Monoclonal antibody characterization through native Orbitrap mass spectrometry leading to improved sensitivity and elucidation of microheterogeneity

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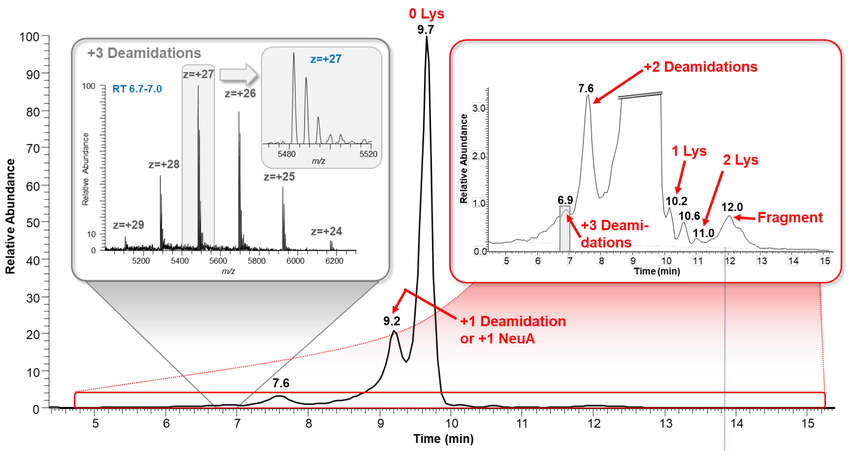
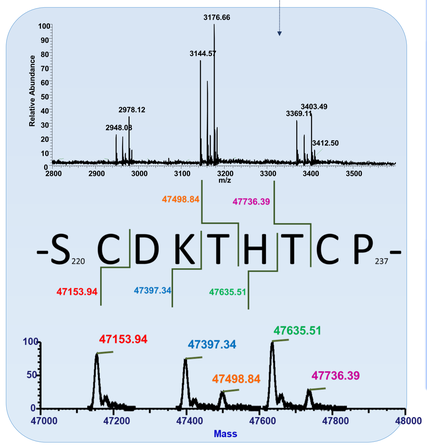
- **Background:** Mass spectrometry (MS) of intact proteins is increasingly used in biopharmaceutical analysis as it is rapid and provides significant structural insights without laborious sample preparation steps that may interfere with endogenous post-translational modifications (PTMs) present on the drug substance. Significant advances in the use of chromatography under native conditions hyphenated with mass spectrometry have been made, including optimized ESI source conditions and the introduction of volatile mobile phases [1]. Charge variant analysis (CVA) can be performed using ion exchange chromatography. Using volatile buffers with low salt concentrations it is possible to provide compatibility with MS, enabling detailed characterization of low abundant modifications that may be more difficult to obtain with size exclusion chromatography (SEC) [2,3]. Sensitivity of the technique can be enhanced by MS detection; indeed, increased sensitivity and performance at higher resolution settings, may provide increased dynamic range and confidence in charge variant identification. Orthogonal techniques, such as peptide mapping and subunit analysis, may be employed to confirm the identity of some very low abundant species.
- **Methodology:** CVA analysis was performed using a Thermo Scientific™ MAbPac™ SCX-10 RS (2.1 x 50 mm) column. Linear gradient using A) 25 mM ammonium bicarbonate, 30 mM acetic acid (pH 5.3) and B) 10 mM ammonium hydroxide (pH 10.9). Separation was performed on a Vanquish™ UHPLC (Thermo Scientific) hyphenated with an Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer. Sample amount: 50 µg injected in triplicate. Data processing was performed using BioPharma™ Finder software v. 4.0, Sliding window option using ReSpect™ algorithm.

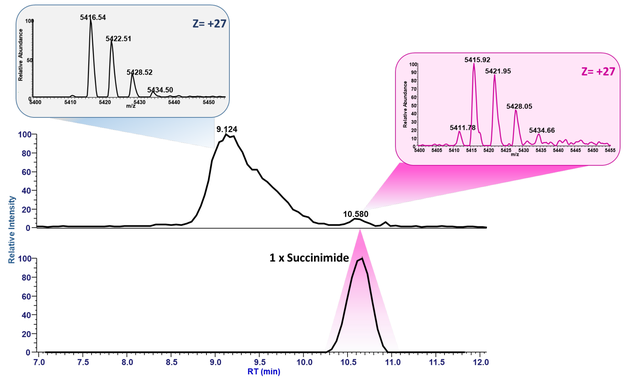
Ipilimumab (IgG1)



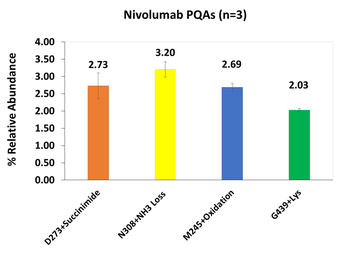
Ipilimumab drug product CVA analysis (above panel) showed a complex profile with one abundant acidic variant and several peaks in both acidic and basic region, all below 3% of relative abundance. The most abundant species corresponds to the deamidated mAb at position N56 as confirmed by peptide mapping analysis. This species co-elutes with a N-glycan variant, presenting one sialic acid. Between the other species, the broad peak at 12.0 mins corresponds to lower molecular weight species constituted by light chain and Fd portion generated upon hydrolysis around the upper hinge region. The panel on the right shows the charge envelope of the peak (up) and the deconvoluted spectrum (down), with a schematic representation of hydrolysis points.



Nivolumab (IgG4)

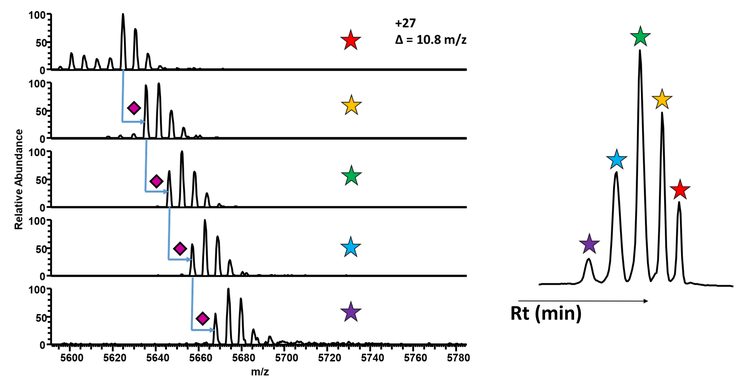


Nivolumab is an IgG4 monoclonal antibody targeting the programmed death receptor (PD-1) and used to treat several types of cancer. Fab region of IgG4 is known to be less stable with temperature and pH changes, potentially causing broadening of the main peak. Nevertheless, several acidic and basic variants could be identified. In the basic region, one species (RT 10.6) with a mass shift of ~17 Da may correspond to succinimide formation on one aspartic acid as suggested by peptide mapping data below.

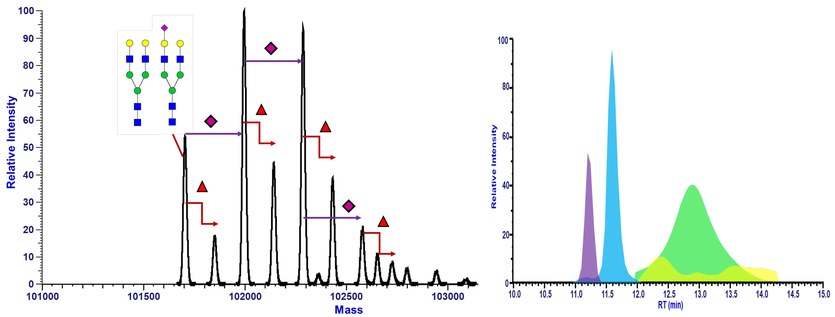


The second peak visible in the basic region has a mass shift of +1 Da. Being in the basic region this shift is not compatible with one deamidation, but could be generated by the simultaneous presence of two variants causing later elution from the chromatographic column, such as the lack of one of the two pyro-Glu present on heavy chains N-terminus and one succinimide. This issue demonstrates the benefits of CVA-MS workflow where both retention time and accurate mass measurements are available to the analyst. Peptide mapping on CVA fractions may be required where component ID is uncertain.

Anti-HIV (bNAb)



The third antibody analysed is a broadly neutralising antibody (bNAb) targeting HIV and produced in CHO cell line. A second N-glycosylation site is present on bNAb light chain, with sialylated non-fucosylated complex N-glycans, which influence CVA profile at intact level (panel above). Only performing CVA analysis on the Fab region obtained after IdeS digestion (lower panel) it was possible to observe fucose presence on the N-glycans present on the light chain.



Conclusions: In the present study 3 mAbs were analysed using CVA analysis hyphenated with new generation Orbitrap mass spectrometry instrument. For all samples, excellent data quality and identification of low abundant variants within 25 ppm was achieved also on very low abundant proteoforms (< 1%).Charge variant analysis on the Orbitrap Exploris 240 MS enables in-depth characterization and confident identification of mAb microheterogeneity due to excellent sensitivity and spectral quality.

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References: [1] Tassi, M. et al. Journal of Separation Science 41, 125-144, (2017). [2] Füssl, F. et al. Analytical Chemistry 90 (7), 4669-4676, (2018). [3] Füssl, F. et al. MABS, 11 (1), 116-128, (2018).