

High sensitivity capillary LC-MS analyses of low amounts of therapeutic antibodies and their subunits

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Goals

- Demonstrate advantages of using low flow liquid chromatography for intact protein analyses and highlight that the quality of data is comparable to high flow experiments
- Demonstrate the column performance through high resolution intact protein separation and overview of intact protein workflows
- Demonstrate the use of capillary format to obtain information on multiple critical quality attributes from very low amounts of material



Introduction

With the rapid growth of the biopharmaceutical industry, there is an increased need for new technologies to provide end users with robust, high throughput, and information rich analyses. To characterize therapeutic antibodies (mAb) to assess product safety, determine batch-to-batch consistency, and assist drug development, state-of-the-art mass spectrometry-based analyses coupled to different separation approaches have become the most important technique in the biopharmaceutical industry. Intact protein analysis with minimal sample preparation facilitates the identification of post-translational modifications (PTMs) of mAbs. Analyzing the subunits, obtained by the digestion with sequence-specific endopeptidases such as IdeS, allows the assignment of PTMs to certain subunits. Furthermore, the heterogeneity of mAbs—which is caused by different PTMs such as glycosylation, methionine

and tryptophan oxidation, deamidation, formation of succinimide from asparagine and glutamine, C-terminal lysine truncation—frequently results in near-isobaric proteoform species. To detect these proteoforms and decipher PTMs, high resolution accurate mass (HRAM) mass spectrometry provides the necessary resolution and accuracy required to reliably distinguish and assign PTMs to subunits.

In the first phase of the early stage of process development, the use of micro bioreactors substantially decreases cost during the optimization of different conditions (such as feeds volume, pH, and temperature) to achieve the highest rate of cell growth and the desired protein attributes. Due to the small volume of such micro bioreactors, the amount of sample that needs to be taken daily to monitor titer and protein attributes is therefore limited. Further to this, additional challenges are raised by the large number of samples taken in the design of experiment (DoE) phase since the aforementioned optimization necessitates performing experiments in multiple micro bioreactors. Thus, applying high-throughput methods delivering the required data quality are highly desired.

By the introduction of the Thermo Scientific™ MAbPac™ RP capillary column, these obstacles, which otherwise slow down process development, are overcome. Owing to the increased sensitivity provided by capillary flow, high-quality MS data can be obtained with a reduced amount of sample. This is especially important since the necessary sample preparation to make the sample amenable to mass spectrometric analyses leads to further reduction of the sample. Hence, due to minimal sample dispersion and improved desolvation in capillary chromatography, using this column can be especially beneficial not only in the early stage of process development. For example, researchers dealing with a scarce quantity of biological samples prepared from cells, tissues, etc. can also greatly benefit from using this column in discovery top-down proteomics. In addition to the superior sensitivity, another hallmark of this column is attributed to high-resolution protein separation attainable in a high-throughput manner.

Experimental Instrumentation

- Capillary chromatography was performed using a Thermo Scientific™ UltiMate™ 3000 RSLCnano System ([ULTIM3000RSLCNANO](#)) with the following modules:

- Thermo Scientific™ UltiMate™ SR-3000 Solvent Rack ([P/N 5035.9200](#))
- Thermo Scientific™ UltiMate™ NCS-3500RS Binary Rapid Separation Nano/Capillary Pump (NC) ([P/N 5041.0010A](#), featuring an NC pump, a loading pump, and a column compartment). The NC pump was equipped with a *Classic* flow meter with a *Capillary* flow selector
- Thermo Scientific™ Dionex™ UltiMate™ 3000 VWD Variable Wavelength Detectors ([P/N 5074.0010](#))
- Thermo Scientific™ Dionex™ UltiMate™ WPS-3000TPL/PL RSLCnano Well Plate Autosamplers ([P/N 5826.0020](#))
- The high flow experiments were carried out using a Thermo Scientific™ Vanquish™ Flex UHPLC System ([IQLAAAGABHFAPUMBJC](#)) with the following modules:
- Thermo Scientific™ Vanquish™ Binary Pump H ([P/N VH-P10-A](#))
- Thermo Scientific™ Vanquish™ Split Sampler HT ([P/N VH-A10-A](#))
- Thermo Scientific™ Vanquish™ Column Compartment H ([P/N VH-C10-A](#))
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer with BioPharma option ([IQLAAEGAAPFALGMBDK](#)) using a Thermo Scientific™ EASY-Spray™ Source ([ES081](#)) for capillary chromatography, or a Thermo Scientific™ Ion Max API Source ([IQLAAEGABBFACTMAJL](#)) with a HESI probe in the high flow experiments

Consumables

MAbPac RP capillary columns with two Thermo Scientific™ nanoViper™ connections (4 µm, 0.15 mm × 150 mm, [P/N 164947](#)) were used in capillary chromatography experiments. In the high flow experiments, a MAbPac RP column (4 µm, 3 mm × 50 mm, [P/N 088645](#)) was applied.

Sample preparation and reactions were performed in either 0.2 mL volume reaction tubes (Applied Biosystems™ MicroAmp™ Reaction Tubes with cap, 0.2 mL, autoclaved, [P/N N8010612](#)) or 2 mL volume microfuge tubes (Invitrogen™, nonstick, RNase-free microfuge tubes, 2.0 mL, [P/N AM12475](#)), depending on the required volume. If controlled temperature was needed during the sample

preparation, the tubes were incubated in a block heater (Fisher Scientific™, Isotemp™ Digital Dry Baths/Block Heater, four blocks, P/N 88860023). Prior to mass spectrometric analysis, samples were cleaned up using centrifugal filter devices (MilliporeSigma™, Amicon® Ultra Centrifugal Filters, 0.5 mL, 10K, Ultracel® 10K, P/N UFC5010BK).

Reagents, chemicals

Mobile phases

- Fisher Scientific™ UHPLC-MS grade water (P/N W8-1)
- Fisher Scientific™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ Optima™ UHPLC-MS grade formic acid (P/N A117-50)

Deglycosylation of intact mAb was carried out using EndoS IgG-specific endoglycosidase (Genovis, IgGZERO® 1000 units, P/N A0-IZ1-010). To generate subunits of monoclonal antibodies, mAbs were first digested with IdeS (Genovis, FabRICATOR® 5000 units, P/N A0-FR1-050) which is a specific cysteine protease that cleaves the mAbs below the hinge and the resulting homogenous pool of (Fab)₂ and Fc/2 fragments were further reduced with Tris(carboxyethyl)phosphine (TCEP) (Thermo Scientific™ Bond-Breaker™ TCEP solution, neutral pH, P/N 77720) to yield Fc, LC (light chain) and Fd' subunits.

A mixture of six recombinant proteins was used to optimize mass spectrometric workflows of intact proteins (Thermo Scientific™ Pierce™ Intact Protein Standard Mix, P/N A33527).

Therapeutic antibodies (mAbs)

Trastuzumab innovator and Fab glycosylated mAbs were obtained from Roche Diagnostics GmbH (Penzberg, Germany). Trastuzumab biosimilar and rituximab innovator were obtained from a collaborator biopharma company. NIST mAb was purchased from National Institute of Standard and Technology (US, Gaithersburg, MD, RM 8671).

Sample preparation

Endo S enzyme is supplied lyophilized in buffer additives necessary to ensure optimal conditions for digestion. The enzyme was first reconstituted in 50 µL distilled water to obtain a concentration of 20 units/µL. Digestion was performed at a ratio of 20 µg mAb to 1 µL enzyme solution;

the solution was incubated at 37 °C for 30 min. After the digestion was complete, the reaction mixture—consisting of salts, deglycosylated intact mAb, detergents from the formulation buffer of mAb, and enzyme—was purified by centrifuging through a 10 kDa cut off filter at 14 000 × g. To ensure the salt and detergent removal is complete, the concentrated solution in the filter was washed three times with 0.5 mL distilled water at 14 000 × g. After recovering the deglycosylated and purified mAbs, concentrations were adjusted with distilled water to approximately 1 mg/mL and aliquots were further diluted 50–100 times prior to analysis.

The lyophilized IdeS enzyme was solubilized in 75 µL of distilled water to obtain enzyme solution at a concentration of 67 units/µL. To generate different subunits of monoclonal antibodies, mAbs were diluted with 1× PBS (pH 7.4) to 1 mg/mL. 100 µL of diluted mAb (consisting of 100 µg mAb) was digested with 1.5 µL of IdeS solution for 1 h at 37 °C. After the digestion was complete, 4.16 µL Bond-Breaker solution (500 mM) was added to the digested mAb solution and was further incubated at 50 °C for 30 min to achieve complete reduction of disulfide bonds. The solutions consisting of the subunits were purified by centrifugation using the 10 kDa cut off filter as already described. After purification and adjustment of concentration with distilled water, the subunits were immediately analyzed. Keeping the purified subunits over a long period of time in distilled water is not recommended due to possible re-oxidation of disulfide bonds.

Liquid chromatography

In the capillary chromatographic experiments, mobile phase “A” was water consisting of 0.1 formic acid and mobile phase “B” was 80% acetonitrile/20% water containing 0.1% formic acid. In all experiments, the flow rate applied was 2 µL/min. Three different gradient programs described by Table 1 were used for the separation of intact protein mixture, intact mAbs, and their subunits. Since separation of intact proteins was carried out under denaturing conditions, employing higher temperature significantly contributes to unfolding of proteins. Analyses of mAbs and their subunits were performed at 75 °C while a lower temperature (60 °C) increased the resolution of six intact proteins from the Intact Protein Standard Mix. In the high flow experiment, the flow rate was 500 µL/min using a 3 mm i.d. column. Separations were carried out at 75 °C, and the gradient program in Table 2 was used.

Table 1. Gradients used in capillary chromatography

Time (min)	Intact mAb [%B]	Subunit [%B]	6 Intact protein [%B]
0.00	25	25	25
15.00	55	60	50
15.50	100	100	100
17.00	100	100	100
17.50	25	25	25
25.00	25	25	25

Table 2. Gradient used in high flow experiment

Time (min)	%B
0.0	20
1.0	20
11.0	50
12.0	50
14.0	90
14.1	20
17.0	20

Mass spectrometric conditions

Experimental conditions for different intact protein analyses are summarized by Table 3. Conditions for the assessment of sensitivity gain in low flow vs. high flow were optimized to give similar distributions of the glycoforms obtained after deconvolution. When analyzing mAbs in high flow, desolvation is promoted by sheath and auxiliary gas flows, which were obviously not available while using the EASY-Spray Source at capillary flow rates. In addition to the two different gas sources in high flow, source CID (SID)

is also available to further improve desolvation. In capillary chromatography, SID (which is a DC offset and applying it results in collisions of the analytes with residual gas molecules in the flatapole region of the instrument) is the only way to further promote desolvation of large molecules. In high flow experiments, the sheath gas flow rate was set at 20 and the flow rate of auxiliary gas was 10 (both in arbitrary units). Additionally, the flow rate applied on the sweep cone was 1. When comparing high flow to capillary flow, in both cases 2.5 kV spray voltage was used.

It is important to emphasize that the analysis of subunits and smaller ($M_w < 40$ kDa) proteins are isotopically resolved in protein mode that can be activated in the tune file. This requires using decreased pressure so in this study a default of 0.2 normalized value (normalized to operating pressure) was applied. This feature is only available with the biopharmaceutical option installed in the MS.

Connecting the column to HPLC outlet and mass spectrometer

The inlet of the MAbPac capillary column (P/N 164947, with two nanoViper fittings) is connected to the line coming from the autosampler valve using a Viper union (P/N 6040.2304) by carefully tightening the nut finger tight using the black knurled screw. The column outlet is connected to a second Viper union. The outlet of the second Viper union is connected to a transfer line (55 cm \times 20 μ m, with 2 nanoViper fittings, P/N 6041.5260), while the other end of the transfer line is connected to the capillary flow emitter (P/N ES994). The emitter is easily plugged into EASY-Spray Source.

Table 3. Mass spectrometric conditions used in all experiments

	Intact mAbs (Normal mode)	Protein mode (Trapping gas 0.2)	Targeted MS ² (4-plex)	Intact mAbs (High flow)
Scan range	1800–3800	700–3000	Fixed first mass at 200	1800–3800
Resolution (Full MS/MS ²)	17,500/n.a.	140,000/n.a.	n.a./140,000	17,500/n.a.
AGC, Full MS	3×10^6	3×10^6	2×10^5	3×10^6
Max injection time (Full MS/MS ²)	200 ms	200 ms	200 ms	200 ms
Isolation window	n.a.	n.a.	6 m/z	n.a.
Microscans	10	5	5	10
Capillary temperature	300	300	300	300
SID (eV)	20	20	20	10
NCE (%)	n.a.	n.a.	20	n.a.

Results and discussion

One of the advantages of using capillary columns for the analysis of biomolecules is the increased sensitivity attributed to the concentrating effect of the narrow bore column and higher ionization efficiency ascribed to a low flow rate.

Figure 1 demonstrates these benefits using the commercially available MAbPac RP column (50 mm × 3 mm) operating at a 500 µL/min flow rate and the novel capillary column (150 mm × 0.15 mm) packed with the same resin, running at 2 µL/min. Tune conditions were optimized in both experiments to yield the same data quality pertaining to glycoform distribution. Injecting a relatively low amount of intact mAb (rituximab) on the high flow column results in a broad peak due to dispersion caused by the 20 times larger column i.d., leading to the dilution of the small amount of analyte (Figure 1A). As a consequence, spectra of reduced quality were obtained (Figure 1B) and signal-to-noise (S/N) ratio calculated by the Root Mean Square (RMS) approach of the peak in the total ion chromatogram (TIC) was 17. Analysis of the same mass of mAb on the capillary column (Figure 1D) significantly

improved peak shape and immensely reduced noise level therefore producing a good quality spectrum (S/N ratio of TIC peak is 626). The two deconvoluted spectra attained by the injection of 15 ng mAb on high flow (Figure 1C) and low flow columns (Figure 1F) appeared to be different in terms of the numbers of identified glycoforms and their distributions. This observation consequently led to a further control experiment at high flow using considerably more (125 ng) intact mAb on the 3 mm column (Figure 1G). This was conducted to exclude the possibility that different source conditions in both experiments were responsible for the abovementioned difference in glycoform distribution. The spectrum acquired in the control experiment (Figure 1H) is of high quality (S/N ratio of TIC peak was 345) and after deconvolution (Figure 1I) shows the same number of identified glycoforms and their very similar distribution to the experiment performed in capillary chromatography by analyzing 15 ng of mAb. The injected 15 ng mAb falls within the loading capacity of the capillary column and the spectrum yielded provides detailed identification of even minor glycoforms. As opposed to the 3 mm column chromatography, the low quantity (15 ng) injected is clearly not enough to generate a spectrum of adequate quality at

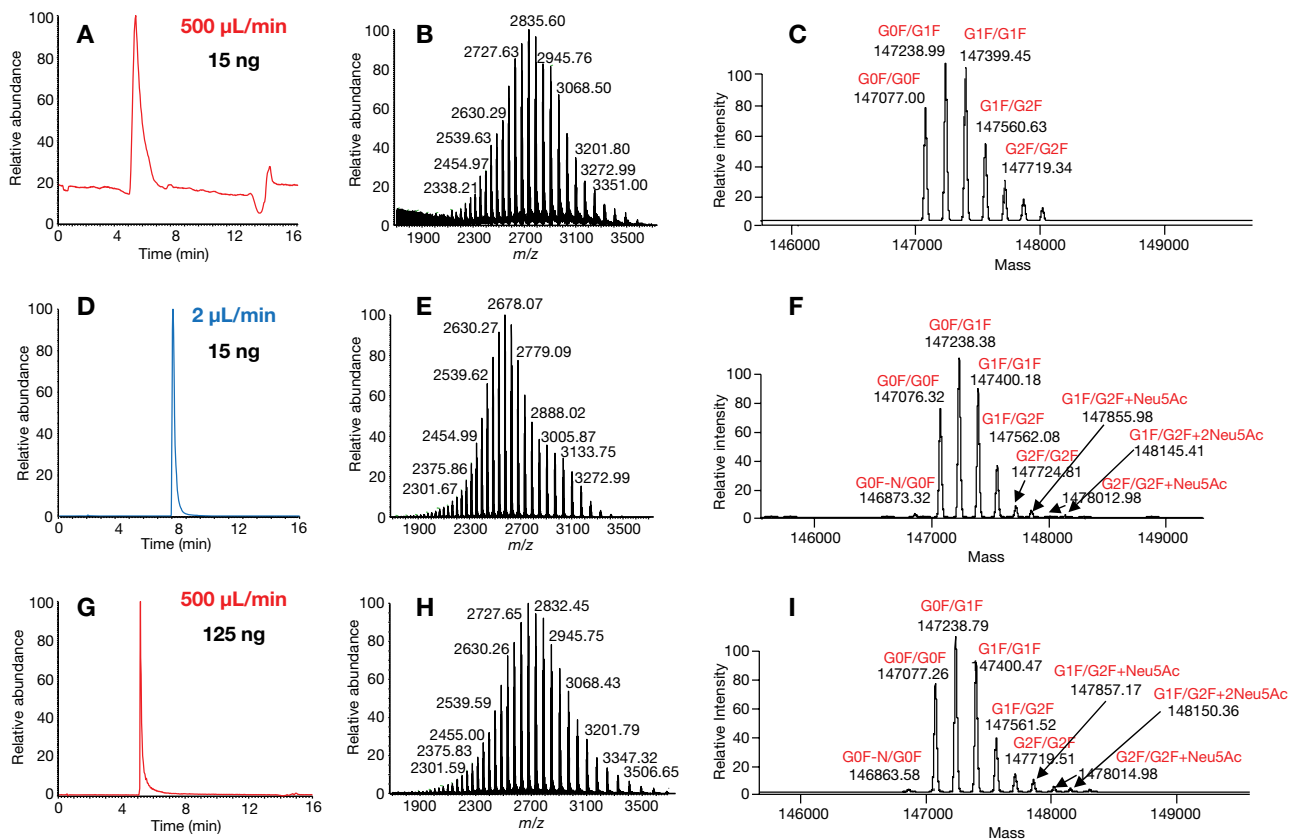


Figure 1. Effect of the flow rate on sensitivity/glycoform distribution. A) TIC of 15 ng rituximab acquired in high flow (500 µL/min) experiments; B) Spectrum of 15 ng rituximab acquired in high flow (500 µL/min) experiments; C) Deconvoluted spectrum of 15 ng rituximab acquired in high flow (500 µL/min) experiments; D) TIC of 15 ng rituximab acquired in capillary chromatography experiment (2 µL/min); E) Spectrum of 15 ng rituximab acquired in capillary chromatography experiment (2 µL/min); F) Deconvoluted spectrum of 15 ng rituximab acquired in capillary chromatography experiment (2 µL/min); G) TIC of 125 ng rituximab acquired in high flow (500 µL/min) experiments; H) Spectrum of 125 ng rituximab acquired in high flow (500 µL/min) experiments; I) Deconvoluted spectrum of 125 ng rituximab acquired in high flow (500 µL/min) experiments

500 $\mu\text{L}/\text{min}$. When analyzing 15 ng of mAb, the peak area of TIC with capillary flow was 140 times higher than in high flow mode. This data highlights the relevance of using this column not only in the early stage of process development but in other fields of discovery proteomics where the amount of sample is often scarce.

In general, liquid chromatography-mass spectrometry (LC-MS) based workflows of intact proteins include the intact mass and top down approaches. Figure 2A shows the TIC of six recombinant proteins acquired in protein mode. Due to the high resolution applied, four proteins (peaks 1, 3, 4, and 5) could be isotopically resolved as demonstrated by Figure 2B where charge states are assigned to each peak of the protein charge envelope. First the source spectrum of these four proteins was generated by averaging multiple scans over a 6–15 min time window of the chromatogram, which was further followed by the deconvolution of the source spectrum using the Xtract algorithm. The deconvoluted spectrum (Figure 2C) displays the highly accurate masses of the proteins (<4 ppm error). The

workflow does not show the masses of peak 2 and 6; they are larger proteins and therefore they cannot be analyzed in protein mode. Hence, it is not possible to determine their monoisotopic masses. Similar to the approach already shown by Figure 1 for an intact mAb, low resolution (17,500) was applied to obtain the average masses of the charge states of these proteins.

To further confirm the sequence of proteins, top-down experiments using a targeted MS^2 method were employed for four selected charge states of each protein listed in a retention time dependent inclusion list. Four quadrupole isolation events were performed for each protein in the retention time window indicated. The first charge state was selected and fragmented in the HCD cell. These product ions were stored in the HCD cell until all charge states were isolated and fragmented. All the product ions were then detected in the Orbitrap mass analyzer, resulting in one fragment ion spectrum (Figure 2D). The fragment ions were assigned to the sequence of the protein, thus creating a fragment map with residue cleavages (Figure 2E).

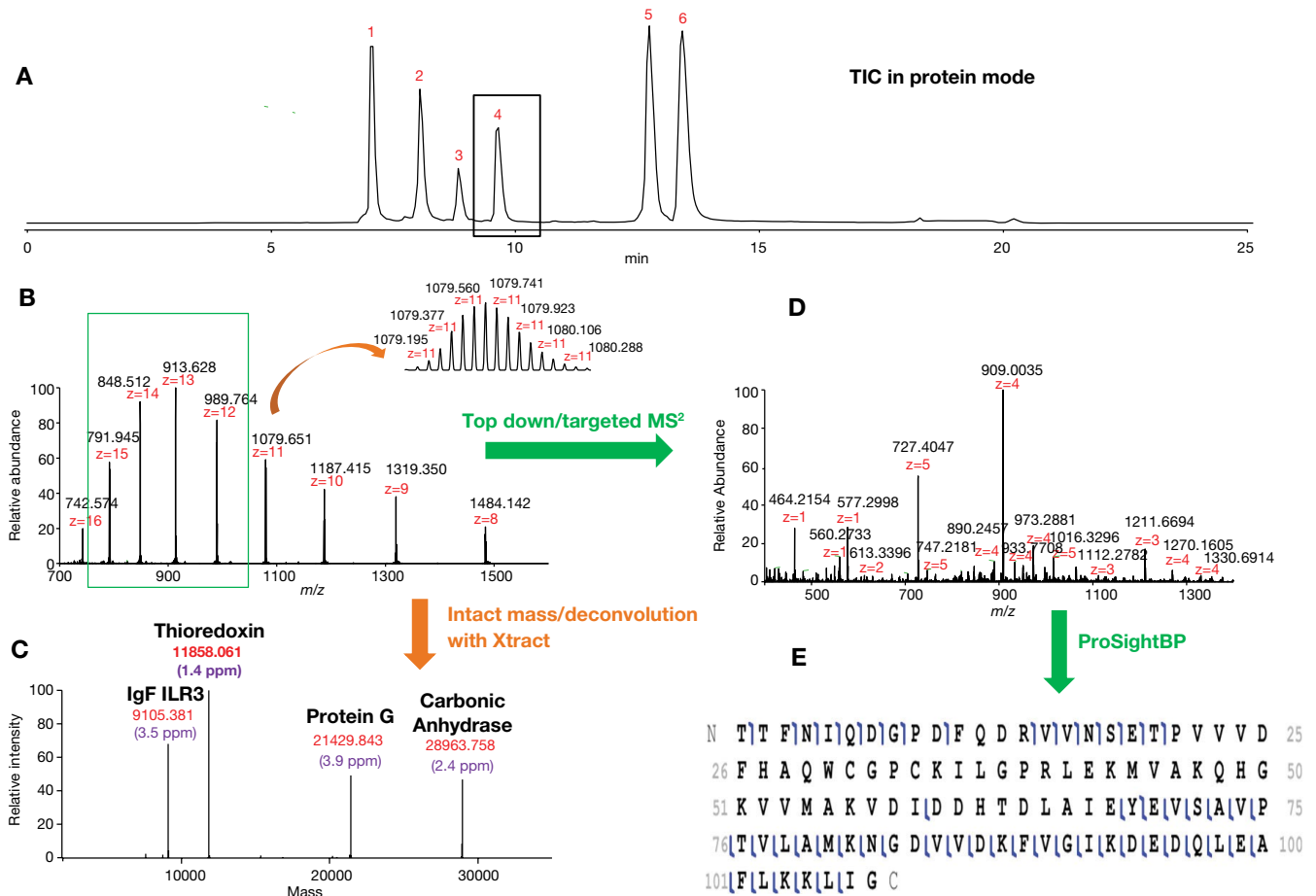


Figure 2. Workflows of intact proteins- intact mass and top down strategies. A) TIC of 15 ng Pierce Intact Protein Standard Mix in protein mode (Peak 1: Protein G, Peak 2: *Streptococcus* protein AG, Peak 3: Human IGF-I, Peak 4: Thioredoxin, Peak 5: Carbonic Anhydrase II, Peak 6: *E. coli* exo-Klenow; B) Spectrum of thioredoxin with isotopically resolved charge states; C) Deconvoluted spectrum of isotopically resolved proteins. The source spectrum generated by averaging over a selected time window of TIC was deconvoluted with the Xtract algorithm; D) Fragment spectrum of thioredoxin acquired by targeted MS^2 by 4-plexing; E) Fragment map shows the assigned fragments to the sequence of thioredoxin with ProSightBP algorithm

MABPac RP columns are well known to possess excellent performance and low carryover for intact mAb samples. As demonstrated by Figure 3, trastuzumab innovator and biosimilar elute at the same time, the differences

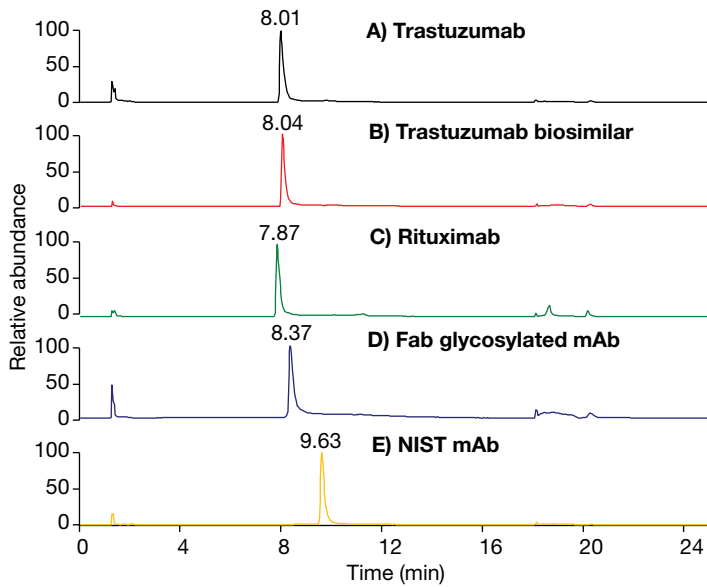


Figure 3. TICs of five intact mAbs in normal mode at 17,500 resolution. From each mAb, 15 ng was injected.

between them are in their glycan distribution on the Fc portion and some PTMs. The most hydrophobic mAb is apparently the NIST mAb, eluting much later than the other four mAbs. Surprisingly, the Fab glycosylated mAb is the second most hydrophobic mAb even though this is the only mAb with two glycosylation sites, heavily occupied with hydrophilic N-glycans. Rituximab elutes slightly earlier than trastuzumab, suggesting this mAb to be the most hydrophilic.

When comparing innovator and biosimilar trastuzumab, no difference was observed in their chromatographic behavior. Therefore, a scrutiny was undertaken to ascertain differences in PTMs both at intact and subunit level.

When comparing the deconvoluted spectra of innovator (Figure 4A) to biosimilar (Figure 4B), one can certainly conclude that innovator trastuzumab shows a higher level of galactosylation than the biosimilar, given the higher intensity of G0F/G1F, G1F/G1F, and G1F/G2F glycoforms. The biosimilar trastuzumab lacks G0/G0F and G2F/G2F glycoforms. Both the innovator and biosimilar drugs show trace level of untruncated proteoforms with C-terminal Lys

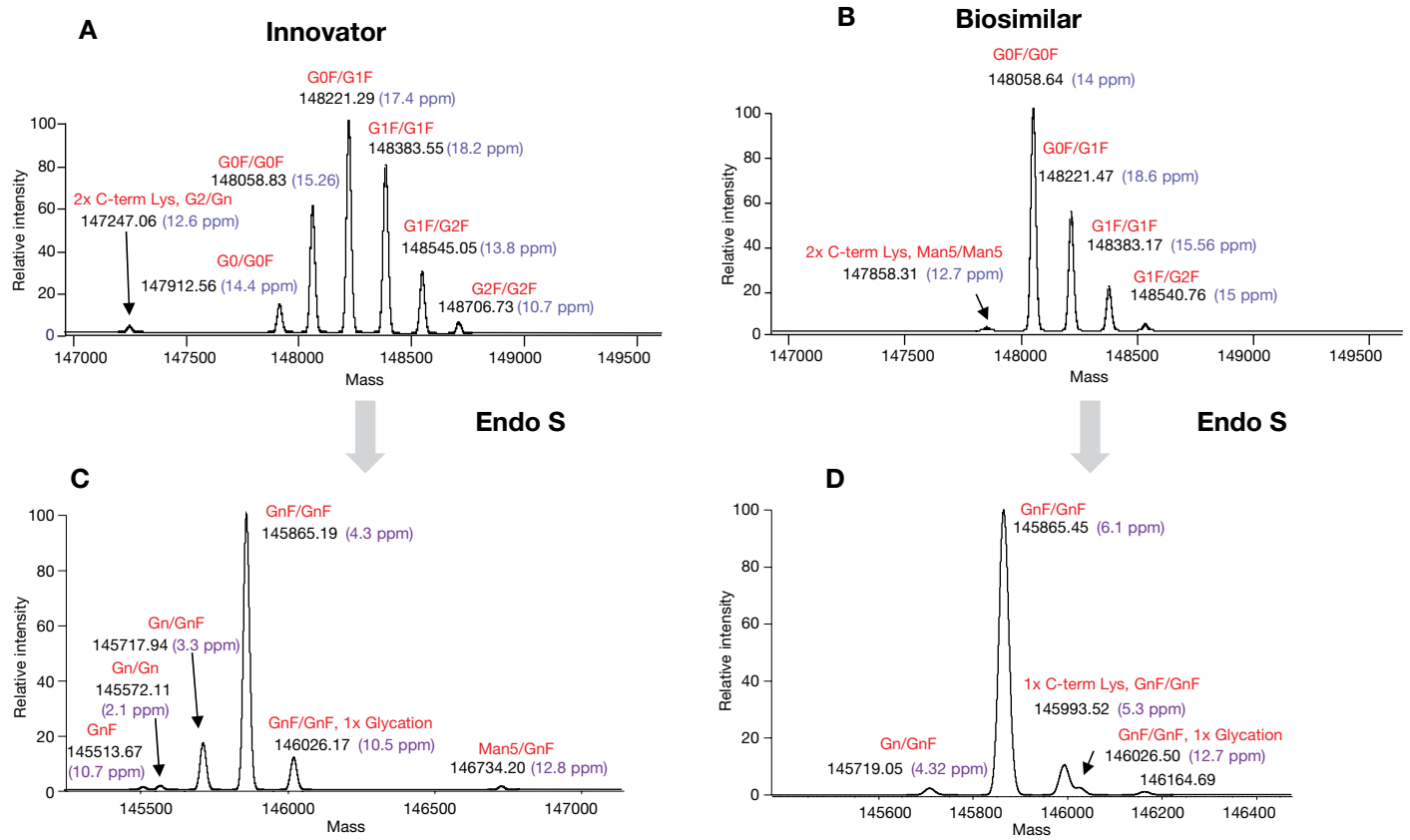


Figure 4. Comparison of trastuzumab innovator to its biosimilar in normal mode at 17,500 resolution. A) Deconvoluted spectrum of the innovator; B) Deconvoluted spectrum of the biosimilar; C) Deconvoluted spectrum of the EndoS digested innovator; D) Deconvoluted spectrum of the EndoS digested biosimilar

residues. Another, important distinctive attribute is the level of afucosylated glycoforms correlated to the effector function of the therapeutic drugs. The presence of G0/G0F glycoform in the innovator indicates that the ADCC function (effector function) of innovator is ranked higher than the biosimilar.

To further verify this statement, the intact innovator and biosimilar mAbs were treated with the endoglycosidase EndoS enzyme that cleaves the β -1,4 linkage between the two GlcNAc units in the core of the glycans on the Fc portions, leaving a GlcNAc unit with or without α -1,6 linked fucose on the protein. With the glycans clipped, the spectral complexity is simplified and some minor details, which would otherwise remain undetectable, became traceable. For instance, as shown by Figures 4C and 4D, digestion with EndoS reveals the presence of non-enzymatic glycation at 146026 in both spectra. Its level is three times higher in the innovator than in the biosimilar. Even at low resolution, the glycated proteoform is resolved from the GnF/GnF proteoform with one C-terminal Lys which is detected at 145993 and was only found in the

biosimilar. The high intensity of Gn/GnF glycoform in the innovator clearly confirms the statement made about the higher level of afucosylated glycans deposited in the Fc domain of the innovator trastuzumab.

Breaking down the mAbs into subunits such as scFc, LC, and Fd' with IdeS digestion followed by reduction helps decipher which of the subunits caused the difference in retention times of the mAbs observed in Figure 3. While retention times are similar between rituximab and trastuzumab subunits (Figure 5), retention time of the Fd' portion of NIST mAb evidences that the highest retention time (most hydrophobic) of NIST mAb can be attributed to the increased hydrophobicity of the Fd' subunit. Regarding fab glycosylated mAb, which was found to be the second most hydrophobic mAb based on Figure 3, the chromatographic behavior of subunits shows contrary to the other four examples in Figure 5, the LC elutes far later indicating this part to be responsible for the increased hydrophobicity of this mAb. The Fd' portion bearing an additional glycosylation site is well resolved from LC and elutes earlier owing to its increased hydrophilic properties.

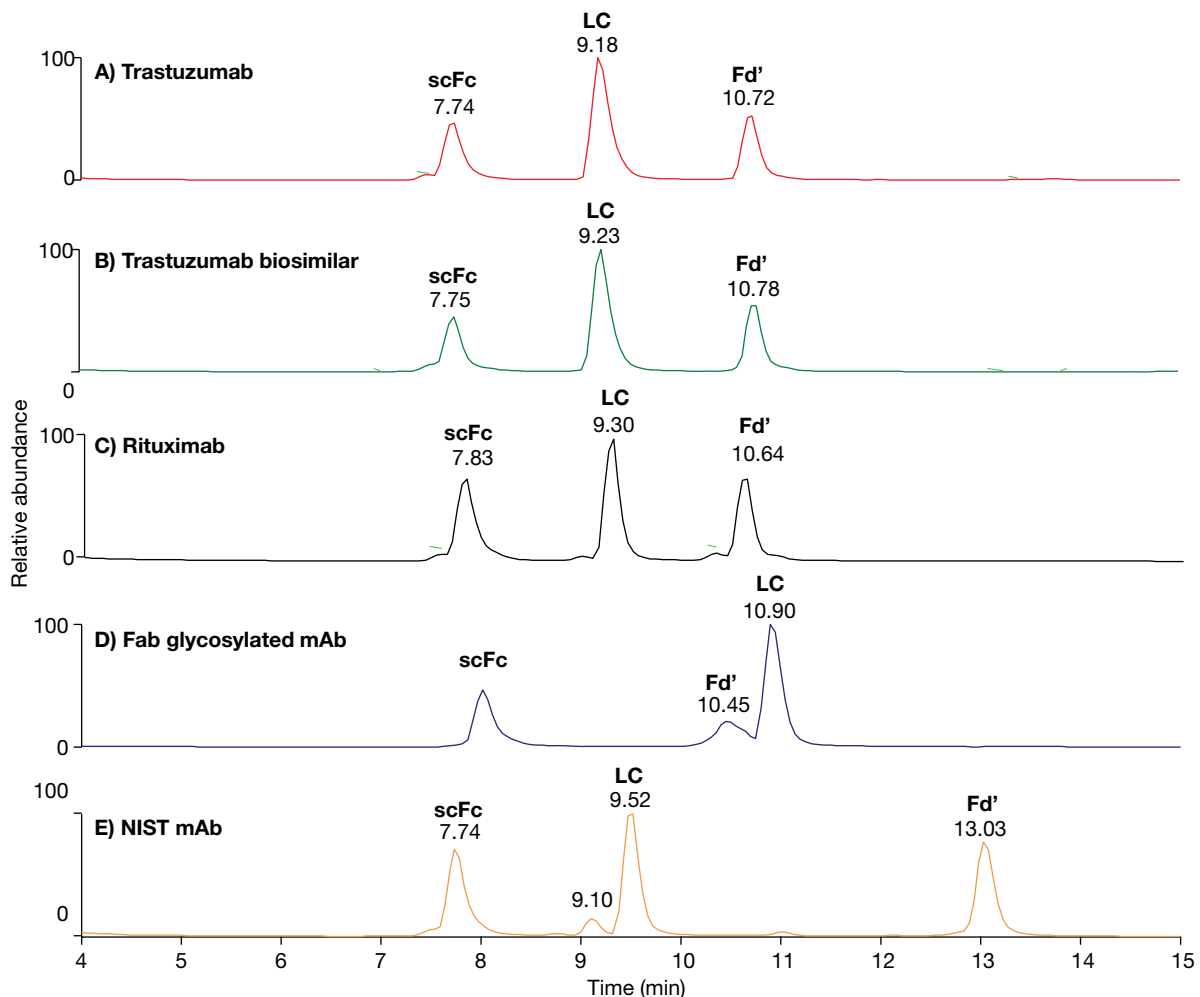


Figure 5. Subunit analysis of 5 mAbs in protein mode at 140,000 resolution. From each digest, 15 ng were injected.

While an intact mass approach requires minimal sample preparation, measuring only the average mass at low resolution makes the identification of low molecular weight or near isobaric PTMs difficult.

Although digesting intact mAbs with EndoS alleviates the identification of additional PTMs such as glycation and core fucosylation levels, the accuracy and resolving power supported by the protein mode at high resolution is still desired for the reliable identification of low abundant, low molecular weight PTMs. Resolving the mAb subunits isotopically in protein mode enables the highly accurate, more reliable identification of PTMs and which region of the mAb that they reside. Figure 6 shows the mirror image of Fc glycoforms of innovator and biosimilar trastuzumab. As shown by this comparison, 11 glycoforms can be distinguished. Their identification relies on the highly

accurate (<5 ppm) masses obtained after deconvolution. Major afucosylated glycoforms, such as G0, G1, and G2 are more abundant on the innovator.

The power of high resolution in subunit analyses was indeed harvested when minor PTMs located on LC and Fd' were identified (Figure 7). Deamidation of asparagine results in an increase of 0.985 Da and detecting it at protein level requires HRAM mass spectrometry in protein mode. As reported by Figure 7A, there are two minor peaks appearing at 23590.575 and 23591.561, where the difference in mass is 0.986 Da allowing one to reliably assign deamidation to the glycosylated proteoform. The Fd' portion of innovator contains an additional glycation and two Cys oxidations compared to biosimilar (Figure 7B and Figure 7D).

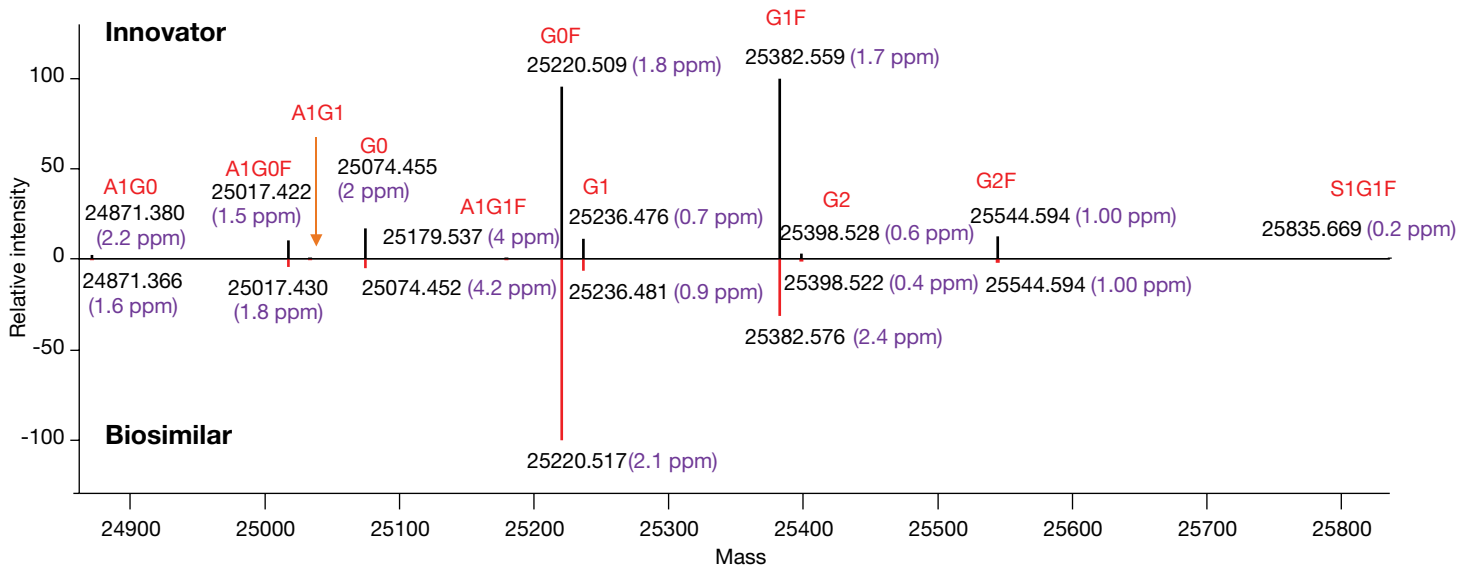


Figure 6. Comparison of glycoform distribution on scFc subunit of trastuzumab innovator vs. biosimilar

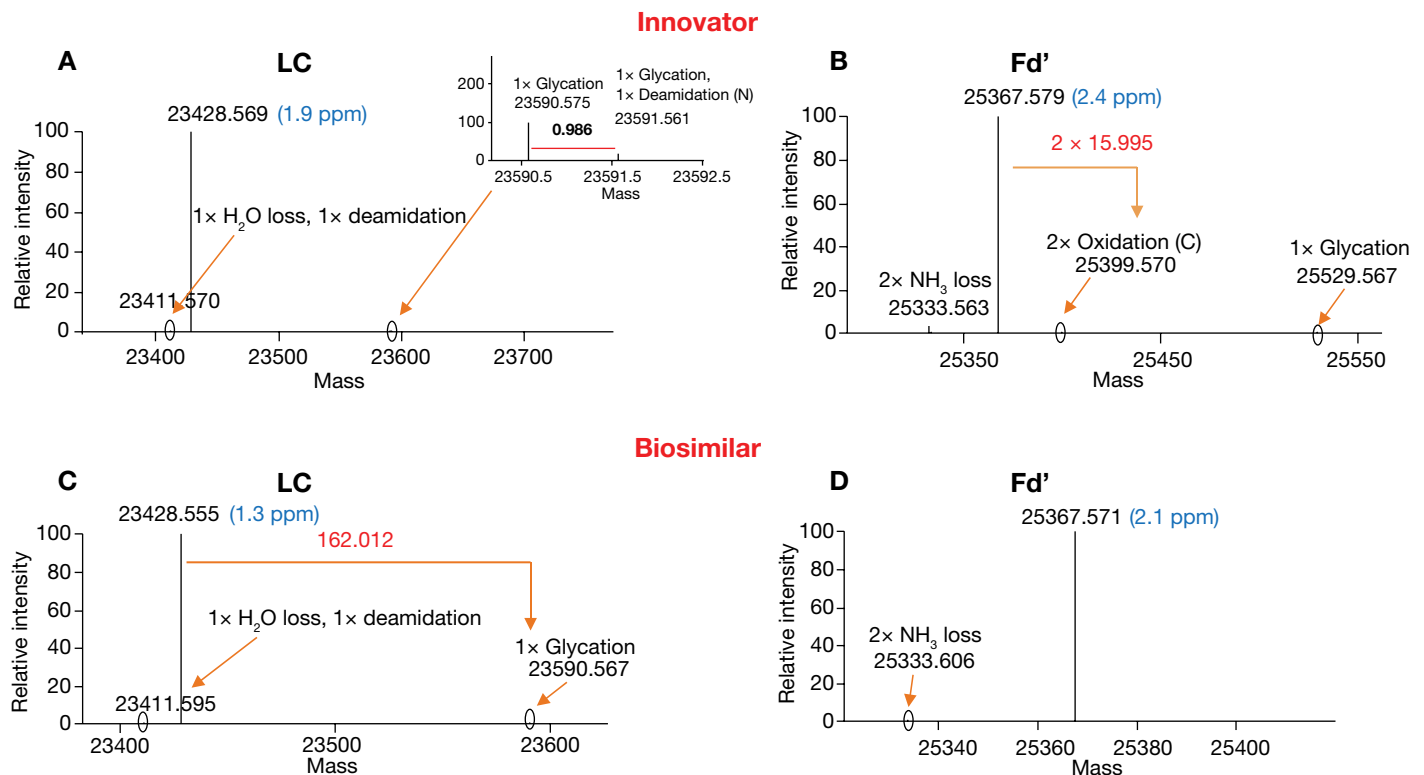


Figure 7. Characterization of microheterogeneity on LC and Fd' trastuzumab subunits-innovator vs. biosimilar. A) Deconvoluted spectrum of light chain (LC) of innovator; B) Deconvoluted spectrum of Fd' fragment of innovator; C) Deconvoluted spectrum of light chain (LC) of biosimilar; D) Deconvoluted spectrum of Fd' fragment of biosimilar

Conclusion

The use of the MAbPac RP capillary column offers utmost sensitivity and excellent selectivity for the mass spectrometric analyses of intact proteins and mAb subunits. As demonstrated, it supports all the intact protein workflows using small amounts of sample. Due to the extreme sensitivity achieved in capillary flow, using this column is anticipated to greatly alleviate difficulties arising from frequent sampling from low volume micro bioreactors at the early stage of process development in the biopharmaceutical industry. Even though only ng quantities of samples was used for each workflow, multiple

critical attributes have been determined with high accuracy. Furthermore, miniaturizing the column chemistry using the proper and robust hardware design still maintains the excellent resolving power of the high flow MAbPac RP columns towards proteins. The workflows demonstrated above can also be easily adapted in discovery proteomics when samples are available at limited quantity.

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