

SCIENTIFIC WHITE PAPER

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Host cell proteins (HCPs) are common impurities in biologic drug products and are an obligatory critical quality attribute (CQA). The individual protein contaminants that make up the HCP profile, however, may vary significantly among individual biologic products and expression systems. As such, the HCP profile must be analyzed to identify even low levels of potentially concerning species in a biologic drug to ensure safety and efficacy. This whitepaper reviews fundamental aspects of HCPs pertaining to biologic drug development and current and emerging approaches to HCP analysis.

Host Cell Proteins

Biologic drugs are produced using living biological systems, which have highly complex milieus containing several thousand to tens of thousands of unique macromolecular species. The majority of biotherapeutics today are recombinant proteins expressed in cultured cells, while others are cellular products. Because biologics are derived from cellular systems, host cell proteins (HCPs) that are co-produced with the biotherapeutic are abundant at harvest and represent the major class of impurities in the final product. As such, HCPs are an obligatory critical quality attribute (CQA) of biologic drug products because these residual impurities can affect product quality, efficacy and safety as well as induce or enhance immunogenicity. To ensure consistency of product manufacture and shelf-life stability and to minimize potential adverse reactions, significant

attention must be paid to identifying HCPs that remain in a biologic product following purification. Once identified, HCPs often are monitored, and the levels controlled to consistently yield a safe and efficacious product.^{1,2,3} While ELISA methods have historically been the main approach to detecting HCPs, LC-MS based HCP detection approaches have increasingly become an expected orthogonal standard in successful biologic development. The specific advantages and application of ELISA and LC-MS methods for HCP profiling are discussed below (Table 1).

The specific components and amounts of HCPs in a drug product depend on numerous factors, including the type of host cell used for production (bacteria, yeast, insect, mammalian, plant), culture media conditions and purification approaches.^{2,4-6} As such, residual HCP profiles can vary widely, particularly with different hosts, and

impurities from individual hosts can result in unique risks in clinical practice. Production in *E. coli* or yeast (e.g., *S. cerevisiae*, *P. pastoris*) leads to a smaller pool of potential HCPs due to the relative simplicity of these organisms, which have approximately 4300 and 5300 protein-encoding genes, respectively, compared to mammalian cells (e.g., CHO) with approximately 30,000 genes and plants, such as tobacco, which can have over 60,000 protein-encoding genes. The type of proteins expressed in microbial or plant cells are more distantly related to human proteins and have potential to be recognized as foreign by a recipient's immune system, and could lead to suspension of a clinical trial due to immune reactions to the foreign HCPs.^{10,11,12} On the other hand, problems can also arise from HCP impurities that are closely related to the biologic drug. The presence of a homologous protein from the host cell in a drug product can diminish potency and efficacy, stemming from competitive binding, and has led to serious reactions such as induction of neutralizing anti-drug antibodies (ADA) to the recombinant therapeutic product.^{11,13,14} In a few specific cases, further complications have occurred due to ADA cross-reactivity with the patient's endogenous protein.^{11,13} This type of HCP is a common concern, particularly for enzyme replacement therapies (ERT), where homologous or closely related host cell proteins to the intended therapeutic product also are expressed in the host cell. Because the homologous host protein is so similar, separation is difficult and co-purification with the recombinant therapeutic protein often occurs.^{10,12,15} The ability to distinguish such HCPs from the intended protein product, therefore, is a key aspect of developing an effective HCP assay. An additional consideration is that post-translational modifications vary among different host cells. Glycan modifications, for example, are heterogeneous inherently and differences in occupancy and relative amounts of individual

species can result from different expression systems. The glycan composition profile can affect pharmacokinetics, pharmacodynamics and biodistribution of a protein,^{16,17} for example, by affecting clearance rate, but more concerning is that small differences can alter immunogenic potential. Some host cells may generate foreign PTMs that potentially promote undesirable immunological reactions, for example unique glycan structures on HCPs may be present that are not observed in humans and may be recognized as foreign.^{11,16,18} In such cases, detection of the relevant impurity would require not only detection of the protein but recognition of the specifically modified form by the HCP assay. Modifications often appear at low-levels, and consequently, both specificity and sensitive detection are needed to identify and monitor modified species.

Within a specific host cell type (e.g., CHO cells) there is more consistency, and studies have reported significant overlap among HCP species detected in recombinant protein products, providing some general awareness about effects of a few defined process-related impurities.^{4,7,19} For example, mAbs are typically purified using Protein A resin, which can leach into the product during purification and cause toxicity as well as result in retention of similar HCPs.⁷ However, it also has been observed that most HCPs found in purified products accompany the active molecule despite extensive purification because of appreciable binding to it. Consequently, differences in HCP content among even very closely related biotherapeutic molecules (e.g., two IgG1 mAbs) can be significant and meaningfully alter clinical response.⁸ Substantive differences in HCPs also have been reported for the same molecule following altered process changes, which often occur during the normal progression of drug development, scale up and post-market.^{2,6}

	Method	Advantages	Limitations
Immunoassays	HCP-specific immunoassay	High sensitivity (LOQ <0.1 ppm)	Lengthy development time (months)
		High throughput	Requires generation and validation of custom immunoreagents
		QC compatible	Finite shelf-life of immunoreagents; assay requires periodic redevelopment
Mass spectrometry	Multiple reaction monitoring (MRM); Targeted absolute quantitation	Sensitive detection (single digit ppm)	Isotopically labeled peptides or protein required
		Excellent accuracy and precision	Analysis takes weeks
	Select top peptides for quantitation	Fast estimate of concentration	Significant margin of error (within ~2-fold of actual concentration)
	Spectral counting for relative quantitation	Rapid assessment of relative amounts	Lower precision than other MS approaches
Effective comparison among samples			

One important consideration in HCP assessment is that not all HCPs are equal with respect to pharmaceutical stability and clinical impact.² Specific HCPs have been shown to diminish the integrity of biologic products in distinct ways and, recently, have been responsible for adverse reactions leading to the termination of two clinical trials.^{9,10} Not only do individual HCP components need to be identified but the level at which each is present should be determined and, whenever possible, correlated with risk. Residual enzymes present at very low levels in drug products have been reported to cause product degradation.^{6,20} Several studies have identified proteases, such as Cathepsin D, as the source of fragmentation of active protein therapeutics.²¹⁻²⁴ Protein disulfide isomerase and other proteins with reactive cysteine residues can promote thiol/disulfide exchange, and these residual contaminants have been found to cause aggregation of proteins.^{25,26}

Other enzymes have been reported to degrade stabilizing surfactants.²⁷ This finding is broadly applicable because the majority of protein therapeutics are monoclonal antibodies (mAbs), and the majority of mAb products are formulated with polysorbate 20 (PS20) or 80 (PS80). Two lipases, lipoprotein lipase (LPL) and phospholipase B-like 2 (PLBL2), have been shown to hydrolyze PS20/80, cleaving them into fatty acid and alcohol components.²⁷ Polysorbates are added to protect biologics from surface-induced aggregation and their degradation can result in increased aggregate formation as well as phase separation and/or increased interaction of the degradants with the biologic molecule.²⁸⁻³⁰ Depending on the extent of breakdown and the specific product, this may reduce shelf-life, diminish potency and/or promote an immune reaction. Clinically, specific HCPs may pose a greater risk than others. In some cases, this may manifest as reduced efficacy due

to chemical degradation or loss of active to aggregation or safety concerns resulting from induction of a direct immunological reaction to a specific HCP or via HCP-induced aggregation of the biologic drug, which can then promote an immune response.^{6, 22-24}

Analytical Approaches to HCP Evaluation

While there is no single specified level of HCP content that is applicable for all products due to differences in dosing, for mAbs typically achieving less than 100 ppm total HCP has been considered generally acceptable,² and most biologic products contain 10-1000 ppm HCPs,^{1,34} with most current processes resulting in HCP levels less than 100 ppm. To detect such low levels, sensitive methods of detection are required to analyze HCPs and confirm individual residual components are reduced to sufficiently low levels in therapeutic products.³ Two techniques have emerged as primary tools for assessing HCPs, enzyme-linked immunosorbent assays (ELISAs) and liquid

chromatography coupled to mass spectrometry (LC-MS). Traditionally, ELISAs have been used to monitor HCPs in biologic products (Figure 1A) because they are easy to implement, fast and inexpensive once developed, making them amenable for use in a QC environment and as a release assay. However, the approach is susceptible to inadequate coverage and artifacts (especially with complex samples) and cannot identify individual HCP species without use of orthogonal techniques. Consequently, MS-based analysis is being developed more and more commonly as a bridging, correlating and/or supporting method for HCP analysis.³⁵ While the sophistication of MS approaches and analysis are not yet supported sufficiently for use in QC/release, MS can be used very effectively to identify specific differences in HCP profiles and inform understanding of process-derived residual impurities and to enable development of appropriate control strategies.^{36,37} They can also be used to evaluate HCP following process changes.

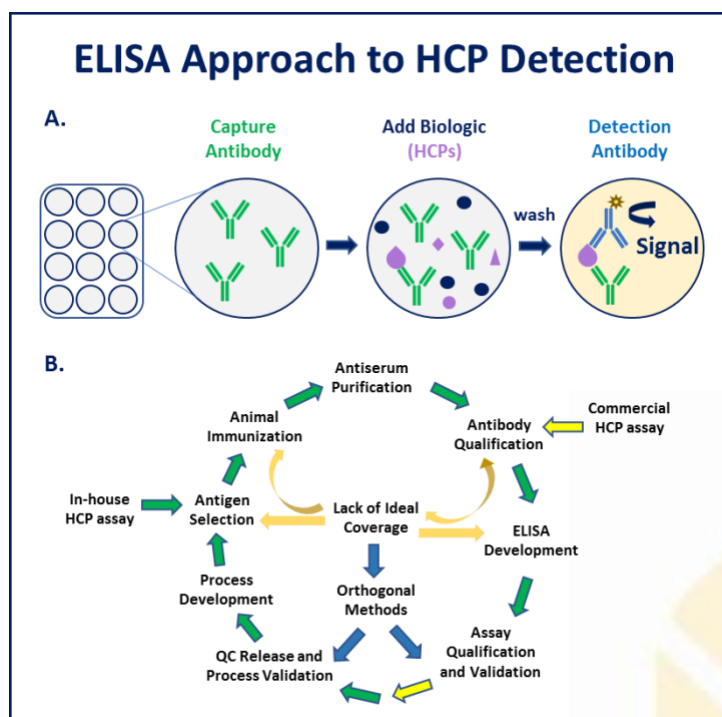


Figure 1. Schematic of a typical ELISA, showing how custom immunoreagents (blue and green) are needed to specifically recognize individual HCPs (purple droplet; Panel A). Panel B depicts the iterative processes involved in generating an ELISA for detecting HCPs in a biologic product (Panel B modified from figure in Wang, et al.¹).

ELISA Methods

ELISA assays are straightforward to perform (Figure 1A) and are typically consistent and reproducible once established, as they are carried out in a standard 96-well plate format in an automated fashion, which makes them highly compatible with the QC environment. ELISAs, once developed, are inexpensive to deploy. Nonetheless, development of each new ELISA requires substantial investment of time and resources to initially construct and validate. Prior to that point, development of an ELISA method involves iterative production, assessment and validation of custom immunoreagents and a significant number of permutations to evaluate assay conditions because numerous variables at each step in the assay need to be analyzed (Figure 1B). Initially, it is a significant undertaking to confirm the polyclonal pool of Capture Antibodies achieves sufficiently broad and sensitive coverage of HCPs relevant to the product. Frequently, incubation and washing steps depend on the specific sample being analyzed and can vary substantially for analyzing samples throughout the purification process, from cell culture harvest (HCCF) through drug substance (DS) to drug product (DP), which requires further optimization and validation of detection accuracy. Complete coverage is not possible to obtain with a single ELISA and understanding of the HCPs and their potential risks is important for ensuring product safety. An additional consideration of ELISA for a specific product is that the crucial custom immunoreagents have a limited shelf-life and must be reproduced and re-validated periodically to support ongoing analysis of marketed products.

For convenience in early development, commercial or platform ELISA kits are used for HCP screening, but they risk inadequate coverage or underestimation of total HCPs for specific processes. In later stages, more specialized process- and HCP-specific ELISAs are developed in house to better characterize the HCP profile of the product. These too require substantial

development effort and long lead times to allow for production and characterization of custom immunoreagents derived from animal sources, plus validation of adequate HCP coverage (Figure 1B).¹ For confirmation of identity, orthogonal approaches, such as 2D gel, Western blot and mass spectrometry (MS) analysis, are used in combination with ELISAs. Staining of 2D gels or Western blotting with polyclonal immunoreagents helps correlate total HCP signal with component proteins by general chemical attribute (size and charge), whose identities can be further determined by peptide digestion and MS analysis (Figure 2). Even without complete identification of individual protein components, differences between 2D gels (DIGE) can highlight changes between different processes and lots. While this may be sufficient to establish consistency among batches and processes, the presence of unknown components in a drug product increases clinical risk, which could be mitigated by MS analysis. As such, LC-MS methods are often used as an orthogonal technique to profile HCPs and identify individual impurities since ELISA cannot directly reveal the identity of an HCP.

Substantive differences in the proteomes of distinct host types demand that ELISA methods utilize a host-cell specific assay to analyze HCPs from the appropriately matched cell source. Because host cell protein expression is further influenced by culture conditions and methods as well as other aspects of living cells, such as age, in practice, the generic ELISA only provides about 75% coverage of HCPs and is effective as an initial assay for guiding development approximately 50% of the time. One common problem inherent in ELISA-based assays is that many production hosts produce impurities that are closely related to the desired product. For example, production of recombinant human tissue plasminogen activator (rhTPA) in CHO may also yield a drug substance that contains endogenous hamster TPA derived from the cellular host. Although the hamster sequence is not identical to human, it is very challenging to separate the products. In such a

case, the ELISA will produce false positive results for HCPs and this type of artifact is very difficult to remediate. The correct approach for mitigating such artifacts in ELISA-based HCP assays is to perform a blank run in which the host cell contents are analyzed in the absence of the recombinant biotherapeutic (blank vector) to establish background levels. Implementation of a blank run strategy is extremely expensive and time consuming, typically costing over \$2 million and taking a year to complete. This is in part because four to five blank production runs (at a cost of \$250-\$500k per run) may be needed to generate and isolate sufficient material for immunization of animals and then the immunoreagents and assay must be qualified and validated for use. A complicating aspect is that, in some cases, differences in the ability to recognize the HCP antigens have been noted between immunoreagents generated from blank and product-containing runs. As such, an entirely different approach may be preferential for effective evaluation of HCPs.

LC-MS Methods

Using modern, advanced instrumentation and approaches, HCP profiling by LC-MS provides comprehensive identification and relative quantitation of components down to low single-digit ppm.³⁸ Like ELISA, MS profiling of HCPs requires initial development of the method but has the benefit that no custom immunoreagents are needed. MS analysis involves sophisticated analytical instrumentation and advanced technical expertise of the analyst to conduct and, like ELISA, the approach requires significant upfront investment of resources. However, once the LC-MS method is developed, it can be applied routinely, without consumption of costly immunoreagents. While ELISAs are considered well-suited for product release testing because of their simplicity and convenience, LC-MS provides consistent, accurate and thorough coverage of HCPs to enable decision making in process development and to eliminate the need for

periodic re-validation.³⁹ Sample preparation is an important step for achieving high-quality data and results, which is a central aspect of LC-MS method development. While much is being done to move MS-based approaches toward a QC environment, presently the quality of MS results obtained depends on the skill of the mass spectrometrist and, as such, is utilized to inform ELISA-based HCP analysis and guide critical CMC, process development and control strategy decisions made during development. MS-based approaches to HCP analysis are being utilized more and more commonly and at earlier stages, because once a sensitive method is established, it can be applied in perpetuity without the need for repeated generation and validation of custom reagents. When applied early in development, MS can replace HCP-specific and process-specific ELISAs, eliminating the time-consuming and costly need to generate and validate custom immunoreagents.

LC-MS analysis of HCPs has quickly developed into a set of approaches, which have varying degrees of robustness and practicality for clinical development of biologic drugs, and that can be performed to profile the diverse set of species and target quantitation of specific components.^{36,39-42} The approach typically involves subjecting the sample to enzymatic digestion, followed by LC separation with high-resolution MS detection (Figure 2). The differences among the methods primarily are in the MS detection approach utilized. Data-dependent MS acquisition targets known peptides for identification from a pre-existing library and is used because it is fast, robust and sensitive, to at least 50 ppm and often to 10 ppm, which is well matched for monitoring impurities found at low levels. Data independent acquisition (DIA) is used to identify untargeted HCPs, and the results of DIA can be verified and provide quantitation using parallel reaction monitoring (PRM). A combination of the two approaches also has been applied to characterize HCPs.⁴¹ The LC separation approach also may differ.

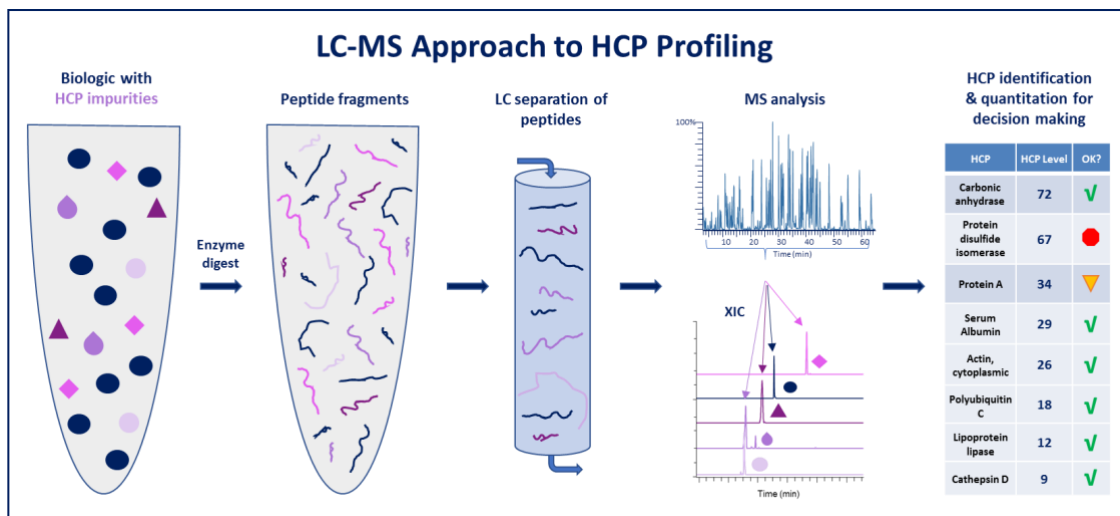


Figure 2. Approach to HCP analysis using LC-MS. The biologic sample that contains low levels of HCPs is subjected to enzymatic digestion, the resulting fragments are separated using liquid chromatography and signal acquired by mass spectrometry approaches is analyzed to enable identification and quantitation of HCP impurities for decision making in process development.

Reverse-phase LC (RP-LC) is most commonly used and 1D UPLC-MS analysis of HCPs was developed to support fast bioprocess development.^{36,38} With 2D LC-MS an additional LC step is added before the LC-MS analysis is performed, and this additional separation significantly improves sensitivity, enabling better detection of low-levels of HCPs in a drug substance or product.^{19,44} In addition, strategies such as the affinity depletion approach reported by Madsen et al.⁴⁴ have been developed to capture and enrich HCPs in order to improve detection. Following identification, it is desirable to determine the amounts of individual HCPs in a sample. Commonly, relative quantitation is performed to establish the proportion of an HCP relative to the total pool of protein. To calculate relative amounts of HCPs from MS data, peak areas for unique peptides are determined from extracted ion chromatograms (XIC) of precursor or fragment ions and used in the calculation. Relative quantitation is well suited for quickly monitoring differences in levels of HCPs and can be applied for evaluating the effects of process changes on HCP levels and for assessing comparability of impurity profiles among multiple runs (Figure 3). Absolute quantitation may be needed in some cases, for example when an adverse event results from a particular HCP and levels of that component must

be precisely established to ensure product safety. Absolute quantitation may be achieved by establishing a standard curve across the relevant concentration range, which is typically done by spiking in protein standards covering a range of concentrations.

Although several MS approaches for HCP detections have been published,^{36,37,39-43} application of complex approaches often is not practical in the context of biologic drug development and a simplified MS workflow seems to be sufficient for achieving reproducible HCP detection and relative quantitation.³⁸ Reproducible HCP detection may be achieved by using a combination of three analyses as indicated in Figure 3, in which relative levels are further determined from the peak areas of the HCP peptides. The comprehensive, accurate detection and quantitation of HCPs using MS de-risks development by providing specific information about HCPs throughout the process of developing a biologic product to inform decisions that impact clinical performance. As such, MS-based analysis increasingly has become a standard tool for identification and quantitation of HCPs in biologic drug development.

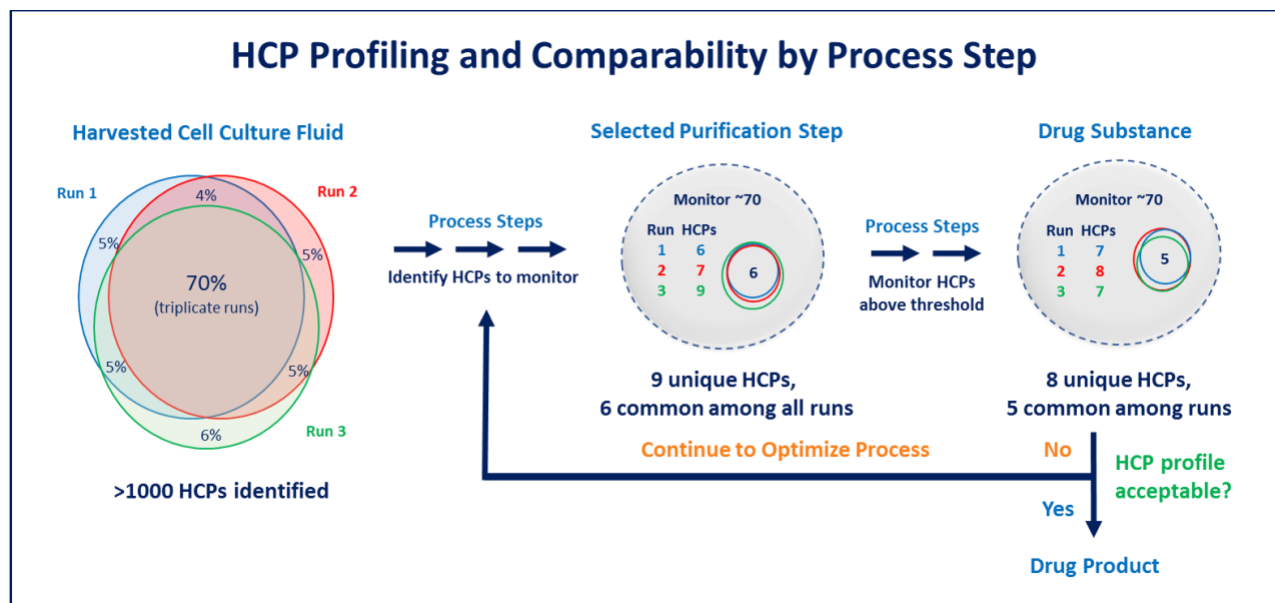


Figure 3. HCP comparability assessment. LC-MS is applied to biotherapeutic samples at various stages of purification to identify HCP impurities and quantify levels to enable process-related decision-making during product development

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