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Host Cell Proteins Clearance in recombinant human EGF Purification

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Abstract

Host-cell proteins (HCPs) are critical process-related impurities in the manufacture of recombinant biopharmaceuticals because residual HCPs may compromise product stability, efficacy, and patient safety. Consequently, regulatory authorities require effective monitoring and control of HCPs contamination throughout downstream processing. In this study, the performance of two orthogonal polishing operations, hydrophobic interaction chromatography (HIC) and tangential-flow filtration (TFF), was evaluated for HCPs removal during the purification of recombinant human epidermal growth factor (rh-EGF). HIC using Toyopearl Butyl-650C resin achieved a 6.5-fold reduction in HCPs content while maintaining 97% product purity and 71% recovery. Subsequent TFF using sequential 30 and 2 kDa membrane cassettes operated at 12 diavolumes further reduced HCPs content to 50 ng mg^{-1} product while preserving 97% purity and 67% recovery. The integrated purification process resulted in an overall yield of 11.6 mg rh-EGF L^{-1} fermentation broth. Sequential application of HIC

and TFF reduced residual HCPs levels below the commonly accepted threshold of 100 ng mg^{-1} protein. These results demonstrate that HIC and TFF provide complementary and orthogonal mechanisms for impurity clearance and constitute a robust downstream strategy for controlling HCPs contamination during laboratory-scale rh-EGF production. The process establishes a foundation for future scale-up and industrial implementation.

Keywords: recombinant human epidermal growth factor; host-cell proteins; hydrophobic interaction chromatography; tangential-flow filtration.

1. Introduction

Recombinant proteins constitute a major class of biopharmaceutical products and are commonly produced using expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae*, and mammalian cells. During cell cultivation and product recovery, host cells release a diverse population of endogenous proteins known as host-cell proteins (HCPs), which represent one of the most important process-related impurities. Although initial capture and intermediate purification operations substantially reduce the HCPs burden, certain HCPs species may persist throughout downstream processing because of specific interactions with the target protein or similarities in physicochemical properties, including molecular size, charge, and hydrophobicity [1,2]. Residual HCPs in the final drug substance may compromise product quality, adversely affect stability, reduce therapeutic efficacy, and potentially induce undesired immune responses in patients. Consequently, HCPs are considered critical quality attributes (CQAs) in the manufacture of recombinant therapeutic proteins. The International Council for Harmonisation (ICH) guideline Q6B recommends the establishment of appropriate specifications and validated analytical methods to monitor process- and product-related impurities throughout manufacturing. Regulatory agencies, including the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), therefore expect manufacturers to demonstrate the robustness and

effectiveness of impurity-clearance strategies during downstream processing. Although no universally accepted specification exists for residual HCPs content, levels below 100 ng HCPs per mg of purified product are commonly considered an acceptable target in the biopharmaceutical industry [3,4]. The development of efficient purification strategies capable of achieving stringent HCPs clearance requirements while maintaining high product recovery remains a major challenge in recombinant protein manufacturing. Because individual purification techniques rely on distinct separation mechanisms, combining complementary unit operations can improve impurity removal and enhance overall process robustness. Such approaches are commonly referred to as orthogonal purification strategies because they exploit different physicochemical properties to remove contaminant populations that may not be efficiently cleared by a single purification mechanism. In the purification process developed for recombinant human epidermal growth factor (rh-EGF), hydrophobic interaction chromatography (HIC) was evaluated as an intermediate purification step, whereas tangential-flow filtration (TFF) was implemented as a final polishing operation. HIC separates biomolecules according to differences in surface hydrophobicity under high-salt conditions and is particularly effective for removing hydrophobic contaminants, protein variants, and residual process impurities. In contrast, TFF primarily separates molecules on the basis of size-dependent membrane transport while simultaneously enabling concentration and buffer exchange. Appropriate selection of membrane molecular-weight cut-off and tangential flow filtration conditions allows efficient removal of low-molecular-weight contaminants as well as residual HCPs that remain after chromatographic purification [5-8]. The combination of HIC and TFF provides an attractive orthogonal purification strategy because the two operations are governed by fundamentally different separation mechanisms. HIC relies on differential hydrophobic interactions between proteins and the chromatographic matrix, whereas TFF achieves impurity removal through membrane-based size exclusion and convective transport. The integration of these complementary operations is therefore expected to improve HCPs clearance efficiency and reduce the probability of impurity

breakthrough during downstream processing. The objective of this study was to evaluate the performance of HIC and TFF as orthogonal purification operations for the removal of HCPs during rh-EGF purification. Process performance was assessed in terms of HCPs reduction, product recovery, and purity, while the quality of the purified rh-EGF was confirmed using complementary analytical techniques. Particular emphasis was placed on quantifying the contribution of each unit operation to impurity clearance and on establishing operating conditions suitable for future process scale-up and industrial implementation.

2. Materials and methods

2.1 Starting biological material

The starting material was obtained from the primary purification of multiple fermentation batches containing recombinant human epidermal growth factor (rh-EGF), secreted in soluble form by *Saccharomyces cerevisiae* transformed with an expression plasmid carrying the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter and terminator, together with the α-factor secretion signal.

The upstream purification process included a capture step based on cation-exchange chromatography (CEC) using SP Sepharose Fast Flow resin under conditions previously described by Rodríguez et al. [9].

Briefly, the conductivity of the CEC eluate was adjusted to 7.5 mS cm^{-1} and the pH was verified at approximately 5.6. The solution was filtered through Sartobran-P capsules (0.45/0.2 µm) and loaded onto an XK 26/40 column packed with 212 mL of DEAE Sepharose Fast Flow resin. Protein loading was maintained at 2 g L^{-1} resin with a residence time of 23 min and a bed height of 40 cm.

After sample application, the column was washed with approximately 10 column volumes (CV) of 50 mmol L^{-1} sodium acetate, 5 mmol L^{-1} EDTA, and 50 mmol L^{-1} NaCl (pH 5.6). Protein elution was subsequently performed using 2 CV of 50 mmol L^{-1} sodium acetate, 5 mmol L^{-1} EDTA, and 300 mmol L^{-1} NaCl (pH 5.6).

The resulting anion-exchange chromatography (AEC) eluate was used as the starting material for all experiments described in this study.

2.2 Evaluation of rh-EGF adsorption conditions on Butyl-650C resin

A three-factor, three-level Box-Behnken response surface design was employed to investigate the effects of protein loading, ammonium sulfate concentration, and pH on rh-EGF adsorption efficiency. Experimental design generation and statistical analyses were performed using STATGRAPHICS Centurion XVII.II.

The design consisted of 15 experimental combinations performed in duplicate, resulting in a total of 30 analytical-scale experiments conducted in batch mode using Toyopearl Butyl-650C resin.

The concentrations of sodium acetate, EDTA, and sodium chloride present in the AEC elution buffer were maintained constant throughout all experiments.

The evaluated factors and levels were:

- Protein loading: 1.0, 5.5, and 10.0 g protein L^{-1} resin
- Ammonium sulfate concentration: 0.10, 0.55, and 1.00 mol L^{-1}
- pH: 5.60, 6.05, and 6.50

The AEC eluate was adjusted to the corresponding experimental conditions and clarified through sterilizing-grade Sartobran-P filters (0.45/0.2 μm).

For each experiment, 0.5 mL of settled resin was transferred into individual centrifuge tubes and equilibrated with the corresponding adsorption buffer. Following centrifugation at 3000 × g for 5 min, the supernatant was discarded and the equilibrated resin was used for adsorption studies.

The sample volume applied to the resin was calculated according to Equation 1 as a function of protein concentration and target protein loading. Samples were incubated with the resin for 10 min under orbital agitation at 300 rpm and subsequently centrifuged under the same conditions. The supernatant, corresponding to the non-adsorbed fraction, was collected and analyzed.

$$V_{ml} = \frac{Q \cdot V_{resina}}{C_{total}} \qquad (Eq.\ 1)$$

Where:

Q: load (g of protein/L of resin)

Vresin: resin volume (L)

Total concentration: protein concentration (g/L)

2.3 Laboratory-scale hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) was optimized using a decreasing ammonium sulfate gradient to maximize HCPs removal while maintaining product recovery, purity, and the desired rh-EGF51/rh-EGF52 isoform ratio. Based on the adsorption study, the AEC eluate was adjusted by controlled dilution (1:4) using 2.5 mol L^{-1} ammonium sulfate. The adjustment was performed by gradual dropwise addition over 20 min under constant stirring to prevent local protein precipitation. Chromatographic experiments were conducted using an XK 16/10 column packed with

20 mL of Toyopearl Butyl-650C resin. The column was equilibrated with five column volumes of buffer A corresponding to the selected adsorption conditions. The adjusted sample was loaded at a residence time of approximately 5 min. Fraction collection commenced upon detection of an increase in UV absorbance and continued until the signal decreased to 10% of the maximum recorded value.

Following sample loading, a stepwise decreasing ammonium sulfate gradient was applied. The gradient consisted of sequential transitions corresponding to 100%, 60%, 40%, and 0% ammonium sulfate, using 10 column volumes for each intermediate step. The target protein was finally eluted under salt-free conditions and collected until the UV signal declined to 10% of its maximum value.

Five independent chromatographic runs were performed to assess process reproducibility.

Process performance was evaluated through determination of total protein concentration, rh-EGF concentration, purity, recovery, and residual HCPs content. Total protein and rh-EGF concentrations were quantified by Bradford assay and ELISA, respectively. Product purity and isoform distribution were determined by RP-HPLC. Residual HCPs content was quantified using the Immunodot assay. After each run, the chromatographic matrix was sanitized and stored in 20% (v/v) ethanol.

2.4 Optimization of tangential-flow filtration operating conditions

Tangential-flow filtration (TFF) was investigated as a polishing operation to maximize HCPs removal while maintaining rh-EGF recovery.

Two TFF strategies were evaluated:

Strategy I

- 10 kDa membrane followed by 2 kDa membrane concentration.

Strategy II

- 30 kDa membrane followed by 2 kDa membrane concentration.

In both cases, continuous TFF was performed using 10 mmol L^{-1} phosphate buffer (pH 7.0). Following completion of diafiltration, the retentate was concentrated to obtain a final protein concentration of 0.2-0.5 g L^{-1}.

The influence of diafiltration volume was studied using 4, 6, 8, 10, and 12 Dia volumes (DV), where one DV corresponds to a buffer volume equivalent to the initial feed volume. Three independent experiments were performed for each condition, generating a total of 30 experimental runs.

The feed material consisted of HIC eluate obtained under the optimized chromatographic conditions described above and derived from a 5-L fermentation batch. The material was filtered through Sartobran-P capsules (0.45/0.2 µm) prior to TFF processing.

Experiments were performed using a SARTOFLOW® TFF system equipped with Slice 200 Hydrosart® stabilized cellulose cassettes (membrane area: 0.1 m^2). Hydrosart membranes were selected based on preliminary studies demonstrating approximately 1.5-fold higher rh-EGF recovery than comparable polyether sulfone membranes.

Controlled operating parameters included:

- Transmembrane pressure: 1.0×10^5-1.5×10^5 Pa
- Feed flow rate: 7 mL min^{-1} (10 and 30 kDa cassettes)
- Feed flow rate: 20 mL min^{-1} (2 kDa cassette)
- Diafiltration buffer flow rate: 8 mL min^{-1}
- Agitation speed: 150 rpm

- Temperature: ambient (22-25°C)

Process performance was assessed in terms of HCPs removal efficiency and product recovery. Samples obtained from permeate and retentate streams were analyzed by Bradford assay, ELISA, RP-HPLC, and Immunodot analysis. After identifying the optimal operating conditions, the purified rh-EGF was subjected to additional characterization analyses.

2.5 Analytical methods

Total protein concentration was determined using the Bradford assay [10]. Recombinant human EGF concentration was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) employing specific capture and detection antibodies [11].

Product purity and isoform distribution were analyzed by RP-HPLC using a C18 column. Chromatographic separation was achieved by gradient elution with increasing organic solvent concentration. Purity was calculated as the combined area percentage corresponding to the rh-EGF51 and rh-EGF52 molecular species.

Residual HCPs content was determined using an Immunodot assay based on specific anti-HCPs antibodies. Bound antibodies were detected using a diaminobenzidine chromogenic substrate system. Although Immunodot analysis provides a rapid and sensitive method for monitoring impurity clearance during process development, complementary HCPs-specific ELISA assays are generally recommended to achieve more comprehensive impurity characterization.

Biological activity was evaluated using a dose-dependent proliferation assay employing murine NIH/3T3 clone A31 fibroblasts. The assay quantified the capacity of purified rh-EGF to stimulate cellular proliferation relative to a reference standard.

Molecular integrity was assessed by electrospray ionization mass spectrometry (ESI-MS) using a QTOF-2 hybrid orthogonal mass spectrometer equipped with a Z-spray ionization source. Analyses were performed to confirm the expected molecular mass and correct disulfide-bond formation.

All analytical procedures were conducted according to established quality-control protocols for biotechnology products and complied with applicable biosafety requirements and good laboratory practice standards.

3. Results and discussion

3.1 Evaluation of rh-EGF adsorption conditions on Butyl-650C resin

The effects of protein loading, ammonium sulfate concentration, and pH on rh-EGF adsorption efficiency were evaluated using a Box-Behnken response surface design. The standardized Pareto chart presented in Figure 1 identifies the factors that significantly influenced adsorption efficiency at the 95% confidence level.

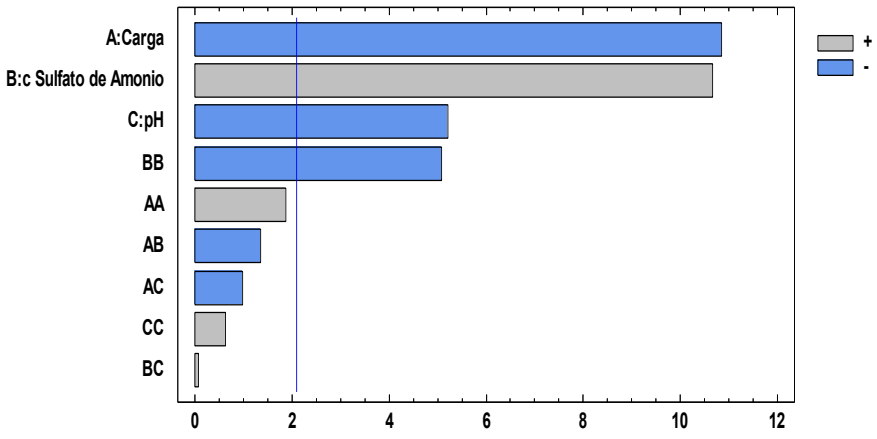


Figure 1. Standardized Pareto chart showing the effects of protein loading (A), ammonium sulfate concentration (B), and pH (C) on rh-EGF adsorption efficiency.

The analysis revealed that protein loading (A), ammonium sulfate concentration (B), the quadratic effect of ammonium sulfate concentration (BB), and pH (C) significantly affected adsorption efficiency. Protein loading, pH, and the quadratic term of ammonium sulfate concentration exerted negative effects, whereas ammonium sulfate concentration showed a positive linear effect. In contrast, the interaction terms (AB, AC, and BC) and the quadratic terms AA and CC were not statistically significant within the evaluated experimental region.

Following identification of the significant factors, the regression model was simplified by removing non-significant terms. The resulting model explained 91.83% of the observed variability in adsorption efficiency ($R^2 = 0.9183$), indicating a strong relationship between the selected variables and process performance. The adjusted model for the response variable is:

$$\text{Adsorption Eficiency} = 143{,}993 - 3{,}30556 \cdot \text{A} + 90{,}022 \cdot \text{B} - 15{,}8333 \cdot \text{C} - 52{,}2928 \cdot \text{B}^2 \quad (\text{Eq. } 2)$$

The adequacy of the model was confirmed through residual analysis. The Durbin-Watson statistic (DW = 1.91; *P* = 0.34) indicated the absence of significant autocorrelation in the residuals, satisfying an important model assumption. Furthermore, the standard deviation of the residuals was 5.64, and the mean absolute error (MAE) was 4.28, indicating that model predictions deviated from experimental values by an average of approximately 4.3 percentage points. Collectively, these indicators demonstrate that the model provides a reliable representation of the adsorption process and can be used to predict adsorption efficiency within the experimental design space.

The main effects plot shown in Figure 2 illustrates the magnitude and direction of the influence of each factor on adsorption efficiency.

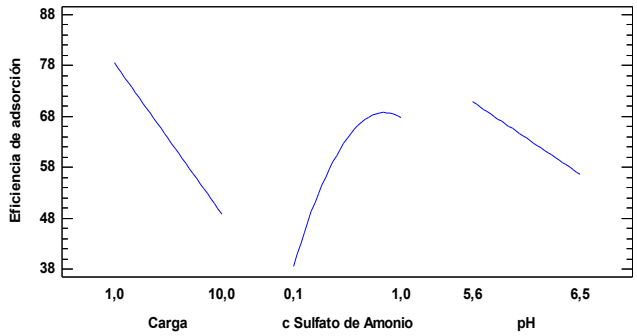


Figure 2. Main effects plot showing the influence of protein loading, ammonium sulfate concentration, and pH on rh-EGF adsorption efficiency.

Adsorption efficiency decreased as protein loading increased. The highest adsorption values were obtained at the lowest loading condition evaluated (1 g protein L^{-1} resin), suggesting that sufficient binding capacity remained available under these conditions. At higher loadings, progressive saturation of hydrophobic binding sites likely reduced protein retention efficiency.

Ammonium sulfate concentration exhibited a positive effect on adsorption up to an optimum region. Increasing salt concentration promotes protein adsorption by decreasing protein solvation and enhancing hydrophobic interactions between rh-EGF and the butyl ligands of the stationary phase [5,12]. This well-known "salting-out" effect increases exposure of hydrophobic protein regions and strengthens retention on HIC media. However, the negative quadratic coefficient indicates that adsorption efficiency declines beyond the optimum salt concentration. At excessive ammonium sulfate concentrations, competition between salt ions and protein molecules, combined with saturation of available hydrophobic binding sites, may limit further increases in adsorption efficiency [12].

The decrease in adsorption efficiency observed at increasing pH values may be explained by alterations in protein surface characteristics that weaken hydrophobic interactions with the

chromatographic matrix. Under these conditions, a larger fraction of rh-EGF remains in the flow-through stream, reducing overall protein capture. Moreover, elevated pH values may alter the conformational stability of rh-EGF, whose biological activity depends on the correct formation of three disulfide bridges. The response surface generated from the fitted model is presented in Figure 3.

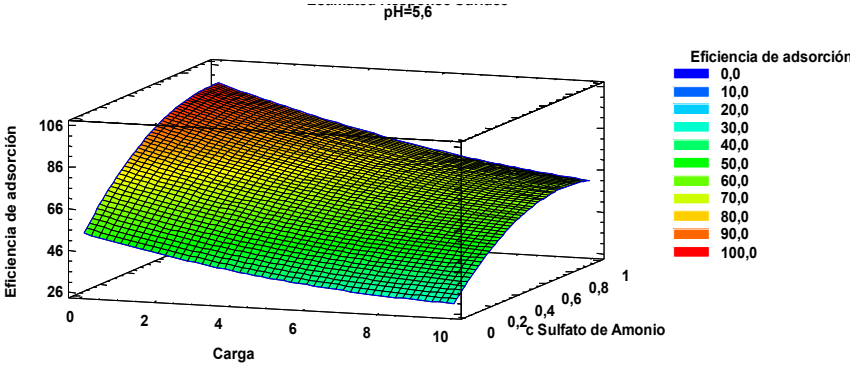


Figure 3. Response surface describing the combined effects of protein loading, ammonium sulfate concentration, and pH on rh-EGF adsorption efficiency.

The model predicted maximum adsorption efficiency values of approximately 91% at a protein loading of 1 g protein L^{-1} resin, an ammonium sulfate concentration of approximately 0.85 mol L^{-1}, and pH 5.6. These conditions were therefore considered optimal within the evaluated experimental domain.

Although 0.85 mol L^{-1} ammonium sulfate was identified as the predicted optimum, a lower concentration (0.55 mol L^{-1}) was also evaluated because of its potential advantages for process economics, buffer preparation, and large-scale operation. A comparative experiment was therefore conducted under identical pH (5.6) and loading (1 g protein L^{-1} resin) conditions.

The comparison was performed using one-way analysis of variance (ANOVA) with five independent replicates for each condition. As shown in Figure 4A, no statistically significant difference was

detected between the two ammonium sulfate concentrations (P = 0.897). Mean adsorption efficiencies were approximately 97% under both conditions. The overlap of the 95% confidence intervals further supports the absence of a significant effect.

Similarly, the box-and-whisker plot presented in Figure 4B demonstrates comparable distributions, similar variability, and the absence of outliers for both operating conditions.

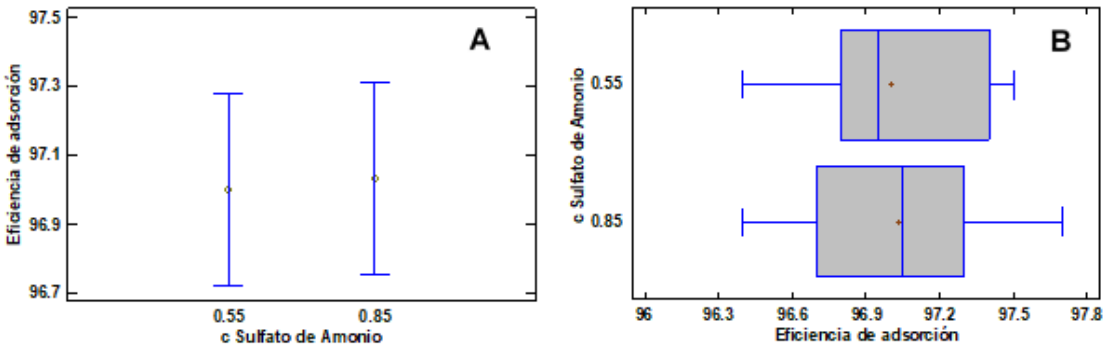


Figure 4. Comparison of adsorption efficiencies obtained using 0.55 and 0.85 mol L^{-1} ammonium sulfate. (A) Mean plot with 95% confidence intervals. (B) Box-and-whisker plot.

The combined statistical analysis demonstrated that reducing the ammonium sulfate concentration from 0.85 to 0.55 mol L^{-1} does not significantly compromise adsorption performance. Consequently, 0.55 mol L^{-1} was selected for subsequent process development because it reduces salt consumption, minimizes downstream desalting requirements, and improves process scalability without adversely affecting protein binding.

3.2 Laboratory-scale hydrophobic interaction chromatography

Hydrophobic interaction chromatography was subsequently evaluated under the optimized adsorption conditions identified during the previous study. Chromatographic separations were performed using a stepwise decreasing ammonium sulfate gradient designed to maximize HCPs removal while maintaining the desired rh-EGF isoform distribution.

The optimized adsorption conditions resulted in an average binding efficiency of approximately 99% at laboratory scale, demonstrating excellent reproducibility and confirming successful scale-up from batch adsorption experiments to column chromatography. The chromatographic profile obtained during HIC is shown in Figure 5.

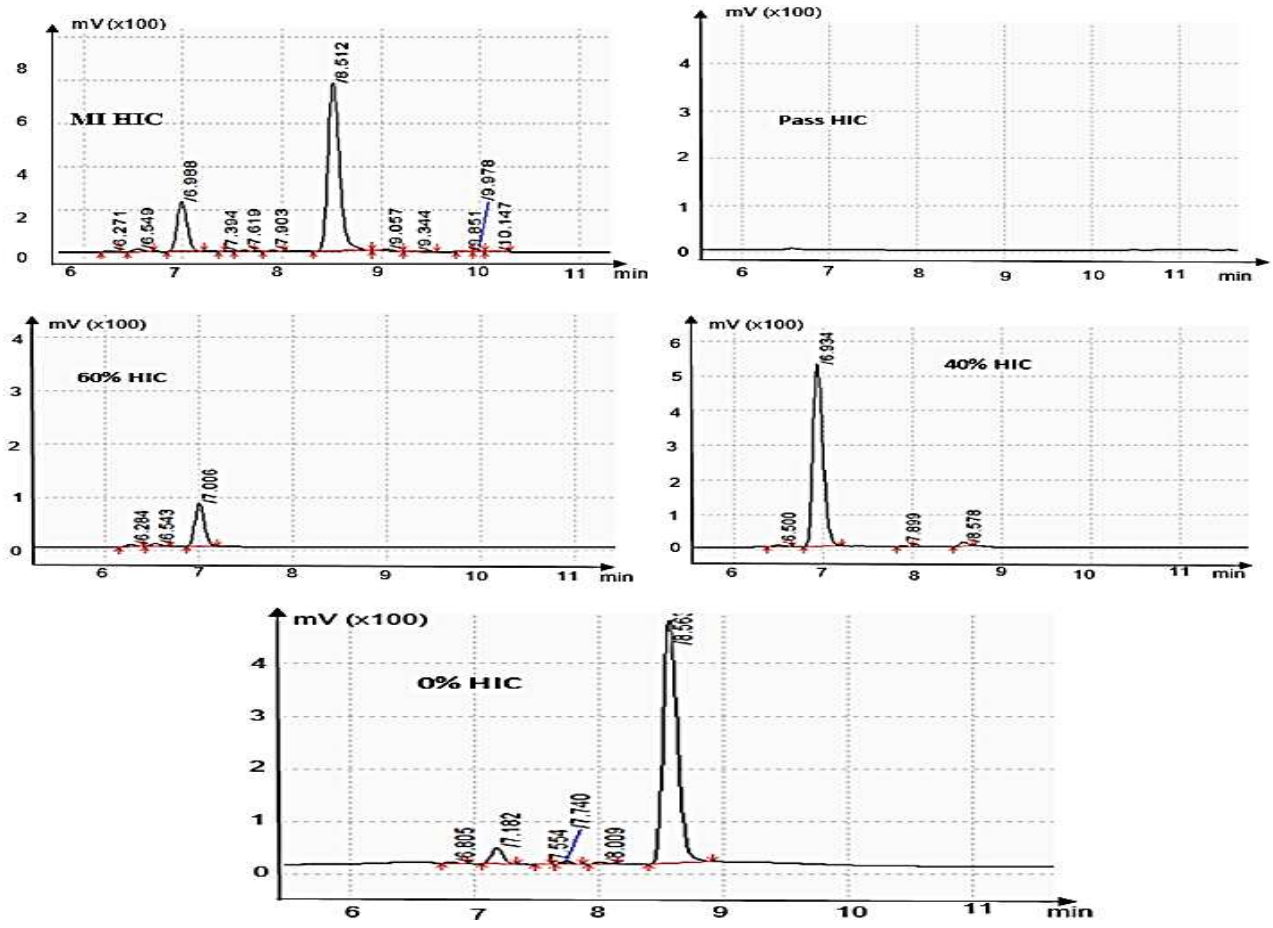


Figure 5. HIC chromatograms obtained using a decreasing ammonium sulfate gradient. Selective washing at 40% ammonium sulfate enabled separation of rh-EGF51, whereas rh-EGF52 was predominantly recovered during elution under salt-free conditions.

No significant UV absorbance signal was observed during the 60% ammonium sulfate wash step. However, RP-HPLC analysis demonstrated the removal of impurities and the partial separation of the rh-EGF51 isoform. During the 40% ammonium sulfate step, rh-EGF51 was selectively removed,

whereas rh-EGF52 remained bound to the chromatographic matrix. Consequently, this step effectively functioned as a selective wash stage that improved product quality and facilitated compliance with the predefined rh-EGF51/rh-EGF52 acceptance criterion ($0.01 \leq$ rh-EGF51/rh-EGF52 ≤ 0.54).

The final elution step, performed in the absence of ammonium sulfate, yielded a highly enriched rh-EGF52 fraction with only a minor contribution from rh-EGF51. Under the selected operating conditions, the HIC process generated product with 97% purity and 71% recovery, satisfying established quality specifications.

The separation achieved is attributable to subtle differences in the surface hydrophobicity of the rh-EGF isoforms. Although both isoforms differ by only a single amino acid residue, the resulting changes in surface hydrophobicity were sufficient to generate distinct retention behavior under decreasing salt-gradient conditions. Similar selectivity has been reported for the separation of monoclonal antibody variants and structurally related protein species using hydrophobic interaction chromatography, highlighting the ability of HIC to provide separation mechanisms complementary to charge-based chromatographic operations [6,13]. In addition to isoform control, HIC significantly improved impurity clearance. Residual HCPs content was reduced by approximately 6.5-fold relative to the AEC eluate, demonstrating that hydrophobic selectivity contributed substantially to host-cell proteins removal while preserving product recovery and purity. These findings confirm that HIC represents an effective intermediate purification step capable of simultaneously controlling product-related variants and process-related impurities.

3.3 Optimization of tangential-flow filtration operating conditions

Tangential-flow filtration was evaluated as a polishing operation to further reduce HCPs content and improve overall product quality. The effects of membrane molecular-weight cutoff (MWCO) and diafiltration volume on rh-EGF recovery and HCPs clearance are summarized in **Figure 6**.

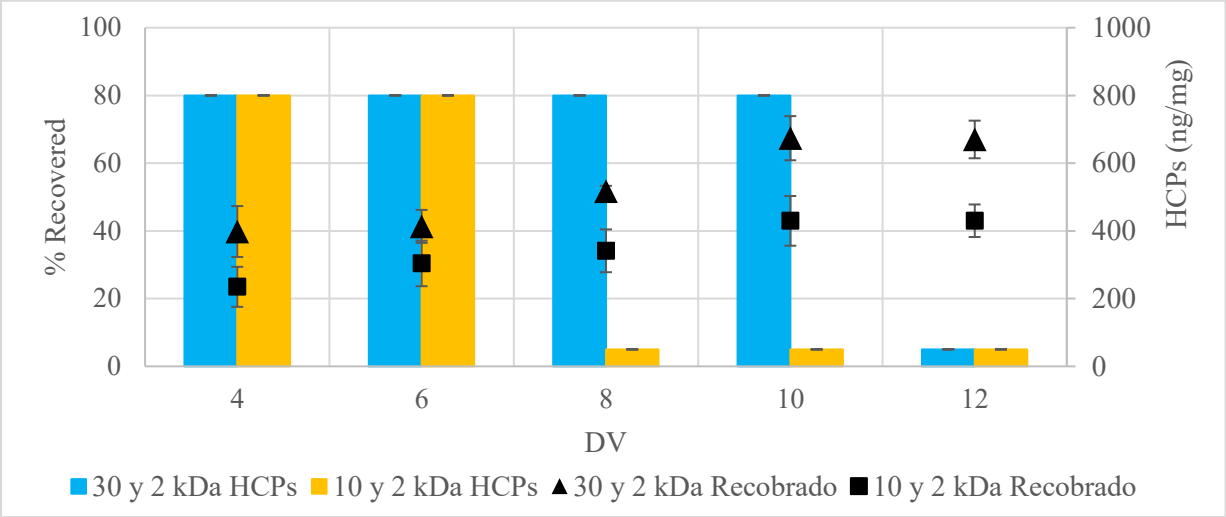


Figure 6. Effects of diafiltration volume and membrane configuration on rh-EGF recovery and residual HCPs content.

For both membrane configurations, rh-EGF recovery increased progressively with increasing diafiltration volume. However, the strategy employing a 30 kDa membrane followed by a 2 kDa concentration module consistently achieved higher recoveries across the investigated operating range.

Recoveries approached 70% at 10-12 diavolumes when using the 30/2 kDa configuration. This improvement is likely associated with the lower steric restriction and higher hydraulic permeability of the 30 kDa membrane, which reduce nonspecific protein adsorption and facilitate transport of rh-EGF into the permeate stream.

Host-cell proteins removal exhibited a strong dependence on the number of diavolumes applied. At low washing volumes (4-6 DV), both membrane configurations exhibited comparable impurity levels.

However, substantial improvement in HCPs clearance was observed as diafiltration progressed. The 10 kDa membrane achieved residual HCPs concentrations of approximately 50 ng mg^{-1} product after 8 DV, whereas a comparable level of impurity removal was obtained after 12 DV when the 30 kDa membrane was used.

The results highlight a typical downstream-processing trade-off between impurity clearance and product recovery. Although the 10 kDa membrane achieved faster HCPs removal, this benefit was offset by lower rh-EGF recovery. In contrast, the 30/2 kDa configuration provided superior overall process performance by maintaining high product recovery while still achieving target HCPs levels. From a bioprocess engineering perspective, the TFF optimal operating condition was therefore identified as diafiltration using a 30 kDa membrane followed by concentration with a 2 kDa membrane at 12 diavolumes. This configuration achieved the best balance between process yield, impurity clearance, and operational robustness while minimizing the risk of product losses during large-scale implementation.

Following identification of the optimal TFF conditions, HCPs removal was quantified throughout the purification process. Results obtained from four independent purification runs are presented in Figure 7.

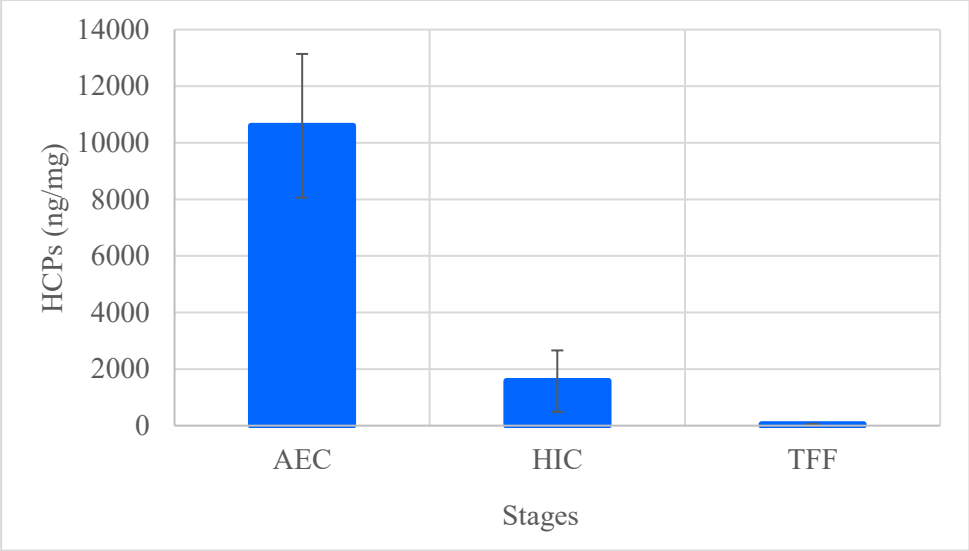


Figure 7. Residual HCPs content following AEC, HIC and TFF stages. Data represent mean ± standard deviation (n = 4).

A progressive reduction in HCPs concentration was observed after each purification step. Hydrophobic interaction chromatography reduced HCPs content by approximately 6.73-fold relative to the AEC eluate, whereas TFF provided an additional 31.5-fold reduction.

The present results confirm the orthogonal nature of HIC within the rh-EGF purification platform. Unlike the preceding ion-exchange chromatography step, which primarily exploits differences in protein charge, HIC separates proteins according to differences in surface hydrophobicity. The use of distinct physicochemical mechanisms increases process robustness and reduces the probability of impurity co-elution across multiple purification stages. Similar benefits of orthogonal chromatographic selectivity have been described for the purification and characterization of biopharmaceutical proteins and monoclonal antibody variants [12,13].

The final HCPs concentration was reduced to approximately 50 ng mg^{-1} rh-EGF, well below the commonly accepted target level of 100 ng mg^{-1} for recombinant protein products. These findings

demonstrate that the proposed purification sequence provides effective control of process-related impurities while maintaining acceptable product recovery.

The overall process yield achieved under the optimized operating conditions was 11.6 mg rh-EGF L^{-1} fermentation broth, indicating efficient recovery of the target protein throughout downstream processing.

3.4 Final product characterization

The quality of purified rh-EGF obtained using the optimized downstream process was assessed through complementary physicochemical and biological analyses. Product characterization included purity assessment, potency determination, molecular-mass verification, and confirmation of native disulfide-bond formation.

RP-HPLC analysis demonstrated a purity of 97%, with the predominant peak corresponding to the rh-EGF52 isoform (Figure 8A). The measured rh-EGF51/rh-EGF52 ratio was 0.01, confirming effective control of product heterogeneity during HIC purification. Consistent results were obtained by SDS-PAGE (Figure 8B), which showed a single protein band migrating at the expected molecular weight and no evidence of aggregation or degradation products.

The agreement between chromatographic and electrophoretic analyses confirms the high purity of the final product and demonstrates the effectiveness of the purification sequence.

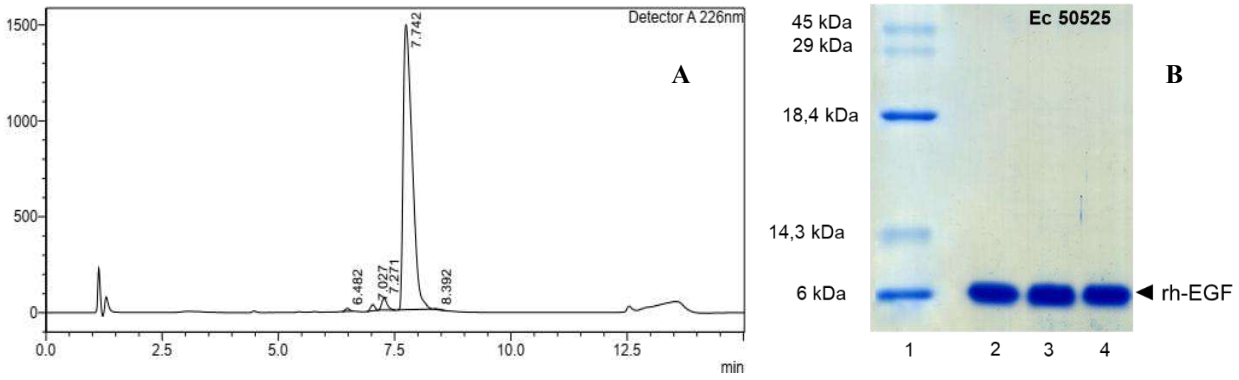


Figure 8. Results of the rh-EGF purity determination obtained with the new process. A. Chromatographic profile in RP-HPLC . B. Electrophoresis profile . 1. Molecular mass standard; 2. rh-EGF; 3. rh-EGF after final filtration (0.2 µm); 4. rh-EGF as homemade reference material.

The biological activity of purified rh-EGF was 12.76 × 10^5 IU mL^{-1}, corresponding to 34.77 × 10^5 IU mg^{-1} protein. This value falls within established product specifications and is consistent with previously reported activity values for recombinant human EGF. The dose-response curves presented in Figure 9 demonstrate parallelism between the analyzed samples and the reference preparation, validating the potency determination and confirming preservation of biological function throughout purification.

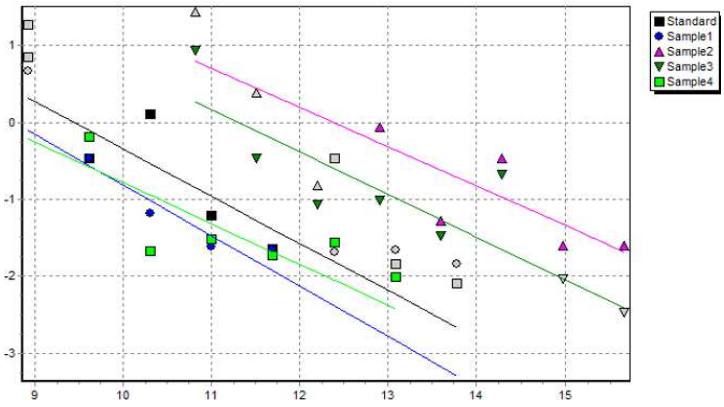


Figure 9. Dose–response curves of the cell proliferation assay. Where: Standard. Homemade Reference material; Sample 1. Positive control; Sample 2. rh-EGF replicate 1; Sample 3. rh-EGF

replicate 2; Sample 4. rh-EGF replicate 3. The parallelism between the samples and the standard of reference validates the calculation of relative potency

Mass spectrometric analysis of the intact protein (Figure 10) yielded an experimental molecular mass of 6059.83 Da, corresponding to the expected rh-EGF52 molecular species. The measured mass differed from the theoretical value by only 0.0002%, reflecting excellent analytical accuracy and confirming molecular integrity.

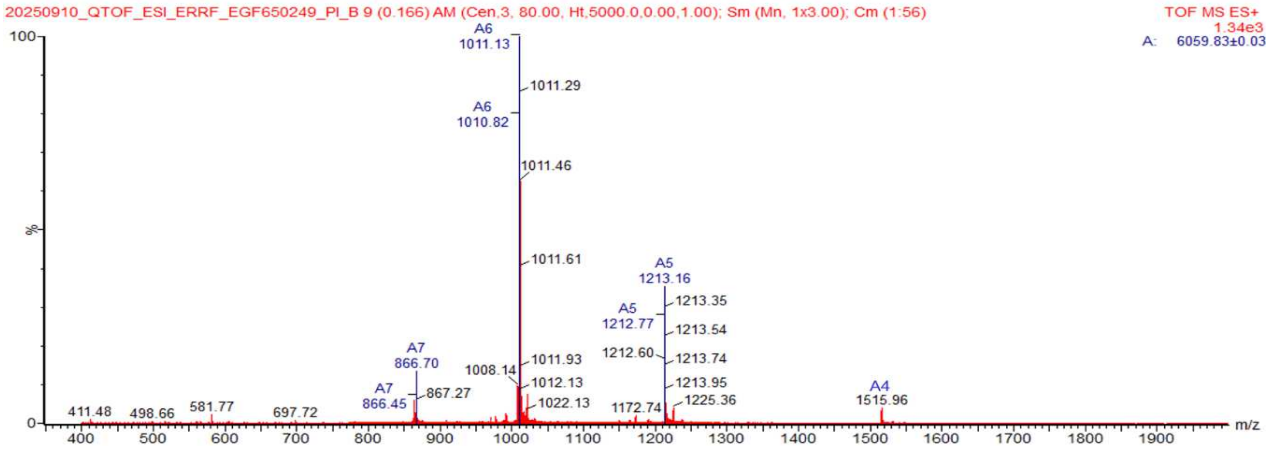


Figure 10. ESI-MS spectrum of the intact protein. Multicharged ions are identified with the labels A4 (4+), A5 (5+), A6 (6+), and A7 (7+). The experimental mass obtained from rh-EGF52 is shown in the upper right corner, indicated by the letter A.

Correct disulfide-bond formation was further verified by analysis of partially hydrolyzed protein fragments (Figure 11, Table 1). The identified peptide species confirmed the presence of the three native disulfide bridges linking Cys6-Cys20, Cys14-Cys31, and Cys33-Cys42. These structural features are essential for maintenance of the characteristic tertiary structure and biological activity of rh-EGF.

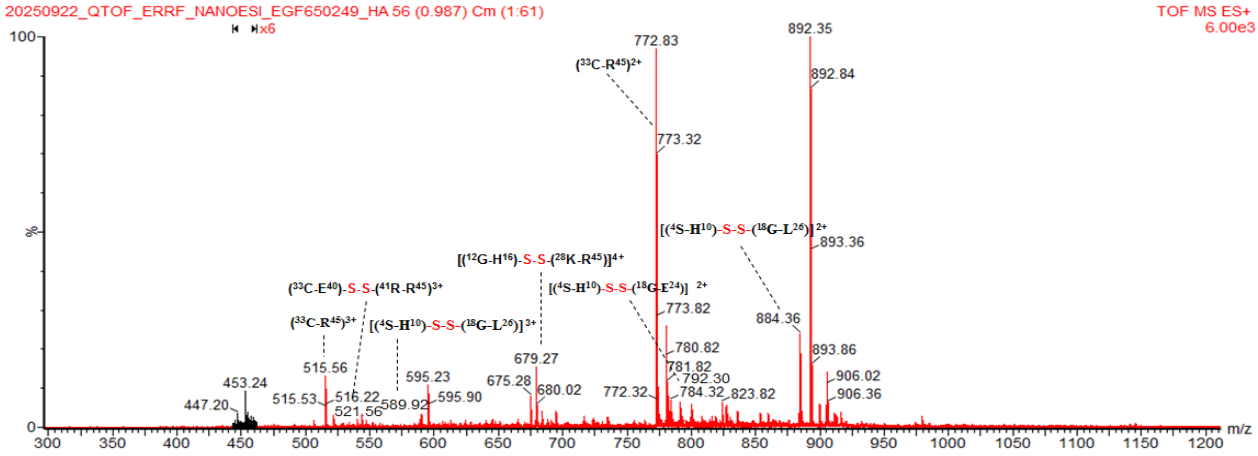


Figure 11. ESI-MS spectrum of the partial acid hydrolysis of rh-EGF. In the spectrum, the peptides that form disulfide bonds in the molecule are identified with dashed black lines.

Table 1 shows the m/z values of the generated peptides. Assignments were made according to agreement between theoretical data, experimental data, and the specificity of the chemical cleavage performed.

Table 1. Summary of peptides that form disulfide bonds in the rh-EGF protein

Theoretical m/z	z	Experimental m/z	Assignment
515.25	3	515.22	C ^{33}-R ^{45}
772.36	2	772.32	Peptide with an intramolecular disulfide bond between Cys ^{33} - Cys ^{42}
589.92	3	589.92	[(^{4}SH ^{10})-SS-(^{18}GL ^{26})]
884.38	2	884.36	Signal assigned to two peptides (^{4}SH ^{10} and ^{18}GL ^{26}) linked by an intermolecular disulfide bond between Cys ^{6}-Cys ^{20}
792.35	2	792.30	[(^{4}SH ^{10})-SS-(^{18}GE ^{24})]

			Signal assigned to two peptides (^{4}SH ^{10} and ^{18}GE ^{24}) linked by an intermolecular disulfide bond between Cys ^{6} - Cys ^{20}
678.81	4	678.78	[(^{12}GH ^{16})-SS-(^{28}KR ^{45})] Signal assigned to two peptides (^{12}GH ^{16} and ^{28}KR ^{45}) linked by an intermolecular disulfide bond that links Cys ^{14} - Cys ^{31} and also contains an intramolecular disulfide bond that links Cys ^{33} - Cys ^{42}

Collectively, the purity, potency, and mass spectrometry data demonstrate that the optimized purification process preserves the structural integrity and biological function of rh-EGF while achieving stringent control of host-cell proteins contamination. The combination of HIC and TFF therefore constitutes a robust orthogonal downstream processing strategy that is suitable for further scale-up and evaluation under industrial manufacturing conditions.

4. Conclusions

This study demonstrated the suitability of combining hydrophobic interaction chromatography (HIC) and tangential-flow filtration (TFF) as orthogonal downstream processing operations for the purification of recombinant human epidermal growth factor (rh-EGF) and the control of host-cell proteins (HCPs) impurities.

Response surface optimization identified a protein loading of 1 g protein L^{-1} resin, 0.55 mol L^{-1} ammonium sulfate, and pH 5.6 as appropriate operating conditions for rh-EGF adsorption on

Toyopearl Butyl-650C resin. Under these conditions, the model predicted an adsorption efficiency of 91%, which was subsequently confirmed during laboratory-scale chromatographic operation. The optimized HIC process generated rh-EGF with 97% purity and 71% recovery while reducing HCPs content by approximately 6.5-fold relative to the anion-exchange chromatography eluate. In addition, the selected stepwise ammonium sulfate gradient enabled effective control of the rh-EGF51/rh-EGF52 isoform distribution while simultaneously contributing to impurity clearance.

The subsequent TFF polishing stage demonstrated that diafiltration using a 30 kDa membrane followed by concentration with a 2 kDa membrane at 12 diavolumes provided the most favorable balance between product recovery and impurity removal. Under these conditions, residual HCPs content was reduced to 50 ng mg^{-1} product while maintaining 67% rh-EGF recovery.

The sequential implementation of HIC and TFF provided complementary and orthogonal separation mechanisms based on differences in protein hydrophobicity and membrane-selective transport. The integration of these distinct purification principles significantly enhanced overall impurity clearance and process robustness, resulting in final HCPs levels below the commonly accepted target for recombinant biopharmaceutical products.

The optimized downstream process achieved an overall yield of 11.6 mg rh-EGF L^{-1} fermentation broth while preserving product purity, biological activity, molecular integrity, and the native disulfide-bond architecture required for therapeutic functionality. Collectively, these findings demonstrate that the proposed purification strategy constitutes a robust platform for laboratory-scale rh-EGF manufacturing and provides a strong foundation for future process scale-up.

Future studies should focus on evaluating process robustness under scale-up conditions, resin and membrane reuse performance, scale-dependent mass-transfer phenomena, and implementation of

a validated product-specific HCPs ELISA assay to further strengthen impurity characterization and support industrial and regulatory requirements for commercial manufacturing.

Declarations

CRediT authorship contribution statement

Yadaimis Rodríguez Hernández: Conceptualization, Methodology, Validation, Formal Analysis, Data curation, writing – original draft, writing – review & editing, visualization.

Ana Ibis Paz Li: Conceptualization, Methodology, Validation, Formal Analysis, Data curation, writing – review & editing, visualization.

Madelin Mayeta Aguilera: Analytical assays contribute figures 7 - 8.

Edgar Rey Rodríguez Figueroa: Analytical assays prepare figures 10 – 11 and Table 1.

Joaquín González Amador: Analytical assays prepare figure 9.

Alexis Musacchio Lasa: Methodology and Data curation.

Dayana Soler Sánchez: Methodology and Data curation

Ania Cabrales Rico: Methodology and Data curation

Odalys Ruiz Hernández: Methodology and Data curation.

Miladys Limonta Fernández: Conceptualization, Methodology, Validation, Formal Analysis, Data curation, writing – review & editing, visualization, supervision, project administration.

All authors reviewed the manuscript

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Declaration of competing interest

“The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.”

Data availability

The manufacturing datasets analyzed in this study are not publicly available because they contain proprietary process information and coded industrial manufacturing variables.

Ethics statement

The biological activity assay used the commercially available murine 3T3 fibroblast cell line, clone A31, and did not involve human participants or live animals. Accordingly, approval by a human or animal research ethics committee was not required.

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