In-depth characterization of monoclonal antibodies using intact mass analysis and middle-down approaches on an Orbitrap Ascend BioPharma Tribrid Mass Spectrometer

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Abstract

Purpose: To assess the performance of the Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer for comprehensive monoclonal antibody (mAb) characterization using intact and middle-down mass spectrometry (MS) approaches.

Methods: The middle-down methods were developed using a combination of ion activation techniques including electron transfer dissociation (ETD), electron transfer higher energy collision dissociation (EThcD), ultraviolet photodissociation (UVPD) and proton-transfer charge reduction (PTCR).

Results: In-depth sequence coverage of mAb subunits was achieved using the advanced MS/MS techniques on Orbitrap Ascend BioPharma Tribrid MS.

Introduction

In-depth characterization of mAbs and their post-translational modifications (PTMs) is critical to ensure the safety and efficacy of biotherapeutics. Intact mass analysis can be used to obtain accurate masses of mAbs.[1,2] The utilization of the middle-down MS technique has gained traction as an encouraging method for the characterization of biotherapeutics.[3-5] In this work, we performed intact mass analysis and middle-down MS to characterize Trastuzumab and its subunits on an Orbitrap Ascend BioPharma Tribrid mass spectrometer equipped with Native MS option, using different MS/MS fragmentation approaches.

Materials and methods

Sample preparation

Trastuzumab was aliquoted into 10 µg/µL stock solution for native LC-MS experiments. For intact mass analysis under denaturing conditions with reverse phase column, the antibody was diluted with 0.1% formic acid to a series of concentrations at 10 ng/µL, 50 ng/µL, 100 ng/µL, 500 ng/µL and 1 µg/µL. For reverse phase LC-MS/MS middle-down experiments, Trastuzumab subunits were prepared at 1 µg/µL final concentration using DTT for reduction combined with or without previous IdeS digestion according to manufacturer's protocol.

Methods

Native MS experiments were performed using a size exclusion column and an isocratic gradient with 50 mM ammonium acetate. Intact mass analysis (denatured) and middle-down MS experiments were conducted using a reverse-phase column. For intact mAb analysis under the native and denaturing conditions, an Orbitrap resolving power setting of 30,000 at 200 *m/z* was used. An Orbitrap resolving power setting of 240,000 at *m/z* 200 was used to acquire isotopically resolved full MS spectra of the Fc/2, light chain (LC) and Fd' subunits and setting of 7,500 at 200 *m/z* was used to acquire full MS spectra of heavy chain (HC) subunit. For middle-down analysis of all subunits, a quadrupole isolation window of 100 *m/z* centered around *m/z* 900 was used. Replicate ETD, EThcD and UVPD data were acquired using five different reaction/activation times.

Data analysis

All data analyses were performed using Thermo Scientific™ BioPharma Finder™ software version 5.2.

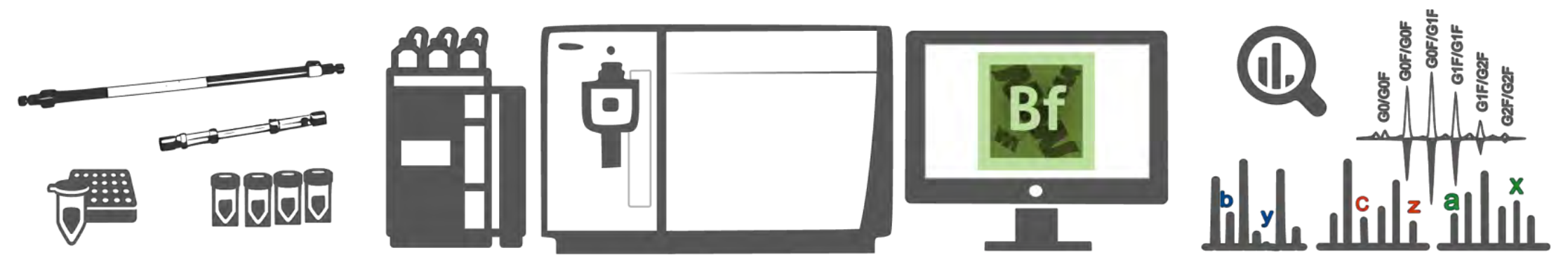


Figure 1. Major LC-MS components used for biopharmaceutical characterization. Experiments were using the Thermo Scientific™ Vanquish™ Flex UHPLC system and Orbitrap Ascend Tribrid mass spectrometer, together with the MAbPac™ reverse phase (P/N 088648) and MAbPac™ SEC-1 size exclusion (P/N 077592) columns for LC separation, and BioPharma Finder software for data processing.

Results

Overview

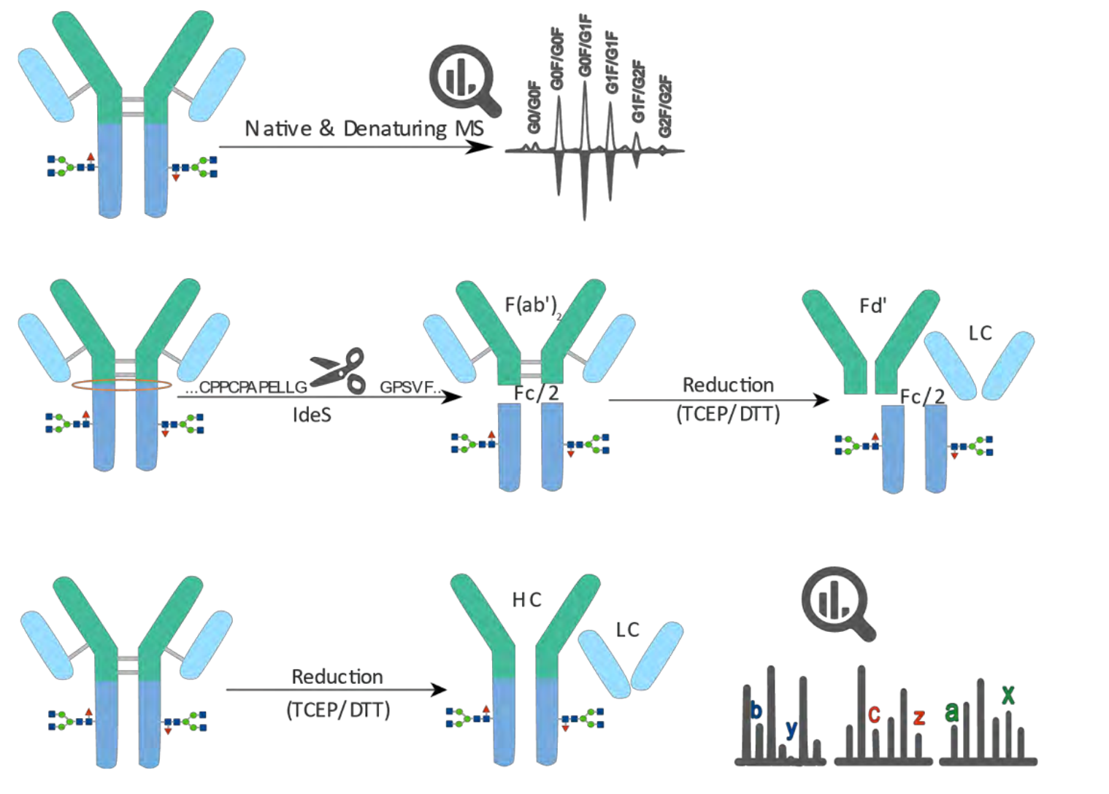


Figure 2. Illustration of trastuzumab sample preparation for intact mass and middle-down analyses. Top – intact mAb was diluted and analyzed under native and denaturing conditions; Middle – IdeS digestion and TCEP/DTT reduction to yield Fc/2, LC and Fd' subunits; Bottom – TCEP/DTT reduction to generate LC and HC subunits.

Intact mass analysis

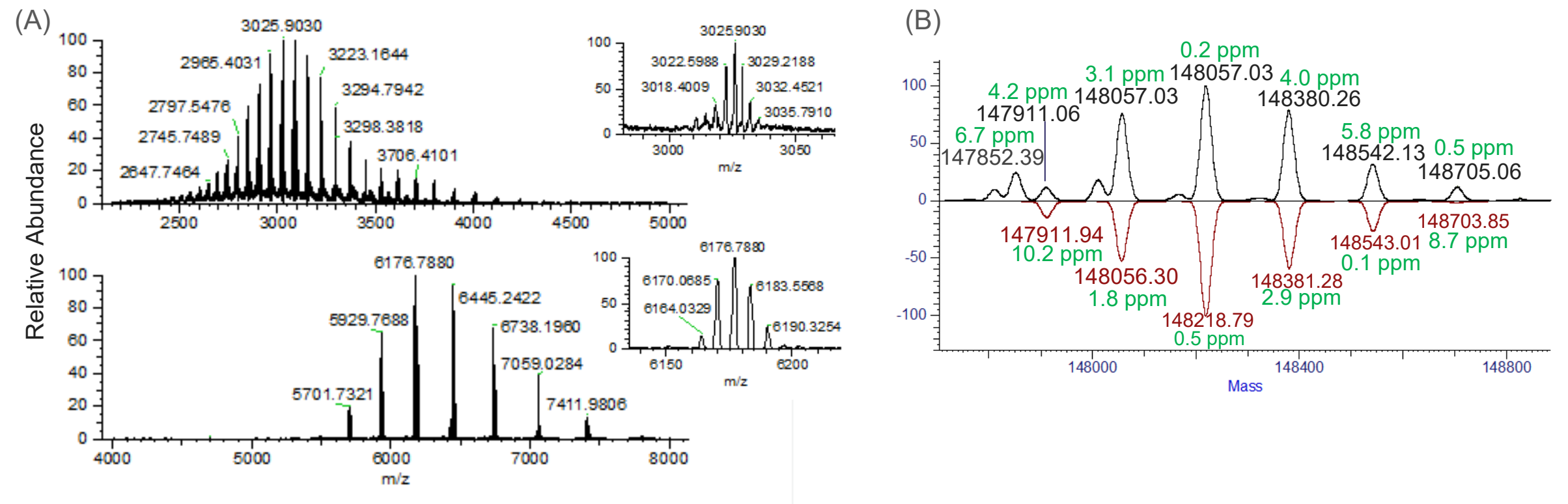


Figure 3. Intact mass analysis of trastuzumab under the denaturing and native conditions. A) Full MS spectra acquired from intact trastuzumab under the denaturing (top) and native (bottom) conditions. The insets show an expanded view of the most abundant charge state with baseline resolved glycoforms. B) Deconvoluted masses measured for intact trastuzumab under the denaturing (top) and native (bottom) conditions using the ReSpect and Sliding Window algorithm

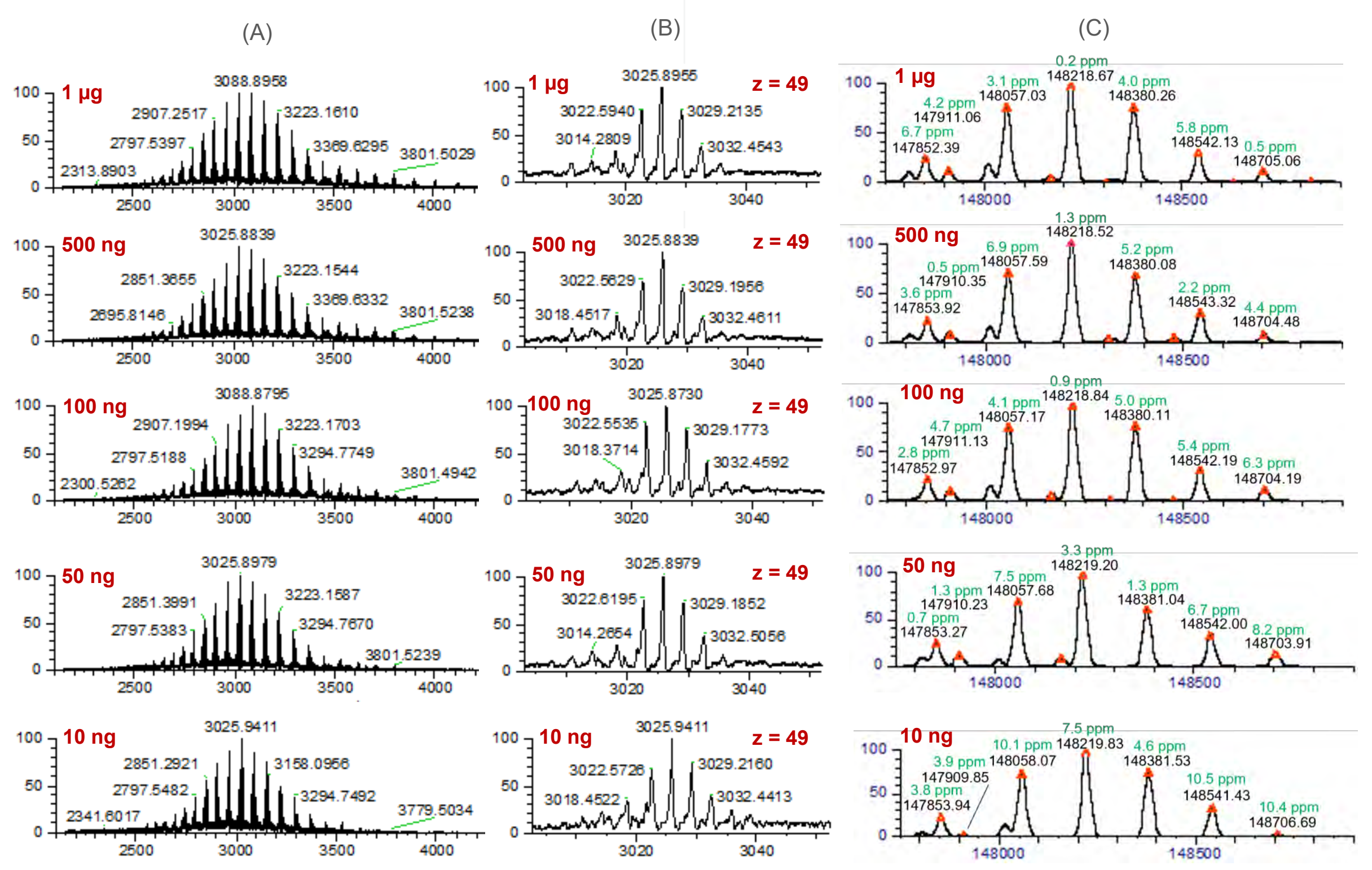
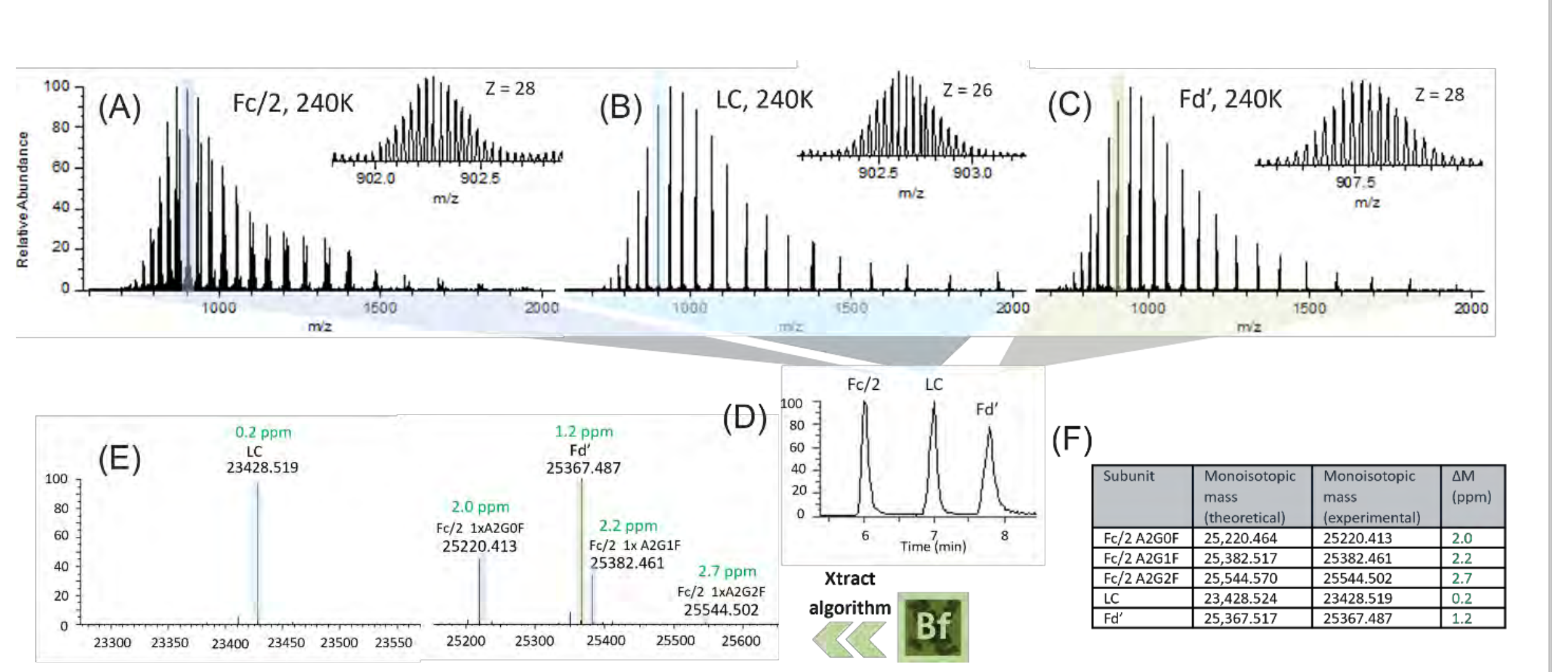


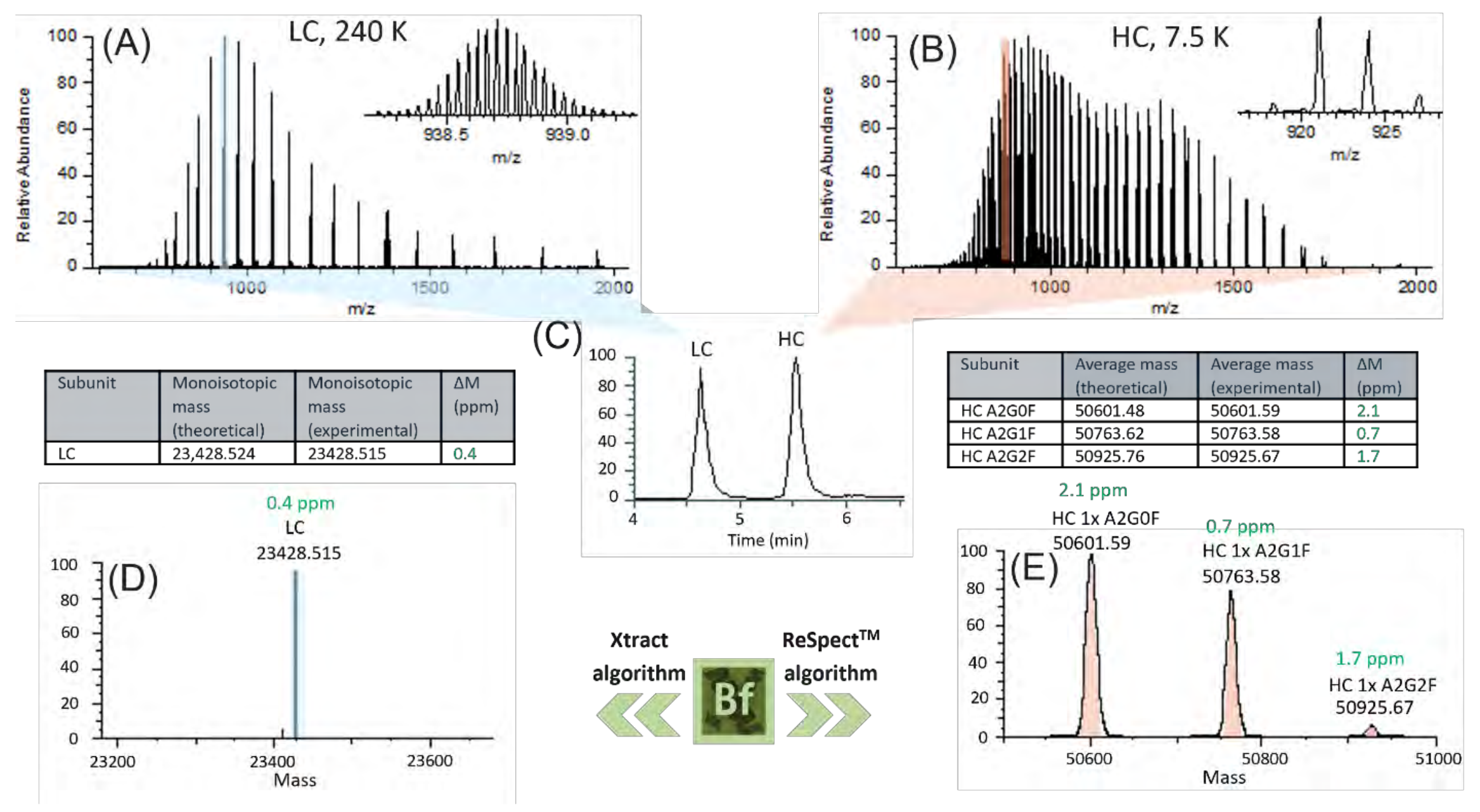
Figure 4. Intact mass analysis of trastuzumab dilution series under the denaturing condition. A) Full MS spectra of denatured trastuzumab with 10 ng to 1 µg loaded on column; B) Zoomed in spectra of the 49+ charge state with baseline resolved glycoforms; C) Deconvoluted results of trastuzumab obtained using BioPharma Finder ReSpect sliding window algorithm.

Subunit mass analysis



Subunit	Monoisotopic mass (theoretical)	Monoisotopic mass (experimental)	ΔM (ppm)
Fc/2 A2G0F	25,220.464	25220.413	2.0
Fc/2 A2G1F	25,382.517	25382.461	2.2
Fc/2 A2G2F	25,544.570	25544.502	2.7
LC	23,428.524	23428.519	0.2
Fd'	25,367.517	25367.487	1.2

Figure 5. Subunit analysis of Fc/2, LC, Fd' subunits from IdeS digestion of trastuzumab. A-C) Full MS spectra of the Fc/2, LC and Fd' subunits acquired at a resolution setting of 240,000 and 2 µscans. The insets show the isotopically resolved charge state at m/z ~900 Da (z=28 for Fc/2 and Fd' and z=26 for LC); D) Total ion chromatogram of Fc/2, LC and Fd' subunits; E-F) Deconvoluted results of three trastuzumab subunits obtained using the Xtract and sliding windows algorithm with BioPharma Finder software. Excellent mass accuracies (<3 ppm) were obtained for all three subunits.



Subunit	Monoisotopic mass (theoretical)	Monoisotopic mass (experimental)	ΔM (ppm)
LC	23,428.524	23428.515	0.4

Subunit	Average mass (theoretical)	Average mass (experimental)	ΔM (ppm)
HC A2G0F	50601.48	50601.59	2.1
HC A2G1F	50763.62	50763.58	0.7
HC A2G2F	50925.76	50925.67	1.7

Figure 6. Subunit analysis of the LC and HC subunits from trastuzumab. A) Full MS spectrum of the LC subunit acquired at a resolution setting of 240,000 and 2 µscans. The inset shows the isotopically *resolved* charge state (z=25) around m/z 939; B) Full MS spectrum of HC subunit acquired at a resolution setting of 7,500 and 10 µscan. The inset shows the isotopically *unresolved* charge state (z=55) around m/z 922; C) Total ion chromatogram of the LC and HC subunit showing baseline separation of two subunits; D) Excellent mass accuracy of <3 ppm was obtained for the LC subunit from the deconvolution of isotopically resolved data using the Xtract algorithm within BioPharma Finder software; E) Excellent mass accuracies of <3 ppm were obtained for three major glycoforms of the HC subunit from the deconvolution of the isotopically unresolved data using the ReSpect and sliding windows algorithm within BioPharma Finder software.

Middle-down MS analysis

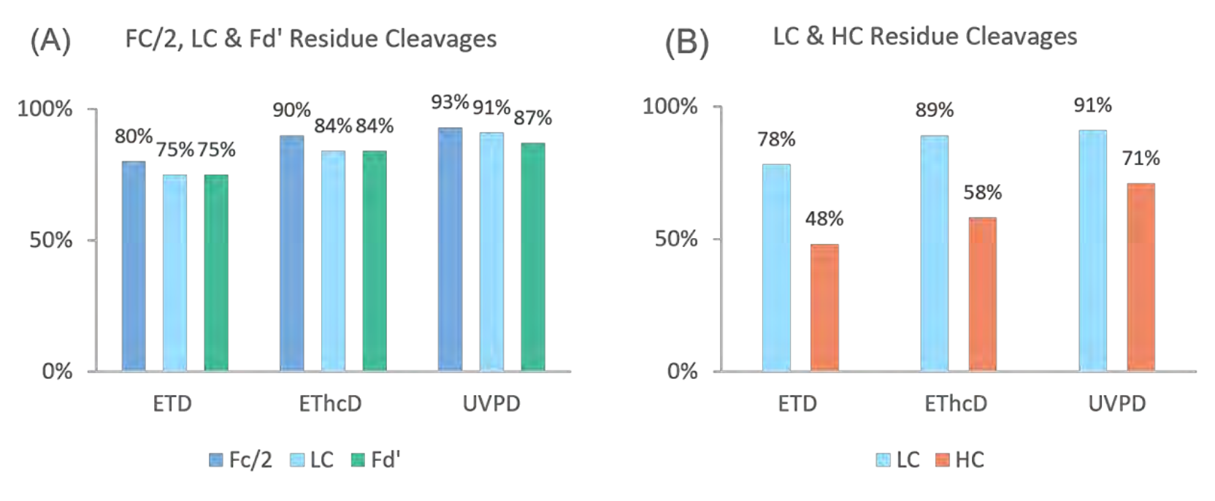


Figure 7 (A-B). Middle-down analyses of trastuzumab subunits using ETD, EThcD, and UVPD. A) Bar chart showing sequence coverage of the Fc/2, LC and Fd' subunits from IdeS digestion of trastuzumab using ETD, EThcD, and UVPD, with 10 files combined; B) Bar chart showing sequence coverage of the LC and HC subunits from reduction of trastuzumab using ETD, EThcD, and UVPD, with 10 files combined.

Figure 7 (C-H). Middle-down analyses of trastuzumab subunits using ETD, EThcD, and UVPD C-E) Fragmentation maps of the Fc/2, LC and Fd' subunits obtained from five experiments using ETD, EThcD, and UVPD, with two replicates for each condition; F-H) Fragmentation maps of the LC and HC subunits obtained from five experiments using ETD, EThcD, and UVPD, with two replicates for each condition. High sequence coverage was obtained using all fragmentation techniques. EThcD and UVPD provided an improved sequence coverage than ETD due to the formation of additional fragment types.

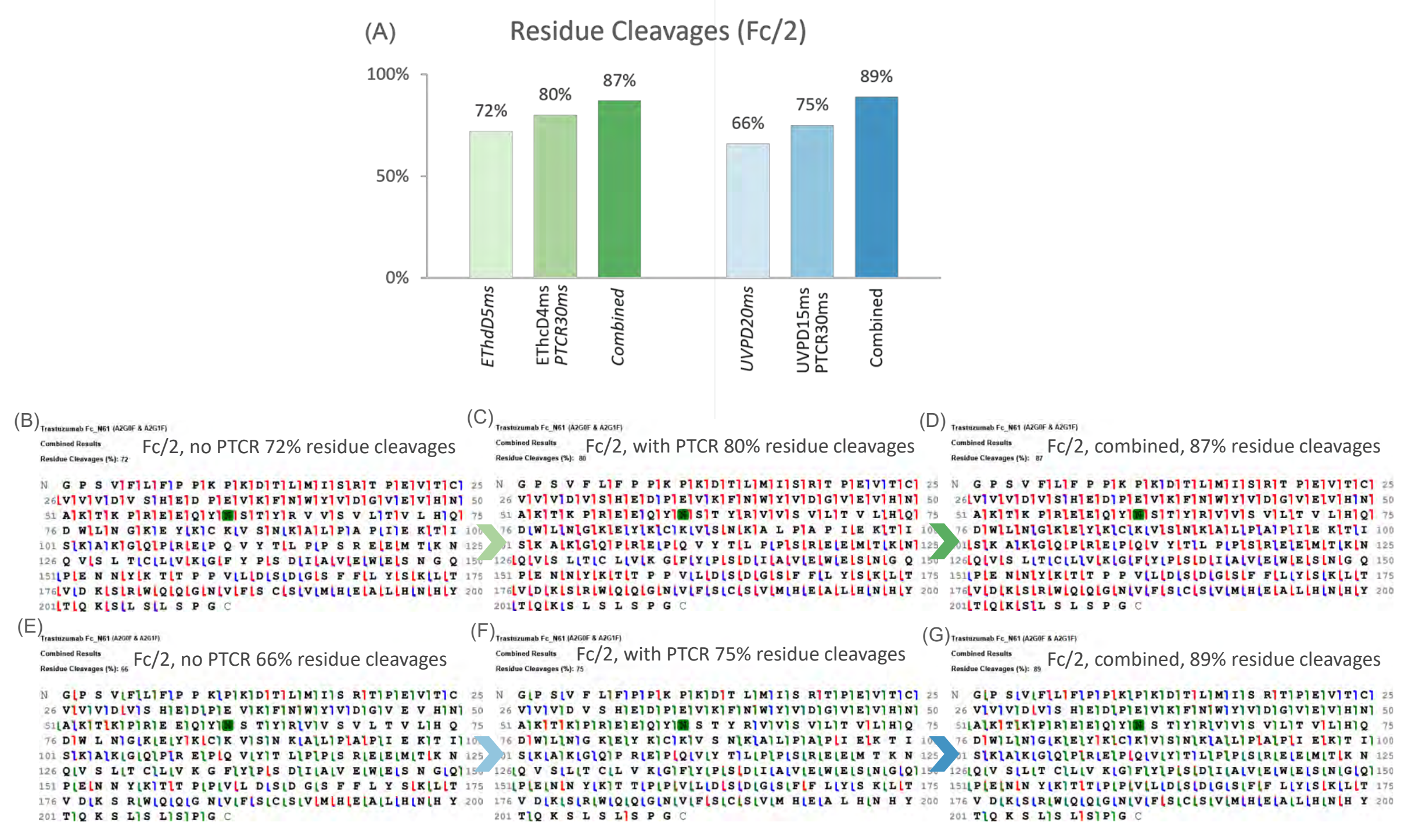


Figure 8. Combined PTCR ion activation with other fragmentation methods benefits residue coverage by decluttering the congested MS2 spectra. A) bar chart representing the improved residue coverage for Fc/2 subunit from 72% to 80% for EThcD fragmentations, and 66% to 75% for UVPD fragmentations, from two replicates of the selected experiment condition as labeled in the figure. When combined all the replicate results from experiments with and without PTCR, EThcD experiments achieved 87% coverage for Fc/2 subunit and UVPD experiments achieved 89% coverage. B.) fragmentation map of Fc/2 subunit for EThcD fragmentation from 5 ms reagent reaction time with two replicates (left). C.) fragmentation map of Fc/2 subunit from EThcD 4ms plus PTCR 30ms method with two replicates (middle) and D.) cumulative fragmentation map of Fc/2 subunit from (B) and (C) (right). E.) fragmentation map of Fc/2 subunit for UVPD fragmentation from 20 ms activation time with two replicates (left). F) fragmentation map of Fc/2 subunit from UVPD 15ms plus PTCR 30ms method with two replicates (middle) and G.) cumulative fragmentation map of Fc/2 subunit from (E) and (F) (right).

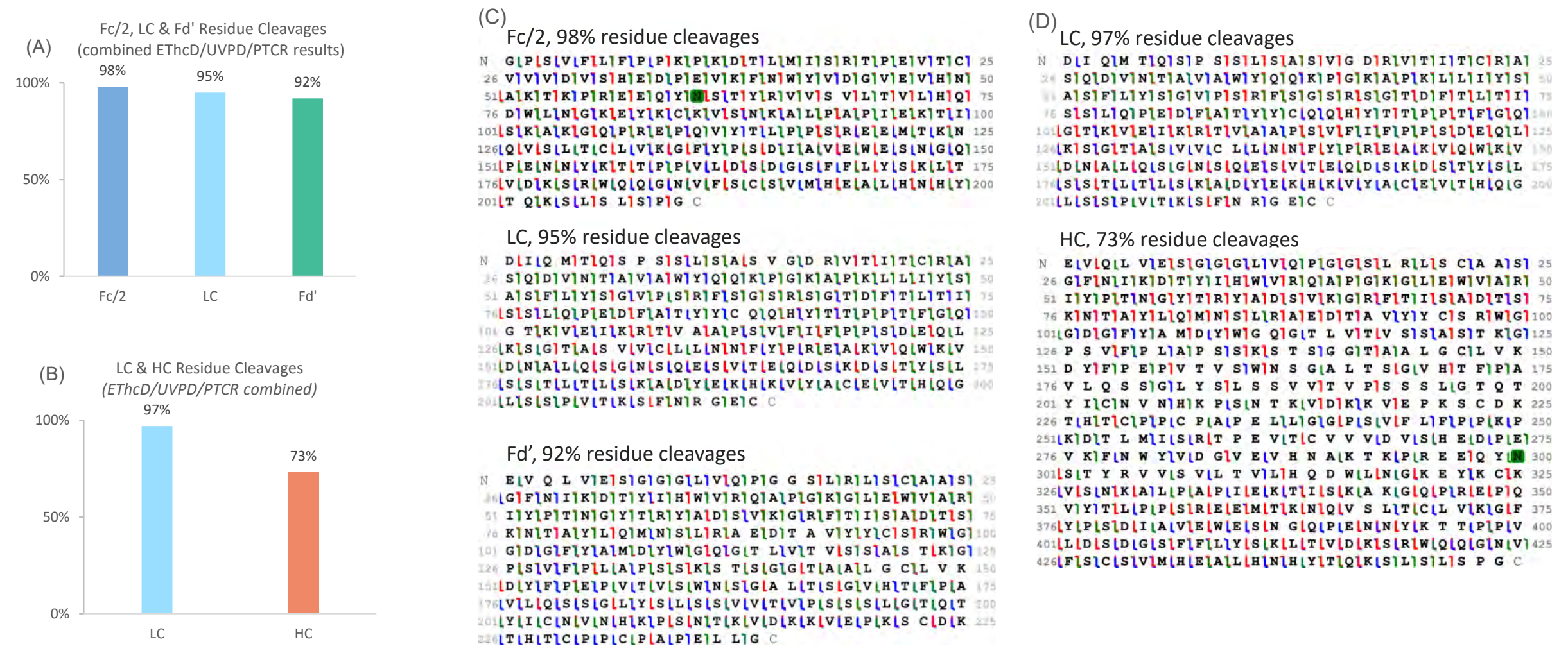


Figure 9. Combined results of EThcD, UVPD, and PTCR middle-down analyses provided excellent sequence coverage of trastuzumab subunits. A.) Bar chart showing a nearly complete sequence coverage of Fc2, LC and Fd' subunits obtained from the combined results of EThcD, UVPD and PTCR raw files (10 in total); B) Bar chart showing a nearly complete sequence coverage of the LC subunit (97%) and high sequence coverage of the HC subunit (73%) when combining the raw files of EThcD, UVPD and PTCR (10 in total); C) Fragmentation maps of the Fc/2, LC and Fd' subunits from the combined results of 10 raw files; D) Fragmentation maps of the LC and HC subunits from the combined results of 10 raw files.

Conclusions

- The high mass accuracy and high resolution offered by the Orbitrap technology provides precise mass measurement for both intact mAbs (native and denaturing) and their subunits
- Compared to ETD, EThcD and UVPD provide an improved sequence coverage of mAb subunits due to the formation of additional fragment types. The combination of PTCR with EThcD and UVPD led to further increase in sequence coverage of mAb.
- The combination of EThcD, UVPD and PTCR offers a nearly complete sequence coverage (>90%) for subunits in the size of 20-25 kDa (Fc/2, LC and Fd' subunits) and high sequence coverage (>70%) for the HC subunit (~50 kDa)

References

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