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Antibody subunit analysis workflow on a quadrupole-Orbitrap mass spectrometer: from optimized sample preparation to data analysis

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Keywords

Antibody subunits, Orbitrap, Q Exactive BioPharma, sample preparation, middle-down, IdeS digest, reduction

Goal

The goal of this application note is to discuss the importance of proper sample preparation for mAb subunit analysis and to provide optimized LC/MS conditions to achieve conclusive information on the mass of the subunits as well as for performing middle-down analysis for sequence confirmation.

Key highlights

- Provides a full workflow for subunit analysis: sample preparation conditions, chromatographic separation, mass spectrometric analysis, and data interpretation for subunit molecular weight confirmation and sequence elucidation applying a middle-down approach.
- Demonstrates the importance of proper sample preparation for subunit analysis.
- Highlights the benefits of using the Intact Protein Mode provided on the Thermo Scientific™ Q Exactive™ BioPharma mass spectrometry platform for high-resolution, accurate mass MS analysis of mAb subunits.

Introduction

Mass spectrometric analysis of antibodies at the protein and peptide level is critical during development and production of biopharmaceuticals. Intact mass and peptide mapping analyses have become essential techniques in mAb characterization throughout the development and production process. The analysis of antibody subunits provides additional and complementary information with the advantage of requiring only very little sample preparation.[1]

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Reduction of an intact mAb is one option to generate the light and heavy chain subunits. When reduction is performed after enzymatic digestion with IdeS enzyme, the scFc, LC, and Fd′ subunits, all ~23–25 kDa in MW are generated (Figure 1). Subunits in that molecular weight range can easily be separated on a chromatographic scale, enabling very accurate mass determination based on high-resolution full-scan mass analysis and are most amenable to middle-down analysis for providing sequence coverage.

Figure 2 shows an overview of the mAb subunit LC/MS analysis workflow: Thermo Scientific™ MAbPac™ RP columns provide excellent chromatographic separation of subunits with minimal carryover. The Thermo Scientific™ Vanquish™ UHPLC platform supports fast, robust, reproducible, and sensitive analysis. MS data acquisition on the Thermo Scientific™ Q Exactive™ BioPharma platform provides sensitive analysis resulting in baseline-resolved spectra with excellent mass accuracy. Thermo Scientific™ BioPharma Finder™ software provides the data analysis platform for the deconvolution of the spectra obtained from subunit for MW determination as well as for the assignment of fragment ions obtained from middle-down analyses for sequence verification.

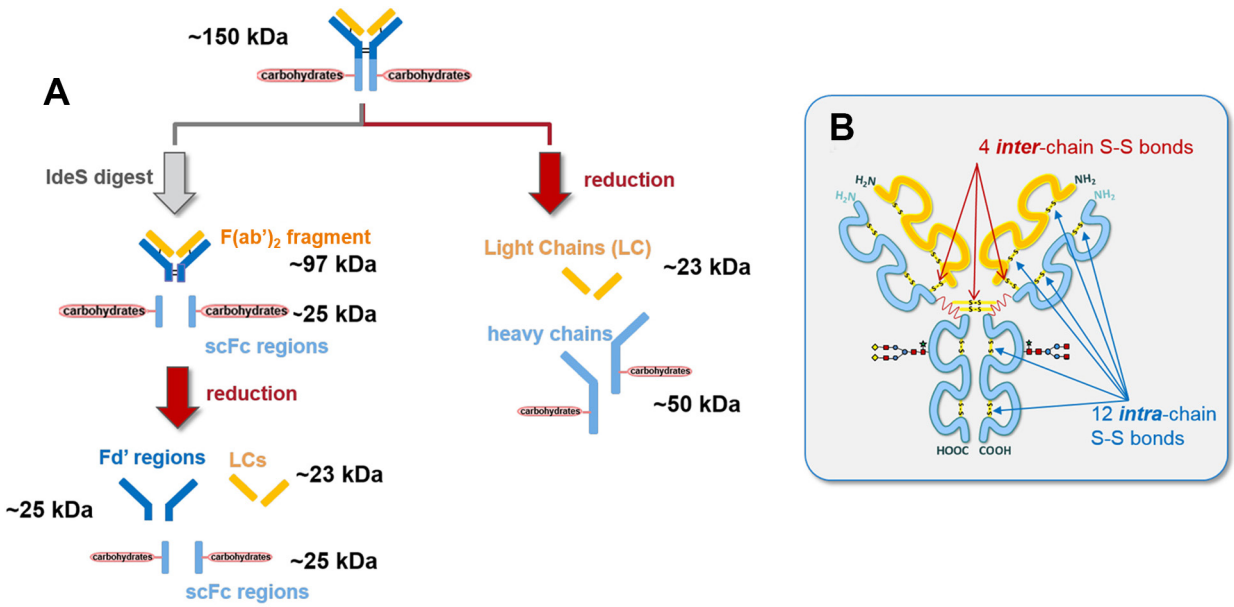


Figure 1. (A) Schematic representing mAb subunit generation via digestion using the IdeS enzyme resulting in F(ab′)$_2$ and scFc subunits. With subsequent reduction Fd′, scFc, and light chain subunits are obtained (left branch). Upon straight reduction without prior enzymatic IdeS digestion the separated light and heavy chains are obtained (right branch). (B) Schematic of an IgG class 1 showing the two light chains (yellow) and the two heavy chains (blue) connected via four inter-chain disulfide bonds. Each of the light chains contains two intra-chain S-S-bonds, and each of the heavy chains contains 4 intra-chain S-S-bonds, summing up to a total 16 disulfide bonds.

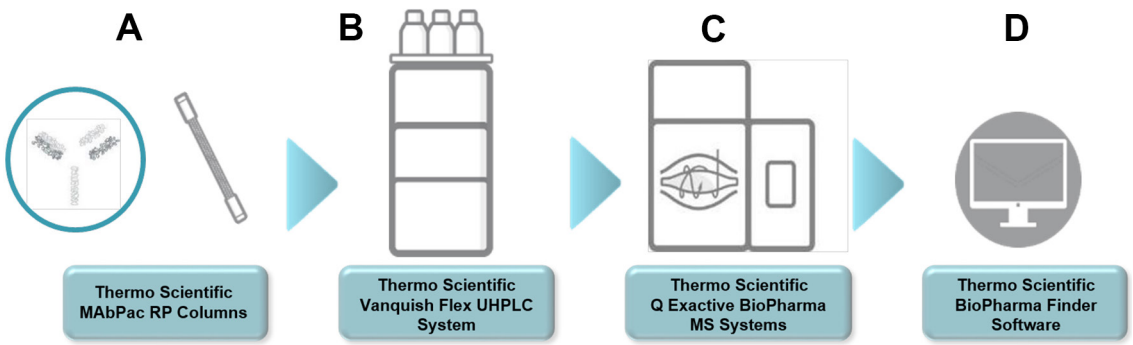


Figure 2. The subunit analysis workflow consists of a) sample preparation, b) liquid chromatography for separation, c) mass spectrometric analysis, and d) data analysis.

Here we have applied the subunit analysis workflow described above for the analysis of three commercially available monoclonal antibodies under different reduction conditions aiming at optimized conditions for complete reduction of inter- and intra-chain disulfide bridges for unambiguous determination of accurate masses of the subunits and most efficient fragmentation and sequence coverage in middle-down experiments. In many cases the inter-chain disulfide bonds separating the light and heavy chains are rather easy to break whereas the intra-chain disulfide bonds require more stringent conditions for complete reduction and the stability of these bonds shows significant variability amongst different antibodies. Thus, sample preparation is a critical step in the entire workflow as incomplete reduction is not necessarily obvious on a chromatographic scale but results in mass differences of ≥2 Da when comparing to the mass of the fully reduced species. Moreover, for top/middle-down analysis the presence of disulfide bridges is generally a limiting factor preventing efficient fragmentation in particular in the regions between two cysteine residues involved in a disulfide bridge.

Experimental

The three commercially available monoclonal antibodies trastuzumab, infliximab, and bevacizumab were used for all experiments. They were obtained either dissolved in formulation buffer or as powder and then dissolved according to the manufacturer's protocol.

Sample preparation

For the analysis of light and heavy chains (LC, HC), samples were reduced with one of the following conditions, performed at room temperature or 57 °C:

a) 50 mM DTT

b) 50 mM DTT in the presence of 4 M guanidine hydrochloride (GdHCl)

c) 50 mM TCEP

d) 50 mM TCEP in the presence of 4 M GdHCl (final concentration)

For LC, scFc, and Fd′ subunit analysis, antibodies were first digested with IdeS enzyme (FabRICATOR®, Genovis) according to manufacturer's protocol and then reduced using conditions a-d listed above. IdeS (immunoglobulin-degrading enzyme from *Streptococcus pyogenes*) is an engineered recombinant protease overexpressed in *Escherichia coli*. The protease cleaves specifically below the hinge region to yield $F(ab')_2$ and scFc fragments (Figure 1).

It is important to note that denaturation upon addition of GdHCl requires thorough mixing for about 15 s on a vortexer at high speed as the densities of the protein solutions and the denaturating agent are very different. A quick manual shake of the sample tube is not sufficient for mixing and will result in only partial denaturation of the protein and thus prevents successful and complete reduction.

Chromatography

Reversed-phase chromatography of all samples was performed on a Thermo Scientific™ Vanquish™ Flex UHPLC system using a 2.1 × 50 mm MAbPac RP column with a gradient separation using solvent A consisting of water 0.1% formic acid and solvent B consisting of acetonitrile/0.1% formic acid. The column temperature for all experiments was set to 80 °C. Details on the gradient are provided in Table 1.

LC conditions

Table 1. Overview of columns, flow rates, solvents, and gradients used for LC/MS analysis of reduced mAbs and IdeS digests with and without subsequent reduction. For infliximab the optimized gradient of IdeS digest with subsequent reduction for efficient separation of all subunits is provided.

IdeS digest without reduction	
Column:	MAbPac RP 2.1 × 50 mm
Column temp.:	80 °C
Flow rate:	250 µL/min
Solvent A:	Water/0.1% formic acid
Solvent B:	ACN/0.1% formic acid

Time [min]	*%B*
0	25
1	25
9	35
10	80
12	80
12	25
20	25

IdeS digest (reduced) optimized for infliximab	
Column:	MAbPac RP 2.1 × 50 mm
Column temp.:	80 °C
Flow rate:	250 µL/min
Solvent A:	Water/0.1% formic acid
Solvent B:	ACN/0.1% formic acid

Time [min]	*%B*
0	25
1	25
16	32
16.5	80
17.5	80
18.5	25
25	25

IdeS digest with reduction	
Column:	MAbPac RP 2.1 × 50 mm
Column temp.:	80 °C
Flow rate:	250 µL/min
Solvent A:	Water/0.1% formic acid
Solvent B:	ACN/0.1% formic acid

Time [min]	*%B*
0	25
1	25
7	35
8	80
9	80
9.5	25
20	25

Reduced mAb	
Column:	MAbPac RP 2.1 × 50 mm
Column temp.:	80 °C
Flow rate:	250 µL/min
Solvent A:	Water/0.1% formic acid
Solvent B:	ACN/0.1% formic acid

Time [min]	*%B*
0	25
1	25
13	32
14	80
16	80
16.5	25
25	25

Mass spectrometry

The mass spectrometers used for all experiments were the Thermo Scientific™ Q Exactive™ HF and Thermo Scientific™ Q Exactive™ HF-X mass spectrometers equipped with the BioPharma Option, which includes the Intact Protein Mode and High Mass Range (HMR) Mode. The mass spectrometers were operated with Thermo Scientific™ Xcalibur™ 4.0 software.

The MS parameter settings are summarized in Table 2.

Intact Protein Mode

For the analysis of proteins there are many factors that play a key role: sample preparation conditions (buffers, solvents, and additives), the mass spectrometer's ion source conditions, and also the physical environment inside the instrument.[2,3] The Q Exactive Plus, Q Exactive HF, and Q Exactive HF-X mass spectrometers have previously been introduced with the BioPharma Option, providing the choice of running experiments in a) Standard Mode, suitable for peptides and all types of small molecules, b) Intact Protein Mode, suitable for the analysis of proteins with a MW of up to ~100 kDa on the intact or top- and middle-down level, and lastly c) the HMR Mode suitable for analysis of intact proteins with a MW above 100 kDa under both native and denaturing conditions. The different modes were enabled by introduction of an electronically controlled valve for regulating the nitrogen gas in the HCD cell for 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 molecule application as well as peptides) and ions are cooled in the C-trap. The trapping gas pressure setting is 1, which corresponds to a high vacuum pressure delta (ΔHV) of ~3.1 e-5 mbar. The ΔHV is defined as the difference between HV with HCD gas on minus HV with HCD gas off.

Table 2. Parameter settings for all experiments described in this application note regarding source and method parameters

MS conditions	Reduced mAb	IdeS digest without reduction	IdeS digest with reduction	Top Down analysis
Method Type	Full MS [two segments]	Full MS [two segments]	Full MS	PRM
Total run time	25 min [0–9.9/9.9–25 min]	20 min [0–5.5/5.5–20 min]	20 min	20 min
Scan range [*m/z*]	600–2400	800–2600/1400–3500	700–2800	Fixed first mass: 300 *m/z*
Resolution*	240,000/15,000	240,000/15,000	240,000	120,000
AGC targt value (Full MS/MS2)	3e6	3e6	3e6	3e6/1e6
Max inject time [FullMS/MS2]	200 ms	200 ms	200 ms	250 ms
Isolation window (MS2)	-	-	-	5/20/300 Th
Microscans (Full MS/MS2)	5 / 10	5 / 10	5	5
SID [eV]	0	0	0	0
(N)CE [%]	-	-	-	CE 20 or stepped NCE 10/13/16
Intensity threshold	-	-	-	-
Dynamic exclusion	-	-	-	-
Lock mass used for internal calibration	-	-	-	-
Source settings				
Probe heater temperature [°C]	150	150	150	150
Source voltage [kV]	3.6	3.8	3.8	3.8
Capillary temperature [°C]	320	320	320	320
S-lens RF level**	80/40	60/40	60/40	60/40
Sheath gas flow rate	25	25	25	25
Aux gas flow rate	10	10	10	10
Sweep gas flow rate	0	0	0	0

*Resolution settings listed apply to the Q Exactive HF/HF-X mass spectrometers and relate to settings 17,500 and 140,000/280,000 on the Q Exactive Plus/Q Exactive Plus with BioPharma Option mass spectrometers. ** S-lens RF levels are provided for both, the Q Exactive HF/ Q Exactive HF-X mass spectrometers that require different settings due to differences in hardware: S-lens vs. electrodynamic ion funnel.

In Intact Protein Mode the default trapping gas pressure setting is 0.2. This corresponds to a ΔHV that is 5× lower than in Standard Mode. And in addition, ions have a longer flight path as they are transferred and cooled in the HCD cell compared to ion collection in the C-trap performed in Standard Mode.

The combination of reduced C-trap and HCD cell gas pressures, and trapping of the ions in the HCD cell prior to the ion detection process, extends the life time of protein ions. As a result, an increased signal intensity of isotopically resolved species is obtained. Moreover, as higher charge states are encountering a prolonged lifetime, a shift of the charge envelope to lower *m/z* values corresponding to higher charge states is observed when comparing intact protein spectra acquired in Standard Mode vs. Intact Protein Mode.

Intact Protein Mode can be toggled on/off in the Instrument Status view in the Tune window (Figure 3).

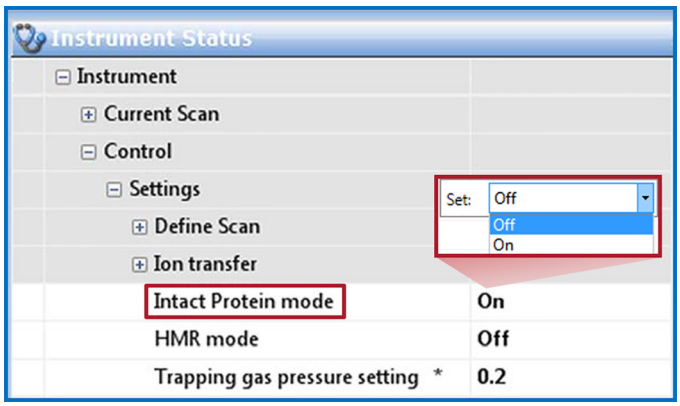


Figure 3. Instrument Status view from the Tune window highlighting where to toggle Intact Protein Mode on/off and set the trapping gas pressure. Q Exactive Plus, Q Exactive HF, and Q Exactive HF-X instruments equipped with BioPharma Option also list HMR Mode as shown in this picture.

The trapping gas pressure for the selected mode is set and saved in the tune file. Since a method allows for segmentation using different tune files, different pressure settings can be used within one LC/MS run.

Calculation of theoretical monoisotopic and average masses

For calculation of theoretical monoisotopic and average masses of the mAb subunits, the element masses provided in Table 3A were used.

To demonstrate the mass difference between monoisotopic and average masses, Table 3B provides the elemental composition and the resulting theoretical masses for trastuzumab light chain. The difference between the two masses in this example is ~14.4 Da. For very small proteins, the difference is only a few Da, whereas for intact mAbs it is typically ~90 Da. These mass differences simply result from the differences between the monoisotopic vs. average masses of the individual elements that appear to be small, but when multiplied by the number of atoms that proteins consist of—typically 3000 up to ~20,500 for intact mAbs—these small differences add up to several tens of Da. However, the difference between the monoisotopic and average mass is irrelevant as only one of the masses is considered for calculating the mass accuracy of experimental data, depending on whether data acquisition was performed with high resolution to achieve monoisotopic masses or with low resolution providing average masses after deconvolution.

It is important to note that the theoretical average mass cannot be calculated with high precision and only theoretical monoisotopic masses are 100% precise. For the average masses of the elements there is no common

Table 3A. Theoretical monoisotopic and average masses of the elements used for the calculation of theoretical masses of the mAb subunits

	C	H	N	O	S
Monoisotopic mass	12	1.00782503	14.0030740	15.9949146	31.9720707
Average mass	12.01074	1.00794	14.00674	15.9994	32.06608

Table 3B. Example of theoretical monoisotopic and average mass calculation based on the elemental composition of trastuzumab light chain using the element masses from Table 3A

Elemental compositions →	C	H	N	O	S	MW (monoisotopic)	MW (average)
Trastuzumab light chain	1032	1603	277	335	6	23,428.52384	23,442.9

agreement on how many digits to use after the decimal point, and there are discrepancies among every software package currently available on the market.[4] This results in an ambiguity of the theoretical average mass that increases the more atoms a molecule contains. This ambiguity is entirely unrelated to experimental data and the type of instrument used. For large molecules such as intact mAbs, the variation in theoretical average mass can be up to ~10 Da, whereas for a small protein such as a mAb subunit it is typically below 0.5 Da. This fact should be taken into account when comparing results from different software packages and when considering if monoisotopic or average masses would be better to obtain from a sample analysis.

Data analysis

Raw data files were analyzed with Thermo Scientific™ BioPharma Finder™ 3.0 software for intact protein and subunit spectra deconvolution as well as for middle-down analysis.

Results and discussion

Reduced mAb

The levels of inter- and intra-chain disulfide bond reduction of trastuzumab were evaluated resulting from sample preparation using DTT and TCEP at room temperature, at 57 °C, and with and without denaturation.

The level of reduction can be derived by reviewing the number of peaks obtained in the chromatogram and also based on the shape of the charge envelope on the Full MS level. Ideally the chromatogram would show two peaks representing the light and heavy chain each in its fully reduced state. Performing the reduction in the absence of denaturing agent results in the detection of up to four or six chromatographic peaks upon LC/MS analysis and is a clear indication of incomplete reduction of the intra-chain disulfide bonds.

On the mass spectral level the presence or absence of disulfide bridges can also be easily assessed. As Figures 4B, 7B, and 8A,B demonstrate, spectra obtained from subunits containing intact intra-chain disulfide bridges show charge envelopes with bimodal distributions with a wide stretch of charge states starting at higher *m/z*. Spectra obtained from fully reduced species, however, show a rather symmetrical and Gaussian shape of the charge envelope with the center being shifted to lower *m/z,* overall representing higher charge states. The shift towards higher charge states and lower *m/z* is also supported by the Intact Protein Mode providing a longer flight path and reduced pressure for ion trapping (see section on Intact Protein Mode in the experimental section). However, when comparing the spectra obtained here with different levels of reduction, the difference in

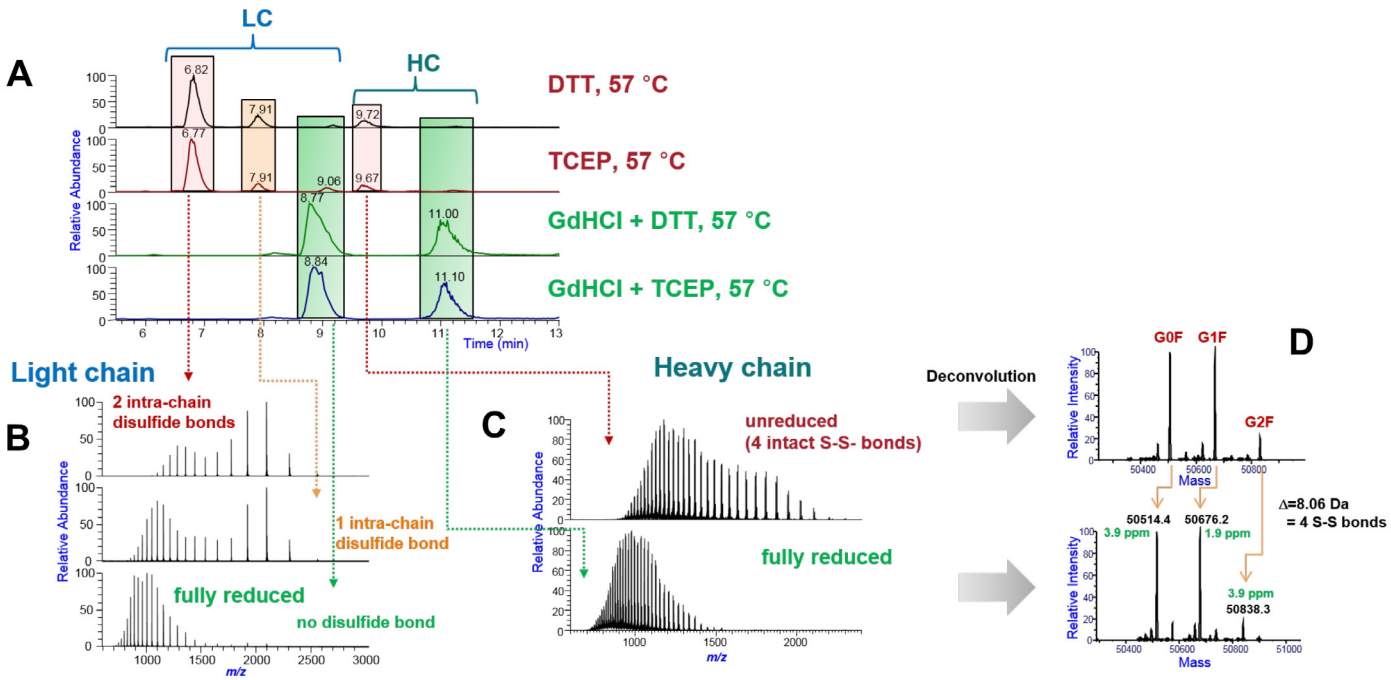
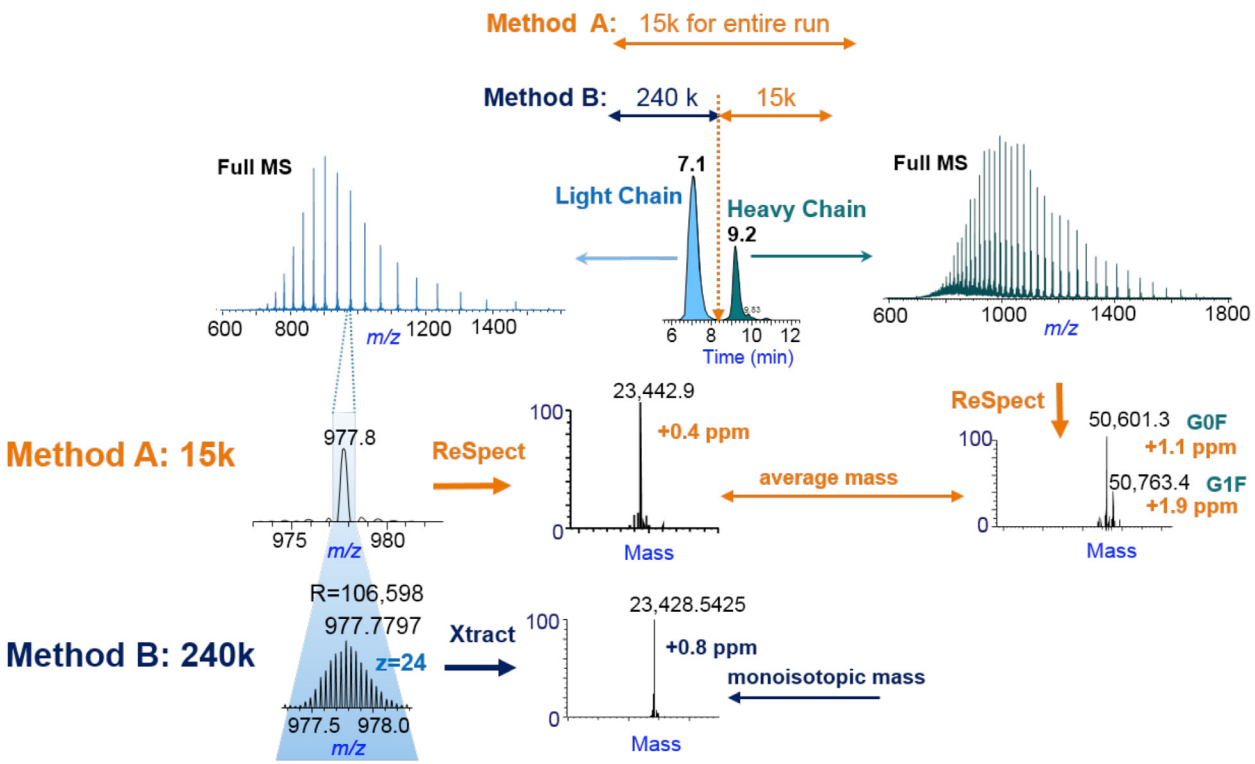


Figure 4. (A) Chromatograms of trastuzumab obtained after reduction under different conditions: DTT or TCEP at 57 °C (top and second from top traces), DTT or TCEP at 57 °C in the presence of GdHCl (traces 3 and 4 from top). Resulting species are highlighted in colors and relate to light and heavy chains in unreduced (pink), semi-reduced (orange), and fully reduced (green) forms as also shown in the full MS spectra (B, C) and the resulting masses after deconvolution (D).

appearance and look of the charge envelopes can be solely explained by different three-dimensional structures resulting from the presence or absence of disulfide bridges as all spectra were acquired in Intact Protein Mode with the exact same pressure setting.

In order to achieve complete reduction of all disulfide bridges entailed in a mAb, the addition of guanidine hydrochloride to a final concentration of 4 M is essential for providing denaturing conditions making the molecule amenable for efficient and complete reduction, both with DTT or TCEP. Upon complete reduction two chromatographic peaks are obtained (Figure 4A, lower two traces) and theoretical masses of the fully reduced species are confirmed upon deconvolution (Figure 4D) of the full MS spectra (Figure 4C).

For MS data acquisition two options regarding resolution settings are recommended. If average masses for both heavy and light chains are desired, the lowest resolution setting of 15,000 (respectively 17,500 for the Thermo Scientific™ Q Exactive™ Plus instrument) is best to set in the acquisition method. An alternative is the use of two different resolution settings starting with 240,000 (respectively 140,000 for the Q Exactive Plus instrument) for the first part of the analysis aiming at isotopically resolved spectra for the earlier eluting light chain. The resolution setting is then switched to 15,000 right before the heavy chain starts to elute, resulting in isotopically unresolved spectra for the heavy chain. Upon deconvolution the monoisotopic mass for the light chain and the average masses for the isoforms of the heavy chain are obtained (Figure 5, Method A and B). The second approach using Method B is successful under two prerequisites: a) the light and heavy chain species are chromatographically well resolved and b) the retention times or rather the valley between the eluting species are known in order to specify the time for switching the resolution setting in the method.



trastuzumab	LC - mono. mass	LC - av. mass	HC G0F - av. mass	HC G1F - av. mass
theoretical mass (mono./av.)	23,428.5238	23,442.87	50,601.4	50,763.5
experimental mass	23,428.5425	23,442.88	50,601.3	50,763.4
Δ ppm	0.8 ppm	0.4 ppm	1.1 ppm	1.9 ppm

Figure 5. Chromatogram obtained for trastuzumab after complete reduction under optimal conditions resulting in two peaks representing the light and heavy chains (top center) and corresponding Full MS spectra obtained for the light chain (top left) and heavy chain (top right). Two data acquisition strategies are: Method A applies a single resolution setting of 15,000 for the entire run resulting in average masses upon deconvolution. Method B applies a high-resolution setting of 240,000 for the light chain and switches to low resolution of 15,000 before the heavy chain elutes from the column, resulting in a monoisotopic mass for the light chain and average masses for the glycoforms of the heavy chain upon deconvolution. The table insert reflects theoretical monoisotopic (mono.) and/or average (av.) masses as well as the experimental masses for the light and heavy chains and resulting mass accuracies.

Subunit analysis of IdeS digested and reduced mAbs

For the assessment of optimal reduction conditions to derive fully reduced scFc, LC, and Fd′ mAb subunits, trastuzumab was first digested with the IdeS enzyme resulting in $F(ab')_2$ and scFc subunits (Figure 1A).

For the reduction of IdeS digested trastuzumab, different conditions were evaluated applying DTT or TCEP at room temperature or 57 °C for 30 min in the presence or absence of 4 M GdHCl as denaturing agent (Figure 6A-H).

Both DTT and TCEP without any further additives are reducing inter-chain disulfide bonds very efficiently, but to only a very small extent also the intra-chain disulfide bonds resulting in three abundant chromatographic peaks eluting at ~3.7, ~4.7, and ~6.0 min, each of them followed by a smaller peak eluting ~0.3–0.4 min later (Figure 6A-D). TCEP was found to have slightly stronger reducing capabilities as the later eluting peaks corresponding to the fully reduced subunits are in slightly higher abundance (Figure 6B,D). However, achieving complete reduction of all disulfide bonds requires denaturation. Additionally, the temperature applied during reduction also plays a role, as room temperature is not sufficient for complete reduction of the scFc subunits (Figure 6E-F).

In conclusion, optimal conditions for achieving complete reduction of an IdeS digested mAb is denaturation in 4 M GdHCl and reduction with either 50 mM TCEP or DTT at elevated temperature (Figure 6G-H).

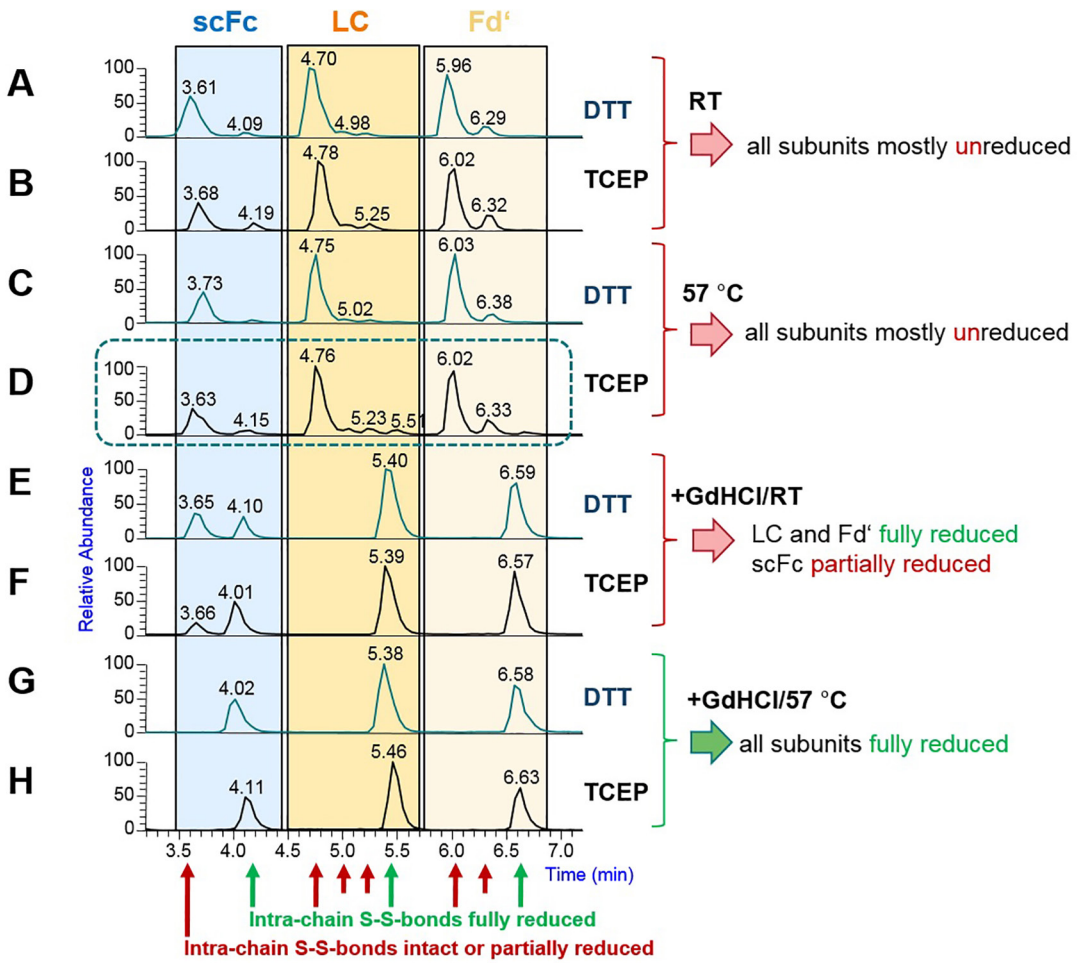


Figure 6. Total ion chromatograms of trastuzumab subunits after IdeS digest, followed by reduction using DTT or TCEP at room temperature or 57 °C, in the presence or absence of 4 M GdHCl

To assess the different species obtained using reduction with TCEP at 57 °C (Figures 6D and 7A) and in particular the three small peaks eluting in the RT window between 5 and 5.6 min, the full MS spectra are displayed in Figure 7B-C. Similar patterns are observed on the light and heavy chain as already discussed in the previous section. Earlier eluting species are showing bimodal distributions of the charge envelope, whereas the later eluting species show a more compact, Gaussian distribution with a shift towards higher charge states and lower *m/z* values. There are four species related to the light chain at RT 4.5–5.6 resulting in three different masses upon deconvolution: the unreduced light chain with a monoisotopic mass of 23,424.5490 Da, the fully reduced light chain with a monoisotopic mass of 23,428.5631 Da, and 2 species both with a mass ~±2 Da off compared to the mass of the fully reduced as well as fully unreduced species. The reason why these two species elute at slightly different retention times cannot be assessed on the subunit level as the obtained masses are nearly identical, thus it requires further analysis, e.g. by middle-down analysis, and will be discussed in the next section.

For optimized parameters of the LC/MS analysis of reduced IdeS digest, chromatographic runtimes of 9–10 minutes for the gradient will in most cases be sufficient to baseline separate the three subunits as shown for trastuzumab and bevacizumab in Figure 8. For infliximab a slightly longer separation time is required to separate the lysine variants of the scFc subunit and the LC and Fd′ subunits. Extending the gradient from 9 to 16.5 minutes is sufficient to achieve good separation of all subunits, also for infliximab (Figure 8B). It is important to note that the appearance of closely eluting peaks in the case of infliximab is not due to incomplete reduction but rather the occurrence of two abundant Lys-variants of the scFc subunit, a fact that is well known for infliximab.

For the MS method there are two options available for resolution settings. However, as all subunits are in the same MW range of 23–25 kDa, one resolution setting can be applied for the entire run to achieve either average masses using a low resolution setting of 15,000 or monoisotopic masses by applying a high resolution setting of 240,000.

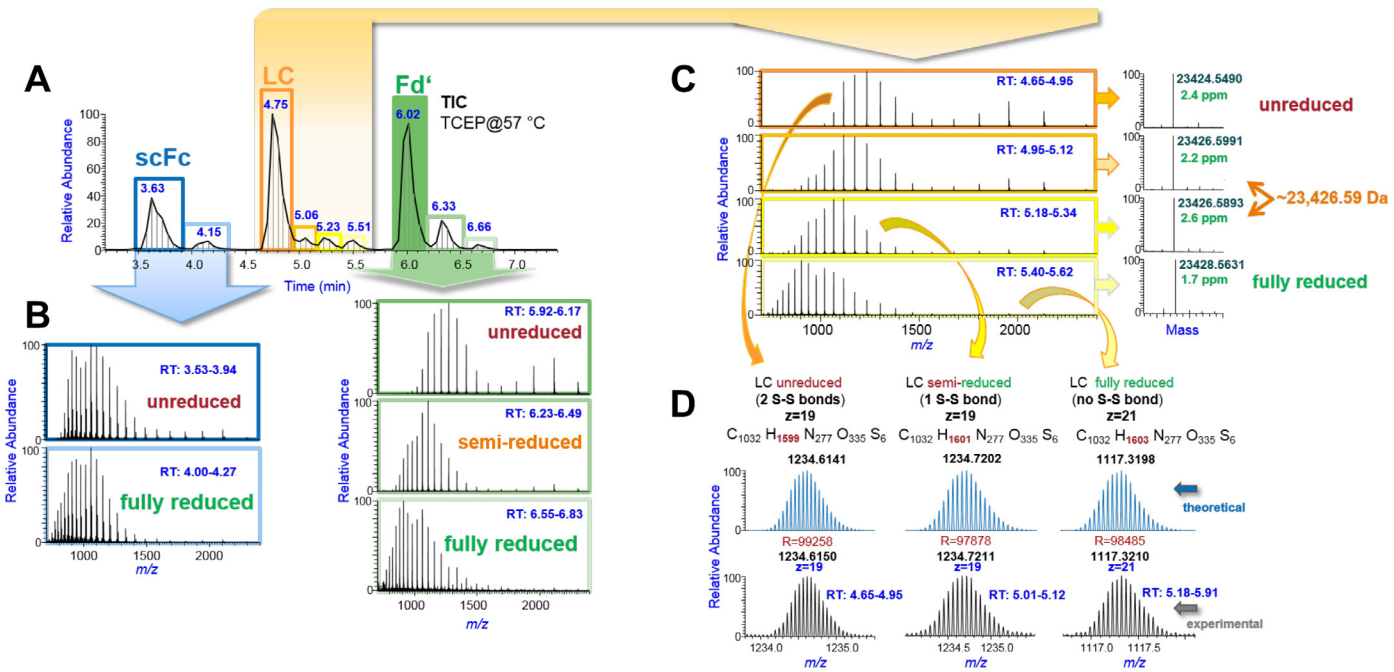


Figure 7. (A) Total ion chromatograms of trastuzumab subunits after IdeS digest, followed by reduction using TCEP at 57 °C without denaturation resulting in a mixture of reduced, semi-reduced, and fully reduced species deducted from the shape of the charge envelopes as well as the monoisotopic masses obtained after deconvolution (B). The four peaks associated to the different forms of the light chain highlighted by the yellow boxes result in three different masses after deconvolution (C) and are assigned as non-reduced, semi-reduced (one disulfide bond intact, one disulfide bond open), and fully reduced forms. Assignments are also supported by comparison of the theoretical and experimental isotope patterns (D).

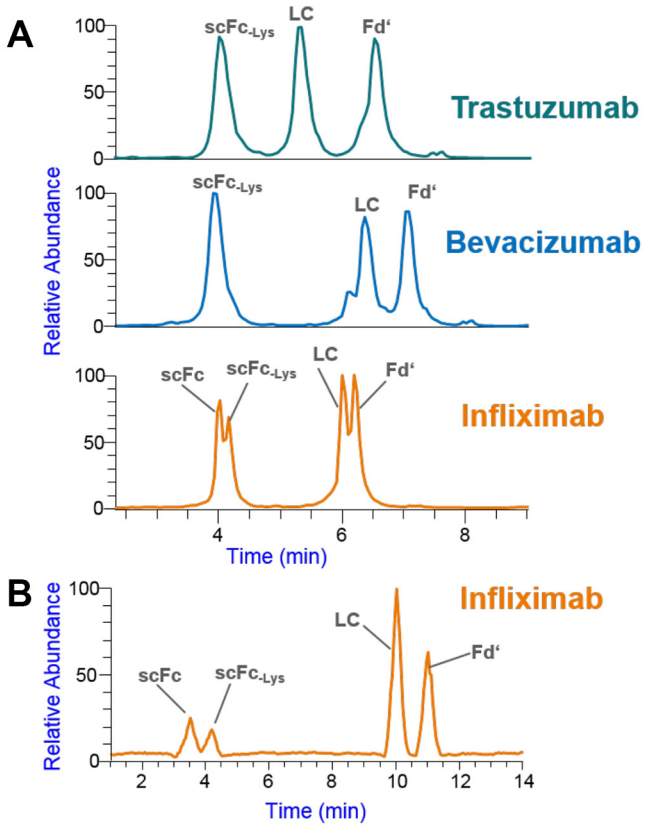


Figure 8. Total ion chromatograms obtained for IdeS digested trastuzumab, bevacizumab, and infliximab samples followed by reduction applying optimized conditions with TCEP at 57 °C in the presence of 4 M GdHCl (A). To achieve baseline-resolved peaks for the scFc, LC, and Fd′ subunits of infliximab a slightly extended gradient was applied (B).

Middle-down analysis of mAb subunits

Top- and middle-down analysis is a useful approach for the confirmation of a protein's sequence and to investigate the amino acid sequence in case of mismatches between theoretical and experimental masses. The benefit is that no additional sample preparation is required, and the elucidation of the subunit's intact mass and middle-down analysis can even be performed in a single experiment. Different approaches can be chosen and several options on the particular method setup and parameter settings are available, some of which are presented in this section. The two parameters in the MS method setup with the highest impact on the obtained result are the precursor isolation window and the collision energy.

Moreover, top- and middle-down analysis strongly benefits from complete reduction of the proteins as disulfide bonds prevent efficient HCD fragmentation, in particular in the regions of the sequence located between the entailed cysteine residues. Proteins containing disulfide bridges will show two effects: in the Full MS spectrum the charge envelope has a bimodal distribution (Figure 9A) as mentioned previously, and secondly, resulting MS/MS spectra will typically provide only a small number of fragment ions and a very low sequence coverage (Figure 9C). Indication of efficient fragmentation is the detection of fragments with a variety of charge states covering a wide *m/z* range (Figure 9D).

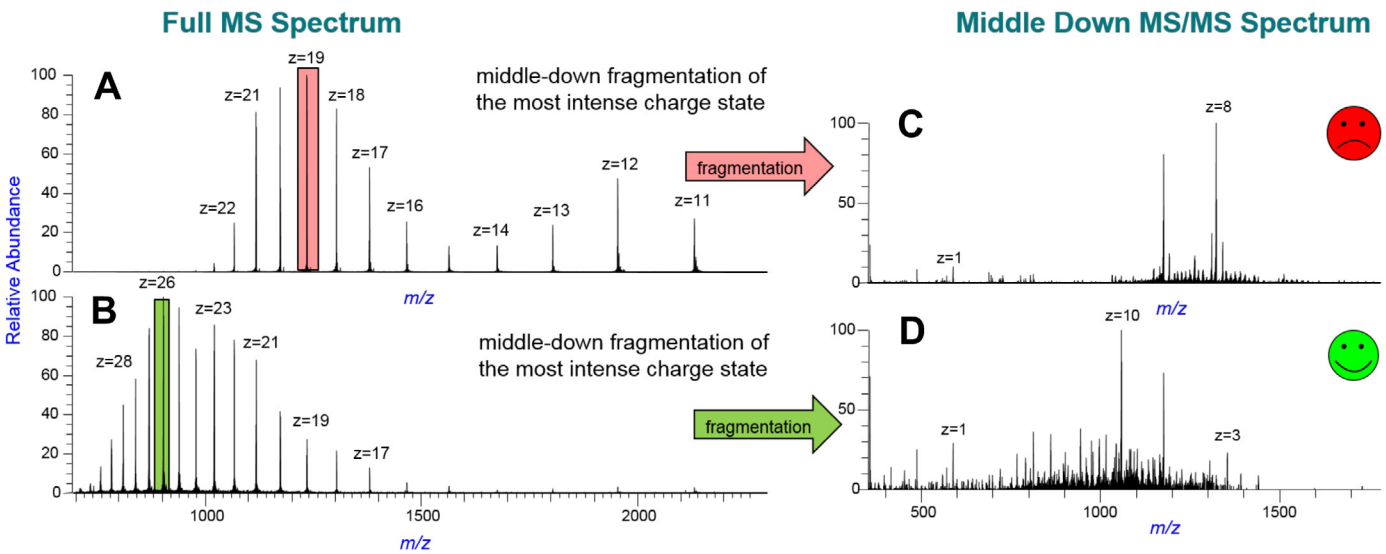


Figure 9. Full MS spectra obtained from the unreduced (A) and fully reduced (B) light chain and resulting middle-down spectra obtained upon fragmentation of the most abundant charge state (C, D). All spectra were acquired in Intact Protein Mode with a resolution setting of 120,000.

The width of the isolation window in middle-down experiments strongly depends on the complexity and purity of the sample and on the scope of the experiment. As illustrated in Figure 10, a 5 Th window isolates one isoform of one charge state, whereas a 20 Th window selects, for example, three isoforms of one charge state and a 200 or 300 Th wide window isolates a range of about five consecutive charge states with all isoforms entailed. The wider the isolation window is set, the less specific the result will be in regard to possible modifications and the higher the risk is to co-isolate other co-eluting species. For low-complexity samples such as mAb subunits, with the goal to confirm the protein sequence, the wider isolation window will support a higher sequence coverage. A wide isolation window also supports the analysis of proteins without prior knowledge of the exact *m/z* of the charge states. In the method, a precursor mass in the target mass list of 950 *m/z* with an isolation window of 300 Th, for example, generally covers a good range of charge states for mAb subunits.

The next associated parameter is the collision energy. In top- and middle-down experiments there is no single optimal collision energy that produces small and large fragments at the same time. Thus, a variation of collision energies will lead to the desired result of a mixture of large and small fragments in higher and lower charge states as demonstrated in Figure 11.

Implementation of a variety of collision energies in a method can be achieved by 1) running the sample multiple times with methods consisting of a single PRM (Parallel Reaction Monitoring) scan event at different collision energies; 2) using stepped collision energy that allows using up to three different energy settings. This results in composite spectra based on successive ion injections and fragmentations at different energy levels and conjoint detection of all generated fragments in a single scan; 3) composing a method with individual PRM scan events with different collision energies resulting in individual scans within one experiment (Figure 11 insert).

Reviewing the different species obtained upon incomplete reduction (as discussed in Figure 6D) by middle-down fragmentation, reveals the difference between the species eluting at 5.06 and 5.23 minutes. As both species were detected with the same molecular mass with a ±2 Da difference compared to the unreduced and reduced species, it suggests that both species contain one reduced and one unreduced disulfide bond (Figure 12A-B). Fragments obtained for the earlier eluting species do not cover the sequence between Cys134 and Cys194, whereas for the later eluting species no fragments are obtained between Cys23 and Cys88 (Figure 12C-D). This leads to the conclusion that the earlier eluting peak contains the species with an intact disulfide bond in position Cys134–Cys194, whereas the slightly later eluting peak contains the species with an intact disulfide bond in position Cys23–Cys88.

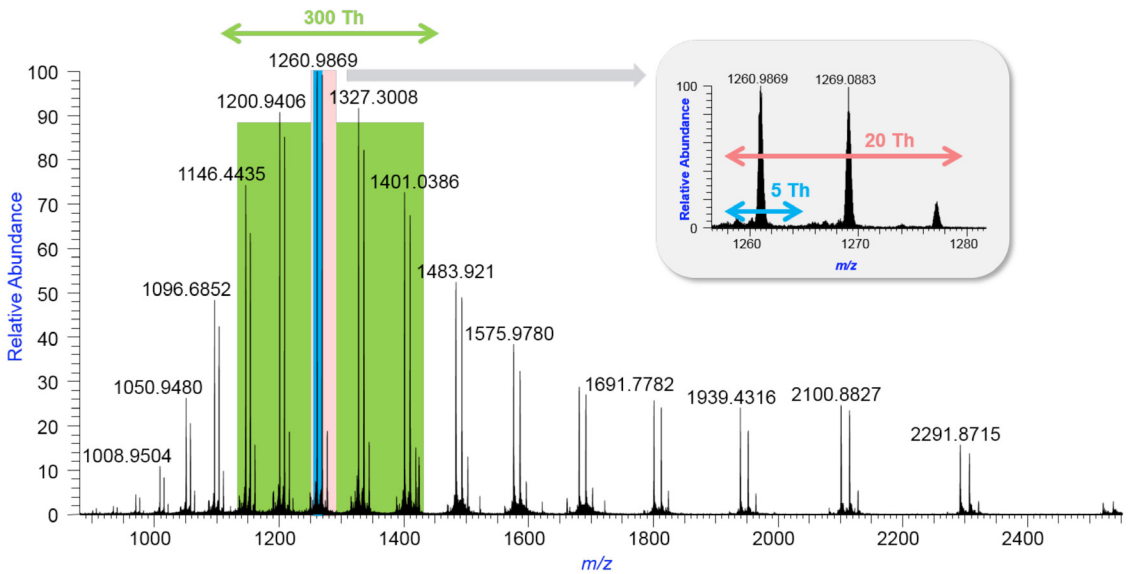


Figure 10. For top- and middle-down fragmentation different widths of isolation windows can be applied to either select a single charge state and a single glycoform in a 5 Th window (blue), or selection of all glycoforms of one charge state in a 20 Th window (pink), or in a wide window selecting several charge states and all associated glycoforms in a 300 Th window (green).

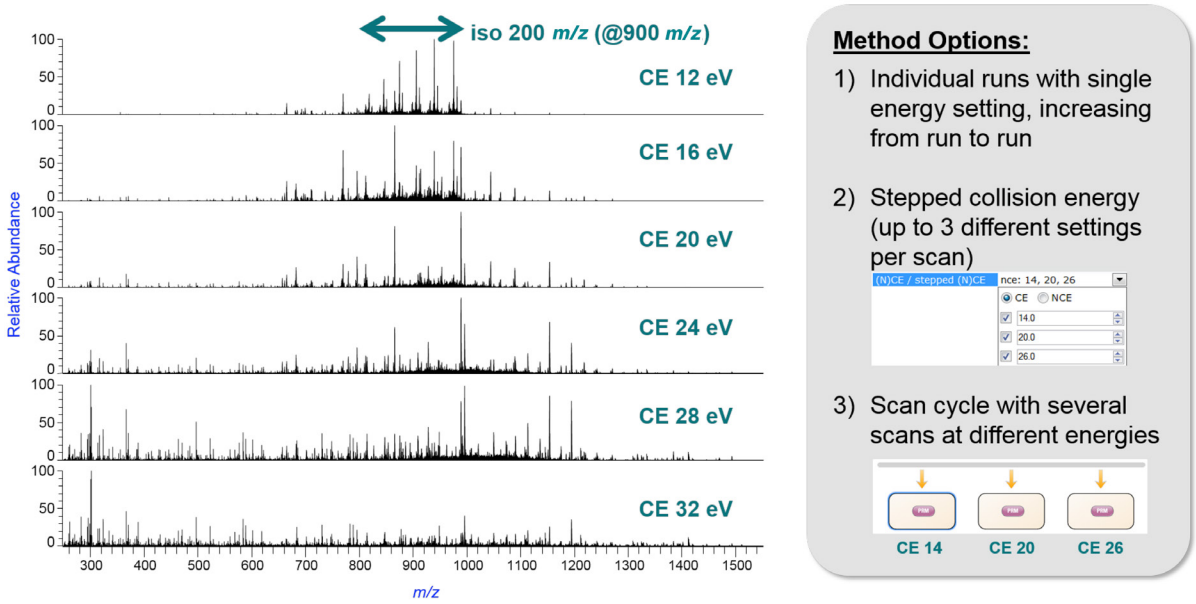


Figure 11. Demonstration of different method options. Method 1: Individual runs applying increasing energy settings. Resulting spectra as shown on the left demonstrate the decrease of intact species and increase of peaks representing fragment ions. Method 2: An alternative method applies stepped collision energies with a choice of three different energy setting for each acquired scan. Method 3: Separate scan events applying a different energy setting, e.g., CE 14, CE 20, and CE 26.

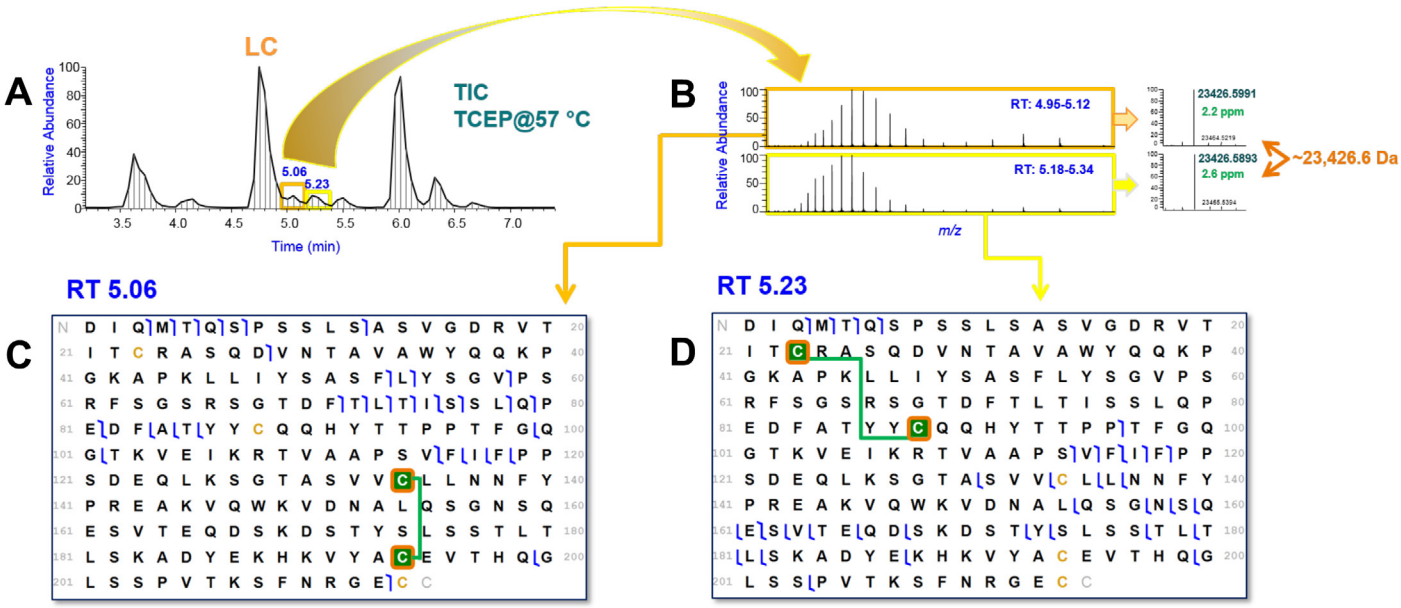


Figure 12. (A) Total ion chromatogram of IdeS digested trastuzumab followed by reduction with TCEP in the absence of GdHCl. (B) Full MS spectra of the two low abundant species eluting right after the unreduced light chains at RT 5.06 and 5.23. Middle-down fragmentation and assignment of fragment ions obtained from a single LC run reveals regions between Cys134 and Cys194 and between Cys23 and Cys88, respectively, that are not covered by fragment ions, suggesting intact disulfide bonds preventing fragmentation in these sequence sections (C, D).

The last example provided on IdeS digested infliximab is a summary of all optimized conditions for sample preparation using IdeS digestion and reduction, chromatographic separation with an optimized gradient (Figure 13A), generation of Full MS data for mass analysis as well as middle-down analysis for sequence confirmation (Figure 13B-E). Full MS spectra of the four abundant peaks in the chromatogram and their middle-down spectra acquired with a resolution setting of 120,000, a wide isolation window of 200 Th around a center mass of 950 *m/z*, stepped collision energies with NCE 10, 13, and 16. Mass analysis as well as middle-down analysis of the two first eluting peaks confirm the scFc regions with and without the C-terminal lysine. Sequence (or bond) coverages obtained from a single LC run are 32% for the scFc, 20% for scFc-Lys, 33% for the light chain, and 19% for the Fd' subunit.

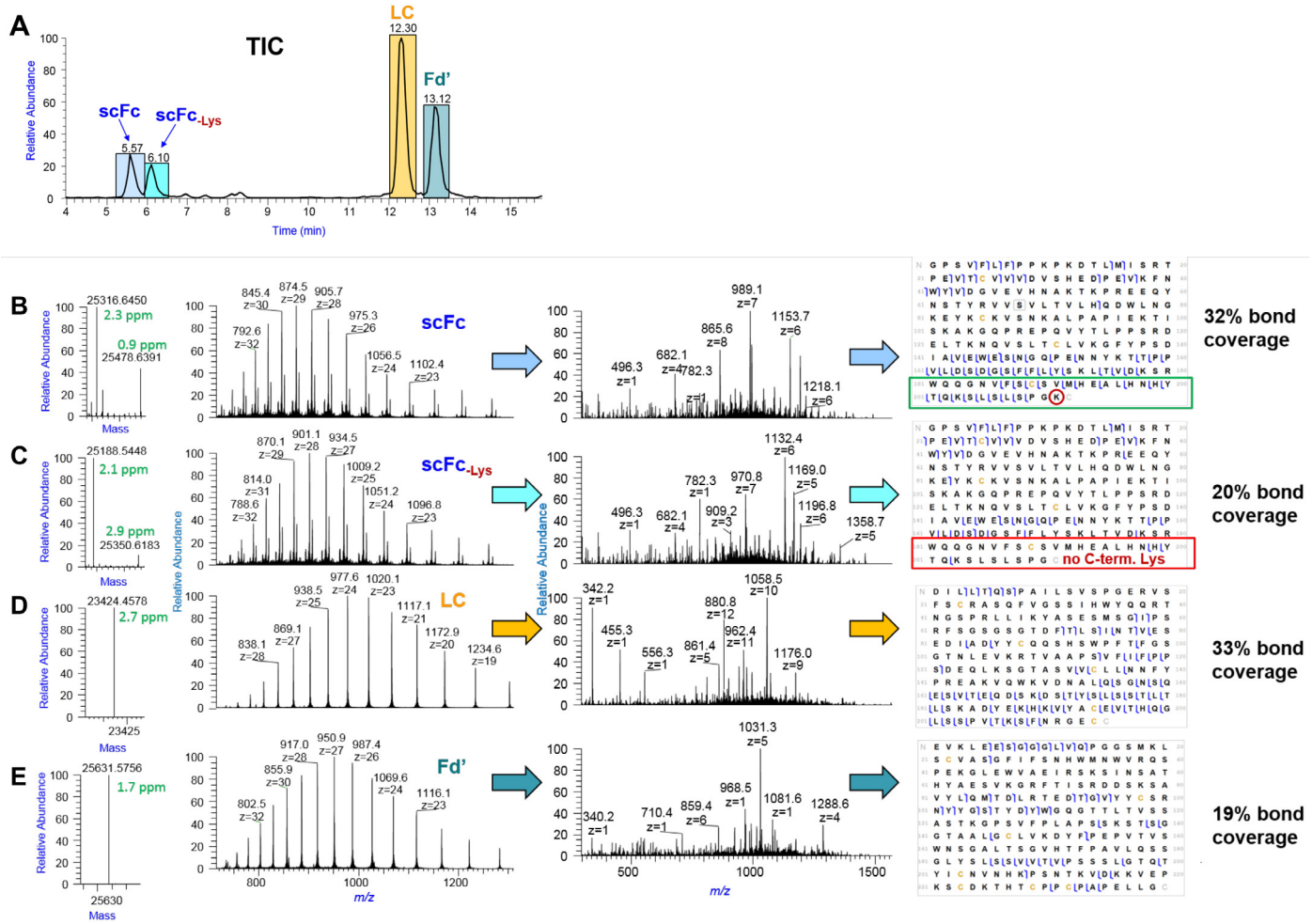


Figure 13. (A) Total ion chromatogram of IdeS digested and reduced infliximab applying an extended gradient to separate the scFc subunits with and without terminal Lys-residues. (B, C, D, E) Full MS spectra of the four abundant peaks in the chromatogram and their middle-down spectra acquired with a resolution setting of 120,000, a wide isolation window of 200 Th around a center mass of 950 *m/z*, stepped collision energies with NCE 10, 13, and 16. Bond coverages obtained from a single LC run are: 32% for the scFc, 20% for scFc-Lys, 33% for LC, and 19% for the Fd' subunits.

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Conclusions

- Denaturation using a denaturing agent such as GdHCl is required for complete reduction of mAb subunits. It is not sufficient to only use reducing reagents like DTT or TCEP even at elevated temperatures. 4 M GdHCl in the presence of 50 mM DTT or TCEP at elevated temperatures of 57 °C were found to be appropriate conditions to achieve complete reduction of all inter- and intra-chain disulfide bridges. These conditions apply to an intact mAb as well as an IdeS digested mAb.
- Complete reduction using either DTT or TCEP, elevated temperature and 4 M GdHCl are essential for achieving an unambiguous mass for antibody subunits and significantly improves the quality of middle-down HCD spectra in regard to the number of fragments detected, supporting increased sequence confirmation.
- Doublet peaks in a chromatogram can either represent subunits with various numbers of intact disulfide bonds or may also represent variants such as scFc regions with and without Lys-clipping.
- The Q Exactive BioPharma platform offers three modes of operation—Standard Mode, Intact Protein Mode, and High Mass Range Mode—that predominantly relate to the ion trapping path and pressure regimes inside the mass spectrometer to cover all major workflows for the characterization of biopharmaceuticals.
- The high resolution settings of 120,000 and 240,000 are essential for the mass and middle-down analyses of mAb subunits to obtain accurate monoisotopic masses and for amino acid sequence confirmation.

References

1. Bobaly, B.; D'Atri, V.; Goyon, A.; Colas, O.; Beck, A.; Fekete, S.; Guillarme, D. Protocols for the analytical characterization of therapeutic monoclonal antibodies. II – Enzymatic and chemical sample preparation, J. *Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2017,** *1060,* 325–335. https://doi.org/10.1016/j.jchromb.2017.06.036
2. Scheffler K.; Damoc, E. Full characterization of heterogeneous antibody samples under denaturing and native conditions on a Q Exactive BioPharma mass spectrometer; Thermo Scientific Application Note 72348; June 2017. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/AN-72348-LC-MS-Heterogeneous-Antibody-Samples-AN72348-EN.pdf
3. Scheffler, K.; Viner, R.; Damoc, E. High resolution top-down experimental strategies on the Orbitrap platform, *J. Proteomics* **2018,** *175,* 42–55. https://doi.org/10.1016/j.jprot.2017.03.028
4. Farrell, A.; Carillo, S.; Scheffler, K.; Cook, K.; Bones, J. Monoclonal antibody sequence assessment using a hybrid quadrupole-Orbitrap mass spectrometer, *Anal. Methods*, **2018,** *10,* 3100–3109. https://doi.org/10.1039/c8ay00582f
5. Carillo, S.; Jakes, C.; Zaborowska, I.; Bones, J. IdeS-cleaved mAb subunit analysis with LC-HRAM-MS: a quick and accurate comparison of biosimilar and originator biotherapeutics; Thermo Scientific Application Note 21806; May 2018. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-21806-lc-ms-ides-cleaved-mab-subunit-an21806-en.pdf

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