

Impact of High-Pressure Homogenization on the Technological Properties of *A. platensis* (Spirulina) Proteins for Food Applications

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This study investigates the extraction efficacy on *Arthrospira platensis* (Spirulina) proteins by chemical (alkaline) extraction, and High-pressure homogenization (HPH) extraction at three pressure levels (200, 500, and 1000 bar). The extraction yield, solubility, water-holding capacity (WHC), oil-holding capacity (OHC), foaming properties, and gelling characteristics of the extracted proteins were then measured. HPH treatment at 500 bar resulted in a significant ($p < 0.05$) increase in protein extraction yield, exceeding by approximately 124% the value obtained through the alkaline method. The highest solubility was observed at 500 bar, while a marked decrease occurred at 1000 bar, probably due to protein aggregation phenomena. Additionally, the HPH extracts demonstrated the best values for OHC and gelling capacity. However, WHC and solubility were significantly higher in the chemical extract. Overall, HPH treatment proved to be an effective method for protein extraction. However, selecting appropriate pressure levels is crucial to prevent protein denaturation, which could adversely affect its technological properties.

1. Introduction

The increasing world population and growing demand of nutritious (high in proteins), less impacting, more sustainable foods are driving the research for alternative and sustainable protein sources. Recently, significant efforts have been made to increase interest in proteins that mitigate environmental impacts in terms of greenhouse gas emissions as well as water and soil consumption. Coupled with these environmental concerns there is an increased awareness among consumers of their own health. However, the widespread use of alternative proteins is followed by the challenge to provide technological properties that are comparable to those of animal proteins (Diaz et al., 2023). Microalgae seem to be promising candidates in novel alternative protein sources due to their several advantages, like higher growth rate, low water consumption, no competition for arable land, carbon-neutral emissions, and the possibility to produce a wide range of bioactive compounds (Silva et al., 2022). Among these, the blue-green cyanobacterium *Arthrospira platensis* (*A. platensis*, Spirulina) is of increasing interest primarily due to its high protein content (up to 60-70 % of dry mass) including phycobiliproteins (e.g., phycocyanin and allophycocyanin), which are valuable for their nutritional and bioactive properties, making Spirulina proteins an excellent ingredient for innovative health food (Mrtek et al., 2024). Chemical extraction is the most common method applied for Spirulina protein extraction, which involves breaking down the cell walls of the microalgae under alkaline conditions to release intracellular proteins (Ladjal-Ettoumi et al., 2024; Purdi et al., 2023; Mohammadi et al., 2022). This method is preferred for its cost-effectiveness and simplicity. However, it encounters several limitations for large-scale production, such as low yields, reduced purity and functionality, as well as long process time (Hadidi et al., 2023). The use of physical technologies for disrupting the cell walls of microalgae is being explored to exploit the full potential of the biomass for protein release preserving their functionality. HPH is a mechanical technology where pressurized fluid is forced through a small orifice, leading to cell fragmentation and particle deformation (Hadidi et al., 2023). HPH exhibits high efficiency and continuous production that is advantageous for industrialised production. To

the best of the authors' knowledge, only a limited number of studies have investigated the technological properties of Spirulina proteins extracted using HPH. Magpsuao et al. (2021) highlighted the effectiveness of HPH in altering the microstructure of Spirulina, which significantly impacts the rheological properties of the whole biomass. Similarly, Shkolnikov Lozober et al. (2021) demonstrated that HPH enhances surface hydrophobicity and improves the gelling properties of Spirulina protein concentrate obtained through isoelectric precipitation. Building on these findings, the present study aims to evaluate the effect of HPH at three different pressure levels on key technological properties of Spirulina proteins, including solubility, WHC, OHC, foaming and gelling properties. Additionally, the study provides a comparative analysis of protein yield extraction via HPH and alkaline methods to assess their relative effectiveness.

2. Materials and Methods

2.1 Materials

The dried *A. platensis* (Spirulina) biomass (SB) was purchased from Sevenhills Wholefoods (Sheffield, UK). As stated by the producer, the powder was dried at 30–45 °C for 24 h, with a maximum water content of 8 %. The Pierce™ Bradford Protein Assay Kit was purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA) and was used to determine the soluble protein content. The palm oil used for oil binding capacity was purchased from Euronut s.p.a (Avellino, Italy). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germania).

2.2 Protein Extraction

2.2.1 Alkaline method

The SB was mixed at 8 % (w/v) in a 0.5 M NaOH solution - pH 10 and stirred for 2 h at room temperature according to Shkolnikov Lozober et al. (2021). The mixture was then centrifuged (10000 RCF, 30 min, 25 °C) and the supernatant was collected. The pellet was re-suspended in NaOH as described above. The two supernatants were merged, and the pH was decreased to the protein isoelectric point (pH 3) (Silvia et al., 2022). After centrifugation (10000 RCF, 30 min, 25 °C) the Spirulina Protein Extract (SPE) was neutralized and freeze-dried.

2.2.2 High-pressure homogenization (HPH) method

The SB was dispersed in distilled water at 2 % (w/v) and gently stirred overnight at 4°C for complete hydration (Magpusao et al. 2021). Then the cooled suspension was submitted to HPH (PandaPlus 2000, GEA Italia, Parma, Italy) at three different pressures (200, 500 and 1000 bar) with two passes. After the treatment, the collected samples were centrifuged (4000 RCF, 10 min, 25 °C) and the supernatants were acidified until the isoelectric point (pH 3) was reached. After centrifugation (10000 RCF, 30 min, 25 °C) the SPE was neutralized and freeze-dried.

2.3 Protein Extract Characterization

2.3.1 Determination of the total protein content

The total protein content of both SB (P_{SB}) and SPEs (P_{SPEs}) was measured by nitrogen content (N), using the standard Kjeldahl procedure with 6.25 as conversion factor (AOAC 981.10).

2.3.2 Protein solubility

The solubility of SPEs was determined according to Zhang et al. (2023) with slight modifications. The SPEs 0.1 % (w/v) was solubilized in distilled water, stirred for 1 h at room temperature and then centrifuged (2000 RCF, 15 min, 25 °C). The supernatant was collected, and the soluble protein content was measured with Bradford's assay method. The calibration curve was constructed using bovine serum albumin (BSA) as standard. The absorbance was measured at 595 nm using a VWR V-3000PC spectrophotometer (VWR International, Milano, Italy). The solubility of SPEs was calculated using Eq(1):

$$\text{Solubility} (g_{P_{\text{Soluble}}}/100g_{P_{\text{SPEs}}}) = \frac{P_{\text{Soluble}}}{P_{\text{SPEs}}} \cdot 100 \quad (1)$$

where P_{Soluble} and P_{SPEs} are the concentration of soluble protein and the total protein content of SPEs, respectively.

2.3.3 Extraction and Protein Yield

The efficacy of the alkaline and HPH extraction methods was evaluated by extraction yield and protein yield according to the Eq(2) and Eq(3):

$$\text{Extraction Yield (\%)} = \frac{m_{SPEs}}{m_{SB}} \cdot 100 \quad (2)$$

$$\text{Protein yield (\%)} = \frac{m_{SPEs} P_{SPEs}}{m_{SB} P_{SB}} \cdot 100 \quad (3)$$

where m_{SPEs} is the mass of the SPEs obtained through alkaline method and HPH at three different pressures and m_{SB} is the biomass mass used for the extraction; while P_{SPEs} and P_{SB} represent the total protein content of SPEs and biomass, respectively.

2.4 Water Holding Capacity and Oil Holding Capacity

The WHC and OHC were determined according to Liu et al. (2020) with minor modifications. Briefly, approximately 0.1 g of SPEs was weighed in a centrifuge tube. Then, distilled water or palm oil was gradually added to the centrifuge tube to cover the sample and were blended with a vortex mixer for 1 min (REAX 2000, Heidolph GmbH, DE). After blending, the sample was rested for half an hour at room temperature and then centrifuged (3200 RCF, 15 min, 20 °C) and the supernatant was decanted. WHC and OHC were calculated by using Eq(4) and Eq(5), respectively:

$$\text{WHC (} g_{H_2O}/g_{SPEs} \text{)} = \frac{m_2 - m_1}{m_0} \quad (4)$$

$$\text{OHC (} g_{oil}/g_{SPEs} \text{)} = \frac{m_2 - m_1}{m_0} \quad (5)$$

where m_0 is the mass of SPEs, m_1 is the initial mass of the centrifuge tube with SPEs and m_2 is the total mass of the centrifuge tube and SPEs after decanting the supernatant.

2.5 Foaming Capacity and Stability

The foaming capacity (FC) and stability (FS) of SPEs were investigated according to the method proposed by Purdi et al. (2023) with some modifications. The SPEs suspensions at 1.5 % (w/v) in distilled were homogenized using a T25 Ultra-Turrax homogenizer (IKA, Staufenim Breisgau, DE) for 1 min at 10000 RCF. The volumes of the SPEs suspensions before and after homogenization were measured using a graduated cylinder. The volume of the suspension remaining after 15 min was recorded. FC and FS were calculated as follows by Eq(6) and Eq(7), respectively:

$$\text{FC(\%)} = \frac{V_2 - V_1}{V_1} \cdot 100 \quad (6)$$

$$\text{FS(\%)} = \frac{V_3 - V_1}{V_1} \cdot 100 \quad (7)$$

where V_1 and V_2 are the volume of SPEs suspension before and after homogenization, respectively, while V_3 is the volume of SPEs at 15 min after homogenization.

2.6 Least gelation concentration (LGC)

The LGC was determined according to Yang et al. (2021) with some modifications. SPEs suspensions were prepared at a concentration ranging from 2.5 to 30 % (w/v) in distilled water in sealed test tubes. All samples were stirred for 30 min at ambient temperature before heating in a water bath at 90 °C for 30 min. Then, the samples were immediately cooled with an ice bath and then stored overnight at 4 °C. After 1 h at room temperature, the test tubes were inverted and the sample with the lowest concentration that did not flow was considered to be the LGC.

2.7 Statistical Analysis

Results were reported as mean \pm standard deviation calculated from three replicates. All results were statistically analysed using one-way analysis of variance (ANOVA), and multiple comparisons were carried out using Tukey's test to evaluate whether differences among the samples were statistically significant ($p < 0.05$), using JMP statistical software (SAS Institute. Inc. Cary, NC, USA).

3. Results and Discussion

3.1 Effect of extraction methods on the *A.platensis* suspensions

Table 1 shows the impact of alkaline and HPH methods on the extraction yield, protein yield and solubility of SPEs. The protein content measured in Spirulina biomass was 63.96 ± 1.36 g/100gdb, consistent with the values reported in literature for Spirulina biomass (Kaferbock et al., 2020; Ilter et al., 2018). The comparison between the two investigated extraction methods highlighted a higher extraction and protein yield for HPH at all tested pressures compared to the alkaline method. Among the three pressures evaluated, the highest extraction yield was observed at 1000 bar, likely due to almost complete cell wall fragmentation, which resulted in the release of all intracellular components into the extraction medium. Conversely, the highest protein yield was achieved at 500 bar. This finding can be explained by the phycobiliproteins protein fraction in Spirulina, which includes C-phycoerythrin, allophycocyanin, and phycoerythrin, representing 10–20% of the total proteins. These proteins exhibit high solubility even at the isoelectric point (pH 3) used for the recovery of the extract (Pez Jaeschke et al., 2021). This hypothesis is supported by the reduced solubility of proteins in the extract at 1000 bar and the total protein content observed in the HPH-1000 supernatants compared to those at lower pressures (data submitted elsewhere). The highest solubility was recorded for 500-SPE, corresponding to an increase of approximately 18.3% compared to A-SPE, while the lowest was at 1000 bar due to the loss of soluble proteins in supernatant as commented above. Moreover, excessive pressure induces aggregation phenomena, promoting the formation of hydrophobic interactions and cross-links between proteins, favouring re-aggregation and compromising dissolution capacity (Yan et al., 2024).

Table 1: Extraction Yield, Protein Yield and Solubility of Spirulina Protein Extracts. A-SPE refers to the extract obtained through alkaline method. 200-SPE, 500-SPE and 1000-SPE resulting from High-pressure homogenization at the three pressures investigated.

	Extraction Yield $g_{SPEs}/100 g_{SDBd}$	Protein Yield $g_{P_{SPEs}}/100 g_{P_{SB}}$	Solubility $g_{P_{Soluble}}/100 g_{P_{SPEs}}$
A-SPE	28.15 ± 1.69^d	31.21 ± 0.83^c	42.88 ± 0.25^b
200-SPE	52.61 ± 1.17^c	55.82 ± 1.24^b	39.05 ± 0.39^c
500-SPE	63.05 ± 0.85^b	68.02 ± 0.92^a	50.74 ± 0.95^a
1000-SPE	76.16 ± 0.39^a	58.42 ± 0.30^b	31.60 ± 0.54^d

Different letters in the columns indicate significant differences ($p < 0.05$) among samples.

3.2 Foaming properties

The foaming capacity (FC) of a protein refers to the ability to create an interfacial area at the air-water interface, which can be created by the protein during foaming, while foaming stability (FS) is the ability of a protein to stabilise air bubbles against gravitational stress by reducing surface tension through protein-protein interactions (Ladjal-Ettoumi et al., 2024). The FC and FS of the A-SPE and 200, 500, 1000 - SPE are reported in Figure 1.

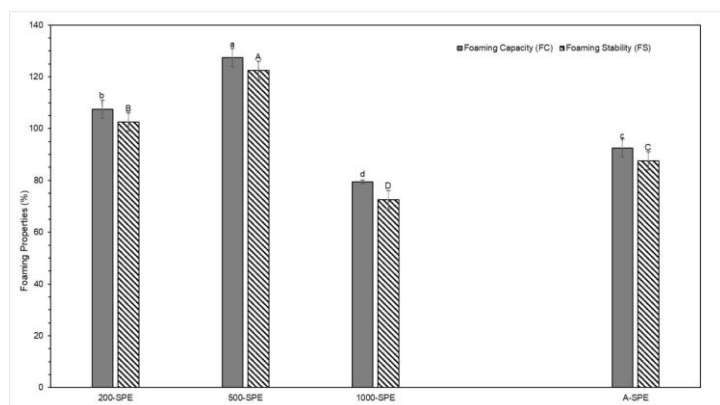


Figure 1 Effect of High-pressure homogenization pressures (200, 500 and 1000 bar) and alkaline treatment on the foaming capacity (FC, %) and stability (FS, %) of Spirulina Protein Extracts. Different letters indicate significant differences ($p < 0.05$) among samples: lowercase (a-c) for foaming capacity and uppercase (A-C) for foaming stability.

The extract obtained at 200 and 500 bar showed higher FC and FS than alkaline extract with the maximum value reached at 500 bar. The 1000-SPE sample showed the lowest foaming properties. The ratio of hydrophobicity and solubility of proteins is a key factor influencing the foaming properties. The degree of hydrophobicity is influenced not only by the amino acids sequence but also by the protein conformation, particularly its folding (Ma et al., 2023). HPH could induce protein unfolding, which reveals more hydrophobic amino acid groups that were hidden within the protein structure, making them available to the protein surface (Yan et al., 2024). Consequently, proteins with higher surface hydrophobicity are expected to exhibit higher adsorption rate at the air-water interface, thus enhancing their ability to foam (Amagliani et al., 2021). Although the soluble protein concentration seems to be a prerequisite for good foaming properties, the protein structure plays a crucial role in determining foaming capacity (Amagliani et al., 2021). At 1000 bar, the severe pressure conditions may lead to extensive protein denaturation and aggregation, reducing their ability to form stable foams. This coupled with the lowest soluble protein content as reported in Table 1 could explain the significantly lower foaming properties measured.

3.3 WHC and OHC

WHC and OHC are important parameters that provide information on the ability of proteins to absorb water and oil respectively influencing the texture and mouthfeel of food products (Purdi et al., 2023). The extraction method highlighted a significant ($p < 0.05$) influence on the results obtained by WHC and OHC, as shown in Table 2. The lower WHC values measured for HPH treatments could be due to the change of the native protein by high pressure with a high exposure of hydrophobic groups, previously hidden within the crude protein structure. These limit protein-water interaction favouring hydrophobic interaction with oil solvent and thus higher OHC values than A-SPE (D'Alessio et al., 2023). These reduced protein-water interactions following HPH treatment could enhance stronger hydrophobic interaction with oil solvent, as reflected by the significantly higher OHC values recorded for 200-SPE and 500-SPE compared to A-SPE. However, at 1000 bar, OHC decreased suggesting loss of oil-binding enhancement achieved at moderate pressures.

Table 2: Water and oil holding capacity (WHC and OHC, respectively) of High-pressure homogenization obtained samples compared to conventional alkaline extracts

	WHC ($g_{H_2O}/100 g_{SPEs}$)	OHC ($g_{oil}/100 g_{SPEs}$)
A-SPE	4.46 ± 0.05 ^a	3.19 ± 0.10 ^c
200-SPE	3.65 ± 0.06 ^c	3.70 ± 0.05 ^b
500-SPE	3.92 ± 0.04 ^b	4.09 ± 0.04 ^a
1000-SPE	3.35 ± 0.05 ^d	3.07 ± 0.03 ^c

Different letters in the columns indicate significant differences ($p < 0.05$) among samples.

3.4 LGC of SPEs

The capability of a protein to create a gel network can be indicated by identifying the LGC. The extract coming from alkaline method formed a gel starting from 18 $g_{SPE}/100$ mL in contrast to the three HPH extracts for which the protein extracts amount was close to 15 $g_{SPE}/100$ mL. In comparison, Benelhadj et al. (2016) reported a LGC of 12% (w/w) for a protein isolate extracted under alkaline conditions followed by isoelectric precipitation. Although slightly lower, this value is in the same order of magnitude and supports the observed gelling capability of *Arthrospira platensis* proteins under similar extraction method.

4. Conclusions

The current research demonstrated the potential of HPH as sustainable method for extracting proteins from *Arthrospira platensis* (Spirulina). At 500 bar a notable enhancement in protein yield compared to alkaline method. These results underscore the efficiency of HPH in overcoming the limitations associated with traditional methods, such as low extraction and protein yields and prolonged processing times. 500-SPE showed the highest functional properties as solubility, foaming capacity and stability, oil-holding capacity and LGC. However, the obtained results also revealed that excessive pressure (1000 bar) can lead to protein aggregation and reduced solubility, emphasizing the importance of optimizing HPH parameters to balance yield and functionality. In conclusion, HPH emerges as a versatile and sustainable technology for processing Spirulina protein, in line with global efforts to develop alternative protein sources for use in new sustainable food formulations. The results presented here provide the basis for future studies aimed at optimising the parameters of HPH and exploring its application in different food systems.

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