A liability study on oxidation using SCIEX OS Software 1.7 for intact MAM

Zoe Zhang Sean McCarthy
SCIEX, USA

During the development of biopharmaceutical therapeutics, it is important to closely identify and monitor product quality attributes (PQAs) from discovery through commercialization because CQAs are tightly related to drug safety and efficacy. Traditionally, tracking different attributes required multiple assays need to be employed. In recent years, the concept of a multiple attribute methodology (MAM) has been introduced which offers one single method capable of monitoring various PQAs simultaneously. This approach increases efficiency and can improve product understanding by replacing or supplementing conventional techniques with mass spectrometry. The use of mass spectrometry plays a pivotal role in a MAM assay, as it provides unparalleled insight into many aspects of a biotherapeutic which can be difficult to determine using other assays.

Subunit analysis is one of the most widely adopted methodologies in biopharmaceutical pipelines for characterization. With the advantage of limited sample preparation steps and short gradient times for data acquisition, the implementation of MAM in subunit analysis has attracted high interest in the biopharmaceutical industry. To enable successful implementation of an intact MAM workflow, SCIEX offers an integrated software solution that can manage all aspects of the workflow, including product quality attribute (PQA) definition, tracking, and quantification.

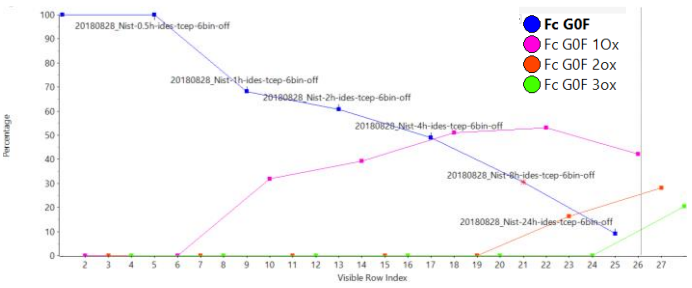


Figure 1. Trending of Fc subunit oxidation over incubation time of 24 hours. Blue: Fc G0F subunit without oxidation. Pink: Fc G0F subunit with one oxidation. Red: Fc G0F subunit with two oxidations. Green: Fc G0F subunit three oxidations.

Presented here is the use of SCIEX X500B QTOF LC-MS/MS System on a liability study to monitor antibody subunit oxidation level as well as the application of the intact MAM workflow for the assessment using SCIEX OS Software 1.7, including the definition of attributes, defining custom calculations and setting Pass/Fail criteria for each attribute.

Key Features of X500B QTOF System and SCIEX OS Software 1.7 for intact MAM analysis

- SCIEX OS Software 1.7 provides a complete and compliant software solution for intact MAM workflows
- SCIEX X500B System is a high resolution mass spectrometer for a wide range of biopharmaceutical applications
- Powerful product attribute definition, tracking, and quantitation
- Flexible custom calculations for attribute-level assessment based on specific user needs
- Easy to use hardware and software accessible for a wide range of users

Methods

Sample Preparation: A total amount of 900 µg NIST sample was incubated with 0.03% H_2O_2 at room temperature for 24 hours. The high sample amount was used to support this study and adjacent studies simultaneously. An aliquot of 150 µg sample was taken out at 0.5, 2, 4, 8, and 24 hours. Aliquots 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). 50 µg of the stressed sample was digested with IdeS at 37°C for 2 hours and further reduced with TCEP.

Chromatography: Separation of the prepared samples was accomplished using an ExionLC™ System fitted with an Agilent PLRP-S column (2.1 x 50 mm, 300Å, 5µm). Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The column temperature was set to 80°C. The gradient is shown in Table 1.

Table 1. Chromatographic Conditions.

Time [min]	Flow Rate [ml/min]	%A	%B
Initial	*0.25*	*85*	*15*
3.0	*0.25*	*85*	*15*
9.0	*0.25*	*10*	*90*
11.4	*0.25*	*10*	*90*
11.5	*0.25*	*85*	*15*
17.0	*0.25*	*85*	*15*

Mass Spectrometry: The samples were analyzed using a SCIEX X500B System fitted with an IonDrive™ Turbo V Source with Twin Sprayer ESI probe. Table 2 describes the mass spectrometry parameters used. The data was processed and analyzed using SCIEX OS Software 1.7.

Row	IS	Gro...	Name	Expected MW (Da)	m/z Range for XIC (Da)	Retention Time (m...	Reconstru... Start Mass...	Reconstru... Stop Mass...	IS Name	Experiment Index
1	☐	LC	LC	23127.20	700 - 2500	5.09	22000.00	25000.00		1 +TOF MS (400 - 30...
2	☐	LC	LC 1ox	23144.00	700 - 2500	5.10	22000.00	25000.00		1 +TOF MS (400 - 30...
3	☐	Fc	Fc G0F	25235.70	700 - 2500	4.88	24000.00	27000.00		1 +TOF MS (400 - 30...
4	☐	Fc	Fc G0F 1Ox	25251.50	700 - 2500	4.90	24000.00	27000.00		1 +TOF MS (400 - 30...
5	☐	Fc	Fc G0F 2ox	25268.00	700 - 2500	4.86	24000.00	27000.00		1 +TOF MS (400 - 30...
6	☐	Fc	Fc G0F 3ox	25284.70	700 - 2500	4.86	24000.00	27000.00		1 +TOF MS (400 - 30...
7	☐	Fab	Fab	25688.40	700 - 2500	5.43	24000.00	27000.00		1 +TOF MS (400 - 30...
8	☐	Fab	Fab 1ox	25704.90	700 - 2500	5.47	24000.00	27000.00		1 +TOF MS (400 - 30...
9	☐									

Figure 2. Definition of oxidation attributes on three NISTmAb subunits.

Table 2. Mass Spectrometry Conditions.

Parameter	Value	Parameter	Value
Curtain gas	*45*	*Time bins to sum*	*6*
Ion source gas 1	*50*	*TOF start mass (Da)*	*400*
Ion source gas 2	*50*	*TOF stop mass (Da)*	*3000*
Temperature(°C)	*400*	*Accumulation time*	*0.5 sec*
Ionspray voltage	*5000*	*CAD gas*	*7*
Scan type	*TOF MS*	*Declustering potential (V)*	*150*
Polarity	*Positive*	*Collision energy (V)*	*10*
Declustering potential spread (V)	*0*		

Results

NISTmAb was stressed by 0.03% H_2O_2 at room temperature for 24 hours. 150 µg of sample was taken out at 0.5, 2, 4, 8, and 24 hours and treated as described in the methods. SCIEX OS Software 1.7 was used to quantify oxidation levels on each subunit, light chain, Fc region and Fab. Oxidation events were defined as different components in SCIEX OS Software 1.7 as shown in Figure 2. The calculation of each attribute is defined and considers the potential for multiple oxidation events (Figure 3). The calculations for targeted attributes can be customized based on the users' needs.

For each defined attribute, pass/fail criteria may be individually specified as shown in Figure 4. After data processing, SCIEX OS Software 1.7 provides a detailed results table to enable rapid review of the oxidation level changes. The results can be sorted by sample, targeted attribute or by the modification event (Figure 5). In addition, a metric plot can be generated to rapidly assess the response of each attribute for each subunit across different time points, which is a great visualization tool to easily identify trends. The underlying data for each subunit was reviewed using SCIEX OS Software 1.7. As an example, the chromatographic TIC, raw spectrum and reconstructed spectrum are displayed in the same interface to ensure correct and accurate identification and integration of each attribute (Figure 5). In addition, integration parameters can be optimized in the same interface to achieve accurate quantitation of each attribute.

Figure 3. Definition of percentage calculations. (a) Peak area for each component individually. (b) Sum all peak areas in each subunit group. (c) Percentage calculation for each component.

Attributes which fall outside of the defined criteria in this oxidative stress liability study are indicated by red and blue highlights (Figure 6). Red highlighting represents values above the criteria while blue highlights those values that fall below the criteria. As shown, the oxidation level increases to 32% on Fc region after only 1 hour incubation, while the overall oxidation on the Fab increased to 25% after 2 hours incubation. The extent of oxidation of the light chain was found to be lower than the other two subunits with only an increase to 18% oxidation at the 24 hour time point. This is likely related to protein structure in solution and is consistent with data seen in peptide map studies under the same conditions.[1]

In this study, only methionine oxidation was considered as other susceptible amino acids, such as tryptophan, are known to be

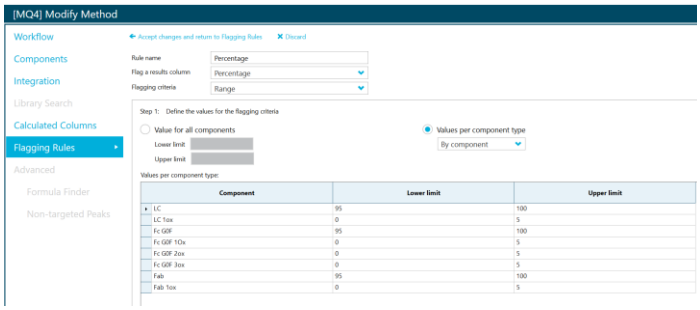


Figure 4. Definition of assay pass/fail criteria for each oxidation attribute on NISTmAb subunits.

oxidized via photooxidation.[2] In the Fc region, there are three methionines in the sequence, Met-255, Met-361 and Met 431. In previous site specific oxidation study, Met-255 and Met 431 showed the most significant oxidation increase after stress because they are located on the surface of the folded structure.[1] Both methionines are likely more susceptible to oxidation under H_2O_2-incubation conditions. The observation in subunit analysis is consistent with peptide level study[1] that the number of oxidation events increases quickly from zero to three and the percentage of the unoxidized form decreases to 9% after the 24 hour incubation period.

The Fab region contains three methionine residues as well, however only Met-101, located in complementarity determining region (CDR) 3, is significantly solvent exposed and highly susceptible to H_2O_2 oxidation. Supporting this, only one oxidation event was observed during the stability study, which matches the previous observation that only Met-101 shows significant increase after the incubation.[1] Methionine residues on the light chain of NISTmAb are internal to the protein structure, therefore, they are less accessible to solvent, which leads to more resistance to oxidation. Thus, only 18% of light chain was oxidized after 24 hour of H_2O_2 stress.

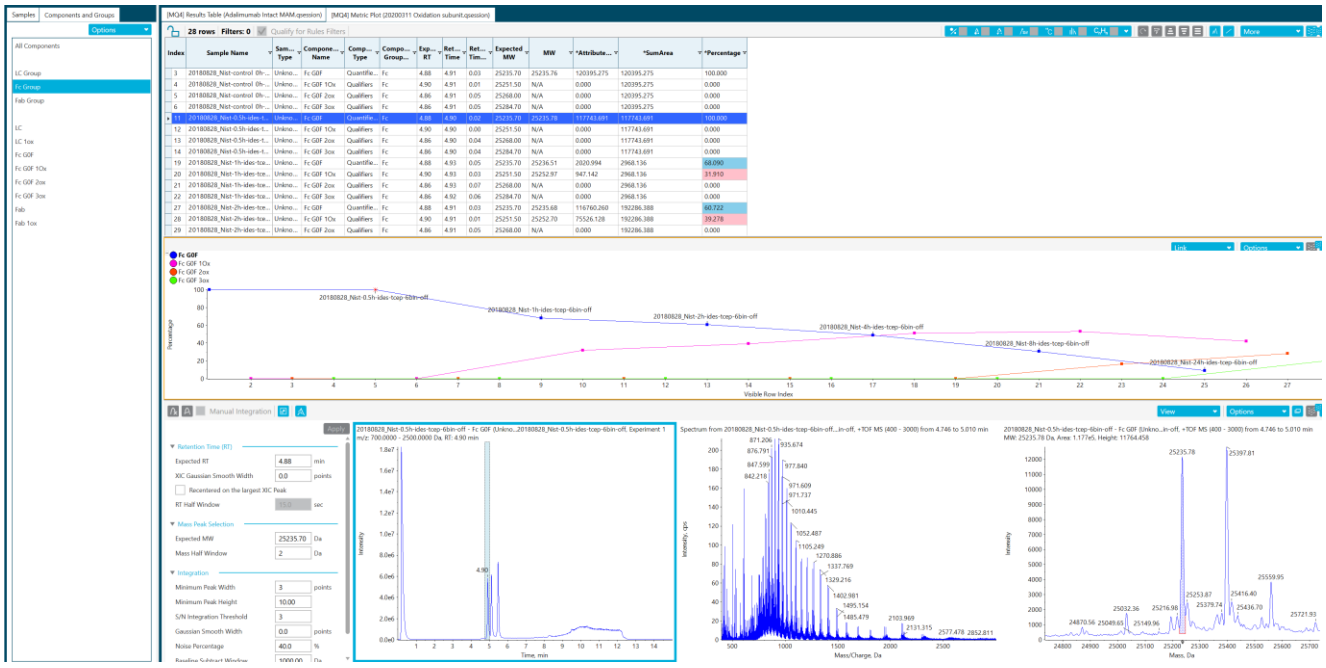
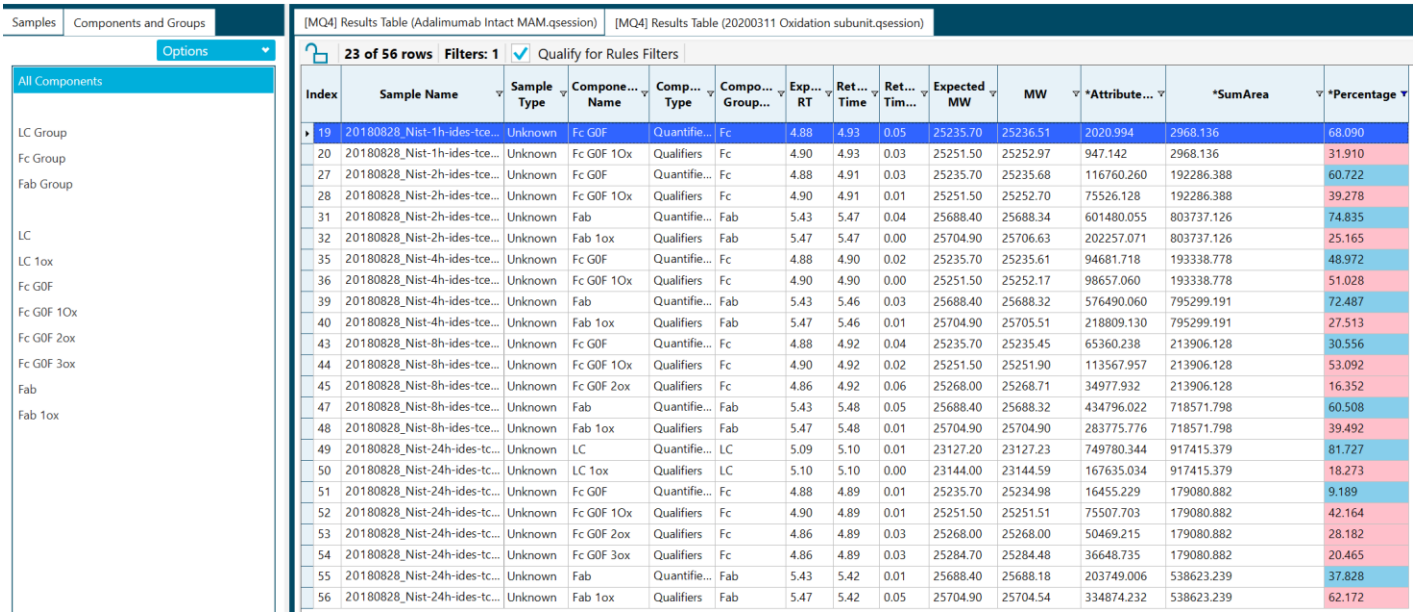


Figure 5. Detailed results of oxidation study for Fc G0F subunit for rapid review. The results are summarized in a table and can be easily sorted (top). Metric plots can be generated to assess the response of each attribute versus different time points (middle). XIC, TOF-MS raw spectra and reconstruction results can also be displayed for full confidence (bottom).



Index	Sample Name	Sample Type	Compone... Name	Comp... Type	Compo... Group...	Exp... RT	Ret... Time	Ret... Tim...	Expected MW	MW	*Attribute...	*SumArea	*Percentage
19	20180828_Nist-1h-ides-tce...	Unknown	Fc G0F	Quantifie...	Fc	4.88	4.93	0.05	25235.70	25236.51	2020.994	2968.136	68.090
20	20180828_Nist-1h-ides-tce...	Unknown	Fc G0F 1Ox	Qualifiers	Fc	4.90	4.93	0.03	25251.50	25252.97	947.142	2968.136	31.910
27	20180828_Nist-2h-ides-tce...	Unknown	Fc G0F	Quantifie...	Fc	4.88	4.91	0.03	25235.70	25235.68	116760.260	192286.388	60.722
28	20180828_Nist-2h-ides-tce...	Unknown	Fc G0F 1Ox	Qualifiers	Fc	4.90	4.91	0.01	25251.50	25252.70	75526.128	192286.388	39.278
31	20180828_Nist-2h-ides-tce...	Unknown	Fab	Quantifie...	Fab	5.43	5.47	0.04	25688.40	25688.34	601480.055	803737.126	74.835
32	20180828_Nist-2h-ides-tce...	Unknown	Fab 1ox	Qualifiers	Fab	5.47	5.47	0.00	25704.90	25706.63	202257.071	803737.126	25.165
35	20180828_Nist-4h-ides-tce...	Unknown	Fc G0F	Quantifie...	Fc	4.88	4.90	0.02	25235.70	25235.61	94681.718	193338.778	48.972
36	20180828_Nist-4h-ides-tce...	Unknown	Fc G0F 1Ox	Qualifiers	Fc	4.90	4.90	0.00	25251.50	25252.17	98657.060	193338.778	51.028
39	20180828_Nist-4h-ides-tce...	Unknown	Fab	Quantifie...	Fab	5.43	5.46	0.03	25688.40	25688.32	576490.060	795299.191	72.487
40	20180828_Nist-4h-ides-tce...	Unknown	Fab 1ox	Qualifiers	Fab	5.47	5.46	0.01	25704.90	25705.51	218809.130	795299.191	27.513
43	20180828_Nist-8h-ides-tce...	Unknown	Fc G0F	Quantifie...	Fc	4.88	4.92	0.04	25235.70	25235.45	65360.238	213906.128	30.556
44	20180828_Nist-8h-ides-tce...	Unknown	Fc G0F 1Ox	Qualifiers	Fc	4.90	4.92	0.02	25251.50	25251.90	113567.957	213906.128	53.092
45	20180828_Nist-8h-ides-tce...	Unknown	Fc G0F 2ox	Qualifiers	Fc	4.86	4.92	0.06	25268.00	25268.71	34977.932	213906.128	16.352
47	20180828_Nist-8h-ides-tce...	Unknown	Fab	Quantifie...	Fab	5.43	5.48	0.05	25688.40	25688.32	434796.022	718571.798	60.508
48	20180828_Nist-8h-ides-tce...	Unknown	Fab 1ox	Qualifiers	Fab	5.47	5.48	0.01	25704.90	25704.90	283775.776	718571.798	39.492
49	20180828_Nist-24h-ides-tc...	Unknown	LC	Quantifie...	LC	5.09	5.10	0.01	23127.20	23127.23	749780.344	917415.379	81.727
50	20180828_Nist-24h-ides-tc...	Unknown	LC 1ox	Qualifiers	LC	5.10	5.10	0.00	23144.00	23144.59	167635.034	917415.379	18.273
51	20180828_Nist-24h-ides-tc...	Unknown	Fc G0F	Quantifie...	Fc	4.88	4.89	0.01	25235.70	25234.98	16455.229	179080.882	9.189
52	20180828_Nist-24h-ides-tc...	Unknown	Fc G0F 1Ox	Qualifiers	Fc	4.90	4.89	0.01	25251.50	25251.51	75507.703	179080.882	42.164
53	20180828_Nist-24h-ides-tc...	Unknown	Fc G0F 2ox	Qualifiers	Fc	4.86	4.89	0.03	25268.00	25268.00	50469.215	179080.882	28.182
54	20180828_Nist-24h-ides-tc...	Unknown	Fc G0F 3ox	Qualifiers	Fc	4.86	4.89	0.03	25284.70	25284.48	36648.735	179080.882	20.465
55	20180828_Nist-24h-ides-tc...	Unknown	Fab	Quantifie...	Fab	5.43	5.42	0.01	25688.40	25688.18	203749.006	538623.239	37.828
56	20180828_Nist-24h-ides-tc...	Unknown	Fab 1ox	Qualifiers	Fab	5.47	5.42	0.05	25704.90	25704.54	334874.232	538623.239	62.172

Figure 6. Time course study for different subunits. Tabular results from the time course study of oxidation stress on NISTmAb subunits at 0, 1h, 2h, 4h, 8h, and 24h. Highlighted columns were outside pass criteria (red: percentage above upper limit; blue: percentage below lower limit).

Conclusions

A oxidative stress stability study using subunit analysis was performed using the intact MAM workflow in SCIEX OS Software 1.7. This software provides a streamlined and compliant solution, from data acquisition to data analysis. With advanced integration, integrated protein reconstruction, and custom calculations and flagging capability, SCIEX OS Software provides a superior MAM solution which can fulfill the needs in both upstream discoveries and downstream development. The launch of SCIEX OS Software 1.7 revolutionizes the current MAM workflow, by offering a solution with less sample manipulation and an integrated software package to realize the intact mass MAM implementation in regulated and non-regulated environments.

References

1. Time Course Study of Oxidation Stress Using SCIEX Solution for MAM with the X500B QTOF System. SCIEX Tech Note RUO-MKT-02-9483-A.
2. McCormick JP, Thomason T (1978) Near-ultraviolet photooxidation of tryptophan. Proof of formation of superoxide ion. J. Am. Chem. Soc., **100 (1)**, pp 312–313.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices