Antibody Affinity Extraction Outperforms Native Digestion as a Sample Preparation Method for LC-MS Detection of Host Cell Proteins in Drug Substances

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INTRODUCTION

Host cell proteins (HCPs) co-purify with biological drug substances (DS) and pose potential risks for both patients and drug manufacturers. While many HCPs are benign, some are immunogenic, others may interact with the drug substance (DS) and impact its efficacy, and others can interfere with DS stability. Thus, the quantity and nature of residual HCPs in DS are generally considered a critical quality attribute (CQA). The enzyme-linked immunosorbent assay (ELISA) is the gold standard analytical method for measuring total HCP levels to support both in process testing and product release. To better understand the HCP properties and thus more effectively remove HCPs, liquid chromatography-mass spectrometry (LC-MS) has emerged as an orthogonal tool for HCP analysis.

Identification of HCPs by mass spectrometry (MS) is a powerful complementary method to demonstrate the suitability of an HCP ELISA. When combined with antibody affinity extraction (AAE™) using an HCP ELISA Antibody, MS can be used to demonstrate that an HCP ELISA is suitable for the purpose of monitoring purification process consistency and product lot release [1].

While coverage to the total HCP mixture present in a cell culture harvest stream has been traditionally requested by regulatory agencies, it is the HCPs that persist through a given purification process that are the most important with respect to patient safety and drug efficacy and stability. A major challenge for LC-MS-based methods for identification of HCP in DS is that there can be a more than 5 orders of magnitude difference in the concentration between HCPs and DS, for example a therapeutic antibody, in solution, which precludes the effective identification of low abundance HCPs.

To overcome this challenge, efforts have been made to optimize the sample preparation to improve the dynamic range, such as online or offline fractionation [2–9], removal of mAbs by affinity depletion [10–13] or molecular weight cut-off [14], and HCP enrichment [15–17].

The native digestion (ND) protocol introduced by Huang et al. has become a popular method for HCP analysis [18]. Compared to the traditional bottom-up proteomics approach (denaturing digestion), it is simpler, quicker and more robust with a limit of detection (LOD) of as low as <10 ppm when using analytical flow LC [8,18-21]. However, there is risk that the recovery of a small subset of low-level HCPs may not be favored during the native digestion.

Developed by Cygnus Technologies in 2013, AAE is highly effective at enriching HCPs and depleting DS. Figure 1 shows the relative abundance of DS and HCPs according to their extracted ion chromatograms that were normalized to one and graphed in a stacked bar chart. Pre-AAE (left) shows the DS heavy chain (DS HC) in orange, the DS light chain (DS LC) in light blue and HCPs in assorted colors beneath demonstrating that most peptides in the DS (Pre-AAE sample) belong to DS. Following AAE enrichment (Post-AAE, right), however, the relative abundance of the HCPs dramatically increased and that of the DS HC and LC decreased.

In this study, we compared two HCP enrichment strategies, native digest and antibody affinity extrac-

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tion, to fully characterize HCP profile in two separate DS samples. Our results indicated that AAE-based sample preparation method is superior to native digestion enrichment method.

MATERIALS AND METHODS

AAE

The CHO 3G polyclonal antibody from F550-1 ELISA Kit (Cygnus Technologies) was covalently immobilized on a separate chromatography support. The column was conditioned to prevent significant leaching of the antibody and to minimize non-spe¬cific binding. The HCP-containing DS samples were passed over the column using an ÄKTA 25L fast protein liquid chromatography (FPLC) system (Cytiva) for binding HCPs and collecting elution fractions. All HCP elution fractions were neutralized, pooled, buffer exchanged, and concentrated.

Native Digest

The native digestion procedure was adapted from Huang et al. [18]. In this sample preparation method, 0.1 to 1 mg of DS sample were added to 25mM Tris-HCl (pH 8). Trypsin (Thermo Fisher) was added at 1:400 enzyme to substrate ratio and incubated in a Thermomixer (Eppendorf) at 37°C and 300 RPM for 18 hours. The samples were reduced with 500mM DTT (Thermo Fisher) with a final concentration of 0.5 mg/mL DTT via incubation at 90°C for 10 minutes. Undigested DS was pelleted by centrifugation at 21,100g (max setting) for 10 minutes to pellet the precipitate. The supernatant containing the natively digested HCPs was transferred to a new tube and acidified with 10% Formic Acid. The sample was diluted by adding LC-MS grade water and aliquoted into 100 µL volumes for MS.

LC-MS

The pre-AAE (pre-enrichment DS sample) and post-AAE proteins were precipitated in greater than nine volumes of methanol overnight at -20°C. The precipitated proteins were dissolved in 8M urea, reduced, alkylated, digested with trypsin, desalted, and concentrated.

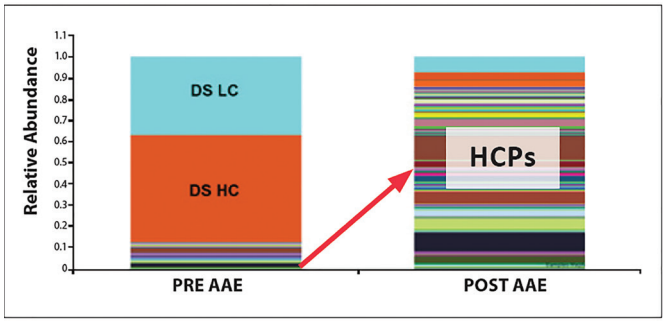


Figure 1. AAE Enriches HCPs and Depletes DS.

LC-MS Data Acquisition

The LC-MS analysis was performed using a Vanquish Horizon UHPLC coupled to an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Scientific) with a factory established limit of detection (LOD) of 0.5-5 ppm. Peptides were loaded onto a C18 column and eluted across a 60-minute gradient before detection in data dependent acquisition (DDA) mode with survey spectrum (m/z range 350-1700) at 240K resolution followed by MS/MS (m/z range 375-2000) of the most intense multiply charged ions using collision induced dissociation.

LC-MS Sequence

The pre-AAE and post-AAE samples were acquired with the LC-MS method independently in technical triplicate and in a randomized sequence. Blank washing runs were implemented in between sample injections to minimize sample carryover.

LC-MS Database Search

Raw spectra were searched against a proprietary CHO database containing common LC-MS protein contaminants using ProteomeDiscoverer version 2.5 (ThermoFisher). HCPs were inferred if at least two unique peptides mapped to them with both peptide- and protein-level false discovery rates of 99%. Search parameters included: protein modifications- Oxidation, Deamidation, and Acetylation; Max. Number of Missed Trypsin Cleavages: 2; Precursor Mass Tolerance: 10 ppm; Fragment Mass Tolerance: 0.6 Da; Number of High Confidence Peptides: 2; and a False Discovery Rate Confidence Threshold: 0.01.

RESULTS

AAE and ND were performed on two different DS samples and compared in parallel to determine the strengths and weaknesses of both approaches. AAE and ND enrichment resulted in the identification of more HCPs than no treatment at all (standard, denaturing condition) which suggests that both techniques effectively enrich for HCPs and deplete the DS leading to deeper proteomic coverage (Table 1&2; Figures 2&3). This result was true for both DS samples despite their containing moderately different numbers of HCPs in total: 16 and 27 for DS

#1 and DS #2, respectively. In both cases, AAE outperformed ND in terms of number of proteins identified. AAE enrichment resulted in 32 HCPs identified, while ND enrichment enabled identification of 20 HCPs in DS #1 (Table 1). Likewise, AAE enrichment resulted in 44 HCPs while ND sample preparation enabled identification of 33 HCPs in DS #2 (Table 2). Additionally, AAE led to the identification of 100% of all HCPs found in the unenriched samples, while ND only captured a subset of those proteins (75% and ~92.6% for DS #1 and DS #2, respectively). All HCPs identified via ND sample preparation method were also identified via AAE sample preparation method, which further supports that both methods enrich for similar populations of HCPs, but AAE does so more effectively.

Table 1. Total, Shared, and Unique HCPs Identified from each Sample Preparation Strategy in DS #1 .

DS #1	AAE Enrichment Sample or Sample Group: Post-AAE	Pre-enrichment Sample or Sample Group: None	Native Digest (ND) Sample or Sample Group: ND
Number of HCPs	**32**	**16**	**20**
Post-AAE(Unique) | None & ND Overlap	8	12 (75%)	
Post-AAE & None Overlap | ND (Unique)	16 (100%)		0
HCPs Identified in All Sample Groups	12 (75%)		

Table 2. Total, Shared, and Unique HCPs Identified from each Sample Preparation Strategy in DS #2

DS #2	AAE Enrichment Sample or Sample Group: Post-AAE	Pre-enrichment Sample or Sample Group: None	Native Digest (ND) Sample or Sample Group: ND
Number of HCPs	**44**	**27_**	**33**
Post-AAE(Unique) | None & ND Overlap	9	25 (~92.6%)	
Post-AAE & None Overlap | ND (Unique)	27 (100%)		0
HCPs Identified in All Sample Groups	25 (~92.6%)		

Table 3. HCP MW, pI and Method Reproducibility

Sample	Metric	AAE Enrichment Sample or Sample Group: Post-AAE	Pre-enrichment Sample or Sample Group: None	Native Digest Sample or Sample Group : ND
DS #1	Molecular Mass (kDA)	37.35	34.00	31.75
DS #2	Molecular Mass (kDA)	38.15	32.3	35.2
DS #1	pI	6.89	7.17	7.06
DS #2	pI	6.69	7.02	6.95
DS #1	CV (%)	5.09	28.00	52.41
DS #2	CV (%)	3.96	27.94	17.40

For each DS sample, the first row in the respective table shows the total number of HCPs identified. The next row shows unique HCPs for Post-AAE and those which overlap between the pre-enrichment (None) and ND samples. The third row shows the overlap between pre-enrichment (None) and post-AAE as well as unique HCPs for the ND samples.

AAE enrichment was superior in terms of variability of relative protein abundance (Table 3). The median coefficient of variance (CV) between technique replicates for HCPs identified by AAE were 5.09% and 3.96% for DS #1 and DS #2, respectively (Table 3). The pre-enriched and ND samples had much higher CVs (~17-52%) which makes protein quantification difficult. No major differences in median molecular masses and isoelectric points were observed between the three Sample Groups. This confirms that AAE antibodies are broadly reactive to diverse HCPs found in DS samples.

Three important metrics were extracted from all HCPs identified by the three sample preparation methods employed in this study. These included shared HCPs, as in the case of Pre-enrichment, there were no unique HCPs in either DS sample. The first two rows display the median molecular masses, rows 3-4 display

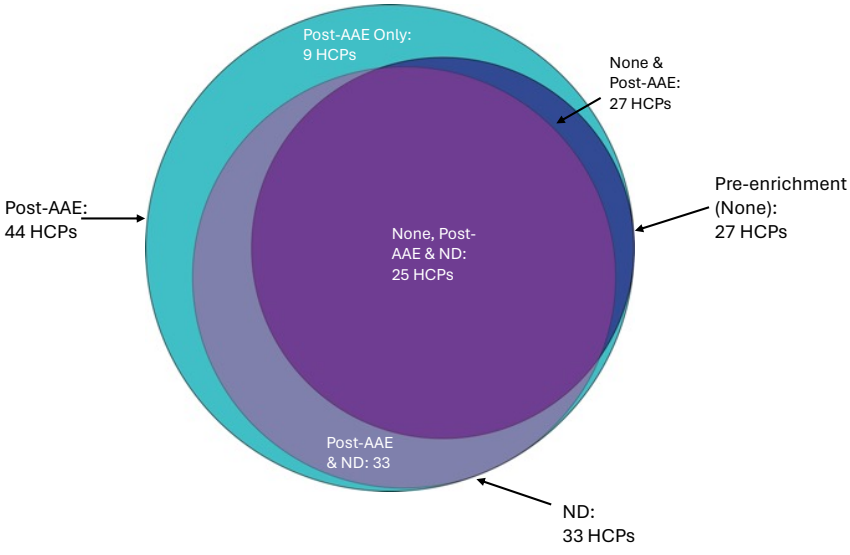
the median isoelectric points, and rows 5-6 show the median coefficient of variance for grouped protein abundance values.

DISCUSSION

Residual HCPs in biopharmaceutics are undesired process-related impurities that need to be well controlled. Besides monitoring the total HCP levels, understanding their physicochemical and biochemical properties is important for achieving maximal HCP removal. The major challenge of LC-MS based methods for HCP characterization in drug substances is the limited dynamic range (3–4 orders of magnitude) that most high-resolution mass spectrometers can achieve [22] compared to the wide dynamic ranges (>5 orders of magnitude) required to detect the low-level HCPs (<10 ppm). The ND method developed by Huang et al. [18] is an efficient way to deplete therapeutic proteins, and therefore reduces the dynamic range requirement and improves the sensitivity for HCP detection. However, while the ND method has been widely adapted for HCP characterization due to its simplicity and high sensitivity, it does not enrich low abundance HCPs that may contribute to the total HCP amount in the final DS as quantified by an HCP ELISA. Our results showed that many HCPs were not detected by the ND but were detected when employing the AAE enrichment sample preparation. These HCPs have been enriched to above the limit of detection of LC-MS method due to their reactivity with the CHO HCP ELISA antibody. It is important to note that these HCPs contribute to the total HCP level as quantified by the corresponding CHO HCP ELISA, 3G (F550-1, Cygnus Technologies). At the same time, the only limitation of the AAE enrichment strategy is that it may miss low-level HCPs with no reactivity to the HCP ELISA Antibody which may still be present in the DS. Despite this limitation, AAE adequately depleted the DS and enriched for many HCPs that it still resulted in a deeper proteomic coverage than ND alone. Our results confirm the superiority of AAE over ND to detect and possibly quantify HCPs in DS samples.

Figure 2. The Quantitative Venn Diagram displays unique identifications of HCPs in the DS #1

Figure 3. The Quantitative Venn Diagram displays unique identifications of HCPs in the DS #2



ABOUT CYGNUS TECHNOLOGIES

Cygnus Technologies, part of Maravai LifeSciences, offers generic HCP ELISA Kits for 23 different expression platforms, advanced orthogonal antibody coverage analysis services, HCP identification in process samples and drug substances by AAE-MS™, generic assay qualification services, and expert process-specific antibody and assay development services. For more information, visit us at: https://www.cygnustechnologies.com/custom-development-services/home

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