Pseudo-affinity capture of K. phaffii host cell proteins in flow-through mode: Purification of protein therapeutics and proteomic study

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Abstract. *K. phaffii* is a versatile expression system that is increasingly utilized to produce biological therapeutics – including enzymes, engineered antibodies, and gene-editing tools – that feature multiple subunits and complex post-translational modifications. Two major roadblocks limit the adoption of *K. phaffii* in industrial biomanufacturing: its proteome, while known, has not been linked to downstream process operations and detailed knowledge is missing on problematic host cell proteins (HCPs) that endanger patient safety or product stability. Furthermore, the purification toolbox has not evolved beyond the capture of monospecific antibodies, and few solutions are available for engineered antibody fragments and other protein therapeutics. To unlock the potential of yeast-based biopharmaceutical manufacturing, this study presents the development and performance validation of a novel adsorbent – PichiaGuard – functionalized with peptide ligands that target the whole spectrum of *K. phaffii* HCPs and designed for protein purification in flow-through mode. The PichiaGuard adsorbent features high HCP binding capacity (~25 g per liter of resin) and successfully purified a monoclonal antibody and an ScFv fragment from clarified *K. phaffii* harvests, affording >300-fold removal of HCPs and high product yields (70 – 80%). Notably, PichiaGuard outperformed commercial ion exchange and mixed-mode resins without salt gradients or optimization in removing high-risk HCPs – including aspartic proteases, ribosomal subunits, and other peptidases – thus demonstrating its value in modern biopharmaceutical processing.

Keywords. *Komagataella phaffii*, chromatography, protein purification, monoclonal antibodies, antibody fragment, proteomics.

1. Introduction. Strain engineering of the *Pichia* genus and similar yeasts has been pursued for more than two decades – recently, as alternative hosts to mammalian cell lines for biomanufacturing due to benefits in enabling sustainability [1,2] – by introducing plasmid designs, promoters, and signal peptides that improve productivity and fidelity. In particular, the species *Komagataella phaffii* (*K. phaffii,* synonymous to *Pichia pastoris*) is a methylotrophic yeast that is currently utilized to express a variety of therapeutic proteins including monoclonal antibodies – either whole and engineered fragments – as well as fusion proteins, virus-like-particles, etc. [3–12]. These efforts have been in concert with systems biology to better understand *K. phaffii*'s genetic repertoire as well as the temporal evolution of mRNA and host cell proteins (HCPs) across the various phases of cell growth under different conditions [7,13–20]. A recent SWOT analysis on the adoption of *K. phaffii* as a platform for protein production highlighted the need to intensify efforts towards improving secretion rates and reducing protease-mediated product degradation [21]. *Biopharmaceutical benchmarks* tracked the growth in the use of *K. phaffii* from its early days [22] to its current adoption in producing five clinically approved products [23]. While capable of both intra- and extra-cellular protein production, *K. phaffii* has been noted for its innately slow secretory trafficking [24,25]. Secretion rates can be increased by tuning gene regulation [26,27] or developing strains with lower proteases content [28], although these modifications can impact cell growth rates and viability [29]. Therefore, strain engineering and cultivation must be adjuvated by purification technologies capable of removing *K. phaffii* HCPs with enzymatic and/or immunogenic activity as early as possible in the manufacturing pipeline [29].

Current biotherapeutic formulations contain a final average of ~20 ng HCP per mg drug product [30,31]. Although *K. phaffii* naturally produces fewer HCPs than its mammalian counterparts, failure to reduce their titer below the prescribed limit puts at risk both process yield and product stability, and thus patient safety. Decades of production of therapeutic monoclonal antibodies (mAbs) in Chinese hamster ovary (CHO) cells has pointed at the presence of HCPs that combine an inherent toxic or immunogenic activity with the ability to escape purification by coeluting with the mAb product. Recent research has assessed the harmful potential of HCPs secreted by non-mammalian cell lines and microbial strains employed – of forthcoming – in biopharmaceutical manufacturing [32,33]. However, the identity and threshold values of potentially detrimental *K. phaffii* HCPs, and their ability to evade clearance by current chromatographic technology have been minimally investigated [34]. For example, variants of *K. phaffii* have been engineered with higher Kex2 expression [35] or the concomitant expression of isomerases and proteases to improve secretion; the CHO counterparts of these HCPs, however, are known as immunogenic and difficult-to-remove, while other evidence points to the interference of similar HCPs with downstream processing [26,27,36,37].

Current processes for the purification of *K. phaffii*-secreted proteins rely on a train of operations including cell separation, multiple chromatographic steps, final concentration or filtration [37–40]. An initial ultrafiltration/diafiltration step is often included to adjust buffer conditions due to the methanolic growth conditions as well as pH adjustment or product concentration [31,41–43]. As in the CHO pipeline, the chromatographic segment typically comprises an affinity-based capture step followed by polishing via ion exchange and hydrophobic interaction [36,44–48] (the purification processes of clinically approved products expressed by *K. phaffii* are outlined in **Table S1**). As the cells are harvested at high density, a liter of culture fluid may contain up to few grams of HCPs [49], whose distribution of physicochemical and biochemical properties can vary with product identity and culture conditions. Such process- and product-dependent diversity of the *K. phaffii* secretome, combined with the limited knowledge of its impact on purification [29,37] and the lack of affinity technology dedicated to the removal of *K. phaffii* HCPs, both may increase the number of purification/polishing steps in the downstream train, and increases the risk of contaminants present in the final product.

Developing a true platform process, capable of sustaining the diversity in titer and biomolecular make-up of industrial feedstocks, can benefit significantly by adopting resins with affinity-driven capture of HCPs and host cell DNA (hcDNA). In prior work, we introduced the paradigm of "flow-through affinity chromatography" and developed an adsorbent – named LigaGuard™ [50] – functionalized with peptide ligands designed to target the CHO proteome spectrum. Our team demonstrated LigaGuard™ performance by purifying therapeutic mAbs from an ensemble of seven CHO harvests: when utilized alone, LigaGuard™ afforded a logarithmic HCPs removal value (HCP LRV) ranging between 1.5-2.5; when coupled with an affinity resin operated in bind-and-elute mode, the two-step process afforded cumulative HCP and hcDNA LRVs ~ 4, corresponding to a final HCP level of 8 ppm. Additionally, LigaGuard™ managed to clear high-risk and hard-to-remove HCPs – such as Cathepsins and Phospholipases – thus demonstrating its potential to de-risk and simplify the chromatographic pipeline of protein purification.

In this study, we sought to expand our efforts towards yeast expression systems, with *K. phaffii* as the model organism. To expand our bioprocess-relevant understanding of *K. phaffii* HCPs, we conducted a proteomics survey of their cultivation harvests to identify species that can cause product degradation via proteolysis or instability via excipient degradation, or present the potential to trigger an immunogenic response in patients. While such data has previously been curated for other commonly used cell lines used for therapeutic production [51,52], we utilized this list pertaining to *K. phaffii* as a guidance to validate the development of a novel purification strategy (described below).

We present the development of a pseudo-affinity adsorbent – PichiaGuard – for removing *K. phaffii* HCPs and hcDNA. We leveraged the knowledge of HCP physicochemical properties to design and screen a peptide library against the *K. phaffii* secretome to identify HCP-targeting peptides under competing conditions with a model product (herein, polyclonal IgG). Following the identification of the peptide ligands and the formulation of PichiaGuard, we evaluated its global HCP binding capacity and conducted proteomics analyses of the flow-through fractions to evaluate the removal of individual HR-HCPs. We finally demonstrated the application of PichiaGuard by purifying ScFv and a full mAb in flow-through mode from clarified *K. phaffii* harvests. Globally, our results indicate that PichiaGuard outperforms state-of-the-art chromatographic resins in terms of both capture of *K. phaffii* HCPs – notably, among them, aspartic proteases, peptidases, ribosomal subunit proteins, heat shock proteins etc. – demonstrating potential for the purification of high-value biotherapeutics produced by yeast hosts.

2. Materials and Methods

2.1. Materials. Fmoc-protected amino acids Fmoc-Gly-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Phe-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Trp(Boc)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Leu-OH, the coupling agent azabenzotriazole tetramethyl uronium hexafluorophosphate (HATU), diisopropylethylamine (DIPEA), piperidine, and trifluoroacetic acid (TFA) were sourced from ChemImpex International (Wood Dale, IL, USA). The Toyopearl AF-Amino-650M (TP-650M) and HW-50F (TP-50F) resins were obtained from Tosoh Bioscience (Tokyo, Japan). Ammonium bicarbonate, phosphate buffered saline at pH 7.4, sodium deoxycholate, iodoacetamide (IAA), calcium chloride ($CaCl_2$), triisopropylsilane (TIPS), 1,2-ethanedithiol (EDT), anisole, Zwittergent 3-16, Protease Inhibitor Cocktail, Kaiser test kits, and 3 kDa and 10 kDa MWCO centrifugal filters were from Millipore Sigma (St. Louis, MO, USA). N,N'-dimethylformamide (DMF), dichloromethane (DCM), methanol, N-methyl-2-pyrrolidone (NMP), sodium phosphate (monobasic), sodium phosphate (dibasic), hydrochloric acid, sodium acetate, glacial acetic acid, formic acid, Tris-HCl at pH 7.5 and 8, trypsin (100 µg), Dithiothreitol (DTT), 660 nm Histidine-rich protein quantification and bicinchoninic acid (BCA) assay reagents were obtained from Fisher Chemicals (Hampton, NH, USA). Human IgG was obtained from Athens Bio (Atlanta, GA). Vici Jour PEEK chromatography columns (2.1 mm ID, 30 mm length, 0.1 mL volume) and polyethylene frits were obtained from VWR international (Radnor, PA, USA). The 10-20% Tris-Glycine HCl SDS-PAGE gels, urea, and Coomassie blue stain were purchased from Bio-Rad Life Sciences (Carlsbad, CA, USA). Alexa Fluor 488 and 594 dyes, and Pierce dye removal columns were obtained from Thermo Fisher Scientific. HiPrep Desalting 26/10 column and Sepharose CM ion exchange resin were obtained from Cytiva.

2.2. Proteomic analysis. A label-free relative quantification workflow was developed to analyze the *K. phaffii* secretome using a bottom-up approach. Filter-aided sample preparation (FASP) [53] was performed using 30 kDa MWCO filtration units passivated with 20 µL of 0.1 M Tris HCl at pH 8. A BCA assay was initially conducted to determine the total protein titer in each 200 µL sample. Addition of 15 µL of 50 mM dithiothreitol or DTT (in 0.1 M Tris HCl at pH 8) to each sample - with incubation for 30 minutes at 56°C - served to cleave disulfide bonds in the proteins. This was followed by further protein denaturation by adding 200 µL of 8 M urea (in 0.1 M Tris HCl at pH 8) to each sample, with vortex and spinning down. Each sample was then transferred onto a separate, passivated, 30 kDa MWCO filtration unit for FASP, and concentrated by centrifugation at 12,000g for 15 minutes at 21°C; the flow-through was discarded. An additional 200 µL of 8 M urea was added to each filtration unit, and immediately followed by the addition of 64 µL of 200 mM iodoacetamide or IAA (in 8 M urea), resulting in a total solution volume of 264 µL and IAA concentration of 50 mM. Incubation for 60 minutes at room temperature in the dark ensured the alkylation of free sulfhydryl groups of cysteine residues displayed by the HCPs. This was followed by centrifugation at 12,000g for 15 minutes at 21°C. Subsequent washing steps were performed involving multiple rounds of centrifugation (12,000g for 15 minutes at 21°C) and resuspension into Tris-based buffers (3 rounds of 2 M Urea and 10 mM $CaCl_2$ in 0.1 M Tris HCl at pH 8 and 3 rounds in digestion buffer 0.1 M Tris HCl pH 7.5) before the addition of trypsin to the MWCO filters (1:50 enzyme/protein ratio – based on BCA assay results – in 0.1 M Tris HCl at pH 7.5) for overnight proteolytic digestion. All flow-through solutions up to this point were discarded.

The overnight digestion was disrupted using a solution of 0.001% v/v Zwittergent in aqueous 1% v/v formic acid (quench buffer). This quench buffer was added in steps (with centrifugation at 12,000g for 15 minutes at 21°C in between steps, combining and collecting the flow-through solutions from each sample) up to a total volume of 450 µL of tryptic peptide solution per sample. All samples were then lyophilized and stored at -20°C until nanoLC-MS/MS. Upon reconstitution in 200 µL of Mobile Phase A (MPA: 98% v/v water, 1.9% v/v acetonitrile, 0.1% v/v formic acid), reverse-phase separation of the tryptic peptide fragments was carried out using a PepMap 100 C18 trap column (3 µm particle size, 75 µm ID, 20 mm length) in series with an EASY-Spray C18 analytical column (2 µm particle size, 75 µm ID, 250 mm length) - in a 'trap-and-elute' configuration - installed on an EASY nanoLC-1200 instrument (Thermo Scientific, San Jose, CA) interfaced with an Orbitrap-Exploris-480 (Thermo Scientific, Bremen, Germany). The flowrate was maintained at 300 nL/min. Peptides were eluted using a 105 minutes solvent gradient, ramping from 5% to 25% Mobile Phase B (MPB: 80% v/v acetonitrile, 19.9% v/v water, 0.1% v/v formic acid) over 75 min, followed by another ramp to 40% MPB over 10 min, and then a steep ramp to 95% MPB in 1 min, at which point MPB was maintained at 95% for 17 minutes for column washing. Eluted tryptic peptides were ionized by subjecting them to 1.9 kV in the ion source for electrospray ionization. The ion transfer tube temperature was maintained at 275°C. The peptides were interrogated by full MS scan and data-dependent acquisition (DDA) MS/MS. DDA was performed as a 105-minute nanoLC-MS/MS method. Scan parameters for full MS were: 120,000 resolution, RF Lens of 40%, maximum injection time of 120 msec, scan range of 375-1,600 m/z, with normalized AGC setting at 300%; dynamic exclusion (with 20 sec exclusion duration) was used to minimize re-interrogation of pre-sampled precursors; cycle time was set at 1.5 sec; charge states 2-6 were included in the analysis; and the intensity threshold of $1.5\ 10^4$ was set for ddMS^2. Scan parameters for ddMS^2 were: 15,000 resolution, inclusion window of 1.5 m/z, maximum injection time of 21 msec, HCD collision energy of 30% (fixed), with normalized AGC setting at 100%. Quality control in nanoLC-MS/MS analyses involved blank runs, as well as standard BSA digest and standard HeLa digest runs at regular intervals within the length of the sample queue.

Post-acquisition data analysis and automated LFQ was performed by Proteome Discoverer v. 2.4 (PD, Thermo Scientific, San Jose, CA) with appropriate grouping variables. The raw nanoLC-MS/MS files were interrogated against the *Pichia pastoris* / *Komagataella phaffii* protein database (4,983 sequences) downloaded from UniProt. The data was also searched against a contaminants database (69 sequences) to identify potential contaminants during the experiments. These databases were searched with the following parameters: trypsin (full) as the digesting enzyme, a maximum of 3 missed trypsin cleavage sites allowed, 5 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, dynamic modifications on N-terminus (acetyl), methionine (oxidation, met-loss+acetyl, and met-loss), and static carbamidomethyl modifications on cysteine residues. The SEQUEST HT algorithm was employed in data interrogation: the algorithm compares the observed peptide MS/MS spectra and theoretically derives spectra from the target database(s) to assign appropriate quality scores. These scores and other important predictors are combined in the algorithm, which assigns a composite score to each peptide. The overall confidence in protein identification is increased with increasing number of distinct amino acid sequences identified. Therefore, proteins are normally categorized into a different priority group depending on whether they have only one or multiple unique tryptic peptide sequences of the required peptide identification confidence. Percolator peptide validation was based on the q-value (adjusted p-value) and minimal false discovery rate (FDR) < 0.01 was selected as a condition for successful tryptic peptide assignments.

2.3. Design of K. phaffii strain X-33 producing ScFv13R4. The DNA sequence encoding the ScFv13R4 protein [54] was first codon-optimized for efficient expression in *Pichia pastoris* wild type X-33 (Life Technologies Ltd, Grand Island, NY). The gene was amplified via PCR using primers scFv13R4 αF1 (GCATGAATTCATGGCAGAAGTCCAATTAG) and scFv13R4 αR1 (GAATGCGGCCGCTCATAA-GACTGCCAACTTAGTACC) carrying the extra restriction sites EcoRI and NotI (New England Biolabs, Beverly, MA), respectively. The PCR product was cloned in the vector pPICZα-A (Life Technologies Ltd, Grand Island, NY) at EcoRI and NotI sites generating a fusion protein ScFv13R4 to both His and C-myc tags present on the expression vector. After electroporation in *E. coli* Stbl4 and selection on LB agar plates supplemented with zeocin at 25 µg/mL, positive recombinant plasmids were identified using colony PCR. One recombinant plasmid was linearized with the restriction enzyme SacI and introduced into *Pichia pastoris* X-33 via electroporation. Selection was performed on YPD plates supplemented with zeocin at a concentration of 1000 µg/mL and positive clones were screened with colony PCR.

2.4. Cultivation of K. phaffii WT (null-X-33) and ScFv13R4 cultures. Overnight culture was initiated from a single colony in 3 mL of YPD medium and incubated at 30°C under agitation at 350 rpm. A volume of 1 mL of this culture was used as the inoculum to initiate a new 50 mL culture in YPD medium, which was grown at the same conditions for 24 hours. In order to adapt the cells to the production medium, cells were centrifuged at 3000g for 10 minutes and resuspended in 50 mL of BFM21 media containing glycerol at 40 g/L and supplemented with trace elements. This media was developed and optimized [55] as a part of BTEC's training and education program; its full composition is provided in **Table S4**. Cell growth was allowed to proceed for 24 hours at 30°C under agitation at 350 rpm. This culture was in turn used to inoculate a 1-L working volume bioreactor containing BFM21 supplemented with PTM4 trace metal solution (12 ml/L) medium and glycerol (40 g/L) starting at low optical density (OD_{600nm} ~ 2). Fermentation was conducted in multiple bioreactors using the following parameters: pH controlled at 5.0 using 28% v/v ammonium hydroxide and 80% v/v phosphoric acid; temperature set point at 30°C; and dissolved oxygen maintained at 30% in cascade with agitation. The initial glycerol was exhausted within 24 hours of fermentation, after which a glycerol feed phase was initiated, and the fermentation was continued until the fluid reached an OD_{600nm} ~ 100. At this point, the reactor was harvested and centrifuged at 4000g for 30 minutes to remove the wet cell mass, while ensuring minimal cellular rupture. For ScFv13R4 production, a methanol feed phase was started in a bioreactor grown similarly, by adding it at a concentration of 0.1% v/v at 0.3 mL/min while maintaining the dissolved oxygen at 30%, to induce ScFv13R4 production. After 48 hours of fermentation in methanol and upon achieving a cell density of OD_{600nm} ~ 300, the cells were harvested via centrifugation at 4000g for 30 minutes. The supernatant was then filtered through a 0.45 µm filter before further use. For experiments with mAbs, a known concentration of human polyclonal IgG was spiked into the aforementioned null culture fluid to simulate real in-process CCF.

2.5. Peptide synthesis. A tetrameric one-bed one-peptide (OBOP) library in the format X_1-X_2-X_3-X_4-GSG was prepared on HMBA-ChemMatrix resin via direct solid-phase Fmoc/tBu peptide synthesis using a Syro I automated peptide synthesizer (Biotage, Uppsala, Sweden), as described in prior work [50,56]. The library was constructed using 10 of the 20 natural amino acids by selecting those capable of imparting the full range of interaction modes (*i.e.*, electrostatic, hydrophobic, polar, hydrogen bonding, etc.) to the 4-mer peptide ligands. The amination of Toyopearl HW-50F resin was performed by incubating 1 g of resin with 2 mmol of carbonyldiimidazole in 30 mL of acetone for 30 minutes at 25°C under mild agitation, followed by rinsing in acetone and incubation with 2 mmol of tris(2-aminoethyl) amine under the same conditions, to obtain an amine density of ~0.2 mmol per gram of resin. The peptide sequences RYWV-GSG, QEKK-GSG, VWHH-GSG, EWAK-GSG, RYWK-GSG, YHKH-GSG, RWYQ-GSG, and WYKK-GSG were conjugated on Toyopearl AF-Amino-650M (TP650M) and aminated HW-50F resins (TP50F) following the same protocol using an Alstra automated peptide synthesizer (Biotage, Uppsala, Sweden). Upon completing chain elongation, the peptides were deprotected using TIPS deprotection cocktail (95% v/v TFA, 2.5% v/v TIPS, 2.5% v/v water). Each sequence was conjugated to the Toyopearl beads at the loading of ~0.2 mmol of peptide per gram of resin. The PichiaGuard-TP650M and PichiaGuard-TP50F adsorbents were finally formulated by mixing the eight peptide-functionalized resins.

***2.6. Protein labeling and formulation of a screening mix*.** Human IgG and the HCPs contained in the *P. pastoris* (X-33 strain) cell culture fluid and were labeled using NHS ester AlexaFluor dyes AF488 (green) and AF594 (red), respectively. Specifically, each dye was initially dissolved in dry DMSO at the concentration of 10 mg/mL. Following diafiltration of the X-33 cell culture fluid into phosphate buffer saline at pH 7.4 using 3 kDa MWCO filters, 100 µL of *K. phaffii* HCPs at the concentration of 0.2 mg/mL were incubated with 1 µL of AF594; in parallel, 100 µL of a solution of IgG at 5 mg/mL in PBS at pH 7.4 was incubated with 1 µL of AF594. The labeling reactions were allowed to proceed for 3 hours at 25°C under mild agitation. Spin columns were used to remove the unreacted dyes. The labeled proteins were then mixed to obtain a screening mix comprising AF488-IgG at 2 mg/mL and AF594-HCPs at 0.1 mg/mL. A volume of 20 µL of OBOP library beads were then incubated with 1 mL of screening mix overnight at 4°C under mild agitation and washed with PBS before proceeding with the screening protocol.

***2.7. Library screening*.** The OBOP tetrameric library was screened against the screening mix (see *Section 2.6)* using a microfluidic device developed by our team [57]. Briefly, the microfluidic device comprises an imaging chamber which hosts individual library beads in rapid sequence and is installed in a DMi8 microscope (Leica, Wetzlar, Germany) equipped with an ORCA-Flash4.0 V3 Digital CMOS camera with W View Gemini image splitting optics (Hamamatsu, Shizuoka, Japan). A MATLAB® GUI developed to operate this screening system was implemented to visualize the library beads under the green (human IgG; excitation/emission: 488/496 nm) and red (*K. phaffii* HCPs; excitation/emission: 594/617 nm) channels. Library beads with green-only or green-and-red fluorescent emission were discarded, whereas beads with strong red-only fluorescent emission were collected as positive leads and analyzed via Edman Degradation using a PPSQ 33-A Protein Sequencer (Shimadzu, Kyoto, Japan) to identify the peptide sequences carried thereon.

2.8. Diafiltration of feedstocks. Prior to chromatographic purification, the clarified null X-33 (non-producing, for control studies) cell culture harvests were diafiltered to remove small media component, chiefly glycerol remnants and unreacted protease inhibitors, using a HiPrep™ 26/10 column (Cytiva, Marlborough, MA) into two different buffers, namely 20 mM sodium acetate at pH 5.7- and 25-mM sodium phosphate at pH 7.1. While these buffers were chosen to investigate the effects of pH on HCP capture (pH 5.7 to mimic the cell culture environment and pH 7.1 as a neutral control condition), the HCP titers were varied respectively between 0.3 mg/mL and 0.5 mg/mL in both the acetate-conditioned and phosphate-conditioned feedstocks to study in detail the purification performance of PichiaGuard resins.

2.9. Static and dynamic HCP binding capacity measurements. Static binding experiments were conducted by incubating aliquots of 30 µL of settled PichiaGuard-TP650M or PichiaGuard-TP50F resin in a 12-well plate (WP) with acetate-conditioned and phosphate-conditioned null X-33 feedstocks featuring HCP titers of 0 – 1 mg/mL for 3.5 hours at 25°C under mild agitation (300 rpm). The plate was then spun down at 3000 rpm for 10 minutes and the supernatants were collected and analyzed to determine the residual HCP concentration via BCA. Finally, the values of static binding capacity were calculated via mass balance. The dynamic binding capacity was measured via breakthrough experiments ($DBC_{20\%}$) by loading ~6 mL of acetate-conditioned or phosphate-conditioned X-33 feedstocks featuring a HCP titer of ~0.4 mg/mL onto 0.1 mL column packed with chromatographic resin – namely, PichiaGuard-TP650M and PichiaGuard-TP50F resin, Capto Q or CaptoAdhere (Cytiva), or CHO LigaGuard™ (LigaTrap Technologies, Raleigh, NC) – pre-equilibrated with the respective buffers – namely, 20 mM sodium acetate at pH 5.7 and 25 mM sodium phosphate at pH 7.1 – at a residence time (RT) of 1 minute until saturation was reached. The effluent was continuously monitored via spectrophotometry at 280 nm and apportioned in 0.5 mL fractions that were finally analyzed to determine the residual HCP titer via BCA.

2.10. Purification of ScFv and mAb via flow-through chromatography using PichiaGuard. PichiaGuard resin was initially packed in a 0.1 mL column and equilibrated with 20 mM sodium acetate at pH 5.7 at the flowrate of 0.2 mL/min for 15 minutes. Two *K. phaffi* harvests were utilized as feedstock in this study – the first one featuring a ScFv titer of ~50 mg/L and an HCP titer of ~0.6 mg/mL; the second one featuring a (spiked) mAb titer of ~0.4 mg/mL and an HCP titer of ~0.5 mg/mL – both conditioned in 20 mM sodium acetate at pH 5.7. A volume of 4 mL of harvest was loaded on the column at the RT of 1 minute and flow-through fractions of 0.3 mL were collected durig the load and the final column wash for analytical characterization (*Sections 2.2, 2.11, and 2.12*). All purification studies were performed using an ÄKTA pure (Cytiva, Chicago, IL, USA) while monitoring the effluents using UV spectroscopy at 280 nm.

2.11. Reverse phase (C18 RP-HPLC) analysis for the quantification of ScFv13R4 titer and purity. The titer of ScFv13R4 in the collected chromatographic fractions was measured via C18 RP-HPLC using a Zorbax C18-SB column (Agilent, Santa Clara, CA) installed on a Waters Alliance 2690 HPLC. The column was equilibrated by flowing 95% mobile phase B (MPB: 0.5% v/v TFA in acetonitrile) and 5% mobile phase A (MPA: 0.5% v/v TFA in water), at a flowrate of 0.5 mL/min. A 20-minutes gradient method (95 – 45% MPB) was conducted at the constant flow rate of 0.5 mL/min to achieve separation. Pure ScFv13R4 expressed in *E. coli* cells was utilized to identify the product retention time and calculate the ScFv13R4 purity in the chromatographic fractions.

2.12. Other Analytical Assays. The total protein and *P. pastoris* HCP titers were measured respectively via BCA assay (Thermo Fisher Scientific, Waltham, MA) and *Pichia pastoris* HCP G.2 ELISA kit (Cygnus Technologies, Southport, SC) following the manufacturer's protocol. Product purity was evaluated via BCA assay (reading wavelength: 660 nm) for histidine-rich proteins and SDS-PAGE analysis under non-reducing conditions. The mAb titer was measured using analytical Protein A HPLC as described previously [50].

3. Results

3.1. Discovery of K. phaffii HCP-targeting peptide ligands and classification of host cell proteins identified via proteomics approaches. A sequence-based bioinformatics analysis of HCPs identified in the harvest fluid via proteomics approaches (**Figure S1A**) was utilized to curate a list of HCPs/proteins (**Table S2**) with an assigned risk factor to each protein depending on their immunogenic propensity or similarity to known problematic HCPs (**Figure S2**). The HCPs identified to possess significant sequential similarity to known CHO HCPs with a registered detrimental effect on downstream processes has also been curated and presented in **Table 1**. The bioinformatics workflow has been described in detail in the *supplemental information*. Biochemically, these proteins presents a rather unique landscape of physicochemical properties (**Figure S1B**; *note:* all values are calculated based on the amino acid sequence of the HCPs): *(i)* the molecular weight distribution, centered at around 46.14 kDa, is significantly sharper (standard deviation, σ ~29.03 kDa) and a narrower (7 – 193 kDa) compared to its CHO counterpart (σ ~ 35.91, 5 – 776 kDa, [50]); *(ii)* the values of isoelectric point feature a distinct bimodal distribution peaking at the values of pH 5 and 9.5, with 40% of the species being anionic and 60% cationic at the physiological culture pH, which represents a second sharp difference compared to CHO HCPs (61% anionic and 39% cationic); *(iii)* the values of grand average hydropathy (GRAVY), varying between -1.84 and 0.49 (σ ~ 0.37), indicate that *K. phaffii* HCPs are more hydrophilic than CHO HCPs (-1.90 to 0.97; σ ~ 0.26); and *(iv)* an average polarity of 50.1% across all *K. phaffii* HCPs, with most species between a range of 42 – 62%, indicate a strong propensity to form hydrogen-bond networks.

This analysis informed the design of a library whence peptides could be selected that target the whole spectrum of the *K. phaffii* secretome: firstly, both positively and negatively charged amino acids (Glu, Lys, His, Arg; Asp was excluded from the library due to the abundance of aspartyl proteases in *K. phaffii* harvests) were included to address anionic and cationic ensembles of HCPs; furthermore, given the abundance of polar species, neutral amino acids that are hydrogen bond-forming (Gln and the spacer Ser) were included to ensure the formation of hydrogen-bonding interactions; for the same reason, only one aliphatic amino acid (Val; Ile and Leu were excluded) and two aromatic amino acids capable of forming hydrogen-bonds (Tyr and Trp; Phe was excluded) were included; finally, two amino acid linkers, the flexible Gly and the semi-rigid Ala (the rigid Pro was excluded) to sample the broadest possible range of conformations. The multi-polar composition of the library was inspired by that of the ligands forming the CHO LigaGuard™, among which multi-polar peptides hold a prominent role as key contributors to the capture of high-risk and persistent CHO HCPs [56]. A short peptide length of 7 total residues in the format X1-X2-X3-X4-G-S-G, wherein the 4 residues on the N-terminus form the HCP-binding segment and are varied within the library, while the Gly-Ser-Gly tripeptide on the C-terminus acts as a flexible spacer to enhance ligand display. This format was inspired by prior studies [58], where we observed that most of the energy of protein:peptide binding is contributed by the 4 residues on the N-terminus, and the recommendation of using PichiaGuard as a disposable – and ideally single-use – adsorbent, which requires moderate cost and thus short, easy-to-synthesize peptides.

***Table 1**: List of K. phaffii host cell proteins flagged as "high-risk" (HR-HCPs) due to their homology to known CHO HR-HCPs. While the full classification and list has been issued in the supplemental information, this table summarizies protein entries that were found in the K. phaffii secretome with significant similarities to previously identified CHO HCPs and their observed effects on downstream processing trains (DSP).*

Name	Homologous to CHO	Effects on DSP
Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl4Ap (C4QV16)		Recurrent observation in downstream processes
Heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70) (C4QV89)		Sorting and degradation of proteins
Ubiquitin-specific protease that deubiquitinates complexes(C4QV95)		Recurrent observation in downstream processes
Phosphoglycerate kinase (C4QY07)	Phosphoglycerate Kinase 1	Recurrent observation in downstream processes
Protein component of the large (60S) ribosomal subunit (C4QZL4)	60S Ribosomal Subunit Protein	Recurrent observation in downstream processes
Endoplasmic reticulum chaperone BiP (C4QZS3)	ER Chaperone BiP Precursor	Increases aggregation propensity
Proteasome endopeptidase complex (C4R080)	Alpha Enolase	Leads to drug product modification via catalysis of dehydration reaction
Alpha 6 subunit of the 20S proteasome (C4R080)	proteasome subunit alpha type 7 isoform x1	Recurrent observation in downstream processes
Kex2 proprotein convertase (C4R095)		Signal peptidase for secretion, often overexpressed with recombinant gene
Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3 (C4R0P1)	Glyceraldehyde-3 Phosphate Dehydrogenase	Recurrent observation in downstream processes
GTP-binding nuclear protein (C4R0Q2)		Recurrent observation in downstream processes
Acetyl-coenzyme A synthetase (C4R1P7)	Glutaredoxin-2	Recurrent observation in downstream processes
Pyruvate Kinase (C4R1P9)	Pyruvate Kinase PKM isoform X1	Potential immunogenicity due to glycolytic action
FK506-binding protein (C4R2G3)	Peptidyl-prolyl cis-trans isomerase	Increases aggregation propensity by influencing folding and assembly
Histone H3 (C4R2J7)		Recurrent observation in downstream processes
Catalase (C4R2S1)		Recurrent observation in downstream processes
Enolase I, a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphopyruvate (C4R3H8)	Beta-enolase isoform x3	Can lead to drug product modification via catalysis of dehydration reaction
Peptidase A1 domain-containing protein (C4R3Q7)		Protease activity
ATPase involved in protein folding and the response to stress (C4R3X8)	Heat shock cognate protein	Recurrent observation in downstream processes
Aspartic protease, attached to the plasma membrane via GPI (C4R458)		Protease activity
Putative GPI-anchored aspartic protease (C4R4F5)		Protease activity
Tetrameric phosphoglycerate mutase (C4R5P4)	Phosphoglycerate mutase 1	Recurrent observation in downstream processes
Triosephosphate isomerase (C4R626)		Recurrent observation in downstream processes
Vacuolar aspartyl protease (Proteinase A) (C4R6G8)	Cathepsin E	
Protein component of the large (60S) ribosomal subunit (C4R6P3)		Recurrent observation in downstream processes
Lysophospholipase (C4R703)	Lysosomal Phospholipase A2 (LPLA2)	Leads to polysorbate degradation by cleaving acyl ester bonds of glycerophospholipids
40S ribosomal protein S4 (C4R7A1)		Recurrent observation in downstream processes
Lon protease homolog, mitochondrial (C4R7W9)		Recurrent observation in downstream processes
Protein component of the small (40S) ribosomal subunit (C4R7Y4)		Recurrent observation in downstream processes
Aspartic protease, attached to the plasma membrane via (GPI) anchor (C4R8B8)		Protease activity
Cytoplasmic thioredoxin isoenzyme of the thioredoxin system (C4R8R1)	Periredoxin-1 family	
Protein disulfide isomerase, multifunctional protein in the endoplasmic reticulum lumen (C4R938)	Protein Disulfide Isomerase	Affects drug product quality by reduction of protein disulfide bonds

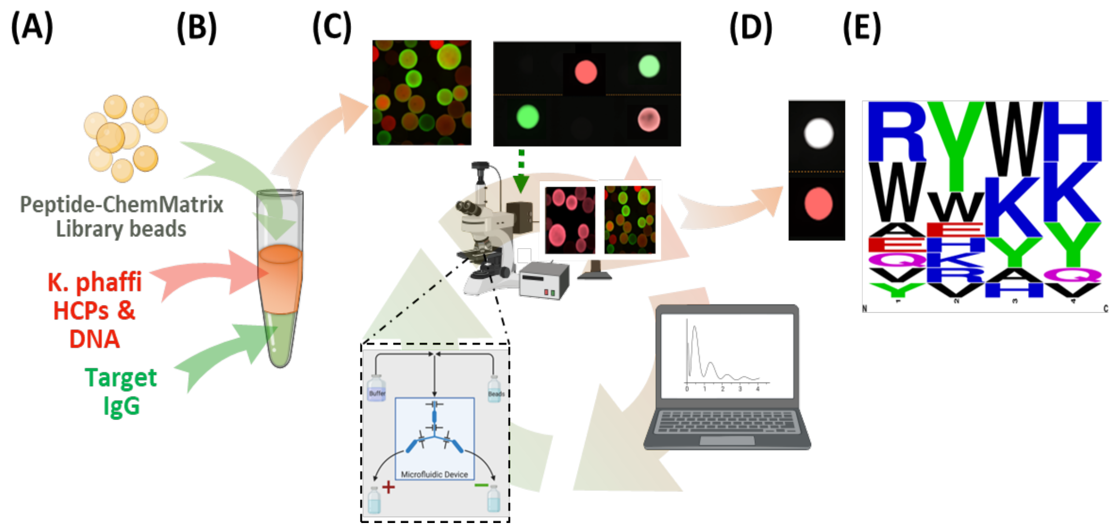

Figure* 1.** *Identification of HCP-binding peptides.* ***(A) *A screening mix was formulated containing red-fluorescently labeled K. phaffi HCPs at the titer of 0.1 mg/mL and green fluorescently labeled IgG at the titer of 2 mg/mL;* ***(B)*** *aliquots of 10 µL of 4-mer peptide-ChemMatrix library beads equilibrated in PBS at pH 7.4 were incubated with the screening mix for overnight at room temperature;* ***(C)*** *the beads were washed and fed to a microfluid bead-sorting device installed in a fluorescent microscope:* ***(D)*** *every bead that displays high red fluorescence emission and high red-to-green signal ratio was retained, while all other beads were discarded;* ***(E)*** *the selected beads were washed with 30% acetonitrile in water (v/v), and individually analyzed by Edman degradation using a PPSQ-33A protein sequencer (Shimadzu, Kyoto, Japan) to identify the peptide sequences.*

These design criteria were implemented in the synthesis of a One-Bead-One-Peptide (OBOP) combinatorial library on ChemMatrix™ resin, whose translucent, porous, hydrophilic beads are ideal for library selection in competitive mode [56]. To ensure identifying HCP-selective ligands capable of purifying cultures producing a variety of protein products – such as full mAbs or engineered fragments [59] in flow-through mode, competitive screening was ensured (HCPs and product). Our discovery workflow which is based on fluorescence screening using a bead-imaging-and-sorting device [57], utilizes an automated bead-selection algorithm that processes images of the beads in real time, thus enabling the sampling of a large portion of the library and utilizes multiple fluorescence emission wavelengths to select leads with high target-binding strength and selectivity. To that end, a library screening feedstock mimicking industrial harvests was prepared by combining *K. phaffii* HCPs and human IgG – including whole antibody, Fc/Fab fragments, and nanobody fragments – at a titer of 0.2 mg/mL and 2 mg/mL respectively. The HCPs and IgG species were collectively labeled with a red and a green-fluorescent dye, respectively. The library beads displaying high-intensity red-only fluorescent emission (*i.e.*, strong HCP-only capture behavior at thermodynamic equilibrium) were selected and the peptides carried thereupon were sequenced via Edman degradation (**Figure 1**). The resulting sequences were analyzed to identify position-based homology of residues (**Figure S3A**) and amino acid frequency in the identified peptides (**Figure S3B**).

The sequences present a strong enrichment in aromatic and cationic/H-bonding residues, and a depletion in anionic and aliphatic residues. The physicochemical properties of the candidate ligands are complementary to those of *K. phaffii* HCPs, thus providing confidence in the outcome of library screening. We note that, while the enrichment of cationic residues was anticipated, given the abundance of anionic HCPs, the presence of a population of cationic HCPs (**Figure S1B**) seemed to warrant some enrichment of Asp, which was not registered. Accordingly, to ensure the broadest possible HCP-binding activity, eight peptides were selected – namely, RYWV, QEKK, VWHH, EWAK, RYWK, YHKH, RWYQ, WYKK – that feature a diverse amino acid arrangement and composition and are thus expected to encompass a broad spectrum of binding modalities.

***3.2. Capture of K. phaffii HCPs via flow-through affinity chromatography using PichiaGuard*.** The selected peptides were conjugated on Toyopearl beads, a polymethacrylate-based chromatographic resin whose mechanical rigidity, particle size (65 µm), ~~and~~ pore diameter (100 nm) and no non-specific binding (**Figure S7**), are ideal for protein purification via flow-through affinity chromatography [50]. The *K. phaffii* X-33 cell culture fluid (CCF) utilized to evaluate the HCP binding activity of PichiaGuard was initially diafiltered to *(i)* remove residual glycerol and methanol that interfere with the assays for HCP quantification (**Figure S3C**; *note:* while adopted in this study to ensure analytical accuracy, diafiltration may not be required in a regular purification train); *(ii)* adjust the HCP titer to different concentrations, ranging from 0.01 to 1.5 mg/mL to study the effect of protein concentration on binding kinetics and capacity; and *(iii)* test buffer compositions of either 20 mM sodium acetate at pH 5.7 (2.6 mS/cm) or 20 mM sodium phosphate at pH 7.1 (7 mS/cm) to study the effect of ionic strength and pH on binding kinetics and capacity. These buffers were adopted owing to their kosmotropic character that ensures a native, folded protein state, while promoting affinity interactions; furthermore, the pH chosen to prepare the acetate buffer matches that of *K. phaffii* culture conditions, while the neutral pH of the phosphate buffer matches that utilized for the CHO HCP-targeting LigaGuard™ and provides a control to interpret the HCP capture results.

Static binding studies were initially conducted by incubating the PichiaGuard resin with the conditioned harvests and the residual HCP titer in solution were measured to calculate the equilibrium binding. The resulting adsorption points were fitted against Langmuir isotherm curves (**Figures 2A** and **2B**), from which the values of maximum binding capacity (Q_{max}) and dissociation constant (K_D) for the peptide mixture were derived. The difference in the Q_{max} of 25.4 and 18.3 mg HCP per mL resin respectively registered in acetate and phosphate buffers can be attributed in part to the different ionic strength of the buffers, where the higher conductivity of phosphate buffer shields Coulomb interactions; however, the observation that cationic peptide ligands provide lower binding at pH 7, where the anionic HCPs should be more accentuated, suggests that the electrostatic component of binding is one, but not the dominant, component of the HCP:peptide interaction in this case. The difference in Q_{max} can also be imputed to the different positions of acetate and phosphate ions in the Hofmeister series, wherein acetate (less Kosmotropic) may be promoting the formation of ligand-accessible cavities/pockets on HCPs (*i.e.*, salting in), and thus potentiate target sites for hydrophobic or aromatic and polar residues in the PichiaGuard peptides to respectively form hydrophobic or π-π/hydrogen bond interactions. On the other hand, the acetate and phosphate ions are not expected to perturb hydrogen bonds and salt bridges between HCPs and peptides [60–62] at their (low) concentrations in the buffers. The combination of these effects results in a strong HCP-binding activity by the peptide-functionalized resin, and ultimately a high binding capacity.

The values of K_D – 17.5 µM and 30 µM measured in acetate and phosphate buffer, respectively – indicate a moderate HCP:peptide affinity. These values, however, result from treating the *K. phaffii* HCPs as a single species, a characteristic trait of the evaluation of a chromatographic technology against fluids featuring varying HCP titers and profiles, and commonly found in the literature. On the other hand, HCP capture is driven by the values of K_D of the single HCP:peptide binding event, and since each HCP is present at very low concentration (≤ 1 nM), the binding affinity of the selected ligands is actually high, as observed in prior work on LigaGuard™ ligands [63].

***3.3. Clearance of K. phaffii HCPs in flow-through mode: PichiaGuard vs. ion exchange resins*.** The purification of biopharmaceuticals expressed by *K. phaffii* relies almost ubiquitously on ion exchange (IEX) and mixed-mode (MM) chromatography. In particular, the post-capture steps of product polishing are being increasingly conducted in flow-through mode, which combines speed of operation with lower capital and operational costs. In this context, the IEX and MM resins employed in flow-through operations often include a quaternary amine (Q) moiety, which, as a strong cationic group, provides high binding capacity and strength of HCPs over a broad range of pH and conductivity values [36,64]. Owing to their high capacity, IEX resins are often utilized at the capture step when affinity resins are not available.

On the other hand, these resins lack binding selectivity and must be combined in a series of orthogonal chromatographic steps to ensure high product purity, which requires extensive process optimization and is often achieved at the expense of yield [65]. The paradigm of flow-through affinity chromatography as a step dedicated to abating process-related impurities holds strong promise to overcome these challenges and offers an ideal route to de-risk platform processes for established products as well as products that do not benefit from a dedicated affinity resin. In prior work on mAb purification in flow-through mode, we observed that LigaGuard™ resin outperform commercial IEX and MM resins on clarified and unconditioned CHO cell culture harvests, especially due to their lack of needing process optimization [56,63,66,67]. In this study, we resolved to conduct an analogous comparison between PichiaGuard, LigaGuard™ [63], and a commercial anion exchanger (AEX, Capto Q) and cationic MM (CaptoAdhere) resins by tracking HCP capture upon continuous loading of a clarified *K. phaffii* harvest [68].

To study the effect of pore size, the PichiaGuard was conjugated on two different polymethacrylate-based resins, one with pore diameter of 100 nm (Toyopearl 650M, TP-650M) and one with pore diameter of 5 nm (Toyopearl HW-50F, TP50F). Engineered antibody fragments, such as the Fab and ScFv often produced in *K. phaffii* hosts [69], feature hydrodynamic radii ~2-4 nm [70,71].

Accordingly, we sought to explore the combination of size exclusion of the product (based on pore size) and pseudo-affinity capture of HCPs by the PichiaGuard peptides as a means to improve product yield and purity under optimal conditions of loading flowrate [72]. Accordingly, we resolved to operate the flow-through process at the bioprocess-relevant value of residence time (RT) of 1 minute. Finally, similar to static binding tests, the harvest was conditioned in either 20 mM phosphate buffer at pH 7.1 or 20 mM acetate buffer at pH 5.7 (whose pH closely mimics that of the *K. phaffii* harvest). The flow-through effluent was continuously collected and apportioned in fractions at regular intervals to evaluate the temporal profiles of protein binding – both as global HCP titers and single species via proteomics analysis.

The results presented in **Figure 2** provide a comparison of the HCP-clearing performance of the peptide-functionalized resin (PichiaGuard) *vs.* AEX, CHO LigaGuard™ and MM resins as a function of load in the two buffer systems. The PichiaGuard outperformed both LigaGuard™ and commercial resins when operated in acetate buffer at the RT of 1 min, providing a cumulative HCP LRV > 2 (*i.e.*, > 100-fold reduction of HCPs) when loaded with up to 10 CVs, followed by LRVs of 1.5 - 2 (*i.e.*, 30-100-fold reduction) for up to 20 CVs, 1.3 - 1.5 (*i.e.*, 20-30-fold reduction) for up to 40 CVs, and plateauing at 1.3 for up to 100 CVs; LigaGuard™ resin afforded the second best HCP clearance profile, with LRVs of 1 - 1.5 for up to 10 CVs, 0.75 - 1 for up to 20 CVs, and plateaued to 0.45-0.55 CV when loaded up to 40 CVs. The reference AEX and MM resins provided a rather poor HCP clearance (without keen buffer or process optimization), consistently below a 10-fold reduction and plateauing to a LRV ~ 0.5 (3-fold reduction) when loaded up to 40 CVs. A similar performance ranking was observed in phosphate buffer, with the sole exception of the AEX resin, which performed on par with the PichiaGuard resin across the entire loading range (40 CVs). The magnitude of HCP LRVs, however, decreased for all resins, thus mirroring the static binding results discussed above.

A number of conclusions can be drawn from these results: *(i)* peptide ligands significantly outperformed simple MM or IEX ligands, likely due to their broader range of non-covalent interactions and conformations; and *(ii)* library selection targeted to *K. phaffii* HCPs proved successful in producing an ensemble of bespoke ligands. The LigaGuard™ peptides, selected for CHO HCP capture, delivered a higher HCP capture than commercial resins (**Table 2**).

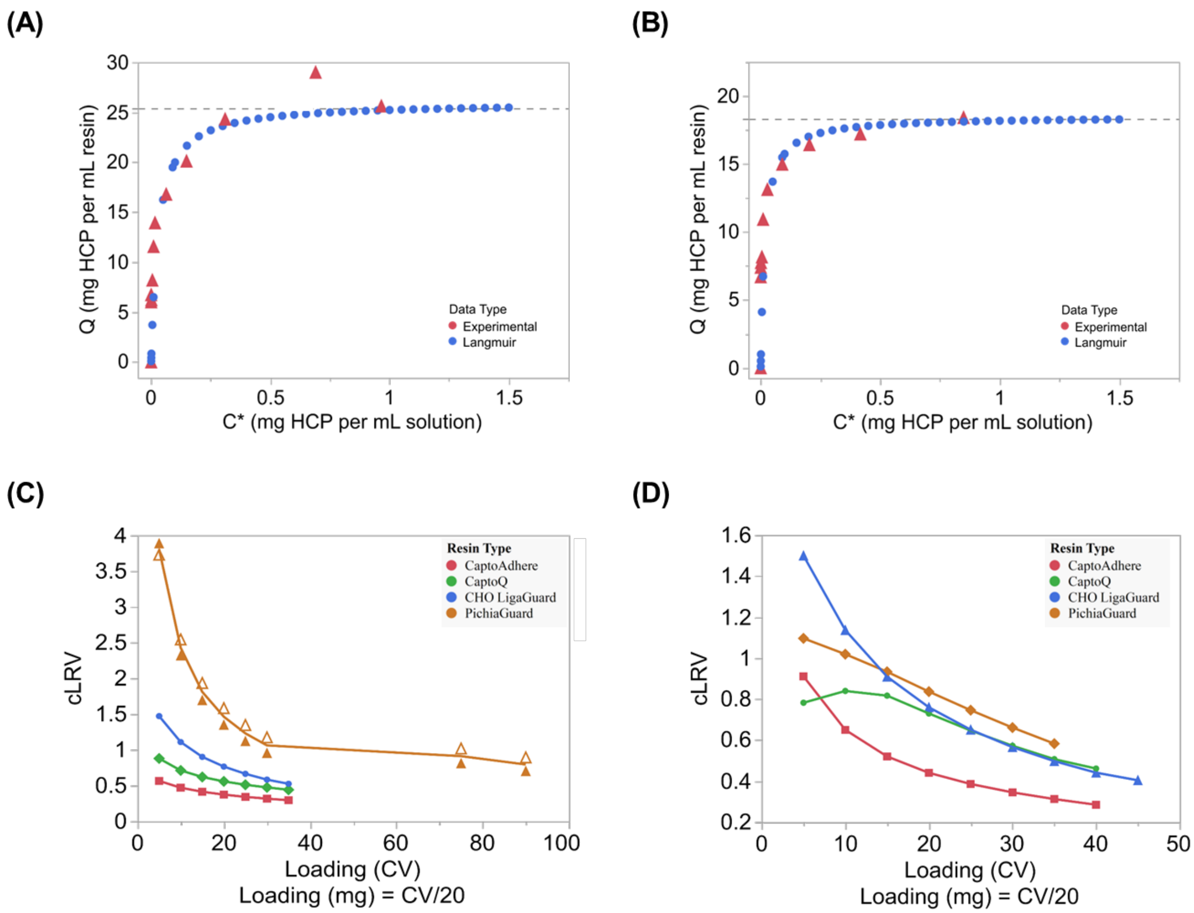

Figure 1: *HCP binding isotherms obtained by incubating PichiaGuard TP-650M resin with solutions of K. phaffii HCPs at concentrations ranging between 0 – 1 mg/mL in either* ***(A)*** *20 mM sodium acetate at pH 5.7 or* ***(B)*** *25 mM sodium phosphate at pH 7.1. Values of cumulative logarithmic removal of K. phaffii HCPs afforded by CaptoAdhere, CaptoQ, CHO LigaGuard, and PichiaGuard TP-650M resins loaded (residence time: 1 minute) with X-33 feedstocks with HCP titer of ~0.4 mg/mL and conditioned in either* ***(C)*** *20 mM sodium acetate at pH 5.7 (with and without protease inhibitors) or* ***(D)*** *25 mM sodium phosphate at pH 7.1. N=2 for all.*

In turn, the PichiaGuard peptides outperformed both LigaGuard™ and the MM and IEX ligands, demonstrating a biorecognition activity that is superior to that of conventional mixed-mode ligands when evaluated under the same conditions and grants them the status of (pseudo-)affinity ligands. Furthermore, *(iii)* the PichiaGuard peptides can be utilized under conditions that mimic the native environment of *K. phaffii* harvests, namely acidic pH. Finally, *(iv)* around the 30th CV of loading, we observed the cumulative LRV plateau at ~1 and the corresponding fractional LRV become lower than 0.5 (**Figure S4E**), indicating that the adsorbent had reached saturation. The 30th CV mark corresponds to a load ratio of ~18 g of HCPs per L of resin, which corresponds to ~75% of Q_{max} (see *Section 3.3*). This is a reasonable outcome given the relationship between dynamic *vs.* static binding and can be improved by adjusting the residence time as well as composition and pH of the running buffer. Nonetheless, contrary to affinity resins that operate in bind-and-elute mode, the loading of 'Guard' resins is intended to exist between the values of $DBC_{10\%}$ and Q_{max}.

Table 2. *Number of K. phaffi HR-HCPs captured by PichiaGuard, CHO LigaGuard™, and CaptoQ resins loaded with X-33 feedstocks (residence time: 1 minute) with HCP titer of ~0.4 mg/mL and conditioned in either 20 mM sodium acetate at pH 5.7- or 25-mM sodium phosphate at pH 7.1.*

Acetate buffer at pH 5.7				
	CaptoQ	**CHO LigaGuard™**	**PichiaGuard-TP50F**	**PichiaGuard-TP650M**
Undetected	19	18	19	18
Not captured	15	19	5	10
Captured	10	11	20	16
%	40%	42%	80%	62%
Phosphate buffer at pH 7.1				
	CaptoQ	**CHO LigaGuard™**	**PichiaGuard-TP50F**	**PichiaGuard-TP650M**
Undetected	0	8	0	0
Not captured	29	20	17	8
Captured	15	15	27	36
%	34%	41%	61%	82%

Further improvement of HCP clearance from *K. phaffii* harvests using PichiaGuard resin can be achieved via process optimization. Conversely, achieving comparable purification under given conditions from fluids that differ substantially in the profile of physicochemical properties of their HCPs is far from a foregone conclusion. We therefore resolved to measure the global HCP removal from two fluids – produced respectively with and without the addition of protease inhibitors to the cell culture medium – whose distribution of HCP molecular weights differed radically (**Figures 2C** and **2D**). To that end, we implemented a design-of-experiments (DOE) algorithm to explore systematically the effects of both categorical (*i.e.*, buffer and resin) and continuous (*i.e.*, buffer conductivity and pH, load ratio, and RT) variables to identify parameters that maximize HCP capture (**Figure S4 A-D**). The choice of resin, buffer, and interaction effects between these parameters were indeed found to be significant. The effect of RT appeared to be the most pronounced when using phosphate buffer: the highest HCP reduction (LRV ~3) was obtained at the RT of 1 min, which was therefore adopted for the remainder of this study. Notably, the combination of phosphate buffer and a smaller pore diameter of the resin led to higher HCP clearance (LRV ~ 0.8 - 1) than its acetate counterpart (LRV ~ 0.7 – 0.9), possibly due to effects of phosphate counterions on protein folding and increased ability to diffuse into smaller pores; however, limiting the total capture capacity. PichiaGuard-TP650M, however, afforded a superlative HCP reduction (LRV ~ 3) and emerged as the resin of choice. As observed above, performing flow-through purification in acetate buffer consistently achieved a higher HCP clearance, thus providing conclusive evidence for the adoption of acetate buffer for resin equilibration and, ideally, conditioning of the feedstock.

3.4. Tracking the removal of high-risk HCPs from K. phaffii cell culture harvests by PichiaGuard. In the established biopharmaceutical practice, the validation of a batch of therapeutic proteins consists in certifying that the residual HCP and hcDNA titers are below an acceptable limit for monoclonal antibodies (mAbs), these values are 100 ppm and 10 ppb, respectively [73]. The measurement of residual HCP titer has been conducted for decades with ELISA kits. Like all assays that relies on polyclonal antibodies raised against multiple antigens, most ELISAs do not provide a full coverage of HCPs, but only of those that are abundant or more immunogenic to the animal host; furthermore, HCP aggregation and the formation of HCP:product complexes – a widespread phenomenon in bioprocess fluids – can impair assay readout [74].

These elements, combined with the variability among assay lots and operator's performance, question the accuracy of HCP quantification provided by ELISA assays. Furthermore, in recent years, the growth of proteomics in biomanufacturing has shown that product batches with acceptable global level of impurities can contain amounts of single high-risk HCPs (HR-HCPs) that pose a risk to patient health (*e.g.*, are toxic or immunogenic) or can degrade the mAb or its excipients during storage, resulting in reduced efficacy or harmful products [74,75]. Recent studies have amply documented that commercial chromatographic resins may struggle to remove particular HR-HCPs: a number of these "persistent" HR-HCPs have been reported on both a process- and product-basis and have been linked to delays in clinical trials and process approval or the recall of drug batches.

Under this premise, we resolved to complement the measurements of global residual HCPs presented above with tracking the individual HCPs captured by PichiaGuard via proteomics analysis. To this end, the effluents obtained in both acetate and phosphate buffers at the RT of 1 min were analyzed via LC-MS/MS by implementing the proteomics workflow outlined above for top-speed data dependent acquisition (DDA) [76]. Spectral interrogation using the Proteome Discoverer Suite was used to perform label-free quantification (LFQ) of HCPs in the flow-through fractions *vs.* the corresponding feedstocks (*note:* due to the lower number of proteins identified in *K. phaffii* harvests compared to CHO harvests, a 'match between runs' was implemented to preserve information from low-intensity peptide hits that that may have been masked by abundant contaminants like trypsin and any media components) [77]. Briefly, *(i)* data processing prior to analysis of variance (ANOVA) involved the exclusion of contaminants such as trypsin and keratin as well as the HCPs found in the effluents but not the load sample; *(ii)* the abundance of individual proteins was used to calculate the HCPs removal values [78]. We noted that certain proteins were identified in some flow-through fractions to have an abundance ratio > 1 (defined as the ratio of HCP abundance in the effluent sample *vs*. the load sample), suggesting that their presence in the load was masked by more abundant peptides with similar sequence or charge that were not excluded.

We adopted three main indicators to evaluate the HCP-capture performance of different adsorbents, namely *(a)* global concentration and reduction; *(b)* between-group analysis, which presents the abundance trends of every identified HCP; and *(c)* clearance or persistence of HR-HCPs. These indicators capture different variations within our study group and have been used collectively to make inferences. **Figure 3** presents the global landscape of the HCPs identified across all control experiments. The between-group significance analysis (group: individual resin tested at an assigned composition/pH) was conducted using an iterative Tukey's HSD (honestly significant difference) test, known as Newman-Kuels method, to construct a 'connected-color' plot that connects fractions with a similar normalized mean abundance using the same color and ensures discontinuity with significant 'within group' difference (p-value > 0.01). This test provides an at-a-glance presentation of changes in global HCP titer compared to the load and other fractions (**Table S3**), while other statistics and visualization techniques have been employed to track changes across individual species.

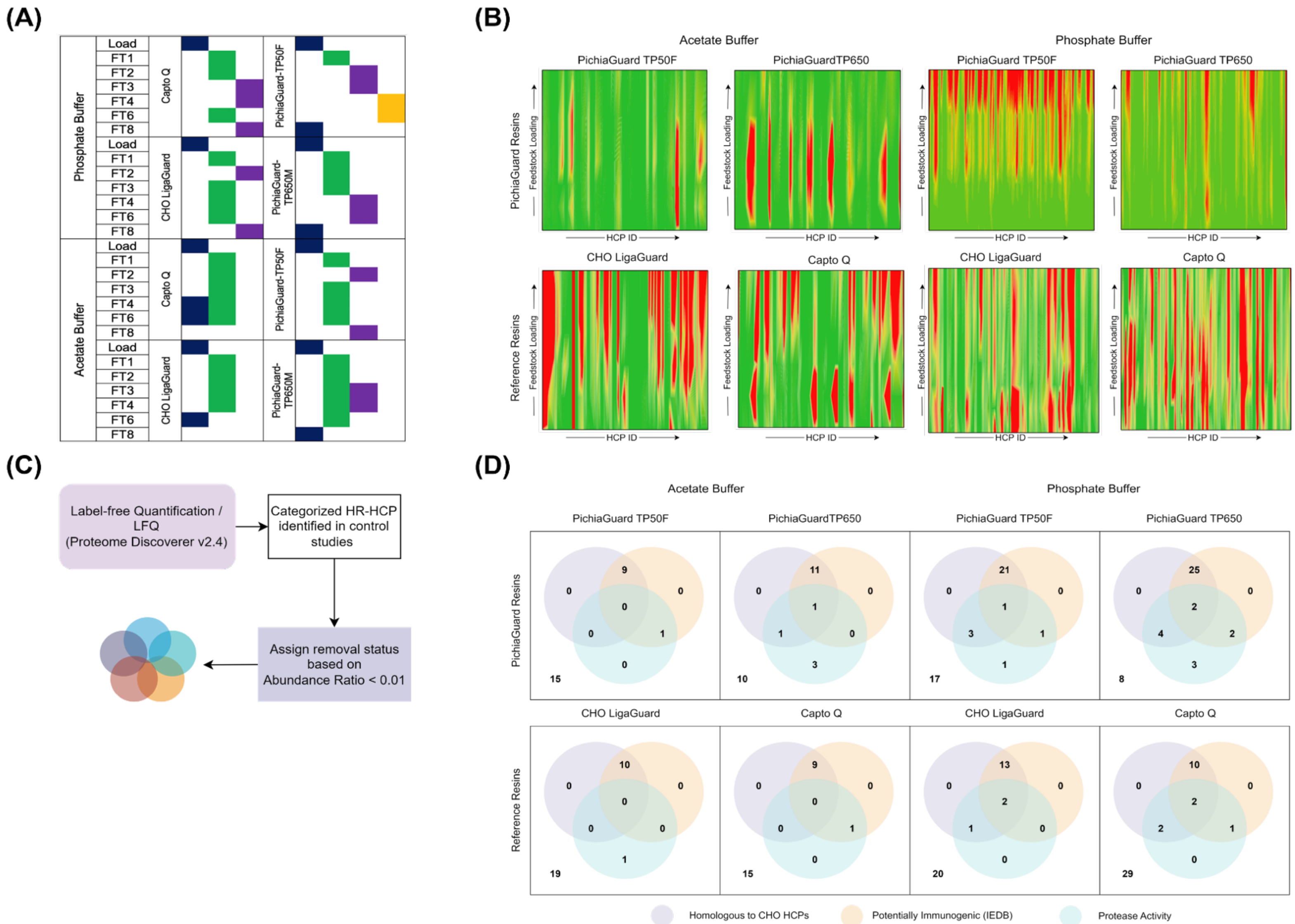

Figure 2: ***(A)*** *Connected colors plot indicating significant differences in mean HCP abundance among flow-through fractions; groups connected with different colors feature significantly different HCP abundances calculated as an average value for the group;* ***(B)*** *Contour plots of K. phaffii HCP abundance ratios registered in the effluents obtained by loading CaptoQ, LigaGuard, and PichiaGuard resins loaded (residence time: 1 minute) with X-33 feedstocks with HCP titer of ~0.4 mg/mL and conditioned in either 20 mM sodium acetate at pH 5.7 or 25 mM sodium phosphate at pH 7.1: individual HCPs are aligned on the x-axis, while the number of collected flow-through fractions is on the y-axis; red to green indicates high to low ratio of individual HCP in the effluent vs. feedstock as detected by nanoLC-MS;* ***(C)*** *Flowchart describing the designation of removal status of 'high-risk' HCPs (HR-HCPs);* ***(D)*** *Number of individual HR-HCPs removed by CaptoQ, LigaGuard, and PichiaGuard resins grouped by risk category; the values outside the Venn diagrams indicate the number of HCPs that were not removed.*

To visualize and compare HCPs in successive flow-through fractions within and across buffer groups – (acetate, phosphate) and adsorbents (PichiaGuard-TP650M, PichiaGuard-TP50F resins, CHO LigaGuard™ and Capto Q resin) – **Figure 3B** presents a reduced-dimension view of individual HCP abundance (Uniprot Accession numbers, left-to-right) *vs.* volume of effluent (flow-through fraction, bottom-to-top). Specifically, abundance ratios (fraction by load) are represented as contour plots wherein ratios ≥ 1 (in red) mark HCPs that were not cleared or whose presence was masked in the feedstock by other species but detected in a flow-through fraction, whereas ratios ≤ 1 (veering from yellow to green as the value decreases) mark HCPs that were captured in the process.

These results illustrate the ability of PichiaGuard resins to capture most HCPs within both buffer systems compared to reference commercial resins. The projected contour plots provide an effective visualization method combining results from the chromatographic process and proteomics analyses, while permitting a clear comparison between resin subjects tested at similar experimental conditions. These resulting plots provide a visual indication of individual HCPs that were not captured throughout a significant part of the load due to insufficient HCP:ligand binding strength or possibly due to lack of charge/polarity of HCPs at the pH condition of the experiment, based on their isoelectric points.

Notably, the proteomics analysis of the effluents allowed us to track the capture of *K. phaffii* HR-HCPs by the various adsorbents (**Figure 3C**), specifically focusing on proteins that are *(i)* homologous to known high-risk and persistent CHO HCPs; *(ii)* immunogenic, either reported or predicted based on their ability to generate MHC-II binding peptides; and *(iii)* possess proteolytic or other enzymatic activity, as outlined in *Section 3.1*. The number of bound HR-HCPs across groups were classified according to their risk subtypes/bins (**Figure 3D**). The corresponding logarithmic values of abundance variation of all identified HR-HCPs are presented in **Figures S5A** and **S5B**, whereas the number of captured HR-HCPs as a function of loaded volume across all groups is reported in **Figure S5C**.

From these results, the following key observations were drawn. Firstly, the PichiaGuard resins (**Figure 3B**, top row) outperformed both LigaGuard™ and AEX (Capto Q) resins in retaining a higher number of *K. phaffii* HCPs, thus corroborating the results of total HCP quantification obtained from the total protein assays (see *Section 3.5*). It is noteworthy that this behavior is observed across all flow-through fractions for the HCPs, warranting the claim of pseudo-affinity capture. As shown in **Figure S5C**, of the 187 HCPs identified in the acetate-conditioned feedstock, 180 were captured by PichiaGuard resins throughout the entire feedstock loading, while 3 – 5 were not captured; similarly, of the 318 HCPs in phosphate-conditioned feedstock (for a total of 335 unique *K. phaffii* HCPs identified), 302 were captured, while 8 – 12 were not captured. Secondly, PichiaGuard-TP650M and PichiaGuard-TP50F resin performed similarly in acetate buffer, whereas in phosphate buffer PichiaGuard-TP650M clearly outperformed its TP50F counterpart. The contour plots also show a greater extent of HCP dissociation from PichiaGuard resins in acetate media - especially from TP650M resins, likely a combined effect of pore size and residence time. Thirdly, PichiaGuard demonstrated the ability to clear up to 82% of HR-HCPs – including various aspartic proteases, ion protease homologs, etc. – whereas LigaGuard™ and CaptoQ resins only bound up to 42% of them. This corroborates our claim that ion-exchange resins, despite their satisfactory global HCP capture under optimized conditions [79], can fail to clear a number of HR-HCPs and are therefore unlikely to serve effectively as an HCP-scrubbing step prior to, or in lieu of, the affinity-based product capture step. Conversely, PichiaGuard makes an excellent tool for orthogonal HCP removal, thus safeguarding the performance and lifetime of product-capture resins as well as promoting product quality and stability.

As is to be expected with any chromatographic adsorbent, process optimization in terms of loading ratio, flow rate, and buffer composition is to some extent needed. In the context of this study, however, these results demonstrate the potential of PichiaGuard resin to increase the performance and robustness of current processes as well as provide a route towards platform processes with altogether novel design for isolating biopharmaceutics from *Pichia* harvests.

***3.5. Purification of ScFv13R4 and mAb from K. phaffii harvests via flow-through affinity chromatography using PichiaGuard*.** Purification processes fully operated in flow-through mode are the ideal embodiment of continuous downstream biomanufacturing: their design minimizes process footprint and the number of tanks for storing service buffers, simplifies the pipework and control systems, and accelerates operations, thus increasing productivity. Straight-through processes combining column chromatography and filtration unit operations have been proposed for the continuous manufacturing of mAbs from CHO cell harvests [31,68]. These processes can be successfully designed based on the size of the target products (*e.g.*, full mAbs), which are often larger than most of the process-related impurities. Conversely, small proteins are significantly more challenging products (listed in **Table S1**), as they exclude filtration as an orthogonal separation method, thus narrowing the purification toolbox to chromatography alone. In this context, pseudo-continuous processes can be utilized by integrating periodic counter-current (PCC) or simulated moving bed (SMB) modes [80] encompassing ion exchange chromatography, whose complexity increases both capital and operation costs. An effective alternative is offered by leveraging resins specifically tasked with HCPs and hcDNA clearance, like LigaGuard™ and PichiaGuard, which provide a product-agnostic route (from the standpoint of specificity to type of therapeutic product), towards fully chromatographic straight-through processes for the purification of both mAb and non-mAb drugs.

Under this premise, having demonstrated the broad HCP-capture activity of PichiaGuard, we moved to evaluate its ability to purify therapeutic proteins from *K. phaffii* harvests in flow-through mode. To this end, we adopted an antibody fragment (ScFv13R4, MW ~30 kDa and pI of 8.36) and a full antibody (referred to as 'mAb' hereon, MW ~150 kDa and pI of 7.56) as model targets. Most importantly, to demonstrate the robustness of the technology, the *K. phaffii* harvests were loaded on PichiaGuard until reaching a protein load of >15 grams per liter of resin. The analyses of the flow-through fractions – namely, global clearance of HCP and other impurities estimated and product yield – are reported in **Table 3**.

The purification of ScFv13R4 returned considerable values of impurity removal, with HCP clearance ranging from LRV > 2.5 (~320-fold removal) to LRV > 1.5 (~32-fold removal) when loading respectively up to 3.9 and 7.5 grams per liter of resin, finally reaching LRV ~ 1 upon loading 14.4 mg/mL resin, at which a DNA LRV ~1.23 was also achieved. The electrophoretic and proteomics analyses of the flow-through fractions in **Figure 4** provide at-a-glance presentation of impurity removal by PichiaGuard: particularly noteworthy is the comparison between the feedstock and effluents containing the ScFv13R4 (**Figure 4C**), which demonstrate the removal of contaminants both heavier and lighter than the product. Notably, the proteomics analysis of the effluents (**Figure 4A**, **B**) showed that, of the total number of HCPs identified in the feedstock, the number of escaping species increased from 11 at the initial collection point of 4 g/L to merely 34 at the final pool, with 10 not being captured throughout. This corresponds to > 84% of HCPs completely removed – chiefly among them, 38 out of 44 HR-HCPs – while the remainder ~15% HCPs were partially cleared. The reverse phase chromatograms show that the product-to-impurity ratio in the effluent increases as the loading progresses up to the value of 14 mg/mL resin, translating in a growing product enrichment. In earlier work, we reported a weak-partitioning effect with peptide ligands for CHO HCP capture [66], where a higher load of culture leads to better separation of the mAb product from HCP contaminants. However, this beneficial effect fades at the load value of > 14 mg/mL resin with PichiaGuard and this ScFv culture, likely due to the relatively low product titer in the feed (~0.06 g of ScFv13R4 per liter of *K. phaffi* harvest *vs.* >1 g of mAb per liter of CHO harvest) and the formation of HCP-HCP and HCP-product complexes observed in the effluent (**Table 3** and **Figure S6B**).

Table 3. *Results of ScFv13R4 and mAb purification obtained by loading PichiaGuard-TP650 resin loaded (residence time: 1 minute) with X-33 K. phaffi feedstock with an ScFv13R4 titer of ~ 60 mg/L, and mAb at pH 5.7, HCP titer of ~0.4 mg/mL.*

	Loading (mg proteins per mL resin)	Ratio of product *vs.* non-product peaks	HCP cLRV	DNA cLRV	cYield	HCP (ppm)
ScFv13R4	Load	0.19				
	2.10	n.d.	3.00			
	3.90	n.d.	2.55			
	5.10	2.06	1.92	1.23		
	6.30	2.83	1.55		75.3%	
	7.50	1.77	1.34			
	9.30	1.40	1.14			
	14.40	1.09	1.01			
		Product Titer (mg/mL)				
mAb	Load	0.41				69643.9
	16.0	0.40	1.14	1.40	80.1%	1206.3
	Wash	0.22	1.18			

The yield of ScFv13R4 reached ~75% at the end of the loading phase (estimated using 660 nm BCA for 6x-His tagged ScFv13R4) but was increased to 85% by 'chasing' the load with a high-conductivity buffer (**Figure S6A**). The relationship between HCP-capture using PichiaGuard resins as a function of ScFv13R4 product concentration will be studied as the next step. Similar results were obtained from the purification of a full mAb: overall HCP clearance of 1.15 LRV was obtained up to a load of 15 mg/mL HCP with a mAb product recovery of 80% estimated using an analytical Protein A HPLC assay.

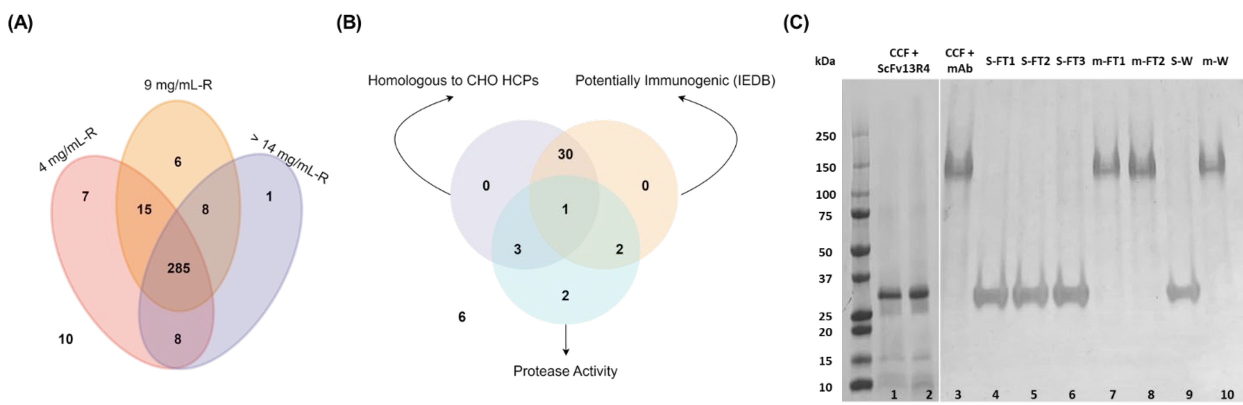

Figure 3:* *(A) *Total number of HCPs removed from a X-33 K. phaffi feedstock (ScFv13R4 titer of ~ 60 mg/L, HCP titer of ~0.4 mg/mL, 20 mM sodium acetate at pH 5.7) by PichiaGuard-TP650 resin operated at the residence time of 1 minute as a function of resin loading (namely, 4, 9, and >14 mg of proteins per mL of resin); the number of removed HCPs was determined via proteomic analysis of the flow-through fractions via nanoLC-MS/MS;* ***(B)*** *Number of individual HR-HCPs removed by PichiaGuard-TP650 resin grouped by risk category; the values outside the Venn diagrams indicate the number of HCPs that were not removed;* ***(C)*** *SDS-PAGE gel (native conditions) of the K. phaffi cell culture harvest containing ScFv13R4 and mAb (lanes 2 – 4), and the flow-through fractions generated by loading the harvests on PichiaGuard-TP650 resin at the residence time of 1 minute (5 – 7 for ScFv13R4; 8 and 9 for mAb) and final column wash (lane 8 for scFv13R4 and lane 9 for mAb). Peak tailing in lanes 3 – 10 results from sample overloading, which was intended to provide visual proof that no protein other than the product can be visualized in the gel, thus demonstrating the effective removal of K. phaffi HCPs by the PichiaGuard resin.*

Collectively, these results provide proof-of-concept of PichiaGuard as a specific, impurity-removal purification technology for *K. phaffii* cultures in a product-agnostic manner. At the same time, improvement in binding selectivity via optimizing ligand combinations or pretreatment of these cultures needs to be explored, along with the development of additional analytics.

4. Discussion and Conclusions. The success of *K. phaffii* in pharmaceutical biomanufacturing is scripted in its rapid growth in chemically defined media, low susceptibility to virus contamination [81], facile expression inducibility, and ability to secrete human proteins with correct post-translational modifications at high titer and purity [15,98]. These upstream-related benefits call for convergent efforts in the downstream toolbox, especially in the context of continuous processing and isolation of products for which dedicated affinity adsorbents are not available. In this context, new chromatographic tools are needed that provide orthogonality in removing process-related impurities and/or complementary to commercial adsorbents in clearing residual impurities across a spectrum of concentrations to ensure the safety of patients treated with complex biological drugs [83,84]. Our group has contributed to this field by introducing 'Guard' resins, whose HCP and hcDNA capture activity provides an orthogonal step to affinity-based resins for product capture or an alternative to IEX and MM resins for product polishing [63,66]. In this context, we leveraged the heuristic power of designed peptide libraries coupled with high-throughput dual-fluorescence screening to identify peptide ligands that can capture persistent and high-risk contaminants; furthermore, we integrated advanced analytics, such as proteomics, to demonstrate the potential of these ligands in biomanufacturing. Our initial efforts, conducted in the domain of full mAb and CHO HCPs, were supported by an established portfolio of analytical technologies and a wealth of literature highlighting target HCPs.

In this study, we extended the 'Guard' technology to *K. phaffii* HCPs by introducing PichiaGuard, the first pseudo-affinity adsorbent dedicated to the clearance of process-related impurities present in *Pichia* cell culture harvests. While the selection of *K. phaffii* HCP-binding peptides was streamlined by a robust ligand identification technology, the evaluation of the resulting PichiaGuard adsorbent faced challenges related to the quantification of product recovery and contaminant removal. In particular, the shortage of relevant literature on the toxicological, immunological, bioprocess-relevant aspects of *Pichia* HCPs encouraged us to formulate criteria for identifying species that pose – or are expected to pose – a risk to patient's health (*e.g.*, immunogenicity via B-cell activation [85]) or product stability, and can compromise the efficiency of the purification process (*e.g.*, co-elution with the product or persistence throughout the downstream train [51]). Having established an analytical panel, we demonstrated that PichiaGuard affords up to 99% reduction of HCPs and hcDNA concentration from a native *K. phaffii* cell culture supernatant even when challenged with a harvest containing a therapeutic product – a full mAb or an ScFv fragment – thus providing product enrichment through impurity removal. Most importantly, PichiaGuard's ability to clear potentially immunogenic HCPs (*i.e.*, species documented to trigger an immune response or homologous to immunogenic species found in other organisms, such as 40S ribosomal proteins, Histone H3, Thioredoxin peroxidase, Phosphatidylinositol transfer protein, Translation initiation factor eIF4G etc.) as well as proteolytic or enzymatically active HCPs (*e.g.*, Lon protease, Aspartic proteases – vacuolar, GPI-anchored and plasma membrane-attached forms, Lysophospholipase, Catalase, Peptidase A1, Ydj1p co-operating heat shock protein etc.) is key to process robustness and product safety.

These results warrant a path forward for PichiaGuard, chiefly its integration with existing purification pipelines, and into a straight-through process especially in combination with commercial resins for product isolation. A two-column process comprising polishing or scrubbing *K. phaffii* cultures followed by affinity capture or focusing of the target biologic can significantly de-risk and improve purification processes by reducing protease-mediated product alteration, improvising formulation stability, and increasing the performance and lifetime of the subsequent chromatographic steps via early HCP removal.

Furthermore, the discovery of these peptides as the biorecognition elements also provide the opportunity of using them as membrane functionalized entities – therefore providing both separation and filtration at the same time [86,87].

Accordingly, future studies on PichiaGuard will focus on *(i)* flexibility – whether it can be utilized in a fully product-agnostic manner; *(ii)* robustness to variability in HCP titer, complexity and composition as they result from the upstream process conditions, as discussed in *Section 3.1*; *(iii)* robustness to variability in conductivity pH, and product titer in the feedstocks; *(iv)* ability to increase product recovery by adding an optimized 'chasing' wash; and *(v)* lifetime – namely, how effectively it can be regenerated and re-used safely over a high number of cycles. Furthermore, observations of commonalities in the HCP repertoire among yeasts used in biomanufacturing [20] will provide both confidence and opportunity to evaluate the use of PichiaGuard across these strains for their wider adaptability in purification processes. Setting the stage for such work, this study presents a cogent message that the PichiaGuard technology holds potential to de-risk platform processes, reduce the cost and time-to-market of established therapeutics, and bring to fruition emerging medicines (*e.g.*, cytokines, enzymes, engineered antibodies, VLP-based vaccines) [3,21,88]. In so doing, the Guard peptide ligands are not anticipated to impose major financial burdens, being manufactured affordably at large scale, or regulatory concerns, given their safety and ease of clearance (small size). This strengthens our confidence in their adaptability in next-generation, truly continuous processes that can affordably and reliably meet the growing global demand [31,89,90].

Acknowledgements. The authors wish to acknowledge the generous support by the Novo Nordisk Foundation (AIM-Bio Grant NNF19SA0035474) and the North Carolina Viral Vector Initiative in Research and Learning (NC-VVIRAL) at NC State University.

Conflicts of Interest. The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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