

Tuning the extraction methodology targeting protein-enriched fractions from red algae

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ABSTRACT

Alternative protein sources are being studied to substitute animal protein production and decrease the environmental issues that are inherent to the production of animal-based foods.

This study investigates the potential of red macroalgae (*Gracilaria verrucosa*, *Porphyra dioica*, and *Palmaria palmata*) as an eco-friendly alternative protein source. Using the Osborne fractionation methodology, proteins were extracted under varying parameters (pH and grinding) and techniques (ultrasound and enzymatic extractions). Seaweed proteins exhibited a strong affinity for water and alkaline environments. Optimal extraction yields were obtained with H₂O for *G. verrucosa* (33.24 ± 1.81 g protein/100 g algae protein) and *P. palmata* (29.56 ± 1.73 g_{protein}/100 g_{algae} protein), while *P. dioica* showed highest yields with aqueous 0.1 M NaOH (26.28 ± 5.90 g_{protein}/100 g_{algae} protein). Molecular weight patterns revealed aggregates (> 200 kDa) in *G. verrucosa* and *P. palmata* in the 0.1 M NaOH fraction. H₂O extracts displayed peaks (48–84 kDa) linked to phycobiliproteins, showcasing good emulsifying properties. Solubility varied with solvents, with saline and 70 % EtOH dissolving lower molecular weight proteins. Aqueous 0.1 M NaOH extracts demonstrated promising texturizing capacity.

Overall, proteins from red macroalgae show potential for food product development, offering an alternative with lower environmental impact compared to conventional animal-based proteins.

1. Introduction

It is expected that the world population will reach around 9.7 billion in 2050, causing an increase in food demand by 70%, leading to an increase in global meat production (Bleakley and Hayes, 2017; Geada et al., 2021; Mæhre et al., 2014; O' Brien et al., 2022). However, meat production involves highly negative environmental issues which leads to the need to find new protein sources for food application. In this sense, algae biomass is a promising alternative non-animal protein source with several advantages when compared to terrestrial biomass: (i) high grow rate; (ii) no competition for arable land; (iii) low water consumption; and (iv) carbon-neutral emissions (Bleakley and Hayes, 2017; Geada et al., 2021). Also, since the oceans represent about 70 % of the earth surface, it is easy to find a wide range of seaweeds species with application in several branches of industry, namely for the extraction of hydrocolloids (alginate, carrageenan and agar) or pharmaceutical

compounds (Bleakley and Hayes, 2017; Gressler et al., 2010; Mæhre et al., 2016; Ścieszka and Klewicka, 2019). On the other hand, they can be applied directly into food and feed (e.g., salads and soups), having also interesting contents of antioxidants, vitamins, proteins, minerals, fibers, and essential fatty acids.

In general, macroalgae (or seaweed) are classified as red (Rhodophyta), brown (Phaeophyta), or green (Chlorophyta) according to their chemical composition and mainly due to the presence of specific pigments (phycobilins, fucoxanthin, and chlorophyll, respectively). Red seaweeds are characterized as having low-fat content (between 1 and 5 % of dry weight) and high content in ash (between 10 and 40 % dry weight) and carbohydrates (generally higher than 40 % dry weight) (Gamero-Vega et al., 2020; Mišurcová, 2011). Moreover, they also possess a significant amount of minerals (e.g., calcium, magnesium, sodium, and potassium salts) due to their capacity to absorb inorganic substances from the environment, which is directly related to the

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presence of cell wall polysaccharides. However, the content of each mineral will depend on the species, seasonal variation, and cell wall constitution. For example, *P. palmata* shows a high quantity of Zn and Mn (37.0 and 27.5 mg/kg, respectively) while *Porphyra tenera* have a high content of Fe and Mn (1833.0 and 260.0 mg/kg, respectively) (Mišurcová, 2011). Vitamins of red seaweeds are mostly included in the fat-soluble group, namely by the presence of carotenoids (and thus α - and β -carotene) which possess good antioxidant properties and reduce the risk of cardiovascular diseases (Mišurcová, 2011). *Gracilaria changi* is reported to have content β -carotene of 5.2 mg/100 g, which is compared with the 6.8 mg/100 g found in carrots (Mišurcová, 2011). The low lipidic fraction found in red seaweeds is mainly attributed to polyunsaturated ω -3 (eicosapentaenoic acid, EPA) and ω -6 (arachidonic acid, AA) fatty acids that are also intervenient in the reduction of several heart diseases. Regarding the protein content, red seaweed (e.g., *Porphyra dioica*, *Gracilaria verrucosa*, *Palmaria palmata*, and *Chondrus crispus*) possess high protein levels, typically ranging from 30 to 40 % of their dry weight. These values are similar to those of some protein-rich vegetables (e.g., soy), surpassing the protein content of green and brown algae species (Aasen et al., 2022; Cian et al., 2015; Steinbruch et al., 2023; Yanshin et al., 2021). The *Porphyra* genus is reported as the algae which contain higher protein content, reaching an average of 29.3 g/100 g while *Gracilaria* and *Palmaria* genera are indicated to have contents lower than 25 g/100 g (Cian et al., 2014; Cofrades et al., 2010; Gamero-Vega et al., 2020). The main proteins that are present in red algae are phycobiliproteins, mainly phycoerythrin, phycocyanin, allophycocyanin, and phycoerythrocyanin, representing up to 50 % of the total protein content. They possess different molecular sizes and tend to aggregate, forming a basic unit that can have different arrangements and higher molecular weights (Cian et al., 2015).

As happens for terrestrial biomass, the digestibility and/or bioavailability of seaweed nutrients may be limited (Vasconcelos et al., 2023). Therefore, pre-treatment and/or fractionation of the seaweed biomass may be needed to allow their efficient use in nutritional applications as a protein source both in feed and food.

Furthermore, these proteins, either in isolated or concentrated form, are gaining much attention because they can have specific technological and biological functions. Besides the possibility of being used as a natural coloring agent, due to the red color of their main phycobiliprotein (e.g., phycoerythrin), they may also be used as ingredients in many food processes due to their improved solubility, gelling, emulsifying, and foaming properties (Jayasinghe et al., 2016). On the other hand, these alternative proteins must maintain or improve the quality and acceptability of food products. These food additives/protein sources should have satisfactory intrinsic properties, such as acceptable flavor, color, and texture, which are directly related to their composition, structure, conformation, interaction with other food components, nature of the surrounding environment, isolation methods, among others (Geada et al., 2021; Kinsella, 1976; Lafarga et al., 2020; Mæhre et al., 2014). Therefore, to make using seaweed as a protein source feasible, it is important to establish efficient protein recovery strategies.

Moreover, concerning protein content, amino acid (AA) composition seems to be important regarding the nutritional value of the protein for the body, as well as the ability to be digested, absorbed, and retained. Animal protein sources are generally significant in essential AAs (EAA), whilst vegetable proteins, are frequently regarded as incomplete protein sources due to a lack of some EAA. This problem is partially solved because algae have better AA composition nutritionally than terrestrial plants. However, protein content and AA balance in marine macroalgae can vary seasonally. As a result, harvesting should take place when the protein content is higher. Additionally, it's important to note that the EAA profile in seaweeds is better compared to that of terrestrial plant sources often used in vegetarian diets, such as soybeans and chickpeas, and roughly equivalent to that of animal sources, such as turkey and beef. In general, algae showed the same potential to reach WHO's EAA requirements for healthy individuals (Geada et al., 2021; Koyande et al.,

2019). In the case of red seaweeds, Thr, Leu, Phe, and Lys are the main AA present and are found in higher percentages when compared to other matrices (e.g., soy and turkey) and possibly equivalent to beef (Geada et al., 2021).

The extraction of proteins from macroalgae is hampered by the physical barrier formed by the cell wall, which impedes the release of proteins (O' Connor et al., 2020; Ummat et al., 2021). This is complicated by the fact that some proteins are bound to other molecules through ionic interactions. These are mainly attractive interactions, between proteins and polysaccharides, that interfere with the release of proteins from the polysaccharide-rich biomass (Cermeño et al., 2020; Geada et al., 2021; Show et al., 2015). Further, the high viscosity and ionic interactions arising from the presence of cell wall and intracellular polysaccharides also difficult the extraction process. Thus, cell disruption and the inclusion of specific reagents during extraction have been employed to improve macroalgal protein extraction efficiency. Conventional protein extraction methods are based on physical processes by mechanical disintegration (e.g., bead milling, ultrasounds, high-pressure homogenization, and microwaves) and non-mechanical extraction (e.g., osmotic shock, enzymatic hydrolysis, chemical extraction, and dry fractionation). The physical approach, besides preventing chemical contamination and preserving a good percentage of the internal cell components (while allowing protein yields between 15 and 80 %), represents a lack in the scalability of the process due to high energy inputs (e.g., ultrasounds, high-pressure homogenization) and the possibility of cell damage by the liberation of free radicals that represent the need of adding more substances to the medium (Cermeño et al., 2020; Geada et al., 2021; Show et al., 2015). On the other hand, regarding the non-physical methodologies, chemical extraction procedures (either by the addition of acids, bases, or surfactants) are established as effective methods for hydrophobic protein solubilization and degradation of chemical bonds (Cermeño et al., 2020; Geada et al., 2021). However, the traditional extraction processes that rely on the use of strong solvents (e.g. concentrated alkali solutions), cause major changes in protein conformation during the whole process. Thus, the application of novel technologies (e.g., ohmic heating) or a combination of different approaches for protein extraction must be done, sometimes needing to add an extra step of pre-treatment (e.g., ultrasounds or grinding) to improve protein release during the extraction (O' Connor et al., 2020; Ummat et al., 2021). The chosen of the most indicated method should be done having in consideration: (i) the nature and constitution of each cell wall; (ii) the intended objective, having in account if it implies the existence of native biomolecules or if some degradability is possible; (iii) economic and environmental impact associated with the methodology; (iv) scalability of the process (Geada et al., 2021; O' Connor et al., 2020; Rawiwan et al., 2022).

Thus, in order to help design adequate recovery procedures and predict how algae proteins will react in food systems, it is important to assess which kinds of proteins are present in different species and their properties (Kinsella, 1976; Steinbruch et al., 2023). The Osborne fractionation method has been applied to different vegetable matrices (e.g., rice and wheat) and relies on the use of different solutions (i.e., dH₂O, NaCl, EtOH, and NaOH) to extract different protein fractions (albumin, globulin, prolamin and glutelin, respectively) (Chanput et al., 2009; Rodríguez-Restrepo et al., 2020; Teixeira et al., 2014). Though the method is specific for protein fractions from terrestrial biomass, and it has never been applied to algae, it could be used in algae biomass to fractionate the proteins according to their solubility in different solvents (and thus polarity).

In this context, the overall aim of this work was to examine and compare different protein extraction methods applied to three different red seaweeds in order to obtain fractions with diverse protein profiles. For this, different solvents (chosen from the Osborne method), pH, and several cell rupture methods (i.e., grinding, US, and enzymes) were used to assess the suitability for recovering seaweed proteins, and the product of the best methodology was studied in terms of functional properties.

2. Materials and methods

2.1. Materials

Different red macroalgae species (*Gracilaria verrucosa*, *Porphyra dioica*, and *Palmaria palmata*) were provided by AlgaPlus company (Ílhavo, Portugal). The collected seaweeds were washed with water to remove epiphytes, sand, and debris, dried at 60 °C, and then milled into flakes (1.5–10 mm). All the chemicals were of analytical grade and purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA).

2.2. Algae characterization

2.2.1. Total protein content

Protein content of different algae species 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 4.59 (Lourenço et al., 2002; Mišurcová, 2011; Mlambo et al., 2022). All determinations were run at least twice, and results were reported as percentage of protein from dry biomass.

2.2.2. Lipids

Lipid content was 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 firstly subjected to ultrasound (10 min) to promote cell wall disruption and then incubated on a heating block at 30 °C for 30 min. Thus, the mixture was centrifuged at 2000 rpm for 10 min, and the organic phase was collected in a pre-weighed glass tube. This procedure was repeated until the solvent had no pigmentation. For the removal of contaminants, the initial extract was re-dissolved in 2 mL chloroform and 1 mL of methanol. 750 µL of water was added to promote phase separation and the organic phase was collected. The combined organic phases were dried under an N₂ stream and weighed. Each sample was analyzed in triplicate and the results were presented in percentage of total lipids in a dry weight basis.

2.2.3. Carbohydrates

Carbohydrates content in algal biomass was assessed by the NREL official protocol (NREL/TP-5100-60957) (Van Wychen and Laurens, 2013). Briefly, 3 mg of each alga was mixed with 3 mL of 72 % H₂SO₄, and placed into a water bath at 30 ± 3 °C for 60 min. Then, each sample was diluted to a 4 % H₂SO₄ final concentration and autoclaved for 1 h at 121 °C. Finally, the hydrolysates were filtered through 0.22 µm membranes and 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 H₂SO₄ at a flow rate of 0.6 mL/min. 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 2 g/L. All experiments were run in triplicate and the results are presented as percentages of total carbohydrates in a dry weight basis.

2.2.4. Ash and moisture

The moisture and ash content in algal biomass were determined according to NREL procedures (NREL/TP-510-42618) (Sluiter et al., 2008). Briefly, 1 g of 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 14–16 h and weighed after cooling down to room temperature to determine ash content. All determinations were run in triplicate and the results are presented as a percentage of total ash in a dry weight basis.

2.3. Protein characterization

2.3.1. Fractionation by the Osborne method

The determination of protein profile from studied macroalgae species was carried out by fractionating the macroalgae proteins with the Osborne methods, as described by Chanput et al. (2009). For this, a sequential extraction with dH₂O, aqueous 0.5 M NaCl solution, EtOH, and 0.1 M NaOH was applied to obtain different protein fractions. Thus, 4 g of dried macroalgae were mixed with 40 mL of dH₂O for 1 h to obtain the first fraction. The residue from the first step was mixed with 40 mL of NaCl 0.5 M for 60 min to obtain the second protein fraction. Then, the residue was treated for 60 min with 40 mL of ethanol 70 % (v/v) and finally with NaOH 0.1 M (60 min). All the extractions were carried out with a shaking of 200 rpm. To improve the protein recovery, each fractionating step was repeated twice, and the supernatants were recovered by centrifugation at 4000×g for 30 min and combined. Protein extracts were kept at -20 °C until further use.

2.3.2. Quantification using Lowry's method

Protein content was determined by the Lowry method optimized for microplates and using a calibration curve with BSA as standard (0 – 2500 µg/mL, R² = 0.99) (Lowry et al., 1951). For this, 20 µL of samples or BSA were added to a microplate followed by the addition of 200 µL of Lowry's solution and 20 µL of Folin-Ciocalteu reagent. Extraction solvents were used as a control. The microplate was then incubated for 4 h at 42 °C with agitation (180 rpm). The absorbance was measured at 750 nm after 10 s of shaking. Results were expressed in mg per g of seaweed dry weight (mg/g_{seaweed}) and mg per g of protein fraction (mg/g_{protein}).

2.3.3. Gel permeation chromatography

The molecular weight distribution of the proteins present in each fraction was evaluated by HPLC gel permeation chromatography (GPC) on a PolySep-GFC-P-4000 column (300×7.8 mm, Phenomenex®, USA). Lyophilized extracts were redissolved in deionized water at 1 mg mL⁻¹ and eluted in 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. The x-axis represents the retention time (min) and the y-axis represents the intensity of UV signals (mV) as an indicator of the relative concentration of each protein (Gómez-Ordóñez et al., 2012).

2.4. Tuning the extraction procedure

2.4.1. pH variation

The studied macroalgae were subjected to pH variations in order to verify the possibility of improving protein solubilization. For that, using the solid-liquid ratio of 1:10 (same as Section 2.3.1), the procedure was done at pH 7, 8, and 10 as follows: algae were mixed with dH₂O, and the pH was adjusted to 7 and kept overnight with constant slow stirring (200 rpm). Thus, the pH was readjusted to 7, 8, and 10 followed by a step of grinding with a blender and the solution was kept with stirring for 30 min. Then, all solutions were centrifuged at 4000×g for 30 min and the supernatants were recovered and kept at 4 °C until further use.

2.4.2. Grinding

The influence of an initial step of grinding in protein solubilization was investigated in all three algae, for the best pH condition achieved in the previous section. Thus, algae were mixed with dH₂O (1:10 (w/v)), grounded and pH was adjusted to 7 with NaOH 4 M. The solutions were kept at 200 rpm for 1 h. Then, the solutions were centrifuged at 4000×g for 30 min and the supernatant was recovered. The resulting residues were used for a new extraction with 0.1 M NaOH for 1 h at 200 rpm and the supernatants were also recovered by centrifugation.

2.4.3. Enzymatic extraction

An enzymatic-assisted extraction using Viscozyme® L (composed of

a wide range of carbohydrases, including arabanase, cellulase, β -glucanase, hemicellulase, and xylanase) (Sigma-Aldrich Chemical Co. Ltd., St. Louis, MO, USA) was evaluated to try to improve protein recovery, by partially degrading the cell wall and making protein more accessible. For this, 5 g of algae was mixed with 50 mL of enzyme in water (50 FPU) at pH 4.5 and 50 °C according to the manufacturer's specifications. Protein solubilization was determined using Lowry's method after 24 h and 48 h of incubation. The procedures were performed in triplicate.

2.4.4. Ultrasound

For the ultrasound-assisted extraction, 50 mL of dH₂O was added to 5 g of algae, and the mixture was then placed in an ultrasound water bath (200 W, 45 kHz, VRW®, Pennsylvania, USA) for 15 min. Then, the sequential dH₂O/0.1 M NaOH extraction was performed for 1 h followed by 30 min of centrifugation at 4000×g. The supernatant was recovered and kept at -20 °C until further use. All assays were done in duplicate.

2.4.5. Rheological characterization: gelation profile and dynamic viscosity

A preliminary assessment of the technological potential as a texturizing agent of the extracts from *Porphyra dioica* was made through rheological tests. The gelation ability of H₂O extracts and apparent dynamic viscosity of 0.1 M NaOH extracts of *Porphyra dioica* (as an indicator of the thickening potential as they did not gel at the tested conditions) were determined using a controlled stress rheometer HR-1 (TA instruments, New Castle, USA) fitted with a plate (40 mm diameter, gap 1000 μ m) and a cone geometry (2° angle, 60 mm diameter), respectively. Regarding gelation measurements, a solution with 10 % of the H₂O extract was stabilized at 25 °C for 10 s and then the temperature was elevated to 85 °C with a ramp rate of 2 °C/min, a strain of 10 % and a frequency of 1.0 Hz. After 60 s at the final temperature, a temperature-decreasing ramp at the same conditions started until the sample reached 25 °C. To avoid evaporation, the plate was covered with silicone oil during the experiments. The contributions of elastic and viscous properties were analyzed based on storage (G') and loss (G'') moduli, respectively.

For the apparent viscosity, the experiment was performed at different extract concentrations (3, 5, and 10 %). First, a sample conditioning step was introduced for 60 s and then the first flow-ramp was done for 120 s with a shear rate ranging from 0.1 to 100/s⁻¹. After another sample conditioning step for 30 s, the second flow ramp was done for 120 s with a shear rate of 100 – 0.1 s⁻¹. All the experiments run at 25 °C.

2.4.6. Statistical analysis

Results were presented as mean $\pm$ standard deviation (SD) of at least two independent experiments. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA). Differences between samples were tested using a T-Test and the results were considered statistically different for values of $p < 0.05$.

3. Results and discussion

3.1. Characterization of different macroalgae species

In general, the composition of algae is highly variable due to differences in sampling, geographical location, and growth conditions, namely related to temperature, time, and salinity (Gokulakrishnan et al., 2015; Gomes-Dias et al., 2020). Thus, when dealing with recovering fractions from seaweed biomass, it is important to assess the composition of the batch in use.

In this work, three species of red macroalgae were analyzed in terms of composition and the results are presented in Table 1. As we can see, all algae have a moisture content of less than 12 %. This parameter is important to ensure good storage conditions, specifically to prevent microbiological degradation. All studied red macroalgae have a high carbohydrate content, i.e., *P. palmata* (58 %_{DW}), *P. dioica* (47 %_{DW}) and

Table 1

Characterization of different red macroalgae species.

Seaweed	Moisture (%)	Ash (%)	Protein (%)	Carbohydrates (%)	Lipids (%)
<i>Gracilaria verrucosa</i>	10.05 $\pm$ 0.06	28.72 $\pm$ 1.15	16.28 $\pm$ 0.05	45.69 $\pm$ 0.65	4.25 $\pm$ 0.79
<i>Porphyra dioica</i>	11.64 $\pm$ 0.24	21.57 $\pm$ 0.23	27.68 $\pm$ 0.05	46.76 $\pm$ 0.20	5.77 $\pm$ 0.43
<i>Palmaria palmata</i>	9.77 $\pm$ 0.19	26.66 $\pm$ 0.93	11.83 $\pm$ 0.18	58.19 $\pm$ 2.48	< 1

G. verrucosa (46 %_{DW}). In terms of protein content, the algae *P. dioica* presents a higher concentration (29 %_{DW}) compared to the other two algae used in this work (17 %_{DW} for *G. verrucosa* and 13 %_{DW} for *P. palmata*, respectively). The inorganic matter (ash content) of the three red algae ranges from 22 %_{DW} to 29 %_{DW}. These results are in accordance with reported by Echave et al. (2021) for *P. palmata* and *Porphyra purpurea* (16–24 %). Moreover, the authors report that the main minerals present in these red algae are Ca, K, P, Cl, and Mg. The lipid contents were relatively low in all the analyzed species, which was expected since seaweeds are known for their low fat content (less than 5 %_{DW}) (Rodríguez et al., 2015). These results are in line with previous works using macroalgae of the genus *Rhodophyta*. For example, in a study conducted by Mcdermid and Stuercke (2003) significant differences between main principal components of each studied red macroalgae were observed, i.e. 2.7–21.2 % protein, 22.7–52.9 % ash, 1.3–4.4 % lipids and 9.2–33.4 % of soluble carbohydrates. Recently, Álvarez-Viñas and coworkers summarized information about economically relevant red seaweed species, and their work reported values that corroborate our research data for the chemical composition of used species (Álvarez-Viñas et al., 2019). As reported before, some differences that can eventually appear may be partly explained by geographical and seasonal variation, as well as by methodological differences, such as the conversion factor used for the determination of total nitrogen content.

Considering the results of the algae composition, the protein content appears to be similar to that obtained for different legumes (e.g., 19–22 % for peas or beans) or meat (18–25 %) which makes raw materials interesting from an economic point of view, for obtaining products of high commercial value (Bleakley and Hayes, 2017; Rodríguez et al., 2015). In terms of nutrition, our results indicate that these biomasses are suitable to be incorporated in protein-balanced diets, becoming a promising alternative to current protein sources such as meat, legumes, and cereals. On the other hand, it can be beneficial for vegetarian and vegan diets.

3.2. Protein fractionation using the Osborne method

Currently, some seaweed species like *P. palmata* and *Porphyra tenera* are already highly consumed in Asia and Western countries (e.g., for sushi production), due to their high protein content and delectable flavor (da Costa et al., 2018; Felix et al., 2021). Most seaweed proteins present poor digestibility in their raw and unprocessed form, which leads to the need to develop improved methods for algal protein extraction and isolation/concentration (Bleakley and Hayes, 2017; Sardari et al., 2019). On the other hand, although there are already some established methods for protein extraction, most of the experimental studies focus on obtaining a single fraction. Moreover, different proteins will have different properties and qualities when applied to food products. Most protein properties are dependent on environmental and molecular factors, which makes its characterization essential before food formulations (Rodríguez-Restrepo et al., 2020; Van Lill et al., 1993). In this sense, the use of Osborne fractionation allows understanding of the type of proteins present in each macroalgae species. Using this

methodology, proteins are sequentially recovered using different extraction solvents with different chemical properties which leads to the solubilization of different types of proteins based on their solubility. Results on the extraction yields achieved with this fractionation are presented in Table 2. For all the analyzed species, the main overall extraction occurred using dH₂O as extraction solvent (36.32 ± 2.11 , 40.50 ± 6.72 , and 48.77 ± 11.23 %_{dw} for *P. dioica*, *G. verrucosa* and *P. palmata*, respectively). Using an aqueous solution of NaCl 0.5 M as solvent allowed further solubilization of protein from a significant part of the algae protein, while EtOH 70 % was the solvent that showed to be least effective for the extraction of red seaweeds in general. Finally, using 0.1 M NaOH as extraction solvent allowed more solubilization being the second-best performing solvent (18.40 ± 1.69 , 11.75 ± 2.15 , 17.43 ± 3.01 %_{dw} for *P. dioica*, *G. verrucosa* and *P. palmata*, respectively). Besides being all part of the group of red seaweeds, different species present specific components that can explain the differences in the results. It is well known that alga belonging to the *Rhodophyta* phylum are mainly composed of proteins and carbohydrates. In terms of proteins, water-soluble phycobiliproteins (i.e., phycoerythrin, phycocyanin, and allophycocyanin) are the major components. However, red seaweeds have a unique polysaccharide composition that confers a higher or lower cell wall resistance. For all species, the most important polysaccharide in the internal cell wall is cellulose which is found linked to water-soluble polysaccharides (galactans) such as carrageenans present in a lot of species (e.g., *Gigartina*, *Chondrus crispus*, *Eucheuma*, *Hypnea* and others), agarans (e.g. *Gelidium*, *Gracilaria*) and/or other galactans such as porphyrans (*Pyropia* and *Porphyra*), agarans being cell wall constituents only soluble in hot water (Cian et al., 2015). Moreover, crosslinking between proteins and polysaccharides is common and both are being extracted to the aqueous phase. During the extraction of these molecules from algae matrices, some secondary metabolites (e.g., phenolic compounds and carotenoids) are also extracted (for example, using EtOH as solvent) and responsible for the existence of interesting biological activities that advent from their high antioxidant activity (through exerting a scavenging action) that is related with other bio-activities (e.g., antihypertensive, antidiabetic, antiviral and hypocholesterolemic) (Jimenez-Lopez et al., 2021; Rosa et al., 2020; Safafar et al., 2015).

3.3. Protein quantification using Lowry’s method

As previously mentioned, the highest extraction yields for all seaweeds were obtained using dH₂O and 0.1 M NaOH as solvents, which indicated that most of the proteins present good solubility in water and alkaline environments and not in saline and EtOH solutions. The protein content of each extract was determined using Lowry’s method and the obtained results are listed in Table 3. Taking these values into account, together with the total protein content of the biomass (quantified by the Kjeldahl method), the protein extraction yield (expressed in grams of recovered protein per gram of total algae protein) was calculated, and the results are presented in Fig. 1. The protein quantification using this method can be influenced by some factors, namely (i) matrix nature and properties, (ii) the chosen extraction method, and (iii) the extract’s color (Mæhre et al., 2018). In a previous work, Mæhre et al. (2018) studied

Table 2
Overall extraction yields (g_{solids}/100 g_{algae}) were obtained for red macroalgae species after the Osborne fractionating method.

Algae	H ₂ O fraction	0.5 M NaCl fraction	70 % EtOH fraction	0.1 M NaOH fraction
<i>Gracilaria verrucosa</i>	40.50 ± 6.72	16.80 ± 4.90	8.80 ± 3.36	11.75 ± 2.15
<i>Porphyra dioica</i>	36.32 ± 2.11	19.02 ± 1.69	9.08 ± 2.91	18.40 ± 1.69
<i>Palmaria palmata</i>	48.77 ± 11.23	18.29 ± 3.18	10.57 ± 1.95	17.43 ± 3.01

Table 3
Content of protein (g_{protein}/g_{extract}) in the crude extract obtained for each fraction.

Algae	Soluble fraction	Protein (g _{protein} /g _{extract})
<i>Gracilaria verrucosa</i>	dH ₂ O	0.83 ± 0.03
	0.5 M NaCl	0.37 ± 0.14
	70 % EtOH	< 0.15
	0.1 M NaOH	0.19 ± 0.02
<i>Porphyra dioica</i>	dH ₂ O	0.17 ± 0.04
	0.5 M NaCl	0.16 ± 0.01
	70 % EtOH	0.18 ± 0.06
	0.1 M NaOH	0.43 ± 0.13
<i>Palmaria palmata</i>	dH ₂ O	0.08 ± 0.03
	0.5 M NaCl	0.06 ± 0.02
	70 % EtOH	0.07 ± 0.01
	0.1 M NaOH	0.17 ± 0.02

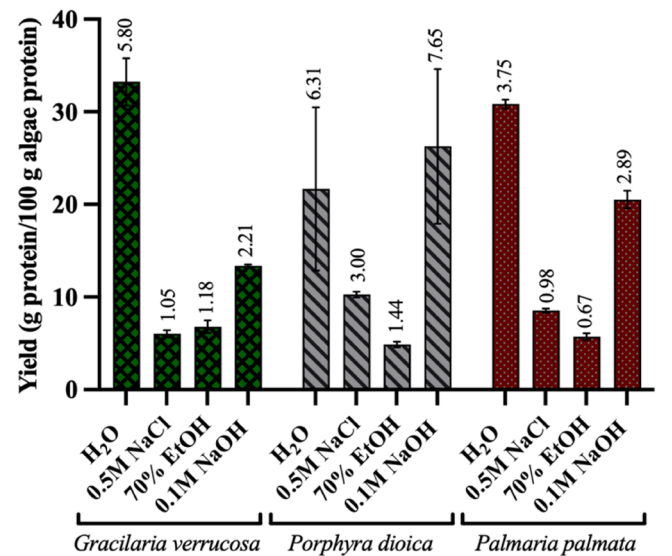


Fig. 1. Protein yields in terms of g_{protein}/100g_{total algae protein} for each obtained fraction. The number on top of the bars represents protein yields in terms of g_{protein}/100g_{dried algae}.

how the determination of protein content in several foods (i.e., cod, salmon, shrimp, dulse, and whole/white flour), with different nutritional compositions, was affected as a result of the choice of the extraction process and the analytical quantification method used (Kjeldahl, Bradford and Lowry’s methods). Authors reported, that for the same extraction procedure (i.e., salt/alkaline protein extraction), red seaweed extracted fraction presented 152.1 ± 10.0 , 48.4 ± 3.9 , and 89.5 ± 15.9 g protein/kg raw material for Kjeldahl, Bradford and Lowry, respectively. These results clearly demonstrated the variation between different protocols used for quantification and also can be related to the conversion factor of Nitrogen to protein used (the generic 6.25, though the recommended values for algae are generally lower range, e.g., 4.59 for red seaweed (Mišurcová, 2011; Mlambo et al., 2022; Trigueros et al., 2021)). Nevertheless, though the overall protein content should be regarded with caution, the methods are useful for comparison purposes, e.g., between different extraction conditions or different seaweeds.

Regarding the red seaweeds used in this work, the highest content of protein was extracted in the dH₂O soluble fraction of *G. verrucosa* and *P. palmata* (33.24 ± 1.81 and 29.56 ± 1.73 g_{protein}/100 g_{algae protein}) (Fig. 1). On the other hand, using 0.1 M NaOH aqueous solution as extraction solvent, yields were better for *P. dioica* (26.28 ± 5.90 g_{protein}/100g_{algae protein}) when compared to the ones obtained from *G. verrucosa* and *P. palmata* (12.65 ± 1.05 and 22.75 ± 3.20 g_{protein}/100g_{algae protein}, respectively). These results are in accordance with the previous ones

(see Section 3.2) and it is confirmed that saline solutions (NaCl) and EtOH do not extract high amounts of protein when compared to the other solvents and should not be used as extraction solvents. Also, around 83 % of the dH₂O extract of *G. verrucosa* (due to the low agar solubility in cold water) and 43 % of the 0.1 M NaOH extract of *P. dioica* correspond to the protein fraction, which indicates good protein extraction and protein selectivity using these solvents (Table 3). Further, in the case of *Gracilaria* (an agarophyte), the H₂O fraction presents high percentage of protein and can be considered a protein concentrate and used in several food technologies without the need for further treatments. This probably occurs because the main soluble polysaccharide (agar) of *Gracilaria*, which represents more than 45 % of the total alga content, is only soluble using high temperatures and was not co-extracted with these methodologies (Martínez-Sanz et al., 2019). Other researchers have already studied the extraction of protein from red seaweeds based on water and alkaline extractions. Stack et al. (2017) investigated the seasonal variation of nitrogenous components of protein hydrolysates (obtained after water and alkaline extraction procedures) from *P. dioica* and obtained higher extraction yields in winter months, reaching a maximum of 47.98 ± 0.8 mg/g DW in December. From this overall yield, 15.04 ± 0.30 mg/g DW corresponded to water extraction and 32.94 ± 1.11 mg/g DW to alkaline extraction, which is in accordance with our results that indicate higher yields in the aqueous 0.1 M NaOH extraction compared to water extraction, using the same red seaweed. Moreover, for *P. palmata*, using the same solvents for protein extraction, different factors that could affect protein content were studied. Within these, an extraction time of 1 h or (which was similar to the extraction time used in this work) led to a protein recovery of around 7 g/100 g of dry weight which was also obtained when the extraction occurred at 20 °C (similar to room temperature used in this work), both representing the sum of water and alkaline extractions (Harnedy and FitzGerald, 2013). In both of the presented works, the obtained protein yields were higher than the ones obtained in this work.

The application of the Osborne method for the fractionation of proteins from algae biomass is still not described in the literature but was widely studied for other matrices like brans (e.g., rice), seeds, and legumes (e.g., lentil, chickpea, and pea). Rodríguez-Restrepo et al. (2020) studied the valorization of rice by-products and used the Osborne method for the protein characterization. In that study, the main proteins present in rice were solubilized in 0.1 M NaOH (8.23 ± 0.11 g/100 g of rice) and dH₂O (0.92 ± 0.03 g/100 g of rice). Also, an older study conducted by Sathe et al. (2012) used seed flour to assess protein solubility in different mediums (dH₂O, 2 M NaCl, 0.1 M NaOH, 70 % (v/v) EtOH) and verified that for this matrix, aqueous 2 M NaCl solution and 0.2 M NaOH did not lead to the solubilization of higher quantity of proteins (50–60 g/100 g). On the other hand, with dH₂O only around 30 g/100 g of proteins were extracted and with EtOH around 10 g/100 g. Regarding legume proteins, it was reported that they can be fractionated based on their solubility in H₂O (10–20 %), NaCl (70 %), and EtOH (Lam et al., 2018; Neji et al., 2022). In these vegetable matrices, the H₂O, NaCl, EtOH, and NaOH fractions correspond to albumin, globulin, prolamins, and glutelin proteins that possess different properties (e.g., antioxidant activity and anti-hypertensive activity). However, since these proteins are not reported to be part of algae matrices, the analogy is not done in this case.

Thus, since a significant amount of algal proteins can be solubilized in H₂O or low-concentration NaOH, it is evident that the red macroalgae used in this study are reliable substitutes for traditional protein matrices and good protein yields (higher or equal to vegetable ones) are obtained, allowing further incorporation in the development of new food products or food supplements.

3.4. Gel permeation chromatography

The molecular weight of all solubilized protein fractions was determined using gel permeation chromatography and the results are shown

in Fig. 2. The results confirm the higher protein content when H₂O and 0.1 M NaOH are used as solvents. For all macroalgae tested, the saline and EtOH fractions possess proteins with higher size variability but low molecular weight (<30 kDa). On the other hand, using H₂O and 0.1 M NaOH as solvent, higher molecular weights were present, around 48–84 kDa and 44–345 kDa for the H₂O and 0.1 M NaOH fractions. Though NaOH eventually could also solubilize the proteins that are soluble in H₂O, those were already removed in the first fractionation step. Alkaline conditions (in sufficient high pH) are expected to saponify the lipid fraction of the membranes causing its dissolution and release of membrane proteins. Furthermore, alkaline conditions may also cause some hydrolytic effects on the biomass, being expected that these conditions may also liberate more proteins that were retained in the seaweed structure and higher molecular weight insoluble proteins, both by making them accessible to the solvent and by slightly hydrolyzing them, allowing their solubilization. Moreover, in all three species, some undefined bands were observed around 4.5–7.5 min of retention time, which can be attributed to the presence of aggregates (1000–300 kDa) of (residual) polysaccharides from the cell wall bound to protein, also in accordance with partial disruption of the cell wall caused by the alkali. The area of these bands (4.5–7.5 min) is low for *Gracilaria*, slightly greater for *Porphyra*, and markedly greater for *Palmaria*. This can be ascribed to the nature of the main galactan present in each seaweed. Agarans from *Gracilaria* are only solubilized at high temperatures and were not extracted at high levels due to the low temperature applied in the extraction process, while Porphyrans for *Porphyra* may dissolve at more moderate temperatures. Thus, these features suggested the presence of polysaccharides aggregates with proteins. The presence of polysaccharides' aggregates was confirmed when the same sample was run with NaNO₃, which impairs aggregation, instead of water, as these peaks disappeared (results not shown).

Some published articles demonstrated the presence of low molecular weight proteins in macroalgae. Harnedy and FitzGerald (2013) studied the extraction of protein from *P. palmata* and found the presence of proteins with molecular weights between 14.7 and 55 kDa for alkaline extraction and 15.5 to 97 kDa for aqueous extraction, which are molecular weights that were also found in this work. Also, phycobiliproteins can be detected in the chromatograms. Phycocyanin has molecular weights typically ranging from 70 to 110 kDa and it is composed of α and β with sizes of around 24 and 15 kDa, respectively. On the other hand, phycoerythrin has around 240 kDa and is composed of α , β and γ subunits with molecular weights of 20, 21, and 31 kDa, respectively (Harnedy and FitzGerald, 2013; Patel et al., 2005). Thus, and regarding the high-water solubility of these proteins, it is possible to see that phycobiliproteins are present in all water chromatograms (75, 84, and 76 kDa for *G. verrucosa*, *P. dioica*, and *P. palmata*, respectively). On the other hand, since no peaks of around 240 kDa were found in the water chromatograms it is possible to see the presence of small peaks that may correspond to phycoerythrin's subunits extracted with aqueous NaCl solution or EtOH (Harnedy and FitzGerald, 2013). Results of gel permeation chromatography indicated that molecular weight ranges of red macroalgae proteins were wide.

Overall, based on the results from Sections 3.2 to 3.4, it is possible to conclude that protein recovery is highest when using aqueous or alkaline environments, with higher molecular weight proteins (with higher technological potential). Therefore, these conditions were chosen to proceed with the work. The use of a saline solution and EtOH as solvents was discontinued due to the low extraction yields. However, since small proteins are typically associated with good biochemical properties (e.g., antioxidant and antihypertensive properties), depending on the intended objective, these extracts can be used (e.g., for nutraceutical applications).

3.5. Tuning the extraction methodology

After characterizing the seaweed's protein profile, it is possible and

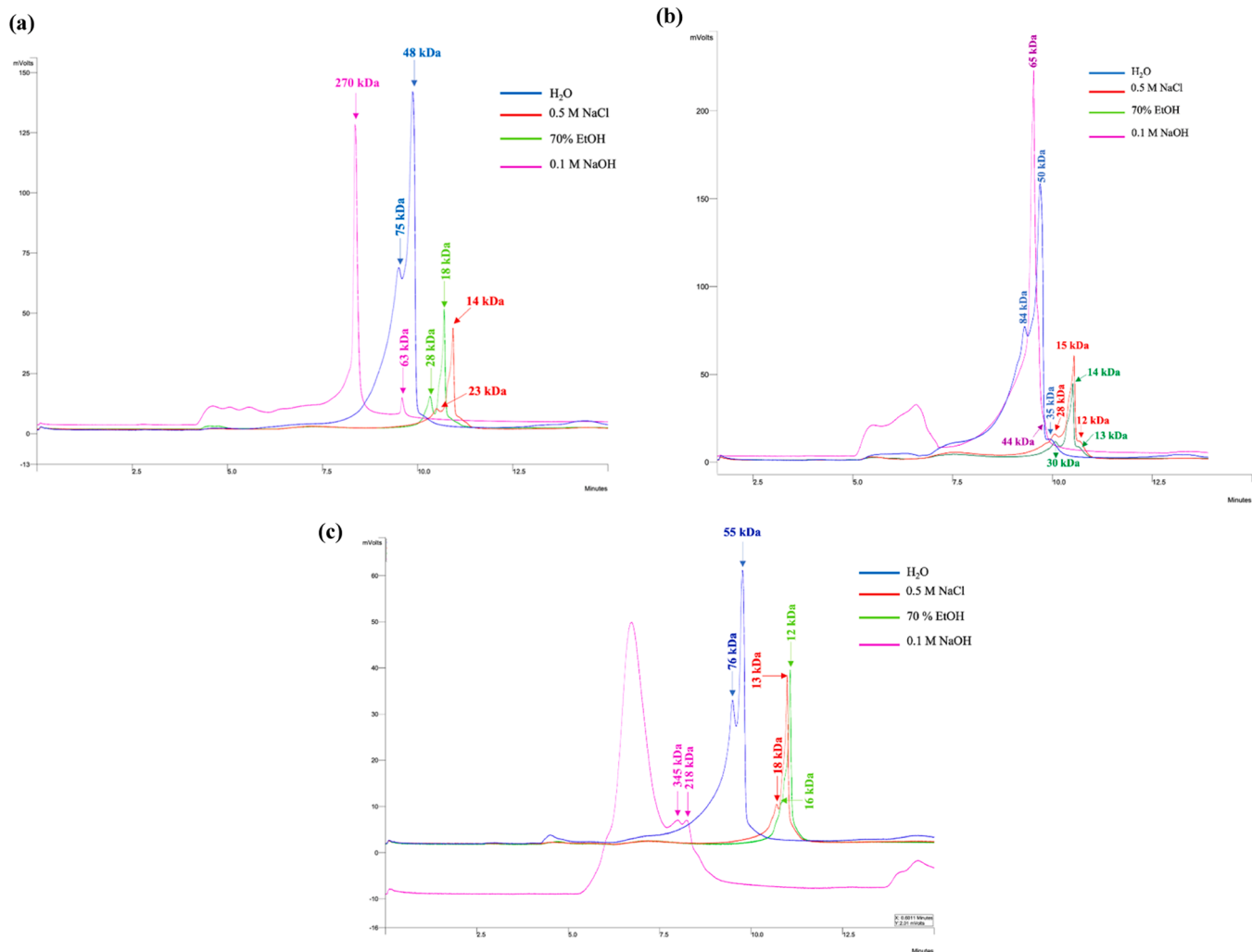


Fig. 2. Molecular weight distributions of red macroalgae protein fractions: (a) *Gracilaria verrucosa*; (b) *Porphyra dioica*; (c) *Palmaria palmata*.

important to tune the extraction procedure to obtain higher recovery efficiency and purer protein-rich fractions. Using the best extraction solvents (dH₂O and 0.1 M NaOH), other variables were studied to try to further improve protein solubilization and establish an appropriate methodology to be applied to this algae biomass. Thus, the effect of solution pH (Table 4), and the performance of a grinding pre-step on sequential extraction using water at pH 7 followed by 0.1 M NaOH were studied (Table 5). Also, enzymatic and ultrasound-assisted extractions were evaluated and the results are summarized in Fig. 3.

For all seaweeds, the protein solubilization at pH 7, 8, and 10 did not show to be significantly different, with protein yields of around 0.40, 0.20, and 0.15 g_{protein}/g_{algae protein} for *G. verrucosa*, *P. dioica*, and *P. palmata*, respectively. Therefore, a pH shift up to 10 did not show a significant effect on the protein solubilization, and using dH₂O and 0.1 M NaOH, sequentially obtaining two different protein extracts (that can be used alone, or mixed at the end, depending on their properties and intended application) was still the best option.

Moreover, the existence of a pre-step of grinding before the protein's sequential extraction did not demonstrate to improve protein solubilization in any case, with a maximum extraction yield obtained for *P. dioica* (27.02 ± 3.82 and 38.73 ± 3.42 g_{protein}/100 g algae protein using dH₂O and 0.1 M NaOH, respectively). Since phycobiliproteins are present in the thylakoid membranes of chloroplasts and are highly soluble in water, their superficial localization in chloroplasts leads to high interaction with the solvent (water) which may explain the similar results obtained with and without the grinding process.

Regarding the assisted extraction, the use of Viscozyme® L did not improve protein solubilization (Fig. 3) when compared to a negative control for *G. verrucosa*, for both incubation times. However, protein solubilization increased for *P. palmata* after 24 h of incubation time (42.58 ± 2.96 g_{protein}/100g_{algae protein}) and increased for *P. dioica* after 48 h of incubation time (37.95 ± 1.82 g_{protein}/100g_{algae protein}). On the other hand, the protein content obtained for *P. palmata* decreased with an increase in the incubation time (42.58 ± 2.96 to 18.08 ± 0.11 g_{protein}/100g_{algae protein}) which indicates that this seaweed protein is more likely to be affected by the environmental temperature (50 °C) or pH (4.5) resulting in lower yields. Besides, though the temperature used in the assay was not high enough to cause extensive protein denaturation, long exposure time (24 and 48 h) can cause an early denaturation process that was triggered by protein structure loss that is observed when protein is subjected to an increase of temperature (see supplementary material). For *G. verrucosa*, the maximum protein was recovered at 24 h (21.98 ± 1.31 g_{protein}/100g_{algae protein}). When compared to the yields obtained using the first step of the Osborne fractionation method (using water as solvent), it is possible to see an increase in protein yields for *P. dioica* (21.67 ± 6.24 to 37.95 ± 1.82 g_{protein}/100g_{algae protein}) and *P. palmata* (29.56 ± 1.73 to 42.58 ± 2.93 g_{protein}/100g_{algae protein}) after 48 h and 24 h of incubation, respectively. This may indicate that the cell walls/structure of *Porphyra* and *Palmaria* are more susceptible to the action of Viscozyme® L. than *Gracilaria* (which is indicated to use agarose and cellulase when protein extraction is intended), eventually due to the nature of their structural polysaccharides, and that enzymatic

Table 4

Effect of the solution pH on the protein extraction yields and on the protein content of the extracts determined by the Kjeldahl method.

Algae		pH 7	pH 8	pH 10	Yield of the 1st step of the Osborne method (g _{protein} /100 g algae protein)
<i>Gracilaria verrucosa</i>	Yield (g _{protein} /100 g algae protein)	39.43 ± 6.94	40.74 ± 2.90	42.89 ± 0.91	33.24 ± 1.81
	Protein content (g _{protein} /g crude extract)	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	
	Yield (g _{protein} /100 g algae protein)	16.32 ± 0.25	17.12 ± 0.34	21.96 ± 5.31	
	Protein content (g _{protein} /g crude extract)	0.13 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	
<i>Porphyra dioica</i>	Yield (g _{protein} /100 g algae protein)	16.21 ± 0.25	13.45 ± 0.46	14.70 ± 2.03	30.76 ± 0.41
	Protein content (g _{protein} /g crude extract)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	
	Yield (g _{protein} /100 g algae protein)	16.21 ± 0.25	13.45 ± 0.46	14.70 ± 2.03	
	Protein content (g _{protein} /g crude extract)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	
<i>Palmaria palmata</i>	Yield (g _{protein} /100 g algae protein)	16.21 ± 0.25	13.45 ± 0.46	14.70 ± 2.03	30.76 ± 0.41
	Protein content (g _{protein} /g crude extract)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	
	Yield (g _{protein} /100 g algae protein)	16.21 ± 0.25	13.45 ± 0.46	14.70 ± 2.03	
	Protein content (g _{protein} /g crude extract)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	

Table 5

Protein yields (g_{protein}/100g_{algae protein}) after the application of a pre-step of grinding.

Algae	Solvent	Without grinding	With grinding
<i>Gracilaria verrucosa</i> ^a	H ₂ O pH 7	13.57 ± 1.42 ^a	12.35 ± 1.80 ^a
	0.1 M NaOH	13.14 ± 2.71 ^a	13.63 ± 2.04 ^a
<i>Porphyra dioica</i>	H ₂ O pH 7	29.71 ± 0.43 ^a	25.25 ± 1.76 ^a
	0.1 M NaOH	36.35 ± 0.74 ^a	31.33 ± 0.50 ^a
<i>Palmaria palmata</i>	H ₂ O pH 7	18.00 ± 1.30 ^a	17.32 ± 0.40 ^a
	0.1 M NaOH	25.52 ± 3.58 ^a	16.89 ± 0.77 ^a

^a A different batch of *G. verrucosa* seaweed was used for this experiment (the protein content (Kjeldahl method) of this alga is 14.7 %).

assisted extraction is able to improve protein recovery from red seaweeds, in particular for *Porphyra* and *Palmaria* (Jeon et al., 2011). A recent work conducted by Naseri et al. (2020) investigated enzyme-assisted protein extraction from *P. palmata* using different enzymes and/or cocktails. In this work, when Viscozyme® L (composed of beta-glucanases, pectinases, hemicellulases, and xylanases) and Celluclast® (composed of cellulases) at 0.4 % enzyme concentration were used, higher extraction efficiency (around 40 %) when compared to Shearzyme® (less than 40 % efficiency), which is mainly composed of xylanases, supporting the previous statement that was made.

Ultrasound-assisted extraction is an innovative “clean technology” that has been gaining interest in recent years. Advantages when compared to traditional technologies such as enzymatic extraction include the use of low solvent volumes, few instrumental requirements, and low economic and environmental impacts. This technique uses ultrasonic waves with frequencies between 20 kHz and 10 MHz to induce the formation, growth, and collapse of bubbles present in the liquid medium that allow the release of intracellular components (Carreir-a-Casais et al., 2021). Regarding seaweed extraction, ultrasound is generally used during 1–2 h with low temperatures applied and using water as solvent. However, in this work, a low exposure time (15 min) was employed since this technique was used just as a pre-step to help increase the extracted protein using the already tested methodology (extraction with water followed by extraction with 0.1 M NaOH). In Fig. 3C it is possible to observe that there are no significant differences (p

> 0.05) between the extraction yield obtained when an ultrasound pre-treatment was applied in comparison with the ones obtained without ultrasound. Fig. 3C presents the results in terms of proteins yields for both ultrasound-assisted extraction and controls with water and 0.1 M NaOH at room temperature and it is possible to verify that the use of sound waves only led to higher protein yields for *P. dioica* (26.08 ± 1.09 and 32.69 ± 3.46 g_{protein}/100g_{algae protein} for H₂O and 0.1 M NaOH, respectively, compared to 21.67 ± 6.24 and 26.28 ± 5.9 g_{protein}/100g_{algae protein} obtained without ultrasound) but the increase was not significant (p > 0.05). On the other hand, for *G. verrucosa* and *P. palmata* higher or similar yields were obtained when the solvents were applied sequentially with no need for a pre-step of physical methods.

A possible use of an enzyme cocktail needs to be considered, namely combining cellulolytic and proteolytic enzymes (e.g., carbohydrase followed by protease) to cause a synergetic effect and cause an intensive cell wall degradation; however, the use of proteases may significantly reduce the molecular weight of the proteins/polypeptides, as often proteolysis exposes hydrophobic patches which leads to aggregation, thus impairing technological applications. Alternatively, a combination of ultrasound and enzymatic extraction can also be tested.

Extracts with higher protein content (*G. verrucosa* and *P. dioica*) were submitted to a gel permeation chromatography and the molecular weight profile was analyzed. According to Figs. 4 and 5, it is possible to verify that all conditions tested in *P. dioica* (enzymatic extraction for 24 and 48 h and ultrasound pre-treatment) led to the solubilization of proteins with high molecular weights (between 79 and 255 kDa). In the case of ultrasound-assisted extraction (Fig. 5C), these results are probably related to a higher release of phycoerythrin (with molecular size between 240 and 260 kDa), as this alga is known for a high content in this protein (also visible by the increase in the intensity of the reddish color). However, in the case of enzymatic extraction, phycoerythrin peaks are not quite visible possibly due to the long exposure time in temperatures that can cause its denaturation (50 °C) and partial hydrolysis. Regarding *G. verrucosa*, molecular weights were lower than the ones obtained in *P. dioica*, and the main difference was observed in ultrasound extraction using H₂O (Fig. 5A and C). Although ultrasound extraction is often associated with mild conditions that cause hydrolysis in polypeptides, in this case, this was not evidenced, which can be explained by the low exposure time. On the other hand, enzymatic extraction using Viscozyme® L seems to cause some protein hydrolysis, as the molecular weight of the main proteins decreased from 24 h to 48 h, though the enzymatic cocktail mainly consists of carbohydrates. Nevertheless, this hydrolysis may be related also to the moderate conditions applied (50 °C) for a long time. Further, this effect seems to be stronger in *G. verrucosa* than in *P. dioica*.

3.6. Rheological analysis

After establishing the best methodology for red seaweed protein solubilization, extracts were analyzed in terms of possible functional properties (i.e., texturizing properties, including gelling and thickening). The storage modulus (G') and loss modulus (G'') for the heating and cooling ramp of *Porphyra dioica* H₂O extracts are depicted in Fig. 6a and 6b, respectively. It is possible to verify that in the initial stages of the experiment, when the temperature increased from 25 °C to approximately 50 °C, the value of G'' always remained higher than G' (Fig. 6a), indicating that in this range of temperatures, the sample remained in the liquid state. However, when 62.7 ± 2.3 °C was reached there was a crossover between G' and G'' , indicating that the sample turned into a gel that remained in the gelled form until the end of the heating ramp (Witek et al., 2021). Since the sample was not purified protein and other molecules were present (i.e., carbohydrates, mainly porphyrans) the gel formation could also be caused by carbohydrates-solvent interaction, which is widely seen in red seaweeds. Rheological properties of *Hypnea musciformis* and *Cryptonemia crenulate* carbohydrates (i.e., agar and carrageenan) extracted using different methodologies (i.e.,

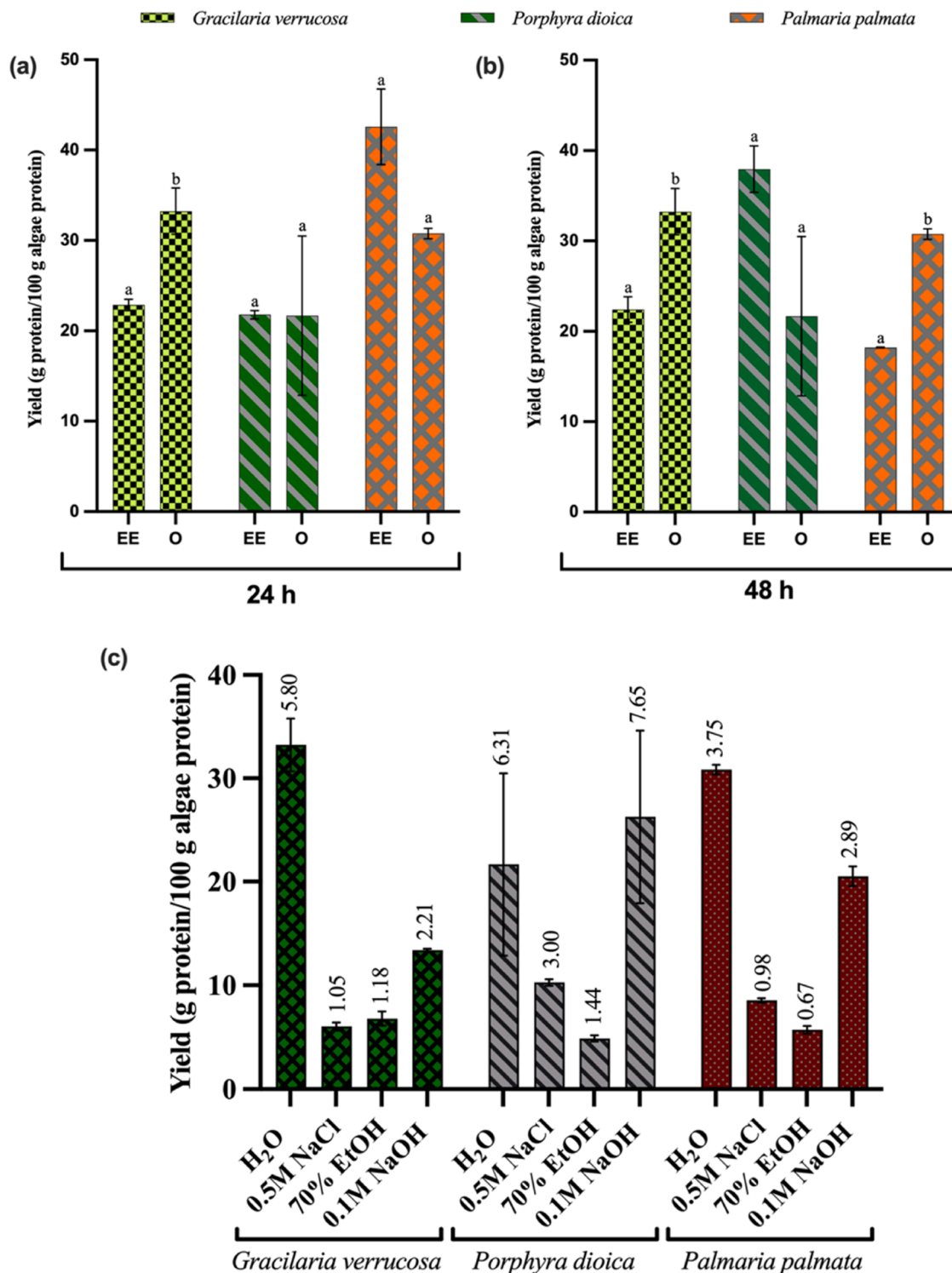


Fig. 3. Protein extraction yields were obtained using different methodologies and conditions: (a) Enzymatic-assisted extraction at 24 h; (b) Enzymatic extraction at 48 h; (c) Comparison between ultrasound pretreatment followed by H₂O and 0.1 M NaOH extraction and negative controls (extraction using H₂O and 0.1 M NaOH at room temperature). EE- Enzymatic extraction; O- Osborne fractionation method. Different letters indicate significant differences ($p < 0.05$) between different extraction methods (or solvents) for each seaweed.

alkali-treatment and water extraction) were studied in a work conducted by Rhein-Knudsen et al. (2017), who demonstrated that gel formation occurred at 33 and 74 °C using water extraction (similarly to this work) for *Hypnea musciformis* and *Cryptonemia crenulate*, respectively. However, typically, carbohydrates form reversible gels in the cooling stage (which occurred at 56 °C for water extraction of *Hypnea musciformis* and

did not occur for *Cryptonemia crenulate*). Similar results were obtained in our work, where melting of the gel was not observed. In contrast, the crossover occurred in the heating ramp and G' remained higher than G'' in the cooling process, indicating that the gel was formed irreversibly, which is what normally occurs when proteins are responsible for gel formation, aggregating after a thermal denaturation step.

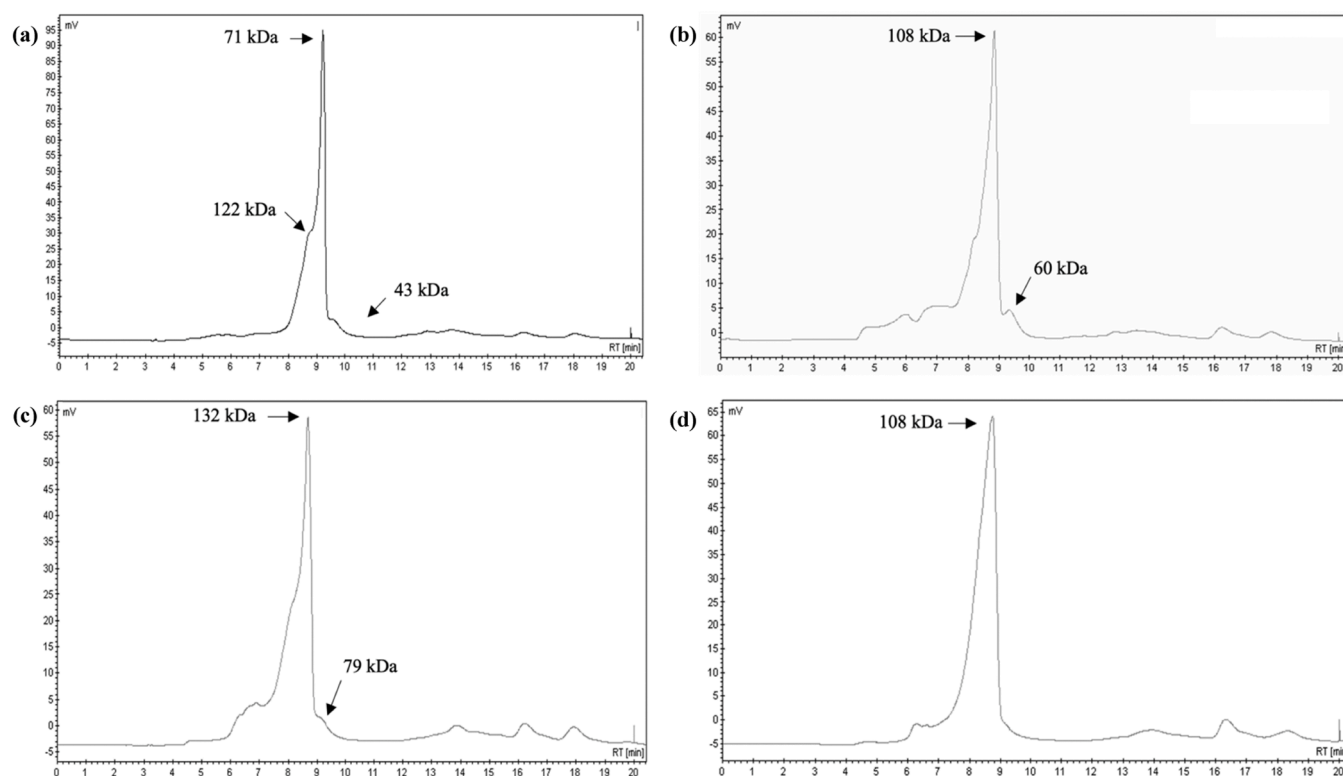


Fig. 4. Molecular weight distribution of the extracts obtained after enzymatic-assisted extraction for (a) *Gracilaria verrucosa*, 24 h incubation; and (b) *Gracilaria verrucosa*, 48 h incubation; (c) *Porphyra dioica*, 24 h incubation and (d) *Porphyra dioica*, 48 h incubation.

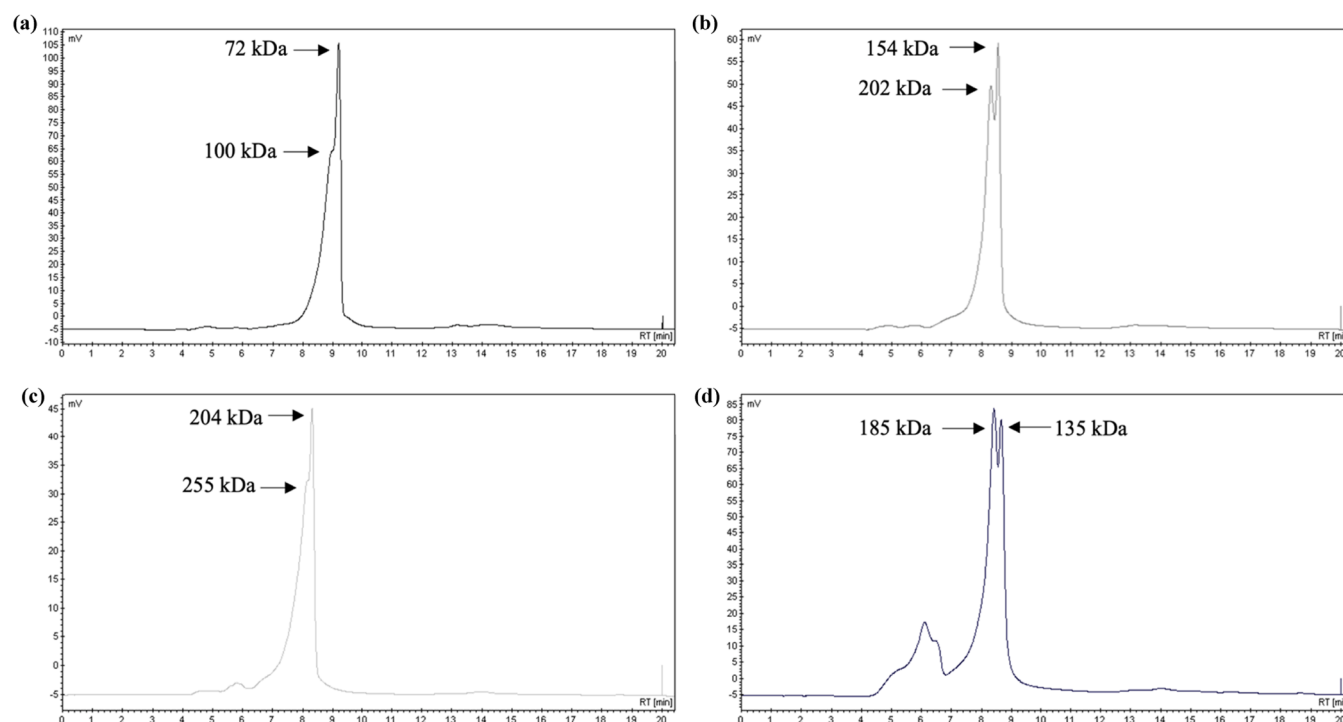


Fig. 5. Molecular weight distribution for extracts obtained with a 15 min of ultrasound pre-treatment followed by H₂O and 0.1 M NaOH extraction at room temperature for (a) *Gracilaria verrucosa*, H₂O extraction; (b) *Gracilaria verrucosa*, 0.1 M NaOH extraction; (c) *Porphyra dioica*, H₂O extraction and (d) *Porphyra dioica*, 0.1 M NaOH extraction.

Since these samples were obtained after a sequential extraction (H₂O followed by 0.1 M NaOH), the 0.1 M NaOH extracts were also tested in terms of gelling properties (results not shown). However, these extracts

did not show the ability to form gels as the H₂O extracts, but they formed thick solutions. Thus, using the same equipment, their ability to act as thickeners was analyzed in different concentrations (3, 5, and 10 %) and

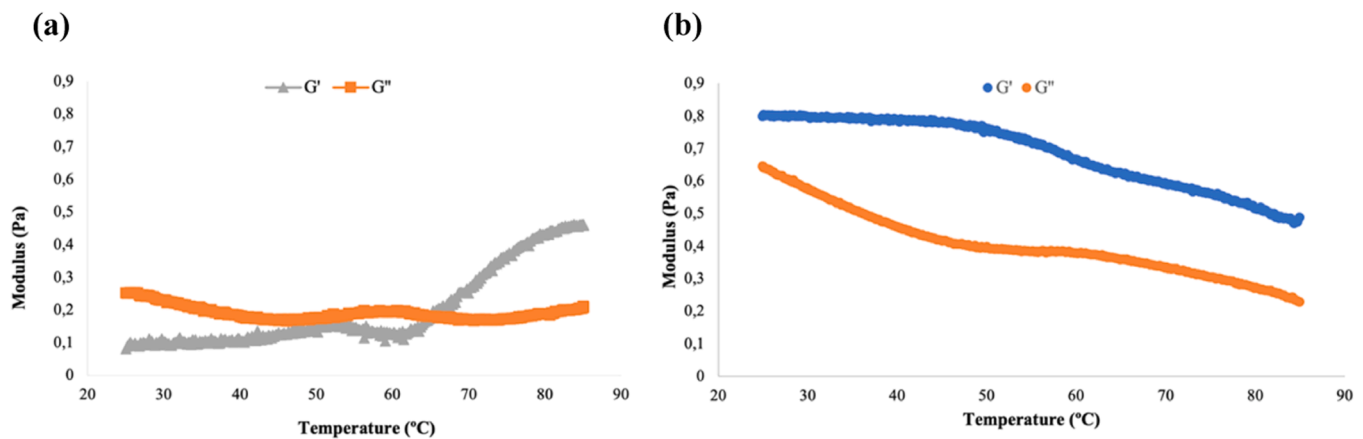


Fig. 6. Rheological properties of *Porphyra dioica* H₂O extracts (a) Storage modulus (G') and (b) Loss modulus (G'') as a function of temperature (25–85 °C) with a ramp rate of 2 °C/min, 10 % strain and frequency of 1.0 Hz.

results are shown in Fig. 7. From the data analysis it can be seen that for all concentrations tested, the higher viscosity was obtained at the lowest shear rate tested and decreased with the increase of the shear rate, showing a shear thinning behavior. Further, for the same shear rate, the apparent viscosity values increased significantly with the extract concentration, indicating thickening ability. For instance, at an initial shear rate of 10 s^{-1} , the apparent viscosity was around 0.012, 0.035, and 0.38 Pa·s for 3, 5, and 10 % of the extract concentration, respectively. Thus, different concentrations can be used to mimic different animal and vegetable-based foods and applied in the development of different food

products. The 3 % extracts reached higher values than cow's and soy milk (0.0022–0.0026 and 0.0026–0.0076 Pa·s, respectively), 5 % extracts presented viscosity values similar to whole eggs (0.0026 Pa·s), and the 10 % NaOH extract presented even higher apparent viscosity values, in the range of the ones found for sauces or other more structured liquid foods (McClements, 2023).

The technological functionalities mentioned above can be directly related to the type of proteins that are solubilized in each solvent. From the analysis of Fig. 8, which represents the chromatograms obtained for each protein extract (H₂O and 0.1 M NaOH in Fig. 8a and 8b,

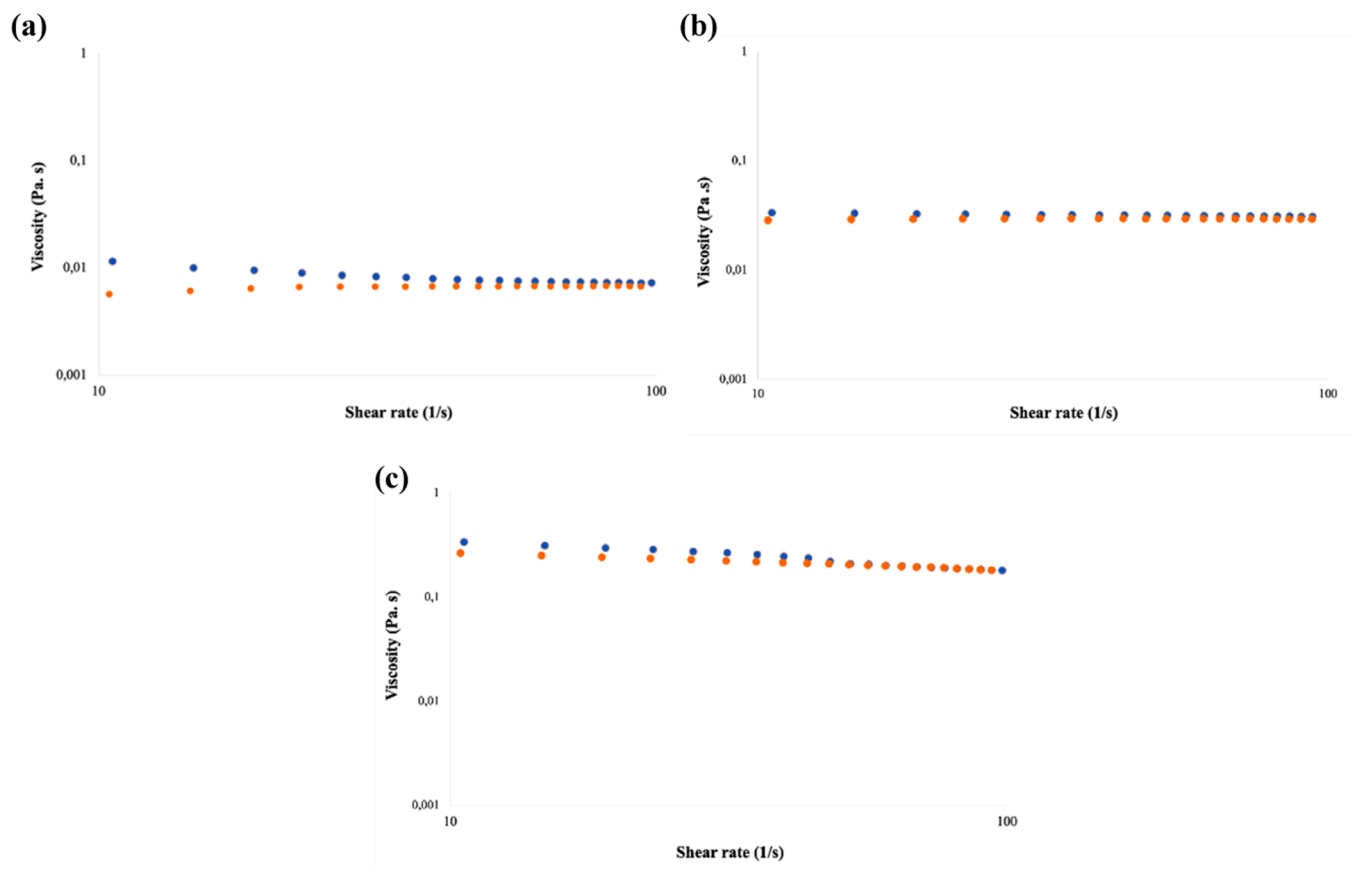


Fig. 7. Dynamic viscosity measurements for *Porphyra dioica* 0.1 M NaOH extracts using different concentrations. (a) 3 %; (b) 5 % and (c) 10 % after rheometer analysis with specific conditions: 60 s conditioning sample; first flow-ramp of 120 s with a shear rate of 0.1–100 s; 30 s conditioning sample; second flow-ramp of 120 s with shear rate of 100 – 0.1 s at 25 °C. Dots represent the temperature flow ramps: blue – 1st flow ramp; orange – 2nd flow ramp.

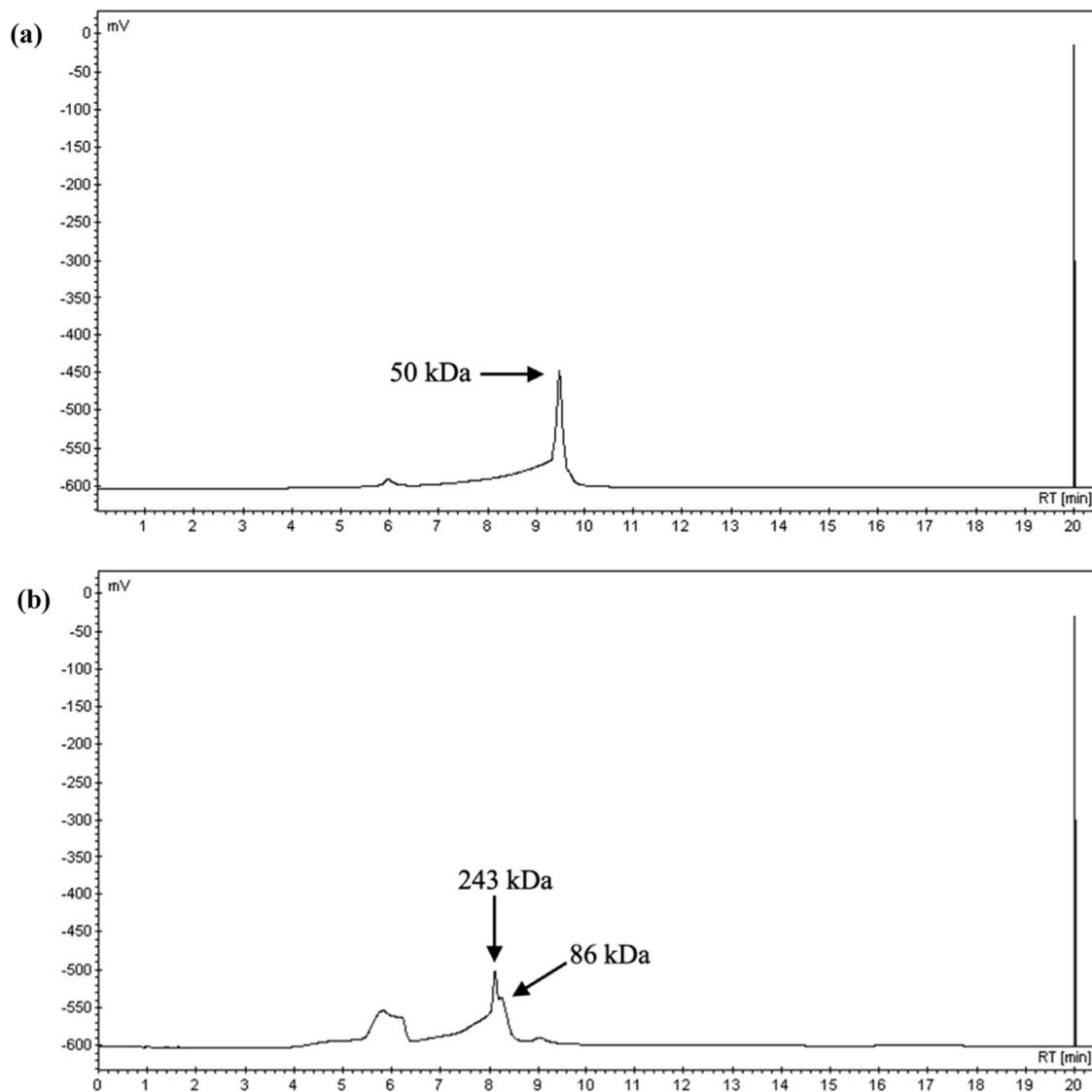


Fig. 8. Protein molecular weight distribution for (a) H₂O and (b) 0.1 M NaOH *Porphyra dioica* extracts after gel permeation chromatography.

respectively), it can be observed that for the H₂O extract an evident peak corresponding to proteins with 50 kDa of molecular weight are visible, while for the 0.1 M NaOH extracts two peaks are obtained (86 and 243 kDa). The presence of a more homogeneous extract in terms of protein size will facilitate the establishment of chemical bonds between molecules which will lead to the presence of a more stable network that induces gel formation. However, these proteins still kept the ability to texturize as they were responsible for thickening the solution.

4. Conclusions and future perspectives

The need for substitutes for animal-based proteins is increasing all over the world, either because of the urgent need for alternative sources due to the continuous growth of the world population, or because of the demand for new and innovative products in the food sector that allow satisfying the needs of the humanity, whether as food or feed.

Macroalgae have emerged as promising sustainable protein sources, due to their high protein content, diverse bioactivities, and techno-functionalities. Proteins derived from seaweed are still scarcely studied, and the development of adequate extraction and purification procedures are essential for their possible industrial application in nutraceuticals, cosmetics, food, and feed. The main obstacles highlighted are the protein solubilization and disruption of the

polysaccharide-rich cell wall of macroalgae (which also has an impact on protein bioavailability). Cell disruption methods are crucial for the successful extraction and enrichment of algal proteins on a larger scale. In addition, protein quantity and quality are highly dependent on factors such as extraction procedure, species, harvest time, location, and growing circumstances.

Our work demonstrates that H₂O and 0.1 M NaOH are appropriate solvents to obtain protein-enriched fractions with a wide range of molecular weights from red macroalgae, *G. verrucosa*, *P. dioica*, and *P. palmata*. Nevertheless, other solvents, such as ethanol or salts, may allow the solubilization of low molecular weight proteins with potential bioactive interest, though the protein recovery yields are extremely low in these cases. The obtained fractions were fully water soluble which is not achieved when leguminous matrices are used for protein concentrates production, allowing their use as a technological agent in different applications (which is not achieved when leguminous matrices are used for protein concentrates production). Regarding *G. verrucosa*, this seaweed is mainly used in the industry sector for the recovery of agar, which is a polysaccharide that is poorly soluble in cold water and 0.1 M NaOH. Thus, a biorefinery approach can be used to apply an integral valorization scheme for this bioresource, resulting in (near-)zero waste. The application of other methodologies to facilitate cell wall rupture, namely ultrasound and enzymatic treatments) did not show to be

effective when compared to the use of the solvents alone. Nevertheless, the behavior was different for different seaweeds and should be adjusted case by case. Enzymatic extraction showed higher protein solubilization only after 24 h (around 40 % against 30 % obtained without the enzymatic treatment) for *P. palmata* and US for *P. dioica* (more evident in the NaOH fraction). Nevertheless, future work addressing the applicability of innovative and effective methodologies is needed.

Understanding the different protein profiles between specie and their behavior towards different solvents, as well as the seaweed species structure and composition, is needed to allow obtaining polypeptides with different molecular sizes and functionalities, including bioactive, nutritional and functional properties (e.g., foaming, gelling, and texturization abilities). In this way, their application can be broadened, and the development of nutritionally enriched food products can be considered. Overall, red seaweeds are underexploited natural resources that can be considered as alternative sources of protein (and other compounds) which makes them interesting as clean-label ingredients for food and feed industries.

Ethical statement

This study did not use animals nor humans.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.fufo.2024.100335](https://doi.org/10.1016/j.fufo.2024.100335).

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