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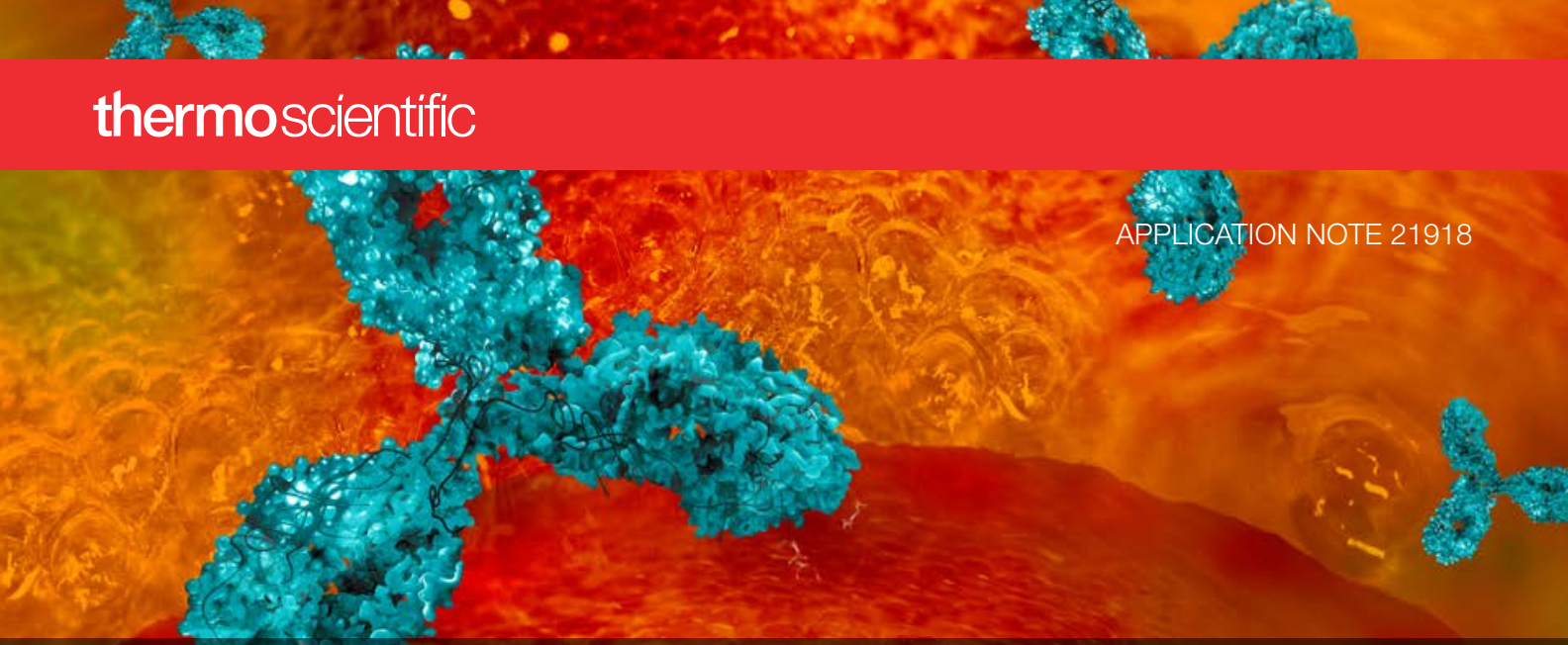

Easy, fast and reproducible analysis of host cell protein (HCP) in monoclonal antibody preparations

Authors

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Keywords

NIBRT, biotherapeutics, CQAs, host cell proteins (HCP), cathepsin L, monoclonal antibodies (mAbs), charge variant analysis (CVA), peptide mapping, bottom-up, Magnetic SMART Digest kit, KingFisher Duo Prime Purification system, Vanquish Flex Binary UHPLC system, Acclaim VANQUISH C18 UHPLC columns, high-resolution accurate mass (HRAM)

Application benefits

- Improved reproducibility and reduced sample preparation time with automated digestion for host cell protein (HCP) analysis
- Easy HCP analysis using the same workflow optimized for standard peptide mapping analysis of monoclonal antibodies
- High confidence in results with excellent data quality using a Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.1 × 250 mm column for HCP and peptide mapping analysis
- Detection of low abundance HCP proteins using automated magnetic bead digestion technology that facilitates excellent recovery of samples with less sample handling, increased reproducibility, and improved reliability of data

Goal

To perform host cell protein analysis using an automated, rapid digestion method based on the Thermo Scientific™ SMART Digest™ kit on a Thermo Scientific™ KingFisher™ Duo Prime purification system. Sample analysis is performed by LC-MS on the Thermo Scientific™ Vanquish™ UHPLC coupled to the Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. To show the flexibility of the automated platform that can be employed for both peptide mapping analysis and HCP characterization.

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To investigate how HCPs could interfere with drug product stability and highlight the importance of monitoring these contaminants along the bioprocess.

Introduction

Chinese hamster ovary (CHO) cells are the most common cell lines used for therapeutic drug production.[1,2] As the biopharmaceutical industry continues to expand, the global demand for monoclonal antibodies (mAbs) and recombinant proteins is increasing. MAbs are produced by recombinant DNA-technology using a non-human host cell.[3,4] Biomanufacturing of these molecules is divided into upstream and downstream unit operations.[4] After cellular expression, the drug substance is purified from cell debris and media components by depth filtration. A considerable number of proteins expressed by the host cell may still be present, and their removal is performed by a number of specific chromatographic polishing steps, such as protein A/G affinity chromatography.[5] The majority of HCPs are removed during these chromatographic purification steps, but some may still be present at very low concentrations in the final drug product, possibly interfering with the product activity and/or stability, and potentially compromising patient safety. The most popular methods for HCP detection are process-specific enzyme linked immunosorbent assays (ELISAs) and protein gel blots.[6,7] Both of these analytical techniques use a semi-quantitative approach that is targeting only a small set of expected proteins. This is bearing the risk of missing any unexpected or unknown protein that might still be present in the final drug product.[8] In addition, some HCPs may be present at levels that exceed the specific anti-HCP antibody available for binding (antigen excess), which would underestimate the amount of those HCPs in the sample.[9]

Consequently, it is a regulatory requirement to monitor HCP impurities in therapeutic mAbs using sensitive analytical methods. LC-MS/MS is a powerful method that facilitates both quantitative and qualitative analysis at these required levels of sensitivity.[10-12]

In this study, we present an LC-MS/MS approach for the determination of HCPs in commercially available adalimumab mAb drug product using the same workflow employed for peptide mapping analysis, without compromising chromatographic resolution or MS sensitivity. HCP analysis allowed us to determine a possible cause of sample instability as observed during charge variant analysis using normal and accelerated aging samples. Fragments were detected that could be derived not only from non-enzymatic hydrolysis but also from enzymatic breakdown. To verify that the fragments could be derived from protease activity caused by a residual host cell protease present in the drug product we performed trypsin digestion and peptide mapping. The SMART Digest kit with magnetic resin was utilized with the KingFisher Duo Prime purification system to automate the digestion. After assessing the performances of our workflow with respect to a standard peptide mapping protocol, we deliberately overloaded the Acclaim VANQUISH column to boost the overall assay sensitivity to enable the analysis of low abundant residual proteins present in the final product formulation.

The efficiency of our workflow was showcased by the identification of 51 host cell proteins present in the mAb product, including the candidate protease contaminant thought to be responsible for the observed fragmentation of the monoclonal antibody.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ•cm conductivity
- 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)
- SMART Digest Trypsin Kit, Magnetic bulk resin option (P/N 60109-101-MB)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 95040450)
- Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm column (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)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Peptide mapping and host cell protein sample preparation

For peptide mapping and host cell protein analysis, samples were run in triplicate. For each analysis different sample concentrations were used: 20 mg/mL for HCP and 2 mg/mL for peptide mapping analysis. Sample and buffer were added to each lane of a KingFisher Deepwell 96 well plate as outlined in Table 1. This correlates to 1 mg protein for the HCP sample and 100 µg for standard peptide mapping in each well. 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 2. Samples were incubated for 45 minutes at 70 °C at medium mixing speed (to prevent sedimentation of beads), with post digestion cooling carried out to 10 °C. Following digestion, disulfide bonds were reduced with 10 mM DTT for 20 minutes at room temperature. All samples were adjusted to 0.1% formic acid. A volume equal to 5 µg and 160 µg of digested protein was loaded on column for peptide mapping and host cell protein analysis, respectively.

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 (2/20 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 digestion 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

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.	MeOH/H_2O, 10:90
Needle wash:	Enabled pre-injection
Gradient:	See Table 3 for details

Table 3. 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

MS conditions

Detailed MS method parameters are shown in Tables 4 and 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 arbitrary units
Auxiliary gas flow	10 arbitrary units
Probe heater temperature	400 °C
Source voltage	3.8 kV
Capillary temperature	300 °C
S-lens RF level	50

Table 5. MS method parameters utilized for peptide mapping analysis

General	Setting	MS^2 Parameters	Setting
Runtime	0 to 65 min	Resolution setting	17,500
Polarity	Positive	AGC target value	1.0×10^5
Full MS parameters		Isolation width	2.0 *m/z*
Full MS mass range	200–2000 *m/z*	Signal threshold	1.0×10^4
Resolution setting	70,000	Normalized collision energy (NCE)	28
AGC target value	3.0×10^6	TopN MS^2	5
Max. injection time	100 ms	Max. injection time	200 ms
Default charge state	2	Fixed first mass	–
In-source CID	0 eV	Dynamic exclusion	7.0 s
Microscans	1	Loop count	5

Table 6. Thermo Scientific™ Biopharma Finder™ 3.0 software parameter settings for peptide mapping data analysis

Component Detection	Setting
Absolute MS signal threshold	1 × 10^6 counts
Typical chromatographic peak width	0.3 min
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 (N) Deamidation (Q) Glycation (K) Oxidation (MW) Isomerization (D)

Host cell protein analysis

HCP analysis data were searched against the *Cricetulus griseus* database from UniProt using PEAKS Studio 7.5 (Bioinformatics Solution Inc., Waterloo, ON, Canada). The parameter mass error tolerance was set to 10 ppm and fragment mass error tolerance was set to 0.01 Da, allowing up to one missed cleavage. Oxidation and deamidation were included as variable modifications and a false discovery rate cut-off of 1% was applied.

Evaluation of chromatographic performance

For chromatographic performance evaluation at different sample loads on the same column, Thermo Scientific™ Chromeleon™ CDS 7.2.9 was used to asses critical parameters such as resolution, asymmetry, and selectivity factor.

Results and discussion

Characterization of mAb drug product via charge variant analysis-mass spectrometry (CVA-MS)

Charge variant analysis (CVA) is commonly used to monitor the surface charge heterogeneity during mAb production from upstream to downstream processing.[13] Due to the high salt content of the mobile phases usually employed for CVA it has been difficult to hyphenate to mass spectrometric detection. Recently, a CVA-MS approach was reported using MS-compatible, volatile buffers to generate a pH gradient able to resolve the charge variants present in a mAb. This approach was used to investigate the drug product profile shown in Figure 1. The complete method for this analysis is described in Reference 13.

The MS data acquired enabled confident annotation of mAb charge variants including lysine truncation, deamidation, isomerization, and succinimide containing variants. Beside the different proteoforms corresponding to intact species, the presence of low molecular weight species was also investigated by filtering the spectra with a narrowed mass range of 2500 to 5000 *m/z*.[13] The analysis revealed three peaks containing lower molecular weight species (Figure 1B, zoom insert). Analysis and deconvolution of the charge envelope of the three peaks, highlighted by the red squares, resulted in the identification of four species corresponding to mAb fragments derived from hydrolysis of two distinct sites within the primary sequence.

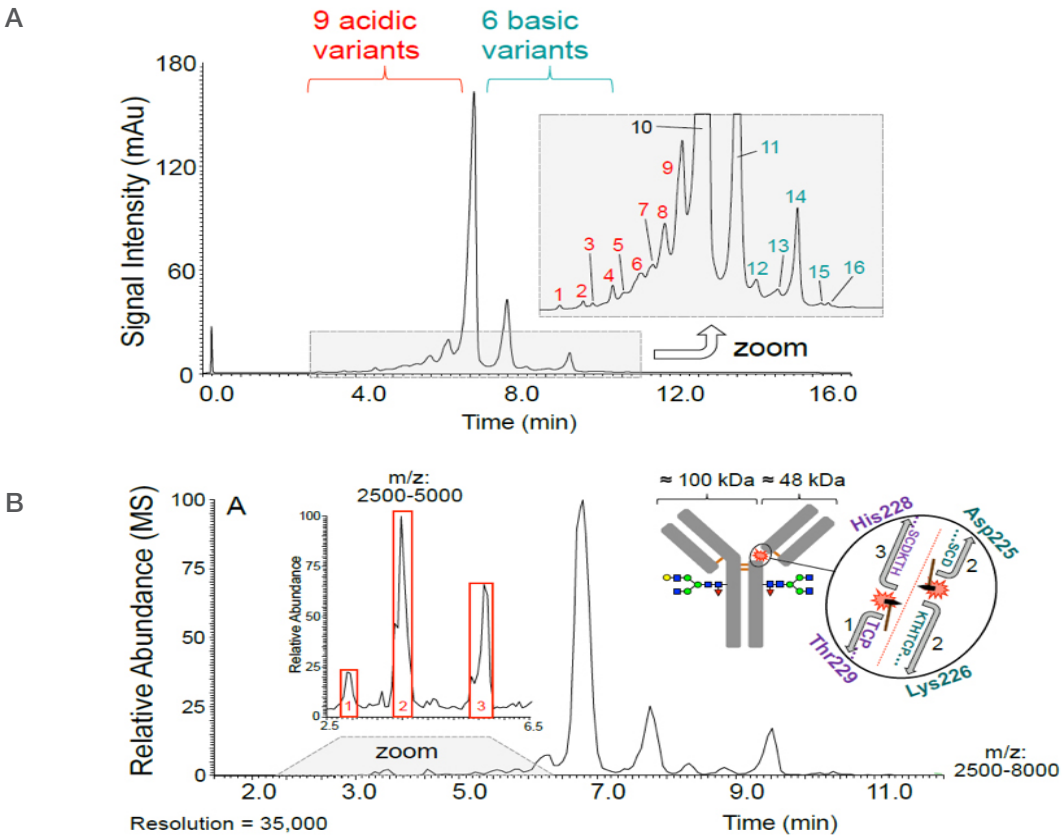

Figure 1. (A) UV-chromatogram of the charge variant separation of the analyzed mAb drug product. Low molecular weight species separated are shown in the zoom window. (B) Base peak chromatogram acquired at the intact protein level at a resolution setting of 35,000. Fragments present in the acidic region of the mAb are shown in red squares. The full-size total ion chromatogram (TIC) represents all species detected across the entire mass range of 2500 to 8000 *m/z*, whereas the insert represents species detected in the mass range from 2500 and 5000 *m/z*. The His-Thr site shown in purple has also been reported as a specific cleavage site of cathepsin L1.

Peptide mapping analysis

To investigate the potential role of residual HCPs with specific protease activity as the cause of fragmentation, we performed HCP analysis on the mAb drug product using an LC-MS/MS approach based on a standard peptide mapping workflow. The digestion of a highly concentrated sample was possible without changing our workflow due to the high trypsin load on SMART Digest magnetic beads. The sample load on column for standard peptide mapping analysis is 5 µg. Here, the column was loaded with 160 µg of digested mAb to increase the sensitivity for low abundant peptides.

Overlaid chromatograms of replicate injections of the resulting base peak chromatograms derived from the 5 µg and 160 µg mAb digest column loads are displayed in Figure 2A-B. To compare the performance of the high load protocol with respect to our standard workflow we considered several chromatographic and MS parameters such as peak area, peak resolution, and asymmetry. Excellent reproducibility was observed across all replicates, demonstrating reliability of the chromatographic separation and high flexibility for HCP analysis. For both sample loads 100% sequence coverage was obtained for the mAb heavy and light chains. We also investigated the number of identified peptides. As expected, we noticed, increased numbers of peptides were identified in the HCP sample (Figure 2C). In more detail, we observed the relative percentage of MS peak area showed small changes in both light and heavy chains for both analysis (Figure 2C). In order to evaluate the efficiency of chromatography performance between a large amount of sample loaded with respect to our standard workflow, we measured several chromatography parameters such as resolution (European Pharmacopeia, EP), asymmetry (EP), and selectivity Factor (α) using Chromeleon CDS 7.2.9 (Figure 2D). Our results show the chromatography resolution efficiency slightly reduced between 20% and 30% during HCP analysis due to the large amount of sample loaded into the column. Despite the large quantity of material injected on to the Acclaim Vanquish C18 reversed phase column, the chromatographic performance remained efficient and demonstrated excellent repeatability.

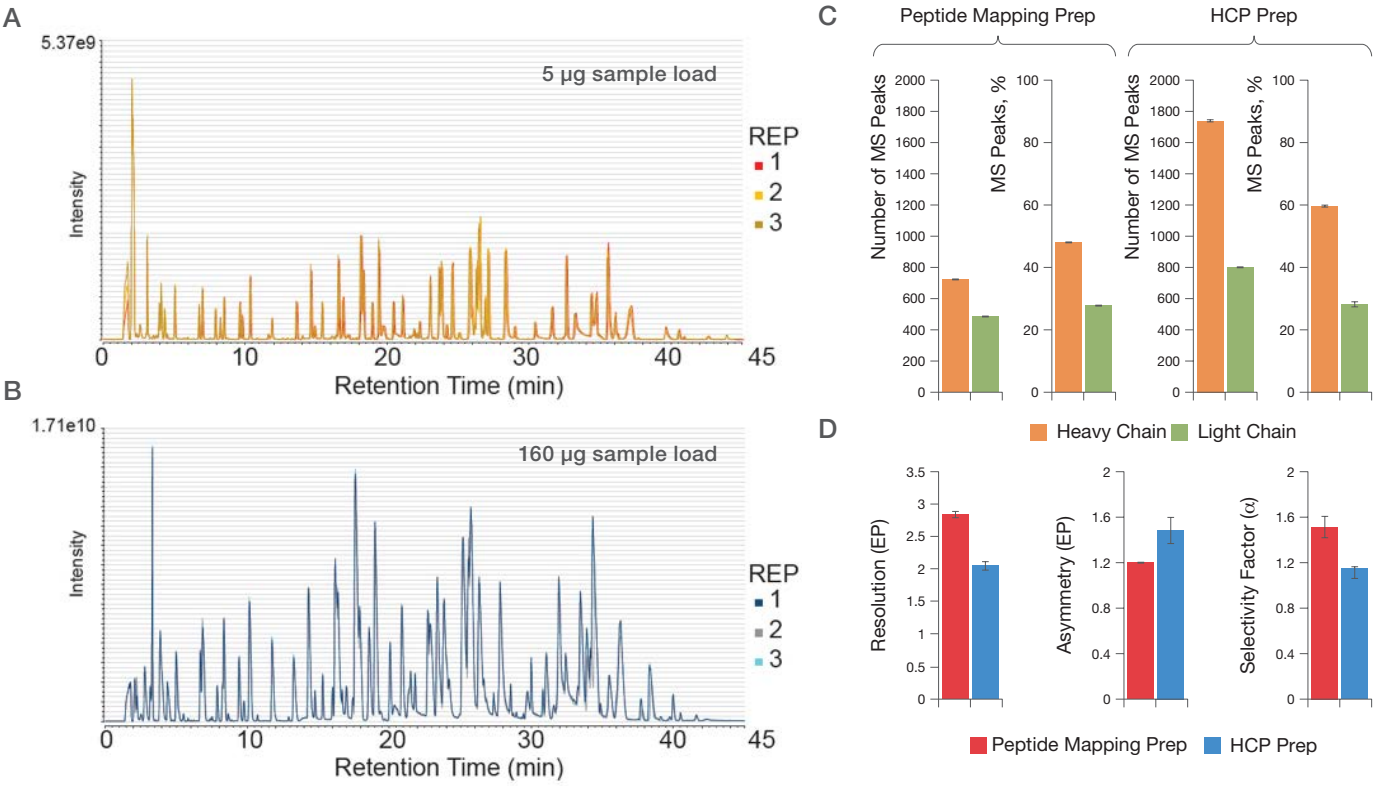

Figure 2. Overlay base peak chromatograms (BPCs) from triplicate injections of the tryptic digest separation with 5 µg sample loading (A) and 160 µg sample loading (B) for HCP and standard peptide mapping analysis. (C) Comparison of MS data for HCP and peptide mapping analysis. Total number of MS peaks detected and relative MS peak area percentage for heavy (orange bars) and light chain (green bars). (D) Chromatographic peak analysis carried out by Chromeleon CDS 7.2.9 providing resolution (EP), asymmetry (EP), and selectivity (α) obtained for peptide mapping analysis with 5 µg sample load (red bars), and HCP analysis based on 160 µg sample load (blue bars).

Investigation of HCPs using a peptide mapping approach

The HCP analysis identified 51 different HCPs, six of which were identified based on two or more unique peptides.[13] From all proteins identified, we found one protein with catalytic activity; cathepsin L1 is a protease with specific activity for the His-Thr motif, which corresponds to the hydrolysis site observed for the fragments identified by CVA-MS analysis. The UniProt database lists cathepsin L1 as an important enzyme for protein degradation in lysosomes. The relative amounts of cathepsin L1 could be crucial for mAb stability and shelf life. Using label free quantitation, we found the concentration of cathepsin L1 in the drug product to be in the range of ~4 ppm (ng HCP per mg therapeutic protein), shown in Table 8. The relative extracted ion chromatograms (XIC) for the three peptides that the identification of cathepsin L1 is based on are shown in Figure 3A-B. Related MS/MS spectra with assigned b- and y-ions for the cathepsin L1 peptides are displayed in Figure 3C.

Table 8. List of CHO proteins identified with at least two unique peptides in a peptide mapping based database search. Quantitation was performed by spiking with rabbit phosphorylase B derived peptides.

Accession	# of Unique Peptides	Confidence Score	Description	ppm (ng HCP/mg Therapeutic Protein)
tr|G3INC5	3	207.4	Cathepsin L1 OS=*Cricetulus griseus* GN=I79_025440 PE=3 SV=1	3.9
tr|G3GR64	2	112.8	Inter-alpha-trypsin inhibitor heavy chain H5 OS=*Cricetulus griseus* GN=I79_000007 PE=4 SV=1	3.2
tr|G3HUA1	2	110.59	Cationic trypsin-3 OS=*Cricetulus griseus* GN=I79_014509 PE=4 SV=1	32.7
tr|G3HUU6	3	286.82	Protein S100-A11 OS=*Cricetulus griseus* GN=I79_014714 PE=4 SV=1	4.5
tr|G3IHL5	2	110.57	Zinc finger and SCAN domain-containing protein 2 OS=*Cricetulus griseus* GN=I79_023307 PE=4 SV=1	0.8
tr|G3IMJ0	2	228.5	Putative uncharacterized protein (Fragment) OS=*Cricetulus griseus* GN=I79_025130 PE=4 SV=1	136.5

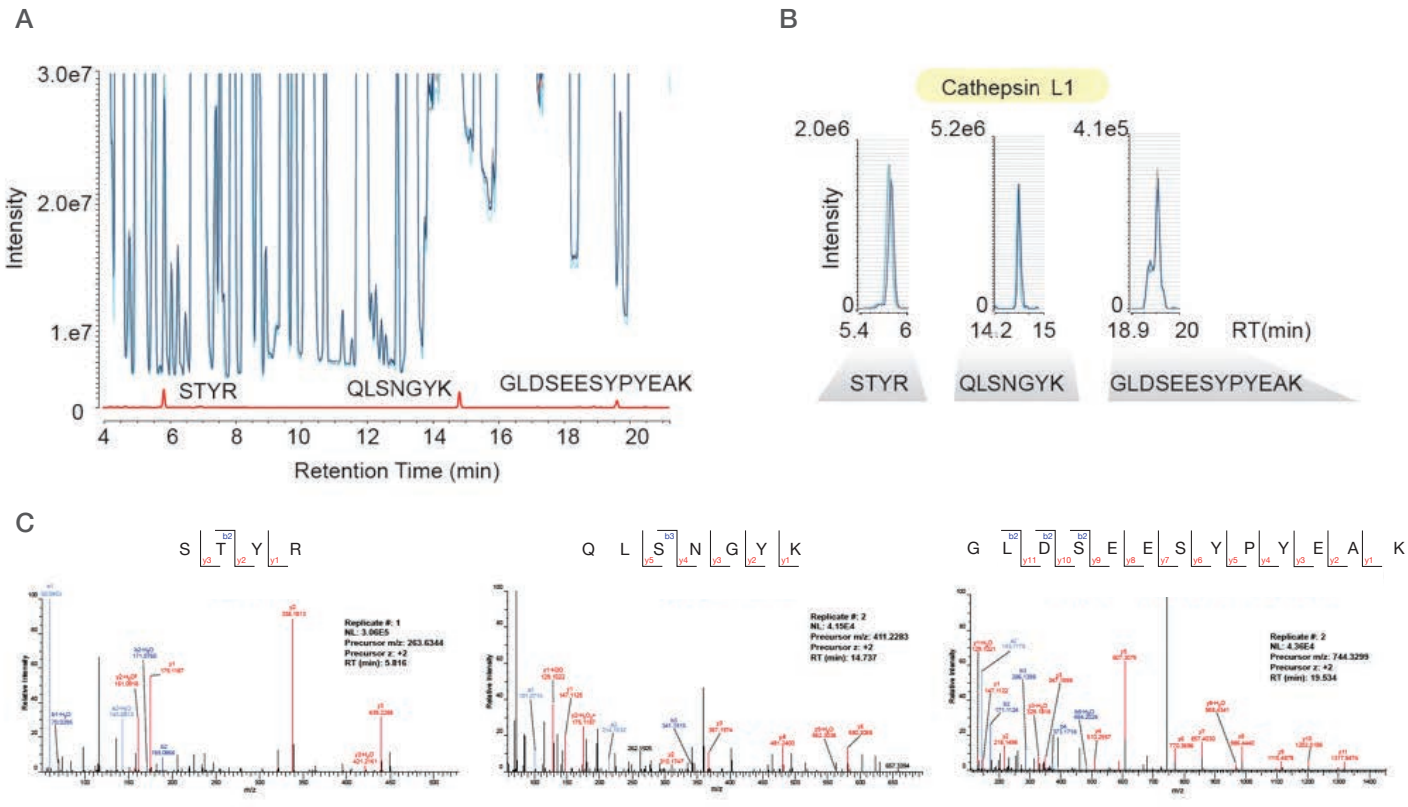

Figure 3. Overlay of the base peak chromatograms (BPCs) from triplicate injections with a 160 µg sample load each used for HCP analysis. A combined extracted ion chromatogram representing the three unique peptides of cathepsin L1 are highlighted by the red trace to indicate their low level of abundance. (B) Extracted ion chromatograms (XICs) for the identified unique cathepsin L1 peptides. (C) MS/MS spectra and fragment ion assignment in blue (a-, b-ions) and red (y-ions) for the peptides "STYR", "QLSNGYK", and "GLDSEESYPYEAK" that were used to identify cathepsin L1 in the mAb drug product.

Conclusions

- The Magnetic SMART Digest kit used with the KingFisher Duo Prime system provides simple, rapid, and highly reproducible digestion of monoclonal antibodies. This has the capacity for the digestion of the large amounts of sample required for host cell protein analysis.

- The Acclaim Vanquish C18 column on the Vanquish Flex UHPLC system provides excellent performance in terms of reproducibility of the peptide separation and stability of the column. High adaptability was observed when switching from HCP analysis to peptide mapping standard workflow on the same analytical platform.

- Deep analysis of HCPs enabled the identification of 51 proteins, including cathepsin L1. Six HCPs were identified based on at least two peptides.[13] Cathepsin L1 may contribute to drug product degradation through hydrolysis at specific His-Thr site in the upper hinge region. This emphasizes the importance of HCP monitoring to maintain product potency and stability over time.

- The Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer has delivered excellent mass accuracy and superb data quality that is very well suitable for host cell protein analysis requiring high sensitivity.[13]

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