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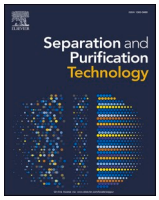

# Multimodal chromatography for the selective capture of pre-miRNA-29b therapeutics

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ABSTRACT

Nucleic acid-based treatments have become increasingly important in addressing diseases linked to genetic defects. In Alzheimer's disease, decreased levels of microRNA-29b lead to the overproduction of the human beta-secretase 1 (hBACE1) enzyme, which is associated with the progression of the condition. Consequently, pre-miR-29b represents a promising therapeutic agent, as restoring its levels could diminish hBACE1 expression and reduce amyloid-beta accumulation in neuronal cells. However, the successful deployment of pre-miRNA-29b as a treatment relies heavily on ensuring its purity, stability, and biological potency. This work evaluates the selective purification of pre-miRNA-29b, recombinantly produced from *Rhodovulum sulfidophilum,* using Capto Q ImpRes, a multi-modal strong anion exchange resin. The research optimizes process parameters, such as NaCl concentration and pH, to maximize the recovery and purity of pre-miRNA-29b through a Central Composite Design (CCD). The results reveal a relationship between recovery and purity, with CCD predicting optimal conditions yielding up to 48.21% recovery and 51.15% purity of pre-miRNA-29b. Overall, this work highlights the potential of using multimodal resins to selectively capture pre-miRNA-29b from complex recombinant samples. This selectivity demonstrates the promise of this technique for the isolation of specific RNA molecules, a crucial step in the development of effective nucleic acid-based therapies. These findings contribute to the ongoing efforts to establish highly effective purification strategies that ensure the quality of therapeutic RNAs.

## 1. Introduction

Nucleic acid-based therapies are emerging as personalized therapeutic approaches, holding substantial promise for addressing a broad spectrum of diseases, particularly those derived from genetic abnormalities. Gene therapy and DNA/RNA vaccination comprise the administration of nucleic acids as biopharmaceuticals, aiming to correct genetic abnormalities or induce an immunological response [1,2]. More recently, RNA has gained increased significance due to the recognition of its extensive involvement in biological functions, particularly through its various regulatory and enzymatic roles. Thus, RNA is considered an essential tool as a biomarker for diagnosis or as a therapeutic agent, capable of modulating the expression of numerous proteins involved in diverse cellular processes [3,4]. Among the various functions, recent studies have demonstrated that a specific RNA sequence, microRNA-29 (miRNA-29), plays a crucial role in the pathological mechanisms associated with Alzheimer's disease. In fact, a decrease in its concentration was associated with the overproduction of the beta-secretase 1 enzyme and the accumulation of amyloid-β peptides, which are implicated in the progression of the disease [5]. Therefore, administrations of miRNA-29 could restore the normal levels of amyloid-β peptides, representing a promising treatment against Alzheimer's disease. Furthermore, miRNA-29 has been implicated in the modulation of neuroinflammation and the activity of microglia, which are essential components of the brain immune response and may impact the progression of the same disease [6–8]. However, producing RNA in large quantities remains a challenge, particularly in meeting the safety and quality criteria imposed by regulatory agencies [9,10]. Actually, specific criteria for the quality requirements of RNA as a therapeutic product are still lacking. Although it is assumed that a high purity degree is required, the characterization of RNA as biopharmaceuticals relies on standards established for other gene-based products. RNAs can be manufactured through diverse techniques, including *in vitro* synthesis, chemical synthesis, and recombinant production. Although the Food and Drug Administration (FDA) has only

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approved the first two approaches, recombinant production demonstrates a higher ability to preserve the structural, functional, and safety characteristics of natural RNAs, thereby offering more promising avenues for large-scale manufacturing [11,12]. In particular, we recently reported the efficient recombinant production of an important precursor of miRNA-29 (*i.e.* pre-miRNA-29b) through perfusion cultures of *Rhodovulum sulfidophilum* [13,14]. This production method requires the extraction and purification of the target RNA from all cellular impurities, including cell debris, endotoxins, proteins, and host genetic material. In this context, chromatography is the technique of choice for purifying biomolecules effectively. Chromatography allows the isolation of various high-value products by exploiting physical–chemical differences in the interaction with chromatographic ligands. The most used chromatographic approaches are ion-exchange, ion-pairing, reversed-phase, affinity, and gel filtration [15–19], which are also used for RNA purification. However, due to the complexity of the mixture recovered from a fermentative process, multiple chromatographic steps are often required. The Capto Q ImpRes support has shown promising results to purify other types of biomolecules [17,20]. Matos *et al.* studied two commercial resins, Capto Adhere and Capto Q ImpRes, with different types of DNA. Single-stranded DNA (ssDNA) was shown to be less retained in Capto Q ImpRes compared to Capto Adhere, as a lower NaCl concentration was sufficient for its elution, which is beneficial when testing these chromatographic supports for biopharmaceutical purposes [17]. The ssDNA has a higher exposure of bases than double-stranded DNA, resembling the RNA structure more closely. Silva *et al.* successfully purified extracellular vesicles (EV) from mesenchymal stromal cells using Capto Q ImpRes, with a recovery of over 85 % and an effective removal of 98 % of proteins and host DNA. This work has proven the applicability of this resin in a process for EV-based therapeutics and its promising usage in large-scale purification processes [20]. Also, in another work by Qu *et al.*, using an Oligo-deoxythymidine (oligo (dT)) ligand-based affinity chromatography for effective mRNA purification, it was possible to achieve a yield of 90 % and purity of 99 % of an *in vitro* transcribed mRNA [21].

To reduce process complexity, downstream processing environment footprint, and costs, this work explores and optimizes the purification of pre-miRNA-29b extracted from *Rhodovulum sulfidophilum* in a single step using multimodal chromatography. Specifically, we relied on the Capto Q ImpRes, a multimodal chromatography resin designed for large-scale bioprocessing applications, particularly for the purification of biomolecules like proteins, monoclonal antibodies, and other biological products [17,22]. It combines quaternary ammonium functional groups, hydroxyl groups and carbon chains that can both establish H-bond and hydrophobic interactions under specific conditions [17]. To optimize the pre-miRNA-29b purification on this resin, an initial screening of binding and elution conditions was performed for intracellular nucleic acid samples. From this preliminary analysis, it was possible to determine a range of sodium chloride concentrations and adequate pH values that could be further explored in a central composite design of the experiments, aimed at defining optimal process conditions to achieve high recovery and purity of the target pre-miRNA-29b. Through this analysis, we provide a general procedure to guide process development and demonstrate the possibility of exploiting multimodal chromatography for the isolation of a recombinant product.

## 2. Materials and methods

### 2.1. Materials

All chemicals and reagents used were of analytical grade. The phototrophic marine alphaproteobacterium *R. sulfidophilum* DSM 1374 (Number 35886, Taxonomy ID1188256) was purchased from ATCC (Virginia, USA). For solid culture media, the following reagents were used: yeast extract, D-glucose anhydrous ($C_6H_{12}O_6$), and sodium chloride (NaCl) from Thermo Fisher Scientific Inc. (Waltham, USA), polipeptone, and magnesium chloride ($MgCl_2$) from Acros (Waltham, USA), LB-Agar from Oxoid (Waltham, USA), zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$) and manganese chloride tetrahydrate ($MnCl_2{\cdot}4H2O$), from Sigma Aldrich (St. Louis, USA), and iron sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) from Thermo Fisher Scientific Inc. (Waltham, USA). For liquid culture media it was used: tryptone, polipeptone, yeast extract, sodium chloride, potassium dihydrogen phosphate ($KH_2PO_4$), and D-glucose anhydrous ($C_6H_{12}O_6$) from Thermo Fisher Scientific Inc. (Waltham, USA), dipotassium hydrogen phosphate ($K_2HPO_4$) from Panreac AppliChem (Barcelona, Spain), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) from Labkem (Barcelona, Spain), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), and ammonium sulfate ($(NH_4)_2SO_4$) from Panreac (Barcelona, Spain), and kanamycin from Thermo Fischer Scientific Inc. (Waltham, USA). Isopropanol (purity 99.99 %) was purchased from Thermo Fisher Scientific Inc. (Massachusetts, USA). Tris (hydroxymethyl)aminomethane (Tris) was acquired from Merck (Darmstadt, Germany). All solutions were prepared with ultra-pure grade water, purified with a Milli-Q system from Merck Millipore (Darmstadt, Germany) treated with 0.01 % of diethyl pyrocarbonate (DEPC) from Sigma-Aldrich (St. Louis, Missouri, USA). The Capto™ Q ImpRes resin and HiScale™ 16/20 column were purchased from Cytiva (Sweden). The Urea-PAGE gel was prepared with urea from Acros (Waltham, USA). Green-Safe and Acrylamide/Bisacrylamide Solutions (37.5:1) were purchased from Grisp (Porto, Portugal). For protein quantification it was used a Dye Reagent Concentrate and bovine serum albumin (BSA), both purchased from Bio-Rad (California, EUA).

### 2.2. Bacterial growth for RNA production

*R. sulfidophilum* was cultured in a solid medium composed of 5 g/L of yeast extract, 10 g/L of glucose, 20 g/L of NaCl, 10 g/L of polypeptone, 4.10 g/L of $MgCl_2$, 15 g/L of agar, 1 mg/L of $ZnSO_4{\cdot}7H_2O$, 10 mg/L of $MnCl_2{\cdot}4H_2O$, 10 mg/L of $FeSO_4{\cdot}7H_2O$, supplemented with 50 μg/mL of kanamycin for 48 h, as previously optimized [14]. Hereafter, pre-fermentation and fermentation were performed using a culture media composed of 10 g/L of tryptone, 5 g/L of polypeptone, 0.6 g/L of yeast extract, 30 g/L of NaCl, 4 g/L of $K_2HPO_4$, 1 g/L of $KH_2PO_4$, 5 % (v/v) of glucose, 1 % (v/v) of $MgSO_4{\cdot}7H_2O$, 1 % (v/v) $CaCl_2{\cdot}2H_2O$, 1 % (v/v) of $(NH_4)_2SO_4$, 0.1 % (v/v) of TES buffer and 50 μg/mL of kanamycin. The medium and oxygen ratio was 1:5. Fermentation process started with flasks with an $OD_{600nm}$ of 0.3 with a duration of approximately 36 h and then the medium was centrifuged at 4 °C and 3900 g, for 10 min to obtain cell pellets which were stored at −20 °C.

### 2.3. Intracellular RNA extraction

RNA extraction was performed by the method of acid guanidinium thiocyanate-phenol–chloroform, as described in the literature [23,24]. First, the *R. sulfidophilum* cell pellets were resuspended with 5 mL of Solution D (4 M guanidinium thiocyanate, 0.025 M sodium citrate pH 7, 0.5 % sodium N-lauroylsarcosinate and 0.1 M β-mercaptoethanol) and incubated in ice for 10 min. After this, 0.5 mL of 2 M sodium acetate pH 4 and 5 mL of phenol were added, homogenizing carefully. Then, 1 mL of chloroform/isoamyl alcohol (49:1) was added followed by vigorous shaking and incubation in ice for 15 min. The suspensions were centrifuged at 10,000 g for 20 min at 4 °C. The upper phase enriched in RNA was carefully transferred to new tubes, avoiding DNA contamination from the bottom phase. To these new tubes, 5 mL of isopropanol were added, and the samples were then centrifuged at 10,000 g for 20 min at 4 °C. RNA pellets were dissolved in 1.5 mL of D Solution, and then 1.5 mL of isopropanol, followed by centrifugation at 10,000 g for 10 min at 4 °C. The resultant pellets were resuspended in 2.5 mL of 75 % ethanol in DEPC water, incubating the samples at RT for 10–15 min, followed by a centrifugation at 10,000 g for 5 min at 4 °C. The pellet was then air-dried for 5–10 min at RT. RNA pellets were finally dissolved in 1 mL of DEPC treated water and incubated at RT for 10–15 min. The concentration of

RNA was measured in the Nano Photometer (IMPLEN, United Kingdom) and integrity of the samples was verified by 1 % agarose gel electrophoresis, being the samples stored at −80 °C.

### 2.4. Chromatographic assays

The chromatographic assays for the purification of the pre-miRNA-29b were carried out on an AKTA Avant® equipment with the software UNICORNTM 6.3 (GE Healthcare Biosciences Uppsala, Sweden) aiming at achieving the best process control and performance. The Capto Q Impres support was packed in a HiScale™ 16/20 column (with 16 mm diameter x 40 mm of height). For initial chromatographic assays, the column was equilibrated with a 10 mM Tris (pH 7 or 8) solution (Buffer A) previously filtered and sonicated, using a flow rate of 1 mL/min. After equilibration, 50 µg of RNA sample (dissolved in binding buffer) were injected in a 100 µL loop. When completed the binding step, a linear or stepwise gradient of increasing salt concentration up to 1.5 M of NaCl in 10 mM Tris (pH 7 or 8) was applied (Buffer B) to analyse the retention patterns and eventual species separation. A final step with 3 M NaCl was performed, to guarantee strip of the column. All experiments were performed at room temperature. The absorbance of eluted species was continuously monitored at 260 nm. The fractions of the elution peaks were recovered and further desalted and concentrated with concentrators Vivaspin 10000 Da (Vivascience) until reaching 100 µL, for further analysis by denaturant polyacrylamide gel electrophoresis. To ensure total cleaning and sanitization of the resin, a regeneration protocol using 0.2 M NaOH was established after every 10 experiments.

### 2.5. Polyacrylamide gel electrophoresis (Urea-PAGE)

Urea-PAGE was performed to verify if the separation conditions used in the chromatographic assays were able to properly separate the pre-miRNA-29 from the rest of the RNA extract. Gels were prepared by mixing 6 g of urea, 5 mL of MilliQ-Water and 1.5 mL of 10 × TBE Buffer (0.89 M tris base, 0.89 M boric acid and 0.02 M ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA). Afterwards, 5 mL of acrylamide were added to the mixture, followed by 25 µL of TEMED and 100 µL of PSA 10 % for gel polymerization. Samples were prepared by mixing 1:1 of RNA sample and formamide followed by denaturation at 60 °C, for 5 min. Then, 20 µL of each sample were injected into the gel, and the electrophoretic run was at 130 V for 60 min. For nucleic acids visualization, the gel was incubated with 0.01 % of Green Safe in TBE buffer and further visualized using ultraviolet (UV) light exposure in the Bio-Rad equipment using the ChemiDoc software.

### 2.6. Central Composite Design for pre-miRNA-29 purification

The Central Composite Design (CCD) was used for the optimization of pre-miRNA-29b purification from the intracellular RNA sample. The factors studied were the NaCl concentration in the first and second chromatographic steps, and the pH (Table 1). Each factor had 2 levels: −1 and 1, with the pH set as a categorical factor. The software used was Design Expert (Version 13), and 28 experiments were performed in two blocks. The responses established were recovery yield (R1) and purity level (R2) of pre-miRNA-29b. The recovery yield was determined by measuring the band intensity of pre-miRNA-29b in the peak of interest (after purification) divided by the corresponding band density in the injected sample. The purity level was determined as the ratio between the intensity of the pre-miRNA-29b band and those of the contaminants present in the peak of interest. To measure the intensity of each band, it was used the Image J software.

**Table 1**
Selected factors for Central Composite Design and respective low and high values for the optimization of intracellular pre-miRNA-29b purification.

| Factors | Levels | |
|---|---|---|
| | −1 | 1 |
| [NaCl] in first step (M) | 0.60 | 0.63 |
| [NaCl] in second step (M) | 0.63 | 1.5 |
| pH | 7 | 8 |

## 3. Results and discussion

### 3.1. Screening of conditions for the selective capture of intracellular pre-miRNA-29

*R. sulfidophilum* was used for target production, highlighting that from a total RNA concentration of 7 µg/mL, only 0.7 µg/L corresponds to pre-miRNA-29b. This fact highlights the importance of developing an effective purification strategy for the biomolecule, the target pre-miRNA represents only 0.01 % of the total RNA extracted [25]. To understand and evaluate the binding and elution pattern of pre-miRNA-29b on the Capto Q ImpRes support, different buffer compositions were studied, by varying the ionic strength and pH values. Given the structure of the ligand (Fig. 1), interactions are supposed to be mainly promoted by the positively charged ammonium group and negative charges on the RNA. However, the ligand's chemical structure also features hydroxyl groups that can participate in hydrogen bonding and alkyl chains that may form hydrophobic interactions. These two types of interactions confer to the support a multimodal nature, since different types of interactions can be favoured depending on buffer composition. Therefore, salt concentration and pH, which, are known to highly influence ionic interactions, were appraised, allowing to determine the range that must be evaluated in the design of experiments, to ultimately find the best purification performance.

As a first approach in this study, a linear gradient of sodium chloride (Fig. 2A) from 0 to 3 M (pH 8) was evaluated in order to verify the general retention pattern. Two distinct peaks were observed throughout the gradient, which already demonstrated a difference in retention time, presumably due to different RNA species. In the corresponding urea-PAGE, it was possible to see that small RNA (sRNA) tended to be less retained since they mainly appeared in the first peak, although almost all the species of the sample eluted in the second peak. Nonetheless, the species present in the sample are highly similar, meaning that the retention profile is also similar, and with a linear gradient, the resolution is compromised. These results suggest that the separation is largely based on overall charge and size differences, rather than specific sequence motifs. What mainly contributes to the selectivity towards the target is the diverse functional groups and moieties of the ligand, which can simultaneously participate in various interactions.

A step gradient was then tested, starting with 0.75 M NaCl and increasing to 1.5 M NaCl after 12 min to better understand the sample distribution under these conditions. As shown in Fig. 2B, the first step led to the elution of the majority of the sample, resulting in a substantial loss of pre-miRNA-29b and consequently, a low recovery in the second peak. Based on these results, the salt concentration in the first step was decreased to 0.60 M of NaCl, with the aim of avoiding the premature elution of the target pre-miRNA-29b. Interestingly, this alteration in salt

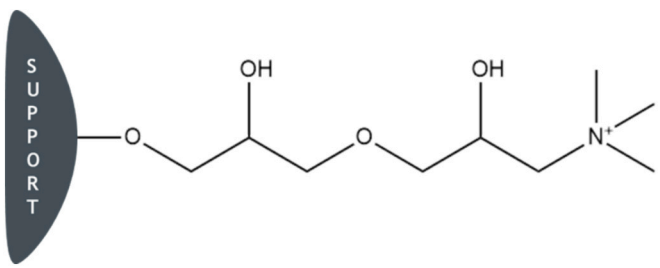


**Fig. 1.** Capto Q ImpRes ligand structure (adapted from manufacturer data file).

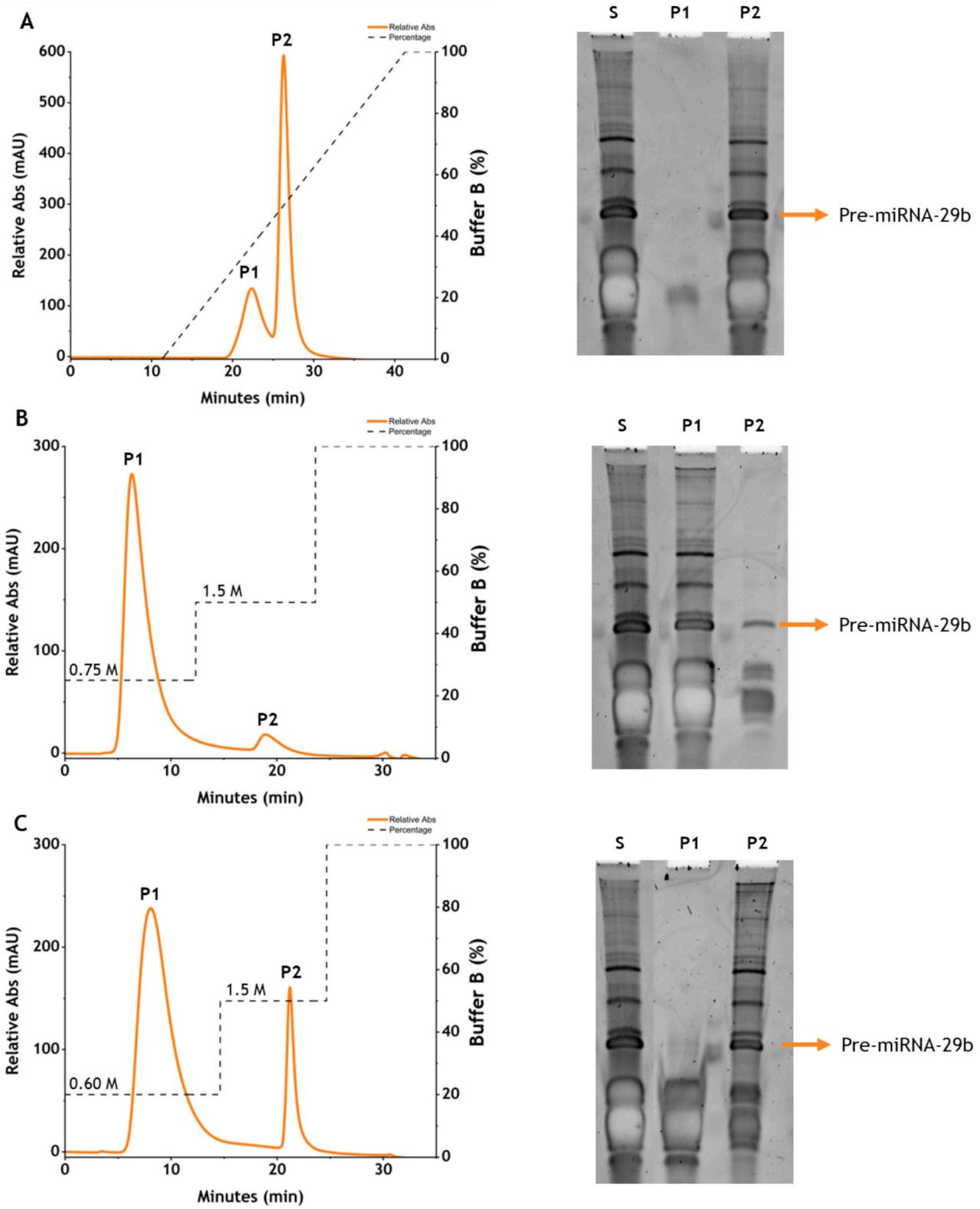


**Fig. 2.** Representative chromatograms of initial assays, evaluating different salt concentrations and gradients for the screening of binding and elution conditions of intracellular RNA sample, and respective Urea-PAGE. A – NaCl linear gradient from 0 to 3 M; B – Stepwise gradient with 0.75 M and 1.5 M NaCl in the first and second steps, respectively; C – Stepwise gradient with 0.60 M and 1.5 M NaCl in the first and second steps, respectively; S – Sample; P1 – Peak 1; P2 – Peak 2.

concentration resulted in a more evident distribution of the different RNA species in the two peaks (Fig. 2C). In peak 1, a considerable amount of sRNA was eluted, consistent with the lower number of charges on these molecules and hence the weaker interaction with the solid phase, while pre-miRNA-29b was only eluted in the second step. These results sustain the tendency for pre-miRNA-29b to bind more strongly to the support, only eluting with higher salt concentrations. Nonetheless, a significant amount of impurities, such as tRNA and rRNA, still co-elute

with the target in the second step, highlighting the need for further process optimization.

In this direction, we decided to explore the addition of an intermediate NaCl step gradient to achieve better selectivity for the target molecule.

Starting with the NaCl concentration (0.60 M) that was demonstrated to be effective in the partial removal of sRNA from the injected sample, the concentration of the second step was then adjusted, in two independent experiments, to 0.68 M and 0.71 M NaCl (Fig. 3A and B, respectively), aiming to achieve a better purification of the target pre-miRNA. This minor change in ionic strength between the first and second steps was shown to have a significant impact on the selectivity of the ligand towards the pre-miRNA-29b. In both assays, the third peak revealed a considerably lower content of contaminants co-eluting with pre-miRNA-29b, compared to what was observed in the previous gradients. Nonetheless, it is still not a sufficient purity level for an intended biopharmaceutical grade. Despite the need for further optimization, this initial screening allowed the establishment of a range of NaCl concentrations in the first and second steps that could be implemented in the CCD. With 0.60 M of NaCl in the first step, a considerable removal of sRNA occurs, and no loss of pre-miRNA-29b is observed which could be set as the lowest value for this factor. Additionally, these screening results revealed that the RNA could interact with the ligands, but increasing sodium chloride concentration the electrostatic interactions between negatively charged RNA and the positively charged ligands are destabilized, leading to the RNA elution. Moreover, due to structural or physical–chemical properties, it was possible to find partial selectivity towards the pre-miRNA. It is worth mentioning that the co-elution of degraded RNA, or other small RNAs can lead to overestimated recovery or underrepresented purity. Although this was partially controlled using Urea-PAGE, more selective detection methods would be beneficial. Urea-PAGE was found to be very effective in routinely distinguishing molecules with different sizes, conformations, or aggregates/fragments (Fig. S1).

Besides the salt concentration, which significantly influences RNA binding and elution pattern in the resin, another crucial factor to evaluate is the buffer pH, considering that the electrostatic interactions are predominant in the RNA retention mechanism. This factor can significantly impact the retention of the target molecule, as it can induce changes in the protonation of biomolecules and the net charge of the chromatographic ligand.

Therefore, two additional screening assays were performed at pH 7 and pH 8 (Fig. 4A and B, respectively). It was demonstrated that this parameter has a significant impact on the retention pattern. At pH 7, the target molecule was less retained, starting to elute in the initial gradient step. On the other hand, at pH 8, the pre-miRNA-29 is more retained,

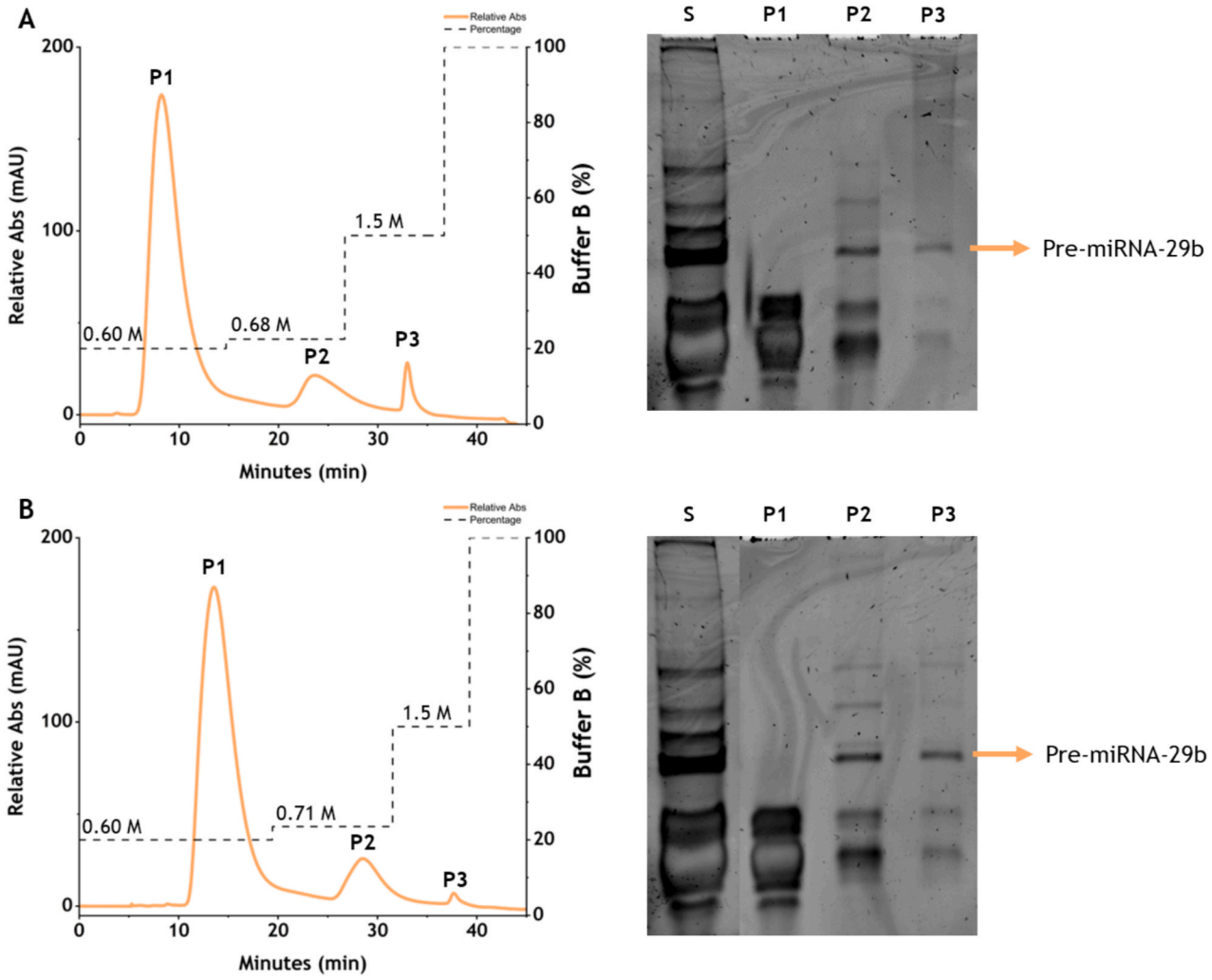


**Fig. 3.** Representative chromatograms of purification conditions with different salt concentrations and gradients for the screening of binding and elution conditions of intracellular RNA sample, and respective Urea-PAGE. A – Stepwise gradient with 0.60 M and 0.68 M NaCl (pH 8) in the first and second steps, respectively; B – Stepwise gradient with 0.60 M and 0.71 M NaCl (pH 8) in the first and second steps, respectively; S – Sample; P1 – Peak 1; P2 – Peak 2; P3 – Peak 3.

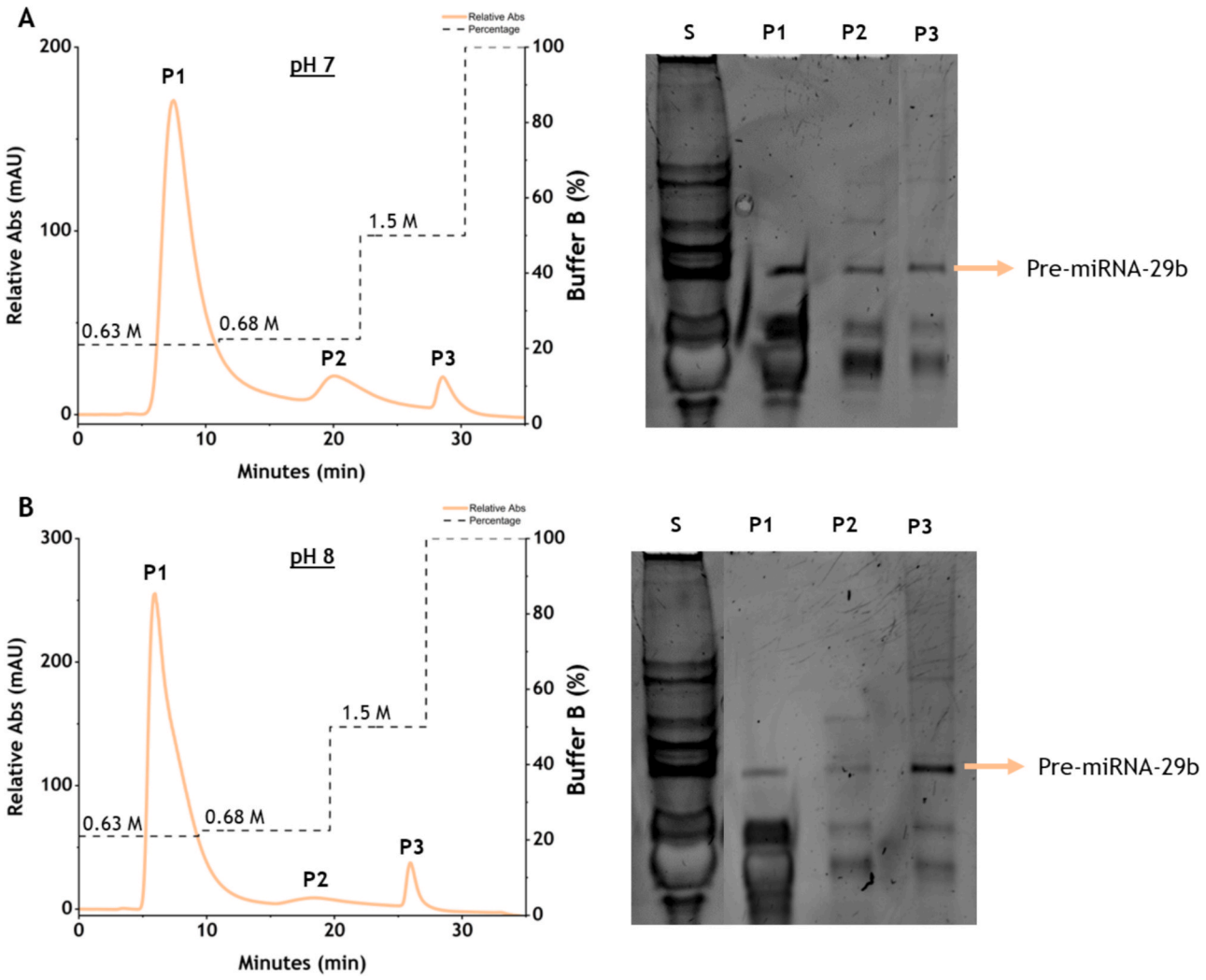


**Fig. 4.** Representative chromatograms of purification conditions in pH 7 (A) and pH 8 (B) for the screening of binding and elution conditions of intracellular RNA sample, and respective Urea-PAGE. A – Stepwise gradient with 0.63 M and 0.68 M NaCl in the first and second steps, respectively, at pH 7; B – Stepwise gradient with 0.63 M and 0.68 M NaCl in the first and second steps, respectively, at pH 8; S – Sample; P1 – Peak 1; P2 – Peak 2; P3 – Peak 3.

mainly eluting with the increase in salt concentration to 1.5 M. If only electrostatic interactions were occurring, it would be expected that the opposite behaviour would occur. In fact, the pKa of this ligand is approximately 9, which means that its net positive charge at pH 8 is lower than at pH 7, meaning that electrostatic interactions are not so preponderant. On the other hand, and given the multimodal character of the ligand, hydrogen bonds can be more relevant in these conditions and can be the reason for a higher retention of the target despite the higher pH [26,27]. At the same time, it was also important to identify a salt concentration at which pre-miRNA-29b started to elute in the first peak, thereby determining the optimal value for the first step in CCD. To address this, the NaCl concentration was slightly increased to 0.63 M in the first chromatographic step. As a matter of fact, with this initial step, the elution of some pre-miRNA-29b was observed (Fig. 4A, P1), and therefore, this concentration was set as the highest value for this factor in the Design of Experiments. With this, we have defined the conditions in which the molecule completely binds to the matrix or completely elutes. Therefore, to establish the parameters for the design of experiments, the range for the salt concentration was defined between 0.63 M and 1.5 M of NaCl. The pH was set as 7 and 8 and maintained as a categorical factor.

### *3.2. Central Composite Design for intracellular pre-miRNA-29 purification*

After performing the initial screening of the effect of NaCl concentration and pH on the binding/elution behaviour of the pre-miRNA-29 onto the Capto Q ImpRes support, an experimental design was established to optimize the purification process. Considering the tested conditions, mild salt concentrations and pH values, and the electrophoretic profiles displaying intact bands without signs of degradation, it can be assumed that the target molecule retains its biological folding following purification. The design chosen was the Central Composite Design, given that the results of the previous screening assays were already promising, and this model is known to be beneficial in optimizing nonlinear systems. As the purification process typically presents a nonlinear relationship between factors, this can be explored with a limited number of experiments in CCD. The analysis of the CCD resulted in a total of 28 experiments, in which recovery yield and purity level were defined as responses (Table 2). From the CCD, the recovery ranged from 0 to 73 % and purity from 0 to 78 %.

Equations **(1) and (2)**, resulting from the 28 performed runs, describe the recovery (Response 1 – R1) and purity level (Response 2 – R2) as a function of different factors.

**Table 2**
CCD generated runs and respective obtained responses.

| Run | [NaCl] in first step (M) | [NaCl] in second step (M) | pH | R1: Recovery (%) | R2: Purity (%) |
|---|---|---|---|---|---|
| 1 | 0.60 | 1.50 | 8 | 50 | 39 |
| 2 | 0.60 | 0.63 | 8 | 0 | 0 |
| 3 | 0.62 | 1.07 | 7 | 41 | 55 |
| 4 | 0.63 | 0.63 | 7 | 0 | 0 |
| 5 | 0.62 | 1.07 | 8 | 54 | 44 |
| 6 | 0.62 | 1.07 | 7 | 60 | 58 |
| 7 | 0.62 | 1.07 | 7 | 34 | 44 |
| 8 | 0.60 | 1.50 | 7 | 46 | 52 |
| 9 | 0.62 | 1.07 | 8 | 43 | 55 |
| 10 | 0.63 | 0.63 | 8 | 0 | 0 |
| 11 | 0.63 | 1.50 | 7 | 24 | 59 |
| 12 | 0.62 | 1.07 | 8 | 55 | 52 |
| 13 | 0.60 | 0.63 | 7 | 0 | 0 |
| 14 | 0.63 | 1.50 | 8 | 73 | 35 |
| 15 | 0.62 | 0.45 | 7 | 0 | 0 |
| 16 | 0.62 | 1.07 | 7 | 55 | 49 |
| 17 | 0.62 | 1.68 | 8 | 60 | 38 |
| 18 | 0.62 | 1.07 | 8 | 45 | 40 |
| 19 | 0.62 | 1.07 | 8 | 49 | 47 |
| 20 | 0.62 | 1.07 | 8 | 45 | 45 |
| 21 | 0.59 | 1.07 | 7 | 27 | 49 |
| 22 | 0.62 | 1.07 | 7 | 42 | 60 |
| 23 | 0.62 | 0.45 | 8 | 0 | 0 |
| 24 | 0.62 | 1.07 | 7 | 54 | 56 |
| 25 | 0.59 | 1.07 | 8 | 65 | 37 |
| 26 | 0.64 | 1.07 | 7 | 13 | 78 |
| 27 | 0.62 | 1.68 | 7 | 36 | 61 |
| 28 | 0.64 | 1.07 | 8 | 35 | 66 |

$$R1,\% = 48.11 - 3.90(A) + 20.68(B) + 5.06(C) - 7.87(A^2) - 13.31(B^2) \quad (1)$$

$$R2,\% = 49.78 + 5.33(A) + 20.30(B) - 4.41(C) - 17.24(B^2) \quad (2)$$

Where A and B are the NaCl concentrations in the first and second step of the gradient, respectively, and C is the pH.

Analysing the resulting equations, it is possible to evaluate which are the main effects that positively or negatively impact each response. Concerning the recovery yield, the NaCl concentration in the first step (A) negatively impacts the output, meaning that higher values of this factor will result in a lower response, i.e. lower pre-miRNA-29b recovery. On the other hand, the NaCl concentration in the second step (B) and the pH (C) have a positive impact on the recovery yield. This is in accordance with the optimization assays, as a higher concentration of NaCl in the first step results in a loss of pre-miRNA-29b in this step, consequently decreasing its recovery in the second step as intended. Also, higher NaCl concentration and pH favour pre-miRNA-29 elution in the second step, regardless of the purity level.

Regarding the purity level, the NaCl concentration in the first and second steps (A and B) has a positive impact on the outcome, while the pH (C) has a negative effect. Altering the pH and salt concentration can affect the surface charge of the ligand, thereby modifying its interactions with various forms of RNA. This factor is highly critical for the selectivity and subsequent purification of pre-miRNA-29b. To better understand these equations and the details provided about the model adjustments to the two responses, it is essential to conduct the analysis of variance (ANOVA) analysis and assess the fitting conditions. When analysing the ANOVA results (Table 3) for the two responses, the key factors to consider are the F-value, p-value, lack of fit, $R^2$, adjusted and predicted $R^2$, and adequate precision.

For the recovery yield, the model is significant due to the F-value of 15.98 and a p-value lower than 0.05 (<0.0001). Also, the lack of statistical significance in the model fit suggests a positive outcome, as it is desirable for the observed values to align with the quadratic model in the case of the recovery. The $R^2$ value indicates the proportion of the total variance in the dependent variable that is accounted for by the model. In this case, the $R^2$ is 0.79, suggesting that the model explains 79 % of the total variability. In contrast, the adjusted $R^2$ considers the number of predictors in the model and adjusts the statistic accordingly. Unlike the standard $R^2$, the adjusted $R^2$ considers only the factors and interactions significantly influencing the response variable. In this case, the predicted $R^2$ of 0.60 is in reasonable agreement with the adjusted $R^2$ of 0.74, given that the difference between them is less than 0.2. Adequate precision measures the signal-to-noise ratio, which should be greater than 4. A value of 11.63 for this model indicates that the signal is adequate and that the model can be used to navigate the design space.

The model is also significant for the purity, as an F-value of 34.16 and a p-value lower than 0.05 (<0.0001) were achieved. However, in this case, the lack of fit p-value of 0.04 is significant, which is not desirable because it means that the model is not explaining the data accurately, and there are patterns or variations in the data that the model is not able to represent. Regardless, the $R^2$ is 0.86, suggesting that the model explains 86 % of the total variability. Also, similar to the previous model, the predicted $R^2$ of 0.76 is in reasonable agreement with the adjusted $R^2$ of 0.84 (difference < 0.2). The adequate precision of 18.33, also above 4, indicates that the signal is adequate.

After statistical analysis, the performed CCD resulted in two different optimal conditions depending on the pH (Fig. 6). Based on these two conditions, as well as the global results obtained from the generated runs, it is clear that recovery and purity follow inverse patterns. This means that the conditions established to achieve higher pre-miRNA-29b purity result in lower recovery, while a higher recovery is always associated with lower purity. Thus, this is a clear parento front, and so the solution selected was to achieve a compromise in the two responses, in which each one is between 50–60 %. Solution 1 (Fig. 5) predicts a pre-miRNA-29b recovery of 60 % and a purity of 52 %, when using 0.62 M of NaCl in the first step, 1.35 M in the second step, and pH 8. This is in accordance with the previous analysis of the model, which predicted that a higher pH would have a positive effect on recovery.

On the other hand, Solution 2 (Fig. 5) predicts a recovery of pre-miRNA-29b of approximately 51 %, with a purity of 60 %, when using 0.62 M NaCl in the first step, 1.36 M NaCl in the second step, and a pH of 7. This is also in accordance with the previous analysis of the models.

Despite the purity not being as high as intended for a biopharmaceutical, Solution 2 presented a higher value compared to Solution 1. Thus, the optimal point was confirmed for the conditions found in Solution 2, and this was experimentally validated.

### 3.3. Optimal conditions validation

The design of experiments model employed in this study was not totally well-suited for forecasting the optimal range of values that would enhance the evaluated response factors (recovery and purity), as the $R^2$ was reasonable but not ideal, and the model for purity optimization presented a significant lack of fit. However, it was possible to predict a range of values for each of the studied factors that were more favorable for attaining optimal results. Fig. 6 represents the experimental result under the conditions obtained for Solution 2 by the CCD.

**Table 3**
ANOVA table for CCD model for the two analysed responses, recovery yield (R1) and purity level (R2).

| Response | F-value | p-value | Lack of fit (p-value) | $R^2$ | Adjusted $R^2$ | Predicted $R^2$ | Adequate Precision |
|---|---|---|---|---|---|---|---|
| R1: Recovery % | 15.98 | <0.0001 | 0.10 | 0.79 | 0.74 | 0.60 | 11.63 |
| R2: Purity % | 34.16 | <0.0001 | 0.04 | 0.86 | 0.84 | 0.76 | 18.33 |

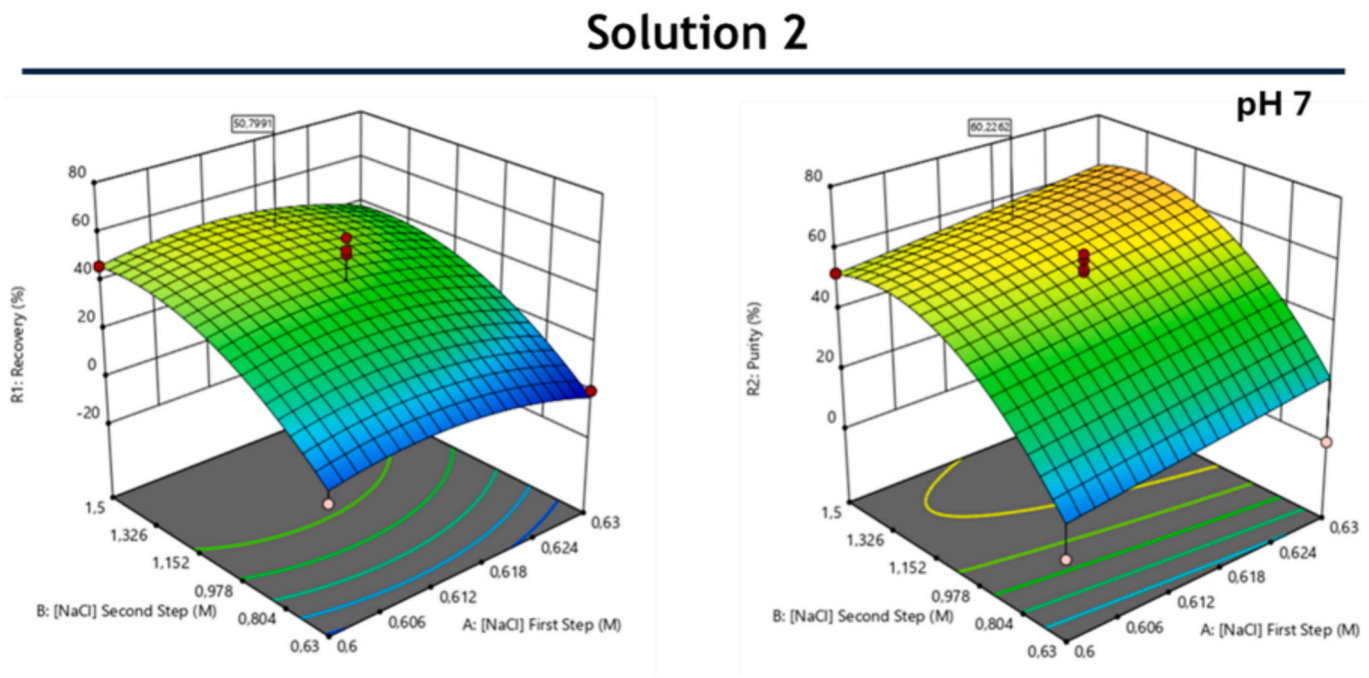


**Fig. 5.** Three-dimensional response surface plot for pH 8 (Solution 1) and pH 7 (Solution 2) of interactions of the sodium chloride concentration on the first and second steps and its effect on the recovery yield (left) and purity level (right).

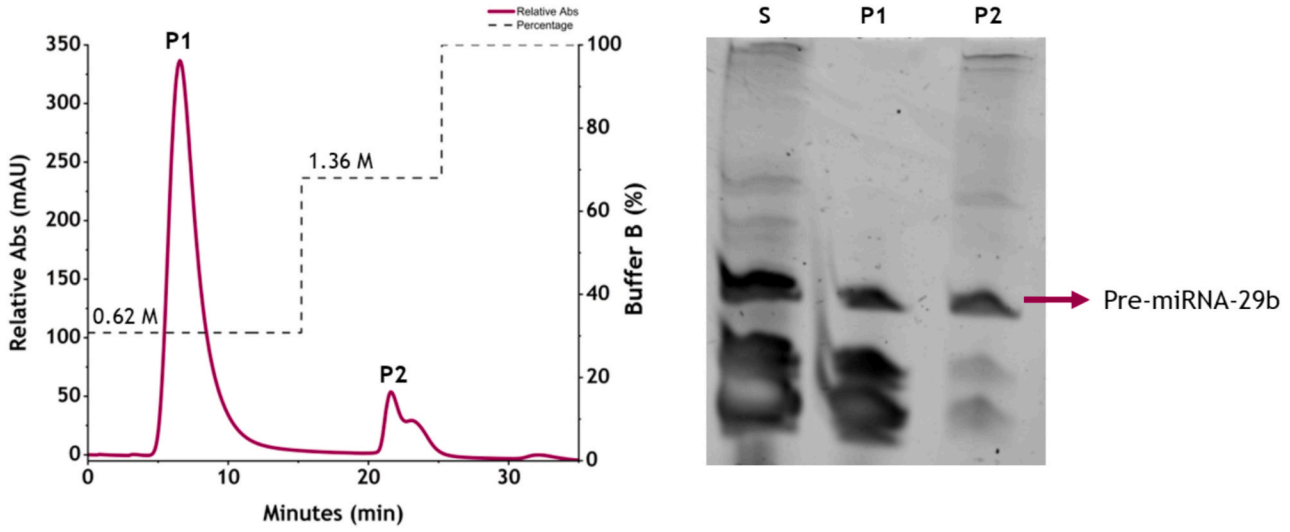


**Fig. 6.** Representative chromatogram of purification conditions for the optimal point (Solution 2) for pre-miRNA-29b purification of intracellular RNA sample and respective Urea-PAGE. Stepwise gradient with 0.62 M and 1.36 M NaCl, at pH 7 in the first and second steps, respectively; S – Sample; P1 – Peak 1; P2 – Peak 2.

The prediction of recovery and purity with this solution was 51 % and 60 %, respectively. In accordance with what was already observed in the initial optimization assays, 0.62 M NaCl in the first step was suitable for substantial removal of contaminants (mainly sRNA) from the initial sample. Nonetheless, it is evident that approximately 50 % of pre-miRNA-29b co-elutes with the contaminants in this step, representing a significant loss of the target during the washing step. Then, using 1.36 M NaCl in the second elution step, an effective recovery of pre-miRNA-29b was observed in peak 2 (Fig. 7). This assay resulted in 48 % and 51 % recovery and purity of pre-miRNA-29b, respectively. These values fall within the range of accepted values of the model for each studied response, validating the prediction of the optimal point. This assay was repeated to confirm the reproducibility of the matrix.

For a better characterization of the RNA samples, the protein quantification was also performed, revealing that even in the initial samples, the protein content was undetectable. The remaining contaminant nucleic acids were mainly low molecular weight RNAs (with no traces of DNA or rRNA). This is due to an efficient extraction protocol that, with the formation of two aqueous phases, enables the effective removal of DNA (bottom phase) and proteins (interface), leading to the recovery of

the RNA in the upper phase. Therefore, for the chromatography strategy, the challenge is to separate the target RNA from other RNA species. It was demonstrated that a significant reduction of other contaminants occurred during the optimized multimodal step; however, some smaller RNAs, such as tRNA, to co-elute with the target. It is worth mentioning that most current gene therapy workflows rely on Oligo(dT) technology for mRNA purification, which also has some limitations, as it only recognises RNAs with poly(A) tails and potentially captures non-target mRNAs. In contrast, multimodal chromatography offers improved selectivity by leveraging diverse interaction mechanisms through ligands with multiple functional groups, allowing broader applicability and greater process robustness. The potential integration of this technology in a downstream processing platform, may rely on the combination with other chromatographic or non-chromatographic techniques, to improve the global performance and achieve higher quality for the target molecules.

## 4. Conclusions

This study highlights the potential of multimodal chromatography for the selective capture of recombinant pre-miRNA-29b, produced by *Rhodovolum sulfidophilum*, a key target for therapeutic applications, particularly in Alzheimer's disease. By optimizing adsorption and elution conditions, including NaCl concentration and pH, a balance between recovery and purity was established. Using a Central Composite Design, recoveries of up to 73 % and a purity of approximately 78 % were achieved, despite the complexity of the sample, in which pre-miRNA-29b accounts for only 0.01 % of the total RNA extracted. The initial sample contains several small RNA species that are very similar to each other in terms of charge and structure, resulting in increased difficulty in achieving both high purity and recovery of the target biomolecule. However, this challenge can be overcome by combining chromatographic strategies. For example, a clarification step could be performed prior to the Capto Q ImpRes protocol. This could reduce the number of species loaded onto the multimodal support, resulting in a more effective polishing strategy, even on a large scale. While results demonstrate the effectiveness of Capto Q ImpRes under specific conditions, further refinements are necessary to improve purity and scalability, making this approach more viable for large-scale biopharmaceutical production.

## CRediT authorship contribution statement

**Rita Carapito:** Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **José Bento:** Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Mattia Sponchioni:** Writing – review & editing, Supervision, Conceptualization. **Mara G. Freire:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Fani Sousa:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

## 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2025.134887.

## Data availability

Data will be made available on request.

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