Sustainable Food Technology

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
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Cite this: *Sustainable Food Technol.*, 2026, **4**, 2159

Fractionation and characterization of marine macroalgae proteins

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Nowadays, we are facing a shift from animal-based protein sources to plant-based alternatives driven by global population growth and environmental issues. Plants and species from the marine environment (*e.g.*, seaweed) are excellent protein sources, offering essential amino acids and health benefits. Seaweed are sustainable protein sources with a low environmental footprint. However, seaweed contains only up to 15% protein, less than crops like soy (35%), making further processing necessary to increase protein content for food use. In this work, milling and size classification were applied to five different seaweeds (*P. palmata*, *G. verrucosa*, *C. crispus*, *U. lactuca*, and *F. vesiculosus*) to produce fractions with different particle sizes and protein profiles. To study the influence of the downstream process, the produced fractions were further solubilized using the Osborne method and analyzed in terms of nutritional composition and optical microscopy, and protein was quantified and characterized using gel permeation chromatography (GPC) and FTIR-ATR. Results showed that the application of milling and size classification had no effect on the protein content of each fraction. However, after the Osborne fractionation methodology, seaweed proteins demonstrated higher affinity to water environments, with a maximum extraction yield obtained for the *F. vesiculosus* whole fraction (32.12 ± 1.56 $g_{protein}$/100 $g_{algae\ protein}$) and a minimum of 10.42 ± 0.52 $g_{protein}$/100 $g_{algae\ protein}$ for the *P. palmata* coarse fraction. Moreover, GPC showed different protein molecular profile patterns between fractions of the same alga, and FTIR-ATR confirmed the presence of high carbohydrate content, as well as proteins.

Received 7th October 2025
Accepted 12th January 2026

DOI: 10.1039/d5fb00666j

rsc.li/susfoodtech

Sustainability spotlight

Nowadays, we are facing a shift from animal-based protein sources to plant-based alternatives driven by global population growth and environmental issues. Plants and species from the marine environment (*e.g.*, seaweeds) are excellent protein sources, offering essential amino acids and health benefits. Seaweeds are sustainable protein sources with a low environmental footprint, requiring no irrigation or pesticides. This work focused on the fractionation of different seaweeds (*i.e.*, red, green, and brown) and further evaluation of the obtained fractions regarding the protein profile as a function of their solubility, aiming at contributing to their future application in the development of novel food products, thus addressing UN SDGs 2 (zero hunger) and 12 (responsible consumption and production).

1 Introduction

The use of meat and dairy foods as protein suppliers is being substituted by other protein sources (*e.g.*, plants) due to the rapid growth of the world population and environmental issues associated with protein production (*e.g.*, biodiversity loss and climate change).[1,2] Plants are excellent sources of proteins, containing sufficient amounts of essential amino acids that can contribute to a balanced diet and decrease the risk of developing cardiovascular diseases. Also, the presence of considerable amounts of fiber helps to maintain good intestinal activity. However, the nutrient bioavailability (including proteins) can decrease, and the antinutrient effect can be more prevalent, leading to the need for processing techniques to solubilize and purify compounds of interest (*e.g.*, proteins) and achieve good technological functionalities that are not needed when animal proteins are used. Following protein production with a low environmental footprint, seaweed emerges as another alternative protein source, associated with a nearly zero-carbon footprint, possessing all essential amino acids, and without the need for irrigation, pesticides, or arable land for their cultivation. Moreover, seaweed may contain good protein levels that can be similar or even higher than those of terrestrial protein crops (*e.g.*, soybean, with 35%$_{dw}$).[2] However, although not as severe as in some plant-based options, limitations are still present in terms of digestibility and absorption, due to the high fiber content. The presence of marine polysaccharides in seaweed cell walls may make protein availability and digestibility difficult since they are often associated with other structural components, and humans do not have the

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appropriate digestive enzymes. Further, to supply human protein needs, a lot of seaweed should be consumed, and some issues related to its color, texture, and flavor can affect consumer acceptance. In some seaweed, relevant iodine contents may also limit the maximum amount that can be used for nutrition. Most of these problems are mitigated by appropriate processing, and protein extraction methodologies may be beneficial. In industry environments, the extraction of proteins from seaweed is usually related to low extraction yields and/or low protein purity, making it essential to apply additional downstream steps (*e.g.*, protein precipitation or alkaline solubilization) to remove unwanted molecules.[2,3] Further, the low protein yields are also associated with the cell anatomy, which makes it important to know in which environments seaweed's proteins present good affinities. Dry and wet fractionations can be employed in order to try to increase protein content and digestibility. Wet fractionation (usually used for the production of meat analogues using soy and pea proteins) is based on the protein solubilization in environments with high pH, low salt concentration, or using a mixture of ethanol and water followed by a further step of protein isolation, which involves the use of chemicals and water and can cause the loss of a significant part of proteins if not all of them dissolve or precipitate.[1,4] On the other hand, dry fractionation is a non-thermal method that produces protein concentrates by mechanically segregating proteins from celluloses and/or starches and other components based on the particle size distribution after size reduction (*e.g.*, milling). This process does not require the use of any additional water or chemicals for separation, has lower energy consumption, and induces less structural changes in proteins.[5–7] However, due to the low efficiency of the separation process, the presence of other biomolecules (*e.g.*, oils and fibers) can influence their functionality and performance during storage and processing. Using this technique, the dried matrix is fragmented by milling, and subsequent mechanical separation of the fragments using air classification or other methods (*e.g.*, tribo-electrostatic separation and size classification) creates two main fractions (*e.g.*, coarse and fine fractions) that are rich in starch/cellulose and proteins.[1,4] Dry fractionation has been successfully used in a wide range of pulses (*e.g.*, peas, chickpeas, faba beans, mung beans, and lentils). A recent study reported the use of dry fractionation for the production of functional fractions from mung bean, yellow pea and cowpea flour and reported that for all matrices, the fine fraction had the highest protein content (independent of the speed), achieving a maximum of around 60 g protein/100 g dry matter for mung bean and a minimum of around 45 g protein/100 g dry matter for cowpea.[8] Moreover, the study also reports that dry fractionation had an impact on the amino acid composition of the fractions, without affecting the ratio between hydrophilic and hydrophobic amino acids. Also, techniques like the Osborne fractionation method are being used for vegetable protein characterization. Using a sequential extraction procedure, different solvents are tested for protein extraction, and the obtained fractions are divided based on their affinity to the solvent. Usually, water, saline solution, ethanol, and alkaline environments are used, and four different protein classes (*i.e.*, albumins, globulins, prolamins, and glutelins) are obtained. In a recent study conducted by Dias *et al.* in 2024, the extraction of lentil proteins was done using the Osborne methodology and alkaline/enzymatic extraction, and its influence on its functional properties was determined.[9] In general, the albumin and globulin fractions showed similar or better results when compared to traditional approaches, with less strong treatments applied. Also, Das *et al.*, 2023 indicated that faba bean proteins are mostly extracted in water and alkaline media (around 80 and 43%, respectively).

However, these fractionation methods were developed for terrestrial plant-based proteins. Further, information regarding seaweed protein dry fractionation and solvent solubility is rare or unavailable. Indeed, seaweed proteins are extremely heterogeneous, comprising structural, enzymatic, storage, and cell-wall-associated proteins. Thus, this work aims to assess the composition of different fractions of several seaweed species obtained after milling followed by size classification for the obtention of biomasses with lower levels of indigestible polysaccharides, minerals, and phenolic compounds that can bind to protein. In general, mechanical disruption allows the obtention of a finer fraction, frequently richer in proteins, and a coarser fraction, more difficult to break and richer in starch granules, fibers, and rigid cell-wall materials. Further, for each fraction and for the whole seaweed, the Osborne fractionation method was used to further characterize the different proteins present.

2 Materials and methods

2.1. Materials

Seaweed *Gracilaria verrucosa, Ulva lactuca, Palmaria palmata, and Chondrus crispus* were provided by ALGAPlus (Ílhavo, Portugal) in the form of dried (at room temperature) and milled flakes (1.5–10 mm). The protein-enriched *Fucus vesiculosus* batch was conditioned under aquaculture conditions and kindly supplied by Green Aqua Vagos and A4f – Algae for Future (Lisbon, Portugal), under the context of the BIOFÁBRICA project (promoted by Green Aqua Vagos). Analytical grade chemicals were purchased from Sigma-Aldrich Chemical Co. Ltd (St. Louis, MO, USA).

2.2. Methods

2.2.1. Nutritional characterization. Seaweed fractions and Osborne sub-fractions were nutritionally characterized as described below:

Protein content was estimated by quantification of total nitrogen after sample acid digestion using a Kjedahl digestor (Foss Analytics, Hilleroed, Denmark), applying the nitrogen conversion factor (N × 4.59, N × 5.13, and N × 5.38 for red, green, and brown algae, respectively).[10,11]

Carbohydrate content was assessed by the NREL official protocol (NREL/TP-5100-60 957).[12] Briefly, 3 mg of each alga was mixed with 3 mL of 72% H_2SO_4, and placed into a water bath at 30 ± 3 °C for 60 min. Then, each sample was diluted to a 4% H_2SO_4 final concentration and autoclaved for 1 h at 121 °C. The

hydrolysates were filtered through 0.22 μm membranes and analyzed using a high-performance liquid chromatograph with a refractive index detector, using an Aminex HPX-87H column at 60 °C with a mobile phase of 0.05 M H_2SO_4 at a flow rate of 0.6 mL min^{-1}. The quantification of monosaccharides was performed using a calibration curve of each standard (glucose, galactose, rhamnose, arabinose, fucose, glucuronic acid, and galacturonic acid) at different concentrations (0.25, 0.5, 0.75, 1, 1.5, and 2 g L^{-1}).

Lipids were determined using the Bligh & Dyer method with modifications. For this, 1 mL of a mixture of chloroform/methanol (2 : 1, v/v) was added to 50 mg of macroalgae biomass. The mixture was first subjected to ultrasound (10 min) to promote cell wall disruption and then incubated on a heating block at 30 °C for 30 min. The mixture was centrifuged at 2000 rpm for 10 min, and the organic phase was collected in a pre-weighed glass tube. The procedure was repeated until the solvent had no pigmentation. The removal of contaminants was conducted by re-dissolving the initial extract in 2 mL of chloroform and 1 mL of methanol. To promote phase separation, 750 μL of deionized water was added, and the organic phase was collected. The combined organic phases were dried under an N_2 stream and weighed.[13]

The ash and moisture contents in algal biomass were determined according to NREL procedures (NREL/TP-510-42618).[14] Briefly, 1 g of algal 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 weighed 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 after cooling down to room temperature to determine the ash content.

All determinations were run in triplicate, and the results are presented as a percentage on a dry weight basis.

2.2.2. Milling and separation of seaweed. Milling of all seaweeds was conducted using a kitchen robot (Vorwerk®, Wuppertal, Germany). For that, 100 g of each seaweed was crushed for 10 s at speed 8. The milled seaweed was recovered and fractionated using test sieves with different mesh sizes (>1 mm; 0.45–1 mm; and 0.1–0.45 mm) (VWR®, Germany), allowing three fractions for each alga to be obtained (indicated as coarse, medium, and fine fractions, respectively). The weight of each resulting fraction was recorded. Due to a low fractionation yield under the present conditions, for *P. palmata*, an additional sieve (1.6 mm) was added, and only two main fractions were considered (>1.6 mm and >1.6 mm). Also, for this algae, three cycles (10 s) at speed 8 were conducted to improve the fractionation degree.

2.2.3. Morphology of the obtained fractions. The morphology of each fraction for the five seaweeds used was assessed using an Olympus BX51-Extreme microscope (Olympus Europa SE & Co. KG, Hamburg, Germany) in bright field with a 40× magnification objective lens acquired by the CellSens software version 1.1.8 (Olympus Corporation). All samples were hydrated overnight, and a single piece was placed on a glass slide and coverslipped.

2.2.4. Osborne fractionation method. The study of the protein profile of the selected seaweed species was conducted by fractionating the macroalgae proteins with the Osborne method, as described by the authors of ref. 15. Different protein fractions were obtained after a sequential extraction with dH_2O, aqueous 0.5 M NaCl solution, 70% EtOH, and 0.1 M NaOH. Thus, dried macroalgae were mixed with dH_2O (1 : 20 solid/liquid ratio, final volume of 100 mL) for 1 h to obtain the first fraction. The residue from the first step was mixed with 100 mL of 0.5 M NaCl for 60 min to obtain the second protein fraction. Then, the residue was treated for 60 min with 100 mL of 70% (v/v) ethanol and finally with 100 mL of 0.1 M NaOH for 60 min. All the extractions were carried out using constant agitation. Each fractionating step was repeated twice to improve protein extraction, and the supernatants were recovered by centrifugation at 4000 × g for 30 min and combined. Extraction yields were quantified gravimetrically, and protein extracts were kept at −20 °C until further use.

2.2.5. Characterization of the extracts from the Osborne fractionation

2.2.5.1. Protein content. The protein content of each extract was quantified by CHN elemental analysis (Elementar, Elemental Analyzer system, GmbH, Hanau, Germany) following the manufacturer's specifications. The final protein content was calculated by multiplying the percentage of nitrogen by 5.38, 4.59, and 5.13 for brown, red, and green seaweed, respectively.[11]

2.2.5.2. Gel permeation chromatography (GPC). The proteins' molecular weight distribution was assessed using GPC on a PolySep-GFC-P-4000 column (300 × 7.8 mm, Phenomenex®, USA) after dissolution of each lyophilized extract in water to achieve a final protein concentration of 1 mg mL^{-1}. The elution occurred with ultrapure water using a flow rate of 0.8 mL min^{-1} at 40 °C with RI and UV detection. Linear regression calibration was performed using standard pullulan kit P-82 (Shodex™, Japan) within a range of 6.3–642 kDa.

2.2.5.3. Fourier Transform Infrared spectroscopy (FTIR-ATR). The study of the functional groups and bonding arrangement, as well as the protein secondary structure of the obtained protein-enriched fractions, was carried out *via* FTIR using an ALPHA II- Bruker spectrometer (Ettlingen, Germany) with a diamond-composite attenuated total reflectance (ATR) cell. The spectra were recorded in the range of 4000–400 cm^{-1} by acquiring 64 scans per sample with a 4 cm^{-1} resolution.

2.2.6. Statistical analyses. 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) (version 9.5.1). A *T*-test was used to test differences between samples, considering values of $p < 0.05$ as statistically significant.

3 Results

3.1. Effect of milling on biomass distribution for downstream processing

After the application of milling, different proportions of seaweed were found in each fraction (coarse, medium, and fine). The breakdown of this is summarized in Fig. 1. For *P. palmata*, *C.*

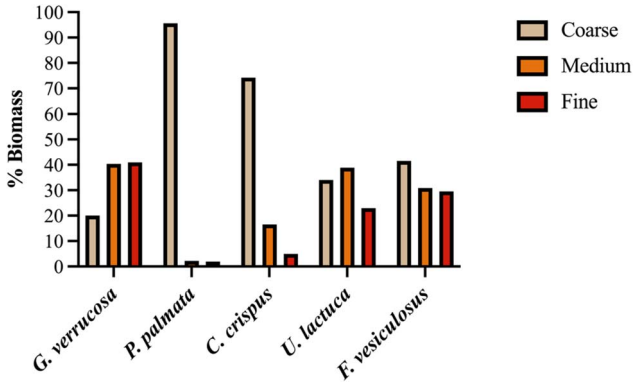

Fig. 1 Particle size distribution across the three main fractions (coarse, medium, and fine) of five different seaweed species (*i.e.*, *G. verrucosa*, *P. palmata*, *C. crispus*, *U. lactuca*, and *F. vesiculosus*).

crispus, and *F. vesiculosus*, the highest proportion of biomass was found in the coarse fraction, with more evidence in *P. palmata* and *C. crispus* (with values of around 95 and 74%, respectively), while very low percentages of the fine fraction were obtained for *P. palmata* and *C. crispus* (between 2 and 5% of the initial biomass). On the other hand, the initial biomass of *G. verrucosa* was mostly and equally distributed between the medium and fine fractions (around 40% of the initial biomass for each fraction). The same equality between medium and fine fractions was also observed in *F. vesiculosus*. The lack of efficient fractionation for *P. palmata* led to the need to add an extra 1.6 mm sieve and an extra step of milling (see section 2.2.2). Thus, for this alga, only two main fractions were considered (higher and smaller than 1.6 mm) in further protein extraction assays.

3.2. Chemical characterization of seaweed and fractions

The red (*i.e.*, *G. verrucosa*, *P. palmata*, and *C. crispus*), green (*i.e.*, *U. lactuca*), and brown (*i.e.*, *F. vesiculosus*) seaweed species used in this research were characterized in terms of nutritional composition, and the results are summarized in Table 1. It was observed that the protein content was highly dependent on the seaweed species, reaching a maximum of 17.76 ± 0.05%$_{dw}$ and a minimum of 6.77 ± 0.10%$_{dw}$ for *F. vesiculosus* and *C. crispus*, respectively. On the other hand, all seaweeds presented a lipid content below 5%$_{dw}$, which was expected since macroalgae are known for their low lipid content. In contrast, the total carbohydrates were found to be the component present in the highest percentage in all samples (higher than 40%$_{dw}$), mainly attributed to the cell wall polysaccharides.[10,16] However, the nutritional composition of seaweed can be highly different depending on the season in which it is harvested. For example, in a recent study conducted by Wirenfeldt *et al.* (2024),[17] the chemical composition of *Ulva* sp. and *F. vesiculosus* was determined, and the researchers found a carbohydrate content of around 60–70%$_{dw}$ and protein content below 10%$_{dw}$, which is very low when compared to the presented results in our work.

The application of dry fractionation led to the production of different fractions of each alga, which could present different behaviors when in contact with solvents aiming at protein extraction. As expected, just like the initial biomass, all fractions presented high carbohydrate content, which represented practically half of the algae composition (on a dry weight basis). Also, lipid content was lower than 5%$_{dw}$, which is also characteristic of seaweed.[10] Regarding ash content, the green seaweed *U. lactuca* demonstrated higher contents of inorganic matter (around 35%$_{dw}$) when compared to the red and brown algae used in this study (which were lower than 30%$_{dw}$) and can be related to the need for more minerals for structural interactions.[18–20]

It was expected that the presence of carbohydrates (including dietary fibers) could be found in higher quantities in fractions with larger particles (coarse fraction) and lower quantities in the

Table 1 Chemical characterization of different fractions of *G. verrucosa*, *P. palmata*, *C. crispus*, *U. lactuca*, and *F. vesiculosus* after the application of milling and size classification. For each column, different letters represent statistically significant differences ($p < 0.05$)

	Fraction	Protein (%)	Lipids (%)	Carbohydrates (%)	Ash (%)
G. verrucosa	Whole	16.28 ± 0.05^{a}	<5	45.69 ± 0.65^{a}	28.72 ± 1.15^{b}
	Coarse	15.34 ± 2.58^{a}		45.67 ± 0.12^{a}	29.27 ± 0.07^{b}
	Medium	19.99 ± 0.46^{a}		53.42 ± 0.00^{c}	21.48 ± 0.33^{a}
	Fine	18.19 ± 0.07^{a}		40.22 ± 0.29^{b}	22.08 ± 0.14^{a}
P. palmata	Whole	11.83 ± 0.18^{a}		44.21 ± 0.72^{a}	26.66 ± 0.93^{a}
	Coarse	13.20 ± 0.14^{a}		45.53 ± 2.07^{a}	26.77 ± 0.61^{a}
	Medium	15.66 ± 1.38^{b}		48.86 ± 1.75^{b}	27.05 ± 0.28^{a}
	Fine	12.47 ± 0.07^{a}		45.69 ± 0.31^{a}	26.71 ± 0.75^{a}
C. crispus	Whole	6.77 ± 0.10^{a}		43.25 ± 0.64^{a}	21.36 ± 1.40^{a}
	Coarse	6.98 ± 0.16^{a}		42.84 ± 0.81^{a}	24.07 ± 0.14^{b}
	Medium	8.29 ± 0.16^{b}		45.68 ± 1.15^{a}	25.39 ± 0.64^{b}
	Fine	9.54 ± 0.26^{b}		49.92 ± 5.43^{a}	27.62 ± 0.54^{c}
U. lactuca	Whole	15.98 ± 0.10^{a}		42.90 ± 1.83^{a}	33.85 ± 0.46^{b}
	Coarse	15.34 ± 0.09^{a}		43.26 ± 1.57^{a}	27.95 ± 1.26^{a}
	Medium	16.00 ± 0.06^{a}		42.03 ± 2.27^{a}	35.44 ± 0.91^{b}
	Fine	14.94 ± 0.38^{a}		49.33 ± 1.30^{b}	34.91 ± 0.52^{b}
F. vesiculosus	Whole	17.76 ± 0.05^{b}		41.52 ± 2.48^{a}	30.41 ± 0.95^{b}
	Coarse	16.91 ± 0.10^{a}		47.75 ± 0.85^{b}	24.94 ± 0.64^{a}
	Medium	18.53 ± 0.06^{c}		46.80 ± 0.82^{b}	22.42 ± 0.90^{a}
	Fine	20.88 ± 0.08^{d}		42.07 ± 1.48^{a}	25.05 ± 1.22^{a}

fine fraction. It was also expected that protein content would increase with a decrease in granulometry.[8] However, in terms of protein, this behavior was only observed for *G. verrucosa*, (16.28 to 18.19%$_{dw}$), *P. palmata* (10.30 to 12.47%$_{dw}$), *C. crispus* (6.77 to 9.54%$_{dw}$), and *F. vesiculosus* (17.76 to 20.88%$_{dw}$) when comparing the biomass and the fine fraction protein content, and not for *U. lactuca*. Further, the decrease in the carbohydrate content in the fine fraction was only observed for *G. verrucosa* and *F. vesiculosus*. This behavior can be explained by the difference in the cell wall composition of green, red, and brown species associated with the cellulose content. Depending on the seaweed group, cellulose is often associated with other polysaccharides (*i.e.*, xylan, mannan, galactans, alginic acid, agar, carrageenan, or others) that, together with cellulose content, make the seaweed cell wall more or less rigid and thus influence its capability to resist tension. In the case of brown seaweed, phlorotannin may also play an important role. For green seaweed, the content of cellulose can range from 1.5 to 34%, followed by 0.85–18%, and 2.2–10.2% for red and brown seaweed, respectively. These results may also be supported by the stability of the carbohydrate (and fiber) content throughout the obtained fractions, indicating that fibers may hinder the access to the intracellular environment, eventually hindering posterior protein extraction. Also, the way that different cells are associated in the seaweed macrostructure will play a major role in cell wall rupture. On the one hand, in the case of red and brown algae, the presence of polysaccharides (in addition to cellulose) that provide structural characteristics causes an increase in resistance to break due to their existence both in the cell wall and in the space between cells. Also, in brown algae, phlorotannins are present and contribute to greater structural strength by interacting with polysaccharides, promoting cross-linking that increases rigidity. In these species, this may increase structural resistance that can make protein extraction difficult. Moreover, it may cause the reduction of protein bioavailability since phlorotannins can bind with them and form complexes and/or aggregates which are less digestible and less accessible to the action of proteolytic/digestive enzymes.[21,22] On the other hand, alginates (in brown algae) and some galactans in red algae, for example, are very abundant in the space between the cells, and are responsible for the gelatinous or "slippery" structure. In green algae such as *U. lactuca*, these structural polysaccharides are not present, which facilitates the break of the cell wall, and thus causes a similar composition between different granulometries, namely regarding protein content.[23]

After analyzing the nutritional composition of each alga and based on the protein content, it can be concluded that despite the significant difference between the fractionation yield of all seaweeds, low yields were obtained for the fine fraction of *P.*

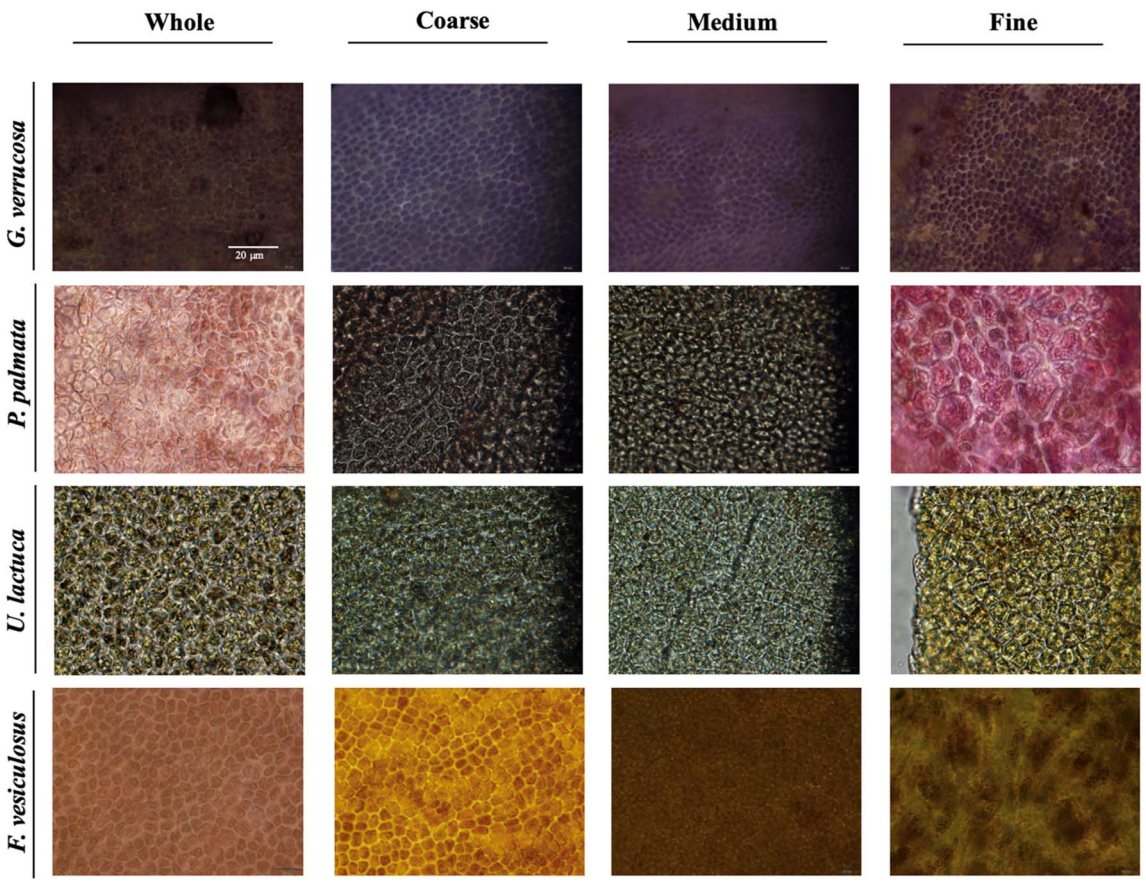

Fig. 2 Morphology of the obtained fraction after milling and size classification of *G. verrucosa*, *P. palmata*, *U. lactuca*, and *F. vesiculosus*. Images were obtained using an amplification of 40×.

palmata and *C. crispus*, and thus, dry fractionation is less promising for these species. This tendency was expected and verified in other field-related studies. Recently, Bahari *et al.* (2021) investigated the influence of different surface areas obtained by mechanical disintegration on carrageenan and phycoerythrin content.[22] Thus, results showed that the application of a low temperature (*e.g.*, 22 °C) did improve the protein extraction yield, whereas higher temperatures (*e.g.*, 45 and 90 °C) caused protein denaturation and, thus, less protein was quantified. This may indicate that, despite the lower fractionation yields, this approach may still be used in these algae if further extraction processes are applied to promote better solubilization yields and production of protein-enriched fractions.

For the next steps of the work, three of the studied seaweed species (*i.e.*, *G. verrucosa*, *P. palmata*, and *F. vesiculosus*) were selected to move forward in the work. Moreover, *C. crispus* was not chosen due to the extremely low protein content in all fractions.

3.3. Microscopy of algal fractions

The effect of milling on the structural morphology of *G. verrucosa*, *P. palmata*, *U. lactuca*, and *F. vesiculosus* was studied using microscopic techniques. When seaweed is used, access to the intracellular environment is hampered by the existence of more complex structures (made up of cellulose and other polysaccharides) in intercellular spaces and in cell walls (with thicker and more resistant cell walls). Thus, more aggressive and specific physical methods (*e.g.*, high-pressure homogenization, ultrasonication, and bead milling) or chemical treatments may be necessary to disrupt cells and access intracellular contents, in the same way that these methodologies are used in the case of protein extraction from legumes, which also present rigid cell walls. On the one hand, as mentioned by Schutyser *et al.*, 2015, dry fractionation techniques, such as milling, can mechanically detach protein bodies and other cellular compounds into flour with particles of different composition that can be separated based on size and density through air classification, leading to a large increase of protein content (*e.g.*, from 27.1 to 50.9, 23.7 to 57.6, and 40.4 to 59.4 g/100 g_{dw} for cowpea, lentil and lupine, respectively), demonstrating good effectiveness in acting on the structural deformation.[23] On the other hand, a recent study conducted by Suchintita Das *et al.* in 2023 studied the effect of different technologies (*e.g.*, ultrasound-assisted extraction and high-pressure homogenization, among others) on protein extraction from faba bean (*Vicia faba* L.),[24] and SEM images of the untreated faba bean powder and residual biomasses after treatment indicated that the application of stronger methodologies (*e.g.*, ultrasonication and high-pressure homogenization) did improve the protein release by disrupting the starch–protein attachments and decreasing the extent of agglomeration of the cellular fragments that were embedding the protein particles. However, despite several research studies on the application of different dry and wet methodologies for cell disruption, mostly in legumes, limited attention has been given to their effect on protein extraction when seaweed is used.[25–28] From Fig. 2 it is possible to see that the chosen parameters for the milling step did not cause any cell wall rupture in either the seaweed and/or fraction, but differences in cell type/structure were found, mostly in the case of *P. palmata* and *F. vesiculosus*, which could explain the significant protein content variation of the obtained fraction for these two algas (see section 3.2).

3.4. Osborne fractionation

The Osborne fractionation method was used to study the protein profile of biomass, coarse, and fine fractions of the selected seaweed (*G. verrucosa*, *P. palmata*, and *F. vesiculosus*) in order to verify the existence of different protein affinities when the granulometry of the seaweed decreases. For that, deionized H_2O, 0.5 M NaCl, 70% EtOH, and 0.1 M NaOH were used as extraction solvents following the methodology described above (see section 2.2.5). It is expected that water will extract highly soluble proteins, located in more accessible sites of the cell and not strongly bond to other structural compounds, as it is the first extraction step. Proteins extracted with NaOH, in the last sequential step, are generally less accessible (eventually physically trapped in the seaweed structure), poorly soluble at neutral pH, and/or strongly associated with other structural compounds within the seaweed cells, requiring stronger extraction conditions. The alkaline pH will help partially disrupt the cell wall and break hydrogen bonds and electrostatic interactions, thus promoting further protein extraction. The solubilization yield in each solvent was quantified, and the results are summarized in Fig. 3. For *G. verrucosa* (Fig. 3A), relevant solubilization yields were achieved with all solvents, which is indicative of the presence of compounds with different chemical properties. However, despite the solubilization yields of H_2O, NaCl, and EtOH extraction being statistically significant ($p < 0.05$) in coarse and fine fractions, the decrease between different granulometries was more evident in the NaOH extraction (10.82 ± 0.06 to 2.44 ± 0.59% from the coarse to fine fraction, respectively) which can be a result of the existence of different interactions between the solvent and *G. verrucosa* filaments or of different compositions of fractions, which result in different solubilization patterns. Moreover, the fine fraction (composed of a material with a small size) eventually presented less content of glycoproteins and more accessible proteins compared with the other fractions, increasing the interaction with the solvent, allowing its extraction and, therefore, making the differences between solvents more easily verified. On the other hand, for *P. palmata* (Fig. 3B), it is clear that higher yields were obtained using water, followed by NaCl. This tendency was already evidenced in a previous study conducted by our research team and is related with the presence of a high content of phycobiliproteins that show affinity to water environments and also by the presence of structural polysaccharides that are more easily solubilized when compared to the ones that are found in *G. verrucosa* (such as agar, that can only be solubilized at high temperatures).[10] On the other hand, the existence of a high level of salts helped to increase the protein solubilization, which also reflected the high solubilization yields in NaCl. Regarding *F. vesiculosus* (Fig. 3C), a brown alga, in all three samples, higher

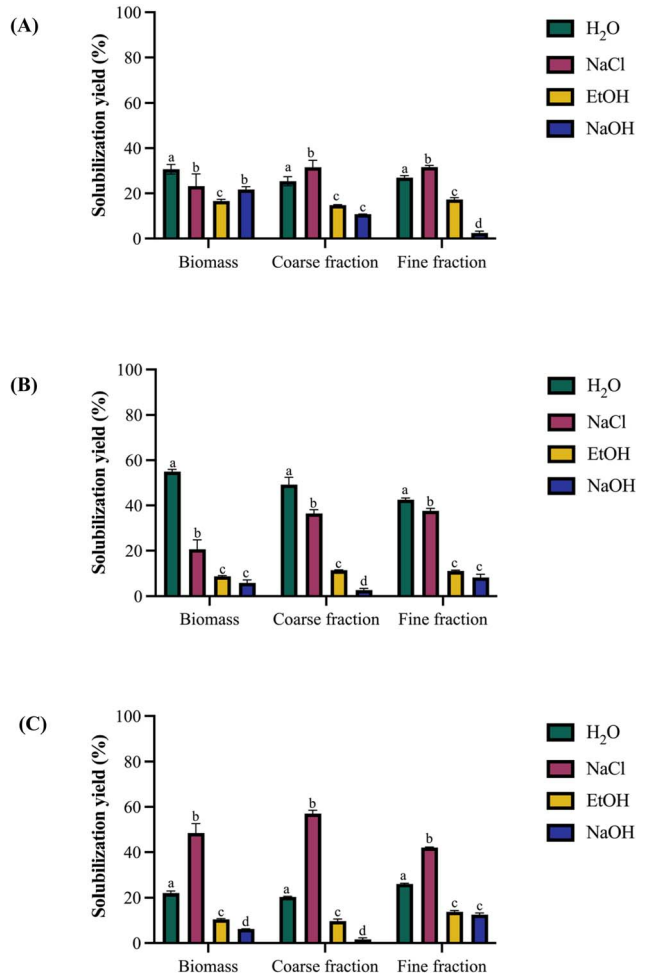

Fig. 3 Solubilization yields obtained for each fraction of the three selected alga: (A) *G. verrucosa*, (B) *P. palmata*, and (C) *F. vesiculosus* after the application of the Osborne fractionation method.

extraction was achieved using saline solution and water, followed by minor percentages using EtOH and NaOH. This behavior was not expected since *F. vesiculosus* has high content in pigments and phlorotannin, which are usually extracted with ethanol but require long extraction times, sequential water/ethanol or acetone solutions, or the use of non-conventional technologies (such as ultrasound and microwaves).[29–31]

The protein content of each extract was then determined, and the results are summarized in Table 2. Regarding the overall results, a clear tendency for protein affinity to water and alkaline environments can be seen for all seaweeds tested. On the other hand, the protein content in the water extracts was significantly higher than that obtained using 0.1 M NaOH, with differences higher than 10%. For *G. verrucosa* and *P. palmata*, protein is mostly extracted in water with no significant differences between fractions. This behavior was expected since red seaweed species are good sources of phycobiliproteins, which are globular proteins with several polar groups (–OH, $–NH_2$, and –COOH) that establish molecular bonding (through hydrogen bonds) with water. Also, the presence of polar and charged amino acids (*e.g.*, aspartic acid, glutamic acid, lysine, and arginine) facilitates the presence of electrostatic interactions with water, favoring their dispersion in aqueous solutions.[32–34] This affinity was also observed for seaweed in other works. A few years ago, Harnedy *et al.* (2013) also studied the extraction of proteins through a similar approach to ours (sequential aqueous and alkaline extraction), allowing the recovery of 6.7 ± 0.2% algal dry weight. More recently, Trigo *et al.* (2025) used aqueous (with surfactants) and alkaline solutions to extract protein from *Ulva fenestrata*, achieving around 60% of N solubility yield, which indicates good protein solubilization.

Nevertheless, it is important to highlight that all extracts had a protein content below the protein content of the whole seaweed, except for *F. vesiculosus*. This probably means that proteins are present mostly in bonded forms, such as glycoproteins, and that higher temperatures were needed to solubilize the polysaccharide fraction and allow protein solubilization. The brown algae *F. vesiculosus* was the one that showed the best results with a recovery of around 30–35%$_{dw}$ of the total protein content present in the initial biomass, mostly extracted with water. On the other hand, the NaOH fraction seemed to have higher protein content when the granulometry of the seaweed decreased (2.41 ± 0.17 to 6.76 ± 0.37 $g_{protein}$/100 $g_{algae\ protein}$, respectively), mostly associated with the increase in the contact area between proteins and the solvent. The higher protein content in the water extract was expected since *Fucus vesiculosus* has a predominant content of globular proteins that are hydrophilic. In a recent study conducted by Ummat *et al.* in 2021, *F. vesiculosus* was used for protein extraction using three different solvents (*i.e.*, deionized water, 0.1 M HCl, and 1% citric acid) in isolated extractions for different times (*i.e.*, 1 h and 2 h) and reported a recovery of around 40% (g protein/100 g dried seaweed),[34] which was higher than the extraction yields presented in this work. However, all solvents were previously heated to 80 °C, which indicates that protein was extracted as a result of the actuation of solvent characteristics together with the thermal effect and not just as an effect of the solvent. This thermal effect may also help in the solubilization of alginates that are poorly soluble at low temperatures, which were probably bound to some proteins, thus increasing the extraction yields.[35] In this work, temperature was not used for protein extraction since technological functionalities are intended, so the protein must be in its native form and not denatured. Instead of water, phosphate-buffered saline or a Tris–HCl solution could be an option to extract protein and prevent protein denaturation.[36] In another study conducted by Golshany *et al.* in 2025, alkaline protein extraction from *F. vesiculosus* was performed using different approaches,[37] in which NaOH was applied at different concentrations (*e.g.*, 2, 4, and 6%), obtaining protein yields of 25.42 ± 1.20%, 31.84 ± 0.82%, and 39.29 ± 1.66%, respectively. These values were similar to the ones obtained in our work when the combination of both water and alkaline fractions is considered. However, the reported work involved the need for further steps of protein precipitation and drying techniques that increase the process time and cost and may cause protein modifications through the use of drying techniques.

Table 2 Quantification of protein content of each obtained fraction after the application of the Osborne fractionation method for *G. verrucosa*, *P. palmata*, and *F. vesiculosus*

Alga	Fraction	Extraction solvent	Protein content/g protein/100 g extract	Protein extraction yield ($g_{protein}/100\ g_{alga\ protein}$)	Total protein extraction yield ($g_{protein}/100\ g_{alga\ protein}$)
G. verrucosa	Whole	H_2O	12.96 ± 0.62	24.40 ± 1.40[a]	29.06
		0.5 M NaCl	0	0	
		70% EtOH	0	0	
		0.1 M NaOH	3.50 ± 0.23	4.66 ± 0.17[b]	
	Coarse	H_2O	13.03 ± 1.07	21.52 ± 1.83[a]	23.41
		0.5 M NaCl	0	0	
		70% EtOH	0	0	
		0.1 M NaOH	2.66 ± 0.23	1.89 ± 0.17[b]	
	Fine	H_2O	14.82 ± 0.58	21.93 ± 0.24[a]	23.66
		0.5 M NaCl	0.46 ± 0.08	0.80 ± 0.12[b]	
		70% EtOH	0	0	
		0.1 M NaOH	6.32 ± 0.66	0.93 ± 0.26[b]	
P. palmata	Whole	H_2O	3.06 ± 0.35	16.28 ± 1.61[a]	20.78
		0.5 M NaCl	0	0	
		70% EtOH	0	0	
		0.1 M NaOH	7.79 ± 0.34	4.49 ± 0.99[b]	
	Coarse	H_2O	2.80 ± 0.04	10.42 ± 0.52[a]	13.21
		0.5 M NaCl	0.46 ± 0.07	1.27 ± 0.23[b]	
		70% EtOH	0	0	
		0.1 M NaOH	5.80 ± 0.99	1.52 ± 0.57[b]	
	Fine	H_2O	3.72 ± 0.58	12.67 ± 1.94[a]	16.89
		0.5 M NaCl	0	0	
		70% EtOH	0.54 ± 0.31	0.48 ± 0.05[c]	
		0.1 M NaOH	5.69 ± 0.31	3.75 ± 0.39[b]	
F. vesiculosus	Whole	H_2O	25.95 ± 1.01	32.12 ± 1.56[a]	36.98
		0.5 M NaCl	0.82 ± 0.06	2.33 ± 0.32[b]	
		70% EtOH	0	0	
		0.1 M NaOH	7.21 ± 0.51	2.52 ± 0.18[b]	
	Coarse	H_2O	25.71 ± 0.93	30.06 ± 0.36[a]	32.43
		0.5 M NaCl	0.50 ± 0.08	1.66 ± 0.21[b]	
		70% EtOH	0	0	
		0.1 M NaOH	6.41 ± 0.34	0.71 ± 0.05[c]	
	Fine	H_2O	21.30 ± 1.53	25.41 ± 1.93[a]	32.17
		0.5 M NaCl	0	0	
		70% EtOH	0	0	
		0.1 M NaOH	11.93 ± 0.29	6.76 ± 0.37[b]	

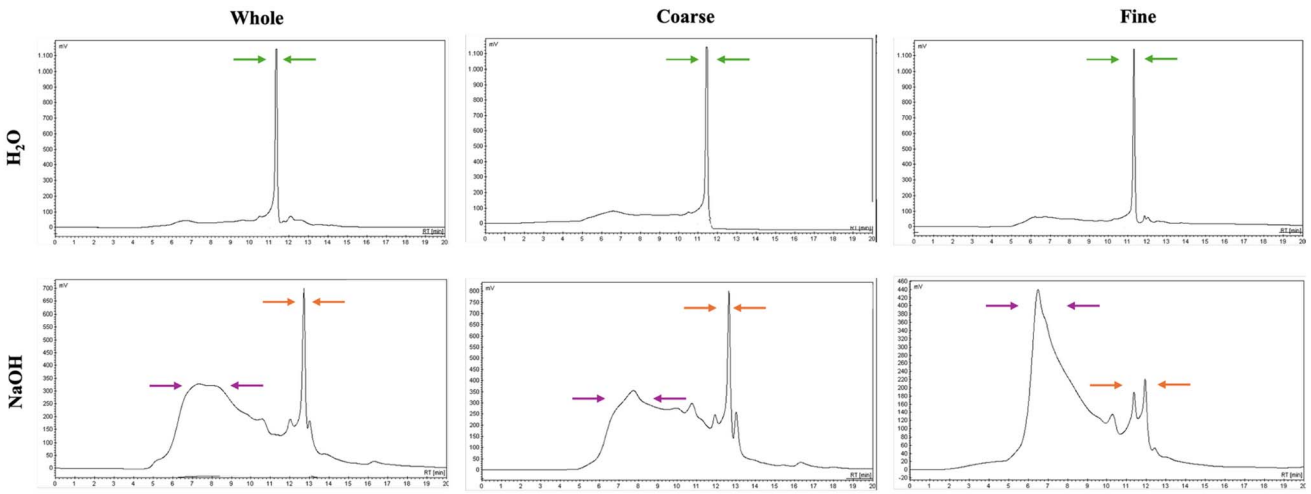

Fig. 4 Influence of milling on the molecular profile of *G. verrucosa* H_2O/NaOH extracts for the whole, coarse, and fine fractions obtained using gel permeation chromatography. Arrows with the same color indicate principal peaks with similar retention times.

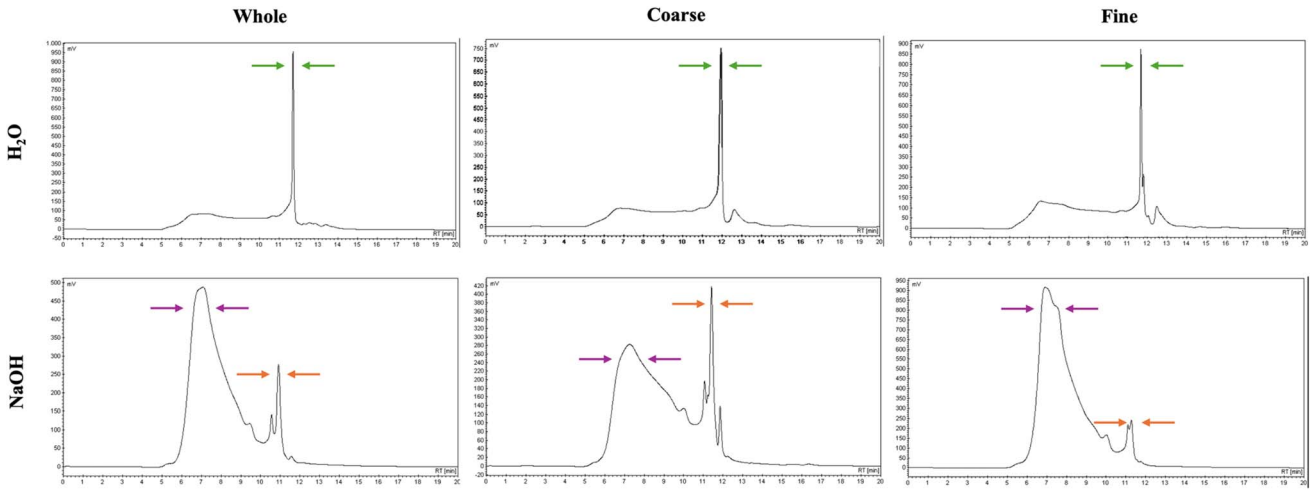

Fig. 5 Influence of milling on the molecular profile of *P. palmata* H_2O/NaOH extracts for the whole, coarse, and fine fractions obtained using gel permeation chromatography. Arrows with the same color indicate principal peaks with similar retention times.

3.5. Protein molecular profile

After the application of the Osborne fractionation method, it was clear that proteins from the studied seaweed were mostly extracted using water. Also, it was shown that the protein content was not significantly different between fractions of the same alga. However, although the content of extracted protein did not differ, the types of proteins that were extracted in each fraction can be significantly different, especially considering that the decrease in the granulometry causes an increase in the surface area of the seaweed that will interact with the solvent. Thus, to determine the protein profile of each fraction, GPC was conducted, and the results are summarized in Fig. 4, 5, and 6. Regarding the red seaweed *G. verrucosa* and *P. palmata*, all of the H_2O spectra demonstrated the presence of a single peak, which is related to the presence of phycobiliproteins (mostly phycoerythrin) since these proteins are extracted with water.[10] On the other hand, the NaOH samples showed a more variable range of molecular sizes that could range from small to large peptides that result from molecular aggregation after the occurrence of the hydrolysis effect. In contrast, in *F. vesiculosus* samples, different molecular patterns were found in the water extracts, with similarity between whole and coarse fractions but a different protein profile in the fine fraction. Further, the results indicate that, unlike the red seaweed, where a single peak was observed, different proteins or proteins with different degrees of association or aggregation are present. Also, the chromatograms of the NaOH extracts showed similar peak identification (namely by the presence of a single peak and a broad peak corresponding to the presence of molecular aggregates), with differences only visible in terms of the intensity of the signal. Thus, regarding this brown alga, the milling of the biomass affected the molecular profile of proteins extracted in water but did not change the protein profile of the alkaline extracts.

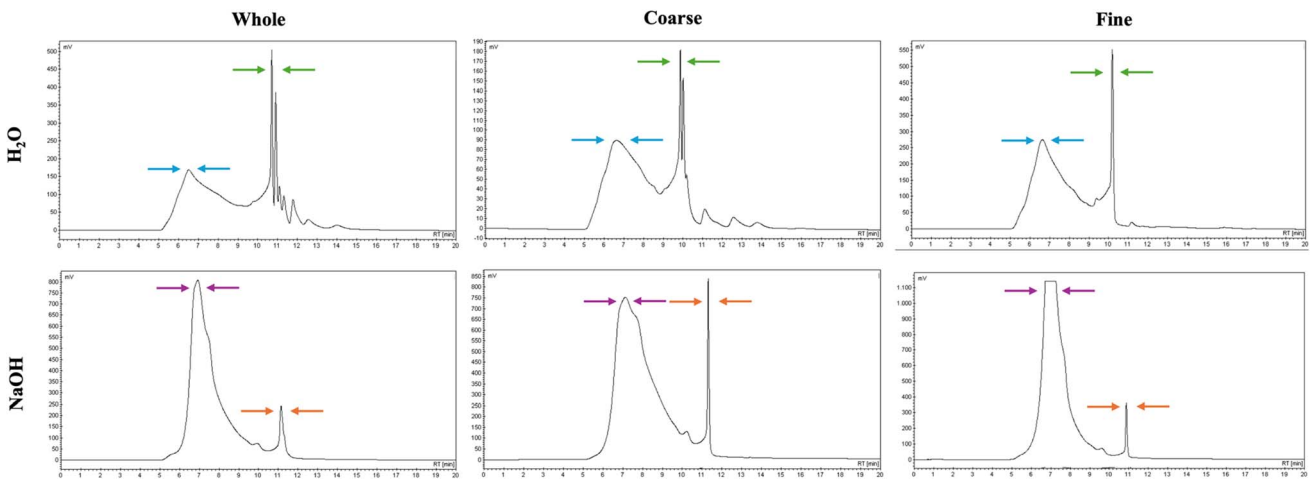

Fig. 6 Influence of milling on the molecular profile of *F. vesiculosus* H_2O/NaOH extracts for the whole, coarse, and fine fractions obtained using gel permeation chromatography. Arrows with the same color indicate principal peaks with similar retention times.

3.6. Functional groups

After the determination of the molecular weight of the extracted protein in all fractions, different characteristics were assessed using FTIR-ATR spectroscopy. Fig. 7 presents the spectra for the three seaweed species, with the main peaks identified. Usually, this technique allows the identification of typical carbohydrate peaks that are indeed extracted together with proteins. Typically, the C–H bending appears between 1465 and 1500 cm^{-1}, N–O stretching between 1550 and 1500 cm^{-1}, O–H bending and carboxylic acid at 1440–1395 cm^{-1}, C–O stretching at 1275–1200 cm^{-1}, S=O stretching at 1070–1030 cm^{-1}, and the anomeric region at 950–700 cm^{-1}. However, the detection of protein can also be achieved using this technique by the identification of amide I and amide II functional groups at 1690–1600 cm^{-1} and 1575–1480 cm^{-1}, respectively.[38] Across all seaweeds, the main differences were found in the coarse and fine NaOH extracts, which showed noticeably stronger C–H bending (around 1500 cm^{-1}). In contrast, these signals were nearly absent in the coarse and fine water extracts of *G. verrucosa* (Fig. 7A). Additionally, the whole NaOH fraction of *F. vesiculosus* did not exhibit higher C–O stretching (around 1200 cm^{-1}), suggesting that this fraction may contain fewer carbohydrates than the other fractions obtained from the same species.[39] All coarse NaOH fractions showed a strong absorption peak at around 600 cm^{-1}, possibly due to the S–O stretching. Regarding protein content, all seaweeds had signals in the wavelength range corresponding to amide I and amide II functional groups, confirming the success of protein extraction. However, different absorption signals are obtained, which can indicate the existence of changes in protein secondary structure.

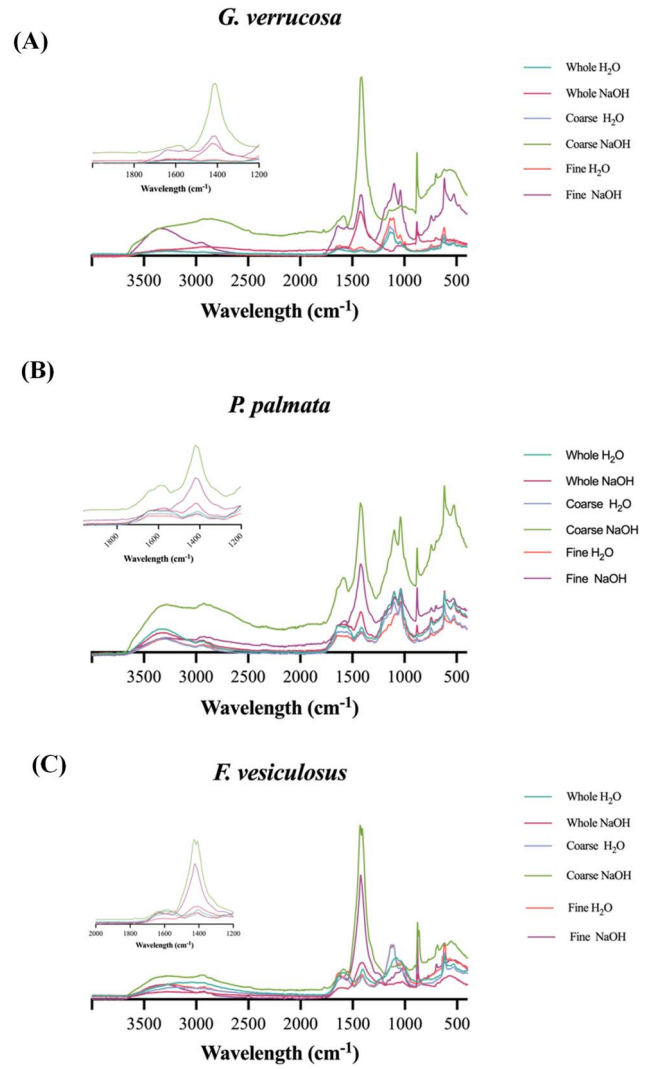

Fig. 7 FTIR-ATR spectra obtained for H_2O and NaOH extracts produced by the Osborne fractionation method: (A) *G. verrucosa*; (B) *P. palmata*; and (C) *F. vesiculosus*. In each spectrum, the amide I and amide II bands related to proteins and recorded between 2000 and 1220 cm^{-1} are clearly identifiable.

4 Conclusions

Macroalgae have been studied as suitable alternatives to traditional protein sources, with the aim of decreasing the negative environmental footprint that is closely associated with animal protein production. Moreover, supplying the right amount of protein and amino acids (which cannot be achieved by simply consuming vegetables) for human nutrition has also been a concern regarding the expected increase in the global population. In this way, seaweed has emerged as a suitable non-animal protein source to be used in the food industry. However, its use in the food industry is facing some issues, namely regarding protein extraction, purity, and digestibility.

Our work focused on the fractionation of different seaweed species (*i.e.*, red, green, and brown) and further evaluation of the obtained fractions regarding the protein profile as a function of their solubility, aiming at their application in the development of novel food products. For that, seaweed with different characteristics (namely regarding the cell wall composition) was used in a preliminary step of milling and size classification, where different fractions of each were produced. In terms of protein content, no significant differences between fractions and the initial biomass were observed. All fractions were then subjected to a further sequential fractionation in different solvents (*e.g.*, water, NaCl, EtOH, and NaOH) in order to determine the protein solubilization profile for each alga. Independent of the species, all of the studied seaweeds had proteins with higher affinity to water environments, and good protein extraction yields were obtained (with a maximum yield for *F. vesiculosus*, in which around 35% of the total algae protein was extracted). Regarding the protein molecular profile, similar patterns were found for each extraction solvent (either H_2O or NaOH) without influence of the granulometry and similar behaviors between species. FTIR-ATR spectrometry allowed us to determine which type of molecular bonds were present in each sample, helping to relate it to previous results (such as high carbohydrate content and the presence of protein molecules). Moreover, the low extraction yields may indicate that the existing proteins are present in the form of strongly bonded (glyco)proteins.

Understanding the influence of the extraction environment as well as the morphology of the seaweed flakes in protein

extraction and profile is an important step that allows scientists to move one step forward towards the final objective of finding different protein sources and helps to predict the behavior of these proteins when in contact with real food products, with the existence of either functional and bioactive properties that arise with the existence of peptides with a wide range of molecular sizes, confirming seaweed protein versatility. This information will support the development of future extraction strategies, including those intended for food-related applications. Also, the performed protein characterization and solubility studies may serve as an initial basis for the optimization of the extraction technology.

Conflicts of interest

There are no conflicts to declare.

Data availability

The authors declare that the data supporting the findings of this study are available within the article's main file. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Acknowledgements

This research work was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of the UIDB/04469/2020 unit (https://doi.org/10.54499/UID/04469/2025), LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems (LA/P/0029/2020, https://doi.org/10.54499/LA/P/0029/2020), and the PhD scholarships SFRH/BD/07527/2020 (Rafaela Nunes) and 2021.05734.BD (Catarina Moreira). This work was also supported by Fundação Calouste Gulbenkian, under the scope of project "MarUMinho – Multidisciplinary research program in Marine Science and Technology from the University of Minho".

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