



Influence of different processing techniques on microalgal protein extraction

Catarina Moreira^a, Pedro Ferreira-Santos^{a,b,c}, Rafaela Nunes^a, Bernardo Carvalho^{d,e}, Hugo Pereira^{d,e}, José A. Teixeira^{a,f}, Cristina M.R. Rocha^{a,f,*}

^a Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

^b Department of Chemical Engineering, Faculty of Science, University of Vigo, As Lagoas, 32004 Ourense, Spain

^c Instituto de Agroecoloxía e Alimentación (IAA), University of Vigo (Campus Auga), As Lagoas, 32004 Ourense, Spain

^d GreenCoLab – Associação Oceano Verde, Campus de Gambelas, University of Algarve, 8005-139 Faro, Portugal

^e CCMAR - Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

^f LBBELS - Associate Laboratory, Guimarães, Braga, Portugal

ARTICLE INFO

Keywords:

Microalgae
Protein
Structure
Cell disrupt methods
Extraction

ABSTRACT

The nutrient-rich composition of microalgae biomass positions it as a highly promising natural food ingredient. This holds the potential to not only enhance the nutritional value of various food products but also simultaneously alter their structural attributes.

This work investigated the effect of five protein extraction techniques such as freeze-thawing, enzymatic-assisted extraction, high-pressure homogenization, ultrasounds-assisted extraction, and pH adjustment (pH 7, pH 10, and pH 13) in protein yield, and subsequent cell and protein structure of three microalgal suspensions, namely, *Chlorella vulgaris*, *Nannochloropsis oceanica*, and *Tetraselmis chui*.

In *Chlorella vulgaris*, freeze-thawing and high-pressure homogenization exhibited a higher effect in terms of protein yield (~26.60 g protein /100 g protein microalgae). The same occurred for *Nannochloropsis oceanica* with also ultrasounds-assisted extractions and pH 7 and 10 having a protein yield above 30%. *Tetraselmis chui* was similar to *Chlorella vulgaris* (>20.00 g protein /100 g protein microalgae) for freeze-thawing, high-pressure homogenization and ultrasound-assisted extraction. Enzymatic-assisted had a the lower protein yield for all the three microalgae (<10.00 g protein /100 g protein microalgae). The majority of proteins extracted from *Chlorella vulgaris* samples had molecular weights exceeding 337 kDa, whereas proteins extracted from *Nannochloropsis* and *Tetraselmis* had molecular weights ranging from 5 to 50 kDa. α -helices occurred in proteins extracted from *Chlorella vulgaris* through freeze-thawing and enzymatic-assisted extraction, while *Nannochloropsis* and *Tetraselmis* only had β -sheet.

In conclusion, for optimal protein yield recovery, methodologies such as freeze-thawing and high-pressure homogenization are the most efficient across all studied microalgae. The method selected for extraction had a greater impact on both the protein yield and structure for spray-dried cells.

1. Introduction

In response to the growing “protein gap” that has emerged in recent decades, researchers have been exploring alternative sources of protein. This gap is primarily a consequence of the rapid increase in the global population, which has led to a heightened demand for food. In addition, the current agricultural practices may not be sustainable in the long term due to their negative environmental impacts, including the generation of greenhouse gases, land clearing, and nutrient run-offs, among

other factors [1].

The rapidly increasing demand for protein has led to a significant interest in microalgae protein. This interest is primarily driven by the attributes of microalgae, including their productivity and efficient resource utilization. Microalgae display a high protein content and an amino acid profile, rich in essential amino acids, as several strains exhibit higher protein content compared to conventional sources like chicken, eggs, and soybeans [2]. Consequently, microalgae protein has emerged as a highly promising and innovative source of protein.

* Corresponding author at: Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

E-mail address: cmrocha@ceb.uminho.pt (C.M.R. Rocha).

<https://doi.org/10.1016/j.algal.2025.103958>

Received 11 March 2024; Received in revised form 7 February 2025; Accepted 7 February 2025

Available online 13 February 2025

2211-9264/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

The high protein content of microalgae like *Chlorella vulgaris* (*C. vulgaris*), *Nannochloropsis oceanica* (*N. oceanica*), and *Tetraselmis chui* (*T. chui*) makes them suitable for use in food products like meat and fish substitutes [3]. However, extracting specific components from microalgae can be challenging due to the structure and rigidity of their cell walls [4].

For example, *C. vulgaris* is characterized by the development of a progressively thickening cell wall as it matures. This process culminates in the formation of a microfibrillar layer consisting of cellulose microfibrils [5]. *N. oceanica* has one distinctive feature: a sturdy bilayered trilaminar sheath outer layer comprising both cellulosic and hydrophobic layers. This unique structural characteristic renders it a unique obstacle when attempting to disrupt cell integrity, primarily because of its rigid and robust nature [6,7]. *T. chui* cell wall is formed by the fusion of cell body scales characteristic of the Prasinophyceae, and primarily comprise mannose and glucose monosaccharides in their composition [8].

An essential initial step involving protein extraction is often required to overcome this natural barrier. This process facilitates access to the intracellular components and streamlines subsequent extraction procedures. In an attempt to overcome the longstanding hurdle in the extraction of microalgae and plant-derived protein, numerous methods, including freezing-thawing (F/T) [9], enzymatic-assisted extraction (ENZ) [10], pH adjustment [11], and other techniques have been employed in the past [12,13]. However, these methods have consistently encountered the barrier of low extraction yields and/or low scalability [4].

The concept of green extraction methods has garnered support from many researchers since it is a paradigm that emphasizes the need to reduce solvent usage, minimise energy consumption, and mitigate environmental pollution while concurrently striving to maximize yield [4,14]. Fueled by the principles of green extraction, novel processing technologies, notably ultrasound-assisted extraction (UAE) and high-pressure homogenization (HPH), have been increasingly employed to effectively extract compounds from plant matrices, significantly increasing the yields. Typically, the efficiency of these techniques is assessed by examining the extraction of a single component before and after the application of the treatment and/or the evaluation or measurement of cellular integrity [15,16].

In addition to the chemical composition of microalgae, their morphological and structural characteristics also significantly influence the efficacy of protein solubilization. Certain proteins present challenges in solubilization due to their hydrophobic nature or the presence of disulfide bonds between protein molecules, resulting in decreased solubility [1,8–10,16,17]. Protein solubility can be enhanced in alkaline media or other denaturing treatments.

A comprehensive literature review does not reveal a clear trend but suggests that each microalgae responds differently to cell lysis, necessitating tailored processes for each species. These investigations predominantly focus on fresh biomasses or those subjected to pre-treatments, such as freeze-drying or freezing. A structured approach and comparable data are imperative to investigate the potential for protein extraction in these microalgae thoroughly.

The central focus of the current study is to assess how different protein extraction techniques affect the extraction of proteins from three different microalgae species in an aqueous medium (*C. vulgaris*, *N. oceanica*, *T. chui*). Different processing techniques (freeze-thawing, high-pressure homogenization, ultrasound-assisted extraction, enzymatic extraction, and pH variation) were employed, and their impact on protein yield, and on the structure of the recovered protein (molecular weight and protein conformation) was evaluated.

2. Materials and methods

2.1. Materials

Chlorella vulgaris (*C. vulgaris*, batch number L201950071) was grown in heterotrophy, washed with decalcified water (1:10 w/w), and processed by microfiltration to obtain a suspension with a concentration of ~60 g/L. *Nannochloropsis oceanica* (*N. oceanica*, batch number L202240131) and *Tetraselmis chui* (*T. chui*, batch number L202130148) were grown autotrophically in reconstituted saline water in tubular photobioreactors in Allmicroalgae Natural Products S.A. (Pataias, Portugal). The biomass was harvested by filtration to obtain a suspension of approximately 60 g/L and the three microalgae pastes were then pasteurized and spray dried.

Viscozyme® L was purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). All chemicals used were analytical grade.

2.2. Microalgae characterization

Microalgae protein content was estimated by quantification of total nitrogen after sample acid digestion using a Kjeldahl digester (Foss Analytics, Hilleroed, Denmark), applying the nitrogen conversion factor ($N \times 4.78$) [18]. Lipids were determined using the Bligh & Dyer method with minor modifications. For this, 1 mL mixture chloroform/methanol (2:1, v/v) was added to 50 mg microalgae biomass, and re-extraction cycles were performed until no pigmentation was detected in the solvent. This step was followed by a re-extraction in 2 mL chloroform and 1 mL methanol, and 750 μ L water were added to promote phase separation and lipid extraction. The organic phase was collected. The combined phases were dried under N_2 steam and weighed. Each sample was analyzed in triplicate, and the results were presented in percentage of biomass dry weight [19–21].

For the moisture and ash content determination 1 g algae biomass was weighed into ceramic crucibles and dried overnight at 105 °C. The crucibles were allowed to cool to room temperature in a desiccator and weighted to calculate the moisture content (%). Samples were then put in a muffle furnace (ECF 12/6, Lenton, UK) at 575 °C for 16 h and weighed (AOAC [22]).

Carbohydrate content was assessed with the following procedure: 3 mg of each microalga was mixed with 3 mL 72 % H_2SO_4 , and placed into a water bath at 30 ± 3 °C for 60 min. Afterwards, each sample was diluted to a 4 % H_2SO_4 final concentration and autoclaved for 1 h at 121 °C. The samples were analyzed by high-performed liquid chromatography with a refractive index detector using the following parameters: Aminex HPX-87H column at 60 °C; mobile phase of 0.05 M H_2SO_4 at a 0.6 mL/min flow rate. The quantification of monosaccharides was performed using a calibration curve of each standard (glucose, galactose, rhamnose, arabinose, fucose, glucuronic acid and galacturonic acid) at concentrations between 0.0625 and 1.5 g/L [23]. A limitation of HPX-87H column is the co-elution of xylose, mannose, and galactose; consequently, the results from compositional analyses were presented as the sum of these three sugars (XMG) [24].

All experiments were run in triplicate, and the results are presented as percentages.

2.3. Microalgae treatments

2.3.1. Freeze/thawing

A volume of 30 mL deionized water (dH_2O) was introduced to 3 g microalgae. This mixture underwent a freezing phase (at -20 °C) lasting 90 min, followed by a thawing phase (at 20 – 25 °C) lasting 180 min. The preliminary assay encompassed a total of 10 cycles, with the optimal outcome emerging after 4 cycles (Table 8- supplementary material). All samples were then centrifuged at $6000 \times g$ for 30 min, and the supernatants were recovered and lyophilized for further use. All experimental procedures were conducted in triplicate.

2.3.2. High pressure homogenization

A quantity of 200 g of powdered of *C. vulgaris*, *T. chui* or *N. oceanica* at a ratio of 1:10 (200 g / 2000 mL of dH₂O) was pumped through the HPH (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) at 1000 ± 100 bar. The process of homogenization was performed in one cycle, and only the first stage of homogenization was utilized [25,26]. The temperature was measured during all the extraction (23 ± 2 °C). After treatment, all solutions were centrifuged at 6000 ×g for 30 min and the supernatants were recovered and lyophilized for further use.

2.3.3. Enzymatic extraction

An enzymatic method using Viscozyme® L (Sigma-Aldrich Chemical Co. Ltd., St. Louis, MO, USA) was performed to evaluate its impact on the recovery of proteins from microalgae. Specifically, 1.5 g algae were combined with 50 mL a 50 FPU enzyme solution in water at pH 4.5 and a temperature of 50 °C, according to the manufacturer's optimum recommended conditions for this enzymatic cocktail. The following incubation times were tested: 1, 2, 3, 4, 5, 6, 24, 48, 72 and 168 h, with the optimal outcome emerging after 48 h. Afterwards, all solutions were centrifuged at 6000 ×g for 30 min and the supernatants were recovered and lyophilized. Extractions were conducted in triplicate.

2.3.4. Ultrasound-assisted extraction

An ultrasound probe (model cv334, Sonics, Newtown, USA) was tested to enhance protein extraction. A volume of 120 mL dH₂O was added to 12 g algae and the following exposure times were tested: 2, 5 and 10 min. The optimal outcome was obtained after 10 min. After, all solutions were centrifuged at 6000 ×g for 30 min and the supernatants were recovered and lyophilized for further use. Experiments were conducted in triplicate.

2.3.5. pH adjustment

The microalgae studied were subjected to pH adjustment to study the impact of pH on protein solubilization for each species. Using the solid-liquid ratio of 1:10 (final volume of 50 mL), the procedure was done with dH₂O, with concentrate solutions of NaOH and HCl used to adjust the pH to the target value (7, 10 and 13). After that, the microalgae suspensions were stirred for 1 h at 200 rpm. After, all solutions were centrifuged at 6000 ×g for 30 min and the supernatants were recovered and lyophilized for further use. Experimental procedures were conducted in triplicate.

2.4. Protein quantification

The protein content was assessed through the quantification of total nitrogen after sample acid digestion using an automatic Kjeldahl digester unit (Foss Analytics, Hilleroed, Denmark), applying the nitrogen conversion factor of 4.78. All determinations were run in triplicate, and results were reported as percentage of protein [18,27].

Bradford method was used for preliminary information regarding the F/T cycles. Briefly, 180 µL extract and 20 mL Bradford solution were mixed and incubated for 15 min. A standard curve was made of BSA (0, 0.0625, 0.125, 0.25, 0.5 and 1 g/L) and absorbance was read at 595 nm [28].

2.5. Fourier Transform Infrared Spectroscopy

The secondary structure in the lyophilized extracts was investigated with FTIR spectroscopy using an ALPHA II-Bruker spectrometer (Ettlingen, Germany) with a diamond-composite attenuated total reflectance (ATR) cell (FTIR-ATR). The FTIR spectra were recorded in the 4000–400 cm⁻¹ range by acquiring 64 scan cycles per sample with a resolution of 4 cm⁻¹. The maximum peaks were assigned with peak picking based on the second derivative as implemented in Bruker OPUS software [29,30].

2.6. 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 dH₂O 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 Shodex™, Japan, over a range spanning from 6.1 kDa to 337 kDa [31].

2.7. Statistical analysis

Results were presented as mean ± standard deviation (SD) of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA). Differences between samples were tested using *t*-test, and the results were considered statistically different for values of *p* < 0.05.

3. Results

3.1. Proximal composition of microalgae biomass

The comparative proximal (see Table 1) analysis demonstrated that *T. chui* exhibited a higher protein content, whereas *C. vulgaris* demonstrated a higher carbohydrate concentration, primarily attributed to glucan levels (see Table 2). Both *N. oceanica* and *T. chui* had comparable lipid and ash levels.

3.2. Protein extraction methods for protein recovery

The protein extraction efficiencies of three microalgae species were evaluated using five different protein extraction techniques (Table 3). F/T and HPH demonstrated the highest efficacy in protein extraction, while enzymatic treatment with Viscozyme® resulted in the lowest protein yield. Application of pH 7 during extraction from *C. vulgaris* and *N. oceanica* did not yield statistically different results compared to UAE. In *N. oceanica*, extraction at pH 10 produced a protein yield equivalent to that of HPH. Extraction under alkaline pH led to the lowest yields in *T. chui*, with pH 13 yielding the least favorable results.

Extracts from *C. vulgaris* treated by F/T, and pH variation demonstrated the highest protein content, while those treated with ENZ exhibited the lowest. *N. oceanica* extracts with higher protein content were F/T, pH 10, pH 13, and HPH. *T. chui* extracts with the highest protein content were HPH, UAE, and pH 7, while ENZ, pH 10, and pH 13 treatments resulted in the lowest protein content (Fig. 1) (See overall extraction yield in Table 7- supplementary material).

3.3. Molecular weight and secondary structure of extracted protein

3.3.1. *Chlorella vulgaris*

The analysis of the MW of *C. vulgaris* protein showed the presence of

Table 1

Chemical composition of *Chlorella vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis chui* biomass expressed in g/100 g of biomass dry weight. Values represent the mean and corresponding standard deviation (*n* = 3).

g/100 g dry weight	<i>C. vulgaris</i>	<i>N. oceanica</i>	<i>T. chui</i>
Protein	27.1 ± 0.2	27.7 ± 0.0	32.3 ± 0.4
Carbohydrates	45.1 ± 0.5	6.4 ± 0.2	9.9 ± 0.4
Lipids	12.2 ± 0.1	21.9 ± 1.1	22.8 ± 0.9
Ashes	4.3 ± 0.1	38.3 ± 0.2	32.9 ± 0.1
Moisture	4.8 ± 0.0	4.5 ± 0.1	4.5 ± 0.0

Table 2

Carbohydrates composition of *Chlorella vulgaris*, *Nannochloropsis oceanica* and *Tetraselmis chui* biomass expressed in g/100 g of biomass dry weight. Values represent the mean and corresponding standard deviation (n = 3).

g/100 g dry weight	<i>C. vulgaris</i>	<i>N. oceanica</i>	<i>T. chui</i>
Glucose	36.1 ± 0.7	1.9 ± 0.0	5.1 ± 0.2
Arabinose	1.2 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
Rhamnose	1.2 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
XMG	3.1 ± 0.1	1.2 ± 0.0	2.4 ± 0.1
Galacturonic acid	3.1 ± 0.7	2.1 ± 0.2	1.2 ± 0.1

aggregates exceeding 337 kDa across all protein extraction techniques employed. In F/T, HPH, ENZ, UAE, and pH 7 methods, aggregates constitute over 90 % of the protein composition. The protein extracted at pH 10 and pH 13 demonstrated having the lowest MW (Tables 3–5).

The FTIR spectra of the microalgae extracts revealed similarities among the five protein extraction techniques with minor variations in the absorption intensity of specific characteristic peaks (Fig. 2A). Particularly for pH 7 and ENZ, higher intensities were observed between 1670 and 1600 cm⁻¹ (amine I) and 1400–1200 cm⁻¹ (amine III). The second derivative spectra indicated the presence of the α -helix form solely in F/T and HPH extracts (Fig. 2B).

3.3.2. *Nannochloropsis oceanica*

The analysis of the MW of *N. oceanica* protein showed a distinct

singular peak for every technique. F/T, and UAE exhibited MW lower than 6 kDa, while HPH, ENZ, pH 7, and pH 10 presented a protein size of 15 kDa, and pH 13 showed MW of 50 kDa.

The FTIR spectra of the *N. oceanica* extracts displayed similarities among the five protein extraction techniques, although in the amine I

Table 4

Molecular weight relative proportions distribution of *Chlorella vulgaris* extracts obtained after F/T, HPH, Enz, UAE, pH 7, pH 10 and pH 13.

kDa	F/T	HPH	ENZ	UAE	pH 7	pH 10	pH 13
<6	3 %	2 %	4 %	5 %	28 %	24 %	23 %
50–20	–	–	–	–	–	16 %	–
100–200	–	–	–	–	7 %	6 %	15 %
>337	97 %	98 %	96 %	95 %	65 %	46 %	62 %

Table 5

Molecular weight relative proportions distribution of *Nannochloropsis oceanica* extracts obtained after F/T, HPH, ENZ, UAE, pH 7, pH 10 and pH 13.

kDa	F/T	HPH	ENZ	UAE	pH 7	pH 10	pH 13
<6	69 %	18 %	24 %	21 %	20 %	26 %	29 %
10–20	–	59 %	55 %	59 %	59 %	46 %	–
20–50	–	–	–	–	–	–	43 %
>337	31 %	23 %	21 %	20 %	21 %	28 %	28 %

Table 3

Protein yields (g protein /100 g total microalgae protein) after the protein extraction techniques application. Different letters within the same microalgae represent statistical differences ($\rho < 0.05$).

Microalgae	F/T	HPH	ENZ	UAE	pH 7	pH 10	pH 13
<i>C. vulgaris</i>	28.0 ± 0.2 ^a	26.6 ± 0.2 ^b	2.2 ± 0.2 ^c	14.3 ± 0.7 ^d	14.4 ± 3.3 ^{d,e}	12.4 ± 0.3 ^e	13.1 ± 0.3 ^e
<i>N. oceanica</i>	34.6 ± 0.2 ^f	31.5 ± 0.2 ^g	9.3 ± 1.8 ^h	29.0 ± 1.3 ⁱ	30.6 ± 0.1 ⁱ	32.0 ± 0.7 ^g	23.6 ± 0.6 ^j
<i>T. chui</i>	25.6 ± 0.2 ^k	28.1 ± 0.1 ^l	6.5 ± 0.8 ^m	23.5 ± 0.1 ⁿ	12.3 ± 2.1 ^o	6.8 ± 1.2 ^m	2.8 ± 0.2 ^p

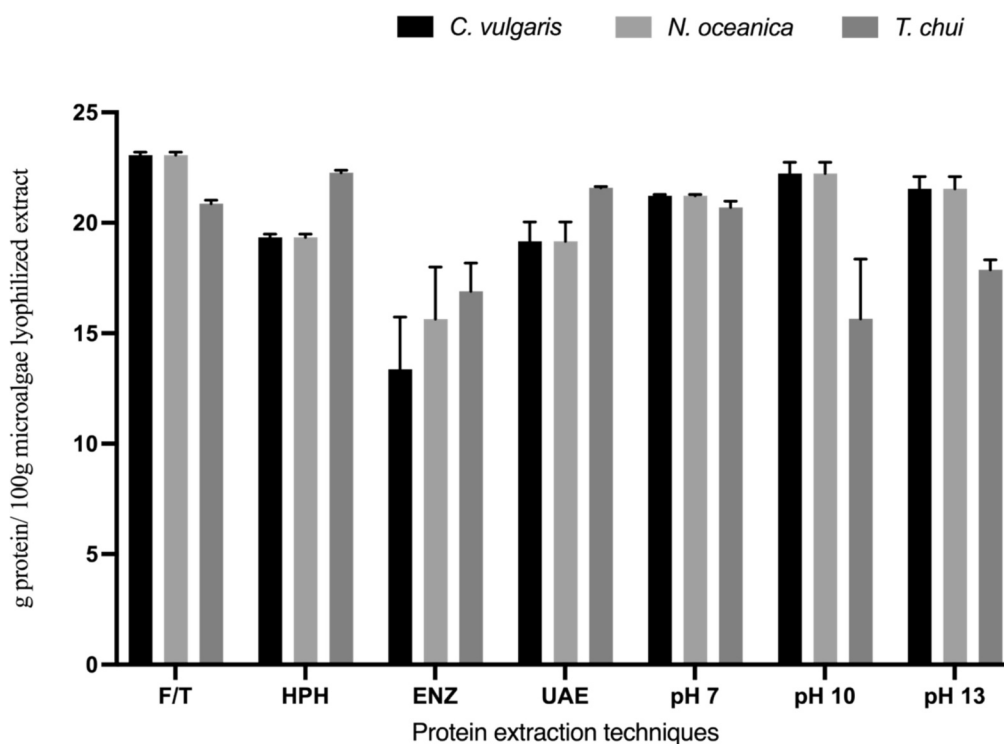


Fig. 1. Protein content in *microalgae* extracts (g protein/ 100 g microalgae lyophilized extract) under five protein extraction techniques (Freeze/Thawing (F/T), High-Pressure Homogenizer (HPH), Enzymatic extraction (ENZ), Ultrasound-Assisted Extraction (UAE), and pH adjustment (pH 7, pH 10, and pH 13). Different letters within the same microalgae represent statistical differences ($\rho < 0.05$).

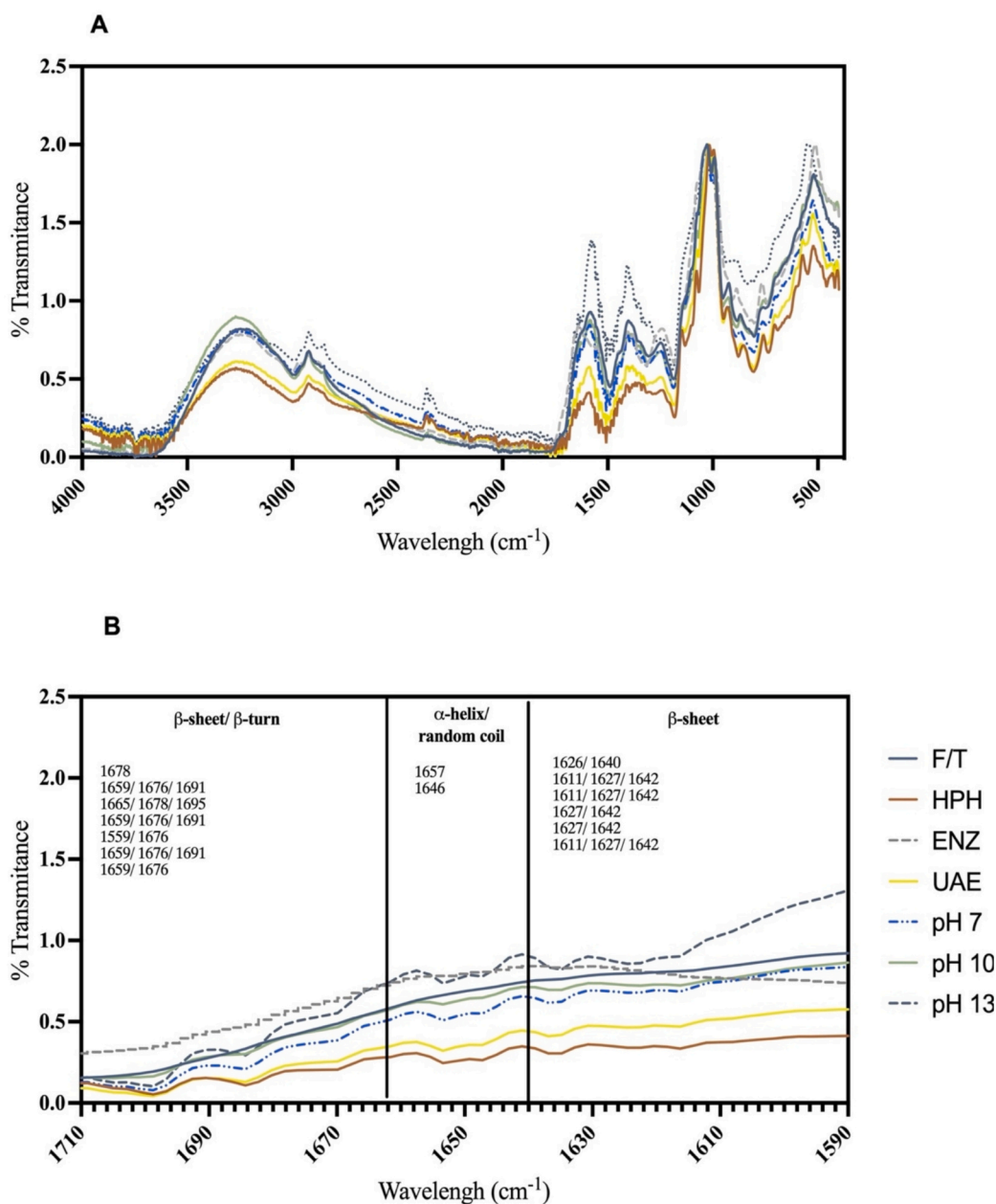


Fig. 2. A) FTIR spectra of *Chlorella vulgaris* extracts (4000–400 cm⁻¹) B) FTIR derivative spectra of *Chlorella vulgaris* extracts (1710–1590 cm⁻¹) under F/T, HPH, ENZ, UAE, pH 7, pH 10 and pH 13 extracts from *Chlorella vulgaris*. Wavenumbers of the FTIR spectra were determined with a peak-picking tool based on the second derivative of these curves. The vertical lines attribute ranges of wavenumbers to specific secondary structures of proteins.

area HPH showed higher intensity than the other four samples, and ENZ the lowest (Fig. 3A). The second derivative spectra analysis indicated the presence of the same secondary structure regardless of the techniques selected (Fig. 3B).

3.3.3. *Tetraselmis chui*

The MW analysis of the *T. chui* protein indicated the presence of aggregates exceeding 337 kDa across all protein extraction techniques (15 %). The lowest MW was observed at pH 7 (<6 kDa), while higher MW were recorded at pH 10 and pH 13 (50 kDa). >50 % of the protein size is distributed between 15 and 50 kDa for all the protein extraction techniques utilized.

The FTIR spectra of the *T. chui* extracts as previously mentioned for *C. vulgaris*, and *N. oceanica*, displayed similarities among the five protein extraction techniques, although in the amine I area HPH showed the lowest intensity and pH 13 the highest (Fig. 4A). In *N. oceanica*, the

second derivative spectra analysis indicated the presence of the same secondary structure regardless of the techniques selected (Fig. 4B).

4. Discussion

4.1. Proximal composition of microalgae biomass

N. oceanica and *T. chui* were grown in marine autotrophic media. Although the protein, carbohydrate and lipid content are generally aligned with previous studies for both microalgae [32–35] high variations are found in literature, depending, e.g., on the growth conditions or harvesting methods. [34] report 22–37 % protein content, 29–40 % carbohydrates content, 15–22 % lipids content and 8–11 % ash for *Nannochloropsis* spp., depending on the residence time in the photobioreactor. Liu et al., 2015 also reported significant variations in the protein (24–30 %) and lipid (42–28 %) content depending on the growth

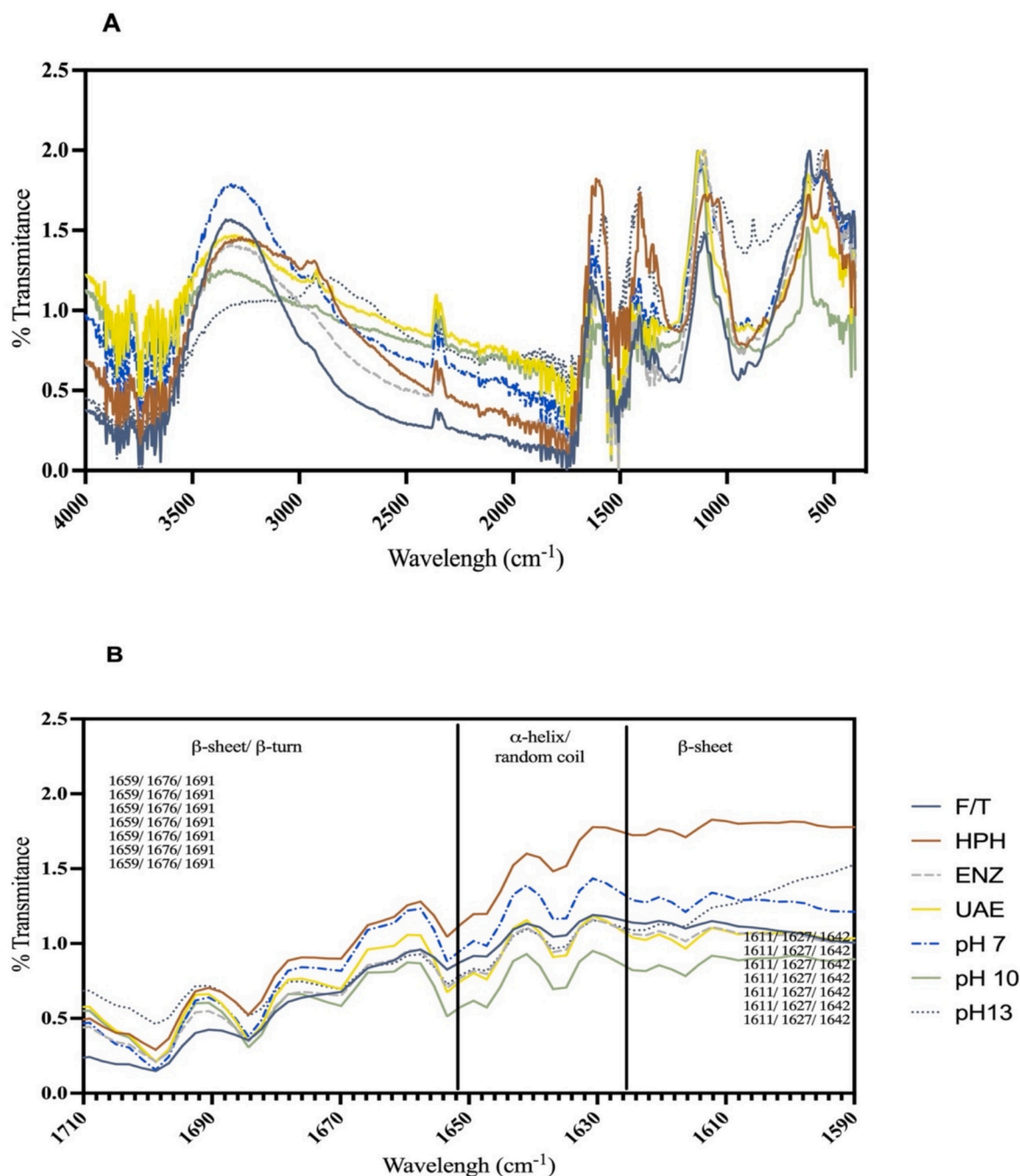


Fig. 3. A) FTIR spectra of *Nannochloropsis oceanica* extracts (4000–400 cm^{-1}) B) FTIR derivative spectra (1710–1590 cm^{-1}) of F/T, HPH, ENZ, UAE, pH 7, pH 10 and pH 13 extracts from *Nannochloropsis oceanica*. Wavenumbers of the FTIR spectra were determined with a peak-picking tool based on the second derivative of these curves. The vertical lines attribute ranges of wavenumbers to specific secondary structures of proteins.

medium composition (nitrogen depleted or repleted), for *N. oceanica*.

Nevertheless, the ash content present in *N. oceanica* and *T. chui* were higher than the ones present on the literature. However, salt content in the final biomass can be strongly influenced by the harvesting methods. For instance, Das et al. [32] have reported ash contents of 19 % and 30 % for *Tetraselmis* sp. harvested by cross-flow filtration or by electrocoagulation, respectively. The biomass utilized in this study was procured from a large-scale supplier based in Europe. The microalgal species *N. oceanica* and *T. chui* were harvested through a filtration process, followed by pasteurization and spray drying. In an industrial context, it is not feasible to achieve complete removal of the growth medium. Specifically, *N. oceanica* and *T. chui* are cultivated in marine

water with a salinity of 30 to 40 g/L. Following filtration, the concentration of microalgae is anticipated to rise from 1 to 2 g/L to 60 g/L, resulting in a substantial increase in the ratio of microalgae to the residual salt in the medium. However, due to the inherent characteristics of the processing, it is expected that approximately 30 % of the final product will consist of ash, which includes salt and various micronutrients, after the spray-drying stage. This phenomenon may account for the observed higher ash content in the microalgae powder compared to existing literature. In a study presented by Magpusao et al. [36] similar ash content was found related to the biomass being resuspended in buffer salt solution before to packing for delivery as a wet paste [36].

The *C. vulgaris* composition is similar to the one present in the

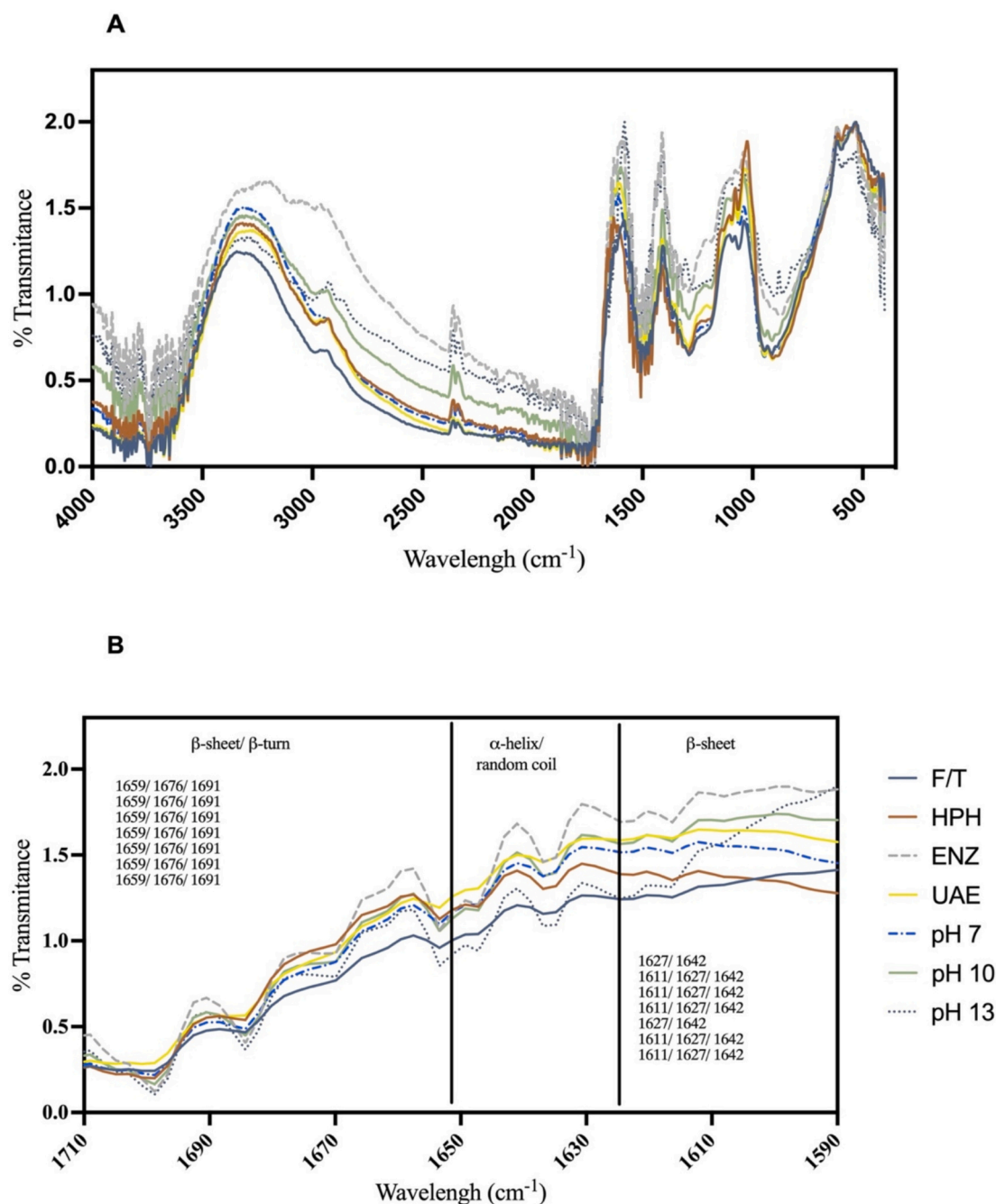


Fig. 4. A) FTIR spectra of *Tetraselmis chui* extracts (4000–400 cm^{-1}) B) FTIR derivative spectra (1710–1590 cm^{-1}) of F/T, HPH, ENZ, UAE, pH 7, pH 10 and pH 13 extracts from *Tetraselmis chui*. Wavenumbers of the FTIR spectra were determined with a peak-picking tool based on the second derivative of these curves. The vertical lines attribute ranges of wavenumbers to specific secondary structures of proteins.

literature for strain growth in a media with glucose [37,38].

The protein content was around 30 % for all the studied microalgae (Table 1). Previous studies has been reported a similar composition in terms of proteins with *C. vulgaris* showing a content of 39.4 %, *N. oceanica* of 35.1 % and *T. chui* 31.1 % [7]. Microalgae's protein content is higher than most vegetables and pulses (e.g., 10 % - 22 % for peas or beans), making them attractive as a food protein source [4].

Some differences may be partly explained by geographical and seasonal variation, as well as by methodological differences, growth conditions, and quantification methodologies [39,40].

4.2. Protein recovery

For *C. vulgaris*, the higher extraction yields were obtained under F/T and HPH methodologies yet, HPH besides being faster than F/T and also a more sustainable process. UAE did not significantly improve the protein extraction yield, since the final value was similar to an extraction at pH 7 (Table 3).

In *T. chui*'s case, HPH showed the best protein extraction yield, with 28 % of the total protein being solubilized, followed by F/T with 25 % and UAE with 23 %. This shows that F/T works almost as well as HPH and UAE for protein extraction for *T. chui*.

In *N. oceanica*, and unlike in *C. vulgaris* and *T. chui*, there appears to be no advantage in using F/T, HPH, UAE, or pH 10 since the gains are

marginal compared to simple extraction or solubilization at pH 7. The only instance where a significant increase was observed was F/T, with an increase of approximately 13 %. Nevertheless, *N. oceanica* stands out as the algae for which the protein extraction yield was higher, probably due to the greater solubility of its proteins.

Subjecting the wet biomass to freezing, initiates the crystallization of intracellular water, and subsequent thawing prompts the expansion of these crystals, resulting in the rupture of cellular structures [41]. Although F/T methods have traditionally been used for their simplicity, they are labour-intensive, time-consuming, and energy-intensive processes at scale, making them unviable for most applications [13].

The ENZ extraction had the lower yields for all algae tested, with a maximum of 9 % of the microalgae protein being solubilized for *N. oceanica*, 6 % for *T. chui*, and 2 % for *C. vulgaris*. Carbohydrases, like the ones present in Viscozyme® L, are involved in the disintegration of cell wall tissue, facilitating protein extraction. The decrease in viscosity caused by the degradation of β -glucan was expected to help in the solubilization and extraction of protein. Microalgae, like *Nannochloropsis* is a source of β -glucan, even though they are not the main constituent of their cell wall [42]. Enzymatic treatments were most effective when used as a pre-treatment to enhance protein extraction. It concluded that the use of carbohydrases was beneficial in improving the extraction yields [43]. This reduced extraction yield could be ascribed to protein denaturation (and consequently insolubilization) due to the long period (48 h) at a moderate temperature (50 °C), causing the protein to remain in the solid phase. This could be a strategy if the intention was to concentrate the protein in the solid phase, while obtaining a liquid extract rich in other compounds (e.g. carbohydrates). Also, a study performed by Safi et al. [44] showed that 35 % of the protein from *N. gaditana* was released using the ENZ approach, a considerably higher value than our results. The authors observed that the use of proteases was not enough to weaken the integrity of the cell wall, so flow cytometry did not detect broken cells. These results can also be observed in our work for all the microalgae studied. In addition, the use of enzymes has resulted in the hydrolysis of the proteins located in the cell wall of *N. gaditana*, which implies that the remaining components of the cell wall remained intact [44].

Alkaline treatments constitute a widely employed non-mechanical procedure for extracting and solubilizing proteins from microalgae. Usually, this type of treatment significantly enhances the yield of extracted proteins. Sodium hydroxide is expected to induce the hydrolysis of ester and hydrogen bonds present among various components (polysaccharides and non-polysaccharides) within microalgae cell walls [45]. *N. oceanica* protein extraction was higher between pH 7 and 10. As the pH increased, the solubility dropped slightly to an average of 27 % between pH 10–13. A pH in the range of 5.5–11 has little impact on the solubility and extraction yield of proteins from *N. oceanica* [46]. The same was observed for *C. vulgaris*, where the results did not show statistical differences in protein extraction between pH 7 and 13. Higher solubility of proteins in alkaline media was attributed to the net electrical charges of the proteins [47]. *T. chui* had a higher protein solubility at pH 7. However, with an increase in pH, the extraction efficiency significantly decreased, reaching 46 % and 78 % at pH 10 and pH 13, respectively, compared to pH 7 [47]. Thus, it seems better to keep the initial pH at 7 for both microalgae, reducing process time and chemicals. [48] studied the influence of the pH in protein solubilization in *Nannochloropsis* spp, and observed that at pH 13 the protein recovery was higher (between 10 and 12 %), although considerably low compared to the results presented in our work [48].

The UAE and HPH techniques have emerged as innovative “clean technology” methods that have garnered increased attention in recent years. They offer several advantages, including minimal solvent usage, limited equipment requirements, and low economic and environmental impacts. UAE employs ultrasonic waves to induce the formation, growth, and subsequent collapse of bubbles within the water, facilitating the release of intracellular components. HPH forces the passage of

a fluid through a valve with narrow stilt, resulting in a substantial increase in pressure, and then striking an impact ring [41]. The resulting mechanical effects, such as turbulence, shear stress, and cavitation, lead to the extraction of cellular structures. HPH usually has a pronounced impact on the microstructural properties of various microalgae suspensions [25]. In a previous study performed with *Nannochloropsis* the protein extraction yield using HPH was 50 % of the total protein. However, this data is not directly comparable with the data from the present work, as there is no information about the quantity of the protein present in the extract. The authors also justify that the release of all proteins was not achieved due to the insoluble nature of some proteins that remained in the pellet [44]. A recent study by Rida et al. [49] on *T. suecica* indicated a total protein content in the extract following HPH at 300 bars of 26.1 ± 0.3 % dry weight [49]. This finding aligns closely with the concentrations obtained in the present research. However, the protein extraction yields reported were significantly higher (61.9 ± 3.9 %). Notably, another investigation carried out by Delran et al. [25] achieved an extraction yield of 80 % for total protein at an HPH of 400 bars, whereas our study recorded only 28.1 % [25]. It is important to note that Delran’s work implemented a desalting step for the initial biomass, which potentially enhanced protein extraction efficiency. Furthermore, in the Rida’s work they utilized the ultrafiltration post-centrifugation step, a method that effectively eliminated most washed minerals along with certain lost extracellular components, including smaller proteins and carbohydrates. The observed differences in extraction yields may also be attributable to the variations in the efficiency of the HPH equipment employed and setting conditions. Also, Safi et al. [51] identified the temperature factor as a significant influence on extraction processes, demonstrating that extractions conducted at 46 °C yielded higher results than those performed at 25 °C. This enhancement is attributed to the ability of elevated temperatures to solubilize various components, particularly starch and protein [51].

The resulting aqueous extracts exhibit protein concentrations consistent with the aforementioned extraction yields, i.e., in the case of *C. vulgaris*, all extracts have a protein content between 19 and 23 %, except for enzymatic extraction, thus confirming that other compounds may have been preferentially extracted when Viscozyme® L was used, and more protein remained unextracted in the solid phase. In the *N. oceanica* and *T. chui* extracts, the same trend was observed, and even with enzymatic extraction, a high percentage of protein was obtained (Fig. 1).

Despite the low protein extraction yield at pH 10 and 13 for *C. vulgaris*, the extract has a protein content similar to F/T. This could be attributed to a reduction in the lipid content of the microalgae due to the saponification process under alkaline treatments [52].

C. vulgaris and *N. oceanica*, while achieving acceptable protein extraction yields through the HPH process, exhibited a lower level of specificity in their protein extracting, leading to extracts with slightly reduced protein percentage. This deviation in protein content is likely attributed to the HPH process’s lower specificity in terms of solubilization. It is probable that other components, such as lipids, carbohydrates, and ashes, were solubilized to a greater extent alongside proteins.

This study represents the first attempt to compare the performance of three distinct microalgae variants characterized by differing cell wall compositions regarding their susceptibility to various protein extraction techniques for protein recovery. This marks the pioneering investigation into the structural analysis of the recovered protein subsequent to application of diverse recovery technologies. Although, the maximum protein extraction yield reach has been 28 %, 35 %, and 28 % for *C. vulgaris*, *N. oceanica*, and *T. chui*, respectively using dH₂O as a solvent at 25 °C, the results are in line with previous studies. For instance, employing mild protein extraction techniques resulted in protein yields of 28–43 % for *C. vulgaris* and *N. oculata* [50], and 7–12 % for *T. impellucida* and *suecica* [53,54].

The purpose of enhanced protein yield through various technological methods is met with several limiting factors. The extraction of proteins

from microalgae is impeded by the presence of a physical barrier created by the cell wall, hindering the release of proteins [55]. Also, certain proteins are bound to other molecules through ionic interactions, specifically with polysaccharides, impeding their extraction [4]. The high viscosity and ionic interactions resulting from the presence of cell walls and intracellular polysaccharides further add complexity to the extraction process also, the use of alkaline solvents has facilitated increased protein extraction from select macroalgae species [56]. The combination of cell-disrupting techniques, such as HPH and pH 13, may hold potential for increasing protein yield. However, depending on the application, different methods can be considered. Further work is needed to study the protein extract from microalgae obtained by different cell-disrupting techniques and its impact on the protein structure and functionality.

4.3. Molecular weight and secondary structure of extracted protein

4.3.1. *Chlorella vulgaris*

The chromatograms of *C. vulgaris* revealing the presence of aggregates across all extraction methods (>337 kDa) (Table 3 and Fig. 5-supplementary material). The chromatograms at pH 10 and pH 13 highlight the presence of MW below 120 kDa. This discrepancy MW distribution suggests that the alkaline conditions at pH 10 and 13 may induce a specific breakdown or alteration in the molecular structure of proteins, resulting in lower MW species. Ursu et al. [52] have also reported an higher eluted peak corresponding to MW higher than 670 kDa and lower peaks (<60 kDa) when exposed to alkaline pH. The presence of higher MW has been identified as the signature of complex soluble aggregates of proteins and chlorophyll, reinforcing this conclusion by the green colour of extracts yet as also reported in this study, alkaline extraction modifies the MW profile compared to others extraction techniques showing MW below 60 kDa [52].

FTIR spectroscopy measures the absorption of IR radiation by a sample and provides data on the wavelength and intensity of the absorption. High polymers' IR spectral data can be understood in terms of the vibrations of a structural repeat unit. The amide I band (1700–1600 cm^{-1}) is the most sensitive spectral region for protein secondary structural components, mainly representing the C=O stretch vibrations of the peptide linkages (about 80 %). The frequencies of the amide I band components closely correlate with each secondary structural element of the proteins. On the other hand, the amide II band mainly originates from in-plane NH bending (40–60 %) and the CN stretching vibration (18–40 %). The secondary structural composition is based on the assumption that any protein can be considered as a linear sum of a few fundamental secondary structural elements. Correlations between IR spectra and protein secondary structures have been established, and assignments of amide I band components to protein secondary structure elements (such as α -helix, β -sheet, β -turn, and random structures) are available for proteins in both H_2O and D_2O media [29].

A comparison of the FTIR spectra of the *C. vulgaris* lyophilized extract (Fig. 2) showed that only protein from F/T methodology was slightly shifted to lower wavenumbers, indicating an increased order in secondary protein structure, namely α -helix ($1656 \pm 2.0 \text{ cm}^{-1}$). β -Sheet structures result in characteristic wavenumbers in the regions $1696 \pm 2.0 \text{ cm}^{-1}$ to $1667 \pm 1.0 \text{ cm}^{-1}$ and $1642 \pm 1.0 \text{ cm}^{-1}$ to $1624 \pm 1.0 \text{ cm}^{-1}$ [30]. The secondary structure of proteins is influenced by the specific sequence of amino acids and the interactions between molecules. Protein extraction techniques induce changes in the secondary structure by disrupting these interactions [29]. In HPH, UAE, and alkaline pH processes, protein molecules unfold, leading to the disruption of α -helices and their conversion into β -sheets. Sonication can induce the formation of aggregates, subsequently causing a decrease in α -helix content and an increase in β -sheet content [57]. Strong absorption signals observed at 1110, 1118 and 1113 cm^{-1} are attributed to the C–O–C band, indicating the presence of polysaccharides, which can support the statement that protein could be aggregated due to their presence [58].

4.3.2. *Nannochloropsis oceanica*

Various conditions such as HPH, ENZ, pH 7, and pH 10 exhibited distinct peaks, indicating the presence of proteins with MW ranging between 10 and 20 kDa (Fig. 6- supplementary material). Conversely, at pH 13, proteins appeared to have MW between 20 and 50 kDa, with lower MW proteins observed under F/T and UAE techniques (5–10 kDa, and < 5 kDa, respectively) (Table 5). The use of sodium hydroxide may have led to the solubilization of insoluble proteins in water, resulting in higher MW proteins. Alkaline conditions are anticipated to have a hydrolytic effect on the biomass, which may have liberated different proteins from the microalgae structure. This was likely due to rendering these proteins accessible to the solvent and slightly hydrolyzing them, thus enabling their solubilization [59].

Absorbance peaks were observed in the range of $3200\text{--}3500 \text{ cm}^{-1}$, indicating the presence of the OH group in cellulose which was the predominant carbohydrate found in the *Nannochloropsis* [60]. Another peak at 1049 cm^{-1} was attributed to the C–O–C band, suggesting the presence of polysaccharides within the $950\text{--}1200 \text{ cm}^{-1}$ range.

The analysis of the second derivative of the absorption spectra (Fig. 3) within the range of $1600\text{--}1700 \text{ cm}^{-1}$ indicated that the predominant components of the spectra were consistently present in all complexes. The samples exhibited an absorption peak at $1627\text{--}1642 \text{ cm}^{-1}$, which was consistently attributed to C=O stretching (amide I) and associated with the protein β -sheets structure ($1623\text{--}1641 \text{ cm}^{-1}$). This observation aligns with the secondary structure composition. This observation suggests that the protein's secondary structure remains largely unaltered, confirming the preservation of the same structural characteristics in all seven extracts [61].

4.3.3. *Tetraselmis chui*

The *T. chui* protein analysis, similar to *N. oceanica*, showed a distinct peak across various conditions: F/T (10–50 kDa), HPH (10–20 kDa), ENZ (5–20 kDa), UAE (5–20 kDa), pH 7 (5–20 kDa), pH 10 (10–50 kDa), and pH 13 (10–50 kDa) (Table 6 and Fig. 7- supplementary material), with smaller aggregates present in all samples studied, exceeding 337 kDa. The presence of low MW proteins in *Tetraselmis* sp. Safi et al. studied the extraction of protein from *Tetraselmis suecica* and found the presence of proteins with MW between 10 and 60 kDa for HPH aqueous extraction, which matches the findings of this work [51]. The results showed that the FTIR spectra of *T. chui* lyophilized extracts (Fig. 4) were very similar, with small differences in the absorption intensity of some characteristic peaks. It indicated that the secondary structure could be the same, with a complete absence of α -helix. The strong absorption peaks are assigned to a C=O stretching (amide I) and correspond to β -sheet structures [58]. The peaks between protein extraction techniques tend to follow the same tendency, which is supported by the unchanged nature of the secondary structure.

The three microalgae species presented a higher proportion of β -sheets and β -turn structures, suggesting a more inflexible and folded globulin configuration. This structural attribute, primarily associated with the prevalence of β -turns, introduces constraints on the conformational entropy of the peptide chain [62]. Therefore, this heightened rigidity may contribute to the comparatively lower solubility of microalgae proteins compared to proteins from alternative plant sources. However, the precise mechanistic implications of these structural

Table 6

Molecular weight relative proportions distribution of *T. chui* extracts obtained after F/T, HPH, ENZ, UAE, pH 7, pH 10 and pH 13 and relative proportions.

kDa	F/T	HPH	ENZ	UAE	pH 7	pH 10	pH 13
<6	26 %	24 %	21 %	24 %	33 %	20 %	27 %
10–20	3 %	–	–	6 %	–	6 %	–
20–30	37 %	47 %	58 %	54 %	41 %	7 %	6 %
50	–	–	–	–	–	37 %	35 %
>337	34 %	29 %	21 %	16 %	26 %	30 %	32 %

variances in protein aggregates and their repercussions on solubility remain the subject of ongoing scientific discourse. For instance, a study posited that protein aggregation was correlated with a reduction in water solubility [63], while another reported that these aggregates were water-soluble [64].

5. Conclusion

This research has shown that methodologies like F/T and HPH were the most efficient for optimal protein yield recovery across various microalgae species, in particular for *T. chui* and *C. vulgaris*. Increased alkalinity showed a tendency to promote protein aggregation. In terms of protein characterization, *N. oceanica* and *T. chui* exhibited lower MW, while *C. vulgaris* tended to aggregate. Although results were obtained with cells dried by spray drying, the findings may not be applicable to fresh cells. Yet, these findings highlight the importance of carefully selecting extraction methods to maximize the efficiency and quality of protein extraction from microalgae, considering both protein yield and structural characteristics.

Based in this study, it can be concluded that the chosen extraction method significantly impacts both protein yield and structure. Understanding the differences in cell wall composition and structure among species is crucial for obtaining desirable proteins with varying molecular sizes and functionalities. This knowledge can expand their applications and support the development of nutritionally enriched food products.

CRedit authorship contribution statement

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

Funding

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UIDB/04469/2020 unit and LA/P/0029/2020 (LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems). This study was also supported by “Pacto da Bioeconomia Azul” (Project No. C644915664-0000026) within the WP5 Algae Vertical, funded by Next Generation EU European Fund and the Portuguese Recovery and Resilience Plan (PRR), under the scope of the incentive line “Agendas for Business Innovation” through the funding scheme C5-Capitalization and Business Innovation. Catarina Moreira, Rafaela Nunes, and Bernardo Carvalho are recipients of Ph.D. scholarships supported by FCT (Ref. 2021.05734.BD, SFRH/BD/07527/2020, and 2022.12615.BDANA, respectively). Pedro Ferreira-Santos would like to express gratitude to the Spanish Ministry of Science, Innovation and Universities and the European Union - NextGenerationEU/PRTR - for financial support (FJC2021-046978-I).

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2025.103958>.

Data availability

Data will be made available on request.

References

- [1] A. Krishna Koyande, V. Tanzil, H. Murrally Dharan, M. Subramaniam, R.N. Robert, P.-L. Lau, I. Khoiroh, P.-L. Show, Integration of osmotic shock assisted liquid biphasic system for protein extraction from microalgae *Chlorella vulgaris*, *Biochem. Eng. J.* 157 (2020) 107532, <https://doi.org/10.1016/j.bej.2020.107532>.
- [2] A.K. Koyande, P.-L. Show, R. Guo, B. Tang, C. Ogino, J.-S. Chang, Bio-processing of algal bio-refinery: a review on current advances and future perspectives, *Bioengineered* 10 (1) (2019) 574–592, <https://doi.org/10.1080/21655979.2019.1679697>.
- [3] C. Moreira, P. Ferreira-Santos, J.A. Teixeira, C.M.R. Rocha, Active aroma compounds assessment of processed and non-processed micro-and macroalgae by solid-phase microextraction and gas chromatography/mass spectrometry targeting seafood analogs, *Frontiers in Sustainable Food Systems* 6 (2022) 1011020.
- [4] P. Geada, C. Moreira, M. Silva, R. Nunes, L. Madureira, C.M.R. Rocha, R.N. Pereira, A.A. Vicente, J.A. Teixeira, Algal proteins: production strategies and nutritional and functional properties, *Bioresour. Technol.* 332 (March) (2021), <https://doi.org/10.1016/j.biortech.2021.125125>.
- [5] C. Safi, B. Zebib, O. Merah, P.Y. Pontalier, C. Vaca-Garcia, Morphology, composition, production, processing and applications of *Chlorella vulgaris*: a review, *Renew. Sust. Energ. Rev.* 35 (2014) 265–278, <https://doi.org/10.1016/j.rser.2014.04.007>.
- [6] M. Alhattab, A. Kermanshahi-Pour, M.S.-L. Brooks, Microalgae disruption techniques for product recovery: influence of cell wall composition, *J. Appl. Phycol.* 31 (1) (2019) 61–88, <https://doi.org/10.1007/s10811-018-1560-9>.
- [7] T.M.M. Bernaerts, L. Gheysen, C. Kyomugasho, Z. Jamsazzadeh Kermani, S. Vandionant, I. Foubert, M.E. Hendrickx, A.M. Van Loey, Comparison of microalgal biomasses as functional food ingredients: focus on the composition of cell wall related polysaccharides, *Algal Res.* 32 (2018) 150–161.
- [8] L. Anjos, J. Estêvão, C. Infante, L. Mantecón, D.M. Power, Extracting protein from microalgae (*Tetraselmis chuii*) for proteome analysis, *MethodsX* 9 (2022) 101637, <https://doi.org/10.1016/j.mex.2022.101637>.
- [9] L. Ji, Y. Liu, J. Luo, J. Fan, Freeze-thaw-assisted aqueous two-phase system as a green and low-cost option for analytical grade B-phycoerythrin production from unicellular microalgae *Porphyridium purpureum*, *Algal Res.* 67 (2022) 102831, <https://doi.org/10.1016/j.algal.2022.102831>.
- [10] Y.W. Sari, M.E. Bruins, J.P.M. Sanders, Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals, *Ind. Crop. Prod.* 43 (2013) 78–83, <https://doi.org/10.1016/j.indcrop.2012.07.014>.
- [11] M. Taghavijelouidar, D.Y. Kebria, P. Yaqoubnejad, Simultaneous harvesting and extracellular polymeric substances extrusion of microalgae using surfactant: promoting surfactant-assisted flocculation through pH adjustment, *Bioresour. Technol.* 319 (2021) 124224, <https://doi.org/10.1016/j.biortech.2020.124224>.
- [12] P. Ferreira-Santos, S.M. Miranda, I. Belo, G. Spigno, J.A. Teixeira, C.M.R. Rocha, Sequential multi-stage extraction of biocompounds from *Spirulina platensis*: combined effect of ohmic heating and enzymatic treatment, *Innovative Food Sci. Emerg. Technol.* 71 (2021) 102707, <https://doi.org/10.1016/j.ifset.2021.102707>.
- [13] P. Ferreira-Santos, R. Nunes, F. De Biasio, G. Spigno, D. Gorgoglione, J.A. Teixeira, C.M.R. Rocha, Influence of thermal and electrical effects of ohmic heating on *C. phycoerythrin* properties and biocompounds recovery from *Spirulina platensis*, *Lwt* 128 (May) (2020), <https://doi.org/10.1016/j.lwt.2020.109491>.
- [14] F. Chemat, M. Abert-Vian, A.S. Fabiano-Tixier, J. Strube, L. Uhlenbrock, V. Gunjevic, G. Cravotto, Green extraction of natural products. Origins, current status, and future challenges, *TrAC Trends Anal. Chem.* 118 (2019) 248–263, <https://doi.org/10.1016/j.trac.2019.05.037>.
- [15] J. Ahmed, V. Kumar, Effect of high-pressure treatment on oscillatory rheology, particle size distribution and microstructure of microalgae *Chlorella vulgaris* and *Arthrospira platensis*, *Algal Res.* 62 (2022) 102617, <https://doi.org/10.1016/j.algal.2021.102617>.
- [16] J.A. Gerde, M. Montalbo-Lombay, L. Yao, D. Grewell, T. Wang, Evaluation of microalgae cell disruption by ultrasonic treatment, *Bioresour. Technol.* 125 (2012) 175–181, <https://doi.org/10.1016/j.biortech.2012.08.110>.
- [17] S. Jubeau, L. Marchal, J. Pruvost, P. Jaouen, J. Legrand, J. Fleurence, High pressure disruption: a two-step treatment for selective extraction of intracellular components from the microalga *Porphyridium cruentum*, *J. Appl. Phycol.* 25 (2013) 983–989.
- [18] *Britannica, Kjeldahl method*, 2016 (T. Editors of Encyclopaedia).
- [19] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 (8) (1959) 911–917, <https://doi.org/10.1139/o59-099>.
- [20] A.R.P. Figueiredo, E. da Costa, J. Silva, M.R. Domingues, P. Domingues, The effects of different extraction methods of lipids from *Nannochloropsis oceanica* on the contents of omega-3 fatty acids, *Algal Res.* 41 (2019) 101556, <https://doi.org/10.1016/j.algal.2019.101556>.

- [21] T. Melo, A.R.P. Figueiredo, E. da Costa, D. Couto, J. Silva, M.R. Domingues, P. Domingues, Ethanol extraction of polar lipids from *Nannochloropsis oceanica* for food, feed, and biotechnology applications evaluated using lipidomic approaches, *Mar. Drugs* 19 (11) (2021) 593, <https://doi.org/10.3390/md19110593>.
- [22] A.O.A.C. Official Method, 985.29., Total Dietary in Foods—Enzymatic Gravimetric Method, Official Methods of Analysis, AOAC International, Gaithersburg, 1995, p. 16.
- [23] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of sugars, byproducts, and degradation products in liquid fraction process samples, *Golden: National Renewable Energy Laboratory* 11 (2006) 65–71.
- [24] B.-H. Um, G.P. van Walsum, Mass balance on green liquor pre-pulping extraction of northeast mixed hardwood, *Bioresour. Technol.* 101 (15) (2010) 5978–5987, <https://doi.org/10.1016/j.biortech.2010.03.004>.
- [25] P. Delran, C. Frances, F. Guihéneuf, J. Peydecaasting, P.Y. Pontalier, L. Barthe, Tetraselmis suecica biofilm cell destruction by high-pressure homogenization for protein extraction, *Bioresour. Technol.* 21 (November 2022) (2023), <https://doi.org/10.1016/j.biortech.2023.101372>.
- [26] J. Magpusao, S. Giteru, I. Oey, B. Kebede, Effect of high pressure homogenization on microstructural and rheological properties of *A. platensis*, *Ischrysis*, *Nannochloropsis* and *Tetraselmis* species, *Algal Res.* 56 (2021) 102327, <https://doi.org/10.1016/j.algal.2021.102327>.
- [27] D.W. Templeton, L.M.L. Laurens, Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed and fuel, *Algal Res.* 11 (2015) 359–367.
- [28] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1) (1976) 248–254, [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- [29] J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, *Acta Biochim. Biophys. Sin.* 39 (8) (2007) 549–559, <https://doi.org/10.1111/j.1745-7270.2007.00320.x>.
- [30] M. Monge-Morera, M.A. Lambrecht, L.J. Deleu, T. Godefroid, P. Goos, F. Rousseau, J. Schymkowitz, J.A. Delcour, Drying mode and hydrothermal treatment conditions govern the formation of amyloid-like protein fibrils in solutions of dried hen egg white, *Food Hydrocoll.* 112 (September 2020) (2021) 106276, <https://doi.org/10.1016/j.foodhyd.2020.106276>.
- [31] C. Castro-Ferreira, J.S. Gomes-Dias, P. Ferreira-Santos, R.N. Pereira, A.A. Vicente, C.M.R. Rocha, *Phaeodactylum tricornutum* extracts as structuring agents for food applications: physicochemical and functional properties, *Food Hydrocoll.* 124 (2022) 107276, <https://doi.org/10.1016/j.foodhyd.2021.107276>.
- [32] P. Das, S. Khan, M. Thaher, M. AbdulQuadir, S.K. Hoekman, H. Al-Jabri, Effect of harvesting methods on the energy requirement of *Tetraselmis* sp. biomass production and biocrude yield and quality, *Bioresour. Technol.* 284 (2019) 9–15, <https://doi.org/10.1016/j.biortech.2019.03.118>.
- [33] A. Niccolai, G. Chini Zittelli, L. Rodolfi, N. Biondi, M.R. Tredici, Microalgae of interest as food source: biochemical composition and digestibility, *Algal Res.* 42 (2019) 101617, <https://doi.org/10.1016/j.algal.2019.101617>.
- [34] M.M. Rebolledo-Fuentes, A. Navarro-Pérez, F. García-Camacho, J.J. Ramos-Miras, J.L. Guill-Guerrero, Biomass nutrient profiles of the microalga *nannochloropsis*, *J. Agric. Food Chem.* 49 (6) (2001) 2966–2972, <https://doi.org/10.1021/jf0010376>.
- [35] L. Zanella, F. Vianello, Microalgae of the genus *Nannochloropsis*: chemical composition and functional implications for human nutrition, *J. Funct. Foods* 68 (2020) 103919, <https://doi.org/10.1016/j.jff.2020.103919>.
- [36] J. Magpusao, I. Oey, B. Kebede, Chemical, rheological, and volatile profiling of microalgae *Arthrospira*, *Ischrysis*, *Nannochloropsis*, and *Tetraselmis* species, *Food Innovation and Advances* 3 (2) (2024) 75–87, <https://doi.org/10.48130/fia-0024-0007>.
- [37] S.-H. Ho, S.-W. Huang, C.-Y. Chen, T. Hasunuma, A. Kondo, J.-S. Chang, Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E, *Bioresour. Technol.* 135 (2013) 157–165, <https://doi.org/10.1016/j.biortech.2012.10.100>.
- [38] Y. Liang, N. Sarkany, Y. Cui, Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions, *Biotechnol. Lett.* 31 (7) (2009) 1043–1049, <https://doi.org/10.1007/s10529-009-9975-7>.
- [39] A. Baidya, T. Akter, M.R. Islam, A.K.M.A. Shah, M.A. Hossain, M.A. Salam, S. I. Paul, Effect of different wavelengths of LED light on the growth, chlorophyll, β -carotene content and proximate composition of *Chlorella ellipsoidea*, *Heliyon* 7 (12) (2021) e08525, <https://doi.org/10.1016/j.heliyon.2021.e08525>.
- [40] H. Khatoun, N. Abdu Rahman, S. Banerjee, N. Harun, S.S. Suleiman, N.H. Zakaria, F. Lananan, S.H. Abdul Hamid, A. Endut, Effects of different salinities and pH on the growth and proximate composition of *Nannochloropsis* sp. and *Tetraselmis* sp. isolated from South China Sea cultured under control and natural condition, *Int. Biodeterior. Biodegradation* 95 (2014) 11–18, <https://doi.org/10.1016/j.ibiod.2014.06.022>.
- [41] F. Pagels, R.N. Pereira, A.A. Vicente, A.C. Guedes, Extraction of pigments from microalgae and cyanobacteria—a review on current methodologies, *Applied Sciences (Switzerland)* 11 (11) (2021), <https://doi.org/10.3390/app11115187>.
- [42] A.H. Rojo-Cebreros, L. Ibarra-Castro, J.M. Martínez-Brown, G. Velasco-Blanco, M. A. Martínez-Téllez, M.A. Medina-Jasso, M. Nieves-Soto, D. Quintana-Zavala, Potential of *Nannochloropsis* in beta glucan production, in: *Nannochloropsis: Biology, Biotechnological Potential and Challenges*, 2017 (Issue October).
- [43] X. Guan, H. Yao, Optimization of Viscozyme L-assisted extraction of oat bran protein using response surface methodology, *Food Chem.* 106 (1) (2008) 345–351, <https://doi.org/10.1016/j.foodchem.2007.05.041>.
- [44] C. Safi, L. Cabas Rodríguez, W.J. Mulder, N. Engelen-Smit, W. Spekking, L.A.M. van den Broek, G. Olivieri, L. Sijtsma, Energy consumption and water-soluble protein release by cell wall disruption of *Nannochloropsis gaditana*, *Bioresour. Technol.* 239 (2017) 204–210, <https://doi.org/10.1016/j.biortech.2017.05.012>.
- [45] Z. Gao, P. Shen, Y. Lan, L. Cui, J.-B. Ohm, B. Chen, J. Rao, Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate, *Food Res. Int.* 131 (2020) 109045, <https://doi.org/10.1016/j.foodres.2020.109045>.
- [46] L.R. Cavonius, E. Albers, I. Undeland, pH-shift processing of *Nannochloropsis* oculata microalgal biomass to obtain a protein-enriched food or feed ingredient, *Algal Res.* 11 (2015) 95–102, <https://doi.org/10.1016/j.algal.2015.05.022>.
- [47] A. Schwenzfeier, P.A. Wierenga, H. Gruppen, Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp, *Bioresour. Technol.* 102 (19) (2011) 9121–9127, <https://doi.org/10.1016/j.biortech.2011.07.046>.
- [48] H.G. Gerken, B. Donohoe, E.P. Knoshaug, Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production, *Planta* 237 (1) (2013) 239–253, <https://doi.org/10.1007/s00425-012-1765-0>.
- [49] H. Rida, J. Peydecaasting, H. Takache, A. Ismail, P.-Y. Pontalier, Concentration and desalting of *Tetraselmis suecica* crude extract by ultrafiltration, *Desalin. Water Treat.* 317 (2024) 100209, <https://doi.org/10.1016/j.dwt.2024.100209>.
- [50] C. Safi, M. Charton, A.V. Ursu, C. Laroche, B. Zebib, P.-Y. Pontalier, C. Vaca-Garcia, Release of hydro-soluble microalgal proteins using mechanical and chemical treatments, *Algal Res.* 3 (2014) 55–60, <https://doi.org/10.1016/j.algal.2013.11.017>.
- [51] C. Safi, D.Z. Liu, B.H.J. Yap, G.J.O. Martin, C. Vaca-Garcia, P.-Y. Pontalier, A two-stage ultrafiltration process for separating multiple components of *Tetraselmis suecica* after cell disruption, *J. Appl. Phycol.* 26 (6) (2014) 2379–2387, <https://doi.org/10.1007/s10811-014-0271-0>.
- [52] A.-V. Ursu, A. Marcati, T. Sayd, V. Sante-Lhoutellier, G. Djelveh, P. Michaud, Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*, *Bioresour. Technol.* 157 (2014) 134–139, <https://doi.org/10.1016/j.biortech.2014.01.071>.
- [53] E.S. Garcia, J.J.A. van Leeuwen, C. Safi, L. Sijtsma, L.A.M. van den Broek, M.H. M. Eppink, R.H. Wijffels, C. van den Berg, Techno-functional properties of crude extracts from the green microalga *Tetraselmis suecica*, *J. Agric. Food Chem.* 66 (29) (2018) 7831–7838, <https://doi.org/10.1021/acs.jafc.8b01884>.
- [54] E. Teuling, P.A. Wierenga, J.W. Schrama, H. Gruppen, Comparison of protein extracts from various unicellular green sources, *J. Agric. Food Chem.* 65 (36) (2017) 7989–8002.
- [55] V. Ummat, S.P. Sivagnanam, G. Rajauria, C. O'Donnell, B.K. Tiwari, Advances in pre-treatment techniques and green extraction technologies for bioactives from seaweeds, *Trends Food Sci. Technol.* 110 (December 2020) (2021) 90–106, <https://doi.org/10.1016/j.tifs.2021.01.018>.
- [56] R. Nunes, P. Ferreira-Santos, C. Moreira, J.A. Teixeira, C.M.R. Rocha, Tuning the extraction methodology targeting protein-enriched fractions from red algae, *Future Foods* 9 (November 2023) (2024), <https://doi.org/10.1016/j.future.2024.100335>.
- [57] P.B. Stathopoulos, G.A. Scholz, Y.-M. Hwang, J.A.O. Rumpfheldt, J.R. Lepock, E. M. Meiering, Sonication of proteins causes formation of aggregates that resemble amyloid, *Protein Sci.* 13 (11) (2004) 3017–3027, <https://doi.org/10.1110/ps.04831804>.
- [58] Y. Chen, J. Chen, C. Chang, J. Chen, F. Cao, J. Zhao, Y. Zheng, J. Zhu, Physicochemical and functional properties of proteins extracted from three microalgal species, *Food Hydrocoll.* 96 (886) (2019) 510–517, <https://doi.org/10.1016/j.foodhyd.2019.05.025>.
- [59] C. Moreira, L. Machado, M. Silva, R. Nunes, R.N. Pereira, C.M.R. Rocha, P. Geada, J.A. Teixeira, in: P.B.T.-S.F.S.-A.C.A. Ferranti (Ed.), 3.14 - Algal Proteins, Elsevier, 2023, pp. 173–194, <https://doi.org/10.1016/B978-0-12-823960-5.00090-1>.
- [60] W. Jian Cedric Sow, J. Du, Effects of high-pressure homogenization and ultrasound on the composition, structure, and physicochemical properties of proteins extracted from *Nannochloropsis oceanica*, *Ultrason. Sonochem.* 105 (2024) 106851, <https://doi.org/10.1016/j.jultsonch.2024.106851>.
- [61] A. Alboresi, C. Le Quiniou, S.K.N. Yadav, M. Scholz, A. Meneghesso, C. Gerotto, D. Simonato, M. Hippler, E.J. Boekema, R. Croce, T. Morosinotto, Conservation of core complex subunits shaped the structure and function of photosystem I in the secondary endosymbiont alga *Nannochloropsis gaditana*, *New Phytol.* 213 (2) (2017) 714–726, <https://doi.org/10.1111/nph.14156>.
- [62] L. Cui, N. Bandillo, Y. Wang, J.-B. Ohm, B. Chen, J. Rao, Functionality and structure of yellow pea protein isolate as affected by cultivars and extraction pH, *Food Hydrocoll.* 108 (2020) 106008, <https://doi.org/10.1016/j.foodhyd.2020.106008>.
- [63] S.M. Beck, K. Knoerzer, J. Sellahewa, M.A. Emin, J. Arcot, Effect of different heat-treatment times and applied shear on secondary structure, molecular weight distribution, solubility and rheological properties of pea protein isolate as investigated by capillary rheometry, *J. Food Eng.* 208 (2017) 66–76.
- [64] J.-L. Messian, N. Sok, A. Assifaoui, R. Saurel, Thermal denaturation of pea globulins (*Pisum sativum* L.) molecular interactions leading to heat-induced protein aggregation, *J. Agric. Food Chem.* 61 (6) (2013) 1196–1204.