Improving Intact Antibody Characterization by Orbitrap Mass Spectrometry

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Introduction

Recombinant monoclonal antibodies have gained significant importance in diagnostic and therapeutic applications over the past years. In order to verify the correctness of the overall molecule to provide a reproducible, safe and effective biological drug compound, the correct protein sequence as well as the presence and relative abundance of different glycoforms have to be confirmed.

Here we present an approach to analyze an intact monoclonal antibody in non-reduced and reduced condition by LC-MS using the Thermo Scientific Dual Pressure Linear Ion Trap Orbitrap Hybrid mass spectrometer. The intact antibody respectively the separated light and heavy chains were analyzed in Full MS experiments as well as with top-down experiments using in-source CID (SID), CID, HCD and ETD fragmentation techniques making use of the ultrahigh resolution of the mass spectrometer. For data evaluation Thermo Scientific ProSightPC 2.0 and Thermo Scientific Protein Deconvolution 1.0 software packages were used.

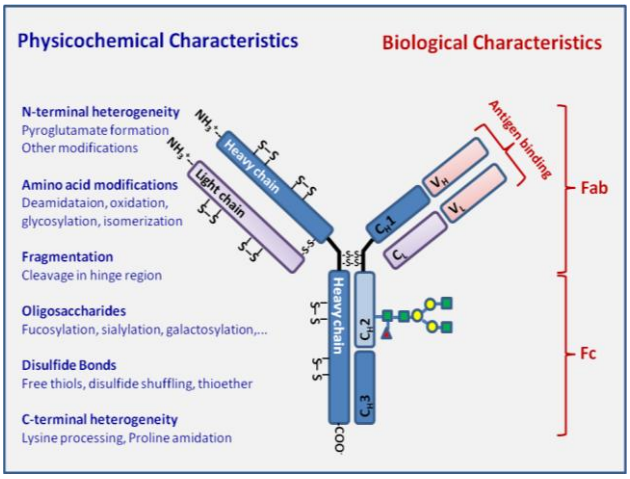

FIGURE 1: General structure of mAbs and their biological and physico-chemical characteristics**.**

Methods

Sample Preparation:

Humira® (adalimumab, Figure 2) [1]: The intact antibody (144 kDa) was dissolved in 0.1 % FA to 1 µg/µL; 5 µg Humira were loaded onto the column.

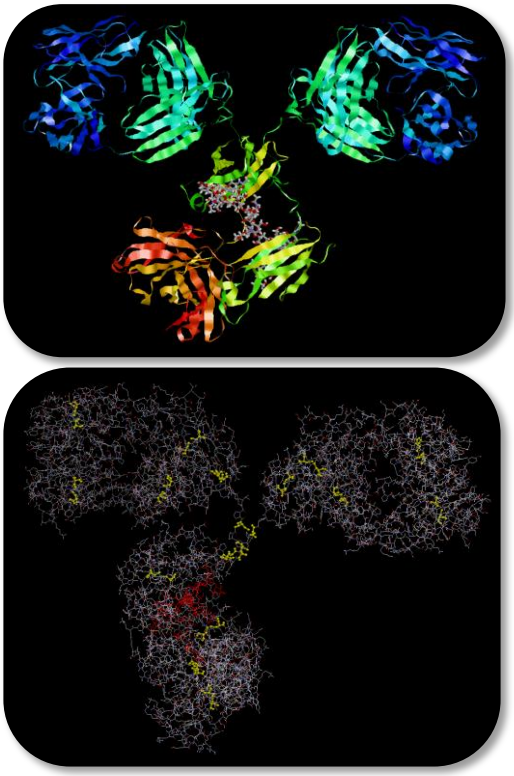

FIGURE 2: 3D structure of Humira highlighting the attached glycans and cystein residues forming inter- and intra-chain disulfide bridges.

For analyzing Humira light chain (24 kDa) and heavy chain (51 kDa) separately, 50 µg Humira was reduced with DTT (20-fold molar excess, 56°C for 1 h) and alkylated with iodoacetamide (50-fold molar excess, room temperature for 30 min in the dark).

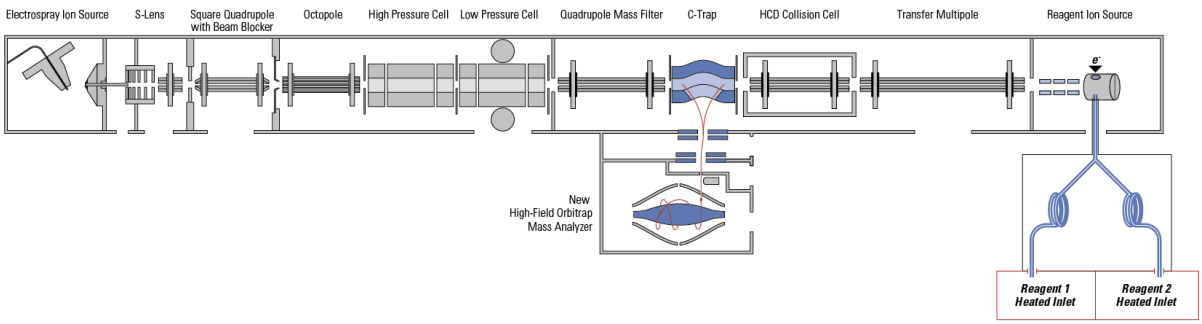

FIGURE 3: Schematics of the Orbitrap Elite hybrid mass spectrometer equipped with an ETD source.

Instrument:

A Thermo Scientific Surveyor MS Pump Plus was coupled to an Orbitrap EliteTM ETD mass spectrometer (Figure 3) [2].

Samples were purified on a Thermo Scientific BioBasic-C4 column(150 x 1 mm, 5 µm particles, solvent A: 0.1 % FA, 2 % ACN in H2O, solvent B: 0.1 % FA in ACN. The LC gradient was 7 min 20–40 % B, 3 min 40–80 % B at a flow rate of 100 µL/min.
Data analysis was done using Protein DeconvolutionTM 1.0 and ProSightPCTM 2.0.

Results

The analysis of large proteins of the size of intact antibodies (~150 kDa) using the Orbitrap detector has been significantly improved over the past years. Large molecules like mAbs show only very short transient life-times due to their relatively big cross section. Thus the method of choice for intact antibodies is to use the shortest transient duration (48 ms) available on the Orbitrap Elite hybrid mass spectrometer (Figure 4).

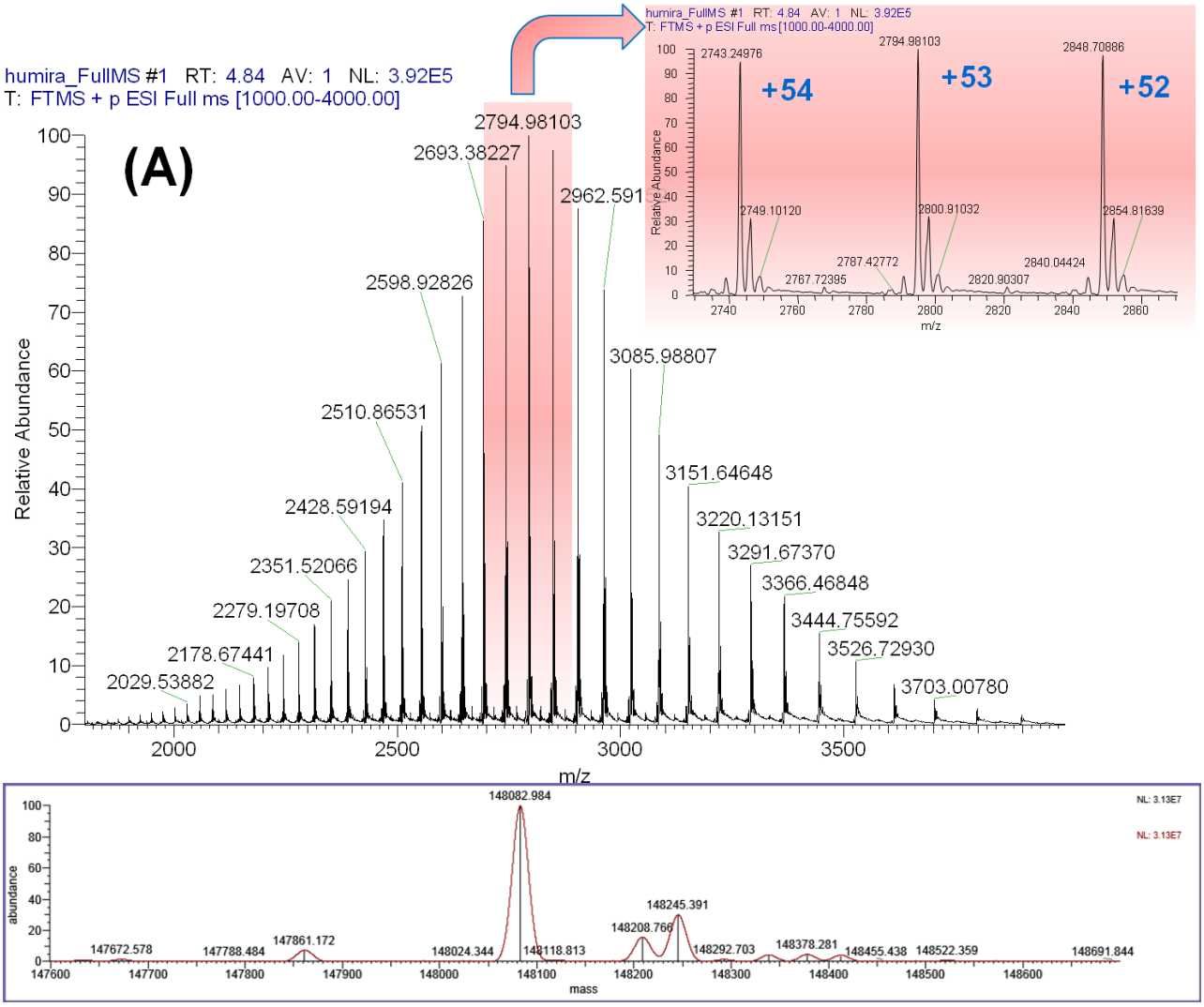

FIGURE 4: (A) Full MS spectrum of intact Humira. The insert shows a zoom into the three most abundant charge states z= 52,53,54. (B) Spectrum after deconvolution.

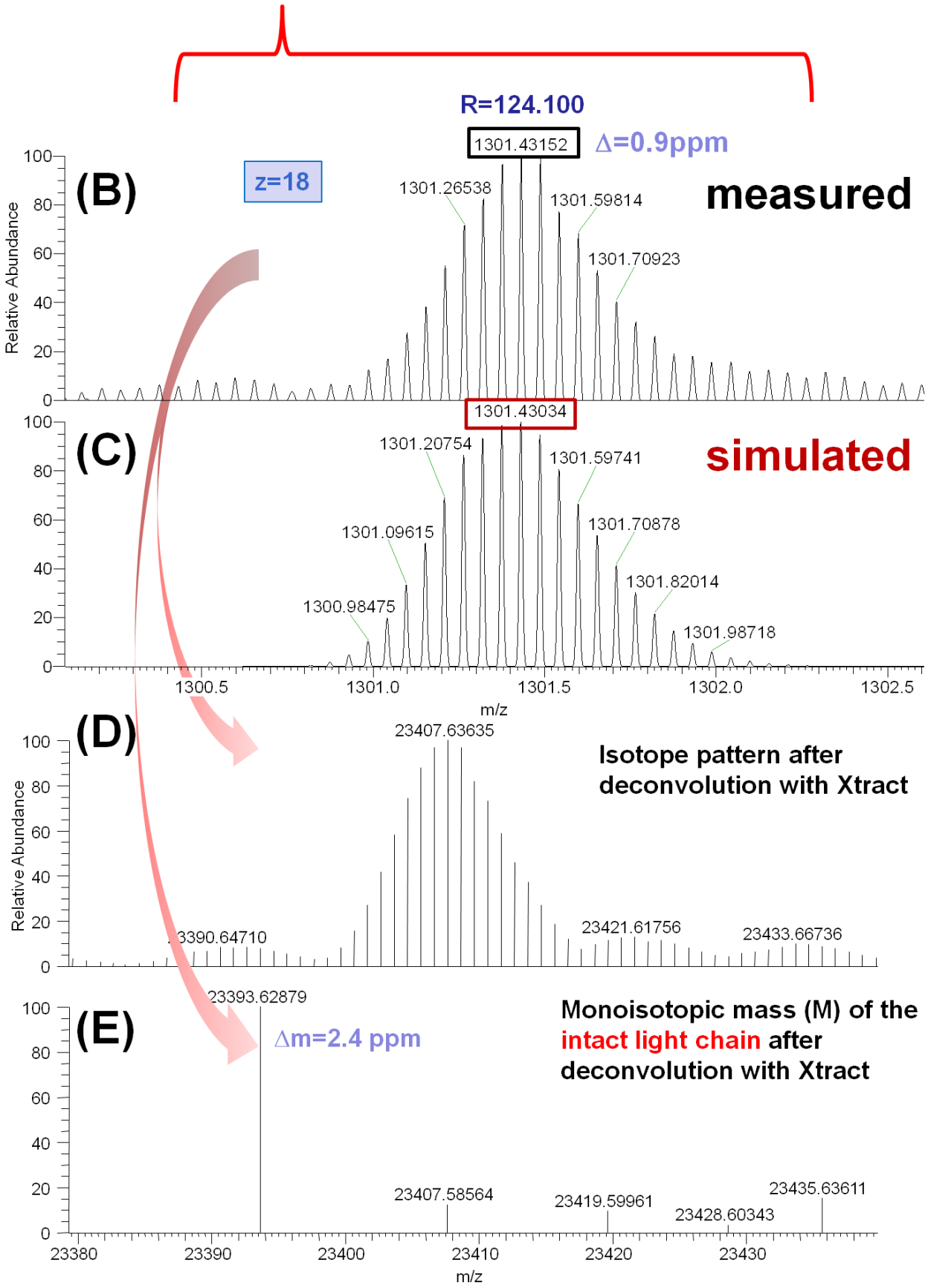

FIGURE 5. (A) Full MS spectrum of intact light chain of Humira. (B) Zoom into +18 charge state of intact light chain. (C) Simulation of isotope pattern of +18 charge state. (D) Isotope pattern of intact light chain after deconvolution. (E) Monoisotopic mass (M) of the measured light chain of Humira obtained after deconvolution with Xtract.

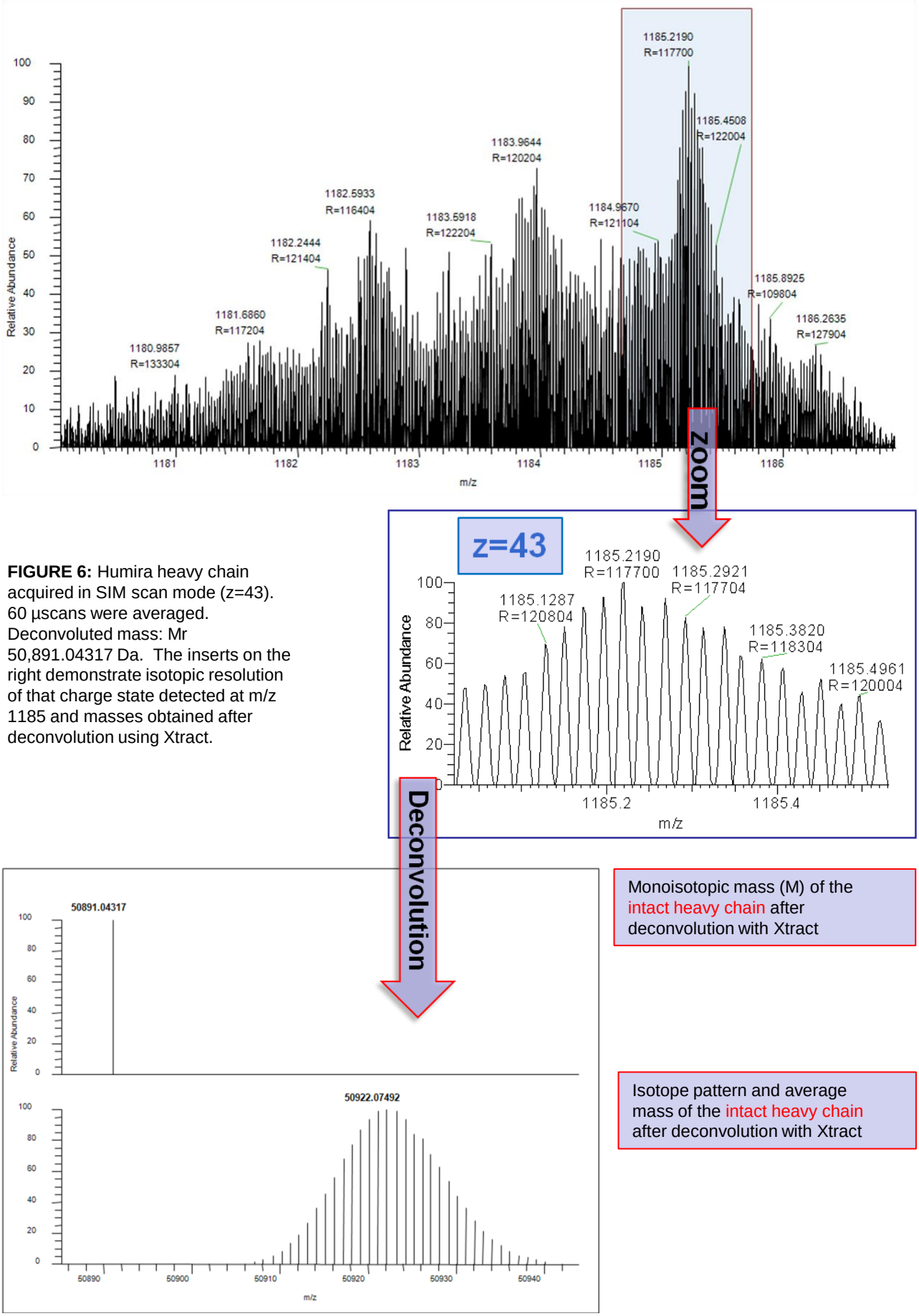

FIGURE 6: Humira heavy chain acquired in SIM scan mode (z=43). 60 µscans were averaged. Deconvoluted mass: Mr 50,891.04317 Da. The inserts on the right demonstrate isotopic resolution of that charge state detected at m/z 1185 and masses obtained after deconvolution using Xtract.

Monoisotopic mass (M) of the intact heavy chain after deconvolution with Xtract

Isotope pattern and average mass of the intact heavy chain after deconvolution with Xtract

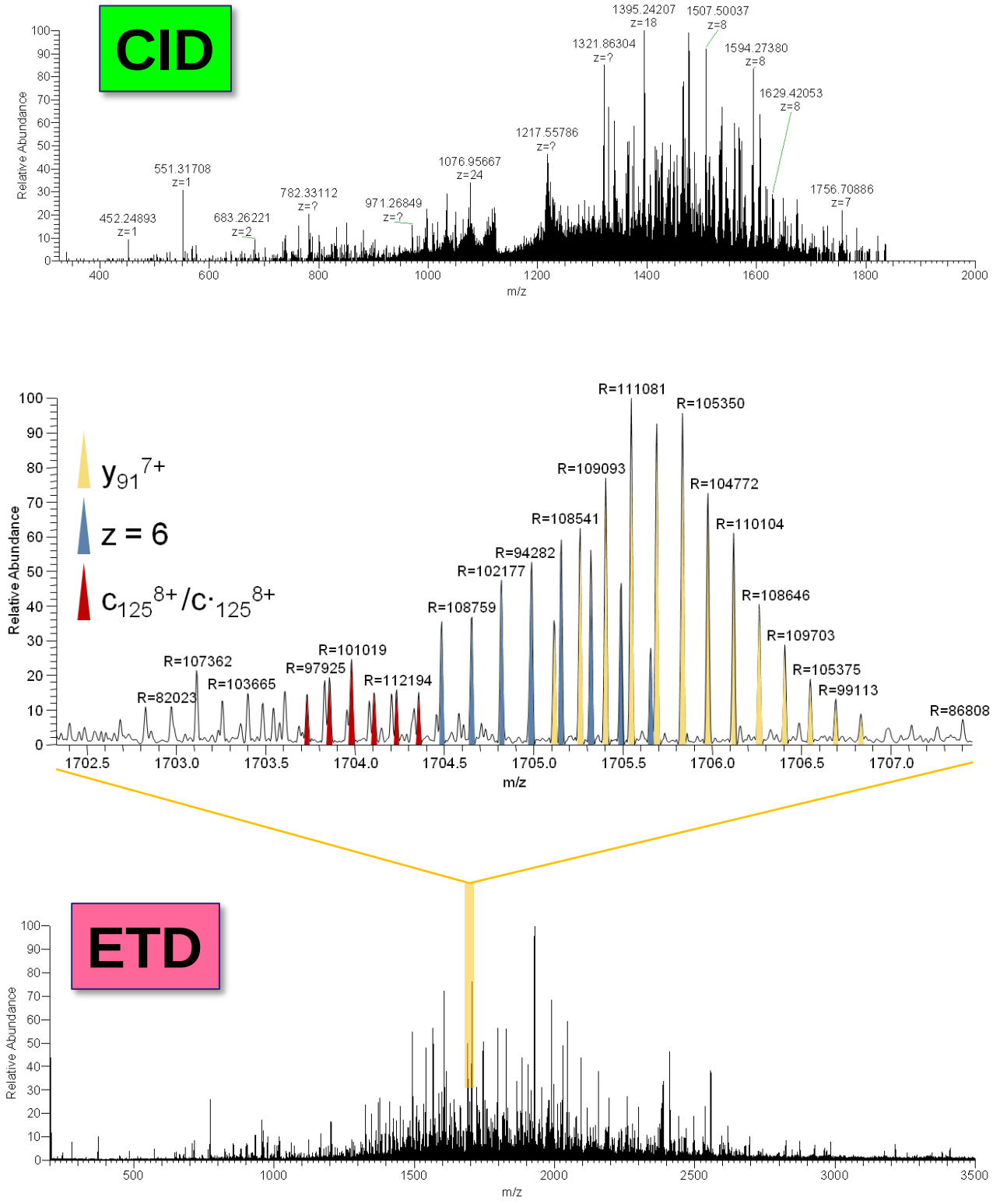

FIGURE 7: (A) CID spectrum and (C) ETD spectrum of **intact** Humira antibody. (B) Zoom in into the ETD fragment ion spectrum of **intact** Humira showing the need for highest resolution possible.

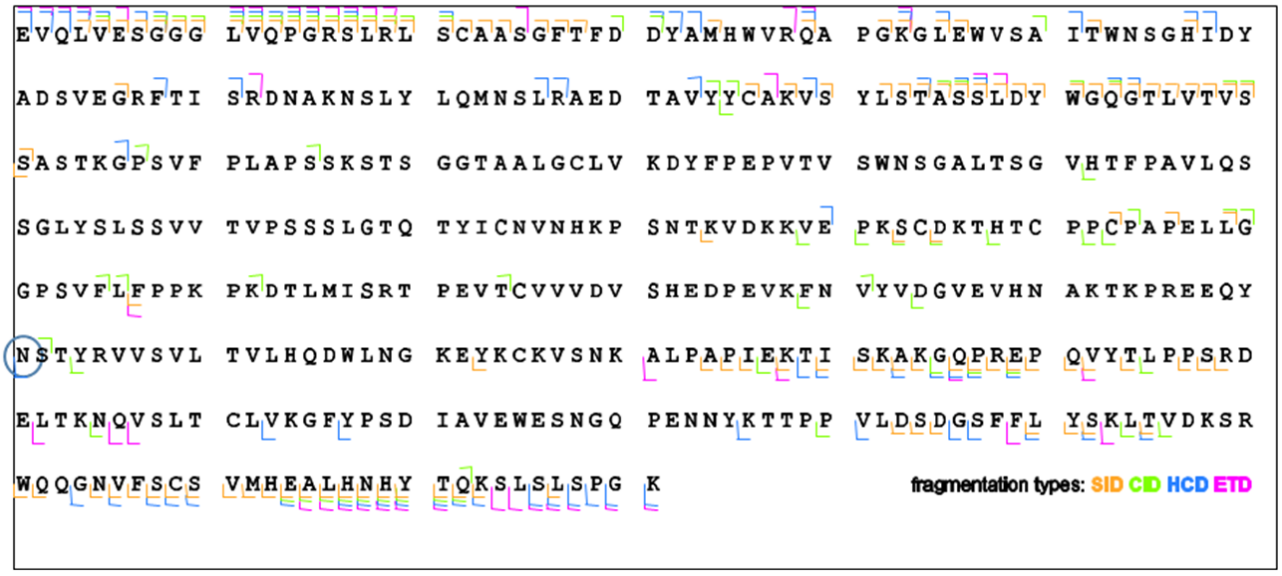

FIGURE 8: Summarized sequence coverage of the Humira heavy chain using fragmentation techniques SID, CID, HCD, and ETD. Optimized conditions: trapping under high pressure settings. N: Putative glycosylation site.

Conclusion

- The analysis of intact and reduced antibodies on the Orbitrap Elite mass spectrometer provides the accurate molecular weight as well as valuable information about the presence and abundance of glycoforms.
- Analysis of the reduced antibody provides isotopically resolved mass spectra for both light and heavy chain.
- The combination of multiple fragmentation techniques in top-down analysis (SID, CID, HCD and ETD) generates comprehensive sequence coverage and enables fast localization of modifications with minimum sample preparation.
- For measurements of intact light and heavy chain as well as for the detection of fragment ion spectra from top-down experiments ultra-high resolution as provided by the Orbitrap Elite mass spectrometer is essential.

Abbreviations

ACN, acetonitrile; CID, collision-induced dissociation; C-trap, curved linear trap; DTT, dithiothreitol; ETD, electron transfer dissociation; FA, formic acid; HCD, higher energy collision-induced dissociation; mAb, monoclonal antibody; µS, micro-scan; SID, *in-source* decay; SIM, single ion monitoring.

References

1. Bondarenko, P.V., Second, T.P., Zabrouskov, V., Makarov, A. & Zhang, Z. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. *Journal of the American Society for Mass Spectrometry* **20**, 1415-24 (2009).

2. Michalski, A. *et al.* Ultra high resolution linear ion trap Orbitrap mass spectrometer (Orbitrap Elite) facilitates top down LC MS/MS and versatile peptide fragmentation modes. *Molecular & cellular proteomics : MCP* (2011).doi:10.1074/mcp.O111.013698

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