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Increasing sensitivity of high throughput host cell protein analysis on a novel high-resolution accurate mass platform

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ABSTRACT

Purpose: Evaluate the performance of the new Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer for fast and sensitive identification of low ppm host cell proteins (HCPs) in biotherapeutics using a combination of non-denaturing trypsin digestion, fast chromatographic separation, sensitive and fast HRAM MS2 detection, and advanced data processing to reduce the dynamic range challenge and maximize the identification rate.

Methods: (1) Chromatographic separation of non-depleted vs. depleted samples, (2) data independent acquisition (DIA) using narrow isolation windows (3Th), and (3) data processing with Proteome Discoverer software using the Chimerys algorithm.

Results: Demonstrated excellent performance of the new Orbitrap Astral mass spectrometer for identification of HCPs at sub-ppm level with increased throughput.

INTRODUCTION

Recombinant biotherapeutics are produced using non-human host cell lines. Released proteins can detrimentally affect safety and efficacy of the final drug product if not removed during downstream purification. While ELISA is commonly used to assess total HCP content, differential immunogenic relevance and effect on product stability of individual HCPs cannot be addressed. LC-MS has emerged as a complementary method allowing for quantification of individual HCP levels. However, the intrasample dynamic range (>six orders of magnitude) between HCPs and the drug product poses a great challenge to detect lowest concentrations of HCPs in biotherapeutics. Here, we demonstrate the use of a novel HRAM platform to improve the dynamic range for identification of HCPs at sub-ppm level with increased throughput.

MATERIALS AND METHODS

Proteolytic NISTmAb digests were generated upon digestion with the Thermo Scientific™ SMART Digest™ kit containing immobilized trypsin a) for 30 min at 70 ˚C; and b) for 3 hrs at 37 ˚C (Claydon *et al.*, Huang *et al.)* and removal of undigested protein via precipitation at 95 ˚C for 15 min, followed by centrifugation at 14,000 x g for 5 min (Fig. 1). Recovered supernatant from both digests was reduced with DTT solution (final concentration of 10 mM) for 45 min at 57 ˚C. 200-500 ng of peptides were separated by reversed phase UHPLC using a 150 µm x 15 cm Thermo Scientific™ EASY-Spray™ PepMap™ Neo column with a 20.2 min gradient on a Thermo Scientific™ Vanquish™ Neo system operated in Trap & Elute mode at a flow rate of 0.8 µl/min. The new mass spectrometer was operated in data independent acquisition (DIA) mode using 3 Th isolation windows and acquisition speed up to 200 Hz (Fig. 2). Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 3.1 software using Chimerys algorithm and UniProt *Mus musculus* database.

Figure 1. Overview of the HCP analysis workflow

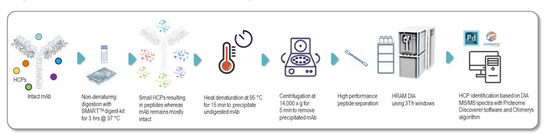
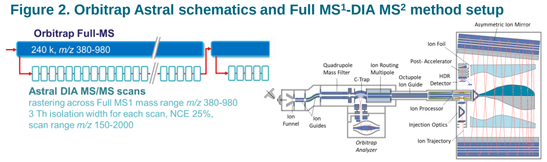


Figure 2. Orbitrap Astral schematics and Full MS¹-DIA MS² method setup

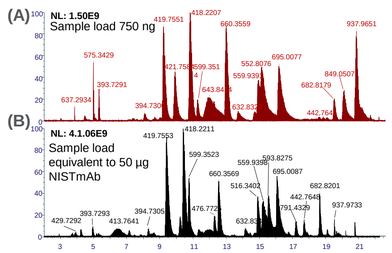


RESULTS

Qualitative analysis of trace level HCPs in NISTmAb

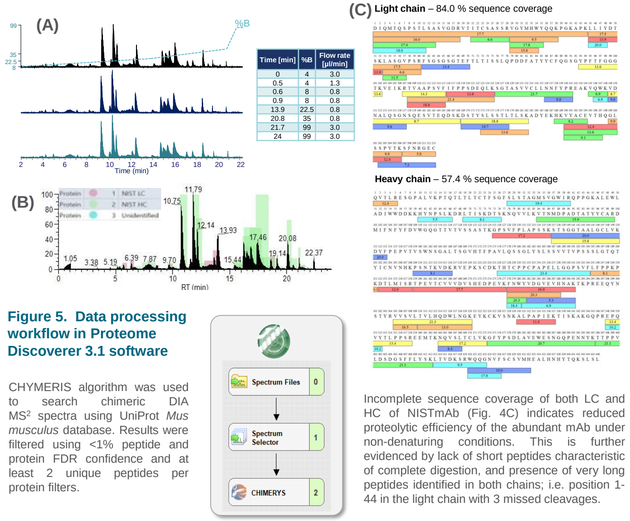
Detection of trace level HCP impurities in biotherapeutics, which present themselves in low levels (sub-ppm to 100-200 ppm), is often cumbersome due to wide dynamic range of proteinaceous species. Highly abundant mAb-derived peptides hinder HCP peptides, thus challenging both their chromatographic separation, as well as the MS instrument´s ability to detect their signal at the noise level. Here deployed non-denaturing proteolysis facilitates detection of HCPs and improves detection limit by exploiting the resistance of native mAb protein structure to proteolysis and thus reducing the dynamic range of peptides present in the sample.

Figure 3. Base peak chromatograms obtained from digests (A) under denaturing conditions (70 ˚C, 30 min) vs. (B) prolonged non-denaturing conditions (37 ˚C, 3 hrs) following denaturation and centrifugation.



Applying the SMART Digest kit under non-denaturing conditions by reducing the temperature to 37 ˚C, resulted in a significantly reduced digestion level of the mAb compared to the denaturing digest (Fig 3). Qualitative comparison of the chromatograms shows reduced presence of mAb peptides, particularly obvious at retention times 3-5, 9-11, 15-17 and 19-21 min. In addition, clear correlation of overall signal intensity and reduction of dynamic range due to presence of mAb peptides is observed when comparing sample loads and overall intensities between the traces. Chromatogram in panel 3B was obtained from >60x higher sample load (calculated based on initial mAb sample concentration) resulting in only half an order of magnitude increase in overall signal intensity.

Figure 4. (A) Base peak chromatograms of triplicate injections of NISTmAb peptides obtained upon the digest under non-denaturing conditions using a 20.2 min gradient (B) and chromatogram shading showcasing peaks representing peptides associated to light and heavy chains demonstrating incomplete sequence coverage.



Time [min]	%B	Flow rate [µl/min]
0	4	3.0
0.5	4	1.3
0.6	8	0.8
0.9	8	0.8
13.9	22.5	0.8
20.8	35	0.8
21.7	99	3.0
24	99	3.0

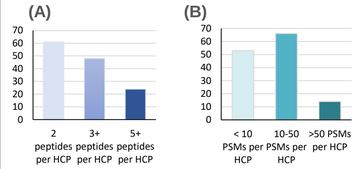
Figure 5. Data processing workflow in Proteome Discoverer 3.1 software

CHYMERIS algorithm was used to search chimeric DIA MS² spectra using UniProt *Mus musculus* database. Results were filtered using <1% peptide and protein FDR confidence and at least 2 unique peptides per protein filters.

Incomplete sequence coverage of both LC and HC of NISTmAb (Fig. 4C) indicates reduced proteolytic efficiency of the abundant mAb under non-denaturing conditions. This is further evidenced by lack of short peptides characteristic of complete digestion, and presence of very long peptides identified in both chains; i.e. position 1-44 in the light chain with 3 missed cleavages.

This study identified over 130 HCPs in NISTmAb with at least two unique peptides, in at least two out of three replicate injections (Fig 6.), at <1% peptide and protein FDR. Importantly, these results were obtained in ~5x shorter analysis time (20.2 min gradient, 60 Samples Per Day (SPD) method) than standard methods,[1,2,3] While the number of identified HCPs in our study is comparable to what has been recently reported in the literature[3], the LC-MS setup applied here allows for a significantly increased throughput.

Figure 6. High confidence NISTmAb HCP identifications: number of unique peptides (A) and number of PSMs per HCP (B)



The excellent quality of the single scan-based MS² spectra (Fig. 7) acquired on the Orbitrap Astral mass spectrometer is the basis for identification of HCPs with high confidence across a wide concentration range. Panel 7A showcases a spectrum supporting the identification of a peptide associated to the low abundant programmed cell death protein (0.589 ng/mg mAb; Acc. P56812). In contrast, panel B relates to a peptide associated to the highly abundant Fructose bisphosphate aldolase A (170 ng/mg mAb; Acc. P05064).

Figure 7. Examples demonstrating MS² spectral quality for HCPs of low and high abundance

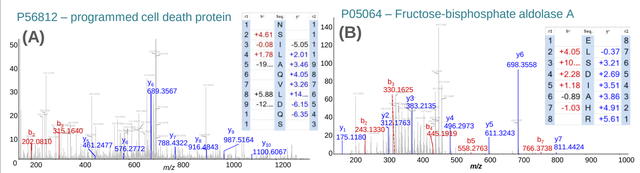


Figure 8. Overlap of host cell proteins identified (A) by DIA in Beaumal *et al.* (2023) (blue) vs. our study (red); and (B) by Beaumal *et al.* entire study (DDA, FAIMS DDA, DIA and GPF DIA) vs. our study.

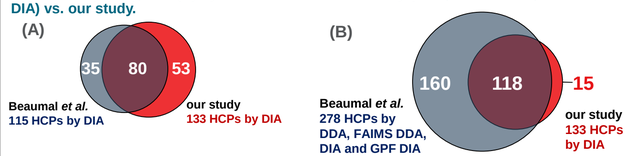


Table 1. HCPs identified in this study and not reported by Beaumal *et al.*

HCP	Unique Peptides	PSMs
Liprin-beta-2	2	2
VIP36-like protein*	2	2
Adaptin ear-binding coat-associated protein 2	2	2
Immunoglobulin kappa variable 6-13	2	3
Treacle protein*	2	3
E3 ubiquitin-protein ligase DTX3L*	2	3
DNA ligase 1 OS=Mus musculus	2	5
Alpha-N-acetylgalactosaminidase*	2	5
Serine/arginine repetitive matrix protein 1*	2	11
Jupiter microtubule associated homolog 2*	3	11
Albumin OS=Mus musculus	2	12
H3.4 histone OS=Mus musculus	2	21
RIKEN cDNA 2210010C04 gene	2	21
Peptidyl-prolyl cis-trans isomerase B*	6	34
Predicted gene 7324 OS=Mus musculus	4	50

** Reported in Johnson et al.[4]*

Figure 9. Representative MS² spectrum of Peptidyl-prolyl cis-trans isomerase B (Acc. P24369) identified in this study.

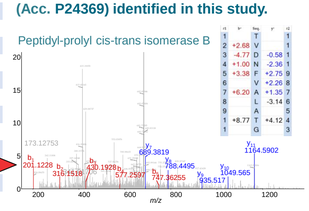


Table 2. Subset of 40 HCPs identified in this study matched against measured ng of HCP per mg of mAb shown by Beaumal *et al.*

Host Cell Protein	Measured ng of HCP per mg of mAb, ppm (Beaumal *et al.*)[3]
Glutathione S-transferase P	0.007
Allograft inflammatory factor	0.007
Nucleoside diphosphate kinase B	0.012
Phosphoglycerate kinase 1	0.020
39S ribosomal protein L12, mitochondrial	0.020
Peroxiredoxin-1	0.040
Ubiquitin-associated protein 2	0.045
Histone H2A type 1-O	0.052
Signal recognition particle receptor subunit alpha	0.080
Serine/arginine-rich splicing factor 1	0.095
40S ribosomal protein S12	0.106
Golgi SNAP receptor complex member 2	0.204
B-cell receptor-associated protein 31	0.212
RNA-binding protein EWS	0.222
THO complex subunit 4	0.230
Heterogeneous nuclear ribonucleoprotein A1	0.310
UMP-CMP kinase	0.414
Programmed cell death protein 5	0.589
GTPase-activating protein GIT1	0.598
Malate dehydrogenase, mitochondrial	0.684
ATP-dependent zinc metalloprotease YME1L1	0.714
Eukaryotic translation initiation factor 3 subunit A	0.907
ATPase inhibitor, mitochondrial	0.989
Transketolase	1.049
Low affinity immunoglobulin gamma Fc region receptor II	1.070
Hepatocyte growth factor-like protein	1.175
Peptidyl-prolyl cis-trans isomerase FKBP2	1.275
Syntaxin-12	1.943
Eukaryotic translation initiation factor 4B	2.978
Heterogeneous nuclear ribonucleoproteins A2/B1	3.043
Methionine--tRNA ligase, cytoplasmic	3.071
NSFL1 cofactor p47	3.118
Prostaglandin reductase 1	4.376
Progranulin	5.261
Protein ABHD11	6.467
Beta-2-macroglobulin	19.049
Glucose-6-phosphate isomerase	29.354
Protein disulfide-isomerase A6	69.000
Fructose-bisphosphate aldolase C	85.843
Fructose-bisphosphate aldolase A	169.387

The HCPs identified in this study correlate well with recently published results by Beaumal *et al.*[3] (Fig 8). Table 2 provides the list of 40 HCPs that have been successfully matched against measured ng of HCP per mg of mAb shown by Beaumal *et al.*. The concentration of these HCPs spans over the wide range from **7 ppb to 169 ppm.**

The correlation with the quantitative results highlights the successful deployment of the HCP analysis workflow applied in this study encompassing sample preparation under non-denaturing conditions, fast chromatographic separation, mass spectrometric detection with highest sensitivity at ultra-fast detection rate, combined with advanced database search capabilities.

CONCLUSIONS

- In this work, we demonstrated outstanding performance of the new Orbitrap Astral mass spectrometer for fast and sensitive host cell proteins analysis.
- The new Astral mass analyzer provides ultra-fast and sensitive HRAM DIA MS² scans enabling the reproducible identification of more than 130 HCPs with a significant reduction in analysis time.

REFERENCES

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4. Johnson B., *et al.*,. Anal. Chem. 2020, 92, 15, 10478–10484.

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TRADEMARKS/LICENSING

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