Detection of Intact Antibody Impurities on the SCIEX X500B

High sensitivity detection of low level protein impurities on the SCIEX X500B QTOF

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INTRODUCTION

Monoclonal antibodies (mAb) based biotherapeutics are a large class of protein biologics. Antibodies allow highly specific targeting of disease markers which is leading to a great deal of interest and investment in the field. Understanding the primary structure, heterogeneity, modifications and potential contaminants of these biologics is key to ensure product safety and quality.

LC-MS analysis has become a highly useful technique for the identification, characterization and quantification of intact proteins as well as characterization of protein clippings and impurities. The X500B system has been developed for biologics characterization, allowing the user to boost analytical capacity, simplify workflows and accelerate throughput. Utilizing the new SCIEX OS software with intuitive icon-based interface, the system brings powerful biologics characterization into the hands of scientists at any skill level without the need for mass spectrometry experts.

This technical note describes the LCMS method using the SCIEX X500B QTOF system on two commonly available monoclonal antibodies, the NIST monoclonal antibody standard, and Trastuzumab. The Trastuzumab was spiked into the NIST mAb standard[1] at different relative concentrations to represent an intact impurity. Additionally, NIST mAb standard treated by the enzyme FabRICATOR (IdeS), were spiked in to represent lower molecular weight protein impurities, again at varying concentrations. This data was collected both with and without treatment by PNGase F to remove glycans.

MATERIALS AND METHODS

Sample preparation: Intact NIST mAb, Humanized IgG1κ Monoclonal Antibody (RM) and Trastuzumab were serially diluted in 0.1 %FA (formic acid) and 15 % acetonitrile down to 0.1 µg/µl. NIST mAb (10 µg/µl) was also treated with FabRICATOR (IdeS, Genovis) for 4 hours at 37°C to separate the Fc from the Fab regions. Samples were treated with PNGase F (Sigma) to remove glycosylation on the heavy chain of the antibodies.

Samples were spiked with Trastuzumab or digested NIST mAb (with and without glycans for both) from 0.1 µg/µl down to 0.0001 µg/µl into final NIST mAb concentration of 0.1 µg/µl. For IdeS digest, the concentration is total protein concentration not individual species.

LIQUID CHROMATOGRAPHY

Samples were injected at 10 µl onto an Agilent PLRP-S column (300 Å ,3 µM, 50 x 2.1 mm) using the HPLC gradient in Table 1 using an Eksigent® ExionLC™ AD system. Column temperature was maintained at 80°C. Mobile phase A was 100 % water with 0.1 % FA and mobile phase B was 100 % acetonitrile with 0.1 %FA.

Table 1: HPLC Gradient Used in Impurity Analysis

Time [min]	*Flow [mL/min]*	*B.Conc [%]*	*B.Curve*
0.50	0.5000	25.0	0
5.50	0.5000	55.0	0
6.20	0.5000	95.0	0
7.00	0.5000	95.0	0
7.10	0.2000	25.0	0
8.10	0.2000	25.0	0

Mass Spectrometry: MS analyses were performed using a SCIEX X500B QTOF. The MS method used is displayed in Table 2. A minimum of three replicate injections were performed for each serial dilution.

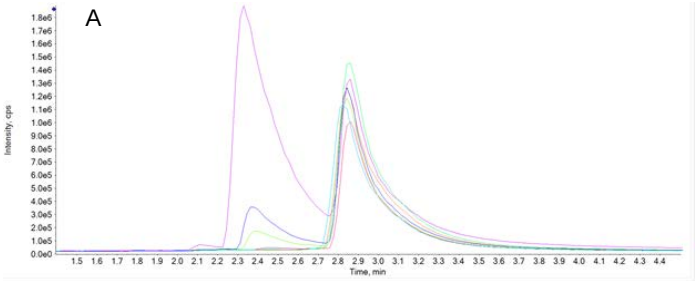
Table 2: MS and Source Parameters

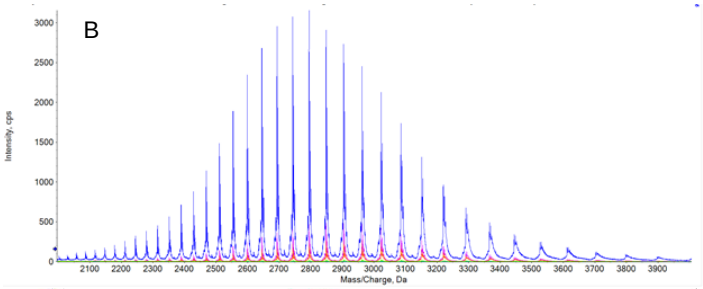
Parameter	Value	Parameter	Value
Curtain gas:	45	Time bins to sum	120
Ion Source Gas 1 (psi)	50	TOF start mass (Da)	900
Ion Source Gas 2 (psi)	50	TOF stop mass (Da)	5000
Temperature (°C)	500	Accumulation time	1
Scan type:	TOF MS	Declustering potential (V)	250
Polarity	Positive	Collision energy (V)	15
Ionspray Voltage (V)	5000	CAD gas	7

RESULTS AND DISCUSSION

DETECTION OF INTACT IMPURITIES

Trastuzumab was spiked into the NIST mAb at varying concentrations to determine the detection limit of intact impurities.





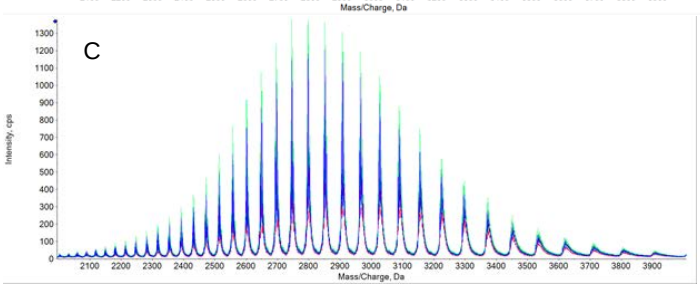


Figure 1 A) Raw ion chromatograms across all 27 injections (3 x 9 repeats), B) Non-normalized raw data for Trastuzumab impurity across all injections C) Raw data for NIST mAb across all 27 injections (3 x 9 repeats) injections.

The MS spectra were analyzed using the BioToolKit micro app in the Explorer™ Software feature of SCIEX OS. The NIST mAb elutes at 2.8 minutes on this column whilst the impurity (Trastuzumab) elutes at 2.3 minutes. Figure 1A shows the total ion chromatograms of injections with all levels of impurity, Figure 1B shows the raw data over the impurity peak (Trastuzumab) for all injections and Figure 1C shows the same for the NIST mAb. The impurity was detectable down to 0.001µg/µl as shown in Figure 2. Figure 2A shows the raw data and 2B shows the intact reconstruction of Trastuzumab.

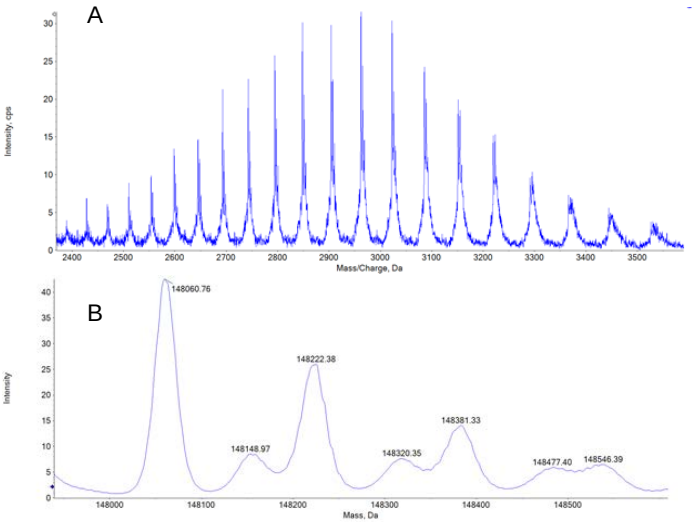


Figure 2. A) Raw data of Trastuzumab impurity at 0.1% concentration to main peak. B) Reconstruction of data in figure 2 A.

DETECTION OF INTACT IMPURITIES ON DEGLYCOSYLATED SAMPLES

Antibodies are natively glycosylated on the Fc region of the heavy chain; this can complicate the mass spectra by having several different potential glycoforms. Removal of these glycans with enzymes like PNGase F not only simplifies the mass spectra, it also increases the intensity of the protein as the ions are no longer split between several *m/z* peaks. Through deglycosylation of the samples the limit of accurately detecting the impurity was lowered by a factor of ten down to 0.0001 µg/µl.

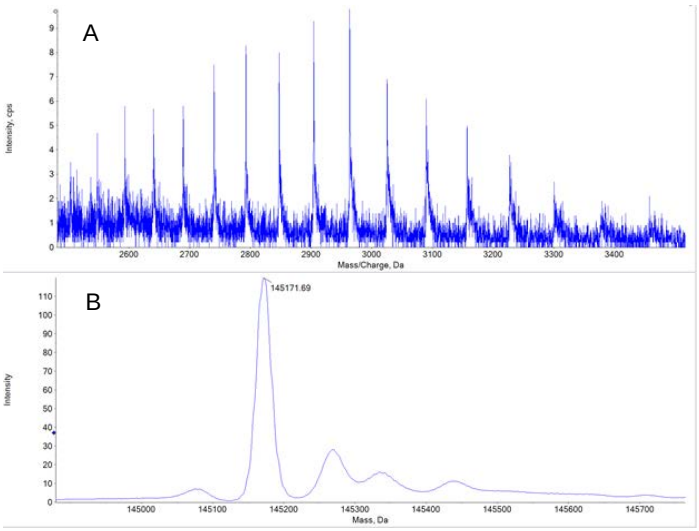
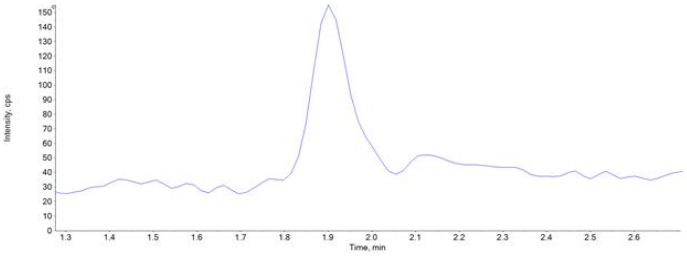
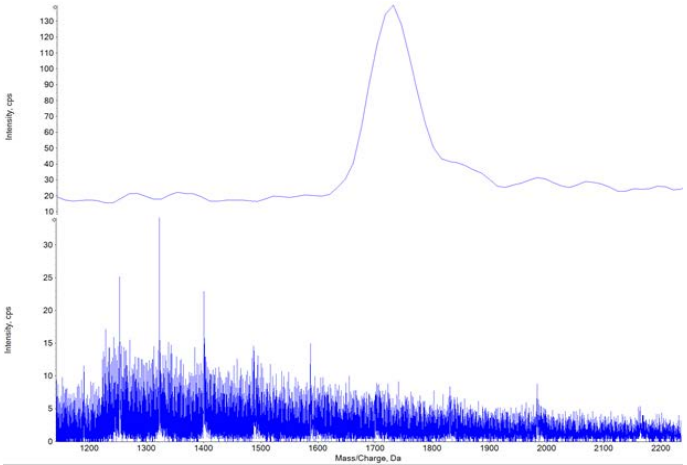


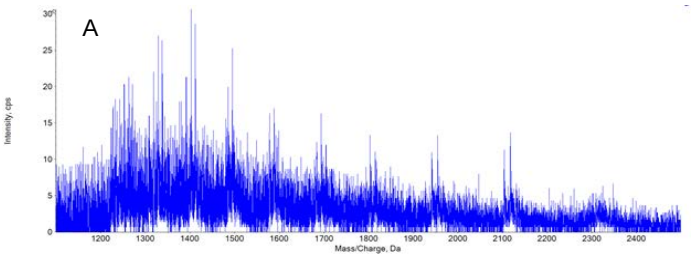
Figure 3. A) Raw data of deglycosylated Trastuzumab impurity at 0.01% concentration to main peak. B) Reconstruction of protein.

DETECTION OF LOWER MOLECULAR WEIGHT INTACT IMPURITIES

Not all impurities are of a similar molecular weight to the target biologic; some may be half constructs (such as heavy or light chains) or co-purified proteins. The mass spectrometry conditions that are optimal for the target may be non-ideal for these molecules leading to fragmentation or poor ionization.







Figur B) Sum extracted ion chromatogram for ten most intense charg es of Fc region impurity at 0.05 % relative concentration to ma... peak. (0.005 µg on column). B) Raw data of Fc region impurity at 0.05 % concentration to main peak. C) Sum extracted ion chromatogram for ten most intense charge states of deglycosylated Fc region impurity at 0.01 % relative concentration to main peak. (0.001 µg on column). D) Raw data of deglycosylated Fc region impurity at 0.01 % concentration to main peak.

Looking at low abundance IdeS forms of NIST as an impurity in the C NIST sample, both the Fc region and the degly...ated forms can be identified with ease, with the deglycosylated Fc region being identified down to 0.01 % of the relative concentration of the intact NIST standard.

CONCLUSION

This work has shown how the X500B can detect intact and semi impuri... in samples down to 0.1 % (0.01 % with degly(D ation) relative to the protein of interest. The workflow was developed to analyze the NIST mAb standard and detect impurities of similar mass without additional optimization.

REFERENCES

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