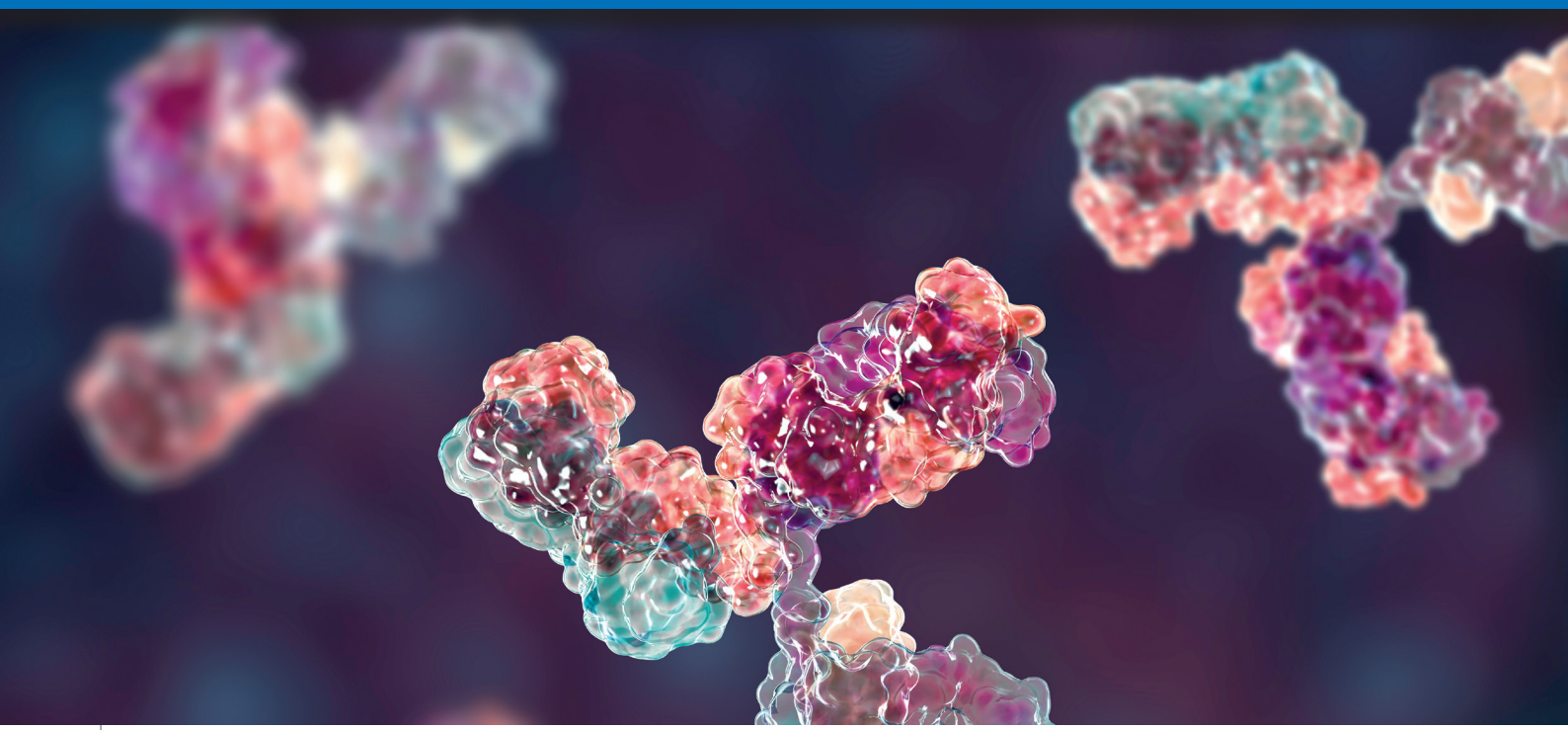

# Protein Clipping Variant Analysis of Vedolizumab using dedicated workflows in BioPharma Compass

Proteolytic degradation of biopharmaceuticals during their life cycle can pose health risks and require dedicated analysis.


## Abstract

In this work we used enzymatically introduced antibody truncation variants as a case study. These clipping variants were detected based on accurate mass measurements using a maXis II ETD and confirmed by LC-ETD and LC-CID Middle-Down protein sequencing. Typically, ETD yielded higher sequence coverages of the proposed clipping candidates. However, CID Middle-Down spectra provided sufficiently long terminal sequence tags to confirm candidates as well.

Dedicated clipping variant analysis workflows in BioPharma Compass 2021 enabled the detection and confirmation of these variants rapidly and easily.


## Introduction

For Biopharmaceuticals, degradation of the active drug substance during production, formulation or storage poses a threat to drug efficacy and safety and is a critical quality attribute (CQA). The detection of such truncated protein species – clipping variants – can be difficult to achieve through classic peptide mapping with trypsin.


*Keywords:*
*Proteoform analysis, protein degradation, enzyme specificity, Middle-Up, Middle-Down, sequence analysis, Elute_UPLC, maXis II ETD, BioPharma Compass 2021*



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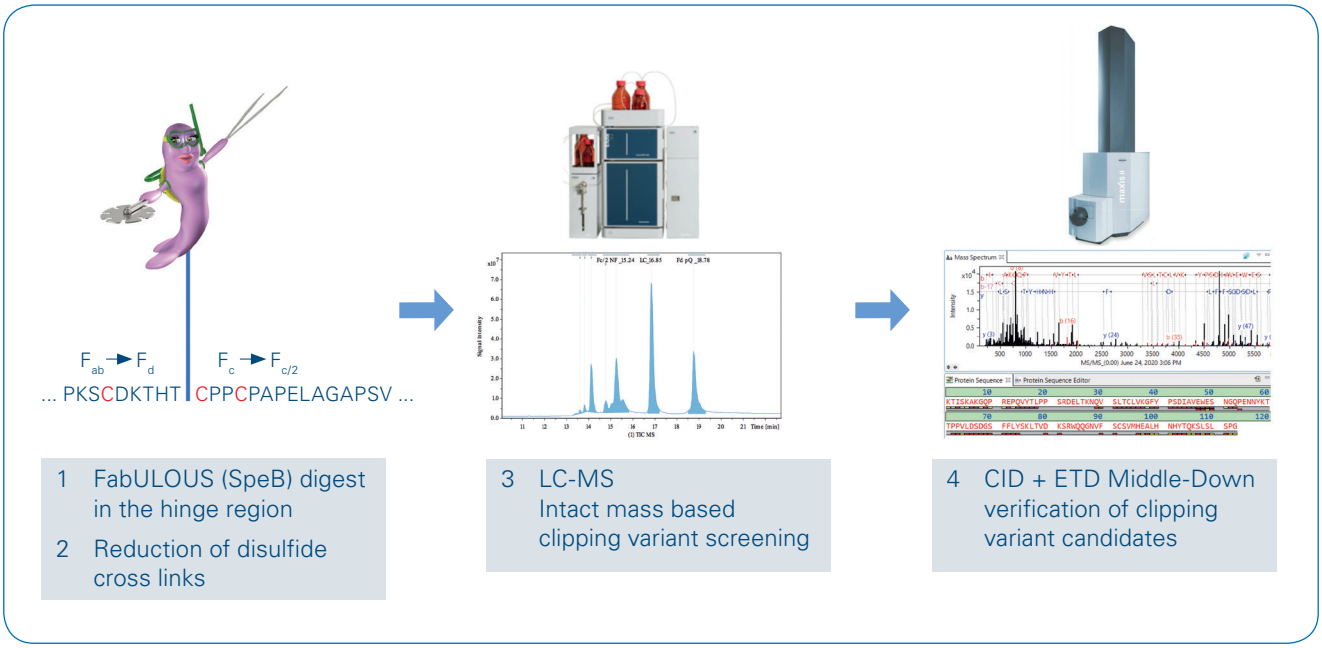


*Figure 1: Experimental design for the Vedolizumab protein clipping analysis. SpeB digestion produced Fab and Fc fragments. After reduction Fd, Fc, and LC subunits were analyzed by LC-MS, yielding the major expected subunits plus some low abundant fragments. Protein clipping analysis in BioPharma Compass provided candidates based on accurate mass and isotope pattern. They were confirmed by direct sequence analysis of the clipping product candidates by ETD and CID.*

Vedolizumab is a recombinant, humanized IgG1 monoclonal antibody directed against the human lymphocyte α4β7 integrin, a key mediator of gastrointestinal inflammation. It is used in the treatment of moderate to severe active ulcerative colitis and Crohn's disease.

In this case study, a Vedolizumab biosimilar was characterized after SpeB treatment, which cleaves IgG1 predominantly in the hinge region at ...KTHT | CPPCPAPE... corresponding to T229|C230 on Vedolizumab's heavy chain. Minor cleavage products with uncharacterized specificity have also been observed.

We analyzed the reduced SpeB digest products with LC-MS using an ultrahigh resolution maXis II ETD QTOF instrument to characterize the enzyme specificity and evaluate the performance of the BioPharma Compass 2021 clipping variants detection workflow. Middle-Down ETD and CID sequence validation measurements were subsequently performed to confirm the findings.

## Experimental

### Samples

A Vedolizumab biosimilar candidate drug substance (Polpharma Biologics) was measured after treatment with FabULOUS and IgGZERO enzyme kits (Genovis) followed by reduction using TCEP. Concentration after dilution was approximately 0.5 mg/mL. The resulting major subunits are named following the usual IgG subunit nomenclature: Fd, Fc/2 and LC.

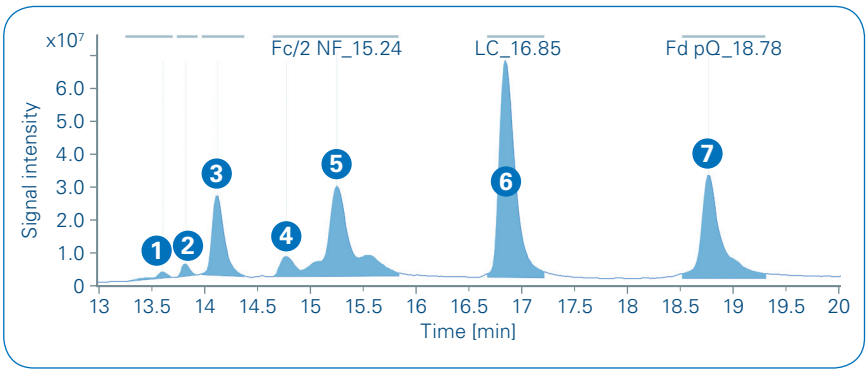


*Figure 2: Top: Total Ion Chromatogram of the SpeB-digested, reduced and deglycosylated Vedolizumab with the annotated subunits peaks ⑤-⑦. Peaks ①-④were further characterized as putative protein clipping variants, which elute earlier in the chromatogram.*

### LC-MS and MS/MS

A Bruker Elute UPLC with Waters Protein BEH C4, 300Å 1.7 µm, 2.1 x 100 mm column was used for the protein separation.

Solvents: A: 0.1% Formic Acid in Water;
B: 0.1% Formic Acid in ACN

Gradient:
0 min 10% B
1.5 min 10% B
2.5 min 15% B
34 min 45% B
35 min 95% B
40 min 95% B
40.2 min 10% B
45 min 10% B

A Bruker maXis II ETD Ultrahigh Resolution QTOF was used for MS spectra acquisition in the 150-2800 *m/z* range. The samples were also subjected to ETD and CID fragmentation for Middle-Down protein sequence analysis using a mass range from 100-2400 *m/z*.

The precursor ion intensity for ETD experiments was controlled in all experiments using these parameters:

*ICC Target Intensity Controlling*: 1000 Mio
*ICC Time Accumulation Analyte min*: 1 ms
*ICC Time Accumulation Analyte max*: 600 ms
*Injection Time*: 16 - 22 ms
*Extended Reaction time*: 4 - 6 ms

For CID, a 50/50 Stepping with 25 eV and 30 eV collision energy was applied to all precursor ions.

### Data Analysis

All datasets were transferred to BioPharma Compass 2021 for data processing and analysis. The Vedolizumab LC and HC sequences were imported from [1] as FASTA file. N-terminal pyro Q was defined as variable modification together with G0F, G1F and G2F as major glycoforms. The **MAM Protein Screening** workflow was used for intact mass analysis of the SpeB proteolysis products and the **Top-Down Sequencing ESI** workflow for the sequence analysis of the CID and ETD spectra. Both workflows allowed to specifically analyze protein clipping variants. All molecular weights calculated were based on the monoisotopic masses determined using the SNAP algorithm in BioPharma Compass.

## Results

### The regular SpeB cleavage products

The cleavage products resulting from Vedolizumab IgGZERO deglycosylation, SpeB treatment and reduction predominantly yielded the expected Fd [1-229], Fc/2 [230-450] and LC [1-219] subunits (peaks 5-7). In addition 4 additional peaks (1-4) were observed by LC-MS analysis (Figure 2).

Chromatographic peaks 5-7 contained predominantly – but not exclusively, see Figure 9 – the expected subunits as confirmed by the maximum entropy-deconvoluted mass spectra (Figure 3).

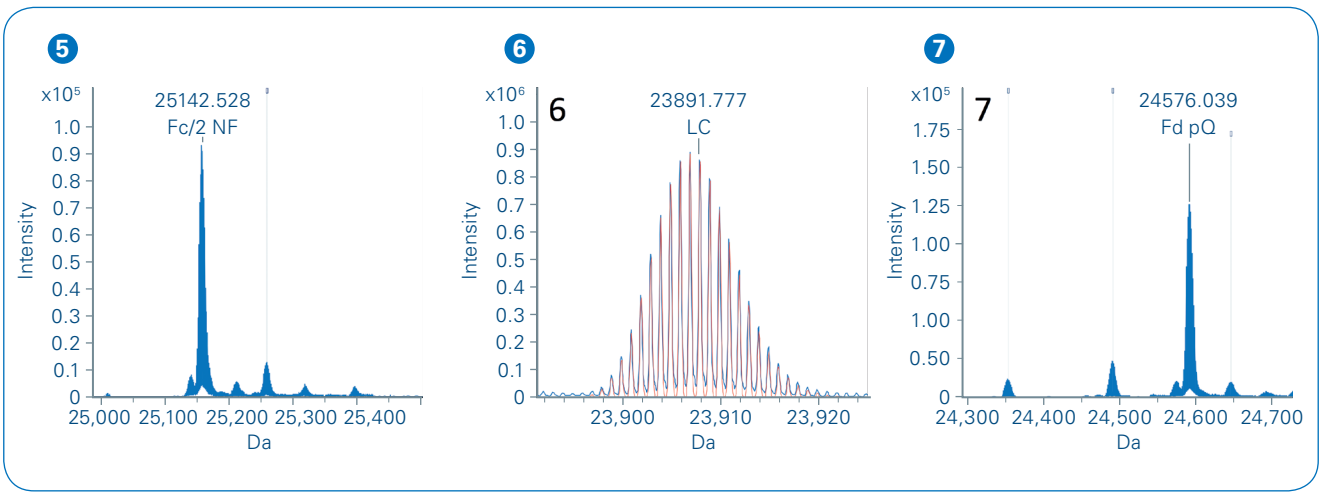


*Figure 3: MaxEnt deconvoluted MS spectra of chromatogram peaks ⑤-⑦, containing the IgGZERO deglycosylated Fc/2, the LC and pyro-glutamylated Fd subunit. All spectra were isotopically resolved and the calculated isotope patterns (red, LC in peak 6) show perfect alignment with the mass spectra. Minor additional peaks are indicative of, e.g., potential protein clipping products of the drug sample (see Figure 9). Monoisotopic molecular weights are annotated to the peaks.*

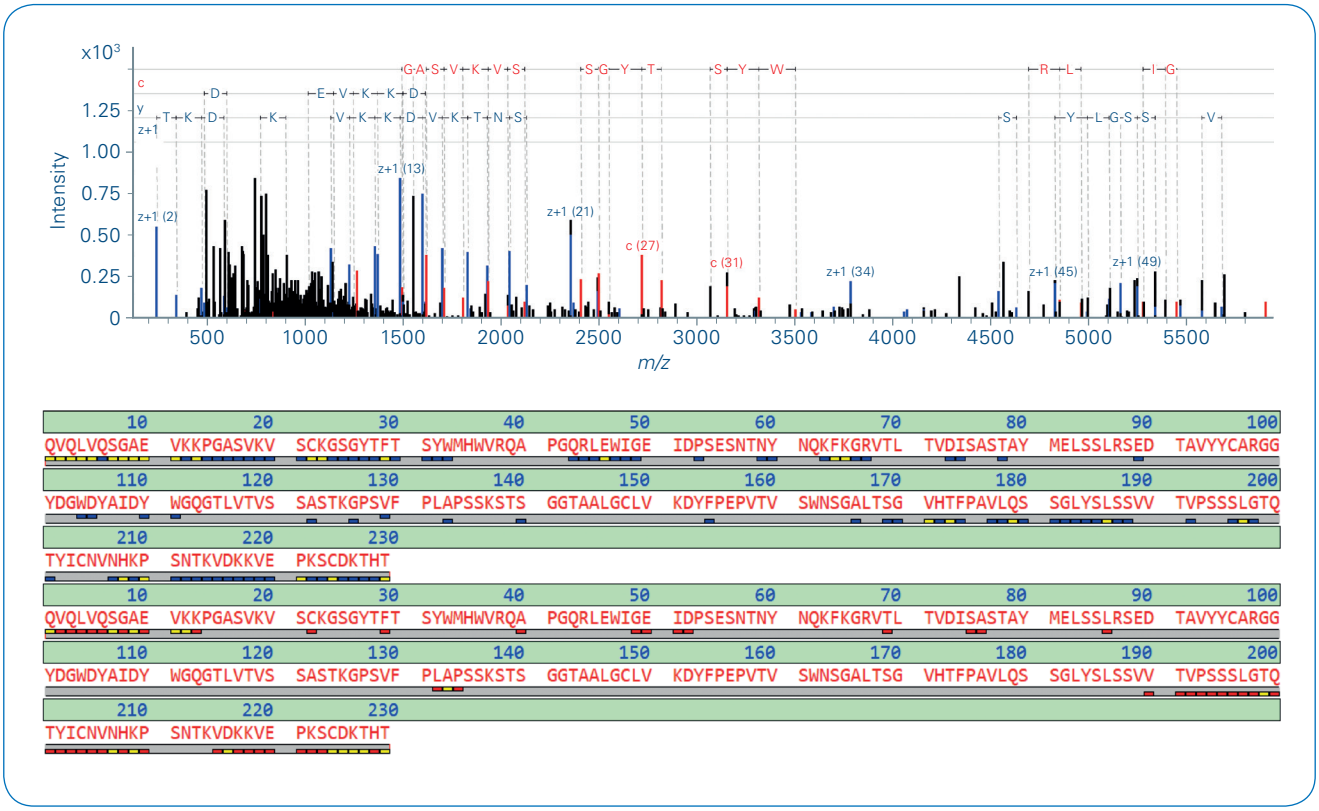


*Figure 4: Top: ETD Middle-Down sequencing spectrum of the Fd subunit in peak 7; c-, y- and z+1 fragment ions were annotated. Center: Sequence coverage map of the ETD fragments (blue); top row of bricks represents observed c-ions, bottom row represents matching y- and z+1-ions. Bottom: Sequence coverage map of the CID fragments (red) from the CID spectrum (not shown); top row of bricks represents observed a- and b-ions, bottom row represents matching y-ions. The yellow bricks indicate tolerated gaps in the observed fragment ion ladders for the calculation of the SVP [2].*

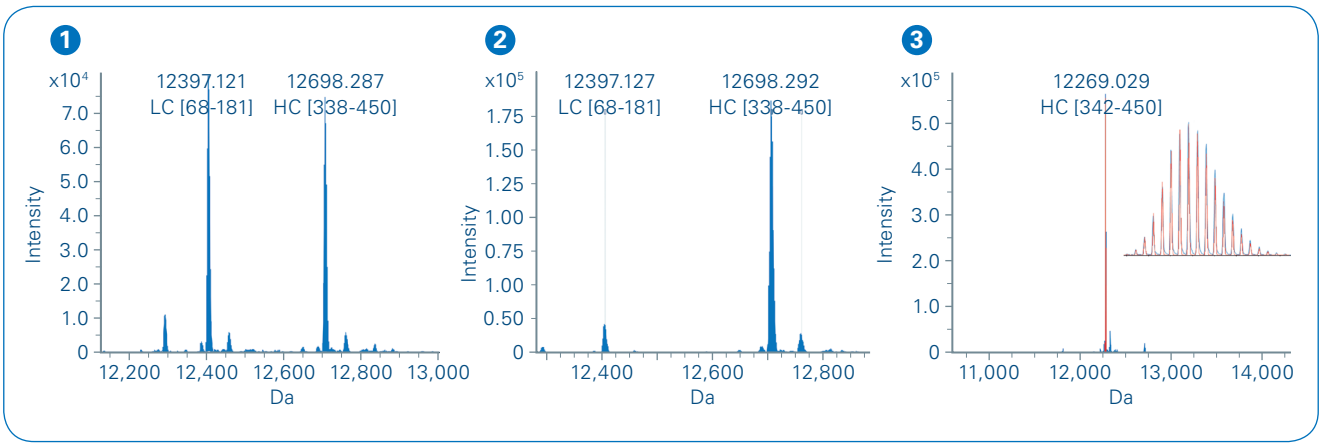


*Figure 5: MaxEnt deconvoluted MS spectra of chromatogram peaks ①-③. Some clipping candidates are annotated, some aren't even visible here such as HC [246-450] in peak 3 at 23605.831 Da (ΔMr -0.86 ppm).*

Subsequent LC-ETD and LC-CID analyses of each of the chromatographic peaks provided sequence information of the fragmented protein subunits, which were automatically calculated based on the Vedolizumab protein sequence. Sequence Coverage (SC) and Sequence Validation Percentage (SVP) [2] were determined; the SVP calculation tolerated a terminal gap size of 15 and internal gap size of 3 missing fragments in the ion series. The observed SVP and SC values obtained for ETD were Fc/2:41/27%, LC: 60/43% and Fd:48/36%, and for CID were Fc/2:36/29%, LC:47/38% and Fd:28/21%, respectively. In average, 50% SVP were obtained from LC-ETD analysis for the 3 subunits and 37% SVP from LC-CID. The results confirmed the Vedolizumab subunit sequence even in case of the weakest spectra of the Fd (Figure 4).

## Clipping variant analysis

The initial list of proposed clipping candidates based on intact mass can be quite extensive if somewhat relaxed mass tolerances, e.g., 10 ppm in this case, are applied to match sequence candidates (Figure 6). However, the applied calibration provided for a mass accuracy better than 2 ppm, which coincided with the only sequence identified by ETD analysis in peak 2 (Figure 7). LC [68-181] in Figure 5, amongst other candidates, was ruled out based on poor mass accuracy (Figure 6) and the absence of a match with the respective ETD spectrum.

Info | Expected | Unidentified | Average DAR Calculation

| Clipping | Annotation | Mr Ref | Mr Sample | Δ Mr [ppm] | Int. [a.u.] | Rt Sample [min] |
|---|---|---|---|---|---|---|
| ✓ | LC [69-182] | 12397.0765 | 12397.1266 | 4.04 | 2.097E+04 | 13.83 |
| ✓ | LC [68-181] | 12397.0765 | 12397.1266 | 4.04 | 2.097E+04 | 13.83 |
| ✓ | LC [37-148] | 12397.2467 | 12397.1266 | -9.69 | 2.097E+04 | 13.83 |
| ✓ | LC [68-184] | 12698.2403 | 12698.2916 | 4.04 | 1.746E+05 | 13.83 |
| ✓ | LC [104-219] | 12698.3025 | 12698.2916 | -0.86 | 1.746E+05 | 13.83 |
| ✓ | HC [338-450] | 12698.3119 | 12698.2916 | -1.60 | 1.746E+05 | 13.83 |
| ✓ | HC [158-277] | 12698.3610 | 12698.2916 | -5.47 | 1.746E+05 | 13.83 |
| ✓ | HC [127-249] | 12698.3617 | 12698.2916 | -5.52 | 1.746E+05 | 13.83 |
| ✓ | HC [31-147] | 12752.1081 | 12752.2018 | 7.34 | 1.489E+04 | 13.83 |

*Figure 6: Proposed clipping variants in peak 2 after application of a 10 ppm mass tolerance. The 2 entries at 12698.3 Da. with <2 ppm errors are the best candidates and were further analyzed by ETD and CID.*

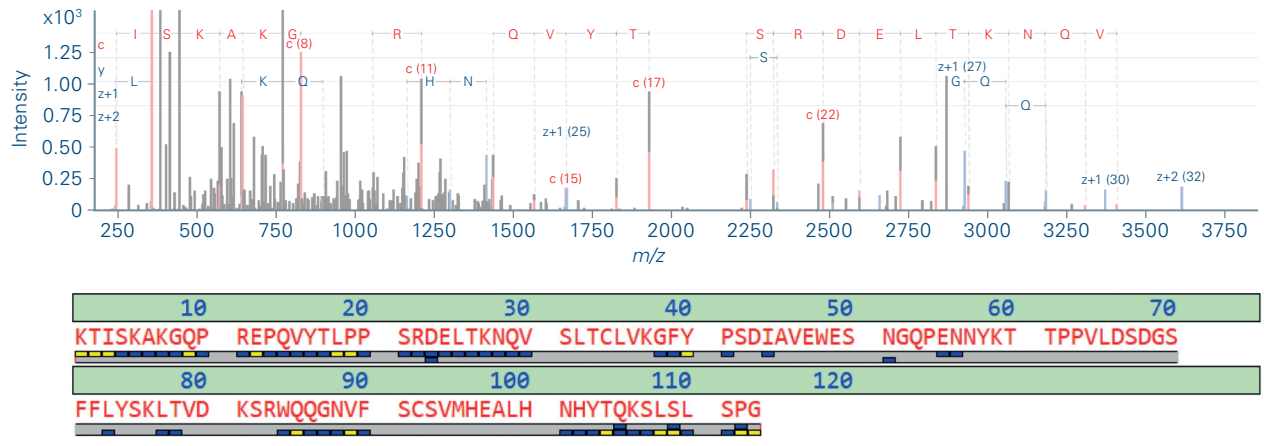


*Figure 7: ETD spectrum of the 12698.3 Da protein in peak 2 matched the sequence HC [338-450]. None of the other candidate sequences at 12698.3 Da were identified by Middle-Down sequencing.*

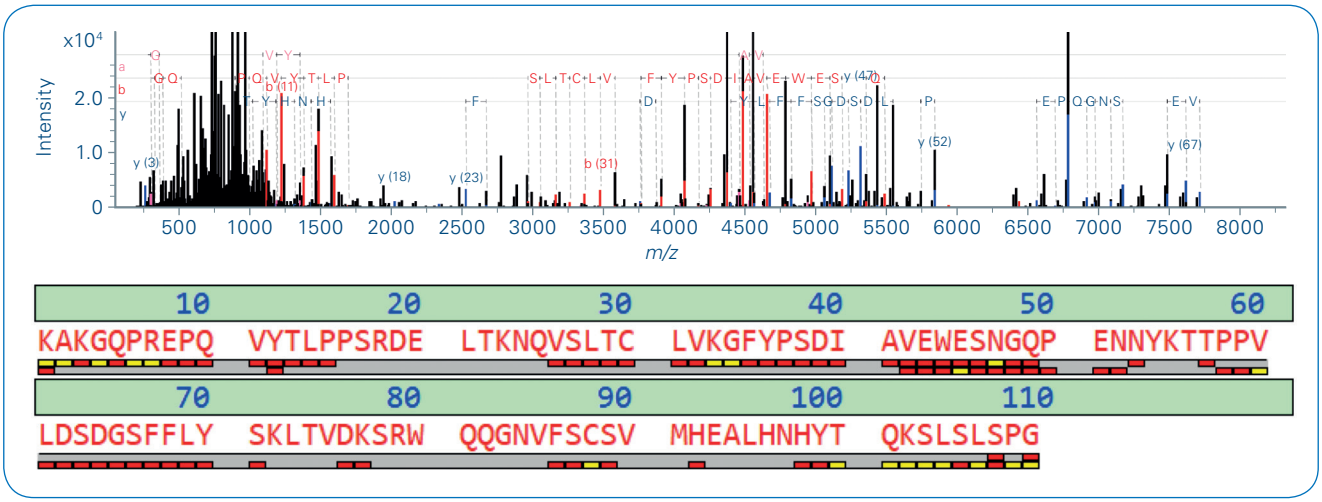


*Figure 8: CID spectrum of the 12269.029 Da protein in peak 3 matching the sequence HC [342-450]. None of the other candidate sequences at 12698.3 Da were identified by Middle-Down sequencing.*

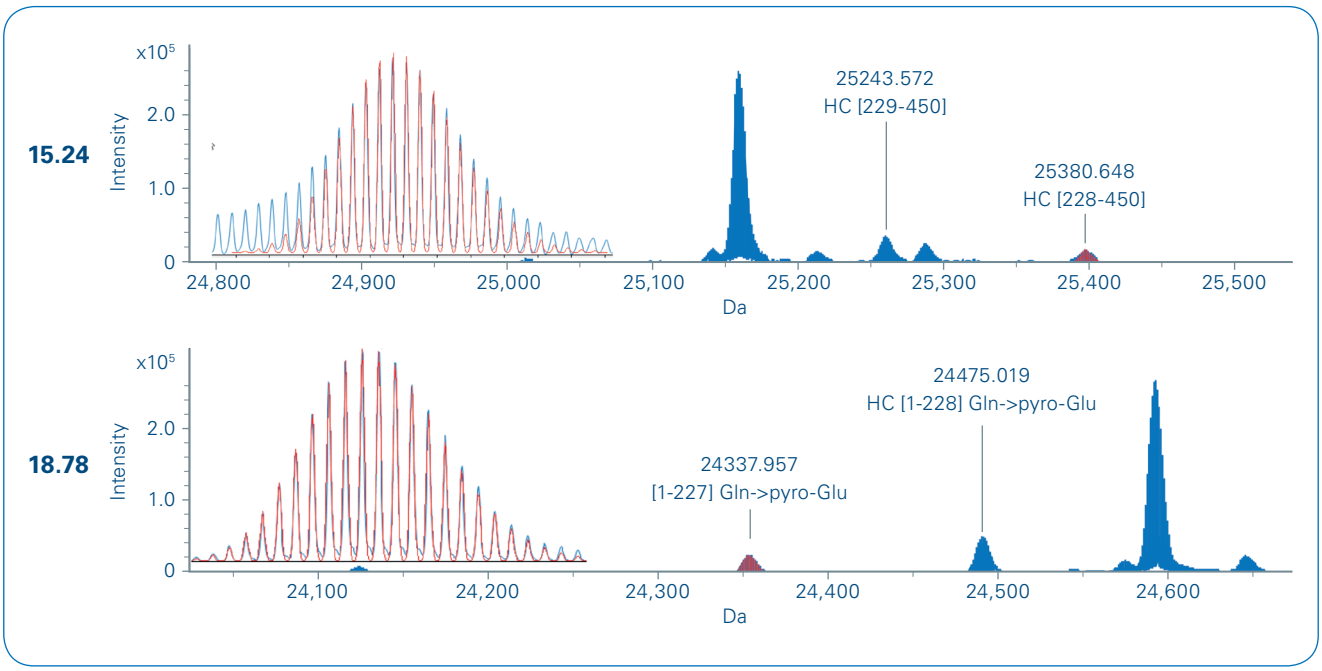


*Figure 9: Intact mass spectra from chromatographic peaks 5 (top) and 7 (bottom). The annotated peaks show cleavage behind positions HC 227 and HC 228. All peaks show a perfect overlap with the calculated isotope pattern (red) with a mass error between 0.4 and 1.5 ppm. The HC [228-450] was matched by the SNAP algorithm even against a significant background of chemical noise.*

The HC [342-450] clipping product at 12269.029 Da in peak 3 was confirmed by ETD and CID, the CID spectrum is shown in Figure 8.

The Mr 24348.231 base peak in the MaxEnt spectrum of Peak 4 was confirmed by ETD as HC [238-450] with a 29% SVP (data not shown).

In addition, peaks were proposed as clipping candidates, which weren't directly confirmed by Middle-Down sequencing but represented the N-terminal and C-terminal fragments of the same cleavage sites: HC [1-227]|HC [228-450] and HC [1-228]|HC [229-450], adjacent to the theoretical SpeB cleavage site HC [1-229]|HC [230-450] (Figure 10). All 4 peaks were matched with an average mass accuracy of 0.8 ppm and well matching isotope patterns.

## Summary

Three LC-MS runs of SpeB-digested and reduced Vedolizumab (LC-MS, LC-ETD, LC-CID) confirmed the expected cleavage site in the hinge region. In addition, the combined approach to qualify candidates by intact mass measurements for Middle-Down sequencing yielded 6 more, minor, cleavage sites, 3 of which were confirmed by direct ETD and CID analysis (Figures 7, 8), 2 were validated by their complementary nature and mass accuracy (Figure 9,) and one by mass accuracy and isotopic fidelity alone, in the absence of alternative possible matches (Figure caption 5). The results are summarized in Figure 10. In case of Vedolizumab, ragged ends of the Fc/2 and the Fd were obtained in the hinge region; and an overall of 6 unspecific cleavage sites were observed with the implemented new workflows in BioPharma Compass.

The identity and amount of the observed cleavage products were comparable between the Vedolizumab originator and bio-similar drug substances (data not shown), which indicates that clipping analysis might become a useful **protein-based MAM approach** in comparability, stability and aging studies.

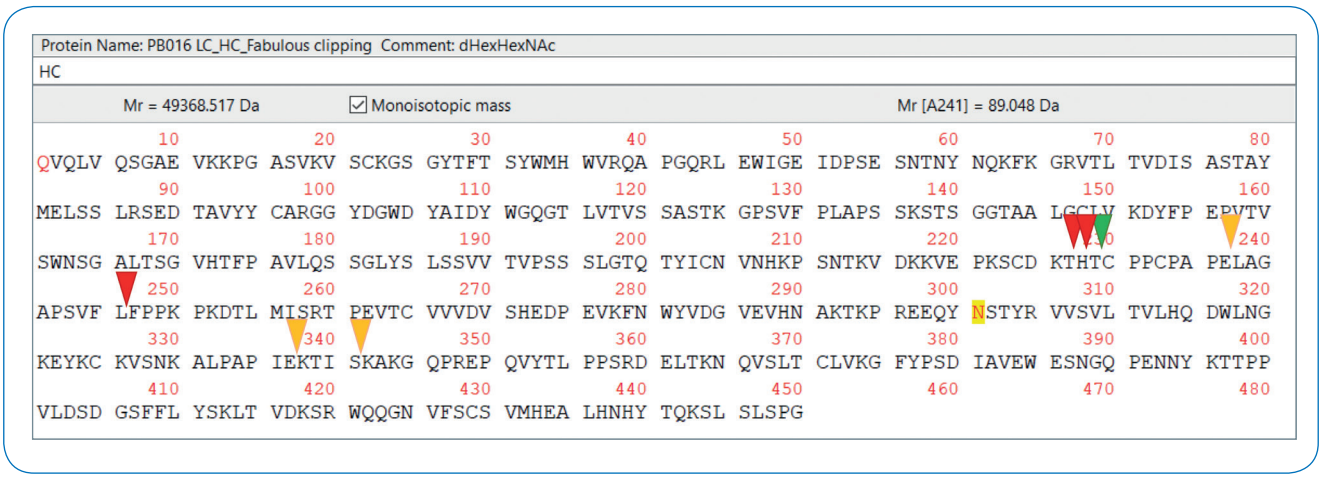


*Figure 10: Vedolizumab HC sequence from [1] with the confirmed main cleavage site (green), CID/ETD-confirmed sites (orange) and sites where the identification was based on accurate mass and isotopic pattern matching (red).*

## Conclusions

- A workflow was described to identify antibody clipping variants based on subunit mass measurements and Middle-Down sequencing.
- A mass accuracy of well below 2 ppm was important to provide sufficient specificity to define candidate sequences. This was achieved with monoisotopic mass assignments using the SNAP algorithm.
- ETD and CID on the maXis II ETD UHR-QTOF were suitable to verify all clipping variants subjected to MS/MS analysis, though ETD typically provided 20-35% more sequence information.
- BioPharma Compass 2021 Clipping workflows, both for the intact mass-based prediction as well as for the Middle-Down sequencing facilitated the prediction and confirmation of clipping variants significantly to speed up potential drug substance clipping analysis.

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**References**

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[2] Resemann A, et al. (2016). *Full validation of therapeutic antibody sequences by middle-up mass measurements and middle-down protein sequencing.* MAbs. **8**(2):318-30. www.ncbi.nlm.nih.gov/pmc/articles/PMC4966597/


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