

# High-Throughput Peptide Mapping with the Vanquish UHPLC System and the Q Exactive HF Mass Spectrometer

Martin Samonig<sup>1</sup>, Kai Scheffler<sup>2</sup>, Remco Swart<sup>1</sup>, and Jonathan Josephs<sup>3</sup>

<sup>1</sup>Thermo Fisher Scientific, Germering, Germany

<sup>2</sup>Thermo Fisher Scientific, Dreieich, Germany

<sup>3</sup>Thermo Fisher Scientific, San Jose, CA, USA

## Key Words

Monoclonal Antibodies, Acclaim C18 RSLC Column, Q Exactive HF Mass Spectrometer, Biocompatible UHPLC, Biotherapeutics Characterization, Biopharma

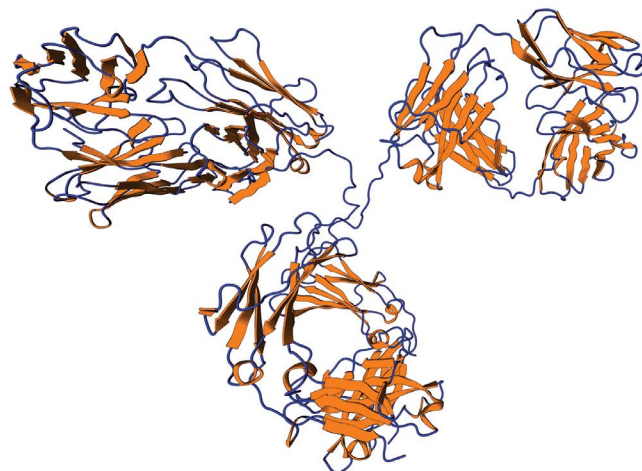
## Goal

Report on the benefits of a fast analytical platform employing highly efficient chromatography in combination with fast and high-resolution quadrupole Thermo Scientific™ Orbitrap™ mass spectrometry technology as a tool for fast identification and quantification of sequence truncations, glycosylation and post-translational or artificial modification of recombinant monoclonal antibodies.

## Introduction

Monoclonal antibodies, or mAbs, (Figures 1 and 2) are the major element in the fastest growing sector of biopharmaceuticals within the pharma industry. By 2016, eight of the top ten drugs will be therapeutic proteins. Their manufacture is accomplished in bacterial or eukaryotic expression systems, requiring extensive purification of the target product. During drug development and production, the quality of biotherapeutics needs to be closely monitored.

Various analytical methods have been used to study quality attributes such as structural integrity, aggregation, glycosylation pattern or amino acid degradation. Because of their high information content and versatility, characterization methods based on high-performance liquid chromatography and mass spectrometry are among the most powerful protein characterization techniques. Proteins can be enzymatically digested to obtain peptides enabling their analysis by means of peptide mapping experiments.



Here, we report a fast and sensitive approach that combines enzymatic digestion, fast chromatographic separation, high-resolution mass spectrometry, and rapid data processing to handle the large amount of samples in diverse biopharma workflows. In this study we have analyzed two commercially available drug products: rituximab (trade names MabThera and Rituxan®) and denosumab (trade names Prolia® and XGEVA®).

## Experimental

The two drug products rituximab and denosumab were denatured for 30 min in 7 M urea and 50 mM Tris HCl at pH 8.0. The samples were reduced with 5 mM DTT for 30 min at 37 °C, alkylation was performed with 10 mM IAA for 30 min at room temperature, and the reaction was quenched by addition of 10 mM DTT. Thermo Scientific™ Pierce™ Trypsin Protease (MS Grade) was added and digestion allowed to proceed overnight at 37 °C. Digests were stopped by addition of TFA to approximately pH 3.0.

Abbreviations	
ACN: Acetonitrile	mAb: Monoclonal antibody
DTT: Dithiothreitol	PTMs: Post translational modifications
FA: Formic acid	TFA: Trifluoroacetic acid
IAA: Iodoacetamide	

A Thermo Scientific™ Vanquish™ UHPLC system with a 2.1 x 250 mm i.d. Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm column and gradients of water and acetonitrile (ACN) with 0.1% formic acid (FA) each were used to separate the peptide mixtures. Five different separation times were applied and compared: 5, 8, 13, 20, and 30 min for the gradient ramping from 4% to 55% eluent B (0.1% FA in 8:2 acetonitrile/water (v/v)). Flow rates were adapted accordingly using 1.1 (5 min), 1.0 (8 min), 0.6 (13 min), 0.4 (20 min), and 0.4 mL/min (30 min). The Thermo Scientific™ Q Exactive™ HF mass spectrometer (MS) equipped with a HESI-II probe was used for mass spectrometric detection.

### Equipment

Vanquish UHPLC system consisting of:

- System Base (P/N VH-S01-A)
- Binary Pump H (P/N VH-A10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer

### Experimental Conditions - HPLC

Column	Acclaim RSLC 120, C18, 2.2 µm, Analytical (2.1 x 250 mm, P/N 074812)
Mobile Phase	A: 0.1% FA in water (P/N FA 28905) B: 0.1% FA in 8/2 acetonitrile/water (v/v) (P/N acetonitrile TS-51101)
Gradient 1	0–30 min: 4–55% B, 30–30.1 min: 55–100% B, 30.1–35 min: 100% B, 35.0–35.1 min: 100–4% B, 35.1–40 min: 4% B
Gradient 2	0–20 min: 4–55% B, 20–20.1 min: 55–100% B, 20.1–25 min: 100% B, 25–25.1 min: 100–4% B, 25.1–30 min: 4% B
Gradient 3	0–13 min: 4–55% B, 13–13.1 min: 55–100% B, 13.1–16 min: 100% B, 16–16.1 min: 100–4% B, 16.1–30 min: 4% B
Gradient 4	0–8 min: 4–55% B, 8–8.1 min: 55–100% B, 8.1–10 min: 100% B, 10–10.1 min: 100–4% B, 10.1–12 min: 4% B
Gradient 5	0–5 min: 4–55% B, 5–5.1 min: 55–100% B, 5.1–7 min: 100% B, 7–7.1 min: 100–4% B, 7.1–9 min: 4% B
Flow Rate	Gradient 1: 0.4 mL/min Gradient 2: 0.4 mL/min Gradient 3: 0.6 mL/min Gradient 4: 1.0 mL/min Gradient 5: 1.1 mL/min
Temperature	60 °C
Injection Volume	2 µL
Detection	214 nm Data Collection Rate: 10 Hz Response Time 0.4 s
Flow Cell	10 mm LightPipe

Experimental Conditions - MS				
Source	HESI-II			
HPLC	0.4	0.6	1.0/1.1	mL/min
Sheath Gas Pressure	25	60	75	psi
Auxiliary Gas Flow	10	20	30	arb units
Capillary Temperature	320 °C			
Probe Heater Temperature	350 °C			
S-lens RF Voltage	60 V			
Source Voltage	3.5 kV			
Full MS Parameters		MS <sup>2</sup> Parameters		
Full MS Mass Range	200–2000 <i>m/z</i>	Resolution Setting	15,000	
Resolution Setting	60,000	Target Value	1e5	
Target Value	3e6	Isolation Width	2.0 Da	
Max Injection Time	100 ms	Signal Threshold	1e4	
Default Charge State	2	Normalized Collision Energy (HCD)	27	
SID	0 eV	TopN MS <sup>2</sup>	5	
		Max Injection Time	100 ms	
		Fixed First Mass	140.0 <i>m/z</i>	
		Dynamic Exclusion (s)	10.0	

## Physicochemical Characteristics Biological Characteristics

### N-terminal heterogeneity

Pyroglutamate formation  
Other modifications

### Amino acid modifications

Deamidation, oxidation,  
glycosylation, isomerization

### Fragmentation

Cleavage in hinge region

### Oligosaccharides

Fucosylation, sialylation,  
galactosylation,...

### Disulfide bonds

Free thiols, disulfide shuffling, thioether

### C-terminal heterogeneity

Lysine processing, Proline amidation

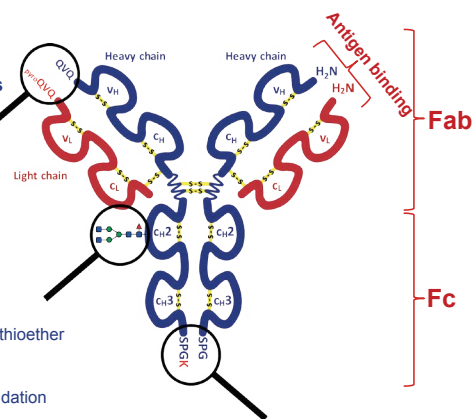


Figure 1. General structure of mAbs and their biological and physicochemical characteristics.

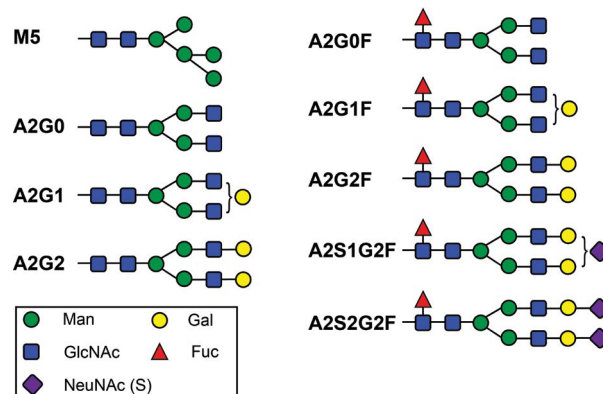


Figure 2. Nomenclature of carbohydrate structures commonly observed on antibodies.

## Data Analysis

The data were acquired with Thermo Scientific™ Xcalibur™ 3.0 software in combination with Thermo Scientific SII for Xcalibur 1.1 software. Data analysis was performed using Thermo Scientific™ PepFinder™ 2.0 and FreeStyle™ 1.0 software packages.

## Results and Discussion

Peptide mapping experiments were performed using the rituximab digest for assessing the sequence coverage for light and heavy chain, as well as for identification and (relative) quantification of a specific set of modifications: a) oxidation, b) glycosylation and c) deamidation.<sup>1,2</sup> For all five gradient times from 30 min down to 5 min, a very good separation was achieved (Figure 3) and resulting sequence coverages of 100% were obtained from all separation times both for light and heavy chain, even for the very short gradient of 5 min. The sequence coverage map (Figure 4) shows the overlap of the different peptides

identified in different intensities and in different lengths due to missed cleavages. Table 1 shows the identification and comparison of a subset of monitored modifications across the different separation times applied. A tilde (~) before the modification indicates the modification was found on the tryptic peptide, but could not be localized on a specific amino acid with MS/MS spectra. The modification is labeled with recovery “Good” when the total peak area, including modified and unmodified forms of the peptide, is at least 10% of the most abundant peptide from the same protein. The recovery “Fair” means it is at least 1%. The relative abundance of the detected modifications in the five different methods has a standard deviation of 0.19% and shows that important information regarding post-translational modifications (PTMs) can be obtained equally and accurately at all separation times.

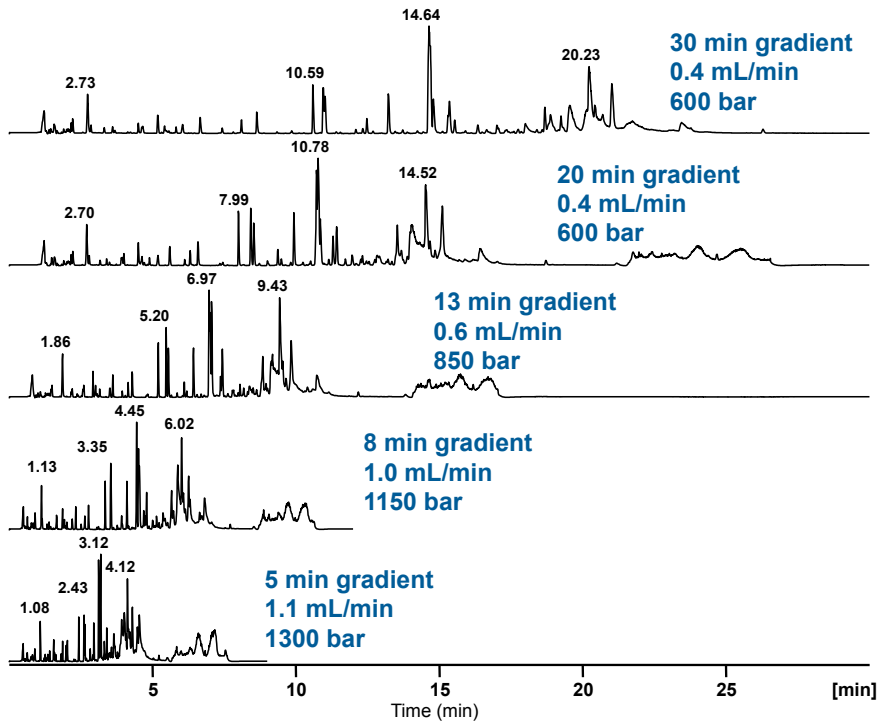
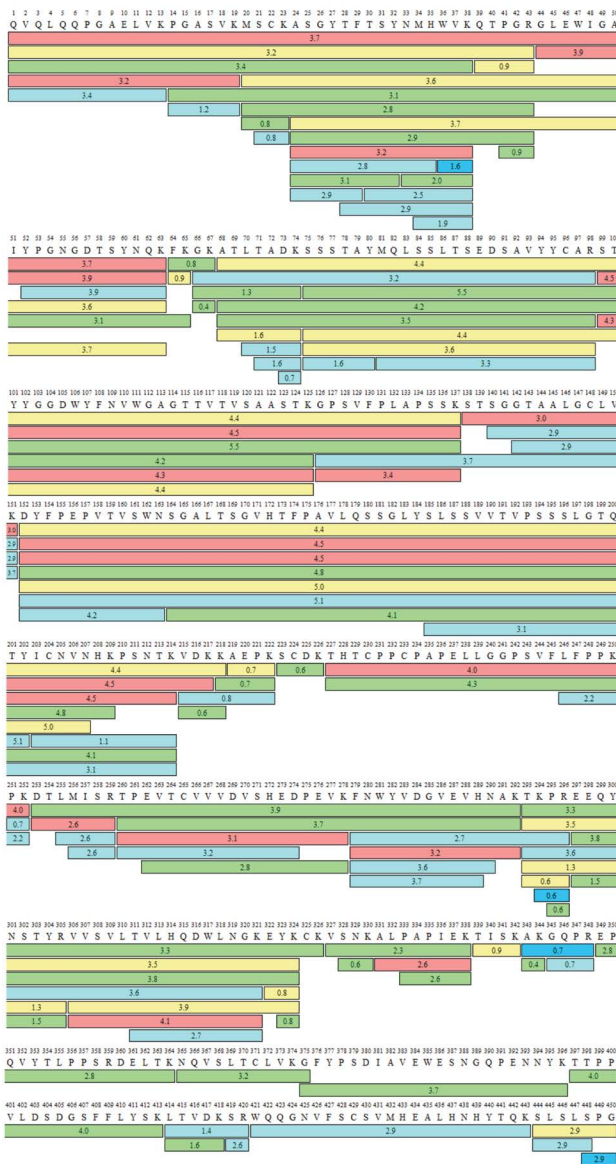


Figure 3. Total ion chromatograms obtained from peptide mapping experiments of rituximab applying gradient lengths of 30, 20, 13, 8, and 5 min. Flow rates and resulting pressures are indicated in the individual traces.

### Rituximab Heavy Chain



### Rituximab Light Chain

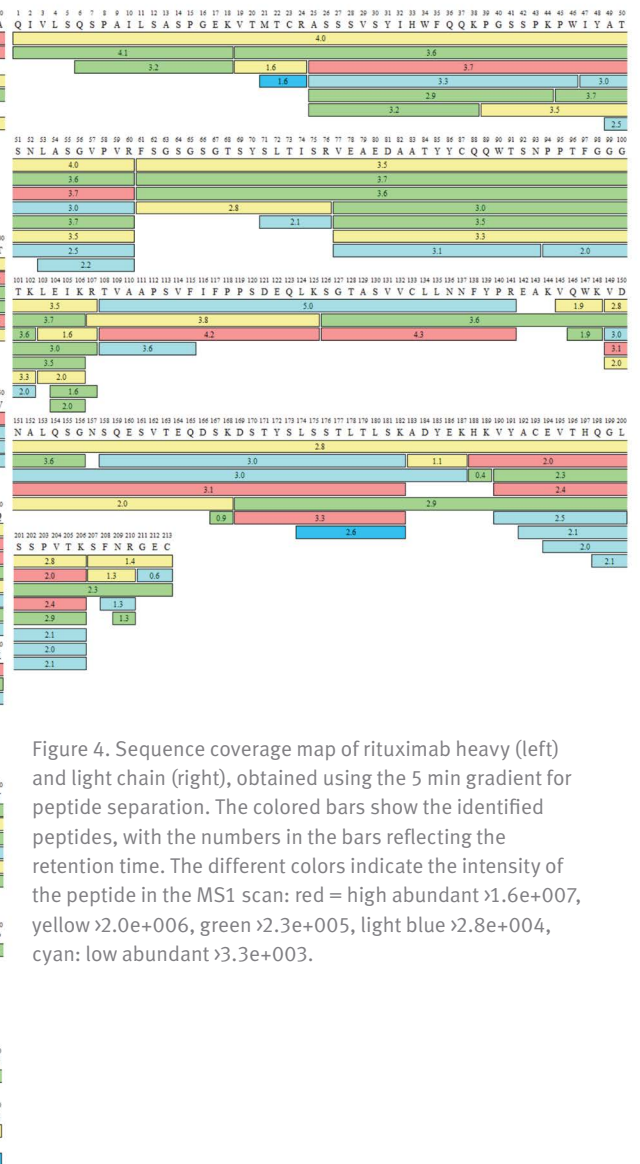


Figure 4. Sequence coverage map of rituximab heavy (left) and light chain (right), obtained using the 5 min gradient for peptide separation. The colored bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the intensity of the peptide in the MS1 scan: red = high abundant >1.6e+007, yellow >2.0e+006, green >2.3e+005, light blue >2.8e+004, cyan: low abundant >3.3e+003.

Table 1. Comparison of the oxidation, deamidation, and glycosylation modifications identified in runs obtained from the different gradient times.

Protein Chain	Modification	Recovery	Gradient Time					$\sigma$
			30 min	20 min	13 min	8 min	5 min	
			Abun.	Abun.	Abun.	Abun.	Abun.	
Rituximab_LC	Q1+NH <sub>3</sub> loss	Good	91.95%	91.17%	89.69%	90.93%	26.57%	28.80%
Rituximab_HC	~Q1+NH <sub>3</sub> loss	Good	99.62%	99.67%	99.61%	99.68%	99.69%	0.04%
Rituximab_HC	N33+Deamidation	Good	0.52%	0.51%	0.58%	-	0.51%	0.03%
Rituximab_HC	M34+Oxidation	Good	1.64%	1.54%	1.73%	1.42%	1.45%	0.13%
Rituximab_HC	N301+A1G0F	Fair	4.32%	4.42%	3.83%	3.52%	3.38%	0.46%
Rituximab_HC	N301+A1G1F	Good	1.87%	1.91%	1.72%	3.32%	1.46%	0.73%
Rituximab_HC	N301+A2G0	Good	1.09%	1.02%	1.02%	-	0.98%	0.05%
Rituximab_HC	N301+A2G0F	Good	37.88%	37.11%	38.59%	40.48%	43.12%	2.41%
Rituximab_HC	N301+A2G1F	Good	42.06%	41.89%	43.42%	43.20%	43.35%	0.75%
Rituximab_HC	N301+A2G2F	Good	10.23%	10.17%	9.81%	10.36%	10.05%	0.21%
Rituximab_HC	N301+A2S1G0F	Fair	0.83%	0.86%	-	-	-	0.02%
Rituximab_HC	N301+A2S1G1F	Fair	2.14%	-	-	-	-	-
Rituximab_HC	N301+A3Sg1G0	Fair	1.30%	-	-	-	-	-
Rituximab_HC	N301+M5	Good	1.61%	1.59%	1.66%	1.87%	1.86%	0.14%
Rituximab_HC	N301+Unglycos.	Good	0.54%	0.90%	0.76%	0.83%	0.97%	0.16%
Rituximab_HC	G450+Lys	Fair	3.57%	3.56%	3.92%	3.40%	3.15%	0.28%
							<b>Median 0.19%</b>	

Since the quantification of modified peptides performed in PepFinder 2.0 software requires their identification based on MS/MS spectra, special care has to be taken in choosing the appropriate ion injection times in the method setup enabling the acquisition of high quality MS/MS spectra required for a positive identification. This is especially true for glycopeptides analyzed using HCD fragment ion spectra, which contain exclusively ions representing sequential loss of glycan residues and no fragment ions representing the decomposition of the peptide as shown in Figure 5. The identification of peptides and modified peptides using PepFinder 2.0

software is based on the comparison of a simulated and the measured spectrum. The strength of the implemented algorithm for spectra and especially fragment ion intensity prediction is displayed in Figure 5 showing the MS/MS spectrum of the low abundant glycosylated peptide TKPREEQFN\*STFR (\*=A2G0) identified in the 5 min run with the typical fragmentation pattern: the two oxonium ions 204 (HexNAC), 366 (Hex-Hex-NAC), and the sequence ladder of the fragmented glycan attached to the intact peptide. The precursor ion of this glycopeptide with 735.58 *m/z* and a +4 charge state is indicated with the dotted blue line in the measured MS/MS spectrum.

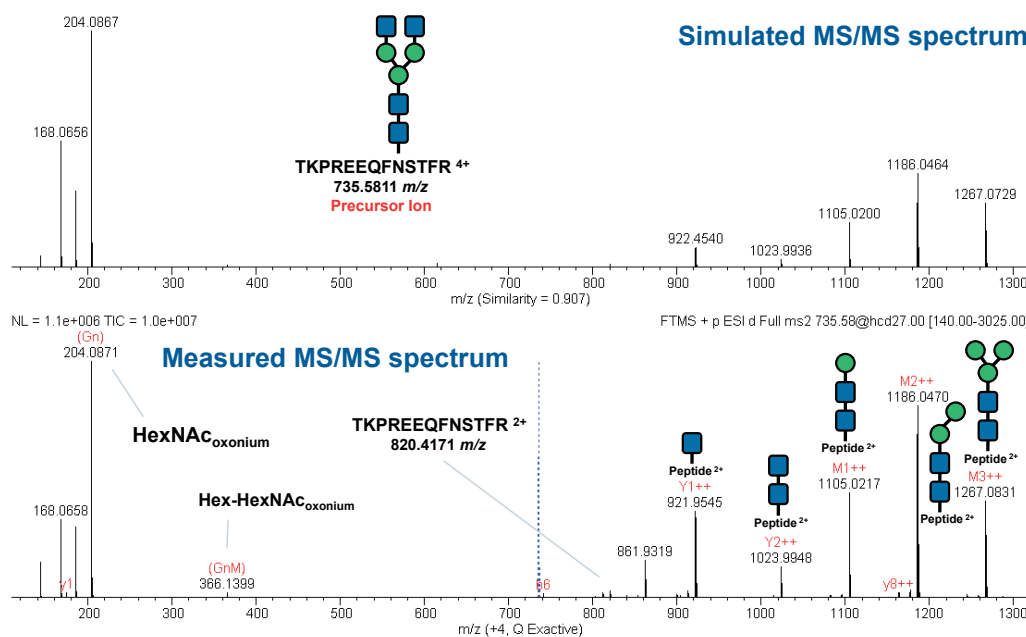


Figure 5. Simulated (top) and measured (bottom) MS/MS spectra of the glycopeptide aa 290-302 (TKPREEQFN\*STFR, \*=A2G0) in the 5 min gradient run.



Even with ultra-short gradients down to 5 min, as shown in Figure 6A, spectacular separation efficiency with average peak width at half maximum ( $w_{1/2}$ ) of 1.41 s were obtained. Figure 6B highlights the number of scans obtained across one chromatographic peak. Typically six Full MS spectra and 25 (5 x Top5) MS/MS spectra were acquired over a chromatographic peak of 2.4 s width. The achieved scan speed is key to the success in obtaining full sequence coverage.

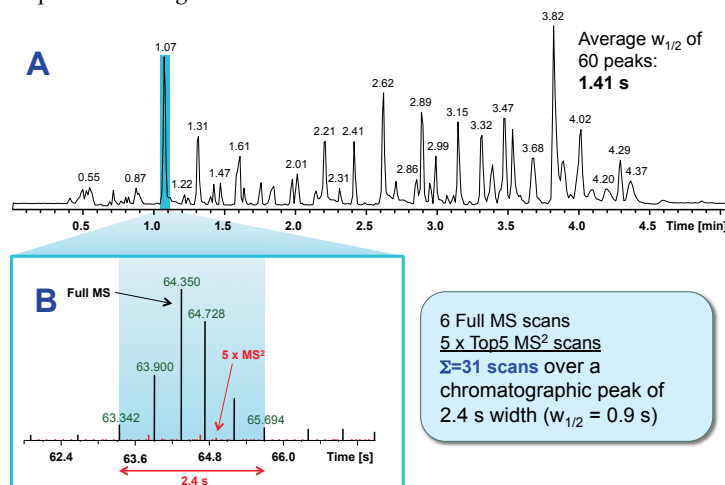


Figure 6. A) Total ion chromatogram of a five minute gradient separation of denosumab and B) data point distribution for a Full MS / ddMS<sup>2</sup> Top5 method during a representative chromatographic peak.

## Conclusion

- The applied hardware setup chosen for the experiments consisting of the Vanquish UHPLC system with an Acclaim RSLC 120 2.1 x 250 mm column installed with Thermo Scientific™ Viper™ Fingertight Fitting connections, attached online to the Q Exactive HF mass spectrometer equipped with the HESI-II source, provides a very robust system allowing for very high reproducibility and long term stability.
- Flow rates between 0.4 and 1.1 mL/min, depending on the chosen gradient lengths between 5 and 30 min, delivered chromatograms with peak widths at half maximum of less than 1 second.
- The accelerated scan speed of the Q Exactive HF mass spectrometer delivered sufficient data points over a chromatographic peak and clearly illustrates the compatibility with fast UHPLC separations.
- For all gradient times ranging from 5 to 30 min, 100% sequence coverage for the light and heavy chains for both rituximab and denosumab was obtained.
- The analysis of the most commonly targeted modifications such as the presence/absence of a C-terminal Lys, the N-glycosylation of asparagine on the heavy chain, the N-terminal pyro-glutamine formation on heavy and light chains, oxidations and deamidations, were successfully identified and relatively quantified. However, the obtained results suggest using the slightly longer gradient times of 20 to 30 min for in-depth analyses to also capture the very low abundant modifications.
- The data presented in this study clearly demonstrate the capability of the applied LC-MS setup to significantly speed up peptide mapping experiments enabling high throughput analyses as required e.g. during clone selection in the development phase of biopharmaceuticals.

## References

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