Biopharmaceutical peptide mapping; addressing the challenges with simple fast, and reproducible workflows

By 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 Reinach, Switzerland;
[3]Thermo Fisher Scientific, Hemel Hempstead, United Kingdom; [4]Thermo Fisher Scientific, Runcorn, United Kingdom

Characterisation of biomarker and biotherapeutics is for many a new and challenging task. Many biomarker/biotherapeutic proteins are complex in nature and are present at low levels typically in complex biological matrices which interfere with analyses. This complexity combined with the continued drive for faster, more sensitive quantitation is exacerbated by the need for reproducible, cost effective and high quality results. One of the key areas which effect these requirements is sample preparation. We will describe how advances in sample preparation technologies help address these issues and how they impact the full workflow to provide significant improvements in simplicity, speed of analysis, reproducibility, sensitivity, accuracy and interpretation of results.

Introduction

Peptide mapping is a common technique in the biopharmaceutical industry to characterise monoclonal antibodies (mAbs) for the determination of product identity and stability.

The quality of the sample preparation often determines the quality and reproducibility of the analytical results. For example, the actual proteolysis or digestion method applied has a high impact on the overall outcome. Current protocols tend to be lengthy, often conducted over night, and are labour intensive, requiring steps to facilitate protein denaturation that can introduce artifacts or compromise digest efficiency [1].

An example is the carbamylation of arginine and lysine side chains by urea, a chaotrope commonly used to facilitate protein unfolding prior to its digestion. These modified amino acid residues not only complicate the peptide map and make accurate quantification more difficult, they also can prevent the trypsin cleavage at these sites thereby diminishing the digestion efficiency. The slightly alkaline conditions that allow for optimal trypsin cleavage harbour an additional risk to introduce artificial modifications with deamidation of asparagine and glutamine being among the most prominent events followed by pyroglutamate formation at the proteins N-terminus.

As a consequence, accurate protein characterisation (peptide mapping) or quantitation of peptides can be severely impacted. A number of options have been investigated to make protein digestion more reproducible and robust in order to meet the industry's needs. Areas which have been identified as having high potential are heat stable enzymes and immobilised enzyme designs. The former enables the use of elevated temperatures to unfold the protein rather than chemicals. This can allow for a reduction in chemically induced post translational modifications (PTM's), and for an increase in speed of digestion. Immobilised enzyme design can help mitigate against autolysis. The combination of these has led to the development of novel digestion technologies (Figure 1) which combine a heat-stable, immobilised enzyme design, which are able to deliver

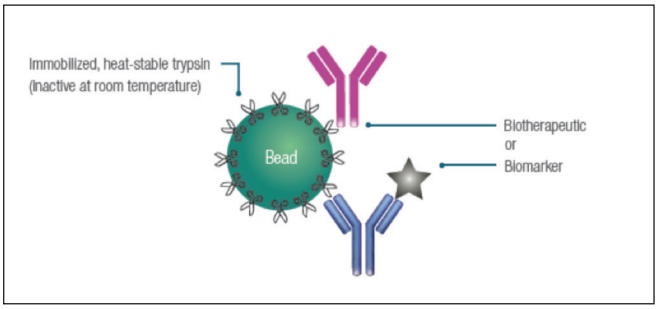

Figure 1: Novel heat-stable, immobilised trypsin product immobilised enzyme design as used in Thermo Scientific™ SMART Digest™ kits

digestion of proteins in minutes or hours compared to overnight [2], are far simpler to use which combined with the reduced number of chemicals required results in a far more reproducible results and are also more amenable to high throughput environments [3].

The following study demonstrates the advantage of the new digestion approach provided by the SMART Digest kit, compared to two classic in-solution digestion procedures for characterisation of a therapeutic recombinant mAb [4]. 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 samples prepared with the SMART Digest kit was performed to assess the influence of digestion time on modification formation. (This involved digesting the sample for a predetermined time i.e. 15, 30, 45 and 75 minutes prior to sample analysis)

Experimental

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 (PN 60109-101) 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 of the SMART digest resin slurry, corresponding to 14 µg of heat-stable, immobilised 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. Reduction/alkylation is performed post digestion with SMART digest as this produced the best results with the technology.

Instrumentation

Separations were performed using a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system and Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.2 µm, 2.1 × 250 mm column coupled to a Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer. The data was acquired with the Thermo Scientific™ Chromeleon™ Chromatography Data System, and processed with Thermo Scientific™ BioPharma Finder™ software

Results and discussion

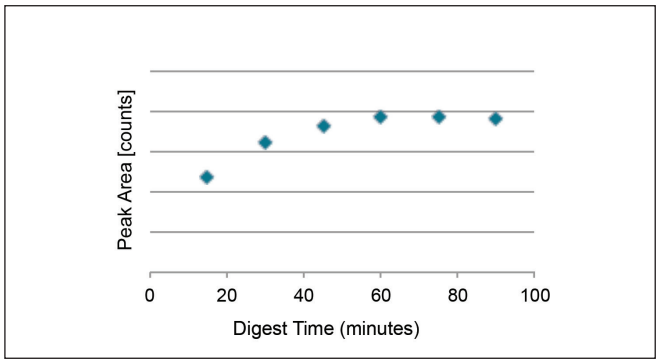

Figure 2. IgG digest profile, monitoring the mAb peptide VVSVLTVLHQDWL-NGK for digestion times from 15–90 min using the SMART Digest kit (3).

The SMART Digest product provides fast and simple protein digestion with high levels of reproducibility, and digestion completeness for the mAb sample 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 overlap with an average RSD for the peak area of less than 5%. These results highlight the reproducibility that can be achieved when using this novel digestion technique in combination with

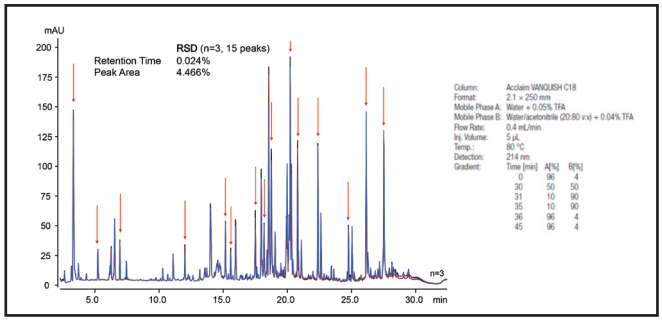

Figure 3. UV chromatogram overlay from three separate SMART digestions from the same mAb, conducted by three individual operators. The 15 marked peptides in each sample were used for inter-user/inter-day RSD value calculations.

new UHPLC systems featuring intelligent sample pre-compression technology (Thermo Scientific™ SmartInject™) for high levels of retention time reproducibility.

Comparing the total ion current (TIC) chromatograms of an in-solution-digested sample and a digestion of sample with the heat-stable, immobilised trypsin approach (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 '1:88-206 + alkly .

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 optimise the digestion efficiency at elevated temperatures.

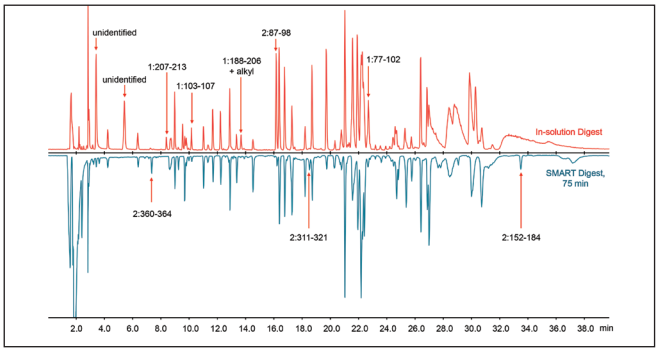

Figure 4. Mirror plot of the TIC chromatogram for the in-solution-digested sample denatured with heat (In-Solution, Heat) and the reduced SMART Digest sample (SMART Digest, 75 min). Peak labels give annotation to light (1) or heavy (2) chains, respectively, and sequence position.

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 solid phase extraction (SPE) [5].

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 1. 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 2).

Table 1. 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 2. 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, localisation, 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 (NH3 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 samples digested with the SMART Digest kit. 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, their amount in the reduced samples prepared with the SMART Digest kit were lower compared to the in-solution-digested samples even when a 75 min (over-)digestion with the SMART digest kit was applied.

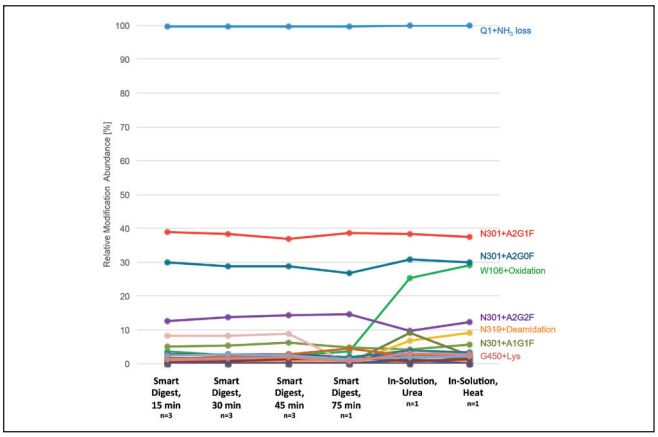

Figure 5. Relative abundance of 85 identified modifications including oxidation, double oxidation, glycation, glycosylation, NH3 loss, isomerisation, lysine truncation, methylation, dimethylation, and carbamylation.

The monoclonal antibody rituximab used in this study consists of 1328 amino acid residues including 16 disulfide bonds [6].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 3 summarises 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 the identified deamidations, the maximum RSD value was 3.2% and with an average RSD of 0.9%. 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

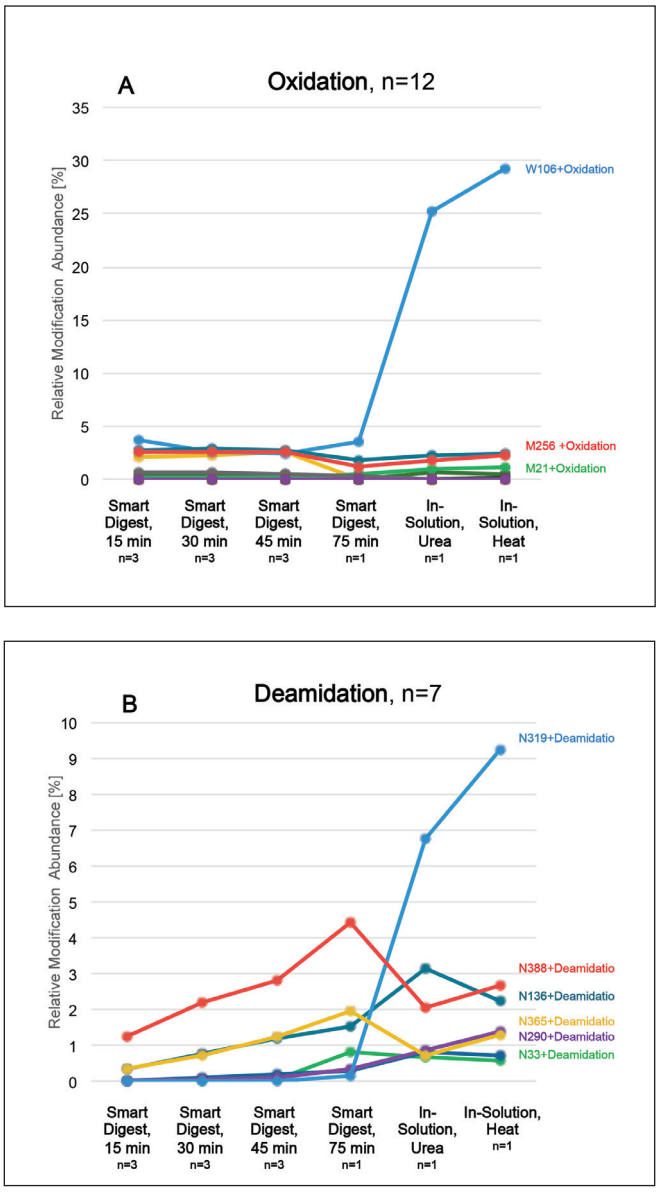

Figure 6. Relative abundance of 12 identified oxidations (A) and 7 deamidations (B) in different runs with various digestion methods.

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 kit 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 [7]. 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 product samples, the average carbamylation was considerably lower in the ppt range or not detectable at all (Table 3). 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 pyroglutamine formation on heavy and light chains, and lysine glycations are listed in Table 3. In total, six lysine glycations and 12 glycosylations of

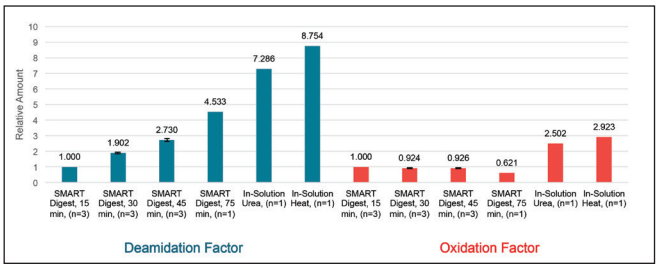

Figure 7. Relative amount of total deamidation and oxidation modifications measured for the six different digest conditions (Normalization to SMART Digest, 15 min).

Table 3. 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	0.060	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	0.057	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	0.726	0.489	0.188	0.847	N301+M5
96.850	0.066	96.802	0.208	96.946	0.486	96.798	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	2.694	0.867	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	0.063	0.336	0.069	0.455	0.015	0.001	0.817	0.445	0.153	0.424	-M34+Oxidation
0.248	0.061	0.189	0.037	0.164	0.013	0.549	0.826	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	0.480	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

N301 could be identified and (relatively) quantified with an average RSD value of 0.4%. 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 sample produced with the SMART Digest kit 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 [8]. One way to minimise the degree of induced deamidation is to lower the pH of the digestion buffer. Digestion with the SMART Digest

kit 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 minimised 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 samples prepared with the SMART Digest Kit product, 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 product 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-K321 (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 humanised 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 [9].

Figure 8 shows the TIC chromatogram for the sample prepared with the SMART Digest Kit (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 product (45 and 75 min digestion time) for the PENNY peptide 2:G375-K396 (Table 4), but the lowest value of 1.3% 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 product. 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 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

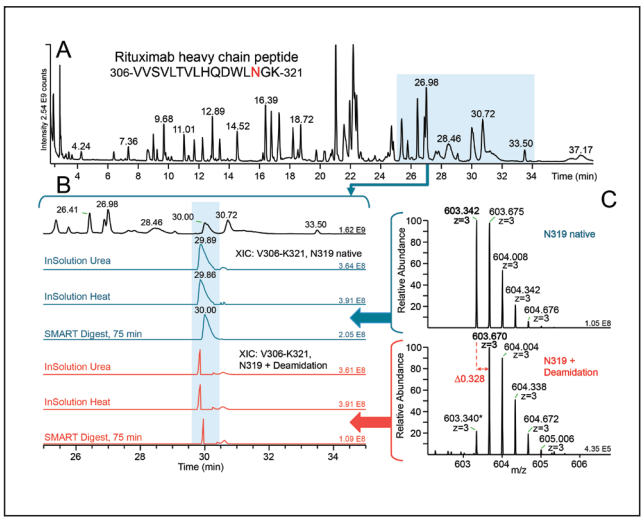

Figure 8. Total ion current chromatogram of the reduced SMART Digest sample, 75 min (A), and extracted ion current chromatograms (B) for the peptide V306-K321 in the native and the deamidated form for the different runs. A comparison of the isotopic distributions of the [M+3H]3+ ions (C) for the native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

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. Optimisation of the incubation time can be used to further minimise 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 not required when using the SMART Digest kit protocol, 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 heat-stable, immobilised trypsin design provided in the SMART Digest Kit make it ideal for peptide mapping workflows and have significant advantages over traditional in-solution digest protocols

1. 1. Ren, Da; Pipes, Gary D; Liu, Dingjiang; Shih, Liang-Yu; Nichols, Andrew C.; Treuheit, Michael J.; Brems, David N.; Bondarenko, Pavel V., An improved trypsin digestion method minimizes digestion-induced modifications on proteins. Analytical Biochemistry, 2009, 392, 12–21.

2. 2. Freije, J. R.; Mulder, P. P.; Werkman, W.; Rieux, L.; Niederlander, H. A.; Verpoorte, E.; Bischoff, R. Chemically modified, immobilized trypsin reactor with improved digestion efficiency. J Proteome.Res. 2005, 4, 1805-1813. Freije, R.; Bischoff, R.; Enzymes with modified amino acids. 2005; US Patent: 8,124,7812.

3. 3. Thermo Scientific SMART Digest Kit Technical Guide, Runcorn, UK, 2015.

4. 4. Thermo Scientific Application Note 1159: SMART Digest compared to classic in-solution digestion of rituximab for in-depth peptide mapping characterization, Gerering, Germany, 2016.

5. 5. Thermo Scientific Application Note 21198: Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow. Runcorn, UK, 2015.

6. 6. Nebija, D.; Kopelent-Frank, H.; Urban, E.; Noe, C. R.; Lachmann, B. Comparison of two-dimensional gel electrophoresis patterns and MALDI-TOF MS analysis of therapeutic recombinant monoclonal antibodies trastuzumab and rituximab. Journal of Pharmaceutical and Biomedical Analysis, 2011, 56, 684–91.

7. 7. Kollipara, L.; Zahedi, R.P. Protein carbamylation: in vivo modification or in vitro artefact? Proteomics, 2013, 13, 941-944.

8. 8. Dick, L.W.; Mahon, D.; Qiu, D.; Cheng, K.C., Peptide mapping of therapeutic monoclonal antibodies: Improvements for increased speed and fewer artifacts. Journal of Chromatography B, 2009, 877, 230-236.

9. 9. Visser, J.; Feuerstein, I.; Stangler, T.; Schmiederer, T.; Fritsch, C.; Schiestl, M. Physicochemical and Functional Comparability Between the Proposed Biosimilar Rituximab GP2013 and Originator Rituximab. BioDrugs, 2013, 27, 495–507.