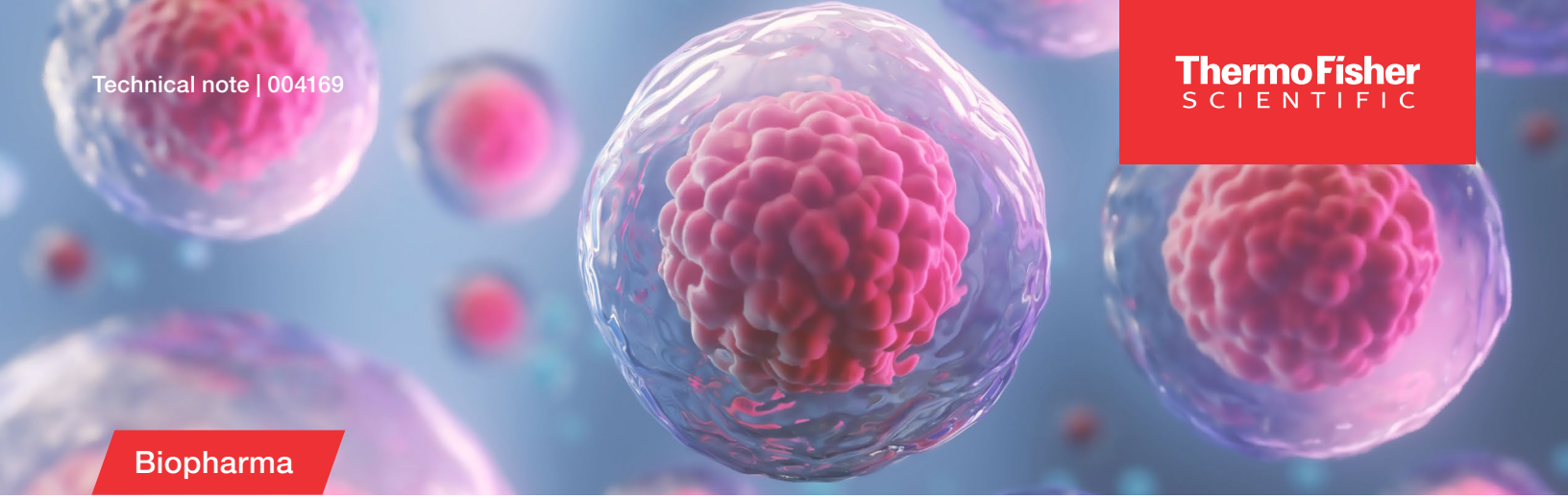

Technical note | 004169

Biopharma

High-sensitivity host cell protein (HCP) analysis on an Orbitrap Excedion Pro Biopharma hybrid mass spectrometer

Authors

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Keywords

HCP, Orbitrap Excedion Pro Biopharma hybrid mass spectrometer, NISTmAb, native digestion, Proteome Discoverer software

Goal

To demonstrate the capability of the new Thermo Scientific™ Orbitrap™ Excedion™ Pro Biopharma Hybrid Mass Spectrometer coupled with the Thermo Scientific™ Vanquish™ Horizon UHPLC System for highly sensitive HCP identification.

Introduction

Host cell proteins (HCPs) are a diverse group of proteins derived from host cell lines used in the production of recombinant biopharmaceutical products.[1] These unintended protein impurities can co-purify with the therapeutic protein during the manufacturing process. HCPs originate from the host cell itself, including enzymes, structural proteins, and other cellular components released during cell lysis or secretion.

HCPs can elicit immune responses in humans, leading to adverse reactions. Even low levels of certain HCPs can be immunogenic, potentially causing allergic reactions or other immune-mediated effects. The presence of HCPs can interfere with the therapeutic action of the biopharmaceutical product. They may inhibit the activity of the product, reduce its potency, or alter its intended function. Additionally, HCPs can affect the stability of the biopharmaceutical product. Proteolytic enzymes among the HCPs can degrade the therapeutic proteins, reducing their shelf life and efficacy.

Therefore, regulatory agencies such as the FDA and EMA require stringent control and quantification of HCPs in biopharmaceutical products.[2] Compliance with these regulations is necessary for product approval and market release.

Monitoring HCPs is an integral part of the quality assurance process in biopharmaceutical manufacturing. Ensuring low levels of HCPs contributes to the overall quality and purity of the product, maintaining consistent production standards. While ELISA is commonly used to assess total HCP amount, it cannot address the differential immunogenic relevance and effect on product stability of individual HCPs. LC-MS has emerged as a complementary approach, allowing for identification and monitoring of individual HCP.[3]

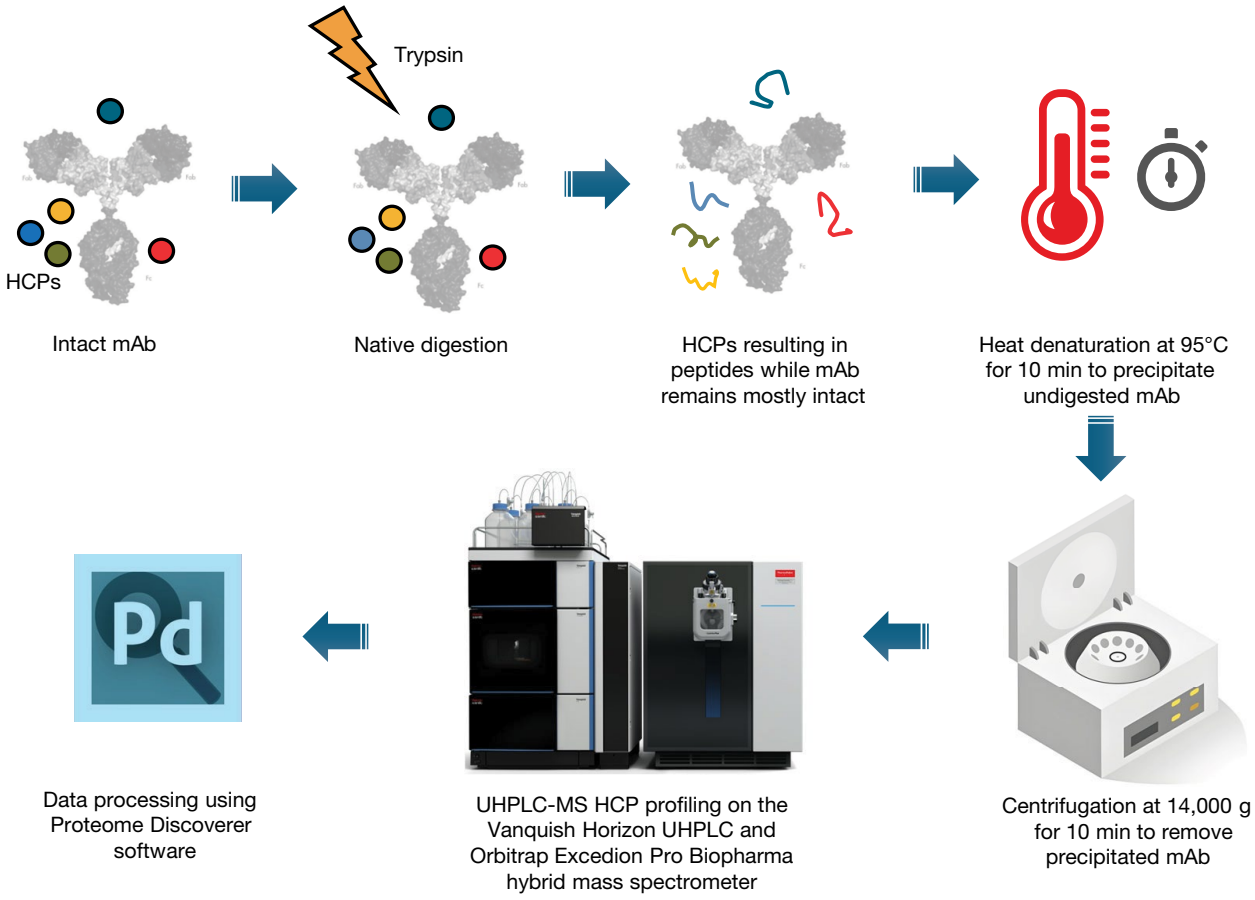


Figure 1. The workflow of NISTmAb HCP profiling on the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer.

However, the intrasample dynamic range (greater than six orders of magnitude) between HCPs and the drug product poses a significant challenge to detect the lowest concentrations of HCPs in biotherapeutics. Here, we demonstrate the capability of the new Orbitrap Excedion Pro Biopharma hybrid mass spectrometer coupled with the Vanquish Horizon UHPLC system for highly sensitive HCP identification.

Experimental

Sample

- The NIST monoclonal antibody (NISTmAb) reference material RM 8671 was ordered from Sigma-Aldrich.

Reagents and consumables

- Fisher Scientific™ Water with 0.1% formic acid (v/v), Optima™ LC/MS grade, Cat. No. LS118-1
- Fisher Scientific™ Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS Grade, Cat. No. LS120-1
- Thermo Scientific™ Formic acid, LC-MS grade, Cat. No. 28905
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5, Cat. No. 15567027
- Thermo Scientific™ Pierce™ Trypsin Protease, MS grade, Cat. No. 90058
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format, Cat. No. A39255
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 UHPLC Column (2.1 mm × 250 mm, 2.2 µm), Cat. No. 074812-V

Sample preparation

1. A 100 µL aliquot of 10 µg/µL NISTmAb was diluted to 500 µL using 50 mM Tris-HCl (pH 7.5).
2. The sample (500 µL) was digested at 37°C for two hours using a solution of trypsin enzyme at a 1:800 enzyme/protein ratio.
3. The digest mixture was reduced with 10 µL of 0.5M DTT for 10 min at 95°C, followed by centrifugation at 14,000 g for 10 minutes to precipitate undigested mAb.
4. The supernatant was acidified with 0.5 µL of formic acid then transferred to a sample vial for LC-MS analysis.

Chromatography

For all experiments, the Thermo Scientific™ Vanquish™ Horizon UHPLC System was used, consisting of the following modules:

- Thermo Scientific™ System Base Vanquish™ Horizon/Flex (Cat. No. VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (Cat. No. VH-P10-A-02)
- Thermo Scientific™ Vanquish™ Split Sampler HT (Cat. No. VH-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (Cat. No. VH-C10-A-03)
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector VWD-F (Cat. No. VF-D40-A)
- Thermo Scientific™ Viper™ MS Connection Kit for Vanquish LC systems (Cat. No. 6720.0405)

The UHPLC conditions are listed in Table 1.

Table 1. UHPLC conditions.

Parameter	Value	
Column	Acclaim VANQUISH C18 UHPLC column (2.1 mm × 250 mm, 2.2 µm)	
Mobile phase A	Water with 0.1% formic acid (v/v)	
Mobile phase B	Acetonitrile with 0.1% formic acid (v/v)	
Flow rate	300 µL/min	
Column temperature	60°C	
Autosampler temperature	10°C	
Sample injection volume	80 µL (approximately 176 µg NISTmAb, if digestion was complete)	
Gradient	Linear	
	Time (min)	% B
	0.0	3
	1.0	3
	90.0	35
	95.0	85
	100.0	85
	105.0	3
	110.0	85
	115.0	85
	115.1	3
	135.0	3

Mass spectrometry

For this study, the new Orbitrap Excedion Pro Biopharma hybrid mass spectrometer was used. The MS parameters are summarized in Table 2, and the data-dependent acquisition workflow is described in Figure 2.

Table 2. MS parameter settings.

Parameter	Value
Source parameters	
Spray voltage (V)	+3,500
Sheath gas (Arb)	40
Aux gas (Arb)	10
Ion transfer tube temp. (°C)	325
Vaporizer temp. (°C)	250
Application mode	Peptide
Full MS	
Orbitrap resolution	120,000
Scan range (*m/z*)	350–1,200
Maximum injection time (ms)	50
AGC target	Custom
Normalized AGC target (%)	300
RF lens (%)	40
Maximum injection time mode	Custom
Include charge states	2–6
ddMS2	
Number of dependent scans	15
Isolation window (*m/z*)	2
Activation type	HCD
HCD collision energies (%)	30
Orbitrap resolution	30,000
First mass (*m/z*)	120
Maximum injection time (ms)	150
AGC target	Custom
Normalized AGC target (%)	100
Maximum injection time mode	Custom

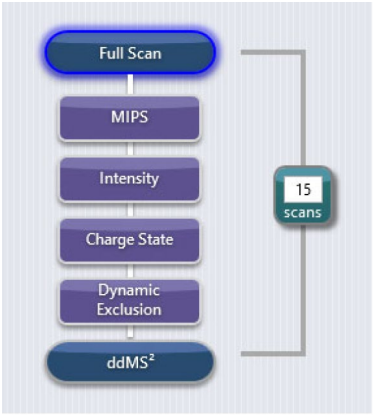


Figure 2. The data-dependent acquisition workflow on the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer.

Data processing

Database searching was performed using Thermo Scientific™ Proteome Discoverer™ Software, version 3.2 (Figure 3). Both SEQUEST™ HT search engine and CHIMERYS™ intelligent search algorithm were used to search DDA MS2 spectra with UniProt Mus musculus database (25,623 entries, TaxID = 10090, 2024/12/16).

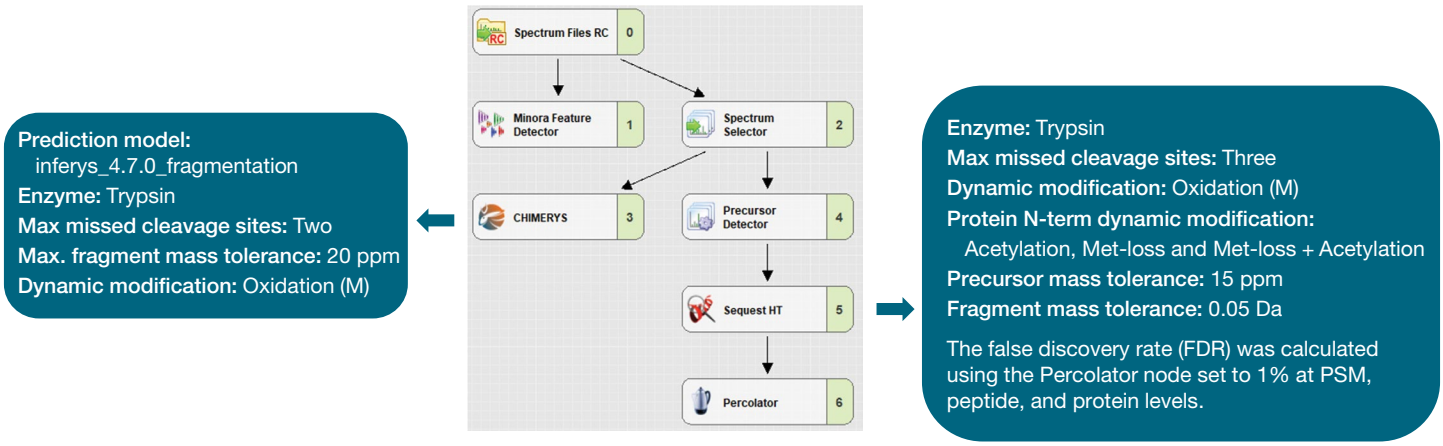


Figure 3. Database searching workflow and parameters.

Results

Reproducible LC separation is important for scientific research, quality control, and regulatory compliance. In this study, the reproducibility of LC separation is also essential for reliable HCP identification and quantification. To overcome the challenge of dynamic range, an optimized native tryptic digestion protocol was utilized.[5] A 25 cm long UPLC column was also used for increasing the loading capacity of the NISTmAb while maintaining peptide separation efficiency. Figure 4 displays the highly reproducible base peak chromatogram (BPC) profiles of three replicate injections of NISTmAb using native digestion. For each injection, 80 µL (approximately 176 µg, assuming digestion was complete) of NISTmAb was loaded onto the column.

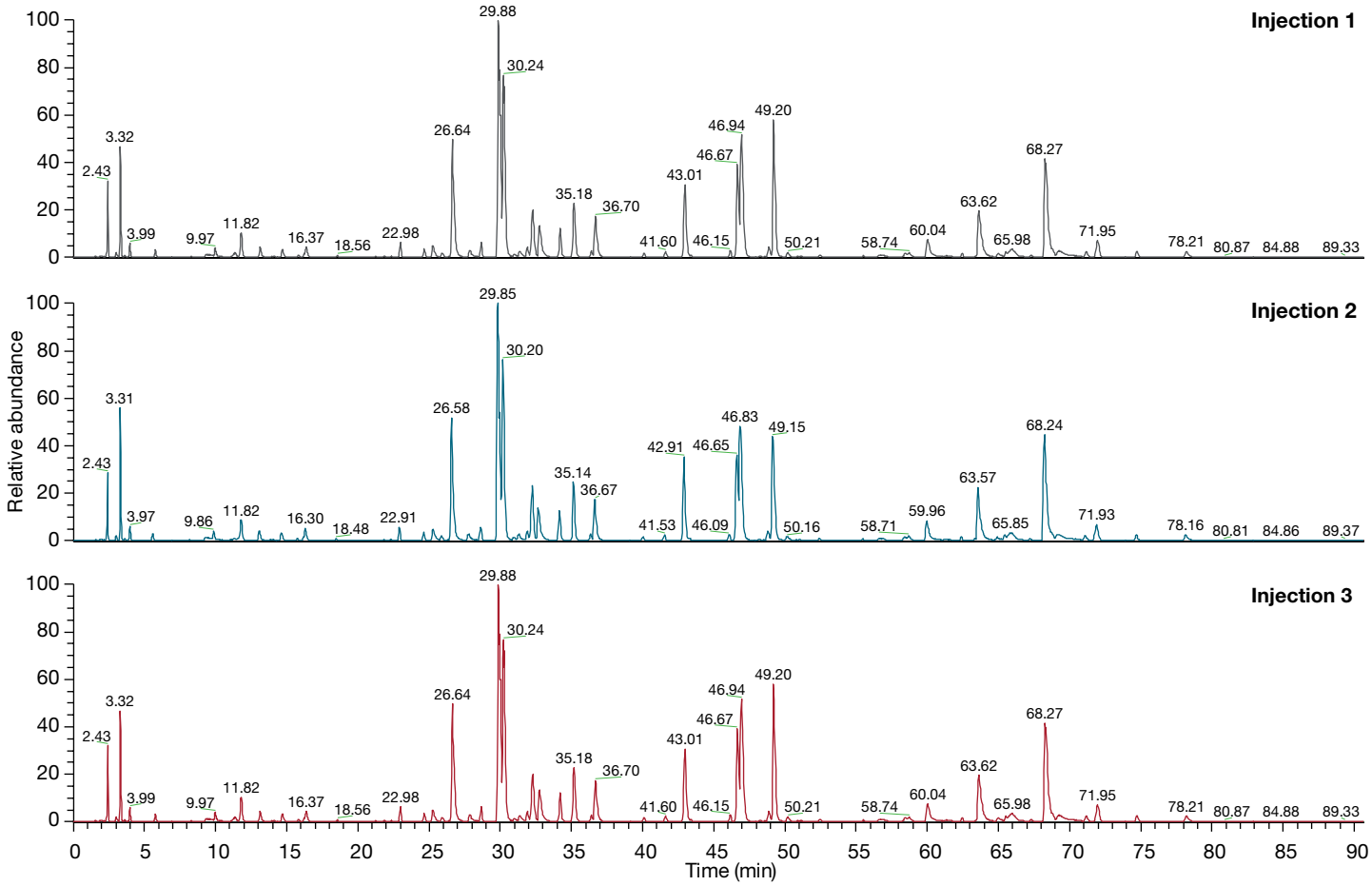


Figure 4. Highly reproducible BPC profiles of three replicate injections of NISTmAb using native digestion.

During this study, 163 HCPs in total were identified in three replicate injections of NISTmAb. The search results were filtered using a unique protein in each protein group, <1% peptide and protein FDR confidence, and at least two unique peptides per protein filters. Both SEQUEST HT and CHIMERYS intelligent search algorithms were used to search DDA MS2 spectra using the UniProt Mus musculus database. Performing different algorithms can help cross-verify results, reduce the likelihood of errors, increase HCP identification coverage, and improve the overall accuracy of the data processing (Figure 5).

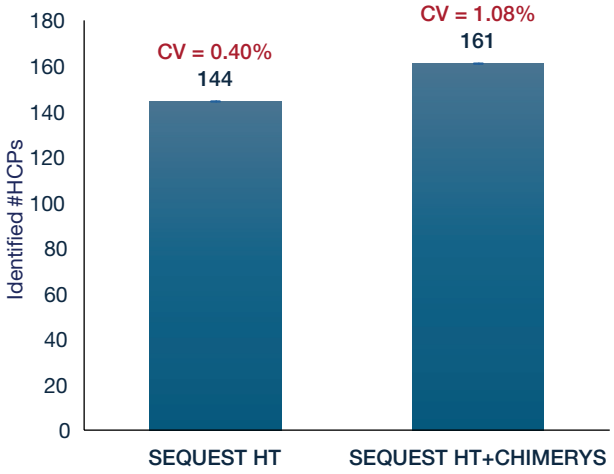


Figure 5. Average numbers of HCPs identified from three replicate injections using SEQUEST HT and CHIMERYS search algorithms. The numbers of identified HCPs shown here are the average of three replicate injections. SEQUEST HT only and SEQUEST HT+CHIMERYS searching were performed, respectively, and SEQUEST HT+CHIMERYS gave more identifications compared to SEQUEST HT only. The CVs of identified HCPs among three replicate injections were 0.40% (SEQUEST HT only) and 1.08% (SEQUEST HT+CHIMERYS), demonstrating excellent reproducibility of the results.

The UHPLC-HRAM MS-ddMS2 method employed for HCP identification in this study showed excellent reproducibility with 159/163 HCPs identified in all three replicates.

We compared our results with a recent HCP publication by Beaumal *et al.*[4] and found 128 HCPs in common with their findings. Their work employed nano flow rate separation followed by DDA MS2 with and without FAIMS, identifying 173 and 103 of HCPs, respectively. By combining DDA MS2, DDA MS2 with FAIMS, and DIA, 188 HCPs were identified in their publication.[4]

Using the relative quantification results in the Beaumal *et al.* publication as reference, the identified HCPs were present across a wide relative abundance range, from >100 ppm (fructose-bisphosphate aldolase A) to 0.007 ppm (glutathione S-transferase P). Most identified HCPs (67%) are present at the sub-ppm level (Figure 6), proving the reproducibility and sensitivity of the platform. The 20 most abundant and least abundant HCPs identified in this study are listed in Table 3 and Table 4, respectively, matched against measured ng of HCP per mg of mAb as shown by Beaumal *et al* using DIA. High-risk HCPs are labeled with "*" and hard-to-remove HCPs are labeled with "#"; more details of these HCPs can be found in Table 6.

Sixty HCPs from our study were newly detected compared to the results published by Beaumal *et al.* using DDA MS2 (Table 5). These newly identified HCPs include some enzymes that may affect product stability, indicating the necessity of using highly accurate and sensitive methods/platforms for potential high-risk HCP identification.

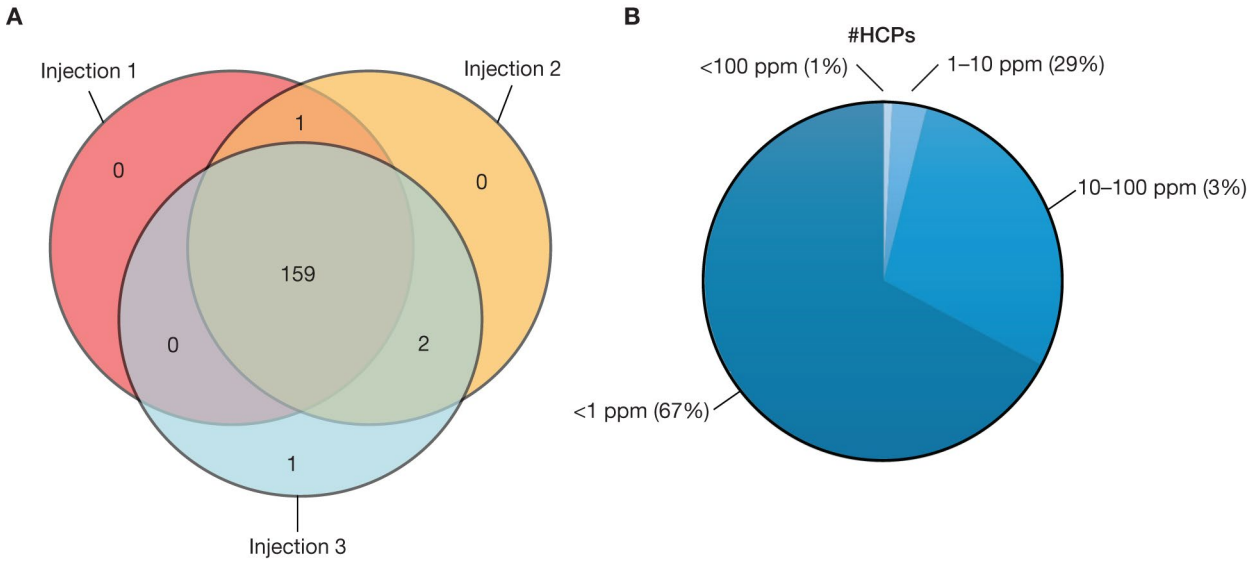


Figure 6. (A) The overlap of HCPs identified in three replicate injections, and (B) the abundance range of identified HCPs. Most of the HCPs distributed in the sub-ppm range (67%) and 29% are between 1 and 10 ppm.

Table 3. Subset of the most abundant 20 HCPs identified in this study matched against the measured ng of HCP per mg of mAb shown by Beaumal *et al*. with DIA in the publication. High-risk HCPs are labeled with "*". More details are included in Table 6.

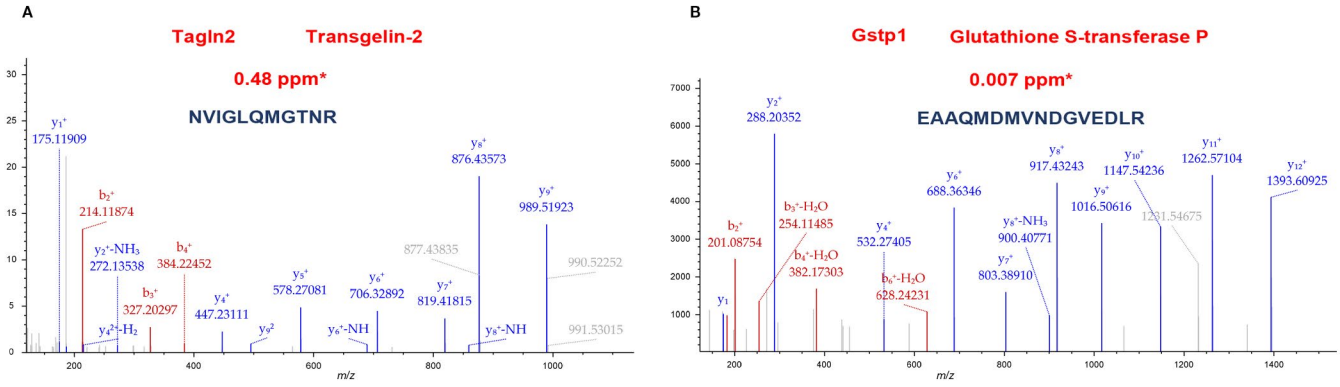
Gene name	Description	MW (Da)	Measured ng of HCP per mg of mAb, ppm (Beaumal *et al*.)
Aldoa	Fructose-bisphosphate aldolase A	39,356	169.387
Aldoc	Fructose-bisphosphate aldolase C	39,395	85.843
Pdia6*	Protein disulfide-isomerase A6	48,100	69.000
Gpi	Glucose-6-phosphate isomerase	62,767	29.354
B2m	Beta-2-microglobulin	13,779	19.049
Abhd11*	Protein ABHD11	33,561	6.467
Grn	Progranulin	63,458	5.261
Ptgr1	Prostaglandin reductase 1	35,560	4.376
Sema4b	Semaphorin-4B	91,392	4.075
Sf3a1	Splicing factor 3A subunit 1	88,545	3.968
Srsf7	Serine/arginine-rich splicing factor 7	30,818	3.670
Papln	Papilin	138,904	3.589
Nsfl1c	NSFL1 cofactor p47	40,710	3.118
Mars1	Methionine--tRNA ligase, cytoplasmic	101,431	3.071
Hnrnpa2b1	Heterogeneous nuclear ribonucleoproteins A2/B1	37,403	3.043
Eif4b	Eukaryotic translation initiation factor 4B	68,840	2.978
Clint1	Clathrin interactor 1	68,513	2.407
Galnt6	Polypeptide N-acetylgalactosaminyltransferase 6	71,537	2.346
Blvrb	Flavin reductase (NADPH)	22,197	2.164
Ak2	Adenylate kinase 2, mitochondrial	26,469	2.115

Table 4. Subset of the least abundant 20 HCPs identified in this study matched against the measured ng of HCP per mg of mAb shown by Beaumal *et al*. with DIA in the publication. High-risk HCPs are labeled with "*" and hard to remove HCPs are labeled with "#". More details re included in Table 6.

Gene name	Description	MW (Da)	Measured ng of HCP per mg of mAb, ppm (Beaumal *et al*.)
Rps12	40S ribosomal protein S12	14,525	0.106
Srsf1	Serine/arginine-rich splicing factor 1	27,745	0.095
Iscu	Iron-sulfur cluster assembly enzyme ISCU, mitochondrial	18,098	0.085
Eef1a1	Elongation factor 1-alpha 1	50,114	0.084
Dnajc2	DnaJ homolog subfamily C member 2	71,722	0.082
Srpra	Signal recognition particle receptor subunit alpha	69,623	0.080
Ldha	L-lactate dehydrogenase A chain	36,499	0.071
Ptk2	Focal adhesion kinase 1	119,243	0.065
Stmn1	Stathmin	17,274	0.054
Prdx1#	Peroxiredoxin-1	22,176	0.040
Ctsd*	Cathepsin D	44,954	0.029
Cfl1	Cofilin-1	18,560	0.025
Hmgb2	High mobility group protein B2	24,162	0.025
Fkbp3*	Peptidyl-prolyl cis-trans isomerase FKBP3	25,148	0.022
Mrpl12	39S ribosomal protein L12, mitochondrial	21,708	0.020
Pgk1	Phosphoglycerate kinase 1	44,550	0.020
Prdx5#	Peroxiredoxin-5, mitochondrial	21,897	0.016
Cacybp	Calcyclin-binding protein	26,510	0.013
Nme2	Nucleoside diphosphate kinase B	17,363	0.012
Gstp1*	Glutathione S-transferase P 1	23,609	0.007

High-quality MS and ddMS2 data are critical for accurate and reliable characterization. High-quality MS data provide precise mass measurements, which are essential for the accurate identification of peptides. High-quality MS2 data are crucial for elucidating the peptide sequence and confirming the identity of peptide and protein.

For peptides associated with low abundant HCPs, MS2 spectra with high S/N still can be acquired for confident identification (Figure 7), indicating the excellent sensitivity of the new Orbitrap Excedion Pro Biopharma hybrid mass spectrometer. The wide dynamic range of this instrument can facilitate the identification of proteins distributing from high to low concentrations.



** Beaumal C., et al., Proteomics 2023 May 6, doi: 10.1002/pmic.202300172.*

Figure 7. The MS2 spectra of peptides associated with two low abundant HCPs. (A) The MS2 spectrum of a peptide from transgelin-2 (0.48 ppm), and (B) the MS2 spectrum of a peptide from glutathione S-transferase P (0.007 ppm). Abundances for identified HCPs were matched with a previous publication by Beaumal *et al.*[4]

Table 5 (part 1). HCPs identified in this study and not reported by Beaumal *et al*. using DDA MS2 in the publication. High-risk HCPs are labeled with "*". More details of the high-risk HCPs are included in Table 6.

Gene name	Description	# Unique peptides	# PSMs
Nme2	Nucleoside diphosphate kinase B	6	76
Nme1	Nucleoside diphosphate kinase A	3	61
Grn	Progranulin	5	57
Cacybp	Calcyclin-binding protein	6	51
Atp5if1	ATPase inhibitor, mitochondrial	4	39
Alyref	THO complex subunit 4	4	35
Eprs1	Bifunctional glutamate/proline--tRNA ligase	7	34
Cnbp	CCHC-type zinc finger nucleic acid binding protein	4	33
Septin7	Septin-7	4	33
Hnrnph1	Heterogeneous nuclear ribonucleoprotein H	4	31
Krt10	Keratin, type I cytoskeletal 10	2	30
Rps12	Small ribosomal subunit protein eS12	5	30
Itih5	Inter-alpha-trypsin inhibitor heavy chain H5	6	29
Vapa	Vesicle-associated membrane protein-associated protein A	2	28
Nherf1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	6	26
Crisp3	Cysteine-rich secretory protein 3	5	24
Dnajc2	DnaJ homolog subfamily C member 2	5	24
Prrc2c	Protein PRRC2C	3	24
Dnase2	Deoxyribonuclease-2-alpha	5	23
Ldha	L-lactate dehydrogenase A chain	5	23

Table 5 (part 2). HCPs identified in this study and not reported by Beaumal *et al*. using DDA MS2 in the publication. High-risk HCPs are labeled with "*". More details of the high-risk HCPs are included in Table 6.

Gene name	Description	# Unique peptides	# PSMs
Ppif*	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	5	22
Pafah1b3	Platelet-activating factor acetylhydrolase IB subunit alpha1	4	22
Krt1	Keratin, type II cytoskeletal 1	4	18
Ppt1	Palmitoyl-protein thioesterase 1	5	18
Rpap3	RNA polymerase II-associated protein 3	4	18
Bclaf1	Bcl-2-associated transcription factor 1	3	17
Dbnl	Drebrin-like protein	3	17
Eif3a	Eukaryotic translation initiation factor 3 subunit A	3	17
Rps15	Small ribosomal subunit protein uS19	2	17
Stmn1	Stathmin	3	17
Hspa8	Heat shock cognate 71 kDa protein	5	16
Krt2	Keratin, type II cytoskeletal 2 epidermal	2	16
Tcof1	Treacle protein	3	16
Eif5b	Eukaryotic translation initiation factor 5B	2	15
Atxn2l	Ataxin-2-like protein	3	14
Lbr	Delta(14)-sterol reductase LBR	3	14
Cltb	Isoform 2 of Clathrin light chain B	3	14
Mocs1	Molybdenum cofactor biosynthesis protein 1	3	14
Nipbl	Nipped-B-like protein	4	14
B4galt1	Beta-1,4-galactosyltransferase 1	2	12
Gpkow	G-patch domain and KOW motifs-containing protein	2	12
Krt5	Keratin, type II cytoskeletal 5	2	12
Vps4a	Vacuolar protein sorting-associated protein 4A	2	12
Rps27a	Ubiquitin-ribosomal protein eS31 fusion protein	2	11
Ubxn4	UBX domain-containing protein 4	2	11
Adh5	Alcohol dehydrogenase class-3	3	10
Mt2	Metallothionein-2	3	10
Phf5a	PHD finger-like domain-containing protein 5A	2	10
Lman2l	VIP36-like protein	3	10
Mmp11	Stromelysin-3	2	9
Cfl1	Cofilin-1	2	8
Nelfa	Negative elongation factor A	2	8
Stk10	Serine/threonine-protein kinase 10	2	8
Pfdn6	Prefoldin subunit 6	2	7
Cdv3	Protein CDV3	2	7
Alpl	Alkaline phosphatase, tissue-nonspecific isozyme	2	6
Gatd3	Glutamine amidotransferase-like class 1 domain-containing protein 3, mitochondrial	2	5
Ighg1	Ig gamma-1 chain C region, membrane-bound form	2	4
Tcerg1	Transcription elongation regulator 1	2	4
Hexb	Beta-hexosaminidase subunit beta	2	2

Table 6. Details of high-risk and hard-to-remove HCPs identified in this study matched against the measured ng of HCP per mg of mAb shown by Beaumal *et al*. using DIA in the publication. High-risk HCPs are labeled with "*" and hard to remove HCPs are labeled with "#".

Gene name	Description	# Unique peptides	ppm	Function	Impact	Type of impact	Select references
Pdia6*	Protein disulfide-isomerase A6	11	69.000	Catalyzes the formation and breakage of disulfide bonds	Drug quality	Aggregation of drugs	Aboulaich *et al*., 2014; Gilgunn & Bones, 2018; Goey *et al*., 2018; Maeda *et al*., 2007
Abhd11*	sn-1-specific diacylglycerol lipase	15	6.467	Formulation	Drug quality	Degradation of polysorbates	
Fkbp2*	Peptidyl-prolyl *cis-trans* isomerase	8	1.275	Catalyzes the formation and breakage of disulfide bonds	Drug quality	Aggregation of drugs	Aboulaich *et al*., 2014; Gilgunn & Bones, 2018; Goey *et al*., 2018; Maeda *et al*., 2007
Prdx1*#	Peroxiredoxin-1	10	0.040	Regulates the intracellular concentration of hydrogen peroxide	Immunogenicity	Immunogenic response	Albrecht *et al*., 2018; Jawa *et al*. 2016; Joucla *et al*., 2013; Park *et al*., 2017
Ctsd*	Cathepsin D	6	0.029	Aspartyl protease with activity in both acidic and neutral pH	Drug quality	Fragmentation of drug	Bee *et al*., 2017; Lima *et al*., 2018; Robert *et al*., 2009; Yang *et al*., 2019
Fkbp3*	Peptidyl-prolyl *cis-trans* isomerase	3	0.022	Catalyzes the *cis–trans* isomerization of peptide bonds N-terminal to proline residues	Drug Quality	Aggregation of drugs	Aboulaich *et al*., 2014; Gilgunn & Bones, 2018; Goey *et al*., 2018; Maeda *et al*., 2007
Prdx5*#	Peroxiredoxin-5, mitochondrial	8	0.016	Regulates the intracellular concentration of hydrogen peroxide	Immunogenicity	Immunogenic response	Albrecht *et al*., 2018; Jawa *et al*. 2016; J oucla *et al*., 2013; Park *et al*., 2017
Gstp1*	Glutathione S-transferase P 1	7	0.007	Conjugates reduced glutathione to exogenous and endogenous hydrophobic electrophiles	Immunogenicity	Immunogenic response	Goey *et al*., 2018; Jawa *et al*., 2016
Ppif*	Peptidyl-prolyl *cis-trans* isomerase F, mitochondrial	5	-	Catalyzes the *cis–trans* isomerization of peptide bonds N-terminal to proline residues	Drug quality	Aggregation of drugs	Aboulaich *et al*., 2014; Gilgunn & Bones, 2018; Goey *et al*., 2018; Maeda *et al*., 2007
Ppid*	Peptidyl-prolyl *cis-trans* isomerase D	4	-	Catalyzes the *cis–trans* isomerization of peptide bonds N-terminal to proline residues	Drug quality	Aggregation of drugs	Aboulaich *et al*., 2014; Gilgunn & Bones, 2018; Goey *et al*., 2018; Maeda *et al*., 2007

Conclusion

In this study, we demonstrated the capability of a new Thermo Scientific Orbitrap Excedion Pro Biopharma hybrid mass spectrometer for HCP profiling.

- A total of 163 HCPs were identified in NISTmAb using an optimized native digestion protocol, UHPLC-ddMS2 method, and stringent database search parameters.
- The developed UHPLC-HRAM MS-ddMS2 method exhibited excellent reproducibility with 159/163 HCPs identified in all three replicates.
- Most identified HCPs (67%) were present at the sub-ppm level, proving the sensitivity of the instrument and the developed method.
- One of the identified HCP, glutathione S-transferase P at a concentration of 0.007 ppm, was consistently identified with at least two unique peptides in all three injections, highlighting the instrument's high ddMS2 data quality for confident detection and identification of very low abundant HCP proteins.
- All experiments were conducted under analytical flow UHPLC conditions, which are user-friendly, easy to maintain, robust, and provide excellent separation reproducibility.[5]

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