## **Drug Discovery and Development**



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

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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 a 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 BioPharmaView<sup>™</sup> 3.0 software, including the definition of attributes, defining custom calculations and setting Pass/Fail criteria for each attribute.

SCIEX X500B QTOF System



## Key Feature of X500B QTOF and BioPharmaView<sup>™</sup> software 3.0

- 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
- Complete MAM solution including both characterization and attribute monitoring



Figure 1. Comparison of oxidation profile for Met-255 between control and 24h stressed NISTmAb digest by TIC, XIC, MS and MSMS



#### **Table 1. Chromatographic Conditions**

Parameter	Value
Stationary phase	Agilent ZORBAX 300 SB-C18 column1.8 μm, 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 ℃
Injection volume	3 µL

#### **Table 3. Mass Spectrometry Conditions**

Parameter	Value	Parameter	Value
Curtain gas:	45	Time bins to sum:	4
lon source gas 1:	35	TOF start mass (Da):	300
lon source gas 2:	35	TOF stop mass (Da):	1800
Temperature(°C):	250	Accumulation time:	0.25 sec
lonspray 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 NIST sample 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

%A 99	<mark>%В</mark> 1	
99	1	
99	1	
90	10	
65	35	
40	60	
10	90	
10	90	
99	1	
99	1	
10	90	
10	90	
99	1	
99	1	
10	90	
10	90	
99	1	
99	1	
	65   40   10   99   99   10   10   99   99   10   10   99   99   10   10   99   99   10   10   99	60 10   65 35   40 60   10 90   10 90   99 1   99 1   10 90   99 1   99 1   10 90   99 1   99 1   90 90   99 1   99 1   99 1   99 1   99 1   99 1   99 1   99 1   99 1

The NIST 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 iodoacetomide at 20 mM for 20 minutes in the dark at room temperature. The alkylation was quenched with 4 uL of 50 mM DTT, and desalted using BioSpin-6 column followed by the instruction. The sample was then 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 lonDrive<sup>™</sup> Turbo V source with TwinSpray coupled with ExionLC<sup>™</sup> 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 BioPharmaView<sup>™</sup> software 3.0..



Assay Information	Sequence Fei	atures	Intact Prof	tein Pep	tide Mappir	ng Quality At	tributes Batch Parameters							
Add Delete Import •	Export	Name	e Oxidatio	n-Light cha	ain M4		Calculated Value: 13	.58 %	<b>√</b> %		Clear Form	ula 💌 🗆	Insert Function	• Insert Set
Attribute Name	Value											_		
1 Oxidation-Light chain M4	13.58 %	Su	Im (Ox	idized	I T1 li	aht chai	n)/Sum(Total ]	1 light ch	ain)					
2 Oxidation M255	75.00 %		(		••••	g	//	- ng	,					
3 Oxidation M34	8.02 %													
4 Oxidation light chain M32	0.00 %												Edit. Ad	d., Delete.,
5 Oxidation W49/W54/W55	0.80 %		0	ridinad T	× Tot	al T1 E 🗙 🗙								•
6 Oxidation M87	0.16 %		~	401264	^ _	· · · · ·	*							
7 Oxidation M101	45.83 %		Batch Usage	Chaird	Peptide	AA Index	Sequence	Modifications	Mono, Mass	Matched	Mono. m/z	Charge	XOC Area	Resension
8 Oxidation W280	0.01 %	1.1	Onional	14	T1	1,18	DIOMTOSPSTI SASVGDR	Ovidarion@ara	1907 8895	1	054.0520	2	2 181745	12.92
9 Oxidation W316	0.02 %		Orrigonal	1.4	T1	1-18	DIOMTOSPSTI SASVGDB	Oviderice Bala	1907 8895	3	054 0520		8.8535#2	13.02
10 Oxidation W384	0.00 %		Onional	1.4	T1	1-18	DIOMTOSPSTI SASVGDR	Ovidarion Bala	1017.8805		A86.0704	-	2.746845	12.01
11 Oxidation M431	41.99 %	1	Optional	1.4	*1	1.18	CACALITOCRETI FADACOR	Conduction (\$ 111)	1007 8807		414.070.0		1.0215-1	11.00
			Opuura	1.4	14	1.10	DIQMIQ3P31L3H3YOUN	Congregation market	EPV//007/	*	000.2104		1744/07	10.00
		Рер	tide Set C	Juery										d. Delete.
			Use	Column		Value								
		1	~	Sequence		DIQMTQS	PSTLSASVGDR"							
		2	~	Modificati	ons	Oxidation(	04(4)							
		2		Use for Qu	ant	"Use"   Uni	known							
		4	~	Retention	Time	11-20								
		5	~	Charge		2-3								
		1.0												

Figure 2 Definition of oxidation attribute on NISTmAb

Assay I	Information	Sequence Features	Intact Protein	Peptide Mapping	Quality Attributes	Batch Parameters					
▼ Quality Attributes Pass/Fail Criteria											
Apply	Attribute Name	Calculated (Standard)	Reference Value	Relative to Reference	Pass 🗸	Marginal 🔺					
<ul><li>✓</li></ul>	Oxidation-Light chain M	4 0.22 %	0.22 %		< 💙 2.00 %	< 3.00 %					
<	Oxidation M255	1.68 %	1.68 %		< 💙 3.00 %	< 5.00 %					
✓	Oxidation M34	0.00 %	0.00 %		< 💙 2.00 %	< 3.00 %					
<	Oxidation light chain M	32 0.00 %	0.00 %		< 💙 1.00 %	< 2.00 %					
✓	Oxidation W49/W54/W5	5 0.57 %	0.57 %		< 💙 1.00 %	< 2.00 %					
✓	Oxidation M87	0.00 %	0.00 %		< 💙 1.00 %	< 2.00 %					
✓	Oxidation M101	0.52 %	0.52 %		< 💙 3.00 %	< 5.00 %					
✓	Oxidation W280	0.01 %	0.01 %		< 💙 0.50 %	< 1.00 %					
✓	Oxidation W316	0.02 %	0.02 %		< 💙 0.50 %	< 1.00 %					
✓	Oxidation W384	0.00 %	0.00 %		< 💙 0.50 %	< 1.00 %					
✓	Oxidation M431	2.78 %	2.78 %		< 💙 2.00 %	< 5.00 %					

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, BioPharmaView3<sup>™</sup> software 3.0 was used to identify and

quantify each oxidation. For each site, a calculation to determine the oxidation level was defined as shown in Figure 2. and Pass/Fail criteria were specified as shown in Figure 3. After data processing, BiopharmaView<sup>™</sup> software 3.0 generates a result table (Figure 4) showing the oxidation level on each defined oxidation site. The result were found to be consistent across all seven injections, demonstrating robustness and reproducibility of the assay.

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. Defined oxidation levels were tracked using BioPharmaView™ software 3.0. The results of time course study are presented in Figure 5. As shown, the oxidation level increases for nearly all 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 H2O2incubation 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 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, BioPharmaView<sup>™</sup> software 3.0. As an example, shown in Figure 1, the unmodified and oxidized methionine located in the heavy chain T21 peptide is shown. From the mirror plot of TIC, the decrease of unmodified peptide at 10.9 min and increase of oxidized peptide at 9.1 min is clearly observed. In addition, selecting peptides of interest in BioPharmaView<sup>™</sup> software 3.0,

Peptide Mapping Batch Results Samples Quality Attributes Processing Parameters											
Open Batch Results reproducibility study before stress											
View	Quality Attributes	Nist_tryp_2ug_TOFMS	Nist_tryp_2ug_TOFMS	Nist_tryp_2ug_TOFMS	Nist_tryp_2ug_SWATH	Nist_tryp_2ug_SWATH	Nist_tryp_2ug_SWATH	Nist_tryp_2ug_ID	Value or Range for Pass		
1	Oxidation light chain M3	0.20 % 🖋	0.19 % 🖋	0.33 % 🖋	0.14 % 🖋	0.14 % 🖋	0.13 % 🖋	0.23 % 🖋	<1.00 %		
2	Oxidation M101	0.38 % 🖋	0.34 % 🖋	0.50 % 🖋	0.37 % 🖋	0.33 % 🖋	0.33 % 🖋	0.57 % 🖋	<3.00 %		
3	Oxidation M255	1.22 % 🖋	1.30 % 🖋	1.29 % 🖋	1.49 % 🎺	1.65 % ✔	1.12 % 🖋	1.21 % 🖋	<3.00 %		
4	Oxidation M34	0.27 % 🖋	0.28 % 🖋	0.52 % 🖋	0.40 % 🖋	0.21 % 🖋	0.25 % 🖋	0.29 % 🖋	<2.00 %		
5	Oxidation M431	0.48 % 🖋	0.52 % 🖋	0.47 % 🖋	0.33 % 🖋	0.37 % 🖋	0.37 % 🖋	0.52 % 🖋	< 5.00 %		
6	Oxidation M87	0.17 % 🖋	0.16 % 🖋	0.16 % 🖋	0.12 % 🖋	0.13 % 🖋	0.12 % 🖋	0.18 % 🖋	<1.00 %		
7	Oxidation W280	0.02 % 🖋	0.02 % 🖋	0.02 % 🖋	0.00 % 🖋	0.00 % 🖋	0.00 % 🖋	0.07 % 🖋	<0.50 %		
8	Oxidation W316	0.04 % 🖋	0.04 % 🖋	0.03 % 🖋	0.02 % 🖋	0.02 % 🖋	0.02 % 🖋	0.03 % 🖋	<0.50 %		
9	Oxidation W384	0.02 % 🖋	0.02 % 🖋	0.02 % 🖋	0.00 % 🖋	0.00 % 🖋	0.00 % 🎺	0.02 % 🖋	<0.50 %		
10	Oxidation W49/W54/W5	0.04 % 🖋	0.30 % 🖋	0.39 % 🖋	0.02 % 🖋	0.00 % 🖋	0.00 % 🖋	0.29 % 🖋	<1.00 %		
11	Oxidation-Light chain M4	0.11 % 🖋	0.13 % 🖋	0.14 % 🖋	0.10 % 🖋	0.13 % 🖋	0.10 % 🎺	0.15 % 🖋	<2.00 %		

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



Рер	Peptide Mapping Batch Results Samples Quality Attributes Processing Parameters										
Open Batch Results MAM time course study final											
	View	Quality Attributes	NIST-0h_IDA.wiff2	NIST-0.5h_IDA.wiff2	NIST-2h_IDA.wiff2	NIST-4h_IDA.wiff2	NIST-8h_IDA.wiff2	NIST-21h_IDA.wiff2	NIST-24h_IDA.wiff2	Value or Range for Pass	
1		Oxidation-Light chain M4	0.15 % 🎺	0.32 % 🎺	1.21 % 🎺	1.12 % 🎺	2.21 % 📥	5.56 % 🔵	5.66 % 🔵	<2.00 %	
2		Oxidation M255	1.91 % 🖋	7.18 % 🔵	17.22 % 🔵	23.41 % 🔵	37.21 % 🔵	67.20 % 🔵	70.63 % 🔵	<3.00 %	
3		Oxidation M34	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	2.79 % 📥	<2.00 %	
4		Oxidation light chain M3.	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	2.53 % 🔵	<1.00 %	
5		Oxidation W49/W54/W5!	0.04 % 🎺	0.02 % 🖋	0.17 % 🎺	0.01 % 🎺	0.00 % 🎺	0.08 % 🖋	0.01 % 🖋	<1.00 %	
6		Oxidation M87	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	<1.00 %	
7		Oxidation M101	0.52 % 🖋	1.75 % 🎺	5.55 % 🔵	9.01 % 🔵	20.16 % 🔵	47.23 % 🔵	66.89 % 🔵	<3.00 %	
8		Oxidation W280	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	<0.50 %	
9		Oxidation W316	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	0.00 % 🎺	<0.50 %	
10		Oxidation W384	0.00 % 🎺	0.00 % 🎺	0.27 % 🎺	0.00 % 🎺	0.00 % 🎺	0.10 % 🎺	0.00 % 🎺	<0.50 %	
11		Oxidation M431	0.00 % 🎸	0.00 % 🎺	0.00 % 🎺	4.52 % 🎺	15.26 % 🔵	26.77 % 🔵	45.52 % 🔵	<5.00 %	

Figure 5. Time course study of oxidation stress on NISTmAb at 0, 00.5h, 2h, 4h, 8h, 21h and 24h.

from the list of identified components provides a detailed view of the underlying MS and MS/MS data. By selecting multiple components simultaneously, it is possible to display data using mirror plots. As shown in Figure 1, both the MS and MSMS data enable rapid assessment of mass shifts and profiles for each selected component. In addition, the MS/MS spectra are annotated with identified b and y ions to expedite review of peptide mass spectra.

#### Conclusions

- BioPharmaView<sup>™</sup> software 3.0 is a streamlined software tool for comprehensive attributes monitoring
- The MAM workflow in BioPharmaView<sup>™</sup> software 3.0 is generally applicable over a range of applications throughout the biopharmaceutical development process
- The X500B QTOF system coupled to the ExionLC<sup>™</sup> system provides highly reproducible data for assessment of protein post translational modifications

## References

 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-02-8702-A



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