Fast, Efficient, Disulfide Bond Mapping Using BioPharmaView™ Software

Automated mapping of inter- and intra-chain disulfide bonds

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INTRODUCTION

Accurate disulfide bond mapping is essential for correctly establishing structure-function relationships as well as for monitoring the structural integrity of recombinant monoclonal antibodies (mAbs) throughout their production. Inappropriate disulfide bonds can affect a mAb's stability, potency; aggregation and may also signal errors in the cell culture or purification process. By following a mAb's disulfide patterns over time, manufacturers can quickly detect production problems and then correct them as early as possible.

Correctly assigning disulfide bonds in a mAb can be challenging and time-consuming due to the heterogeneity, large size, and multiple cysteine residues found in these biomolecules. Traditional approaches for disulfide mapping are based on fast liquid chromatography-mass spectrometry analysis; however, these methods can be inefficient and usually involve digestion with multiple enzymes, tedious data processing, and intensive manual inspection of chromatograms for the identification of any possible disulfide linkages.

As the biotherapeutics industry develops and expands, there is an urgent need for software tools that can rapidly facilitate and accelerate the higher-order structural characterization of biopharmaceutical products. To meet these needs, SCIEX has developed BioPharmaView™ Software, a data processing suite that can reduce the complexity of the massive data sets generated during large macromolecule analysis. BioPharmaView Software uses rapid processing tools to accelerate critical characterization assays–such as peptide mapping and disulfide bond identification–by automating peak assignments, simplifying data processing, and streamlining the reporting process.

To identify and sequence peptides, BioPharmaView Software automatically scores b- and y- ion annotations; and then the highest scoring experimental peaks are compared to a list of theoretical masses automatically generated by the software. The peak assignment process is further enhanced by predicting the theoretical fragment ion masses for non-reduced, disulfide-linked

peptides prior to comparison with experimental data. Including other criteria in the ion selection process–such as MS/MS scoring, multiple charge states, and a retention time (RT) filter–can also help reduce the time needed for peptide mapping experiments, enabling manufacturers to meet regulatory requirements more quickly during the production and marketing of a new biotherapeutic product.

In this technical note, we successfully developed an efficient and automated workflow that comprehensively identified every disulfide linkage in the Fab region of a mAb. By using BioPharmaView Software to process accurate mass data sets obtained on a hybrid triple quadrupole time-of-flight (TOF) mass spectrometer, we were able to identify the location of five disulfide linkages in the Fab region of a mAb. The use of the high-speed, TripleTOF® 5600 System further contributed to time-savings during disulfide analysis by permitting accurate mass MS and MS/MS information to be collected simultaneously, providing the high-resolution data necessary for differentiating closely related species and confirming structural assignments, all at the fast chromatographic speeds needed for efficient analysis.

MATERIALS AND METHODS

HPLC Conditions: Samples of a mAb raised against lupine seeds were digested with trypsin under both reducing and non-

reducing conditions. Native and reduced tryptic digests were separated using a Shimadzu UFLCXR system equipped with a Kinetex C18 column (Phenomenex, 2.1 x 100 mm, 3 µm). Solvent A consisted of 2% acetonitrile and 0.1% formic acid, and solvent B consisted of 98% acetonitrile with 0.1% formic acid. Samples were injected and analyzed under high-flow conditions (0.2 mL/min).

Mass Spectrometry: LC-MS/MS analysis of peptides separated under high-flow conditions was completed using a TripleTOF® 5600 System (SCIEX, CONCORD, ON) coupled to a DuoSpray™ Ion Source. A generic information dependent acquisition (IDA) workflow was employed as follows: 1) an MS scan was acquired in high-resolution mode using an accumulation time of 250 ms per spectrum; 2) followed by the acquisition of 20 MS/MS scans of 50 ms each; 3) after peak selection, each previously acquired ion was placed on a dynamic exclusion list for 5s. Rolling collision energy and a collision energy spread of 5 V were used.

Data Processing: Experimental accurate mass data was compared to a list of theoretical peptide masses generated within a pre-defined mass error tolerance for the automatic identification of disulfide bond pairings using BioPharmaView Software. Peptide sequence information and any corresponding post-translational modifications were assigned to each peak during the analysis. Additional criteria in the peptide ion selection process, such as MS/MS scoring, inclusion of multiple charge states and retention time (RT) filtering, were used to ensure the correct assignment of disulfide bonds.

RESULTS

To investigate the pattern of disulfide bonds in the Fab region of a mAb, native and reduced tryptic peptides were analyzed using the TripleTOF 5600 System, a high-speed hybrid instrument that acquired accurate mass MS and MS/MS data simultaneously using an IDA workflow. This high-resolution accurate mass information provides increased depth to the structural characterization process and permits researchers to differentiate more easily between closely related species and to confirm structural details more quickly. BioPharmaView Software was able to assign identity to each disulfide-containing peak using the high-quality, high-resolution structural information acquired through IDA experiments on the TripleTOF System.

The comparative profile of the reduced and non-reduced digest (Figure 1) predominantly mirror each other; however, there are unpaired peaks in the non-reduced and native profiles, which indicate the presence of disulfide-linked peptides and reduced peptides that were prior disulfide partners, respectively (Figure1).

In total, five disulfide pairs in the mAb Fab region were identified: two intra-light-chain pairs (C194-C134 and C23-C88), two intra-

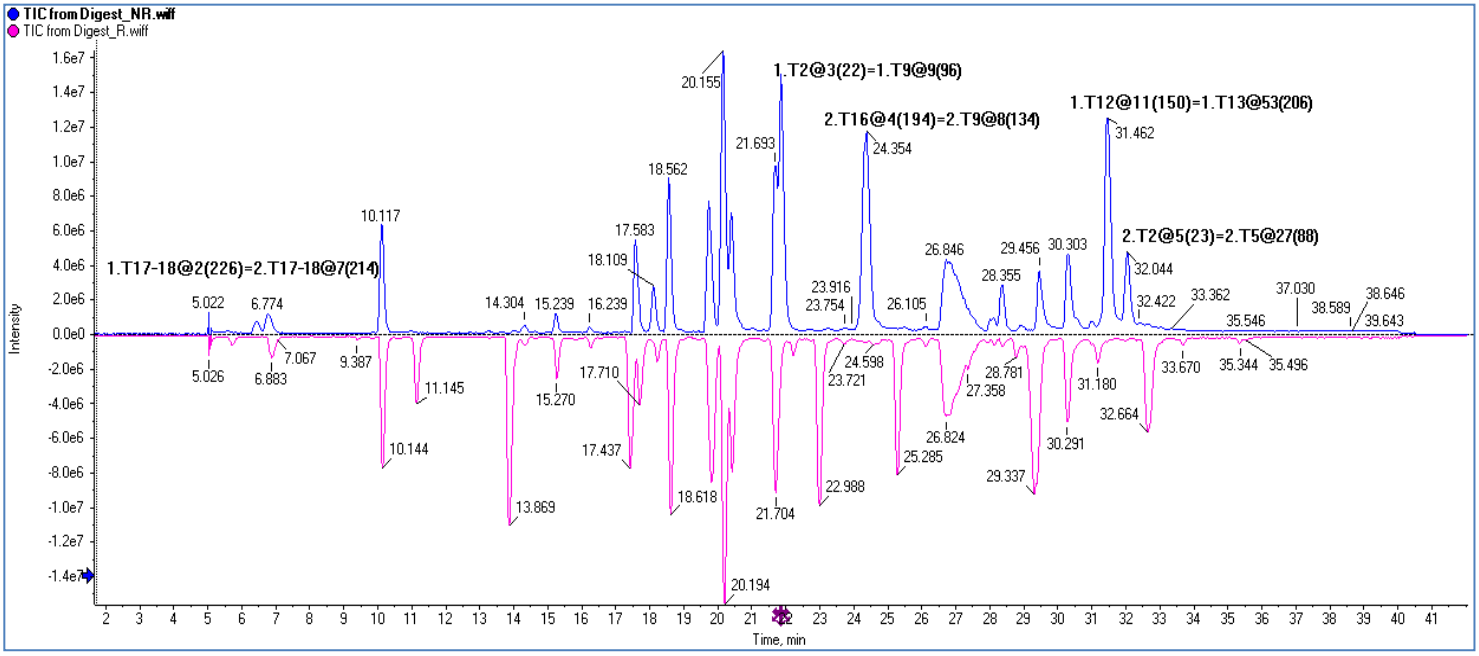

Figure 1. The comparative profiling of tryptic peptides from a mAb under reducing (pink trace) and non-reducing (blue trace) conditions is shown. Non-reduced peaks that do not align with a peak in the reduced trace indicate the presence of disulfide-linked peptides.

heavy-chain pairs (C22-C96 and C150-C206), and one inter-

chain disulfide bond between the heavy and light chain (C226-C214).

Included below are three representative examples of accurate-mass MS spectra and the corresponding MS/MS data that were used to characterize disulfide bond pairs from different regions of the Fab fragment:

- A disulfide bond located within the light chain (C194-C134, Figure 2)
- A disulfide located in the heavy chain (C22-C96, Figure 3)
- A disulfide located between the light and heavy chain (C226-C214, Figure 4)

Figure 2. Automated identification of the disulfide bond C194-C134 in the light chain of the Fab. An extracted ion chromatogram (XIC) trace displays peaks obtained during TOF MS analysis of the tryptic digest (upper panel). BioPharmaView™ Software automatically identified four charge states for the C194-C134 disulfide-containing peptide with a good MS/MS score (middle panel, +3, +4, +5, and +6). A high-resolution TOF MS spectrum of the disulfide-containing peptide is displayed (lower left panel), and the corresponding MS/MS spectrum shows the fragment ions for that peptide (lower right panel).

Figure 3. Automated identification of the disulfide bond C22-C96 in the heavy chain of the Fab. An extracted ion chromatogram (XIC) trace displays peaks obtained during TOF MS analysis of the tryptic digest (upper panel). BioPharmaView™ Software automatically identified three charge states for the C22-C96 disulfide-containing peptide with a good MS/MS score (middle panel, +3, +4 and +5). A high-resolution TOF MS spectrum of the disulfide-containing peptide is displayed (lower left panel), and the corresponding MS/MS spectrum shows the fragment ions for that peptide (lower right panel).

The extracted chromatograms or base peak chromatograms (upper panels, Figures 2-4) highlight the relevant species that were evaluated for disulfide bonding. In the middle panels, peak characteristics–such as RT, theoretical and observed m/z, MS/MS spectra scores, and the predicted charge-state–are catalogued in a table. Because of the high quality of the MS and MS/MS data, multiple charge states can be identified for each peptide, which builds significant

confidence in the disulfide-bond assignments.

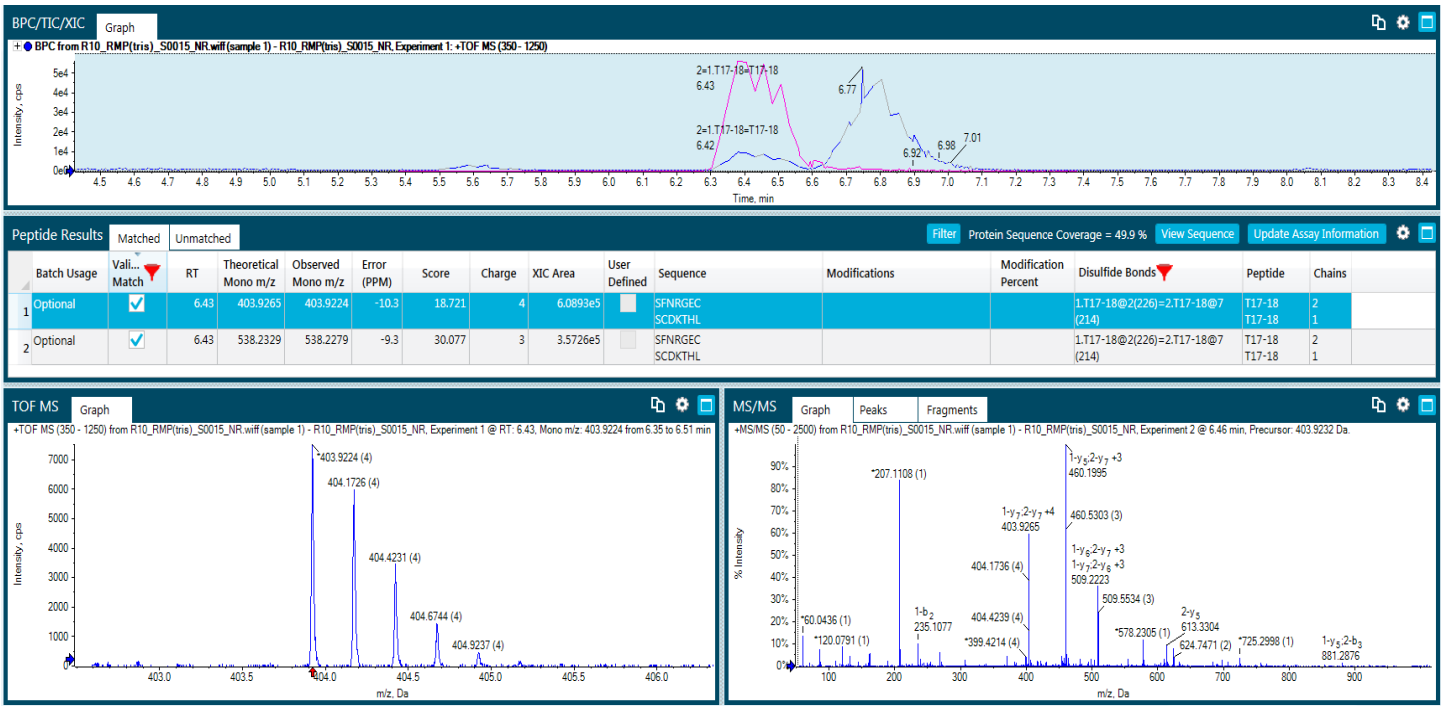

Figure 4. Automated identification of the disulfide bond C226-C214 between the heavy and light chain of the Fab. An extracted ion chromatogram (XIC) trace displays peaks obtained during TOF MS analysis of the tryptic digest (*upper panel*). BioPharmaView™ Software automatically identified two charge states for the C226-C214 disulfide-containing peptide with a good MS/MS score (*middle panel*, +3 and +4). A high-resolution TOF MS spectrum of the disulfide-containing peptide is displayed (*lower left panel*), and the corresponding MS/MS spectrum shows the fragment ions for that peptide (*lower right panel*).

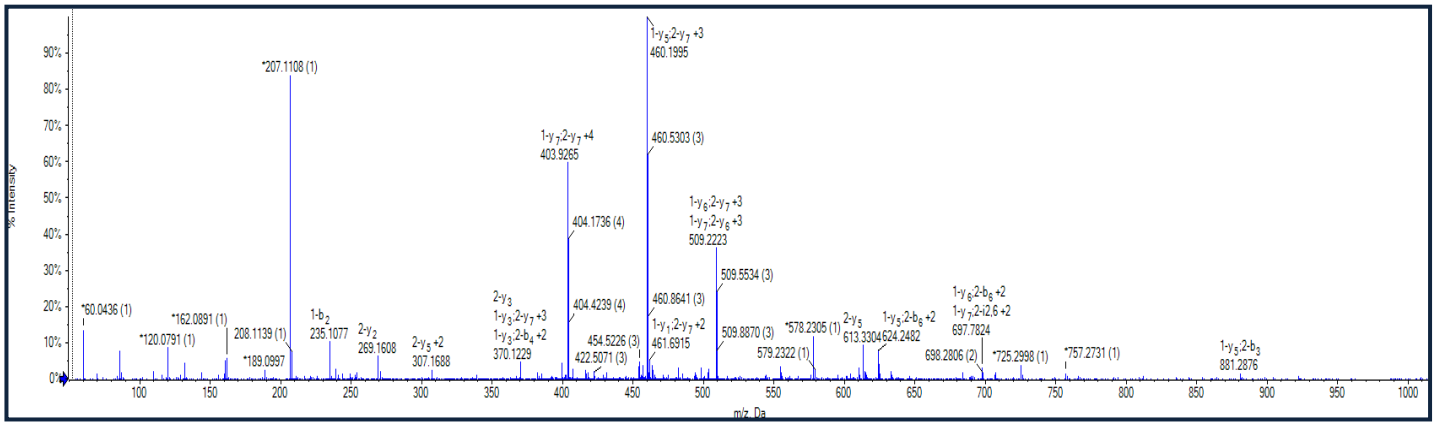

Figure 5: MSMS annotation of Disulfide linkage C226-C214. The b- and y- ions of individual as well as bonded peptide provides the high level of confidence in identification and localization of linkage.

BioPharmaView Software automates the peak assignment based on the MS information uses and MSMS fragment ion information for scoring. The scoring algorithm is based on b & y fragment ions annotation, mass accuracy along with many other parameters. The user has the flexibility to set the threshold value of the score for auto-validation of the data, thereby providing an automatic way for the software to identify the higher quality matches (middle panels, Figures 2-4). The coverage of individual b and y ions with or without the disulfide bond for disulfide linkage C226-C214 is shown in Figure 5 & table 1,

which is usually sufficient for high level of confidence in identification and assignment of the disulfide linkage position.

CONCLUSION

An automated and efficient peptide mapping workflow in BioPharmaView Software was used to successfully locate and identify all disulfide-containing linkages in the Fab region of a mAb. To pinpoint the location of the five disulfide-containing peptides, the profiles for reduced and native tryptic peptides were compared in PeakView® Software, and non-aligning peaks were further sequenced and structurally characterized in BioPharmaView Software. BioPharmaView Software automatically evaluated multiple factors before validating the identity for each peptide–such as b and y ion annotation, MS/MS scoring, multiple charge states, retention time, and mass accuracy. In aggregate, this workflow for disulfide-bond analysis benefits from the combination of high-quality accurate-mass MS and MS/MS data obtained simultaneously on a high-resolution TripleTOF System and the remarkable automation of the peak assignment by BioPharmaView Software, thus simplifying data processing, reporting and therefore reducing the overall processing time.

Fragment	Type	Mono. m/z	Error (Da)	Charge	Mono. Mass	Nomenclature	Fragment
RGEC[*1] / SC[*1]D	b,y	384.13	0.032	2	766.24	1-y4; 2-b3, +2	RGEC[*1] / SC[*1]D
C[*1] / C[*1]DKTHL	2y	418.18	-0.004	2	834.34	1-y1;2-y6, +2	C[*1] / C[*1]DKTHL
C[*1] / SC[*1]DKTHL	y	461.69	-0.004	2	921.37	1-y1;2-y7, +2	C[*1] / SC[*1]DKTHL
SFNRGEC[*1] / SC[*1]DKTHL	y	538.23	-0.003	3	1611.68	1-y7; 2-y7, +3	SFNRGEC[*1] / SC[*1]DKTHL
NRGEC[*1] / SC[*1]DKTH	b,y	624.25	-0.006	2	1246.48	1-y5; 2-b6, +2	NRGEC[*1] / SC[*1]DKTH
SFNRGEC[*1] / C[*1]DKT	b,y	629.25	-0.010	2	1256.49	1-y6; 2-b5, +2	SFNRGEC[*1] / C[*1]DKT
FNRGEC[*1] / SC[*1]DKT	b,y	629.25	-0.010	2	1256.49	1-y7; 2-i2,5, +2	FNRGEC[*1] / SC[*1]DKT
NRGEC[*1] / SC[*1]DKTHL	y	689.80	-0.004	2	1377.58	1-y5; 2-y7, +2	NRGEC[*1] / SC[*1]DKTHL
SFNRGEC[*1] / SC[*1]DKTH	b	741.30	-0.005	2	1480.58	1-y7;2-2-b6	SFNRGEC[*1] / SC[*1]DKTH
C[*1] / C[*1]DKTHL	2y	835.34	-0.006	1	834.34	1-y1;2-y6	C[*1] / C[*1]DKTHL
NRGEC[*1] / SC[*1]D	b,y	881.29	0.003	1	880.28	1-y5;2-b3	NRGEC[*1] / SC[*1]D
C[*1] / SC[*1]DKTHL	y	922.38	-0.005	1	921.37	1-y1;2-y7	C[*1] / SC[*1]DKTHL
SFNRGEC[*1] / SC[*1]	b	1000.36	0.001	1	999.35	1-y6;2-b2	SFNRGEC[*1] / SC[*1]
SFNRGEC[*1] / C[*1]D	b,y	1028.36	0.006	1	1027.35	1-y6;2-b3	SFNRGEC[*1] / C[*1]D
FNRGEC[*1] / SC[*1]D	b,y	1028.36	0.006	1	1027.35	1-y7;2-i2,3	FNRGEC[*1] / SC[*1]D
SFNRGEC[*1] / SC[*1]D	b	1115.39	0.004	1	1114.38	1-y7;2-b3	SFNRGEC[*1] / SC[*1]D
SFNRGEC[*1] / C[*1]DK	b,y	1156.45	-0.022	1	1155.44	1-y6;2-b4	SFNRGEC[*1] / C[*1]DK
FNRGEC[*1] / SC[*1]DK	b,y	1156.45	-0.022	1	1155.44	1-y7;2-i2,4	FNRGEC[*1] / SC[*1]DK

Table 1: The HL Chain C226-C214 bonded peptide fragment ion information is shown. The MS/MS fragment Ion assignment provided by the BioPharmaView Software helps with accurate location of disulfide bonds.

ACKNOWLEDGEMENTS

We would like thank Dr. Anita Krishnan, Dilip K Reddy and Shirish Patel at LUPIN Limited Biotech Division, Pune, India for helpful discussions during the sample analysis and data processing.

Document number: RUO-MKT-02-3213-A

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