APPLICATION NOTE

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

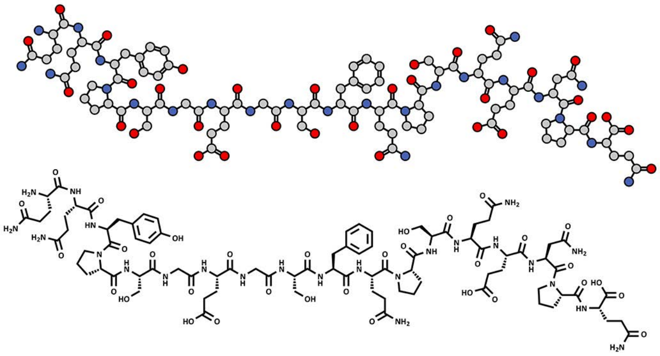
**Authors:** Martin Samonig[1], Alexander Schwahn[2], Ken Cook[3], Mike Oliver[4], and Remco Swart[1]

[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 |

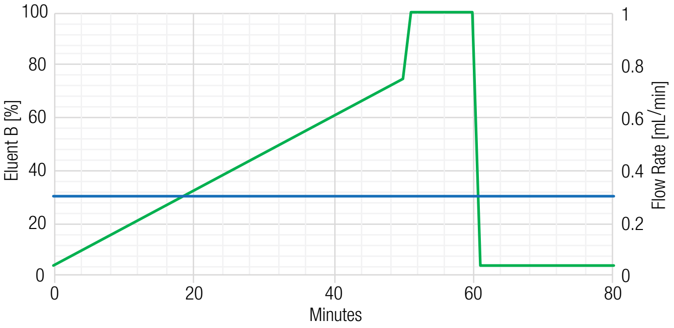


**Figure 1. LC gradient.**

## 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 | $1.0 \times 10^5$ |
| Target Value | $3.0 \times 10^6$ | Isolation Width | 2.0 Da |
| Max Injection Time | 100 ms | Signal Threshold | $5.0 \times 10^3$ |
| Default Charge State | 2 | Normalized Collision Energy (HCD) | 27 |
| SID | 0 eV | Top-N $MS^2$ | 5 |
| Microscans | 2 | Max Injection Time | 200 ms |
| | | Fixed First Mass | *m/z* 140.0 |
| | | Dynamic Exclusion | 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 |
|---|---|
| Absolute MS Signal Threshold | $3.00 \times 10^5$ counts |
| **Identification** | **Setting** |
| Mass Accuracy | 5 ppm |
| Minimum Confidence | 0.80 |
| Maximum Number of Modifications for a Peptide | 2 |
| Unspecified Modification | -58 to +162 Da |
| *N*-Glycosylation | CHO |
| Protease Specificity | High |

| Variable Modifications | Setting |
|---|---|
| N Terminal | Carbamylation<br>Gln -> Pyro-Gln |
| C Terminal | Lys |
| Side Chain | Carbamidomethylation (C)<br>Carbamylation (K)<br>Deamidation (N)<br>Dimethylation (K)<br>Double Oxidation (MWC)<br>Glycation (K)<br>Methylation (K)<br>Oxidation (MWC) |

## 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

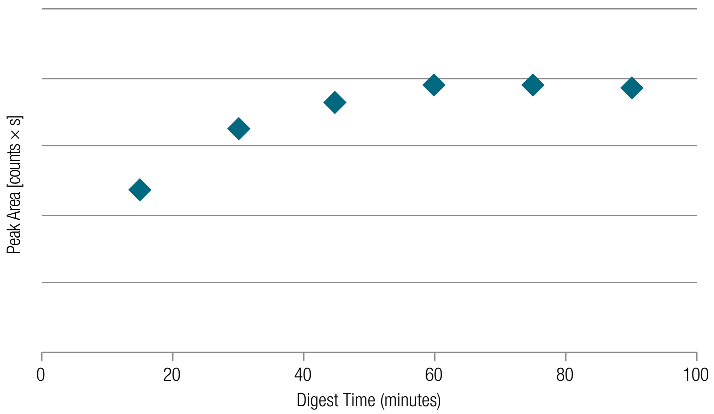
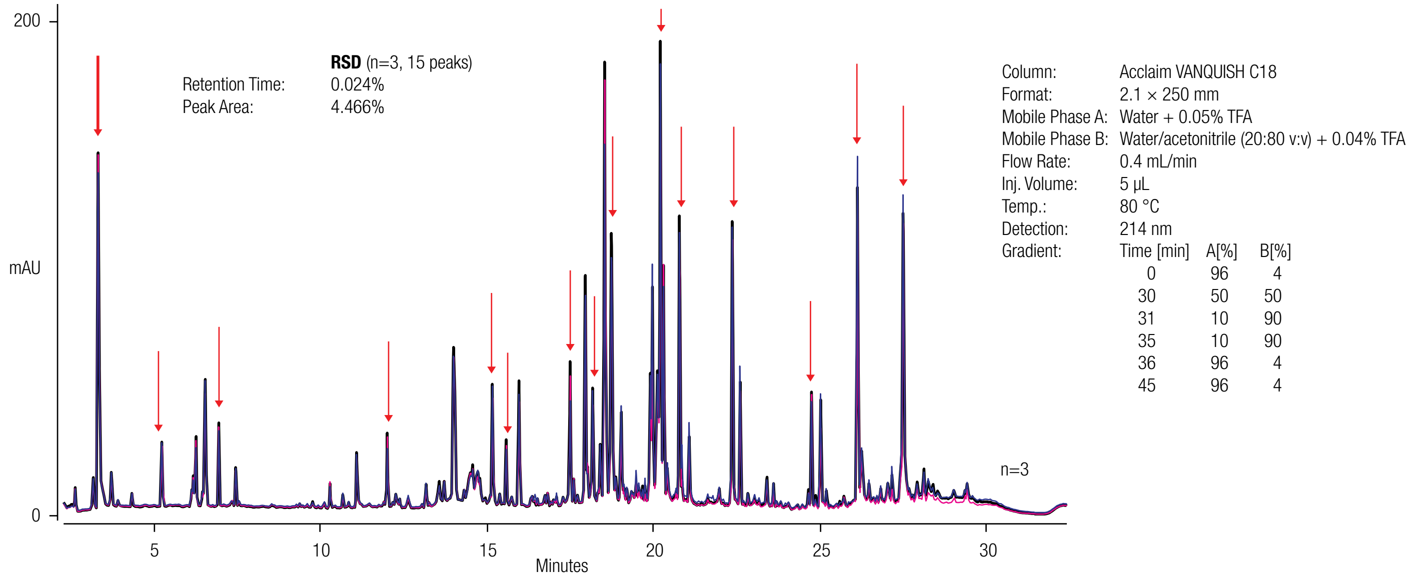


**Figure 2. IgG digest profile, monitoring the mAb peptide VVSVLTVLHQDWLNGK for digestion times from 15–90 min using the SMART Digest kit.[2]**



**RSD** (n=3, 15 peaks)

| | |
|---|---|
| Retention Time: | 0.024% |
| Peak Area: | 4.466% |

| | |
|---|---|
| Column: | Acclaim VANQUISH C18 |
| Format: | 2.1 × 250 mm |
| Mobile Phase A: | Water + 0.05% TFA |
| Mobile Phase B: | Water/acetonitrile (20:80 v:v) + 0.04% TFA |
| Flow Rate: | 0.4 mL/min |
| Inj. Volume: | 5 µL |
| Temp.: | 80 °C |
| Detection: | 214 nm |

Gradient:

| Time [min] | A[%] | B[%] |
|---|---|---|
| 0 | 96 | 4 |
| 30 | 50 | 50 |
| 31 | 10 | 90 |
| 35 | 10 | 90 |
| 36 | 96 | 4 |
| 45 | 96 | 4 |

**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.

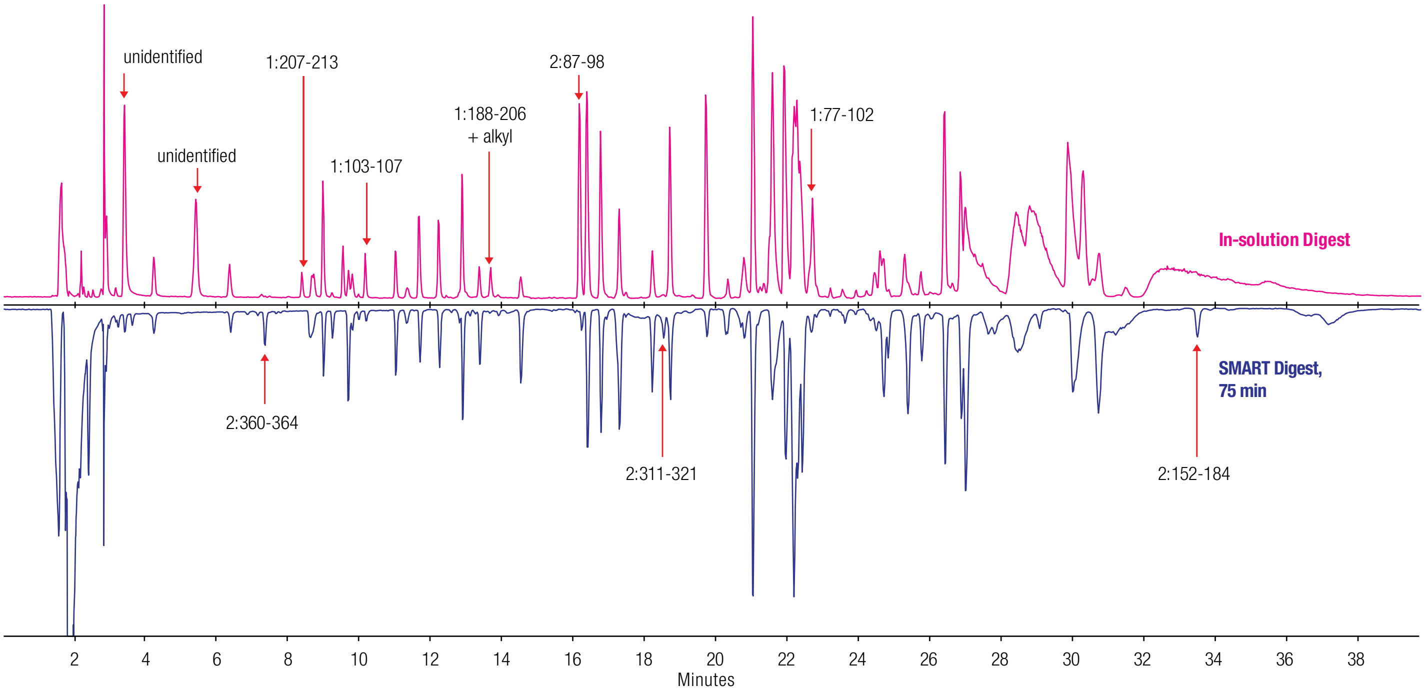


**Figure 4. Mirror plot of the TIC chromatogram for the in-solution-digested sample denatured with heat (*In-Solution, Heat*) and the reduced SMART Digest sample (*SMART Digest, 75 min*).** Peak labels give annotation to light (1) or heavy (2) chains, respectively, and sequence position.

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 | $3.48 \times 10^9$ | *SMART Digest, 15 min* |
| 1678 | $4.12 \times 10^9$ | *SMART Digest, 30 min* |
| 1688 | $3.96 \times 10^9$ | *SMART Digest, 45 min* |
| 1551 | $3.13 \times 10^9$ | *SMART Digest, 75 min* |
| 1171 | $3.65 \times 10^9$ | *In-Solution, Urea* |
| 1145 | $4.04 \times 10^9$ | *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

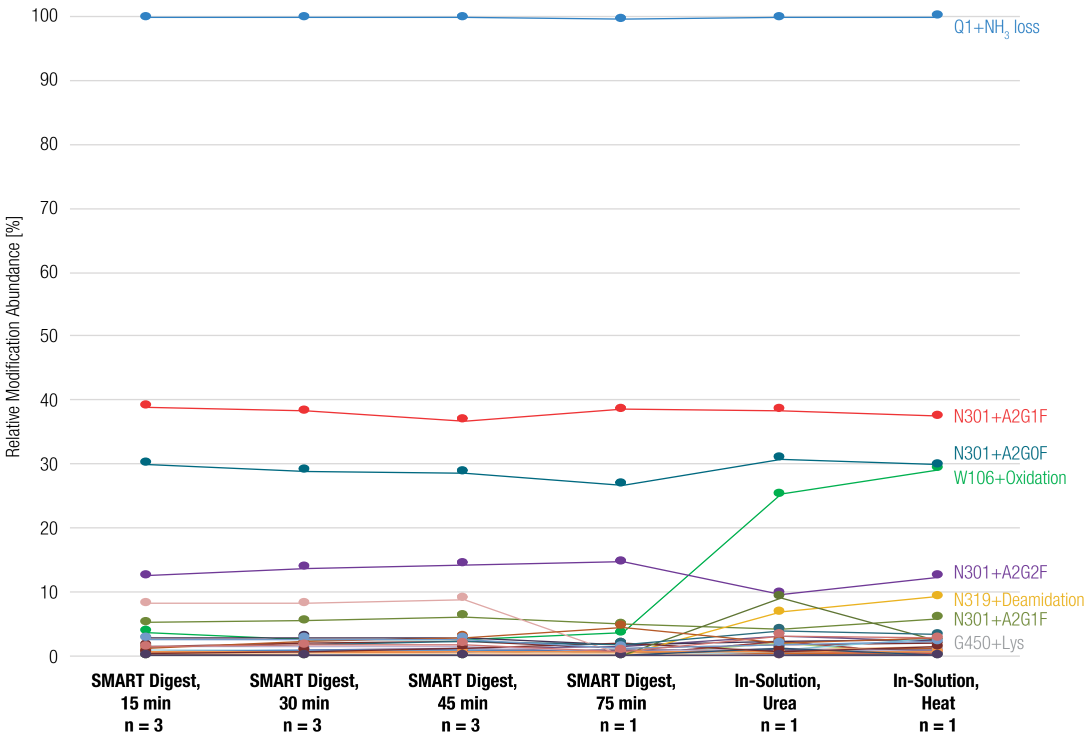


Figure 5. Relative abundance of 85 identified modifications including oxidation, double oxidation, glycation, glycosylation, $NH_3$ loss, isomerization, lysine truncation, methylation, dimethylation, and carbamylation.

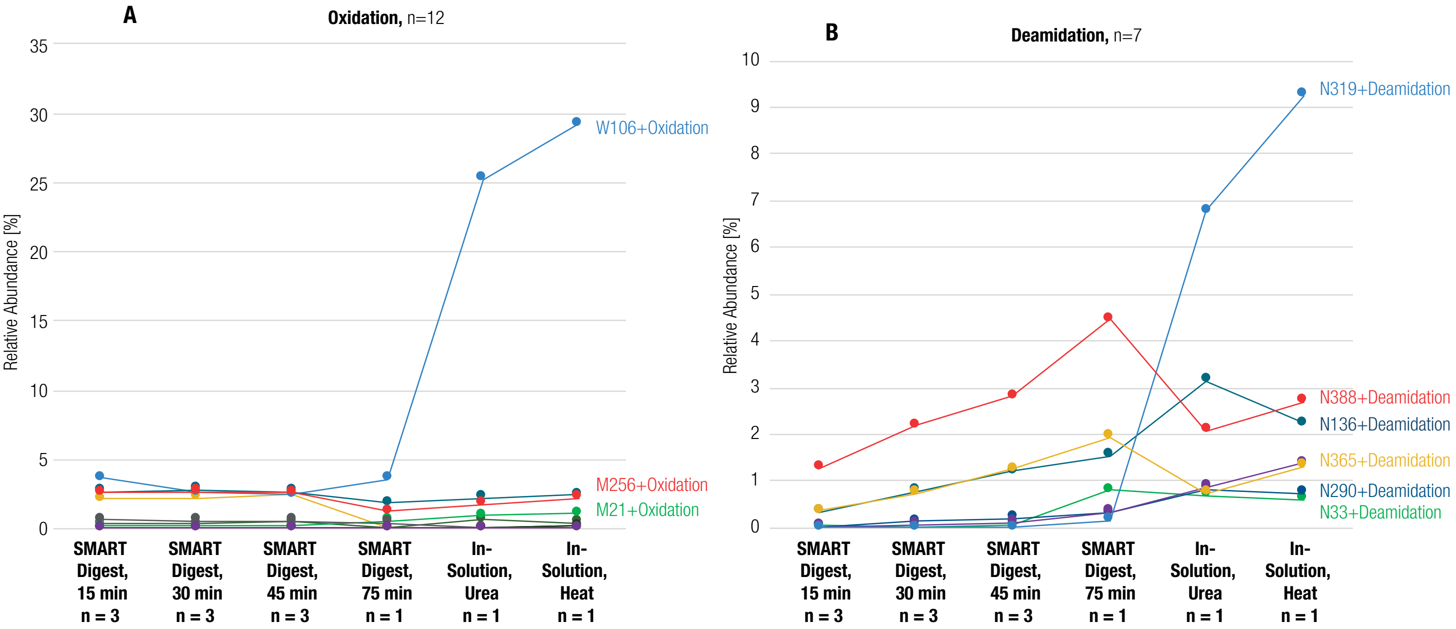


Figure 6. Relative abundance of 12 identified oxidations (A) and 7 deamidations (B) in different runs with various digestion methods.

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

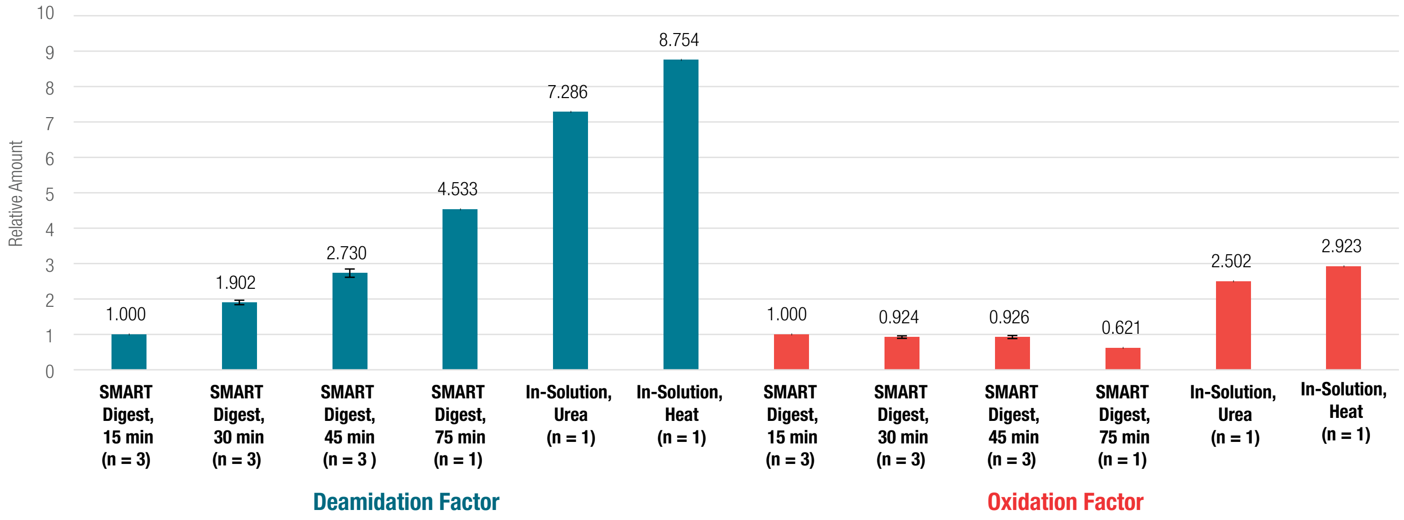


**Figure 7. Relative amount of total deamidation and oxidation modifications measured for the six different digest conditions (Normalization to *SMART Digest, 15 min*).**

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

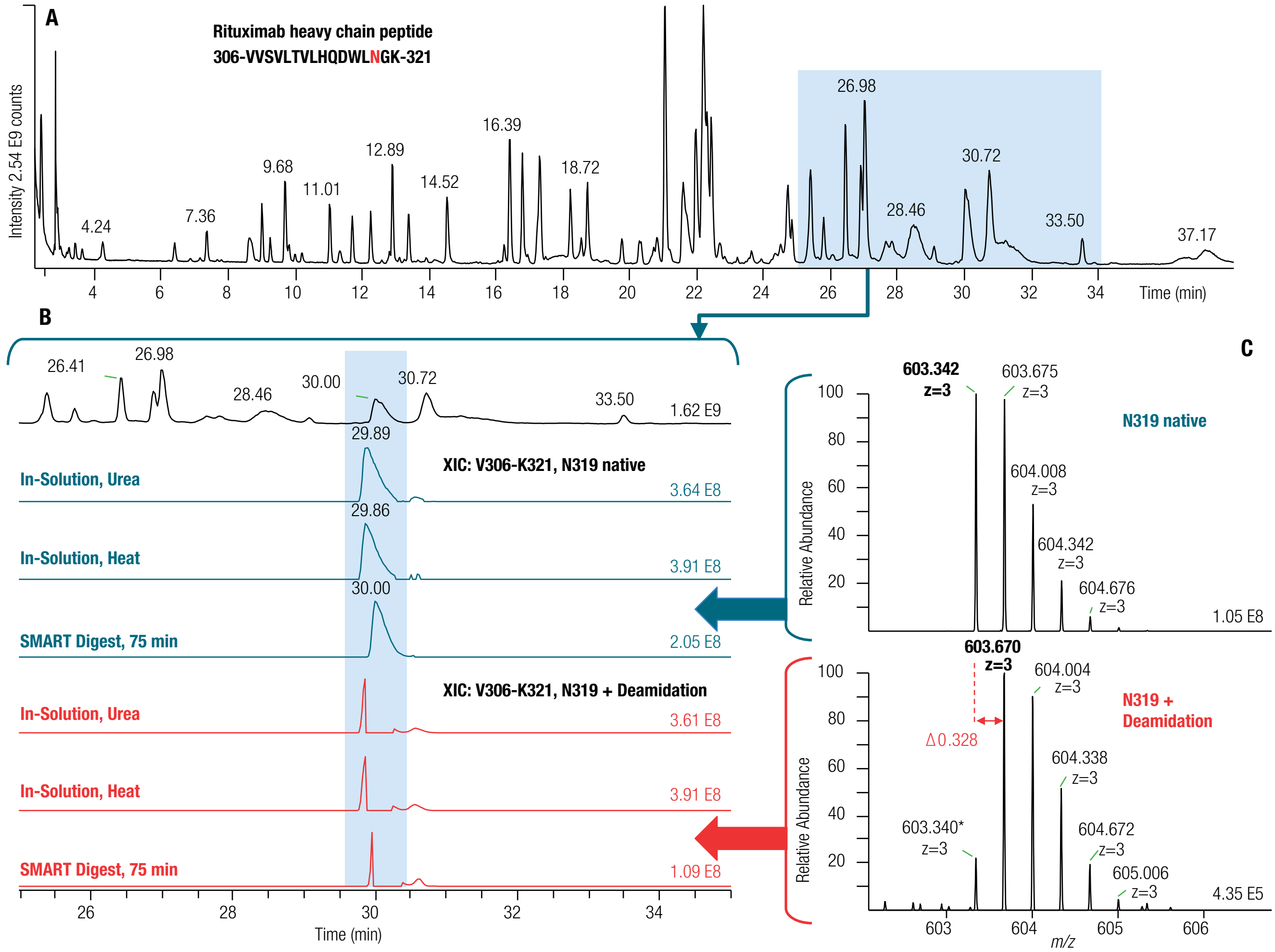


**Figure 8. Total ion current chromatogram of the reduced SMART Digest sample, 75 min (A), and extracted ion current chromatograms (B) for the peptide V306-K321 in the native and the deamidated form for the different runs.** A comparison of the isotopic distributions of the $[M+3H]^{3+}$ ions (C) for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

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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