

# Time Course Study of Oxidation Stress Using SCIEX Solution for MAM with the X500B QTOF System

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## SCIEX X500B QTOF System



During the development of a biopharmaceutical it is critical to closely monitor critical quality attributes, which may be related to safety and efficacy. Traditionally, arrays of assays were employed to track different attributes. In recent years, the concept of a multiple attribute methodology (MAM) has been introduced which has the potential to expand the use of mass spectrometry for tracking and quantification of attributes. The use of mass spectrometry plays a pivotal role in a MAM assay. It provides unparalleled insight into many aspects of biotherapeutics which can be difficult to determine using other assays. In short, the concept of MAM consists of characterization, attribute definition and monitoring, quantification, and purity assessment via new component detection. Presented here is the use of SCIEX X500B QTOF System to monitor each potential oxidation site on an antibody. Presented here is the application of the MAM workflow for liability assessment using SCIEX OS Software 1.5, including the definition of attributes, defining custom calculations and setting Pass/Fail criteria for each attribute.

## Key Feature of X500B QTOF and SCIEX OS 1.5

- High resolution mass spectrometer for a wide range of biopharmaceutical applications
- Compact benchtop footprint reduces laboratory space requirements
- Easy to use hardware and software accessible for a wide range of users
- A compliant MAM solution for attribute monitoring

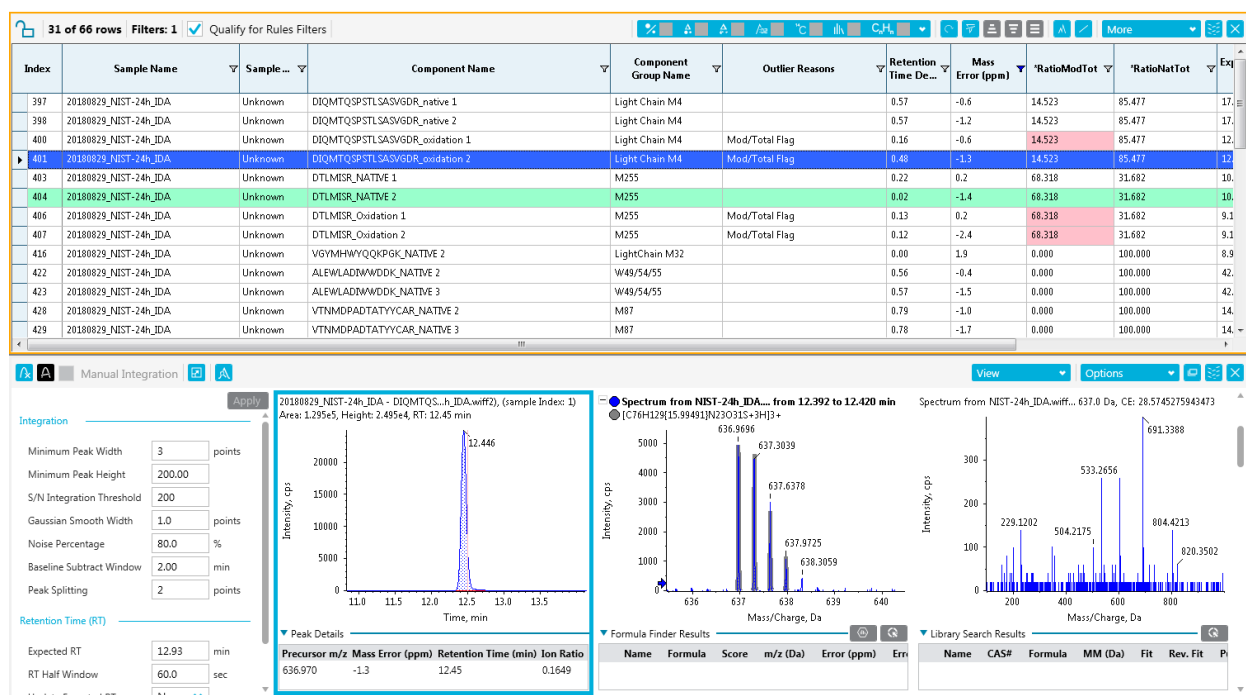


Figure 1. Real time monitor of oxidation profile for Met-4 on light chain in 24h stressed NISTmAb digest by XIC, MS and MSMS

**Table 1. Chromatographic Conditions**

Parameter	Value
Stationary phase	Agilent ZORBAX 300 SB-C18 column 1.8 $\mu$ m, 2.1mm X 150 mm
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile
Flow rate	0.3 mL/min
Column temperature	50 °C
Injection volume	3 $\mu$ L

**Table 3. Mass Spectrometry Conditions**

Parameter	Value	Parameter	Value
Curtain gas:	45	Time bins to sum:	4
Ion source gas 1:	35	TOF start mass (Da):	300
Ion source gas 2:	35	TOF stop mass (Da):	1800
Temperature(°C):	250	Accumulation time:	0.25 sec
Ionspray voltage:	5200	CAD gas:	7
Scan type:	IDA MS	Declustering potential (V):	20
Polarity:	Positive	Collision energy (V):	4
IDA setting			
Maximum candidates ion	10	Intensity threshold exceeds (counts/s)	100
TOF start mass (Da):	100	TOF stop mass (Da):	1000
Accumulation time (s)	0.05	Declustering potential (V)	50
Declustering potential spread (V):	0	Collision energy spread(V):	5

## Methods

### Sample Preparation:

A total amount of 990  $\mu$ g NISTmAb was incubated with 0.03% H<sub>2</sub>O<sub>2</sub> at room temperature for 24h. An aliquot of 150  $\mu$ g sample was taken out at different time point (0.5h, 2h, 4h, 8h, 21h, 24h). The samples were quenched with an equal volume of 250 mM Methionine, followed by a buffer exchange with 12.5 mM L-histidine (pH 6.0), using Amicon centrifugal filter (Millipore, 10K, R8EA69651). Samples are subsequently stored at 4 °C before reduction or digestion.

**Table 2. Chromatographic Gradient**

Time (min)	Flow Rate (ml/min)	%A	%B
Initial	0.3	99	1
5.0	0.3	99	1
6.0	0.3	90	10
50.0	0.3	65	35
55.0	0.3	40	60
56.0	0.3	10	90
60.0	0.3	10	90
62.0	0.3	99	1
64.0	0.3	99	1
66.0	0.3	10	90
70.0	0.3	10	90
72.0	0.3	99	1
74.0	0.3	99	1
76.0	0.3	10	90
80.0	0.3	10	90
82.0	0.3	99	1
95.0	0.3	99	1

The NISTmAb samples were diluted to 1 mg/mL with the denaturing buffer (7.0 M Guanidine HCl, 100 mM Tris, pH 8.3). The denatured protein was subjected to reduction with DTT at 10 mM DTT at room temperature for 30 minutes, followed by alkylation with iodoacetamide at 20 mM for 20 minutes in the dark at room temperature. The alkylation was quenched with 4  $\mu$ L of 50 mM DTT and desalted using BioSpin-6 column followed by the instruction. The sample was digested with Trypsin/LysC at a ratio of 1:10 (Roche, sequence grade) for overnight at 37 °C. The digestion was subsequently quenched by adding 10% TFA to a final concentration of 1%. Samples were stored at -20 °C before injected into LC-MS analysis.

The sample was analyzed by SCIEX X500B system fitted with a IonDrive™ Turbo V source with TwinSpray coupled with ExionLC™ system. Table 1 and Table 2 describe the liquid chromatography conditions and gradient used. Table 3 describes the mass spectrometry parameters used. The data was processed using SCIEX OS Software 1.5...

[MQ4] Modify Method

Workflow Select or verify the analyte and internal standard names and masses.

Components

Integration

Library Search

Calculated Columns

Flagging Rules

Advanced

Formula Finder

Non-targeted Peaks

Row	IS	Group	Name	Chemical Formula	Adduct/Ch...	Precursor (Q1) Mass (Da)	XIC Width (ppm)	Retention Time Mode	Retention Time (min)	IS Name	Experiment Index
20		LightChain...	VGVMHWYQQKPGK_NATIVE 2	VGVMHWYQQK...	[M+2H] <sup>2+</sup>	911.40094	9.99506	RT value	8.90		1 • TOF MS (300 - 1800)
21		LightChain...	VGVMHWYQQKPGK_NATIVE 3	VGVMHWYQQK...	[M+3H] <sup>3+</sup>	541.26965	12.8088	RT value	9.30		1 • TOF MS (300 - 1800)
22		LightChain...	VGVMHWYQQKPGK_Oxidation 1	VGVMHWYQQK...	[M+4H] <sup>4+</sup>	410.20278	11.99407	RT value	7.89		1 • TOF MS (300 - 1800)
23		LightChain...	VGVMHWYQQKPGK_Oxidation 2	VGVMHWYQQK...	[M+2H] <sup>2+</sup>	919.39829	9.99514	RT value	7.89		1 • TOF MS (300 - 1800)
24		LightChain...	VGVMHWYQQKPGK_Oxidation 3	VGVMHWYQQK...	[M+3H] <sup>3+</sup>	546.60129	12.00144	RT value	7.89		1 • TOF MS (300 - 1800)
25		W49/54/55	ALEWLADWDDK_NATIVE 1	ALEWLADWDD...	[M+H] <sup>+</sup>	1660.80058	12.00024	RT value	42.34		1 • TOF MS (300 - 1800)
26		W49/54/55	ALEWLADWDDK_NATIVE 2	ALEWLADWDD...	[M+2H] <sup>2+</sup>	930.90393	11.99999	RT value	42.09		1 • TOF MS (300 - 1800)
27		W49/54/55	ALEWLADWDDK_NATIVE 3	ALEWLADWDD...	[M+3H] <sup>3+</sup>	554.27171	11.99773	RT value	42.09		1 • TOF MS (300 - 1800)
28		W49/54/55	ALEWLADWDDK_Oxidation 1	ALEWLADWDD...	[M+H] <sup>+</sup>	1676.79549	11.99908	RT value	38.20		1 • TOF MS (300 - 1800)
29		W49/54/55	ALEWLADWDDK_Oxidation 2	ALEWLADWDD...	[M+2H] <sup>2+</sup>	938.90329	12.00379	RT value	38.20		1 • TOF MS (300 - 1800)
30		W49/54/55	ALEWLADWDDK_Oxidation 3	ALEWLADWDD...	[M+3H] <sup>3+</sup>	559.60335	12.00851	RT value	38.20		1 • TOF MS (300 - 1800)
31		M87	VTNMDPADTATYCAR_NATIVE 4	VTNMDPADTAT...	[M+4H] <sup>4+</sup>	462.95273	12.00987	RT value	14.70		1 • TOF MS (300 - 1800)
32		M87	VTNMDPADTATYCAR_NATIVE 2	VTNMDPADTAT...	[M+2H] <sup>2+</sup>	924.89819	12.00132	RT value	14.70		1 • TOF MS (300 - 1800)
33		M87	VTNMDPADTATYCAR_NATIVE 3	VTNMDPADTAT...	[M+3H] <sup>3+</sup>	616.93455	11.99479	RT value	14.69		1 • TOF MS (300 - 1800)
34		M87	VTNMDPADTATYCAR_Oxidation 2	VTNMDPADTAT...	[M+2H] <sup>2+</sup>	932.89565	11.99493	RT value	11.14		1 • TOF MS (300 - 1800)
35		M87	VTNMDPADTATYCAR_Oxidation 3	VTNMDPADTAT...	[M+3H] <sup>3+</sup>	608.59667	11.99493	RT value	11.13		1 • TOF MS (300 - 1800)
36		M87	VTNMDPADTATYCAR_Oxidation 4	VTNMDPADTAT...	[M+4H] <sup>4+</sup>	456.69492	11.99926	RT value	11.13		1 • TOF MS (300 - 1800)
37		M101	DMIFNFYFDWVGQTTVTSSAST...	DMIFNFYFDW...	[M+2H] <sup>2+</sup>	1401.15999	11.9972	RT value	45.49		1 • TOF MS (300 - 1800)
38		M101	DMIFNFYFDWVGQTTVTSSAST...	DMIFNFYFDW...	[M+3H] <sup>3+</sup>	934.44242	11.99646	RT value	46.79		1 • TOF MS (300 - 1800)
39		M101	DMIFNFYFDWVGQTTVTSSAST...	DMIFNFYFDW...	[M+4H] <sup>4+</sup>	701.08363	11.99572	RT value	45.72		1 • TOF MS (300 - 1800)
40		M101	DMIFNFYFDWVGQTTVTSSAST...	DMIFNFYFDW...	[M+2H] <sup>2+</sup>	1409.15745	12.00008	RT value	42.38		1 • TOF MS (300 - 1800)
41		M101	DMIFNFYFDWVGQTTVTSSAST...	DMIFNFYFDW...	[M+3H] <sup>3+</sup>	939.77406	12.00289	RT value	42.38		1 • TOF MS (300 - 1800)
42		M101	DMIFNFYFDWVGQTTVTSSAST...	DMIFNFYFDW...	[M+4H] <sup>4+</sup>	705.08236	11.9986	RT value	42.27		1 • TOF MS (300 - 1800)

Process & Close

Figure 2 Definition of oxidation attribute on NISTmAb

[MQ4] Modify Method

Workflow

Components

Integration

Library Search

Calculated Columns

Flagging Rules

Advanced

Formula Finder

Non-targeted Peaks

Rule name: Mod/Totol Flag

Flag a results column: RatioModTot

Flagging criteria: Range

Step 1: Define the values

Value for all components:  Lower limit  Upper limit

Lower limit: Value (+/- %)

Upper limit: Value (+/- Deviation)

Values per component type:  Automatic Mean (+/- %)

Component	Lower limit	Upper limit
SASVIGR_native 1	95	100
SASVIGR_native 2	95	100
SASVIGR_native 3	95	100
SASVIGR_oxidation 1	0	5
SASVIGR_oxidation 2	0	5
SASVIGR_oxidation 3	0	5
ME 1	95	100
ME 2	95	100
ME 3	95	100
ption 1	0	5
ption 2	0	5
ption 3	0	5
FTLTLCTFSGFILSTAGMVGWBR_NATIVE 3	98	100

Step 2: Apply the values to the following sample types

Unknowns

Only if the sample name contains... Type comma separated text

Standards

Only if the sample name contains... Type comma separated text

QCs

Only if the sample name contains... Type comma separated text

Blanks

Solvent  Blank  Double blank

Treat N/A values as: Error

Process & Close Close Help

Figure 3 Definition of assay Pass/Fail criteria for each oxidation attribute on NISTmAb

## Results and Discussion

Before oxidation liability assessment, the robustness and reliability of MAM assay was validated. A total 7 replicates of NISTmAb tryptic digest were injected and acquired using TOF-MS, IDA and SWATH® acquisition. For data processing, SCIEX OS™ software 1.5 was used to quantify each oxidation. The

calculation of each attribute is performed using three charge states as shown in Figure 2. and Pass/Fail criteria can be individually specified as shown in Figure 3. After data processing, SCIEX OS™ software 1.5 was used to compare the oxidation level on each defined site (Figure 4). The result was consistent across all seven injections, demonstrating robustness and reproducibility of the assay.

Attribute Name	Nist SWATH01	Nist SWATH02	Nist SWATH03	Nist TOF01	Nist TOF02	Nist TOF03	Nist IDA01
<b>Light Chain M4</b>	0.09	0.12	0.10	0.12	0.12	0.13	0.16
<b>M255</b>	1.49	1.62	1.19	1.21	1.25	1.23	1.19
<b>M34</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>LightChain M32</b>	0.15	0.14	0.14	0.18	0.18	0.35	0.21
<b>W49/54/55</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>M87</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>M101</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>W280</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>W316</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>W384</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>M431</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Figure 4. Validation on a MAM assay using seven replicate injections of NISTmAb tryptic digest

NISTmAb was stressed by 0.03% H<sub>2</sub>O<sub>2</sub> at room temperature for 24 hours. 150ug of sample was taken out at different time (0, 0.5h, 2h, 4h, 8h, 21h and 24h) using the protocol defined. Targeted oxidation levels were tracked using SCIEX OS Software 1.5. The results of time course study are presented in Figure 5. As shown, the oxidation level increases for multiple methionine residues in NISTmAb, however tryptophan residues were largely unchanged under the conditions tested. Met-255 and Met 431 which are located in the Fc region, and Met 101 located in complementarity determining region (CDR) 3 were found to be the most susceptible to oxidation under H<sub>2</sub>O<sub>2</sub>-incubation conditions. This finding is expected as they are known to be solvent exposed and located on the outside of protein folded structure. Met 34 on CDR1 region and methionine residues on the light chain of NISTmAb were found to be more

resistant to oxidation. This is attributed to these residues being less accessible to solvent as they are internal to the protein structure. Tryptophan level remains the same (0%) across the incubation time which is consistent with previous literature that tryptophan oxidation is mainly induced by photooxidation<sup>1</sup>.

The underlying data for each oxidation site was reviewed using, SCIEX OS Software 1.5. As an example, shown in Figure 1, SCIEX OS Software 1.5 is able to perform real time monitor of unmodified and oxidized peptides, like methionine-4 located in the light chain T1 peptide. The decrease of unmodified peptide at 17 min and increase of oxidized peptide at 12 min is clearly observed. In addition, selecting peptides of interest in SCIEX OS Software 1.5, from the list of components provides a detailed view of the underlying TIC, MS and MS/MS data. In the same

Attribute Name	0h	0.5h	2h	4h	8h	21h	24h
<b>Light Chain M4</b>	0.44	0.83	3.50	2.83	<b>*5.47</b>	<b>*13.70</b>	<b>*14.52</b>
<b>M255</b>	1.88	<b>*6.91</b>	<b>*17.01</b>	<b>*23.06</b>	<b>*36.85</b>	<b>*66.99</b>	<b>*68.32</b>
<b>M34</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>LightChain M32</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>W49/54/55</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>M87</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>M101</b>	0.00	0.00	0.00	<b>*11.76</b>	<b>*29.85</b>	<b>*61.97</b>	<b>*78.29</b>
<b>W280</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>W316</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>W384</b>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<b>M431</b>	0.00	0.00	0.00	<b>*5.06</b>	<b>*9.22</b>	<b>*33.71</b>	<b>*45.79</b>

Figure 5. Time course study of oxidation stress on NISTmAb at 0, 0.5h, 2h, 4h, 8h, 21h and 24h.

interface, SCIEX OS Software 1.5 provides the capability to real-time optimize integration parameter along data review.

## Conclusions

- SCIEX OS™ software 1.5 is a streamlined and compliant software tool for comprehensive attributes monitoring
- The MAM workflow in SCIEX OS Software 1.5 is generally applicable over a range of applications throughout the biopharmaceutical development process including quality control.
- The X500B QTOF system coupled to the ExionLC System provides highly reproducible data for assessment of protein post translational modifications

## References

1. J.P. McCormick, Thomas Thomason Near-ultraviolet photooxidation of tryptophan. Proof of formation of superoxide ion *J. Am. Chem. Soc.*, **1978**, *100* (1), pp 312–313

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Document number: RUO-MKT-02-9483-A



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