SWATH –Data Independent Acquisition Strategies for Ultimate Confidence in Peptide Mapping and Post Translational Modifications Quantitation for Biotherapeutics Characterization

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ABSTRACT

Biotherapeutics is an ever increasing market with its own specialized requirements for analysis. Of these, protein characterization at all levels is a fundamental requirement. This can include intact, subunit analysis and peptide mapping. Peptide mapping allows for identification of slight changes in the sequence or modifications to allow for confidence in the product being produced. Here, we evaluated both data dependent (DDA) and data independent strategies (DIA), in this case, SWATH ® for biotherapeutic peptide mapping. Both DDA and DIA acquisition strategies give comprehensive coverage of the target sample of trastuzumab using MS and MSMS data. In-depth analysis of both DDA and DIA data using BioPharmaView™ software showed identification of low abundant attributes with higher degree of confidence with high MSMS data quality. This ability to identify low abundant attributes is of vital interest to the biotherapeutics market as it allows for identification of slight changes in protein or environment which may have an adverse affect on the safety and efficacy of the final product.

INTRODUCTION

Peptide mapping by mass spectrometry is an integral part of biologics characterization and batch process monitoring. It monitors primary structural characterization and detection of critical quality attributes. Most commonly, data dependent acquisition (DDA) is used to monitor peptide mapping. However this technique sometimes is limited in terms of filtering criteria for the precursor selection, low intensity of precursor ions and peak picking of co-eluting precursors, which in the case of looking for contaminants, degradation or even CQAs, is not useful.

With the technological advancements in instrumentation, it has become possible to use data independent acquisition (DIA) which enables acquisition of MS/MS data of all the possible *m/z* within the selected sample range, thus providing broader dynamic range of detected signals, improved reproducibility for identification, and better overall sensitivity. SWATH® acquisition is a DIA approach that allows you to create a digital record of the sample, which can be used not just at the time to characterize the biotherapeutic, but allows for re-interrogation later for other attributes that weren't being monitored. SWATH acquisition allows for comprehensive peptide mapping with an increase in sensitivity and data quality for low abundant peptides and critical quality attributes. Here we evaluated both DDA and SWATH acquisition with replicate analysis to identify low abundance modifications and their subsequent MSMS data for confirmation using BioPharmaView™ software.

MATERIALS AND METHODS

Sample Preparation:
Trastuzumab was purchased from Myoderm (Norristown, PA, USA). Tris-HCl, iodoacetamide, DTT and TFA were purchased from Sigma (St. Louis, MI, USA), ProteaseMax™ and trypsin were purchased form Promega (Madison WI, USA). Premixed mobile phases were purchased from VWR (Radnor, PA, USA).

50 ug of trastuzumab was diluted into 50 mM Tris pH 7.8 buffer. 2 µl of 1% ProteaseMax™ (Promega, Madison WI, USA) was added to denature the mAb. 1 µl of 0.5 M DTT in 50 mM Tris buffer pH 7.8 was added to the trastuzumab and the mixture was incubated at 56˚ C for 20 min. A total of 2.7 µl of 0.55 M iodoacetamide was added to the cooled mixture and the solution was incubated in the dark for 15 min. A second 1 µl of 1% Protease Max was added to the solution before 2 µl of trypsin at 1 µg/µl was added to a ratio of 1:25. The digest was incubated for 3 hours before acidifying with TFA to a final concentration of 0.5%. Samples were centrifuged to removed any particulate before putting them into autosampler vials for analysis.

HPLC Conditions:
An ExionLC™ system with a Phenomenex Kinetex 1.7 µm C18 100 Å 50 x 2.1 mm column at 40˚ C with a gradient of mobile phase A: water + 0.1 % formic acid and mobile phase B: acetonitrile + 0.1 % formic acid was used at a flow rate of 250 µl/min. A gradient of 5 to 40 % B over 25 min was used. The injection volume was set to 8µl.

MS Conditions:
SCIEX X500B QTOF system with IonDrive™ source and Electrospray Ionization (ESI) probe was used. Data was acquired in both data dependent (DDA) and data independent (DIA) modes in replicates. For DDA analysis, MS scan was acquired in high resolution mode using 150 ms accumulation time followed by 20 MSMS scans of 50 ms each and charge states 1 to 7. For SWATH® MS data independent acquisitions, the same MS scan time was used followed by 28 SWATH at 50 ms.

Data Processing:
Both DDA and DIA data was processed using BioPharmaView™ software using similar processing parameters.. A reference digest was used for comparing all 6 injections. Samples were processed in a batch and both MS and MSMS data were compared to the software to identify differences between the two acquisition techniques.

RESULTS

Trastuzumab was acquired in triplicate using DDA and SWATH keeping the same LC conditions and all the replicates were batch processed using same parameter settings.

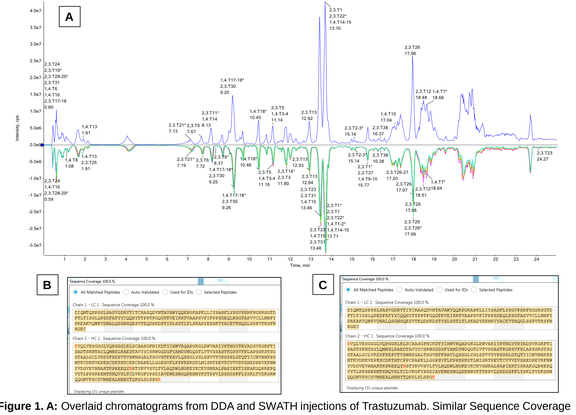

Figure 1. A: Overlaid chromatograms from DDA and SWATH injections of Trastuzumab. Similar Sequence Coverage Obtained with DDA (**B**) and DIA (**C**) approach

All 6 injections using the same chromatographic gradient overlay extremely well with each other, showing robustness of the LC system as well as confirming that the TOF –MS data for the 6 injections results in the same profile. Both the approaches DDA and SWATH showed comprehensive coverage between 99 and 100% of trastuzumab within the defined mass tolerance of 5ppm using BioPharmaView software.

However thorough investigation of the low abundant attributes showed better data quality and sensitivity in SWATH mode of data acquisition. One of the example shown here is the low levels of deamidation found in peptide EEQYNSTYR. This peptide is of particular importance due to the location and heterogeneity of glycosylation at this position. Very low levels of deamidation was identified at this particular site and was confirmed by SWATH in all the three replicates.

The deamidation of EEQYNSTR was not found in any of the DDA acquisition strategies probably due to the fact that the deamidated precursor being very low intense was not picked up for fragmentation under the defined filtering criteria's.

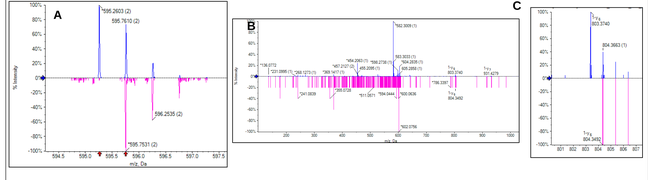

Figure 2. MS (A) and MSMS (B) and (C) y6 -fragment ion from the non-deamidated (blue) and deamidated (pink) forms of EEQYNSTR.

Similarly, investigation of the glycosylation heterogeneity of the peptide EEQYNSTR in both DIA and DDA approaches using BioPharmaVIew™ identified and confirmed the presence of low levels of mannose-5 present in the sample.

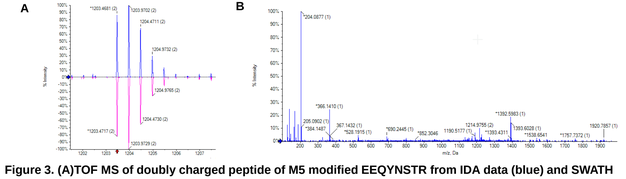

Figure 3. (A)TOF MS of doubly charged peptide of M5 modified EEQYNSTR from IDA data (blue) and SWATH data (pink). (B) TOF MSMS data doubly charges peptide of M5 modified EEQYNSTR SWATH data.

The SWATH-DIA acquisition doesn't require any set fragmentation criteria and provides high quality MSMS data irrespective of the precursor intensity thus resulting in high confidence in the peptide identification and glysosylation profiling.

Another example for very low level of deamidation was identified on another peptide with the sequence FNWYVDGVEVHNAK using BioPharmaView™

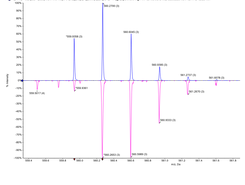

Figure 5. TOF MS of the triply charged peptide with non-deamidated (blue) and deamidated (pink) shown in relative intensities.

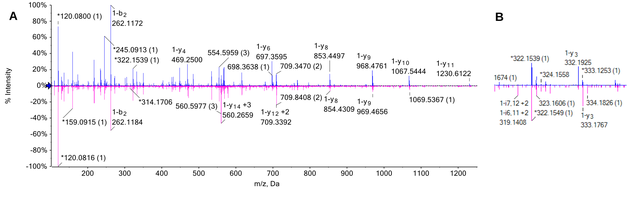

Figure 6. SWATH acquisition (A) for FNWYVDGVEVHNAK peptide showing non-deamidated (blue) and deamidated (pink) MSMS data with expanded spectra (B) showing exact deamidation site.

SWATH acquisition acquired MSMS data for all three replicates. The level of deamidation was extracted out of BioPharmaView™ and the resulting percent modification was calculated, showing that SWATH acquisition was able to acquire MSMS data on a very low modified attribute as well as it's highly abundant counterpart.

Table 1: Calculation of Percent Deamidation from Trastuzumab.

Sample	Non-deamidated XIC area	Deamidated XIC area	% modification
DDA-1	1.50E+07	8.04E+04	0.54
DDA-2	1.51E+07	8.06E+04	0.53
DDA-3	1.50E+07	8.66E+04	0.58
SWATH-1	1.39E+07	8.61E+04	0.62
SWATH-2	1.36E+07	9.23E+04	0.68
SWATH-3	1.25E+07	8.93E+04	0.71

CONCLUSIONS

Both acquisition strategies DDA and SWATH provide comprehensive wealth of information with almost 100% coverage for trastuzumab. However, SWATH-MSMS provided high resolution XICs on fragment ions, with reduced chance of interferences, greater degree of confidence in identification and confirmation of low abundant modifications. This ability to obtain high quality MSMS data on such low abundance attributes is very important for increasing the confidence in assigning the sequence identity as well as identification of potential critical quality attributes within the scope of the analysis.

REFERENCES

1. MS/MSALL with SWATH™ Acquisition – Comprehensive Quantification with Qualitative Confirmation using the TripleTOF® 5600+ System. AB SCIEX Technical note, 3330111-03.

TRADEMARKS/LICENSING

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