Comparative Multi-Supplier Lot Analysis of Trastuzumab using Subunit Analysis on the X500B QTOF System

Trastuzumab Subunit Analysis using Benchtop X500B QTOF Mass Spectrometer

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Introduction

Routine characterization of biosimilars or batch comparison of manufactured biologic samples is critical to maintain quality for biotherapeutics throughout development and production. The large size and inherent heterogeneity of biologics make these drugs highly complex, requiring robust mass analysis techniques to ensure accuracy in detection of the protein backbone composition as well as the presence of post-translational modifications. Mass spectrometry is a powerful and flexible tool for biotherapeutic characterization with the ability to analyze both large and small molecules with high mass accuracy.

Here we demonstrate the use of a fast, reproducible and robust method for biologic subunit analysis on the X500B QTOF system with streamlined and rapid batch processing and product comparison using the BioPharmaView™ software.

Materials and methods

Biosimilar trastuzumab therapeutic was obtained from two different manufacturing sources (labeled TRAST-1 and TRAST-2). Reduction was performed using 50 mM TCEP (0.1% v/v) at 65°C for 30 mins.

1 mg/ml of trastuzumab pH 7.5 was incubated with IdeS protease (IdeZ, Promega, Madison, WI, USA) at 37°C for 30 mins. Samples were further reduced with the addition of another 0.1 v/v of 50 mM TCEP at 65°C for 30 mins.

All samples were diluted with 0.2% formic acid before analysis.

High flow chromatography

A total of 0.5 µg of protein was injected onto the ExionLC™ and separated using a Waters Acquity UPLC® Protein BEH C4 column, 300A 1.7 um, 2.1mm x 50mm column 40°C. Standard mobile phases were used (Mobile Phase A: 0.1% formic acid in water, Mobile Phase B: 0.1% formic acid in acetonitrile) with a gradient of 15 – 40% B over 20 min and a total run time of 30 min using moving flow rate of 0.2 mL/min. An integrated divert valve was used to flush to waste for the first 0.5 mins of each injection.

Mass spectrometry

Acquisition was performed on X500B QTOF with a Turbo V™ ion source using large protein mode acquisition and decreased detector voltage selected over a range from 600-4000 m/z. Electrospray parameters were as follows:

Curtain gas:	35
Ion source gas 1 (psi):	50
Ion source gas 2 (psi):	50
Temperature (°C):	400
Declustering Potential:	150
Bins to sum:	60

Data processing

Data was processed in BioPharmaView using a standardized sample of trastuzumab as reference.

Results and Discussion

Tastuzumab is a recombinant IgG1 monoclonal antibody used to treat Her2 positive breast cancer. For this study, trastuzumab from two different manufacturing sources were obtained for comparison and characterization. Two methods were used for subunit preparation: reduction and IdeS/reduction.

Reduced Trastuzumab

Raw LC-MS data for reduced antibody therapeutics can be complex to interpret. Subunit analysis using a partial reduction reduces complexity by reducing interchain disulfide linkages, yielding a light chain and a heavy chain species. LC-MS data for the light chain from TRAST-1 and TRAST-2 show multiple charge states as expected (Figure 1A). Reconstructed mass of the light chain between the samples was consistent (Figure 1B) confirming that the light chains are consistent with each other.

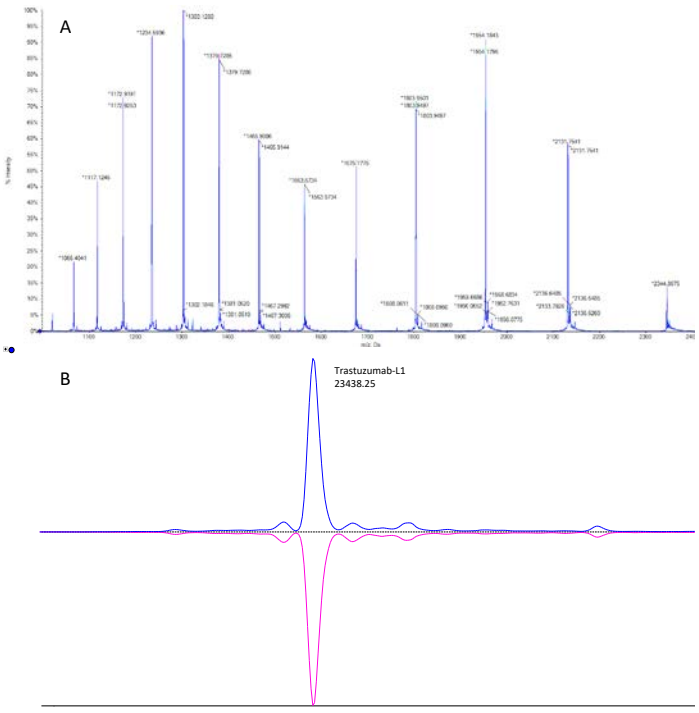

Figure 1: Rapidly comparing multiple biotherapeutic samples with the highly accurate data acquisition. Raw data (1A) and reconstructed mirror plots (1B) for trastuzumab light chain from TRAST-1 (blue) and TRAST-2 (pink), indicating comparable protein mass.

Analysis of the heavy chain was to assess comparability between the two samples. Raw mass spectral data for the heavy chain is shown in Figure 2. Overall each sample produces similar species with m/z values that agree, however the relative intensity of each species is different.

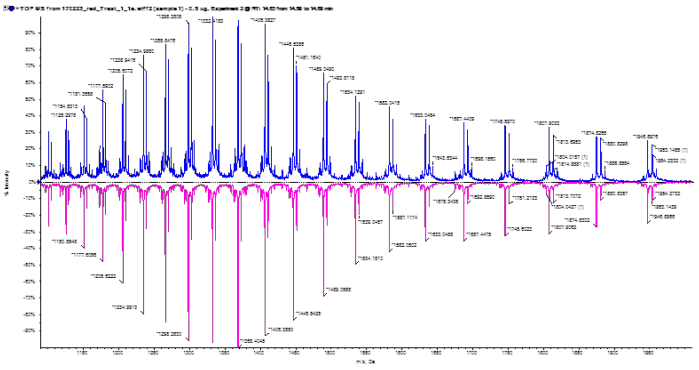

Figure 2: Comparison of raw data charge state envelope of TRAST-1 (blue) and TRAST-2 (pink) showing slight variations in glycoforms across the protein.

Detection of differences between the two samples is visible from the raw data (Figure 2), but more easily observed when the m/z axis is expanded, focusing on a few charge states (Figure 3).

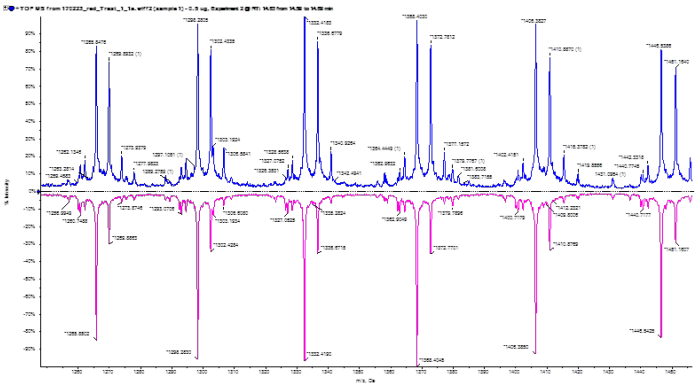

Figure 3: Expanded view of overlaid raw data from TRAST-1 (blue) and TRAST-2 (pink) showing changes in the glycoform distribution across the m/z range.

Reconstruction of the raw mass spectral data to obtain the heavy chain was accomplished using BioPharmaView software which allows for the assignment of defined post translational modifications of each sample. The high-resolution accurate mass X500B QTOF system provides high-quality mass spectral data which is reproducible, allowing for accurate comparison of the two samples (Figure 4).

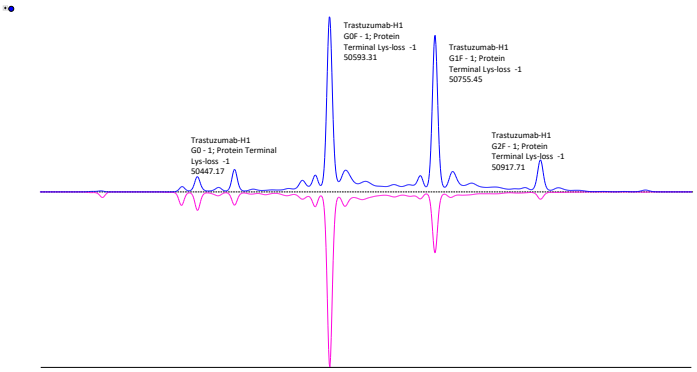

Figure 4: Reconstructed heavy chain of TRAST-1 (blue) and TRAST-2 (pink) highlighting the differences in the glycoforms.

Relative intensities of the glycoforms show a distinct difference between the two samples. Some such as G0F and G1F are very noticeable (Figure 4) and can be identified visually. Others such as M5 are more difficult to see in the reconstructed spectrum. Batch data processing by BioPharmaView software yields easy-to-view graphs for rapidly pinpointing the exact glycoforms altered between the two samples (Figure 5).

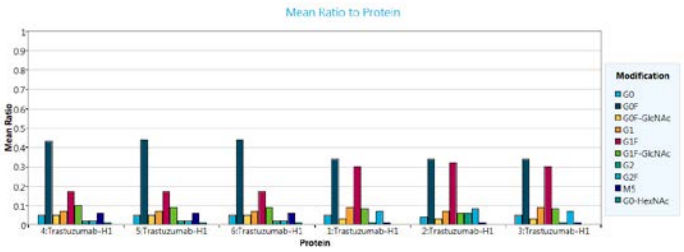

Figure 5: Graphic representation of glycoform abundances allow for fast identification of heavy chain differences between the two trastuzumab lots. Relative abundances of modifications for TRAST-1 (right 1:Trastuzumab-3:Trastuzumab) and TRAST-2 (left 4:Trastuzumab – 6:Trastuzumab) are visualized. Changes for all post translational modifications are graphically represented and the mean ratio value can be easily obtained by hovering over the modification.

This data confirms changes in the relative abundance of G0F and G1F between the two samples, and indicates the M5 glycoform is detectable at the subunit level in both samples. Additionally, there were more minor changes in the abundance of G0F HexNac, G0F-GlcNAc, G1F –GlcNAc, and G2F glycoforms that were observed.

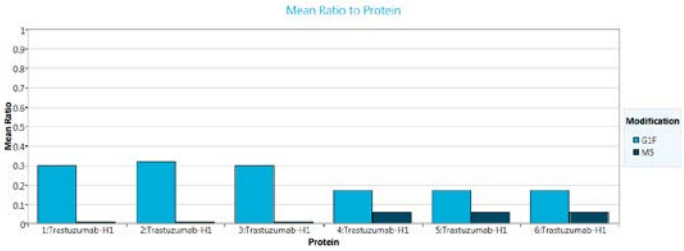

Figure 5: Rapid identification of alterations in M5 and GIF between sample sets are identified with the graphical representations from the BioPharmaView Software.

Relative modification ratios are calculated automatically by the software based on known, present glycoforms in the samples to ensure the mean ratio is not skewed by the amount of biotherapeutic protein injected. In this case the M5 glycoform is barely detectable in the TRAST-1 sample set (1:Trastuzumab-H1 – 3:Trastuzumab-H1) while present in TRAST-2 sample set (4:Trastuzuma-H1 – 6:Trastuzumab-H1).

IdeS Digested and Reduced Trastuzumab

A secondary subunit analysis was performed using IdeS protease, an enzyme which cleaves at the hinge region of the mAb, at the conserved CPAPELLG / GPSVF sequence. The resulting protein subunits were treated using a reducing agent to produce three fragments; Light chain (Figure 6), Fab chain (Figure 7) and Fc chain (Figure 8) for LC-MS analysis.

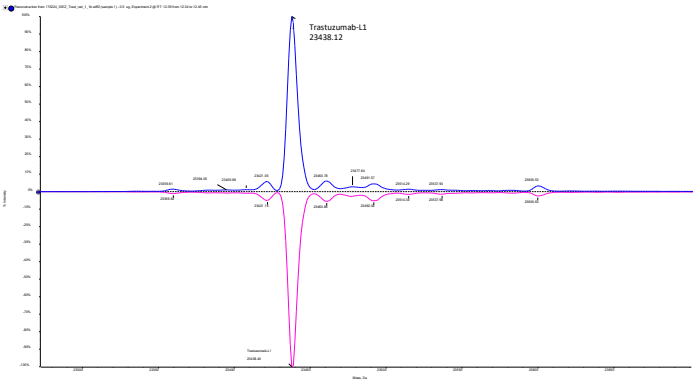

Figure 6: Reconstructed light chain from IdeS and reduction. TRAST-1 (blue) and TRAST-2 (pink) show consistent mass profile for the light chain.

The light chain fragments correlate well with each other as seen in the mirror plot (Figure 6). The Fab fragment (Figure 7) from TRAST-1 and TRAST-2 also correlated very well with each other.

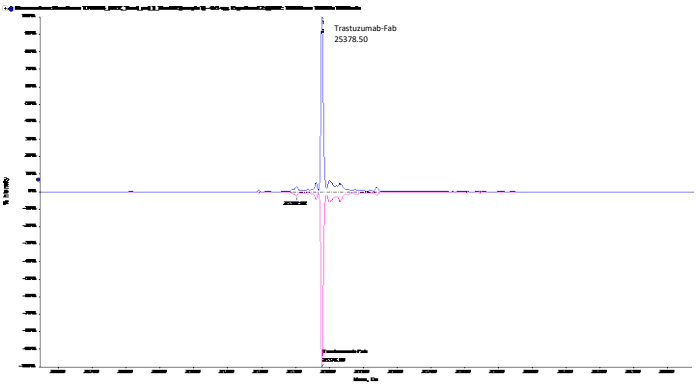

Figure 7: Reconstructed Fab fragment from TRAST-1(blue) and TRAST-2(pink) treated with IdeS and reduced. Good correlation between the two Fab fragments.

Other low level species are present in the Fab fragment, which correlate well between the two samples and are components of the Fc species present as seen in Figure 8.

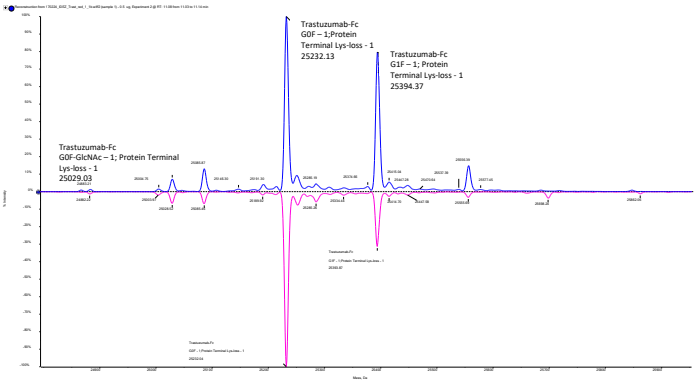

Figure 8: Reconstructed Fc fragments from TRAST-1 (blue) and TRAST-2 (pink) with differing intensities on the present glycoforms.

The Fc fragment is of the most interest as it contains the variable glycosylation region within trastuzumab. There is a difference in the glycoforms of major intensity such as the G1F and the G2F but some small differences in intensities in the minor glycoforms as well. Overall however, comparatively, most glycoforms are present, giving consistent but different mass profiles for both. One of the major differences was the barely detectable level of M5 from TRAST-1 which was seen in both the reduced and the IdeS/reduced samples. A summary of the relative ratios of M5 to G1F were calculated for both sets of experiments.

Table 1: Relative ratio of glycoforms M5 to G1F for reduced samples and reduced IdeS samples.

Sample	M5 Reduced	G1F Reduced	M5/G1F Reduced	M5 IdeS	G1F IdeS	M5/G1F IdeS
TRAST-1-1	0.01	0.30	*0.033*	*0.01*	*0.28*	*0.036*
TRAST-1-2	0.01	*0.32*	*0.031*	*0.01*	*0.27*	*0.037*
TRAST-1-3	*0.01*	*0.3*	*0.033*	*0.01*	*0.28*	*0.036*
MEAN	*0.01*	*0.31*	*0.032*	*0.01*	*0.28*	*0.037*
TRAST-2-1	*0.06*	*0.17*	*0.35*	*0.06*	*0.22*	*0.27*
TRAST-2-2	*0.06*	*0.17*	*0.35*	*0.06*	*0.22*	*0.27*
TRAST-2-3	*0.06*	*0.17*	*0.35*	*0.06*	*0.21*	*0.28*
MEAN	*0.06*	*0.17*	*0.35*	*0.06*	*0.22*	*0.27*

Automated extraction of the mean ratio numbers into a table allows for fast confirmation of glycoform expression alterations between the two samples. Both methods show consistent glycoform ratios including the low abundance of M5 in TRAST-1 and calculation of the relative M5/G1F ratios from both methods gives consistent results. Using the relative area ratios for the samples, the results of the M5/G1F calculation is consistent across the two analytical methods.

Conclusion

The high-quality mAb subunit data obtained by the X500B QTOF System, and rapid data processing in BioPharmaView Software, allows for accurate and efficient identification of relative changes in post translational modifications between the two lots of trastuzumab. Results obtained from the sample reduction procedure agreed well with data acquired following IdeS protease digestion and reduction, showing no bias between the sample preparation methods. The simple sample reduction method is highly efficient as a single sample preparation step, but the IdeS digestion and reduction method can provide additional specificity in locating and identifying post-translational modifications since the subunits are smaller in size. The mass accuracy, resolution and robustness of the X500B QTOF System was necessary to provide high quality data that could identify and confirm complex changes in glycoprotein forms between the two trastuzumab samples.

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