Putting Peptides on the Map

Please click the circles to navigate

Digestion

Separation

Mass Detection

Data Interpretation

Introduction

Navigating Peptides

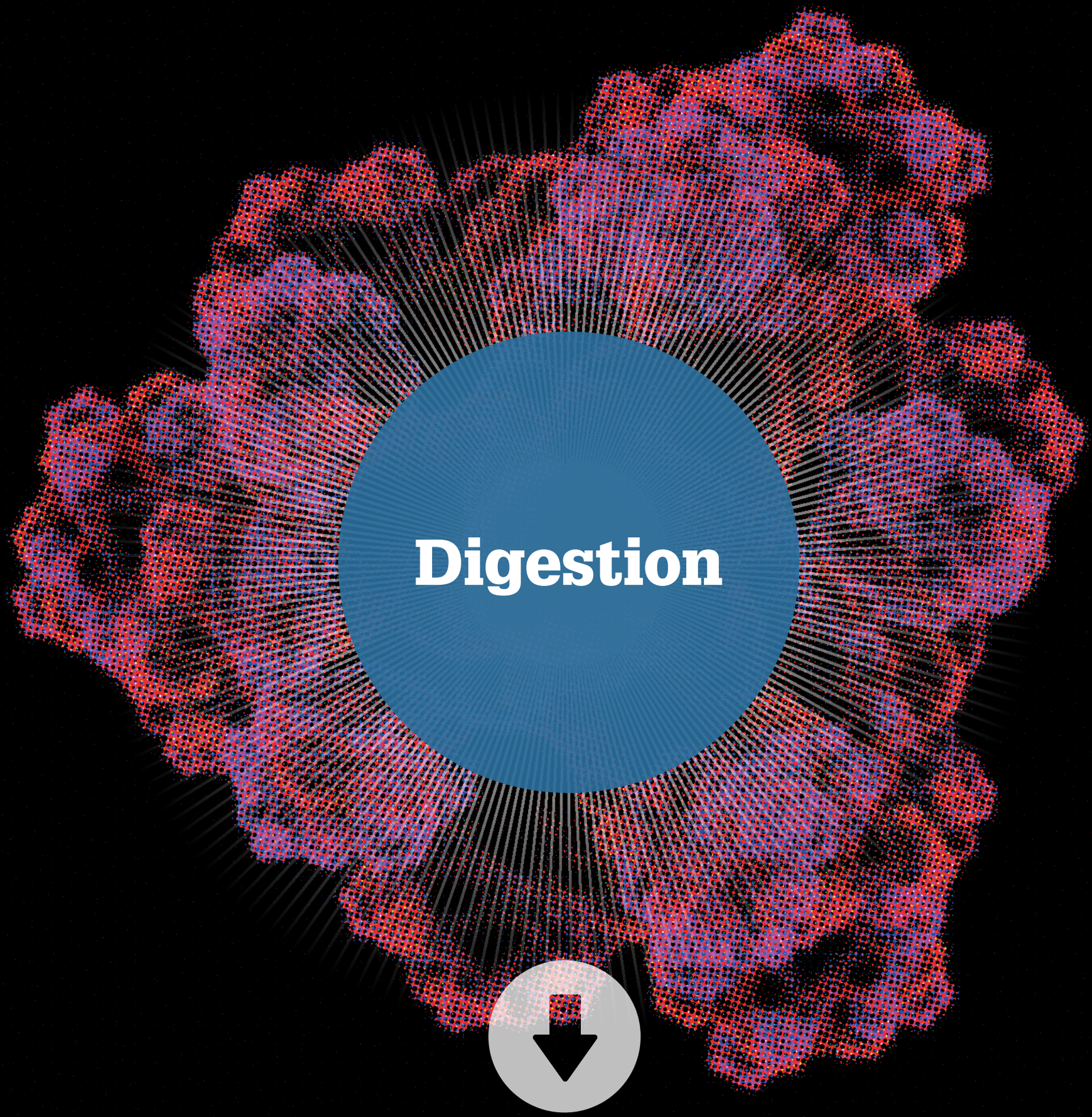
Streamlined workflows help scientists plot a course for painless peptide mapping

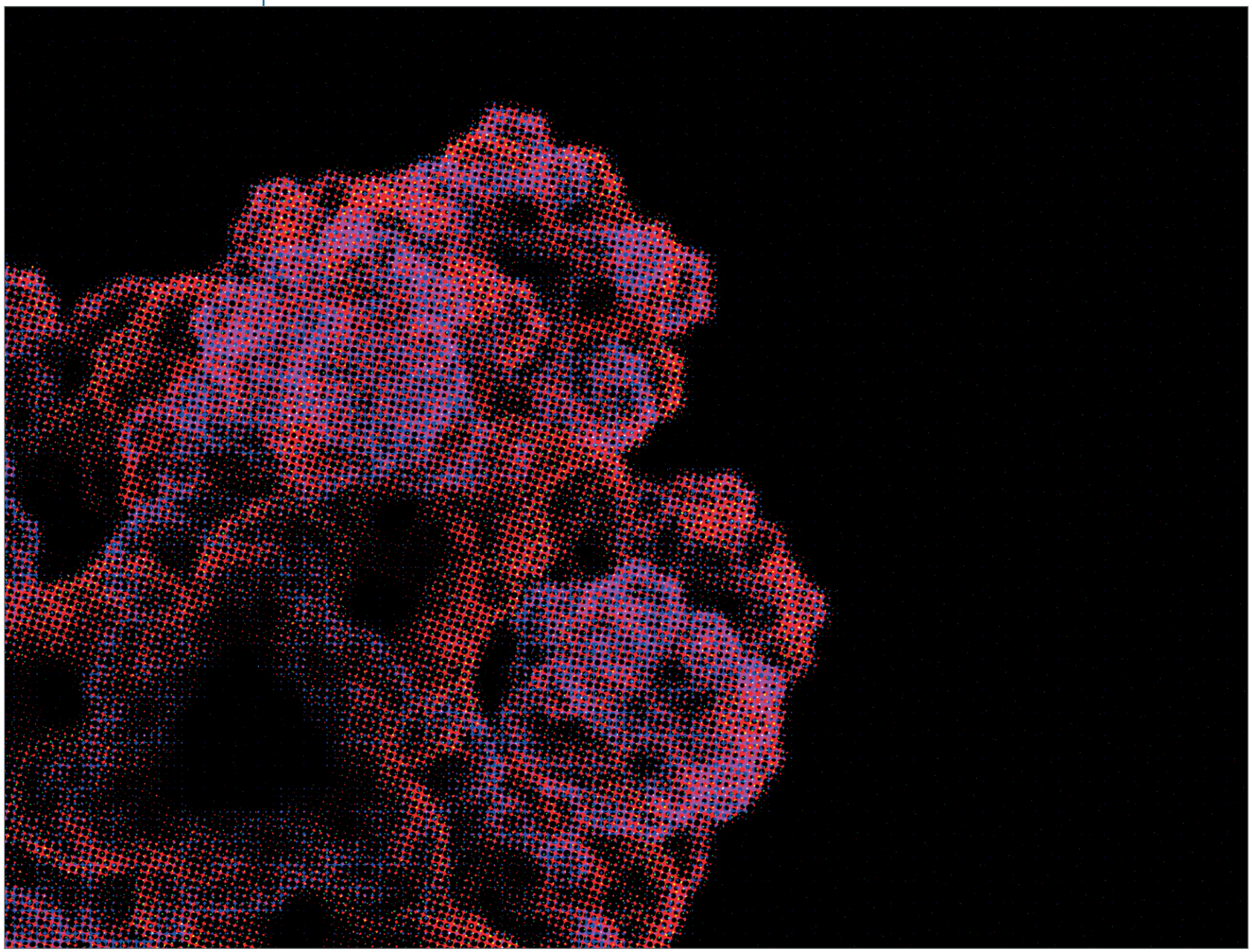
To ensure patient safety, it is vital that biopharmaceuticals are comprehensively characterized. Peptide maps that detail the entire protein are required to prove molecular structure as well as determine post-translational modifications and sequence variants – a level of detail that can't be obtained by intact mass analyses.

Peptide mapping has a reputation as a slow and laborious process, but new technology is making it easier to get robust and reproducible results. Monoclonal antibodies and antibody–drug conjugates pose unique challenges, and require high peak capacity, high-resolution separations, reliable detection, and confident data analysis.

In the following pages, Thermo Fisher Scientific presents a series of case studies, detailing how advances in digestion, separation, mass detection and data interpretation are removing roadblocks in protein characterization. In each section, you'll also find links to a wide range of resources, including videos, infographics and online articles.

Charlotte Barker
Editor


Digestion



SMART Digest compared to classic in-solution digestion of rituximab for in-depth peptide mapping characterization

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Key words
SMART Digest, tryptic digestion, in-solution protein digestion, monoclonal antibody, mAb, Vanquish, reversed phase, mass spectrometry, Q Exactive, Orbitrap, biopharmaceutical, biomolecules, peptide mapping

Goal

To compare the results achieved by using the newly developed Thermo Scientific™ SMART Digest™ kit to those obtained from classic in-solution protein digestion methods, focusing on protein sequence coverage and identified post-translational modifications (PTMs), including deamidation, oxidation, and glycosylation. A Thermo Scientific™ Acclaim™ VANQUISH™ C18 column with conventional water/acetonitrile-based gradients and the Thermo Scientific™ Vanquish™ Flex UHPLC system were used for separation in combination with the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer.

Introduction

Peptide mapping is a common technique in the biopharmaceutical industry to characterize monoclonal antibodies (mAbs) for the determination of product identity and stability. Many conventional sample preparation methods are time consuming with digestion times of several hours and can introduce modifications such as deamidation, oxidation, and carbamylation[1]. In this study, two classic in-solution digestion approaches were compared to the recently developed SMART Digest kit method to quantify the extent

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Video
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MS Source Parameters	*Setting*
Source	Ion Max source with HE-SI-II probe
Sheath Gas Pressure	45 psi
Auxiliary Gas Flow	12 arbitrary units
Probe Heater Temperature	350 °C
Source Voltage	3.5 kV
Capillary Temperature	350 °C
S-lens RF Voltage	60 V

Table 1A. Q_Exactive HF mass spectrometer source parameters.

of post-translational and chemical modifications of a therapeutic recombinant mAb. The critical requirements for each method were the complete sequence coverage of the heavy and light chain and the accurate identification and (relative) quantification of the glycans attached to the asparagine 301 on the heavy chain. Deamidation, oxidation, and carbamylation are induced primarily during sample preparation and were thus monitored for a direct comparison of the different digestion methods. A time course experiment for the SMART Digest was performed to assess the influence of digestion time on modification formation.

Experimental

Consumables

- Thermo Scientific Acclaim VANQUISH C18, 2.2 µm,
- 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific SMART Digest Kit (P/N 60109-101)
- Fisher Scientific™ LCMS Grade Water (P/N W/011217)
- Fisher Scientific™ LCMS Grade Acetonitrile
- (P/N A/0638/17)
- Fisher Scientific™ Optima™ LCMS Trifluoroacetic Acid
- (P/N 10125637)
- Thermo Scientific™ Pierce™ Formic Acid LCMS Grade
- (P/N 28905))
- Thermo Scientific™ Pierce™ Trypsin Protease MS Grade
- (P/N 90057)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol),
- No-Weigh™ Format (P/N 20291)
- Thermo Scientific™ Pierce™ Urea (P/N 29700)
- Thermo Scientific™ Pierce™ Iodoacetamide (P/N 90034)
- Thermo Scientific™ Invitrogen™ UltraPure™ Tris Hydrochloride (P/N 15506017)

Sample pretreatment and sample preparation

A commercially available monoclonal antibody rituximab drug product (Hoffmann La Roche, Basel, Switzerland) was supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.7 mg/mL polysorbate 80, 7.35 mg/mL sodium citrate dehydrate, 9 mg/mL sodium chloride, and sterile water adjusted to pH 6.5 using sodium hydroxide.

In-solution digestion protocol using urea for denaturation

400 µg rituximab were denatured for 75 min in 7 M urea and 50 mM tris hydrochloride (HCl) at pH 8.0, followed by a reduction step using 5 mM dithiothreitol (DTT) for 30 min at 37 °C. Alkylation was performed with 15 mM iodoacetamide (IAA) for 30 min at room temperature, and the reaction was quenched by addition of 9 mM DTT. The sample was then diluted 1:10 (v/v) with 50 mM tris HCl pH 8.0 to reach a final urea concentration below 1 M. Trypsin was added with a protein/protease ratio of 40:1 (w/w) and digestion was allowed to proceed overnight at 37 °C. Digestion was stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.5%. (Sample name: In-Solution, Urea)

In-solution digestion protocol using heat for denaturation

400 µg rituximab were denatured in 50 mM tris HCl at pH 8.0 and 70 °C for 75 min, followed by a reduction step using 5 mM DTT for 30 min at 70 °C. Alkylation was performed with 15 mM IAA for 30 min at room temperature, and the reaction was quenched by addition of 9 mM DTT. The sample was then diluted 1:10 (v/v) with 50 mM tris HCl pH 8.0. Trypsin was added with a protein/protease ratio of 40:1 (w/w) and digestion was allowed to proceed overnight at 37 °C. Digestion was stopped by addition of TFA to a final concentration of 0.5%. (Sample name: In-Solution, Heat)

SMART Digest kit protocol

50 µL rituximab sample, adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the SMART Digest buffer provided with the kit. It was

Time [min]	A[%]	B[%]	Flow Rate [mL/min]
0.0	96	4	0.3
50.0	25	75	0.3
51.0	0	100	0.3
60.0	0	100	0.3
61.0	96	4	0.3
80.0	96	4	0.3

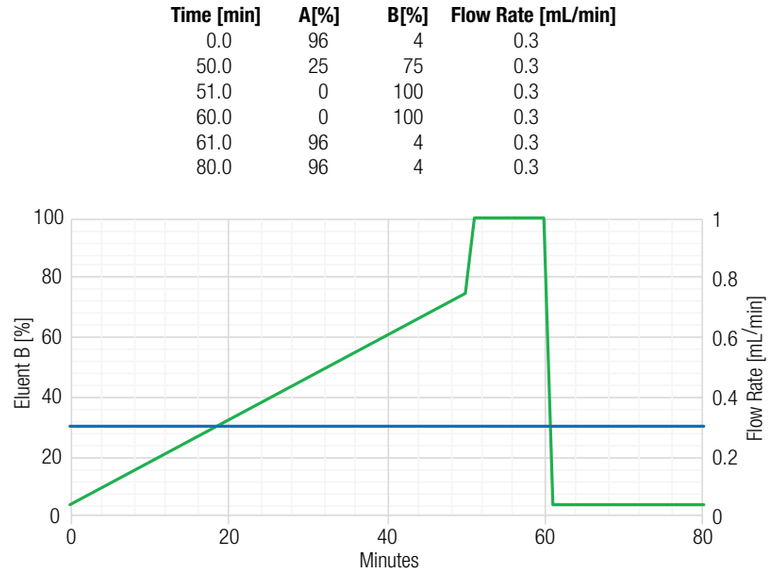

Figure 1. LC gradient.

Full MS Parameters	*Setting*	*MS2 Parameters*	*Setting*
Full MS Mass Range	*m/z* 140–2000	Resolution Settings	15,000 (FWHM at *m/z* 200)
Resolution Settings	60,000 (FWHM at *m/z* 200)	Target Value	1.0×10^5
Target Value	3.0×10^6	Isolation Width	2.0 *m/z*
Max Injection Time	100 ms	Signal Threshold	5.0×10^3
Default Charge State	2	HCD Normalized Collision Energy (NCE)	27
SID	0 eV	Top N MS2	5
Microscans	2	Max Injection Time Fixed First Mass Dynamic Exclusion	200 ms *m/z* 140.0 10.0 s

Table 1B. MS method parameters.

LINKS

Online Article
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Video
BioPharMoore Episode 2: SMARTening up on Protein Digestion

Component Detection	*Setting*	*Variable Modifications*	*Setting*
Absolute MS Signal Threshold	3.00 × 10^5 counts	N Terminal	Carbamylation
Identification	*Setting*	C Terminal	Lys
Mass Accuracy	5 ppm	Side Chain	Carbamidomethylation (C)
Minimum Confidence	0.8		Carbamylation (K)
Maximum Number of Modifications for a Peptide	2		Deamidation (N)
Unspecified Modification	-58 to +162 Da		Dimethylation (K)
N-Glycosylation	CHO		Double Oxidation (MWC) Glycation (K)
Protease Specificity	High		Methylation (K)

Table 2. BioPharma Finder parameter settings for all samples.

Proteins	*Number of MS Peaks*	*MS Peak Area*	*Sequence Coverage*	*Relative Abundance*	*Sample*
1: Rituximab Light Chain	521	26%	100%	40%	SMART Digest, 15 min
	532	24%	100%	38%	SMART Digest, 30 min
	526	22%	100%	38%	SMART Digest, 45 min
	516	19%	100%	36%	SMART Digest, 75 min
	404	28%	100%	37%	In-Solution, Urea
	407	31%	100%	38%	In-Solution, Heat
2: Rituximab Heavy Chain	827	43%	100%	54%	SMART Digest, 15 min
	833	47%	100%	56%	SMART Digest, 30 min
	827	45%	100%	55%	SMART Digest, 45 min
	855	37%	100%	59%	SMART Digest, 75 min
	638	54%	100%	62%	In-Solution, Urea
	619	52%	100%	61%	In-Solution, Heat

Table 3. Sequence coverage with different digestion methods.

then transferred to a reaction tube containing 15 µL of the SMART Digest resin slurry, corresponding to 14 µg of heat-stabile, immobilized trypsin. A time course experiment was performed and tryptic digestion was allowed to proceed at 70 °C for 15, 30, 45, and 75 min at 1400 rpm; a digestion time of 45–60 min was found to be sufficient to achieve digestion completeness for mAb samples (Figure 2). After the digestion, the reaction tube was centrifuged at 7000 rpm for 2 min, the supernatant was transferred to a new tube, and the centrifugation step was repeated. Disulfide bonds were reduced by incubation for 30 minutes at 37 °C with 5 mM DTT. (Sample names: SMART Digest, 15, 30, 45, 75 min)

All samples were diluted with 0.1% formic acid (FA) in water to a final protein concentration of 100 ng/µL, and 2.5 µg were loaded on the column for all runs.

LC Conditions

Instrumentation

- Vanquish Flex Quaternary UHPLC System consisting of:
- Flex 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 (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A) (not used in the LC-MS experiments)
- Static Mixer for 200 µL mixing volume (P/N 6044.5110)
- MS Connection Kit Vanquish (P/N 6720.0405)

Separation conditions (unless noted otherwise in the text)

Column:	Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm
Mobile Phase A:	Water + 0.1% formic acid
Mobile Phase B:	Water/acetonitrile (20:80 v/v) + 0.1% formic acid
Flow Rate:	0.3 mL/min
Temperature:	50 °C, Forced air mode
Gradient:	See Figure 1

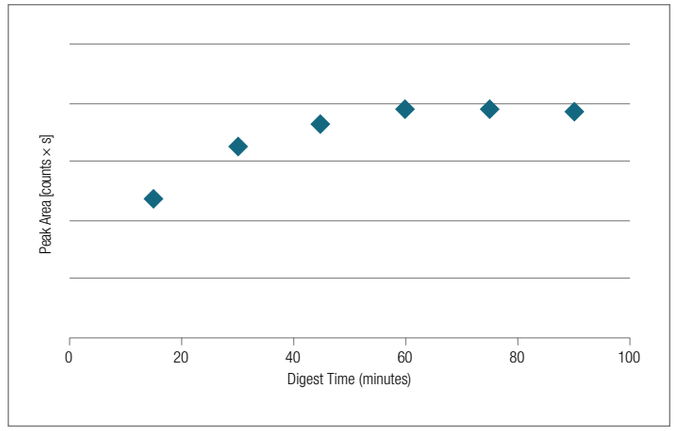

Figure 2. IgG digest profile, monitoring the mAb peptide VVSVLTVLHQDWLNGK for digestion times from 15–90 min using the SMART Digest kit.[2]

MS conditions

Instrumentation

The Thermo Scientific Q Exactive HF mass spectrometer (MS) was used for detection. The detailed MS source and method parameters are given in Tables 1A and 1B.

Data processing

The data were acquired with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR4. Thermo Scientific™ BioPharma Finder™ software, version 1.0 SP1, was used for data analysis. The algorithm parameters defined in Table 2 were identical for all samples.

Results and discussion

The SMART Digest kit provides fast and simple protein digestion with outstanding reproducibility, and digestion completeness for mAb samples is typically achieved within 45–60 min (Figure 2). Here, the relative standard deviation (RSD) was used to evaluate reproducibility, as demonstrated in Figure 3. Three separate digestions of the same mAb sample were conducted by three different analysts on different days. The peptide maps generated perfectly overlap with an average RSD for the peak area of less than 5%. These results impressively highlight the

reproducibility that can be achieved when using this novel digestion technique in combination with the Vanquish Flex UHPLC system featuring SmartInject technology for class-leading retention time reproducibility.

Comparing the total ion current (TIC) chromatograms of an in-solution digested sample and a SMART Digest sample (Figure 4) shows the similarity of the two digestion methods. The 75 min time point was chosen to mirror the elongated incubation time of an overnight digest. In general, the peptide pattern is homogenous and most of the detected peptides are aligned. Differences in the two chromatograms and identified peptides are highlighted. For some peptides, the intensity slightly differs between the two SMART and in-solution digest runs, for example, peptides "1:103–107" and "2:87–98". Others appear in only one of the two digestion methods, such as alkylated peptides "1:188–206 + alkyl". The injection peak eluting with the void volume of the SMART Digest sample is higher in comparison to the in-solution digested sample and is caused by salt components included in the SMART Digest buffer to optimize the digestion efficiency at elevated temperatures. This peak did not affect the result of the peptide map but could be easily removed if required. One option is to use a post-column diverter valve prior to the MS ion source. Another is to use Thermo Scientific™ SOLAμ™ SPE plates that allow highly reproducible post-digestion desalting of peptide samples by solid phase extraction (SPE).[3]

In peptide mapping analysis of mAbs, 100% sequence coverage for the heavy and light chains must be achieved. The sequence coverages for the different digest conditions are shown in Table 3. For all six methods, including the fast digestion methods of 15 and 30 min, 100% coverage was achieved for light as well as heavy chains. Strikingly, an incubation time of only 15 min is sufficient to achieve 100% sequence coverage for both the heavy and light chains of the antibody when the SMART Digest kit is used. The number of detected MS peaks in the samples digested with the SMART Digest kit were generally higher than in the in-solution digested samples. The same trend was observed when the number of identified components, including all peptides and charge states, and the total MS ion count were compared (Table 4).

Peptide mapping experiments can provide the identification, localization, and (relative) quantification of various post translational and chemical modifications (PTMs) that might be present on the amino acid residues. The relative abundance of all identified modifications (n=85) in the different runs are plotted in Figure 5. The relative abundance of the major modifications, including the pyro-glutamate formation (NH_3 loss) on the N-terminal glutamine of heavy as well as light chain and the most abundant glycoforms attached to the asparagine 301 of the heavy chain (A2G1F, A2G0F and A2G2F), are shown in Figure 5. Sixteen cysteine carbamidomethylation sites were exclusively identified in the samples derived from the in-solution digested samples but not in the SMART Digest. This is consistent with the modification being caused by the alkylation with IAA during the sample preparation. For simplicity, the carbamidomethylation sites are not shown in Figure 5. Overall, similar levels for all modifications were detected for all digest protocols and no significant trend of an increased or decreased amount in any of the conditions tested was observed. Noteworthy, for many modification sites, e.g. deamidation of N319 and oxidation of W106, the amount in the reduced SMART Digest samples were lower compared to the in-solution-digested samples even when a 75 min (over-)digestion with the SMART Digest was applied.

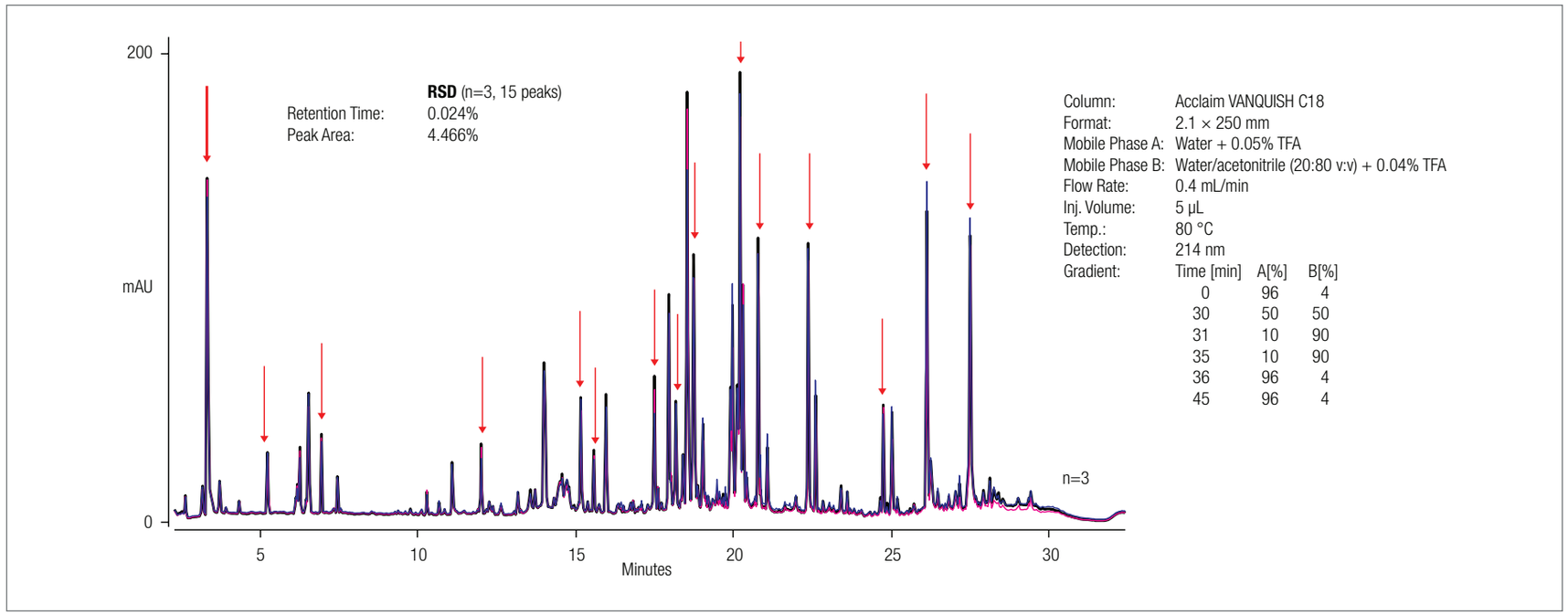

Figure 3. UV chromatogram overlay from three separate SMART digestions from the same mAb, conducted by three individual operators. The 15 marked peptides in each sample were used for inter-user/inter-day RSD value calculations.

The monoclonal antibody rituximab used in this study consists of 1328 amino acid residues includ-ing 16 disulfide bonds.4 Amongst several potential PTMs of amino acids, deamidation of aspara-gine or glutamine and oxidation of methionine or tryptophan represent common chemical modifications for mAbs during downstream processing and storage. Figures 6A and 6B show the extent of amino acid oxidation, and deamidation, respectively, for oxidations for the different digestion methods. Table 5 summarizes the quantification results for the individual modification sites. The variance between the six digestion methods is expressed as the RSD of the measured relative abundance for each modification with each of the digestion protocols. With the exception of the oxidation of W106 that was high in the in-solution digested samples, all results are comparable, resulting in RSD values ≤ 1%. For for the identified deamidations, the maximum RSD value was 3.164% and with an average RSD of 0.913%. While a clear trend of increased deamidations with increasing sample incubation time could be observed between the six digestion methods (Figure 6B), less or equal amounts of deamidation were observed when the SMART

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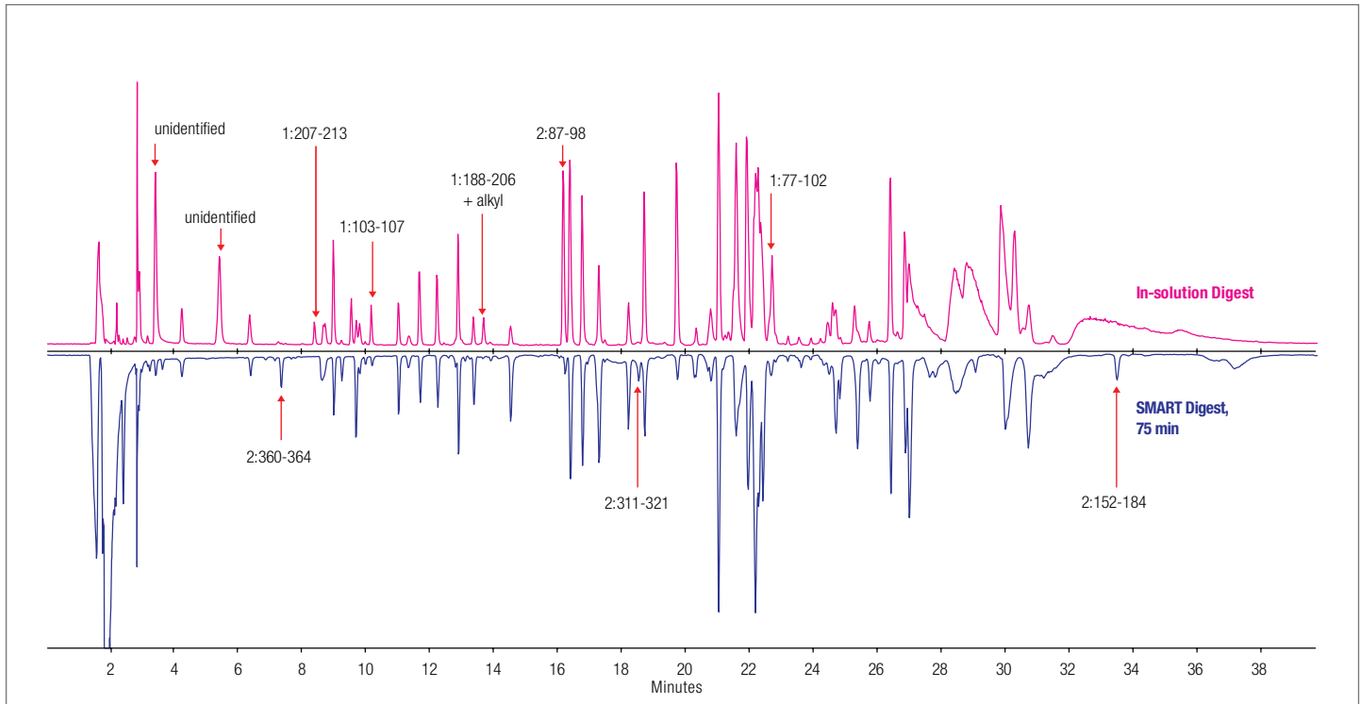

Figure 4. Mirror plot of the TIC chromatogram for the in-solution digested sample denatured with heat (In-Solution, Heat) and the reduced SMART Digest sample (SMART Digest, 75 min). Peak labels give annotation to light (1) or heavy (2) chains, respectively, and sequence position.

Digest kit was used at the recommended digestion time of ≤ 45 min (Figure 6B and Figure 7). Only two deamidation sites (N236 and N388) were more prone to undergo deamidation under the SMART Digest conditions and required a reduced incubation time of 30 min. Another critical modification is the carbamylation of lysine residues and protein N-termini (+43.006 Da), which is a non-enzymatic PTM that has been related to protein aging.5 It can be artificially introduced during sample preparation using urea as the protein denaturing agent. For in-solution tryptic digest with urea in the sample preparation, the average carbamylation of lysine was ≤ 1% relative abundance (n=6). For the SMART Digest samples, the average carbamylation was considerably lower in the ppt range or not detectable at all (Table 5). Other 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-glutamate formation on heavy and light chains, and lysine glycations are listed in Table 5. In total, six lysine glycations and 12 glycosylations of N301 could be identified and (relatively) quantified with an average RSD value of 0.423%.

Based on all identified oxidations (n=12) and deamidations (n=7), the deamidation and oxidation factor was calculated for each individual sample (Figure 7). The in-solution digested sample with heat denaturation had the highest induced modification rate of the compared methods, with a deamidation factor of 8.754 and an oxidation factor of 2.923. In contrast, the SMART Digest samples that were reduced on peptide level showed the lowest levels of deamidation and oxidation compared to both in-solution digestion samples. The degree of deamidation increases with extended

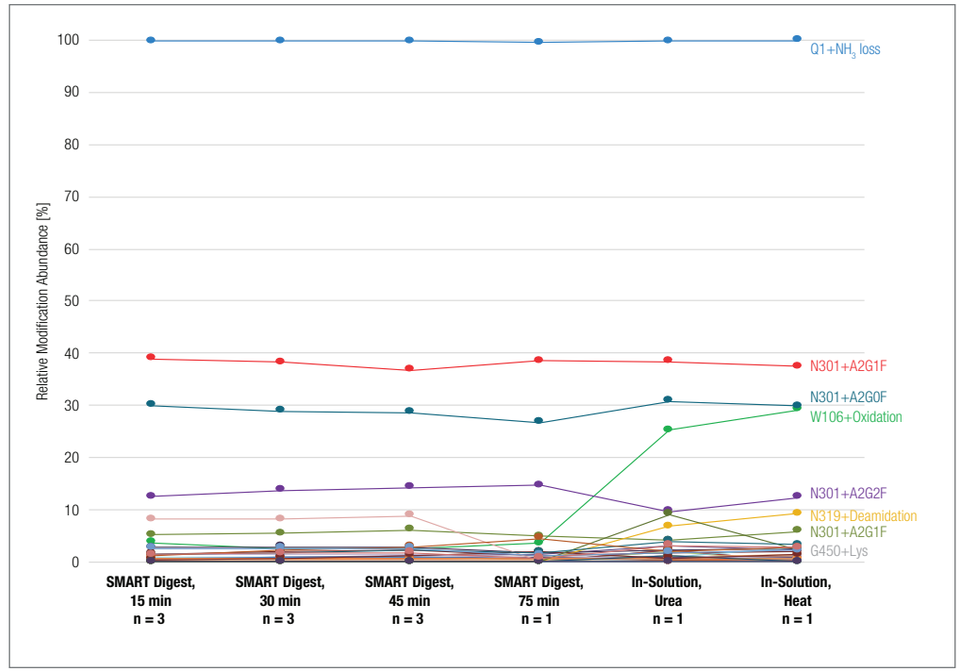

Figure 5. Relative abundance of 85 identified modifications including oxidation, double oxidation, glycation, glycosylation, NH_3 loss, isomerization, lysine truncation, methylation, dimethylation, and carbamylation.

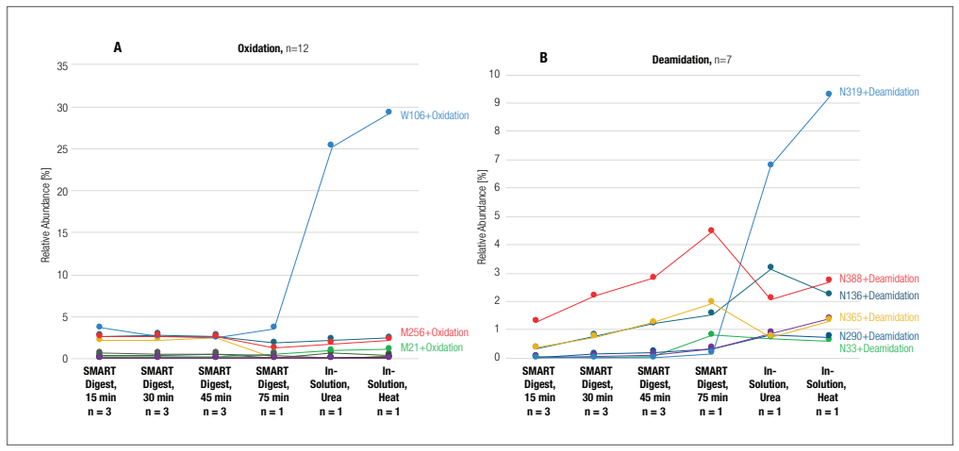

Figure 6. Relative abundance of 12 identified oxidations (A) and 7 deamidations (B) in different runs with various digestion methods.

# Identified Components	Total MS area [counts × s]	Sample
1702	3.48×10^9	SMART Digest, 15 min
1678	4.12×10^9	SMART Digest, 30 min
1688	3.96×10^9	SMART Digest, 45 min
1551	3.13×10^9	SMART Digest, 75 min
1171	3.65×10^9	In-Solution, Urea
1145	4.04×10^9	In-Solution, Heat

Table 4. Number of identified components and TIC area for the different runs.

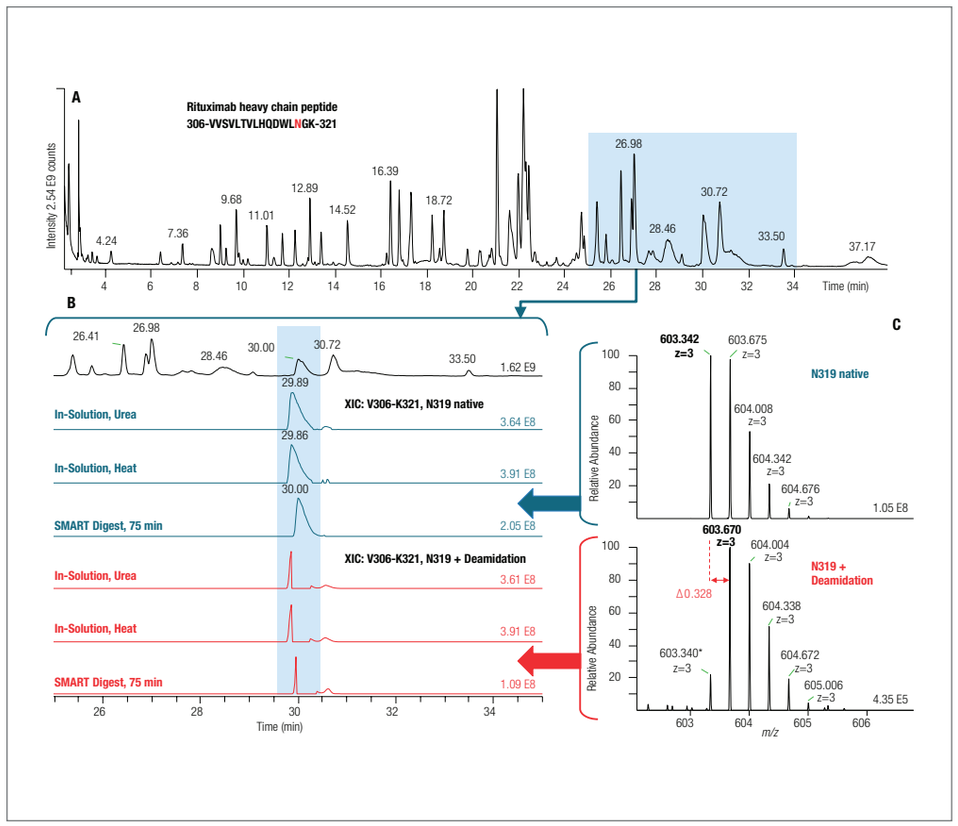

Figure 8. Total ion current chromatogram of the reduced SMART Digest sample, 75 min (A), and extracted ion current chromatograms (B) for the peptide V306-K321 in the native and the deamidated form for the different runs.
A comparison of the isotopic distributions of the [M+3H]3+ ions (C) for the native and deamidated V306-K321 peptide.

digestion times, and the lowest deamidation rate was observed for the sample digested for 15 min using the SMART Digest kit. Deamidation is, in general, accelerated at high temperatures and high pH values.[6] One way to minimize the degree of induced deamidation is to lower the pH of the digestion buffer. SMART Digest is performed at elevated temperatures but at a pH of 7.2, which is much lower than the pH of classical in-solution digestion methods. Thus, deamidation is minimized and is comparable to that observed for standard in-solution digests at 37 °C. Figure 5 also demonstrates that the extent of deamidation is location dependent. For some positions, lower levels of deamidation are observed for the SMART Digest, even when compared to the urea-treated in-solution digest (e.g. N33, N136, N319). For others, higher levels are observed with the SMART Digest and digestion times ≥ 45 min (e.g. N388).

Two of the tryptic peptides from rituximab have been identified as the most susceptible to deamidation under stress conditions.[7] The peptides 2:V306-K 321 (VVSVLTVLHQDWLNGK), containing N319, and 2:G375-K396 (GFYPSDIAVEWESNGQPENNYK), containing N388, are both located within the Fc region of the heavy chain, which shares the same sequence with other human or humanized mAbs. More than one asparagine is present in the sequences, but the asparagines highlighted in bold are identified as deamidation hot spots.[7] The second peptide is known as the "PENNY peptide", but both peptides are a decent indicator for induced deamidation of mAbs.[7]

Figure 8 shows the TIC chromatogram for the SMART Digest sample (Figure 8A) and extracted ion current (XIC) chromatograms with a 5 ppm mass extraction window for the different samples (Figure 8B). The XIC traces in blue are derived from the native 2:V306-K321 peptide present in all runs. The traces in red are the corresponding deamidated forms of the peptide (N319) eluting prior to the native peptide in the chromatogram. The relative abundance, based on all charge states of the deamidated peptide, is lowest in the 15 min digested SMART Digest sample at 0.001%. In contrast, a higher amount of deamidation (N388) was observed with the SMART Digest (45 and 75 min digestion time) for the PENNY peptide 2:G375-K396 (Table 5), but the lowest value of 1.267% could be observed with the 15 min method.

As shown in Figure 8C, the isotopic distribution of the triply charged native peptide is different from its deamidated form. The monoisotopic peak is highlighted in bold and, due to coelution of the two species, the monoisotopic peak (*; *m/z* 603.340) of the native peptide is also visible in the lower mass spectrum. A deamidation leads to a theoretical mass increase for the monoisotopic peak of 0.984 Da, which results in a mass shift of 0.328 Da for the triply charged signal and nicely correlates with the measured value.

Conclusion

The direct comparison of the SMART Digest kit with the conventional in-solution protein digestion methods conducted in this study showed no substantial difference for the mAb rituximab between the different approaches with respect to the data quality and information content obtained. Protein sequence coverage of 100% for rituximab was achieved with all six digestion methods tested and could be achieved in only 15 min when using the SMART Digest kit. The most common PTMs targeted for analysis, such as the presence/absence of a C-terminal Lys, the N-glycosylation of asparagine on the heavy chain, and the N-terminal pyro-glutamate formation on heavy and light chains, were identified, relatively quantified, and compared between the different digestion methods. Overall, the extent of chemical modifications detected was similar for all digestion methods. The elevated temperatures during enzymatic digestion using the SMART Digest kit did not increase the amount of induced deamidation compared to in-solution digested samples. In fact, the calculated deamidation (and oxidation) factors were lower or identical to the urea-treated samples, and heat-denaturation combined with in-solution digestion resulted in slightly increased modification levels. Optimization of the incubation time can be used to further minimize the introduction of chemical modification during digestion using the SMART Digest kit. For Rituximab,a digestion time of 15 min is feasible and results in complete sequence coverage and accurate relative quantification of PTMs. In contrast, prolonged digestion times >45 min can increase the amount of chemical modifications. Interestingly, some positions were more prone to undergo deamidation in one condition compared to the others, but no correlation with a specific digest condition was seen. Since the use of urea is omitted during the SMART Digest, lysine carbamylation was absent in SMART Digest and urea-treated samples. This contributed to a less complex but comprehensive peptide map.

The huge time-saving potential, ease of use, and outstanding reproducibility of the SMART Digest make it the heart of

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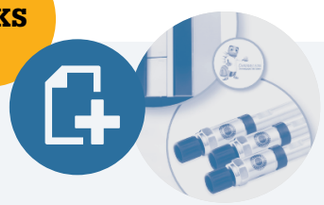

UHPLC Columns
VANQUISH UHPLC Columns

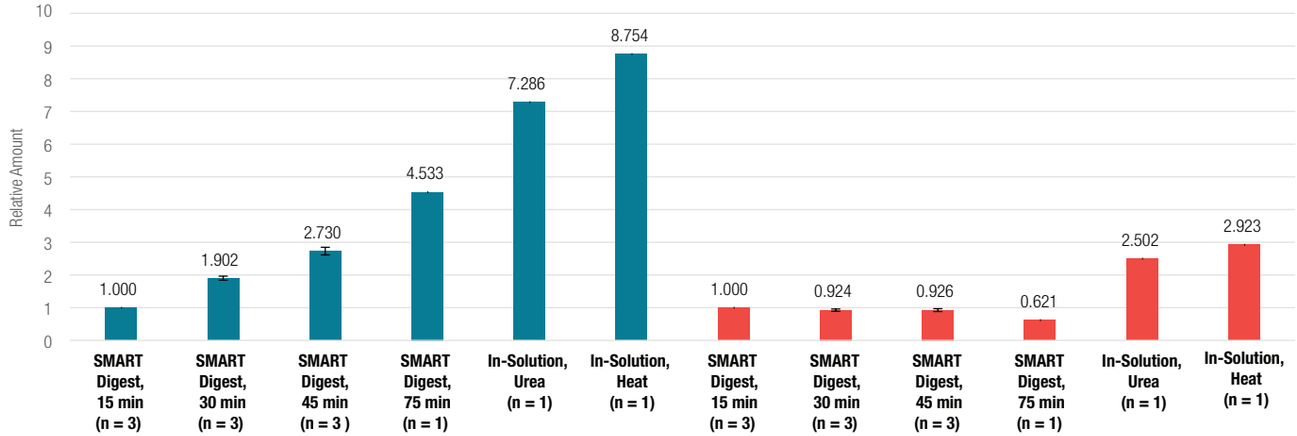

Figure 7. Relative amount of total deamidation and oxidation modifications measured for the six different digest conditions (Normalization to SMART Digest, 15 min).

a comprehensive peptide mapping workflow as applied in this study. When combined with the Vanquish Flex UHPLC system, Orbitrap-based mass spectrometer, and the simple yet powerful tools within Chromeleon and BioPharma Finder software, SMART Digest kit facilitates standardized, fast, and reproducible peptide mapping workflows.

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Relative Abundance [%]											
SMART Digest, 15 min (n=3)	SMART Digest, 15 min, RSD (n=3)	SMART Digest, 30 min (n=3)	SMART 30 min, RSD (n=3)	SMART Digest, 45 min (n=3)	SMART Digest, 45 min, RSD (n=3)	SMART Digest, 75 min (n=1)	In-Solution, Urea (n=1)	In-Solution, Heat (n=1)	RSD (%)*	Median (%)*	Modification
0	0	0.002	0.003	0	0	0.01	0.14	0.063	0.042	0	K63+Glycation
0.039	0.009	0.12	0.004	0.2	0.019	0.233	0	0	0.085	0.118	K102+Glycation
0.144	0.02	0.136	0.006	0.142	0.005	0.036	0	0	0.06	0.138	K137+Glycation
0.208	0.024	0.288	0.03	0.339	0.012	0.017	0.403	0.248	0.101	0.274	K148+Glycation
0.075	0.008	0.085	0.008	0.087	0.006	0.121	0.58	0.197	0.144	0.088	K168+Glycation
0.325	0.178	0.631	0.009	0.626	0.019	0.55	0.529	0.49	0.151	0.581	K182+Glycation
0.411	0.033	0.48	0.014	0.515	0.012	0.632	0.244	0.177	0.125	0.473	N301+A1G0
12.448	0.899	13.703	0.618	14.255	0.08	14.672	9.657	12.41	1.462	13.467	N301+A2G2F
5.141	0.373	5.476	0.186	6.166	0.148	4.852	4.088	5.777	0.642	5.458	N301+A1G1F
0	0	0	0	0	0	0	0.322	0.307	0.123	0	N301+A1S1F
0.703	0.05	0.776	0.035	0.796	0.029	0.928	0.895	0.88	0.08	0.781	N301+A2G0
30.052	2.351	28.838	1.471	28.667	0.971	26.689	30.825	29.838	1.652	29.229	N301+A2G0F
0.363	0.036	0.451	0.004	0.49	0.021	0.576	0.396	0.302	0.079	0.449	N301+A2G1
38.932	3.324	38.235	1.881	36.765	1.84	38.505	38.349	37.45	1.999	37.701	N301+A2G1F
1.386	0.055	1.496	0.067	1.435	0.047	0.714	1.093	0.739	0.288	1.404	N301+A2S1G1F
0.838	0.079	0.816	0.038	0.836	0.031	0.003	0.584	0.306	0.274	0.813	N301+A2S2F
0.278	0.005	0.302	0.03	0.291	0.03	0.268	0	0	0.114	0.274	N301+M4
0.753	0.068	0.994	0.104	0.966	0.088	0.577	0.728	0.489	0.188	0.847	N301+M5
96.85	0.066	96.802	0.208	96.946	0.486	96.788	97.655	97.886	0.428	96.815	Q1+Gln→Pyro-Glu
99.824	0.015	99.81	0.009	99.815	0.003	99.586	99.853	99.92	0.077	99.816	Q1+Gln→Pyro-Glu
1.348	0.537	1.685	0.036	1.778	0.045	0.673	3.083	2.684	0.667	1.735	G450+Lys
0.043	0.012	0.034	0.013	0.069	0.015	0.818	0.699	0.608	0.302	0.056	N33+Deamidation
0.334	0.069	0.778	0.054	1.213	0.027	1.545	3.159	2.233	0.842	1.014	~N136+Deamidation
0.035	0.008	0.132	0.004	0.219	0.004	0.321	0.823	0.734	0.261	0.175	~N137+Deamidation
0.034	0.005	0.07	0.037	0.115	0.02	0.343	0.879	1.399	0.428	0.103	N290+Deamidation
0.001	0	0.002	0.001	0.002	0.001	0.168	6.786	9.248	3.164	0.002	N319+Deamidation
0.368	0.038	0.747	0.019	1.257	0.045	1.951	0.738	1.314	0.486	0.757	N365+Deamidation
1.267	0.137	2.198	0.183	2.811	0.134	4.462	2.089	2.694	0.905	2.304	N388+Deamidation
2.177	0.04	2.211	0.125	2.522	0.059	0.065	0	0.001	1.043	2.2	M21+Oxidation
0.342	0.093	0.336	0.069	0.455	0.015	0.001	0.617	0.445	0.153	0.424	~M34+Oxidation
0.248	0.061	0.189	0.037	0.164	0.013	0.549	0.926	1.113	0.327	0.21	M81+Oxidation
3.654	0.683	2.56	0.348	2.435	0.152	3.556	25.225	29.209	9.505	3.064	W106+Oxidation
0.63	0.15	0.591	0.042	0.562	0.023	0.378	0.008	0	0.241	0.553	~W111+Oxidation
0	0	0	0	0	0	0	0.034	0.199	0.057	0	C133+Double Oxidation
0	0	0	0	0	0	0	0.011	0.179	0.051	0	C148+Double Oxidation
0	0	0	0	0	0	0	0.032	0.192	0.055	0	C193+Double Oxidation
2.673	0.158	2.843	0.254	2.686	0.243	1.82	2.215	2.407	0.344	2.578	M256+Oxidation
0	0	0	0	0	0	0	0.019	0.057	0.017	0	C265+Double Oxidation
0.016	0.004	0.021	0.002	0.033	0.002	0.068	0	0	0.018	0.02	C371+Double Oxidation
2.591	0.179	2.646	0.188	2.558	0.029	1.218	1.76	2.243	0.46	2.545	M432+Oxidation
0	0	0	0	0.001	0.001	0	2.192	0	0.633	0	~K38+Carbamylation
0	0.001	0	0	0	0	0	0.087	0	0.025	0	K38+Carbamylation
0.124	0.008	0.19	0.011	0.232	0.016	0.001	0	0	0.092	0.155	K102+Carbamylation
0.003	0.003	0.003	0.002	0.007	0.004	0.004	0.9	0.019	0.258	0.005	K278+Carbamylation
0	0	0	0	0	0	0	0.202	0	0.058	0	K321+Carbamylation
0	0	0.001	0	0.001	0.001	0	1.254	0	0.362	0.001	K338+Carbamylation

Table 5. Comparison of the oxidation, deamidation, and carbamylation modifications identified with the different digestion methods. **Between 6 digestion methods*

Separation

Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments

Mauro De Pra, Ken Cook, Mike Oliver, and Carsten Paul
Thermo Fisher Scientific

Key Words
Acclaim VANQUISH, Biocompatible UHPLC, Biopharma, Biotherapeutics Characterization, Monoclonal Antibodies, Protein Digest, Vanquish UHPLC System

Goal

Provide an ultra-high retention time and peak area precision example of the separation of a mAb digest.

Introduction

Peptide mapping of digested proteins are of high importance when characterizing biotherapeutics. Peptide maps are utilized to confirm the expression of the intended amino acid sequence, to confirm genetic stability or to identify post-translational modifications, especially when interfaced with mass spectrometry. Reversed phase separation in combination with only UV detection is, however, still very common in stability studies, for in process measurements and quality assurance. In these cases peak areas, peak area ratio and retention times are sufficient to provide the required information. For highest confidence in the qualitative and quantitative results of such assays, the retention time as well as the peak area has to be extremely stable.

The Thermo Scientific™ Vanquish™ UHPLC system features a binary pump with extremely low pulsation ripple due to a brand new pump concept. In addition, the Vanquish UHPLC system SmartInject feature results in a highly stable flow delivery. Thanks to these benefits, the Vanquish UHPLC system is capable of providing unmatched retention time precision. This retention time precision accompanied with a high peak area precision guarantees the analytical success for even challenging shallow gradient separations by a reliable peptide identification and quantification. In this work, the separation of peptides obtained from a therapeutic protein is provided. The retention time and peak area precision is evaluated for repeated injections.

Equipment

Vanquish UHPLC system consisting of:

- Binary Pump H (P/N VH-A10-A)
- System Base (P/N VH-S01-A)
- Mixer Kit, 200 µL, VH-P1 (6268.5120)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active pre-heater (6732.0110)
- Post column cooler, 1 µL (6732.0510)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe™ flow cell, standard (10 mm; P/N 6083.0100)

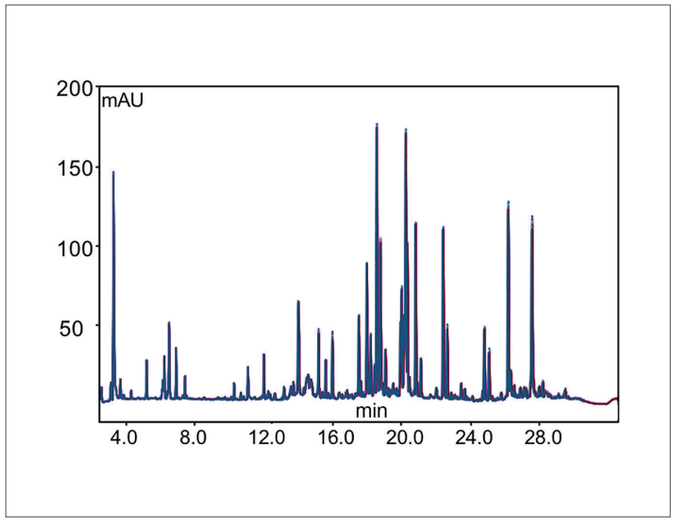
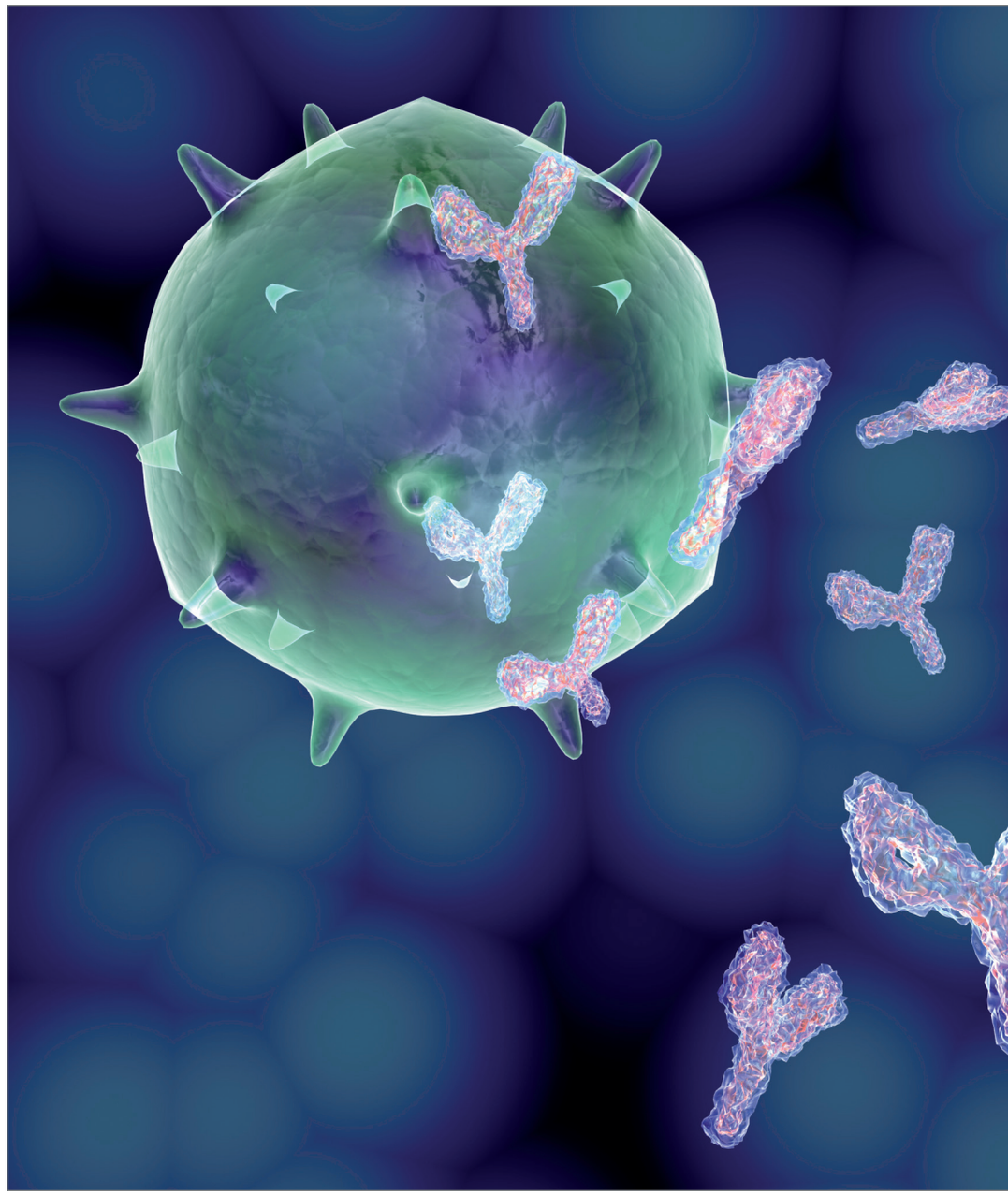


Figure 1. Overlaid chromatogram of 13 repeated injections of the mAb tryptic digest.



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Video
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Online Article
Piecing Together Protein Analysis

Thermo Scientific™ Chromeleon™
Chromatogaphy Data System (CDS) software, version 7.2

Protein Digestion

SMART Digest Kit (P/N 60109-101)

Experimental

Sample Preparation

- Cetuximab® monoclonal antibody (5 mg /mL) was diluted 1:4 with the SMART Digest buffer to a final volume of 100 µL
- The diluted sample was then added to a SMART Digest tube and left for 60 minutes at 70 °C
- The digested sample was then centrifuged at 10,000g for 5 minutes and the supernatant was removed for chromatographic analysis

Conditions

Column:	Acclaim VANQUISH C18, C18, 2.2 µm Analytical (2.1 × 250 mm), P/N 074812
Mobile Phase:	A: 0.05% TFA in water, P/N TFA 85183 B: 0.04% TFA in 8/2 acetonitrile/water (v/v), P/N acetonitrile TS-51101
Gradient:	0–30 min: 4–50% B, 30-31 min: 50–90% B, 31–35 min: 90% B, 35–36 min: 90–4% B, 36–45 min: 4% B
Flow Rate:	0.4 mL/min
Maximal Pressure:	384 bar
Temperature:	80 °C; Forced Air Mode
Injection Volume:	5 µL
Detection:	214 nm Data Collection Rate: 20 Hz Response Time 0.2 sec
Flow Cell:	10 mm LightPipe™

Results and Discussion

The digestion was achieved utilizing the SMART Digest kit. Using this approach the sample preparation time could be reduced significantly and total preparation time of the monoclonal antibody (mAb) digest was lower than 75 minutes.

The separation of the resulting peptides was obtained with a 30 minutes gradient, and a total analysis time of

45 minutes, including column wash with high organic eluent, and re-equilibration at initial conditions. Figure 1 shows the overlay of 13 consecutive injections of the same sample of mAb digest.

The results show excellent reproducibility across the whole chromatogram. On average, standard deviation (SD) was of the order of 0.13 seconds (0.00214 minutes). SD for some peaks was as low as 0.065 seconds; and did not exceed 0.3 seconds for any peptide.

The relative standard deviation was consistently extremely low (Figure 2). Out of 110 peaks automatically integrated by Chromeleon CDS, 34 had RSD smaller than 0.0100%, reaching the minimum value of 0.0060% for the peak at retention time 23.057 minutes. Please note that the early eluting peaks naturally have the highest retention time RSDs because of a mathematical disadvantage in the calculation.

In addition, Table 1 gives the peak area reducibility as relative standard deviation.

The relative standard deviation of the peak areas was below 1.0% for all peptides. The average reproducibility was 0.4% highlighting the highly reliable sample injection and peak integration at challenging conditions.

Conclusion

Stability of retention time and peak areas is critical for a confident evaluation of chromatographic results and to avoid any misinterpretation. The Vanquish UHPLC system is extremely reproducible in both retention time and peak area reproducibility. The retention time precision provided by the system enables the analyst to deduce any change in retention time to an actual change of the sample structure. As shown, the peak area reproducibility provided by the Vanquish UHPLC or Vanquish system will result in a maximal confidence of quantitative result. Consequently, the Vanquish system meets the requirements of demanding peptide mapping analysis.

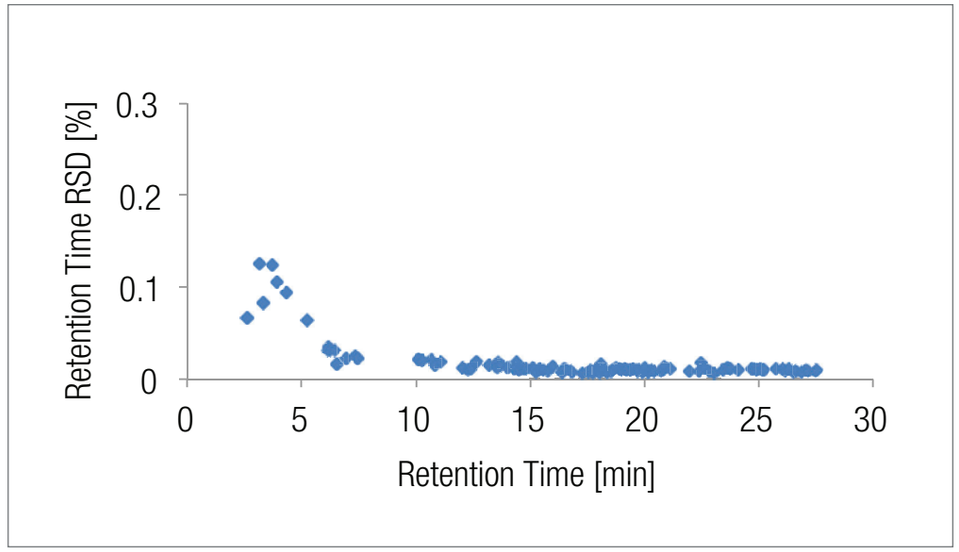


Figure 2. Retention Time RSD (%) relative standard deviation measured for 13 repeated injections of a mAb digest.

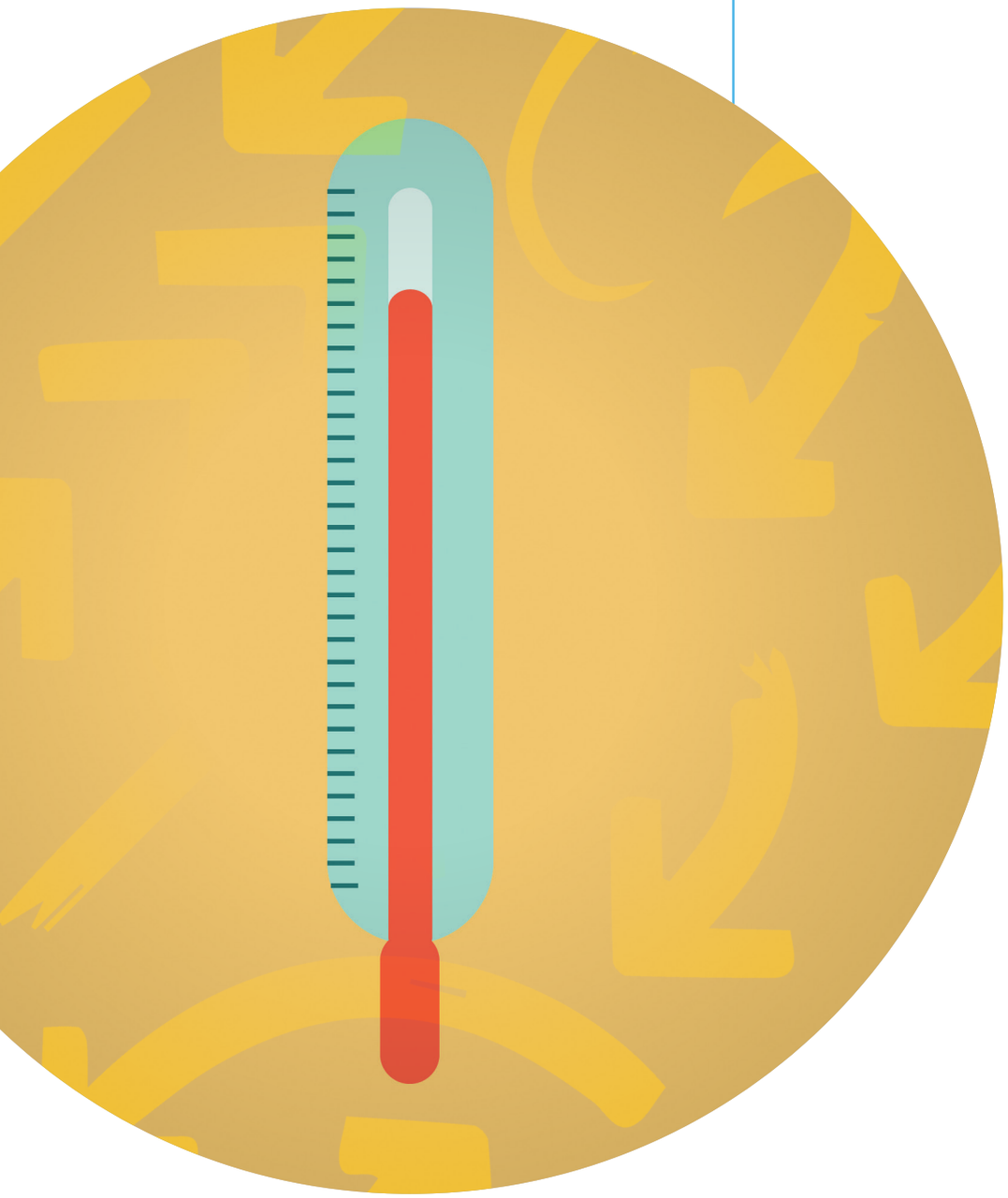
Retention Time (min)	*Average Area (mAU*min)*	*RSD Area (%)*
5.23	1.04	0.1
10.29	0.36	0.22
13.07	0.05	0.94
15.96	0.96	0.49
22.39	5.17	0.14
24.68	0.25	0.61

Table 1. Peak area of six selected peaks eluting over the entire gradient and spanning a wide concentration range.

LINKS

Video
Biopharmaceutical characterization demands advanced detection capabilities

Video
The Vanquish UHPLC: A Fantastic Addition for BioPharma



Column Temperature Control in Peptide Mapping

Maximizing Peak Capacity by Keeping Your Column Hot the Right Way

Separation efficiency and peak capacity are important to analyze complex samples (e.g., peptide mapping). They depend on column plate number, system performance, and column temperature. In this article a new binary biocompatible UHPLC system is described that provides two column thermostatting modes, namely forced-air and still-air. For complex peptide mixtures of monoclonal antibody digests, still-air mode generates higher peak capacity compared to forced-air. Interestingly, the capacity gain is more evident at high temperature.

Reversed phase chromatography peptide mapping is extensively used for characterization and control of critical quality attributes of biothera-peutic proteins. In combination with MS, peptide mapping can provide the primary sequence of the protein of interest. Detection and quantitation of targeted peptides can be easily achieved by UV or MS detection. The high efficiency re-quired to resolve the complexity of protein digests is provided by UHPLC columns. Long and shallow gradients can be applied to increase the number of resolved peptides. Although this is a well-known and proven approach to achieve higher peak capacity, unfortunately, long gradient times are hardly compatible with the throughput requirements of biopharmaceutical laboratories.

Other method tuning strategies are preferred to increase peptide peak capacity. An effective and recognized approach is to run the analysis at high temperature. The mass transfer kinetic of the relatively large peptide molecules is thereby improved, and better resolution achieved. Column compartment temperature can be controlled by two main approaches: forced-air or still-air mode, with the latter being the most common implementation on commercial UHPLC platforms. Forced-air thermostatting is preferred for method portability, which is in most cases, the transfer of a previously developed HPLC method to the UHPLC domain. Still-air is usually preferred to maximize the efficiency instead. With the new Thermo Scientific™ Vanquish ™ UHPLC Platform any of the two thermostatting modes are available in the same system thanks to the double functionality column compartment; the thermostatting mode can be set in the instrument method, based on the requirements and purpose of the analysis. Here we show how temperature, and the way of controlling it, affects the resolution of complex peptide mixtures, for a typical biopharmaceutical sample such as the tryptic digest of a monoclonal antibody.

Experimental

LC system: Vanquish UHPLC. Column: Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm Analytical 2.1x250 mm. Mobile phase A: TFA 0.05% in water; mobile phase B: 0.04% TFA in 8/2 acetonitrile/water. Gradient program: from 4% to 55% B in 30 minutes. Injection volume: 1 µl. Sample: monoclonal IgG tryptic digest 2 mg/ml.

Peak capacity was calculated by the peak width at half height. All peaks were selected to calculate capacity even if co-eluting, unless the coelution generated excessive peak distortion. Peak capacity was calculated by using the formula It is well known from the literature that peak capacity in peptide mapping benefits from high temperature. The results shown here merely confirm this well-established knowledge. However it should be noticed that a peak capacity on the order of 400 was achieved at 80 °C, which is remarkable for a 30 minute gradient run (total run time 45 minutes, including column wash and re-equilibration).

$$n_c = \frac{t_w}{1.7 \cdot W_{1/2}} + 1$$

The coretool to obtain high resolution separation of complex mixtures is the column. The system is equally important, as it must generate the least possible extra column dispersion to ensure that the potential of the column is fully met. Among all the parts of a UHPLC that play a role on extra column band dispersion, the mobile phase pre-heater is a critical component for separation at high temperature. The pre-heater must precisely adjust the temperature of the mobile phase prior to accessing the column. Any temperature mismatch can cause thermal inhomogeneity within the column that may be detrimental to efficiency. At the same time, the band dispersion generated by the additional volume of the pre-heater must be negligible. The low-dispersion active pre-heater

LINKS

Blog
Finding HOPE Using Peptide Mapping?

Video
Thermo Scientific Vanquish Flex UHPLC – The Evolution of a Biocompatible Masterpiece

Designed by the Medicine Maker

installed in the UHPLC system allowed achieving high capacity in relatively short time.

Thermostatting Mode vs. Peak Capacity

The column thermostatting mode and its influence on peak capacity were evaluated at three temperature levels, namely 40, 60 and 80 °C (fig. 1). The forced-air mode allows fast column compartment temperature control, however when significant viscous heating is generated during the UHPLC run, this approach tends to cause a radial temperature gradient across the column, which causes retention and radial diffusion rate inhomogeneity. This produces additional band dispersion. The still-air mode approach instead, is clearly a less efficient heat-removal strategy, when significant viscous heating is generated. The slower viscous heating removal will create a temperature gradient with longitudinal orientation rather than radial, i.e. the column temperature will be lower at the inlet and higher at the outlet, but essentially constant for any given cross section. With this approach the column in-trinsic efficiency is better exploited. When the UHPLC method is transferred from HPLC, retention discrepancies are very likely to arise due to temperature difference between inlet and outlet.

The analysis of peak capacity at different temperatures with still-air and forced-air thermostatting, both showed the typical behavior, which is increase of peptide resolution with temperature. It also showed the expected efficiency increase that still-air mode thermal control allows in comparison to forced-air. However a closer look at the plot of figure 1 shows an interesting and also un-expected outcome: the efficiency benefits by still-air mode are increasingly higher with tempera-ture. For instance, the capacity difference between still-air and forced-air is about 2.9% at 80 °C, 1.5% at 60 °C, but at 40 °C there is no actual capacity difference. This is unexpected as the most viscous-heating should be generated at 40 °C, where maximum back pressure was on the order of 650 bar. At 80 °C, where maximum pressure was on the order of 350 bar, the viscous heating effect was expected to be less important.

At 80 °C the average peak width at half height was about 2.64 seconds for still-air mode and 2.56 seconds for forced-air mode. The difference is not dramatic, but it was consistent throughout the retention window. The improvement may turn out to be useful in resolving critical pairs in the case of challenging separations. Detailed view of the chromatogram of Figure 2 shows several instances of better resolved peak pairs with still-air mode.

Final considerations

Temperature control is crucial in peptide mapping. High temperature separations are preferred as peak capacity will increase thanks to the improved mass transfer kinetics. Another consequence of the temperature increase is that the retention of the vast majority of peptides will decrease. This effect will be beneficial to the elution of highly hydrophobic components, which would be otherwise detected as co-eluting wash-peaks with low-temperature methods. A moderate but significant improvement of peak capacity was observed with still-air mode column compartment, particularly at high temperature. However forced-air mode still generated excellent results. The use of one or the other heating mode should be carefully considered in case of method transfer. For instance, when HPLC methods need to be converted to UHPLC (or the other way around), forced-air mode is the safest approach as it minimizes the chances of retention shifts due to viscous heating.

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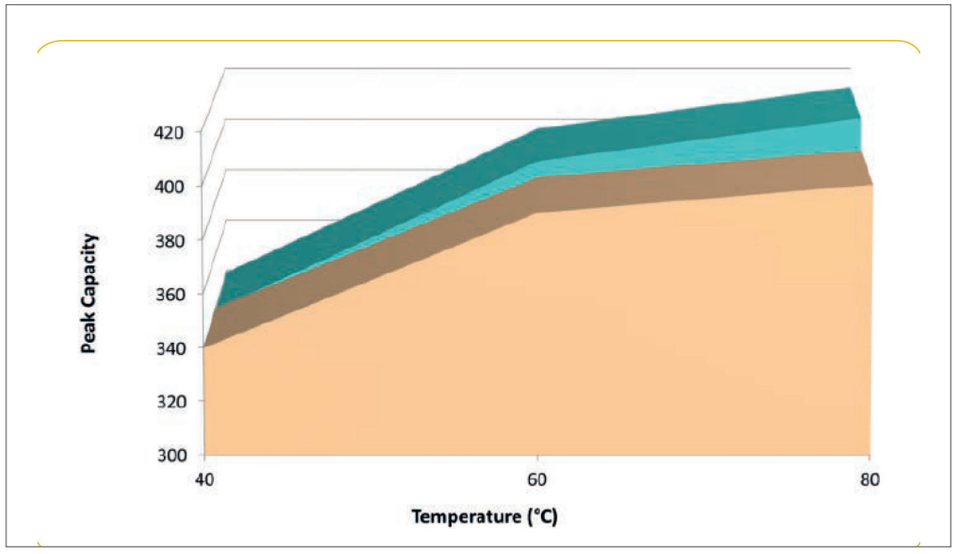


Fig. 1: Peak capacity obtained for gradient separation 30 minutes long. Still-air mode thermostatting (green) delivered higher peak capacity than forced-air mode thermostatting (tan) at 60 and 80 °C.

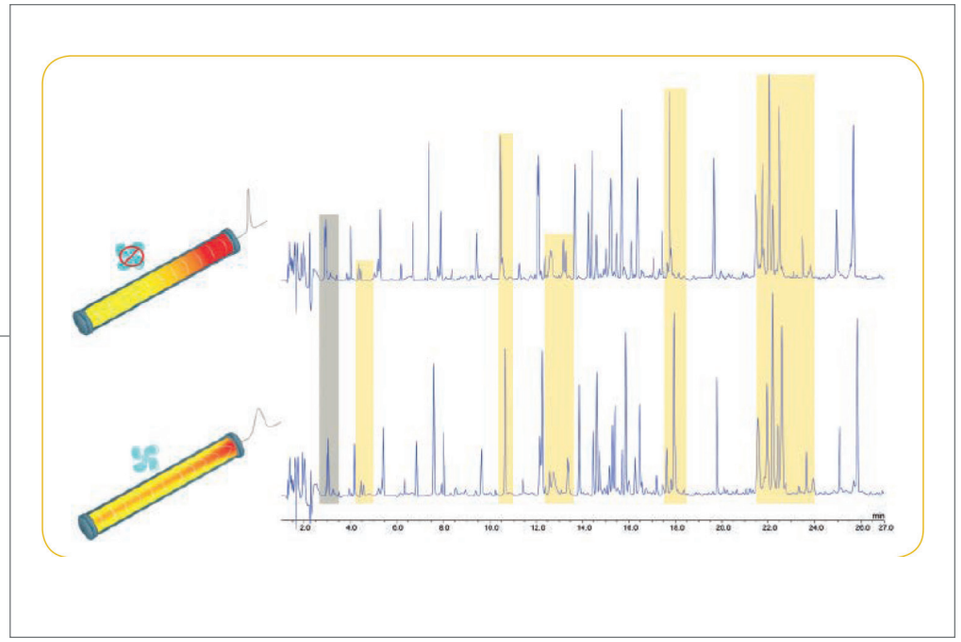


Fig. 2: Typical separation of a IgG digest at 80 °C with different thermostatting modes. The yellow boxes highlight the area in the chromatograms where the narrower peak width obtained with still-air thermostatting resulted in better resolution of closely eluting peaks. The grey area highlights where better resolution was found for forced-air thermostatting as the consequence of different selectivity, rather than peak-width improvement. On the left, the schematic view of the effect of viscous heating and temperature distribution in columns thermostatted by still-air (above) and forced-air (below). The color gradient from yellow to red indicates transition from low to high temperature.

Online Article
Ultra-Engineering UHPLC from Scratch

Video
Biopharmaceuticals are complex; analyzing them just got a lot easier

Mass Detection

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

Martin Samonig, Remco Swart
Thermo Fisher Scientific

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

Goal

Prove the suitability of a Thermo Scientific™ Vanquish™ Flex UHPLC 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, fully biocompatible flow and SmartInject technology. 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.

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)

Experimental Conditions - HPLC

Column	Thermo Scientific™ Acclaim™ VANQUISH™ 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),

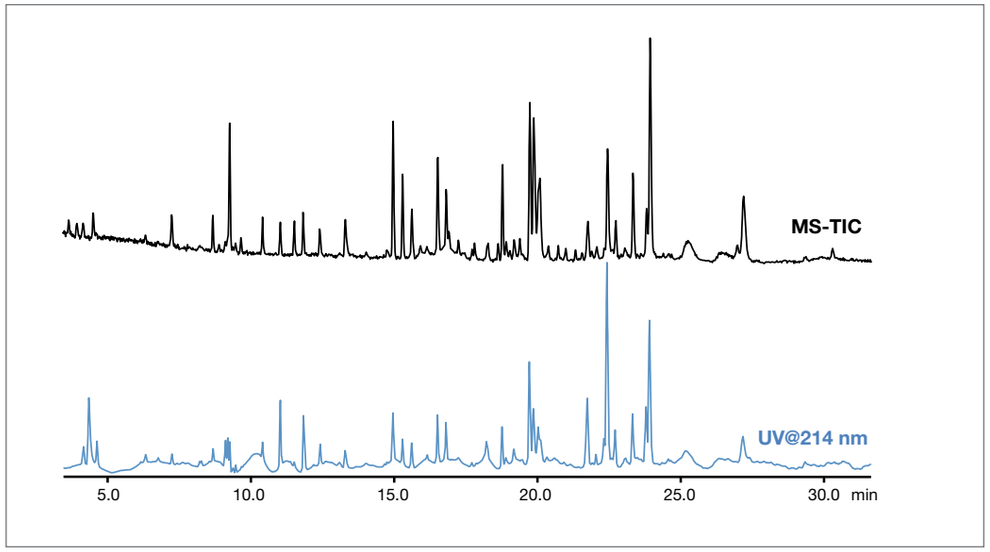


Figure 1. Overlaid chromatograms of the total ion current (TIC) and the UV trace at 214 nm of a SMART Digest Kit digested rituximab sample with subtracted blank baseline.

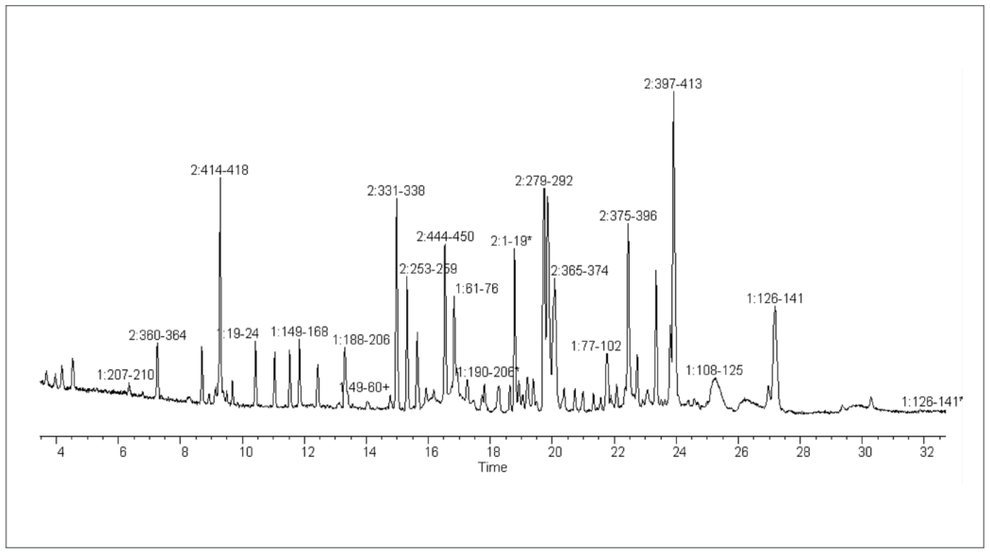
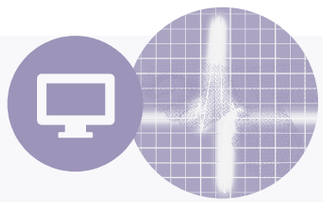


Figure 2. Peak assignment of the tryptic peptides from rituximab. Peak labels with 1 correspond to the light chain, and those with 2 correspond to the heavy chain of the mAb. The number after the colon indicates the amino acid region of this particular tryptic peptide.

LINKS

Online Article
Orbitrap Against All Odds



Online Article
Keeping a Finger on the Biosimilar Pulse

Video
A Journey in Biotherapeutic Peptide Mapping

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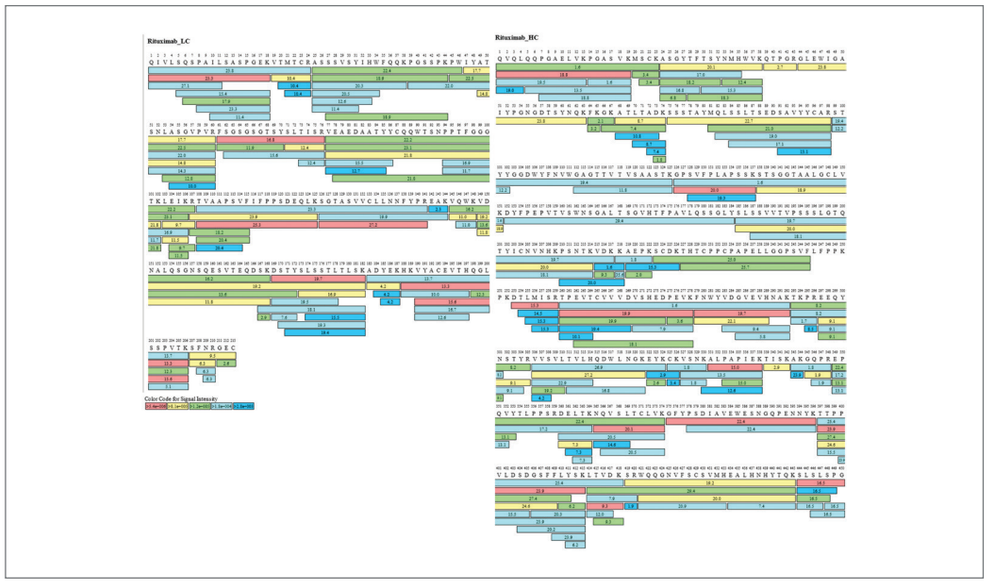

Figure 3. Sequence coverage map of the heavy (right) and light chain (left).

Protein	*Modification*	*Recovery*	*Abundance*
Rituximab_LC	Q1+NH3 loss	Good	87.81%
Rituximab_LC	W90+Oxidation	Good	2.06%
Rituximab_HC	~Q1+NH3 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	3.27%

Table 1. Peak area of six selected peaks eluting over the entire gradient and spanning a wide concentration range.

	(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	MS2 Parameters
Full MS Mass Range	200–2000 *m/z*
Resolution Settings	15.000
Resolution Settings 60000	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	TopN MS2 5
	Max Injection Time 100 ms

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™ BioPharma™ Finder mass informatics software.

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, BioPharma Finder 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.

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%.

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.*

LINKS

Infographic
Points of Interest on a Peptide Map

Poster Note
Complete Characterization of a Cysteine linked Antibody-Drug

Online Article
The Biologics Boost

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the Medicine Maker

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

Martin Samonig, Kai Scheffler, Remco Swart, and Jonathan Josephs
Thermo Fisher Scientific

Key Words
Monoclonal Antibodies, Acclaim VANQUISH C18 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®).

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

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.

A Thermo Scientific™ Vanquish™ UHPLC system with a 2.1 x 250 mm i.d. Thermo Scientific™ Acclaim VANQUISH C18, 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.

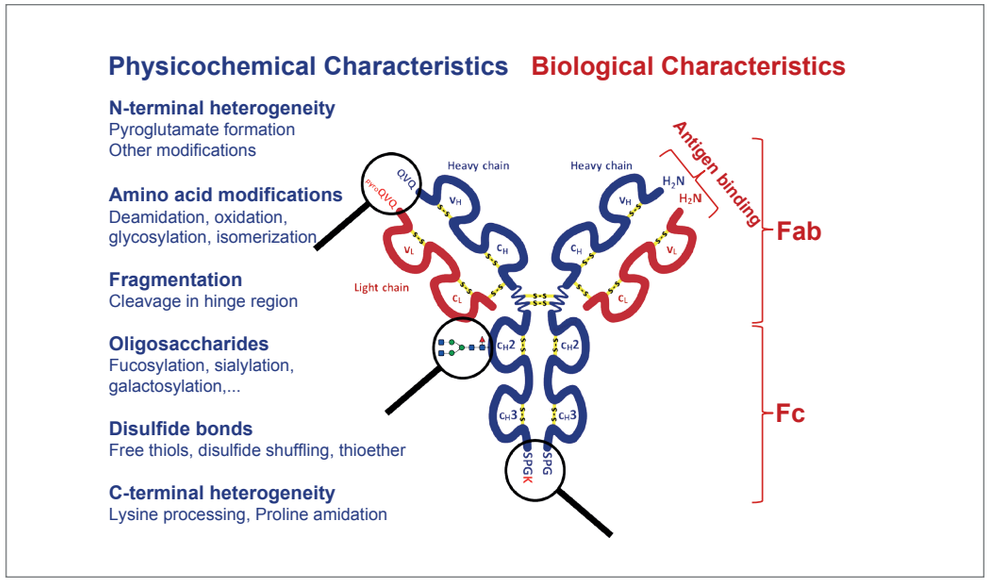

Figure 1. General structure of mAbs and their biological and physicochemical characteristics.

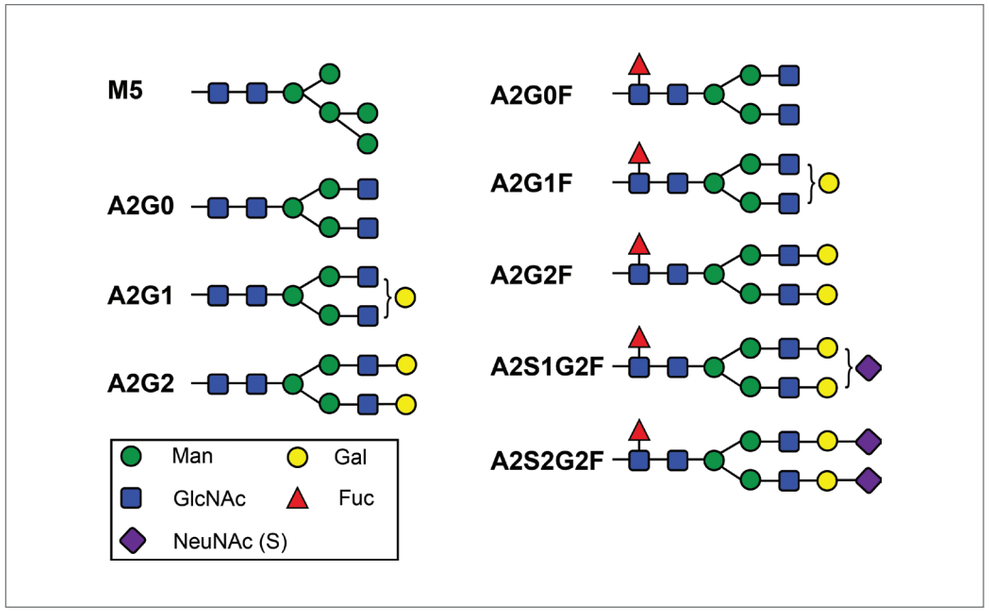

Figure 2. Nomenclature of carbohydrate structures commonly observed on antibodies.

LINKS

Poster Note
Full Characterization of Heterogeneous Antibody Samples

Online Article
High-Throughput, High-Resolution Peptide Maps

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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 VANQUISH 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

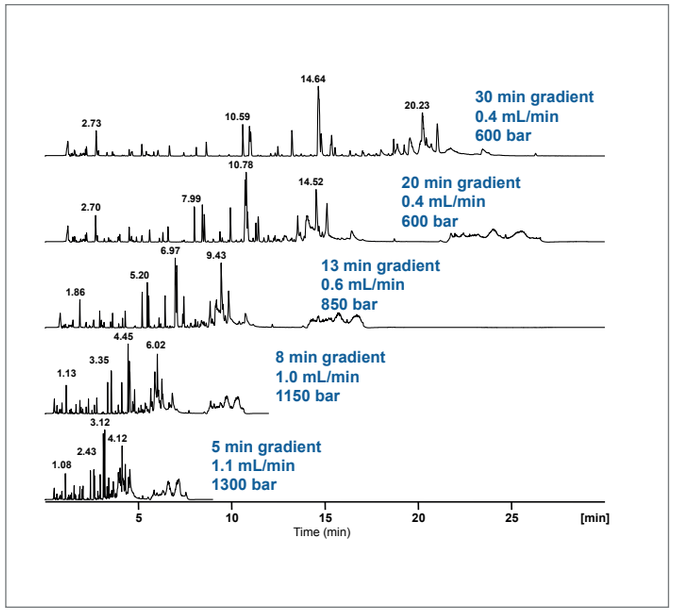


Figure 3. Simulated (top) and measured (bottom) MS/MS spectra of the glycopeptide aa 290-302 (TKPREEQFN*STFR, *=A2G0) in the 5 min gradient run.

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			

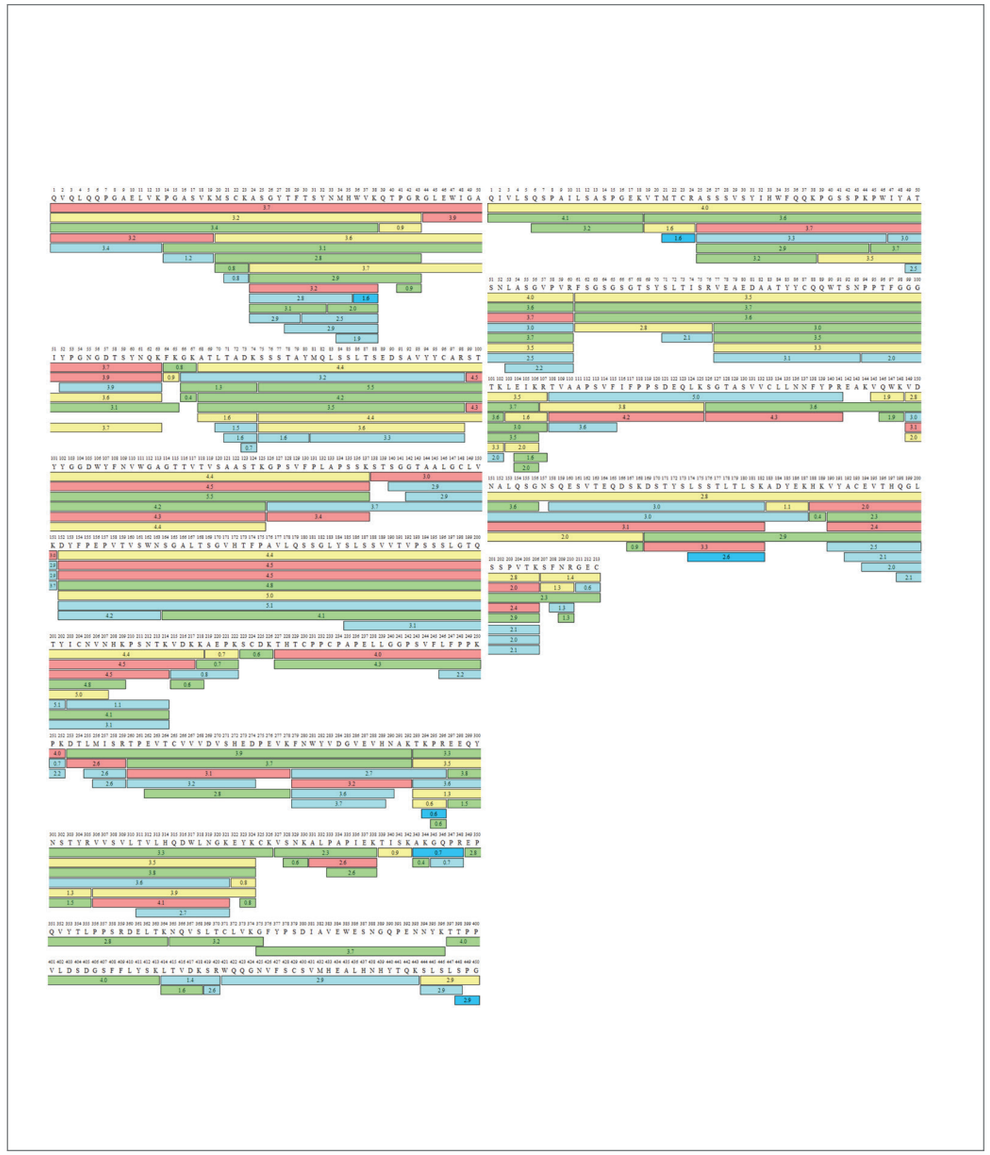


Figure 4. Sequence coverage map of rituximab heavy (left) and light chain (right), obtained using the 5 min gradient for peptide separation. The colored bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the intensity of the peptide 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

LINKS

Blog
Not All Who Wander Are Lost

Tool
Online Digestion Calculator

Full MS Parameters

Full MS Mass Range	200–2000 *m/z*
Resolution Setting	60000
Target Value	3E6
Max Injection Time	100 ms
Default Charge State	2
SID	0 eV

MS2 Parameters

Resolution Setting	15,000
Target Value	1E5
Isolation Width	2.0 Da
Signal Threshold	1E4
Normalized Collision Energy (NCE)	27
TopN MS2	5
Max Injection Time	100 ms
Fixed First Mass	140.0 *m/z*
Dynamic Exclusion (s)	10.0

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™ BioPharma Finder™ mass informatics software.

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.[1,2] 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.

Since the quantification of modified peptides performed in BioPharma Finder 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 BioPharma Finder 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.

Even with ultra-short gradients down to 5 min, as shown in Figure 6A, spectacular separation efficiency with average peak width at half maximum (w½) 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.

Conclusion

- The applied hardware setup chosen for the experiments consisting of the Vanquish UHPLC system with an Acclaim VANQUISH C18 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

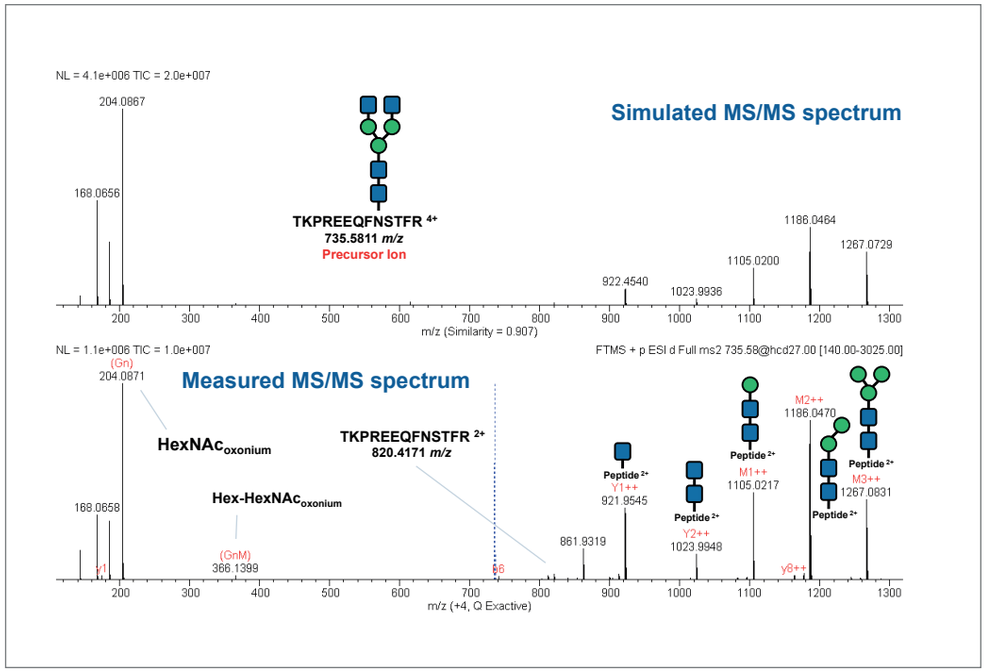
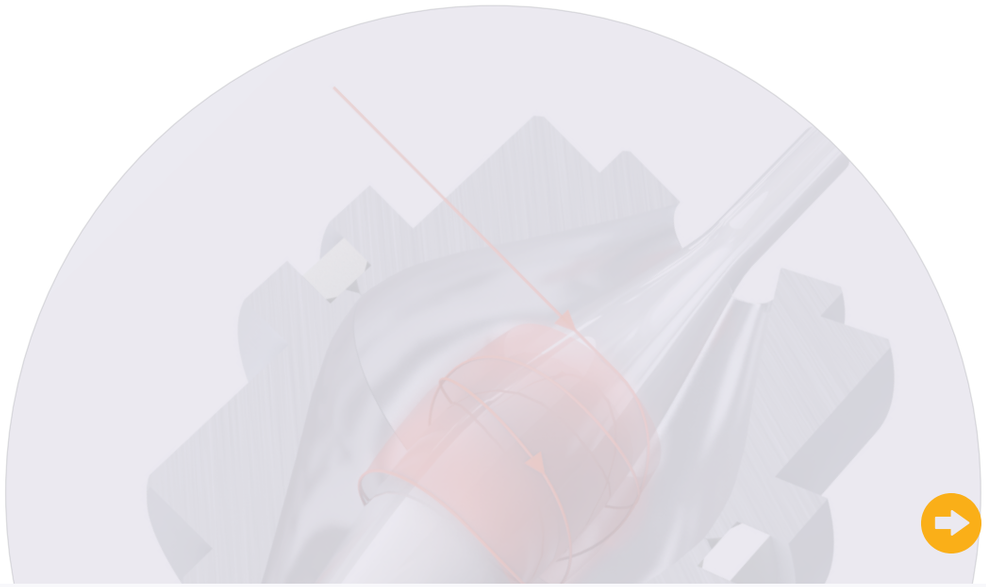


Figure 5. Simulated (top) and measured (bottom) MS/MS spectra of the glycopeptide aa 290-302 (TKPREEQFN*STFR, *=A2G0) in the 5 min gradient run.



LINKS

Video
High throughput Peptide Mapping by UHPLC-MS

Online Article
The Role of Mass Spectrometry in Bottom-up Characterization

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Protein Chain	*Modification*	*Recovery*	*Gradient Time*					σ
			30 min	*20 min*	*13 min*	*8 min*	*5 min*	
			Abun.	*Abun.*	*Abun.*	*Abun.*	*Abun.*	
Rituximab_LC	Q1+NH3 loss	Good	91.95%	91.17%	89.69%	90.93%	26.57%	28.80%
Rituximab_HC	~Q1+NH3 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%
								Median 0.19%

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

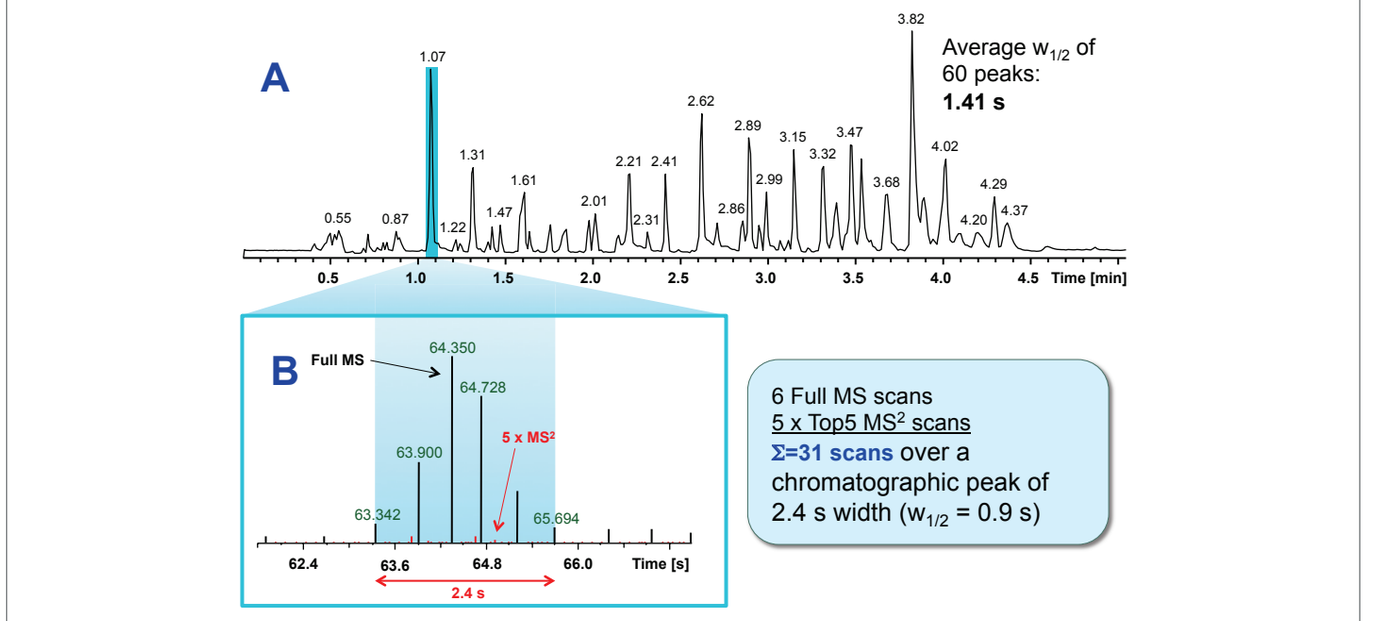

Figure 6. A) Total ion chromatogram of a five minute gradient separation of denosumab and B) data point distribution for a Full MS / ddMS2 Top5 method during a representative chromatographic peak.

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-glutamate 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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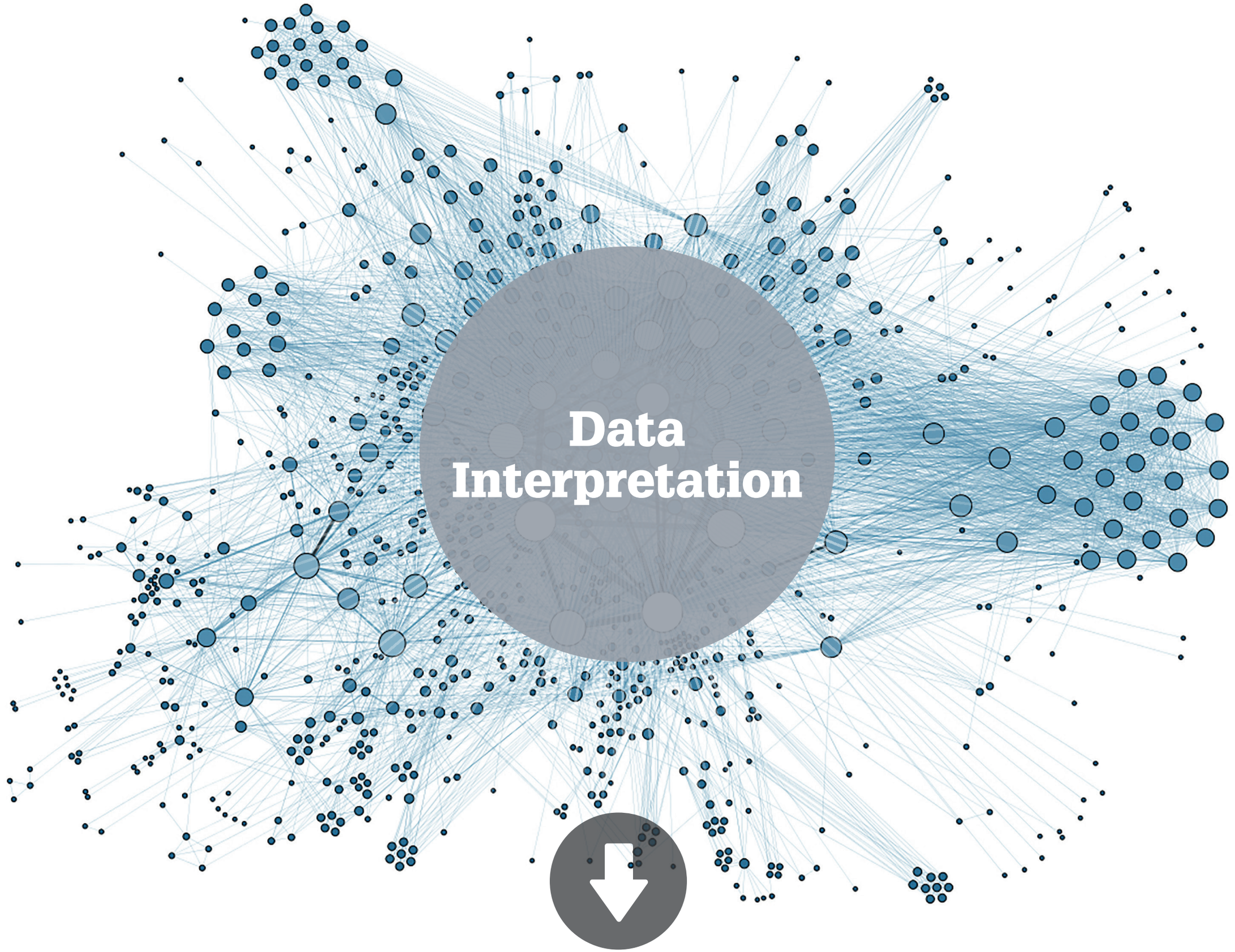
2. *Shah, B.; Jiang, X.G.; Chen, L.; Zhang, Z., LC-MS/MS peptide mapping with automated data processing for routine profiling of N-glycans in immunoglobulins.J.Am. Soc. Mass. Spectrom. 2014, 25, 999-1011.*


Data
Interpretation

New Integrated Informatics Solution for Protein Biotherapeutics Characterization

Stephane Houel, Jennifer Sutton, Terry Zhang, Jonathan Josephs & Mark Sanders
Thermo Fisher Scientific

Overview

Purpose: Integrated informatics solution for protein biotherapeutics characterization. Two software packages Thermo Scientific™ Protein Deconvolution and Thermo Scientific™ PepFinder™ software are integrated into one software platform.

Methods: Intact and subunit mass analysis and peptide mapping were performed to characterize trastuzumab. The new Thermo Scientific™ BioPharma Finder™ software was used for data processing.

Results: One integrated software solution was used for full characterization of trastuzumab. Intact protein mass was determined and all major glycoforms were identified using the intact protein analysis (Protein Deconvolution). Subunit analysis used a novel sliding window algorithm which improves peak detection in complex mixtures, to generate deconvoluted masses for the Fc/2, Fd and light chain molecules. A simulated stress study is automatically processed using the peptide mapping workflow (PepFinder software) where expected modifications are identified and a relative amount is determined automatically. New visualization for data mining and data interpretation enable both expert and beginner users to be successful with this new software while providing more confident results.

Introduction

Increasing requirements to fully characterize complex protein biotherapeutics for safety and efficacy place analytical scientists under pressure. In spite of this, the discovery and development of protein biotherapeutics continues to thrive and demands faster and better tools. Here we present a new, powerful software that can leverage chromatographic separations and High Resolution Accurate Mass (HRAM) analysis for the characterization of biotherapeutics.

Methods

Sample: Trastuzumab was used for both intact analysis and tryptic peptide mapping analysis.
LC: Thermo Scientific™ Vanquish™ UHPLC system
Column:
Thermo Scientific™ MAbPac™ RP, (50 mm * 2.1 mm; 4 mm)
Thermo Scientific™ Accucore™, C18 (100mm * 2.1 mm; 1.7 mm)
Mass Spectrometry: Thermo Scientific™ Orbitrap Fusion™ Tribrid™ MS
Data Analysis: Raw files were processed with Thermo Scientific™ BioPharma Finder software.

Results

BioPharma Finder software allows users to organize and store protein sequences with the Protein Sequence Manager and then the user can attach the sequence to the intact and peptide mapping methods (Figure 1). This simple interface allows users to easily navigate through the software seamlessly from one workflow to another.

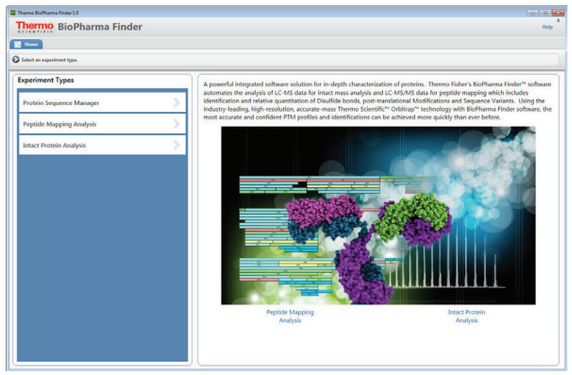


Figure 1. BioPharma Finder software homepage

For the intact protein and sub-unit analyses, Trastuzumab was analyzed using the Orbitrap Fusion MS at 17K and 120K resolutions respectively. The deconvolution processing method used the ReSpect™ algorithm for the intact protein and Xtract™ algorithm was used for sub-unit study. MS scans were processed as "static" for the intact or "sliding window" for the sub-unit. All major glycoforms were identified and annotated. Average masses, sum intensities, matched delta masses and identification as well as abundances are reported in an exportable table. Sub-unit raw file was processed automatically using the sliding window tool eliminating the need to define manually the time range for each chromatographic peak.

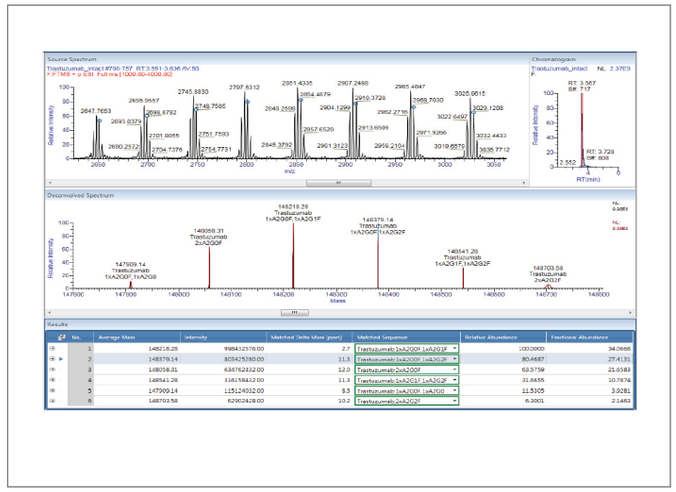


Figure 2. Intact mass analysis of Trastuzumab.

Displayed in the software is the source mass spectrum used for deconvolution with interactive graphics for manual interrogation of the results, chromatogram for reviewing elution profile, interactive deconvoluted spectrum and a results table which contains all of the relevant information.

The width and the overlap between consecutive sliding window boxes for deconvolution is user defined. During processing the box will move across the chromatogram providing real time visualization. All of the subunits were identified with a delta mass error below 1ppm.

LINKS

Software Brochure
Workflow driven informatics for complete biotherapeutic characterization

Guide
BioPharma Finder 'How To Guide'

Workflow Brochure
Upgrade Your Maps

Designed by
the Medicine Maker

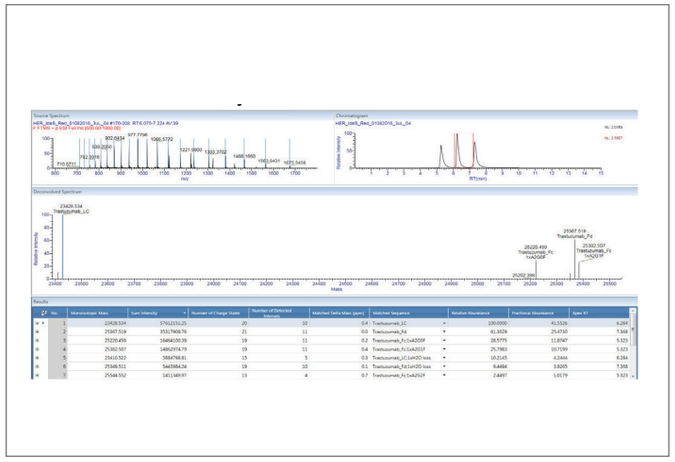


Figure 3. Subunit analysis of Trastuzumab.

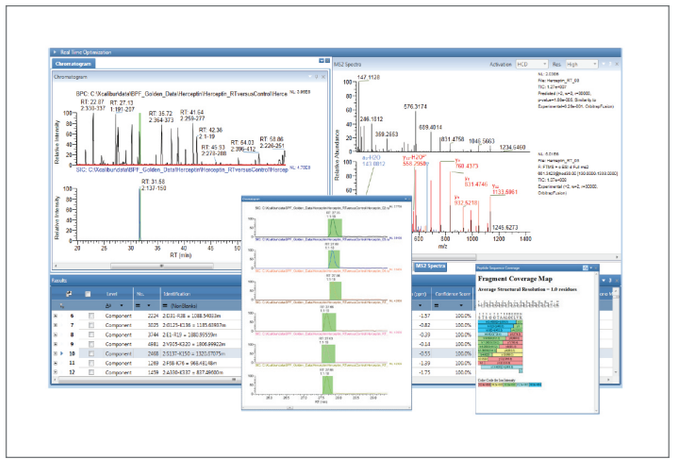


Figure 4. Peptide Mapping analysis of Trastuzumab. Main process & review page in BioPharma Finder software with interactive plots and tables for user friendly data mining.

A "stress" sample was generated by storing at room temperature for 48 hrs a tryptic digest sample. For peptide mapping analysis, raw files were divided in two groups, control and stress. Data were acquired using an Orbitrap Fusion mass spectrometer. Data processing was performed using the peptide mapping workflow in BioPharma Finder software. The core algorithms for peptide mapping are from the PepFinder software. However, BioPharma Finder software has an updated user interface which provides a significantly improved user experience for data mining and data interpretation. Figure 4 shows the process & review page of the peptide mapping workflow. The main page has an interactive table, chromatogram plotting capability with 6 different types of plots and the ability to stack chromatograms from multiple raw files allowing the user to view peptides across samples. All of the plots and tables can be floated on the screen or moved to a second monitor for custom layout.

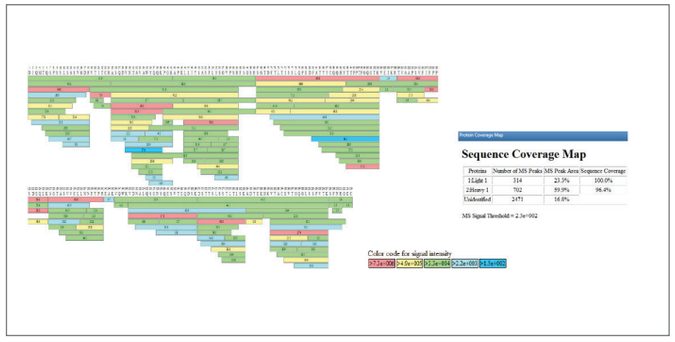


Figure 5. Sequence coverage map

The sequence coverage map is automatically generated and provides a visual display of the depth of identification that is achieved in the peptide mapping workflow. Each peptide is colored based on abundance and a summary report is generated for each data file.

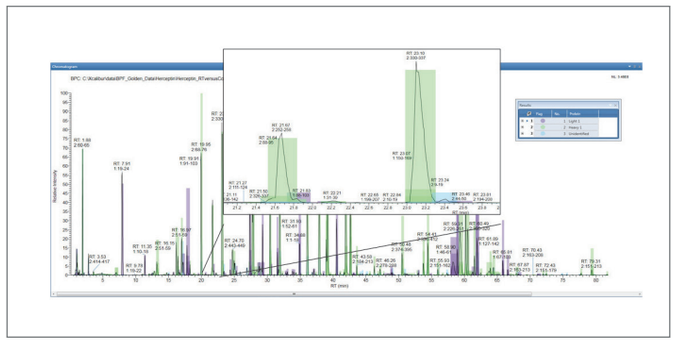


Figure 6. Novel chromatographic shading plot

A new feature in BioPharma Finder software peptide mapping workflow is shown in Figure 6. The basic peak chromatogram is shaded using different colors based on the protein identification. The purple are peptides from the light chain, green are from heavy chain and blue are unidentified. This interactive plot provides the user an image where they can quickly see non-identified peaks, which peptides are the most abundant and displays co-eluting peptides.

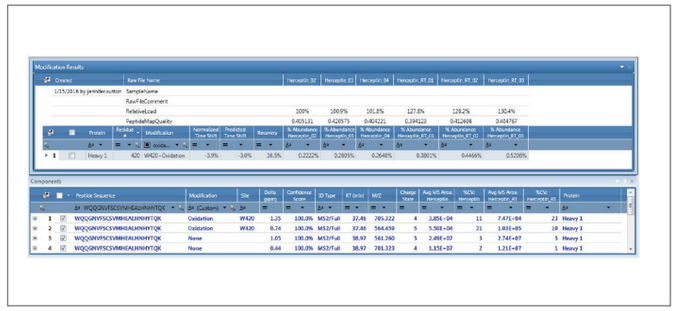


Figure 7. Modification table for the oxidation on W420 of the heavy chain.

Figure 7 is a dispay of the new and improved modification summary report which was part of PepFinder software. This report is now an interactive report in which the user can select a specific modification on the top table and see the components used in the abundance calculation. Normalized time shift, a new feature, is the comparison of the modified and non-modified peptides retention times. The normalized time shift can be compared to the predicted time times. The normalized time shift can be compared to the predicted time shift providing an extra level of confidence for the identification.

Conclusions

BioPharma Finder software provides:

- Confident deconvoluted molecular weight of proteins in denaturing and native conditions.
- Extra confidence in peptide identification by using a novel MS/MS predictive algorithm.
- Quantification of modifications.
- Characterization of disulfide linkages.
- Low level impurities - sequence variants identification.
- Sequence alteration – stress samples, level of deamidation or oxidation.

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