Development of new methods to measure and improve HCP ELISA reagent coverage

DISSERTATION

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Summary

Protein-based biopharmaceuticals are produced in living host cells, such as Chinese hamster ovary (CHO) cells. Besides the desired product, other host cell proteins (HCPs) are secreted into the supernatant or released through cell lysis. Since these inevitable process-related impurities possibly have immunogenic effects, may destabilize the product or induce adjuvant activity, they need to be removed largely by appropriate purification methods. The most commonly used method to monitor HCP removal over the entire biopharmaceutical manufacturing and to quantify HCPs in the final monoclonal antibody (mAb) product is the Enzyme Linked Immunosorbent Assay (ELISA). This method offers the required sensitivity, sample throughput, selectivity, dynamic range, cost efficiency and ease in application. Reliable HCP quantification relies mainly on the ability of the polyclonal anti-HCP ELISA antibodies to detect the heterogeneous HCP population. In this regard, the ratio of HCPs detected by the polyclonal antibodies (pAbs) relative to the complete HCPs is referred to as coverage. ELISA pAbs are produced by immunizing an appropriate host animal with an HCP standard, containing the vast majority of HCPs present in the current manufacturing process. The resulting pAbs need to be tested regarding their coverage by appropriate analytical methods and for their performance in ELISA based HCP quantification.

A major objective in this work was to better understand the influence of the quality of the applied anti-HCP pAbs on their performance in ELISA. The commonly applied method to determine the coverage of HCP-ELISA pAbs, the two-dimensional (2D) western blot (WB), exhibits in general detection gaps. Exact causes for these detection gaps have not yet been clear, but it was suspected that some of these detection gaps originated from inherent technical limitations of the 2D-WB method itself, rather than from a real absence of HCP-specific pAbs. A detailed analysis indicated that the major cause for the detection gaps is the loss of conformational epitopes through protein denaturation during 2D polyacrylamide gel electrophoresis hindering HCP – antibody recognition. Hence, more precise coverage determination can be achieved by using native conditions.

An orthogonal method to 2D-WB, the affinity purification based liquid chromatography-tandem mass spectrometry (AP-MS) method, allows pAb – HCP binding under native conditions. Coupled with the sensitivity of MS, it is therefore a promising method to get a deeper understanding of pAb coverage. However, the AP-MS methods used so far have some limitations, which can lead to false positive hits. An optimized AP-MS based method was developed to circumvent the non-specific binding issues to the matrix by separating the immune-complexed HCPs from the matrix prior to digestion. The method proved to

identify covered HCPs unambiguously and therefore can enhance the understanding of antibody binding behavior of single HCPs.

The analytical toolbox developed within this work is very helpful to shed some light on the details of ELISA antibody coverage and facilitates the selection of the most suitable pAbs for reliable HCP quantification by ELISA. It helps to achieve the other main objective of this work, which is to examine the potential for improving coverage by changing the species or even apply a mixture of species. Thus, the influence of the host animal species on coverage was investigated in detail. Comparison of sheep, goat, donkey, rabbit, and chicken derived anti-CHO-HCP pAbs exhibited similar results for all species besides chicken, which clearly fell short in pAb coverage and ELISA performance. The multispecies pAb mixture achieved slightly improved coverage, but did not perform better in the ELISA for HCP quantification of various samples and therefore provided no clear advantage over the single species pAbs.

Overall, 2D-WB to determine coverage should be supplemented by orthogonal methods, which better represent the ELISA conditions for antibody binding and provide additional information. In combination with a performance evaluation of pAbs in ELISAs, this detailed characterization is necessary to make reliable decisions about the suitability of HCP ELISAs, as demonstrated in this work. In turn, the use of appropriate HCP ELISAs helps to increase patient safety by reducing the risk of overlooking individual HCPs or underestimating the total HCP content.

Zusammenfassung

Biopharmazeutika auf Proteinbasis werden in lebenden Wirtszellen, wie z. B. Ovarialzellen des chinesischen Hamsters (Chinese hamster ovary [CHO]), hergestellt. Neben dem gewünschten Produkt werden auch andere Wirtszellproteine (host cell protein [HCPs]) in den Überstand sezerniert oder durch Zelllyse freigesetzt. Da diese unvermeidbaren prozessbedingten Verunreinigungen möglicherweise immunogene Wirkungen haben, das Produkt destabilisieren oder eine Adjuvant-Aktivität induzieren können, müssen sie durch geeignete Reinigungsverfahren weitestgehend entfernt werden. Die gängigste Methode zur Überwachung der HCP-Entfernung während des gesamten biopharmazeutischen Herstellungsprozesses und zur Quantifizierung der HCPs im monoklonalen Antikörper (monoclonal antibody [mAb]) Endprodukt ist der Enzyme Linked Immunosorbent Assay (ELISA). Diese Methode bietet die erforderliche Empfindlichkeit, den Probendurchsatz, die Selektivität, den dynamischen Bereich, die Kosteneffizienz und eine einfache Anwendung. Eine zuverlässige HCP-Quantifizierung hängt hauptsächlich von der Fähigkeit der polyklonalen Anti-HCP-ELISA-Antikörper ab, die heterogene HCP-Population zu erkennen. In diesem Zusammenhang wird das Verhältnis der von den polyklonalen Antikörpern (polyclonal antibody [pAbs]) nachgewiesenen HCPs im Verhältnis zur vollständigen HCP Population als Abdeckung bezeichnet. Die ELISA-pAbs werden durch Immunisierung eines geeigneten Wirtstieres mit dem HCP-Standard hergestellt, welcher die überwiegende Mehrheit der im aktuellen Herstellungsprozess vorhandenen HCPs enthält. Die resultierenden pAbs müssen mit Hilfe geeigneter Analysemethoden auf ihre Abdeckung der HCPs und auf ihre Leistung bei der ELISA-basierten HCP Quantifizierung getestet werden.

Ein Hauptziel dieser Arbeit war es, den Einfluss der Qualität der verwendeten Anti-HCP-pAbs auf deren Leistung im ELISA besser zu verstehen. Die üblicherweise angewandte Methode zur Bestimmung des Abdeckungsgrads von HCP-ELISA pAbs, der zweidimensionale (2D) Western Blot (WB), weist im Allgemeinen Nachweislücken auf. Die genauen Ursachen für diese Nachweislücken waren noch nicht klar, aber es wurde vermutet, dass einige dieser Lücken auf inhärente technische Beschränkungen der 2D-WB-Methode selbst zurückzuführen sind und nicht auf ein tatsächliches Fehlen von HCP-spezifischen pAbs. Eine detaillierte Analyse ergab, dass die Hauptursache für die Detektionslücken der Verlust von Konformationsepitopen durch Proteindenaturierung während der 2D-Polyacrylamid-Gelelektrophorese ist, durch welchen die HCP - Antikörper Erkennung verhindert wird. Deshalb kann eine genauere Bestimmung des Abdeckungsbereichs durch die Verwendung nativer Bedingungen erreicht werden.

Eine orthogonale Methode zum 2D-WB, die auf Affinitätsreinigung basierende Flüssigchromatographie-Tandem-Massenspektrometrie (AP-MS) Methode, ermöglicht die pAb-HCP-Bindung unter nativen Bedingungen. In Verbindung mit der Empfindlichkeit der MS ist sie daher eine vielversprechende Methode, um ein tieferes Verständnis der pAb-Abdeckung zu erhalten. Die bisher verwendeten AP-MS-Methoden haben jedoch einige Nachteile, die zu falsch positiven Treffern führen können. Deshalb wurde eine optimierte AP-MS-Methode entwickelt, welche die unspezifischen Bindungsprobleme zur Matrix umgeht, indem die immunkomplexierten HCPs vor dem Verdau von der Matrix getrennt werden. Die Methode hat bewiesen, dass sie abgedeckte HCPs eindeutig identifiziert und daher das Verständnis des Antikörperbindungsverhaltens einzelner HCPs verbessern kann.

Die im Rahmen dieser Arbeit entwickelten analytischen Werkzeuge sind sehr hilfreich, um die Details für ELISA-Antikörperabdeckung zu beleuchten und erleichtern damit die Auswahl der am besten geeigneten pAbs für eine zuverlässige HCP-Quantifizierung mittels ELISA. Sie tragen zur Erreichung des anderen Hauptziels dieser Arbeit bei, nämlich der Untersuchung des Verbesserungspotenzials bei der Abdeckung durch Änderung der Tierspezies oder auch durch Verwendung einer Mischung von verschiedenen Tierspezies. Daher wurde der Einfluss der Tierspezies auf die Abdeckung im Detail untersucht. Der Vergleich der von Schaf, Ziege, Esel, Kaninchen und Huhn stammenden Anti-CHO-HCP pAbs ergab für alle Tierspezies ähnliche Ergebnisse, mit Ausnahme des Huhns, das in Bezug auf die pAb-Abdeckung und der ELISA-Leistung deutlich zurückfiel. Die Multispezies-pAb-Mischung erzielte eine leicht verbesserte Abdeckung, schnitt aber im ELISA für die HCP-Quantifizierung verschiedener Proben nicht besser ab und bot daher keinen eindeutigen Vorteil gegenüber den pAbs von einzelnen Tierspezies.

Insgesamt sollten bei der Bestimmung der Abdeckung der 2D-WBs durch orthogonale Methoden ergänzt werden, welche die ELISA-Bedingungen für die Antikörperbindung besser repräsentieren und zusätzliche Informationen liefern. In Kombination mit einer Leistungsbewertung der pAbs in ELISAs ist diese detaillierte Charakterisierung notwendig, um eine verlässliche Entscheidung über die Eignung von HCP-ELISAs treffen zu können, wie in dieser Arbeit gezeigt wurde. Die Anwendung geeigneter HCP-ELISAs trägt wiederum zur Erhöhung der Patientensicherheit bei, da das Risiko, einzelne HCPs zu übersehen oder den Gesamtgehalt an HCPs zu unterschätzen, sinkt.

List of published manuscripts

This dissertation is composed of the following manuscripts:

1. Adapted with permission from "**Seisenberger, C.**, Graf, T., Haindl, M., Wegele, H., Wiedmann, M., & Wohlrab, S. (2021). Questioning coverage values determined by 2D western blots: A critical study on the characterization of anti-HCP ELISA reagents. *Biotechnol Bioeng*, *118*(3), 1116-1126. https://doi.org/10.1002/bit.27635"

2. Adapted with permission from "**Seisenberger, C.**, Graf, T., Haindl, M., Wegele, H., Wiedmann, M., & Wohlrab, S. (2022). Toward optimal clearance: A universal affinity-based mass spectrometry approach for comprehensive ELISA reagent coverage evaluation and HCP hitchhiker analysis. *Biotechnol Prog*, *38*(3), e3244. https://doi.org/10.1002/btpr.3244"

3. Adapted with permission from "**Seisenberger, C.**, Graf, T., Sticht, S., Haindl, M., Mohn, U., Wegele, H., Wiedmann, M., & Wohlrab, S. (2022). The agony of choice: Impact of the host animal species on the ELISA performance for HCP quantification. *Biotechnol Bioeng*. https://doi.org/10.1002/bit.28265"

Publication with personal contribution during the PhD period, which is not part of this dissertation:

4. Graf, T., **Seisenberger, C.**, Wiedmann, M., Wohlrab, S., & Anderka, O. (2021). Best practices on critical reagent characterization, qualification, and life cycle management for HCP immunoassays. *Biotechnol Bioeng*, *118*(10), 3633-3639. https://doi.org/10.1002/bit.27881

Personal contribution to the manuscripts

Publication 1

The concept for this research was designed by myself, Markus Haindl, Michael Wiedmann and Stefanie Wohlrab. Data acquisition was performed by myself. Only the identification of proteins from a preparative 2D gel using LC-MS/MS was conducted as a contract analysis by the company PROTAGEN (see 2.3.6 in this thesis). Analysis and interpretation of data was performed by myself, Tobias Graf, Markus Haindl, Michael Wiedmann and Stefanie Wohlrab. The publication was written by myself and Tobias Graf, with input from all the co-authors. All authors contributed to the final proofreading.

Publication 2

The research was designed by myself, Tobias Graf, Markus Haindl, Michael Wiedmann and Stefanie Wohlrab. Data curation and visualization was performed by myself. The original draft was written by Tobias Graf and myself. All authors contributed to reviewing and editing of the manuscript.

Publication 3

The concept for this research was designed by myself, Tobias Graf, Markus Haindl, Ulrich Mohn, Michael Wiedmann and Stefanie Wohlrab. Formal analysis was performed by myself and Tobias Graf. Investigation was conducted by myself and Sarah Sticht (she supported the antibody purification and provided the data for HCP-ELISA development, validation and measurement as master student under my supervision). The original draft was written by myself and Tobias Graf. All authors contributed to reviewing and editing of the manuscript.

Publication 4

All authors designed the concept for this publication. Visualization was performed by myself and Oliver Anderka. All authors contributed to writing, reviewing and editing of the manuscript.

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1 General Introduction

1.1 Biopharmaceuticals and therapeutic monoclonal antibodies

Biopharmaceuticals are protein- or nucleic acid based therapeutic molecules produced in living cells (Kesik-Brodacka, 2018; Walsh, 2018). Prominent examples of biopharmaceuticals are hormones, vaccines, blood factors and recombinant therapeutic proteins. Among the latter, monoclonal antibodies (mAbs) have been the most impactful class in the last years (Kesik-Brodacka, 2018; Rader, 2008). The foundation for the production of mAbs was laid by the development of the hybridoma cell line by Köhler and Milstein (1975), which allows the production of antibodies with predefined specificity in large amounts. Just eleven years later, in 1986, muromonab-CD3 (Orthoclone OKT3) was the first approved therapeutic antibody produced by the hybridoma technology (Emmons & Hunsicker, 1987; Kung et al., 1979). Despite adverse effects like immunogenicity due to its murine origin, muromonab-CD3 was used to treat acute renal allograft rejection until the discontinuation of the production in 2010 (Cosimi, 1987; Cosimi et al., 1981; Norman et al., 1987; Reichert, 2012). Subsequent technology improvements like the humanization of mAbs by recombinant deoxyribonucleic acid (DNA) technology further improved compatibility, thus facilitating the success of mAbs in therapeutic use (Gorman & Clark, 1990; Morrison & Oi, 1984; Walsh, 2000). To date, over 100 therapeutic mAbs have been approved by the U.S. Food and Drug Administration (Kaplon et al., 2022; Mullard, 2021) and the global market accounted for about $111 billion in 2021 (Joshi, 2022). Therapeutic antibodies approved in the United States or European Union are nowadays used mainly to treat cancer (45%) and immune-mediated diseases (27%) (Kaplon et al., 2022). The majority of therapeutic mAbs belong to the Immunglobulin G (IgG) class that is a Y-shaped 150 kDa molecule with two identical 50 kDa heavy and 25 kDa light chain units (Posner et al., 2019). IgGs originate naturally from B-lymphocytes and bind with high affinity and specificity to the cell surface or secreted targets (Mullard, 2021). Over the years, not only traditional Y-shaped IgGs have been used as therapeutics, but also fragments (Fab fragments, single-chain variable regions), bispecific antibodies (engineered molecules with two different binding sites that recognize two independent antigens or epitopes) or antibody drug conjugates (small molecule drugs covalently linked to mAbs) constantly emerge (Bedzyk et al., 1990; Ford et al., 1983; Fudenberg et al., 1964; Kim et al., 2002; Labrijn et al., 2019; Posner et al., 2019).

1.2 Production of therapeutic antibodies

The production of therapeutic antibodies (Figure 1.1) is divided in two major steps, the upstream-processing (USP) and the downstream-processing (DSP).

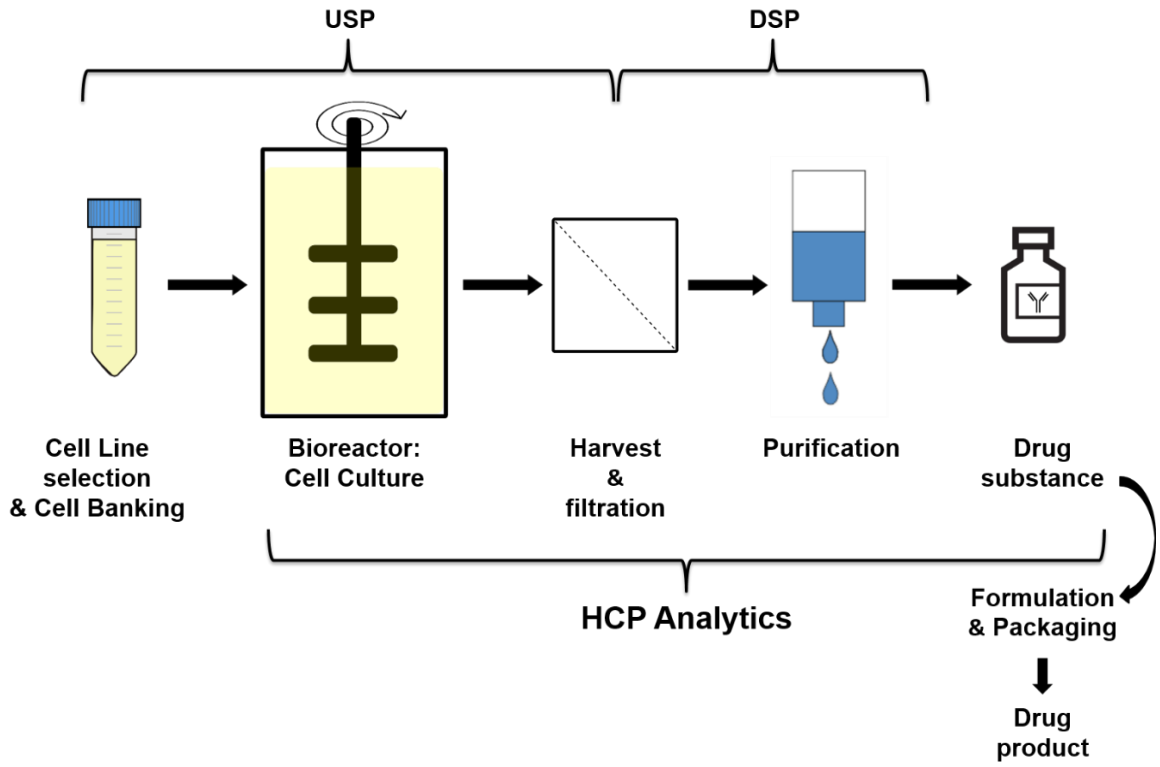

Figure 1.1: Production process of mAb pharmaceutical.

The upstream-process (USP) includes the development of the cell line, selection of the best clone, cell banking, cell culture development in bioreactors, fermentation and harvest of the mAb-containing supernatant. During the downstream-process (DSP) the mAb is purified and filtered in several steps. The drug substance (DS) is formulated in a protein stabilizing buffer and packed into appropriate devices e.g. glass vials, which then results in the final drug product (DP). Host cell protein (HCP) Analytics are applied over the entire DS-production process, to support the development of suitable USP and DSP strategies and ensure process consistency.

The first step of the USP contains the preparation of media and cell line development, followed by several cultivation steps of the cells in bioreactors and ends with the harvest of the cell culture fluid. The aim of the DSP is to increase purity and concentration of the desired active pharmaceutical ingredient (API) by application of different filtration and chromatography steps. Additionally virus inactivation and clearance via filtration is performed. After production of the therapeutic mAbs, the quality is tested using different

analytical methods. The drug substance (DS) is brought in a stable final formulation and packed into appropriate containers (Allmendinger et al., 2014; Berkowitz et al., 2012; Freitag, 2014; Gronemeyer et al., 2014; Kornecki et al., 2017; Rathore & Rajan, 2008).

In general, prokaryotic and eukaryotic cells can be used as recombinant expression systems in cell culture during USP. Prokaryotic bacterial strains such as *Escherichia coli (E. coli)* are fast, easy and cheap to cultivate. However, the downsides of bacterial strains are incorrect protein folding, lack of post-translational modifications (e.g. glycosylation, phosphorylation, methylation) and the accumulation of proteins in inclusion bodies, which requires solubilization steps after cell disruption (Burgess, 2009; Schmidt, 2004; Walsh & Jefferis, 2006). Eukaryotic mammalian cell lines overcome these obstacles, as they are able to fold the antibodies correctly and introduce post-translational modifications like glycosylation, which reduces their immunogenicity in humans. A further benefit of mammalian expression systems over prokaryotic ones is the secretion of antibodies into the medium. This allows simpler purification of mAbs directly from the cell supernatant as most of the cells remain intact (Dumont et al., 2016; Durocher & Butler, 2009; Frenzel et al., 2013).

Among the eukaryotic mammalian expression systems murine myeloma cell lines (NS0, Sp2/0) and Chinese Hamster Ovary (CHO) are primarily chosen. The latter is by far the most used cell line (Dhara et al., 2018; Posner et al., 2019; Walsh, 2014) as it provides the following advantages: the fermentation using CHO cells yields high titers of up to 10 g/l (Donaldson et al., 2021), the simple cultivation of the cells in serum-free medium lowers the risk to introduce infectious agents (Glassy et al., 1988; Li et al., 2010), the availability of various selection systems like metabolic selection, which is possible due to lacking dihydrofolate reductase or glutamine synthetase (Page & Sydenham, 1991; Rita Costa et al., 2010; Wernicke & Will, 1992) and the growth in suspension cultures allows large-scale cultures with higher cell densities (Zhang et al., 2015). Furthermore, the CHO cell line is well known to the regulatory agencies and has been proven to be safe over years in clinical studies, which could be an advantage for approval (Kim et al., 2012).

After fermentation, different methods were applied for the purification of CHO-derived recombinant proteins in DSP. The purification starts after removal of the cells via centrifugation or filtration steps during harvest. For the initial capture step the most widely used Protein A-affinity chromatography helps to isolate, concentrate and stabilize the mAb product. Protein A is a cell wall component of *Staphylococcus aureus* known to bind several IgG types via their Fc part (Forsgren & Sjöquist, 1966; Hjelm et al., 1972; Jensen, 2007; Kronvall, 1973; Shukla et al., 2007). Although protein A binds the IgGs highly specific,

it is not possible to avoid co-purification of some impurities that bind either non-specifically to the mAbs themselves or to the column material. Additionally low levels of leached Protein A ligand can co-elute with the mAbs. The type of resin backbone plays an important role for non-specific binding, but also the chemistry used for coupling the protein A ligand to the resin. For example, Agarose is known for lower unspecific binding than glass backbones or Sepharose. Today, engineered Protein A ligands have been developed to minimize leaching of the ligand and increase their pH-stability for extended column lifespans (Aboulaich et al., 2014; Batalla et al., 2012; Beattie et al., 2021; Fahrner et al., 1999; Hahn et al., 2006; Shukla & Thömmes, 2010; Tarrant et al., 2012; van Sommeren et al., 1993). Despite known challenges of Protein A, this single purification step allows the removal of over 98% of the impurities (Shukla & Thömmes, 2010). Different numbers and types of polishing steps were applied after Protein A-affinity chromatography to remove remaining impurities as good as possible. These steps can include anion-exchange (AEX), cation-exchange (CEX), hydrophobic-interaction (HIC) or mixed-mode chromatography (Gronemeyer et al., 2014; Pezzini et al., 2011; Schwaminger et al., 2022; Shukla et al., 2007; Shukla & Thömmes, 2010).

Remaining contaminants are monitored by different analytical techniques over the entire production process to ensure process consistency and sufficient quality of the mAb therapeutic.

1.3 Critical quality attributes during production of Biopharmaceuticals

Therapeutic antibodies have an inherent complex structure and even minor alterations in this structure can lead to changes in potency or induce unwanted side effects such as immunogenicity (Kuriakose et al., 2016). Modifications in USP and DSP parameters have an influence on product quality. Hence, monitoring composition and structure of the DS is crucial to ensure consistent high quality (Yu et al., 2014). Therefore, different characterization methods have to be applied, which include physicochemical characterization (e.g., appearance, identity, pH, osmolality, protein content, and purity), product-related impurity characterization (e.g., product variants, aggregates, fragments), process-related impurity measurement (cellular components, process material), sterility testing (e.g., microbes, fungi) and excipient analysis (e.g., polysorbate, poloxamer). Some of these attributes are considered Critical Quality Attributes (CQAs), as defined in the ICH guidelines Q8 as “a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality” (ICH, 2009). Risk assessment and application of a control strategy

(e.g. Quality by design) help to define individual CQAs, which have the greatest potential for a direct impact on drug product (DP) safety and efficacy (Alt et al., 2016; ICH, 2006, 2009; Rathore & Winkle, 2009; Yu, 2008). Among the CQAs, nonconforming glycosylation, aggregation of DS and host cell proteins (HCPs) are the most difficult to control and monitor (Oshinbolu et al., 2018).

1.4 Host cell proteins

Besides host cell DNA, cell culture media components, Protein A chromatography leachables and selection markers, HCPs are a major group of process related impurities (F. Wang, 2015; Gronemeyer et al., 2014; Liu et al., 2010). HCPs are produced by the expression cell line alongside the desired recombinant therapeutic antibody and ensure the functionality of the cells, making them inevitable process-related impurities (Bracewell et al., 2015; Pilely et al., 2022).

The nature of the expression cell line and the fermentation conditions influence the number and identity of produced HCPs (Jin et al., 2010). While *E. coli* cells contain about 4,300 (Blattner et al., 1997), CHO cells even carry about 24,000 genes (Lewis et al., 2013; Xu et al., 2011). Due to the mammalian origin of CHO cells, these cells are able to produce way more complex proteins than bacterial cells, comprising post-translational modifications and complex molecular foldings (Hirschberg & Snider, 1987; Kamionka, 2011; Neurath, 1989). Fermentation conditions that are likely to influence the HCP content are e.g., medium composition, cell culture temperature, pH, feeding strategy, cell density, cell age and viability (Goey, Bell, et al., 2018; Goey et al., 2017; Hogwood et al., 2013; Jin et al., 2010; Tait et al., 2012; Valente et al., 2015).

The purification steps in the DSP must be robust enough to resist slight variations in the USP. Harvesting conditions can also affect the composition and amount of HCPs (Jin et al., 2010). While *E. coli* cells have to be lysed to release the protein of interest, CHO cells are solely separated from the mAb-containing supernatant, which results in a reduced amount of HCPs. The complexity of the HCP profile prior to purification and the choice of the initial purification steps significantly affects the success of the HCP depletion during DSP (Aboulaich et al., 2014; Hogwood et al., 2013; Schenauer et al., 2013; Valente et al., 2014). Monitoring the HCP content during the purification process helps to develop the most suitable purification strategy and to demonstrate process consistency (Grampp & Ramanan, 2013; Liu et al., 2010). Nevertheless, HCPs are in any case a complex mixture of proteins with different physicochemical properties, which makes purification challenging (Bracewell et al., 2015; Jin et al., 2010; Nogal et al., 2012). To this end, complex

purification strategies were applied during DSP to remove HCPs as good as possible. A commonly accepted level of total HCPs in mAb therapeutics is below 100 ppm (100 ng HCP/ mg DS), even if there exists no fixed level (Champion et al., 2005; J. H. Chon & G. Zarbis-Papastoitsis, 2011; Eaton, 1995; Wang et al., 2009). Specification of the acceptable HCP limit for an individual mAb can be affected by several criteria like, frequency of dosing, maximum dose, route of administration (e.g. subcutaneous, intravenous, intramuscular), patient population, pre-clinical and clinical data (de Zafra et al., 2015; Shahrokh et al., 2016; Vanderlaan et al., 2018). There are significantly lower limits for certain HCPs, as even residual amounts of HCPs may have immunogenic effects, exhibit adjuvant activity, be biologically active or destabilize the product (Bracewell et al., 2021; Vanderlaan et al., 2018). For example, Clusterin is a highly immunogenic glycoprotein that directly binds to IgGs, making it difficult to remove from DS (Wilson & Easterbrook-Smith, 1992). Ribose Phosphate Isomerase (RPI) was found to have an adjuvant effect and increases unwanted anti-drug antibody (ADA) production to the human growth hormone (hGH) therapeutic (Bierich, 1986; Eaton, 1995; Vicens-Calvet et al., 1986). In addition, HCPs can be biologically active enzymes or cytokines that have an inherent risk of unwanted activity in humans or destabilization of the API. In recent years, particle formation in DP due to degradation of polysorbate by host cell hydrolases like Lysosomal Acid Lipase (LIPA) was discovered (Beatson et al., 2011; Graf, Tomlinson, et al., 2021; Vanderlaan et al., 2018). Polysorbate 20 and 80 are frequently used nonionic surfactants to stabilize protein based APIs.

1.5 Host cell protein analytics

HCP analytics are well known to be challenging for several reasons. In the first place, HCP analytics have to detect the majority of the heterogeneous protein mixture, with variable physicochemical properties (Jin et al., 2010). In cases where a HCP has similar physicochemical properties as the DS or interacts directly with the DS (so called hitchhiker), it can be difficult to remove and monitor. Furthermore, the wide dynamic range of up to six orders of magnitude between HCP and DS concentration makes the detection of HCPs complicated (Doneanu et al., 2012; Huang et al., 2017; Wu & Han, 2006).

Tracking the HCP content during production of biopharmaceuticals is important to ensure consistency of the production process, sufficient purity of the DS and patient safety (Bracewell et al., 2021; Champion et al., 2005; Pilely et al., 2022). To monitor HCP removal and quantify the HCP levels in the final DS, different analytical strategies could be applied. Technologies for HCP characterization include electrophoretic, western blot (WB), chromatographic, proteomic and immunoassay methods (USP <1132>, 2016).

The electrophoretic methods comprise Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS), two-dimensional difference gel electrophoresis (2D-DIGE), SDS polyacrylamide gel electrophoresis (PAGE) and two-dimensional (2D) SDS-PAGE. CE-SDS and SDS-PAGE were used to screen for non-product related peaks or protein bands, but are rarely used for HCP analysis due to lack of sensitivity, unless one of these methods is part of the routine control system and the data are available anyway. However, Zhu-Shimoni et al. (2014) has published a case in which a HCP impurity was observed by CE-SDS, but was not detected by immuno-based assays due to lack of immunogenicity. CE-SDS is less sensitive compared to SDS-PAGE, but is rapid and capable of high throughput testing. SDS-PAGE is often combined with WB to identify a single HCP by using the corresponding antibody or for quantification of HCPs. A further method to visualize HCPs is 2D-DIGE, primarily used to compare various HCP samples on the same gel by labeling each sample with a different fluorescent dye (Grzeskowiak et al., 2009; Jin et al., 2010). 2D-DIGE is a widespread method to monitor the stability of the HCP immunogen at room temperature or to several freeze-thaw cycles. As several samples could be analyzed on one single gel, 2D-DIGE is able to overcome inherent reproducibility, expenditure of time and quantitation issues of 2D-PAGE (Kondo & Hirohashi, 2009; Unlü et al., 1997).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) based methods are applied, as they are able to detect, identify and quantify single HCPs. This technology benefits from several methods developed in the context of proteomics (USP <1132>, 2016). The general workflow starts with the sample preparation, comprising denaturation, reduction, alkylation and digestion (e.g. tryptic) of the protein. The derived peptides were separated on high-performance liquid chromatography (HPLC) columns, analyzed in mass spectrometers and the associated proteins were identified in a database search (Wohlrab et al., 2018). Since the correctness of LC-MS/MS results depends on the quality of genomic databases used, the recently published improved genome sequence data for CHO (Rupp et al., 2018) could help to ameliorate database results. A major problem is here again, the tremendously larger number of product proteins compared to HCPs. Current LC-MS/MS workflows are limited to a dynamic range of 3-4 order of magnitude, however, up to six order of magnitude are required, especially for the very clean DS samples (Esser-Skala et al., 2020; Huang et al., 2017; Wu & Han, 2006). Different solutions were applied to solve this issue, like improvement of the separation techniques before the mass spectrometry measurement to reduce sample complexity by separating product and HCP related peptides. Additionally, removal of the DS by affinity purification chromatography (Protein A) or concentration of HCPs with anti-HCP pAbs leads to a reduction of the dynamic range (Doneanu et al., 2012; Esser-Skala et al., 2020; Graf,

Tomlinson, et al., 2021; Huang et al., 2017; Madsen et al., 2015; Reisinger et al., 2014; Tscheliessnig et al., 2013). The additional steps make those methods very time consuming. A faster method (native digestion), recently developed by Huang et al. (2017), omits the denaturation step at the beginning of the sample preparation and starts directly with the tryptic digestion. As intact antibodies are resistant to tryptic digestion, they remain intact while the HCPs are digested. The undigested antibody can be removed afterwards by heat denaturation and precipitation (Huang et al., 2017; Kufer et al., 2019). With this method, the dynamic range for HCP measurement by mass spectrometry was reduced by 1 to 2 orders of magnitude, compared to the classical denaturing trypsin digestion (Huang et al., 2017). Due to the mentioned properties, this method is also very well suited to investigate antibody-binding proteins. However, mass spectrometry based methods are in general very complex, often time consuming, need skilled people for their execution, require expensive equipment and are not yet high-throughput capable (Bracewell et al., 2015; Shahrokh et al., 2016).

Although LC-MS/MS based analyses are increasingly used as complementary methods, immunoassay based methods like the enzyme-linked immunosorbent assays (ELISAs), are still the method of choice for HCP analytics.

1.6 ELISA

The first immunoassay based technique for highly sensitive detection of analytes was the radioimmunoassay (RIA), using radioactive labeled antigens or antibodies (Lequin, 2005; Yalow & Berson, 1960). It formed the basis for the later by Engvall and Perlmann (1971) developed ELISA, which benefits from the less dangerous enzymatic detection system. At the same time, Van Weemen and Schuurs (1971) described the enzyme immunoassay (EIA) and used the enzyme horseradish peroxidase (HRP) as reporter label instead of Alkaline phosphatase (AP) used by Engvall and Perlmann (1971). The basic ELISA workflow involves immobilization of the antigen to a certain matrix, which is either a microtiter plate (MTP) or the capture antibody coated on a MTP and detection with the help of an enzyme labeled antibody. Finally, the enzyme converts the added substrate and the resulting reaction product can be measured by either chromogenic, fluorescent or chemiluminescent detection. The resulting numerical value is compared to a standard curve, generated by serial dilutions of a sample with known antigen concentration (Aydin, 2015; Gaastra, 1984; Lequin, 2005).

The launch of standardized 96well MTPs commonly made of polystyrene was another important step for the success of the ELISA. Hydrophobic interactions between the plastic

of the MTPs and the non-polar protein residues ensure binding through passive adsorption of the protein to the MTPs. The standardized format of the plate facilitates the development of automated assay steps, like plate washing, pipetting or readout, hence making this method high throughput capable and user friendly (Lequin, 2005).

A distinction is made between four different ELISA types. The direct, indirect, competitive or sandwich ELISAs were performed with diverse antigen immobilization and detection techniques (Figure 1.2).

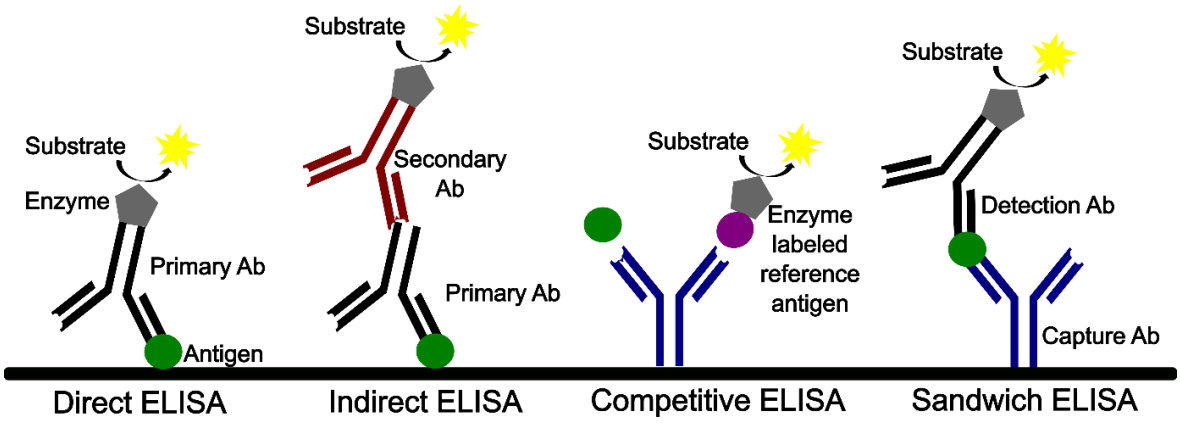

Figure 1.2: ELISA types.

Four standard enzyme-linked immunosorbent assay (ELISA) types are known, namely the direct, indirect, competitive and sandwich ELISA. The green circle represents the antigen of interest that either is coated onto a plate (direct and indirect ELISA) or is captured by an antibody (Ab) immobilized on a plate (competitive and sandwich ELISA). The reporter enzyme, depicted as gray polygon, converts the added substrate into a detectable reaction product (spiked yellow star). Adapted from Van Gool et al. (2020)

Direct and indirect ELISA share the immobilization of the antigen to the surface of the MTP. Whereas the former method uses a specific antibody directly conjugated with the enzyme to detect the antigen, the latter involves a two-step process with antigen binding by an unlabeled primary antibody and detection using a secondary antibody labeled with the enzyme. The direct ELISA is a very rapid method, without the risk of cross-reactivity to the secondary antibody, but displays low sensitivity (e.g. due to lacking signal amplification possibilities) and is expensive as specific antibodies have to be labeled for each ELISA. Indirect ELISAs, on the other hand, allow the same secondary antibodies to be used for different assays, which makes them cost-effective and flexible. At the same time, they are very sensitive, as signal amplification is possible with the help of the secondary antibody. Sometimes the use of secondary antibodies can lead to high background issues due to

cross-reactivity between them. A competitive ELISA measures the antigen content by detecting signal interference. Therefore, a MTP is coated with a defined amount of antigen-specific antibody, generating a limited number of binding sites. The antigen of interest competes with an enzyme-labeled reference antigen for binding to the immobilized antibody. Since the amount of enzyme activity is inversely proportional to the concentration of the unlabeled antigen of interest, the antigen concentration can be determined by comparison of the detected signal with a standard curve. This method can be adapted for competitive detection of unknown antibody amounts. The competitive ELISA is especially suitable for small antigens with few epitopes. Sandwich ELISAs are the most commonly used format, especially for complex samples. The analyte to be measured is bound between the capture and the detection antibody, requiring at least two different epitopes on the same antigen, each detected by one of the antibodies. First, the capture antibody is immobilized to the MTP and after binding of the antigen, the added detection antibody facilitates either direct or indirect measurement of the analyte concentration. A disadvantage of this method is the higher effort required for method optimization, since antibody pairs must be identified that bind different epitopes on the same antigen but do not show cross-reactivity with each other. Due to its high sensitivity, flexibility and specificity the sandwich ELISA is very popular (Aydin, 2015; Gan & Patel, 2013; O'Kennedy et al., 1990; Shah & Maghsoudlou, 2016).

HCP analytics deals with very complex samples and needs to cover a wide dynamic range, making the sandwich ELISA the format of choice. A distinction between different assay types, such as the process-specific, platform or generic HCP-ELISAs, is made. As the name implies, process-specific assays are developed with HCPs unique to a certain DS production process, making it only applicable for this specific process. In contrast, the platform HCP-ELISA could be used to monitor several products with the same set of HCP standard and antibodies, but is also developed by an individual manufacturer. The prerequisite is that the production processes are carried out with the same host organism and similar upstream as well as downstream processes. Finally, the generic assays are commercially available HCP-ELISA test kits and work broadly for similar host organisms. The HCP standard is often prepared by using a combination of upstream isolates. Since generic assays may not cover all HCPs present in the current process, they are only recommended to be used for early phases in process development. If these are nevertheless used for late stage processes, then it must be carefully checked whether the HCPs are representative of the current manufacturing process. Another point to consider for the use of generic assays is the lack of control over the consistency and quality of the reagents, if they are not produced in-house. Overall, process-specific assays are therefore

considered superior, especially for late process phases (Ph. Eur. 2.6.34, 2017; USP <1132>, 2016).

Different detection and antibody labeling strategies could be applied for the sandwich ELISA (Figure 1.3).

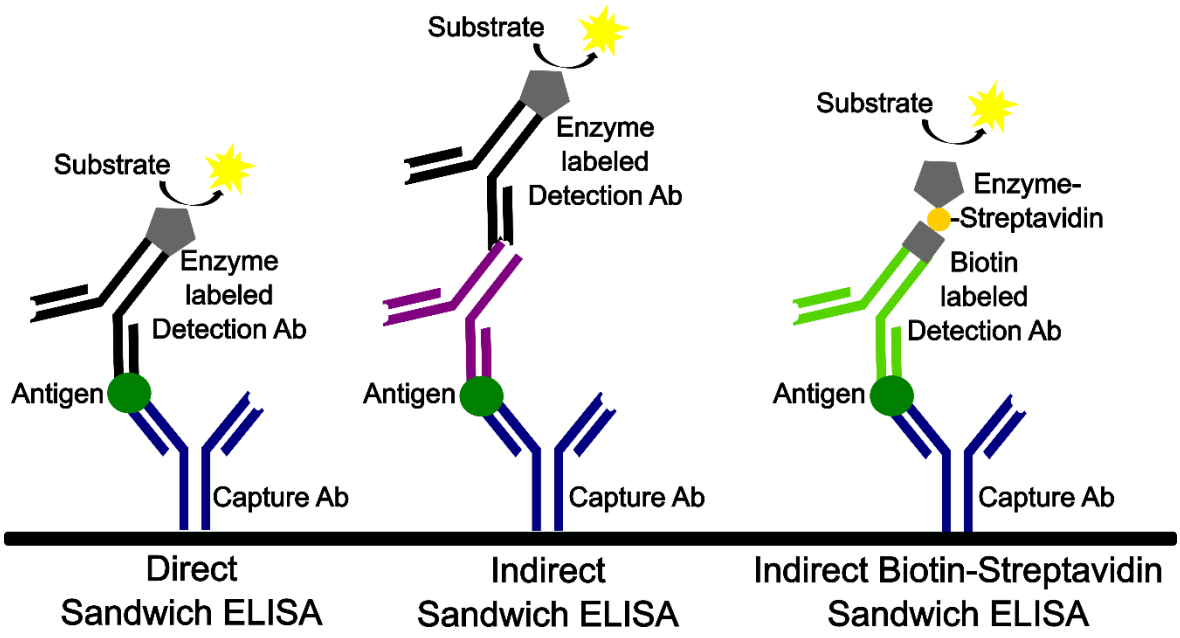

Figure 1.3: Different Sandwich-ELISA types.

For all depicted sandwich enzyme-linked immunosorbent assays (ELISA) formats, the capture antibody (Ab) is immobilized onto a microtiterplate and binds the antigen. However, differences exist in the detection Abs. They are either directly labeled with the enzyme for conversion of the substrate in the detection process (Direct Sandwich ELISA). Alternatively, the same unlabeled Abs were used for capture and detection, to form the sandwich. The sandwich is detected with a secondary enzyme-conjugated Ab (Indirect Sandwich ELISA). Finally, the detection Ab can be labeled with Biotin and the binding of a Streptavidin-enzyme complex enables signal enhancement (Indirect Biotin-Streptavidin Sandwich ELISA). Adapted from Stanker and Hnasko (2015), Nimse et al. (2016) and Jiang et al. (2021).

While chemiluminescent imaging is the most sensitive detection strategy and offers a wide dynamic range, just like fluorescent detection, which is additionally highly reproducible between plates, chromogenic detection allows direct visualization in combination with high plate-to-plate reproducibility. A downside of the chemiluminescent substrates is the great variability in signal intensity and fast signal decay. Despite somewhat lower sensitivity, chromogenic detection is the favorite method because no special or expensive equipment

is required for the readout (just standard absorbance plate readers) and it is applicable for kinetic studies. Horseradish peroxidase (HRP) and alkaline phosphatase are the most popular enzyme tags, with substrates for all three detection strategies available for the former only. HRP is more stable than alkaline phosphatase and more colorimetric substrates are available for it. The enzyme is either directly attached to the detection antibody, an extra Streptavidin-enzyme conjugate is used to bind to the biotin-labeled detection antibody, or an enzyme labeled secondary detection antibody is used to bind to the unlabeled first detection antibody (Aydin, 2015; Nygren et al., 1980; Tabatabaei & Ahmed, 2022; Walker et al., 1992).

1.7 Critical reagents of the HCP-ELISA

The HCP-ELISA requires complex reagents of biological origin, which are considered critical if they are decisive for the performance of the test. In this regard, two critical reagents, the HCP standard and the associated anti-HCP antibodies are needed for the HCP-ELISA (Figure 1.4) (Graf, Seisenberger, et al., 2021).

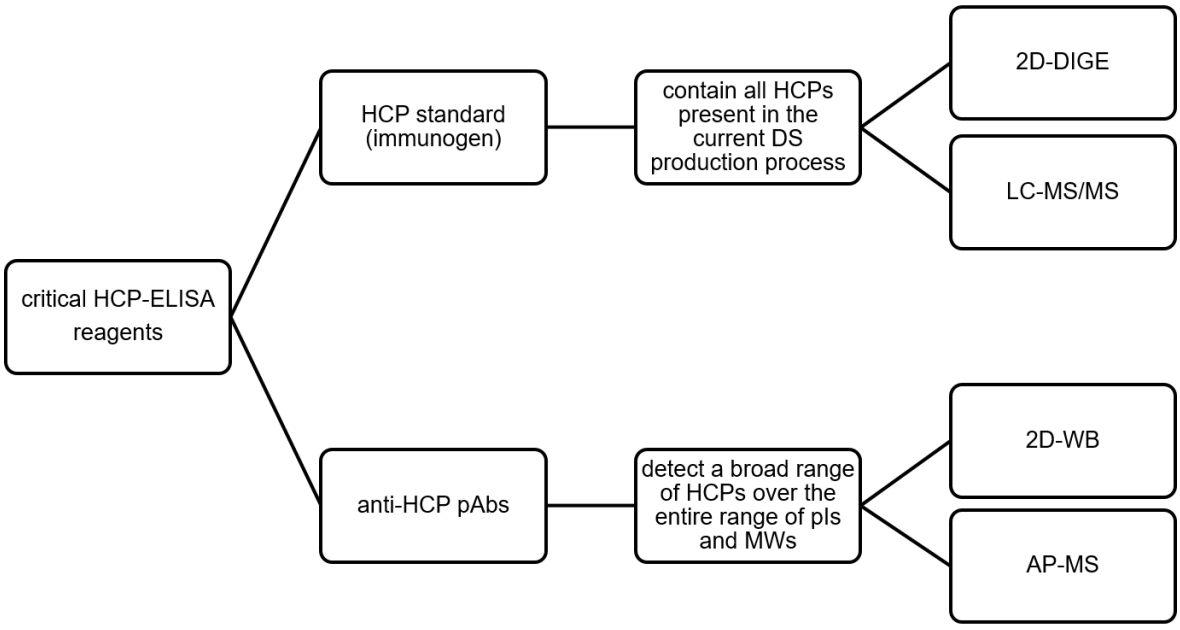

Figure 1.4: The critical HCP-ELISA reagents.

The quality of the host cell protein (HCP) enzyme-linked immunosorbent assay (ELISA) depends on two critical reagents, the HCP standard and the anti-HCP polyclonal antibodies (pAbs). Suitability of the former is demonstrated by either two-dimensional difference gel electrophoresis (2D-DIGE) or liquid chromatography-tandem mass spectrometry (LC-MS/MS). The coverage of the anti-HCP pAbs is determined primarily by two-dimensional-western blot (2D-WB) or more recently by affinity-purification based liquid chromatography-tandem mass spectrometry (AP-MS) based methods. Adapted from Wohlrab et al. (2018).

The HCP standard is typically used as antigen for the generation of the polyclonal anti-HCP antibodies and should contain the totality of all HCPs that could occur in the current DS production process. Ways to demonstrate that a broad range of HCPs of the standard is identical to the HCPs present in the process are electrophoresis methods like 2D-DIGE or LC-MS/MS methods. Another important point to consider is preventing cross-reactivity in the ELISA. Therefore, one has to avoid DS contamination in the HCP standard used for the production of the ELISA antibodies. Hence, the HCP standard is commonly prepared by mimicking the fermentation process either by using the established expression cell line alone or transfected with a vector lacking the product-coding gene. The advantage of this so-called mock-transfection is that e.g. selection markers are expressed and thus are covered by the ELISA. Other factors that may influence the composition of the HCPs are the fermentation conditions (e.g. temperature, pH, feeding strategy, medium), the cell viability at the time of harvest and the exact cell line within a species. When using mammalian CHO cells, the supernatant, which contains all proteins of interest, can be separated easily from the cells, concentrated, re-buffered and filtered after the fermentation is completed. The resulting HCP standard is tested for its protein content to define a certain protein concentration for future use and to ensure consistency from batch to batch in the case of reagent resupply (Graf, Seisenberger, et al., 2021; Gunawan et al., 2018; Jin et al., 2010; Obrstar et al., 2018; Tait et al., 2012; Wohlrab et al., 2018; Yuk et al., 2015).

Since the HCP standard is injected into host animals to produce the anti-HCP antibodies, the buffer used should be able to stabilize the HCPs and be suitable for immunization (e.g. without potassium). Depending on the type and origin of antigen, an appropriate host animal species is chosen for immunization. Whether an animal species is suitable for the production of ELISA pAbs depends, among other things, on the phylogenetic distance to the source of the antigen, the ease of blood sampling, the effort for animal husbandry, the required amount of pAbs and the intended mode of use. The most widely used host animal species for HCP-ELISA pAbs are sheep, goat and rabbit. Before starting the immunization, the protocols and procedures need to be ethically reviewed and approved by the appropriate authorities of the respective government. In addition, pre-bleeds should be examined to rule out possible cross-reactivity with the immunogen. The immunization protocol needs to be planned carefully. In general, the immunization starts with an initial injection for priming and is followed by several booster injections. Therefore the immunogen is mixed with an appropriate adjuvant (e.g. Freund`s adjuvant or Titer-Max®) and is routinely injected subcutaneously. The amount of the antigen and the injection volume depends on various factors of the immunization strategy (e.g. animal species,

molecular weight of the immunogen). Blood samples are drawn regularly (e.g. weekly or monthly depending on the size of the animal species), and the volume of these samples should not exceed 1-1.5% of the animal's weight. The reaction of each single animal to the immunogen needs to be verified by a suitable analytical method such as western blot. Animals lacking immune response to the immunogen must be excluded from further immunizations. The antibody response of the immunized animals should be monitored during the whole immunization period. Antibody titer tests are applied to determine the amount of specific pAbs in blood sera. Therefore, the antigen-antibody binding is monitored for example with an indirect ELISA, in which a defined amount of the antigen is immobilized onto a MTP and the intensity of color change measured by light absorbance is compared for serial dilutions of the raw sera. A combination of 2D-PAGE with WB (2D-WB) could be applied to characterize the HCP-specific antibodies in raw sera regarding their coverage over the whole range of molecular weights (MWs) and isoelectric points (pIs). Coverage in this context means number of HCPs detected by the anti-HCP pAb relative to the complete HCPs (Baldus et al., 2017; Delahaut, 2017; Frey et al., 1998; Gunawan et al., 2018; Hartman et al., 2018; Henry et al., 2017; Hogwood et al., 2013; Karpinski et al., 1987; Leenaars & Hendriksen, 2005; Lundström et al., 2014; Rey & Wendeler, 2012; Shukla et al., 2008; Thomson et al., 2017; Tscheliessnig et al., 2013; Wang et al., 2009; Wohlrab et al., 2018).

After completion of the immunization, the resulting polyclonal antibodies are purified from the blood sera by either Protein A/G affinity chromatography or HCP column affinity chromatography, which uses the immobilized HCP-standard. While the former binds all IgGs via their Fc part, the latter binds only the HCP specific IgGs. The Protein A/G affinity chromatography results in total IgG pools with low amounts of HCP specific antibodies, but is a well-established and reproducible standard purification method. In contrast, the HCP affinity purification method requires more effort to prepare the column material and make the method reproducible as the column material is no commercial available standard material However, it enriches especially the high affinity anti-HCP IgGs, which are beneficial for the use in ELISAs (Baldus et al., 2017; USP <1132>, 2016).

To avoid false positive HCP concentration results, the generated anti-HCP antibodies need to recognize as many HCPs in the heterogeneous HCP population as possible, but not the DS itself. Another important point is sufficient coverage. This could be demonstrated either by 2D-WB or recently also by an affinity purification based liquid chromatography-tandem mass spectrometry (AP-MS) method. Although several technical problems are known (e.g. overall low reproducibility, sensitivity issues), 2D-WB is still the

method of choice to determine the coverage of HCP-ELISA pAbs. With redards to 2D-WB, different approaches to improve its reproducibility exist. In the past, for example, two separate 2D-PAGE copies were needed, one to detect the HCP spots using silver or SYPRO™ Ruby staining and the other for the transfer of the protein spots onto a membrane to allow subsequent immunostaining with the anti-HCP pAbs. Due to inherent run-to-run variabilities of the 2D-PAGE, the protein spots of the gel need to be aligned to the immunostained protein spots on the WB to enable coverage determination. Besides being very laborious, the need of two separate gels reduces the reproducibility of the method. Today, the application of fluorescent cyanine dyes allows total protein detection and immunostaining with only one gel. This approach also facilitates the verification of transfer efficiency in WB. Further progress in reproducibility could be made by application of pre-cast gels, standardized workflows and software tools for image analysis (e.g. SpotMap, TotalLab or Delta2D, Decodon Software UG). However, as ELISAs are performed under native protein conditions and the related pAbs are accordingly produced by immunization of non-denatured HCPs, the main issue with using 2D-WB for coverage determination is probably impaired protein binding due to the loss of conformational epitopes caused by the denaturing condition during 2D-PAGE (Berkelman et al., 2015; Gunawan et al., 2018; Hogwood et al., 2017; Meleady, 2018; Posch et al., 2013; Posch et al., 2021; Tscheliessnig et al., 2013; Zhu-Shimoni et al., 2014).

An attempt to overcome protein denaturation issues for ELISA pAb coverage determination is the application of an AP-MS based method. The basic idea for this method came from the company Cygnus Technology (Bishop, 2016; Cygnus; Hoffman & Bishop, 2015; Isaac et al., 2019; Zilnerman A., 2019) that immobilized ELISA antibodies on chromatography support. HCPs were passed over the column and the bound proteins (covered HCPs) were eluted. While in the original method, covered HCPs were analyzed by 2D-PAGE, later attempts used LC-MS/MS for this purpose, which allows the identification of individual HCPs. Around the same time, Henry et al. (2017) published a similar method, but instead of chromatography columns, magnetic beads were used to immobilize the ELISA pAbs. Both methods are dependent on error-prone elution of the bound HCPs. Pilely et al. (2020) meanwhile worked with 96-well ELISA plates for immobilization of the ELISA pAbs and digested the HCPs bound to the antibodies directly in the plate. In addition to the advantage of HCP binding under native conditions, AP-MS methods allow the identification of individual protein IDs and thus a better understanding of the reasons for the missing coverage. Limitations of the current AP-MS methods for ELISA pAb coverage determination are e.g. the application of error prone elution steps, limited sensitivity of the LC-MS/MS method due to the large dynamic range between pAb

and HCP-derived peptides or/and unspecific binding issues. The latter could occur to the matrix and to the anti-HCP pAbs. Omission of unspecific bound HCPs would lead to a great underestimation of the overall coverage. Whether an HCP is specifically or non-specifically bound has so far been determined by using a threshold above which enrichment of the HCP peptides in the pAb bound fraction is considered specific (Henry et al., 2017; Pilely et al., 2020; USP <1132>, 2016; Waldera-Lupa et al., 2021). The fact that a reliable threshold value for specific binding is difficult to establish, was recently confirmed by Waldera-Lupa et al. (2021), who obtained different coverage values with their AP-MS method depending on the threshold value applied.

A general limitation of immuno-based assays is that weakly or non-immunogenic proteins are hardly or even not covered at all and therefore 100% coverage can never be anticipated. Nevertheless, it is essential to strive for the highest possible coverage in order to minimize the risk of missing single HCPs and thus increase patient safety (Baldus et al., 2017; Goey, Alhuthali, et al., 2018; Grzeskowiak et al., 2009; Jin et al., 2010; Krawitz et al., 2006; Ph. Eur. 2.6.34, 2017; Tait et al., 2012; USP <1132>, 2016; Wohlrab et al., 2018; Zhu-Shimoni et al., 2014).

1.8 Quality improvement strategies for anti-HCP ELISA antibodies

The quality of ELISA reagents and mainly the pAbs are responsible for the performance of the HCP-ELISA. There are several strategies to improve the performance of ELISA antibodies. One important factor is the grade of the antigen used for immunization. Furthermore, proper formulation is crucial, meaning that the used buffer needs to stabilize the HCPs and protease inhibitors should be added to prevent degradation of the proteins. Additionally, the antigen needs to be stable to several freeze-thaw cycles and suitable for storage at 4°C for a certain time, as this is necessary prior to immunization. Finally, it should not contain any toxic substances or DS contaminants (Delahaut, 2017; Leenaars et al., 1995; Shahrokh et al., 2016).

The whole immunization strategy is key to the quality of pAbs. In this context, the immune response evoked in the host animal depends among other factors on the amount of antigen used for immunization, frequency of injection and mode of application (Hanly et al., 1995; Leenaars & Hendriksen, 2005). It has been shown that the choice of the adjuvant has an impact on the immune response for pAb production (Fodey et al., 2008; Leenaars et al., 1995; Stills, 2005) and also, more specifically, on the anti-HCP pAb coverage (Lundström et al., 2014). “Freund´s complete adjuvant”, composed of mineral oil and mycobacteria, is

still considered as the adjuvant of choice for the generation of high-quality antibodies in animals (Karachaliou et al., 2021; Stills, 2005). The immunization site and thus the husbandry of the animals, as well as the competence of the staff in charge, is crucial to achieve sufficient quality. If possible, the use of specific pathogen-free animals is advisable (Eaton, 1995). The inherent immunogenicity of the immunized protein determines the immune response evoked in the host animal. Several factors contribute to the immunogenicity. For example, good degradability of the antigen enhance the immunogenicity of an antigen, as it allows the molecule to be easily phagocytosed and presented to T cells on the surface of antigen-presenting cells by major histocompatibility complex (MHC) molecules. An elaborate protein structure, heterogeneous composition (heteropolymers) and the chemical composition of the molecule are factors that influences immunogenicity. The physical form has also an impact on immunogenicity. In this regard, antigens in particulate form elicit a stronger immune response compared to soluble antigens, as they e.g. lead to enhanced B-cell activation or increased antigen presentation to T cells. Furthermore, the size is important as the number of epitopes increases proportional with the molecular weight of the protein and larger molecules are more easily phagocytosed. Hence, immunogens with higher molecular weight are more prone to elicit stronger immune responses than small proteins. Another factor that contributes to the immunogenicity of the antigen is the degree of foreignness of the antigen (Brodsky & Guagliardi, 1991; De Groot & Scott, 2007; Dintzis et al., 1976; Greenfield et al., 2021; Mantegazza et al., 2013; Rüde, 1971; Snapper, 2018; Ziegler et al., 1987). Major differences in protein sequences are expected especially in the case of larger phylogenetic distance of the source species to the immunized host species (Delahaut, 2017). Hence, the choice of the host animal species plays an important role for the quality of the ELISA pAbs. Most of the previously published CHO-HCP ELISA pAbs are derived from rabbit, goat, and sheep (Baldus et al., 2017; Gunawan et al., 2018; Haemmig et al., 2017; Henry et al., 2017; Rey & Wendeler, 2012; Shukla et al., 2008; Thomson et al., 2017; Waldera-Lupa et al., 2021). As foreignness of the antigen is considered a key factor for immunogenicity, the source of the antigen, here Chinese hamster is compared with the three frequently used host animal species regarding their phylogenetic relation (Figure 1.5).

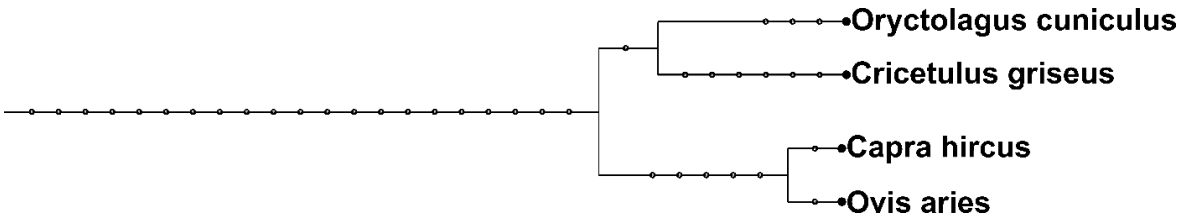

Figure 1.5: Phylogenetic tree of frequently used host animal species for CHO-HCP ELISA pAb generation.

Rabbit (Oryctolagus cuniculus), Chinese hamster (Cricetulus griseus), goat (Capra hircus) and sheep (ovis aries) are compared regarding their phylogenetic distance. Chinese hamster ovary cells are the source of the HCP-ELISA antigen for the pAb production. While sheep and goat are phylogenetically close to each other, they are further away from Chinese hamster compared to rabbit, which itself is phylogenetically closest related to hamster. This phylogenetic tree is based on the NCBI taxonomy and does not contain branch lengths or clade support values. Internal nodes, which have only one child, were removed. The tree topology was created with the phyloT tree generator (Letunic, 2015). Abbreviations: CHO, Chinese hamster ovary; HCP, host cell protein; ELISA, enzyme-linked immunosorbent assay; pAb, polyclonal antibody ; NCBI, National Center for Biotechnology Information.

Rabbit and Chinese hamster show the lowest phylogenetic distance. Goat and sheep are very close to each other but, compared to rabbit, phylogenetically more distant to Chinese hamster. Nevertheless, all those species are mammals and therefore they are phylogenetically very close to each other. This raises the question whether an improvement in the coverage of ELISA pAbs could be achieved by using a phylogenetically more distant species.

Purification of the ELISA antibodies from crude sera is another factor influencing the quality of the final reagent. Baldus et al. (2017) who compared different purification strategies reported that pAbs purified with Protein G (total IgG) and HCP affinity purification (only HCP-specific pAbs) chromatography exhibited similar 2D-WB coverage and ELISA performance. It is important to avoid the loss of HCP-specific antibodies during purification to achieve maximum yield. To this end and for enhanced reproducibility, fewer purification steps are preferable. Reducing the required purification steps of the previously applied method was already achieved as part of a master thesis (Sticht, 2020) within this project. The resulting method for purification of HCP-specific pAbs from crude sera contains a delipidation, HCP-affinity purification and size exclusion-chromatography step.

Further pre-purification steps like ammonium sulfate precipitation or anion-exchange chromatography, which are prone to pAb losses, could be omitted.

1.9 Aim of the thesis

HCPs, being a very complex and heterogeneous mixture of proteins, are in general challenging to analyze. Multianalyte ELISAs are still the workhorse for measuring HCP content in DS and their quality is tightly linked to the coverage of the associated pAbs, always featuring certain detection gaps. This work aims to understand and minimize these gaps by improving the coverage of the HCP-ELISA antibodies and related analytics. For this purpose, CHO-derived HCPs were used as practical application examples. To better understand the relationship between the applied method for coverage determination and the resulting coverage values, the methods used so far were examined closely to see whether the values obtained really reflect the actual coverage or whether the coverage tends to be overestimated or underestimated. For this purpose, on the one hand, the coverage values derived from the most frequently used method, the 2D-WB, were scrutinized in a critical study. On the other hand, non-specific binding issues of previously published AP-MS methods were addressed by optimizing this method. The newly established method should contribute to a more reliable determination of pAb coverage under non-denaturing protein-binding conditions and be an orthogonal method to the 2D-WB. With these methods in hand, the influence of host species on coverage was also investigated. Therefore, ELISA pAbs derived from five different host animal species, namely sheep, goat, donkey, rabbit and chicken were compared regarding their coverage and performance in HCP-ELISA.

2 Questioning coverage values determined by 2D Western Blots – a critical study on the characterization of anti-HCP ELISA reagents

2.1 Abstract

Host cell proteins (HCPs) constitute a major class of process-related impurities, whose substantial clearance must be demonstrated by suitable analytical methods to warrant product quality and reduce potential safety risks for patients. In this regard, enzyme linked immunosorbent assays (ELISAs), which primarily rely on the quality of the HCP reference standard (immunogen) and HCP-specific polyclonal antibodies, are considered the gold standard for HCP monitoring. For the qualification of the employed antibodies, two-dimensional western blots (2D-WBs) are the preferred technique to determine the coverage, though a number of practical constraints are well recognized. By using several orthogonal approaches, such as affinity-based mass spectrometry and indirect ELISA, the present study revealed potential detection gaps (i.e., non-covered HCPs) of conventional 2D-WBs, which can be primarily attributed to two different root causes: (i) low amounts of proteins or antibodies being unable to overcome the detection limit and (ii) western blot artifacts due to the loss of conformational epitopes through protein denaturation hindering HCP-antibody recognition. In contrast, the lack of specific antibodies against certain (particularly, low molecular weight) HCPs, as proposed in previous studies, seems to play only a minor role. Together, these findings imply that CHO-HCP ELISA antibodies are better than qualification studies by 2D-WBs indicate.

2.2 Introduction

Protein-based bio-therapeutics, a rapidly evolving field within the pharmaceutical industry, are derived from living cells, of which Chinese Hamster Ovarian (CHO) cell lines have become the preferred choice, in particular for the production of glycoproteins such as monoclonal antibodies (mAbs) (John H. Chon & Gregory Zarbis-Papastoitsis, 2011; Estes & Melville, 2014; Walsh, 2018). Due to their manufacturing conditions, constituents originating from the host cell are inevitably introduced during expression and may persist downstream processing. Among these endogenous contaminants, host cell proteins (HCPs) constitute the most abundant and diverse group of process-related impurities and are generally considered a critical quality attribute (CQA), because of their potential impact

on product quality and stability, as well as on patient's safety (Baik & Lee, 2016; Bracewell et al., 2015; Dixit et al., 2016; Fischer et al., 2017; Wang et al., 2009). To safeguard the efficient removal of HCPs over the entire purification process to the final drug substance, multi-analyte enzyme linked immunosorbent assays (ELISAs) are in general the method of choice due to their unrivaled combination in terms of sensitivity, selectivity, dynamic range, sample throughput capacity and cost efficiency (Bracewell et al., 2015; Krawitz et al., 2017; Tscheliessnig et al., 2013; Zhu-Shimoni et al., 2014).

The performance of ELISAs for determining the HCP content primarily depends on the quality of the employed critical reagents, namely the HCP ELISA reference standard (also called immunogen) and the HCP-specific polyclonal antibodies (pAbs). The immunogen, which is usually generated through mock fermentation (i.e., cells transfected with the vector of a production cell line lacking the product-coding gene), comprises a mixture of HCPs reflecting in its composition the HCP population from the current process. For the qualification of the immunogen, two-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS)-based approaches are the recommended methods to evaluate the degree of similarity (Wohlrab et al., 2018).

The second critical reagent, the HCP-specific pAbs, is in general generated through the immunization of host animals using the HCP reference standard as an immunogen (Baldus et al., 2017; Bracewell et al., 2015). Optimally, these pAbs exhibit a broad immune reactivity against the entire HCP population (comprising the whole molecular weight and pI range) present in the product manufacturing process (Ph. Eur. 2.6.34, 2017; USP <1132>, 2016). In this context, coverage refers to the ratio of HCP recognized by the pAbs relative to the totality of all detected HCPs. A widely applied technique to determine this value of HCP-specific pAbs is the 2D western blot (2D-WB), even if its usage is linked with a number of technical drawbacks (Berkelman et al., 2015; Zhang et al., 2014). These include variations in transfer efficiency, quick decay of the detection signal, high background, subjectivity of spot counting, overall bad reproducibility, too low stoichiometric amounts of proteins (from the HCP reference standard) or the corresponding reagent antibodies and impaired recognition ability of the primary antibody (capture antibody, specific to the HCP) towards the proteins due to the loss of conformational epitopes of the protein caused by the denaturing conditions during 2D-PAGE (Bass et al., 2017; Ghosh et al., 2014; Gunawan et al., 2018; Mishra et al., 2017; Tino, 2000; Wohlrab et al., 2018).

With all the named challenges for 2D-WB, this study intended to distinguish method-induced interferences from actual detection gaps arising from the lack of specific antibodies against single HCPs. For this purpose, our in-house CHO-HCP ELISA

antibodies were thoroughly characterized by applying orthogonal methods such as affinity-based mass spectrometry (MS) and indirect ELISA as well as identification of non-covered 2D-WB spots by MS analysis. Furthermore, the impact of protein size, the employed amount of proteins or specific antibodies and denaturing conditions as commonly used during 2D-WB on the occurrence of detection gaps were assessed. Based on the results presented herein, we could demonstrate that the number of covered HCPs is considerably higher than the characterization results by 2D-WBs may suggest.

2.3 Materials and Methods

Buffers, solvents and media were prepared with filtered water from a water purification system (Milli-Q® Reference, Millipore). Chemicals for ELISA, gel electrophorese and western blot analysis, were ordered from Invitrogen or SERVA except as noted otherwise and had molecular biological grade.

2.3.1 CHO-HCP standard preparation

The CHO K1 DXB11 cell line (Urlaub & Chasin, 1980) was cultivated in a mock fermentation run with in-house serum free medium at a 1000 L scale by a fed-batch process. After the harvest at a cell viability of approximately 65%, ammonium sulfate (Merck Chemicals GmbH) was added to the cell free supernatant and the precipitate was collected using two times Sartopure® PP2 20“ 1.2 µm (1.2 m²) filter cartridges (Sartorius Stedim Biotech). To resolve the precipitate, the filter was flushed backwards with salt buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). The protein solution was concentrated and diafiltrated against salt buffer using four times Sartocon Slice Hydrosart (5 kD cutoff, Sartorius Stedim Biotech) yielding a final concentration of 6.7 g/L. Finally, the protein solution was sterile filtrated (0.2 µm) and stored at –80°C.

2.3.2 CHO-HCP specific antibody preparation

Five sheep were immunized with the CHO-HCP standard using complete Freund`s adjuvant. The protocol and procedures of the sheep immunizations were ethically reviewed and approved by the District Government of Upper Bavaria (Az 211-2532-43/04). The experiments were performed in accordance with relevant institutional and national guidelines for the care and use of experimental animals (German Animal Welfare act and related regulations). Whole blood was harvested 6 months after the first boost, followed by weekly harvests for another 24 months. A total of 116.9 L whole blood was collected, pooled and stored in 2 L batches at –20°C. Those pooled raw sera were purified with an aerosol delipidation step, an ammonium sulfate precipitation and a short acetic acid

treatment for virus removal. Afterwards, a negative DEAE-Sepharose (DEAE Sepharose® Fast Flow, GE Healthcare) chromatography was conducted, for further removal of impurities. The CHO-HCP specific IgGs were purified from the pure total IgG pool from the previous step, with at least three affinity purification steps, for which the CHO-HCP standard was coupled to NHS-Sepharose (NHS Act Sepharose® 4 Fast Flow, GE Healthcare). A double negative immune adsorption onto LongR3-IGF-I-Sepharose (in-house) was performed for removal of still remaining unspecific IgGs, followed by a preparative SEC (HiLoad 26/600 Superdex 200 pg, GE Healthcare) to remove aggregates. Purified CHO-HCP specific antibodies were stored at –80°C.

2.3.3 2D-PAGE and western blot

Sample preparation and protein separation by 2D-PAGE

For separating the CHO-HCP standard based on the isoelectric point, isoelectric focusing (IEF) was performed. Therefore, a buffer exchange to DIGE buffer (aq, 7 M urea, 2 M thio-urea, 30 mM Tris, 4% [w/v] CHAPS, pH 8.5) was conducted using Amicon Ultra filters (0.5 ml centrifugal filters, Ultracel 3 K, Millipore). Protein samples were labeled with Cy-dye (Cy5, GE Healthcare) following the manufacturer's instruction. The IEF was performed with the IEF 100 system (SERVA) following the manufacturer's procedure. In brief, immobilized pH-gradient IPG stripes (ZOOM® Strip 7 cm pH 3-10L, Invitrogen) were rehydrated with sample in 140 µl rehydration buffer (DeStreak rehydration solution, GE Healthcare; adding 0.5% [w/v] SERVALYT™ 3-10) over night. The IEF was carried out with the following voltages for the 7 cm stripes: 30 min gradient 0-250 V, 90 min gradient 250-2250 V, 120 min constant 2250 V. Following the IEF, stripes were incubated 20 min in equilibration buffer (200 mM dithiotreitol, 2.5 M urea, 2% [w/v] sodium-dodecylsulfate) and, in addition, 20 min in alkylation buffer (250 mM iodaceteamide, 2.5 M urea, 2% [w/v] sodium-dodecylsulfate).

For protein separation by mass, a sodium-dodecylsulfate poly-acrylamide gel electrophoresis (SDS-PAGE) was performed. 2D gels (NuPAGE Novex 4%-12% Bis-Tris ZOOM® Gel, Invitrogen) and 7 cm IPG stripes were run with Running Buffer (NuPAGE MES SDS Running Buffer, Invitrogen) according to the manufacturer's instructions.

After SDS-PAGE, the 2D gel was imaged using the Cy5 fluorophore settings of the Octoplus QPLEX (NH Diagnostics) imager.

Western blot

The 2D gels were blotted onto a 0.2 µm polyvinylidene difluoride (PVDF) membrane (Immobilion - P^{SQ} Transfer Membrane, Merck Millipore) by Tank-Blotting (NuPAGE, Invitrogen) according to the manufacturer's instructions. After the protein transfer to the membrane, the blot was imaged using the Cy5 fluorophore settings of the Octoplus QPLEX imager.

The membrane was covered with blocking buffer for 1 h at room temperature (RT). Subsequently, the blocked membrane was incubated with 10 ml of the appropriate CHO-HCP specific antibodies (10 µg/ml diluted in blocking buffer) at RT overnight. The membrane was washed three times for 10 min with tris buffered saline and 0.1% [v/v] polysorbate 20 (TBST), incubated with 10 ml anti-sheep (H+L) horseradish peroxidase (HRP)-conjugated antibodies (diluted 1:1000 in blocking buffer) at RT for 2 h and washed again. For enhanced chemiluminescence (ECL) protein detection, the membrane was covered with Lumi-Light substrate solution (Roche Applied Sciences) and images were recorded at different exposure times with the Octoplus QPLEX imager.

Data evaluation

Images at different exposure times were compared visually and the images with the highest number of resolved protein spots were used for evaluation. The images were evaluated with the SpotMap software (Version 2.5.0.0, TotalLab Limited). For coverage determination, total protein was defined by Cy5 labeled protein spots on the 2D-SDS-PAGE and compared with immunostained spots from the western blot. Therefore, the gel and western blot images were aligned, followed by automated spot detection on both images. Subsequently, spots were reviewed and marked as absent or present. For percent coverage calculation, the following formula was used:

$$\%\ \text{coverage} = \frac{\text{Total number of immuno detected spots}}{\text{Total number of protein spots}}\ \text{x}\ 100$$

2.3.4 Size based fractionation of the CHO-HCP standard

The CHO-HCP standard was separated in two size based fractions (<40 kDa and >40 kDa) with a preparative SEC column (HiLoad 26/600 Superdex 75 pg, GE Healthcare) on an ÄKTA pure system (GE Healthcare). After calibration of the column, according to the manufacturer`s protocol, 53 mg of CHO-HCP standard in phosphate buffered saline (PBS)

was loaded onto the column. Fractions were collected (2.2 ml per fraction) and analyzed on a 4%-12% Bis-Tris gel (NuPAGE™, Invitrogen) under reducing conditions. The fractions containing the less than 40 kDa proteins were pooled, concentrated by 3 kDa ultrafiltration and re-chromatographed on the SEC column. After another analysis via gels, the appropriate fractions were pooled, concentrated and stored at –80°C.

2.3.5 Affinity-based mass spectrometry: Native coverage

The iST kits for proteomic sample preparation were obtained from PreOmics GmbH. Chemicals and chromatographic solvents used for mass spectrometry methods were liquid chromatography–mass spectrometry (LC-MS) grade.

Affinity based separation of covered HCP

Anti-CHO-HCP antibodies (1.2 mg/100 µl beads) were crosslinked to magnetic protein G sepharose beads (GE healthcare) according to the manufacturer`s protocol. For HCP binding, 100 µl of beads were incubated with an excess of the CHO-HCP standard under slow end-over-end mixing at 4°C overnight. The beads were washed several times with wash buffer (1x TBS pH 7.5). The specific bound proteins were denatured and digested with the PreOmics iST kit.

PreOmics digestion

PreOmics preparation was carried out according to the manufacturer's protocol. Each sample with up to 100 µg protein was lysed with 1x lyse buffer and the system suitability standard (SST) containing 200 µg protein was lysed with 2x lyse buffer, denatured at 95°C at 1000 rpm for 10 min and subsequently digested with trypsin. This reaction was stopped, peptides were washed, eluted from the cartridge and dried using a speed-vacuum at 45°C. Finally, the peptides were dissolved in LC-load solution and sonicated.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Ultra high performance liquid chromatography (UHPLC)-MS/MS, protein identification and data analysis was conducted as described previously (Kufer et al., 2019). Briefly, the UHPLC-MS/MS was performed with a Thermo Fisher Scientific Vanquish-H UHPLC System coupled to a Triple TOF 6600 mass spectrometer (AB Sciex). About 65 µg of peptides were separated on a CSH130 C18 (1.7 µm, 2.1 mm x 1550 mm, Waters) column, with mobile phase A consisting of 0.1% formic acid in water and mobile phase B 0.1% formic acid in acetonitrile, using a 65 min gradient. Tandem mass spectrometric analysis was performed using data dependent acquisition (DDA). Proteins were identified by the ProteinPilot Software (AB Sciex, Version 5.0) using a customized CHO database.

Acceptance criteria for positive protein identification were at least two unique peptides at a confidence of 95% and a false discovery rate of less than 1%. Internally developed software packages were used for further filtering and comparison of the obtained results.

2.3.6 Protein identification by LC-MS/MS conducted by PROTAGEN

Identification of covered and non-covered spots was performed by Protagen Protein Services, Germany. In-house CHO-HCP standard was separated by high resolution analytical 2D-PAGE (20 x 20 cm). Proteins were visualized by silver stain in the gel and colloidal gold staining, as well as immunodetection in the subsequent western blot. Resulting images were overlaid and evaluated for coverage determination. After identification of potential detection gaps, a preparative 2D-SDS-PAGE was prepared and subsequently stained with Coomassie. Exemplarily, 13 non-covered and two covered (positive control) protein spots in the 2D-WB were picked, enzymatically digested and protein identification was carried out by LC-MS/MS measurement.

2.3.7 Enzyme-linked immunosorbent assay (ELISA)

In-house CHO-HCP ELISA pAbs were used as primary antibodies, with a HRP conjugated anti-sheep antibody as secondary antibody and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Roche Applied Sciences) as substrate for detection.

Unless otherwise stated, 1 µg protein, diluted in coating buffer (50 mM sodium carbonate pH 9.6), was incubated under shaking (350 rpm) overnight at 4°C in a 96-well Microtiter plate (nunc Maxi Sorp, Thermo Scientific). After several wash steps with 1x PBST (0.05% Tween 20) buffer, the wells were filled with Blocking buffer (50 mM potassium phosphate, 0.1% gelatin, 0.05% polysorbate 20) and agitated (350 rpm) for 3 h at RT. Following washing, the primary antibodies were added and the plates incubated overnight at RT under agitation (350 rpm). Followed by another wash step, the secondary antibody was added and the mixture shaked (350 rpm) for 2 h at RT. After the final wash step, ABTS substrate was added and the absorbance was read at 405 nm with a microplate reader (VersaMax, Molecular Devices). Samples being compared were always measured on one plate to avoid the influence of plate-to-plate variability. Data evaluation was performed with an internal software using the four-parameter fit model.

The denatured and reduced CHO-HCP standard was produced by buffer exchange to DIGE buffer (aq, 7 M urea, 2 M thio-urea, 30 mM Tris, 4% [w/v] CHAPS, pH 8.5) using Amicon Ultra filters (0.5 ml centrifugal filters, Ultracel 3 K, Millipore, Germany) and reduction with 200 mM DTT. Recombinant Glutathione S-transferase P (GSTP) protein (from mouse, Novus Biologicals), which possess 91% sequence similarity to GSTP from

CHO, was processed similar to the CHO-HCP standard, when referred to as denatured and reduced.

2.4 Results

2.4.1 2D-WB of in-house CHO-HCP ELISA reagents

Detection gaps in 2D-WB stem reportedly from a number of technically inherent problems (Gunawan et al., 2018; USP <1132>, 2016; Wohlrab et al., 2018) as described in the introduction. For this reason, a number of precautionary measures should be taken to eliminate or, at least, minimize the impact of these factors on 2D-WB performance: (i) careful selection of the blotting membrane and transfer conditions, (ii) evaluating the transfer efficiency, (iii) choosing the best blocking solution to reduce background signal and iv) adjusting the enzyme-substrate-ratio to obtain a wide dynamic detection range.

According to these recommendations, method parameters, such as the employed blocking solution, washing steps and antibody concentration, were optimized in a first step (data not shown). Following this, transfer conditions were adjusted, including transfer time, method of transfer (capillary vs. electro blotting), type of membrane (nitrocellulose vs. PVDF) and transfer buffer, whose significance has been reported previously (Bass et al., 2017; Mahmood & Yang, 2012; Mishra et al., 2017). For evaluation of the transfer efficiency, CHO-HCPs were fluorescently labeled with Cy5-dye and the obtained protein spots on the gel compared with the spots on the respective membrane using the SpotMap software. By applying the herein delineated method parameters, a complete transfer (100%) onto the membrane could be achieved as shown in Figure 2.1a and b, which was considered essential for the following experiments to exclude the chance of potential detection gaps originating from poor transfer efficiency.

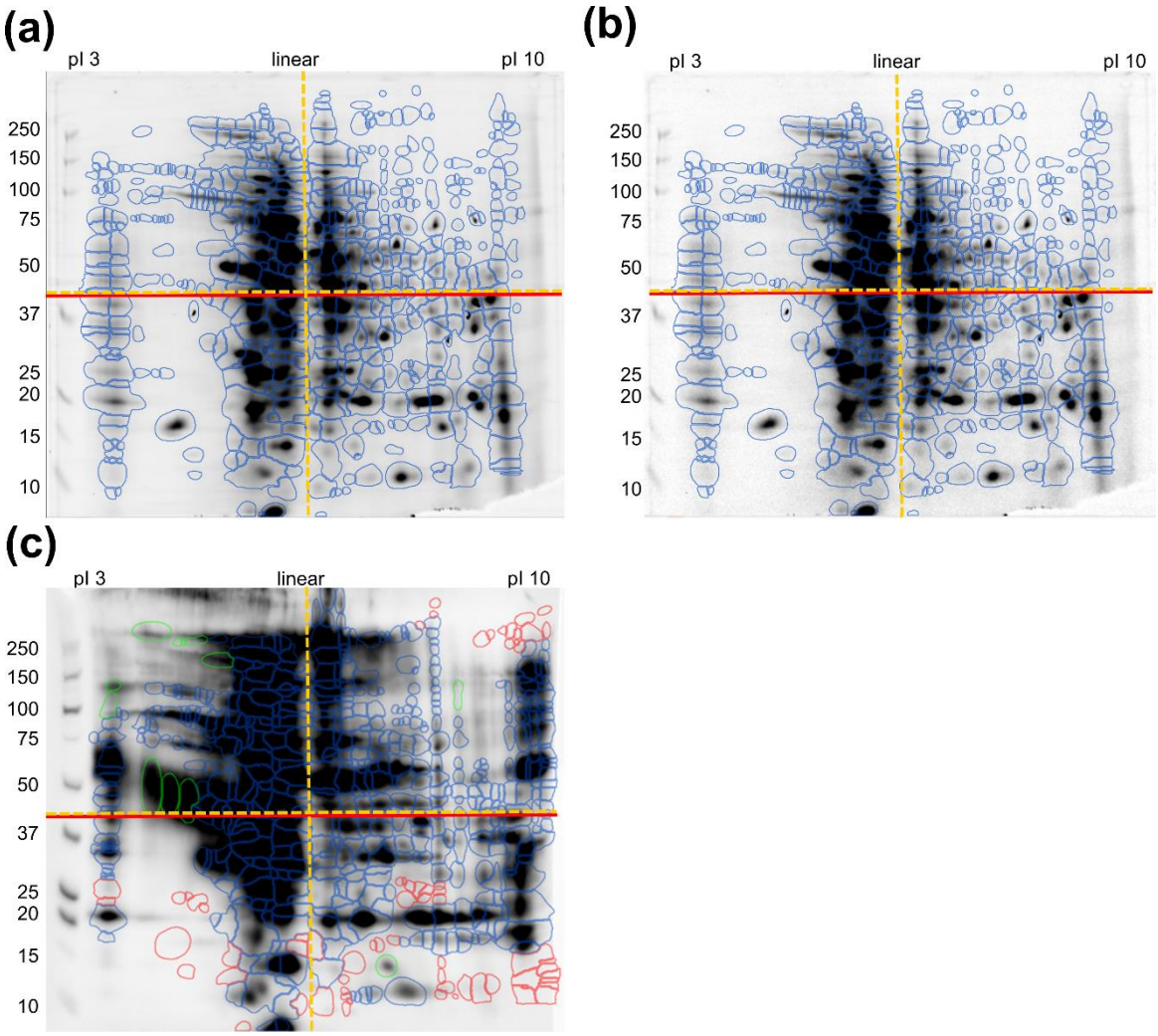

Figure 2.1: 2D-PAGE (a), 2D-WB fluorescence (protein spots) after blotting (b) and 2D-WB ECL detection (c), of in-house CHO-HCP ELISA reagents.

Cy5 labeled CHO-HCP standard (100 µg) was separated by 2D PAGE and transferred onto a PVDF membrane using the herein described method parameters. Transfer efficiency was monitored by comparing protein spots of the 2D-PAGE ***(a)*** *and 2D-WB* ***(b)****. The 2D-WB was immunodetected with CHO-HCP specific antibodies using ECL detection* ***(c)*** *and the coverage determined by comparing the number of spots to the results from the 2D-PAGE* ***(a)****. Spots present in both the protein gel and western blot (i.e., covered) are marked with blue frames, while uniquely detected spots are marked with red frames (protein gel) or green frames (western blot), respectively. For visualization, (protein) spots are divided into four quadrants (indicated by dashed yellow lines) according to their molecular weight (40 kDa threshold marked by red line) and pI. 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 2D-WB, two-dimensional western blot; CHO, Chinese Hamster Ovarian; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; HCP, host cell protein; PVDF, polyvinylidene difluoride.*

Besides the risk to miss certain proteins, unspecific binding to the immunogen, as especially observed in association with pre-immunization IgGs, can also distort 2D-WB results, since it might generate false positive hits leading to an overestimation of the overall coverage (Zilnerman A., 2019). For both antibody detection systems evaluated herein, namely combinations of pre-immunization sheep IgG/HRP labeled rabbit-anti sheep antibodies or human IgG/HRP labeled goat anti-human IgG as primary/secondary antibodies, no indications of this effect could be detected (data not shown). We ascribed this feature to our rigorous internal purification process for pAbs involving several affinity steps (i.e., CHO-HCP chromatography) to remove non-specific antibodies.

After the elimination of confounding factors arising from the experimental procedure, a 2D-WB was performed to assess potential coverage gaps of our CHO-HCP specific pAbs. Therefore, the total number of protein spots on the 2D-SDS-PAGE was compared with the detected spots on the western blot, which resulted in good coverage (88%, Supporting information Table S 7-1). For a more detailed evaluation, the 2D-WB was divided into quadrants (Figure 2.1c) to classify the respective spots according to their molecular weight (MW) and isoelectric point (pI). As shown in Table S 7-1, the LMW region (Q3 and Q4) exhibits significantly lower coverage (77% and 62%) compared with the HMW region (100% and 92%, respectively). In contrast, the pI seems to have only a minor impact, since the coverage for Q1/Q3 and Q2/Q4 is fairly similar.

2.4.2 Enrichment of LMW proteins in the CHO-HCP ELISA reference standard

For further investigation of this observation, the LMW species of the in-house CHO-HCP ELISA reference standard were separated by preparative SEC and the obtained fractions analyzed by SDS-PAGE to determine the molecular weight. Fractions containing protein spots less than 40 kDa were pooled and compared with the CHO-HCP ELISA reference standard by performing a 2D-DIGE, for which the proteins were labeled with Cy2 (LMWs) and Cy5 (CHO-HCP ELISA reference standard). Assessing the LMW region (<40 kDa) of the respective gels (Figure S 7-1a and S 7-1b), the number of detected spots were notably increased in the pooled LMW fraction, indicating an enrichment of several HCPs, which were below the detection limit for the CHO-HCP ELISA reference standard.

Based on these results, we next addressed the question if the coverage of LMW species could be improved by blending the CHO-HCP ELISA reference standard and the LMW fraction. Therefore, two separate 2D-PAGEs were run with and without the admixture of the LMW fraction and the proteins subsequently blotted onto a membrane to perform a 2D-WB (Figure 2.2).

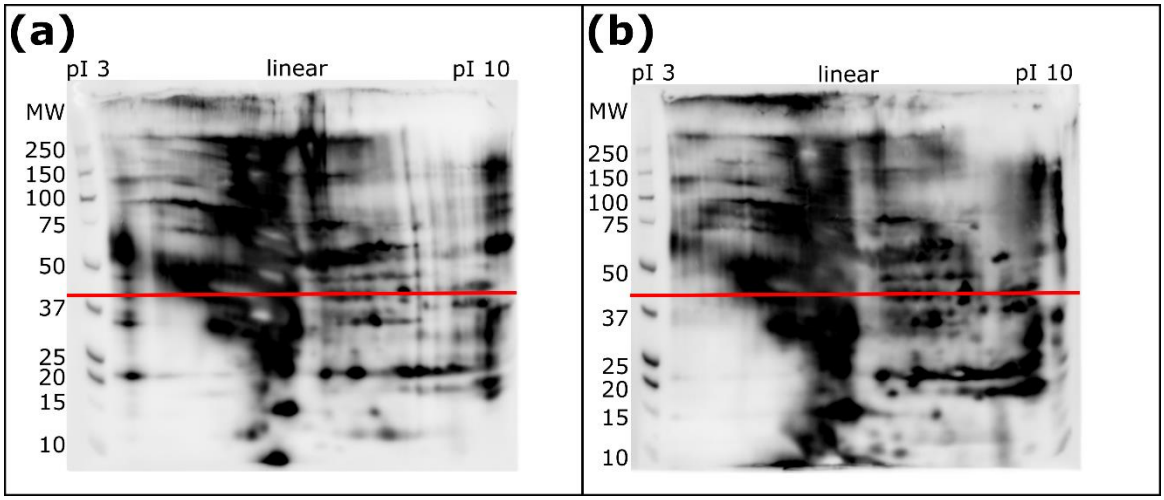

Figure 2.2: 2D-WBs without and with addition of <40 kDa CHO-HCP fraction.
Comparison of 2D-WB without ***(a)*** *and with* ***(b)*** *1:2 (100 µg complete CHO-HCP + 100 µg LMW CHO-HCP) admixture of LMW CHO-HCPs. Proteins were separated by 2D-PAGE, transferred onto a PVDF membrane and immunostained with CHO-HCP specific ELISA antibodies using ECL detection. Exposure time for both blots was 30 sec. LMW region (<40 kDa) is indicated by the horizontal red line. 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 2D-WB, two-dimensional western blot; CHO, Chinese Hamster Ovarian; ELISA, enzyme-linked immunosorbent assay; HCP, host cell protein; LMW, low molecular weight; PVDF, polyvinylidene difluoride.*

Compared with the CHO-HCP ELISA reference standard, a multitude of additional spots distributed across large parts of the LMW region (as indicated by the red line in Figure 2.2b) was observed for the produced CHO-HCP mixture resulting in a considerable increase of the protein spot count. In accordance with these results, the overall coverage increased from 88% to 96% when adding the LMW fraction, which can be primarily attributed to the enhanced coverage in the LMW region rising from 70% to 92%.

2.4.3 Characterization of ELISA reagents by native coverage

Apart from protein amounts below the detection limit, impaired antibody binding due to the loss of conformational epitopes caused by protein denaturation constitutes another potential cause for detection gaps in 2D-WB. Hence, we established an orthogonal method for determining the coverage of our in-house CHO-HCP specific pAbs while preserving the native state of the antigens by an affinity-based MS approach (referred to as native coverage). For this, the CHO-HCP specific pAbs were crosslinked to magnetic Protein G beads and incubated with the CHO-HCP ELISA reference standard. After several washing steps followed by tryptic digestion, the samples were analyzed by LC-MS/MS enabling the

identification of bound HCPs. In addition, unprimed Protein G beads as well as beads after conjugation with unspecific (pre-immunization) IgGs were treated analogously to evaluate the degree of unspecific binding facilitated by interactions with the solid carrier or the respective attached proteins as recently reported by Henry et al. (2017) using a similar approach. Consistent with the observed behavior in this study, both control bead samples exhibited a number of positive protein IDs, which could impair the validity of the results. To address this issue, a threshold value (i.e., at least log-2-fold changes in protein abundance) was defined that allows for the discrimination of specific HCP-antibody interactions from promiscuous binding. In addition, the untreated (i.e., not contacted with beads) CHO-HCP ELISA reference standard was digested and analyzed by LC-MS/MS for benchmarking purposes.

In the CHO-HCP ELISA reference standard, a total of 1526 proteins were detected (≥2 peptides at a confidence of 95%), whereas the native coverage experiment yielded in 1029 covered (bound) HCP IDs, thus leaving 497 proteins (33%) undetected. In this regard, the resulting native coverage (67%) of the affinity-based LC-MS/MS approach was considerably lower than the coverage determined by 2D-WB (88%). However, it must be stated that the total number of spots in the 2D-WB (548) is substantially lower than the identified proteins by LC-MS/MS leading to somewhat biased data (see obtained values in Table S 7-2). To address this concern and allow for a more adequate comparison of both approaches, the result table from the native coverage experiment was shortened to the Top 500 Hits. In this case, a very similar coverage could be observed for both methods as displayed in Figure 2.3 and Table S 7-2.

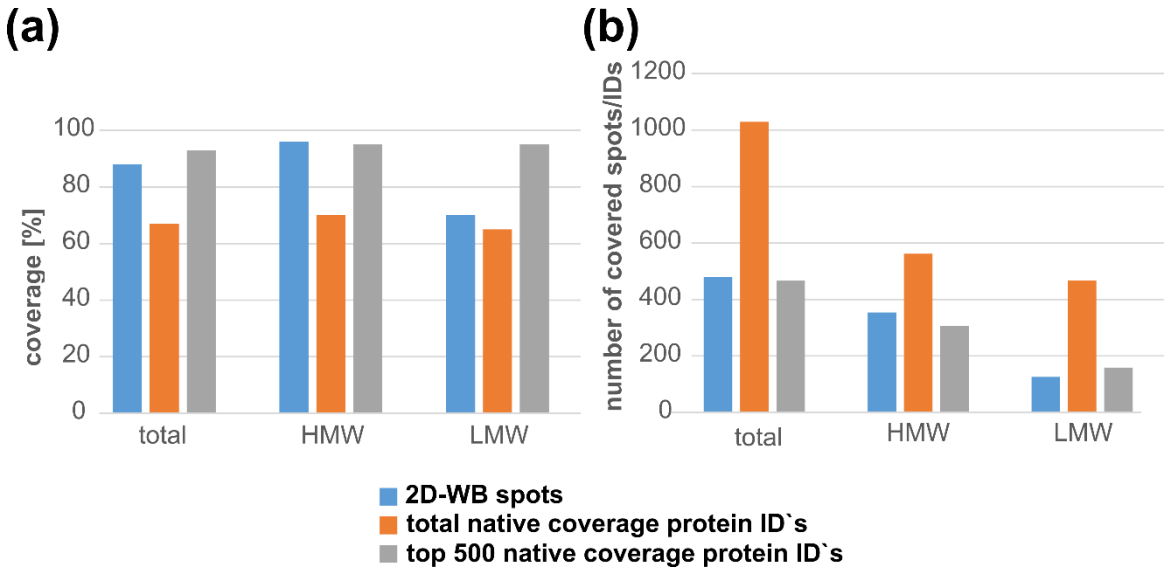

Figure 2.3: Comparison 2D-WB and native coverage results.

*Visual comparison of coverage values in percent **(a)** and number of covered protein spots or IDs **(b)** for 2D-WB and affinity-based MS (native coverage) of the in-house CHO-HCP ELISA reagents. Identified proteins are divided into the total molecular weight (total), HMW (>40 kDa) and LMW (<40 kDa) range. 2D-WB, two-dimensional western blot; CHO, Chinese Hamster Ovarian; ELISA, enzyme-linked immunosorbent assay; HCP, host cell protein; HMW, high molecular weight; LMW, low moleclar weight.*

Analogous to the approach for 2D-WB, the Top 500 Hits were additionally split into HMW (>40 kDa) and LMW (<40 kDa) HCPs as derived from the protein sequences of the database entries. Overall, a total of 307 out of 332 HMW and 159 out of 168 LMW proteins were detected, translating into coverage values of 92% and 95%, respectively. Notably, the relative ratio of HMW and LMW proteins remains almost unaffected when comparing the Top 500 Hits with the 2D-WB results (see Table S 7-2), indicating that there is no bias in their distribution after applying the filter criteria for native coverage.

For better visual comparison to our 2D-WB results, virtual 2D gels and Venn Diagrams of Top 500 native coverage hits were prepared with in-house software, which are depicted in the supporting information (Figure S 7-2).

2.4.4 Indirect ELISA of native and denatured CHO-HCP ELISA reference standard

As an orthogonal method for evaluating the impact of protein denaturation on the binding behavior of the CHO-HCP specific pAbs, an indirect ELISA method was developed. Therefore, the CHO-HCP ELISA reference standard was treated according to the sample

preparation conditions employed for 2D-PAGE, namely exchanging buffer to DIGE buffer (aq, 7 M urea, 2 M thio-urea, 30 mM Tris and 4% [w/v] CHAPS, pH 8.5) and sample reduction with DTT. After both unprocessed and denatured/reduced CHO-HCP ELISA reference standard samples were coated onto a plate and incubated with a serial dilution of CHO-specific pAbs (ranging from 0.2 pg to 10 µg), antigen-antibody recognition was monitored using a HRP-ABTS detection system. For evaluation, the signal intensity was plotted against the log concentration (Figure 2.4) and the inflection point as well as the resulting titer were calculated.

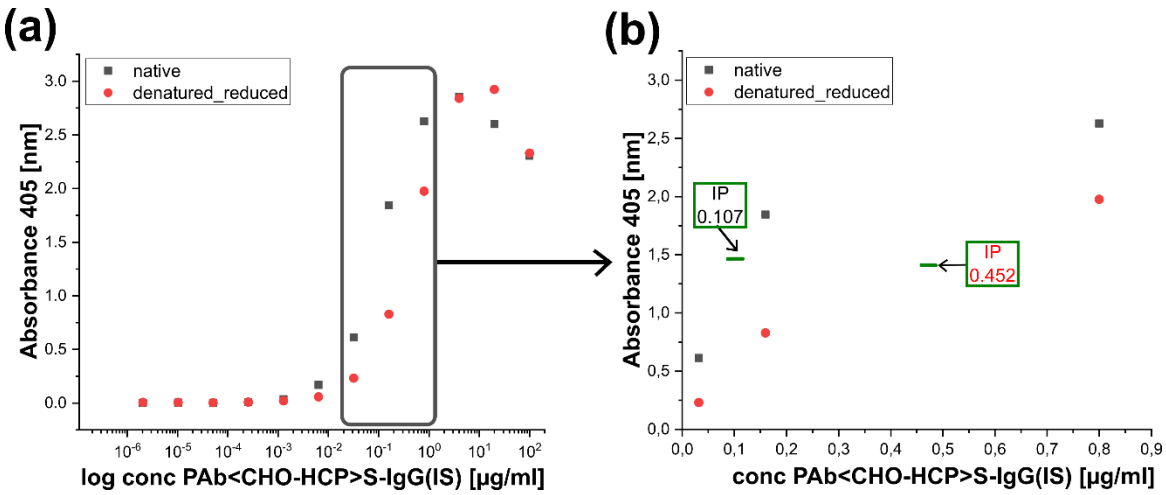

Figure 2.4: Indirect ELISA for assessing the response rate of the in-house CHO-HCP ELISA reagents under both native and denatured/reduced conditions.

*The log of the 12 employed pAbs concentrations are plotted against the absorbance at 405 nm **(a)** and the resulting sigmoidal curves evaluated by using a 4-parameter fit model. Figure **b** shows the zoom-in of the plots around the inflection point. The green lines indicate the obtained inflection points and the corresponding pAbs concentrations are shown in green boxes. CHO, Chinese Hamster Ovarian; ELISA, enzyme-linked immunosorbent assay; HCP, host cell protein.*

Compared with native conditions, the amount of antibodies to reach the inflection point was about four times higher when analyzing the denatured/reduced CHO-HCP standard.

2.4.5 Identification of missed protein spots in 2D-WB by MS

Since the previous experiments only allowed for a high-level overview, we next focused on the identification of 2D-WB gaps at the protein level. This is of particular relevance to resolve the question whether there are actually HCPs non-covered by specific antibodies. For this, the 2D-WB detection gaps found in our CHO-HCP ELISA reagents were analyzed

externally by LC-MS/MS after extraction of the respective 2D-WB spots. An overview of the selected 13 non-covered and, for control purposes, two covered proteins as well as their coverage by 2D-WB and native coverage is provided in Table 2.1.

Table 2.1: List of identified protein spots by LC-MS/MS. 15 protein spots (13 non-covered and 2 covered by 2D-WB) were extracted and externally analyzed by LC-MS/MS. The obtained protein IDs were compared with the results from the native coverage experiments.

identified protein	Molecular weight [kDa]	pI	2D-WB signal	Native coverage protein identified
14-3-3 protein epsilon isoform X2	29.17	4.63	no	yes
40S ribosomal protein S12	14.51	6.81	no	yes
Adenine phosphoribosyltransferase	20.77	5.66	no	yes
Cofilin-1	18.53	8.22	no	yes
Eukaryotic translation initiation factor 2 subunit 3 isoform X2	51.11	8.66	no	yes
Glucose-regulated protein precursor	71.88	5.04	no	yes
Glutathione S-transferase P	25.00	8.24	no	yes
Heterogeneous nuclear ribonucleoprotein F	45.15	5.12	no	yes
Proteasome subunit beta type-1	26.30	7.67	no	yes
Proteasome subunit beta type-4	29.17	5.31	no	yes
Rho GDP-dissociation inhibitor 1	23.42	5.10	no	yes
Ubiquitin-conjugating enzyme E2 N-like protein	17.93	8.76	no	yes
Putative UPF0556 protein C19orf10 like protein	15.90	5.02	no	no
L-lactate dehydrogenase A chain	36.52	7.01	yes	yes
Peroxiredoxin-1	22.26	8.22	yes	yes

Abbreviations: 2D-WB, two-dimensional western blot; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

For 12 out of 13 selected spots, missed proteins by 2D-WB were identified as covered by native coverage. In addition, a single HCP was selected (Glutathione S-Transferase P, GSTP) that was missed by 2D-WB, but covered by the native coverage approach, and subjected to analysis by indirect ELISA in both the native and the denatured/reduced state using our CHO-HCP specific pAbs (Figure 2.5).

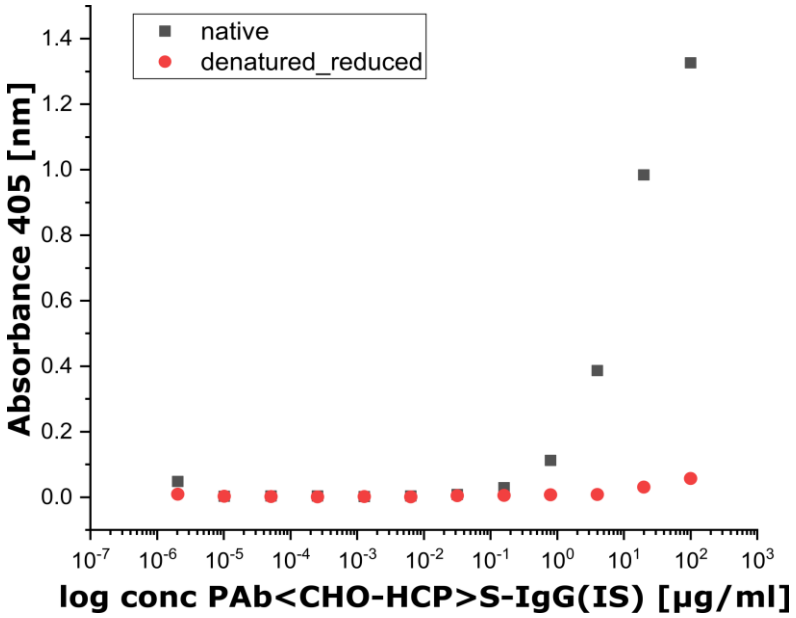

Figure 2.5: Indirect ELISA of recombinant glutathione S-transferase P (GSTP) under both native and denatured/reduced conditions.

The absorbance at 405 nm is plotted against the log of the 12 employed pAbs concentrations. pAbs, polyclonal antibodies.

While denatured/reduced GSTP only resulted in an insufficient signal to calculate the inflection point, a proper signal was observed for native GSTP.

2.5 Discussion

Detection gaps of 2D-WB in the LMW region (<40 kDa) have been repeatedly reported over the last years (Bass et al., 2017; Butler et al., 2019; Gilda et al., 2015; Henry et al., 2017; Mishra et al., 2017), suggesting the absence of specific antibodies against the respective proteins. In this regard, it was proposed, that LMW proteins are generally underrepresented in the immunogen (Müller et al., 2010) and possess a smaller number of epitopes (Dintzis et al., 1976), both aspects translating into a theoretically reduced immunogenicity compared to high-abundant HMW proteins. However, also the inherent technical limitations of 2D-WB could affect the recognition or detection of certain proteins

that are actually covered by specific antibodies. In this study, the impact of these factors on 2D-WB performance were evaluated by using orthogonal approaches.

2.5.1 Impaired 2D-WB signal by an insufficient amount of LMW HCPs

LMW proteins are reportedly underrepresented in the CHO-HCP ELISA reference standard due to their tendency of getting lost during protein separation and concentration procedures (Müller et al., 2010). In accordance with this notion, separation of the CHO-HCP ELISA reference standard by preparative SEC demonstrated that the LMW fraction (<40 kDa) only accounts for 5% of the total protein amount (mg/mg). Therefore, it is easily conceivable that the corresponding signals for 2D-WB fall outside the dynamic range even if specific antibodies are generally available. A similar effect is known for fluorescently labeled proteins, when certain spots are exclusively detected by western blots, but not in the corresponding SDS-PAGE, which can be explained by the different sensitivity of fluorescence (CyDye™ DIGE fluors: 25 pg of a single protein required) and ECL (Lumi-Light ECL Plus: 1-5 pg of antigen required) detection. Correspondingly, this could also be true for the dynamic range of 2D-WBs, if the differences in proteins concentration of different HCPs exceed a certain threshold. To prove this theory, the pooled LMW fraction was mixed with the CHO-HCP reference standard to perform a 2D-WB. Compared to the reference experiment (using merely the CHO-HCP reference standard), a significantly improved coverage, especially in the LMW region, could be observed, corroborating the hypothesis that a large number of the observed detection gaps stems from an insufficient amount of LMW proteins rather than the lack of specific pAbs targeted against them. A potential shortcoming of the herein described approach to evaluate the coverage is that by using small gels (7 cm) the overall number of protein spots is considerably lower than for 2D-WBs performed on large gels (25 cm), which may lead to an overestimation of the % coverage. However, due to the limited amount of the enriched LMW fraction available, the application of small gels - which is common practice within the HCP community (Jin et al., 2010; Tait et al., 2012) - appeared as a reasonable compromise, projecting the general behavior when a higher proportion of LMW proteins is present in the CHO-HCP reference standard.

2.5.2 Effect of protein denaturation on 2D-WB coverage

Protein denaturation can lead to impaired antibody binding due to the loss of conformational epitopes on the surface of antigens, which are substantial for antigen-antibody recognition (Anderer & Ströbel, 1972; Barlow et al., 1986; Forsström et al., 2015; Gilda et al., 2015; Tino, 2000). For this reason, it is generally recommended to adhere to the conditions used for the immunization protocol (i.e., detection of denatured proteins

should be performed by employing antibodies that were generated with a denatured immunogen and *vice versa*). However, as HCP ELISAs are routinely performed without a denaturation step and thus the antibodies are raised against the immunogen in its native form, there is an inherent risk of missing a variety of proteins when performing 2D-WB under denaturing conditions. Evidently, this effect is more pronounced for LMW proteins due to the (by mere chance) smaller number of potential epitopes compared with HMW proteins, which could be a further explanation for the observed detection gaps in the LMW region. To evaluate the impact of protein denaturation on antibody binding in more detail, an affinity-based LC-MS/MS workflow was implemented in this study, which allows for monitoring antigen-antibody interactions under native conditions. Taking into account all identified proteins, the obtained coverage (67%) was well below the overall value determined by 2D-WB (88%), which was attributed to the superior sensitivity of MS detection leading to a total number of 1526 identified proteins compared to 548 resolved protein spots in the 2D-WB (see Table S 7-2). For a more meaningful comparison of both approaches, the results table from the native coverage experiment was shortened to the Top 500 Hits. We justified this strategy on two counts. First, the selected Top 500 Hits provides a statistically significant number of identified proteins, which corresponds with the detected spots on the 2D-WB. Second, by focusing on proteins that were identified with a relatively high number of unique peptides, the overall robustness of the method is improved resulting in highly reliable and reproducible data. Using the adapted Top 500 list, an overall coverage of 93% was determined with nearly equal distribution for the HMW and LMW region as opposed to the considerably higher number of detection gaps for LMW proteins in the 2D-WB. To further corroborate the hypothesis that the coverage is largely unaffected by the molecular weight of the respective HCPs when the analysis is performed under native conditions, an indirect ELISA was conducted under both native and denatured conditions. As shown herein, the amount of antibodies required to reach the inflection point was about four times higher for the denatured/reduced CHO-HCP reference standard compared to the untreated sample, indicating impaired binding of the CHO-HCP specific pAbs due to the loss of conformational epitopes after protein denaturation.

2.5.3 Undetected proteins caused by the absence of specific pAbs

Having revealed two technical root causes for the relatively low coverage of 2D-WBs in the LMW region, we finally pursued the question to what extent the lack of specific pAbs contributes to the occurrence of detection gaps. To accomplish this, 13 protein spots, which remained undetected by 2D-WB, were extracted and analyzed by LC-MS/MS. Interestingly, the vast majority (92%) of the identified proteins was covered by our native

coverage approach, suggesting that missed proteins in 2D-WB are in most cases related to the employed denaturing conditions, but not due to the absence of the respective specific antibodies. Among the identified proteins, GSTP, a basic protein with a MW of approx. 25 kDa, was additionally analyzed by the herein indirect ELISA under both native and denatured/reduced conditions. In accordance with the results from the native coverage, the native protein elicited a considerably higher signal, indicating the presence of specific pAbs that are specifically targeted against the protein in its native state, but show impaired binding after loss of conformational epitopes through protein denaturation.

Taken together, these results suggest that detection gaps observed in 2D-WB can in many cases be attributed to practical constraints of this technique, rather than the actual absence of specific antibodies, leading to an overall underestimate for the coverage determination. On that account, 2D-WB remains a valid method for the characterization of ELISA antibodies to demonstrate that the vast majority of HCPs in the current process are covered, but it is highly recommended to use orthogonal methods overcoming the drawbacks of Western Blot approaches. In this context, native coverage emerged as a powerful tool to show suitability of the applied CHO-HCP specific pAbs, even if affinity-based MS methods feature some drawbacks themselves, such as unspecific binding issues. Thus, combining both methods leads ultimately to improved knowledge about the quality of these antibodies, which is essential for the development of a reliable HCP ELISA assay.

3 Toward optimal clearance - A universal affinity based mass spectrometry approach for comprehensive ELISA reagent coverage evaluation and HCP hitchhiker analysis.

3.1 Abstract

In the control strategy for process related impurities in biopharmaceuticals the enzyme linked immunosorbent assay (ELISA) is the method of choice for the quantification of host cell proteins (HCP). Besides two-dimensional (2D) - western blots (2D-WB), the coverage of ELISA antibodies is increasingly evaluated by affinity purification based liquid chromatography–tandem mass spectrometry (AP-MS) methods. However, all these methods face the problem of unspecific binding issues between antibodies and the matrix, involving the application of arbitrarily defined thresholds during data evaluation. To solve this, a new approach (optimized AP-MS) was developed in this study, for which a cleavable linker was conjugated to the ELISA antibodies enabling the subsequent isolation of specifically interacting HCPs. By comparing both approaches in terms of method variability and the number of false positive or negative hits, we could demonstrate that the optimized AP-MS method is very reproducible and superior in the identification of antibody detection gaps, while previously described strategies suffered from over- or underestimating the coverage. As only antibody associated HCPs were identified, we demonstrated that the method is beneficial for hitchhiker analysis. Overall, the method described herein has proven as a powerful tool for reliable coverage determination of ELISA antibodies, without the need to arbitrarily exclude HCPs during the coverage evaluation.

3.2 Introduction

Monoclonal antibodies (mAbs) for therapeutic use are produced in living cells and the most commonly employed hosts for this purpose are Chinese hamster ovary (CHO) cells. Expressed besides the desired product during manufacturing, host cell proteins (HCPs) belong to the group of process related impurities. HCPs are a critical quality attribute (CQA), since they have potential immunogenic effects (inherently or by inducing the formation of anti-drug antibodies) or are biologically/ enzymatically active affecting product quality and stability (Bracewell et al., 2015; Vanderlaan et al., 2018; Wang et al., 2009). By using efficient purification methods, HCPs are largely removed. As a result, HCPs occur

in most cases only in (sub-)ng quantities as opposed to the mAb present in mg quantities (Guiochon & Beaver, 2011; Shukla et al., 2007).

The method of choice to quantify HCPs for downstream process monitoring and release testing is the enzyme linked immunosorbent assay (ELISA). ELISAs should cover the majority of HCPs over a broad range of molecular weights and isoelectric points (pIs) as recommended by the Pharmacopoeias (Ph. Eur. 2.6.34, 2017; USP <1132>, 2016). In this regard, 2D-western blot (2D-WB) is the most commonly used method to demonstrate adequate coverage of the polyclonal antibodies (pAbs) used in the ELISA (Graf, Seisenberger, et al., 2021). However, this method has some disadvantages, such as poor reproducibility, subjectivity in data analysis, relatively low sensitivity and the loss of conformational epitopes due to the denaturing conditions employed during 2D-PAGE (Bracewell et al., 2021; Ghosh et al., 2014; Gunawan et al., 2018; Mishra et al., 2017; Seisenberger et al., 2021; Tino, 2000; Wohlrab et al., 2018).

To complement 2D-WB, affinity purification based approaches have increasingly been applied recently. These methods generally follow the same principle of immobilization of ELISA antibodies to a matrix, the addition of HCPs and the analysis of the bound proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or gel-based methods, but differ in their technical details: For example, ELISA antibodies were immobilized either directly via the amino groups, the Fc-part or after conjugation with biotin on different matrices, such as magnetic beads, ELISA plates or resins in FPLC columns (Henry et al., 2017; Isaac et al., 2019; Pilely et al., 2020; Seisenberger et al., 2021; Waldera-Lupa et al., 2021). These methods are of particular relevance - especially when combined with LC-MS/MS analysis - as they have the following advantages over 2D-WBs: (i) unambiguous identification of single HCPs and thus a better understanding of the ELISA coverage e.g. by finding common properties of uncovered HCPs or facilitating the search of appropriate ELISA antibodies for a particular process, (ii) binding under native conditions similarly to those used for the ELISA and (iii) allowing unimpaired detection of HCP binding even in the presence of product antibodies (Henry et al., 2017; Pilely et al., 2020; Waldera-Lupa et al., 2021).

All previous affinity-purification LC-MS/MS (AP-MS) (Henry et al., 2017; Pilely et al., 2020; Seisenberger et al., 2021; Waldera-Lupa et al., 2021) methods have the common drawback of non-specific binding to the matrix. In order to distinguish between specific and non-specific binding, the common solution was determination of a threshold value above which enrichment in the antibody bound fraction is considered as specific. Total exclusion of HCPs identified in both the specific and unspecific bound fraction would certainly lead

to a gross underestimation of the coverage. As verified in the present work, it proves difficult to define the appropriate enrichment factor for specific binding, especially for low abundant HCPs only identified in low- to mid-single peptide digits.

To overcome this potential bias, a novel method eliminating the effects of non-specific binding to the matrix was developed in this work, named optimized AP-MS. For this purpose, the ELISA antibodies were labeled site-specifically with a cleavable Biotin-Linker, enabling the release of the immuno-complexed antibodies from the streptavidin beads by reductive cleavage. As a consequence, the immuno-complexed antibodies were digested in the absence of the matrix, which allowed the exclusive identification of antibody bound HCPs after LC-MS/MS measurement.

Another shortcoming of some of the recently described affinity based mass spectrometry methods is the need to elute the HCPs from the antibodies, whereby complete elution of the bound HCPs is difficult to achieve (USP <1132>, 2016) and may lead to variable recovery between different HCPs. Using the method presented herein evades this error-prone step, as the immunocomplexed HCPs are released from the matrix utilizing a cleavable linker. Also, applying denaturing conditions for digesting the bound HCPs directly from the antibody affects the sensitivity by LC-MS/MS analysis due to a large excess of antibody peptides in the digested sample. In contrast, a tryptic digestion using native conditions reduces the dynamic range of the sample resulting in improved sensitivity, as demonstrated in several recent publications (Huang et al., 2017; Kufer et al., 2019; Molden et al., 2021).

All crucial steps of the optimized AP-MS method were carefully evaluated in terms of functionality and efficiency. The effect of a threshold application on false negative and false positive coverage results was analyzed in detail. In addition, the new method was applied to determine the coverage of two different CHO-HCP ELISA antibodies and compared with 2D-WB results. Finally, the optimized AP-MS method was used to perform a hitchhiker analysis with an in-house mAb instead of the ELISA antibody, to identify HCPs that are non-specifically bound to the mAbs and thus often difficult to remove in the mAb purification process.

3.3 Materials and Methods

Buffers, solvents and media were prepared with filtered water from a water purification system (Milli-Q® Reference, Millipore).

Antibodies

All antibodies used were produced and purified in-house.

3.3.1 Affinity based separation of covered HCP using protein G beads (established method)

The affinity based separation of covered HCPs using magnetic Protein G beads was performed as described previously (Seisenberger et al., 2021). In brief, CHO-HCP-ELISA antibodies were crosslinked to magnetic protein G beads and incubated with the CHO-HCP ELISA reference standard. After several wash steps, the bound proteins were isolated from the beads by denaturation, tryptically digested and the peptides were identified with LC-MS/MS analysis.

3.3.2 Affinity based separation of covered HCP using click chemistry (optimized method)

Antibody modification

The functionalization of the antibody with azido groups was carried out according to the manufacturer's protocol using the GlyCLICK Azide Activation kit (Genovis AB). Briefly, conserved N-glycans of the Fc-part of the antibody were hydrolyzed to the innermost N-Acetylglucosamine (GlcNac) by immobilized GlycINATOR (EndoS2) enzyme. In a second step the UDP-N-azidoacetylgalactosamine (UDP-GalNAz) was attached site-specific to the GlcNac by the galactosyltransferase (GalT) Y289L (Duivelshof et al., 2020).

Conjugation of cleavable biotin-linker by using click-chemistry

Click chemistry for binding the cleavable biotin-linker to the azide-modified antibody was performed using Diazo Biotin-PEG3-DBCO (BroadPharm). Therefore, about 450 µg of azide-modified antibody was incubated with 11 µl of 20 mM Diazo Biotin-PEG3-DBCO dissolved in DMSO and filled up with tris buffered saline (TBS) pH 7.5 to a total volume of 476 µl. The mixture was incubated overnight at room temperature (RT) with slow end-over-end mixing. Excess of non-bound Diazo Biotin-PEG3-DBCO was removed in two steps.

First, the solution was purified with Sephadex-G25 columns (PD-Minitrap G-25, Cytiva) according to the manufacturer's protocol. Second, the sample was passed through Centrifugal Filter Devices (Amicon® Ultra-0.5 Centrifugal Filter Devices 50 kDa MWCO, Merck). The concentration of the antibody was determined spectroscopically and the degree of labeling (DOL) was determined as described below.

HCP binding

250 µg of the biotin-labeled antibody was incubated overnight at RT under slow end-over-end mixing with (16-fold) excess of the HCP standard. The antibody-HCP complex was bound to 250 µl magnetic streptavidin beads (Streptavidin Mag Sepharose, Cytiva) and subsequently washed three times using phosphate buffered saline, 0.1% [v/v] Polysorbate 20 (PBST) pH 7.5, followed by three wash steps with TBS buffer pH 7.5 (ELISA like washing conditions).

Cleavage of biotin linker

The cleavage of antibodies from the streptavidin beads was performed for 1 h under slow end-over-end mixing at RT using 100 µl of a 25 mM Sodium dithionite solution.

3.3.3 SDS-PAGE

Samples were mixed with NuPAGE™ LDS Sample Buffer (Invitrogen, Thermo Fisher Scientific) and NuPAGE™ Reducing Agent (Invitrogen, Thermo Fisher Scientific), according to the manufacturers protocol. The samples were loaded onto a NuPAGE™ Bis-Tris mini gel (Invitrogen, Thermo Fisher Scientific) after incubation for 10 min at 70°C. The gel run was conducted for 35 min while remaining the voltage constant at 200 V. The gels were washed three times for 5 min in purified water, imaged using the Cy5 fluorophore settings of the Octoplus QPLEX (NH Diagnostics) imager and stained with Coomassie brilliant blue.

3.3.4 Determination of the degree of labeling

The DOL of the antibody was calculated by spectrophotometric measurement of DBCO absorption (DBCO λ_{max} = 309 nm) and antibody concentration (λ_{max} = 280 nm) and was calculated as follows:

$$DOL = (A_{max} * \varepsilon_{280}) / ((A_{280} - \varepsilon_{max} * CF) * \varepsilon_{max})$$

$$CF = CF_{280} \text{ for DBCO} = 0.04$$

3.3.5 Denaturing PreOmics digestion

PreOmics digestion was carried out according to the manufacturer's protocol and as described elsewhere (Kufer et al., 2019; Seisenberger et al., 2021). Briefly, the protein sample (up to 200 µg) was lysed, denatured at 95°C for 10 min and loaded onto an iST cartridge. Trypsin was added and the sample was digested for 2 h at 37°C. The reaction was stopped and the peptides were purified in several wash steps. After elution of the peptides from the iST cartridge, the eluate was dried using a speed-vacuum concentrator at 45°C. The peptides were dissolved in LC-load solution and sonicated for 5 min. At this point, the sample was measured directly or stored at –80°C until measurement.

3.3.6 Native digestion with peptide clean-up

The protocol was adapted from the method described by Huang et al. (2017) and Kufer et al. (2019). In short, the volume of the protein sample was adjusted to 185 µl with purified water and the pH was adjusted with 5 µl 1 M Tris/HCl buffer (pH 8.0). Trypsin was added and the mixture was incubated for 2 h at 37°C (300 rpm). Samples were reduced with 4 µl of 100 mM DTT solution and denatured at 95°C for 10 min. The denatured antibody was precipitated in a centrifugation step (2 min, 14,000 rcf) and the supernatant was transferred in a new reaction tube. After acidification of the supernatant with 0.1% TFA, a peptide clean-up was carried out with the Pierce Peptide Desalting Spin columns, according to the manufacturer's protocol. For sample volumes greater than 185 µl the sample volume was adjusted to 370 µl and all other reagent volumes were doubled as well.

3.3.7 LC-MS/MS analysis and data analysis

The LC-MS/MS analysis was performed as described in Kufer et al. (2019), with the following variation. Addition of an extra column wash step with wash buffer (25% acetonitrile, 25% methanol, 25% isopropanol and 0.1% formic acid) directly after each sample.

In short, up to 65 µg of peptides were separated on a CSH130 C18 (1.7 µm, 2.1 mm x 1550 mm, Waters) column using a 65 min gradient, with mobile phase A consisting of 0.1% formic acid in water and mobile phase B 0.1% formic acid in acetonitrile. The measurement was performed with a Thermo Fisher Scientific Vanquish-H UHPLC System coupled to a Triple TOF 6600 mass spectrometer (AB Sciex) using data dependent acquisition (DDA).

Proteins were identified using the ProteinPilot Software (Version 5.0, AB Sciex) with a customized CHO database. At least two unique peptides at a confidence of 95% and a false discovery rate of <1% were required for positive protein identification. An internally developed software package was used for filtering and comparison of the results, as well as for preparation of virtual 2D-gels. Relative protein quantification was performed as described elsewhere (Pilely et al., 2020) by using Skyline (MacLean et al., 2010) for determination of the sum of MS peak areas for all peptides mapped to a unique protein (SumAll peptide area) (Pilely et al., 2020).

3.3.8 2D PAGE and Western blot

Sample preparation and protein separation by 2D PAGE.

The CHO-HCP standard was separated by differences in the isoelectric point. First, a buffer exchange to DIGE buffer (aq, 7 M urea, 2 M thio-urea, 30 mM Tris, 4% [w/v] CHAPS, pH 8.5) was conducted using Amicon Ultra filters (0.5 ml centrifugal filters, Ultracel 3K, Millipore). Protein samples were labeled with Cy-dye (Cy5, GE Healthcare) following the manufacturer instruction. The isoelectric focusing (IEF) was performed with the IEF 100 system (SERVA) following the manufacturers procedure. In a nutshell, immobilized pI - gradient (IPG) stripes (IPG BlueStrip 24 cm pH 3-10 L, SERVA) were rehydrated with sample in 500 µl rehydration buffer (DeStreak rehydration solution, GE Healthcare; adding 0.5% [w/v] SERVALYT™ 3-10) over night. The IEF was carried out with the following voltages for the 24 cm stripes: 3 h step 250 V, 3 h step 500 V, 6 h gradient 1000 V, 1 h gradient 10,000 V, step 10,000 V up to 52,000 Vh, maximal 10 h step 1000 V. Following the IEF, stripes were incubated 20 min in equilibration buffer (200 mM dithiothreitol, 2.5 M urea, 2% [w/v] sodium-dodecyl sulfate) and additional 20 min in alkylation buffer (250 mM iodoacetamide, 2.5 M urea, 2% [w/v] sodium-dodecyl sulfate).

For protein separation by mass, a sodium-dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) was performed. The HPE FlatTop Tower system with 2D HPE Large Gel NF 12.5% Kit (SERVA) was used for the 24 cm IPG stripes. Both systems were applied following the manufacturer's guidance.

Western blot

The 2D gels were blotted onto a 0.2 µm PVDF membrane (Immobilon - PSQ Transfer Membrane, Merck Millipore) by dry blotting using capillary force. After the protein transfer to the membrane, the protein was detected with enhanced chemiluminescence (ECL).

The membrane was covered with a blocking buffer for 1 h at room RT, for ECL western blotting. Accordingly, the blocked membrane was incubated with 10 ml of the biotinylated in-house CHO-HCP ELISA antibodies (10 µg/ml diluted in blocking buffer) at RT overnight. The membrane was washed three times for 10 min with tris buffered saline and 0.1% [v/v] polysorbate 20 (TBST), incubated for 1 h with 0.1 U/ml Streptavidin-POD conjugate (Roche Applied Sciences) solution and washed three times with TBST. For ECL protein detection, the membrane was covered with Lumi-Light substrate solution (Roche Applied Sciences) and images were recorded with the Octoplus QPLEX (NH Diagnostics).

The images were evaluated with SpotMap (Version 2.5.0.0, TotalLab Limited). Coverage determination was performed as described in Seisenberger et al. (2021).

3.4 Results and Discussion

The scope of this study was to develop an improved affinity-purification based approach for characterizing the HCP coverage under native conditions, which resolves the problems associated with nonspecific binding of HCPs to the matrix. For this purpose, a diazobenzene cleavable linker was conjugated to the antibodies facilitating the separation of the proteins nonspecifically interacting with the matrix from those specifically bound to the antibody (Figure 3.1).

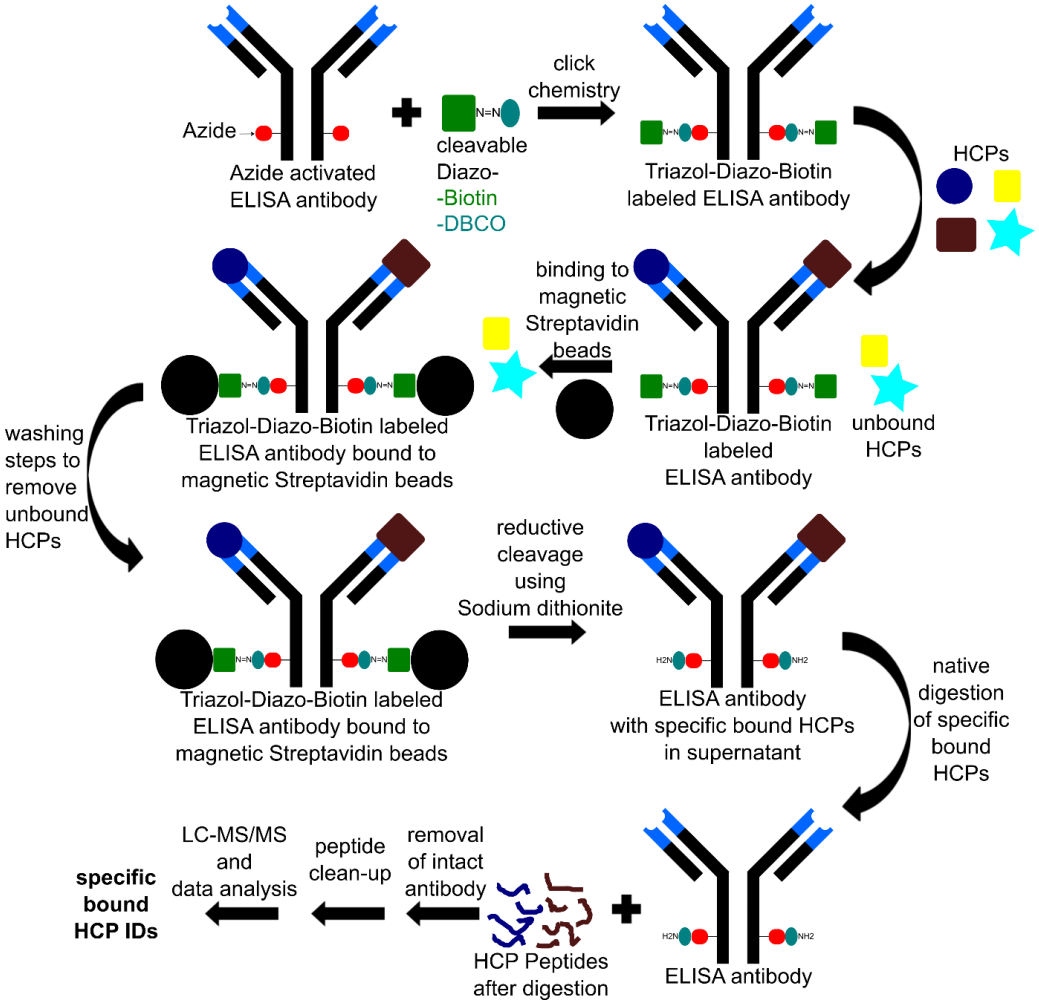

Figure 3.1: Workflow of the optimized AP-MS method.

Polyclonal HCP ELISA antibodies were functionalized site-specifically with an azide-label via the glycan structure in the Fc part, after removal of all N-glycans to the innermost N-acetylglucosamine. A cleavable Diazo-Biotin-DBCO linker was conjugated under physiological conditions to the azide-label using click-chemistry, more precisely by a strain-promoted azide-alkyne cycloaddition (SPAAC). The CHO-HCP ELISA standard (antigen) was bound and the immunocomplexed antibodies were coupled to magnetic streptavidin beads, facilitating the removal of unbound HCPs. After several wash steps using conditions as commonly applied for ELISAs, the diazo-spacer arm was cleaved reductively with sodium dithionite resulting in the release of the immunocomplexes from the matrix. The immunocomplexed antibodies were digested under native conditions, in which bound HCPs are preferably digested while the antibodies remain mostly intact. After digestion, the intact ELISA antibodies were removed by heat denaturation and precipitation. Finally, specifically bound HCPs were purified using a peptide clean-up and identified by LC-MS/MS analysis. AP-MS, affinity purification-based liquid chromatography-tandem mass spectrometry; CHO, Chinese hamster ovary; ELISA, enzyme linked immunosorbent assay; HCPs, host cell proteins.

Here, a diazobenzene linker was used, which can be cleaved chemoselectively under mild reductive conditions using sodium dithionite (Landi et al., 2010; Verhelst et al., 2007; Yang et al., 2010). To prepare the antibodies for conjugation with the cleavable linker, labeling with an azide functionality was required. There exist different ways to attach labels to an antibody. Whereas the conjugation via amino or thiol groups is usually associated with the risk of masking the complementarity-determining region (CDR), site-specific functionalization in the Fc part under physiological conditions can be achieved by conjugating the azide-label via the glycan structure (Kristensen et al., 2019). For this purpose, all conserved N-glycans after the innermost GlcNac were hydrolyzed enzymatically and subsequently UDP-GalNAz (Azide) was attached site-specifically using GalT. The introduced azide functionality was used to conjugate the alkyne (Diazo-Biotin-DBCO comprising a cleavable linker) via a strain-promoted azide-alkyne cycloaddition (SPAAC), which can be performed in the absence of catalysts, such as Cu(I) (Eeftens et al., 2015; Sletten & Bertozzi, 2009). In the next step, the functionalized antibodies were incubated with the CHO-HCP ELISA standard (antigen), since immunocomplexation in solution proved superior compared to their formation after bead conjugation (Henry et al., 2017). After immobilization of the biotin-labeled antibodies onto Streptavidin beads, several washing steps were performed using similar conditions as commonly applied for ELISAs and the Diazo-spacer arm was cleaved reductively facilitating the release of the immunocomplexes from the matrix. All steps described herein were performed under mild conditions to ensure that the antibody remained in its native form. Finally, the specifically bound HCPs were prepared for LC-MS/MS analysis using a native digestion method, in which HCPs are preferably digested while the antibodies remain mostly intact (Huang et al., 2017; Kufer et al., 2019). In the course of the method development, the crucial steps were evaluated as described below.

3.4.1 Method development

To confirm the successful conjugation of the azide and Diazo-Biotin-DBCO onto the antibody, the DOL was evaluated. To determine the DOL of the Diazo-Biotin-DBCO a spectrophotometric measurement at 309 nm was conducted. A DOL of two is expected since the complete deglycosylation of the IgG to the inner most GlcNAc moiety using the Fc specific endoglycosidase generates homogenous starting conditions, with exactly two site-specific incorporation sites for the azide functionality and accordingly two conjugation sites per antibody for the alkyne moieties by click chemistry (Duivelshof et al., 2020). Additionally drug-antibody rates of two were published in several publications (Deslignière et al., 2021; Duivelshof et al., 2020; Kristensen et al., 2019) working with antibody-drug

conjugates (ADCs). Based on this knowledge, the threshold for the DOL to proceed with the conjugated antibodies was set to two for each of the following experiments.

Since bead-based systems have limited antibody-binding capacity, the next step was the evaluation of the ideal antibody amount to achieve reliable results. By using the optimized AP-MS approach, different antibody concentrations were screened. Based on the results, a concentration of 250 µg Diazo-Biotin-DBCO labeled ELISA antibody proved to be an appropriate concentration to achieve reliable coverage with a reasonable amount of employed antibodies (data not shown).

To demonstrate that the immunocomplexed antibodies were quantitatively removed from the streptavidin beads, the efficiency of the reductive cleavage was monitored using Cy5-labeled Streptavidin. Therefore, the ELISA antibodies were labeled with the cleavable Diazo-Biotin linker and Streptavidin-Cy5 (SA-Cy5) was bound. For the removal of non-bound SA-Cy5, the ELISA antibody was bound to magnetic Protein G beads and washed thoroughly. Subsequently, the Biotin-Streptavidin-Cy5 was cleaved three consecutive times using Sodium dithionite and the beads were heated with LDS-sample-buffer after the third cleavage step to release non-cleaved Biotin-Streptavidin-Cy5. All samples were prepared for SDS-PAGE under reducing conditions and analyzed on SDS-PAGE. Figure 3.2 shows the workflow scheme (a) and the experimentally obtained SDS-PAGE of the reduced proteins by Coomassie staining (b) as well as in-gel fluorescence scanning (c).

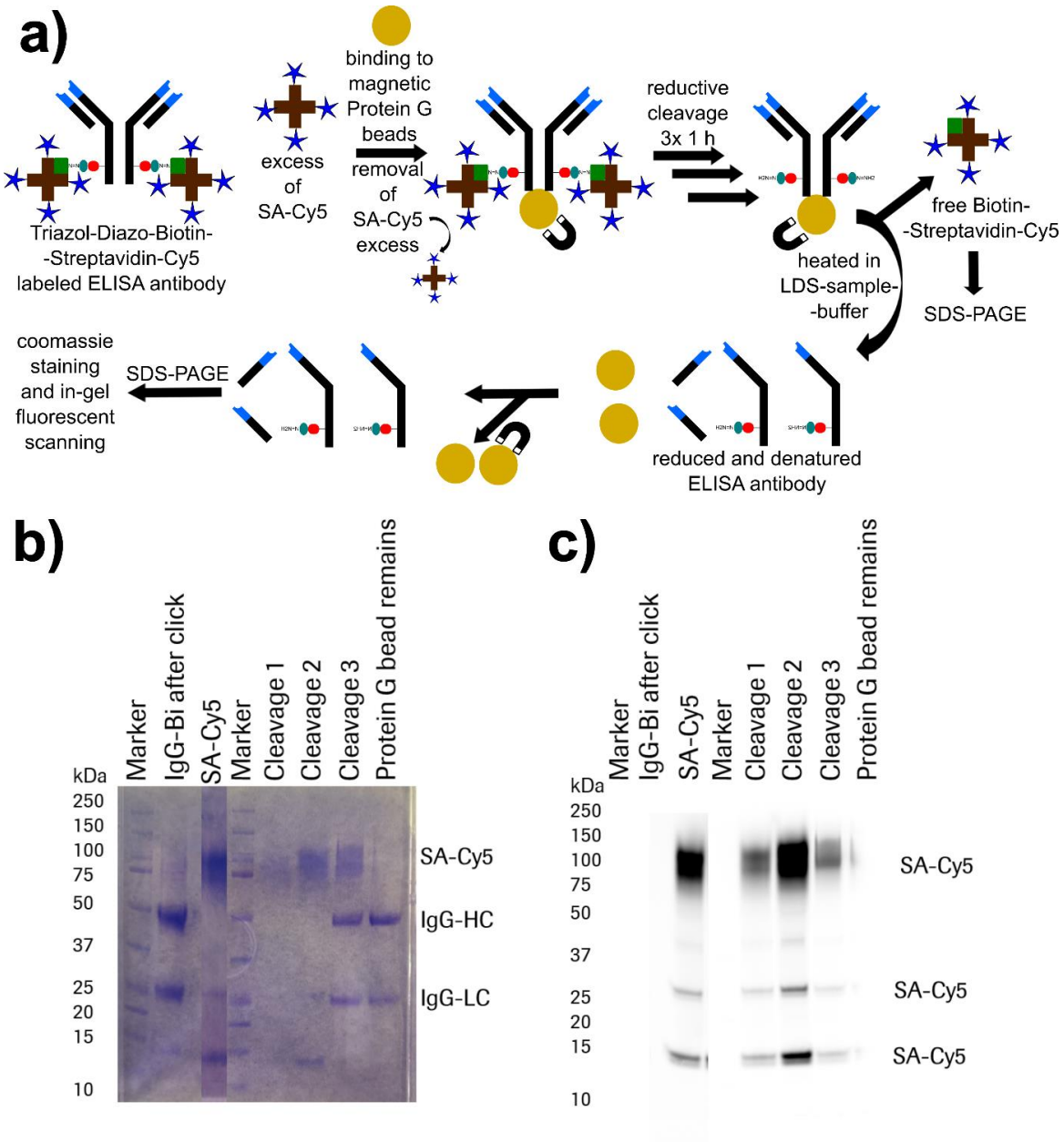

Figure 3.2: Reductive cleavage efficiency of the Diazo-Linker.

(a) *Overview of the workflow,* ***(b)*** *sodium-dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) Coomassie stain, and* ***(c)*** *SDS-PAGE in gel fluorescence scanning. Polyclonal antibodies (IgG) were labeled with a cleavable Diazo-Biotin-DBCO linker using click-chemistry (IgG-Bi after click) and subsequently connected with Streptavidin-Cy5 (SA-Cy5, pure reagent on the gel). This complex was incubated with Protein G magnetic beads and unbound SA-Cy5 was washed away. Reductive cleavage (cleavage 1-3) of the Diazo linker using Sodium dithionite leads to the release of SA-Cy5 into the supernatant. Remaining Protein G beads after the third cleavage were heated in an LDS-Sample buffer (Protein G bead remains). All samples were reduced before loading onto the SDS-PAGE.*

The best cleavage efficiency was obtained for the second cleavage reaction, while faint SA-Cy5 bands were visible for the first and third reduction step. No SA-Cy5 band was visible in the bead fraction, indicating that the third cleavage step led to the release of the remaining SA-Cy5. In contrast, an increased release of heavy and light chains due to the reduction of the Immunoglobulin G (IgG) could be observed in the third cleavage step. As a result, the cleaving steps were performed in all the following experiments twice to keep the amount of reduced IgGs relatively low, which may in turn impair the sensitivity of the LC-MS/MS analysis.

After testing the efficiency of the reductive cleavage, the performance of the native digestion was assessed. For the native digestion of HCPs, trypsin is directly added to the therapeutic antibody solution, whereby the HCPs are digested and the antibody remains mostly intact. The antibody is then precipitated by heat treatment and the supernatant containing the HCP peptides is analyzed by LC-MS/MS, thus reducing the dynamic range between HCPs (sub ng levels) and antibodies (mg) (Huang et al., 2017). Concerns with the use of the native digestion method in this study were a lack in digestion efficiency, as HCPs usually bind specifically and therefore with considerably higher affinity to polyclonal ELISA antibodies than to mAbs, possibly affecting the accessibility of these HCPs for trypsin. Hence, the efficiency of the native digestion method was assessed by digesting the same amount of the HCP-ELISA reference standard under the following conditions: native digestion in the absence (i) and after binding to the ELISA antibodies (ii), denaturing digestion in the absence (iii) and after binding to the ELISA antibodies (iv) (Figure 3.3a i-iv).

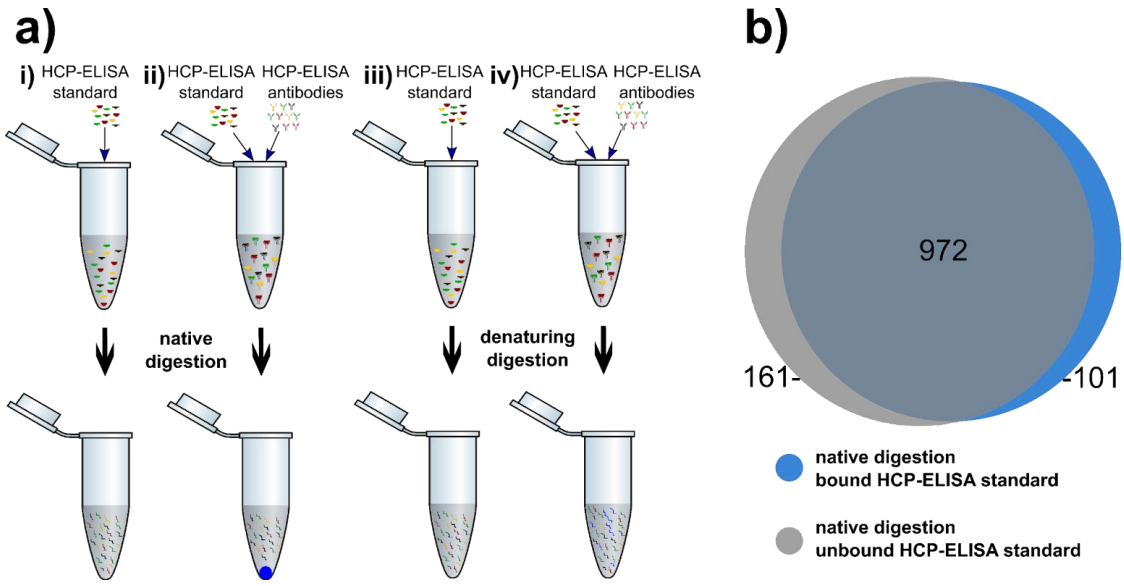

Figure 3.3: Efficiency of the native digestion.

Assessed by digesting the same amount of the HCP-ELISA reference standard (100 µg) under the following conditions: ***(a)*** *native digestion in the absence (i) and after binding to the ELISA antibody (130 µg) (ii), denaturing digestion in the absence iii) and after binding to the ELISA antibody (130 µg) (iv). The blue circle at the bottom of the Eppendorf cup in (ii) represents the denatured and precipitated HCP-ELISA antibody after the native digestion.* ***(b)*** *Venn diagram for comparison of identified HCPs with native digestion of bound (blue) and unbound (gray) HCP-ELISA reference standard. ELISA, enzyme linked immunosorbent assay; HCPs, host cell proteins.*

With the denaturing digestion, 1040 HCP IDs for the bound and 1249 for unbound HCP-ELISA standard were identified (see Venn diagram in Figure S 7-3). 1073 bound HCP IDs as well as 1133 unbound HCPs were identified using the native digestion (Figure 3.3b). The number of identified proteins was comparable within the method variation for the native digestion, indicating that the specific binding of the HCPs to pAbs did not affect the digestion efficiency. In the presence of antibodies, the number of identified HCPs was slightly lower for the denaturing digestion, which can be attributed to the large amounts of antibody peptides in the digested sample reducing the sensitivity of the LC-MS/MS analysis. Together, these results indicate that the native digestion method is suitable for immunocomplexed proteins.

3.4.2 Qualification of the method

In the course of the method qualification, the reproducibility of the optimized AP-MS method (Supporting information, Table S 7-3) and unspecific binding issues were

systematically investigated. First, the variability of the complete method including the cleavage reaction was assessed for two different in-house HCP-ELISA antibody sets (1 and 2). For both antibodies the coverage (number of covered HCP IDs) was determined after performing the optimized AP-MS in three independent experiments. Total number of specifically bound HCP IDs were compared for each replicate and the resulting CV (coefficient of variation) varied between 11% (HCP ELISA antibody 1) and 7% (HCP ELISA antibody 2).

Afterwards, the influence of the digestion procedure in combination with the instrumental variability on the method reproducibility was evaluated. A CV of 3% was observed for a sample (in-house HCP-ELISA reference standard) from one stock-solution, digested seven times independently and analyzed by LC-MS/MS on different days. The instrumental variability analyzing five aliquots of one sample on the same day was determined previously and resulted also in a CV of 3%. These results indicate that the influence of the digestion is very low, since the observed CV is similar to that of the instrument variability.

Overall, the optimized method proved to be very reproducible for all evaluated steps. Compared to the results reported by Piehowski et al. (2013), the CV values for the instrumental variance in this study are slightly lower, which can be attributed to the different evaluation strategy based on the analysis on protein level in this study instead of peptide level in the other. As mentioned in the introduction, the 2D-WB has some inherent technical drawbacks, some of which may also have an impact on reproducibility, like subjective counting of spots or variations in protein transfer to the WB membrane. To the best of our knowledge, no studies investigating the reproducibility of qualitative 2D-WB are published but 2D gel studies show that only 6.8% of the spots were detected and matched on 12 gel replicates (Corzett et al., 2006). Based on these assumptions we expect a better or at least comparable reproducibility for the optimized AP-MS.

The main purpose of this work was the reduction of non-specific binding, which can either occur to the antibodies or the matrix. To illustrate the benefits of our new approach in this regard, the optimized AP-MS and the previously described method (Seisenberger et al., 2021) were compared. Unspecific binding to the antibodies was assessed by using normal sheep IgGs (total IgGs of non-immunized sheep) instead of HCP-ELISA antibodies for both methods. By applying our previously described method, 23% of the bound HCPs were identified as non-specifically bound to normal sheep IgG primed beads, which corresponds to 17% of the total HCP-ELISA standard population. These results are in accordance with recently published data (Table 3.1) reporting 21% (Henry et al., 2017) and 28% (normal

goat IgG) (Pilely et al., 2020) unspecific binding of the immunocaptured HCPs. For the optimized AP-MS method, the non-specific binding to normal IgGs amounted to 8% (5% of the total HCP ELISA standard) using the herein described ELISA like washing conditions. Although non-specific binding to normal IgGs could not be completely prevented, it was considerably reduced applying the optimized method.

Assessment of our previously described AP-MS method resulted in 89% unspecific binding to the matrix, as 914 out of 1029 HCP-ELISA antibody bound HCPs were also attached to the uncoated Protein G magnetic beads (Table 3.1).

Table 3.1: Unspecific binding to IgGs and the matrix in percentage, calculated by using the number of ELISA-antibody bound HCPs as 100% value.

unspecific binding	our established method	our optimized method	Henry et al. (2017)	Pilely et al. (2020)	Waldera-Lupa et al. (2021)
to the IgG	23%	8%	21%	28%	not tested
to the matrix	89%	0%	51%	16.2 - 21.4%	N/A

Abbreviations: ELISA, enzyme linked immunosorbent assay; HCPs, host cell proteins; N/A, not applicable.

Using the same calculation, Henry et al. (2017) identified 51% of the immunocaptured HCPs non-specifically bound to unprimed Streptavidin magnetic beads and Pilely et al. (2020) found 16.2-21.4% interacting non-specific with uncoated ELISA plates.

For the optimized method, the unspecific binding to the matrix is irrelevant, because the matrix is separated from the immunocomplexed ELISA antibodies before the specific bound HCPs are digested. Nevertheless, further analysis was carried out to assess the benefits of this method. The beads were digested after removing the cleaved immunocomplexed antibodies, resulting in the identification of 359 HCPs in the subsequent LC-MS/MS analysis. 315 of those HCPs were also identified in the ELISA antibody bound fraction (825 HCPs in total) and accordingly 44 HCPs solely in the bead fraction. Previous methods need workarounds e.g. an enrichment threshold to decide whether these HCPs belong to the covered fraction potentially leading to false coverage results. In the present case 315 HCPs could be incorrectly assigned as non-covered (false negative) and 44 HCPs as covered (false positive), while all these HCPs are assigned correctly with the optimized AP-MS approach.

To substantiate the limitation of false coverage results the commonly used threshold of more than twofold enrichment (SumAll peptide area) by HCP-ELISA antibodies was applied as evaluation method for our previously described method. Several proteins (e.g. Proteasome-associated protein ECM29-like, Ras-related protein Rab-14, 26S proteasome non-ATPase regulatory subunit) were found to be falsely identified as covered using this threshold, while they were actually only bound to the beads. A fundamental problem, which makes threshold determination difficult, is the reproducibility of the protein quantity between sample replicates. Five replicates measured in one sequence were compared regarding the reproducibility of the SumAll peptide area of 100 HCPs with peptide numbers between 2 and 43. Fold-change was calculated with maximum SumAll peptide area divided by minimum SumAll peptide area and resulted in fold-changes ranging from 6 to 46 (Figure 3.4).

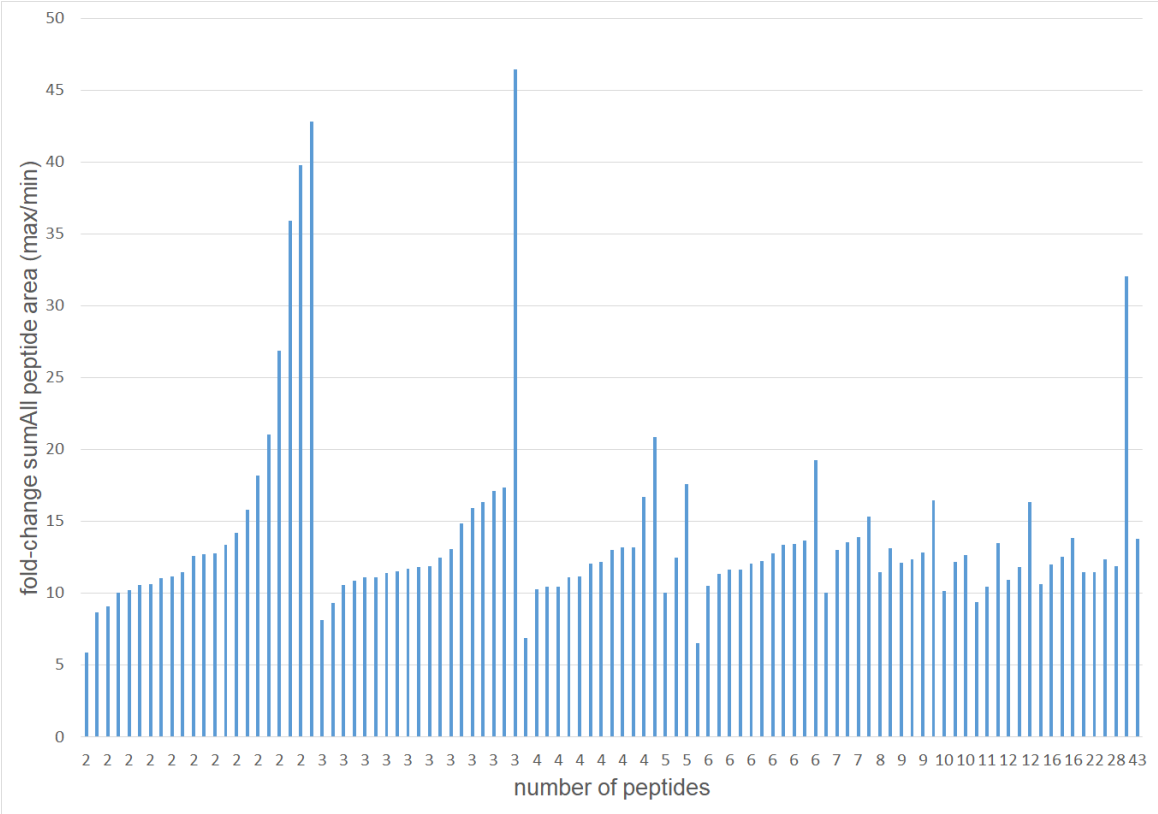

Figure 3.4: SumAll peptide area replicate comparison.

One hundred host cell proteins of five replicates measured in one sequence were compared regarding their relative protein quantity. Therefore, the sum of all peptide areas (areas under the peak) assigned to each protein was calculated. Maximum and minimum SumAll peptide areas were divided to calculate fold-change in protein abundance of the five replicates. Each bar is related to a protein and the number of peptides sorted in ascending order is plotted against the fold change.

In this regard, the method revealed high variability in relative SumAll peptide abundance despite very good method reproducibility on protein level, hampering the definition of an appropriate threshold. In accordance, Waldera-Lupa et al. (2021) commonly observed different coverage values when varying enrichment thresholds. By using the optimized AP-MS application of a threshold value for matrix associated HCPs is not required, making it superior in terms of detection gap identification. This avoids the risk of falsely declaring HCPs as covered and consequently overlooking them in the process monitoring.

3.4.3 Coverage analysis of two different CHO-HCP ELISA antibodies

As a proof of concept, the coverage of two different in-house CHO-HCP ELISAs (ELISA 1 and 2) was determined using the optimized AP-MS method. Coverage in percentage refers in this context to the number of HCPs recognized by the respective HCP-ELISA antibody (covered) in relation to all identified HCPs in the CHO-HCP ELISA reference standard (ELISA standard). The HCPs in the respective ELISA standard were identified by LC-MS/MS analysis using a digestion under native conditions, resulting in an average of 1102 HCPs (n = 9) for ELISA standard 1 and 1087 HCPs (n = 3) for ELISA standard 2. The HCPs were in both cases distributed over the entire MW and pI range (Figure 3.5). The AP-MS analysis in triplicates resulted in a mean coverage of 68% (752 HCP IDs) for ELISA antibody 1 and 76% (823 HCP IDs) for ELISA antibody 2.

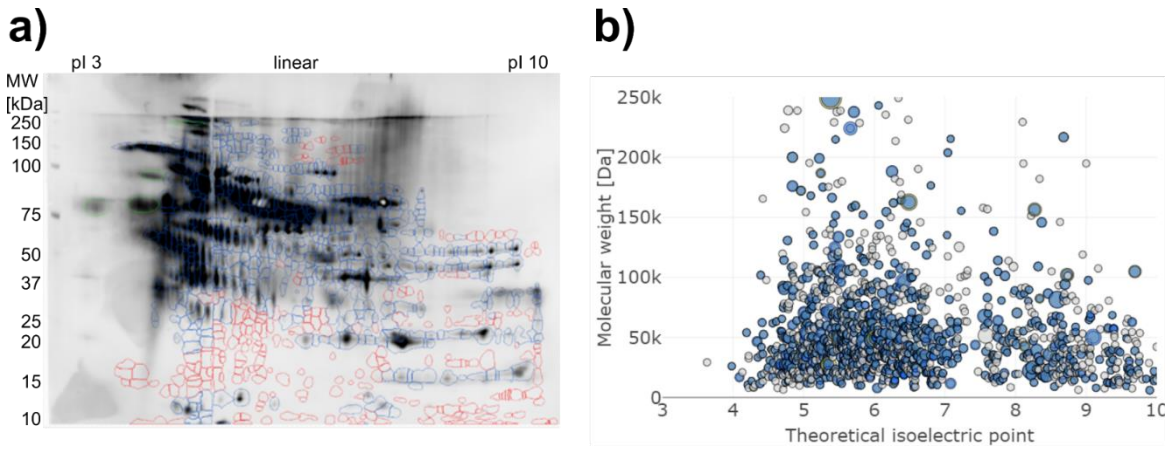

Figure 3.5: 2D-WB coverage analysis.

2D-WB of HCP ELISA antibody 1 ***(a)*** *and virtual 2D-WB of the same antibody generated after analysis with the optimized AP-MS method* ***(b)****. In the 2D-WB (a) spots present in both the protein gel and western blot (i.e., covered) are marked with blue frames, while uniquely detected spots are marked with red frames (protein gel) or green frames (western blot), respectively. As a consequence, blue and red frames represent all protein spots detected in the 2D protein gel. For the virtual 2D-WB the theoretical calculated pI was plotted against the calculated MW and spot size is scaled relative to protein abundance. Blue spots symbolize covered HCPs, while gray spots are non-covered HCPs. 2D-WB, two dimensional-western blots; ELISA, enzyme linked immunosorbent assay; HCPs, host cell proteins.*

Figure 3.5 shows the comparison of the previous standard method, 2D-WB (Figure 3.5a), and the virtual 2D-WB (Figure 3.5b) using the optimized AP-MS data of ELISA antibody 1. The 2D-WB revealed a coverage of 71% (total of 779 protein spots and 551 covered), which is comparable to the AP-MS results. In contrast to several detection gaps observed in the LMW region of the 2D-WB, the covered HCPs are more evenly distributed over the entire molecular weight range using the AP-MS method. Inferior detection of LMW proteins is mainly a consequence of impaired antibody binding through denaturing 2D-WB conditions (Seisenberger et al., 2021). On the contrary, overexposure of certain parts in the HMW acidic regions of the 2D-WB stems from the signals obtained by highly immunoreactive HCPs potentially superimposing other protein spots. The main focus of the present work was to develop an AP-MS method with improved performance and reliability. However, traditional 2D-WB and the method described herein are comparable regarding the expenditure of time, whereas antibody consumption is lower with the AP-MS method.

3.4.4 Binding of HCPs to in-house mAb product

HCPs non-specifically bound to mAb products, known as hitchhikers, are often difficult to remove during downstream purification (Aboulaich et al., 2014; Doneanu et al., 2012; Levy et al., 2014; Liu et al., 2019; Zhang et al., 2016). It was assumed that the optimized AP-MS method is particularly suitable for hitchhiker analysis, since it allows the removal of proteins interacting with the matrix facilitating the unambiguous identification of antibody-associated HCPs. An in-house human mAb product, with HCP levels below the detection limit, was used for hitchhiker analysis applying the optimized AP-MS method. After conjugation of the cleavable Diazo-Biotin-DBCO linker as described above, the functionalized antibody was incubated with the CHO-HCP standard. The HCP-mAb complex was cleaved, separated from the matrix, digested under native conditions and analyzed using LC-MS/MS. 14% (176 HCPs) out of 1287 HCP found in the respective HCP standard (n = 3) were identified as non-specifically bound to the mAb. Furthermore, the identified HCPs were compared with a list of 82 currently published potential hitchhiker HCPs from several publications (Table S 7-4). The relevance of the investigated HCPs can be substantiated when compared with a recently published list of high risk HCPs from Jones et al. (2021), sharing 16 out of 21 HCPs. Some HCPs identified in the present work, such as 78 kDa glucose-regulated protein, Actin cytoplasmic 1, Alpha-enolase, Clusterin, Glyceraldehyde-3-phosphate dehydrogenase have already been described as mAb associated in the literature. Other well-known HCPs like Putative phospholipase B-like 2, suspected of being hitchhikers, have not been identified here. However, non-specific binding to the antibody is also dependent on the type and structure of the mAb. In contrast to previously published hitchhiker HCPs (Levy et al., 2014; Liu et al., 2019), the identified HCP in the optimized AP-MS method are unambiguously assigned to the mAb and not to the matrix.

3.5 Conclusions

In summary, this work describes an improved method for coverage determination of ELISA antibodies and product mAb hitchhiker analysis under native conditions, which overcomes unspecific matrix binding issues by facilitating the separation of the matrix from the immunocomplexed HCP-ELISA antibodies prior to LC-MS/MS analysis. Furthermore, error-prone elution steps are not required, as the separation from the matrix is achieved via a cleavable linker. A stable DOL of two Diazo-Biotin-DBCO molecules per antibody, efficient reductive cleavage and native digestion could be verified during method development. The qualification of the method demonstrated high reproducibility, especially concerning the digestion and instrument variability. Considerable improvements have

been achieved with regards to unspecific binding issues, reducing the number of incorrectly assigned proteins of the bound HCP standard to 8%, while previously described methods revealed unspecific binding up to 28% for antibody associated proteins and 89% for matrix bound HCPs. This is due to the isolation of specifically bound HCPs prior to the analysis instead of applying arbitrarily defined threshold values. As demonstrated in this work, proper threshold settings are almost impossible because of low replicate reproducibility in relative protein quantification.

In general, the optimized AP-MS is a powerful method to complement or even replace at some point the 2D-WB, since in contrast to the 2D-WB, the HCPs were bound under native conditions similar to the ELISA and unambiguous protein identification improves the understanding of antibody binding behavior of single HCPs. In addition, the method can also be applied to contribute substantially to the understanding of HCP coverage, unspecific binding to product antibodies and HCPs, which are difficult to remove in the downstream purification process. Specifically, improved knowledge with regards to the coverage of “high-risk” (Jones et al., 2021) or known problematic HCPs by comparing different ELISA antibody reagents and selecting the most suitable antibodies could clearly contribute to optimal clearance of these HCPs.

4 The agony of choice: Impact of the host animal species on the ELISA performance for HCP quantification

4.1 Abstract

Host cell proteins (HCPs) are inevitable process related impurities in biotherapeutics commonly monitored by Enzyme linked immunosorbent assays (ELISAs). Of particular importance for their reliable detection are the anti-HCP polyclonal antibodies (pAbs), supposed to detect a broad range of HCPs. The present study focuses on the identification of suitable host animal species for the development of high performance CHO-HCP ELISAs, assuming the generation of pAbs with adequate coverage and specificity. Hence, antibodies derived from immunization of sheep, goats, donkeys, rabbits and chickens were compared concerning their amount of HCP specific antibodies, coverage and performance in a sandwich ELISA. Immunization of sheep, goats, donkeys and rabbits met all test criteria, whereas the antibodies from chickens cannot be recommended based on the results of this study. Additionally, a mixture of antibodies from the five host species was prepared to assess if coverage and ELISA performance can be improved by a multi-species approach. Comparable results were obtained for the single- and multi-species ELISAs in different in-process samples, indicating no substantial improvement for the latter in ELISA performance while raising ethical and financial concerns.

4.2 Introduction

Biotherapeutics are produced in living cells, such as Chinese hamster ovary (CHO) cells. Despite elaborate purification processes, it is inevitable that trace levels of host cell proteins (HCPs) remain in the final drug product (Krawitz et al., 2017). HCPs are generally considered a critical quality attribute (CQA) due to their potential immunogenicity and impact on product stability (Bracewell et al., 2015; ICH, 2009).

HCP depletion during downstream purification and release of the final product is routinely monitored by enzyme linked immunosorbent assays (ELISAs), which are particularly suitable for this purpose due to their sensitivity, potential scalability to high throughput analysis, and compliance to good manufacturing practice (GMP) principles. The performance of an ELISA relies primarily on the quality of two critical reagents, namely the ELISA standard and the polyclonal antibodies (pAbs) used for HCP detection. In this context, HCPs occurring in the respective manufacturing process should be represented by the ELISA standard and the corresponding antibodies have to cover the majority of the

complex HCP mixture over a broad range of molecular weights and pIs. Those pAbs are produced by immunizing an appropriate host animal species with the HCP-ELISA standard (Ph. Eur. 2.6.34, 2017; USP <1132>, 2016; Wohlrab et al., 2018; Zhu-Shimoni et al., 2014).

Hence, choosing the optimal adjuvant, host animal species, immunization site and quality of HCP mixture for immunization plays an important role in generating suitable pAbs for HCP ELISA development (Delahaut, 2017; Leenaars & Hendriksen, 2005; Shahrokh et al., 2016). In general, the decision for the most appropriate host animal species often depends on the amount of antiserum needed, the phylogenetic distance to the source of the antigen (here: Chinese hamster), and the ease of blood sampling (Delahaut, 2017; Leenaars & Hendriksen, 2005). To the best of our knowledge, different host animal species were so far only compared in one study using the same HCPs as immunization standard. In this work, Haemmig et al. (2017) immunized rabbits and goats for a newly developed CHO-HCP platform ELISA. Antibodies from goats were characterized by lower titers, greater animal-to-animal variability and considerable discrepancies in coverage and intensity of the HCP immunostaining. Despite differences in individual titer and coverage, the purified goat and rabbit anti-HCP antibodies exhibited similar ELISA performance. Another study (Pilely et al., 2020) compared commercial goat and rabbit anti-*Escherichia coli* HCP antibodies from different vendors regarding their coverage. However, a head-to-head comparison by using an affinity-purification based mass spectrometry (AP-MS) method remained inconclusive because different antigens were used for immunization. Other publications focused on anti-HCP antibodies from a single animal species to address HCP related issues, whereby antibodies from goats, rabbits, or sheep were predominantly used (Baldus et al., 2017; Gunawan et al., 2018; Henry et al., 2017; Lundström et al., 2014; Rey & Wendeler, 2012; Seisenberger et al., 2021, 2022; Shukla et al., 2008; Thomson et al., 2017). Besides identifying the optimal host species, the coverage may be further enhanced by a mixture of the antibodies derived from different hosts, as certain HCPs not covered by one species could be covered by another (e.g. due to their phylogenetic distance to the origin of the HCP used). However, it is still unclear whether the generation of a mixture of anti-HCP antibodies from more than two different species is generally feasible and whether this can lead to an increased HCP-coverage and ELISA performance in practice.

Rabbits, goats, sheep, chickens and donkeys were immunized by using the same CHO-HCP antigen. The resulting pAbs were characterized individually and as a multi-species mixture for their coverage, suitability in ELISAs and performance for HCP quantification using different in-process control samples from several drug products.

4.3 Materials and Methods

Buffers, solvents and media were prepared with filtered water from a water purification system (Milli-Q® Reference, Millipore). Chemicals for ELISA, gel electrophoresis and western blotting, were derived from SERVA or Invitrogen / Thermo Fisher Scientific if not stated otherwise and had molecular biological grade.

4.3.1 CHO-HCP ELISA standard and antibody preparation

The CHO-HCP standard for immunization was prepared as described elsewhere (Seisenberger et al., 2021). Briefly, an ammonium sulfate precipitation, diafiltration (5 kDa cutoff filter) and sterile filtration (0.2 µm) were conducted to purify the HCPs of a mock fermentation run using a CHO K1 DXB11 cell line (Urlaub & Chasin, 1980).

For generation of anti-CHO-HCP antibodies sheep, goats, donkeys, rabbits and chickens were immunized using Freund's adjuvant for the initial and monthly booster injections. For the monthly sera pools blood was collected weekly from sheep, goats and donkeys and monthly from rabbits, for 1 year. Eggs from the chickens were collected over 1 year starting from Day 31 with crude purification of total IgY in 5 or 24 days (from Month 5 on) batches at Lampire Biological Laboratories. The purified IgYs were pooled monthly, after determination of the ideal egg collection period.

The protocol and procedures of the animal experiments with sheep were ethically reviewed and approved by the District Government of Upper Bavaria (reference number Az 211-2532-43/04). The experiments were performed in accordance with relevant institutional and national guidelines for the care and use of experimental animals (German Animal Welfare act and related regulations).

The protocol and procedures of the animal experiments with goats, donkeys and chickens at Lampire Biological Laboratories were ethically reviewed and approved by the Institutional Animal Care and Use Committee under the USDA registration number 23-R-0122 and 23-B-0020 as mandated by the Animal Welfare Act of the United States Government. The experiments were performed in accordance with the institutional procedures utilizing the NIH Guide for the Care and Use of Laboratory Animals.

The protocol and procedures of the animal experiments with rabbits at Charles River France were ethically reviewed and approved by the French Ministry of Research (APAFIS#5626-2016061011167092 v4). The experiments were performed in accordance

with relevant institutional and national guidelines for the care and use of experimental animals (French Animal Welfare for Scientific Use of Animals act and related regulations).

Sera and total chicken IgY batches were analyzed by western blot (WB) and ELISA titer determination.

Serum and total chicken IgY batches collected over 1 year were pooled per species (total of five pools) and the pools were pre-purified with an aerosil delipidation step. CHO-HCP specific antibodies were purified directly from pre-purified serum-pools and the total chicken IgY pool, using affinity purification with the CHO-HCP standard coupled to NHS-Sepharose (NHS Act Sepharose® 4 Fast Flow, GE Healthcare). Aggregates were removed using a preparative SEC (HiLoad 26/600 Superdex 200 pg, GE Healthcare). Purified CHO-HCP specific antibodies were stored at –80°C.

4.3.2 Titer determination

Serum titers were determined by indirect ELISA. Each well of a 96-well Microtiter plate (nunc Maxi Sorp, Thermo Scientific, Germany) was coated with 1 µg HCP-ELISA standard diluted in coating buffer (50 mM sodium carbonate pH 9.6) and incubated under shaking (350 rpm) overnight at 4°C. After several wash steps with 1x PBST (0.05% polysorbate 20) buffer, the wells were filled with Blocking buffer (50 mM potassium phosphate, 0.1% gelatin, 0.05% polysorbate 20) and agitated (350 rpm) for 2 h at room temperature (RT). Following washing, serial five-fold dilutions of the sera or total chicken IgY samples were added, starting at a dilution of 1:2 in Blocking buffer and incubated overnight at RT under agitation (350 rpm). The secondary antibody was added and the mixture shaken (350 rpm) for 2 h at RT, after thorough washing. Finally, ABTS substrate was added and the absorbance was read at 405 nm with a microplate reader (VersaMax, Molecular Devices). Samples were measured in triplicates and data evaluation was performed with an internal software using a four-parameter fit model.

4.3.3 Western blot

For sodium-dodecylsulfate poly-acrylamide gel electrophoresis (SDS-PAGE), 10 µg reduced (NuPAGE™ Reducing Agent) and denatured (10 min, 70°C) CHO-HCP ELISA reference standard in LDS Sample Buffer (NuPAGE™) was loaded onto each lane of the gel (NuPAGE™ Bis-Tris Mini Gel). Protein separation by mass was conducted with Running Buffer (NuPAGE™ MES SDS Running Buffer) according to the manufacturer's instructions. Protein spots were subsequently blotted onto a PVDF membrane (Immobilion - P^{SQ} Transfer Membrane, Merck Millipore) in accordance to the manufacturer's

specifications by using a wet tank transfer system (XCell SureLock™ Mini-Cell). The membrane was covered for 1 h with blocking buffer (2 g ECL Prime™ blocking agent in 100 ml tris buffered saline and 0.1% [v/v] polysorbate 20 [TBST]) at RT. Followed by incubation with diluted sera (in blocking buffer) or total chicken IgY (with blocking agent) overnight at RT. The membranes were washed three times for 10 min with TBST (1x) and covered with 10 ml anti-host-species (H + L) HRP-conjugated antibodies (diluted 1:1000 in blocking buffer) for 2 h at RT. After final wash steps, Lumi-Light substrate solution (Roche Applied Sciences) was used for enhanced chemiluminescence (ECL) protein detection using the Octoplus QPLEX (NH Diagnostics) imager.

4.3.4 Two-dimensional-western blot (2D-WB)

Sample preparation, protein separation by 2D-PAGE, WB and data evaluation was conducted as described previously (Seisenberger et al., 2021, 2022). Briefly, 250 µg of the Cyanine-dye (G-Dye 300, NH Diagnostics) labeled CHO-HCP standard was separated on immobilized pI-gradient (IPG) stripes (IPG BlueStrip 24 cm pH 3–10 L, SERVA) and resolved by mass using the HPE FlatTop Tower system with 2D HPE Large Gel NF 12.5% Kit (SERVA). The proteins were blotted onto a membrane and the blot was incubated either with diluted sera, purified IgY or affinity purified anti-HCP antibodies. Followed by incubation with the respective anti-species (H + L) horseradish peroxidase (HRP)-conjugated antibodies (diluted 1:1000 in blocking buffer) and 0.1 U/ml Streptavidin-POD conjugate (Roche Applied Sciences) solution. For ECL protein detection the membrane was covered with Lumi-Light substrate solution (Roche Applied Sciences).

4.3.5 CHO-HCP ELISA

A sandwich ELISA format with a biotin-streptavidin system and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Roche Applied Sciences) as substrate was used for the CHO-HCP ELISA. Briefly, the CHO-HCP specific capture antibodies were coated onto 96-well plates (Nunc Maxisorp Microtiter plates, Thermo Fisher Scientific) overnight. All incubation steps were conducted at RT, under agitation (350 rpm) and afterwards, the plate was washed using 1x PBST (0.1% polysorbate 20) buffer in a Skan Washer 400 device. The plates were blocked for 1 h with blocking buffer (1x PBS, 0.1% polysorbate 20, 1% [w/v] bovine plasma albumin-1 [BPLA1, Roche Applied Sciences]) and afterwards incubated with samples diluted in ELISA buffer (1x PBS, 0.1% polysorbate 20, 1% w/v BPLA1) overnight. Followed by incubation with the biotinylated detection antibody for 3 h and addition of Streptavidin-POD conjugate (Roche Diagnostics GmbH) for 1 h. Finally, ABTS (Roche Diagnostics GmbH) was added. The colorimetric readout was performed at 405 nm using a microplate reader (VersaMax, Molecular Devices). Each run

includes a control measured in duplicates; standards were measured in triplicates and test samples at least in two dilutions. The HCP concentration of the samples was calculated with the help of the CHO-HCP standard curve ranging from 2 to 100 ng/ml by applying a four-parameter curve-fitting function in the software SoftMaxPro (Version 5.4.1).

4.3.6 Optimized AP-MS

The affinity based mass spectrometry method was carried out as described elsewhere (Seisenberger et al., 2022). Briefly, the Fc-part of the HCP-ELISA antibodies was labeled with a cleavable Biotin linker and immobilized to Streptavidin magnetic beads. After binding of the HCP-ELISA standard, the immunocomplexed antibodies were reductively cleaved from the beads and tryptically digested under native conditions. The bound (covered) HCPs were identified by LC-MS/MS analysis. Additionally the proteins of the total HCP-ELISA standard were identified after digestion under native conditions.

4.3.7 Native digestion

The protocol for the digestion under native conditions was performed as described in Kufer et al. (2019) and Seisenberger et al. (2022).

4.4 Results and Discussion

To identify appropriate single or a combination of multiple species for the development of a high coverage and well performing CHO-HCP-ELISA, rabbits, goats, sheep, donkeys and chickens served as hosts for pAb production based on the following justification:

Rabbits are commonly used due to their production of high-affinity antibodies at high titers, relatively long lifespan (5-8 years), and ease of housing (even under specific pathogen-free conditions) as well as manipulation for blood sampling or booster injections (Delahaut, 2017). Whereas the use of rabbits is restricted by their low antisera volume, farm animal species like goats, sheep or donkeys deliver larger blood volumes. Another advantage of farm animals is their even longer lifespan increasing the blood collection time and volume (Ascoli & Aggeler, 2018). Finally, chickens have the largest phylogenetic distance to (Chinese) hamsters and produce large amounts of IgY in the egg yolk, which makes blood collections unnecessary and thereby reduces the stress on the animal (Delahaut, 2017; Schade et al., 2005).

4.4.1 Polyclonal antibody preparation and characterization

Blood samples of the animals were drawn before the immunization and screened by WB analysis for pre-existing antibodies against the CHO-HCP ELISA standard (Supporting Information: Figure S 7-4, pre-bleed). All animals were tested negative by WB, as no

distinct bands were visible, indicating their suitability for immunization. WBs of the monthly test bleeds or total chicken IgY samples of each single animal allowed to monitor the immune response throughout the entire process. The resulting WBs of the first and the fourth test bleed of each individual animal are depicted in Supporting Information: Figure S 7-4 (bleed 1 and bleed 4). Several bands occurred predominantly in the high molecular weight (HMW) region after immunization, demonstrating an immune reaction of each single animal to the immunogen. The fourth bleed WBs showed a clear increase in protein bands, also for low molecular weight (LMW) HCPs, indicating an increase in coverage or titer. All single bleeds and total chicken IgY samples belonging to the same host species were pooled for subsequent purification of the HCP-specific pAbs by antigen-affinity-chromatography. Their detailed characterization described below enabled monitoring of the purification process for possible losses of certain antibodies.

The titer levels (amount of HCP-specific antibodies) for each host species were determined in an indirect ELISA by using serial dilutions (X) of the sera or total chicken IgY. Titer values correspond to the dilution (1:X) where the inflection point in a sigmoidal curve fit was reached, whereby a higher dilution translates into a larger amount of HCP-specific antibodies in the respective sample. Chicken antibodies resulted in the lowest titer of 1:4521, followed by antibodies from rabbits (1:15,787), goats (1:38,577), donkeys (1:50,950) and sheep with the highest antisera titer (1:120,159) (Table 4.1).

Table 4.1: Resulting serum titers and antibody coverage determined by 2D-WB using blood sera or IgY pools of the five host animal species as well as yield and purity levels of the HCP-specific antibodies after affinity-purification from blood sera or IgY pools.

	Chicken	**Rabbit**	**Goat**	**Donkey**	**Sheep**
Titer blood sera / IgY pool	1:4521	1:15,787	1:38,577	1:50,950	1:120,159
2D-WB coverage [%] blood sera / IgY pool	40	54	58	67	69
LMW (<40 kDa) 2D-WB coverage [%] blood sera / IgY pool	22	30	32	45	46
Yield HCP-specific pAbs [g/L]	0.96	1.80	1.26	2.69	2.89
Purity [%] of HCP-specific pAbs	99.61	99.83	99.59	99.87	98.65

Abbreviations: 2D-WB, two-dimensional western blot; HCP, host cell protein; LMW, low molecular weight; pAbs, polyclonal antibodies.

Although total chicken IgY was purified from egg yolk and thus should theoretically occur with higher purity and concentration than the IgG in mammal blood sera, it showed the lowest titer values. The titers obtained for immunized goats are in accordance with the results published by Haemmig et al. (2017), while for rabbits the titers are at least by a factor of six lower in the present study. Reasons for titer differences could be the antigen amount, immunization frequency, adjuvant, or other immunization-related issues. Additionally, different methods used for titer determination could have an influence on the results. ELISA data were analyzed herein by a four-parameter sigmoidal curve fit, while the other study (Haemmig et al., 2017) applied the endpoint titer method, for which the highest dilution giving a signal above a defined cutoff level is set as titer (Frey et al., 1998; Hartman et al., 2018; Karpinski et al., 1987; Rodbard & Frazier, 1975). We hypothesized that larger amounts of HCP-specific antibodies in a sample are associated with higher functional activity, a better recognition of different epitopes, and of course lower material requirements. However, it is questionable if higher titers are tantamount to a broader antibody coverage, as it could originate from one particularly immunogenic HCP leading to large amounts of antibodies against this protein. As high titers are only one aspect that should be considered when selecting appropriate immunization hosts, antibodies of all species were used for the subsequent antibody coverage studies.

Nonspecific pAbs and other proteins, present in sera and total chicken IgY pools may affect 2D-WB coverage and ELISA performance. Hence, the HCP specific antibodies were purified using the immobilized HCP standard for antigen affinity chromatography. As listed in Table 4.1, all purified antibodies exhibited a purity higher than 99.5% by SEC-HPLC after removal of aggregates. While the sera from sheep yielded the highest amount of HCP specific pAbs (2.89 g/L serum), chicken resulted in only 0.86 g/L total chicken IgY, which is in good agreement with the findings of the titer test.

To monitor possible losses of HCP-specific pAbs, the coverage was assessed before and after the purification process. First, coverage of the HCP immunogen was tested in dependence on their MW and pI, by preparing 2D-WBs for each of the crude antisera pools (Supporting Information: Figure S 7-5). Broad coverage with values up to 69% was achieved for all species except chicken (40%) (Table 4.1). For the LMW region of the 2D-WBs, values of 22%, 30%, 32%, 45%, and 46% were obtained for pAbs from chicken, rabbits, goats, donkeys, and sheep, respectively (Table 4.1). It has to be mentioned that 2D-WB preparation with total chicken IgY was optimized as described below, due to low signal to background noise when using our standard 2D-WB protocol. Several confounding factors that can influence 2D-WB quality were tested, including weak antigen- antibody interactions at low salt concentrations (Carlander et al., 1999; Hoffmeister & Voss, 1974);

inhibition of the ECL signal through sodium azide (added to the total chicken IgY as preservative); overall too low concentrations of specific IgY; impact of the blocking agent or too fast reaction of the immunostaining during the readout. In this regard, high salt conditions (1.5 M NaCl) and switching to a fluorescence detection system, as well as omission of sodium azide did not improve the 2D-WB results. Eventually, total chicken IgY was used undiluted with blocking agent added directly to maximize the HCP-specific IgY concentration and the readout was done immediately after addition of the substrate, to achieve satisfying quality of the chicken 2D-WBs. No improvement of the coverage in the LMW region was observed (Table 4.1), which is contrary to our expectation that chicken should exhibit a stronger immunologic reaction to small, thus less immunogenic, HCPs due to the larger phylogenetic distance to mammals. Other possible reasons for inferior coverage of the chicken derived pAbs could be related to the immunization strategy (e.g., type of adjuvant, boost frequency, housing condition, antigen amount, application method, and preparation) or the purification of the IgY from egg yolk.

Furthermore, the coverage of the HCP population after purification was assessed by both 2D-WB and AP-MS. In four out of five cases, the 2D-WB coverage was slightly improved (Table 4.2) and no gaps in the respective regions of the blots were detected.

Table 4.2: Obtained coverage values by AP-MS and 2D-WB for the HCP-specific antibodies derived from each of the five host animal species and the multi-species mixture.

Host animal species	HCP IDs covered MS (duplicates, total 1311 HCPs)	Coverage [%] AP-MS	Spots covered 2D-WB	Coverage [%] 2D-WB	Coverage [%] LMW region (<40 kDa)
Chicken	656	**50**	Covered 414 (total 976)	**42**	**21**
Rabbit	876	**67**	Covered 537 (total 896)	**60**	**34**
Goat	914	**70**	Covered 652 (total 978)	**67**	**44**
Donkey	973	**74**	Covered 598 (total 966)	**64**	**40**
Sheep	1005	**77**	Covered 551 (total 779)	**71**	**46**
Multispecies	1191	**91**	Covered 746 (total 920)	**81**	**58**

Abbreviations: AP-MS, affinity purification based mass spectrometry; 2D-WB, two-dimensional western blot; HCP, host cell protein; LMW, low molecular weight.

Only donkey 2D-WB coverage decreased slightly from 67% to 64% after purification. Purified sheep antibodies achieved the highest coverage with 2D immunoblots (71%), followed by goat (67%), donkey (64%), rabbit (60%), and chicken (42%). While the coverage values obtained by AP-MS for sheep (77%), donkey (74%), goat (70%), rabbit (67%), and chicken (50%) showed overall a similar trend, the results were consistently higher compared to 2D-WBs. This can be attributed to inherent technical drawbacks of the 2D-WB technique like impaired protein binding under denaturing conditions or differing detection limits especially for low abundant HCPs (Berkelman et al., 2015; Seisenberger et al., 2021; Zhang et al., 2014).

While rabbit derived antibodies proved superior over those from goats in the study performed by Haemmig et al. (2017) determining the 2D WB coverage, the opposite trend was observed by Pilely et al. (2020) per AP-MS. In contrast to these results, no considerable coverage difference were observed between both species in the present study, regardless of the method used. However, comparison of percentage coverage values determined in different labs should be handled with care, as the results can be affected by the experimental setup. This includes the analytical method, the method parameters (for 2D-WB, e.g., the evaluation method [spot counting], staining technique, gel format, and for the AP-MS method, e.g., the matrix for pAb immobilization, dealing with non-specific binding issues) and the preparation of the pAb itself (e.g., immunization method, antigen, purification). The present study allows a valid comparison, as the same antigen and analytical methods, were used for all species.

Besides evaluating the coverage for the single hosts separately, a comparison of the five different anti-CHO-HCP pAbs with each other was prepared, to assess if these species complement each other resulting in an improved coverage of the mixture. For this purpose, an overlay of the previously obtained 2D-WBs was created (Figure 4.1) utilizing the sheep 2D-WB as base image due to the highest coverage of all tested species.

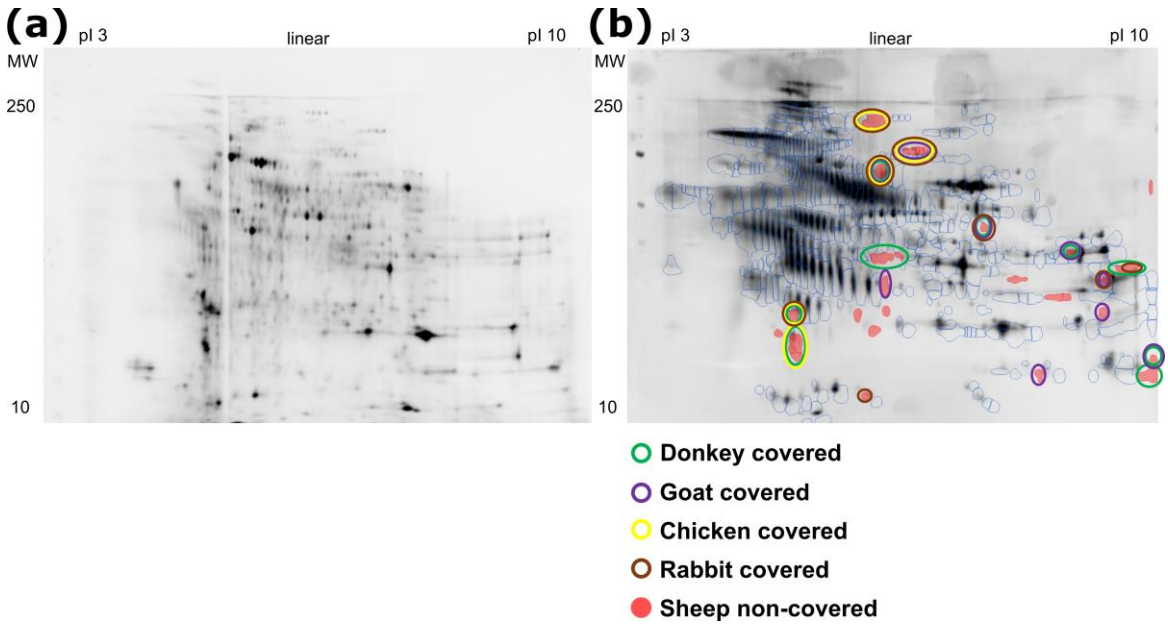

Figure 4.1: (a) 2D-PAGE of the CHO-HCP ELISA standard and (b) overlay of 2D-WBs obtained by single-species pAbs.

Two hundred and fifty micrograms of Cy-labeled CHO-HCP standard were separated by 2D-PAGE. After transferring the protein onto a membrane, the 2D-WBs were immunostained by using affinity purified pAbs (1 mg for each blot) of the respective animal species as primary Ab and the herein described method parameters for ECL detection. The sheep 2D-WB was used as base image and the 2D-WBs of the other four species were aligned. The filled red spots represent host cell protein spots non-covered by sheep pAbs. If detected by pAbs from another species, the 2D WB spots were marked with circles in the following colors: green for donkey, blue for goat, yellow for chicken, and brown for rabbit. 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; CHO-HCP, Chinese hamster ovary-host cell protein; ELISA, enzyme linked immunosorbent assay; 2D-WBs, two-dimensional western blots; pAbs, polyclonal antibodies; ECL, enhanced chemiluminescence.

The majority of spots that were missing for sheep were covered by one of the remaining species, while only six remained undetected. Besides 2D-WB analysis, differences between the identified HCPs for each species were assessed by AP-MS. Only few individual HCPs were identified as solely covered by one species (Supporting Information: Table S 7-5), with rabbit having the most uniquely covered HCPs. None of the HCPs is known as a high risk or so called hitchhiker HCP (Jones et al., 2021; Seisenberger et al., 2022). To detect any synergistic effect between different species, a systematic comparison of all possible combinations regarding their coverage values was conducted. Therefore,

the raw data files from duplicate measurements for each species were merged by using the combined database function from Protein Pilot, resulting in combinations of two to five species as shown in Supporting Information: Figure S 7-6a. The coverage raised as the number of combined animal species increased, with the combination of all five species achieving the highest coverage (91%, Supporting Information: Figure S 7-6a). Overall, the rise in coverage plateaued as the number of species increased. Combinations containing chicken-derived pAbs consistently achieved the lowest coverage values for combinations of up to three species, while its deficiencies were compensated from four species upwards. It needs to be noted, however, that the merging of different data sets by a combined database search leads to a certain bias of the results, as an increasing number of files are combined with each additional species. To estimate the magnitude of this effect, the observed gain in coverage for combinations from 2 to 5 species was compared to the increase of identified proteins when combining the corresponding number of data files originating from the analysis of the HCP standard (Figure S 7-6b). For example, the combination of two species measured in duplicates amounts to a total of 4 combined data files. Combining the same number of measurements of our HCP standard in a combined database search led to an increase of 10% in identified proteins, which can be attributed to the run-to-run variability of the LC-MS/MS method, especially for low abundant HCPs. Consequently, the positive effect on coverage due to the combination of different species is much smaller than one would assume based on the numerical increase. To assess the benefit of combining different species on a more experimental basis, the purified pAbs of the five species were mixed in equal amounts and the mixture was characterized by 2D-WB. Indeed the multi-species 2D-WB showed 10% higher coverage (81%) compared to the best single species (sheep, 71%) and the coverage in the LMW region was improved from 46% to 58% (Table 4.2). However, the validity of a direct comparison between the coverage values obtained for the single- and multi-species ELISA is limited, as the amount of employed antibodies was quintupled for the latter. This can in turn facilitate the detection of low-abundant or low-immunogenic HCPs that are otherwise below their detection limit. To investigate whether higher coverage correlates with better performance, ELISAs for all single species and the multi-species pAbs were developed.

4.4.2 ELISA development and validation

Appropriate HCP-ELISA antibodies are supposed to recognize a broad range of HCPs over the entire range of pI and molecular size. Additionally, they should be sensitive (low ppm range), specific (e.g., no product cross-reactivity), exhibit high affinity (stable antigen-antibody binding, e.g., during wash steps), and low background (not sticky to each other or the matrix) (Ph. Eur. 2.6.34, 2017; USP <1132>, 2016).

To this end, a sandwich ELISA format was developed using the same antibody stock solution for capture and detection antibodies (Figure 4.2).

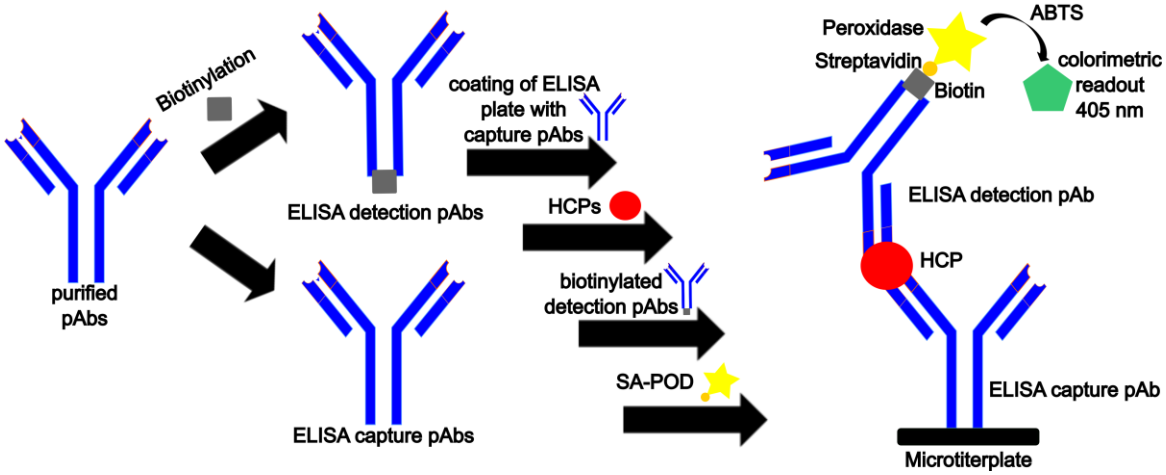

Figure 4.2: Illustration of the applied sandwich ELISA format.

Purified pAbs are separated in two fractions. One fraction is labeled with Biotin and used as detection pAbs, while the other is used as capture pAbs. The HCPs are added directly after the capturing of pAbs. Addition of the biotinylated detection antibody forms the sandwich complex, which binds SA-POD. The Peroxidase reacts with the added ABTS and the colorimetric reaction is measured at 405 nm. ELISA, enzyme-linked immunosorbent assay; pAbs, polyclonal antibodies; HCPs, host cell proteins; SA-POD, Streptavidin-peroxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

The detection pAbs were conjugated with biotin to allow sequential detection via Streptavidin--peroxidase (SA-POD) and the chromogenic substrate ABTS. Labeling efficiency (mono- and poly-biotinylation) for all biotinylated pAb species was determined by evaluating the complex formation between the biotinylated-pAb and Streptavidin-Fluorescein at 280 nm and 494 nm during gel filtration chromatography (data not shown).

For method development, the ELISA parameters were optimized by checkerboard titration (Posthuma-Trumpie et al., 2008; Wang et al., 2005) of the assay reagents, including the capture and detection antibodies, as well as the SA-POD conjugate. Therefore, only one component was tested in different concentrations, while the others were kept constant, with the goal to achieve a maximum signal-to-noise ratio (titration example Figure S 7-7). The resulting ELISA parameters are listed in Table 4.3.

Table 4.3: Final concentration of the rare reagents and incubation time with ABTS until readout at 405 nm selected for ELISA measurements using the respective pAbs.

Host animal species for pAbs	SA-POD conjugate [U/ml]	Capture pAbs [µg/ml]	Biotinylated detection pAbs [µg/ml]	ABTS incubation time [min]
Chicken	0.1	1	2	35
Rabbit	0.05	2	10	10
Goat	0.05	10	5	9
Donkey	0.05	5	5	8
Sheep	0.05	10	10	6
Multi – species	0.05	5	10	6

Abbreviations: ELISA, enzyme-linked immunosorbent assay; pAbs, polyclonal antibodies; SA-POD, Streptavidin-peroxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

Interestingly, chicken antibodies reached the maximum signal-to-noise ratio at very low concentrations (1 µg/ml and 2 µg/ml for capture and detection pAb, respectively) due to rapidly increasing background signals. This indicates cross-reactivity between the capture and the detection antibody, since interactions with the buffer components, inefficient blocking and pAb aggregates were excluded (data not shown). Furthermore, the SA-POD concentration for the chicken ELISA had to be doubled (0.1 U/ml) compared to the other species (0.05 U/ml), to achieve the ABTS color change in a feasible timespan. For the other ELISAs, the capture antibody concentrations were 2 µg/ml (rabbit), 5 µg/ml (donkey/multi-species) or 10 µg/ml (goat/sheep), and the detection antibody was used with 5 µg/ml (goat/donkey) or 10 µg/ml (rabbit/sheep/multi-species).

The performance of the single species and the multi-species formats was validated according to ICH guidelines (ICH, 1995) and internal acceptance criteria, to ensure that the assays meet the established specifications for HCP-ELISAs. The ELISA validation parameters included limit of quantitation (LOQ), linearity, accuracy, repeatability, intermediate precision and assay range (Table 4.4).

Table 4.4: Method validation for ELISA using pAbs from single species and the corresponding multi-species mixture.

Test para-meter	**Acceptance criteria**	**Multi – species**	**Sheep**	**Goat**	**Donkey**	**Rabbit**	**Chicken**
Limit of quanti-fication	**Accuracy at LOQ: 70-130% Repeatability at LOQ: RSD ≤ 20%**	1.0 ng/ml	2.0 ng/ml	2.0 ng/ml	2.0 ng/ml	1.0 ng/ml	2.0 ng/ml
Linearity	**R ≥ 0.97**	1.00	1.00	1.00	1.00	1.00	1.00
Accuracy	**70-130%**	91-107%	88-103%	83-110%	90-98%	80-99%	83-104%
Repeat-ability	**RSD ≤ 20%**	4-13%	1-6%	1-17%	2-11%	0-14%	1-15%
Inter-mediate precision	**RSD ≤ 30%**	10%	11%	9%	11%	10%	19%
Sample dilution linearity	**RSD ≤ 20%**	0-14%	0-12%	0-13%	0-10%	0-10%	1-20%
Range	**Linearity: R ≥ 0.97 Accuracy: 70-130% Repeatability: RSD ≤ 20%**	1-100 ng/ml	2-100 ng/ml	2-100 ng/ml	2-100 ng/ml	1-100 ng/ml	2-100 ng/ml

Abbreviations: ELISA, enzyme-linked immunosorbent assay; LOQ, limit of quantitation; pAbs, polyclonal antibodies; RSD, Relative standard deviation; R, correlation coefficient.

Acceptance criteria were set as described elsewhere (Leiss et al., 2015). Linearity within a given range ensures that the achieved ELISA results are directly proportional to the quantity of analyte in the sample (ICH, 1995) and linearity is therefore important to ensure accurate HCP content determination (USP <1132>, 2016). It was assessed by analyzing triplicates of diluted drug substance formulation buffer (sample buffer) spiked with various concentrations (2 ng/ml–100 ng/ml) of the CHO-HCP ELISA standard. Sample dilution linearity was tested with two different samples from two in-house mAb projects in four serial dilutions. Samples after different downstream process units varying in their HCP content and complexity were chosen, to demonstrate suitability throughout the entire purification process. Spike recovery experiments, using CHO-HCP ELISA standard added at three levels (2 ng/ml, 50 ng/ml and 100 ng/ml) into sample buffer and mAb product samples, were used to demonstrate HCP assay accuracy, which is defined as the closeness of the test result to the true value (USP <1132>, 2016). The data of the accuracy experiments served also to assess the repeatability and the LOQ. To evaluate the intermediate precision, which takes the variability of the day of measurement and equipment into account, the control samples were measured in two dilutions in a total of six independent runs on different days. The assay range was defined based on assessing the linearity, accuracy and repeatability. Finally, specificity of the anti-HCP antibodies was demonstrated by using the accuracy data, as no interference to the matrix was identified. While the acceptance criteria were met for all species, the sample dilution linearity (Table 4.4) was only barely within the defined specifications when using the chicken ELISA, which is in line with the low coverage of the chicken antibodies in the 2D-WB. Chicken antibodies also exhibited problems with high background signals. As LOQs of 1 ng/ml and 2 ng/ml were obtained, the assay range was set to 1 ng/ml-100 ng/ml for the rabbit as well as multispecies ELISAs and to 2 ng/ml -100 ng/ml for the other ELISAs (Table 4.4). These results demonstrate that all ELISA formats achieved suitable sensitivity and a broad assay range to serve as method for HCP impurity testing. All six ELISAs were successfully validated and therefore ready for use.

4.4.3 Determination of HCP content using the single and multi-species sandwich HCP-ELISAs

The six developed ELISAs were used to quantify the HCP content of in-process samples from five in-house projects with a broad range of impurity levels. To assess the correlation between the results for the different assays, the HCP concentrations of each sample were plotted against the sheep ELISA HCP levels (Figure 4.3).

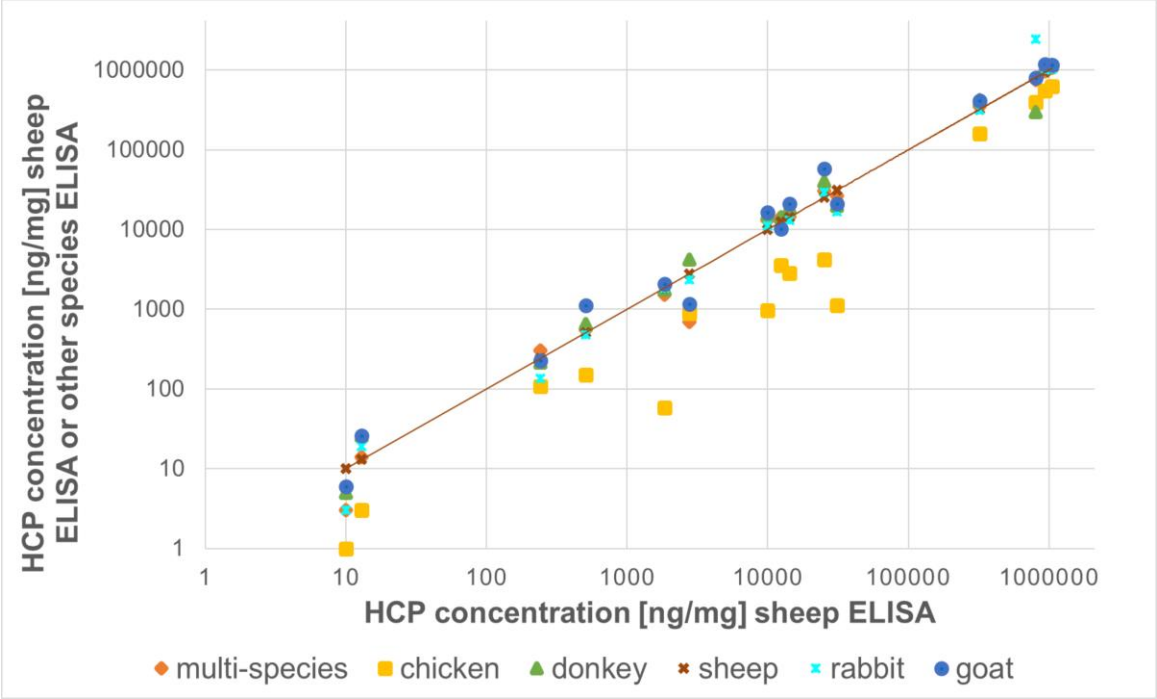

Figure 4.3: Correlation for the HCP quantification between the five single-species and the multi-species ELISA.

The HCP content of n = 15 different in process samples with varying purity levels and from five in-house projects was determined with each of the six ELISAs and the resulting HCP concentrations of each sample were plotted against the sheep ELISA HCP levels. All samples were measured in four serial dilutions to reach the point where nonlinear behavior was no longer observed. The mean value of the HCP concentration measured in two different dilutions was reported. A control sample in two dilutions (triplicates) and the CHO-HCP ELISA standard dilution series (triplicates) were included on each plate. HCP, host cell proteins; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary.

The measured HCP content of the rabbit, goat, donkey, and the multi-species ELISAs were in good agreement with the sheep ELISA, indicating comparable performance for quantifying HCPs and monitoring the consistency of a manufacturing process. This observation is in accordance with previous reports (Graf, Seisenberger, et al., 2021; Haemmig et al., 2017), that slight variations in ELISA antibody coverage do not necessarily affect ELISA performance. Results obtained with the chicken ELISA however generally showed lower HCP levels, supposing a direct influence on ELISA performance when the anti-HCP pAb coverage falls below a certain threshold. In contrast, higher 2D-WB coverage with the multi-species antibodies compared to sheep-derived antibodies does not directly translate into higher ELISA results. Even if higher coverage is achieved with the multi-species mixture, obtaining 100% coverage is practically impossible, as some

proteins do not elicit an immune reaction in the host animal. Therefore, orthogonal methods like LC-MS/MS-based impurity testing are essential to complement ELISA HCP quantification to detect less or non-immunogenic proteins, marginalizing the impact of subtle variations in overall coverage values. In addition, the multi-species approach is associated with some disadvantages, as more animals for immunization are required, making it problematic from an ethical and financial perspective. From our point of view, the efforts of developing a multi-species ELISA and its poor reproducibility in the event of reagent resupply outweigh the benefit of increased coverage.

4.5 Conclusions

Five different host animal species (sheep, goat, donkey, rabbit, and chicken) were immunized with the same CHO-HCP antigen to conduct a systematic study on the influence of the host species on HCP-ELISA pAb coverage and performance. The single species as well as the mixture derived of them were assessed regarding their immunogen coverage using two orthogonal methods (2D-WB and AP-MS) and their performance in the respective ELISAs. While the obtained sheep, goat, donkey, and rabbit pAbs featured resembling titers and coverage values and performed similarly in the developed ELISAs, only chicken pAbs proved deficient. As chicken antibodies exhibited an unexpected low coverage and ELISA values, they proved unsuitable for our CHO-HCP ELISA because of the increased risk of missing single HCPs or underestimating the overall content. The feasibility of a multi-species ELISA was demonstrated, but although the coverage of the antibodies was slightly higher, they did not perform superior in HCP quantification for the herein-tested samples and therefore provided no clear advantage over single-species ELISAs.

5 Comprehensive Summary, Discussion, Conclusions and Outlook

5.1 Comprehensive summary

HCPs are inevitable process related impurities monitored usually by HCP-ELISAs. As the presence of HCPs in the final DP has potentially a negative influence on drug stability and patient safety, working with high quality HCP ELISAs is very important. The performance of the HCP-ELISA for HCP quantification is tightly linked with the quality of the critical ELISA reagents (HCP standard and anti-HCP pAbs). In this context, the anti-HCP pAbs in particular play a crucial role, therefore this work focuses on them. The HCP-ELISA pAbs are produced by immunizing a host animal with the HCP standard, which contains a heterogeneous mixture of HCPs with highly variable immunogenicity. Consequently, weakly immunogenic HCPs could be poorly or not detected by the HCP immunoassay. It is, however, required by the United States and European Pharmacopeias (USP<1132> and Ph. Eur. 2.6.34) that the anti-HCP pAbs cover (detect) the majority of the diverse HCP mixture present in the current production process (Bracewell et al., 2015; Ph. Eur. 2.6.34, 2017; Tscheliessnig et al., 2013; USP <1132>, 2016; Wang et al., 2009; Wohlrab et al., 2018; Zhu-Shimoni et al., 2014). The aim of this thesis was to understand and minimize CHO-HCP ELISA detection gaps by improving the coverage of the anti-HCP pAbs and the related analytics. The strategies to achieve this goal are depicted in Figure 5.1.

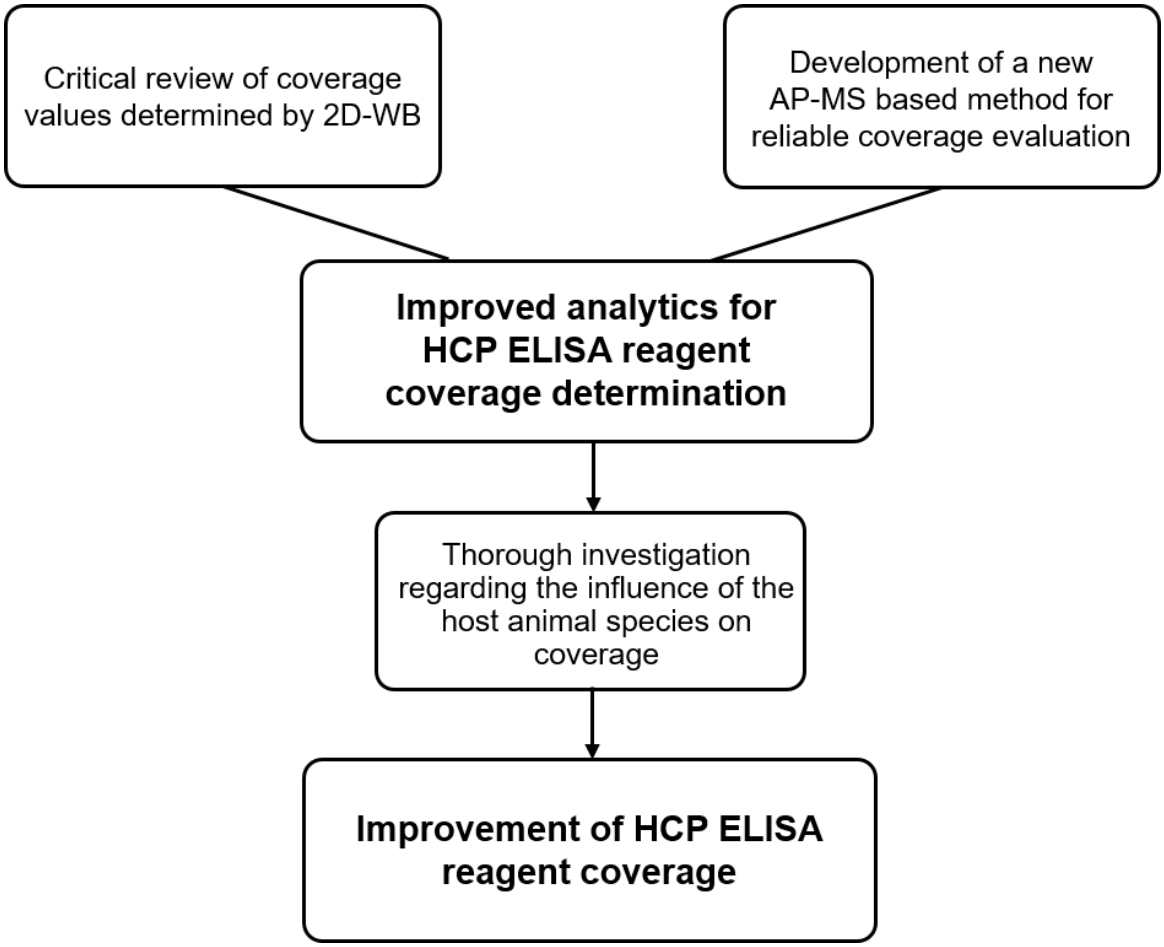

Figure 5.1: Strategies to measure and improve the HCP ELISA reagent coverage and the related analytics.

Abbreviation: HCP, host cell protein; ELISA, enzyme linked immunosorbent assay; AP-MS, affinity purification based mass spectrometry; 2D-WB, two-dimensional western blot.

It was claimed that the performance of ELISAs for HCP quantification depends on the overall coverage and specificity of the associated pAbs (Shahrokh et al., 2016). Reliable analytical methods are indispensable to identify coverage gaps and thus get a precise overview of actual detection gaps. The 2D-WBs are still the method of choice for determination of coverage gaps, although some problems are discussed (Berkelman et al., 2015; Seisenberger et al., 2021; Zhang et al., 2014). In chapter 2 of this work the coverage values obtained by 2D-WB are questioned by a critical study on the root causes of 2D-WB detection gaps during the characterization of ELISA antibody reagents. A 2D-WB with the tested CHO-HCP ELISA reagents showed that the detection gaps are located mainly in the LMW region (below 40 kDa) of the blot, while the pI does not seem to play a major role. There are three possible causes for the limited detection of the LMW HCPs. First, the

concentration of the LMW proteins could be outside the dynamic range of the 2D-WB, even if specific pAbs are generally available. Enrichment of the LMW fraction and preparation of a 2D-WB with and without blending the total HCP standard with the LMW fraction confirmed this issue, as the detection gaps could actually be reduced by admixing the LMW HCPs. The second cause for the detection gaps could be the absence of specific pAbs. To investigate this, the HCPs of individual undetected protein spots of the 2D-WB were identified by LC-MS/MS. However, 92% of these HCPs could be identified as covered under native protein-binding conditions with an AP-MS based method. This indicates that the observed detection gaps can rather be ascribed to impaired antibody binding due to the denaturing conditions of the western blot approach than to the actual absence of specific antibodies. Hence, this third cause for the 2D-WB detection gaps was examined closer. The effect of protein denaturation on 2D-WB coverage was tested by application of the indirect ELISA with the native and the denatured HCP standard. Additionally a single HCP which was non-covered with the 2D-WB, but covered with the AP-MS method, was measured under native and denatured conditions in the indirect ELISA. The elicited signal was always considerably higher with the native proteins, verifying impaired antibody binding after the loss of conformational epitopes through protein denaturation.

After some inherent problems in the 2D-WB method were confirmed, orthogonal methods to overcome 2D-WB limitations and correctly determine the anti-HCP pAb detection gaps should be developed. In contrast to the traditionally applied 2D-WB, the AP-MS provides the identity of covered HCPs under native conditions, similar to the ELISA. A new AP-MS method (referred to as native coverage) to overcome undesired elution errors is presented in this work (chapter 3). Briefly, ELISA pAbs were immobilized via their Fc part to magnetic protein G beads and stably cross-linked. This allows direct denaturation of the bound HCP from the pAb, while keeping the antibody widely stable. However, it was demonstrated that all previously described approaches lead to false positive and false negative hits due to unspecific binding of the HCPs, compromising the validity of the obtained results. In order to overcome unspecific binding issues, a universal affinity-based mass spectrometry approach for comprehensive ELISA reagent coverage evaluation and HCP hitchhiker analysis was developed. Instead of relying on arbitrarily determined exclusion criteria to distinguish between specific and unspecific bound HCPs, the optimized AP-MS is capable of eliminating this problem and therefore allows simple and reliable data evaluation. This was achieved by conjugation of a diazobenzene-cleavable linker to the ELISA antibodies facilitating the separation of HCPs interacting with the matrix from those specifically bound to the antibody. During qualification of the optimized AP-MS high reproducibility and superior identification of detection gaps could be demonstrated, compared to the

previously published methods (Henry et al., 2017; Pilely et al., 2020; Waldera-Lupa et al., 2021). Furthermore, application of the optimized AP-MS method for hitchhiker analysis contributes substantially to the understanding of antibody HCP interactions as it only identifies specifically bound proteins. Identification of those high-risk HCPs enables tailored selection of column wash conditions during downstream purification, improving product quality and process knowledge. As HCP ELISA antibody coverage is the key factor to ensure the functionality and confidence in the reliability of the assay, the unambiguous coverage determination for each single HCP in the herein developed method supports the selection of the most appropriate antibodies. Likewise, reliable evaluation of all covered HCPs ensures the suitability of the ELISA for a certain process, which in turn helps to secure product quality and patient safety.

With the knowledge of the 2D-WB limitations and the availability of new orthogonal methods for reliable coverage determination, the improvement of pAb coverage could be addressed now. Purification and immunization methods are key to the improvement of the antibody coverage. As already mentioned, the purification of pAbs from crude sera has already been optimized as part of a master thesis (Sticht, 2020) within this project and thus is state of the art. Within the immunization method, the choice of animal species is, among other factors, crucial for the quality and coverage of the resulting pAbs (Delahaut, 2017). Hence, the fourth chapter deals with the impact of the host animal species on ELISA performance for HCP quantification. HCP-specific pAbs are commonly produced by immunizing an appropriate host animal species with the HCP standard. In general, the host animal species should be able to provide sufficient amounts of antibodies, making small animals such as mice and guinea pigs unsuitable for this purpose. Larger phylogenetic distance between the source of the HCP and the species used for the anti-HCP antibody production is considered beneficial for stronger reactions of the immune system to the immunogen. A phylogenetic tree (Figure 5.2), comparing the Chinese hamster as source of the antigen, to chicken, rabbit, donkey, goat and sheep, indicates that chicken is phylogenetically furthest from hamster.

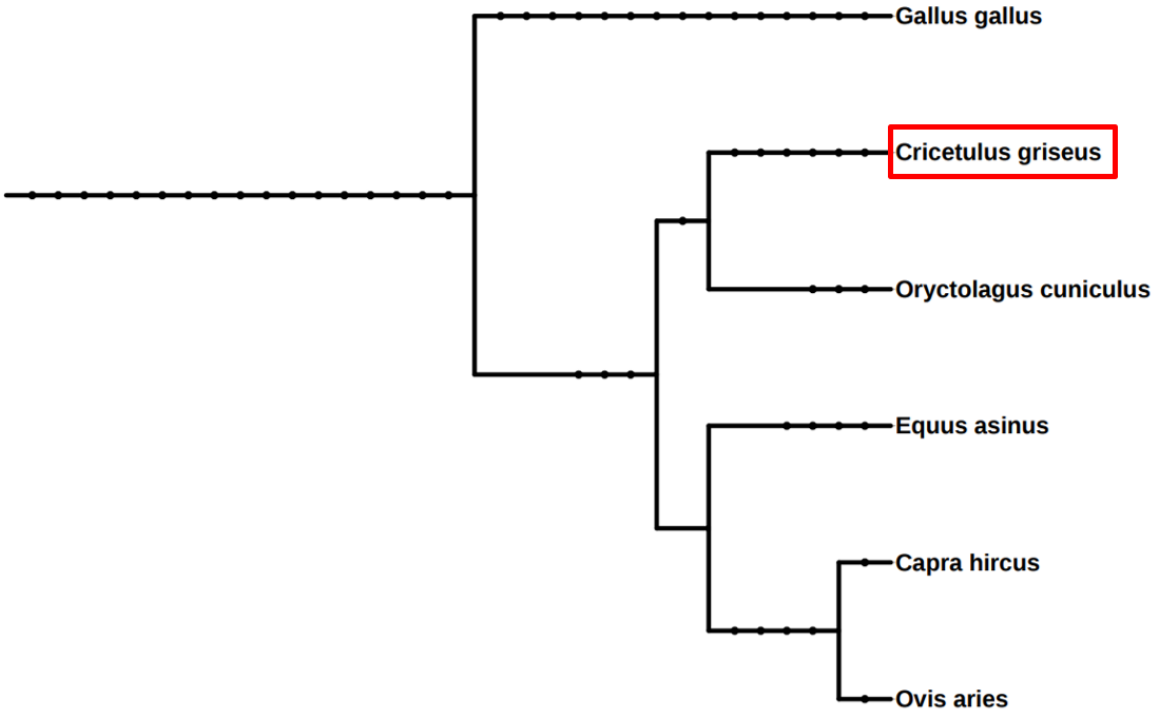

Figure 5.2: Phylogenetic tree of the five immunized host animal species and Chinese hamster.

Chicken (Gallus gallus), rabbit (Oryctolagus cuniculus), donkey (Equus asinus), goat (Capra hircus) and sheep (ovis aries) were compared to the source of the host cell protein immunogen, the Chinese hamster (Cricetulus griseus, red frame), regarding their phylogenetic distance. Chicken is phylogenetically furthest from hamster, followed by sheep, goat and donkey. Rabbit is phylogenetically most related to hamster. The tree topology was created with the phyloT tree generator (Letunic, 2015).

Due to the lack of systematic studies using the same antigen for immunization and consistent analytical techniques for reliable comparison, antibodies derived from five different host animal species (sheep, goat, donkey, rabbit and chicken) were compared with respect to their coverage of the HCP standard and ELISA performance. Additionally the question was addressed whether a mixture of the antibodies derived from the five host animal species can improve the ELISA performance for HCP quantification, assuming a higher coverage compared to single species derived pAbs. Employing the pAbs from the five different host animal species and the multi-species mixture resulted in similar ELISA performance with the sole exception of chicken pAbs. In general, slight variations in ELISA antibody coverage did not influence the ELISA performance, if the anti-HCP pAb coverage did not fall below a certain threshold. A comprehensive dataset could be provided, bringing new insights into the selection of the host animal species during ELISA development.

5.2 Discussion

HCP analytics is challenging, due to the diversity and the wide concentration range between DS and HCPs. Despite some well-known disadvantages, such as coverage limitations and yielding just sum parameters of HCP contents, ELISA is still the workhorse for HCP detection (Tscheliessnig et al., 2013; Zhu-Shimoni et al., 2014). Orthogonal methods like mass spectrometry are often routinely used to complement ELISA results. Those methods have some advantages over ELISA such as fewer detection gaps, higher flexibility, and faster development possibilities, because time-consuming production of HCP-specific pAbs is not required. However, LC-MS based methods have also some downsides such as the need of skilled operators with a lot of experience to perform the analysis and interpret the data in a reliable manner. For example, the detection of the peptides is dependent on their ionization capabilities and the digestion efficiency. The wide dynamic range between DS and HCP is challenging, especially for low abundant HCPs, as e.g. algorithms that sort out spectra to achieve better time-efficiencies can lead to false positive hits or incorrect assignments during data interpretation. Due to the complexity of the method and the required equipment, data integrity is hard to achieve (Matuszewski et al., 2003; Wu & Han, 2006). LC-MS based methods do not detect HCPs depending on their immunogenicity but, conversely, do not allow any conclusions to be drawn about them, but immunogenic HCPs pose a higher risk for patient safety (Rane et al., 2019; Robotham & Kelly, 2020). Both ELISA and mass spectrometry-based methods have benefits and limitations. However, ELISAs have decisive advantages that are very important for the use in the pharmaceutical industry. HCP ELISAs can be applied under GMP conditions in quality control labs, which is required for their use as GMP release test to show adequate DS purity. This is very difficult to achieve with mass spectrometry-based methods due to technical challenges such as instrument and method variability, lacking robustness of the software and issues with method transferability. Additionally regulatory and compliance concerns regarding e.g. challenges in method validation, harder to achieve data integrity requirements, large data volume and the complex nature of the whole method makes it more prone to out-of-specification errors (Háda et al., 2018; Zhang & Guo, 2017). ELISA, on the contrary, is a high-throughput and easily automatable method, making it ideally suited for, and predominantly used in purification development and as process consistency marker (Rey & Wendeler, 2012; Zhu-Shimoni et al., 2014). When automating HCP ELISAs, it is possible to either continue with the 96-well plate format (Rey & Wendeler, 2012) or even increase the throughput capability further by using other formats e.g. based on beads (Bielefeld-Sevigny, 2009; Leiss et al., 2015), microfluidics technology (Liu et al., 2017; Manen-Brush et al., 2020) or bio-layer interferometry technology (Singh et al., 2019). A

higher degree of automation reduces the need for qualified lab workers and increases reproducibility (Holland & Davies, 2020). In this regard, ELISA-based methods are significantly more advanced than LC-MS/MS methods. Since there will be no adequate replacement for the HCP-ELISA in the near future, improvement of this method is still very important.

Furthermore, the question arises whether a method such as ELISA, which depends on the immunization of host animals for reagent production, is still required. A multianalyte HCP-ELISA needs the use of pAbs to be able to detect the diverse protein mixture (Wang 2015) and pAb production relies on the immunization of host animals with the respective immunogen. The resulting pAbs are a heterologous mixture of IgGs derived from different B-cells that recognize multiple epitopes on the target protein with high sensitivity. In contrast, hybridoma-based mAbs react only to a single epitope of the target antigen, but have the advantage of high reproducibility and specificity, as the same B lymphocyte clone produces them. However, the generation of mAbs is more expensive than it is for pAbs (Ascoli & Aggeler, 2018; Lipman et al., 2005). In addition, a mAb cannot detect two epitopes simultaneously, which would prevent the use of the same mAb for capture and detection in the preferred sandwich ELISA format. Presumably, mAbs are not suitable for frequently occurring small changes in the HCP population after process adjustments, due to the reasons already mentioned and the accompanying lack of flexibility.

As the immunization of host animals is unavoidable for the production of pAbs, the principle of the Three Rs (reduction, refinement and replacement), first described by Russell and Burch (1959), should be applied for animal welfare reasons. In this regard, chicken was a promising host species, as it produces large amounts of IgY in the egg yolk (100 mg/egg yolk with 1-10% specific IgY), making painful blood collection needless and such replacements were demanded by the European Centre for the Validation of Alternative Methods. Besides the already mentioned larger phylogenetic distance to Chinese hamster, chicken are cheaper in housing, compared to the eligible mammals. IgYs do not activate the mammalian complement system nor interact with rheumatoid factors or bacterial and human Fc receptors (Dias da Silva & Tambourgi, 2010; Schade et al., 2005; Schade et al., 1996; Spillner et al., 2012). The latter could be challenging for purification, as the frequently used Protein G is not suitable for IgY (Akerström et al., 1985). However, chicken did not provide the expected results in this work and yielded the lowest amount of HCP-specific pAbs, compared to the other four tested species. Another reason for the poor performance of chicken-derived antibodies could be due to their deviating structure. While the light chains are the same for IgY and IgG, chicken IgY has an additional constant domain in the heavy chain (CH4) (Figure 5.3).

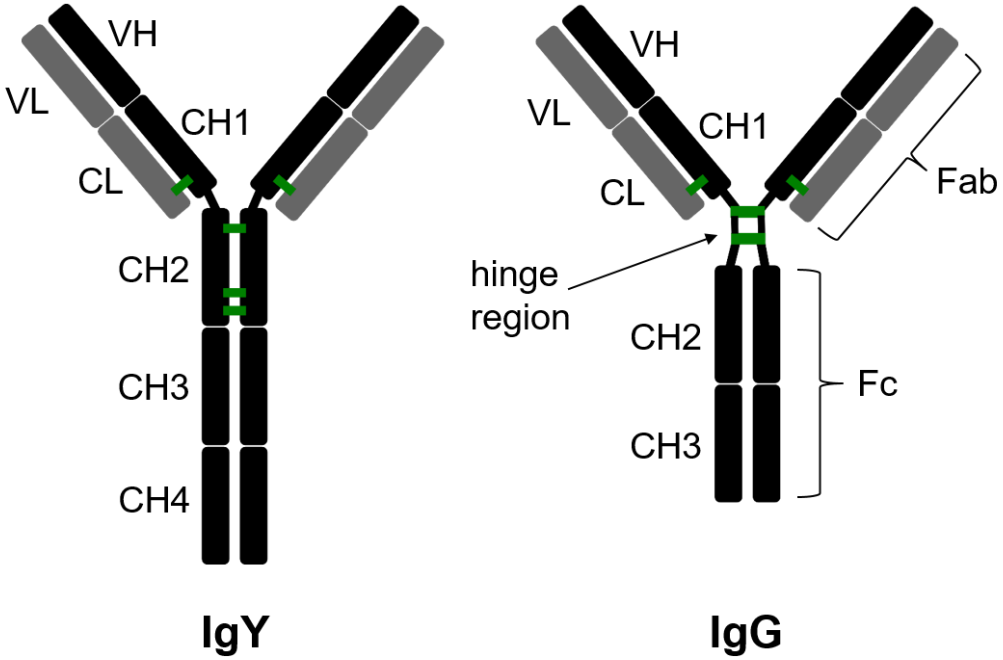

Figure 5.3: Structure of chicken IgY and mammalian IgG.
Chicken immunoglobulin(Ig) Y is composed of four domains in the constant region of the heavy chain (CH1-4), while mammalian IgG comprise only three. The flexible hinge region of the IgG is missing in the IgY. Both immunoglobulins are composed of similar structural organizations of the Fab parts with the variable domains of the light (VL) and heavy (VH) chains, and the constant domains (VL, VH) respectively. Adapted from Ferreira Júnior et al. (2021).

This additional domain results in an increased molecular weight of about 180 kDa for the IgY, compared to IgG (~150 kDa) (Cser et al., 1982; Noll et al., 1982; Shimizu et al., 1992). IgY and IgG are both bivalent, but due to the absence of the hinge region, the former is more rigid as a result of the varying amino acid sequence (proline/glycine residues) and this is probably a reason for the characteristic behavior during antigen binding (Dias da Silva & Tambourgi, 2010; Narat, 2003; Warr et al., 1995). Furthermore IgY is more hydrophobic due to its larger Fc part and has a pI of 5.7-7.6 (IgG pI: 6.1-8.5) (Dávalos-Pantoja et al., 2000; Sun et al., 2001). The lower pI of chicken antibodies may necessitate an adjustment of the buffers used. Indeed, Clarke et al. (1995) reported pH-dependent sensitivity (maximum at pH 7) and specificity (lower specificity at pH 6, higher specificity at pH 8) when using chicken pAbs in ELISAs. Hence, the buffers used in the present work for 2D-WB and ELISA, which had pH values between 7.0-7.5, were suitable for the use in chicken IgY-based immunodetection methods. In addition, other studies reported poorer performance of chicken-derived IgYs compared to mammalian IgGs in ELISAs and other

immunoassays. It has been demonstrated that chicken-derived IgYs are less sensitive to the target protein, compared to mammal-derived IgGs (rabbit or goat), resulting in up to 17-fold higher detection limits for IgYs (Bauwens et al., 1988; Clarke et al., 1995; Doth et al., 1996; Ohnishi et al., 2000). Titer values were approximately a factor 1.5-2.0 lower in chicken egg yolk compared to mammal sera (rabbit, sheep) when using the same immunization protocol and antigen (Svendsen Bollen et al., 1996; Woolley & Landon, 1995). Despite using only single protein species for immunization of chicken, the previously described studies had problems with lower sensitivity and titer values in chicken-derived pAbs. The study described in the present thesis, however, used a complex protein mixture, which makes an immune response to all proteins harder to achieve. Svendsen Bollen et al. (1996) compared different adjuvants for their efficacy in immunizations of chicken and rabbits, with complete Freund´s adjuvant (CFA) resulting in the highest titer and antibody avidity for both species. This is in accordance with our internal experience and was the reason for the application of Freund`s adjuvant in this study. CFA, composed of mineral oil and mycobacteria, is known to boost the immune reaction to a wide range of proteins and especially to LMW proteins in unexcelled manner (Altman & Dixon, 1989; H S Warren et al., 1986; Munoz, 1964; Smith et al., 1992).

After performance of the chicken-derived pAbs in HCP-ELISA fell short and to apply the principles of the Three Rs, alternative reagents without the need to immunize host animals, might have the potential to replace polyclonal antibodies in ELISAs. Aptamers or peptide ligands could act as possible alternative HCP binders. Aptamers are short nucleic acid fragments (single-stranded DNA or RNA) with high specificity and affinity to the target molecule, enabled by their unique tertiary structure (Ellington & Conrad, 1995; Famulok et al., 2000; Gold et al., 2002; Zhu & Chen, 2018). The strong binding to the target is facilitated by a combination of non-covalent interactions (e.g. hydrogen bonding, Van der Waals force, stacking interactions) (Huang et al., 2003; Lebars et al., 2008; Long et al., 2008; Toh et al., 2015). Aptamers are produced either by the systematic evolution of ligands by exponential enrichment (SELEX) process (Ellington & Szostak, 1990; Robertson & Joyce, 1990; Tuerk & Gold, 1990) or a non-SELEX method like non-equilibrium capillary electrophoresis of equilibrium mixture (NECEEM)-based partitioning (Berezovski et al., 2006). SELEX repeats several times the following steps; binding of a randomized oligonucleotide library to the immobilized target, removal of unbound nucleic acid fragments, elution of the bound oligonucleotides and their amplification by polymerase chain reaction (PCR). As the application of SELEX is very time-consuming, non-SELEX methods omit the oligonucleotide amplification by PCR for fast selection of interacting aptamers (Berezovski et al., 2006; Parashar et al., 2022). The main advantages of

aptamers over pAbs are their high reproducibility and independency of the target protein immunogenicity, as they are produced via chemical synthesis (Bradbury & Pluckthun, 2015; McGivney et al., 2018; Schonbrunn, 2014; Toh et al., 2015). McGivney et al. (2018) who carried out the first systematic study on the application of aptamer reagent sets for HCP detection, hold a patent on this method (Csordas et al., 2016). They prepared 321 CHO-HCP-binding DNA aptamers grouped in 32 sequence family sets and compared their performance with commercially available anti-HCP pAbs. The aptamers were selected in two SELEX rounds and a single particle display process. The CHO-HCPs needed to be labeled with biotin for conjugation of the fluorescence probes to enable sorting of HCP-binding aptamers with fluorescence-activated cell sorting (FACS) during particle display. On the one hand, there is a risk that not all HCPs can be labeled with biotin, and on the other hand, sorting out HCP-binding aptamers involves the risk of narrowing the sequence diversity and consequently resulting in limited detection of the heterologous HCP mixture. The latter was indeed observed (McGivney et al., 2018), as the fraction with the highest concentration of HCP-binding aptamers, generated in three rounds of enrichment (particle display), exhibited limited diversity of sequence populations. The HCP coverage of the aptamers and pAbs was tested by an AP-MS based method using NHS-activated sepharose in a column for immobilization of the HCP-binders. Although comparable numbers of covered HCPs were identified for both, some known high-risk HCPs (Aboulaich et al., 2014; Vanderlaan, 2015) were not covered by the aptamers and the authors acknowledged that this could be due to failed biotinylation of these HCPs. Another weakness of this approach is that the aptamers were not tested with DS-containing HCP samples, although the large concentration difference between them is one of the main difficulties in HCP analytics. McGivney et al. (2018) admitted that cross-reactivity between DS and aptamers is possible. In summary, aptamers are not yet valid replacements for pAbs in HCP-ELISAs, as they have not yet been shown to detect the diverse HCP population with its varying abundance in a high-throughput format with actual IPC samples.

The other possible ELISA-pAb alternative are peptide ligands, to which the following experiments already exist: selection of peptide-based high-affinity ligands (Diehnelt et al., 2010; Gupta et al., 2011), preparation of peptide-based synthetic antibodies called Synobodies (Williams et al., 2009) and the creation of peptides that mimic antibody binding (Sachdeva et al., 2019). While the application of peptide ligands in ELISAs for the detection of single targets has already been established (Heyduk et al., 2018; Liu et al., 2021), they are still missing for the analysis of protein mixtures. In the context of HCP analytics, peptide ligands were only used in DSP purifications to take off particularly high risk or difficult to remove HCPs from DS (Lavoie, di Fazio, Blackburn, et al., 2019; Lavoie, di Fazio, Williams,

et al., 2019; Menegatti et al., 2019) or as alternative to protein A chromatography for superior removal of HCPs during the purification of mAbs (Reese et al., 2020). Peptide ligands are theoretically an alternative, but have similar limitations as aptamers, primarily caused by the protein diversity of HCPs.

Instead of generating new pAbs to the complete HCP population, it may be beneficial to close some individual detection gaps to increase the overall coverage. The so-called GAPex^{SM} technology, which generates peptide specific pAbs to a single HCP, can be used for this purpose (Cansizoglu et al., 2018; Mreyen M. et al., 2018). Briefly, this method identifies the detection gaps of the ELISA pAbs with a 2D-WB. The non-covered protein spots were picked from a separate 2D-PAGE and the respective HCPs were identified with the help of mass spectrometry. This part was already done for some non-covered HCPs in the course of the present thesis (see 2.3.6). In addition to the existing GAPex^{SM} workflow, the identified detection gaps could be verified by the optimized AP-MS method. The next step of the GAPex^{SM} method involves screening for immunogenic peptides in the sequence of the non-covered HCPs and synthesis of those peptides. Calis et al. (2013) identified some factors for increased immunogenicity of peptides such as presence of amino acids with large and aromatic side chains and amino acid composition of the middle part of the peptide (P4-P6) presented on MHC molecules. The use of peptides instead of the completely recombinant protein allows faster results, as synthesis of correctly folded, soluble and stable recombinant proteins if often challenging (Bhatwa et al., 2021; Rosano & Ceccarelli, 2014; Shanmugasundaram et al., 2021; Sørensen & Mortensen, 2005). The peptides themselves are not immunogenic, because of their small size and therefore have to be coupled to highly immunogenic carrier proteins such as keyhole limpet hemocyanin (KLH). The KLH-peptide complex is immunized and the resulting HCP-specific antibodies were purified. It still needs to be tested whether the pAbs produced in this way actually bind reliably and with high affinity to the HCPs. Furthermore, it is not clear in which amount these pAbs should be added to the already existing ELISA pAbs to get a balanced mixture. Presumably, these pAbs are more suitable for the detection of individual problematic HCPs in a custom-built ELISA.

Besides improvement of the pAbs themselves, the analytical methods to determine their coverage were tested for their reliability and an orthogonal method to the 2D-WB was developed. Two major shortcomings of 2D-WBs were identified. First, too low amounts of HCPs and associated pAbs are not able to overcome the detection limit and second, the western blot artifacts due to the denaturing conditions of the 2D-PAGE. The detection limit issue could possibly be solved by increasing the dynamic range of the 2D-WB with the application of improved detection strategies. For example, by applying Streptavidin

conjugated with Poly-horseradish peroxidase (PolyHRP) as published recently by Mishra et al. (2019). They tested different Streptavidin-PolyHRP conjugates bound to biotinylated secondary antibodies and achieved up to 110-fold increase in sensitivity compared to traditional western blots using secondary antibodies with directly conjugated HRP molecules. Disadvantages of this approach may be the necessity of additional blocking steps to avoid nonspecific binding to endogenous biotin molecules (Grant et al., 2019; Mishra et al., 2019; Vaitaitis et al., 1999) and the lack of tests regarding the suitability for 2D-WBs. For the second limitation, namely, 2D-WB artifacts caused by impaired antibody binding of the HCPs due the denaturing conditions, no solution is currently available. Native PAGE is not suitable due to insufficient resolution of the heterogeneous HCP mixture. AP-MS based methods have recently been developed to address the denaturation issues. However, all previously published AP-MS methods for coverage determination (Henry et al., 2017; Pilely et al., 2020; Waldera-Lupa et al., 2021) had non-specific binding issues. The newly developed optimized AP-MS method is a reliable method to determine the coverage of HCP-ELISA pAbs under native protein-binding conditions that overcomes non-specific binding issues to the matrix. The unspecific binding to the antibodies could be reduced to 8%, while previously described methods revealed unspecific binding up to 28%. This small amount of nonspecific binding to the pAbs could possibly be monitored via stable isotope labeling methods. Label-based quantification methods are in general regarded as more accurate for relative protein quantification, compared to the previously applied label-free quantification methods to solve unspecific binding issues. After digestion, the peptides could be chemically labeled with isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tags (TMT). Nevertheless, this method also has some disadvantages, such as high cost, impurities from the reporter ions and heterogeneity in variance depending on peak intensity (Anand et al., 2017; Hsu et al., 2003; Karp et al., 2010; Thompson et al., 2003; Wiese et al., 2007). In addition, the question arises whether nonspecific binding to the pAbs is actually a problem. This kind of binding is obviously stable enough to resist the ELISA wash conditions. Since the same set of pAbs is applied for capture and detection, it should be possible to form the sandwich for the ELISA measurement and detect HCPs. The previously mentioned properties make optimized AP-MS particularly suitable for hitchhiker HCP identification, as only the binding to the tested mAb product is relevant for this analysis, regardless of its nature.

5.3 Conclusions

Since aptamers and peptide ligands have not yet been developed to the point where they can serve as pAb alternatives and all orthogonal methods or alternative pAb production platforms discussed in the present work have their own inherent problems, it will still take some time before they come close to the reliability and practicability of the typical HCP-ELISA. Therefore, it is important to implement the knowledge gained here to ensure the preparation of the best possible HCP-ELISAs and thus increase patient safety.

First of all, the suspected weaknesses of the 2D-WB based antibody coverage determination were confirmed within this thesis and orthogonal AP-MS based methods were developed to overcome these limitations. The first AP-MS method, described in chapter 2 is a simple and fast method to analyze the covered HCPs, without reliable discrimination between specifically and unspecifically bound (to the magnetic Protein G beads or the IgGs) HCPs. Nevertheless, the method can be useful to monitor e.g. low abundant HCPs present in DSP process samples, in which only the totality of HCPs present in the sample is relevant and not the ones specifically bound to the ELISA pAbs. For this purpose, the low abundant HCPs can be enriched by adding the DSP samples several times to the anti-HCP pAb-beads. The newly developed AP-MS method (chapter 3), avoiding unspecific binding to the matrix, is helpful for a better understanding of the covered HCP population and hitchhiker HCPs in DS samples. For the latter, the mAb drug itself is used for HCP binding, instead of the ELISA pAbs. Better understanding of the reasons for unspecific binding of HCPs to the DS enables their removal by targeted application of additional purification steps and thus the optimization of the purification strategy. Reliable pAb coverage determination with the optimized AP-MS allows selection of most appropriate HCP-ELISA pAbs for best possible ELISA performance.

With improved analytical techniques for ELISA pAb coverage determination, the influence of host species on ELISA performance for HCP quantification could be studied thoroughly. Chicken was inferior, compared to the mammal host species, as their pAbs exhibited lower coverage and underestimated HCP levels of various samples in ELISA measurement. Consequently, rabbit, goat, sheep and donkey are equally well suited for the production of CHO-HCP ELISA pAbs. The mixture of the pAbs derived from the five animal species exhibit slightly increased coverage, but did not perform superior in ELISA, making it not advisable from an ethical point of view and exhibited overall no clear advantage.

The insights gained in this work can be applied to other fields besides CHO-HCP ELISAs, as HCPs have to be monitored in all newly developed APIs derived from living cells (e.g. cell and gene therapy, vaccines, reagents for diagnostic tests).

5.4 Outlook

There are further possibilities to improve the quality of ELISA reagents besides the ones already achieved in this work. For example application of the antigen in either immunogenicity or size-based fractions could help to close detection gaps. The principle of the former was first published by Thalhamer and Freund (1984) and is based on the principle that covered proteins are removed in order to immunize only the less immunogenic or non-immunogenic proteins. For such an affinity cascade immunization, the CHO-HCP pAbs are immobilized onto a matrix (e.g. NHS-Sepharose) and CHO-HCP antigen is passed over the column multiple times (negative immunosorption see Figure 5.4a). In between, the bound HCPs are eluted and discarded. The flow through containing the HCPs, to which no or only few antibodies exist, is concentrated and immunized again, using either the same animal species or a different one. Changing the animal species could have the advantage that HCPs, non-immunogenic in one animal species, might be immunogenic in another. An alternative solution to reduce ELISA detection gaps is immunization of size-based fractions of the whole HCP-standard (Figure 5.4b) using SEC, as performed in chapter 2.4.2 in the present work. That LMW proteins are underrepresented could be verified in this thesis (chapter 2) and therefore generation of sufficient amounts of pAbs is more difficult to achieve. Size-based fractions could be useful, as it is assumed that small proteins are less immunogenic (Dintzis et al., 1976; Müller et al., 2010). Coupling those small HCPs to an immunogenic carrier protein (e.g. Keyhole limpet hemocyanin (KLH) or bovine serum albumin) in order to increase their immunogenicity may help to get better results. Generation of ELISA antibodies by immunization with HCP fractions is already offered by some companies (e.g. Biogenes, Rockland), but only without changing the host animal species. The admixture ratio of anti-HCP pAbs from cascade immunization to the existing pAbs requires precise balancing to avoid over- or underestimation of individual HCPs.

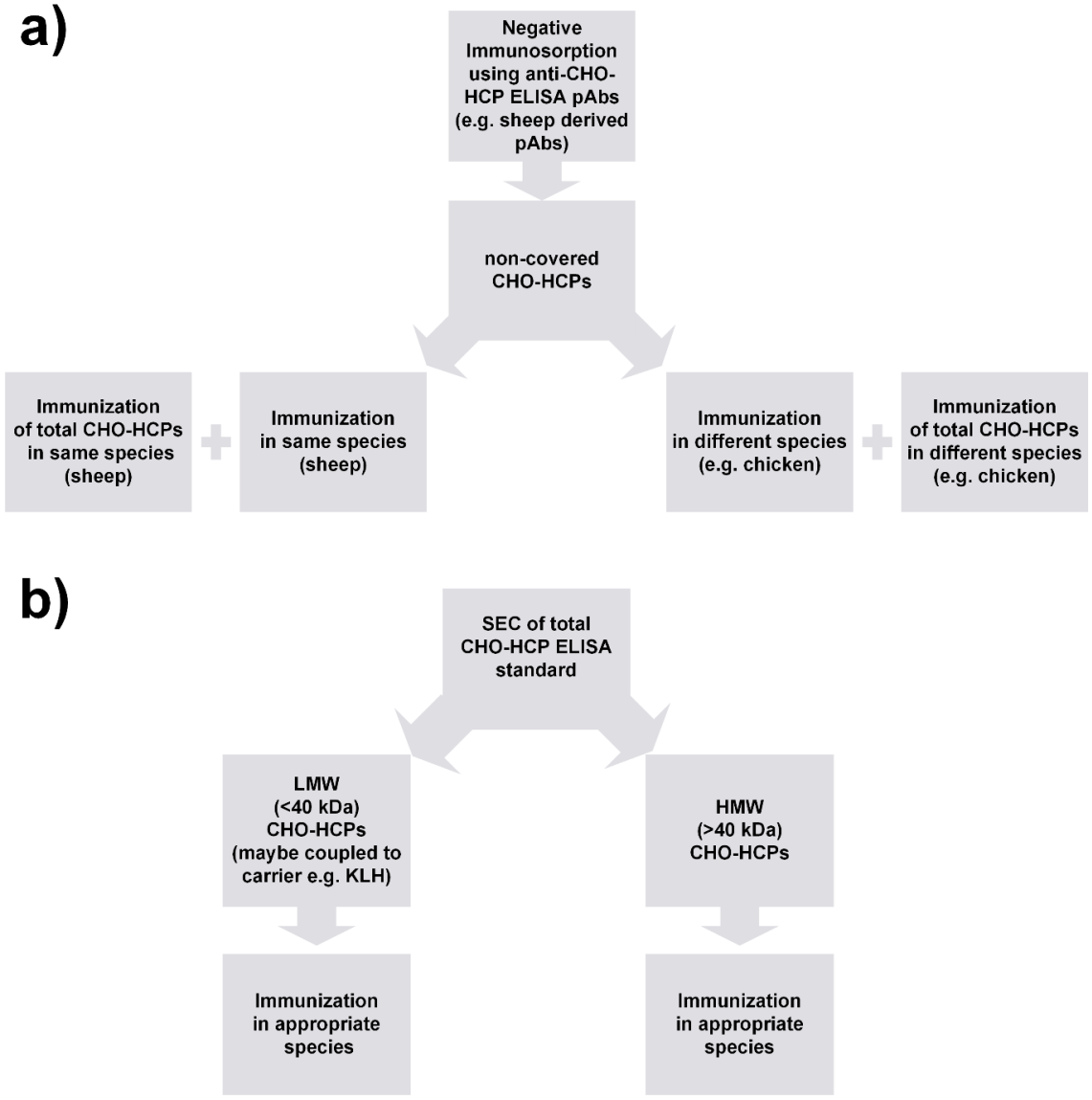

Figure 5.4: Additional immunization strategies.

a) Cascade immunization and b) immunization of size-based fractions. For the cascade immunization a negative immunosorption is conducted, meaning immobilization of polyclonal antibodies (pAbs) against Chinese hamster ovary-host cell proteins (CHO-HCPs) from a certain species (e.g. sheep) to column material, followed by several chromatography steps with the CHO-HCP standard, whereby the unbound CHO-HCPs are loaded repeatedly onto the column and bound HCPs are discarded. The unbound HCPs (non-covered) are immunized in the same species (e.g. sheep) and in another species (e.g. chicken). Additionally the total CHO-HCP standard is immunized in each species. For the immunization of the size-based fractions, the CHO-HCP standard is separated in two size-based fractions (e.g. smaller and larger 40 kDa) by size-exclusion chromatography (as described in chapter 2).The low molecular weight (LMW) fraction could be coupled to a highly immunogenic carrier e.g. Keyhole limpet hemocyanin (KLH). Both size-based fractions are immunized, either in one or two host animal species.

With the data generated by the AP-MS method, gene ontology and functional analysis could be applied to get a better overview of biological function and location of frequently identified or problematic HCPs (Chiverton et al., 2016; Kumar et al., 2020; Molden et al., 2021; Park et al., 2017). In general, the AP-MS method could be used to investigate binding behavior of any target protein or mixture thereof and the corresponding antibody. Application of surface plasmon resonance (SPR) could help to increase the knowledge of HCP-pAb binding behavior for the different species derived pAbs. Furthermore, the optimized AP-MS could be applied in a similar attempt as published by Graf, Tomlinson, et al. (2021), where polysorbate 20-degrading HCPs were enriched using an anti-HCP affinity chromatography column and subsequently identified by mass spectrometry. Recently, problems with particle formation in mAb DPs were described frequently in the literature and hydrolytically active HCPs were suspected as cause due to polysorbate 20 degradation (Chen et al., 2021; Dixit et al., 2016; Evers et al., 2021; Graf, Tomlinson, et al., 2021; Li et al., 2021; Li et al., 2022; Martos et al., 2017; Zhang et al., 2022; Zhang et al., 2021). With the optimized AP-MS, HCPs present in final DS could be captured, concentrated and identified.

6 References

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7 Supporting information

7.1 Supporting information chapter 2

Table S 7-1: Coverage values of the in-house CHO-HCP ELISA reagents after dividing the western blot into quadrants according to the molecular weight and pI of the detected protein spots as illustrated in Figure 2.1. The overall coverage of the 2D-WB is 88%.

Q1 pI 3-7 **>40 kDa** **100% coverage**	Q2 pI 7-10 **>40 kDa** **93% coverage**
Q3 pI 3-7 **<40 kDa** **77% coverage**	Q4 pI 7-10 **<40 kDa** **62% coverage**

Abbreviations: 2D-WB, two-dimensional western blot; CHO-HCP, Chinese hamster ovary-host cell protein; ELISA, enzyme linked immunosorbent assay; pI, isoelectric point; 2D-WBs, two-dimensional western blots

Table S 7-2: Total, covered and non-covered number of spots (2D-WB) or identified proteins (native coverage) as well as the determined %-coverage for total (0-250 kDa), HMW (>40 kDa) and LMW (<40 kDa) proteins of the in-house CHO-HCP ELISA standard. Native coverage results representing the Top 500 approach are marked in yellow.

	complete number of		**covered number of**		**non-covered number of**		**coverage [%]**	
	2D-WB spots	**native cover-age protein ID`s**	**2D-WB spots**	**native cover-age protein ID`s**	**2D-WB spots**	**native cover-age protein ID`s**	**2D-WB spots**	**native cover-age protein ID`s**
total	548	1526	480	1029	68	497	88	67
		500		466		34		93
HMW	369	808	354	562	15	246	96	70
		332		307		25		92
LMW	179	718	126	467	53	251	70	65
		168		159		9		95

Abbreviations: 2D-WB, two-dimensional western blot; CHO-HCP, Chinese hamster ovary-host cell protein; ELISA, enzyme linked immunosorbent assay; ID, identity; HMW, high molecular weight; LMW, low molecular weight.

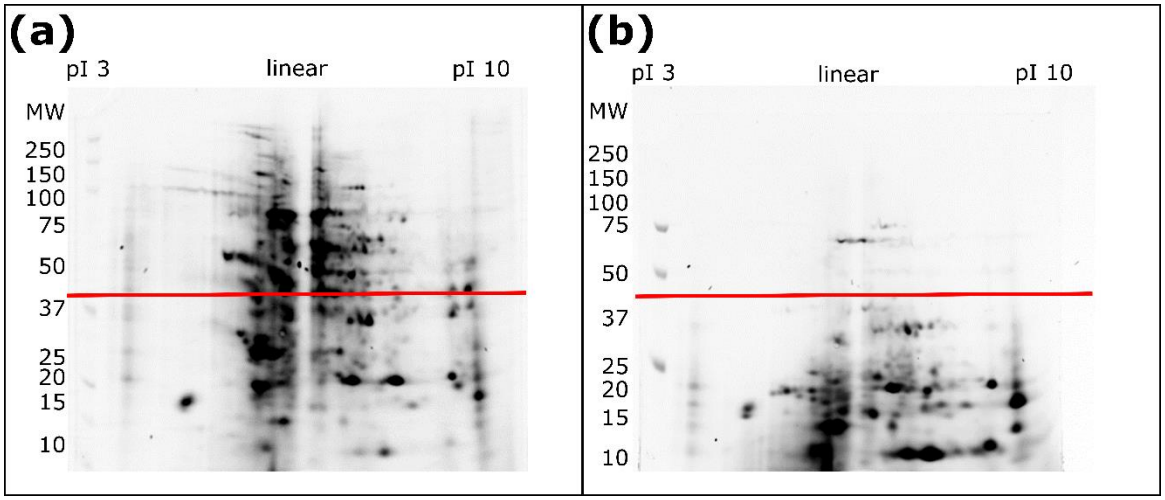


Figure S 7-1: 2D-DIGE of Cy5 labeled in-house CHO-HCP standard (a) and of Cy2 labeled LMW (<40 kDa) fraction (b). *Abbreviations: 2D-DIGE, two-dimensional difference gel electrophoresis; CHO-HCP, Chinese hamster ovary-host cell protein; LMW, low molecular weight.*

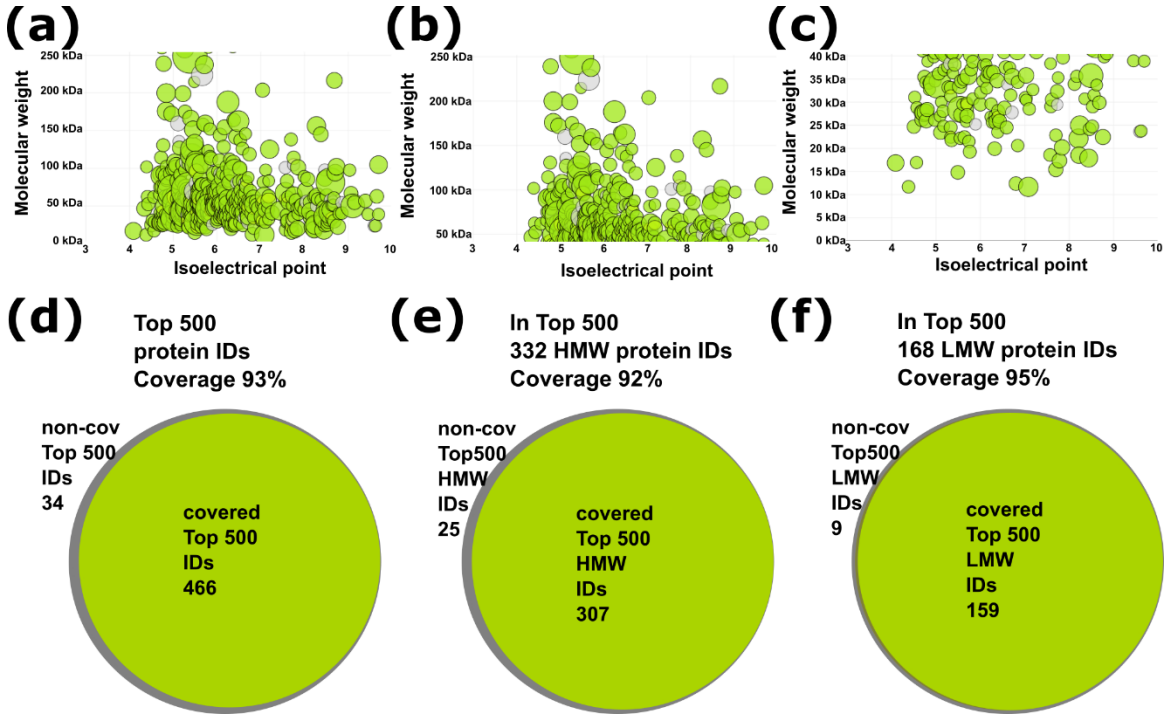


Figure S 7-2*: Affinity-based MS (native coverage) results of in-house CHO-HCP ELISA standard.* *Virtual 2D-PAGEs were prepared separately for the total molecular weight **(a)**, HMW **(b)** and LMW range **(c)** of the Top 500 CHO-HCPs, where non-covered proteins are depicted as gray and covered ones as green spots (The gel range from pI 3-10 and MW 0-250 kDa was chosen to have a similar size as the real 2D-WB). Theoretical pIs and MWs were calculated based on the protein sequences of the database entries. The size of the protein spots correlates with the relative abundance of the respective proteins. Native coverage results visualized as Venn diagrams of Top 500 **(d)**, HMW proteins in Top 500 **(e)** and LMW proteins in Top 500 **(f)**. Covered/non-covered proteins are shown in total numbers and percent coverage. Abbreviations: MS, mass spectrometry; CHO-HCP, Chinese hamster ovary-host cell protein; ELISA, enzyme linked immunosorbent assay; ID, identity; ; HMW, high molecular weight; LMW, low molecular weight, pI; isoelectric point; MW, molecular weight; 2D-WB, two-dimensional western blot.*

7.2 Supporting information chapter 3

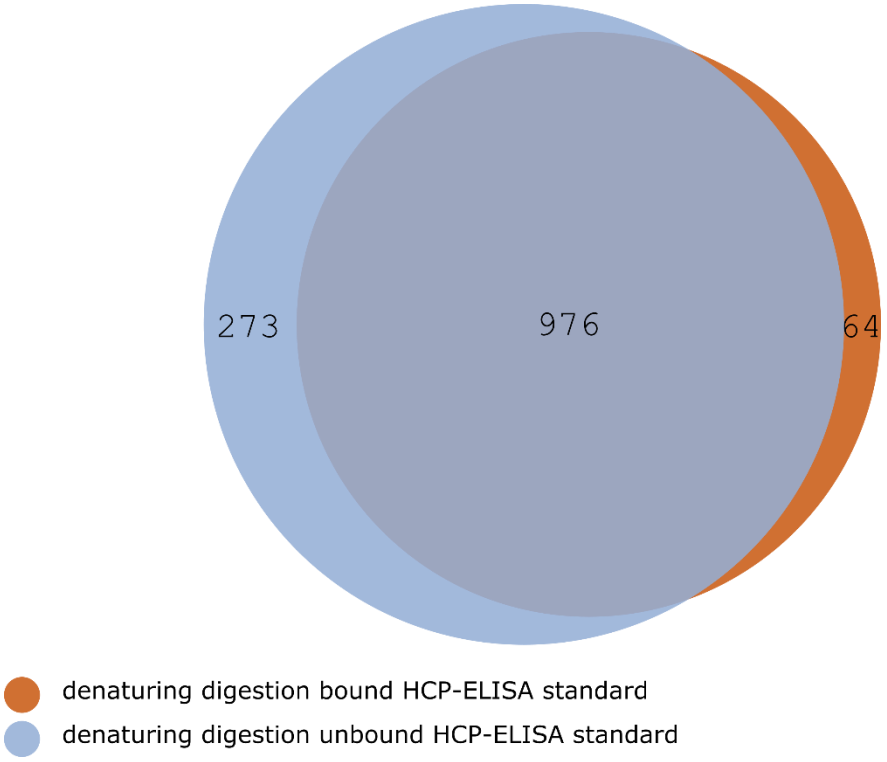


Figure S 7-3: Denaturing digestion efficiency for bound and unbound HCPs. *Venn diagram for comparison of identified HCPs with denaturing digestion of bound to 130 µg ELISA antibodies (orange) and unbound (blue) HCP-ELISA standard. For each condition, the same amount of HCP-ELISA reference standard (100 µg) was digested. Abbreviations: HCP, host cell protein; ELISA, enzyme linked immunosorbent assay.*

Table S 7-3: Reproducibility optimized AP-MS method

variability	coefficient of variation [%]
complete optimized AP-MS method	7-11
digestion and instrument (n=7, different days)	3
instrument (n=5, one sample, same day)	3

Abbreviation: AP-MS, affinity purification based mass spectrometry.

Table S 7-4: Comparison previously published potential hitchhiker and identified hitchhiker in our in-house mAb.

HCP ID	MW	pI	host-orga-nism	hitch-hiker in-house mAb	previously published by	info
14-3-3 protein epsilon~	7.89	6.52	CHO	yes	~Aboulaich et al. 2014	
40S ribosomal protein S6^	174.48	10.83	CHO	no	^Migani et al. 2017	
40S ribosomal protein S15a~	14.83	10.13	CHO	no	~Aboulaich et al. 2014	
40S ribosomal protein S17~	15.51	9.85	CHO	no	~Aboulaich et al. 2014	
40S ribosomal protein S20~	15.21	9.94	CHO	yes	~Aboulaich et al. 2014	
40S ribosomal protein S21~	9.14	8.32	CHO	yes	~Aboulaich et al. 2014	
40S ribosomal protein SA~	19.72	9.29	CHO	yes	~Aboulaich et al. 2014	
60S acidic ribosomal protein P0~.*	29.87	8.51	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al.	
60S acidic ribosomal protein P2~	11.67	4.54	CHO	no	~Aboulaich et al. 2014	
60S ribosomal protein L12~	14.69	9.55	CHO	no	~Aboulaich et al. 2014	
60S ribosomal protein L23a~	23.11	10.45	CHO	no	~Aboulaich et al. 2014	
78 kDa glucose-regulated protein~.*.†.‡.‴	72.33	5.16	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al. / ‡Levy et al. /‴Liu et al. 2019	subcellular location: cytoplasm, endo-plasmic reticulum lumen, melano-some
Acid trehalase-like protein 1~	29.46	5.62	CHO	no	~Aboulaich et al. 2014	
Actin. alpha cardiac muscle 1~.*.†.‡	41.99	5.39	CHO	no	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al. / ‡Levy et al.	

Actin. cytoplasmic 1~.*.†.‡.‴	41.71	5.39	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al. / ‡Levy et al. /‴Liu et al. 2019	subcellular location: cytoplasm, cytoskeleton
Alpha-enolase~.*.†.‡	46.67	6.16	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al. / ‡Levy et al.	
Annexin A2~	27.02	5.97	CHO	no	~Aboulaich et al. 2014	
Aspartyl aminopeptidase~	51.91	7.12	CHO	no	~Aboulaich et al. 2014	
ATP-dependent RNA helicase DDX39‴	73.21	6.73	CHO	no	‴Liu et al. 2019	subcellular location: cytoplasm, nucleus
Biglycan~	41.61	7.31	CHO	no	~Aboulaich et al. 2014	
Catalase‴	63.10	8.29	CHO	yes	‴Liu et al. 2019	subcellular location: peroxisome
Cathepsin B~	37.48	6.13	CHO	no	~Aboulaich et al. 2014	potential risk to cause enzymatic degradation
Cathepsin D[i] ¨ º	44.11	6.54	CHO	no	[i]Bee et al. 2015 /¨Gao et al. 2011 / ºRobert et al. 2009	polysorbate degradation
Clusterin~.*.†.‡.ª.¯	51.72	5.74	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al. / ‡Levy et al./ ªWilson MR et al. 1992 / ¯Singh et al. 2019	
Cofilin-1~.†	18.52	8.09	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al.	

Cullin-associated NEDD8-dissociated protein 1~	133.54	5.74	CHO	no	~Aboulaich et al. 2014	
Dystroglycan^	96.90	8.72	CHO	no	^Migani et al. 2017	a complex involved in numerous processes such as cell survival and migration and membrane assembly
Elongation factor 2~.*.†	95.26	6.83	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al.	
Eukaryotic translation initiation factor 2 subunit 1~	36.13	5.14	CHO	yes	~Aboulaich et al. 2014	
Galectin-3~	32.42	7.37	CHO	yes	~Aboulaich et al. 2014	
Glutathione S-transferase P 1~.*	23.62	7.80	CHO	no	~Aboulaich et al. 2014 / *Zhang et al.	
Glyceraldehyde-3-phosphate dehydrogenase~.*.†.‴	35.73	8.34	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al. /‴Liu et al. 2019	subcellular location: cytoplasm, cytosol, nucleus, perinuclear region, membrane, cytoskeleton
GTP-binding nuclear protein Ran~	24.47	8.34	CHO	yes	~Aboulaich et al. 2014	
Guanine nucleotide-binding protein beta-2-like 1~	30.44	7.39	CHO	no	~Aboulaich et al. 2014	
Heat shock cognate 71 kDa protein~.†.‡	70.76	5.36	CHO	no	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
Histidine-tRNA ligase~	57.39	6.95	CHO	no	~Aboulaich et al. 2014	

Histidyl-tRNA synthetase. cytoplasmic~	40.67	5.08	CHO	no	~Aboulaich et al. 2014	
Histone H2A type 1~	28.45	11.03	CHO	no	~Aboulaich et al. 2014	
Histone H2A type 4~	14.24	11.02	CHO	no	~Aboulaich et al. 2014	
Histone H2B type 1-N~	13.93	10.32	CHO	no	~Aboulaich et al. 2014	
Insulin-like growth factor-binding protein 3¯	31.47	8.83	CHO	no	¯Singh et al. 2019	
kinase cAMP-dependent protein kinase catalytic subunit gamma^	46.13	8.70	CHO	no	^Migani et al. 2017	
Lactadherin~	16.14	9.41	CHO	no	~Aboulaich et al. 2014	
L-lactate dehydrogenase A chainv	36.50	7.42	CHO	no	~Aboulaich et al. 2014	
Lipoprotein Lipase¯	54.58	7.94	CHO	yes	¯Singh et al. 2019	
Matrix metalloproteinase-19~	58.90	7.88	CHO	no	~Aboulaich et al. 2014	potential risk to cause enzymatic degradation
Metalloproteinase inhibitor 1~. ‡.¯	22.39	8.47	CHO	no	~Aboulaich et al. 2014 / ‡Levy et al. / ¯Singh et al. 2019	
Neuraminidase^	41.96	6.82	CHO	no	^Migani et al. 2017	the protease involved in cleaving glycosidic linkages in neuraminic acids
Nidogen-1~.†.‡.¯	30.07	8.07	CHO	no	Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al./ ¯Singh et al. 2019	
Nucleoside diphosphate kinase A	17.18	6.33	CHO	no	~Aboulaich et al. 2014	

Nucleoside diphosphate kinase B~	17.33	7.99	CHO	no	~Aboulaich et al. 2014	
Out at first protein-like~	17.72	8.06	CHO	no	~Aboulaich et al. 2014	
Peptidyl-prolyl cis-trans isomerase A ~.*	17.89	8.28	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al.	
Peroxiredoxin-1~.*.†.‡	22.25	8.05	CHO	yes	~Aboulaich et al. 2014 / *Zhang et al. / †Doneanu et al. / ‡Levy et al.	
Peroxiredoxin-2‴	21.81	5.35	CHO	yes	‴Liu et al. 2019	
Phosphoglycerate mutase 1~.*	20.19	8.12	CHO	no	~Aboulaich et al. 2014 / *Zhang et al.	subcellular location: cytoplasm
Phosphotyrosine protein phosphatase~	17.90	6.01	CHO	no	~Aboulaich et al. 2014	
Plectin-1~	83.37	8.54	CHO	no	~Aboulaich et al. 2014	
procollagen C-endopeptidase enhancer 1^	55.23	8.63	CHO	yes	^Migani et al. 2017	involved in cartilage and bone formation
Proteasome subunit alpha type-7~	23.76	8.48	CHO	yes	~Aboulaich et al. 2014	
Protein disulfide-isomerase A6~	28.38	4.91	CHO	no	~Aboulaich et al. 2014	potential risk to cause enzymatic degradation
Protein-glutamine gamma-glutamyl transferase E‴	75.81	6.33	CHO	no	‴Liu et al. 2019	subcellular location: extracellular, plasma membrane, cytosol
Protein S100-A6~	10.00	5.48	CHO	no	~Aboulaich et al. 2014	
Putative phospholipase B-like 2~.ª	65.50	6.28	CHO	no	~Aboulaich et al. 2014 / ªVanderlaan et al. 2015	

Pyruvate kinase isozymes M1/M2~.†.‡	51.53	7.68	CHO	yes (like-protein)	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
Septin-2~	37.05	6.18	CHO	yes	~Aboulaich et al. 2014	
Serine protease HTRA1~.†.‡	28.70	7.03	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	potential risk to cause enzymatic degradation
Serpin H1~	46.53	8.68	CHO	no	~Aboulaich et al. 2014	
SPARC¯	73.12	4.53	CHO	no	¯Singh et al. 2019	
Sulfated glycoprotein 1~.†.‡	27.36	5.49	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
T-complex protein 1 subunit alpha~.†.‡	60.30	5.99	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
T-complex protein 1 subunit beta~.†.‡	57.45	6.46	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
T-complex protein 1 subunit delta~.†.‡	42.11	8.27	CHO	no	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
T-complex protein 1 subunit eta~.†.‡	54.87	7.42	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
T-complex protein 1 subunit gamma~.†.‡	60.58	6.64	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
T-complex protein 1 subunit theta~.†	22.21	4.91	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al.	

T-complex protein 1 subunit zeta~.†.‡	57.94	6.90	CHO	yes	~Aboulaich et al. 2014 / †Doneanu et al. / ‡Levy et al.	
Transforming Growth Factor- b1 (TGF-b1)°	46.88	7.90	CHO	no	°Beatson et al. 2011	
Triosephosphate isomerase~	16.42	8.19	CHO	yes	~Aboulaich et al. 2014	
Ubiquitin‴	17.28	6.75	CHO	no	‴Liu et al. 2019	subcellular location: cytoplasm, nucleus
Vimentin~.‡	44.50	4.81	CHO	yes	~Aboulaich et al. 2014 / ‡Levy et al.	
V-type proton ATPase subunit C 1~.‡	43.89	7.46	CHO	no	~Aboulaich et al. 2014 / ‡Levy et al.	

Abbreviations: mAb, monoclonal antibody; CHO, Chinese hamster ovary ; HCP, host cell protein; ID, identity; ; MW, molecular weight, pI; isoelectric point.

7.3 Supporting information chapter 4

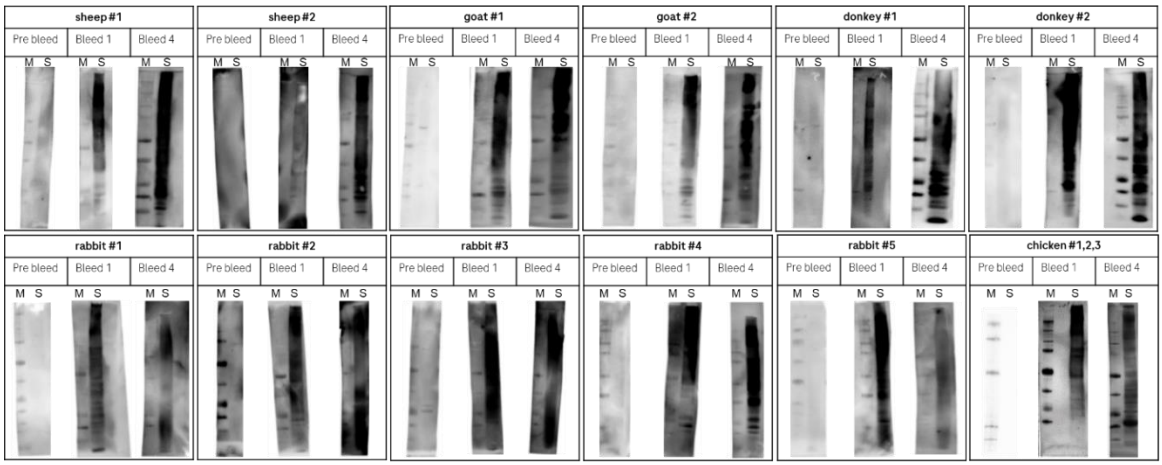


Figure S 7-4: Western blots of the pre immunization and two test bleeds. *10 µg reduced CHO-HCP ELISA standard were applied onto each lane. As primary antibodies, the blood sera or purified chicken IgY samples were diluted 1:3 in ECL prime blocking solution. The secondary antibodies were 1:1000 diluted anti-host-species (HRP-labeled) antibodies. ECL detection was applied for visualization of the protein bands. Each blot contains two lanes, the molecular weight marker (M) and sample (S), respectively. Abbreviations: CHO-HCP, Chinese hamster ovary-host cell protein; ELISA, enzyme linked immunosorbent assay; IgY, Immunoglobulin Y, ECL, enhanced chemiluminescence; HRP, horseradish peroxidase.*

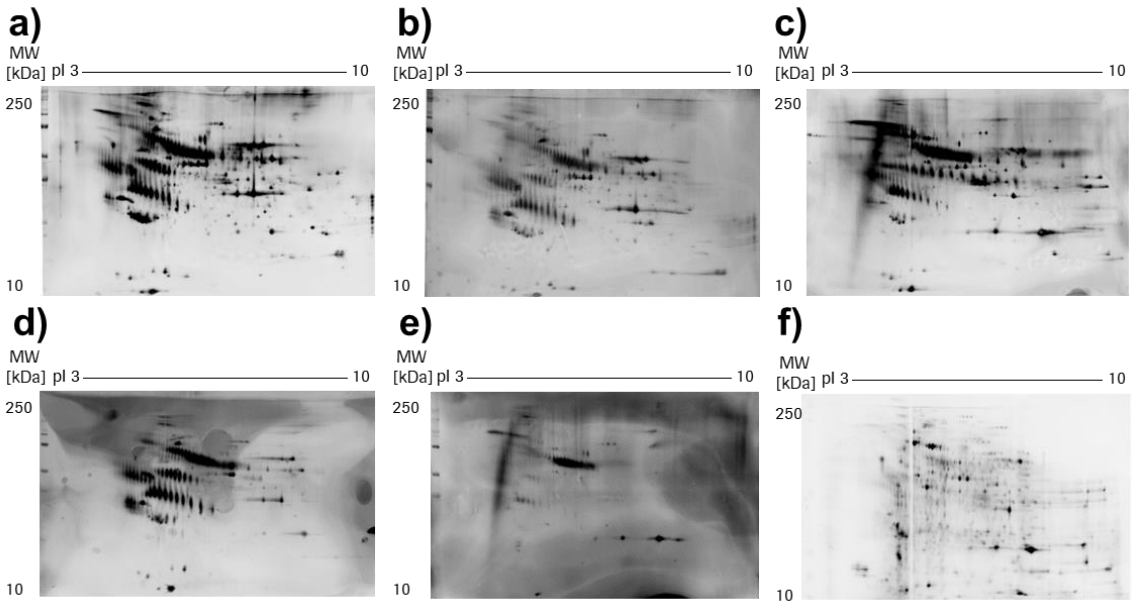
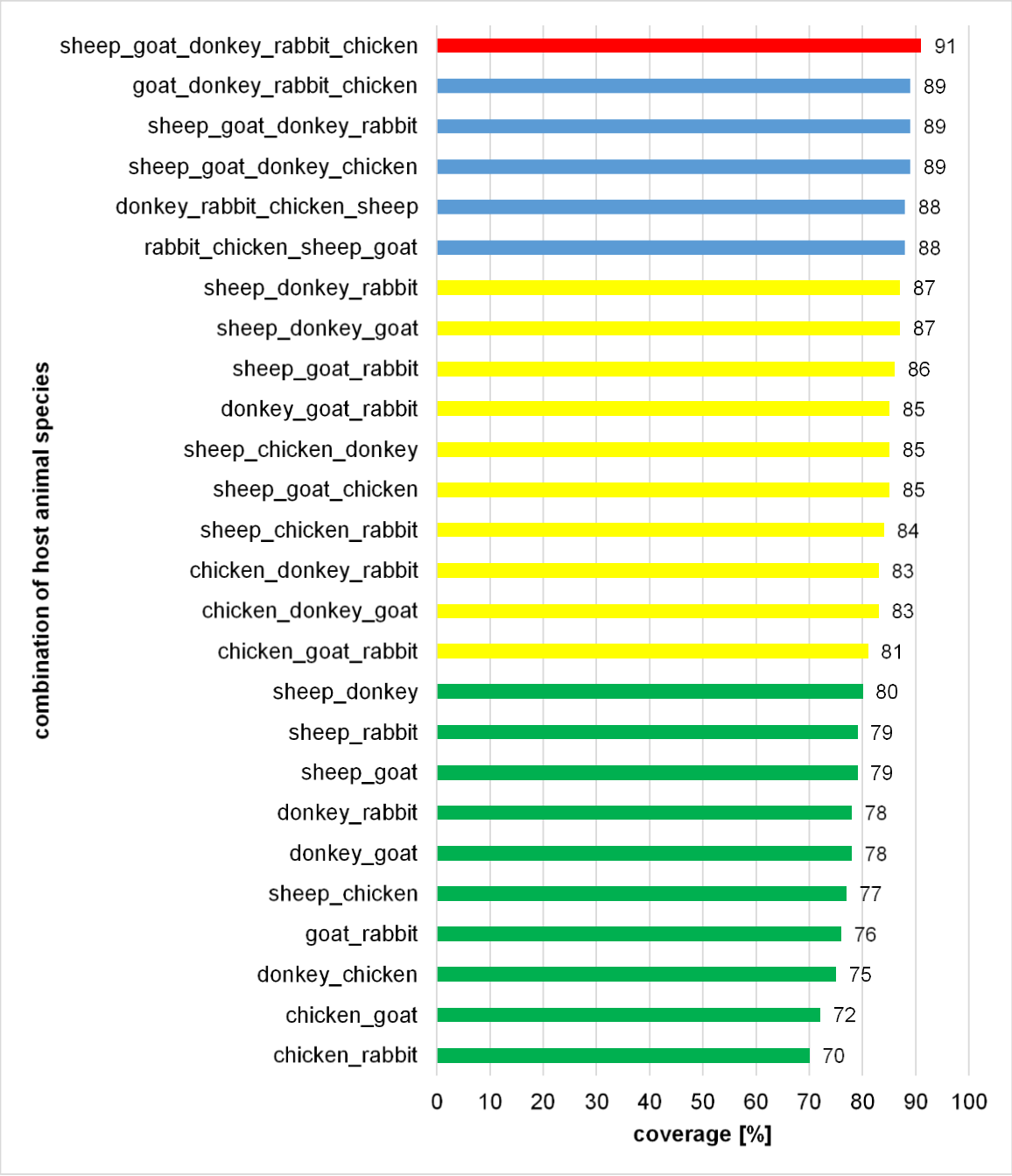


Figure S 7-5: 2D-WB of crude antisera pools of each host animal species*; a) sheep, b) donkey, c) goat, d) rabbit, e) chicken and f) 2D gel. The Cy-labeled CHO-HCP ELISA standard was separated by 2D-PAGE and transferred onto a membrane. The 2D-WBs were immunostained by using crude antisera pools of the respective animal species as primary antibody, the respective anti-species (H+L) HRP-conjugated antibodies as secondary antibody and Streptavidin-POD conjugate. Lumi-Light substrate solution was applied for ECL protein detection with an Octoplus QPLEX camera system. Abbreviations: CHO, Chinese hamster ovary ; HCP, host cell protein; ELISA, enzyme linked immunosorbent assay; ID, identity; MW, molecular weight, pI; isoelectric point; 2D, two-dimensional, PAGE, polyacrylamide gel electrophoresis, 2D-WB, two-dimensional western blot, HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; POD, peroxidase.*

a)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rabbit_chicken_sheep_goat 88
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sheep_goat 79
donkey_rabbit 78
donkey_goat 78
sheep_chicken 77
goat_rabbit 76
donkey_chicken 75
chicken_goat 72
chicken_rabbit 70
combination of host animal species
0 10 20 30 40 50 60 70 80 90 100
coverage [%]

b)

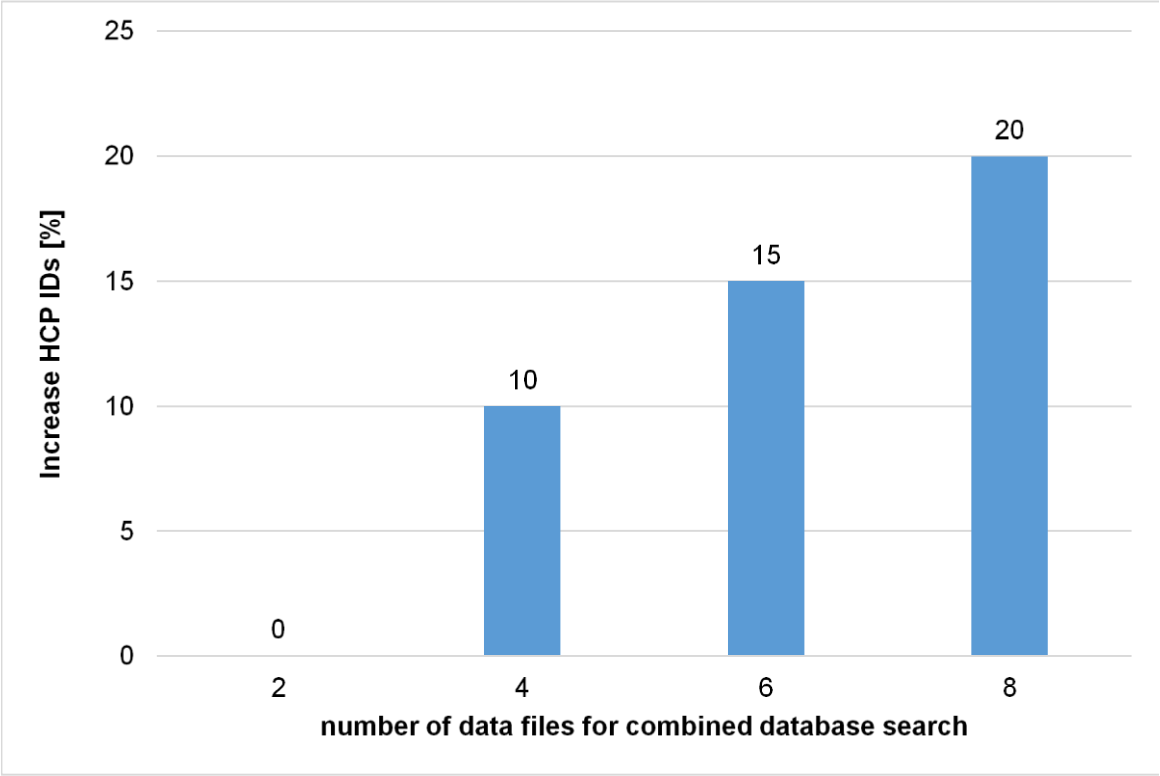

Figure S 7-6: a) Theoretical AP-MS analysis for the combination of the investigated host animal species. *Covered HCPs for the pAbs of each species were determined by performing the AP-MS method with the HCP-ELISA standard in duplicates. Database searches were performed by merging the results of each possible combination containing two to five species using a combined search in ProteinPilot, where always two data files per species were used. The coverage increases with each additional species and consequently the combination of five species reaches the highest coverage. However, the increase in coverage plateaued with increasing numbers of species. As the results of the theoretical AP-MS could possibly be biased by the number of files combined in database search, this effect was tested by combining increasing numbers of data files derived from different LC-MS/MS sequences of the same HCP standard sample (b). For example, the combination of two species measured in duplicates amounts to a total of four combined data files. Indeed, the number of identified HCPs increases with the number of combined files, which can be attributed to the run-to-run variability of the LC-MS/MS method, especially for low abundant HCPs. To sum it up, the positive effect on coverage due to the combination of more species appears larger based on numerical increase than it actually is. Abbreviation: pAbs, polyclonal antibodies; HCP, host cell protein; ELISA, enzyme linked immunosorbent assay; ID, identity; AP-MS, affinity purification based mass spectrometry; LC-MS/MS, Liquid chromatography-tandem mass spectrometry.*

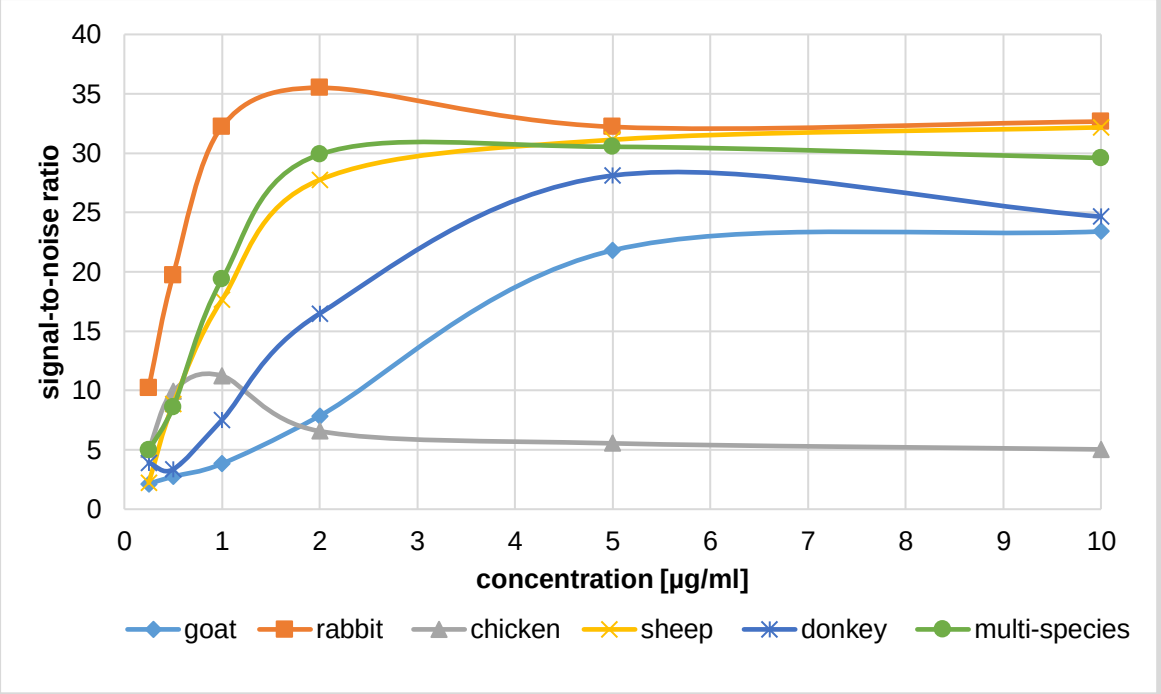

Figure S 7-7: Example for method optimization performing titration of capture pAbs. *Several concentrations of the capture pAbs ranging from 0.25 µg/ml to 10 µg/ ml were tested, while the other ELISA components (detection pAbs, SA-POD) remained constant. The concentration exhibiting the maximum signal-to-noise ratio was selected for each animal species to be used in the final ELISA. The optimal concentration for the detection antibodies was determined analogously. For the SA-POD conjugate, the concentration allowing the readout within an appropriate time span (ideally 5-10 min) after addition of the ABTS substrate, which not necessarily corresponds to the concentration achieving maximum signal-to-noise value, was chosen. Signal-to-noise ratios were in the same range for all tested ELISA components and chicken ELISA exhibited always the lowest signal-to-noise ratio. Abbreviation: pAbs, polyclonal antibodies; HCP, host cell protein; ELISA, enzyme linked immunosorbent assay; SA-POD, Streptavidin-peroxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).*

Table S 7-5: Number of HCPs uniquely covered by each of the host animal species. *Covered CHO-HCPs were derived from duplicate measurements using the optimized AP-MS method. All HCPs from the CHO-HCP ELISA standard identified after affinity purification were counted as covered by the pAbs from the specific species. Only the HCPs found in both samples of the duplicate measurements were scored. Each animal species exhibited solely covered HCPs, with rabbit having the most. None of the solely covered HCPs is a published high risk or hitchhiker HCP (Jones et al., 2021; Seisenberger et al., 2022). Abbreviation: CHO, Chinese hamster ovary; HCP, host cell protein; ELISA, enzyme linked immunosorbent assay; AP-MS, affinity purification based mass spectrometry; ID, identity.*

Animal species	Number of solely covered HCP IDs (identified in each of the duplicate samples)
Chicken	1
Sheep	3
Goat	5
Donkey	5
Rabbit	19

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