

Overview

Intact protein analysis provides useful global protein information. It is possible to identify modifications such as oxidation, glycosylation, etc. based on intact protein analysis. However, for large proteins such as IgG, it is difficult to identify small changes in mass resulting from the modifications. We were able to identify galactose heterogeneity (162 Da) for intact IgG, but were unable to identify other modifications such as alkylation (57 Da) and oxidation (16 Da) which have smaller mass shifts. The reason for that is the theoretical peak width for intact IgG is equal to or greater than the mass differences caused by those modifications, which makes the detection very difficult. Denaturing SEC-MS is an online method that uses organic solvent in the eluting buffer for SEC separation before the analytes get detected in the mass spectrometer. The organic solvent used in this method serves as a denaturing reagent to detach IgG light chains and heavy chains before they are separated by SEC. Instead of detecting intact IgG, the mass spectrometer measures IgG heavy chain (50 kDa) and light chain (25 kDa). We were able to identify glycosylation on the heavy chain at higher resolution and also detect oxidation, alkylation, and combination of oxidation and alkylation using the IgG light chain mass spectrometry data.

Experimental

System Components:

Waters® 2796 Bioseparations Module
Waters® Micromass® Q-ToF 2™ Mass Spectrometer

Column:

Waters® BioSuite™ 250, 5 µm HR SEC (7.8 mm X 300 mm)

MS Conditions:

-Source = ESI(+)
-Capillary (kV) = 3.3
-Cone (V) = 35
-Temperature (°C)
 -Source = 150
 -Desolvation = 400
-Gas Flow (L/Hr)
 -Cone = 50
 -Desolvation = 500
-Scan Mode
 -MS Mode
-Collision Energy (V) = 10

HPLC Conditions:

-Isocratic
-Mobile phase: 25 mM ammonium formate, 1% formic acid in 50% acetonitrile
-Flow Rate: 1 mL/min. 200 µL/min to mass spectrometer after split
-Run time: 20 minutes

Results

Traditional SEC methods use ammonium phosphate buffer for separations. In this research, we replaced it with ammonium formate, which is mass spectrometry compatible. By adding acetonitrile to the buffer, it does not only help to denature antibody molecules, but also increase ionization efficiency. Figure 1 is the denaturing SEC-MS spectrum of reduced and alkylated monoclonal IgG 1 from mouse.

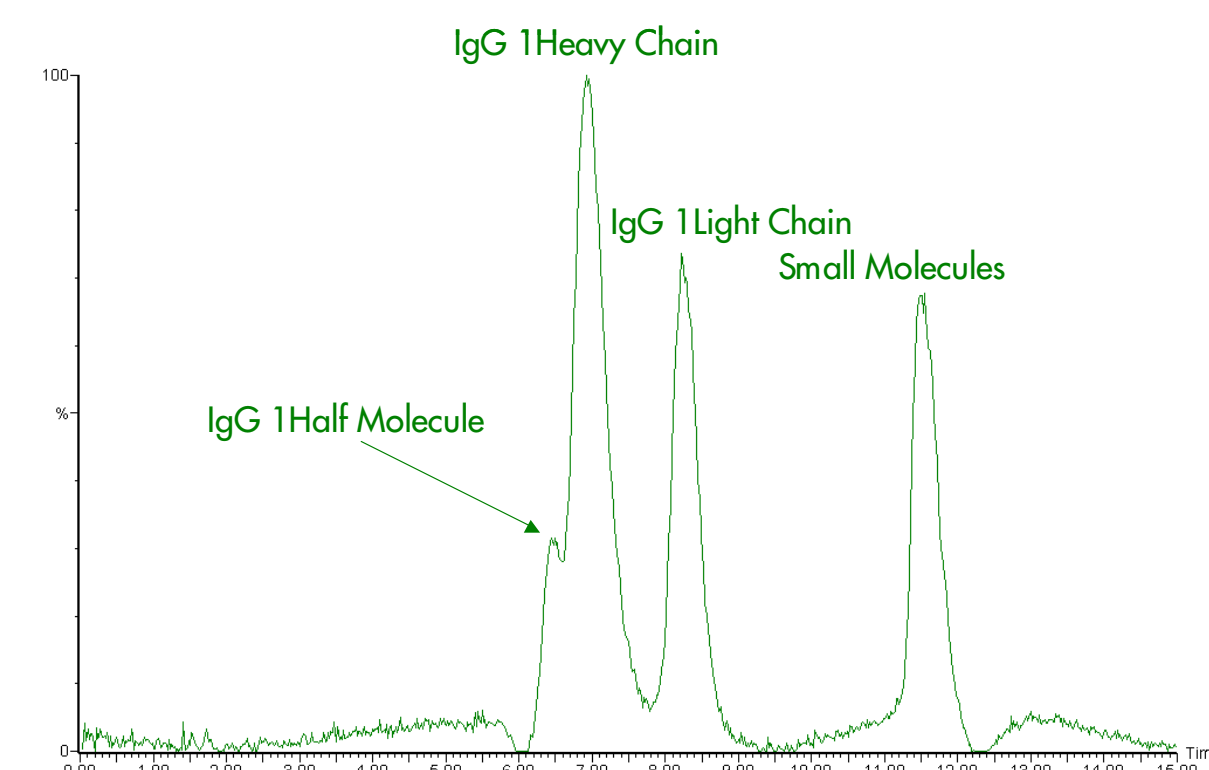


Figure 1. Denaturing SEC-MS Chromatograph of IgG 1

From the above mass chromatograph, we were able to identify half molecule, heavy chain, light chain, and some small molecules from reduced and alkylated IgG 1 molecule. Figure 2 to Figure 7 show ESI charge envelopes of the above molecules and their corresponding deconvoluted spectra.

There are three variations of glycosylation in monoclonal IgG 1 depending on how many galactose are retained on biantennary ends, they are named as G2, G1, and G0 as shown below.

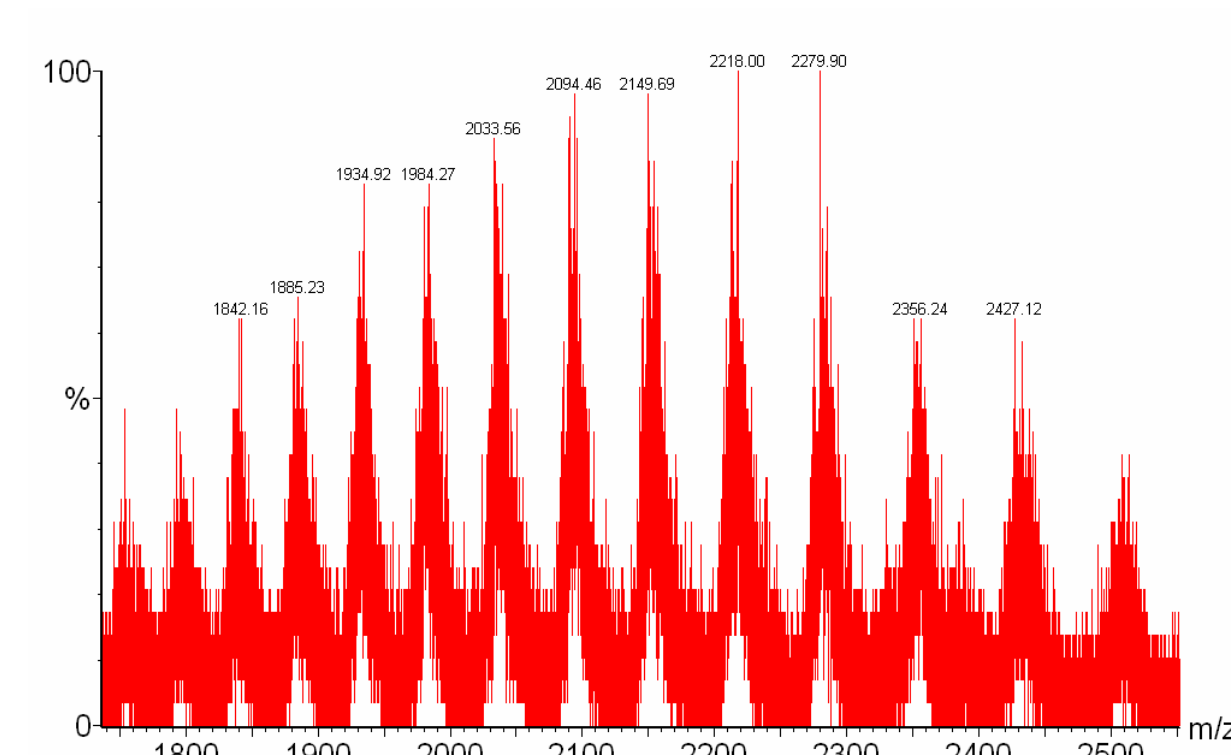
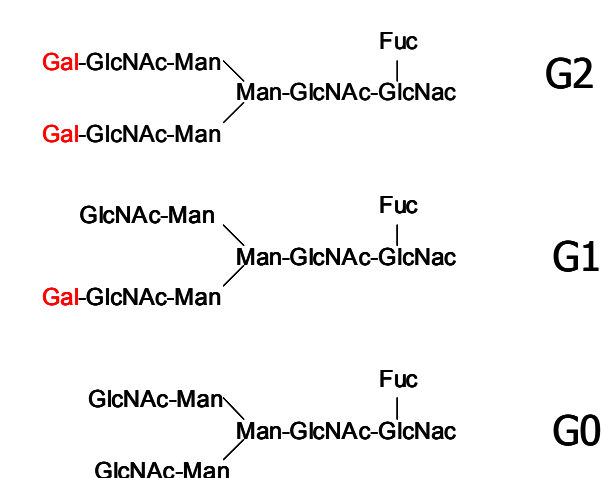


Figure 2. ESI Mass Spectrum of IgG 1 Half Molecule

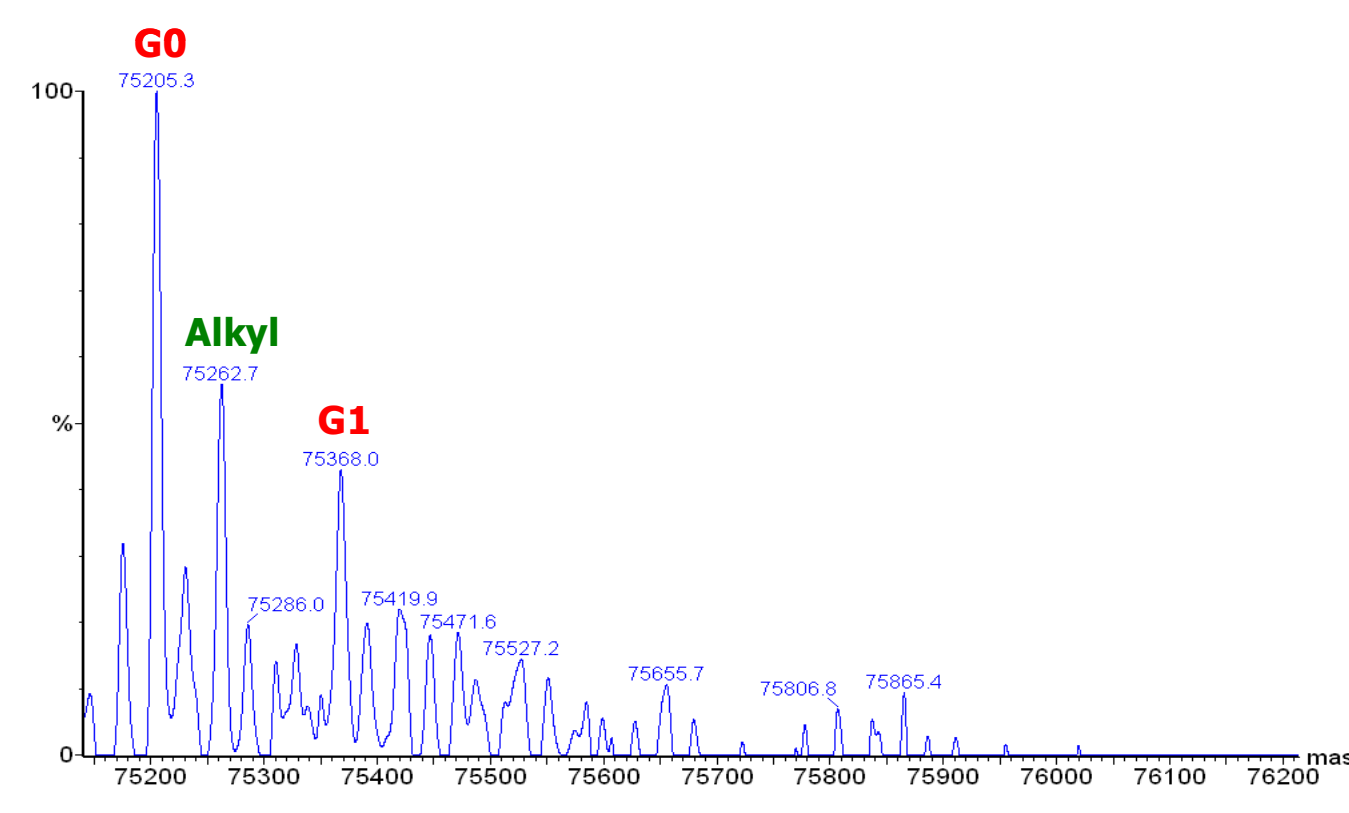


Figure 3. MaxEnt 1 Deconvoluted Spectrum of IgG 1 Half Molecule

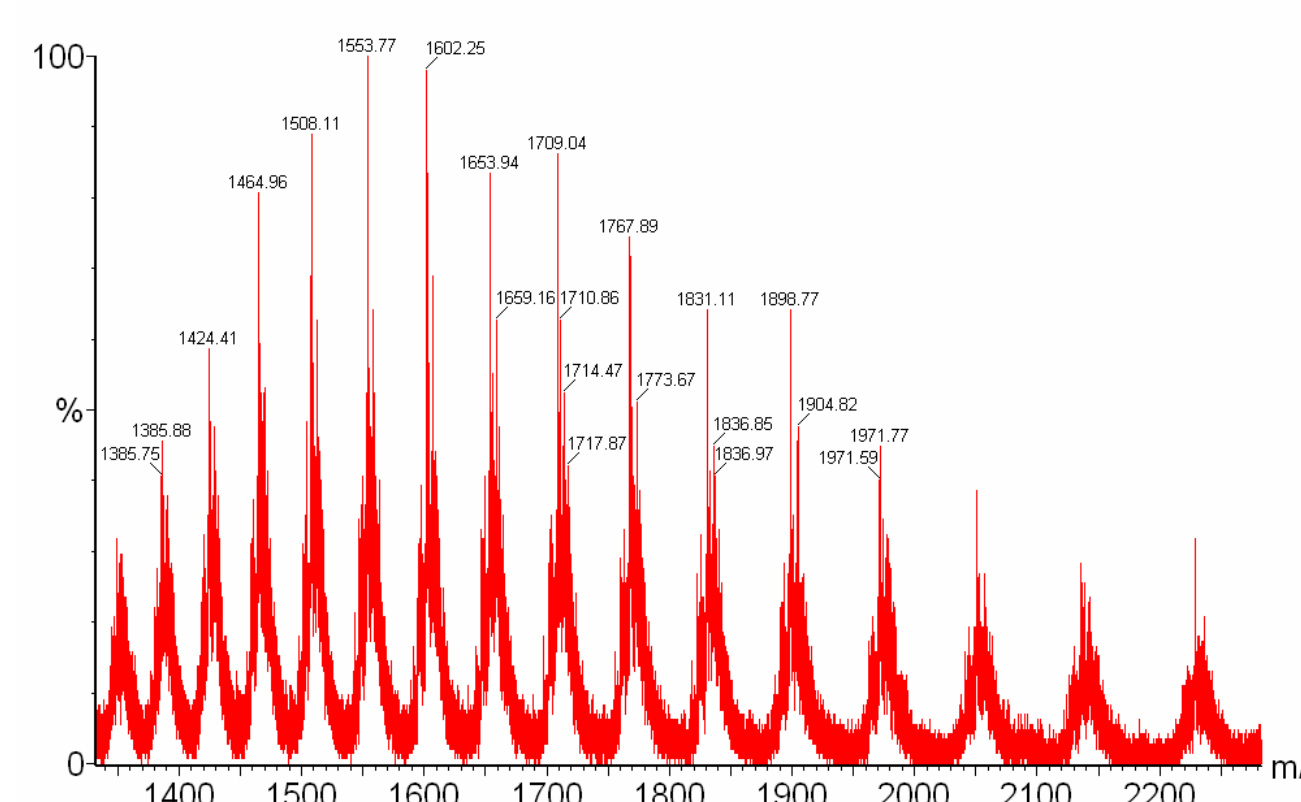


Figure 4. ESI Mass Spectrum of IgG 1 Heavy Chain

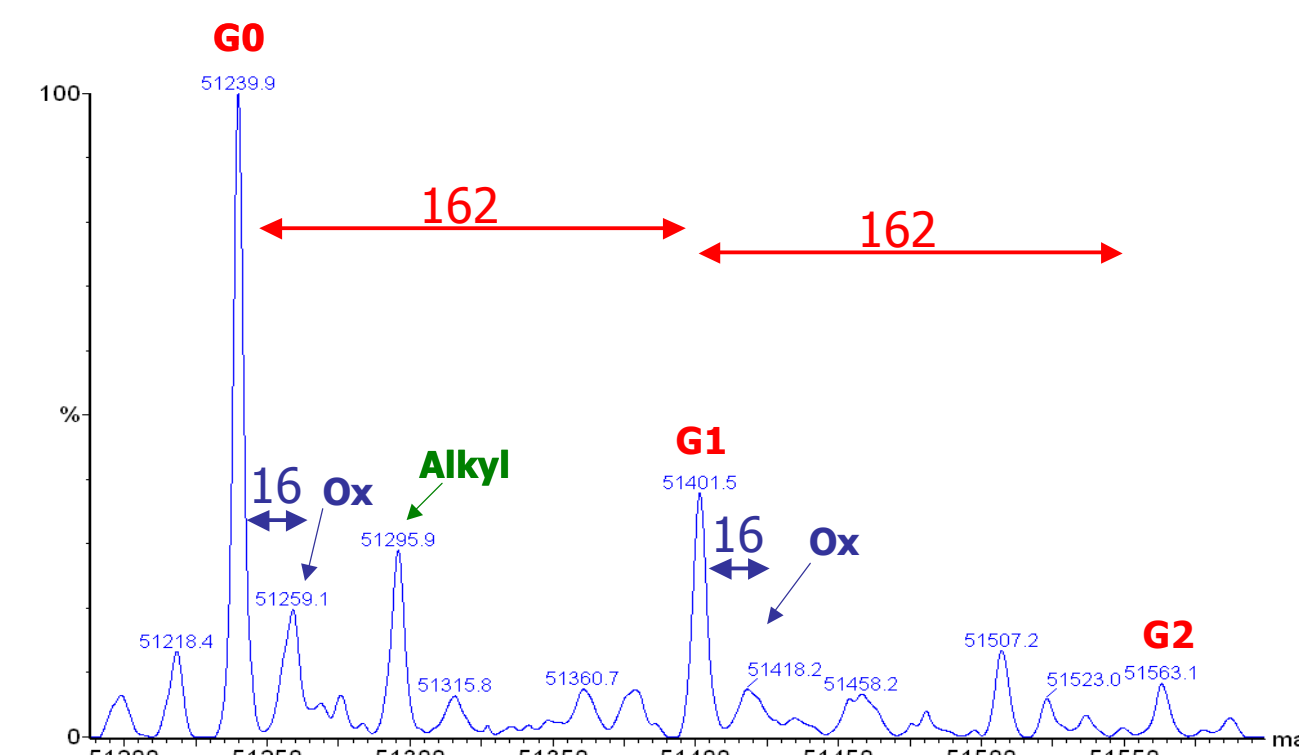


Figure 5. MaxEnt 1 Deconvoluted Spectrum of IgG 1 Heavy Chain

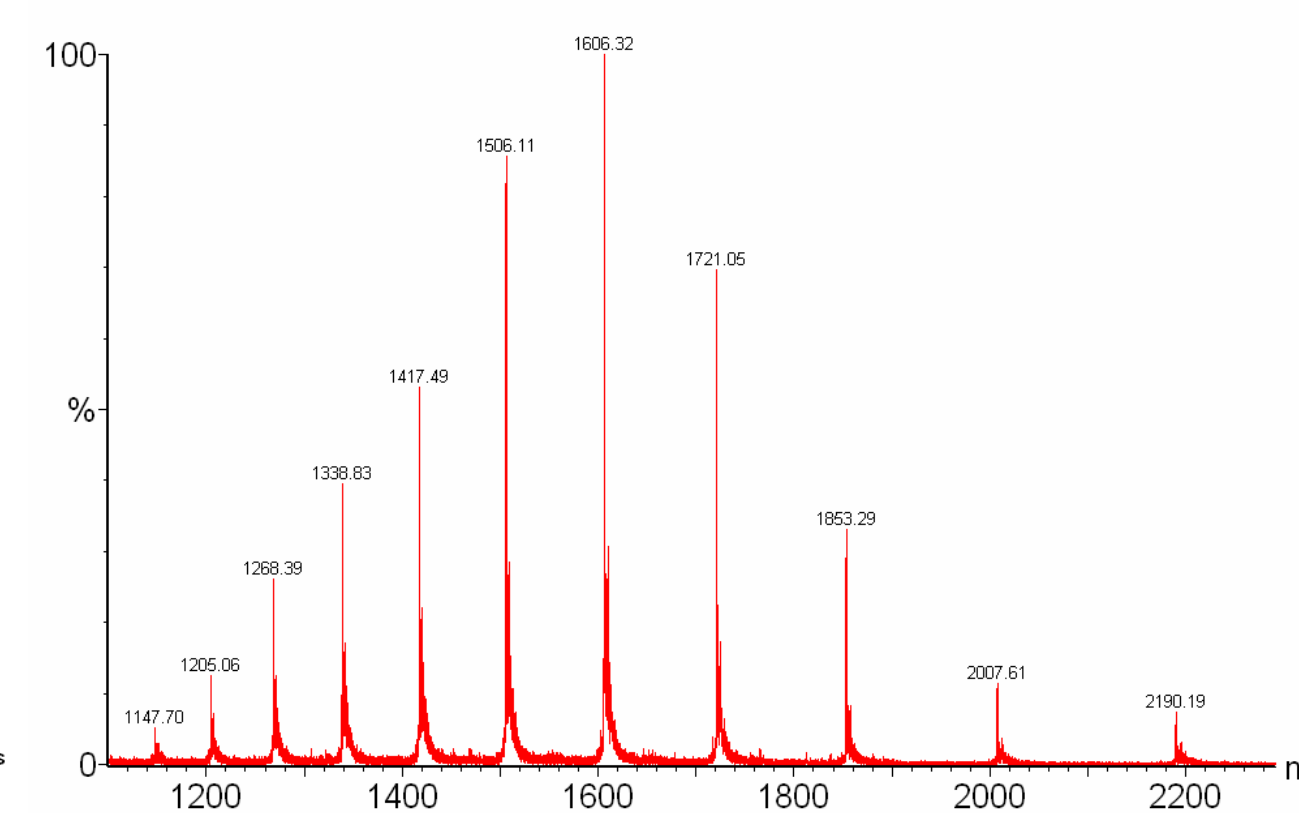


Figure 6. ESI Mass Spectrum of IgG 1 Light Chain

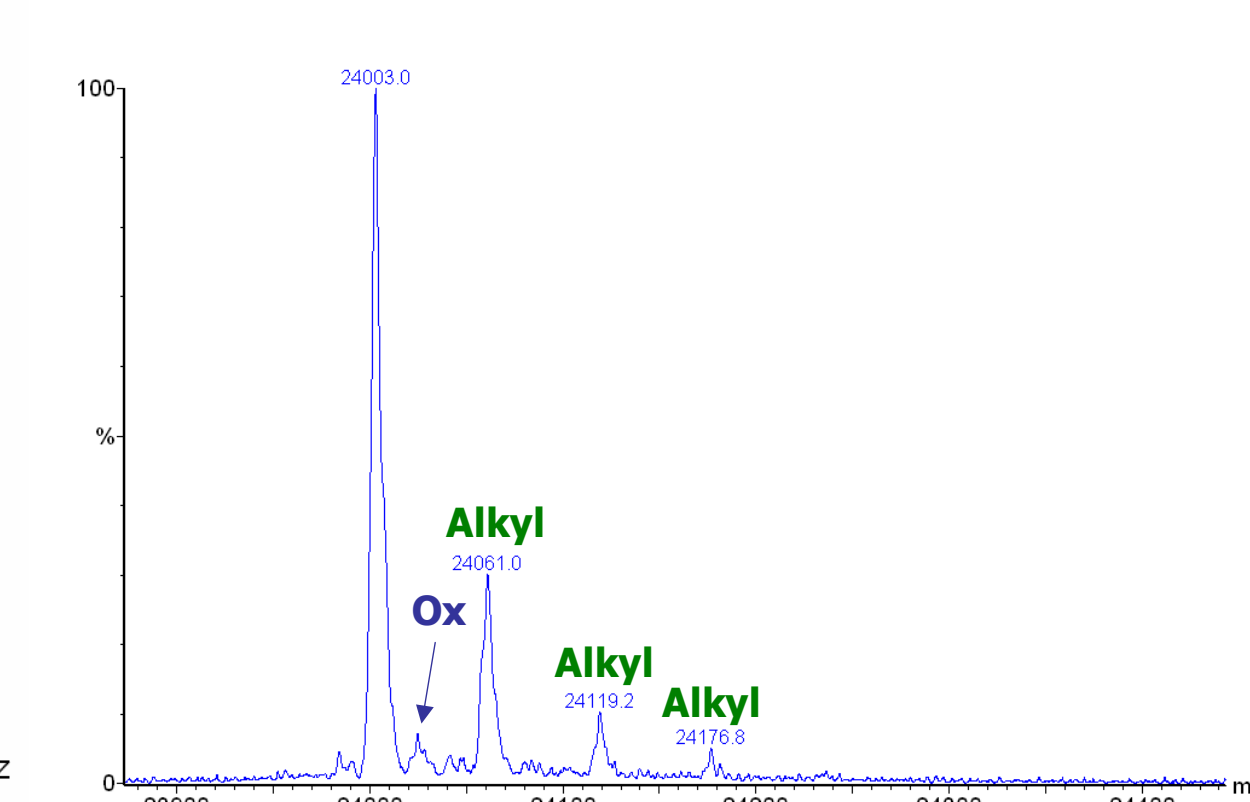


Figure 7. MaxEnt 1 Deconvoluted Spectrum of IgG 1 Light Chain

In the MaxEnt 1 deconvoluted spectra of IgG 1 half molecule, heavy chain, and light chain, we identified several post translational modifications other than glycosylation, which was observed at the intact IgG 1 mass spectrum. Mass shift caused by oxidation is only 16 Da and the theoretical peak width of a protein at 150 kDa is around 25 Da. If we also consider instrument peak width and the relative intensity of oxidation product, it is almost impossible to detect oxidation in intact IgG 1 mass spectrum. By using the denaturing SEC-MS method, we successfully separated an antibody heavy chain and light chain before analyzing them in a mass spectrometer. This reduces the challenge from measuring 150 kDa mass to measuring 50 kDa and 25 kDa masses. Formic acid and acetonitrile in the mobile phase help the ionization, too.

Conclusions

- Denaturing SEC-MS enables quick online mass measurement on protein mixtures with different sizes
- Mobile phase used in this method facilitates both denaturing and ionization
- Complete separation of IgG 1 heavy chain and light chain makes it possible for the detection of post translational modifications with smaller mass shifts