Application Note 1134

LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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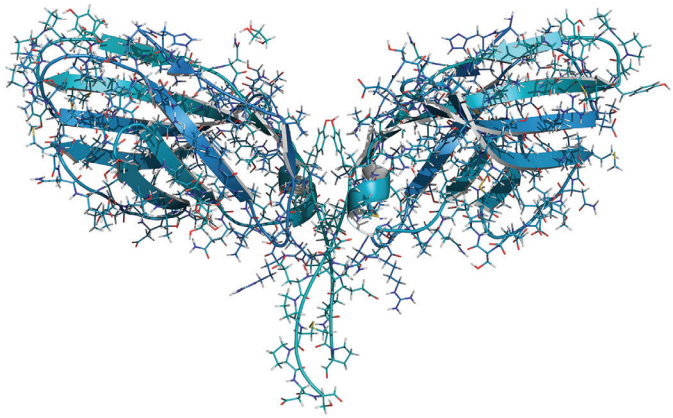
Key Words
Monoclonal Antibodies, Acclaim C18 RSLC Column, Q Exactive HF Mass Spectrometer, Biocompatible UHPLC, SMART Digest Kit, Biotherapeutics Characterization, Biopharma

Goal
Prove the suitability of a Thermo Scientific™ Vanquish™ Flex system for efficient and reliable peptide mapping method development with a LC-UV-MS setup.



Introduction
Peptide mapping is one of several routine methods to characterize biopharmaceutical proteins. For research environments, this technique, if combined with mass spectrometry (MS), is utilized for the characterization and confirmation of the primary sequence of monoclonal antibodies. In addition, peptide mapping can help to identify, localize, and quantitate post-translational modifications (PTMs). Peptide mapping methods are often developed and evaluated with combined UV and MS detection, to simplify the transfer to routine environments where UV detection is used alone. In high-throughput workflows, peptide mapping experiments are performed for antibody identity confirmation, PTM characterization, and stability studies.

The new Vanquish Flex UHPLC system features a quaternary pump[1] for highest application flexibility and fully biocompatible flow path. In addition, similar to the Thermo Scientific Vanquish UHPLC system[2], the sample is pressurized prior to the injection into the high pressure flow path. This results in a highly stable flow delivery and thus significantly improved retention time precision, increasing the confidence in peak assignment in peptide mapping experiments with UV detection.[3]

In this work, the separation of peptides obtained from a monoclonal antibody digest is demonstrated with a LC-UV-MS setup.

Experimental
The commercially available monoclonal antibody rituximab (F. Hoffmann-La Roche, Ltd) was digested using the Thermo Scientific™ SMART Digest™ kit. It is designed for applications that require highly reproducible, sensitive, and fast analyses, due to its optimized, heat stable, immobilized trypsin design. The sample was 1:4 diluted with the SMART digestion buffer included in the kit, and enzymatic digestion was allowed to proceed at 70 °C for 75 min and 1400 rpm. Disulfide bonds were reduced by incubation for 30 minutes at 60 °C with 5 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP). The separation of the tryptic digest was achieved with a 30 min gradient and a total analysis time of 56 min, including the column wash with high organic eluent, and re-equilibration at initial conditions. The Vanquish Flex system was coupled to the Thermo Scientific™ Q Exactive™ HF mass spectrometer using the MS connection kit for Vanquish systems. With this setup, simultaneous UV and MS detection is feasible.

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Equipment

Vanquish Flex UHPLC system consisting of:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (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

SMART Digest Kit (P/N 60109-101)

Data Analysis

Thermo Scientific™ Xcalibur™ software version 3.0 in combination with the Thermo Scientific Standard Instrument Integration (SII) for Xcalibur 1.1 SR2 was used for data acquisition and the data analysis was performed using Thermo Scientific™ PepFinder™ software version 2.0.

Results and Discussion

Peptide mapping experiments were performed with UV as well as MS detection. Figure 1 shows the overlay of the UV trace at 214 nm and the total ion current (TIC) chromatogram obtained from the mass spectrometer, which allows confident peak assignment (Figure 2).

To assess the sequence coverage, PepFinder software was used to analyze the data. The sequence coverage map (Figure 3) shows the overlap of the different peptides identified in different intensities, indicated with the color of the bar (red = high abundant, blue = low abundant), and in different lengths due to missed cleavages with sequence coverage for heavy and light chain of 99.2%. The number in the bar shows the retention time of the particular peptide.

Experimental Conditions - HPLC	
Column	Thermo Scientific™ 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	0–30 min: 4–55% B 30–31 min: 55–100% B 31–35 min: 100% B 35–36 min: 100–4% B 36–56 min: 4% B
Flow Rate	0.3 mL/min
Temperature	50 °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		
Sheath Gas Pressure	35 psi		
Auxiliary Gas Flow	10 arbitrary units		
Capillary Temperature	300 °C		
S-lens RF Voltage	60 V		
Source Voltage	3.5 kV		
Full MS Parameters		**MS^2 Parameters**	
Full MS Mass Range	200–2000 *m/z*	Resolution Settings	15.000
Resolution Settings	60.000	Target Value	1e5
Target Value	3e6	Isolation Width	2.0 Da
Max Injection Time	200 ms	Signal Threshold	1e4
Default Charge State	2	Normalized Collision Energy (HCD)	27
SID	0 eV	Top-N MS^2	5
		Max Injection Time	100 ms

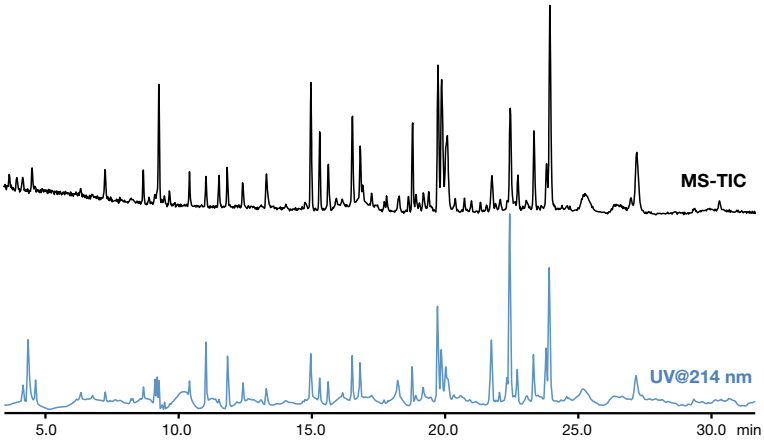

Figure 1. Overlaid chromatograms of the total ion current (TIC) and the UV trace at 214 nm of a SMART Digest Kit digested rituximab sample with subtracted blank baseline.

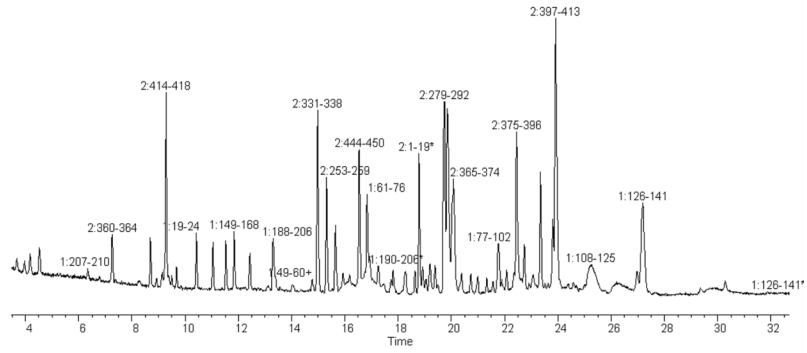

Figure 2. Peak assignment of the tryptic peptides from rituximab. Peak labels with 1 correspond to the light chain, and those with 2 correspond to the heavy chain of the mAb. The number after the colon indicates the amino acid region of this particular tryptic peptide.

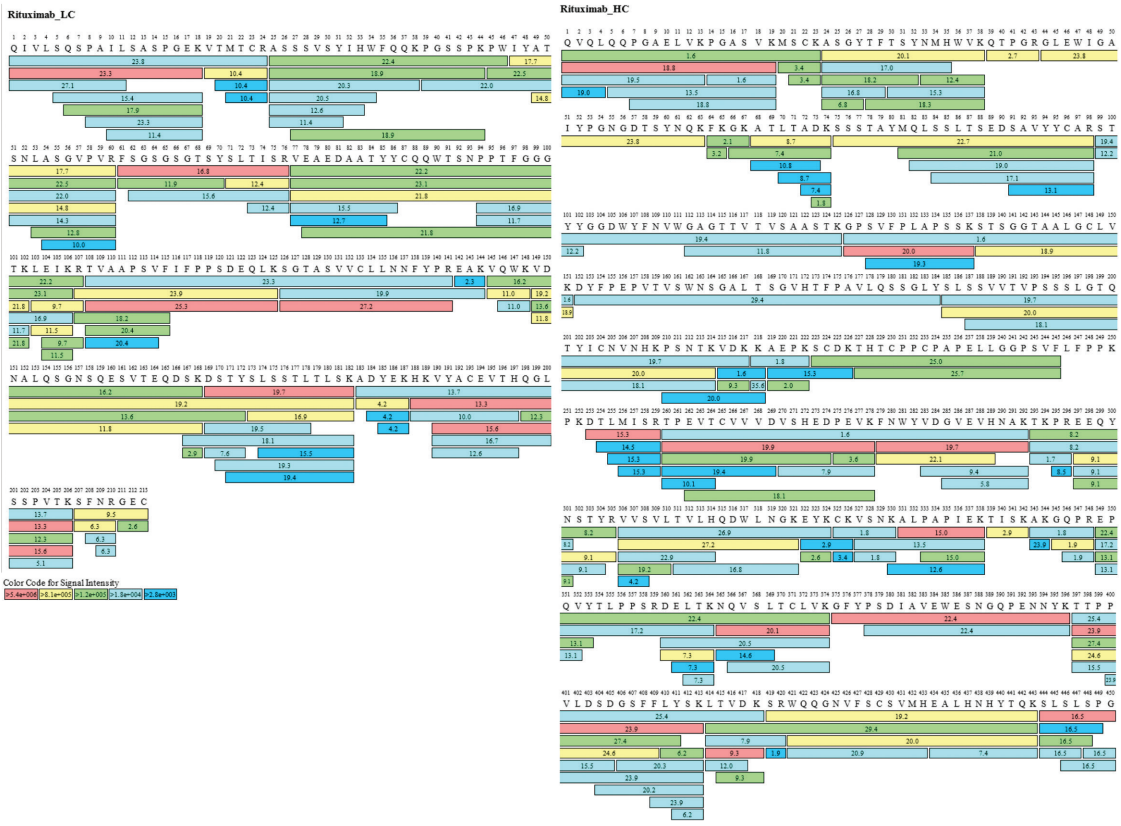

Figure 3. Sequence coverage map of the heavy (right) and light chain (left).

Table 1 shows the identification and relative quantification of a subset of monitored modifications on the light and heavy chain of rituximab, respectively. The selected modifications are deamidations, oxidations, pyro-Gln formations on the N-terminus of heavy and light chain, glycosylation of the N301 on the heavy chain, and sequence variants like C-terminal Lys (K+ variant). 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%.

Table 1. Identification and (relative) quantification of a specific set of modifications (oxidation, glycosylation and deamidation) on the mAb.

Protein	Modification	Recovery	Abundance
Rituximab_LC	Q1+NH_3 loss	Good	87.81%
Rituximab_LC	W90+Oxidation	Good	2.06%
Rituximab_HC	~Q1+NH_3 loss	Good	100.00%
Rituximab_HC	W281+Oxidation	Good	4.98%
Rituximab_HC	N301+A1G0F	Fair	2.87%
Rituximab_HC	N301+A1G1F	Fair	1.22%
Rituximab_HC	N301+A2G0	Fair	1.30%
Rituximab_HC	N301+A2G0F	Fair	37.69%
Rituximab_HC	N301+A2G1F	Fair	44.86%
Rituximab_HC	N301+A2G2F	Fair	10.77%
Rituximab_HC	N301+M5	Fair	1.07%
Rituximab_HC	N365+Deamidation	Good	2.72%
Rituximab_HC	W385+Oxidation	Good	5.37%
Rituximab_HC	G450+Lys	Good	3.2683%

Conclusion

For peptide mapping, especially the combination of UV and MS detection, the Vanquish Flex setup chosen for the experiments, consisting of column size of 2.1 x 250 mm coupled with Thermo Scientific™ Viper™ Fingertight Fitting connections and a flow rate of 0.3 mL/min combined with the HESI-II source on the mass spectrometer, delivers a very robust setup allowing straightforward method transfer to UV-based QC applications. The SMART Digest Kit compliments this by delivering highly reproducible digestion of samples allowing for easier and more confident data interpretation.

References

1. Thermo Scientific Technical Note 108: Reliable Solvent Mixing in UHPLC. Sunnyvale, CA, 2011.
2. Thermo Scientific Oral Technical Presentation 71659: High Throughput Peptide Mapping with the Vanquish UHPLC System and the Q Exactive HF Mass Spetrometer. Germering, Germany, 2015.
3. Thermo Scientific Application Note 1132: Reliable Results in Peptide Mapping Using the Vanquish Flex UHPLC System. Germering, Germany, 2015.

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