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ABSTRACT

Direct protein purification from raw materials by chromatographic media represents an enormous challenge, especially when particulate solids and high charge solute are present. In this sense, protein purification from cheese whey is an excellent opportunity to test new chromatographic matrices that can be applied to direct protein isolation. The present work shows the utility of novel multimodal chitosan-based chromatographic matrices for obtaining lactoferrin (LF) and a whey protein isolate (WPI) directly from cheese whey without any pretreatment. A central composite experimental design was used to optimise an operative sequence. This sequence involved LF capture using sulfanilic acid-modified chitosan mini-spheres followed by the capture of the massive remnant proteins using glycidyl trimethylammonium-modified chitosan mini-spheres. Interestingly, a yield of 68% and 70% purity degree was obtained for LF, and 2.71 mg of WPI mL^{-1} whey was obtained in the WPI recovery process, revealing a potential industrial use of the developed matrices and processes.

1. Introduction

Sweet whey is usually applied to food and feed products or commercialised as whey powders; however, a growing interest in its valorisation is mainly due to the high nutritional, biological, and technological value of its proteins and the large volumes produced (Doultani, Turhan, & Etzel, 2003; Mollea, Marmo, & Bosco, 2013; Urtasun et al., 2018).

One of the minor proteins present in cheese whey is lactoferrin (LF), which has a concentration of 0.02–0.2 g L^{-1} and an isoelectric point (pI) higher than 8 (Dizaji, 2016; Etzel, 2004). This protein is commercially interesting because of its role in the innate defence system and its antimicrobial, antioxidant, antiviral, immunomodulating, anti-inflammatory, and anticarcinogenic activities (Garcia-Montoya, Cendon, Arevalo-Gallegos, & Rascon-Cruz, 2012; González-Chávez, Arévalo-Gallegos, & Rascón-Cruz, 2009; Legrand, 2016; Manzoni, 2016; Wakabayashi, Oda, Yamauchi, & Abe, 2014). Due to its high pI compared with other proteins present in cheese whey (Urtasun et al., 2018), it can be selectively adsorbed on cation-exchange materials, which is why ion exchange chromatography is the most common choice for LF purification (Doultani et al., 2003; Pochet et al., 2018; Wakabayashi, Yamauchi, & Takase, 2006). The processing of the remaining whey not only brings economic benefits to the producers but also has a beneficial impact on the environment since the dumping of huge volumes of whey causes environmental pollution (Bacenetti, Bava, Schievano, & Zucali, 2018; Nicolás, Ferreira, & Lassalle, 2019; Price, 2019).

Nowadays, two protein products from cheese whey are marketed: whey protein concentrates (WPC) and whey protein isolates (WPI). Protein content in WPC usually varies from 35 to 85%, whereas WPI presents the advantage of having more than 90% protein and almost no carbohydrate content (Kilara & Vaghela, 2018; Nicolás, Ferreira, & Lassalle, 2018). Because of this characteristic, the greatest WPI market is currently intended for sports nutrition, since proteins play an important role in the

recovery after arduous exercise and consumers appreciate the absence of carbohydrates in these products (Price, 2019).

When processing complex starting materials, such as cheese whey, a pre-treatment is usually necessary before membrane filtration or column chromatography to avoid the fouling of the membranes or the column clogging (Ganju & Gogate, 2017). Some of the strategies used involve chemical, thermal, and ultrasound pre-treatments (Ganju & Gogate, 2017). The use of pre-treatments makes the processing more expensive, which is why using a separation method that avoids this step represents a great advantage. In this regard, our group has already developed several chitosan-based matrices that can be used in batch systems without any pre-treatment due to their size and density (Baieli et al., 2017b; Baieli, Urtasun, Miranda, Cascone, & Wolman, 2014b; Urtasun et al., 2017). Not only do these matrices make the process cheaper but their recovery is facilitated after each step as well.

Chitosan is a linear copolymer of 2-acetamide-2-dioxy-β-D-glucose and 2-amino-2-dioxy-β-D-glucose residues randomly distributed. It is the total or partial N-deacetylated derivative form of chitin, which is the second most abundant polysaccharide in nature after cellulose (Kumar, 2000; Kumari, Kumar Annamareddy, Abanti, & Kumar Rath, 2017; Roh & Kwon, 2002). Chitosan presents the advantage of being biodegradable, biocompatible, non-toxic, and highly available (Baieli et al., 2017a; Roh & Kwon, 2002; Shariatinia & Jalali, 2018). In addition, it has many interesting physicochemical properties, like pH sensibility, since it is soluble in dilute acidic media and insoluble in neutral or basic media (Baieli et al., 2017a; Kumar, 2000). This characteristic makes chitosan easy to handle for preparing fibres, hydrogels, micro/nanoparticles, or membranes. Moreover, it presents free amino and hydroxyl groups, which facilitate the immobilisation of various ligands, and is therefore often selected as a support material for chromatographic purposes (Baieli et al., 2017a; Shariatinia & Jalali, 2018; Yang et al., 2016). Regarding this chromatographic purpose, it is well known that chitosan beads or mini spheres formed by the acid/base phase separation possess an open pore internal structure, consisting in macro

and meso pores which facilitates protein binding (Subramanian & Hommerding, 2005). Because of its diffusive aspects, regarding protein binding dynamics, this type of chromatographic support has interesting applications in batch operation processes, especially for raw material processing (Baieli, Urtasun, Miranda, Cascone, & Wolman, 2014a).

Even though ion exchange chromatography is mostly used in the purification of LF from cheese whey, an appealing alternative is multimodal chromatography (Kallberg, Johansson, & Bulow, 2012; Yang & Geng, 2011; Zhang & Liu, 2016). This type of chromatography consists of ligands that interact with the target proteins in different ways, such as hydrophobic and Van der Waals interactions, in addition to the charge interactions (Johansson et al., 2003; Zhao, Dong, & Sun, 2009). In a previous work, a new multimodal matrix by sulfanilic acid immobilisation on chitosan mini-spheres was developed and characterised (Hirsch et al., 2018a,b). Sulfanilic acid is a precursor in the synthesis of triazine dyes and it presents an aromatic ring in addition to the sulfonic group. Good results in affinity purification processes with these dye-ligands have been studied and reported (Baieli et al., 2014b; Urtasun et al., 2017). An important difference between triazine dyes and their precursor is that the latter does not have regulatory restrictions as a ligand if the purified product or the matrix itself is applied to the food industry. The multimodal matrix developed was successfully tested for lysozyme purification from egg white in a previous study (Hirsch et al., 2018a). Given that LF has a high pI and a complex source, in this study the purification process parameters were optimised through statistical analysis for its purification from cheese whey using the sulfanilic acid-modified chitosan mini-spheres. Another matrix with the opposite charge was also developed to recover WPI. In this sense, quaternary amino groups were introduce in the chitosan mini-spheres by the immobilisation of glycidyl trimethylammonium as the ligand (Prado & Matulewicz, 2014; Senra, Campana-Filho, & Desbrières, 2018).

The response surface methodology (RSM) has been implemented to optimise the process (Das & Mishra, 2017; Yoshida, 1989)'. RSM combines mathematical and statistical techniques to improve processes by maximising or minimising (according to the objective set) the response of a variable to several factors (Martínez Álvarez, Lo Balbo, Mac Cormack, & Ruberto, 2015; Ravikumar, Krishnan, Ramalingam, & Balu, 2007). One of the main designs used in chromatography is faced-centred central composite design (CCF) (Kumar Gupta, Agarwal, Asif, Fakhri, & Sadeghi, 2017; Shojaeimehr et al., 2018). RSM is a particularly interesting approach for optimising the chromatographic parameters when using multimodal matrices since many factors are affecting the protein-matrix interactions.

Although LF is usually co-purified with lactoperoxidase (LP), this work focuses on LF purification since the market demand for this protein is bigger and it is found at a higher concentration in cheese whey. Despite the number of papers and patents for LF purification, it is still a challenge to purify LF from cheese whey at an industrial scale since not all the proposed technologies are cost-effective. The focus of this work was to purify this protein and take advantage of the remaining whey to further process it through the quantitative isolation of the other whey proteins and study their potential differential elution.

2. Materials and methods

2.1. Materials

Chitosan (low viscosity, Lot# BCBF7885V, acetylation degree 41%), sulfanilic acid (4-aminobenzenesulfonic acid), and glycidyl trimethylammonium chloride (GTMA) were from Sigma-Aldrich (St. Louis, MO, USA). LF standard (95% purity, 20% iron saturation) was from Friesland Campina Domo® (Amersfoort, The Netherlands). Epichlorohydrin was from Fluka Analytical (Buchs SG,

Switzerland). The Bicinchoninic Acid (BCA) protein assay kit for total protein determination was from Thermo Scientific (Rockford, IL, USA). The Bovine Lactoferrin ELISA Quantification kit was from Bethyl Laboratories Inc. (Montgomery, TX, USA). All other reagents were analytical reagent grade.

2.2. Sweet whey preparation

Milk was kindly donated by Perassolo y Cia. S.A. (Rojas, Buenos Aires, Argentina). Sweet whey was prepared as described in a previous work (Urtasun et al., 2017). Briefly, 0.075 g of chymosin (Sigma-Aldrich) was added to 1 L of milk at 37 °C until coagulation (30 min). Chymosin was inactivated by raising the temperature to 55 °C for 15 min. The resulting whey was centrifuged at 4 °C, 3000 x *g*, for 30 min to remove the residual fat and the precipitated casein.

2.3. Response surface methodology

The adsorption and elution steps for the two processes studied were optimised by the CCF design using the Design Expert software (version DX7). Every independent variable was studied in three coded levels (+1, 0, and -1) as shown in Tables 1 and 2. Eq. (1) explains the relation between the coded and the real values:

$$x_i = (X_i - X_0)/X \quad (1)$$

where X_i is the real value of the independent variable, x_i is the dimensionless coded value for X_i, i = -1, 0, +1, X_0 is the mid-point of X_i, and X is the step change value.

The best-fitting model was selected. Analysis of variance was implemented to validate the model accuracy. Mean squares, lack of fit, sum of squares, F-value, and *P*-value were the parameters analysed to check the efficacy of the model. *P*-values confirmed the model from the statistical point of

view. According to the variance analysis, *P*-values lower than 0.05 represented that either the model or the variables were significant from the statistical point of view (95% significance level).

For the optimisation of multiple responses, the desirability function (D(x)) was maximised according to Eq. (2):

$$D(x) = (Y1*Y2*Y3 \dots Yn)^{1/n} \quad (2)$$

where Y*i* (i = 1, 2 … *n*) are the selected responses and *n* is the total number of responses evaluated. The value of D is between zero (least desirable) and one (most desirable) at the goal set for each response.

2.4. Matrix synthesis and characterisation

2.4.1. Matrix synthesis

Chitosan mini-spheres (1.32 ± 0.14 mm diameter) were obtained according to the procedure described in previous studies (Baieli et al., 2014b; Hirsch et al., 2018a). Briefly, a chitosan solution was prepared by dissolving 2% of low viscosity chitosan powder (acetylation degree 41%) in 2% acetic acid (Kasaai, Arul, & Charlet, 2000). This solution was dripped through a 30 G needle on a 2 M NaOH solution under continuous soft stirring (Baieli et al., 2014b). The resulting mini-spheres were crosslinked with a 250 mM epichlorohydrin solution at 60 °C for 4 h under continuous gentle stirring. The matrix designed for the purification of LF was then activated by a second treatment with epichlorohydrin, using a 2.55 M solution (60 °C, 16 h, pH 10.0) followed by the immobilisation of sulfanilic acid by the reaction with a 0.46 M sulfanilic acid solution, pH 10.0, stirred at 60 °C for 40 h. In addition, for the matrix designed for recovering WPI, the 250 mM epichlorohydrin crosslinked mini-spheres were directly incubated with a 250 mM GTMA solution in the presence of 0.25% pyridine, pH 10.0, stirred at 60°C for 24 h.

2.4.2. Nuclear magnetic resonance

Solid-state nuclear magnetic resonance (ss-NMR) experiment data were acquired with a Bruker Avance-III HD spectrometer equipped with a 14.1 T narrow bore magnet operating at Larmor frequencies of 600.09 MHz and 150.91 MHz for ^{1}H and ^{13}C, respectively. Powdered samples were packed into a 3.2 mm ZrO_2 rotor and rotated at room temperature at the magic angle spinning (MAS) rate of 15 kHz. The contact time during crosspolarisation (CP) was 2000 µs. The SPINAL64 sequence (small phase incremental alternation with 64 steps) was used for heteronuclear decoupling during acquisition. Spectral editing with the pulse sequence for cross-polarisation with polarisation inversion (CPPI) was used according to previous reports (Algarra et al., 2019; Wu & Zilm, 1993). ^{13}C natural abundance direct polarisation experiments with proton decoupling (SPINAL64) during acquisition were conducted for the GTMA-chitosan sample. An excitation pulse of 4.0 µs and a recycling time of 100 s were used and 4000 scans were accumulated to obtain quantitative and good signal-to-noise ratio.

2.4.3. Zeta potential

The ζ potential study was performed as previously reported (Hirsch et al., 2018a). Briefly, the matrices were first disrupted mechanically (100 mg 10 mL^{-1} distilled water) with a spatula and then kept under magnetic stirring for 48 h to obtain a homogeneous microparticle suspension. The particle size was homogenised by filtrating the suspensions (1.2 mm nitrocellulose membrane, Osmonics). Aliquots (2 mL) were diluted 1:1 with distilled water, and NaCl was added until a 10 mM solution concentration was obtained. An adequate volume of 50 mM HCl or NaOH solution was added to adjust the pH value in a range between 2.0 and 9.0.

The hydrodynamic size (D_h), size distribution (polydispersity index, PDI), and ζ potential of the different samples were assayed by dynamic light scattering using a Zeta sizer Nano-ZS (Malvern Instruments, Malvern, UK) at a scattering angle of 173° and a fixed measurement position of 4.65 mm.

214 The temperature was controlled at 25 ± 0.1 °C (all samples). Viscosities ranged between 0.8875 and
215 0.8888 cP (25 °C), and the refractive index (RI) was 1.33.
216
217 *2.5. Lactoferrin purification*
218
219 *2.5.1. Lactoferrin adsorption isotherms*
220 LF solutions (1 mL) of different concentrations (0.063–10 mg mL^{-1}) were incubated with 50 mg of
221 hydrated and buffer-equilibrated matrix with gentle agitation (16 h, 20 °C). The LF solutions were
222 prepared at different pH values using 20 mM phosphate buffer for pH 6.0, 7.0, and 8.0, and 20 mM
223 carbonate buffer for pH 9.0 with gentle agitation (16 h, 20 °C). The adsorbed equilibrium protein
224 concentration was calculated from the difference between the initial and final LF concentrations
225 measured at 280 nm using the extinction coefficient factor of 1.51. The results were analysed by the
226 Langmuir model (Langmuir, 1917).
227 Langmuir isotherm can be expressed by the following equation (3):
228 $q = q_m C/(K_d + C)$ (3)
229 where q is the adsorbed protein mass at equilibrium, q_m is the maximum adsorption capacity, C is the
230 protein concentration in the solution at equilibrium, and K_d is the dissociation constant.
231 All determinations were performed in triplicate, and the results were expressed as the average ±
232 SD.
233
234 *2.5.2. Adsorption studies from whey*
235 For the adsorption, three independent variables were taken into account: amount of hydrated
236 matrix (mg) (A), adsorption time (h) (B), and whey pH (C).

Six central points (0,0,0) were included in the analysis, resulting in 20 experiments to be performed (Supplementary material Table S1). Only one response was analysed: LF adsorption (R1LFA). The response was defined by the following equation (4):

*R1LFA (%) = [LF adsorbed (mg) * 100] / [LF in the initial whey (mg)]* (4)

The objective was to optimise this process by maximising R1LFA. The mini-spheres were first equilibrated with a buffer set at the same pH as that of the adsorption step, followed by the adsorption at the condition determined by the design. All experiments were performed at 11 °C to reduce the growth of microorganisms. The results were determined by the Bovine Lactoferrin ELISA Quantification kit and the BCA protein assay kit for total protein determination.

2.5.3. Elution studies

For the elution step, three independent variables were taken into account: elution buffer pH (A), NaCl molar concentration (B), and propylene glycol (PG) percentage (C). The pH and NaCl concentration would mainly affect the ionic interactions between the matrix and the protein; however, the PG and the pH would alter the hydrophobic interactions.

As for adsorption, six central points (0,0,0) were included in the analysis, resulting in 20 experiments (Table S1). Two responses were analysed: LF elution (R2LFE) and total proteins eluted (R3LFE). These responses were defined by the following equations (5 & 6):

*R2LFE (%) = [LF eluted (mg) * 100] / [LF adsorbed (mg)]* (5)

*R3LFE (%) = [Total proteins eluted (mg) * 100] / [Total proteins in the initial whey (mg)]* (6)

The objective was to optimise this process by maximising R2LFE while R3LFE was set at a target of 1.59%, which is the amount of LF in the original whey, to achieve higher purity in the eluent.

The mini-spheres were first equilibrated with 20 mM phosphate buffer, pH 7.0, followed by the adsorption at the optimal condition previously determined in the adsorption study. Four washing steps

were included after the adsorption using 20 mM phosphate buffer, pH 7.0, and the elution buffer was determined according to the experimental design. The results were measured as previously described.

2.5.4. Purification of lactoferrin from cheese whey

To verify the results obtained from the RSM study, a purification process under the optimal conditions found by RSM for adsorption and elution was performed in batch mode. The mini-spheres (100 mg mL^{-1} of whey) were first equilibrated using 20 mM phosphate buffer, pH 6.7. The adsorption step was performed at the original pH of the cheese whey (6.6), 11 °C for 4 h with gentle stirring. Four washing steps were carried out with the equilibration buffer, and 20 mM phosphate buffer (pH 7.5) solution with 1.45 M NaCl and 31.33% of PG were used for the elution. LF and total protein concentration were determined as previously described and analysed by SDS-PAGE.

2.6. Whey protein isolate recovery

2.6.1. Adsorption studies

For the adsorption, two independent variables were taken into account: amount of hydrated matrix (mg) (A) and adsorption time (h) (B).

Five central points (0,0) were included in the analysis, resulting in 14 experiments to be performed (Supplementary material Table S2). Only one response was analysed: Total protein adsorption (R4WPIA), which was defined by the following equation (7):

R4WPIA (%) = [*Total proteins adsorbed (mg)* * 100] / [*Total proteins in the initial whey (mg)*] (7)

The objective was to optimise this process by maximising R4WPIA. All experiments were performed at 11 °C. The results were determined by the BCA protein assay kit.

285 *2.6.2. Elution studies*

286 For the elution, three independent variables were taken into account: elution buffer pH (A),
287 NaCl molar concentration (B), and PG percentage (C). Six central points (0,0,0) were included in the
288 analysis, resulting in 20 experiments to be performed (Supplementary material Table S1). Only one
289 response was analysed: total proteins eluted (R5WPIE). This response was defined by the following
290 equation (8):

291 *R5WPIE (%) = [Total proteins eluted (mg) * 100] / [Total proteins in the initial whey (mg)]* (8)

292 The objective was to optimise this process by maximising R5WPIE. The mini-spheres were first
293 equilibrated with 20 mM phosphate buffer, pH 7.0, followed by the adsorption at the optimal condition
294 found in the adsorption study at 11 °C. Four washing steps were included after the adsorption using the
295 equilibration buffer, and the elution buffer was selected according to the experimental design. Total
296 protein was measured using the BCA protein assay kit. In addition, the eluents were analysed by HPLC to
297 determine if any tested condition allowed the elution of a specific protein.

298

299 *2.6.3. Recovery of whey protein isolate from cheese whey*

300 The optimum conditions found in the adsorption and elution RSM studies were verified in a
301 recovery process. The mini-spheres (1000 mg mL^{-1} of whey) were first equilibrated using 20 mM sodium
302 phosphate buffer, pH 7.0. The adsorption step was performed at 11 °C with gentle stirring for 0.5 h. Four
303 washing steps were conducted using the equilibration buffer. A 20 mM sodium acetate buffer, pH 4.0,
304 with 1.57 M NaCl and 50.0 % of PG was used for the elution. LF and the total protein concentration were
305 determined as previously described and analysed by SDS-PAGE. The lactose concentration was
306 measured in the eluents by an enzymatic methodology.

307

308 *2.7. Lactose determination*

309

310 The lactose determination was performed according to the enzymatic methodology (Ansari,
311 Satar, Kashif Zaidi, & Ahmad, 2014; Mariotti, Yamanaka, Araujo, & Trevisan, 2008). These samples were
312 incubated at 45 °C for 30 min, an aliquot of 10 µL was taken from each sample, and the glucose
313 concentration was determined using an enzymatic glycaemic kit. The glucose concentration was also
314 determined for the samples evaluated without β-galactosidase; all sample absorbances were read at
315 505nm in UV-Visible spectrophotometer (Shimadzu Inc., Kyoto, Japan). Glucose concentration was
316 calculated by comparison with standard glucose concentrations. The lactose moles on each sample were
317 determined according to the following equation (9):

318 *Lactose moles of the sample = Glucose moles from the sample with β galactosidase – Glucose*
319 *moles of the sample without treatment* (9)

320 All determinations were performed in triplicate, and the results were expressed as the average ±
321 SD.

322

323 **3. Results and discussion**

324

325 *3.1. Matrix synthesis and characterisation*

326

327 It is worth mentioning that, from the same original material, two similar syntheses resulted in
328 different chitosan-based chromatographic materials (Fig. 1), providing evidence of the expanding uses of
329 this natural polymer in chromatographic applications.

330 To obtain chemical information about the modification of chitosan with GTMA, ^{13}C ss-NMR
331 experiments were done and the results are shown in Fig. 2. The ^{13}C CP-MAS spectrum for the GTMA-
332 modified chitosan mini-spheres shows the carbon resonance signals for the chitosan structure (C_{1-8}) and

333 those for the crosslinking with epichlorohydrin ($C_{9\text{-}10}$) (Hirsch et al., 2018a) and with the reaction with
334 GTMA ($C_{11\text{-}14}$). Particularly, the ^{13}C CPPI experiment allowed us to unequivocally assign the methyl
335 carbons of the trimethylammonium group of the GTMA at a carbon chemical shift of 54.9 ppm
336 (Supplementary material Fig. S1). Considering that this signal is representative of the GTMA moieties in
337 the chitosan mini-spheres, a ^{13}C direct polarisation experiment was conducted using a sufficiently long
338 recycle delay for all the ^{13}C to relax. In this sense, the quantitative amount of GTMA related to the
339 chitosan structure can be estimated from the deconvolution of the ^{13}C-NMR line (Supplementary
340 material Fig. S1). The quantitative information for the GTMA mini-spheres estimated that every three
341 monomeric units of chitosan one was modified. The difference observed between the ^{13}C CP-MAS and
342 ^{13}C DP spectra stemmed from the intensity of the NMR lines from the ordered or crystalline regions
343 enhanced in the cross-polarisation experiment (Lázaro-Martínez, Rodríguez-Castellón, Vega, Monti, &
344 Chattah, 2015). However, the direct-polarisation experiment with an adequate long recycle delay can
345 bring quantitative information from the NMR lines of the entire system (ordered or disordered regions).
346 In the latter region, the only disadvantage is the time consumed by the spectrometer to obtain a good
347 signal-to-noise ratio (5 days for the GTMA-chitosan sample).

348 The ζ potential curves versus pH (Fig. 3) reveal that the incorporation of the different ligands
349 was successful. The immobilisation of the sulfonic groups onto the chitosan mini-spheres showed a
350 charge inversion at a pH-value between 5 and 6. However, the immobilisation of the GTMA showed an
351 increase in the charge in almost all the pH spectrum.

352

353 *3.2. Lactoferrin purification*

354

355 *3.2.1. Equilibrium adsorption isotherms*

Maximum adsorption capacity (q_m) was determined following the Langmuir model from the equilibrium adsorption isotherms. To characterise the interaction between LF and the matrix, q_m was determined at different pH values at which the matrix has a net negative charge (6.0–9.0). Fig. 4 shows that the highest q_m was reached at pH 8.0 (112.4 ± 6.347 mg g^{-1}). However, there was no linear relation between the pH value and the measured q_m, which may be attributed to the interaction between the matrix and the protein not being entirely mediated by ion exchange, thus revealing other types of interactions that are not negligible.

Compared with other ion exchange matrices, the maximum adsorption capacity obtained was high. In a previous work, LF isolation from bovine colostrum was studied using a cation exchange resin SPEC 70 SLS from Pall Corporation (Port Washington, New York, USA). In this case, the maximum adsorption capacity resulted in 21.73 mg g^{-1} resin at pH 7.0 (Liang, Wang, Wu, & Zhu, 2011).

3.2.2. Adsorption studies

The response, R1LFA, was analysed as shown in Fig. 5 and it followed Eq. 10, in terms of codified factors (A = amount of hydrated matrix (mg); B = adsorption time (h); C = whey pH).

$$R1LFA = +52.68 + 24.79 * A + 16.86 * B + 9.51 * C - 3.14 * A * B - 5.82 * A * C - 7.78 * B * C - 6.33 * A^2 - 9.12 * B^2 - 0.72 * C^2 \quad (10)$$

Supplementary material Table S3 presents the ANOVA results for R1LFA. The optimum adsorption conditions were obtained using numerical optimisation of RSM, to attain the maximum LF adsorption (R1LFA). The optimum amount of hydrated matrix was 100 mg, adsorption time 4 h, and pH 6.57. The predicted value for R1LFA was 78.00%. As the pH of the cheese whey is 6.60, this factor was left unmodified. At pH 6.57, the matrix and most of the proteins were charged negatively, while the target protein had the opposite charge (Urtasun et al., 2018). This factor is also seen as important in Fig. 4 since it influenced the maximum capacity of the matrix.

When analysing the other two variables (adsorption time and amount of matrix), it is important to take into account that adsorption time is usually limited to increase the process productivity and to reduce the growth of microorganisms in the remaining whey. For this reason, the maximum adsorption time studied was 4 h. Noteworthy, when working with batch systems, diffusion is the main time limitation since the target molecule has to diffuse from the solution to the hydrated film around the adsorbent, then inside the pore, and afterward interact according to its binding kinetics (Urtasun et al., 2018). Given that LF is at a low concentration compared with other proteins in the cheese whey, the limitation of 4 h for the adsorption may influence the amount of matrix needed to accomplish the adsorptive step, which could explain not only the significance of this variable but also the fact that the optimum for both variables was near the higher level.

3.2.3. Elution studies

The first response analysed was R2LFE, as shown in Fig. 6A, and it followed Eq. 11, in terms of coded factors (A = elution buffer pH; B = NaCl molar concentration; C = propylene glycol percentage).

$$R2LFE = +68.95 + 12.54 * A + 24.64 * B + 13.85 * C + 13.57 * A * B - 0.26 * A * C + 11.82 * B * C - 7.94 * A^2 - 25.56 * B^2 - 13.75 * C^2 \quad (11)$$

Supplementary material Table S4 presents the ANOVA results for R2LFE. The second response was R3LFE and it followed Eq. 12 in terms of the same codified factors described for Eq. 11 (Fig. 6B).

$$R3LFE = +1.99 + 0.42 * A + 1.27 * B + 0.30 * C + 0.48 * A * B + 0.70 * A * C + 0.46 * B * C + 0.52 * A * B * C \quad (12)$$

Supplementary material Table S5 shows the ANOVA results.

The optimum pH was 7.53, NaCl concentration was 1.33 M, and PG was 32.42%. The predicted values for R2LFE and R3LFE were 84.98% and 2.97%, respectively, with desirability of 0.840 as shown in Fig. 6C. Fig. 6A,B indicates that, for the same pH value, both responses were maximised using the

maximum PG and NaCl concentrations; however, since the curvatures of the plots differ from one another, it was possible to obtain a result with good desirability. Even though the composition of the starting material is very important in the optimisation of these variables and a study is required in each individual case, the information from this study, in particular, allows a proper definition of the limits of each variable to obtain more certain information and reduce the time and number of experiments required for process development.

The fact that the terms NaCl and PG were significant for R2LFE suggests that the interaction between the mini-spheres and the target protein is mediated both by ion exchange and hydrophobic interactions. However, in Eq. 11, the coefficient for the NaCl factor is larger than that for the PG factor, which means that ion exchange forces are predominant over the hydrophobic ones. The variable NaCl is the most significant for both responses, as shown by Eqs. 11 and 12, the NaCl (B) terms have the larger number and are positive for both responses. This makes it more difficult to set the optimum for this variable to accomplish the objective, which is to maximise one response while the other one is targeted at 1.59%. For this reason, for the optimisation step, the two responses were given different levels of importance: R2LFE maximisation was highly important (+++++) while R3 minimisation was left at a medium setting of importance (+++) (Fan, Duquette, Dumont, & Simpson, 2018). Given that R2LFE presents B^2 as a significant term (Supplementary material Table S4) with a negative effect on the response, the optimum for this term was not the highest but 0.33 in coded factors.

Regarding the role of pH in the elution, although this variable is not significant for R2LFE, the interaction between the pH and the PG was significant for R3LFE. As the term in Eq. 12 is positive, if this were the only response considered and the objective was to minimise it (because the target was low), one of the two factors (A or C) would need to have a negative coded number. Nonetheless, because of the greater importance established for R2LFE, this information is not seen in the optimum reached.

3.2.4. *Purification of lactoferrin from cheese whey*

The predicted optimums obtained from the RSM for the adsorption and the elution were used in a process to verify the correspondence between the predicted and the actual values. The differences between the predicted value and the actual responses were as follows: 1.49% for Adsorption R1LFA (78.00% and 76.51%, respectively), 4.49% for Elution R2LFE (84.98% and 89.47%, respectively), and 0.46% for Elution R3LFE (2.97% and 2.51%, respectively). These results show that there was no significant difference between the actual and the predicted values ($p > 0.05$). The complete purification process showed a purification factor of 27.52 ± 0.21 and a yield of 67.99 ± 1.12 %. Fig. 7 shows the SDS-PAGE of the process. A 70 % purity degree was estimated for LF by gel densitometry, which was similar to the commercial food-grade product (lane 1).

In comparison with other ion-exchange matrices developed, the results obtained for the mini-spheres were similar to previous results obtained from different methods, but in this case no pre-treatment was necessary and the recovery of the matrix after each purification step was easily done with a sieve. For instance, other authors used carboxymethyl ion-exchange chromatography for LF purification from acid cheese whey and obtained a yield of 88 mg of LF from 1 L of acid whey (Yoshida & Ye, 1991). However, in their work, hydrophobic chromatography was performed before the ion-exchange chromatography as whey pre-treatment. In the present case, the obtained amount of LF was 120 mg from 1 L of cheese whey and no pre-treatment was required. Fractionation of LF was also studied using a microporous membrane containing immobilised sulfonic acid moieties (Chiu & Etzel, 1997). A 50 ± 5% yield was obtained, which is also lower than that obtained with the mini-spheres here presented. In addition, the purification using microporous membranes required a vacuum filtration through a 0.7 µm glass filter before the adsorption step.

3.3. *Whey protein isolate recovery*

452

453 *3.3.1. Adsorption studies*

454 The response, R4WPIA, was analysed and optimised according to the obtained model. The
455 response followed Eq. 13, in terms of coded factors (A = amount of hydrated matrix (mg); B = adsorption
456 time (h)).

457 $R4WPIA = +46.53 + 20.83 * A + 2.54 * B$ (13)

458 Supplementary material Table S6 presents the ANOVA results for R4WPIA.

459 The optimum conditions for the adsorption were obtained by RSM numerical optimisation, to
460 attain the maximum total protein adsorption (R4WPIA). The optimum amount of hydrated matrix was
461 1000 mg and the adsorption time was 0.5 h. The predicted value for R4WPIA was 64.82%. This optimum
462 can be analysed from Fig. 8, which clearly shows that, for total proteins to be maximised, the most
463 important factor to be taken into account is the utilisation of the maximum amount of matrix. If the
464 amount of matrix used for the WPI recovery process is compared with the optimum amount for the LF
465 purification, the amount of matrix is only 10 times greater while the total number of whey proteins is by
466 far more than 10 times the total amount of LF (cheese whey has 0.02–0.2 g L^{-1} of LF and 6 g L^{-1} of total
467 proteins). Even though a higher amount of matrix is needed to obtain higher adsorption rates, the
468 proper agitation of the system is not possible with a greater amount of matrix. Other authors working in
469 WPI recovery used similar amounts of resin to process an equivalent amount of whey (Gerberding &
470 Byers, 1998).

471

472 *3.3.2. Elution studies*

473 The response analysed followed Eq. 14, in terms of codified factors (A = elution buffer pH; B =
474 NaCl molar concentration; C = propylene glycol percentage) (Fig. 9).

475 $R5WPIE = +60.19 – 3.25 * A + 27.76 * B + 1.44 * C – 0.047 * A * B – 1.71 * A * C + 0.99 * B * C –$

476 $2.18 * A^2 – 25.46 * B^2 – 1.29 * C^2$ (14)

477 Supplementary material Table S7 presents the ANOVA results for R5WPIE. The optimum

478 adsorption conditions were obtained by numerical optimisation of RSM, to attain the maximum total

479 protein elution. The optimum pH was 4.00, the NaCl concentration was 1.57 M, and the PG was 50.0%.

480 The predicted value for R5WPIE was 71.27%.

481 The fact that the term NaCl was significant for R5WPIE suggests that the interaction between

482 the mini-spheres and the target proteins is mainly by ion exchange. As expected, this model also

483 suggests that using higher pH for the eluent minimises the total proteins eluted. When analysing the PG

484 effect on the elution, we noted that this factor had a low effect on the response. However, if a

485 constraint were to be set to minimise this factor, the response would decrease its predicted value to

486 63.81%. If this process were scaled-up, an economic evaluation would have to be made to analyse which

487 option would result in a higher profit.

488 Further studies were made on the eluted fractions to determine if any tested condition resulted

489 in the purification or enrichment of a single whey protein. This analysis was done by HPLC, but none of

490 the conditions tested resulted in a single peak chromatogram (data not shown). All the fractions kept

491 the same peak profiles, where only differences in the total number of proteins were evident.

492

493 *3.3.3. Recovery of whey protein isolate from cheese whey*

494 The predicted optimums obtained from the RSM for the adsorption and elution were used in a

495 process to verify the correlation between the prediction and the experimental values. The differences

496 between the predicted value and the actual responses were 2.11% for Adsorption R4WPIA (64.82% and

497 62.71%, respectively) and 5.44% for Elution R5WPIE (71.27% and 65.83%, respectively). These results

498 show that there was no significant difference between the actual and the predicted values ($p > 0.05$).

499 The complete purification process showed a yield of 65.82 ± 2.20%. Fig. 10 shows the SDS-PAGE of the
500 process. The lactose concentration in the eluent was zero.

501 To establish the whole process sequence, the LF content and the total number of whey proteins
502 were evaluated. Interestingly, during the adsorption, 43.81% of the total LF was adsorbed to the matrix.
503 This information was vital in determining the correct sequence of processes for these matrices; thus, the
504 adsorption of LF was defined as the first step of the whole process.

505

506 *3.4. Sequence purification of lactoferrin and whey protein isolate*

507

508 Due to the high loss of LF in the WPI adsorption step and the higher market value of this product
509 in comparison with WPI, the purification of LF was considered the first step in whey processing. The
510 optimum conditions obtained from each purification process were used in the sequence purification,
511 using the pass-through of the LF adsorptive step as a starting material for WPI recovery. Table 3 shows
512 the results of the entire process, where the addition of the LF purification process prior to the WPI
513 purification did not significantly affect the yield of this second process. As can be estimated from this
514 table, a yield of 62.31% was obtained for LF. Regarding WPI, 2.71 mg of whey protein was obtained per
515 ml processed. It is worth mentioning that during LF adsorption, about 0.69 mg of the total protein
516 content was bound to the sulfanilic matrix, and this could explain the slight reduction in the yield
517 obtained for WPI, a reduced content in the initial total protein amount in the LF depleted cheese whey.
518 Lactose concentration was determined for the eluents of the processes, with the absence of this
519 carbohydrate found for both LF and WPI.

520

521 **4. Conclusions**

522

The present work provides a useful tool to infer the optimum conditions for the purification process of LF from cheese whey. For this purpose, the matrix developed was applied to use this protein as an ingredient in other formulations and allow the rest of the whey to continue with its usual processing, such as WPI production. The depletion of LF from the cheese whey did not significantly affect the yields of the WPI process, resulting in a higher profit of the total cheese whey processing. This work also focuses on the addition of commercial value of natural polymers, such as chitosan, and its potential use in industrial whey processing. In addition, the fact that the LF purification process has little effect on the composition of the remaining whey, its usual industrial processing and other purification strategies to produce WPI or other products can be considered. Furthermore, the experimental procedure using RSM could be applied to other proteins from other sources using other types of chromatographic supports, being especially interesting for multimodal matrices. The developed mini-spheres allow the cheese whey to be processed without any previous conditioning, to have good mechanic resistance, and to be easily recovered after adsorption/washing and elution steps.

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690

Figure legends

Fig. 1. Scheme of synthesis reactions for mini-spheres.

Fig. 2. ^{13}C CP-MAS spectra for chitosan-minispheres (A), crosslinked chitosan-minispheres (B) and GTMA-modified chitosan-minispheres (C). The numbers above spectra C correspond to the numbers assigned for the different carbon atoms shown to the right.

Fig. 3. ζ potential at different pH values of disrupted (■) chitosan mini-spheres, (●) chitosan mini-spheres with GTMA and (▲) chitosan mini-spheres with sulfanilic acid

Fig. 4. Maximum adsorption capacity of sulfanilic modified mini-spheres for pure LF at different pH values.

Fig. 5. LF adsorption (%) as function of the matrix amount (mg) and whey pH, keeping the time of adsorption constant at 4 h.

Fig. 6. Elution (%) of (A) LF and (B) total proteins as function of the NaCl concentration (M) and PG concentration (%), keeping the elution buffer pH constant at the determined optimum, pH 7.53 and (C) desirability of the optimisation by maximising LF elution while the total proteins eluted were settled at a target of 1.59%.

Fig. 7. SDS-PAGE (12%) of the LF purification process: lane 1, commercial LF 0.5 mg mL^{-1}; lane 2, molecular mass marker; lane 3, whey 4×; lane 4, pass-through 4×; lane 5, desalted eluate 4×.

Fig. 8. Total whey protein adsorption (%) as function of the matrix amount (mg) and time of adsorption (h).

Fig. 9. Total whey proteins eluted (%) as function of the NaCl concentration (M) and elution buffer pH, keeping the PG concentration constant at the determined optimum of 50%.

Fig. 10. SDS-PAGE (12%) of the WPI recovery: lane 1, molecular mass marker; lane 2, desalted eluate; lane 3, pass-through; lane 4, whey.

Table 1

Factor codification for the optimisation of the LF purification process.

Factor	Low level (-1)	Medium level (0)	High level (+1)
Adsorption			
Hydrated matrix amount (A)	25 mg	62.5 mg	100 mg
Adsorption time (B)	0.5 h	2.25 h	4 h
pH (C)	6.0	7.5	9.0
Elution			
pH (A)	3.0	6.0	9.0
NaCl (B)	0.0 M	1.0 M	2.0 M
Propylene glycol (C)	0.0%	25.0%	50.0%

Table 2

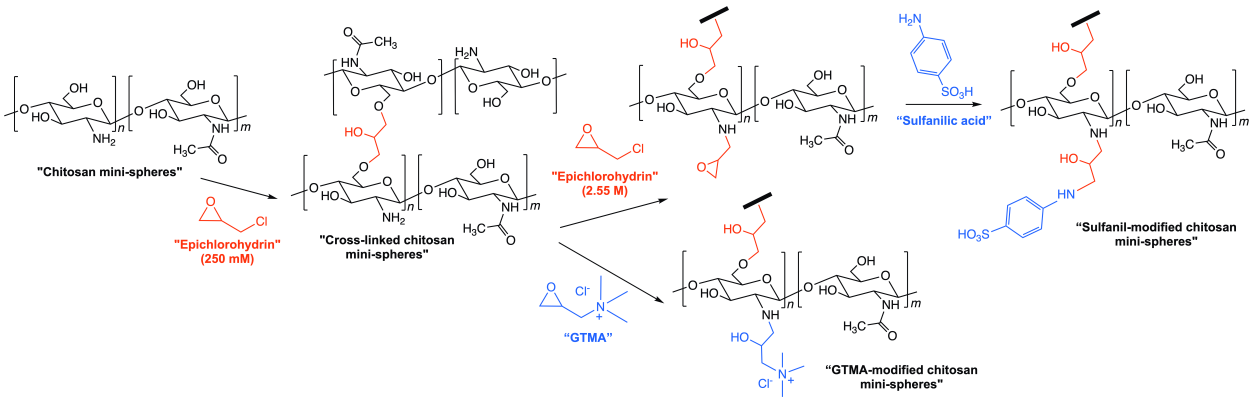
Factor codification for the optimisation of the WPI recovery process.

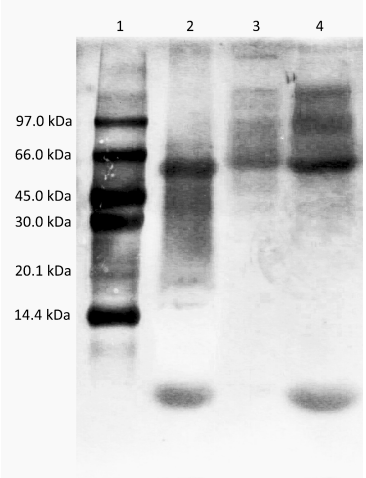
Factor	Low level (-1)	Medium level (0)	High level (+1)
Adsorption			
Hydrated matrix amount (A)	100 mg	550 mg	1000 mg
Adsorption time (B)	0.5 h	2.25 h	4 h
Elution			
pH (A)	4.0	5.5	7.0
NaCl (B)	0.0 M	1.0 M	2.0 M
Propylene glycol (C)	0.0%	25.0%	50.0%

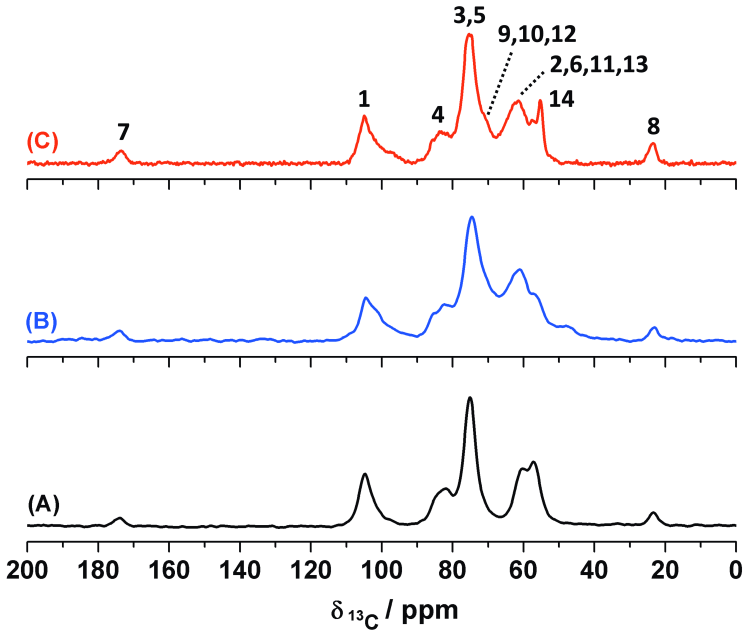
Table 3

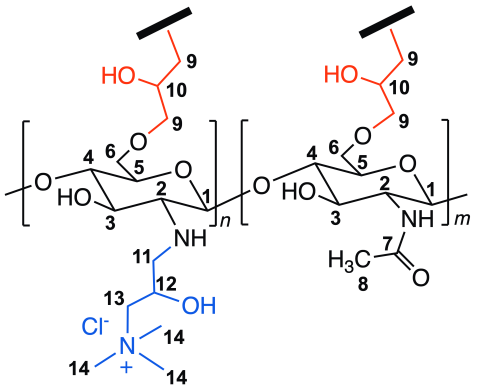
Sequence purification process results.

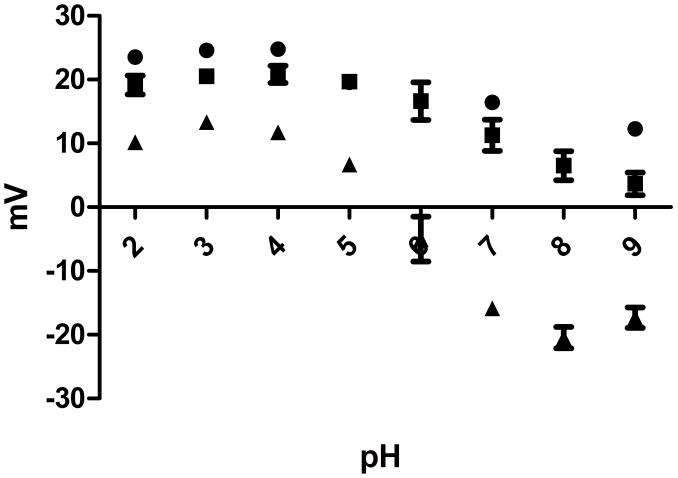
Step	Initial total protein (mg mL^{-1})	Initial LF (mg mL^{-1})	Adsorption (%)	Elution (%)
LF purification step	7.29 ± 0.52	0.14 ± 0.01	75.35 ± 5.79	82.73 ± 1.05
WPI recovery step	5.97 ± 0.09	-	52.09 ± 1.34	87.30 ± 6.13

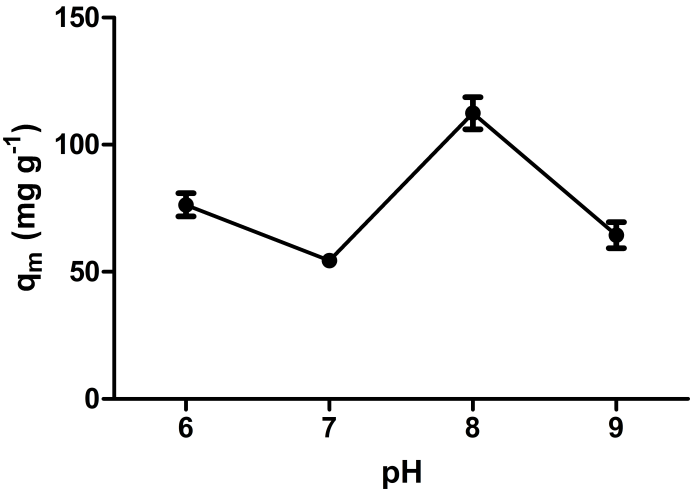


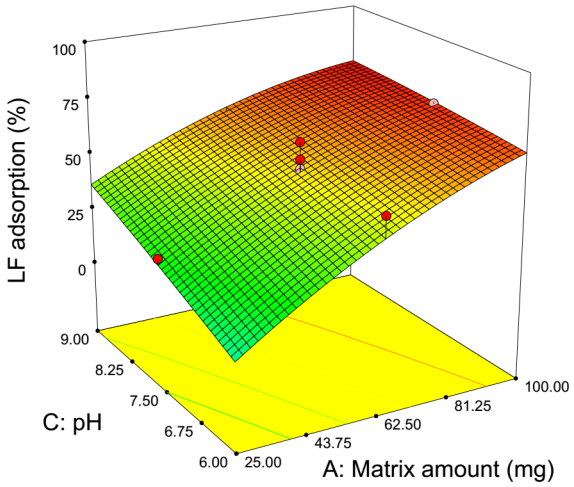


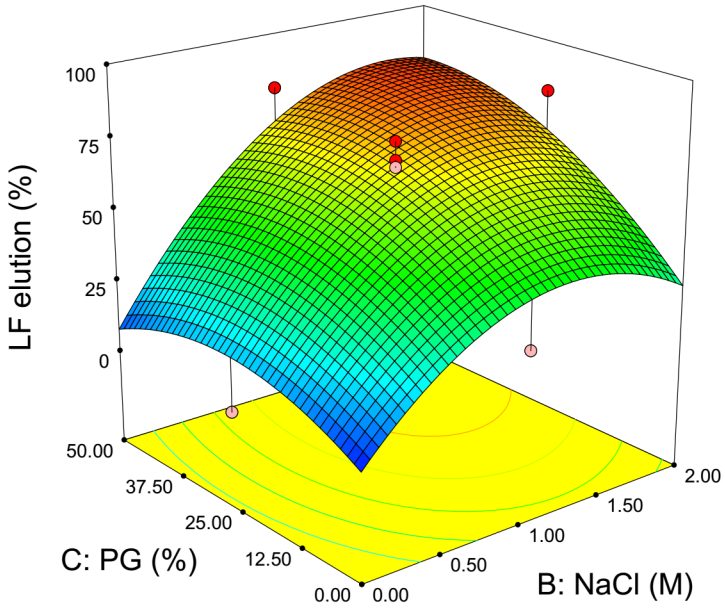


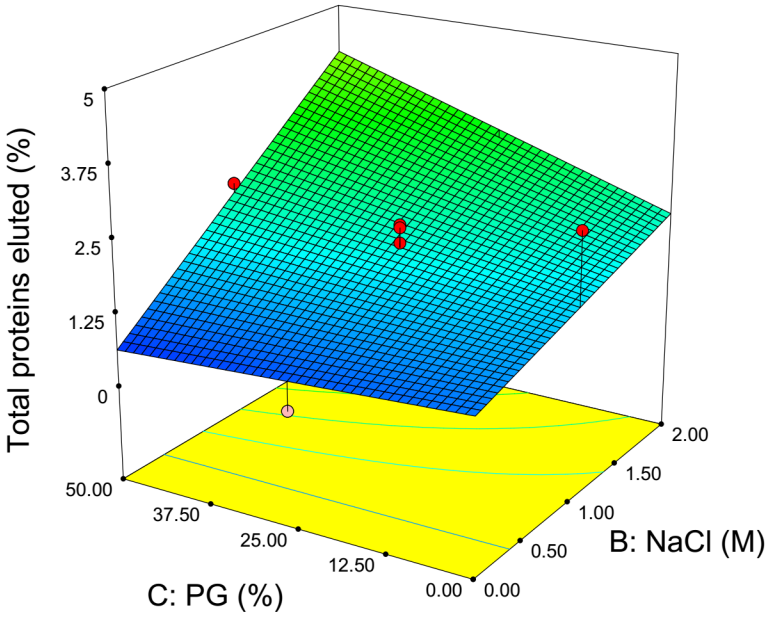


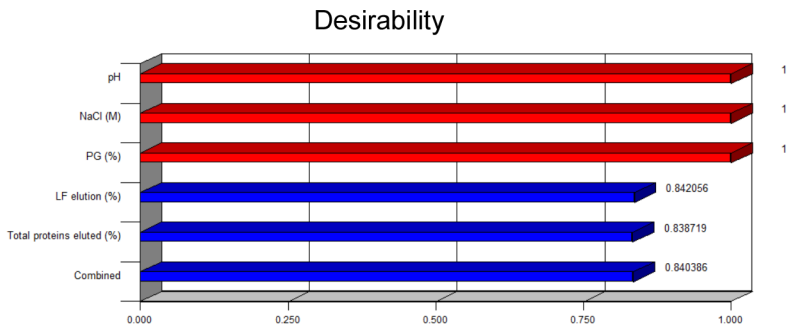


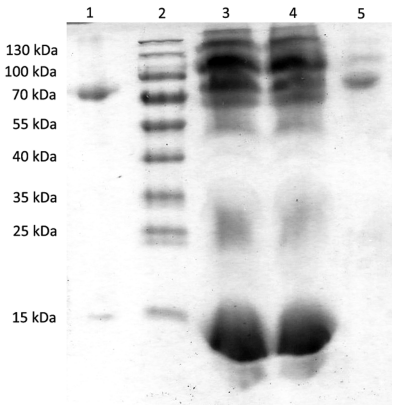


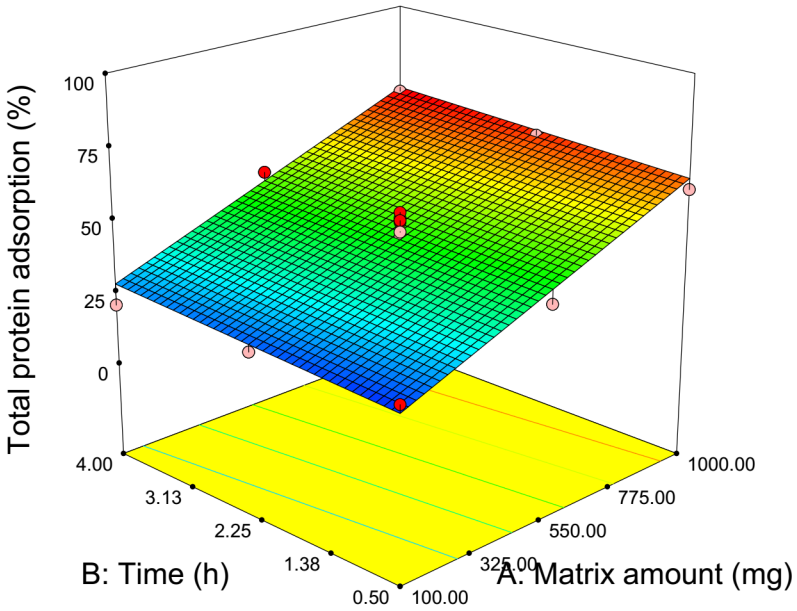


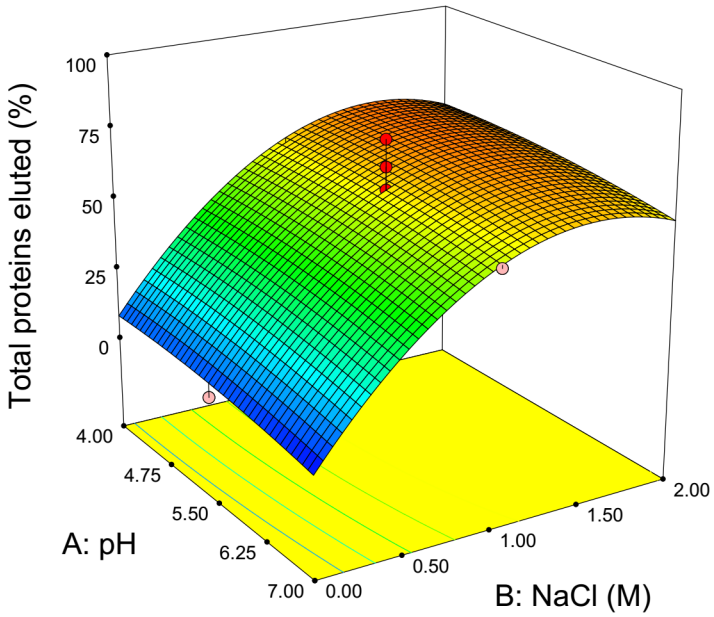












Author contributions

DBH, conceptualisation, design, experimentation, writing; FJW, conceptualisation, design, manuscript review; LMMA, statistical analysis; MFB, NU, data analysis and validation. JMLM, RJG, experimentation; MVM, OC, manuscript review.