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High-precision, Automated Peptide Mapping of Proteins

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ABSTRACT

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.

INTRODUCTION

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 labour 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 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™ kit magnetic beads and the Thermo Scientific™ KingFisher™ Duo Prime 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.

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.

Figure 1. SMART digest kit with additional sample clean-up option.

MATERIALS AND METHODS

Sample Preparation

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

Infliximab 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.

Figure 2. Kingfisher Du 12-tip comb (p/n 97003500).

Magnetic SMART Digest, Automated Digestion Protocol

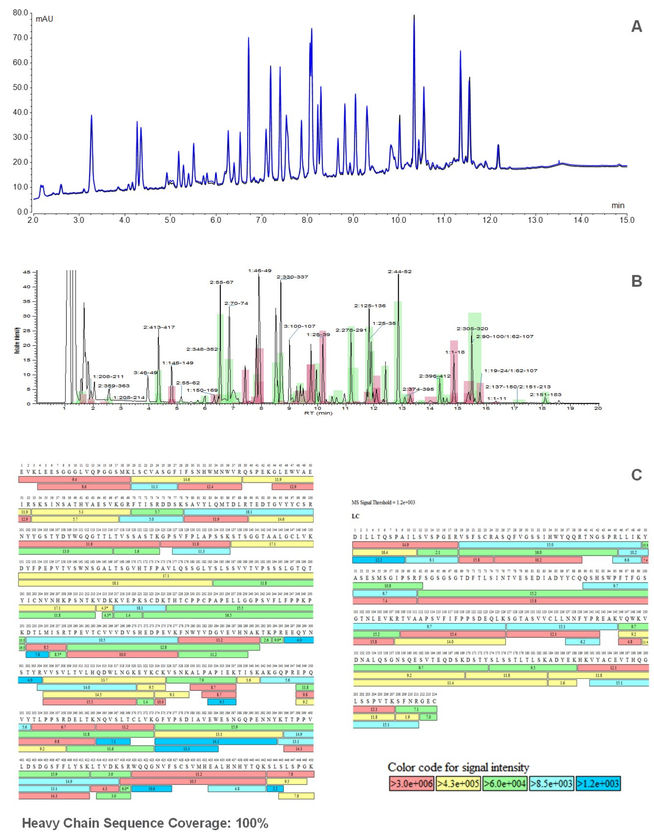
The KingFisher Duo Prime purification system was used to automate the protein digestion. Digests of infliximab and cytochrome c were carried out.

- SMART Digest magnetic resin slurry was diluted and uniformly suspended in SMART Digest 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.
- Thermo Scientific™ BindIt™ software (version 4.0) was used to control the KingFisher Duo Prime system
- 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 the reaction and the digest solution was actively cooled to 15 °C.

RESULTS

The applicability of the automated protein digestion with the KingFisher Duo Prime purification system was tested with infliximab and cytochrome C. Replicate digests of cytochrome c were conducted and the generated peptides were separated and analyzed by UHPLC-UV. All proteins were readily digested using the automated SMART Digest kit protocol resulting in complete digestion. 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.

Figure 3. Automated digestion of infliximab drug product using SMART Digest magnetic kit resin. Panel A: Overlaid peptide maps for two digests of infliximab antibody. 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.



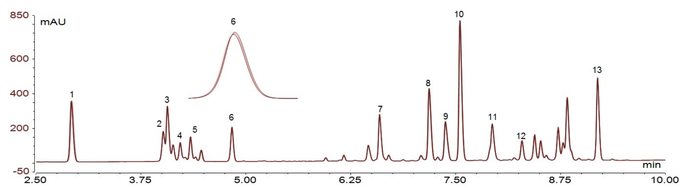
Heavy Chain Sequence Coverage: 100%

The manual method has shown to be reproducible, robust, and efficient even in the hands of multiple users with varying experience1. 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 method1. 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.

Digestion of a monoclonal antibody biotherapeutic 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 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.

Figure 4. Cytochrome C digest: Retention time (TR) and Peak Area (Arel) reproducibility (n = 5).



Peak	1	2	3	4	5	6	7	8	9	10	11	12	13
%RSD (t_R)	0.12%	0.05%	0.03%	0.04%	0.02%	0.02%	0.02%	0.03%	0.03%	0.02%	0.01%	0.01%	0.01%
%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%

Table 1. Chromatographic conditions.

	For UV	For MS
Column	Thermo Scientific™ Hypersil™ Gold 1.9 µm 2.1 × 150 mm	
Mobile Phase A	Water + 0.05% TFA	Water + 0.1% formic acid
Mobile Phase B	Water/acetonitrile/TFA (20/80/0.04 v/v/v)	Acetonitrile + 0.1% formic acid
Flow Rate	0.5 mL/min	0.3 mL/min
Column Temperature	70 °C	70 °C
Injection Volume	5 µL	5 µL
UV absorbance	214 nm	–

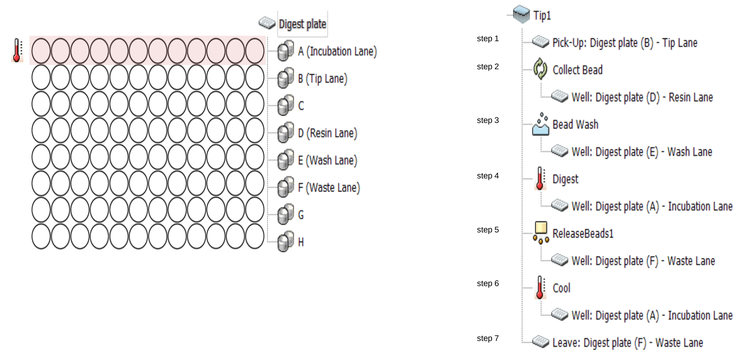
Table 2. Mass spectrometry conditions.

Ionization	HESI positive
Scan range	140 – 2000 m/z
Source temperature	350 °C
Spray voltage	3.4 kV
Resolution (full MS)	70,000 at 200 (FWHM)
Resolution (full MS2)	17,500 at 200 (FWHM)
Top-N MS2	5
Collision energy	27 (arb)
Max inject time	100 ms

CONCLUSIONS

- 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.

Figure 5. Kingfisher Duo method program.



REFERENCES

1. Thermo Scientific Application Note 21682, High-precision, automated peptide mapping of proteins.

TRADEMARKS/LICENSING

ThermoFisher SCIENTIFIC

PO21860-EN 0718S