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SMART Digest Peptide Mapping and Quantitation Compendium

Table of Contents

Introduction

Faster and More Sensitive Protein Characterization and Quantitation

Easier Digestion

Faster Digestion

Highly Reproducible Digestion

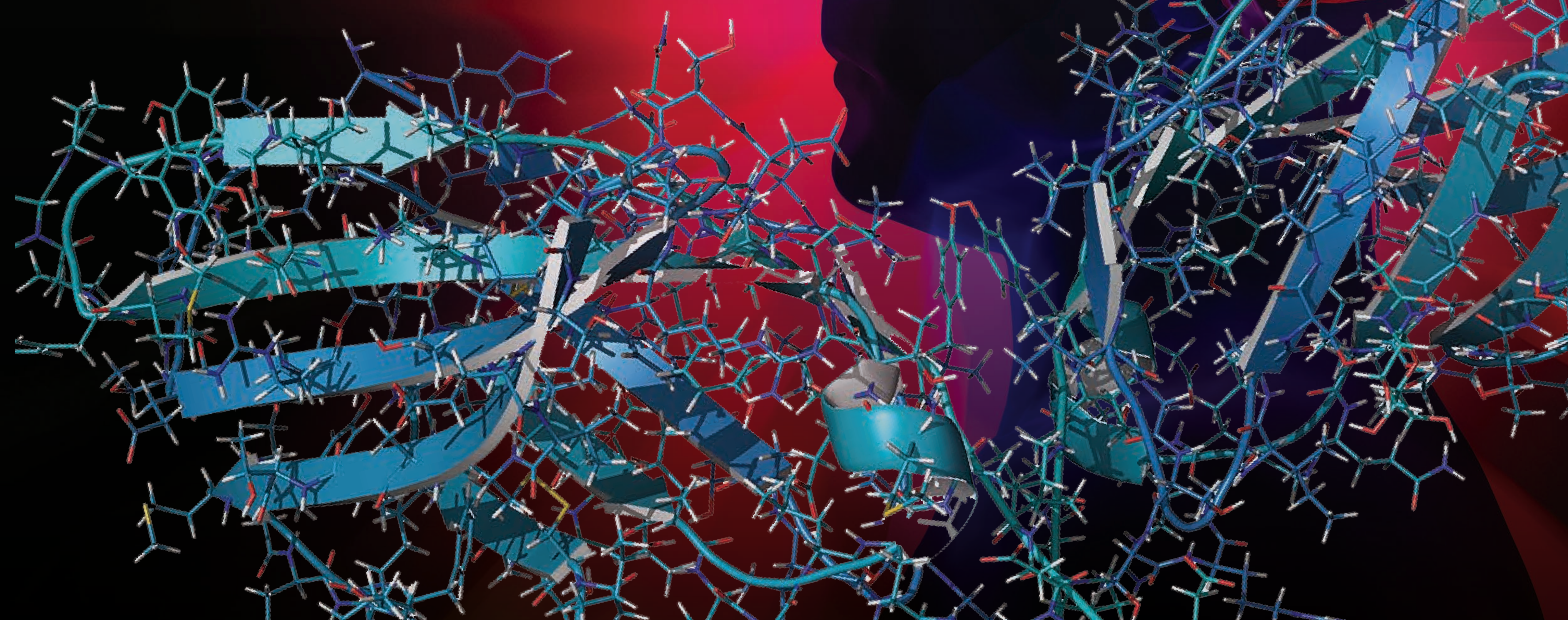
Automation of Digestion

Improving Sensitivity and Speed

Peptide Mapping

Peptide Quantitation

Product and Method Development Considerations



Peptide Mapping and Quantitation – Easier, Faster and More Reproducible

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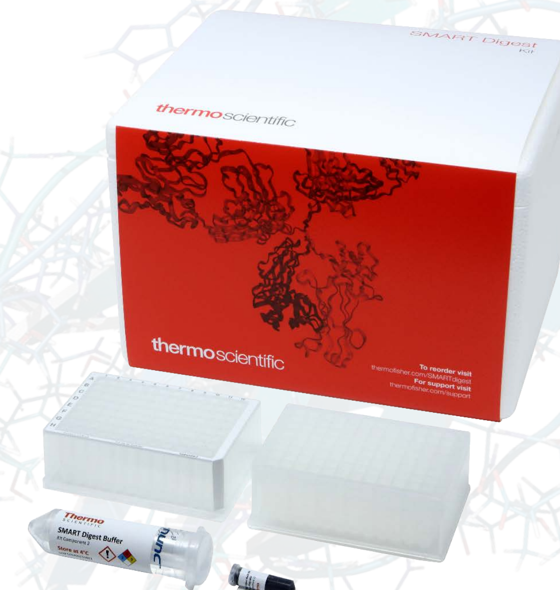
Table of Contents

Introduction 2
Faster and more sensitive protein characterization and quantitation 3
Easier digestion 4
Faster digestion 5
Highly reproducible digestion 6
Automation of digestion 7
Improving sensitivity and speed 10

Peptide Mapping 13
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies 13
High-precision, automated peptide mapping of proteins 24
Automated chymotrypsin peptide mapping of proteins by LC-MS automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies 31
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications PTMs) of monoclonal antibodies 37
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis 54
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis 67
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform 77
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization 88
LC-UV-MS peptide mapping development for easy transfer to LC-UV QA/QC 97
Providing the highest retention time and peak area reproducibility for maximal confidence in peptide mapping experiments 100
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses 102

Peptide Quantitation 108
Analysis of the low-abundance plasma biomarker klotho in less than four hours 108
Simplifying immunoaffinity capture workflow 111
Improvement in speed and reproducibility of protein digestion and peptide quantitation, utilizing novel sample preparation technology in a full solution workflow 116

Product and Method Development Considerations 120
Fast digestion method optimization using innovative trypsin technology 120
Enhanced digestion for improved biomarker identification 123
Increased efficiency of biomolecule identification by optimization of trypsin digestion buffers 126
Impact on tryptic digestion when detergents and chaotropes are essential: A case study with ribonuclease A 129
Generic SPE protocol for peptide clean-up and concentration 134
Selecting buffers to remove uncertainty in tryptic digestion 137



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ThermoFisher SCIENTIFIC

Table of Contents
Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations

Faster and More Sensitive Protein Characterization and Quantitation

In 1996 the first antibody based drug was introduced to the market and since then the growth of the biopharmaceutical market has continued at a rapid pace. Today approximately 20% of the overall pharmaceutical market is based on biological drugs with the market continuing to grow at more than 8% per year, whereas the market based on classic synthetic drugs exhibits little if any growth. The growth of biopharmaceuticals comes from their greater success rate and speed of development. This is in part due to their evolution where engineered fully human monoclonal antibodies (mAbs) blend perfectly with the body's own antibody repertoire to avoid an unwanted immune response, over antibody-drug-conjugates that allow the directed delivery of a small molecule drug to a limited population of carefully defined target cells, e. g. cancer cells, or the combination of different target structures in antibody-fusion proteins or bi-specific antibodies to harness a combinatorial effect, resulting in higher selectivity and efficacy.

As with synthetic drugs, biotherapeutics require rigorous analytical characterization and quantitation to ensure efficacy and safety for the patient. The analytical challenges especially in terms of characterization of bio-therapeutics (peptide mapping) have been exacerbated due to their tremendous structural complexity. Adding to this are post translational modifications (PTMs) of the protein such as glycosylation or the specific formation of disulfide-bridges that are crucial requirements for the functionality of the protein. This complexity however can give rise to a virtually unlimited number of potential different combinations of modifications. Within bioanalyitcal workflows (where the drug is evaluated in terms of its absorption, distribution, metabolism and excretion (ADME) in the target organism) the challenges are from complex matrices which cause suppression of signal in mass spectrometers and need to be effectively removed, the drive towards higher efficacy drugs which require greater sensitivity in analytical workflows and the nature of protein based bio-therapeutics which are prone to absorption and nonspecific binding.

These challenges are compounded by sample preparation approaches required in these workflows where the protein is typically digested into its constituent peptides normally via an in-solution based enzyme reaction. These approaches are often subject to a number of issues that are not aligned to the needs of the modern biopharmaceutical laboratories, which are tasked with providing high quality analytical results, often in high throughput, regulated environments. These issues include:

1. Complex procedures–prior to digestion the protein must be denatured. This is done in order to unravel and open the protein structure to allow for optimal enzyme interaction. This requires a multiple steps including disulfide bond reduction, and free sulphydryl alkylation.
2. Reproducibility of results–due to the complexity of the process and the chemicals involved (potential for introduction of chemically induced post translational modifications [PTMs]). There is the potential for poor reproducibility which has a significant effect on the confidence in the analytical results produced.
3. Speed of analysis–depending on the size and complexity of the protein being analyzed, the digestion process can take up to 24 hours to complete. This combined with the complexity of the process does not lend itself to automation and high throughput processing.

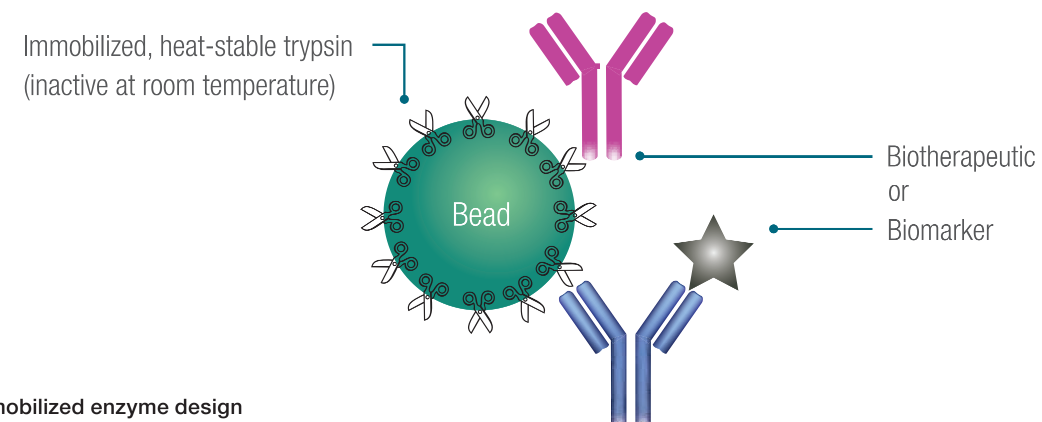


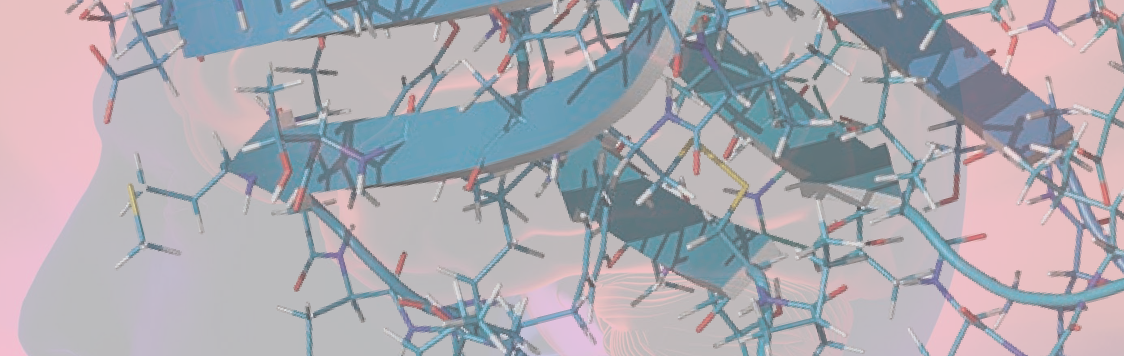
Figure 1. Heat-stable, immobilized enzyme design

Bioanalytical workflows are further complicated by the use of a complicated immunoaffinity step to remove the protein of interest from complex matrices e.g. plasma prior to digestion.

In order to meet these challenges a number of sample preparation options have shown promise, in particular the Thermo Scientific™ SMART Digest™ Kit, which is a heat stable, immobilized resin based enzyme design (Figure 1). The SMART Digest Kit offers significant advantages over traditional in-solution based protocols in terms of:

- Ease of use - significantly simplified procedure compared to in-solution digestion
- Speed - most mAb's take less than an hour to completely digest
- Reproducibility of results - high levels of reproducibility are achieved, user to user, day to day, lab to lab
- Automation - the simple procedure allows for easy automation

Table of Contents
Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations



Easier Digestion

Trypsin is the enzyme most commonly used for proteolytic digestion due to its high specificity. Although a widely accepted technique, in-solution trypsin digestion protocols required for sample preparation are labor intensive and prone to manual errors. These errors affect the quality of the analytical data compromising the ability to reproducibly characterize a protein to the required standard. In the most critical cases where workflows only employ UV detection without confirmation by MS, robust and stable sample preparation and separation methods are critical. The digestion must be reproducible and chromatography must be extremely stable to allow unambiguous peptide identification based on chromatographic retention time.

The SMART Digest kit provides a highly simplified protocol (Figure 2) which is highly amenable to automation and helps provide highly reproducible results.

In addition to its complexity in-solution digestion typically requires an overnight procedure. This does not fit with the high speed and throughput of analysis required within biopharmaceutical environments. Due to the excess of resin-based, heat stable enzyme in the SMART Digest Kit the speed of digestion can be greatly accelerated, providing procedures which can be performed in just a few minutes or hours compared to overnight.

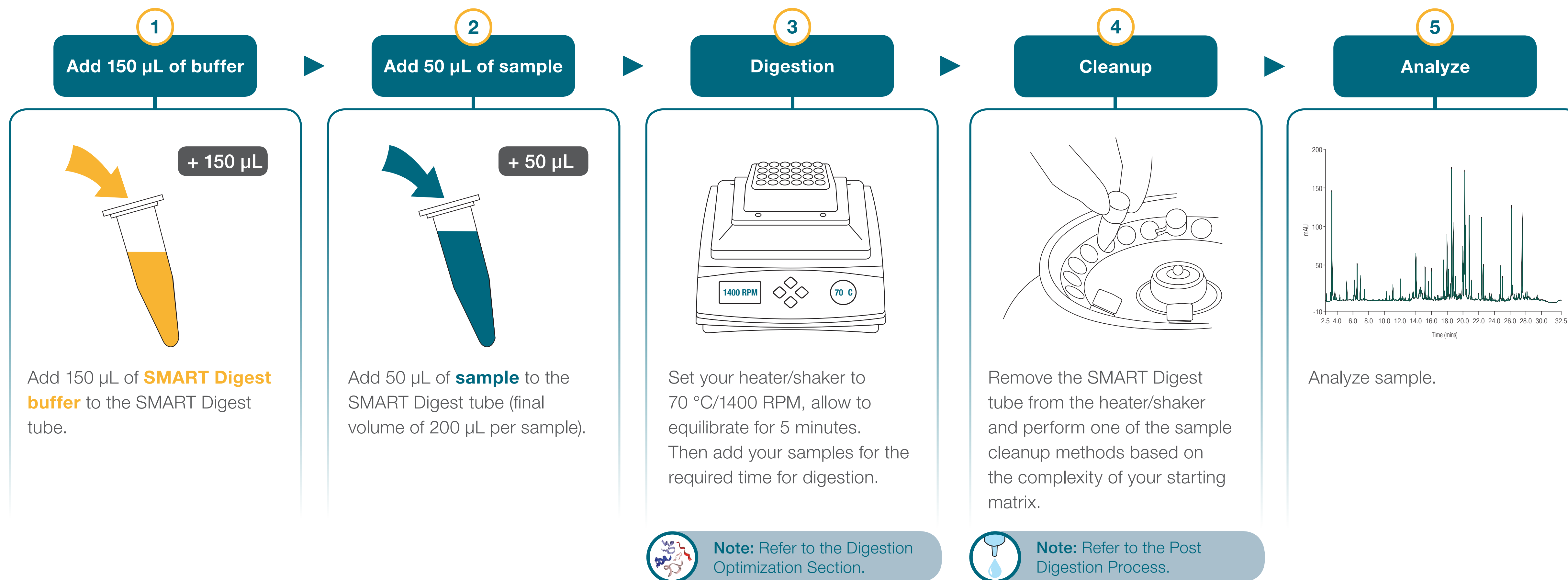


Figure 2. Typical SMART Digest procedure

Table of Contents
Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations

Faster Digestion

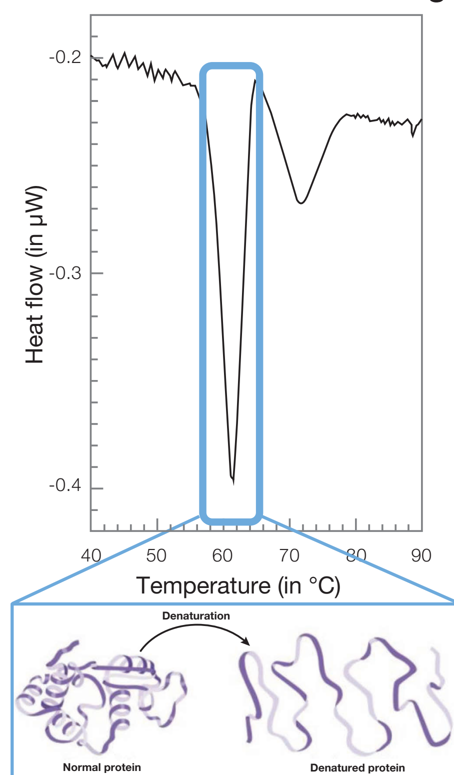
In order to demonstrate the speed of the workflow offered by the SMART Digest kit compared to an in-solution based protocol, a commercially available monoclonal antibody rituximab drug product (Hoffmann La Roche, Basel, Switzerland) was digested via in-solution based protocols using urea and heat respectively for denaturation with the SMART Digest kit procedure and the data compared.

Both in-solution digest protocols required reduction and alkylation steps prior to overnight digestion with trypsin at 37 °C prior to addition of trifluroacetic acid (TFA) to stop the reaction.

The SMART Digest procedure involved adding the sample and pre-made SMART Digest buffer 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). 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).

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 3). Here, the relative standard deviation (RSD) was used to evaluate reproducibility, as demonstrated in Figure 4. Three separate digestions of the same mAb sample were conducted by three different analysts on different days.

Thermal denaturation of IgG



DSC thermogram of IgG (6 mg/ml; mouse IgG2b) in a 10 mM phosphate buffer pH 8.1; 0.5°C/min [*]

* Vermeer & Norde (2000), Biophysical Journal 78: 394 – 404

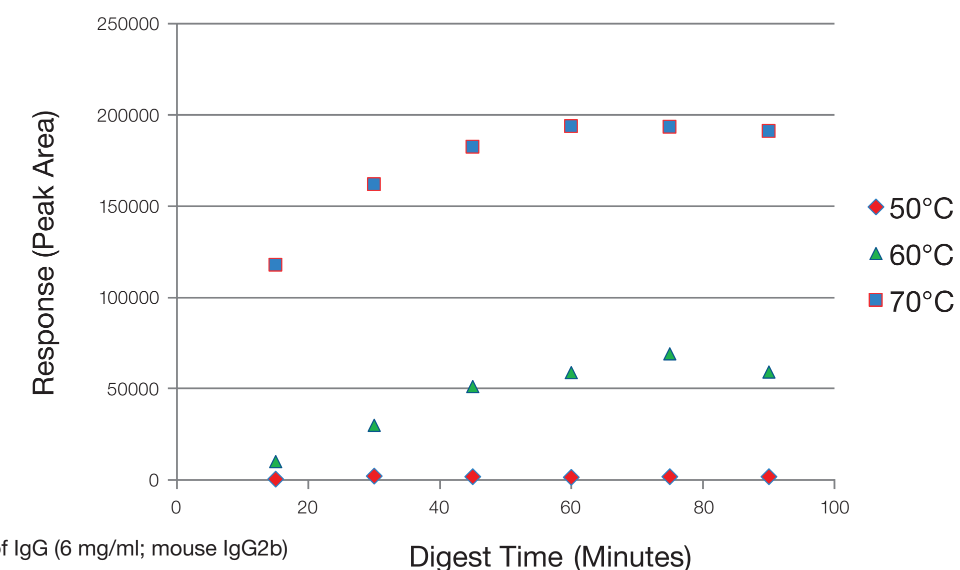


Figure 3: IgG digestion profile, monitoring the mAb peptide VVSVLTVLHQDWLNGK for digestion times using the SMART Digest kit.

thermoscientific

Table of Contents

Introduction

Faster and More Sensitive Protein Characterization and Quantitation

Easier Digestion

Faster Digestion

Highly Reproducible Digestion

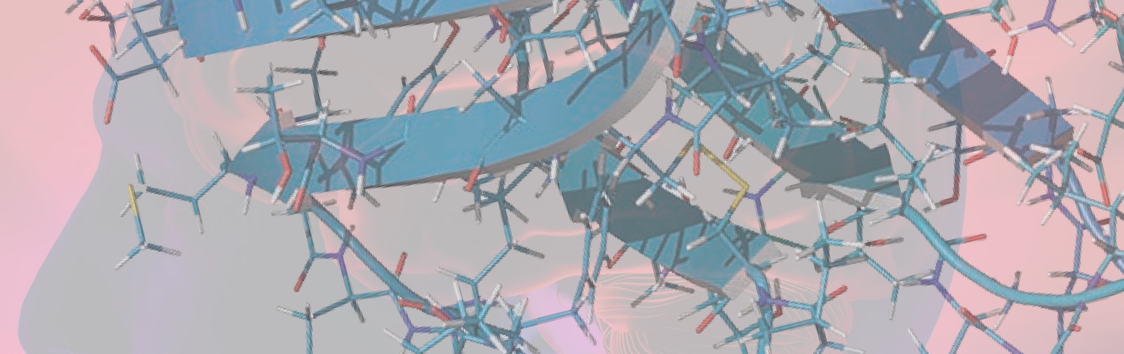
Automation of Digestion

Improving Sensitivity and Speed

Peptide Mapping

Peptide Quantitation

Product and Method Development Considerations



Highly Reproducible Digestion

The peptide maps generated perfectly 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 the Thermo Scientific™ Vanquish™ Flex UHPLC system featuring Thermo Scientific™ SmartInject (intelligent sample pre-compression technology) for high levels of retention time reproducibility.

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 was 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).

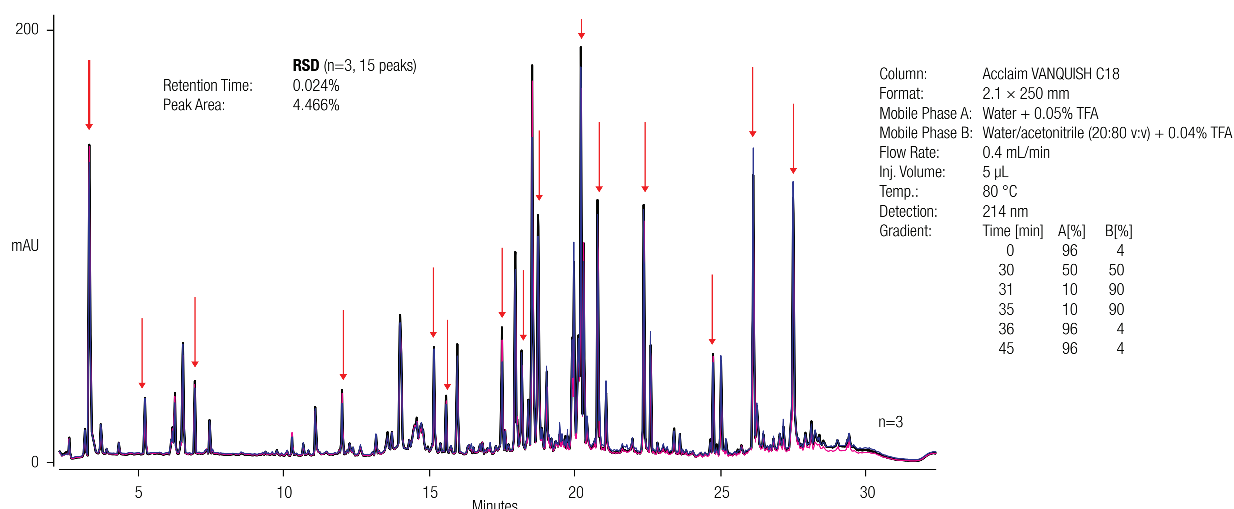


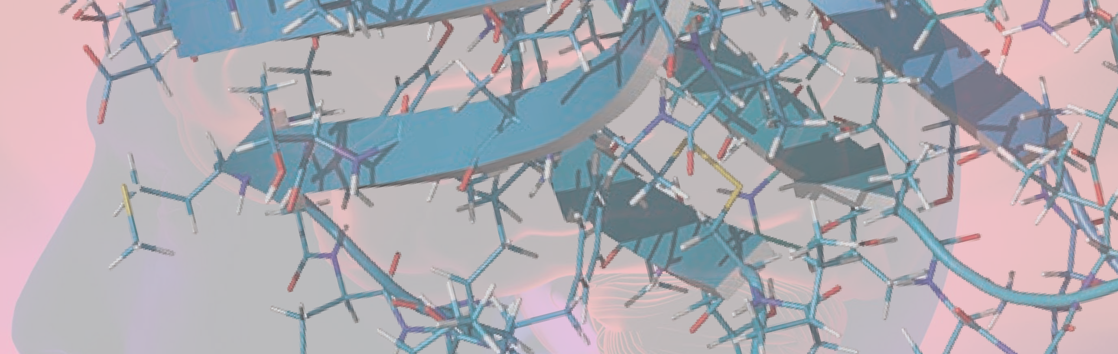
Figure 4. UV chromatogram overlay from three separate SMART digestions from the same mAb, conducted by three individual operators. The 15 marked peptides in each sample were used for inter-user/inter-day RSD value calculations.

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*

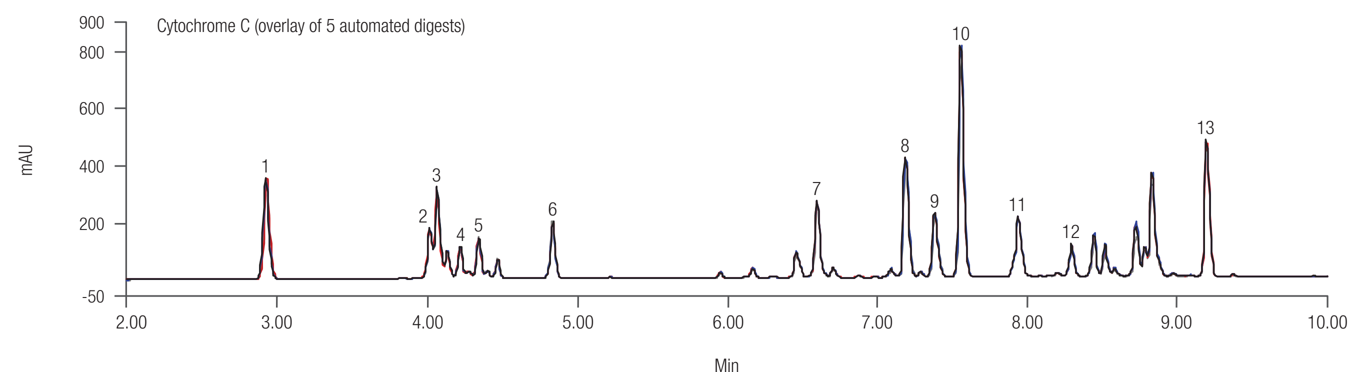


Automation of Digestion

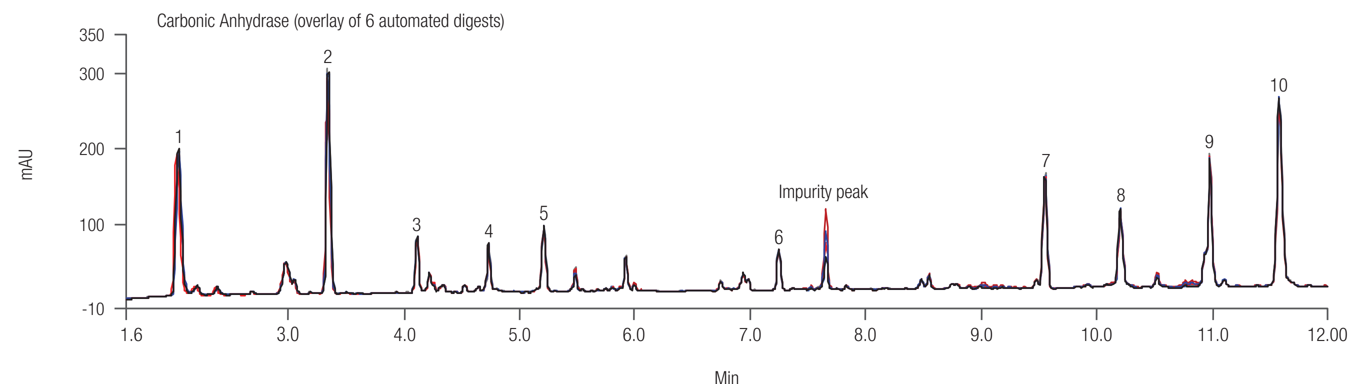
Automation of the digestion procedure is highly desirable helping to streamline the sample preparation process, provide reproducible results and free up resources. Due to their complex nature in-solution digestion is difficult to automate. The simple SMART Digest procedure is highly amenable to automation and the magnetic version of the kit is easily combined with the Thermo Scientific™ KingFisher™ DUO™ purification system to provide high quality, reproducible peptide mapping data.

Digests of proteins infliximab, somatotropin, cytochrome c, and carbonic anhydrase were carried out using the magnetic version of the SMART Digest Kit in combination with a KingFisher Duo Prime purification system to provide an automated solution. This was compared to a manual digestion procedure using a standard SMART Digest Kit.

Replicate digests of cytochrome c and carbonic anhydrase were conducted with the KingFisher Duo and the generated peptides were separated and analyzed by UHPLC-UV. The corresponding peptide maps are shown as an overlay in Figure 5. Both cytochrome c and carbonic anhydrase were readily digested using the automated SMART Digest Kit protocol resulting in complete digestion of the proteins. An average RSD for relative peak area of 2.08% was achieved for the peaks annotated with cytochrome c; several of these peaks had peak area RSD values of 1% and below. Carbonic anhydrase gave similar highly reproducible results with an average area RSD value of 1.8.



Peak	1	2	3	4	5	6	7	8	9	10	11	12	13
%RSD (A_{rel})	2.75	1.87	2.45	0.71	1.27	1.90	3.60	2.09	2.35	3.92	1.11	0.72	2.42
%RSD (t_R)	0.12	0.03	0.05	0.04	0.03	0.02	0.03	0.03	0.03	0.02	0.01	0.01	0.01



Peak	1	2	3	4	5	6	7	8	9	10
%RSD (A_{rel})	2.12	1.46	1.53	3.03	1.71	1.26	1.51	2.60	1.14	1.64
%RSD (t_R)	0.32	0.1	0.03	0.04	0.02	0.02	0.01	0.01	0.01	0.01

Figure 5. Automated digestion of cytochrome c and carbonic anhydrase using SMART Digest magnetic resin with the KingFisher Duo Prime system. Overlaid peptide maps of different digests of cytochrome c (upper panel) and carbonic anhydrase (lower panel). Digest solutions of 5 µL were injected without further purification and peptides were separated using separation condition A. %RSD values for relative peak area (upper) and retention time (lower) and are given for the peaks indicated.

Table of Contents
Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations

Automation of Digestion

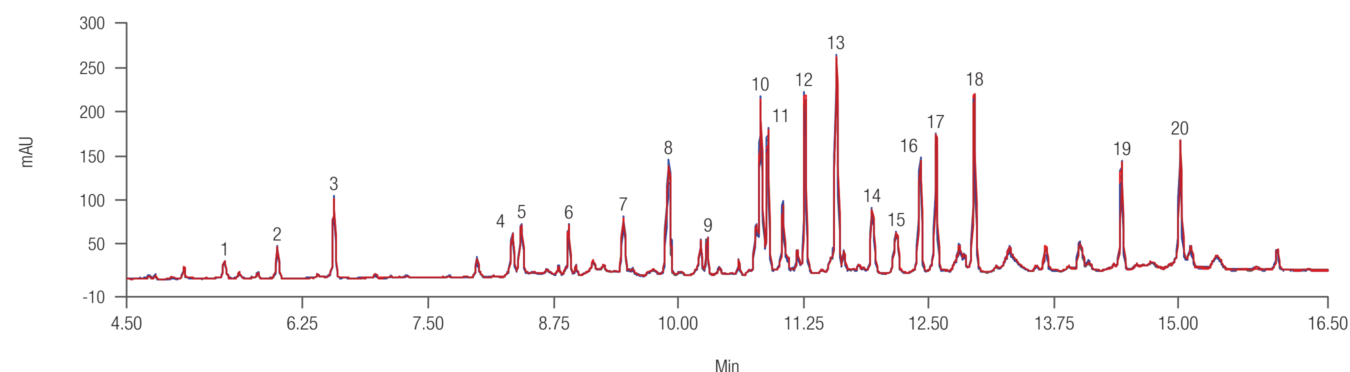
This level of reproducibility can be visualized by the high consistency of the Vanquish Horizon UHPLC system gradients and injection accuracy, which gives identical chromatography and makes integration and interpretation of the peaks easier. This level of reproducibility in protein digestion has never been reported before so the degree of influence between different users was characterized.

To assess the robustness and ease of use of the SMART Digest Kit protocol in general between different users, an experiment was performed during a protein chromatography workshop with five different people performing a manual digestion using the SMART Digest Kit, some of whom had never performed a protein digestion before. The results of this experiment are shown in Figure 6.

The results show an average RSD value for peak area of 2.74 over 20 different peaks in a complex chromatogram. Considering that this result was achieved from a protein digestion of a large monoclonal antibody performed by five people, the robustness of the protocol between different users is very apparent. The ease of use is also demonstrated in that some of the digestions were done by people who have no experience with protein digestion techniques.

The new SMART Digest Kit automated protocol was further evaluated by LC-MS using infliximab drug product as a test sample.

A 45 min incubation at 70 °C enabled the complete digestion of the infliximab antibody and resulted in a close to identical UV peptide map of the two parallel digestion reactions (Figure 7, upper panel). Analysis by LC-MS confirmed complete sequence coverage of 100% for both the light and heavy chain of the antibody (Figure 7, lower panel). This result demonstrates reproducible, complete digestion of infliximab, and with the additional reproducibility studies, that the SMART Digest Kit when automated is readily applicable for the characterization and quality control of modern biopharmaceuticals.



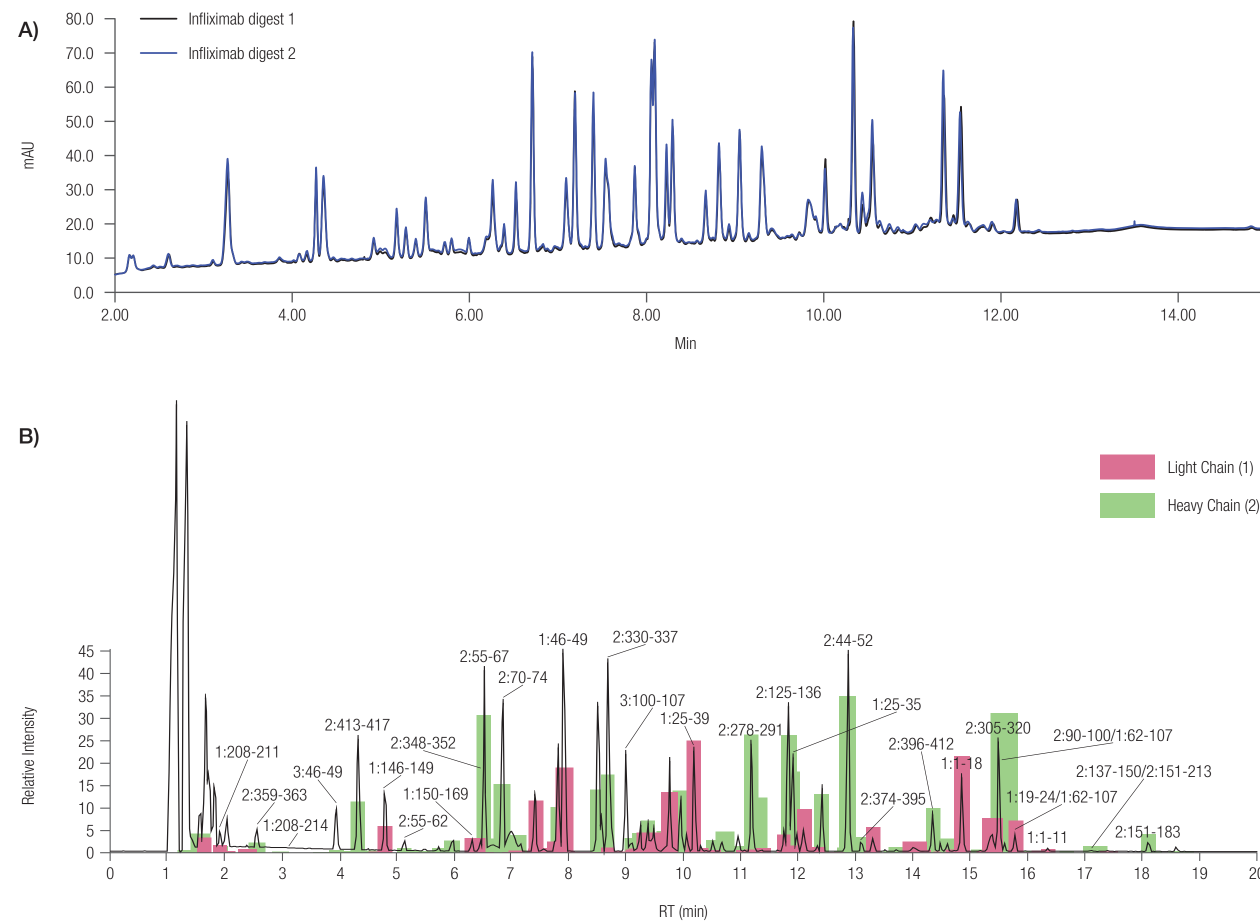
Peak	1	2	3	4	5	6	7	8	9	10
%RSD (A_{rel})	2.54	2.41	1.89	3.39	3.53	2.16	4.41	2.10	2.10	3.65
Peak	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
%RDS (A_{rel})	1.96	3.5	3.72	2.26	2.91	1.97	3.28	2.62	3.16	1.20

Figure 6. Manual digestion of rituximab performed by 5 different people. Overlaid peptide maps of the monoclonal antibody rituximab. 5 µL of digest solutions were injected without further purification and peptides were separated using the gradient and conditions in Table 1. Percentage relative standard deviation (%RSD) values for relative peak area are given for the peaks indicated.

Table of Contents
Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations

Automation of Digestion

Both the manual and automates SMART Digest procedures provide highly reproducible, robust and fast results even in the hands of multiple users with varying experience. The combination of the SMART Digest magnetic resin with the KingFisher automation system minimizes the manual handling required for protein digestion. This yields a further increase in reproducibility.



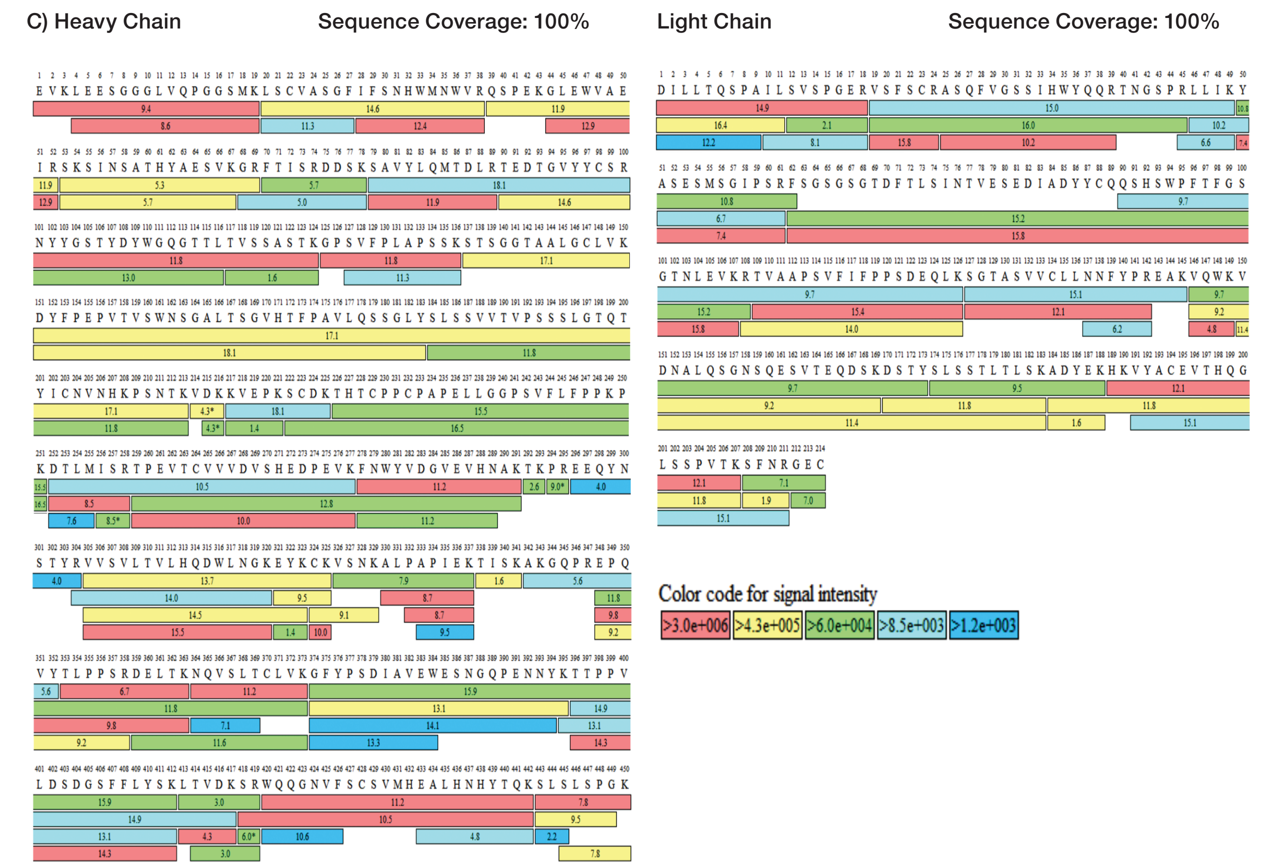
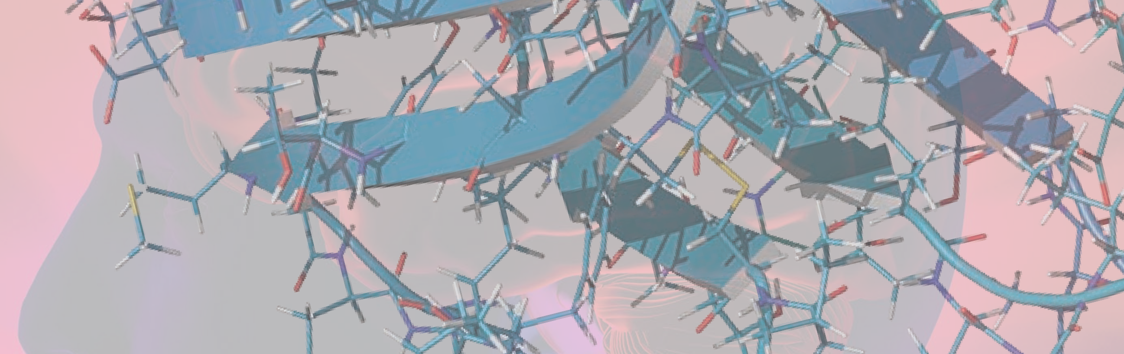


Figure 7. Automated digestion of infliximab drug product using SMART Digest magnetic resin. Panel A: Overlaid peptide maps for two digests of infliximab antibody. Digest solutions of 5 µL were injected without further purification and peptides were separated using separation conditions listed in Table 3. Panel B: Total ion chromatogram from infliximab indicating the peptide origin to light (1) and heavy chain (2). Position numbers are given together with the peptide chain annotation, the heavy chain in green and the light chain in red highlights. Panel C: Sequence coverage map of the automated infliximab using the SMART Digest magnetic kit. Lines containing peptides with signal intensity > 4.3 e5 are shown.

Table of Contents
Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations

Improving Sensitivity and Speed for Protein Quantitation from Complex Matrices



As mentioned protein characterization is just one of the analytical workflows within the biopharmaceutical arena that requires digestion. The other is bioanalysis, where there are additional challenges due to the low abundance of the protein biomarker/biotherapeutic and the complex biological matrices e.g. plasma in which they reside. The ability to perform accurate and robust quantitative bioanalysis of therapeutic monoclonal antibodies (mAbs) is critical to successfully bring new drugs to market.

Accurate data on pharmacokinetics must be available as early as possible in the development process as it contributes to the final success or failure of the compound. The initiation of early absorption, distribution, metabolism, and excretion (ADME) screening has dramatically decreased the proportion of compounds failing in clinical trials. The main aim of preclinical ADME is to eliminate weak drug candidates in the early stages of drug development; this allows resources to be focused on more promising potential drug candidates. Undesirable pharmacokinetic properties such as poor absorption or extensive first-pass metabolism are major contributors to the failure of many drug candidates in early stages of drug development programs.

Early-stage studies, typically within the drug discovery or development phase, have adopted the use of generic methodologies, or assay starting points, to quickly and cost effectively develop analytical methods. Studies in these phases have the goal of generating blood concentration data as quickly as possible in order to make fast decisions whether to progress a drug candidate to the next phase, or to 'fail quick, fail cheap' for candidates that, for example, do not show the expected ADME performance.

It is difficult to justify extensive method development times as short studies and quick data turn-around is required to efficiently progress candidates. The use of enabling technologies to simplify the workflow and help to reduce failure rates are vital tools for the bioanalytical scientist.

Many pharmaceutical companies and clinical research organizations (CRO), with limited bio-molecule experience, are now being asked to perform accurate quantitative analyses of biotherapeutics in a variety of matrices across the drug's lifecycle, from discovery through to clinical studies. While small molecule scientists are experts in techniques required for small molecule analysis, they often have little hands-on experience with quantifying large molecules.

One of the most common methods for protein quantitation is the surrogate peptide approach, particularly in mass spectrometry (MS)-based analyses. Proteins are digested, or broken down into smaller peptides, which can be easier to analyze and interpret than the intact proteins. Peptides are generally more amendable to triple quadrupole MS detection, which is still the tool of choice for fast and sensitive bioanalytical quantitation. This approach, however, can be time-consuming and complex due to the number of steps and reagents required for sample preparation.

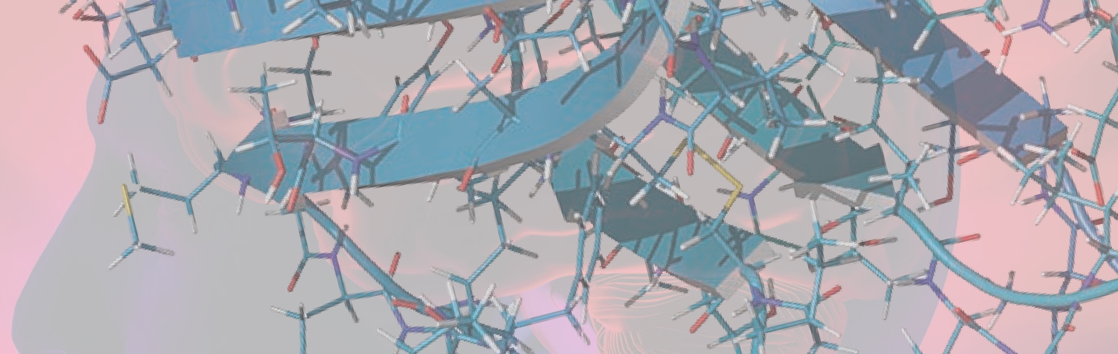
After the protein has been digested (typically through the use of trypsin), one or more peptides with good selectivity and specificity are chosen as a surrogate measure for the protein and analyzed by liquid chromatography (LC)-MS or LC-MS/MS. However, direct digestion of plasma is often not advisable due to its lack of selectivity. The final extract will contain peptides derived from every protein in the sample, such as abundant immunoglobulins. This can have a dramatic effect on the level of sensitivity that can be achieved.

In these workflows immunoaffinity capture is typically employed prior to digestion to increase sensitivity by purifying the low level proteins from the complex biological matrix. This step is then followed by protein digestion. As a consequence these workflows have all the challenges faced when using in-solution digestion protocols, but with the added complexity of additional steps that add labor, the potential for error and that are not amenable to automation. The SMART Digest ImmunoAffinity (IA) kits offer a solution to these issues. The SMART Digest IA kits have all the advantages previously outlined for fast, easy and reproducible protein digestion for quantitation and characterization applications, with the added advantage of combining an immunocapture and the digestion process into a single well. This is achieved due to their unique design where the immunoaffinity reagents (either streptavidin, protein A or protein G) and heat activated thermally stable trypsin are co-immobilized onto a single bead).

Following the binding of a capture reagent to the bead, and enrichment of the target, the enzyme is activated at elevated temperatures for accelerated digestion under protein denaturing conditions. The resulting workflow is therefore significantly simplified and provides a process where the sample is ready for analysis in just a few hours rather than overnight (Figure 8). The following example highlights the advantages of using the SMART Digest IA Streptavidin kit for the quantitation of a biomarker (human interferon a14) in human plasma, compared to a traditional immunocapture and digestion method.

Table of Contents
Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations

Improving Sensitivity and Speed for Protein Quantitation from Complex Matrices



Expose biotinylated capture antibody to biological sample

Conventional protein enrichment and digestion

Incubate with affinity capture resin

Sepharose bead

Conventional tryptic digest
Reduction, TCEP: 60 °C, 30–60 min
Alkylation, IAA: RT, 30 min
Quenching, ACC: RT, 15 min
Digestion: 37 °C, 12–16 h

Sepharose bead

Total time
15–24 hours

Protein enrichment and digestion with the SMART Digest IA Kit

Use SMART Digest IA resin for affinity capture. Incubate at room temperature.

Bead

Digestion with SMART Digest IA resin
70 °C, 1 h

Bead

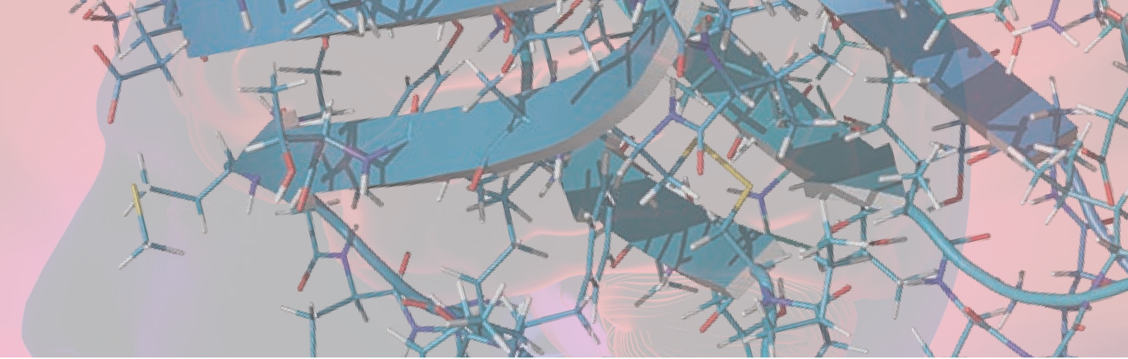
Total time
3–4 hours

Figure 8. Comparison of SMART Digest IA Kit workflow with a conventional workflow.

Table of Contents

Introduction
Faster and More Sensitive Protein Characterization and Quantitation
Easier Digestion
Faster Digestion
Highly Reproducible Digestion
Automation of Digestion
Improving Sensitivity and Speed
Peptide Mapping
Peptide Quantitation
Product and Method Development Considerations

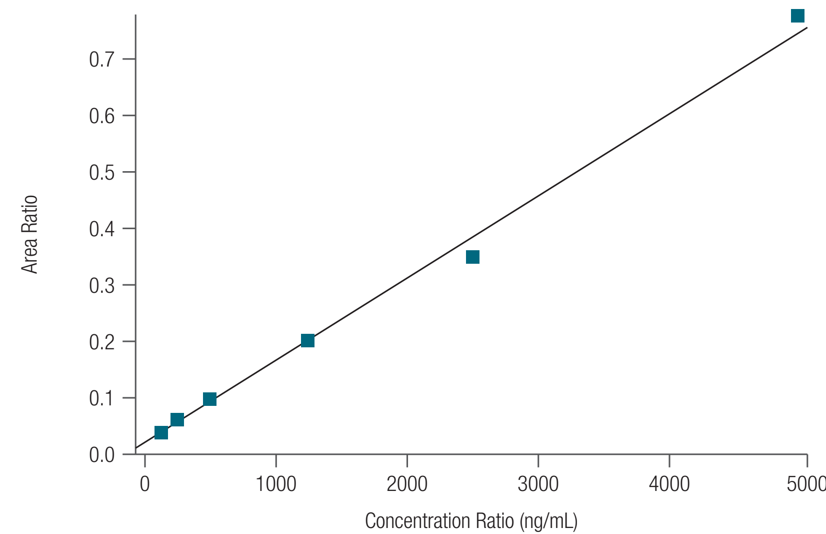
Improving Sensitivity and Speed for Protein Quantitation from Complex Matrices



The SMART Digest IA protocol used involved an immunocapture step, taking 2 hours, followed by a 1 hour, high-temperature digestion with immobilized trypsin. This is compared to immunocapture with a high capacity streptavidin gel followed by overnight tryptic digestion of the biomarker protein. A SIL peptide was spiked into the samples to act as an internal standard.

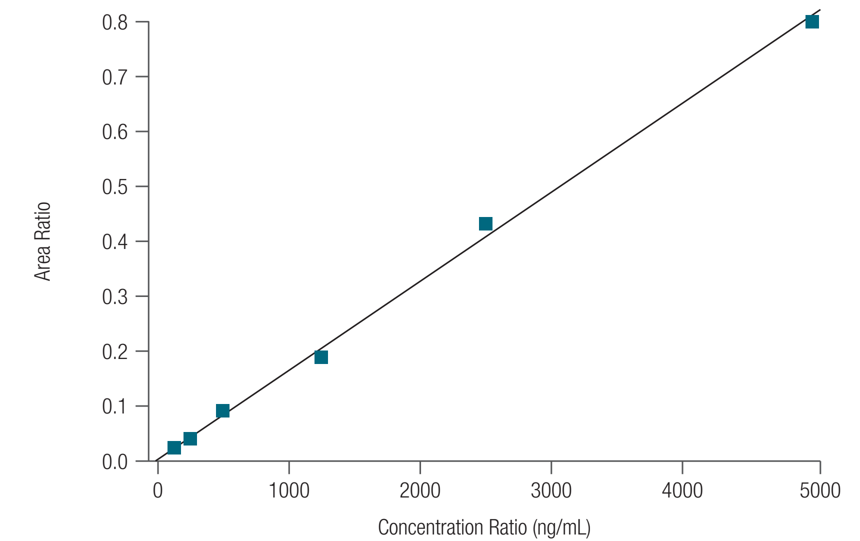
The results show that using the SMART Digest IA Kit (Figure 9 and Table 3) good linearity is achieved over a wide dynamic range, with better % CVs and higher recovery than for the traditional approach (Figure 10 and Table 4).

For the demands of the modern biopharmaceutical company where characterization and quantitation workflows prove challenging due to the complexity of the workflow, speed of analysis and quality of results the new innovations offered by SMART Digest and SMART Digest IA Kits provide a step change in protein sample preparation by providing workflows which are significantly faster, easier to use highly reproducible and sensitive.



Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	93	116.5			
250	107	266.3	250	11.5	90.2
500	106	531.1			
1250	100	1247	1250	7.4	99.1
2500	90	2251			
5000	104	2251			

Figure 9. Calibration curve for the detection biomarker (human interferon a14), in human plasma using the SMART Digest IA Streptavidin kit.



Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	105	131			
250	90	225	250	14.5	111.2
500	109	544			
1250	92	1149	1250	4.1	104.8
2500	106	2654			
5000	99	4922			

Figure 10. Calibration curve for the detection biomarker (human interferon a14), in human plasma using a conventional streptavidin agarose process.

Recovery with SMART Digest IA Kit	
500 ng/mL spike	7330 (cps)
Recovery	64%

Table 3. Recovery data for biomarker (human interferon a14), in human plasma using the SMART Digest IA Streptavidin kit.

Recovery with conventional approach	
500 ng/mL spike	2778 (cps)
Recovery	35%

Table 4. Recovery data for biomarker (human interferon a14), in human plasma using a conventional streptavidin agarose process.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations
Find out more...

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

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Application benefits

- A simple and rapid protein digestion approach to peptide mapping analysis has been identified using the Thermo Scientific™ SMART Digest™ Kit with digestion taking only 45 min with subsequent reduction and alkylation taking a further 30 min each
- 100% sequence coverage was attained for all the studied digestion protocols and low levels of sample preparation-induced post-translational modifications (PTMs) were observed for the SMART Digest Kit approach
- The proposed SMART Digest Kit approach allows easily automated peptide mapping resulting in less sample handling, increased productivity, and improved reproducibility

Goal

To compare peptide mapping data acquired using the SMART Digest Kit and the SMART Digest Trypsin kit, Magnetic Bulk Resin option, to those obtained by alternative digestion methods including an in-solution protocol and a rapid protein digestion method. The study focused on reproducibility, protein sequence coverage and identification of post-translational modifications (PTMs), including deamidation and oxidation.

Introduction

Due to their long half-life in humans and high specificity to target antigens, monoclonal antibodies (mAbs) are the leading biotherapeutics used for the treatment of multiple disease states, including cancer, inflammatory, autoimmune, cardiovascular, and infectious diseases.[1] Sales of €125 billion worldwide by the year 2020 are predicted at the current approval rate.[2] However, due to their complexity and the possibility of protein degradation, extensive characterization of mAbs must be carried out prior to clinical trials and for routine quality control assessments.[3]

Peptide mapping is a "gold standard" tool in biotherapeutic characterization, used to measure critical quality attributes (CQAs) of mAbs to ensure product quality, efficacy, and safety. Peptide mapping consists of the enzymatic or chemical treatment of a protein, resulting in peptide fragments that are then separated, detected, and interpreted. The protease trypsin is most frequently employed to digest therapeutic mAbs for peptide mapping analysis due to its specificity for cleavage at lysine and arginine residues. This results in the production of peptides with the preferred mass range for mass spectrometry detection.

Various approaches to peptide mapping have been developed, utilizing different protein denaturation procedures and digestion times. However, there can often be a lack of reproducibility and confidence in results with commonly used digestion protocols for peptide mapping. This leads to the incurring of excessive cost and reduced productivity due to the laborious sample handling procedures and long digestion times (up to several hours). Furthermore, modifications such as deamidation and oxidation may be induced by sample preparation methods, thereby distorting results.[4] Hence, a simple, robust and reproducible method is essential for peptide mapping.

In this study, a traditional overnight trypsin digestion method and a recently applied rapid digestion protocol[5] were compared to two SMART Digest Kit options (the standard kit and the magnetic bulk resin option). These kits contain thermally stable, immobilized trypsin that enables high temperature protein denaturation without a requirement for addition of denaturants. The SMART Digest trypsin kit (magnetic bulk resin option) was used in conjunction with the Thermo Scientific™ KingFisher™ Duo Prime purification system, which allows easy automation of magnetic bead based applications and is therefore suitable for high-throughput, reproducible sample analysis.[6]

Two monoclonal antibodies were used to assess the alternative digestion methods: adalimumab, the world's top selling biotherapeutics product[2] and the reference monoclonal antibody NISTmAb RM 8671 (NISTmAb), a standard commonly applied for evaluation of analytical method performance.[7] All samples were analyzed on a high-resolution analytical platform consisting of a Thermo Scientific™ Vanquish™ Flex Binary UHPLC and a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer with BioPharma Option.

Four sample preparation methods for peptide mapping were investigated to evaluate the impact of sample preparation on reproducibility and PTMs detected. For each preparation method evaluated (reference experimental section), samples were prepared by two different analysts, with varying sample preparation experience, over three different days and analyzed by liquid chromatography-mass spectrometry (LC-MS) detection. Data analysis was performed using Thermo Scientific™ BioPharma Finder™ software. The analytical platform enabled excellent reproducibility of chromatographic data enabling 100% sequence coverage of mAbs.

Peptide reproducibility and PTMs induced by sample preparation were investigated, highlighting the benefits of using a heat stable immobilized trypsin for sample preparation compared to other trypsin formulations that require high pH buffers for protein denaturation. In addition, sample reproducibility was found to be further improved using trypsin immobilized on magnetic beads in combination with an automated handling system, e.g. KingFisher Duo Prime purification system.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
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Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ-cm resistivity
- Water, Fisher Chemical™ Optima™ LC/MS grade (P/N 10505904)
- Acetonitrile, Fisher Chemical™ Optima™ LC/MS grade (P/N 10001334)
- Water with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10118464)
- Trifluoroacetic acid (TFA), Fisher Chemical™ (P/N 10294110)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- SMART Digest Trypsin Kit (P/N 60109-102)
- Dithiothreitol (DTT), Fisher Bioreagents™ (P/N 10386833)
- Iodoacetic acid (IA), Acros Organics™ (P/N 10235940)
- MS Grade Trypsin Protease, Thermo Scientific™ Pierce™ (P/N 13464189)
- 8M Guanidine-HCl, Thermo Scientific™ Pierce™ (P/N 10167783)
- 1M Triethylammonium bicarbonate (TEAB), Thermo Scientific™ (P/N 90114)
- Tris-HCl, Fisher Chemical™ (P/N 10142400)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Equipment

- KingFisher Duo Prime Purification System (P/N 5400110)
- Vanquish Flex Binary Flex UHPLC system including:
  - Binary Pump F (P/N VF-P10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A)
  - System Base Vanquish Horizon (P/N VH-S01-A)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer with BioPharma Option (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Sample pre-treatment

Commercially available monoclonal antibody adalimumab drug product was supplied at a concentration of 50 mg/mL. NISTmAb reference material 8671, was supplied by National Standard of Standards and Technology (NIST), also at 10 mg/mL.

Sample preparation

Monoclonal antibody samples were prepared in triplicate by different analysts on different days.

In-solution digestion protocol using heat for denaturation (in solution, heat)

Samples were diluted to 2 mg/mL in water. Reduction of disulfide bonds was carried out by the addition of 10 mM DTT in 100 mM TEAB, pH 8.5, to the mAb samples with incubation at 70 °C for 75 minutes. Alkylation was carried out by the addition of 20 mM IA. The samples remained in darkness for 30 minutes, and 11 mM DTT was added to quench IA. Trypsin was added to mAb samples at a protein/trypsin ratio of 1:50, and tryptic digestion was carried out at 37 °C for 16 hours, followed by quenching with 10% TFA.

Alternative rapid digestion protocol (alternative rapid digest)

A peptide mapping sample preparation method described by Rogers et al.[5] was used for this analysis. Samples were diluted to 2 mg/mL, denatured, and reduced using 10 mM DTT in 7.5 M guanidine, pH 8.3, for 30 minutes at ambient temperature. Following reduction, samples were alkylated in 20 mM IA and incubated for 20 minutes in darkness. The reaction was quenched with 11 mM DTT. Sample solutions were desalted using BioSpin™-P6 columns (Bio-Rad) and eluted in 100 mM Tris Buffer, pH 8.0. Protein concentration was measured with a NanoDrop 2000 Spectrophotometer. Trypsin was added to samples at a trypsin/protein ratio of 1:10 and incubated at 37 °C for 30 minutes, followed by reaction quenching with 10% TFA.

Protocol for sample preparation using a SMART Digest trypsin kit (SMART Digest)

Samples were diluted to 2 mg/mL in water. Then, 150 µL SMART Trypsin Buffer was added to SMART Digest vials, followed by 50 µL of sample (100 µg). MAb samples were incubated at 1400 rpm at 70 °C for 45 minutes. Following tryptic digestion, samples were spun down for 2 minutes at 7000 rpm and supernatant placed in a fresh tube. Reduction of disulfide bonds was carried out with 10 mM DTT for 30 minutes at 57 °C. Samples were alkylated in 20 mM IA for 30 minutes in darkness. Quenching of IA and trypsin was carried out with 11 mM DTT and 10% TFA, respectively. (Note: use of IA and TFA is not required as part of the SMART Digest protocol, however, these were added to ensure that peptides induced by these reagents would be uniform across all sample preparations).

Protocol for sample preparation using a SMART Digest trypsin kit, magnetic bulk resin option (Magnetic SMART Digest)

Samples were diluted to 2 mg/mL in water. For each sample digest, sample and buffers were added to each lane of a KingFisher Deepwell 96 well plate as outlined in Table 1. Bead “wash buffer” was prepared with by diluting SMART Digest buffer 1:4 (v/v) in water. Bead buffer was neat SMART Digest buffer. Digestion was performed using a KingFisher Duo Prime purification system with Thermo Scientific™ BindIt™ software (version 4.0), using the protocol outlined in Table 2. Samples were incubated for 45 minutes at 70 °C on medium mixing speed (to prevent sedimentation of beads), with post-digestion cooling carried out to 10 °C. Following digestion,

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

disulfide bond reduction was performed with 10 mM DTT for 30 minutes at 57 °C and subsequently alkylated with 20 mM IA in darkness for 30 minutes. The reaction was quenched with 11 mM DTT followed by 10% TFA. (Note: use of IA and TFA is not required as part of the SMART Digest protocol, however, these were added to ensure that peptides induced by these reagents would be uniform across all sample preparations).

Table 1. KingFisher Duo Prime plate layout utilized for sample preparation. Reagents and associated volumes placed in each well are outlined.

Lane	Content	Volume Applied to Each Well (µL)
A	SMART Digest buffer	150
	Sample (2 mg/mL)	50
B	Tip Comb	
C	Empty	
D	Magnetic SMART Beads	15
	Bead Buffer (SMART Digest buffer)	100
E	Bead Wash Buffer (SMART Digest buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

Table 2. Protocol for automated peptide mapping using a KingFisher™ Duo Prime system

Step	Release Bead	Mixing	Collect Beads	Temperature	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	45 min Medium Mix	3 count, 15 s	During digestion: 70 °C Post-digestion: 10 °C	A
Release Beads	Yes, Fast	–	–	–	F

LC conditions

Mobile phase A: Water with 0.1% formic acid (v/v)
Mobile phase B: Acetonitrile with 0.1% formic acid (v/v)
Flow rate: 0.3 mL/min
Column temperature: 25 °C (Still air mode)
Autosampler temperature: 5 °C
Injection volume: 10 µL
Injection wash solvent: Methanol/water, 10:90
Needle wash: Enabled pre-injection
Gradient: See Table 3 for details

Data processing

Acquisition software: Thermo Scientific™ Xcalibur™ software 4.0
MS data analysis: Thermo Scientific™ BioPharma software: Finder 3.0

MS conditions

Detailed MS method parameters are shown in Tables 4 and 5.

Table 3. Mobile phase gradient for UHPLC separation of peptides

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

Table 4. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Thermo Scientific™ Ion Max source with HESI II probe
Sheath gas pressure	40 psi
Auxiliary gas flow	10 arbitrary units
Probe heater temperature	400 °C
Source voltage	3.8 kV
Capillary temperature	320 °C
S-lens RF voltage	50 V

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Table 5. MS method parameters utilized for peptide mapping analysis

General	Setting	MS² Parameters	Setting
Runtime	0 to 65 min	Resolution settings	17,500
Polarity	Positive	AGC target value	1.0×10^5
Full MS parameters	**Setting**	Isolation width	2.0 *m/z*
Full MS mass range	200–2000 *m/z*	Signal threshold	1.0 × 104
Resolution settings	70,000	Normalized collision energy (HCD)	28
AGC target value	3.0×10^6	Top-N MS²	5
Max injection time	100 ms	Max injection time	200 ms
Default charge state	2	Fixed first mass	–
SID	0 eV	Dynamic exclusion	7.0 s
Microscans	1	Loop count	5

MS data processing

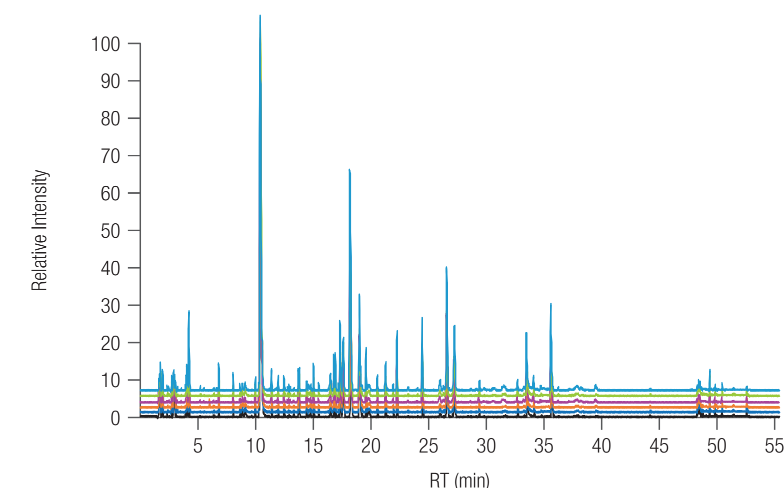
Detailed parameter settings are shown in Table 6.

Table 6. BioPharma Finder software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS signal threshold	1.60×10^5 counts
Identification	**Setting**
Mass accuracy	5 ppm
Minimum confidence	0.8
Maximum number of modifications for a peptide	1
Unspecified modification	-58 to +162 Da
N-glycosylation	CHO
Protease specificity	High
Static Modifications	**Setting**
Side chain	Carboxymethylation
Variable Modifications	**Setting**
Side chain	Deamidation (NQ)
	Glycation (K)
	Oxidation (MW)

Results and discussion

Figure 1 shows chromatograms generated for six different sample preparations of NISTmAb using all four protein digestion methods, highlighting both the reproducibility of the sample preparation method and LC-MS platform.



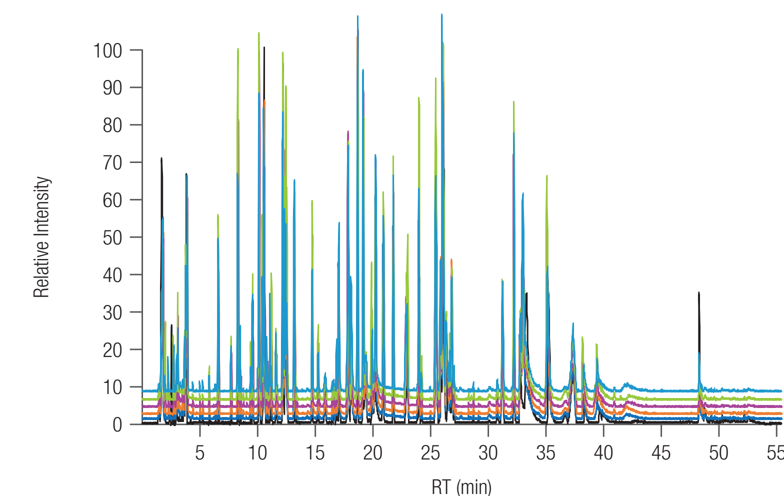


Figure 1A. Base Peak Chromatograms (BPC) overlay from six peptide mapping sample preparations of NISTmAb using in solution, heat digest (top) and alternative rapid digest (bottom). Samples were prepared by two individual operators on different days.

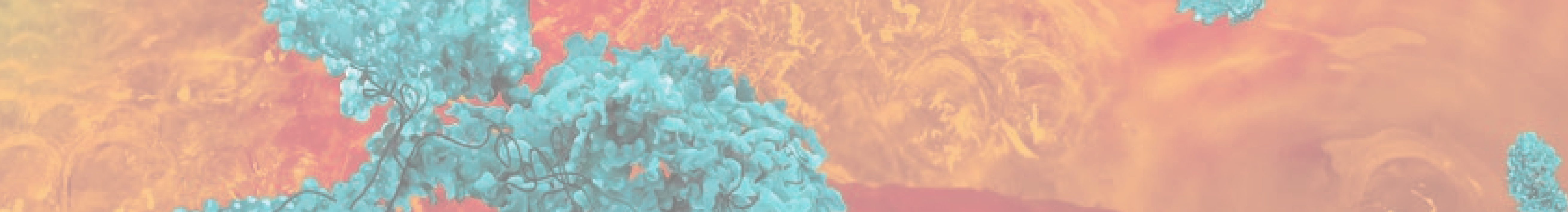


Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Peptide mapping analysis provides relative quantification of post-translational and chemical modifications; therefore, very high sequence coverage for both mAb light and heavy chains is essential. For nearly all protein digestion methods 100% coverage was achieved (Table 7). Both the rapid digestion protocol and SMART Digest protocol resulted in full sequence coverage using digestion times of < 45 minutes.

Table 7. Sequence coverage determined for adalimumab and NISTmAb following sample preparation with various digestion methods

Proteins	Number of MS Peaks	Sequence Coverage	Relative Abundance	Sample, n=6
NISTmAb RM8671 Light Chain	425	100%	16.01%	Magnetic SMART Digest, 45 minutes
	268	100%	22.77%	SMART Digest, 45 minutes
	493	100%	46.61%	Alternative rapid digest
	549	100%	26.52%	In-solution, heat
NISTmAb RM8671 Heavy Chain	1340	100%	83.94%	Magnetic SMART Digest, 45 minutes
	1045	100%	77.23%	SMART Digest, 45 minutes
	1002	100%	53.40%	Alternative rapid digest
	1652	100%	73.48%	In-solution, heat
Adalimumab Light Chain	753	100%	38.14%	Magnetic SMART Digest, 45 minutes
	627	100%	51.88%	SMART Digest, 45 minutes
	510	100%	23.30%	Alternative rapid digest
	911	100%	49.40%	In-solution, heat
Adalimumab Heavy Chain	1461	99.10%	61.86%	Magnetic SMART Digest, 45 minutes
	1409	99.10%	48.13%	SMART Digest, 45 minutes
	870	100%	45.48%	Alternative rapid digest
	1167	100%	50.60%	In-solution, heat

It has previously been reported that certain aspects of sample preparation may induce modifications, such as deamidation and oxidation of amino acid residues. Ren et al. reported that both the buffer pH and length of digestion time may artificially induce deamidation modifications.[4] Similarly, oxidation may be induced by sample preparation or digestion. To determine the effect of the sample preparation methods evaluated on oxidation and deamidation modifications, the percentage relative abundance of each modified form of a peptide identified in each sample was determined using BioPharma Finder software. The average percent relative abundance of each deamidated and oxidized peptide from different sample preparations and replicate injections was calculated and is shown in Tables 8 and 9 for adalimumab and NISTmAb, respectively. For both mAbs, the percentage relative abundance of PTMs, including deamidations, oxidations, glycation, and N-glycosylation were examined. To demonstrate and compare the relative abundance, plots of oxidation and deamidation modifications are shown for all digestion methods for adalimumab and NISTmAb. For both oxidation and deamidation modifications, the alternative rapid digest method, the SMART Digest, and the magnetic SMART Digest Kits demonstrated the lowest amount of modified peptide forms for the studied mAbs, except for adalimumab where SMART digestion showed similar levels of total deamidation than In-solution, heat digestion (44.30% and 44.32%, respectively). Adalimumab showed higher levels of modifications than NISTmAb for all studied digestion methods. In-solution, heat digestion showed the highest levels of oxidation and deamidation for adalimumab and NISTmAb.

SMART digest protocols were performed close to neutral pH (pH 7.2), while the in solution, heat method and alternative rapid digest method were carried out in basic conditions (pH 8.5 and pH 8.3, respectively). Studies have shown high buffer pH and long digestion times may lead to higher levels of deamidated peptides. As Figures 2 and 4 show, a combination of elevated buffer pH and digestion times over a number of hours lead to dramatically increased abundance of some deamidated peptide forms (N319/N318 and N329/N328 for adalimumab and NISTmAb, respectively).

The presence of certain deamidated peptides at high levels resulted in a significant increase in the relative total amount of deamidated species detected for the in-solution, heat method, in particular the presence of deamidated form of N318 (11.94%) in the in-solution, heat digests for NISTmAb and N319 (16.23%) in the in-solution, heat samples for adalimumab. Certain asparagine residues are more susceptible to sample preparation induced modifications. For example, N319 displayed low levels of deamidation for adalimumab when prepared using the SMART Digest Kit (5.22%) and Magnetic SMART Digest Kit (1.93%).

A therapeutic protein's higher order structure may have an effect on the proportion of artificially induced modifications observed following sample preparation with different protein digestion methods.

High levels of methionine oxidation were found following sample preparation using the in-solution, heat digestion method that were not observed following sample processing with the SMART Digest Kit, Magnetic SMART Digest Kit and the alternative rapid digestion protocol. For example, M256 was found to be 10.79% modified, contributing to the larger oxidation based relative abundance of this method for the adalimumab sample (Figure 3). The analysis of NISTmAb did not result in large percent relative oxidation modifications with only 3.69% of average modification for M431 when using the in-solution, heat digestion method. Similar profiles were observed for the percentage relative oxidized forms of M255

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

following sample preparation using the SMART Digest Kit (1.52%), Magnetic SMART Digest Kit (2.17%), and alternative rapid digestion method (1.54%).

Other commonly targeted modifications are lysine (K) glycations, which are listed in Tables 8 and 9. In total between 6 (SMART Digest method) to 26 (alternative rapid method) lysine glycations could be identified and relatively quantified for NISTmAb < 2.20% and for adalimumab sample < 0.90%.

One of the most noted PTMs for therapeutic mAbs is their varied N-linked glycan structures, which include galactosylation, fucosylation, mannosylation, and sialylation. High abundance of glycosylation of the heavy chain is also observed for the studied mAbs at the Fc part at position N301 for adalimumab or N300 for NISTmAb. The main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the reducing end of N-acetylglucosamine (A2G0F, A2G1F, A2G2F and A1G0F), afucosylated biantennary (A1G0, A2G0), and high mannose (M5, M6, and M7) structures (Tables 8 and 9).

The different approaches to peptide mapping sample preparation vary greatly in the number of preparation steps and amount of sample handling required. For example, the use of guanidine in the alternative rapid digest protocol results in the requirement for a desalting step in the sample preparation process, which causes a 30% loss in sample recovery. A lengthy and manual sample preparation protocol has the potential to lead to variations in peptide samples generated. To evaluate the reproducibility of sample preparation for each method, standard deviation (stdev) values were investigated for oxidation and deamidation modifications. For the data shown, low stdev values for modified peptides were observed for samples digested with the SMART Digest Kits. The ease of automation for the digest method, and therefore the potential for high-throughput analysis of samples, make these methods ideal for a QC environment where precise and robust high-throughput analysis of samples is required.

Overall, the data shows that while 100% sequence coverage may be achieved using different methods, the method of denaturation and buffers used for sample preparation have a potentially large impact on the level of deamidation and oxidation modifications reported. Use of heat stable trypsin enzyme, high temperature protein denaturation and neutral pH reaction buffers may dramatically reduce sample preparation induced protein modifications when compared to in-solution, heat digestion method. The data shows a high level of reproducibility with SMART Digest Kits and low levels of PTMs induced by sample preparation when compared to alternative methods evaluated.

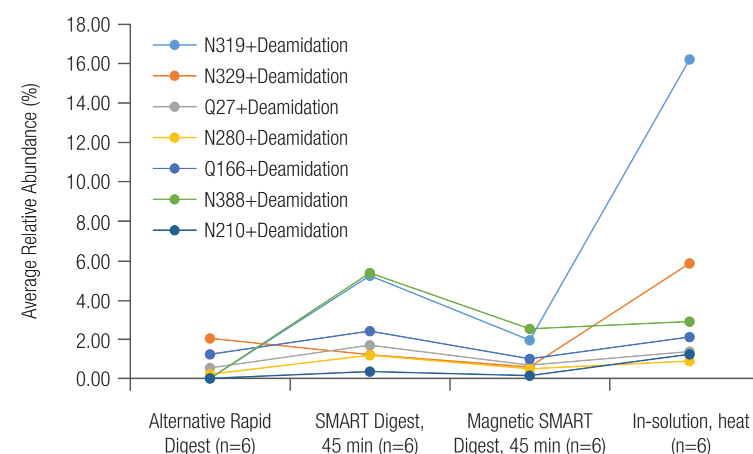


Figure 2. Average relative abundance of seven identified deamidation modifications for adalimumab with various digestion methods

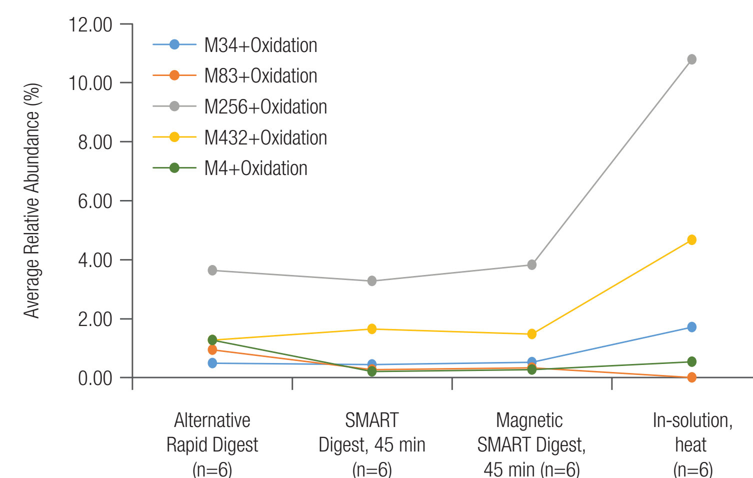


Figure 3. Average relative abundance of five identified oxidation modifications for adalimumab with various digestion methods

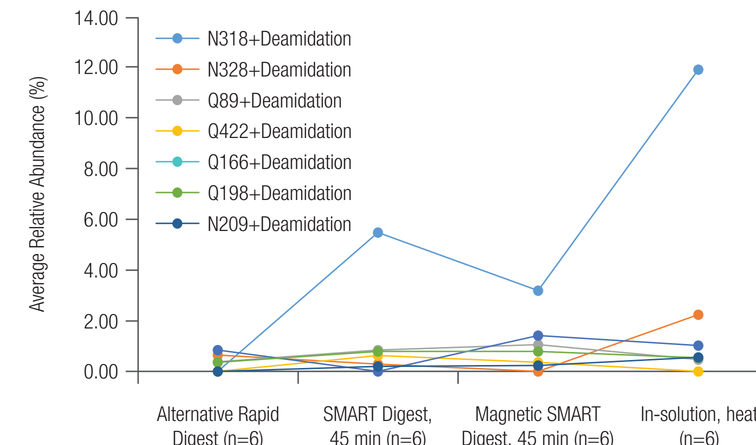


Figure 4. Average relative abundance of seven identified deamidation modifications for NISTmAb with various digestion methods

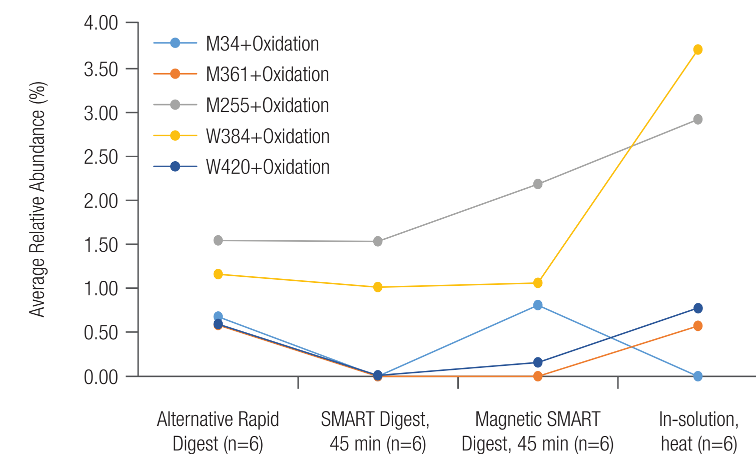


Figure 5. Average relative abundance of five identified oxidation modifications for NISTmAb with various digestion methods

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Table 8A. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for adalimumab. A tilde (~) before the modification indicates the modification was found on the tryptic peptide but could not be localized on a specific amino acid with MS/MS spectra.

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
~Q13+Deamidation	1.654	2.693	–	1.999
N74+Deamidation	–	–	–	0.805
N77+Deamidation	0.224	3.498	1.595	–
Q82+Deamidation	0.514	1.414	0.652	–
N84+Deamidation	0.259	1.359	0.577	–
Q113+Deamidation	0.567	–	–	–
N163+Deamidation	–	–	0.661	–
N290+Deamidation	0.218	1.174	0.496	0.873
N301+Deamidation	0.051	0.285	0.149	–
N319+Deamidation	–	5.221	1.933	6.229
N329+Deamidation	2.041	3.875	0.586	5.855
Q346+Deamidation	0.873	–	–	–
N365+Deamidation	0.127	1.880	0.910	0.170
Q366+Deamidation	0.423	1.268	0.571	0.905
~N388+Deamidation	–	5.367	2.523	2.892
~Q423+Deamidation	–	1.062	–	–
~N393+Deamidation	2.430	–	–	–
~Q3+Deamidation	1.016	1.224	1.046	–
~Q6+Deamidation	0.830	1.325	0.330	–
Q27+Deamidation	0.540	1.682	0.664	1.366
N31+Deamidation	0.131	–	0.571	–
~Q37+Deamidation	1.144	–	1.131	1.421
~Q38+Deamidation	–	1.476	–	–
Q55+Deamidation	0.787	1.263	0.327	0.726
Q100+Deamidation	0.689	1.941	0.541	1.195
~N137+Deamidation	0.388	2.088	0.588	1.510
Q147+Deamidation	0.661	1.049	0.387	0.801
~N158+Deamidation	–	1.777	–	1.741

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Table 8B. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for adalimumab

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
~Q155+Deamidation	–	–	0.436	1.431
~Q160+Deamidation	0.911	–	0.601	–
Q166+Deamidation	1.215	2.397	0.997	2.101
Q199+Deamidation	0.603	1.315	0.557	1.083
N210+Deamidation	–	0.338	0.148	1.220
M34+Oxidation	0.491	0.434	0.523	1.714
W53+Oxidation	0.084	–	–	–
W162+Oxidation	0.439	–	–	–
M256+Oxidation	3.636	3.278	3.828	10.798
~M432+Oxidation	1.268	1.654	1.477	4.670
M4+Oxidation	1.267	0.202	0.267	0.535
K76+Glycation	0.095	0.091	0.079	–
K137+Glycation	0.116	0.190	0.211	–
~K292+Glycation	0.135	0.214	0.224	–
K321+Glycation	0.046	0.031	–	–
~K330+Glycation	0.212	0.180	0.301	0.097
K338+Glycation	–	–	–	0.029
~K39+Glycation	0.364	–	–	–
K103+Glycation	0.194	0.265	0.177	0.217
K107+Glycation	0.041	–	–	–
K145+Glycation	0.050	–	–	–
K149+Glycation	0.209	–	0.361	0.252
K169+Glycation	0.127	–	–	–
~K183+Glycation	0.410	–	–	0.399
K188+Glycation	0.897	–	0.327	0.396
K190+Glycation	0.050	0.845	–	0.047
K207+Glycation	0.063	–	–	0.023
N301+A1G0	0.324	0.359	0.532	–
N301+A1G0F	3.238	6.531	11.534	4.942
N301+A1G1F	0.806	1.114	1.466	0.462
N301+A2G0	0.841	0.681	0.802	0.617
N301+A2G0F	66.339	67.081	61.945	75.617
N301+A2G0FB	0.223	–	0.338	–
N301+A2G1	0.028	–	0.098	-
N301+A2G1F	15.589	12.881	14.879	15.932
N301+A2G2F	1.003	0.796	0.988	1.071
N301+Gn	0.151	0.350	0.332	-
N301+M3	0.591	0.953	0.791	0.147
N301+M4	0.611	0.706	0.953	0.351
N301+M5	5.535	5.076	4.639	3.897
N301+M6	2.645	2.342	1.876	1.662
N301+M7	1.225	0.904	0.794	0.726
N301+M8	0.467	0.285	0.316	0.262
N301+Unglycosylated	1.631	3.606	3.471	0.425

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

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Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
Q1+Deamidation	0.041	–	–	0.041
N62+Deamidation	0.039	–	–	–
N78+Deamidation	0.116	0.584	0.690	–
Q79+Deamidation	0.380	0.845	1.059	0.477
~N279+Deamidation	–	–	–	0.469
N86+Deamidation	0.108	–	–	–
~N206+Deamidation	–	1.180	–	–
~N204+Deamidation	–	–	1.407	–
N289+Deamidation	0.180	1.056	0.869	0.591
N300+Deamidation	-	0.230	0.106	–
N318+Deamidation	-	5.502	3.206	11.942
N328+Deamidation	0.650	0.294	–	2.253
N364+Deamidation	0.040	1.849	0.440	0.288
Q365+Deamidation	0.304	0.853	1.694	0.324
~N392+Deamidation	3.002	–	4.429	2.736
~Q3+Deamidation	0.389	–	–	–
~Q6+Deamidation	0.508	–	–	–
~N387+Deamidation	–	4.579	–	
~Q422+Deamidation	–	0.625	0.353	–
~Q36+Deamidation	0.690	1.553	1.440	–
~N136+Deamidation	0.273	1.027	0.932	0.618
Q146+Deamidation	0.284	0.724	0.603	0.310
~Q154+Deamidation	0.517	0.244	0.664	0.464
~Q159+Deamidation	0.537	–	–	-
~Q165+Deamidation	0.854	–	1.418	1.019
Q198+Deamidation	0.367	0.786	0.784	0.544
~N157+Deamidation	–	1.478	0.977	0.840
N209+Deamidation	–	0.195	0.241	0.555
~M34+Oxidation	0.674	–	0.803	–
M87+Oxidation	0.752	–	0.495	–
W161+Oxidation	0.245	–	–	–
M255+Oxidation	1.537	1.524	2.173	2.905
M361+Oxidation	0.583	–	–	0.568
~M431+Oxidation	1.157	1.007	1.055	3.691
M4+Oxidation	0.790	–	0.369	0.412
M32+Oxidation	0.589	0.013	0.160	0.774
K58+Glycation	0.601	–	0.430	–
K66+Glycation	0.369	–	–	–
K73+Glycation	1.036	0.964	0.998	0.796
K77+Glycation	0.281	–	–	–
K136+Glycation	0.309	–	0.360	–
K216+Glycation	0.336	–	–	–
K249+Glycation	0.590	–	–	–
~K291+Glycation	0.400	0.527	0.571	0.255
K320+Glycation	0.125	–	0.082	0.096
~K323+Glycation	0.167	–	2.198	–
K325+Glycation	0.703	–	0.716	0.317
K329+Glycation	0.360	0.429	0.422	0.256

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Table 9B. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for NISTmAb RM8671

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
K395+Glycation	0.080	–	–	–
K341+Glycation	–	–	–	0.036
K417+Glycation	0.048	–	–	–
K38+Glycation	0.124	–	0.207	–
~K41+Glycation	0.625	–	–	–
K44+Glycation	0.294	–	–	–
K52+Glycation	0.469	–	0.552	0.472
K106+Glycation	0.140	–	–	–
K144+Glycation	0.163	–	–	–
K148+Glycation	0.480	0.481	0.697	0.461
K168+Glycation	0.306	–	–	–
~K182+Glycation	0.498	–	–	0.724
K187+Glycation	0.855	–	0.554	0.910
K189+Glycation	0.158	2.060	–	0.131
K206+Glycation	0.104	0.024	–	0.085
K337+Glycation	–	–	0.101	0.089
K363+Glycation	–	–	0.123	–
~K208+Glycation	–	–	–	0.109
N300+A1G0	0.668	0.613	0.676	0.501
N300+A1G0F	3.549	6.003	12.143	3.349
N300+A1G0M4F	0.603	–	–	–
N300+A1G0M5F	1.332	–	–	0.879
N300+A1G1	0.098	-	0.066	0.047
N300+A1G1F	3.297	3.881	6.208	2.745
N300+A1G1M4F	1.512	0.985	1.400	1.084
N300+A1G1M5	0.231	–	–	–
N300+A1G1M5F	0.207	–	–	–
N300+A1S1M5	0.095	–	–	0.051
N300+A1Sg1	–	–	–	1.054
N300+A1Sg1F	1.049	0.968	1.006	0.797
N300+A2G0	0.118	0.097	0.168	–
N300+A2G0F	38.644	40.615	34.173	44.163
N300+A2G1	–	–	0.180	–
N300+A2G1F	36.308	34.641	33.831	38.902
N300+A2G2F	9.012	8.433	8.060	9.019
N300+A2G2M4F	1.836	–	–	–
N300+A2G2M5F	–	–	0.718	0.582
N300+A2Ga1G1F	1.637	1.670	2.052	1.739
N300+A2Ga2F	0.882	0.819	0.960	0.915
N300+A2S1G0	–	–	–	0.488
N300+A2S1G1	0.247	0.326	–	0.343
N300+A2Sg1G0F	0.304	–	0.383	0.161
N300+A2Sg1G1F	0.393	–	0.477	0.321
N300+A2Sg1Ga1F	0.563	0.430	–	0.300
N300+A3G0F	–	–	–	0.176
N300+A3G1F	0.558	–	–	0.422
N300+A3G2F	0.460	–	0.492	0.428
N300+A3G3F	0.257	–	0.294	–

ThermoFisher SCIENTIFIC

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Table 9C. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for NISTmAb RM8671

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
N300+Gn	–	0.062	0.038	–
N300+M3	–	0.070	0.176	–
N300+M4	–	0.015	0.138	–
N300+M5	1.199	1.267	0.949	0.823
N300+M6	0.072	–	0.041	0.041
N300+Unglycosylated	0.708	1.610	1.043	0.498

Conclusions

- SMART Digest Kits provide simple and rapid protein digestion for peptide mapping analysis in comparison with in solution digestion methods. The SMART Digest Kit and Magnetic SMART Digest Kit allow digestion in 45 min with little manual sample preparation.
- Analysis of monoclonal antibodies gives excellent quality data with high confidence in results. Excellent sequence coverage (100%) and low levels of sample preparation-induced post-translational modifications (PTMs) were observed with both versions of the SMART Digest Kits (deamidation <5.5% and oxidation <3.8% for both adalimumab and NISTmAb).
- Easily automated peptide mapping resulting in less sample handling, increased productivity and improved reproducibility.

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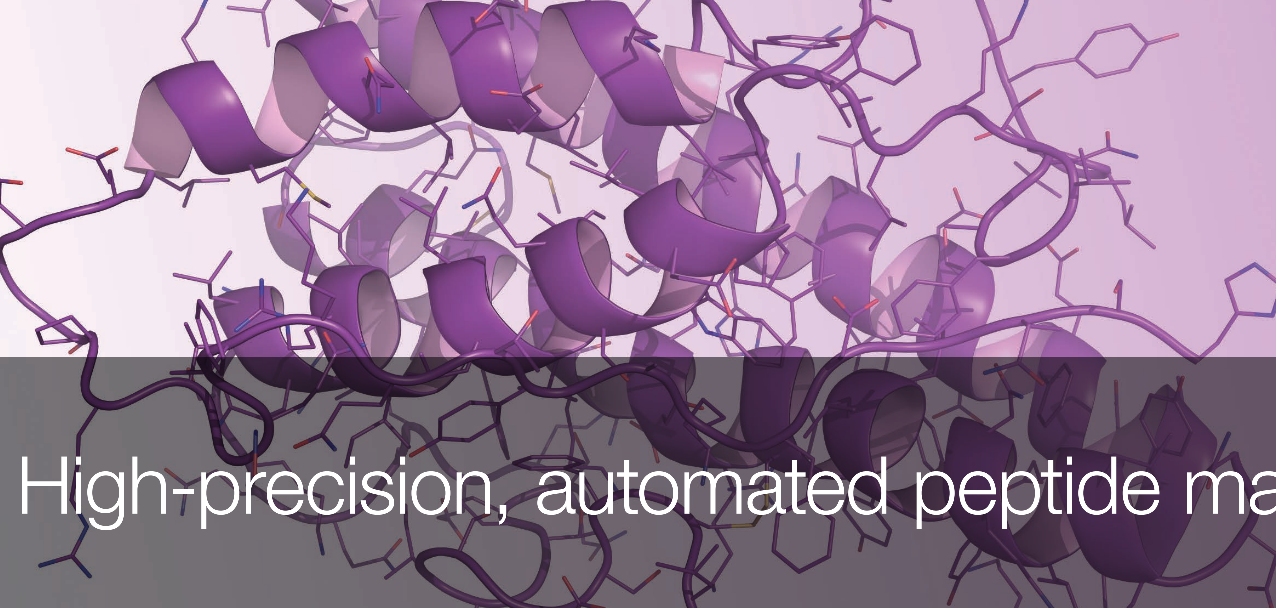
thermoscientific

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations
Find out more...



High-precision, automated peptide mapping of proteins

Amy Farrell[1], Jonathan Bones[1], Ken Cook[2], Suraj Patel[2], Alexander Schwahn[2], Jon Bardsley[2]
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[2]Thermo Fisher Scientific, Reinach, Switzerland; Runcorn, UK; Hemel Hempstead, UK

Application benefits

- High-precision digestion carried out in under one hour including preparation time. This represents a significant time saving (up to 24 fold) compared to traditional digestion techniques.
- Reproducible results that are user-independent with less than 3.1% RSD in peptide area for six independent digests and a sequence coverage of 100%.
- Associated ease-of-use through automation.

Goal

To develop a robust and reproducible, high-precision, automated, digestion workflow that is appropriate as an easy-to-use, general approach to peptide mapping characterization with both LC-only and LC-MS processes. To confirm the peptide map quality with multiple examples of proteins and show high sequence coverage is possible using high-resolution, accurate-mass mass spectrometry.

Introduction

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biotechnological production, there are many attributes that need to be analyzed to guarantee their safety and efficacy.

Peptide mapping is used to measure several critical quality attributes (CQA) required for the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and to check for post-translational and chemical modifications.

Mass spectrometry (MS) is coupled to liquid chromatography (LC) for peak identification and confirmation of the sequence. However, many quality control (QC) methods use detection by ultraviolet (UV) absorption only after the peaks identities have been confirmed.[1]

Trypsin is the enzyme most commonly used for proteolytic digestion due to its high specificity. Although a widely accepted technique, in-solution trypsin digestion protocols required for sample preparation are labor intensive and prone to manual errors. These errors affect the quality of the analytical data compromising the ability to reproducibly characterize a protein to the required standard. In the most critical cases where workflows only employ UV detection without confirmation by MS, robust and stable sample preparation and separation methods are critical. The digestion must be reproducible and chromatography must be extremely stable to allow unambiguous peptide identification based on chromatographic retention time.

This work details the automated peptide mapping of cytochrome c, recombinant somatotropin, and infliximab drug product. These proteins were chosen to investigate the applicability and reproducibility of the automated digestion protocol and subsequent analysis. The combination of the Thermo Scientific™ SMART Digest™ magnetic beads and the Thermo Scientific™ KingFisher™ Duo purification system was used to automate the digestion process to produce high quality, reproducible peptide mapping data.

Magnetic beads are a proven support used for many purification and sample preparation approaches in life science research and biotechnology. The KingFisher purification system enables robotic handling and easy automation of any magnetic bead based application resulting in superior performance and reproducibility.[2]

The Thermo Scientific™ Vanquish™ Horizon UHPLC system was subsequently used to analyze the samples by UHPLC-UV and, additionally, coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer for MS confirmation of the peptide sequence.

Experimental

Consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ HPLC grade water (P/N 10449380)
- Fisher Scientific LC/MS grade acetonitrile (P/N 10489553)
- Fisher Scientific™ Optima™ LC/MS grade water with 0.1% formic acid (P/N 10429474)
- Fisher Scientific Optima LC/MS grade acetonitrile with 0.1% formic acid (P/N 10468704)
- Fisher Scientific Optima LC/MS trifluoroacetic acid (P/N 10125637)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- SMART Digest Trypsin Kit, with filter/collection plate (P/N 60109-102)
- KingFisher Deepwell, 96 well plate (P/N 95040450)

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

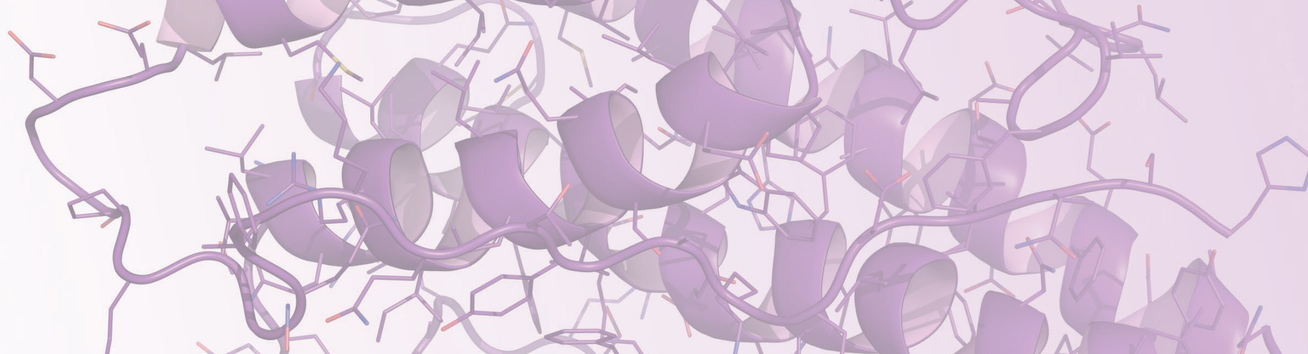
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations



- KingFisher Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Hypersil GOLD™ column 3 µm, 2.1 × 150 mm (P/N 25003-152130)

Equipment

- KingFisher Duo Prime Purification System (P/N 5400110)
- Thermo Scientific™ Hypersep™ 96 well Positive Pressure System (P/N 60103-357)
- Vanquish Horizon UHPLC System including:
  - Binary Pump H (P/N VH-P10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
  - System Base Vanquish Horizon (P/N VH-S01-A)
- MS Connection Kit Vanquish (P/N 6720.0405)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (P/N IQLAAEGAAPFALGMBDK)

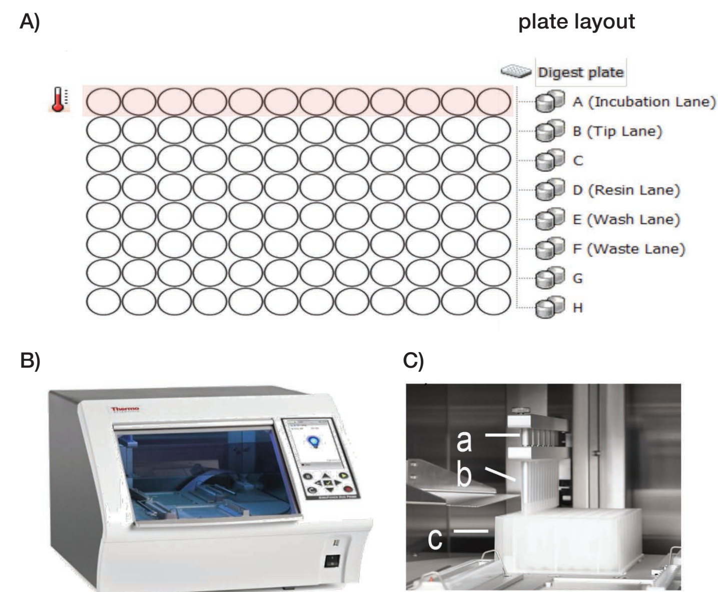
Sample preparation

- Lyophilized powder of cytochrome c, carbonic anhydrase, and recombinant somatotropin were dissolved in deionized water and adjusted to a final concentration of 10 mg/mL.
- Infliximab and rituximab drug product was reconstituted in water to a concentration of 10 mg/mL with gentle swirling to aid in solubilization as directed from the manufacturer's product insert information.

SMART Digest, manual digestion protocol

The comparison of the manual and automated SMART Digest protocol was conducted with somatotropin and rituximab using 100 µg recombinant protein per digestion reaction.

- Proteins were adjusted to 2 mg/mL with deionized water.
- The solution was further diluted 1:4 with the SMART Digest buffer.
- 200 µL of this solution was directly transferred to each reaction tube (containing 15 µL of the SMART Digest standard resin slurry).
- Digestion was conducted in a heater/shaker at 70 °C, 1200 rpm (to prevent sedimentation of the immobilized trypsin beads).



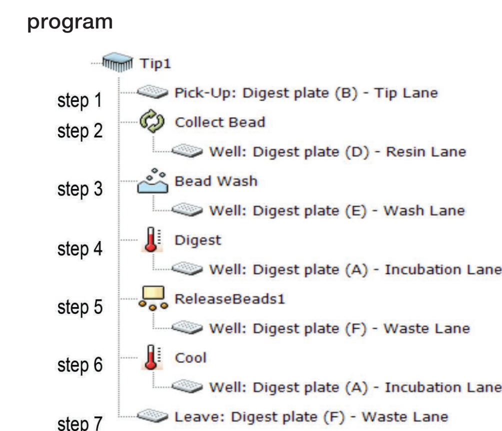


Figure 1. Automated SMART Digest configuration using the KingFisher Duo Prime purification system. • A) Schematic of plate layout and digestion program.• B) The KingFisher Duo Prime Purification System.• C) Robotic handling compartment: 12 magnetic rods (a), disposable comb tip (b), 96 DW plate (c).

- Digestion incubation times of 15 minutes for somatotropin and 45 minutes for rituximab were used as optimal times to ensure complete digestion of each protein in the shortest time.
- Immobilized resin was removed by filtration with the filtration plate provided with the SMART Digest Kit using a positive pressure manifold.

Magnetic SMART Digest, automated digestion protocol

The KingFisher Duo Prime purification system was used to automate the protein digestion. Digests of infliximab, somatotropin, cytochrome c, and carbonic anhydrase were carried out.

- SMART Digest magnetic resin slurry was diluted and uniformly suspended in SMART Digest buffer to create a suspension of 15 µL original resin into 100 µL of buffer in each well of the dedicated "resin lane" of a KingFisher Deepwell 96 well plate.
- 200 µL of 1:4 diluted SMART Digest buffer was prepared in each well of a separate row of the plate as the optional wash buffer.
- 50 µL of the sample solution was diluted into 150 µL of SMART Digest buffer in the dedicated "incubation lane" that allows for heating and cooling (row A).
- Thermo Scientific™ BindIt™ software (version 4.0) was used to control the KingFisher Duo Prime system with the program outlined in Figure 1 and Tables 1 and 2.
- The digestion step was completed at 70 °C.
- Sedimentation of beads was prevented by repeated insertion of the magnetic comb using the mixing speed setting "medium".
- An incubation time of 15 min for somatotropin, 20 min for cytochrome c, and carbonic anhydrase with 45 min for infliximab were used as optimal times to ensure complete digestion of each protein in the shortest time.
- Immediately after incubation, the magnetic beads were collected and removed from the reaction and the digest solution was actively cooled to 15 °C.

Table 1. Plate layout showing the volumes and solutions in each well.

Lane	Content	Volume (µL)
A	Buffer	150
	Sample	50
B	Tip Comb	
D	Beads	15
	Bead Buffer	100
E	Bead Wash Buffer	200
F	Waste Lane	250

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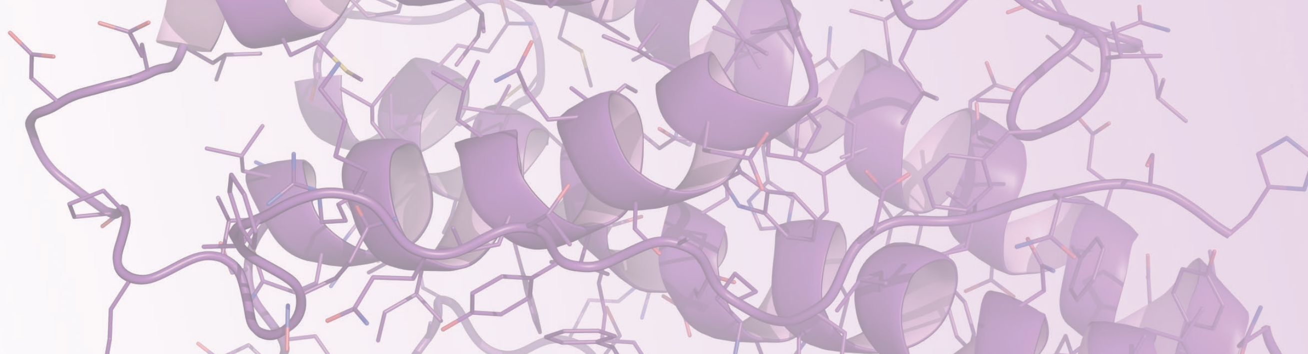

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Table 2. Protocol step details.

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	8 min 30 s Medium Mix	3 count, 15 s	70 °C heating while mixing 5 °C post temp.	A
Release Beads	Yes, Fast	–	–	–	F

UHPLC-UV separation conditions - cytochrome c, infliximab, rituximab, and carbonic anhydrase

Column: Hypersil GOLD 1.9 µm, 2.1 × 150 mm
Mobile phase A: Water + 0.05% trifluoroacetic acid
Mobile phase A B: Water/acetonitrile/trifluoroacetic acid (20:80:0.04 v/v/v)
Flow rate: 0.5 mL/min
Column temperature: 70 °C (still air mode)
Injection volume: 5 µL
UV wavelength: 214 nm
Gradient: Table 3

Table 3. Mobile phase gradient.

Time (min)	%A	%B	Flow (mL/min)	Curve
0.0	95	5	0.5	5
15.0	45	55	0.5	5
15.1	0	100	0.5	5
17.0	0	100	0.5	5
17.1	95	5	0.5	5
22	95	5	0.5	5

Data processing and software

Chromatographic software: Thermo Scientific™ Chromeleon™ CDS 7.2 SR4

UHPLC-UV and UHPLC-MS separation conditions - somatotropin and infliximab

Columns: Hypersil GOLD 1.9 µm, 2.1 × 150 mm
Mobile phase A: Water + 0.1% formic acid
Mobile phase B: Acetonitrile + 0.1% formic acid
Flow rate: 0.3 mL/min
Column temperature: 70 °C (still air mode)
Injection volume: 5 µL
UV wavelength: 214 nm
Gradient: Table 4

Table 4. Mobile phase gradient.

Time (min)	%A	%B	Flow (mL/min)	Curve
0.0	96	4	0.3	5
30	25	75	0.3	5
30	0	100	0.3	5
35	0	100	0.3	5
35	96	4	0.3	5
45	96	4	0.3	5

MS conditions

The Q Exactive Plus mass spectrometer equipped with a HESI-II probe was used for mass spectrometric detection using a full MS / dd-MS2 (Top5) experiment.

Ionization: HESI Positive ion
Scan range: 140 to 2000 *m/z*
Source temperature: 350 °C
Sheath gas pressure: 45 psi
Auxiliary gas flow: 10 arb
Spray voltage: 3.4 kV
Capillary temperature: 320 °C
Resolution (Full MS) at *m/z* 200 (FWHM): 70,000
Resolution (MS2) at *m/z* 200 (FWHM): 17,500
Top-N MS2: 5
S-lens RF level: 60
Max inject time: 100 ms
Collision energy (CE): 27

Results and discussion

The applicability of the automated protein digestion with the KingFisher Duo Prime purification system was tested with cytochrome c and carbonic anhydrase. Replicate digests were conducted and the generated peptides were separated and analyzed by UHPLC-UV. The corresponding peptide maps are shown as an overlay in Figure 2. Both cytochrome c and carbonic anhydrase were readily digested using the automated SMART Digest Kit protocol resulting in complete digestion of the proteins. An average RSD for relative peak area of 2.08% was achieved for the peaks annotated with cytochrome c; several of these peaks had peak area RSD values of 1% and below. Carbonic anhydrase gave similar highly reproducible results with an average area RSD value of 1.8.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
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Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
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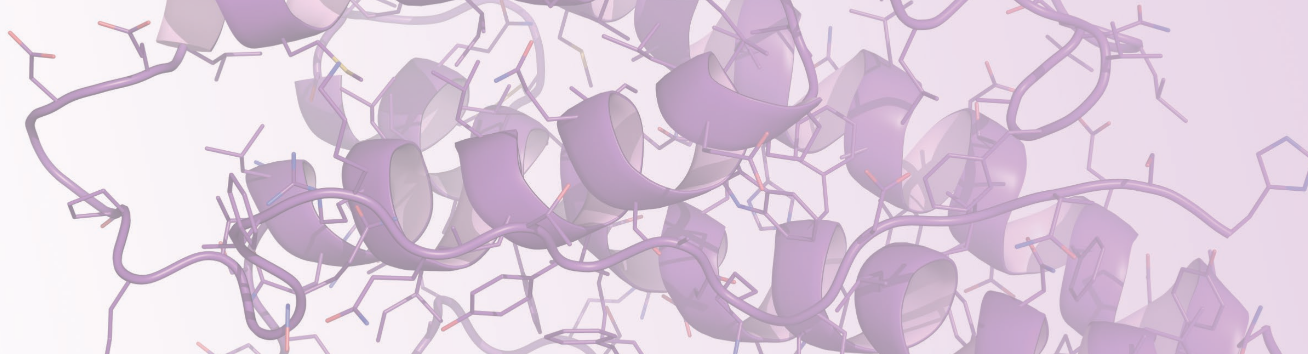
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



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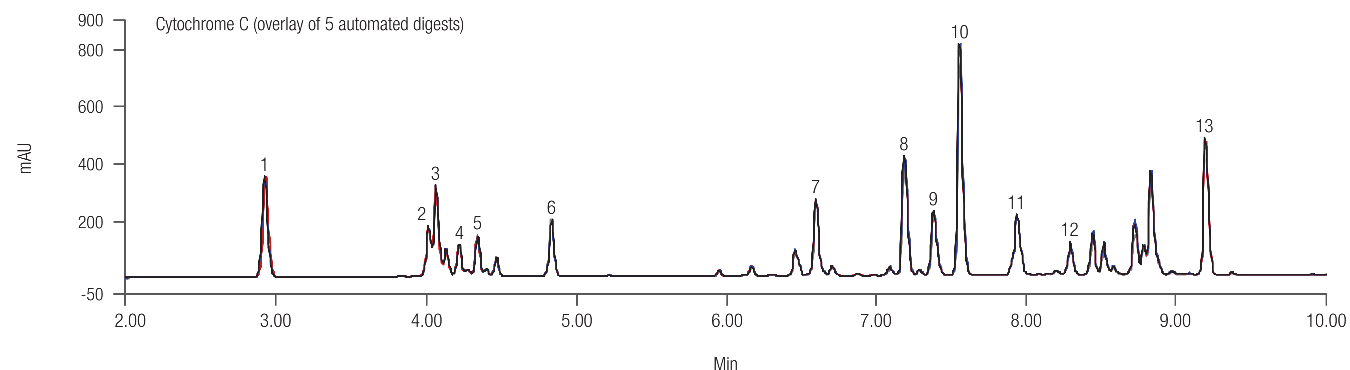
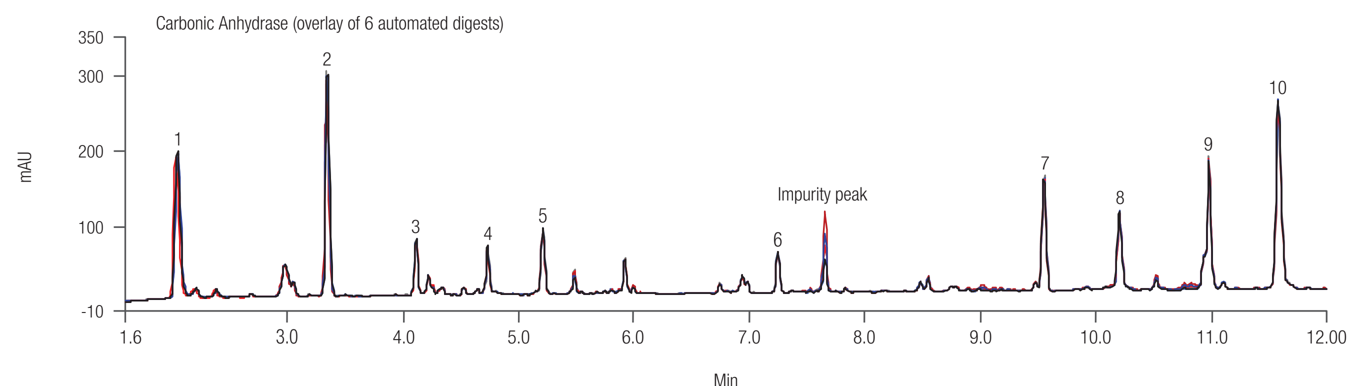


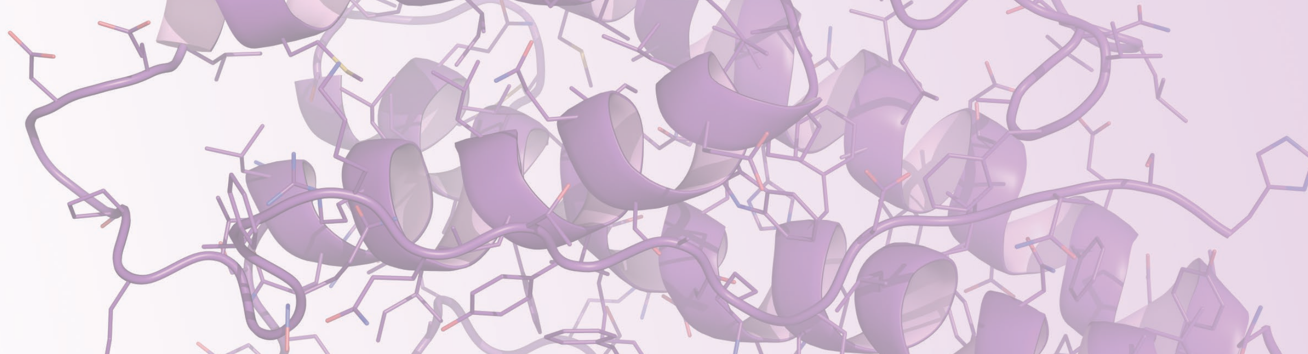
Peak	1	2	3	4	5	6	7	8	9	10	11	12	13
%RSD (A_{rel})	2.75	1.87	2.45	0.71	1.27	1.90	3.60	2.09	2.35	3.92	1.11	0.72	2.42
%RSD (t_R)	0.12	0.03	0.05	0.04	0.03	0.02	0.03	0.03	0.03	0.02	0.01	0.01	0.01



Peak	1	2	3	4	5	6	7	8	9	10
%RSD (A_{rel})	2.12	1.46	1.53	3.03	1.71	1.26	1.51	2.60	1.14	1.64
%RSD (t_R)	0.32	0.1	0.03	0.04	0.02	0.02	0.01	0.01	0.01	0.01

Figure 2. Automated digestion of cytochrome c and carbonic anhydrase using SMART Digest magnetic resin with the KingFisher Duo Prime system. Overlaid peptide maps of different digests of cytochrome c (upper panel) and carbonic anhydrase (lower panel). Digest solutions of 5 µL were injected without further purification and peptides were separated using separation condition A. %RSD values for relative peak area (upper) and retention time (lower) and are given for the peaks indicated.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



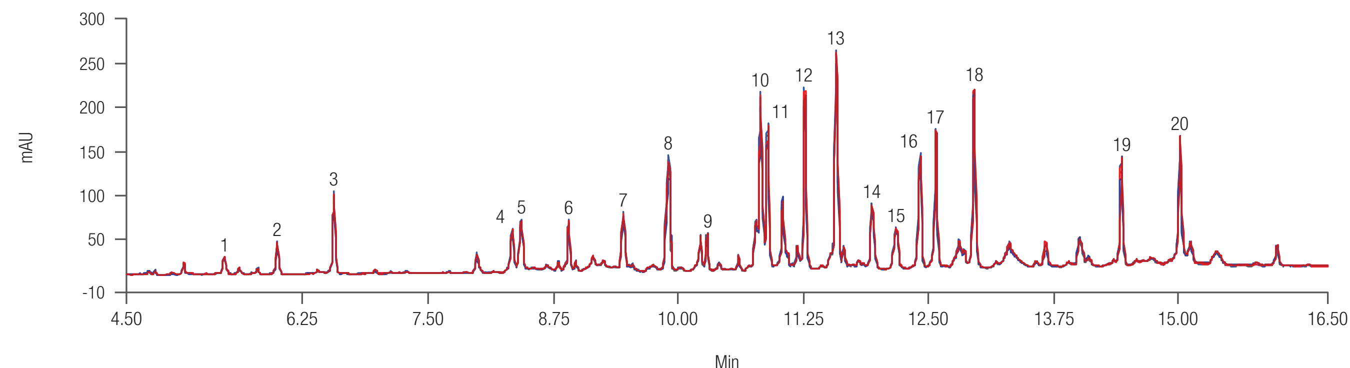
This level of reproducibility can be visualized by the high consistency of the Vanquish Horizon UHPLC system gradients and injection accuracy, which gives identical chromatography and makes integration and interpretation of the peaks easier. This level of reproducibility in protein digestion has never been reported before so the degree of influence between different users was characterized.

To assess the robustness and ease of use of the SMART Digest Kit protocol in general between different users, an experiment was performed during a protein chromatography workshop with five different people performing a manual digestion using the SMART Digest Kit, some of whom had never performed a protein digestion before. The results of this experiment are shown in Figure 3.

The results show an average RSD value for peak area of 2.74 over 20 different peaks in a complex chromatogram. Considering that this result was achieved from a protein digestion of a large monoclonal antibody performed by five people, the robustness of the protocol between different users is very apparent. The ease of use is also demonstrated in that some of the digestions were done by people who have no experience with protein digestion techniques.

The new SMART Digest Kit automated protocol was further evaluated by LC-MS using infliximab drug product as a test sample.

A 45 min incubation at 70 °C enabled the complete digestion of the infliximab antibody and resulted in a close to identical UV peptide map of the two parallel digestion reactions (Figure 4, upper panel). Analysis by LC-MS confirmed complete sequence coverage of 100% for both the light and heavy chain of the antibody (Figure 4, lower panel). This result demonstrates reproducible, complete digestion of infliximab, and with the additional reproducibility studies, that the SMART Digest Kit when automated is readily applicable for the characterization and quality control of modern bio-pharmaceuticals.



Peak	1	2	3	4	5	6	7	8	9	10
%RSD (A_{rel})	2.54	2.41	1.89	3.39	3.53	2.16	4.41	2.10	2.10	3.65
Peak	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
%RDS (A_{rel})	1.96	3.5	3.72	2.26	2.91	1.97	3.28	2.62	3.16	1.20

Figure 3. Manual digestion of rituximab performed by 5 different people. Overlaid peptide maps of the monoclonal antibody rituximab. 5 µL of digest solutions were injected without further purification and peptides were separated using the gradient and conditions in Table 1. Percentage relative standard deviation (%RSD) values for relative peak area are given for the peaks indicated.

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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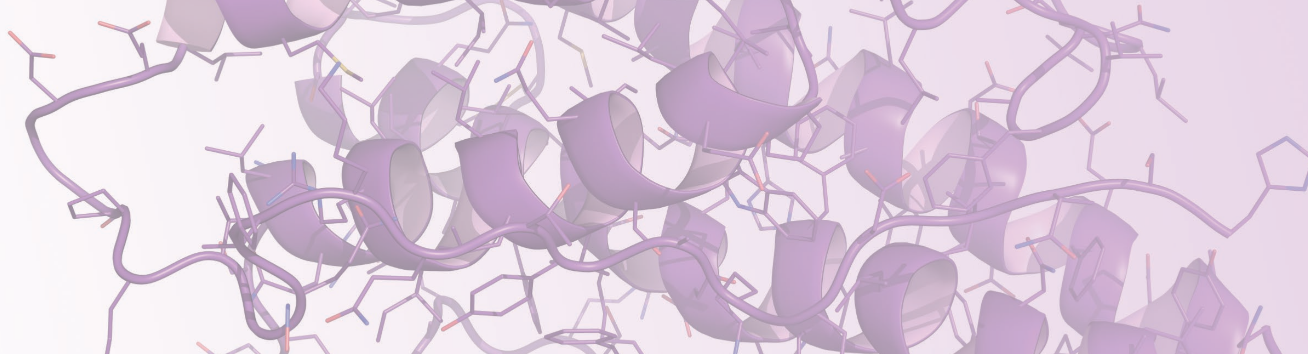
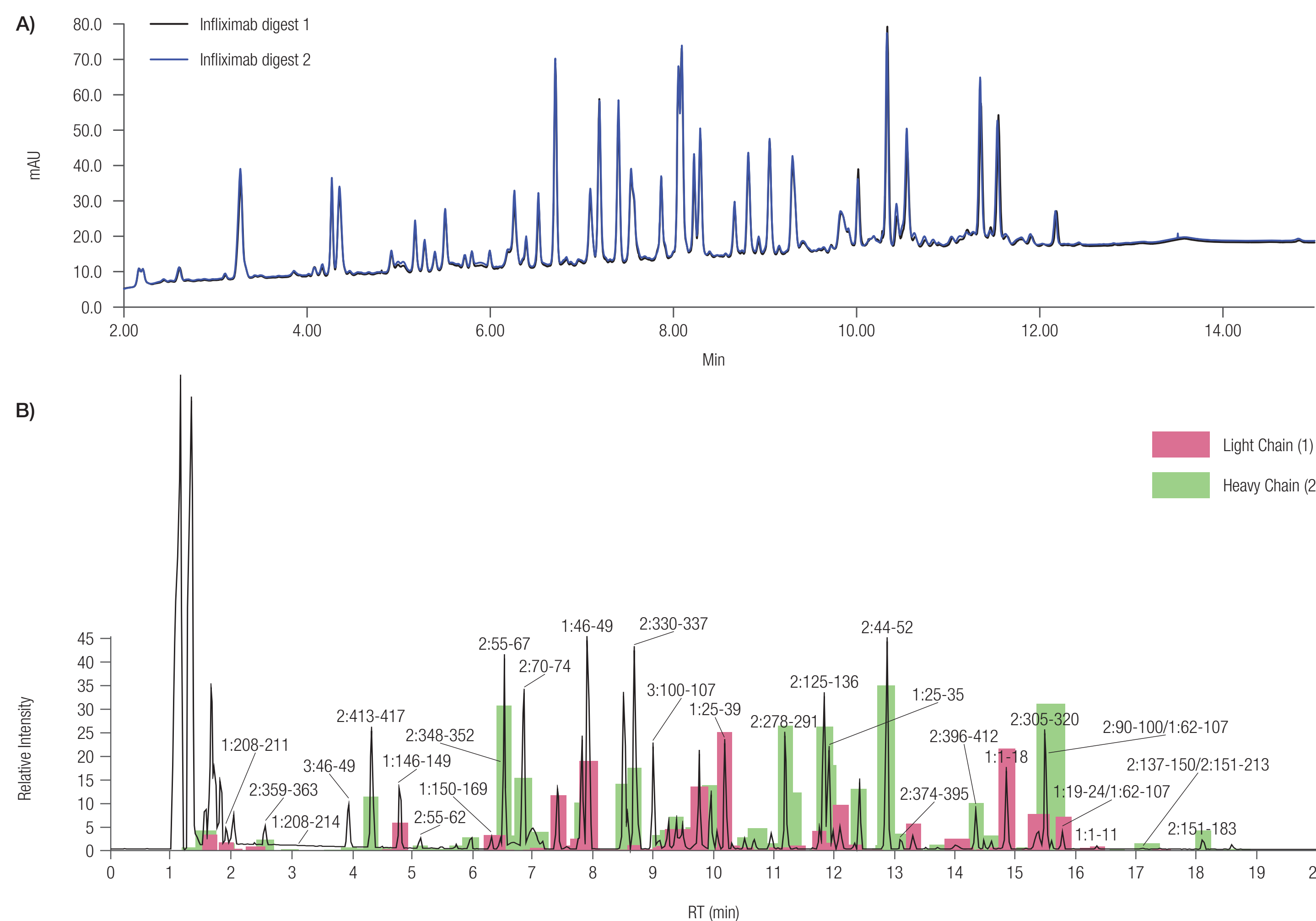
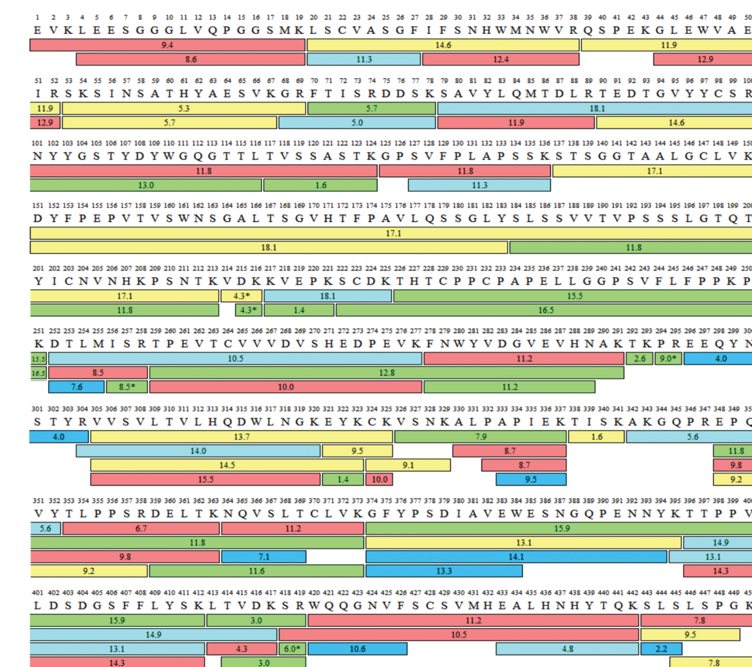
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations







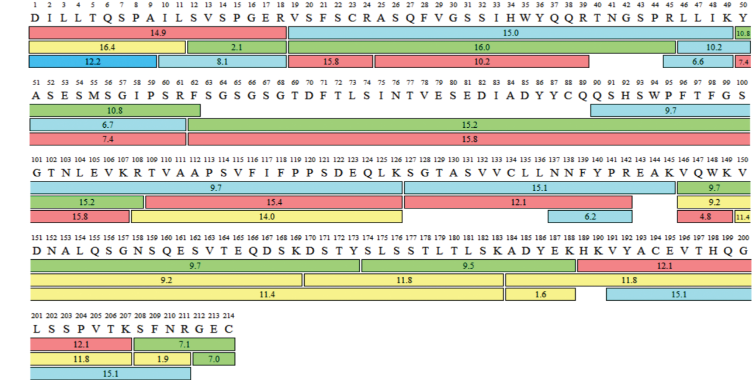


Figure 4. Automated digestion of infliximab drug product using SMART Digest magnetic resin. Panel A: Overlaid peptide maps for two digests of infliximab antibody. Digest solutions of 5 µL were injected without further purification and peptides were separated using separation conditions listed in Table 3. Panel B: Total ion chromatogram from infliximab indicating the peptide origin to light (1) and heavy chain (2). Position numbers are given together with the peptide chain annotation, the heavy chain in green and the light chain in red highlights. Panel C: Sequence coverage map of the automated infliximab using the SMART Digest magnetic kit. Lines containing peptides with signal intensity > 4.3 e5 are shown.

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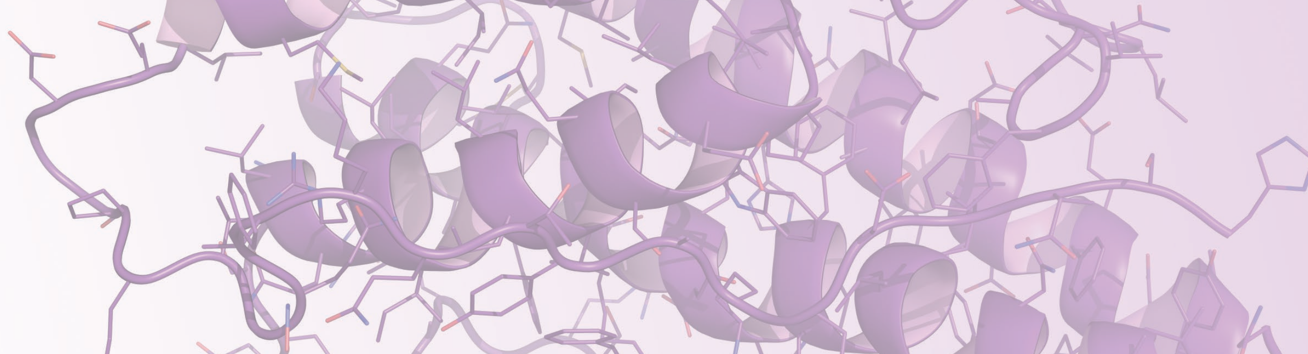

A direct comparison of the standard and the SMART Digest magnetic resins and protocols were conducted using recombinant somatotropin in quadruplicate digestions with LC-MS-UV. The MS data was used to ensure that the digest conditions used were optimal in both the manual and the automated protocols. The sequence coverage using both digestion methods showed 100% sequence coverage, and the identified peptides showed complete digestion had been achieved for both digestions [data not shown]. Identical UV peptide patterns were generated with both digestion approaches. However, for a more qualitative comparison, the major peaks from the UV data were examined more closely with linear regression (Figure 5).

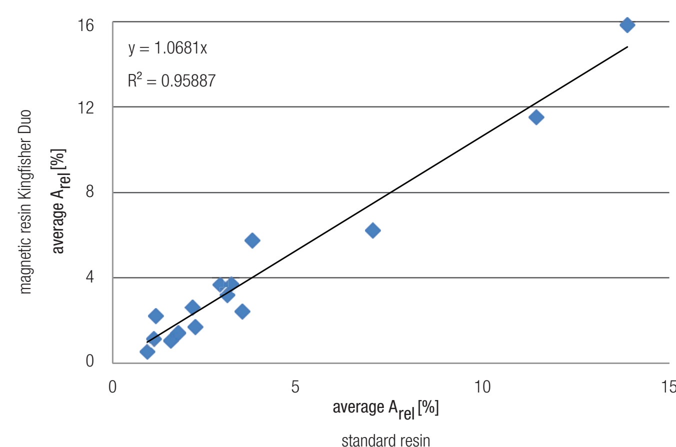

Figure 5. Linear regression curves comparing the peak area results from an automated digestion using the SMART Digest magnetic kit to a manual digestion using the SMART Digest Kit.

Correlation of the relative peak areas observed for the different digestion methods leads to linear regression curves with a slope of 1, indicating the equivalence of the obtained digestion results in both cases.

Comparison of the average variance between the digest replicates demonstrates the benefit of automation for the reproducibility of the digestion results. The pre-aliquoted standard SMART Digest Kit, although already shown to give good reproducibility in Figure 3, resulted in higher relative standard deviations for absolute peak area and peak height compared to results from the SMART Digest magnetic resin kit when automated. The use of the KingFisher Duo Prime system for automation resulted, on average, in 1.5 times less variance (% RSD) compared to the manually processed standard resin.

Conclusions

We have studied in detail two versions of the SMART Digest Kit.

- The manual method has shown to be reproducible, robust, and efficient even in the hands of multiple users with varying experience.

- The combination of the SMART Digest magnetic resin with the KingFisher automation system minimizes the manual handling required for protein digestion. It also ensures that the timing of the reactions are perfect for each sample and reduces the time at which the proteins and peptides are exposed to elevated temperatures, reducing the possibility of post translational modifications to a minimum. This yields a further increase in reproducibility of the obtained digestion results from that already seen with the manual SMART Digest Kit protocols.

- Several proteins have been used in this work to emphasize the more global applicability of the method. It should be noted that the digestion times for each of these proteins were different. This is dependent on the heat stability of the target protein to be digested and as such each protein to be studied should have the time of digestion optimized. The digestion should be long enough to obtain complete digestion of the protein into peptides with stable peak areas, but not longer than necessary, to avoid the slow build-up of some possible post translational modifications.

- Digestion of two monoclonal antibody biotherapeutics was readily achieved with outstanding reproducibility, creating a peptide map that covers the entire amino acid sequence of both chains.

- The combination of this automated digestion process with the class leading retention time stability offered by the Vanquish UHPLC systems[5,6] provides a truly robust and stable peptide mapping workflow for the detailed characterization of modern biotherapeutics.

- The workflow is equally suitable for the in-depth product characterization that becomes possible with modern HRAM Orbitrap mass spectrometry systems or a quality control approach that relies on UV absorbance and pattern recognition only.

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6. Thermo Scientific Application Note 1132, Reliable Results in Peptide Mapping Using the Vanquish Flex UHPLC System (2015), Thermo Fisher Scientific Germering, Germany.

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations
Find out more...

Automated chymotrypsin peptide mapping of proteins by LC-MS

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Application benefits

- An easily automated proteolytic digestion using chymotrypsin
- Orthogonal digestion to trypsin
- High reproducibility from a robust protocol and automation

Goal

To demonstrate an easy-to-use, robust, high-precision, automated approach to a chymotrypsin peptide mapping characterization workflow that provides alternative digestion selectivity to trypsin digestion. Additionally, to show confirmation of a peptide map with high sequence coverage using high-resolution, accurate-mass mass spectrometry. To further display that it is possible to develop a highly reproducible, automated digestion procedure for use in high level characterization of biotherapeutic proteins.

Introduction

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biotechnological production, there are many attributes that need to be analyzed to guarantee their safety and efficacy.

Peptide mapping is used to measure several critical quality attributes (CQAs) required for the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and to check for post-translational and chemical modifications (PTMs). Mass spectrometry (MS) is coupled to liquid chromatography (LC) for peak identification and sequence confirmation.

Trypsin is the most commonly used proteolytic digestion enzyme due to its high specificity for cleavage at arginine and lysine residues. However, there are proteins that do not digest well with trypsin due to too many or too few of the specific trypsin cleavage sites in the sequence. There are also cases where the cleavage sites can be too close together, producing very short hydrophilic tryptic peptides that do not retain on the reversed-phase HPLC columns and are therefore difficult to detect.

Chymotrypsin is a protease that has alternative cleavage specificity to trypsin. Chymotrypsin will cleave primarily at the hydrophobic aromatic amino acid residues of tryptophan, tyrosine, and phenylalanine. However, chymotrypsin is also known to additionally cleave at other sites such as leucine, histidine, and methionine, but with a lower level of specific activity. The consequence of chymotrypsin's selectivity is that the aromatic amino acids are among the first peptide bonds to be broken with other sites being cleaved at a slower rate.

Unlike trypsin, which has a high specificity for only two amino acid residues, the chymotrypsin digestion pattern alters as the digestion time increases due to a slower rate of activity at its alternative digestion sites. Unless the time of digestion is carefully controlled, errors can occur that will compromise the ability to reproducibly characterize a protein to the required standard. This is especially true where workflows only employ UV detection without peptide confirmation by MS. The digestion must be reproducible and chromatography must be extremely stable to allow unambiguous peptide identification based on chromatographic retention time.

This work details the automation of a chymotrypsin digestion of recombinant somatotropin as a model protein. The applicability and reproducibility of an automated chymotrypsin digestion protocol and subsequent analysis was investigated. In addition, this work also shows the effects of digestion time on chymotrypsin activity.

Magnetic beads are a proven support used for many purification and sample preparation approaches in life science research and biotechnology. The Thermo Scientific™ KingFisher™ Duo purification system enables robotic handling and easy automation of any magnetic-bead-based application resulting in superior performance and reproducibility. The combination of Thermo Scientific™ Magnetic SMART Digest™ beads and the KingFisher Duo purification system was used to automate the digestion process to produce high quality, reproducible peptide mapping data using an alternative protease.

The Thermo Scientific™ Vanquish™ Horizon UHPLC system was subsequently used to analyze the samples and coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer for MS confirmation of the peptide sequence.

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
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Product and method development considerations

Experimental

Consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ HPLC grade water (P/N 10449380)
- Fisher Scientific™ LCMS grade acetonitrile (P/N 10489553)
- Fisher Chemical™ Optima™ LC/MS grade water with 0.1% formic acid (v/v) (P/N 10429474)
- Fisher Chemical™ Optima™ LC/MS grade acetonitrile with 0.1% formic acid (v/v) (P/N 10468704)
- Fisher Chemical™ Optima™ LC/MS trifluoroacetic acid (P/N 10125637)
- SMART Digest Chymotrypsin Kit, Magnetic Bulk Resin option (P/N 60109-104-MB)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 UHPLC column, 2.1 × 250 mm (P/N 074812-V)

Equipment

- KingFisher Duo Prime purification system (P/N 5400110)
- Thermo Scientific™ Hypersep™ 96 well positive pressure system (P/N 60103-357)
- Vanquish Horizon UHPLC system, including:
  - Binary Pump H (P/N VH-P10-A)
  - Column Compartment H with (P/N VH-C10-A)
    - Active Pre-heater VH-C1 (P/N 6732.0110)
    - Post-column Cooler 1 µL VH-C1 (P/N 6732.0510)
  - Split Sampler HT (P/N VH-A10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
- MS Connection Kit Vanquish (P/N 6720.0405)
- Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (P/N IQLAAEGAAPFALGMBDK)

Sample preparation

Lyophilized powder of recombinant somatotropin was dissolved in deionized water and adjusted to a final concentration of 10 mg/mL.

Magnetic SMART Digest, automated digestion protocol

- Magnetic SMART Digest resin slurry was diluted and uniformly suspended in SMART Digest buffer to create a suspension of 15 µL original resin into 100 µL of buffer in each well of the dedicated “resin lane” of a KingFisher Deepwell 96 well plate (96 DW plate).
- 200 µL of 1:4 diluted SMART Digest buffer was prepared in each well of a separate row of the plate as the optional wash buffer.
- 50 µL of the sample solution was diluted into 150 µL of SMART Digest buffer in the dedicated “incubation lane” that allows for heating and cooling (row A).
- Thermo Scientific™ BindIt™ software (version 4.0) was used to control the KingFisher Duo Prime with the program outlined in Figure 1.
- The digestion step was completed at 70 °C.
- Sedimentation of beads was prevented by repeated insertion of the magnetic comb using the mixing speed setting “medium”.
- Incubation times for somatotropin are shown in the text; 15 minutes was used as the optimal time to ensure complete digestion of the protein in the shortest time period.
- Immediately after incubation the magnetic beads were collected and removed from the reaction, and the digest solution was actively cooled to 15 °C.

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

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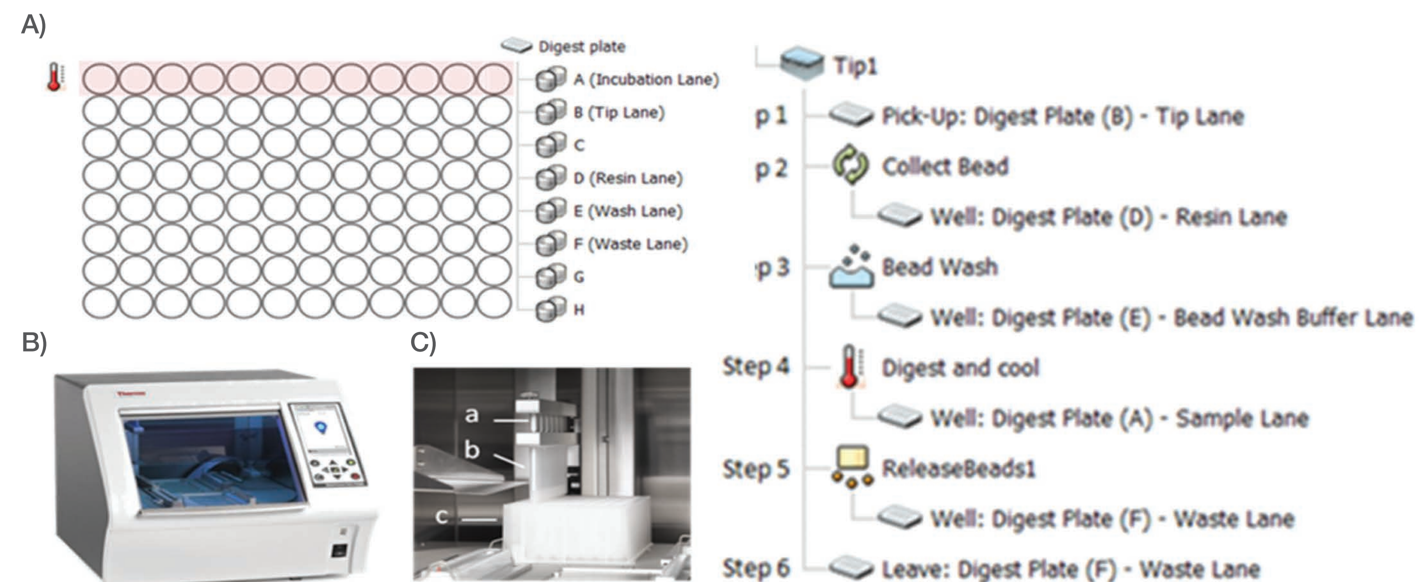
Peptide Quantitation

Product and method development considerations

Figure 1. Automated SMART Digest configuration using the KingFisher Duo Prime purification system

A) Schematic of plate layout and digestion program.
B) The KingFisher Duo Prime Purification System.
C) Robotic handling compartment: 12 magnetic rods (a), disposable comb tip (b), 96 DW plate (c)
D) Plate layout showing the volumes and solutions in each well
E) Protocol step details



D) Plate Layout:

Lane	Content	Volume (µL)
A	Buffer	150
	Sample	50
B	Tip Comb	
D	Beads	15
	Bead Buffer	100
E	Bead Wash Buffer	200
F	Waste Lane	250

E) Protocol Step Details:

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	8 min, 30 s Medium Mix	3 count, 15 s	70 °C heating while mixing 5 °C post temperature	A
Release Beads	Yes, Fast	–	–	–	F

UHPLC-MS separation conditions

Column:	Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.2 µm, 2.1 × 250 mm
Mobile phase:	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow rate:	0.3 mL/min
Column temperature:	70 °C (still air mode)
Post column cooler:	50 °C
Injection volume:	5 µL
UV wavelength:	214 nm
Gradient	Table 2

Table 2. Mobile phase gradient.

Time (min)	%A	%B	Flow (mL/min)	Curve
0.0	96	4	0.3	5
30	25	75	0.3	5
30	0	100	0.3	5
35	0	100	0.3	5
35	96	4	0.3	5
45	96	4	0.3	5

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

MS conditions

The Q Exactive Plus mass spectrometer equipped with a HESI-II probe was used for mass spectrometric detection using a full MS / dd-MS2 (Top5) experiment.

Ionization:	HESI Positive ion
Scan range:	140 to 2000 *m/z*
Source temperature:	350 °C
Sheath gas pressure:	45 psi
Auxiliary gas flow:	10 Arb
Spray voltage:	3.4 kV
Capillary temperature:	320 °C
Resolution [fullMS/MS2]:	70,000/17,500
Top-N MS2:	5
S-lens RF level:	60
Max inject time:	100 mS
Collision energy:	27 eV

Data processing and software

MS data acquisition	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
Analysis	Thermo Scientific™ BioPharma Finder™ 2.0 software

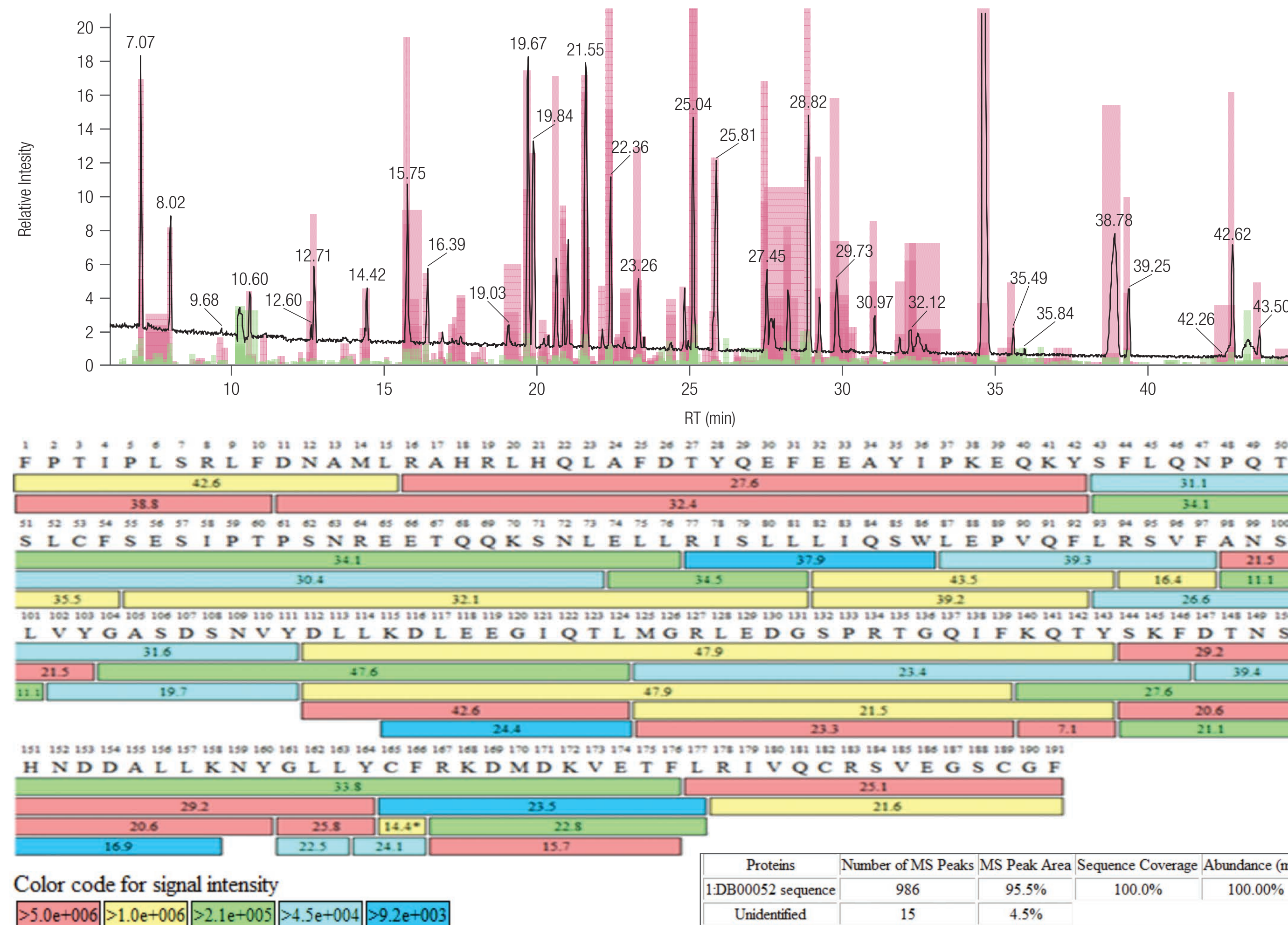
Results and discussion

The applicability of the automated protein chymotrypsin digestion with the KingFisher Duo Prime purification system was tested with somatotropin. Three replicate digests were conducted for 15 minutes and the generated peptides were separated and analyzed by UHPLC-MS. The corresponding peptide map is shown in Figure 2 with the sequence coverage at 100%.

Somatotropin was readily digested using the automated SMART Digest protocol with chymotrypsin, resulting in complete digestion of the protein. The magnetic SMART chymotrypsin cleaves efficiently at the aromatic amino acids, tyrosine, phenylalanine, and tryptophan, with no missed cleavages observed with somatotropin; however, there are also a significant number of cleavages after some leucine residues.

Extra cleavages increase with longer incubation times to eventually include all the leucine residues, some methionine, histidine, and even an arginine residue (Figure 3). This produces an increasingly complex pattern with more peptides than required for full coverage. Care must be taken when optimizing the length of digestion time to avoid this. This can easily be achieved however because of the reproducibility of the automated SMART digestion process, which allows for precise timing and therefore reproducible digestion. This is more difficult to achieve with traditional in-solution digests.

The increasing number of peptides released during a digestion time course is depicted in Figure 3 over a 30 minute time period for the C-terminus of somatotropin. Initial cleavage is restricted to sites following aromatic amino acid residues. However, at 15 minutes digestion time, cleavage at lysine residues starts to occur, with less specific cleavage sites appearing at 30 minutes digestion. Automation with the KingFisher Duo Prime purification system allows optimization with a very specific time of digestion, in this case 15 minutes. The number of cleavage sites produced during the digestion can therefore be controlled to suit the protein of interest.



Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:DB00052 sequence	986	95.5%	100.0%	100.00%
Unidentified	15	4.5%		

Figure 2. Sequence coverage of somatotropin with magnetic chymotrypsin digestion. The upper trace shows the base peak chromatogram (BPC) with the sequence coverage underneath. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant > yellow > green > light blue > cyan = low abundant.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

The part of the sequence shown in Figure 3 has two cleavage sites very close together at Y 164 and F 166 producing a short dipeptide (highlighted with a red circle). If a short dipeptide is produced from a trypsin digest, one of the amino acids would be charged [R or K] almost certainly, making the peptide very hydrophilic and not retained on the reversed-phase separation column. The elution in the column void would make the peptide very difficult, if not impossible, to detect. As chymotrypsin cleaves after hydrophobic aromatic amino acids, the peptides are usually retained and detectable with the higher UV absorbance of the aromatic amino acid. The short $C^{165}F^{166}$ peptide can be seen to elute in 14.4 minutes in the peptide gradient shown in Figure 2. This is another advantage with chymotrypsin digestions.

In addition to peptide coverage, reproducibility was investigated over three replicates of the 15 minute digestion. Measurements were made using six selected peptide peaks from the chromatogram. An average percentage relative standard deviation (% RSD) of 3.56% was archived for the peaks annotated in Figure 4. Three of these peak areas had % RSD values of 2.2% and below. This level of reproducibility is enhanced by the consistency of the Vanquish Horizon UHPLC system gradient generation and injection accuracy allowing near identical chromatography, which makes integration and peak interpretation easier.

Figure 3. The controlled appearance of sites of cleavage from SMART chymotrypsin over the C-terminal sequence of somatotropin. A time course of 5, 10, 15, 20, and 30 minutes is shown with increasing numbers of cleavages over time.

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

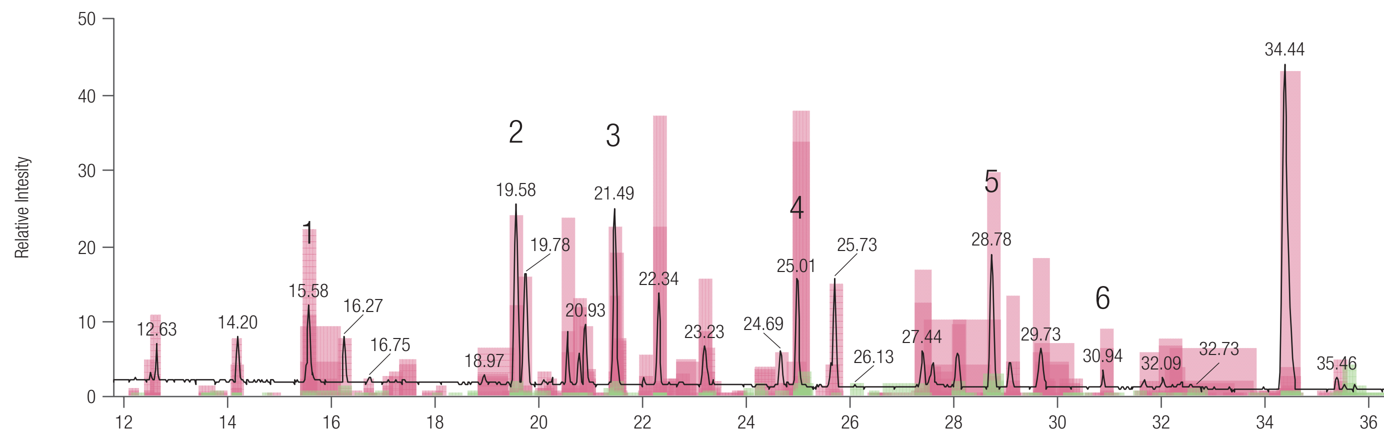
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations



Peak	1	2	3	4	5	6
RSD	6.1%	1.2%	6.6%	3.4%	1.9%	2.2%

Figure 4. Overlaid base peak chromatograms (BPCs) of three replicates showing reproducibility of automated chymotrypsin digestion using magnetic beads. Overlay of n = 3 different digestions.

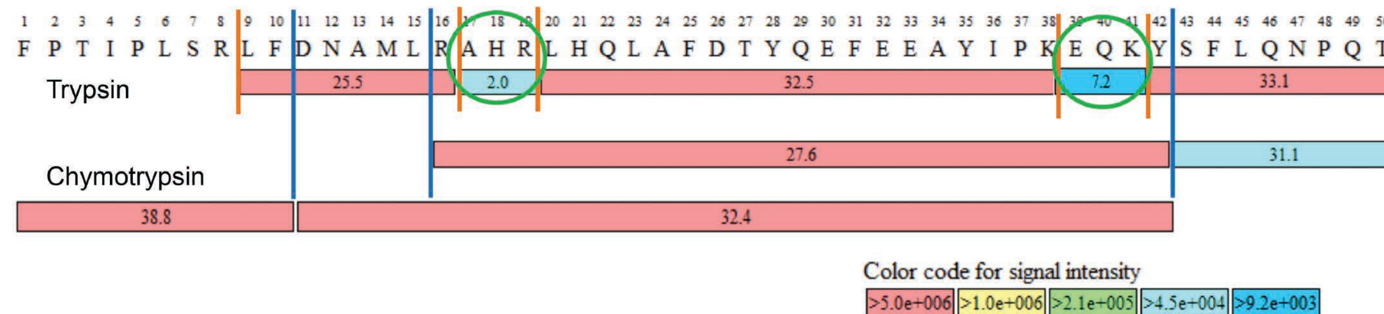


Figure 5. Comparisons of magnetic SMART trypsin and chymotrypsin cleavage over part of the somatotropin sequence. Trypsin cleavage sites are shown with a red line and chymotrypsin cleavage sites are shown with a blue line. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant > yellow > green > light blue > cyan = low abundant.

The complimentary cleavage patterns for trypsin and chymotrypsin allow overlapping sequence analysis, which is useful to confirm parts of the sequence that could be missed through the presence of cleavage sites too close together or too far apart to give good sequence coverage with one enzyme alone. It can also be used to find the correct order of peptides for an unknown protein sequence. Figure 5 shows part of the somatotropin sequence that produces two short peptides (marked with a green circle) following a trypsin digestion that are difficult to detect. In comparison, chymotrypsin digestion produces two longer, high abundant peptides that contain the sequence for both of these short trypsin cleavage peptides, allowing unambiguous sequence coverage with the two digestion protocols.

The results show that an automated chymotrypsin digestion can be easily reproduced with careful optimization of the digestion time. There are instances where the application of an alternative protease activity to the commonly used trypsin can be extremely useful.

Conclusions

- Automated magnetic SMART chymotrypsin digestion can be reproducibly undertaken with ease.
- Optimization of the time the digestion is allowed to proceed is important.
- Reproducibility is further improved with chymotrypsin when automated.
- Chymotrypsin is a viable alternative digestion protocol to trypsin.
- There are useful applications for this alternative digestion enzyme.

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

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

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An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Silvia Millán-Martín, Craig Jakes, Giorgio Oliviero, Sara Carillo, Jonathan Bones Characterisation and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Foster Avenue, Mount Merrion, Blackrock, Co., Dublin, Ireland

Application benefits

- Speed and simplicity of data preparation in addition to automation or highly reproducible peptide mapping results
- Simple and rapid protein digestion for a peptide mapping workflow of different monoclonal antibodies (chimeric, humanized, and fully human) in less than two hours
- Automated magnetic bead technology allows excellent recovery of samples with great reproducibility and efficiency with less hands-on time
- High confidence in results with excellent quality data; excellent coverage and low levels of sample preparation-induced post translational modifications (PTMs)

Goal

Report on the benefits of automated high-throughput trypsin digestion for highly reproducible peptide mapping of five top-selling monoclonal antibodies (chimeric, humanized and fully human) using the magnetic Thermo Scientific™ SMART Digest™ Trypsin Kit as a bulk resin option on the Thermo Scientific™ KingFisher™ Duo Prime purification system. The study focused on reproducibility, protein sequence coverage, and identification of post-translational modifications (PTMs), including deamidation, oxidation, N-terminal pyroglutamination, C-terminal lysine loss, glycation, and glycosylation.

Introduction

Complex glycoproteins, specifically monoclonal antibodies, are currently the most prevalent type of biotherapeutics in development. Monoclonal antibodies (mAbs), which offer high specificity and low side effects, are used to treat many types of cancer, autoimmune and inflammatory diseases, infections, and metabolic disorders, yielding their impressive success as human medicines.[1] These large proteins have molecular weights near 150 kDa and are composed of two identical
~50 kDa heavy chains (HC) and two identical ~25 kDa light chains (LC). They also contain at least 16 disulfide bonds that maintain three-dimensional structure and biological activity. Although they share similar secondary protein structures, different mAbs vary greatly in the sequence of variable regions.

Since the commercialization of the first therapeutic mAb product in 1986, this class of biopharmaceutical products has grown significantly so that, as of December 1st, 2017, seventy-six mAb products have been approved in the US or Europe for the treatment of a variety of diseases, and many of these products have also been approved for other global markets.[2,3] Rituximab (Rituxan®), cetuximab (Erbitux®), bevacizumab (Avastin®), trastuzumab (Herceptin®), and adalimumab (Humira®) were five of the top-ten selling mAbs in 2012.[4]

Post-translational modification (PTM) refers to the covalent and generally enzymatic modification of proteins during or after protein biosynthesis. Proteins are synthesized by ribosomes translating mRNA into polypeptide chains, which may then undergo PTM to form the mature protein product. PTMs are important components in cell signaling and can occur on the amino acid side chains or at the protein's C- or N- termini. They can extend the chemical repertoire of the 20 standard amino acids by modifying an existing functional group or introducing a new one. PTMs affect structural and functional aspects of therapeutic proteins, and the effects can be detrimental, that is, heterogeneity and immunogenicity, even though the modification may originally be required for functional activity of the polypeptide.[5]

Most therapeutic proteins approved or in development bear at least one or more PTMs. Variants of proteins produced for medicinal purposes can occur during manufacturing, handling, and storage, and can impact the activity and stability of the biotherapeutic. Their characterization is challenging due to their size and inherent heterogeneity caused by PTMs, among which glycosylation is probably the most prominent.[6,7]

Peptide mapping is used routinely to study PTMs and is capable of pinpointing the amino acid residue within the sequence at which the modification has occurred. Peptide mapping is commonly used in the biopharmaceutical industry to establish product identity by confirming the primary structure of a product.[8,9] For recombinant protein pharmaceuticals, peptide mapping is used for the initial "proof of structure" characterization. It confirms expression of the desired amino acid sequence and characterizes any PTM, for subsequent lot-to-lot identity testing, in support of bioprocess development and clinical trials. It is also used as the current method of choice for monitoring the "genetic stability" of recombinant cell lines.

This application note describes a full workflow solution for peptide mapping of five different monoclonal antibodies using the Magnetic SMART Digest resin option on a KingFisher Duo Prime purification system, in combination with the high-resolution, accurate-mass (HRAM) capabilities of the Thermo Scientific™ Q Exactive™ Plus mass spectrometer, and high-resolution chromatographic separation with the Thermo Scientific™ Vanquish™ Flex UHPLC system. Thermo Scientific™ BioPharma Finder™ software was used to interrogate the high-quality data sets.

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
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Experimental

Consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical™) (P/N 10505904)
- Acetonitrile, Optima LC/MS grade (Fisher Chemical) (P/N 10001334)
- Water with 0.1% formic acid (v/v), Optima LC/MS grade (Fisher Chemical) (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Optima LC/MS grade (Fisher Chemical) (P/N 10118464)
- Trifluoroacetic acid (TFA) (Fisher Chemical) (P/N 10294110)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Dithiothreitol (DTT) (Fisher Bioreagents™) (P/N 10386833)
- Iodoacetic acid, sodium salt 99% (IA) (Acros Organics™) (P/N 10235940)
- Thermo Scientific™ KingFisher™ Deepwell plates, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ vial identification system (P/N 60180-VT100)

Equipment

- KingFisher Duo Prime purification system (P/N 5400110)

Vanquish Flex Binary UHPLC system including:

- Binary Pump F (P/N VF-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A)
- System Base Vanquish Horizon (P/N VH-S01-A)

Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (P/N IQLAAEGAAPFALGMBDK)

Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Samples (mAbs)

Five commercially available monoclonal antibodies were supplied at different concentrations (Table 1).

Table 1. Monoclonal antibodies used in the study

Drug	Brand name	Concentration	Type
Bevacizumab	Avastin®	25 mg/mL	Recombinant IgG1 humanized mAb
Cetuximab	Erbitux®	25 mg/mL	Recombinant IgG1 kappa, chimeric murine/human mAb
Adalimumab	Humira®	5 mg/mL	Recombinant fully human mAb
Rituximab	MabThera®	10 mg/mL	Recombinant IgG1 kappa, chimeric murine/human mAb
Trastuzumab	Herceptin®	15 mg/mL	Recombinant IgG1 kappa, humanized mAb

Sample preparation

Monoclonal antibody samples were prepared in triplicate on different days. For cetuximab biotherapeutic nine replicates were prepared by different analysts on different days.

Protocol for sample preparation using a SMART Digest trypsin kit, magnetic bulk resin option (Magnetic SMART Digest)

Samples were diluted to 2 mg/mL in water. For each sample digest, sample and SMART Digest buffer were added to each lane of a KingFisher Deepwell 96-well plate as outlined in Table 2. Bead "wash buffer" was prepared by diluting SMART Digest buffer 1:4 (v/v) in water. Bead buffer was neat SMART Digest buffer. Digestion was performed using Kingfisher Duo Prime purification system with Thermo Scientific™ BindIt™ software (version 4.0), using the protocol outlined in Table 3. Samples were incubated for 45 minutes at 70 °C on medium mixing speed (to prevent sedimentation of beads), with post-digestion cooling carried out to 10 °C. Following digestion, disulfide bond reduction was performed with 10 mM DTT for 30 minutes at 57 °C and subsequently alkylated with 20 mM IA in darkness for 30 minutes. The reaction was quenched with 15.45 µL of 100 mM DTT followed by 15.64 µL 10% TFA (final concentration 11 mM DTT and 1% TFA). Samples were then injected immediately into the LC-MS.

Table 2. KingFisher Duo Prime plate layout utilized for sample preparation. Reagents and associated volumes placed in each well are outlined.

Lane	Content	Volume Applied to Each Well (µL)
A	SMART Digest buffer	150
	Sample (2 mg/mL)	50
B	Tip Comb	
C	Empty	
D	Magnetic SMART Beads	15
	Bead Buffer (SMART Digest buffer)	100
E	(SMART Digest buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

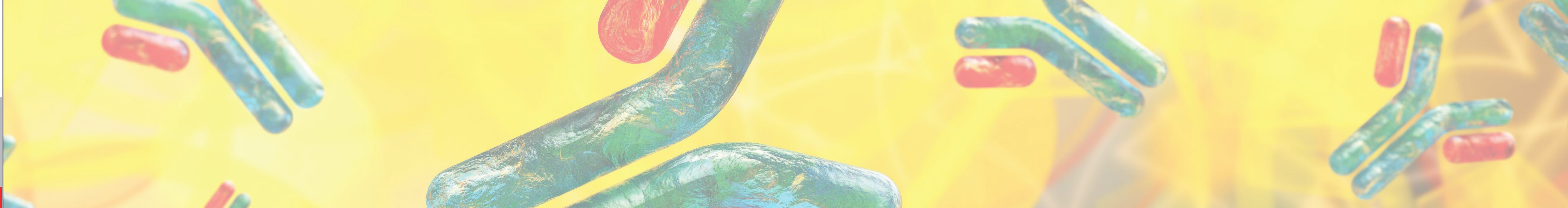


Table 3. Protocol for automated peptide mapping using a KingFisher Duo Prime system

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	45 min Medium Mix	3 count, 15 s	70 °C heating while mixing 10 °C post temperature	A
Release Beads	Yes, Fast	–	–	–	F

LC conditions

Mobile phase A: Water with 0.1% formic acid (v/v)
Mobile phase B: Acetonitrile with 0.1% formic acid (v/v)
Flow Rate: 0.3 mL/min
Column Temperature: 25 °C (Still air mode)
Autosampler Temp: 5 °C
Injection Volume: 10 µL
Injection Wash Solvent: Methanol/water, 10:90 (v/v)
Needle Wash: Enabled pre-injection
Gradient: See Table 4 for details

Table 4. Mobile phase gradient for UHPLC separation of peptides

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

Data processing and software

Acquisition software: Thermo Scientific™ Xcalibur™ software version 4.0 MS data analysis software:
Thermo Scientific™ BioPharma Finder™ version 3.0

MS Conditions

Detailed MS method parameters are shown in Tables 5 and 6.

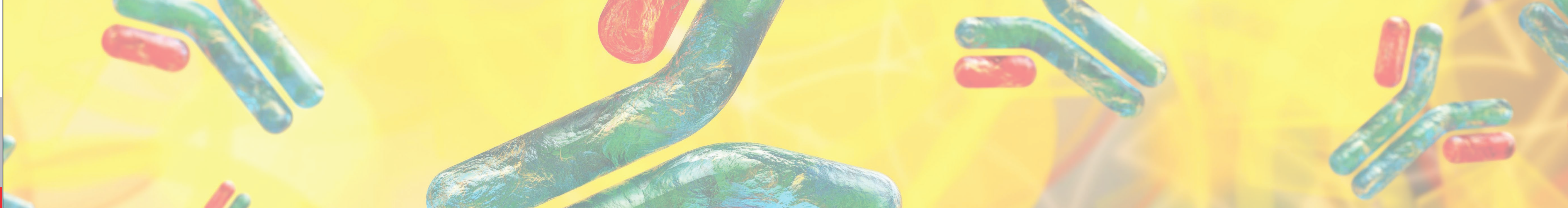
Table 5. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Ion Max source with HESI II probe
Sheath Gas Pressure	40 psi
Auxiliary Gas Flow	10 arbitrary units
Probe Heater Temperature	400 °C
Source Voltage	3.8 kV
Capillary Temperature	320 °C
S-lens RF Voltage	50 V

Table 6. MS method parameters utilized for peptide mapping analysis

General	Setting	MS² Parameters	Setting
Run Time	0 to 65 min	Resolution Settings	17,500
Polarity	Positive	AGC Target Value	1.0×10^5
Full MS Parameters	**Setting**	Isolation Width	2.0 *m/z*
Full MS Mass Range	200–2,000 *m/z*	Signal Threshold	1.0 × 104
Resolution Settings	70,000	Normalized Collision Energy (HCD)	28
AGC Target Value	3.0×10^6	Top-N MS²	5
Max Injection Time	100 ms	Max Injection Time	200 ms
Default Charge State	2	Fixed First Mass	–
SID	0 eV	Dynamic Exclusion	7.0 s
Microscans	1	Loop Count	5

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



MS data processing

Detailed parameter settings are shown in Table 7.

Table 7. Biopharma Finder software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS Signal Threshold	8.0 x 10^4 counts
Typical Chromatographic Peak Width	0.3
Relative MS Signal Threshold (% base peak)	1
Relative Analog Threshold (% of highest peak)	1
Width of Gaussian Filter (represented as 1/n of chromatographic peak width)	3
Minimum Valley to Be Considered as Two Chromatographic Peaks	80.0%
Minimum MS Peak Width (Da)	1.2
Maximum MS Peak Width (Da)	4.2
Mass Tolerance (ppm for high-res or Da for low-res)	4.00
Maximum Retention Time Shift (min)	1.69
Maximum Mass (Da)	30,000
Mass Centroiding Cutoff (% from base)	15
Identification	**Setting**
Maximum Peptide Mass	7,000
Mass Accuracy	5 ppm
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	1
Unspecified Modification	-58 to +162 Da
N-Glycosylation	CHO
Protease Specificity	High
Static Modifications	**Setting**
Side Chain	Carboxymethylation
Variable Modifications	**Setting**
N Terminal	Gln→Pyro Glu
C Terminal	Loss of lysine
Side Chain	Deamidation (N) Deamidation(Q) Glycation (K) Oxidation (MW) Oxidation (C)

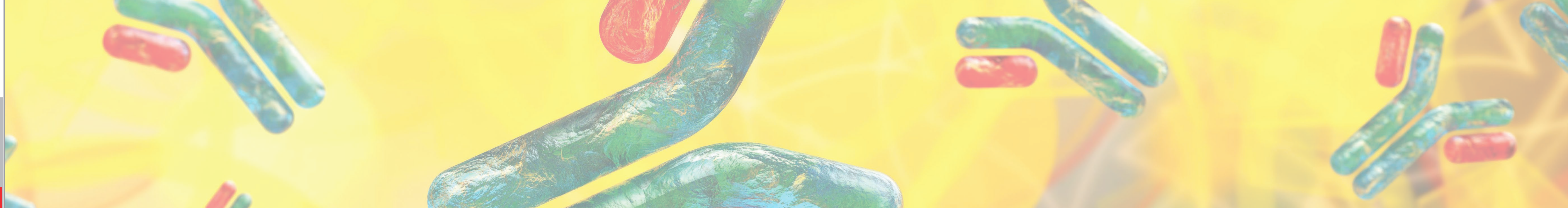
Results and discussion

Changes to the mAb structure introduced during the manufacturing process or storage may influence the therapeutic efficacy, bio-availability, clearance, and immunogenic properties, thus altering drug safety and use. Peptide mapping is a widely utilized technique to characterize monoclonal antibodies for the purpose of product identity and as an important stability indicating assay. Peptide mapping by liquid chromatography-mass spectrometry (LC-MS) analysis of enzymatically digested mAb is a powerful method for PTMs characterization to ensure mAb drug function and quality.

Using the magnetic SMART Digest Kit in combination with the KingFisher Duo Prime system simplifies the process and reduces the time needed for peptide mapping sample preparation. This methodology provides significant improvements in reproducibility over existing protocols, resulting in fewer sample failures, higher throughput, and the ability to more easily interrogate data. The following chromatogram (Figure 1) shows nine overlaid traces of cetuximab (Erbitux®) peptides, digested with Magnetic SMART Digest Kit. Excellent retention time reproducibility ≤ 0.14 RSD with an average of 0.083% is achieved when using the Vanquish Flex Binary UHPLC system, as indicated in Table 8.

The same peptide mapping workflow was performed using five top-selling mAbs of different categories such as chimeric (rituximab and cetuximab), humanized (trastuzumab and bevacizumab), and fully human (adalimumab) for assessing the sequence coverage for light and heavy chain, as well as for identification and relative quantification of a specific set of modifications: deamidation, oxidation, pyroglutamination, glycation, Lys loss, and glycosylation. Five top mAbs were analyzed after performing an automated SMART digest resulting in peptide mixtures. The obtained base peak chromatograms are similar but show distinct differences (Figure 2). Each protein to be mapped presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity. All antibodies can be identified with 100% sequence coverage when analyzed separately (Table 9).

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



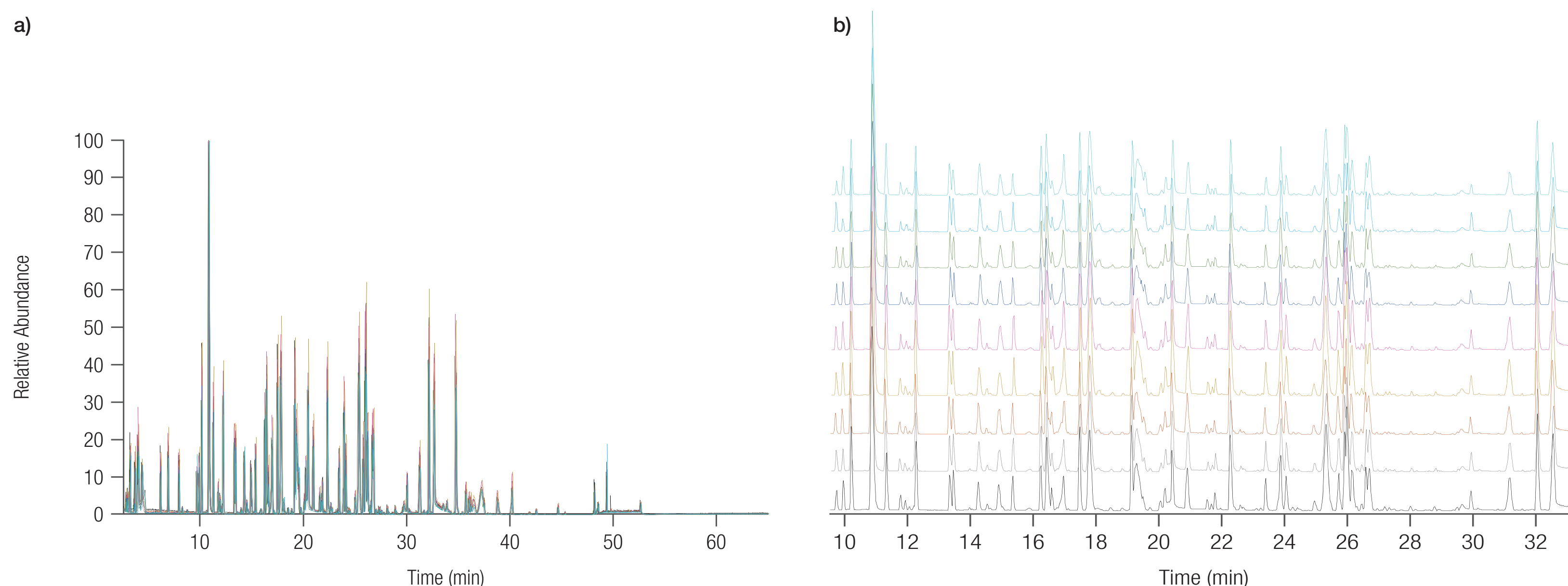


Figure 1. Overlaid base peak chromatograms (BPCs) of nine technical replicates for cetuximab (Erbitux®) Magnetic SMART digested samples (a) and the stacked expanded time region between 9.5 and 33.5 minutes (b)

Table 8. Retention time repeatability (n=9) for fourteen peptides from cetuximab after Magnetic SMART digestion on the King Fisher Duo prime system

Protein	Identification	Peptide Sequence	Average RT (min)	RSD (%)
Cetuximab HC	1:L412-K416	LTVDK	10.138	0.137
Cetuximab HC	1:L67-K71	LSINK	12.216	0.138
Cetuximab HC	1:G33-R38	GVHWVR	14.981	0.139
Cetuximab HC	1:Q1-K5	QVQLK	17.473	0.099
Cetuximab HC	1:A329-K336	ALPAPIEK	17.796	0.109
Cetuximab LC	2:Y50-R61	YASESISGIPSR	19.309	0.108
Cetuximab HC	1:347-R357	EPQVYTLPPSR	20.416	0.078
Cetuximab LC	2:A25-R39	ASQSIGTNIHWYQQR	22.299	0.072
Cetuximab HC	1:S28-R38	SLTNYGVHWVR	23.430	0.064
Cetuximab HC	1:G124-K135	GPSVFPLAPSSK	25.363	0.068
Cetuximab LC	2:D170-K183	DSTYSLSSTLTLSK	26.643	0.046
Cetuximab LC	2:R108-K126	RTVAAPSVFIFPPSDEQLK	29.716	0.065
Cetuximab HC	1:G373-K394	GFYPSDIAVEWESNGQPENNYK	31.242	0.053
Cetuximab HC	1:T395-K411	TTPPVLDSDGSFFLYSK	32.126	0.058

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

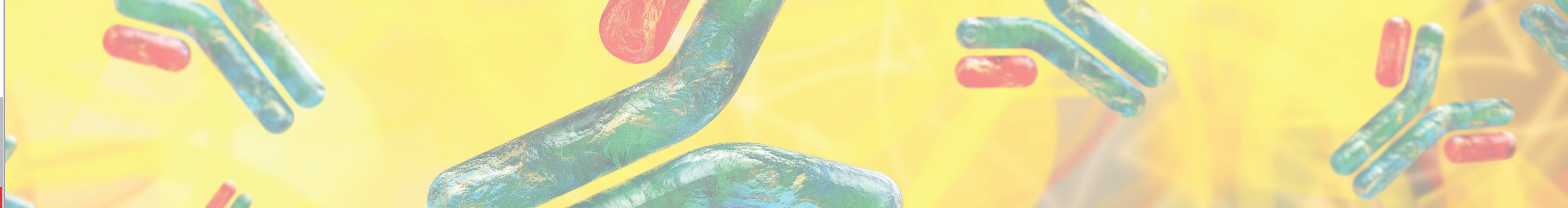
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations



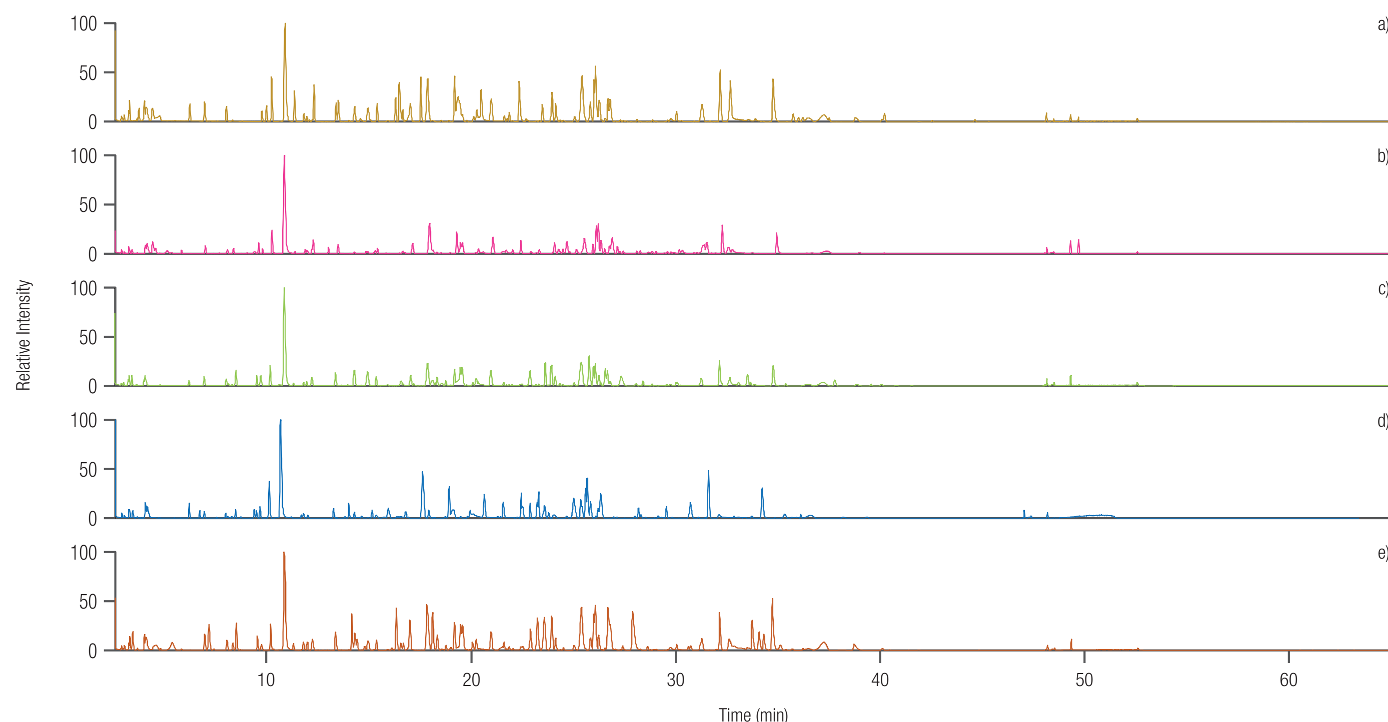


Figure 2. BPCs obtained from peptide mapping experiments of a) cetuximab, b) rituximab, c) bevacizumab, d) trastuzumab, and e) adalimumab after Magnetic SMART digestion with KingFisher Duo Prime system

Table 9. Sequence coverage for the studied recombinant IgG1 mAbs

Proteins	Sample	Number of Peaks	Sequence Coverage (%)
Heavy chain	Cetuximab (Erbitux®)	1457	100.00
	Rituximab (MabThera®)	1187	100.00
	Trastuzumab (Herceptin®)	1169	100.00
	Bevacizumab (Avastin®)	946	100.00
	Adalimumab (Humira®)	1384	100.00
Light chain	Cetuximab (Erbitux®)	533	100.00
	Rituximab (MabThera®)	532	100.00
	Trastuzumab (Herceptin®)	375	100.00
	Bevacizumab (Avastin®)	361	100.00
	Adalimumab (Humira®)	686	100.00

The number of detected MS peaks in the samples digested with Magnetic SMART Digest Kit on the Kingfisher Duo Prime system varied between the different mAbs from 361 to 686 for the light chain and 946 to 1457 for the heavy chain. The chimeric drug product cetuximab showed the largest number of detected MS peaks for heavy chain, while bevacizumab, a humanized IgG1 showed the lowest number of detected MS peaks.

For biotherapeutic protein identity, a peptide map needs high specificity, and complete or nearly complete sequence coverage is important because there maybe not prior knowledge of the alterations to the protein. The sequence coverage for studied mAbs are shown in Table 9. For all five top mAbs, 100% sequence coverage was achieved for light and heavy chains.

The sequence coverage map (Figure 3) shows the overlap of the different peptides identified with different intensities and in different lengths due to missed cleavages and nonspecific peptides that do not follow protease's rule. As an example, a sequence coverage map is shown for bevacizumab drug product. The colored bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the intensity of the peptide in the MS1 scan.

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

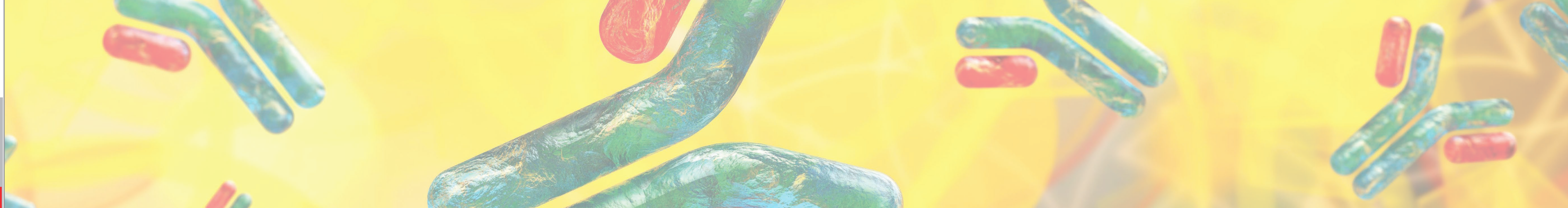
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations



Bevacizumab heavy chain

Bevacizumab light chain

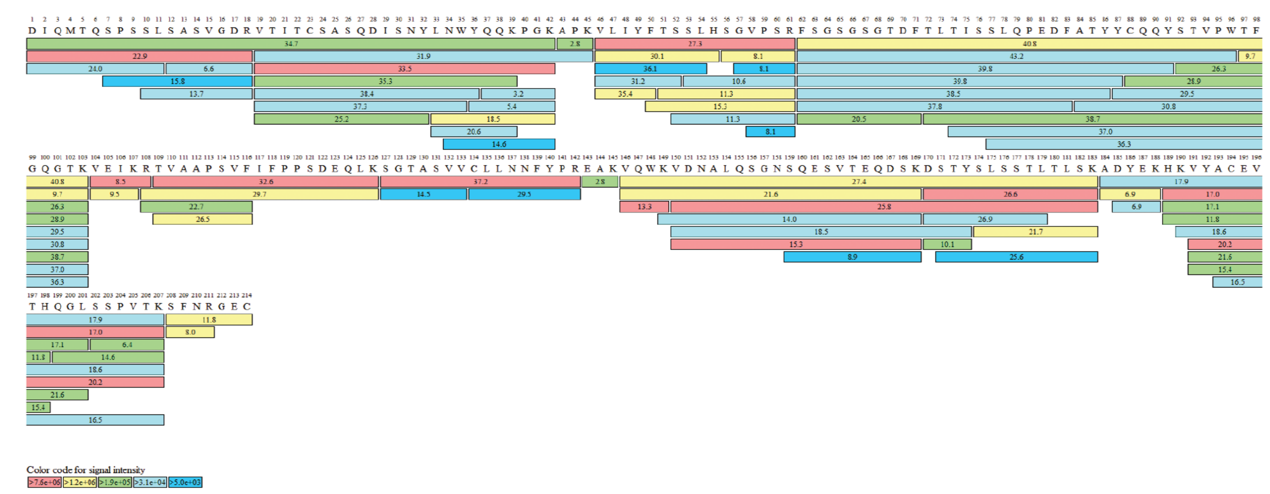


Figure 3. Sequence coverage map of bevacizumab heavy (top) and light chain (bottom), obtained using a 65 min gradient for peptide separation on an Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm column. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant > 7.6e+06, yellow > 1.2e+06, green > 1.9e+05, light blue > 3.1e+04, cyan = low abundant > 5.0e+03.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

To ensure high confidence in the identified peptides, all peptide sequence matches were required to have ±5 ppm of MS mass error, confidence score ≥ 95, and full MS and MS/MS spectra. Figure 4 shows an example of the selected ion chromatogram (SIC) and corresponding MS/MS spectra for selected peptide ALPAPIEK present on the five top mAb heavy chain, which elutes at 17.80 min. The combination of high-quality MS and MS/MS data ensures a reliable peptide matching.

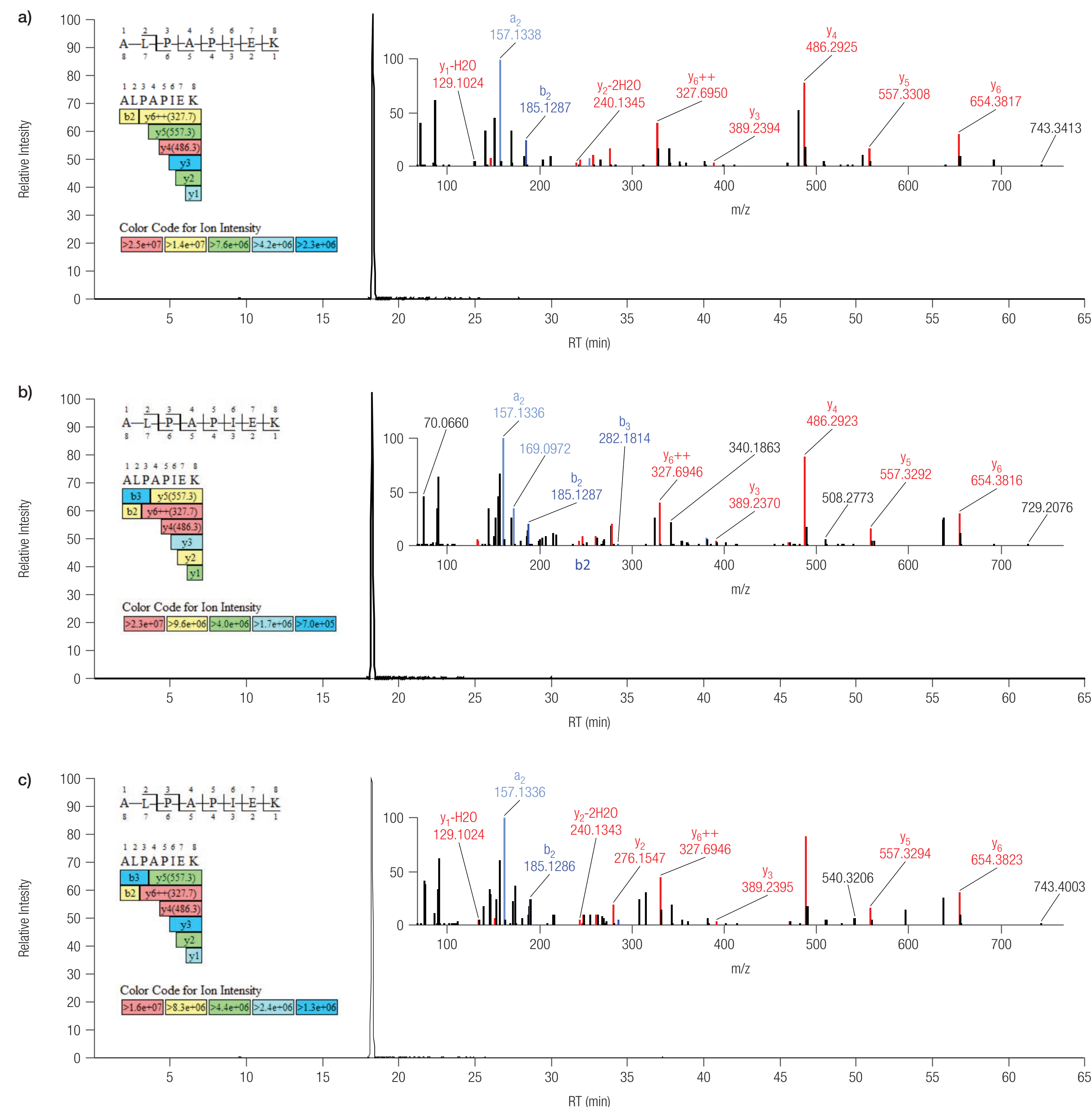


Figure 4. Representative SIC of HC peptide ALPAPIEK, MS/MS spectra, and fragment coverage map from digested (a) cetuximab, (b) rituximab and (c) bevacizumab

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

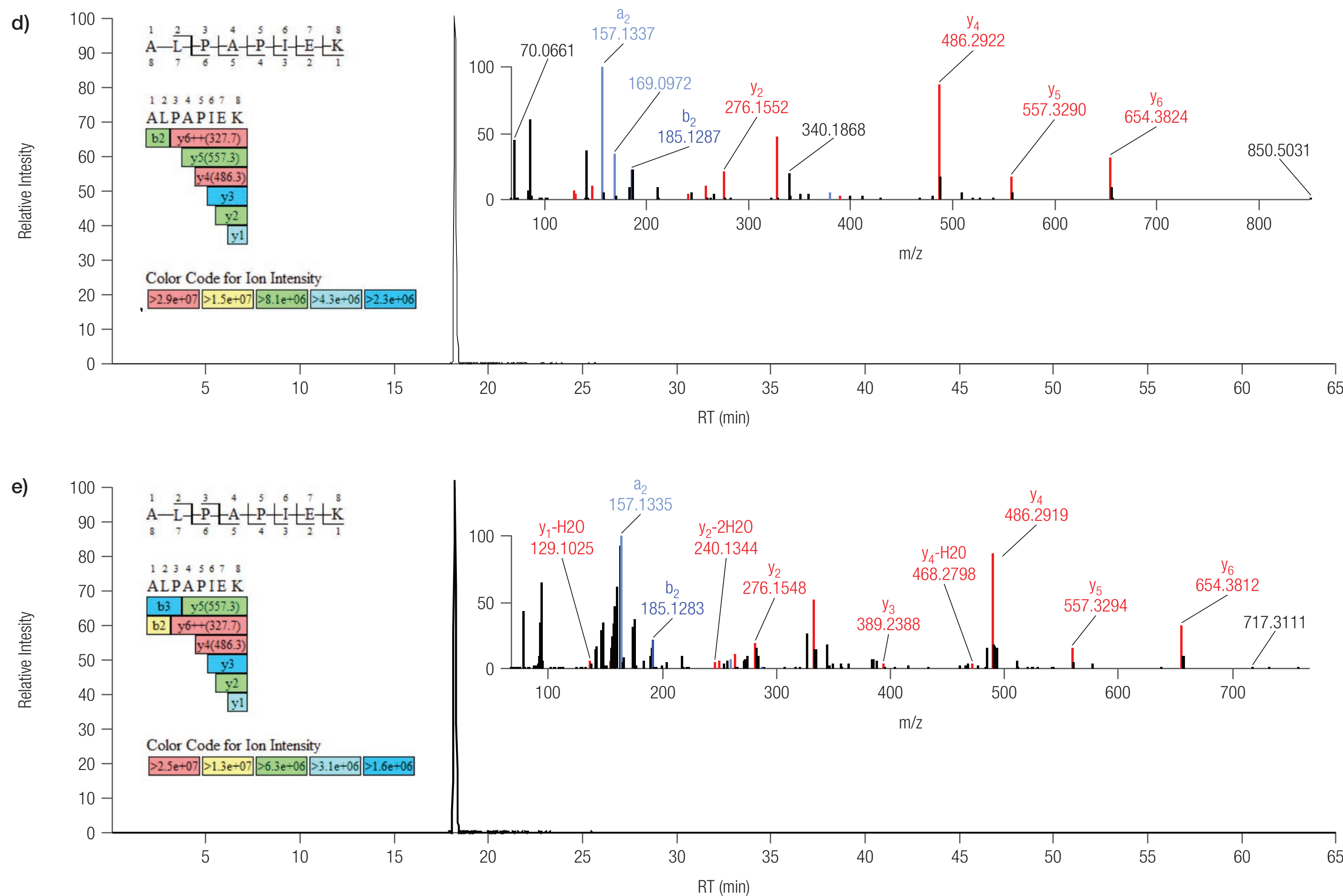


Figure 4. (continued). Representative SIC of HC peptide ALPAPIEK, MS/MS spectra, and fragment coverage map from digested (d) trastuzumab and (e) adalimumab biotherapeutics

Peptide mapping analysis allows the identification and quantification of PTMs. Many common PTMs cause a shift in reversed-phase LC retention relative to the native peptide. In combination with direct MS and MS/MS analysis it can be used to interrogate modifications with relative large mass shifts, such as C-terminal lysine (128 Da), glycation (162 Da), and small mass differences such as deamidation (1 Da), oxidation (16 Da), or pyroglutamination (17 Da), between others.

Tables 10–14 show the identification and comparison of a subset of monitored modifications across the different mAbs studied. PTMs such as N-terminal pyroglutamination, deamidation, oxidation, glycation, C-terminal lysine clipping, and glycosylation are confidently identified in many variations based on MS1 and MS/MS spectra. A tilde (~) before the modification indicates the modification was found on the tryptic peptide but could not be localized on a specific amino acid with MS/MS spectra. The relative abundance of the detected modifications in the five different mAbs usually have relative standard deviations < 10% except for a few modifications above 15% (cetuximab Q37 and N158; and trastuzumab Q199), which were in low abundance. Overall, the method shows that important information regarding PTMs can be obtained equally and accurately at all separation times.

A common structural modification of recombinant proteins is observed through the non-enzymatic deamidation of glutamine (Gln) and asparagine (Asn) residues. The latter occurs in a variety of protein-based pharmaceuticals, including monoclonal antibodies with varying effects on the activity or stability of the therapeutic protein. Thus, determining the deamidation of Asn residues in recombinant proteins is a significant challenge for analytical and protein chemists in the quality control and process departments at biotechnology and pharmaceutical companies.[10] Deamidation of Gln proceeds at a much slower rate than deamidation of Asn residues at peptide level. Liu et al[11] investigated Gln deamidation of a recombinant monoclonal IgG1 antibody, showing Gln residues at different locations had different susceptibilities to deamidation and it is a highly pH-dependent modification. Figure 5 shows the average relative abundance of four to six of the most abundant deamidation modifications for the five top-studied mAbs. A common deamidation to all the studied mAbs was observed for the residue Q199/Q198 of the LC, with variable levels that go from 0.064% (trastuzumab) to 0.884% (adalimumab). In relation to Asn deamidation, residue N321/N319/N318/N317 (VVSVLTVLHQDWLNGK) seems to be more susceptible to PTMs, being present in the five mAbs at a relative abundance between 1.3% (bevacizumab) and 2.1% (adalimumab). The most abundant deamidation levels were detected for the N55 site from rituximab heavy chain (7.05%) and for the Q27 site from trastuzumab light chain (4.77%).

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

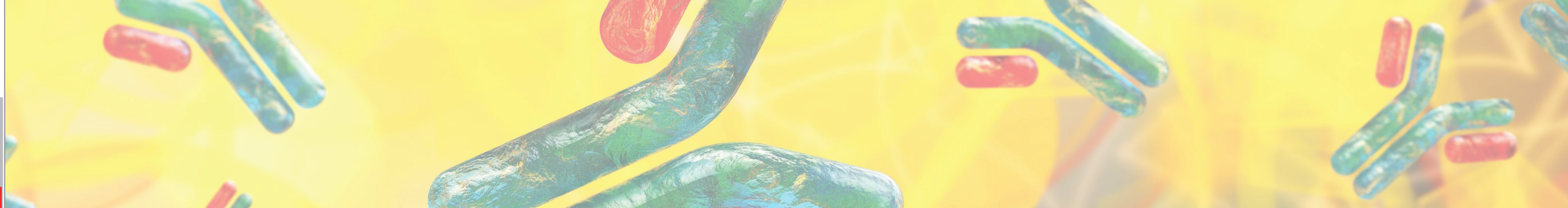
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations



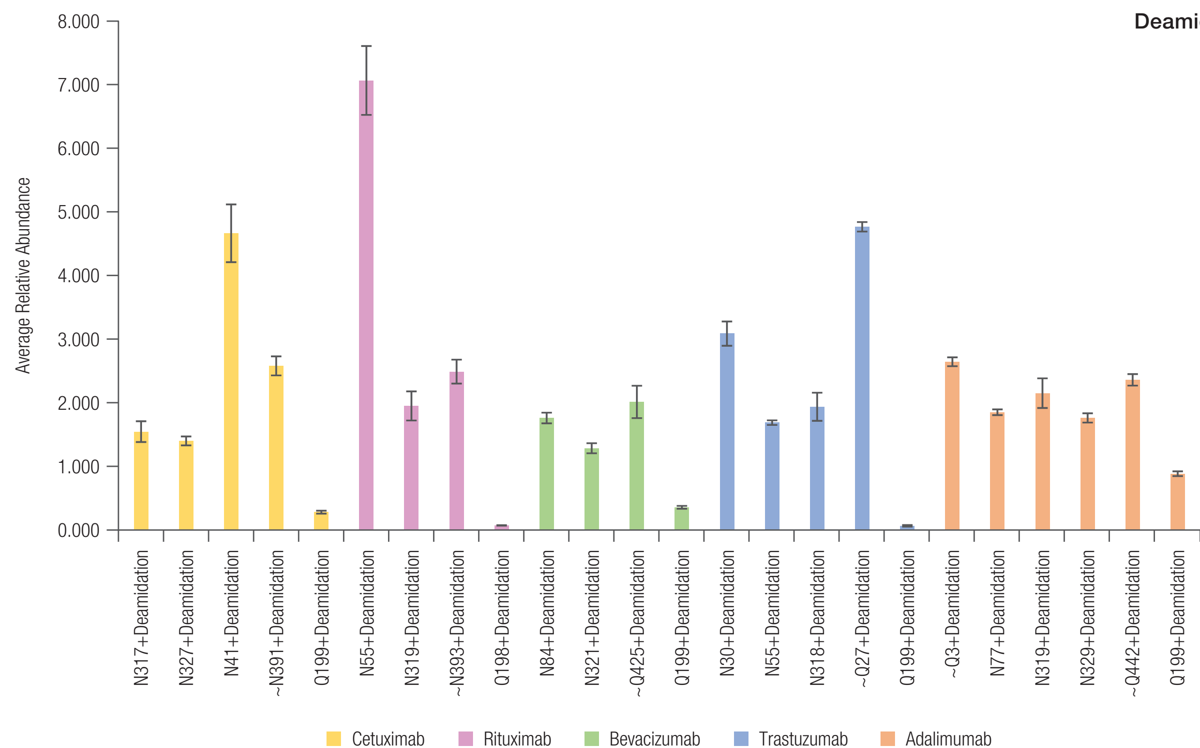


Figure 5. Average relative abundance (n=3) of some identified deamidation modifications for trastuzumab, rituximab, cetuximab, bevacizumab, and adalimumab

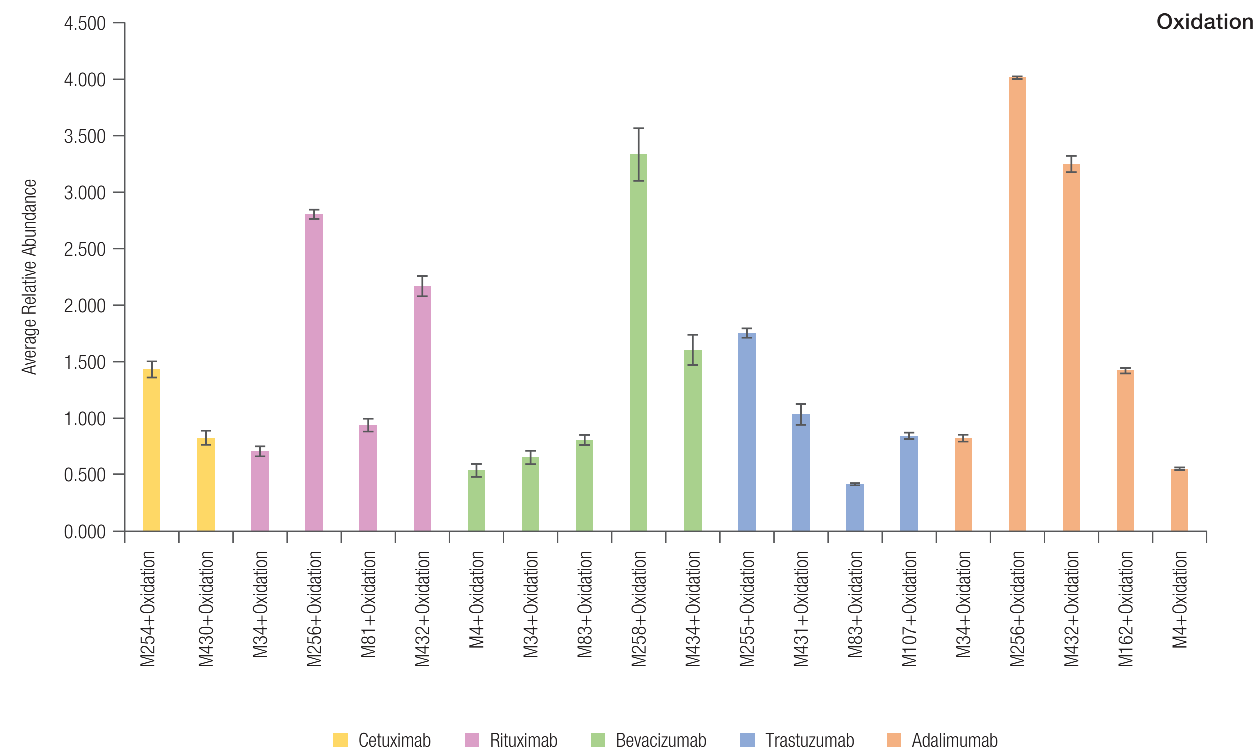


Figure 6. Average relative abundance (n=3) of identified oxidation modifications for cetuximab, rituximab, bevacizumab, trastuzumab, and adalimumab

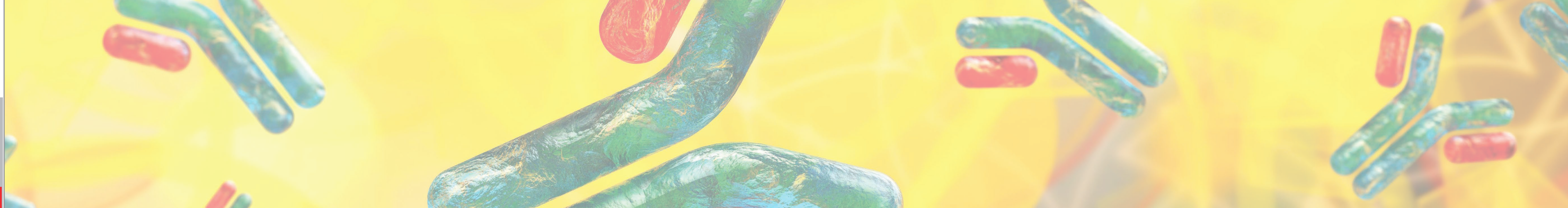
On the other hand, protein and peptide microheterogeneity can sometimes be attributed to oxidation of tryptophan (Trp) or methionine (Met) residues. This is also a common PTM observed in proteins and peptides. *In vivo* oxidation is caused by oxygen radicals and other biological factors (e.g., exposure to certain oxidizing drugs or other compounds). *In vitro* oxidation can be due to conditions encountered during purification or formulation. Oxidation can also occur during storage and from frequent freeze-thawing cycles. Protein chemists in process development and quality control are concerned with oxidation because it can adversely impact the activity and stability of biotherapeutics.[12] The studied mAbs in the present work show low oxidation levels (<4.0%). Residue M254/M255/M256/M258 (on the peptide sequence DTLMISR) detected in the heavy chain for all the five studied mAbs seems to be more susceptible for oxidation (Figure 6). Adalimumab showed the highest levels for heavy chain M256 (4.0%) and M432 (3.2%), and bevacizumab for M258 (3.3%).

One of the most important PTMs to characterize and quantify is glycoslyation, which is critical for therapeutic efficacy and safety of the drug. N-glycans have important structural functions as they stabilize the CH2 domain of IgGs. Deglycosylation makes mAbs thermally less stable and more susceptible to unfolding and they are more prone to aggregation. Moreover, functionality of the IgG is influenced by the attached N-glycans and their size.[13] Glycosylation renders a peptide more hydrophilic, meaning it can be easily resolved from its non-glycosylated counterpart. High abundance of glycosylation of the heavy chain is also observed for the five studied mAbs at the Fc part at position N299 (cetuximab), N301 (rituximab and adalimumab), N300 (trastuzumab), or N303 (bevacizumab), where the main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the reducing end of N-acetylglucosamine (A2G0F, A2G1F, A2G2F and A1G0F), afucosylated biantennary (A2G0), and high mannose (M5) structures (Figure 7).

Cetuximab drug product shows another N-glycosylation site at the position N88 on the Fab region (Table 10). The glycan structures observed correspond to several non-human glycan motifs containing *α-1,3-Gal-epitopes* (A2Ga2F, A2Ga1G1F, A3Ga3F, A3Ga2G1F) and the glycan moiety N-glycolylneuraminic acid (A2Sg1G1F, A2Sg1Ga1F, A2Sg2F), which have been reported to cause anaphylaxis in over 30% of patients that received cetuximab.[14] Those findings highlight the importance and need for reliable glycosylation analysis.

The C-terminal lysine (Lys) truncation variant is commonly observed in monoclonal antibodies and recombinant proteins. Although the effect that this variation has on protein activity does not seem to impact the potency or safety profile,[15] the degree of heterogeneity of C-terminal Lys variants reflect the manufacturing consistency and should be monitored for product consistency. Lys loss is detected in all the five studied mAbs with the lowest % of modification for cetuximab (67.13%) and the highest level of modification detected for bevacizumab (97.45%) and trastuzumab (97.73%).

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



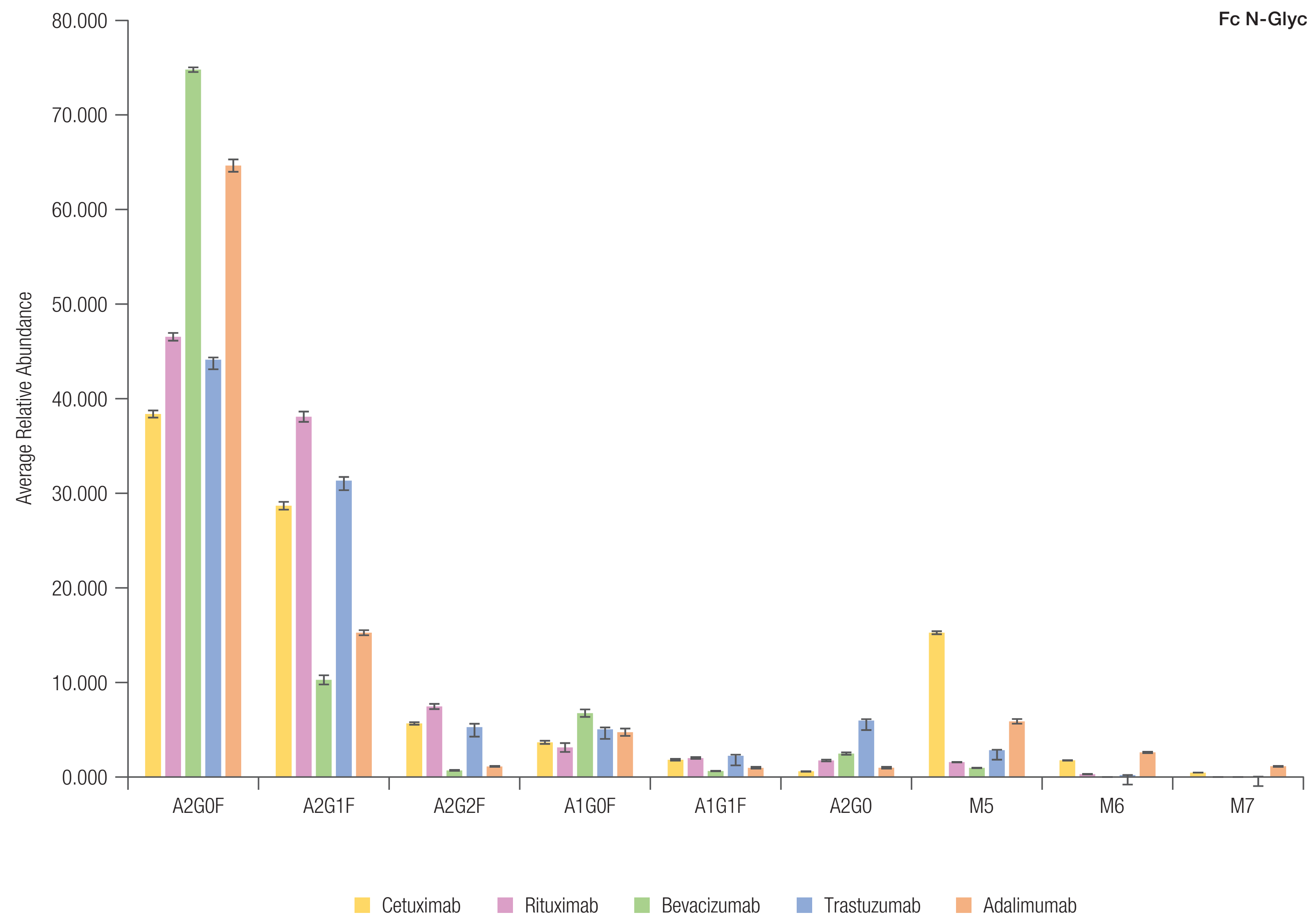


Figure 7. Average relative abundance (n=3) of identified N-glycosylation on the Fc region for cetuximab, rituximab, bevacizumab, trastuzumab, and adalimumab

Another commonly targeted modification is lysine (K) glycations which are listed in Tables 10–14. In total, between eight and twelve lysine glycations could be identified and relatively quantified < 1.58% with an average RSD value ≤ 15%. Overall, similar levels were observed for the different studied IgG1 mAbs.

Almost half the antibodies reported in the literature contain a glutamic acid residue at the N-terminus of the light or the heavy chain. As studied by *Chelius D. et al.*[16] the formation of pyroglutamic acid from N-terminal glutamic acid in the heavy chains and light chains of several antibodies, indicate that it is a non-enzymatic reaction that occurs very commonly. For therapeutic mAbs, pyroGlu can be one of many PTMs or transformations observed during production and storage. N-terminal pyroglutamination was observed for cetuximab and rituximab in high abundance (>85%).

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Table 10. Summary of PTMs identified and quantified for cetuximab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest Kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=9)	RSD (n=9)
CETUXIMAB (chimeric IgG1)				
Heavy chain	Q1+Gln→Pyro-Glu	QVQLK	99.778	0.027
Heavy Chain	N31+Deamidation	SLTNYGVHWVR	0.172	8.421
Heavy Chain	N70+Deamidation	LSINK	0.078	5.746
Heavy Chain	N73+Deamidation	LSINKDNSK	0.939	8.572
Heavy Chain	Q77+Deamidation	SQVFFK	0.173	8.224
Heavy Chain	N288+Deamidation	FNWYVDGVEVHNAK	0.492	5.948
Heavy Chain	N299+Deamidation	EEQYNSTYR	0.130	11.260
Heavy Chain	N317+Deamidation	VVSVLTVLHQDWLNGK	1.546	10.566
Heavy Chain	N327+Deamidation	CKVSNK; VSNK	1.399	5.030
Heavy Chain	N363+Deamidation	NQVSLTCLVK	1.210	6.686
Heavy Chain	Q364+Deamidation	NQVSLTCLVK	0.233	15.143
Heavy Chain	~N391+Deamidation	GFYPSDIAVEWESNGQPENNYK	2.537	6.047
Heavy Chain	~Q421+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	0.459	7.148
Heavy Chain	~N436+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	1.221	8.461
Light Chain	Q6+Deamidation	DILLTQSPVILSVSPGER	0.296	13.542
Light Chain	Q27+Deamidation	ASQSIGTNIHWYQQR	0.420	13.158
Light Chain	~Q37+Deamidation	ASQSIGTNIHWYQQR	0.157	17.755
Light Chain	N41+Deamidation	TNGSPR	4.664	9.743
Light Chain	~N137+Deamidation	SGTASVVCLLNNFYPR	0.691	9.221
Light Chain	Q147+Deamidation	VQWK	0.012	5.418
Light Chain	~Q155+Deamidation	VDNALQSGNSQESVTEQDSK	0.405	10.058
Light Chain	~N158+Deamidation	VQWKVDNALQSGNSQESVTEQDSK;	0.688	18.898
Light Chain	Q199+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.280	8.257
Heavy Chain	M254+Oxidation	DTLMISR	1.431	4.975
Heavy Chain	M430+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK;	0.827	7.532
Heavy Chain	K75+Glycation	DNSKSQVFFK	0.060	14.765
Heavy Chain	K135+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.156	5.155
Heavy Chain	~K290+Glycation	FNWYVDGVEVHNAKTKPR	0.182	3.349
Heavy Chain	K328+Glycation	VSNKALPAPIEK	0.245	3.073
Light Chain	K49+Glycation	LLIKYASESISGIPSR	0.151	7.557
Light Chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.346	3.625
Light Chain	K188+Glycation	ADYEKHKVYACEVTHQGLSSPVTK; ADYEKHK	0.206	9.077
Heavy Chain	K449+Lys Loss	SLSLSPGK	67.128	2.015
Heavy Chain	N299+A1G0	EEQYNSTYR	0.421	5.738
Heavy Chain	N299+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	3.676	9.192
Heavy Chain	N299+A1G0M4	EEQYNSTYR	0.256	5.985
Heavy Chain	N299+A1G0M5	EEQYNSTYR	0.602	2.687
Heavy Chain	N299+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.700	5.358
Heavy Chain	N299+A1G1M4F	EEQYNSTYR	1.710	8.178
Heavy Chain	N299+A1G1M5	EEQYNSTYR	0.832	3.161
Heavy Chain	N299+A1G1M5F	EEQYNSTYR	1.979	2.623
Heavy Chain	N299+A1S1M5	EEQYNSTYR	0.188	4.132
Heavy Chain	N299+A2G0	EEQYNSTYR; TKPREEQYNSTYR	0.616	2.710
Heavy Chain	N299+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	38.399	1.069
Heavy Chain	N299+A2G1	EEQYNSTYR	0.298	2.476

Protein	Modification	Sequence	Relative Abundance (%) (n=9)	RSD (n=9)
CETUXIMAB (chimeric IgG1)				
Heavy Chain	N299+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	29.404	1.738
Heavy Chain	N299+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	6.134	1.468
Heavy Chain	N299+A2Ga1G1F	EEQYNSTYR	0.724	2.554
Heavy Chain	N299+A2Ga2F	EEQYNSTYR	0.285	9.907
Heavy Chain	N299+A2S1G1	EEQYNSTYR	0.091	11.504
Heavy Chain	N299+Gn	EEQYNSTYR	0.254	7.589
Heavy Chain	N299+M3	EEQYNSTYR	0.110	27.945
Heavy Chain	N299+M4	EEQYNSTYR	0.718	15.189
Heavy Chain	N299+M5	EEQYNSTYR; TKPREEQYNSTYR	14.502	1.228
Heavy Chain	N299+M6	EEQYNSTYR; TKPREEQYNSTYR	1.760	1.520
Heavy Chain	N299+M7	EEQYNSTYR; TKPREEQYNSTYR	0.426	5.671
Heavy Chain	N299+Unglycosylated	EEQYNSTYR	1.093	8.454
Heavy Chain	N88+A1G0F	MNSLQSNDTAIYYCAR	0.969	5.826
Heavy Chain	N88+A1G1F	MNSLQSNDTAIYYCAR	0.482	3.863
Heavy Chain	N88+A1G1M4F	MNSLQSNDTAIYYCAR	1.305	3.604
Heavy Chain	N88+A1G1M5F	MNSLQSNDTAIYYCAR	0.801	2.973
Heavy Chain	N88+A1S1M5	MNSLQSNDTAIYYCAR	0.192	11.107
Heavy Chain	N88+A2G0F	MNSLQSNDTAIYYCAR	2.491	4.836
Heavy Chain	N88+A2G1F	MNSLQSNDTAIYYCAR	4.433	2.141
Heavy Chain	N88+A2G2F	MNSLQSNDTAIYYCAR	6.832	2.530
Heavy Chain	N88+A2Ga1G1F	MNSLQSNDTAIYYCAR	7.452	1.915
Heavy Chain	N88+A2Ga2F	MNSLQSNDTAIYYCAR	34.807	1.159
Heavy Chain	N88+A2S1Ga1F	MNSLQSNDTAIYYCAR	0.993	1.954
Heavy Chain	N88+A2Sg1G0F	MNSLQSNDTAIYYCAR	0.722	2.152
Heavy Chain	N88+A2Sg1G1F	MNSLQSNDTAIYYCAR	3.991	4.522
Heavy Chain	N88+A2Sg1Ga1F	MNSLQSNDTAIYYCAR	17.037	1.641
Heavy Chain	N88+A2Sg2F	MNSLQSNDTAIYYCAR	2.490	2.532
Heavy Chain	N88+A3G1F	MNSLQSNDTAIYYCAR	0.511	17.567
Heavy Chain	N88+A3G2F	MNSLQSNDTAIYYCAR	1.579	1.789
Heavy Chain	N88+A3G3F	MNSLQSNDTAIYYCAR	0.457	13.301
Heavy Chain	N88+A3Ga1G2F	MNSLQSNDTAIYYCAR	1.159	3.235
Heavy Chain	N88+A3Ga2G1F	MNSLQSNDTAIYYCAR	1.725	1.849
Heavy Chain	N88+A3Ga3F	MNSLQSNDTAIYYCAR	3.784	1.967
Heavy Chain	N88+A3S1Ga2F	MNSLQSNDTAIYYCAR	0.573	2.850
Heavy Chain	N88+A3Sg1G2F	MNSLQSNDTAIYYCAR	0.637	2.161
Heavy Chain	N88+A3Sg1Ga1G1F	MNSLQSNDTAIYYCAR	1.127	1.682
Heavy Chain	N88+A3Sg1Ga2F	MNSLQSNDTAIYYCAR	2.098	2.101
Heavy Chain	N88+A3Sg2G1F	MNSLQSNDTAIYYCAR	0.192	4.822
Heavy Chain	N88+A3Sg2Ga1F	MNSLQSNDTAIYYCAR	0.289	7.464
Heavy Chain	N88+Gn	MNSLQSNDTAIYYCAR	0.112	6.637
Heavy Chain	N88+GnF	MNSLQSNDTAIYYCAR	0.160	4.803
Heavy Chain	N88+M4	MNSLQSNDTAIYYCAR	0.093	13.101
Heavy Chain	N88+M5	MNSLQSNDTAIYYCAR	1.991	3.211

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thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

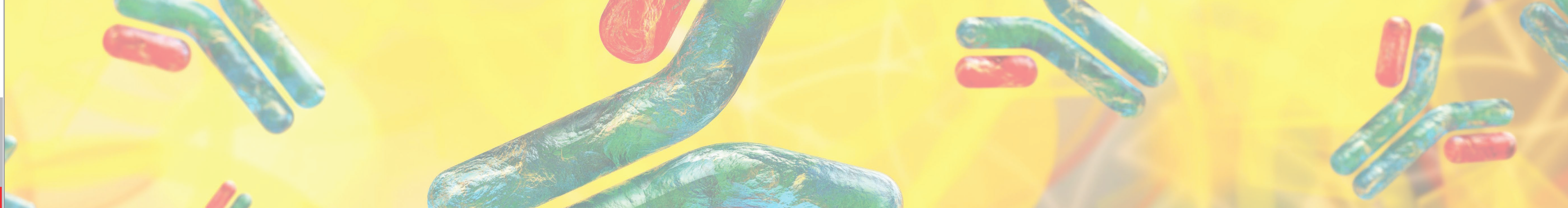

Table 11. Summary of PTMs identified and relatively quantified for rituximab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest Kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
RITUXIMAB (chimeric IgG1)				
Heavy chain	Q1+Gln→Pyro-Glu	QVQLQQPGAELVKPGASVK	99.919	0.002
Light chain	Q1+Gln→Pyro-Glu	QIVLSQSPAILSASPGEK	85.693	1.158
Heavy chain	N33+Deamidation	ASGYTFTSYNMHWVK; NMHWVK	0.439	5.608
Heavy chain	N55+Deamidation	GLEWIGAIYPGNGDTSYNQK	7.067	7.666
Heavy chain	N163+Deamidation	DYFPEPVTVSWNSGALTSGV HTFPAVLQSSGLY	0.457	4.674
Heavy chain	N290+Deamidation	FNWYVDGVEVHNAK	0.520	1.756
Heavy chain	N301+Deamidation	EEQYNSTYR	0.095	2.323
Heavy chain	N319+Deamidation	VVSVLTVLHQDWLNGK; TVLHQDWLNGK	1.950	11.737
Heavy chain	N365+Deamidation	NQVSLTCLVK	1.211	3.802
Heavy chain	Q366+Deamidation	NQVSLTCLVK	0.273	4.272
Heavy chain	~N393+Deamidation	GFYPSDIAVEWESNGQPENNYK	2.489	7.556
Heavy chain	~Q423+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	0.551	12.770
Heavy chain	~N438+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	1.300	9.579
Light chain	~Q36+Deamidation	ASSSVSYIHWFQQKPGSSPKPW	0.224	3.650
Light chain	~Q88+Deamidation	VEAEDAATYYCQQWTSNPPTFGGGTK	0.387	12.866
Light chain	~N136+Deamidation	SGTASVVCLLNNFYPR	0.711	4.549
Light chain	Q146+Deamidation	VQWK	0.285	3.753
Light chain	~Q159+Deamidation	VDNALQSGNSQESVTEQDSK	0.319	12.837
Light chain	Q198+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.071	5.828
Heavy chain	M34+Oxidation	ASGYTFTSYNMHWVK; TFTSYNMHWVK; NMHWVK	0.706	6.209
Heavy chain	M81+Oxidation	SSSTAYMQLSSLTSEDSAVYYCAR	0.938	6.038
Heavy chain	M256+Oxidation	DTLMISR	2.805	1.457
Heavy chain	M432+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	2.168	4.135
Heavy chain	M21+Oxidation	VTMTCR	0.203	12.689
Heavy chain	K137+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.302	9.471
Heavy chain	K252+Glycation	PKDTLMISR	0.045	7.924
Heavy chain	~K292+Glycation	FNWYVDGVEVHNAKTKPR	0.431	2.588
Heavy chain	K321+Glycation	VVSVLTVLHQDWLNGKEYK	0.085	2.214
Heavy chain	K330+Glycation	VSNKALPAPIEK	0.536	7.005
Light chain	K148+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.690	12.726
Light chain	K182+Glycation	DSTYSLSSTLTLSKADYEK	0.458	7.301

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
RITUXIMAB (chimeric IgG1)				
Light chain	K187+Glycation	ADYEKHKVYACEVTHQGLSSPVTK; ADYEKHK	0.585	3.770
Light chain	K189+Glycation	HKVYACEVTHQGLSSPVTK	0.077	10.802
Heavy chain	K451+Lys Loss	SLSLSPGK	96.512	0.037
Heavy chain	N301+A1G0	EEQYNSTYR	0.342	5.911
Heavy chain	N301+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	5.062	2.841
Heavy chain	N301+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.797	11.967
Heavy chain	N301+A1S1F	EEQYNSTYR	0.208	1.153
Heavy chain	N301+A2G0	EEQYNSTYR; TKPREEQYNSTYR	1.611	3.774
Heavy chain	N301+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	40.775	0.845
Heavy chain	N301+A2G1	EEQYNSTYR	0.632	0.804
Heavy chain	N301+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	39.803	2.049
Heavy chain	N301+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	7.511	2.256
Heavy chain	N301+A2S1G0F	EEQYNSTYR	0.483	8.957
Heavy chain	N301+A2S1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.005	11.177
Heavy chain	N301+A2S2F	EEQYNSTYR; TKPREEQYNSTYR	0.478	10.156
Heavy chain	N301+A3G1F	EEQYNSTYR	0.157	3.359
Heavy chain	N301+M5	EEQYNSTYR; TKPREEQYNSTYR	1.454	0.280
Heavy chain	N301+M6	EEQYNSTYR	0.295	7.111
Heavy chain	N301+Unglycosylated	EEQYNSTYR	1.336	17.487

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Table 12. Summary of PTMs identified and relatively quantified for trastuzumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest Kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
TRASTUZUMAB (humanized IgG1)				
Heavy chain	~Q13+Deamidation	EVQLVESGGGLVQPGGSLR	0.299	14.640
Heavy chain	N28+Deamidation	LSCAASGFNIK	0.051	10.428
Heavy chain	N55+Deamidation	IYPTNGYTR	1.693	2.128
Heavy chain	N77+Deamidation	NTAYLQMNSLR	0.662	1.892
Heavy chain	Q82+Deamidation	NTAYLQMNSLR	0.242	15.735
Heavy chain	N289+Deamidation	FNWYVDGVEVHNAK	0.264	5.494
Heavy chain	N300+Deamidation	EEQYNSTYR	0.073	5.561
Heavy chain	N318+Deamidation	VVSVLTVLHQDWLNGK; TVLHQDWLNGK	1.937	11.390
Heavy chain	N328+Deamidation	CKVSNK	2.545	4.699
Heavy chain	N364+Deamidation	NQVSLTCLVK	1.164	4.098
Heavy chain	Q365+Deamidation	NQVSLTCLVK	0.154	15.557
Heavy chain	~N387+Deamidation	GFYPSDIAVEWESNGQPENNYK	2.376	3.210
Heavy chain	~Q421+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	0.295	13.616
Heavy chain	~N437+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	1.191	6.289
Light chain	~Q27+Deamidation	ASQDVNTAVAWYQQKPGK	4.766	1.565
Light chain	N30+Deamidation	ASQDVNTAVAWYQQKPGK; ASQDVNTAVAWYQQKPGKAPK	3.087	6.181
Light chain	~Q38+Deamidation	ASQDVNTAVAWYQQKPGK	0.222	9.985
Light chain	~N137+Deamidation	SGTASVVCLLNNFYPR	0.608	2.685
Light chain	Q199+Deamidation	VYACEVTHQGLSSPVTK	0.064	19.876
Heavy chain	M83+Oxidation	NTAYLQMNSLR	0.415	2.390
Heavy chain	M107+Oxidation	WGGDGFYAMDYWGQGTLVTVSSASTK	0.844	3.392
Heavy chain	M255+Oxidation	DTLMISR	1.753	2.351
Heavy chain	M361+Oxidation	EEMTKNQVSLTCLVK; EEMTK; EPQVYTLPPSREEMTK	0.164	8.929
Heavy chain	M431+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	1.034	8.911
Light chain	M4+Oxidation	DIQMTQSPSSLSASVGDR	0.381	8.610
Heavy chain	K30+Glycation	LSCAASGFNIKDTYIHWVR	0.820	1.723
Heavy chain	K76+Glycation	FTISADTSKNTAYLQMNSLR	0.179	6.451
Heavy chain	K136+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.497	5.148
Heavy chain	K251+Glycation	PKDTLMISR	0.088	9.273

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
TRASTUZUMAB (humanized IgG1)				
Heavy chain	~K291+Glycation	FNWYVDGVEVHNAKTKPR	0.482	2.329
Heavy chain	K320+Glycation	VVSVLTVLHQDWLNGKEYK	0.116	8.646
Heavy chain	K323+Glycation	EYKCK	0.104	9.495
Heavy chain	K325+Glycation	CKVSNK	1.580	12.067
Heavy chain	K329+Glycation	VSNKALPAPIEK; CKVSNKALPAPIEK	0.335	1.391
Heavy chain	K337+Glycation	ALPAPIEKTISK	0.063	3.309
Heavy chain	K363+Glycation	EEMTKNQVSLTCLVK	0.080	6.908
Light chain	K103+Glycation	SGTDFTLTISSLQPEDFATYYCQQHY TTPPTFGQGTKVEIK	1.152	1.359
Light chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.919	4.916
Light chain	K183+Glycation	DSTYSLSSTLTLSKADYEK	0.369	13.651
Light chain	K188+Glycation	ADYEKHK	0.939	6.465
Light chain	K190+Glycation	HKVYACEVTHQGLSSPVTK	0.113	4.871
Heavy chain	K450+Lys Loss	SLSLSPGK	97.730	0.098
Heavy chain	N300+A1G0	EEQYNSTYR; TKPREEQYNSTYR	2.297	2.306
Heavy chain	N300+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	6.976	9.383
Heavy chain	N300+A1G1	EEQYNSTYR	0.594	1.401
Heavy chain	N300+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	2.180	6.721
Heavy chain	N300+A1S1	EEQYNSTYR	0.091	5.173
Heavy chain	N300+A1S1F	EEQYNSTYR	0.401	2.875
Heavy chain	N300+A2G0	EEQYNSTYR; TKPREEQYNSTYR	5.378	1.718
Heavy chain	N300+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	39.879	0.616
Heavy chain	N300+A2G1	EEQYNSTYR	1.938	0.475
Heavy chain	N300+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	32.283	2.911
Heavy chain	N300+A2G2	EEQYNSTYR	0.165	1.872
Heavy chain	N300+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	5.190	3.141
Heavy chain	N300+A2S1G0F	EEQYNSTYR	0.442	10.083
Heavy chain	N300+A2S1G1F	EEQYNSTYR	0.647	1.787
Heavy chain	N300+A2S2F	EEQYNSTYR	0.234	6.664
Heavy chain	N300+A3G1F	EEQYNSTYR	0.161	4.784
Heavy chain	N300+Gn	EEQYNSTYR	0.069	6.463
Heavy chain	N300+M3	EEQYNSTYR	0.165	17.312
Heavy chain	N300+M4	EEQYNSTYR	0.153	0.493
Heavy chain	N300+M5	EEQYNSTYR; TKPREEQYNSTYR	2.874	2.319
Heavy chain	N300+M6	EEQYNSTYR	0.204	3.781
Heavy chain	N300+Unglycosylated	EEQYNSTYR	1.443	4.976

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

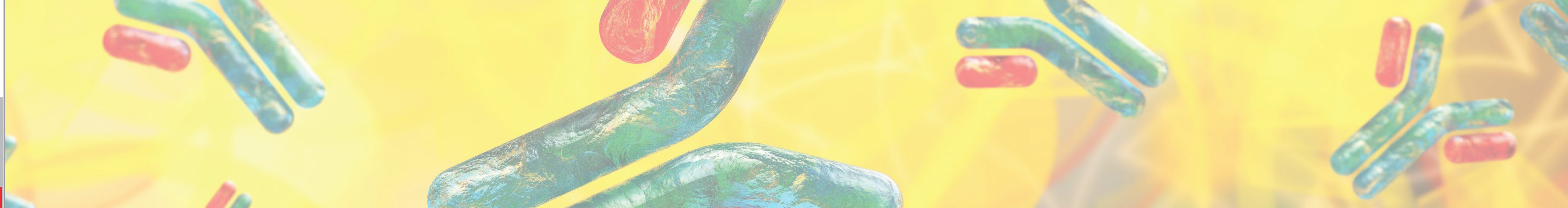

Table 13. Summary of PTMs identified and relatively quantified for bevacizumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest Kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
BEVACIZUMAB (humanized IgG1)				
Heavy chain	~Q3+Deamidation	EVQLVESGGGLVQPGGSLR	0.889	7.378
Heavy chain	N52+Deamidation	GLEWVGWINTYTGEPTYAADFK	0.172	1.118
Heavy chain	Q82+Deamidation	STAYLQMNSLR	0.800	4.216
Heavy chain	N84+Deamidation	STAYLQMNSLR	1.760	4.745
Heavy chain	N292+Deamidation	FNWYVDGVEVHNAK	0.400	4.808
Heavy chain	N303+Deamidation	EEQYNSTYR	0.213	6.696
Heavy chain	N321+Deamidation	VVSVLTVLHQDWLNGK	1.285	6.153
Heavy chain	N367+Deamidation	NQVSLTCLVK	1.165	2.714
Heavy chain	Q368+Deamidation	NQVSLTCLVK	0.493	9.825
Heavy chain	~N395+Deamidation	GFYPSDIAVEWESNGQPENNYK	1.256	13.661
Heavy chain	~Q425+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	2.013	12.658
Light chain	~Q3+Deamidation	DIQMTQSPSSLSASVGDR	0.081	14.929
Light chain	~Q6+Deamidation	DIQMTQSPSSLSASVGDR	0.671	3.767
Light chain	~N34+Deamidation	VTITCSASQDISNYLNWYQQKPGK	0.769	6.008
Light chain	~N137+Deamidation	SGTASVVCLLNNFYPR	0.651	5.199
Light chain	Q147+Deamidation	VQWK	0.412	7.233
Light chain	~Q160+Deamidation	VQWKVDNALQSGNSQESVTEQDSK; VDNALQSGNSQESVTEQDSK	0.877	9.020
Light chain	Q199+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.356	6.204
Heavy chain	M34+Oxidation	LSCAASGYTFTNYGMNWVR; TFTNYGMNWVR	0.653	9.110
Heavy chain	M83+Oxidation	STAYLQMNSLR	0.807	5.659
Heavy chain	M258+Oxidation	DTLMISR	3.333	6.960
	M434+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	1.604	8.337
Light chain	M4+Oxidation	DIQMTQSPSSLSASVGDR	0.538	10.628
Heavy chain	K65+Glycation	GLEWVGWINTYTGEPTYAADFKR	0.230	9.207
Heavy chain	K98+Glycation	AEDTAVYYCAKYPHYY; AEDTAVYYCAKYPHYYGSSHW	0.415	5.397
Heavy chain	K139+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.425	1.092
Heavy chain	K254+Glycation	PKDTLMISR	0.204	9.839
Heavy chain	~K294+Glycation	FNWYVDGVEVHNAKTKPR	0.505	1.635
Heavy chain	K332+Glycation	VSNKALPAPIEK	0.575	0.299
Light chain	K103+Glycation	TFGQGTKVEIK	1.227	2.171
Light chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.918	3.678
Light chain	K169+Glycation	VDNALQSGNSQESVTEQDSKDST YSLSSTLTLSK	0.239	3.940

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
BEVACIZUMAB (humanized IgG1)				
Light chain	K183+Glycation	DSTYSLSSTLTLSKADYEK	0.439	13.595
Light chain	K188+Glycation	ADYEKHKVYACEVTHQGLSSPVTK; ADYEKHK	0.782	0.929
Light chain	K190+Glycation	HKVYACEVTHQGLSSPVTK	0.135	5.153
Heavy chain	K453+Lys Loss	SLSLSPGK	97.454	0.016
Heavy chain	N303+A1G0	EEQYNSTYR	0.837	3.039
Heavy chain	N303+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	6.607	9.799
Heavy chain	N303+A1G1F	EEQYNSTYR	0.761	4.044
Heavy chain	N303+A2G0	EEQYNSTYR; TKPREEQYNSTYR	2.335	3.762
Heavy chain	N303+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	74.864	0.893
Heavy chain	N303+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	10.769	1.695
Heavy chain	N303+A2G2F	EEQYNSTYR	0.735	0.551
Heavy chain	N303+M5	EEQYNSTYR	0.873	3.126
Heavy chain	N303+Unglycosylated	EEQYNSTYR	5.348	1.247

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

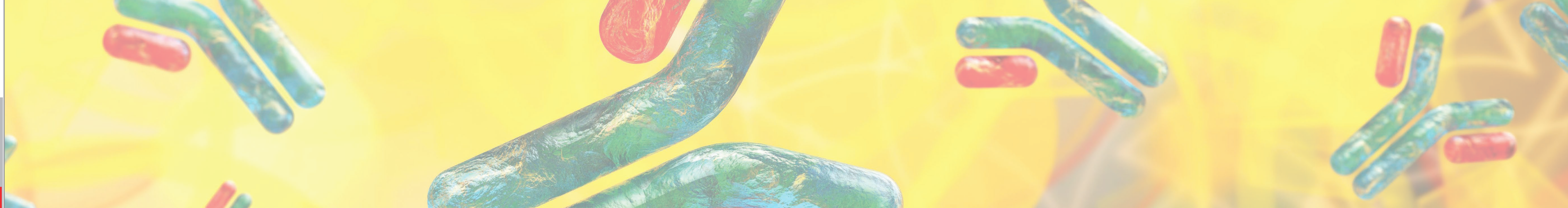

Table 14. Summary of PTMs identified and relatively quantified for adalimumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest Kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
ADALIMUMAB (fully human IgG1)				
Heavy Chain	~Q3+Deamidation	EVQLVESGGGLVQPGR	2.644	2.644
Heavy Chain	N77+Deamidation	NSLYLQMNSLR	1.851	2.437
Heavy Chain	Q82+Deamidation	NSLYLQMNSLR	0.952	5.733
Heavy Chain	N84+Deamidation	NSLYLQMNSLR	0.267	2.713
Heavy Chain	Q113+Deamidation	VSYLSTASSLDYWGQGTLVTVSSASTK	1.493	3.818
Heavy Chain	N290+Deamidation	FNWYVDGVEVHNAK	0.691	5.938
Heavy Chain	N301+Deamidation	EEQYNSTYR	0.240	3.262
Heavy Chain	N319+Deamidation	VVSVLTVLHQDWLNGK	2.150	10.848
Heavy Chain	N329+Deamidation	CKVSNK	1.761	4.136
Heavy Chain	N365+Deamidation	NQVSLTCLVK	1.246	1.989
Heavy Chain	Q366+Deamidation	NQVSLTCLVK	0.824	5.030
Heavy Chain	~N388+Deamidation	GFYPSDIAVEWESNGQPENNYK	1.062	6.606
Heavy Chain	~Q423+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	1.957	9.794
Heavy Chain	~Q442+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	2.360	3.829
Light Chain	~Q3+Deamidation	DIQMTQSPSSLSASVGDR	1.303	1.031
Light Chain	~Q6+Deamidation	DIQMTQSPSSLSASVGDR	1.280	5.373
Light Chain	Q27+Deamidation	ASQGIR	1.078	1.777
Light Chain	N31+Deamidation	NYLAWYQQKPGK	0.975	1.194
Light Chain	~Q37+Deamidation	NYLAWYQQKPGK	2.119	1.391
Light Chain	Q55+Deamidation	LLIYAASTLQSGVPSR	1.165	1.969
Light Chain	Q79+Deamidation	FSGSGSGTDFTLTISSLQPEDVATY	0.821	9.288
Light Chain	Q100+Deamidation	APYTFGQGTK; YNRAPYTFGQGTK; APYTFGQGTKVEIKR	0.911	2.157
Light Chain	~N137+Deamidation	SGTASVVCLLNNFYPR	1.169	2.671
Light Chain	Q147+Deamidation	VQWK	0.883	2.261
Light Chain	~N152+Deamidation	VQWKVDNALQSGNSQESVTEQDSK	0.418	8.678
Light Chain	~Q160+Deamidation	VQWKVDNALQSGNSQESVTEQDSK; VDNALQSGNSQESVTEQDSK	2.309	1.920
Light Chain	Q166+Deamidation	VDNALQSGNSQESVTEQDSK	0.821	8.929
Light Chain	Q199+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.884	4.186

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
ADALIMUMAB (fully human IgG1)				
Heavy Chain	M34+Oxidation	LSCAASGFTFDDYAMHWVR	0.823	3.711
Heavy Chain	~W53+Oxidation	GLEWVSAITWNSGHIDYADSVEGR	0.083	11.215
Heavy Chain	M83+Oxidation	NSLYLQMNSLR	0.673	5.697
Heavy Chain	M256+Oxidation	DTLMISR	4.014	0.284
Heavy Chain	M432+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	3.249	2.223
Light Chain	M4+Oxidation	DIQMTQSPSSLSASVGDR; DIQMTQSPSSLSASVGDRVTITCR	0.553	2.021
Heavy Chain	K76+Glycation	DNAKNSLYLQMNSLR	0.090	3.475
Heavy Chain	K137+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.275	2.992
Heavy Chain	~K292+Glycation	FNWYVDGVEVHNAKTKPR	0.242	1.760
Heavy Chain	K330+Glycation	VSNKALPAPIEK	0.330	2.494
Light Chain	K103+Glycation	APYTFGQGTKVEIK; APYTFGQGTKVEIKR	0.198	1.793
Light Chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.454	1.797
Light Chain	~K188+Glycation	ADYEKHKVYACEVTHQGLSSPVTK	0.444	7.008
Heavy Chain	K451+Lys Loss	SLSLSPGK	78.254	0.559
Heavy Chain	N301+A1G0	EEQYNSTYR	0.511	7.201
Heavy Chain	N301+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	5.562	3.165
Heavy Chain	N301+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.115	2.578
Heavy Chain	N301+A2G0	EEQYNSTYR; TKPREEQYNSTYR	0.972	7.113
Heavy Chain	N301+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	64.244	0.718
Heavy Chain	N301+A2G0FB	EEQYNSTYR	0.244	5.530
Heavy Chain	N301+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	15.473	1.181
Heavy Chain	N301+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	1.122	2.580
Heavy Chain	N301+Gn	EEQYNSTYR	0.362	1.247
Heavy Chain	N301+GnF	EEQYNSTYR	0.060	1.931
Heavy Chain	N301+M3	EEQYNSTYR; TKPREEQYNSTYR	0.793	5.631
Heavy Chain	N301+M4	EEQYNSTYR; TKPREEQYNSTYR	0.973	7.936
Heavy Chain	N301+M5	EEQYNSTYR; TKPREEQYNSTYR	5.501	2.622
Heavy Chain	N301+M6	EEQYNSTYR; TKPREEQYNSTYR	2.526	2.694
Heavy Chain	N301+M7	EEQYNSTYR; TKPREEQYNSTYR	1.127	2.949
Heavy Chain	N301+M8	EEQYNSTYR; TKPREEQYNSTYR	0.386	6.322
Heavy Chain	N301+Unglycosylated	EEQYNSTYR	3.464	1.341

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Conclusions

- The Magnetic SMART Digest Kit provides simple and rapid protein digestion for peptide mapping analysis and PTM investigation of mAbs.
- The analysis of five top monoclonal antibodies using the described global workflow solution results in excellent quality data with high confidence in results. Excellent sequence coverage (100%) and low levels of sample preparation-induced post-translational modifications (PTMs) were observed with the Magnetic SMART Digest Kit.
- Easily automated peptide mapping results in less sample handling, increased productivity, and improved reproducibility.
- The combination of the KingFisher Duo Prime system with the Magnetic SMART Digest Kit offers a global automated option for biotherapeutic digestions, simplifying the process for reproducible method transfer and reducing the time needed for sample preparation. This not only speeds up the biotherapeutic peptide mapping workflow for high-throughput analysis, but also significantly reduces method development time.
- The Vanquish Flex UHPLC delivers outstanding retention time and peak area precision. The system offers a high-pressure flow path and sample pressurization prior to injection ensuring high peak capacity, retention time stability, and peak area precision, ideal for peptide mapping applications.
- The Q Exactive Hybrid Quadrupole-Orbitrap system has been proven to deliver excellent mass accuracy and highly sensitive MS results for protein identification and detailed peptide mapping. BioPharma Finder 3.0 software can provide automatic data processing, peptide sequence matching, and protein sequence coverage mapping accurately and with high confidence.

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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Find out more...

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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Application benefits

- Rapid, automated digestion leading to highly reproducible results for innovator and biosimilar comparability studies with less hands on time
- Simple reproducible protein digestion with minimal user intervention for peptide mapping analysis
- High confidence in workflow results with excellent quality data; excellent coverage and low levels of post-translational modifications (except for M34+oxidation site where noticeable levels were observed)

Goal

Biosimilars are an increasingly important area of interest for the pharmaceutical industry worldwide as patents for the first biologics derived from recombinant technology, including monoclonal antibodies (mAbs), are expiring. The development and demonstration of biosimilarity represents a significant challenge and is required to show the presence or absence of differences resulting from the manufacturing process by investigating the physicochemical and biological properties of a biosimilar candidate molecule compared to the corresponding reference product (innovator). This study evaluated the use of the Thermo Scientific™ SMART Digest™ Trypsin Kit, with Magnetic Bulk Resin option (Magnetic SMART Digest), protocol in combination with the Thermo Scientific™ KingFisher™ Duo Prime purification system to investigate PTMs in the innovator and biosimilar drug substances. The efficiency and reproducibility of the platform was evaluated with a specific focus on the determination of protein sequence coverage and identification of post-translational modifications (PTMs), including deamidation, oxidation, lysine clipping, glycation, and glycosylation.

Introduction

Monoclonal antibodies (mAbs) are an important class of therapeutic proteins and the fastest growing class of therapeutic agents due to their high specificity to target antigens, long serum half-life in humans, and ability to treat a wide range of ailments. Biologics are among the highest-cost treatments on the global market today, which implies the need for low-cost alternatives.[1] As the patents of many biologics expire, the development of biosimilar products with similar quality, safety and efficacy profiles to the original biologics should improve the accessibility of biotherapeutic drugs to patients. In emerging markets, biosimilars already offer more affordable prices, which are of high importance to economies where expensive treatments are not financially feasible.[2]

Regulatory bodies worldwide have already prepared guidelines to regulate the development of biosimilar products.[3,4] Biosimilars or follow-on biologics are biologic products that receive authorization based on an abbreviated regulatory application containing comparative quality and nonclinical and clinical data that demonstrate similarity to a licensed biological product. Regulatory authorities have generally reached the consensus that extrapolation of biosimilarity from one indication to other approved indications of the reference product can be permitted if it is scientifically justified[5] and is an important way to simplify biosimilar development.

The similarity between a proposed biosimilar product and the reference product (innovator) can be affected by many factors. MAb products exist as a mixture of heterogeneous variants due to post-translational modifications that arise during cell culture, purification, and storage. Some of these modifications can alter the biological activity, drug metabolism and pharmacokinetics (DMPK), and immunogenicity and thus may pose a risk to the patient. In this way, extensive analytical testing platforms are needed for in-depth characterization and to ensure product stability, proper in-process controls, safety, and efficacy. During the development and production of therapeutic monoclonal antibodies, characterization of structural variants is a critical challenge. The rigors of biotherapeutic development and analysis have clearly indicated a need for control over every stage of development. The biopharmaceutical industry requires fast and robust analytical platforms to fulfill regulatory requirements involved in the Biologics License Applications (BLA) process.

Infliximab is a tumor necrosis factor (TNF-α) blocker and a chimeric monoclonal IgG1 antibody composed of human constant (75%) and murine variable (25%) regions. It is produced by recombinant cell line cultured by continuous perfusion. TNF-α is a key proinflammatory cytokine involved in chronic inflammatory diseases.[6]
Its hyperactivity and enhanced signaling pathways can be observed in inflammatory diseases where it activates further pro-inflammatory cascades. By binding to both the soluble subunit and the membrane-bound precursor of TNF-α, infliximab disrupts the interaction of TNF-α with its receptor and may also cause lysis of cells that produce TNF-α.[7]

Infliximab was first approved by the United States Food and Drug Administration (FDA) in 1998 as an intravenous injection. It is indicated for the treatment of various inflammatory disorders such as adult or pediatric Crohn's disease, adult or pediatric ulcerative colitis, rheumatoid arthritis in combination with methotrexate, ankylosing spondylitis, psoriatic arthritis, and plaque psoriasis.[8] In clinical trials, multiple infusions of infliximab resulted in a reduction of signs and symptoms of inflammatory diseases and induction of remission in patients who have had an inadequate response to alternative first-

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

line therapies for that disorder.

In September 2013, the European Medicines Agency (EMA) approved the first two biosimilars of infliximab. Now, those two biosimilars together incur more sales than all the other biosimilars on the market combined.[9] In the US market, there are currently two biosimilars of infliximab available that demonstrate a high degree of similarity to the reference product. They are approved for all eligible indications of the reference product.[9,10]

This application note presents the benefits of using the recently developed Magnetic SMART Digest method to perform a comparability study of PTMs for infliximab innovator and biosimilars. An efficient approach that combines automated enzymatic digestion using the Magnetic SMART Digest Kit on a KingFisher Duo Prime purification system, analysis with the high-resolution, accurate-mass (HRAM) capabilities of the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer, and high-resolution chromatographic separation with the Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.2 µm, 2.1 × 250 mm column on a Thermo Scientific™ Vanquish™ Flex binary UHPLC system. Thermo Scientific™ BioPharma Finder™ software was used to interrogate the high-quality data sets.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ•cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical) (P/N 10505904)
- Acetonitrile, Optima™ LC/MS grade (Fisher Chemical) (P/N 10001334)
- Water with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 10118464)
- Trifluoroacetic acid (TFA) (Fisher Chemical) (P/N 10294110)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Iodoacetic acid, sodium salt, 99% (IA) (Acros Organics) (P/N 10235940)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Acclaim VANQUISH C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Equipment

- KingFisher Duo Prime Purification system (P/N 5400110)
- Vanquish Flex Binary UHPLC system including:
  - Binary Pump F (P/N VF-P10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A)
  - System Base Vanquish Horizon (P/N VH-S01-A)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Table 1. Monoclonal antibodies used in the study

Drug	Specifications	Concentration	Type
Infliximab DP1	Sp2/0 cell line	10 mg/mL	Recombinant chimeric IgG1 mAb
Infliximab DP2	Sp2/0 cell line	10 mg/mL	Recombinant chimeric IgG1 mAb
Infliximab BS1	In-house CHO expressed	9.3 mg/mL	Recombinant chimeric IgG1 mAb
Infliximab BS2	In-house HEK expressed	10.7 mg/mL	Recombinant chimeric IgG1 mAb

Sample preparation

Commercially available infliximab monoclonal antibody products (DP1 and DP2) were supplied at different concentrations and two biosimilar (BS1 and BS2) were produced in house using CHO and HEK expression systems, respectively (Table 1). Monoclonal antibody samples were prepared in triplicate.

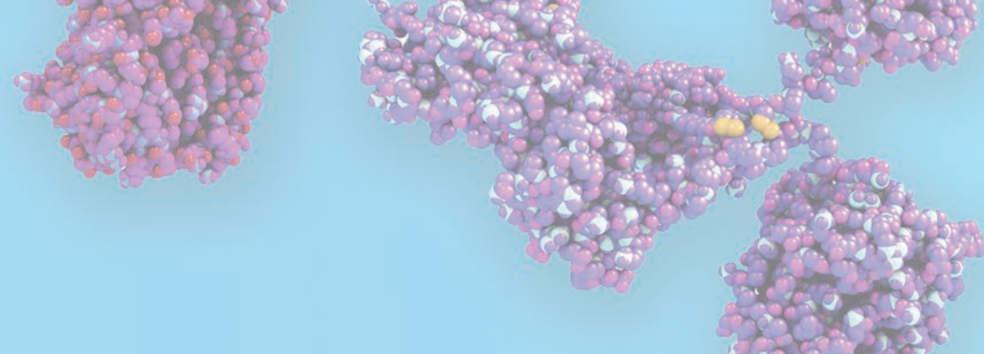
Infliximab biosimilar in-house production

ExpiCHO-S™ Cells (Gibco, #A29127) were derived from a non-engineered subclone that has been screened and isolated from Chinese hamster ovary (CHO) cells. Expi293F™ Cells (Gibco, #A14527) were derived from the human HEK293 cell line, adapted to suspension culture and high-density growth. Cells were cultured in suspension in serum-free, chemically defined media (Gibco), and transiently transfected with plasmid DNA encoding particular monoclonal antibody using a lipid-based transfection system (Gibco). The vectors (pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hk) were purchased from InvivoGen. Following transfection, the cells were harvested, and samples of clarified media were passed through a HiTrap™ Protein A column (GE Healthcare) then washed with phosphate buffered saline (PBS). mAbs were eluted from the Protein A column using 100 mM citric acid, pH 3.2. mAbs solutions were buffered exchanged in PBS and protein concentration was evaluated with a Nanodrop 2000 Spectrophotometer.

Sample preparation using a SMART Digest trypsin kit, magnetic bulk resin option (Magnetic SMART Digest)

Samples were diluted to 2 mg/mL in water. For each sample digest, sample and buffers were added to each lane of a KingFisher Deepwell 96 well plate as outlined in Table 2. Bead "wash buffer" was prepared by diluting SMART Digest buffer 1:4 (v/v) in water. Bead

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



buffer was neat SMART Digest buffer. Digestion was performed using the Kingfisher Duo Prime Purification System with Thermo Scientific™ BindIt™ software (version 4.0), using the protocol outlined in Table 3. Samples were incubated for 45 minutes at 70 °C on medium mixing speed (to prevent sedimentation of beads), with post-digestion cooling carried out to 10 °C. Following digestion, disulfide bond reduction was performed with 10 mM DTT for 30 minutes at 57 °C and subsequently alkylated with 20 mM IA in darkness for 30 minutes. The reaction was quenched with 15.45 µL of 100 mM DTT followed by 15.64 µL 10% TFA (final concentration 11 mM DTT and 1% TFA). Samples were then injected immediately into the LC-MS (3 µg).

Table 2. KingFisher Duo Prime plate layout utilized for sample preparation. Reagents and associated volumes placed in each well are outlined.

Lane	Content	Volume Applied to Each Well (µL)
A	SMART Digest buffer	150
	Sample (2 mg/mL)	50
B	Tip Comb	
C	Empty	
D	Magnetic SMART Beads	15
	Bead Buffer (SMART Digest buffer)	100
E	Bead Wash Buffer (SMART Digest buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

LC conditions

Column:	Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm
Mobile Phase A:	0.1% formic acid aqueous solution
Mobile Phase B:	0.1% formic acid solution in acetonitrile
Flow Rate:	0.3 mL/min
Column Temperature:	25 °C (Still air mode)
Autosampler Temp.:	5 °C
Injection Volume:	10 µL
Injection Wash Solvent:	MeOH:H_2O, 10:90 (v/v)
Needle Wash:	Enabled pre-injection
Gradient:	See Table 4 for details

Table 4. Mobile phase gradient for UHPLC separation of peptides

Time (minutes)	Flow (mL/min)	% Mobile Phase B	Curve
0.00	0.30	2.0	5
45.0	0.30	40.0	5
46.0	0.30	80.0	5
50.0	0.30	80.0	5
50.5	0.30	2.0	5
65.0	0.30	2.0	5

MS conditions

Detailed MS method parameters are shown in Tables 5 and 6.

Table 5. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Thermo Scientific™ Ion Max source with HESI-II probe
Sheath Gas Pressure	40 psi
Auxiliary Gas Flow	10 arbitrary units
Probe Heater Temperature	400 °C
Source Voltage	3.8 kV
Capillary Temperature	320 °C
S-lens RF Voltage	50 V

Table 3. Protocol for automated peptide digestion with the KingFisher Duo Prime system

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	45 min Medium Mix	3 count, 15 s	During digestion: 70 °C Post-digestion: 10 °C	A
Release Beads	Yes, Fast	–	–	–	F

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Table 6. MS method parameters utilized for peptide mapping analysis

General	Setting	MS² Parameters	Setting
Run Time	0 to 65 min	Resolution Settings	17,500
Polarity	Positive	AGC Target Value	1.0×10^5
Full MS Parameters	**Setting**	Isolation Width	2.0 *m/z*
Full MS Mass Range	200–2,000 *m/z*	Signal Threshold	1.0×10^4
Resolution Settings	70,000	Normalized Collision Energy (HCD)	28
AGC Target Value	3.0×10^6	Top-N MS²	5
Max Injection Time	100 ms	Max Injection Time	200 ms
Default Charge State	2	Fixed First Mass	–
SID	0 eV	Dynamic Exclusion	7.0 s
Microscans	1	Loop Count	5

Data processing and software

Thermo Scientific™ Xcalibur™ software version 4.0.27.13 (Cat. No. OPTON-30487) was used for data acquisition and analysis. For data processing, Thermo Scientific™ Biopharma Finder™ software version 3.0 was applied. Detailed parameter settings are shown in Table 7.

Results and discussion

Biosimilar medicines must be produced in accordance with the specific requirements established by the regulatory agencies to prove comparability to the reference product in terms of efficacy, quality, and safety. Thus, the biosimilar is authorized for all or some of the indications approved for the reference product. Dosage and route of administration must be the same as the innovative biological. Biosimilars are complex biological molecules and the required studies to prove biosimilarity are much more challenging than those requested for a generic drug. Comparability studies naturally arise because subtle manufacturing variations may yield micro-heterogeneities that can affect product potency and/or toxicity. Extensive physicochemical characterization of innovator products and the proposed biosimilar should be performed.[10]

Through the physicochemical and functional level comparisons emerged the 'fingerprint' concept from FDA guidelines on biosimilars (2012).[11] This concept is the candidate biosimilar should be highly similar with fingerprint-like similarity. The bases of this program are a set of analytical assessments able to demonstrate that the quality attributes for the biosimilar candidate are highly similar to the quality attributes for the reference product. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) established guidelines in 2004 that describe a comparability exercise to ascertain whether a biologically manufactured product retained its quality despite changes in manufacturing.[12,13] The 1999 ICH Q6B guideline describes how to use analytical procedures to establish acceptance criteria for proteins made by cell culture expression systems.[14] Guidelines outline the basic underlying principles used to set specifications, including characterization, analysis, and manufacturing controls.[15] Further, prespecified quantitative parameters, e.g., primary structure, glycosylation, disulfide structure, charge variants, size variants, biophysical characterization (secondary structure, tertiary structure, and thermal stability), and biological characterization must be identified to determine acceptability of raw materials, excipients and final product.[16]

Two candidate biosimilar mAbs were compared to two commercially available chimeric IgG1 products by peptide mapping analysis using the magnetic SMART trypsin digestion with subsequent LC-MS analysis of the generated peptides. This provided a powerful method for PTMs characterization to ensure mAb quality.

A peptide map is a characteristic fingerprint of a protein and the end product of several processes that provide a comprehensive understanding of the protein being analyzed. Peptide mapping is a routine analysis for the characterization of monoclonal antibodies; however, it often involves tedious sample preparation steps, e.g., pre-treatment, digestion, and clean-up procedures, which might reduce reproducibility due to differences among techniques, technicians, or different partner labs. This variation can be specially challenging when it is necessary to compare different product batches or biosimilar products across months or years. As the data quality is imperative, variation in results might jeopardize product quality, affecting the ultimate product efficacy and safety.

Using the Magnetic SMART Digest Kit automated with the KingFisher Duo Prime system simplifies the process and reduces the time needed for peptide mapping sample preparation. This approach provides significant improvements in reproducibility over existing protocols, which results in fewer sample failures, higher throughput, and the ability to more easily interrogate data. Figure 1 shows four chromatograms of peptides from infliximab drug products and biosimilars, digested with the Magnetic SMART Digest Kit. Obtained base peak chromatograms are very similar but show distinct differences (Figure 1). Each protein to be mapped presents unique characteristics that must be well understood so that the validated development of a peptide map provides sufficient specificity for characterization.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

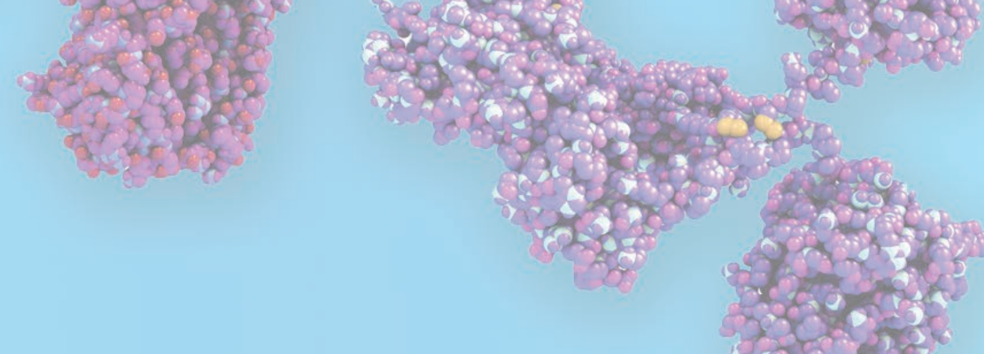
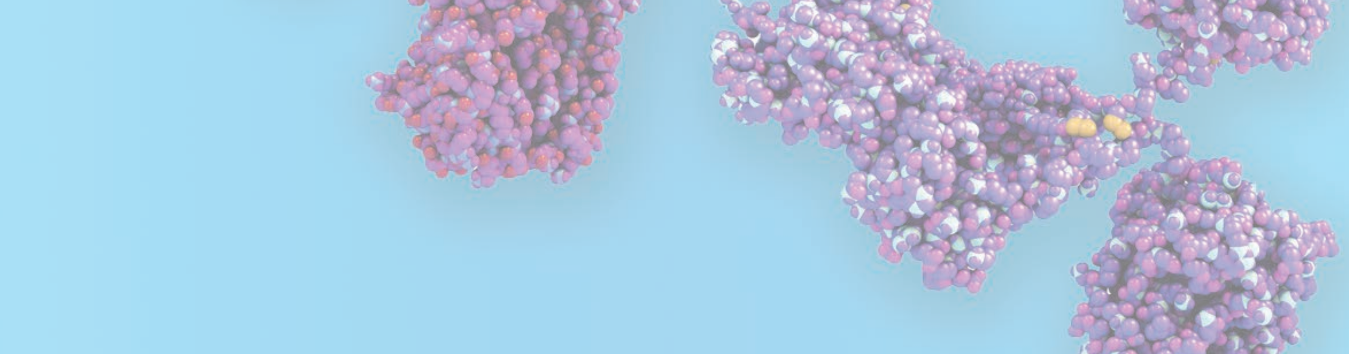


Table 7. Biopharma Finder 3.0 software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS Signal Threshold	8.0 x 10^4 counts
Typical Chromatographic Peak Width	0.3 s
Relative MS Signal Threshold (% base peak)	1.00
Relative Analog Threshold (% of highest peak)	1.00
Width of Gaussian Filter (represented as 1/n of chromatographic peak width)	3
Minimum Valley to Be Considered as Two Chromatographic Peaks	80.0
Minimum MS Peak Width (Da)	1.20
Maximum MS Peak Width (Da)	4.20
Mass Tolerance (ppm for high-res or Da for low-res)	4.00
Maximum Retention Time Shift (min)	1.69
Maximum Mass (Da)	30,000
Mass Centroiding Cutoff (% from base)	15
Identification	**Setting**
Maximum Peptide Mass	7,000
Mass Accuracy	5 ppm
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	1
Unspecified Modification	-58 to +162 Da
N-Glycosylation	CHO
Protease Specificity	High
Static Modifications	**Setting**
Side Chain	Carboxymethylation
Variable Modifications	**Setting**
N Terminal	Gln→Pyro Glu
C Terminal	Loss of lysine
Side Chain	Deamidation (N) Deamidation(Q) Glycation (K) Oxidation (MW)



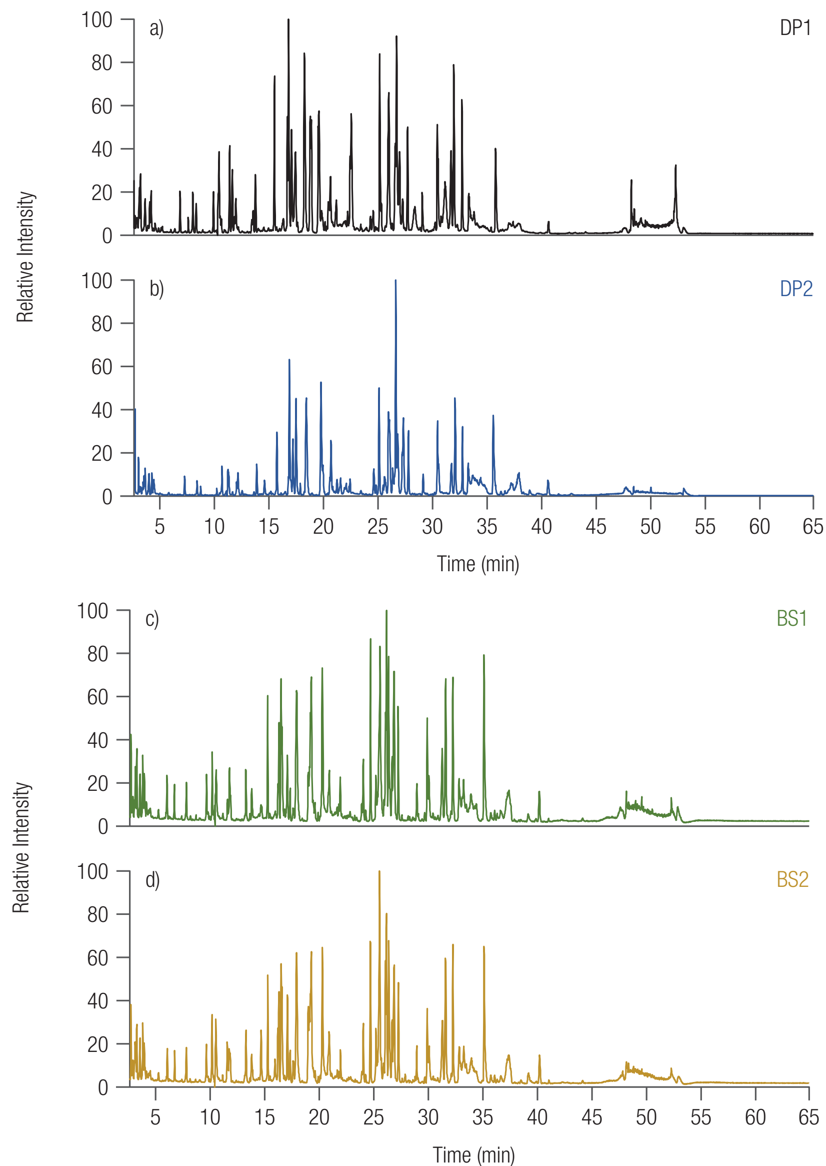


Figure 1. Base peak chromatograms (BPCs) obtained from peptide mapping experiments of a) DP1, b) DP2, c) in-house CHO expressed infliximab, and d) in-house HEK expressed infliximab, after Magnetic SMART digestion with the KingFisher Duo Prime system

Table 8. Sequence coverage for the studied recombinant IgG1 mAbs

Proteins	Sample	Number of Peaks	Sequence coverage (%)
Recombinant Chimeric IgG1			
Heavy Chain	DP1	1116	100.00
	DP2	1473	100.00
	BS1	864	100.00
	BS2	914	100.00
Light Chain	DP1	487	100.00
	DP2	573	98.60
	BS1	312	98.60
	BS2	326	98.60

The first drug product (DP1) can be identified with 100% sequence coverage while DP2, BS1, and BS2 can be identified with 100% sequence coverage for HC and 98.60% for LC (Table 8). The missing peptide corresponds to a tripeptide 2:E143-K145 (sequence EAK), which has probably not been detected due to poor column retention and low intensity signals where confirmatory MS2 data could not be obtained. The same peptide was identified successfully for DP1 as part of a missed cleavage peptide 2:R108-K145, eluting at 24.7 minutes with low signal intensity <4.9e+04.

A sequence coverage map (Figure 2) shows the overlap of the different peptides identified with different intensities and the different lengths due to missed cleavages. As an example, the sequence coverage map is shown for the drug product DP1. The colored bars show the identified peptides, and the numbers in the bars reflect the retention time. The different colors indicate the intensity of the peptide in the MS1 scan.

The number of detected MS peaks in the samples varied between 304 and 535 for light chain and 804 and 1397 for heavy chain. The chimeric commercially available biosimilar DP2 showed the biggest number of detected MS peaks for both heavy and light chain.

thermo scientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

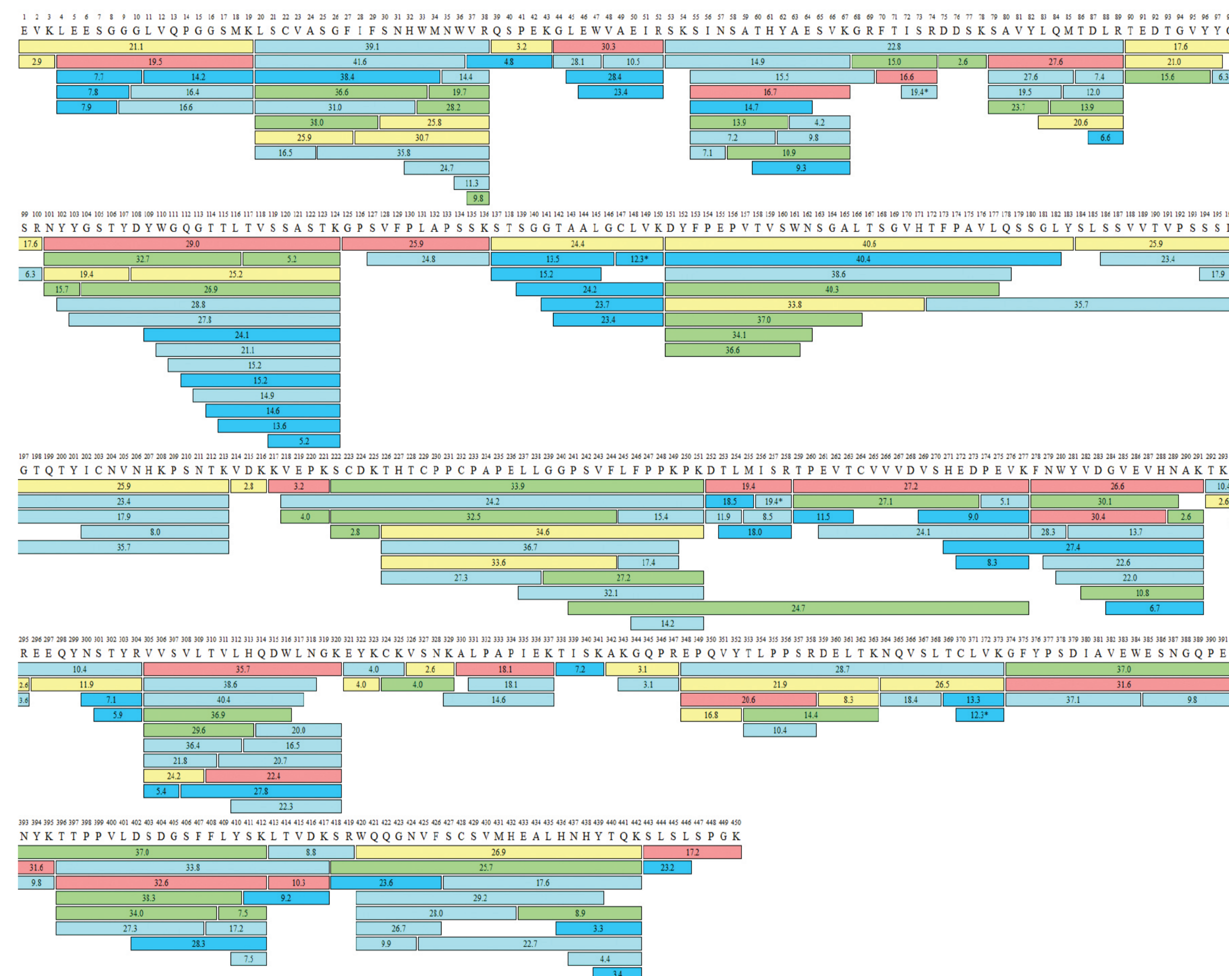
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Heavy chain



Light chain

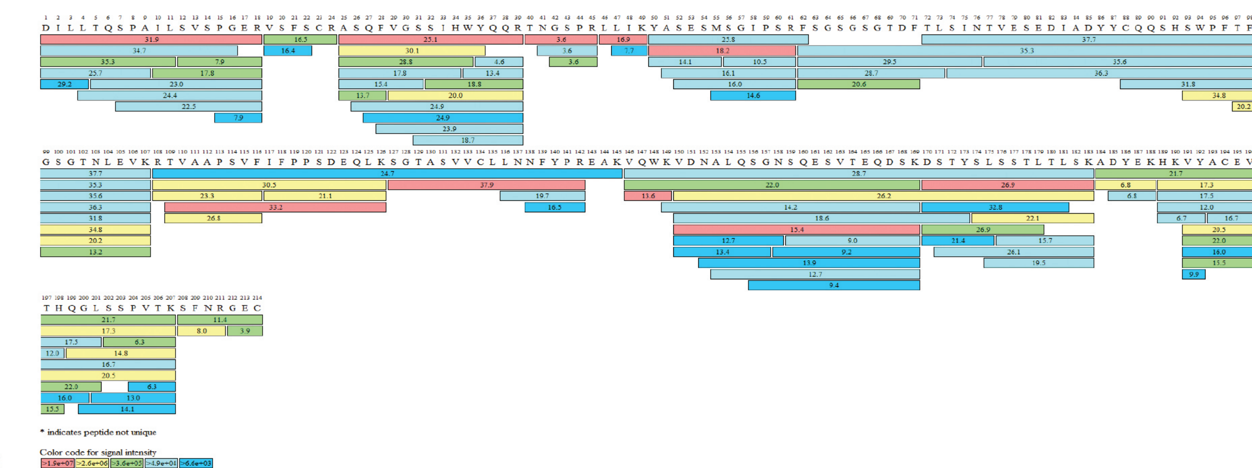


Figure 2. Sequence coverage map of DP1 heavy (top) and light chain (bottom), obtained using a 65 min gradient for peptide separation on an Acclaim VANQUISH C18, 2.2 µm, 2.1 x 250 mm column. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant >1.9e+07; yellow >2.6e+06; green >3.6e+05; light blue >4.9e+04; cyan=low abundant >6.6e+03.

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

In regard to the peptide identification with high confidence, all matched peptides were expected to have ≤ 5 ppm and ≥ 5 ppm of MS mass error, confidence score ≥ 95, and/or confirmatory MS/MS spectra. Figure 3 shows an example of the selected ion chromatograms (SIC) and corresponding MS/MS spectra for the selected peptide LEESGGGLVQPGGSMK present on the four infliximab heavy chains, which elutes at 19.3 min. The combination of high-quality MS and MS/MS data provides a more confident peptide match. The experimental MS/MS spectrum displays an inverted triangle marker at the top of the spectral line for the theoretical precursor ion (773.382 *m/z*, z=2). The labels appear in color for the identified peaks and show their fragment ion assignments and charge states, for example, "b2", "y6", or "y12". The peptide fragment coverage map displays the peptide sequence with corresponding modification, and the peptide sequence with the numbered amino acid sequence and the identified fragment lines, and finally the identified fragment ions using a color-coded code for ion intensity (red, yellow, green, cyan, and blue), with red as most intense and blue as the least intense.

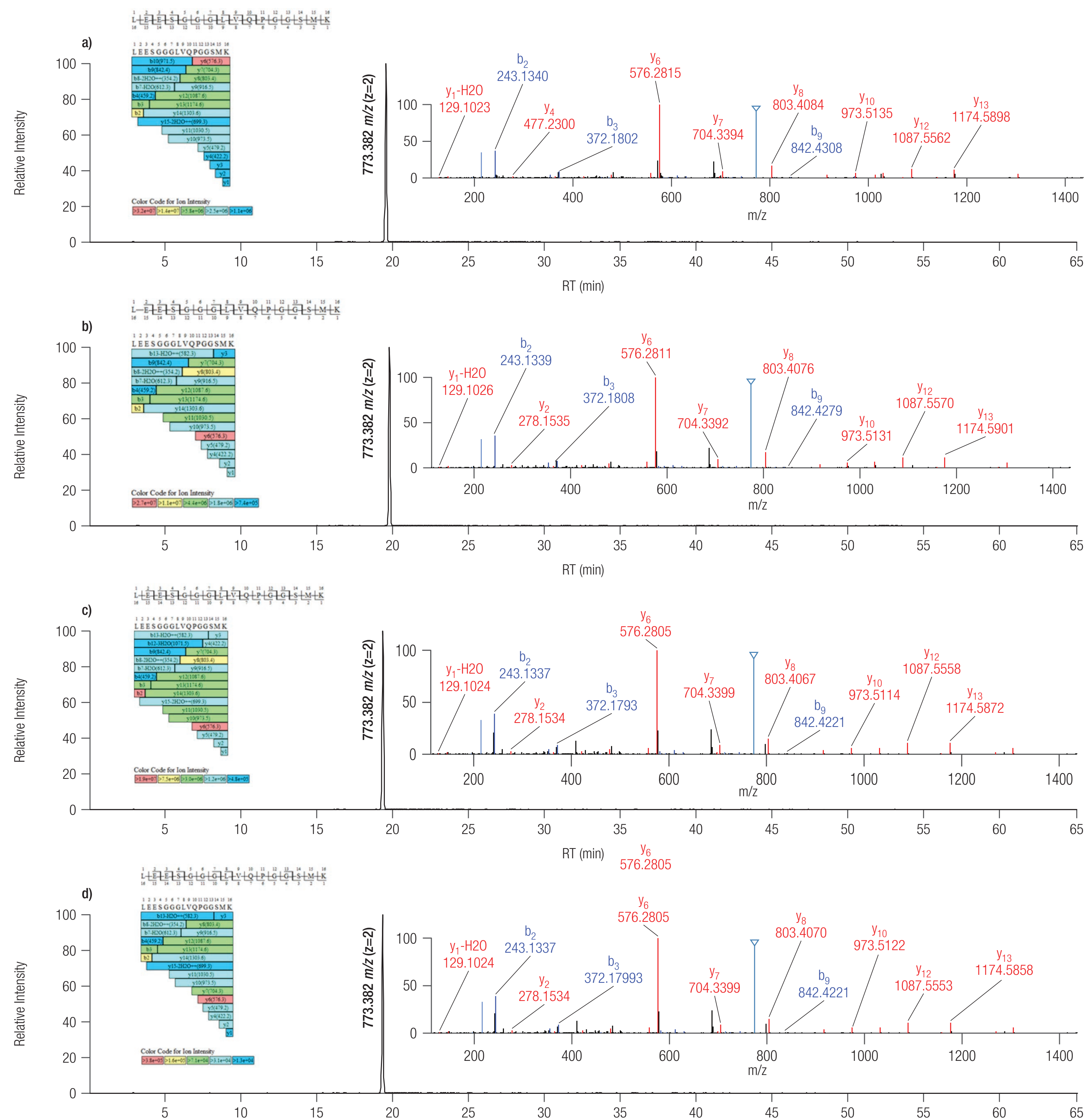


Figure 3. Representative extracted ion chromatogram (XIC) of HC peptide LEESGGGLVQPGGSMK, MS/MS spectra and fragment coverage map from digested (a) DP1, (b) DP2, (c) BS1, and (d) BS2 biotherapeutics. NL indicates normalized level (NL) intensity. The color for the lines and labels for the identified ions in the experimental spectrum vary based on the ion type, as follows: dark blue for "b" ions with a charge on the N-terminal side and red for "y" ions with a charge on the C-terminal side.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Deamidation

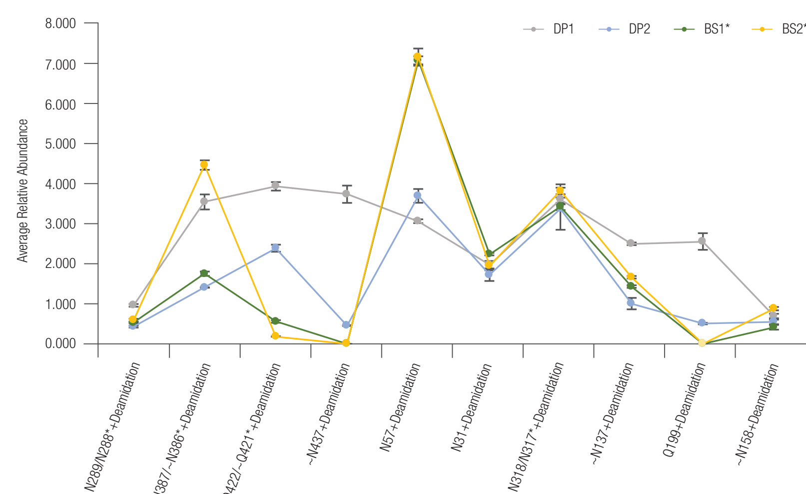


Figure 4. Average relative abundance (n=3) of 10 identified deamidation modifications for infliximab DP1, DP2, and in-house biosimilars BS1 and BS2, respectively. * indicates the different position in biosimilars of corresponding modification in the drug products.

Peptide mapping analysis also facilitates identification and quantitation of PTMs. Many common PTMs cause a shift in reversed-phase LC retention relative to the native peptide. In combination with direct MS and MS/MS analysis it can be used to interrogate modifications with relatively large mass shifts, such as C-terminal lysine (128 Da), glycation (162 Da) and small mass differences such as deamidation (1 Da), oxidation (16 Da), and others.

Table 10 summarizes the identification and relative quantification of a subset of monitored modifications across the infliximab innovator and biosimilar candidates studied. PTMs such as deamidation, oxidation, glycation, C-terminal lysine clipping, and glycosylation are confidently identified based on MS1 and/or MS/MS spectra. A tilde (~) before the modification indicates the modification was found on the parent tryptic peptide but could not be localized on a specific amino acid within the MS/MS spectra. The relative abundance of the detected modifications in the four infliximab products has a relative standard deviation < 10% in most cases. Overall, the method shows that important information regarding PTMs can be obtained reproducibly and accurately.

Deamidation of asparagine (Asn, N)[17] and glutamine (Gln, Q)[18] residues is a common degradation of proteins and it can significantly impact protein structure and function. The rate of deamidation depends on protein sequence and conformation, as well as external factors such as temperature, pH, and time. Figure 4 shows the average relative abundance of eight of the most abundant deamidation modifications for the infliximab products studied. N387/N386 and N57 residues were detected for all the studied mAbs and seem to be more susceptible to PTM showing the most abundant deamidation levels (4.5% for the infliximab biosimilar HEK expressed and around 7% for both studied biosimilars).

In relation to glutamine (Q) deamidation, residue Q422/Q421 of HC seems to be the most susceptible to PTMs, being present at a low level in the biosimilars (<0.5%) and in higher abundances for the drug products between 3.93 and 2.38%.

Microheterogeneity can sometimes be attributed to oxidation of tryptophan (W) or methionine (M) residues. This is another common PTM observed in proteins and peptides. Oxidation of methionine occurs in mAbs during purification, formulation, and storage processes.[19] Oxidation can also occur from frequent freeze-thawing cycles. *In vivo* oxidation is caused by oxygen radicals and other biological factors (e.g., exposure to certain oxidizing drugs or other compounds). *In vitro* oxidation can be due to conditions encountered during purification or formulation. Protein chemists in process development and quality control are concerned with oxidation as it can adversely impact the activity and stability of biotherapeutics.[20] The studied infliximab products in the present work show low oxidation levels between 0.2 and 3.0% except for M34 which is potentially susceptible to oxidation (Figure 5). This was a common modification for all the four infliximab studied products, showing highest levels for the biosimilars produced in-house by CHO and HEK expression systems (32.47% and 27.99%, respectively) and for DP1 (13.66%). It is noteworthy to mention higher levels have been found in the literature for the studied DP infliximab products, which could be attributed to the fact that this PTM is susceptible to change and varies between production batches. Pisupati *et al.*[21] have recently published a multidimensional analytical comparison of Remicade and the biosimilar Remsima/Inflectra where the levels of oxidation were remarkably similar for both mAbs, showing highest oxidation levels for the M34 site (26.44% and 28.69%, respectively), M255 site (20.80% and 21.53%, respectively), and M18 (6.71% and 6.94%, respectively).

Oxidation

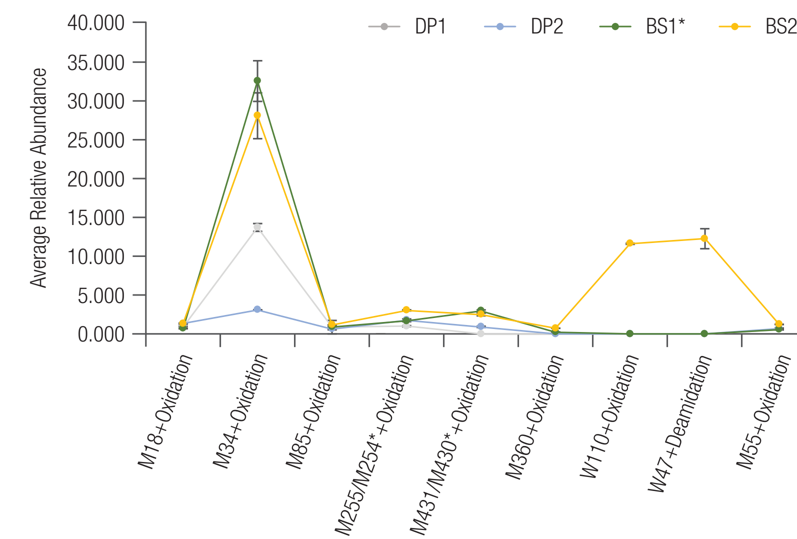


Figure 5. Average relative abundance (n=3) of nine identified oxidation modifications for infliximab drug products (DP1 and DP2) and two biosimilars produced in-house by CHO (BS1) and HEK (BS2) expression systems, respectively. * indicates the different position in biosimilars of corresponding modification in the drug products.

These differences highlight the importance of monitoring CQAs along the production batches.

Some of the most noted PTMs for therapeutic mAbs are their varied *N*-linked glycan structures, which include galactosylation, fucosylation, mannosylation, and sialylation. Glycosylation is a highly variable and heterogeneous process that depends on such factors as clonal variation, production cell line, media, and culture conditions[22-24] and their characterization and quantification are critical to ascertain therapeutic efficacy and safety of the drug. *N*-glycans have important structural functions as they stabilize the CH2 domain of IgGs. Deglycosylation makes mAbs thermally less stable and more susceptible to unfolding and they are more prone to aggregation. Moreover, functionality of the IgG is influenced by the attached *N*-glycans and their size.[25] MAbs produced by Chinese hamster ovary (CHO) cells typically have complex

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

biantennary structures with no bisecting *N*-acetylglucosamine (GlcNAc) and a high level of core fucosylation. Overexpression of *N*-acetylglucosaminetransferase III in such cell lines increases bisecting GlcNAc and nonfucosyated oligosaccharides on mAbs and thus raises ADCC (antibody-dependent, cell-mediated cytotoxicity).[26]

N-Glycosilation

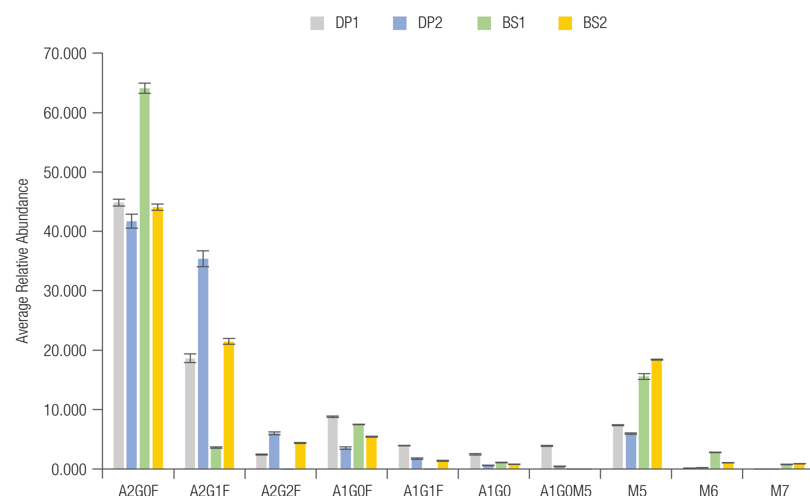


Figure 6. Average relative abundance (n=3) of identified *N*-glycosylation on the Fc region for infliximab drug products and two biosimilars produced in-house by CHO and HEK expression systems, respectively

High abundance of glycosylation of the heavy chain is also observed for the four studied infliximab products in the Fc region at position N300 (DP1 and DP2) or N299 (in-house produced infliximab biosimilars expressed by CHO and HEK cell lines), where the main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the reducing end of *N*-acetylglucosamine (A2G0F, A2G1F, A2G2F, and A1G0F). Also present at lower levels are afucosylated biantennary (A1G0) and high mannose (M5, M6, and M7) structures (Figure 6). Core fucosylation is relatively quantified between 75.16% (biosimilar produced in-house CHO expressed) and 88.31% (DP2).

Glycosylation of the Fc region is also important for maintaining a long catabolic half-life.[27] IgGs containing high-mannose glycans have shown increased serum clearance,[28] and in addition to terminal sialic acid leads to upregulation of the surface expression of the FcγRIIb on inflammatory cells, thereby initiating the anti-inflammatory cascade.[29] Mannose-5 (M5) N-glycans are detected in high abundance for infliximab biosimilars produced in-house by CHO (15.58%) and HEK (18.38%) cell lines. BS1 and BS2 also contain low levels of higher mannose structures such as M7 and M8 (0.33–0.86%), which
have not been detected for DP1 and DP2 (Table 10). According to the literature, high levels of M5 were observed during development of a therapeutic mAb produced in CHO cell line and correlated to the increase of cell culture medium osmolality levels and culture duration.[30] *N*-acetylneuraminic acid (Neu5Ac or NANA) is detected only for DP1 and DP2 at levels < 0.5%. Those infliximab samples also showed low levels of immunogenic *N*-glycolylneuraminic acid (Neu5Gc or NGNA) between 0.10% and 2.88%. The presence of Neu5Gc in recombinant therapeutic proteins expressed in nonhuman cell cultures may be immunogenic and potentially relevant to half-life, efficacy, and adverse events.[31,32] Figure 7 shows the comparison between high mannose, non-galactosylated and galactosylated glycans relatively quantified for the four studied infliximab drug products. Non-galactosylated biantennary *N*-glycan structures are detected with the highest relative abundances (60.76% for DP1 and 76.21% for infliximab biosimilar produced in-house CHO expressed), while the highest levels of galactosylation are detected for infliximab drug product DP2 (45.17%) and infliximab biosimilar produced in-house HEK expressed (27.81%). Regarding high mannose content there is a noticeable variability between the four studied samples, DP2 contained the lowest high mannose content (6.86%) and infliximab biosimilars in-house CHO and HEK expressed with the highest levels (20.03% and 20.75%, respectively).

More detailed information on the glycosylation of infliximab can be found in Application 3648 in the Thermo Scientific™ AppsLab Library of Analytical Applications where a detailed *N*-glycosylation from commercial chimeric IgG1 mAb (infliximab) is shown.[33] The Vanquish Horizon UHPLC system was used for the high-resolution determination of 2-AA (anthranilic acid) labelled N-glycans released from infliximab DP. Separation was performed on a 150 mm Thermo Scientific™ Accucore™ 150 Amide HILIC column with fluorescence detection giving separation in less than 30 minutes.

The C-terminal lysine (Lys) variant is a very common modification observed in monoclonal antibodies and recombinant proteins.

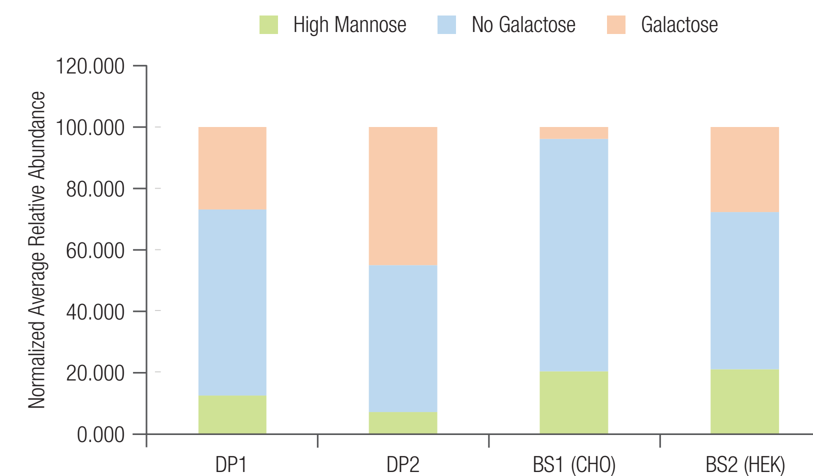


Figure 7. Normalized average relative abundance (n=3) of identified high-mannose, non-galactosylated and galactosylated *N*-glycans for infliximab drug products, and two biosimilars produced in-house by CHO and HEK expression systems, respectively

Although the effect this variation has on protein activity does not seem to impact potency or safety profile,[34] the degree of heterogeneity of C-terminal Lys variants reflect the manufacturing consistency and should be monitored for product consistency. Lys loss is detected in only three of the four infliximab samples and at different levels (Table 10), with the lowest % of modification for DP1 (38.89%) and the highest level of modification detected for the infliximab biosimilars produced in-house from CHO (94.21%) and HEK (98.77%).

Other commonly targeted modifications are lysine (K) glycations, which are listed in Table 10. In total between 3 and 14 lysine glycations could be identified and relatively quantified for DP1 (<0.13%) and DP2 (< 0.84%), while infliximab biosimilar produced from CHO shows the highest levels of glycation (<2.98%) with more than 20 lysine residues modified. Interestingly, this modification was not observed for infliximab biosimilar from HEK.

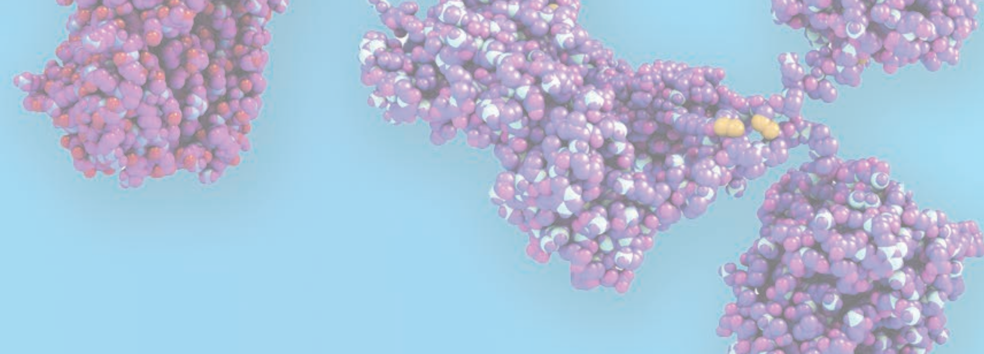


Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Table 10. Comparison of the deamidation, oxidation, glycation, lysine-loss, and glycosylation modifications identified for the different infliximab drug products and biosimilars studied. (n=3). * indicates the different position in biosimilars of corresponding modification in the drug products.

	Relative Abundance			
Modification	DP1 (n=3)	DP2 (n=3)	BS1* (n=3)	BS2* (n=3)
Q13+Deamidation	1.071	0.779	0.156	-
N31+Deamidation	1.972	1.718	2.236	1.945
Q39+Deamidation	0.833	0.639	-	0.212
N57+Deamidation	3.057	3.688	7.066	7.146
Q84+Deamidation	0.905	0.628	0.142	0.262
N101+Deamidation	0.882	-	0.969	1.259
Q112+Deamidation	0.997	-	-	-
N162/N161*+Deamidation	0.599	0.787	-	0.432
N279+Deamidation	0.142	-	-	-
N289/ N288*+Deamidation	0.960	0.430	0.529	0.590
N318/N317*+Deamidation	3.603	3.368	3.424	3.811
N328+Deamidation	-	0.159	-	-
N364/ N363*+Deamidation	1.728	0.939	0.240	1.240
Q365/ Q364*+Deamidation	2.626	0.458	0.995	0.154
~N387/~N386*+Deamidation	3.537	1.404	1.748	4.456
~N392+Deamidation	-	1.985	-	-
~Q422/~Q421*+Deamidation	3.926	2.384	0.558	0.183
~N437+Deamidation	3.729	0.456	-	-
Q6+Deamidation	1.152	0.734	0.128	0.231
~Q27+Deamidation	-	-	0.115	-
~Q37+Deamidation	1.668	1.269	0.261	0.671
~N137+Deamidation	2.494	1.003	1.428	1.660
Q147+Deamidation	0.864	0.449	-	0.169
~N158+Deamidation	0.694	0.547	0.413	0.878
~Q166+Deamidation	1.833	1.109	-	-
Q199+Deamidation	2.551	0.506	-	-
N210+Deamidation	0.255	0.217	-	-
~Q155+Deamidation	-	1.075	-	0.247
M18+Oxidation	0.875	1.324	0.679	1.310
M34+Oxidation	13.660	3.062	32.446	27.992
M85+Oxidation	0.861	0.605	0.898	1.142
M255/M254*+Oxidation	0.991	1.745	1.652	3.017
~M360+Oxidation	-	-	0.232	0.723
M431/M430*+Oxidation	-	0.905	2.934	2.487
M55+Oxidation	0.566	0.702	0.543	1.217
W47+Oxidation	-	-	-	12.214
W110+Oxidation	-	-	-	11.564
K67+Glycation	0.027	0.201	0.878	-
K149+Glycation	0.100	0.842	2.266	-
K188+Glycation	0.129	0.152	2.983	-
K54+Glycation	-	0.037	0.327	-
K136/K135*+Glycation	-	0.290	1.272	-
K150+Glycation	-	0.419	-	-
K251/~K250*+Glycation	-	0.080	0.521	-
~K291/~K290*+Glycation	-	0.235	1.306	-
K320/K319*+Glycation	-	0.140	0.403	-
K329/K328*+Glycation	-	0.341	1.377	-
K49+Glycation	-	0.660	1.468	-
K169+Glycation	-	0.188	0.637	-

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
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Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

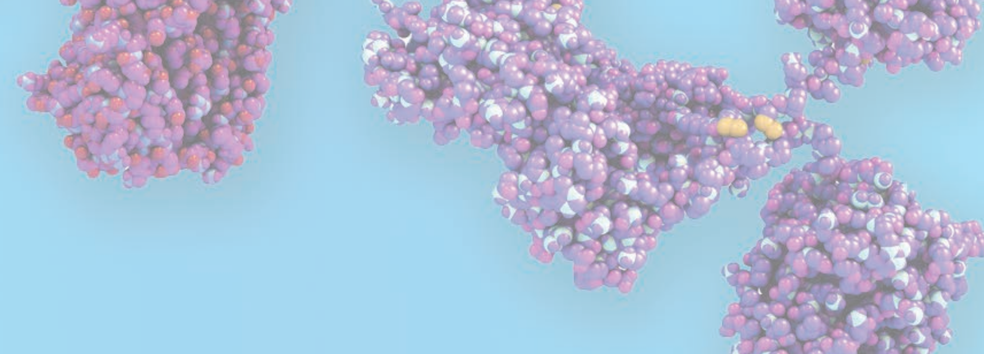

Table 10 (continued). Comparison of the deamidation, oxidation, glycation, lysine-loss, and glycosylation modifications identified for the different infliximab drug products and biosimilars studied. (n=3). * indicates the different position in biosimilars of corresponding modification in the drug products.

	Relative Abundance			
Modification	DP1 (n=3)	DP2 (n=3)	BS1* (n=3)	BS2* (n=3)
K3+Glycation	-	-	0.235	-
K19+Glycation	-	-	0.082	-
K78+Glycation	-	-	0.383	-
K207+Glycation	-	-	0.336	-
K224+Glycation	-	-	0.517	-
K322+Glycation	-	-	0.238	-
K336+Glycation	-	-	0.239	-
K362+Glycation	-	-	0.195	-
K145+Glycation	-	-	0.268	-
K190+Glycation	-	-	0.305	-
K450/K449*+Lys Loss	38.892	-	94.212	98.768
N300/N299*+A1G0	2.466	0.551	1.065	0.800
N300/N299*+A1G0F	8.785	3.523	7.471	5.410
N300+A1G0M4	1.705	0.241	-	-
N300+A1G0M5	3.866	0.417	-	-
N300+A1G0M5F	1.831	-	0.568	-
N300+A1G1F	3.908	1.713	3.573	1.359
N299+A2G1FB	-	-	-	0.282
N300+A1G1M5	1.359	-	-	-
N300+A1G1M5F	0.524	-	-	-
N300+A1S1M5	0.512	0.400	-	-
N300+A1Sg1	0.318	0.099	-	-
N300+A1Sg1F	2.100	0.983	-	-
N300/N299*+A2G0	1.660	0.822	0.818	0.204
N300/N299*+A2G0F	44.860	41.700	64.118	44.047
N300+A2G1	0.370	0.422	-	-
N300/N299*+A2G1F	18.605	35.393	3.573	21.467
N300/N299*+A2G2F	2.401	5.985	-	4.349
N299+A3G0F	-	-	1.295	-
N300+A2Sg1G0F	0.757	2.628	-	-
N300+A2Sg1G1F	-	2.881	-	-
N299*+A2S1G1F	-	-	-	0.238
N300+A2Ga1G1F	-	0.344	-	-
N300+A2Sg2F	-	0.260	-	-
N300+M4	0.094	0.053	-	-
N300/N299*+M5	7.329	5.917	15.581	18.378
N300/N299*+M6	0.121	0.208	2.767	1.036
N299/N299*+M7	-	-	0.756	0.861
N299/N299*+M8	-	-	0.537	0.326
N300/N299*+Unglycosylated	0.390	0.517	1.222	0.738

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Conclusions

- The Magnetic SMART Digest Kit provides simple and rapid protein digestion for peptide mapping analysis and PTM investigations for comparability studies between innovator and biosimilar monoclonal antibodies.
- Analysis of four infliximab samples gave excellent quality data with high confidence in the results.
- Excellent sequence coverage (~100%) was observed.
- Low levels of post-translational modifications (PTMs) were observed with the Magnetic SMART Digest Kit, except for the M34+oxidation site, where noticeable levels were relatively quantified.
- Peptide mapping was easily automated, resulting in less sample handling, increased productivity, and improved reproducibility, even with peptides at low levels. This will allow confident transfer of methods between laboratories.
- Infliximab biosimilarity of the primary structure and PTMs was achieved successfully by the analytical approach using automated magnetic SMART digestion for peptide generation and subsequent LC-MS analysis. The Thermo Scientific peptide mapping workflow provided reproducible results with excellent mass accuracy and high sensitivity.
- BioPharma Finder software can provide automatic data processing, peptide sequence matching, and protein sequence coverage mapping accurately and with high confidence.

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Find out more...

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

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Application benefits

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

Goal

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

Introduction

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

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

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

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

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

The NISTmAb is a recombinant humanized IgG1κ expressed in murine suspension culture, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process related impurities. It is a ≈150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds. The molecule has a high abundance

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

of N-terminal pyroglutamination, C-terminal lysine clipping, and glycosylation of the heavy chain sub-units. The protein also has low abundance PTMs including methionine oxidation, deamidation, and glycation.[8] In this study, both light and heavy chain sequence coverage was assessed, as well as the identification and relative quantification of a specific set of PTMs: oxidation, glycosylation, and deamidation.

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

Experimental

Recommended consumables

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

Equipment

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

Sample preparation

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

SMART Digest Kit protocol

Duplicate 50 µL mAb samples were taken and adjusted to 2 mg/mL with water. This was further diluted 1:4 (v/v) with the SMART Digest buffer provided with the kit. The solution was transferred to a reaction tube containing 15 µL of the SMART digest resin slurry, corresponding to 14 µg of heat-stabile, immobilized trypsin. A time course experiment was carried out where tryptic digestion was performed at 70 °C for 15, 30, 45, 60, and 75 min with agitation at 1400 rpm. After the digestion the reaction tube was centrifuged twice at 7000 rpm for 2 min to remove any solid residue. Disulfide bonds were then reduced by addition of 1 M to a final concentration of 5 mM DTT and incubation for 30 minutes at 37 °C. All samples were diluted with 0.1% formic acid (FA) in water and 3 µg were loaded on the column for all runs.

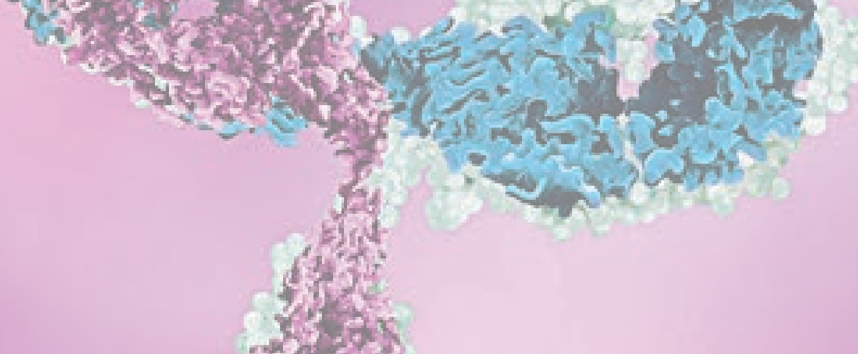
LC conditions

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

Table 1. Mobile phase gradient for UHPLC separation of peptides

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

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



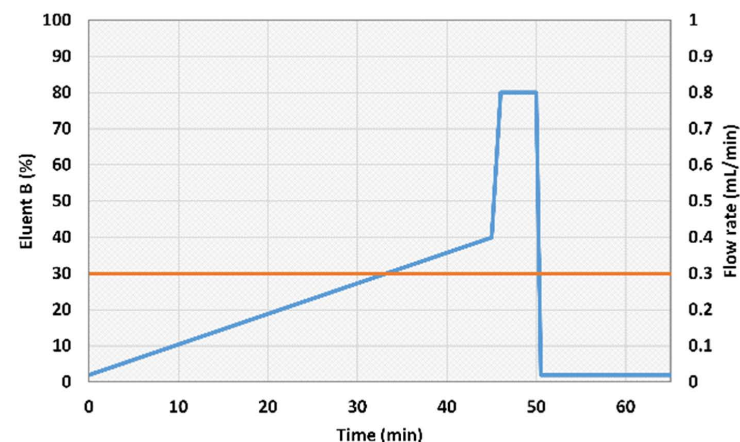


Figure 1. Mobile phase gradient for UHPLC separation of peptides

MS Conditions

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

Table 2. MS source and analyzer conditions

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

Data processing

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

Table 3. MS method parameters utilized for peptide mapping analysis

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

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

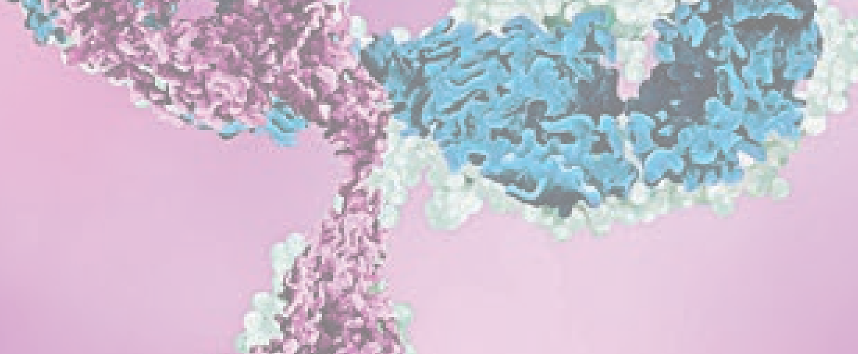


Table 4. Biopharma Finder 3.0 software parameter settings for analysis of peptide mapping data

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

Results and discussion

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

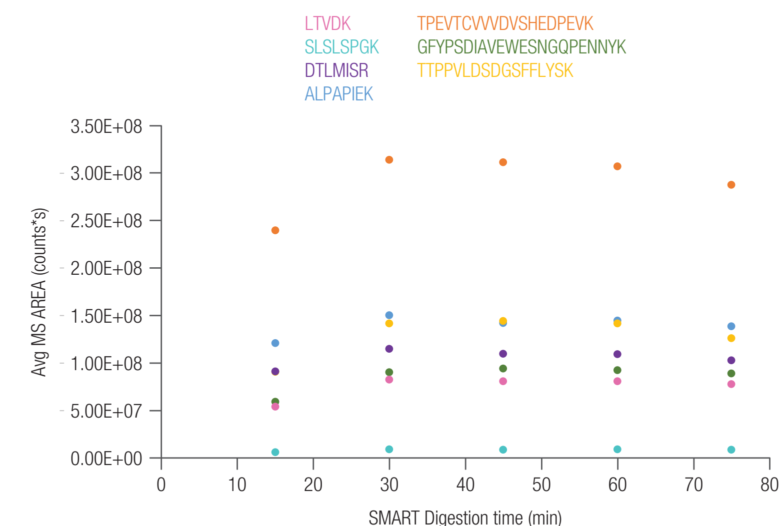


Figure 2. NISTmAb resulted peptides after digestion times from 15 to 75 min using the SMART Digest Kit: LTVDK (10.49 min), SLSLSPGK (17.35 min), ALPAPIEK (18.17 min), DTLMISR (19.45 min), TPEVTCVVVDVSHEDPEVK (26.40 min), GFYPSDIAVEWESNGQPENNYK (31.46 min), and TTPPVLDSDGSFFLYSK (32.49 min) The graph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

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

Figure 4 illustrates extracted ion chromatograms (XICs) of seven identified peptides of NISTmAb, which has been digested with immobilized trypsin of the SMART Digest Kit. Identified peptides

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

are labeled with their corresponding sequence number and chromatographic retention time. Using the Vanquish Flex Binary UHPLC system, stable retention times were observed for all measured peptides as shown in Figure 4. The retention time precision (RSD) for the monitored peptides was <0.2%.

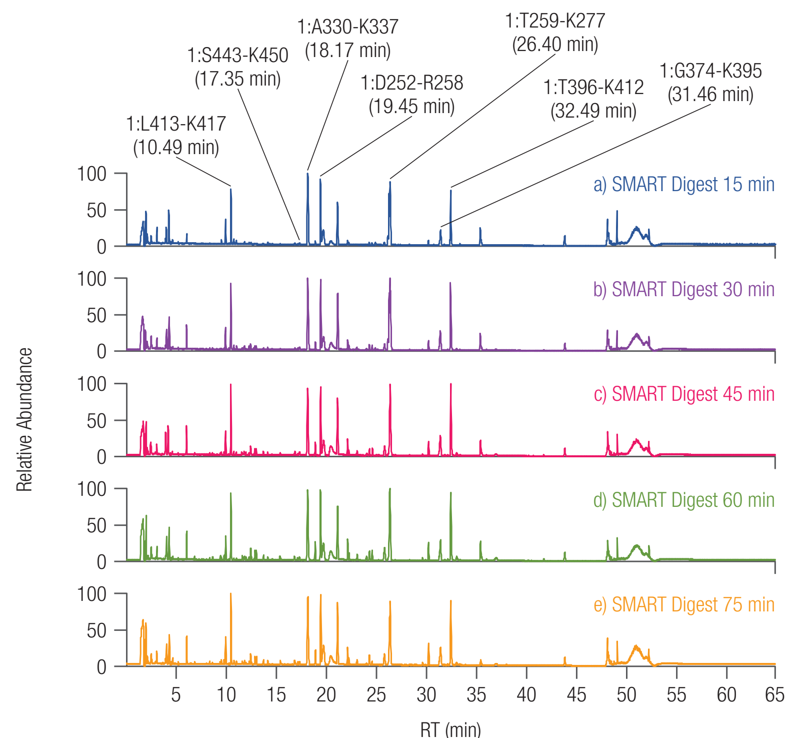


Figure 3. Base peak chromatograms (BPC) for the time course experiment SMART Digested NISTmAb sample: a) Smart Digest, 15 min; b) Smart Digest, 30 min; c) Smart Digest, 45 min; d) Smart Digest, 60 min; e) Smart Digest, 75 min

The sequence coverages for the different digest conditions are shown in Table 5. For all evaluated methods using the SMART Digest Kits, 100% coverage was achieved for light and heavy chains in all cases for all studied digestion times between 15 and 75 minutes. As confidence in the results is imperative, only results with ≥ 95% confidence were accepted, and identification was based on full MS and MS² spectra. These criteria will support strongly the confident identification of peptide sequences.

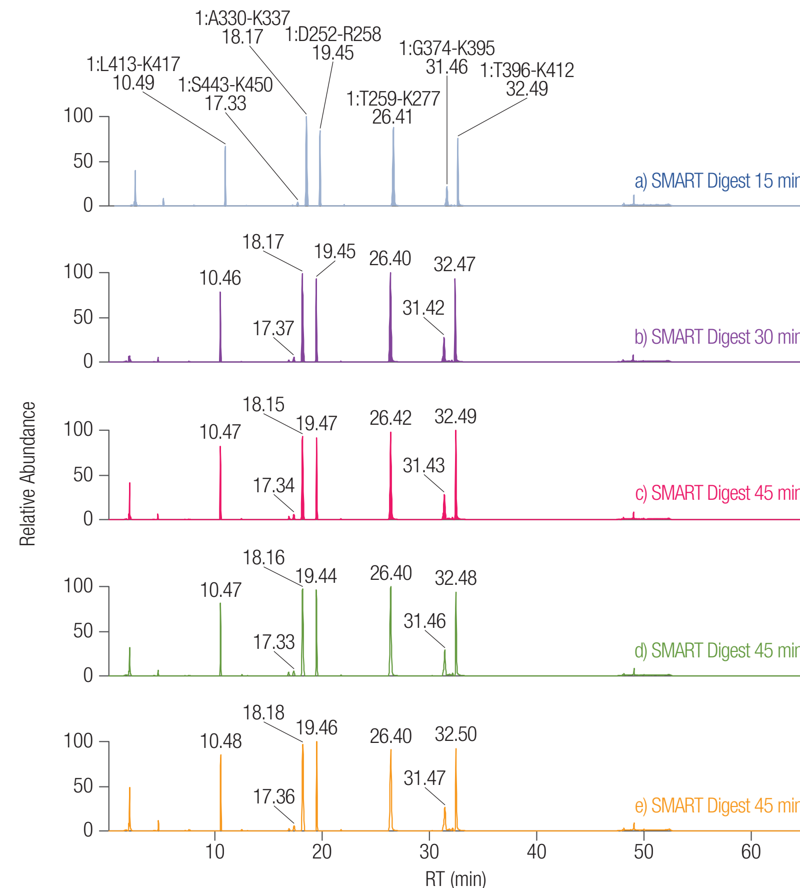


Figure 4. Extracted ion chromatograms of seven identified peptides for the time course experiment SMART digested NISTmAb sample highlighting the retention time precision: a) SMART Digest, 15 min; b) SMART Digest, 30 min; c) SMART Digest, 45 min; d) SMART Digest, 60 min; e) SMART Digest, 75 min

The sequence coverage map based on a 30-minute digest (Figure 5) shows the overlap of the different peptides identified with different intensities and in different lengths due to missed cleavages. The colored bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the intensity of the peptide in the MS1 scan: red = high abundance, >1.7e+007; yellow, >1.5e+006; green, >1.4e+005; light blue, >1.2e+004; cyan = low abundance, >1.0e+003.

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

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

The number of detected MS peaks in the samples digested were generally over 1000, obtaining maximum values for digestion times of 30–45 minutes (~1360 identified components). The same trend was observed when the number of identified components, including all peptides and charge states, and the total MS ion count were compared (Table 6).

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

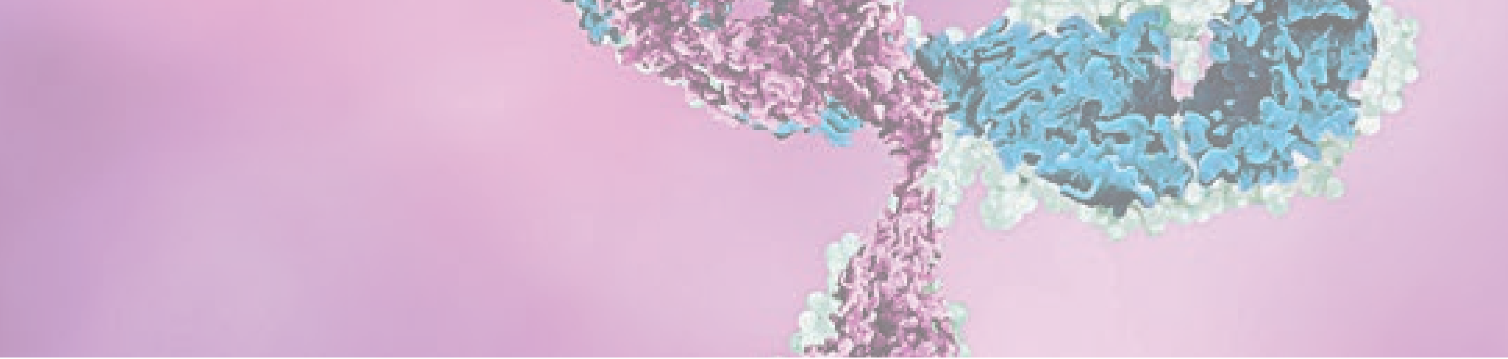


Figure 5. Sequence coverage map of NISTmAb heavy (upper panel) and light (lower panel) chain, obtained using a tryptic SMART Digest time of 30 minutes

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

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

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

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

ThermoFisher SCIENTIFIC

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
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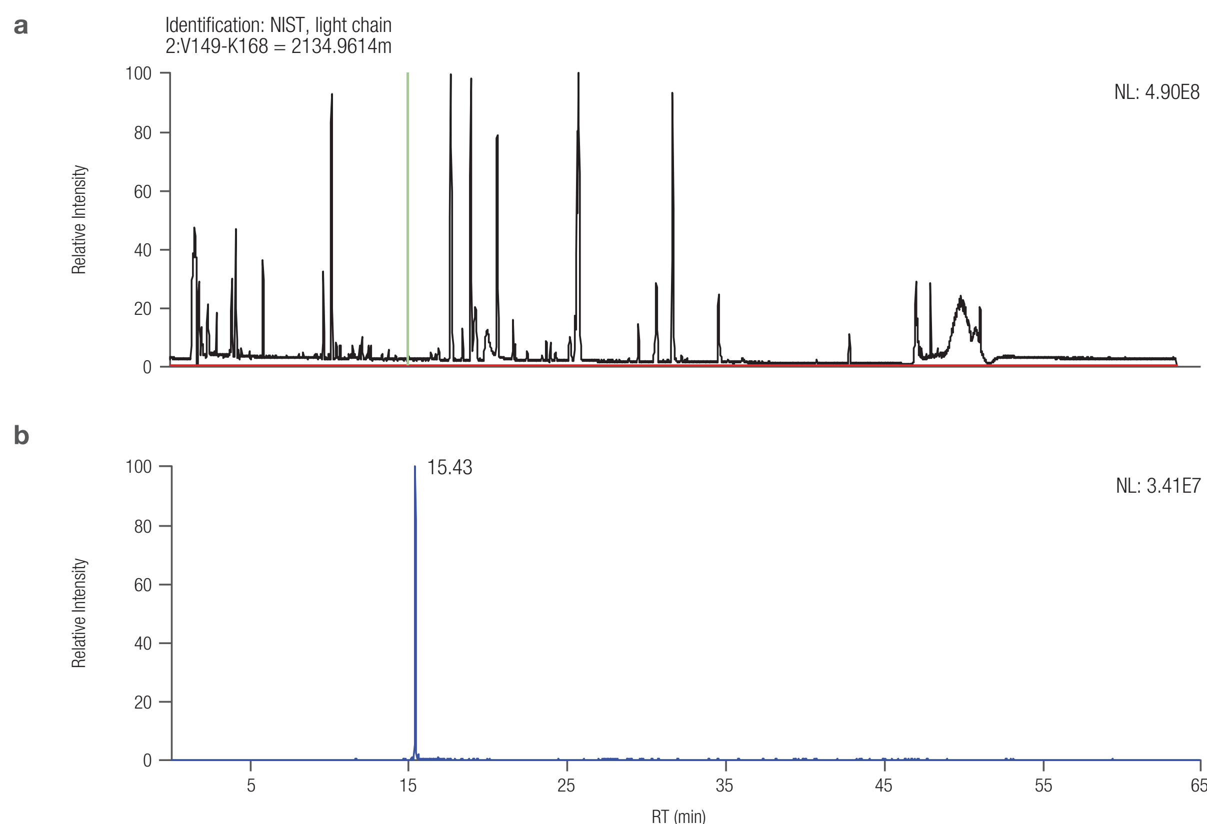
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



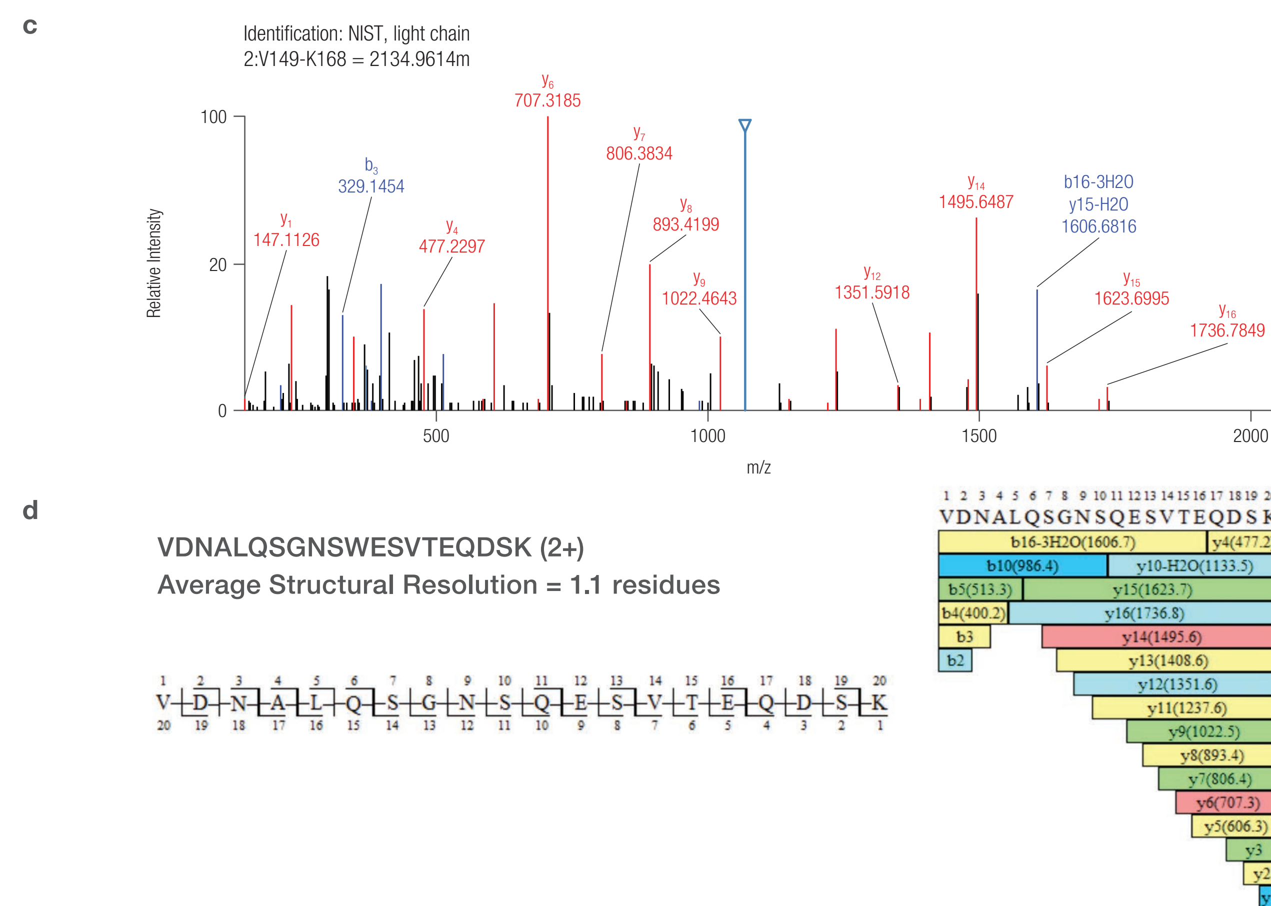
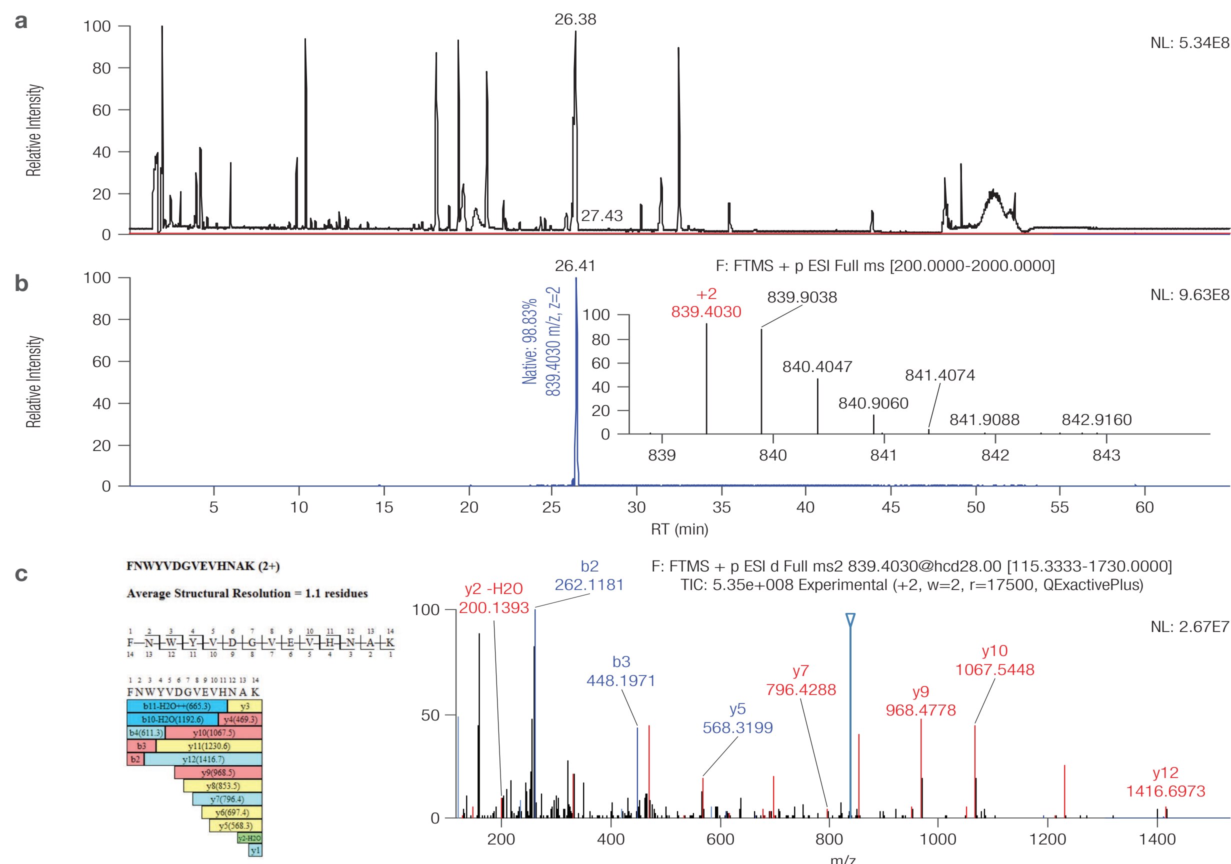


Figure 6. Representative BPC chromatogram (a), XIC (b). NL indicates normalized level (NL) intensity. (c), and fragment coverage map (d) of a light chain peptide from digested NIST mAb.

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

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

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



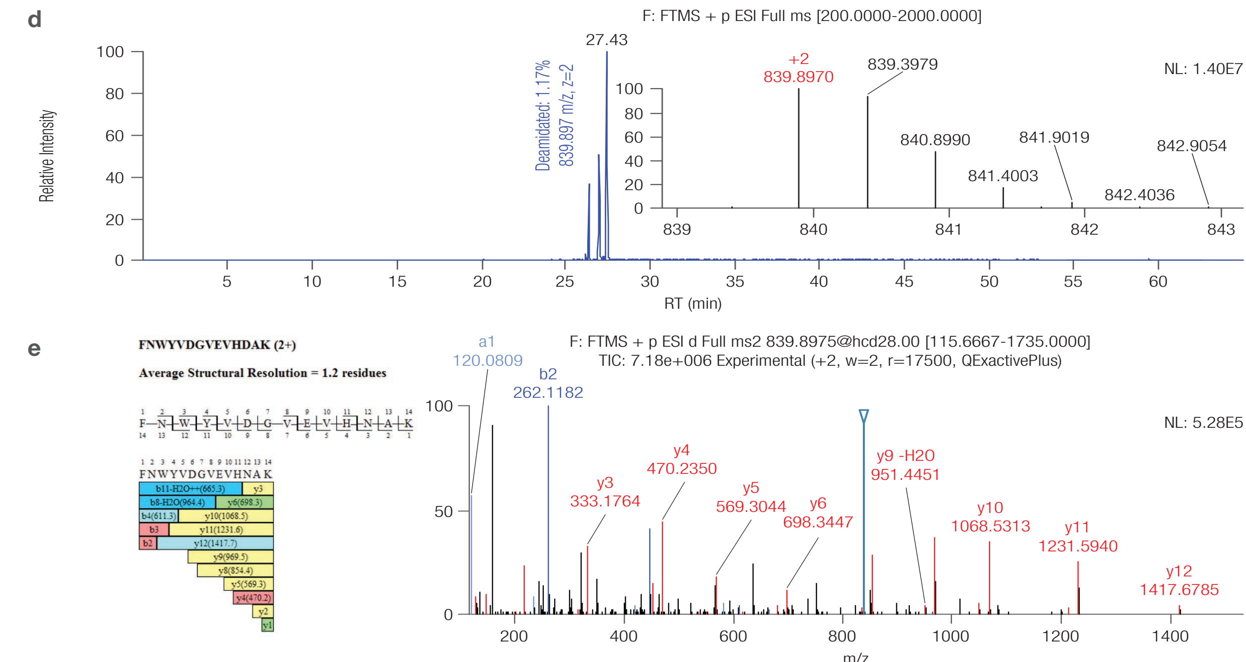


Figure 7. Post-translational modification (N289 asparagine deamidation) analysis for a heavy chain peptide (1:F278-K291) from NISTmAb. BPC (a), XIC for native (b) and deamidated peptide (d) and MS/MS spectra for native (c) and deamidated form (e). NL indicates normalized level (NL) intensity.

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

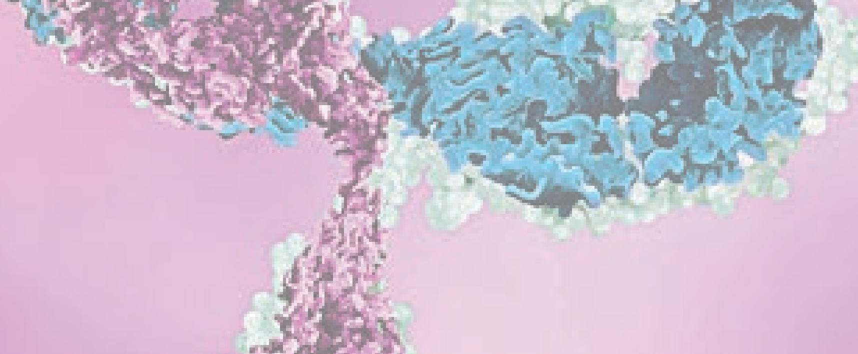
High abundance of glycosylation of the heavy chain is also observed where the main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the reducing end of N-acetylglucosamine (A2G0F, A2G1F, and A2G2F) and high mannose structures (M5).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

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

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

The degree of deamidation increases with extended digestion time. The lowest deamidation rate was observed for the sample digested for 15 min using the SMART Digest Kit. Deamidation is, in general, accelerated at high temperatures and high pH values,[15] which could explain the increase in the relative total amount of deamidated

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



peptides when increasing digestion time. Oxidation was presented in low levels with similar relative abundances between the different digested samples. Methionine (M) oxidation was <2.95%, while tryptophan (W) oxidation was <2.7% for the studied digestion times.

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

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

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

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

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

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Conclusions

- Using the Thermo Scientific SMART Digest Kit provides a simple and rapid protein digestion protocol for peptide mapping analysis, which is more efficient and reproducible than traditional methods.
- The analysis of NISTmAb RM 8671 produced excellent quality data with high confidence in results. Sequence coverage was high and sample preparation-induced post-translational modifications (PTMs) while observed were low, except for deamidation of N318 and N387 residues, which are potentially more susceptible to deamidation (as previously described in AN21782).
- The time course experiment comparing increasing digestion times with the NISTmAb, showed no substantial difference between the different approaches for either data quality or data information content. Protein sequence coverage of 100% was achieved for the five digestion times tested.
- The analysis of the most commonly targeted modifications were successfully identified and relatively quantified. The elevated temperatures during enzymatic digestion using the SMART Digest Kit showed only a very slight predictable increase over time in the amount of induced deamidation except for deamidation of N318 where 10.62% modification was observed when using a 75-minute digestion time.. It was also shown that optimization of the incubation time can be used to further minimize the introduction of chemical modification during digestion.
- The data presented in this study clearly demonstrate the capability of the applied SMART Digest protocol when combined with the Vanquish Flex UHPLC system and Orbitrap-based LC-MS to significantly speed up peptide mapping experiments enabling high throughput analyses, even with low abundant samples, as required during the development phase of biopharmaceuticals.

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Find out more...

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

Amy Farrell[1], Kai Scheffler[2], Ken Cook[2], Martin Samonig[2], David Munoz[2], Alexander Schwahn[2], and Jonathan Bones[1]
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Application benefits

- Native and denatured intact protein analysis and peptide mapping on a single platform
- The entire workflow for the peptide mapping assay, including the digestion step, was completed in a little over one hour with minimal effort from the user
- The accurate and reproducible confirmation of the sequence with 100% sequence coverage, verification of the correct disulfide bond linkages, and quantification of several post-translational modifications
- Associated ease-of-use through automation

Goal

To demonstrate the applicability of a single Thermo Scientific™ LC-MS platform for extensive characterization of biotherapeutic proteins, by peptide mapping and intact protein analysis on the recombinant protein somatotropin.

Introduction

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biological production by living cells, there are many attributes that need to be analyzed to guarantee their safety and efficacy. This can involve multiple analytical techniques based on several different instrument platforms. There is an industry desire to simplify the processes, produce multi-attribute methodologies, and increase reproducibility between laboratories.[1] Here we use a single instrument platform and software with multiple characterization workflows that generate data for multiple quality controlled attributes.

Peptide mapping is one of the most important assays in the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and as a check for several post-translational modifications. High-resolution mass spectrometry is coupled to chromatography for peak identification and quantitation. Many QC methods use solely UV detection after the peak identities have been confirmed to simplify the method for a QC environment. However, UV-only data limits the attributes that can be measured and does not give absolute positive identification of the peptides. Here we show the possibility to use a simple, easy-to-implement LC-MS method with evidence of the benefits of such a system over UV-only detection.

Trypsin is the most commonly used proteolytic digestion enzyme due to its high specificity. However, many trypsin-based protocols and kits that have been developed for proteolytic digestion of proteins are labor intensive, prone to manual errors and may also introduce post-translational modifications during digestion.[2,3] Reproducible digestion is imperative for peptide mapping sample preparation, yet often leads to difficulty during method transfer. The digestion method used here is simplified and improved using immobilized heat stable trypsin.[4,5]

Intact protein analysis confirms that product with the correct molecular weight has been expressed and is an important characterization step for biotherapeutic proteins. High-resolution, accurate-mass (HRAM) Orbitrap mass spectrometry has been shown to be essential for this technique.[6] The same instrument platform, incorporating an easily automated change of columns and separation gradient, was used for the peptide mapping analysis. The intact molecular weight analysis was performed under native and denaturing conditions at high resolution to give isotopically resolved mass spectra.

Somatotropin is a small recombinant biotherapeutic protein used here as a model protein to describe the use of a new improved single instrument platform for extensive protein characterization analysis.

It is essential to detect, characterize, and quantify any undesirable modifications and confirm the correct product identity of recombinant proteins. In this application note, we demonstrate that typical protocols used for this type of characterization can be simplified and made more reproducible with new workflows performed on the same system. Peptide mapping is the most common analytical method employed for this purpose and delivers a wealth of information from correct amino acid sequence to the presence, location, and quantification of several post-translational modifications. Multiple quality attributes can be defined by peptide mapping analysis. Although a widely accepted and powerful technique, the digestion protocols for sample preparation are labor intensive and prone to manual errors and unwanted modifications. This can affect the quality of the analytical data and creates a source of irreproducibility. Incomplete digestion may render the accurate quantification of modifications impossible, however, a small amount of missed cleavage may enable 100% sequence coverage. A careful balance of digestion completion needs to be maintained in a very reproducible manner.

The Thermo Scientific™ SMART Digest™ Kit was used for the sample preparation for peptide mapping analysis. This protocol greatly simplifies the digestion process and increases reproducibility. Intact protein analysis is a complimentary technique used to ensure the correct molecular weight of the protein biotherapeutic. Both these techniques can be performed on the same analytical platform with

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
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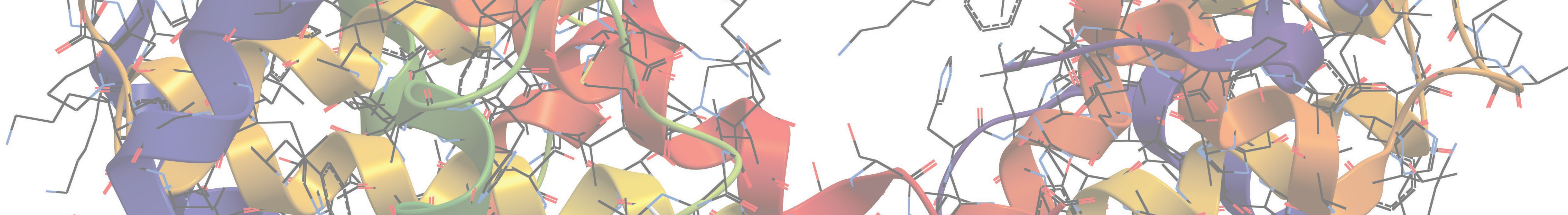
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



no change in the eluents used for chromatography. The Thermo Scientific™ BioPharma Finder™ software combines the identification and quantitation tools for peptide mapping with the deconvolution software used for intact protein analysis. Use of the described workflows on a single platform for this extensive characterization easily facilitates implementation in the laboratory.

Experimental

A Thermo Scientific™ Vanquish™ Flex UHPLC system connected to a Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer equipped with the BioPharma option was the LC-MS platform used for the analysis. The same system was utilized for the intact protein analysis, which was performed under both native and denaturing conditions with the data for all techniques analyzed using the both native and denaturing conditions. The data for all techniques was analyzed using BioPharma Finder 2.0 software.

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Ammonium acetate LC-MS grade (P/N A11450)
- Fisher Scientific TCEP Tris [2-carboxyethyl]phosphine (P/N 20490)
- SMART Digest Kit (P/N 60109-101)
- Fisher Scientific LC-MS grade water (P/N W/011217)
- LC-MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ formic acid LC-MS grade (P/N 28905)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ MAbPac™ RP column, 4 µm, 2.1 × 100 mm (P/N 088647)
- Thermo Scientific™ Acclaim™ SEC-300 column, 5µm, 4.6 x 300 mm (P/N 079723)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Sample pre-treatment

Somatotropin dry stock was made to a final concentration of 5 mg/mL in water with gentle swirling to aid in solubilization.

Digestion for peptide mapping

The sample was submitted to the SMART Digest protocol: 250 µg of somatotropin in 50 µL formulation buffer (5 mg/mL) was diluted to 200 µL with SMART Digest buffer. This was incubated for 5 and 15 minutes at 70 °C and 1400 rpm in an Eppendorf ThermoMixer®. The immobilized trypsin beads were then removed by spinning down in a micro-centrifuge for 5 minutes at 1,000g. The sample was gently removed and the supernatant carefully aspirated from the beads. The sample was split in half. One aliquot was reduced by adding DTT to a final concentration of 5 mM and incubated at 25 °C for 30 min. The other half remained untreated and was used for the detection of disulfide-bridged peptides and disulfide bond analysis.

Separation conditions

Instrumentation

Thermo Scientific Vanquish Flex Quaternary UHPLC system equipped with:

- System Base Vanquish Flex (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)
- Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Mobile phase

Eluent A:	Water + 0.1% formic acid
Eluent B:	80% acetonitrile + 0.1% formic acid
Eluent C:	50 mM ammonium acetate
Flow rate:	0.3 mL/min
Column temperature:	70 °C
UV:	214 nm
Run conditions:	Tables 1, 2, and 3

Table 1. Gradient for peptide mapping with the Acclaim VANQUISH C18 column.

Retention time (min)	Flow (mL/min)	%B
0.0	0.3	4.0
30.0	0.3	70.0
31.0	0.3	100.0
34.0	0.3	100.0
35.0	0.3	4.0
60.0	0.3	4.0

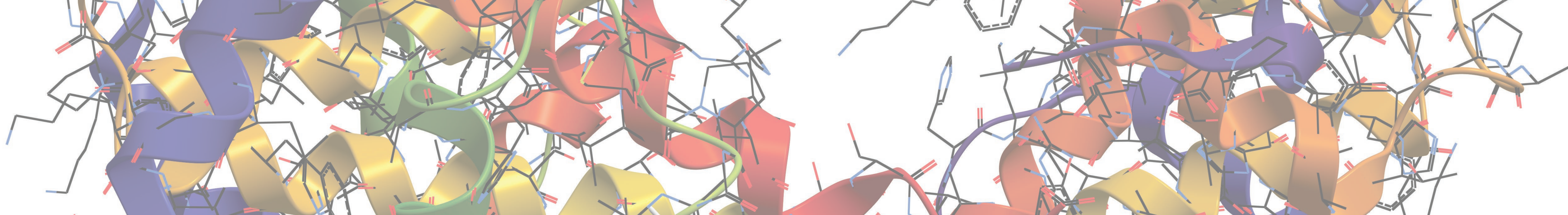
Table 2. Gradient for intact protein analysis under denaturing conditions with the MAbPac RP column.

Retention time (min)	Flow (mL/min)	%B
0.0	0.3	10
0.1	0.3	10
10.0	0.3	55
10.1	0.3	90
11.0	0.3	90
11.1	0.3	10
15.0	0.3	10

Table 3. Run conditions for native intact protein analysis with the Acclaim SEC-300 column.

Flow (mL/min)	%C
0.25	100

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



MS conditions

Table 4. Q Exactive BioPharma MS parameter settings.

Parameter	Peptide mapping	Intact native/ denaturing conditions
Source probe	HESI II	HESI II
Polarity	Positive	Positive
Spray voltage	4.0 kV	4.0 kV
Vaporizer temp.	300 °C	320 °C/300 °C
Sheath gas flow rate [arb. units]	45	25/25
Auxiliary gas flow rate [arb. units]	12	10/5
Capillary temp.	320 °C	275 °C
Resolution (Full MS/MS^2)	60k/15k	120k/240k
Top-N MS^2	5	n.a.
S-lens RF level	50	80
Max inject time (Full MS/MS^2)	100 ms/200 ms	150 ms

Data processing

BioPharma Finder 2.0 software was used for analysis of all data acquired on the peptide and protein level. For deconvolution of isotopically resolved mass spectra of the intact proteins under native and denaturing conditions, the Xtract algorithm was used with a signal-to-noise threshold of 2, a fit factor of 80%, and a remainder of 25%.

For peptide mapping, searches were performed using a single-entry protein FASTA database with oxidation and deamidation set as variable modifications, 5 ppm mass accuracy, and a confidence level of 0.8 for MS/MS spectra.

Results and discussion

The sequence for somatotropin outlined in Figure 1 shows the expected cleavage positions for trypsin in red and the position for the disulfide bond linkages in yellow. This protein represents a good model system for peptide mapping and intact protein analysis with 18 peptides expected of varying size and two disulfide bond linkages (20 peptides when reduced). There are also several sites available for possible post-translational modifications by deamidation, isomerization, and oxidation.

```
      12 14
  1 FPTIPLSRLF DNAMLRAHRL HQLAFDTYQE FEEAYIPKEQ KYSFLQNPQT
 51 SLCFSESIPT PSNREETQQK SNLELLRISL LLIQSWLEPV QFLRSVFANS
                          125
101 LVYGASDSNV YDLLKDLEEG IQTLMGRLED GSPRTGQIFK QTYSKFDTNS
                                                     149
151 HNDDALLKNY GLLYCFRKDM DKVETFLRIV QCRSVEGSCG F
                       170
```

Figure 1. Amino acid sequence of somatotropin with cysteine residues involved in disulfide bonds marked in yellow and asparagines and methionine residues prone for oxidation and deamination marked in green.

Peptide Mapping

The samples obtained after performing digestion with and without subsequent reduction were subjected to LC-MS analysis under the conditions outlined above. The base peak chromatograms obtained for the reduced and unreduced samples are shown in Figure 2.

The chromatogram of the non-reduced somatotropin digest sample shows two extra peaks with retention times of 7.96 min and 16.50 min labelled with a black star in the upper panel of Figure 2. These correspond to the two disulfide-linked peptides present in the native somatotropin. The SMART Digest protocol does not use upfront reduction or alkylation as the proteins are digested in heat denaturing conditions at 70 °C. Thus, the disulfide linked peptides still maintain the covalent linkage following the digestion procedure. This linkage can be broken by reduction with DTT at the peptide level following digestion and the effects are visualized in the bottom panel of Figure 2. The two peaks representing the disulfide-linked peptides in the chromatogram of the non-reduced sample disappear with the subsequent appearance of four additional peptides that are not initially observed in the non-reduced sample. The released peptides from the broken disulfide bonds are marked in the lower panel with stars. The peptides at retention times 4.28 min and 10.32 min relate to one disulfide linkage, and the peptides at retention times 15.45 min and 16.21 min relate to the other. The direct confirmation of the disulfide bond linkages is given by the BioPharma Finder software, which can identify the presence of the linked peptides in the non-reduced digestion by accurate parent ion mass alone. Further verification of the di-peptide can be achieved based on MS^2 spectra. This is an advantage of the described workflow, as an effective digestion of the unreduced protein can prove difficult to achieve with some proteins using standard in-solution digestion protocols.

thermo scientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

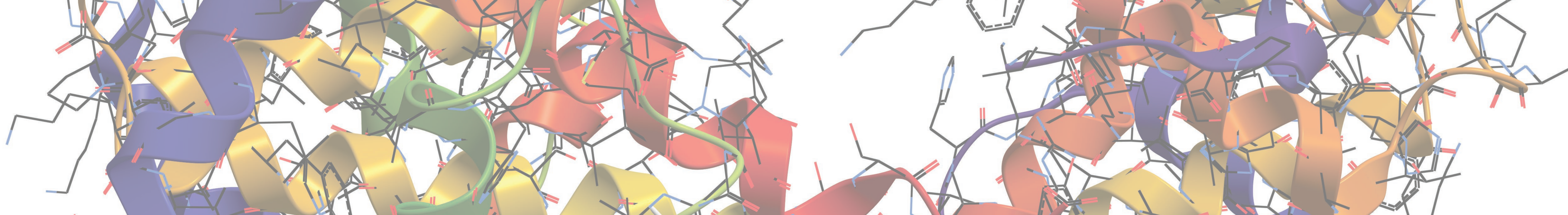
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations



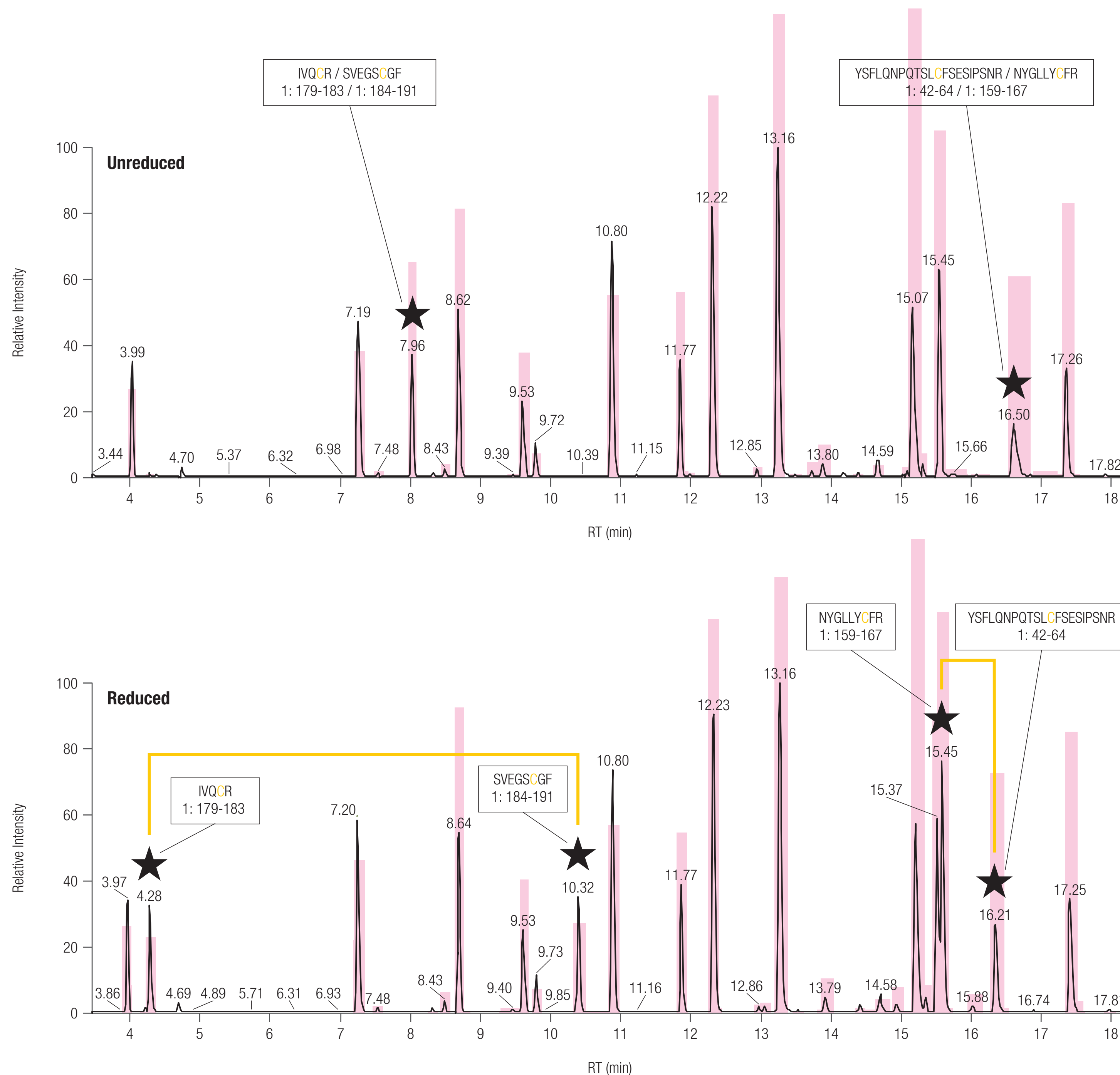


Figure 2. Base peak chromatograms obtained for the unreduced (top) and reduced (bottom) somatotropin trypsin digest. Cysteine containing peptides are labelled with black stars indicating four disconnected peptides in the reduced sample and two peptides in the unreduced sample bearing disulfide bonds.

The disulfide bond assignments in Figure 3 of the chromatogram are marked with a star for clarity. Unlike the other peptides that have a peak label indicating the position of the peptide in the sequence, the peak label for the disulfide-linked peptides show sequence positions for both peptides. The first to elute contains the tryptic peptides at positions 179-183 linked to the peptide at position 184-191. The second has 159-167 and 42- 64 linked together by a disulfide bond. This is also shown in the sequence coverage map highlighted with yellow and red boxes, respectively. The yellow boxes indicate the peptides in the first eluting disulfide-linked peptide. The retention times indicate that they are eluting at the same retention time of 8.0 min due to the covalent linkage connecting them together. The last eluting disulfide-linked peptides are marked with a red box showing the identical elution time of 16.5 min. BioPharma Finder has this disulfide bond assignment method as a default choice from the menu. The results were obtained without any further manipulation of the disulfide bond default method. Using the standard built-in methods sequence coverage of 100% was achieved for the peptide map of somatotropin without reduction of the digested peptides.

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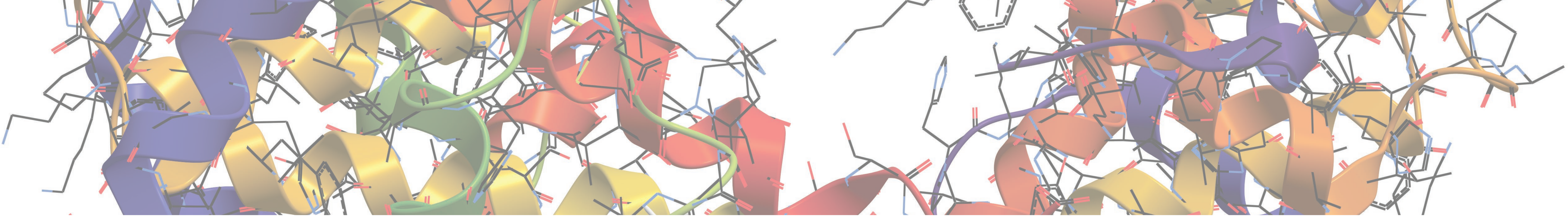
Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



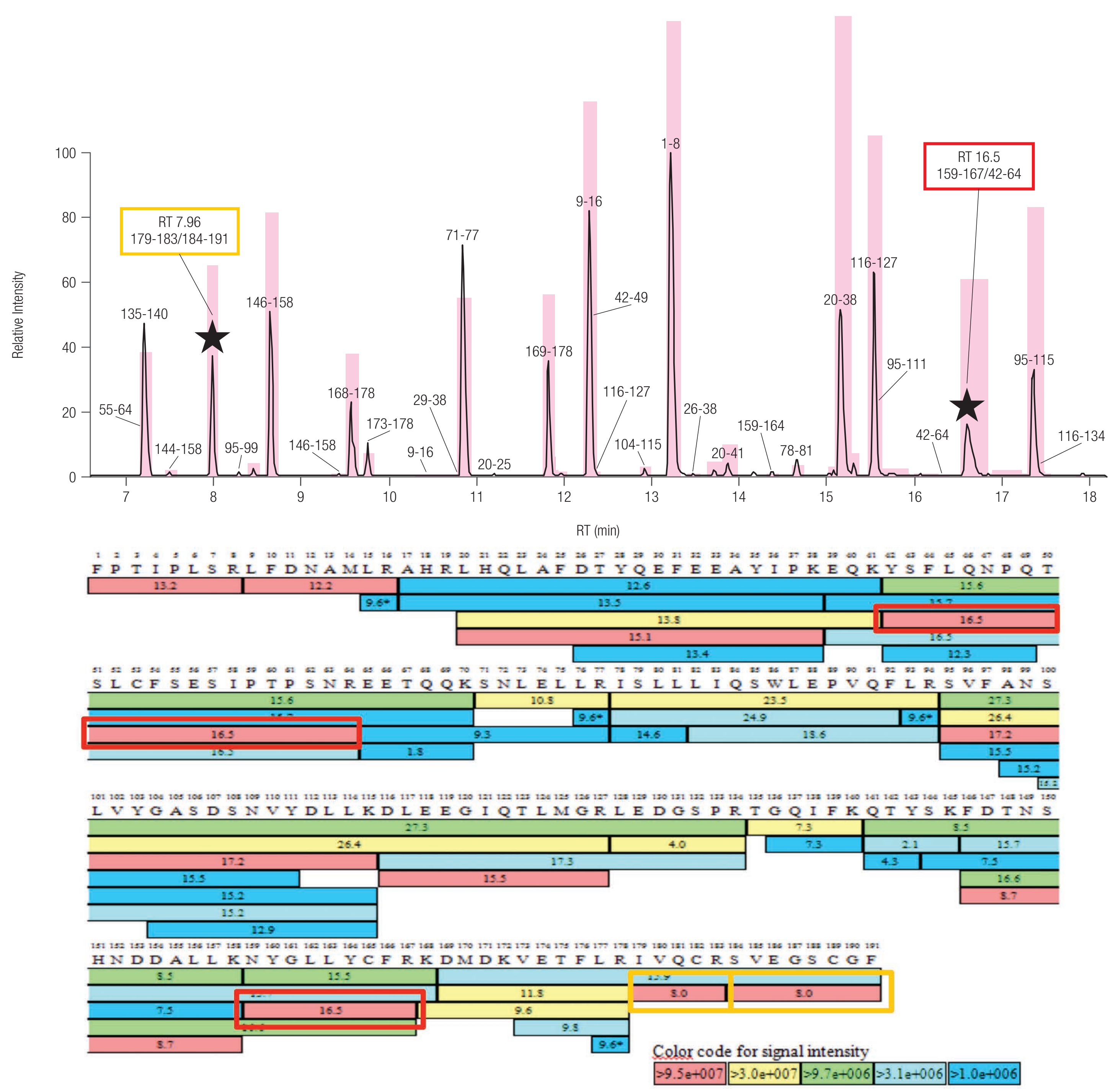


Figure 3. Assignment of peaks in the chromatogram representing peptides with intact disulfide bonds and location of the peptides involved in disulfide bonds in the protein sequence highlighted with boxes in red for the peptide aa 159-167/42-64 and with boxes in yellow for the linked aa peptides 179-183/184-191.

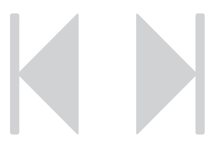
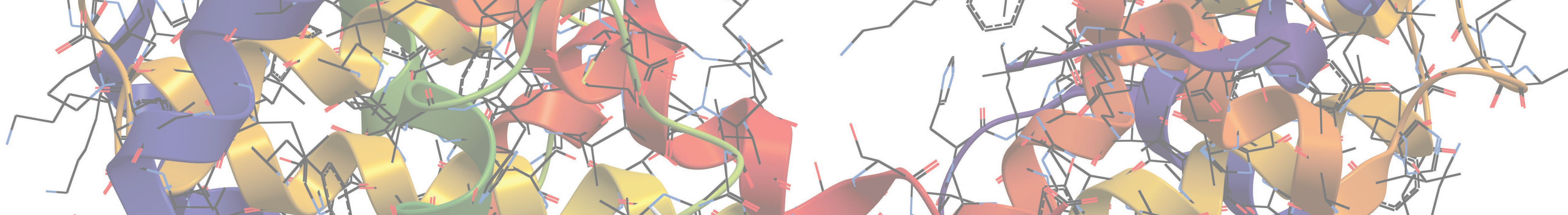


Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



Proteins	Number of MS peaks	MS peak area	Sequence coverage	Abundance (mol)
Somatotropin	457	90.9%	100.0%	100.0%
Unidentified	14	9.1%		

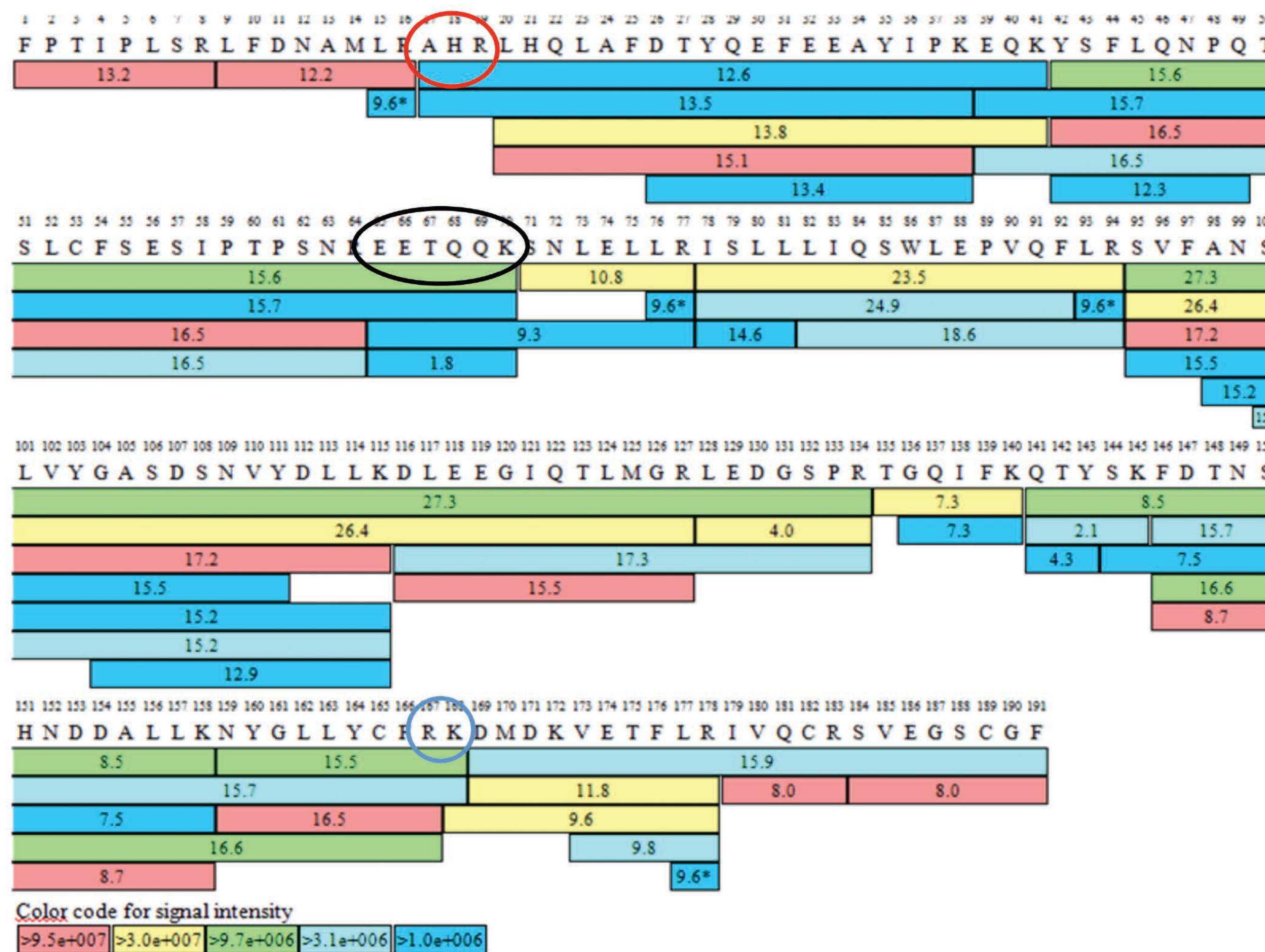


Figure 4. Sequence coverage map for somatotropin showing 100% coverage. Full MS and MS/MS data were used. The bars underneath the sequence represent peptides with colors indicating the precursor intensities and the numbers in the bars representing the retention time.

A small amount of a missed cleavage product is required to see a very hydrophilic peptide EETQQK, which is circled in black in Figure 4. The full cleavage product is difficult to see without a high sensitivity MS system and impossible with UV detection only, as it does not ionize well and elutes in the column void at a retention time of 1.8 min (Figure 4). Another short peptide is circled in red with the sequence AHR, which is also identified by a very low abundant missed cleavage product. The full cleavage product elutes very early in the column void at 1.3 min but does not ionize well at all (Figure 4). If the digestion time is increased to 60 minutes, full digestion occurs at this site and the low level missed cleavage product used in the confirmation of the AHR peptide sequence is lost (data not shown). Somatotropin also has two trypsin cleavage sites adjacent to each other at positions R167 and K168. This is circled in blue in Figure 4 with the two resulting abundant peptides eluting at 9.6 min and 11.8 min (yellow bars). Both peptides contain an unusually abundant missed cleavage site at position K172, a position just after the adjacent R167 and K168 sites and in front of a methionine at M170, which is susceptible to oxidation (Table 5). This missed cleavage is possibly due to the acidic nature of the glutamate and aspartate residues near the cleavage site. The other trypsin cleavage sites throughout the somatotropin sequence are cleaved perfectly using the SMART Digest Kit, showing high abundant peptides that are easily identified with BioPharma Finder software and can be converted to a method using UV absorption only for detection.

An additional benefit of the LCMS methodology is the identification of post-translational modifications. There are several sites in the sequence that could be prone to deamidation, oxidation, and isomerization of aspartic acid. These can be identified in BioPharma Finder software by simply adding the modifications that are interesting from a built-in list to the sequence manager. In this case, we looked for all the above modifications in the somatotropin sample. Results are indicated in Table 5 for a 5 and 15 minute trypsin digest using the SMART Digest Kit.

thermo scientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

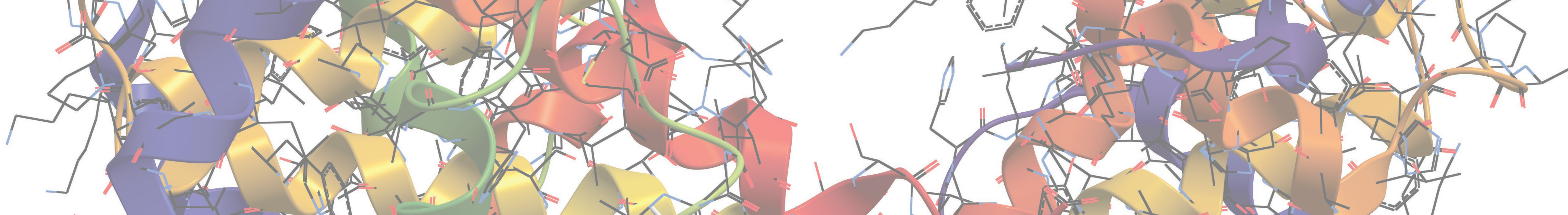


Table 5. Post-translational modifications identified and quantified from the somatotropin SMART Digest samples after 5 min and 15 min digestion time.

Position	Sequence	Retention time [mod./ unmod.]	Modification	% (5 min digest)	% (15 min digest)
N149	FDTNSHNDDALLK	9.43 / 8.68	Deamidation	1.05	1.64
M170	DMDKVETFLR	9.58 / 10.31	Oxidation	0.59	0.60
M14	LFDNAMLR	10.41 / 12.28	Oxidation	1.52	1.34
M125	DLEEGIQTLMGR	12.40 / 15.48	Oxidation	0.18	0.21
D130	LEDGSPR	3.95 / 4.06	Isomerization	0.18	0.55

There is one deamidation site, three oxidation sites, and one isomerization site identified, all at very low levels. The low levels alone would make these modifications difficult to impossible to quantify by UV only, especially in the presence of significant numbers of high abundant peptides. In addition, the isomerization site modified peptide did not separate well from the unmodified form at the peptide level with the short reversed-phase gradient chromatography used in this example. This would make their identification and quantification impossible by UV detection only with the chromatographic conditions applied. The mass differences for isomerization and deamidation modifications are also very small, which makes the use of high-resolution mass spectrometry the correct choice for confident results.

The results in Table 5 show a small increase in the levels of deamidation and isomerization modifications with time, and this has been noticed previously.[2,4] This increase is still relatively small over the digestion period with the SMART Digest Kit even if digestion times are increased to 60 minutes.[4] Optimization of the time of digestion should be considered, however, to minimize any digestion-induced modifications. For somatotropin, a 15 minute digestion seems optimal, providing a good balance between complete digestion suitable also for quantification while keeping induced modifications to a minimum.

In addition, a robust and complete digestion process is critical for the identification of modifications using high-resolution, accurate-mass (HRAM) mass spectrometry. If complete digestion is not achieved then missed cleavages around the modification site will lead to more than one peptide that contains the same modification eluting at different positions, making accurate quantification difficult or impossible. Table 6 shows the effects of a short digestion time of 5 minutes, creating a small level of an additional missed cleavage peptide for the deamidation at N149. A digestion site in front of the peptide has not been cleaved, creating an additional longer peptide with five additional amino acids, QTYSK, at the front of the sequence. In this example, the SMART Digest procedure still gives a credible result as the missed cleavage product is only present in relatively small amounts. This is made more important as the percentage levels of the deamidation product in the missed cleavage peptides are shown here to be different. An incomplete digestion would lead to several peptide products containing the same modification at different levels with the modified and unmodified peptides for deamidation all eluting at different retention times. This applies considerable pressure on obtaining an efficient reliable digestion for the assay. With less predictable and difficult to use digestion procedures this will cause a problem with accurate reproducible quantification.

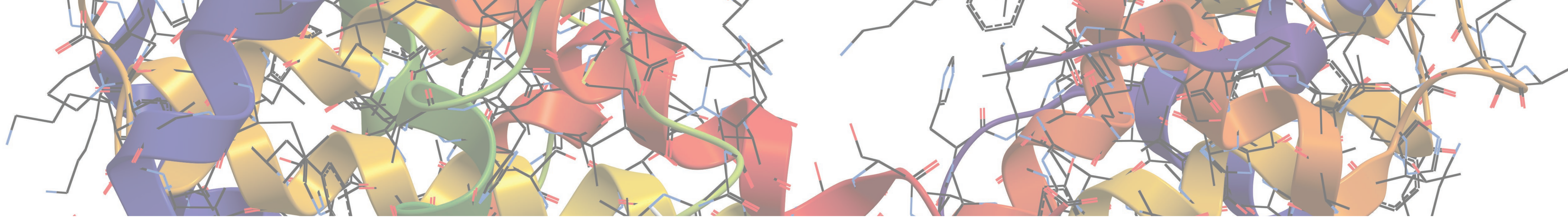
Table 6. Quantification result of the deamidation level of asparagine at position 149 obtained from a sample after 5 min digestion time. Two peptides are shown, one of which includes a missed cleavage site.

Position	Sequence	Retention time	Modification	%
Native	FDTNSHNDDALLK	8.69	None	
N149	FDTNSHNDDALLK	9.43	Deamidation	1.05
Native	QTYSKFDTNSHNDDALLK	8.48	None	
N149	QTYSKFDTNSHNDDALLK	9.11	Deamidation	1.69
			Average	0.99

In this series of experiments, the procedure using the SMART Digest Kit was found to be extremely reproducible and efficient. Optimization is simple to obtain a complete digest that gives a small enough number of missed cleavages to help with sequence coverage but not enough not to interfere with the accurate determination of modifications.

The use of HRAM MS can be daunting to implement in the standard QC environment where HPLC systems with UV or fluorescence detection are the standard instruments and there is often little experience in high-end mass spectrometry. In this setting, a simple high-resolution MS-only instrument can be implemented in much the same way as another detector for the HPLC system. This adds a detector with minimal functional settings to give an additional mass trace in the chromatogram running under the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software. The additional online mass analysis enables all the advantages of quantification of some post-translational modifications as well as positive identification of the peptide sequence as demonstrated above. Figure 5 shows a peptide map of somatotropin with identification of the peptides using the intact peptide mass as measured in the MS1 scan only. Confident identification can be done in the Chromeleon CDS software, combining accurate mass and retention time with this simple system configuration. In the example in Figure 5, sequence coverage of 100% was achieved with standard settings in BioPharma Finder software. The peptides produced from the workflow give high abundant (red and yellow shading), perfectly cleaved tryptic peptides making identification easy. Missed cleavage peptides are in very low abundance (blue shading) and do not interfere with the quantitative analysis of the modifications.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



Proteins	Number of MS peaks	MS peak area	Sequence coverage	Abundance (mol)
Somatotropin	205	89.6%	100.0%	100.0%
Unidentified	4	10.4%		

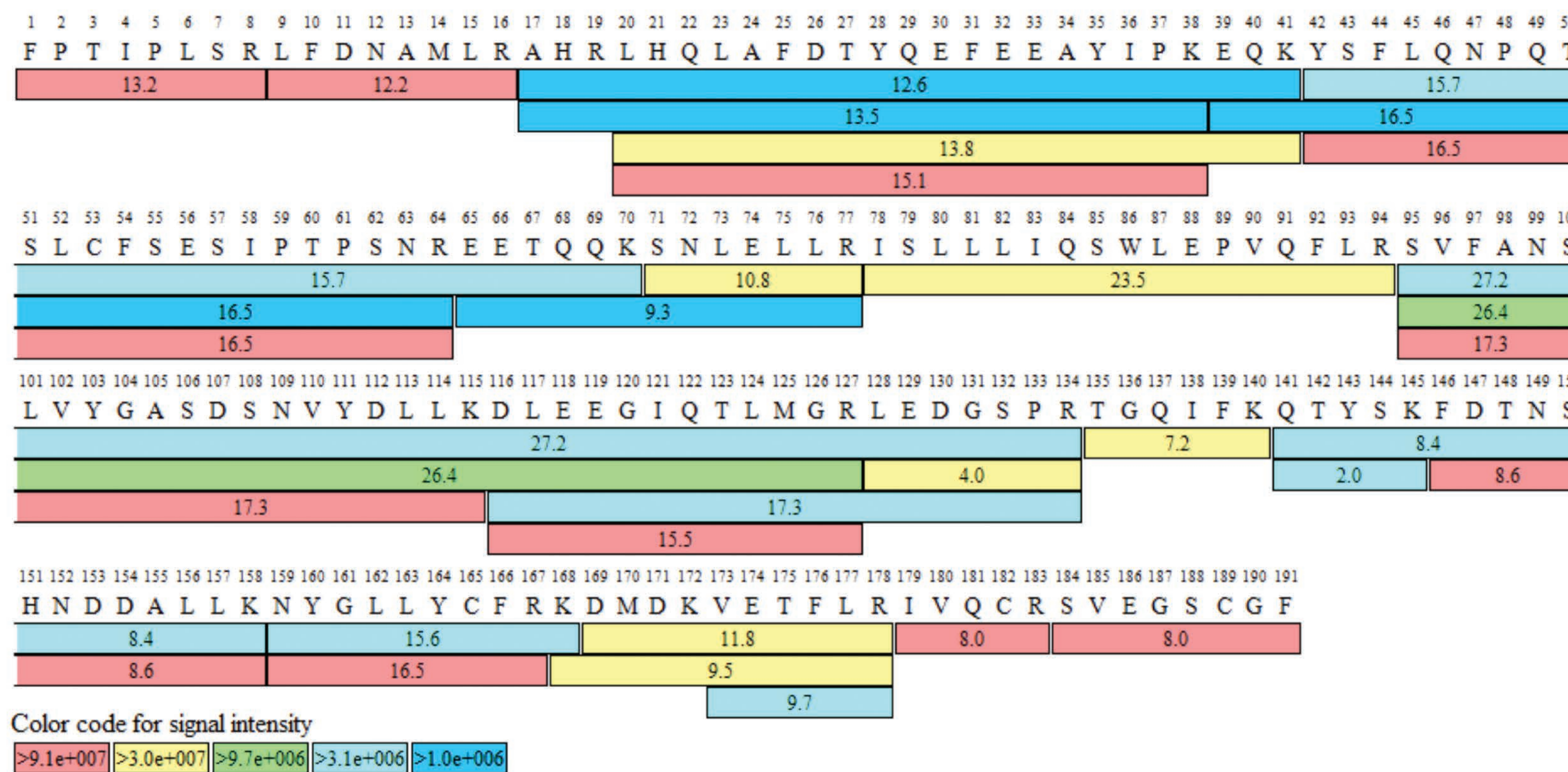
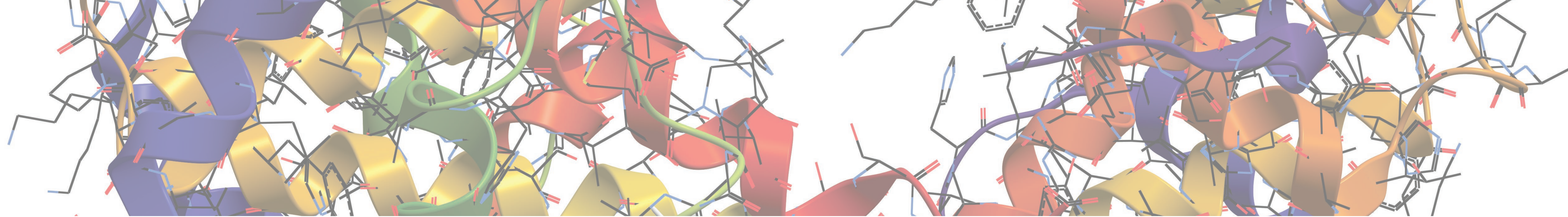


Figure 5. Sequence coverage map of somatotropin of identified peptides based on MS full scan data only.

The same system configuration can be used for denatured intact protein analysis as for the peptide mapping analysis, with a simple column change that may be performed automatically with a column change valve. The column of choice for intact mass analysis is the MAbPac RP column, a polymeric column with large pore size that exhibits high resolution of intact proteins with very little to no carryover. The eluents are the same for both analyses, with a slightly different gradient in the chosen intact analysis chromatography method. The example in Figure 6 shows the intact analysis of somatotropin with the deconvoluted monoisotopic mass at 22,111.0920 Da. The mass difference of ~4 Daltons corresponds to the four missing hydrogens lost from the cysteine residues involved in the two disulfide bonds, which were still intact in this sample. The theoretical mass with the disulfide bonds intact is 22,111.0409 Da, which is with 2.31 ppm deviation in very close agreement with the experimental value. The excellent resolution provided by the Orbitrap mass analyzer of this relatively small protein can easily be isotopically resolved at a resolution setting of 120,000. The charge envelope obtained for somatotropin analyzed under denaturing conditions spans charge states from 10 to 20 with the most abundant charge state detected at *m/z* 1,476.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



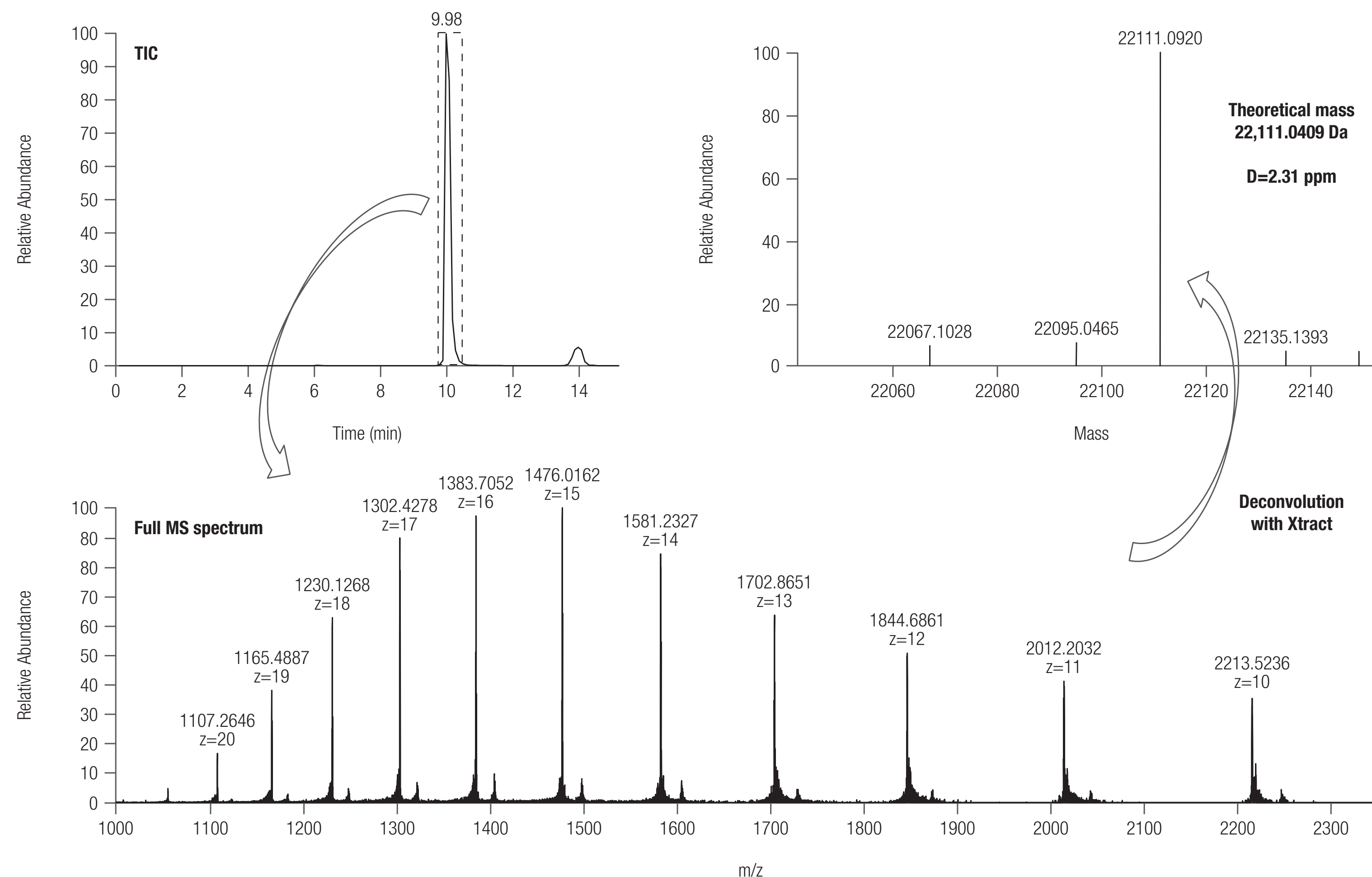
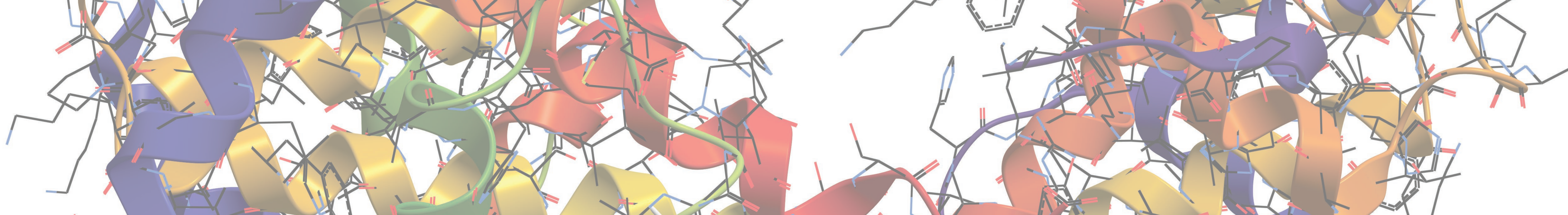


Figure 6. Mass analysis of intact somatotropin under denaturing conditions showing the total ion chromatogram, the Full MS spectrum, and the mass obtained after deconvolution with Xtract.

The mass of the native protein can also be characterized using this instrument platform. The analysis in the native form can give additional information that is not available after the protein is denatured. Binding characteristics in the native form and any higher-order structural variations can be studied with this approach. As the protein will be in its native folded state, the charges available on the surface of the protein are much lower in number than in the fully unfolded, denatured state, resulting in a reduced charge distribution on the protein and so the mass-to-charge ratio elevates to a higher *m/z* range. The reduced charge distribution can help with spectral resolution between modifications that could overlap with different variants from different charge states. For this analysis, an Acclaim SEC-300 column was utilized to introduce the intact protein to the MS, while maintaining low salt concentration. This is a polymeric size-exclusion chromatography (SEC) column that shows very little secondary interaction with proteins even at low salt eluent conditions. This was preferred over a silica-based SEC column as it requires less salt in the eluent system to maintain excellent chromatography under size-exclusion conditions. The low salt concentration is beneficial to the mass spectrometry system for reduction of the background signal and greatly reduces the signal suppression inherent with salt gradients, resulting in overall increased sensitivity in the MS.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



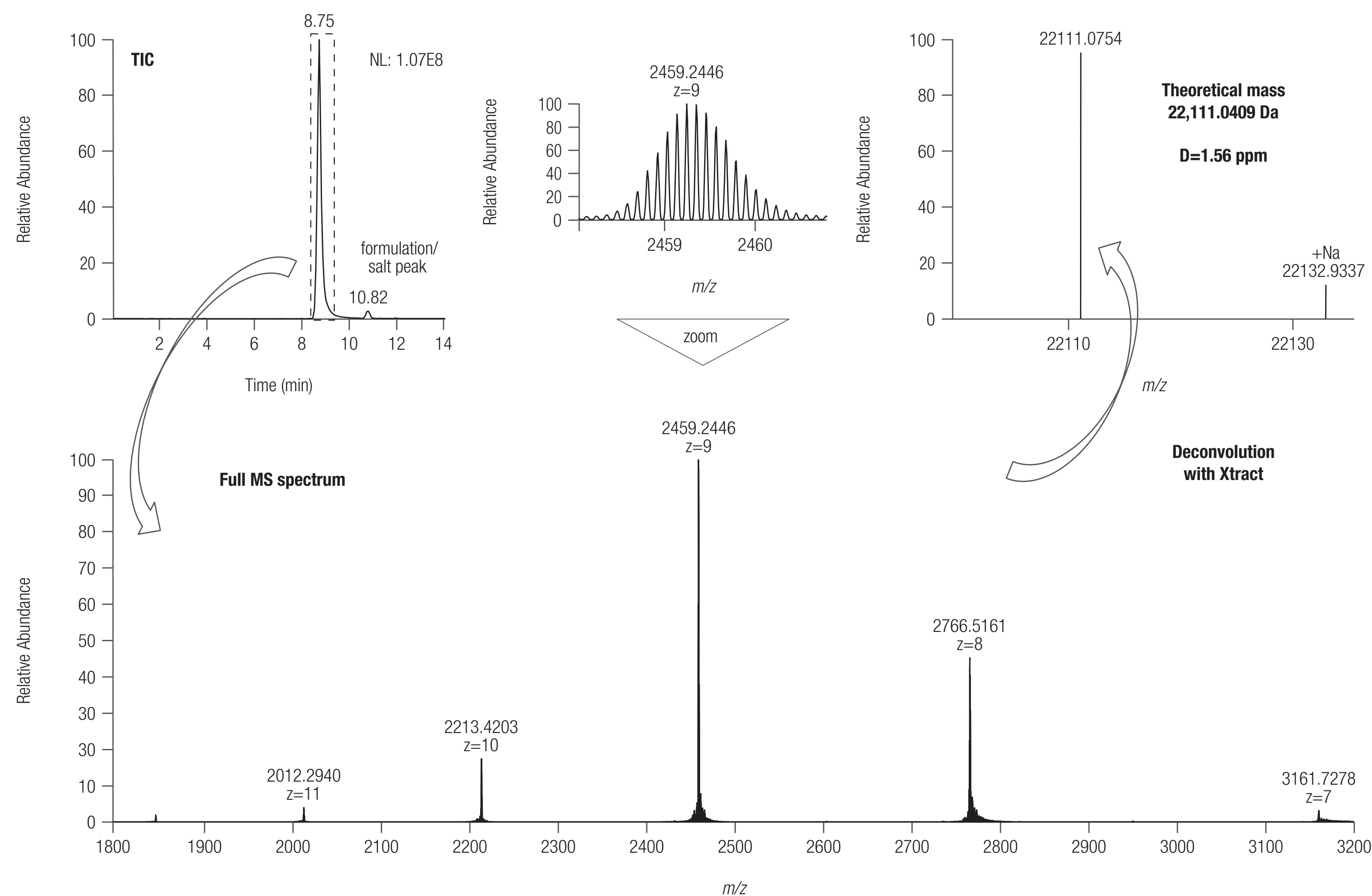


Figure 7. Analysis of intact somatotropin under native conditions showing the total ion chromatogram, the Full MS spectrum with a zoom into the most abundant charge state, and the mass obtained after deconvolution with Xtract.

Figure 7 shows the base peak chromatogram, the full MS spectrum with a zoom into the most abundant charge state at *m/z* 2459.2446, and the deconvoluted spectrum for somatotropin acquired under native conditions. The distribution shows a smaller number and a shift to lower charge states due to the native form of the protein that is amenable to accepting fewer charges when compared to the analysis under denaturing conditions. The monoisotopic mass was calculated at 22,111.0754 Da, (1.56 ppm mass deviation) also in excellent agreement to the expected mass for somatotropin with the disulfide bonds intact.

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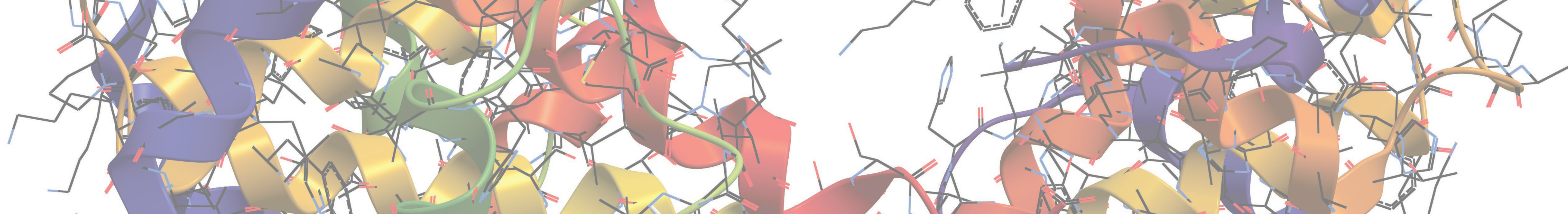
Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



Conclusions

An extensive characterization of the biotherapeutic protein somatotropin was successfully achieved using a single instrument platform. The peptide mapping workflow was simplified with excellent and reproducible data generated using the SMART Digest workflow, which showed consistent and complete digestion and good specificity for cleavage at the correct trypsin cleavage sites. This produced a simple peptide map that was easy to interpret using the BioPharma Finder software. The digestion efficiency and reproducibility was shown to be an important factor for the combination of total sequence coverage and accurate determination of posttranslational modifications. The information from the peptide mapping experiment included the following:

- Confirmation of the sequence with 100% sequence coverage
- Verification of the correct disulfide bond linkages
- Quantification of several post-translational modifications

The accuracy and reproducibility of the procedure using the SMART Digest Kit allows the user to simply modify the digestion by time to allow the production of small amounts of missed cleavage products that may be required for complete sequence coverage and ensure correct conditions for complete digestion to allow accurate measurement of post-translational modifications. This is very difficult to achieve reproducibly with other digestion techniques.

The entire workflow for the peptide mapping assay, including the digestion step, could be accomplished in a little over one hour with minimal effort from the user. This assay could be implemented in a routine environment with little prior knowledge or experience in mass spectrometry using Chromeleon software to control the entire LC-MS system with the acquisition of Full MS data only.

In addition to the information from peptide mapping experiments, the verification of the correct monoisotopic mass of the intact protein based on isotopically resolved mass spectra can be done using the same system configuration. This can be achieved by analyzing the protein in the native or denatured state.

The described instrument platform and workflows show the possibilities for routine use to measure several critical quality attributes in routine characterization of biotherapeutics. The system configuration of UHPLC/MS with BioPharma Finder software has proven to be a simple, versatile, and powerful platform for the analysis of somatotropin, which could be extended to any biotherapeutic protein product.

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Acknowledgement

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thermo scientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Find out more...

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

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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

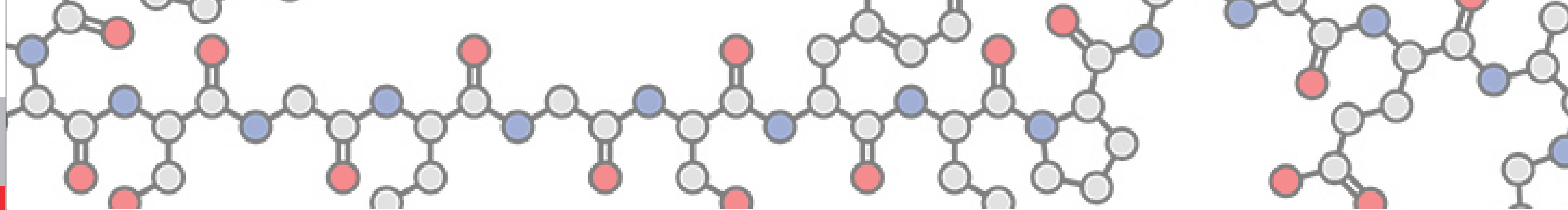
400 µg rituximab were denatured in 50 mM tris HCl at pH 8.0 and 70 °C for 75 min, followed by a reduction step using 5 mM DTT for 30 min at 70 °C. Alkylation was performed with 15 mM IAA for 30 min at room temperature, and the reaction was quenched by addition of 9 mM DTT. The sample was then diluted 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.

Thermo Fisher
SCIENTIFIC

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



(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

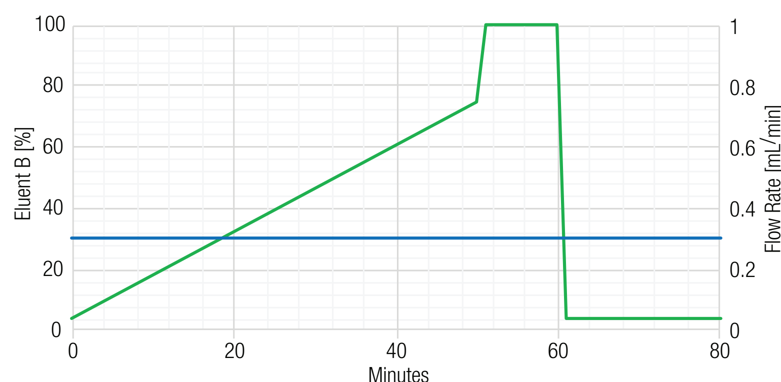


Figure 1. LC gradient.

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 of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

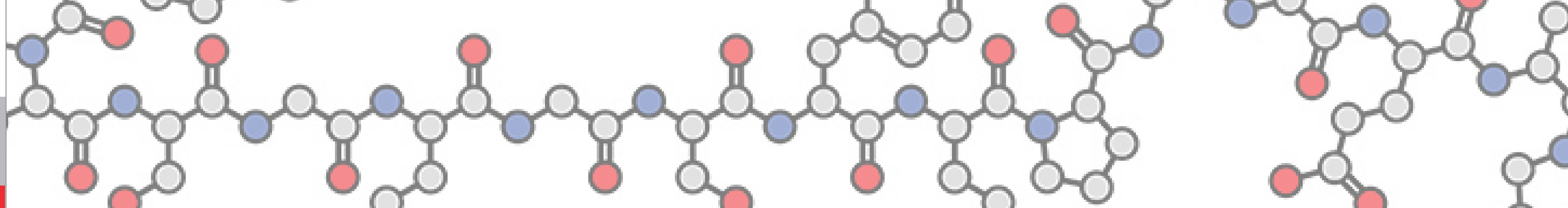


Table 2. BioPharma Finder parameter settings for all samples.

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		

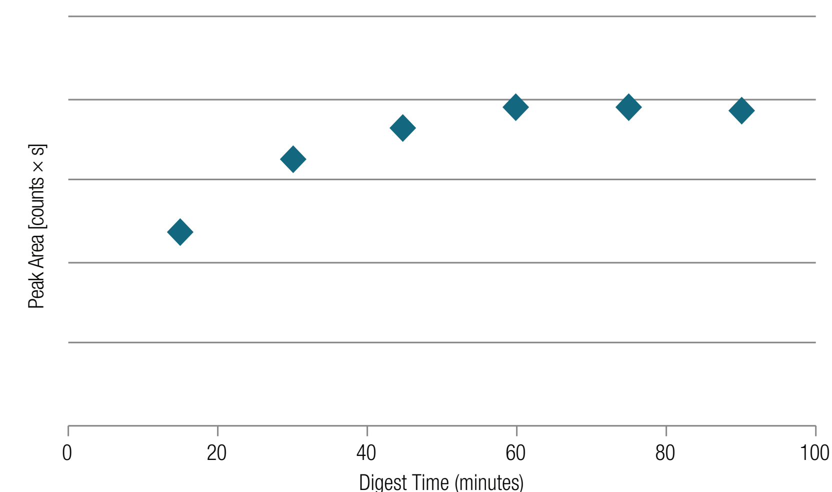


Figure 2. IgG digest profile, monitoring the mAb peptide VVSVLTVLHQDWLNGK for digestion times from 15–90 min using the SMART Digest Kit.[2]

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 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 of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
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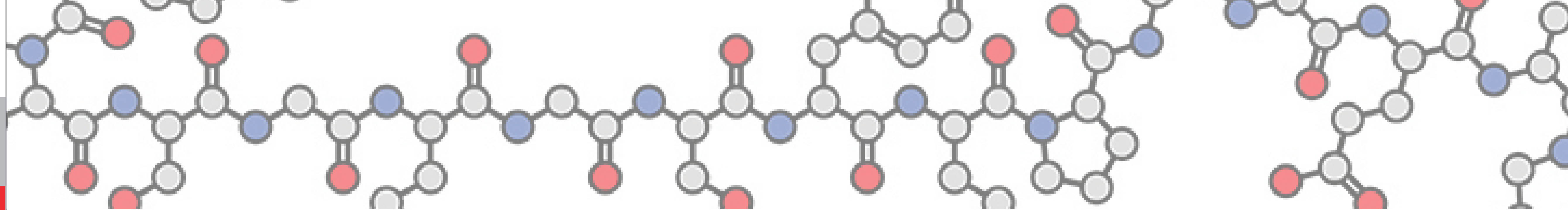
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



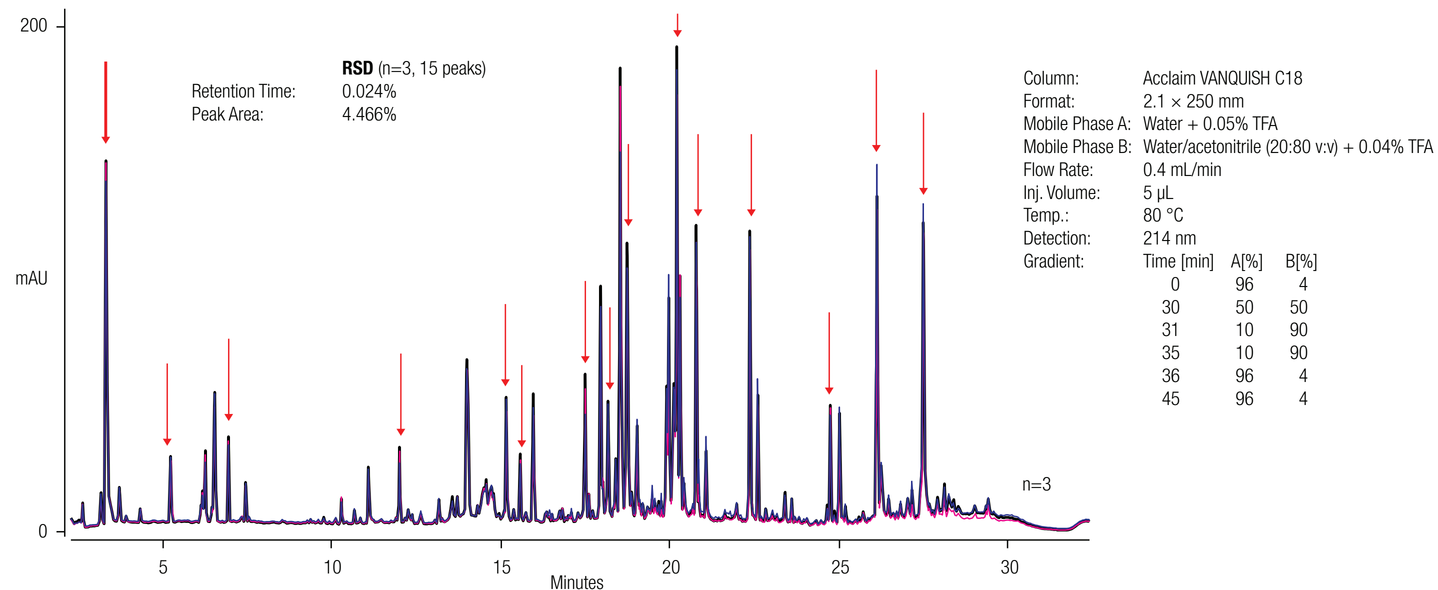


Figure 3. UV chromatogram overlay from three separate SMART digestions from the same mAb, conducted by three individual operators. The 15 marked peptides in each sample were used for inter-user/inter-day RSD value calculations.

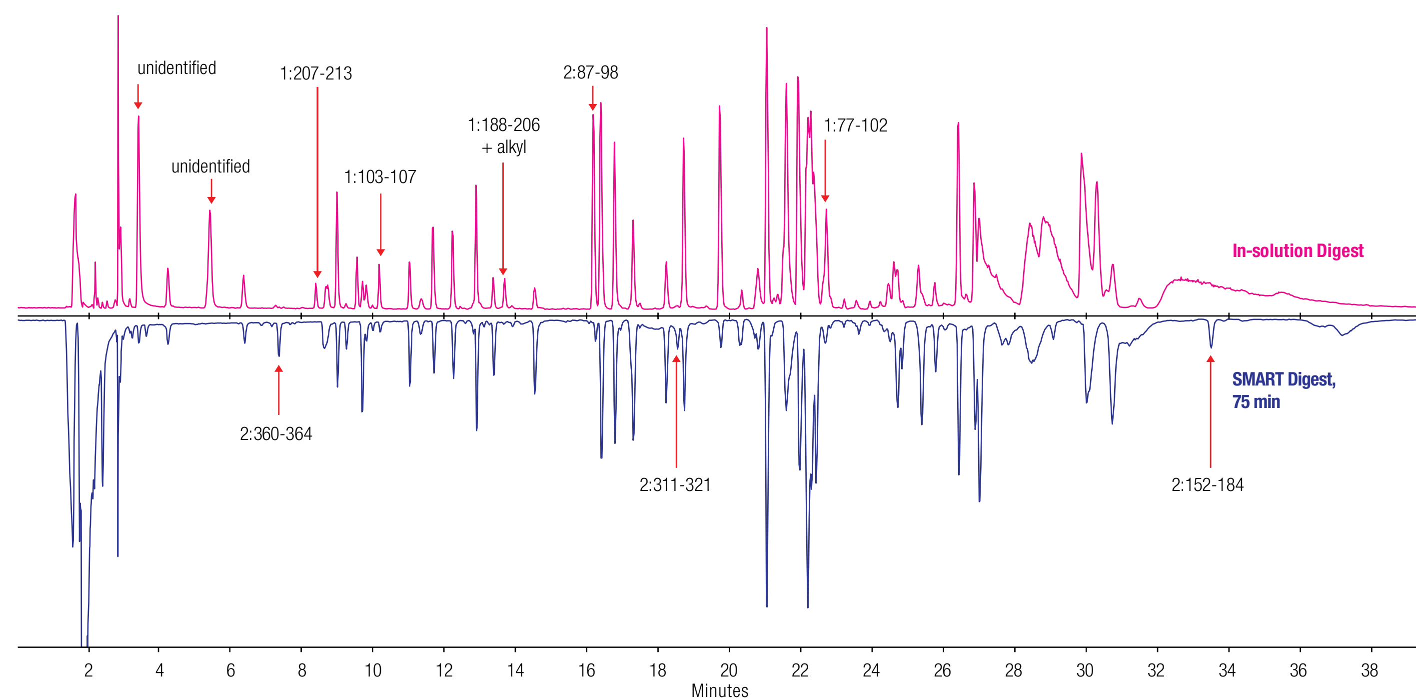


Figure 4. Mirror plot of the TIC chromatogram for the in-solution-digested sample denatured with heat (*In-Solution, Heat*) and the reduced SMART Digest sample (*SMART Digest, 75 min*). Peak labels give annotation to light (1) or heavy (2) chains, respectively, and sequence position.

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

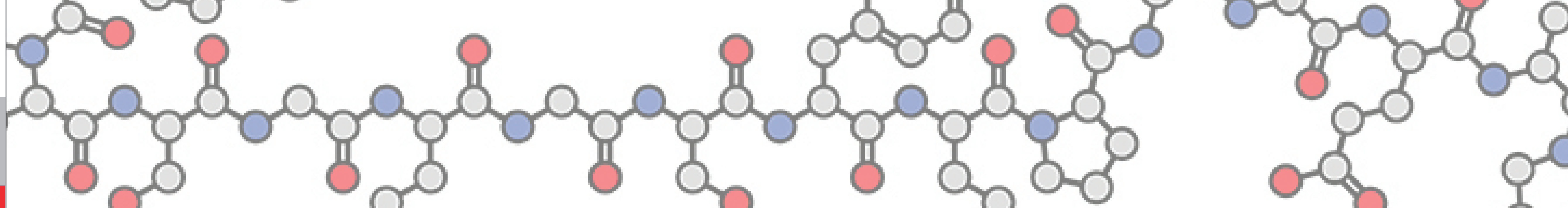


Table 3. Sequence coverage with different digestion methods.

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*

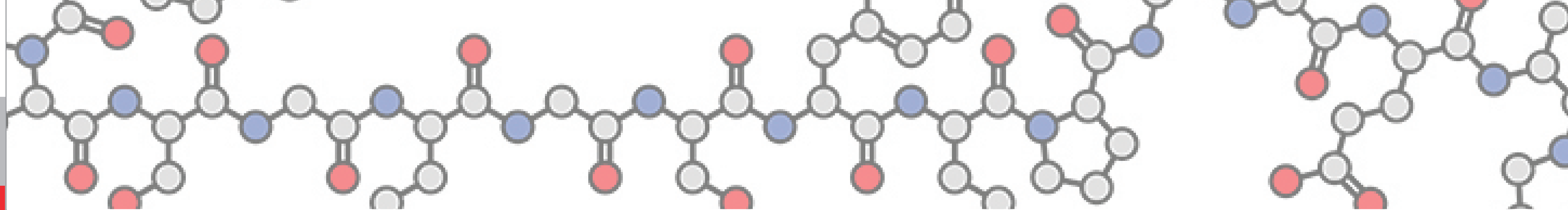
Table 4. Number of identified components and TIC area for the different runs.

# 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 d 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 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 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%.

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



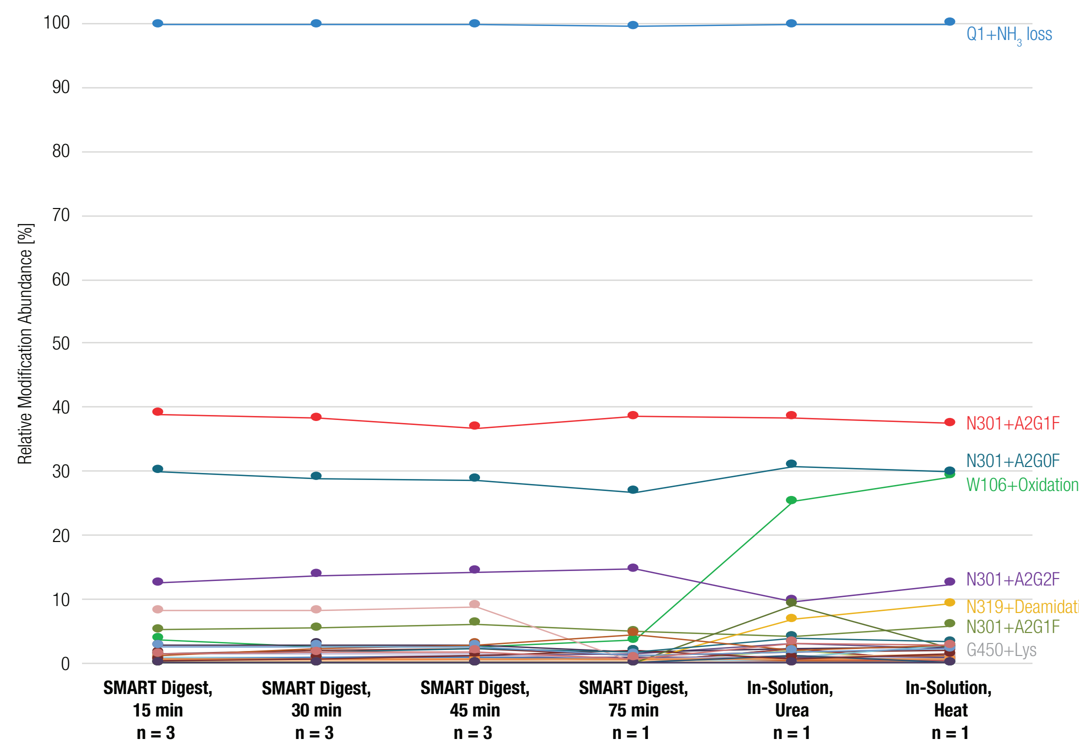


Figure 5. Relative abundance of 85 identified modifications including oxidation, double oxidation, glycation, glycosylation, NH_3 loss, isomerization, lysine truncation, methylation, dimethylation, and carbamylation.

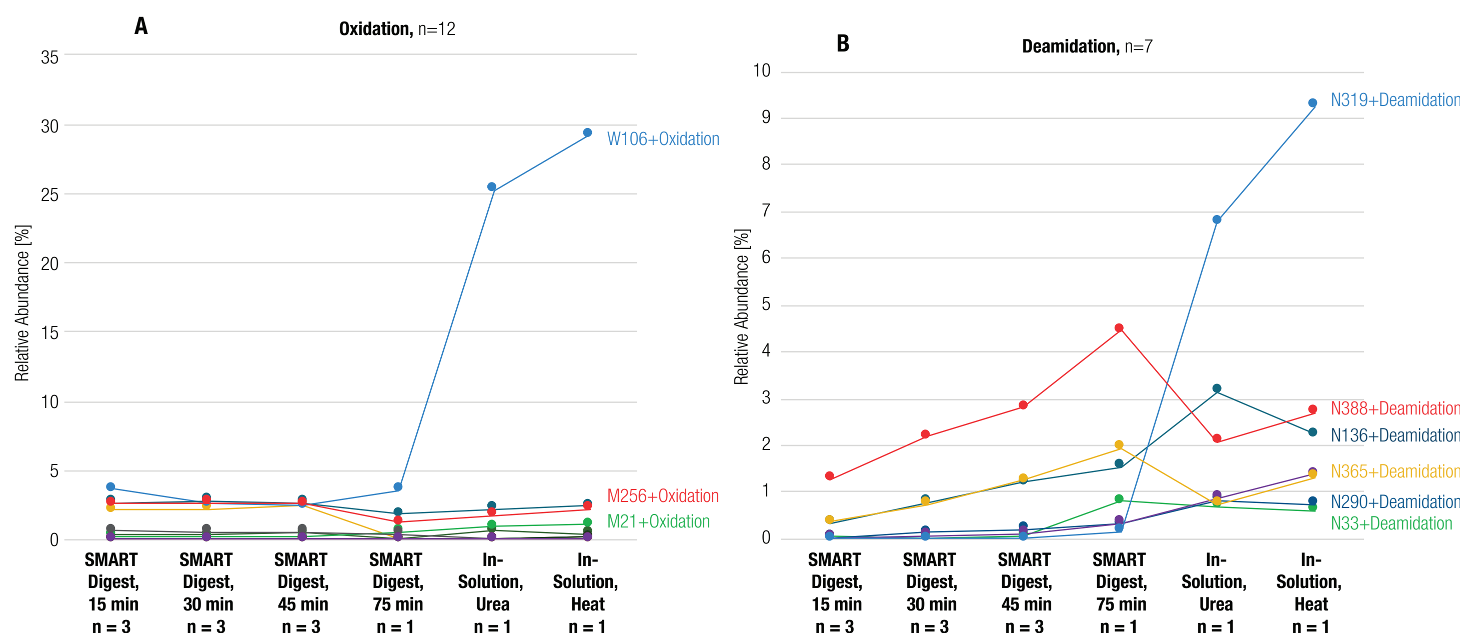


Figure 6. Relative abundance of 12 identified oxidations (A) and 7 deamidations (B) in different runs with various digestion methods.

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).

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
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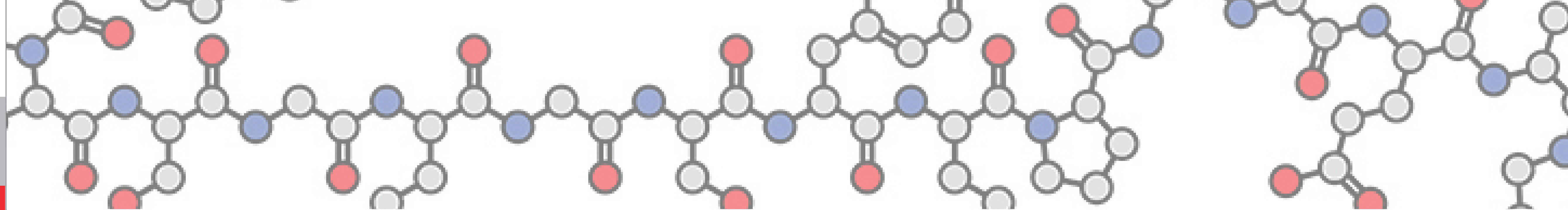
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



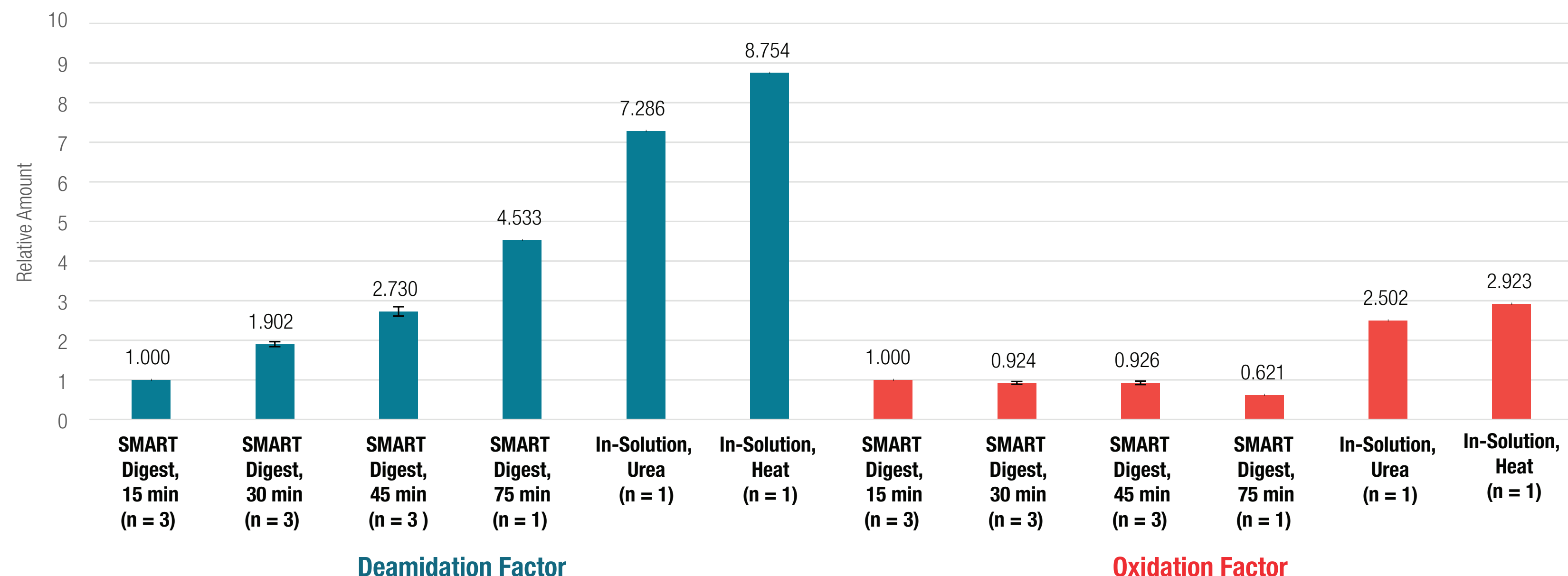


Figure 7. Relative amount of total deamidation and oxidation modifications measured for the six different digest conditions (Normalization to *SMART Digest, 15 min*).

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]

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.

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

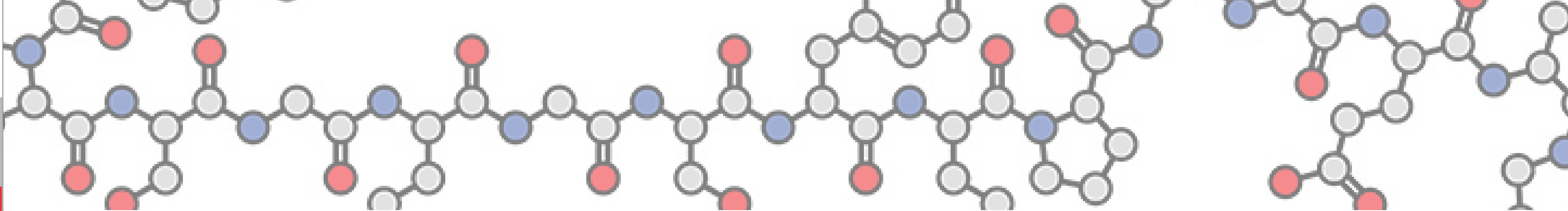

Table 5. Comparison of the oxidation, deamidation, and carbamylation modifications identified with the different digestion methods.

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

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 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

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Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

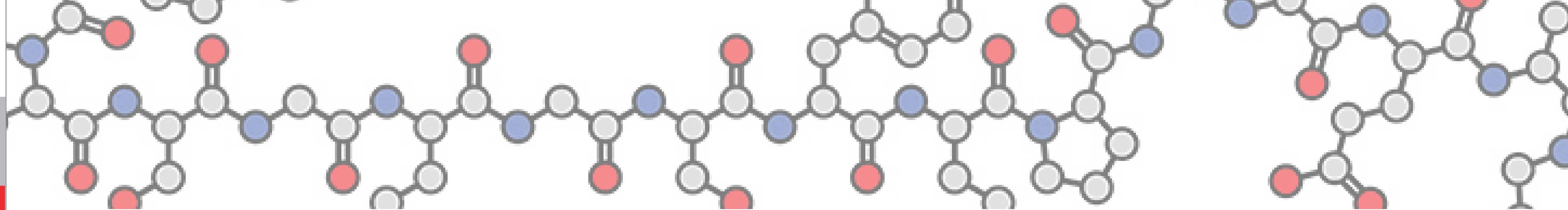
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations



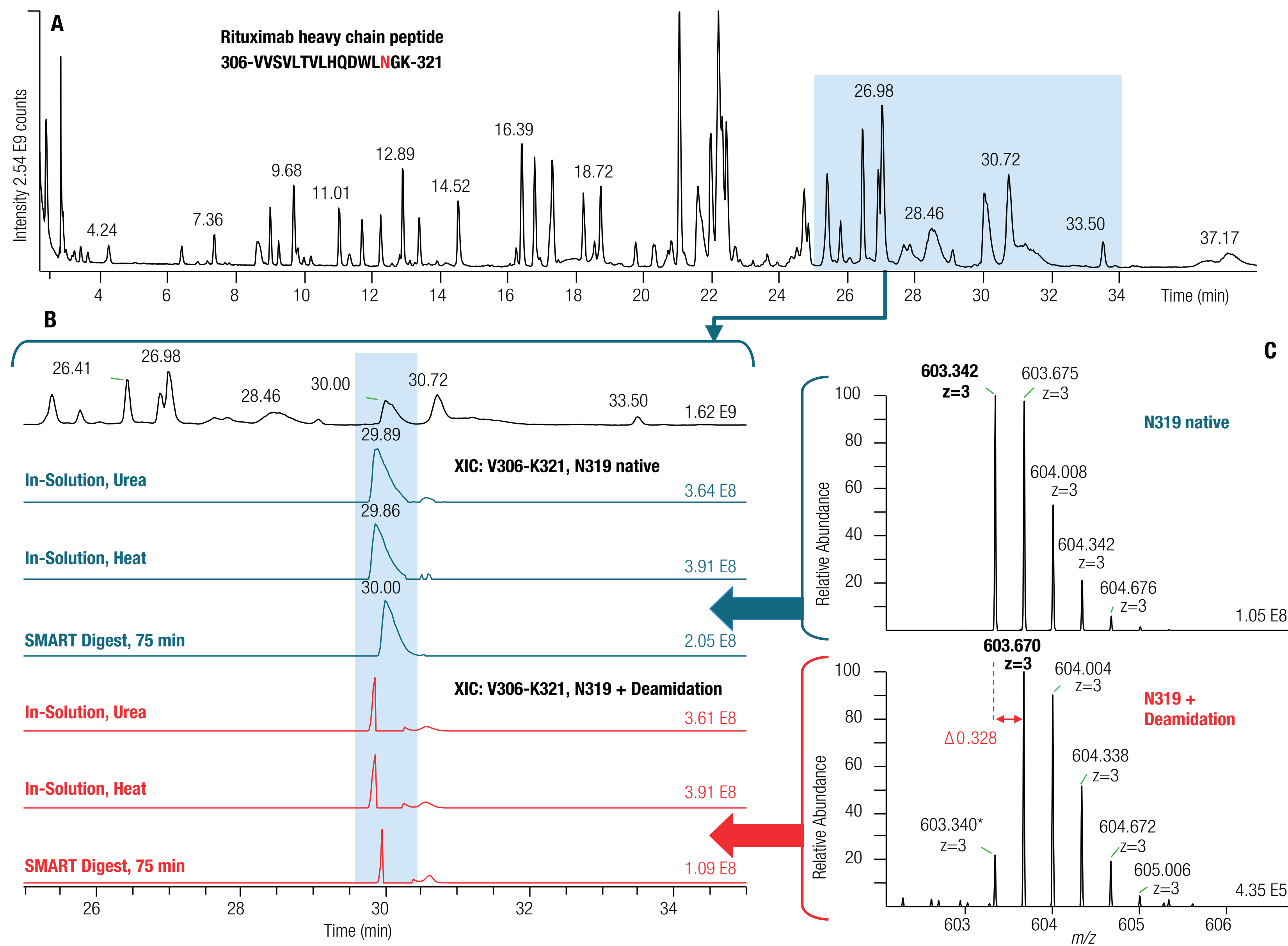


Figure 8. Total ion current chromatogram of the reduced SMART Digest sample, 75 min (A), and extracted ion current chromatograms (B) for the peptide V306-K321 in the native and the deamidated form for the different runs. A comparison of the isotopic distributions of the $[M+3H]^{3+}$ ions (C) for the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

Chromeleon and BioPharma Finder software, SMART Digest Kit facilitates standardized, fast, and reproducible peptide mapping workflows.

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thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

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Find out more...

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

Martin Samonig, Remco Swart
Thermo Fisher Scientific, Germering, Germany

Goal

Prove the suitability of a Thermo Scientific™ Vanquish™ Flex system for efficient and reliable peptide mapping method development with a LC-UV-MS setup.

Introduction

Peptide mapping is one of several routine methods to characterize biopharmaceutical proteins. For research environments, this technique, if combined with mass spectrometry (MS), is utilized for the characterization and confirmation of the primary sequence of monoclonal antibodies. In addition, peptide mapping can help to identify, localize, and quantitate post-translational modifications (PTMs). Peptide mapping methods are often developed and evaluated with combined UV and MS detection, to simplify the transfer to routine environments where UV detection is used alone. In high-throughput workflows, peptide mapping experiments are performed for antibody identity confirmation, PTM characterization, and stability studies.

The new Vanquish Flex UHPLC system features a quaternary pump1 for highest application flexibility and fully biocompatible flow path. In addition, similar to the Thermo Scientific Vanquish UHPLC system2, the sample is pressurized prior to the injection into the high pressure flow path. This results in a highly stable flow delivery and thus significantly improved retention time precision, increasing the confidence in peak assignment in peptide mapping experiments with UV detection.3

In this work, the separation of peptides obtained from a monoclonal antibody digest is demonstrated with a LC-UV-MS setup.

Experimental

The commercially available monoclonal antibody rituximab (F. Hoffmann-La Roche, Ltd) was digested using the Thermo Scientific™ SMART Digest™ Kit. It is designed for applications that require highly reproducible, sensitive, and fast analyses, due to its optimized, heat stable, immobilized trypsin design. The sample was 1:4 diluted with the SMART digestion buffer included in the kit, and enzymatic digestion was allowed to proceed at 70 °C for 75 min and 1400 rpm. Disulfide bonds were reduced by incubation for 30 minutes at 60 °C with 5 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP). The separation of the tryptic digest was achieved with a 30 min gradient and a total analysis time of 56 min, including the column wash with high organic eluent, and re-equilibration at initial conditions. The Vanquish Flex system was coupled to the Thermo Scientific™ Q Exactive™ HF mass spectrometer using the MS connection kit for Vanquish systems. With this setup, simultaneous UV and MS detection is feasible.

Equipment

Vanquish Flex UHPLC system consisting of:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)|
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
- Vanquish MS Connection Kit (P/N 6720.0405)

Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer
SMART Digest Kit (P/N 60109-101)

Experimental Conditions - HPLC

Column	Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm Analytical (2.1 × 250 mm, P/N 074812)
Mobile Phase	A: 0.1% FA in water (P/N FA 28905) B: 0.1% FA in 8/2 acetonitrile/water (v/v), (P/N acetonitrile TS-51101)
Gradient	0–30 min: 4–55% B 30–31 min: 55–100% B 31–35 min: 100% B 35–36 min: 100–4% B 36–56 min: 4% B
Flow Rate	0.3 mL/min
Temperature	50 °C
Injection Volume	2 µL
Detection	214 nm Data Collection Rate: 10 Hz Response Time 0.4 s
Flow Cell	10 mm LightPipe

Experimental Conditions - MS

Source	HESI-II
Sheath Gas Pressure	35 psi
Auxiliary Gas Flow	10 arbitrary units
Capillary Temperature	300 °C
S-lens RF Voltage	60 V
Source Voltage	3.5 kV

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Full MS Parameters	
Full MS Mass Range	200–2000 *m/z*
Resolution Settings	60.000
Target Value	3e6
Max Injection Time	200 ms
Default Charge State	2
SID	0 eV

MS2 Parameters	
Resolution Settings	15.000
Target Value	1e5
Isolation Width	2.0 Da
Signal Threshold	1e4
Normalized Collision Energy (HCD)	27
Top-N MS^2	5
Max Injection Time	100 ms

Data Analysis

Thermo Scientific™ Xcalibur™ software version 3.0 in combination with the Thermo Scientific Standard Instrument Integration (SII) for Xcalibur 1.1 SR2 was used for data acquisition and the data analysis was performed using Thermo Scientific™ PepFinder™ software version 2.0.

Results and Discussion

Peptide mapping experiments were performed with UV as well as MS detection. Figure 1 shows the overlay of the UV trace at 214 nm and the total ion current (TIC) chromatogram obtained from the mass spectrometer, which allows confident peak assignment (Figure 2). To assess the sequence coverage, PepFinder software was used to analyze the data. The sequence coverage map (Figure 3) shows the overlap of the different peptides identified in different intensities, indicated with the color of the bar (red = high abundant, blue = low abundant), and in different lengths due to missed cleavages with sequence coverage for heavy and light chain of 99.2%. The number in the bar shows the retention time of the particular peptide.

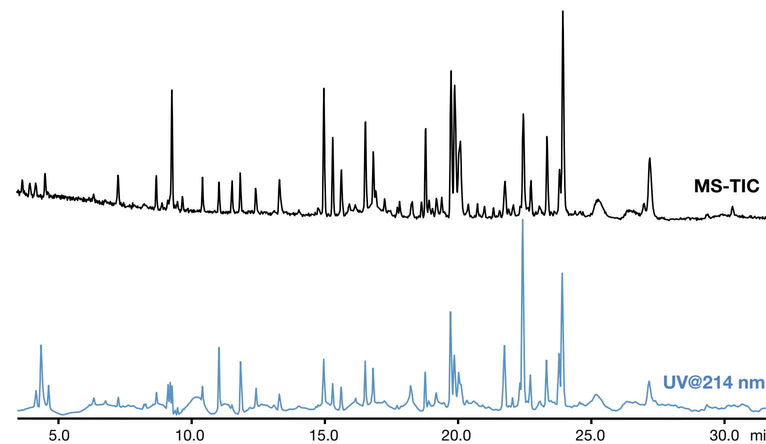


Figure 1. Overlaid chromatograms of the total ion current (TIC) and the UV trace at 214 nm of a SMART Digest Kit digested rituximab sample with subtracted blank baseline.

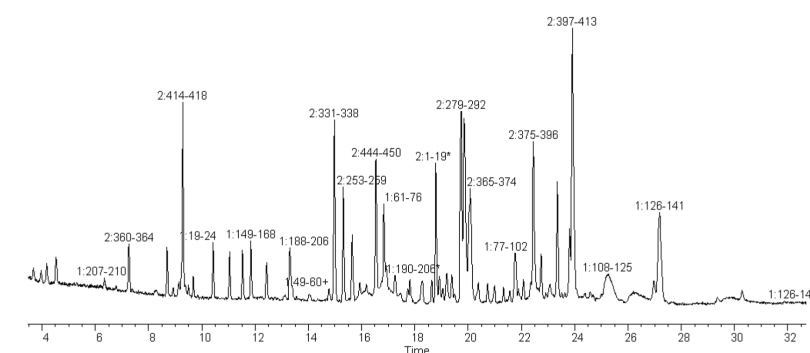
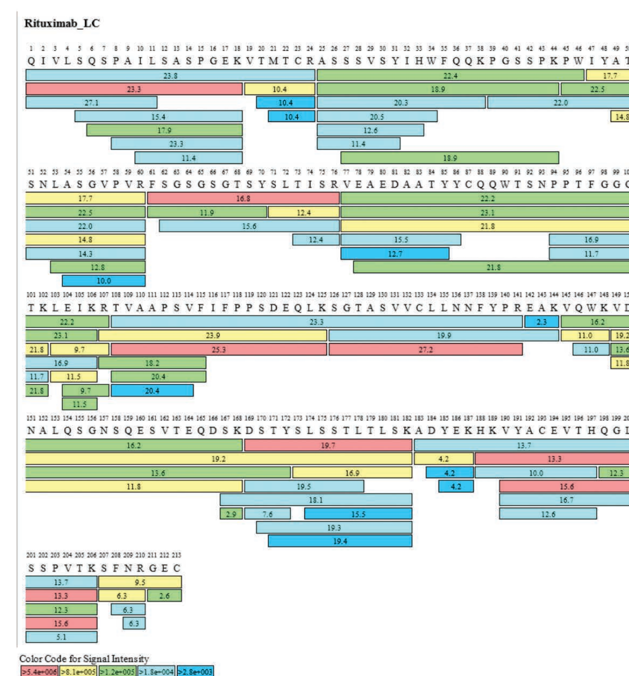


Figure 2. Peak assignment of the tryptic peptides from rituximab. Peak labels with 1 correspond to the light chain, and those with 2 correspond to the heavy chain of the mAb. The number after the colon indicates the amino acid region of this particular tryptic peptide.



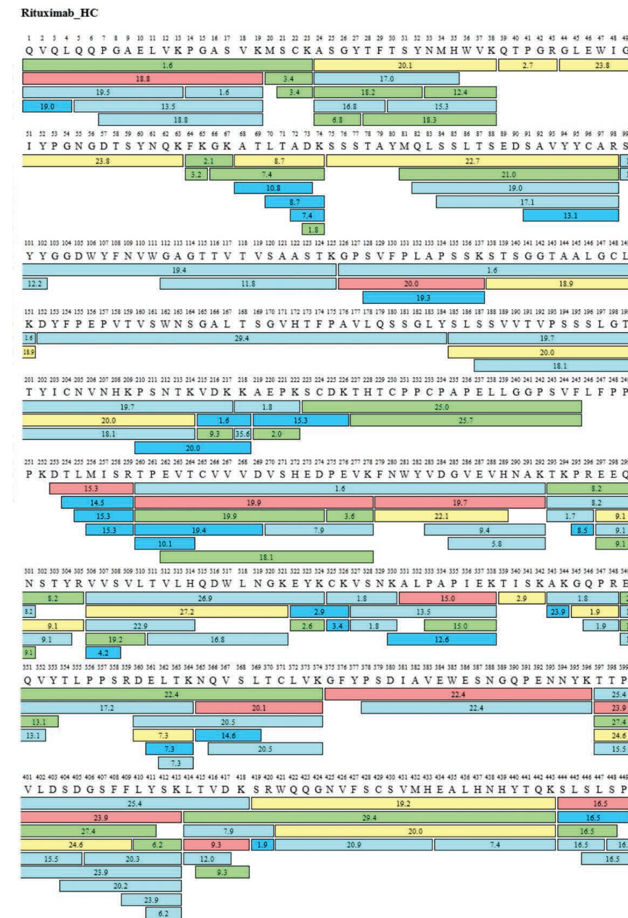


Figure 3. Sequence coverage map of the heavy (right) and light chain (left).

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

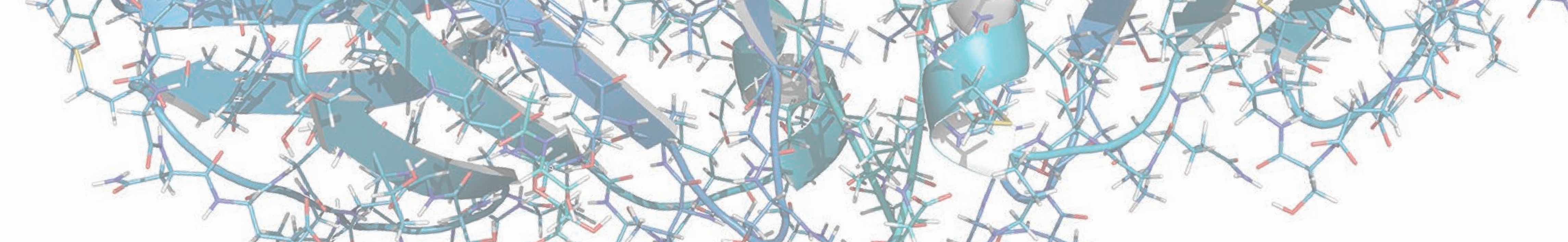


Table 1 shows the identification and relative quantification of a subset of monitored modifications on the light and heavy chain of rituximab, respectively. The selected modifications are deamidations, oxidations, pyro-Gln formations on the N-terminus of heavy and light chain, glycosylation of the N301 on the heavy chain, and sequence variants like C-terminal Lys (K+ variant). A tilde (~) before the modification indicates the modification was found on the tryptic peptide, but could not be localized on a specific amino acid with MS/MS spectra. The modification is labeled with recovery "Good" when the total peak area, including modified and unmodified forms of the peptide, is at least 10% of the most abundant peptide from the same protein. The recovery "Fair" means it is at least 1%.

Table 1. Identification and (relative) quantification of a specific set of modifications (oxidation, glycosylation and deamidation) on the mAb.

Protein	Modification	Recovery	Abundance
Rituximab_LC	Q1+NH_3 loss	Good	87.81%
Rituximab_LC	W90+Oxidation	Good	2.06%
Rituximab_HC	~Q1+NH_3 loss	Good	100.00%
Rituximab_HC	W281+Oxidation	Good	4.98%
Rituximab_HC	N301+A1G0F	Fair	2.87%
Rituximab_HC	N301+A1G1F	Fair	1.22%
Rituximab_HC	N301+A2G0	Fair	1.30%
Rituximab_HC	N301+A2G0F	Fair	37.69%
Rituximab_HC	N301+A2G1F	Fair	44.86%
Rituximab_HC	N301+A2G2F	Fair	10.77%
Rituximab_HC	N301+M5	Fair	1.07%
Rituximab_HC	N365+Deamidation	Good	2.72%
Rituximab_HC	W385+Oxidation	Good	5.37%
Rituximab_HC	G450+Lys	Good	3.2683%

Conclusion

For peptide mapping, especially the combination of UV and MS detection, the Vanquish Flex setup chosen for the experiments, consisting of column size of 2.1 x 250 mm coupled with Thermo Scientific™ Viper™ Fingertight Fitting connections and a flow rate of 0.3 mL/min combined with the HESI-II source on the mass spectrometer, delivers a very robust setup allowing straightforward method transfer to UV-based QC applications. The SMART Digest Kit compliments this by delivering highly reproducible digestion of samples allowing for easier and more confident data interpretation.

References

1. Thermo Scientific Technical Note 108: Reliable Solvent Mixing in UHPLC. Sunnyvale, CA, 2011.
2. Thermo Scientific Oral Technical Presentation 71659: High Throughput Peptide Mapping with the Vanquish UHPLC System and the Q Exactive HF Mass Spetrometer. Germering, Germany, 2015.
3. Thermo Scientific Application Note 1132: Reliable Results in Peptide Mapping Using the Vanquish Flex UHPLC System. Germering, Germany, 2015.

thermo scientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Find out more...

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

Mauro De Pra,[1] Ken Cook,[2] Mike Oliver,[3] and Carsten Paul[1]
[1]Thermo Fisher Scientific, Germering, Germany
[2]Thermo Fisher Scientific, Hemel Hempstead, United Kingdom
[3]Thermo Fisher Scientific, Runcorn, United Kingdom

Goal

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

Introduction

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

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

Equipment

Vanquish UHPLC system consisting of:

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

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

Protein Digestion

SMART Digest Kit (P/N 60109-101)

Experimental

Sample Preparation

1. Cetuximab® monoclonal antibody (5 mg /mL) was diluted 1:4 with the SMART Digest buffer to a final volume of 100 µL

2. The diluted sample was then added to a SMART Digest tube and left for 60 minutes at 70 °C

3. The digested sample was then centrifuged at 10,000g for 5 minutes and the supernatant was removed for chromatographic analysis

Conditions

Column:	Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm Analytical (2.1 × 250 mm), P/N 074812
Mobile Phase:	A: 0.05% TFA in water, P/N TFA 85183 B: 0.04% TFA in 8/2 acetonitrile/water (v/v), P/N acetonitrile TS-51101
Gradient:	0–30 min: 4–50% B, 30-31 min: 50–90% B, 31–35 min: 90% B, 35–36 min: 90–4% B, 36–45 min: 4% B
Flow Rate:	0.4 mL/min
Maximal Pressure:	384 bar
Temperature:	80 °C; Forced Air Mode
Injection Volume:	5 µL
Detection:	214 nm Data Collection Rate: 20 Hz Response Time 0.2 sec
Flow Cell:	10 mm LightPipe™

Results and Discussion

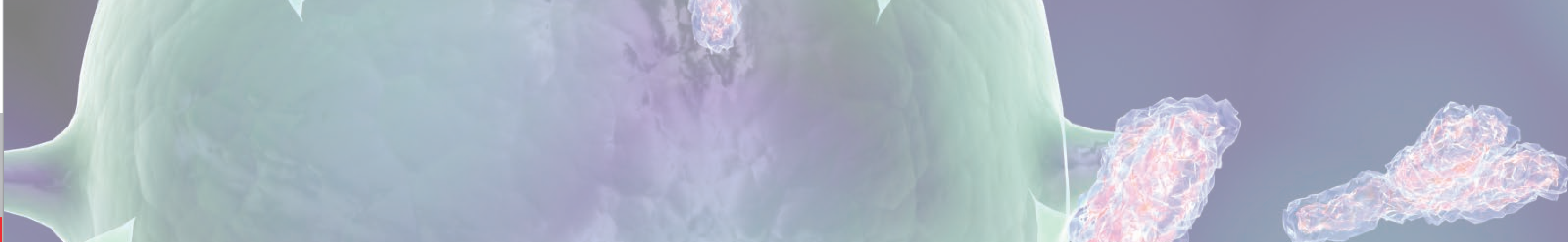
The digestion was achieved utilizing the SMART Digest Kit. Using this approach the sample preparation time could be reduced significantly and total preparation time of the monoclonal antibody (mAb) digest was lower than 75 minutes.

The separation of the resulting peptides was obtained with a 30 minutes gradient, and a total analysis time of 45 minutes, including column wash with high organic eluent, and re-equilibration at initial conditions. Figure 1 shows the overlay of 13 consecutive injections of the same sample of mAb digest.

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

ThermoFisher SCIENTIFIC

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations



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

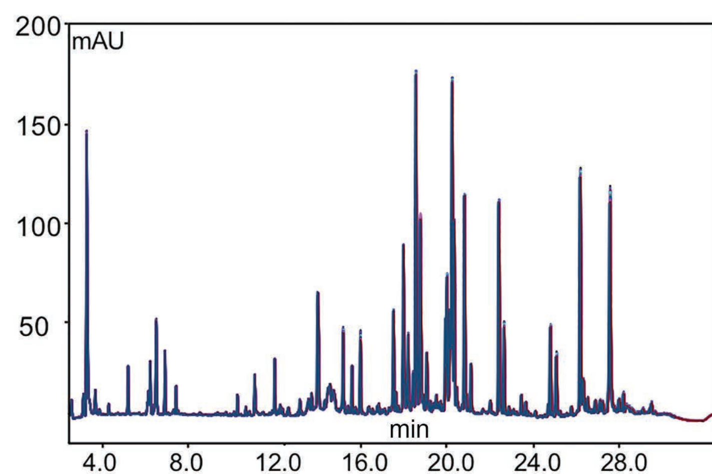


Figure 1. Overlaid chromatogram of 13 repeated injections of the mAb tryptic digest.

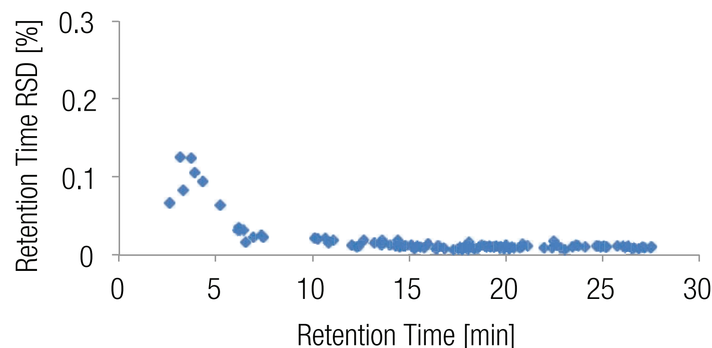


Figure 2. Retention Time RSD (%) relative standard deviation measured for 13 repeated injections of a mAb digest.

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

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

Retention Time (min)	Average Area (mAU*min)	RSD Area (%)
5.23	1.04	0.10
10.29	0.36	0.22
13.07	0.05	0.94
15.96	0.96	0.49
22.39	5.17	0.14
24.68	0.25	0.61

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

Conclusion

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

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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Peptide Quantitation

Product and method development considerations

Find out more...

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Martin Samonig, Sabrina Patzelt, Carsten Paul, Martin Rühl, and Remco Swart
Thermo Fisher Scientific, Germering, Germany

Goal

To demonstrate the use of the new Thermo Scientific™ Vanquish™ Duo UHPLC system and enable tandem analysis with two columns in parallel, addressing productivity and throughput improvement of existing LC-MS methods.

Introduction

Common liquid chromatography (LC) methods with gradient elution can be segmented into an analytical gradient section and a reconditioning section. The gradient section is responsible for the actual chromatographic separation, while the reconditioning section is used for the column wash and re-equilibration for the next injection (Figure 1). The process of column re-equilibration involves replacing the mobile phase between the particles (inter-particle), within the pores of the particles (intra-particle), and in the interfacial region between the mobile phase and stationary phase.[1] Good and accepted practice suggests using at least five column volumes to sufficiently equilibrate the analytical column.[2] If a column is required to be equilibrated with a buffered mobile phase or with a mobile phase containing an ion pair reagent, the required equilibration time is even longer. Depending on the column dimensions, gradient length, and flow rate, typically 10–60% of the total runtime is consumed by these column reconditioning steps within the gradient method.

Many UHPLC peptide mapping methods require lengthy periods of column washing and equilibration between separations. To possibly increase throughput and mitigate these delays without changing the chromatographic gradient section, a tandem LC approach with a two-pump setup and column switching capabilities can be implemented. In this setup one column is used for the

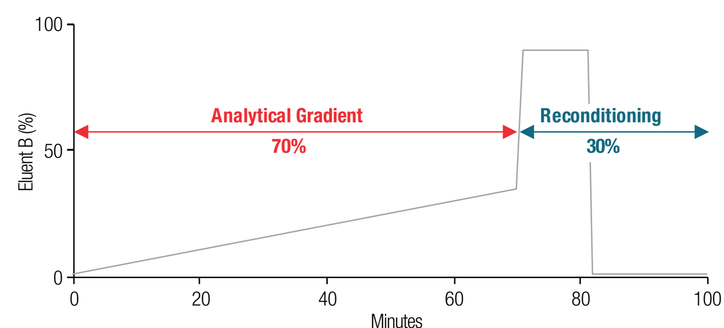

Figure 1. Gradient and reconditioning section of a common LC method.

ongoing separation, while the second column is switched offline from the mass spectrometer (MS) and simultaneously washed and conditioned for the next injection (Figure 2). The technique provides several benefits. First, throughput can be increased without changing existing (validated) methods. Second, with the latest instrument technology, a system suitable for the technique does not occupy any additional bench space (compared to a second LC-MS system). Third, laboratories can increase throughput without additional staff to operate multiple instruments.

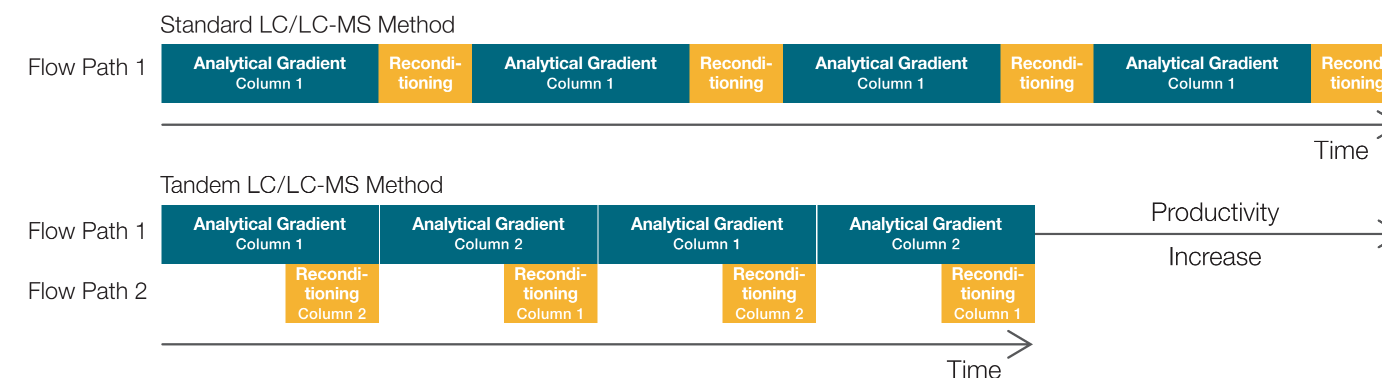

Figure 2. Standard LC-MS method compared to tandem LC-MS method.

Experimental

Consumables

- 2 × Thermo Scientific™ Acclaim™ VANQUISH™ C18, column 2.1 × 250 mm, 2.2 µm, (P/N 074812-V)
- Fisher Scientific™ LC/MS grade water (P/N W/011217)
- Fisher Scientific LC/MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ LC/MS grade formic acid (P/N 28905)
- Thermo Scientific™ SMART Digest™ Kit (P/N 60109-101)

Sample pretreatment and sample preparation

A commercially available monoclonal antibody infliximab drug product (Hospira® UK Limited, Leamington Spa, United Kingdom) was supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.05 mg/mL polysorbate 80, 50 mg/mL sucrose, 0.22 mg/mL monobasic sodium phosphate monohydrate, 0.61 mg/mL dibasic sodium phosphate dihydrate, and sterile water adjusted to pH 7.2 using sodium hydroxide or hydrochloric acid.

ThermoFisher SCIENTIFIC

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

SMART Digest Kit protocol

A 50 µL infliximab sample, adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the SMART Digest buffer provided in the kit. The solution was then transferred to a reaction tube containing 15 µL of the SMART Digest resin slurry, corresponding to 14 µg of heat-stable immobilized trypsin. Tryptic digestion was allowed to proceed at 70 °C for 45 min at 1400 rpm. 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.

The non-reduced sample was diluted with 0.1% formic acid (FA) in water to a final protein concentration of 100 ng/µL, and 1.0 µg was loaded on the column for all runs.

LC conditions

Instrumentation

Thermo Scientific™ Vanquish™ Horizon Duo UHPLC system for Tandem LC workflows consisting of the following:

- System Base Vanquish Horizon/Flex (P/N VF-S01-A-02)
- 2x Binary Pump H (P/N VH-P10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Variable Wavelength Detector F (P/N VF-D40-A)
- Flow Cell Semi-Micro, 2.5 µL, 7 mm light path (SST) (P/N 6077.0360)
- MS Connection Kit Vanquish (P/N 6720.0405)
- Vanquish Duo for Tandem LC Kit (P/N 6036.2020)

Figure 3 shows the Vanquish Duo system for Tandem LC-MS workflows chosen for this setup, consisting of two binary high pressure gradient pumps (HPG) used as an analytical pump and a reconditioning pump. The setup is configured for best chromatographic performance using the high-end Vanquish Binary Pump H, but is not limited to this particular pump type and can be also set up using other pump modules (e.g. Vanquish Binary Pump F (P/N VF-P10-A-01) or Vanquish Quaternary Pump F (P/N VF-P20-A)). All required capillaries and additional parts for this setup are defined in Table 1.

Table 1. Parts used for the Tandem LC-MS setup. Using a Vanquish VWD for reconditioning monitoring is a very specific use-case, so additional capillaries were needed for this setup. For all other Tandem LC or LC-MS configurations (independent from the pump type) the Vanquish Duo for Tandem LC Kit (P/N 6036.2020) contains all required parts and capillaries.

#	Amount	Product	PN
1	2	Biocompatible 2-position/6-port (2p6p) column switching valve	6036.1560
2	2	Viper Capillary, MP35N, biocompatible, 0.1 × 65 mm	6042.2306
3	2	Viper Capillary, MP35N, biocompatible, 0.1 × 150 mm	6042.2320
4	1*	Viper Capillary, MP35N, biocompatible, 0.1 × 250 mm	6042.2330
5	2	Viper Capillary, MP35N, biocompatible, 0.1 × 450 mm	6042.2350
6	1	Viper Capillary, MP35N, biocompatible, 0.1 × 750 mm	6042.2390
7	1	Viper Capillary, MP35N, biocompatible, 0.18 × 350 mm	6042.2337
8	2*	Active Pre-heater, 0.1 × 380 mm	6732.0110

* 1 already included in System Base Vanquish Ship Kits

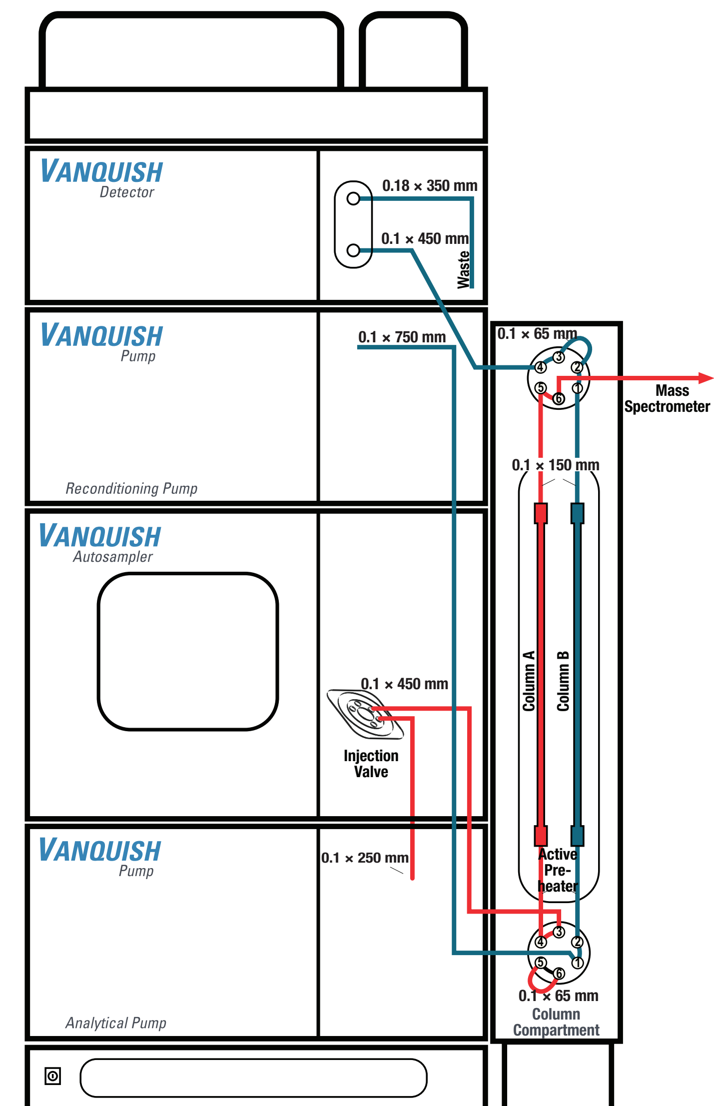

Figure 3. Vanquish Horizon Duo UHPLC system for Tandem LC workflows with 2-position/6-port (2p6p) valve configurations and required fluidic connections (for details see Table 1). * The recommended capillary to connect the LC to individual mass spectrometer depends on the setup and is defined in the Vanquish MS Connection Kit.

thermoscientific

Table of Contents

Introduction

Peptide Mapping

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

High-precision, automated peptide mapping of proteins

Automated chymotrypsin peptide mapping of proteins by LC-MS

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Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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

Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform

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

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

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

Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses

Peptide Quantitation

Product and method development considerations

Separation conditions

Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Water/acetonitrile (10:90 v/v) + 0.1% formic acid
Flow rate:	See Table 2
Temperature:	60 °C, forced air
Detection:	214 nm
Gradient:	See Table 2 and Figure 4

Table 2. LC gradient conditions for the separation of the mAb digest.

Analytical Pump			
Time [min]	A1 [%]	B1 [%]	Flow Rate [mL/min]
0.0	99	1	0.4
40.0	55	45	0.4
40.1	99	1	0.4
43.0	99	1	0.4
Reconditioning Pump			
Time [min]	A1 [%]	B1 [%]	Flow Rate [mL/min]
0.0	99	1	0.4
1.0	10	90	0.4
6.0	10	90	0.4
7.0	99	1	0.4
10.0	99	1	0.4
12.0	99	1	0.4
13.0	10	90	0.4
18.0	10	90	0.4
19.0	99	1	0.4
43.0	99	1	0.4

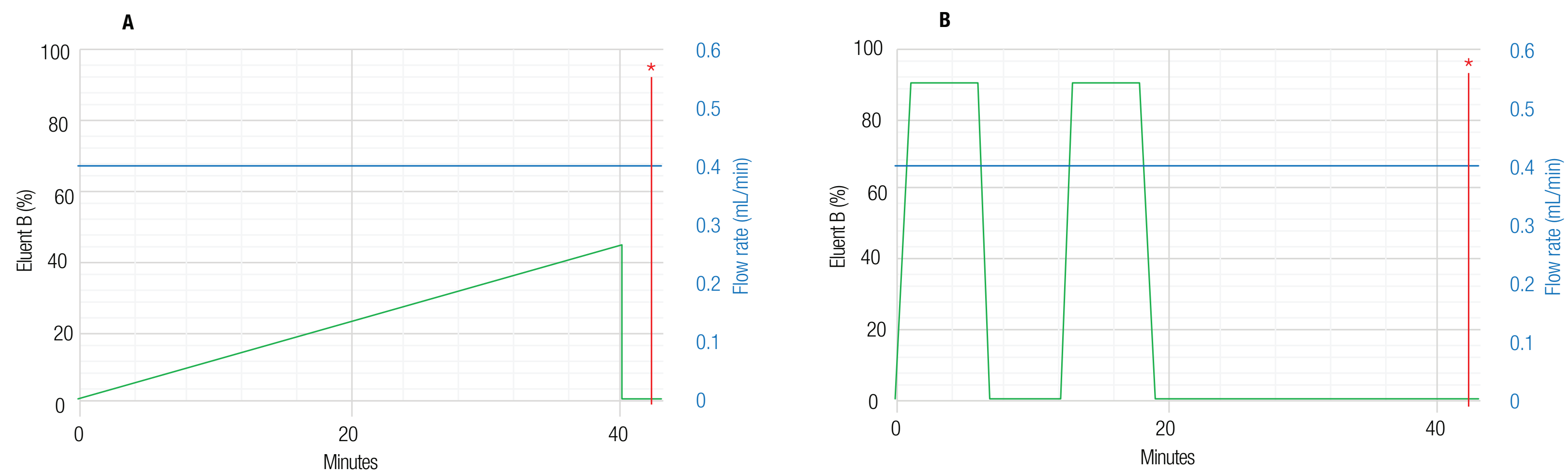

Figure 4.(A) Gradient Analytical Pump including the void volume purge at 40.0 min and (B) Gradient Reconditioning Pump including a multi-step wash section. The method was created using the Tandem LC method wizard implemented in Chromeleon. *Upper and lower valve switched position simultaneously at 40.9 min, with the "ColumnComp. NextColumn" command which is automatically inserted in the method from the Tandem LC method wizard.

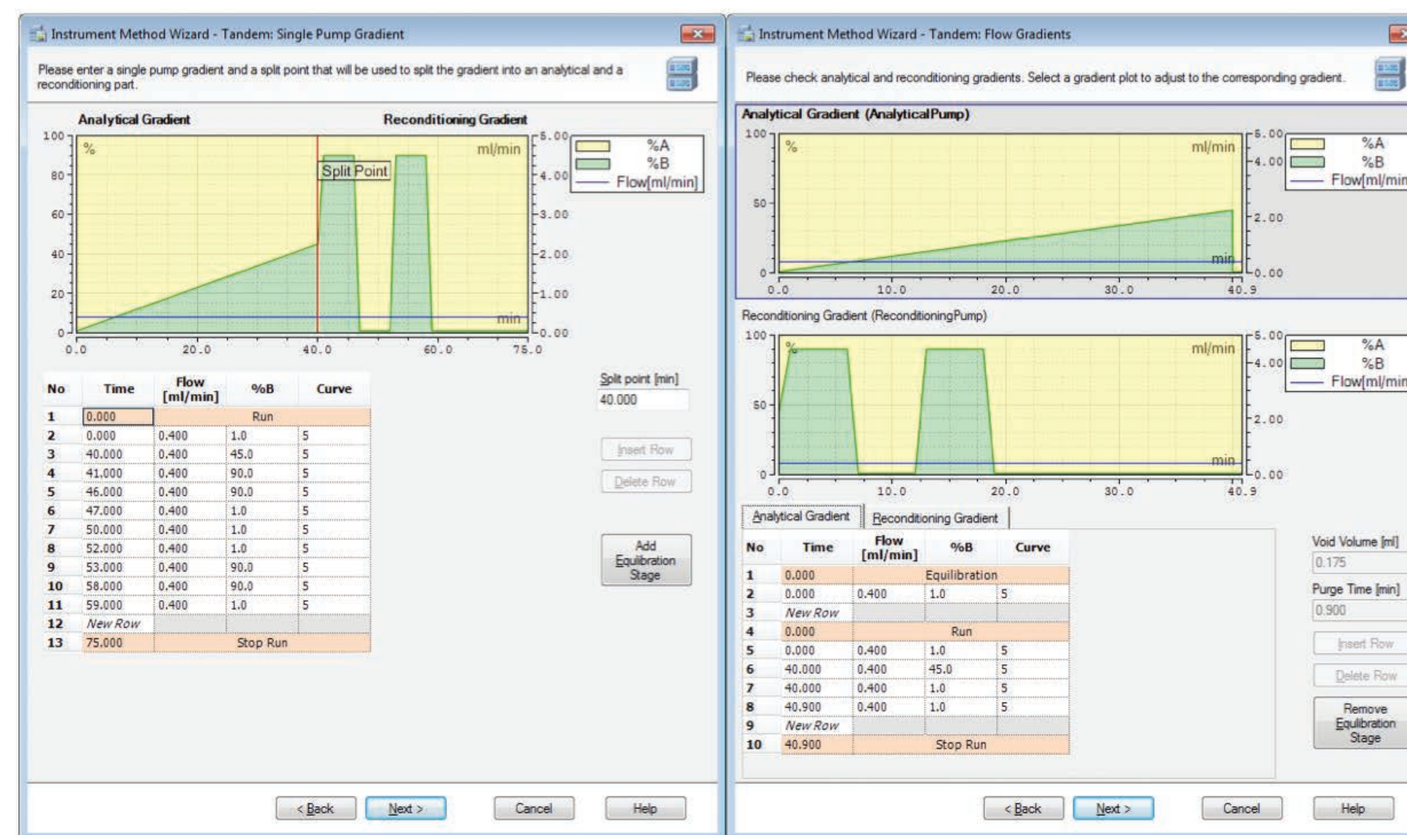

Figure 5. Chromeleon Tandem LC method wizard.

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Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

To enable the Tandem LC workflow, the corresponding fluidic description has to be selected within the Thermo Scientific™ Chromeleon™ Chromatography Data System software. Implemented in Chromeleon is also a specific Tandem LC method wizard (Figure 5), which enables straight forward transformation of existing methods into Tandem LC methods. The lower and upper switching valve of the Thermo Scientific™ Vanquish™ Thermostatted Column Compartment (VTCC) was used to switch between the two flow paths and two analytical columns (Figure 6). The analytical pump was utilized to deliver a water/acetonitrile +0.1% formic acid gradient (Table 2) to separate the peptides on one column. Simultaneously, the second column, offline from the mass spectrometer, was subject to a multi-step wash and equilibration gradient delivered by the reconditioning pump (Table 2) prior to being switched online for the next injection. A multi-step wash section with repeated up and down gradients was used to increase the washing efficiency and to reduce carryover for very big and non-polar tryptic peptides.[3] At the end of the gradient, the analytical pump was set to initial conditions at 40.0 min to perform a void volume purge and equilibrate the fluidics from the analytical pump to the lower switching valve for the next injection. At 40.9 min, the lower and upper switching valve changed the position and the next sample was immediately injected on the pre-equilibrated analytical column. In LC-MS setups a UV detector is not always needed. This setup used the Thermo Scientific™ Vanquish™ Variable Wavelength Detector (VWD) to monitor the reconditioning step to ensure that no peptides were eluting from the column during this stage, and to confirm proper column equilibration.

MS conditions

The Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap mass spectrometer was used for detection. The detailed MS source and method parameters are given in Table 3.

Data processing

The data were acquired and analyzed with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2.8.

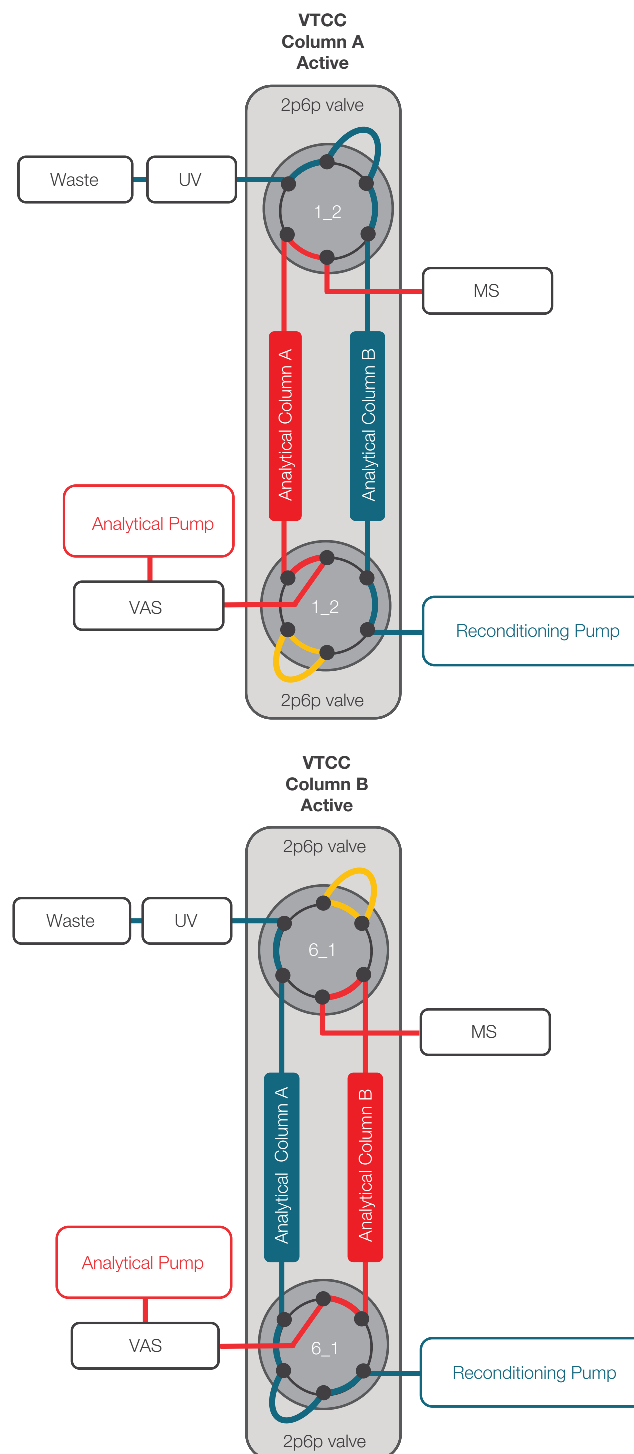

Figure 6. Flow schematic for tandem operation with two flow paths. One for analysis (red) and one for off-line column wash and re-equilibration (blue).

Results and discussion

Using the Vanquish Horizon Duo system for Tandem LC-MS peptide mapping experiments, or more precisely for the separation of the tryptic digested monoclonal antibody infliximab, gave reproducible and confident results as demonstrated in the total ion current (TIC) chromatogram overlay of five replicates (Figure 7) on two analytical columns with automated alternating column regeneration.

Table 3. MS source and method parameters.

MS Source Parameters	Setting
Source	Ion Max source with HESI-II probe
Sheath gas pressure	45 psi
Auxiliary gas flow	12 arbitrary units
Vaporizer temperature	350 °C
Capillary temperature	350 °C
S-lens RF voltage	60 V
Source voltage	3.5 kV
MS Method Parameters	**Setting**
Method type	Full MS only
Full MS mass range	140–2000 *m/z*
Resolution settings	15.000 (FWHM at *m/z* 200)
Target value	3e6
Max injection time	200 ms
Microscans	1
SID	0 eV

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
Investigating process-related post-translational modifications in NISTmAb RM 8671 using high-throughput peptide mapping analysis
Robust and reproducible peptide mapping and intact mass analysis workflows on a single instrument platform
SMART Digest compared to classic in-solution digestion of rituximab or in-depth peptide mapping characterization
LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC
Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

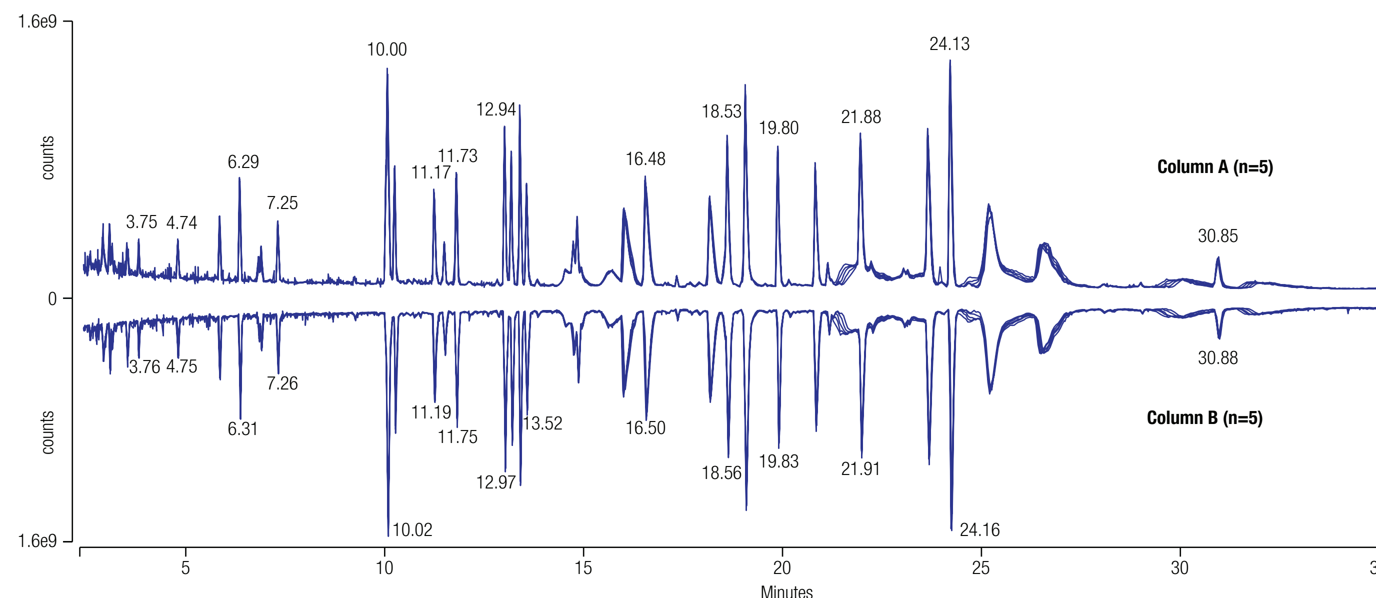

Figure 7. Reproducible results for the Vanquish Horizon Duo system for Tandem LC - Q Exactive HF setup, showing the overlay of five TIC chromatograms for the separation of digested infliximab using the SMART Digest Kit.

Table 4. Reproducible results for the Vanquish tandem LC - Q Exactive HF setup with detailed RSD values for infliximab tryptic peptides for column A/B in tandem and single column operation based on the TIC chromatograms shown in Figure 7.

Column A			Column B					Column A/B	
RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	Abs. RT Shift Column A to B [min]	Rel. RT Shift Column A to B [%]	RT RSD [%] n=10	Area RSD [%] n=10
3.75	0.18	2.72	3.76	0.13	1.64	0.005	0.13	0.16	2.12
4.74	0.054	3.53	4.75	0.11	5.05	0.010	0.21	0.14	4.24
6.29	0.072	2.33	6.31	0.037	1.14	0.020	0.32	0.18	2.19
7.25	0.018	4.94	7.26	0.033	4.72	0.016	0.23	0.12	4.94
10.00	0.032	3.05	10.02	0.037	1.75	0.023	0.23	0.12	2.35
11.17	0.040	3.96	11.19	0.047	2.70	0.022	0.20	0.11	3.22
11.73	0.043	1.64	11.75	0.007	2.59	0.025	0.21	0.12	2.15
12.94	0.014	4.19	12.97	0.012	1.61	0.023	0.18	0.10	3.03
13.49	0.028	1.66	13.52	0.025	3.11	0.024	0.18	0.10	2.36
16.48	0.056	1.02	16.50	0.031	0.78	0.024	0.14	0.087	0.91
18.53	0.019	1.94	18.56	0.020	1.90	0.027	0.15	0.080	2.35
19.80	0.019	0.50	19.83	0.016	0.78	0.029	0.15	0.078	0.62
21.88	0.028	4.35	21.91	0.0075	1.71	0.033	0.15	0.083	3.78
24.13	0.025	1.52	24.16	0.030	0.60	0.031	0.13	0.072	1.09
30.85	0.039	1.56	30.88	0.039	2.00	0.031	0.10	0.064	1.74
Average	**0.045**	**2.59**		**0.039**	**2.14**	**0.023**	**0.18**	**0.11**	**2.47**

Retention time relative standard deviation (RSD) values below 0.11% were achieved for the UHPLC system in tandem column operation compared to 0.045% and 0.039% for the single column setup. Polar tryptic peptides eluting between 0 and 14 min had the highest RSD values up to 0.18%, and the heavy chain peptide (D151-Y183) at 30.85 min had the lowest with 0.064% (Table 4). The average absolute retention time shift between column A and column B was 0.023 min (relative, 0.18%) and shows that peak assignment based on retention time is not impaired. An average peak area RSD value of 2.47% demonstrates the suitability for quantitative data analysis using the tandem LC setup.

The advanced wash and reconditioning method used in this study enables significant reduction of protein/peptide column carryover and can also be individually optimized by reducing or increasing the flow rate during the method. The UV trace used to exclusively monitor the wash and equilibration step of the reconditioning pump showed reproducible results for all runs (Figure 8).

Table of Contents
Introduction
Peptide Mapping
Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies
High-precision, automated peptide mapping of proteins
Automated chymotrypsin peptide mapping of proteins by LC-MS
An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies
Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis
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Providing the Highest Retention Time and Peak Area Reproducibility for Maximal Confidence in Peptide Mapping Experiments
Tandem UHPLC operation for high-throughput LC-MS peptide mapping analyses
Peptide Quantitation
Product and method development considerations

Conclusions

The Vanquish Horizon Duo UHPLC system for Tandem LC or LC-MS workflows enables a throughput increase up to 40% without changing the actual gradient of the existing peptide mapping method. The retention time RSD values are below 0.11% for the tandem and single column operation. In this study, peptide mapping methods were used to demonstrate the capabilities of a tandem LC setup, but it can be applied to other methods and samples as well. Both Chromeleon version 7.2.8 and Thermo Scientific™ SII for Xcalibur™ version 1.4 support the Vanquish Duo Tandem LC workflow.

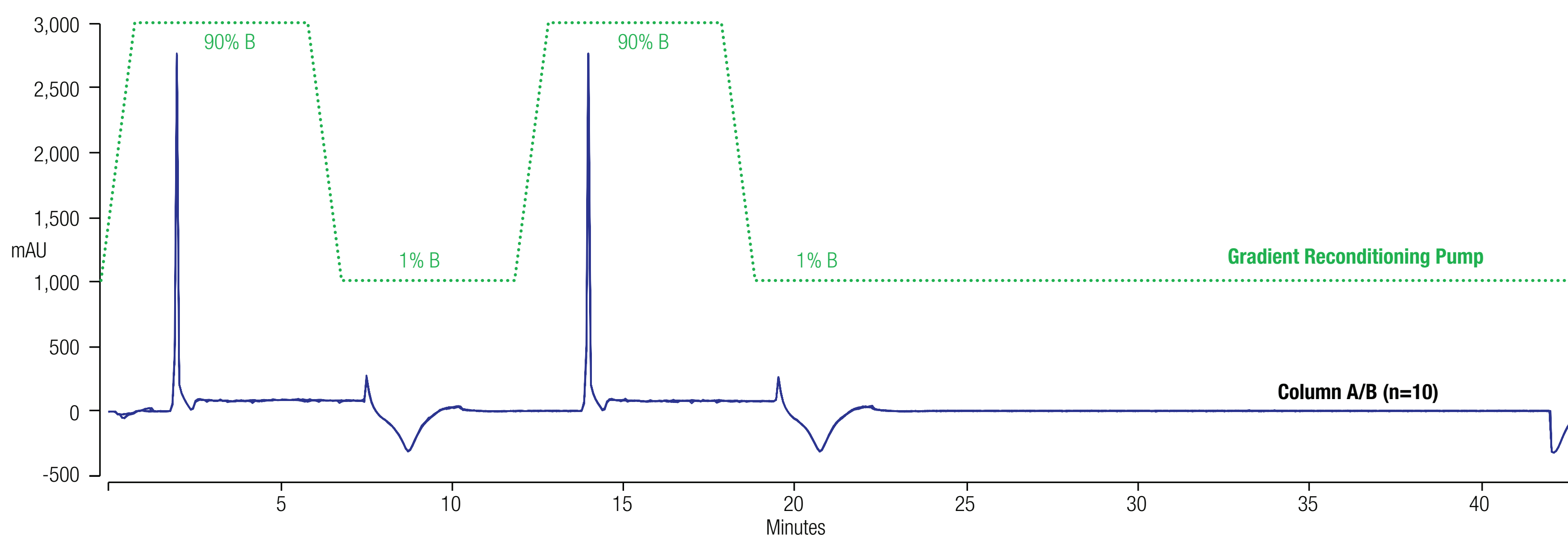

Figure 8. Overlay of ten chromatograms of the tandem LC reconditioning step.

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations
Find out more...

Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours

Brian King, Mike Oliver Thermo Fisher Scientific, Runcorn, UK

Goal

To demonstrate the rapid quantitation of the low-level biomarker Klotho from plasma by LC-MS/MS. using the Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) Kit, which combines the immunoaffinity capture and digestion process into a single well.

Introduction

Klotho, a transmembrane β-glucuronidase, is an important biomarker in aging research.[1-3] LC-MS/MS analysis provided single-run multiplexed quantitation of two specific peptides formed by the digestion of Klotho. The amount of the biomarker present was determined by quantitation of these signature peptides. An immunoaffinity step prior to digestion was used to purify the target biomarker for increased sensitivity and was able to purify all the isoforms of the biomarker.[4]

The SMART Digest IA Kit is designed for biomarker quantitation. Since biomarker proteins are often present at low levels in complex biological matrices, it is often necessary to use immunoaffinity capture to reduce interferences and thus increase sensitivity. This step is often followed by protein digestion and subsequent quantitation of known unique signature peptides.

The SMART Digest IA Kits are designed to remove issues often associated with sample preparation of proteins by delivering a process that is:

- Fast
- Simple
- Highly reproducible
- Sensitive
- Compatible with automation

The SMART Digest IA Kits achieve this with their unique design, where the immunoaffinity reagents (either streptavidin, protein A, or protein G) and heat-activated, thermally stable trypsin are co-immobilized onto a single bead. Following the binding of a capture reagent to the bead and enrichment of the target, the enzyme is activated at elevated temperatures for accelerated digestion under protein denaturing conditions.

This results in a fast, easy-to-implement, and sensitive workflow (Figure 1). Magnetic and non-magnetic versions of the beads are available.

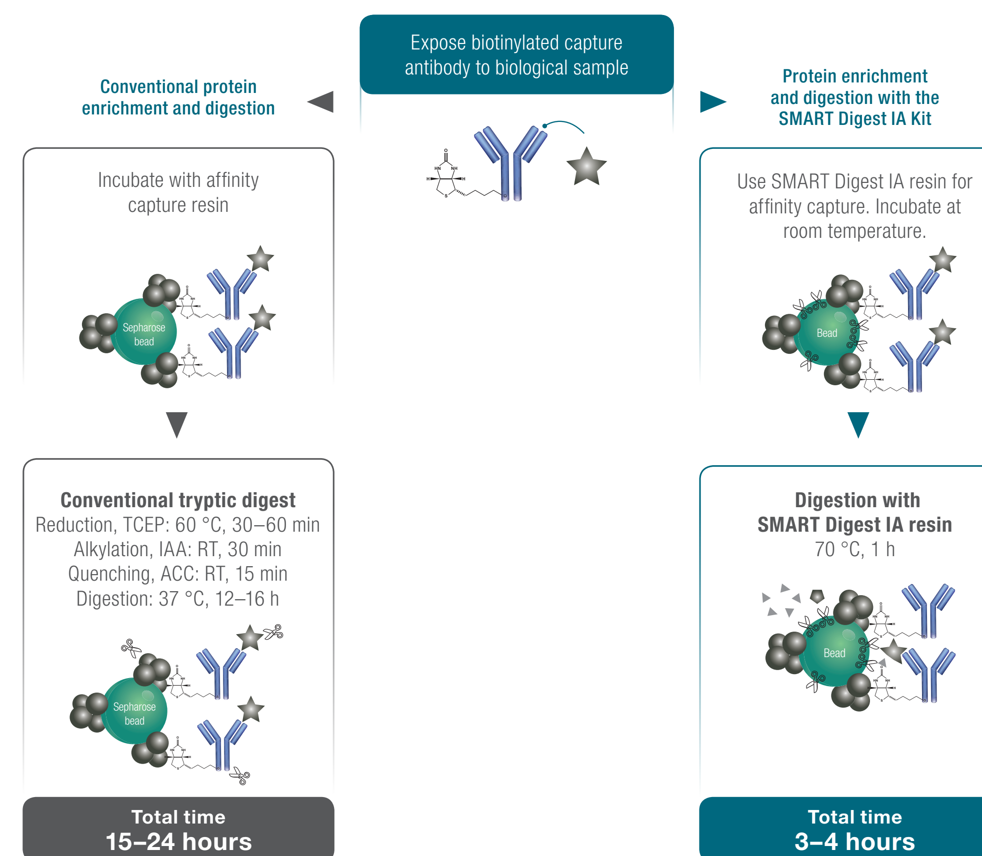

Figure 1. Comparison of SMART Digest IA Kit workflow with a conventional workflow.

Consumables

Affinity / Digestion

- SMART Digest IA Streptavidin Non-Magnetic Kit (P/N 60110-101)

Chemicals

- Deionized water, 18.2 MΩ/cm resistivity
- Fisher Scientific™ Optima™ Acetonitrile (ACN) (P/N A955-4)
- Fisher Scientific™ Formic Acid (FA) (P/N F/1900/PB08)
- Thermo Scientific™ Pierce™ Dimethylsulfoxide (DMSO), LC-MS Grade (P/N 85190)
- Thermo Scientific™ EZ-Link™ NHS-Biotin (P/N 20217)
- Human Klotho antibody from a reputable supplier
- Recombinant human Klotho (rhKlotho) from a reputable supplier
- Murine plasma from a reputable supplier

Sample Handling

- Thermo Scientific™ Pierce™ Microcentrifuge Tubes, 1.5 mL (P/N 69715)
- Thermo Scientific™ Mass Spec™ Certified 2 mL clear vial with blue bonded PTFE silicone cap (P/N MSCERT5000-341W)

Sample Handling Equipment

- Thermo Scientific™ MicroCL 17 microcentrifuge (P/N 75002449)
- Thermo Scientific™ LP Vortex Mixer (P/N 88880018)
- Heater/shaker equipped with heated block and lid

Antibody Pretreatment

Antibody biotinylation: 500 µL of 100 µg/mL Klotho antibody was biotinylated by the addition of 2.5 µL of 0.5 mg/mL NHS-biotin in DMSO. The solution was then incubated for 2 hours at room temperature (RT) on a heater/shaker set at ambient temperature and 1400 RPM before storage at 4 °C until subsequent use.

Standard Sample Preparation

rhKlotho was spiked into mouse plasma in varying concentrations ranging from 1.58 ng/mL to 500 ng/mL.

Immunoaffinity Purification Using the SMART Digest IA Kit

Purification: 500 µL of sample, 1 µg (10 µL) of biotinylated Klotho antibody, and 30 µL of SMART Digest IA resin were added to a 1.5 mL microcentrifuge tube, placed in a heater/shaker and incubated for two hours at room temperature and 1400 RPM. Following the capture step, 500 µL of supernatant was decanted, 500 µL of wash buffer was added to the samples before they were vortexed, and then centrifuged at 13,200 RPM (16,100 RCF) for 1 minute. This was repeated for a total of seven washes.

Digestion Using the SMART Digest IA Kit

After the final centrifugation, 460 µL of supernatant was removed, carefully with no removal of beads, by the pipetting step. 150 µL of SMART Digest buffer was added and samples were incubated at 70 °C and 1400 RPM for 90 minutes.

Separation Conditions

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system equipped with:

- SRD-3600 Solvent racks with degasser (P/N 5035.9230)
- DGP-3600RS Rapid Separation Pump (P/N 5040.0066)
- WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)

Column

Thermo Scientific™ Accucore™ C18 column
2.1 mm × 50 mm, 2.6 µm (P/N 17126-052130)

LC Settings

Mobile Phase A	0.1% formic acid in 98% water and 2% acetonitrile
Mobile Phase B	0.1% formic acid in 5% water and 95% acetonitrile
Gradient	See Table 1
Flow Rate	0.5 mL/Min
Column Temperature	40 °C
Injection Details	5 µL

Table 1. LC gradient conditions.

Time (min)	A %	B %
0	98	2
1	98	2
6	50	50
6.1	10	90
7.5	10	90
7.51	98	2
9	98	2

MS Conditions

Instrumentation

Thermo Scientific™ Velos Pro™ Ion Trap Mass Spectrometer

MS Settings

Mode	Positive
Heater Temp	350 °C
Sheath Gas	60
Aux Gas	20
Spray Voltage	4 kV
Capillary temp	375 °C
S-Lens RF Level	55%

Data Processing

The Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System SR2 was used for data acquisition and analysis. MS instrument control was by Thermo Scientific™ Xcalibur™ software.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations

Results and Discussion

Using the SMART Digest IA Streptavidin kit and high flow LC-MS/MS, the method was able to achieve a LLOQ of 1.58 ng/mL in mouse plasma. CVs at the LLOQ were 6.3% and 12.5% without the use of isotopic standards. Additionally, as seen in Figure 2, the data exhibited strong linearity across the dynamic range tested with excellent linearity at low levels of FISWAR marker.

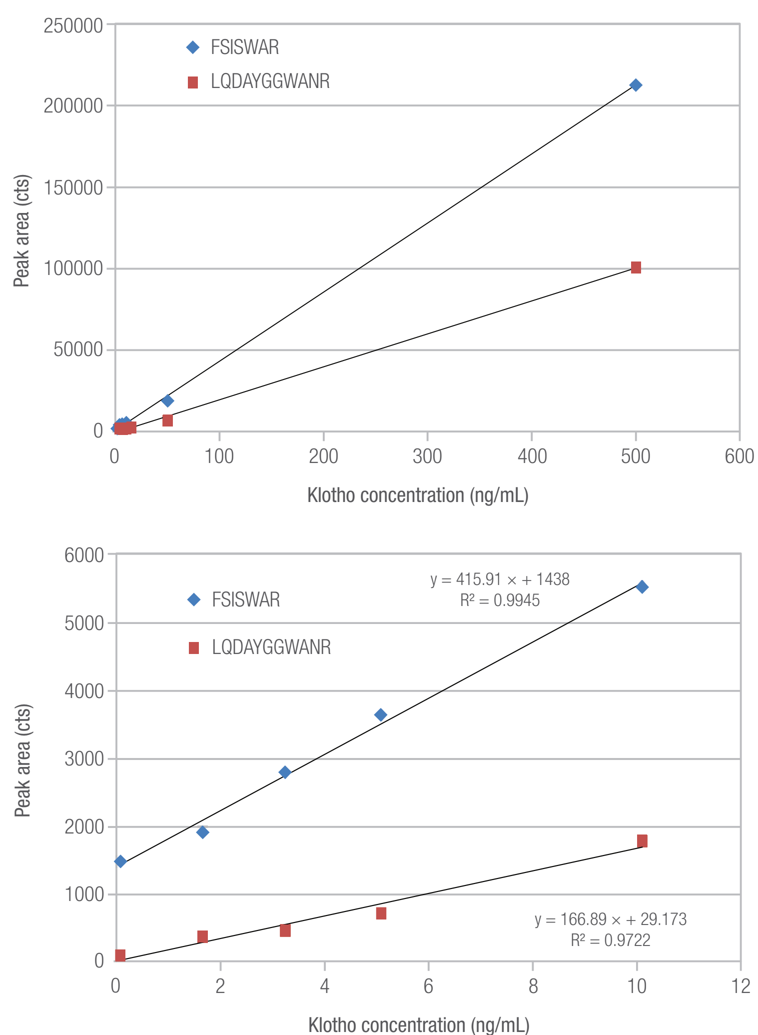

Figure 2. Calibration curves for the detection of Klotho from plasma using SMART Digest IA Streptavidin kit. Samples were run in triplicate. Figure 2A shows the full range of the calibration curve. Figure 2B shows the lower concentrations with linear fit.

The SMART Digest IA Kit enabled the preparation of assay and calibration samples in 3 to 4 hours. The samples were analyzed using LC-MS/MS, with linearity coefficients of > 0.97 and CVs of < 17% for the two marker peptides being achieved across the calibration range.

Table 2. Determination of peptide at low, medium, and high levels of quantitation. A weighted fit was used (1/x where x is the analyte concentration).

Compound	Linearity Range (ng/mL)	R^2	CV (%) at LLOQ (n=3)	CV (%) at MLOQ (n=3)	CV (%) at HLOQ (n=3)
FSISWAR	1.58–500	0.9945	6.3	10.0	9.7
LQDAYGGWANR	1.58–500	0.9722	12.5	17.0	10.5

Conclusions

- A simple, robust method for the quantification of Klotho was developed using the SMART Digest IA Kit.
- The kit combines immunoaffinity enrichment and digestion in a single reactor to produce results in less than four hours.
- The full biological range of Klotho and its isoforms, as well as other low-abundance biomarkers, can be accessed using this generic strategy.

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations
Find out more...

Simplifying ImmunoAffinity Capture Workflow

Rapid, Sensitive, LC-SRM Quantitative Analysis of Proteins in Plasma

As pharmaceuticals grow more efficacious, reporting regulations grow more rigorous. With more low abundance proteins shown to be key biomarkers the need for high sensitivity assays that are easy to perform and amenable to high-throughput workflows gain importance. For protein biomarkers in particular, effective, rapid digestion and detection strategies are becoming increasingly critical to analytical workflows. Current methods can be laborious, time consuming and often hard to reproduce.

The Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) Kits enable a simple combined workflow of protein enrichment by immunoaffinity capture (IAC) and high estion under protein denaturing conditions. temperature proteolytic digestion in a single, easy-to-use reagent.

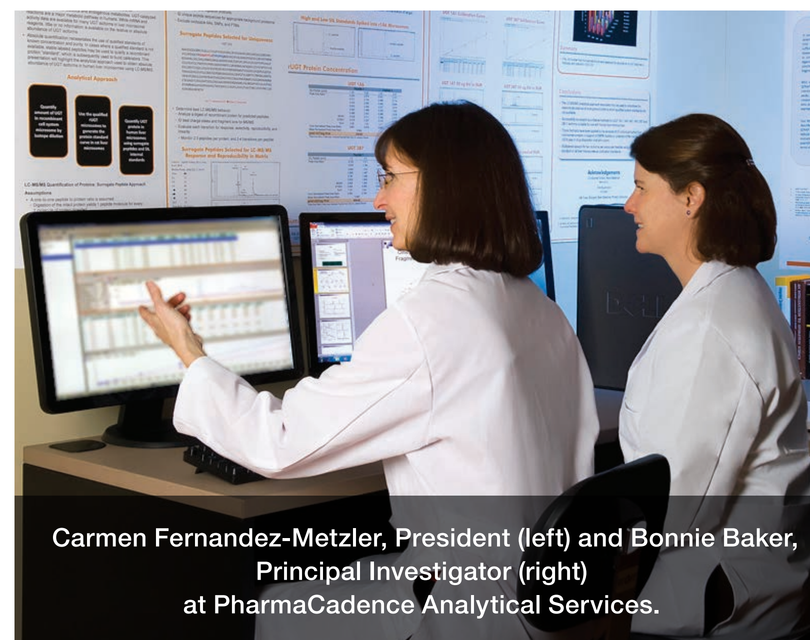
Introduction

PharmaCadence Analytical Services is a professional analytical services organization for pharmaceutical and life sciences with expertize in LC-MS services. PharmaCadence specialize in high quality quantitative Liquid Chromatography Mass Spectrometry (LC-MS) bioanalytical methods for research and early development. This includes services in biomarker quantification such as protein and peptide quantitative analysis by Liquid Chromatography Single Reaction Monitoring (LC-SRM).

Rapid and Easy Protein Enrichment and Digestion

Dr. Fernández-Metzler, President, and Dr. King, Laboratory Director, at PharmaCadence are leading projects to deliver quantitative information on biological therapeutics and low level biomarkers. The increased efficacy and low abundance of these compounds is driving the analytical community to deal with extremely low levels of analyte in complex biological matrices.

The most sensitive LC-MS protein quantitation and biomarker analytical procedures utilize an immunoaffinity enrichment step to concentrate the target analyte and reduce matrix background. The captured target protein is then digested to yield surrogate peptides amenable to sensitive detection by LC-SRM. The IAC LC-SRM workflow is ideal for maximizing the signal to noise from a minimum amount of sample while providing the selectivity and sensitivity needed to achieve the lowest possible limits of quantification.



Carmen Fernandez-Metzler, President (left) and Bonnie Baker, Principal Investigator (right) at PharmaCadence Analytical Services.

Dr. Fernández-Metzler and Dr. King identified the affinity capture and subsequent protein digestion as being a time consuming, multistep process which causes a significant bottleneck in a standardized IAC LC-SRM workflow. The multiple steps and the additive imprecision were found to affect the quality of their analytical results. In order to overcome these challenges the team at PharmaCadence implemented SMART Digest ImmunoAffinity Kits that allow protein enrichment and rapid proteolytic cleavage to be performed in a single, easy to use reactor in less than 4 hours. This has significantly improved their throughput, reduced assay complexity and maintained or improved the overall quality of the results.

"Coupling thermally stable trypsin and co-immobilized streptavidin allows for a simplified workflow with unprecedented speed and sensitivity from raw sample to purified digest..."

—Carmen Fernandez-Metzler, President

PHARMACADENCE
Analytical Services, LLC

The Method

The team at PharmaCadence used the SMART Digest IA Streptavadin kit sample preparation protocol to prepare a circulating biomarker protein targeted for LC-MS analysis.

The SMART Digest IA Kit combines immunoaffinity capture and digestion of the protein into a single well. This is achieved by immobilizing the streptavidin and heat-stable trypsin onto the same bead (Figure 1). Using this procedure immunoaffinity capture was achieved in 2 hours. The biomarker is captured onto the magnetic bead, washed and solvent exchanged to the SMART Digest buffer, followed by a 1 hour digestion. The immobilized trypsin being activated by elevation to high-temperature (70 °C) and addition of the pre-prepared SMART Digest buffer supplied with the kit.

The results obtained with this novel approach were compared to a conventional immunoaffinity capture procedure. This was achieved by using a high capacity streptavidin gel to capture the circulating biomarker in plasma, followed by tryptic digestion of the enriched protein.

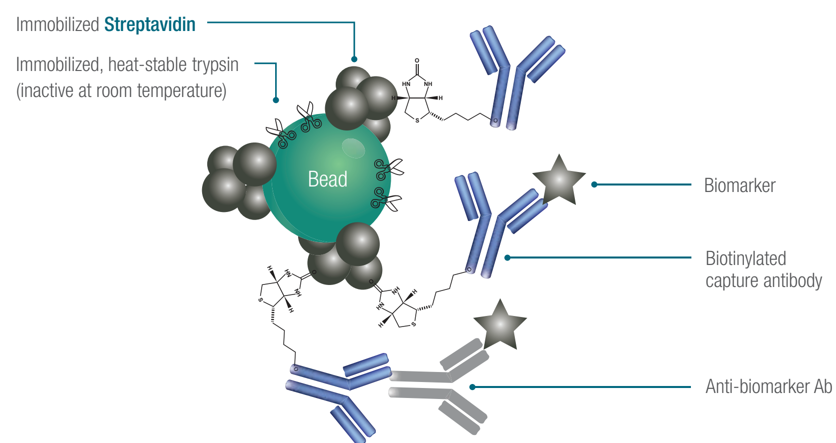


Figure 1. SMART Digest IA heat-stable, immobilized enzyme design combined with immunoaffinity capture.

Table 1. Materials.

Material	Function	Supplier
SMART Digest IA, Streptavidin magnetic bead kit (P/N 60110-104)	Protein enrichment and digestion	Thermo Fisher Scientific
High capacity Streptavidin Agarose	Protein enrichment	Thermo Fisher Scientific (Pierce™)
Trypsin	In-solution digestion	Promega Trypsin Gold
Human plasma	Biological matrix	BioreclamationIVT

Table 2. LC-MS.

UHPLC Conditions		
Column	C18, 300 Å, 3 µm, 1 × 50 mm	
Mobile Phase	A: water containing 0.1% formic acid	
	B: acetonitrile containing 0.1% formic acid	
Gradient	Time (min)	%B
	0	5
	0.2	5
	0.5	5
	4.5	20
	5.5	45
	6.5	95
	7.5	95
	8	5
	10	5
Flow Rate	50 µL/min	
Detection	SRM on a trap Mass Spectrometer	
Ionization	Electrospray	

Table 3. Preparation of Standard Curves and Quality Controls.

Recombinant protein standard curve and QCs were prepared in 4% BSA and diluted in human plasma.
Calibration curve from 125 to 500 ng/mL.
QCs in replicate.
Recovery was calculated by spiking a known level of protein into a blank sample before digestion.
Calibration curve read back concentrations and back calculated QC values were determined using the peak area ratios of target peptide and the corresponding stable isotope labeled (SIL) -peptide internal standard.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations

Workflow Time Reduction

The co-immobilized streptavidin and heat-activated temperature stable trypsin technology provided by the SMART Digest IA Kit enabled Dr. Fernández-Metzler and Dr. King to dramatically accelerate their workflow (Figure 2) with the added benefit of achieving very high sensitivities with excellent reproducibility. In the example shown the workflow was reduced from 21 hours to 3–4 hours, with an increase in sensitivity of up to 3 times.

The ability to perform digestion on the same resin as the immunoaffinity removed the need to perform a lengthy subsequent protein digestion. This dramatically reduces the amount of manual handling required and thus eliminates potential sources for error and sample loss. This directly contributes to the improved recovery and repeatability observed with the new approach. Additionally, the thermally stable trypsin allowed them to digest their samples in only 1 hour, significantly faster than the traditional over-night protocol they had employed before. Coupling these two advancements allowed the PharmaCadence team to routinely increase the speed, sensitivity and precision of their workflow.

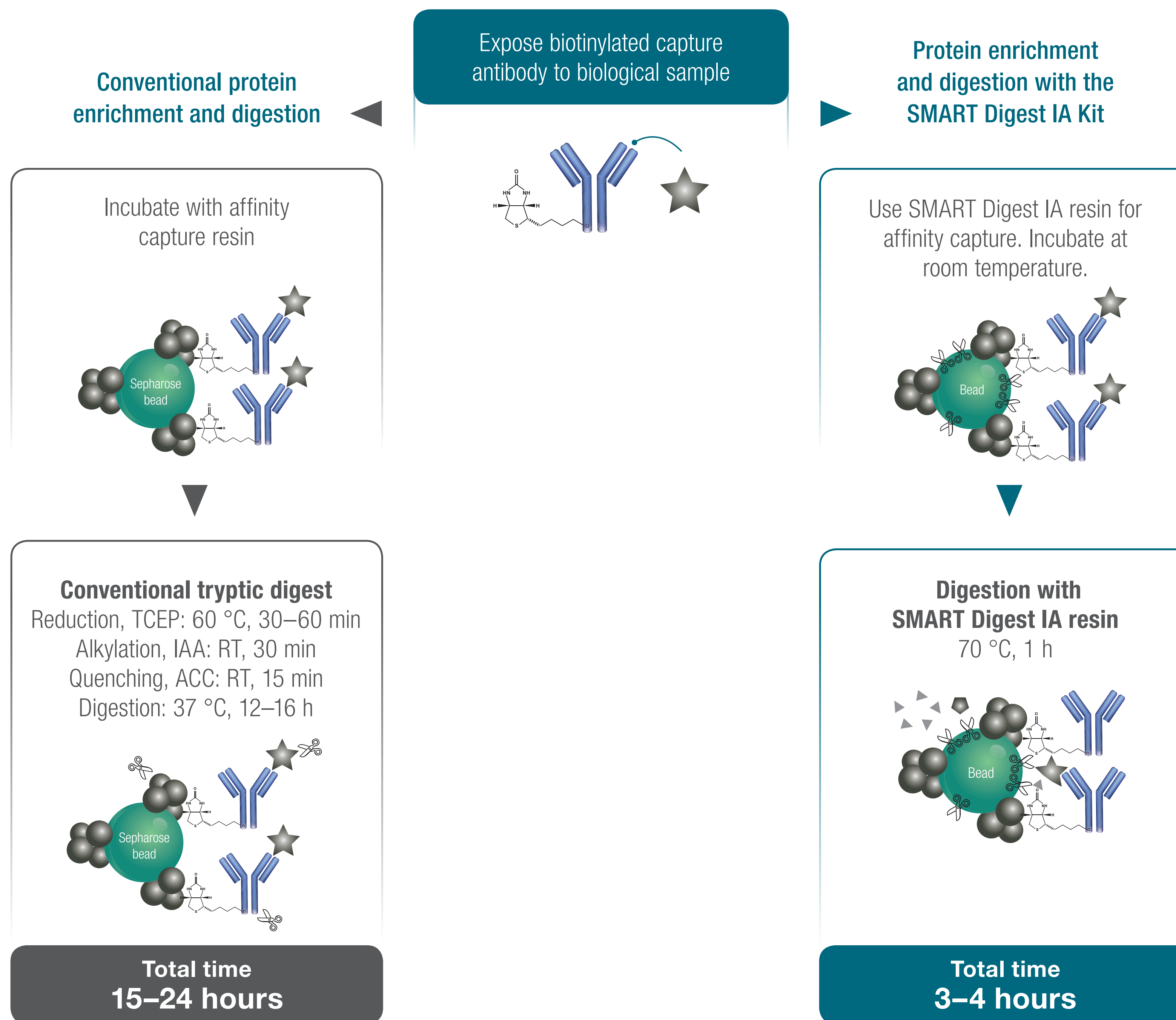


Figure 2. SMART Digest IA Kit analytical approach compared to a conventional enrichment and digestion approach.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations

IAC LC-SRM Platform Technology

A critical part of protein analysis in pharmaceuticals, food, agriculture, and many other industries is developing detection methods with good sensitivities that can be used as a standard platform. The capture protocols previously available to PharmaCadence often involve a large number of pipetting and washing steps that are best performed by an experienced and skilled analyst. The ability to purify protein from plasma and perform a complete digestion in less than 4 hours with the SMART Digest IA Kits dramatically simplifies the overall IAC method. The simplified method not only takes less time and skill to perform but consistently yields excellent precision and LLOQ (Figure 3 and 5, Table 4). The general utility of the streptavidin-biotin binding and the rapid protein digestion afforded by the SMART Digest IA Kits improves upon what the team could previously achieve (Figure 4 and 5, Table 5). They greatly enhanced efficiency making the SMART Digest IA Kit an ideal platform technology for the IAC LC-SRM.

> "The general utility, high reliability and ease of use make the SMART Digest IA Kits an ideal platform technology for IAC LC-SRM methods..."
>
> **—Bonnie Baker, Principal Investigator**

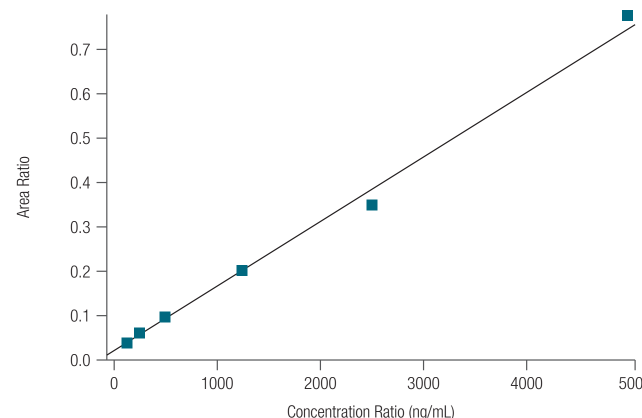


Figure 3. SMART Digest IA streptavidin kit. Human plasma calibration curve.

Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	93	116.5			
250	107	266.3	250	11.5	90.2
500	106	531.1			
1250	100	1247	1250	7.4	99.1
2500	90	2251			
5000	104	5212			

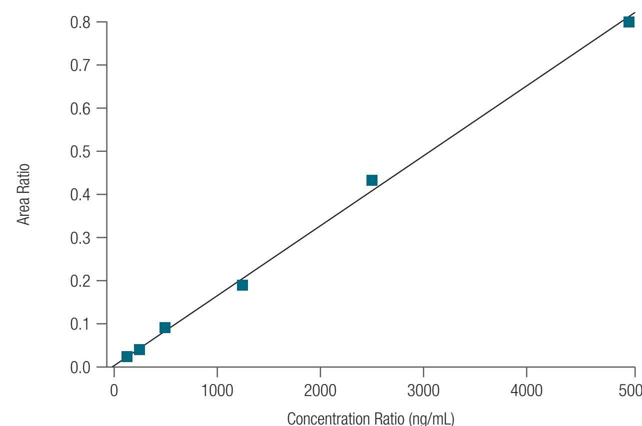


Figure 4. Conventional streptavidin IAC workflow. Human plasma calibration curve.

Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	105	131			
250	90	225	250	14.5	111.2
500	109	544			
1250	92	1149	1250	4.1	104.8
2500	106	2654			
5000	99	4922			

Table 4. SMART Digest IA streptavidin kit. Biomarker recovery from human plasma.

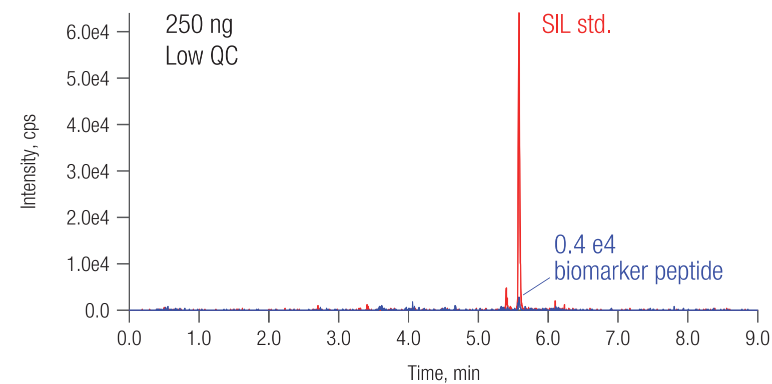
Recovery with SMART Digest IA Kit	
500 ng/mL spike	7330 (cps)
Recovery	64%

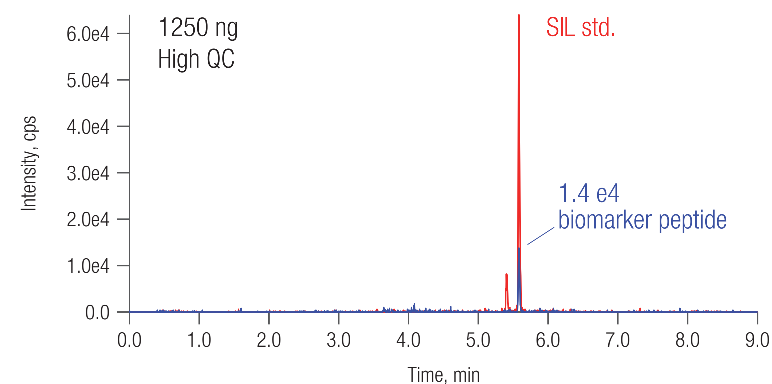
Table 5. Conventional streptavidin IAC. Biomarker recovery from human plasma.

Recovery with conventional approach	
500 ng/mL spike	2778 (cps)
Recovery	35%

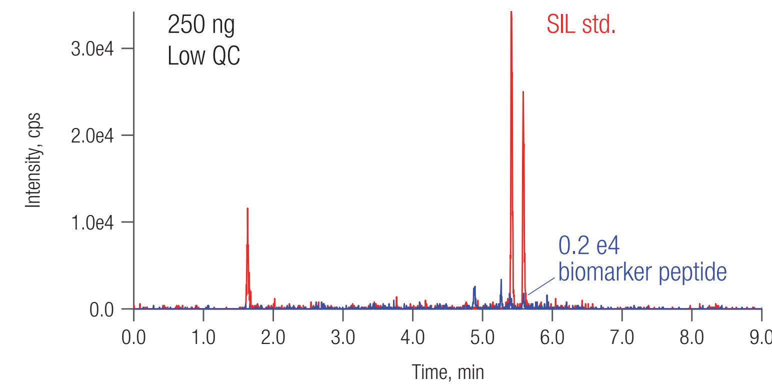
Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations

SMART Digest IA kit





Conventional streptavidin agarose process



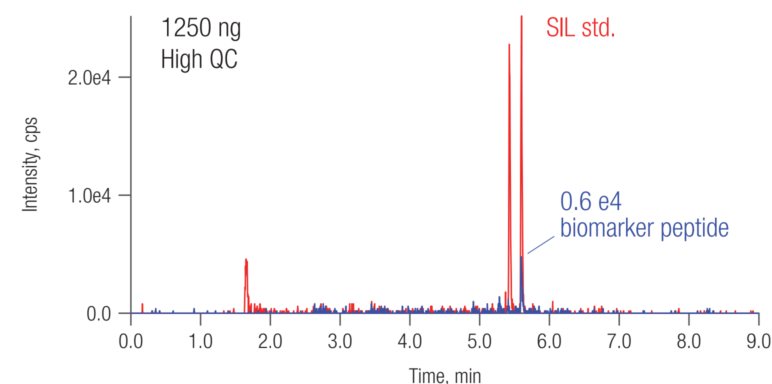


Figure 5. MS chromatographic response at low and high QC levels for sample prepared with SMART Digest IA Kit, compared to a conventional protein enrichment and digestion approach. This shows improved method recovery with samples prepared with the SMART Digest IA Kit.

Conclusions

The PharmaCadence team have experienced that as pharmaceuticals grow more efficacious, reporting regulations on food and agriculture grow more rigorous. Also, as more low abundance proteins are shown to be key biomarkers the need for simple, rapid and robust methods for IAC LC-SRM are required for success.

Dr. Fernández-Metzler, Dr. King and their team found that the co-immobilized streptavidin and heat-activated temperature stable trypsin in the SMART Digest ImmunoAffinity Kits is capable of significantly improving efficiency while delivering high quality results to allow them to keep pace with the increasing application demands.

"Coupling thermally stable trypsin and co-immobilized streptavidin allows for a generic protein quantification workflow with performance equivalent to traditional methods that can be performed routinely in a fraction of the time typically required..."

—Rick King, Laboratory Director

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations
Find out more...

Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow

Jon Bardsley, Joanne Jones, Phillip Humphryes,
Thermo Fisher Scientific, Runcorn, UK

Goal

To describe a rapid and precise protocol for peptide analysis utilizing immobilized, heat stable trypsin and micro-elution solid-phase extraction (SPE) coupled with next generation ultra-high pressure liquid chromatography and high resolution mass spectrometry (UHPLC-HRMS). Traditional trypsin digest protocols are time consuming, with some protocols taking up to one and a half days to complete and involving multiple steps including evaporation and reconstitution. This adds potential for a high degree of variability. The Thermo Scientific™ SMART Digest™ Kit was used to digest cytochrome C with a 10 minute digestion protocol. This was followed by Thermo Scientific™ SOLAμ™ SPE plates to clean and concentrate the digest prior to injection onto the UHPLC system. Following separation on a Thermo Scientific™ Acclaim™ C18 RSLC analytical column, high-resolution mass spectrometry was performed. A high-throughput, reproducible workflow was achieved that can be applied to nontargeted, semitargeted, or targeted quantitative environment for peptide analysis.

Introduction

A fundamental requirement of peptide mapping and quantitative analysis workflows is reproducibility. This enables users to confidently assign data differences to the sample and not the methodological conditions used.

The current approach for peptide mapping and quantitative analysis involves in-solution trypsin digestion of proteins overnight. This protocol is time consuming and requires a number of different steps, including protein assay, denaturation, alkylation, and reduction, which can differ between laboratories and make method transfer and data analysis between user groups problematic.

Additionally, due to the number of steps required, in-solution digestion protocols are highly laborious, increasing the potential for user error. As a result, in-solution digestion often leads to irreproducible protein cleavage which manifests in variation in the chromatographic profile. This prevents the adoption of robust, generic methodologies resulting in reduced throughput and return on investment.

The SMART Digest Kit eliminates these issues by providing a protocol that is:

- Highly reproducible
- Quick and easy to use
- Detergent free
- Less prone to chemically-induced post translational modification (PTMs)
- Autolysis-free
- Highly amenable to automation

Prior to analyzing digested samples it is common to perform sample cleanup such as centrifugation, filtration, or solid-phase extraction (SPE). This removes unwanted chemicals (such as detergents) that can interfere with the downstream mass spectrometric detection.

Centrifugation cannot always provide the level of cleanup required and exposes the detection system to unwanted levels of contaminants. Consequences can be physical, such as a blocked injection needle, as well as analytical, such as reduced column life or detection variability.

Filtration can be used to prevent the physical issues from occurring but offers little in the removal of excess buffers and reagents. Furthermore, the apparatus used in filtration can increase assay variability due to unpredictable binding of molecules to sample handling devices.

SPE provides a solution to both issues by filtering the digest while selectively removing the reagents. A generic method can be employed for a nontargeted workflow, removing only the unwanted reagents from the digest while maintaining a high recovery and reproducibility.

SOLAμ SPE plates provide reproducibility, robustness, and ease of use at low elution volumes by utilizing the revolutionary Thermo Scientific™ SOLA™ Solid-Phase Extraction technology. This removes the need for frits while delivering a robust, reproducible format which ensures highly consistent results at low elution volumes providing:

- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples which are limited in volume
- Improved stability of bio-molecules by reduction of adsorption and solvation issues

SOLAμ HRP is a micro-elution SPE device built on a polymeric backbone, containing both reversed-phase and polar-retentive moieties. In addition to providing high levels of recovery and reproducibility, it enables an additional concentration factor to be achieved without the need for evaporation and reconstitution.

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Table of Contents

Introduction

Peptide Mapping

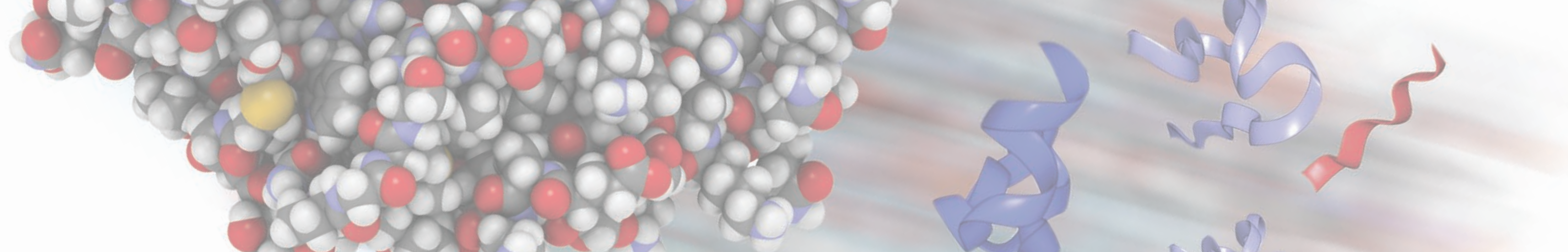
Peptide Quantitation

Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours

Simplifying ImmunoAffinity Capture Workflow

Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow

Product and method development considerations



Removal of the evaporation step reduces non specific binding when compared to traditional-scale SPE where evaporation and reconstitution are required.

Here we compare two protocols of sample cleanup following protein digestion with the SMART Digest Kit: filtration and SOLAμ HRP SPE. A total of eight peptides were assessed, four from a cytochrome C digest (to assess the SMART Digest) and four exogenous peptides spiked in post digest (to assess the reproducibility of the cleanup methods). Recovery and reproducibility of all eight peptides were measured, allowing an assessment of each stage of the workflow.

Experimental

Digestion

- SMART Digest Kit (P/N 60109-101)
- SMART Digest with SOLAμ HRP kit (P/N 60109-103)

Chemicals

- Water
- Acetonitrile (ACN)
- Formic acid (FA)
- Trifluoroacetic acid (TFA)

Cytochrome C, leu_enkephalin, angiotension 1, angiotensin 2, and neurotensin were purchased from reputable sources.

Sample Handling Equipment

- 96 well square well microplate
- 96 well positive pressure manifold

Separation

- Acclaim RSLC 120, C18, 2.2 µm analytical column (2.1 × 100 mm) (P/N 068982)

It is also recommended that a heater/shaker equipped with PCR block be used to perform the digest.

Sample Preparation

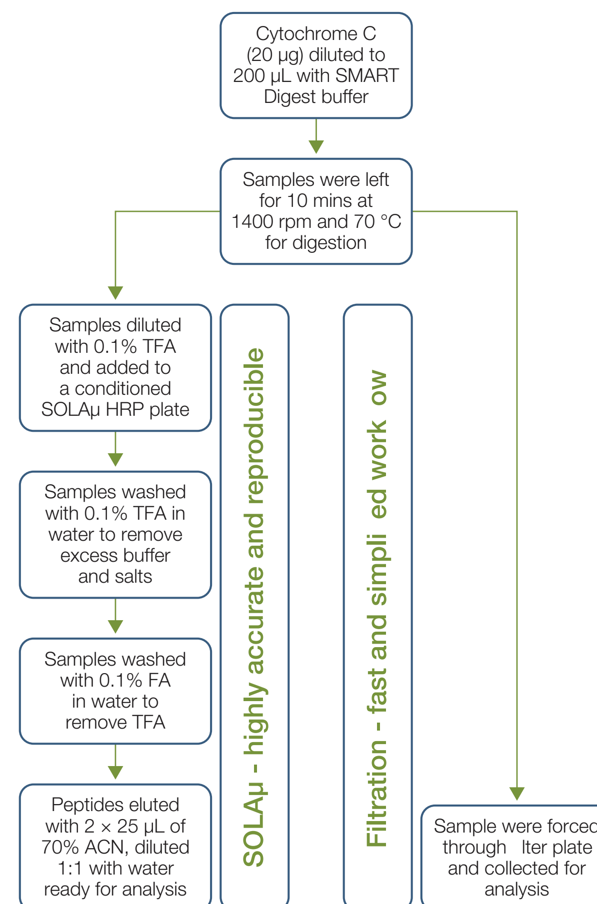


Table 1. LC gradient conditions.

Time (min)	Flow (mL/min)	B (%)
0.00	0.5	0
15.00	0.5	50
15.01	0.5	100
20.00	0.5	100
20.01	0.5	0
25.00	0.5	0

Data Processing

High Resolution Mass Spectrometer Quantitation Software software was used for data processing. Details are provided in Table 2.

Table 2. Compound transition details used for data processing.

Sample	Amino Acid Sequence	Precursor *(m/z)*
Cytochrome C peptide	EDLIAYLK	483.27301
	GITWGEETLMEYLENPKK	711.33099
	MIFAGIK	779.44641
	TGPNLHGLFGR	390.21155
Leu_Enkephalin	YGGFL	556.27526
Angiotensin_I	DRVYIHPFHL	432.8987
Angiotensin_II	DRVYIHPF	523.77349
Neurotensin	ELYENKPRRPYIL	558.30907

Results and Discussion

A total of eight peptides were assessed when comparing SOLAμ HRP and filtration as methods of post-digest sample cleanup. Four well-characterized peptides derived from cytochrome C were used for assessment of the SMART Digest Kit, selected for their high abundance and stable retention times. Four exogenous peptides were also spiked in post digest. This allowed assessment of the reproducibility of the SMART Digest Kit, along with an independent assessment of the cleanup procedures (Figure 1).

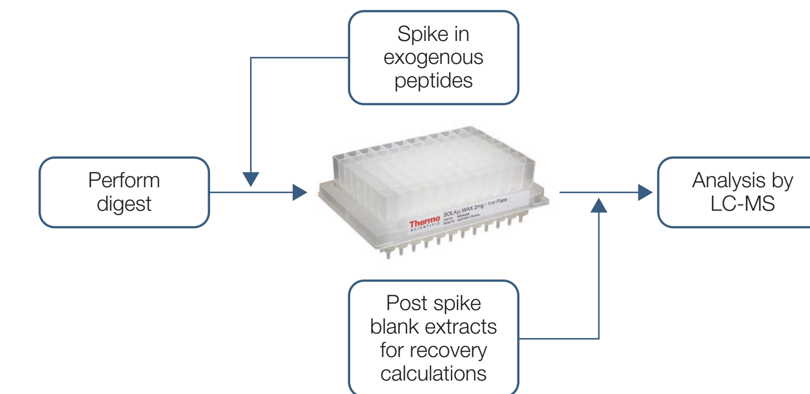
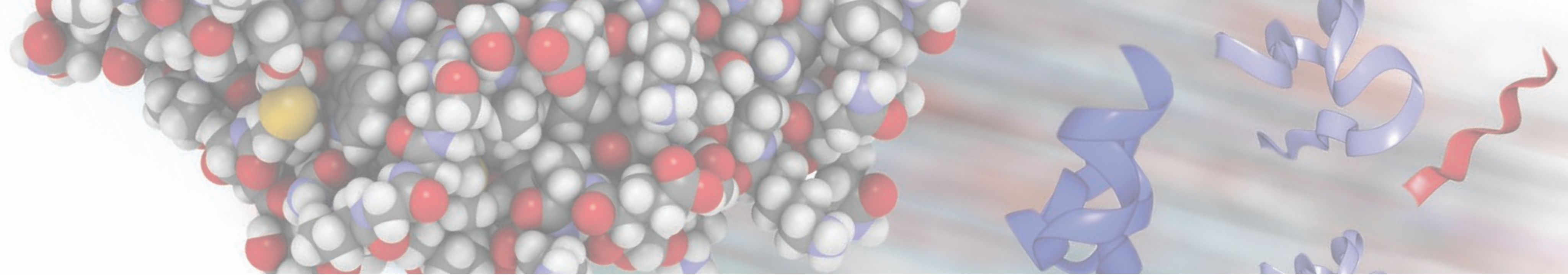


Figure 1. Flow chart of workflow used to calculate peptide recovery from cleanup method.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations



The separation of all eight peptides is provided (Figure 2). This separation was obtained using an Acclaim C18 RSLC analytical column with a 15 minute LC gradient.

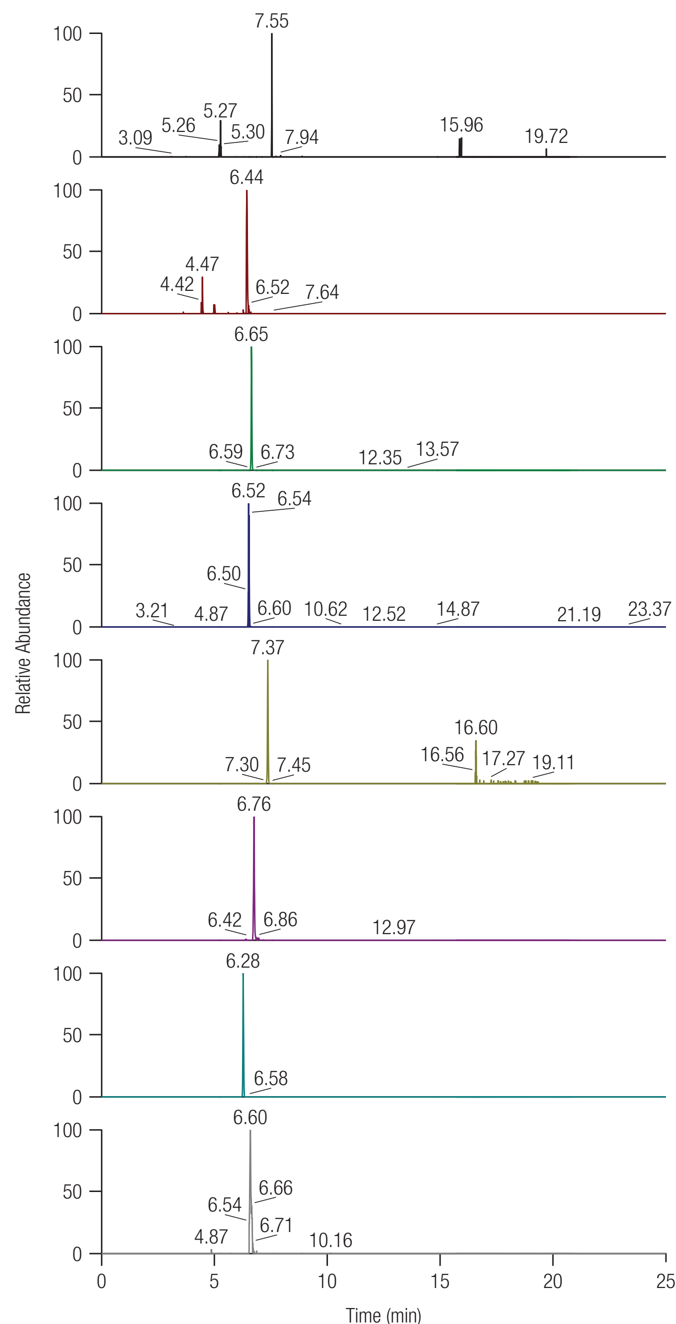


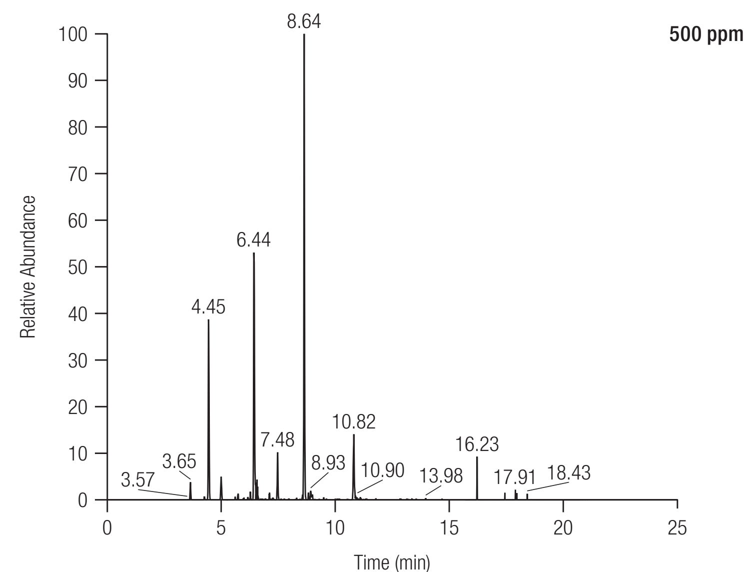
Figure 2. Separation of the eight peptides using an Acclaim C18 RSLC analytical column.

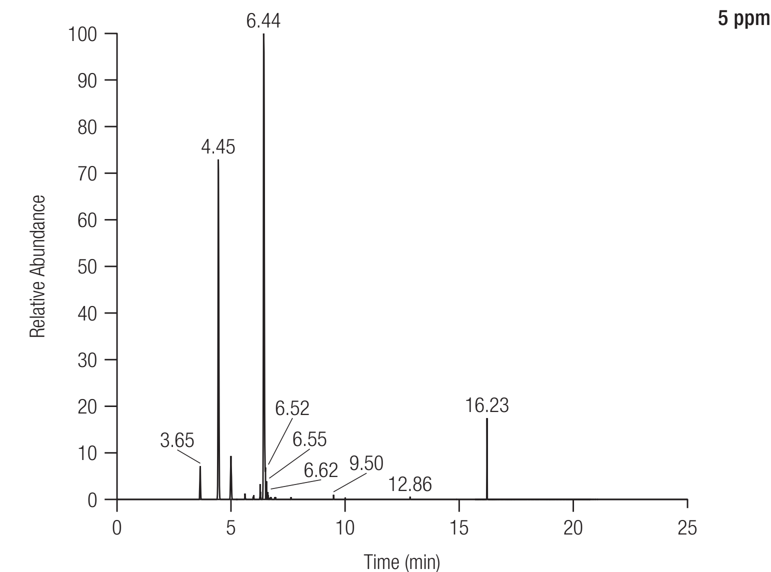
Stable retention times were observed for all measured peptides as shown in Table 3.

Table 3. Retention times (RT) of each peptide.

Sample	Amino Acid Sequence	RT (min)	%RSD of RT (n=6)
Cytochrome C peptide	EDLIAYLK	7.55	0.123%
	GITWGEETLMEYLENPKK	6.44	0.186%
	MIFAGIK	6.65	0.203%
	TGPNLHGLFGR	6.52	0.179%
Leu_Enkephalin	YGGFL	7.37	0.127%
Angiotensin_I	DRVYIHPFHL	6.76	0.179%
Angiotensin_II	DRVYIHPF	6.28	0.156%
Neurotensin	ELYENKPRRPYIL	5.60	0.0589%

Accurate mass data was collected from the 'High Resolution mass spectrometer. Mass accuracy was assessed at 500 ppm, 5 ppm, and 2 ppm for assessment. Figure 3 shows the benefits of accurate mass for peptide sequence GITWGEETLMEYLENPKK. Increasing the accuracy of the measurement from 500 to 2 ppm shows clear advantages in reducing background noise levels and ultimately increasing signal-to-noise values of targeted analytes. This also adds further accuracy when measuring unknown samples for identification purposes.





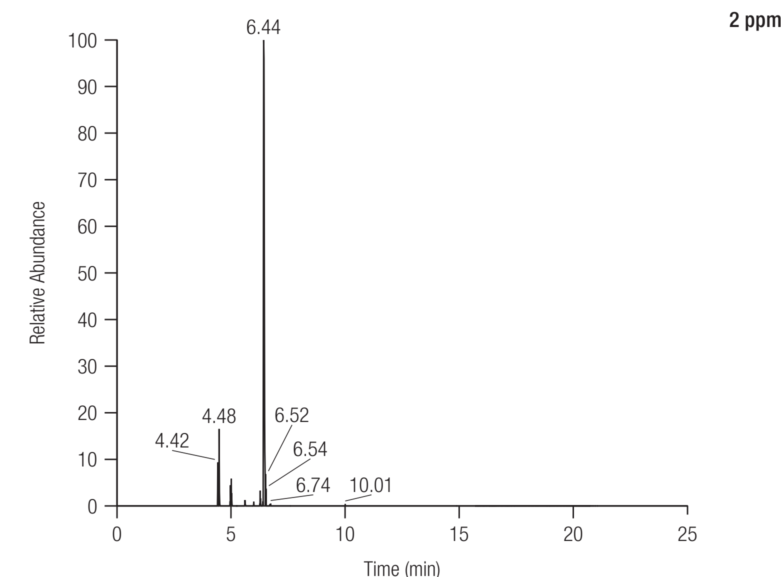
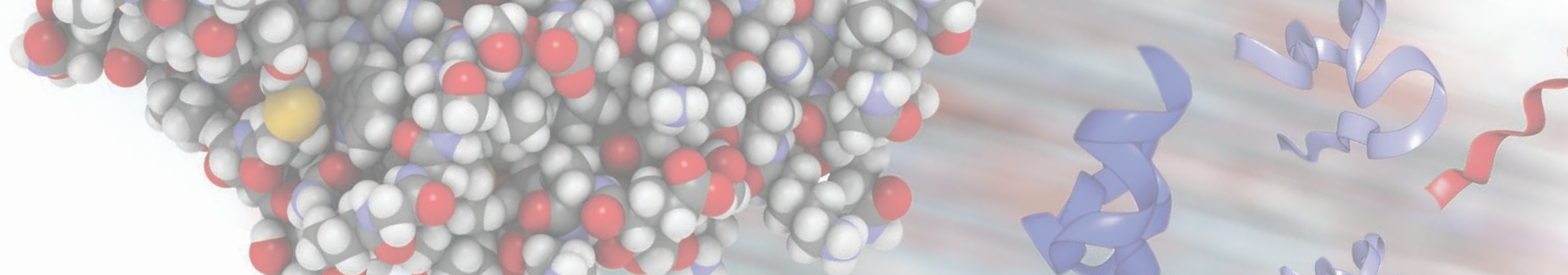


Figure 3. Peptide sequence GITWGEETLMEYLENPKK with accurate mass setting of 500 ppm, 5 ppm, and 2 ppm, RT 6.4 mins.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours
Simplifying ImmunoAffinity Capture Workflow
Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow
Product and method development considerations



The recovery and precision of all eight peptides is summarized in Figure 4. This is a direct comparison between SOLAμ HRP and filtration when used as post digestion cleanup methods. The SOLAμ HRP method showed higher levels of recovery on seven out of eight peptides, with higher levels of precision on every peptide.

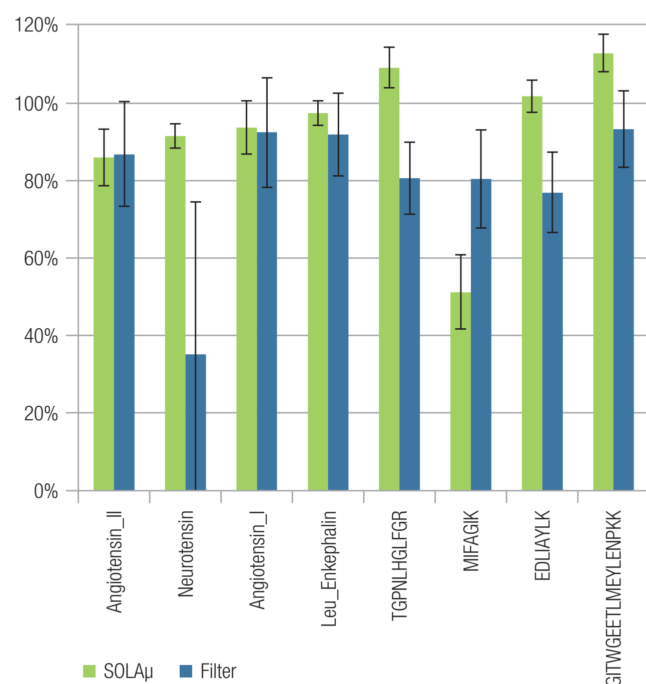


Figure 4. Average recovery values for each peptide with %RSD (n=6).

Some selectivity differences are observed between the two protocols, in particular for neurotensin and MIFAGIK, where recoveries are lower on the SOLAμ HRP for the former and noticeably higher for the latter. Despite the differences in recovery levels, the precision of the data obtained from the SOLAμ HRP is much higher than that obtained with filtration as seen in Figure 5.

The percent relative standard deviation (RSD) of the exogenous peptides gives us the level of precision of the cleanup method, along with the subsequent LC and detection. The RSD of the cytochrome C peptides gives us the level of precision of the entire process including the reproducibility of the digest. Comparing precision data between the exogenous and cytochrome C peptides allows assessment of the reproducibility of the digestion (Figure 6).

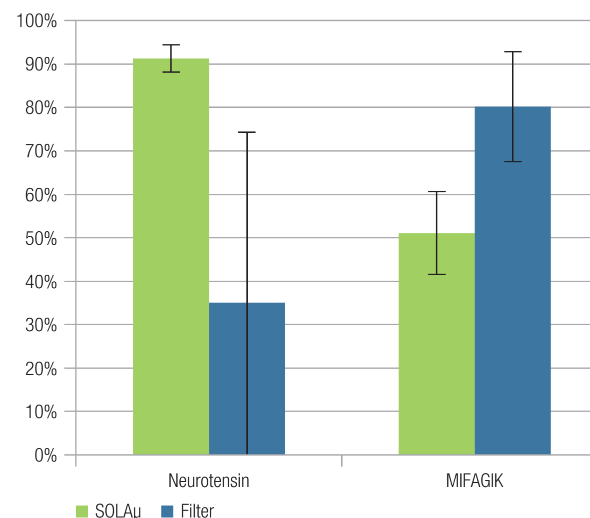


Figure 5. Selectivity differences between SOLAμ HRP and filter.

Comparable levels of precision for both sets of peptides were observed. This indicates the following:

- The entire analytical workflow is precise (RSD of exogenous peptides = 5%)
- The reproducibility using the SMART Digest Kit is precise (RSD of cytochrome C peptides = 6%)

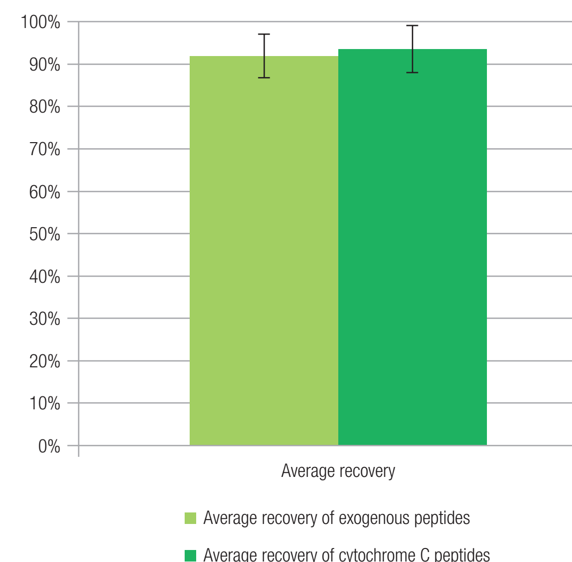


Figure 6. Recovery and %RSD of exogenous and cytochrome C peptides (n=6) both using SOLAμ HRP method.

Conclusion

This analysis demonstrates that use of the SMART Digest Kit offers:

- A highly reproducible digest protocol
- Quick and easy use
- Detergent free digestion

Post digest sample cleanup was achieved using filtration and SOLAμ HRP SPE:

- Filtration provides a simple, fast workflow but offers limited sample cleanup
- SPE provides a high precision workflow with optimized sample cleanup, offering an additional sample concentration factor where required

The benefits outlined above clearly demonstrate the advantages of the SMART Digest Kit with regards to workflow efficiency and reproducibility. A choice of sample clean-up is also demonstrated depending on the important factors for the analyst: speed and simplicity, or accuracy and precision of data. The workflow described allows for the introduction of fast, generic, and robust analytical methods within a high-throughput, biopharmaceutical environment.

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion
Find out more...

Fast Digestion Method Optimization Using Innovative Trypsin Technology

Valeria Barattini, Thermo Fisher Scientific, Runcorn, UK

Goal

To describe how to construct a simple time course experiment to identify the digestion endpoint for a given protein by monitoring degradation of the intact protein. The method is simple and uses LC-UV detection. Ribonuclease A, a small, heat stable protein, and mouse IgG, a large immunoglobulin, are used as test probes.

Introduction

Standard in-solution protocols for triptic digestion are lengthy, multifaceted, and prone to irreproducibility. Thermo Scientific™ SMART Digest™ Kits eliminate these issues by providing a protocol that is:

- Highly reproducible
- Quick and easy to use
- Detergent free
- Highly amenable to automation

When working with a new target protein, it is important to identify the optimum SMART digestion time to achieve complete digestion. A simple way to identify this is to monitor the disappearance of the intact protein from the sample by LC-UV. It is crucial that an appropriate stationary phase is selected.

The Thermo Scientific™ MAbPac™ RP column is a polymeric reversed-phase (RP) LC column designed for monoclonal antibody (mAb) characterization including separation of intact mAbs, mAb fragments, and large biomolecules. The unique column chemistry provides excellent performance under a broad range of pH, temperature, and mobile phase composition.

Here we show proof of principle experiments on how to identify the optimum SMART digestion time using ribonuclease A and mouse IgG as test probes. Ribonuclease A is a small, heat stable protein with a molecular weight of 13.7 kDa, and IgG is an antibody of approximately 150 kDa molecular weight. While the two proteins differ largely in size, their digestion presents a number of challenges. Ribonuclease A is particularly resistant to tryptic digestion due to its heat stability and ability to quickly refold,1 while IgG presents a considerable challenge due to its large size.

Digestion progress using the SMART Digest Kit is monitored to completion by running a simple time-course experiment and monitoring the disappearance of the intact protein and the stability of the peptides produced. The method is simple and robust and applicable to any extracted target protein sample.

Experimental

Digestion
- SMART Digest Kit with 96-well filter plate (P/N 60109-102)

Chemicals
- Deionized water, 18.2 MΩ/cm resistivity
- Fisher Scientific™ Optima™ acetonitrile (ACN) (P/N 10001334)
- Fisher Scientific™ trifluoroacetic acid (TFA) (P/N 10294110)
- Mouse IgG Isotype Control (3 mg/mL solution) (P/N 10400C)

Ribonuclease A from bovine pancreas was purchased from a reputable source.

Sample Handling
- 2 mL Chromatography Certified 96-well square plates (P/N 60180-P202)
- Thermo Scientific™ WebSeal™ Silicone/PTFE mats (P/N 60180-M122)

Sample Handling Equipment
- 96-well positive pressure manifold (P/N 60103-357)

It is also recommended that a heater/shaker equipped with PCR block be used to perform the digestion.

Sample Pretreatment

Ribonuclease A

To each SMART Digest trypsin tube 75 µL of SMART buffer were added. Ribonuclease A was subsequently added in 25 µL aliquots (2 mg/mL, 50 µg). To ensure a uniform starting point across all the tubes, the aliquots were added using an 8-channel pipette. Samples were digested using a heater shaker set at 70 °C and 1400 rpm shaking. Tubes were subsequently removed after 15, 30, 60, 90, 120, 150, 180, and 210 minutes. Each time point was prepared in triplicate.

Mouse IgG

To each SMART Digest trypsin tube 75 µL of SMART buffer were added in 25 µL aliquots (3 mg/mL, 75 µg). To ensure a uniform starting point across all the tubes, the aliquots were added using an 8-channel pipette. Samples were digested using a heater shaker set at 70 °C and 1400 rpm shaking. Tubes were removed after 15, 30, 45, 60, 90, 120, 150, and 180 minutes. Each time point was prepared in triplicate.

For both sample sets, at the required time point digestion was stopped by simply forcing the sample through the filter plate using positive pressure to remove any immobilized trypsin beads. The eluent was analyzed using LC-UV.

ThermoFisher SCIENTIFIC

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Samples corresponding to t=0 minutes were prepared by diluting 25 µL of ribonuclease A or IgG solutions with 75 µL of SMART buffer. No mixing with trypsin beads was applied. The whole sample was then forced through the filter plate using positive pressure.

Separation Conditions

Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system equipped with: SRD-3600 Solvent Racks with Degasser (P/N 5035.9230) DGP-3600RS Rapid Separation Pump (P/N 5040.0066) WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020) TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000) DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082.0020)
Column:	MabPac RP, 50 × 2.1 mm (P/N 088648)
Mobile Phase A:	Water + 0.1 % TFA
Mobile Phase B:	Acetonitrile + 0.08% TFA
Gradient:	See Table 1
Flow Rate:	0.5 mL/min
Column Temperature:	80 °C
Autosampler Settings:	The autosampler pick-up settings were optimized for this analysis. Settings described in Table 2 were applied. These settings may differ depending on the autosampler type and manufacturer.
Injection Wash:	Water + 0.1% TFA Solvent
UV Detector:	Wavelengths selected were 214 nm and 280 nm. Peak width was set to 0.1 min and recommended values were selected for the data collection settings.

Table 1. LC gradient conditions.

Time	%B	Curve
0	0	5
1	0	5
11	50	5
11.01	100	5
15	100	5
15.01	0	5
20	0	5

Table 2. Autosampler settings.

Parameter	Setting
Injection Volume	5 µL
Draw Speed	0.05 µL/s
Draw Delay	3.0 s
Dispense Speed	10.0 µL/s
Dispense Delay	2.0 s
Dispense to Waste Speed	1.0 µL/s
Sample Height	2.0 mm

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 SR2 Chromatography Data System was used for data acquisition and analysis.

Results and Discussion

The MAbPac RP column is packed with supermacroporous polymer resin. Its large pore size (~ 1,500 Å) is suitable for separation of large proteins. In addition, large biomolecule analysis by reversed-phase liquid chromatography requires high temperatures such as 70 to 80 °C. The polymeric nature of the MAbPac RP column provides both pH and temperature stability, making it an ideal column for protein analysis.

First, a separation method was developed for intact ribonuclease A and IgG. A single peak was identified for both analytes (Figure 1).

Digested and filtered ribonuclease A and IgG samples were subsequently analyzed with the same method. To monitor digestion progress, the chromatograms of each time point were overlaid (Figures 2 and 3).

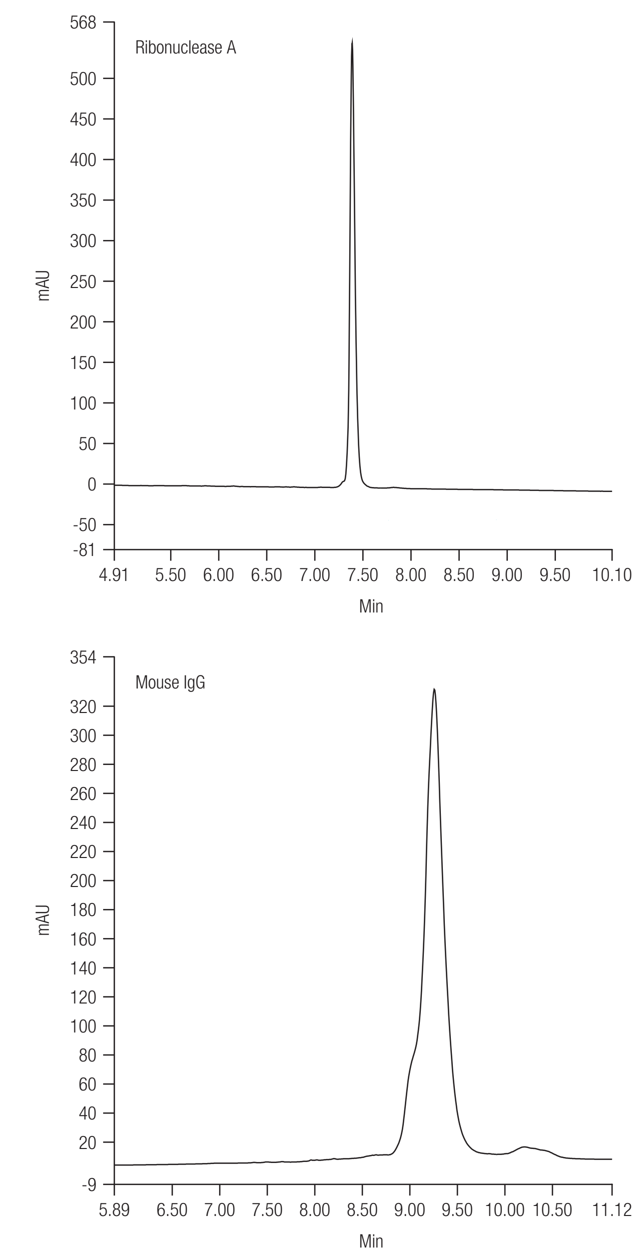

Figure 1. Chromatography of ribonuclease A and mouse IgG at 214 nm on a MabPac RP column.

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ThermoFisher SCIENTIFIC

thermoscientific

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

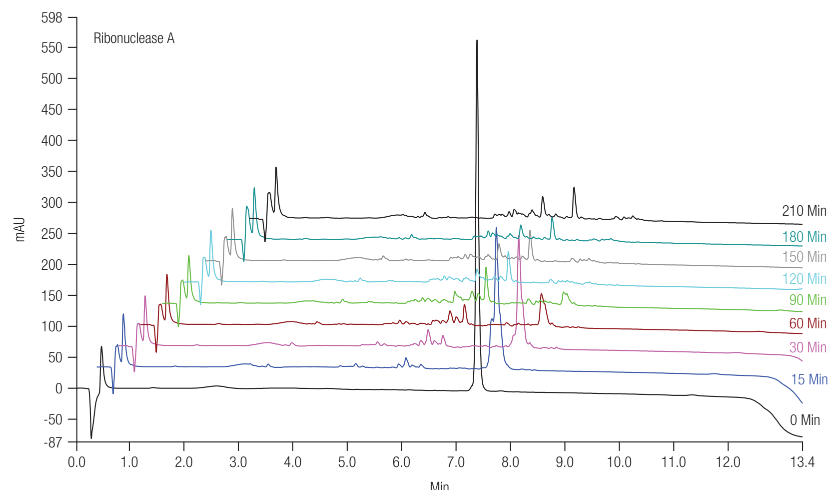

Figure 2. Chromatogram overlay of the digestion time points for ribonuclease A. Complete digestion is achieved in 120 minutes, when the peptide fingerprint is stable and the intact peak is no longer visible.

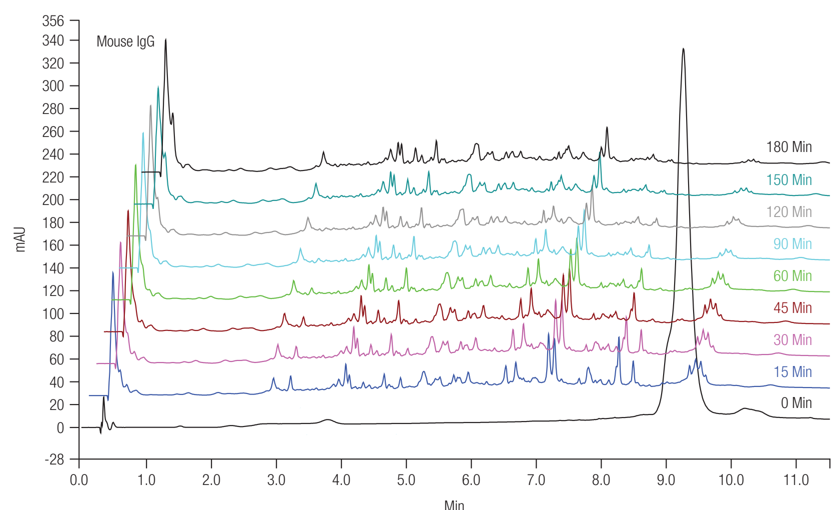

Figure 3. Chromatogram overlay of the digestion time points for mouse IgG. Initial digestion proceeds very fast and most of the sample is digested in the first 15 minutes. After 60 minutes the peptide fingerprint is stable and the intact peak is visible only in trace amounts.

Ribonuclease A is a small protein with a molecular weight of only 13.7 kDa. However, it is known to be very resilient to conventional tryptic digestion due to its heat stability and refolding ability.1 Inspection of the chromatographic data shows that ribonuclease A, with the SMART Digest Kit, is fully digested within 2 hours (Figure 2).

Compared to ribonuclease A, the digestion of mouse IgG proceeds much faster despite its considerably larger size. Most of the sample is digested in the first 15 minutes; after 60 minutes the peptide fingerprint is stable.

The unique features of the SMART Digest Kit result in complete, fast digestions of normally challenging analytes. Table 3 lists the digestion times of a number of proteins previously tested. Even challenging samples such as thyroglobulin are digested in as little as 4 hours.

Table 3. Typical SMART digestion times of a series of protein.

Protein	SMART Digestion Time
Insulin	4
BSA	<5
Carbonic Anhydrase	<5
Lysozyme	<5
Apo-B	30
IgG in 50 µL plasma	75
Thyroglbulin	240
C-reactive protein	240

Conclusion

- A simple method has been developed to identify the digestion endpoint with the SMART Digest Kit.
- Complete SMART digestion method optimization is achieved in less than a day from start to finish.
- Ribonuclease, a protein known to be challenging to digest, is completely lysed within 2 hours.
- The method is applicable to any extracted protein sample.

Reference

1. Lang, K, Use of a trypsin-pulse method to study the refolding pathway of ribonuclease, Eur. J. Biochem., 1986, 159, 275-281.

Useful Links

AppsLab Library
The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:
www.thermofisher.com/appslab

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Enhanced Digestion for Improved Biomarker Identification

Valeria Barattini, Phillip Humphryes,
Thermo Fisher Scientific, Runcorn, UK

Goal

To demonstrate how the Thermo Scientific™ SMART Digest™ Kit makes it possible to quickly digest even challenging biomarkers such as thyroglobulin, while significantly improving sensitivity over previously described methods.

Introduction

Biomarker identification is analytically challenging due to sample complexity and inherently low levels of many important biomarkers. Clean-up and enrichment strategies have resulted in significant gains in analytical sensitivity. However, complete digestion of the protein can also improve sensitivity and generate more peptides.

Biomarkers digest at different rates based on their properties; some are completely digested in a few hours while others are only partially digested after 48 hours.[1] Incomplete digestion leads to decreased sensitivity and sample to sample variability.

Protein digestion is a fundamental technique employed in biopharmaceutical and proteomic applications. It is used to analyze the sample and post translational modifications (PTMs). Despite its widespread use, protein digestion still provides many analytical challenges. The optimum trypsin digestion should provide conditions that accomplish the following:

- Cleave the proteins after every lysine and arginine
- Unfold the protein/proteins of interest
- Completely denature the protein to be digested yet not affect trypsin itself
- Use the minimum amount of trypsin to protein ratio to prevent partial digestion

Thyroglobulin is a large protein of significant interest. However, it is difficult to digest and the current methodology is unreliable. This protein provides an opportunity to establish the viability of new digestion techniques to improve speed and sensitivity.

Currently, the most reliable method of determining thyroglobulin levels is to perform a protein digestion of the plasma, followed by affinity purification using anti-peptide antibodies with subsequent analysis by liquid chromatography/mass spectrometry system. In this way, the sample complexity can be reduced without interference from native anti-thyroglobulin antibodies.

The inherent difficulty in this workflow is the limited ability to efficiently obtain reproducible and accurate results when digesting proteins from a complex matrix. Here we present a method for performing the digestion of thyroglobulin in an efficient and reproducible manner using an immobilized, thermally stable form of trypsin. The SMART Digest Kit removes uncertainty associated with conventional solution-based tryptic digestion protocols, resulting in higher reproducibility and sample characterization.

In the first set of experiments, peptide mapping of thyroglobulin was run to identify the biomarkers of interest. These were chosen based on previously published results.[2] After these peptides were identified, time-course studies were run to optimize the digestion time of thyroglobulin in murine plasma. Finally, a calibration curve was measured for digested samples with no further treatment.

In the second set of experiments a previously published in-solution digest protocol2 was followed and the results compared with data from SMART-digested samples.

Experimental

Digestion

- SMART Digest Kit (P/N 60109-101)

Chemicals

- Fisher BioReagents™ tris buffered saline (TBS) (P/N 10648973)
- Fisher Scientific™ Optima™ LC-MS Water (P/N 10095164)
- Fisher Scientific Optima acetonitrile (ACN) (P/N 10001334)
- Fisher Scientific Optima isopranol (IPA) (P/N 10091304)
- Fisher Scientific dimethyl sulfoxide (DMSO) (P/N 10500151)
- Fisher Scientific Optima methanol (MeOH) (P/N 10031094)
- Fisher BioReagents Tween 20 (P/N 10113103)
- Thermo Scientific™ Pierce™ octyl-beta-glucoside (P/N 28310)
- Fisher Scientific Optima formic acid (P/N 10596814)
- Fisher BioReagents ammonium bicarbonate (P/N 10532775)
- Fisher BioReagents dithiothreitol (P/N 10386833)
- Acros Organics™ iodoacetamide (P/N 10346660)
- Pierce trypsin protease, MS grade (P/N 13454189)
- Pierce octyl-beta-glucoside (P/N 28310)
- Fisher BioReagents tris buffered saline (TBS), 10X solution, pH 7.4 for molecular biology (P/N 10153103)
- Human thyroglobulin and murine plasma were purchased from reputable sources.

Sample Handling Equipment

NSC Mass Spec Certified 2 mL clear vial with blue bonded PTFE silicone cap MSCERT4000-34W

Heater/shaker equipped with PCR block and heated lid

Separation

Thermo Scientific™ Accucore™ C18 column (50 × 2.1 mm, 2.6 µm particle) (P/N 17126-052130)

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Experiment 1: Digestion time optimization and calibration curve measurement

Thyroglobulin peptide mapping

Samples were prepared by adding 20 µg of thyroglobulin to each well containing 150 µL SMART Digest buffer,
50 µL of water, and 0.1 wt% octyl-β-glucoside. These were incubated at 70 °C and 1400 rpm for 2 hours before analysis.

Digestion

Samples were prepared by adding 20 µg of thyroglobulin to each well containing 150 µL SMART Digest buffer and 50 µL murine plasma. These were incubated at 70 °C and 1400 rpm. Wells were sampled every 30 minutes and diluted 10-fold in tris buffer saline (TBS) before analysis.

Calibration curve sample preparation

Samples were prepared to cover a concentration range of 4–4000 µg/mL. The thyroglobulin was spiked in various weights into each well of three SMART Digest strips containing 150 µL SMART Digest buffer and 50 µL murine plasma. These were incubated at 70 °C and 1400 rpm. Samples were collected at 210 minutes and diluted 10-fold in TBS before analysis.

Experiment 2: In-solution digestion protocol

Comparison to existing methods[2]

Reduction buffer was made containing 125 mM NH3HCO3, 0.0086% Tween 20, and 12.5 mM dithiothreitol. Samples of 50 µL of murine plasma were spiked with 4 µg/mL of thyroglobulin to match the high end of the linearity tests performed with the SMART Digest Kit and then diluted with 0.2 mL of reduction buffer before being reduced for 1 hour at 37 °C with agitation. Samples were then alkylated by the addition of 5 µL of 200 mM iodoacetamide before incubation in the dark at room temperature for 1 hour. At this point, 3 µg of sequencing grade trypsin was added and the samples were incubated for 4 hours at 37 °C with agitation. Subsequently, an additional 10 µg of sequencing grade trypsin was added and the sample was incubated for another 16 hours at 37 °C with agitation. Samples were diluted 10-fold prior to analysis.

Separation Conditions

Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system interfaced with a Thermo Scientific™ Velos Pro™ ion trap mass spectrometer
Mobile phase A:	2% acetonitrile, 98% water, 0.1% formic acid
Mobile phase B:	90% acetonitrile, 10% water, 0.1% formic acid
Gradients:	LC Gradient 1 (Table 1) was used in the peptide mapping experiments. All other analyses were run using Gradient 2 (Table 2).
Flow rate:	0.5 mL/min
Column temp:	40 °C
Injection details:	5 µL (for peptide mapping and calibration curve development) 2 µL (for digestion time course analysis)

Table 1. LC Gradient 1 conditions.

Time (min)	% A	% B
0	98	2
1	98	2
61	50	50
61.1	10	90
63	10	90
63.1	98	2
65	98	2

Table 2. LC Gradient 2 conditions.

Time (min)	% A	% B
0	95	5
1	95	5
6	40	60
6.1	10	90
8	10	90
8.01	95	5
10	95	5

Software

Thermo Scientific™ Xcalibur™ software 2.0, Qual browser version 2.0.3.2

Results and Discussion

Three peptides were selected for study. These were chosen on initial screening results as well as literature surveys. The SRM transitions for each selected peptide are listed in Table 3.

Table 3 Monitored peptide sequences for thyroglobulin.

Peptide Sequence	Precursor Ion (m/z)	Fragment Ion (m/z)
VIFDANAPVAVR	636.5	541.3
LGDQEFIK	475.4	836.37
FPLGESFLVAK	604.5	850.4

Running a digestion time course, a maximum sensitivity was obtained following 210 minutes of digestion using the temperature stable immobilized enzyme. Figure 1 shows the trend observed for the peptide LGDQRFIK with an m/z of 475.4. This trend was confirmed for the other selected peptides (data not shown).

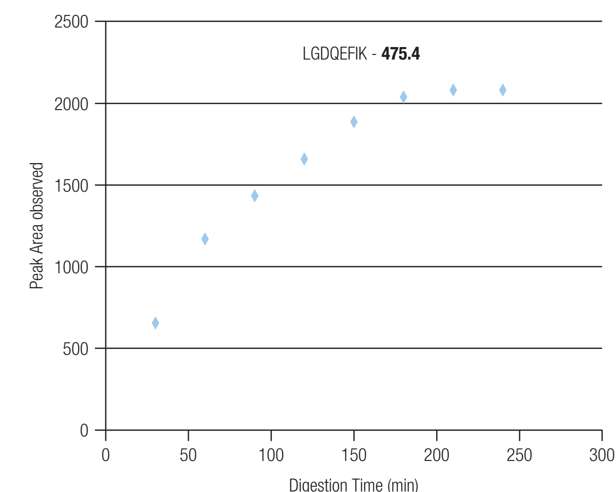

Figure 1. Digestion time course of thyroglobulin examining the generation of the LGDQEFIK peptide.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Following the time course analysis, a calibration curve was run to determine an approximate LLOQ of thyroglobulin in a plasma matrix. Triplicates were run for statistical accuracy. While conventional methods would involve an immune-affinity purification to increase sensitivity, no further sample preparation was applied after digestion.

The concentration range of thyroglobulin was from 4 µg/mL to 4 mg/mL in plasma. The peptide VIFDANAPVAVR is commonly used for thyroglobulin quantitation. Initial studies on this peptide showed it to have a good signal at the high end of the calibration curve; however, this was also accompanied by low S/N values. The peptide LGDQEFIK was chosen as an alternative biomarker. This peptide showed strong linearity through the entire calibration curve (Figure 2), with CVs <10% down to 40 µg/mL, below which instrumentation variance becomes a significant factor (Table 4).

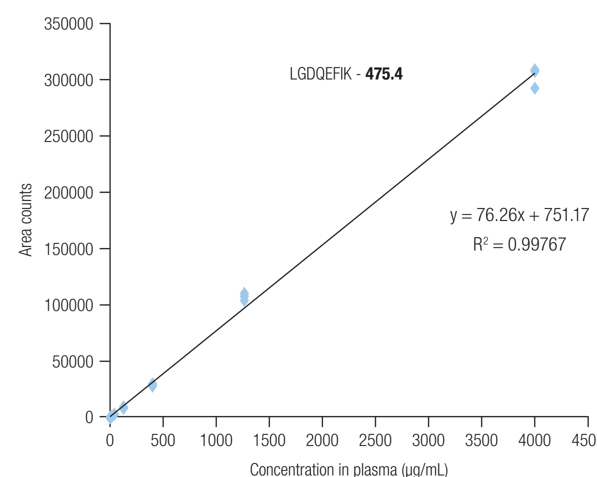

Figure 2. Calibration curve for thyroglobulin in murine plasma using the LGDQEFIK peptide.

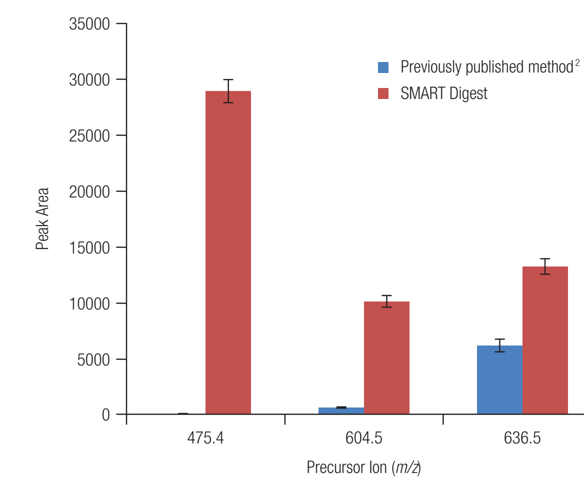

Figure 3. Comparison of the SMART Digest protocol with a previously published in-solution digestion protocol.[2]

Table 4. Calibration curve for the LGDQEFIK peptide in murine plasma.

LGDQEFIK - 475.4/836.37 Calibration Curve in Murine Plasma						
Concentration (µg/mL)	Rep1	Rep2	Rep3	Average	StDev	CV
0	0	0	0	0	0	0
4	330	247	347	308.0	53.5	17.4
12.64	951	771	608	776.7	171.6	22.1
40	2696	2876	2963	2845.0	136.2	**4.8**
126.4	9303	9476	8010	8929.7	801.1	**9.0**
400	29911	27844	29048	28934.3	1038.2	**3.6**
1264	103781	107545	110303	107209.7	3273.9	**3.1**
4000	292450	307302	308959	302903.7	9091.0	**3.0**

Comparison to previously published methods2 using in-solution digestion showed less than half the signal response for each of thethree peptides studied, with LGDQEFIK being entirely undetectable at a concentration of 500 µg/mL in plasma (Figure 3).

The well-known limitations of conventional digestion protocols suggest that the lower signal achieved by in-solution digestion of thyroglobulin is largely due to incomplete lysis of the protein. This possibility is particularly troublesome in a quantitative assay. Even though peptide internal standards are able to offset sample to sample variation due to matrix effects, they are not able to account for the variance due to poor digestion efficiency. This will significantly influence the results if digestion is only partially completed.

Conclusion

The SMART Digest Kit allows increased biomarker identification by providing a fast, simple, and clean tryptic digestion protocol. A comparison between the SMART Digest protocol and published solution-based digestion protocol was run for the quantitation of thyroglobulin from whole murine plasma. Complete digestion was achieved in 3.5 hours with a SMART Digest Kit, compared to 22 hours required for the solution-based protocol. The signal response of key SRM-peptides was found to be doubled when using the SMART Digest Kit. Excellent linearity was observed for the key SRM thyroglobulin peptides.

References

1. Kilpatrick, E.L. and Bunk, D.M. Reference measurement procedure development for C-reactive protein in human serum, Analytical Chemistry, 2009, 81, 8610-8616.
2. Clarke, N.J.; Zhang, Y.; Reitz, R.E. A novel mass spectrometry-based assay for the accurate measurement of thyroglobulin from patient samples containing antithyroglobulin autoantibodies, Journal of Investigative Medicine, 2012, 60, 1157-1163.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
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Find out more...

Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers

Phillip Humphryes, Valeria Barattini
Thermo Fisher Scientific, Runcorn, UK

Goal

To demonstrate how the Thermo Scientific™ SMART Digest™ Kit removes the uncertainty associated with conventional solution-based tryptic digestion protocols, resulting in higher efficiency and sample characterization.

Introduction

Protein insolubility can be a major impediment to traditional bottom-up proteomic methods becauses it reduces the efficiency of enzymatic digestions and makes analysis problematic. Insolubility can be caused by a variety of factors, including the salt concentration, the presence of various organic solvents, temperature, or simply the nature of the protein itself. In some cases, this insolubility can be useful for purification purposes as is seen with immunoprecipitation or pre-pelleting protocols. However, in most cases, precipitation of the protein out of solution is undesirable.

The digestion itself can increase the solubility of a protein as the resulting peptides, due to their smaller size, are more soluble than their parent proteins or corresponding polypeptide chains. Protein solubility can be enhanced by the addition of small amounts of solvents, detergents, and/or chaotropes; however, many of these methods are also detrimental to trypsin digestion.

The SMART Digest buffer was developed to maintain protein solubility while increasing the rate of digestion. This was achieved by optimizing the solubility buffer then the digestion buffer.

Experimental

Digestion

- SMART Digest Kit (P/N 60109-101)

Chemicals

- Fisher BioReagents™ bovine serum albumin (BSA) (P/N 128516300)
- Thermo Scientific™ Pierce™ trypsin protease (P/N 13464189)
- Fisher BioReagents Tris buffered saline (TBS) (P/N 10648973)
- Fisher Scientific™ Optima™ LC-MS water (P/N 10095164)
- Fisher Scientific Optima acetonitrile (ACN) (P/N 10001334)
- Fisher Scientific Optima isopranol (IPA) (P/N 10091304)
- Fisher Scientific dimethyl sulfoxide (DMSO) (P/N 10500151)
- Fisher Scientific Optima methanol (MeOH) (P/N 10031094)
- Fisher BioReagents 2,2,2-trifluoroethanol (TFE) (P/N 10468733)
- Thermo Scientific Pierce guanidine HCl (P/N 11821365)
- Fisher BioReagents Tween 20 (P/N 10113103)
- Thermo Scientific Pierce octyl glucoside (P/N 28310)
- Fisher BioReagents deoxycholate (P/N 10346653)

Sample Handling Equipment

Heater/shaker equipped with PCR block and heated lid

Experiment 1: Solubility buffer comparison

Bovine serum albumin (BSA) was dissolved in the following buffers:

- Tris buffered saline (TBS)
- TBS with trypsin (1:5 trypsin to protein ratio)
- An optimized proprietary solubility buffer

Final concentrations of BSA of 1.25, 2.5, 3.75. 6.25, and 12.5 mg/mL were produced.

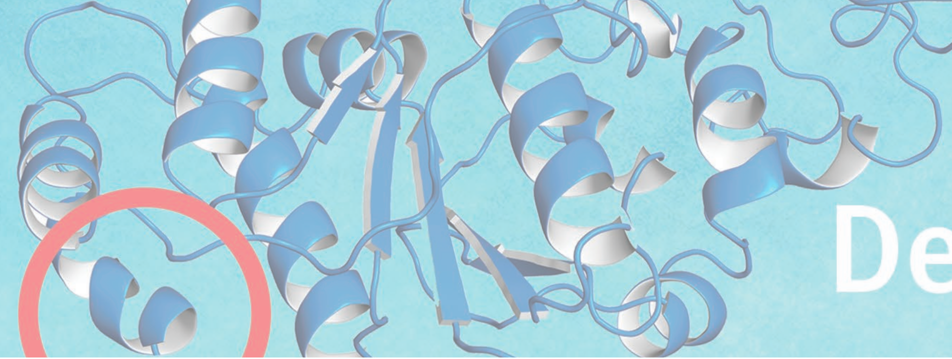
Experiment 2: Digestion buffer comparison

A 12.5 mg/mL solution of BSA in the optimized digestion buffer was prepared and compared to BSA in optimized digestion buffer with the organic solvents, chaotropes, surfactants, and detergent additives listed in Table 1. Samples were then digested using the SMART Digest Kit at 1400 rpm and 70 °C using a range of different incubation times (30, 60, 90, and 210 minutes).

Table 1. List of additives and concentrations used to assess BSA solubility.

Additives	Concentrations
Optimized Buffer	Undiluted
ACN	1, 5, 10, 20%
IPA	1, 5, 10, 20%
DMSO	1, 5, 10, 20%
MeOH	1, 5, 10, 20%
TFE	1, 5, 10, 20%
Guanidine HCl	0.5M
Tween 20	0.005%, 0.05%
Octylglucoside	Undiluted
Deoxycholate	Undiluted

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion



Denaturation

Results and Discussion

Experiment One: Solubility Buffer Comparison

Data in Figure 1 shows BSA solubility up to 1.25 mg/mL in the TBS solution, which further increased to 2.5 mg/mL with the addition of trypsin (Figure 2) for the ≥ 15 min time points. This increase in solubility on addition of trypsin can largely be attributed to the effect of digestion of the protein to its constituent peptides, which are generally more soluble than the parent protein.

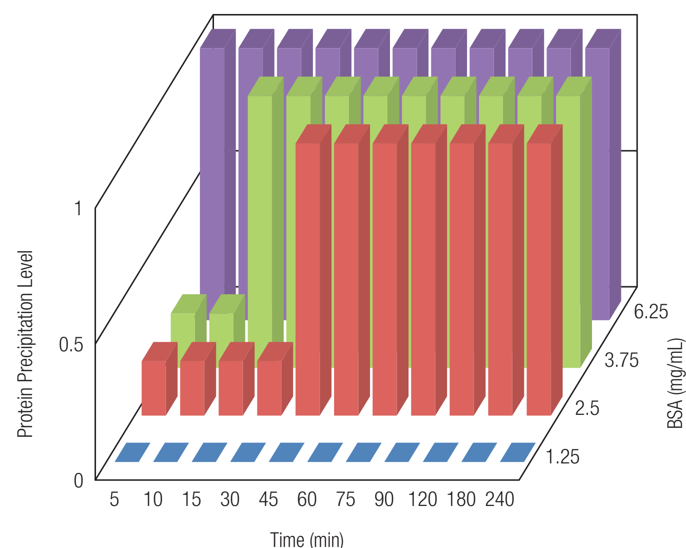


Figure 1. BSA solubility in TBS solution.

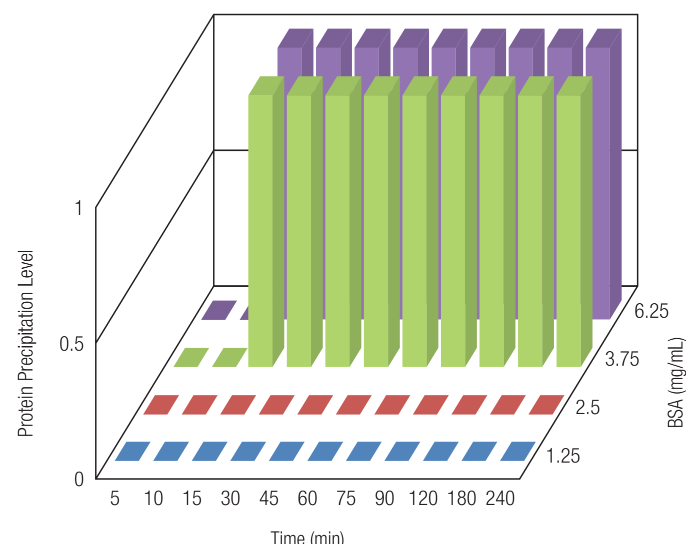


Figure 2. BSA solubility in TBS solution with trypsin.

Concentrations of BSA greater than 2.5 mg/mL in TBS/trypsin solutions (Figure 2) displayed precipitation after only 10 minutes of incubation. This can be attributed to the denaturation of the trypsin, due to the high temperature, limiting the extent to which digestion of the protein could be achieved.

By contrast, the optimized solubility buffer was capable of solubilizing all concentrations of BSA up to as high as 12.5 mg/mL (Figure 3). This is five times higher than the other approaches.

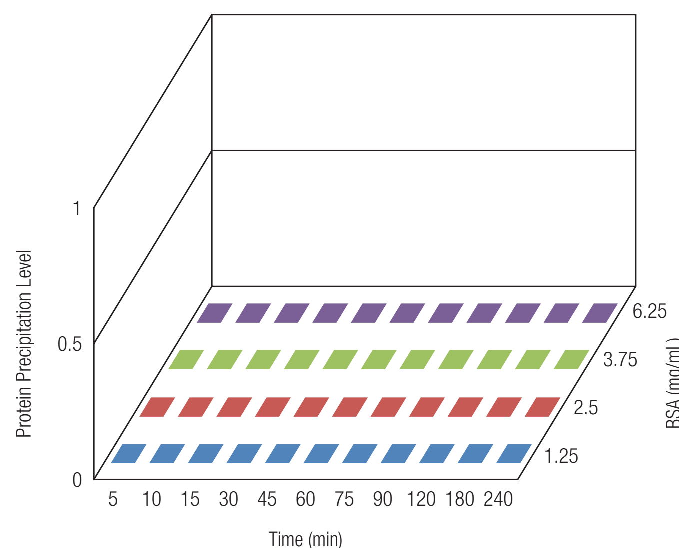
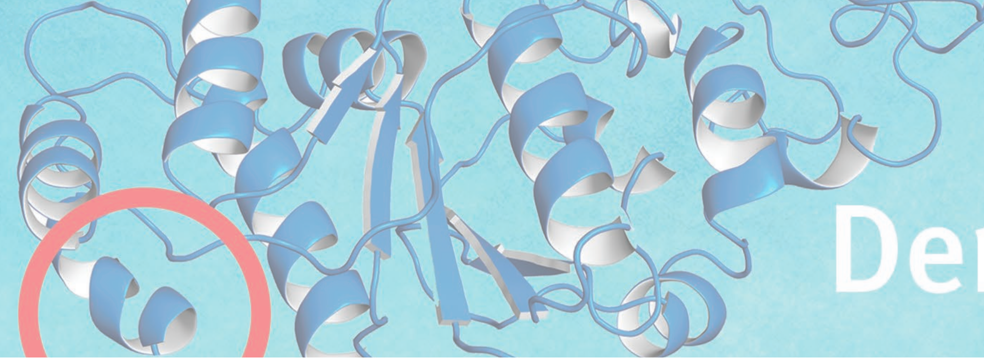


Figure 3. BSA solubility in optimized buffer.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion



Denaturation

Experiment Two: Digestion Buffer Comparison
The solubility of BSA in digestion buffer samples was assessed after digestion with the SMART Digest Kit (Figure 4). The digestion buffer showed excellent solubility with incubation times ranging from 30 to 210 minutes.

Conclusion

Based on this analysis, the two most effective buffers were combined to create the SMART Digest buffer, which does the following:

- Enables BSA to be kept in solution, both with and without the addition of trypsin, at concentrations up to 12.5 mg/mL.
- Allows higher concentrations of proteins to be kept in solution for longer periods of time, than standard digestion buffers.
- Permits use with high-temperature (up to 70 °C) digestions, enabling digestion and denaturation of proteins to be performed in one step.

Figure 4. BSA solubility after digestion with SMART Digest Kit.

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion
Find out more...

Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A

Valeria Barattini, Thermo Fisher Scientific, Runcorn, UK

Goal

To investigate the effect of four common additives on the digestion of ribonuclease A, a small, digestion-resistant, heat-stable protein, using the Thermo Scientific™ SMART Digest™ Kit.

Introduction

Standard in-solution protocols for trypsin digestion are lengthy, multifaceted, and prone to irreproducibility. The SMART Digest Kit eliminates these issues by providing a protocol that is:

- Highly reproducible
- Quick and easy to use
- Highly amenable to automation

The SMART Digest Kit is shown to provide a fast, efficient, and reproducible tryptic digestion in a fraction of the time compared to a standard in solution digest method.

The SMART Digest buffer has been optimized to provide optimum digestion efficiency for most protein samples, without the need for addition of chaotropes or detergents. Occasionally, proteins may be resistant to digestion and require these additives. The main reasons for this include:

- Solubility – very large, hydrophobic proteins, such as membrane proteins, may be difficult to solubilize efficiently and thus require the addition of large quantities of detergents
- Heat stability/folding stability – proteins featuring a very stable secondary and tertiary structure require larger quantities of chaotropes to aid the unfolding process

In this study we have investigated the effect of three commonly used detergents (RIPA, CHAPS, and OGS) and one common chaotrope (urea) on the digestion of ribonuclease A. This protein is particularly resistant to tryptic digestion due to its heat resistance and ability to re-fold to its native structure even when subjected to temperatures as high as 90 °C.1 RIPA and urea were chosen as representative of additives commonly used in cell lysis.

Ribonuclease A was monitored during digestion with the SMART Digest Kit, with and without the addition of each chaotrope in various concentrations. The rate of ribonuclease A depletion was compared under all conditions.

Experimental

Digestion

• SMART Digest Kit with 96 well filter plate (P/N 60109-102)

Consumables

• Deionized water, 18.2 MΩ·cm resistivity
• Fisher Scientific™ Optima™ acetonitrile (ACN) (P/N 10001334)
• Fisher Scientific trifluoroacetic acid (TFA) (P/N 10294110)
• RIPA Lysis and extraction buffer (P/N 89900)
• Urea, 99%, ACS Reagent (P/N 10462985)
• CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) (P/N 28300)
• OGS (Octyl-beta glucoside) (P/N 28310)

Ribonuclease A from bovine pancreas was purchased from a reputable source.

Sample Handling

• 2 mL 96 well square plates (P/N 60180-P202)
• Thermo Scientific™ WebSeal™ mats (P/N 60180-M122)

Sample Handling Equipment

• 96 well positive pressure manifold (P/N 60103-357)

It is also recommended that a heater/shaker equipped with PCR block and lid is used to perform the digestion.

Sample Pretreatment

Ribonuclease A stock solution:

A stock solution of ribonuclease A from bovine pancreas was prepared in water at a concentration of 2 mg/mL. The solution was sonicated to ensure complete dissolution of the protein.

SMART Digest buffer + urea:

All urea solutions in SMART Digest buffer were prepared at a concentration 25% higher than that required for the digestion study. This was applied in order to take into account for a 25% dilution factor applied when the ribonuclease A stock solution was added to the SMART Digest buffer prior to digestion.

A 5.33 M stock solution of urea in SMART Digest buffer was prepared and subsequently diluted in SMART Digest buffer according to Table 1.

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thermoscientific

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Table 1. Dilution series for the preparation of urea in SMART Digest buffer.

Solution	Concentration of Urea (Before Digestion Start)	Concentration of Urea (Digestion Reaction)	Dilution Protocol
A	0.67 M	0.5 M	250 µL stock solution + 1.750 mL SMART Digest buffer
B	0.27 M	0.2 M	50 µL stock solution + 950 µL SMART Digest buffer
C	0.133 M	0.1 M	200 µL solution A + 800 µL SMART Digest buffer
D	0.067 M	0.05 M	100 µL solution A + 900 µL SMART Digest buffer

SMART Digest buffer + RIPA/CHAPS/OGS

Solutions of each detergent were prepared from 10% w/v stock solutions, as described in Table 2.

Table 2. Preparation of detergent solutions.

Solution	Concentration of Detergent for Digestion Reaction (v/v)	Preparation for 1 mL (10 mL for 0.01% stock)
A	0.50%	5 µL stock + 995 µL SMART Digest buffer
B	0.20%	2 µL stock + 998 µL SMART Digest buffer
C	0.10%	1 µL stock + 999 µL SMART Digest buffer
D	0.05%	1 µL stock + 1.999 mL SMART Digest buffer
E	0.01%	100 µL solution C + 900 µL SMART Digest buffer

Sample Preparation

Ribonuclease A digestion

First, 75 µL of SMART Digest buffer, with or without additive, were pipetted into each SMART Digest trypsin tube. Then, 25 µL of the 2 mg/mL ribonuclease A was added to each tube (50 µg final protein amount). To ensure a uniform starting time across all the tubes, the aliquots were added using an 8 channel pipette. Samples were digested using a heater shaker set at 70 °C and 1400 rpm shaking. Tubes were subsequently removed after 15, 30, 60, 90, 120, and 150 minutes for analysis.

For all sample sets, digestion was stopped by simply passing the sample through the filter plate using positive pressure. This removed the sample from the immobilized trypsin beads. The eluent was subsequently analyzed using LC-UV.

Standards corresponding to t=0 minutes, containing the maximum ribonuclease A concentration, were prepared by diluting 25 µL of ribonuclease A solutions with 75 µL of SMART Digest buffer with the corresponding additive.

Separation Conditions

Instrumentation: Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system equipped with:

SRD-3600 Solvent Racks with Degasser (P/N 5035.9230)

DGP-3600RS Rapid Separation Pump (P/N 5040.0066)

WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020)

TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)

DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082.0020)

Column:	Thermo Scientific™ MAbPac™ RP, 50 × 2.1 mm (P/N 088648)
Mobile Phase A:	Water + 0.1 % TFA
Mobile Phase B:	Acetonitrile + 0.08% TFA
Gradient:	See Table 3
Flow Rate:	0.5 mL/min
Column Temp.:	80 °C
Autosampler Settings:	The autosampler settings were optimized for the WPS 3000 TRS (see Table 4)
Injection Wash Solvent:	Water + 0.1% TFA
UV Detector:	Wavelengths selected were 214 nm and 280 nm. Peak width was set to 0.1 min and recommended values were selected for the data collection settings.

Table 3. LC gradient conditions.

Time (min)	%A	%B
0	100	0
1	100	0
11	50	50
11.01	0	100
15	0	100
15.01	100	0
20	100	0

Table 4. Autosampler settings.

	SMART Digest Buffer SMART Digest Buffer + Urea SMART Digest Buffer + RIPA	SMART Digest Buffer + CHAPS	SMART Digest Buffer + OGS
Injection Volume (µL)	5	5	5
Draw Speed (µL/s)	0.05	3	1
Draw Delay (s)	3.0	3	3
Dispense Speed (µL/s)	10.0	20	20
Dispense Delay (s)	2.0	2	2
Dispense to Waste Speed (µL/s)	1.0	20	20
Sample Height (mm)	2.0	2	2

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Table of Contents

Introduction

Peptide Mapping

Peptide Quantitation

Product and method development considerations

Fast Digestion Method Optimization Using Innovative Trypsin Technology

Enhanced Digestion for Improved Biomarker Identification

Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers

Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A

Generic SPE Protocol for Peptide Clean-up and Concentration

Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 SR2 Chromatography Data System was used for data acquisition and analysis.

Results and Discussion

Figure 1 shows the progress of the ribonuclease A digestion with no additives at different time points. Digestion is complete at 120 minutes. This can be determined as the intact protein peak is no longer visible and the tryptic peptides are consistent.

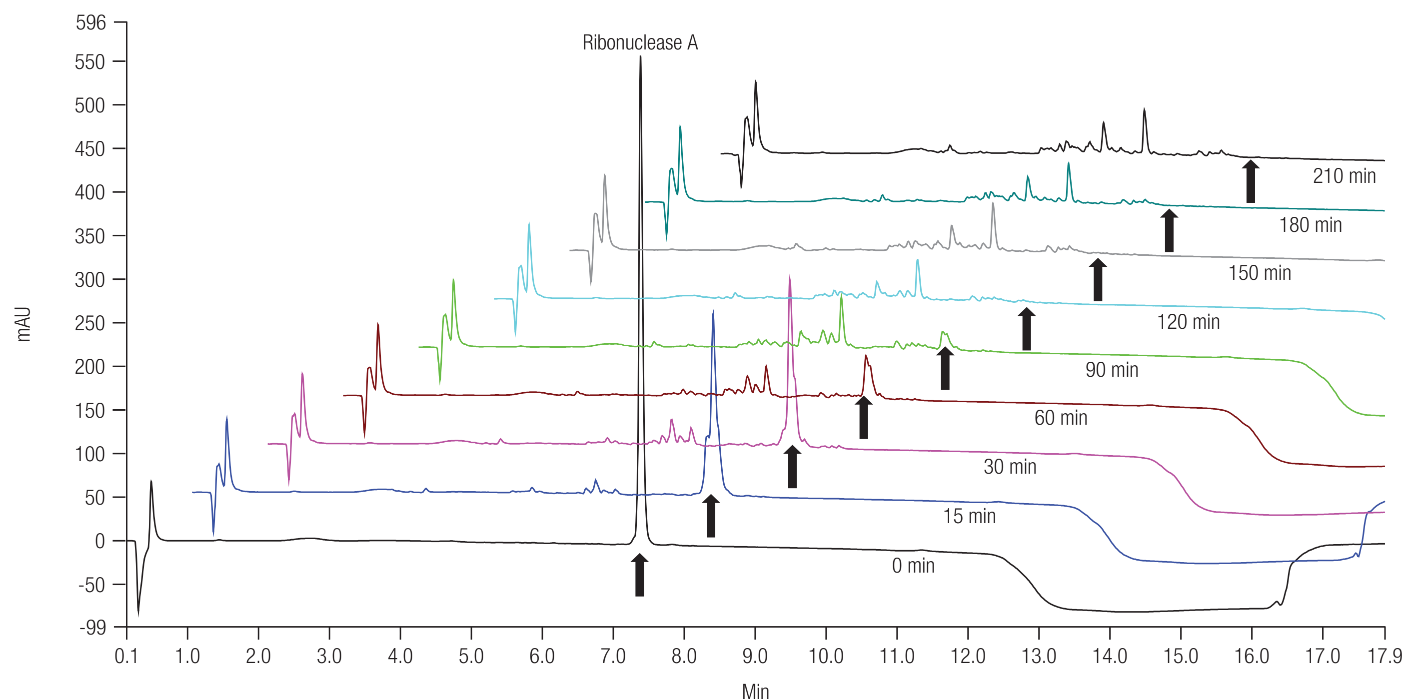

Figure 1. Chromatogram overlay of the digestion time points for ribonuclease A (no additive).

It is evident that the addition of urea in this case results in a small reduction in the digestion rate in the first 60 minutes, but has no significant impact on the overall digestion completion time.

Urea is commonly used in very high concentrations (4–8 M) to efficiently lyse cell cultures for subsequent digestion.

The peak area of ribonuclease A was measured for each time point in each condition studied. To compare digestion progress with each additive, the rate of decrease of ribonuclease A peak area was monitored. For simplicity, each condition was monitored against digestion in SMART Digest buffer without additives.

A concern in these cases is that in excessive concentrations, urea will readily precipitate once heated to 70 °C, as required for tryptic digestion with the SMART Digest Kit. The results above indicate that a simple dilution of the sample to a concentration of 0.5 M urea or below can be applied and prevent this.

SMART Digest Buffer + Urea

Figure 2 shows the peak area decrease of ribonuclease A with the addition of urea. For simplicity, all areas have been normalized with respect to the peak area at t=0 in the same condition.

SMART Digest Buffer + RIPA

Figure 3 shows the area depletion of ribonuclease A with the addition of RIPA. For simplicity, all areas have been normalized with respect to the peak area at t=0 in their the same condition.

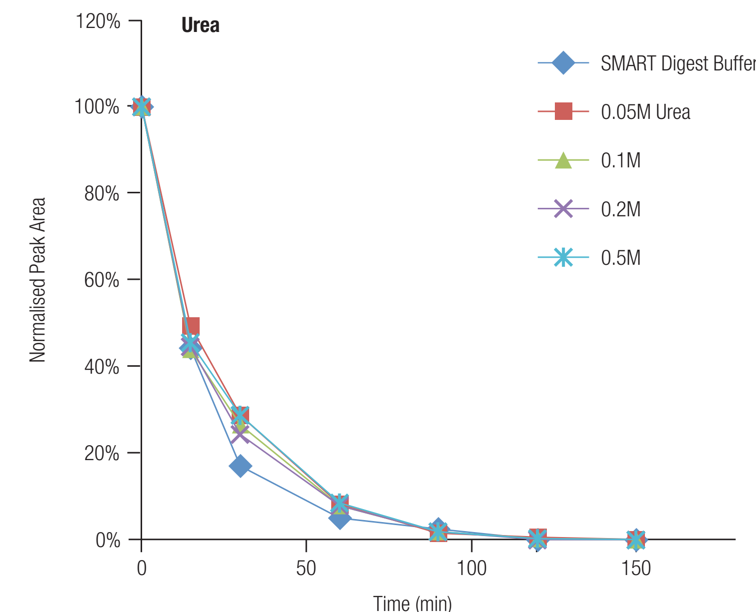

Figure 2. Area depletion of ribonuclease A with the addition of urea in concentrations up to 0.5 M.

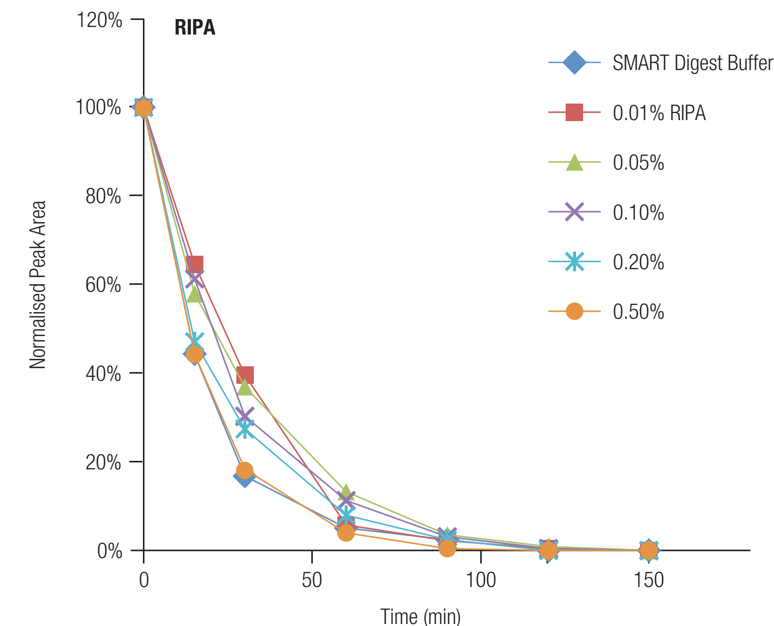

Figure 3. Area depletion of ribonuclease A with the addition of RIPA up to 0.5% w/v.

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

The effect of RIPA on the digestion of ribonuclease A is largely concentration dependent. A significant reduction of up to 20% in digestion rate is observed in the first hour for concentrations up to 0.1%. However, beyond this concentration the rate is comparable to digestion in SMART Digest buffer without additives. A possible explanation for this is that beyond a concentration of 0.1% RIPA, the additives present in this buffer provide an additional denaturing effect on ribonuclease A, which favor the digestion to a greater extent than any partial trypsin inhibition.

SMART Digest Buffer + CHAPS

The effect of CHAPS on the digestion of ribonuclease A was found to be uniformly negative, with a reduction in digestion rate of up to 32% (Figure 4). Unlike RIPA, this trend is not concentration-dependent.

SMART Digest Buffer + OGS

The effect of adding OGS to the digestion of ribonuclease A was found to have a mild positive effect on the digestion rate. In the first 15 minutes of digestion, OGS showed a 10–20% increase in ribonuclease A depletion (Figure 5). The effect is particularly pronounced at smaller concentrations of OGS (up to 0.1%).

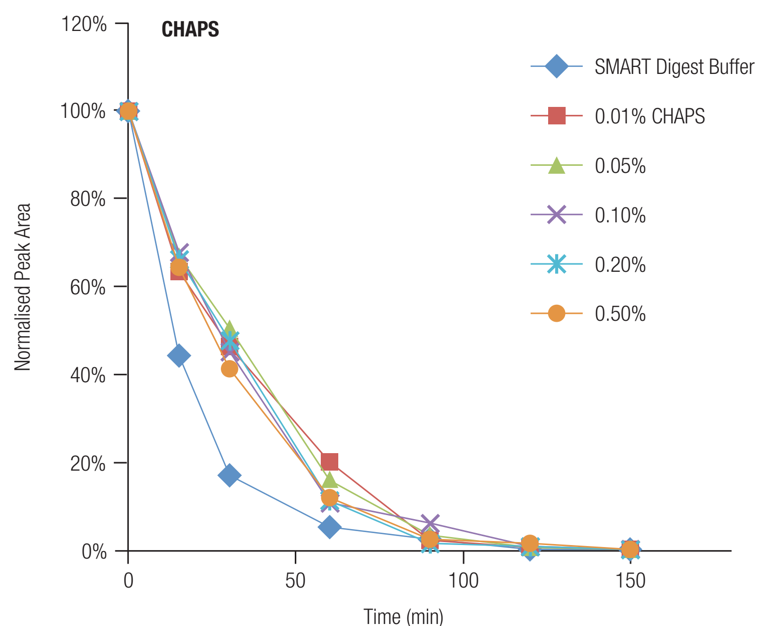

Figure 4. Area depletion of ribonuclease A with the addition of CHAPS up to 0.5% w/v.

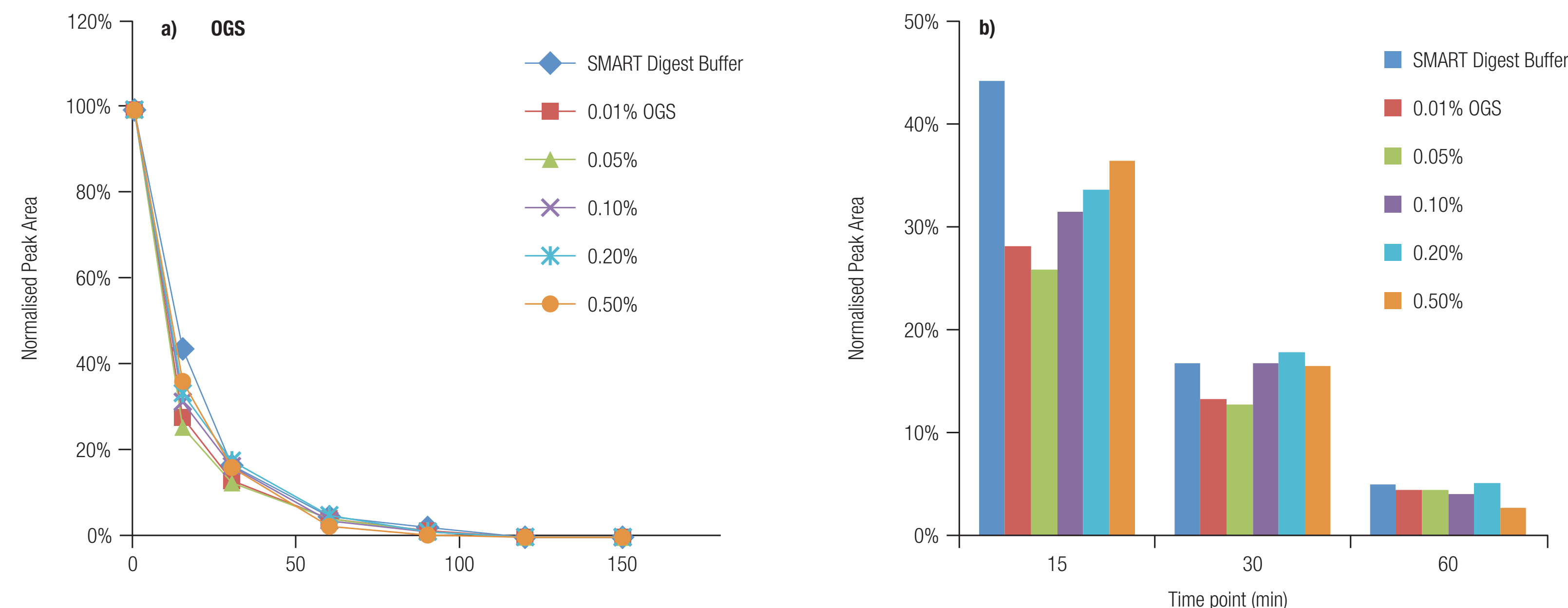

Figure 5. a) Area depletion of ribonuclease A with the addition of OGS up to 0.5% w/v. b) Individual time point bar chart view for time points up to 60 minutes.

To verify if the digestion rate increase was statistically significant, the time points were repeated in triplicate; for simplicity only three concentrations were studied (SMART Digest buffer, 0.1% and 0.5% OGS). Figure 6 shows the measured digestion time points for the triplicate experiments. The time curve did not show an obvious rate increase (Figure 6a); however, the detailed view of the 15 minute time point shows a statistically significant increase in digestion of approximately 7% (Figure 6c). At 30 minutes an apparent increase of approximately 5% was observed, but this was found to be within the measured error and therefore not significant.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

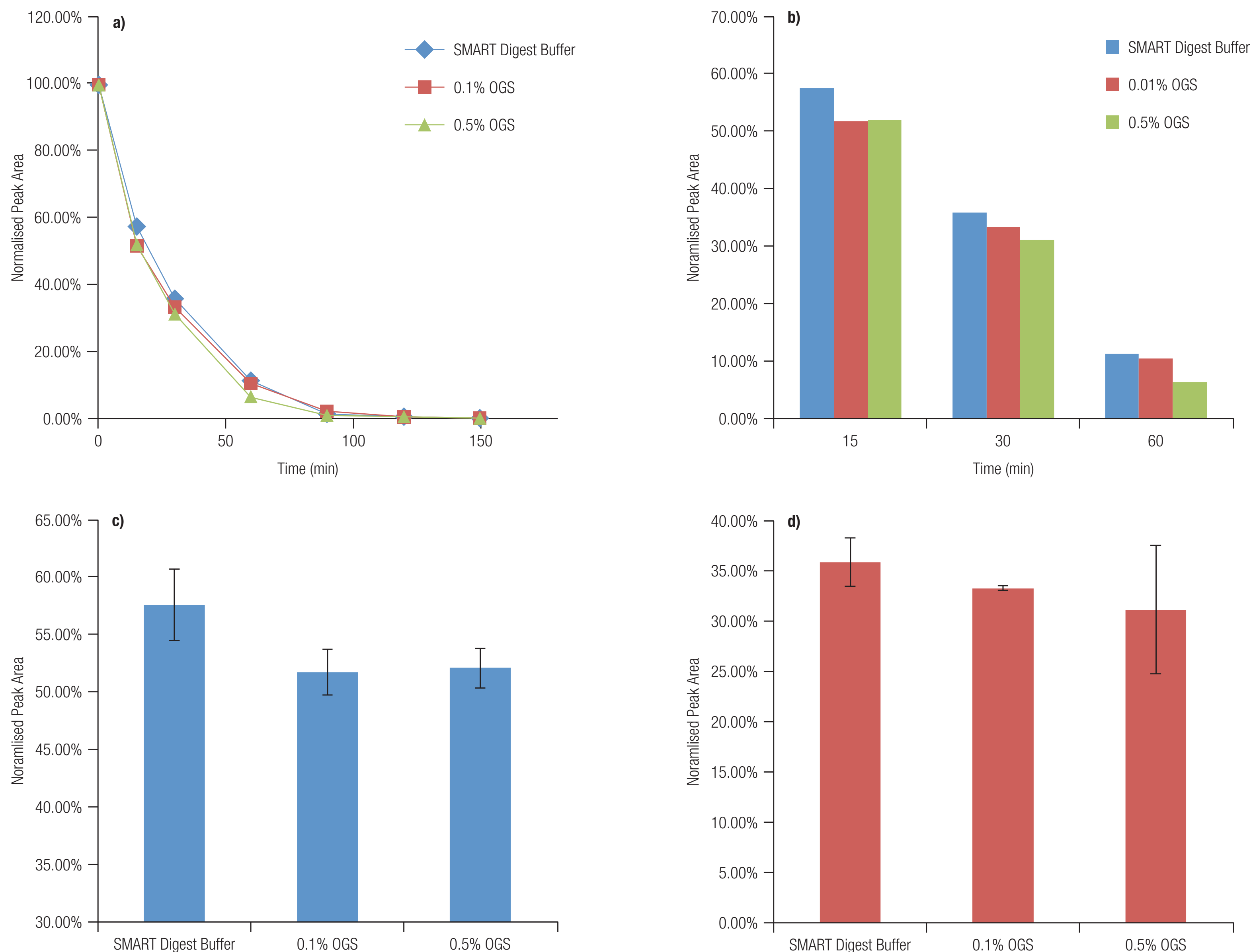

Figure 6. a) Average area depletion for n=3 of ribonuclease A with the addition of OGS (0, 0.1% and 0.5%)
b) Individual time point bar chart view for time points up to 60 minutes
c) Detailed view of 15 minutes, including %RSD above and below the average
d) Detailed view of 30 minutes, including %RSD above and below the average

In cases where troublesome analytes are to be digested, the addition of OGS forms a particularly promising prospect. The additive does not inhibit digestion and can lead to an improved digestion rate.

Conclusion

Using a case study with ribonuclease A, we have shown that:

- Addition of up to 0.5 M urea does not significantly impact the digestion rate.
- In cases where high urea amounts are usually used for cell lysis, a reduction in concentration to 0.5 M prevents precipitation.
- The addition of OGS has a slight rate enhancement effect.
- The addition of RIPA, for ribonuclease A digestion, results in a concentration-dependent effect, where initial enzyme inhibition is overcome by improved substrate solubilization at higher concentrations only.
- The addition of CHAPS as a detergent leads to an overall decrease in ribonuclease A digestion rate.

Reference

1. Lang, K. Use of a trypsin-pulse method to study the refolding pathway of ribonuclease, Eur. J. Biochem., 1986, 159, 275-281.

Useful Links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:
www.thermofisher.com/appslab

thermoscientific

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion
Find out more...

Generic SPE Protocol for Peptide Clean-up and Concentration

Jon Bardsley, Thermo Fisher Scientific, Runcorn, UK

Goal

Description of a generic, reproducible and robust procedure that can be used to clean up and concentrate peptide samples. The procedure can remove unwanted buffer, reagents, and any physical particulates from the sample while maintaining high levels of analyte recovery and reproducibility. An additional benefit is the ability to concentrate the sample prior to analysis.

The method is performed using micro-elution solid phase extraction (SPE) and, therefore, benefits from removal of post-extraction sample evaporation and reconstitution required by traditional scale SPE. Removal of these steps eliminates issues with solubility and non-specific binding (NSB) that are often associated with peptide analysis.

Introduction

Peptide analysis can present a number of issues that affect detector response, due to the presence of unwanted interferences from either the matrix or from reagents and other additives used to facilitate protein digestion. Sample preparation techniques employed to remove these interferences are required to be quick, simple, and generic. Reproducibility is also important as this enables users to confidently assign data differences to the sample and not the methodological conditions used.

Generic micro-elution SPE methods can be successfully employed for a nontargeted workflow, removing only the unwanted reagents and particulates from the digested sample while maintaining a high recovery and extraction reproducibility of the peptides. An additional concentration factor can be applied to assist with analysis.

The Thermo Scientific™ SOLAμ™ micro-elution SPE device is built on a polymeric backbone containing both reversed-phase and polar retentive moieties. SOLAμ SPE provides reproducibility, robustness, and ease of use at low elution volumes by utilizing the revolutionary Thermo Scientific™ SOLA™ Solid Phase Extraction technology. This removes the need for frits by delivering a robust, reproducible format that ensures highly consistent results at low elution volumes providing;

- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples that are limited in volume
- Improved stability of bio-molecules by reduction of adsorption and solvation issues

Here we show a proof of concept for a generic clean-up protocol. Eight well-characterized peptides were processed and monitored for recovery and reproducibility. Four of the peptides were derived from digested cytochrome c. Four additional peptides were spiked into the digested sample before processing in order to broaden the range of peptides used for assessment of the protocol.

Experimental

Sample Preparation

- SOLAμ HRP 96 well plate (P/N 60209-001)
- Thermo Scientific™ SMART Digest™ Kit with SOLAμ HRP (P/N 60109-103)

Chemicals

- Fisher Scientific™ Optima™ water (P/N10095164)
- Fisher Scientific Optima acetonitrile (ACN) (P/N 10001334)
- Thermo Scientific™ Pierce™ formic acid (FA) (P/N 10628654)
- Fisher Scientific trifluoroacetic acid (TFA) (P/N 10294110)

Cytochrome c, leu-Enkephalin, angiotensin 1, angiotensin 2, and neurotensin were purchased from reputable sources

Sample Handling

- 96 well square well microplate (P/N 60180-P202)

Separation

- Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm Analytical (2.1 × 100 mm) (P/N 068982)

Sample Handling Equipment

- 96 well positive pressure manifold (P/N 60103-357)

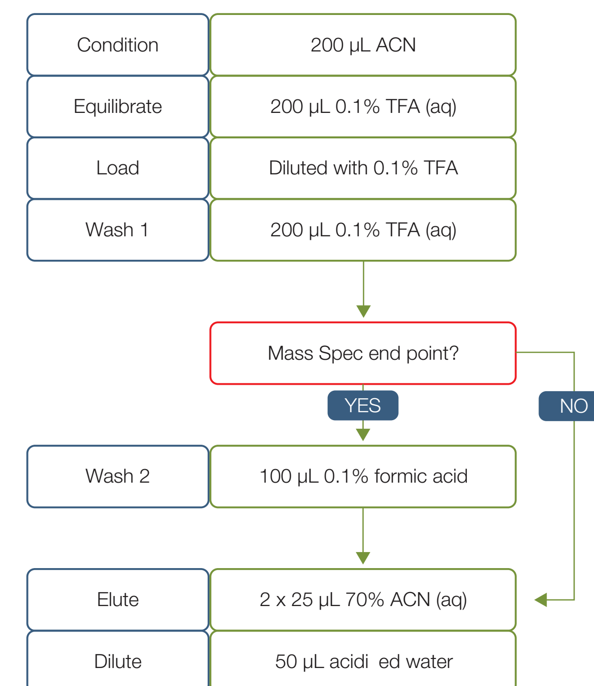

Figure 1. Clean-up workflow.

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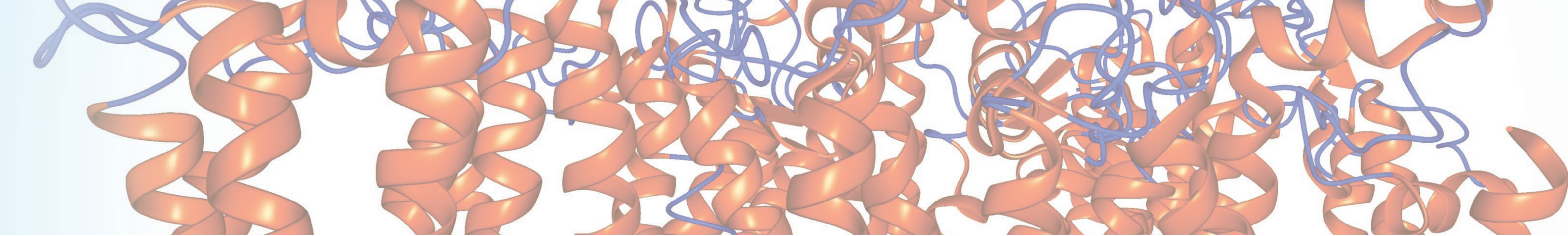
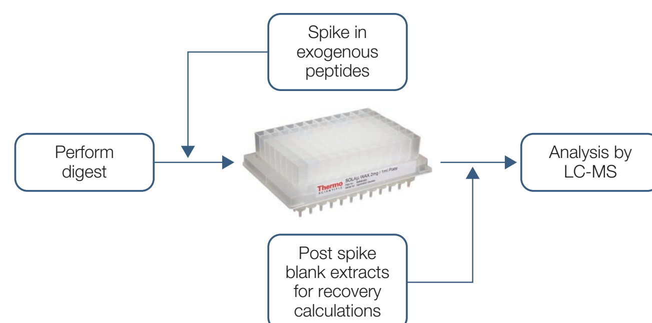
Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion



Separation Preparation

Additional exogenous peptides were added to digested cytochrome c (20 µg) samples. Both the cytochrome c derived and the exogenous peptides were processed and analyzed (Figure 1). Results were compared to reagent blanks that were processed and spiked with peptide standards post extract (Figure 2).



$$\text{Recovery} = \frac{\text{Average Response of Sample}}{\text{Average Response of Overspike}} \times 100$$

Figure 2. Recovery calculation method.

Analytical Conditions

All samples were analyzed using the Thermo Scientific™ Vanquish™ UHPLC system. Separation on an Acclaim RSLC analytical column was achieved with a linear gradient of 0.1% formic acid in water to 0.1% formic acid in acetonitrile over 15 minutes.

Detection was performed in full scan mode on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer, and the data was processed using Thermo Scientific™ Xcalibur™ Quant software version 3.0.63 with the mass to charge ratios (m/z) in Table 1.

Data Analysis

Thermo Scientific™ Pepfinder™ 2.0 was used to analyze the resulting data set.

Results and Discussion

A total of eight peptides were analyzed for assessment of the method. Four well-characterized peptides derived from cytochrome c, along with four well-characterized exogenous peptides, were spiked in post digestion. Table 1 and Figure 3 show recovery levels of each peptide assessed along with the reproducibility (n=6). Recovery levels of 86% or higher were achieved in seven out of eight peptides with single digit %RSD on all peptides. This demonstrates a high level of reproducibility across the range of peptides assessed.

Table 1. Peptide recovery data.

Sample	Amino Acid Sequence	MS (m/z)	Recovery (%)	Precision (%RSD)
Cytochrome c peptide	EDLIAYLK	483.27301	101%	4%
	GITWGEETLMEYLENPKK	711.33099	113%	5%
	MIFAGIK	779.44641	51%	9%
	TGPNLHGLFGR	390.21155	109%	6%
Leu_Enkephalin	YGGFL	556.27526	97%	3%
Angiotensin_I	DRVYIHPFHL	432.8987	93%	7%
Angiotensin_II	DRVYIHPF	523.77349	86%	7%
Neurotensin	ELYENKPRRPYIL	558.30907	91%	3%

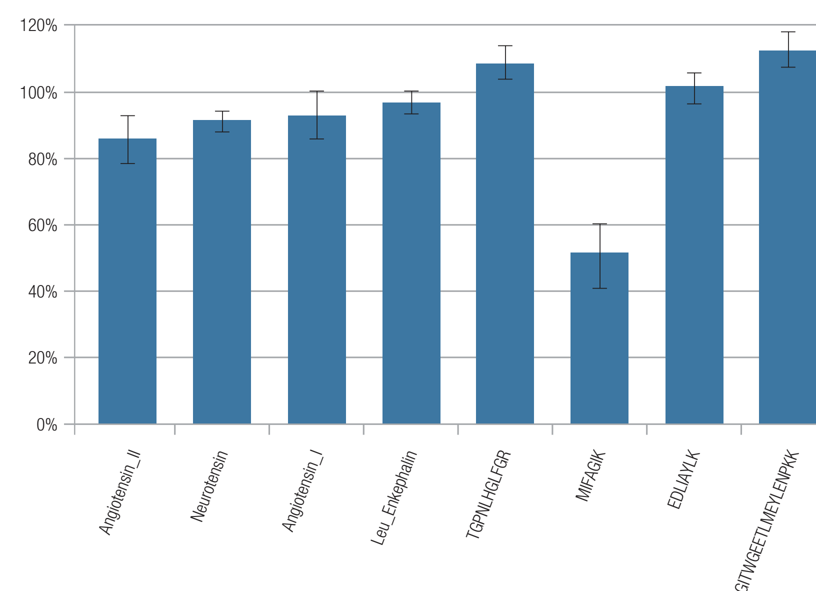


Figure 3. Peptide recovery data.

The final extracts demonstrated 85% protein coverage. This was calculated using PepFinder software against the theoretical number of peptides possible from cytochrome c. The peptide coverage map is shown in Figure 4.

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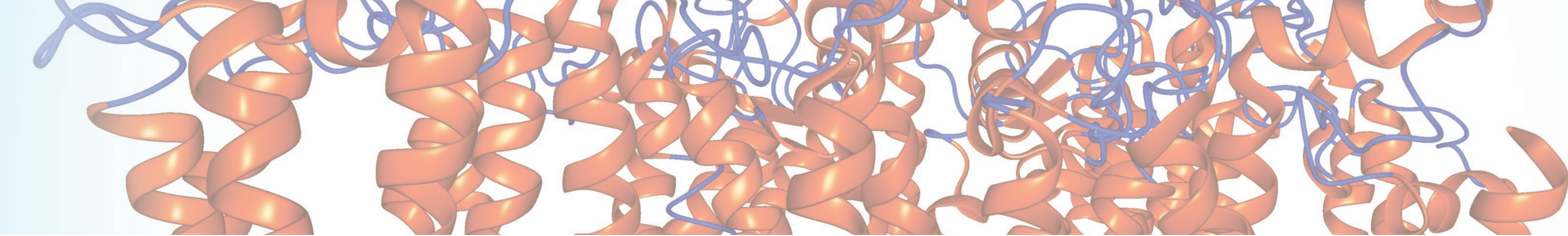


Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

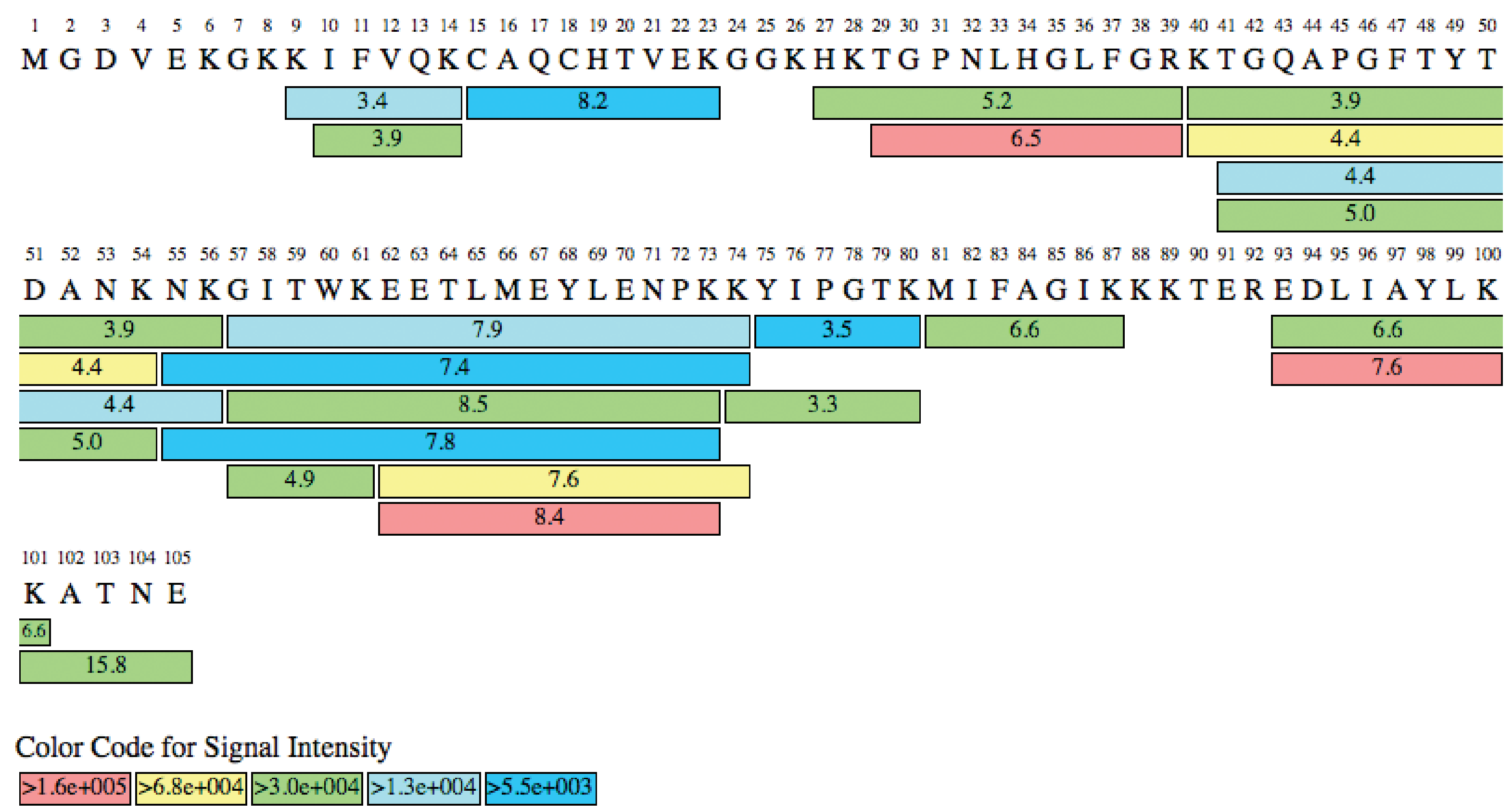


Figure 4. Peptide coverage data for cytochrome c. *Sequence of cytochrome c is shown. Each peptide is identified here by amino acid sequence, color coded for detection intensity. The chromatographic retention time is denoted by the number within each box (minutes).

Conclusion

- Evaluation of the generic clean-up procedure shows high levels of recovery and reproducibility of the peptides used for assessment. An increase in detector response can be achieved through sample concentration.
- Despite one low recovery value for MIFAGIK, levels of precision remained high throughout. Furthermore, an assessment of sequence coverage revealed that this protocol maintains a high overall percentage of peptides, demonstrating the use of this method as a nontargeted approach for peptide analysis.
- The described workflow both cleaned and concentrated the digest sample. Due to the format, up to 96 samples can be processed in as little as 15 minutes, making this workflow well suited to a high-throughput environment.

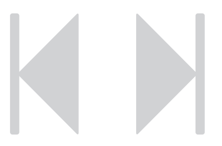


Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion
Find out more...

Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Valeria Barattini, Phillip Humphryes, Thermo Fisher Scientific, Runcorn, UK

Goal

To demonstrate how Thermo Scientific™ SMART Digest™ Kits remove uncertainty associated with conventional solution-based tryptic digestion protocols, resulting in higher reproducibility and sample characterization.

Introduction

Protein digestion is a fundamental technique employed in biopharmaceutical and proteomic applications. It is used to analyze the sample and identify structural features or post-translational modifications (PTMs). Despite its widespread use, protein digestion still provides many analytical challenges. The optimum digestion should provide conditions that accomplish the following:

- Cleave the proteins after every lysine and arginine
- Unfold the protein/proteins of interest
- Completely denature the protein to be digested yet do not affect trypsin itself
- Use the minimum amount of trypsin to protein to prevent partial digestion

Since optimization of all steps listed above is required, the process can be lengthy and complex and often leads to poor reproducibility.

The SMART Digest Kit mitigates lengthy and complex method development by providing a protocol with the following attributes:

- Highly reproducible
- Quicker and easier to use
- Requires no detergent
- Temperature-induced denaturing of proteins through the use of a heat-stable trypsin
- Reduced autolysis through the use of immobilized trypsin
- Results in fewer chemically-induced PTMs

Trypsin digestion of insulin using the SMART Digest Kit and a series of commonly used buffer species and additives were compared. In order to determine the impact of various buffers and additives on the trypsin digestion, a partial digestion of insulin was performed.

Insulin provides an ideal candidate for this study, as it creates a very simple peptide map. The partial digestion was used to assess variance of enzyme activity. When exposed to trypsin, human insulin forms two major products. There is a C-terminal peptide sequence and a larger N-terminal sequence (Figure 1). These two peaks are easily resolved from the intact protein using reversed phase chromatography (Figure 2).

Protein digestions are often performed in the presence of chaotropes, surfactants, salts, and organic solvents. Due to the general robustness of trypsin, it is often assumed that activity is negligibly affected by these additives. In this study, the activity of trypsin from the SMART Digest Kit was assessed by varying the digestion buffer conditions and comparing these to the SMART Digest buffer. The results show that trypsin activity varies significantly depending on the buffer used. Additives such as guanidine HCl and urea have a concentration-dependent adverse effect on the digest efficiency. Furthermore, buffer species such as phosphate buffer saline (PBS) and ammonium bicarbonate (ABC) were also found to adversely affect digestion.

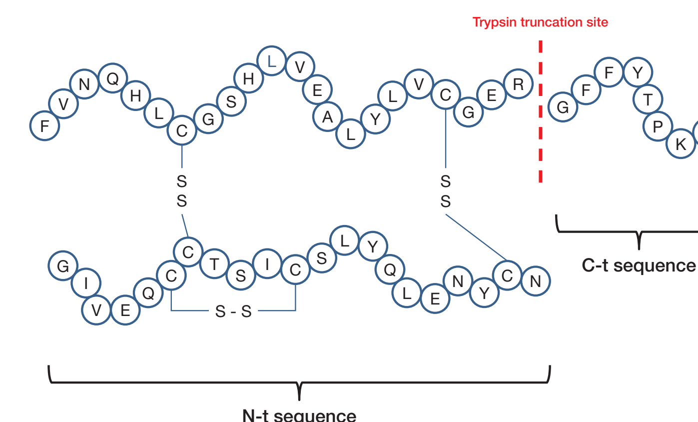


Figure 1. The amino acid sequence of insulin.

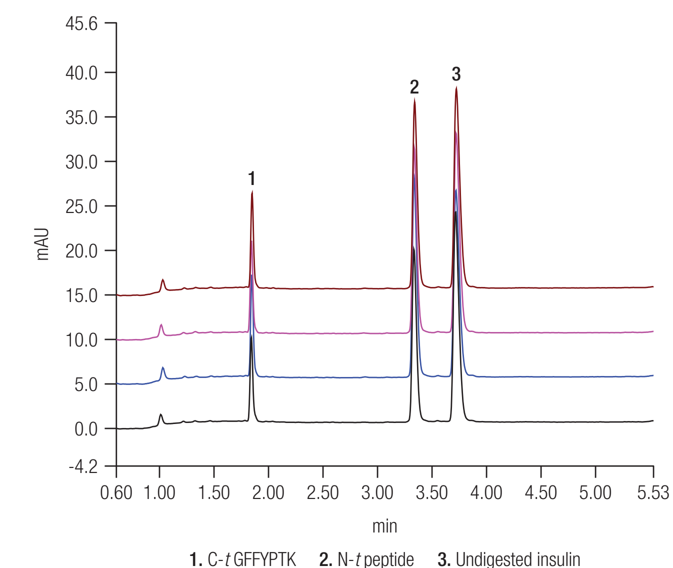


Figure 2. Typical chromatogram obtained for the LC/UV analysis of insulin partial digest.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Digestion

- SMART Digest Kit (P/N 60109-101)

Sample Handling

- 96-well collection plate provided in SMART Digest Kit

Column

- Thermo Scientific™ Accucore™ C18 (50 × 2.1 mm, 2.6 µm particle) (P/N 17126-052130)

Chemicals

- Fisher BioReagents™ tris buffered saline (TBS) (P/N 10648973)
- Fisher Scientific™ Optima™ LC-MS water (P/N 10095164)
- Fisher Scientific Optima acetonitrile (ACN) (P/N 10001334)
- Fisher Scientific Optima isopranol (IPA) (P/N 10091304)
- Fisher Scientific dimethyl sulfoxide (DMSO) (P/N 10500151)
- Fisher Scientific Optima methanol (MeOH) (P/N 10031094)
- Fisher BioReagents 2,2,2-trifluoroethanol (TFE) (P/N 10468733)
- Fisher BioReagents formamide (P/N 10440464)
- Thermo Scientific Pierce guanidine HCl (P/N 11821365)
- Fisher BioReagents™ Tween™ 20 (P/N 10113103)
- Pierce octyl-beta-glucoside (P/N 28310)
- Fisher BioReagents deoxycholate (P/N 10346653)
- Fisher Scientific analytical grade trifluoroacetic acid (P/N 10112740)
- USP-grade insulin was purchased from a reputable supplier.

Sample Handling Equipment

Heater/shaker equipped with PCR block and heated lid.

Digestion Protocol

As listed in Table 1, 100 µg/mL solutions of insulin were prepared in a variety of buffers.

Experiments were designed to compare the performance of the SMART Digest buffer against six variables commonly used in solution based digests. The variables studied are also listed in Table 1.

Table 1. Buffer systems used to prepare insulin solutions.

Buffer System Studied		Variable Studied
SMART Digest buffer		n/a
PBS ABC Tris (0.05–0.5 M) HEPES		Buffering ion
4.50	NaCl (0.1–2 M)	Electrolyte
	pH 7–9	pH
	Gdn HCl (0.5–6 M) Urea (0.5–8 M) OGS (0.05–0.2%)	Chaotropes
Tris + 10 mM $CaCl_2$ +	Tween (0.01–0.1% v/v) Zwitt (0.1–1%) SDC (0.1–1%)	Detergents
	DMSO (5–20% v/v) ACN (5–20% v/v) MeOH (5–20% v/v) IPA (5–20% v/v) Formamide (5–20% v/v) TFE (5–20% v/v)	Co-solvents

Each insulin solution was subsequently digested by adding 200 µL of the solution to the SMART Digest immobilized trypsin tube. The mixture was incubated with agitation at 70 °C for 1 minute. This provided a partial digestion.

The peak area of the C-t sequence was measured in all buffer systems for comparison. Following incubation, the digestion reaction was quenched by means of acidification.

Separation Conditions

Insulin digestion products were analyzed by LC/UV using the gradient outlined in Table 2. Separation was achieved on an Accucore C18 column, 2.6 µm particle, 50 × 2.1 mm, as shown in Figure 2.

Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC System
Column:	Accucore C18 (50 × 2.1 mm, 2.6 µm particle) (P/N 7126-052130)
Mobile phase A:	2% acetonitrile, 98% water, 0.1% TFA
Mobile phase B:	90% acetonitrile, 10% water, 0.1% TFA
Flow rate:	0.5 mL/min
Column temp.:	40 °C
Injection details:	25 µL

Table 2. LC gradient conditions.

Time (min)	% A	% B
0.01	90	10
1	75	25
6	50	50
6.01	10	90
7.5	10	90
7.51	90	10
9	90	10

Software

The Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System was used for data acquisition and analysis.

Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Results and Discussion

The various additives and their concentrations had a dramatic effect on the digestion efficiency of the SMART Digest trypsin. Figure 3 summarizes the results found.

A small number of additives showed minor impact. Among the buffering ion screening (Figure 3a), HEPES and Tris up to 0.1 M gave a less than 20% reduction in tryptic activity. Equally, the chaotrope octylglucoside (Figure 3d) showed to have only a small impact on the digestion efficiency; the effect was not found to be concentration-dependent.

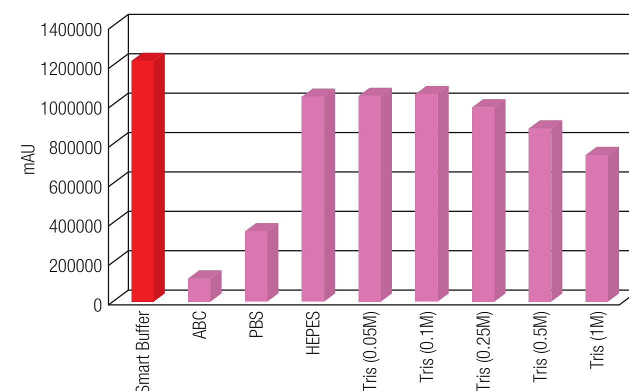
The buffering ion, however, was found to be crucial for optimal activity. Ammonium bicarbonate (ABC) and phosphate buffer saline (PBS) showed a much reduced rate of digestion, giving a 90% and 70% product reduction, respectively (Figure 3a).

When the pH of the buffer was scrutinized (Figure 3c), tris buffer at pH 8 showed comparable performance to the results obtained using the SMART Digest Kit. However, the higher pH is known to be a likely cause of chemically-induced PTMs and, therefore, deviations in the SMART Digest buffer pH away from pH 7.4 were not made in order to prevent this effect.

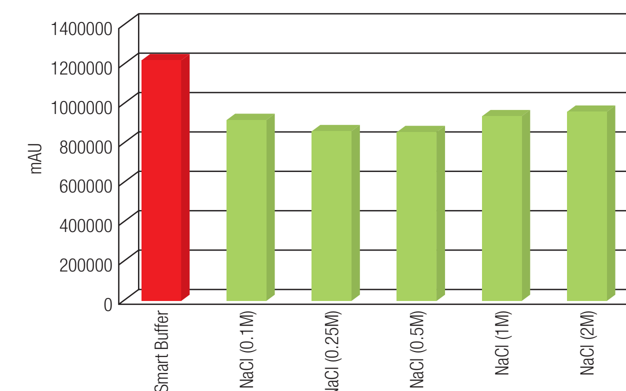
Addition of sodium chloride was found to give activity with a uniform decrease of 20–30%, regardless of the concentration studied (Figure 3b).

The addition of detergents in solution-based tryptic digests is common and serves the purpose of aiding protein solubility. The quantity of detergent, however, is crucial in being able to subsequently extract the triptic peptides. The most pronounced negative effect was observed with the addition of zwitterionic detergent (Zwitt) and sodium deoxycholate (SDC) (Figure 3e). In both cases, minimal quantities resulted in almost complete absence of digestion product.

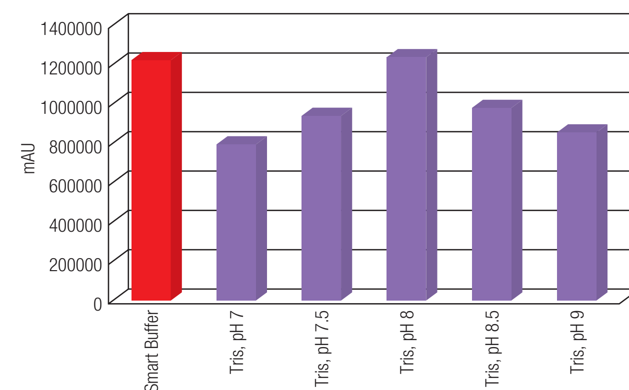
A Effect of buffering ion



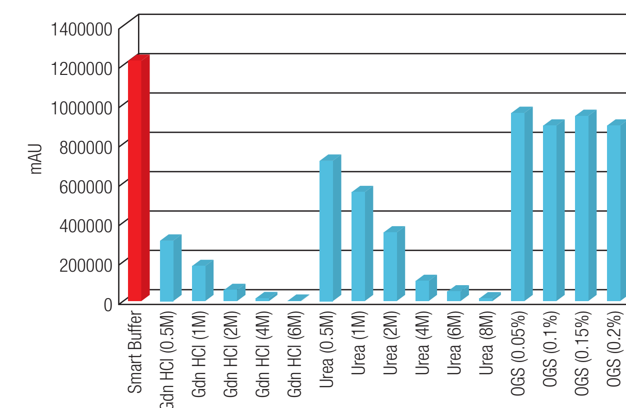
B Effect of salt concentration



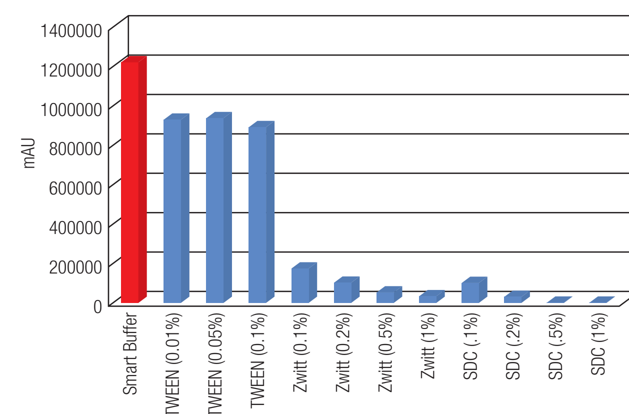
C Effect pf pH



D Effect of chaotropes



E Effect of detergents



F Effect of co-solvents

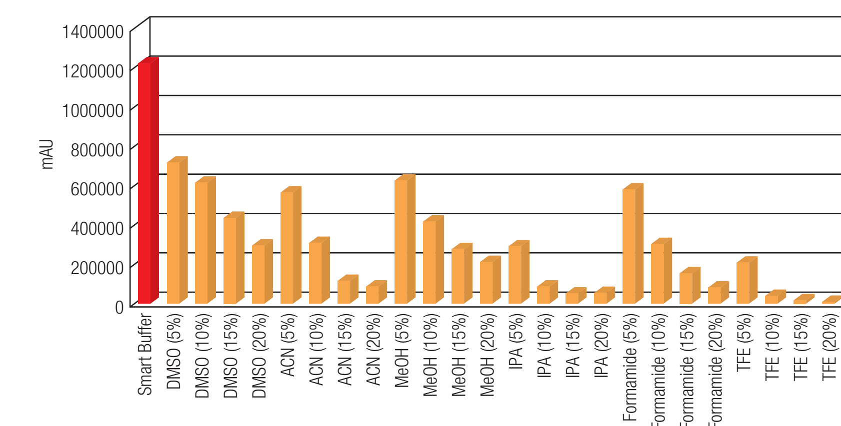


Figure 3. Peak areas of C-t peptide (GFFYTPKT) generated from the digestion of the protein using the SMART Digest Kit as a function of a) variation of buffering ion; b) salt concentration; c) pH; d) chaotropes; e) detergents; and f) organic co-solvents. The SMART Digest buffer result is shown as a red bar in each figure.

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Table of Contents
Introduction
Peptide Mapping
Peptide Quantitation
Product and method development considerations
Fast Digestion Method Optimization Using Innovative Trypsin Technology
Enhanced Digestion for Improved Biomarker Identification
Increased Efficiency of Biomolecule Identification by Optimization of Trypsin Digestion Buffers
Impact on Tryptic Digestion when Detergents and Chaotropes are Essential: A Case Study with Ribonuclease A
Generic SPE Protocol for Peptide Clean-up and Concentration
Selecting Buffers to Remove Uncertainty in Tryptic Digestion

Digestion in the presence of guanidine hydrochloride (Gdn HCl) or urea (both Figure 3d) was found to be negatively affected, with a pronounced concentration-dependent effect. At concentrations above 2M, these additives were both found to result in almost no digestion product. A similar trend was observed when organic co-solvents were studied (Figure 3f).

Conclusion

The SMART Digest Kit outperforms conventional solution-based tryptic digestion buffer systems, while providing a fast, simple, and clean method. Fewer steps are required to achieve complete digestion of samples.

In this application note, trypsin digestion of insulin was compared to the SMART Digest buffer and a series of commonly used buffer species and additives. From the results it is obvious that the following are true:

- Digestion efficiency is highly dependent on the buffer and additives used.
- The SMART Digest buffer outperforms all 6 variables compared.

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For more information on Smart Digest visit **thermofisher.com/smartdigest**

To talk to an expert, request a quote or get a product demo, **click here.**

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