APPLICATION NOTE

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

Authors: Martin Samonig[1], Alexander Schwahn[2], Ken Cook[3], Mike Oliver[4], and Remco Swart[1]

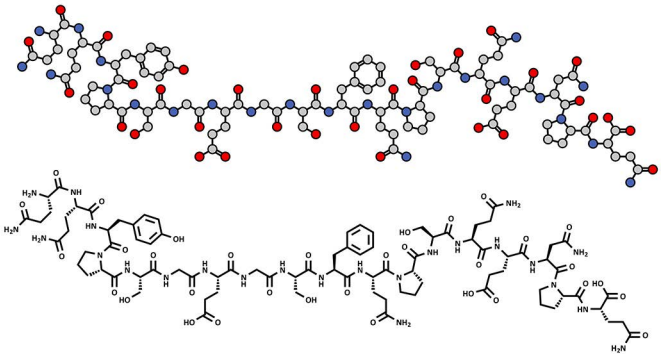
[1]Thermo Fisher Scientific, Germering, Germany; [2]Thermo Fisher Scientific, Basel, Switzerland; [3]Thermo Fisher Scientific, Hemel Hempstead, United Kingdom; [4]Thermo Fisher Scientific, Runcorn, United Kingdom

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 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 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 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)

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

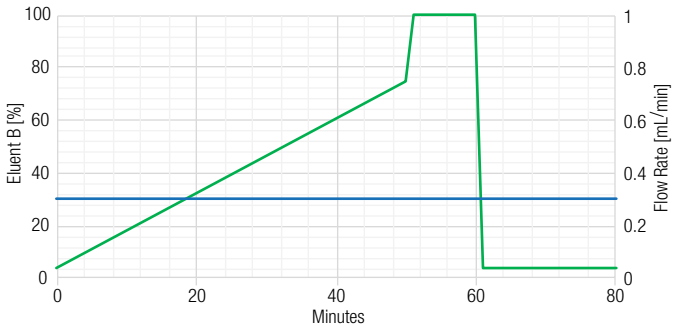


Figure 1. LC gradient.

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% FA
Mobile Phase B:	Water/acetonitrile (20:80 v/v) + 0.1% FA
Flow Rate:	0.3 mL/min
Temperature:	50 °C, Forced air mode
Gradient:	See Figure 1

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.

Table 1A. Q Exactive HF mass spectrometer source parameters.

MS Source Parameters	Setting
Source	Ion Max source with HESI-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 1B. MS method parameters.

Full MS Parameters	Setting	MS^2 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 Da
Max Injection Time	100 ms	Signal Threshold	5.0×10^3
Default Charge State	2	Normalized Collision Energy (HCD)	27
SID	0 eV	Top-N MS^2	5
Microscans	2	Max Injection Time Fixed First Mass Dynamic Exclusion	200 ms *m/z* 140.0 10.0 s

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.

Table 2. BioPharma Finder parameter settings for all samples.

Component Detection	Setting	Variable Modifications	Setting
Absolute MS Signal Threshold	3.00 × 10^5 counts	N Terminal	Carbamylation Gln -> Pyro-Gln
Identification	**Setting**	C Terminal	Lys
Mass Accuracy	5 ppm	Side Chain	Carbamidomethylation (C) Carbamylation (K) Deamidation (N) Dimethylation (K) Double Oxidation (MWC) Glycation (K) Methylation (K) Oxidation (MWC)
Minimum Confidence	0.80		
Maximum Number of Modifications for a Peptide	2		
Unspecified Modification	-58 to +162 Da		
N-Glycosylation	CHO		
Protease Specificity	High		

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, the intelligent sample pre-compression 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

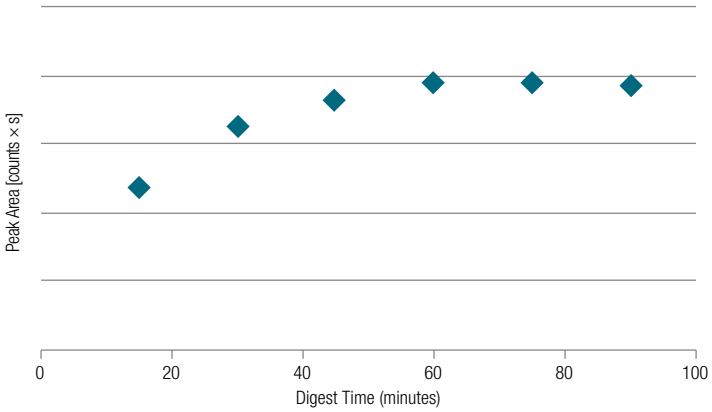


Figure 2. IgG digest profile, monitoring the mAb peptide VVSVLTVLHQDWLNGK for digestion times from 15–90 min using the SMART Digest kit.[2]

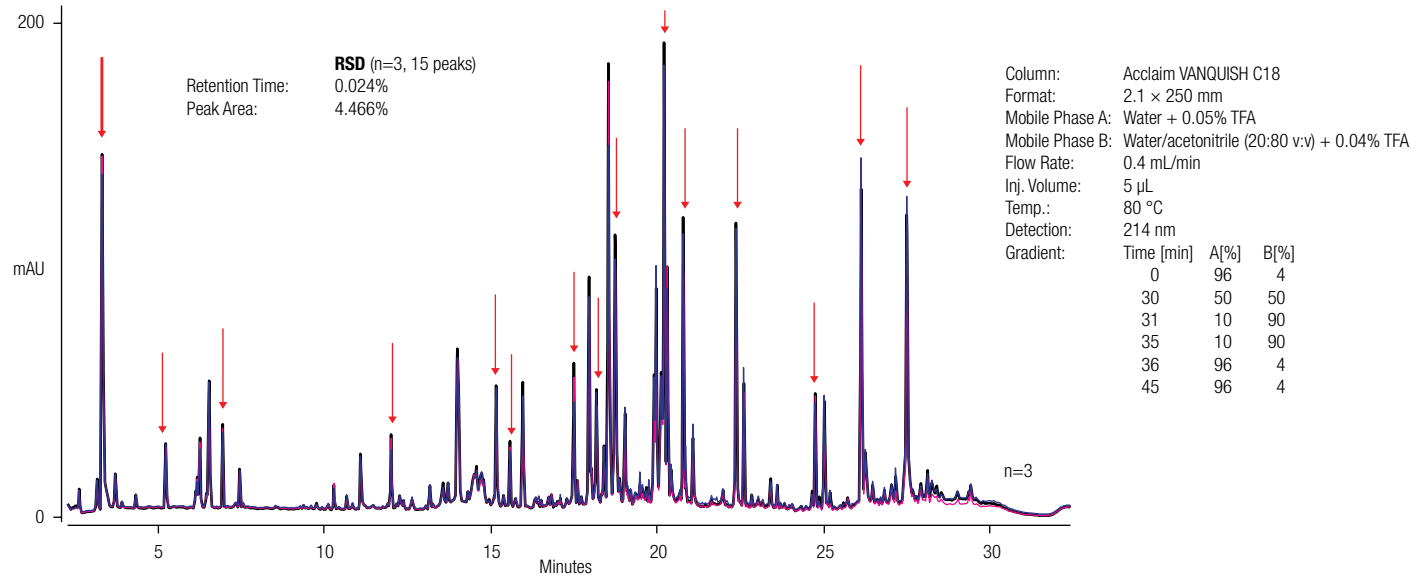


Figure 3. UV chromatogram overlay from three separate SMART digestions from the same mAb, conducted by three individual operators. The 15 marked peptides in each sample were used for inter-user/inter-day RSD value calculations.

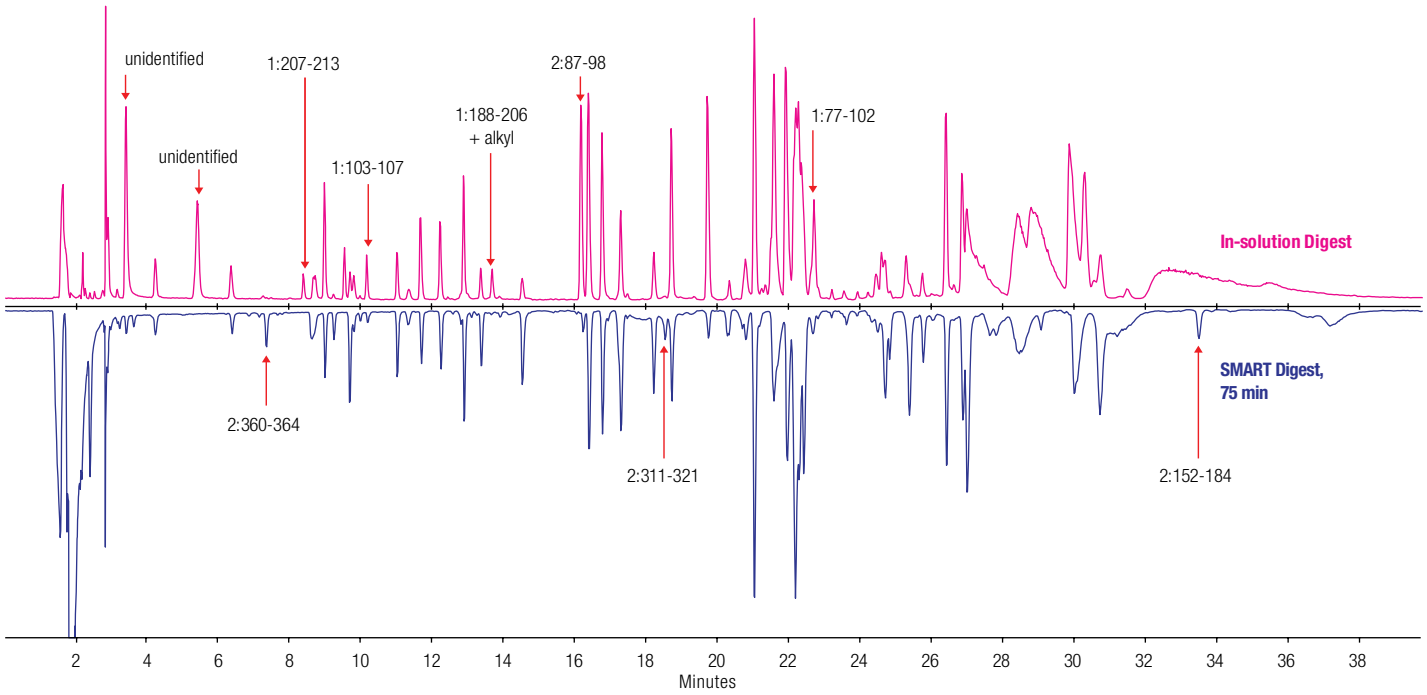


Figure 4. Mirror plot of the TIC chromatogram for the in-solution-digested sample denatured with heat (*In-Solution, Heat*) and the reduced SMART Digest sample (*SMART Digest, 75 min*). Peak labels give annotation to light (1) or heavy (2) chains, respectively, and sequence position.

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).

Table 3. Sequence coverage with different digestion methods.

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

# 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*

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.

The monoclonal antibody rituximab used in this study consists of 1328 amino acid residues including 16 disulfide bonds.[4] Amongst several potential PTMs of amino acids, deamidation of asparagine 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

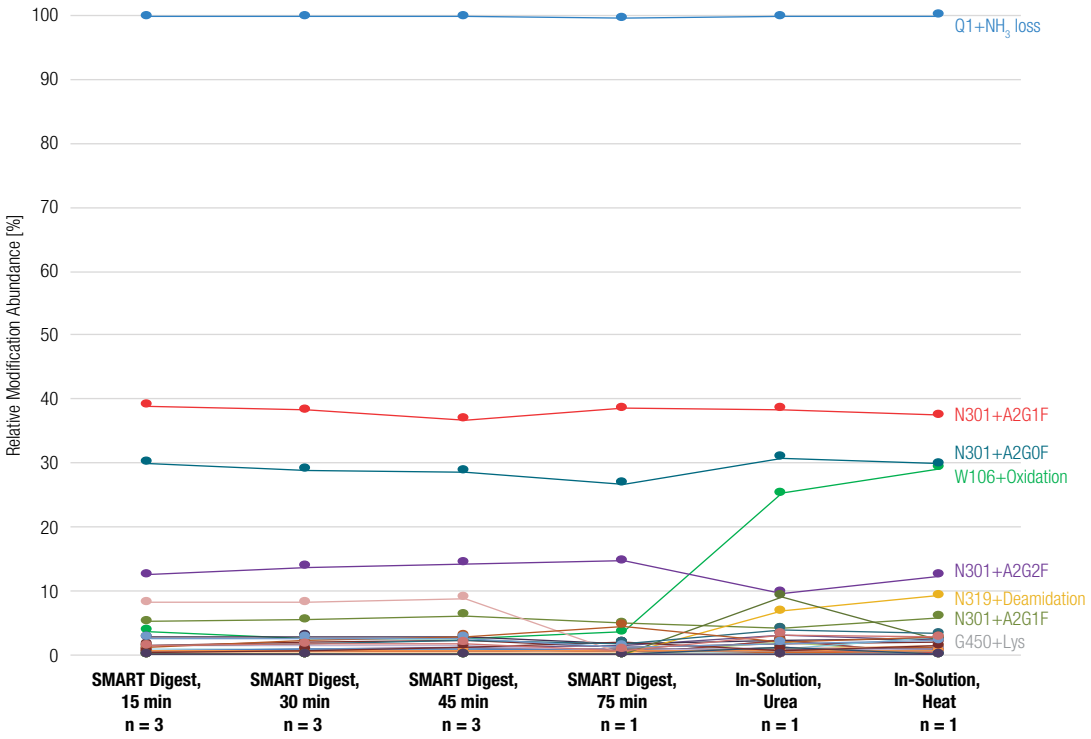


Figure 5. Relative abundance of 85 identified modifications including oxidation, double oxidation, glycation, glycosylation, NH_3 loss, isomerization, lysine truncation, methylation, dimethylation, and carbamylation.

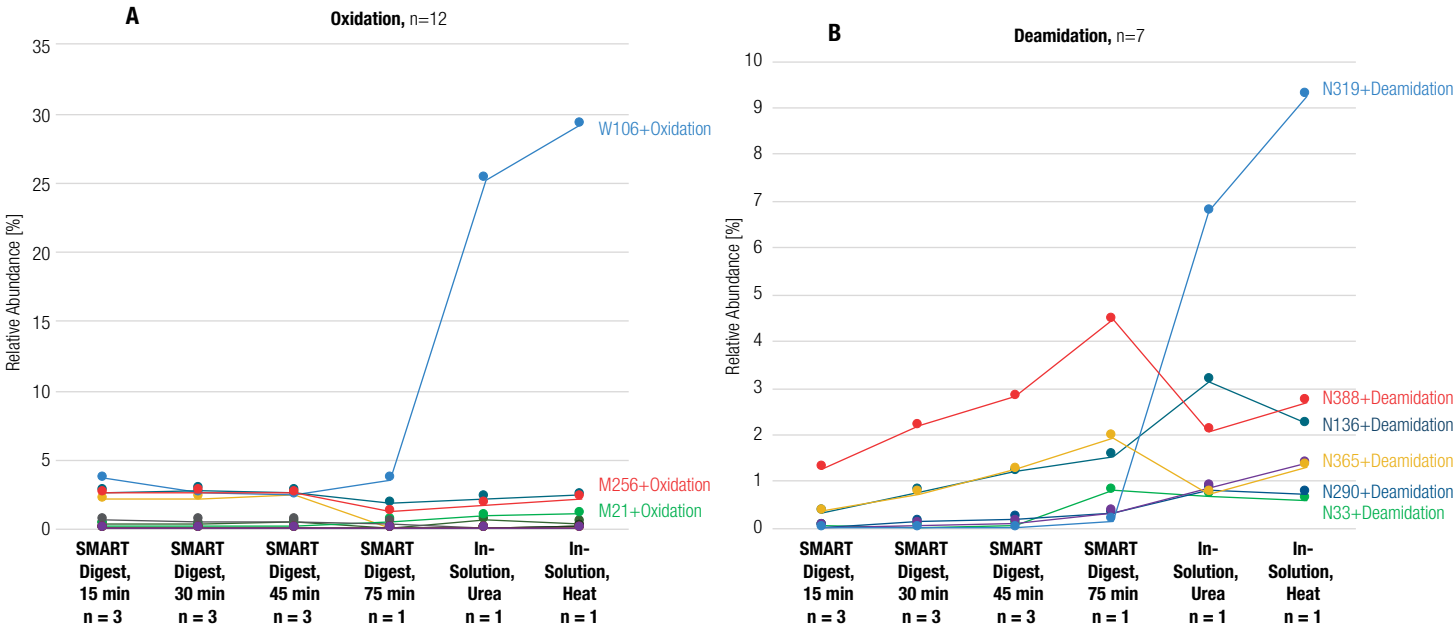


Figure 6. Relative abundance of 12 identified oxidations (A) and 7 deamidations (B) in different runs with various digestion methods.

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 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

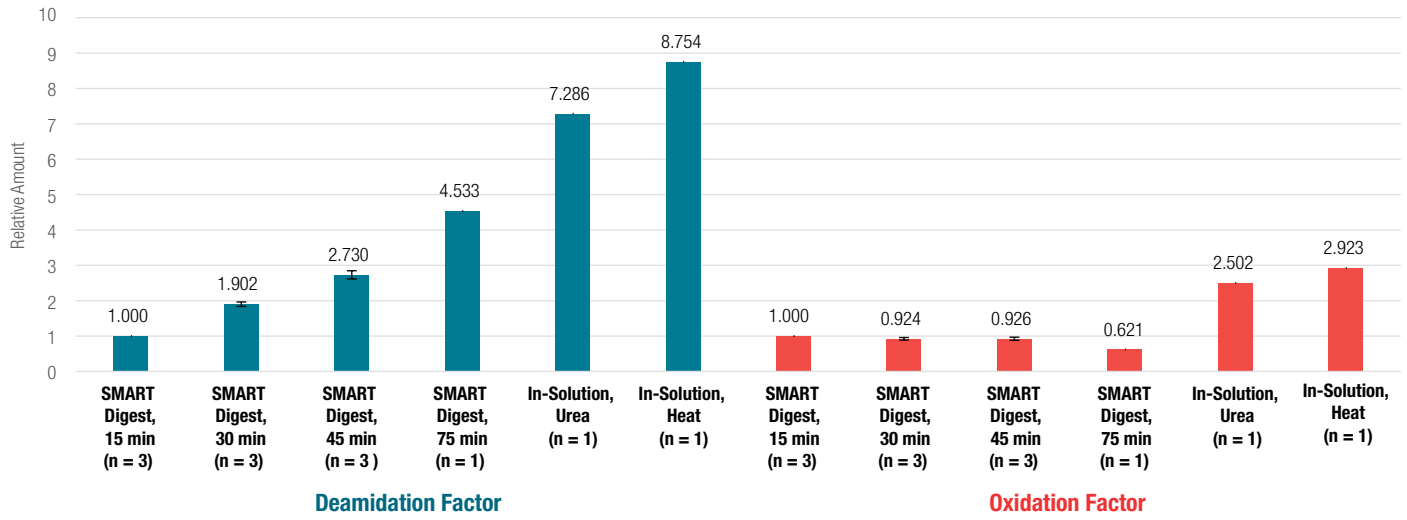


Figure 7. Relative amount of total deamidation and oxidation modifications measured for the six different digest conditions (Normalization to *SMART Digest, 15 min*).

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-glutamine 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 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 (VVSVLTVLHQDWL**N**GK), containing N319, and 2:G375-K396 (GFYPSDIAVEWES**N**GQPENNYK), 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]

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

Relative Abundance [%]											
SMART Digest, 15 min (n=3)	SMART Digest, 15 min, RSD (n=3)	SMART Digest, 30 min (n=3)	SMART Digest, 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
0.000	0.000	0.002	0.003	0.000	0.000	0.010	0.140	0.063	0.042	0.000	K63+Glycation
0.039	0.009	0.120	0.004	0.200	0.019	0.233	0.000	0.000	0.085	0.118	K102+Glycation
0.144	0.020	0.136	0.006	0.142	0.005	0.036	0.000	0.000	0.060	0.138	K137+Glycation
0.208	0.024	0.288	0.030	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	0.580	0.197	0.144	0.088	K168+Glycation
0.325	0.178	0.631	0.009	0.626	0.019	0.550	0.529	0.490	0.151	0.581	K182+Glycation
0.411	0.033	0.480	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	0.080	14.672	9.657	12.410	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
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.322	0.307	0.123	0.000	N301+A1S1F
0.703	0.050	0.776	0.035	0.796	0.029	0.928	0.895	0.880	0.080	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	0.490	0.021	0.576	0.396	0.302	0.079	0.449	N301+A2G1
38.932	3.324	38.235	1.881	36.765	1.840	38.505	38.349	37.450	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	0.030	0.291	0.030	0.268	0.000	0.000	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
96.850	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	99.810	0.009	99.815	0.003	99.586	99.853	99.920	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	0.070	0.037	0.115	0.020	0.343	0.879	1.399	0.428	0.103	N290+Deamidation
0.001	0.000	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	0.040	2.211	0.125	2.522	0.059	0.065	0.000	0.001	1.043	2.200	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	0.210	M81+Oxidation
3.654	0.683	2.560	0.348	2.435	0.152	3.556	25.225	29.209	9.505	3.064	W106+Oxidation
0.630	0.150	0.591	0.042	0.562	0.023	0.378	0.008	0.000	0.241	0.553	~W111+Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.034	0.199	0.057	0.000	C133+Double Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.179	0.051	0.000	C148+Double Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.032	0.192	0.055	0.000	C193+Double Oxidation
2.673	0.158	2.843	0.254	2.686	0.243	1.820	2.215	2.407	0.344	2.578	M256+Oxidation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.057	0.017	0.000	C265+Double Oxidation
0.016	0.004	0.021	0.002	0.033	0.002	0.068	0.000	0.000	0.018	0.020	C371+Double Oxidation
2.591	0.179	2.646	0.188	2.558	0.029	1.218	1.760	2.243	0.460	2.545	M432+Oxidation
0.000	0.000	0.000	0.000	0.001	0.001	0.000	2.192	0.000	0.633	0.000	~K38+Carbamylation
0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.087	0.000	0.025	0.000	K38+Carbamylation
0.124	0.008	0.190	0.011	0.232	0.016	0.001	0.000	0.000	0.092	0.155	K102+Carbamylation
0.003	0.003	0.003	0.002	0.007	0.004	0.004	0.900	0.019	0.258	0.005	K278+Carbamylation
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.202	0.000	0.058	0.000	K321+Carbamylation
0.000	0.000	0.001	0.000	0.001	0.001	0.000	1.254	0.000	0.362	0.001	K338+Carbamylation

Between 6 digestion methods

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-glutamine formation on heavy and light chains, were identified, relatively quantified, and compared between the different digestion methods. Overall, the

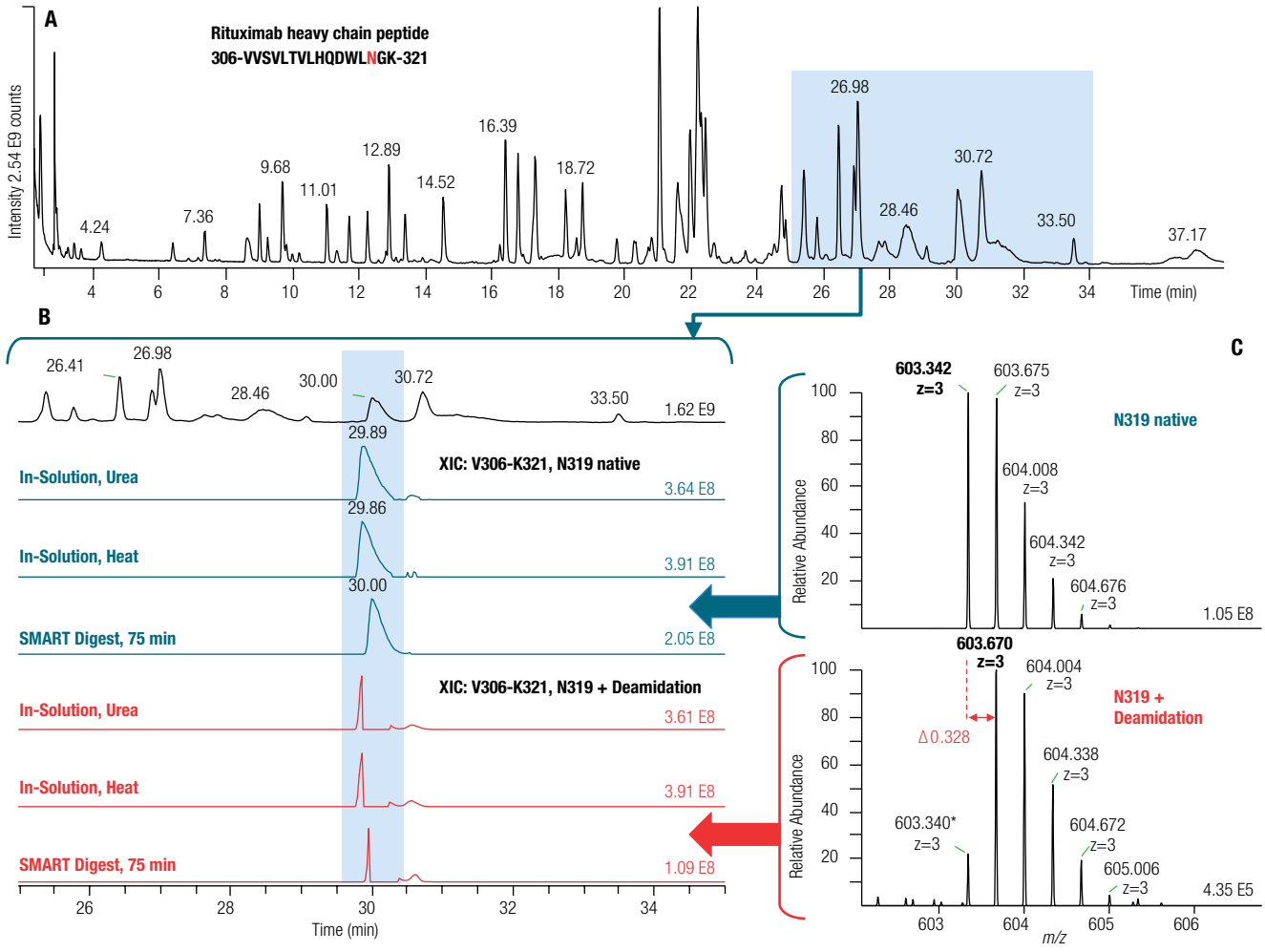


Figure 8. Total ion current chromatogram of the reduced SMART Digest sample, 75 min (A), and extracted ion current chromatograms (B) for the peptide V306-K321 in the native and the deamidated form for the different runs. A comparison of the isotopic distributions of the $[M+3H]^{3+}$ ions (C) for the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

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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 virtually 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 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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