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Production and characterization of protein-rich extracts from the red macroalga *Gelidium corneum* and its industrial agar extraction residues

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ABSTRACT

The red macroalga *Gelidium corneum* is a species commercially exploited for agar extraction, though with enormous potential for other industrial applications. This work aimed to produce protein-rich extracts from the crude alga and from residues of the industrial agar extraction process by applying different extraction and recovery procedures, and to characterize the obtained protein extracts.

The sequential implementation of two water extractions followed by two alkaline extractions resulted in the recovery of 12.0 ± 1.2 % and 15.4 ± 0.9 % of the total protein contents of the crude alga and industrial residues, respectively. Two sequential water extractions assisted by Celluclast® and Alcalase® enzymes followed by two alkaline extractions recovered 52.1 ± 1.7 % and 36.5 ± 1.3 % of the protein contents of the crude alga and industrial residues, respectively. Extracts produced by these processes were characterized focusing on proximal composition, protein accessibility, nutrition and antioxidant potential, showing value as aquaculture feed ingredients.

As all protein products revealed high carbohydrate contents and, aiming at enriching protein content, different protein precipitation methods were assessed, namely through ammonium sulfate precipitation and pH-shift protocols. The pH-shift method in sequential mode proved to be simple and fast with low reagent consumption. The purification of extracts by this precipitation mode allowed precipitating up to 72 % of the extracted protein.

The results obtained suggest that protein concentrates extracted from *Gelidium corneum* appear to be promising sustainable sources for fish feed production, owing to their essential amino acid content, protein bioaccessibility and antioxidant properties.

Abbreviations: AA, amino acid; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) acid; ANOVA, analysis of variance; BIO, bioaccessible fraction; DPPH, 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazine; dw, dry weight; EDTA, ethylenediaminetetraacetic acid; FAO, Food and Agriculture Organization of the United Nations; G, crude *Gelidium corneum*; GA, water extracts from *Gelidium corneum*; GB, enzyme-assisted extracts from *Gelidium corneum*; MW, molecular weight; MWCO, molecular weight cut-off; NBIO, non-bioaccessible fraction; NF, nanofiltration; NREL, National Renewable Energy Laboratory (U.S.); RG, residues of *Gelidium corneum*; RGA, water extracts from residues of *Gelidium corneum*; RGB, enzyme-assisted extracts from residues of *Gelidium corneum*; SD, standard deviation; SEC, size exclusion chromatography; TFF, tangential flow filtration; TMP, transmembrane pressure; UF, ultrafiltration; VCF, volumetric concentration factor.

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1. Introduction

According to the United Nations Department of Economic and Social Affairs [1], the global population will reach 9.4×10^9–10.0×10^9 people in 2050, rising to 8.9×10^9–12.4×10^9 in 2100 (figures given with 95 % probablility). This rapid growth is expected to lead to an increased demand for food and energy, which will result in the depletion of agricultural resources and fossil fuels. This in turn will cause irreversible damage to the environment [2]. Undernourished people were estimated to reach 841.4 million (9.8 % of the world population) by 2030 [3]. This awareness has sparked a growing interest in finding alternative and sustainable sources of feed, food and energy.

Due to their intrinsic characteristics, macroalgae, commonly known as seaweed, are excellent sustainable resources in a biorefinery context. They have numerous advantages when compared to terrestrial plant biomass: rapid growth, high photosynthetic efficiency, low nutrient requirements and no competition with agricultural and other land uses [4].

Regarding protein content, macroalgae are considered a novel promising source, with some species presenting similar or higher contents than those of conventional protein sources, such as eggs, soybean, fish, meat, and cereals [5]. In general, seaweeds from *Rhodophyta* present a higher protein content (20–30 %) when compared to *Chlorophyta* (9–26 %) and *Phaeophyceae* (3–15 %) on a dry weight basis [6]. Besides this, proteins and peptides derived from marine macroalgae have demonstrated additional value due to their nutraceutical, pharmaceutical, and cosmeceutical properties, such as antioxidant, antihypertensive, immune-modulatory, and anticoagulant functions [7]. Thus, macroalgal proteins are potential raw materials for producing protein-based food ingredients with both technological and biofunctional applications [8].

Methods for industrial macroalgal protein extraction are still underdeveloped [9], when compared to polysaccharide extraction, especially for the red macroalgal species used in the phycocolloid industry, such as *Gelidium corneum* (formerly *Gelidium sesquipedale*). The greatest challenge in macroalgal protein extraction is the complexity and rigidity of the cell wall. The presence of polysaccharide-bound cell wall mucilage, comprising anionic or neutral polysaccharides and polyphenols, reduces protein extractability and requires additional adaptation of the fractionation and purification steps [10]. To improve macroalgal protein extraction efficiency, cell disruption methods and the use of specific reagents have been tested. To date, the most common approaches for disrupting macroalgal cells include osmotic shock, mechanical grinding, ultrasonic treatment and polysaccharidase-aided digestion [11]. Although enzyme-assisted extraction has been identified as a promising method [12], its efficiency depends on the algal cell wall composition, requiring careful selection of specific enzymes for each species. Generally, the traditional cell disruption method is employed for the solubilisation of the aqueous protein fraction. In order to recover other protein fractions, specific tunable reagents (such as subcritical water with organic modifiers, aqueous two-phase systems, deep eutectic solvents and ionic liquids) must be used. Alkaline solutions have long been recognized as an effective method for solubilizing highly water-insoluble hydrophobic macroalgal proteins when used after an initial aqueous extraction involving cell disruption by osmotic shock [13]. Other protein fractions that can be isolated from macroalgae include acid, low-salt and high-salt water-soluble extracts.

Industrial agar extraction, on the other hand, requires boiling seaweeds in excess of water even though recent developments point to the possibility of replacing this high-temperature soaking step with short-time and low-temperature microwave-mediated extraction, among other emerging techniques [14,15]. Careful addition of acid to adjust the pH to 6.3–6.5 is usually needed. Extraction under pressure reduces the processing time while increasing the agar yield. These conditions can degrade the extracted agar and, as a result, optimal extraction conditions must be established for each type of seaweed. The dissolved agar is afterwards filtered to remove residual seaweed fragments, and the hot filtrate is then cooled to form a gel. The gel may be bleached (e.g., with sodium hypochlorite) to reduce any colour [16]. Seeking the valorisation of marine bioresources, it is important to profit from both the protein and the agar components of the hydrocolloid producing seaweeds. In this context, the impact of prior protein extraction on the agar content and quality, and vice versa, deserves investigation.

The objective of this study was firstly to develop an effective protein extraction protocol adequate for up-scaled application to crude *Gelidium corneum* and its residues after agar extraction, aiming at a zero-waste process within a biorefinery concept. A second objective was to characterize the protein-rich extracts, contributing to the exploitation of macroalgae and of *Gelidium corneum* in particular as a new renewable and sustainable resource for the food and feed industries.

2. Materials and methods

2.1. Biomass

Gelidium corneum biomasses – crude, whole seaweed and its residues after agar extraction – were kindly provided by IBERAGAR S.A. Both biomasses were provided dry ground to powder (using a mill from Almacinha Lda., Porto Portugal, coupled to a 3 mm cut-off sieve, from Cisa Cedaceria Industrial S.L., Barcelona, Spain). Microscopy images of the powders revealed very heterogeneous particle size. For the dry crude alga, sizes of about 0.86 mm × 6.3 mm to about 0.82 mm × 1.4 mm could be found, while for the residues, the measured sizes were 1.3 mm × 1.9 mm to 71 μm × 90 μm and even lower. No particle size distribution measurements were provided or performed.

To minimize potential safety concerns on the use of these raw materials for feed production, the contents of toxic trace mineral elements in the seaweeds were evaluated by an external certified analytical laboratory (LAIST, Lisbon, Portugal). The total levels of arsenic, cadmium, chromium and mercury found in the raw materials were (in $mg/kg_{\ dry\ alga}$): As<4.0, Cd–1.1, Cr–8.8, and Hg<0.02 for crude *G. corneum*; and As<4.0, Cd–0.99, Cr–11, and Hg–0.05 for *Gelidium* industrial residues. The analytical methods used were hydride generation atomic absorption spectroscopy (for As), inductively coupled plasma atomic emission spectroscopy (for Cd and Cr), and atomic fluorescence spectrometry (for Hg).

2.2. Chemicals

The chemicals used in this work were purchased from recognized manufacturers for science, services and industry and their purity was graded “for analysis”, except if otherwise specified. Ultrapure water was employed, obtained from Milli-Q systems (or from equivalent brands).

2.3. Experimental methods

2.3.1. Protein extraction

The two algal biomasses were used for protein extraction following two different methods (a scheme is shown in Fig. S1 of the supplementary material).

Procedure A: For water extraction, 100 g of algal biomass powder was suspended in 2 L of deionized water. The suspension was stirred at 600 rpm for 16 h at 4 °C. Afterwards, the suspension was centrifuged (10,000 ×*g* for 30 min) at 4 °C. The supernatant was collected and the pellet was re-suspended in 2 L of deionized water, the stirring and centrifugation steps being repeated. For subsequent alkaline soluble protein extraction, the pellet from water extraction was re-suspended in 1 L of 0.1 M NaOH, stirred at 600 rpm for one hour at room temperature and centrifuged under the previously described conditions. The supernatant was collected and the alkaline process was repeated on the pellet.

Procedure B: For enzyme-assisted water extraction, 75 g of algal biomass powder was suspended in 1.5 L of deionized water previously

supplemented with Celluclast® (0.2 $g_{enzyme}/100g_{algal\ biomass}$). The pH was adjusted to 4.5 with HCl 1 M. The suspension was stirred at 600 rpm for 16 h at 50 °C and centrifuged (10,000 ×*g* for 30 min) at 4 °C. The supernatant was collected and the pellet was re-suspended in 1.5 L of deionized water containing Alcalase® (0.2 $g_{enzyme}/100g_{algal\ biomass}$), a saturated Na_2CO_3 solution being then used to adjust the pH to 8.0. The suspension was stirred and centrifuged under the conditions described for the first enzymatic step. Both enzyme preparations were supplied by Novozymes (Denmark). For subsequent alkaline soluble protein extraction, the pellet from enzyme-assisted water extraction was taken through the method described in procedure A (with 1000 mL of 0.1 M NaOH).

In both procedures, after each extraction step, samples were withdrawn from the supernatant, for protein analysis. The remaining collected supernatants were pooled together and their pH was adjusted to 7.0. For crude alga biomass, samples were also withdrawn from the pellet at each extraction step, for carbohydrate analysis. These pellet samples were placed in a convection oven at 40 °C (Function Line, Heraeus) for 7 days for complete drying.

2.3.2. Protein concentration by ultrafiltration / nanofiltration

The protein extracts (pooled supernatants from extraction procedures A and B) were concentrated by tangential flow filtration (TFF) with retentate recycling and permeate removal occurring at the same time.

Ultrafiltration (UF) of the extracts from procedure A was carried out using a lab-scale QuixStand Benchtop TFF system, coupled to a hollow fibre membrane cartridge with a nominal molecular weight cut-off (MWCO) of 10 kDa and 110 cm^2 of effective filtration area. The transmembrane pressure (TMP) was kept at 0.8–1.0 bar. The final volumetric concentration factor (VCF) achieved was 10.

Due to the use of the Alcalase® proteolytic enzyme, the extracts from procedure B were expected to contain low molecular weight peptides and thus they were concentrated by nanofiltration (NF). A flat-plate disc-shaped stainless steel module 0.23 m in diameter housing two polyamide membrane discs (830 cm^2 total filtration area) with 400 Da of NMWCO (FilmTec™ NF270, Dupond) was used. The TMP was maintained at 20–30 bar, through a piston pump (Rannie, Denmark) and a needle valve at the retentate outlet. The operation was carried to the minimum achievable retentate volume, resulting in a VCF of approximately 5.

Samples of the final retentates and permeates were taken for protein analysis. The concentrated extracts (final retentates) were frozen at −80 °C and freeze dried during 48 h.

These steps are also shown in Fig. S1 of the supplementary material.

2.3.3. Protein precipitation

2.3.3.1. Ammonium sulfate precipitation. The protein extract (pooled supernatants, without UF concentration and before freeze drying) from extraction procedure A applied to the crude alga was further subjected to ammonium sulfate precipitation (a scheme is shown in Fig. S2 of the supplementary material).

In order to find the suitable ammonium sulfate concentration for protein precipitation, this salt was directly dissolved in 30 mL of the extract to attain concentrations in the range of 70 % to 85 % by weight, and left for 16 h at 4 °C, with gentle stirring to induce precipitation. Thereafter, the suspension was centrifuged at 15,000 ×*g* for 30 min at 4 °C. The supernatants were collected for protein analysis and the pellets were dissolved in 15 mL of Tris–HCl buffer (10 mM, pH 7.0) for subsequent removal of the remaining salt, by diafiltration with this same buffer.

Stepwise diafiltration was carried out using Amicon Ultra-15 Centrifugal Units (3 kDa MWCO), the centrifugations being performed at 3220 × g and 4 °C. Ten millilitres of the dissolved pellet were loaded onto the membrane and 5 mL of diafiltration buffer were added. Approximately 5 mL of permeate were collected by centrifugation and the same volume of diafiltration buffer was further added to the retentate. This procedure was repeated until about 50 mL of permeate were cumulatively collected. The diafiltered retentate was further concentrated on the same UF unit by centrifugation at 3220 ×*g* at 4 °C for repeated 5-min periods until the retentate volume was reduced to approximately 3 mL.

2.3.3.2. pH-shift precipitation. The concentrated protein extracts (pooled supernatants after UF/NF and before freeze drying) from extraction procedures A and B applied to the crude alga were further subjected to pH-shift precipitation (a scheme is shown in Fig. S3 of the supplementary material). The pH-shift protein precipitation method developed for these extracts was adapted from a previous study on protein isolation from the brown seaweed *Saccharina latissima* [17].

For both extraction procedures (A and B), HCl 6 M was slowly added to 30-mL aliquots of the extract, with stirring and at room temperature, to adjust the pH to a range of values between 1.0 and 6.0, one of the 30-mL aliquots being left at pH 7.0. The mixtures were incubated for 30 min with stirring, at 4 °C, and were subsequently centrifuged at 10,000 ×*g* for 10 min at 4 °C. The supernatants were collected for protein analysis.

2.3.3.3. Consecutive pH-shifts precipitation. The concentrated protein extracts (pooled supernatants after UF/NF and before freeze drying) from extraction procedures A and B applied to the crude alga were further subjected to pH-shift precipitation in an alternative, consecutive mode (a scheme is shown in Fig. S3 of the supplementary material). In this, precipitated proteins are consecutively recovered from successive pH adjustment steps done on the same extract portion. The pH ranges tested were 4.0–1.5 and 7.0–3.5, for extracts from procedures A and B, respectively.

For both extraction procedures (A and B) 30-mL aliquots of the extract were transferred to 50-mL conical Falcon tubes. With the addition of HCl 1 M, the pH value of the extract was adjusted to the highest value of tested pH range and subsequently incubated and centrifuged as described above for the simple pH-shift protocol. Before the pH of the supernatant was adjusted to the following value, the precipitate pellet was recovered and a sample was collected from the supernatant for protein analysis. The process was repeated stepwise, in 0.5 pH units, down to the last value of the tested pH range.

2.4. Analytical methods

The original dry biomasses and the powders resulting from freeze drying were stored in air-tight plastic bags protected from light at room temperature (25 °C) for further analyses.

2.4.1. Moisture, solids and ash

The total solids content (dry weight, dw) was determined by oven drying 100-mg samples of freeze dried protein extracts, dry crude alga or alga residues, in a convection oven at 60 °C and atmospheric pressure for 18 h. After cooling in a desiccator, the sample weight was recorded. The ash content was determined by combusting the oven-dried samples at 600 °C for 16 h in a muffle. After cooling in a desiccator, the weight of the remaining ash was recorded. These analytical procedures are fully described in online accessible methods from the U.S.A. National Renewable Energy Laboratory (NREL) “Determination of total solids and ash in algal biomass” [18].

2.4.2. Protein

Total protein content was determined in dried or freeze dried samples using an FP-528 DSP LECO combustion N analyser (LECO Corporation, St. Joseph, MI, USA). Briefly, 70–80 mg of each sample is placed in the loading head of the analyser, where it is sealed and purged of any atmospheric gases. Thereafter, the sample is dropped into a hot furnace

and flushed with pure oxygen for very rapid combustion, covalently bound nitrogen (N) being converted into nitrogen gas (N_2). The N_2 content is detected by passing the gas through a thermal-conductivity cell. An air blank was carried out and the calibration standard curve was performed with ethylenediaminetetraacetic acid (EDTA) (LECO 502–896, St. Joseph, MI, USA). Protein values were calculated as N × 4.59 conversion factor [19] for the estimation of the protein content of seaweeds more accurately. Analyses were performed ($n = 3$) and the results were expressed as a percentage of dry weight (% dw).

Protein concentration in aqueous samples was determined using the Lowry method [20]. Briefly, to 0.1 mL of sample, 0.1 mL of 2 N NaOH were added. Hydrolysis followed for 10 min at 100 °C in a heating block. After cooling to room temperature, 1 mL of freshly prepared complex-forming reagent was added. The latter was composed of 2 % (*w/v*) Na_2CO_3, 1 % (w/v) $CuSO_4 \bullet 5H_2O$ and 2 % (w/v) sodium potassium tartrate (all salts dissolved in water), mixed in a 100:1:1 (by volume) proportion. After incubation for 10 min, 0.1 mL of 1 N Folin reagent were added and the mixture was incubated at room temperature for 30 min. The absorbance of the final mixture was read at 750 nm in a DR3900 spectrophotometer (Hach Lange, USA). A calibration curve was prepared for this procedure using bovine serum albumin standards in the concentration range of 0.0–0.5 g/L.

2.4.3. Lipids

The content in lipids was determined in dried or freeze dried samples by gravimetry after solvent extraction and evaporation.

Lipid content was determined using the Folch technique [21]. Briefly, 200 ± 1 mg of the dehydrated samples ($n = 3$) were homogenized with 3 mL of a 2:1 mixture of chloroform and methanol. The mixture was then agitated on an orbital shaker (Orb—B, Ibx Instruments) at 350 rpm for 15 min. To this mixture, 3 mL of 0.1 M HCl and 300 μL of 0.5 % (*w/v*) $MgCl_2$ were added and rigorously shaken in a vortex for 30 s. Subsequently, a centrifugation at 2000 ×*g* for 5 min at 4 °C was performed. The organic phase was recovered, filtered through anhydrous sodium sulfate and evaporated using a rotary evaporator (Buchi 461, Switzerland). After evaporation, the pear-shaped flasks were weighed and the lipid content thus determined was expressed as percentage of dry weight (% dw).

2.4.4. Total carbohydrates

To assess possible co-extraction of carbohydrates, along with the algal proteins, the carbohydrate content of dry or freeze dried samples of algal material and extracts was determined according to NREL's "Determination of Total Carbohydrates in Algal Biomass" [22]. To 0.5 g of algal material, 5 mL of 72 % (*w/w*) sulfuric acid were added, and the mixture was incubated for 1 h at 30 °C with orbital agitation at 250 rpm. Afterwards, by adding 138.5 mL of Milli-Q water, the sulfuric acid concentration was reduced to 4 % (*w/w*) and the resulting mixture was autoclaved for 1 h at 121 °C. After vortexing, aliquots of 10 mL were withdrawn and their pH was adjusted to a value of 6–8 with the addition of calcium carbonate. The aliquots were centrifuged for 10 min at 4000 ×*g*, and 200 μL of each resultant supernatants were diluted with 200 μL of 50 mM H_2SO_4 and centrifuged again under the same conditions. The supernatants were 1:10 diluted with 50 mM H_2SO_4 to a final volume of 1 mL. Chromatographic analysis of the prepared supernatants (20 μL per injection) was carried out in a Hitachi LaChrom Elite® HPLC system, using 5 mM H_2SO_4 for elution at a flow rate of 0.5 mL/min. Calibration curves were prepared by injecting the same volume of standard solutions of the target analytes, specifically in the ranges of 0.0–3.64 g/L for glucose and 0.0–3.50 g/L for galactose, and plotting the respective chromatographic peak areas against analyte concentrations.

The cellulose plus starch fraction and agar fraction contents in the analysed algal material were determined using Eq. (1) and Eq. (2), respectively.

$$Cellulose + starch\ (\%) = \frac{m_{glucose} \bullet \frac{162}{180}}{m_{algal\ material\ (dw)}} \times 100 \quad (1)$$

$$Agar\ (\%) = \frac{\left(m_{galactose} + 1.27 \bullet m_{galactose}\right) \bullet \frac{162}{180}}{m_{algal\ material\ (dw)}} \times 100 \quad (2)$$

where *m* is the analyte or algal material (dry weight) mass, *162* is the molar mass (in g) of the glucose and galactose monomeric units in polymeric glucan starch and galactan, *180* is the molar mass (in g) of glucose and galactose and *1.27* is the mass ratio between L-3,6-anhydro galactose (AHG) and D-galactose in agar, (0.44 % D-galactose and 0.56 % L-AHG) [23].

The total carbohydrate concentration in the freeze dried protein products (extracts) was determined using the DuBois method [24] with some minor modifications. Briefly, to 200 μL of water-dissolved sample or standard solution, 200 μL of 5 % phenol solution and 1 mL of 96 % sulfuric acid solution were rapidly added under vortex agitation and the mixture was incubated at room temperature for at least 60 min. Afterwards, the absorbance of the final solution was read at 485 nm. A calibration curve was prepared with glucose standard solutions in the concentration range of 0.0–100 mg/L.

2.4.5. Protein and peptide molecular size profiles

Freeze dried protein extracts (obtained using the enzyme-assisted procedure B) from both algal biomasses, were re-solubilized to achieve a concentration of 10 mg/mL and the respective solutions were analysed by size exclusion chromatography (SEC). Two different SEC columns (Cytiva™) and two different sets of protein/peptide calibration standards were selected for the analyses. The first column was a Superdex 75 5/150 GL (5 mm × 150 mm) for separation in the molecular weight (MW) range of 3 kDa–70 kDa. This column operated at room temperature, with a flow rate of 0.5 mL/min, using PBS as the eluent, and an injection volume of 50 μL. The calibration standards and their respective SEC elution volumes were: conalbumin (1.5 mg/mL), 75 kDa, 1.5 mL; albumin (4 mg/mL), 67 kDa, 1.7 mL; carbonic anhydrase (1,5 mg/mL), 29 kDa, 1.9 mL; chymotrypsin (3 mg/mL), 25 kDa, 2.0 mL; cytochrome *c* (1 mg/mL), 12.4 kDa, 2.2 mL. The second column was a Superdex Peptide 10/300 GL (10 mm × 310 mm) for separation in the MW range of 100 Da–7 kDa. This column also operated at room temperature, with a flow rate of 0.5 mL/min, utilizing a 30 % acetonitrile solution with 0.1 % TFA as the eluent, and an injection volume of 500 μL. The calibration standards and their respective SEC elution volumes were: cytochrome *c* (1 mg/mL), 12.4 kDa, 9.3 mL; aprotinin (2 mg/mL), 6.5 kDa, 10.0 mL; angiotensin I (1 mg/mL), 1.3 kDa, 13.0 mL; hexaglycine (1 mg/mL), 360 Da, 15.4 mL. In both sets, Blue Dextran 2000 (1.0 mg/mL) was used for estimating the void volume, V_0.

The calibration was established by plotting the decimal logarithm of the MW of a standard solute against the respective K_{av} in the SEC run [Eq. (3)], in the same conditions as those used for the sample run:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad (3)$$

where V_e is the solute elution volume and V_t is the total volume of the SEC column.

From the chromatograms obtained (spectrophotometric detection at 280 nm) for the algal extract under analysis, the mass percent of proteins/peptides in a certain range of MW was estimated using Eq. (4) as follows:

$$\text{Proteins and peptides in a MW range (\%wt)} = 100 \times \frac{\text{Area of SEC peaks found for the sample in the MW range}}{\text{Sum of the areas of total SEC peaks found for the sample}} \quad (4)$$

2.4.6. Protein bioaccessibility

Protein bioaccessibility was determined on dried or freeze dried

samples of algal material and extracts by an in vitro digestion procedure, described in detail by Marmelo et al. [25]. The in vitro digestion models recreate the digestion process of the human gastrointestinal tract by using physiologically based conditions, such as the chemical composition of digestive fluids, pH, temperature, and residence times typical for each digestion stage. The composition of the solutions used to mimic the human gastrointestinal process may be consulted in Table S1. Briefly, 300 ± 5 mg of each sample were digested in triplicate (Nalgene™ high-speed PPCO centrifuge tubes) at 37 °C using a Rotary Tube Mixer with Disc (25 rpm; LSCI, Portugal) in an incubator (Select 400 W). The digestion procedure involved three distinct phases: (1) oral phase, where 4 mL of saliva fluid at pH 7.0 ± 0.2 was added to each sample and incubated for 5 min; (2) gastric phase, where 8 mL of gastric fluid at pH 2.0 ± 0.2 was added to the samples, which were then incubated for 2 h; and (3) intestinal phase, where 8 mL of duodenal fluid and 4 mL of bile fluid were combined at pH 7.0 ± 0.2, added to the samples and incubated for 2 h. The digestion fluids were prepared immediately before the start of the procedure to avoid degradation/inhibition of the enzymes. The pH was adjusted using NaOH (1 M) or HCl (1 M). At the end of the digestion process, the reaction tubes were promptly placed on ice to stop the digestion reactions. Subsequently, the tubes were centrifuged at 2750 ×*g* and 10 °C for 10 min to separate the bioaccessible fraction (BIO) from the sample residues (non-bioaccessible fraction - NBIO). Negative controls, involving the digestion fluids without the sample, were also conducted.

2.4.7. Amino acid profiles

The amino acid analysis was performed on freeze dried samples (0.2 g) of the crude seaweeds and protein-based extracts from *G. corneum*. First, acid hydrolysis of the matrices was undertaken in a closed-vessel microwave digestion system (Milestone ETHOS 1 Series), as indicated in Motta et al. [26]. Thereafter, amino acids were quantified chromatographically following pre-column derivatisation and separation methods developed elsewhere [27].

2.4.8. Antioxidant and chelating activities

The reducing power, the antioxidant activity and the metal ion chelating power of the freeze dried protein extracts were analysed. The methods used are described in Henriques et al. [28] and Sapatinha et al. [29] and summarized below. The extracts to be tested were solubilized in distilled water to concentrations in the range of 1.0–20 mg/mL.

The reducing power, reflecting the ability of bioactive compounds to donate electrons, was measured through the reduction of Fe^{3+} to Fe^{2+}. The method used the spectrophotometric detection of a coordination complex formed between Prussian blue and Fe^{3+}. The reducing power was expressed as the sample concentration (mg/mL) producing a value of 0.500 absorbance units (A0.5) in standardized conditions.

To determine antioxidant activities, the ability of extracts for scavenging coloured stable free radicals was assessed. The spectrophotometric methods with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) acid (ABTS) and 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazine (DPPH) were both used. Antioxidant activities were expressed as the sample concentration (mg/mL) producing 50 % inhibition of the test oxidation reaction (EC_{50}), through scavenging of the respective free radical.

Free cuprous and ferrous ions mediate pro-oxidative reactions and therefore are undesirable in many formulations. These metal ions form complexes with specific chelators producing chromophores that are quantified spectrophotometrically. The presence of extracts having Cu^{2+} and/or Fe^{2+} chelating activity will decrease the extent of chelation with their specific chelators. The chelation activity standardized methods here followed used pyrocatechol violet for Cu^{2+} and ferrozine for Fe^{2+}. Ion metal chelation activities were expressed as the sample concentration (mg/mL) producing 50 % reduction of the extent of chelation with their respective chelators.

2.5. Statistical data treatment

MS Excel was used for statistical treatment of the results. Data are presented as average values ± standard deviation (SD), with all experiments and/or analyses performed in triplicate ($n = 3$), unless otherwise indicated. A one-way analysis of variance (ANOVA) was used when comparing two or more sets of experimental data, with a significance level of $p = 0.05$.

3. Results and discussion

3.1. Chemical composition of the crude algal biomass and its industrial residues

The compositions of the studied biomass resources, namely crude *G. corneum* and its residues after industrial agar extraction are shown in Table 1. The total carbohydrate content found in crude *G. corneum* (62.1 ± 1.7 % dw) is in agreement with previous reports indicating that carbohydrates comprise 50 % to 60 % of red algae on a dry weight basis [30]. Regarding the industrial residues, their total carbohydrate content (34.9 ± 3.4 % dw) is lower than that of the crude alga, as expected from the prior agar extraction step. A higher carbohydrate content value of 44.2 % dw has been reported for *G. corneum* waste biomass following agar extraction [31], determined using the same quantification method. The protein content obtained for the crude *G. corneum* (14.8 ± 0.2 % dw) is also below the average values reported for red macroalga, generally in the 20–30 % dw range [6]. Even though these values are not comparable to those in high-protein vegetables, they are still close to those of protein content in cereals (7–15 % dw). In the industrial residues, as the carbohydrate fraction was partially withdrawn, the protein content (22.3 ± 1.1 % dw) was higher than that of the crude alga. A very similar value of 21 ± 1 % dw was obtained for residues of the same seaweed species by an elemental analysis and use of a 4.9 nitrogen-to-protein conversion factor [32]; a higher protein content value of 25 % dw had also been reported in *G. corneum* residues after agar extraction [33], however this might also result from the nitrogen-to-protein conversion factor of 6.25 used by the authors with the Kjeldahl method, higher than the 4.59 value [19] used in the present work.

The lipid content obtained is in agreement with the literature, which reported lipid fraction values between 0.7 % and 2.2 % dw in *Gelidium* species [34]. The ash fraction in red algae species is much higher than that found in terrestrial vegetables, partially due to the high sodium content caused by contact with seawater. A study with 34 red algae strains showed an average ash content of 22.9 ± 11 % dw [35], and the results of the present study fall within this range of values.

3.2. Evaluation of the protein extraction procedures

The amounts of extracted protein, in grams per 100 g of initial algal biomass dry weight, obtained after each step of the two extraction procedures, A and B, are shown in Fig. 1a and Fig. 1b, respectively.

Overall, the sequential water and alkaline extractions of procedure A yielded 3.16 ± 0.01 g protein/100 g algal biomass (corresponding to circa 15.4 % of protein recovery) for the industrial residues, and 1.78 ± 0.03 g protein/100 g algal biomass dw (circa 12.0 % of protein recovery) for the crude alga (Fig. 1a). It is worth mentioning that proteins yields are approximate, as two distinct protein quantification methods were used for the analyses (in partner laboratories), one for the algal biomasses, another for the sequential extracted fractions. These yield values agree with those of protein recovery yield reported earlier for *Gelidium corneum* extraction, 14.7 ± 2.3 % [36]. Comparing the water-soluble and the alkaline-soluble protein fractions in the crude alga, the protein amount extracted during the first two process steps was significantly higher ($p < 0.05$) than that recovered in the last two steps. This suggests that most proteins are water-soluble, particularly the protein-pigment complexes, like the phycobiliproteins, since the obtained extracts

Table 1
Solids and moisture contents and chemical compositions (dry weight basis) of the crude *Gelidium corneum* biomass and of its industrial residues, and of the protein products obtained from them by extraction procedures A and B with further concentration by UF/NF and freeze drying. Values are expressed as average ± SD ($n = 3$).

Analysed components	Seaweed raw materials		Extracts from the seaweed raw materials			
			Crude *G. corneum*		Industrial residues	
Names ➔	Crude *G. corneum*	Industrial Residues	*Procedure A*	*Procedure B*	*Procedure A*	*Procedure B*
(Acronyms) ➔	(G)	(RG)	(GA)	(GB)	(RGA)	(RGB)
Total solids (%)	92.8 ± 0.3	89.4 ± 0.2	95.2 ± 0.4	95.0 ± 0.5	92.7 ± 0.9	95.9 ± 0.1
Moisture (%)	7.5 ± 0.2	10.6 ± 0.2	4.8 ± 0.4	5.0 ± 0.5	7.3 ± 0.9	4.1 ± 0.1
Total carbohydrates (%dw)	62.1 ± 2.5	34.9 ± 3.4	36.3 ± 2.5	29.7 ± 1.2	23.3 ± 2.4	22.5 ± 0.9
Cellulose+starch	10.5 ± 1.4	22.4 ± 1.3	*nd*	*nd*	*nd*	*nd*
Agar	51.6 ± 1.2	12.5 ± 3.0	*nd*	*nd*	*nd*	*nd*
Protein (% dw)	14.7 ± 0.2	22.3 ± 1.1	19.6 ± 0.7	10.6 ± 0.03	19.6 ± 0.2	11.6 ± 0.7
Lipids (% dw)	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	8.0 ± 0.3	1.7 ± 0.1	7.0 ± 0.4
Ash (% dw)	19.8 ± 0.9	18.5 ± 0.7	37.8 ± 0.6	48.4 ± 0.4	30.4 ± 0.1	33.7 ± 0.1

nd – not determined

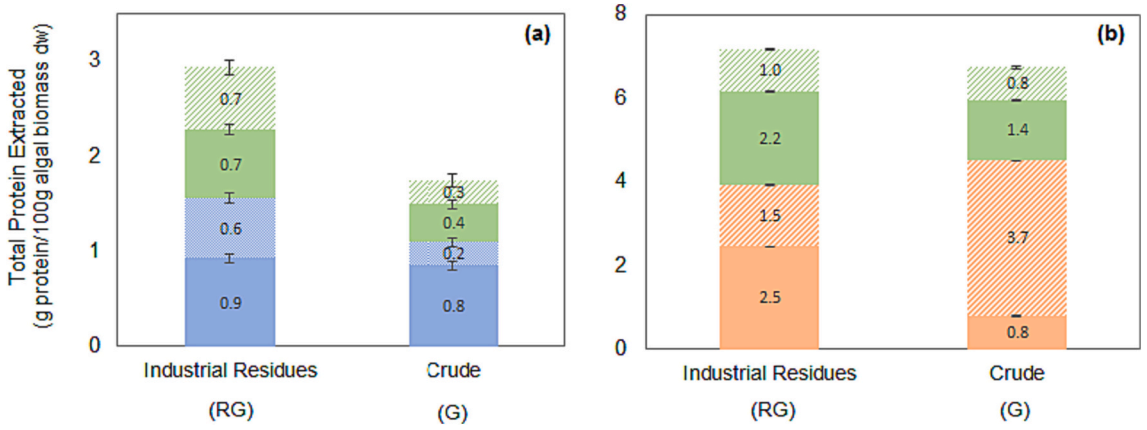

Fig. 1. Total protein extracted using procedure A (two water extractions and two alkaline extractions) (a) and procedure B (two enzyme-assisted water extractions and two alkaline extractions) (b), in grams of protein per 100 g of algal biomass (dry weight), from crude *G. corneum* and its industrial residues ($n = 3$). Values are expressed as average ± SD in the error bar ($n = 2$). ■ - first water extraction; ■ - second water extraction ■ - enzyme-assisted extraction with Celluclast®; ■ - enzyme-assisted extraction with Alcalase®; ■ - first alkaline extraction; ■ - second alkaline extraction.

exhibited a reddish colour.

The sequential extractions of procedure B, led to overall protein yields of 7.2 ± 0.01 g/100 g algal biomass (circa 36.5 % of protein recovery) and 6.7 ± 0.02 g/100 g algal biomass (circa 52.0 % of protein recovery) for industrial residues and crude alga, respectively (Fig. 1b). Overall protein extraction yields were significantly higher than those attained using procedure A ($p < 0.01$), but were not significantly different between the two algal biomasses ($p > 0.05$). It is however observable that both enzymes produced different outcomes for each type of biomass. The results suggest that the industrial agar extraction procedure markedly affects cell wall integrity, resulting in an improved action of the cellulase enzymes (Celluclast®) on the residues as compared to the crude alga. The results obtained with extraction procedure B agree with those previously obtained for *G. corneum* following a similar procedure (38.5 ± 3.5 % of protein recovery yield) [36].

The collected supernatants from each extraction procedure A or B were combined and the pH was adjusted to 7.0. To concentrate the extracts, ultrafiltration was performed to the extracts from procedure A, while nanofiltration was chosen to concentrate the extracts resulting from procedure B, as this method used Alcalase® and thus smaller peptides were expected to be present. The concentrated extracts were freeze dried to produce the final protein products which were subsequently characterized (data in Table 1).

3.3. Characterization of the protein products

3.3.1. Chemical composition

The compositions of the four protein products, namely the freeze dried extracts of crude *G. corneum* and its residues obtained through procedures A and B, are shown in Table 1.

The protein content found in the freeze dried extracts was lower than expected given the content in the starting materials and the contents in commercial protein-rich plant extracts (e.g., *Spirulina* meal, 71.3 % [37]. Analyses showed that the performed extractions also partially extracted the carbohydrate fraction. Procedure A followed by ultrafiltration for purification and concentration retained the proteins most efficiently for both biomass sources, whereas procedure B followed by nanofiltration resulted in products with lower protein contents than their respective biomass sources. Tighter NF membranes could have been used to concentrate the extracts; however, a significant decrease of the already low permeation fluxes would occur, increasing the process time and energy consumption.

The lipid content was significantly higher ($p < 0.01$) in the extracts obtained with procedure B than in the initial biomass sources, whereas this difference was not significant for procedure A. The enzyme-assisted extractions, performed at 50 °C, possibly enhanced lipid solubilisation.

The lowest ash content was found in the extracts obtained from the industrial residues biomass. Nevertheless, the ash content was significantly higher ($p < 0.01$) in the extracts than in their respective biomass sources, indicating that the extraction procedures added and co-extracted minerals to the resultant protein solutions.

3.3.2. Protein bioaccessibility

Protein products were in vitro digested, simulating the human digestion process, to assess protein bioaccessibility. The method was also applied to the algal biomasses, crude *G. corneum* and its industrial

residues, to verify the effect of the protein extraction procedures on this important parameter. Fig. 2 shows the obtained results.

All protein products showed improved protein bioaccessibility when compared to their respective initial biomass. The high fibre content (17–33 %) of the algal cell wall is considered to be one of the primary reasons for reduced protein digestibility in unprocessed seaweed, since it can block the access of digestive enzymes and particularly impair the action of proteases [38]. Similarly, phenolic compounds released by plant cells into their cell wall are known to become insoluble by reacting covalently with multiple local compounds including structural proteins [39], decreasing their bioaccessibility. Tibbetts et al. [40] showed a clear evidence of a negative correlation between in vitro protein digestibility and total phenolic content of wild and cultivated seaweeds. The algal industrial residues show significantly lower protein accessibility than the crude alga. This suggests that the agar extraction process causes structural differences in the alga components, jeopardizing digestive access to the protein fraction, and/or that the most accessible proteins had been already co-extracted with the agar, leaving in the residual biomass the most inaccessible proteins (i.e., those protected by the layer of fibres of the cell walls).

The results of procedure A on the crude *G. corneum* are comparable to those reported in the literature for this type of extract, e.g., values of 87 % and 95 % bioaccessibility were measured in protein extracts from *Ulva pinnatifida* and *Ulva pertusa*, respectively [41]. Even though lower bioaccessibility values were found in the present study for the protein products from crude *Gelidium* and *Gelidium* residues, they are still comparable to those measured in commonly consumed plant products, such as grains (69 %–84 %), legumes (72 %–92 %), fruits (72 %–92 %), and vegetables (68 %–80 %) [40]. Nevertheless, other extraction conditions could be investigated to improve these results, namely through the use of other enzymes and/or solution modulators.

3.3.3. Protein and peptide molecular size profiles of enzymatic extracts

To gain an insight on the molecular weight distribution of proteins, peptides and even free amino acids released from the algal biomasses during the performed enzyme-assisted extractions, profile analyses by size exclusion chromatography were undertaken. Two distinct SEC columns were used which differed in working volume and mainly in the effective molecular size range for molecular separation. Since the detection of elution peaks was done by UV spectrophotometry at 280 nm, and not by refractive index measurements, the profile results should not be influenced by the carbohydrate polymers present in the protein samples.

The obtained results are depicted in Fig. 3. It can be observed that none of the used size-exclusion media were perfectly adjusted to the molecular size distribution in the analysed products. Even so, the use of column Superdex 75 5/15 GL gave the indication that both products had high percentage of small-sized proteins, since <10 % of the proteins showed molecular weights (MW) above 30 kDa and around a third sat in the MW range of 7 Da to 30 Da. The column Superdex Peptide 10/300 GL did not contradict the former chromatographic result, as 38 % to 47 % of the peptide chains showed MW values above 200 Da. It also confirmed that the enzymes employed to assist protein extraction were effective, since 62 % to 53 % of the detected molecules had MW values smaller than 200 Da, i.e., they were mainly single amino acids or eventually 2-monomer oligopeptides. Apparently, no obvious differences resulted in the obtained peptide profiles between the two *Gelidium* products.

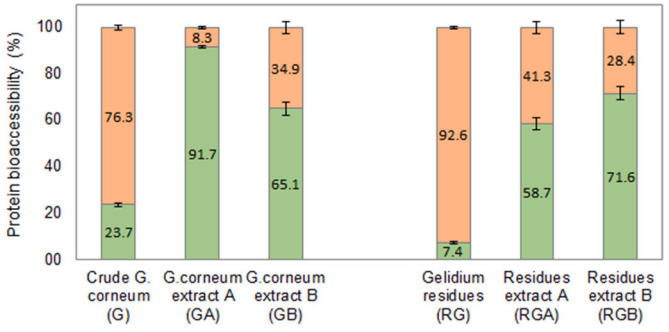

Fig. 2. Protein bioaccessibility (%) measured in the initial biomass (crude alga and industrial residues) and in the protein products obtained after their extraction using procedure A (two water extractions, two alkaline extractions) and B (two enzyme-assisted water extractions with Celluclast® and Alcalase®, two alkaline extractions). Values are expressed as average ± SD in the error bar ($n = 3$). ▯ - bioaccessible; ▯ - not bioaccessible.

3.3.4. Amino acid profiles in the protein extracts

Both types of algal biomass used in this study (crude *G. corneum* and its residues from industrial agar extraction) and the protein products extracted from the crude alga using both procedures A and B were analysed with respect to amino acid (AA) content. The obtained results are shown in Fig. 4 and Table 2, and therein are compared with the profiles of protein-based ingredients marketed for inclusion in animal diets. The individual AA quantifications are also shown in the supplementary material (Table S2).

These values indicate that EAA were in lower concentrations compared to NEAA in all products. Despite the quantitative differences observed for the individual amino acids, all products presented a complete profile of EAA and the two most abundant EAA were leucine and lysine. The third most abundant in G and RG was phenylalanine while in GA and GB was valine. Overall, methionine, and histidine presented the lower EAA levels. Regarding NEAA, the most abundant in all products were glutamine-plus-glutamic acid and asparagine-plus-aspartic acid.

In our study, the essential and non-essential amino acid profiles in the algae and analysed derived products were very similar (Fig. 4) and the percentage of EAA lies in the range of 34 %–39 % of the sum of 17 analysed AA. For comparison, AA profiles of common protein-based ingredients in fish farming feeds (animal diets in aquaculture) were retrieved from the literature [37]. Four typical ingredients were selected, namely, soy protein concentrate (SPC), soybean meal (SBM), Spirulina meal (SM) and United States menhaden fish meal (FM-M).

It should be noted that in Fig. 4 and Tables 2 and S2, the attributes of 'essential' and 'non-essential' amino acids considered those of human nutrition. However, arginine is known as a EAA for fish and other aquatic animals [42]. Therefore, in view of these species' nutrition, the percentage of EAA in the AA profile of our products is a little higher (to 39 %–44 %), as also in the foodstuffs for animal diets used for comparison.

Our methodology for AA analysis was based on acid hydrolysis, so that all AA were released from oligopeptides and proteins and could be individually quantified by ultra-high performance liquid chromatography. The downside of this less-complex procedure when compared to that used by Li and Wu [37] is that tryptophan (Trp) is degraded and asparagine (Asn) and glutamine (Gln) are converted to aspartate (Asp) and glutamate (Glu), respectively. Therefore, although our products do contain these natural AAs, in our AA-analytical reports the Trp was not detected and Asn and Gln were not distinguished from Asp and Glu, respectively.

The AA mass profiles of the selected feed ingredients (Fig. 4, C and D) seem to indicate that the proteins, peptides and free AA pools of these materials are richer in all the 17 (of the 20 standard) AA than the algal materials studied in this work (Fig. 4, A and B), even though the comparison was made on the basis of total protein mass. This result could be related to the different analytical methodologies or to a higher content in the algal materials of AA other than the quantified standards. Nevertheless, the percentage of essential AA in the selected feed ingredients is very similar to that measured in our seaweed and seaweed-derived products (in the range of 34 %–39 %; Table 2), albeit the proportions of Asn plus Asp and Gln plus Glu seem particularly high. Also,

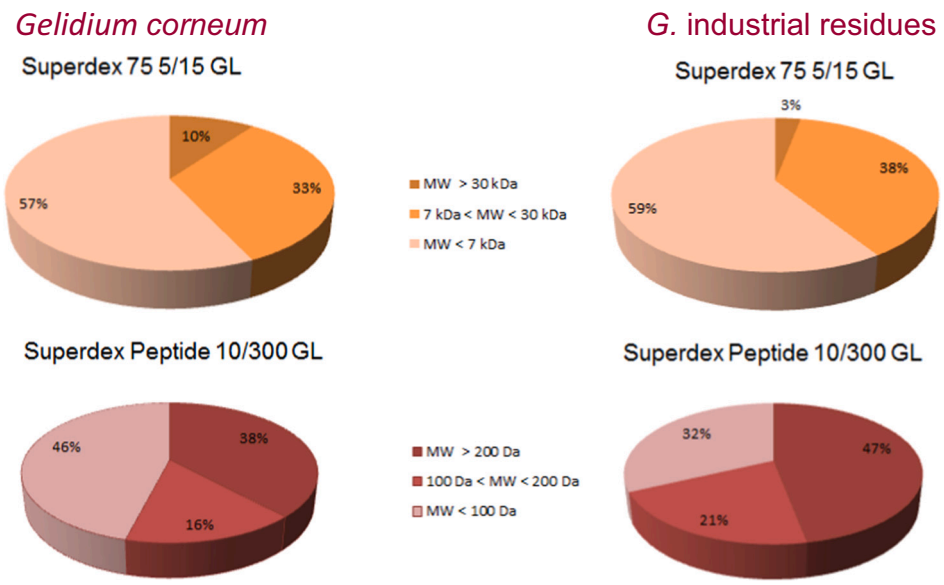

Fig. 3. Molecular weight profiling of seaweed protein extracts by size exclusion chromatography (SEC). The analysed products were obtained through enzyme-assisted extraction (procedure B) followed by nanofiltration and freeze drying, performed on crude *G. corneum* (left pie charts) or on their residues after industrial agar extraction (right pie charts). Top pie charts – protein characterization SEC; Bottom pie charts – peptide characterization SEC.

according to the 'BRF Ingredients News ' [43], lysine is a limiting AA in fish diet. Our analytical results (*c.f.* Table S2; Supplementary material) indicate that the lysine contents of the crude *Gelidium* and of the *Gelidium* residues (but not those of the extracts) fall in the range of 5.5–6.5 % of total protein, as recommended by BRF Ingredients.

Another aspect that needs to be pointed out is the very high total protein content (52 %–71 % dw; Table 2), of the protein-rich feed ingredients selected for comparison, which emphasizes the need to increase the protein content in the products extracted from *Gelidium* biomass, if one intends to meet market demands.

3.3.5. Antioxidant and chelating activities

The functional properties determined for the protein products obtained in the present study from crude *G. corneum* and its industrial residues are depicted in Table 3.

The biological activities evaluated in these products lie within the range of values found for many fish hydrolysates [28]. However, these authors mention specialty fish hydrolysates with EC_{50} values for antioxidant activity (0.76 ± 0.34 mg/mL and 1.12 ± 0.03 mg/mL, for DPPH and ABTS, respectively) which are one order of magnitude lower than those found in the present study, showing that much lower amounts of such hydrolysates are needed to retain antioxidant activity, when compared to our products. Reducing power levels of our seaweed derived extracts are also one third to one half of those measured for most of the fish hydrolysates evaluated in the cited reference. However, the water extracts from industrial residues of *G. corneum* (RGA in Table 3) did show very good ferrous ions chelation properties, comparable to and even better than the best reported for the fish hydrolysates, which were 0.26 ± 0.00 to 0.53 ± 0.01 mg/mL [28]. The antioxidant and chelating properties were also evaluated by Sapatinha et al. [29] in extracts resulting from the contact of red and brown macroalgae with hot water and with several warm enzyme solutions. The extracts thereof also showed activities comparable with the ones found in the present work. The obtained values are however dependent on the extraction conditions and specific alga. It is relevant to mention that those authors analysed extracts from two red seaweeds (one of them also used for hydrocolloid production), but none was from the *Gelidium* genus. In those studies, there was no attempt to enrich the protein content of extracts; all non-volatile extracted molecules were kept in the extracts after dehydration. Phenolic compounds from the macroalgae are known to have good antioxidant capacity and some may have been co-extracted with the proteins. In the present work, the contents of such polyphenols were certainly reduced in the final powdered products due to the implemented UF/NF concentration procedures. Even though the levels of functional properties in our extracts are lower or similar to those measured in other animal feed ingredients, seaweeds are clearly abundant resources worth exploiting for these functionalities, since they do not rely on wild fish captures, like the majority of sea-derived ingredients for aquaculture.

3.4. Upgrading of protein extracts

3.4.1. Carbohydrate co-elution in protein extraction

Carbohydrate co-elution occurred during both of the tested extraction procedures, as evidenced by the carbohydrate content present in the obtained protein products (Table 1). Even if the resulting extracts exhibit attractive nutritional value and bioactivity levels, when it comes to *Gelidium corneum* processing in a biorefinery context, the conservation of the agar fraction is imperative, as well as the valorisation of other carbohydrate components, in order to design a "no waste" agar extraction industrial process.

Samples were withdrawn from the residual biomass pellets obtained after each extraction step in both the A and B procedures applied to crude *G. corneum*, and oven-dried. Carbohydrate quantification by the NREL method was performed on the dry samples and the results are given in Fig. 5.

The most substantial losses in the cellulose contents (eventually including starch) occurred during the alkaline extractions in both procedures. Some losses also occurred in the enzyme-assisted extraction step with Celluclast® pertaining to procedure B. It should be noted that the cellulose content (eventually including starch) measured on the crude *G. corneum* was 10.5 ± 1.4 % dw (Table 1). In addition, a significant difference ($p < 0.01$) was noted between the alkaline re-extraction steps of the two procedures, indicating that the cellulose (+ starch) content is significantly different in the two final residues.

Each step in both protein extraction procedures produced some agar loss. No significant differences were observed ($p > 0.05$) when comparing the agar contents (Fig. 5) obtained after water extraction (procedure A) and after enzyme-assisted extraction (procedure B). This suggests that the enzymatic procedure performed at 50 °C did not

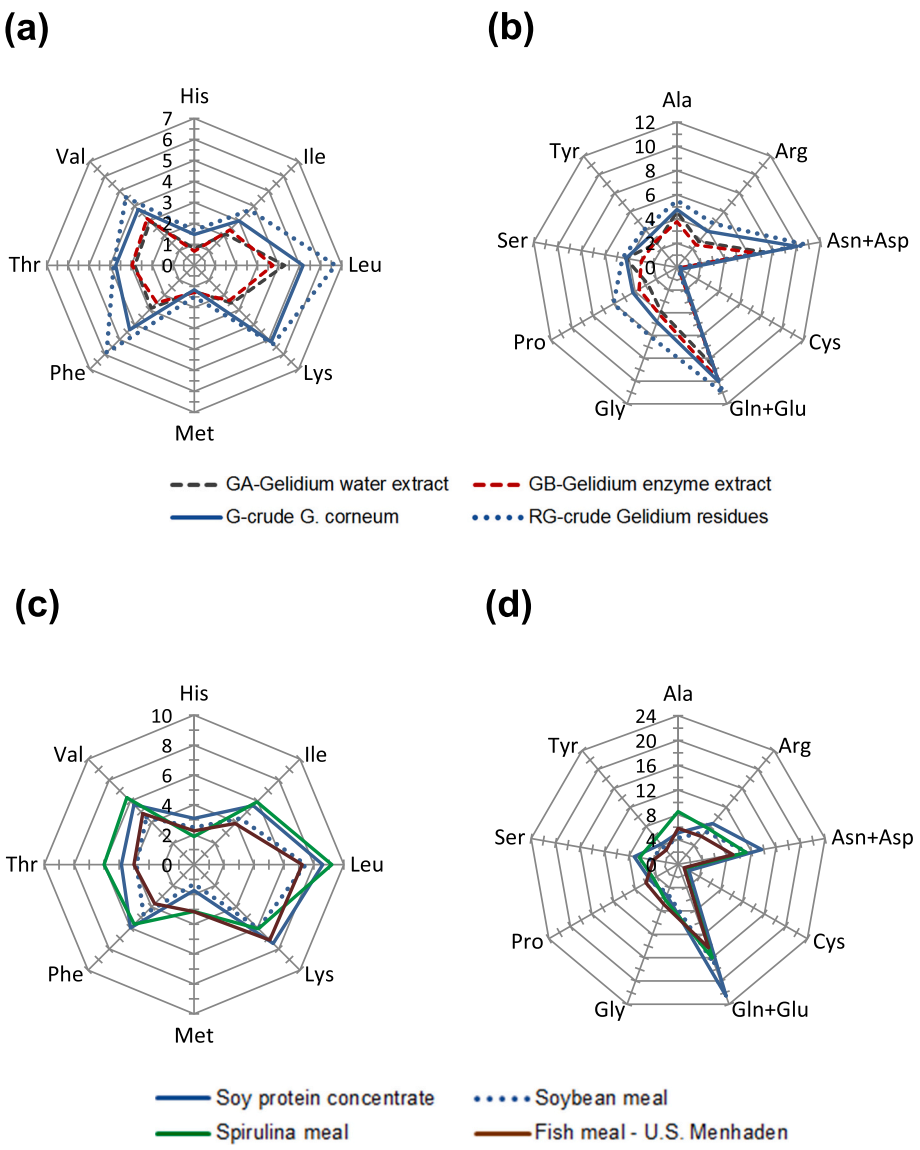

Fig. 4. Amino acid profiles of *G. corneum* biomasses in this study and their extracts (top graphs) and of protein-rich animal feedstuffs (data retrieved from Li and Wu [37]). Plotted data: essential amino acids (a; c) and non-essential amino acids (b; d). Values in gAA/100gprotein.

enhance agar co-elution, presumably because the optimal conditions for agar extraction from *Gelidium corneum* are a temperature close to the water boiling point and a pH value close to 6 [16]. No significant difference is observed in the agar content of the algae residue after the two protein extraction procedures ($p > 0.05$), with on average 8 % (g agar/g initial algae biomass dw) being lost during both procedures.

Despite the reduced agar content in the alga biomasses after the tested protein extraction procedures, when compared to the initial crude seaweed, the values are still similar to those found in other *Gelidium* species exploited for industrial agar extraction [44]. The properties of the hydrocolloids remaining in the biomasses would however need to be investigated to ascertain the true impacts of the protein extraction steps when applied prior to agar extraction from *G. corneum*.

3.5. Protein precipitation

Aiming to reach even higher protein concentrations in the extracts that had already been concentrated by UF or NF, and also to reduce time and energy requirements for final dehydration into powder form, protein precipitation trials were carried out, comparing salting-out with ammonium sulfate (Fig. 6) with acidifying the protein solution to pH values in the range of 7.0 to 1.0 (Fig. 7), targeting the isoelectric points of proteins and peptides in the extracts.

3.5.1. Ammonium sulfate precipitation

To determine the ammonium sulfate concentration able to induce a protein precipitation yield suitable for further processing (purification and dehydration), the pooled extracts obtained from the crude alga through procedure A (without following concentration by UF) were supplemented with increased ammonium sulfate concentrations, to achieve values in solution of 70 % to 85 % of the saturation concentration. The protein precipitation yield values were calculated from protein content measurements (Lowry method) in the initial extract and in the supernatants after separation of the precipitates by centrifugation (Fig. 6).

A saturation of 85 % led to the highest protein precipitation yield (45.5 ± 0.4 %), and significant differences were observed between the yields obtained with saturation levels of 70 % and 85 % ($p < 0.01$), 75 % and 85 % ($p < 0.01$) and 80 % and 85 % ($p < 0.05$). These results are also significantly higher than those reported before for *Gelidium corneum* protein precipitation after enzyme-assisted and alkaline extraction (identical to procedure B of the present study), which was of 24.6 ± 4.9

Table 2
Protein and amino acid contents of *G. corneum* biomasses and their extracts (water – Procedure A; enzymatic – Procedure B), and of marketed feedstuffs for animal feeds. Values are expressed as average ± SD ($n = 3$).

Materials	Protein content	ΣEAA[a]	ΣNEAA[b]	ΣAA[a+ b]
	(g/100 g dw)	(g $_{AA}$/100 g $_{protein}$)		
Gelidium biomass				
Crude *G. corneum* (G)	14.8 ± 0.2	27.8 ± 0.5	45.8 ± 1.0	73.6 ± 1.5
G. corneum industrial residues (RG)	22.3 ± 1.1	33.2 ± 1.0	53.1 ± 1.3	86.3 ± 2.2
Gelidium extracts				
Crude *G. corneum* water extract (GA)	19.6 ± 0.7	19.6 ± 0.6	36.9 ± 0.9	56.5 ± 1.5
Crude *G. corneum* enzymatic extract (GB)	10.6 ± 0.7	19.1 ± 1.2	36.4 ± 3.2	55.5 ± 4.3
Feedstuffs for animal diets[c]				
Soy protein concentrate	70.8 ± 0.1	43.0 ± 0.4	68.5 ± 0.3	111.5 ± 0.7
Soybean meal	52.0 ± 0.1	31.9 ± 0.2	56.6 ± 0.3	89.9 ± 0.9
Spirulina meal	71.3 ± 0.1	44.2 ± 0.3	62.1 ± 0.4	106.3 ± 0.7
Fish meal – U.S. menhaden	67.3 ± 0.1	35.6 ± 0.3	51.6 ± 0.4	87.2 ± 0.7

[a] ΣEAA – sum of the essential amino acids - His, Ile, Leu, Lys, Met, Phe, Thr, and Val.
[b] ΣNEAA – sum of the non-essential amino acids – Ala, Arg, Asn + Asp, Cys, Gln + Glu, Gly, Pro, Ser, and Tyr.
[c] Data retrieved from [37]; only values pertaining to the amino acids also analysed in this study were summed-up.

Table 3
Biological activities measured on *G. corneum* protein extracts (water extraction – Procedure A; enzymatic extraction – Procedure B). Antioxidant activities (ABTS, DPPH), reducing power (measured on Fe^{3+}) and metal ion chelation power. Values are expressed as average ± SD ($n = 3$).

	ABTS EC_{50} (mg/mL)	DPPH EC_{50} (mg/mL)	Reducing Power A0.5 (mg/mL)	Cu^{2+} chelation EC_{50} (mg/mL)	Fe^{2+} chelation EC_{50} (mg/mL)
Extracts from crude *G. corneum*					
GA	15.66 ± 1.99	42.01 ± 10.41	15.27 ± 0.8	**3.52 ± 0.16**	0.25 ± 0.01
GB	23.73 ± 0.52	32.16 ± 3.0	15.21 ± 0.98	5.20 ± 0.19	0.50 ± 0.01
Extracts from *G. corneum* industrial residues					
RGA	9.92 ± 0.23	56.60 ± 4.83	**9.20 ± 0.12**	9.45 ± 0.65	**0.21 ± 0.0**
RGB	**8.56 ± 0.50**	**15.22 ± 0.13**	10.87 ± 0.35	4.72 ± 0.97	1.40 ± 0.04

Note: values in bold are the best activity levels for the specific test among the seaweed extracts analysed.

% at 85 % saturation [36]. This result could have been due to the extract from procedure B containing small peptides from protease action, hampering protein-protein interactions during salting-out.

3.5.2. pH-shift precipitation

Extracts obtained from crude *G. corneum* using procedures A and B (without UF/NF concentration) were also used in protein precipitation trials by the pH-shift. Protein precipitation yield values obtained for each adjusted pH value are shown in Fig. 7.

Regarding the extract from procedure A, the highest precipitation yields were obtained when the pH was adjusted to 2.0 and 3.0, namely 33.9 ± 0.4 % and 30.2 ± 0.9 %, respectively, which do not differ significantly ($p > 0.05$). The other values were significantly different from these ($p < 0.05$), and the yield values decreased with the increase of the pH value. This method was applied before to crude *Saccharina latissima* by Veide Vilg and Undeland [17], who observed that 34.5 % of proteins which were solubilized at pH 12 were precipitated at pH 2. Although ammonium sulfate salting-out at 85 % saturation yielded 45.5 % ± 0.4 % of protein precipitation (Fig. 6), the precipitate still requires the desalting process, where more protein mass is lost.

Using the extract from procedure B, it is impossible to identify a consistent trend in the relation between the pH value and the protein precipitation yield, probably because of the use of the Alcalase® enzyme. Its protease activity results in a variety of peptides and amino acids with a large diversity of isoelectric points (pI), making it impossible to precipitate >37 % of the initial protein using a single step of pH adjustment. Nevertheless, pH 7.0 yielded the best result, with 36.6 ± 0.6 % of the protein fraction precipitated, a result which is not statistically different ($p > 0.05$) from the results at pH 4.0 and pH 6.0, namely 33.8 ± 0.6 % and 33.3 ± 2.2 %, respectively.

Naseri et al. [45] also used the pH-shift method to recover the protein fraction from *Palmaria palmata* after an enzyme-assisted extraction with Celluclast® and Alcalase®, followed by alkaline treatment, alike to procedure B in the present work. Adjusting the pH value to 2, allowed the recovery of approximately 20 % of the protein, a lower yield than that obtained in the current work (30.7 ± 2.0 %).

Comparing protein precipitation from crude *G. corneum* extracts obtained through procedure B, using the pH-shift process (present study) or ammonium sulfate precipitation [36], more promising results were obtained with the pH-shift method, since 85 % ammonium sulfate saturation only resulted in 24.6 ± 4.9 % of protein recovery.

3.5.3. Consecutive pH-shift precipitation

Consecutive pH-shifts were implemented to optimize the recovery of proteins by precipitation, using extracts obtained from crude *G. corneum* by water extraction (procedure A) and enzyme-assisted extraction (procedure B). The objective was to investigate the possibility of recovering protein fractions with different pI values.

Using a one-time pH adjustment, proteins precipitated at intermediate pH values between the initial and target value would re-solubilize as the latter deviated from their pI, unless they were separated while still insoluble. From the results of the simple pH-shift precipitation performed at 1 pH unit intervals (Fig. 7), the best range of pH values corresponding to the highest precipitation yields were identified for both extracts. Therefore, the pH ranges selected for the pH-shift precipitation experiments in consecutive mode were 4.0–1.5 and 7.0–3.5, in 0.5 pH unit steps, for extracts from procedures A and B, respectively, The obtained results are shown in Fig. 8a and Fig. 8b.

The overall, cumulative protein mass recovered and corresponding precipitation yield was significantly higher in the consecutive mode, for both extracts, with respect to the simple pH-shift method. Using the pooled extract from procedure A, an excess of 2 ± 0.2 mg of protein were recovered, from pH 4.0 to 1.5, resulting in a precipitation yield approximately 11 % points higher than that of the simple pH-shift method. With the pooled extract from procedure B, a total amount of 29 ± 0.8 mg of recovered protein was reached, from pH 7.0 to 3.5, which is twice the highest precipitated amount obtained with the simple pH-shift method, and represents an increase of 40 % points (Fig. 8b) over the best yield value of 33.9 ± 0.4 % in the latter method (Fig. 7).

The combination of extraction procedure B, a protease-assisted procedure, with pH-shift precipitation in consecutive mode seems interesting for forthcoming studies, since it permits much higher protein extraction yield together with a very good precipitation yield of the extracted proteins, thus promoting a marked reduction in the subsequent dehydration cost. Furthermore, since the addition of chemicals is much reduced with respect to the salting-out method, possibly no

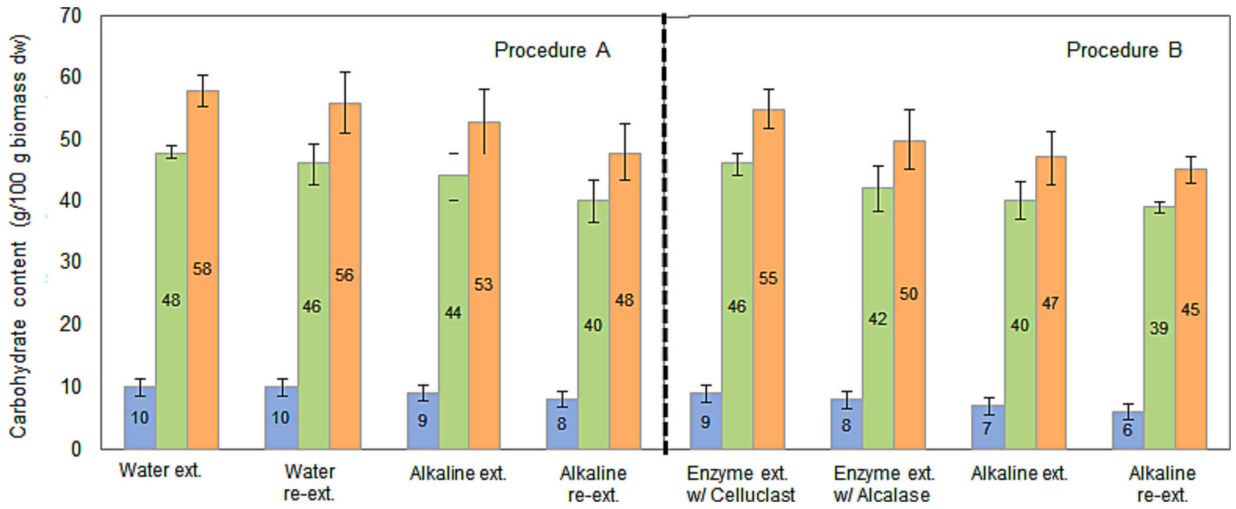

Fig. 5. Cellulose (eventually including starch), agar and total carbohydrate contents, in grams of carbohydrate per 100 g of initial crude *G. corneum* biomass (dw), calculated from analyses to the residual pellets after each protein extraction step from procedures A (two water extractions, two alkaline extractions) and B (two enzyme-assisted water extractions with Celluclast® and Alcalase®, two alkaline extractions). Values are expressed as average ± SD in the error bar ($n = 3$). - Cellulose (+ starch); - Agar; - Total.

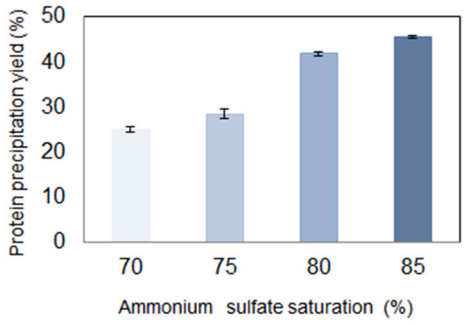

Fig. 6. Protein precipitation yields obtained from crude *G. corneum* extracts (procedure A) by salting-out using different ammonium sulfate concentrations. Protein content was quantified in the respective supernatants after centrifugation using the Lowry method. Values are expressed as average ± SD in the error bar ($n = 3$).

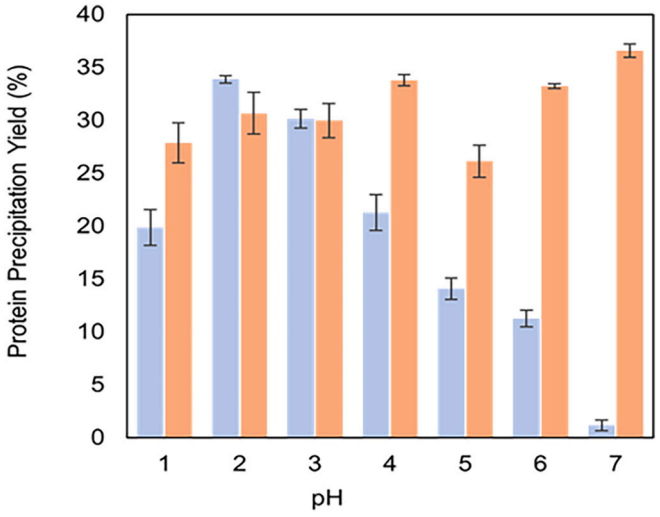

Fig. 7. Protein precipitation yields obtained from crude *G. corneum* extracts (procedures A and B) by pH-shift. Protein was quantified in the respective supernatants after centrifugation using the Lowry method. Values are expressed as average ± SD in the error bar (n = 3). - extract from procedure A; - extract from procedure B.

desalting step is needed. Nevertheless, it should be noted that this consecutive pH-shift process is significantly more time-consuming than simple pH adjustment, and more difficult to adapt to an industrial-scale process. A transition to continuous operation, involving a battery of precipitation-separation units, should be considered. Also, confirmation of the protein recovery results should first be obtained with the batch-wise method described herein scaled up to a minimum of 500 mL working volume in the precipitation stage.

4. Conclusions

Protein extractions from *G. corneum*, crude and its industrial residues, performed by two different approaches – with and without enzyme assistance – were conducted on 75–100 g of dry algae, rendering initial extraction pools of 4.5–6.0 L, respectively. The dry powders obtained, after concentration by membrane filtration and freeze drying, exhibited characteristics suitable for inclusion in aquaculture feeds taking into account the protein contents and their bioaccessibilty, amino acid profiles, and some additional interesting functional properties.

Similar extraction yield values were obtained for the different starting biomasses, dependent on the extraction process used (A or B). Sequential water (without enzyme assistance) and alkali extractions (procedure A) permitted protein recoveries of circa 12.0 % of the original protein in the crude alga and circa 15.4 % of total protein in *G. corneum* industrial residues. For sequential extractions in which the first water extraction steps were enzyme-assisted (procedure B), protein recoveries were circa 36.5 % and 52.0 % for the industrial residues and crude alga, respectively. Despite the higher recovery yields in the latter process, future enzyme-assisted process designs could probably benefit of a different enzyme combination when using the industrial residues, because the Alcalase® enzymatic step did not facilitate the protein release as much as it did for the crude alga. The use of Alcalase® would have also to be decided based on the final use of the protein-rich powder to be produced. It has protease activity which is necessary when small peptides and free amino acids are the desired nutrients, rather than large peptides or the whole proteins. This is the case of nutrition for fish larvae in the fish-farming nurseries or even in a pharmaceutical context for the production of human therapeutic nutrition. However, production cost-effectiveness is a must. In that regard, one possible way of conducting

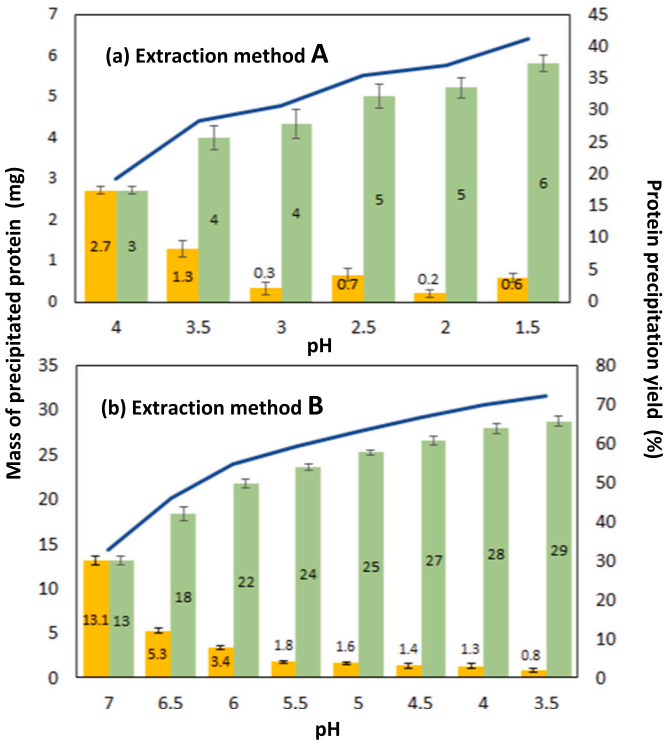

Fig. 8. Consecutive pH-shift protein precipitation profiles using extracts produced from crude *G. corneum*. Values in the vertical axes correspond to precipitated protein mass (mg; yellow bars; left axis), to cumulative protein mass (mg; green bars; left axis) and to cumulative protein precipitation yield (%; blue line; right axis), all obtained after consecutive pH-shifts starting from the highest pH value of the range (pH 4.0 in (a); pH 7.0 in (b)), down to the pH values indicated in the horizontal axis. (a) extract from procedure A; (b) extract from procedure B. Protein was quantified in the respective supernatants after centrifugation using the Lowry method. Values are expressed as average ± SD in the error bar ($n = 3$).

further research would be to use only cellulase activity during an enzyme-mediated extraction, followed by a protein concentration by pH-shift precipitation. Then, a protease treatment would be applied to the recovered and re-dissolved protein, for a controlled hydrolysis of the proteins. However, this procedure would have to be tested, since the extraction of structural proteins covalently bound to the seaweed cell wall might be impaired if protease activity is not used during the extraction step. In both extraction procedures A and B using crude *Gelidium,* a fraction of the alga carbohydrates was lost in each protein extraction step, with a total loss of approximately 20 % of the total carbohydrate content, sustaining the high carbohydrate content found in the protein products.

The percentage of bioaccessible proteins in the crude seaweed and in its industrial residues without further processing is very low (in vitro analyses) – values of 23.7 % and 7.4 % for crude alga and for its residues, respectively. The extraction processes permitted to obtain protein powder products with considerable higher bioaccessibilities (best values: 91.7 % for extracts from procedure A over crude seaweed; 71.6 % for extracts from procedure B over their industrial residues). The total protein content of the products obtained by extraction, membrane filtration (UF or MF) and final freeze drying was low (up to 22 g/100 g dw), hardly competing with the typical values of protein-rich ingredients found in the market for aquafeed formulations (up to 70 g/100 g powder dw).

The measurement of amino acid profiles for the two types of extracts produced from crude *Gelidium corneum* permitted a comparison with common protein rich aquafeed ingredients, namely some of those based on wild-fish captures. The contents of the several natural amino acids (in g/100 g protein) in these two extract products are similar to those in the referred competing aquafeed ingredients, when the differences in analytical methods employed are taken into account. Also similar are the ratios between total essential and total non-essential natural amino acids among the several protein powders.

The extract products obtained herein showed interesting values for antioxidant, reducing power and iron and copper ions chelating properties, even though the extracting processes were not designed to especially extract and/or preserve those properties.

Complementary protein precipitation methods were assessed on the extracts obtained from the crude *G. corneum*. The precipitation yield obtained with 85 % saturated ammonium sulfate solution (≈46 %) was higher than that of single pH-shift but it still requires a subsequent desalting process, where some protein is also lost. The pH-shift precipitation seems to be a good alternative, as it is simple, straightforward to implement at industrial scale and does not require high reagent consumption. In fact, single pH step allowed to precipitate up to 34 and 37 % of the extracted protein, while consecutive pH-shifts allowed reaching 41 % and 72 % from extraction procedures A (non-enzymatic) and B (enzyme-assisted), respectively. These methods show promise for attaining higher protein contents in future process designs for aquafeed ingredients from the macroalga.

In conclusion, this work proves the potential of using *Gelidium corneum* for "zero waste" industrial processing, providing insights into the valorisation of this macroalgae species that go much beyond agar extraction. Analysis of the process economics of seaweed-derived protein ingredients is however required to establish the impact and affordability of these products in the context of circular economy.

CRediT authorship contribution statement

Marília Mateus: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Rita Mão de Ferro:** Writing – original draft, Investigation, Formal analysis, Data curation. **Helena M. Pinheiro:** Writing – review & editing, Project administration. **Remígio Machado:** Investigation. **M. Manuela R. da Fonseca:** Writing – review & editing, Funding acquisition. **Maria Sapatinha:** Investigation. **Carla Pires:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Isa Marmelo:** Writing – review & editing, Investigation, Formal analysis, Data curation. **António Marques:** Writing – review & editing, Methodology, Conceptualization. **Maria Leonor Nunes:** Methodology, Funding acquisition, Conceptualization. **Carla Motta:** Investigation. **M. Teresa Cesário:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability

The authors declare that the data supporting the findings of this study are available within this paper, including its Appendix (supplementary materials).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103420.

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