

# Middle-down Approach for Monitoring Monoclonal Antibody Variants and Deglycosylation

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## ABSTRACT

With the recent growth of the biopharmaceutical industry, sensitive and fast methods are required to monitor microheterogeneity and PTMs during all stages of process development to guarantee product safety and efficacy. Therapeutic mAbs, such as rituximab, trastuzumab, infliximab, and bevacizumab, are mostly produced from mammalian cells. These biological products are heterogeneous, containing multiple charge variants and glycosylation forms. Additional modifications such as oxidation can be introduced during the manufacturing process. In the current study, mAbs are broken down into several large fragments using reduction reagent and IdeS enzyme. A fast LC/MS separation method is employed for the following two applications: 1) monitoring mAb fragments containing charge variants and oxidation variants; 2) confirming complete deglycosylation.

## INTRODUCTION

The monoclonal antibody (mAb) therapeutics market is growing at a rapid rate owing to increasing demand for targeted treatments. Therapeutic mAbs are mostly produced from mammalian cells. These biological products are heterogeneous due to post-translational modifications (Figure 1). Additional modifications such as oxidation can be introduced during the manufacturing process. A comprehensive characterization of mAb purity, aggregates, and variants is required for the final biopharmaceutical product approval and subsequent manufacturing processes. There is a growing trend to obtain intact mass information as well as the glycan profile in the QC of mAbs using reverse phase chromatography coupled with high resolution mass spectrometry detection. LC/MS analysis of mAb fragments such as light chain (LC), heavy chain (HC), Fc, Fab, scFc and F(ab)<sub>2</sub>, can accurately reveal the location, as well as nature, of the modification. Moreover, in most QC environments, LC/UV analysis of mAb fragments has been established as a high throughput assay.

The reversed phase column (MABPac RP) used in the fast LC/MS assays is based on wide-pore 4 μm polymer particles that are stable at extreme pH (0–14) and high temperature (up to 110 °C). The wide-pore size of polymeric particles (1,500 Å) enables efficient separation of large protein molecules with low carry-over. This study focuses on two application areas: 1) monitoring scFc fragments containing charge variants and oxidation variants; 2) confirming complete deglycosylation of HC.

## MATERIALS AND METHODS

### Chemicals and reagents

FabRICATOR™ (IdeS) protease was purchased from Genovis (Lund, Sweden). PNGase F was purchased from Prozyme (Hayward, CA).

### Sample preparation

**Reduction:** reduction of inter-chain disulfides in a mAb (4 mg/mL) was achieved by incubation of mAb with 20 mM DTT at 37 °C for 30 min.

**IdeS Digestion:** IdeS protease was added at 1 unit enzyme per 1 μg of mAb ratio. The digestion was carried out in 50 mM sodium phosphate, 150 mM NaCl (pH 6.6) buffer at 37 °C for 30 min.

**Deglycosylation:** glycoprotein solutions were prepared at 10 mg/mL concentration in PBS buffer. Prior to deglycosylation, buffer containing HEPES at pH 7.9, reductant (TCEP) and detergent (proprietary) were added to the glycoprotein solutions. This was followed by heat denaturation at 95 °C for 5 min. PNGase F was added to the mixture followed by incubation at 50 °C for 15 min. Deglycosylated proteins were injected onto Thermo Scientific™ MABPac™ RP analytical columns without further sample cleanup.

**Oxidation:** dilute the mAb solution (5 mg/mL) in half with the 2X oxidation buffer (360 mM sodium chloride, 10 mM sodium acetate, pH 5.0). Then add H<sub>2</sub>O<sub>2</sub> to a final concentration of 0.01% (v/v) and incubate the sample for 24h at room temperature.

### High Performance Liquid Chromatography

Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system was used for mAb fragment separation. The column used was the MABPac RP analytical column (3.0 x 50 mm, P/N 088645; 2.1 x 100 mm, P/N 088647). The following mobile phase A and B are used for LC/UV experiment: H<sub>2</sub>O/TFA (99.9 : 0.1 v/v) and MeCN/ H<sub>2</sub>O/TFA (90 : 9.9 : 0.1 v/v/v). The following mobile phase A and B are used for LC/MS experiment: H<sub>2</sub>O/FA/TFA (99.88 : 0.1 : 0.02 v/v/v) and MeCN/ H<sub>2</sub>O/FA/TFA (90 : 9.88 : 0.1 : 0.02 v/v/v/v).

### Mass Spectrometry conditions

The Thermo Scientific™ Q Exactive™ Plus Orbitrap™ mass spectrometer was used for this study. Intact mAb or mAb fragments were analyzed by ESI-MS. H-ESI II probe was used. The resolution was set at 17.5 k (FWHM) at *m/z* 200, see Table 1.

### Data processing

Full MS spectra of intact mAbs and mAb fragments were analyzed using Thermo Scientific™ Protein Deconvolution™ software 4.0 that utilizes the ReSpect algorithm for molecular mass determination.

Table 1. MS conditions

Instrument Conditions	mAb and mAb Fragments
Mass range	<i>m/z</i> 1,000–4,000
Spray voltage	3.9 kV
Sheath gas	45 arb. units
Auxiliary gas	15 arb. units
Capillary temperature	320 °C
S-lens level	55
In-source CID	40 eV
Microscans	10
AGC target	3 × 10 <sup>6</sup>
Maximum IT	200 ms
Resolving power	17,500
Probe temperature	300 °C

Figure 1. Structure of IgG and typical forms of heterogeneity.

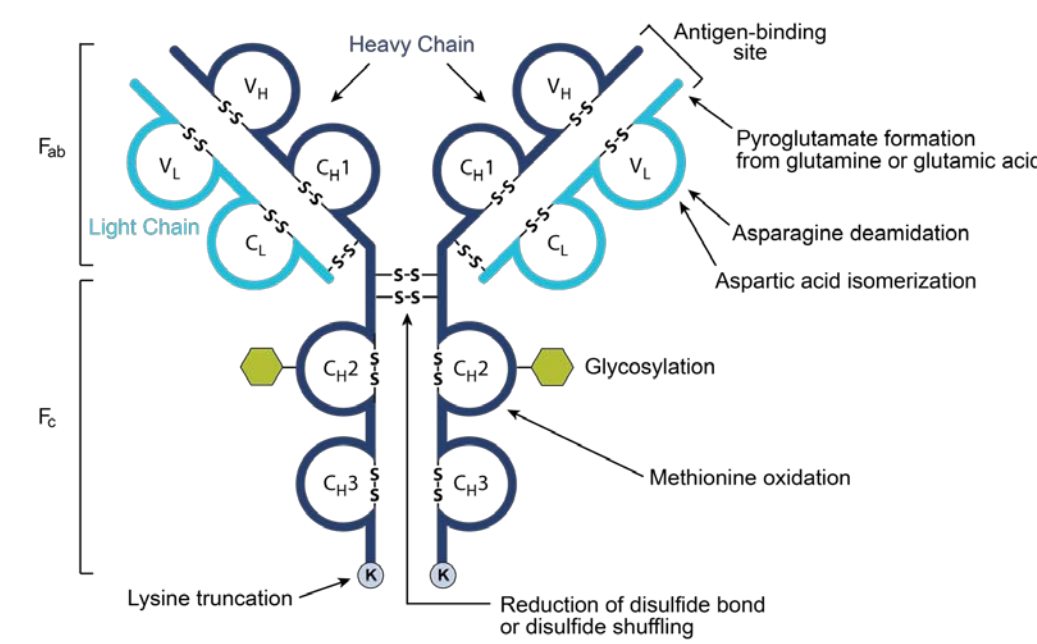
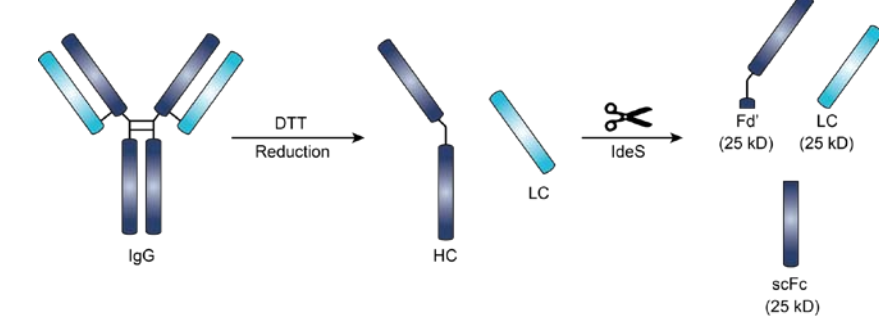


Figure 2. mAb reduction and IdeS digestion flowchart



## RESULTS

### mAb Charge Variant Analysis

A workflow to generate smaller mAb fragments scFc, LC, and Fd' is shown in Figure 2. Each of these fragments has approximate 25 kDa molecular weight. LC and Fd' are single polypeptide chain. scFc contains the N-glycan modification site and therefore more heterogeneous. mAb charge variants often include C-terminal lysine variant. Figure 3 shows the baseline separation of scFc, LC, and Fd' of infliximab on a MABPac RP column. In addition, there is sufficient spatial separation of lysine containing scFc fragment from the unmodified scFc when using mobile phase containing 0.1% TFA ion-pairing reagent. Figure 4 shows a lesser separation of lysine modified scFc and unmodified scFc when using a lower concentration of ion-pairing reagent and a steeper organic gradient. Figure 5 shows the mass spectra of scFc and scFc-Lys at +10 charge state. Deconvoluted spectra show the mass difference between scFc-Lys and scFc is 128 Da, corresponding to the residue mass of a lysine (Figure 6).

Figure 3. LC/UV analysis of mAb fragments containing lysine charge variants

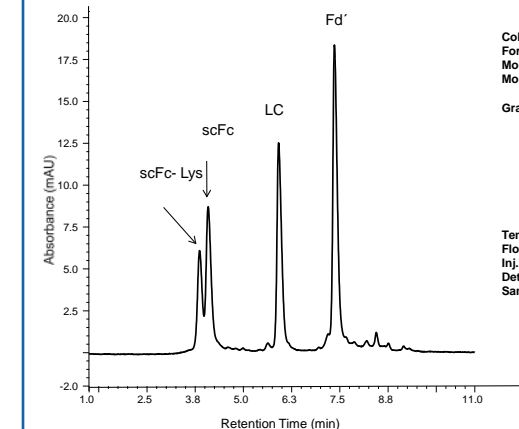


Figure 4. LC/MS analysis of mAb fragments containing lysine charge variants

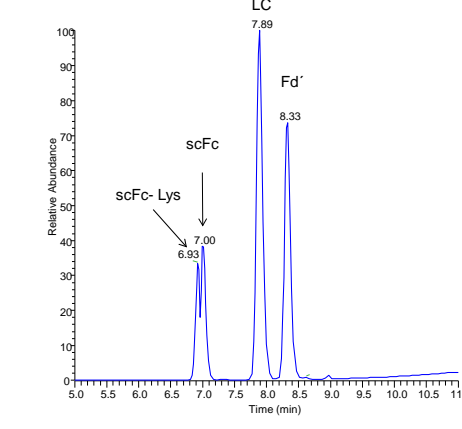


Figure 5. Mass spectra of scFc, LC, and Fd' fragments

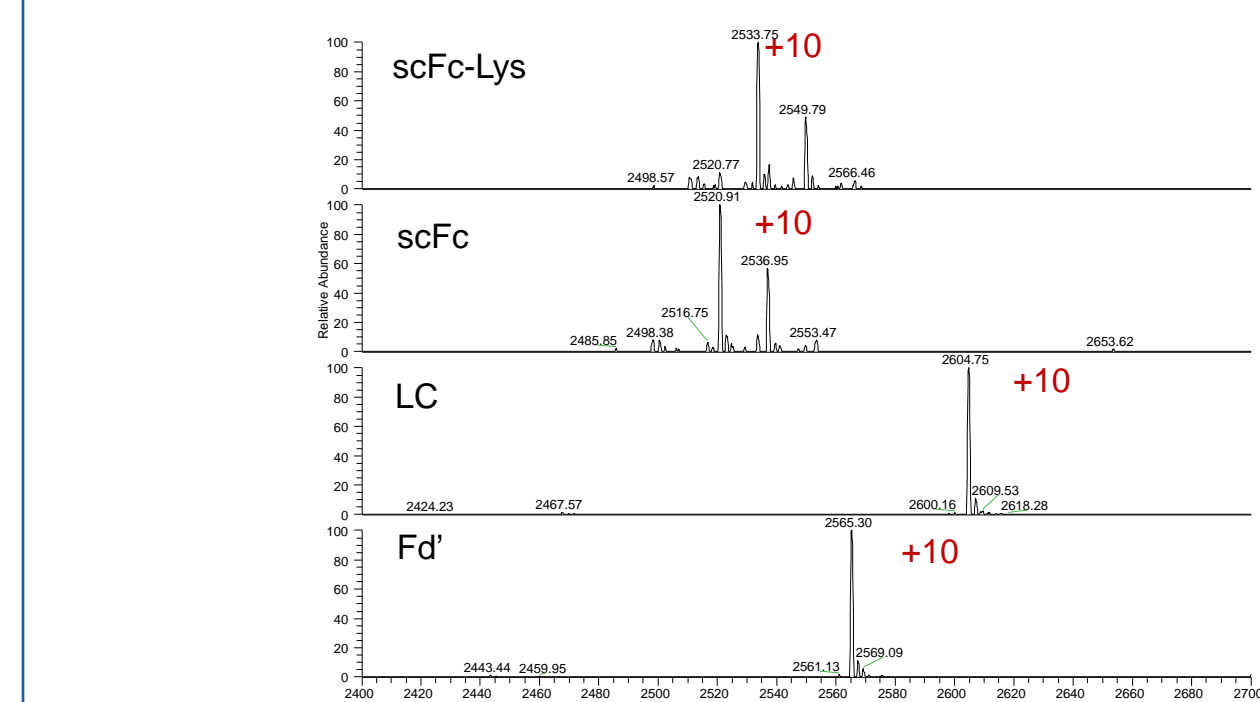
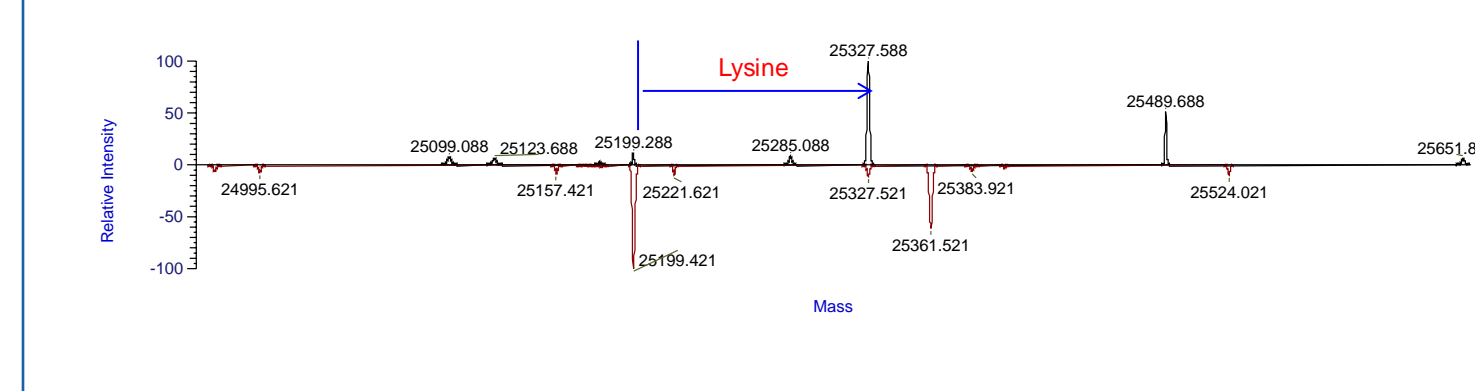


Figure 6. Deconvoluted spectra of infliximab scFc fragments containing lysine charge variants



### mAb Oxidation Variant Analysis

Met oxidation is one of the critical quality attributes required to be closely monitored. The two Met residues in the CH2-CH3 domain interface of recombinant humanized and fully human IgG1 antibodies were found susceptible to oxidation [1]. It is desirable to monitor the progress of the Met oxidation without complete digestion of mAb. Infliximab was treated with H<sub>2</sub>O<sub>2</sub>, resulting in the oxidation of LC and HC. Figure 7 shows the separation of scFc, LC, and Fd' fragments as well as its oxidized forms on a MABPac RP column. Figure 8 shows a lesser separation of oxidized fragments from the unmodified fragments when using a lower concentration of ion-pairing reagent and a steeper organic gradient. Figure 9 shows the mass spectra of oxidized scFc at +10 charge state. Figure 10 shows the mass spectra of oxidized LC and Fd' at +10 charge state. Deconvoluted spectra of scFc and LC show the mass difference between the oxidized form and the unmodified fragment is 16 Da, corresponding to the residue mass of an oxygen (Figure 11 and Figure 12).

Figure 7. LC/UV analysis of mAb fragments containing oxidation variants

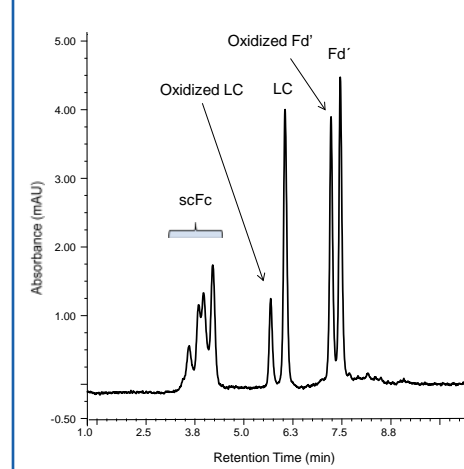


Figure 8. LC/MS analysis of mAb fragments containing oxidation variants

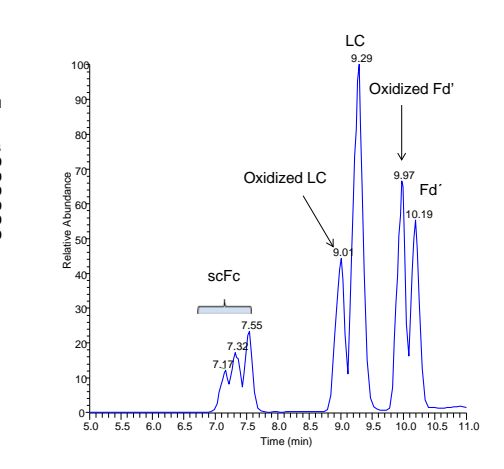


Figure 9. Mass spectra of scFc fragments

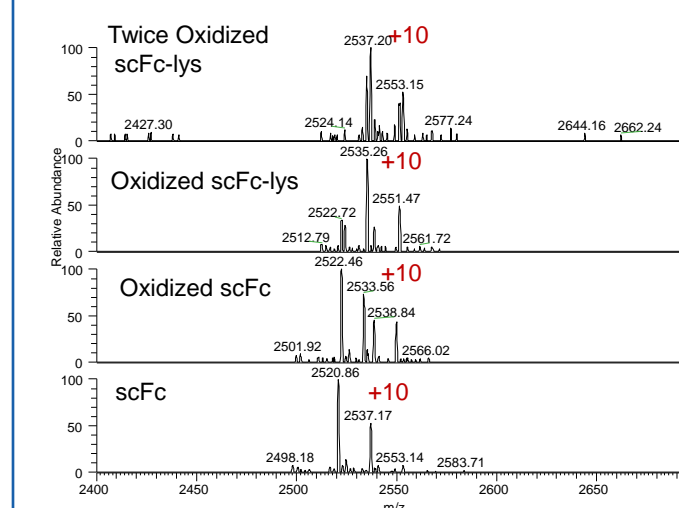


Figure 10. Mass spectra of LC and Fd' fragments

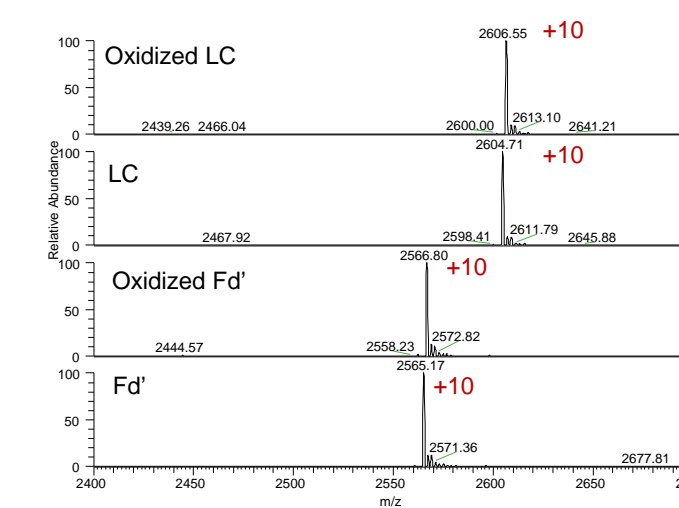


Figure 11. Deconvoluted spectra of infliximab scFc fragments containing a single oxidation site

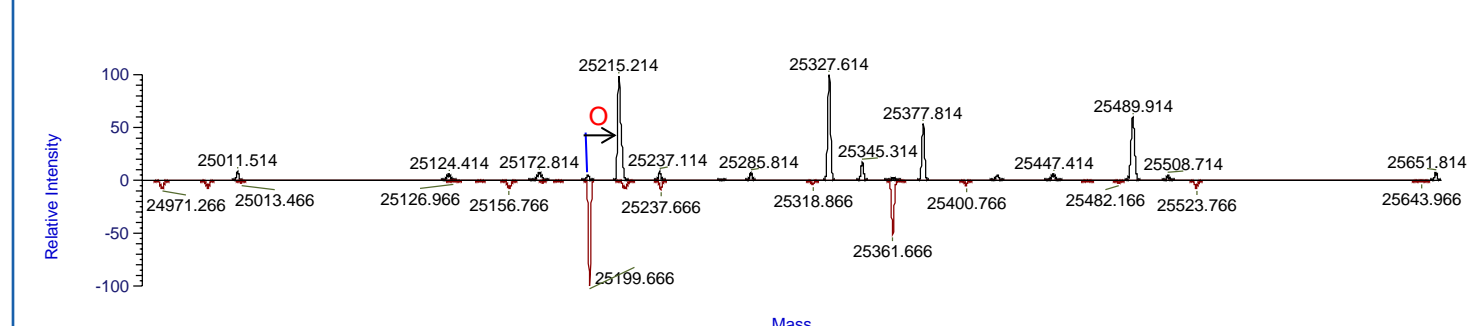
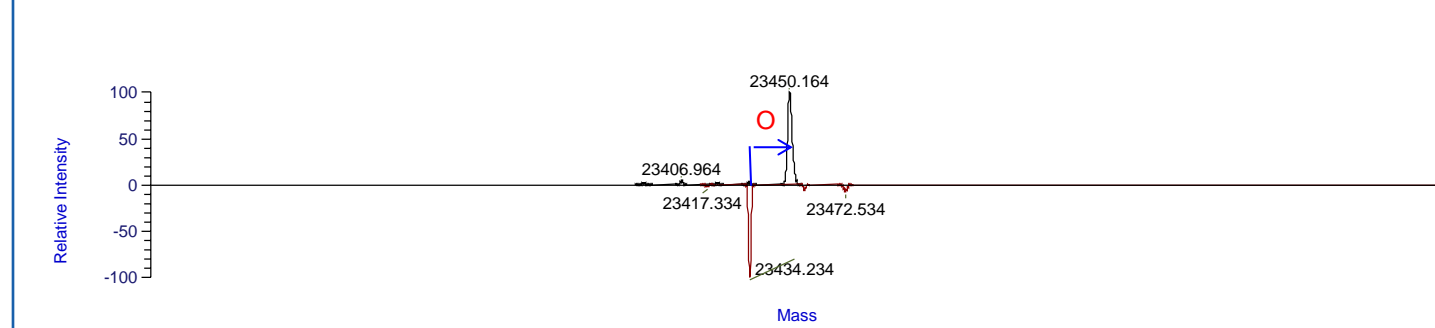


Figure 12. Deconvoluted spectra of LC fragments containing a single oxidation site



### mAb Deglycosylation Analysis

A fast workflow has been developed to remove N-linked glycans in 15 min. Figure 13 shows the separation of LC and HC of trastuzumab before and after deglycosylation. The deglycosylated HC elutes later (retention time at 16.238 min) than the glycosylation HC (retention time at 15.821 min). Figure 14 shows the mass spectra of glycosylated HC and deglycosylated HC. Deconvoluted spectrum of deglycosylated HC shows a single polypeptide of 48,157 Da (Figure 15). The deconvoluted result agrees well with the calculated MW based on the sequence of trastuzumab HC.

Figure 13. LC/UV analysis of trastuzumab LC and HC fragments before and after deglycosylation

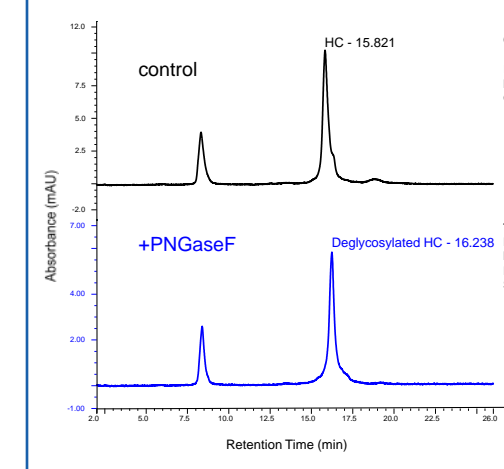


Figure 14. Mass spectra of trastuzumab HC before and after deglycosylation

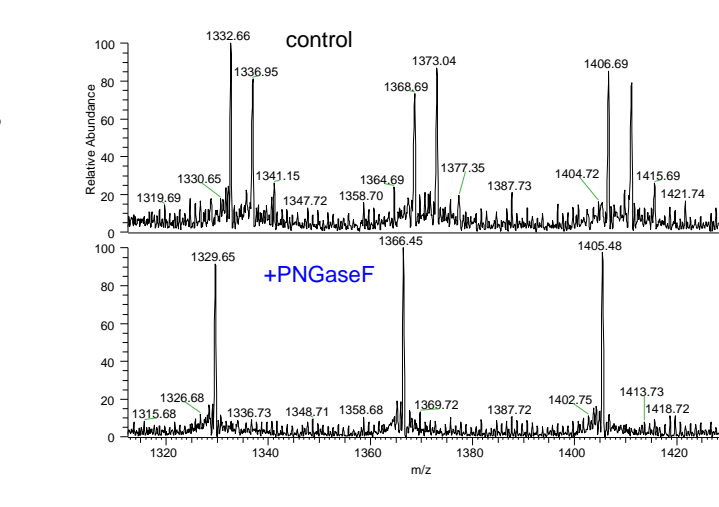
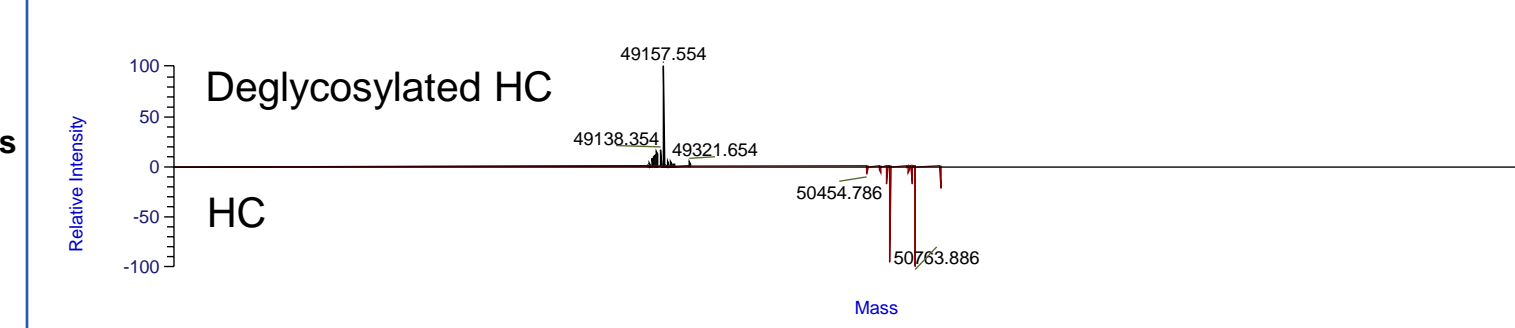


Figure 15. Deconvoluted spectra of trastuzumab HC before and after deglycosylation



## CONCLUSIONS

•mAb fragments scFc, LC, and Fd' are baseline separated on a 3.0 x 50 mm MABPac RP column. Spatial separation of lysine variant and oxidation variant from the unmodified scFc is achieved.

•LC/MS analysis of the scFc, LC, and Fd' fragments successfully pinpoints the location of lysine and oxidation modifications.

•A workflow has been developed to successfully remove N-glycan in 15 min. Trastuzumab HC and its deglycosylated form are baseline separated on a longer (2.1 x 100 mm) MABPac RP column.

## REFERENCES

- Liu H., Gaza-Bulseco G., and Zhou L. Mass Spectrometry Analysis of Photo-Induced Methionine Oxidation of a Recombinant Human Monoclonal Antibody. J. Am. Soc. Mass. Spectrom., 2009, 20, 525-528

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