

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



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## OVERVIEW

Modification of free cysteines in monoclonal antibodies (mAbs) is not widely reported, but studies have suggested that free or modified cysteine in the Fab region may lead to changes in the structural integrity of the mAb, which may alter the potency of the drug product. Described here is an optimized five-minute FabRICATOR<sup>®</sup> digestion protocol under non-reducing conditions, followed by five-minute analysis using the BioAccord<sup>™</sup> LC-MS System.

## INTRODUCTION

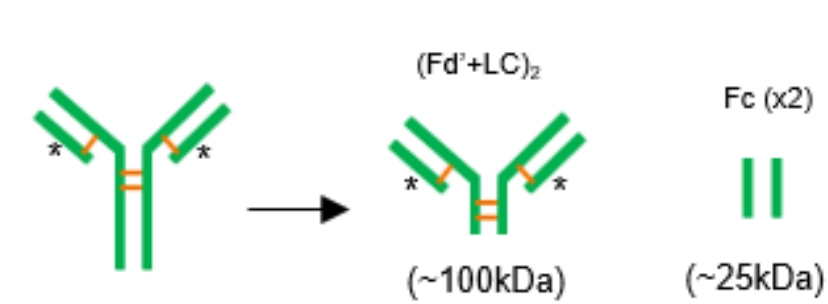
- Monoclonal antibodies (mAbs) have made up a major part of successful biopharmaceutical drug products over the past decade. With many of the older products approaching loss of market exclusivity, many companies are investing in the development of biosimilar mAb products that share the same protein sequences as the innovators but may exhibit differences in modifications due to the manufacturing cell line or process parameter differences.
- mAbs are comprised of two heavy chains (HC) and two light chains (LC) connected via interchain disulfide bridges between cysteine residues.<sup>1</sup> Some mAbs may contain additional cysteine residues in the hypervariable region which are unpaired. This has been shown to cause instability leading to aggregation, which then translated to a loss of biological activity.<sup>2-3</sup> These cysteine modifications must be monitored and quantified, and to support process development, 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, for in reducing the disulfides to aid analysis, they also remove the very modifications which need to be analyzed. To achieve an efficient and rapid unpaired cysteine modification screening workflow, we opted for a non-reduced mAb subunit analysis.

## METHODS

### Sample Preparation

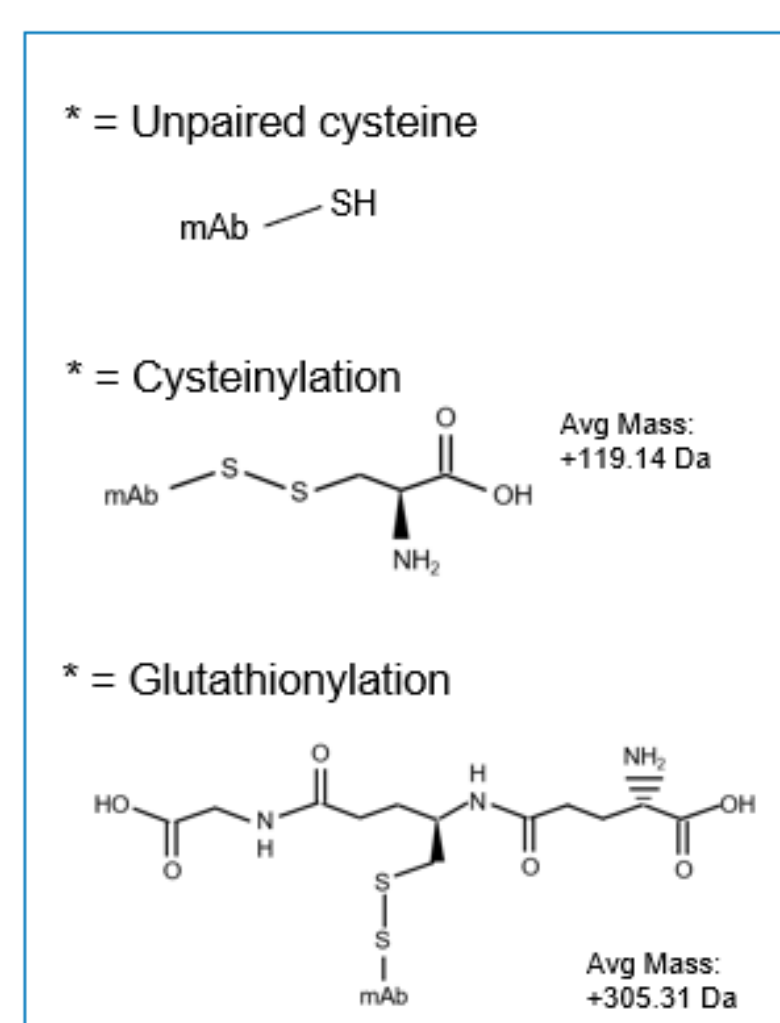
Subunit mAb:

#### Non-Reduced Subunit Sample Prep



FabRICATOR<sup>®</sup> Digestion: < 10 min

- 5-min digestion



Peptide Mapping: Innovator reference sample was denatured and free cysteines alkylated with iodoacetamide before dilution and digestion with RapiZyme Trypsin (P/N 186010108).

### Experimental Conditions

Subunit mAb Analysis: A BioAccord<sup>™</sup> LC-MS System with an ACQUITY<sup>™</sup> Premier UPLC<sup>™</sup> System equipped with a 2.1 x 50 mm ACQUITY Premier BEH<sup>™</sup> C4 (1.7 μm) (P/N 186010326) was used for all subunit analyses (250 ng injections), using an optimized 5-minute LC-MS method. The gradient separation was performed from 20% B to 80% B over 3 min.



Peptide Mapping: An ACQUITY Premier UPLC System coupled to a Xevo<sup>™</sup> G3 Mass Spectrometer was equipped with a 2.1 x 100 mm ACQUITY Premier CSH<sup>™</sup> C18 300 Å (1.7 μm) (P/N 186009488) was used for all peptide mapping experiments, employing a targeted LC-MS/MS method which was abbreviated to focus only on the peptides of interest with a DDA method that used a precursor ion inclusion list and mass-dependent collision energy ramp for fragmentation. The gradient separation was performed from 15 %B to 20 %B over 7 min, following an initial hold at 15 %B for 2.5 min.



## RESULTS

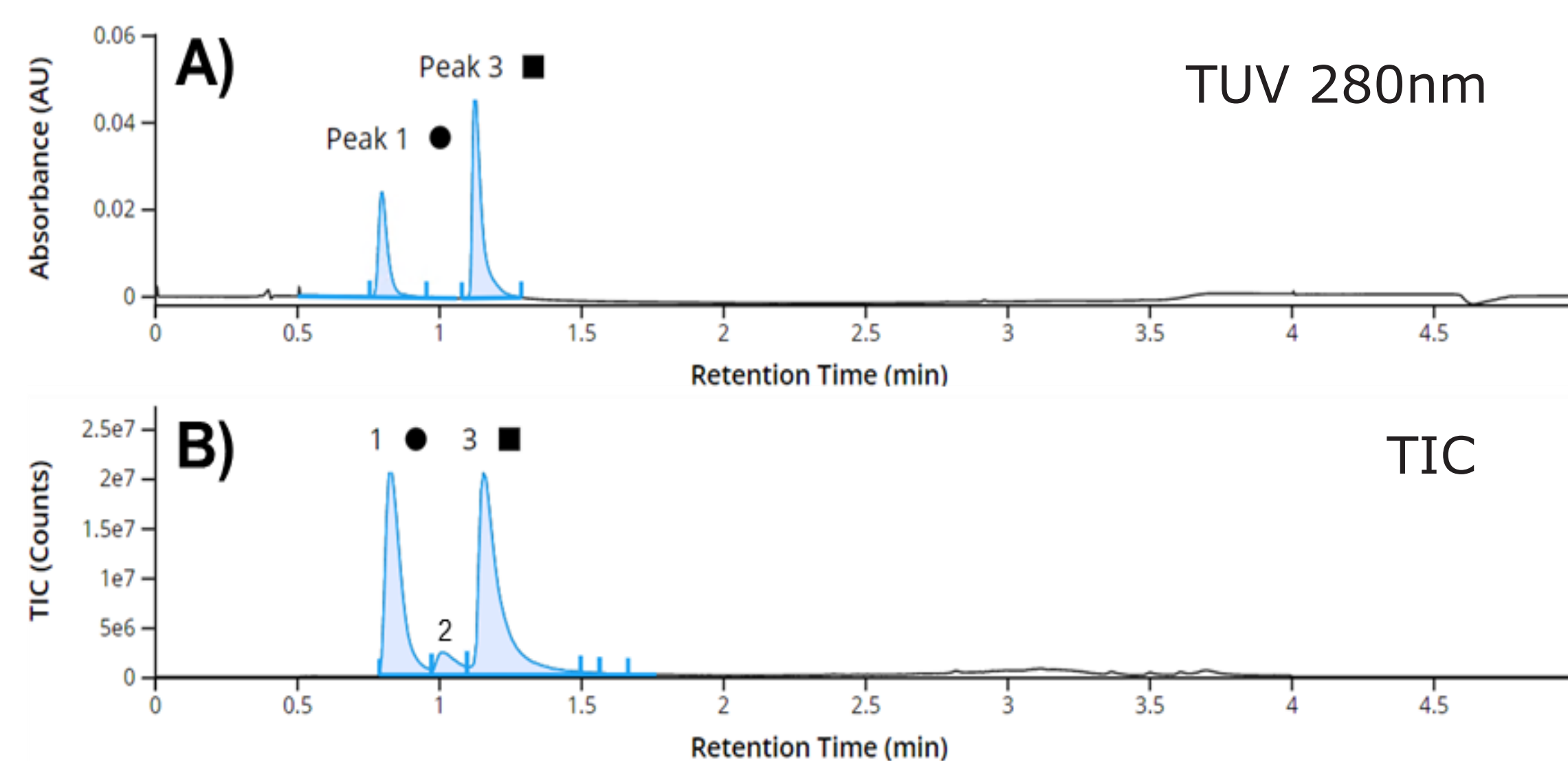


Figure 1. UV (Panel A) & TIC (Panel B) chromatograms for 250 ng injection of non-reduced FabRICATOR digest of the innovator mAb in a five-minute LC-MS analysis. Peak 1 corresponds to the Fc (~25kDa) species and Peak 3 corresponds to the (Fd+LC)<sub>2</sub> (~100 kDa) species. (The small peak (#2) has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

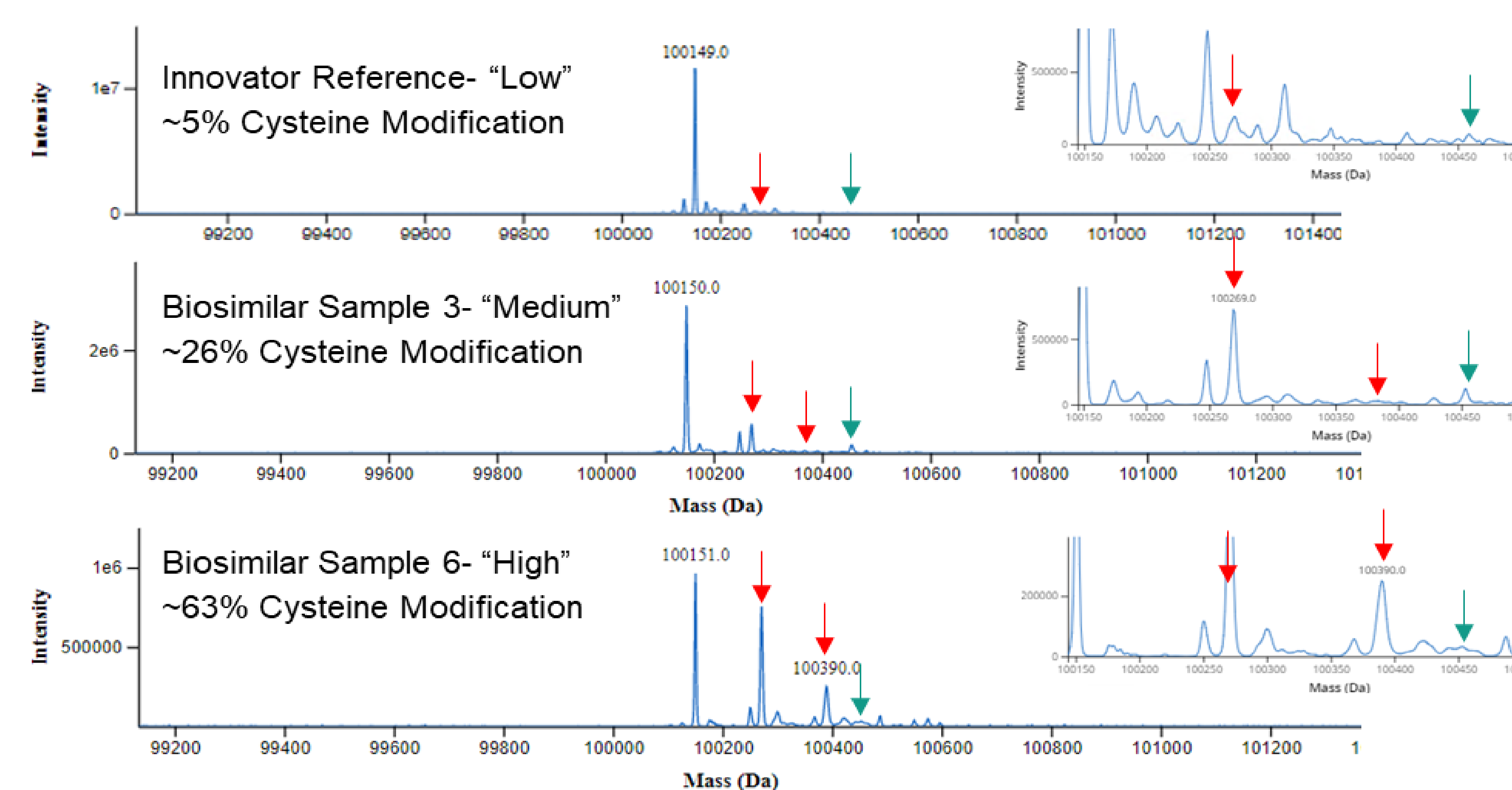


Figure 2. Deconvoluted spectra for (Fd+LC)<sub>2</sub> species of non-reduced FabRICATOR digestions for the Innovator Reference, Biosimilar Sample 3, and Biosimilar Sample 6, with "low", "medium", and "high" levels of cysteine modification, respectively, with a zoomed section showing the modifications. Cysteinylated and glutathionylated peaks are noted with red and green arrows, respectively. Deconvoluted via Auto Deconvolution setting in the Intact Mass App. \*Low level glutathionylation assignment for the innovator is supported by the confident assignment in biosimilar samples with elevated levels.

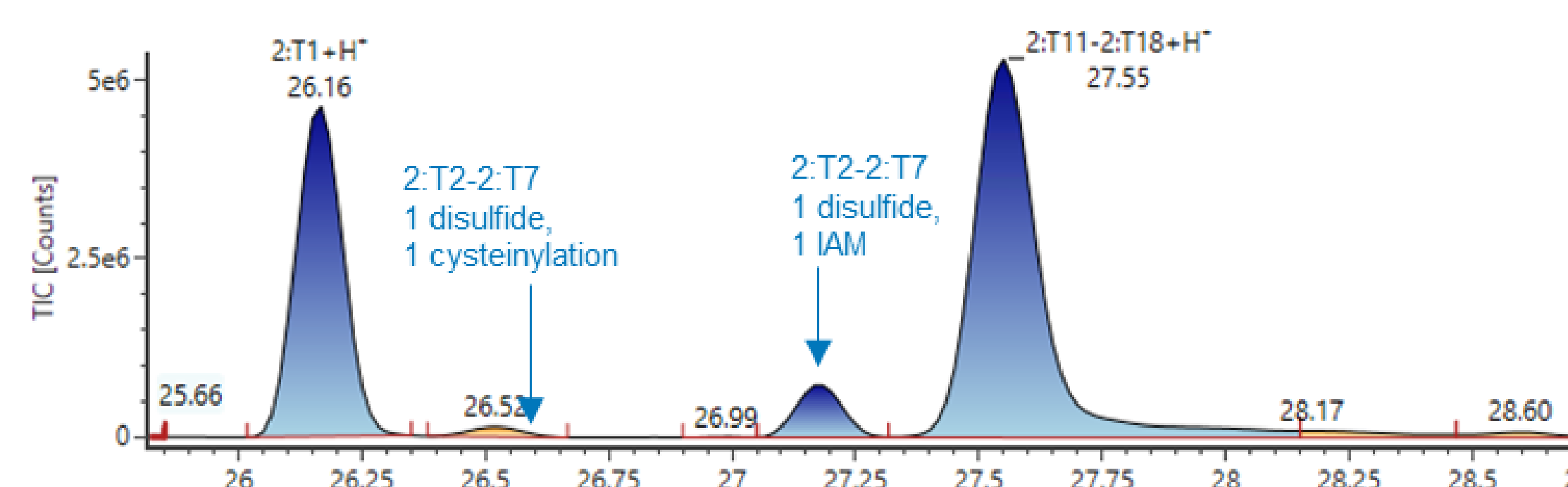


Figure 3. Non-reducing peptide mapping results. This figure displays a zoomed section of the integrated TIC chromatogram where the peptides containing the expected unpaired cysteine elute. The cysteinylated species co-eluted with another peptide in the TIC chromatogram (26.5 min) but is clearly distinguishable in the resulting deconvoluted MS spectra.

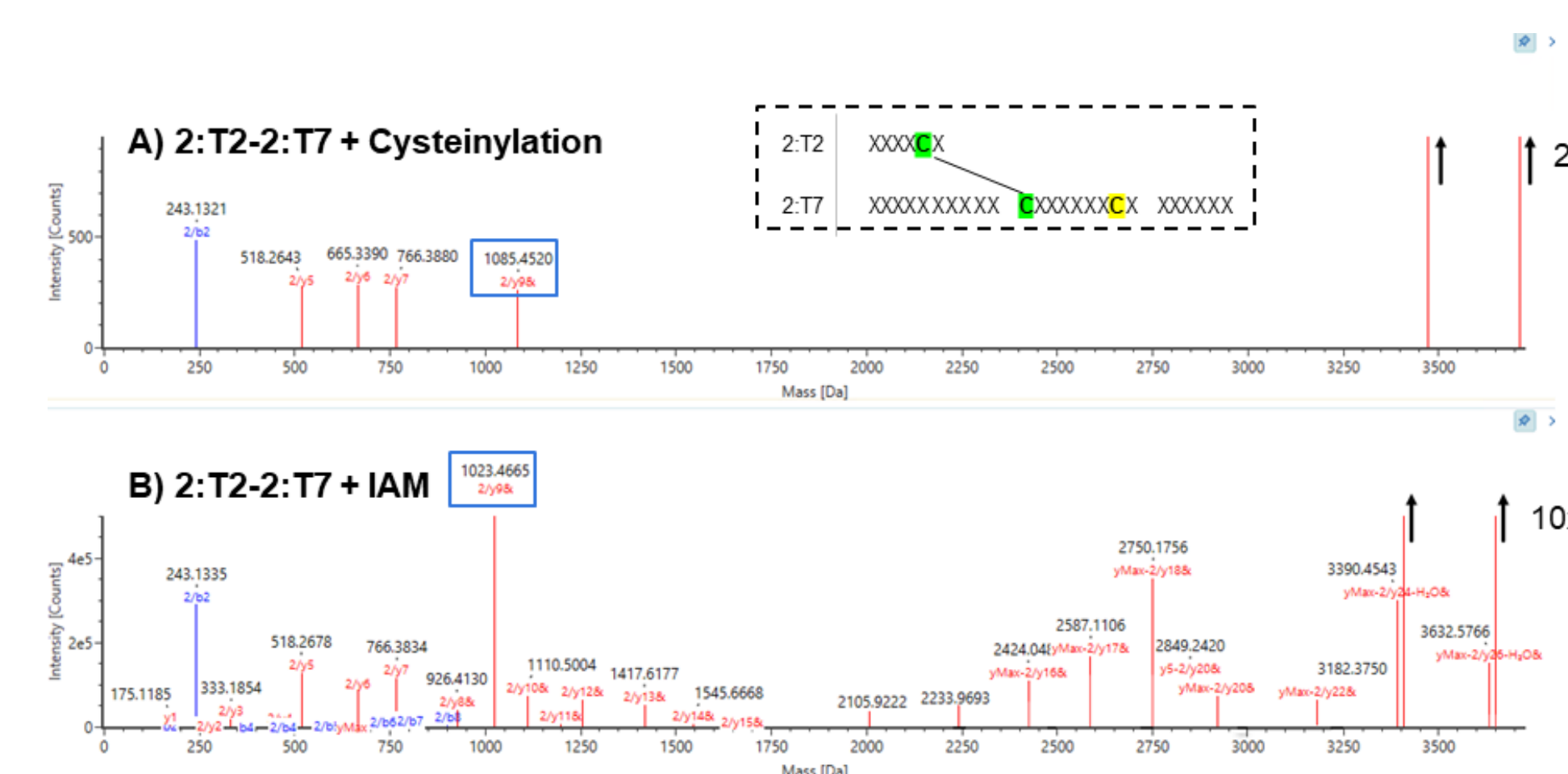


Figure 4. Fragmentation map of matched fragments from the disulfide linked peptides (2:T2-2:T7) containing the expected unpaired cysteine (highlighted in yellow). Panel A: cysteinylated modified and Panel B: alkylated form with IAM. The location of the unpaired cysteine was confirmed to be on the expected unpaired cysteine through the examination of y fragment ladder, with the modification mass difference series beginning at the cysteine corresponding to the y9 fragment and continuing through the y-max fragment.

## CONCLUSIONS

- Successful method optimization of a rapid screening workflow to measure levels of cysteinylated & glutathionylation on unpaired cysteines in the Fd' or LC of IgG1 mAbs.
- The workflow utilized a five-minute FabRICATOR digestion under non-reducing conditions, followed by LC-MS analysis via a BioAccord LC-MS system operated under the compliance-ready waters\_connect Informatics Platform. Utilization of the INTACT Mass App automated deconvolution, mass assignment, and reporting capabilities provided a streamlined workflow for going from sample to report.
- In this case, the study of innovator and biosimilar mAb samples from Similis Bio, a wide range (5 - 63%) of cysteine modification levels was observed using the non-reduced subunit method, with the unpaired modified cysteine residue confirmed on the predicted site by a targeted non-reduced peptide mapping of the innovator reference sample.
- This optimized subunit mAb LC-MS workflow for free and modified cysteine site analysis can be easily adopted and implemented to support product and process development for innovator or biosimilar candidates.

## REFERENCES:

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. McSherry T, McSherry J, Ozaeta P, Longnecker K, Ramsay C, Fishpaugh J, Allen S. Cysteinylated of a monoclonal antibody leads to its inactivation. mAbs. 2016; 8 (4): 718-725.
3. Banks DD, Gadgil HS, Pipes GD, Bondarenko PV, Hobbs V, Scavezze JL, Kim J, Jiang X, Mukku V, Dillon TM. Removal of Cysteinylated 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.

1. FabRICATOR is a registered trademark of Genovis AB. BioAccord, RapiZyme, waters\_connect, ACQUITY, BEH, CSH, Xevo, and RDa are trademarks of Waters Technologies Corporation.