# HIGH THROUGHPUT CYSTEINYLATION SCREENING AT MAB SUBUNIT LEVEL USING LC-MS SCREENING WORKFLOW

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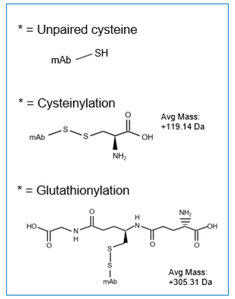
## INTRODUCTION

**Monoclonal antibodies (mAbs) have made up a major part of successful biopharmaceutical drug products over the past decade. mAbs are comprised of two heavy chains (HC) and two light chains (LC) connected via interchain disulfide bridges between cysteine residues. Some mAbs may contain additional cysteine residues Fab region which are unpaired. This may cause instability leading to aggregation, which then translated to a loss of biological activity. These modifications must be monitored and quantified during process development, which means the method of analysis must be quick, easy, and robust. The added challenge to the analysis for cysteine modifications is the inability to use any reducing reagents in the sample preparation, which is very common in typical LC-MS analyses. Reducing the disulfides to subunits aids in the analysis, but it also removes the very modifications which need to be analyzed. To achieve a sensitive, efficient and rapid unpaired cysteine modification screening workflow, we opted for a non-reduced mAb subunit analysis for a biosimilar mAb characterization.**




## METHODS

### Non-Reduced mAb Subunit:

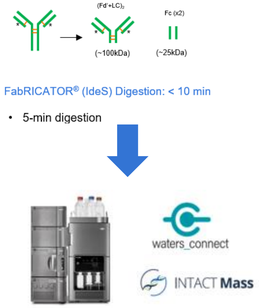
LC-MS System: BioAccord™ LC-MS System with ACQUITY™ Premier System

Column: ACQUITY Premier BEH™ C4 column (1.7 µm, 2.1 x 50mm) (P/N 186010326)

Gradient: 20-80 %B over 3 min (5-min full method)

MPA: 0.1% Formic acid in water; MPB: 0.1% Formic acid in acetonitrile

Data acquired and processed through INTACT Mass App within the waters_connect™ Informatics Platform.




### Non-Reduced Targeted Peptide Mapping:

LC-MS System: Xevo™ G3 QTof Mass Spectrometer with ACQUITY Premier System

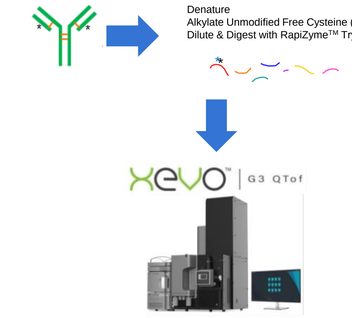
Column: ACQUITY Premier CSH™ C18 300 Å column (1.7 µm, 2.1 x 100mm) (P/N 186009488)

Gradient: Targeted 15-20 %B over 7 min (15-min full method)

MPA: 0.1% Formic acid in water; MPB: 0.1% Formic acid in acetonitrile

MS Method: DDA method that used a precursor ion inclusion list and mass-dependent collision energy ramp for fragmentation

Data acquired and processed through UNIFI™ App within the waters_connect Informatics Platform.




## RESULTS & DISCUSSION

### Non-Reduced mAb Subunit Analysis:

The non-reduced mAb subunit workflow implemented here offers a path forward for rapid screening of unpaired cysteine residues and their potential modifications. Under non-reducing conditions, the FabRICATOR enzyme yields a covalently linked $(Fd'+LC)_2$ species (~100 kDa) and disassociated Fc chains (~25 kDa), as observed in the UV (Figure 1A) and TIC (Figure 1B) chromatograms. The Fc species (Peak 1) contained the N-glycoforms and unprocessed C-terminal lysine which would obscure the cysteinylation and glutathionylation results if intact mAb analysis was performed. With the Fc modification complexity removed, the $(Fd'+LC)_2$ species (Peak 3) then only showed possible glycation, unpaired cysteine modifications, and/or oxidation.

The deconvoluted spectra (Figure 2) are displayed for a subset of the samples—the innovator with "low" level of cysteine modification, plus two biosimilar samples containing a "medium" and "high" level of cysteine modification. The +1 and +2 cysteinylations are noted with red arrows, and the +1 glutathionylation is noted with green arrows (+2 glutathionylation was not detected for any of the samples). The detailed results for the sample set are reported in Table 1. This subunit mAb method provided confident relative quantitation of individual species down to 0.5% based on these results.

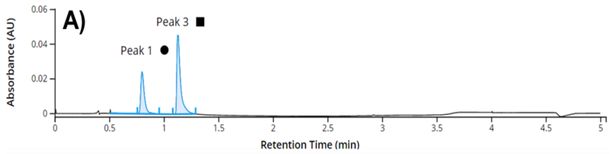



*Figure 1. UV (Panel A) & TIC (Panel B) chromatograms for 250 ng injection of non-reduced FabRICATOR (IdeS) digest of the innovator mAb in a five-minute LC-MS analysis. Peak 1 corresponds to the Fc (~25kDa) species and Peak 3 corresponds to the $(Fd'+LC)_2$ (~100 kDa) species. (The small peak (#2) has a mass corresponding to the FabRICATOR (IdeS) enzyme.)*

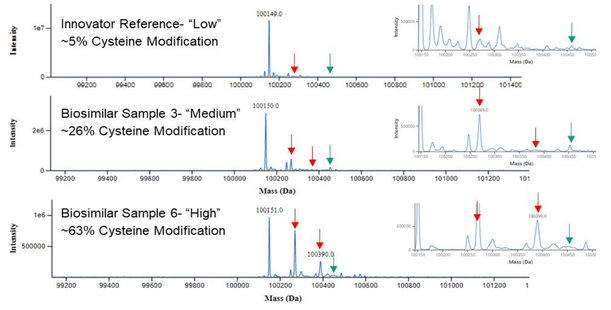



*Figure 2. Deconvoluted spectra for $(Fd'+LC)_2$ species of non-reduced FabRICATOR (IdeS) digestions for the Innovator Reference, Biosimilar Samples 3 and 6, with "low", "medium", and "high" levels of cysteine modification, respectively. Cysteinylation is noted with red arrows and glutathionylation with green arrows.*

| Sample Name | 0x Cysteine Mod (Free Cysteine) (%) | Total Cysteinylation (%) | Total Glutathionylation (%) |
|---|---|---|---|
| Innovator (Ref) | 87.9 | **4.2** | **0.9** |
| Biosimilar Sample 1 | 81.4 | **14.9** | **1.6** |
| Biosimilar Sample 2 | 75.1 | **10.4** | **3.6** |
| Biosimilar Sample 3 | 69.3 | **19.6** | **6.0** |
| Biosimilar Sample 4 | 64.4 | **26.0** | **4.7** |
| Biosimilar Sample 5 | 36.8 | **55.2** | **3.7** |
| Biosimilar Sample 6 | 35.0 | **60.2** | **3.1** |

*Table 1. Summary of unpaired cysteine modification results for Innovator and Biosimilar samples originating from various manufacturing processes. The "Total" cysteinylation and glutathionylation values are a sum of the $(Fd'+LC)_2$ species with one unpaired cysteine modified and both unpaired cysteines modified. The remaining 2 - 11% of $(Fd'+LC)_2$ species deconvoluted MS signal from glycated species.*

### Non-Reduced Targeted Peptide Mapping:

To confirm that the cysteinylation observed in the innovator sample was indeed located on the expected unpaired cysteine in the light chain, non-reduced peptide mapping analysis was performed. The peptide containing the assumed unpaired cysteine also contains a second cysteine residue which is involved in an intrachain disulfide bridge. Thus, the species of interest was two peptides connected via disulfide bridge, labeled "2:T2-2:T7". This peptide species has either the +1 cysteinylation modification or the +1 "IAM" (pre-alkylation via iodoacetamide), meaning that this was the unpaired, unmodified cysteine.

A short 15-min LC-MS method with a focused gradient was developed for a targeted analysis (data not shown). The retention times and m/z for the modified cysteine (m/z 928.9) and unmodified cysteine (m/z 913.4) peptide species were used to create a targeted DDA (data dependent acquisition) with an inclusion list to ensure the detection and fragmentation of only these peptides of interest. The resulting fragmentation data (Figure 3) from the cysteinylated and alkylated peptide species were compared to determine the location of the modification. The presence of y9 + cysteine in the cysteinylated peptide species (and corresponding y9 + IAM alkylation in the unpaired cysteine peptide species) confirms the location as the expected unpaired cysteine (highlighted in yellow).

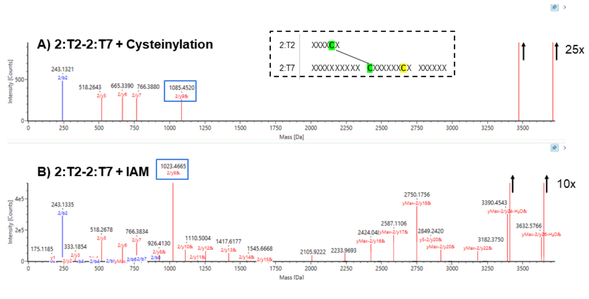



*Figure 3. Fragmentation map of matched fragments from the disulfide linked peptides (2:T2-2:T7) containing the expected unpaired cysteine (yellow). Panel A: cysteinylation modified and Panel B: alkylated form with IAM. The cysteinylation was confirmed to be on the expected unpaired cysteine through the examination of y fragment ladder, beginning at the cysteine corresponding to the y9 fragment and continuing through the y-max fragment.*

## CONCLUSION

- **Rapid screening workflow to measure levels of modification of unpaired cysteines in the Fab region of IgG1 mAbs using fast 5-min FabRICATOR (IdeS) digestion under non-reducing conditions, followed by a 5-min LC-MS method using BioAccord System.**
- **INTACT Mass App for automated deconvolution, mass assignment, and reporting capabilities**
- **Unpaired modified cysteine residue confirmed on the predicted site by a targeted non-reduced peptide mapping of the innovator reference sample.**

**References**

1. 1. Liu H, May K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. mAbs. 2012; 4(1): 17-23.
2. 2. Biosimilar Basics for Patients. FDA website. <https://www.fda.gov/drugs/biosimilars/biosimilar-basics-patients> Accessed 7 Nov 2023.
3. 3. Banks DD, Gadgil HS, Pipes GD, Bondarenko PV, Hobbs V, Scavezze JL, Kim J, Jiang X, Mukku V, Dillon TM. Removal of Cysteinylation from an Unpaired Sulfhydryl in the Variable Region of a Recombinant Monoclonal IgG1 Antibody Improves Homogeneity, Stability, and Biological Activity. J Pharm Sci. 2008; 97:2, 775-790.
4. 4. Ippoliti S, Yu YQ, Ranbaduge N, Chen W. Establishment of a Robust mAb Subunit Product Quality Attribute Monitoring Method Suitable for Development, Process Monitoring, and QC Release. Waters Application Note 720071291en. 2021.
5. 5. Sokolowska I, Mo J, Dong J, Lewis M, Hu P. Subunit mass analysis for monitoring antibody oxidation. mAbs. 2017; 9:3, 498-505.
6. 6. Sokolowska I, Mo J, Pirkolachahi F, McVean C, Meijer L, Switzar L, Balog C, Lewis M, Hu P. Implementation of a High-Resolution Liquid Chromatography-Mass Spectrometry Method in Quality Control Laboratories for Release and Stability Testing of a Commercial Antibody Product. Anal Chem. 2020; 92, 2369-2373.
7. 7. Nägeli A, Ekemohn M, Nyhlén H. Automated Middle-level Analysis of Therapeutic mAbs in Complex Protein Samples. Genovis Application Note. AN0056.
8. 8. Gadgil HS, Bondarenko PV, Pipes GD, Dillon TM, Banks D, Abel J, Kleemann GR, Treuheit MJ. Identification of cysteinylation of a free cysteine in the Fab region of a recombinant monoclonal IgG1 antibody using Lys-C limited proteolysis coupled with LC/MS analysis. Anal Biochem. 2006; 355, 165-174.
9. 9. Ippoliti S, Zampa N, Yu YQ, Lauber MA. Versatile and Rapid Digestion Protocols for Biopharmaceutical Characterization Using RapiZyme Trypsin. Waters Application Note 720007840en. 2023.
10. 10. DeLaney K, Ippoliti S, Reid L, Cornwell O, Yu YQ, Harry E, Towers M. Applying Peptide Mapping and Multi-Attribute Method (MAM) Workflow for Biosimilar mAb Drug Products Comparison on the Xevo G3 QTof Platform. Waters Application Note. 720007632en. 2022.



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