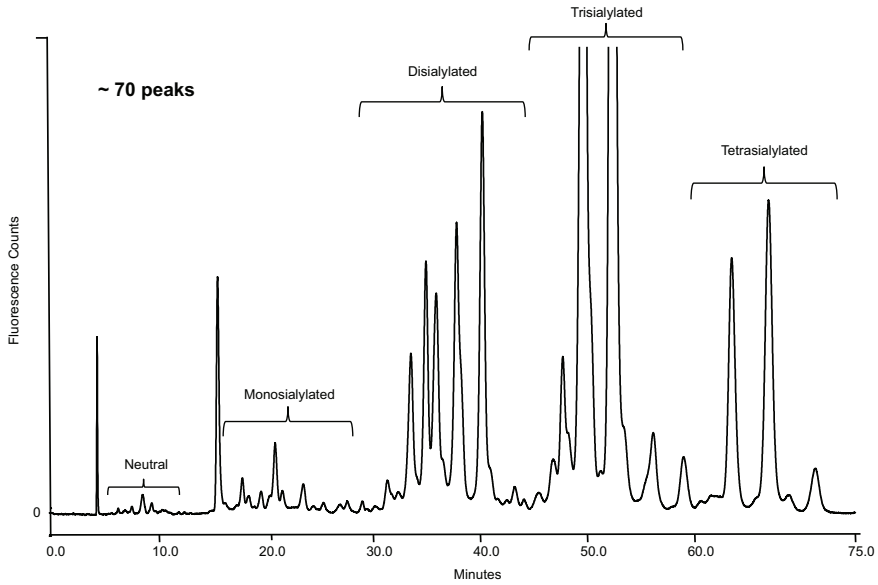

Figure 2: Separation of 2AA-labeled *N*-linked glycans from bovine fetuin by charge, isomerism and size using a binary gradient without acetonitrile on a GlycanPac AXR-1 column

Time (min)	%B	%C
-10	90	10
0	90	10
1	90	10
45	40	60
60	30	70
75	30	70

Table 2: Binary gradient conditions for Figure 2

Figure 2 repeats the chromatography using a binary gradient, beginning with a lower ammonium formate concentration, employing a shallower (slower) gradient rate and eliminating acetonitrile from the eluent system. Here, slightly better resolution of several peaks is observed due to the lower initial salt concentration and gradient slope. In this case the chromatogram was completed in 60 minutes.

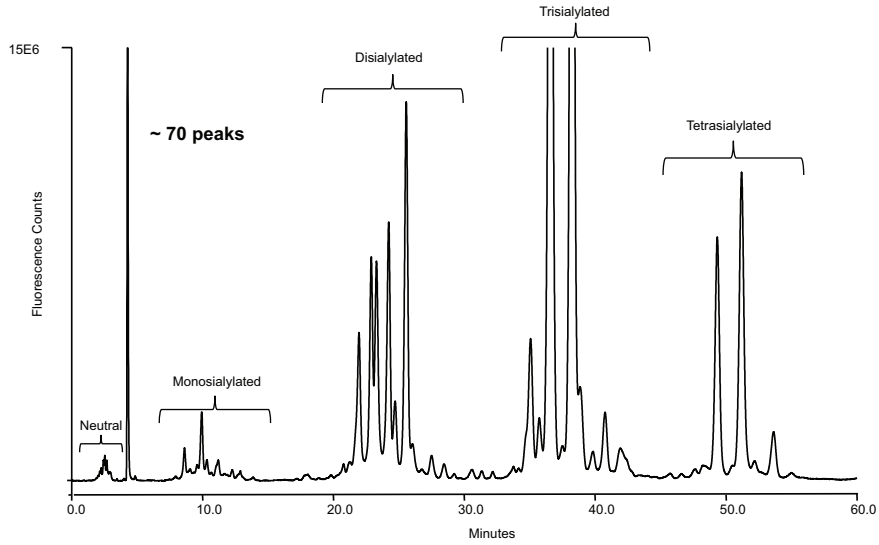

Figure 3: Separation of 2AB-labeled *N*-linked glycans from bovine fetuin by charge, isomerism and size using a binary gradient without acetonitrile on a GlycanPac AXR-1 column

Time (min)	%B	%C
-10	95	5
0	95	5
1	95	5
50	50	50
60	50	50

Table 3: Binary gradient conditions for Figure 3

Due to 2AA-labeling, a formal negative charge was introduced onto each glycan, increasing all glycan negative charges by 1. For example, glycans that are neutral when labeled with 2AB become negatively charged when labeled with 2AA. This produces greater retention on the GlycanPac AXR-1 column. Similarly mono-sialylated glycans acquire a negative-2 charge; di-sialylated glycans acquire a negative-3 charge, etc. Thus 2AA-labeled *N*-linked glycans (Figure 2) from fetuin require higher ionic strength for elution than the 2AB-labeled *N*-linked glycans from fetuin. However, 2AA-labeling promotes better retention and thus resolution of neutral glycans than the same glycans labeled with 2AB, using the GlycanPac AXR-1 column. For this comparison, Figure 3 depicts the chromatography of 2AB-labeled *N*-linked glycans from bovine fetuin using significantly reduced ammonium formate concentrations. In figure 2, the ammonium formate concentration runs from 10 to 70 mM, while in Figure 3, the range is 5 to 50 mM.

In Figure 1, the ammonium formate concentration runs from 15 to 30 mM in 25 min, and later-eluting glycans are eluted with an acetonitrile gradient. This limits the volatile salt concentration and supports better performance of the MS system. Thus, the ternary gradient condition is preferred for mass spectrometric applications with 2AA-labeled *N*-linked glycans.

Conclusion

- The GlycanPac AXR-1 is a high- resolution, silica-based mixed-mode HPLC column for simultaneous separation of glycans by isomeric structure, charge and size.
- The GlycanPac AXR-1 column provides improved selectivity and excellent resolution of 2AA-labeled *N*-linked glycans released from bovine fetuin.
- Binary and ternary gradient conditions for the separation of both 2AA-labeled *N*-linked glycans from fetuin are illustrated.
- The GlycanPac AXR-1 works well for the direct injection of purified 2AA-labeled *N*-linked glycans from bovine fetuin under fully aqueous conditions.

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Direct Determination of Sialic Acids in Glycoprotein Hydrolyzates by HPAE-PAD

Thermo Fisher Scientific, Inc.

INTRODUCTION

Sialic acids are critical in determining glycoprotein bioavailability, function, stability, and metabolism.[1] Although over 50 natural sialic acids have been identified,[2] two forms are commonly determined in glycoprotein products: *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Because humans do not generally produce Neu5Gc and have been shown to possess antibodies against Neu5Gc, the presence of this sialic acid in a therapeutic agent can potentially lead to an immune response.[3] Consequently, glycoprotein sialylation, and the identity of the sialic acids, play important roles in therapeutic protein efficacy, pharmacokinetics, and potential immunogenicity.

Sialic acid determination can be performed by many methods. Typically, sialic acids are released from glycoproteins by acid hydrolysis or by enzymatic digestion before analysis. Once the sialic acids are liberated, there are many options for quantification. Numerous spectroscopic methods exist, although interferences in these methods can cause overestimation of the concentration of sialic acids in many samples. Therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred.[4] Among the chromatographic methods, there are those that require further sample derivatization for analyte detection, such as fluorescent labeling followed by high-performance liquid chromatography (HPLC), and direct detection methods such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD).[5] Of these methods, HPAE-PAD offers the advantage of direct analysis without sample derivatization.

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis followed by HPAE-PAD. Sialic acid determination by HPAE-PAD on a Thermo Scientific™ Dionex™ CarboPac™ PA20 column is specific and direct, eliminating the need for sample derivatization after sample preparation. The use of a disposable gold on polytetrafluoroethylene (Au on PTFE) working electrode simplifies system maintenance compared to conventional gold electrodes while providing consistent response with a four-week lifetime. The rapid gradient method discussed separates Neu5Ac and Neu5Gc in under 10 min with a total analysis time of 16.5 min, compared to 27 min using the Dionex CarboPac PA10 column by a previously published method.[6,7] By using the Dionex CarboPac PA20 column, the total analysis time is reduced, eluent consumption and waste generation are reduced, and sample throughput is improved.

Thermo SCIENTIFIC

EQUIPMENT

Thermo Scientific Dionex ICS-3000 or Thermo Scientific Dionex ICS-5000 Ion Chromatography (IC) system including:

- SP Single Pump or DP Dual Pump module
- DC Detector/Chromatography module
- Thermo Scientific Dionex AS Autosampler

Dionex ICS-3000/5000 ED Electrochemical Detector

Electrochemical Cell

Disposable Gold Working Electrode, Au on PTFE

Reference Electrode (Ag/AgCl)

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System, Workstation 7

Polypropylene injection vials, 0.3 mL, with caps

Polypropylene injection vials, 1.5 mL, with caps

Thermo Scientific Nalgene 1000 mL 0.2 µm nylon filter units

Polypropylene microcentrifuge screw-cap tubes, 1.5 mL

Dry block heater

REAGENTS AND STANDARDS

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

Sodium hydroxide, 50% (w/w)

Sodium acetate, anhydrous

Acetic acid

N-Acetylneuraminic acid

N-Glycolylneuraminic acid (Neu5Gc, NGNA)

Micro BCA™ Protein Assay Kit

α (2→3,6,8,9) Neuraminidase, proteomics grade from *Arthrobacter ureafaciens*

SAMPLES

Five glycoproteins were selected for analysis:

- Calf fetuin
- Bovine apo-transferrin (b. apo-transferrin)
- Human transferrin (h. transferrin) (Sigma P/N T8158)
- Sheep α_1-acid glycoprotein (s. AGP) (Sigma P/N G6401)
- Human α_1-acid glycoprotein (h. AGP) (Sigma P/N G9885)

CONDITIONS

Columns:	Dionex CarboPac PA20, 3 × 150 mm Dionex CarboPac PA20 Guard, 3 × 30 mm
Eluent Gradient:	70–300 mM acetate in 100 mM NaOH from 0–7.5 min, 300 mM acetate in 100 mM NaOH from 7.5–9.0 min, 70 mM acetate in 100 mM NaOH from 9.0–9.5 min, 7 min of equilibration at 70 mM acetate in 100 mM NaOH.
Eluents:	A: NaOH, 100 mM B: Sodium acetate, 1.0 M, in 100 mM NaOH
Flow Rate:	0.5 mL/min
Temperature:	30 °C (column and detector compartments)
Inj. Volume:	10 µL
Detection:	Pulsed amperometric, disposable Au on PTFE electrode
Background:	18–25 nC (using the carbohydrate waveform)
Noise:	~15–30 pC
System Backpressure:	~3000 psi

Carbohydrate 4-Potential Waveform for the ED

Time(s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000 IC system, but not used in older Dionex systems.

Reference electrode in Ag mode (Ag/AgCl reference). See Application Update 141 for more information.[6]

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solutions

Prepare 1 L of 100 mM sodium hydroxide by adding 5.2 mL of 50% (w/w) NaOH to 994.8 mL of degassed DI water.

Prepare 1 L of 1 M sodium acetate in 100 mM sodium hydroxide by dissolving 82.0 g of anhydrous sodium acetate in ~800 mL of DI water. Filter and degas the acetate solution through a 0.2 µm nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% (w/w) NaOH, and fill the flask with degassed DI water. See Dionex (now part of Thermo Scientific) Technical Note 71 for detailed information on eluent preparation for HPAE-PAD applications.[8]

Acetic Acid, 4 M

Transfer 22.5 mL of glacial acetic acid to a polyethylene bottle containing 77.5 mL of DI water.

Sodium Acetate Buffer, 0.1 M, pH 5 for Neuraminidase Digestions

Prepare a 0.3 M sodium acetate stock solution by dissolving 12.31 g of sodium acetate in 500 mL of DI water. Transfer 68.21 g (68.3 mL) of 0.3 M sodium acetate to a 250 mL polypropylene bottle. Add 1.8 mL of 4 M actetic acid to the solution. Dilute to a total of 249.62 g (250 mL).

Stock Standard Solutions

Dissolve 149.8 mg of Neu5Ac in 50 mL DI water and 41.0 mg Neu5Gc in 50 mL of DI water. This results in 9.68 mM and 2.52 mM stock solutions, respectively. Dilute 500 µL of 9.68 mM Neu5Ac and 130 µL of 2.52 mM Neu5Gc to 48.4 mL total with DI water. Aliquot this mixed stock of 0.10 mM Neu5Ac and 6.8 µM Neu5Gc into 1.5 mL cryogenic storage vials and store at -40 °C.

Working Standard Solutions

Prepare calibration standards by diluting the standard stock solution as detailed in Table 1. For example, 10 µL of the stock solution were added to 990 µL of DI water to prepare a calibration standard of 1.0 µM Neu5Ac, or 10 pmol/10 µL injection. Prepare standards daily from the stocks stored at -40 °C.

Protein Stock Solutions, 4.0 mg/mL Nominal

Dissolve 3.44 mg of sheep α_1-acid glycoprotein in 860 µL of DI water. Gently swirl to thoroughly mix the solution. Prepare 200 µL aliquots of the solution in microcentrifuge vials to minimize freeze/thaw cycles when the stock is needed. Store all protein solutions at -40 °C. Repeat this process as follows. Dissolve 8.8 mg (b. apo-transferrin), 8.6 mg (h. transferrin), and 8.1 mg (fetuin) of the glycoprotein in individual aliquots of 2.0 mL DI water. Dissolve 2.2 mg of h. AGP in 0.56 mL of DI water. Each glycoprotein will be at a nominal concentration of 4 mg/mL.

Working Stock Protein Solutions

Pipet 250 µL of a protein stock solution into 1750 µL DI water to prepare a working stock solution. Aliquot 400 µL of the working stock into individual microcentrifuge tubes and store the working stock solutions at -40 °C. Protein may be lost both during freeze/thaw cycles and by adsorption to surfaces. Therefore, it is important to measure the working stock protein concentrations before hydrolysis by using a colorimetric BCA protein assay kit. Values listed in Table 2 are results from BCA assay of the working stock solutions.

Table 1. Sialic Acid Standards Used for Sample Analysis

Volume of Combined Stock Standard (µL) Diluted to 1000 µL	Neu5Ac Concentration (nM)	Neu5Gc Concentration (nM)	Neu5Ac Amount (pmol/10µL)	Neu5Gc Amount (pmol/10 µL)
1.0	100	7.8	1.0	<LOQ*
2.5	250	20	2.5	<LOQ*
5.0	500	39	5.0	0.39
10	1000	78	10	0.78
25	2500	200	25	2.0
50	5000	390	50	3.9
75	7500	580	75	5.8
100	10000	780	100	7.8

*Not used for Neu5Gc calibration

Acetic Acid Hydrolysis of Proteins

Add 14 µg (fetuin), 20 µg (h. transferrin), 25 µg (b. apo-transferrin), 13 µg (h. AGP), and 7 µg (s. AGP) of the glycoprotein to individual 1.5 mL microcentrifuge vials with a total of 200 µL of 2 M acetic acid as detailed in Table 2. For example, pipet 50 µL of the working fetuin stock, 50 µL of DI water, and 100 µL of 4 M acetic acid to prepare the solution for hydrolysis. Hydrolyze the protein solutions for 2 h by the method of Varki et al.[9] to preserve *O*-acetylated sialic acids for comparison to UHPLC-FLD method using DMB derivatization.[10] After hydrolysis, lyophilize and resuspend 50 µL of sample hydrolyzates in 500 µL of DI water; prepare more concentrated hydrolyzates (0.70 µg/µL of protein) by diluting the hydrolyzate 1:80 with DI water. Note that this acid hydrolysis method may not be optimized for complete release of all sialic acids without degradation of the free sialic acids. Optimization of the hydrolysis conditions for a given sample and analysis method is highly recommended. Additional hydrolysis conditions may be found in Technical Note 41.[7]

Neuraminidase Digestion of Proteins

Add 50 µL of DI water to a 25 mU vial of neuraminidase. Add 2 µL of this stock to 148 µL of 0.1 M sodium acetate buffer to prepare a 1 mU/mL neuraminidase solution. Add 14 µg, 20 µg, 25 µg, 13 µg, and 7 µg of fetuin, h. transferrin, b. transferrin, h. AGP, and s. APG, respectively, to individual 1.5 mL microcentrifuge vials with this solution and incubate at 37 °C for 18 h. After incubation, centrifuge the samples and dilute them with an additional 300 µL of DI water prior to analysis.

RESULTS AND DISCUSSION

Figure 1 shows the separation of Neu5Ac and Neu5Gc on the Dionex CarboPac PA20 column with a 70–300 mM acetate gradient in 100 mM NaOH. The peaks are well separated and easily quantified. Additionally, the Neu5Ac peak is well separated from the void, which is an important consideration when analyzing acid hydrolyzed samples that may contain additional poorly retained compounds.

Linear Range, Limit of Quantification, Limit of Detection, and Precision

Table 3 shows the calibration results for Neu5Ac and Neu5Gc. In both cases, response is linear for the range studied. The limit of detection (LOD) and limit of quantification (LOQ) were confirmed by standard injections that resulted in a response of 3× and 10× the noise, respectively. Neu5Ac was determined to have an LOD of 0.17 pmol on column and an LOQ of 0.50 pmol. Similarly, Neu5Gc limits were found to be 0.08 pmol and 0.30 pmol. During this work, 2 and 3 mil gaskets were installed with the disposable electrodes and evaluated in terms of analyte linearity and LOQ. The results listed were determined with a 3 mil gasket. Injections of LOD and LOQ standards with a 2 mil gasket installed yielded equivalent results. When establishing an assay, standardize on one gasket size and specify it in the standard operating procedure.

Retention time and peak area precisions of standards were determined by seven injections of a mid-range standard. In both cases, precision was excellent, with an RSD of <0.2 for retention time for both sialic acids and peak area RSDs of 1.08 and 1.01 for Neu5Ac and Neu5Gc, respectively.

Table 2. Protein Hydrolyzate Concentrations

Protein	BCA Measured Working Soln. Conc. (µg/mL)	Volume Protein (µL)	Amount of Protein (µg)	Volume DI Water (µL)	Volume 4M Acetic Acid (µL)	Protein Conc. (µg/µL)
Fetuin	280	50	14	50	100	0.07
h. Transferrin	400	50	20	50	100	0.10
b. apo-Transferrin	500	50	25	50	100	0.12
h. AGP	260	50	13	50	100	0.06
s. AGP	140	50	7.0	50	100	0.04

Table 3. Linearity, LOD, LOQ, and Precision of Sialic Acid Determination

Analyte	Range (pmol)	Coeff. of Determination (r^2)	RT (min)	RT Precision (RSD)	Peak Area Precision[a] (RSD)	LOQ[b] (pmol)	LOD (pmol)
Neu5Ac	1.0–100	0.9997	4.08	0.18	1.08	0.5	0.17
Neu5Gc	0.39–7.8	0.9995	7.18	0.09	1.01	0.3	0.08

[a]Precision was measured by 7 injections of 25 pmol Neu5Ac, 2.0 pmol Neu5Gc.

[b]LOD and LOQ are confirmed by injections at the concentrations listed and measuring response at 3× and 10× the noise, respectively.

Sample Analysis, Precision, and Accuracy

Figure 2 shows the separation of hydrolyzed and lyophilized protein samples. In each case, Neu5Ac is well separated from early eluting components of the hydrolyzed sample. In each case, Neu5Ac is present, and as expected, Neu5Gc is not detected in human forms of the glycoproteins. Using fetuin as an example, 0.07 µg/µL of protein were hydrolyzed. After lyophilizing 50 µL of hydrolyzate and dissolving it in 500 µL of DI water (a 10-fold dilution), a 10 µL full-loop injection loads the equivalent of 0.07 µg of protein on the column. After preparation by dilution, 10 µL of 0.75µg/µL hydrolyzate are diluted to a total of 800 µL of sample with the equivalent of 0.09 µg of protein injected with 10 µL. In both cases, there is enough sample for multiple sets of triplicate full-loop injections.

For glycoproteins that are highly glycosylated, such as α_1-acid glycoproteins, the amount of protein that is hydrolyzed can easily be reduced. In the example of s. AGP, 0.04 µg/µL of protein were hydrolyzed. Because of the high degree of glycosylation, the average determined amount for a single day of sample analysis was 20 pmol of Neu5Ac (Table 4). In comparison, an average of 19 pmol of Neu5Ac was determined in fetuin hydrolyzates, even though nearly twice as much protein was hydrolyzed. Based on the amount of Neu5Gc in the fetuin hydrolyzate and the determined LOQs for Neu5Ac and Neu5Gc, a lower concentration of fetuin is not recommended. However, the concentration of s. AGP can be reduced by a factor of 10 before reaching the LOQ of Neu5Gc and by a factor of 40 before reaching the LOQ of Neu5Ac. Depending on the degree of sialylation, the amount of protein hydrolyzed can be reduced to low ng/µL concentrations and still allow efficient sialic acid determination. Designing experiments that routinely release amounts of analyte near the LOQ is not recommended. However, this evaluation of potential conditions highlights both the sensitivity of the method and the importance of considering the approximate protein sialylation amount when designing acid hydrolysis experiments.

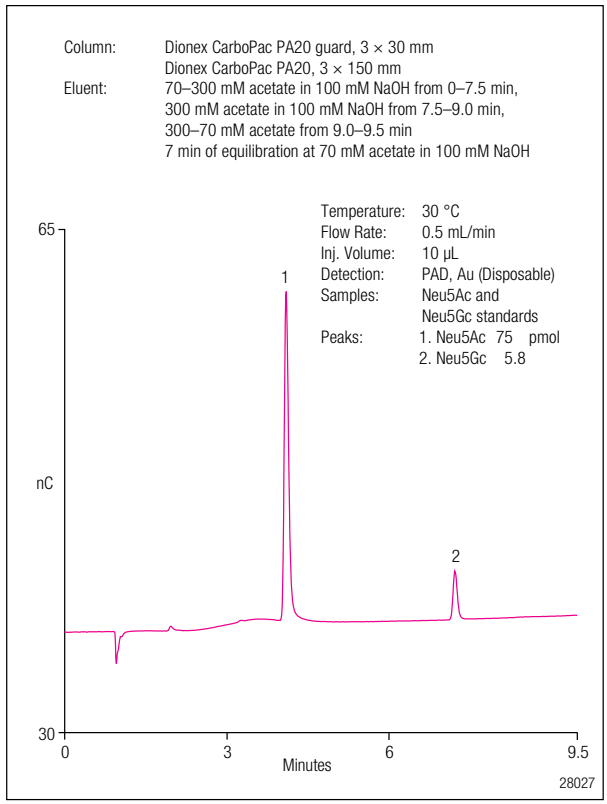

Figure 1. Separation of sialic acid standards on the Dionex CarboPac PA20 column.

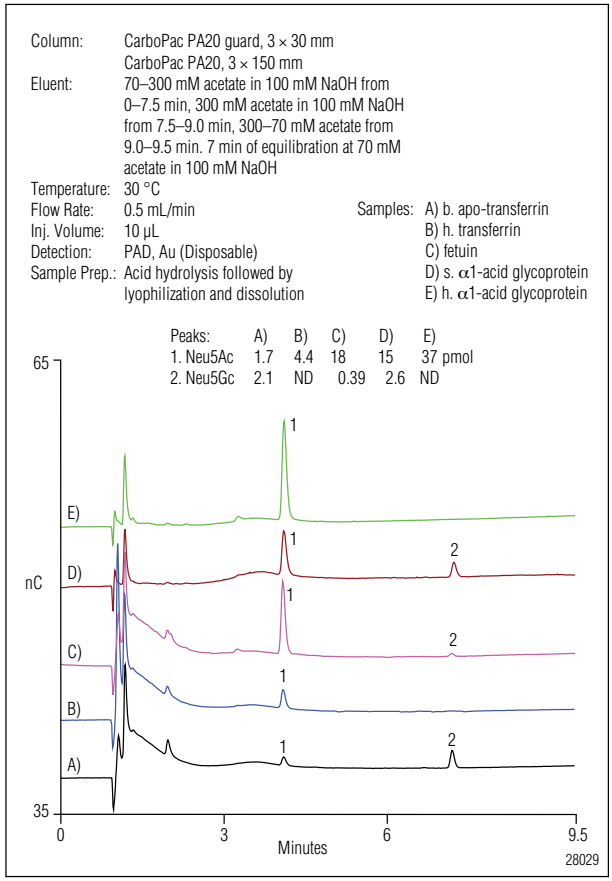

Figure 2. Sialic acid determination of five glycoprotein acid hydrolyzates. A 10% signal offset has been applied.

Table 4. Results from Analysis of Triplicate Protein Acid Hydrolysis, n=3 per Hydrolyzed Sample

Sample (replicate #)	Analyte	Amount (pmol)	Peak Area (nC*min)	Peak Area Precision (RSD)	RT (min)	RT Precision (RSD)
Fetuin (1)	Neu5Gc	0.45	0.017	1.76	7.18	0.13
	Neu5Ac	20	0.404	2.76	4.04	0.24
Fetuin (2)	Neu5Gc	0.54	0.020	2.20	7.18	<0.01
	Neu5Ac	23	0.479	0.54	4.04	<0.01
Fetuin(3)	Neu5Gc	0.35	0.014	1.44	7.19	0.12
	Neu5Ac	15	0.313	2.23	4.06	0.21
h. Transferrin (1)	Neu5Gc	ND				
	Neu5Ac	4.4	0.095	2.39	4.08	0.20
h. Transferrin (2)	Neu5Gc	ND				
	Neu5Ac	4.5	0.096	2.66	4.08	<0.01
h. Transferrin (3)	Neu5Gc	ND				
	Neu5Ac	3.9	0.084	2.25	4.07	0.12
b. Transferrin (1)	Neu5Gc	2.6	0.099	0.59	7.20	0.08
	Neu5Ac	2.0	0.044	2.66	4.09	0.14
b. Transferrin (2)	Neu5Gc	2.2	0.083	2.34	7.21	0.07
	Neu5Ac	1.6	0.036	2.22	4.09	0.12
b. Transferrin (3)	Neu5Gc	2.4	0.090	1.01	7.21	0.07
	Neu5Ac	1.8	0.039	2.19	4.10	0.12
h. AGP (1)	Neu5Gc	ND				
	Neu5Ac	42	0.876	1.55	4.10	<0.01
h. AGP (2)	Neu5Gc	ND				
	Neu5Ac	41	0.820	3.52	4.10	<0.01
h. AGP (3)	Neu5Gc	ND				
	Neu5Ac	42	0.865	1.60	4.10	<0.01
s. AGP (1)	Neu5Gc	3.7	0.139	1.31	7.21	0.07
	Neu5Ac	21	0.431	1.39	4.10	<0.01
s. AGP (2)	Neu5Gc	3.4	0.128	0.86	7.21	0.07
	Neu5Ac	19	0.396	0.73	4.10	<0.01
s. AGP (3)	Neu5Gc	3.4	0.131	0.52	7.21	0.07
	Neu5Ac	19	0.403	0.38	4.10	0.12

Sample	Analyte	Acid Hydrolysis Average (mol analyte/mol protein)	Intraday Precision Between Replicates (RSD)	Between-Day Precision (RSD)	Neuraminidase Digestion Average (mol analyte/ mol protein)
Fetuin	Neu5Gc	0.32	22	14	0.30
	Neu5Ac	14	21	13	19
h. Transferrin	Neu5Gc	ND	ND	ND	ND
	Neu5Ac	3.4	7.8	8.6	4.8
b. apo-Transferrin	Neu5Gc	1.6	8.6	7.9	1.4
	Neu5Ac	1.2	9.5	9.4	1.9
h. AGP	Neu5Gc	ND	ND	ND	ND
	Neu5Ac	25	1.7	8.9	30
s. AGP	Neu5Gc	4.5	4.6	12	3.1
	Neu5Ac	26	4.6	13	25

Table 4 presents the results from one day of triplicate analysis. Retention time precision was similar to that determined by injecting standards, with retention time RSDs ranging from <0.01–0.24. Variability in the absolute retention time may be expected based on the batch of manual eluent prepared. Peak area precision for triplicate injections, as measured by RSD, is generally good, ranging from 0.38–3.52.

Table 5 shows the calculated results of sialic acid analysis for the proteins studied, as well as intraday precision for one day of analysis, as in Table 4, and between-day precision for three days of triplicate analysis. Variability between sample replicates can be large, with RSDs ranging from 1.7 to 22; therefore, optimization of the digestion for individual glycoproteins is highly recommended. Between-day precision, as RSD, ranges from 7.9 to 14, with an average of 11.

Comparison of the determined amounts between acid hydrolysis and neuraminidase digestion suggest that either the hydrolysis is not complete using the mild hydrolysis or that acid degradation of the free sialic acids has occurred. Acid hydrolysis is a complex balance between release of the sialic acids from the glycoprotein and degradation of the released analytes. The efficiency of the hydrolysis will depend on the hydrolysis temperature, acid concentration, type of sample being hydrolyzed, and the relative concentrations of acid and the sample. Because of these interdependent factors—which can impact the hydrolysis—variability between sample preparations can be expected. For the best accuracy, either optimized acid hydrolysis or neuraminidase digestion is recommended. For methodology to optimize acid hydrolysis, see Fan et al.[11] However, the amounts of sialic acids determined in the protein samples are consistent with literature results for the glycoproteins.[12-16] Method accuracy was investigated by spiking protein acid hydroylzates with known amounts of Neu5Ac and Neu5Gc equal to the determined amounts. For human glycoproteins, which did not contain Neu5Gc, 0.38 pmol of Neu5Gc was added. Recoveries were evaluated for both sample preparation by lyophilization and by dilution. Recoveries for Neu5Ac ranged from 83–103% by dilution and 75–82% by lyophilization (Table 6). Recoveries for Neu5Gc were similar, ranging from 76–100% by dilution and 75–86% by lyophilization. Where protein amounts are not limited, dilution is recommended for both ease of sample preparation and improved recoveries.

Glycoprotein Hydrolyzate Stability

A set of glycoprotein hydrolyzates was re-analyzed after 14 days of storage at -40 °C. These samples were stored in solution after lyophilization. The comparative results of these stored samples quantified with freshly prepared working standards are shown in Table 7. Values across replicates can be more variable after storage; however, overall, the determined amounts are generally within 10% of the original values.

Table 6. Recoveries, Triplicate Hydrolyzes

Sample	Analyte	Amount Added (pmol)	Average Recovery (Dilution) (%)	Average Recovery (Lyophilization) (%)
Reagent blank	Neu5Gc	0.50	92.7	75.9
	Neu5Ac	5.0	91.3	78.7
Fetuin	Neu5Gc	0.60	99.0	86.4
	Neu5Ac	25	94.7	81.6
h. Transferrin	Neu5Gc	0.50	99.8	74.9
	Neu5Ac	5.0	77.4	74.6
b. apo-Transferrin	Neu5Gc	2.5	76.3	84.9
	Neu5Ac	2.5	83.2	78.9
h. AGP	Neu5Gc	0.50	98.8	74.9
	Neu5Ac	50	102	74.6
s. AGP	Neu5Gc	5.0	88.9	84.9
	Neu5Ac	30	87.2	78.9

Table 7. Stability of Samples Stored at -40 °C

Sample (Replicate)	Analyte	Initial Determined Amount (pmol)	Amount After 14 Days of Storage at -40 °C (pmol)	Difference (%)
h. AGP (1)	Neu5Gc	ND	ND	
	Neu5Ac	42±0.6	45±0.8	7.4
h. AGP (2)	Neu5Gc	ND	ND	
	Neu5Ac	41±1.7	38±0.8	-6.9
h. AGP (3)	Neu5Gc	ND	ND	
	Neu5Ac	42±0.7	35±1.4	-15
s. AGP (1)	Neu5Gc	3.7±0.5	3.6±0.04	-2.3
	Neu5Ac	21±0.3	20±0.2	-4.4
s. AGP (2)	Neu5Gc	3.3±0.03	3.2±0.03	-3.8
	Neu5Ac	19±0.1	18±0.07	-4.4
s. AGP (3)	Neu5Gc	3.4±0.02	3.4±0.03	<0.1
	Neu5Ac	19±0.07	19±0.3	<0.1

CONCLUSION

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis release and HPAE-PAD. Determination of the sialic acids Neu5Ac and Neu5Gc by HPAE-PAD on a Dionex CarboPac PA20 column is specific and direct. After sample hydrolysis or enzymatic treatment, there is no need for further sample derivatization. Disposable Au on PTFE working electrodes with a four-week lifetime simplify system maintenance compared to conventional gold electrodes. The gradient method discussed separates Neu5Ac and Neu5Gc with a total analysis time of 16.5 min, which is faster than previous methods, allowing greater sample throughput.

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Application Update: 181

Rapid Screening of Sialic Acids in Glycoproteins by HPAE-PAD

Deanna Hurum and Jeff Rohrer

- Neuraminic Acids
- Anion Exchange
- Pulsed Amperometric Detection
- CarboPac PA20 Fast Sialic Acid Column
- High-Throughput Analysis

Introduction

Glycoprotein sialylation has been shown to be critical to bioavailability, stability, metabolism, and immunogenicity of therapeutic proteins.[1-4] As a result, such proteins are routinely analyzed to determine sialylation amount and identity. Although over 50 forms of sialic acid have been identified,[5-6] two forms of this carbohydrate are routinely determined, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Of these, Neu5Gc is generally not found in human proteins.[7] Due to this lack of Neu5Gc in healthy human tissue and the natural occurrence of antibodies against Neu5Gc, this sialic acid has the potential to cause an immune response in patients when present in a glycoprotein therapeutic.[8]

Many therapeutic proteins are produced via expression of the protein in a cell line chosen to maximize protein yield. Because the final glycoprotein sialylation amount and identity depend on the expression cell line and growth conditions for that cell line,[9-11] expression experiments and production optimization have the potential to generate large numbers of samples requiring analysis. In this case, high-throughput screening assays are valuable for quick product evaluation relative to expression cell lines and growth conditions.

Sialic acid determination can be performed by many methods. Typically, sialic acids are released from glycoproteins by acid hydrolysis or by enzymatic digestion before analysis. Once the sialic acids are liberated, there are multiple options for quantification. Numerous spectroscopic methods exist, although interferences in these methods can overestimate the sialic acid concentrations in many samples, and therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred.[12] Among the chromatographic methods, some require further sample derivatization for analyte detection, such as fluorescent labeling followed by high-performance liquid chromatography (HPLC). Others use direct detection methods, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD).[13] Of these two methodologies, HPAE-PAD offers the advantage of direct analysis without sample derivatization.

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis followed by HPAE-PAD. Sialic acid determination by HPAE-PAD using the Thermo Scientific Dionex CarboPac PA20 Fast Sialic Acid column is specific and direct, eliminating the need for sample derivatization after hydrolysis. The use of a disposable gold on polytetrafluoroethylene (PTFE) working electrode simplifies system maintenance while providing consistent response with a lifetime of four weeks. The rapid gradient method discussed separates Neu5Ac and Neu5Gc in <3 min with a total analysis time of 4.5 min. By using the Dionex CarboPac™ PA20 Fast Sialic Acid column, total analysis time is reduced, per sample eluent consumption and waste generation are reduced, and sample throughput is improved.

Equipment

Thermo Scientific Dionex ICS-3000 or Dionex ICS-5000 Ion Chromatography system including:
- SP Single Pump or DP Dual Pump module
- DC Detector/Chromatography module
- AS Autosampler

Electrochemical Detector (P/N 061719)

Electrochemical Cell (P/N 061757)

Disposable Gold Electrode, Au on PTFE (P/N 066480)

Reference Electrode (P/N 061879)

Thermo Scientific Dionex Chromeleon 7 Chromatography Data System software

Polypropylene injection vials with caps, 0.3 mL (P/N 055428)

Polypropylene injection vials with caps, 1.5 mL (P/N 061696)

Nalgene™ 1000 mL, 0.2 µm nylon filter units (Fisher Scientific P/N 09-740-46)

Polypropylene microcentrifuge screw cap tubes, 1.5 mL (Sarstedt P/N 72.692.005)

Dry block heater (VWR P/N 13259-005)

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Reagents and Standards

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

Sodium hydroxide, 50% (w/w) (Fisher Scientific P/N SS254-500)

Sodium acetate, anhydrous (P/N 059326)

Acetic acid (JT Baker P/N 9515-03)

N-Acetylneuraminic acid (Neu5Ac, NANA) Ferro Pfanstiehl

N-Glycolylneuraminic acid (Neu5Gc, NGNA) Ferro Pfanstiehl

Micro BCA™ Protein Assay Kit, (Thermo Scientific P/N 23235)

Samples

Five glycoproteins were selected for analysis:

Calf fetuin (Sigma P/N F2379)

Bovine apo-transferrin (b. apo-transferrin) (Sigma P/N T1428)

Human transferrin (h. transferrin) (Sigma P/N T8158)

Sheep α_1-acid glycoprotein (s. AGP) (Sigma P/N G6401)

Human α_1-acid glycoprotein (h. AGP) (Sigma P/N G9885)

Conditions

Column:	Dionex CarboPac PA20 Fast Sialic Acid Column, 3 × 30 mm (P/N 076381)
Eluent Gradient:	70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min. 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Eluents:	A: 100 mM NaOH B: 1.0 M sodium acetate in 100 mM NaOH
Flow Rate:	0.5 mL/min
Inj. Volume:	4.5 µL, (full loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au on PTFE working electrode
Background:	18–25 nC (using the carbohydrate waveform)
Noise:	~15–30 pC
Sys. Backpress.:	~750 psi

Carbohydrate 4-Potential Waveform for the ED

Time(s)	*Potential (V)*	*Gain Region**	*Ramp**	*Integration*
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000, but not used in older Dionex systems. Reference electrode in Ag mode (Ag/AgCl reference). See Thermo Scientific (formerly Dionex) Application Note 141 and Technical Note 41 for more information regarding sialic acid determination.[14,15]

Preparation of Standards and Samples

Eluent Solutions

Prepare 1 L of 100 mM sodium hydroxide by adding 5.2 mL of 50% (w/w) NaOH to 994.8 mL of degassed DI water.

Prepare 1 L of 1 M sodium acetate in 100 mM sodium hydroxide by dissolving 82.0 g of anhydrous sodium acetate in ~800 mL of DI water. Filter and degas the acetate solution through a 0.2 µm nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% (w/w) NaOH, and fill the flask with degassed DI water.

See Thermo Scientific (formerly Dionex) Technical Note 71 for detailed information on eluent preparation for HPAE-PAD applications.[16]

Acetic Acid, 4 M

Transfer 22.5 mL of glacial acetic acid to a polyethylene bottle containing 77.5 mL of DI water.

Stock Standard Solutions

Dissolve 149.8 mg of Neu5Ac in 50 mL DI water and 41.0 mg Neu5Gc in 50 mL of DI water. This results in 9.68 mM and 2.52 mM stock solutions, respectively. Add 20 µL 9.68 mM Neu5Ac to 949 µL DI water to prepare a 0.20 mM solution, and 8.0 µL 2.52 mM Neu5Gc to 992 µL DI water to prepare a 0.020 mM solution of Neu5Gc. Add 500 µL of 0.20 mM Neu5Ac and 500 µL of 0.020 mM Neu5Gc to a 1.5 mL cryogenic storage vial to prepare a combined stock of 0.10 mM Neu5Ac and 10 µM Neu5Gc and store at -40 °C.

Working Standard Solutions

Prepare calibration standards by diluting the stock standard solution as detailed in Table 1. For example, add 5.0 µL of the stock solution to 195 µL of DI water to prepare a calibration standard of 2.5 µM Neu5Ac and 0.25 µM Neu5Gc (11 pmol Neu5Ac and 1.1 pmol Neu5Gc per 4.5 µL injection). Prepare working standards daily from the stocks stored at -40 °C.

Volume of Combined Stock Standard (µL) Diluted to 1000 µL	Neu5Ac Concentration (µM)	Neu5Gc Concentration (nM)	Neu5Ac Amount (pmol/4.5 µL)	Neu5Gc Amount (pmol/4.5 µL)
0.5	0.25	25	1.1	0.11*
1.0	0.5	50	2.3	0.23
5.0	2.5	250	11.0	1.1
10.0	5.0	500	23.0	2.3
20.0	10.0	1000	45.0	4.5
30.0	15.0	1500	68.0	6.8
40.0†	20.0	2000	90.0	9.0

*Not used for routine Neu5Gc calibration
† Not used for Neu5Ac calibration

Table 1: Sialic acid standards used for sample analysis.

Protein	Working Solution Conc (mg/mL)	Volume Protein (µL)	Amount of Protein (µg)	Volume DI Water (µL)	Volume 4 M Acetic Acid (µL)	Protein Conc (µg/µL)
Fetuin	2.3	35	80	65	100	0.40
s. AGP	1.0	35	35	65	100	0.18
h. AGP	2.9	35	100	65	100	0.51
b. apo-Transferrin	5.0	35	180	65	100	0.88
h. Transferrin	4.1	35	140	65	100	0.72

Table 2: Protein hydrolyzate concentrations.

Protein Stock Solutions, 4.0 mg/mL Nominal

Dissolve 2.4 mg of sheep α_1-acid glycoprotein in 400 µL of DI water to prepare a 4 mg/mL solution. Gently swirl to thoroughly mix the solution. Prepare 200 µL aliquots of the solution in microcentrifuge vials to minimize freeze/thaw cycles when the stock is needed. Store all protein solutions at -40 °C. Repeat this process as follows. Dissolve 8.8 mg (b. apo-transferrin) and 8.6 mg (h. transferrin) in individual 2 mL aliquots of DI water. Dissolve 19.0 mg of fetuin in 4.75 mL of DI water. Dissolve 2.2 mg of h. AGP in 0.60 mL of DI water. Protein may be lost both during freeze/thaw cycles and by adsorption to surfaces. Therefore, it is important to measure the working stock protein concentrations before hydrolysis using a colorimetric BCA protein assay kit. Values listed in Table 2 are results from BCA assay of the working stock solutions.

Acetic Acid Hydrolysis of Proteins

Add 80 µg fetuin, 140 µg h. transferrin, 175 µg b. apo-transferrin, 100 µg h. AGP, and 35 µg s. AGP to individual 1.5 mL microcentrifuge vials with a total of 200 µL of 2 M acetic acid, as detailed in Table 2. For example, pipet 35 µL of the fetuin stock, 65 µL of DI water, and 100 µL of 4 M acetic acid to prepare the solution for hydrolysis. Hydrolyze the protein solutions for 3 h at 80 °C.[17] After hydrolysis, dilute the hydrolyzate 1:100 with DI water. Please note that this acid hydrolysis method may not be optimized for complete release of all sialic acids without degradation of the free sialic acids. Optimization of the hydrolysis conditions for a given sample and analysis method is highly recommended. Additional hydrolysis conditions may be found in Thermo Scientific (formerly Dionex) Technical Note 41.[15]

Precautions

The Dionex CarboPac PA20 Fast Sialic Acid column has been tested for glycoprotein hydrolyzates only. More complex matrixes may not separate acceptably with the conditions presented here. For greater sensitivity and sample stability, lyophilization followed by dissolution in DI water is recommended. See Thermo Scientific (formerly Dionex) Application Update 180 for more information on stability of lyophilized hydrolysates that have been dissolved in DI water.[18] To avoid underestimation of sialic acid content due to acid catalyzed degradation, perform analysis of samples prepared by dilution within 24 h of hydrolysis.

Absolute mass detection limits will depend on the sample injection volume. For this reason, a calibrated injection loop was used. Prepare a 4.5 µL sample loop by measuring approximately 3.7 in of 0.010 in. i.d. tubing. Verify the volume of the loop by first weighing the empty tubing, fill the tube with DI water, then reweigh the filled tube and calculate the volume. The total sample volume should be ~4.5 µL. Due to the high-throughput nature of this method, service requirements on the autosampler and injection valves will increase. If replicate injections show poor precision, check the autosampler needle assembly, transfer line, and the injection valve to ensure each is in good condition.

Analyte	Range (pmol)	Coeff of Determination (r^2)	Retention Time (min)	Retention Time Precision[a] (RSD)	Peak Area Precision (RSD)	LOQ[b] (pmol)	LOD (pmol)
Neu5Ac	0.27–68	0.9995	0.745	0.88	1.36	0.34	0.11
Neu5Gc	0.23–11	0.9997	2.58	0.32	1.38	0.18	0.058

[a]Precision was measured by seven injections of 11 pmol Neu5Ac and 1.1 pmol Neu5Gc.
[b] LOD and LOQ are confirmed by injections at the concentrations listed and measuring response at 3× and 10× the noise, respectively.

Table 3: Linearity, limit of detection (LOD), limit of quantification (LOQ), and precision of sialic acid determination.

Results and Discussion

Figure 1 shows the separation of Neu5Ac and Neu5Gc on the Dionex CarboPac PA20 Fast Sialic Acid column with a 70–300 mM acetate gradient in 100 mM NaOH. The peaks are well separated and easily quantified. Additionally, the Neu5Ac peak is well separated from the void, which is an important consideration because a large void volume peak can interfere with quantification. Neu5Gc elutes in <3 min under these conditions, allowing a short run time.

Linear Range, Limit of Quantification, Limit of Detection and Precision

Table 3 shows the calibration results for Neu5Ac and Neu5Gc. In both cases, response is linear for the range studied. The LOD and LOQ were confirmed by standard injections that resulted in a response of 3× and 10× the noise, respectively. Neu5Ac had an LOD of 0.11 pmol on column and an LOQ of 0.34 pmol. Similarly, Neu5Gc limits were 0.058 pmol and 0.18 pmol. Retention time and peak area precisions of standards were determined by seven injections of a mid-range standard. In both cases, precision was excellent, with an RSD of <0.9 and a standard deviation of <0.008 min for retention time for both sialic acids and peak area RSDs of 1.36 and 1.38 for Neu5Ac and Neu5Gc, respectively.

Sample Analysis, Precision, and Accuracy

Figures 2 and 3 illustrate the separation of sialic acids from acid hydrolyzed and diluted protein samples. In each case, Neu5Ac is well separated from early eluting components of the hydrolyzed sample and, as expected, Neu5Gc is not detected in the human glycoproteins. The amount of protein necessary for sialic acid determination depends on the individual protein. For glycoproteins that are highly sialylated, such as α_1-acid glycoproteins, the amount of protein that is hydrolyzed easily can be reduced. In the example of s. AGP, 35 µL of 0.18 µg/µL protein solution are hydrolyzed, which is equivalent to 7.9 ng of protein per injection. The average determined amount for a single day of triplicate sample analysis in the hydrolyzate for s. AGP is 6.1 pmol of Neu5Ac and 1.1 pmol of Neu5Gc. Based on the determined LOQs for Neu5Ac and Neu5Gc, and because of the high degree of sialylation, the concentration of s. AGP can be reduced by a factor of 5 before reaching the LOQ of Neu5Gc and a factor of 17 before reaching the LOQ of Neu5Ac.

Conversely, b. apo-transferrin (39 ng per injection), as shown in Figure 3, contains less total sialic acid, and lower hydrolysis amounts are not recommended. Depending on the degree of sialylation, the amount of protein hydrolyzed can be reduced to low-ng/µL concentrations and still allow efficient sialic acid determination. Designing experiments that release amounts of analyte routinely near the LOQ is not recommended. However, this evaluation highlights both the sensitivity of the method and the importance of considering the approximate protein sialylation amount when designing acid hydrolysis experiments.

Table 4 presents the results from one day of triplicate

Column: Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
Eluent: 70–300 mM sodium acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300 70 mM acetate from 2.9–3.0 min, 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Flow Rate: 0.5 mL/min
Inj. Volume: 4.5 µL (Full loop)
Temperature: 30 °C
Detection: PAD, Au on PTFE, 2 mil gasket
Samples: Neu5Ac and Neu5Gc standard

Peaks: 1. Neu5Ac 11 pmol
2. Neu5Gc 1.1

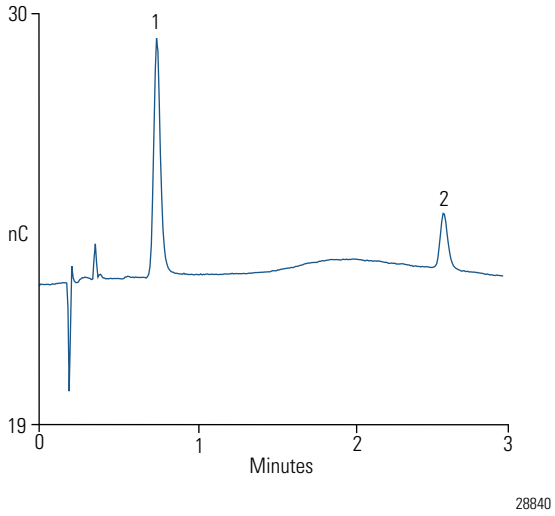

Figure 1: Separation of sialic acid standards on the Dionex CarboPac PA20 Fast Sialic Acid column.

Column:	Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
Eluent:	70-300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Inj. Volume:	4.5 µL (full loop)
Detection:	PAD, Au on PTFE, 2 mil gasket
Samples:	A) h. α_1-acid glycoprotein, 1:100 dilution (23 ng protein) B) calf fetuin hydrolyzate, 1:100 dilution (18 ng protein) C) s. α_1-acid glycoprotein hydrolyzate, 1:100 dilution (7.9 ng protein)

Peaks:	A	B	C	
1. Neu5Ac	9.8	6.3	5.6	pmol
2. Neu5Gc	—	0.17	0.93	

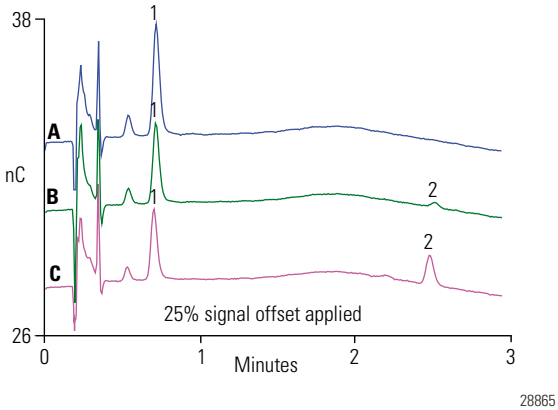

Figure 2: Separation of fetuin, h. AGP, s. AGP hydrolyzates (1:100 dilution) on the Dionex CarboPac PA20 Fast Sialic Acid column.

Column:	Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
Eluent:	70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Inj. Volume:	4.5 µL (full loop)
Detection:	PAD, Au on PTFE, 2 mil gasket
Samples:	A) h. transferrin hydrolyzate, 1:100 dilution (32 ng protein) B) b. apo-transferrin hydrolyzate, 1:100 dilution (39 ng protein)

Peaks:	A	B	
1. Neu5Ac	1.4	0.50	pmol
2. Neu5Gc	—	0.71	

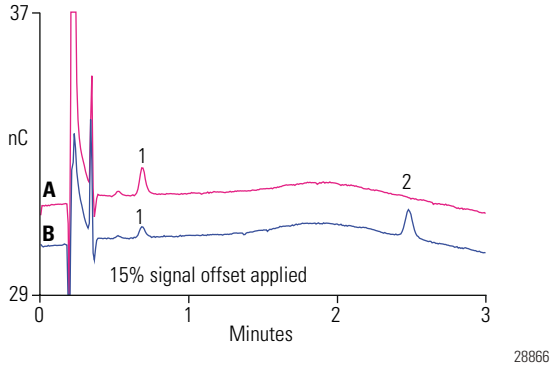

Figure 3: Separation of h. and b. apo-transferrin hydrolyzates (1:100 dilution) on the Dionex CarboPac PA20 Fast Sialic Acid column.

sample analysis. Retention time precision is similar to that determined by injecting standards, with retention time RSDs ranging from <0.01–1.18. Differences in the absolute retention time can be expected, depending on the eluent preparation. Peak area precision for triplicate injections, as measured by RSD, is generally good, ranging from 0.13–6.14. As expected, larger peak area RSD is observed near the LOQ.

Table 5 lists the calculated results of sialic acid determination for the studied proteins, as well as both intraday precision for one day of analysis and between-day precision for three days of triplicate analysis. The amounts of sialic acids determined in the protein samples are generally consistent with literature results for the glycoproteins.[19-24] However, as shown in Table 5, sample replicate precision RSDs can be greater than chromatographic precision, with intraday RSDs ranging from 2.4–11 and between-day RSDs ranging from 3.9–17. For this reason, optimization of the acid hydrolysis for individual glycoproteins is highly recommended.

Acid hydrolysis is a complex balance between release of the sialic acids from the glycoprotein and degradation of the released analytes. The efficiency of the hydrolysis depends on the hydrolysis temperature, acid concentration, type of sample being hydrolyzed, and the relative concentrations of acid and the sample. Because of these interdependent factors, which can impact the hydrolysis, variability between sample preparations must be expected. For the best accuracy, either an optimized acid hydrolysis or neuraminidase digestion is recommended. For methodology to optimize acid hydrolysis, see Fan et al.[25]

Method accuracy was investigated by spiking protein acid hydroylzates with known amounts of Neu5Ac and Neu5Gc in similar concentration as the determined amounts (Table 6). For human glycoproteins, which lack Neu5Gc, 0.22 pmol of Neu5Gc was added. Recoveries for Neu5Ac ranged from 81–96% and recoveries for Neu5Gc were similar, ranging from 82–106%.

Sample (replicate #)	Analyte	Amount (pmol)	Retention Time (min)	Retention Time Precision (RSD)	Peak Area Precision (RSD)
Fetuin (1)	Neu5Gc	0.18*	2.58	0.19	3.35
	Neu5Ac	5.78	0.74	0.65	2.59
Fetuin (2)	Neu5Gc	0.17*	2.58	0.19	5.25
	Neu5Ac	6.31	0.75	0.65	1.23
Fetuin (3)	Neu5Gc	0.18*	2.58	<0.01	6.14
	Neu5Ac	7.44	0.74	0.65	0.99
h. Transferrin (1)	Neu5Gc	ND			
	Neu5Ac	1.42	0.72	0.67	2.62
h. Transferrin (2)	Neu5Gc	ND			
	Neu5Ac	1.52	0.72	0.67	1.75
h. Transferrin (3)	Neu5Gc	ND			
	Neu5Ac	1.64	0.72	0.67	2.50
b. apo-Transferrin (1)	Neu5Gc	0.91	2.51	<0.01	2.75
	Neu5Ac	0.61	0.70	0.68	3.23
b. apo-Transferrin (2)	Neu5Gc	0.91	2.51	0.19	1.49
	Neu5Ac	0.63	0.71	<0.01	2.93
b. apo-Transferrin (3)	Neu5Gc	0.88	2.51	0.19	2.23
	Neu5Ac	0.61	0.71	<0.01	2.14
h. AGP (1)	Neu5Gc	ND			
	Neu5Ac	15	0.72	1.34	1.14
h. AGP (2)	Neu5Gc	ND			
	Neu5Ac	13	0.72	1.16	0.98
h. AGP (3)	Neu5Gc	ND			
	Neu5Ac	12	0.72	0.67	0.95
s. AGP (1)	Neu5Gc	1.0	2.52	0.57	1.18
	Neu5Ac	5.8	0.71	0.68	2.33
s. AGP (2)	Neu5Gc	1.2	2.52	0.57	1.70
	Neu5Ac	6.6	0.71	1.18	2.64
s. AGP (3)	Neu5Gc	1.0	2.52	0.33	0.13
	Neu5Ac	5.9	0.71	0.68	2.52

*Neu5Gc calibration range extended from 0.11 pmol–11 pmol, $r^2 = 0.9995$.

Table 4: Sialic acid determination from five glycoprotein acid hydrolyzates.

Sample	Analyte	Acid Hydrolysis Average (mol analyte/mol protein)	Intraday Precision Between Replicates (RSD)	Between-Day Precision (RSD)
Fetuin	Neu5Gc	0.33	7.2	7.3
	Neu5Ac	15	8.8	7.0
h. Transferrin	Neu5Gc	ND	—	—
	Neu5Ac	3.1	6.7	17
b. apo-Transferrin	Neu5Gc	1.4	2.4	3.9
	Neu5Ac	1.1	3.5	13
h. AGP	Neu5Gc	ND	—	—
	Neu5Ac	29	11	14
s. AGP	Neu5Gc	4.7	6.2	8.7
	Neu5Ac	26	6.4	9.0

Table 5: Triplicate sample analysis results of between-day precision over three days.

Sample	Analyte	Average Native Amount (pmol)	Added Amount (pmol)	Recovery (%)
Hydrolyzate blank	Neu5Ac	ND	2.2	94 ± 5.8
	Neu5Gc	ND	0.22	92 ± 7.1
Fetuin	Neu5Ac	5.8	2.2	84 ± 1.0
	Neu5Gc	0.18	0.22	86 ± 2.2
h. Transferrin	Neu5Ac	1.6	2.2	95 ± 4.1
	Neu5Gc	ND	0.22	94 ± 2.4
b. Apo-transferrin	Neu5Ac	0.35	1.8	87 ± 5.0
	Neu5Gc	0.45	0.90	95 ± 3.0
h. AGP	Neu5Ac	4.5	3.6	91 ± 1.5
	Neu5Gc	ND	0.36	89 ± 3.4
s. AGP	Neu5Ac	5.8	4.5	94 ± 1.9
	Neu5Gc	1.0	0.45	98 ± 6.9

Table 6: Accuracy of analysis as measured by recovery (n = 3).

Conclusion

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis release followed by HPAE-PAD. The method is both specific and direct, eliminating the need for sample derivatization common in other chromatographic methods. Good recoveries, precision, and linear detection for Neu5Ac and Neu5Gc are demonstrated, indicating the method is appropriate for glycoprotein analysis. Using the Dionex CarboPac PA20 Fast Sialic Acid column, this rapid method separates Neu5Ac and Neu5Gc with a total analysis time of <5 min, providing high-throughput sample analysis while reducing eluent consumption and waste generation.

Suppliers

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Integrated LC/MS Workflow for the Analysis of Labeled and Native N-Glycans from Proteins Using a Novel Mixed-Mode Column and a Q Exactive Mass Spectrometer

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Application Note 595

Key Words
GlycanPac AXH-1, HILIC, WAX, glycomics, glycoproteins, glycopeptides, glycans, labeled *N*-glycans, Q Exactive, SimGlycan software

Goal
Develop a comprehensive method for the structural characterization of released glycans from proteins. The described integrated method covers sample preparation, separation, mass spectrometry data acquisition, and analysis.

Introduction
Glycans are widely distributed in biological systems in 'free state' as well as conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They play significant roles in many biological and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development.[1] Glycans can affect efficacy and safety of protein based drugs. For example, recombinant proteins and monoclonal antibodies (mAb) are often dependent on the structure and types of glycans attached to the proteins.[2] The structures of glycans are diverse, complex, and heterogeneous due to post-translational modifications (PTMs) and physiological conditions. Minor changes in glycan structure can result in striking differences in biological functions and clinical applications. The structural characterization of glycans is essential in bio-therapeutics and bio-pharmaceutical projects.[3] In addition to the characterization of the sugar sequence, the analysis must elucidate linkages and separate all isomeric, charge, and branching variations of glycans.

Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans. Hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine, or zwitterionic-based packing materials are often used for glycan analysis. These HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and composition-based separation. A limitation of this approach is that identification of the glycan charge state is not possible due to the fact that glycans of different charge states are intermingled in the separation envelope.

The Thermo Scientific™ GlycanPac™ AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural analysis of glycans, either labeled or native, by LC-fluorescence or LC/MS methods.The GlycanPac AXH-1 column is based on innovative mixed-mode surface chemistry combining both weak anion-exchange (WAX) and HILIC retention mechanisms. The WAX functionality provides retention and selectivity for negatively charged glycans, while the HILIC mode facilitates the separation of glycans according to their charge, polarity, and size. As a result, the GlycanPac AXH-1 column provides unparalleled separation capabilities for glycans.

LC-MS/MS analysis of glycans requires the processing of large sets of data. The incorporation of SimGlycan® software (PREMIER Biosoft) alleviates this issue, thus enabling the development of a true high-throughput workflow.

This application note presents a step-by-step method for the release, labeling, separation, and structural elucidation of *N*-glycans from proteins by LC-MS/MS.

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Experimental Conditions

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific™, AC610010040)
- LC/MS grade formic acid (Fisher Scientific, A117-50)
- Ammonium formate (Fisher Scientific, AC40115-2500)
- Thermo Scientific Premium 2 mL vial convenience kit, 60180-600
- PNGase F (New England BioLab, P0705L)
- Bovine fetuin (Sigma-Aldrich®, F2379)
- Thermo Scientific™ Hypercarb™ cartridge, 6 mL, 60106-403
- Trifluoracetic acid (Fisher Scientific, 28904)
- Sodium cyanoborohydride (Fisher Scientific, AC16855-0500)
- Anthranilamide (2AB) (Fisher Scientific, AC10490-5000)
- Glacial acetic acid (Fisher Scientific, AA36289AP)
- Dimethylsulfoxide (DMSO) (Fisher Scientific, D128500LC)
- Sodium hydroxide (NaOH) (Fisher Scientific, S318-100)
- Ammonium acetate (Fisher Scientific, A637-500)
- SEC column, 0.9 x 50 cm Sephadex® (GE Healthcare, G-10-120)
- GlykoClean™ G Cartridges, Prozyme, GC250
- 2-mercaptoethanol (Fisher Scientific, O3446I-100)

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 system, including pump: LPG-3400RS, thermal compartment: TCC-3000RS, pulled-loop well plate auto sampler: WPS-3000TRS, fluorescence detector with Dual-PMT: FLD3400RS, and 2µL micro flow cell: 6078.4330
- Q Exactive hybrid quadrupole-Orbitrap mass spectrometer
- Thermo Scientific™ SpeedVac™ Concentrator
- Thermo Scientific Lyophilizer (Labconco® FreeZone® -105 °C 4.5 L benchtop freeze dry system) 16-080-207
- Thermo Scientific 24-Port SPE vacuum manifold, 60104-233

Buffer Preparation

- Ammonium formate (80 mM, pH 4.4):
  Dissolve 5.08 ± 0.05 g of ammonium formate (crystal) and 0.60 g of formic acid in 999.6 g of DI water. Sonicate the resulting solution for 5 min.
- 0.1 M sodium phosphate buffer, pH 7.25:
  Add 102.24 mg of Na_2HPO_4 and 38.14 mg of NaH_2PO_4 to 10 mL of DI water. Vortex to mix the solid completely. Verify that the pH of the solution is 7.25 ± 0.02.

Release of *N*-Glycans from Proteins

1. Dissolve 1 mg of the bovine fetuin protein in 500 µL of 0.1 M sodium phosphate buffer, pH 7.2 ± 0.05, in an Eppendorf tube.
2. Add 0.5 µL of 2-mercaptoethanol to this solution.
3. Finally, add 50 U (units) of PNGase F and incubate total solution at 37 °C water bath for 18 h.
4. Cool to room temperature and purify the released glycans as described in the next section.

Purification of *N*-Glycans

Purify free glycans after digestion using a Hypercarb cartridge as follows:

1. Attach a single Hypercarb cartridge per reaction to a designated port in the SPE manifold.
2. Slowly, and with a consistent flow rate, pre-treat each cartridge with the following volumes of reagents in the order described: 15 mL of 1M NaOH, 15 mL of HPLC grade water, 15 mL of 30% acetic acid, 15 mL of HPLC grade water.
3. Prime the cartridge with 15 mL of 50% acetonitrile/0.1% trifluoroacetic acid (TFA), followed by 15 mL of 5% acetonitrile/0.1% TFA.
4. Load the entire sample volume into the cartridge and let it permeate into the resin by pulsing the vacuum on and off quickly.
5. Rinse the reaction tube with ~50 µL of HPLC grade water, transfer into the cartridge, and pulse the vacuum again.
6. Wash the cartridge with 15 mL of HPLC grade water, followed by 15 mL of 5% acetonitrile/0.1% TFA.
7. Elute the glycans with 4 x 2.5 mL of 50% acetonitrile/ 0.1% TFA into a labeled 15 mL conical tube.
8. Immediately freeze samples on dry ice and then lyophilize to dryness (16–24 h).
9. After lyophilization, dissolve the solid in 1 mL of water, dry the samples again in a 1.5 mL Eppendorf tube, and store at -20 °C.

2AB Labeling Reaction

Carry out the labeling reaction using a modified reported procedure.[4]

1. Prepare the 2AB labeling reagent (100 µL): Dissolve 2-aminobenzamide (4.6 mg) in 70 µL of DMSO.
2. Add 30 µL of glacial acetic acid (100%) to the mixture.
3. Transfer the complete solution to a black or light-protected, screw-cap, 1.5 mL Eppendorf tubes containing 6.4 mg of sodium cyanoborohydride.
4. Incubate the solution at 60 °C for 10 min to dissolve sodium cyanoborohydride completely. Occasionally vortex the solutions. When all the solids are completely dissolved, the 2AB labeling reagent is ready to use for the labeling reaction.
5. Add 20 µL of 2AB labeling reagent to 50 µg of free glycans and vortex to mix the solution. Then, incubate the mixture at 60 °C for 3 h.

Clean Up of Labeled Glycans

1. After completion of the 2AB reaction, add 250 µL of acetonitrile to the vial at room temperature.
2. Purify the samples using a GlykoClean G cartridge; pre-equilibrate the column with the following solutions in the order they appear: wash with 3 mL of deionized water, 3 mL acetonitrile, 3 mL of 96% acetonitrile.
3. Add the labeled glycans to the pre-equilibrated column.
4. Wash with 96% acetonitrile.
5. Elute the glycans with 5 mL of DI water.
6. Lyophilize the solution to dryness.
7. Upon dryness, dissolve the sample in 500 µL of water.
8. Further purify the labeled glycans using a size-exclusion chromatography (SEC) Sephadex® column to get highly pure labeled oligosaccharides.
9. Inject the samples onto an SEC column connected to a UV detector. Equilibrate the column with 10 mM ammonium acetate at a flow rate of 0.35 mL/min until a steady baseline of 205 nm is achieved.
10. Run the column with 10 mM ammonium acetate for 90 min and collect glycan containing fractions using UV detection at 205 nm.
11. Dry the combined fractions by lyophilization, re-suspend with 1 mL of DI water. Quantify the glycans[5] and then store the remaining sample at -20 °C for future use.
12. Ready for use as 2AB labeled *N*-glycan from fetuin.

Sample Preparation for Injection

1. Mix 25 µL of purified labeled glycans at 0.2 nmol/µL in DI water with 75 µL of acetonitrile.
2. Transfer the total solution to the auto sampler vial for analysis.

Note: Store the standard at -20 °C.

Separation Conditions

Column	GlycanPac AXH-1, 2.1 x 150 mm, 1.9 µm
Mobile phase	A: acetonitrile + water (80:20, v/v)
	B: ammonium formate (80 mM, pH 4.4)
Flow rate (µL/min)	400
Column temperature (°C)	30
Sample volume (injected) (µL)	1
Mobile phase gradient	Refer to Table 1

Table 1. Mobile phase gradient

Time (min)	% A	%B	Flow (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

MS Conditions

MS instrument	Q Exactive hybrid quadrupole-Orbitrap MS
Source	HESI-II probe
Ionization mode	Negative ion
Full MS	
MS scan range (*m/z*)	380–2000
Resolution	70,000
Microscans	1
AGC target	1×10^6
Max IT (ms)	60
dd-MS2	
dd-MS2 resolution	17,500
Microscans	3
MS/MS AGC target	2×10^5
MS/MS max IT (ms)	250–1000
Isolation window (*m/z*)	2
NCE	35
Stepped NCE	8%
Dynamic exclusion (s)	90

Source Conditions

Source position	C
Sheath gas flow rate (arb units)	20
Auxillary gas flow rate (arb units)	5
Sweep gas flow rate	0
Spray voltage (kV)	3.30
Capillary temperature (ºC)	275
S-lens RF level	50
Heater temperature (ºC)	300

Data Processing and Software

Chromatographic software	Thermo Scientific™ Chromquest™ v 5.0 Chromatography Data System
MS data acquisition	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
MS/MS data analysis	SimGlycan software v 4.5

SimGlycan Search Parmeters

Ion mode	Negative
Adduct	H
Chemical derivatization	Underivatized
Match fragment ion for charge state	< Precursor *m/z* charge state
Precursor ion *m/z*	10 ppm
Fragment ion	0.05 Da
Modification	2AB
Class	Glycoprotein
Sub class	*N*-glycan (Intact Core)
Biological source	Bovine Fetuin
Pathway	Unknown
Search structure	All
Glycan type	All
% of evident glycosidic linkages	2
Fragmentation pattern	Specify Expected Fragments in the Spectra
Glycosidic	B: Yes; C: Yes; Y:Yes; Z:Yes
Cross-ring	A:Yes; X:Yes
Glycosidic/Glycosidic	Z/Z: Yes; Y/Y: Yes; B/Y or Y/B: Yes; C/Z or Z/C: No; Z/Y or Y/Z: No; B/Z or Z/B: No; C/Y or Y/C: Yes
Cross-ring/Glycosidic	A/Y or Y/A: Yes; A/Z or Z/A:Yes; X/Y or Y/X: Yes; X/Z or Z/X: No; X/B or B/X: Yes; X/C or C/X: Yes

Results and Discussion

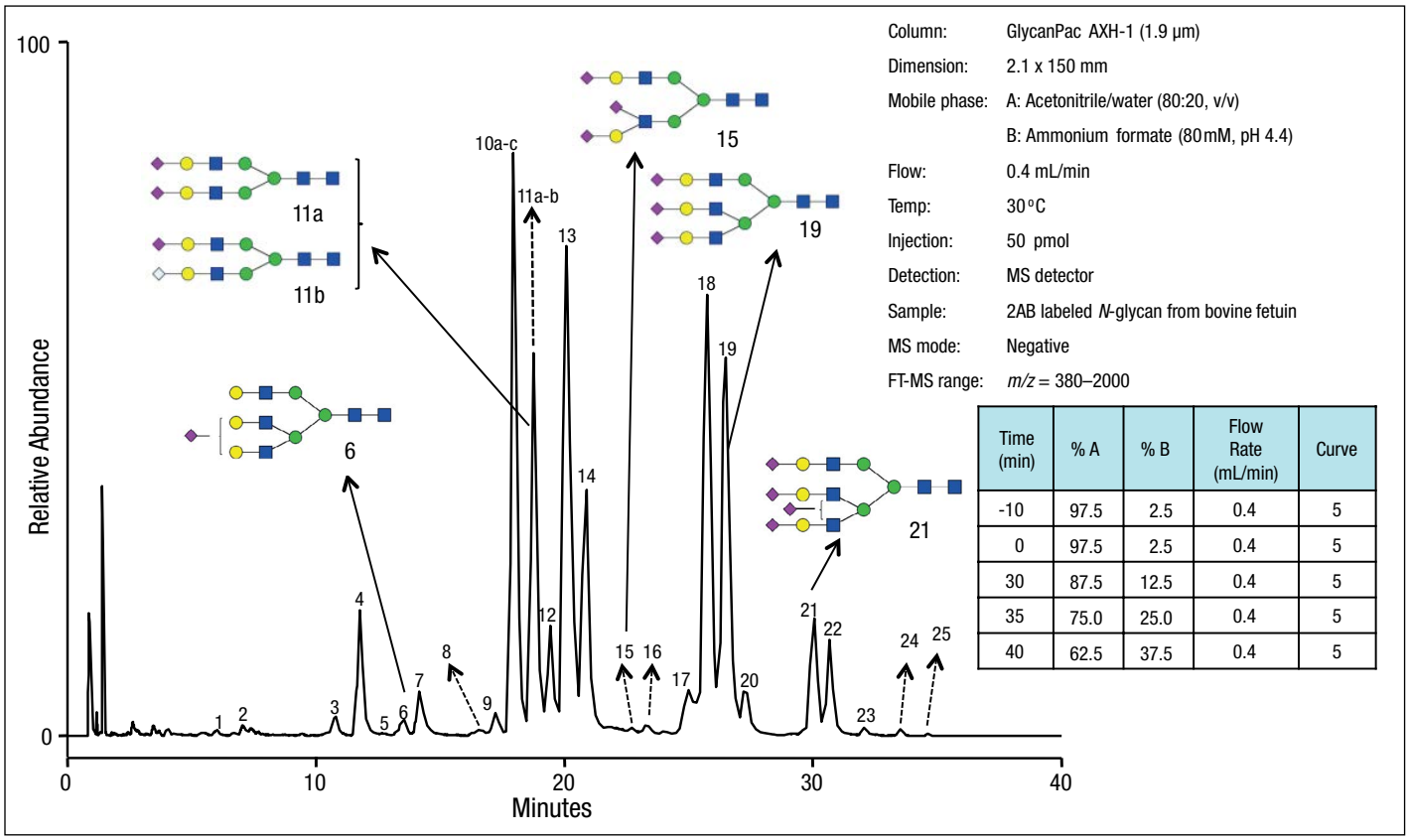
The protocol outlined in this application note yields detailed information on the set of glycans present in proteins including mAbs. The protocol describes a fully integrated workflow that combines novel column technology (GlycanPacAXH-1 column), mass spectrometry (Q Exactive mass spectrometer), and a bioinformatics tool (SimGlycan software). This fully integrated workflow is demonstrated for *N*-glycans released from bovine fetuin glycoprotein, but can be used for released *N*-glycans from any glycoprotein.

The GlycanPac AXH-1 column described in this application note can be used for qualitative and quantitative characterization of neutral and charged glycans present on proteins. The elution of glycans is based on charge: the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated to penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. Separation of glycans based on charge, size, and polarity–combined with MS–provides complete structural and quantitative information.

2AB labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXH-1 column and analyzed on a Q Exactive mass spectrometer (Figure 1). Data-dependant MS/MS spectra were acquired on all precursor ions ($z \geq 2$), and SimGlycan software was used for structural elucidation. A representative example of the analysis is shown in Figure 2. The Q Exactive mass spectrometer was selected for these experiments because of its 140,000 FWHM resolution at *m/z* 200, high scan speeds at all resolution settings, and sensitivity. All of these contribute to the detection of minor glycan species and generation of high-quality MS/MS spectra even for low-abundance glycans.

Additionally, the Q Exactive mass spectrometer has the ability to generate higher-energy collisional dissociation (HCD) with high-resolution, accurate-mass (HR/AM) fragment ions. This allows for differentiation of near-mass fragment ions, which were observed to be useful for correctly assigning branching and linkage. The variation of collision energy can provide different fragment ions within the mass spectrometer. To maximize both glycosidic and cross-ring fragments, normalized stepped collision energy (NSCE) was incorporated. This provided optimum conditions for generation of a maximum number of both cross-ring and glysodic cleavages in a single spectrum, thereby increasing confidence in the identification (Figure 2). The detailed structural information obtained from the MS/MS data shown in Table 2 further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, and polarity.

The use of LC-MS/MS for glycan analysis increases the complexity of data analysis due to the large number of MS/MS spectra generated. SimGlycan software was incorporated to simplify data analysis.[6,7] SimGlycan software predicts the structure of a glycan from the MS^n data. It accepts the raw MS^n files, matches them with its own database of theoretical fragmentation of over 22,000 glycans, and generates a list of potential glycan structures. Each proposed structure is assigned a score to reflect how closely it matches with the experimental data.



Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Figure 1. LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin by GlycanPac AXH-1 column with MS detection

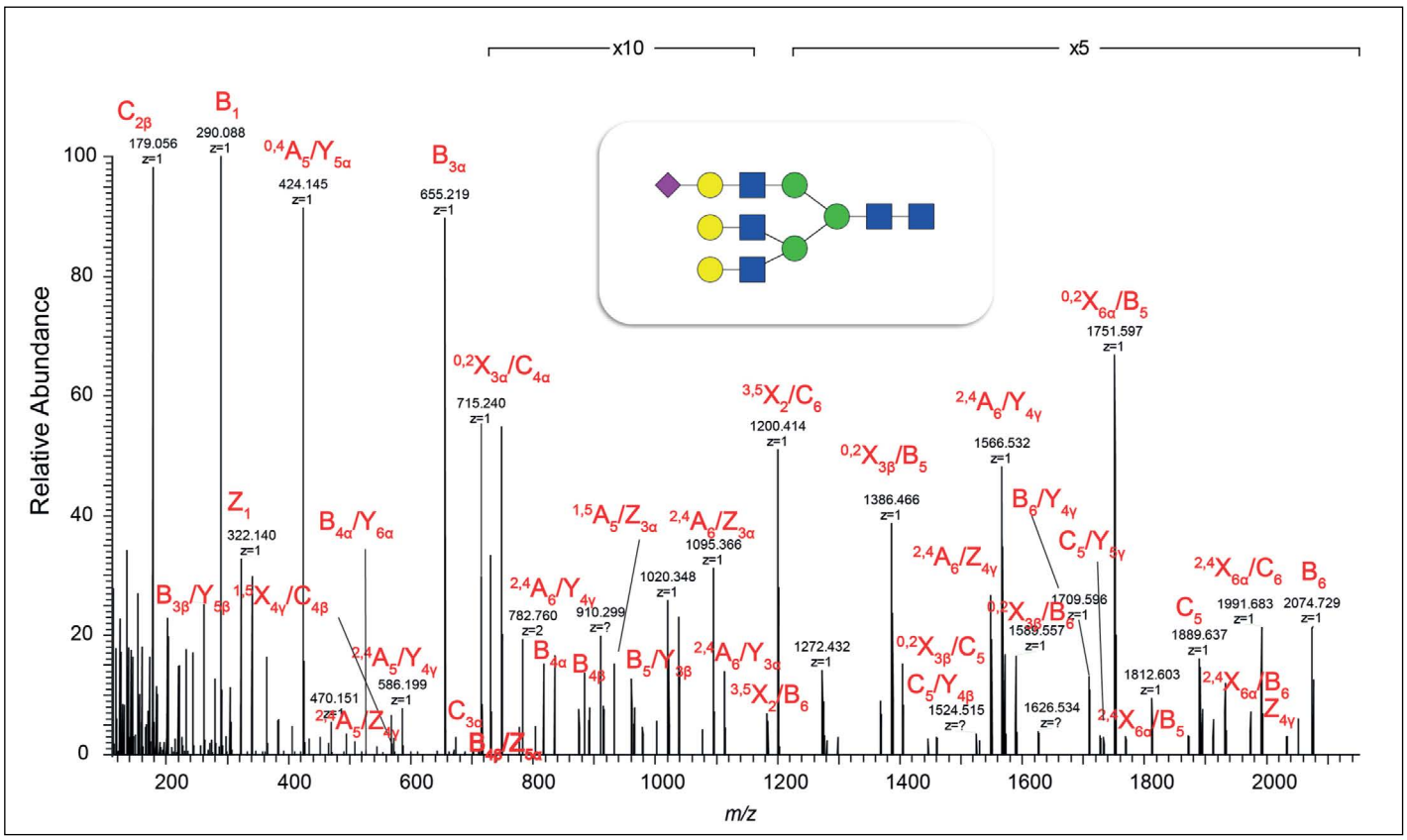
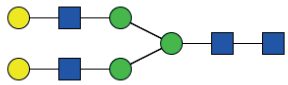
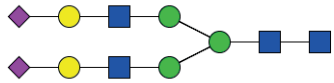
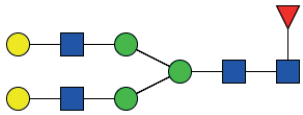
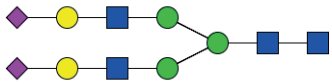
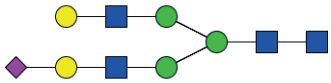
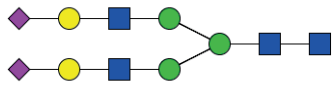
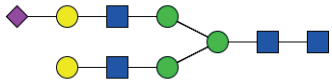
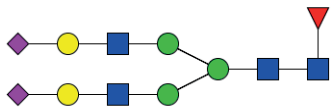
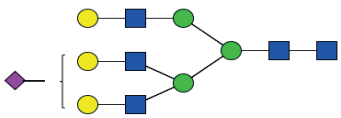
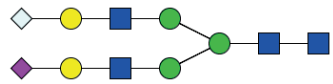
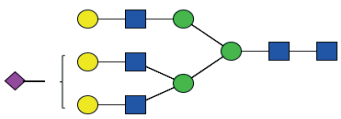
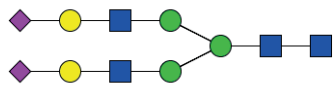
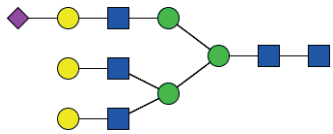
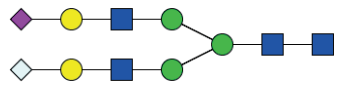
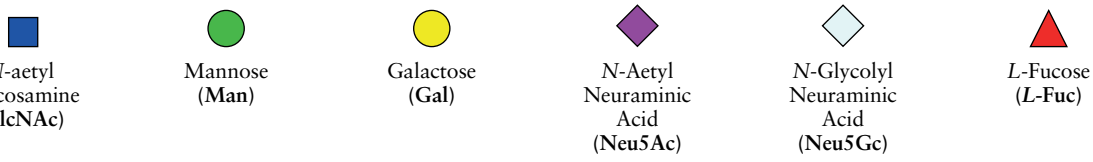
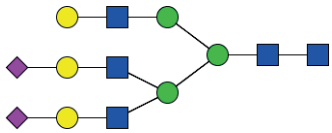
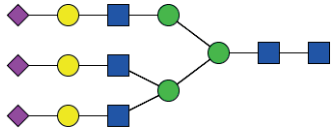
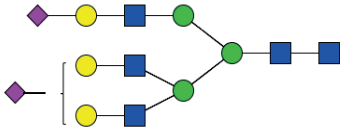
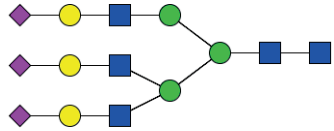
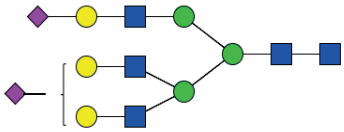
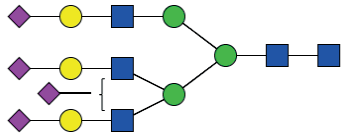
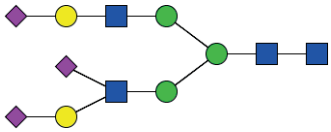
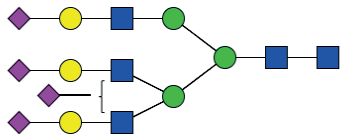
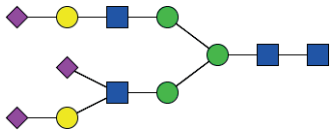
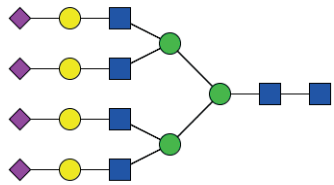
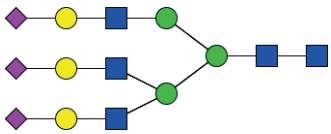
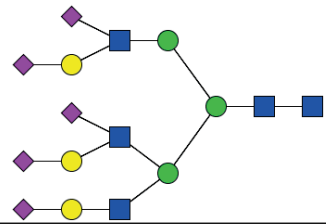
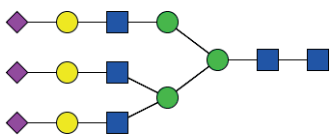
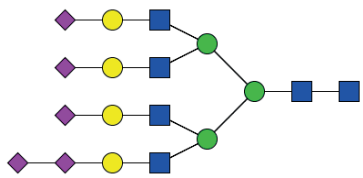


Figure 2. HCD MS/MS spectrum of a 2AB-labeled monosialylated triantennary *N*-glycan from bovine fetuin

Table 2. Structural identification of glycans present in each peak by the separation of 2AB labeled *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

Peak (Figure 1)	Compound structure (2AB labeling is not shown)	Peak (Figure 1)	Compound structure (2AB labeling is not shown)
1		8	
2		9	
3		10a	
4		10b	
5		10c	
6		11a	
7		11b	

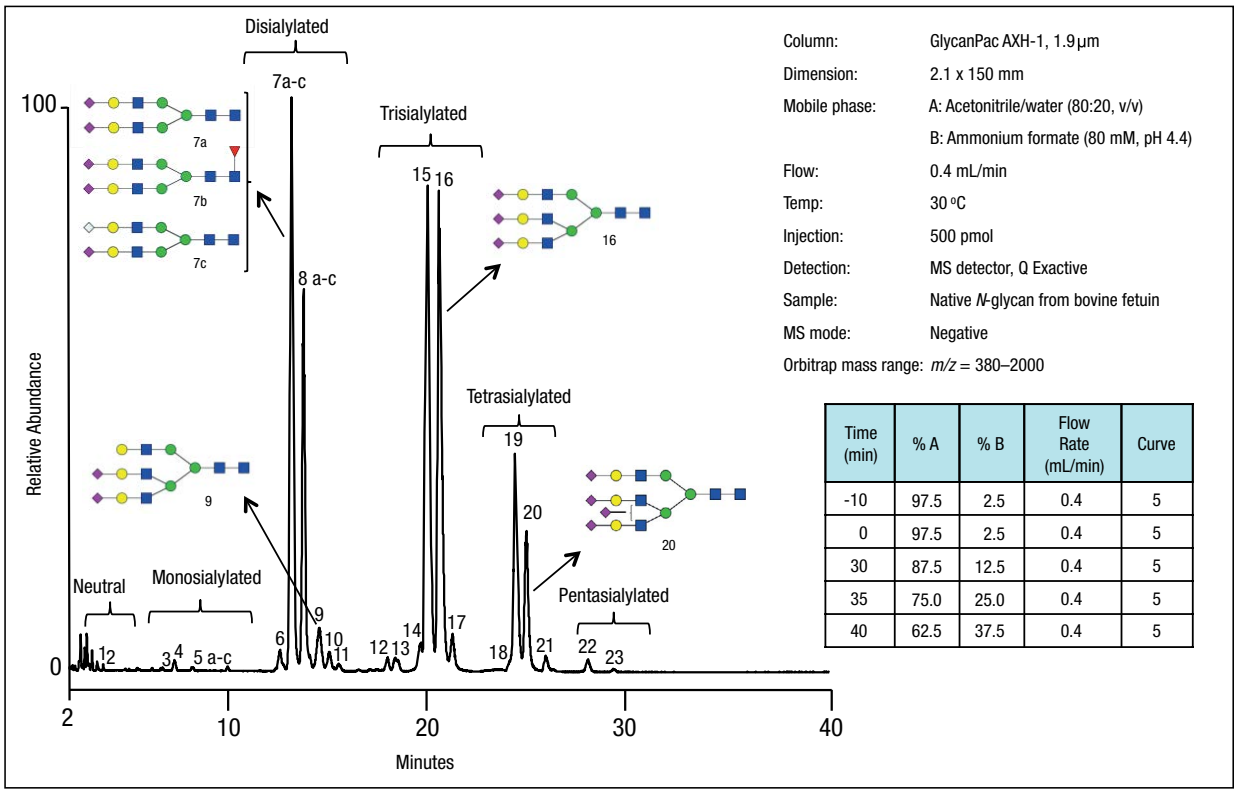


12		19	
13		20	
14		21	
15		22	
16		23	
17		24	
18		25	

LC-MS Analysis of Native *N*-Glycans Released from Proteins

The GlycanPac AXH-1 column is also suitable for analysis of native glycans. Analyzing unlabeled glycans not only eliminates the extra reaction step and cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

Figure 3 shows the LC/MS analysis of native *N*-glycans from bovine fetuin using the GlycanPac AXH-1 column. Detailed information is in Table 3. A representative MS/MS spectrum for a trisialylated triantennary glycan is shown in Figure 4.



Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Figure 3. LC/MS analysis of native *N*-glycan from bovine fetuin

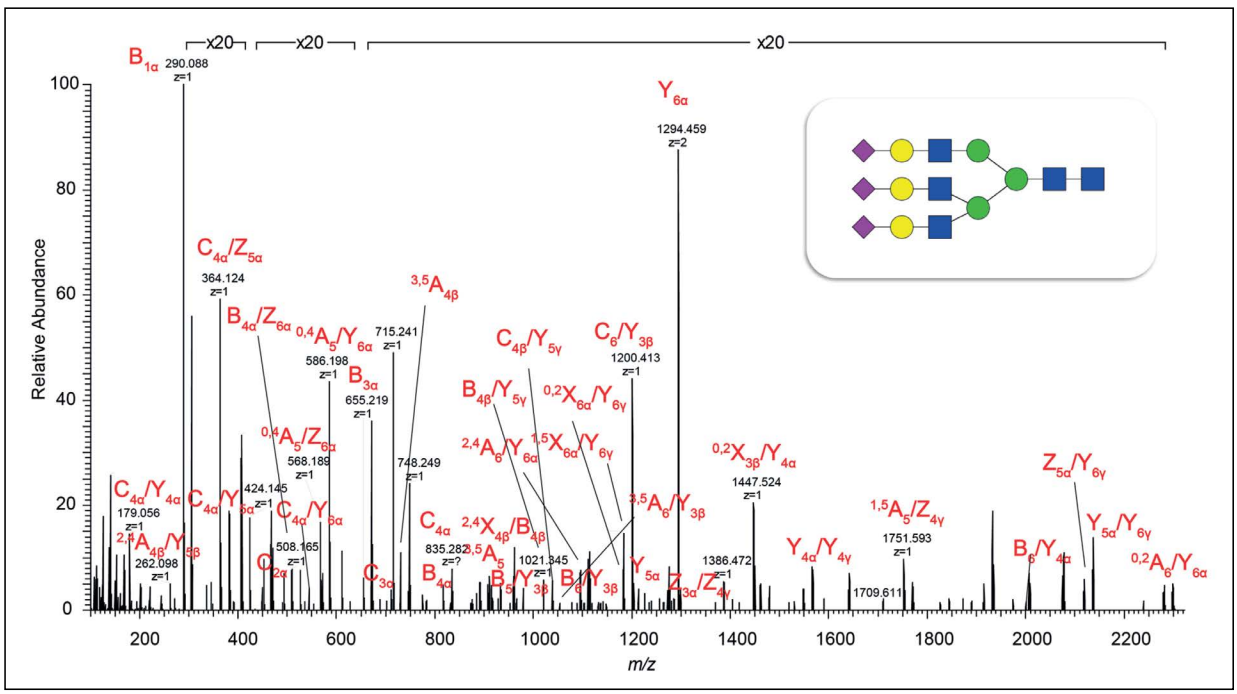


Figure 4. MS/MS spectra for a native trisialylated triantennary *N*-glycan released from bovine fetuin

Table 3. Structural identification of glycans present in each peak by the separation of native *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

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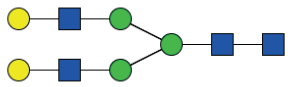
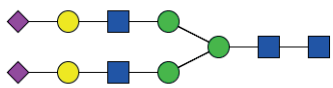
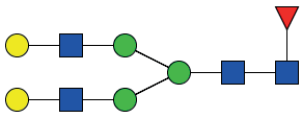
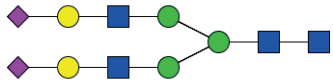
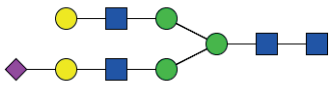
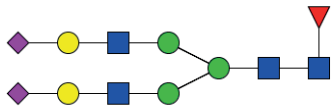
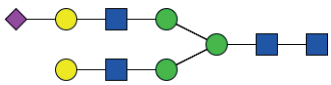
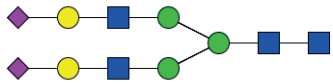
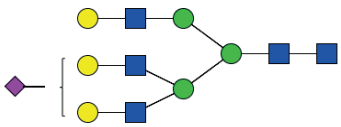
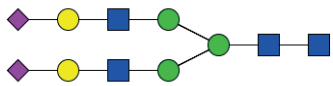
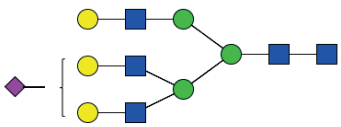
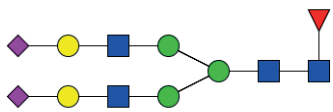
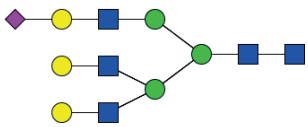
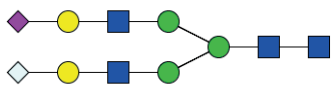
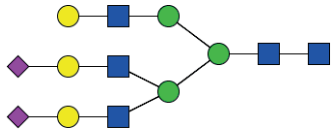
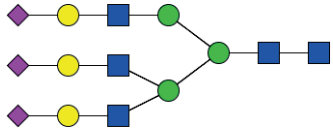
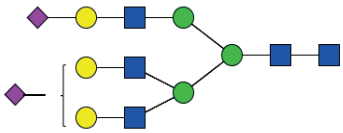
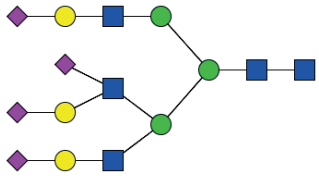
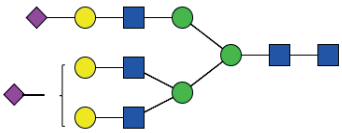
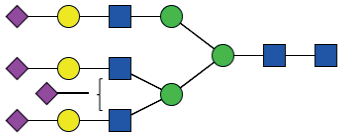
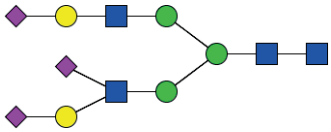
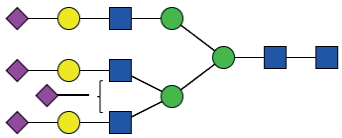
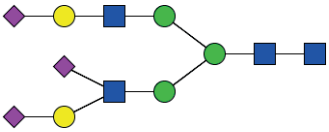
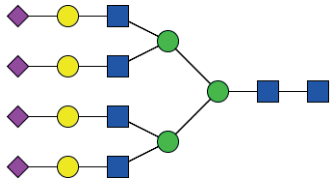
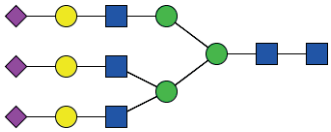
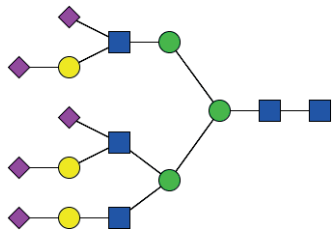
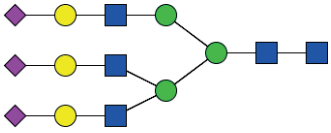
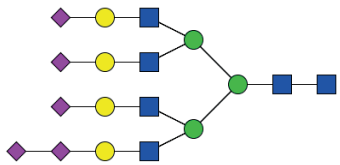
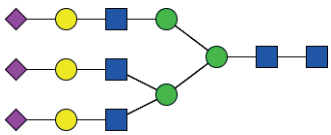
1		6	
2		7a	
3		7b	
4		7c	
5a		8a	
5b		8b	
5c		8c	

9		17	
10		18	
11		19	
12		20	
13		21	
14		22	
15		23	
16			

Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially for glycans containing multiple sialic acids (Figure 3). However, labeled glycans require smaller amounts (10 times) of samples for MS analysis as compared to native glycans. Thus, the GlycanPac AXH-1 column is useful for the analysis of biologically relevant glycans including glycans from antibodies, either labeled or native, by LC-fluorescence or LC-MS methods. If the amount of the sample is not extremely limited, analysis of unlabeled glycans using the GlycanPac AXH-1 is highly feasible.

Conclusion

- A fully integrated workflow for structural characterization of native and fluorescently labeled *N*-glycans released from proteins was demonstrated successfully.
- Novel GlycanPac AXH-1 column demonstrated excellent separation of released N-glycans especially forsilalylated species. It allowed for their sensitive detection by the Q Exactive mass spectrometer and identification by SimGlycan software.
- This LC-MS integrated technology is also useful for the separation and structural characterization of reduced *O*-linked glycans from proteins, mucins, and the analysis of charged and neutral glycosylaminoglycans and glycolipids.

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An Ultra High Resolution Glycan Column for Isomeric Separation and the Structural Identification of Labeled *N*-Glycans from Proteins Including Antibodies

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Overview

Purpose: Separation and identification of various complex *N*-linked glycans from proteins using a novel high resolution mixed-mode column and an Orbitrap Fusion Tribrid mass spectrometer.

Methods: Fluorescently labeled glycans from various proteins were separated and analyzed on a Thermo Scientific™ GlycanPac™ AXR-1 column coupled to a Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ mass spectrometer. Data analysis was performed using SimGlycan® software.

Results: The GlycanPac AXR-1 column coupled to Orbitrap Fusion mass spectrometer enabled resolution of twice as many peaks and identification of four times as many structures for 2AB-labeled *N*-linked glycans from bovine fetuin compared to other commercially available column technologies. Furthermore, this column allows direct injection of aqueous samples.

Introduction

Glycans are involved in a wide range of biological and physiological processes including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development. The functions of glycans are often dependent on the structure and types of glycans attached to the proteins. *N*-linked glycans are commonly investigated as important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficacy are affected by glycosylation. Understanding, measuring, and controlling glycosylation in glycoprotein-based drugs, the glycan content of glycoprotein products, as well as thorough characterization of biosimilars have become increasingly important. The structures of glycans are highly diverse, complex and heterogeneous due to post-translational modifications. Thus, it is challenging to comprehensively characterize glycan profiles and determine their structures [1].

Various modes of HPLC separation have been developed for the analysis of glycans [2-4]. Glycans are highly hydrophilic polar substances, and therefore one common separation mode utilizes amide hydrophilic interaction liquid chromatography (HILIC columns), which separates glycans based on hydrogen bonding, resulting in a size and composition-based separation. Amide HILIC columns are particularly useful for the separation of 2AA-labeled *N*-linked glycans released from antibodies, such as mAbs, where the majority of the glycans are neutral. However, amide HILIC amide columns do not provide a good separation when glycans are highly charged (charge≥ 2) such as sialylated *N*-linked glycans. Here glycans of different charge states are intermingled in the separation envelope.

Recently, we have developed a novel mixed-mode column (GlycanPac AXH-1) with both weak anion-exchange (WAX) and HILIC properties [5], which separate *N*-linked glycans based on charge, size, and polarity. The GlycanPac AXH-1 column provides unique charge-based separation and broader applicability in term of qualitative and quantitative structural analysis of 2AB and 2AA labeled as well as native *N*-linked glycans from proteins by both fluorescence and mass spectrometry (MS) detection [6, 7]. Here we describe the new GlycanPac AXR-1 mixed-mode column which provides an even higher resolution separation based on isomeric structure along with separation based on charge, size, and polarity.

The GlycanPac AXR-1 column is based on novel mixed-mode column chemistry, combining both WAX and reversed-phase (RP) retention mechanisms for optimal selectivity and high resolving power. The WAX functionality provides retention and selectivity for negatively charged glycans, while the reversed-phase mode facilitates the separation of glycans of the same charge according to their isomeric structure, polarity, and size. As a result, the GlycanPac AXR-1 column provides exceptional resolution with more than 4 times the glycan structures identified compared to existing commercial columns, including amide HILIC columns and the GlycanPac AXH-1 column, for 2AB-labeled *N*-linked glycans released from bovine fetuin. The GlycanPac AXR-1 column is designed for HPLC and UHPLC methods using either fluorescence or MS detection, and uses volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) and acetonitrile, presenting the eluting glycans ready for introduction into MS instruments.

Methods

Sample Preparation

Glycans were released from glycoproteins with PNGase F enzyme (New England BioLabs). The released glycans were labeled with 2-aminobenzamide (2AB) and

2-amino benzoic acid (2AA) with slight modification from the reported procedure of Bigge *et. al.*,[8] Prior to analysis, samples were dissolved in 100 µL D.I. water in a 250 µL auto sampler vial.

Liquid Chromatography

All glycans were separated on a GlycanPac AXR-1 column (1.9 µm, 2.1 × 150 mm) by a Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC instrument with either a fluorescence or MS detector.

Mass Spectrometry

MS analysis was performed using an Orbitrap Fusion Tribrid mass spectrometer in negative ion mode. LC-MS^2 and LC-MS^3 experiments were conducted for structural elucidation.

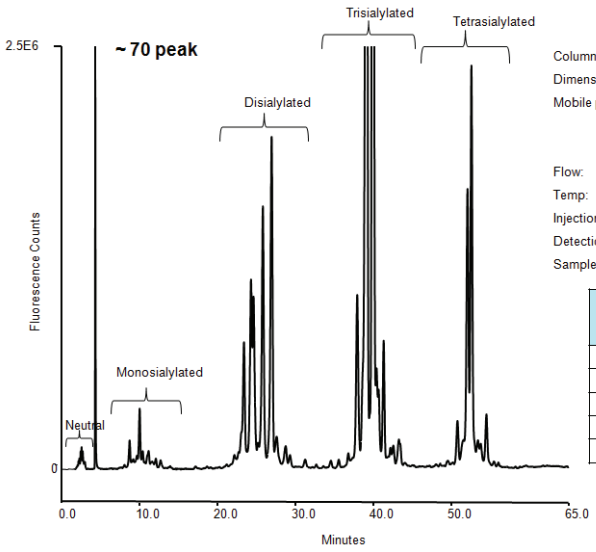
Data Analysis

SimGlycan® 4.5 software (PREMIER Biosoft) was used for MS/MS data analysis.

Results

The GlycanPac AXR-1 column is designed for high-resolution separation of neutral and charged glycans (native and labeled) present in glycoproteins, glycolipids and glycopolymers. However, it should be noted that for neutral glycans, such as those released from antibodies, it is advantageous to use 2AA-labeling technique to enhance retention as well as selectivity on the GlycanPac AXR-1 column. Figure 1 shows the separation of neutral and acidic 2AB-labeled *N*-linked glycan from bovine fetuin using a GlycanPac AXR-1 (1.9 µm, 2.1 × 150 mm) column. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, close to the void, followed by monosialylated, disialylated, trisialylated, tetrasialylated,and finally pentasialylated species. Peaks in each cluster represent the glycans of the same charge separated by ion exchange interaction. Within each cluster, glycans containing the same charge are further separated according to their isomeric structures, sizes, and polarity by reversed-phase interaction. The GlycanPac AXR-1 (1.9 µm) column provides ≥ 70 resolved peaks with ≥ 1% intensity for 2AB-labeled *N*-linked glycans from bovine fetuin. *N*-linked glycan structures present in each peak were identified using LC-MS^2 and LC-MS^3 data.

FIGURE 1. Separation of 2AB-labeled *N*-linked glycans from bovine fetuin by charge, size, polarity and isomeric structure using GlycanPac AXR-1 (1. 9µm) column by ternary gradient condition.



Column:	**GlycanPac AXR-1 (1.9 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: acetonitrile (100 %)
	B: water
	C: ammonium formate (100 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 °C
Injection:	100 pmoles
Detection:	fluorescence at 320/420 nm
Sample:	2AB labeled *N*-glycan from bovine fetuin

Time (min)	% A	% B	% C	Flow Rate (mL/min)	Curve
-10	0	95	5	0.4	5
0	0	95	5	0.4	5
1	0	95	5	0.4	5
30	1	74	25	0.4	5
65	20	50	30	0.4	5

LC-MS/MS Analysis of 2AB-Labeled *N*-linked Glycans from Bovine Fetuin using GlycanPac AXR-1 Column

The coupling of the GlycanPac AXR-1 column to MS is particularly attractive because MS enables in-depth analysis of complex glycans due to its ability to provide structural information. 2AB-labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXR-1 column and analyzed on an Orbitrap Fusion mass spectrometer.

The LC-MS profile of the GlycanPac AXR-1 column showed the highest number of resolved peaks (≥ 70) for bovine fetuin glycans ever achieved (Figure 2), more than doubling the number the existing commercially available stationary phases can resolve. The commercially available HILIC amide column (1.7 µm, Figure 2) was only able to resolve 26 peaks. Most commercial stationary phases are poor for separating glycan structural isomers. A single LC peak using these columns can have many structural isomers. So in most instances mixed MS^2 spectrum are generated that contain fragment ions from multiple glycans making it extremely difficult to assign correct structures. The GlycanPac AXR-1 column can resolve structural isomers (Figure 4). The ability to resolve structural isomers introduces complexity to analysis. Namely, far more MS/MS spectra need to be triggered in a single LC-MS^2 analysis. Additionally, wider dynamic range and sensitivity are needed from MS to detect and generate good quality MS^2 spectra not only for the most abundant glycans but the low abundant species as well (Figure 5). Orbitrap Fusion with it's wide dynamic range and ultrahigh mass resolution of makes it the ideal platform for looking deeper into the glycome and confidently identifying low-abundance glycans. Overall, 135 unique glycan structures were identified using a combination of GlycanPac AXR-1 column and Orbitrap Fusion

FIGURE 2. LC-MS analysis of 2AB-labeled *N*-linked glycans from bovine fetuin by GlycanPac AXR-1 column with MS detection.

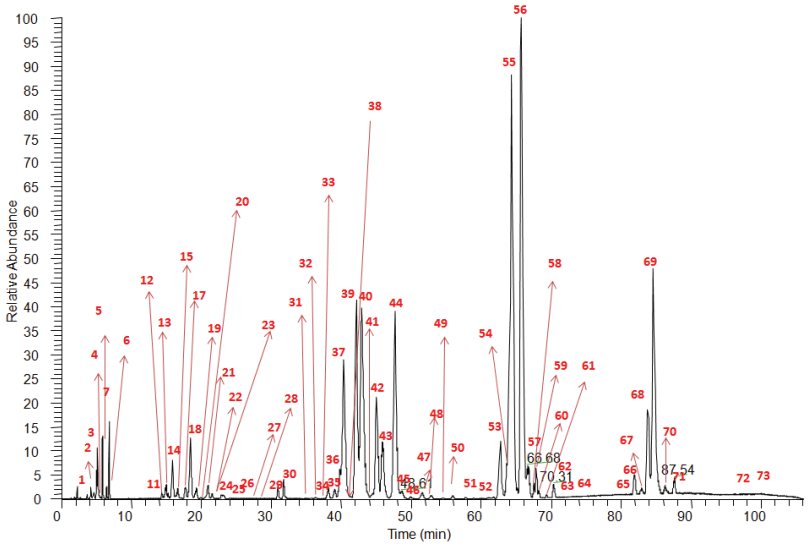
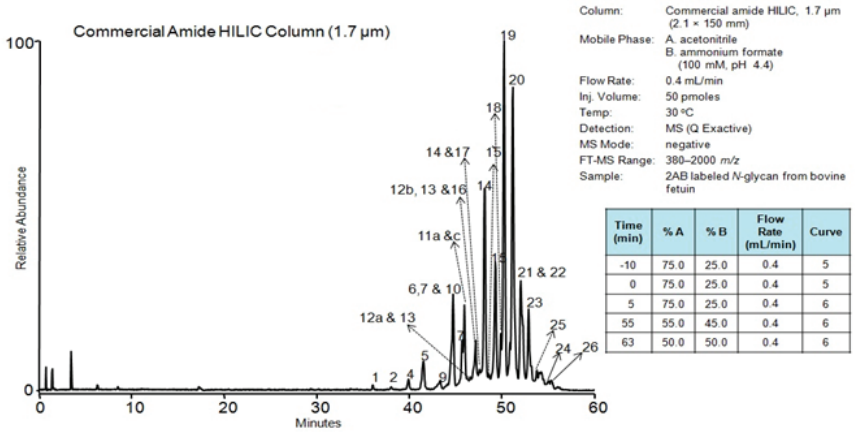


FIGURE 3. LC-MS analysis of 2AB-labeled *N*-linked glycans from bovine fetuin by a commercial amide HILIC column (1.7 µm) with MS detection.



Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	75.0	25.0	0.4	5
0	75.0	25.0	0.4	5
5	75.0	25.0	0.4	6
55	55.0	45.0	0.4	6
63	50.0	50.0	0.4	6

FIGURE 4. Separation of disialylated biantennary glycans from bovine fetuin based on α2-3 and α2-6 sialic acid linkage by the GlycanPac AXR-1 column.

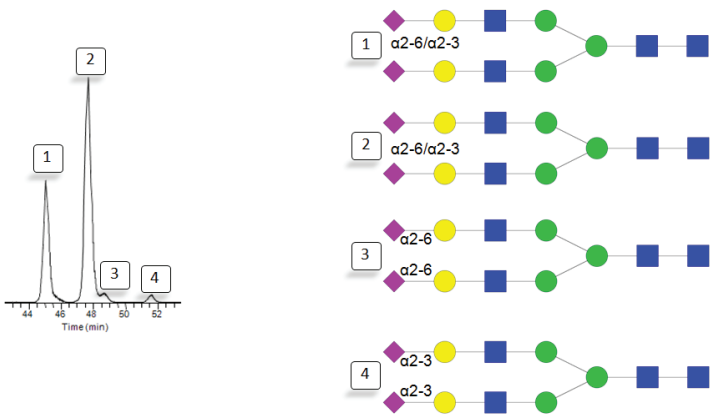
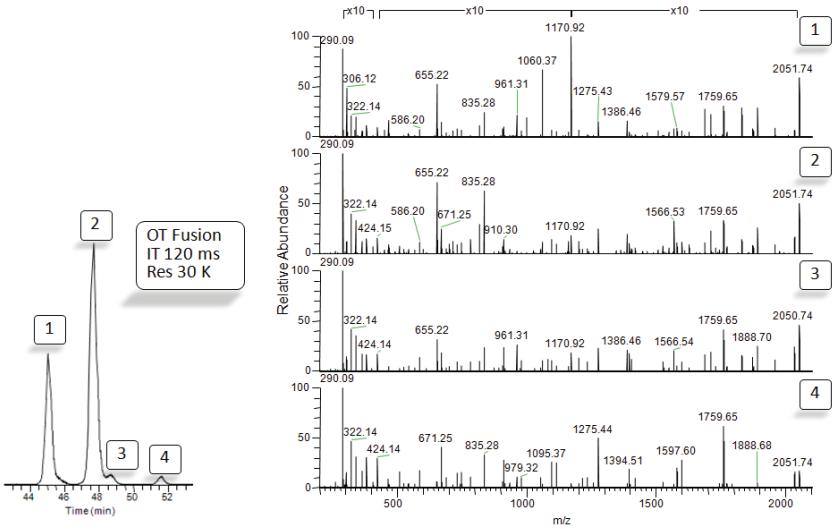


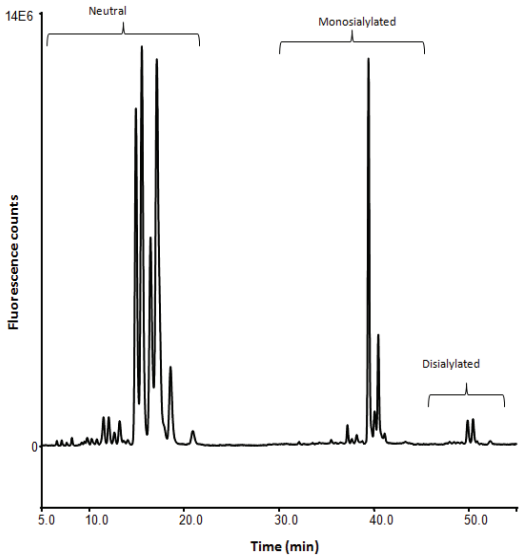
FIGURE 5. LC-MS/MS (HCD) spectra of 2AB-labeled *N*-linked glycans from bovine fetuin.



Analysis of 2AA-Labeled Antibody Glycans

Unlike 2AB, 2AA labeling introduces a formal negative charge to each glycan. This promotes greater binding to the GlycanPac AXR-1 column, thus improving retention of both neutral and negatively charged glycans. Antibodies are the most common proteins developed for therapeutics, and are under development for the treatment of numerous diseases. However, antibody glycosylation is a major source of heterogeneity with respect to both structure and therapeutic function. Glycosylation variants are primary factors in batch-to-batch antibody variation, altering product stability in vivo, and significantly influencing Fc effector functions in vivo. Both the U.S. FDA and European regulations require understanding of glycan profiles in these proteins because of their profound influence on safety and efficacy of biopharmaceuticals. Figure 6 shows the separation of neutral and acidic 2AA-labeled *N*-Linked glycans from a human IgG using a GlycanPac AXR-1 (1.9μm, 2.1 x 150 mm) column. As with the fetuin sample in the previous figures, the IgG-derived glycan elution profile consists of clusters of peaks in which the neutral glycans elute first, followed by monosialylated and disialylated forms. Analytes in each cluster represent the glycans of the same charge. Within each cluster, the glycans having the same charge are further separated according to their isomerism and size by reversed-phase interactions. As shown in Figure 6, 2AA-labeled neutral glycans elute between 5 and 22 min, 2AA-labeled monosialylated glycans elute between 30 and 45 min and 2AA-labeled disialylated glycans elute between 45 and 55 min. More than 40 peaks are identified from the separation of 2AA-labeled *N*-glycans from this human IgG.

FIGURE 6. Separation of 2AA-labeled *N*-linked glycans from human IgG by charge, isomers, and size using a GlycanPac AXR-1 (1.9 µm) column



Column:	**GlycanPac AXR-1 (1.9 µm)**
Dimensions:	2.1 x 150 mm
Mobile phase:	A: Acetonitrile
	B: water
	C: 50 mM Ammonium Formate, pH 4.4
Flow:	0.3 mL/min
Temp:	30 °C
Injection:	100 pmoles
Detection:	Fluorescence at 320/420 nm
Sample:	2AA labeled *N*glycan from human IgG

Time (min)	% A	% B	% C	Flow Rate (mL/min)	Curve
-10	0	92	8	0.3	5
0	0	92	8	0.3	5
1	0	92	8	0.3	5
25	0	92	8	0.3	5
26	0	50	50	0.3	5
60	10	40	50	0.3	5

Conclusion

- The GlycanPac AXR-1 column separates glycans based on charge, isomeric structure, size, and polarity, providing twice the number of resolved peaks and more than 4 times the glycan structures identified compared to existing commercial amide HILIC columns for 2AB-labeled *N*-linked glycans released from bovine fetuin.
- Faster Orbitrap enables higher scan rates at higher resolution. This translates to increased sensitivity and better quality MS/MS data for both abundant and low abundance glycans. This also enables LC-MS^3 workflows, providing additional stage of information for glycans structural elucidation

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Evaluating Protein Glycosylation in Limited-Quantity Samples by HPAE-PAD

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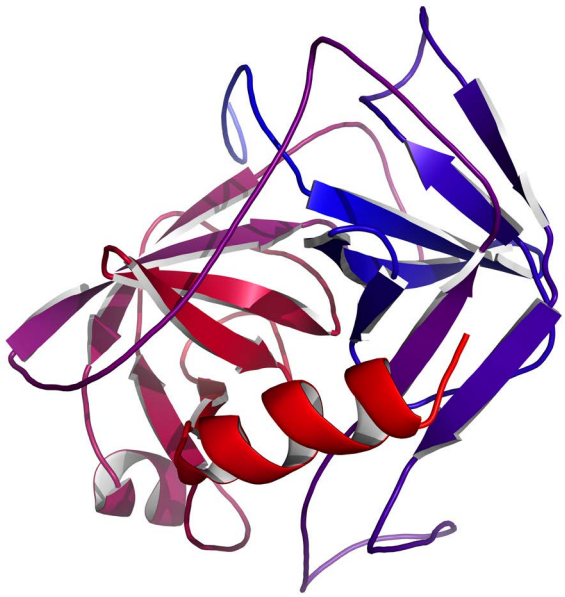
Key Words
Prostate-Specific Antigen (PSA), Transferrin, *N*-Glycans, *O*-Glycans, Dionex CarboPac Column



Introduction

Changes in protein glycosylation are frequently studied in cancer research to identify potential biomarkers. Factors investigated include differences in overall oligosaccharide (glycan) content, monosaccharide content changes, differences in sialylation amount and sialylation linkages, the degree of fucosylation, and differences in glycan branching.

PSA is an example of a well known glycoprotein cancer marker. The glycosylation of this protein is of importance as researchers seek to understand the changes that occur in this protein during carcinogenesis and tumor growth to provide a greater understanding of the disease. PSA is a 28.7 kD protein with a single glycosylation site at Asn-69 (Asn-45 after cleavage of the signal peptide and propeptide regions).[1] It is typically 8% carbohydrate by mass.[2]

Recent investigations that study glycan changes in PSA for the purpose of understanding changes that occur between benign and cancerous states have identified a number of potential glycan biomarkers. Key differences investigated include sialylation and sialyl linkages,[3-4] fucosylation,[5] and the elevated presence of *O*-glycans which are involved in regulation of cellular growth and function.[6] Further investigations into the mechanisms of *O*-glycan activity have recently been suggested for tumor growth as well as apoptosis regulation.[7-8]

Many methods are currently available for identification and quantification of glycans. Chromatographic methods include gas chromatography (GC), GC-mass spectrometry (MS), high-performance liquid chromatography (HPLC), LC-MS, capillary electrophoresis, and HP anion exchange chromatography with pulsed amperometric detection (HPAE-PAD). For many of these methods, glycan derivatization is a common step to enhance detection sensitivity and improve chromatographic retention and resolution. For GC methods, derivatization is needed to increase the volatility of the glycans for GC separation.

In contrast to these methods, HPAE-PAD does not require modification of the glycans before analysis. As is true of many biomarkers, the amounts of glycoproteins available for research are limited, adding additional challenges to work assessing changes in glycosylation. The sensitivity of PAD allows this analysis to be performed with sample quantities as low as 3–10 µg of protein. Specificity is ensured by both the separation and the electrochemical detection that has been optimized for carbohydrate analysis.

Thermo SCIENTIFIC

In this study, the glycosylation of human transferrin (as a model of PSA) and PSA are investigated by HPAE-PAD using <10 µg of protein. Two methods are utilized to evaluate protein glycosylation. One method is used to investigate the *N*-linked oligosaccharides and glycan sialylation. A second method evaluates the potential presence of *O*-linked glycans by monitoring monosaccharide composition. By using a combination of acid hydrolysis and enzymatic digestion steps prior to HPAE-PAD analysis, information about the glycosylation, as well as linkages within the glycans, is determined.

These methods provide a cost-effective option for investigating glycans without the derivatization required by many other methods. Electrochemical detection determines the glycans directly without the potential loss of sialylation or linkage rearrangement that may occur during analysis with other methods. HPAE-PAD provides a direct-detection method without the additional time and reagent costs needed for methods that rely on derivatization for effective separation and detection.

Goal

To develop HPAE-PAD methods to evaluate glycoprotein glycosylation using <10 µg amounts of protein

Equipment

- Thermo Scientific™ Dionex™ ICS-3000/5000⁺ system, including:
  - SP Single Pump or DP Dual Pump
  - DC Detector/Chromatography Compartment
  - Dionex AS or AS-AP Autosampler
  - EG Eluent Generator
  - ED Electrochemical Detector (without Cell, P/N 079830)
  - ED Cell with Reference Electrode and Spacer Block (P/N AAA-061756)
  - Disposable Electrodes for Carbohydrates (P/N 060139): for oligosaccharide analysis
  - Gold on PTFE Disposable Electrode (P/N 066480): for monosaccharide analysis
  - pH-Ag/AgCl Reference Electrode (P/N 061879)
  - 10 µL PEEK™ Sample Loop (P/N 042949)
- Thermo Scientific™ Savant™ SPD131DDA SpeedVac™ Concentrator
- Fisher Scientific™ accuSpin™ Micro17 Microcentrifuge (Fisher Scientific P/N 13-100-675)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software was used for all data acquisition and processing.

Additional Equipment for Monosaccharide Determination Using Eluent Generation

- Thermo Scientific Dionex EGC III KOH Eluent Generator Cartridge (P/N 074532)
- Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column (P/N 060477)
- EG Vacuum Degas Conversion Kit (P/N 063353)

Additional Equipment for Protein Sample Concentration Determination

- Thermo Scientific™ NanoDrop™ 2000c Spectrophotometer (P/N ND-2000c)

Consumables

- Vial Kit, 0.3 mL Polypropylene with Caps and Septa (P/N 055428)
- Vial Kit, 1.5 mL Polypropylene with Caps and Septa (P/N 079930)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane (1000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- Polypropylene Microcentrifuge Screw Cap Tubes, 1.5 mL (Sarstedt® P/N 72.692.005)
- 10 KD MWCO Centrifugal Filters, Amicon® Ultra-0.5 mL (Millipore P/N UFC501024)
- 30 KD MWCO Centrifugal Filters, Amicon Ultra-0.5 mL (Millipore P/N UFC503024)

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better
- Sodium Hydroxide, 50% w/w (Fisher Scientific P/N SS254-500)
- Sodium Acetate, anhydrous, electrochemical grade (P/N 059326)
- Hydrochloric Acid, 6N, 1 mL ampules (Thermo Scientific P/N 24308)
- Sodium Chloride (Fisher Scientific P/N S671-500)
- Sodium Azide (Fisher Scientific P/N BP922I-500)
- G0 (NGA2) (Fisher Scientific P/N NC0145854)
- G1F (NA2G1F) (Fisher Scientific P/N NC9603959)
- MAN-6 (Fisher Scientific P/N NC9071406)
- G2bF (NA2FB) (Fisher Scientific P/N NC0145860)
- MAN-9 (Fisher Scientific P/N 50-355-869)
- A1F (Fisher Scientific P/N NC9603958)
- A2F (Fisher Scientific P/N NC9698597)
- PNGase F, 15,000 units (equivalent to 500,000 U/mL). A unit is defined as the amount of enzyme required to remove >95% of carbohydrate from 10 µg of denatured RNase B in 1 h at 37 °C in a total reaction volume of 10 µL (Fisher Scientific P/N 50-811-832). Enzyme is supplied with: 10 × glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol), 10 × G7 buffer (0.5 M sodium phosphate, pH 7.5 at 25 °C), and 10% NP-40.

- Neuraminidase, 2000 units (equivalent to 50,000 U/mL). One unit is defined as the amount of enzyme required to cleave >95% of terminal α-Neu5Ac from 1 nmol Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-7-amino-4-methyl-coumarin (AMC) in 5 min at 37 °C in a total reaction volume of 10 µL (Fisher Scientific P/N 50-811-834, cloned from *Clostridium perfringens* and overexpressed in *E. coli*).
- α2-3 Neuraminidase, 2500 units (equivalent to 50,000 U/mL). One unit is defined as the amount of enzyme required to cleave >95% of the α-Neu5Ac from 1 nmol of AMC in 1 h at 37 °C in a total reaction volume of 10 µL (Fisher Scientific P/N 50-811-844, cloned from *Salmonella typhimurium* LT2 and overexpressed in *E. coli*).
- α-L-Fucosidase from bovine kidneys, 1 unit (13 units/mg). One unit will hydrolyze 1.0 µmole of p-nitrophenyl α-L-fucoside to p-nitrophenol and L-fucose per min at pH 5.5 at 25 °C (Sigma-Aldrich® P/N F5884).

Samples

- Human Transferrin, 10 mg/mL as supplied (Fisher Scientific P/N NC9583465)
- Human Transferrin (Sigma-Aldrich P/N T8158)
- Human Prostate Specific Antigen, 5.9 mg/mL as supplied (Lee Biosolutions P/N 497-11)
- Human Transferrin, spiked with porcine mucin (Sigma-Aldrich P/N M-2378)
- Pierce™ Human Polyclonal IgG (Thermo Scientific P/N PA1-31154)

Conditions: Oligosaccharides

Columns:	Thermo Scientific™ Dionex™ CarboPac™ PA200 Guard, 3 × 50 mm (P/N 062895) Dionex CarboPac PA200 Analytical, 3 × 250 mm (P/N 062896)
Eluents:	A) DI water B) NaOH, 100 mM C) Sodium acetate, 200 mM, in 100 mM NaOH
Eluent Gradient:	50–100 mM NaOH from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM NaOAc in 100 mM NaOH from 35 to 50 min, 200 mM NaOAc from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL (partial loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au electrode, carbohydrate certified
Background:	~18 nC (using the carbohydrate 4-potential waveform, Table 1)[9]
Noise:	~50 pC
System Backpressure:	~2900 psi

Table 1. Carbohydrate 4-potential waveform for the ED.

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000+ system but not used in older Dionex systems
Reference electrode in Ag mode (Ag/AgCl reference)

Preparation of Solutions and Reagents

Eluent Solutions: Oligosaccharides

NaOH, 100 mM

Add 5.2 mL of 50% w/w NaOH to 994.8 mL of degassed deionized water to prepare 1 L of 100 mM NaOH. Proportion the 100 mM hydroxide solution with DI water to produce the described hydroxide gradient.

Sodium acetate, 200 mM, in 100 mM NaOH

Dissolve 16.4 g of sodium acetate in 400 mL of DI water. Once the solid has dissolved, dilute the solution with an additional 400 mL of DI water. Filter degas the solution through a 0.2 µm nylon filter unit and transfer the solution to a 1 L polypropylene volumetric flask. Pipette 5.2 mL of 50% w/w NaOH into the volumetric flask. Fill the flask to the mark with degassed DI water. Promptly transfer the solution to the Dionex ICS-5000+ system and maintain an inert headspace of ultrahigh purity (UHP)-grade nitrogen or UHP helium gas. See Dionex (now part of Thermo Scientific) Technical Note 71 for detailed information on eluent preparation.[10]

Reagents

Sodium chloride, 100 mM

Dissolve 74.75 mg of sodium chloride, anhydrous, in 12.75 g of DI water.

Sodium azide, 1 mg/mL

Dissolve 13.25 mg of sodium azide in 13.25 g of DI water.

Sodium acetate buffer, 25 mM, pH 5

Dissolve 2.50 g of sodium acetate in 100.27 g (100.27 mL) of DI water to prepare a 0.3 M sodium acetate stock solution. Dilute 22.5 mL of glacial acetic acid with 77.5 mL of DI water to prepare a 4.0 M acetic acid stock solution. Add 17.0 mL of 0.3 M sodium acetate and 0.45 mL of 4.0 M acetic acid to a 250 mL volumetric flask. Dilute to the mark with DI water.

Human transferrin, 4 mg/mL

Gently dissolve 8.3 mg/mL human transferrin in 2.0 mL of DI water. If the transferrin is supplied dissolved in a buffer, dilute a 10 mg/mL solution by adding 200 µL of transferrin solution to 300 µL of DI water to prepare a 4 mg/mL solution.

Mucin, 2.5 mg/mL

Gently dissolve 13.14 mg of porcine mucin in 1.3 mL of DI water to prepare a viscous suspension of 10 mg/mL mucin. Dilute 250 µL of the 10 mg/mL suspension to a total of 1 mL with DI water to prepare a nominal 2.5 mg/mL solution of mucin.

Conditions: Monosaccharides[13]

Columns:	Thermo Scientific™ Dionex™ AminoTrap,™* 3 × 30 mm (P/N 060146) Dionex CarboPac PA20 Analytical, 3 × 150 mm (P/N 060142)
Eluent:	10 mM potassium hydroxide (KOH)
Eluent Source:	Dionex EGC III KOH Cartridge with Dionex CR-ATC Continuously Regenerated Anion Trap Column
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL (partial loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au on PTFE electrode
Background:	~30 nC (using the carbohydrate 4-potential waveform, Table 1)
Noise:	~20 pC
System Backpressure:	~2200 psi

*Installed before the Dionex CarboPac PA20 column

The waveform used for monosaccharide analysis is the same as applied for oligosaccharides (Table 1).

Eluent Solutions: Monosaccharides

Generate the KOH eluent on line by pumping high-quality degassed DI water through the Dionex EGC III KOH cartridge. The Chromeleon CDS software will track the amount of KOH used and calculate the remaining cartridge lifetime.

If the Dionex ICS-3000/5000+ system does not have eluent generation and the degas conversion kit has not been installed, eluents for monosaccharide analysis can be manually prepared. See Dionex (now part of Thermo Scientific) Technical Note 71 for detailed information on eluent preparation.[10]

Stock Standard Solutions: Monosaccharide Standards

Dissolve the contents of one Thermo Scientific™ Dionex™ MonoStandard™ 100 nmol vial in 1.0 mL of DI water and mix to prepare a stock standard solution containing 0.1 mM (100 pmol/µL) of each monosaccharide. Dilute this stock to prepare standards at concentrations of 0.05 µM (0.25 pmol), 0.10 µM (0.50 pmol), 0.50 µM (2.5 pmol), 1.0 µM (5.0 pmol), 5.0 µm (25 pmol), and 10 µM (50 pmol). For example, add 100 µL of the stock solution to 900 µL of DI water to prepare a 10 µM standard that equates to 50 pmol when 5 µL are injected.

Immediately freeze unused stock standard at <–10 °C.

Sample Preparation: Oligosaccharides

Sample preparation was performed by modified versions of previously described protocols.[11-13]

PNGase F digestion with denaturing: large scale

The PNGase F solution is supplied as 30 µL of solution. Add 270 µL of DI water to the PNGase F solution to prepare 300 µL of 50,000 U/mL enzyme.

Digestion control sample

Add 752 µL of DI water to a 1.5 mL microcentrifuge vial. Add 96.0 µL of 100 mM sodium chloride to match the saline content of many buffered protein solutions. Add 57 µL of 1 mg/mL sodium azide to match the preservative concentration in many buffered protein solutions. Add 13 µL of denaturant provided with the PNGase F.

Protein samples

Add 688 µL of DI water to a 1.5 mL microcentrifuge vial. Add 64.0 µL of 4 mg/mL human transferrin solution. If not already present, add 96.0 µL of 100 mM sodium chloride and 57 µL of 1 mg/mL sodium azide as with the digestion control sample described above. Add 13 µL of denaturant provided with the PNGase F.

Prepare a mucin sample by replacing the 64.0 µL of human transferrin with 10 µL of mucin solution and 54 µL of DI water.

Prepare a transferrin sample spiked with mucin by reducing the amount of DI water by 10 µL and adding 10 µL of mucin solution.

Incubate each sample at 100 °C for 10 min to denature protein.

After protein denaturation, treat each sample as follows:

- Add 9.6 µL of NP40 to protect the PNGase F from denaturation.
- Add 9.6 µL of 10 × G7 followed by 22.4 µL of 10-fold diluted PNGase F. Gently mix the solution.
- Incubate the samples at 37 °C for 20 h (overnight).

PNGase F digestion with denaturing: small scale

Digestion control sample

Add 44.7 µL of DI water to a 1.5 mL microcentrifuge vial. Add 9.0 µL of 100 mM sodium chloride to match the saline content of buffered protein solutions. Add 3.6 µL of 1 mg/mL sodium azide to match the preservative concentration in many buffered protein solutions. Add 0.8 µL of denaturant provided with the PNGase F.

PSA samples

Add 56.8 µL of DI water to a 1.5 mL microcentrifuge vial. Add 3.0 µL of 5.9 mg/mL human PSA solution. Add 0.8 µL of denaturant as provided with the PNGase F. Incubate each sample at 100 °C for 10 min.

After protein denaturation, add 0.70 µL of NP40 to each sample. Then:

- Add 0.70 µL of G7 followed by 1.4 µL of 10-fold diluted PNGase F to each sample.
- Briefly centrifuge the sample to ensure the solutions mix.
- Incubate the samples at 37 °C for 20 h.

Separation of released glycans from protein

Glycans were separated from the remaining protein by a two-step process:

- Remove detergents and salts remaining from the PNGase F digest by ultracentrifugation in a 10 kD MWCO filter. Transfer the retentate (proteins and glycans) from this filter to a 30 kD MWCO filter with an additional 40 µL of DI water.
- Centrifuge the solution to separate the glycans (filtrate) from the proteins in the retentate. Confirm protein removal by UV absorbance at 280 nm using a 2 µL sample and a NanoDrop 2000c spectrophotometer.

Glycan retention by the filter was confirmed by recovery experiments of glycans released from human transferrin by PNGase F. These samples were released without denaturing so that the glycan recovery could be determined by injections of PNGase F-released transferrin glycans prior to filtration followed by injections of the filtrate and retentate. Although some glycans pass through the filter and are detected in the filtrate (typically between 7–23%), greater than 76% of the transferrin glycans were retained. The loss of transferrin glycans from the rententate did not appear to be correlated with the size or charge, suggesting nonselective loss (data not shown).

This method provided the best recoveries for these limited-quantity samples compared to other methods to desalt, remove detergents, and remove the proteins prior to HPAE-PAD analyses.

Precautionary Note: This method has been tested and applied for proteins with primarily branched sialylated glycans. For proteins that are known to contain neutral glycans, other methods are available and should be followed.[11-12]

Centrifugal filter preparation

Assemble the filter unit by inserting the filter into the provided centrifuge tube. Load each filter with 500 µL of DI water and centrifuge at 14,000 ×g for 10 min. Invert the filter and reinsert it into the centrifuge tube. Centrifuge briefly at 2000 ×g to remove the retentate. Discard the water wash. Repeat this process a second time to ensure removal of any humectants present in the filter.

PNGase F digest sample preparation

For each sample, load 60 µL (or in the case of the small-scale protein digest, load the entire sample) into a 10 kD MWCO filter unit. Centrifuge the sample at 14,000 ×g for 15 min. Collect the retentate (20 µL) for further preparation. Transfer the retentate into a 30 kD MWCO filter unit. Add an additional 40 µL of DI water to the filter unit with the retentate. Centrifuge the sample for 15 min at 14,000 ×g for 15 min. Collect the filtrate for injection and further work. The retentate contains the PNGase F and the protein of interest. This may be saved for other analyses if desired.

α2-3/2-6 Neuraminidase digestion of released glycans

Dilute the 40 µL solution of neuraminidase 1:10 by adding 360 µL of 25 mM sodium acetate, pH 5.0, to prepare a solution of 5000 U/mL. Add 5.0 µL of the diluted neuraminidase to 5.0 µL of the glycans isolated as described above. Incubate the samples at 37 °C for 1 h. Transfer the samples to a 300 µL autosampler vial and keep at 6 °C until injection. Store unused neuraminidase at –40 °C.

α2-3 Neuraminidase digestion of released glycans

In a 300 µL autosampler vial, add 0.5 µL of α2-3 neuraminidase to 10 µL of glycans isolated as described in the PNGase F digest sample preparation section. Ensure that the enzyme and sample are well mixed by gently tapping the vial to move all solution to the bottom of the vial and remove any air bubbles that may be present. Alternatively, centrifuge the sample briefly. Incubate the samples at 37 °C for 1 h. Keep the samples at 6 °C until injection.

α-L-Fucosidase digestion of released glycans

Add 2000 µL of DI water to the supplied fucosidase suspension of 1 unit in 80 µL of 3.2 M $(NH_4)_2SO_4$, 10 mM NaH_2PO_4, and 10 mM citrate (pH 6.0) to prepare a suspension of 0.04 U/mL in 120 mM $(NH_4)_2SO_4$, 0.38 mM NaH_2PO_4, and 0.38 mM citrate. Add 5 µL of this diluted fucosidase suspension to 5 µL of PSA PNGase F digest and incubate the solution for 20 h at 37 °C. Store samples at –40 °C, if necessary, before analysis.

Sample Preparation: Monosaccharides

Acid hydrolysis

Open a fresh ampule of 6 N hydrochloric acid.

Add 100 µL of 6 N hydrochloric acid to a 1.5 mL microcentrifuge vial. Add 5 µL of released glycans from PSA to the hydrochloric acid. Similarly, add 5 µL of glycans released from transferrin, 5 µL of glycans released from transferrin spiked with mucin, and 5 µL of mucin solution to individual 1.5 microcentrifuge vials containing 100 µL of 6 N hydrochloric acid. Prepare two control samples: 5 µL of PNGase control that has been prepared in the same manner as the samples, and 5 µL of DI water. For comparison to the released glycans, hydrolyze samples of 0.5 µL of PSA (2.9 µg of protein) in 100 µL of 6 N hydrochloric acid.

Hydrolyze samples for 4 h at 100 °C. After hydrolysis, dry the samples in a SpeedVac concentrator equipped with an acid trap. Dissolve the samples in 30 µL of DI water prior to analysis.

Note: These conditions are optimized for release of amino sugars and will significantly degrade other monosaccharides. See Thermo Scientific Technical Note 40 for alternate hydrolysis methods.[13]

Precautions

- Carbohydrates have limited stability unless sterility is maintained. Store solutions and samples at –40 °C. Avoid multiple freeze/thaw cycles to preserve the carbohydrates.
- Thoroughly remove glycerol or polyethylene glycol from filters before use to avoid interferences in HPAE-PAD analyses. Similarly, be sure to use glycerol- (glycerin-) free PNGase F.
- The buffers used during the enzymatic digests can lead to column overload due to high salt concentrations. If larger sample volumes are injected, this must be considered and control experiments are recommended to evaluate column loading. Desalting of PNGase F digests may be necessary if sample preparation methods other than those described in this work are used.
- When using a Dionex ICS-3000/5000⁺ EG Eluent Generator, it is critical to install the Vacuum Degas Conversion Kit (P/N 063353). This degasser will remove gasses generated by the EG and help maintain a stable baseline. This kit is not necessary when preparing eluents manually.

Health Precaution

When working with human- or animal-derived fluids, appropriate Biosafety Level protocol must be followed. For guidance, see the Center for Disease Control publication *Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition.*[14]

Results and Discussion

Human PSA was analyzed following the scheme in Figure 1. Human transferrin was used as a model protein to assess the method following PNGase F digestion. Although human transferrin is a larger protein—with a MW of 77 kD compared to PSA at 28.7 kD—it has two glycosylation sites leading to a similar total glycosylation amount of ~6–9% by weight compared to 8% for PSA.[2,15] In both cases, the glycans are dominated by mono- and disialylated biantennary glycans with minor amounts of neutral glycans,[5,15] although higher-order branched glycans have been identified in human transferrin.[16]

Transferrin was analyzed as a model protein to determine monosaccharide content as well as to profile the *N*-linked glycans. The monosaccharide content of PSA was determined for both the intact protein and for the isolated *N*-glycans. A selection of *N*-linked glycan structures commonly investigated in human proteins is shown in Table 2. For the sake of brevity, one glycan, G0, is shown afucosylated. However, glycans may be present fucosylated or afucosylated. Carbohydrate structure schematics are based on the nomenclature of the Center for Functional Glycomics.[17]

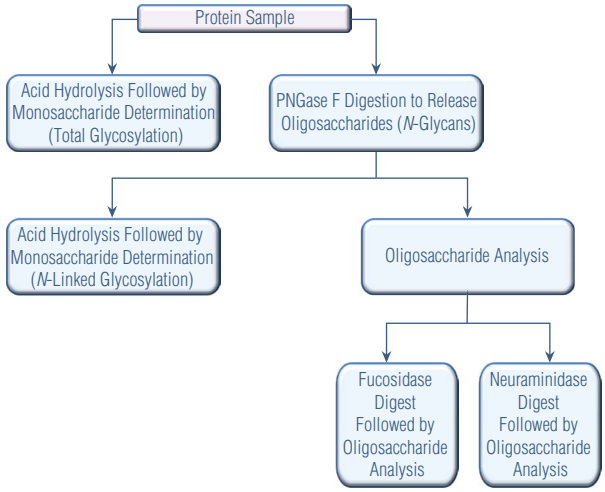


Figure 1. Schematic of glycan analysis workflow for PSA samples.

Table 2. A selection of commonly investigated glycans and their nomenclature. Afucosylated glycans of the shown fucosylated structures, as well as other branched glycans, may be found on glycoproteins. Structures adapted from Center for Functional Glycomics (CFG) standards.

Glycan (Oxford)	mAb Acronym	Structure (Adapted from CFG)
NGA2F (FA2G0)	G0F	
NA2G1F (FA2G1)	G1F	
NA2F (FAG2)	G2F	
NA2FB (FABG2)	G2bF	
G2FA1 (FA2G2S1)	A1F	
G2FA2 (FAG2S2)	A2F	
NGA2 (A2G0)	G0	
Man3	M3	
Man5	M5	
Man6	M6	

- *N*-acetylglucosamine (GlcNAc)
- Fucose (Fuc)
- Mannose (Man)
- Galactose (Gal)
- *N*-acetylneuraminic acid (Neu5Ac)

Comparative Monosaccharide Determination

As an indicator of *O*- and *N*-glycosylation differences, the amounts of galactosamine (GalN) and glucosamine (GlcN) were determined by hydrochloric acid hydrolysis of both the intact proteins and released glycans after protein PNGase F digestion, followed by glycan separation from the remaining protein. To test the viability of this approach, glycans were released from human transferrin via PNGase F and then hydrolyzed with HCl. At the same time, human transferrin that had been spiked with porcine mucin was treated similarly. Porcine mucin primarily contains abundant *O*-glycans with potential *N*-linked glycan sites that have been identified.[18] The addition of mucin to transferrin will simulate changes in *O*-glycosylation (three sites) or contamination of a sample with 8% (by weight) of a highly *O*-linked glycoprotein.

Figure 2 shows the separation of monosaccharides released from transferrin by hydrochloric acid to investigate the amino sugars present. As shown in Chromatogram 2C, the transferrin digest contained primarily glucosamine with no detectable galactosamine. When mucin was treated similarly, there was much less carbohydrate content overall; however, there were significant amounts of galactosamine and glucosamine (Chromatogram 2A). When the mixed transferrin/mucin sample was analyzed, glucosamine and galactosamine were both present, indicating that the presence of mucin-type glycans, and therefore *O*-linked glycans, can be identified (Chromatogram 2B).

By repeating this process for two samples of PSA—one that has been treated with PNGase F and one that has not—the potential presence of *O*-glycans can be determined. Figure 3 illustrates the separation of monosaccharides present in PSA and PSA that has been treated with PNGase F. In both cases, both glucosamine and galactosamine were present in roughly the same molar ratio of 0.07. This suggests that for this PSA sample, the galactosamine was not a result of *O*-glycosylation but is associated with the PSA *N*-glycans, or that there was a contaminating protein with *O*-glycosylation that is not removed during the sample preparation to separate protein from released *N*-glycans.

Oligosaccharide Analysis

In order to investigate the neutral glycans that may be present and to better identify the neutral glycans after neuraminidase digestion, a two-part gradient method was developed. The first portion of this gradient is a 50–100 mM sodium hydroxide gradient that focuses on separating the neutral glycans that may be present. The second portion of the gradient is a 0–200 mM acetate gradient in 100 mM sodium hydroxide. This portion of the gradient elutes the larger oligomannose species and the charged glycans. If individual neutral glycans are not of interest, a simpler method of a sodium acetate gradient in 100 mM NaOH can be used, as has been previously published using human transferrin[11] and IgG,[12] as examples. This method separates the charged glycans well, providing a rugged method for profiling the sialylated glycans.

Columns:	Dionex CarboPac PA20, 3 × 150 mm Dionex AminoTrap, 3 × 30 mm
Eluent:	10 mM KOH, 10 min 100 mM KOH wash followed by 10 min of equilibration at 10 mM KOH
Eluent Source:	Dionex EGC III Cartridge and Dionex CR-ATC Column
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp:	30 °C
Samples:	Hydrochloric acid hydrolyzate of PNGase F digests of A) mucin, B) human transferrin spiked with mucin, C) human transferrin, and D) monosaccharide standards

Peaks:		A	B	C	D
	1. Galactosamine	0.5	0.4	ND	5 pmol
	2. Unknown	—	—	—	—
	3. Glucosamine	1.4	13	14	5
	4. Galactose	N/A	N/A	N/A	5
	5. Glucose	N/A	N/A	N/A	5
	6. Mannose	N/A	0.5	0.7	5

ND = Not Determined

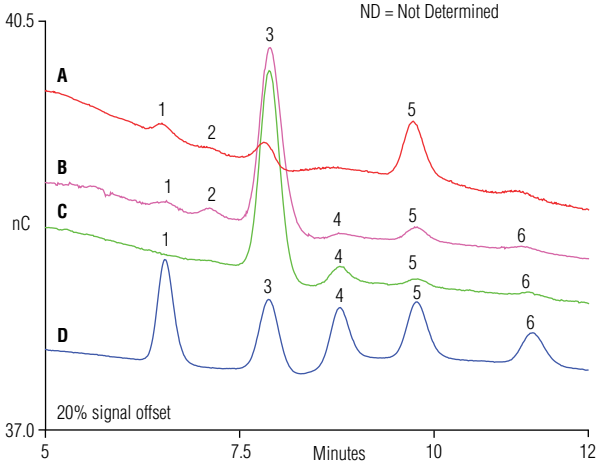


Figure 2. Separation of transferrin acid hydrolysates on a Dionex CarboPac PA20 column. The presence of contaminating mucin as an *O*-glycan source can be identified by the presence of galactosamine in Chromatogram B.

Columns:	Dionex CarboPac PA20, 3 × 150 mm Dionex AminoTrap, 3 × 30 mm
Eluent:	10 mM KOH, 10 min 100 mM KOH wash followed by 10 min of equilibration at 10 mM KOH
Eluent Source:	Dionex EGC III Cartridge and Dionex CR-ATC Column
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp:	30 °C
Samples:	Hydrochloric acid hydrolyzates of A) 0.67 µg PSA, B) PSA glycans after PNGase F release (equivalent to 0.09 µg of protein), and C) monosaccharide standards

Peaks:		A	B	C
	1. Fucose	ND	ND	5 pmol
	2. Galactosamine	1.6	0.3	5
	3. Unknown	—	—	—
	4. Glucosamine	22	4.5	5
	5. Galactose	N/A	N/A	5
	6. Glucose	N/A	N/A	5
	7. Mannose	ND	ND	5

ND = Not Determined

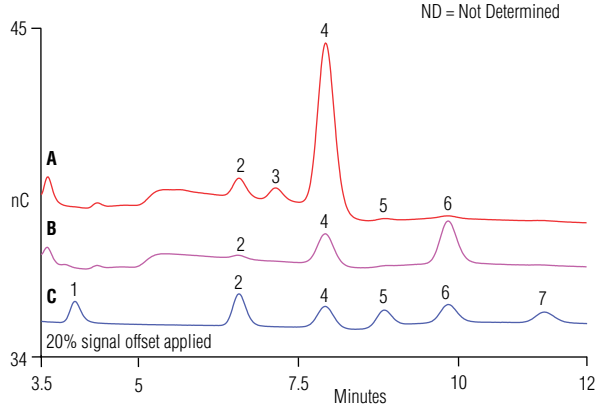


Figure 3. Separation of PSA acid hydrolysates on a Dionex CarboPac PA20 column. Both the total protein hydrolysis and the PSA-*N*-linked glycans have a similar ratio of galactosamine/glucosamine of 0.07. The hydrolysis conditions here do not allow reliable quantification of galactose and glucose.

Columns:	Dionex CarboPac PA200 Guard, 3 × 50 mm Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent:	A) 100 mM NaOH B) 1M Sodium Acetate in 100 mM NaOH
Gradient:	20–150 mM Sodium Acetate in 100 mM NaOH from 0 to 75 min, 20 mM Sodium Acetate in 100 mM NaOH from 75.1 to 100 min
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp:	30 °C
Samples:	A) Neutral glycan standards B) Transferrin glycans after treatment as with PSA glycans, equivalent of 1.3 µg of protein injected C) PSA glycans after PNGase F release detergent removal, equivalent of 1 µg of protein injected
Peaks:	1. G0 2. G1F isomers 3. Man6 4. G2bF 5. Man9

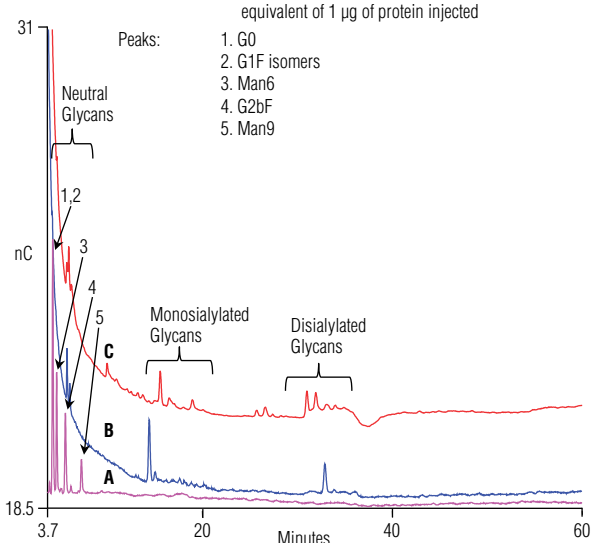


Figure 4. Charged glycan profiling using the Dionex CarboPac PA200 column with a 20–150 mM sodium acetate gradient in 100 mM NaOH. Neutral glycan standards are at a concentration of 5 pmol each. The same neutral glycan standards are also shown in Figure 5.

Columns:	Dionex CarboPac PA200 Guard, 3 × 50 mm Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent:	A) DI water B) 100 mM NaOH C) 200 mM Sodium Acetate in 100 mM NaOH
Gradient:	50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp:	30 °C
Samples:	A) Neutral glycan standards B) Neutral glycan-rich polyclonal IgG PNGase F digest, equivalent of 4.5 µg of protein injected
Peaks:	1. G0F 2. G0 3. G1F isomers 4. G1F/G0bF 5. Man6 6. G2F 7. G1bF 8. G2bF 9. Man9 10. A1F isomers 11. A2F isomers

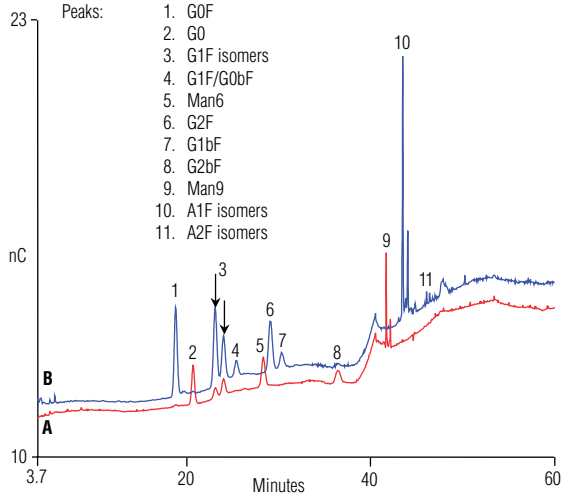


Figure 5. Improved resolution for neutral glycan profiling using the Dionex CarboPac PA200 column with a hydroxide gradient followed by an acetate gradient. Note the improved resolution between neutral glycans compared to the method shown in Figure 4. Human IgG is shown here due to its high proportion of neutral glycans compared to the other glycoproteins investigated for this study.

Figure 4 illustrates the separation of charged glycans with a 20–150 mM acetate gradient in 100 mM sodium hydroxide. In Chromatogram 4A, a standard of neutral glycans has been injected for comparison. The neutral glycans were partially resolved but elute in a region prone to interferences from other reagents in digested samples. Chromatograms 4B and 4C show PSA and transferrin glycans, respectively. The similarity between the glycans of the two proteins is evident in this profile; however, they are not identical.

For both proteins, monosialylated and disialylated glycans were present. The distribution within these classes of glycans show some differences, including a peak present in PSA PNGase F digests that eluted between chromatographic regions associated with the mono- and disialylated glycans, potentially a glycan modified with sulfate or phosphate that will be discussed further in the description of Figure 8. For both proteins, identification of neutral glycans was hindered by the presence of a large weakly retained peak. If charged glycans are of primary interest, the separation with an acetate gradient, as illustrated in Figure 4, provides superior resolution of charged glycans.

Separation of neutral glycans was evaluated using multiple gradients, including weak acetate gradients with constant hydroxide concentration[19] and methods that combine isocratic and gradient separations within a method.[20] The final approach chosen for this work—a hydroxide gradient followed by an acetate gradient—was selected based on glycan resolution, the method analysis time, and eluent preparation convenience.

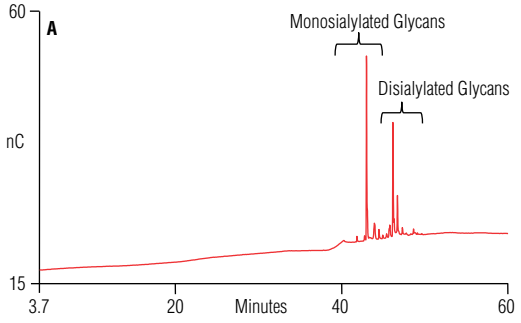
The method illustrated in Figure 5, which used a hydroxide gradient followed by an acetate gradient, allowed excellent separation of the neutral glycans, as illustrated by the chromatogram of a PNGase F digest of polyclonal human IgG. Chromatogram 5A shows the same neutral glycan standard illustrated in Chromatogram 4A. With the method described here, these glycans are well resolved and retained past the void, reducing the potential for interferences from other reagents used during sample digestion and preparation.

Glycans released by PNGase F were identified by comparison to known standards and by enzymatic digestion to determine core glycan structures. The work described here was performed with minimal protein to meet the need to conduct multiple experiments with a total protein sample of 10 µg. Greater amounts of protein, if available, can be digested by PNGase F and analyzed with better sensitivity. If the protein concentration in the digest is increased, the amount of PNGase F must also be increased.

The conditions used for this work were selected based on control experiments with human transferrin that would allow efficient glycan release with minimal denaturant and enzyme. PNGase F and other enzyme digest protocols are available for greater protein amounts.[11] Figure 6 shows examples of human transferrin digests to illustrate the improved signal-to-noise ratio by methods optimized for neutral (Chromatogram 6A) and charged (Chromatogram 6B) glycans by injecting released glycans from the equivalent of 4 µg of protein.

As shown in Figure 7, neutral, monosialylated, and disialylated glycans were present in the PSA sample. These digests were treated with α2-3 neuraminidase in order to investigate the linkages of the terminal sialic acids as well as measure the released sialic acid relative to the amount of protein used in the initial digest (Chromatogram 8B). After the α2-3 neuraminidase treatment, 3.6 mol Neu5Ac/mol protein were released. At the same time, peaks correlating to G1F, G2F, and G2 increased in relative peak area, suggesting that the Neu5Ac had been present in a α2-3 linkage on the G1F and G2F termini (Chromatogram 9B).

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm
Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent: A) DI water
B) 100 mM NaOH
C) 200 mM Sodium Acetate in 100 mM NaOH
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL
Temp: 30 °C
Sample: PNGase F digestion of human transferrin
Note: Denaturation was not used for these samples; therefore, glycan release is not complete.



Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm
Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent: A) 100 mM NaOH
B) 1 M Sodium Acetate in 100 mM NaOH
Gradient: 20–150 mM Sodium Acetate in 100 mM NaOH from 0 to 75 min, 20 mM Sodium Acetate in 100 mM NaOH from 75.1 to 100 min
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL
Temp: 30 °C
Sample: PNGase F digestion of human transferrin
Note: Denaturation was not used for these samples; therefore, glycan release is not complete.

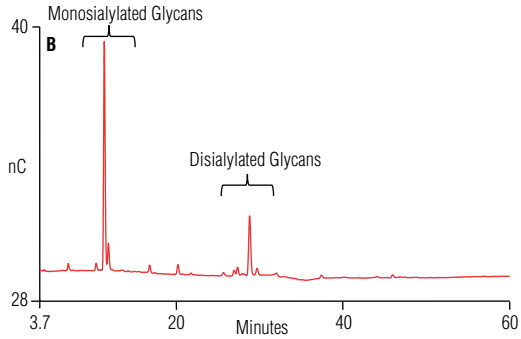


Figure 6. Illustration of typical baseline for injections of human transferrin samples. The equivalent of 4 µg of protein is injected in both chromatograms. Note the improved resolution of charged glycans in Chromatogram B for samples that do not typically contain neutral glycans.

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm
Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent: A) DI water
B) 100 mM NaOH
C) 200 mM Sodium Acetate in 100 mM NaOH
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL
Temp: 30 °C
Samples: A) PSA glycans after PNGase F release detergent removal, equivalent of 1 µg of protein injected
B) PNGase F control sample
Peaks: 1. G0F
2. G1F isomers
3. G1 isomers
4. G2F
5. G2bF
6. A1F isomers
7. Charged glycans
8. A2F isomers

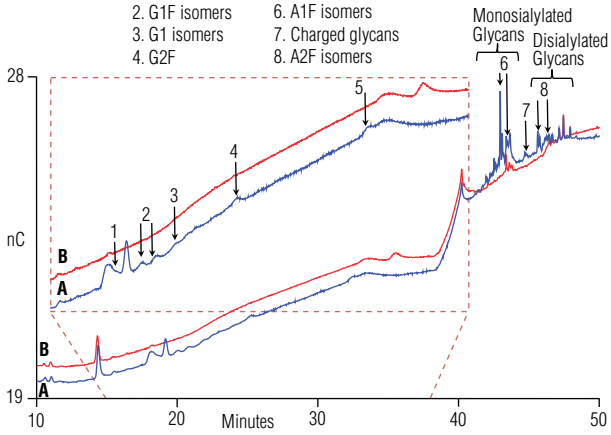
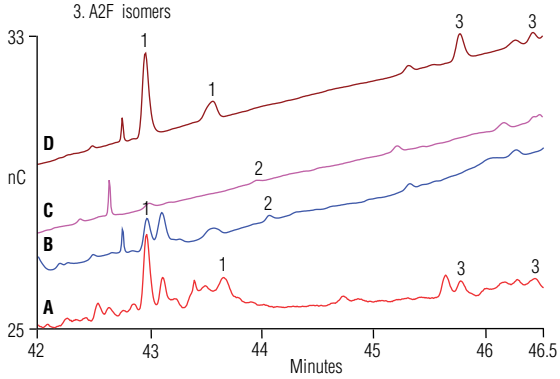


Figure 7. Released PSA oligosaccharides separated on a Dionex CarboPac PA200 column. The elution region of neutral *N*-linked glycans is expanded in the inset. Charged glycans are further expanded in Figure 8.

Further digestion of this sample with a α2-3/α2-6 neuraminidase released an additional 2.8 mol Neu5Ac/mol protein, removed most of the peaks present in the charged glycan region of the chromatogram, and increased the relative peak area of G2F (Figure 9). This suggests that the Neu5Ac present on remaining glycans are α2-6 linked and present as A2F and A1F. Additionally, a single peak persisted that was resistant to sialidase digestion. The glycan eluting at this position is potentially a charged glycan that is not sialylated but may be modified with phosphate or sulfate (Peak 2 in Figure 8).

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm
Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent: A) DI water
B) 100 mM NaOH
C) 200 mM Sodium Acetate in 100 mM NaOH
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH
from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH
from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH
from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL
Temp: 30 °C
Samples: A) PSA glycans after PNGase F release
B) Glycans after α2-3 neuraminidase digestion, equivalent of 1.6 µg of protein injected
C) Glycans after α2-3/2-6 neuraminidase digestion; equivalent of 0.8 µg of protein injected
D) A1F and A2F glycan standards
Peaks: 1. A1F isomers
2. Neuraminidase-resistant charged glycan
3. A2F isomers



10% signal offset applied. Time axis normalized to A1F for chromatograms from different days.

Figure 8. Separation of charged glycans after PNGase F digest followed by neuraminidase digestions. A charged glycan standard of A1F and A2F is shown in Chromatogram D. Note the dramatic reduction of charged glycans after neuraminidase treatment, with the exception of peak 2.

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm
Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent: A) DI water
B) 100 mM NaOH
C) 200 mM Sodium Acetate in 100 mM NaOH
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH
from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH
from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH
from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL
Temp: 30 °C
Samples: A) PSA PNGase F-released glycans
B) PSA glycans after α2-3 neuraminidase, equivalent of 1.6 µg of protein injected
C) PSA glycans after α2,3/2-6 neuraminidase, equivalent of 0.8 µg of protein injected
Peaks: 1. G0F 5. G2F
2. G0 6. G2
3. Neu5Ac 7. G2bF
4. G1F isomers

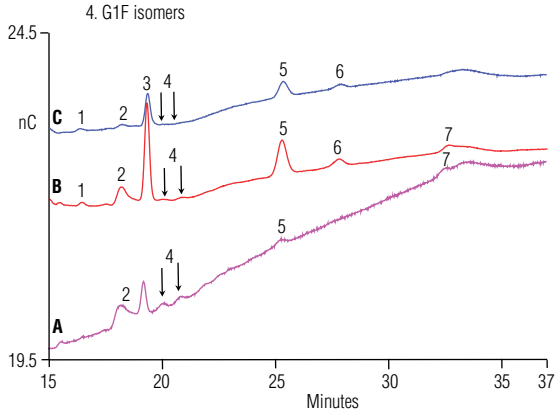


Figure 9. Separation of neutral glycans after PNGase F digest followed by neuraminidase digestions. Note the increase in the relative amounts of neuraminidase and G2F (Peak 5), confirming the presence of A1F and A2F as dominant sialylated glycan species. An increase in G2 (Peak 6) is also observed, indicating the presence of afucosylated sialylated glycans.

Fucosidase digestion of the released glycans further indicated the presence of G2F in the PNGase F-released glycans. Peaks in Chromatogram 10B (G1F isomers, G2F) were removed after fucosidase digestion and a corresponding relative increase in G2 observed, as shown in Chromatogram 10A. Although G1F isomers were present in the released glycans, it is not an abundant glycan, and the combination of dilution during the enzyme digest and the presence of isomers reduced overall sensitivity.

The expected presence of G1 isomers was not confirmed. Peak assignments were made after a corresponding fucosidase digestion of a known sample and standards containing G1F and G2F. Similarly, peaks from charged glycans shifted to longer retention times after fucosidase digestion, suggesting that the majority of the sialylated glycans were also fucosylated, as shown in Figure 11. In this figure, the major mono- and disialylated peaks shift to longer retention times, which is consistent with the behavior observed when treating sialylated glycan standards with fucosidase.

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm
Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent: A) DI water
B) 100 mM NaOH
C) 200 mM Sodium Acetate in 100 mM NaOH
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH
from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH
from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH
from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL
Temp: 30 °C
Samples: A) Neutral PSA glycans after fucosidase digestion, equivalent of 0.5 µg of protein injected
B) Neutral PSA glycans after PNGase F release, equivalent of 1.0 µg of protein injected
Peaks: 1. G0F 5. G2F
2. G0 6. G2
3. Neu5Ac 7. G2bF
4. G1F isomers

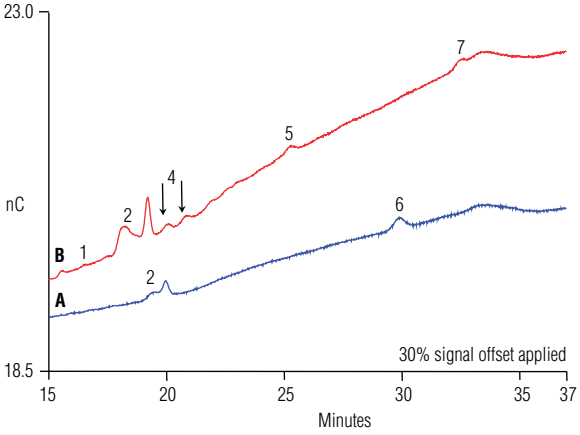


Figure 10. Neutral PSA glycans treated with α-L-fucosidase. Note the shifting of the G2F peak to later retention correlating with G2. Note that data collected on different days may show retention time shifts with different eluent preparations. For this work, G0 and other neutral glycan standards were used to confirm peak identity (data not shown).

Columns: Dionex CarboPac PA200 Guard, 3 × 50 mm
Dionex CarboPac PA200 Analytical, 3 × 250 mm
Eluent: A) DI water
B) 100 mM NaOH
C) 200 mM Sodium Acetate in 100 mM NaOH
Gradient: 50–100 mM Sodium Hydroxide from 0 to 30 min, 100 mM NaOH
from 30 to 35 min, 0–200 mM Sodium Acetate in 100 mM NaOH
from 35 to 50 min, 200 mM Sodium Acetate in 100 mM NaOH
from 50 to 60 min; equilibration at 50 mM NaOH for 15 min before injection
Flow Rate: 0.5 mL/min
Inj. Volume: 5 µL
Temp: 30 °C
Samples: A) Charged PSA glycans after fucosidase digestion, equivalent of 0.5 µg of protein injected
B) A1F and A2F standards
C) Charged PSA glycans after PNGase F release, equivalent of 1.0 µg of protein injected
Peaks: 1. A1F isomers
2. A1 isomers
3. A2F isomers
4. A2 isomers

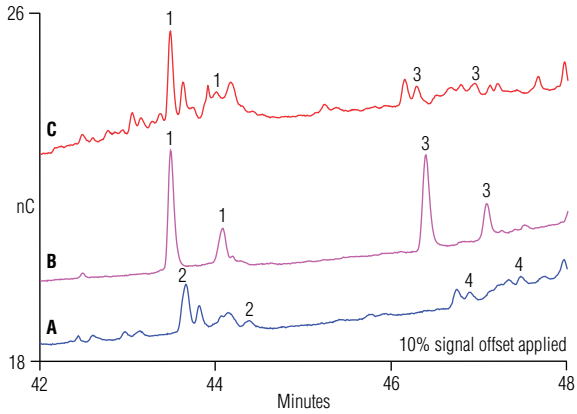
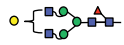
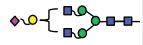
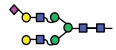
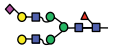
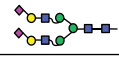
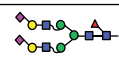


Figure 11. Charged PSA glycans treated with α-L-fucosidase. Note the shift and reduced number of peaks after fucosidase digest (Chromatogram A), compared to peaks present before fucosidase digestion (Chromatogram C), indicating that both fucosylated and afucosylated sialylated glycans are present in PSA. A fucosylated and sialylated standard (Chromatogram B) is included for comparison.

Identified glycans from seminal plasma PSA were compared to those previously determined by Tabares et al., with the comparative results presented in Table 3.[4] Although the results are not identical, some trends are consistent. In both studies, charged glycans greatly dominated the population of glycans on the protein. These glycans were mostly fucosylated, as indicated by the increase in the concentration of G2F when the glycans were treated with neuraminidase, as well as peak shifting to later retention times after fucosidase digestion. Potential desialylation of the protein during handling is always a risk. The samples investigated seem to be less sialylated than those previously studied; however, there is much debate in the literature regarding the PSA glycan composition.[3-5, 21,22]

Table 3. PSA glycans identified in this work compared to those noted in previously published work.

mAb Acronym	% Present Found by Current Study (by Peak Area)	% Present in Seminal PSA (Tabares et al.)[4]	Structure (Adapted from CFG)
G0	2.5		
Neutral afucosylated glycan	5		
G1F	1.5		
G2F	5		
G2bF	9		
	33 (monosialylated glycans, excluding A1F)	6	
A1		18	
A1F	10	25	
Sialidase-resistant charged glycan	5		
A2F+A2	29	51	 

- *N*-acetylglucosamine (GlcNAc)
- Fucose (Fuc)
- Mannose (Man)
- Galactose (Gal)
- *N*-acetylneuraminic acid (Neu5Ac)

Conclusion

By combining HPAE-PAD analysis with acid hydrolysis and enzymatic digestion of protein glycans, information about the glycan identity as well as terminal carbohydrate linkage isomers can be determined from small amounts of protein (0.5–1.6 µg per injection). This study investigated human PSA to evaluate the potential presence of *O*-linked glycans and provided a detailed set of experiments to identify the *N*-linked glycans present on the protein. The methods described here directly determine the carbohydrates present, without additional labeling steps that are often needed for other analysis methods, thus saving time and reagent costs.

Acknowledgement and References

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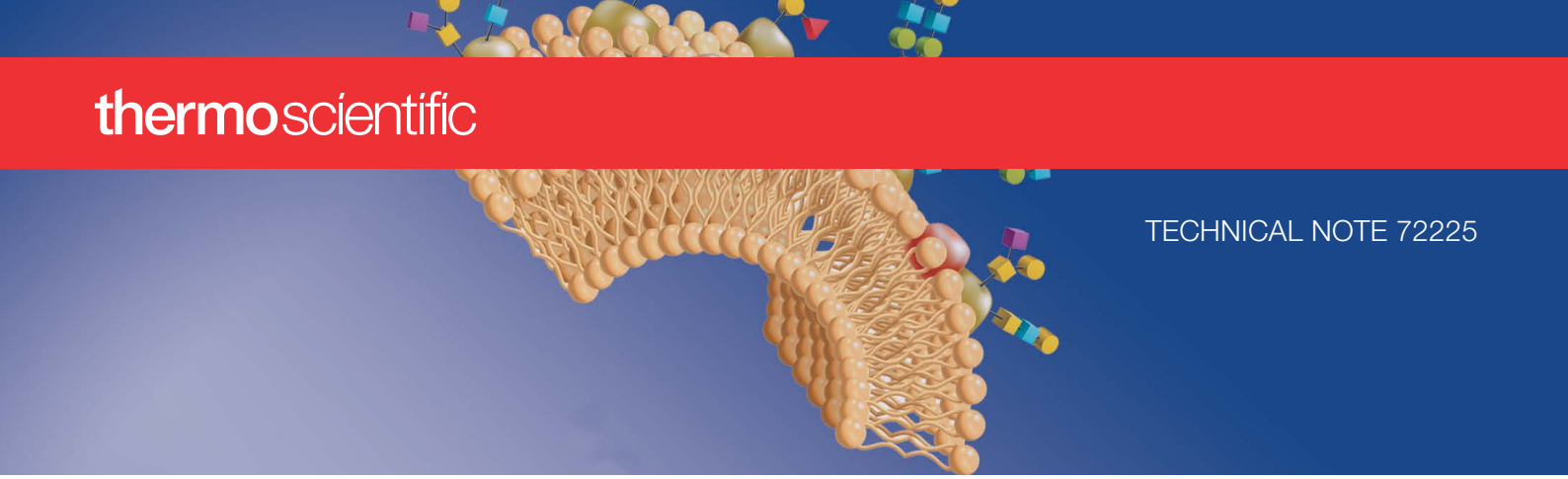

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TECHNICAL NOTE 72225

Glycoprotein monosaccharide analysis using HPAE-PAD with manually prepared eluent

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Keywords
Glycoprotein, Monosaccharide, HPAE-PAD, Manually prepared eluent, Dionex CarboPac columns, Dionex AminoTrap column

Goal

To present an accurate method of determining monosaccharides in glycoproteins using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) and manually prepared eluent

Introduction

Glycosylation plays an important part in protein structure and function.[1] Factors such as cell age, culture conditions, and purification affect the nature of protein glycosylation. As understanding of the role glycoproteins play in many biological processes[2,3] increases, the number of protein-based therapeutics will likely grow rapidly. In the near future, patents will be expiring for several key biotherapeutic glycoproteins. This will create an increased emphasis on the production of biosimilars[4] and need for methods to characterize protein glycosylation for quality control.

Two major types of protein glycosylation are observed in nature. The first type is *N*-linked glycosylation, which involves attachment of oligosaccharide chains to a side-chain nitrogen of the amino acid asparagine. The second type of glycosylation is *O*-linked, involving attachment through the hydroxyl-containing side chains of amino acids like serine and threonine. Overall, *N*-linked glycosylation is more clearly understood than *O*-linked glycosylation.

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Determination of the monosaccharide composition of a glycoprotein pharmaceutical is a typical quality control assay in the pharmaceutical industry. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for glycoprotein carbohydrate analysis. HPAE-PAD separates carbohydrates with specific interactions between their hydroxyl and carboxyl groups based on charge, size, composition, isomerism, and linkages. Therefore, HPAE-PAD is the best method for determining monosaccharides, sialic acids, and other carbohydrates. It allows for direct detection without sample derivatization, thereby reducing analyst time, expense, and exposure to hazardous chemicals. It is a selective technique with sensitive detection. Fast separations can be performed without loss of resolution using Thermo Scientific™ Dionex™ CarboPac™ PA20 columns.

The goal of this work is to describe an HPAE-PAD method for monosaccharide composition analysis using manually prepared eluent. Monosaccharide analysis using electrolytically generated eluent has been described before.[5] Here, three commercially available proteins, IgG, fetuin, and alpha-1-acid glycoprotein (AGP), were individually subjected to two hydrolysis conditions using 1) HCl, for the amino sugars galactosamine and glucosamine, and 2) TFA, for the neutral sugars mannose, glucose, and galactose. Results for method linearity, robustness, and accuracy for monosaccharide quantification are discussed here.

Experimental

Conditions

Columns:	Dionex CarboPac PA20, 3 × 150 mm (P/N 060142), Thermo Scientific™ Dionex™ AminoTrap™ 3 × 30 mm column (P/N 060146)
Column Temperature:	30 °C
Compartment Temperature:	30 °C
Flow Rate:	0.5 mL/min
Eluent:	A) DI water B) 0.2 M sodium hydroxide
Working Electrode:	Gold disposable on PTFE (P/N 066480)
Sampler Tray Temperature:	4 °C
Injection Volume:	10 µL (push_partial_LS)
Typical Backpressure:	2600 psi
Sample Loop Size:	20 µL
Elution Conditions:	10 mM NaOH for 12 min, 200 mM NaOH for 10 min, 10 mM NaOH for 10 min (Table 1)

Table 1. Elution conditions.

Time (min)	Solution A (%)	Solution B (%)	Elution
0	95	5	10 mM NaOH
12	95	5	10 mM NaOH
12.001	0	100	200 mM NaOH
22	0	100	200 mM NaOH
22.001	95	5	Start re-equilibration
32	95	5	End

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free Ion Chromatography (RFIC™) system was used in this work. The Dionex ICS-5000+ HPIC system is a modular ion chromatograph that includes:
  - DP dual pump module (P/N 079975) with degas option
  - DC standard bore detector compartment (P/N 075943) with dual temperature zones, two injection valves
  - Electrochemical detector (P/N 072042) and Cell (P/N 072044)
  - pH-Ag/AgCl reference electrode (P/N 061879)
  - Carbohydrate disposable Au working electrode, pack of 6 (two 2.0 mil gaskets included) (P/N 066480)
- AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Sterile assembled microcentrifuge tubes with screw cap, 1.5 mL (Sarstedt® P/N 72.692.005)
- Nalgene Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (Thermo Scientific P/N 164-0020)

Table 2 describes the carbohydrate four-potential waveform for the electrochemical detector.

Table 2. Carbohydrate four-potential waveform for the ED.
Reference electrode used in Ag mode (Ag/AgCl reference).

Time (s)	Potential (V)	Gain	Ramp Region	Integration
0	0.1	Off	On	Off
0.2	0.1	On	On	On
0.4	0.1	Off	On	Off
0.41	-2	Off	On	Off
0.42	-2	Off	On	Off
0.43	0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.5	-0.1	Off	On	Off

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Sodium hydroxide, 50% w/w (Fisher Scientific™ P/N SS254-500)
- Human serum IgG (Sigma® P/N I4506)
- Bovine serum fetuin (Sigma P/N F2379)
- Alpha-1-acid glycoprotein from human plasma (Sigma P/N G9885)
- Thermo Scientific™ Pierce™ Micro BCA™ Protein Assay Kit (P/N 23235)
- Thermo Scientific Pierce Trifluoroacetic acid (TFA), sequencing grade (P/N 28904)
- Thermo Scientific Pierce Hydrochloric acid (P/N 24308)

Preparation of eluent and reagents

200 mM sodium hydroxide eluent

Dilute 10.4 mL of a 50% (w/w) sodium hydroxide solution into 1 L of DI water to prepare a 0.2 M sodium hydroxide solution. After preparation, keep the eluent blanketed under UHP-grade nitrogen (5.0) or UHP-grade helium (5.0) at 34 to 55 kPa (5 to 8 psi) at all times. Note: Hydroxide eluents for HPAE-PAD should be prepared only from commercial 50% (w/w) sodium hydroxide solutions. Please see Technical Note 71[6] for more information on preparing eluents for HPAE-PAD.

Carbohydrate standards

Dissolve the contents of one Thermo Scientific™ Dionex™ MonoStandard 100 nmol vial in 1.0 mL of DI water and mix to prepare a stock standard solution containing 0.1 mM (100 pmol/µL) of each monosaccharide. Immediately freeze unused stock standard at < -10 °C. Avoid repeated freeze/thaw cycles. Deterioration can occur within 24–48 h at room temperature.

Methods

TFA and HCl hydrolysis

1. Human serum IgG
   - HCl hydrolysates: Prepare HCl hydrolysates for IgG by combining 400 µL of 6 M HCl with 20 µL of 3 mg/mL IgG in a 1.5 mL microcentrifuge tube.
   - TFA hydrolysates: Prepare TFA hydrolysates of IgG by combining 200 µL of 0.3 mg/mL IgG, 140 µL of DI water, and 60 µL of neat TFA in a 1.5 mL microcentrifuge tube.
2. Bovine fetuin and AGP
   - TFA hydrolysates: Prepare TFA hydrolysates of fetuin and AGP by combining 20 µL of 3 mg/mL protein solution, 150 µL DI water, and 30 µL of neat TFA in a 1.5 mL microcentrifuge tube.
   - HCl hydrolysates: Combine 400 µL of 6 M HCl with 20 µL of 3 mg/mL fetuin solution in a 1.5 mL microcentrifuge tube.
3. Heat the solutions for 4 h at 100 °C and then dry for 3 h at room temperature in a Thermo Scientific™ Savant™ SpeedVac™ concentrator equipped with an acid trap.
4. Reconstitute each vial with 300 µL of DI water.
5. Vortex for 30 s and centrifuge for 5 min. Inject 10 µL of the supernatant (2 µg protein per injection) into the ion chromatography system.

Dionex BorateTrap column

Borate is a known contaminant in laboratory water supplies. In chromatography, borate contamination of HPLC eluents can come from degrading (i.e. poorly maintained) deionized water systems or it may come from leaching from glass eluent bottles, which should not be used for HPAE-PAD. We have found that if borate is present in the eluent, it forms anionic complexes with carbohydrate analytes. Because the carbohydrate-borate complex is less efficiently eluted by hydroxide from the anion exchanger than the carbohydrate itself, peak tailing occurs. Analytes with vicinal hydroxyl groups, such as sugar alcohols and mannose, show severe chromatographic tailing when borate is present in the eluents. This tailing causes the peak to differ from a Gaussian distribution (where peak asymmetry = 1), making it difficult to identify and quantify the carbohydrate analytes. If peak tailing of mannose (or alditols) is observed, the Thermo Scientific™ Dionex™ BorateTrap™ Inline Trap column may be used to remove borate from the eluent stream. The Dionex BorateTrap column should be installed between the HPLC pump and the sample injector. After installation, the mannose peak should appear symmetric. With prolonged use, if the capacity of the Dionex BorateTrap is exceeded, peak tailing of mannose may become apparent. If this occurs, the Dionex BorateTrap should be replaced.

Dionex AminoTrap column

The Dionex AminoTrap column delays the elution of amino acids and small peptides found in glycoprotein hydrolysates. The Dionex AminoTrap column is used in place of a guard column before the Dionex CarboPac PA20 column. Install the Dionex AminoTrap column after the injection valve and condition by flushing with 100 mM KOH at 0.5 mL/min for 2 h. Although slight peak broadening and longer retention times are expected with the addition of the Dionex AminoTrap column (compared to those obtained with the analytical column), the six monosaccharides will be well resolved. Please see Technical Note 125[7] for guidance on successful use of Dionex AminoTrap columns.

Note: Do not pump water through the Dionex AminoTrap column; it will cause irreversible damage to the column.

Results and discussion

Separation

Separation of monosaccharides was achieved using a Dionex CarboPac PA20 column (3 × 150 mm) with a Dionex AminoTrap guard column using isocratic elution conditions, followed by a step change to higher eluent concentration that was used to remove contaminant species, including carbonate, still bound to the column. The Dionex AminoTrap column delays the elution of the amino acids and small peptides from protein acid hydrolysis that could interfere with monosaccharide peak integration and response. Figure 1 shows a typical separation of a 10 µL injection of the Dionex MonoStandard, containing fucose, galactosamine, glucosamine, galactose, glucose, and mannose, each at 10 µM concentration (100 pmol each). The peaks are baseline resolved and elute within a window of 13 min. The total run time is 32 min to allow for washing and re-equilibration after the column regeneration step. The chromatogram shows not only the region where the monosaccharides elute, but also the column wash and re-equilibration regions.

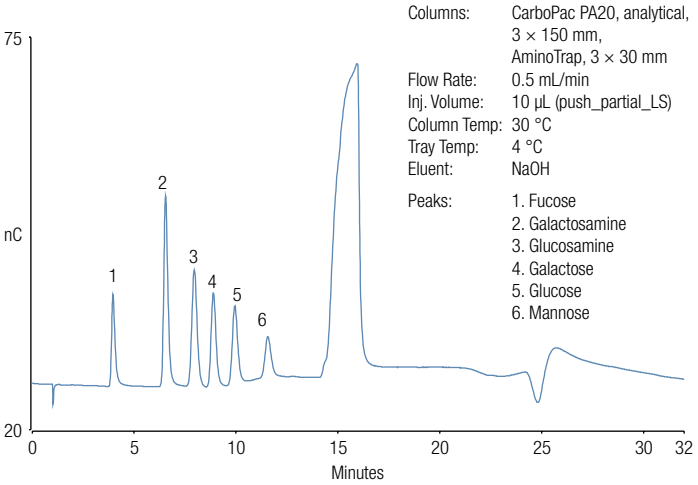

Figure 1. Separation of a 10 µM Dionex MonoStandard injection containing 10 µM each of the six monosaccharides.

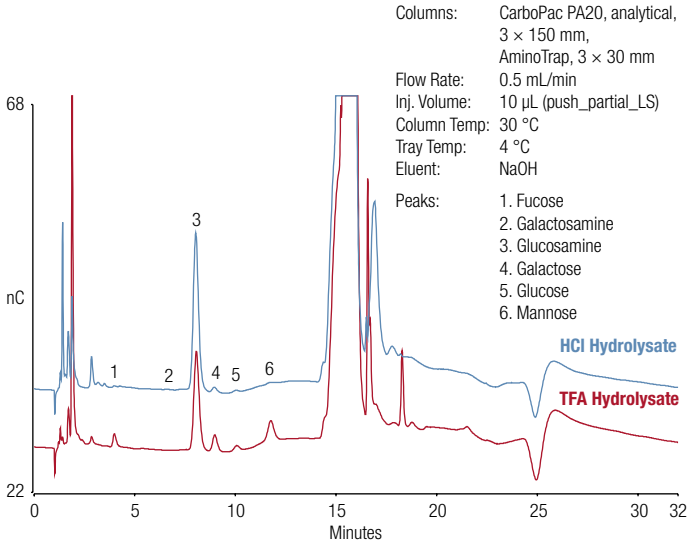

Figure 2. Analysis of human serum IgG TFA and HCl hydrolysates.

For each of the three proteins used in this study, hydrochloric acid (HCl) and trifluoroacetic acid (TFA) hydrolysates were prepared and injected directly after drying. Each injection contained 2 µg protein. Figure 2 shows typical injections of human serum IgG TFA as well as HCl hydrolysates. Human serum IgG has lower carbohydrate content compared to most mammalian glycoproteins.

The low concentration of carbohydrate makes monosaccharide determination more challenging because there is a higher concentration of peptides and amino acids in the acid hydrolysate, relative to glycoproteins when there is more glycosylation. However, the proposed method is sensitive enough to determine monosaccharides in this sample without derivatization. The TFA hydrolysis is done to determine the neutral sugars, fucose, galactose, and mannose. The yield of the amino sugars, galactosamine and glucosamine, is not 100% (commonly estimated to be 95%), but many scientists use these or similar hydrolysis conditions to determine amino sugars.

To improve amino sugar accuracy, some scientists use HCl hydrolysis, as we have done here. HCl hydrolysis conditions destroy a majority of the neutral sugars. While glucose is observed in the hydrolysates, it is nearly always a contaminant as it is not typically present in glycoprotein oligosaccharides. Note the difference in the column cleaning section of the chromatogram compared to the chromatogram of the standard in Figure 1.

Figure 3 presents the HPAE-PAD chromatograms of bovine fetuin TFA and HCl hydrolysates. The monosaccharide peaks are baseline resolved. The neutral monosaccharides are seen at higher concentrations in the TFA hydrolysate, and the amino sugars at a higher concentration in the HCl hydrolysate, as expected. As fetuin is more glycosylated than IgG, note the increase in the ratio of the size of the monosaccharide peaks to the peaks in the column cleaning section of the chromatogram compared to human serum IgG.

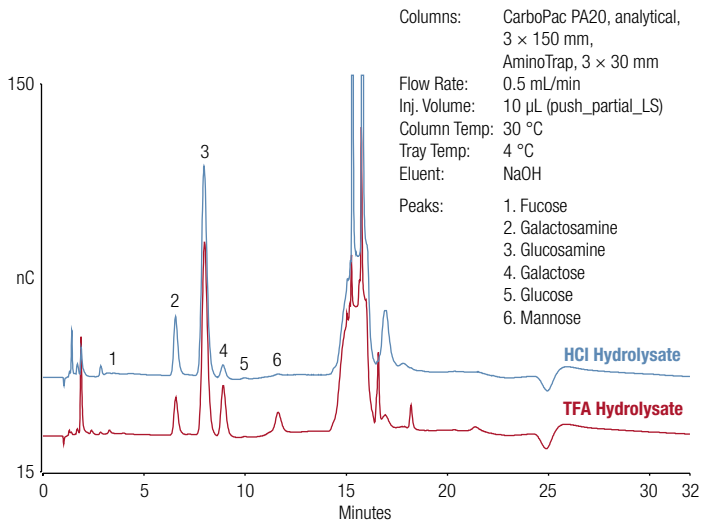

Figure 3. Analysis of bovine fetuin TFA and HCl hydrolysates.

Figure 4 shows HPAE-PAD chromatograms from human alpha-1-acid glycoprotein (AGP) hydrolysates. AGP has the highest level of glycosylation among the three proteins studied here. Both TFA and HCl hydrolysates show well-resolved amino and neutral sugars.

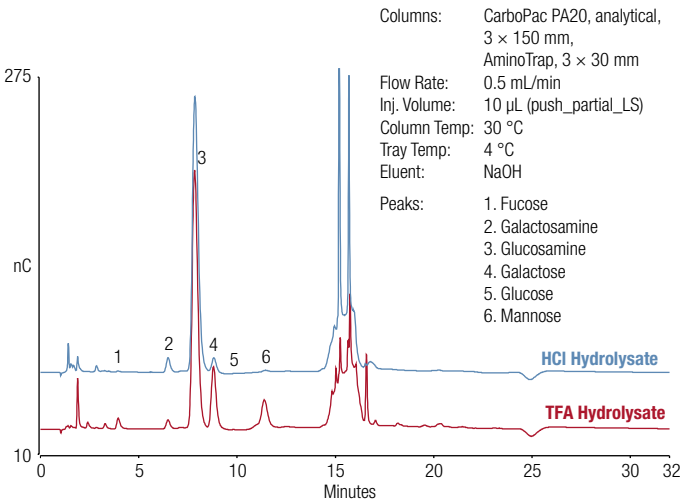

Figure 4. Analysis of AGP TFA and HCl hydrolysates.

Linearity and precision

The linearity of monosaccharide determination was studied by generating peak area response curves for all six monosaccharides using a monosaccharide standard mix containing 1.56 to 300 µM of each of the six monosaccharides, except galactosamine for which the linearity range was from 1.56 to 50 µM. The results included in Table 3 show that the coefficients of determination ranged from 0.983 to 0.999 for all six monosaccharides. A linear curve fit was used for five of the six monosaccharides, and a second order polynomial curve fit was used for glucosamine. Figure 5 shows calibration plots obtained for each of the six monosaccharides.

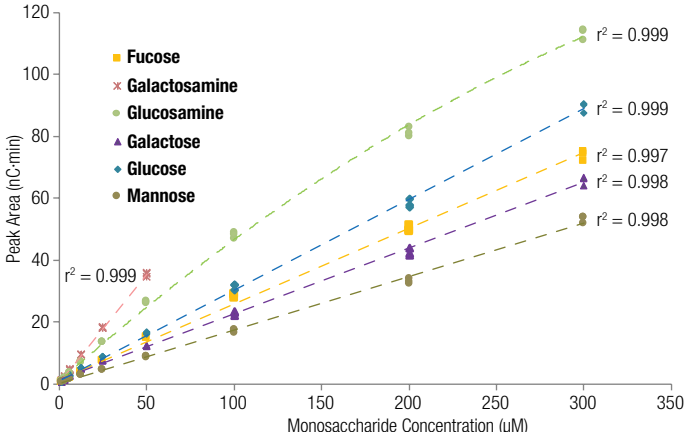

Figure 5. Calibration plots generated for each of the six monosaccharides.

The method precision was determined in two ways. First, method repeatability was determined at three concentrations of the six Dionex MonoStandard mix with three replicates of each sample to give nine total injections. The results included in Table 4 show excellent peak area as well as retention time precision for all three concentration levels tested with all RSD values below 2%. The intermediate precision was determined by assaying eight replicate injections of the 10 µM Dionex MonoStandard each day for three consecutive days. The results of this experiment contained in Table 5 show excellent retention time as well peak area precision. The highest RSDs for retention time and peak area were 0.58% and 3.39%, respectively.

Table 3. Calibration data for monosaccharides (n=3).

Peak No.	Peak Name	Retention Time (min)	Concentration Range (µM)	Levels	Coefficient of Determination
1	Fucose	3.91	1.56-300	9	0.999
2	Galactosamine	6.48	1.56–50	6	0.999
3	Glucosamine	7.87	1.56–300	9	0.983
4	Galactose	8.83	1.56–300	9	0.997
5	Glucose	9.87	1.56–300	9	0.998
6	Mannose	11.42	1.56–300	9	0.998

Table 4. Method precision determined at three concentrations (n=3).

Standard Conc	Retention Time and Peak Area RSDs											
	Fucose		Galactosamine		Glucosamine		Galactose		Glucose		Mannose	
	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area
3.12 µM	0.12	1.14	0.07	1.84	0.01	1.57	0.01	1.78	0.05	1.89	0.07	0.73
12.5 µM	0.13	1.12	0.00	0.91	0.06	0.92	0.01	1.06	0.05	1.08	0.05	0.95
100 µM	0.00	1.67	0.00	1.46	0.06	1.42	0.01	1.22	0.00	1.33	0.01	1.07

Table 5. Intermediate precision determined using 10 µM Dionex MonoStandard samples over three days (n=8).

Days	Retention Time and Peak Area RSDs											
	Fucose		Galactosamine		Glucosamine		Galactose		Glucose		Mannose	
	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area
1	0.29	2.08	0.41	2.21	0.49	2.09	0.34	1.91	0.45	2.6	0.58	1.93
2	0.09	3.39	0.06	3.37	0.07	3.4	0.06	2.92	0.04	3.01	0.06	3.43
3	0.1	3.02	0.09	2.63	0.05	2.55	0.08	1.81	0.06	2.59	0.05	2.12

Detection limits

The monosaccharide detection limits for this HPAE-PAD assay under the conditions described was set at monosaccharide concentrations that resulted in a signal-to-noise ratio of 10:1. A series of monosaccharide standards were prepared and analyzed. The signal-to-noise ratios were calculated using the peak height for each monosaccharide; the noise level was calculated from a stable portion of the baseline where no peak elutes. Table 6 contains limits of detection for all six monosaccharides determined in this study.

Table 6. Method sensitivity determination (n=3).

Monosaccharide	Detection Limit (µM)	Amount Injected (pmoles)	S/N Ratio
Fucose	0.25	2.5	13.4
Galactosamine	0.1	1.0	10.9
Glucosamine	0.2	2.0	9.7
Galactose	0.25	2.5	13.7
Glucose	0.25	2.5	11.7
Mannose	0.2	2.0	9.5

Accuracy

Accuracy of the assay was determined by spiking a known amount of monosaccharides into each of the dried and reconstituted acid hydrolysates prepared for the three glycoproteins used in the study. For each monosaccharide, 20% to 150% spike levels based on calculated endogenous monosaccharide concentration were used. The monosaccharides present below the lowest calibration standard were not quantified and hence were not spiked. The results in Table 7 show excellent recoveries of the spiked monosaccharides with all the recoveries falling between 80% and 120%.

Table 7. Recovery of monosaccharide spikes in to the acid hydrolysates prepared for all three glycoproteins used in this study (n=3).

Sample	Parameter	AGP HCl Digest	AGP TFA Digest	Fetuin HCl Digest	Fetuin TFA Digest	IgG HCl Digest	IgG TFA Digest
Fucose	Base Conc. (µM)	-	5.3	-	-	-	-
	Spike%	-	37.5	-	-	-	-
	%Recovery	-	120	-	-	-	-
Galactosamine	Base Conc. (µM)	3.2	1.9	6.7	4.7	-	-
	Spike%	62.1	104	89.3	129	-	-
	%Recovery	98.3	94.5	87.4	76.9	-	-
Glucosamine	Base Conc. (µM)	162.5	142.9	45.9	44.9	11.0	6.2
	Spike%	49.0	55.0	130.8	66.8	72.9	129.03
	%Recovery	90.1	102	93.2	102	79.3	98.9
Galactose	Base Conc. (µM)	8.6	58.3	-	20.1	-	-
	Spike%	23.2	137	-	150	-	-
	%Recovery	84.7	104	-	94.1	-	-
Mannose	Base Conc. (µM)	2.1	55.3	-	21.7	-	7.5
	Spike%	94.0	145	-	138	-	107
	%Recovery	113	108	-	97.8	-	105

Robustness

Assay robustness was determined on three columns, two new columns from the same lot and a 6-month-old column from a different lot. Figure 6 shows representative chromatograms of the 10 µM monosaccharide standard on all three columns used. The robustness was studied by introducing ±10% variation in common chromatographic parameters. The parameters varied in this study were: initial eluent concentration, final eluent concentration, column temperature, and flow rate. Method performance under these conditions was evaluated by calculating percent difference in three key chromatographic parameters: retention time, peak asymmetry, and resolution.

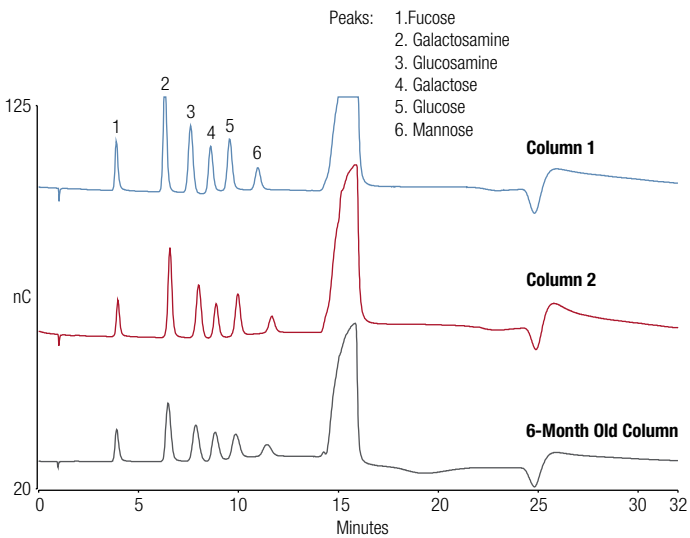

Figure 6. Separation of a 10 µM Dionex MonoStandard injection containing 10 µM each of the six monosaccharides on three different columns used for robustness.

The results are included in Tables 8, 9, and 10 for column 1, column 2, and column 3 (the 6-month-old column), respectively. For the first two columns, none of the experimental variations tested here resulted in significant disruption of the three target chromatography parameters. The highest impact was observed when the column temperature was reduced to 27 °C, which resulted in approximately 18% reduction in resolution between glucosamine and galactose. Even under these conditions, the resolution between these two peaks was 1.7 for column 1 and 1.9 for column 2. This level of resolution remains good for quantitative analysis.
In the robustness studies using the 6-month-old column, the highest impact was also observed for the reduced-temperature condition. This condition resulted in the highest percent difference of 16.6 as compared to the non-stressed method. The lowest resolution between glucosamine and galactose is 1.33 for the low temperature condition, which is not ideal for peak quantification. The impact of lower temperature is more severe for the older column because it has reduced retention of the target monosaccharides at the start of the experiment compared to columns 1 and 2, as can be observed in Figure 6. This difference is due to the natural loss of column capacity with injection of samples that can sometimes be reversed with an aggressive column cleaning. This cleaning was not done for column 3 before the start of the robustness experiment. Refer to Dionex CarboPac PA20 column manual[8] section 5.3.1 for column cleaning methods.

Table 8. Results of robustness study performed on column 1 using 10 µM Dionex MonoStandard samples containing 10 µM of each monosaccharide (n=3).

Condition	Percent Difference (%)																
	Retention Time						Asymmetry						Resolution				
	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C																	
0.5 mL/min, 9 mM/200 mM NaOH, Column Temp. 30 °C	1.5	2.7	3.3	2.5	3.0	4.0	3.5	0.9	1.6	0.6	-2.1	10.3	2.3	2.4	-6.5	4.6	7.1
0.5 mL/min, 11 mM/200 mM NaOH, Column Temp. 30 °C	-0.7	-1.7	-2.2	-1.5	-1.9	-2.6	3.0	2.4	2.9	1.8	1.8	-7.5	-2.0	-2.4	6.8	-3.3	-4.3
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 27 °C	2.2	5.6	5.9	4.1	4.9	5.0	1.7	3.0	0.0	2.8	1.2	-7.5	3.9	-2.1	-18.5	5.9	-0.5
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 33 °C	-2.7	-6.2	-6.5	-4.5	-5.3	-5.7	5.0	6.3	5.3	3.1	2.1	-8.6	-6.5	0.0	21.7	-6.5	-0.3
0.45 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	12.0	12.4	12.7	12.4	12.6	13.1	5.0	6.6	5.9	5.6	2.4	-9.4	2.4	3.4	0.1	4.0	7.2
0.55 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	-8.4	-8.3	-8.06	-8.3	-8.1	-7.8	4.2	5.4	3.6	3.4	4.9	-7.5	-2.4	-1.3	-5.5	-0.4	0.8
0.5 mL/min, 10 mM/180 mM NaOH, Column Temp. 30 °C	0.2	-0.0	0.00	-0.06	-0.03	0.02	5.5	7.2	5.6	4.0	4.6	-0.5	-1.4	-0.2	-0.9	0.1	0.6
0.5 mL/min, 10 mM/220 mM NaOH, Column Temp. 30 °C	0.3	0.2	0.32	0.1	0.2	0.3	6.7	7.6	4.9	3.7	3.9	1.6	-1.2	0.1	-2.3	0.8	1.3

Table 9. Results of robustness study performed on column 2 using 10 µM Dionex MonoStandard samples containing 10 µM of each monosaccharide (n=3).

Condition	Percent Difference (%)																
	Retention Time						Asymmetry						Resolution				
	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C																	
0.5 mL/min, 9 mM/200 mM NaOH, Column Temp. 30 °C	1.3	2.5	3.1	2.3	2.7	3.7	3.7	2.1	-0.3	0.2	-1.1	5.2	1.4	2.7	-7.1	4.6	5.5
0.5 mL/min, 11 mM/200 mM NaOH, Column Temp. 30 °C	-1.1	-2.3	-2.9	-2.1	-2.6	-3.4	0.0	1.6	1.5	0.5	0.5	-0.5	-3.5	-3.3	5.2	-4.9	-6.7
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 27 °C	2.0	5.5	5.8	3.9	4.8	4.9	-0.6	0.0	-1.5	0.5	-0.8	-0.2	3.8	-2.2	-18.0	6.1	-1.1
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 33 °C	-2.6	-5.9	-6.3	-4.5	-5.3	-5.5	1.7	5.4	6.3	2.8	1.4	1.9	-8.6	-1.8	15.7	-7.3	-2.4
0.45 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	11.0	11.0	11.1	11.0	11.0	11.1	5.1	7.9	5.1	4.3	3.3	0.2	-2.0	-0.4	-0.9	-0.4	-0.3
0.55 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	-9.2	-9.6	-9.6	-9.7	-9.6	-9.7	-2.4	1.6	1.8	1.7	0.0	-0.5	-4.1	-3.1	-3.9	-2.1	-2.8
0.5 mL/min, 10 mM/180 mM NaOH, Column Temp. 30 °C	-0.3	-0.7	-0.8	-0.9	-0.9	-1.0	2.0	4.3	3.6	4.0	1.9	4.4	-4.0	-2.6	-2.5	-1.6	-2.8
0.5 mL/min, 10 mM/220 mM NaOH, Column Temp. 30 °C	-0.5	-1.1	-1.2	-1.4	-1.4	-1.4	1.5	5.4	3.0	4.0	3.9	1.6	-4.9	-2.7	-4.6	-1.9	-2.8

Table 10. Results of robustness study performed on column 3 (old column) using 10 µM Dionex MonoStandard containing 10 µM of each monosaccharide (n=3).

Condition	Percent Difference (%)																
	Retention Time						Asymmetry						Resolution				
	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C																	
0.5 mL/min, 9 mM/200 mM NaOH, Column Temp. 30 °C	1.3	2.8	3.5	2.5	3.1	4.2	-0.7	-1.9	-1.5	2.6	-3.6	1.3	2.5	2.9	-8.1	5.7	7.3
0.5 mL/min, 11 mM/200 mM NaOH, Column Temp. 30 °C	-1.1	-1.8	-2.3	-1.7	-2.1	-2.7	-0.9	-1.3	1.5	0.0	0.5	-3.2	-1.7	-2.6	4.8	-3.4	-4.1
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 27 °C	2.1	5.7	6.0	4.1	4.9	5.1	-3.9	-2.7	-5.7	0.6	-2.2	-5.6	5.4	0.1	-16.6	6.9	0.2
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 33 °C	-3.0	-6.2	-6.6	-4.7	-5.5	-5.8	2.3	2.9	6.3	1.1	1.4	-0.5	-8.2	-3.8	16.4	-8.8	-2.7
0.45 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	10.9	10.9	10.8	15.2	7.0	10.8	2.1	2.4	2.7	2.0	-1.9	1.3	-1.4	-1.8	-0.2	9.9	-9.5
0.55 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	-9.1	-9.1	-9.1	-9.2	-9.2	-9.2	-2.7	-1.3	0.3	1.1	0.0	-1.3	-2.2	-2.3	-3.7	-1.9	-1.6
0.5 mL/min, 10 mM/180 mM NaOH, Column Temp. 30 °C	-0.3	-0.4	-0.4	-0.4	-0.4	-0.5	0.9	1.6	1.5	0.6	1.1	0.3	-2.1	-2.2	-1.9	-1.9	-2.7
0.5 mL/min, 10 mM/220 mM NaOH, Column Temp. 30 °C	-0.9	-1.3	-1.4	-1.7	-1.7	-1.7	1.6	0.8	1.2	2.0	1.4	0.3	-2.7	-2.5	-4.0	-1.3	-1.9

Conclusion

This study describes an HPAE-PAD assay for determination of the monosaccharide composition of a glycoprotein. A single injection provides analysis of six monosaccharides. This assay for monosaccharide quantification was validated according to the analytical performance characteristics outlined in USP General Chapter <1225>, Validation of Compendial Procedures.[9] The results show that the method can provide baseline separation for all the monosaccharides in HCl as well TFA hydrolysates. Even for IgG, which has low glycosylation and, therefore, a greater protein-to-carbohydrate ratio compared to many other glycoproteins, the separation is good. The method shows excellent precision for retention time as well as peak area. The method was shown to accurately measure the monosaccharide concentration in complex matrices such as acid-hydrolyzed proteins. Moreover, the method is robust to experimental condition variations that may occur during routine use.

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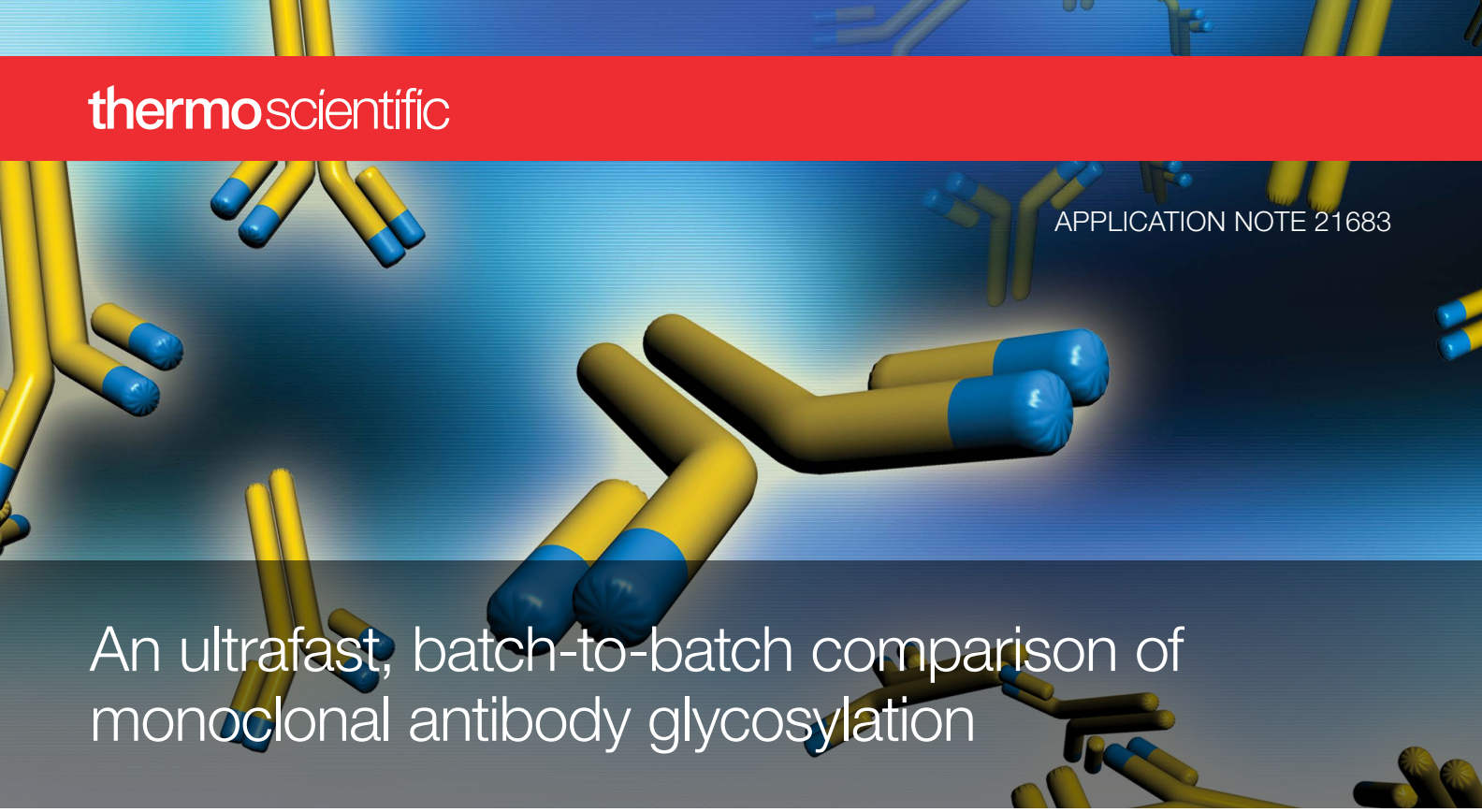

An ultrafast, batch-to-batch comparison of monoclonal antibody glycosylation

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Keywords

NIBRT, Biopharmaceutical, Bioproduction, QA/QC, Biotherapeutic, IgG, Monoclonal antibody (mAb), Critical quality attribute, Glycoprotein, Glycosylation, Glycoform, Released N-glycan, 2-AA, Rituximab, Solid core, Accucore Amide HILIC, Vanquish Horizon UHPLC, Q Exactive, HRAMS MS

Application benefits

- High-throughput screening method for unambiguous identification of glycoforms
- 20 times reduction in separation time compared to standard profiling methods
- Excellent batch-to-batch reproducibility for method development of novel mAbs and biosimilar products

Goal

To develop a high-throughput screening method using HILIC UHPLC separation of 2AA-labelled glycans in a model mAb as a proof of concept for a more general approach to mAb glycoprofiling capable of identifying differences in high abundance gylcoforms. The separation must be rapid but requires sufficient resolution to allow batch-to-batch differences in the glycan profile to be identified and subsequently characterized by high-resolution, accurate-mass mass spectrometry.

Introduction

One of the fastest growing fields in the pharmaceutical industry is the market of therapeutic glycoproteins (glycosylated proteins), which are produced by living cell systems.[1,2] These include monoclonal antibodies (mAbs) and other

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recombinant protein products (e.g., fusion proteins, growth factors, cytokines, therapeutic enzymes, and hormones), which are approved or under development as therapeutics. Glycosylation is a critical quality attribute (CQA) for development and manufacturing of therapeutic mAbs in the biopharmaceutical industry and, therefore, needs to be assessed to ensure desired product quality, safety, and efficacy.[3] Different glycosylation variants have been shown to affect stability, pharmacokinetics, serum half-life, immunogenicity, and effector functions.[4,5,6]

Glycoforms on biopharmaceutical glycoproteins are affected by the culture conditions as well as the cell type by which they are produced.[7,8] Biopharmaceutical glycosylation monitoring for correct structure during production and for quality control is required by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA).[9] Current glycoanalytical methods are laborious and time-consuming; therefore, more rapid and high-throughput (HTP) methods are required. HTP techniques for in-depth monitoring of glycoform distributions must be integral components for the implementation of quality by design (QbD) approaches. To meet this need, novel approaches toward HTP monitoring are required.

Rituximab (MabThera®) is a genetically engineered chimeric mouse/human monoclonal antibody representing a glycosylated immunoglobulin with human IgG1 constant regions and murine light-chain and heavy-chain variable region sequences. The antibody is produced by mammalian (Chinese hamster ovary) cell suspension culture and purified by affinity chromatography and ion exchange, including specific viral inactivation and removal procedures.

This application note presents a proof of concept for an ultrafast *N*-glycan analysis approach to glycoprofiling of the main glycoforms of mAbs by HILIC chromatography and the Vanquish Horizon UHPLC system with fluorescence detection. Time for analysis is reduced considerably in comparison to the standard method applied for full detailed characterization (the standard methods can take up to 55 minutes).[10] The analysis is completed in 2.5 minutes and can be applied for the comparison of mAbs glycosylation expressed under various cell culture conditions, as well as for the evaluation of antibody culture clones and various production batches.

Experimental

Chemicals and reagents

- Deionized (DI) water, 18.2 MΩ•cm resistivity
- Fisher Scientific™ Acetonitrile, HPLC grade (P/N 10407440)
- Fisher Scientific™ Optima™ Formic acid, LC-MS (P/N 10596814)
- Fisher Scientific Ammonium hydroxide (P/N 10508610)
- Fisher Scientific Ammonium bicarbonate (P/N 10207183)
- Thermo Scientific™ Virtuoso™ Vial kit (P/N 60180-VT402)
- PNGase F (purchased from a reputable supplier)
- Ultra 0.5 mL centrifugal filters MWCO 10 kDa (purchased from a reputable supplier)
- Fisher Scientific Sodium cyanoborohydride (P/N 10082110)
- Fisher Scientific Glacial acetic acid (P/N A/0360/PB17)
- Fisher Scientific Dimethylsulfoxide (DMSO) (P/N 10213810)
- Anthranilic acid (2-AA) (purchased from a reputable supplier)
- Fisher Scientific Tris(hydroxymethyl)methylamine hydrochloride (P/N 10060390)
- Fisher Scientific Urea (P/N 10132740)

Equipment

Thermo Scientific™ UltiMate™ 3000 RS system, including pump:

- LPG-3400RS Rapid Separation Quaternary Pump (P/N 5040.0036)
- WPS-3000TRS Rapid Separation Thermostatted Well Plate Autosampler (P/N 5840.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- FLD-3400RS Rapid Separation Fluorescence Detector with Dual-PMT (P/N 5078.0025)
- SR-3000 Solvent Rack (P/N 5035.9200)
- 2 µL Micro Flow Cell (P/N 6078.4330)

Thermo Scientific™ Vanquish™ Horizon UHPLC system, including:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A),
- Column Compartment H (P/N VH-C10-A),
- Split Sampler HT (P/N VH-A10-A) with 25 µL (V=50 µL) Sample Loop
- Fluorescence Detector F (P/N VF-D50-A)

Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer

Thermo Scientific™ Accucore™ 150-Amide-HILIC 2.6 µm, 2.1 × 50 mm (P/N 16726-052130)

Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 150 mm (P/N 16726-152130)

Thermo Scientific™ SpeedVac™ Concentrator (P/N SPD121p)

Buffers preparation

- Ammonium formate (50 mM, pH 4.4): 1.8 g of formic acid was dissolved in 1 L of DI water. pH was adjusted to pH 4.4 with ammonium hydroxide solution.
- 8 M urea in 0.1 M Tris-HCl, pH 8.5 (UA buffer): 1.57 g of Tris-HCl was dissolved in 100 mL DI water and pH adjusted to 8.5 with 1 M NaOH. 24 g of urea was dissolved in 50 mL 0.1 M Tris-HCl, pH 8.5.
- Ammonium bicarbonate (50 mM, pH 7.8): 0.39 g of ammonium bicarbonate was dissolved in 100 mL of DI water.

N-glycan release, labelling, and clean up

1. 100 µg of protein was denatured using 8 M urea in 0.1 M tris buffer pH 8.0 (UA solution) and subsequently reduced and alkylated using 10 mM DTT and 50 mM IAA prepared in UA solution, respectively.
2. Following buffer exchange into 50 mM ammonium bicarbonate using 10 kDa MWCO filters, *N*-glycan release was performed by incubation of the reduced and alkylated sample with 1000 units of PNGase F overnight at 37 °C.
3. Released glycans were collected from the deglycosylated proteins by centrifugation through 10 kDa MWCO filters and subsequently reduced to dryness via vacuum centrifugation.
4. Glycans were converted to reducing aldoses by reconstitution in 50 µL of 1% formic acid and reduced to dryness.
5. Dried glycans were derivatized with 5 µL 2-aminobenzoic acid (2-AA) via reductive amination with sodium cyanoborohydride in 30% (v/v) acetic acid in DMSO at 60 ºC for 5 hours.
4. Excess labelling reagent was removed by HILIC purification using an UltiMate 3000 RS system. Samples were loaded in 80% acetonitrile / 20% 50 mM ammonium formate pH 4.4 (v/v) onto an Accucore 150-Amide-HILIC 2.1 × 50 mm column at 0.5 mL/min for 2.5 minutes. Labelled glycans were eluted in 20% aqueous acetonitrile for 2.5 minutes, monitored by fluorescence detection, λex/em = 350/425 nm, and evaporated to dryness.

UHPLC-fluorescence *N*-glycan profiling

Labelled *N*-glycans were separated by hydrophilic interaction UHPLC-FLD on a Vanquish Horizon UHPLC. 2AA-labelled glycans were separated using a linear gradient. Glycans were injected in 5 µL 80% v/v acetonitrile and stored at 10 ºC prior to injection.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 50 mm (P/N 16726-052130)
Mobile phase A:	Ammonium formate 50 mM, pH 4.5
Mobile phase B:	Acetonitrile
Flow rate:	2.2 mL/min 395 backpressure at starting conditions
Column temperature:	60 ºC
Injection volume:	5 µL

Table 1. Mobile phase gradient for UHPLC-fluorescence N-glycan profiling.

Time (min)	%A	%B	Flow (mL/min)	Curve
0	25	75	2.2	5
2.00	33	67	2.2	5
2.01	25	75	2.2	5
2.50	25	75	2.2	5

N-glycan analysis by LC-MS

Glycan samples were injected into a Q Exactive Plus MS equipped with a HESI ion source. Samples were diluted in 75% acetonitrile prior to analysis.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 µm, 2.1 x 150 mm (P/N 16726-152130)
Mobile phase A:	Ammonium formate 50 mM, pH 4.5
Mobile phase B:	Acetonitrile
Flow rate:	0.4 mL/min
Column temperature:	40 ºC
Injection volume:	11 µL

Table 2. Mobile phase gradient.

Time (min)	%A	%B	Flow (mL/min)	Curve
0	25	75	0.4	5
30.0	50	50	0.4	5
30.5	55	45	0.4	5
32.0	55	45	0.4	5
32.5	25	75	0.4	5
40.0	25	75	0.4	5

MS conditions

Ionization:	HESI Negative Ion
Scan range:	500 to 2000 *m/z*
Source temperature:	300 °C
Sheath gas flow:	20 Arb
Auxiliary gas flow:	10 Arb
Spray voltage:	3.8 kV
Capillary temperature:	320 °C

Data processing and software

Chromatographic software:	Thermo Scientific™ Chromeleon™ CDS 7.2 SR4
MS data acquisition:	Thermo Scientific™ Xcalibur™ software 2.2 SP1.48

Results and discussion

A commercial chimeric IgG1 mAb was analyzed on an Accucore 150-Amide-HILIC HPLC column (2.1 × 50 mm) within 2.5 min, offering an ultrafast method for glycoprofiling of the main glycoforms. Figure 1 shows the separation of the eight most abundant 2AA-labelled *N*-glycans, which include bi-antennary structures with variable degrees of core fucosylation and galactosylation and a high mannose structure. The structural assignment for each peak was confirmed by accurate MS data.

Retention time precision is essential in labelled glycan analysis for peak identification. Precision of retention time was determined for seven consecutive injections of rituximab 2-AA *N*-glycans. Figure 2 shows excellent retention time precision for the eight dominant glycan structures.

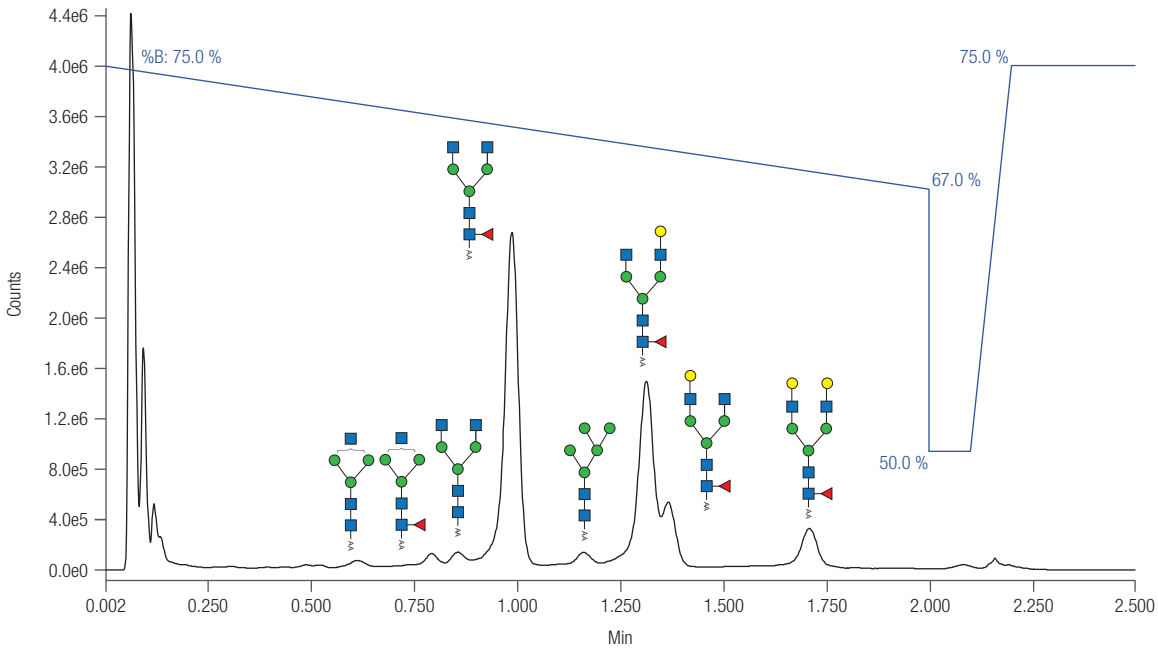

Figure 1. Chromatographic separation of commercial chimeric IgG1 mAb (rituximab) 2AA-labelled *N*-glycans on an Accucore 150-Amide-HILIC column (2.1 × 50 mm, 2.6 µm).

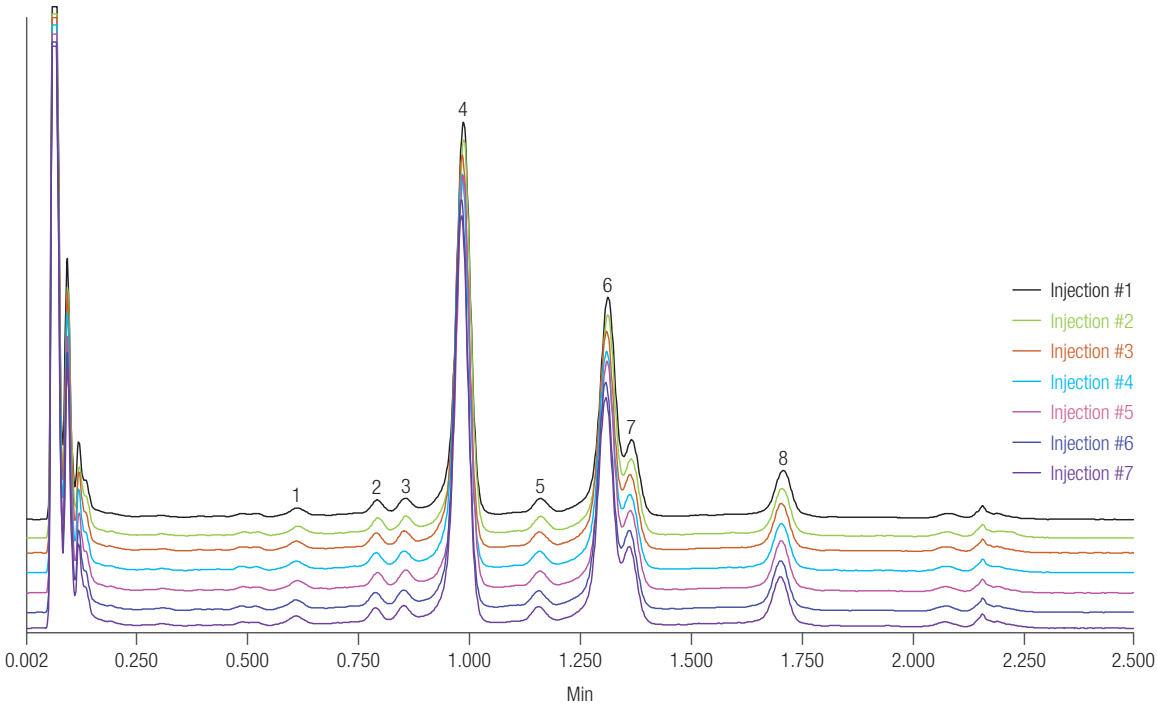

Figure 2. Repeated injections of rituximab 2-AA *N*-glycans on an Accucore 150-Amide-HILIC column (2.1 × 50 mm, 2.6 µm).

The developed method was applied for batch-to-batch comparison of rituximab glycosylation. One of the batches corresponded to the drug product perfusion solution in water for injection (10 mg/mL) opened in-house. The other two batches were the surplus products obtained at the hospital after patients' treatment, which were donated to the research institute for research purposes. Figure 3 shows glycan profile comparison of the three batches. Chromatographic profiles were normalized to the main signal (peak 4). Differences are noticeable in the relative abundance for peaks 6, 7, and 8 for both surplus batches compared to the freshly opened drug product.

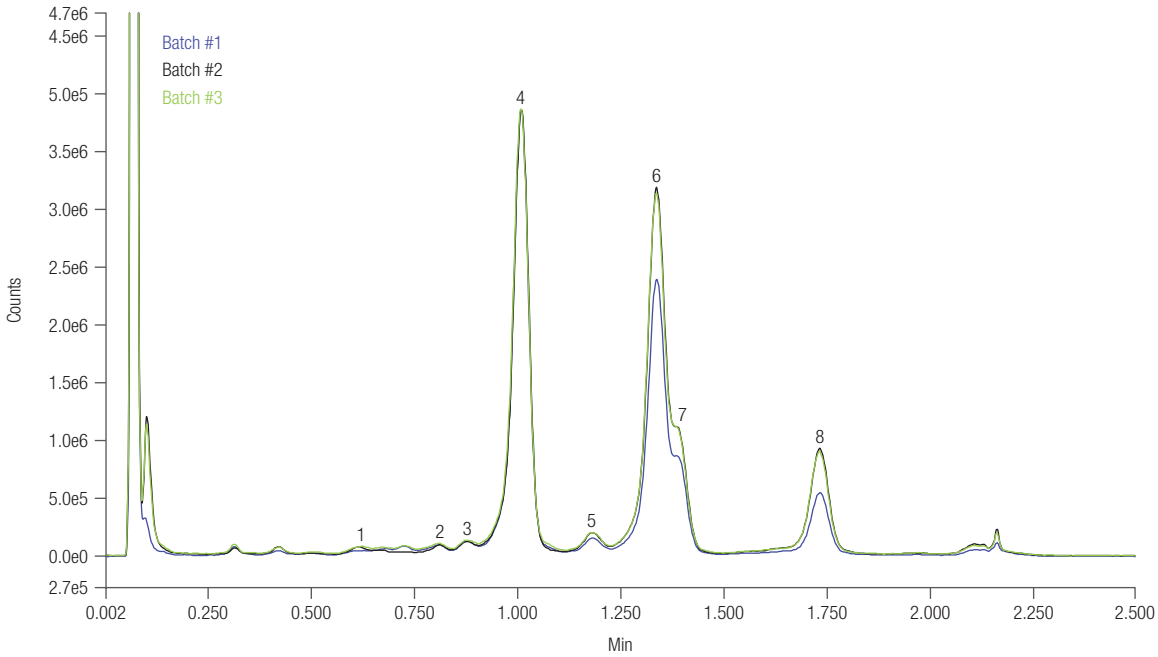

Figure 3. Glycan profile batch-to-batch comparison of rituximab on an Accucore 150-Amide-HILIC column (2.1 × 50 mm, 2.6 µm).

Conclusions

- A fully integrated workflow for glycan profiling was successfully demonstrated using the combination of the Vanquish Horizon UHPLC system equipped with fluorescence detection and the Accucore 150-Amide-HILIC column to provide rapid resolution of *N*-glycoforms from biotherapeutics for ultrafast glycan profiling.

- This process was effectively applied to the rapid separation of labeled glycans for batch-to batch glycoprofiling comparison of a model mAb, offering a potential approach to process development of novel mAbs and biosimilar products.

- Orthogonal structural confirmation of glycans using the Q Exactive Plus MS allowed rapid and unambiguous profiling.

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APPLICATION NOTE 21651

Comprehensive protein glycosylation comparison of an innovator monoclonal antibody to a candidate biosimilar by HILIC UHPLC analysis

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Keywords

Glycosylation, Glycoprotein, Released *N*-glycan, Glycoform, Biosimilar, Innovator, Monoclonal Antibody, mAbs, Biotherapeutics, IgG, Trastuzumab, Vanquish UHPLC, Accucore Amide HILIC, Solid Core, 2-AA, HRAMS MS, Q Exactive Plus

Goal

- To apply a fast, hydrophilic interaction UHPLC approach to the comprehensive glycan profiling of 2-AA labelled candidate biosimilar and an innovator mAb
- To determine variations in glycan profile are observable between samples
- To confirm the glycan profiles by exoglycosidase enzyme digestion and high-resolution, accurate-mass (HRAM) mass spectrometry

Introduction

Biosimilars are biologic products that receive authorization based on an abbreviated regulatory application containing comparative quality, non-clinical and clinical data that demonstrate similarity to a licensed biological product (ICH Q5E/Q6B, USP 129). Regulatory authorities have generally reached the consensus that extrapolation of similarity from one indication to other approved indications of the reference product can be permitted if it is scientifically justified.[1] Monoclonal antibody (mAb) products are extraordinarily heterogeneous due to the presence of a variety of enzymatic and chemical modifications, such as deamidation, isomerization, oxidation, glycosylation, glycation, and terminal cyclization. The modifications in different domains of the antibody molecule can result in different biological consequences.

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Therefore, characterization and routine monitoring of domain-specific modifications are essential to ensure the quality of the therapeutic antibody products.

Glycosylation is considered a critical quality attribute (CQA) and therefore, should be within an appropriate limit, range or distribution to ensure desired product quality, safety and efficacy.[2] Different glycosylation variants have been shown to influence the pharmacodynamics and pharmacokinetic behavior, while other glycan structures may be involved in adverse immune reactions.[3-5] Comprehensive glycan profiling may be achieved using a variety of techniques, including use of oligosaccharide standards, enzymatic digests and lectin affinity in combination with liquid chromatography, or capillary electrophoresis coupled with fluorescence detection and mass spectrometry.[6]

Trastuzumab (Herceptin®) is a commercially available recombinant IgG1 kappa, humanized monoclonal antibody biotherapeutic produced in Chinese hamster ovary (CHO) cell culture. The early stage development biosimilar candidate has been produced using transient expression in a CHO cell line (Thermo Scientific™ ExpiCHO™ expression system). In this work, comprehensive glycan profiling of 2-AA labelled mAbs was achieved using HILIC chromatography coupled to a Thermo Scientific™ Vanquish™ UHPLC system with fluorescence (FLD) detection and subsequent structural confirmation by exoglycosidase digestions and high-resolution, accurate-mass mass spectrometry.

Experimental

Chemicals and reagents

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Acetonitrile, HPLC grade (P/N 10407440)
- Fisher Scientific™ Optima™ Formic acid, LC-MS (P/N 10596814)
- Fisher Scientific Ammonium hydroxide (P/N 10508610)
- Fisher Scientific Ammonium bicarbonate (P/N 10207183)
- Thermo Scientific™ Virtuoso™ Vial kit (P/N 60180-VT402)
- PNGase F (purchased from a reputable supplier)
- Amicon® Ultra 0.5 mL centrifugal filters MWCO 10 kDa (purchased from a reputable supplier)
- Fisher Scientific Sodium cyanoborohydride (P/N 10082110)
- Fisher Scientific Glacial acetic acid (P/N A/0360/PB17)
- Fisher Scientific Dimethylsulfoxide (DMSO) (P/N 10213810)
- Anthranilic acid (2-AA) (purchased from a reputable supplier)
- Fisher Scientific Tris(hydroxymethyl)methylamine hydrochloride (P/N 10060390)
- Fisher Scientific Urea (P/N 10132740)
- Fisher Scientific Ethanol (P/N 10644795)
- ABS Sialidase/NANase III (purchased from a reputable supplier)
- BKF α(1-2,3,4,6) Fucosidase (bovine kidney) (purchased from a reputable supplier)
- SPG beta(1-4)-Galactosidase (*Streptococcus pneumoniae*) (purchased from a reputable supplier)
- GUH β-*N*-Acetylhexosaminidase / Hexase I (purchased from a reputable supplier)

Equipment

Thermo Scientific™ UltiMate™ 3000 RS system, including:

- LPG-3400RS Rapid Separation Quaternary Pump (P/N 5040.0036)
- WPS-3000TRS Rapid Separation Thermostatted Well Plate Autosampler (P/N 5840.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- FLD-3400RS Fluorescence Detector with Dual-PMT (P/N 5078.0025)
- SR-3000 Solvent Rack (P/N 5035.9200)
- 2 µL Micro Flow Cell (P/N 6078.4330)

Thermo Scientific™ Vanquish™ Horizon UHPLC system, including:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler HT (P/N VH-A10-A) with 25 µL (V=50 µL) sample loop
- Fluorescence Detector F (P/N VF-D50-A)

Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer

Thermo Scientific™ Accucore™ 150-Amide-HILIC 2.6 µm, 2.1 × 150 mm (P/N 16726-152130)

Thermo Scientific™ SpeedVac™ Concentrator (P/N SPD121p)

Preparation of buffers

- Ammonium formate (50 mM, pH 4.4): 1.8 g of formic acid was dissolved in 1 L of DI water. pH was adjusted pH to 4.4 with ammonium hydroxide solution.
- 8 M urea in 0.1 M Tris-HCl, pH 8.5 (UA buffer): 1.57 g of Tris-HCl was dissolved in 100 mL DI water and pH adjusted to 8.5 with 1 M NaOH. 24 g of urea was dissolved in 50 mL 0.1 M Tris-HCl, pH 8.5.
- Ammonium bicarbonate (50 mM, pH 7.8): 0.39 g of ammonium bicarbonate was dissolved in 100 mL of DI water.

Release and 2-AA labeling of released *N*-glycans

1. 100 µg of protein was denatured using 8 M urea in 0.1 M tris buffer pH 8.0 and subsequently reduced and alkylated using 10 mM DTT and 50 mM IAA prepared in UA solution, respectively.
2. Following buffer exchange into 50 mM ammonium bicarbonate using 10 kDa MWCO filters, *N*-glycan release was performed by incubation of the reduced and alkylated sample with 1000 units of PNGase F overnight at 37 °C.
3. Released glycans were collected from the deglycosylated proteins by centrifugation through 10 kDa molecular weight cut-off (MWCO) filters and subsequently reduced to dryness via vacuum centrifugation.
4. Dried glycans were reconstituted in 50 µL of 1% (v/v) aqueous formic acid to ensure complete conversion to the reducing sugar form prior to derivatization and subsequently reduced to dryness.
5. Glycan samples were derivatized with 5 µL 2-aminobenzoic acid (2-AA) via reductive amination with sodium cyanoborohydride in 30% v/v acetic acid in DMSO at 60 ºC for 5 hours.
6. Excess labelling reagent was removed by HILIC purification using an UltiMate 3000RS system. Samples were loaded in 80% acetonitrile, 20% 50 mM ammonium formate pH 4.4 (v/v) onto an Accucore 150-Amide-HILIC 2.1 × 50 mm column at 0.5 mL/min for 2.5 minutes. Labeled glycans were eluted in 20% aqueous acetonitrile for 2.5 minutes, monitored by fluorescence detection, $\lambda_{ex/em}$ = 350/425 nm and evaporated to dryness.

Exoglycosidase digestions

All exoglycosidase digestions were performed in 50 mM ammonium acetate buffer, pH 5.5 in a final volume of 10 µL at 37 °C overnight. Amounts of enzyme are indicated in Table 1.

1. For each enzyme digestion, 5 µL of labelled *N*-glycan pool was placed in a 0.2 mL PCR tubes (for GUH digest sample was dried in vacuum centrifuge and re-suspended in 3 µL water).
2. Required volume of buffer (1 µL), water, and enzyme(s) (Table 1) was added to each tube mixing by pipette after each addition (10 µL final volume).
3. Samples were incubated at 37 °C overnight (16 hours).
4. Cleanup was performed by ethanol precipitation: 90 µL ethanol (-30 °C) was added to the sample tubes and mixed thoroughly. Samples were kept at -30 °C for 30 min and spun for 10 min at 16.1 x 1000 rcf, 10 °C. Supernatant was placed into a new tube and sample was dried completely in a vacuum centrifuge.
5. Digested samples were re-suspended in 5 µL DI water and 20 µL acetonitrile for UHPLC-FLD analysis.

Table 1. Enzyme specificity and required volume per exoglycosidase digestion.

Enzyme	Specificity	Volume per Digest
ABS	Releases α(2-3), α(2-6) and α(2-8) lined non-reducing terminal sialic acids (NeuNAc and NeuNGc)	1 µL
BKF	Releases α(1-2) and α(1-6) linked non-reducing terminal fucose residues more efficiently than α(1-3) and α(1-4) linked fucose. Used for release of core α(1-6) fucose residues, can also remove α(1-3), but less efficiently.	1 µL
SPG	Hydrolyses non-reducing terminal β(1-4) linked galactose residues	2 µL
GUH	Recombinantly expressed in *E. coli*. Releases β-linked GlcNAc but not bisecting GlcNAc β(1-4) Man	2 µL

UHPLC-fluorescence *N*-glycan profiling

Labeled *N*-glycans were separated by hydrophilic interaction UHPLC-FLD on a Vanquish Horizon UHPLC. 2-AA labelled glycans were separated using a linear gradient. Glycans were injected in 20 µL 80% v/v acetonitrile and stored at 10 °C prior to injection.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 150 mm (P/N 16726-152130)
Mobile phase A:	Ammonium formate 50 mM, pH 4.5
Mobile phase B:	Acetonitrile
Flow rate:	1.3 mL/min
Column temperature:	60 °C
Sample volume:	20 µL
Backpressure:	Approximately 500 bar

Table 2. Mobile phase gradient for UHPLC-fluorescence N-glycan profiling.

Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1.3	5
31.05	40	60	1.3	5
32.23	50	50	1.3	5
33.08	50	50	1.3	5
33.15	20	80	1.3	5
35.00	20	80	1.3	5

N-glycan analysis by LC-MS

Glycan samples were injected on a Q Exactive Plus hybrid quadrupole-Orbitrap MS equipped with H-ESI ion source. Samples were diluted in 75% acetonitrile prior to analysis.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 µm, 2.1 x 150 mm
Mobile phase A:	Ammonium formate 50 mM, pH 4.5
Mobile phase B:	Acetonitrile
Flow rate:	0.4 mL/min
Column temperature:	40 ºC
Sample volume:	11 µL

Table 3. Mobile phase gradient for *N*-glycan analysis by LC-MS.

Time (min)	%A	%B	Flow (mL/min)	Curve
0	25	75	0.4	5
30.0	50	50	0.4	5
30.5	55	45	0.4	5
32.0	55	45	0.4	5
32.5	25	75	0.4	5
40.0	25	75	0.4	5

MS conditions

Ionization:	HESI Negative Ion
Scan range:	500 to 2000 *m/z*
Source temperature:	300 °C
Sheath gas flow:	20 Arb
Auxiliary gas flow:	10 Arb
Spray voltage:	3.8 kV
Capillary temperature:	320 °C

Data processing and software

Chromatographic software:	Thermo Scientific™ Chromeleon™ CDS 7.2 SR4
MS data acquisition:	Thermo Scientific™ Xcalibur™ software 2.2 SP1.48

Results and discussion

A candidate biosimilar mAb was compared in detail to a commercially available IgG1 innovator product. Separation of *N*-glycan structures for innovator (black trace, a) and biosimilar candidate produced in house (blue trace, b) are displayed as a mirror chromatogram in Figure 1. Thirteen *N*-glycan features were assigned for commercial IgG1 mAb (trastuzumab, Herceptin) and 16 *N*-glycan features for the biosimilar candidate.

Annotation of the *N*-glycans structures and glycosidic linkages present in each chromatographic peak were deduced using exoglycosidase arrays as detailed in Figures 2 and 3. Use of exoglycosidase arrays involves the application of a panel of enzymes with high specificity for different monosaccharides to samples of released glycans. Here, a panel of four exoglycosidases, namely sialidase (ABS), α(1-2,3,4,6) fucosidase (BKF), beta(1-4)-galactosidase (SPG), and β-*N*-acetylhexosaminidase (GUH), were applied to facilitate removal of sialic acid, fucose, galactose, and hexose glycan constituents, respectively. After removal of specific monosaccharides, differences in retention time and peak area were observed upon LC analysis, as is shown in Figures 2 and 3, enabling the structural annotation of glycan species in the mAb samples under analysis. The oligosaccharide composition, outlined in Table 4, was also confirmed by accurate-mass MS data.

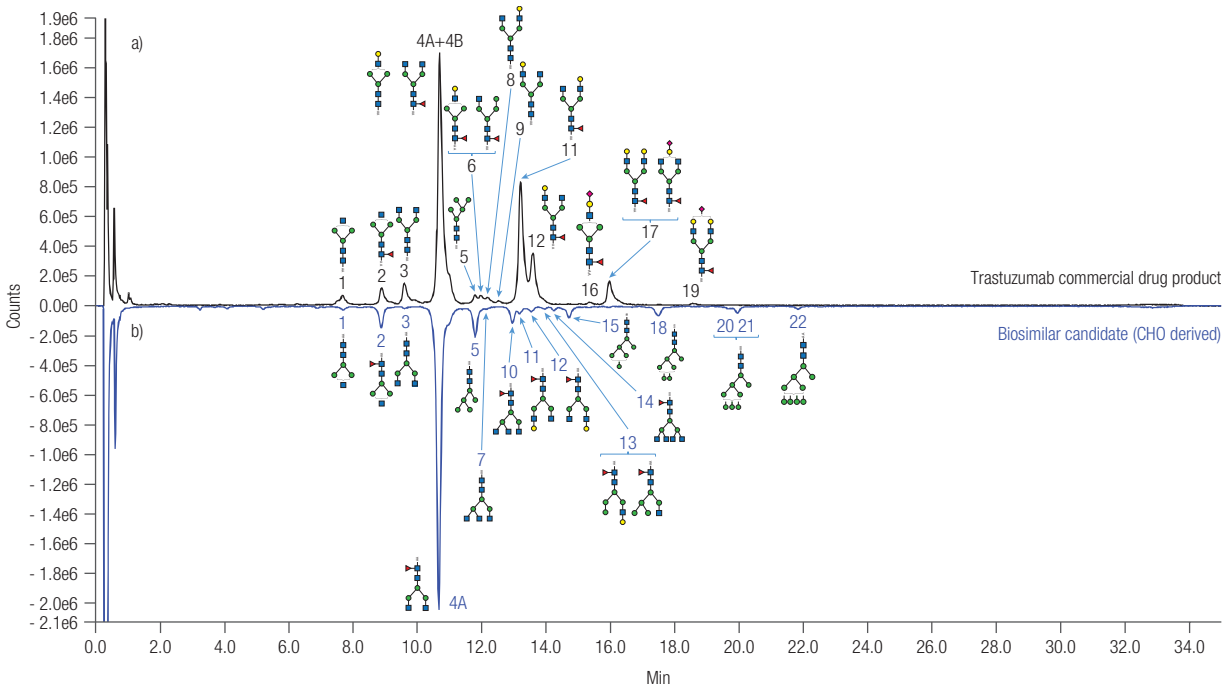


Figure 1. Mirror plot for the chromatographic separation on an Accucore 150-Amide-HILIC column (2.1 × 150 mm, 2.6 µm) of *N*-glycans from commercial IgG1 mAb (a) and biosimilar candidate (b) produced in CHO cell lines.

Relative areas for the identified glycan peaks showed few important differences in terms of glycosylation as defined in Table 4. Innovator drug product showed 92.5% of core fucosylated *N*-glycan mainly bi-antennary structures and 5.6% of sialylated structures. The biosimilar candidate produced 'in house' showed 80.9% of core fucosylation and a high content of high mannose *N*-glycan structures (14.5%). Non-sialylated structures were detected for the biosimilar candidate.

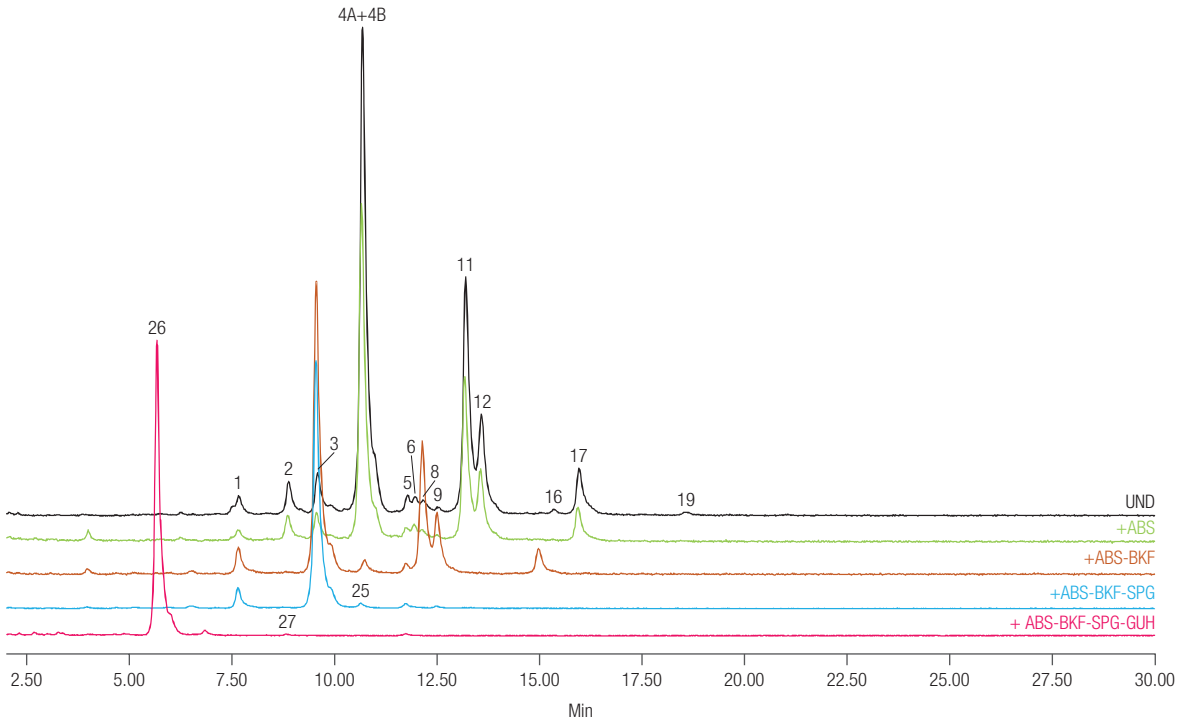


Figure 2. HILIC chromatograms of trastuzumab commercial drug product 2-AA labelled *N*-glycan pool (undigested, UND) and after digestion with a range of exoglycosidase enzymes on an Accucore 150-Amide-HILIC 2.1 × 150 mm column. ABS removes all sialic acids (α2-3, -6 and -8), BKF removes α(1-6) linked core fucose and outer arm α(1-2 and 1-6) linked fucose, SPG removes β(1-4) linked galactose, and GUH removes β linked GlcNAc.

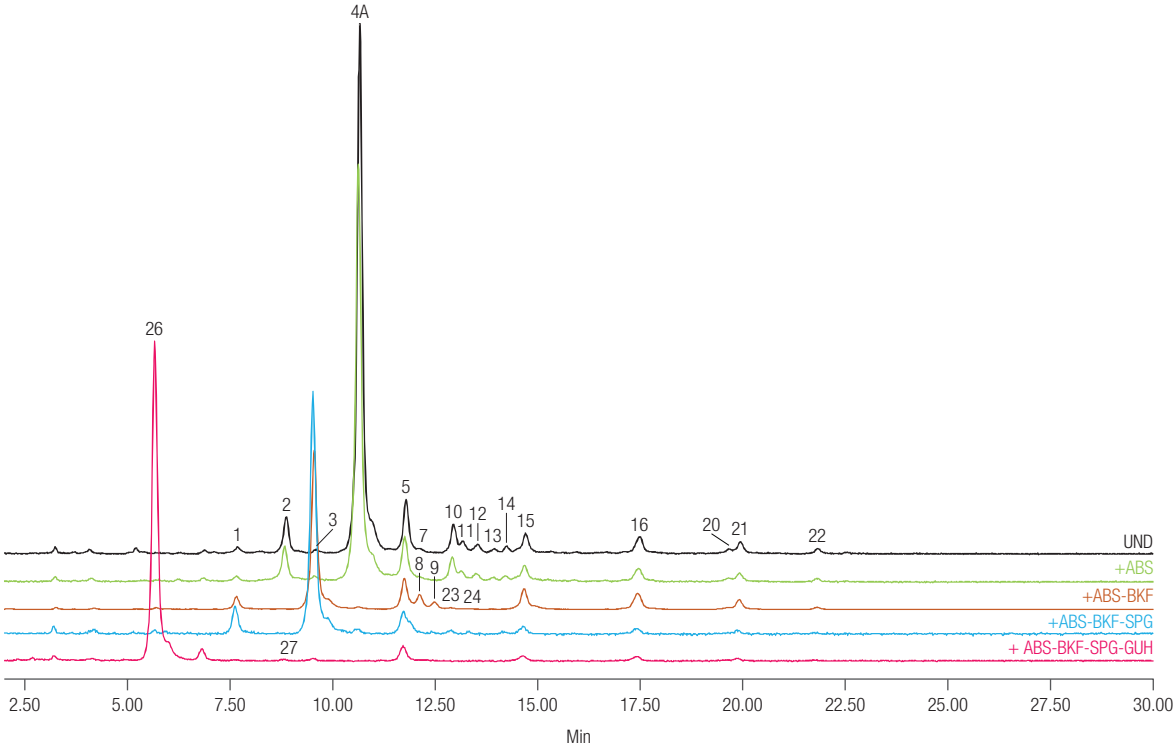
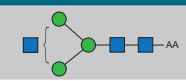
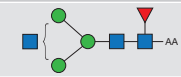
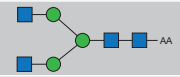
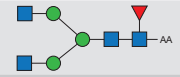
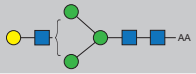
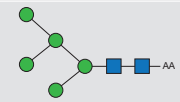
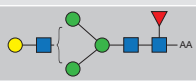
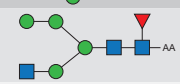
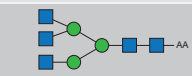
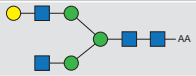
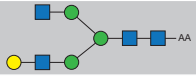


Figure 3. HILIC chromatograms of trastuzumab biosimilar candidate 2-AA labelled *N*-glycan pool (undigested, UND) and after digestion with a range of exoglycosidase enzymes on Accucore 150-Amide-HILIC 2.1 × 150 mm column. ABS removes all sialic acids (α2-3, -6 and -8), BKF removes α(1-6) linked core fucose and outer arm α(1-2 and 1-6) linked fucose, SPG removes β(1-4) linked galactose, and GUH removes β-linked GlcNAc.

Table 4. Structural identification of 2-AA labeled *N*-glycans from trastuzumab commercial drug product and related biosimilar candidate using an Accucore 150-Amide-HILIC column, exoglycosidase digestions, and offline Q Exactive Plus MS. Green shading signifies the presence of that glycan moiety in either mAb, whereas red shading indicates the absence of a glycan species in either mAb sample.

Glycan peak number	Glycan structure	Present in commercial mAb	Present in candidate biosimilar	
1				
2				
3				
4A		A + B	A	B
4B				
5				
6A		A + B	A + B	
6B				
7				
8				
9				

Glycan peak number	Glycan structure	Present in commercial mAb	Present in candidate biosimilar
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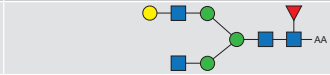
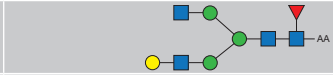
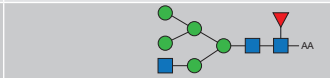
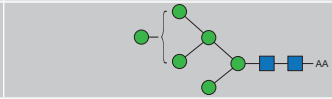
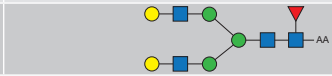
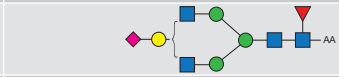
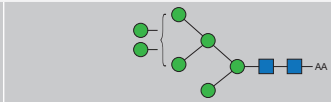
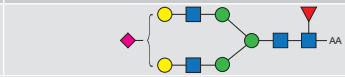
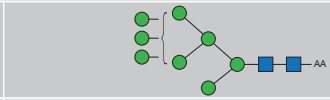
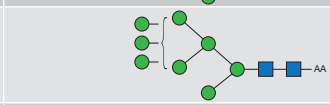
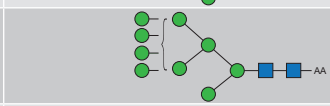
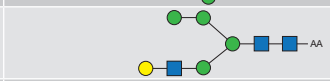
11			
12			
13 A		A + B	A + B
13 B			
14			
15			
16			
17 A		A + B	A + B
17 B			
18			
19			
20			
21			
22			
23 A		A + B	A + B
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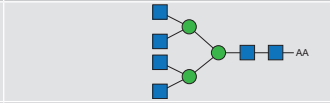
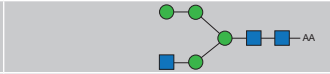
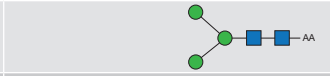
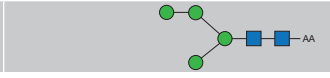
24			
25			
26			
27			

Fucose | *N*-Acetylglucosamine | Mannose | Galactose | *N*-Acetyl Neuraminic acid

Conclusions

- A fully integrated workflow for glycan profiling for an innovator and candidate biosimilar was successfully demonstrated and differences between biomolecules were identified.

- The combination of the Vanquish Horizon UHPLC system equipped with fluorescence detection and the Accucore 150-Amide-HILIC column provided fast resolution of *N*-glycoforms from biotherapeutics for comprehensive glycan profiling.

- Structural confirmation of glycans with exoglycosidase enzyme digestion followed by determination using the Q Exactive Plus Hybrid Quadrupole-Orbitrap MS allowed rapid and unambiguous profiling.

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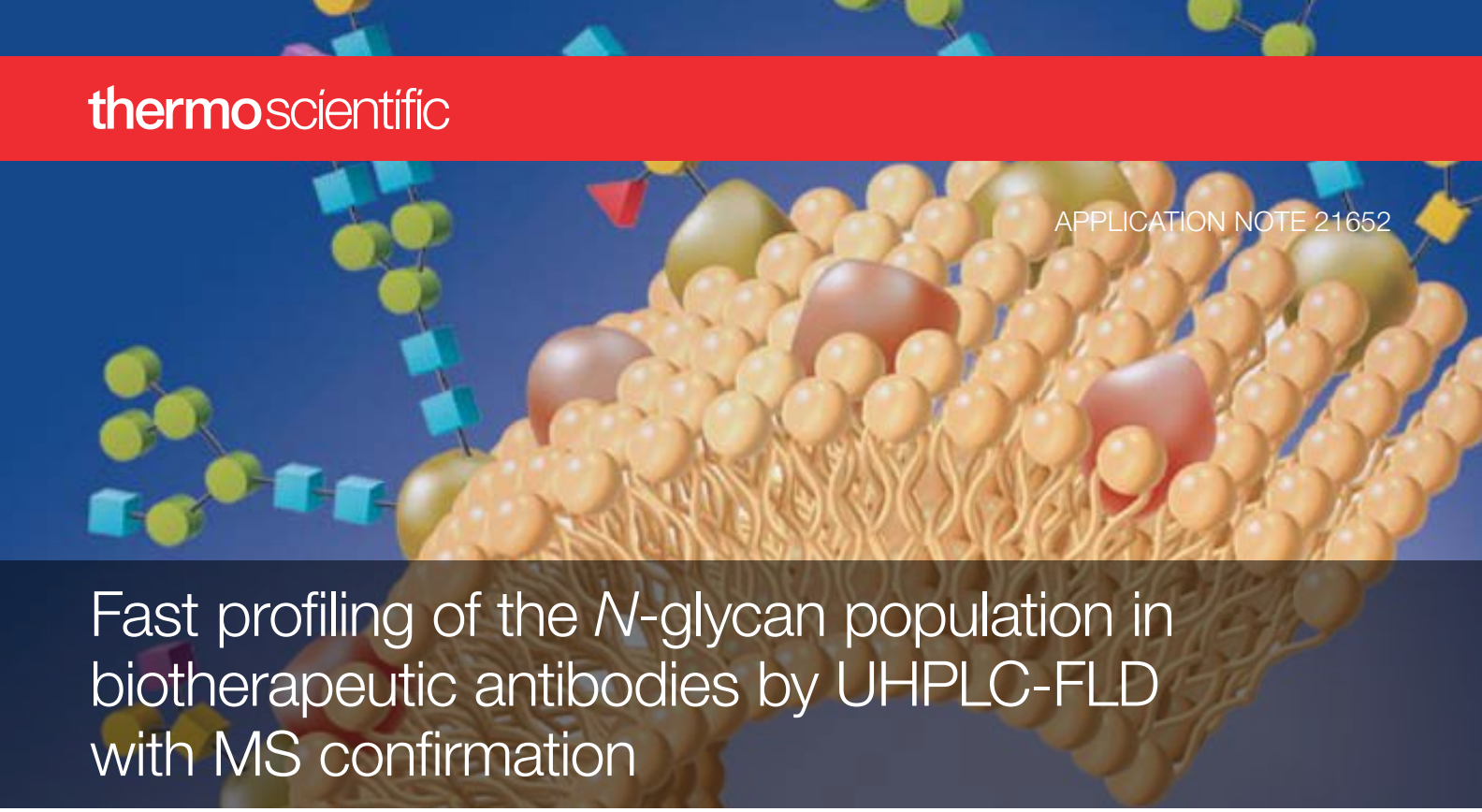

Fast profiling of the *N*-glycan population in biotherapeutic antibodies by UHPLC-FLD with MS confirmation

Authors

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Keywords

Glycosylation, Glycoprotein, Released *N*-glycan, Glycoform, Biosimilar, Innovator, Monoclonal Antibody, mAb, Biotherapeutics, IgG, Vanquish UHPLC, Accucore Amide HILIC, Solid Core, 2-AA, 2-AB, HRAMS MS, Q Exactive, Infliximab

Goal

- To develop a widely applicable UHPLC approach to fast, comprehensive profiling of 2-AA and 2-AB labeled glycans in IgG antibodies
- To validate the approach with a human serum IgG and a commercial chimeric IgG1 mAb (infliximab)
- To confirm the glycan profiles by exoglycosidase enzyme digestion and high-resolution, accurate-mass mass spectrometry (HRAMS MS)
- To increase throughput with assay optimization

Introduction

Glycans play an essential role in many biological processes, including cell development and differentiation, cell-cell or cell-matrix communication, and pathogen-host recognition. The glycan components of biotherapeutics can be important determinants of biological activity and therapeutic efficacy[1-3] and hence characterization of the glycan profile of a biomolecule is required under regulatory guidelines (ICH Q5E/Q6B and USP 129).[4-5] Therapeutic antibodies must be demonstrated to meet applicable quality requirements to ensure continued safety, purity, and potency of a drug product. An intensive biochemical characterization of the antibody itself is required, which includes a thorough examination of glycan distribution and potential

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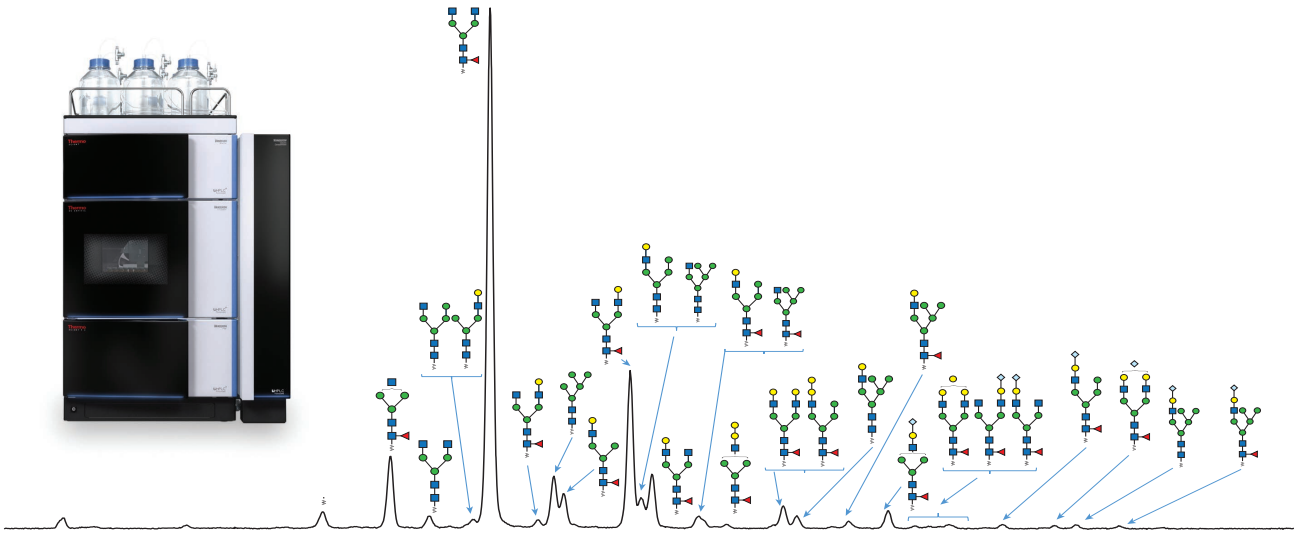
impacts of glycoforms on mAb function.[6] On the other hand, differences in glycan profiles between healthy and diseased states[7] are utilized for clinical diagnosis[8], providing targets for many novel classes of therapeutics including cancer chemotherapy, diabetes treatment, and antibiotic and anti-viral medicine.

The 'gold standard' for studying IgG glycosylation relies on enzymatic *N*-glycan release, subsequent fluorescent labeling by reductive amination, and analysis of the labeled glycans by high-performance liquid chromatography with fluorescence detection (HPLC-FLD), using hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection. In addition to characterization of the sugar sequence, the analysis must elucidate linkages and separate all isomeric, charge, and branching variations of glycans.

HILIC columns commonly used for glycan analysis are based on amide, amine, or zwitterionic packing materials. These columns separate glycans mainly by hydrogen bonding, resulting in separations based on size and composition. The Thermo Scientific™ Accucore™ 150-Amide-HILIC HPLC column is a solid particle core phase designed for the separation of hydrophilic biomolecules in HILIC mode, which offers an excellent choice for glycan separation.

This application note presents a step-by-step method for release, labeling, separation, and exoglycosidase-based structural elucidation of *N*-glycans from human serum IgG and commercial chimeric IgG1 mAb (infliximab) using Thermo Scientific™ Vanquish™ Horizon UHPLC-FLD and confirmation of the structures by a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer.



Experimental

Chemicals and reagents

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Acetonitrile, HPLC grade (P/N 10407440)
- Fisher Scientific™ Optima™ Formic acid, LC-MS (P/N 10596814)
- Fisher Scientific Ammonium hydroxide (P/N 10508610)
- Fisher Scientific Ammonium bicarbonate (P/N 10207183)
- Thermo Scientific™ Virtuoso™ Vial kit (P/N 60180-VT402)
- PNGase F (New England Biolabs®, Ipswich, MA, USA)
- Amicon® Ultra 0.5 mL centrifugal filters MWCO 10 kDa (purchased from a reputable supplier)
- Fisher Scientific Sodium cyanoborohydride (P/N 10082110)
- Fisher Scientific Glacial acetic acid (P/N A/0360/PB17)
- Fisher Scientific Dimethylsulfoxide (DMSO) (P/N 10213810)
- Anthranilamide (2-AB) (purchased from a reputable supplier)
- Anthranilic acid (2-AA) (purchased from a reputable supplier)
- Fisher Scientific Tris(hydroxymethyl)methylamine hydrochloride, (P/N 10060390)

- Fisher Scientific Urea (P/N 10132740)
- Fisher Scientific Ethanol (P/N 10644795)
- ABS Sialidase/NANase III (purchased from a reputable supplier)
- BKF α(1-2,3,4,6) Fucosidase (bovine kidney) (purchased from a reputable supplier)
- SPG beta(1-4)-Galactosidase (*Streptococcus pneumoniae*) (purchased from a reputable supplier)
- GUH β-*N*-Acetylhexosaminidase / Hexase I (purchased from a reputable supplier)

Equipment

Thermo Scientific™ UltiMate™ 3000 RS system, including:

- LPG-3400RS Rapid Separation Quaternary Pump (P/N 5040.0036)
- WPS-3000TRS Rapid Separation Thermostatted Well Plate Autosampler (P/N 5840.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- FLD-3400RS Fluorescence Detector with Dual-PMT (P/N 5078.0025)
- SR-3000 Solvent Rack (P/N 5035.9200)
- 2 µL Micro Flow Cell (P/N 6078.4330)

Thermo Scientific™ Vanquish™ Horizon UHPLC system, including:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler HT (P/N VH-A10-A) with 25 µL (V=50 µL) sample loop
- Fluorescence Detector F (P/N VF-D50-A)

Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer

Thermo Scientific™ SpeedVac™ Concentrator (P/N SPD121p)

Accucore 150-Amide-HILIC, 2.6 µm, 2.1 × 50 mm (P/N 16726-052130)

Accucore 150-Amide-HILIC, 2.6 µm, 2.1 × 150 mm (P/N 16726-152130)

Accucore 150-Amide-HILIC, 2.6 µm, 2.1 × 250 mm (P/N 16726-252130)

Preparation of buffers

- Ammonium formate (50 mM, pH 4.4): 1.8 g of formic acid was dissolved in 1 L of DI water. pH was adjusted pH to 4.4 with ammonium hydroxide solution.
- 8 M urea in 0.1 M Tris-HCl, pH 8.5 (UA buffer): 1.57 g of Tris-HCl was dissolved in 100 mL DI water and pH adjusted to 8.5 with 1 M NaOH. 24 g of urea was dissolved in 50 mL 0.1 M Tris-HCl, pH 8.5.
- Ammonium bicarbonate (50 mM, pH 7.8): 0.39 g of ammonium bicarbonate was dissolved in 100 mL of DI water.

Release of *N*-glycans from proteins

1. 100 µg of protein were denatured using 8 M urea in 0.1 M tris buffer pH 8.0 (UA solution) and subsequently reduced and alkylated using 10 mM DTT and 50 mM IAA prepared in UA solution.
2. Following buffer exchange into 50 mM ammonium bicarbonate using 10 kDa MWCO filters, *N*-glycan release was performed by incubation of the reduced and alkylated sample with 500 units of PNGase F overnight at 37 °C.
3. Released glycans were collected from deglycosylated proteins by centrifugation through 10 kDa molecular weight cut-off (MWCO) filters and subsequently reduced to dryness via vacuum centrifugation.
4. Dried glycans were reconstituted in 50 µL of 1% (v/v) aqueous formic acid to ensure complete conversion to the reducing sugar form prior to derivatization and subsequently reduced to dryness

2-AA labeling reaction of released *N*-glycans

1. 2-AA labeling reagent (100 µL) was prepared by dissolving 2-aminobenzoic acid (5 mg) and sodium cyanoborohydride (6 mg) in 70/30 DMSO/glacial acetic acid.
2. 5 µL of 2-AA labeling reagent solution were added to the mixture (dried *N*-glycans released from 100 µg glycoprotein).
3. The solution was incubated at 60 °C for 5 hours.

2-AB labeling reaction of released *N*-glycans

1. 2-AB labeling reagent (100 µL) was prepared by dissolving 2-aminobenzamide (5 mg) and sodium cyanoborohydride (6 mg) in 70/30 DMSO/glacial acetic acid.

2. 10 µL of 2-AB labeling reagent solution were added to the mixture (dried *N*-glycans released from 100 µg glycoprotein).
3. The solution was incubated at 65 °C for 2 hours.

Cleanup of 2-AB/2-AA labeled *N*-glycans

1. Excess labeling dye removal was carried out by HILIC purification using an UltiMate 3000RS system.
2. Samples were loaded in 85% acetonitrile, 15% 50 mM ammonium formate pH 4.4 (v/v) (for 2-AB labeled *N*-glycans) or in 80% acetonitrile, 20% 50 mM ammonium formate pH 4.4 (v/v) (for 2-AA labeled *N*-glycans) onto an Accucore 150-Amide-HILIC 2.1 × 50 mm column at 0.5 mL/min for 2.5 minutes.
3. Labeled glycans were eluted in 20% aqueous acetonitrile for 2.5 minutes, monitored by fluorescence detection, $\lambda_{ex/em}$ = 330/420 nm (for 2-AB labeled *N*-glycans) or $\lambda_{ex/em}$ = 350/425 nm (for 2-AB labeled *N*-glycans), and evaporated to dryness.

Sample preparation for analysis using UHPLC-FLD

1. 5 µL of purified labeled *N*-glycans re-suspended in DI water (at 2.5 µg/µL) were mixed with 20 µL of acetonitrile.
2. The total solution was transferred to the auto-sampler vial for analysis. Note: Store the standard at -20 °C.

Exoglycosidase digests

All exoglycosidase digestions were performed in 50 mM ammonium acetate buffer, pH 5.5 in a final volume of 10 µL at 37 °C overnight. Amounts of enzyme are indicated in Table 1.

1. For each enzyme digestion, 5 µL of labeled *N*-glycan pool were placed in a 0.2 mL PCR tube (for GUH digest, sample was dried in vacuum centrifuge and re-suspended in 3 µL water).
2. Required volume of buffer (1 µL), water, and enzyme(s) (Table 1) were added to each tube mixing by pipette after each addition (10 µL final volume).
3. Samples were incubated at 37 °C overnight (16 hours).
4. Cleanup was performed by ethanol precipitation: 90 µL ethanol (-30 °C) were added to the sample tubes and mixed thoroughly. Samples were kept at -30 °C for 30 min and spun for 10 min at 16.1 x 1000 rcf, 10 °C. Supernatant was placed into a new tube and sample was dried completely in a vacuum centrifuge.
5. Digested samples were re-suspended in 5 µL DI water and 20 µL acetonitrile for UHPLC-FLD analysis.

Table 1. Enzyme specificity and required volume per exoglycosidase digestion.

Enzyme	Specificity	Volume per Digest
ABS	Releases α(2-3), α(2-6) and α(2-8) lined non-reducing terminal sialic acids (NeuNAc and NeuNGc)	1 µL
BKF	Releases α(1-2) and α(1-6) linked non-reducing terminal fucose residues more efficiently than α(1-3) and α(1-4) linked fucose. Used for release of core α(1-6) fucose residues, can also remove α(1-3), but less efficiently.	1 µL
SPG	Hydrolyses non-reducing terminal β(1-4) linked galactose residues	2 µL
GUH	Recombinantly expressed in E. coli. Releases β-linked GlcNAc but not bisecting GlcNAc β(1-4) Man	2 µL

Separation conditions

Columns:	Accucore 150-Amide-HILIC #1: 16726-252130 (2.1 × 250 mm) #2: 16726-152130 (2.1 × 150 mm) #3: 16726-052130 (2.1 × 50 mm)
Mobile phase A:	Ammonium formate 50 mM, p 4.4
Mobile phase B:	Acetonitrile
Flow rate (mL/min):	Refer to Tables 2–4
Column temperature:	60 °C
Sample volume:	33 µL (2.1 × 250 mm) 20 µL (2.1 × 150 mm) 11.5 µL (2.1 × 50 mm)
Mobile phase gradient:	Refer to Tables 3–5

Table 2. Mobile phase gradient for column #1 (P/N 16726-252130).

Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1.4	5
48.10	40	60	1.4	5
49.88	50	50	1.4	5
51.19	50	50	1.4	5
51.31	20	80	1.4	5
55.00	20	80	1.4	5

Table 3. Mobile phase gradient for column #2 (P/N 16726-152130).

Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1.3	5
31.05	40	60	1.3	5
32.23	50	50	1.3	5
33.08	50	50	1.3	5
33.15	20	80	1.3	5
35.00	20	80	1.3	5

Table 4. Mobile phase gradient for column #3 (P/N 16726-052130).

Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1	5
13.45	40	60	1	5
13.97	50	50	1	5
14.33	50	50	1	5
14.37	20	80	1	5
15.00	20	80	1	5

N-glycan analysis by LC-MS

Glycan samples were injected on a Q Exactive Plus Hybrid Quadrupole-Orbitrap MS equipped with a HESI source. Samples were diluted in 75% acetonitrile prior to analysis.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 150 mm
Mobile phase A:	Ammonium formate 50 mM, pH 4.4
Mobile phase B:	Acetonitrile
Flow rate:	0.4 mL/min
Column temperature:	50 °C
Sample volume:	11 µL

Table 5. Mobile phase gradient for *N*-glycan analysis by LC-MS.

Time (min)	%A	%B	Flow (mL/min)	Curve
0	25	75	0.4	5
30.0	50	50	0.4	5
30.5	55	45	0.4	5
32.0	55	45	0.4	5
32.5	25	75	0.4	5
40.0	25	75	0.4	5

MS conditions

Ionization:	HESI Negative Ion
Scan range:	500 to 2000 *m/z*
Source temperature:	300 °C
Sheath gas flow:	20 Arb
Auxiliary gas flow:	10 Arb
Spray voltage:	3.8 kV
Capillary temperature:	320 °C

Data processing and software

Chromatographic software:	Thermo Scientific™ Chromeleon™ CDS 7.2 SR4
MS data acquisition:	Thermo Scientific™ Xcalibur™ software 2.2 SP1.48

Results and discussion

Separation of 2-AB-labeled glycan from human IgG

A sample of human serum IgG glycans was analyzed on Accucore 150-Amide-HILIC HPLC columns of different lengths. The glycan separation and elution is based on size and polarity by HILIC interaction. Figure 1 shows the separation of neutral and acidic 2-AB labeled *N*-glycans from human IgG on different column lengths. FLD chromatograms reveal 18 well-resolved *N*-glycan peaks for 2.1 × 250 and 2.1 × 150 mm columns. Despite some loss in peak resolution when column length is reduced to 50 mm, it may be sufficient for fast analysis of less-complex glycan profiles, enabling high-throughput analysis within 15 minutes. The structural assignment for each identified peak was confirmed by accurate MS data and listed in Table 6. The oligosaccharides present in the polyclonal IgG, attached at Asn297, are of the complex bi-antennary type and are comprised of a chitobiose core with variable addition of fucose, galactose, bisecting *N*-acetylglucosamine, and sialic acid. Sialylation is modest, with < 15% of structures being monosialylated or disialylated.

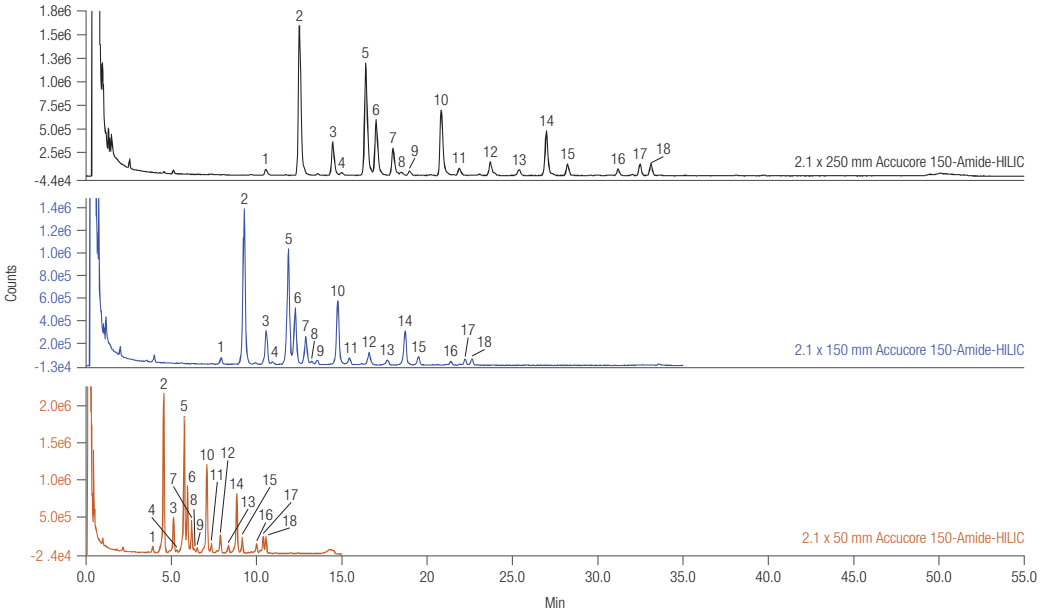
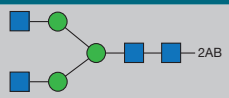
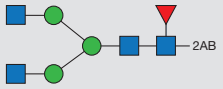
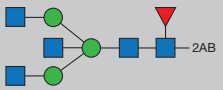
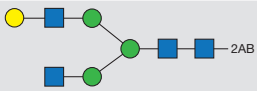
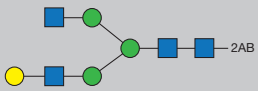
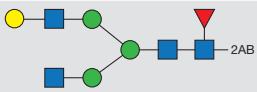
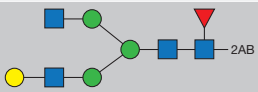
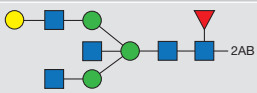
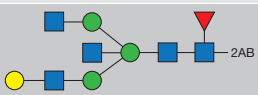
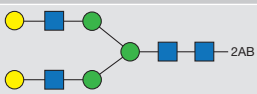


Figure 1. Chromatographic separation of human serum IgG 2-AB-labeled *N*-glycans on Accucore 150-Amide-HILIC columns of varying lengths.

Table 6. Structural identification of 2-AB labeled *N*-glycans from human IgG using Accucore 150-Amide-HILIC columns and a Q Exactive Plus mass spectrometer.

PEAK number (Figure 1)	Glycan structure
1	
2	
3A	
3B	
4	
5	
6	
7	
8	
9	

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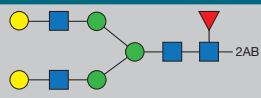
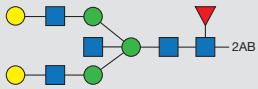
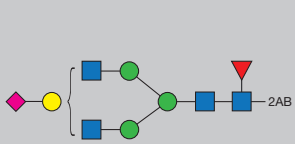
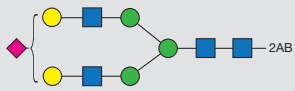
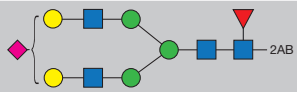
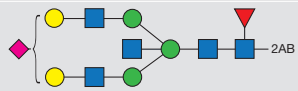
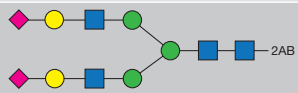
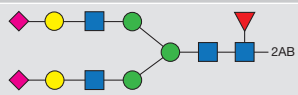
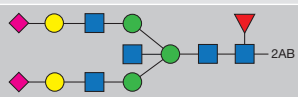
10	
11	
12	
13	
14	
15	
16	
17	
18	

▼ Fucose ■ *N*-Acetylglucosamine ● Mannose ○ Galactose ◆ *N*-Acetyl Neuraminic acid

Separation of 2-AA-labeled glycan from commercial chimeric IgG1

A commercial chimeric IgG1 was also analyzed on Accucore 150-Amide-HILIC columns with the aim to demonstrate appropriate separation for a more complex glycan sample. Figure 2 shows the separation of neutral and acidic 2-AA labeled *N*-glycans from chimeric IgG1 on different column lengths. FLD chromatograms reveal 24 *N*-glycan peaks for 2.1 × 250 and 2.1 × 150 mm columns. Annotation of the *N*-glycans structures and glycosidic linkages present in each chromatographic peak were deduced using exoglycosidase arrays as detailed in Figure 3 and Table 7. The oligosaccharide composition was also confirmed by high-resolution, accurate-mass (HRAM) MS data and listed in Table 7. Thirty-eight *N*-glycan structures were annotated, including high mannose, hybrid and complex bi-antennary glycans with variable degrees of core fucosylation, galactosylation, sialylation, and galactose α1-3 linked galactose epitopes.

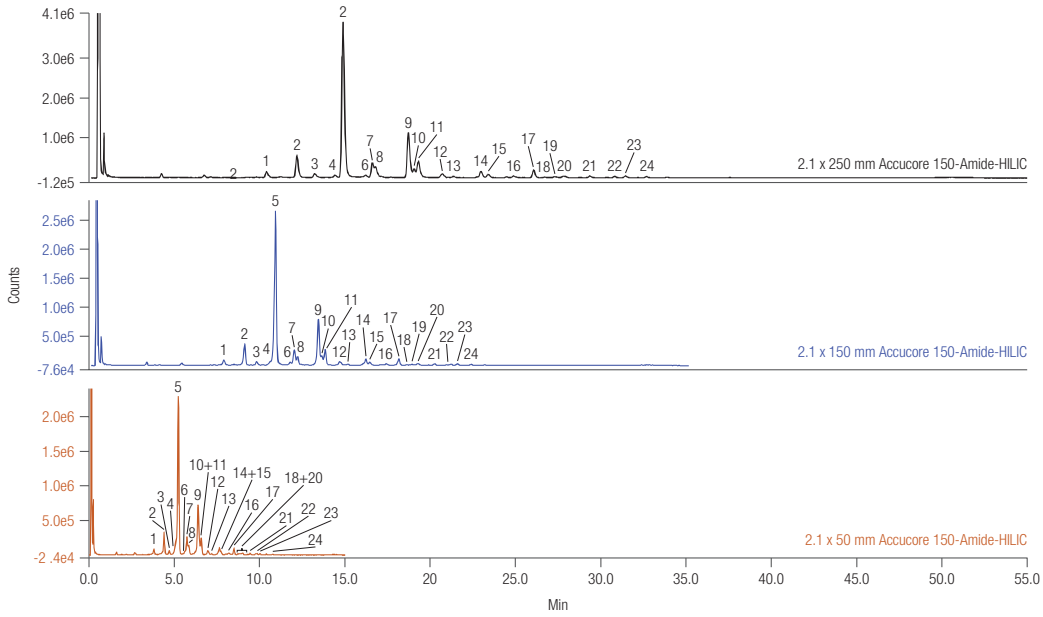
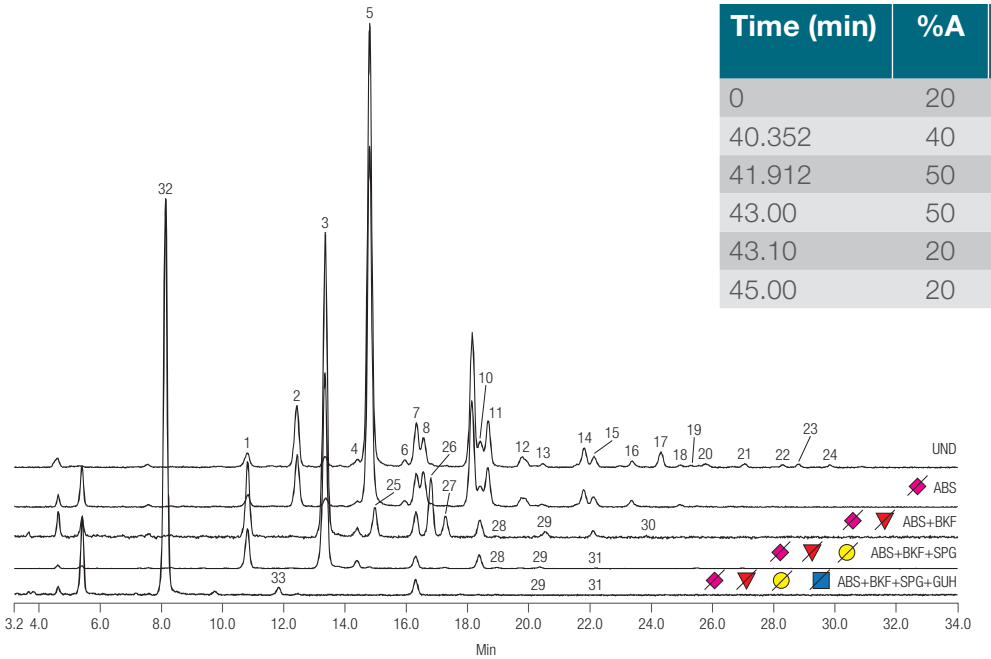


Figure 2. Chromatographic separation of commercial chimeric IgG1 mAb (infliximab) 2-AA-labeled *N*-glycans on Accucore 150-Amide-HILIC columns of varying lengths.



Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1.0	5
40.352	40	60	1.0	5
41.912	50	50	1.0	5
43.00	50	50	1.0	5
43.10	20	80	1.0	5
45.00	20	80	1.0	5

Figure 3. HILIC chromatograms of the infliximab 2-AA labeled *N*-glycan pool (undigested, UND) and after digestion with a range of exoglycosidase enzymes. Separations were performed on an Accucore 150-Amide-HILIC 2.1 × 150 mm column using the gradient conditions outlined in the figure legend table.

ABS removes all sialic acids (α2-3, -6 and -8), BKF removes α(1-6) linked core fucose and outer arm α(1-2 and 1-6) linked fucose, SPG removes β(1-4) linked galactose, and GUH removes β linked GlcNAc.

Table 7. Structural identification of 2-AA labeled *N*-glycans from commercial chimeric IgG1 mAb (infliximab) using Accucore 150-Amide-HILIC columns, exoglycosidase digests, and an offline Q Exactive Plus mass spectrometer.

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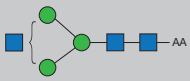
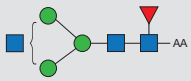
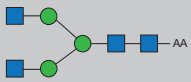
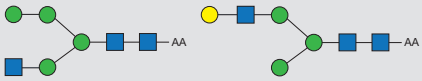
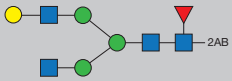
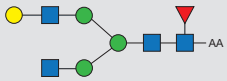
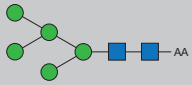
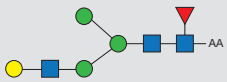
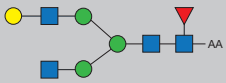
1	
2	
3	
4	
5	
6	
7	
8	
9	
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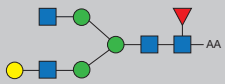
11	
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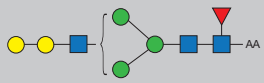
13	
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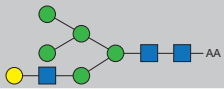
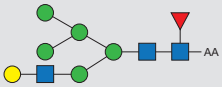
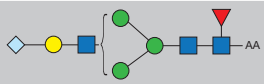
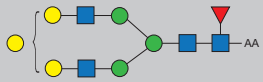
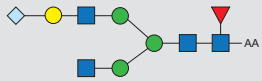
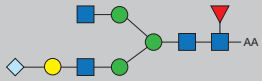
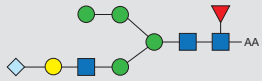
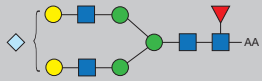
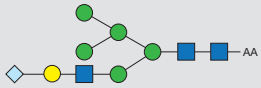
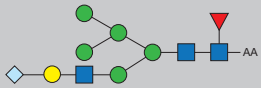
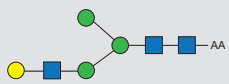
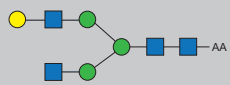
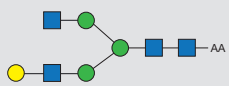
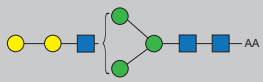
15	
16	
17	

PEAK Number (Figures 2 & 3)	Glycan structure
18	
19	
20	
21	
22	
23	
24	
25	
26	
27	
28	
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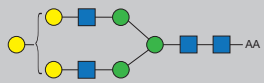
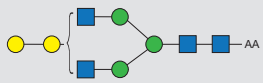
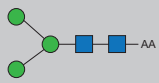
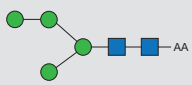
30	
31	
32	
33	

Fucose *N*-Acetylglucosamine Mannose Galactose *N*-Acetyl Neuraminic acid

Conclusions

- A fully integrated workflow for glycan profiling and structural characterization of fluorescently labeled *N*-glycans released was demonstrated successfully.
- The combination of the Vanquish Horizon UHPLC system equipped with fluorescence detection and the Accucore 150-Amide-HILIC column provided the opportunity for assay speed up and associated increase in throughput without compromising the quality of the analytical data.
- The chromatographic resolution, reproducibility, and sensitivity enabled analysis of minor glycoforms, which are otherwise challenging to assign.
- Structural confirmation of glycans with exoglycosidase enzyme digestion followed by determination using the Q Exactive Plus Hybrid Quadrupole-Orbitrap MS allowed rapid and unambiguous profiling.

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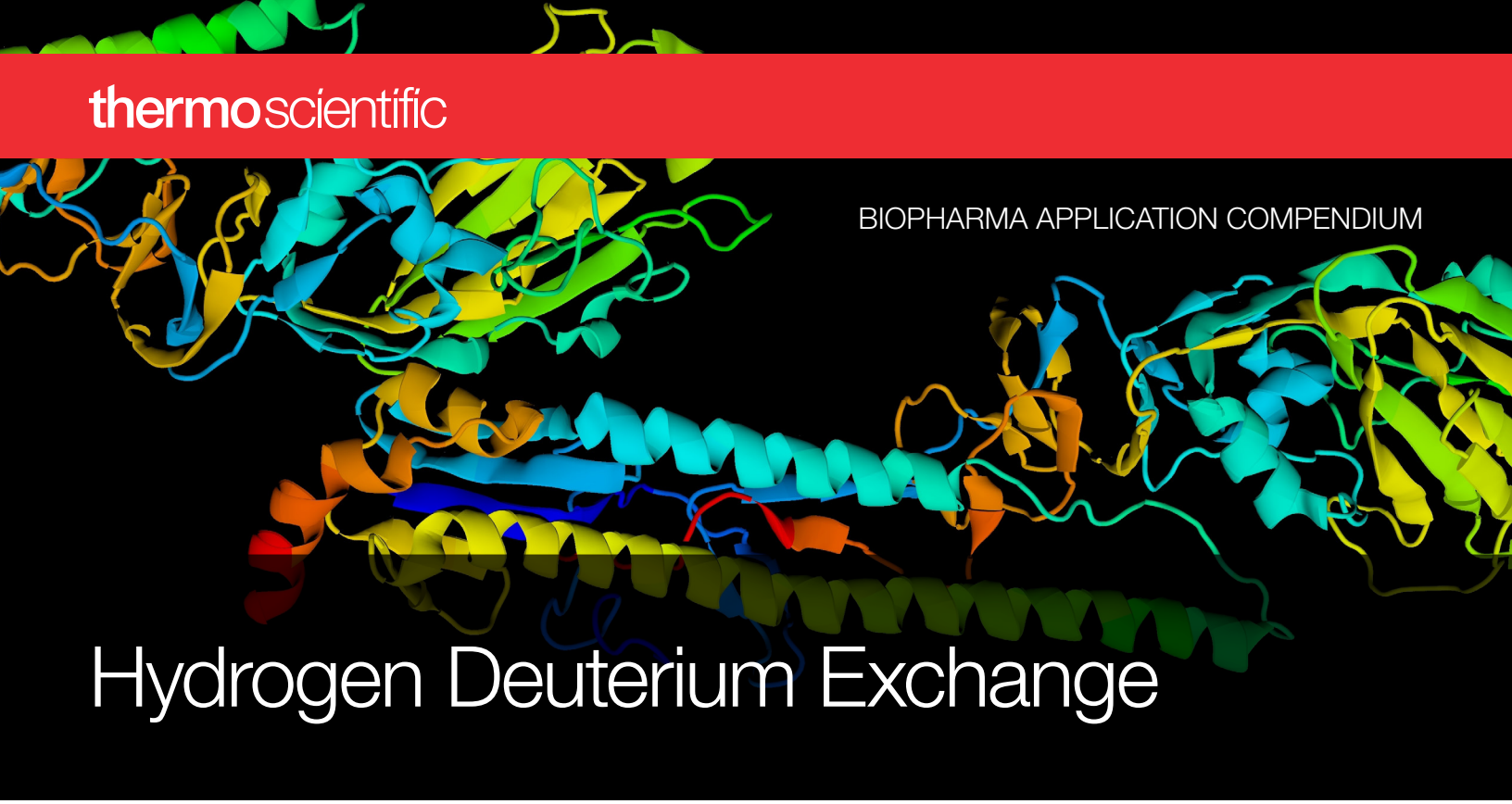

Hydrogen Deuterium Exchange

- Workflow
- Data processing

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Characterization of the Conformation of Therapeutic Antibody Oxidation Variants with Optimized Hydrogen/Deuterium Exchange Mass Spectrometry

Terry Zhang,[1] David Horn,[1] Shanhua Lin,[2] Xiaodong Liu[2] and Jonathan Josephs[1]
[1]Thermo Fisher Scientific, San Jose, CA, U. S.A; [2]Thermo Fisher Scientific, Sunnyvale, CA

Poster Note 64465

Overview

Purpose: Probe the conformation of herceptin and its oxidation variants.

Methods: Fully automatic hydrogen/deuterium exchange mass spectrometry

Results: There are no significant conformational changes for most regions of herceptin and its oxidation variants. However, local solvent exposure differences in the vicinity of the peptides containing methionine oxidation were observed.

Introduction

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of diseases. Characterization of chemical degradation of mAb-based drugs is a primary concern for biopharmaceutical development due to the subtle but critical local conformational changes that may impact safety and efficacy.[1,2] It is thus important to have an analytical tool that can detect these minor conformational changes. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. In this study, an optimized HDX workflow was developed and used to probe the conformation of Herceptin and its oxidation variants.

Methods

Therapeutic antibody, Herceptin, was partially oxidized with 0.01% H_2O_2 overnight. Both non-oxidized and oxidized mAb were diluted (1 to 9 ratio) with labeling buffer and incubated for multiple time points. The samples were then quenched with 4M guanidine, 200mM citric acid (pH 2.7) at 0.5 °C and subject to online pepsin digest at 8 °C for three minutes at 50 µL/min flow rate in a fully automated manner using H/D-X PAL™ (LEAP Technology). The digested peptides were injected into a Thermo Scientific™ PepMap™ trapping column washed for one minute and eluted to a Thermo Scientific™ Hypersil™ Gold C18 reverse phase column. A Thermo Scientific™ Ultimate™ 3000 nano pump system was employed to separate the digested peptides with 5% to 40% mobile phase B in 6 minutes gradient at flow rate of 40 µL/min. The separated peptides MS analysis was performed with Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. The data dependent MS/MS HCD spectra were collected using undeuterated protein for peptides identification first. And MS full scan at 60K was collected for HDX analysis. Figure 1 is the HDX work station set up. Figure 2 is HDX experimental workflow.

Liquid Chromatography

Thermo Scientific™ online pepsin Column: 2.1 x 3 mm

Thermo Scientific™ Dionex™ trapping Column: 500 µm x 15mm, C18 PepMap300, 5µm

Hypersil Gold analytical Column: 0.5 mm x 100mm, 3µm

Data Analysis

Data was processed with Proteome Discoverer 1.4™ software for peptide identification. Peptide mapping and PTM analysis was performed with PepFinder 2.0™ software. HDX experimental data were analyzed with HDExaminer and the Mass Analyzer HDX algorithm.[3,4]

FIGURE 1. HDX Work Station

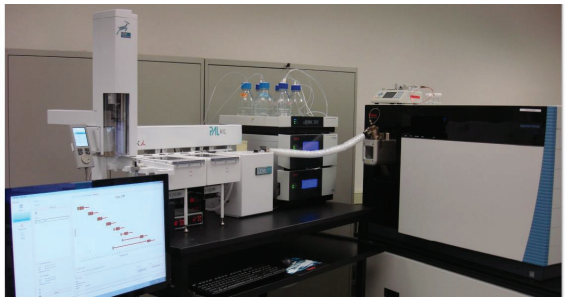
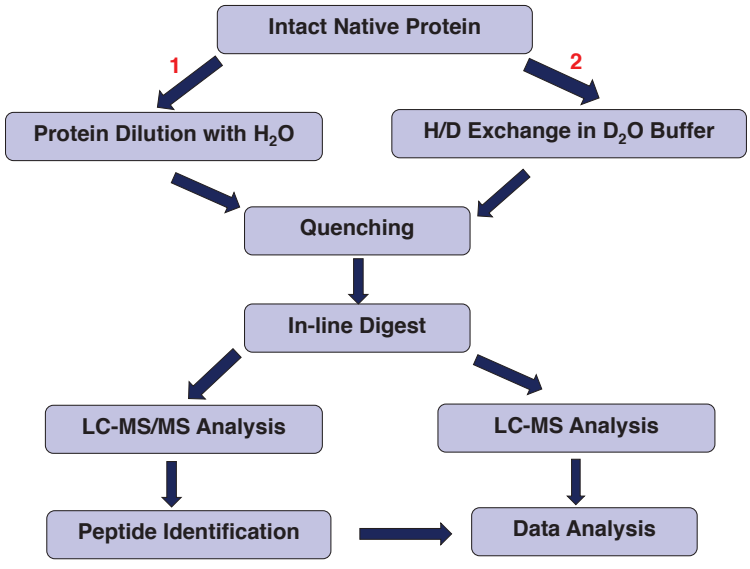


FIGURE 2. HDX experimental workflow



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Results

Peptide mapping of Hercetin

MS/MS experiments were first performed using non-deuterated Herceptin for peptide identification. Nearly 100% sequence coverage was achieved for both the Herceptin and oxidized Herceptin samples. Figure 3 is the peptide map of Herceptin generated by the Pepfinder software. More than 200 peptides generated by online pepsin digestion from the optimized HDX workflow were identified. These were subsequently used to probe the conformation of the two samples by HDX.

FIGURE 3. Peptide map of Herceptin

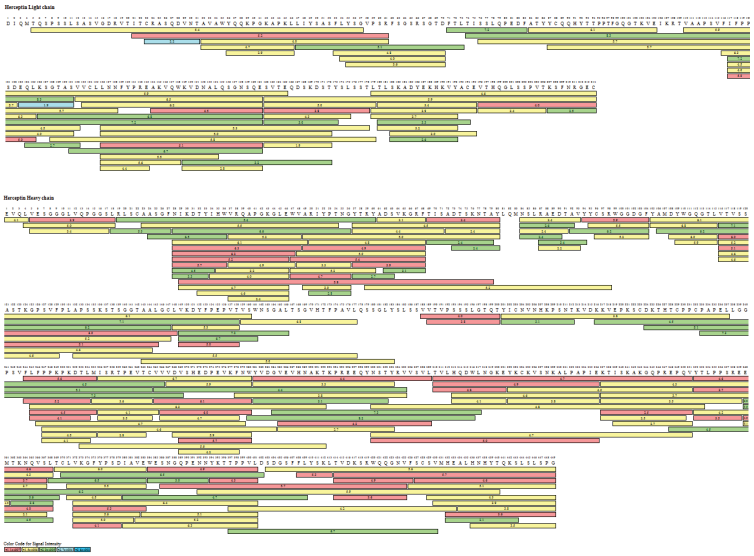
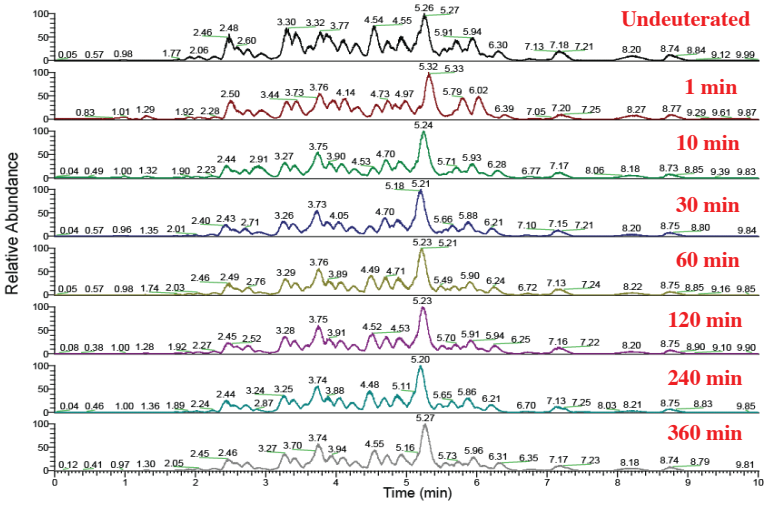


FIGURE 4. Herceptin HDX experimental base peak chromatogram



Conformation of Herceptin and its oxidized variants

Multiple time points of HDX experiment were performed for both Herceptin and oxidized Herceptin samples. Highly reproducible chromatograms were obtained for the various experimental time points (Figure 4). MS full scan spectra were collected to measure the deuterium uptake to probe the conformation of the therapeutic antibody and its variants. The deuterium uptake information were processed by HDExaminer.

FIGURE 5. a) Light chain deuterium uptake mirror plot of Herceptin and Herceptin variants. b) Deuterium uptake information modeled to Herceptin light chain crystal structure (PDB 1N8Z). The relative percent deuterium incorporation is shown at 30, 600, 3600 and 7200 seconds respectively.

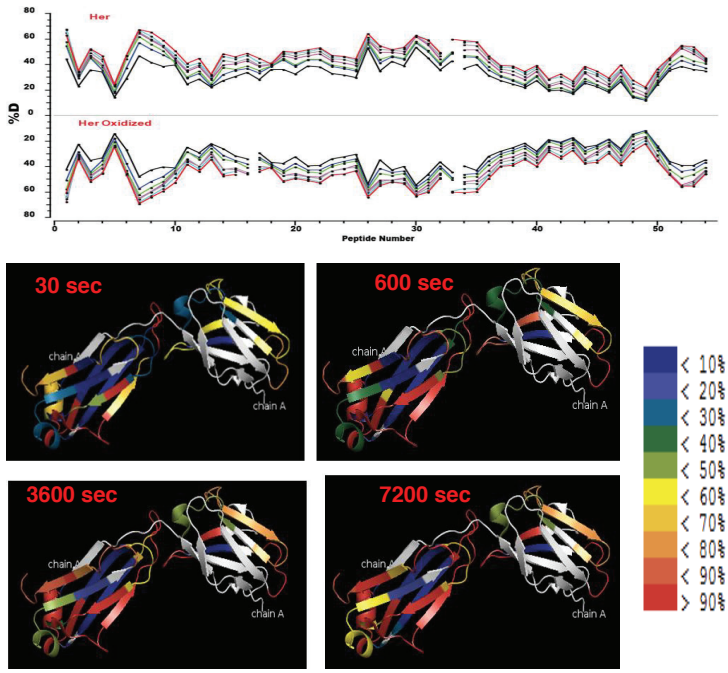
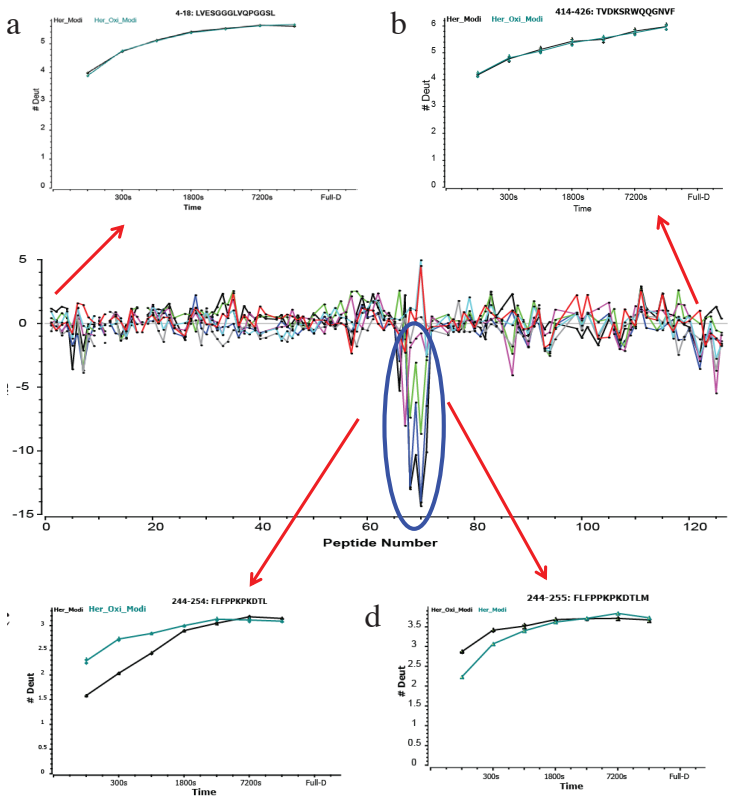


Figure 5 shows the light chain deuterium uptake measurement information. The very similar deuterium uptake patterns of the two samples indicate that there is no significant light chain conformational differences between Herceptin and Herceptin variants samples as shown in Figure 5a). The deuterium uptake measurements were exported to PMOL software and incorporated with the available Herceptin crystal structure as shown in Figure 5 b). The deuterium incorporation difference provides the information understanding the conformation dynamics of the light chain.

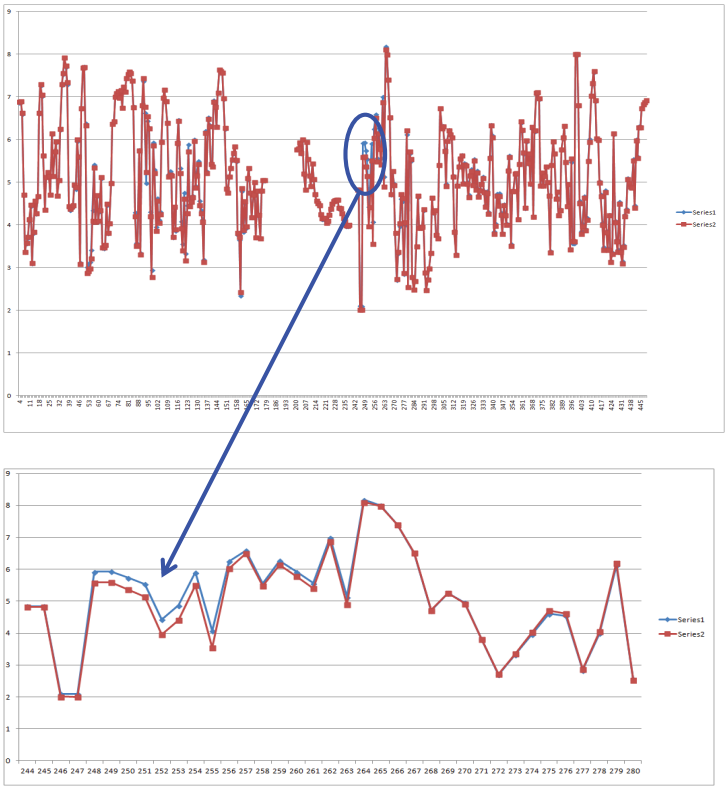
FIGURE 6. Herceptin versus Herceptin variants heavy chain deuterium uptake residual plot. Inserts a, b, c d are the specific peptides deuterium uptake plots of Herceptin and its variants



In Figure 6, heavy chain deuterium uptake difference between Herceptin and its oxidized variants is plotted vs. peptide number. The data were obtained with HDExaminer from MS full scan at various deuterium exchange time points. At most regions the difference is minimal (inserts a, b), except at the specific region where in the vicinity of methionine (residue 255), which is the amino acid that is oxidized. The inserts (c, d) of Figure 6, deuterium uptake plots of peptide FLFPPKPKDTL and FLFPPKPKDTLM, show the different kinetic behavior of deuterium uptake of Herceptin and its oxidized forms; after oxidation, the deuterium uptake is faster. Structurally, it is more sterically accessible for solvent exchange when methionine's SCH_3 terminal is oxidized to $SOCH_3$ or SO_2CH_3.

In Figure 7 protection factors for each residue in heavy chain of Herceptin and its variants were plotted. Mass Analyzer HDX algorithm was used to calculate the protection factor at the amino acid level. HDX model is built to simulate the whole deuterium labeling and back exchange processed during the digestion and analysis. The HDX model utilized the maximal information of the entire HDX MS data set (both the HDX kinetics and the labeling information from all overlapping peptides)[4]. 400 simulation was employed for this data set. Similar to findings shown in Figure 6, the protection factors are identical for most of the residues except in the region where methionine (residue 255) is involved. The oxidized variants have lower values compared to the original form, consistent with results obtained with HDExaminer.

FIGURE 7. (top): the average value of protection factors for each residue in heavy chain of Herceptin and its variants. (bottom): zoom in of the specific region with significant protection factor change



Conclusion

•A fully automated HDX workflow was developed and successfully applied to the study of conformational changes of Herceptin upon oxidation.

•The workflow was reliable and able to pinpoint the subtle but significant changes in the methionine region.

•The MS data were analyzed by two independent packages HDExaminer and Mass Analyzer and the conclusions are consistent.

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Poster Note 64771

Characterization of Conformation of Therapeutic Antibody Aggregation with Optimized Hydrogen/Deuterium Exchange Mass Spectrometer

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[1]Thermo Fisher Scientific, San Jose, CA; [2]Thermo Fisher Scientific, Sunnyvale, CA

ABSTRACT

Protein aggregation is one of the major critical quality attributes (CQA). To determine the structural change of antibody in connection with aggregation is important. In this study, a therapeutic antibody, Herceptin®, was treated with different acidic solutions (pH 0.5, 1.5 and 2.5) to induce aggregation. The aggregation was quantified by UV 280 absorption. The conformation of Herceptin and its aggregation was characterized by an optimized hydrogen/deuterium exchange mass spectrometer (HDX). The HDX results revealed the more significant conformation change regions when aggregation was induced.

INTRODUCTION

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of cancer and other diseases. Characterization of critical quality attributes (CQA) of mAb-based drugs is a primary concern for biopharmaceutical development. Structural characterization is used to assess the CQAs of biopharmaceutical products. Protein aggregation is a major CQA during monoclonal antibody (mAb) production. The formation of aggregates may impact safety and efficacy of mAbs. It is thus important to understand the mechanism of aggregation and the conformational changes of the aggregates. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. In this study, an optimized HDX workflow was used to probe the conformation of Herceptin and its aggregation.

MATERIALS AND METHODS

Instrument and Materials

A fully automated HDX platform, based on the H/D-X PAL™ system (LEAP Technologies) and the Thermo Scientific™ UltiMate 3000 pump system coupled online with an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer, was used. Figure 1 shows the HDX work station. It is composed of an autosampler equipped with individually temperature-controlled sample plate and labeling plate. The syringe head and pepsin column can also be independently temperature-controlled. Chronos™ control software is fully integrated with the Thermo Scientific™ Xcalibur™ platform and the user interface is shown in Figure 2. Figure 3a) describes the three valve configurations at various stages of the experiment inside the cooling chamber. The flexibility allows for maximized productivity. Figure 3 b) shows the dual heads high pressure mixing pump on the left and ternary loading pump on the right. Flow rates and solvents can be changed on the fly during the experiment. The execution of the whole experiment was completely automated and controlled by Chronos™ software.

Therapeutic antibody (Herceptin) aggregation was induced by adjusting the pH to 0.5, 1.5 and 2.5 with HCl and by incubating each sample at room temperature for 30 min. The pH of the samples was subsequently adjusted to pH 9. Both non aggregated and aggregated mAbs were diluted with labeling buffer and incubated for multiple time points. The samples were then quenched and digested online with a pepsin column in a fully automated manner using the H/D-X PAL system. The digested peptides were injected into a Thermo Scientific™ Acclaim™ Pepmap™ C18 reverse phase column with a 7 min gradient. MS analysis was performed with the Orbitrap Fusion mass spectrometer.

Data Analysis

Peptide identification was performed with Thermo Scientific™ Proteome Discoverer™ 1.4 software . Peptide mapping and PTM analysis was performed with Thermo Scientific ™ BioPharma Finder™ 1.0 software. HDX experimental data were analyzed with HDExaminer™ software (Sierra Analytics) and the HDX tool in Mass Analyzer (licensed from Amgen).[1,2].

FIGURE 1. HDX work station coupled online to the Orbitrap Fusion mass spectrometer

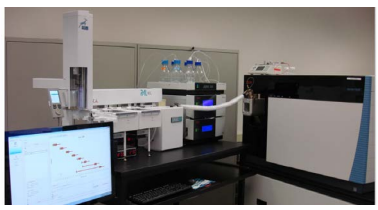


Figure 2. Chronos software interface, integrated with Xcalibur software

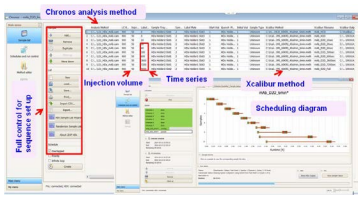


Figure 3. a). Flexible three valve configuration for carrying out different experimental tasks simultaneously in the cooling chamber, b). NCS-3500RS with binary rapid separation micro flow pump with ternary loading pump

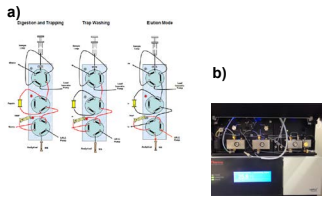


Figure 4. UV absorption spectrum of Herceptin and pH stressed samples

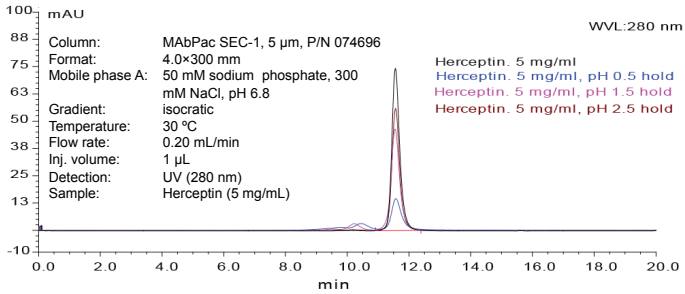
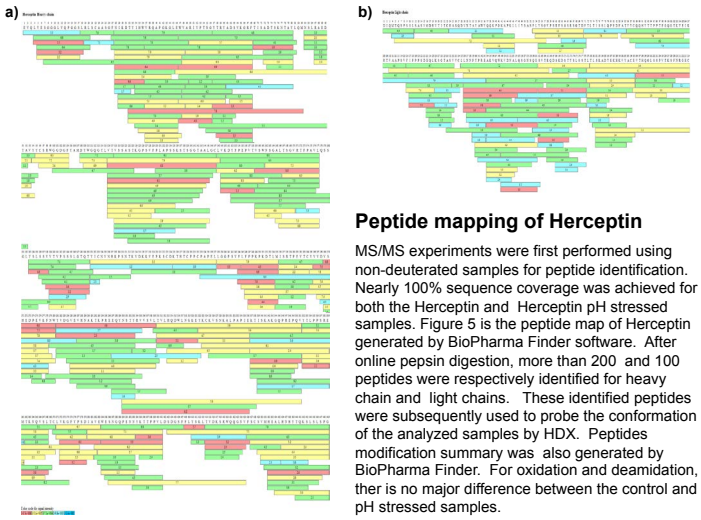


Figure 5. Peptide map of Herceptin by BioPharma Finder a) Heavy chain b) Light Chain



Peptide mapping of Herceptin

MS/MS experiments were first performed using non-deuterated samples for peptide identification. Nearly 100% sequence coverage was achieved for both the Herceptin and Herceptin pH stressed samples. Figure 5 is the peptide map of Herceptin generated by BioPharma Finder software. After online pepsin digestion, more than 200 and 100 peptides were respectively identified for heavy chain and light chains. These identified peptides were subsequently used to probe the conformation of the analyzed samples by HDX. Peptides modification summary was also generated by BioPharma Finder. For oxidation and deamidation, ther is no major difference between the control and pH stressed samples.

RESULTS

Herceptin and Herceptin Aggregation

Herceptin and its pH stressed samples were quantified by measuring UV 280 absorption. Figure 4 is the overlay absorption spectra of four samples. Aggregations around 25% for pH 0.5, 6% for pH 1.5 and 0% for pH 2.5 were observed.

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Herceptin and its Aggregation conformation characterization

Multiple time point HDX experiments were performed for both Herceptin and pH stressed Herceptin samples. Highly reproducible chromatograms were obtained for the various experimental time points. MS full scan spectra were collected to measure the deuterium uptake to probe the conformation.

Overall, the control and pH stressed samples had similar deuterium uptake profile but for most of the pH stressed sample peptides showed slightly more deuterium uptake, around 2 to 5%, than for the non-stressed sample. The differences could reach up to 10 to 15% for different regions for the light chain and could be even higher for the heavy chain. The sample stressed at pH = 0.5 showed more deuterium uptake than the pH = 1.5 sample. Finally, there was no significant deuterium uptake change between Herceptin and the stressed sample pH=1.5 (data not shown).

Figure 6. a): Herceptin and pH 0.5 stressed sample light chain deuterium uptake light chain residual plot. b), c): Example of specific peptides deuterium uptake plots of Herceptin and its pH 0.5 stressed sample

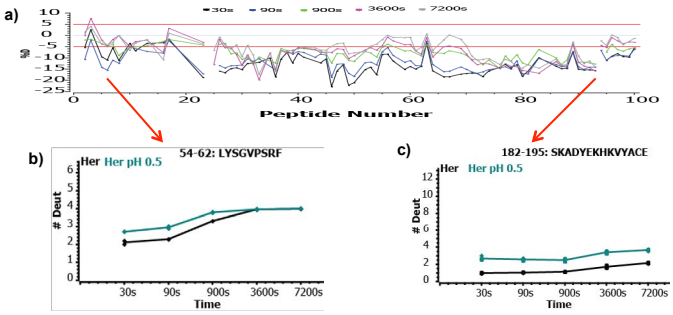


Figure 6 a) is the light chain deuterium uptake residual plot whereas figure b) and , c) are peptides uptake plots. In figure 6 b) peptide showed more differences for short labeling time but would reach to similar levels at longer time points, while in figure 6 c) peptide showed the significant differences persistent along all the time points. Protection factors were calculated and shown in figure 7. Certain regions have similar protection factors while other regions have significant differences, i.e., between amino acids160 and 190 for the heavy chain. These results were consistent with HDExaminer's analysis.

Figure 7. Herceptin and pH 0.5 stressed sample light chain protection factor comparison

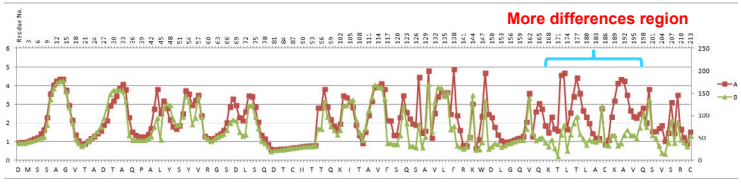
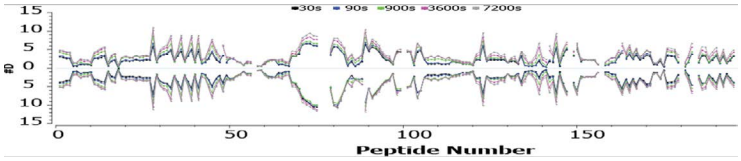


Figure 8. Herceptin and pH 0.5 stressed sample heavy chain deuterium uptake mirror plots



Similar deuterium uptake profiles of Herceptin and its pH stressed sample were observed for heavy chain and are shown in figure 8. In general, the stressed sample showed more deuterium uptake, and the amount of uptake increased with degree of aggregation. This implies that aggregation would impact the 3D structure of Herceptin and more exposed to solvents, i.e., more deuterium uptake. Furthermore, the increase in deuterium uptake was not uniform across all the peptides. Example deuterium uptake plots were shown in figure 9. In these examples, different peptides showed different deuterium uptake dynamics and levels. With mass spectrometry, the regions with such increase can be pinpointed. A pronounced deuterium uptake increase (up to 40%, see left panel of figure 10) was observed from amino acid 120 to 200 and 240 to 255 of the heavy chain for the sample stressed at pH=0.5. The differences could be up to 5 daltons, see figure 10, right panel. These results indicated that aggregation would cause more conformational change for the Fab region and smaller changes for the Fc region.

Figure 9. Herceptin and pH 0.5 stressed sample heavy chain peptides deuterium uptake plots

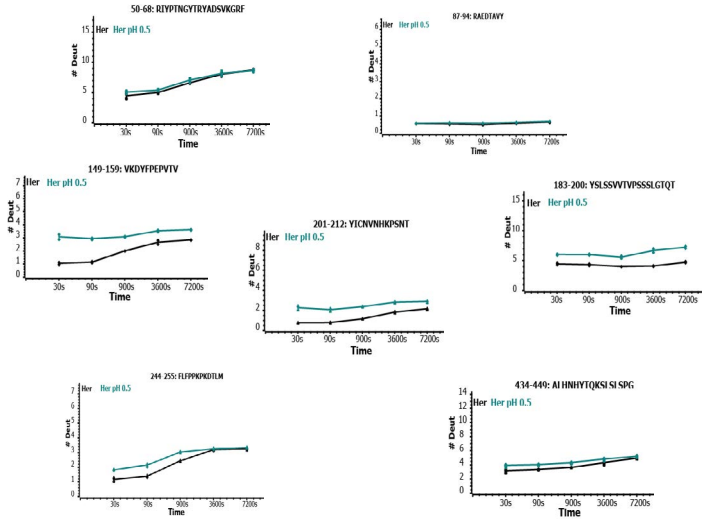


Figure 10. Herceptin and pH 0.5 stressed sample heavy chain deuterium uptake residual plots, Left: %D Right: #D

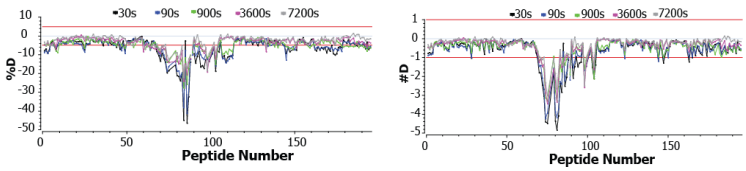
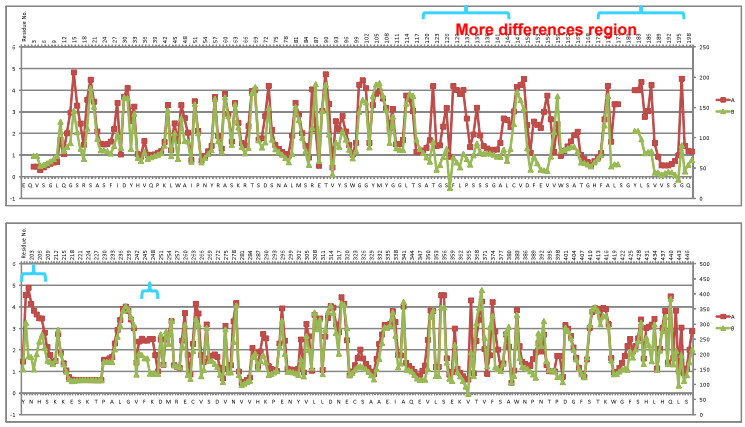
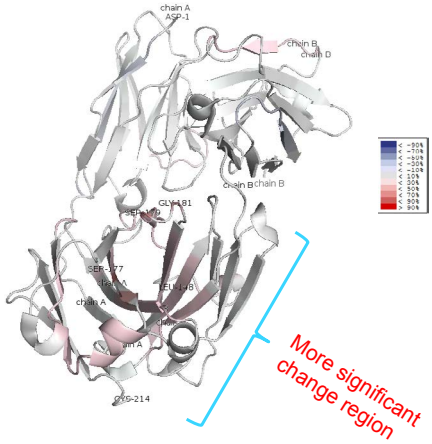


Figure 11. Herceptin and pH 0.5 stressed sample heavy chain protection factor comparison. Top: residual 1-200 Bottom: residual 201-449



To further understand the conformational dynamics, single amino acid residue level protection factors were calculated using Mass Analyzer HDX, (see figure 11). In most of the regions Herceptin showed slightly higher protection factor than the pH 0.5 stressed sample. From amino acid 110 to 210 and 240 to 255, Herceptin had a much higher protection factor, which is consistent with the deuterium uptake residual plots obtained from HDExaminer.

Figure 12. Herceptin and pH 0.5 stressed sample 1hr deuterium labeling Fab differential exchange map on crystal structure (PDB: 1N8Z)



Antibody aggregation is a complex, multistep process[3]. The conformation change with more deuterium uptake observed for the sample stressed at pH 0.5 agreed with the proposed aggregation mechanism[3,4] and the reported results[5] in the literature. The differential deuterium uptake of Herceptin and the sample stressed at pH 0.5 for1 hour labeling was mapped with Herceptin Fab crystal structure (PDB:1N8Z) and shown in figure 12. Most of the significant change regions were in the C_L, C_H and part of Fc C_H2 domains.

CONCLUSIONS

•A fully automated HDX workflow was successfully applied to the study of conformational changes of Herceptin upon aggregation.

•In this study, we were able to pinpoint the subtle but significant changes around the C_L, C_H and C_H2 regions.

•The MS data were analyzed by two independent software packages (HDExaminer and Mass Analyzer) and the conclusions were consistent.

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POSTER NOTE

No. 64890

Evaluation of different Hydrogen/Deuterium Exchange data processing software by looking at conformational changes induced by mAb aggregation

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Xiaodong Liu[2] and Jonathan Josephs[1]
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ABSTRACT

Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. One of the biggest challenges in HDX experiment is data processing. In this study, three HDX processing software, HDExaminer (Sierra Analytics), HDX WorkBench (Scripps) and Thermo Scientific ™ BioPharma Finder 2.0 were compared and used to characterize the conformation of Trastuzumab and its aggregation.

INTRODUCTION

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of cancer and other diseases. Characterization of critical quality attributes (CQA) of mAb-based drugs is a primary concern for biopharmaceutical development. Structural characterization is used to assess the CQAs of biopharmaceutical products. Protein aggregation is a CQA for monoclonal antibody (mAb) product. The formation of aggregates may impact safety and efficacy of mAbs. Antibody aggregation is a complex, multistep process[1]. Thus, it is important to understand the mechanism of aggregation and the conformational changes of the aggregates. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. One of the biggest challenges in HDX is data processing. Accurately measuring the deuterium uptake would yield more precise information of the protein conformation and conformation dynamics. In this study, three HDX process software, HDExaminer (Sierra Analytics), HDX WorkBench (Scripps) and Thermo Scientific ™ BioPharma Finder 2.0 were compared and used to characterize the conformation of Trastuzumab and its aggregation.

MATERIALS AND METHODS

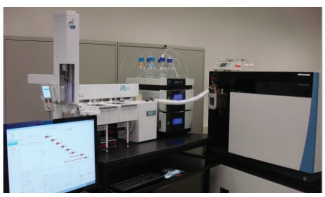
A fully automated HDX platform, based on the H/D-X PAL™ system (LEAP Technologies) and the Thermo Scientific™ UltiMate 3000 pump system coupled online with an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer, was used. Figure 1 shows the HDX work station. It is composed of an autosampler equipped with individually temperature-controlled sample plate and labeling plate. The syringe head and pepsin column can also be independently temperature-controlled. Chronos™ control software is fully integrated with the Thermo Scientific™ Xcalibur™ platform and the user. The execution of the whole experiment is completely automated and controlled by Chronos™ software.

Therapeutic antibody Trastuzumab (Herceptin) aggregation was induced by adjusting the pH to 0.5, 1.5 and 2.5 with HCl and by incubating each sample at room temperature for 30 min. The pH of the samples was subsequently adjusted to pH 9. Both non aggregated and aggregated mAbs were diluted with labeling buffer and incubated for multiple time points. The samples were then quenched and digested online with a pepsin column in a fully automated manner using the H/D-X PAL system. The digested peptides were injected into a Thermo Scientific™ Acclaim™ Pepmap™ C18 reverse phase column with a 7 min gradient. MS analysis was performed with the Orbitrap Fusion mass spectrometer.

Data Analysis

Peptide identification, mapping, PTM analysis and HDX data analysis were performed with Thermo Scientific ™ BioPharma Finder™ 2.0 software. HDX experimental data were also analyzed with HDExaminer™ software (Sierra Analytics) and HDX WorkBench (Scripps, Florida)

FIGURE 1. HDX work station coupled online to the Orbitrap Fusion mass spectrometer



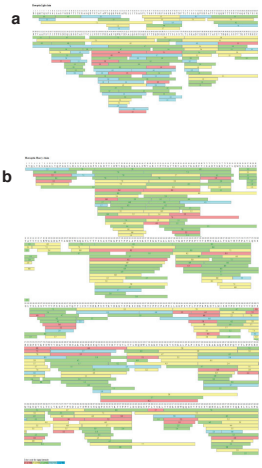


Figure 2. Peptide map of Herceptin by BioPharma Finder a) Light chain b) Heavy Chain

RESULTS

Herceptin and Herceptin Aggregation

Herceptin (Her) and its pH (Her pH0.5) stressed samples were quantified by measuring UV 280 absorption. Aggregations around 25% for pH 0.5, 6% for pH 1.5 and 0% for pH 2.5 were observed. The HDX data from the control Her and Her stressed at pH 0.5 samples were used for this study.

MS/MS experiments were first performed using non-deuterated samples for peptide identification. Nearly 100% sequence coverage was achieved for both the Herceptin and Herceptin pH stressed samples. Figure 2 is the peptide map of Herceptin generated by BioPharma Finder software. . After online pepsin digestion, around 200 and 100 peptides were respectively identified for heavy and light chains. These identified peptides were subsequently used to probe the conformation of the analyzed samples by HDX.

Overall, the control and pH stressed samples showed similar deuterium uptake. However, most of the pH stressed sample peptides showed slightly more deuterium uptake, between 2 and 5%, than for the control sample. The differences could reach up to 15% for different regions of the light chain and could be even higher for the heavy chain. The sample stressed at pH = 0.5 showed more deuterium uptake than the pH = 1.5 sample. Finally, there was no significant deuterium uptake change between Herceptin and the stressed sample pH=1.5,as reported previously[2].

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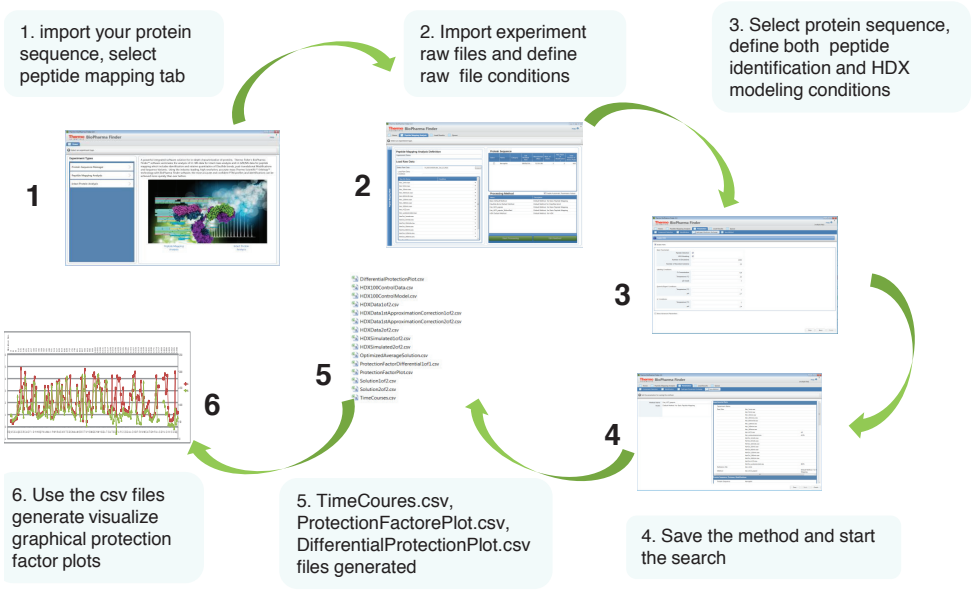
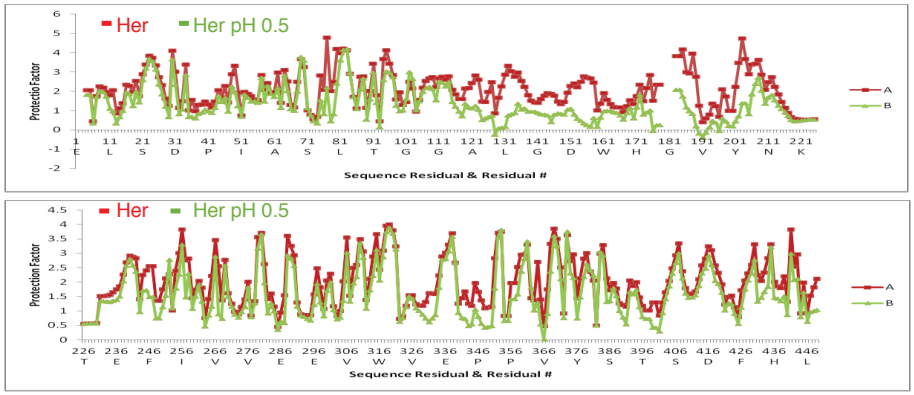
Figure 3. Biopharma Finder HDX data analysis workflow



The HDX tool in mass analyzer[3] has been implemented into BioPharma Finder 2.0. The HDX MS data can be processed in an automated fashion by BioPharma Finder 2.0. The unlabeled protein samples must be analyzed with data-dependent MS/MS for peptide identification. Deuterium labeling is performed at different time intervals to obtain time courses for each condition. The software uses a 5-parameter equation to fit the curves to calculate the variance in the data[3]. Ideally, a 0% and 100% deuteration control should be performed for appropriate back exchange modeling. Figure 3 shows the HDX data process workflow of BioPharma Finder. First, import the protein sequence in fasta format, including all chains of the protein into the protein sequence manager, then select peptide mapping analysis tab. Browse in all the raw files and define the experiment name, the file conditions and search parameters. There are two major steps involved once the data analysis process started, peptide detection and HDX modeling. The peptide detection including feature exaction, retention time alignment, gap-fillling and peptide identification. The HDX modeling including deuterium level calculation, back exchange modeling and full HDX modeling. HDXData, TimeCourses, HDXSilulation, ProtectionFactorPlot, Differential Protection Plot csv result files were created for analyzed protein including all the chains, ie, light and heavy chains for mAb. To evaluate the overall protein conformation properties at different conditions, visualized deuterium uptake and protection factor plots can be generated from the csv files.

Figure 4. Herceptin and pH 0.5 stressed sample heavy chain protection factor comparison Top: residual 1-225 Bottom: residual 226-449



The HDX data from Her and stressed Her samples were processed by Biopharma Finder. The heavy chain protection factor plots of Her and stressed Her samples were generated from the BioPharma Finder searched csv file as shown in figure 4. In most of the regions Herceptin showed slightly higher protection factor than the stressed sample. The differences were not uniform along the sequence. From amino acid 110 to 210 and 240 to 255, Herceptin had a much higher protection factor than Herceptin pH stressed sample which would reflect in figure 5 the deuterium uptake profile. One peptide with less deuterium uptake difference, peptide 49-60, and two peptides with more significant deuterium uptake changes , 149-159 and, 244-255, were selected to evaluate the deuterium uptake profiles as shown in figure 5. The deuterium uptake differences range from 0.1 to around 2 Daltons. These results indicated that aggregation would induce more conformational changes in the Fab region and less changes for the Fc region.

Figure 5. Example deuterium uptake plots generated by Biopharma Finder

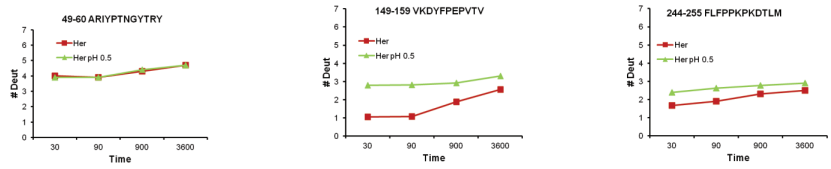


Figure 6. HDExaminer HDX data analysis workflow

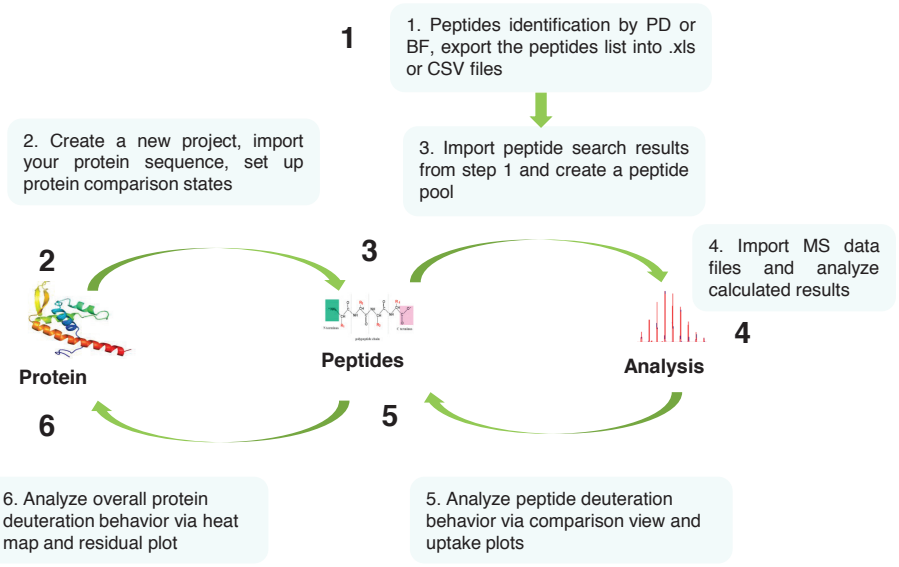
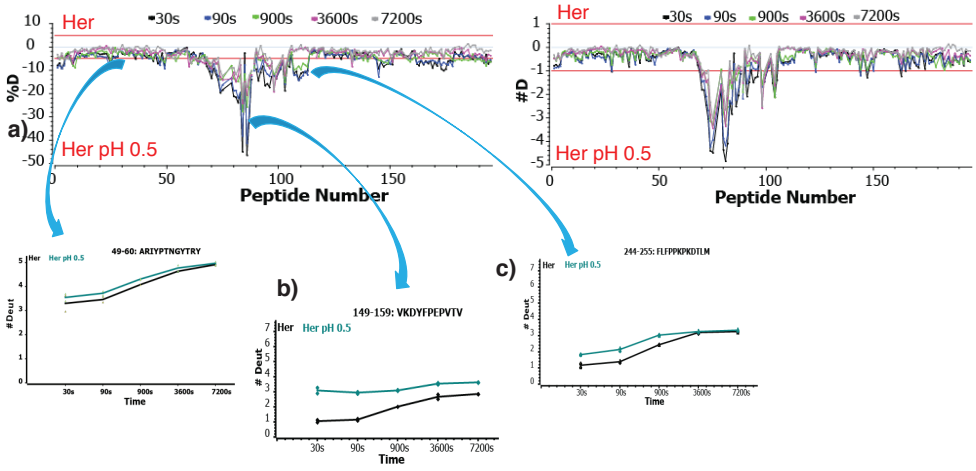


Figure 6 is the HDX data process workflow of HDExaminer. To process the HDX data with HDExaminer, a .xsl or csv file had to be generated by exporting the peptide identification list from the peptide identification search results. Similar to BioPharma Finder, the protein sequence had to be defined first in the protein tab (the software would only process one protein at a time, the different chains of the same protein would be treated as different protein and would be processed separately), the .xsl or csv peptide list would be directly imported into a peptide pool for peptide deuterium uptake measurement in the peptides tab. The undeuterated and deuterated raw files at different time points would be loaded in the analysis tab to start the data analysis. The deuterium uptake heat map of the protein, the peptide deuterium uptake plots, different protein states deuterium uptake mirror and residual plots would be created to probe the protein conformation and conformational changes.

The peptide identification list csv file of Her and Her stressed samples from the BioPharma Finder was exported and directly used to process the HDX data by HDExaminer. The heavy chain peptides deuterium uptake residual plots in both %D (top left) and absolute #D (top right) were shown in figure 7. In general, the Herceptin had less deuterium uptake compare to stressed sample and the differences were not uniform. Peptides with more significant change, were identified between amino acides 70 and 100 and this observation was consistent with the BioPharma Finder protection factor prediction shown in figure 4. Figure 7 a), b), c) were the deuterium uptake plots from the same three peptides shown in figure 5. The deuterium uptake of the three selected peptides was very similar to the plots shown in figure 5.

Figure 7. Herceptin and pH 0.5 stressed sample heavy chain deuterium uptake residual plots, Top left: %D Top right: #D Bottom: peptide deuterium uptake plots a), b), c)



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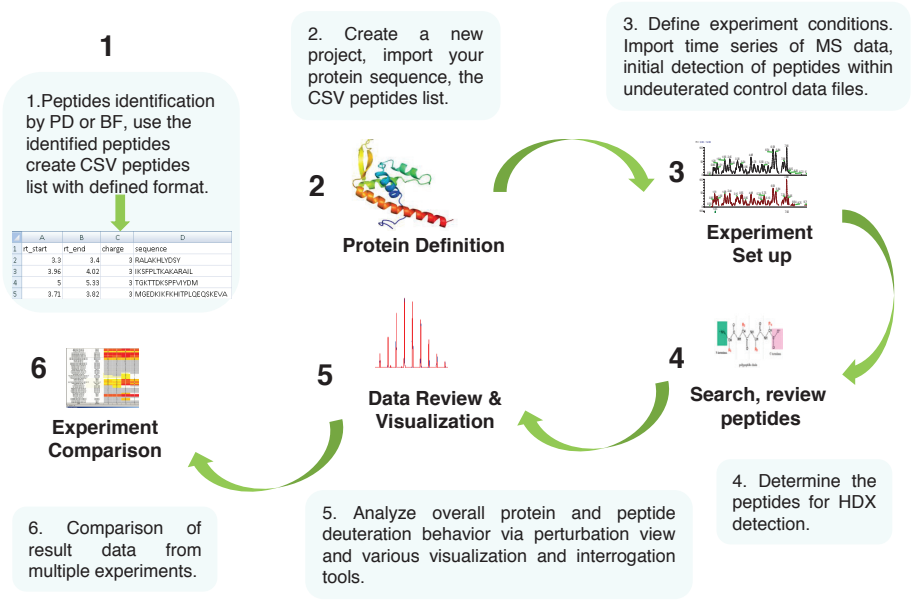
1. D. Weis et al, Hydrogen Exchange Mass Spectrometry of Proteins
2. T. Zhang et al, ThP180, ASMS 2016
3. Z. Zhang et al, Anal. Chem. 2012, Vol. 84, 4942-493

Figure 8. HDX WorkBench data analysis workflow



HDX WorkBench from Scripps is an integrated software platform for analysis of HDX mass spectrometry data. Figure 8 was the HDX WorkBench HDX data analysis workflow. The first step was to build a specific format csv peptide list based on the peptide identification results (the list from BioPharma Finder) as indicated in step 1. The next step was to define the protein and to import the csv peptides list . The protein could be edited to add secondary structure features. The experimental conditions and labeling time series of the identified peptide in the undeuterated sample would be defined in the experimental set up. The different chains of the protein, heavy and light chain of mAb would be treated as two proteins under the same experiment. Only the identified peptides from the undeuterated sample would be used to start the HDX data analysis.The software would create a very comprehensive results dashboard including: peptide list, chromatogram, peptide deuterium update plot, peptide spectra, and time points statistics as shown in figure 9.

A csv file with the specific format was generated by using the BioPharma Finder search results and used in HDX WorkBench to process the data from the Her and Her stressed samples. Similar results were obtained with HDX Workbench and the two previous software packages. The same three peptide deuterium uptake plots were shown in figure 10. Figure 11 was the single residual consolidated perturbation sequence coverage of the heavy chain.

Figure 9. HDX WorkBench Results Dashboard

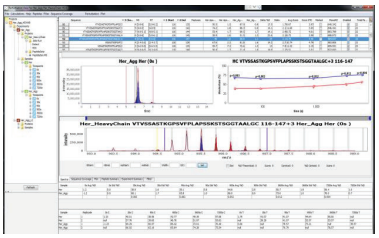


Figure 10. Her and Her pH 0.5 stressed samples heavy chain peptides deuterium uptake plots

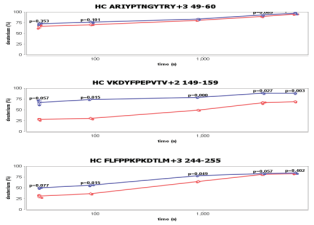
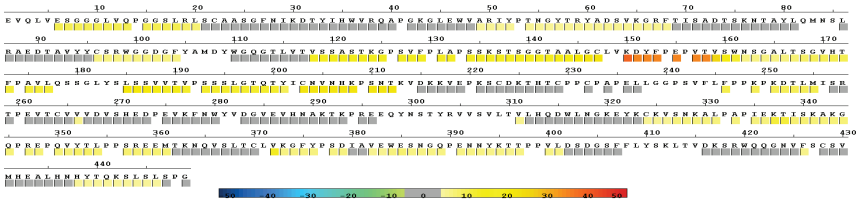


Figure 11. Herceptin and pH 0.5 stressed sample heavy chain consolidated perturbation sequence coverage



CONCLUSIONS

•The HDX data from Her and Her pH stressed samples were successfully processed by all three software packages and the results were consistent.
•The BioPharma Finder is a comprehensive software package that can perform both peptide identification and HDX data analysis. HDX model simulates the H/D exchange and back exchange process at the single residue level. The protection factor plot can be used to evaluate the whole protein conformational property.
•HDExaminer can directly import the .xsl or csv files from a peptides identification results. The HDX data process is straight forward and results are easy to implement. The peptides deuterium uptake residual plots can be used to probe the protein deuterium uptake behavior.
•HDX WorkBench generates a very comprehensive results dashboard. It can yield a consolidated amino acid level perturbation sequence coverage map.

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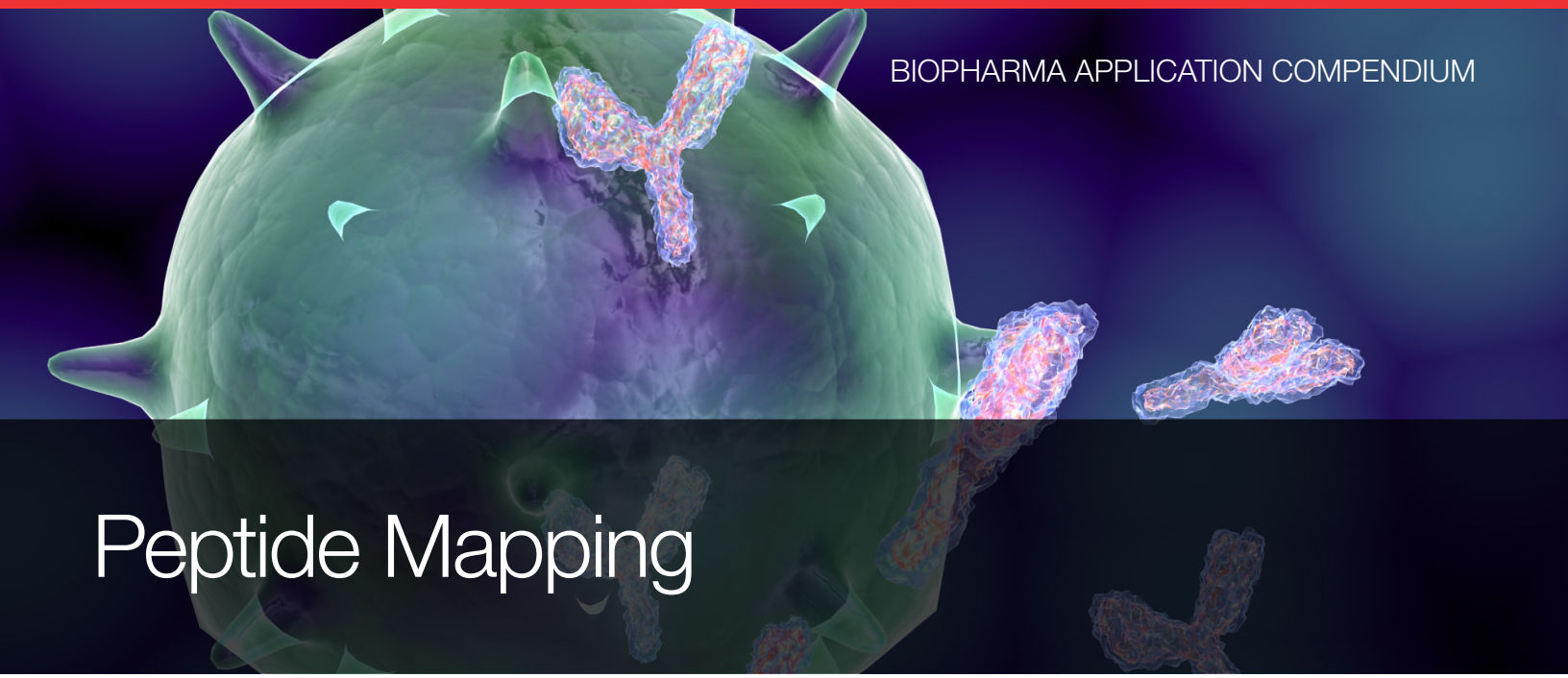

Peptide Mapping

- Protein digestion
- LC-UV
- LC-MS
- Automated peptide mapping
- LC-MS to LC-UV method transfer

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APPLICATION NOTE

SMART Digest compared to classic in-solution digestion of rituximab for in-depth peptide mapping characterization

Authors: Martin Samonig[1], Alexander Schwahn[2], Ken Cook[3], Mike Oliver[4], and Remco Swart[1]

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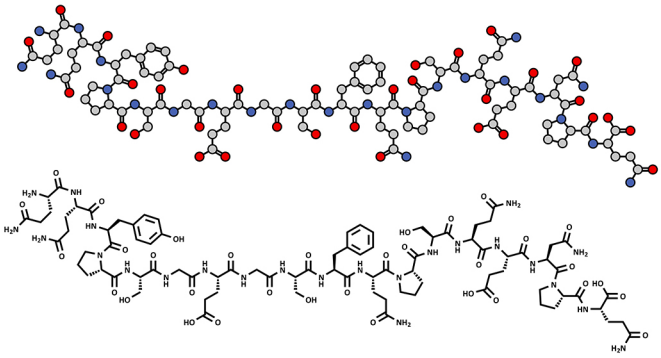
Key words

SMART Digest, tryptic digestion, in-solution protein digestion, monoclonal antibody, mAb, Vanquish, reversed phase, mass spectrometry, Q Exactive, Orbitrap, biopharmaceutical, biomolecules, peptide mapping

Goal

To compare the results achieved by using the newly developed Thermo Scientific™ SMART Digest™ kit to those obtained from classic in-solution protein digestion methods, focusing on protein sequence coverage and identified post-translational modifications (PTMs), including deamidation, oxidation, and glycosylation. A Thermo Scientific™ Acclaim™ VANQUISH™ C18 column with conventional water/acetonitrile-based gradients and



the Thermo Scientific™ Vanquish™ Flex UHPLC system were used for separation in combination with the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer.

Introduction

Peptide mapping is a common technique in the biopharmaceutical industry to characterize monoclonal antibodies (mAbs) for the determination of product identity and stability. Many conventional sample preparation methods are time consuming with digestion times of several hours and can introduce modifications such as deamidation, oxidation, and carbamylation.[1] In this study, two classic in-solution digestion approaches were compared to the recently developed SMART Digest kit method to quantify the extent of post-translational and chemical modifications of a therapeutic recombinant mAb. The critical requirements for each

method were the complete sequence coverage of the heavy and light chain and the accurate identification and (relative) quantification of the glycans attached to the asparagine 301 on the heavy chain. Deamidation, oxidation, and carbamylation are induced primarily during sample preparation and were thus monitored for a direct comparison of the different digestion methods. A time course experiment for the SMART Digest was performed to assess the influence of digestion time on modification formation.

Experimental

Consumables

- Thermo Scientific Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific SMART Digest Kit (P/N 60109-101)
- Fisher Scientific™ LCMS Grade Water (P/N W/011217)
- Fisher Scientific™ LCMS Grade Acetonitrile (P/N A/0638/17)
- Fisher Scientific™ Optima™ LCMS Trifluoroacetic Acid (P/N 10125637)
- Thermo Scientific™ Pierce™ Formic Acid LCMS Grade (P/N 28905))
- Thermo Scientific™ Pierce™ Trypsin Protease MS Grade (P/N 90057)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Thermo Scientific™ Pierce™ Urea (P/N 29700)
- Thermo Scientific™ Pierce™ Iodoacetamide (P/N 90034)
- Thermo Scientific™ Invitrogen™ UltraPure™ Tris Hydrochloride (P/N 15506017)

Sample pretreatment and sample preparation

A commercially available monoclonal antibody rituximab drug product (Hoffmann La Roche, Basel, Switzerland) was supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.7 mg/mL polysorbate 80, 7.35 mg/mL sodium citrate dehydrate, 9 mg/mL sodium chloride, and sterile water adjusted to pH 6.5 using sodium hydroxide.

In-solution digestion protocol using urea for denaturation

400 µg rituximab were denatured for 75 min in 7 M urea and 50 mM tris hydrochloride (HCl) at pH 8.0, followed by a reduction step using 5 mM dithiothreitol (DTT) for 30 min at 37 °C. Alkylation was performed with 15 mM iodoacetamide (IAA) for 30 min at room temperature, and the reaction was quenched by addition of 9 mM DTT. The sample was then diluted 1:10 (v/v) with 50 mM tris HCl pH 8.0 to reach a final urea concentration below 1 M. Trypsin was added with a protein/protease ratio of 40:1 (w/w) and digestion was allowed to proceed overnight at 37 °C. Digestion was stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.5%. (Sample name: In-Solution, Urea)

In-solution digestion protocol using heat for denaturation

400 µg rituximab were denatured in 50 mM tris HCl at pH 8.0 and 70 °C for 75 min, followed by a reduction step using 5 mM DTT for 30 min at 70 °C. Alkylation was performed with 15 mM IAA for 30 min at room temperature, and the reaction was quenched by addition of 9 mM DTT. The sample was then diluted 1:10 (v/v) with 50 mM tris HCl pH 8.0. Trypsin was added with a protein/protease ratio of 40:1 (w/w) and digestion was allowed to proceed overnight at 37 °C. Digestion was stopped by addition of TFA to a final concentration of 0.5%. (Sample name: In-Solution, Heat)

SMART Digest kit protocol

50 µL rituximab sample, adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the SMART Digest buffer provided with the kit. It was then transferred to a reaction tube containing 15 µL of the SMART digest resin slurry, corresponding to 14 µg of heat-stabile, immobilized trypsin. A time course experiment was performed and tryptic digestion was allowed to proceed at 70 °C for 15, 30, 45, and 75 min at 1400 rpm; a digestion time of 45–60 min was found to be sufficient to achieve digestion completeness for mAb samples (Figure 2). After the digestion, the reaction tube was centrifuged at 7000 rpm for 2 min, the supernatant was transferred to a new tube, and the centrifugation step was repeated. Disulfide bonds were reduced by incubation for 30 minutes at 37 °C with 5 mM DTT. (Sample names: SMART Digest, 15, 30, 45, 75 min)

All samples were diluted with 0.1% formic acid (FA) in water to a final protein concentration of 100 ng/µL, and 2.5 µg were loaded on the column for all runs.

LC Conditions

Instrumentation

- Vanquish Flex Quaternary system consisting of:
  - Flex System Base (P/N VF-S01-A)
  - Quaternary Pump F (P/N VF-P20-A)
  - Split Sampler FT (P/N VF-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Active Pre-heater (P/N 6732.0110)
  - Diode Array Detector HL (P/N VH-D10-A) (not used in the LC-MS experiments)
  - Static Mixer for 200 µL mixing volume (P/N 6044.5110)
  - MS Connection Kit Vanquish (P/N 6720.0405)

Time [min]	A[%]	B[%]	Flow Rate [mL/min]
0.0	96	4	0.3
50.0	25	75	0.3
51.0	0	100	0.3
60.0	0	100	0.3
61.0	96	4	0.3
80.0	96	4	0.3

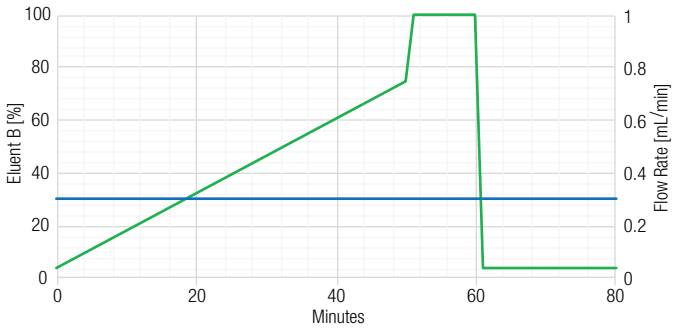


Figure 1. LC gradient.

Separation conditions (unless noted otherwise in the text)

Column:	Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm
Mobile Phase A:	Water + 0.1% FA
Mobile Phase B:	Water/acetonitrile (20:80 v/v) + 0.1% FA
Flow Rate:	0.3 mL/min
Temperature:	50 °C, Forced air mode
Gradient:	See Figure 1

MS conditions

Instrumentation

The Thermo Scientific Q Exactive HF mass spectrometer (MS) was used for detection. The detailed MS source and method parameters are given in Tables 1A and 1B.

Table 1A. Q Exactive HF mass spectrometer source parameters.

MS Source Parameters	Setting
Source	Ion Max source with HESI-II probe
Sheath Gas Pressure	45 psi
Auxiliary Gas Flow	12 arbitrary units
Probe Heater Temperature	350 °C
Source Voltage	3.5 kV
Capillary Temperature	350 °C
S-lens RF Voltage	60 V

Table 1B. MS method parameters.

Full MS Parameters	Setting	MS^2 Parameters	Setting
Full MS Mass Range	*m/z* 140–2000	Resolution Settings	15,000 (FWHM at *m/z* 200)
Resolution Settings	60,000 (FWHM at *m/z* 200)	Target Value	1.0×10^5
Target Value	3.0×10^6	Isolation Width	2.0 Da
Max Injection Time	100 ms	Signal Threshold	5.0×10^3
Default Charge State	2	Normalized Collision Energy (HCD)	27
SID	0 eV	Top-N MS^2	5
Microscans	2	Max Injection Time Fixed First Mass Dynamic Exclusion	200 ms *m/z* 140.0 10.0 s

Data processing

The data were acquired with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR4. Thermo Scientific™ BioPharma Finder™ software, version 1.0 SP1, was used for data analysis. The algorithm parameters defined in Table 2 were identical for all samples.

Table 2. BioPharma Finder parameter settings for all samples.

Component Detection	Setting	Variable Modifications	Setting
Absolute MS Signal Threshold	3.00 × 10^5 counts	N Terminal	Carbamylation Gln -> Pyro-Gln
Identification	**Setting**	C Terminal	Lys
Mass Accuracy	5 ppm	Side Chain	Carbamidomethylation (C) Carbamylation (K) Deamidation (N) Dimethylation (K) Double Oxidation (MWC) Glycation (K) Methylation (K) Oxidation (MWC)
Minimum Confidence	0.80		
Maximum Number of Modifications for a Peptide	2		
Unspecified Modification	-58 to +162 Da		
N-Glycosylation	CHO		
Protease Specificity	High		

Results and discussion

The SMART Digest kit provides fast and simple protein digestion with outstanding reproducibility, and digestion completeness for mAb samples is typically achieved within 45–60 min (Figure 2). Here, the relative standard deviation (RSD) was used to evaluate reproducibility, as demonstrated in Figure 3. Three separate digestions of the same mAb sample were conducted by three different analysts on different days. The peptide maps generated perfectly overlap with an average RSD for the peak area of less than 5%. These results impressively highlight the reproducibility that can be achieved when using this novel digestion technique in combination with the Vanquish Flex UHPLC system featuring SmartInject, the intelligent sample pre-compression technology for class-leading retention time reproducibility.

Comparing the total ion current (TIC) chromatograms of an in-solution-digested sample and a SMART Digest sample (Figure 4) shows the similarity of the two digestion methods. The 75 min time point was chosen to mirror the elongated incubation time of an overnight digest. In general, the peptide pattern is homogenous and most of the detected peptides are aligned. Differences in the two chromatograms and identified peptides are highlighted. For some peptides, the intensity slightly differs between the two SMART and in-solution digest runs, for example, peptides “1:103–107” and “2:87–98”. Others appear in only one of the two digestion methods, such as alkylated peptides “1:188–206 + alkyl”. The injection peak eluting with the void volume of the SMART Digest sample is higher in comparison to the

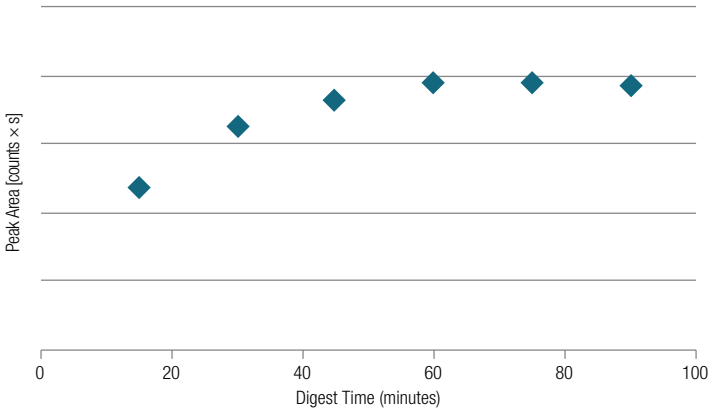


Figure 2. IgG digest profile, monitoring the mAb peptide VVSVLTVLHQDWLNGK for digestion times from 15–90 min using the SMART Digest kit.[2]

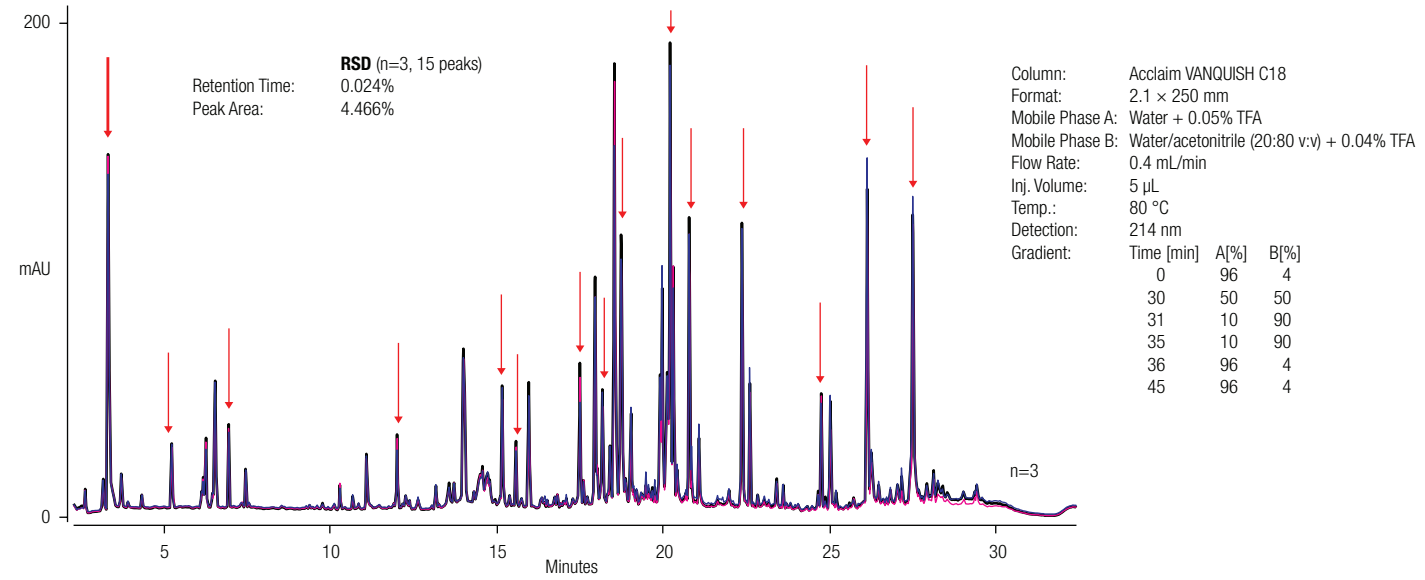


Figure 3. UV chromatogram overlay from three separate SMART digestions from the same mAb, conducted by three individual operators. The 15 marked peptides in each sample were used for inter-user/inter-day RSD value calculations.

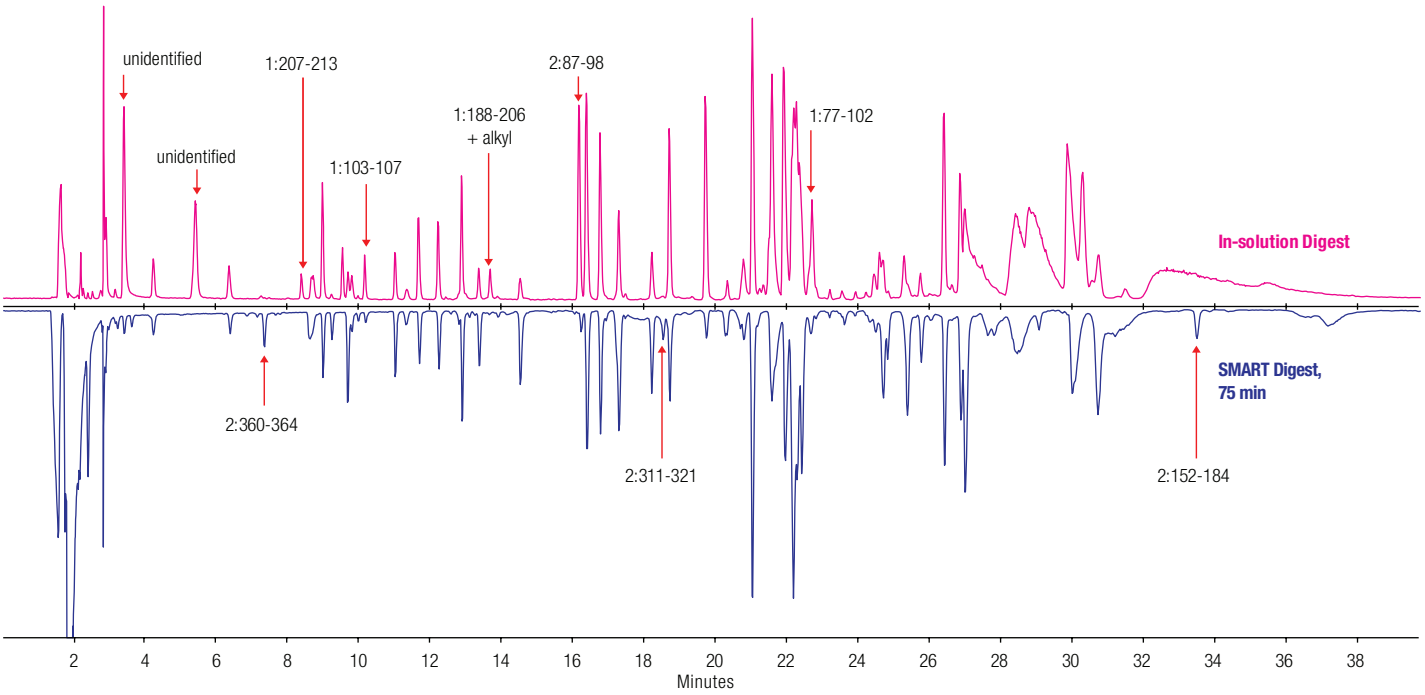


Figure 4. Mirror plot of the TIC chromatogram for the in-solution-digested sample denatured with heat (*In-Solution, Heat*) and the reduced SMART Digest sample (*SMART Digest, 75 min*). Peak labels give annotation to light (1) or heavy (2) chains, respectively, and sequence position.

in-solution-digested sample and is caused by salt components included in the SMART Digest buffer to optimize the digestion efficiency at elevated temperatures. This peak did not affect the result of the peptide map but could be easily removed if required. One option is to use a post-column diverter valve prior to the MS ion source. Another is to use Thermo Scientific™ SOLAµ™ SPE plates that allow highly reproducible post-digestion desalting of peptide samples by solid phase extraction (SPE).[3]

In peptide mapping analysis of mAbs, 100% sequence coverage for the heavy and light chains must be achieved. The sequence coverages for the different digest conditions are shown in Table 3. For all six methods, including the fast digestion methods of 15 and 30 min, 100% coverage was achieved for light as well as heavy chains. Strikingly, an incubation time of only 15 min is sufficient to achieve 100% sequence coverage for both the heavy and light chains of the antibody when the SMART Digest kit is used. The number of detected MS peaks in the samples digested with the SMART Digest kit were generally higher than in the in-solution digested samples. The same trend was observed when the number of identified components, including all peptides and charge states, and the total MS ion count were compared (Table 4).

Table 3. Sequence coverage with different digestion methods.

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Relative Abundance	Sample
1: Rituximab Light Chain	521	26%	100%	40%	*SMART Digest, 15 min*
	532	24%	100%	38%	*SMART Digest, 30 min*
	526	22%	100%	38%	*SMART Digest, 45 min*
	516	19%	100%	36%	*SMART Digest, 75 min*
	404	28%	100%	37%	*In-Solution, Urea*
	407	31%	100%	38%	*In-Solution, Heat*
2: Rituximab Heavy Chain	827	43%	100%	54%	*SMART Digest, 15 min*
	833	47%	100%	56%	*SMART Digest, 30 min*
	827	45%	100%	55%	*SMART Digest, 45 min*
	855	37%	100%	59%	*SMART Digest, 75 min*
	638	54%	100%	62%	*In-Solution, Urea*
	619	52%	100%	61%	*In-Solution, Heat*

Table 4. Number of identified components and TIC area for the different runs.

# Identified Components	Total MS area [counts × s]	Sample
1702	3.48×10^9	*SMART Digest, 15 min*
1678	4.12×10^9	*SMART Digest, 30 min*
1688	3.96×10^9	*SMART Digest, 45 min*
1551	3.13×10^9	*SMART Digest, 75 min*
1171	3.65×10^9	*In-Solution, Urea*
1145	4.04×10^9	*In-Solution, Heat*

Peptide mapping experiments can provide the identification, localization, and (relative) quantification of various post translational and chemical modifications (PTMs) that might be present on the amino acid residues. The relative abundance of all identified modifications (n=85) in the different runs are plotted in Figure 5. The relative abundance of the major modifications, including the pyro-glutamate formation (NH_3 loss) on the N-terminal glutamine of heavy as well as light chain and the most abundant glycoforms attached to the asparagine 301 of the heavy chain (A2G1F, A2G0F and A2G2F), are shown in Figure 5. Sixteen cysteine carbamidomethylation sites were exclusively identified in the samples derived from the in-solution-digested samples but not in the SMART Digest. This is consistent with the modification being caused by the alkylation with IAA during the sample preparation. For simplicity, the carbamidomethylation sites are not shown in Figure 5. Overall, similar levels for all modifications were detected for all digest protocols and no significant trend of an increased or decreased amount in any of the conditions tested was observed. Noteworthy, for many modification sites, e.g. deamidation of N319 and oxidation of W106, the amount in the reduced SMART Digest samples were lower compared to the in-solution-digested samples even when a 75 min (over-)digestion with the SMART Digest was applied.

The monoclonal antibody rituximab used in this study consists of 1328 amino acid residues including 16 disulfide bonds.[4] Amongst several potential PTMs of amino acids, deamidation of asparagine or glutamine and oxidation of methionine or tryptophan represent common chemical modifications for mAbs during downstream processing and storage. Figures 6A and 6B show the extent of amino acid oxidation, and deamidation, respectively, for oxidations for the different digestion methods. Table 5 summarizes the quantification results for the individual modification sites. The variance between the six digestion methods is expressed as the RSD of the measured relative abundance for each modification with each of the digestion protocols. With the exception of the oxidation of W106 that was high in the in-solution-digested samples, all results are comparable, resulting in RSD values ≤ 1%. For for the identified deamidations, the maximum RSD value was 3.164% and with an average RSD of 0.913%. While

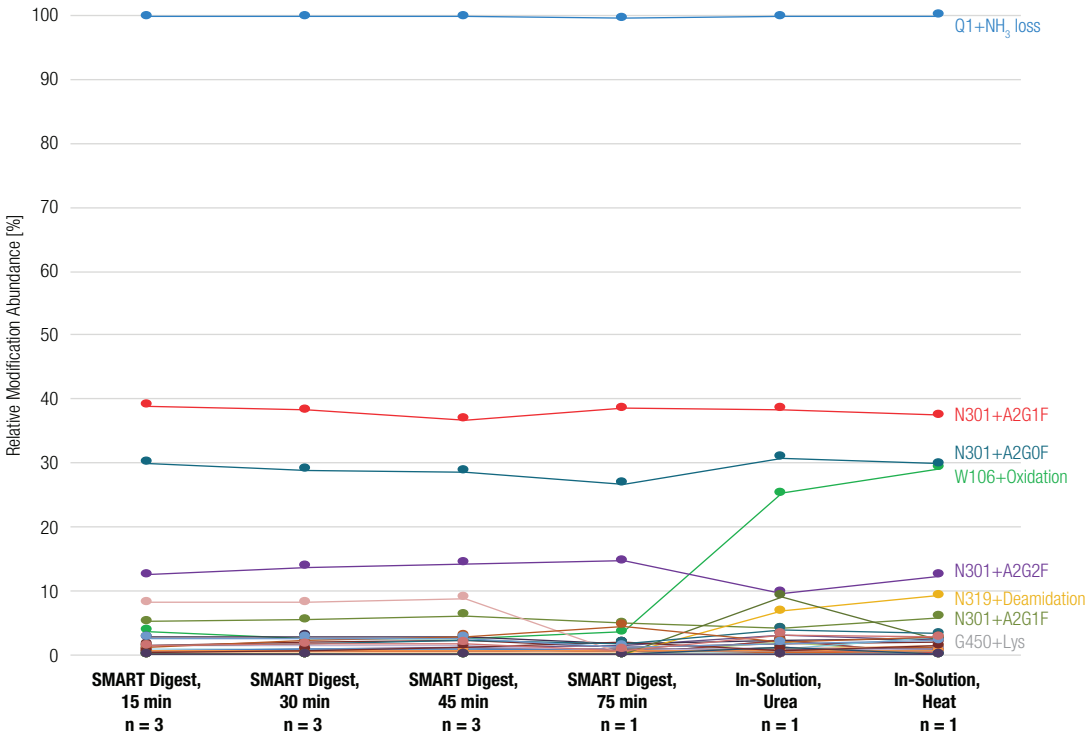


Figure 5. Relative abundance of 85 identified modifications including oxidation, double oxidation, glycation, glycosylation, NH_3 loss, isomerization, lysine truncation, methylation, dimethylation, and carbamylation.

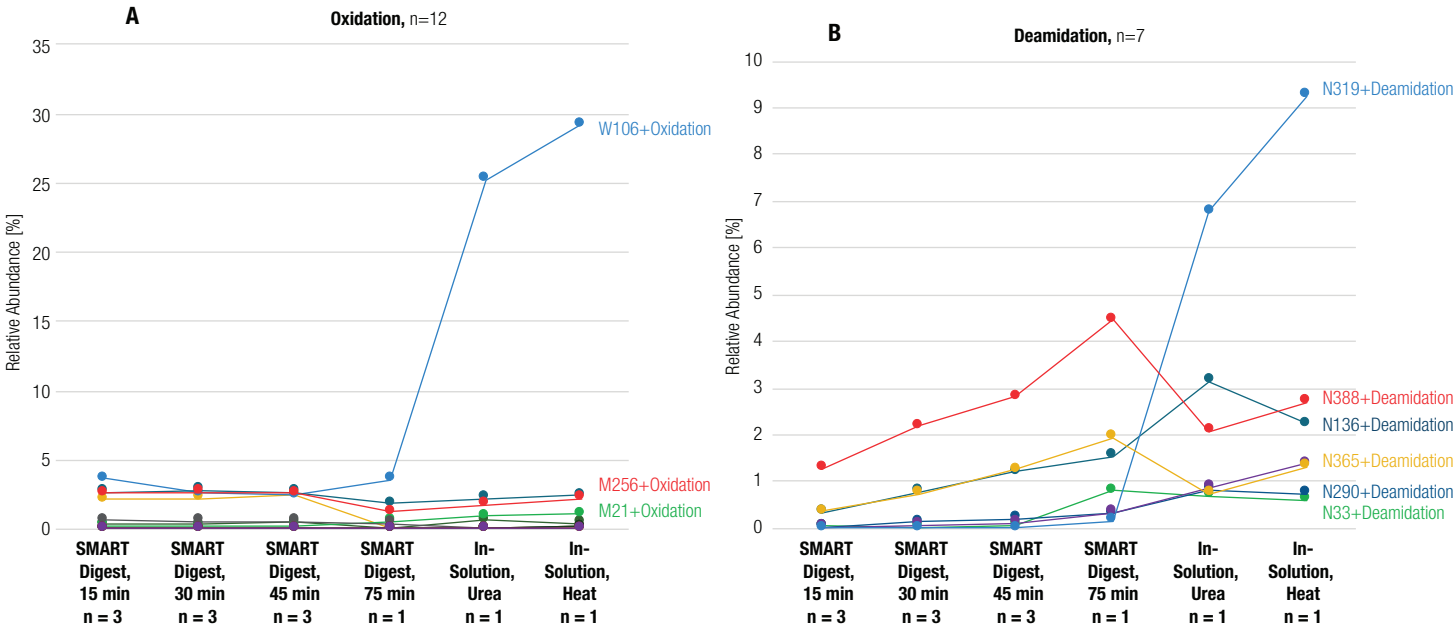


Figure 6. Relative abundance of 12 identified oxidations (A) and 7 deamidations (B) in different runs with various digestion methods.

a clear trend of increased deamidations with increasing sample incubation time could be observed between the six digestion methods (Figure 6B), less or equal amounts of deamidation were observed when the SMART Digest kit was used at the recommended digestion time of ≤ 45 min (Figure 6B and Figure 7). Only two deamidation sites (N236 and N388) were more prone to undergo deamidation under the SMART Digest conditions and required a reduced incubation time of 30 min. Another critical modification is the carbamylation of lysine residues and protein *N*-termini (+43.006 Da), which is a non-enzymatic PTM that has been related to protein aging.[5] It can be artificially introduced during sample preparation using urea as the protein denaturing agent. For in-solution tryptic digest with urea in

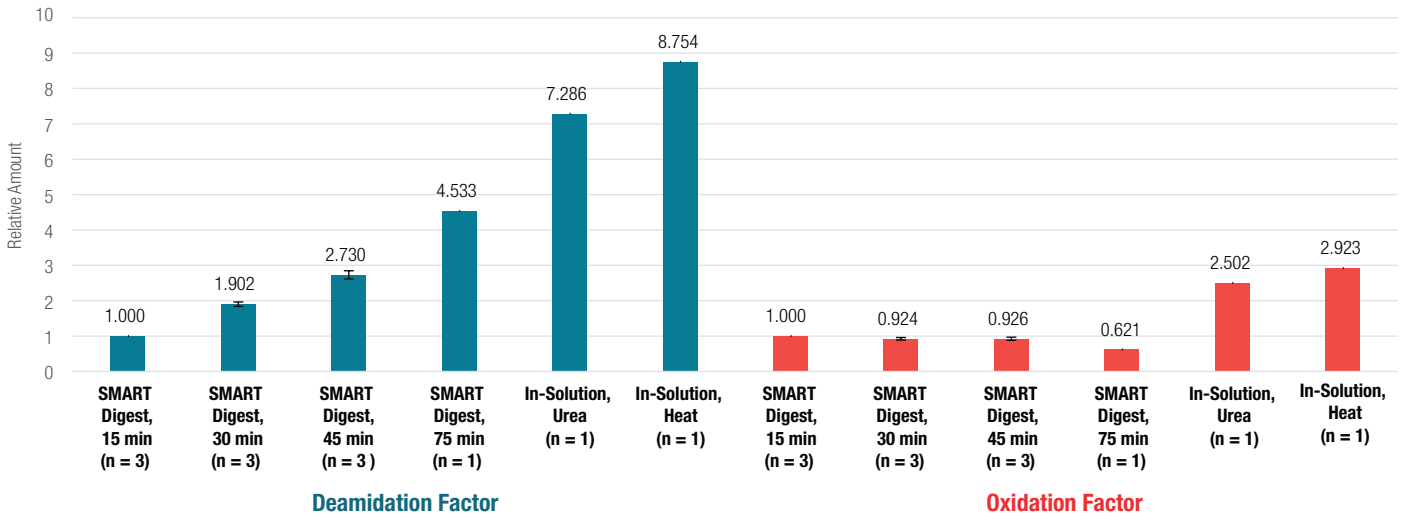


Figure 7. Relative amount of total deamidation and oxidation modifications measured for the six different digest conditions (Normalization to *SMART Digest, 15 min*).

the sample preparation, the average carbamylation of lysine was ≤ 1% relative abundance (n=6). For the SMART Digest samples, the average carbamylation was considerably lower in the ppt range or not detectable at all (Table 5). Other commonly targeted modifications such as the presence/absence of a C-terminal Lys, the *N*-glycosylation of asparagine on the heavy chain, the *N*-terminal pyro-glutamine formation on heavy and light chains, and lysine glycations are listed in Table 5. In total, six lysine glycations and 12 glycosylations of N301 could be identified and (relatively) quantified with an average RSD value of 0.423%.

Based on all identified oxidations (n=12) and deamidations (n=7), the deamidation and oxidation factor was calculated for each individual sample (Figure 7). The in-solution-digested sample with heat denaturation had the highest induced modification rate of the compared methods, with a deamidation factor of 8.754 and an oxidation factor of 2.923. In contrast, the SMART Digest samples that were reduced on peptide level showed the lowest levels of deamidation and oxidation compared to both in-solution-digestion samples. The degree of deamidation increases with extended digestion times, and the lowest deamidation rate was observed for the sample digested for 15 min using the SMART Digest kit. Deamidation is, in general, accelerated at high temperatures and high pH values.[6] One way to minimize the degree of induced deamidation is to lower the pH of the digestion buffer. SMART Digest is performed at elevated temperatures but at a pH of 7.2, which is much lower than the pH of classical in-solution-digestion methods. Thus, deamidation is minimized and is comparable to that observed for standard in-solution digests at 37 °C. Figure 5 also demonstrates that the extent of deamidation is location dependent. For some positions, lower levels of deamidation are observed for the SMART Digest, even when compared to the urea-treated in-solution digest (e.g. N33, N136, N319). For others, higher levels are observed with the SMART Digest and digestion times ≥ 45 min (e.g. N388).

Two of the tryptic peptides from rituximab have been identified as the most susceptible to deamidation under stress conditions.[7] The peptides 2:V306-K 321 (VVSVLTVLHQDWL**N**GK), containing N319, and 2:G375-K396 (GFYPSDIAVEWES**N**GQPENNYK), containing N388, are both located within the Fc region of the heavy chain, which shares the same sequence with other human or humanized mAbs. More than one asparagine is present in the sequences, but the asparagines highlighted in bold are identified as deamidation hot spots.[7] The second peptide is known as the “PENNY peptide”, but both peptides are a decent indicator for induced deamidation of mAbs.[7]

Table 5. Comparison of the oxidation, deamidation, and carbamylation modifications identified with the different digestion methods.

Relative Abundance [%]											
SMART Digest, 15 min (n=3)	SMART Digest, 15 min, RSD (n=3)	SMART Digest, 30 min (n=3)	SMART Digest, 30 min, RSD (n=3)	SMART Digest, 45 min (n=3)	SMART Digest, 45 min, RSD (n=3)	SMART Digest, 75 min (n=1)	In-Solution, Urea (n=1)	In-Solution, Heat (n=1)	RSD (%)*	Median (%)*	Modification
0.000	0.000	0.002	0.003	0.000	0.000	0.010	0.140	0.063	0.042	0.000	K63+Glycation
0.039	0.009	0.120	0.004	0.200	0.019	0.233	0.000	0.000	0.085	0.118	K102+Glycation
0.144	0.020	0.136	0.006	0.142	0.005	0.036	0.000	0.000	0.060	0.138	K137+Glycation
0.208	0.024	0.288	0.030	0.339	0.012	0.017	0.403	0.248	0.101	0.274	K148+Glycation
0.075	0.008	0.085	0.008	0.087	0.006	0.121	0.580	0.197	0.144	0.088	K168+Glycation
0.325	0.178	0.631	0.009	0.626	0.019	0.550	0.529	0.490	0.151	0.581	K182+Glycation
0.411	0.033	0.480	0.014	0.515	0.012	0.632	0.244	0.177	0.125	0.473	N301+A1G0
12.448	0.899	13.703	0.618	14.255	0.080	14.672	9.657	12.410	1.462	13.467	N301+A2G2F
5.141	0.373	5.476	0.186	6.166	0.148	4.852	4.088	5.777	0.642	5.458	N301+A1G1F
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.322	0.307	0.123	0.000	N301+A1S1F
0.703	0.050	0.776	0.035	0.796	0.029	0.928	0.895	0.880	0.080	0.781	N301+A2G0
30.052	2.351	28.838	1.471	28.667	0.971	26.689	30.825	29.838	1.652	29.229	N301+A2G0F
0.363	0.036	0.451	0.004	0.490	0.021	0.576	0.396	0.302	0.079	0.449	N301+A2G1
38.932	3.324	38.235	1.881	36.765	1.840	38.505	38.349	37.450	1.999	37.701	N301+A2G1F
1.386	0.055	1.496	0.067	1.435	0.047	0.714	1.093	0.739	0.288	1.404	N301+A2S1G1F
0.838	0.079	0.816	0.038	0.836	0.031	0.003	0.584	0.306	0.274	0.813	N301+A2S2F
0.278	0.005	0.302	0.030	0.291	0.030	0.268	0.000	0.000	0.114	0.274	N301+M4
0.753	0.068	0.994	0.104	0.966	0.088	0.577	0.728	0.489	0.188	0.847	N301+M5
96.850	0.066	96.802	0.208	96.946	0.486	96.788	97.655	97.886	0.428	96.815	Q1+Gln→Pyro-Glu
99.824	0.015	99.810	0.009	99.815	0.003	99.586	99.853	99.920	0.077	99.816	Q1+Gln→Pyro-Glu
1.348	0.537	1.685	0.036	1.778	0.045	0.673	3.083	2.684	0.667	1.735	G450+Lys
0.043	0.012	0.034	0.013	0.069	0.015	0.818	0.699	0.608	0.302	0.056	N33+Deamidation
0.334	0.069	0.778	0.054	1.213	0.027	1.545	3.159	2.233	0.842	1.014	~N136+Deamidation
0.035	0.008	0.132	0.004	0.219	0.004	0.321	0.823	0.734	0.261	0.175	~N137+Deamidation
0.034	0.005	0.070	0.037	0.115	0.020	0.343	0.879	1.399	0.428	0.103	N290+Deamidation
0.001	0.000	0.002	0.001	0.002	0.001	0.168	6.786	9.248	3.164	0.002	**N319+Deamidation**
0.368	0.038	0.747	0.019	1.257	0.045	1.951	0.738	1.314	0.486	0.757	N365+Deamidation
1.267	0.137	2.198	0.183	2.811	0.134	4.462	2.089	2.694	0.905	2.304	**N388+Deamidation**
2.177	0.040	2.211	0.125	2.522	0.059	0.065	0.000	0.001	1.043	2.200	M21+Oxidation
0.342	0.093	0.336	0.069	0.455	0.015	0.001	0.617	0.445	0.153	0.424	~M34+Oxidation
0.248	0.061	0.189	0.037	0.164	0.013	0.549	0.926	1.113	0.327	0.210	M81+Oxidation
3.654	0.683	2.560	0.348	2.435	0.152	3.556	25.225	29.209	9.505	3.064	W106+Oxidation
0.630	0.150	0.591	0.042	0.562	0.023	0.378	0.008	0.000	0.241	0.553	~W111+Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.034	0.199	0.057	0.000	C133+Double Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.179	0.051	0.000	C148+Double Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.032	0.192	0.055	0.000	C193+Double Oxidation
2.673	0.158	2.843	0.254	2.686	0.243	1.820	2.215	2.407	0.344	2.578	M256+Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.057	0.017	0.000	C265+Double Oxidation
0.016	0.004	0.021	0.002	0.033	0.002	0.068	0.000	0.000	0.018	0.020	C371+Double Oxidation
2.591	0.179	2.646	0.188	2.558	0.029	1.218	1.760	2.243	0.460	2.545	M432+Oxidation
0.000	0.000	0.000	0.000	0.001	0.001	0.000	2.192	0.000	0.633	0.000	~K38+Carbamylation
0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.087	0.000	0.025	0.000	K38+Carbamylation
0.124	0.008	0.190	0.011	0.232	0.016	0.001	0.000	0.000	0.092	0.155	K102+Carbamylation
0.003	0.003	0.003	0.002	0.007	0.004	0.004	0.900	0.019	0.258	0.005	K278+Carbamylation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.202	0.000	0.058	0.000	K321+Carbamylation
0.000	0.000	0.001	0.000	0.001	0.001	0.000	1.254	0.000	0.362	0.001	K338+Carbamylation

Between 6 digestion methods

Figure 8 shows the TIC chromatogram for the SMART Digest sample (Figure 8A) and extracted ion current (XIC) chromatograms with a 5 ppm mass extraction window for the different samples (Figure 8B). The XIC traces in blue are derived from the native 2:V306-K321 peptide present in all runs. The traces in red are the corresponding deamidated forms of the peptide (N319) eluting prior to the native peptide in the chromatogram. The relative abundance, based on all charge states of the deamidated peptide, is lowest in the 15 min digested SMART Digest sample at 0.001%. In contrast, a higher amount of deamidation (N388) was observed with the SMART Digest (45 and 75 min digestion time) for the PENNY peptide 2:G375-K396 (Table 5), but the lowest value of 1.267% could be observed with the 15 min method.

As shown in Figure 8C, the isotopic distribution of the triply charged native peptide is different from its deamidated form. The monoisotopic peak is highlighted in bold and, due to coelution of the two species, the monoisotopic peak (*; *m/z* 603.340) of the native peptide is also visible in the lower mass spectrum. A deamidation leads to a theoretical mass increase for the monoisotopic peak of 0.984 Da, which results in a mass shift of 0.328 Da for the triply charged signal and nicely correlates with the measured value.

Conclusion

The direct comparison of the SMART Digest kit with the conventional in-solution protein digestion methods conducted in this study showed no substantial difference for the mAb rituximab between the different approaches with respect to the data quality and information content obtained. Protein sequence coverage of 100% for rituximab was achieved with all six digestion methods tested and could be achieved in only 15 min when using the SMART Digest kit. The most common PTMs targeted for analysis, such as the presence/absence of a C-terminal Lys, the *N*-glycosylation of asparagine on the heavy chain, and the *N*-terminal pyro-glutamine formation on heavy and light chains, were identified, relatively quantified, and compared between the different digestion methods. Overall, the

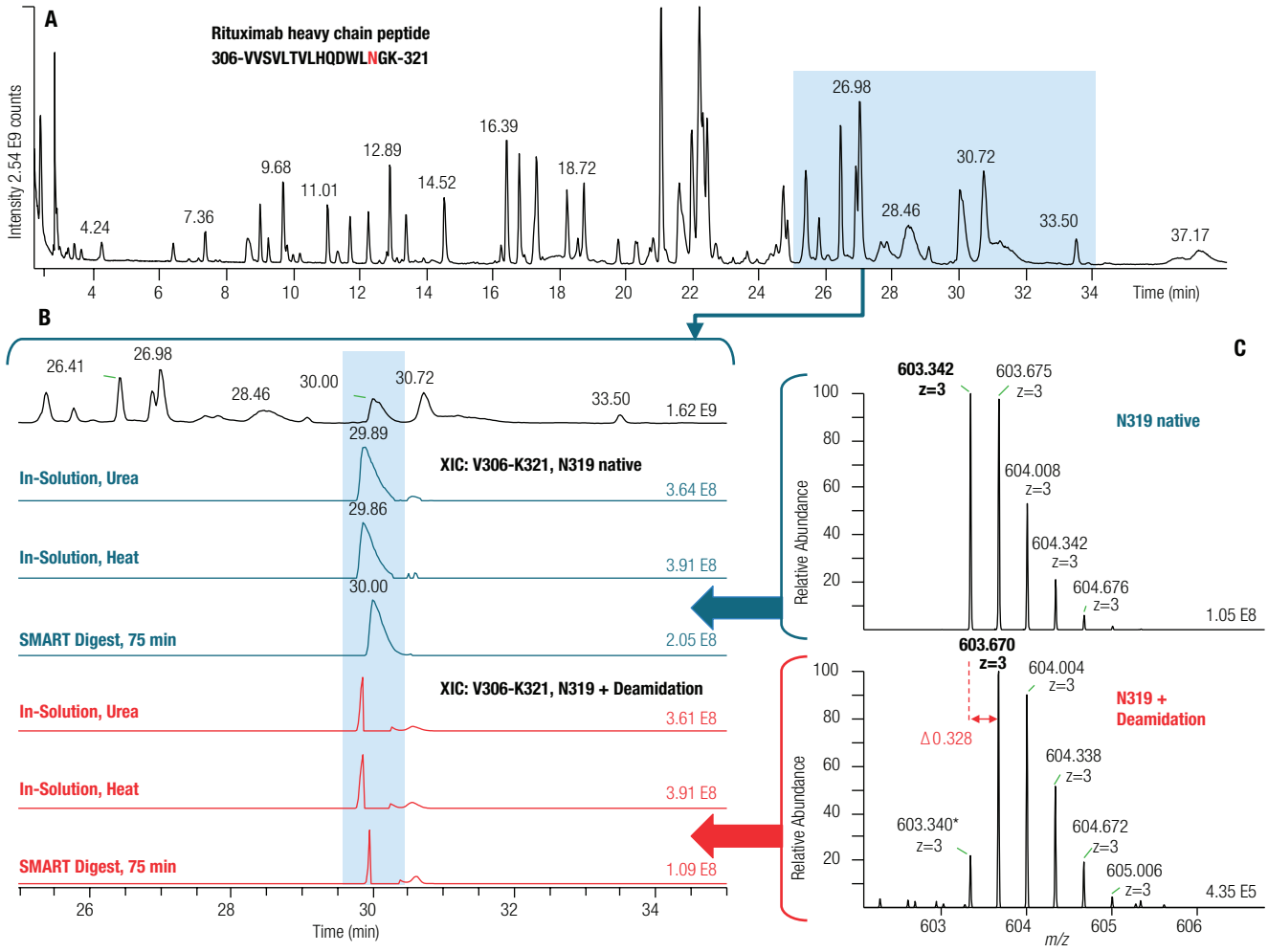


Figure 8. Total ion current chromatogram of the reduced SMART Digest sample, 75 min (A), and extracted ion current chromatograms (B) for the peptide V306-K321 in the native and the deamidated form for the different runs. A comparison of the isotopic distributions of the $[M+3H]^{3+}$ ions (C) for the native and deamidated V306-K321 peptide.

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extent of chemical modifications detected was similar for all digestion methods. The elevated temperatures during enzymatic digestion using the SMART Digest kit did not increase the amount of induced deamidation compared to in-solution-digested samples. In fact, the calculated deamidation (and oxidation) factors were lower or identical to the urea-treated samples, and heat-denaturation combined with in-solution digestion resulted in slightly increased modification levels. Optimization of the incubation time can be used to further minimize the introduction of chemical modification during digestion using the SMART Digest kit. For Rituximab,a digestion time of 15 min is feasible and results in complete sequence coverage and accurate relative quantification of PTMs. In contrast, prolonged digestion times > 45 min can increase the amount of chemical modifications. Interestingly, some positions were more prone to undergo deamidation in one condition compared to the others, but no correlation with a specific digest condition was seen. Since the use of urea is omitted during the SMART Digest, lysine carbamylation was virtually absent in SMART Digest and urea-treated samples. This contributed to a less complex but comprehensive peptide map.

The huge time-saving potential, ease of use, and outstanding reproducibility of the SMART Digest make it the heart of a comprehensive peptide mapping workflow as applied in this study. When combined with the Vanquish Flex UHPLC system, Orbitrap-based mass spectrometer, and the simple yet powerful tools within Chromeleon and BioPharma Finder software, SMART Digest kit facilitates standardized, fast, and reproducible peptide mapping workflows.

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Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments

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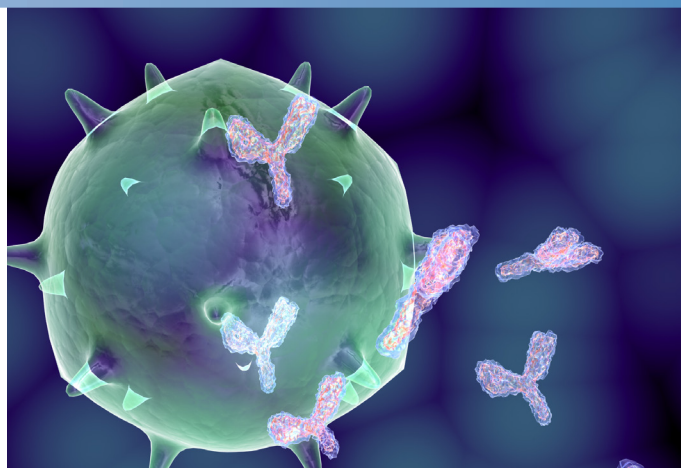
Application Note 1124

Key Words
Acclaim C18 RSLC, Biocompatible UHPLC, Biopharma, Biotherapeutics Characterization, Monoclonal Antibodies, Protein Digest, Vanquish UHPLC System

Goal

Provide an ultra-high retention time and peak area precision example of the separation of a mAb digest.



Introduction

Peptide mapping of digested proteins are of high importance when characterizing biotherapeutics. Peptide maps are utilized to confirm the expression of the intended amino acid sequence, to confirm genetic stability or to identify post-translational modifications, especially when interfaced with mass spectrometry. Reversed phase separation in combination with only UV detection is, however, still very common in stability studies, for in process measurements and quality assurance. In these cases peak areas, peak area ratio and retention times are sufficient to provide the required information. For highest confidence in the qualitative and quantitative results of such assays, the retention time as well as the peak area has to be extremely stable.

The Thermo Scientific™ Vanquish™ UHPLC system features a binary pump with extremely low pulsation ripple due to a brand new pump concept. In addition, the Vanquish UHPLC system pre-compresses the sample prior to the injection which results in a highly stable flow delivery. Thanks to these benefits, the Vanquish UHPLC system is capable of providing unmatched retention time precision. This retention time precision accompanied with a high peak area precision guarantees the analytical success for even challenging shallow gradient separations by a reliable peptide identification and quantification. In this work, the separation of peptides obtained from a therapeutic protein is provided. The retention time and peak area precision is evaluated for repeated injections.

Equipment

Vanquish UHPLC system consisting of:

- Binary Pump H (P/N VH-A10-A)
- System Base (P/N VH-S01-A)
- Mixer Kit, 200 µL, VH-P1 (6268.5120)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active pre-heater (6732.0110)
- Post column cooler, 1 µL (6732.0510)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe™ flow cell, standard (10 mm; P/N 6083.0100)

Thermo Scientific™ Dionex™ Chromeleon™ Chromatogaphy Data System (CDS) software, version 7.2

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Protein Digestion

SMART Digest Kit (P/N 60109-101)

Experimental

Sample Preparation

1. Cetuximab® monoclonal antibody (5 mg /mL) was diluted 1:4 with the SMART Digest buffer to a final volume of 100 µL
2. The diluted sample was then added to a SMART Digest tube and left for 60 minutes at 70 °C
3. The digested sample was then centrifuged at 10,000g for 5 minutes and the supernatant was removed for chromatographic analysis

Conditions

Column:	Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm Analytical (2.1 × 250 mm), P/N 074812
Mobile Phase:	A: 0.05% TFA in water, P/N TFA 85183 B: 0.04% TFA in 8/2 acetonitrile/water (v/v), P/N acetonitrile TS-51101
Gradient:	0–30 min: 4–50% B, 30-31 min: 50–90% B, 31–35 min: 90% B, 35–36 min: 90–4% B, 36–45 min: 4% B
Flow Rate:	0.4 mL/min
Maximal Pressure:	384 bar
Temperature:	80 °C; Forced Air Mode
Injection Volume:	5 µL
Detection:	214 nm Data Collection Rate: 20 Hz Response Time 0.2 sec
Flow Cell:	10 mm LightPipe™

Results and Discussion

The digestion was achieved utilizing the SMART digest kit. Using this approach the sample preparation time could be reduced significantly and total preparation time of the monoclonal antibody (mAb) digest was lower than 75 minutes.

The separation of the resulting peptides was obtained with a 30 minutes gradient, and a total analysis time of 45 minutes, including column wash with high organic eluent, and re-equilibration at initial conditions. Figure 1 shows the overlay of 13 consecutive injections of the same sample of mAb digest.

The results show excellent reproducibility across the whole chromatogram. On average, standard deviation (SD) was of the order of 0.13 seconds (0.00214 minutes). SD for some peaks was as low as 0.065 seconds; and did not exceed 0.3 seconds for any peptide.

The relative standard deviation was consistently extremely low (Figure 2). Out of 110 peaks automatically integrated by Chromeleon CDS, 34 had RSD smaller than 0.0100%, reaching the minimum value of 0.0060% for the peak at retention time 23.057 minutes. Please note that the early eluting peaks naturally have the highest retention time RSDs because of a mathematical disadvantage in the calculation.

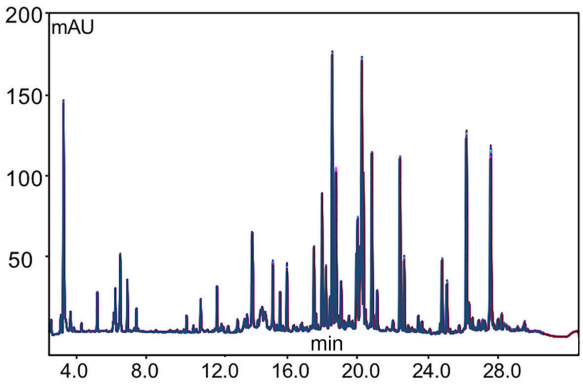

Figure 1. Overlaid chromatogram of 13 repeated injections of the mAb tryptic digest.

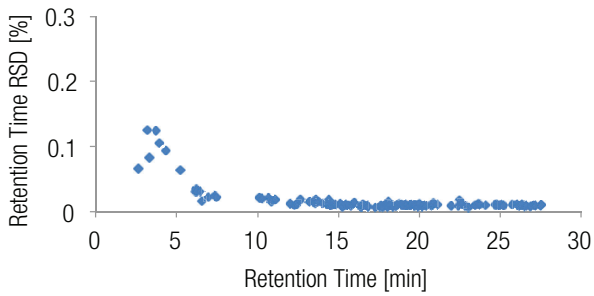

Figure 2. Retention Time RSD (%) relative standard deviation measured for 13 repeated injections of a mAb digest.

In addition, Table 1 gives the peak area reducibility as relative standard deviation.

The relative standard deviation of the peak areas was below 1.0% for all peptides. The average reproducibility was 0.4% highlighting the highly reliable sample injection and peak integration at challenging conditions.

Conclusion

Stability of retention time and peak areas is critical for a confident evaluation of chromatographic results and to avoid any misinterpretation. The Vanquish UHPLC system is extremely reproducible in both retention time and peak area reproducibility. The retention time precision provided by the system enables the analyst to deduce any change in retention time to an actual change of the sample structure. As shown, the peak area reproducibility provided by Vanquish will result in a maximal confidence of quantitative result. Consequently, the Vanquish system meets the requirements of demanding peptide mapping analysis.

Table 1. Peak area of six selected peaks eluting over the entire gradient and spanning a wide concentration range.

Retention Time (min)	Average Area (mAU*min)	RSD Area (%)
5.23	1.04	0.10
10.29	0.36	0.22
13.07	0.05	0.94
15.96	0.96	0.49
22.39	5.17	0.14
24.68	0.25	0.61

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400 650 5118
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High-Throughput Peptide Mapping with the Vanquish UHPLC System and the Q Exactive HF Mass Spectrometer

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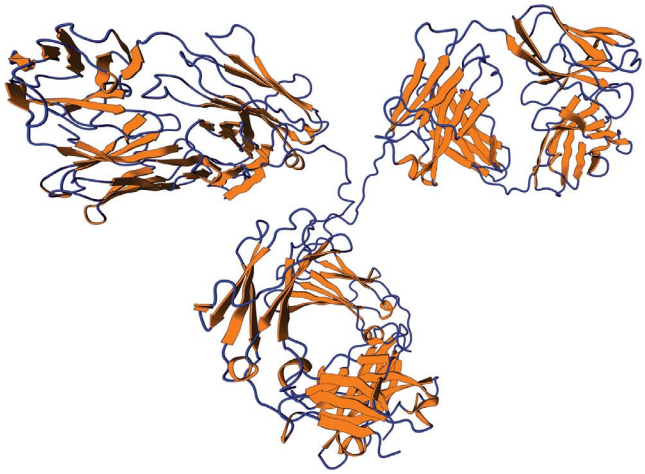
Application Note 1135

Key Words
Monoclonal Antibodies, Acclaim C18 RSLC Column, Q Exactive HF Mass Spectrometer, Biocompatible UHPLC, Biotherapeutics Characterization, Biopharma

Goal
Report on the benefits of a fast analytical platform employing highly efficient chromatography in combination with fast and high-resolution quadrupole Thermo Scientific™ Orbitrap™ mass spectrometry technology as a tool for fast identification and quantification of sequence truncations, glycosylation and post-translational or artificial modification of recombinant monoclonal antibodies.



Introduction
Monoclonal antibodies, or mAbs, (Figures 1 and 2) are the major element in the fastest growing sector of biopharmaceuticals within the pharma industry. By 2016, eight of the top ten drugs will be therapeutic proteins. Their manufacture is accomplished in bacterial or eukaryotic expression systems, requiring extensive purification of the target product. During drug development and production, the quality of biotherapeutics needs to be closely monitored.

Various analytical methods have been used to study quality attributes such as structural integrity, aggregation, glycosylation pattern or amino acid degradation. Because of their high information content and versatility, characterization methods based on high-performance liquid chromatography and mass spectrometry are among the most powerful protein characterization techniques. Proteins can be enzymatically digested to obtain peptides enabling their analysis by means of peptide mapping experiments.

Here, we report a fast and sensitive approach that combines enzymatic digestion, fast chromatographic separation, high-resolution mass spectrometry, and rapid data processing to handle the large amount of samples in diverse biopharma workflows. In this study we have analyzed two commercially available drug products: rituximab (trade names MabThera and Rituxan®) and denosumab (trade names Prolia® and XGEVA®).

Experimental
The two drug products rituximab and denosumab were denatured for 30 min in 7 M urea and 50 mM Tris HCl at pH 8.0. The samples were reduced with 5 mM DTT for 30 min at 37 °C, alkylation was performed with 10 mM IAA for 30 min at room temperature, and the reaction was quenched by addition of 10 mM DTT. Thermo Scientific™ Pierce™ Trypsin Protease (MS Grade) was added and digestion allowed to proceed overnight at 37 °C. Digests were stopped by addition of TFA to approximately pH 3.0.

Abbreviations			
ACN:	Acetonitrile	mAb:	Monoclonal antibody
DTT:	Dithiothreitol	PTMs:	Post translational modifications
FA:	Formic acid	TFA:	Trifluoroacetic acid
IAA:	Iodoacetamide		

Thermo SCIENTIFIC

A Thermo Scientific™ Vanquish™ UHPLC system with a 2.1 x 250 mm i.d. Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm column and gradients of water and acetonitrile (ACN) with 0.1% formic acid (FA) each were used to separate the peptide mixtures. Five different separation times were applied and compared: 5, 8, 13, 20, and 30 min for the gradient ramping from 4% to 55% eluent B (0.1% FA in 8:2 acetonitrile/water (v/v)). Flow rates were adapted accordingly using 1.1 (5 min), 1.0 (8 min), 0.6 (13 min), 0.4 (20 min), and 0.4 mL/min (30 min). The Thermo Scientific™ Q Exactive™ HF mass spectrometer (MS) equipped with a HESI-II probe was used for mass spectrometric detection.

Equipment

Vanquish UHPLC system consisting of:

- System Base (P/N VH-S01-A)
- Binary Pump H (P/N VH-A10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer

Experimental Conditions - HPLC	
Column	Acclaim RSLC 120, C18, 2.2 µm, Analytical (2.1 x 250 mm, P/N 074812)
Mobile Phase	A: 0.1% FA in water (P/N FA 28905) B: 0.1% FA in 8/2 acetonitrile/water (v/v) (P/N acetonitrile TS-51101)
Gradient 1	0–30 min: 4–55% B, 30–30.1 min: 55–100% B, 30.1–35 min: 100% B, 35.0–35.1 min: 100–4% B, 35.1–40 min: 4% B
Gradient 2	0–20 min: 4–55% B, 20–20.1 min: 55–100% B, 20.1–25 min: 100% B, 25–25.1 min: 100–4% B, 25.1–30 min: 4% B
Gradient 3	0–13 min: 4–55% B, 13–13.1 min: 55–100% B, 13.1–16 min: 100% B, 16–16.1 min: 100–4% B, 16.1–30 min: 4% B
Gradient 4	0–8 min: 4–55% B, 8–8.1 min: 55–100% B, 8.1–10 min: 100% B, 10–10.1 min: 100–4% B, 10.1–12 min: 4% B
Gradient 5	0–5 min: 4–55% B, 5–5.1 min: 55–100% B, 5.1–7 min: 100% B, 7–7.1 min: 100–4% B, 7.1–9 min: 4% B
Flow Rate	Gradient 1: 0.4 mL/min Gradient 2: 0.4 mL/min Gradient 3: 0.6 mL/min Gradient 4: 1.0 mL/min Gradient 5: 1.1 mL/min
Temperature	60 °C
Injection Volume	2 µL
Detection	214 nm Data Collection Rate: 10 Hz Response Time 0.4 s
Flow Cell	10 mm LightPipe

Experimental Conditions - MS				
Source	HESI-II			
HPLC	0.4	0.6	1.0/1.1	mL/min
Sheath Gas Pressure	25	60	75	psi
Auxiliary Gas Flow	10	20	30	arb units
Capillary Temperature	320 °C			
Probe Heater Temperature	350 °C			
S-lens RF Voltage	60 V			
Source Voltage	3.5 kV			

Full MS Parameters		MS² Parameters	
Full MS Mass Range	200–2000 *m/z*	Resolution Setting	15,000
Resolution Setting	60,000	Target Value	1e5
Target Value	3e6	Isolation Width	2.0 Da
Max Injection Time	100 ms	Signal Threshold	1e4
Default Charge State	2	Normalized Collision Energy (HCD)	27
SID	0 eV	TopN MS²	5
		Max Injection Time	100 ms
		Fixed First Mass	140.0 *m/z*
		Dynamic Exclusion (s)	10.0

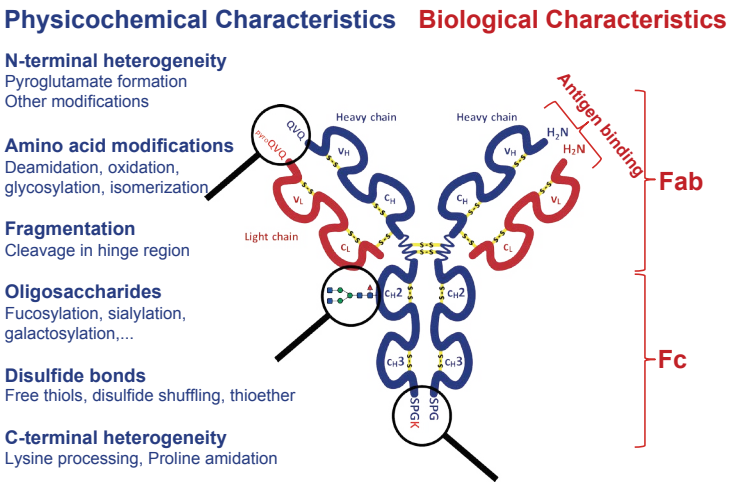


Figure 1. General structure of mAbs and their biological and physicochemical characteristics.

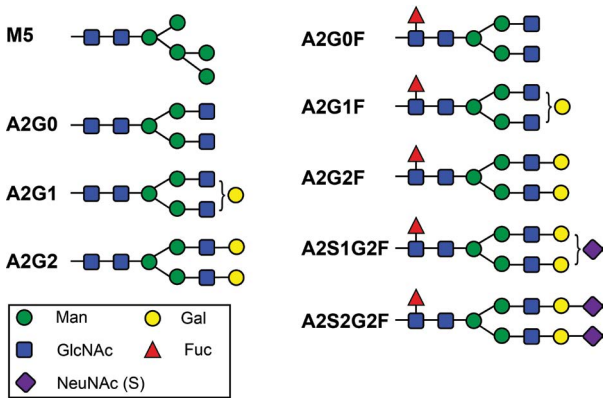


Figure 2. Nomenclature of carbohydrate structures commonly observed on antibodies.

Data Analysis

The data were acquired with Thermo Scientific™ Xcalibur™ 3.0 software in combination with Thermo Scientific SII for Xcalibur 1.1 software. Data analysis was performed using Thermo Scientific™ PepFinder™ 2.0 and FreeStyle™ 1.0 software packages.

Results and Discussion

Peptide mapping experiments were performed using the rituximab digest for assessing the sequence coverage for light and heavy chain, as well as for identification and (relative) quantification of a specific set of modifications: a) oxidation, b) glycosylation and c) deamidation.[1,2] For all five gradient times from 30 min down to 5 min, a very good separation was achieved (Figure 3) and resulting sequence coverages of 100% were obtained from all separation times both for light and heavy chain, even for the very short gradient of 5 min. The sequence coverage map (Figure 4) shows the overlap of the different peptides identified in different intensities and in different lengths due to missed cleavages. Table 1 shows the identification and comparison of a subset of monitored modifications across the different separation times applied. A tilde (~) before the modification indicates the modification was found on the tryptic peptide, but could not be localized on a specific amino acid with MS/MS spectra. The modification is labeled with recovery "Good" when the total peak area, including modified and unmodified forms of the peptide, is at least 10% of the most abundant peptide from the same protein. The recovery "Fair" means it is at least 1%. The relative abundance of the detected modifications in the five different methods has a standard deviation of 0.19% and shows that important information regarding post-translational modifications (PTMs) can be obtained equally and accurately at all separation times.

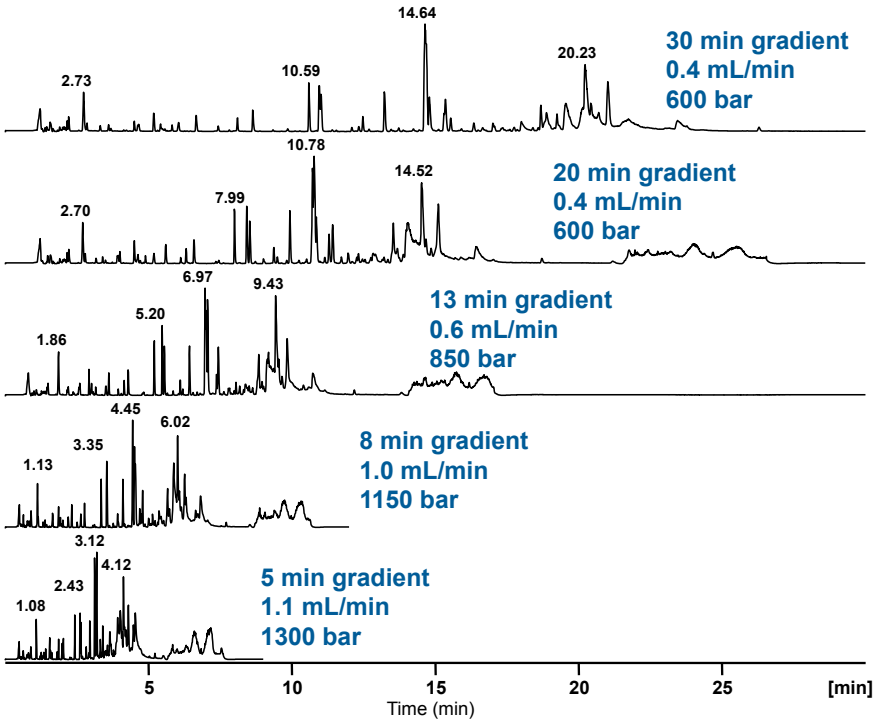


Figure 3. Total ion chromatograms obtained from peptide mapping experiments of rituximab applying gradient lengths of 30, 20, 13, 8, and 5 min. Flow rates and resulting pressures are indicated in the individual traces.

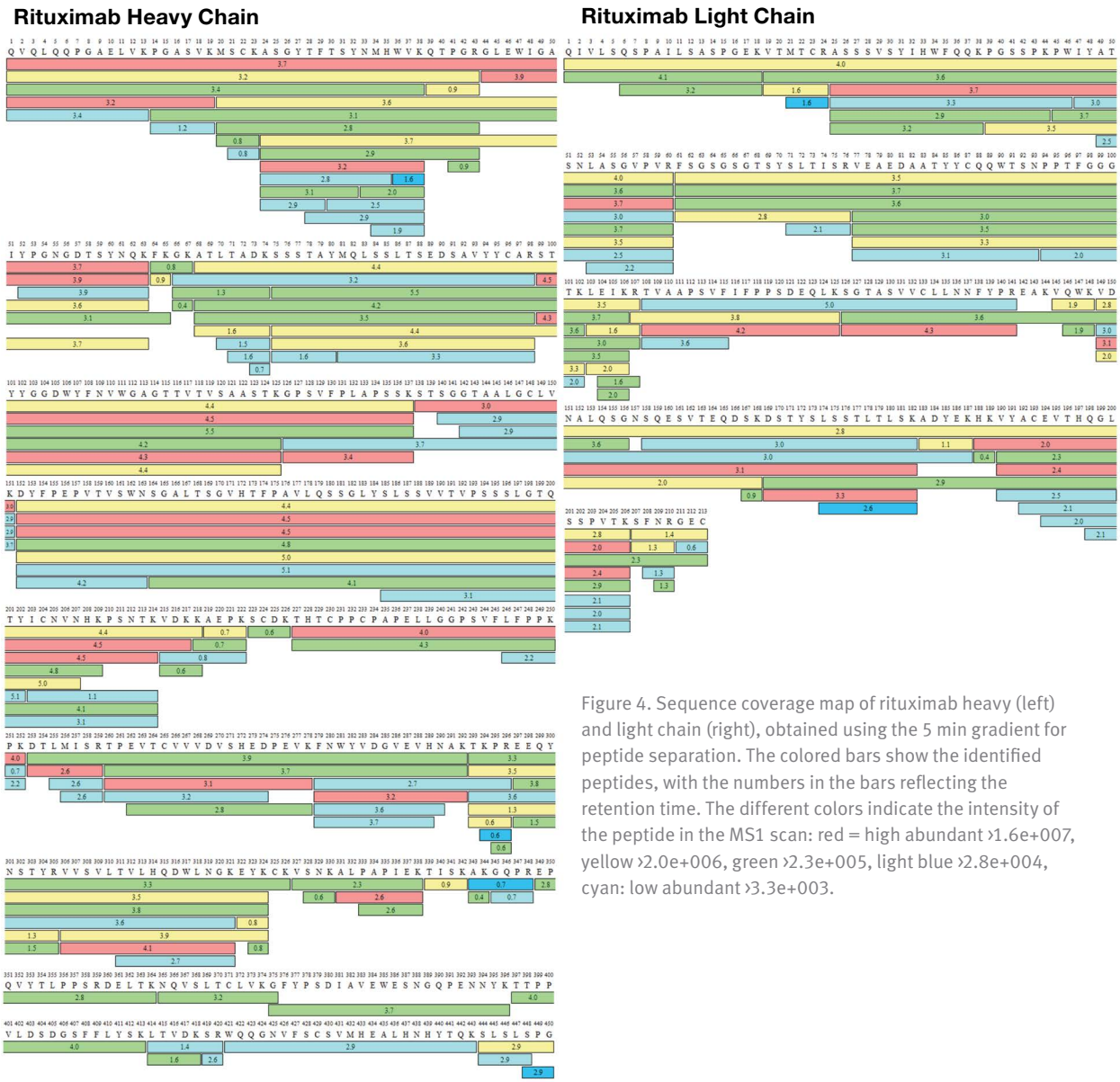


Figure 4. Sequence coverage map of rituximab heavy (left) and light chain (right), obtained using the 5 min gradient for peptide separation. The colored bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the intensity of the peptide in the MS1 scan: red = high abundant ›1.6e+007, yellow ›2.0e+006, green ›2.3e+005, light blue ›2.8e+004, cyan: low abundant ›3.3e+003.

Table 1. Comparison of the oxidation, deamidation, and glycosylation modifications identified in runs obtained from the different gradient times.

Protein Chain	Modification	Recovery	Gradient Time					σ
			30 min	20 min	13 min	8 min	5 min	
			Abun.	Abun.	Abun.	Abun.	Abun.	
Rituximab_LC	Q1+NH_3 loss	Good	91.95%	91.17%	89.69%	90.93%	26.57%	28.80%
Rituximab_HC	~Q1+NH_3 loss	Good	99.62%	99.67%	99.61%	99.68%	99.69%	0.04%
Rituximab_HC	N33+Deamidation	Good	0.52%	0.51%	0.58%	-	0.51%	0.03%
Rituximab_HC	M34+Oxidation	Good	1.64%	1.54%	1.73%	1.42%	1.45%	0.13%
Rituximab_HC	N301+A1G0F	Fair	4.32%	4.42%	3.83%	3.52%	3.38%	0.46%
Rituximab_HC	N301+A1G1F	Good	1.87%	1.91%	1.72%	3.32%	1.46%	0.73%
Rituximab_HC	N301+A2G0	Good	1.09%	1.02%	1.02%	-	0.98%	0.05%
Rituximab_HC	N301+A2G0F	Good	37.88%	37.11%	38.59%	40.48%	43.12%	2.41%
Rituximab_HC	N301+A2G1F	Good	42.06%	41.89%	43.42%	43.20%	43.35%	0.75%
Rituximab_HC	N301+A2G2F	Good	10.23%	10.17%	9.81%	10.36%	10.05%	0.21%
Rituximab_HC	N301+A2S1G0F	Fair	0.83%	0.86%	-	-	-	0.02%
Rituximab_HC	N301+A2S1G1F	Fair	2.14%	-	-	-	-	-
Rituximab_HC	N301+A3Sg1G0	Fair	1.30%	-	-	-	-	-
Rituximab_HC	N301+M5	Good	1.61%	1.59%	1.66%	1.87%	1.86%	0.14%
Rituximab_HC	N301+Unglycos.	Good	0.54%	0.90%	0.76%	0.83%	0.97%	0.16%
Rituximab_HC	G450+Lys	Fair	3.57%	3.56%	3.92%	3.40%	3.15%	0.28%
							Median 0.19%	

Since the quantification of modified peptides performed in PepFinder 2.0 software requires their identification based on MS/MS spectra, special care has to be taken in choosing the appropriate ion injection times in the method setup enabling the acquisition of high quality MS/MS spectra required for a positive identification. This is especially true for glycopeptides analyzed using HCD fragment ion spectra, which contain exclusively ions representing sequential loss of glycan residues and no fragment ions representing the decomposition of the peptide as shown in Figure 5. The identification of peptides and modified peptides using PepFinder 2.0 software is based on the comparison of a simulated and the measured spectrum. The strength of the implemented algorithm for spectra and especially fragment ion intensity prediction is displayed in Figure 5 showing the MS/MS spectrum of the low abundant glycosylated peptide TKPREEQFN*STFR (*=A2G0) identified in the 5 min run with the typical fragmentation pattern: the two oxonium ions 204 (HexNAc), 366 (Hex-Hex-NAc), and the sequence ladder of the fragmented glycan attached to the intact peptide. The precursor ion of this glycopeptide with 735.58 *m/z* and a +4 charge state is indicated with the dotted blue line in the measured MS/MS spectrum.

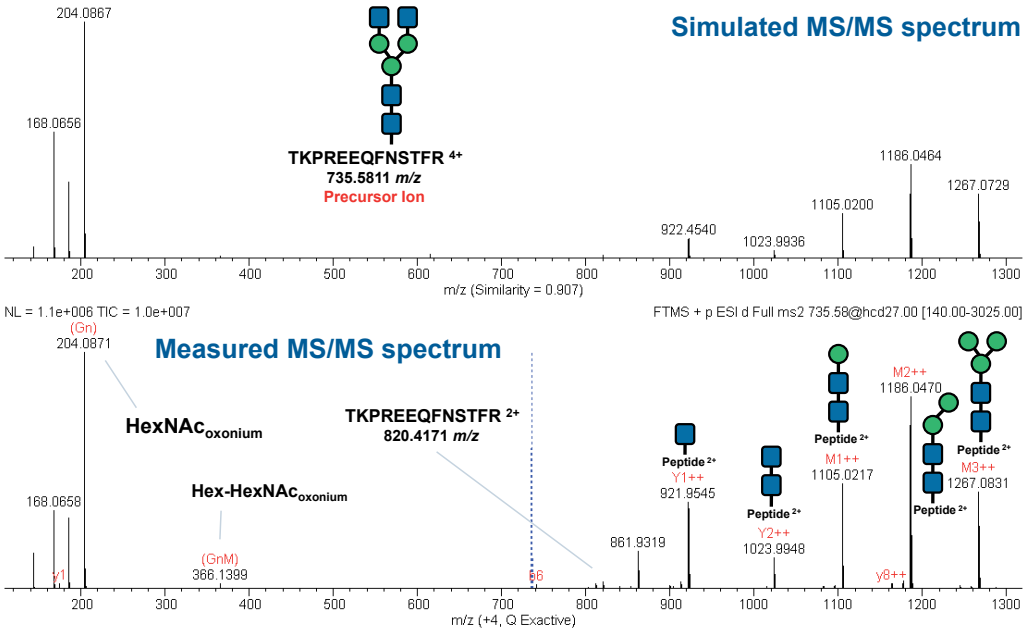


Figure 5. Simulated (top) and measured (bottom) MS/MS spectra of the glycopeptide aa 290-302 (TKPREEQFN*STFR, *=A2G0) in the 5 min gradient run.

Even with ultra-short gradients down to 5 min, as shown in Figure 6A, spectacular separation efficiency with average peak width at half maximum ($w_{1/2}$) of 1.41 s were obtained. Figure 6B highlights the number of scans obtained across one chromatographic peak. Typically six Full MS spectra and 25 (5 x Top5) MS/MS spectra were acquired over a chromatographic peak of 2.4 s width. The achieved scan speed is key to the success in obtaining full sequence coverage.

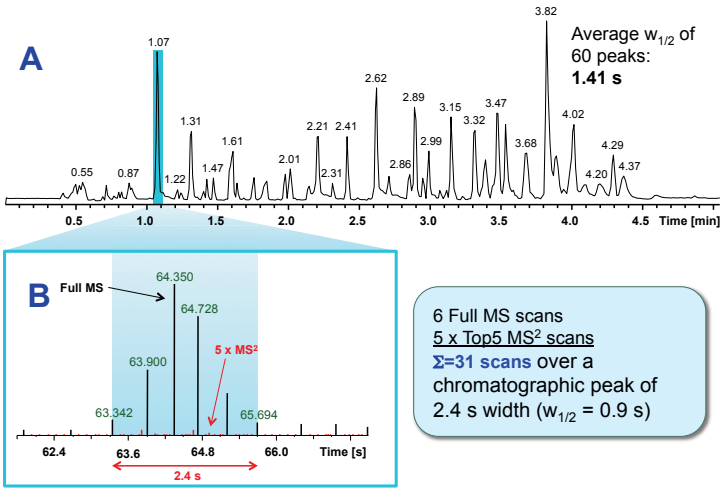


Figure 6. A) Total ion chromatogram of a five minute gradient separation of denosumab and B) data point distribution for a Full MS / ddMS^2 Top5 method during a representative chromatographic peak.

Conclusion

- The applied hardware setup chosen for the experiments consisting of the Vanquish UHPLC system with an Acclaim RSLC 120 2.1 x 250 mm column installed with Thermo Scientific™ Viper™ Fingertight Fitting connections, attached online to the Q Exactive HF mass spectrometer equipped with the HESI-II source, provides a very robust system allowing for very high reproducibility and long term stability.
- Flow rates between 0.4 and 1.1 mL/min, depending on the chosen gradient lengths between 5 and 30 min, delivered chromatograms with peak widths at half maximum of less than 1 second.
- The accelerated scan speed of the Q Exactive HF mass spectrometer delivered sufficient data points over a chromatographic peak and clearly illustrates the compatibility with fast UHPLC separations.
- For all gradient times ranging from 5 to 30 min, 100% sequence coverage for the light and heavy chains for both rituximab and denosumab was obtained.
- The analysis of the most commonly targeted modifications such as the presence/absence of a C-terminal Lys, the N-glycosylation of asparagine on the heavy chain, the N-terminal pyro-glutamine formation on heavy and light chains, oxidations and deamidations, were successfully identified and relatively quantified. However, the obtained results suggest using the slightly longer gradient times of 20 to 30 min for in-depth analyses to also capture the very low abundant modifications.
- The data presented in this study clearly demonstrate the capability of the applied LC-MS setup to significantly speed up peptide mapping experiments enabling high throughput analyses as required e.g. during clone selection in the development phase of biopharmaceuticals.

References

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2. Shah, B.; Jiang, X.G.; Chen, L.; Zhang, Z., LC-MS/MS peptide mapping with automated data processing for routine profiling of N-glycans in immunoglobulins. *J. Am. Soc. Mass. Spectrom*. **2014**, *25*, 999-1011.

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Europe-Other +43 1 333 50 34 0
Finland +358 10 3292 200
France +33 1 60 92 48 00
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India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 6 6885 1213
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Latin America +1 561 688 8700
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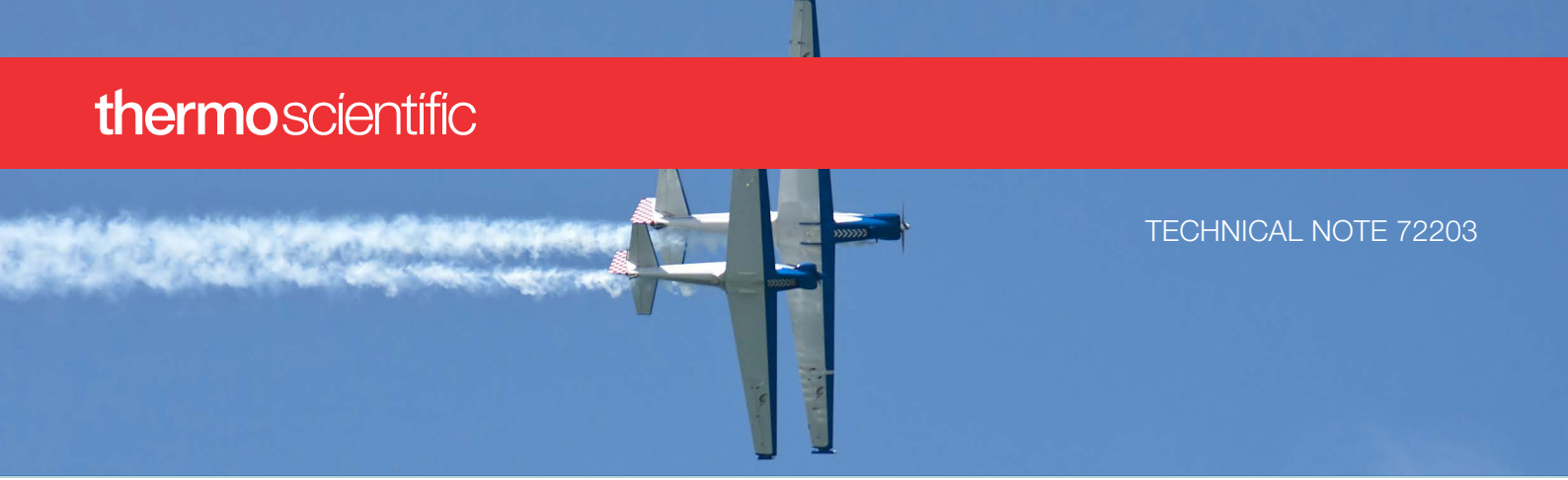

TECHNICAL NOTE 72203

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

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Thermo Fisher Scientific, Germering, Germany

Keywords
Dual column, dual gradient, offline reconditioning, alternating column regeneration, Vanquish, Q Exactive, monoclonal antibody, biotherapeutics, biosimilar

Goal

To demonstrate the use of a multi-pump UHPLC system and enable tandem analysis with two columns in parallel, addressing productivity and throughput improvement of existing LC-MS methods.

Introduction

Common liquid chromatography (LC) methods with gradient elution can be segmented into an analytical gradient section and a reconditioning section. The gradient section is responsible for the actual chromatographic separation, and the reconditioning section is used for the column wash and re-equilibration for the next injection (Figure 1). The process of column re-equilibration involves replacing the mobile phase between the particles (inter-particle), within the pores of the particles (intra-particle), and in the interfacial region between the mobile phase and stationary phase.[1] Good and accepted practice suggests using at least five column volumes to sufficiently equilibrate the analytical column.[2] If a column is required to be equilibrated with a buffered mobile phase or with a mobile phase containing an ion pair reagent, the required equilibration time is even longer. Depending on the column dimensions, gradient length, and flow rate, typically 10–60% of the total runtime is consumed by these column reconditioning steps within the gradient method.

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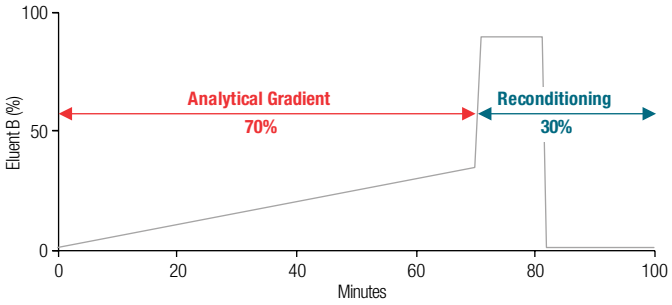

Figure 1. Gradient and reconditioning section of a common LC method.

Many UHPLC peptide mapping methods require lengthy periods of column washing and equilibration between separations. To possibly increase throughput and mitigate these delays without changing the chromatographic gradient section, a tandem LC approach with a two-pump setup and column switching capabilities can be implemented. In this setup one column is used for the ongoing separation, while the second column is switched offline from the mass spectrometer (MS) and simultaneously washed and conditioned for the next injection (Figure 2). The technique provides several benefits. First, throughput can be increased without changing existing (validated) methods. Second, with the latest instrument technology, a system suitable for the technique does not occupy any additional bench space (compared to a second LC-MS system). Third, laboratories can increase throughput without additional staff to operate multiple instruments.

Experimental

Consumables

- 2× Thermo Scientific™ Acclaim™ VANQUISH™ C18, column 2.1 × 250 mm, 2.2 µm, (P/N 074812-V)
- Fisher Scientific™ LC/MS grade water (P/N W/011217)
- Fisher Scientific LC/MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ LC/MS grade formic acid (P/N 28905)
- Thermo Scientific™ SMART Digest™ Kit (P/N 60109-101)

Sample pretreatment and sample preparation

A commercially available monoclonal antibody infliximab drug product (Hospira® UK Limited, Leamington Spa, United Kingdom) was supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.05 mg/mL polysorbate 80, 50 mg/mL sucrose, 0.22 mg/mL monobasic sodium phosphate monohydrate, 0.61 mg/mL dibasic sodium phosphate dihydrate, and sterile water adjusted to pH 7.2 using sodium hydroxide or hydrochloric acid.

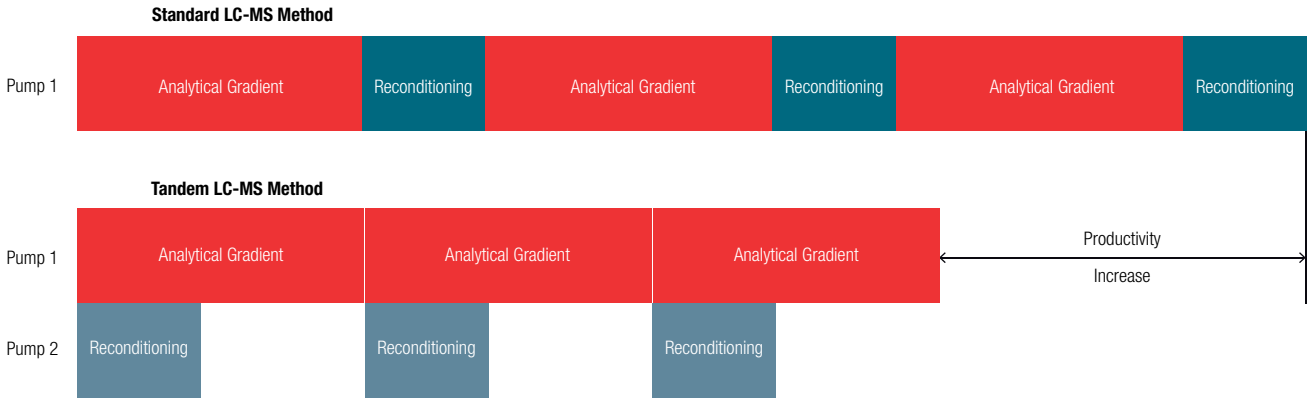

Figure 2. Standard LC-MS method compared to tandem LC-MS method.

SMART Digest Kit protocol

A 50 µL infliximab sample, adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the SMART Digest buffer provided in the kit. The solution was then transferred to a reaction tube containing 15 µL of the SMART Digest resin slurry, corresponding to 14 µg of heat-stable immobilized trypsin. Tryptic digestion was allowed to proceed at 70 °C for 45 min at 1400 rpm. After the digestion, the reaction tube was centrifuged at 7000 rpm for 2 min, the supernatant was transferred to a new tube, and the centrifugation step was repeated.

The non-reduced sample was diluted with 0.1% formic acid (FA) in water to a final protein concentration of 100 ng/µL, and 1.0 µg was loaded on the column for all runs.

LC conditions

Instrumentation

Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of the following:

- System Base Vanquish Horizon (P/N VH-S01-A)
- 2× Binary Pump H (P/N VH-P10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Variable Wavelength Detector F (P/N VF-D40-A)
- Flow Cell Semi-Micro, 2.5 µL, 7 mm light path (SST) (P/N 6077.0360)
- MS Connection Kit Vanquish (P/N 6720.0405)

Figure 3 shows the Vanquish UHPLC system chosen for this setup, consisting of two binary high pressure gradient pumps (HPG) used as an analytical pump and a reconditioning pump. The setup is configured for best chromatographic performance using the high-end Vanquish Binary Pump H, but is not limited to this particular pump type and can be also set up using other pump modules (e.g. Vanquish Binary Pump F (P/N VF-P10-A-01) or Vanquish Quaternary Pump F (P/N VF-P20-A)). All required capillaries and additional parts for this setup are defined in Table 1.

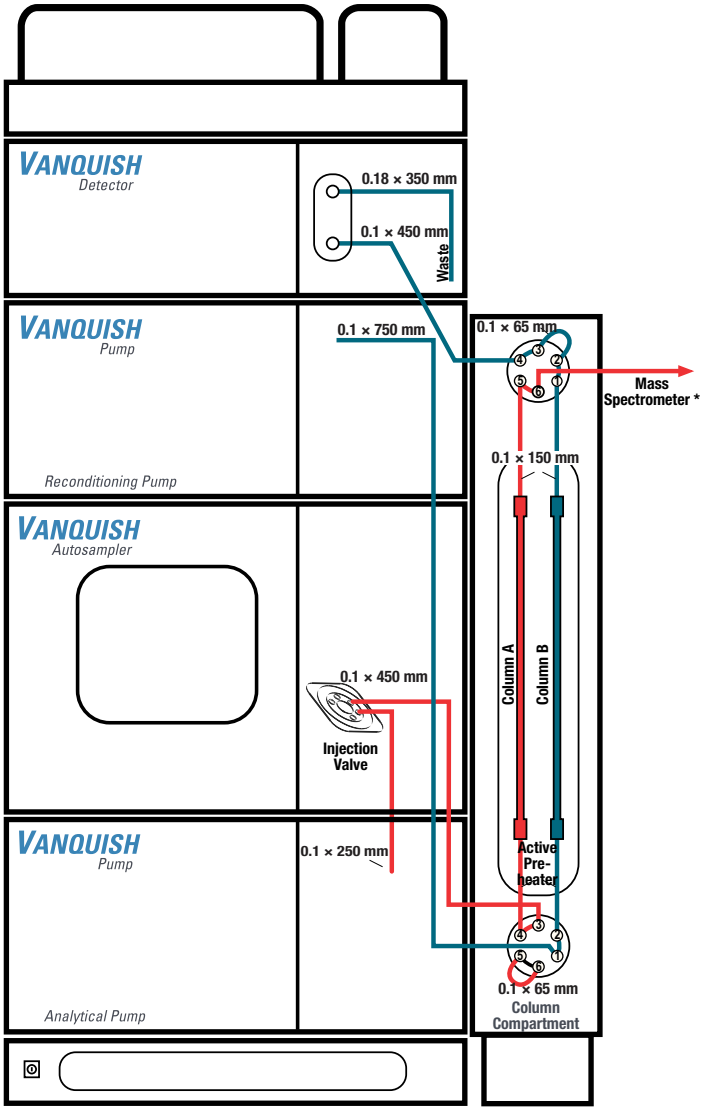

Figure 3. Vanquish UHPLC tandem LC setup with 2-position/6-port (2p6p) valve configurations and required fluidic connections (for details see Table 1). * The recommended capillary to connect the LC to individual mass spectrometer depends on the setup and is defined in the Vanquish MS Connection Kit.

Table 1. Additional parts needed for the tandem LC setup.

#	Amount	Product	PN
1	2	Biocompatible 2-position/6-port (2p6p) column switching valve	6036.1560
2	2	Viper Capillary, MP35N, biocompatible, 0.1 × 65 mm	6042.2306
3	2	Viper Capillary, MP35N, biocompatible, 0.1 × 150 mm	6042.2320
4	1*	Viper Capillary, MP35N, biocompatible, 0.1 × 250 mm	6042.2330
5	2	Viper Capillary, MP35N, biocompatible, 0.1 × 450 mm	6042.2350
6	1	Viper Capillary, MP35N, biocompatible, 0.1 × 750 mm	6042.2390
7	1	Viper Capillary, MP35N, biocompatible, 0.18 × 350 mm	6042.2337
8	2*	Active Pre-heater, 0.1 × 380 mm	6732.0110

* 1 already included in System Base Vanquish Ship Kits

Separation conditions

Mobile phase A: Water + 0.1% formic acid
Mobile phase B: Water/acetonitrile (10:90 v/v) + 0.1% formic acid
Flow rate: See Table 2
Temperature: 60 °C, forced air
Detection: 214 nm
Gradient: See Table 2 and Figure 4

Table 2. LC gradient conditions for the separation of the mAb digest.

Analytical Pump			
Time [min]	A1 [%]	B1 [%]	Flow Rate [mL/min]
0.0	99	1	0.4
40.0	55	45	0.4
40.1	99	1	0.4
43.0	99	1	0.4
Reconditioning Pump			
Time [min]	A1 [%]	B1 [%]	Flow Rate [mL/min]
0.0	99	1	0.4
1.0	10	90	0.4
6.0	10	90	0.4
7.0	99	1	0.4
10.0	99	1	0.4
12.0	99	1	0.4
13.0	10	90	0.4
18.0	10	90	0.4
19.0	99	1	0.4
43.0	99	1	0.4

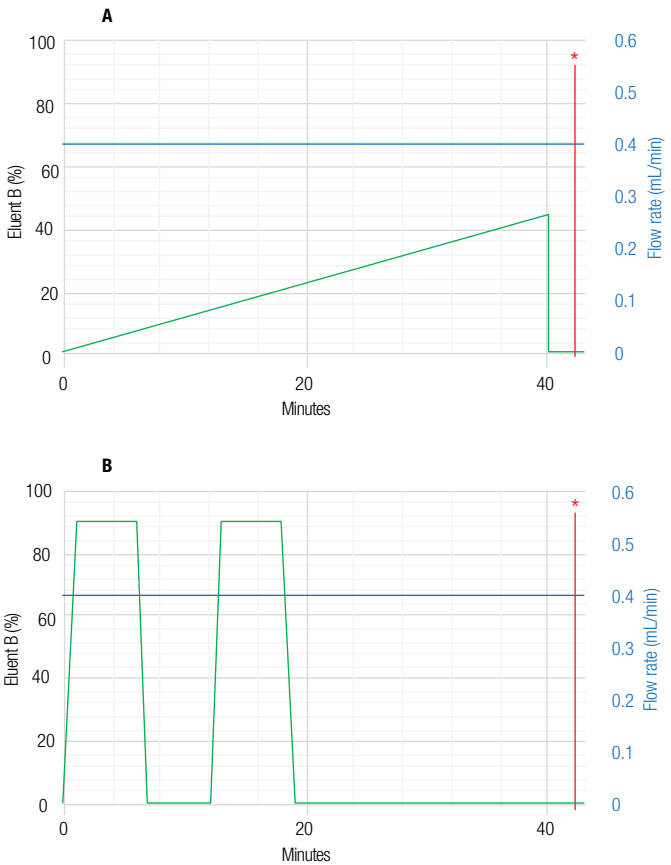

Figure 4. (A) Gradient Analytical Pump including the void volume purge at 40.1 min and (B) Gradient Reconditioning Pump including a multi-step wash section. *Upper and lower valve switched position simultaneously at 42.0 min, the required command is specified in Table 3.

To enable simultaneous valve switching of both 2p6p valves, a specific if-command was inserted in the script editor at 42 min as shown in Table 3. To ensure proper functionality of this specific command, the modules must be named in the instrument configuration as AnalyticalPump and ReconditioningPump, respectively.

Table 3. Valve switching command for the tandem LC setup.

Time	Command	Value
42.000		
If		ColumnComp.LowerValve.CurrentPosition=6_1
	ColumnComp.LowerValve.CurrentPosition	1_2
	ColumnComp.UpperValve.CurrentPosition	1_2
	ColumnComp.Column_A.ActiveColumn	Yes
	ColumnComp.Column_A.SystemPressure	"AnalyticalPump"
	ColumnComp.Column_B.ActiveColumn	No
	ColumnComp.Column_B.SystemPressure	"ReconditioningPump"
Else		
	ColumnComp.LowerValve.CurrentPosition	6_1
	ColumnComp.UpperValve.CurrentPosition	6_1
	ColumnComp.Column_A.ActiveColumn	No
	ColumnComp.Column_A.SystemPressure	"ReconditioningPump"
	ColumnComp.Column_B.ActiveColumn	Yes
	ColumnComp.Column_B.SystemPressure	"AnalyticalPump"
End If		

The lower and upper switching valve of the Vanquish Thermostatted Column Compartment (VTCC) was used to switch between the two flow paths and two analytical columns (Figure 5). The analytical pump was utilized to deliver a water/acetonitrile + 0.1% formic acid gradient (Table 2) to separate the peptides on one column. Simultaneously, the second column, offline from the mass spectrometer, was subject to a multi-step wash and equilibration gradient delivered by the reconditioning pump (Table 2) prior to being switched online for the next injection. A multi-step wash section with repeated up-and-down gradients was used to increase the washing efficiency and to reduce carryover for very big and non-polar tryptic peptides.[3] At the end of the gradient, the analytical pump was set to initial conditions at 40.1 min to perform a void volume purge and equilibrate the fluidics from the analytical pump to the lower switching valve for the next injection. At 42 min, the lower and upper switching valve changed the position (required command, see Table 3), and the next sample was immediately injected on the pre-equilibrated analytical column. In LC-MS setups a UV detector is not always needed. This setup used the Vanquish Variable Wavelength Detector (VVWD) to monitor the reconditioning step, to ensure that no peptides were eluting from the column during this stage, and to confirm proper column equilibration.

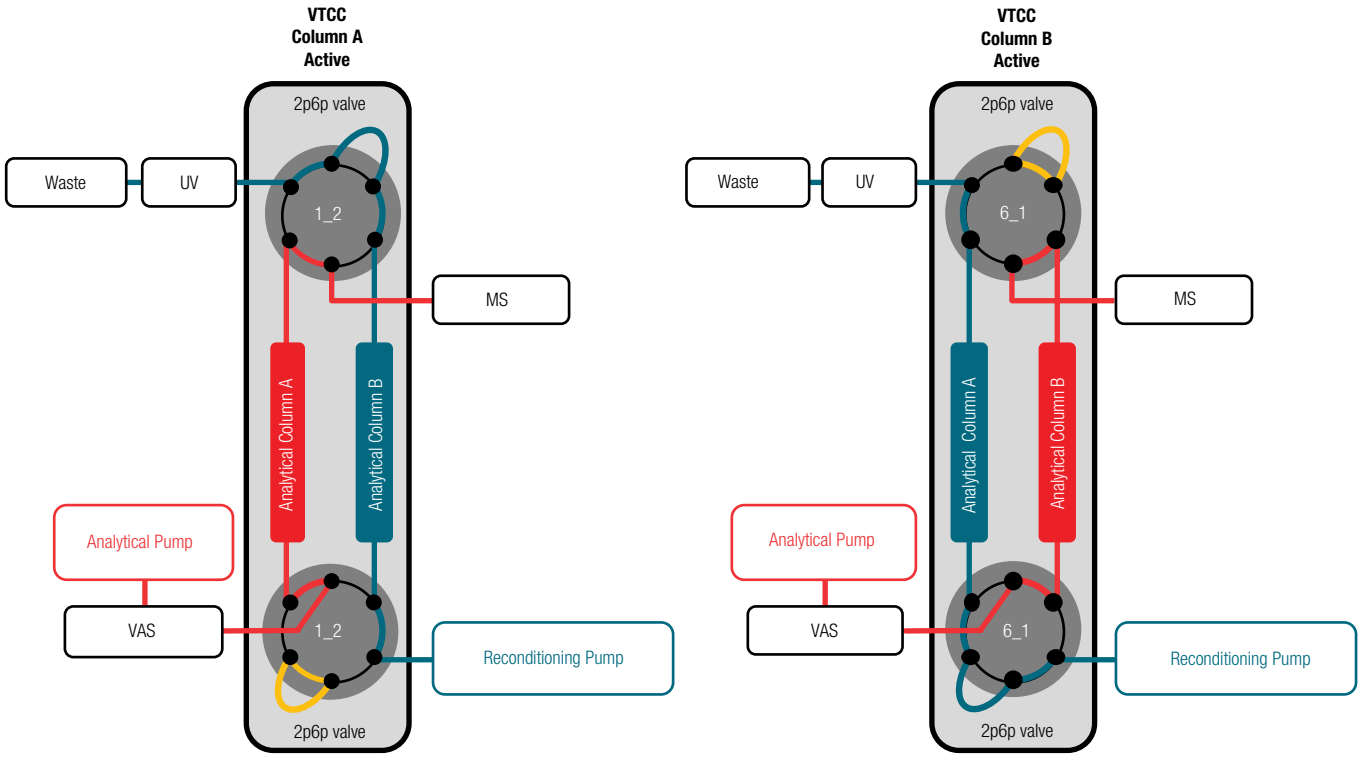

Figure 5. Flow schematic for tandem operation with two flow paths. One for analysis (red) and one for off-line column wash and re-equilibration (blue).

MS conditions

The Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap mass spectrometer was used for detection. The detailed MS source and method parameters are given in Table 4.

Data processing

The data were acquired and analyzed with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR4.

Results and discussion

Using the tandem LC setup for LC-MS peptide mapping experiments, or more precisely for the separation of the tryptic digested monoclonal antibody infliximab, gave reproducible and confident results as demonstrated in the total ion current (TIC) chromatogram overlay of five replicates (Figure 6) on two analytical columns with automated alternating column regeneration.

Table 4. MS source and method parameters.

MS Source Parameters	Setting
Source	Ion Max source with HESI-II probe
Sheath gas pressure	45 psi
Auxiliary gas flow	12 arbitrary units
Vaporizer temperature	350 °C
Capillary temperature	350 °C
S-lens RF voltage	60 V
Source voltage	3.5 kV
MS Method Parameters	**Setting**
Method type	Full MS only
Full MS mass range	140–2000 *m/z*
Resolution settings	15.000 (FWHM at *m/z* 200)
Target value	3e6
Max injection time	200 ms
Microscans	1
SID	0 eV

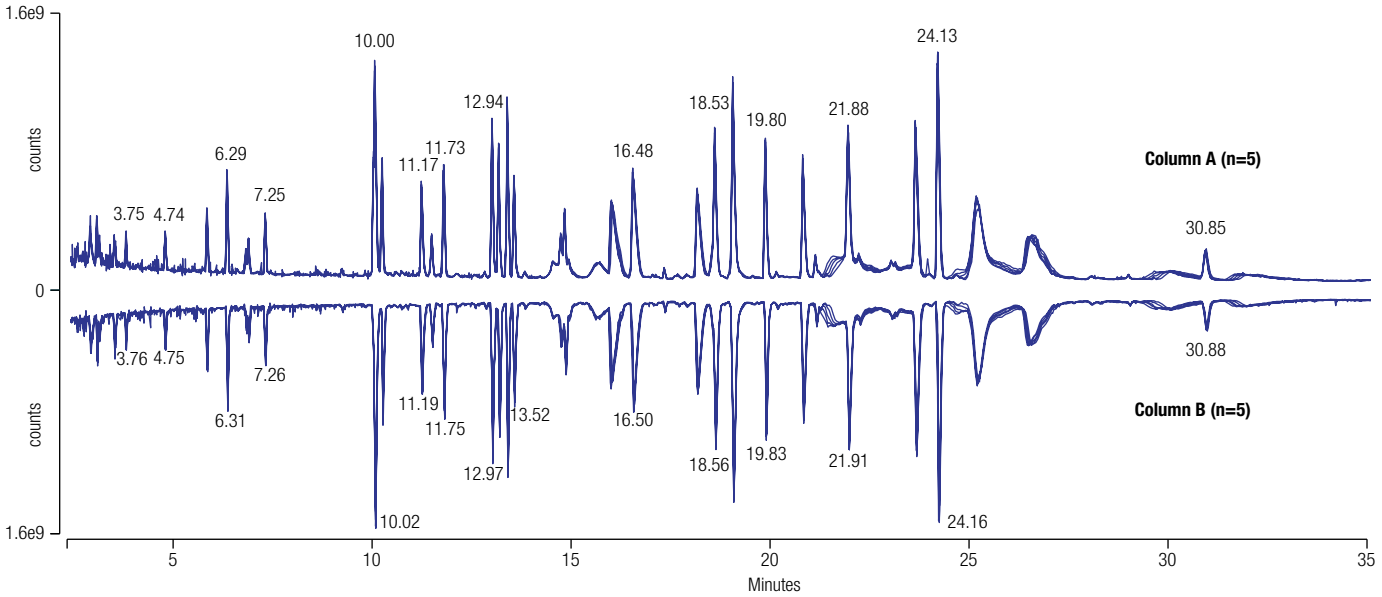

Figure 6. Reproducible results for the Vanquish tandem LC - Q Exactive HF setup, showing the overlay of five TIC chromatograms for the separation of digested infliximab using the SMART Digest Kit.

Retention time relative standard deviation (RSD) values below 0.11% were achieved for the UHPLC system in tandem column operation compared to 0.045% and 0.039% for the single column setup. Polar tryptic peptides eluting between 0 and 14 min had the highest RSD values up to 0.18%, and the heavy chain peptide (D151-Y183) at 30.85 min had the lowest with 0.064% (Table 5). The average absolute retention time shift between column A and column B was 0.023 min (relative, 0.18 %) and shows that peak assignment based on retention time is not impaired. An average peak area RSD value of 2.47% demonstrates the suitability for quantitative data analysis using the tandem LC setup.

Table 5. Reproducible results for the Vanquish tandem LC - Q Exactive HF setup with detailed RSD values for infliximab tryptic peptides for column A/B in tandem and single column operation based on the TIC chromatograms shown in Figure 6.

Column A			Column B					Column A/B	
RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	Abs. RT Shift Column A to B [min]	Rel. RT Shift Column A to B [%]	RT RSD [%] n=10	Area RSD [%] n=10
3.75	0.18	2.72	3.76	0.13	1.64	0.005	0.13	0.16	2.12
4.74	0.054	3.53	4.75	0.11	5.05	0.010	0.21	0.14	4.24
6.29	0.072	2.33	6.31	0.037	1.14	0.020	0.32	0.18	2.19
7.25	0.018	4.94	7.26	0.033	4.72	0.016	0.23	0.12	4.94
10.00	0.032	3.05	10.02	0.037	1.75	0.023	0.23	0.12	2.35
11.17	0.040	3.96	11.19	0.047	2.70	0.022	0.20	0.11	3.22
11.73	0.043	1.64	11.75	0.007	2.59	0.025	0.21	0.12	2.15
12.94	0.014	4.19	12.97	0.012	1.61	0.023	0.18	0.10	3.03
13.49	0.028	1.66	13.52	0.025	3.11	0.024	0.18	0.10	2.36
16.48	0.056	1.02	16.50	0.031	0.78	0.024	0.14	0.087	0.91
18.53	0.019	1.94	18.56	0.020	1.90	0.027	0.15	0.080	2.35
19.80	0.019	0.50	19.83	0.016	0.78	0.029	0.15	0.078	0.62
21.88	0.028	4.35	21.91	0.0075	1.71	0.033	0.15	0.083	3.78
24.13	0.025	1.52	24.16	0.030	0.60	0.031	0.13	0.072	1.09
30.85	0.039	1.56	30.88	0.039	2.00	0.031	0.10	0.064	1.74
Average	**0.045**	**2.59**		**0.039**	**2.14**	**0.023**	**0.18**	**0.11**	**2.47**

The advanced wash and reconditioning method used in this study enables significant reduction of protein/peptide column carryover and can also be individually optimized by reducing or increasing the flow rate during the method. The UV trace used to exclusively monitor the wash and equilibration step of the reconditioning pump showed reproducible results for all runs (Figure 7).

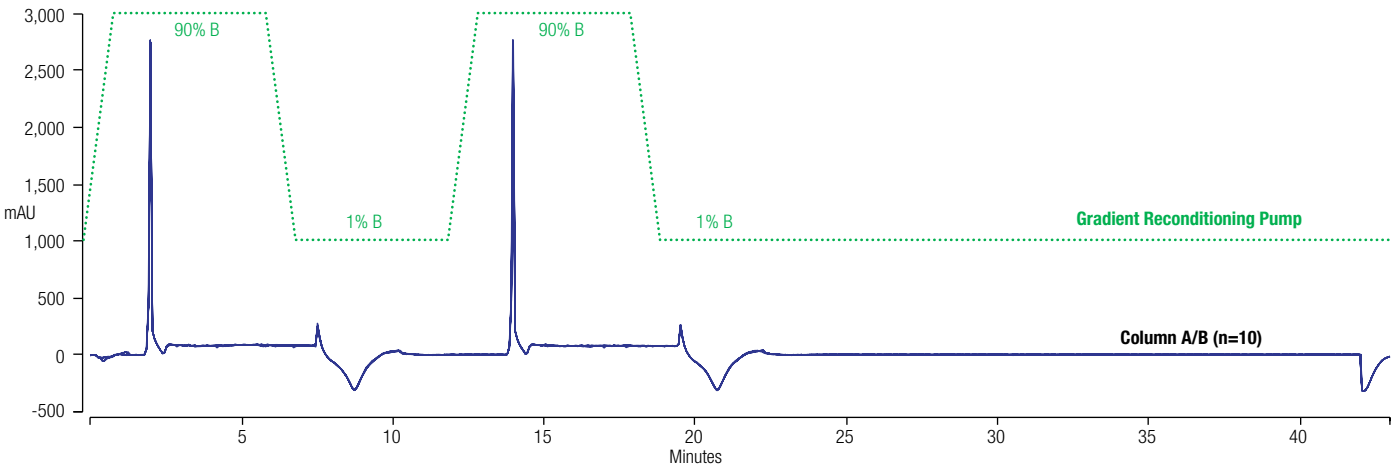

Figure 7. Overlay of ten chromatograms of the tandem LC reconditioning step.

Conclusions

The Vanquish UHPLC systems can be easily configured for tandem LC-MS operation for peptide mapping experiments by adding one additional pump module. This enables a throughput increase up to 40% without changing the actual gradient of the existing method. The retention time RSD values are below 0.11% for the tandem and single column operation. In this study, peptide mapping methods were used to demonstrate the capabilities of a tandem LC setup, but it can be applied to other methods and samples as well. Both Chromeleon version 7.2 and Thermo Scientific™ SII for Xcalibur™ version 1.3 support the Vanquish tandem LC setup.

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2. Van Middlesworth, B. J., Dorsey, J. G., Re-equilibration Time of Superficially Porous Silica Based Columns in Gradient Elution Reversed Phase Liquid Chromatography. *Journal of Chromatography A,* **2011,** *1218*(40), 7158–65. doi:10.1016/j.chroma.2011.08.030.
3. Freiser, H.H., Nowlan, M.P., Schmuck, M.N., The Use of Stabilized Silica Support with a Short Alkyl Chain for Reversed-Phase Preparative Chromatography of Proteins, Micra Scientific, product bulletin number 10, 1988.

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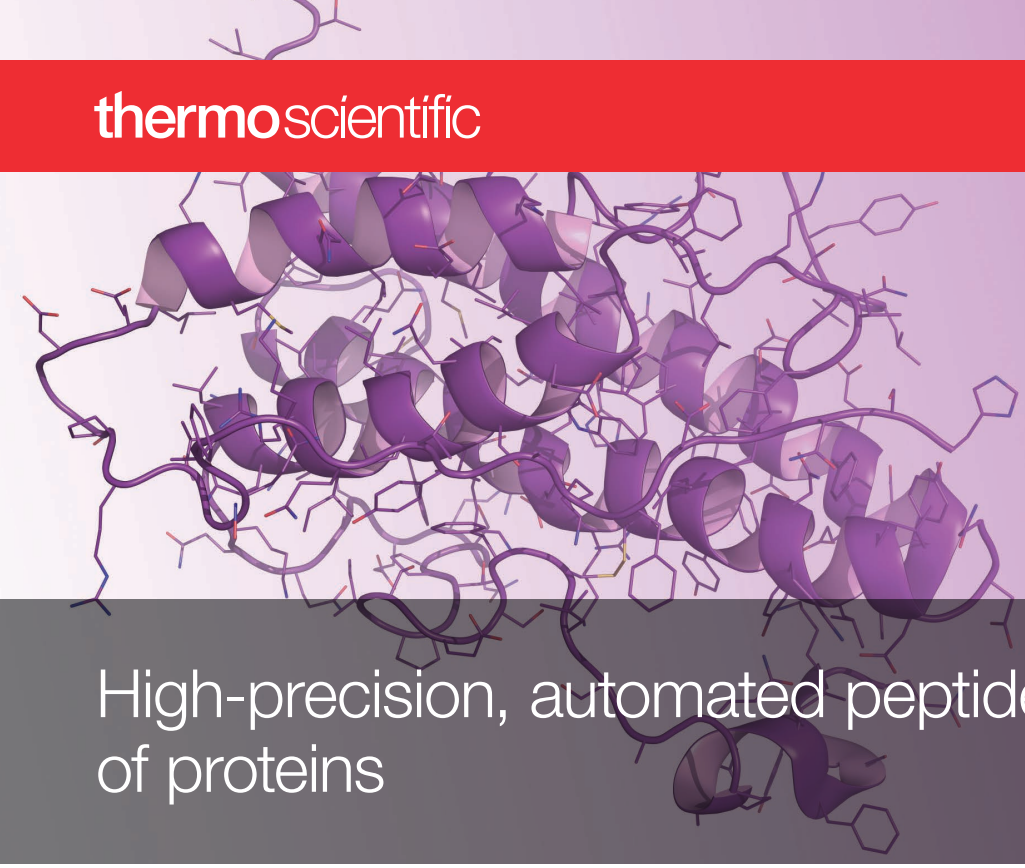

High-precision, automated peptide mapping of proteins

Authors

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Keywords

NIBRT, Biopharmaceutical, QA/QC, Critical quality attribute, Peptide mapping, Cytochrome c, Recombinant somatotropin, Infliximab, Rituximab, Carbonic anhydrase, Hypersil GOLD, Magnetic SMART Digest, KingFisher, Vanquish Horizon UHPLC, Q Exactive Plus MS

Application benefits

- High-precision digestion carried out in under one hour including preparation time. This represents a significant time saving (up to 24 fold) compared to traditional digestion techniques.
- Reproducible results that are user-independent with less than 3.1% RSD in peptide area for six independent digests and a sequence coverage of 100%.
- Associated ease-of-use through automation.

Goal

To develop a robust and reproducible, high-precision, automated, digestion workflow that is appropriate as an easy-to-use, general approach to peptide mapping characterization with both LC-only and LC-MS processes. To confirm the peptide map quality with multiple examples of proteins and show high sequence coverage is possible using high-resolution, accurate-mass mass spectrometry.

Introduction

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biotechnological production, there are many attributes that need to be analyzed to guarantee their safety and efficacy.

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Peptide mapping is used to measure several critical quality attributes (CQA) required for the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and to check for post-translational and chemical modifications.

Mass spectrometry (MS) is coupled to liquid chromatography (LC) for peak identification and confirmation of the sequence. However, many quality control (QC) methods use detection by ultraviolet (UV) absorption only after the peaks identities have been confirmed.[1]

Trypsin is the enzyme most commonly used for proteolytic digestion due to its high specificity. Although a widely accepted technique, in-solution trypsin digestion protocols required for sample preparation are labor intensive and prone to manual errors. These errors affect the quality of the analytical data compromising the ability to reproducibly characterize a protein to the required standard. In the most critical cases where workflows only employ UV detection without confirmation by MS, robust and stable sample preparation and separation methods are critical. The digestion must be reproducible and chromatography must be extremely stable to allow unambiguous peptide identification based on chromatographic retention time.

This work details the automated peptide mapping of cytochrome c, recombinant somatotropin, and infliximab drug product. These proteins were chosen to investigate the applicability and reproducibility of the automated digestion protocol and subsequent analysis. The combination of the Thermo Scientific™ SMART Digest™ magnetic beads and the Thermo Scientific™ KingFisher™ Duo purification system was used to automate the digestion process to produce high quality, reproducible peptide mapping data.

Magnetic beads are a proven support used for many purification and sample preparation approaches in life science research and biotechnology. The KingFisher purification system enables robotic handling and easy automation of any magnetic bead based application resulting in superior performance and reproducibility.[2]

The Thermo Scientific™ Vanquish™ Horizon UHPLC system was subsequently used to analyze the samples by UHPLC-UV and, additionally, coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer for MS confirmation of the peptide sequence.

Experimental

Consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ HPLC grade water (P/N 10449380)
- Fisher Scientific LC/MS grade acetonitrile (P/N 10489553)
- Fisher Scientific™ Optima™ LC/MS grade water with 0.1% formic acid (P/N 10429474)
- Fisher Scientific Optima LC/MS grade acetonitrile with 0.1% formic acid (P/N 10468704)
- Fisher Scientific Optima LC/MS trifluoroacetic acid (P/N 10125637)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- SMART Digest Trypsin Kit, with filter/collection plate (P/N 60109-102)
- KingFisher Deepwell, 96 well plate (P/N 95040450)
- KingFisher Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Hypersil GOLD™ column 3 µm, 2.1 × 150 mm (P/N 25003-152130)

Equipment

- KingFisher Duo Prime Purification System (P/N 5400110)
- Thermo Scientific™ Hypersep™ 96 well Positive Pressure System (P/N 60103-357)
- Vanquish Horizon UHPLC System including:
  - Binary Pump H (P/N VH-P10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
  - System Base Vanquish Horizon (P/N VH-S01-A)
- MS Connection Kit Vanquish (P/N 6720.0405)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (P/N IQLAAEGAAPFALGMBDK)

Sample preparation

- Lyophilized powder of cytochrome c, carbonic anhydrase, and recombinant somatotropin were dissolved in deionized water and adjusted to a final concentration of 10 mg/mL.
- Infliximab and rituximab drug product was reconstituted in water to a concentration of 10 mg/mL with gentle swirling to aid in solubilization as directed from the manufacturer's product insert information.

SMART Digest, manual digestion protocol

The comparison of the manual and automated SMART Digest protocol was conducted with somatotropin and rituximab using 100 µg recombinant protein per digestion reaction.

- Proteins were adjusted to 2 mg/mL with deionized water.
- The solution was further diluted 1:4 with the SMART Digest buffer.
- 200 µL of this solution was directly transferred to each reaction tube (containing 15 µL of the SMART Digest standard resin slurry).
- Digestion was conducted in a heater/shaker at 70 °C, 1200 rpm (to prevent sedimentation of the immobilized trypsin beads).
- Digestion incubation times of 15 minutes for somatotropin and 45 minutes for rituximab were used as optimal times to ensure complete digestion of each protein in the shortest time.
- Immobilized resin was removed by filtration with the filtration plate provided with the SMART Digest kit using a positive pressure manifold.

Magnetic SMART Digest, automated digestion protocol

The KingFisher Duo Prime purification system was used to automate the protein digestion. Digests of infliximab, somatotropin, cytochrome c, and carbonic anhydrase were carried out.

- SMART Digest magnetic resin slurry was diluted and uniformly suspended in SMART Digest buffer to create a suspension of 15 µL original resin into 100 µL of buffer in each well of the dedicated "resin lane" of a KingFisher Deepwell 96 well plate.
- 200 µL of 1:4 diluted SMART Digest buffer was prepared in each well of a separate row of the plate as the optional wash buffer.
- 50 µL of the sample solution was diluted into 150 µL of SMART Digest buffer in the dedicated "incubation lane" that allows for heating and cooling (row A).
- Thermo Scientific™ BindIt™ software (version 4.0) was used to control the KingFisher Duo Prime system with the program outlined in Figure 1 and Tables 1 and 2.
- The digestion step was completed at 70 °C.
- Sedimentation of beads was prevented by repeated insertion of the magnetic comb using the mixing speed setting "medium".
- An incubation time of 15 min for somatotropin, 20 min for cytochrome c, and carbonic anhydrase with 45 min for infliximab were used as optimal times to ensure complete digestion of each protein in the shortest time.
- Immediately after incubation, the magnetic beads were collected and removed from the reaction and the digest solution was actively cooled to 15 °C.

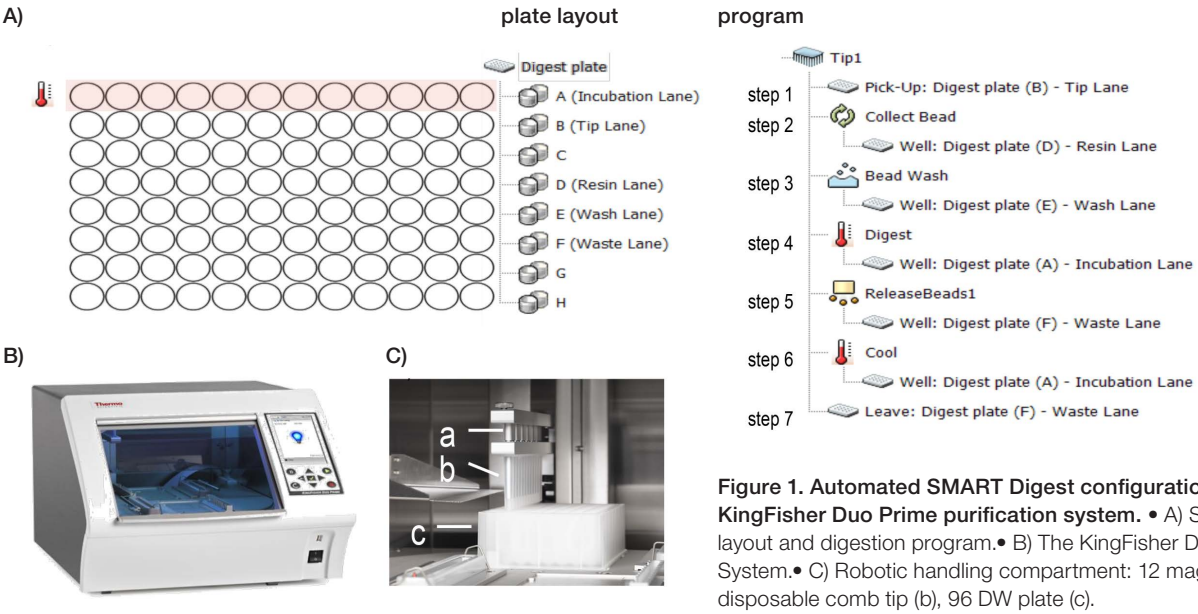


Figure 1. Automated SMART Digest configuration using the KingFisher Duo Prime purification system. • A) Schematic of plate layout and digestion program.• B) The KingFisher Duo Prime Purification System.• C) Robotic handling compartment: 12 magnetic rods (a), disposable comb tip (b), 96 DW plate (c).

Table 1. Plate layout showing the volumes and solutions in each well.

Lane	Content	Volume (µL)
A	Buffer	150
	Sample	50
B	Tip Comb	
D	Beads	15
	Bead Buffer	100
E	Bead Wash Buffer	200
F	Waste Lane	250

Table 2. Protocol step details.

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	8 min 30 s Medium Mix	3 count, 15 s	70 °C heating while mixing 5 °C post temp.	A
Release Beads	Yes, Fast	–	–	–	F

UHPLC-UV separation conditions - cytochrome c, infliximab, rituximab, and carbonic anhydrase

Column:	Hypersil GOLD 1.9 µm, 2.1 × 150 mm
Mobile phase A:	Water + 0.05% trifluoroacetic acid
Mobile phase A B:	Water/acetonitrile/trifluoroacetic acid (20:80:0.04 v/v/v)
Flow rate:	0.5 mL/min
Column temperature:	70 °C (still air mode)
Injection volume:	5 µL
UV wavelength:	214 nm
Gradient:	Table 3

Table 3. Mobile phase gradient.

Time (min)	%A	%B	Flow (mL/min)	Curve
0.0	95	5	0.5	5
15.0	45	55	0.5	5
15.1	0	100	0.5	5
17.0	0	100	0.5	5
17.1	95	5	0.5	5
22	95	5	0.5	5

Data processing and software

Chromatographic software:	Thermo Scientific™ Chromeleon™ CDS 7.2 SR4

UHPLC-UV and UHPLC-MS separation conditions - somatotropin and infliximab

Columns:	Hypersil GOLD 1.9 µm, 2.1 × 150 mm
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Flow rate:	0.3 mL/min
Column temperature:	70 °C (still air mode)
Injection volume:	5 µL
UV wavelength:	214 nm
Gradient:	Table 4

Table 4. Mobile phase gradient.

Time (min)	%A	%B	Flow (mL/min)	Curve
0.0	96	4	0.3	5
30	25	75	0.3	5
30	0	100	0.3	5
35	0	100	0.3	5
35	96	4	0.3	5
45	96	4	0.3	5

MS conditions

The Q Exactive Plus mass spectrometer equipped with a HESI-II probe was used for mass spectrometric detection using a full MS / dd-MS2 (Top5) experiment.

Ionization:	HESI Positive ion
Scan range:	140 to 2000 *m/z*
Source temperature:	350 °C
Sheath gas pressure:	45 psi
Auxiliary gas flow:	10 arb
Spray voltage:	3.4 kV
Capillary temperature:	320 °C

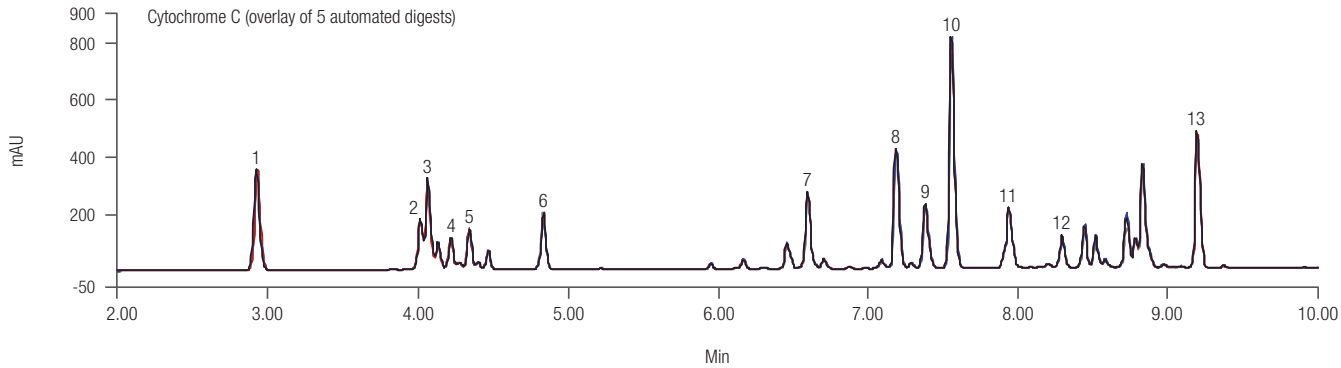
Resolution (Full MS) at *m/z* 200 (FWHM):	70,000
Resolution (MS2) at *m/z* 200 (FWHM):	17,500
Top-N MS2:	5
S-lens RF level:	60
Max inject time:	100 ms
Collision energy (CE):	27

Data processing and software

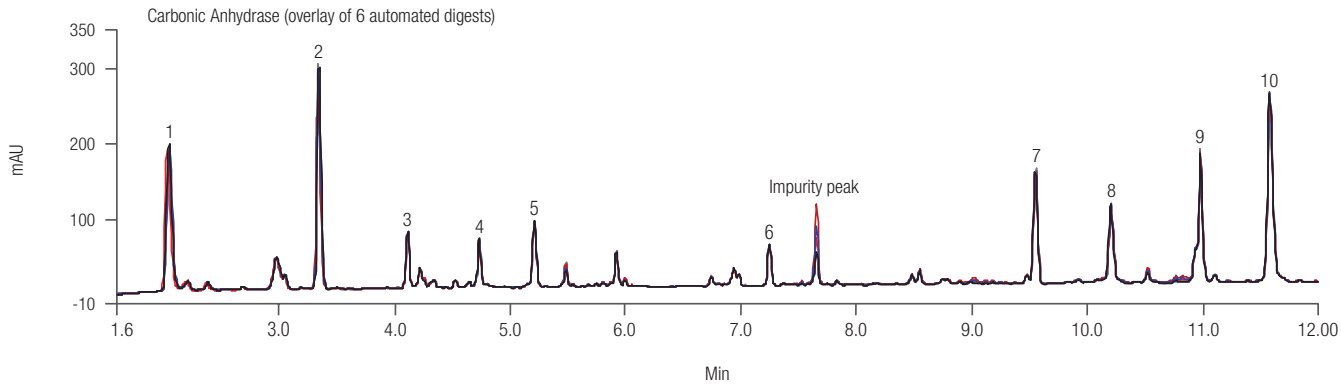
Chromatographic software:	Chromeleon CDS 7.2 SR4
MS data acquisition:	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
Protein characterization software:	Thermo Scientific™ BioPharma Finder™ 2.0 software

Results and discussion

The applicability of the automated protein digestion with the KingFisher Duo Prime purification system was tested with cytochrome c and carbonic anhydrase. Replicate digests were conducted and the generated peptides were separated and analyzed by UHPLC-UV. The corresponding peptide maps are shown as an overlay in Figure 2. Both cytochrome c and carbonic anhydrase were readily digested using the automated SMART Digest kit protocol resulting in complete digestion of the proteins. An average RSD for relative peak area of 2.08% was achieved for the peaks annotated with cytochrome c; several of these peaks had peak area RSD values of 1% and below. Carbonic anhydrase gave similar highly reproducible results with an average area RSD value of 1.8.



Peak	1	2	3	4	5	6	7	8	9	10	11	12	13
%RSD (A_{rel})	2.75	1.87	2.45	0.71	1.27	1.90	3.60	2.09	2.35	3.92	1.11	0.72	2.42
%RSD (t_R)	0.12	0.03	0.05	0.04	0.03	0.02	0.03	0.03	0.03	0.02	0.01	0.01	0.01

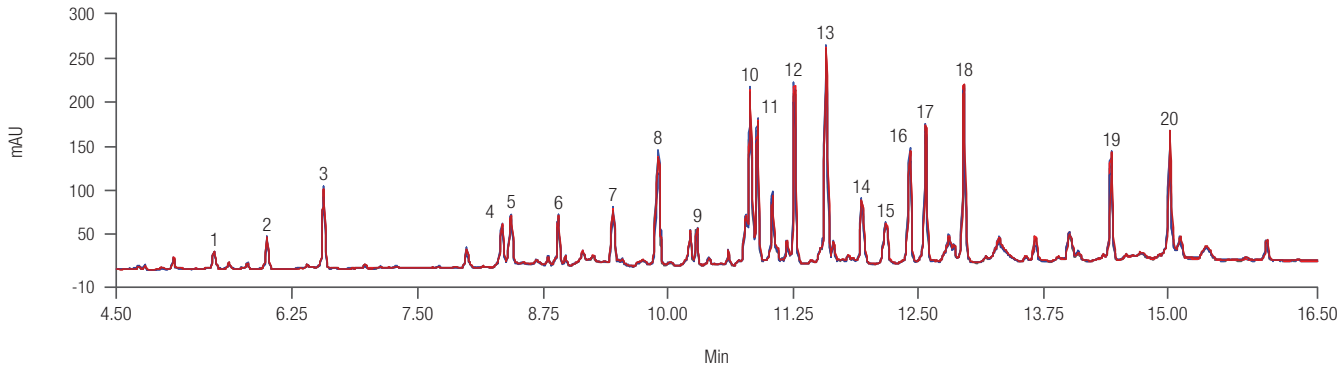


Peak	1	2	3	4	5	6	7	8	9	10
%RSD (A_{rel})	2.12	1.46	1.53	3.03	1.71	1.26	1.51	2.60	1.14	1.64
%RSD (t_R)	0.32	0.1	0.03	0.04	0.02	0.02	0.01	0.01	0.01	0.01

Figure 2. Automated digestion of cytochrome c and carbonic anhydrase using SMART Digest magnetic resin with the KingFisher Duo Prime system. Overlaid peptide maps of different digests of cytochrome c (upper panel) and carbonic anhydrase (lower panel). Digest solutions of 5 µL were injected without further purification and peptides were separated using separation condition A. %RSD values for relative peak area (upper) and retention time (lower) and are given for the peaks indicated.

This level of reproducibility can be visualized by the high consistency of the Vanquish Horizon UHPLC system gradients and injection accuracy, which gives identical chromatography and makes integration and interpretation of the peaks easier. This level of reproducibility in protein digestion has never been reported before so the degree of influence between different users was characterized.

To assess the robustness and ease of use of the SMART Digest kit protocol in general between different users, an experiment was performed during a protein chromatography workshop with five different people performing a manual digestion using the SMART Digest kit, some of whom had never performed a protein digestion before. The results of this experiment are shown in Figure 3.



Peak	1	2	3	4	5	6	7	8	9	10
%RSD (A_{rel})	2.54	2.41	1.89	3.39	3.53	2.16	4.41	2.10	2.10	3.65
Peak	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
%RDS (A_{rel})	1.96	3.5	3.72	2.26	2.91	1.97	3.28	2.62	3.16	1.20

Figure 3. Manual digestion of rituximab performed by 5 different people. Overlaid peptide maps of the monoclonal antibody rituximab. 5 µL of digest solutions were injected without further purification and peptides were separated using the gradient and conditions in Table 1. Percentage relative standard deviation (%RSD) values for relative peak area are given for the peaks indicated.

The results show an average RSD value for peak area of 2.74 over 20 different peaks in a complex chromatogram. Considering that this result was achieved from a protein digestion of a large monoclonal antibody performed by five people, the robustness of the protocol between different users is very apparent. The ease of use is also demonstrated in that some of the digestions were done by people who have no experience with protein digestion techniques.

The new SMART Digest kit automated protocol was further evaluated by LC-MS using infliximab drug product as a test sample.

A 45 min incubation at 70 °C enabled the complete digestion of the infliximab antibody and resulted in a close to identical UV peptide map of the two parallel digestion reactions (Figure 4, upper panel). Analysis by LC-MS confirmed complete sequence coverage of 100% for both the light and heavy chain of the antibody (Figure 4, lower panel). This result demonstrates reproducible, complete digestion of infliximab, and with the additional reproducibility studies, that the SMART Digest kit when automated is readily applicable for the characterization and quality control of modern bio-pharmaceuticals.

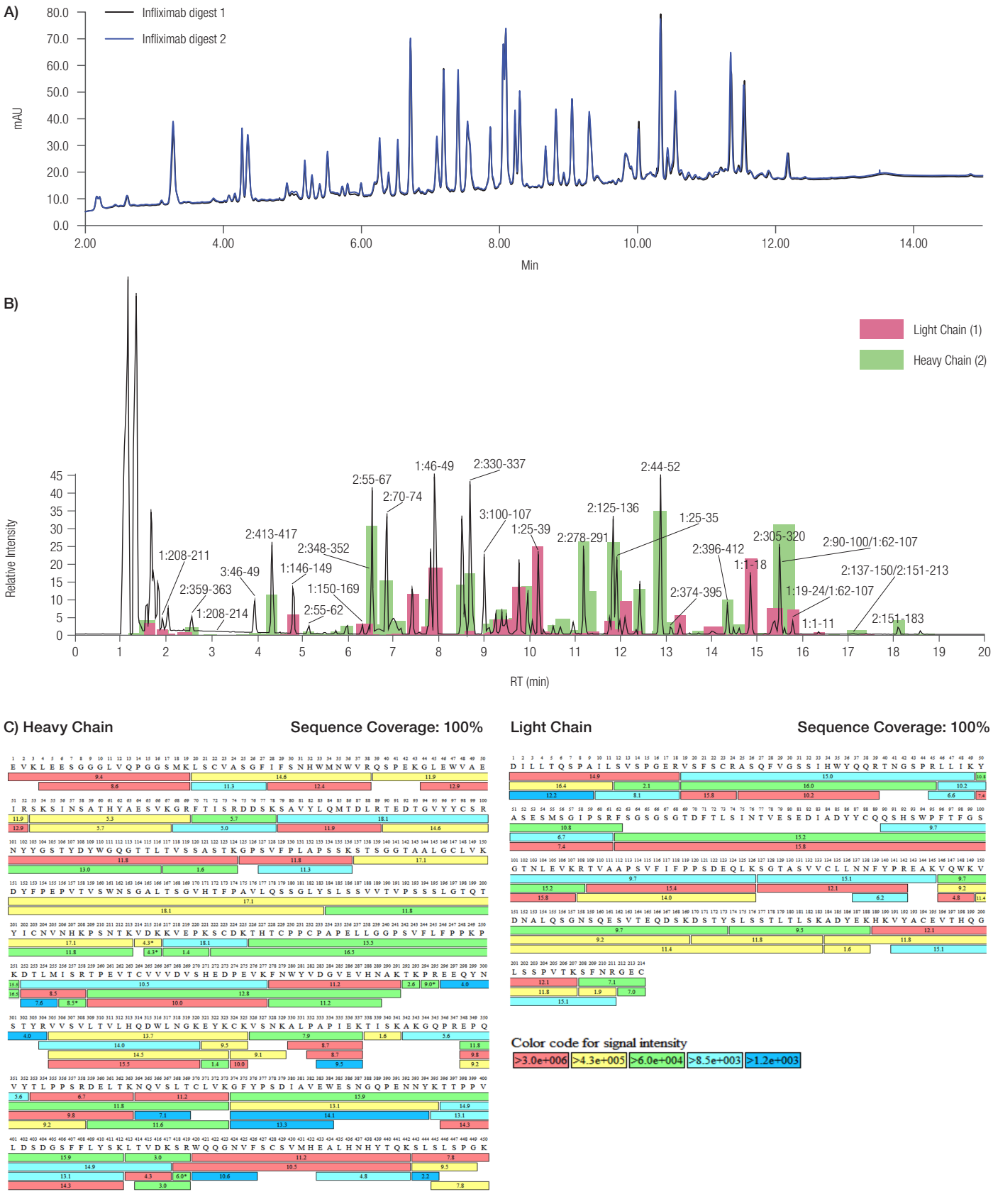


Figure 4. Automated digestion of infliximab drug product using SMART Digest magnetic resin. Panel A: Overlaid peptide maps for two digests of infliximab antibody. Digest solutions of 5 µL were injected without further purification and peptides were separated using separation conditions listed in Table 3. Panel B: Total ion chromatogram from infliximab indicating the peptide origin to light (1) and heavy chain (2). Position numbers are given together with the peptide chain annotation, the heavy chain in green and the light chain in red highlights. Panel C: Sequence coverage map of the automated infliximab using the SMART Digest magnetic kit. Lines containing peptides with signal intensity > 4.3 e5 are shown.

A direct comparison of the standard and the SMART Digest magnetic resins and protocols were conducted using recombinant somatotropin in quadruplicate digestions with LC-MS-UV. The MS data was used to ensure that the digest conditions used were optimal in both the manual and the automated protocols. The sequence coverage using both digestion methods showed 100% sequence coverage, and the identified peptides showed complete digestion had been achieved for both digestions [data not shown]. Identical UV peptide patterns were generated with both digestion approaches. However, for a more qualitative comparison, the major peaks from the UV data were examined more closely with linear regression (Figure 5).

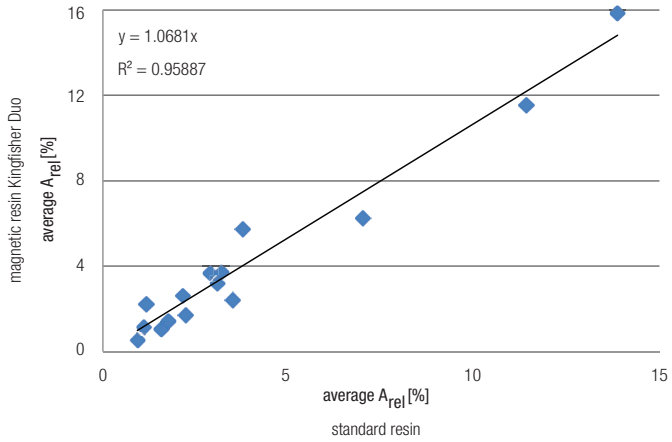


Figure 5. Linear regression curves comparing the peak area results from an automated digestion using the SMART Digest magnetic kit to a manual digestion using the SMART Digest kit.

Correlation of the relative peak areas observed for the different digestion methods leads to linear regression curves with a slope of 1, indicating the equivalence of the obtained digestion results in both cases.

Comparison of the average variance between the digest replicates demonstrates the benefit of automation for the reproducibility of the digestion results. The pre-aliquoted standard SMART Digest kit, although already shown to give good reproducibility in Figure 3, resulted in higher relative standard deviations for absolute peak area and peak height compared to results from the SMART Digest magnetic resin kit when automated. The use of the KingFisher Duo Prime system for automation resulted, on average, in 1.5 times less variance (% RSD) compared to the manually processed standard resin.

Conclusions

We have studied in detail two versions of the SMART Digest kit.

- The manual method has shown to be reproducible, robust, and efficient even in the hands of multiple users with varying experience.

- The combination of the SMART Digest magnetic resin with the KingFisher automation system minimizes the manual handling required for protein digestion. It also ensures that the timing of the reactions are perfect for each sample and reduces the time at which the proteins and peptides are exposed to elevated temperatures, reducing the possibility of post translational modifications to a minimum. This yields a further increase in reproducibility of the obtained digestion results from that already seen with the manual SMART Digest kit protocols.

- Several proteins have been used in this work to emphasize the more global applicability of the method. It should be noted that the digestion times for each of these proteins were different. This is dependent on the heat stability of the target protein to be digested and as such each protein to be studied should have the time of digestion optimized. The digestion should be long enough to obtain complete digestion of the protein into peptides with stable peak areas, but not longer than necessary, to avoid the slow build-up of some possible post translational modifications.

- Digestion of two monoclonal antibody biotherapeutics was readily achieved with outstanding reproducibility, creating a peptide map that covers the entire amino acid sequence of both chains.

- The combination of this automated digestion process with the class leading retention time stability offered by the Vanquish UHPLC systems[5,6] provides a truly robust and stable peptide mapping workflow for the detailed characterization of modern biotherapeutics.

- The workflow is equally suitable for the in-depth product characterization that becomes possible with modern HRAM Orbitrap mass spectrometry systems or a quality control approach that relies on UV absorbance and pattern recognition only.

References

1. Thermo Scientific Application Note 1134, LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC, Thermo Fisher Scientific Germering, Germany.
2. Thermo Scientific Application Note SPALHMolBio1014, Reproducible liquid handling for the molecular biology workflow (2014), Thermo Fisher Scientific, Vantaa, Finland.
3. Thermo Scientific Technical Guide 21544, SMART Digest and SMART Digest ImmunoAffinity (IA) Kit Technical Guide (2016), Thermo Fisher Scientific, Runcorn, UK.
4. Thermo Scientific Technical Note KFDuoPrime_0415, Thermo Scientific KingFisher Duo Prime System (2015), Thermo Fisher Scientific, Vantaa, Finland.
5. Thermo Scientific Application Note 1124, Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments System (2015), Thermo Fisher Scientific Germering, Germany.
6. Thermo Scientific Application Note 1132, Reliable Results in Peptide Mapping Using the Vanquish Flex UHPLC System (2015), Thermo Fisher Scientific Germering, Germany.

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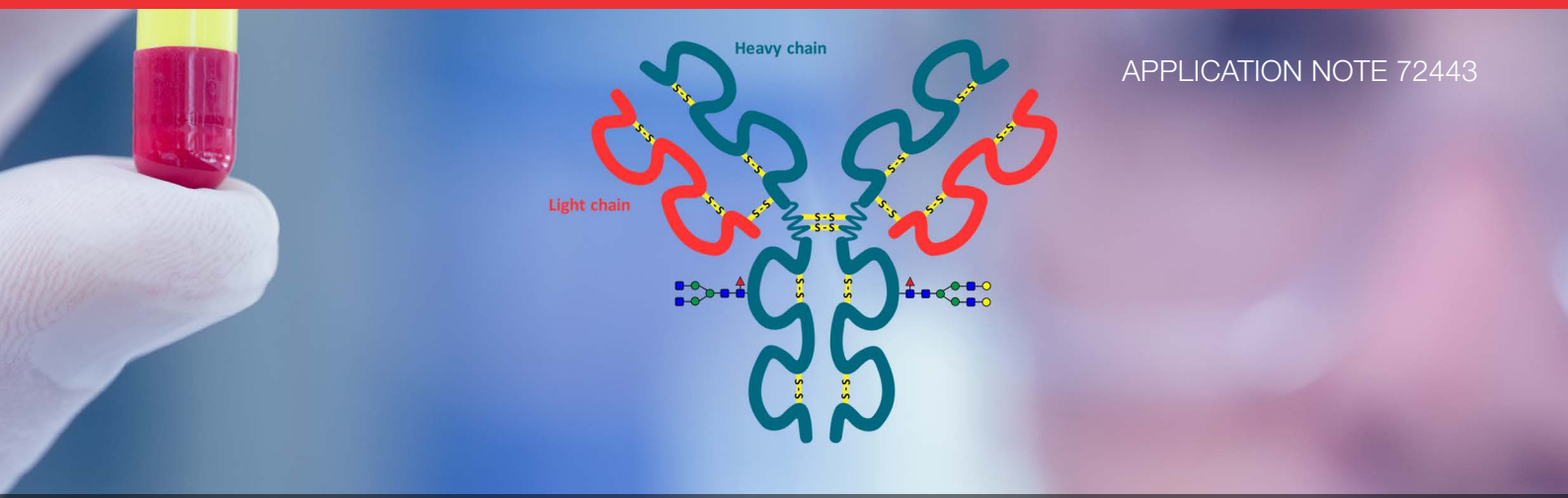

APPLICATION NOTE 72443

HILIC – an alternative separation technique for glycopeptides

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Keywords
Monoclonal antibodies, Accucore, Amide HILIC, Biocompatible UHPLC, SMART Digest, Protein Digest, Biotherapeutics characterization, Biopharma, Vanquish Flex UHPLC

Goal

Demonstrate the suitability of the Thermo Scientific™ Vanquish™ Flex UHPLC system and Thermo Scientific™ Accucore™ 150 Amide HILIC column for efficient and reliable separation of glycopeptides.

Introduction

In this work, an approach to efficiently separate peptides and glycopeptides of a monoclonal antibody (mAb) in a single run is demonstrated. The separation was achieved using hydrophilic interaction liquid chromatography (HILIC) with the Accucore 150 Amide HILIC column. Peptides and glycopeptides were obtained by digesting Infliximab using the Thermo Scientific™ SMART Digest™ kit. The reproducibility of the glycopeptide peaks was evaluated for repeated injections.

Peptide mapping is one of the routine methods for biotherapeutics characterization. This technique, combined with mass spectrometry, is utilized in research environments for the determination of the primary sequence of a mAb and the identification of post-translational modifications (PTMs). Glycosylation affects the potency and efficacy of the biotherapeutic.

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Therefore, during production process optimization, the levels of critical glycosylations are monitored and used as feedback to facilitate development, in order to acheive a given glycan pattern.

After enzymatic digestion, peptide mapping is typically run by reversed-phase (RP) chromatography.[1] However, unlike RP chromatography—which provides very low retention of glycopeptides—HILIC offers significantly more retention for glycopeptides than peptides. This results in a very distinct elution profile between the two, allowing for considerably more resolution than RP. In this study, a HILIC-UV/FLD method was developed and glycan identification was performed with LC-MS experiments. HILIC, paired with UV or fluorescence detection, provides a robust and unique detection tool for routine analysis (e.g. glycoform stability studies)—with retention times as qualitative and peak area as quantitative information.

Remsima® and Inflectra® were the first mAb biosimilars to be approved in the European Union. Remsima and Inflectra are both infliximab biosimilars to the originator Remicade® (Janssen).[2] During their evaluation, as per the ICH Q6B and EMA guidelines[3], these mAbs had to meet a significant number of strict criteria to be granted biosimilarity by the EMA (European Medicines Agency).[4] The evaluation of the glycosolated sites found on the heavy chain of mAbs are among the most critical to characterize, as glycosylation of these sites affects the potency and efficacy of the biotherapeutic.

Many conditions during the up- and downstream processing affect the manifestation of PTMs. Glycosylation is characterized with an array of chromatographic techniques. Depending on the scope of the analysis, glycans may be cleaved from the protein and then analyzed either natively or after fluorescent labelling, mostly with MS or fluorescence detection, respectively. Additionally, glycans can be analyzed at the glycopeptide level after enzymatic digestion of the protein with MS detection.

The column was operated by the Vanquish Flex Quaternary UHPLC system. The Vanquish Flex Quaternary system features a low pressure mixing pump for highest application flexibility. In addition, all Vanquish UHPLC systems feature SmartInject technology. SmartInject technology significantly improves the retention time precision, thereby increasing the confidence in peak assignment.[5]

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Thermo Scientific Accucore Amide HILIC, 2.6 µm, 150 Å, 2.1 × 150 mm (P/N 16726-1520130)
- Thermo Scientific SMART Digest kit (P/N 60109-101)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Thermo Scientific™ Pierce™ C18 spin columns (P/N 89870)
- Thermo Scientific™ formic acid, LC-MS Grade (for pH adjustment) (P/N 85178)
- Thermo Scientific™ 9 mm MS certified clear screw thread kit: (P/N C4000-LV1W)
- Sample vials, with insert and 9 mm vial screw caps with pre-assembled septa
- Fisher Scientific™ trifluoroacetic acid, Optima™ LC-MS Grade (P/N 1015347)
- Fisher Scientific™ ammonium formate, Optima™ LC-MS Grade (P/N A11550)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Fisherbrand™ Premium Microcentrifuge Tubes: 1.5 mL (P/N 05-408-129)

Recommended lab equipment

- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)
- Virtuoso 9 mm Wide Opening SureStop™ Screw Thread Vial Convenience Kit (P/N 60180-VT405)
- Thermo Scientific™ Digital Heating Shaking Drybath (P/N 88880028)
- Thermo Scientific™ Orion Star™ A211 pH Benchtop Meter (P/N1 3-645-519)
- Fisher Scientific™ Microcentrifuge (Benchtop) (P/N 3722L)
- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)

Sample preparation

A commercially available mAb, infliximab drug product (Hospira UK Limited, Leamington Spa, United Kingdom), was supplied at a concentration of 10 mg/mL in formulation buffer. The sample was digested for 45 minutes at 70°C at 1,200 rpm using the Heating Shaking Drybath in conjunction with the SMART Digest kit. Upon completion of the enzymatic digestion, the peptides were reduced of all disulfide linkages by the addition of 5 mM DTT in the final volume. Reduction took place for thirty minutes at room temperature under cover from any source of light radiation. A solid phase extraction purification of the reduced peptides was performed using the C-18 Spin Columns. After each column was washed with 50% acetonitrile and equilibrated with 5% acetonitrile with 0.5% trifluoroacetic acid (TFA), the samples were loaded (150 µL per column) and washed with 5% acetonitrile with 0.5% TFA to remove the SMART Digest buffer and excess DTT. The peptides were finally eluted and solvated in 80% acetonitrile, which provided a sufficiently non-polar solution, suitable for HILIC chromatographic starting conditions allowing effective loading retention and subsequent gradient separation. Diluted samples were aliquoted into sample vials and stored at 4°C in the Vanquish autosampler prior to analysis.

Instrumentation

The separation was achieved in HILIC mode by using the Accucore 150 Amide HILIC column. The column was operated by the Vanquish Flex Quaternary UHPLC system (Table 1 and Table 2). Detection was performed using the Thermo Scientific™ Vanquish™ Diode Array Detector HL with a LightPipe™ 10 mm standard flow cell or the Thermo Scientific™ Vanquish™ Fluorescence Detector F with a 2 µL micro bio flow cell.

- The Vanquish Flex UHPLC system consisted of the following:
  - Flex System Base (P/N VF-S01-A)
  - Quaternary Pump (P/N VF-P20-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A) with 25 µL and 100 µL sample loops
  - Diode Array Detector HL (P/N VH-D10-A) with LightPipe 10 mm standard flow cell (P/N 6083.0100)
  - Fluorescence Detector F (P/N VF-D50-A) with 2 µL micro bio flow cell (P/N 6079.4330)
  - Static Mixer for 200 µL mixing volume (P/N 6044.5110)

The Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer was used for MS detection. The detailed MS source and method parameters are given in Tables 3 and Table 4.

Separation conditions

Table 1. Chromatographic conditions.

UHPLC Experimental Conditions	
Column:	Accucore Amide 150 Å HILIC 2.1 × 150 mm
Mobile Phase:	A: Acetonitrile/water (90:10 v/v) with 10 mM ammonium formate B: 10 mM Ammonium formate, pH 4.4 Buffers filtered through 0.2 µm filter membrane before use.
Gradient:	Full & Short as described in Table 2.
Flow Rate:	0.5 mL/min
Temperature:	50°C still-air
Injection Volume:	24–50 µL of ~ 0.25 µg/µL digested infliximab sample
UV Detection:	280 nm, DAD
FLD Detection:	280 nm excitation 304 nm emission (filter wheel, auto)

Table 2. Chromatographic gradient conditions.

Gradient (Full)		Gradient (Short)	
Time (min)	B	Time (min)	B
0	10%	0	25%
45	60%	1	30%
46	80%	15	35%
50	80%	16	80%
51	10%	20	80%
70	10%	21	25%
		40	25%

Table 3. Mass spectrometer source conditions.

Source:	Ion Max source with HESI-II probe
Sheath Gas Pressure:	25 psi
Auxiliary Gas Flow:	10 arbitrary units
Probe Heater Temperature:	350°C
Capillary Temperature:	320°C
S-Lens RF Voltage:	60 V
Source Voltage:	3.5 kV

Table 4A. Mass spectrometer conditions (full MS).

Full MS Parameters	
Full MS Mass Range:	*m/z* 400–2000
Resolution Settings:	120,000 (FWHM at *m/z* 200)
Target Value:	3e6
Max Injection Time:	100 ms
Default Charge State:	2
SID:	0 eV

Table 4B. Mass spectrometer conditions (MS^2).

MS^2 Parameters	
Resolution Settings:	15,000 (FWHM at *m/z* 200)
Target Value:	5e5
Isolation Width:	2.0 *m/z*
Signal Threshold:	1e4
Normalized Collision Energy (HCD):	27
Top-N MS2:	3
Max Injection Time:	250 ms
Dynamic Exclusion:	5.0 s

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2 SR5, was used for data acquisition and Thermo Scientific™ BioPharma Finder™ software, version 2.0, was used for data analysis.

Results and discussion

The separation of a tryptic digest of infliximab was obtained with a 45 minute gradient and a total analysis time of 75 minutes, including column wash with high buffer content and re-equilibration at initial conditions. Figure 1 shows a UV chromatogram with an overlay of five subsequent 24 µL injections of infliximab tryptic digest, with the glycopeptide region highlighted between 28.0 and 38.0 minutes. Note the separation of the two structural isomers A2G1Fa and A2G1Fb at approximately 32.0 to 32.5 minutes. The repeatability was assessed using the retention time standard deviation (SD) and relative standard deviation (RSD) of all glycopeptides automatically calculated by Chromeleon CDS. The RSD was below 0.05% for all four main glycopeptides (Table 5). All retention time SDs were in the range of 0.013 to 0.017 minutes. This data shows excellent flow delivery and composition precision of a long shallow gradient. It has been previously demonstrated[5,6] that with repeatable gradient delivery as well as improved retention time and high area precision, LC-UV-MS (or LC-FLD-MS) can be used to identify and quantitate peptide mapping results, which can then be easily transferred to an LC-UV (or LC-FLD) QA/QC stability monitoring application. Of course, if FLD is to be used, it is important to ensure that the monitored peptides contain at least one of the three fluorescent amino acids (tryptophan, tyrosine, or phenylalanine).

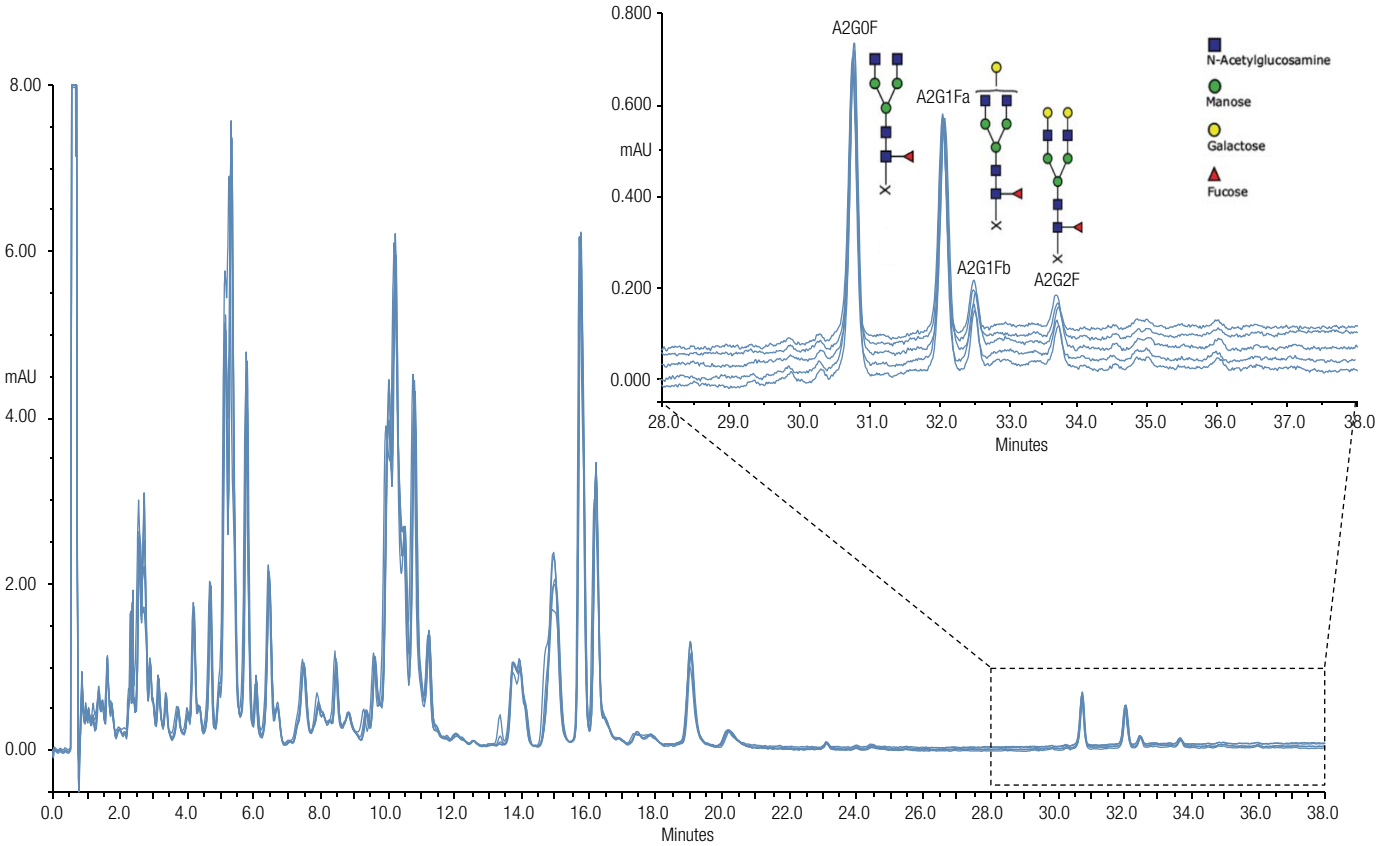


Figure 1. UV chromatogram overlay of five subsequent (24 µL) injections of infliximab tryptic digest, highlighting the excellent retention time precision and the distinct glycopeptide region showing the four most abundant glycoforms.

Table 5. Table of retention time standard deviations of a Vanquish Flex system for the glycopeptide analysis of an Infliximab tryptic digest

Peak	Average Retention Time (min)	Standard Deviation of Retention Time (min)	Relative Standard Deviation of Retention Time (%)
A2G0F	30.761	0.013	0.042
A2G1Fa	32.058	0.016	0.050
A2G1Fb	32.503	0.017	0.052
A2G2F	33.701	0.015	0.045

An optimized short gradient method (Table 2) was also developed to enable the efficient separation of the glycopeptides in the corresponding region of the chromatogram with a significant loss in resolution of the non-glycosylated peptides. Figure 2 shows non-glycosylated peptides eluting between 0.0 and 7.0 minutes followed by the glycopeptide region starting at 7.0 minutes. The short gradient allows the glycosylation to be characterized in a shorter time, saving time and solvents, without a loss in glycopeptide peak resolution.

For further identification of the glycan structures, a 100 µL loop was installed and injections of 50 µL were loaded onto the column. This allowed more peaks to be resolved with UV and sufficient sensitivity for MS1 and MS2 identification. The corresponding UV trace and extracted ion chromatograms (XIC) are shown in Figure 3. Glycopeptides were analysed using high-energy collision dissociation (HCD) fragment ion spectra, which contain ions exclusively representing sequential loss of glycan residues and no fragment ions representing the decomposition of the peptide. All 18 glycopeptide peaks could be identified on the MS1 as well as MS2 level using the Biopharma Finder software (Table 6) and are visualized with XICs in Figure 3. All peaks could also be detected with UV, however quantitation is limited to the four most abundant peaks in the chromatogram.

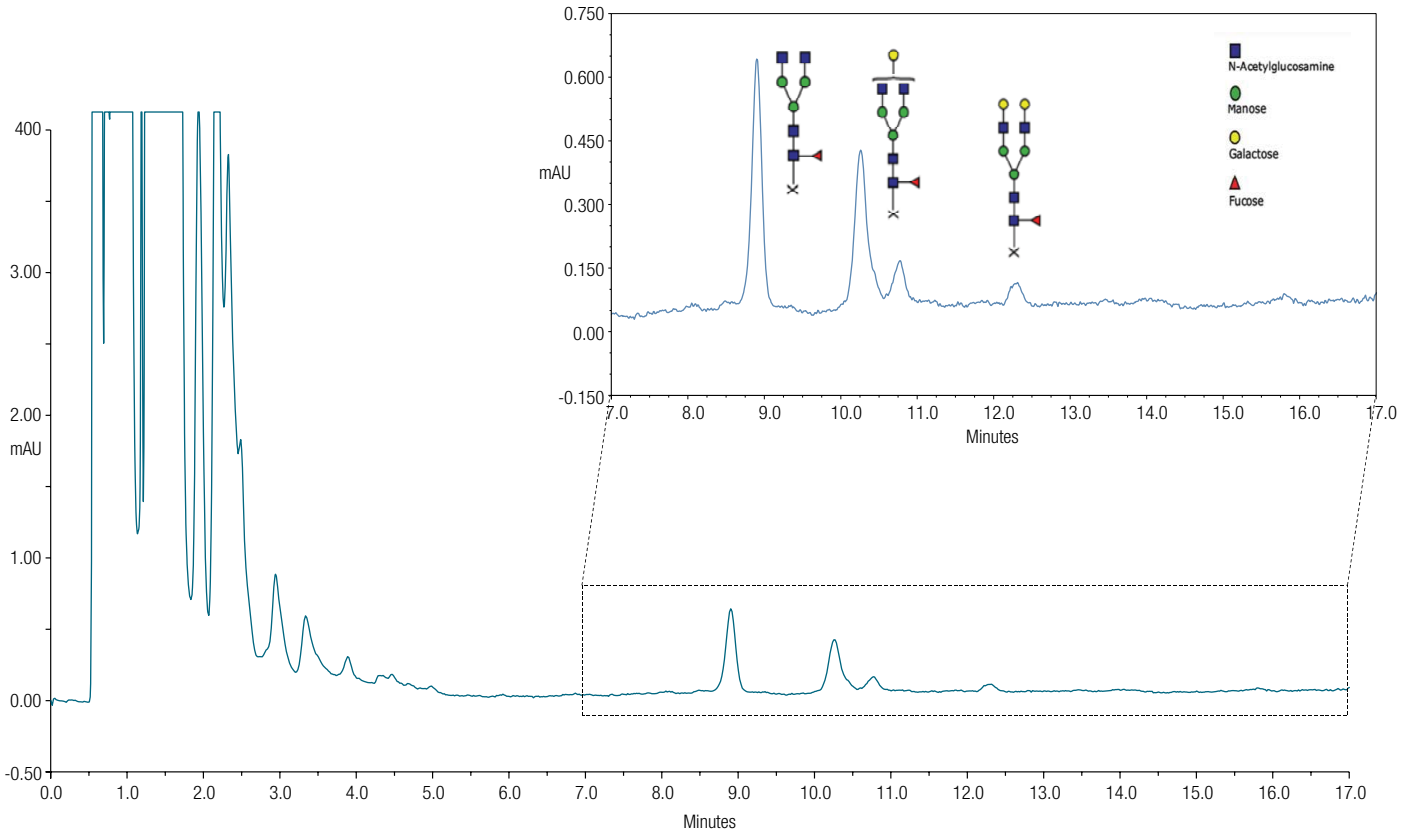


Figure 2. Chromatogram illustrating the short gradient separation of the glycopeptides from the non-glycosylated peptides (24 µL injection).

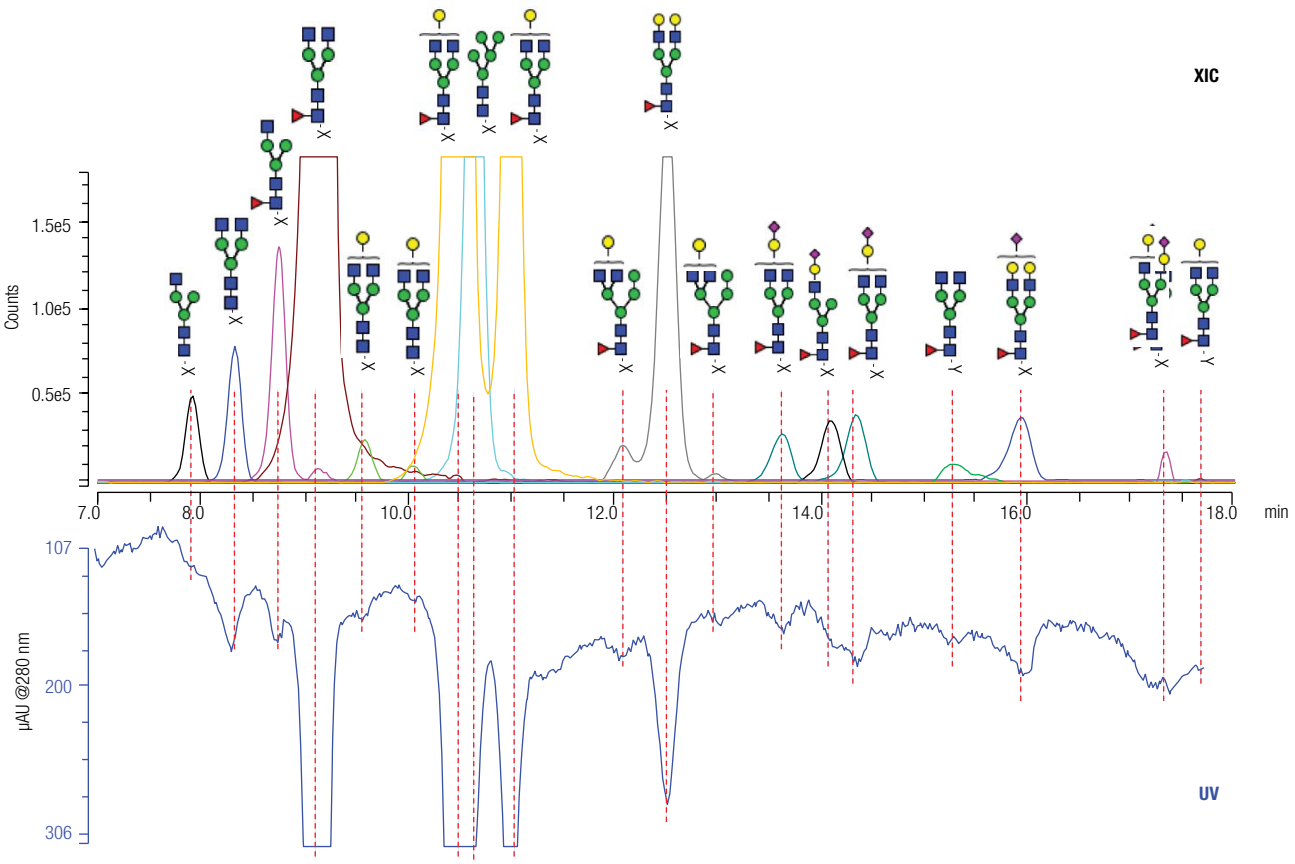
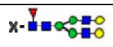
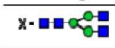
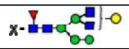
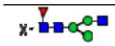
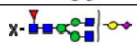
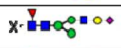


Figure 3. A mirrored overlay of XIC (top) and UV (bottom) detection methods. XIC and UV resulting after 50 µL on-column injections of infliximab tryptic digest, both utilizing the short gradient. Peptide X= EEQYNSTYR and (missed-cleaved) peptide Y = TKPREEQYNSTYR.

Table 6. Glycan structure, name, retention time, and their *m/z* detected.

Structure	Glycan	M+2H	Structure	Glycan	M+2H
	A1G0	1142.9614		A2G2F	1479.5829
	A2G0	1244.5010		A2G1M4F	1479.5839
	A1G0F	1215.9895		A2Sg1G0F	1552.1024
	A2G0F	1317.5295		A1Sg1F	1450.5619
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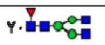
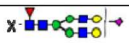
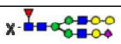
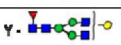
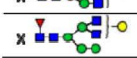
	A2G1	1325.5273		A2G0Fmc	1558.6803
	A2G1F	1398.5566		A2Sg1G1F	1633.1296
	M5	1203.4742		A2Sg1Ga1F	1714.6578
	A2G1F	1398.5566		A2G1Fmc	1720.7315
	A2G1M4F	1479.5839			

LC-UV or LC-FLD can be used when in-depth characterization is not needed (or has already been performed), for instance in stability studies. The data interpretation of these experiments is based on retention time as qualitative and peak area as quantitative information. For this reason—paired with the fact that the tryptic digest of Infliximab contains glycopeptides with fluorescent amino acids (Y - tyrosine)—fluorescence detection was also performed to compare to the LC-UV data. It is easy to overlook that peptides containing tryptophan, tyrosine or phenylalanine can be detected with FLD as well as with UV. The main advantage of FLD over UV detection is increased signal-to-noise at low analyte concentration in addition to a homogeneous response relative to the number of a specific fluorescent amino acid in the peptide sequence. This equates to increased signal for lower abundance peaks and—in particular in this case—since each glycopeptide contains two tyrosine amino acids, enables accurate quantitation using the FLD detector. UV detection is more generally applicable, however the important difference between the homogeneous response of the FLD compared to the inhomogeneous response of UV (because different molecules and functional groups absorb UV light to varying degrees) can be beneficial and should be understood. For this reason, fluorescence detection can be of particular use in many biopharma applications where homogeneous response is needed for quantitation. Figure 4 shows an overlay of two 24 µL injections, one with UV and the other with FLD detection. It is clear that the signal-to-noise ratio is larger for the FLD when compared to the UV detector.

Given the excellent precision displayed with this method, you can have confidence in your qualitative and quantitative information gathered with LC-UV or LC-FLD for the four main glycans, without the need for continued MS (Figures 1, 2, and 4).

As can be seen in Figure 4, when the signal intensities are normalized between FLD and UV detectors for the main four peaks (noting the slightly different responses between the homogeneous and inhomogeneous detectors as described earlier), it is clear that the level of noise is considerably higher for the UV detector. This means that after peaks have been confidently identified using MS, additional peaks can be confidently monitored in stability studies with FLD compared to UV detection.

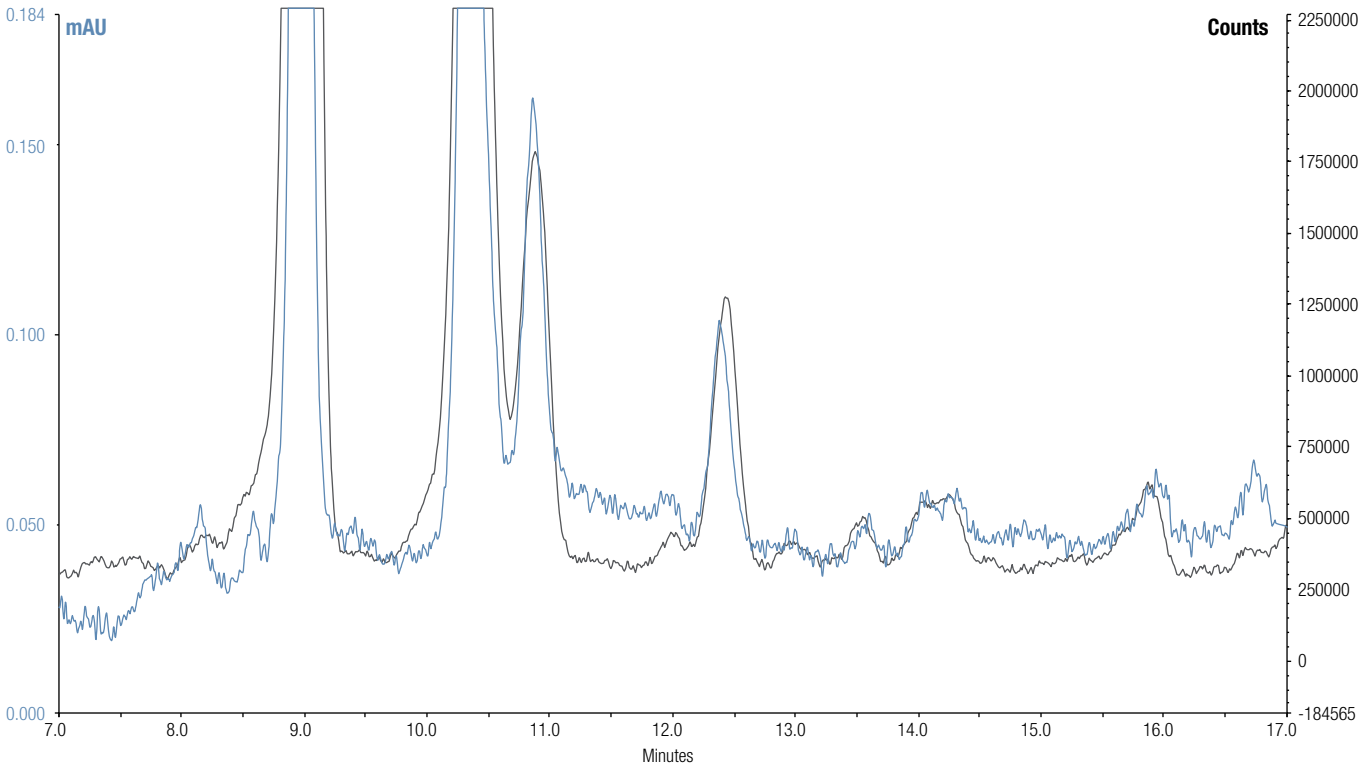


Figure 4. An overlay of UV (blue) and FLD (black) detection methods, both after 24 µL on-column injections of infliximab tryptic digest, both utilizing the short gradient.

Conclusion

- The Vanquish Flex UHPLC system combined with UV or FLD detection and coupled to the Q Exactive HF MS, provides a robust LC-MS setup to characterize and monitor glycosylation on the peptide level of mAbs.
- Using reversed-phase (RP) chromatography, the differences in hydrophobicity are negligible between two glycopeptides with the same amino-acid structure, leading to poor separation. HILIC mode has the considerable advantage in the separation of glycopeptides when compared to traditional RP chromatography, because differences in a given peptide's hydrophilicity—due to differing glycan structures—can be separated with increased resolution. Additionally, with HILIC mode the glycosylated peptides are well separated from the unglycosylated peptides because of the significant difference in hydrophilicity. This allows the unglycosylated peptides to be discarded, by means of method optimization, reducing analysis time and increasing throughput without a loss of resolution.
- A similar glyco-profile was achieved, with a much simplified sample preparation effort (when compared to labelled glycan analysis).
- This method is widely appropriate for the analysis of N-glycans found in many mAbs. However, for more complex glycoproteins (e.g. Enbrel® - etanercept)—which contain many sites of glycosylation including N- and O-linked glycans—may be too complicated to interpret. In these situations, it is worth considering released N-glycan analysis and subsequent O-glycan analysis or consider a post-digestion middle-down mass spectrometry approach.
- The effective glycopeptide separation from other non-glycosylated tryptic peptides of a monoclonal antibody (mAb) has been shown with the use of the Accucore 150 Amide HILIC column. The column provides an effective mode to separate the glycoforms based on their hydrophilic interactions.

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2. European Medicines Agency recommends approval of first two monoclonal-antibody biosimilars: http://www.ema.europa.eu/ema/index.jsp%3Fcurl=pages/news_and_events/news/2013/06/news_detail_001837.jsp%26mid=WC0b01ac058004d5c1
3. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf
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https://appslab.thermofisher.com/App/3911/glycopeptide-hilic

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AN72443-EN 0817S

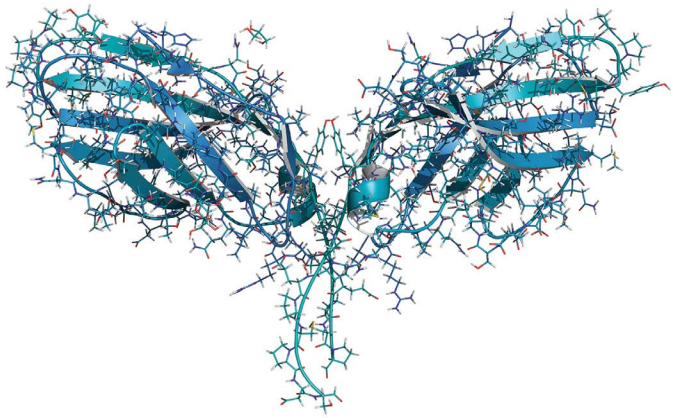
Application Note 1134

LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

Martin Samonig, Remco Swart
Thermo Fisher Scientific, Germering, Germany

Key Words
Monoclonal Antibodies, Acclaim C18 RSLC Column, Q Exactive HF Mass Spectrometer, Biocompatible UHPLC, SMART Digest Kit, Biotherapeutics Characterization, Biopharma

Goal
Prove the suitability of a Thermo Scientific™ Vanquish™ Flex system for efficient and reliable peptide mapping method development with a LC-UV-MS setup.



Introduction
Peptide mapping is one of several routine methods to characterize biopharmaceutical proteins. For research environments, this technique, if combined with mass spectrometry (MS), is utilized for the characterization and confirmation of the primary sequence of monoclonal antibodies. In addition, peptide mapping can help to identify, localize, and quantitate post-translational modifications (PTMs). Peptide mapping methods are often developed and evaluated with combined UV and MS detection, to simplify the transfer to routine environments where UV detection is used alone. In high-throughput workflows, peptide mapping experiments are performed for antibody identity confirmation, PTM characterization, and stability studies.

The new Vanquish Flex UHPLC system features a quaternary pump[1] for highest application flexibility and fully biocompatible flow path. In addition, similar to the Thermo Scientific Vanquish UHPLC system[2], the sample is pressurized prior to the injection into the high pressure flow path. This results in a highly stable flow delivery and thus significantly improved retention time precision, increasing the confidence in peak assignment in peptide mapping experiments with UV detection.[3]

In this work, the separation of peptides obtained from a monoclonal antibody digest is demonstrated with a LC-UV-MS setup.

Experimental
The commercially available monoclonal antibody rituximab (F. Hoffmann-La Roche, Ltd) was digested using the Thermo Scientific™ SMART Digest™ kit. It is designed for applications that require highly reproducible, sensitive, and fast analyses, due to its optimized, heat stable, immobilized trypsin design. The sample was 1:4 diluted with the SMART digestion buffer included in the kit, and enzymatic digestion was allowed to proceed at 70 °C for 75 min and 1400 rpm. Disulfide bonds were reduced by incubation for 30 minutes at 60 °C with 5 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP). The separation of the tryptic digest was achieved with a 30 min gradient and a total analysis time of 56 min, including the column wash with high organic eluent, and re-equilibration at initial conditions. The Vanquish Flex system was coupled to the Thermo Scientific™ Q Exactive™ HF mass spectrometer using the MS connection kit for Vanquish systems. With this setup, simultaneous UV and MS detection is feasible.

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Equipment

Vanquish Flex UHPLC system consisting of:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer

SMART Digest Kit (P/N 60109-101)

Data Analysis

Thermo Scientific™ Xcalibur™ software version 3.0 in combination with the Thermo Scientific Standard Instrument Integration (SII) for Xcalibur 1.1 SR2 was used for data acquisition and the data analysis was performed using Thermo Scientific™ PepFinder™ software version 2.0.

Results and Discussion

Peptide mapping experiments were performed with UV as well as MS detection. Figure 1 shows the overlay of the UV trace at 214 nm and the total ion current (TIC) chromatogram obtained from the mass spectrometer, which allows confident peak assignment (Figure 2).

To assess the sequence coverage, PepFinder software was used to analyze the data. The sequence coverage map (Figure 3) shows the overlap of the different peptides identified in different intensities, indicated with the color of the bar (red = high abundant, blue = low abundant), and in different lengths due to missed cleavages with sequence coverage for heavy and light chain of 99.2%. The number in the bar shows the retention time of the particular peptide.

Experimental Conditions - HPLC	
Column	Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm Analytical (2.1 x 250 mm, P/N 074812)
Mobile Phase	A: 0.1% FA in water (P/N FA 28905) B: 0.1% FA in 8/2 acetonitrile/water (v/v), (P/N acetonitrile TS-51101)
Gradient	0–30 min: 4–55% B 30–31 min: 55–100% B 31–35 min: 100% B 35–36 min: 100–4% B 36–56 min: 4% B
Flow Rate	0.3 mL/min
Temperature	50 °C
Injection Volume	2 µL
Detection	214 nm Data Collection Rate: 10 Hz Response Time 0.4 s
Flow Cell	10 mm LightPipe

Experimental Conditions - MS			
Source	HESI-II		
Sheath Gas Pressure	35 psi		
Auxiliary Gas Flow	10 arbitrary units		
Capillary Temperature	300 °C		
S-lens RF Voltage	60 V		
Source Voltage	3.5 kV		
Full MS Parameters		**MS² Parameters**	
Full MS Mass Range	200–2000 *m/z*	Resolution Settings	15.000
Resolution Settings	60.000	Target Value	1e5
Target Value	3e6	Isolation Width	2.0 Da
Max Injection Time	200 ms	Signal Threshold	1e4
Default Charge State	2	Normalized Collision Energy (HCD)	27
SID	0 eV	Top-N MS²	5
		Max Injection Time	100 ms

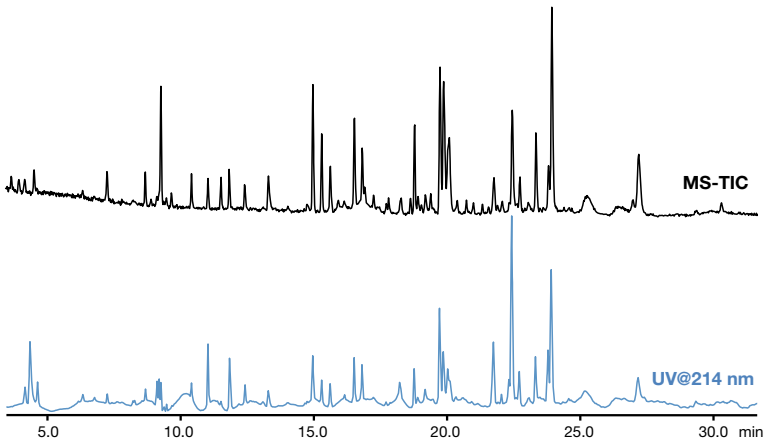

Figure 1. Overlaid chromatograms of the total ion current (TIC) and the UV trace at 214 nm of a SMART Digest Kit digested rituximab sample with subtracted blank baseline.

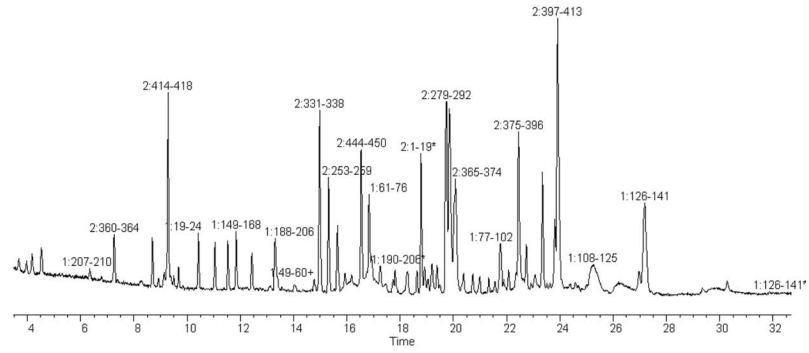

Figure 2. Peak assignment of the tryptic peptides from rituximab. Peak labels with 1 correspond to the light chain, and those with 2 correspond to the heavy chain of the mAb. The number after the colon indicates the amino acid region of this particular tryptic peptide.

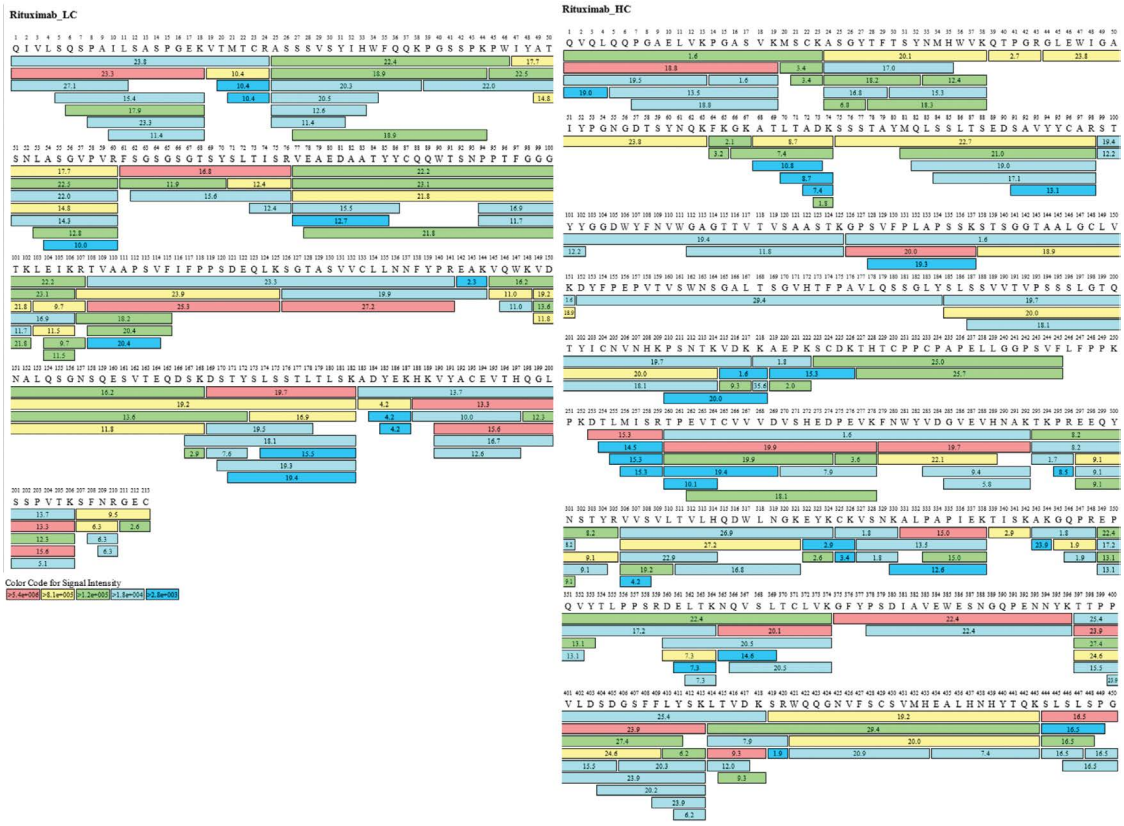

Figure 3. Sequence coverage map of the heavy (right) and light chain (left).

Table 1 shows the identification and relative quantification of a subset of monitored modifications on the light and heavy chain of rituximab, respectively. The selected modifications are deamidations, oxidations, pyro-Gln formations on the N-terminus of heavy and light chain, glycosylation of the N301 on the heavy chain, and sequence variants like C-terminal Lys (K+ variant). A tilde (~) before the modification indicates the modification was found on the tryptic peptide, but could not be localized on a specific amino acid with MS/MS spectra. The modification is labeled with recovery "Good" when the total peak area, including modified and unmodified forms of the peptide, is at least 10% of the most abundant peptide from the same protein. The recovery "Fair" means it is at least 1%.

Table 1. Identification and (relative) quantification of a specific set of modifications (oxidation, glycosylation and deamidation) on the mAb.

Protein	Modification	Recovery	Abundance
Rituximab_LC	Q1+NH_3 loss	Good	87.81%
Rituximab_LC	W90+Oxidation	Good	2.06%
Rituximab_HC	~Q1+NH_3 loss	Good	100.00%
Rituximab_HC	W281+Oxidation	Good	4.98%
Rituximab_HC	N301+A1G0F	Fair	2.87%
Rituximab_HC	N301+A1G1F	Fair	1.22%
Rituximab_HC	N301+A2G0	Fair	1.30%
Rituximab_HC	N301+A2G0F	Fair	37.69%
Rituximab_HC	N301+A2G1F	Fair	44.86%
Rituximab_HC	N301+A2G2F	Fair	10.77%
Rituximab_HC	N301+M5	Fair	1.07%
Rituximab_HC	N365+Deamidation	Good	2.72%
Rituximab_HC	W385+Oxidation	Good	5.37%
Rituximab_HC	G450+Lys	Good	3.2683%

Conclusion

For peptide mapping, especially the combination of UV and MS detection, the Vanquish Flex setup chosen for the experiments, consisting of column size of 2.1 x 250 mm coupled with Thermo Scientific™ Viper™ Fingertight Fitting connections and a flow rate of 0.3 mL/min combined with the HESI-II source on the mass spectrometer, delivers a very robust setup allowing straightforward method transfer to UV-based QC applications. The SMART Digest Kit compliments this by delivering highly reproducible digestion of samples allowing for easier and more confident data interpretation.

References

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BIOPHARMA APPLICATION COMPENDIUM

Antibody Drug Conjugates Analysis

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High-Resolution Separation of Cysteine-Linked Antibody-Drug Conjugate Mimics Using Hydrophobic Interaction Chromatography

Julia Baek and Xiaodong Liu, Thermo Fisher Scientific, Sunnyvale, CA, USA

Application Note 21079

Key Words
Hydrophobic interaction chromatography, HIC, monoclonal antibody, mAb, antibody-drug conjugate, ADC, drug-to-antibody ratio, DAR, MAbPac HIC-Butyl

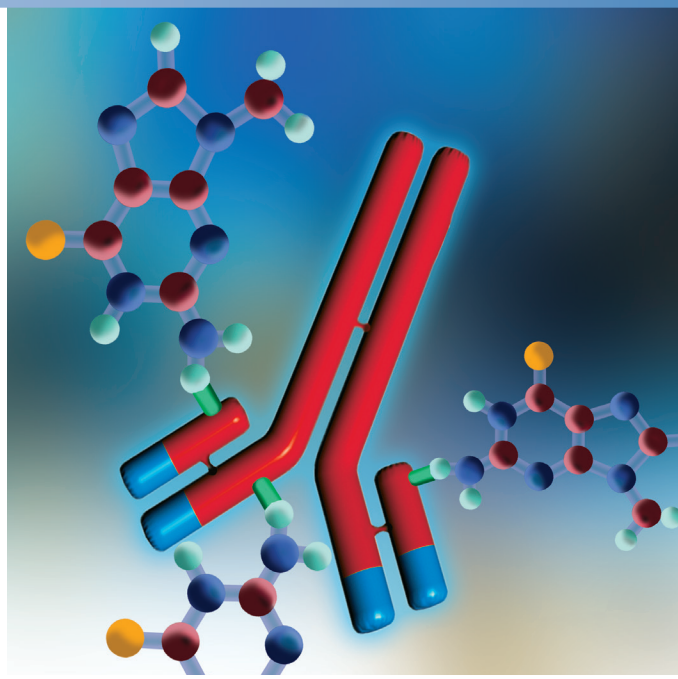
Goal

To demonstrate the analysis of antibody-drug conjugate (ADC) mimics using a Thermo Scientific™ MAbPac™ HIC-Butyl column.

Introduction

Monoclonal antibody drugs are the fastest growing class of biotherapeutics and have become a major part of the biopharmaceutical market. Monoclonal antibodies have demonstrated their effectiveness against autoimmune disorders, cardiovascular diseases, infectious diseases, and cancer.[1] Monoclonal antibodies selectively recognize antigens that are present on the tumor cells and exert their cytotoxic effect by receptor agonist activity, immune response, or vascular and stromal cell ablation. However, most of these mAbs are used in combination with chemotherapy, and many others have shown a lack of clinical efficacy. The success and the limitation of monoclonal antibodies have fueled the development of another form of antibody based therapeutics–antibody-drug conjugates (ADCs).[2] Antibody-drug conjugates utilize the exquisite selectivity of the antibody to achieve targeted delivery of cytotoxic drugs. ADCs have gained tremendous interest among pharmaceutical companies due to their significantly improved clinical efficacy over native monoclonal antibodies.[3]

The conjugation of drugs often results in an ADC molecule that is heterogeneous with respect to both the distribution and loading of cytotoxic drugs on the mAb. The number of drugs attached to the mAb has been shown to directly affect the safety and the efficacy of the drug. Unconjugated mAbs have significantly lower potency, and the ADCs with high drug load are subject to rapid renal clearance. Therefore, it is critical to fully characterize and monitor the heterogeneity of ADCs during development and production.[4]



Hydrophobic interaction chromatography (HIC) is a technique for separation of proteins, including monoclonal antibodies, by hydrophobicity. The HIC mobile phase usually consists of a salting-out agent, which at high concentration retains the protein by increasing hydrophobic interaction between the protein and the stationary phase. Bound proteins are eluted by decreasing the salt concentration.

HIC has been widely used as an orthogonal method to size-exclusion chromatography and ion-exchange chromatography for the characterization of mAb heterogeneity. Analysis of succinimides, antibody fragments, oxidated mAbs, and C-terminal lysine modifications were successfully carried out to monitor the stability and, in some cases, the potency of the drug.[5,6] HIC is also suitable for the separation of ADCs since attachment of cytotoxin alters the hydrophobicity of the antibody. The least hydrophobic unconjugated antibody

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elutes first and as the number of drugs attached increases the elution time increases. Therefore, HIC is considered to be the method of choice to characterize the distribution of ADC molecules with different drug-to-antibody ratios (DARs).[7]

The MAbPac HIC-Butyl column is a polymer-based HIC column designed for separation of mAbs and variants, including ADCs. The hydrophilic nature of polymer particles and the optimal density of butyl functional groups lead to excellent biocompatibility, low carryover, and high resolution. Here we describe the analysis of ADC samples that were conjugated via interchain disulfide bonds on the MAbPac HIC-Butyl column.

Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- 2-Propanol (Fisher Scientific P/N A461-4)
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, ≥98.0%)
- Ammonium sulfate [$(NH_4)_2SO_4$, ≥99.0%]

Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Cysteine-conjugated ADC mimic samples were provided by a customer. 10 mg/mL ADC mimic samples were diluted in half with 2 M $(NH_4)_2SO_4$, 100 mM NaH_2PO_4, pH 7.0 solution. 25 mg/mL control mAb was diluted five-fold with 2 M $(NH_4)_2SO_4$, 100 mM NaH_2PO_4, pH 7.0 solution.

LC Separation

The LC separation conditions were as follows:

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRSLC system equipped with:		
	SR-3000 Solvent Rack (without degasser) (P/N 5035.9200)		
	LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)		
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)		
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)		
	VWD-3400RS Rapid Separation Variable Wavelength Detector) equipped with a micro flow cell (P/N 5074.0010		
Column	MAbPac HIC-Butyl, 4.6 × 100 mm (P/N 088558)		
Mobile phase A	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / 2-propanol (95:5 v/v) Dissolve 6.55 g of $NaH_2PO_4 \cdot H_2O$ and 188.30 g of $(NH_4)_2SO_4$ in 750 mL DI water, adjust the pH to 7.0 using 50% sodium hydroxide (NaOH) solution. Bring the volume to 950 mL with DI water. Then bring the volume to 1000 mL with 2-propanol.		
Mobile phase B	50 mM sodium phosphate, pH 7.0 / 2-propanol (80:20 v/v) Dissolve 5.52 g of $NaH_2PO_4 \cdot H_2O$ in 750 mL DI water, adjust the pH to 7.0 using 50% NaOH solution. Bring the volume to 800 mL with DI water. Then bring the volume to 1000 mL with 2-propanol.		
Gradient	Time (min)	%A	%B
	-5.0	100	0
	0.0	100	0
	1.0	100	0
	15.0	0	100
	20.0	0	100
Flow rate	1.0 mL/min		
Run time	20 min		
Injection volume	5 µL		
Temperature	25 °C		
UV detector wavelength	280 nm		

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

The ADC mimics used in this work were conjugates between a drug mimic and mAb via the sulfhydryl group of interchain cysteine residues (Figure 1a). This procedure results in a mixture of drug-loaded antibody species with 0 to 8 drugs (Figure 1b). The stoichiometry of the drug mimic was varied in the conjugation reaction. Samples are labeled low, moderate, and high based on the average number of the drug mimic attached to the mAb. The typical separation of cysteine-conjugated ADC mimic on a MAbPac HIC-Butyl column is demonstrated in Figure 2. HIC peak identities were assigned by examining the UV spectra of each peak using a diode array detector using a literature method.[7]

Concentration of organic solvent in mobile phases and temperature are critical factors for the resolution and peak shape when analyzing protein samples in HIC. The best peak shape and separation was achieved using 5% 2-propanol in mobile phase A and 20% 2-propanol in mobile phase B at 25 °C. Without organic solvent, DAR species were not well resolved, while addition of more 2-propanol in mobile phase A or mobile phase B resulted in a very broad peak for the 8-drug form, probably due to denaturing of the heavy and light chain. All the ADC species with 0 to 8 drugs were observed with three additional peaks for the 6-drug species. This indicates there are at least four hydrophobic variants of the 6-drug species.

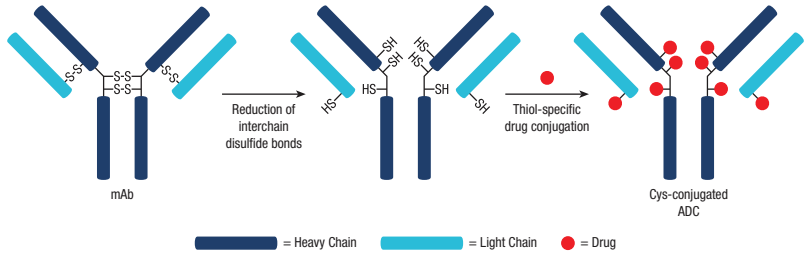

Figure 1a. Schematic representation of conjugation of drug mimic via interchain cysteine residues.

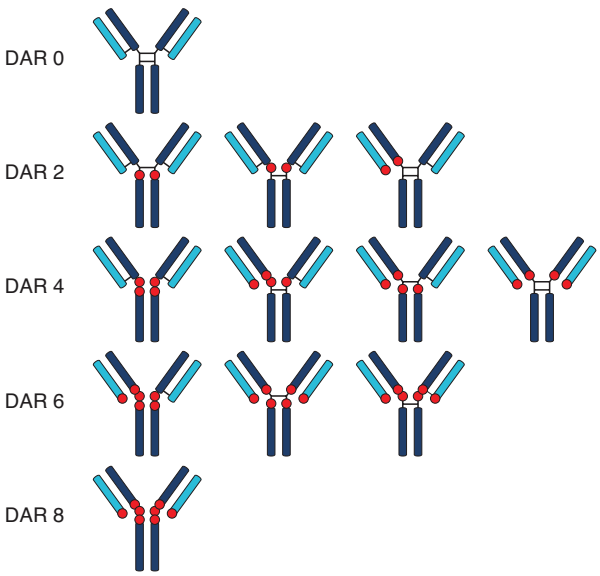

Figure 1b. Heterogeneity of cysteine-conjugated ADCs.

Column:	**MAbPac HIC-Butyl**, 5 µm		
Format:	4.6 × 100 mm		
Mobile Phase A:	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / 2-propanol (95:5 v/v)		
Mobile Phase B:	50 mM sodium phosphate, pH 7.0 / 2-propanol (80:20 v/v)		
Gradient:	Time (min)	%A	%B
	-5.0	100	0
	0.0	100	0
	1.0	100	0
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	5 µL		
Temp.:	25 °C		
Detection:	UV (280 nm)		
Sample:	Cys-conjugated ADC mimic (5 mg/mL)		

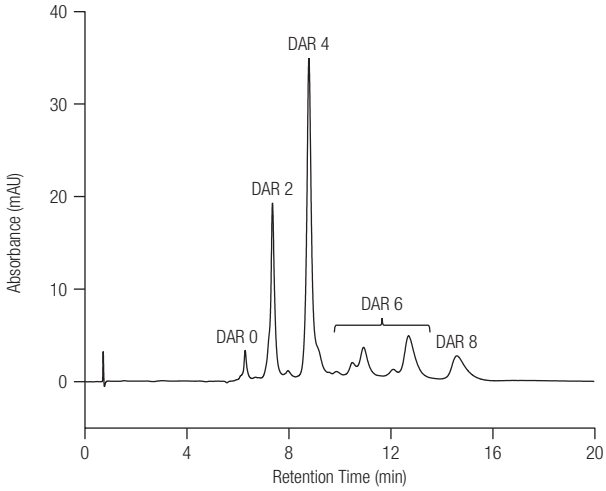

Figure 2. Separation of cysteine-conjugated ADC mimic.

Figure 3 compares the analysis of cysteine-conjugated ADC mimics with different drug loads assuming the UV absorption of the drug mimic is minimal at 280 nm. The mimic with high drug load shows no unconjugated mAb and a higher intensity of the 8-drug form while the low drug load sample contains a significant amount of unconjugated mAb, 2-drug form, and 4-drug form.

Column	**MAbPac HIC-Butyl**, 5 µm		
Dimension:	4.6 × 100 mm		
Mobile Phase A:	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / 2-propanol (95:5 v/v)		
Mobile Phase B:	50 mM sodium phosphate, pH 7.0 / 2-propanol (80:20 v/v)		
Gradient:	Time (min)	%A	%B
	-5.0	100	0
	0.0	100	0
	1.0	100	0
	15.0	0	100
	20.0	0	100
Flow Rate:	1.0 mL/min		
Inj. Volume:	5 µL		
Temp.:	25 °C		
Detection:	UV (280 nm)		
Sample:	a. Unconjugated mAb (5 mg/mL) b. Cys-conjugated ADC mimic (low load; 5 mg/mL) c. Cys-conjugated ADC mimic (moderate load; 5 mg/mL) d. Cys-conjugated ADC mimic (high load; 5 mg/mL)		

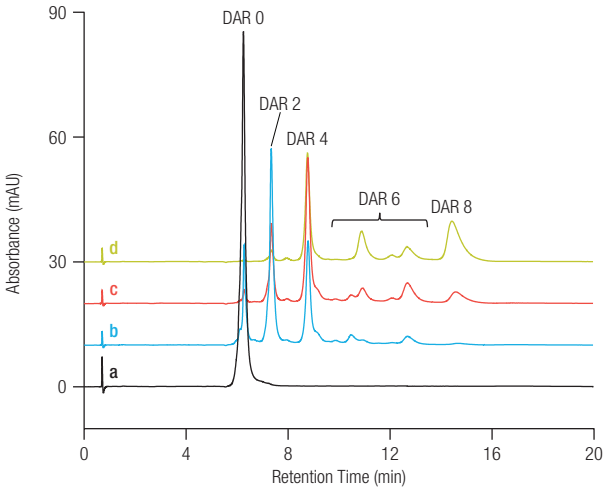

Figure 3. Comparison of cysteine-conjugated ADC mimics with different drug loads

Conclusion

- The MAbPac HIC-Butyl column provides high resolution for unconjugated mAbs and cysteine-linked ADCs with different drug-to-antibody ratios (DARs).
- The drug load distribution can be monitored using the MAbPac HIC-Butyl column.

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Analysis of Monoclonal Antibodies and Antibody-Drug Conjugates Using New Hydrophobic Interaction Chromatography (HIC) Columns

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Thermo Fisher Scientific, Sunnyvale, CA

Poster Note 21218

Overview

Purpose: Demonstrate high resolution separation of mAbs and ADC mimics using Thermo Scientific™ MAbPac™ HIC columns.

Methods: Ammonium sulfate and sodium phosphate mobile phases were used. In some cases, addition of isopropanol to both mobile phases increased the resolution of the chromatogram.

Results: MAbPac HIC columns were developed using advanced surface bonding technology to achieve unique selectivity, high recovery and high efficiency. High resolution separation of proteins and various mAb samples were successfully carried out using MAbPac HIC columns.

Introduction

Various types of monoclonal antibody (mAb) products including intact mAbs, mAb fragments, engineered variants, and antibody-drug conjugates (ADCs) are being developed for the treatment of cancer and other diseases due to their excellent biocompatibility and high selectivity. The proliferation of monoclonal antibody therapeutics and their susceptibility to various biochemical modifications has highlighted the importance of characterizing these highly heterogeneous products for their safety and efficacy.

Hydrophobic interaction chromatography (HIC) is a technique for separation of proteins and has been widely used as an orthogonal method to size exclusion chromatography and ion exchange chromatography for the characterization of mAb variants.

Here we introduce a new family of HIC columns designed for the analysis of mAbs and related biologics. Three different ligand chemistries-polyamide, amide and butyl-were developed for the analysis of a wide range of mAb samples. Separation of mAb aggregates, mAb fragments, oxidized mAbs, and antibody-drug conjugates were successfully carried out with excellent efficiency and high recovery.

Methods

Samples

Monoclonal antibody samples and ADC sample were donated by biotech companies. Proteins and other chemicals were from Sigma-Aldrich®.

Columns

MAbPac HIC-10, 5 µm, 4.6 × 100 mm (P/N 088480)
MAbPac HIC-20, 5 µm, 4.6 × 100 mm (P/N 088553)
MAbPac HIC-20, 5 µm, 4.6 × 250 mm (P/N 088554)
MAbPac HIC-Butyl, 5 µm, 4.6 × 100 mm (P/N 088558)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:

- SR-3000 Solvent Rack (P/N 5035.9200)
- LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
- WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- VWD-3400RS Rapid Separation Variable Wavelength Detector (VWD) equipped with micro flow cell (P/N 5074.0010)
- Chromatography was controlled by Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System .

Mobile phases

Formula 1

Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0

Mobile phase B: 100 mM sodium phosphate, pH 7.0

Formula 2

Mobile phase A: 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)

Mobile phase B: 50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)

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Method Development

Ammonium sulfate and sodium phosphate based mobile phases were used. For the applications with mAb and ADC mimics, mobile phases were optimized by either lowering the starting salt concentration by adjusting the gradient using formula 1 or adding isopropanol into both mobile phase A and mobile phase B (formula 2). For most applications, lower flow rate (0.5 mL/min) using longer gradient increased the resolution.

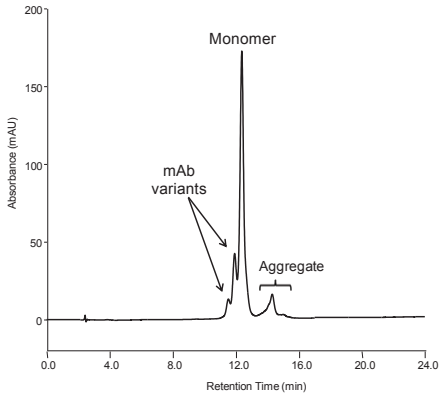
All three MAbPac HIC columns were screened to obtain the best separation.

Results

Analysis of mAb Aggregates

Protein and antibody aggregates are formed either during product expression in cell culture, downstream processing or storage. These aggregates may cause undesirable immune reactions which affect the safety of the drug. SEC is the most widely used technique for the detection and quantification of protein aggregates in biological drug products. However several researchers have reported the use of HIC for the removal of protein aggregates.[1,2] Figure 1 demonstrates the separation of monoclonal antibody aggregates from the monomer form on the MAbPac HIC-10 column. In addition to the separation of aggregates, hydrophilic mAb variants were also detected using the MAbPac HIC-10 column.

FIGURE 1. Separation of mAb aggregates



Column: MAbPac HIC-10, 5 µm
Format: 4.6 × 100 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

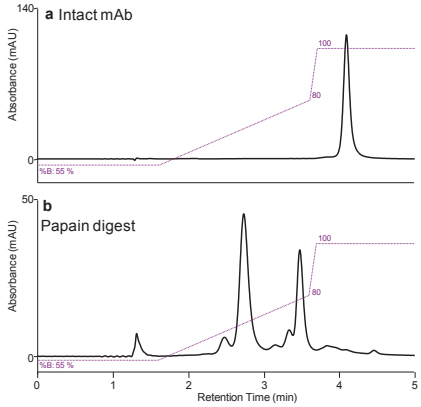
Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
29.0	0	100
34.0	0	100

Temperature: 20 °C
Flow rate: 0.5 mL/min
Inj. volume: 10 µL (4 mg/mL)
Detection: UV (280 nm)
Sample: Monoclonal antibody

Analysis of mAb Fragments

Analysis of antibody fragments is important for both characterization of Fab or Fc based biotherapeutics and localization of the sources of heterogeneities on a monoclonal antibody molecule. HIC can provide the resolution required for the separation of Fab and Fc fragments and their hydrophilic or hydrophobic variants.[3] Figure 2 shows a comparison of an intact mAb and its papain digest on the MAbPac HIC-20 column. Fast and high resolution separation of Fab and Fc fragmenst was achieved by simply optimizing the gradient method. The MAbPac HIC-20 column efficiently separated Fab and Fc fragments and further separated variants of these fragments. These variant peaks imply oxidation or other modifications in these fragments.

FIGURE 2. Separation of papain digested mAb
(a) Intact mAb, (b) Papain digested mAb



Column: MAbPac HIC-20, 5 µm
Format: 4.6 × 100 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

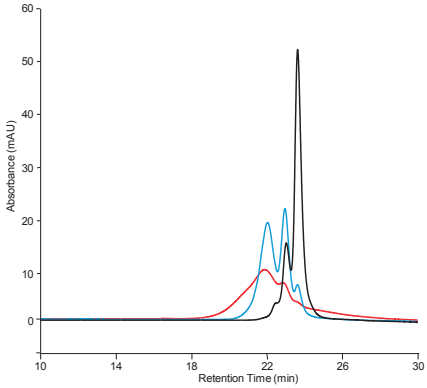
Time (min)	%A	%B
-5.0	45	55
0.0	45	55
2.0	45	80
2.1	0	100
5.0	0	100

Temperature: 30 °C
Flow rate: 1.0 mL/min
Inj. volume: Intact mAb: 12 µL
Papain digest: 12 µL
Detection: UV (280 nm)
Sample: a) Intact mAb (1 mg/mL)
b) Papain digest (1 mg/mL)

Analysis of oxidized mAb

Oxidation of therapeutic mAbs during production or storage is a common degradation mechanism and has become a major concern in mAb production. In many cases, oxidized mAbs have less to no potency compared to their native form.[4] Oxidation of amino acid residues on a mAb can alter the hydrophobic nature of the mAb by either the increase in polarity of the oxidized form or the resulting conformational change. Hydrophobicity-based HPLC methods such as reverse phase chromatography and HIC are typically used to characterize oxidized mAb products. A mAb sample was oxidized using hydrogen peroxide or 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). The MAbPac HIC-20 provided good resolution of oxidized mAb variants from unmodified mAb without fragmentation, or other sample preparation. The best result was obtained using a longer 250 mm column and lower flow rate.

FIGURE 3. Separation of oxidized mAb



Column: MAbPac HIC-20, 5 µm
Format: 4.6 × 250 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-6.0	50	50
0.0	50	50
2.0	50	50
30.0	0	100
35.0	0	100

Temperature: 30 °C
Flow rate: 0.5 mL/min
Inj. volume: Untreated mAb: 20 µL (1.25 mg/mL)
Oxidized mAb: 20 µL (1.25 mg/mL)
Detection: UV (280 nm)
Sample: Untreated mAb
H_2O_2 oxidized mAb
AAPH oxidized mAb

Analysis of Antibody-Drug Conjugate Mimic

Hydrophobic interaction chromatography is often used for the separation of ADCs with different drug-to-antibody ratios (DARs) since attachment of cytotoxin alters the hydrophobicity of the antibody.[5] The least hydrophobic unconjugated antibody elutes first and as the number of attached drugs increases the elution time of each ADC increases as well. Below is the separation of a cysteine-conjugated ADC mimic sample on the MAbPac HIC-Butyl column. The ADC mimics were conjugates between a drug mimic and mAb via the sulfhydryl group of interchain cysteine residues which results in a mixture of drug-loaded antibody species with 0 to 8 drugs (Figure 4a). The unmodified mAb and ADCs with DAR values ranging from 2 to 8 are well resolved by the MAbPac HIC-Butyl column (Figure 4b). The best peak shape and separation were achieved using 5% IPA in mobile phase A and 20% IPA in mobile phase B at 25 ºC. Figure 5 compares the analysis of cysteine-conjugated ADC mimics with different drug loads assuming the UV absorption of the drug mimic is minimal at 280 nm. The mimic with high drug load showed no unconjugated mAb and a higher intensity of the 8-drug form while the low drug load sample contains a significant amount of unconjugated mAb, 2-drug form, and 4-drug form.

FIGURE 4. Separation of Cys-conjugated ADC mimic (a) Schematic representation of conjugation of drug mimic via interchain cysteine residues (b) Separation of Cys-conjugated ADC mimic on MAbPac HIC-Butyl

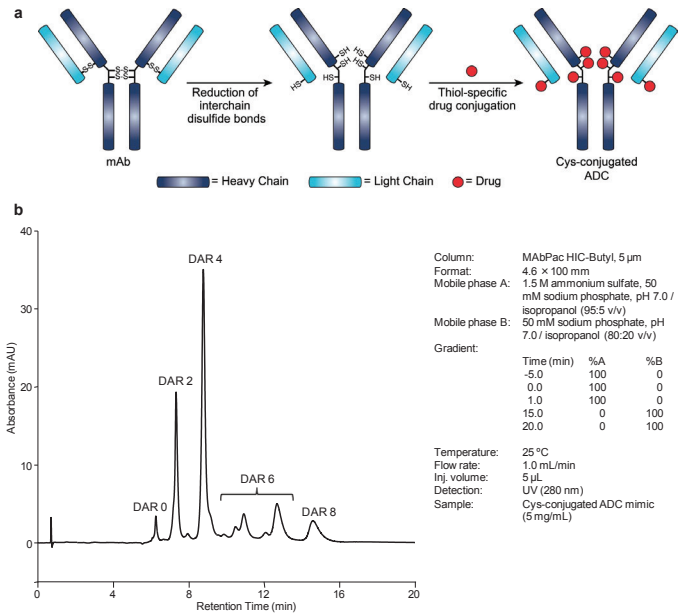
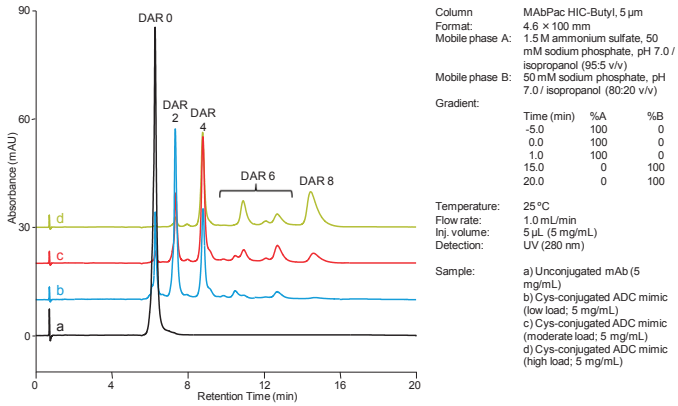


FIGURE 5. Comparison of Cys-conjugated ADC mimics with different drug loads



Conclusion

- MAbPac HIC columns with three different ligand chemistries were developed for the analysis of monoclonal antibodies (mAbs) and related biologics.
- High resolution separation of various mAb samples including mAb aggregates, mAb fragments, oxidized mAb, and ADC mimic were obtained using MAbPac HIC columns.

References

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5. Ouyang, J. *Methods in Molecular Biology* (2013) 1045, 275.

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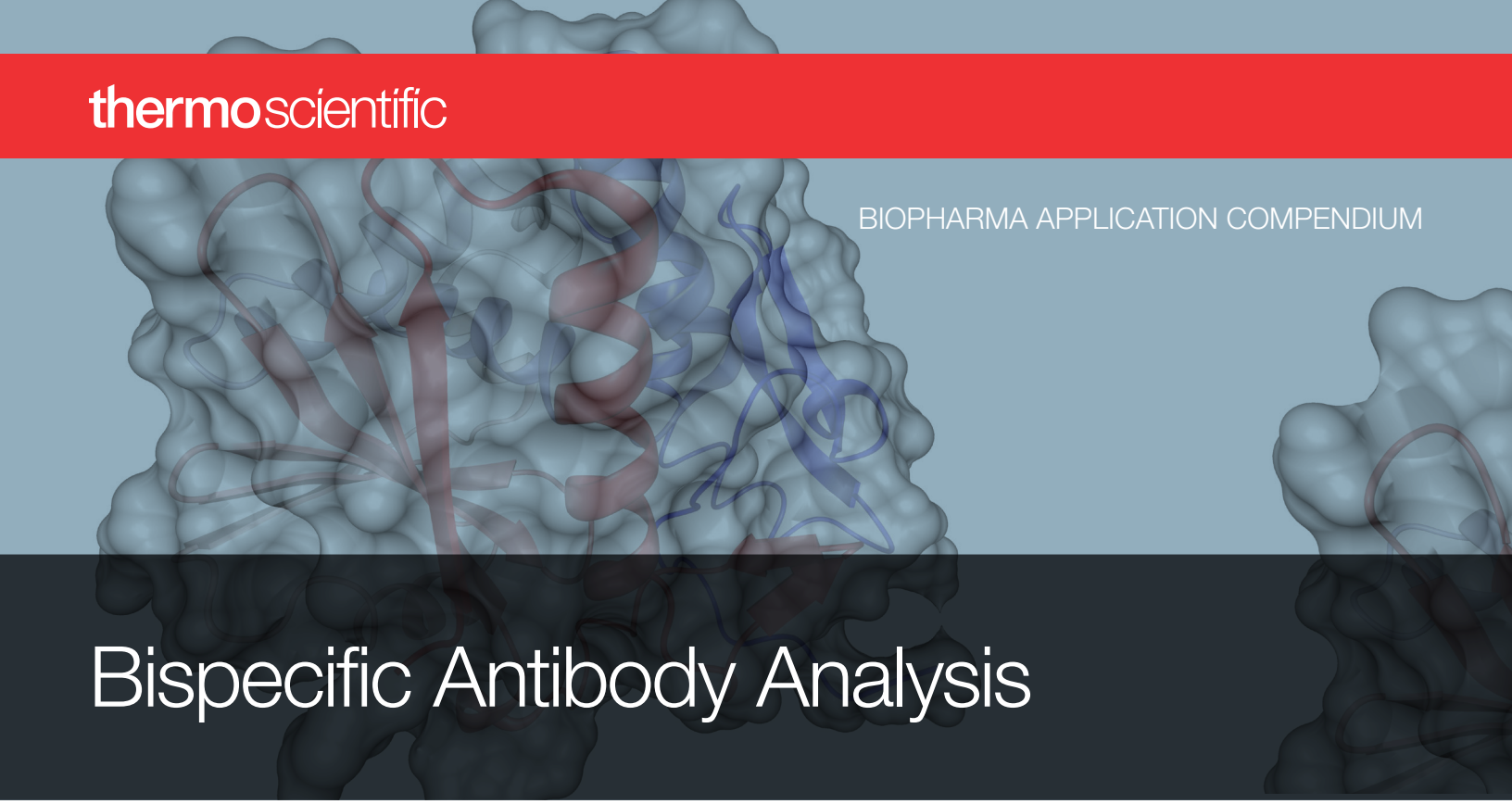

Bispecific Antibody Analysis

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POSTER NOTE

No. 21597

Separation of Bispecific mAbs Using Hydrophobic Interaction Chromatography (HIC)

Julia Baek, Shanhua Lin, and Xiaodong Liu
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ABSTRACT

Purpose: Demonstrate separation of a bispecific mAb from related species generated during the assembly using the Thermo Scientific™ MAbPac™ HIC-20 column.

Methods: Various chromatography methods including size exclusion chromatography (SEC), ion-exchange chromatography (IEC) using pH gradient, reversed-phase (RP) chromatography and hydrophobic interaction chromatography (HIC) were investigated for the separation of four species that are generated during bispecific mAb assembly. For HIC, three different column chemistries were tested using ammonium sulfate and sodium phosphate mobile phases.

Results: Among chromatography methods that were investigated, HIC using MAbPac HIC-20 column provided the best separation of all four species generated during the bispecific mAb assembly.

INTRODUCTION

Advancement of recombinant antibody technologies has enabled the development of many different types of monoclonal antibody (mAb) therapeutics such as antibody-drug conjugates (ADC) and bispecific mAbs.[1] Bispecific mAbs have specificity towards two different antigens which may be utilized to recruit killer T cells to the tumor cell or antagonize two different tumor specific antigens simultaneously.[2] Bispecific mAbs are engineered with two different mAbs that have different antigen binding sites. Therefore it is important to separate and monitor possible mis-assembled mAb biproducts that are produced during the process. Chromatographic separation of multiple species formed during assembly of a bispecific mAb could be challenging due to structural similarities between parental antibody domains and the desired bispecific mAb product. In this study, we have investigated various chromatography methods including size exclusion chromatography (SEC), ion-exchange chromatography (IEC), reversed-phase (RP) chromatography and hydrophobic interaction chromatography (HIC) to separate and analyze four related species that are generated during assembly of a bispecific mAb.

MATERIALS AND METHODS

Sample Preparation

The bispecific mAb samples were donated by a biotech company.

Columns

MAbPac SEC-1, 5 µm, 4.0 × 300 mm (P/N 074696)
MAbPac SCX-10, 10 µm, 4.6 × 250 mm (P/N 074625)
MAbPac RP, 4 µm, 3.0 × 50 mm (P/N 088645)
MAbPac HIC-10, 5 µm, 4.6 × 100 mm (P/N 088480)
MAbPac HIC-20, 5 µm, 4.6 × 100 mm (P/N 088553)
MAbPac HIC-Butyl, 5 µm, 4.6 × 100 mm (P/N 088558)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:

- SR-3000 Solvent Rack (P/N 5035.9200)
- LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
- WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- VWD-3400RS Rapid Separation Variable Wavelength Detector (VWD) equipped with micro flow cell (P/N 5074.0010)
- Chromatography was controlled by Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System.

Chromatography Conditions

Mobile phases are shown in Table 1. Flow rate, gradient and column temperature are shown on the right side of each figure.

Table 1. Mobile Phases Used for Various Chromatography Methods

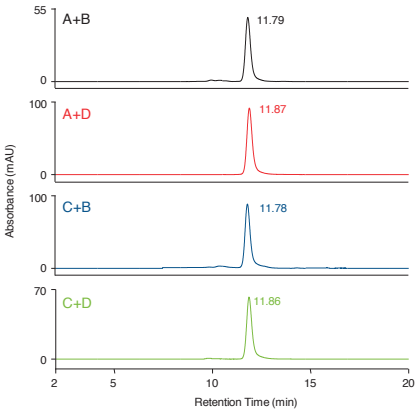
	Mobile phase A	Mobile phase B
MAbPac SEC-1	50 mM sodium phosphate, 300 mM NaCl, pH 6.8	
MAbPac SCX-10	CX-1 pH Gradient Buffer A (P/N 085346)	CX-1 pH Gradient Buffer B (P/N 085348)
MAbPac RP	H_2O/TFA (99.9:0.1 v/v)	MeCN/H_2O/TFA (90:9.9:0.1 v/v/v)
MAbPac HIC-10	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0	100 mM sodium phosphate, pH 7.0
MAbPac HIC-20 (a)	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0	100 mM sodium phosphate, pH 7.0
MAbPac HIC-20 (b)	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)	50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)
MAbPac HIC-Butyl	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0	100 mM sodium phosphate, pH 7.0

RESULTS

SEC Anlaysis of Bispecific mAbs

A bispecific mAb was generated using four domains, which resulted in four possible combinations of the domains. To obtain pure form of the product it is essential to separate these bi-products from the desired species. First, to determine whether these species can be separated based on size, SEC was evaluated. A MAbPac SEC-1 column with sodium phosphate mobile phase was used to analyze four species. As indicated by the retention time of the four species, no separation was observed which implies minimal differences in size of these species.

Figure 1. SEC separation of four species generated during bispecific mAb assembly



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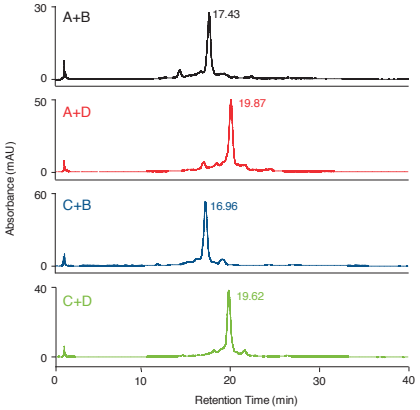
IEC Analysis of Bispecific mAbs Using a Linear pH Gradient Method

Next, IEC using CX-1 pH Gradient Buffers was evaluated for the separation of four bispecific mAb species. After running all samples from 0 to 100% Buffer B, the gradient was optimized to 25 to 55% B for maximum separation. The four species were separated into two groups. A+B and C+B eluted earlier with retention times of 17.42 and 16.96 and A+D and C+D were eluted later with retention times of 19.87 and 19.62. Domains B and D determined the retention behavior of these species.

Figure 2. pH Gradient separation of four species generated during bispecific mAb assembly



Column: MAbPac SCX-10, 10 µm
Format: 4.0 × 250 mm
Mobile phase A: CX-1 pH gradient buffer A, pH 5.6
Mobile phase B: CX-1 pH gradient buffer B, pH 10.2
Gradient:

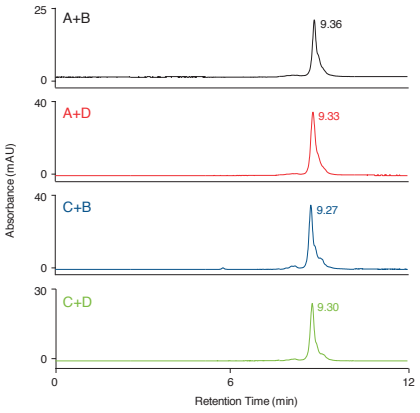
Time (min)	%A	%B
0.0	75	25
1.0	75	25
31.0	45	55
34.0	45	55
34.1	75	25
40.0	75	25

Temperature: 30 °C
Flow rate: 1.0 mL/min
Inj. volume: 20 µL
Detection: UV (280 nm)
Sample: A+B (1.74 mg/mL)
A+D (2.79 mg/mL)
C+B (3.43 mg/mL)
C+D (2.00 mg/mL)
Peak label: Retention time

RP Analysis of Bispecific mAbs

In order to evaluate separation of four bispecific mAb species using differences in hydrophobic interaction with the stationary phase, reversed-phase chromatography and HIC were performed. Figure 3 shows RP chromatograms using standard water to acetonitrile gradient with trifluoroacetic acid as the ion-pair reagent. Even with a shallow gradient (30 to 40% mobile phase B), the retention time of all four species were within 0.10 minute.

Figure 3. RP separation of four species generated during bispecific mAb assembly



Column: MAbPac RP, 4 µm
Format: 3 × 50 mm
Mobile phase A: H_2O/TFA (99.9:0.1 v/v)
Mobile phase B: MeCN/H_2O/TFA (90:9.9:0.1 v/v/v)
Gradient:

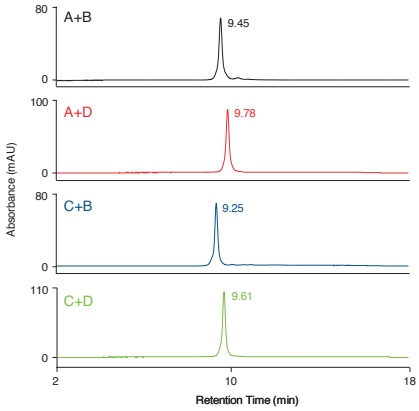
Time (min)	%A	%B
0.0	70	30
1.0	70	30
11.0	60	40
12.0	60	40
14.0	70	30
15.0	70	30

Temperature: 80 °C
Flow rate: 0.5 mL/min
Inj. volume: 2 µL
Detection: UV (280 nm)
Sample: A+B (0.87 mg/mL)
A+D (1.40 mg/mL)
C+B (1.72 mg/mL)
C+D (1.00 mg/mL)
Peak label: Retention time

HIC Analysis of Bispecific mAbs

Column chemistry could significantly affect the selectivity and/or the resolution of analytes. Here three HIC columns with different ligand chemistries-polyamide, amide and butyl-were evaluated (Figure 4, 5 & 6). All three HIC columns showed same order of elution; 1) C+B 2) A+B 3) C+D 4) A+D. However best resolution was achieved using the MAbPac HIC-20 column with an ammonium sulfate gradient from 0.8 to 2 M. Addition of isopropanol to the mobile phases A and B resulted in a separation pattern similar to the IEC using pH gradients which the separation of A+B and C+D was larger but the separation between C+B and A+B and separation between C+D and A+D were significantly reduced (chromatogram not shown; Table 2). Retention times obtained from all the chromatography modes and columns are shown in Table 2. In addition, the retention time differences between C+B and A+B, A+B and C+D and C+D and A+D were calculated for all the chromatograms. Although IEC using pH gradient buffers provided the best separation between A+B and C+D, MAbPac HIC-20 column gave the best separation between all four species.

Figure 4. Separation of four species generated during bispecific mAb assembly on MAbPac HIC-10

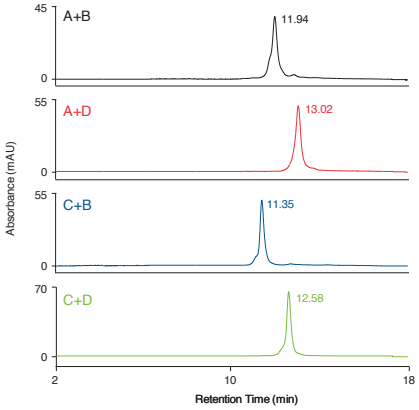


Column: MAbPac HIC-10, 5 µm
Format: 4.6 × 100 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Temperature: 30 °C
Flow rate: 1.0 mL/min
Inj. volume: A+B: 20 µL
A+D: 12 µL
C+B: 10 µL
C+D: 20 µL
Detection: UV (280 nm)
Sample: A+B (0.87 mg/mL)
A+D (1.40 mg/mL)
C+B (1.72 mg/mL)
C+D (1.00 mg/mL)
Peak label: Retention time

Figure 5. Separation of four species generated during bispecific mAb assembly on MAbPac HIC-20

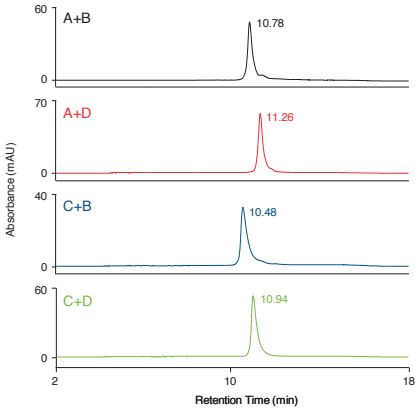


Column: MAbPac HIC-20, 5 µm
Format: 4.6 × 100 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Temperature: 30 °C
Flow rate: 1.0 mL/min
Inj. volume: A+B: 20 µL
A+D: 12 µL
C+B: 10 µL
C+D: 20 µL
Detection: UV (280 nm)
Sample: A+B (0.87 mg/mL)
A+D (1.40 mg/mL)
C+B (1.72 mg/mL)
C+D (1.00 mg/mL)

Figure 6. Separation of four species generated during bispecific mAb assembly on MAbPac HIC-Butyl



Column: MAbPac HIC-Butyl, 5 µm
Format: 4.6 × 100 mm
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B: 100 mM sodium phosphate, pH 7.0
Gradient:

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Temperature: 30 °C
Flow rate: 1.0 mL/min
Inj. volume: A+B: 20 µL
A+D: 12 µL
C+B: 10 µL
C+D: 20 µL
Detection: UV (280 nm)
Sample: A+B (0.87 mg/mL)
A+D (1.40 mg/mL)
C+B (1.72 mg/mL)
C+D (1.00 mg/mL)

Table 2. Retention times and retention time differences of four species generated during bispecific mAb assembly on MAbPac columns

	A+B	A+D	C+B	C+D	Δ[(A+B)-(C+B)]	Δ[(C+D)-(A+B)]	Δ[(A+D)-(C+D)]
MAbPac SEC-1	11.79	11.87	11.78	11.86	**0.01**	**0.07**	**0.01**
MAbPac SCX-10, CX-1 pH Gradient Buffers	17.43	19.87	16.96	19.62	**0.47**	**2.20**	**0.24**
MAbPac RP	9.36	9.33	9.27	9.30	**0.10**	**-0.06**	**0.02**
MAbPac HIC-10	9.45	9.78	9.25	9.61	**0.20**	**0.16**	**0.17**
MAbPac HIC-20 (a)	11.94	13.02	11.35	12.58	**0.59**	**0.64**	**0.44**
MAbPac HIC-20 (b)	7.44	8.42	7.25	8.29	**0.19**	**0.85**	**0.13**
MAbPac HIC-Butyl	10.78	11.26	10.48	10.94	**0.30**	**0.16**	**0.32**

CONCLUSIONS

- For separations of structurally related species generated during assembly of bispecific mAbs, screening of multiple chromatographic methods including SEC, IEC, HIC, and RP, is necessary.
- Among chromatography methods that were investigated, HIC using MAbPac HIC-20 column provided baseline separation of all four species generated during the bispecific mAb assembly.

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2.Kontermann, R. *MAbs* 4, 182–197 (2012).

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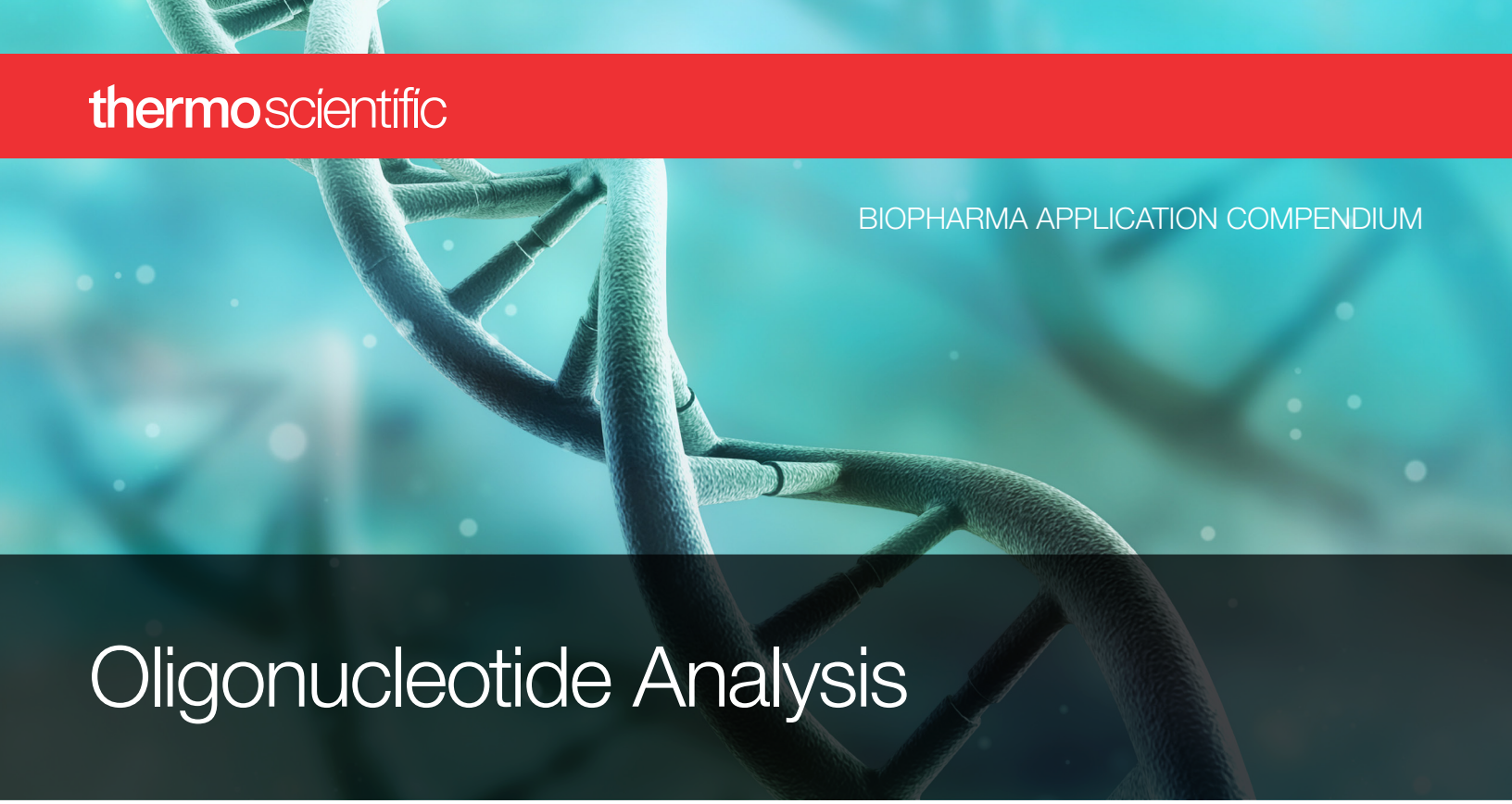

Oligonucleotide Analysis

ThermoFisher SCIENTIFIC

Application Note 21476

Separation of Mixed-Base Oligonucleotides Using a High-Resolution, Reversed-Phase Chromatography Column

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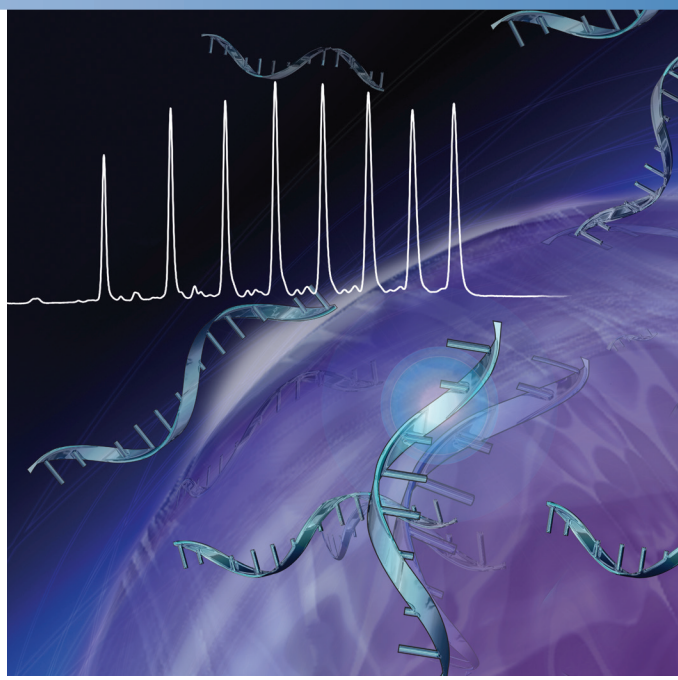
Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words
Ion-pair reversed-phase chromatography, DNA, oligonucleotides, DNAPac RP, HPLC

Goal
To demonstrate the impact of different chromatography conditions to achieve fast and high-resolution separation of mixed-base oligonucleotides (ONs) using a porous reversed-phase chromatography column. Mixed-base ONs were separated using different flow rates, gradient curves, temperatures, and ion-pair reagents.



Introduction
Synthetic ONs are used extensively in laboratories as primers for polymerase chain reactions (PCR) and DNA sequencing, probes to visualize a specific DNA or RNA, tools to study gene function, and biopharmaceutical drugs for treating various diseases.[1-4] ONs are most often synthesized using solid-phase chemistry, which consists of many sequential coupling reactions. Although the yield of each reaction is high, accumulation of minor reaction failures results in truncated ONs in addition to the target ON. These truncated ONs and other impurities (e.g., deprotection failures) must be removed for most molecular biology applications and to ensure efficacy and safety of therapeutic agents. Analyses of synthetic ONs are most commonly performed using ion-pair reversed-phase chromatography (IP-RP).[5-7] IP-RP utilizes the ionic interaction between the analyte and the ion-pair reagent, which also interacts with the stationary phase via its hydrophobic moiety. IP-RP provides high-resolution separation of failure sequences and can be directly coupled to mass spectrometry for identification of target ON and impurities.

Here we describe the use of the Thermo Scientific™ DNAPac™ RP column for the separation of mixed-base ONs. The DNAPac RP column is a wide-pore, polymer-based reversed-phase column, well suited for the separation of a wide range of ON lengths. The polymeric nature of the DNAPac RP column allows the use of high pH and high temperature conditions, which afford alternative selectivities and control of specific ON resolution. In this study, we examine the influence of flow rate, gradient curve, and temperature and compare two ion-pair reagents on retention and resolution of ONs using the DNAPac RP column.

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Experimental

Consumables

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (Fisher Scientific™ P/N A955-4)
- Triethylammonium acetate (TEAA) 2.0 M (Applied Biosystems™ P/N 400613)
- Hexylamine (HA) (Alfa Aesar™ P/N A15663)
- Acetic acid (Fisher Scientific P/N A507-P500)

Oligonucleotides

8-combo DNA

- 12mer: $(GACT)_3$
- 16mer: $(GACT)_4$
- 20mer: $(GACT)_5$
- 24mer: $(GACT)_6$
- 28mer: $(GACT)_7$
- 32mer: $(GACT)_8$
- 36mer: $(GACT)_9$
- 40mer: $(GACT)_{10}$

ONs were purchased from Integrated DNA Technologies, Inc.

Sample Handling Equipment

Vial and closures: Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Stock solutions were prepared by dissolving the ONs to 200 µM with deionized water (DI). Equivalent amounts of each ON were mixed to prepare 25 µM for each ON. The sample was further diluted five-fold with either DI water or mobile phase A to make 5 µM.

Separation Conditions	
Instrumentation	Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:
	System Base (P/N VF-S01-A)
	Quaternary Pump Flex (P/N VF-P20-A)
	Split Sampler FT (P/N VF-A10-A)
	Column Compartment H (P/N VH-C10-A)
	Active Pre-heater (P/N 6732.0110)
	Diode Array Detector HL (P/N VH-D10-A)
	Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
Column(s)	DNAPac RP, 2.1 × 50 mm (P/N 088924)
Mobile Phases	
Set I Mobile Phase A Mobile Phase B	 0.1 M TEAA in water, pH 7.0 0.1 M TEAA in water / acetonitrile (75:25 v/v)
Set II Mobile Phase A Mobile Phase B	 0.1 M HAA in water, pH 7.4 0.1 M HAA in water / acetonitrile (50:50 v/v)
Gradient	As specified in Figures.
Flow Rate	As specified in Figures.
Column Temp.	As specified in Figures.
UV Detector Wavelength	260 nm
Resolution	$R = 1.18 \times \frac{t_{Ref\,Peak\text{-}t_R}}{W_{50\%RefPeak}+W_{50\%R}}$ t_R = *retention time of the current peak* $t_{RefPeak}$ = *retention time of the reference peak for the resolution (the peak after the current peak)* $W_{50\%,R}, W_{50\%,RefPeak}$ = *Widths of the two peaks at 50% of the peak height*

Software

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System

Results and Discussion

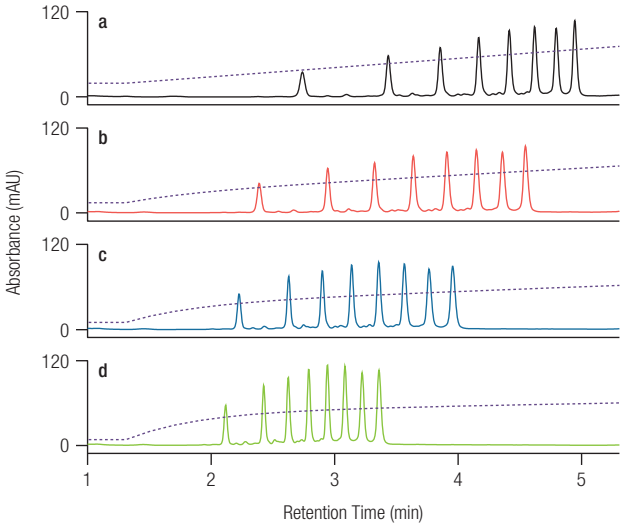
A mixture of eight mixed-base DNA ONs from 12mer to 40mer that differ in the number of GACT units (3–10) was used to study the impact of different chromatographic conditions on mixed-base ONs.

General Considerations

At pH values between 6 and 8, standard oligonucleotides have one negative charge for each phosphodiester bond. Therefore, a terminally non-phosphorylated ON will harbor a charge equal to one less than the number of bases. The amine on the ion-pair reagent interacts with the negatively charged phosphodiester bond "coating" the ONs with a hydrophobic layer. This enhances the hydrophobic interaction with the stationary phase. Since the number of phosphodiesters, and thus the number of IP reagent molecule interactions, is proportional to the length of the ON, longer ONs elute later at neutral pH values. As the ONs get longer, the percent charge difference between ONs decreases, so resolution between ONs decreases with ON length.

Curved Gradients

Since early-eluting ONs are typically well resolved and longer (later-eluting) ONs less well resolved, non-linear gradients can help improve resolutions of the longer ONs, provided early eluting ONs are sufficiently separated. The influence of different gradient types on resolution is shown in Figures 1 and 2. Curved gradients asymptotically reduce the slope of the gradient over time. Figure 1 (a, b, c and d) shows the chromatography of eight ONs using gradient curves with increasing initial gradient rates. The gradient curve number is inversely proportional to initial gradient slope and proportional to the ending gradient slope (Figure 2). In addition to increasing the separation between the longer ONs, gradient curves below 5 can be used to reduce the analysis time. Using the same gradient program, gradient curve 2 provides adequate separations in 3.5 minutes, instead of 5 minutes (30% faster, Figure 1d).



Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 50 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA in water/acetonitrile (25:75 v/v)
Gradient:	Time (min) %A %B
	-3.0 85 15
	0.0 85 15
	4.0 61 39
	4.1 10 90
	6.0 10 90
Gradient Curve:	a) 5 (linear)
	b) 4
	c) 3
	d) 2
Flow Rate:	0.60 mL/min
Inj. Volume:	2 µL
Temp.:	60 °C
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)

Figure 1. Adjustment of gradient curve.

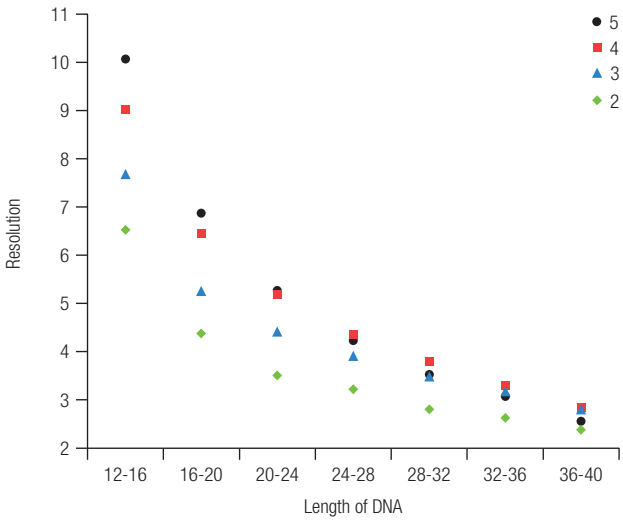
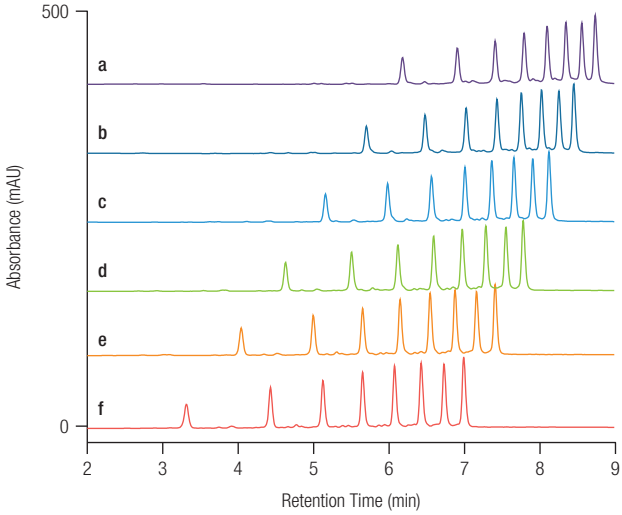


Figure 2. Effect of gradient curve on resolution.

Effect of Temperature

The impact of temperature on retention time and resolution (Figures 3 and 4) was also investigated. Samples were separated at different temperatures using the same gradient program (linear, curve 5). As the temperature increased, ON retention decreased. Since viscosity of the mobile phase decreases with increasing temperature, the pressure also decreases (data not shown). Therefore, ON separation at higher temperatures requires less organic solvent and can employ higher flow rates without exceeding the maximum recommended column pressure. Also, higher ON resolution is observed with increasing temperatures (Figure 4). A change from 30 °C to 80 °C produced more than 40% improvement in resolution. For longer ONs, especially those with internal hydrogen bonds, higher temperatures can minimize those interactions and improve separations between the ON target and its impurities.



Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 50 mm		
Mobile Phase A:	0.1 M TEAA, pH 7.0		
Mobile Phase B:	0.1 M TEAA in water/acetonitrile (25:75 v/v)		
Gradient:	Time (min)	%A	%B
	-3.0	90	10
	0.0	90	10
	8.0	54	46
	8.1	10	90
	10.0	10	90
Flow Rate:	0.40 mL/min		
Inj. Volume:	2 µL		
Temp.:	a) 30 °C		
	b) 40 °C		
	c) 50 °C		
	d) 60 °C		
	e) 70 °C		
	f) 80 °C		
Detection:	UV (260 nm)		
Sample:	8-Combo DNA (5 µM)		

Figure 3. Separation of oligonucleotides at different temperatures.

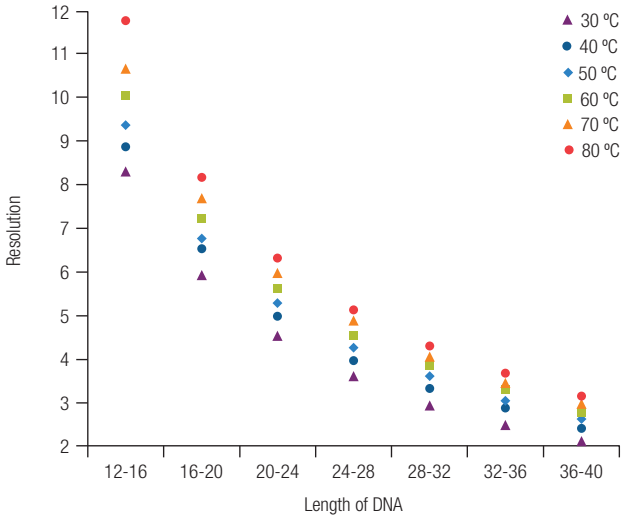


Figure 4. Effect of temperature on resolution.

Effect of Flow Rate

In Figures 5 and 6, the relationship between flow rate and resolution is depicted. The flow rates were varied while the gradient volume was kept constant. A flow rate of 0.2 mL/min produced the best resolution for these ONs. As the flow rate increased, decreased resolution was observed. However, at 0.8 mL/min, the separation of up to 40mer mixed-base DNA can be achieved in 3.5 minutes, as opposed to 0.2 mL/min completing in ~13 minutes, which is a 3.7-fold improvement. For more challenging separations, we suggest the use of a 0.2 mL/min flow rate, and for separations where target ONs are well resolved, we suggest up to 0.8 mL/min and temperatures at or above 60 °C.

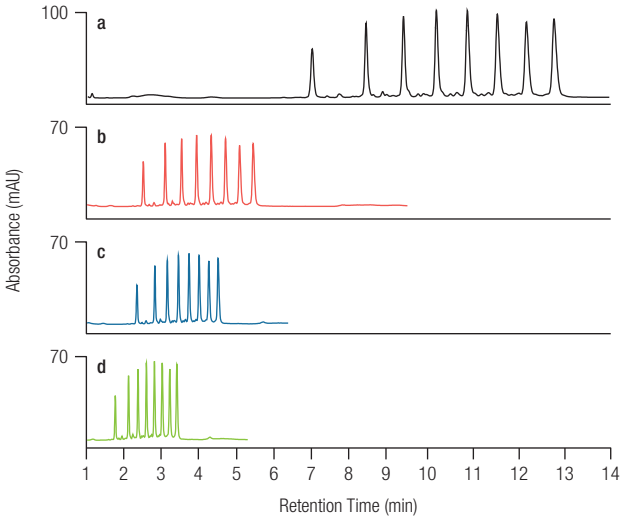


Figure 5. Separation of oligonucleotides at different flow rates.

Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 50 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA in water/acetonitrile (75:25 v/v)
Gradient:	a) 15 to 37%B in 12 min b) 15 to 37%B in 6 min c) 15 to 37%B in 4 min d) 15 to 37%B in 3 min
Gradient Curve:	3
Flow Rate:	a) 0.20 b) 0.40 c) 0.60 d) 0.80
Inj. Volume:	2 µL
Temp.:	60 ºC
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)

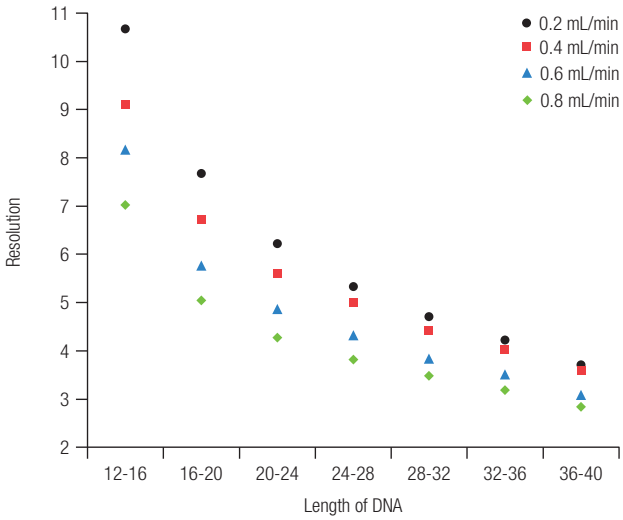


Figure 6. Effect of flow rate on resolution.

Effect of Ion-Pair Reagent

The type of ion-pair reagent has been reported to affect the separation of ONs.[8] Here triethylamine (TEA) and hexylamine (HA) as ion-pair reagents were compared (Figure 7). Using a 3 minute gradient, the resolution obtained using HA was 18–23% higher for the short oligonucleotides and 4–11% higher for the longer oligonucleotides. Both steeper acetonitrile gradients (2% acetonitrile/min for TEA vs 7% acetonitrile/min for HA) and higher solvent concentrations (initial solvent concentrations of 2% with TEA and 11% with HA) are required for HA mobile phase since HA is more hydrophobic and produces greater interactions between the ONs and the stationary phase.

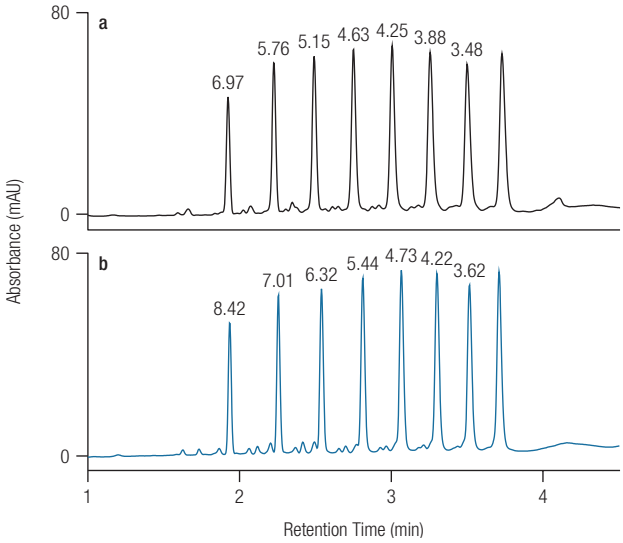


Figure 7. Comparison of TEA and HA as ion-pair reagents.

Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 50 mm
Mobile Phases & Gradient	
a)	
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA in water/acetonitrile (75:25 v/v)
Gradient:	8 to 32% B in 3 min
b)	
Mobile Phase A:	0.1 M HAA, pH 7.4
Mobile Phase B:	0.1 M HAA in water/acetonitrile (50:50 v/v)
Gradient:	23 to 63.5% B in 3 min
Gradient Curve:	3
Flow Rate:	0.80 mL/min
Inj. Volume:	2 µL
Temp.:	80 °C
Detection:	UV (260 nm)
Sample:	8-Combo DNA (5 µM)
Peak Label:	Resolution (ep)

Effect of Oligonucleotide Diluent

In Figure 8, the effect of sample diluents was examined. For some chromatographic systems, diluents must be carefully considered to support optimal peak shape and resolution. In this system, significant impact on peak shape or resolution was not observed when the ON diluent was mobile phase A or DI water.

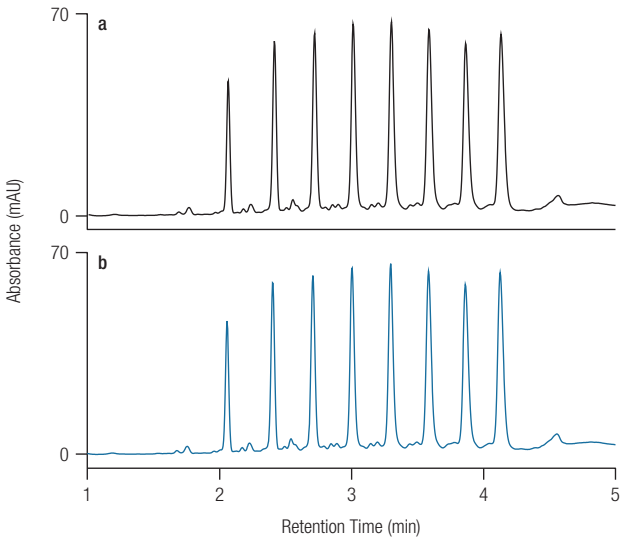


Figure 8. Effect of oligonucleotide diluent.

Column:	**DNAPac RP,** 4 µm		
Format:	2.1 × 50 mm		
Mobile Phase A:	0.1 M TEAA, pH 7.0		
Mobile Phase B:	0.1 M TEAA in water/acetonitrile (75:25 v/v)		
Gradient:	Time (min)	%A	%B
	-2.0	92	8
	0.0	92	8
	3.0	68	32
	3.1	10	90
	5.0	10	90
Gradient Curve:	3		
Flow Rate:	0.60		
Inj. Volume:	2 µL		
Temp.:	60 °C		
Detection:	UV (260 nm)		
Sample:	8-Combo DNA (5 µM)		
Sample diluent:	a) Water		
	b) Mobile phase A		

Conclusion

- The DNAPac RP column delivers fast, high-resolution separation of mixed-base ONs on a stable polymeric stationary phase.
- Gradient curve, temperature, and flow rate can be adjusted to improve resolution and/or reduce analysis time.

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Application Note 21533

Separation of Large Double-Stranded DNA (dsDNA) Fragments Using a Wide-Pore Reversed-Phase Chromatography Column

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Key Words
Ion-pair reversed-phase chromatography, double-stranded DNA (dsDNA), DNAPac RP, HPLC

Goal

To demonstrate the separation of dsDNA fragments and a PCR product using a wide-pore, polymer-based DNAPac RP column.

Introduction

Restriction nucleases are enzymes that generate dsDNA fragments of various sizes, and help researchers construct plasmids for expression of modified or novel proteins. In addition, DNAs that contain sequences for alternative gene editing systems (such as CRISPR-Cas9) may also generate plasmids.[1] Restriction enzymes may also be used to identify single nucleotide polymorphisms (SNPs) where the base change alters the specific restriction nuclease site.[2] For all these applications, restriction nuclease DNA fragments are typically purified prior to subsequent use. Agarose and polyacrylamide gels are most often used for separating and recovering dsDNA fragments. Gel electrophoresis is followed by manual excision of the target dsDNA size from the gel and extraction of the DNA from the excised gel. The method is laborious and time consuming and produces relatively low yields. It also requires handling of toxic materials such as acrylamide or ethidium bromide. HPLC provides reliably higher yields, allows direct DNA quantitation, and can be automated using fraction collection. Here we describe the use of a new reversed-phase column for the separation and recovery of dsDNA molecules.

The Thermo Scientific™ DNAPac™ RP column is based on a 4 µm, wide-pore polymer resin, which is stable at high pH and high temperatures. The wide-pore resin (1000–2000 Å) separates large dsDNA molecules as well as single-stranded oligonucleotides. This application note demonstrates the separation of DNA fragments generated from restriction enzymes and DNA ladder components, with automated purification and collection of the PCR product using ion-paired reversed-phase chromatography on the DNAPac RP column.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (Fisher Scientific™ P/N A955-4)
- Triethylammonium acetate (TEAA) 2.0 M (Applied Biosystems P/N 400613)

Samples

- pBR322-BsuRI digest (Thermo Scientific P/N SM0252)
- ΦX174-BsuRI digest (Thermo Scientific P/N SM0271)
- Thermo Scientific™ FastRuler™ Low Range DNA Ladder, ready-to-use (P/N SM1103)
- Thermo Scientific FastRuler Middle Range DNA Ladder, ready-to-use (P/N SM1113)
- Thermo Scientific FastRuler High Range DNA Ladder, ready-to-use (P/N SM1123)
- WPRE PCR product (Generous gift from Sanjay Vasu)

Sample Handling and Electrophoresis Equipment

Vial and closures: 300 µL PP 8-425 Screw Thread Vial (P/N C4013-11), Pre-Assembled Black PP 8-425 Cap with Pre-Slit PTFE/Silicone Septa (P/N C4013-77A)

Agarose Gels: Thermo Scientific™ Invitrogen™ 1.2% E-Gel (P/N G501801) with Invitrogen E-Gel Base

Power supply: FisherBiotech™ FB200Q Electrophoresis Power Supply or equivalent

Visualization: UV/Vis transilluminator

Sample Preparation

pBR322-BsuRI and ΦX174-BsuRI digests were diluted five-fold with DI water to make 100 µg/mL solutions.

FastRuler DNA Ladders and the PCR product were used as delivered.

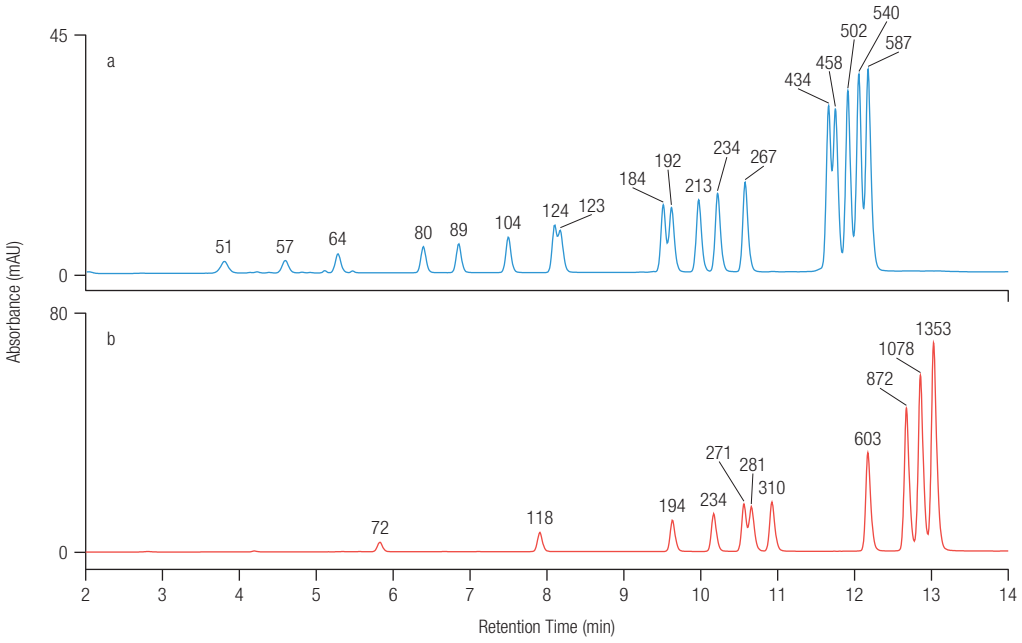
Separation Conditions	
Instrumentation	1. Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:
	System Base (P/N VF-S01-A)
	Quaternary Pump Flex (P/N VF-P20-A)
	Split-Loop Sampler FT (P/N VF-A10-A)
	Column Compartment H (P/N VH-C10-A)
	Active Pre-heater (P/N 6732.0110)
	Diode Array Detector HL (P/N VH-D10-A)
	Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
	2. Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRSLC system equipped with:
	SR-3000 Solvent Rack (without degasser) (P/N 5035.9200)
	LPG-3400RS Biocompatible Quaternary Rapid Separation Pump with degasser (P/N 5040.0036)
	WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection (P/N 5825.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000) equipped with 11 µL pre-column heater (P/N 6723.0252)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a micro flow cell (PN 6074.0300)
Columns	DNAPac RP, 2.1 × 100 mm (P/N 088923) DNAPac RP, 2.1 × 50 mm (P/N 088924)
Mobile Phase A	0.1 M TEAA, pH 7.0
Mobile Phase B	0.1 M TEAA in water/acetonitrile (75:25 v/v)
Gradient	As specified in Figures
Flow Rate	As specified in Figures
Column Temperature	As specified in Figures
UV Detector Wavelength	260 nm

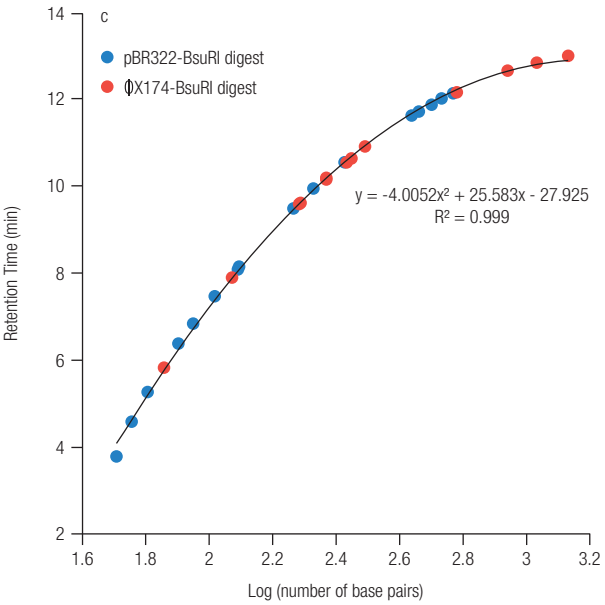
Data Processing

The Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System was used for data analysis.

Results and Discussion

Ion-pair reversed-phase chromatography on the DNAPac RP column was used to separate restriction digest components (Figure 1). At 55 °C, all of the dsDNA digestion fragments from pBR322 plasmid (Figure 1a) and ΦX174 viral DNA (Figure 1b) with BsuRI enzyme were separated. The DNAPac RP column was able to separate fragments differing by as little as a single base pair (bp) up to 100 bp. In addition, the retention of the fragments correlated to the length of the DNA. Retention versus log (number of base pairs) of both pBR322 and ΦX174 digests was plotted in Figure 1c. The data fit well to a second-order polynomial equation, indicating good correlation between the retention time and the length of the fragments.





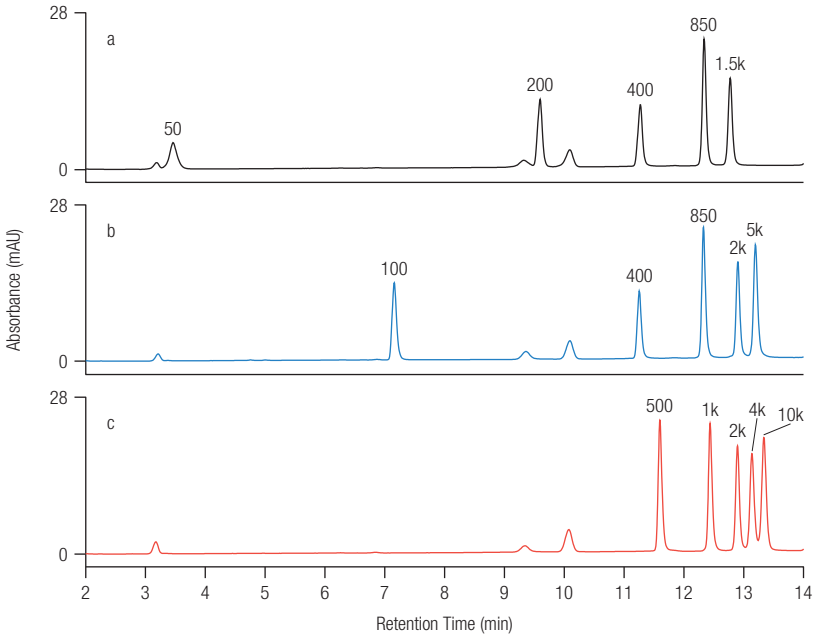
Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water/acetonitrile (75:25 v/v)
Gradient:	

Time (min)	%A	%B
-14.0	64	36
0.0	64	36
12.0	31	69
12.1	5	95
15.0	5	95

Flow Rate:	0.40 mL/min
Inj. Volume:	5 µL
Temp.:	55 °C
Detection:	UV (260 nm)
Sample:	a. pBR322-BsuRI digest (100 µg/mL) b. ΦX174-BsuRI digest (100 µg/mL)
Peak Label:	Base pairs

Figure 1. Separation of restriction enzyme digests.

To further investigate the DNAPac RP column resolution of large dsDNA fragments, DNA ladders with components up to 10,000 base pairs were examined (Figure 2). All of the DNA ladder components were resolved in less than 14 minutes with the resolution of components differing by approximately 10% for fragments up to around 10,000 bp. The DNA ladder retention time can be compared to the retention time of a DNA fragment to estimate its size.



Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water/acetonitrile (75:25 v/v)

Gradient:

Time (min)	%A	%B
-14.0	65	35
0.0	65	35
12.0	30	70
12.1	10	90
15.0	10	90

Flow Rate:	0.40 mL/min
Inj. Volume:	10 µL
Temp.:	55 °C
Detection:	UV (260 nm)
Sample:	DNA ladders a. Low range b. Middle range c. High range
Peak label:	Base pairs

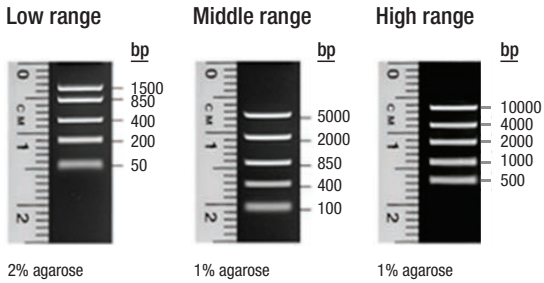


Figure 2. Separation of FastRuler DNA ladders.

Separation of ΦX174-BsuRI digest, which contains components from 72 to 1353 bp, on a DNAPac RP column was compared to competitive columns of similar format (all equivalent 2.1 mm columns, except for brand C, which has a 2.0 mm ID). The same mobile phase and linear velocity were used. The larger pore size DNAPac RP column gave significantly better separation of the larger dsDNA fragments. Differences in separation can be attributed to larger pore size; however, selectivity, particle size, and particle chemistry may have also contributed to the difference in the separation of dsDNA fragments (Figure 3).

The ΦX174-BsuRI digest was used to study the temperature effect on the dsDNA retention and resolution (Figure 4). Resolution and the retention of these dsDNA fragments increased with temperature up to 50 °C without changing the peak intensity pattern. However, at 60 °C, the retention pattern changed and the resolution of the peaks appeared to be higher. Since the intensities of several peaks change at 60 °C, partial denaturation of some dsDNA fragments is likely induced (hyperchromic shift). Two peaks also eluted earlier at 60 °C than at 50 °C, which is consistent with exposure of ssDNA sections within the fragment. At 70 °C, denaturation is obvious as the number of peaks increased from 11 to 18, and many peaks eluted much earlier than at lower temperatures, suggesting full denaturation of some dsDNA into single stranded DNA (ssDNA) forms. At even higher temperatures (80–90 °C), more peaks were observed and all eluted much earlier, indicating full denaturation of all of the dsDNA fragments.

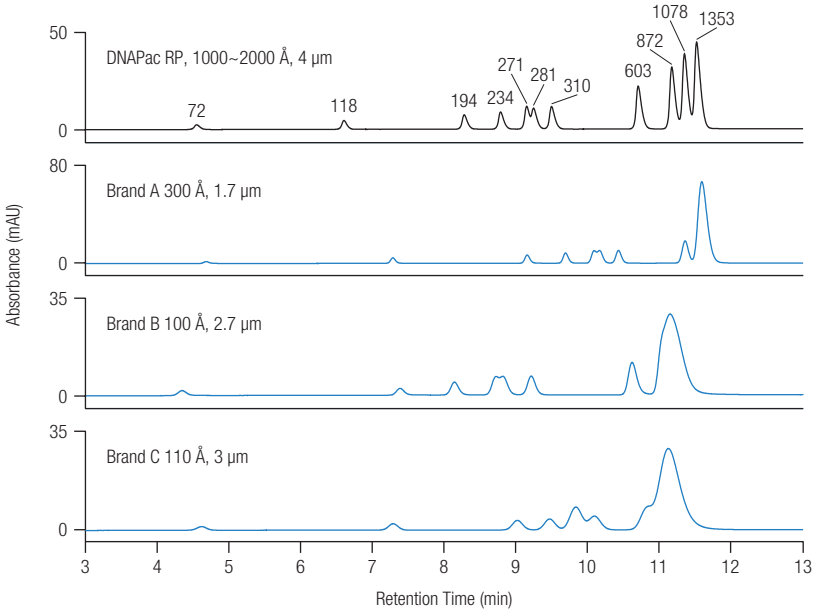
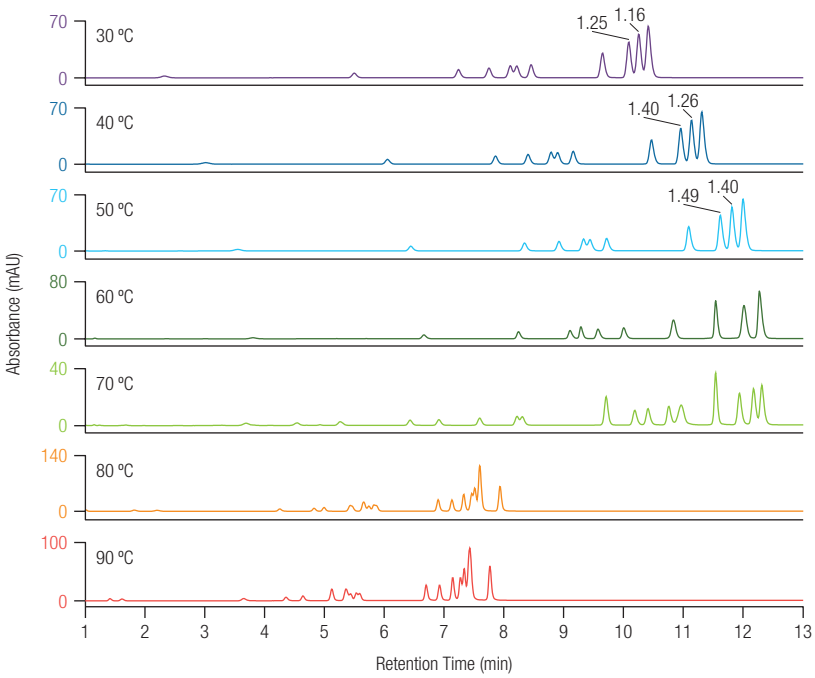


Figure 3. Separation of ΦX174-BsuRI digest on columns with different pore sizes.



Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water/acetonitrile (75:25 v/v)

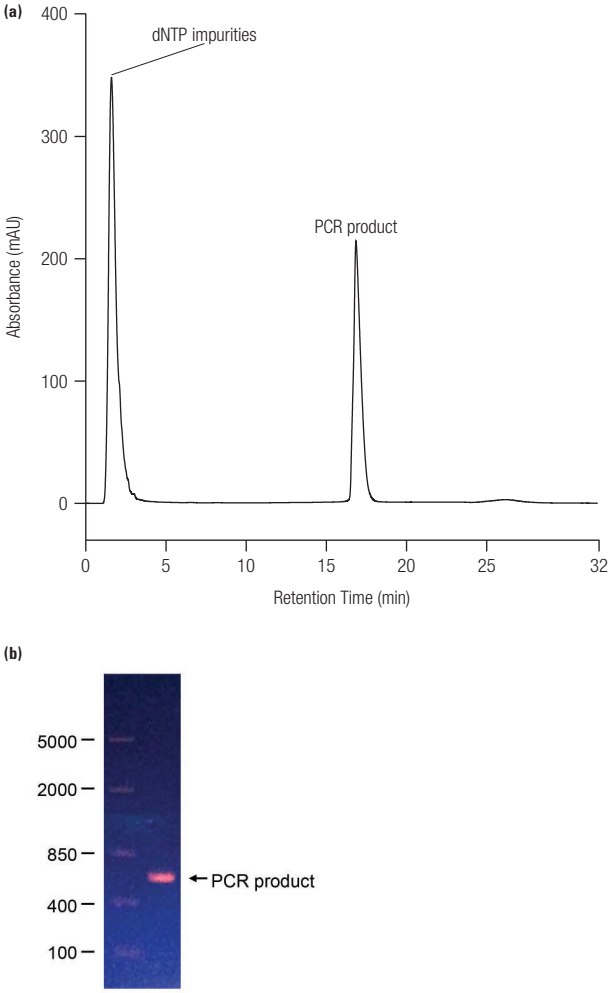
Gradient:

Time (min)	%A	%B
-14.0	60	40
0.0	60	40
12.0	30	70
12.1	5	95
15.0	5	95

Flow Rate:	0.40 mL/min
Inj. Volume:	5 µL
Detection:	UV (260 nm)
Sample:	**ΦX**174-BsuRI digest (100 µg/mL)
Peak label:	Resolution (ep)

Figure 4. Separation of ΦX174-BsuRI digest at different temperatures.

PCR is routinely used in molecular biology labs for amplification of DNA and sequencing. PCR products are typically purified from the dNTPs and other impurities in the reaction prior to use. Using an UltiMate 3000 system with an UltiMate WPS-3000TBFC (Well Plate Autosampler configured for Fraction Collection), a PCR product was separated from its impurities and automatically collected into a well plate (Figure 5).[4,5] The collected sample was then analyzed with a 1.2% agarose E-gel to confirm its purity (Figure 5b). This example demonstrates automated HPLC with fraction collection of dsDNA using the DNAPac RP column, showing facile purification from PCR-related components in 20 minutes.



Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 100 mm		
Mobile Phase A:	0.1 M TEAA, pH 7.0		
Mobile Phase B:	0.1 M TEAA in water/acetonitrile (75:25 v/v)		
Gradient:	Time (min)	%A	%B
	0.0	56	44
	20.0	32	68
	20.1	10	90
	22.0	10	90
	22.1	56	44
	32.0	56	44
Flow Rate:	0.25		
Inj. Volume:	50 µL		
Temp.:	50 °C		
Detection:	UV (260 nm)		
Sample:	PCR product		

Figure 5. Purification of a PCR product.

Conclusion

- The combination of the DNAPac RP column and Vanquish Flex UHPLC system delivers rapid, high-resolution dsDNA fragment separation up to 10,000 bp.
- Elution of the dsDNA fragments is essentially proportional to fragment length (in bp).
- The UltiMate 3000 system equipped with the WPS-3000 TBFC Well Plate Autosampler configured for Fraction Collection facilitates purification and recovery of dsDNA fragments.

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Useful Links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:
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