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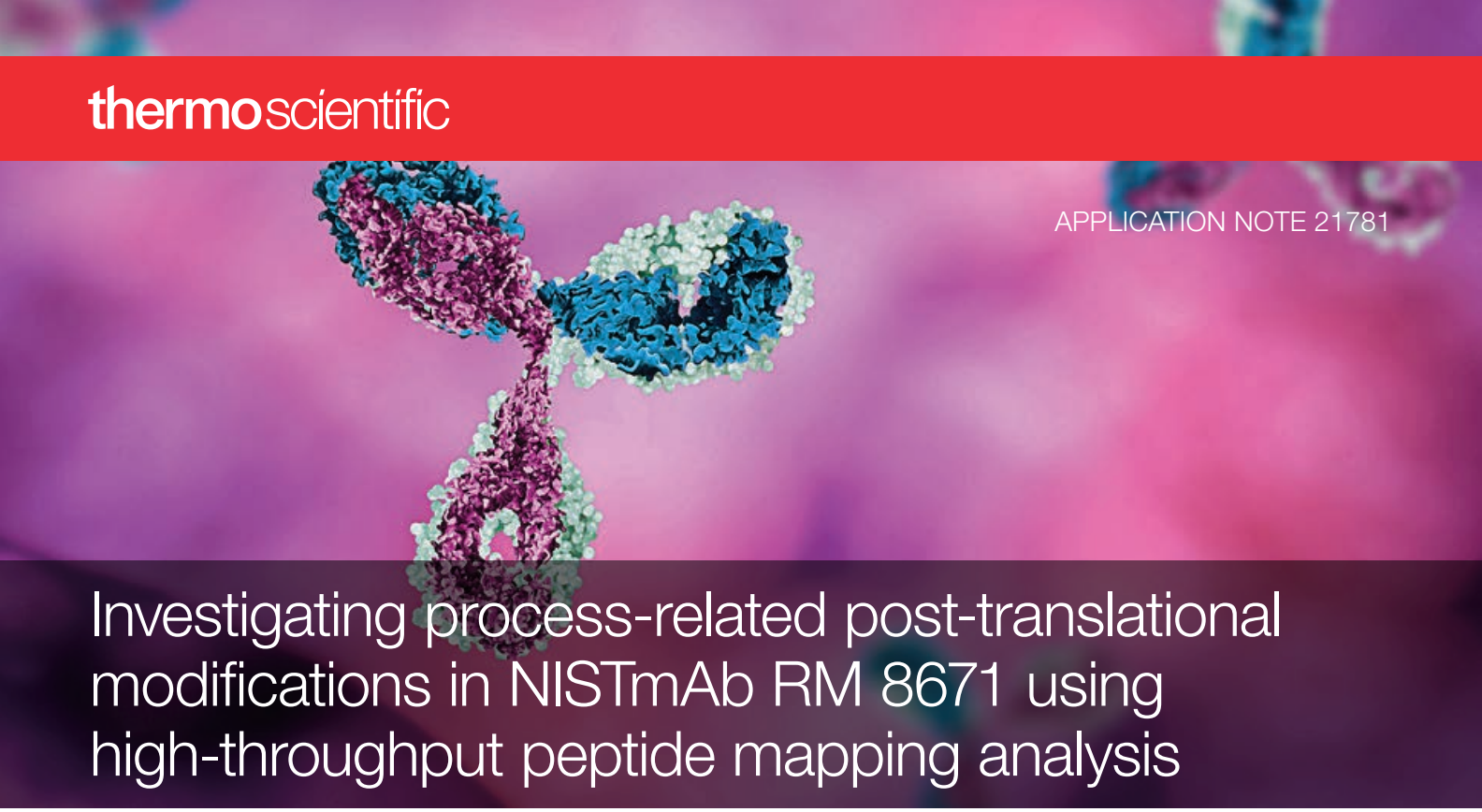

Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis

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Keywords

NIBRT, biopharmaceuticals, biotherapeutics, CQAs, monoclonal antibodies (mAbs), IgG, NISTmAb, post-translational modifications (PTMs), peptide mapping, bottom-up, sequence coverage, SMART Digest kit, high throughput, Vanquish Flex Binary UHPLC system, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer

Application benefits

- A fast and simple protein digestion for peptide mapping analysis that results in low levels of sample preparation-induced post-translational modifications
- Time to complete digestion considerably shorter than traditional methods, making it possible to achieve an efficient digestion even in 30 min
- Workflow achieves 100% sequence coverage with high confidence
- Analytical separation achieves outstanding reproducibility with retention time precision RSD ≤ 0.2%

Goal

This study intended to evaluate post-translational modifications (PTMs) during enzymatic digestion of monoclonal antibodies using the Thermo Scientific™ SMART Digest™ kit. A simple time course study was performed as an efficient means to ensure sufficient digestion for intended use. Its efficiency was studied in terms of the determination of protein sequence coverage and identification of PTMs, including deamidation, oxidation, glycation, and glycosylation.

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Introduction

Monoclonal antibodies (mAbs) and related products are the fastest growing class of human therapeutics.[1] They are a class of recombinant proteins that are susceptible to a variety of enzymatic or chemical modifications during expression, purification, and long-term storage. Unlike small molecule drugs, protein therapeutics are made via DNA expression techniques. This is a highly complex process; hence, protein therapeutics require a close monitoring of their structural characterization and evaluation of their quality in each step[2] to ensure drug safety and efficacy.[3] Therefore, it is vital that biopharmaceuticals are comprehensively characterized.[4]

Peptide mapping is a critical workflow in biotherapeutic protein characterization and is essential for elucidating the primary amino acid structure of proteins. For recombinant protein pharmaceuticals, such as mAbs, peptide mapping is used for proof of identity, primary structural characterization, and quality assurance (QA). Global regulatory agencies, including U.S. Food and Drug Administration (US FDA) and European Medicines Agency (EMA), look to harmonized guidelines from the International Council for Harmonisation (ICH). ICH Q6B[5] covers the test procedures and acceptance criteria for biologic drug products and specifies the use of peptide mapping as a critical quality test procedure for drug characterization used to confirm desired product structure for lot release purposes.

To generate a peptide map, a bottom-up strategy is generally used. The therapeutic protein must first be digested into its constituent peptides via a chemical or enzymatic reaction. Robust separation and identification of the resultant peptides then provides insight into a protein's full sequence information, displaying each amino acid component and the surrounding amino acid microenvironment. Structural characterization at this level highlights PTMs such as site-specific glycosylation, amino acid substitutions (sequence variants), and/or truncations, which may result from erroneous transcription of complementary DNA.[6]

A peptide map is a fingerprint of a protein that provides a comprehensive understanding of the protein being analyzed. Consequently, it is a routine analysis for the characterization of mAbs. Although modern peptide-mapping procedures adequately perform their primary function, they typically consist of many laborious steps, which can vary due to differences among techniques or operators, or even across laboratories. As such they are susceptible to changes that affect reproducibility, reduce assay sensitivity and significantly increase analysis times. These procedures are not easily automated, which can reduce data confidence and potentially introduce sample artefacts due to manual sample processing. This variation can be especially challenging when it is necessary to compare different product batches across months or years. As data quality is imperative, variation in results might jeopardize product quality, ultimately affecting patient safety. All this raises the need to adopt a new method that can overcome all these drawbacks and offer a simple, robust and reliable alternative.

The Thermo Scientific™ SMART Digest™ kit[7] provides a simple alternative for peptide mapping sample preparation. It is a fast and simple procedure that greatly improves intra- and inter-laboratory data reproducibility, assuring absolute confidence in analytical results. In this study, peptide mapping experiments were performed using the NISTmAb (NISTmAb RM 8671) provided by the National Institute of Standards and Technology (NIST). The NISTmAb was chosen because it is a well-characterized, commercially available test material that is expected to greatly facilitate analytical development applications associated with the characterization of originator and follow-on biologics for the foreseeable future.

The NISTmAb is a recombinant humanized IgG1κ expressed in murine suspension culture, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process related impurities. It is a ≈150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds. The molecule has a high abundance of N-terminal pyroglutamination, C-terminal lysine clipping, and glycosylation of the heavy chain sub-units. The protein also has low abundance PTMs including methionine oxidation, deamidation, and glycation.[8] In this study, both light and heavy chain sequence coverage was assessed, as well as the identification and relative quantification of a specific set of PTMs: oxidation, glycosylation, and deamidation.

After digestion with the SMART Digest kit was completed, separation of peptides was performed using the Thermo Scientific™ Vanquish™ Flex Binary UHPLC system with the Thermo Scientific™ Acclaim™

VANQUISH™ C18 analytical column. MS data was interrogated using Thermo Scientific™ BioPharma Finder™ software. A list of peptides was generated from the highly complex data and subsequently matched against the corresponding mAb sequence with a sequence coverage map generated. The use of the SMART Digest kit resulted in a simple, easy to use, fast method, emphasizing its high reproducibility, lower tendency to generate PTMs, higher enzyme stability, and high amenability to automation.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ UHPLC-MS grade water (P/N W/0112/17)
- Fisher Scientific™ Optima™ LC-MS grade acetonitrile with 0.1% formic acid (v/v) (P/N LS120-212)
- Fisher Scientific™ Optima™ LC-MS grade water with 0.1% formic acid (v/v) (P/N LS118-212)
- SMART Digest Kit - Trypsin (P/N 60109-101)
- Fisher Scientific™ Optima™ LC-MS grade methanol (P/N A458-1)
- Fisher Scientific™ Optima™ LC-MS grade acetic acid (P/N A113-50)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Acclaim VANQUISH C18 column, 2.1 × 250 mm, 2.2 µm (P/N 074812-V)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Equipment

- Vanquish Flex Binary UHPLC system including:
  - Binary Pump F (P/N VF-P10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A)
  - System Base Vanquish Horizon (P/N VH-S01-A)
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 spectrophotometer (P/N ND-2000)

Sample preparation

NISTmAb, reference material 8671, was supplied by the National Institute of Standards and Technology (NIST) at 10 mg/mL in sample buffer (12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl, pH 6.0). NISTmAb digested samples were prepared in duplicate for each time course point using the SMART Digest kit.

SMART Digest kit protocol

Duplicate 50 µL mAb samples were taken and adjusted to 2 mg/mL with water. This was further diluted 1:4 (v/v) with the SMART Digest buffer provided with the kit. The solution was 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 carried out where tryptic digestion was performed at 70 °C for 15, 30, 45, 60, and 75 min with agitation at 1400 rpm. After the digestion the reaction tube was centrifuged twice at 7000 rpm for 2 min to remove any solid residue. Disulfide bonds were then reduced by addition of 1 M to a final concentration of 5 mM DTT and incubation for 30 minutes at 37 °C. All samples were diluted with 0.1% formic acid (FA) in water and 3 µg were loaded on the column for all runs.

LC conditions

Column:	Acclaim VANQUISH C18, 2.1 × 250 mm, 2.2 µm
Mobile phase A:	0.1% formic acid aqueous solution
Mobile phase B:	0.1% formic acid solution in acetonitrile
Flow rate:	0.3 mL/min
Column temperature:	25 °C (Still air mode)
Autosampler temperature:	5 °C
Injection volume:	10 µL
Injection wash solvent:	MeOH:H_2O, 20:80
Gradient:	See Table 1 and Figure 1 for details

Table 1. Mobile phase gradient for UHPLC separation of peptides

Time (minutes)	Flow (mL/min)	% Mobile Phase B	Curve
0.000	0.300	2.0	5
45.000	0.300	40.0	5
46.000	0.300	80.0	5
50.000	0.300	80.0	5
50.500	0.300	2.0	5
65.000	0.300	2.0	5

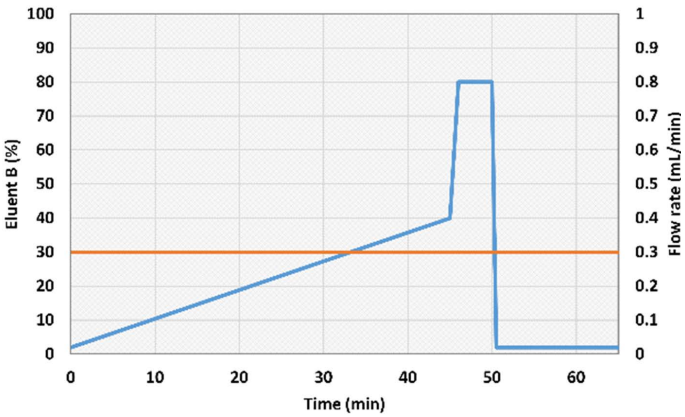

Figure 1. Mobile phase gradient for UHPLC separation of peptides

MS Conditions

Detailed MS method parameters are shown in Tables 2 and 3.

Table 2. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Ion Max source with HESI II probe
Sheath gas pressure	35 psi
Auxiliary gas flow	10 arbitrary units
Probe heater temperature	400 °C
Source voltage	3.8 kV
Capillary temperature	400 °C
S-lens RF voltage	60 V

Data processing

Thermo Scientific™ Xcalibur™ software version 4.0.27.13 (Cat. No. OPTON-30487) was used for data acquisition and analysis. For data processing, Biopharma Finder software version 3.0 was applied. Detailed parameter settings are shown in Table 4.

Table 3. MS method parameters utilized for peptide mapping analysis

General	Setting	MS^2 Parameters	Setting
Runtime	0 to 65 min	Resolution settings	17,500
Polarity	Positive	AGC target value	1.0 × 105
Full MS parameters	**Setting**	Isolation width	2.0 *m/z*
Full MS mass range	200–2000 *m/z*	Signal threshold	1.0 × 104
Resolution settings	70,000	Normalized collision energy (HCD)	28
AGC target value	3.0×10^6	Top-N MS^2	5
Max injection time	100 ms	Max injection time	200 ms
Default charge state	2	Fixed first mass	–
SID	0 eV	Dynamic exclusion	7.0 s
Microscans	1	Loop count	5

Table 4. Biopharma Finder 3.0 software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS signal threshold	8.0 x 10^4 counts
Typical chromatographic peak width	0.30
Relative MS signal threshold (% base peak)	1.00
Relative analog threshold (% of highest peak)	1.00
Width of Gaussian filter (represented as 1/n of chromatographic peak width)	3
Minimum valley to be considered as two chromatographic peaks (%)	80.0
Minimum MS peak width (Da)	1.20
Maximum MS peak width (Da)	4.20
Mass tolerance (ppm for high-res or Da for low-res)	4.00
Maximum retention time shift (min)	1.69
Maximum mass (Da)	30,000
Mass centroiding cut-off (% from base)	15
Identification	**Setting**
Maximum peptide mass	7000
Mass accuracy	5 ppm
Minimum confidence	0.8
Maximum number of modifications for a peptide	1
Unspecified modification	-58 to +162 Da
N-Glycosylation	CHO
Protease specificity	High
Variable Modifications	**Setting**
N Terminal	Gln → Pyro Glu
C Terminal	Loss of lysine
Side chain	Deamidation (NQ)
	Glycation (K)
	Oxidation (MW)

Results and discussion

The SMART digestion procedure assures digestion completeness for mAb samples within 45–60 min, as previously reported for rituximab,[9] however it is also possible to achieve an efficient digestion even in 30 min, as it is observed in Figure 2. Elution time for the monitored individual peptide is shown on the chromatograms in Figure 3.

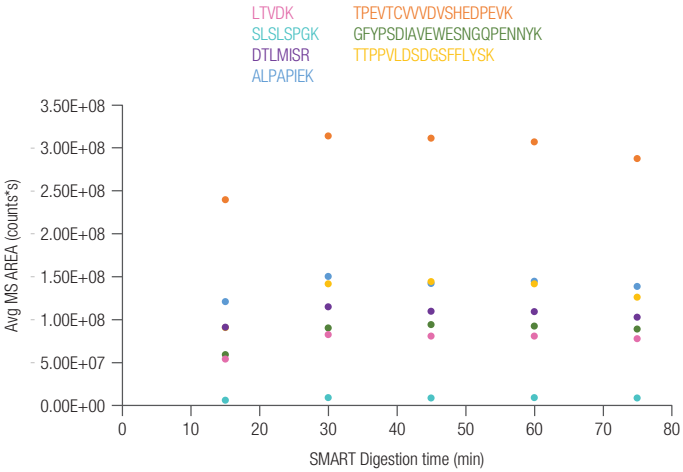

Figure 2. NISTmAb resulted peptides after digestion times from 15 to 75 min using the SMART Digest kit: LTVDK (10.49 min), SLSLSPGK (17.35 min), ALPAPIEK (18.17 min), DTLMISR (19.45 min), TPEVTCVVVDVSHEDPEVK (26.40 min), GFYPSDIAVEWESNGQPENNYK (31.46 min), and TTPPVLDSDGSFFLYSK (32.49 min) The graph shows average results of duplicate samples.

Figure 3 shows base peak chromatograms (BPC) for NISTmAb, obtained in Xcalibur software, which have been digested with the SMART Digest kit containing trypsin using digestion times from 15 to 75 minutes. Visual comparison of the profiles resulting from the different time points revealed some differences related to peak intensities but showed good similarity in the number of detected major peaks. For minor signals, the longer the digestion is performed the more peaks are noticeable along the chromatogram. Peptides monitored in Figure 2 are highlighted on the BPCs.

Figure 4 illustrates extracted ion chromatograms (XICs) of seven identified peptides of NISTmAb, which has been digested with immobilized trypsin of the SMART Digest kit. Identified peptides are labeled with their corresponding sequence number and chromatographic retention time. Using the Vanquish Flex Binary UHPLC system, stable retention times were observed for all measured peptides as shown in Figure 4. The retention time precision (RSD) for the monitored peptides was <0.2%.

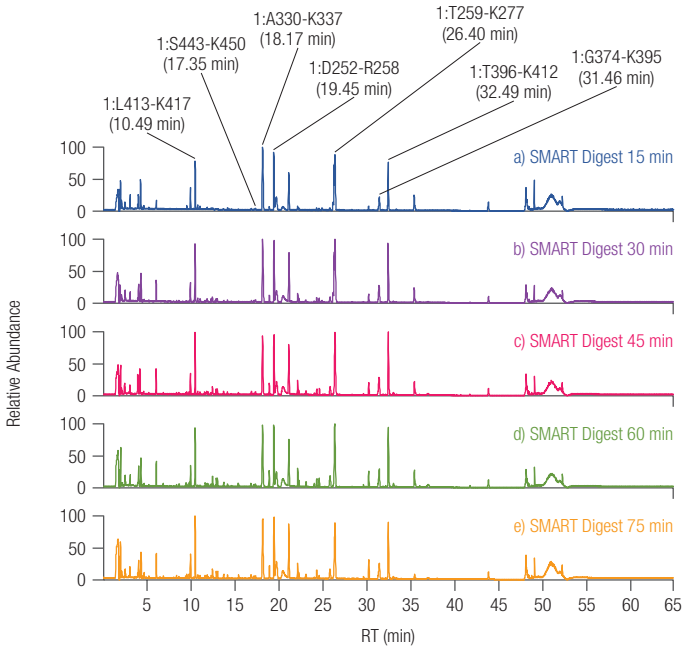

Figure 3. Base peak chromatograms (BPC) for the time course experiment SMART Digested NISTmAb sample: a) Smart Digest, 15 min; b) Smart Digest, 30 min; c) Smart Digest, 45 min; d) Smart Digest, 60 min; e) Smart Digest, 75 min

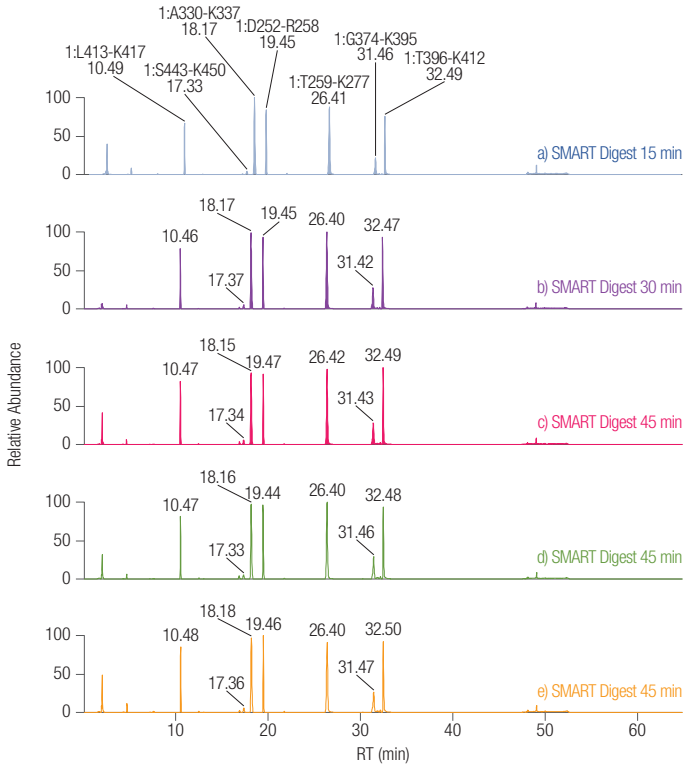

Figure 4. Extracted ion chromatograms of seven identified peptides for the time course experiment SMART digested NISTmAb sample highlighting the retention time precision: a) SMART Digest, 15 min; b) SMART Digest, 30 min; c) SMART Digest, 45 min; d) SMART Digest, 60 min; e) SMART Digest, 75 min

The sequence coverages for the different digest conditions are shown in Table 5. For all evaluated methods using the SMART Digest kits, 100% coverage was achieved for light and heavy chains in all cases for all studied digestion times between 15 and 75 minutes. As confidence in the results is imperative, only results with ≥ 95% confidence were accepted, and identification was based on full MS and MS^2 spectra. These criteria will support strongly the confident identification of peptide sequences.

The sequence coverage map based on a 30-minute digest (Figure 5) shows the overlap of the different peptides identified with different intensities and in different lengths due to missed cleavages. 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 abundance, >1.7e+007; yellow, >1.5e+006; green, >1.4e+005; light blue, >1.2e+004; cyan = low abundance, >1.0e+003.

Table 5. Sequence coverage with different digestion methods for the studied NISTmAb RM 8671.

Proteins	Sample	Number of Peaks	Sequence coverage (%)
NISTmAb RM 8671			
Heavy Chain	SMART Digest, 15 min	1145	100.00
	SMART Digest, 30 min	1180	100.00
	SMART Digest, 45 min	1126	100.00
	SMART Digest, 60 min	1107	100.00
	SMART Digest, 75 min	1067	100.00
Light chain	SMART Digest, 15 min	220	100.00
	SMART Digest, 30 min	265	100.00
	SMART Digest, 45 min	282	100.00
	SMART Digest, 60 min	287	100.00
	SMART Digest, 75 min	281	100.00

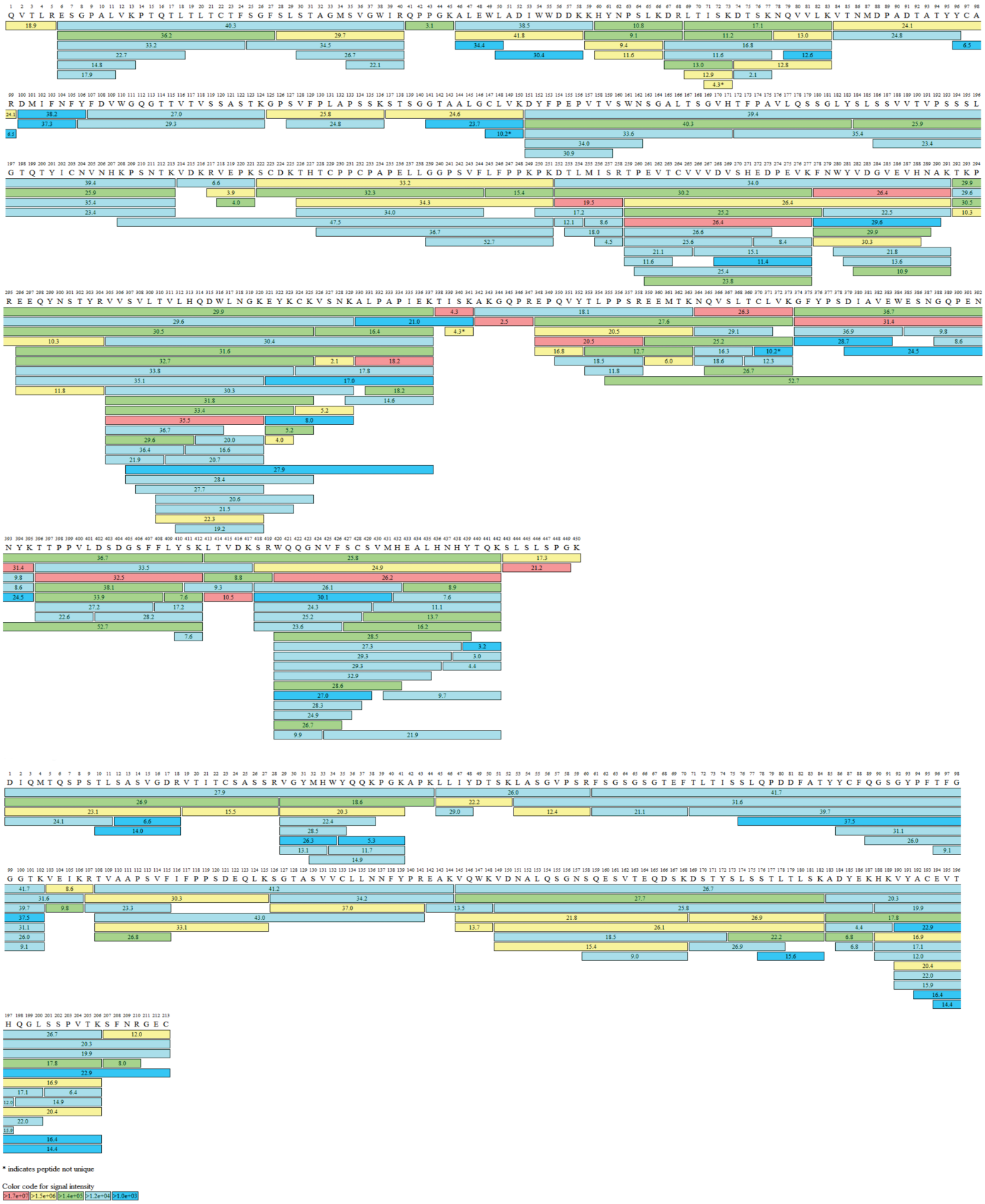

Figure 5. Sequence coverage map of NISTmAb heavy (upper panel) and light (lower panel) chain, obtained using a tryptic SMART Digest time of 30 minutes

The number of detected MS peaks in the samples digested were generally over 1000, obtaining maximum values for digestion times of 30–45 minutes (~1360 identified components). 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 6).

All matched peptides have ≤5 ppm and ≥-5 ppm of MS mass error, a confidence score ≥95, and full MS and confirmatory MS/MS spectra. Combining data from high-quality MS and MS/MS further improves peptide matching.

Figure 6 shows an example of the MS/MS spectra for selected peptide 2:V149-K168 of the light chain from NISTmAb 30 min digest. BPC (a) and XIC (b) show the identified peptide eluting at 15.43 min. Identification displays the identification associated with the component (2:V149-K168) where "2" is the protein ID number (Light Chain for NISTmAb), "V149" is the first amino acid in the peptide sequence and its position number, "K168" is the last amino acid in the peptide sequence and its position number, and "2134.9614m" is the mass of the unmodified peptide (this is neutral and not a charged mass).

Figure 6c shows the experimental spectrum and displays an inverted triangle marker at the top of the spectral line for the theoretical precursor ion. The labels appear in color for the identified peaks and also show their fragment ion assignments and charge states, for example, "b3", "y4", or "y9". The color for the lines and labels for the identified ions in the experimental spectrum vary based on the ion type, as follows: dark blue for "b" ions with a charge on the N-terminal side and red for "y" ions with a charge on the C-terminal side.

Table 6. Number of identified components and average total MS area for the different runs

Sample	Number of Identified Components	Total MS Area (counts×s)
NISTmAb RM 8671		
SMART Digest, 15 min	1300	1.66E+09
SMART Digest, 30 min	1375	2.17E+09
SMART Digest, 45 min	1341	2.12E+09
SMART Digest, 60 min	1304	2.15E+09
SMART Digest, 75 min	1279	1.98E+09

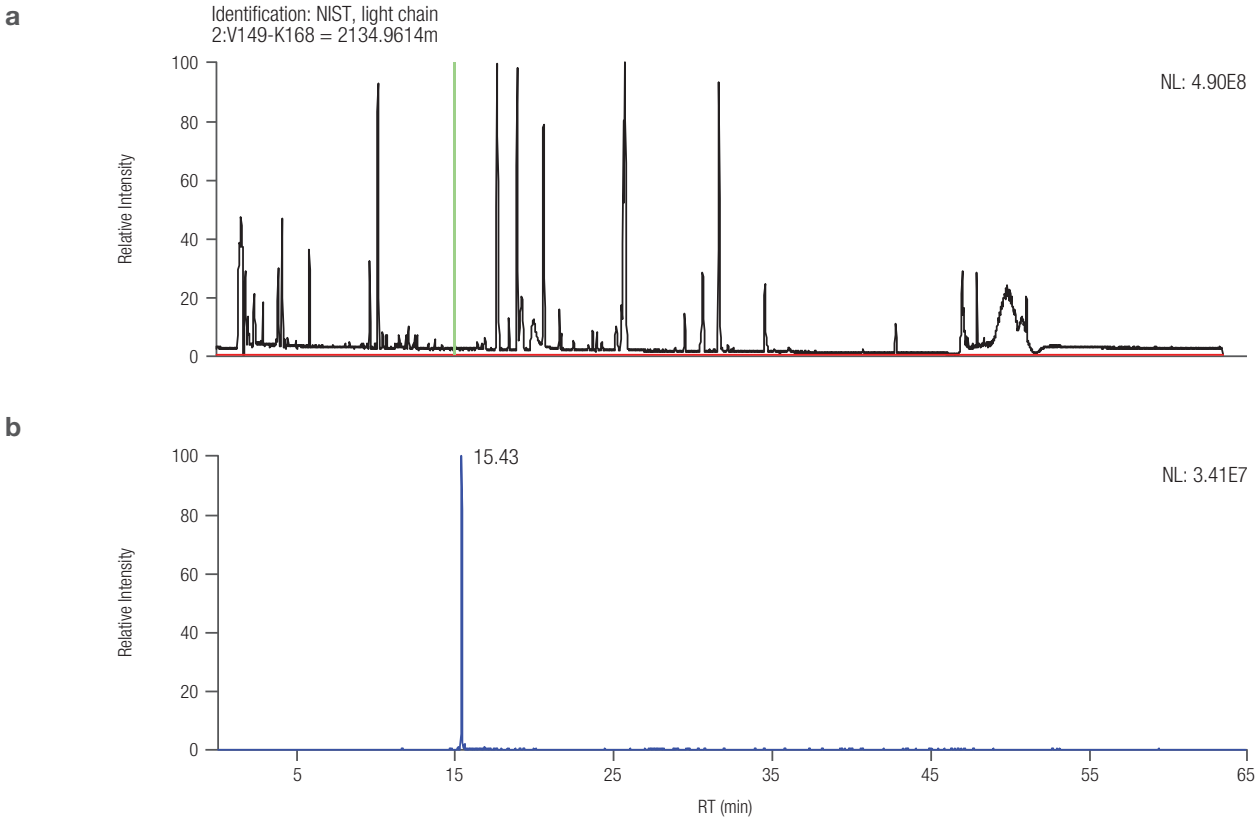

Figure 6. Representative BPC chromatogram (a), XIC (b). NL indicates normalized level (NL) intensity.

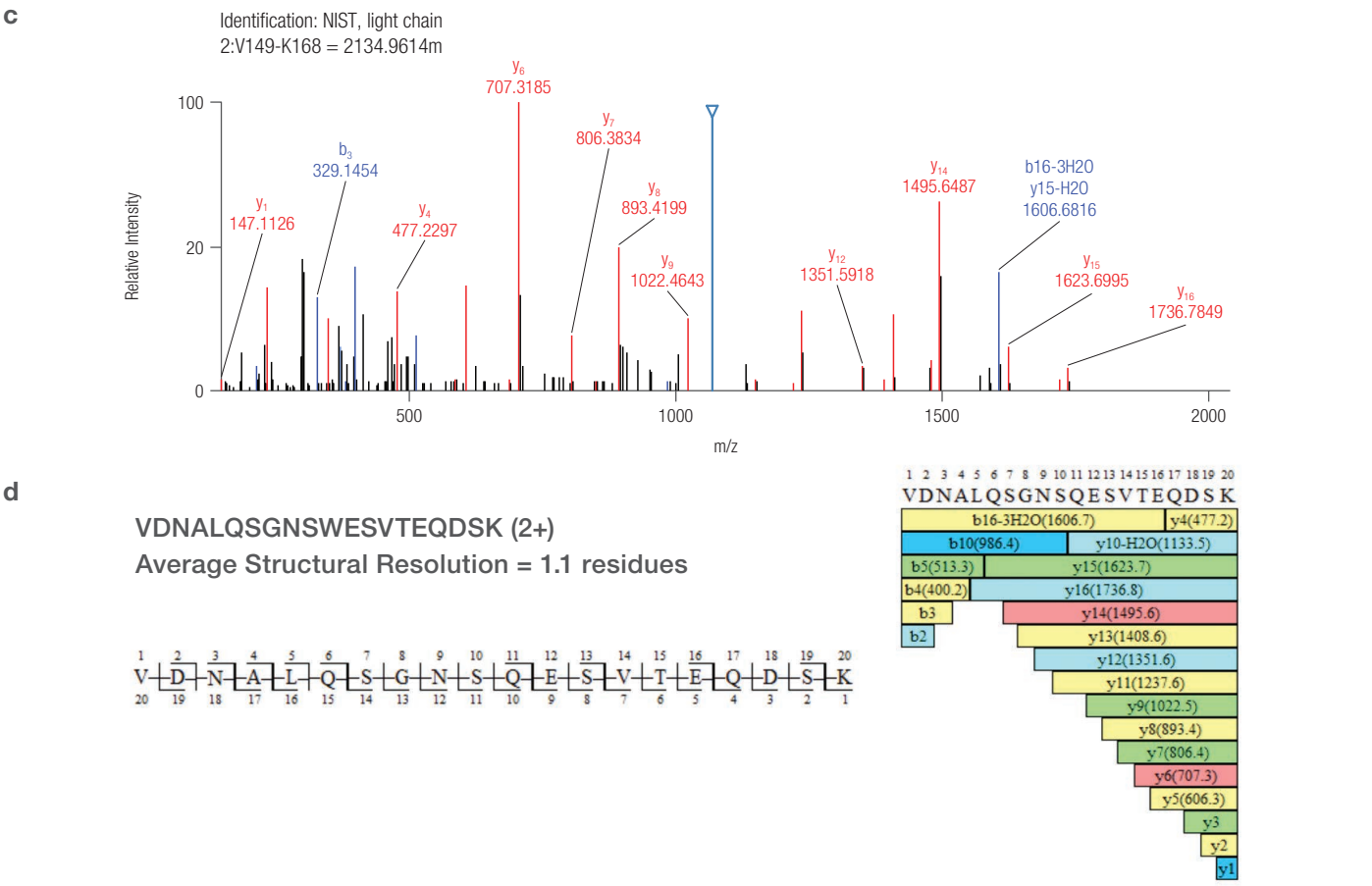

Figure 6 (continued). MS/MS spectra (c), and fragment coverage map (d) of a light chain peptide from digested NIST mAb.

The peptide fragment coverage map (6d) displays the peptide sequence with corresponding modification and charge state, the average structural resolution score in number of residues (total number of amino acids/number of peptide fragments) where a value of 1 indicates the best fit, the peptide sequence with the numbered amino acid sequence and the identified fragment lines, and finally the identified fragment ions using a color-coded scheme for ion intensity (red, yellow, green, cyan, and blue), with red as most intense and blue as the least intense.

Peptide mapping analysis also provided information about the identification, localization, and (relative) quantification of various PTMs that might be present on the amino acid residues, as shown in Figure 7, where deamidation of N289 is shown as an example. BPC (a) and XICs (b and d) show the identified peptide eluting at 26.41 min and 27.43 min for native and deamidated forms, respectively. Identification displays the identification associated with the component (1:F278-K291) where “1” is the protein ID number (Heavy Chain for NISTmAb), “F278” is the first amino acid in the peptide sequence and its position number, “K291” is the last amino acid in the peptide sequence and its position number, and “1676.7947m” is the mass of the unmodified peptide (neutral mass). Full MS isotopic pattern is also shown, highlighting the high resolution power of the Q Exactive MS and the ability to resolve non-deamidated vs. deamidated peptides. Low abundance of the deamidated peptide (1.17%) resulted in the XIC showing few peaks, which correspond to the native form (26.40 min) and most likely the isoaspartic acid isomer for the deamidated form (27.02 min). Figures 7c) and 7e) show the experimental MS/MS spectra and fragment coverage map for native and deamidated forms, respectively. An inverted triangle marker at the top of the spectral line for the theoretical precursor ion (z=2) is shown for both nondeamidated and deamidated peptide 1:F278-K291. Identified ions in the experimental MS/MS spectra show accurately that the mass difference corresponding to the deamidation of N289 (e. g., y5=568.3199 for native and 569.3044 for deamidated peptide form) is 0.98 Da.

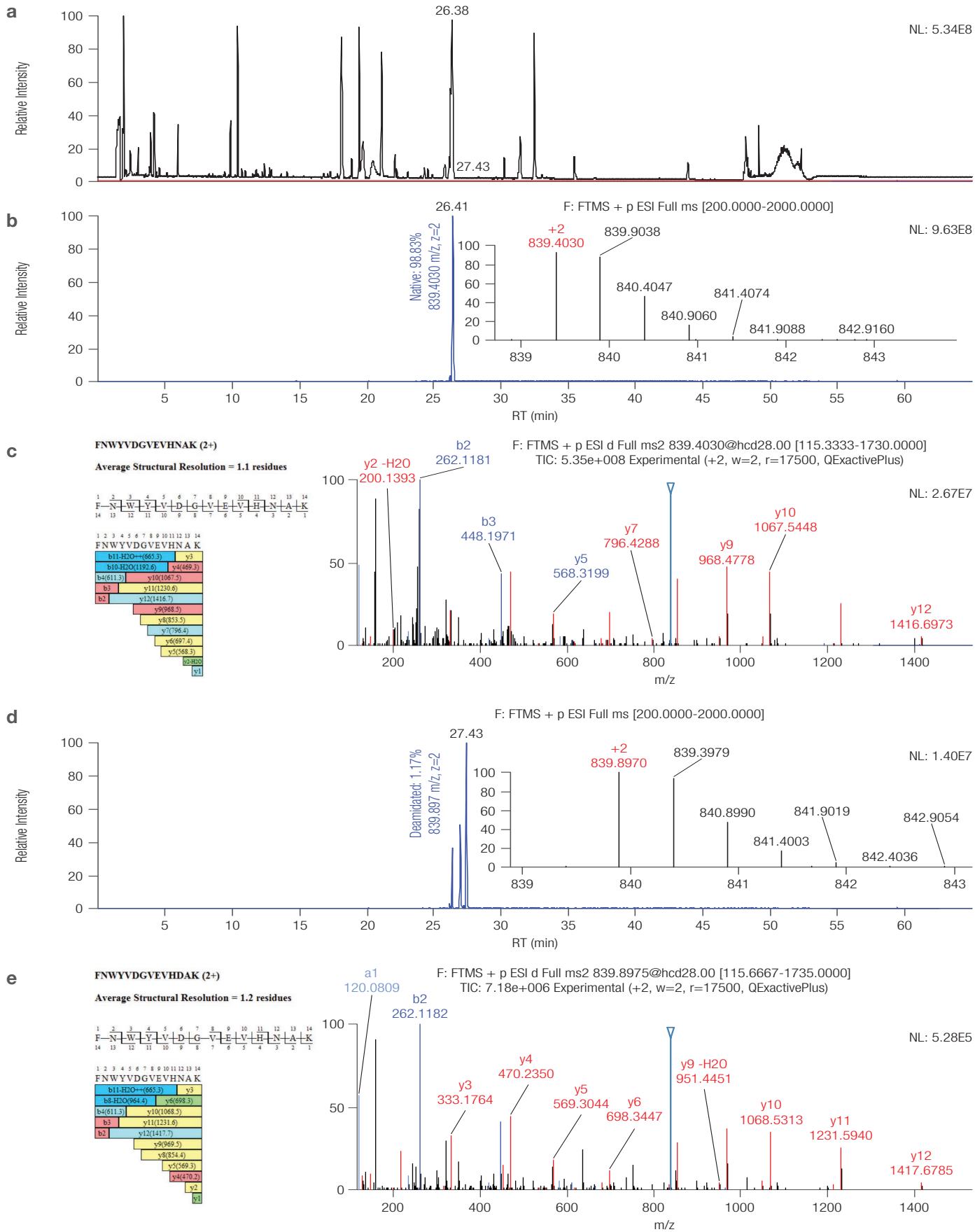

Figure 7. Post-translational modification (N289 asparagine deamidation) analysis for a heavy chain peptide (1:F278-K291) from NISTmAb. BPC (a), XIC for native (b) and deamidated peptide (d) and MS/MS spectra for native (c) and deamidated form (e). NL indicates normalized level (NL) intensity.

Table 7 summarizes the quantification results for the individual modification sites. The NISTmAb molecule has a high abundance of N-terminal pyroglutamination (>99%). Almost half the antibodies reported in the literature contain a glutamic acid residue at the N-terminus of the light or the heavy chain. As studied by Chelius D. et al.,[10] the formation of pyroglutamic acid from N-terminal glutamic acid in the heavy chains and light chains of several antibodies indicates that it is a nonenzymatic reaction that occurs very commonly. For therapeutic mAbs, pyroGlu can be one of many PTMs or transformations observed during production and storage. Because of the loss of a primary amine in the glutamine (Gln) to pyroGlu conversion, mAb becomes more acidic.

High abundance of glycosylation of the heavy chain is also observed where the main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the reducing end of N-acetylglucosamine (A2G0F, A2G1F, and A2G2F) and high mannose structures (M5). According to literature, within a panel of commercially available therapeutics mAbs, IgGs expressed in CHO tend to contain a higher level of G0 glycans compared to recombinant IgGs produced in mouse myeloma cell lines.[11] In contrast, unusual high levels of mannose-5 (Man5) were observed during development of a therapeutic mAb produced in CHO cell line and correlated to the increase of cell culture medium osmolality levels and culture duration.[12]

The NISTmAb also has low abundance of PTMs including methionine oxidation, glycation, and deamidation. Deamidation of asparagine residues is a common degradation of proteins, and it can significantly impact protein structure and function. The rate of deamidation depends on protein sequence and conformation, as wells as on external factors such as temperature, pH, and others.[13] Oxidation of methionine is a common chemical modification that occurs in mAbs during purification, formulation, and storage processes. Met oxidation could decrease bioactivity and stability of IgGs, which results in reduced product serum half-life and limited shelf-life.[14]

Overall, similar levels for all modifications were detected for all digested samples, except for deamidation where the level of modifications slightly increases with digestion time. This was noteworthy for some modification sites, e.g. deamidation of N318 presented 6.95% of deamidated peptide for the 15 min digest sample while it was 10.62% when digestion time increased to 75 min.

The degree of deamidation increases with extended digestion time. 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,[15] which could explain the increase in the relative total amount of deamidated peptides when increasing digestion time. Oxidation was presented in low levels with similar relative abundances between the different digested samples. Methionine (M) oxidation was <2.95%, while tryptophan (W) oxidation was <2.7% for the studied digestion times.

Another commonly targeted modification type is lysine (K) glycation (Table 7). In total, seven lysine glycations could be identified and relatively quantified <1.63% with an average RSD value ≤13% for the SMART Digest time course study (K77 and K189 where higher RSD; 25.8% and 16.8% were observed respectively for two samples). Overall, similar levels were observed for the different digestion times.

The variance values across the five different digestion times, expressed as the %RSD of the measured relative abundance for each modification with each digestion protocol, were overall < 15% for all the identified modifications with the exception of deamidation of N328 and oxidation of M361, M4, and M32, which showed RSD >15% for some of the samples.

Overall the data shows excellent sequence coverages with a low amount of sample loaded onto the column (3 µg). There is a high level of reproducibility and, while observable in some cases, the level of PTMs induced by sample preparation is not significant.

Table 7. Comparison of the oxidation, deamidation, glycation, C-terminal lysine loss, glycosylation, and N-terminal pyroglutamination modifications identified with the different digestion methods, for NISTmAb

Modification	Sequence	SMART Digest. 15 min (n=2)	SMART Digest. 30 min. (n=2)	SMART Digest. 45 min (n=2)	SMART Digest. 60 min. (n=2)	SMART Digest. 75 min (n=2)
Q1+Gln→Pyro-Glu	QVTLR	99.691	99.700	99.692	99.714	99.719
N78+Deamidation	NQVVLK	1.848	2.097	2.301	2.490	2.719
N289+Deamidation	FNWYVDGVEVHNAK	0.791	0.997	1.183	1.396	1.523
N300+Deamidation	EEQYNSTYR	0.173	0.149	0.138	0.136	0.133
N318+Deamidation	VVSVLTVLHQDWLNGK;TVLHQDWLNGK	6.952	7.203	8.201	8.971	10.623
N328+Deamidation	VSNK	0.684	0.955	0.823	1.111	1.150
N364+Deamidation	NQVSLTCLVK	1.667	2.275	2.757	2.958	2.951
~N387+Deamidation	GFYPSDIAVEWESNGQPENNYK	2.819	3.042	3.557	4.069	4.263
~N392+Deamidation	GFYPSDIAVEWESNGQPENNYK	0.930	0.985	1.156	1.187	1.472
~Q36+Deamidation	VGYMHWYQQKPGK	1.042	2.220	1.279	0.742	0.615
~N136+Deamidation	SGTASVVCLLNNFYPR	1.954	2.106	2.531	2.935	3.191
N157+Deamidation	VDNALQSGNSQESVTEQDSK	1.482	1.804	2.023	2.266	2.453
M87+Oxidation	VTNMDPADTATYYCAR	2.945	2.378	2.517	2.439	2.754
M255+Oxidation	DTLMISR	2.440	2.390	2.222	2.509	2.497
W280+Oxidation	FNWYVDGVEVHNAK	0.062	0.054	0.054	0.055	0.057
W316+Oxidation	VVSVLTVLHQDWLNGK	0.059	0.051	0.065	0.055	0.073
M361+Oxidation	EPQVYTLPPSREEMTK; EEMTK	1.258	1.134	0.910	1.128	1.040
M4+Oxidation	DIQMTQSPSTLSASVGDR	1.910	1.580	1.676	2.168	1.820
M32+Oxidation	VGYMHWYQQKPGK	0.907	0.808	0.715	0.892	0.914
K73+Glycation	LTISKDTSK	0.609	0.785	0.866	0.895	0.879
K77+Glycation	DTSKNQVVLK	0.134	0.213	0.257	0.277	0.284
K329+Glycation	VSNKALPAPIEK	0.590	0.684	0.683	0.696	0.691
K337+Glycation	ALPAPIEKTISK	0.096	0.105	0.103	0.105	0.101
K363+Glycation	EEMTKNQVSLTCLVK	0.062	0.102	0.172	0.173	0.165
K148+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.458	0.644	0.668	0.709	0.710
~K189+Glycation	ADYEKHKVYACEVTHQGLSSPVTK	1.631	1.591	1.152	0.898	0.597
K450+Lys Loss	SLSLSPGK	90.547	90.746	90.677	90.734	90.692
N300+A1G0	EEQYNSTYR; TKPREEQYNSTYR	0.749	0.767	0.806	0.847	0.889
N300+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	7.899	8.727	9.115	9.459	9.555
N300+A1G0M4F	TKPREEQYNSTYR	1.799	1.751	1.508	1.682	0.744
N300+A1G1	EEQYNSTYR	0.119	0.111	0.104	0.106	0.103
N300+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	4.595	5.100	5.222	5.058	5.150
N300+A1G1M4F	EEQYNSTYR	1.049	1.033	1.027	1.040	1.002
N300+A1G1M5	EEQYNSTYR	0.229	0.234	0.225	0.239	0.226
N300+A1Ga1F	EEQYNSTYR	0.197	0.211	0.206	0.234	0.229
N300+A1Sg1F	EEQYNSTYR; TKPREEQYNSTYR	1.043	1.063	1.049	1.034	1.057
N300+A2G0	EEQYNSTYR	0.203	0.214	0.212	0.202	0.200
N300+A2G0F	TKPREEQYNSTYR; EEQYNSTYR	37.949	35.962	35.018	35.163	35.607
N300+A2G1F	TKPREEQYNSTYR; EEQYNSTYR	40.515	35.982	34.614	33.691	33.107
N300+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	8.631	8.415	8.495	8.392	8.132
N300+A2G2M5F	TKPREEQYNSTYR	0.793	0.947	1.092	1.096	1.018
N300+A2Ga1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.618	1.642	1.701	1.702	1.678
N300+A2Ga2F	EEQYNSTYR	0.678	0.714	0.720	0.740	0.765
N300+A2Sg1G0F	EEQYNSTYR	0.327	0.336	0.340	0.343	0.357
N300+A2Sg1G1F	EEQYNSTYR	0.502	0.541	0.570	0.583	0.574
N300+A2Sg1Ga1F	EEQYNSTYR	0.452	0.510	0.512	0.513	0.509
N300+A3G0F	EEQYNSTYR	0.130	0.137	0.145	0.140	0.146
N300+A3G1F	EEQYNSTYR	0.418	0.440	0.443	0.439	0.439
N300+M5	EEQYNSTYR; TKPREEQYNSTYR	1.209	1.289	1.379	1.459	1.506
N300+Unglycosylated	EEQYNSTYR	1.787	1.455	1.281	1.233	1.219

Conclusions

- Using the Thermo Scientific SMART Digest kit provides a simple and rapid protein digestion protocol for peptide mapping analysis, which is more efficient and reproducible than traditional methods.

- The analysis of NISTmAb RM 8671 produced excellent quality data with high confidence in results. Sequence coverage was high and sample preparation-induced post-translational modifications (PTMs) while observed were low, except for deamidation of N318 and N387 residues, which are potentially more susceptible to deamidation (as previously described in AN21782).

- The time course experiment comparing increasing digestion times with the NISTmAb, showed no substantial difference between the different approaches for either data quality or data information content. Protein sequence coverage of 100% was achieved for the five digestion times tested.

- The analysis of the most commonly targeted modifications were successfully identified and relatively quantified. The elevated temperatures during enzymatic digestion using the SMART Digest kit showed only a very slight predictable increase over time in the amount of induced deamidation except for deamidation of N318 where 10.62% modification was observed when using a 75-minute digestion time.. It was also shown that optimization of the incubation time can be used to further minimize the introduction of chemical modification during digestion.

- The data presented in this study clearly demonstrate the capability of the applied SMART Digest protocol when combined with the Vanquish Flex UHPLC system and Orbitrap-based LC-MS to significantly speed up peptide mapping experiments enabling high throughput analyses, even with low abundant samples, as required during the development phase of biopharmaceuticals.

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