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HOST CELL PROTEINS

MOVING FROM DETECTION TO KNOWLEDGE-BUILDING

Derrick Zhang and Shankar Sellappan, and Brian Gazaille with Eric Bishop and Alla Zilberman

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Host Cell Proteins

Moving from Detection to Knowledge-Building

by Derrick Zhang and Shankar Sellappan,
and Brian Gazaille with Eric Bishop and Alla Zilberman

As analytical technologies gain in sophistication, process-development scientists are becoming more scrupulous in detecting and identifying process-related impurities, especially host cell proteins (HCPs) that are known to coelute with product proteins beyond protein-A capture. Read this eBook to learn about emerging approaches to HCP identification — and about the kinds of questions that come with improved understanding of HCP behavior.

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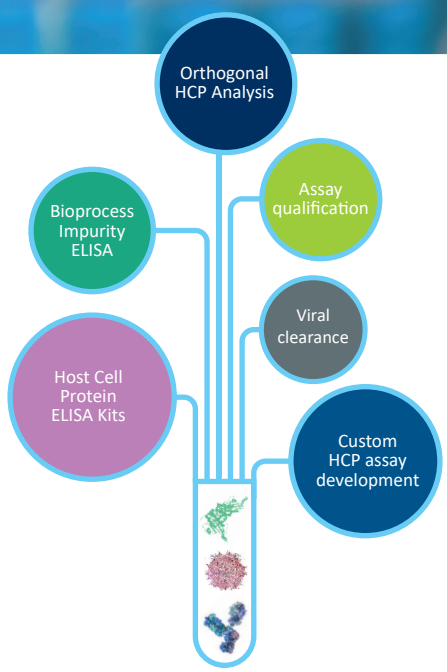

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New Needs for Host Cell Protein Identification

Brian Gazaille

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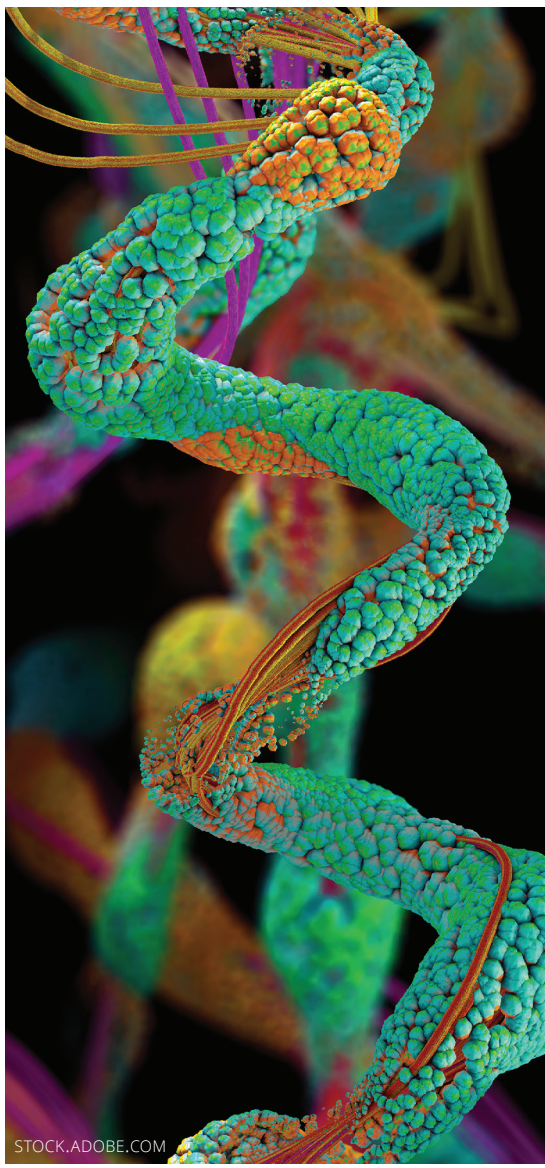

Biopharmaceutical manufacturers have good reasons to worry about process-related impurities being present in monoclonal antibody (mAb) and other recombinant-protein products. Cell-culture media, growth supplements, antibiotics, and even chromatography media all leave residuals that must be cleared from drug substances and/or products to ensure patient safety and product quality. The same is true for cell substrates that are used to express therapeutic proteins. Residual DNA, RNA, and endogenous proteins can persist through harvest and clarification steps — hence the need for capture- and polishing-chromatography processes as well as filtration steps.

Host cell proteins (HCPs) have become particularly notorious among process-related impurities. Purification workflows generally yield highly pure drug substances, and the biopharmaceutical industry has a strong track record of ensuring patient safety. However, some HCPs can coelute with therapeutic proteins into drug substances, and if those "hitchhiker" proteins are not detected and removed, they become unintended components of a final product. Such HCPs can activate immune responses in treated patients, including generation of antidrug antibodies and induction of cross-reactivity with therapeutic proteins (**1–5**). HCPs also can diminish product stability. For instance, some HCPs are known to degrade polysorbates that are used as stabilizers in drug-product formulations, and degradation events can diminish drug efficacy and potency (**6, 7**). Thus, regulatory guidances such as ICH Q6B require developers to detect HCPs and related contaminants that could remain in a drug substance and then to monitor impurity removal during purification processes (**8**). Rigorous risk assessment is a requirement (**9–12**).

Coelution events are not hypothetical possibilities. In a 2016 *AAPS Journal* article, Fischer et al. report on coelution of CHO phospholipase B-like 2 (PLBL2) with lebrikizumab, a humanized immunoglobulin G4 (IgG4) mAb that binds specifically to soluble interleukin 13 (**1**). At that time, the product was undergoing phase 3 clinical studies for treatment of asthma. After discovery of the coelution, the drug's developer initiated a special assessment. The resulting data indicated that ~90% of subjects developed a "specific and measurable immune response to PLBL2," although "no correlation between safety events and anti-PLBL2 antibodies could be made. Additionally, no impact on the incidence of anti-lebrikizumab antibodies was observed, suggesting the lack of an adjuvant effect from PLBL2."

The drug's developer promptly revisited its downstream purification processes and uncovered ways to minimize PLBL2 levels in drug substances. Using the resulting material in clinical trials led to "significantly less and dose-dependent frequency of immune responses to PLBL2" (**1**). But even though downstream scientists were able to ensure patient safety in this case, the coelution event solidified the need for further research into problematic HCPs (see the "Wanted" box) — not to mention the need for industry alignment about expectations for HCP detection and monitoring.

Subsequent efforts to investigate process-related impurities have been fruitful. For instance, in 2021, members of the BioPhorum Development Group HCP Workstream compiled comprehensive information from published data and industry experiences about "high-risk" HCPs that have proven to be difficult to remove by conventional purification approaches (**4**). The authors list problematic HCPs classified by type and level of risk and provide step-by-step recommendations for establishing HCP control strategies.

But as this eBook shows, much work remains for the biopharmaceutical industry regarding high-risk HCPs. Scientists not only need to identify problematic proteins, but also must gain a mechanistic understanding of their origins, ensure their removal during downstream processing, and validate the analytical methods by which they are detected.

Below I tease out implications from two HCP-focused presentations from the 2023 BioProcess International (BPI) Conference and Exhibition in Boston, MA. Those lectures destabilize how downstream scientists generally conceive of HCPs and, in turn, shed light on new approaches to their monitoring and removal. In the article that follows, scientists from the United States Pharmacopeia (USP) explore technologies for HCP detection. The USP writers call attention to the growing importance of liquid chromatography with mass spectroscopy (LC-MS) to HCP analytics

Wanted: Dead or Purified

In a 2021 article for *Biotechnology Progress*, Wilson et al. describe their use of mass spectrometry (MS) to identify a breadth of Chinese hamster ovary (CHO) host cell proteins (HCPs) generated during development of a process for an immunoglobulin G1 (IgG1) monoclonal antibody (mAb) (**5**). The writers also provide useful details about HCP species that are known to coelute with product proteins during protein-A chromatography. Below are some of the proteins that the writers discuss, with basic information about the biological processes in which those proteins are implicated. Additional information is listed for the notorious hamster phospholipase B-like 2 (PLBL2) (**3**).

HCP	Cellular Location	Implicated Processes
78-kDa glucose-regulated protein	Intracellular	Stress response in endoplasmic reticulum, protein folding
Actin cytoplasmic protein 1	Intracellular	Cytoskeletal structure, cell motility
Clusterin	Extracellular	Chaperone, protein folding, apoptosis
Elongation factor 1, α1	Intracellular	Protein translation and biosynthesis
Elongation factor 2	Intracellular	Protein translation and biosynthesis
Glutathione S-transferase P	Intracellular	Stress response, detoxification
Glyceraldehyde-3-phosphate dehydrogenase	Intracellular	Glycolysis
Hamster phospholipase B-like 2	Intracellular	Hydrolysis of phosphatidylcholine and phosphatidylethanolamine
Heat shock cognate 71-kDa protein	Intracellular	Stress response in endoplasmic reticulum, protein folding
Peptidyl-prolyl cis-trans isomerase	Unknown	Cell life cycle, acceleration of protein folding
Peroxiredoxin 1	Intracellular	Stress response to oxidation, cell redox homeostasis
Phosphoglycerate kinase 1	Intracellular	Glycolysis
Pyruvate kinase	Intracellular	Glycolysis
Serine protease HTRA1	Extra- and/or intracellular	Proteolysis

amid industry calls for detailed information about what particular HCPs are present at what quantities in a given sample. Such assays, the writers explain, require effective reference standards, such as peptides labeled with stable isotopes. In the eBook's final chapter, scientists from Cygnus Technologies focus on analytical methods for establishing the accuracy of HCP assays such as enzyme-linked immunosorbent assays (ELISAs) based on polyclonal anti-HCP antibodies. Traditional methods for ensuring sufficient antibody coverage are inadequate, the contributors contend, and more accurate and sensitive technologies now enable biologics manufacturers to gather valuable HCP information for regulatory submissions.

Understanding HCP Persistence

At the 2023 BPI Boston event, Chase E. Herman (an investigator at GSK) presented "Characterization and Clearance of HCP-Rich Aggregates in Monoclonal Antibody Bioprocessing," based on doctoral research that he performed at the University of Delaware. He observed that scientists tend to dichotomize impurities into *process-related impurities* (deriving from cellular and culture components and adventitious viruses) and *product-related impurities* such as aggregates, fragments, and charge variants. Based on those classifications, the industry has settled into platform purification approaches. For instance, protein-A affinity chromatography has become the industry workhorse for reducing HCP content in mAb products, and the method is usually quite effective, often decreasing HCP burdens by three or more orders of magnitude. Herman explained, however, that "it would be useful for us to have a better, more mechanistic understanding of how [certain] HCPs are able to persist" through protein-A capture and even downstream polishing steps. Developing that kind of understanding will require scientists to "reexamine the assumption that process- and product-related impurities are disjointed sets."

Herman pointed to previous studies showing closer ties between the two classes of impurities than might be expected. In a formative article, Gagnon et al. described their off-line analysis of harvested cell-culture fluid by quantitative polymerase chain reaction (qPCR) and two different HCP ELISAs (**13**). Testing revealed that high–molecular-weight (HMW) species in the sample — which normally are treated as aggregates to be removed during polishing steps — contained a complex mixture of chromatin-derived complexes and endogenous proteins. Herman noted that, especially considering protein A chromatography's limitations for aggregate clearance, downstream scientists have good reason to investigate aggregates as mediators of HCP persistence beyond chromatography.

Herman and his colleagues sought to understand how HCP-rich aggregates influence HCP clearance and analysis (**14–16**). In one set of experiments, the team performed size-exclusion chromatography (SEC) to fractionate samples of harvested cell-

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> It would be useful for us to have a better, more mechanistic understanding of how [certain] HCPs are able to persist, and to develop that understanding, I think that it is helpful to **REEXAMINE THE ASSUMPTION** that process- and product-related impurities are disjointed sets.
>
> —C.E. Herman

culture fluid and of protein-A eluate that had undergone viral inactivation. The resulting chromatograms showed that the protein-A step effectively cleared impurities of low molecular weight (LMW), but two distinct aggregate populations that were present in the culture-fluid samples persisted beyond the capture step. The aggregates even presented sharper peaks in the protein-A chromatogram, suggesting that purification to some extent concentrated those impurities.

To learn more about the compositions of those HMW populations, Herman's team performed cross-digest proteomic analysis by MS (**15, 17**). They leveraged both standard and native digestion techniques to facilitate comparison of samples from the culture fluid and protein-A eluate, partly because native-digestion sample preparation is designed to keep mAb products intact. In the cell-culture fluid, most of the identifiable HCPs were part of the aggregate populations. Quantitative proteomics revealed that, in the protein-A eluate, the highest observable HCP concentrations derived from large aggregates. And subsequent comparison of the SEC and proteomic data indicated that ~99% of HCP mass in the eluate material derived from those aggregates. Herman explained, "It is not just that [large aggregates] are implicated in the persistence of HCPs; they appear to mediate the majority of HCP persistence through the capture step." He cautioned, however, that "false negatives" could be a "nontrivial problem" for proteomic analysis of protein-A eluate and related sample types: Scientists can be confident about observed HCPs, but the same cannot be said for undetectable and/or unknown impurities.

Based on such results, Herman's team performed other experiments to explore implications for chromatography processes and materials. Having isolated the HCP content, the team also used dynamic light scattering (DLS) to measure the aggregates' sizes. Assay results indicated that the aggregates had hydrodynamic radii of ~50 nm, which is comparable to the pore sizes of some protein-A resin beads. Thus, resin fouling and capacity loss are plausible explanations for why aggregate-borne HCPs sometimes persist through capture chromatography.

The group also studied aggregate adsorption behavior by applying confocal microscopy to protein-A resin beads exposed to HCP-rich aggregates at different pH levels (**16**). At pH 7, HMW species tended to adsorb to the beads and coelute with product proteins after a low-pH wash. However, aggregates were less likely to coelute under more basic conditions (e.g., pH 10). Thus, high-pH washes could facilitate partial removal of HCP-rich aggregates during mAb capture processes.

That said, clearance capability can differ significantly across protein-A resins. Herman's team observed appreciable differences in aggregate removal by three commercially available anion-exchange (AEX) resins. For all three resins, small aggregates tended to flow through during mAb capture, as was expected. But only one resin appreciably retained large aggregates during mAb

elution. Potential factors included that resin's comparatively high salt sensitivity, large throughpores, branched throughpore structure, and hydrophobic properties.

Herman concluded by noting that evaluations of large-aggregate content could serve as "surrogate measurements" for HCP content and impurity clearance, assuming sufficient validation of the concept. He added that downstream scientists might reconceive of how they address HCP clearance by selecting protein-A resins that can help in retaining large-aggregate species.

Building Mechanistic Knowledge with SEC

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In "SEC in Tandem with MS: A Powerful Tool To Understand HCP Risk," Younghoon Oh (senior scientist in protein active pharmaceutical ingredients at Johnson and Johnson's Innovative Medicine division, formerly Janssen) presented a complementary discussion about the need for improved HCP characterization. Also reporting on doctoral research performed at the University of Delaware, Oh explored how SEC with liquid chromatography and tandem mass spectroscopy (LC-MS/MS) could enhance understanding of mAb HCP profiles and mechanisms underlying HCP persistence (**18, 19**).

Oh highlighted SEC's advantages for HCP detection. In one set of experiments, his team compared LC-MS/MS's effectiveness when using unfractionated samples and materials that underwent SEC fractionation. Samples came from harvested cell-culture fluid from seven mAb processes. For all seven, SEC with LC-MS/MS identified 30–288% more host-cell impurities than were identified by standard LC-MS/MS. Results were similar when analyzing protein-A eluate. Thus, SEC-based fractionation could help analysts to generate more complete HCP profiles than would be possible with standard LC-MS/MS workflows. Oh added that process-development scientists generally should be wary about how many HCPs might remain undetected when LC-MS/MS is applied to unfractionated samples, especially knowing that high-risk HCPs from HMW species can persist through chromatographic purification. He suggested that developers ask, "Can any of the unidentified HCPs in the HMW species cause impacts on product quality or patient safety?"

SEC-LC-MS/MS can enhance both HCP identification and quantitation, Oh continued. He demonstrated how the method could measure the average mass of the top 20 HCPs in different SEC fractions, including those with large HMW species, small HMW species, main monomers, and LMW species. In the large-HMW fraction, for instance, two extracellular-matrix proteins and four chaperone proteins accounted for 21.5% and 7.9% of the total HCP burden, respectively, whereas six chaperone proteins represented the highest HCP burden in the fraction with small HMW species. Chaperone proteins also represented a significant proportion of the impurities in the main-monomer fraction. The LMW fraction, however, contained relatively few chaperone proteins, although those impurities were particular to that SEC fraction.

Using such data, analysts can visualize the uneven distribution of HCPs in a given sample, thereby laying a foundation for understanding those impurities' origins and behaviors. Oh highlighted several areas for further research. Scientists might investigate HCPs that are particular to the mAb-monomer fraction, elucidating whether such proteins are mAb-associated. Future studies also could explore different SEC operating conditions — e.g., by comparing the performance of native SEC and biodenaturing SEC. Whereas the former technique preserves mAb–HCP interactions, the latter is designed to disrupt such interactions. The reasoning, Oh explained, is to determine whether HCPs detected in HMW fractions under native conditions also are detected in mild denaturing conditions. If so, then such HCPs are likely to be strongly bound to mAbs (**19**).

Oh cautioned against the tendency to speak about *host cell protein* as a singular entity. As new methods emerge for HCP detection, identification, and quantitation, it is increasingly clear that such proteins are not monolithic. Rather, each type of HCP exhibits distinctive biophysical properties and molecular associations, making them more complex and more unpredictable than developers might have surmised in the past. SEC-LC-MS/MS could help to tease out some of those complexities, helping drug developers to improve product quality while bolstering patient safety.

To facilitate subsequent HCP research, Oh's group has included with their publications supplemental data about CHO HCPs identified during their studies, including information about the cell lines, culture conditions, and purification parameters that they applied (**18, 19**). With such data, process-development scientists can begin to develop mechanistic understanding of the many HCPs that could be present in drug substances and products.

Oh cautioned against the tendency to speak about *host cell protein* as a singular entity. As new methods emerge for HCP detection, identification, and quantitation, it is increasingly clear that such proteins are **NOT MONOLITHIC.**

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Using Stable-Isotope–Labeled Peptide Analytical Reference Materials

for Quantifying Host Cell Proteins in Protein Therapeutics

Derrick Zhang and Shankar Sellappan

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Protein therapeutics, including monoclonal antibodies (mAbs), are powerful tools for treating myriad illnesses, including cancer, autoimmune disorders, and infectious diseases. However, such products require complex manufacturing processes. Recombinant-protein production usually occurs in a host cell line, a process that involves challenges — e.g., generation of host cell proteins (HCPs) — that do not arise during chemical synthesis of small-molecule pharmaceuticals. HCPs are a diverse group of process-related impurities that can copurify with drug substances (DSs). Some such proteins are immunogenic, and their presence in a final drug product (DP) can harm patients. Therefore, biomanufacturers must monitor HCP levels closely.

Chinese hamster ovary (CHO) cells often are used to express mAbs, other recombinant proteins, and some vaccines because they are easy to culture and can carry out necessary posttranslational modifications (PTMs) (**1**). But CHO cells also generate many HCPs. To date, ~6000 HCPs have been identified in CHO cells, and a subset of those are considered to be "high risk" because they can compromise drug efficacy and/or patient safety (**1, 2**). In addition to being immunogenic, some high-risk HCPs can degrade excipients used in a DP formulation as well as the active pharmaceutical ingredient (API) itself. High-risk HCPs also can decrease drug-product stability, potentially leading to aggregation or degradation and thereby reducing product shelf life. Available literature contains documented cases of HCPs harming patients (**3**), so manufacturers and regulatory authorities are understandably concerned about mitigating HCP-associated risks.

Recent advances in process development have enabled biologic manufacturers to decrease the total number and concentration of HCPs in final products. However, eliminating all HCPs during protein expression is not feasible, so manufacturers must develop analytical methods to measure and monitor the presence of such impurities throughout purification processes. Thus, HCP assays ensure product quality and demonstrate process control, both of which are critical for regulatory approval and commercialization.

To date, **~6000 HOST CELL PROTEINS** have been identified in Chinese hamster ovary cells, and a subset of those are considered to be "high risk" because they can compromise drug efficacy and/or patient safety.

Detecting HCPs with ELISAs

HCP detection is a critical aspect of biopharmaceutical quality control (QC) because it enables manufacturers to identify and mitigate safety risks. Moreover, consistent quantification of HCPs across batches ensures process reliability and product uniformity, thus reducing variability across batches. An HCP assay should be capable of detecting most such proteins early in a workstream (e.g., during cell harvesting) and must have a high sensitivity for contaminants that might be present at low levels in a sample. The latter consideration is particularly important for manufacturers that are focused on accelerating time to market. Assays that lack sufficient HCP coverage or fail to detect high-risk HCPs in a purified and formulated product could prompt regulators to request further assay development, resulting in project delays.

For the past couple of decades, enzyme-linked immunosorbent assays (ELISAs) have been used frequently to detect and measure the presence of HCPs (Figure 1). The method's popularity comes from its high selectivity and specificity, which enable detection of HCPs in concentrations in the low parts-per-million (ppm) range (**4**). ELISAs provide a single value for total HCPs, which is sufficient when the contaminants in a sample pose similar risks. However, designing and validating ELISAs can be challenging due to the diversity of potential HCPs and the lack of exact standards for quantification. Complex samples containing multiple high-risk HCPs can be particularly difficult to characterize in those regards.

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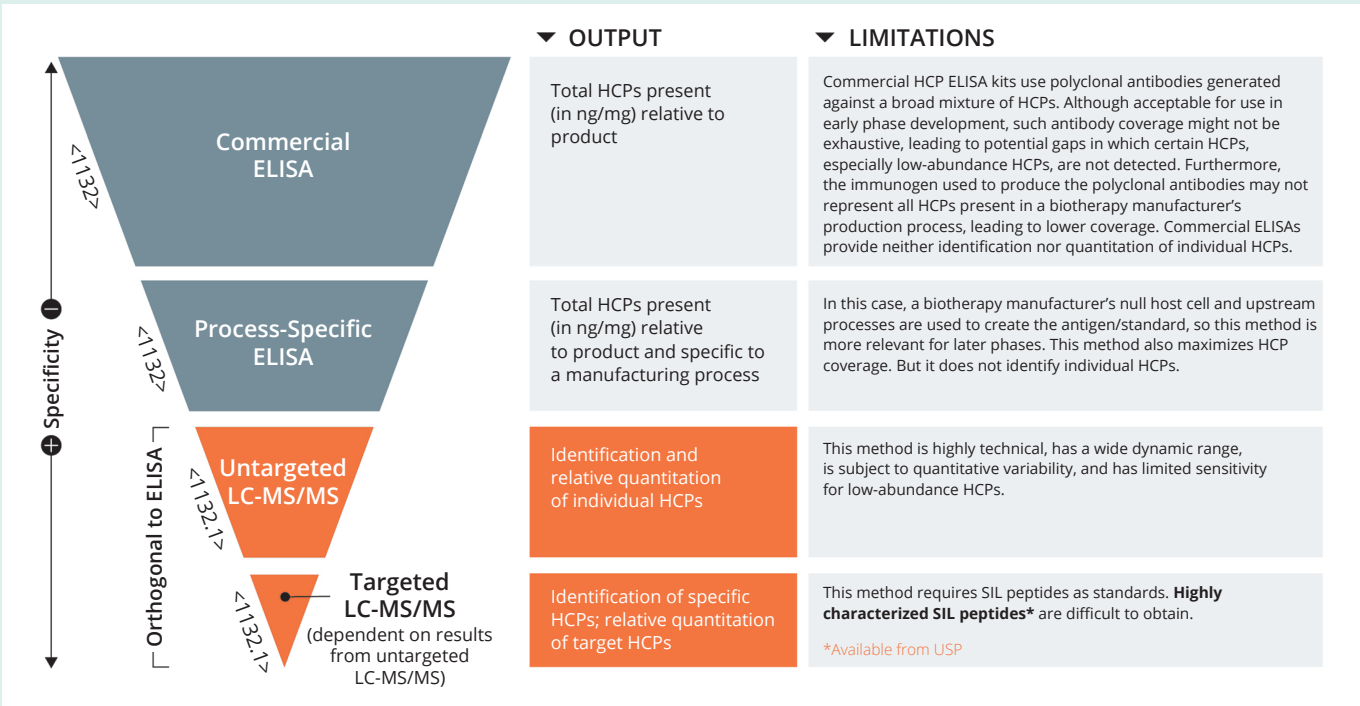
Figure 1: Overview of methods used to assess host cell proteins (HCPs) in therapeutic-protein drug substance; bracketed numbers refer to relevant chapters in the *United States Pharmacopeia–National Formulary*. (ELISA = enzyme-linked immunosorbent assay, LC-MS/MS = liquid chromatography with tandem mass spectroscopy)



Developing an ELISA for each impurity is impractical given the large number of HCPs that have been identified so far (**5**). Some high-risk HCPs even can be present in quantities in the sub-ppm range, which falls outside the ELISA limit of detection (LoD) (**6**). ELISAs also require a primary antibody to target HCPs effectively, necessitating extensive development before the assays can be used on samples.

Identifying Specific HCPs

Until recently, ELISA was the only method sensitive enough to detect HCP impurities in milligram samples of DS (**7**). However, the biopharmaceutical industry has worked to develop and implement improved detection methods based on liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC-MS/MS methods offer several advantages over standard ELISAs, including the ability to gather information about a sample's HCP profile and the ability to identify impurities early in product development. LC-MS/MS methods also can determine relative levels of individual HCPs, including those that cannot be detected or fully quantified by ELISAs due to the lack of specific HCP antibodies. Identification of individual HCPs enables manufacturers to optimize downstream processes before good manufacturing practice (GMP) manufacture of product batches (**8, 9**).

LC-MS/MS HCP methods are faster to develop than ELISAs are, and several LC-MS/MS workflows based on instruments, database-search software, and quantification methods from different vendors have proven to be successful. Such results have convinced many companies to use LC-MS/MS as an orthogonal method to ELISAs, and many contract research organizations (CROs) implement it routinely (**10, 11**). However, MS-based methods have their own specific considerations. For instance, the high sensitivity of MS makes it susceptible to buffer and matrix interference. Thus, sample preparation is a critical step in HCP analysis. The high sensitivity also necessitates use of highly pure reagents.

Stable-Isotope–Labeled (SIL) Peptides

HCP analysis by LC–MS/MS involves a complex workflow with several steps for sample preparation, including denaturation, reduction, alkylation, and tryptic digestion of proteins into peptides (**4**). Then, LC-MS/MS is applied to determine the amino-acid sequences of the peptides produced enzymatically. However, the physicochemical diversity of those peptides complicates accurate HCP quantification: Each peptide has distinct fragment ions and different levels of ionization efficiency and detectability.

SIL peptides have emerged as a powerful tool for overcoming technical barriers associated with LC-MS/MS identification of HCPs. Such peptides are synthesized with amino acids that contain stable isotopes and are designed to be identical to specifically chosen fragments of a target HCP. The peptides' specificity minimizes interference from other sample components, improving

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Stable-isotope–labeled peptides have emerged as a powerful tool for **OVERCOMING TECHNICAL BARRIERS** associated with liquid chromatography–tandem mass spectrometry identification of host cell proteins.

measurement accuracy such that they can serve as internal standards in MS-based quantification methods for measuring HCP levels in complex samples.

Applications for High-Risk HCPs

To help manufacturers identify specific HCPs in their products, the United States Pharmacopeia (USP) has developed several SIL peptides for two of the highest-risk HCPs found in CHO-based products: clusterin and lipoprotein lipase (LPL). Both present manufacturers with unique but equally significant problems. Clusterin is a disulfide-linked heterodimeric glycoprotein associated with apoptosis. Thus, the protein poses a significant immunogenicity risk (**12**). LPL is an enzyme that can degrade polysorbates in DP formulations. It can diminish protein stability, cause particle formation, and reduce DP shelf life (**13**).

USP's SIL peptides can be spiked into samples before or after digestion and used as calibration standards for HCP measurements (Figure 2). Then, manufacturers can analyze their samples using MS methods described in the proposed *United States Pharmacopeia–National Formulary* (*USP–NF*) General Chapter ‹1132.1› "Residual Host Cell Protein Measurement in Biopharmaceuticals by Mass Spectrometry." The documentary standard provides both new and experienced MS users with critical information on steps in a typical LC-MS/MS workflow for HCP analysis, including protocols for sample preparation and ultra/high-performance liquid chromatography (UHPLC and HPLC). The resource also details MS methods such as multiple-reaction

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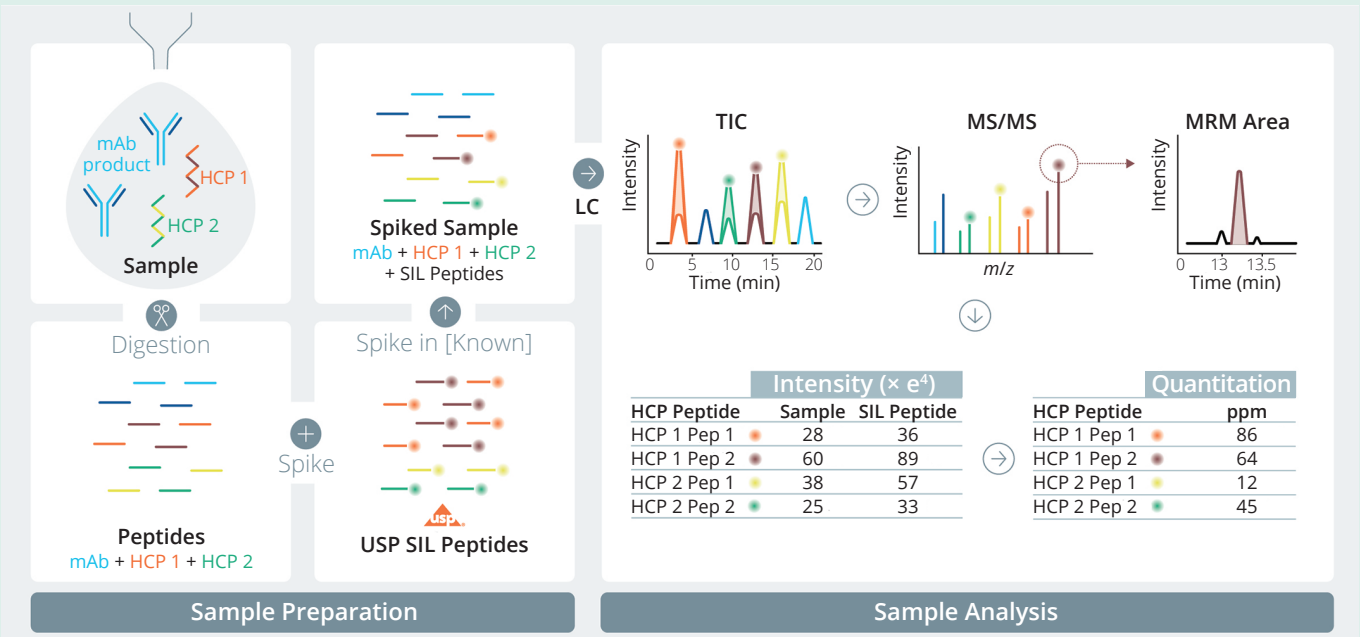
Figure 2: Sample preparation for liquid chromatography with tandem mass spectroscopy (LC-MS/MS) methods used to identify host cell proteins (HCPs) in complex samples; *m/z* = mass:charge ratio, mAb = monoclonal antibody, MRM = multiple-reaction monitoring, SIL = stable-isotope–labeled, TIC = total ion current, USP = United States Pharmacopeia



monitoring (MRM), which relies on specific detection of predefined SIL peptides and their corresponding unlabeled counterparts (**14**). To facilitate critical bridging studies, USP provides manufacturers with guidance on data analysis and best practices for reporting comparisons of ELISA and LC-MS data.

Enhancing HCP Control

ELISAs remain an important tool for high-throughput monitoring of immunoreactive HCPs. Such assays have high sensitivity, with an LoD of ~1 ppm. They are relatively easy to perform in standard analytical laboratories, and they can be validated for GMP release testing. However, ELISAs neither identify nor quantify individual HCPs in complex samples, and such assays come with risks for missing nonimmunoreactive HCPs.

Recent application of LC-MS/MS has shown that protein reagents and therapeutics such as mAbs still might contain unacceptable amounts of high-risk HCP impurities. Together, LC-MS/MS, proteomics technology, and CHO-protein databases are advancing HCP detection. However, some manufacturers find it difficult to generate adequate impurity profiles from DS samples because of their extremely low levels of individual HCPs.

SIL peptides represent new and powerful means for quantification of HCPs in CHO-based protein manufacturing processes. SIL-based methods can cover a wide dynamic range, enabling quantification of low-abundance HCPs in therapeutic-protein samples. To help manufacturers with SIL-peptide adoption, USP has published documentary standards that directly address applications in MS-based methods for identifying HCPs. The publication describes method validation and provides system-suitability recommendations regarding SIL peptides as calibration standards. USP also has developed a set of SIL peptides for two particularly high-risk HCPs. Used together, these tools enable reliable HCP quantification, helping manufacturers demonstrate control over HCP levels in their recombinant-protein and mAb products and thus ensure that such products meet safety and efficacy standards.

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To help manufacturers with SIL-peptide adoption, USP has published **DOCUMENTARY STANDARDS** that directly address applications in MS-based methods for identifying host cell proteins.

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Enhancing HCP-Antibody Coverage Analysis

New Possibilities for Identifying Process-Related Impurities and Demonstrating Immunoassay Suitability

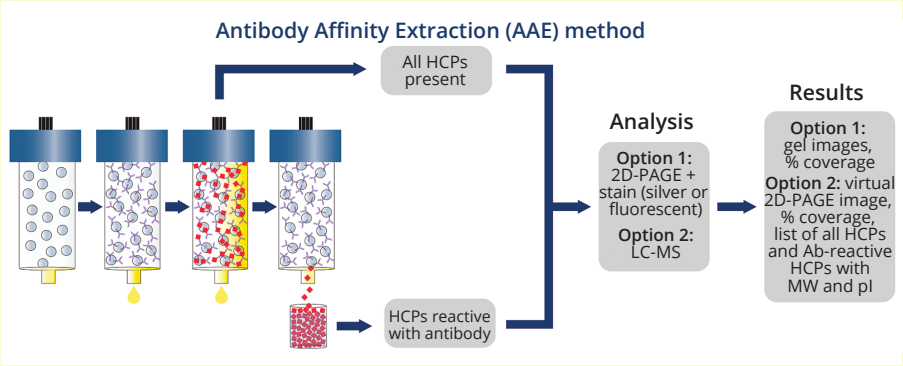
Brian Gazaille with Eric Bishop and Alla Zilberman

Biopharmaceutical manufacturers must demonstrate to regulatory agencies that their downstream purification processes sufficiently remove impurities from drug substances (DSs). Of particular concern are host cell proteins (HCPs), some of which are known to persist through chromatography steps. As the previous chapters of this eBook show, such HCPs can diminish the stability of product proteins and even cause adverse side effects in patients.

To monitor the effectiveness and consistency of downstream purification processes, biomanufacturers rely primarily on HCP enzyme-linked immunosorbent assays (ELISAs). But even ELISAs have analytical limitations, necessitating orthogonal confirmation of their ability to detect HCPs. Specifically, biomanufacturers must show that the type of antibody used in a given ELISA provides adequate detection capability — hence the need for HCP-antibody coverage analyses. Until recently, available methods for coverage assessment have been lacking in accuracy, robustness, and detection capability, all of which are critical to ensuring removal of especially problematic impurities from DSs. To learn about possibilities for HCP-antibody coverage analysis, I corresponded with Eric Bishop (vice president for research and development, R&D) and Alla Zilberman (vice president of technical marketing), both of Cygnus Technologies (part of Maravai LifeSciences).

Bishop and Zilberman described factors accounting for the inadequacy of traditional assessment methods. They also explained how their company's Antibody Affinity Extraction (AAE) immunoaffinity chromatography method can overcome such limitations to provide biomanufacturers and regulatory reviewers with additional information about what impurities are present in a given sample and in what quantities (Figure 1). A key theme in their discussion is the need to reframe the coverage-analysis conversation such that biologics manufacturers are encouraged to obtain information about specific types of HCPs, especially those that can coelute with DSs.

Figure 1: Antibody (Ab) coverage by AAE chromatography with options for analysis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or liquid chromatography with mass spectrometry (LC-MS) analysis; HCPs = host cell proteins, MW = molecular weight, pI = isoelectric point



The Evolution of ELISA Evaluation

Why are ELISAs the industry standard for HCP detection and monitoring? What are some of the method's strengths and limitations, and how do analysts generally apply it? ELISA continues to be the gold-standard method for HCP-related process monitoring and release testing. The most important reason why is that ELISA can measure ng/mL levels of HCPs in the presence of mg/mL levels of product proteins. The assay does not require special expertise to run, and an organization can transfer it easily across laboratories or to a contract development and manufacturing organization (CDMO).

Unfortunately, HCP ELISAs have some limitations. At the end of a run, a plate spectrophotometer takes absorbance measurements for each sample. Those results cannot tell you what HCPs are in a sample or which HCPs the assay reacted to. That is because HCP ELISAs do not measure ng/mL quantities of endogenous proteins as such. Actually, such tests measure an immunological equivalent relative to standards. Therefore, results are subject to change, sometimes significantly, with a change of kit or reagents. And although ELISA is not difficult to run, it requires significant expertise to develop a broadly reactive HCP antibody and then turn it into a sensitive, specific, and robust immunoassay that can recognize close to 2000–3000 types of HCPs. Because of the complexity of that process, it can take 12–14 months to develop a new HCP ELISA and generate requisite reagents.

Such difficulties are not lost on regulatory agencies. As a result, biomanufacturers must prove that reported HCP levels are low because they truly are low and not because of an insensitive assay. That necessitates incorporation of orthogonal methods for HCP-antibody coverage analysis to help assess an antibody's ability to recognize a breadth of HCPs that are present in calibration standards and

that might be present in process samples and DSs.

Historically, how have analysts demonstrated that an ELISA provides sufficient detection of process-specific HCPs? For lack of better coverage-analysis methods, analysts have performed two-dimensional western blot (2D WB), based on large-format two-dimensional polyacrylamide gel electrophoresis (2D PAGE) gels with protein transfer to membranes for 2D WB comparison to silver stain. Because of the recognized sensitivity limitations of 2D WB, the conventional acceptance criteria are that >50% of the total HCP content should be reactive and that the antibody must recognize HCPs in all four quadrants of a 2D PAGE gel — those quadrants representing species of low molecular weight (LMW), high molecular weight (HMW), low isoelectric point (LpI), and high isoelectric point (HpI). Coverage assessment by 2D WB must be performed on null or mock-transfected upstream harvest.

Why are such orthogonal methods inadequate? While using 2D WB, coverage estimation requires upstream harvest samples, in which concentrations of most HCPs still fall within the sensitivity limits of various staining methods. In addition, 2D PAGE's limited loading capacity, destruction of native epitopes by harsh sample treatment, and potential for steric hindrance of HCP-antibody binding epitopes all decrease the sensitivity and specificity of 2D WB, which in turn significantly underestimates true antibody coverage to upstream HCPs. More important is that 2D WB cannot predict quantitatively how an ELISA antibody will react to the most important HCPs, which are those that copurify with DSs.

Unfortunately, regulatory guidelines still refer to 2D WB as an acceptable coverage-assessment method. That said, the *United States Pharmacopeia* (USP) General Chapter <1132> on "Residual Host Cell Protein Measurement in Biopharmaceuticals" and subsection <1132.1> on "Residual Host Cell Protein Measurement in Biopharmaceuticals by Mass Spectrometry" reference newer and more sensitive approaches, such as AAE immunoaffinity chromatography and methods based on mass spectroscopy (MS).

Enhancing Coverage Analysis

What is AAE immunoaffinity chromatography? It is an advanced orthogonal method designed to assess polyclonal-antibody coverage for an array of HCPs that are present in a given process and for downstream, process-specific HCPs that could copurify with DSs. Cygnus Technologies developed the method in 2013 to overcome analytical deficiencies with traditional 2D WB and two-dimensional differential in-blot electrophoresis (2D DIBE) methods used to assess antibody coverage to total HCP content. The AAE method's most important advantage over traditional approaches is that it mimics an ELISA's biophysical environment for interaction between antibodies and HCP antigens. Thus, the AAE approach is more predictive of HCP antibody performance in the corresponding HCP ELISA.

What does a typical AAE process entail? Polyclonal HCP antibodies from a corresponding HCP ELISA are immobilized covalently onto a chromatography support. Next, a column packed with that affinity resin is conditioned to prevent significant antibody leaching and to minimize nonspecific binding. A native, undenatured sample containing all HCPs from a given process is passed over the column for binding (Figure 1). The column is washed to ensure removal of nonspecifically bound HCPs or HCPs that remain on the column because of nonspecific protein–protein interactions. Then, immunoreactive HCPs are eluted with acid. The HCP sample is cycled again over the column, undergoing binding, washing, and elution for four cycles to collect enough HCP content for analysis and to enrich LMW proteins to the point of detection for silver-stain or MS methods. All HCP elution fractions are pooled, buffer-exchanged, and concentrated back to the original sample volume. The final "post-AAE" sample represents all immunoreactive HCPs.

What can analysts do with post-AAE samples, and how do those activities enhance ELISA antibody-coverage analysis? Post-AAE samples can be analyzed using MS or separated by 2D PAGE and analyzed by comparison with a silver stain of a starting, unextracted "pre-AAE" sample containing all HCPs that were present during upstream harvest (Figure 1). Pre-AAE and post-AAE samples also can be analyzed by differential gel electrophoresis (DIGE) using cyanine 3 and 5 (Cy3, Cy5) to label the extracted and starting, unextracted samples. Coverage is assessed by comparing the number of HCPs in the AAE elution fraction (using silver stain or Cy3 labeling) with that seen in the 2D PAGE of the starting antigen sample (using silver stain or Cy5 labeling).

The combination of AAE immunoaffinity chromatography with 2D PAGE or 2D DIGE represents a significant improvement for HCP analysis because it overcomes 2D WB's immunological and specificity limitations — not to mention WB's difficulties with sample preparation and transfers to membranes. In tandem with the AAE method, 2D-PAGE or 2D-DIGE testing can provide gels to assess whether an HCP antibody is broadly reactive — with immunoreactive proteins found in all four gel quadrants (e.g., for LMW, HMW, LpI, and HpI species) — and to estimate antibody-coverage percentage. This approach does not, however, provide information about HCP identity.

Combining the AAE method with mass spectrometry — Cygnus's proprietary AAE-MS technique — for HCP-antibody coverage analysis provides significantly more information. In addition to percentage of coverage, the method identifies both HCPs in harvest material and antibody-reactive HCPs, and it yields protein MW and pI information. Currently, the AAE-MS method is the only available approach that can assess HCP-antibody coverage reliably using samples of a product (e.g., DS) containing harvest material rather than clarified culture fluid (CCF) derived from a null cell line.

How else can the AAE method be applied? Although regulatory agencies traditionally have requested assessment of coverage for the total HCP mixture present in a cell-culture harvest stream, the most important HCPs with respect to patient safety and drug efficacy and stability are those that persist through a given purification process. Complete characterization of downstream HCPs is not part of current regulatory guidelines, but the value of such information to biopharmaceutical companies cannot be overestimated.

That said, certain challenges arise in performing LC-MS identification of HCPs in DSs. HCP abundance can be four to six orders of magnitude lower than that of a therapeutic antibody in solution. That factor precludes effective identification of low-abundance HCPs, necessitating sample preparation or separation strategies to close the gap.

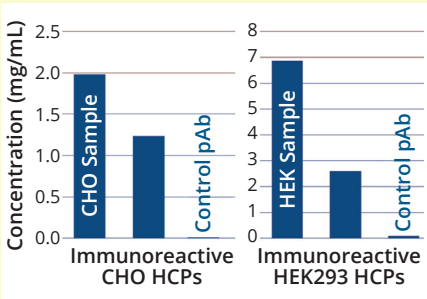
The AAE method is highly effective at enriching HCPs and depleting DS. Performing the AAE-MS technique on a final DS provides in-depth proteomic information on copurifying HCPs, helping biopharmaceutical manufacturers assess HCP-related risks to ensure product safety and efficacy.

Shifting the Coverage Conversation

What questions do AAE users approach you with? One frequently asked question concerns whether regulatory agencies have accepted HCP-antibody coverage analysis by AAE with 2D PAGE or by the AAE-MS method. We have completed more than 300 AAE-based coverage assessments in recent years. Data obtained from those studies have been submitted to regulatory agencies to support drug developers' respective investigational new drug (IND) or biologics license application (BLA) submissions. The value of AAE-based data is not surprising considering that the method has been featured in USP Chapter <1132> since 2017.

For companies that strive to make science-driven decisions, performing the AAE-MS method for coverage analysis is a logical approach. However, our customers often report that regulatory agencies hold them back and ask to supplement their AAE-MS data with results from an antiquated 2D WB-based coverage assessment that has little predictive value as to how an HCP antibody will perform in a corresponding HCP ELISA. We encourage HCP ELISA users as well as regulatory agencies and reviewers of chemistry, manufacturing, and controls (CMC) to reframe the coverage-assessment question from "What is the HCP-antibody coverage percentage?" to "What specific HCPs does your ELISA quantify?"

Figure 2: To assess the AAE method's specificity, (LEFT) a sample with Chinese hamster ovary (CHO) host cell proteins (HCPs) was passed over an F550-1 3G CHO HCP-antibody AAE column and over a nonimmune-goat IgG (negative control) column for fast protein liquid chromatography (FPLC). (RIGHT) Moreover, a sample with human embryonic kidney (HEK293) HCPs was passed over an F650S HEK293 HCP-antibody column and over a nonimmune-rabbit IgG (negative control) column for FPLC. Immunoreactive HCPs were eluted from the columns and quantified by liquid chromatography with mass spectrometry (LC-MS). Total CHO and HEK293 HCPs were quantified using LC-MS.



What advice do you have for scientists who work in HCP analytics? There are several schools of thought in HCP analytics. That includes many opinions about what type of assay to use and about the best way to perform coverage analysis. Keep in mind that tools are available to help us know if we are using the best assay for a given application.

The AAE-MS method provides a solid foundation in that regard. It enables us to understand our coverage analyses and ensures that the antibodies applied in an ELISA are broadly reactive to the total HCPs in a given process (Figure 2). In addition, the technique identifies those HCPs, so you can know which problematic proteins are present and whether your antibodies are reactive to them. With AAE-MS technology, you can identify all HCPs is a DS to perform meaningful risk assessments. Users easily can make a compelling case for their HCP assays when providing such data alongside strong assay qualification/validation data.

What other aspects of HCP analysis merit discussion? To date, regulatory expectations have not kept up with the technologies available for HCP analytics. However, the lack of regulatory expectation does not mean that biopharmaceutical manufacturers can shirk the work of HCP detection and identification. Early application of tools such as the AAE-MS method helps to identify HCPs that eventually could cause problems with product stability, product efficacy, and even patient safety. The better that an organization understands the HCP profile for a particular process, the higher will be its chance of overall success in clinical trials and, ultimately, the market.

Note, however, that even though regulations lag behind the current technology in HCP analytics, regulatory reviewers generally do not. They are scientists, too, and are willing to evaluate the data package presented to determine the suitability of an HCP ELISA or HCP control strategy. Advanced technologies such as the AAE method with MS detection provide valuable data to help drug developers and regulators alike feel comfortable about the selection of an HCP ELISA, therefore reducing risks for delays relating to IND or BLA approval.

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