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Optimisation of Protein Recovery from *Arthrospira platensis* by Ultrasound-Assisted Isoelectric Solubilisation/Precipitation

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Abstract: A response surface methodology was used to optimise the solubilisation and precipitation of proteins from the cyanobacterium *Arthrospira platensis*. Two separate experiments were designed and conducted in a sequential manner. Protein solubilisation was affected by pH, extraction time, and biomass to solvent ratio ($p < 0.001$). Although spray-drying and the osmotic shock suffered when resuspending the dried biomass into distilled water led to a certain degree of cell wall disruption, the amount of protein that could be solubilised without an additional disruption step was in the range 30–60%. Sequential extractions improved protein solubilisation by less than 5%. For this reason, a pre-treatment based on sonication (400 W, 24 kHz, 2 min) had to be used, allowing the solubilisation of 96.2% of total proteins. Protein precipitation was affected by both pH and extraction time ($p < 0.001$). The optimised precipitation conditions, which were pH 3.89 over 45 min, led to a protein recovery of 75.2%. The protein content of the extract was close to 80%, which could be further increased by using different purification steps. The proteins extracted could be used in the food industry as technofunctional ingredients or as a source of bioactive hydrolysates and peptides for functional foods and nutraceuticals.

Keywords: microalgae; Spirulina; sonication; cell wall disruption; novel proteins

1. Introduction

The worlds' population is expected to increase from approximately 7.7 billion today to 9.7 billion in 2030, according to recent estimations reported by the United Nations [1]. This expanded population is expected to consume twice as much protein than is consumed today [2]. Most of the protein consumed today is derived from animal sources, and demand for livestock products is projected to grow by 70% by 2050 [3]. However, climate change has become a significant reality in today's world, and the global livestock sector contributes an important share of anthropogenic greenhouse gases emissions [4]. Adapting protein consumption in countries with high-meat eating habits is the key to achieving sustainability [3]. Moreover, livestock products, in particular red meat and processed meats, have been suggested as risk factors for cardiovascular diseases, type-2 diabetes, and some types of cancer—mainly colorectal [5]. For this reason, together with an increased concern for animals (ethics), the proportion of individuals choosing to follow a vegan diet has increased significantly in recent years, especially in more affluent countries [6].

The worlds' future protein supply needs new initiatives to address the environmental impact of food production and to produce enough quantities of high-quality protein [3]. Since the 1950s, huge efforts have been made by scientists and food processors to explore new protein sources for food

applications, anticipating a predicted insufficient future protein supply [7]. Microalgae are a diverse group of unicellular photosynthetic microorganisms that can grow in a wide range of environmental conditions and convert sunlight, water, and carbon dioxide into microalgal biomass. Due to their abundance and amino acid profile, microalgae-derived proteins have the potential for being used as an alternative protein source in foods [8]. The incorporation of microalgae biomass into foods faces some challenges that still need to be overcome. These include a strong (generally) green colour, a marine-like taste and odour, low production capacities, and high production costs, among other issues [9,10]. However, industrial production of microalgae for food applications is a reality, and the number of microalgae-enriched foods launched into the market is increasing every year, with this trend predicted to continue growing [11].

Microalgal biomass, especially the biomass of cyanobacteria, has high protein content. Indeed, a hierarchical Bayesian analysis of data compiled from 130 scientific publications found in the literature revealed that, when compared to eukaryotic microalgal phyla, cyanobacteria have higher protein and carbohydrate content (43.1% and 21.8%, respectively) and lower lipid and ash content (11.7% and 8.1%, respectively) as a dry weight percentage [12]. Several reports reported that the protein content of *Arthrospira* biomass is around 60% on a dry weight basis [13]. Proteins extracted from food sources have industrial applications in the food industry not only because of their nutritional importance but also as technofunctional ingredients or as sources of bioactive peptides with health-promoting activities [14].

Isoelectric solubilisation/precipitation is a strategy that has been used to recover protein from a range of sources, including seaweed [15], plants [16], fish [17], and terrestrial animals [18]. When applied to plants or algae, because of their strong cell wall, this strategy is generally used in combination with a disruption step to degrade the cell wall and facilitate the release of proteins. One of the most commonly used strategies is sonication, which has shown to be efficient in facilitating the extraction of several valuable microalgae-derived compounds, including lipids [19] and carbohydrates [20]. Other advantages of this technology are that it can be easily scalable and that it avoids the use of chemical compounds that would need to be further recovered.

The aim of the current study was to optimise the conditions needed to first solubilise, and then precipitate proteins from the microalgae *Arthrospira platensis*. *A. platensis*, known and commercialised as *Spirulina*, was selected because of its high protein content and because of its long history of use as food. Although spray-drying and osmotic shock led to a certain degree of cell wall disruption, the amount of protein that could be solubilised was relatively low, and for this reason, the current paper also evaluated the effect of serial extraction steps and the effect of sonication as a pre-treatment to improve protein extraction.

2. Materials and Methods

2.1. Microalgae, Materials, and Reagents

Dried biomass of *A. platensis*, cultured in closed photobioreactors, was kindly provided by Biorizon Biotech (Almería, Spain). The biomass was separated from the culture medium by centrifugation and dehydrated by spray-drying. The protein content of the dried biomass was 55.4 ± 0.2 g/100 g on a dry weight basis, determined using a LECO FP628 protein analyser (LECO Corp., St. Joseph, MI, USA) based on the Dumas method and according to the AOAC method 992.15. The nitrogen-to-protein conversion factor used was 6.25. Sodium hydroxide and hydrochloric acid used for pH adjustment were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Protein Solubilisation

Protein solubilisation was conducted at room temperature 20 ± 1 °C with constant stirring using an Agimatic-N (J.P Selecta, Barcelona, Spain) stirrer operating at 300 rpm. *A. platensis* suspensions were prepared in triplicate in distilled water at the concentrations and pH values listed in Table 1.

The suspensions were homogenised using a T 25 digital ULTRA-TURRAX® homogeniser (IKA, Staufen, Germany) at 14,000 rpm for 30 s. Protein extraction times ranged between 10 and 120 min, as shown in Table 1. After extraction, insoluble material was separated from the solubilised protein using a Sigma 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) operating at 8000× *g* for 10 min.

Table 1. Central composite response surface design for protein solubilisation: M1.

Coded Variables				Actual Variables			Response
Run [1]	A	B	C	Time (min)	pH	Concentration (g/100 mL)	Protein Solubility (%) [2]
1	0	0	0	65	10	12	54.08 ± 0.51
2	1	0	0	120	10	12	54.29 ± 1.07
3	−1	1	−1	10	11	4	45.06 ± 3.39
4	1	−1	−1	120	9	4	54.95 ± 1.87
5	0	0	−1	65	10	4	57.21 ± 1.58
6	0	1	0	65	11	12	56.14 ± 0.41
7	−1	0	0	10	10	12	41.29 ± 0.88
8	1	1	1	120	11	20	37.87 ± 0.73
9	1	1	−1	120	11	4	60.11 ± 2.36
10	0	−1	0	65	9	12	46.60 ± 1.22
11	0	0	0	65	10	12	51.46 ± 1.25
12	0	0	0	65	10	12	47.66 ± 1.02
13	−1	−1	−1	10	9	4	40.11 ± 1.74
14	−1	−1	1	10	9	20	28.12 ± 0.41
15	0	0	1	65	10	20	37.81 ± 0.78
16	1	−1	1	120	9	20	35.12 ± 0.60
17	−1	1	1	10	11	20	33.22 ± 0.38

[1] Run number does not correspond to the order of processing; [2] Results are the average of three independent experiments ± S.D.

The soluble protein content of the supernatant was determined following the method of Lowry using bovine serum albumin as the standard. Solubilised protein was calculated using the equation:

$$\%\ Protein\ solubilised = \frac{V_e \cdot PC_e}{W_s \cdot PC_s} \cdot 100, \quad (1)$$

where V_e is the volume of extract expressed in L, PC_e is the protein content of the extract expressed in g/L, W_s is the weight of *Spirulina* expressed in g, and PC_s is the protein content of the biomass expressed in g of protein/g of biomass on a dry weight basis.

Sequential Extraction and Sonication

Once the optimum solubilisation conditions were obtained, the current study assessed the potential of sequential extractions or sonication as potential strategies to improve protein solubilisation yields. Up to 3 serial extractions were conducted on the precipitate obtained after centrifugation (described in the previous subsection). Moreover, the cell wall of the microalga was disrupted using a UP400S ultrasonic processor (Hielscher Ultrasonics GmbH, Teltow, Germany) operating at 400 W and 24 kHz for either 30, 60, 90, or 120 s. Temperature was kept constant during sonication by recirculating cold water. For the ultrasound-assisted extractions, concentration and pH values were the optimum given by the model but extraction time was reduced to 5 min under constant stirring using an Agimatic-N (J.P Selecta, Barcelona, Spain) stirrer operating at 300 rpm. Centrifugation was conducted using a Sigma 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) operating at 8000× *g* for 10 min.

2.3. Protein Precipitation

Protein precipitation was conducted by pH-shifting using the conditions detailed in Table 2. Briefly, the pH of the supernatant obtained after the previous section, containing the solubilised protein, was adjusted to the values listed in Table 2 using 1 mol/L HCl (0.1 mol/L for fine adjustment).

The mixture was stirred for the listed times, and the insoluble extracts were separated by centrifugation at 8000× *g* for 10 min. Protein content of the precipitate was determined using a LECO FP628 protein analyser (LECO Corp., St. Joseph, MI, USA) based on the Dumas method and according to AOAC method 992.15. A nitrogen-to-protein conversion factor of 6.25 was used, and the percentage of protein recovered was calculated using the equation:

$$\% \, Protein \, recovery = \frac{W_p \cdot PC_p}{W_s \cdot PC_s} \cdot 100 \quad (2)$$

where W_p is the weight of the extract expressed in g, PC_p is the protein content of the extract expressed in g of protein/g of extract, W_s is the weight of *A. platensis* expressed in g, and PC_s is the protein content of the biomass expressed in g of protein/g of biomass on a dry weight basis.

Table 2. Central composite response surface design for protein precipitation: M2.

	Coded Variables		Actual Variables		Response
Run [1]	**A**	**B**	**Time (min)**	**pH**	**Protein Recovery (%)** [2]
1	1	0	45.0	3.25	77.20 ± 0.05
2	−1	−1	10.0	2.50	46.76 ± 3.21
3	1	−1	45.0	2.50	60.41 ± 3.39
4	0	1	27.5	4.00	74.98 ± 0.01
5	0	0	27.5	3.25	73.97 ± 2.11
6	1	1	45.0	4.00	76.59 ± 0.41
7	0	0	27.5	3.25	79.12 ± 1.01
8	0	−1	27.5	2.50	53.31 ± 1.52
9	−1	1	10.0	4.00	73.09 ± 3.86
10	0	0	27.5	3.25	74.01 ± 2.59
11	−1	0	10.0	3.25	61.56 ± 0.93

[1] Run number does not correspond to the order of processing; [2] Results are the average of three independent experiments ± SD.

2.4. Experimental Design

A response surface methodology was used to optimise the extraction and precipitation of protein from *A. platensis*. Two separate experiments were designed and conducted using Design-Expert version 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA) in a sequential manner.

Design model 1 (M1) was conducted to optimise protein solubilisation, investigating the effect of three independent variables (extraction time, pH, and biomass concentration) on solubilised protein yield. Using a central composite face-centred design, the software generated 17 combinations (listed in Table 1). Design model 2 (M2) was conducted to optimise the recovery of the solubilised protein by precipitation using the independent variables of time and pH. In this case, the software generated 11 combinations using a central composite face-centred design.

Combinations generated by the software in M1 and M2 were conducted in triplicate in the lab, and the mean value of the three independent experiments was used as the response. The central points of M1 and M2 were repeated three times to assess the error within the model. Experimental data were fitted to a polynomial response surface, predicted by the following equation:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1+1}^{n} \beta_{ij} \beta X_i X_j, \quad (3)$$

where Y is the dependent variable, β_0 is the centre point of the system, β_i, β_{ii}, and β_{ij} are the coefficients of the linear, quadratic, and interactive effect, respectively, and X_i, X_i^2, and $X_i X_j$ are the linear, quadratic, and interactive effect of the independent variables, respectively. Non-significant terms ($p < 0.05$) were deleted from the second-order polynomial model after ANOVA analysis and a new ANOVA was

performed to obtain the coefficients of the final equation. Both models M1 and M2 were validated in duplicate, and optimisation was done as described previously [21].

2.5. Statistical Analysis

Differences between samples were analysed using analysis of variance with JMP 13 (SAS Institute Inc., Cary, USA). Where significant differences were present, a Tukey pairwise comparison of the means was conducted to identify where the sample differences occurred ($p < 0.05$).

3. Results

3.1. Protein Solubilisation: M1

The design for protein solubilisation M1 generated 17 experimental runs, listed in Table 1. The obtained quadratic models' *F*-value was 54.79, and its coefficient of determination R^2 was 0.9705, while predicted and adjusted R^2 values were in reasonable agreement and were 0.9345 and 0.9528, respectively. The analysis of error indicated that the lack of fit *F*-value of 0.27 was not significant relative to the pure error, confirming the validity of the model (Table 3).

Table 3. ANOVA for response surface reduced quadratic model—M1.

Source	Sum of Squares	Coefficient	Mean Square	*F*-Value	Prob > F
Model	1423.59		237.27	54.79	<0.0001 [a]
A: Time	297.46		297.46	68.69	<0.0001 [a]
B: pH	75.63		75.63	17.46	<0.0001 [a]
C: Concentration	727.61		727.61	168.02	<0.0001 [a]
AC	41.59		41.59	9.6	0.0113 [a]
A^2	64.33		64.33	14.86	0.0032 [a]
C^2	72.39		72.39	16.72	0.0022 [a]
Residual	43.3		4.33		
Lack of fit	22.46		2.81	0.27	0.9276 [b]
Pure error	20.84		10.42		
Cor. Total		1466.9			
Adjusted R^2		0.9528			
Predicted R^2		0.9345			
SD		2.08			
CV		4.53			
PRESS		96.06			
Adequate precision		25.063			

[a] Model terms are significant ($p < 0.05$); [b] Lack of fit is not significant relative to the pure error. Abbreviations used: SD, standard deviation: CV, coefficient of variation; PRESS, predicted residual error sum of squares.

The application of RSM led to the following quadratic equation in terms of actual factors, which represented an empirical relationship between the percentage of protein from *A. platensis* that could be solubilised and the independent variables:

$$\%Protein\ solubilised = 8.9044 + 0.3594 \cdot t + 2.7500 \cdot pH + 1.1039 \cdot C - 5.1818 \cdot 10^{-3} \cdot t \cdot C - 1.5236 \cdot t^2 - 0.0763 \cdot C^2 \quad (4)$$

where t is extraction time expressed in min, pH is the pH value at which the extraction was conducted, and C is the initial concentration of *A. platensis* expressed in g/100 mL. Variables and interactions between variables that were not significant to the model were not considered. Figure 1 represents the 3D contour model graphs generated by Design-Expert software, as well as the perturbation plot of percentage protein solubilisation.

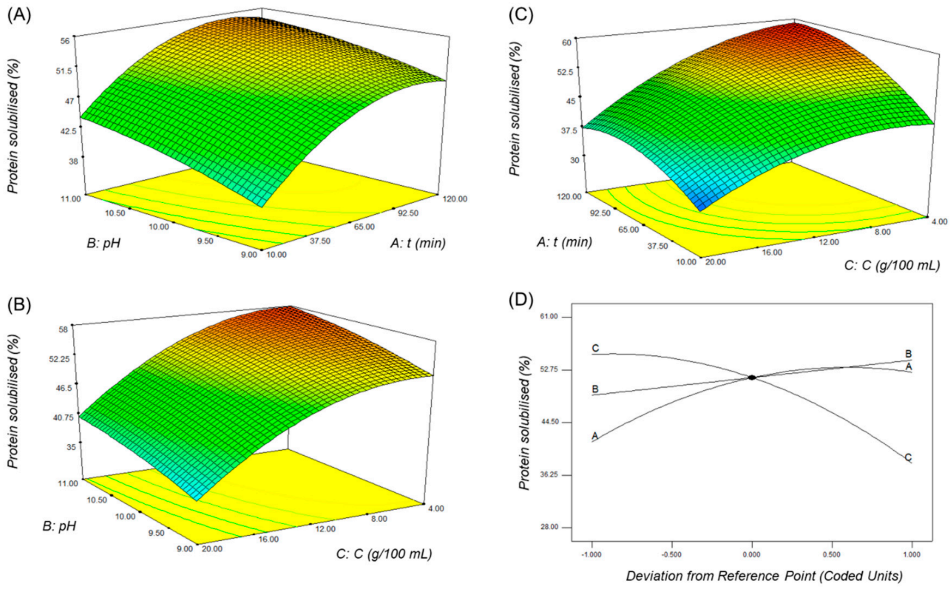

Figure 1. 3D contour plots of percentage protein solubilised from *A. platensis*, (**A**) extraction time and pH; (**B**) concentration and pH; (**C**) extraction time and concentration. (**D**) Perturbation plot of percentage protein solubilisation—M1. The missing variable is fixed to coded zero level. Actual factors: A (extraction time (min)), B, pH, and C (concentration (g/100 mL)).

In order to ensure the quality and reliability of the model developed, M1 was validated using the same volumes and procedures followed to generate the model. Data (shown in Figure 2) demonstrated that the predicted percentage of solubilised protein values were accurate enough to fit the experimental results, with a correlation coefficient of 0.9556. Moreover, the combined conditions (extraction time, pH, and biomass concentration) that led to a higher percentage of protein solubilised were determined.

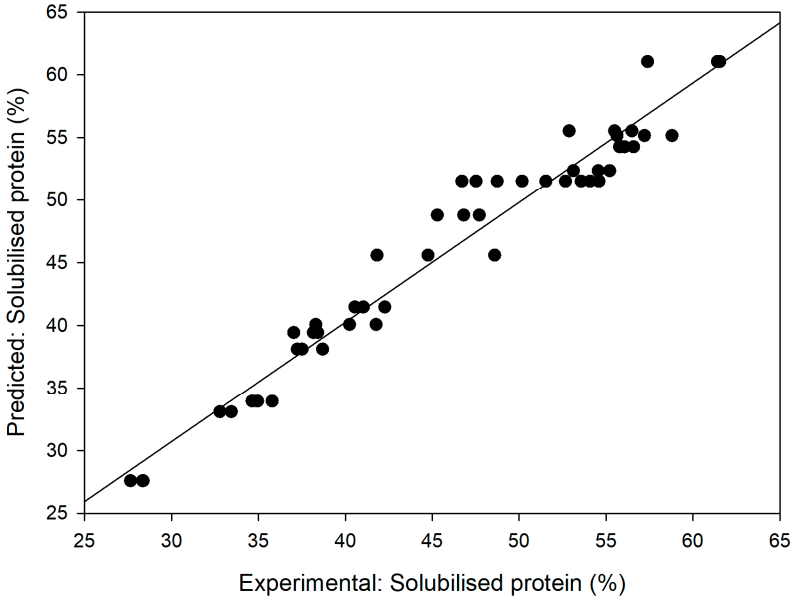

Figure 2. Scatter plot of predicted and experimental protein solubilisation.

Overall, higher protein recoveries were obtained when working at higher pH values and lower initial biomass concentrations for longer extraction times. In addition, the software predicted a number of conditions to maximise the percentage of protein solubilised with a desirability coefficient of 1.00 (that fit within the ranges of the model).

Predicted protein recoveries were approximately 60% for all—the maximum achieved within the studied range without sample pre-treatment. Previous studies suggested that it was necessary to include a disruption step before isolation of bioactive compounds from microalgae. Not all the protein is soluble at the studied conditions because of the rigid cell wall of microalgae, which in the case of *A. platensis*, consists of four layers and is 40–60 nm thick [22]. In the current study, shear stress suffered during production and processing, as well as the dehydration step and the osmotic shock suffered while resuspending the dried biomass into distilled water, led to a percentage of total protein that could be solubilised that was lower than 60%. To increase the amount of protein solubilised, the current paper evaluated the potential of (i) performing several sequential extractions and (ii) including a cell wall disruption step using ultrasound.

Results, shown in Figure 3, suggest that a second extraction can slightly but significantly improve the amount of protein solubilised ($p < 0.05$). However, extraction time and water consumption were nearly doubled, and extra centrifugation and dehydration steps were added to the process rendering it not viable. No improvement in protein recoveries were observed with a third sequential extraction. Figure 3 also shows the effect of sonication (400 W, 24 kHz) on protein solubility and on the integrity of *A. platensis* cells. Sonication, even for 30 s, led to a significant improvement in the amount of protein that was solubilised ($p < 0.05$). After sonication for 120 s, the percentage of the total protein that could be solubilised was 96.2 ± 2.9%. Not only protein but also other cellular and intracellular molecules were solubilised as the amount of insoluble material that could be recovered by centrifugation was negligible. One of the main advantages of adding a sonication step was that, besides increasing the percentage of protein that was solubilised from approximately 60 to 96% ($p < 0.05$), the process duration was reduced from approximately 2 h to 7 min (2 min sonication plus 5 min stirring).

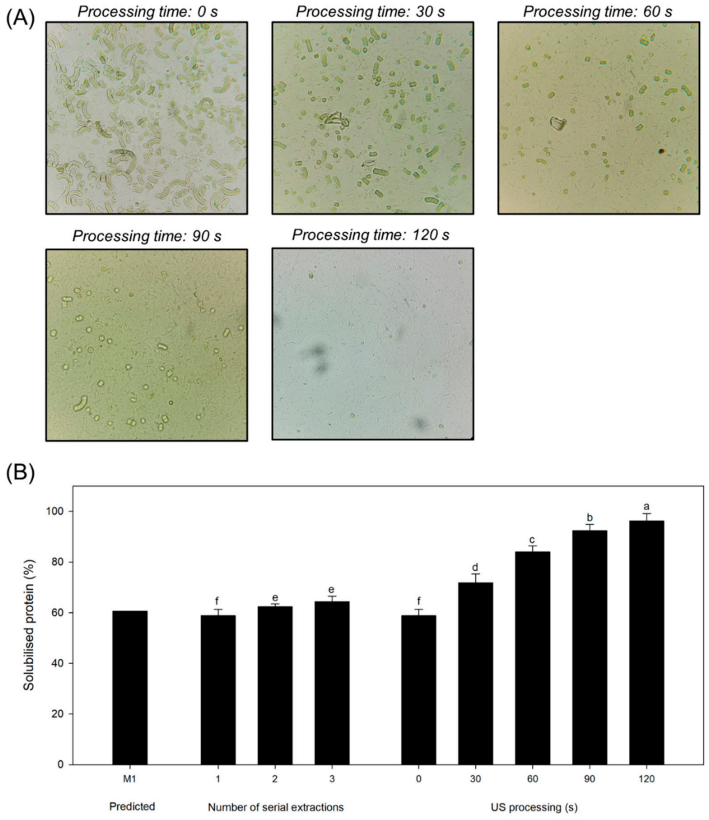

Figure 3. (**A**) Micrographs of untreated and sonicated *A. platensis* suspensions and (**B**) Effect of serial extractions and sonication on protein solubilisation. Values represent the mean of three independent determinations ± SD. Different letters indicate significant differences. The criterium for statistical significance is $p < 0.05$.

3.2. Protein Precipitation: M2

The design for protein precipitation M2 generated 11 experimental runs (Table 2). The percentage of total protein recovered ranged between 55 and 90%, and the protein content of the extracts was in the range 75–85%, which was in line with previous reports [23]. The obtained quadratic models' F-value of 23.47 implied that the model was significant ($p < 0.0005$). Its coefficient of determination R^2 was 0.9096, while predicted and adjusted R^2 values were 0.8637 and 0.8708, respectively, which were in reasonable agreement. The analysis of error indicated that the lack of fit *F*-value of 2.06 was not significant relative to the pure error, confirming the validity of the model (Table 4).

Table 4. ANOVA of independent variables for optimisation of protein precipitation from *A. platensis.*

Source	Sum of Squares	Coefficient	Mean Square	*F*-Value	Prob > F
Model	1085.74		362.91	23.47	0.0005 [a]
A: Time	179.20		179.20	11.62	0.0110 [a]
B: pH	686.51		686.51	44.52	0.0003 [a]
B2	220.03		220.03	14.27	0.0069 [a]
Residual	107.93		15.42		
Lack of fit	90.39		18.08	2.06	0.3582 [b]
Pure error	17.55		8.77		
Cor. Total		1193.67			
Adjusted R^2		0.8708			
Predicted R^2		0.8637			
SD		3.93			
CV		5.75			
PRESS		282.11			
Adequate precision		13.65			

[a] Model terms are significant ($p < 0.05$); [b] Lack of fit is not significant relative to the pure error. Abbreviations used: SD, standard deviation: CV, coefficient of variation; PRESS, predicted residual error sum of squares.

As mentioned previously, Adequate precision values higher than 4.0 indicate that the observed variation is large in relation to the underlying uncertainty of the fitted model. In this case, the adequate precision value was 13.65, suggesting that the model could be used to navigate the design space. The application of RSM leads to the following quadratic equation in terms of actual factors, which represents an empirical relationship between the percentage of protein from *A. platensis* that can be recovered by isoelectric solubilisation/precipitation and the independent variables:

$$\%Protein\ recovery = -150.4300 + 0.3123 \cdot t + 118.0542 \cdot pH - 15.9680 \cdot pH^2 \quad (5)$$

where t is extraction time expressed in min and pH is the pH value at which the precipitation is conducted. As with M1, variables and interactions between variables that were not significant to the model were not considered. Figure 4 represents the 3D contour model graph generated by the software as well as the perturbation plot of percentage protein recovery. In the case of M2, protein recovery was more sensitive to the pH value at which the protein precipitation occurred than to the extraction time, although both variables were significant.

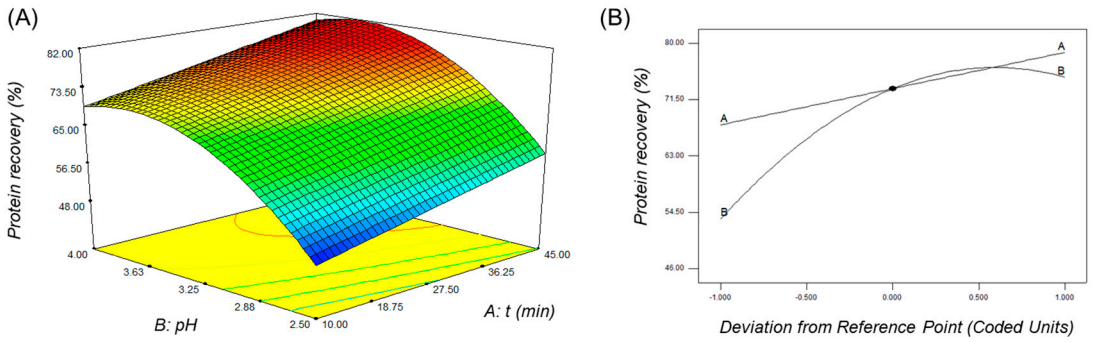

Figure 4. (**A**) 3D contour plot of percentage total protein recovery from *A. platensis* and (**B**) Perturbation plot of percentage protein recovery—M2.

The validation of M2, shown in Figure 5, demonstrates that the predicted percentage of protein recovery values are accurate enough to fit the experimental results with a correlation coefficient of 0.9138. Moreover, the combined conditions (extraction time and pH) that led to the highest protein recoveries were determined. The software predicted the precipitation conditions needed to maximise protein recovery, with a desirability coefficient of 1.00. Overall, the highest predicted protein recoveries were obtained when protein precipitation was conducted at a pH value of 3.89 over 45 min. The software predicted that using these conditions, a predicted protein recovery of 81.24% could be achieved. It is important to highlight that the microalgal biomass was sonicated and most of the *A. platensis* protein was solubilised. These conditions were validated in the lab, and a protein recovery of 75.2 ± 1.5% was achieved, demonstrating the validity of the model once again. In the current study, the isoelectric point of *A. platensis* was determined as 3.89, and the protein content of the extract was 79.1 ± 1.8%. This means that the optimised conditions led to a protein recovery yield of 0.57 ± 0.03 g of extract per g of dried biomass (0.42 ± 0.02 g of protein per g of dried biomass).

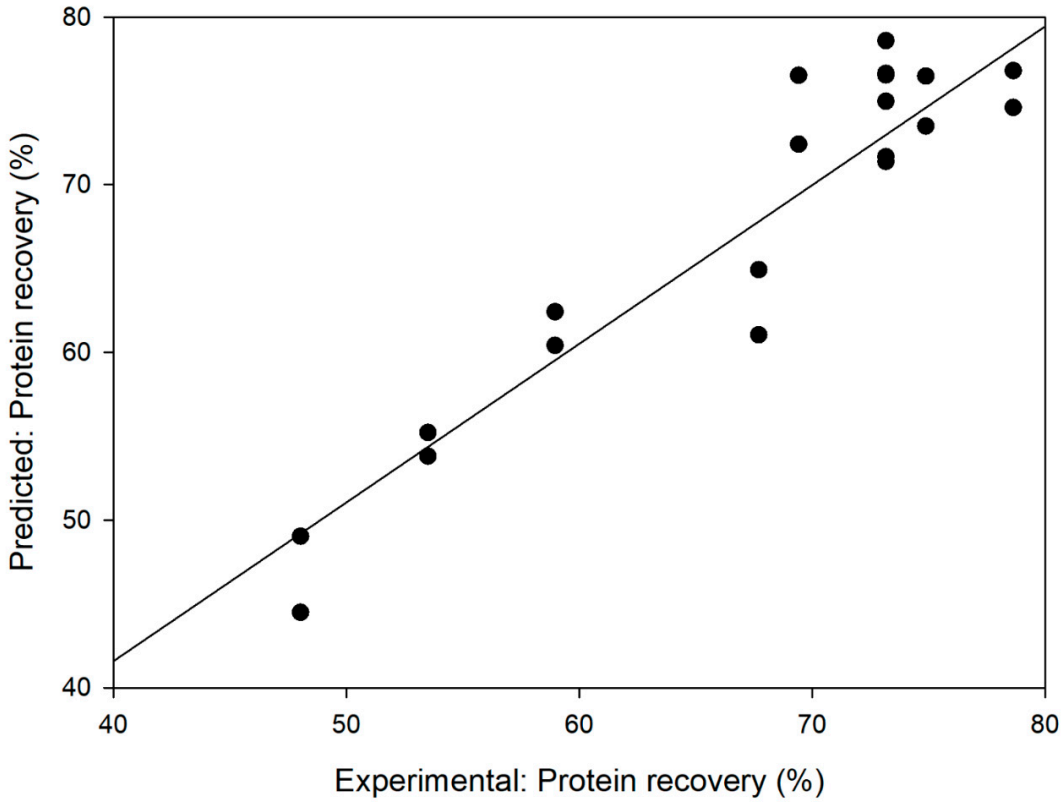

Figure 5. Scatter plot of predicted and experimental protein recovery.

4. Discussion

The obtained quadratic models' *F*-values were 54.79 and 23.47 for M1 and M2, respectively, which implied that both models were significant ($p < 0.0001$). Moreover, adequate precision values higher than 4.0, in this case, 25.3 (M1) and 13.65 (M2), indicated that the variation we observed in both models was large in relation to the underlying uncertainty of the fitted models, suggesting that both could be used to navigate the design spaces. Perturbation plots show the sensitivity of protein solubilisation/precipitation to the independent variables. A sharp curvature or slope is an indication that the response is more sensitive to a certain variable, while a relatively flat line shows that the response is less sensitive to changes in that particular factor [24]. In the case of M1, protein solubility was more sensitive to the *A. platensis* concentration than to extraction time. In the case of M2, protein recovery was more sensitive to the pH value at which the protein precipitation occurred than to the extraction time, although both variables were significant.

Protein solubilisation from *A. platensis* was influenced by pH ($p < 0.0001$). It is accepted that protein solubility increases as the pH value of the solvent moves away from the isoelectric point, either at high or low pH values [25]. Highly acidic or alkaline conditions improve protein solubility by inducing a net charge on amino acid residues. As expected, higher pH values led to higher protein solubilisation ($p < 0.0001$). Previous studies observed higher solubility of *Spirulina*-derived proteins after extraction for 35 min at pH 11.38 [26].

The current model assessed protein solubility within the pH range 9.0–11.0. Intense pH values during extraction can affect the structure and functionality of the end isolates or concentrates. Functional properties are largely influenced by solubility. The most suitable condition to produce emulsions, foams, and films using *A. platensis*-derived proteins has been suggested to be 10.0 [26]. The effect of extraction conditions on protein bioactivity, functionality, and structure will be assessed in future studies. Protein solubilisation from *A. platensis* was also influenced by extraction time ($p < 0.0001$) and biomass concentration ($p < 0.0001$). Higher extraction times led to higher protein solubility ($p < 0.0001$). Preliminary solubilisation trials have suggested that extraction times longer than 120 min did not increase protein recovery yields. These findings were in line with previous publications that suggested protein solubility mainly occurred during the first 60 min of extraction, and extraction times above 120 min has little benefit on protein yields [18]. Lower biomass concentration leads to higher protein solubility. The influence of biomass concentration on protein solubility can be partially attributed to the effect of concentration on viscosity. Higher biomass concentrations lead to higher viscosities when straining insoluble material by centrifugation, potentially leading to the loss of soluble proteins in the insoluble sediment [27]. Moreover, lower viscosity aids agitation and mass transfer and, therefore, protein solubility. It is important to highlight that lower *A. platensis* concentrations lead to higher protein recoveries but also to higher water and energy usage, key factors for the viability of the process. Finally, previous studies suggested that higher extraction temperatures led to higher protein solubilisation and, therefore, higher protein recoveries. However, temperature displayed a milder influence than pH or sample:solvent ratio on soluble protein yields [18]. Moreover, because of the high sensitivity of proteins to temperature and to reduce the energy input for the overall process, the current study conducted all the extractions at a constant temperature of 20 ± 1 °C.

As protein solubility values were relatively low, the current study assessed the potential utilisation of ultrasound as a pre-treatment for disrupting the microalgal cell wall. Ultrasound processing is based on a phenomenon known as transient cavitation, where cycles of pressure form microscopic bubbles that collapse and lead to the formation of spots of extremely high temperature (5000 °C) and pressure (50 MPa), destroying cellular envelopes [28]. Sonication can also improve mass transfer due to microstreaming caused by symmetrical cavitation and promotion of microscopic turbulence of the solid-liquid film [29]. In this work, ultrasound-processing, even for short times, led to a significant improvement in protein solubilisation ($p < 0.05$). Results were consistent with previous studies, where ultrasound processing for under 10 min was enough to achieve the liquefaction of lignocellulosic materials [30]. Sonication also allowed improved protein recoveries from plant [16] and animal [17]

sources previously. Moreover, ultrasound-assisted extraction of lipids [31], carbohydrates [20], or protein [32] from microalgae has also been reported.

In the case of M2, protein recovery yields were significantly affected by pH ($p < 0.001$) and extraction time ($p < 0.05$). As discussed previously, protein solubility is pH-dependent. The isoelectric point of a protein is the pH of the solution at which the net charge of the protein becomes zero where negative and positive charges are balanced, reducing electrostatic forces. Under these conditions, attraction forces are balanced, causing aggregation and precipitation. Highly acidic or alkaline conditions enhance solubility by inducing net charges on their amino acid residues. There is a mixture of proteins within the microalgal cells. Therefore, the aim of the current study was to identify the pH value at which the largest amount of proteins precipitate. The isoelectric point of *A. platensis* proteins, predicted to be 3.89, was in line with previous studies that suggested that the isoelectric point of *A. platensis* varied between 3.0 [26,33] and 4.0 [23]. The protein content of the extract was relatively high, although further purification steps to remove salts and other non-proteinaceous material would lead to higher purities. Further studies will assess the potential of this protein-rich extract for use as a technofunctional ingredient in the food industry. As proteins derived from algae have also been suggested as a potential source of bioactive peptides [34], further studies will also assess the potential of the extract for use as a novel material for the generation of bioactive peptides with potential applications in functional foods and in the pharmaceutical industry.

5. Conclusions

Protein isolation from the microalga *A. platensis* was carried out by isoelectric solubilisation/precipitation. Both the conditions needed to maximise protein solubilisation and protein precipitation were optimised using a response surface methodology. Although spray-drying and the osmotic shock suffered when resuspending the biomass into distilled water led to a certain degree of cell wall disruption, the amount of protein that could be solubilised was relatively low. Sequential extraction steps improved the amount of proteins that could be solubilised although the observed increase was relatively low. For this reason, it was necessary to include a cell wall disruption process based on ultrasound that led to a high amount of proteins solubilised (over 95%). At the optimised conditions, almost 75% of the total proteins could be recovered with a purity of approximately 80%, which could be further increased by dialysis and other purification steps. Further studies will assess the potential of the isolated proteins for being used as technofunctional ingredients in the food industry and as a source of bioactive hydrolysates and peptides with potential applications in the functional foods and pharmaceutical industries.

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