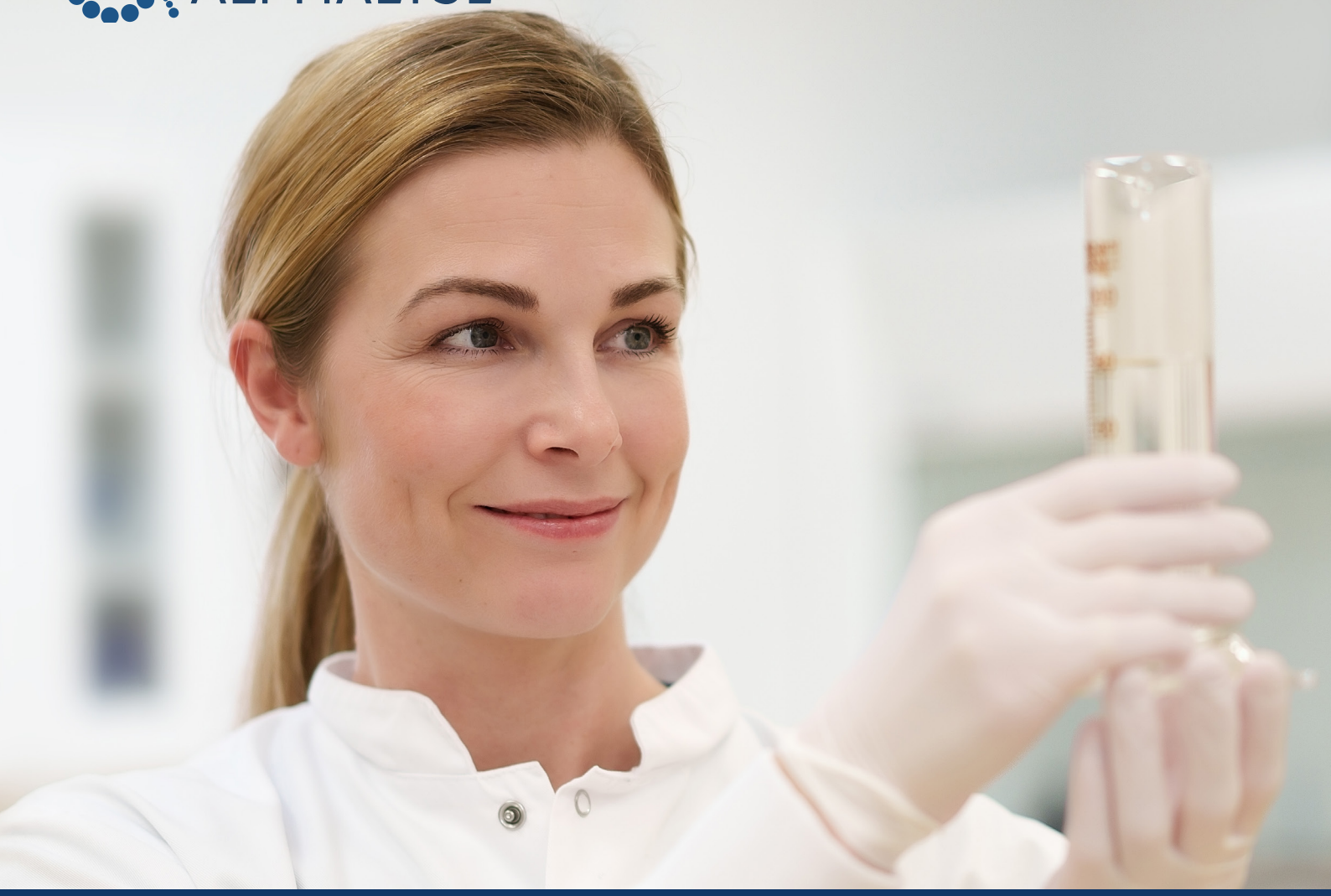
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6 HOST CELL PROTEINS THAT CHALLENGE DRUG DEVELOPMENT

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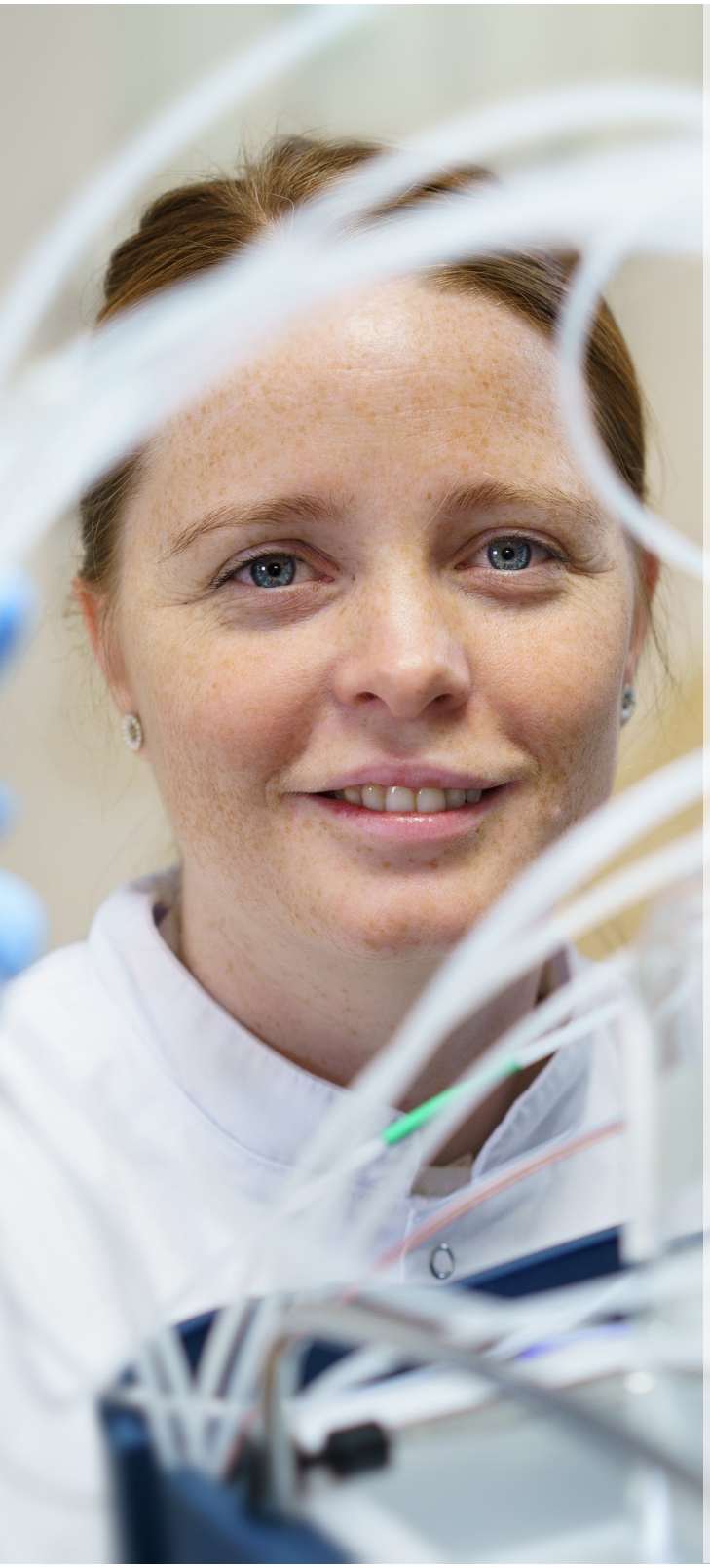


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Introduction

Host Cell Proteins (HCPs) are process-related impurities generated during the production of biotherapeutic drugs. These residual proteins, originating from the expression systems used to manufacture biologics, pose significant risks to product quality, safety, and efficacy if not adequately removed.

Risks of residual HCPs

HCPs can have a wide range of impacts on biopharmaceutical products:

- Immunogenic HCPs may trigger adverse immune responses in patients.
- Biologically active HCPs have homologous activity to endogenous human proteins, potentially interfering with biological processes.
- Enzymatically active HCPs can degrade the drug substance or excipients, leading to reduced product stability and efficacy.

Evolving regulatory expectations

Previously, manufacturers have focused on monitoring and reducing total HCP levels as part of process control. However, health authorities now emphasize the identification and quantification of individual HCPs. This shift reflects an increased understanding of how specific HCPs can influence product quality and patient safety, necessitating more advanced analytical approaches.

Strategies for monitoring HCPs

Advanced orthogonal techniques, particularly Liquid Chromatography-Mass Spectrometry (LC-MS), have become essential tools for comprehensive HCP analysis. LC-MS provides the sensitivity and specificity required to detect, identify, and quantify individual HCPs, even at trace levels. Its application ensures process consistency, facilitates troubleshooting, and supports regulatory compliance.

The importance of LC-MS in HCP analysis is underscored by the recent establishment of the United States Pharmacopeia (USP) General Chapter <1132.1>, *Residual Host Cell Protein Measurement in Biopharmaceuticals by Liquid Chromatography-Mass Spectrometry*. This chapter outlines best practices for quantifying HCPs using LC-MS, reflecting the industry's movement toward more precise and reliable analytical methods.

HCPs of concern

In this ebook, we explore six HCPs of concern from the scientific literature, their harmful potential, and the lessons they offer for biopharmaceutical development.

Meet the contributors



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Ejvind Mortz is the COO and Co-founder of Alphalyse with more than 20 years of managerial experience from biotech companies. His background includes development of protein analysis methods in research & CMC development of protein biologics, including the LC-MS-based HCP analysis used at Alphalyse.



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Victor Chrone is a bioinfomatics specialist working on developing tools to analyze mass spectrometry data at Alphalyse. He has considerable experience with data mining, machine learning, data visualization, and protein chemistry. He is currently pursuing an Industrial PhD from the University of Southern Denmark.



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Cecilie Hovitz Lautrup-Larsen is communication specialist at Alphalyse, working with research publication, information dissemination, and digital communication. Cecilie further holds BScs in Medicine and Biomedical Laboratory Science and has twelve years of hands-on healthcare and medical research experience.

Flagellin

Flagellin, the primary structural protein of bacterial flagella, is abundantly expressed in all flagellated bacteria, including motile strains of *Escherichia coli* frequently used in recombinant biotherapeutic production.

It is a significant HCP impurity in biopharmaceutical manufacturing employing bacterial expression systems.

Immunogenicity concerns

Flagellin is recognized by the innate immune system as a pathogen-associated molecular pattern through its interaction with Toll-like receptor 5 (TLR5). This interaction activates pro-inflammatory pathways, potentially leading to hyperinflammation, tissue damage, or systemic inflammatory responses similar to septic shock.

Even minimal amounts of residual flagellin may provoke unintended immune reactions, posing a serious risk to patient safety.

A CMC worst-case scenario

In one notable case, flagellin contamination of a recombinant drug candidate expressed in *E. coli* led to the suspension of Phase 3 clinical trials after a patient suffered severe adverse effects.

Flagellin likely went undetected in the clinical product characterization because its levels, sufficient to trigger a TLR5 response, were below the detection sensitivity of the commercial, generic *E. coli* HCP-ELISA initially used.

To address this critical gap, the drug developer designed a novel screening assay using cultured mammalian cells expressing TLR5 to detect TLR-binding bacterial impurities.

Following the failed clinical trials, the developer spent more than a decade refining the manufacturing process and eliminating problematic HCPs before successfully bringing the drug to market.

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Lysosomal Acid Lipase

Lysosomal acid lipase (LAL), also referred to as lipase A (LIPA) in some scientific publications, is a particularly problematic HCP co-purifying with monoclonal antibodies (mAbs) and other biologics during standard manufacturing processes, often persisting in the final drug substance.

As an intracellular hydrolase highly expressed on the mRNA level, it is challenging for low cell viability or high cell lysis bioprocesses.

Due to its abundant transcript expression and frequent detection in mAb products, it has been identified as a "high-risk" HCP and is considered a greater concern than other enzymatic HCPs, e.g. lysosomal phospholipase A2 (LPLA2) and carboxylesterases (CES).

Risk to drug stability

Even in trace amounts, LAL poses significant risks to drug stability and patient safety due to its ability to degrade polysorbate (PS) excipients, such as PS20 and PS80.

Polysorbates are nonionic surfactants widely used in biotherapeutic formulations to prevent protein adsorption, denaturation, and aggregation. Degradation of these stabilizers can lead to particle formation, loss of protein stability, and increased immunogenicity, heightening the risk of adverse immune responses in patients.

Polysorbate degradation

Studies have established a strong link between LAL activity and polysorbate degradation.

In PS20, LAL preferentially targets and cleaves higher-order esters, whereas it primarily degrades monoesters in PS80. Since higher-order esters are more effective at solubilizing fatty acids, their degradation causes increased precipitation of free fatty acids, dramatically increasing the likelihood of particle formation.

So, PS80 might be a better choice if the drug product contains trace amounts of LAL. However, the decision must be weighed against other formulation requirements and stability factors.

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Phospholipase B-like 2

Phospholipase B-like 2 (PLBL2) is a lysosomal enzyme commonly found in Chinese hamster ovary (CHO) cells, which are widely used in the production of mAbs. Due to its physicochemical properties, PLBL2 is classified as a "hitchhiker" HCP capable of binding to the therapeutic protein and bypassing polishing steps to the final biopharmaceutical product.

In early publications, PLBL2 was accused of degrading PS excipients, reducing product stability. However, more recent studies have found no correlation between the amount of PLBL2 and the level of PS degradation.

It is now believed the PS degradation found in earlier studies resulted from contamination with Lipoprotein Lipase (LPL), another HCP.

Immunogenicity concerns

CHO-derived PLBL2 shares around 80% of its amino acid sequence with its human ortholog, making it immunogenic in humans.

Clinical studies find that up to 90% of exposed subjects develop a specific and measurable immune response to PLBL2 at levels as low as 0.2-0.4 ppm. While no direct clinical adverse effects have been documented, regulatory expectations and patient safety considerations emphasize minimizing residual PLBL2 levels.

Detection challenges

Historically, PLBL2 was undetectable by enzyme-linked immunosorbent assays (ELISAs) until specific antibodies were developed.

Even today, standard ELISAs may struggle to effectively detect PLBL2. Nonlinear dilution behavior observed in CHO protein ELISAs has been attributed to the presence of PLBL2, suggesting that traditional immunoassays may systematically underestimate its concentration.

Therefore, orthogonal analytics like LC-MS are recommended to control and document purification process efficiency and consistency.

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- Sisi Zhang et al. Putative Phospholipase B-Like 2 is Not Responsible for Polysorbate Degradation in Monoclonal Antibody Drug Products. J Pharm Sci. 2020 Sep;109(9):2710-2718. doi: 10.1016/j.xphs.2020.05.028. Epub 2020 Jun 11. Erratum in: J Pharm Sci. 2020 Dec;109(12):3734. doi: 10.1016/j.xphs.2020.08.022.
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Protein Disulfide Isomerase

Protein Disulfide Isomerase (PDI) is an essential enzyme predominantly located in the endoplasmic reticulum of eukaryotic cells, where it facilitates the formation and rearrangement of disulfide bonds during protein folding.

While vital for maintaining cellular homeostasis, PDI presents notable challenges in therapeutic protein manufacturing.

Purification challenges

PDI is highly abundant in mammalian cells, including CHO cells. Its ability to interact with nascent polypeptides increases its likelihood of co-purifying with recombinant proteins during downstream processing.

This challenge is pronounced for biologics that require complex disulfide bonding, e.g. mAbs, fusion proteins, and glycoproteins.

The strong affinity of PDI for unfolded or partially folded proteins further complicates its removal. Conventional purification methods, like Protein A chromatography, may inadvertently enrich PDI alongside the therapeutic protein, necessitating additional purification steps or orthogonal methods to ensure its complete clearance.

Product stability and aggregation risk

Residual PDI in the final drug product poses significant risks to product quality and stability.

As a multifunctional enzyme, PDI can catalyze unintended disulfide bond rearrangements, potentially altering the structure or activity of the therapeutic protein. Even at trace levels, PDI has been implicated in protein aggregation, a critical quality concern for biopharmaceuticals. Aggregation not only reduces the efficacy of the drug, but also increases the likelihood of immunogenic responses in patients.

Additionally, studies suggest that PDI may contribute to platelet aggregation and thrombus formation, raising further concerns about its potential impact on patient safety.

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Clusterin

Clusterin is is a multifunctional glycoprotein widely expressed in mammalian tissues. It is involved in various physiological processes, including lipid transport, tissue remodeling, and apoptosis regulation, and acts as an extracellular molecular chaperone in vivo, stabilizing misfolded or aggregated proteins.

Its function is critical in protecting cells from proteotoxic and oxidative stresses, which cause upregulation of clusterin expression. Clusterin is also active in regulation of immune responses.

Co-purification with biologics

In 1992, clusterin became the subject of the earliest publication identifying a specific protein binding to IgG.Clusterin has a particularly high affinity for immunoglobulins, binding multivalently to both the Fc and Fab regions of antibodies.

As an HCP, clusterin is highly adhesive and interacts with a wide range of biomolecules, including misfolded proteins, lipids, and cellular debris. Its strong affinity for hydrophobic and aggregated proteins increases the likelihood of clusterin co-purifying with therapeutic proteins during downstream processing and challenges its removal from drug formulations, particularly mAbs.

Clusterin has been found in Protein A-purified mAb products and co-elutes readily with mAbs through CEX and MMC polishing steps.

Immunogenicity concerns

Furthermore, clusterin's immunomodulatory properties exacerbate its risk as an HCP. Clusterin is highly immunogenic and can elicit adverse responses in patients, compromising drug tolerability and safety.

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Heat Shock proteins

Heat Shock Proteins (HSPs) are a family of highly conserved molecular chaperones essential for cellular homeostasis. They assist in protein folding, stabilization, and the prevention of protein aggregation under stress conditions, including changes in temperature, pH, and nutrient availability – conditions commonly encountered during bioprocessing. This makes them abundant in host cell-derived systems.

As molecular chaperones, HSPs have a strong affinity for binding partially folded or misfolded proteins, increasing the risk of their co-purification with therapeutic proteins during downstream processing.

Immunogenicity

HSPs are potent immune modulators. While they have been intentionally used as vaccine adjuvants, their presence as uncontrolled impurities poses significant patient safety concerns.

HSPs can stimulate innate and adaptive immune responses, exacerbate preexisting inflammatory conditions, trigger autoimmunity, and become targets of autoimmune responses themselves.

If they unintentionally increase the immunogenicity of therapeutic proteins, they may also impede drug tolerability.

Thrombosis risk

A study on process- and product-related impurities in a COVID-19 vaccine identified HSPs as the most abundant HCP contaminants.

HSPs exhibit ATPase activity, hydrolyzing ATP to ADP, which is a strong activator of platelets in vivo. The COVID-19 vaccine study found significant ATPase activity in the HSP-contaminated vaccine batches.

Residual ATPase activity in a vaccine could lead to local ADP generation at the injection site and trigger platelet aggregation, increasing the risk of thrombotic events, including vaccine-induced immune thrombotic thrombocytopenia (VITT) – a rare but serious adverse event observed in some COVID-19 vaccine recipients.

References:

- Xiaohui Lu et al. Identification and Quantification of Heat-Shock Protein 70: A Major Host-Cell Protein Contaminant from HEK Host Cells. BioProcess International. 2015 Oct 15. https://www.bioprocessintl.com/viral-clearance/identification-and-quantification-of-heat-shock-protein-70-a-major-host-cell-protein-contaminant-from-hek-host-cells
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HCP analysis by LC-MS

The evolution of complex biotherapeutics and advances in technology have expanded the use of LC-MS to enable developers to detect, identify, and eliminate HCPs of concern.

Limitations of ELISA

Recent advancements also highlight limitations of traditional methods like the ELISA, which has long been the standard method for HCP analysis.

ELISA relies on polyclonal antibodies from immunized animals, which may fail to detect non-immunogenic or weakly immunogenic HCPs. Conversely, highly immunogenic HCPs can dominate the assay signal, leading to overestimation of their levels, while HCPs present in high concentrations can saturate the assay's antibodies, resulting in underestimation.

Another critical limitation of ELISA is its single-value readout, which provides only a cumulative measure of total HCPs without identifying or quantifying individual proteins.

As demonstrated in this ebook, HCPs can exhibit distinct enzymatic or biologic activities, making identification and quantification of individual HCPs essential for effective impurity clearance and process optimization.

Benefits of LC-MS

The LC-MS technology is transforming biopharmaceutical analytics by enabling precise identification and quantification of individual impurities, providing unparalleled insight into product composition, process consistency, and impurity profiling.

LC-MS supports a data-driven approach to process development and quality control, ensuring that manufacturing processes remain predictable, reproducible, and compliant with evolving regulatory expectations. As health authorities increasingly emphasize detailed HCP characterization, LC-MS provides the analytical power to demonstrate robust process control, effective purification, and product stability.

Beyond regulatory alignment, LC-MS plays a critical role in risk mitigation by detecting problematic HCPs before they compromise drug safety or efficacy. By integrating LC-MS into their workflows, biopharmaceutical developers gain deeper process understanding, optimize purification strategies, and accelerate regulatory approval timelines while ensuring the highest level of product quality and consistency.

References:

- United States Pharmacopeia. (2024). USP General Chapter <1132.1>: Residual Host Cell Protein Measurement in Biopharmaceuticals by Liquid Chromatography-Mass Spectrometry. United States Pharmacopeia. https://doi.org/10.31003/USPNF_M17756_02_01

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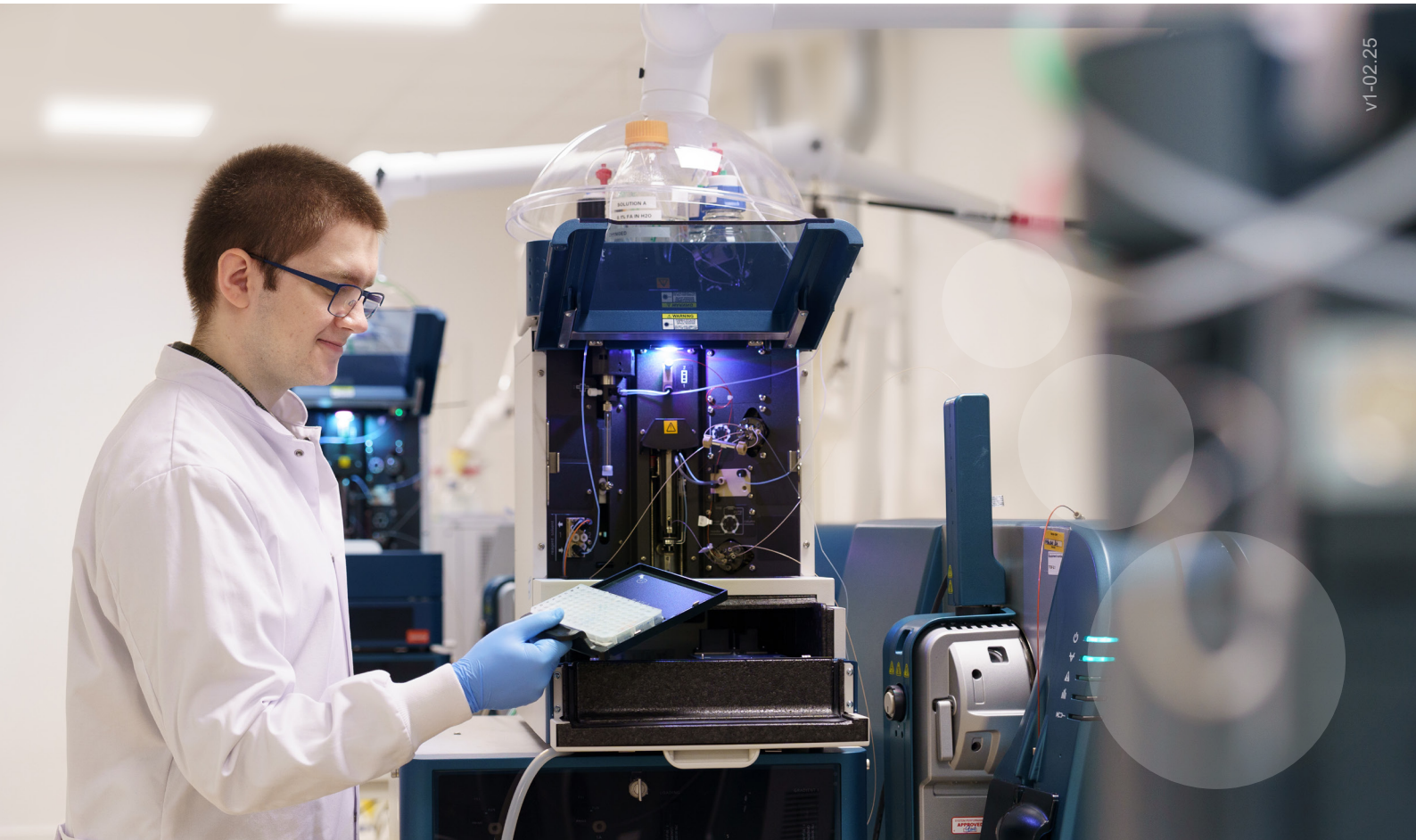
About Alphalyse

Alphalyse is a specialist contract research organization and world leading in HCP analysis by LC-MS, supporting biopharmaceutical companies in process development and manufacturing of patient-safe products.

Since 2002, our scientists have completed over 10,750 protein analyses and 600 HCP projects. Our facilities are cGMP-certified and our analytical methods are validated under ICH Q2(R2) and ICH Q14 guidelines.

We make complex mass spectrometry data accessible – because we believe superior analytics enable better biologics.

More info available on our website
alphalyse.com



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