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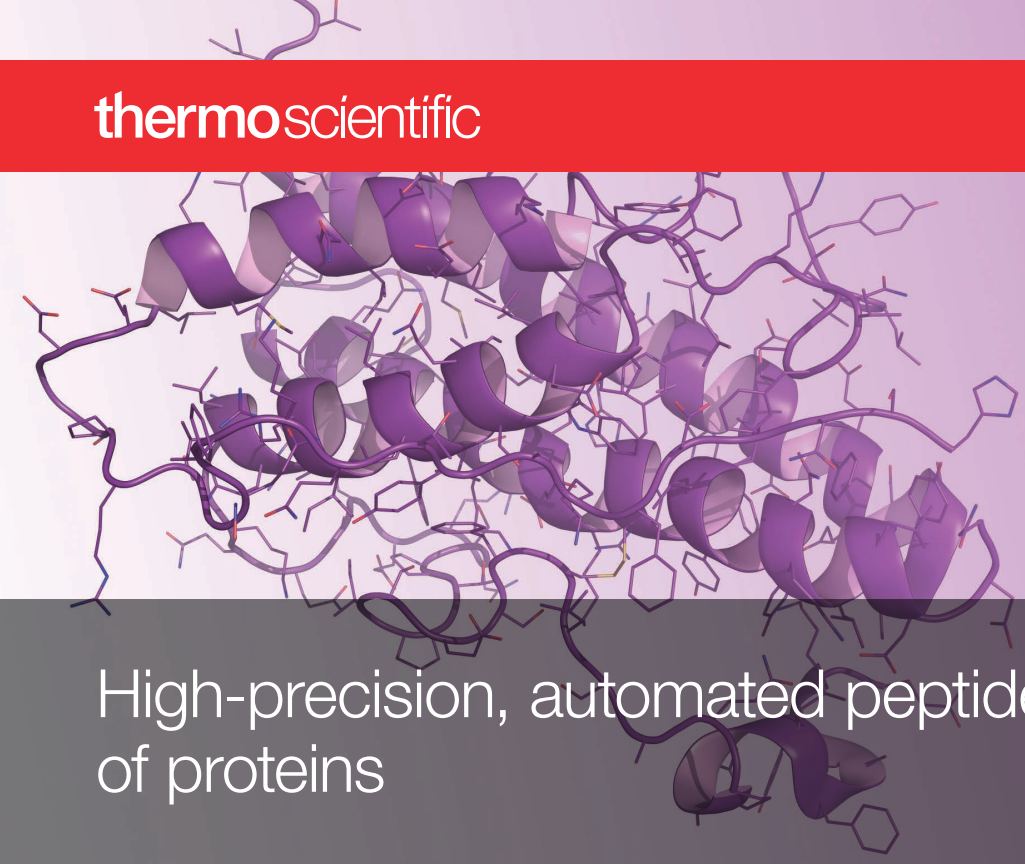

High-precision, automated peptide mapping of proteins

Authors

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Keywords

NIBRT, Biopharmaceutical, QA/QC, Critical quality attribute, Peptide mapping, Cytochrome c, Recombinant somatotropin, Infliximab, Rituximab, Carbonic anhydrase, Hypersil GOLD, Magnetic SMART Digest, KingFisher, Vanquish Horizon UHPLC, Q Exactive Plus MS

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.

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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)
- 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).
- 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.

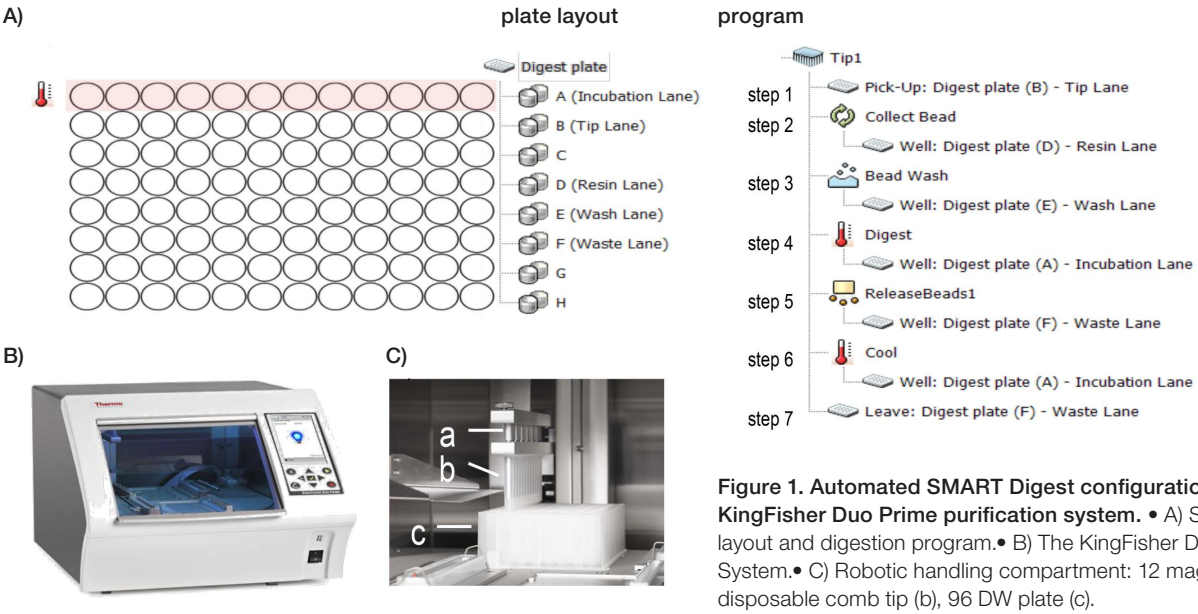


Figure 1. Automated SMART Digest configuration using the KingFisher Duo Prime purification system. • A) Schematic of plate layout and digestion program.• B) The KingFisher Duo Prime Purification System.• C) Robotic handling compartment: 12 magnetic rods (a), disposable comb tip (b), 96 DW plate (c).

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

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

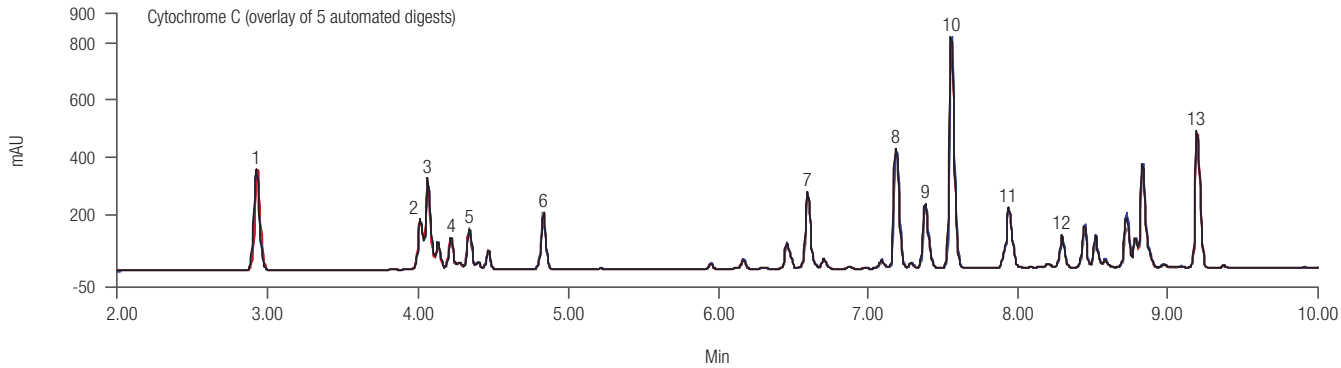
Resolution (Full MS) at *m/z* 200 (FWHM):	70,000
Resolution (MS2) at *m/z* 200 (FWHM):	17,500
Top-N MS2:	5
S-lens RF level:	60
Max inject time:	100 ms
Collision energy (CE):	27

Data processing and software

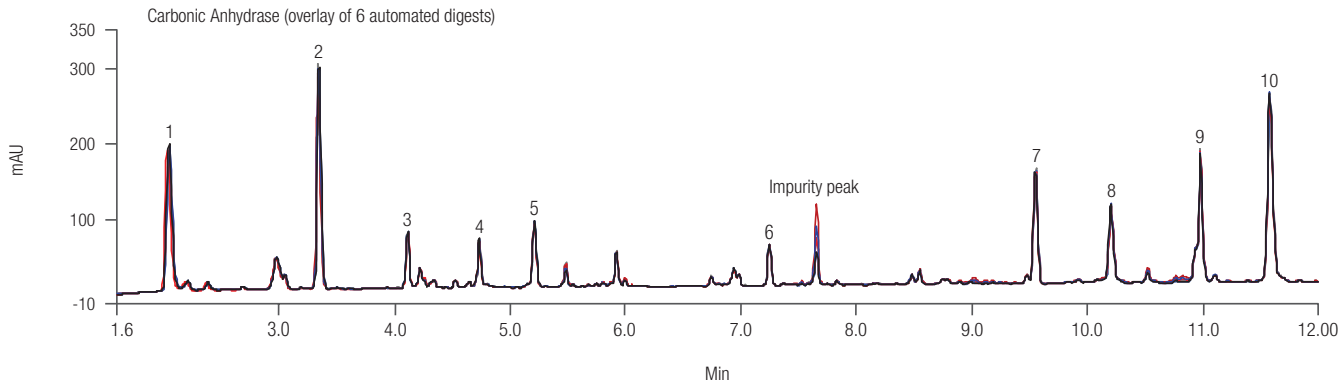
Chromatographic software:	Chromeleon CDS 7.2 SR4
MS data acquisition:	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
Protein characterization software:	Thermo Scientific™ BioPharma Finder™ 2.0 software

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.



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

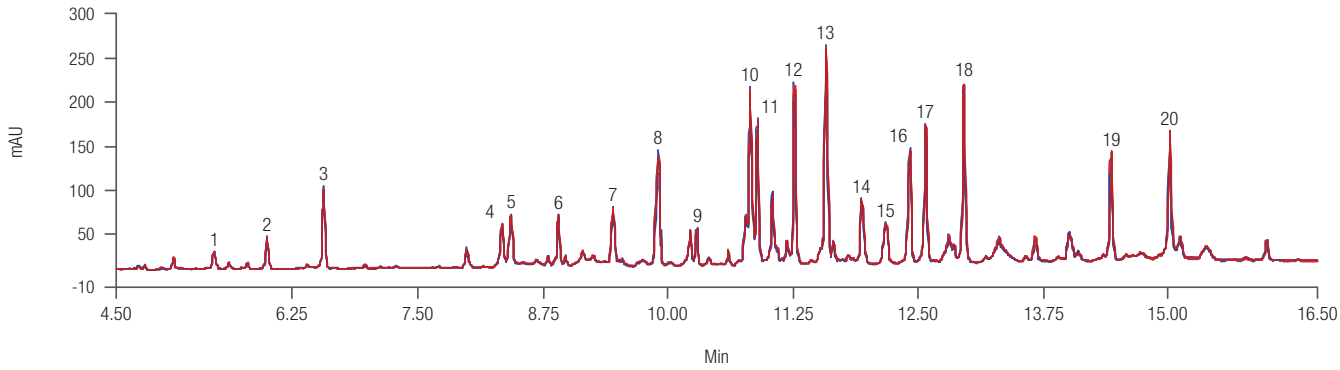


Peak	1	2	3	4	5	6	7	8	9	10
%RSD (A_{rel})	2.12	1.46	1.53	3.03	1.71	1.26	1.51	2.60	1.14	1.64
%RSD (t_R)	0.32	0.1	0.03	0.04	0.02	0.02	0.01	0.01	0.01	0.01

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.

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.



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.

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.

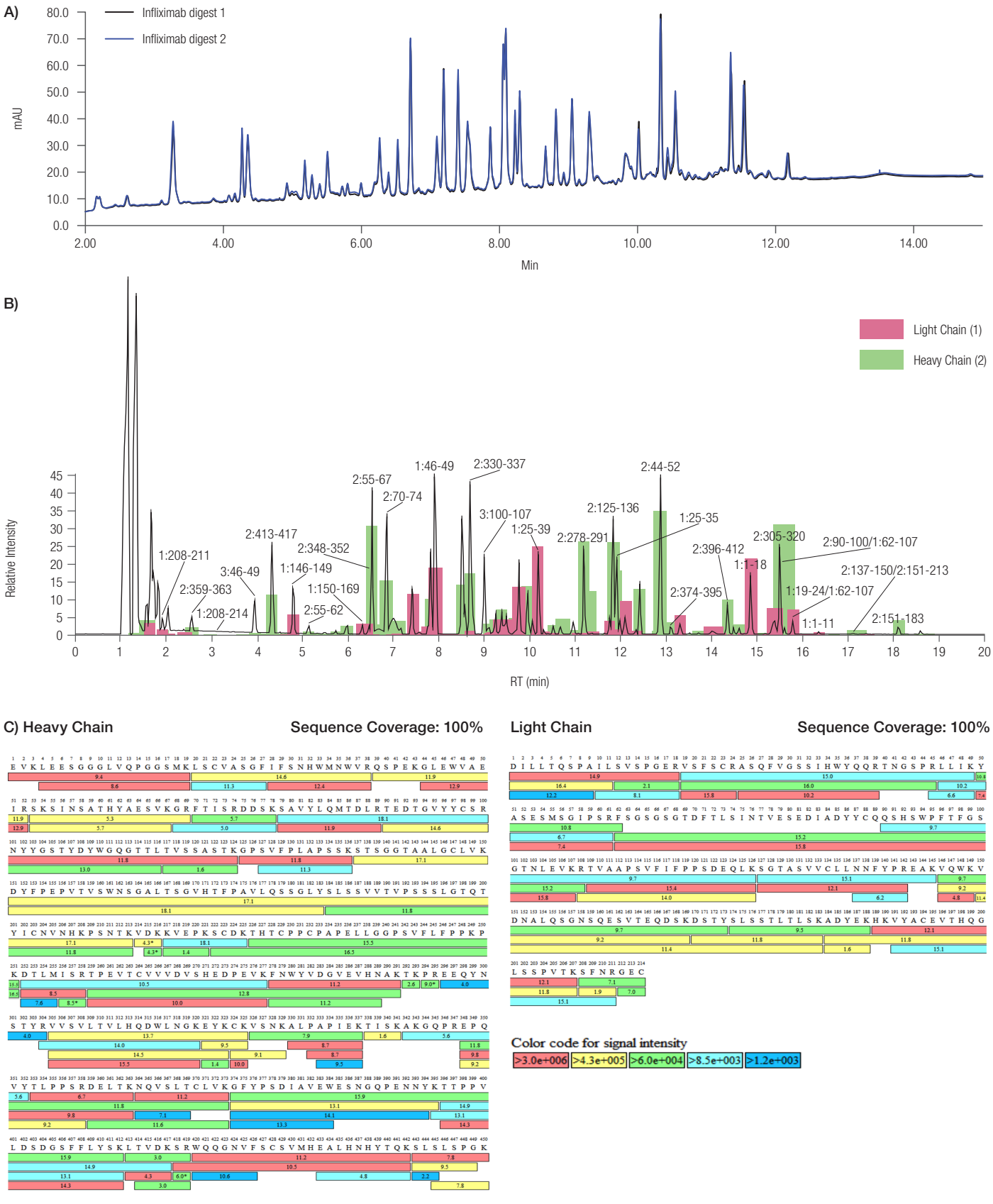


Figure 4. Automated digestion of infliximab drug product using SMART Digest magnetic resin. Panel A: Overlaid peptide maps for two digests of infliximab antibody. Digest solutions of 5 µL were injected without further purification and peptides were separated using separation conditions listed in Table 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.

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).

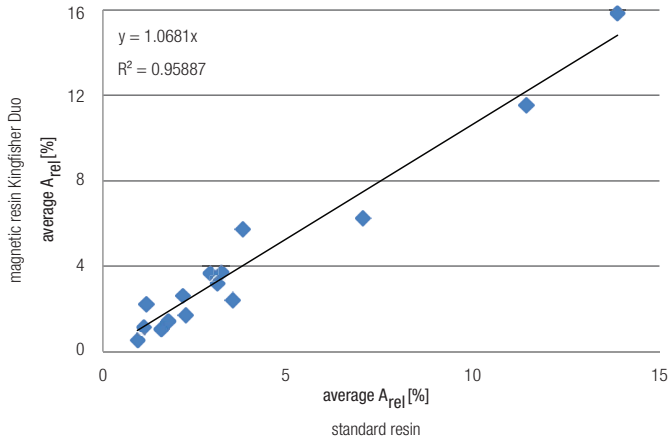


Figure 5. Linear regression curves comparing the peak area results from an automated digestion using the SMART Digest magnetic kit to a manual digestion using the SMART Digest kit.

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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