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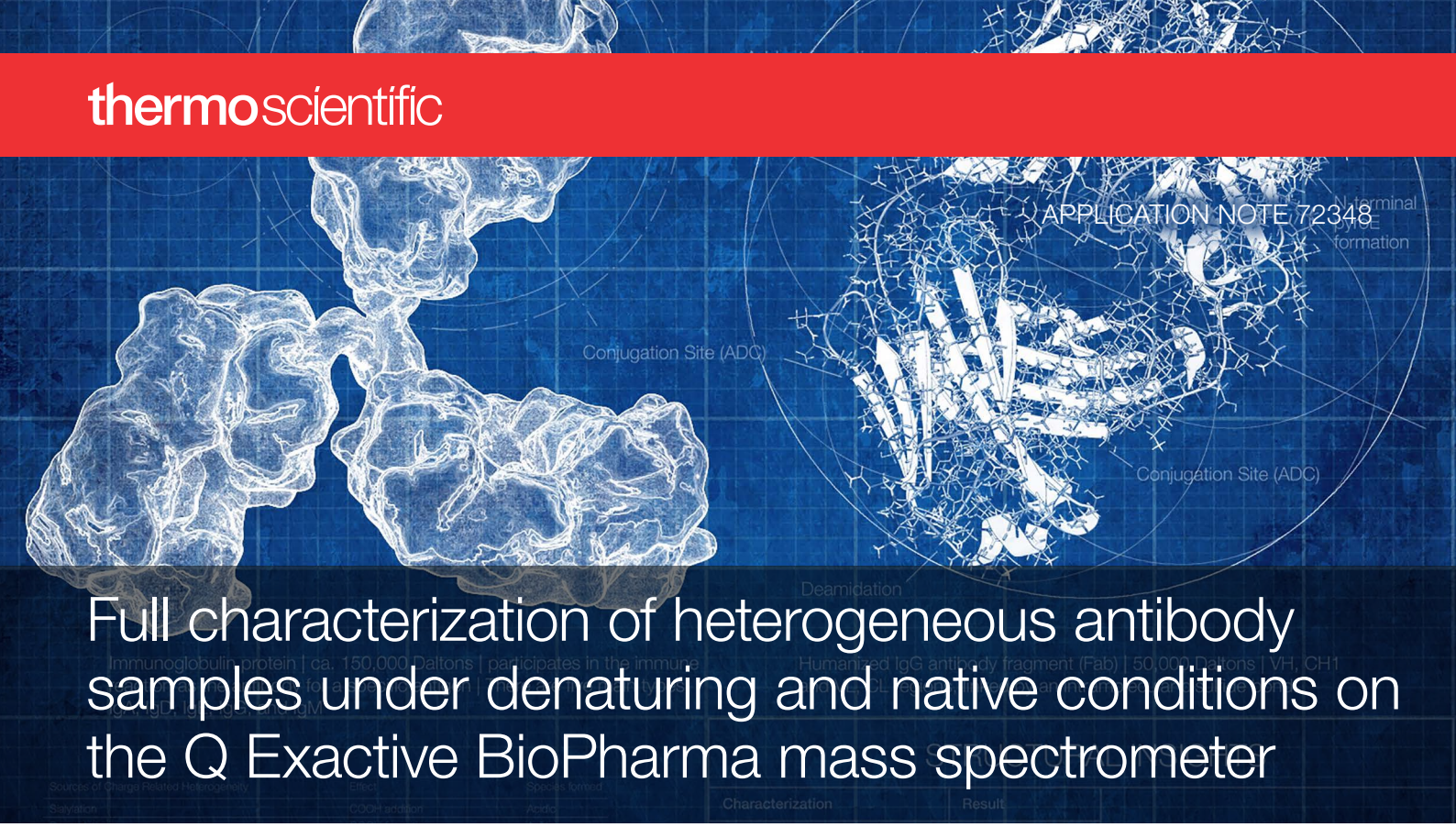

Full characterization of heterogeneous antibody samples under denaturing and native conditions on the Q Exactive BioPharma mass spectrometer

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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.

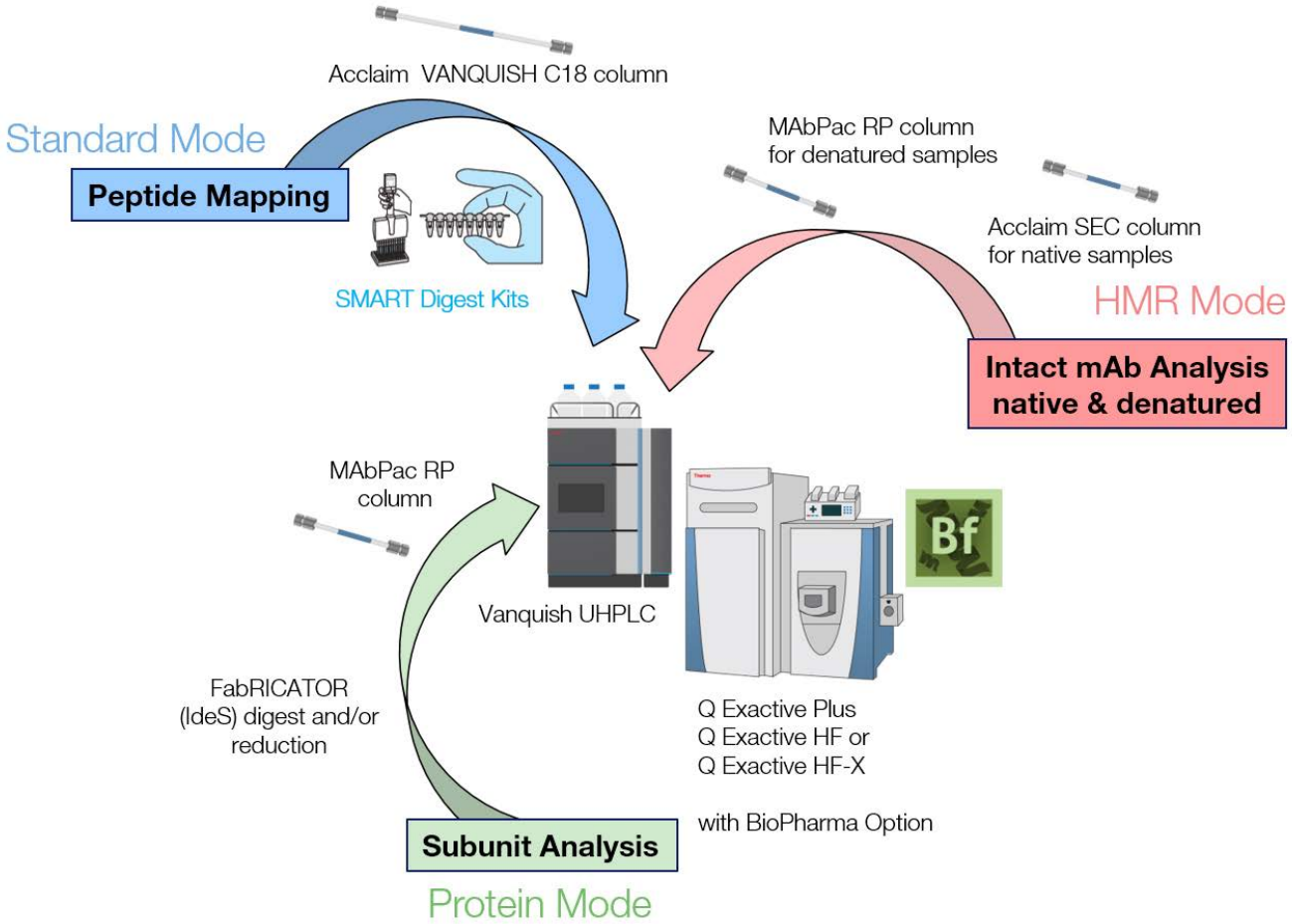


Figure 1. The Q Exactive Plus and Q Exactive HF mass spectrometers equipped with the BioPharma Option provide three different modes to cover the three major workflows in BioPharma.

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.

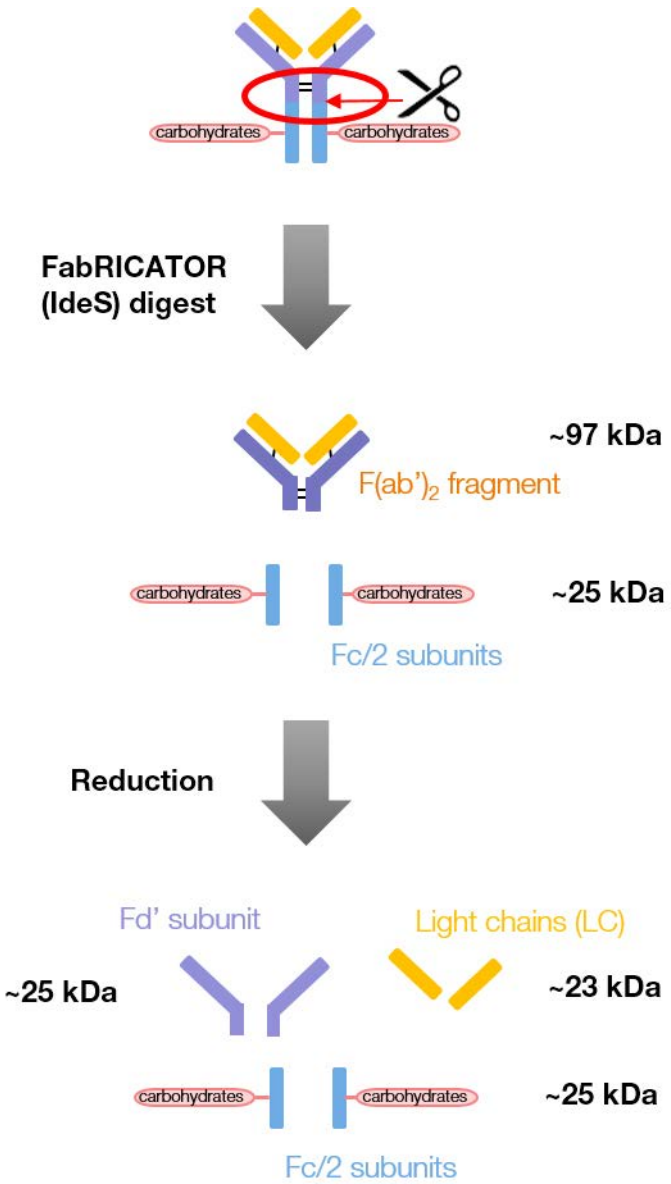


Figure 2. Schematic displaying the subunits obtained after FabRICATOR (IdeS) digest, which are the $F(ab')_2$ fragment and the Fc/2 subunits resulting after reduction in the Fd', LC, and Fc/2 subunits.

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:	Acclaim VANQUISH C18 2.1 × 250 mm		
Column Temp.:	60 °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.

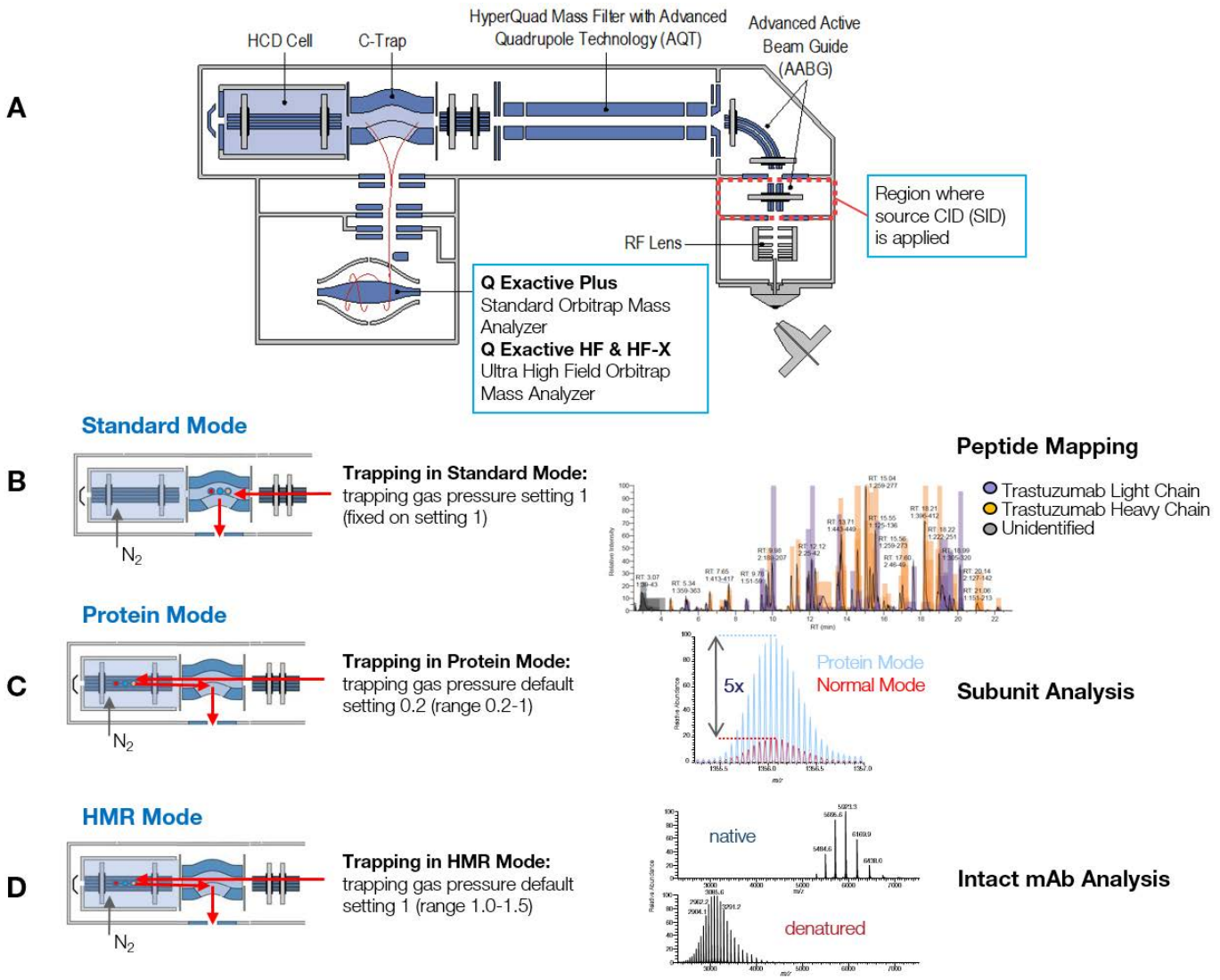


Figure 3. A) Schematic of the Q Exactive Plus and Q Exactive HF mass spectrometers and differences in the trapping path in the three different operating modes available: B) Standard Mode, C) Protein Mode and D) HMR Mode.

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.

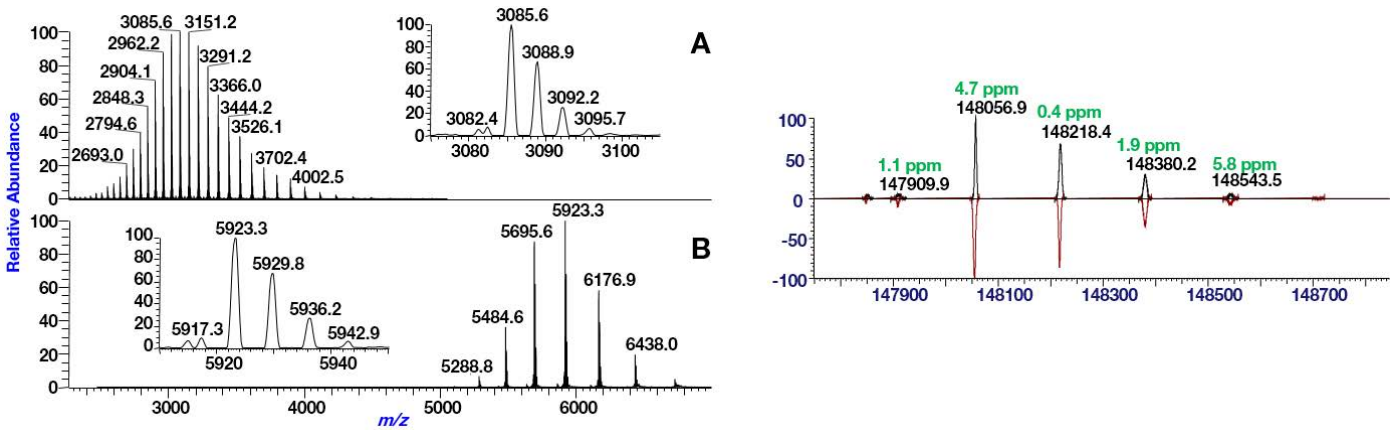


Figure 4. Full MS spectra acquired from intact trastuzumab under denaturing (A) and native conditions (B).

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

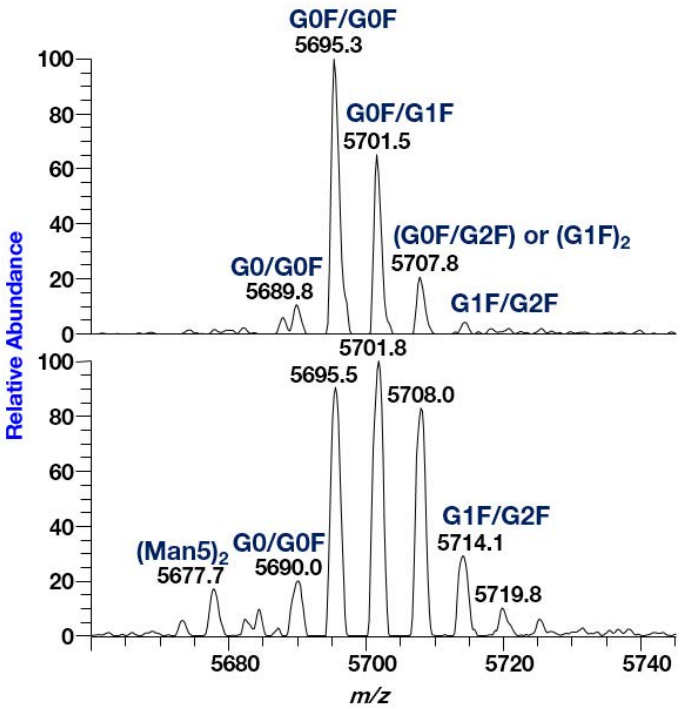


Figure 5. Observed glycoform patterns for two different lots of trastuzumab on the raw data level showing significantly different relative abundances.

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.

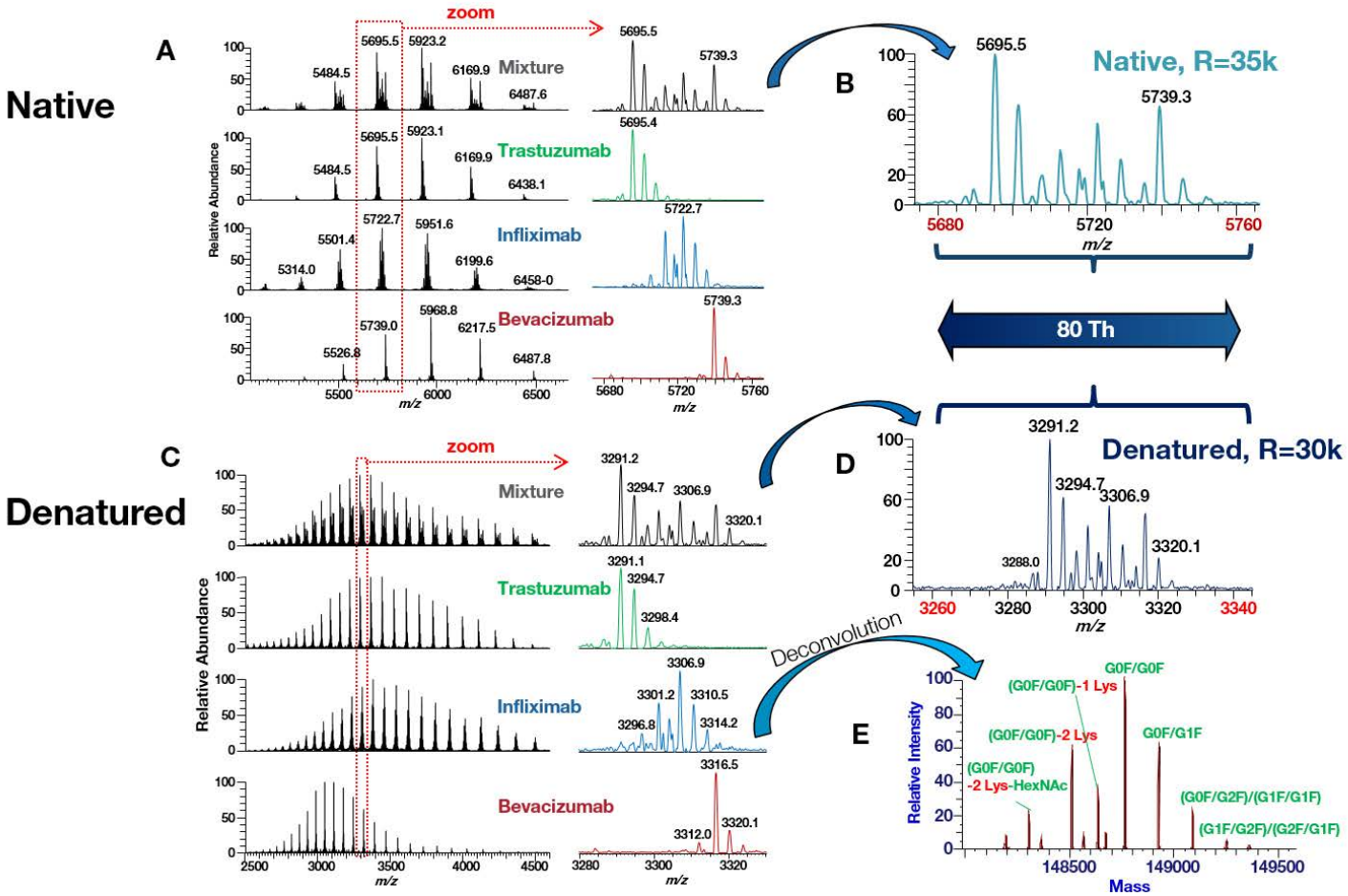


Figure 6. Analysis of the three mAbs trastuzumab, infliximab, and bevacizumab individually, as well as a mixture, under denaturing and native conditions.

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.

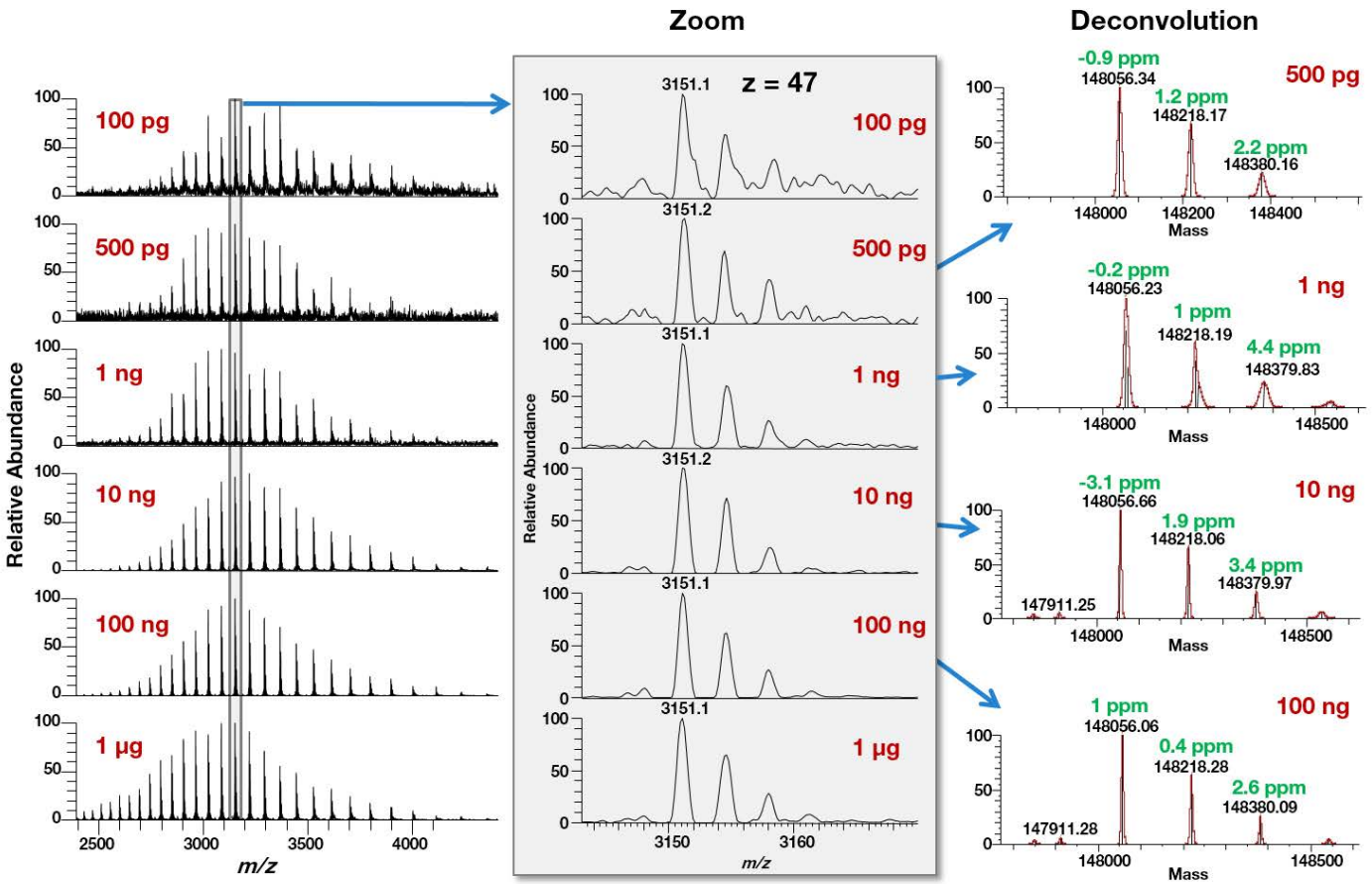


Figure 7. Serial dilution series of trastuzumab ranging from 100 pg to 1 µg total protein loaded on column.

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

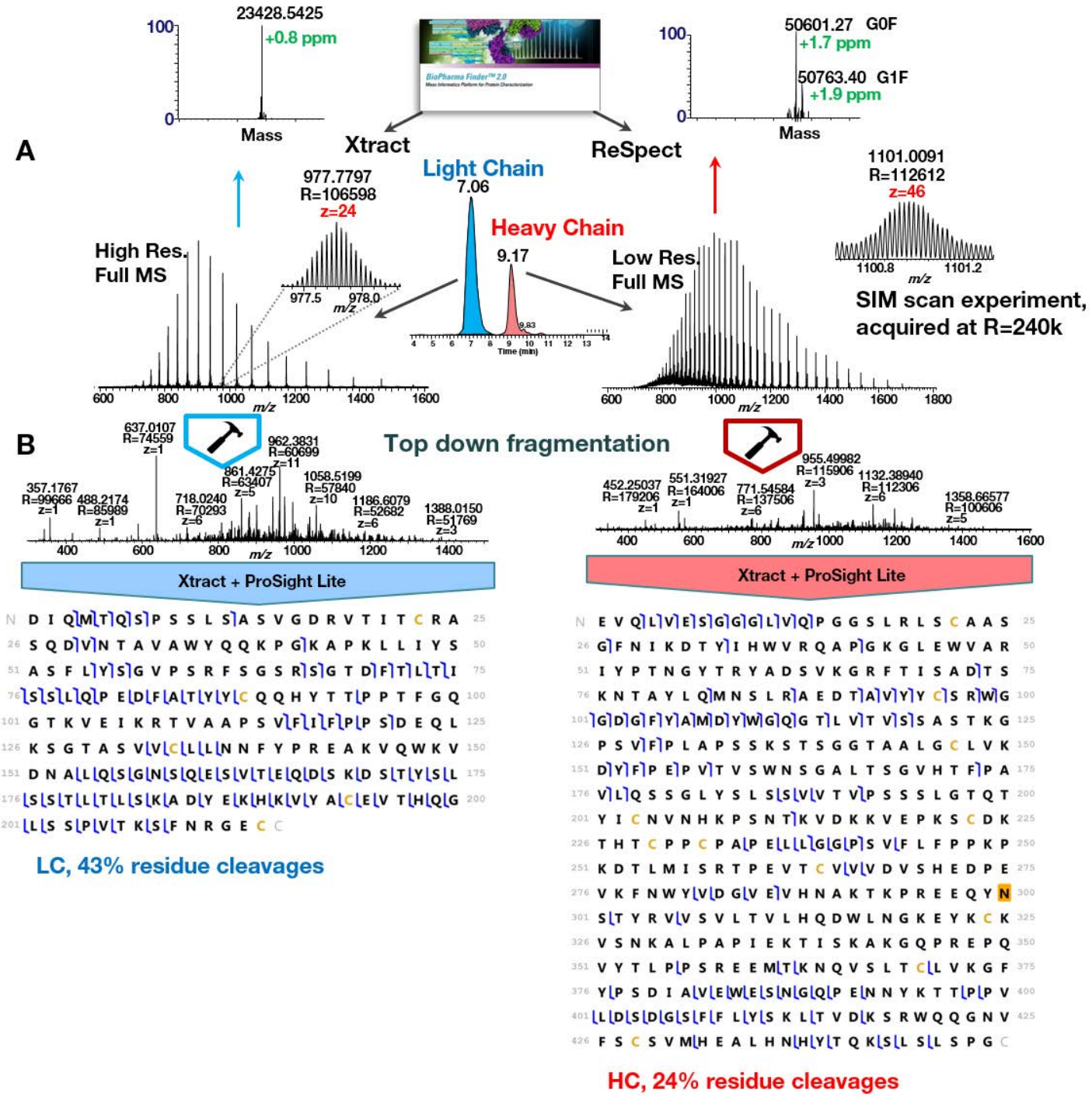


Figure 8. A) Detection of the separated light (LC) and heavy chains (HC) in Full MS as well as a SIM scan experiment of one charge state of one glycoform of the heavy chain providing a baseline resolved isotope pattern. B) Top-down subunit analysis of light and heavy chain of trastuzumab and assignment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

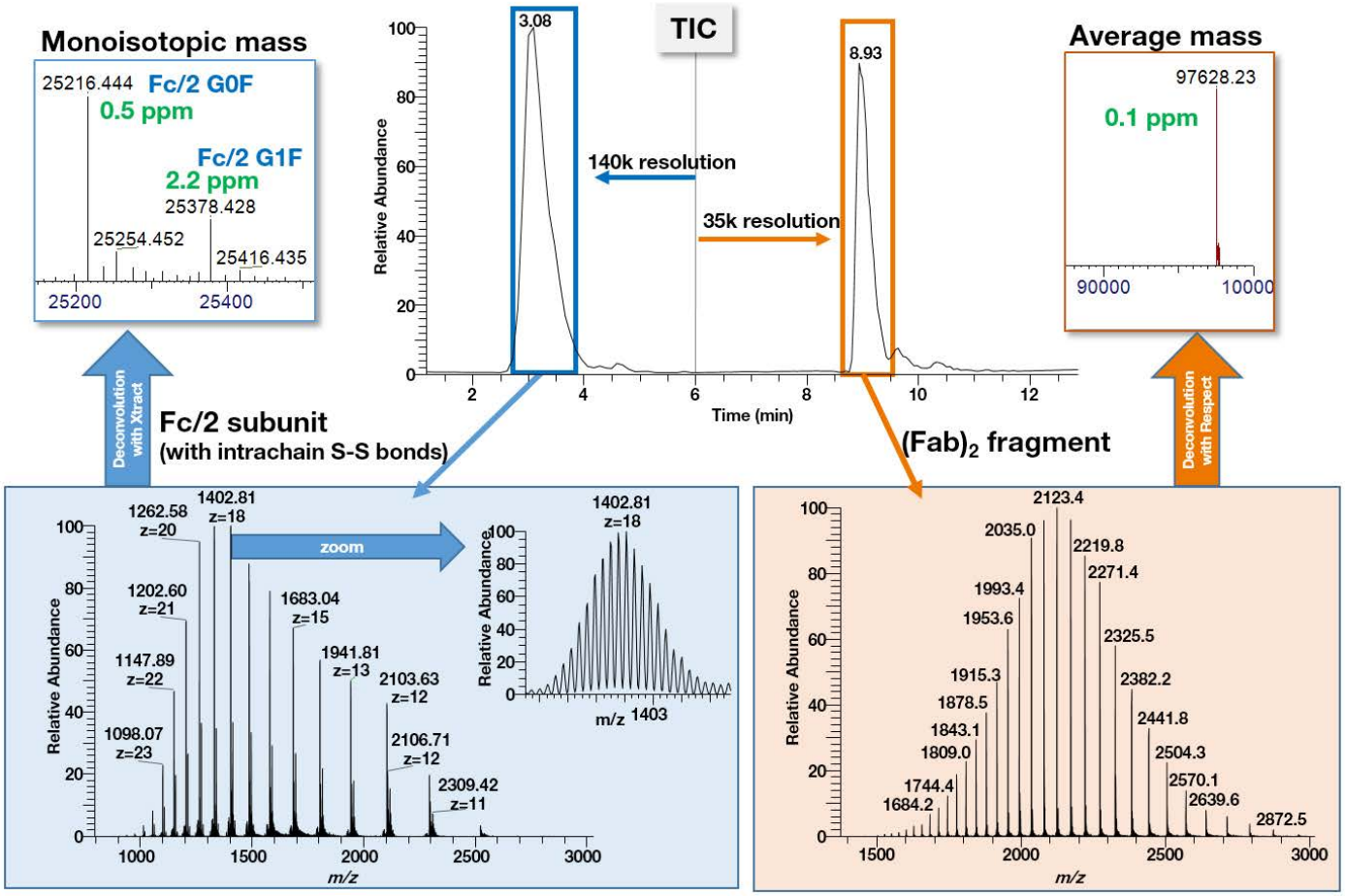


Figure 9. Total ion chromatogram and obtained MS spectra of trastuzumab after FabRICATOR digest without reduction resulting in the Fc/2 and F(ab')$_2$ regions with both subunits bearing intact disulfide bonds, resulting in observed molecular weights of ~25 kDa for the Fc/2 subunits and ~97 kDa for the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

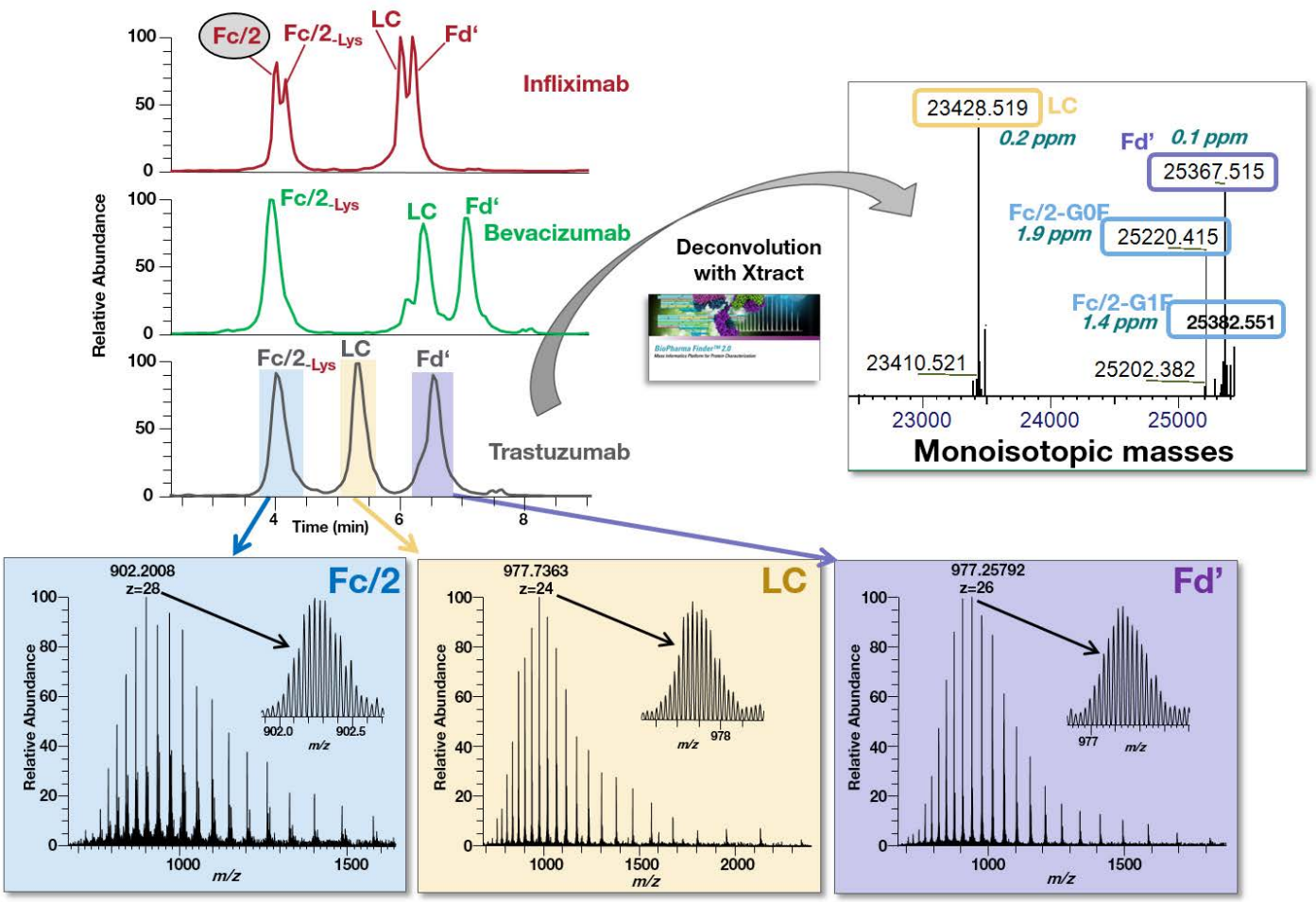
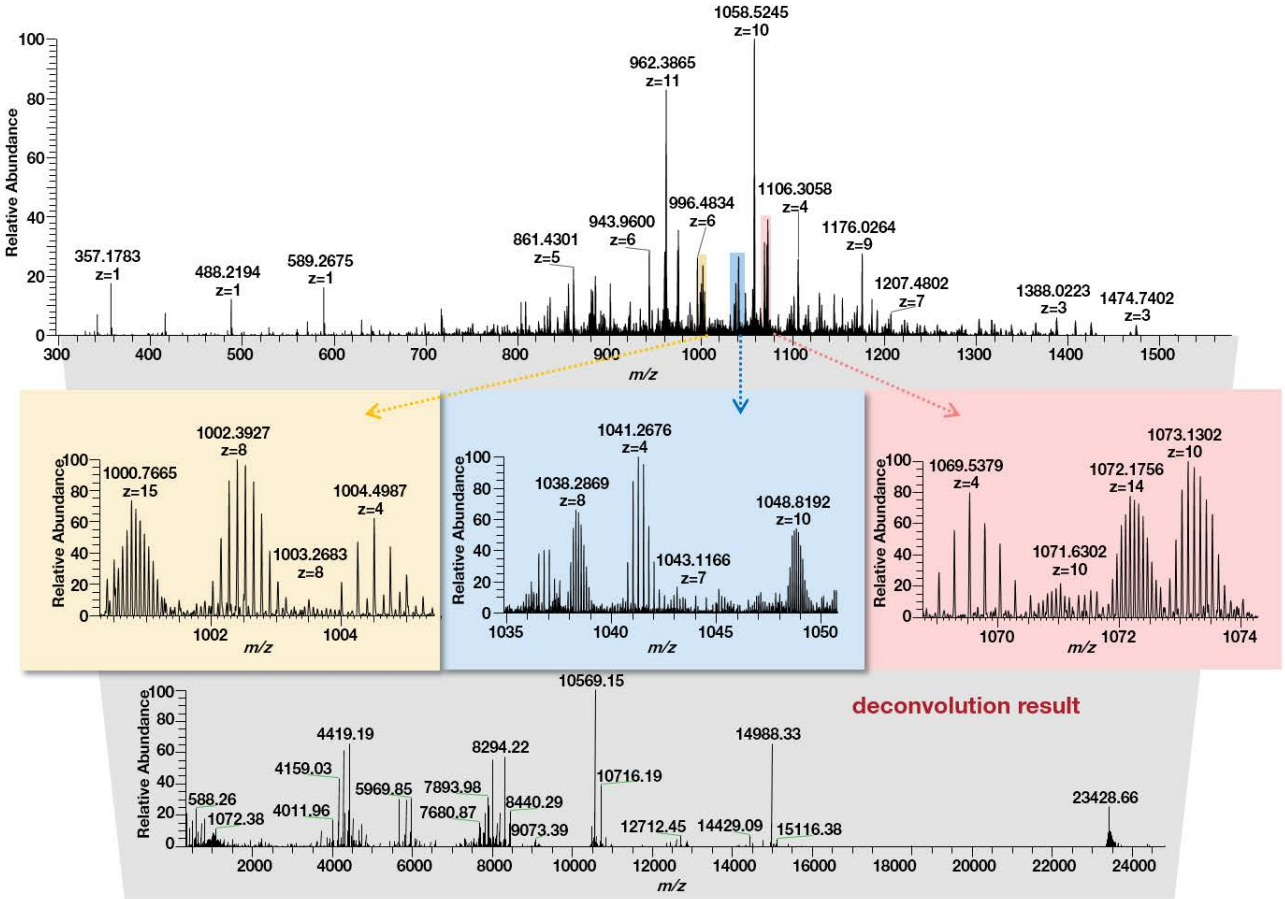


Figure 10. Total ion chromatograms of separated subunits obtained after FabRICATOR digest and reduction from trastuzumab, bevacizumab, and infliximab. Obtained Full MS mass spectra and baseline-resolved isotope patterns of the individual charge states, as well as the result after deconvolution, is showcased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

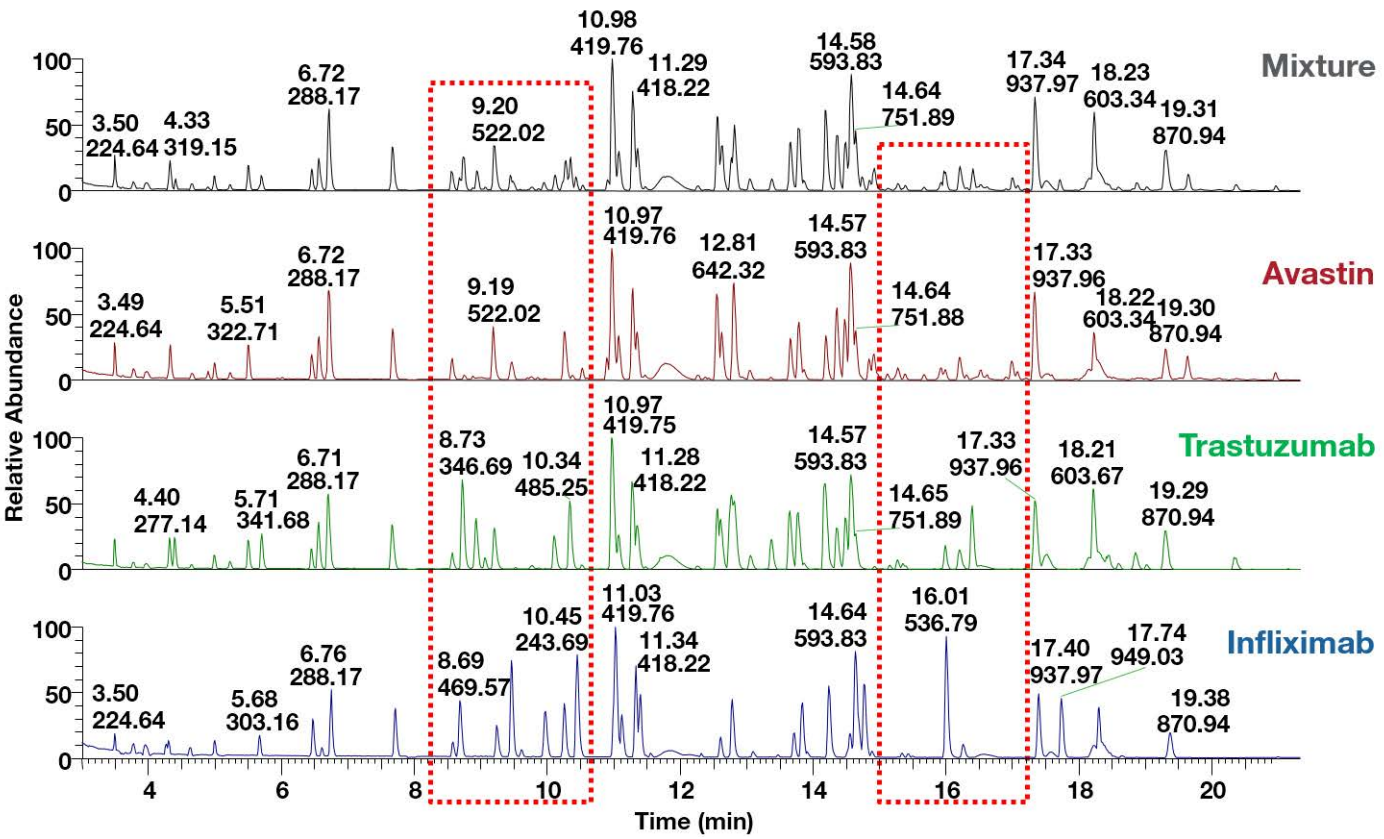


Figure 12. Base peak chromatograms obtained from individual digests of trastuzumab, bavacizumab, and infliximab as well as a mixture of all three. The basic peak pattern is very similar due to high similarity of the amino acid sequences amongst the three mAbs. Areas reflecting distinct differences are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

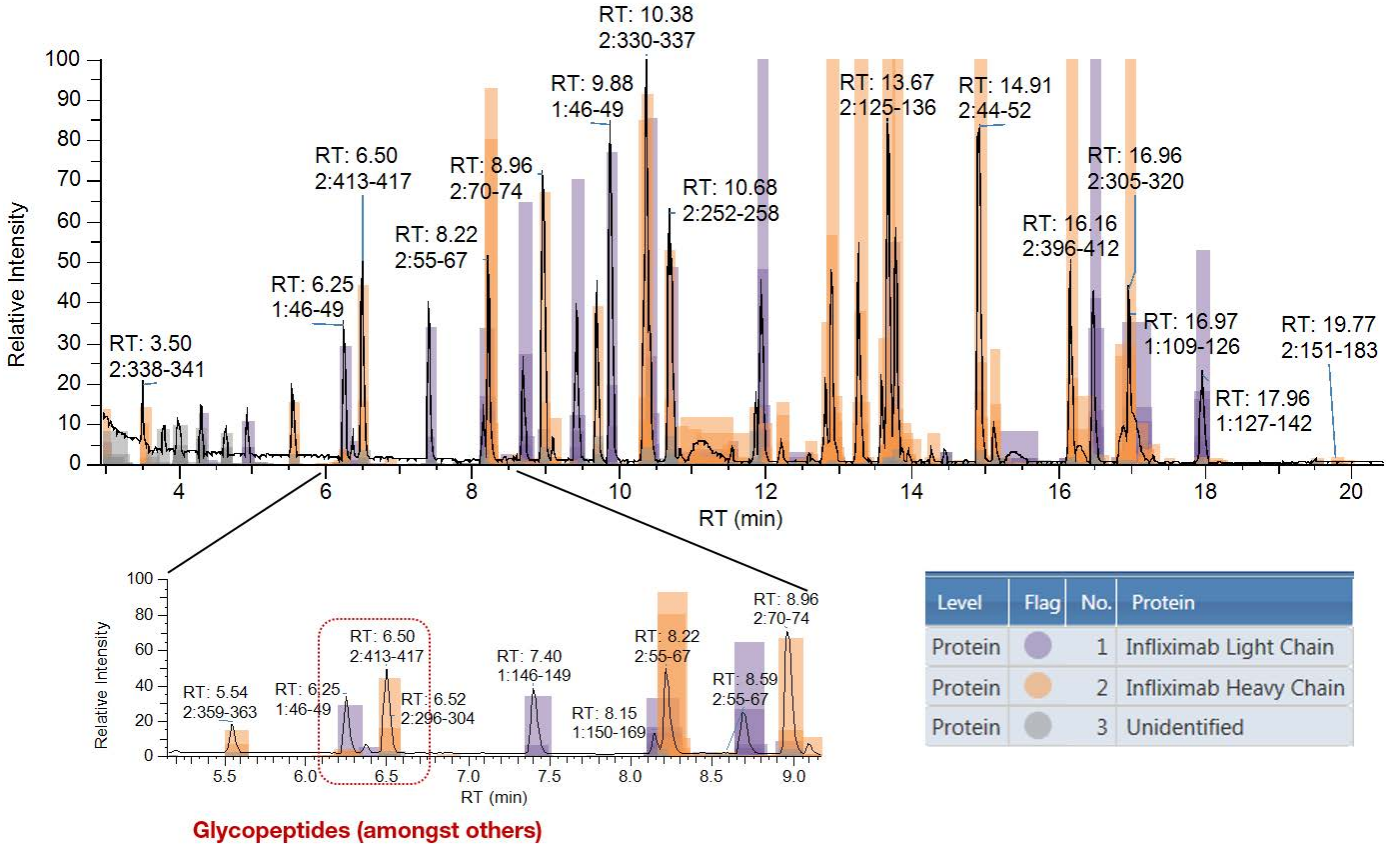


Figure 13. Peak shading provided by BioPharma Finder software based on peptides assigned to the light chain or heavy chain of infliximab. Some peaks remain unidentified, most commonly towards the onset or the end of the chromatogram.

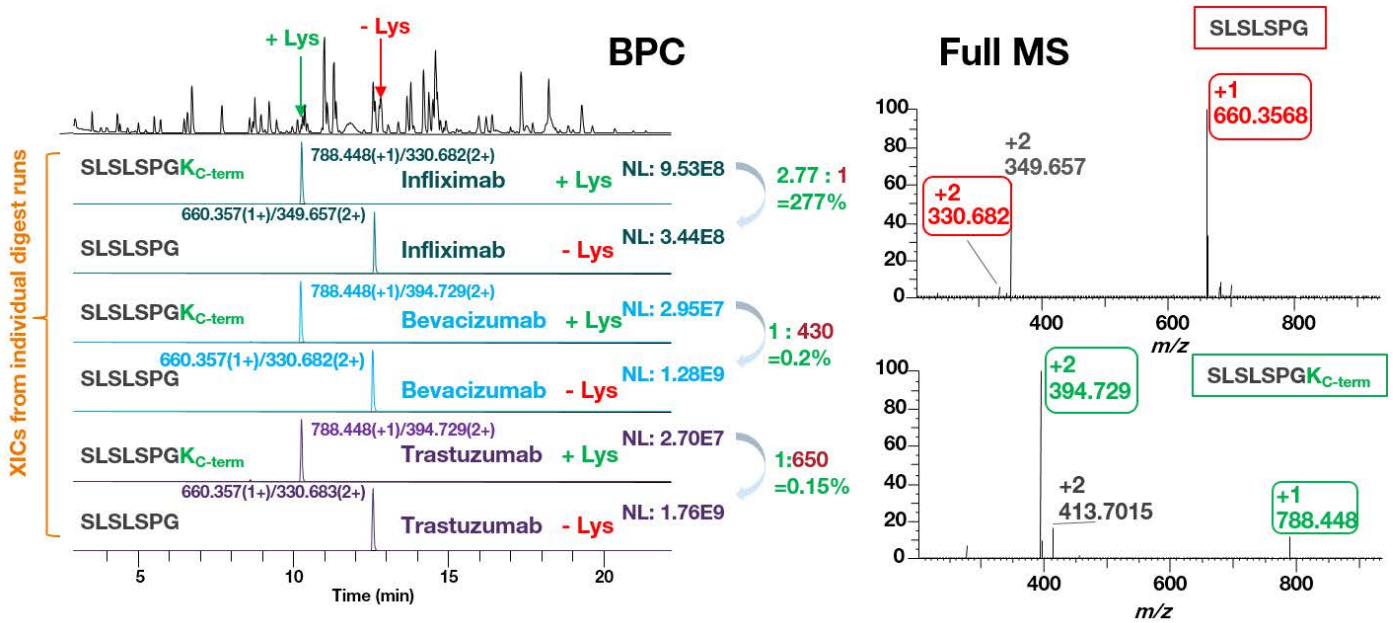
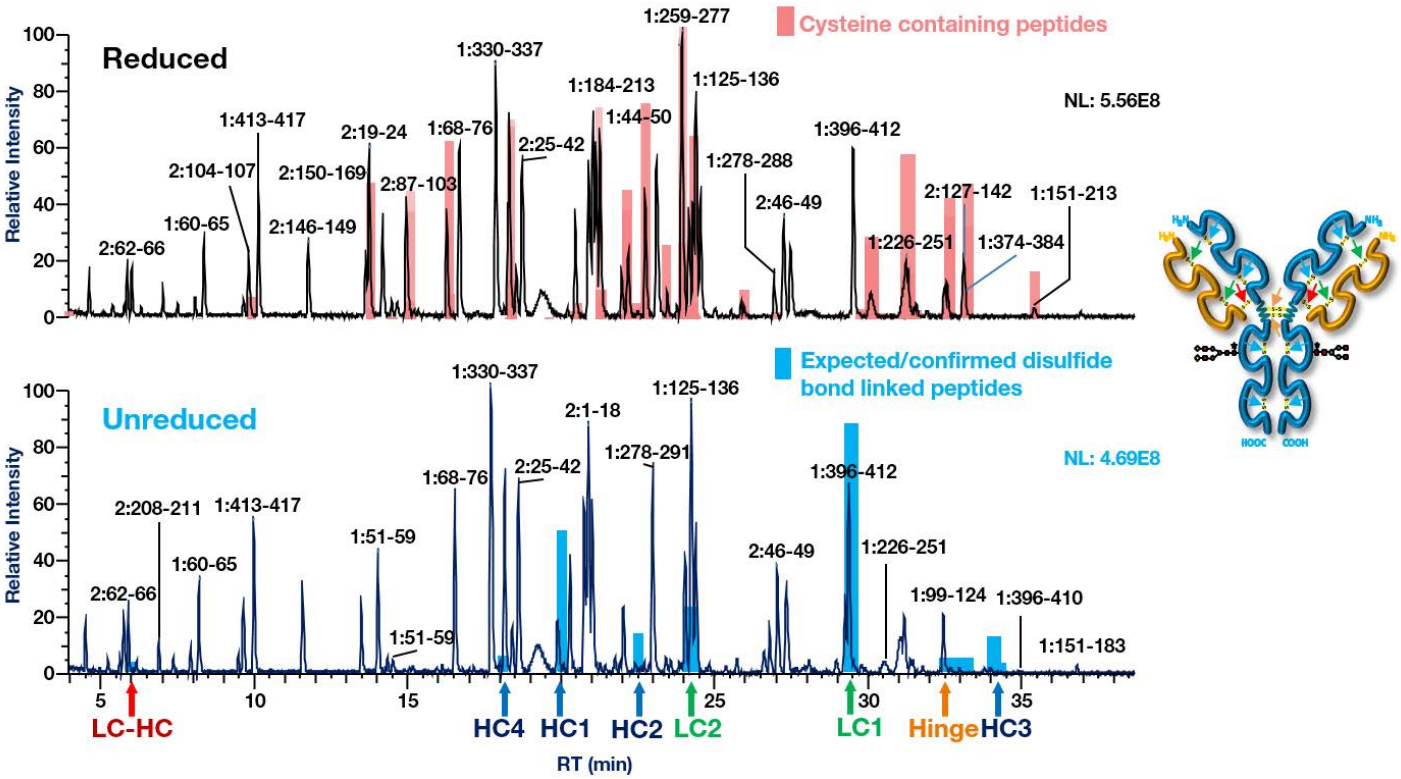


Figure 14. Base peak chromatogram (BPC) of infliximab and XICs for the C-terminal peptides of the heavy chain with and without lysine truncation for infliximab, bevacizumab, and trastuzumab. XICs were taken from the individual digests of the three mAb analyses. The Full MS spectra highlight the relative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

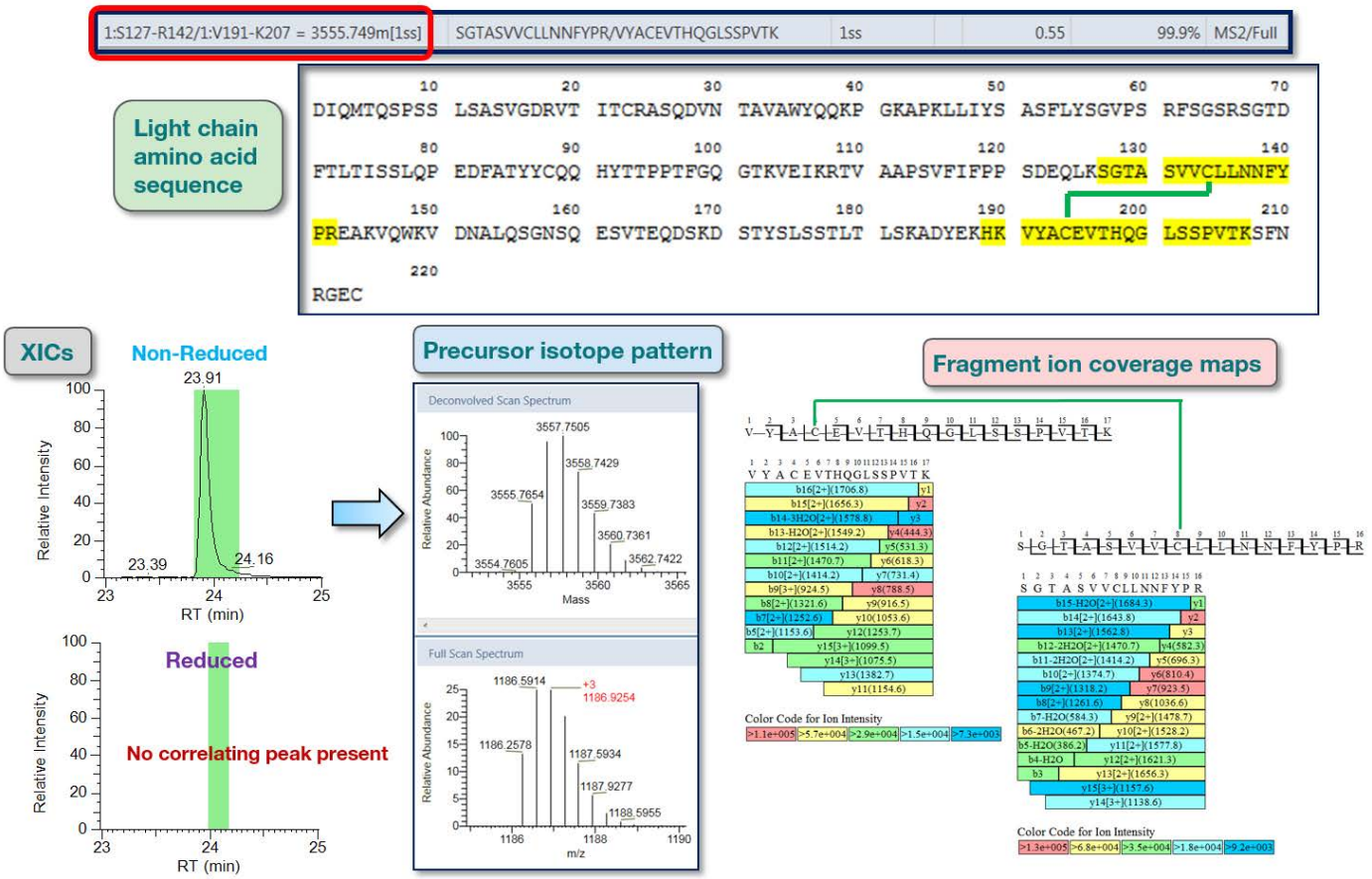


Figure 16. Example of an identified and confirmed intrachain disulfide linked peptide. The XIC based on the linked peptide mass shows the absence upon reduction of the sample. The detected isotope pattern of the un-reduced peptide matches very well with the theoretical isotope pattern. The fragment ion 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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