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Confident LC-MS Identification of Low ppm Host Cell Proteins (HCPs) in Biotherapeutic Monoclonal Antibodies

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ABSTRACT

Purpose: An improved approach for identification of low ppm HCPs in biotherapeutics using non-denaturing tryptic digestion, efficient chromatographic separation, sensitive high-resolution accurate-mass mass spectrometry (HRAM-MS) detection, and advanced data processing.

Methods: Monoclonal antibody (mAb) biotherapeutic, digested under non-denaturing conditions, was separated by reversed-phase UHPLC. Data were acquired with a Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer, followed by processing and confident HCP identification using Thermo Scientific™ Proteome Discoverer™ 2.4 Software.

Results: Non-denaturing protein digestion facilitates detection of HCPs by exploiting the resistance of native mAb protein structure to proteolysis. Over 100 NISTmAb HCPs were confidently identified with two or more peptides, in at least two out of three replicate analyses.

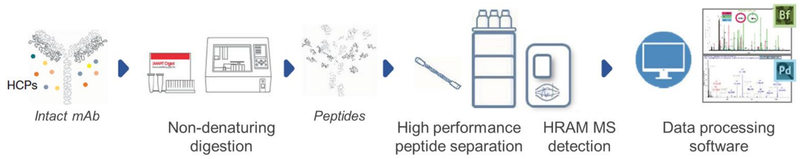
INTRODUCTION

Most mAb-based biotherapeutics are produced using mammalian host cell-lines. Any host cell proteins also synthesized by these cells, that are present in the final drug product, are process-related impurities and therefore critical quality attributes (CQAs). To minimize detrimental effects that could be caused by HCPs, there are multiple purification steps post-harvest, with the aim of reducing HCP levels in the final drug product to a point considered acceptable to regulatory authorities.

The large intrasample dynamic range between HCPs and the active drug product can restrict comprehensive identification by LC-MS. However, incorporating a non-denaturing digestion into the analysis workflow can facilitate identification of low abundance HCPs: Proteolysis of the mAb is limited, thus reducing the dynamic range of the sample introduced for LC-MS analysis.[1]

The Orbitrap Exploris 480 MS provides high sensitivity and faster acquisition rates for improved HCP identification. Increasing the MS^2 isolation window, in combination with a high resolution setting, promotes peptide ion co-isolation and enables acquisition of well resolved chimeric spectra.

MATERIALS AND METHODS



Sample Preparation

NIST monoclonal antibody (NISTmAb) reference material (RM 8671) was concentrated to 80 µg/µL using 3 kDa cut-off spin filters and spiked with a known amount of reference proteins to be used for label-free quantification. 1.44 mg was digested with 10 µL Thermo Scientific™ SMART Digest™ magnetic trypsin beads over 3 hr at 37˚C using an automated purification system. Undigested protein was removed *via* precipitation at 95˚C for 10 min, followed by centrifugation at 14,000 xg. Peptides in the recovered supernatant were reduced with 1 µL Thermo Scientific™ Bond-Breaker™ TCEP Solution.

Test Method

Peptides were separated using a Thermo Scientific™ Acclaim™ VANQUISH™ C18 UHPLC column (2.1 x 250 mm, 2.2 µm) at 60˚C with a Thermo Scientific™ Vanquish™ Horizon UHPLC System: A flow rate of 0.3 mL/min was used, over a 90 min linear gradient (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile)

The Orbitrap Exploris 480 MS was used for data-dependent acquisition (dd-MS^2, Top15) with resolution settings of 120,000 MS^1 and 30,000 for MS^2. The MS^2 isolation window was 4 *m/z*.

Data Analysis

Proteome Discoverer 2.4 software was used for HCP identification. Precursor Detector node was included to process chimeric MS^2 spectra. Peptide Filter node excluded low confidence peptide spectral matches (PSMs) and required a minimum of two peptides per protein for identification. The SEQUEST-HT search algorithm was used, with the UniProt *Mus musculus* database, plus NISTmAb heavy and light chain sequences. A 5 ppm precursor mass tolerance was set, with a 0.02 Da fragment mass tolerance. Precursor Ions Quantifier node was used for TopN label-free quantification of protein abundances.

RESULTS

Non-denaturing digest optimization and reproducibility

For SMART Digest, high temperature is typically used to denature the proteins to aid proteolysis. For a non-denaturing digest the temperature was reduced to 37˚C, resulting in a significant reduction in the level of mAb digestion (Fig. 1). The automated non-denaturing digestion is highly consistent, which is further highlighted by the reproducible separation using the Vanquish Horizon UHPLC system and Acclaim VANQUISH C18 UHPLC column (Fig. 2).

Figure 1. Effect of SMART Digest temperature on mAb digestion

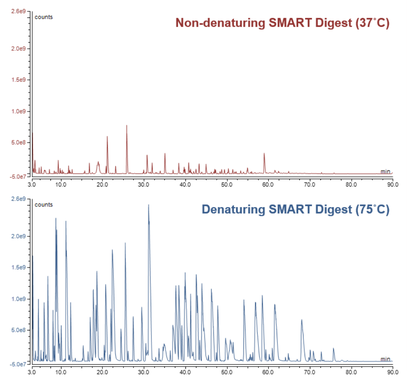
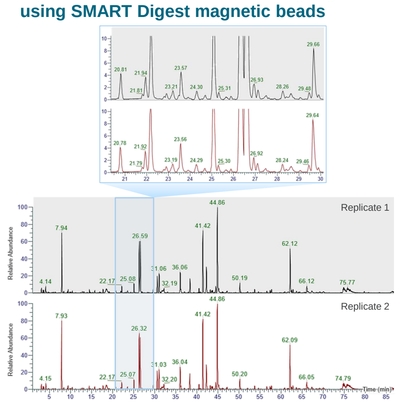


Figure 2. Replicate non-denaturing digests using SMART Digest magnetic beads



NISTmAb HCP Identifications

During this study, over 100 HCPs were identified in NISTmAb with at least two peptides, in at least two out of three replicate injections (Fig. 3). HCPs spanning the previously referenced published abundance range[1] were identified from 222 ppm (fructose-bisphosphate aldolase A) to <0.5 ppm (malate dehydrogenase, serine/arginine-rich splicing factor 1, cystatin-C).

The accurate mass of precursor ions (typically less than 2 ppm) plus excellent MS2 spectral quality provided by the Orbitrap Exploris 480 mass spectrometer (Fig. 4) further increases the confidence in HCP identifications, even for proteins present at very low abundance. The excellent sensitivity and dynamic range capabilities also facilitate the identification of multiple peptides per protein.

Figure 3. High confidence NISTmAb HCP identifications

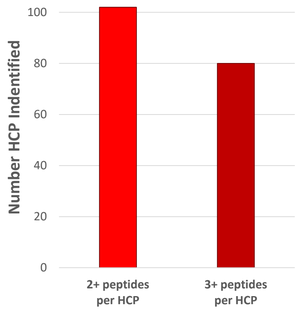
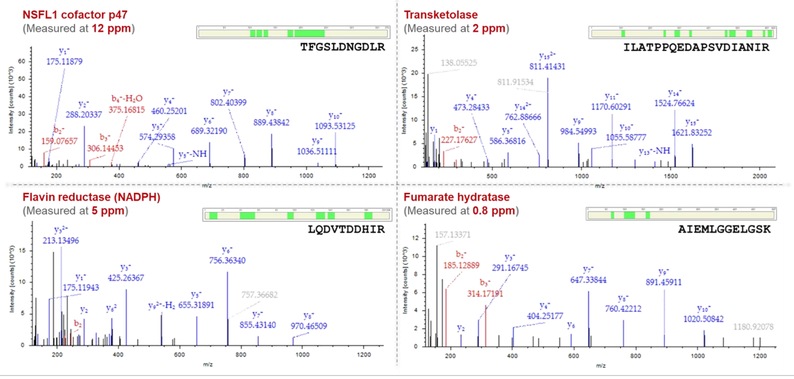


Figure 4. Representative MS^2 spectra highlighting spectral quality, even for low abundant HCPs



Label-free quantification of NISTmAb HCPs

The average abundance values from triplicate injections, calculated by Proteome Discoverer software, for three spiked proteins were used to determine the measured ppm value for HCPs identified in this study. For each HCP, the average abundance from triplicate injections was calculated as a ratio to the corresponding value for each spiked protein, allowing the amount of each HCP (in ng on-column) to be calculated from the known amount (ng on-column) per spiked protein.

For the final measured ppm, the calculated amount of each HCP (ng) was related to the total NISTmAb amount in the digest (mg) to give ng HCP per mg mAb. These values have been compared to measured ppm values from previously published studies of NISTmAb (Table 1).

Table 1. Measured ppm (ng HCP: mg mAb) for a subset of identified HCPs

HCP	Peptides	Measured ppm	Measured ppm (Huang *et al.*)	Measured ppm (Doneanu *et al.*)
Fructose-bisphosphate aldolase A	36	**210**	222	116
Beta-2-microglobulin 1	5	**42**	12 (1 peptide)	7
Glucose-6-phosphate isomerase	29	**36**	20	12
NSFL1 cofactor p47	11	**12**	3	7
Syntaxin-12	9	**7**	4	-
Adenylate kinase 2	9	**6**	2	4
Heterogeneous nuclear ribonucleoprotein A1	12	**6**	4	-
Flavin reductase (NADPH)	6	**5**	3	2
Serine/arginine-rich splicing factor 7	5	**4**	2	5
Prostaglandin reductase 1	5	**3**	5	3
Granulins	3	**3**	-	-
Polypeptide N-acetylgalactosaminyltransferase 2	15	**2**	3	-
Transketolase	12	**2**	1	5
Malate dehydrogenase	6	**1**	<0.5	-
Polyadenylate-binding protein 1	6	**1**	-	-
Semaphorin-7A	6	**0.9**	1 (2 peptides)	-
Fumarate hydratase	5	**0.9**	-	-
Serine/arginine-rich splicing factor 1	4	**0.8**	<0.5 (2 peptides)	-
IgE-binding protein	2	**0.3**	1 (2 peptides)	-
Clathrin light chain B	2	**0.2**	-	-

CONCLUSIONS

- For non-denaturing SMART Digest, the lower temperature minimizes the level of mAb digestion and therefore reduces the dynamic range of the sample
- Successful LC-MS analysis of low abundant HCPs with analytical flow UHPLC
- Improved sensitivity and the increased dynamic range capabilities of the Orbitrap Exploris 480 MS increased HCP detection. Over 100 HCPs were reproducibly identified at sub-1 ppm levels, with identification requiring HCP to be present in at least 2 out of 3 replicate injections, with at least two peptides identified per protein
- Advanced data processing algorithm in Proteome Discoverer 2.4 ensure confidence in HCP identifications. A new node, Precursor Detector, is able to identify proteins from chimeric spectra acquired with wider isolation windows.

REFERENCES

1. Huang, *et al.*, A Novel Sample Preparation for Shotgun Proteomics Characterization of HCPs in Antibodies, *Anal. Chem. 89, 10, 5436-5444,* 2017
2. Doneanu *et al.*, Enhanced Detection of Low-Abundance Host Cell Protein Impurities in High-Purity Monoclonal Antibodies Down to 1 ppm Using Ion Mobility Mass Spectrometry Coupled with Multidimensional Liquid Chromatography, *Anal. Chem. 87, 20, 10283-10291,* 2015

TRADEMARKS/LICENSING

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