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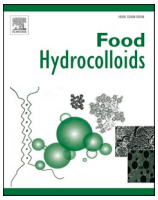

Microalgae protein: A comparison between spray-dryed and frozen paste cells

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ABSTRACT

The need for sustainable protein substitutes is being driven by the growing global population. Because of their high protein content and environmental sustainability, microalgae are a viable source. In this study, proteins extracted from frozen paste and spray-dried biomass from *Nannochloropsis oceanica* and *Tetraselmis chui* were studied.

High-pressure homogenization, followed by ultrafiltration (non-purified, NPS) and ammonium sulfate precipitation (purified, PS), was used to process the protein extracts.

PS extracts showed higher protein concentrations, reaching approximately three-fold higher levels than NPS in spray-dried *N. oceanica* and about 2.7-fold higher in frozen paste samples, while frozen paste *T. chui* exhibited a 2.6-fold increase. Spectroscopic and chromatographic analyses revealed that frozen paste extracts preserved a greater proportion of native protein structures and displayed higher hydrophobic site exposure, whereas spray-drying and purification reduced α-helix content and promoted protein aggregation. Despite their lower protein concentration, frozen paste NPS extracts exhibited more favorable structural characteristics that may support improved techno-functional performance.

These results highlight the importance of selecting processing conditions based on intended applications. Future food formulations could benefit greatly from the use of microalgal proteins, especially those derived from frozen biomass, as adaptable and sustainable ingredients.

1. Introduction

It is projected that the global population may reach 10 billion in the next 30 years, suggesting that food demand will increase correspondingly and, consequently, demand for alternative proteins will be higher than ever (United Nations, 2024). The rising pressure on traditional animal-based protein sources, due to both economic and environmental concerns, has accelerated interest in sustainable alternatives. Among these, plant-based proteins have received significant attention; however, novel sources such as microalgae offer unique advantages that extend beyond conventional plant proteins (Geada et al., 2021).

Microalgae represent a particularly promising biomass as they grow rapidly, have low cultivation costs, do not require arable land, and are widely recognized for their rich profile of bioactive compounds, such as proteins, polyunsaturated fats and polysaccharides (Geada et al., 2021). These features make microalgae an attractive candidate for sustainable protein production. In recent years, microalgae have been incorporated into a variety of traditional food products due to their high nutritional value and health-promoting properties (Y. Chen et al., 2019). The protein content of microalgae generally ranges from 20% to 80%, and these proteins have attracted significant interest because of their high nutritional value compared to other vegetable proteins (such as pea, lupins, bens among others), particularly in terms of amino acid profile composition (Geada et al., 2021).

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When developing non-animal foods, the choice and treatment of protein are crucial, as they determine not only nutritional value but also functional characteristics such as taste, texture, and consumer acceptability. Generally, protein act as emulsier, texture modifier and/or water/oil absorption enhancer in food products (Ahmed et al., 2022; Y. Chen et al., 2019; Samarathunga et al., 2023; Zhao et al., 2024). However, the functional properties of proteins may vary across species and are influenced by numerous factors, such as extraction and preservation methods, which can alter their conformation or molecular size (Y. Chen et al., 2019).

Among preservation strategies, food drying is a key step in stabilizing microalgae for long-term use. Drying reduces water activity, limiting microbial growth and enzymatic degradation, while enabling transport and storage of biomass in powder form (Ratti, 2001). Several drying techniques have been applied to microalgae, each with advantages and limitations. Convective hot-air drying is simple and cost-effective but may lead to nutrient loss due to prolonged heating (Lewicki, 2006). Freeze-drying preserves bioactive compounds effectively but is expensive and energy-intensive (Tang & Pikal, 2004). Spray-drying offers a practical balance of cost and efficiency, converting liquid extracts into stable powders, though thermal and air-interface stresses can denature sensitive proteins and alter secondary structures (α-helix, β-sheet, and random coil) (Adler et al., 2000; Maa et al., 1998).

The choice of drying method is particularly important for microalgae proteins because it affects both their functional properties and overall nutritional quality. For example, studies on *Arthrospira platensis* and *Chlorella* sp. species have shown that freeze-dried powders retain more bioactive compounds and functional protein structures compared to air- or spray-dried powders (Plaza et al., 2009)(Kumar et al., 2016). These differences highlight the need for careful consideration of processing conditions to maximize the utility of microalgae proteins in food applications.

Ammonium sulfate, (NH4)2SO4, is frequently utilized for salting out proteins because of its high solubility, which allows for solutions of very high ionic strength, low price, and broad availability of pure material (Burgess, 2009; Duong-Ly & Gabelli, 2014). Although the influence of processing on functional properties is anticipated, there is limited information regarding the assessment of this effect in microalgae proteins, particularly in these specific microalgae.

The main objectives of this study were:

- To assess the influence of different preservation methods, specifically spray-drying and freezing, on posterior protein extraction and structural integrity of proteins from *Nannochloropsis oceanica* (*N. oceanica*) and *Tetraselmis chui* (*T. chui*);
- To investigate the impact of high-pressure homogenization as a low-energy method for disrupting cell membranes of microalgal cells and extraction, considering the preservation methods cited;
- To examine the effect of subsequent purification through ammonium sulfate precipitation (ASP) on protein conformation in extracts from both *N. oceanica* and *T. chui.*

2. Materials and methods

2.1. Material and reagents

N. oceanica and *T. chui* used in this work were kindly provided by Necton (Olhão, Portugal) in a dried (spray-dryer), and frozen paste format (frozen at −18 °C). The frozen paste solid content was 27.69 g/100 g for *N. oceanica* and 32.33 g/100 g for *T. chui*.

The dH_2O was obtained from Milli-Q equipment (Millipore, Sigma-Aldrich, St. Louis, MO, USA), and the ultrapure H_2O from Water Milli-Q equipment (WonderStatus, Sabrosa, Portugal). The $(NH_4)_2SO_4$ was obtained from Sigma-Aldrich (St. Louis, MO, USA), and the standard pullulan kit P-82 (6.1 to 337 kDa) for HPLC was obtain from ShodexTM, Japan.

2.2. Proteins extract preparation

The spray-dried microalgae species, *N. oceanica* and *T. chui*, were diluted in dH_2O to a solid-to-liquid ratio of 1:10, then stirred magnetically for 4 h at room temperature. The frozen paste biomasses (initial H_2O content between 15 and 20%) were diluted to a final concentration of 10 % in the suspension with H_2O. Thereafter, each of the microalgae species was subjected to High Pressure-Homogenizer (HPH) (Panda Plus 2000; GEA Niro Soavi, Parma, Italy) at a pressure of 1000 ± 100 bar, in a single cycle, at 23 ± 2 °C as described in Moreira et al., 2025). The suspensions were then subjected to a microfiltration step, at room temperature, using a pilot-scale filtration unit, Pellicon® 2 0.65 mm (Merck, Darmstadt, Germany), followed by an ultrafiltration step using a Pellicon® 2 Cassette with Ultracel® 5 kDa Membrane. The concentrated protein solutions from the retentate were collected and store at 4 °C – non-purified sample (NPS).

The NPS was subjected to further purification via ammonium sulfate precipitation (ASP). The process employed was detailed by Duong-Ly and Gabelli (2014) with minor modifications (Duong-Ly & Gabelli, 2014). Proteins were separated from other components by applying a 70% $(NH_4)_2SO_4$ saturation. The mixture was stirred continuously at 4 °C overnight. Subsequently, the resulting protein precipitate was separated by centrifugation at 18000 rpm for a period of 30 min. The resulting pellet was resuspended in an equal initial volume of dH_2O and then subjected to ultrafiltration using the previous filtration system to remove residual salts. The protein solutions from the permeate were collected and kept at 4 °C – purified sample (PS) (see Fig. 1).

2.3. Protein quantification

The protein content was determined by CHN elemental analysis, according to the procedure provided by the manufacturer using an elemental analyzer system (Unicube, Elementar, GmbH, Hanau, Germany). Briefly, lyophilized samples (2 mg) were weighed into small tin capsules and subjected to combustion at temperatures above 1200 °C. Carbonization occurred in the presence of ultrapure oxygen, ensuring complete oxidation of the organic material, while ultrapure helium served as the carrier gas. During this process, carbon (C), hydrogen (H), and nitrogen (N) in the samples were converted into CO_2, H_2O, and N_2, respectively. The resulting signals were automatically recorded and integrated by the analyzer system. Final concentrations of C, H, and N were then determined stoichiometrically based on their percentages obtained from CHN analysis and the total mass of the freeze-dried samples. The final protein content was calculated by multiplying the nitrogen content by the conversion factor (4.78) (Biancarosa et al., 2017). The results are expressed as g/100g of dry weight biomass. All experiments were done in triplicate.

2.4. Microscopy

The morphology of frozen and spray-dried microalgae, before and after HPH, was observed by optical microscopy. The biomass was suspended in distilled water and the pictures were acquired with an Axioscope Z5 microscope coupled to a Zeiss-Axiocam305 RGB (Carl Zeiss Microscopy, Germany), with an amplitude of 40x. Images were treated using Zeiss Zen Lite microscope software (BLUE edition 3.3), and scale bars were added.

2.5. Circular dichroism

The protein's secondary and tertiary structural changes were determined using a Circular dichroism (CD) spectrophotometer Jasco J-1500 (Jasco Inc., Tokyo, Japan). The analyses were performed at room temperature (approximately 22 °C). For the secondary structure study, the following parameters were used: wavelength ranging from 190 nm to 250 nm; scanning speed of 20 nm/min; bandwidth of 1 nm; data pitch of

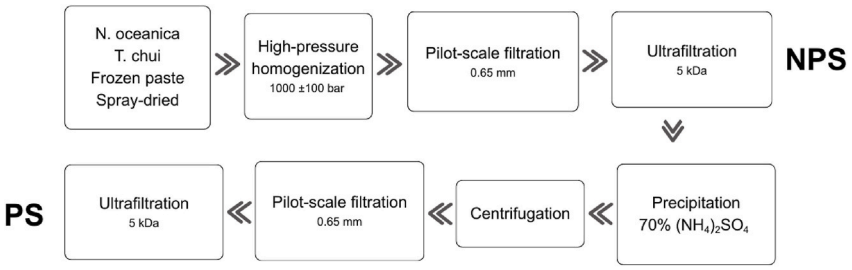

Fig. 1. Flowchart of the process to obtain protein extracts. NPS: non-purified samples; PS: purified samples.

0.5 nm; digital integration time (D.I.T) of 1 s; 5 accumulations. Samples with a concentration of 0.1 mg/mL protein content were analyzed in a 1 mm path length quartz cell. Samples were studied with a gas flow (N_2) of approximately 5 L/min. Potential interference from other compounds was minimized by subtracting a baseline spectrum of the solvent (dH_2O), adjusting all samples to 0.1 mg/mL protein content to reduce variability in protein content, selecting wavelength regions dominated by peptide bond signals (190-250 nm), and normalizing the spectra.

2.6. Intrinsic fluorescence measurements

For the study on the tertiary structure of protein, fluorescence determinations were performed using the Aqualog fluorescence instrument (Horiba-Jobin Yvon, Inc. Japan). Intrinsic fluorescence was measured using a sample solution with a concentration of 1 mg/mL protein. The analysis was conducted by exciting the aromatic pool and amino acids present in the protein with an excitation wavelength of 280 nm to determine the intrinsic fluorescence. The emitted light in the range of 290 nm to 450 nm was collected for analysis.

2.7. Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR analyses were conducted using an ALPHA II- Bruker spectrometer (Ettlingen, Germany) to investigate the wavelength region spanning from 4000 to 400 cm^{-1}. Each sample was scanned 64 times, with a resolution of 4 cm^{-1}. The generated spectra underwent baseline correction and absorbance normalization. The samples were lyophilized to be analyzed under ATR-FTIR.

2.8. Molecular weight

The protein's molecular weight (MW) distribution was assessed through high-performance liquid chromatography (HPLC) gel permeation chromatography (GPC), utilizing a PolySep-GFC-P-4000 column (300 × 7.8 mm, Phenomenex, USA). Lyophilized extracts were diluted in dH_2O at a concentration of 1 mg/mL and then subjected to elution with ultrapure water, employing a flow rate of 0.8 mL/min at a temperature of 40 °C, with refractive index (RI) and ultraviolet (UV) detection methods. Linear regression calibration was executed using the standard pullulan kit P-82 from ShodexTM, Japan, over a range spanning from 6.1 kDa to 337 kDa (Castro-Ferreira et al., 2022).

2.9. Data processing, fitting and analyses

A statistical analysis was conducted to determine significant differences, based on the analysis of variance (One-Way ANOVA) using GraphPad Prism software (version 9.5.1, California, USA). A Tukey test was used for multiple comparisons using statistical hypothesis testing, and a 95 % confidence interval was considered. All spectra for CD, fluorescence and FTIR were the result of at least three accumulations of independent determinations, smoothed using a 10-point adjacent averaging. For CD spectra analysis to obtain secondary structure quantifications, and to estimate the melting temperature (Tm), from CD transition profile, all fitting and calculations were performed individually for each spectroscopic determination, and the results are presented as average values with standard deviations. All experiments were done in triplicate.

3. Results and discussion

3.1. Extracts protein content

The protein content in extracts from the frozen paste is higher than that of extracts obtained from the spray-dried biomass, as shown in Fig. 2. The *N. oceanica* and *T. chui* NPS show protein content of 35.58 % ± 5.69 in frozen paste, contrasted with 25.69 % ± 0.09 from spray-dried cells. Similarly, for *T. chui*, the protein content was observed at 33.32 % ± 2.02 for the frozen paste, compared to 25.19 % ± 0.12 for the spray-dried. This discrepancy in preservation methodologies results in over a 30% increase in protein concentration in the frozen paste following extraction via HPH. After the purification step, the *N. oceanica* PS from frozen paste resulted in a protein concentration of 95.65 % ± 3.82, whereas the spray-dried showed 78.54 % ± 0.52. In the case of *T. chui*, frozen paste PS showed a protein concentration in the extracts of 88.05 % ± 5.33, compared to 59.43 % ± 0.74 in the spray-dried. The most significant difference was observed in *T. chui*, where the protein concentration in PS extracts from frozen cells was 50% greater than that in the spray-dried cells. It is recognized that the hydrophobicity of algae surfaces can indeed affect the extraction, depending also on the solvent's chemical properties. Protein denaturation, which involves changes in the secondary structure and surface hydrophobicity, can significantly impact extraction efficiency. Heat denaturation, for example during spray-drying, can increase the surface hydrophobicity of proteins,

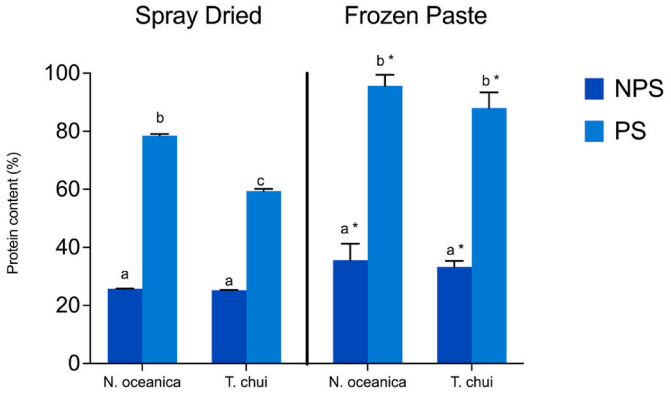

Fig. 2. Protein content of extracts (% dry weight basis) from *N. oceanica* and *T. chui* spray-dried and frozen paste. Each value is represented as an average of three independent measurements. NPS: Non-purified sample; PS: Purified sample. Different letters indicate statistically significant differences ($p < 0.05$) among treatments and algae within either Spray Dried or Frozen Paste. An asterisk (*) indicates statistically significant differences ($p < 0.05$) between Spray Dried and Frozen Paste.

promoting aggregation and potentially reducing extraction efficiency (Wagner & Anon, 1990; Yoshidome & Kinoshita, 2009). Besides, the spray-dryer method by itself could also impact the aggregation of microalgal biomass, leading to reduced solvent accessibility and increased steric hindrance (Li & Meng, 2023; Ryckebosch et al., 2011). This effect is evident in both microalgae studied, where the yield increase is substantially higher compared to the spray-dryer method when using frozen paste.

The frozen step in the frozen paste cells may also help during the extraction phase (Moreira et al., 2025). Theoretically, freezing the biomass initiates the crystallization of intracellular water, and the subsequent thawing leads to the expansion of these crystals, which results in the rupture of cellular structures (Pagels et al., 2021). A study conducted by Izanlou et al. (2023) have reported that freeze-dried samples achieved higher lipid, carbohydrate, and protein yields compared to oven-dried samples for some *Chlorella* strains (Izanlou et al., 2023). Also, Ba et al. (2016) have reported a higher protein content in the extracts when using frozen biomass than the data provided on the literature with dried *Haematococcus pluvialis* (Ba et al., 2016).

Also, the ASP has been demonstrated to be an effective method for protein purification in microalgae. This technique is frequently utilized as an initial step in the purification process due to its capacity to concentrate proteins and precipitate the desired molecules from solution (Grodzki & Berenstein, 2010). In *N. oceanica*, ASP has been successfully employed to purify proteins. For instance, a study demonstrated that using 50% $(NH_4)_2SO_4$ concentration resulted in the highest yield of precipitated total soluble proteins, significantly increasing the recovery of a protein by 1.8-fold compared to lower concentrations (Park et al., 2015). This methodology has been applied to other microalgae to concentrate protein, such as in the purification of C-phycocyanin from cyanobacteria (Moraes & Kalil, 2009).

Generally, in the *N. oceanica* spray-dried samples, the protein concentration in PS was three times higher than in NPS, whereas in the frozen paste, this difference was approximately 2.7 times greater. In the *T. chui* samples, the frozen paste exhibited a similar difference in protein concentration between PS and NPS, with PS being 2.6 times higher. A study conducted by Zhang et al., 2025 have already demonstrated that the protein purity in Spirulina cells increased by 15% in the 10 kDa retentate following a pre-treatment involving ultrasonication and Viscozyme® L, in conjunction with ultrafiltration (Zhang et al., 2025). Notably, this increase in purity is substantially lower than the results achieved in the current study. The presence of water in the frozen paste may facilitate the extraction process, particularly in *T. chui*.

3.2. Morphology

N. oceanica is distinguished by its spherical morphology, measuring 4 to 6 μm in diameter. Its cell wall is predominantly composed of carbohydrates (75%, in the form of cellulose polymers), with minor contributions from other monosaccharides such as rhamnose (Bernaerts et al., 2018). In contrast, *T. chui* cells are oval, measuring 10-15 μm, with mannose and glucose as the primary monosaccharides in their cell walls (Anjos et al., 2022). As illustrated in Fig. 3, *N. oceanica* subjected to spray-drying prior to HPH exhibits a tendency for aggregation, which is not present in the frozen paste, with aggregates showing dimensions between 15 and 20 μm. In the case of *T. chui*, the preservation method does not appear to affect cell size or promote aggregation, although frozen paste cells appear more susceptible to disruption following HPH treatment, which aligns with the extraction yields. HPH exerts a substantial influence on the structural characteristics. For spray dried *N. oceanica*, a clear disaggregation is visible after HPH, while in *T. chui* cell disintegration is the most notorious. A correlation between applied pressure and cell structure has been described for *Tetraselmis, Arthospira platesis, and Isochrysis,* highlighting that these species' cell wall structure has lower resistance to shear-induced damage inflicted by HPH. In contrast, *Nannochloropsis* showed a minimal response, attributable to its robust cell walls and possible steric hindrance effects (Delran et al., 2023; Magpusao et al., 2024).

The cell wall of *Nannochloropsis* is characterized by structural polysaccharides and highly recalcitrant biopolymers. Previous studies have reported the presence of algaenan-like compounds in the outer cell wall layer, which consist of resistant aliphatic polymers resistant to both alkali and acid hydrolysis and aqueous/organic solubilization and, thus contributing to the resistance of the cell wall (Gelin et al., 1997; Scholz et al., 2014).

Literature also show the presence of glucose, rhamnose, and mannose in *Nannochloropsis* cell wall, suggesting the occurrence of cellulose-related and other structural polysaccharides. The presence of these structural components, together with resistant wall polymers described, likely contributes to the mechanical robustness of the cells and explains the need for efficient disruption strategies (Scholz et al., 2014).

Various extraction techniques and pre-treatment methods have been explored in the paste to facilitate the disintegration and degradation of membranes and complex cell walls, including physical methods and chemical treatments, but it seems that HPH is the most promising. (Sow and Du, 2024) conducted an investigation using scanning electron microscopy to examine *Nannochloropsis* sp. after subjecting it to two stages of HPH at 2000 bar. Their observations revealed tiny holes on the cell surfaces (Sow & Du, 2024), consistent with increased permeability. Nonetheless, they reported that these conditions did not lead to complete cell disruption (Sow & Du, 2024).

3.3. Circular dichroism of microalgae extracts

The circular dichroism analysis in the Far-UV region (190–250 nm)

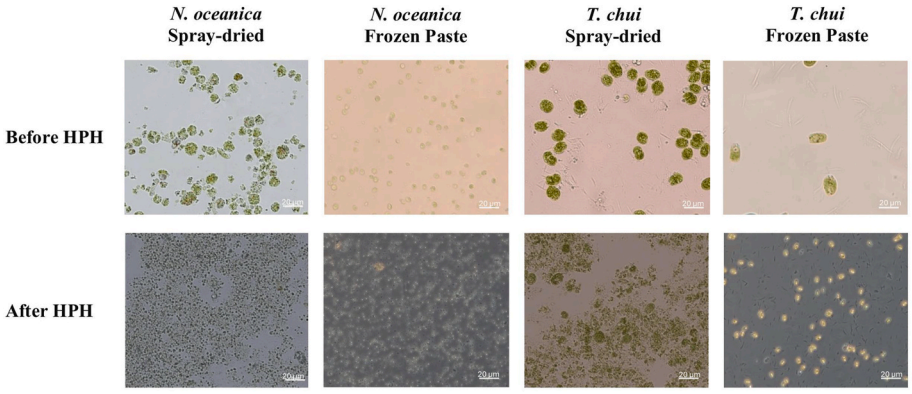

Fig. 3. Optical microscopy of microalgae suspensions (spray-dried and frozen paste), before and after HPH. The images were captured with an amplitude of 40x. HPH: High-pressure homogenization.

was used to study the conformational changes in the protein secondary structure after the purification process. CD study spectroscopy can be used to rapidly evaluate the effects of different treatments on protein secondary structure, allowing its correlation with protein aggregation, unfolding, and binding interactions (Marques et al., 2023). When an α-helix structure is present, the spectrum shows two negative peaks at 208 and 222 nm, while a typical β-sheet structure presents a broad negative band between 218 nm (Kuril et al., 2025).

The obtained spectra for each microalgae extract condition is represented in Fig. 4. From the analysis of Fig. 4A (which represents the CD spectra of *N. oceanica*) there is no marked significant difference between frozen paste and spray-dried NPS since both showed that have maintained the integrity of protein secondary structure. A negative peak at 208 nm and 222 nm was observed, representing a broad n→π* transition, which represents the α-helix configuration, as well as PS frozen paste. On the other hand, the application of a purification step combined with the spray-dryer technique caused a higher loss of secondary structure when compared to the protein from frozen paste PS.

In the case of *T. chui* (Fig. 4B), the most considerable differences were between NPS and PS, as opposed to frozen paste and spray-dried methods. PS samples exhibit an n→π* transition at 208 nm and a very subtle peak at 222 nm, whereas NPS samples display a distinct n→π* transition at 208 nm followed by a curve that appears to represent both α-helix and β-sheet. Consequently, the purification of *T. chui* NPS extracts seems to result in a significant reduction in β-sheet structure.

Furthermore, for both microalgae, the decrease in the amplitude of the peak near 190 nm and shift to slightly higher wavelengths from the non-purified to the purified samples with ASP may be indicative of the presence of more disordered (unfolded) structures (Kuril et al., 2025). Nevertheless, this is also corroborated by the decrease in the 208 and 220 nm peaks, corresponding to a decrease in the helical fraction of the protein, when the purification through precipitation was applied. This indicates that precipitation with $(NH_4)_2SO_4$ causes major changes in the secondary structure of the analyzed proteins. Moreover, preserving the microalgae by spray-drying did not appear to have a significant effect, except for *N. oceanica* PS, where there appears to be a total loss of secondary structure.

The observed variations in the secondary structure of both microalgae can be attributed to the application of a pre-processing step involving HPH. This process typically results in a reduction of α-helix and an increase in β-sheet structures, a phenomenon observed exclusively in *T. chui*. This is likely due to the exposure of proteins to the solvent, as well as the steric hindrance and the extent of cell disruption experienced by *Nannochloropsis* under HPH conditions (W. Chen et al., 2024; Q. Wang et al., 2024). However, the general comparison between the spectral analysis of PS and NPS showed significant differences. The transitions in the PS spectrum were smoother than those in the NPS, especially in *N. oceanica*. The Hofmeister series (Patete et al., 2009) could explain that phenomenon: when $(NH_4)_2SO_4$ binds with water molecules, they become unavailable for interaction with proteins. Consequently, the proteins aggregate through intermolecular interactions and eventually precipitate. Although this process is easy to understand, the precipitation step often results in a loss in protein activity, leading to the loss of structure, which could be the reason why the PS shows smoother transitions in the secondary structure. It is also interesting to note that, as previously mentioned for *T. chui* extracts, the purification step by precipitation is associated with and observable loss of β-sheet structures (Kuril et al., 2025). This could be happening due to molecular rearrangement between protein-protein after and before $(NH_4)_2SO_4$ (Pessoa Jr et al., 2024).

3.4. Intrinsic fluorescence of microalgae extracts

The tertiary structure comprises a protein molecule with a hydrophobic core, hydrogen bonds, and salt bridges. The changes in the tertiary structure of the protein molecule can be measured by the intrinsic fluorescence (IF) of tryptophan (Trp) residues (see Fig. 5). The key determinant factors of the tertiary structure of proteins are Trp residue changes, sulfhydryl content, disulfide bonds, and surface hydrophobicity.

The presence of different temperatures as a pre-treatment, such as during spray drying, and frozen, imposed lower IF intensity for spray-drying and higher IF intensity for frozen, confirming different protein conformations induced since the origin of the biomass (Rodrigues et al., 2020). A distinct difference exists between the frozen PS paste and the other samples. The use of a spray dryer may facilitate aggregation induced by protein thermal unfolding. This process likely increases hydrophobic interactions among exposed hydrophobic amino acids, leading to a decrease in IF. Such heightened protein-protein interactions may hinder the availability of Trp residues (Asen & Aluko, 2023; Avelar et al., 2024). Both *N. oceanica* and *T. chui* samples obtained by the spray-dryer method show considerably lower IF than frozen paste PS. The main difference is on the frozen paste PS *vs*. the frozen paste NPS. Through ASP the aromatic groups of frozen paste PS were exposed to water, increasing the IF, indicating the changes of tertiary conformation and partial unfolding of protein and denaturation (Hu et al., 2019; Jadhav et al., 2024), although promoting protein aggregation (see Fig. 6). In the frozen paste NPS it is possible that the protein is present in its native form, presenting the carbohydrates or lipids connected to the protein on the hydrophobic region, leading to a decrease in the IF (X. Wang et al., 2017).

It is essential to acknowledge that the current microalgae biomass available on the market is often subjected to spray-drying, which, as previously mentioned, can result in protein denaturation and aggregate formation. The pre-treatment involved in the drying of microalgal biomasses significantly influences the structural integrity of the proteins.

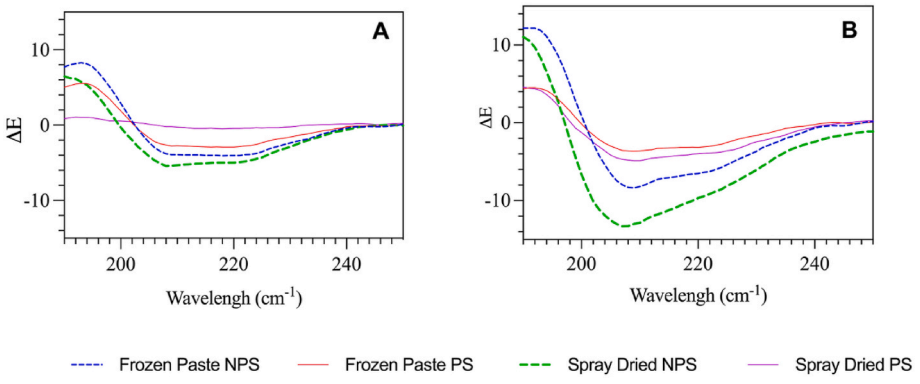

Fig. 4. Circular dichroism spectra obtained for the secondary structure of non-purified protein extracts (NPS) and purified protein extracts (PS) analyzed in Frozen paste and Spray-dried of **A)** *N. oceanica*, and **B)** *T. chui*. Each spectrum is represented as an average of three independent measurements.

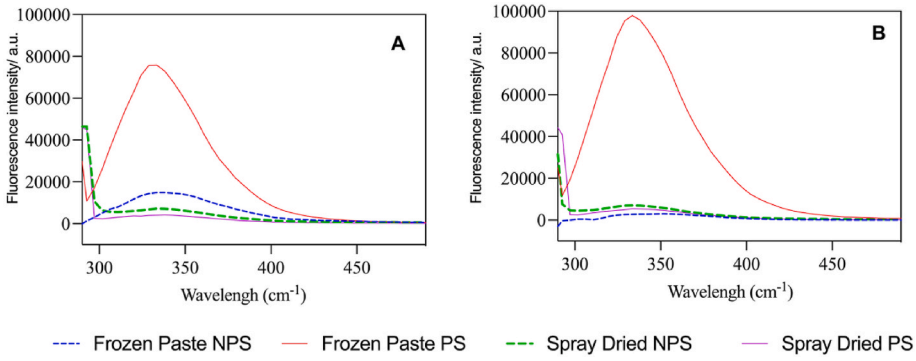

Fig. 5. Intrinsic fluorescence spectrum of non-purified protein extracts (NPS) and purified protein extracts (PS) of A) *N. oceanica*, and B) *T. chui*. Each spectrum is represented as an average of three independent measurements.

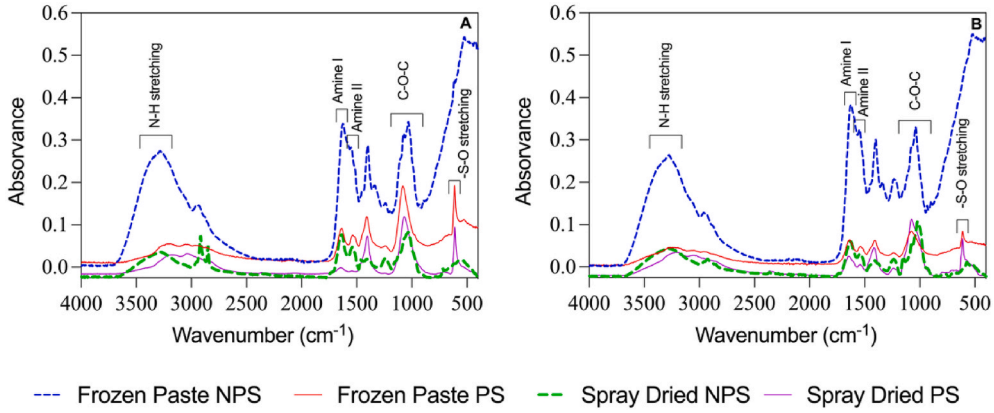

Fig. 6. Fourier transform infrared (FTIR) spectrum of non-purified protein extracts (NPS) and purified protein extracts (PS) of **A)** *N. oceanica*, and **B)** *T. chui*. Each spectrum is represented as an average of three independent measurements.

While for *T. chui* it appears to be inconsequential whether frozen paste biomass or spray-dried biomass is utilized in the NPS, the tertiary structure of *N. oceanica* exhibits considerable differences between these two samples. Such differences could have implications not only at the structural level but also in functional characteristics, such as emulsifying capacity (Avelar et al., 2024).

3.5. ATR-FTIR

The FTIR spectroscopy is a valuable method for analyzing a sample's chemical structure through the absorption of IR radiation at specific wavelengths and intensities. This non-destructive technique requires minimal sample preparation and is adaptable to various conditions (Kong & Yu, 2007). The FTIR spectra of the microalgae extracts, as shown in Fig. 6, showed that the spectra of the PS were very similar between NPS and PS, particularly for the *T. chui* samples, indicating that the secondary structure could be very similar, as visible in Fig. 4B (Y. Chen et al., 2019). Amide I bands (1700-1600 cm^{-1}) are highly related to the secondary structure of protein spectra (Silverstein & Bassler, 1962). The vibrations at 1614 cm^{-1}, 1620 cm^{-1}, 1605 cm^{-1}, and 1589 cm^{-1} are found in the frozen paste NPS, frozen paste PS, spray-dried NPS, and spray-dried PS samples, respectively, from *N. oceanica*, indicating aromatic ring stretching. The same happens in *T. chui* with vibrations at 1612 cm^{-1}, 1612 cm^{-1}, 1597 cm^{-1}, 1676 cm^{-1} for frozen paste NPS, frozen paste PS, spray-dried NPS, and spray-dried PS. In the case of *N. oceanica* (Fig. 6A), the amide I peak observed at 1589 cm^{-1} in the spray-dried PS is almost absent. This peak is primarily associated with the C=O stretching, which correlates with the secondary structure, corroborating the data present in Fig. 4A.

Peaks present in all PS at 1400 cm^{-1} and 3100-3200 cm^{-1} could be attributed to ammonium absorption in both the 3250 cm^{-1} and 1500–1400 cm^{-1} (Anunciado et al., 2023). Additionally, in the PS samples, peaks are observed at 600 cm^{-1} possibly due to the –S–O stretching (Mansour et al., 2022). The spectral peaks observed between 950 cm^{-1} and 1200 cm^{-1} are attributed to carbohydrates linkage, which is observable in the NPS, particularly from frozen paste biomass. Furthermore, the band located at 2920 cm^{-1} is associated with the total sugar content, which was exclusively present in the NPS and corresponds with the composition of the extract (Gomes-Dias et al., 2024).

3.6. Molecular weight

The UV spectrum of the Spray-dried NPS predominantly indicated the presence of large macromolecules or aggregates, which were transformed into smaller macromolecules or aggregates upon combination with purification (PS). In contrast, the frozen paste samples showed that the PS had a higher molecular weight than the NPS. Specifically, in *N. oceanica*, the Mw distribution of the PS was mainly detected between 9 and 10 min, while for the NPS, the higher peak elution occurred between 8 and 11 min. A similar trend was observed in *T. chui*, where the PS eluted between 9 and 11 min, and the NPS eluted between 6 and 11 min. This confirms the results of the intrinsic fluorescence which indicated denaturation and possible aggregation when the purification step was applied.

In spray-dried cells, the purification step may have partially disrupted the polysaccharide-protein linkages, thereby effectively separating low-molecular weight proteins from these complexes. In contrast, for frozen paste, the situation is reversed, as the PS demonstrates a higher degree than aggregation than for NPS (Ba et al., 2016) (see Fig. 7).

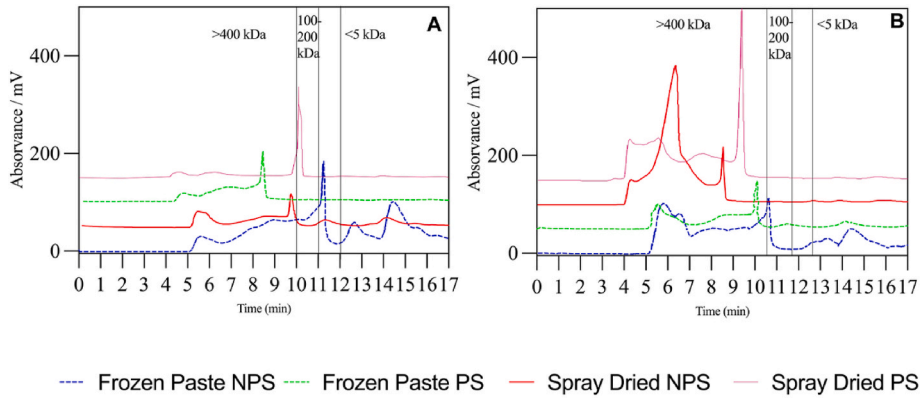

Fig. 7. GPC chromatogram of protein molecular weight of non-purified protein extracts (NPS) and purified protein extracts (PS) of **A)** *N. oceanica*, and **B)** *T. chui*. Each spectrum is represented as an average of three independent measurements (for clearer peak identification, a 50 mV offset between samples was applied).

Previous research has examined the effects of various extraction methods on protein size, indicating that factors such as pH, heat, and ultrasound influence protein aggregation. Moreira et al. (2025) reported lower MWs in water extracts from both *T. chui* and *N. oceanica*; however, the current study also incorporated an extra purification step with the utilization of ultrafiltration membranes, which could potentially further enhance protein aggregation (Moreira et al., 2025). A study conducted by Kim et al. (1993) also indicated that ultrafiltration might lead to protein aggregation, suggesting that the shear forces generated during solvent flow near the membrane surface could unfold protein molecules and consequently promote flocculation through increased particle collisions (Kim et al., 1993), although all samples were subjected to ultrafiltration. Generally, the overall Mw protein profile was shifted to higher Mw when the spray-dryer was applied, both for NPS and PS samples, indicating thermal protein aggregation.

3.7. Potential applications: results overview

This study has demonstrated that processing conditions significantly influenced the structure of the microalgal extracts obtained from *N. oceanica* and *T. chui*. In particular, the PS extracts exhibited higher protein concentrations than the NPS across the different biomass, indicating that the applied purification methodology effectively enriched protein-containing components. The observed differences between spray-dried biomass and frozen paste further highlight the role of processing history in determining extraction efficiency and macromolecular distribution.

From an application perspective, the enrichment of protein in the PS extracts suggests promising opportunities for the development of functional ingredients. Microalgal proteins and associated polysaccharides have been increasingly investigated for their ability to act as natural emulsifiers, foaming agents, or viscosity modifiers in complex food systems (Bertsch et al., 2021; Mosibo et al., 2024; Prates, 2025; Schwenzfeier et al., 2011). Therefore, the extracts obtained in this study may present potential for incorporation into food matrices where both nutritional value and techno-functional properties are desired, such as plant-based beverages, or protein-enriched formulations. Moreover, the presence of structural polysaccharides could contribute to hydrocolloid-like behavior, influencing, viscosity, emulsion stability, and water-binding capacity. However, further work is required to fully assess the techno-functional performance of these fractions in real food systems, including their emulsifying capacity, rheological behavior, and stability under processing conditions relevant to food manufacturing. Such investigations would help clarify the extent to which these microalgal-derived fractions could serve as sustainable multifunctional ingredients in future food formulations.

Despite the growing interest in microalgae as sustainable protein sources, several technological and economic challenges still limit their large-scale application. One of the major bottlenecks is the efficient extraction of proteins, since many microalgal species possess rigid and complex cell walls that hinder protein accessibility and require energy-intensive disruption techniques (e.g., HPH, bead milling, ultrasonication, enzymatic hydrolysis, or pulsed electric fields) (Ali et al., 2025). Recent studies highlight that optimizing extraction methods remains essential to improve protein yield while preserving functional properties. For example, a recent comparative study demonstrated that extraction yields can vary widely among species, ranging from approximately 5 % to over 80 %, depending on the microalgal strain and extraction strategy (Ma et al., 2026). In addition, although microalgae proteins generally have balanced amino acid profiles, their digestibility and bioavailability may vary significantly due to differences in cell wall composition and protein structure (Xu et al., 2024). From an industrial perspective, the scalability and economic feasibility of microalgae protein production remain key challenges, as cultivation, harvesting, drying, and downstream processing considerably increase production costs (Ali et al., 2025). Moreover, recent reviews emphasize that regulatory frameworks, sensory characteristics (e.g., color, flavor, and odor), and consumer acceptance may further limit the integration of microalgae proteins into mainstream food products (Geada et al., 2021; Prates, 2025). Therefore, future research should focus on developing cost-effective extraction technologies and improving protein food formulations that enhance consumer acceptance of microalgae-based protein ingredients.

4. Conclusion

This work showed that both purifying and preservation techniques significantly impact the structural integrity and functionality of microalgal proteins. Compared with spray-dried biomass, frozen paste extracts of *N. oceanica* and *T. chui* showed higher protein content, reduced aggregation, and improved retention of native secondary and tertiary structures.The use ASP successfully concentrated proteins but changed their shape, lowering the α-helix content and promoting partial unfolding.

These structural modifications influence protein functionality: purified samples (PS) are better suited for nutritional enrichment, while non-purified extracts (NPS) are likely to be more adaptable for technical applications. The results show how important it is to select processing conditions based on the intended use, whether it is nutritional or functional.

While the present study focuses on the impact of biomass preservation and processing strategies on protein extraction and structural integrity, further work is required to evaluate the technological functionality of the resulting protein fractions (e.g., emulsifying properties,

foaming capacity and water/oil holding capacity). Such analyses will be important to determine their potential application in food and biotechnological systems.

In conclusion, the higher protein content observed with frozen paste compared to spray-dried biomass can be attributed to the change in the cell structure, avoidance of heat-induced changes, microalgal surface hydrophobicity, steric hindrance, and higher recovery yields in the first extraction step (HPH). However, the specific effects may vary depending on the microalgae species, target compounds, and extraction conditions used.

Overall, microalgal proteins, especially those obtained from frozen biomass, represent a versatile and sustainable source for developing novel food products and may help in the transition toward more resilient food systems.

CRediT authorship contribution statement

Catarina Moreira: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Rafaela Nunes:** Writing – review & editing, Validation, Investigation. **Mariam Kholany:** Writing – review & editing, Validation, Investigation. **Hugo Pereira:** Writing – review & editing, Validation, Resources, Methodology. **José A. Teixeira:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Formal analysis. **Pedro Ferreira-Santos:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Cristina M.R. Rocha:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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