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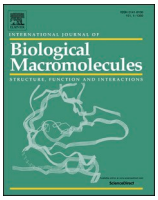

Red seaweed biorefinery: The influence of sequential extractions on the functional properties of extracted agars and porphyrans

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ABSTRACT

Red seaweeds are exploited for their hydrocolloids, but other fractions are usually overlooked. In a novel approach, this study aimed to evaluate cold-water (CWE), ethanolic (EE), and alkaline (SE) extractions, alone and in sequence, to simultaneously: i) decrease the hydrocolloid extraction waste (valorizing bioactive side-streams and/or increasing extraction yield); and ii) increase the hydrocolloids' texturizing properties. It is the first time these extractions' synergetic and/or antagonistic effects will be accessed. For *Porphyra dioica*, a combination of CWE and EE was optimal: a positive influence on the melting temperature (increasing 5 °C to 74 °C) and sulphate content (a 3-fold reduction to 5 %) was observed, compared to a direct porphyran extraction. The same was observed for *Gracilaria vermiculophyla*, recovering two additional bioactive fractions without impacting the hydrocolloid's extraction (agar with 220 g/cm^2 gelling strength and 14 % yield was obtained). The sequential use of CWE, EE, and SE was the most beneficial in *Gelidium corneum* processing: it enhanced agar's texturizing capacity (reaching 1150 g/cm^2, a 1.5-fold increase when compared to a direct extraction), without affecting its 22 % yield or over 88 % purity. Ultimately, these findings clarified the effects of cascading biorefinery approaches from red seaweeds and their pertinence.

1. Introduction

Red seaweeds and their products are currently commercialised as sushi ingredients, gelling agents, grazing fodder, and colourants, among others. Due to their important role in the food, cosmetic, biotechnological, and aquaculture industries, their market value is in the million-dollar range [1]. With the growing concern for climate change and the need to achieve a carbon-neutral society, in the last years, attention towards integral biomass use and zero-waste processing has been rising. There have been some reports targeting biorefinery approaches for their valorisation. However, the available literature refers almost exclusively to the use of raw biomass to produce biofuels and chemicals (such as acetone, butanol, ethanol, biogas, biodiesel, 5-hydroxymethylfurfural and furfural, levulinic, and formic acids), in processes using mild acids or alkaline solvents [2–7]. Although valid from the point of view of offering alternatives to solve the current fuel demand, this direct conversion of whole seaweed to biofuels often dismisses the valorization of marine algae hydrocolloids, the major industrially exploited product from these biomasses.

Agar is commercially extracted and used as a food-safe additive due to its gelling and thickening properties [8]. This polysaccharide is composed of agarose (a linear chain of 3-o-substituted β-D-galactopyranosyl units joined by (1 → 4) linkages to 3,6-anhydro-α-**L**-galactopyranosyl units, being formed by a repeating unit called agarobiose) and agaropectin with sulphate, pyruvate, and other sugar residues [8]. Both fractions present the same galactose backbone, but agaropectin presents a more complex structure since many variants for agaropectin can occur, making it impossible to show one representative chemical formula [9]. Agar has a high gelling capacity because of its low sulphation degree. While the extraction of food-grade agar from *Gelidium* does not require any pretreatment (although for some high-end applications, it is used to increase purity), with *Gracilaria*, a strong alkali modification is employed before the hot-water extraction [10]. A subvariety of agar called porphyran (present in *Porphyra* species) has a high sulphation degree, and a tendency to display methylations on the D-galactopyranosyls that decrease or inhibit its gel formation ability [8]. More

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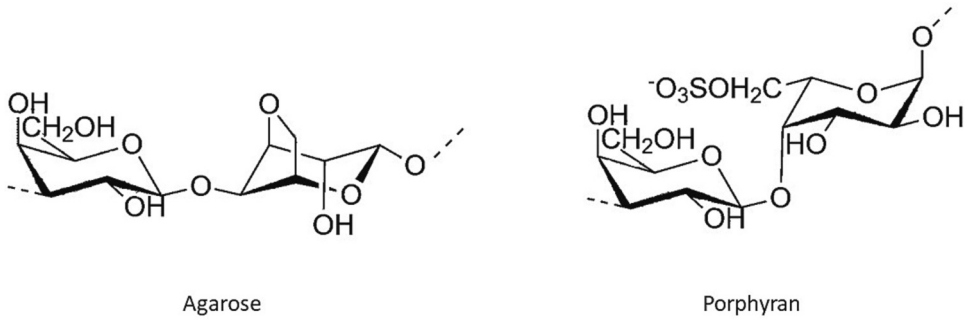

Fig. 1. Structure of the main repeating units in the agarophyte seaweeds studied (left – representation of agarose, the main polysaccharide unit in *Gelidium corneum* and *Gracilaria vermiculophyla*; right - representation of porphyran, the main polysaccharide unit in *Porphyra dioica*).

information about these structures can be observed in Fig. 1 (adapted from Rodríguez Sánchez and coworkers [11]). Scanning electron microscopy of both hydrocolloids revealed they possess an irregular network with hexagonal (and a few rectangular) pores, with the average number of larger pores increasing with increasing the ionic strength [12,13].

Because of their commercial value, several reports regarding the optimization of extraction methods and chemical modifications for the direct extraction of hydrocolloids from these seaweeds have been published, as well as their effects on the bioactive and functional properties of the extracts [14–18]. Alongside these extractions, several studies have been conducted regarding the valorization of hydrocolloid-depleted seaweed biomass. The lower-grade and/or remaining extracted polysaccharides have applications such as the production of natural sweeteners, bio-packaging, and fermentation media [19–23]. Protein and phenolic compound valorization are also possible approaches [19–23].

Nevertheless, not all sequential valorization approaches begin with hydrocolloid extraction. There are reports such as preliminary extraction of proteins [24], phycobiliproteins (R-phycoerythrin) [25], sequential extraction of phycobiliproteins and lipids [26], sequential extraction of iodine, phenolic compounds, carotenoids, chlorophyll, and lipids [27], extraction of lipids (mainly polyunsaturated fatty acids) and phenolics [28]. Despite their existence, they focus mostly on the valorisation process's possible economic and environmental impact, but not on each processing step's influence on the extracted fractions' characteristics and functionality, only considering crude extraction yields [29]. In fact, to the best of the authors' knowledge, only one of the reports investigates the effects of phycobiliprotein recovery on the gelling abilities and composition of the subsequently recovered agar [30]. This results in a substantial knowledge gap that can significantly hinder the much-recommended integral valorization of red seaweeds.

Overall, considering that the phycocolloid industry produces high amounts of by-products that currently end up in low (or no) added-value applications, there is a noteworthy demand for efficient zero-waste approaches that can simultaneously address the environmental issues of waste disposal, but also the economic feasibility of seaweed exploitation. This raise in the red seaweed's value can originate from the valorization of previously discarded fractions, but also from the increased market value of the hydrocolloids, due to their improved functionality.

Thus, this work aims to understand the effects of protein, pigment, and/or lipid recovery on the structure, molecular weight, texturizing, and rheological behaviour of agar and porphyran extracted from representative red seaweeds. Furthermore, the synergetic and/or antagonistic effects of a combination of these prior extractions will be accessed for the first time. Finally, sequential valorization strategies will be suggested for each biomass, targeting the most efficient recovery yield without compromising their commercial value.

2. Materials and methods

2.1. Raw materials

Seaweeds used in this study are from the same lot as the ones previously characterized by Teixeira-Guedes, Gomes-Dias and co-workers [31,32]. Briefly, the composition of the biomasses was determined using calcination (for ash) [33], Kjeldhal method (for protein) [34], quantitative acid hydrolysis (for carbohydrates and acid insoluble residues) [35] and Bligh and Dyer method (for lipids) [36], according to the methods and modifications reported in previous works.

Gelidium corneum (formerly known as *Gelidium sesquipedale*) biomass (kindly supplied by Iberagar-Sociedade Luso-Espanhola de Colóides Marinhos S.A. (Coina, Portugal)) was manually cleaned, washed, cut into smaller pieces, oven-dried at 40 °C, and stored in vacuum-sealed bags at room temperature in a dry and dark place until further use. The biomass used has a reported composition of 14.78 ± 0.73 % ash, 14.52 ± 0.09 % crude protein, 1.47 ± 0.15 % crude lipids, 1.59 ± 0.11 % acid insoluble residue, 37.88 ± 6.71 % galactan and 14.99 ± 1.88 % glucan [32].

Gracilaria vermiculophyla (supplied by AlgaPlus (Ílhavo, Portugal)) was prepared and stored in the same way, having a reported composition of 28.3 ± 0.18 % ash, 15.9 ± 0.07 % crude protein, 1.24 ± 0.06 % crude lipids, 0.92 ± 0.15 % acid insoluble residue, 26.27 ± 0.30 % galactan, 6.14 ± 0.26 % glucan, 4.73 ± 0.05 % arabinan and 2.00 ± 0.01 % uronic acids [31].

Porphyra dioica (supplied by AlgaPlus (Ílhavo, Portugal)) was purchased ready to use. The seaweed has a reported composition of 20.8 ± 0.55 % ash, 26.7 ± 0.07 % crude protein, 2.00 ± 0.14 % crude lipids, 0.92 ± 0.17 % acid insoluble residue, 21.80 ± 0.21 % galactan, 4.59 ± 0.02 % arabinan, 1.47 ± 0.14 % uronic acids and 0.95 ± 0.20 % glucan [31].

2.2. Sequential extraction conditions

Each extraction sequence was initiated with 10 to 12 g of seaweed (on a dry weight basis) and 300 mL of the different solvents. The mixture was then subjected to cold water extraction (CWE), ethanolic extraction (EE), and/or alkaline solvent extraction (SE), followed by hot water extraction. Biomass was washed/neutralized between extractions to remove impregnated residual solvents, reducing the risk of their influence on the subsequent steps. At the end of the extraction cascade, residues were dried and weighed to determine solubilization yield.

2.2.1. Cold water extraction (targeting phycobiliprotein recovery)

Water extraction was performed using distilled water at room temperature (between 20 °C and 25 °C), overnight (circa 16 h), under orbital agitation, and protected from the light. Samples were filtered, and the solid fraction was subjected to further processing. The liquid fraction was centrifugated (10,000 g for 10 min), and the pellet was

discarded. The phycobiliprotein content of the selected samples was calculated using equations originally proposed by Beer and co-workers [37] and optimized by Sampath-Wiley and co-workers [38], based on the absorbances at 564 nm, 618 nm, and 730 nm (measured using a UV-VIS spectrophotometer).

2.2.2. Ethanolic extraction (targeting pigment recovery)

The seaweed biomass was suspended in 96 % *v*/v and incubated in a shaking water bath for 60 min at 60 °C, protected from light. Samples were filtered, and the solid fraction was subjected to further processing. The liquid fraction was centrifugated (10,000 g for 10 min) and the pellet was discarded. The chlorophyll (a and b) and carotenoid content of the selected samples were calculated based on the absorbance at 470 nm, 649 nm, and 664 nm (measured using a UV-VIS spectrophotometer) using equations previously reported [39].

2.2.3. Alkaline solvent extraction (targeting protein recovery)

In this step, the seaweed biomass was suspended in 0.5 M NaOH and incubated in a shaking water bath for 120 min at 60 °C, protected from light, according to the most common conditions reported in the literature for alkaline protein extractions from these biomasses [40,41]. Samples were filtered, and the solid fraction was subjected to further processing.

2.2.4. Hot water extraction (targeting hydrocolloid recovery)

For control purposes, before the hot-water extraction, a standard alkaline pretreatment (PTC) with 6 % (*w*/w) NaOH at 85 °C for 3.5 h, followed by several cycles of tap water washing and neutralization with acetic acid 0.5 % (w/w) for 1 h at room temperature was performed [16]. Equally, a control native agar extraction (NC) without any preliminary extraction step or alkaline pre-treatment was performed for all seaweeds.

Native agar extraction was conducted in a shaking water bath (at 120 rpm) as previously optimized: for *Gracilaria* a solid:solvent ratio of 1:30 for a final volume of 300 mL, using distilled water, during 2 h at 85 °C was used [18], while for *Gelidium* a solid:solvent ratio of 1:25 for a final volume of 300 mL, using distilled water, during 3 h at 95 °C was used [17]. After the agar extractions, the still-hot solution was filtered using a cotton cloth, and the liquid fraction was submitted to a freeze-thaw process [16]. The resulting solid gelling fraction was dehydrated with ethanol (96 %), dried at 60 °C overnight in a ventilated oven, and weighed for agar yield determination (expressed in g of gelling fraction per 100 g of raw material, on a dry basis). Native porphyran extraction was conducted in the same manner, using a solid:solvent ratio of 1:25 for a final volume of 300 mL using distilled water for 3 h at 80 °C [42]. After the extraction, the solution was filtered to remove the insolubilized seaweed, and a 3-fold volume of ethanol (96 % *v*/v) was added to the extract, maintained at 4 °C overnight, and centrifuged (4000 *g* for 15 min) to recover the native porphyran [43]. The resulting solid fraction was dried and weighed for yield determination.

2.3. Hydrocolloid characterization

2.3.1. Texturizing and rheological behaviour

Agars and porphyrans (1.5 % *w*/w) were solubilized with distilled water at boiling temperature until complete dissolution. 15 g of the hot solution was poured into a 30 mm diameter cylindrical container and allowed to rest at room temperature overnight. A texture analyser (TA. HDplus from Stable Micro Systems, Surrey, England) equipped with a cylindrical probe with a 10 mm diameter was used for the gel strength determination. Gel strength (reported in g per cm^2) was the stress required for breaking the gel surface [18].

Gelling and melting temperatures were measured in a controlled stress HR-1 rheometer (TA Instruments, New Castle, USA) fitted with a parallel plate geometry (40 mm diameter, 1000 μm gap), using a constant frequency of 6.28 rad/s and 1.0 % strain throughout. Native agars and porphyrans (1.5 % *w*/w) were solubilized with distilled water at boiling temperature and poured still hot onto the pre-heated (80 °C) plate of the rheometer, using paraffin oil to prevent water loss. A temperature ramp from 80 to 20 °C at the rate of 2 °C/min was applied (for gelling temperature determination), followed by equilibration for 30 min at 20 °C (after which storage and viscous moduli were recorded), and a second ramp from 20 to 99 °C at 2 °C/min (for melting temperature determination) [16].

2.3.2. Structure and chemical composition

Sulphate content was determined by turbidimetry, based on the barium chloride–agarose method, and expressed as a percentage of SO_4^{2-}, on a dry weight basis [17,44]. 3,6-anhydro-L-galactose content (3,6-AG) was determined through the resorcinol–acetal method (using D-fructose as a standard and a conversion factor of 1.087) and is expressed as a percentage [17,45].

Agars and porphyrans were purified by heat solubilization at 0.2 % (w/ w) followed by centrifugation (40 °C, 17000 *g* for 1 h) [16] The pellet formed was discarded, and the supernatant was collected and dried at 60 °C (in the case of agars) or submitted to another precipitation with ethanol (for porphyrans) for determination of purification yield. Purified hydrocolloids' functional groups and bonding arrangements of constituents were determined by Fourier Transform Infrared Spectroscopy (FTIR) using an ALPHA II-Bruker spectrometer (Ettlingen, Germany) with a diamond-composite attenuated total reflectance (ATR) cell. The FTIR spectra were recorded in the range of 4000–400 cm^{-1}, by acquiring 60 scan cycles per sample with 4 cm^{-1} resolution [14].

2.3.3. Molecular weight determination

The molecular weight distribution of hot-water extracted polysaccharides was evaluated by HPLC gel permeation chromatography (refractive index detector; PolySep-GFC-P Linear column at 40 °C; mobile phase of 0.1 M $NaNO_3$ at a flow rate of 0.8 mL/min). The native fractions were solubilized in the mobile phase (at a concentration of 0.5 mg/mL), and the molecular weight distribution was calculated using a standard pullulan kit P-82 within a range of 6.3 kDa to 642 kDa in the same conditions [46,47].

2.4. Bioactive potential characterization

The liquid fractions from the final selected sequential extraction protocols were analysed for total protein by the Bradford method [48], total sugar by the Phenol-Sulphuric method [49], and antioxidant activity by the Ferric Reducing Antioxidant Power (FRAP) [50], 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay [51] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging [52], according to recently modified protocols [18,53]. The extracted polysaccharides from the same selected conditions were also subjected to antioxidant activity assays.

2.5. Statistical analysis

A statistical analysis was conducted to determine significant differences, based on the analysis of variance (One-Way ANOVA) using GraphPad Prism version 8.0.1. A Tukey test was used for multiple comparisons using statistical hypothesis testing, and a 95 % confidence interval was considered. For each seaweed studied, statistical analysis was conducted to determine if the different sequential extraction procedures resulted in significant ($p < 0.05$) differences in the extracted fractions' yield, composition, and functional behaviour. All values are reported as averages ± standard deviation. Unless stated otherwise, at least three replicates were performed.

3. Results and discussion

Seaweeds can be consumed as a whole, with promising nutraceutical

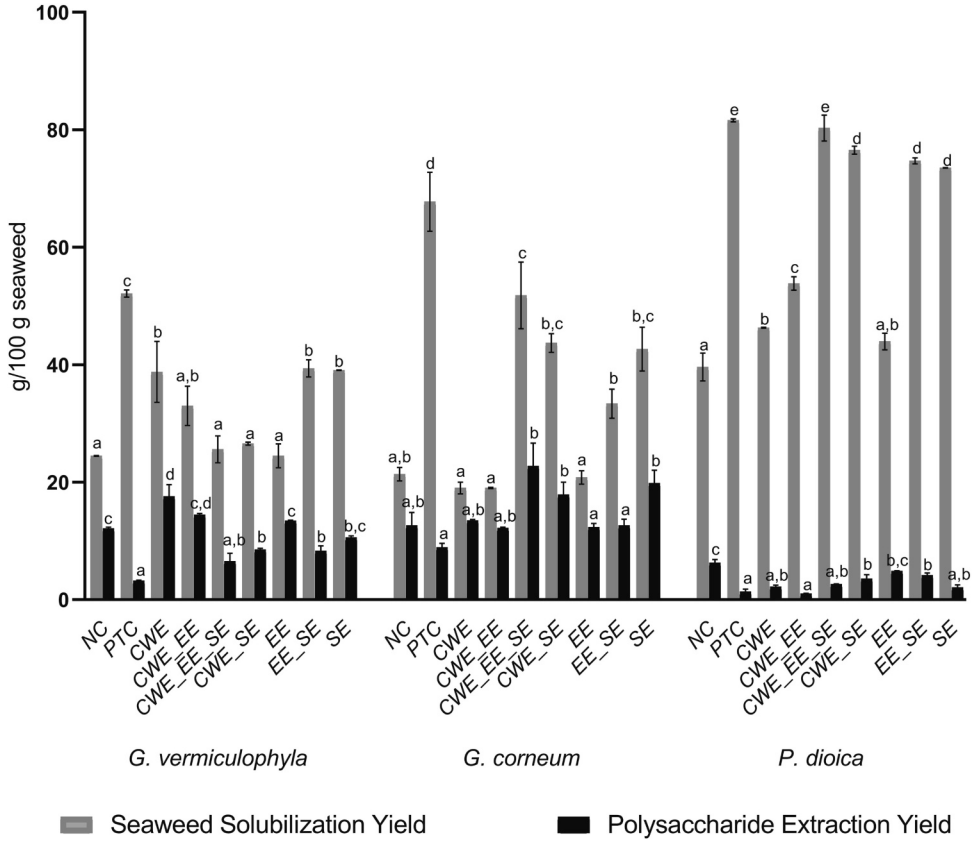

Fig. 2. Effect of sequential extractions on the total seaweed solubilization yield and polysaccharide extraction yield (considering only the recovery of the hot water extraction fraction) of the three studied red seaweeds. For each seaweed and variable, different letters represent statistically significant differences ($p < 0.05$). NC - native control, PTC - traditional alkali-treated control, CWE - cold-water extraction, EE - ethanolic extraction and SE - modified alkaline extraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effects. However, it has been reported that the fractionation of this matrix into several components increases not only its beneficial effects but also its market value and valorization opportunities: i) protein–polysaccharide interactions hinder the digestibility of seaweed proteins, whereas the use of protein extracts increases seaweed biaccessibility up to 3-fold; ii) seaweed dietary fibre has shown promising prebiotic and antiobesogenic effects but because of its low concentration in some seaweeds, the amount of biomass needed to achieve the daily recommended fibre intake is prohibitive, whereas carbohydrate-rich fractions require more feasible doses; iii) similarly, the polyunsaturated fatty acids of seaweeds have nutraceutical properties but it is impractical to achieve the necessary intake of this compounds by consuming the whole biomass; iv) high consumption of whole seaweed leads to an excessive salt intake, with added health hazards; v) heavy metals such as arsenic are often present in seaweeds at concentrations above the recommended amounts, being possible contributions to the increase of some cancers but, since they are mostly present in the form of arsenosugars, seaweed fractionation can decrease the health risk associated with the consumption of the other fractions [54]. Of all seaweed fractions, hydrocolloids are the ones with the most commercial relevance. Furthermore, the market value of this hydrocolloid is intrinsically related to its texturizing behaviour and purity, making the removal of other fractions even more necessary. Since this process is usually performed using time-consuming, energy-demanding chemical processes that significantly increase the environmental burden of the process, simpler and more eco-friendly approaches are essential. Additionally, the disposal of the remaining biomass goes against the recommendations for zero-waste processing and circular economy, special when considering the high potential of these fractions. All things considered, this work aims to understand the effects of seaweed fractionation (i.e. protein, pigment, and/or lipid recovery) on the agars and porphyrans extracted from representative red seaweeds and advise uses for the remaining fractions.

3.1. Solubilization, agar and porphyran yields

The effects of the sequential extraction protocols on total seaweed solubilization and polysaccharide extraction yield (considering only the polysaccharide recovered on the hot water extraction step, expected to be the water-soluble non-cellulosic hydrocolloid component) can be observed in Fig. 2. Regardless of the extraction conditions applied, *Porphyra dioica* presented the highest solubilization yields and lowest hot-water soluble polysaccharide yields of all studied seaweeds, and it was followed by *Gracilaria vermiculophyla* and *Gelidium corneum*, as expected from the initial algae composition. Moreover, it is possible to observe that the different biomasses respond distinctly to the sequential extraction protocols applied. In *P. dioica,* all sequential treatments provide an increase in total solubilization yield when compared to the direct native hot-water soluble polysaccharide extraction, while for *G. vermiculophyla* and *G. corneum,* several conditions do not impact this parameter. This trend may be due to the different seaweeds' carbohydrate compositions. In *P. dioica,* a negative effect on the main soluble polysaccharide extraction yield was observed when applying sequential extractions, regardless of the combination applied, as opposed to direct polysaccharide extraction. This can be explained by the degradation of this polysaccharide with overprocessing and by the easier solubilization of porphyrans when compared to agarans (present in *Graciaria* and *Gelidium* sp.). On the other hand, in *G. vermiculophyla,* the application of a cold-water extraction step resulted in a significant increase in polysaccharide recovery yield. In the case of *G. corneum,* there is a significant

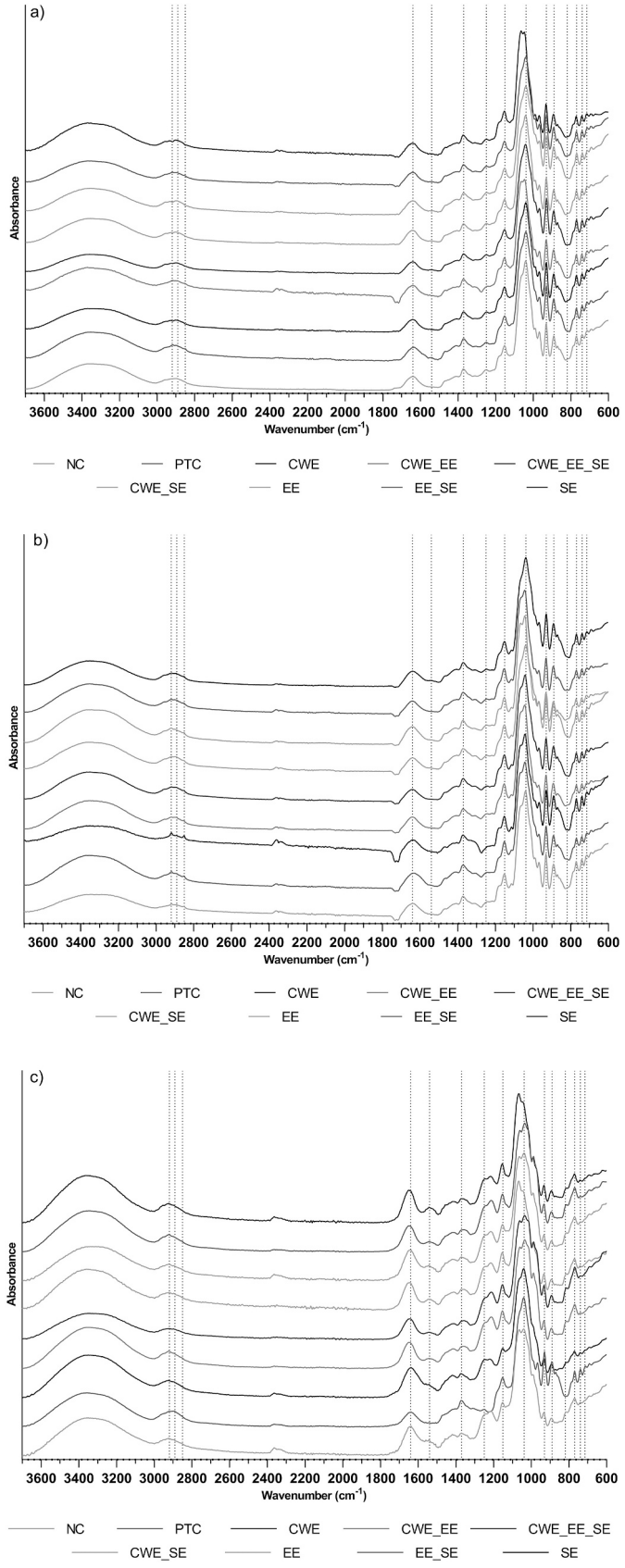

Fig. 3. Effect of sequential extractions on the FTIR-ATR spectra of polysaccharides from *G. vermiculophyla* (a), *G. corneum* (b) and *P. dioica* (c). NC - native control, PTC - traditional alkali-treated control, CWE - cold-water extraction, EE - ethanolic extraction and SE - modified alkaline extraction.

increase in polysaccharide yield in the conditions containing the modified alkali step, versus a conventional alkaline pretreatment control, but the results are not statistically different from the native direct agar extraction. Also, none of the studied sequential treatments resulted in yield loss when compared to the native hydrocolloid extraction. These results are corroborated by the reports available in the literature regarding different processing conditions on the seaweeds' structure (evaluated by Scanning Electron Microscopy). Bahari and co-workers observed that the application of room temperature water (22 °C) was efficient in the recovery of phycobiliproteins from *Chondrus crispus* without hindering the integrity of the cuticle layers of the matrix (keeping the polysaccharides intact in the seaweed matrix, for further extractions), while an increase in temperature to 45 °C results in the peeling off the outermost layer of the seaweed, exposing the cells beneath it [55]. On the other hand, even at room temperature, the use of ethanolic solvents resulted in the reduction of impurities and residual materials but an increased number of pores that allowed the diffusion of compounds to the media [56]. Similar results were observed after protein extraction from *Porphyra umbilicalis* blades, which resulted in increased surface roughness, cavitation, and erosion [24]. Xiao and co-workers also reported the destruction of *Gracilaria lemaneiformis* epidermis and dissolution and destruction of the cell wall of the finless porpoise, thinning of the cortex, cell disorder, originating cell structure loss and cracks on the surface of algae, due to the sulphate removal caused by alkali treatment [57]. Even without considering the effect of the different algae composition and different solvents on the structure of the algae biomass, the effect of temperature applied on the preliminary extraction can be responsible for the loss of cell integrity. Ge and co-workers observed that after 30 min at 90 °C, there is a significant loss in *Porphyra* cell integrity, resulting in the uncontrolled release of intracellular substances [58]. Authors believe the same effect can be present in this study, causing the undesired solubilization of some of the polysaccharides in the ethanolic and alkaline fractions.

3.2. Effects on polysaccharide structure and composition

The different sequential protocols influenced not only the yields but also the composition of the obtained fractions. The correlation between these parameters is detailed in Fig. 3 and Table 1.

Sulphate and 3,6-AG are two of the most important structural components of agar. The lower the sulphates/3,6-AG ratio, the higher the gel strength of the polysaccharide. The peak at 891 cm^{-1}, related to the C—H bending at the anomeric carbon in galactose units is representative of agar frames, while the one at 930 cm^{-1} is due to the vibration of the C-O-C bridge of 3,6-AG [16]. All *G. vermiculophyla* and *G. corneum* agars present strong peaks at these two locations, corroborating the 20 to 30 % 3,6-AG content determined in these, respectively. For *P. dioica*, the peaks are significantly stronger in the PTC condition in comparison with the remaining porphyrans, whose 3,6-AG averages 5 %. The total sulphate content can be correlated with two bands: one at 1250 cm^{-1}, due to ester-sulphate vibrations, and one at 1370 cm^{-1}, due to S═O sulphate stretching [59]. All *G. vermiculophyla* and *G. corneum* agars present a defined small peak in the latter location and a smaller but broader band in the first location. *P. dioica* porphyrans (except PTC) present a different behaviour, with high ester-sulphate vibration bands. Despite the traditional use of alkali pretreatments to decrease the sulphate content of the agars, it is reported that not all seaweed species react to this methodology: Freile-Pelegrín and Murano observed that alkali treatment was effective in removing alkali-labile sulphate in *G. crassissima* but not in *G. cervicornis* and *G. blodgettii* [60]. The results obtained in this work corroborate that observation: *G. vermiculophyla* agars present lower sulphate values in PTC than NC. However, similar decreases in the sulphate content can be obtained in alkaline-free conditions (CWE_EE). The sulphate content of *G. corneum* agars appears not to be influenced by alkaline extractions, being similar in PTC and NC conditions, with the lowest values being obtained in protocols with and without SE. This can

Table 1
Influence of sequential extraction conditions on the composition (sulphate and 3,6-anydrogalactose content), purification yield and molecular weight of the polysaccharides from three red seaweeds. For each seaweed and variable, different letters represent statistically significant differences ($p < 0.05$).

Seaweed	Condition	Extracted Polysaccharide			
		Sulphate content	3,6-AG content	Purification yield	Molecular weight
		%	%	%	kDa
Gracilaria vermiculophyla	NC	2.05 ± 0.34^{b}	25.27 ± 1.52^{a}	75.4 ± 1.2^{b}	$131 \pm 34^{a,b}$
	PTC	0.32 ± 0.03^{a}	25.65 ± 2.85^{a}	82.4 ± 0.3^{c}	84 ± 22^{a}
	CWE	0.82 ± 0.01^{a}	21.06 ± 2.55^{a}	$80.0 \pm 2.8^{b,c}$	160 ± 11^{b}
	CWE_EE	0.36 ± 0.11^{a}	24.67 ± 1.31^{a}	$80.8 \pm 2.7^{b,c}$	162 ± 5^{b}
	CWE_EE_SE	0.61 ± 0.09^{a}	26.75 ± 1.12^{a}	75.1 ± 0.5^{b}	174 ± 10^{b}
	CWE_SE	0.69 ± 0.11^{a}	22.87 ± 1.00^{a}	75.2 ± 3.1^{b}	157 ± 12^{b}
	EE	3.17 ± 0.45^{c}	22.64 ± 0.67^{a}	$76.5 \pm 2.6^{b,c}$	158 ± 21^{b}
	EE_SE	0.54 ± 0.08^{a}	24.27 ± 1.49^{a}	68.9 ± 1.8^{a}	182 ± 28^{b}
	SE	0.21 ± 0.09^{a}	$27.05 \pm 1.54^{a,b}$	$79.6 \pm 2.1^{b,c}$	169 ± 6^{b}
Gelidium corneum	NC	0.49 ± 0.02^{c}	28.15 ± 2.20^{c}	88.0 ± 1.1^{a}	181 ± 38^{a}
	PTC	$0.42 \pm 0.09^{b,c}$	27.80 ± 2.01^{c}	94.7 ± 6.1^{a}	174 ± 19^{a}
	CWE	0.33 ± 0.01^{b}	$22.57 \pm 0.17^{a,b}$	85.5 ± 4.8^{a}	178 ± 28^{a}
	CWE_EE	0.16 ± 0.01^{a}	$24.98 \pm 0.84^{b,c}$	86.1 ± 7.9^{a}	205 ± 32^{a}
	CWE_EE_SE	0.16 ± 0.01^{a}	$25.30 \pm 0.36^{b,c}$	89.4 ± 0.4^{a}	198 ± 17^{a}
	CWE_SE	0.15 ± 0.01^{a}	29.92 ± 0.22^{c}	88.8 ± 4.6^{a}	202 ± 19^{a}
	EE	0.16 ± 0.01^{a}	19.99 ± 0.60^{a}	91.2 ± 0.7^{a}	225 ± 20^{a}
	EE_SE	$0.38 \pm 0.08^{b,c}$	30.72 ± 0.30^{c}	91.8 ± 4.2^{a}	231 ± 45^{a}
	SE	0.16 ± 0.01^{a}	28.37 ± 0.58^{c}	89.6 ± 0.7^{a}	$268 \pm 33^{a,b}$
Porphyra dioica	NC	16.02 ± 3.40^{d}	5.95 ± 0.28^{b}	78.2 ± 1.7^{a}	360 ± 2^{b}
	PTC	0.43 ± 0.12^{a}	27.69 ± 1.62^{c}	89.0 ± 4.4^{b}	$137 \pm 13^{a,b}$
	CWE	6.30 ± 0.23^{b}	$4.07 \pm 0.39^{a,b}$	$76.5 \pm 1.5^{a,b}$	278 ± 27^{b}
	CWE_EE	5.77 ± 0.44^{b}	$4.44 \pm 0.74^{a,b}$	73.8 ± 7.1^{a}	28 ± 1^{a}
	CWE_EE_SE	11.54 ± 0.26^{c}	7.16 ± 0.50^{b}	$81.7 \pm 6.4^{a,b}$	$907 \pm 99^{d,e}$
	CWE_SE	10.44 ± 0.52^{c}	6.06 ± 0.38^{b}	87.2 ± 1.5^{b}	$971 \pm 32^{d,e}$
	EE	10.33 ± 1.33^{c}	3.74 ± 0.01^{a}	85.6 ± 0.7^{b}	658 ± 101^{c}
	EE_SE	12.16 ± 0.67^{c}	6.64 ± 0.84^{b}	84.9 ± 0.8^{b}	832 ± 46^{d}
	SE	10.68 ± 1.27^{c}	$4.81 \pm 0.54^{a,b}$	$83.2 \pm 2.1^{a,b}$	1048 ± 34^{e}

NC - native control, PTC - traditional alkali-treated control, CWE - cold-water extraction, EE - ethanolic extraction and SE - modified alkaline extraction.

Table 2
Influence of sequential extraction conditions on the texturizing behaviour (gelling strength (GS)) and thermal properties (storage modulus (G′), loss modulus (G″), gelling temperature (T_g) and melting temperature (T_m)) of the polysaccharides from three red seaweeds. For each seaweed and variable, different letters represent statistically significant differences ($p < 0.05$). For *Porphyra dioica,* samples marked with a *, rheological behaviour was measured in a 10 % (*w/v*) polysaccharide solution.

Seaweed	Condition	Extracted Polysaccharide				
		Texturizing behaviour	Rheological behaviour			
		GS	G' at 20 °C	G" at 20 °C	T_g	T_m
		g/cm^2	Pa	Pa	°C	°C
Gracilaria vermiculophyla	NC	217 ± 25^{a}	2567 ± 512^{b}	42 ± 11^{a}	39.0 ± 0.9^{b}	88.5 ± 0.5^{a}
	PTC	468 ± 77^{b}	5950 ± 1204^{c}	192 ± 18^{b}	$37.3 \pm 0.1^{a,b}$	87.8 ± 0.4^{a}
	CWE	205 ± 54^{a}	2947 ± 50^{b}	47 ± 5^{a}	$37.4 \pm 0.8^{a,b}$	87.4 ± 0.6^{a}
	CWE_EE	224 ± 23^{a}	$2406 \pm 70^{a,b}$	32 ± 2^{a}	$37.9 \pm 0.3^{a,b}$	88.2 ± 0.3^{a}
	CWE_EE_SE	151 ± 20^{a}	$1912 \pm 162^{a,b}$	31 ± 1^{a}	38.6 ± 0.3^{b}	88.8 ± 0.2^{a}
	CWE_SE	167 ± 34^{a}	1002 ± 11^{a}	30 ± 6^{a}	37.2 ± 0.2^{a}	88.6 ± 0.5^{a}
	EE	189 ± 19^{a}	2567 ± 492^{b}	32 ± 5^{a}	38.7 ± 0.3^{b}	89.1 ± 0.3^{a}
	EE_SE	197 ± 34^{a}	$2103 \pm 203^{a,b}$	54 ± 6^{a}	39.5 ± 0.4^{b}	89.5 ± 0.6^{a}
	SE	205 ± 5^{a}	$2349 \pm 461^{a,b}$	54 ± 7^{a}	38.6 ± 0.1^{b}	88.4 ± 2.0^{a}
Gelidium corneum	NC	767 ± 58^{a}	8838 ± 1087^{a}	180 ± 48^{a}	$38.0 \pm 0.8^{b,c}$	91.7 ± 0.1^{a}
	PTC	$1049 \pm 18^{b,c}$	$18{,}071 \pm 251^{c}$	359 ± 60^{b}	32.3 ± 0.8^{a}	97.5 ± 0.7^{b}
	CWE	939 ± 2^{b}	$10{,}941 \pm 401^{a,b}$	193 ± 39^{a}	$33.7 \pm 1.3^{a,b}$	93.3 ± 1.7^{a}
	CWE_EE	920 ± 32^{b}	8979 ± 36^{a}	183 ± 33^{a}	$38.9 \pm 1.5^{b,c}$	$95.3 \pm 1.2^{a,b}$
	CWE_EE_SE	1150 ± 77^{c}	$17{,}908 \pm 1141^{c}$	$276 \pm 28^{a,b}$	$34.0 \pm 0.1^{a,b}$	$95.0 \pm 1.4^{a,b}$
	CWE_SE	1117 ± 77^{c}	$12{,}810 \pm 1802^{b}$	352 ± 15^{b}	$34.6 \pm 0.3^{a,b}$	$94.6 \pm 1.3^{a,b}$
	EE	935 ± 27^{b}	8388 ± 315^{a}	172 ± 11^{a}	40.9 ± 2.9^{c}	93.1 ± 2.2^{a}
	EE_SE	$1064 \pm 27^{b,c}$	$13{,}638 \pm 619^{b}$	294 ± 21^{b}	$34.3 \pm 1.5^{a,b}$	98.3 ± 0.4^{b}
	SE	1141 ± 12^{c}	$15{,}188 \pm 49^{b}$	$239 \pm 30^{a,b}$	36.9 ± 0.8^{b}	$95.2 \pm 1.1^{a,b}$
Porphyra dioica	NC	–	$*288 \pm 6^{a}$	$*80 \pm 13^{a,b}$	$*32.1 \pm 0.3^{b}$	$*69.8 \pm 0.6^{d}$
	PTC	321 ± 57^{a} | $*611 \pm 25^{b}$	4181 ± 743^{d} | $*29585 \pm 3631^{e}$	63 ± 10^{a} | $*748 \pm 17^{c}$	37.3 ± 1.0^{c} | $*46.1 \pm 1.5^{d}$	79.4 ± 0.6^{g} | $*77.1 \pm 0.4^{f}$
	CWE	–	$*1825 \pm 278^{b}$	$*634 \pm 78^{c}$	$*40.0 \pm 0.2^{c}$	$*63.9 \pm 1.5^{b,c}$
	CWE_EE	–	$*713 \pm 39^{a}$	$*150 \pm 3^{a,b}$	$*37.8 \pm 0.5^{c}$	$*74.5 \pm 0.1^{e}$
	CWE_EE_SE	–	$*3679 \pm 204^{d}$	$*2130 \pm 167^{f}$	$*38.5 \pm 3.1^{c}$	$*62.1 \pm 0.3^{b}$
	CWE_SE	–	$*2886 \pm 192^{c,d}$	$*1223 \pm 109^{d}$	$*30.0 \pm 1.4^{a,b}$	$*65.1 \pm 0.1^{b,c}$
	EE	–	$*681 \pm 32^{a}$	$*295 \pm 10^{b}$	$*26.7 \pm 0.7^{a}$	$*66.2 \pm 0.8^{c}$
	EE_SE	–	$*2694 \pm 154^{c}$	$*1756 \pm 94^{e}$	$*39.9 \pm 1.1^{c}$	$*63.2 \pm 1.7^{b,c}$
	SE	–	$*2330 \pm 262^{b,c}$	$*1279 \pm 58^{d}$	$*29.2 \pm 0.1^{a,b}$	$*57.9 \pm 2.0^{a}$

indicate that either the PTC treatment was not complete in the removal of all alkali-labile sulphates in *G. corneum*, or that these sequential treatments can interact with the alkali-stable forms. Nevertheless, in this case, the sulphate content is already low in the polysaccharide from NC. *P. dioica* hydrocolloid's sulphate content was strongly affected by concentrated alkali content (PTC) but lower concentrations (SE) act in the same way as alkali-free alternatives (e.g., EE). More information about the sulphate content of the samples can be obtained by assessing the 820, 830, and 854 cm^{-1} regions, which are correlated with its location in the C6, C2 or C4 regions of galactose, respectively, while the 805 cm^{-1} region is linked to the sulphate location in the C2 region of 3,6-AG [16]. The PTC porphyran of *P. dioica* clearly illustrates the behaviour reported by Yarnpakdee and co-workers: the decrease in amplitude at 1250 cm^{-1}, accompanied by the loss of the band around 820 cm^{-1} and the increase of the band intensity around 930 cm^{-1}, indicate the conversion of sulphate substitution at C-6 to 3,6-AG among 4-linked residues since 6-sulphate-α-l-galactose is known to be a precursor of 3,6-anhydro-α-l-galactose [61]. More information about the influence of the extraction conditions applied on the hydrocolloids can be extracted from the 1730 cm^{-1} area of the spectra. These peaks are correlated with the carboxylic acid ester form of the carbonyl group, promoting cross-linking, and aiding in gel strength, while simultaneously indicating the oxidation of the polysaccharides. Overall, since no significant increase in these small peaks can be observed when compared to native conditions, it can be concluded that the studied sequential protocols do not promote the oxidation of the hydrocolloids [62].

3.3. Effects on texturizing behaviour and commercial value

The texturizing capacity of the hydrocolloids is a key factor in their commercial application and value. Results regarding the texturizing behaviour (gelling strength and thermal properties) can be observed in Table 2. The gelling temperature (Tg) is reported to as the temperature for the initial development of gel networks, through the formation of helices and junction zones upon cooling, while the melting temperature (Tm) corresponds to the dissociation of the most cross-linked junction zones of gel networks while melting [63].

The effects of composition on the gelling and melting temperatures of agar and porphyran gels have been reported several times but with contradictory information, making it impossible to conclude the influence sulphate and sugar content have. There are reports associating the use of alkali treatments (and the subsequential decrease in sulphate content and increase in 3,6-AG content) with an increase in both gelling and melting temperatures of hydrocolloids but reports of no correlation between these parameters or a decrease in temperatures are also frequent [9]. The same can be said about the effects of different extraction technologies and methodologies [17,64]. With the data obtained in this work, it was not possible to observe any correlation between sulphate or 3,6-AG content and the rheological behaviour of the hydrocolloids obtained. On the other hand, it was possible to conclude that the sequential treatments applied did influence these parameters.

When it comes to *G. vermiculophyla*, none of the sequential extraction conditions significantly affected the gelling strength of the extracted agars (all averaging 200 g/cm^2) compared to the control native extraction. Furthermore, all gelling temperatures are below 40 °C, and all melting temperatures are superior to 85 °C, placing them within the range defined by the United States Pharmacopeia for agar standards [65]. On the other hand, the values of the viscoelastic moduli present significant differences between extraction conditions, being higher for PTC conditions and lower for the modified alkali solvent extraction conditions (with or without combination with other solvents). This indicates a significant difference in resistance to stretching and viscous flows [46]. Nonetheless, all agars present a substantial gel-forming ability (ranging from 151 to 224 g/cm^2). Furthermore, lower gelling temperatures were observed for the agars extracted with a cold-water pretreatment, although the mechanism behind these effects is not clear.

For *G. corneum,* all sequential extractions had a significant increase in gelling strength when compared to NC, being as good as PTC. This behaviour is more relevant in agars obtained by the modified alkali step (either alone or in combination), whose gelling strength surpassed 1000 g/cm^2, while having higher polysaccharide extraction yields. This is reflected in the viscoelastic properties, which present higher storage and loss moduli, lower gelling temperatures, and higher melting temperatures. On the other hand, the use of ethanolic extractions (alone or in combination with cold water extraction) had no improvement in the texturizing properties of the agars, resulting in the highest gelling temperatures and lowest viscoelastic moduli. This is drastically pertinent in the EE, whose gelling value superior to 40 °C impedes the use of the agar as a gelation standard.

For *P. dioica*, only the PTC porphyran presented measurable gelling strength at a 1.5 % concentration. Thus, to better assess the effect of the sequential extraction conditions on the rheological behaviour of these hydrocolloids, it was necessary to increase the polysaccharide concentration. For that purpose, a series of increasing concentrations were tested (Fig. S1), with 10 % chosen as the ideal concentration to pursue testing. In this concentration, the storage modulus is significantly higher than the loss modulus, indicating a potential gelling ability. As expected, the viscoelastic moduli of all non-pretreated *P. dioica* hydrocolloids are significantly lower than the ones observed for the gelling polysaccharides from the remaining sources. Furthermore, the storage modulus is only 2 to 5 times superior to the loss modulus, indicating a very weak gel even at 10 % concentration. However, gel formation was obtained in all conditions, indicating that the obtained polysaccharide can be used as a texturizing agent. There appears to be a strong correlation between the use of moderate alkali conditions and the increase in the loss modulus. On the other hand, the use of ethanolic pretreatment appears to cause a decrease in the storage modulus. The hydrocolloid obtained using cold water and ethanolic pretreatment was the one that presented the most industrially interesting temperature profile (between the ones observed for NC and PTC), due to the gelling temperature below 40 °C and the highest melting temperature of the lot. This is also the one with the highest difference between the viscoelastic moduli, indicating once again the beneficial effect of this combination. The combined use of cold water and mild solvent also presents a promising alternative, due to the lower gelling temperature and intermediate melting temperature combined with higher viscoelastic moduli.

NC - native control, PTC - traditional alkali-treated control, CWE - cold-water extraction, EE - ethanolic extraction and SE - modified alkaline extraction.Thermal hysteresis, calculated as the temperature difference between melting and gelling temperatures, is an expected consequence of aggregation and gives an insight into the sol-gel and gel-sol transitions of the hydrocolloids [66]. This value ranges from 23 °C to 42 °C in *P. dioica*, to 47 °C to 51 °C in *G. vermiculophyla,* and 52 °C to 65 °C in *G. corneum*. Once again, this corroborates the low influence of the sequential extraction conditions on the *G. vermiculophyla* agars, as opposed to *P. dioica.* Since this phenomenon has been attributed to the high stability of the structures, which are disrupted at temperatures much higher than those at which they are formed upon cooling, it indicates the changes occurring during the gelation process of the hydrocolloids. Generally, it is accepted that the gelation process occurs in two steps: a first step with the transition from a random coil conformation in solution at high temperatures to a rigid, ordered structure of double helices upon cooling, that provide the junction zones necessary for the gel network formation; and a second step, at lower temperatures, where the helices aggregate to form thick bundles, resulting in the formation of strong gels [67]. Furthermore, the number of cross-linking sites is directly dependent on the sulphate content of the polymers, since these compounds allow molecular chains to change their counterpart for double helix formation, while the number of double helices per junction zone may decrease primarily with increasing molecular size [63]. Thus, the higher hysteresis observed in *G. corneum* is typical of

agarose hydrogels, being attributed to the formation of large aggregates of double helices, which remain stable at temperatures much higher than those at which they start associating on cooling. Since values of thermal hysteresis as high as 64 °C can be obtained in the new proposed sequential treatments (versus a value of 65 °C for the conventional PTC treatment), this proves that the proposed sequences, when properly optimized, can be promising alternatives.

3.4. Integrated discussion on the influence of the different pretreatments

In general, the seaweeds' composition proved to have a significant effect on the result of the sequential treatments applied. *Gelidium corneum* biomass presents the highest amount of galactans (37 %) and glucans (14 %) in its composition, but the lowest percentage of water extractives (28 %). Further, the galactans (agar) from *Gelidium* sp. will solubilize only in hot water at temperatures higher than 90 °C and form stronger networks (Table 2). Therefore, it is expected that the agar present is harder to reach and extract and will resist mild processing conditions without leaking in the pre-treatment steps. The opposite is observed for *Porphyra dioica*, where a lower amount of galactans (21 %) and glucans (1 %) and a higher percentage of water extractives (32 %) were determined. These galactans presented high sulphate content, low melting temperatures and gelling strength. These differences strongly affected the outcome of the sequential treatments employed: while in *G. corneum* the use of extraction methodologies with more steps was beneficial, helping break down the seaweeds cellulolytic wall and making the polysaccharide more accessible for extraction without prematurely solubilizing them, in *P. dioica*, due to its high initial availability to begin with and to the lack of cellulose to protect its integrity, all sequential treatments applied result in an undesired loss of this compound. At the same time, in this seaweed, a positive effect of all sequential treatments on sulphate reduction was observed. This can have occurred since the polysaccharide was already exposed to come in contact with the used solvents, as opposed to the other seaweeds. *G. vermiculophyla* biomass presents an intermediate composition, with 26 % of galactans, 6 % of glucans and 32 % of water-extractives. This leads the seaweed to be moderately protected against over-processing, with negative effects only being observed in the combined use of EE and SE, while simultaneously being more susceptible to extraction when CWE is applied. Concurrently, red algae are known for their content in phycobiliproteins, which are generally water-soluble, and *P. dioica* has a much higher protein content (26 %) than *G. vermiculophyla* and *G. corneum* biomasses (averaging 15 %). This influenced not only extraction yield but also the composition of the hydrocolloids: porphyrans presented a higher molecular weight in alkali-pretreated samples (CWE_EE_SE, CWE_SE, EE_SE, and SE), due to a presumed aggregation of the polysaccharide with extracted protein, while agars were not affected.

The sequential pretreatments applied did not hinder the size of extracted agars from *G. vermiculophyla* nor altered its sulphate and 3,6-AG content. This is visible in the gelling strength, all presenting values averaging 200 g/cm^2, and in the thermal hysteresis, in the range of 49 ± 2 °C. The exception is the CWE_SE condition, which results in a desirable decrease in gelling temperature but also an adverse decrease in the viscoelastic moduli. Moreover, EE negatively impacted the hydrocolloid sulphate content, resulting in a decrease in the purification yield (EE_SE) and extraction yields (CWE_EE_SE). On the other hand, CWE and CWE_EE proved to have a positive effect on the overall solubilization and polysaccharide yields. Nonetheless, the feasibility of incorporating the studied sequences with the conventionally used alkali pretreatment in applications where higher gelling strength is required should be assessed in future works through techno-economic and life cycle assessment studies.

In *G. corneum*, agars obtained by the modified alkali step (either alone or in combination), have gelling strengths surpassing 1000 g/cm^2, higher storage and loss moduli, lower gelling temperatures, and higher melting temperatures. Since no change in molecular weight was observed, and the lowest sulphate contents were achieved regardless of employing the alkali step, these parameters do not appear to be responsible for this behaviour. At the same time, no significant change in purity was observed which, alongside the low protein content of the biomass, makes it less likely to be a result of protein interactions. Thus, the slight increase in 3,6-AG content visible in these conditions seems to be the cause of this beneficial effect on agar's behaviour. Due to its recognition as a premium agar source, the obtained results regarding the recovery of this hydrocolloid can make a significant contribution to its industrial exploitation.

Once again, *P. dioica* presents very distinct results. Despite the presumed effect of SE treatments on the molecular weight due to protein aggregation, these treatments are not capable of removing all the alkali-liable sulphate of the hydrocolloids, not being sufficient to promote gelling at lower concentrations. However, since all sequential protocols result in a significant decrease in sulphate content and an increase in storage modulus, it is expected that only a higher concentration of polysaccharide will permit achieving a texturizing effect. Additionally, CWE_EE resulted in a significant increase in melting temperature as well as the highest ratio between viscoelastic moduli, both positive indicators of commercially desirable hydrocolloids. However, it should be noted that despite their texturizing capacity, these hydrocolloids are not capable of gelling at low concentrations without the application of a strong alkali pretreatment, which should be considered.

3.5. Optimal sequential extraction protocols and possible valorization of side-fractions

Overall, it was possible to observe that each seaweed responded differently to the sequential extraction protocols proposed.

Due to its positive influence on extraction yields, and the fact that water and ethanol are food-grade solvents with different polarities, the authors consider CWE_EE to be the most promising *G. vermiculophyla* valorization strategy. This sequential approach allows for the maximization of opportunities for bioactive compound recovery without impairing the technological features of the main soluble polysaccharide. Additionally, the valorization of protein-rich fraction after the CWE_EE hydrocolloid recovery should be pursued since only 11 % of the seaweed protein content is recovered in the liquid fractions.

CWE_EE_SE was chosen as the most promising approach for *G. corneum* seaweed due to the balance between the number of recovered streams and the rheological behaviour of the galactan recovered fraction. This is of extreme relevance since extracts from this seaweed have reported anti-carcinogenic, anti-inflammatory, immunomodulatory, neuroprotective, anti-obesity, and antidiabetic activity [68]. Furthermore, this processing approach allowed for the recovery of 29 % of the seaweed's protein content. The high affinity towards this compound is expected to be associated with the reported effects of alkaline solvents in protein solubilization [69].

CWE_EE was selected as the most promising strategy for porphyran recovery from *P. dioica* due to the ameliorated rheological behaviour. Alternative texturizing applications, such as the thickening of jams, sauces, and other food products, or simply its use as a soluble fibre are possible valorization opportunities for this macromolecule. Furthermore, the phycobiliproteins from this seaweed present in the cold-water fraction are expected to have hepatoprotective, antioxidant, anti-ageing, and anti-inflammatory effects, besides its natural colouring ability, while its phenolic compounds can protect cells from UV damage [70]. This is significant since this fraction would usually be lost during conventional seaweed hydrocolloid processing. On the other hand, the alternative use of the CWE_SE sequence should not be discarded due to its significantly higher purity and sulphate content, which can be beneficial for bioactive applications. Allied with the thickening effect, the incorporation of this expectedly bioactive hydrocolloid in food products can result in the development of new nutraceutical

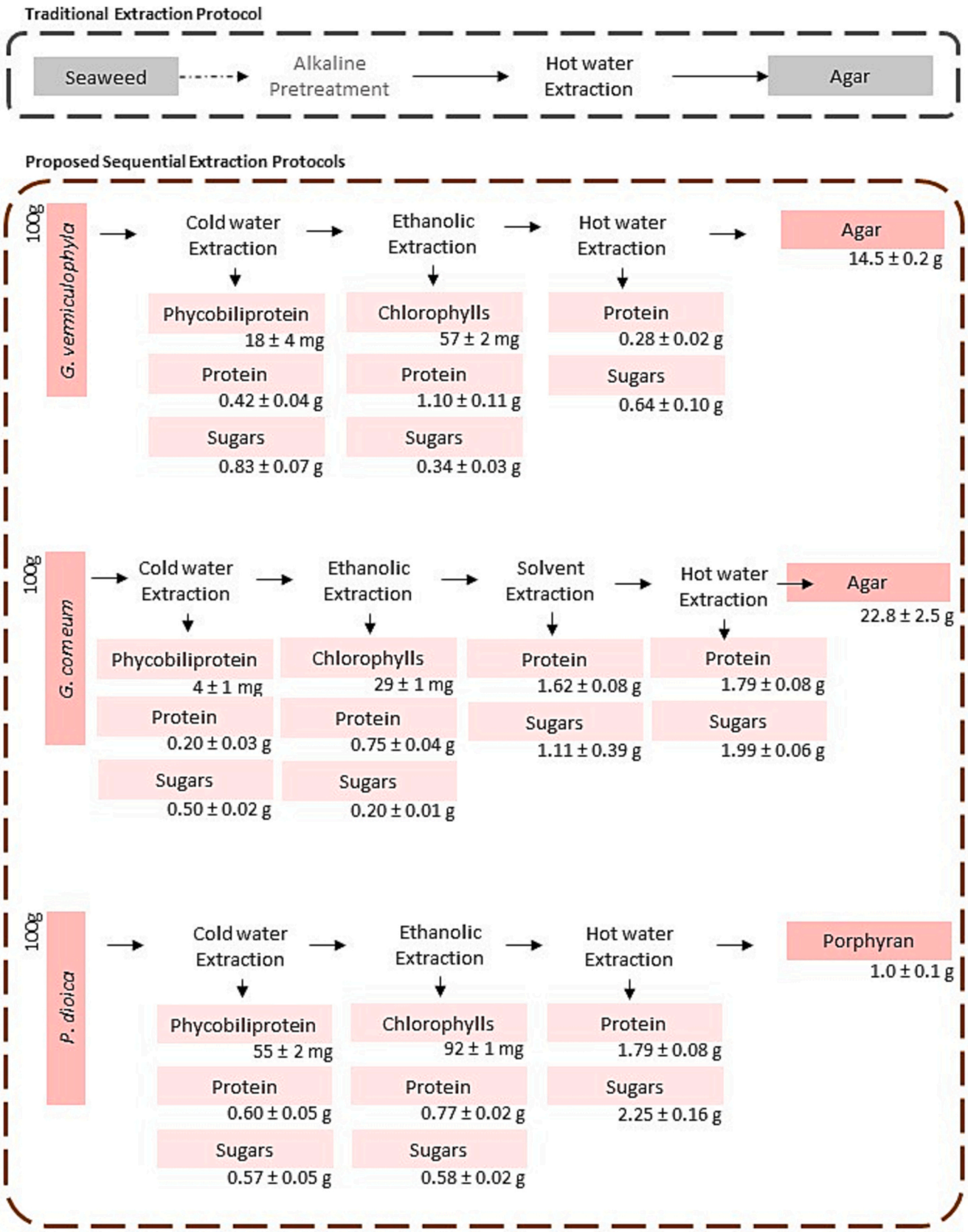

Fig. 4. Proposed sequential extraction protocols aimed at maximum polysaccharide valorization for *G. vermiculophyla*, *G. corneum* and *P. dioica*, and proximal characterization of remaining liquid fractions obtained thereof.

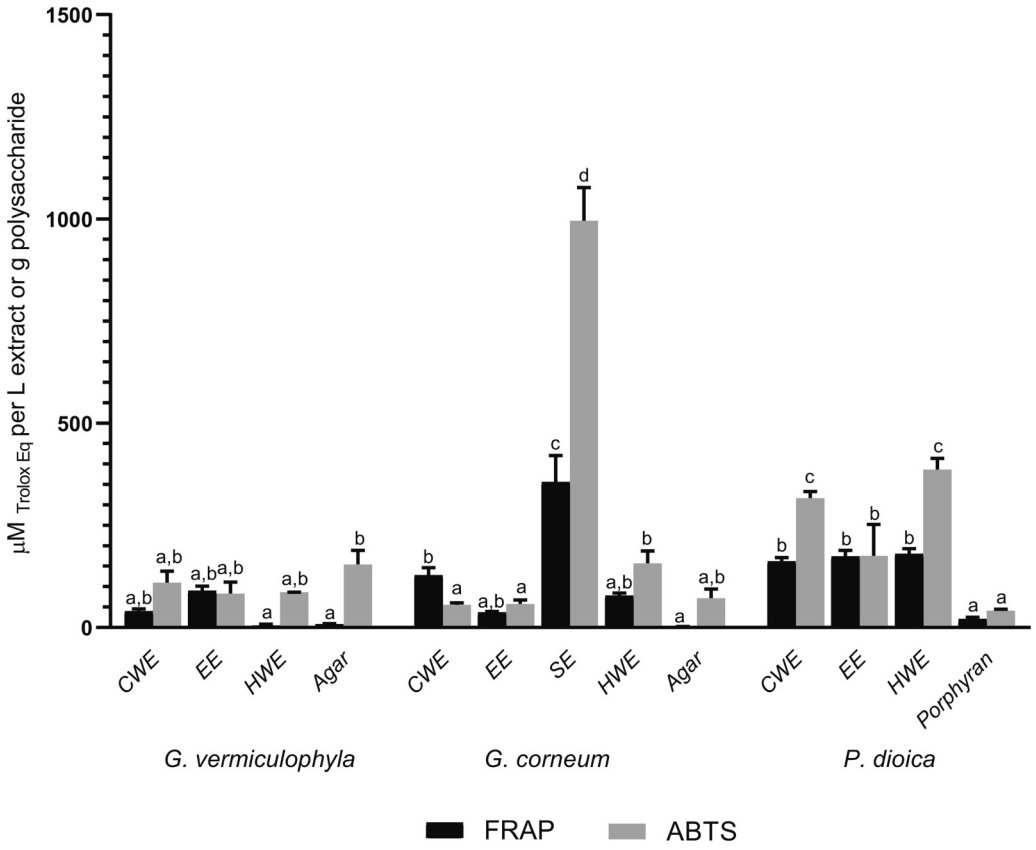

Fig. 5. Potential bioactivity of the selected fractions obtained from the optimized sequential extractions for the three studied seaweeds. For seaweed and each antioxidant method, different letters represent statistically significant differences ($p < 0.05$). CWE - cold-water extraction, EE - ethanolic extraction and SE - modified alkaline extraction.

formulations with additional health benefits for consumers.

The proximate composition of all fractions recovered, in the final chosen conditions, from the three studied red seaweeds, can be observed in Fig. 4. The selected protocol for *P. dioica* results in a significant recovery of proteins, and pigments, whereas in *G. vermiculophyla* and *G. corneum,* agar is the main valuable product. This information is corroborated by the absorbance spectra of the CWE and EE extracts of the seaweeds (Fig. S3). To further assess the applicability of the remaining fractions, the bioactive potential of the selected extracts was determined, and the data is expressed in Fig. 5. None of the samples presented significant antioxidant potential using the DDPH method. On the other hand, all extracts presented some antioxidant potential using the ABTS and FRAP methods, with SE extract from *G. corneum* and extracts from *P. dioica* presenting the most promising values.

Along with the advantages of the proposed sequential treatments previously discussed, the economic impact of the processing conditions must not be overlooked. While whole seaweed prices are in the 25–125 $/ton range, the price of agar is in the 17,000 $/ton range [71,72], indicating more than a 100-fold increase in market value with seaweed processing, being proportional to agar's texturizing behaviour and yield. Considering the high yields obtained using the optimal processing condition for *G. corneum* and *G. vermiculophyla,* the process is expected to be economically feasible, even when considering the production costs (these costs are already present in the currently used industrial extraction of the hydrocolloids and are not considered prohibitive). Furthermore, the processing cost for the cold-water extraction step is expected to be minimal, since only the energetic input for agitation and posterior filtration and water cost are present, whereas the phycobiliproteins extracted have a value of 0.1$/mg in their unpurified form, increasing 1000-fold for highly purified molecular markers [73]. This is extremely relevant for the processing of *P. dioica* due to the higher extraction yield of this compound, and the lack of information regarding the market price of porphyran. Nevertheless, it is expected to be within the range of other low-gelling hydrocolloids such as pectin or starch (840–17,000 $/ton) [72]. Also not be overlooked are the environmental gains associated with these proposed sequential treatments: instead of a high-solvent approach and the creation of effluents and residues, using mild chemicals the same results can be obtained, resulting in food-grade fractions with high potential (and market value). This results in a significant process intensification, since it leads to an ameliorated productivity but also a significant improvement in other environmental and economic metrics (i.e. energy consumption, carbon footprint) [74].

For the optimized sequential strategies, a more thorough economic and life cycle assessment must be performed before considering the implementation of these conditions on an industrial scale. Despite the expected increase in revenues (from the increased market value of the ameliorated hydrocolloid fraction and possible revenues from new streams), new processing steps must be included (resulting in longer processing times, higher energy demands, and requiring the acquisition of further chemicals). Thus, it should be guaranteed that the alterations cause a positive economic balance without negatively impacting the overall environmental footprint.

4. Conclusions and future prospects

This study successfully aided in shortening the knowledge gap surrounding the integral valorization of red seaweeds. The effects of protein, pigment, and/or lipid recovery (alone and in combination) on the structure, molecular weight, texturizing, and rheological behaviour of agar and porphyran were elucidated for the first time. Overall, the pre-processing of the seaweeds before hydrocolloid extraction is a beneficial approach that, when properly optimized, leads to the recovery of

additional valuable side streams while improving the functional properties of the main product (hydrocolloid). Additionally, the recovered fractions, composed of phycobiliproteins, chlorophylls, proteins, and sugars, resulted in significant antioxidant potential and, subsequently, improved market value. Thus, this strategy not only increases the economic potential of each seaweed but also decreases the amount of hydrocolloid-depleted seaweed that results from the process. Moreover, it was proven that polysaccharide sources are influenced by the sequential extractions in different ways: for *P. dioica*, and *G. vermiculophyla* a combination of CWE and EE was the most beneficial, while in *G. corneum* the CWE_ EE_SE extraction was the most promising approach.

Nonetheless, the proposed approach also presents some drawbacks. Despite the low alkaline concentrations and use of food-grade solvents, the environmental impact of these chemicals should be assessed. Furthermore, due to the low phycobiliprotein yields in *Gelidum* and *Gracilaria* seaweeds, a techno-economic analysis should be performed on an industrial scale, to guarantee this step results in a profit, taking into consideration the possible need for purification of these fractions. In the case of *Porphyra*, the opposite is true. Despite the promising phycobiliprotein yields, the low gelling ability obtained indicates that alternative texturizing applications or further depolymerisation of the hydrocolloid fraction into prebiotic oligosaccharides should be accessed to ameliorate the overall techno-economical balance.

Despite the contribution of the present work, several steps are still required to shorten the knowledge gap regarding seaweed biorefineries. Thus, future works should evaluate the possible effects of alternative and greener processing technologies (e.g., ohmic heating, hydrothermal treatments, microwaves) on each of the selected sequential extraction protocols. Moreover, in a true biorefinery scheme, the production of energy (e.g., biogas, bioethanol) from the remaining residue should be optimized and implemented. At the same time, the effect of these sequential extraction protocols on the downstream processing aimed at bleaching and improving agar's performance, as well as their necessity, should be accessed.

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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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References

[1] M. Griffiths, S.T.L. Harrison, M. Smit, D. Maharajh, Major Commercial Products from Micro- and Macroalgae, 2016, pp. 269–300, https://doi.org/10.1007/978-3-319-12334-9_14.

[2] A.P. Pereira, T.J. Woodman, C.J. Chuck, An integrated biorefinery to produce 5-(hydroxymethyl)furfural and alternative fuel precursors from macroalgae and spent coffee grounds, Sustain Energy Fuel 5 (2021) 6189–6196, https://doi.org/10.1039/d1se01142a.

[3] M.D.N. Meinita, A. Amron, D. Harwanto, A. Trianto, G.T. Jeong, The production of levulinic acid and formic acid from red macroalga Kappaphycus alvarezii using methanesulfonic acid, Bioresour Technol Rep. 17 (2022), https://doi.org/10.1016/j.biteb.2022.100954.

[4] A. Schultze-Jena, R.C. Vroon, A.K.A. Macleod, G.Ó. Hreggviðsson, B. T. Adalsteinsson, N.P.E. Engelen-Smit, T. de Vrije, M.A.W. Budde, H. van der Wal, A.M. López-Contreras, M.A. Boon, Production of acetone, butanol, and ethanol by fermentation of Saccharina latissima: cultivation, enzymatic hydrolysis, inhibitor removal, and fermentation, Algal Res. 62 (2022), 102618, https://doi.org/10.1016/j.algal.2021.102618.

[5] R. Ramaraj, P. Junluthin, N. Dussadee, Y. Unpaprom, Potential evaluation of biogas production through the exploitation of naturally growing freshwater macroalgae Spirogyra varians, Environ. Dev. Sustain. (2022), https://doi.org/10.1007/s10668-021-02051-2.

[6] Y. AP, M. Farghali, I.M.A. Mohamed, M. Iwasaki, S. Tangtaweewipat, I. Ihara, R. Sakai, K. Umetsu, Potential of biogas production from the anaerobic digestion of Sargassum fulvellum macroalgae: influences of mechanical, chemical, and biological pretreatments, Biochem. Eng. J. 175 (2021). doi:https://doi.org/10.1016/j.bej.2021.108140.

[7] E. Vimali, S. Gunaseelan, V. Chitra Devi, S. Mothil, M. Arumugam, B. Ashokkumar, I.M. Ganesh Moorthy, A. Pugazhendhi, P. Varalakshmi, Comparative study of different catalysts mediated FAME conversion from macroalga Padina tetrastromatica biomass and hydrothermal liquefaction facilitated bio-oil production, Chemosphere 292 (2022), https://doi.org/10.1016/j.chemosphere.2021.133485.

[8] N. Rhein-Knudsen, A.S. Meyer, Chemistry, gelation, and enzymatic modification of seaweed food hydrocolloids, Trends Food Sci. Technol. 109 (2021) 608–621, https://doi.org/10.1016/j.tifs.2021.01.052.

[9] K. Nishinari, Y. Fang, Relation between structure and rheological/thermal properties of agar. A mini-review on the effect of alkali treatment and the role of agaropectin, Food Structure. 13 (2017) 24–34, https://doi.org/10.1016/j.foostr.2016.10.003.

[10] K. Alba, V. Kontogiorgos, Seaweed polysaccharides (agar, alginate carrageenan), in: Encyclopedia of Food Chemistry, Elsevier, 2018, pp. 240–250, https://doi.org/10.1016/B978-0-08-100596-5.21587-4.

[11] R.A. Rodríguez Sánchez, M.C. Matulewicz, M. Ciancia, NMR spectroscopy for structural elucidation of sulfated polysaccharides from red seaweeds, Int. J. Biol. Macromol. 199 (2022) 386–400, https://doi.org/10.1016/j.ijbiomac.2021.12.080.

[12] J. Rahbani, A.R. Behzad, N.M. Khashab, M. Al-Ghoul, Characterization of internal structure of hydrated agar and gelatin matrices by cryo-SEM, Electrophoresis 34 (2013) 405–408, https://doi.org/10.1002/elps.201200434.

[13] B.M. Khan, H.M. Qiu, S.Y. Xu, Y. Liu, K.L. Cheong, Physicochemical characterization and antioxidant activity of sulphated polysaccharides derived from Porphyra haitanensis, Int. J. Biol. Macromol. 145 (2020) 1155–1161, https://doi.org/10.1016/j.ijbiomac.2019.10.040.

[14] F. Silva-Brito, S.G. Pereira, C.M.R. Rocha, E. da Costa, M.R. Domingues, A. Azevedo, A. Kijjoa, H. Abreu, L. Magnoni, Improving agar properties of farmed Gracilaria gracilis by using filtered sunlight, J Appl Phycol. 33 (2021) 3397–3411, https://doi.org/10.1007/s10811-021-02497-x.

[15] G. Azevedo, M.D. Torres, I. Sousa-Pinto, L. Hilliou, Effect of pre-extraction alkali treatment on the chemical structure and gelling properties of extracted hybrid carrageenan from Chondrus crispus and Ahnfeltiopsis devoniensis, Food Hydrocoll. 50 (2015) 150–158, https://doi.org/10.1016/j.foodhyd.2015.03.029.

[16] C.M.R. Rocha, A.M.M. Sousa, J.K. Kim, J.M.C.S. Magalhães, C. Yarish, M.P. do Gonçalves, Characterization of agar from Gracilaria tikvahiae cultivated for nutrient bioextraction in open water farms, Food Hydrocoll. 89 (2019) 260–271, https://doi.org/10.1016/j.foodhyd.2018.10.048.

[17] J.S. Gomes-Dias, S.G. Pereira, J.A. Teixeira, Cristina M.R. Rocha, Hydrothermal treatments – a quick and efficient alternative for agar extraction from Gelidium sesquipedale, Food Hydrocoll. 132 (2022), 107898, https://doi.org/10.1016/j.foodhyd.2022.107898.

[18] S.G. Pereira, C. Teixeira-Guedes, G. Souza-Matos, É. Maricato, C. Nunes, M. A. Coimbra, J.A. Teixeira, R.N. Pereira, C.M.R. Rocha, Influence of ohmic heating in the composition of extracts from Gracilaria vermiculophylla, Algal Res. 58 (2021), 102360, https://doi.org/10.1016/j.algal.2021.102360.

[19] V. Cebrián-Lloret, M. Metz, A. Martínez-Abad, S.H. Knutsen, S. Ballance, A. López-Rubio, M. Martínez-Sanz, Valorization of alginate-extracted seaweed biomass for the development of cellulose-based packaging films, Algal Res. 61 (2022), https://doi.org/10.1016/j.algal.2021.102576.

[20] A. Bianchi, V. Sanz, H. Domínguez, M.D. Torres, Valorisation of the industrial hybrid carrageenan extraction wastes using eco-friendly treatments, Food Hydrocoll. 122 (2022), https://doi.org/10.1016/j.foodhyd.2021.107070.

[21] E. Trigueros, M.T. Sanz, A. Filipigh, S. Beltrán, P. Riaño, Enzymatic hydrolysis of the industrial solid residue of red seaweed after agar extraction: extracts characterization and modelling, Food Bioprod. Process. 126 (2021) 356–366, https://doi.org/10.1016/j.fbp.2021.01.014.

[22] S.L. Baptista, A. Romaní, C. Oliveira, S. Ferreira, C.M.R. Rocha, L. Domingues, Galactose to tagatose isomerization by the L-arabinose isomerase from Bacillus subtilis: a biorefinery approach for Gelidium sesquipedale valorisation, LWT 151 (2021), https://doi.org/10.1016/j.lwt.2021.112199.

[23] S. Tůma, J.K. Izaguirre, M. Bondar, M.M. Marques, P. Fernandes, M.M.R. da Fonseca, M.T. Cesário, Upgrading end-of-line residues of the red seaweed Gelidium

sesquipedale to polyhydroxyalkanoates using Halomonas boliviensis, Biotechnology Reports. 27 (2020), e00491, https://doi.org/10.1016/j.btre.2020.e00491.
[24] N. Wahlström, H. Harrysson, I. Undeland, U. Edlund, A strategy for the sequential recovery of biomacromolecules from red macroalgae Porphyra umbilicalis Kützing, Ind. Eng. Chem. Res. 57 (2018) 42–53, https://doi.org/10.1021/acs.iecr.7b03768.
[25] F. Mensi, Agar yield from R-phycoerythrin extraction by-product of the red alga Gracilaria verrucosa, J Appl Phycol. (2019) 741–751. doi:https://doi.org/10.1007/s10811-018-1533-z/Published.
[26] R.S. Baghel, N. Trivedi, V. Gupta, A. Neori, C.R.K. Reddy, A. Lali, B. Jha, Biorefining of marine macroalgal biomass for production of biofuel and commodity chemicals, Green Chem. 17 (2015) 2436–2443, https://doi.org/10.1039/c4gc02532f.
[27] N. Nunes, S. Valente, S. Ferraz, M.C. Barreto, M.A.A. Pinheiro De Carvalho, Nutraceutical potential of Asparagopsis taxiformis (Delile) Trevisan extracts and assessment of a downstream purification strategy, Heliyon 4 (2018) 957, https://doi.org/10.1016/j.heliyon.2018.
[28] U. Cid, P. Rodríguez-Seoane, B. Díaz-Reinoso, H. Domínguez, Extraction of fatty acids and phenolics from mastocarpus stellatus using pressurized green solvents, Mar. Drugs 19 (2021), https://doi.org/10.3390/md19080453.
[29] R.S. Baghel, N. Trivedi, C.R.K. Reddy, A simple process for recovery of a stream of products from marine macroalgal biomass, Bioresour. Technol. 203 (2016) 160–165, https://doi.org/10.1016/j.biortech.2015.12.051.
[30] P. Zhao, X. Wang, J. Niu, L. He, W. Gu, X. Xie, M. Wu, G. Wang, Agar extraction and purification of R-phycoerythrin from Gracilaria tenuistipitata, and subsequent wastewater treatment by Ulva prolifera, Algal Res. 47 (2020), 101862, https://doi.org/10.1016/j.algal.2020.101862.
[31] C. Teixeira-Guedes, J.S. Gomes-Dias, S.A. Cunha, M.E. Pintado, R. Pereira, J. A. Teixeira, C.M.R. Rocha, Enzymatic approach for the extraction of bioactive fractions from red, green and brown seaweeds, Food Bioprod. Process. (2023), https://doi.org/10.1016/j.fbp.2022.12.005.
[32] J.S. Gomes-Dias, A. Romaní, J.A. Teixeira, C.M.R. Rocha, Valorization of seaweed carbohydrates: autohydrolysis as a selective and sustainable pretreatment, ACS Sustain. Chem. Eng. 8 (2020) 17143–17153, https://doi.org/10.1021/acssuschemeng.0c05396.
[33] A. Sluiter, B. Hames, R. Ruiz, C. Scarllata, J. Sluiter, D. Templeton, Determination of Ash in Biomass, Technical Report NREL/TP-510-42622, 2008.
[34] B. Hames, C. Scarlata, A. Sluiter, Determination of Protein Content in Biomass, Technical Report NREL/TP-510-42625, 2008.
[35] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of Structural Carbohydrates and Lignin in Biomass, Technical Report NREL/TP-510-42618. http://www.nrel.gov/docs/gen/fy13/42618.pdf, 2012.
[36] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[37] S. Beer, A. Eshel, Determining Phycoerythrin and Phycocyanin Concentrations in Aqueous Crude Extracts of Red Algae, 1985.
[38] P. Sampath-Wiley, C.D. Neefus, An improved method for estimating R-phycoerythrin and R-phycocyanin contents from crude aqueous extracts of Porphyra (Bangiales, Rhodophyta), J. Appl. Phycol. (2007) 123–129, https://doi.org/10.1007/s10811-006-9118-7.
[39] H.K. Lichtenthaler, A.R. Wellburn, Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents, Biochem. Soc. Trans. (1983) 591–592.
[40] R.S. Baghel, P. Suthar, T.K. Gajaria, S. Bhattacharya, A. Anil, C.R.K. Reddy, Seaweed biorefinery: a sustainable process for valorising the biomass of brown seaweed, J. Clean. Prod. 263 (2020), https://doi.org/10.1016/j.jclepro.2020.121359.
[41] H.K. Mæhre, L. Dalheim, G.K. Edvinsen, E.O. Elvevoll, I.J. Jensen, Protein determination—method matters, Foods 7 (2018), https://doi.org/10.3390/foods7010005.
[42] M. Dong, Y. Jiang, C. Wang, Q. Yang, X. Jiang, C. Zhu, Determination of the extraction, physicochemical characterization, and digestibility of sulfated polysaccharides in seaweed-Porphyra haitanensis, Mar. Drugs 18 (2020), https://doi.org/10.3390/md18110539.
[43] B.M. Khan, H.M. Qiu, S.Y. Xu, Y. Liu, K.L. Cheong, Physicochemical characterization and antioxidant activity of sulphated polysaccharides derived from Porphyra haitanensis, Int. J. Biol. Macromol. 145 (2020) 1155–1161, https://doi.org/10.1016/j.ijbiomac.2019.10.040.
[44] S.G. Jackson, E.L. McCandless, Simple, rapid, turbidometric determination of inorganic sulfate and/or protein, Anal. Biochem. 90 (1978) 802–808, https://doi.org/10.1016/0003-2697(78)90171-9.
[45] W. Yaphe, G.P. Arsenault, Improved resorcinol reagent for the determination of fructose, and of 3,6-anhydrogalactose in polysaccharides, Anal. Biochem. 13 (1965) 143–148, https://doi.org/10.1016/0003-2697(65)90128-4.
[46] C. Castro-Ferreira, J.S. Gomes-Dias, P. Ferreira-Santos, R.N. Pereira, A.A. Vicente, C.M.R. Rocha, Phaeodactylum tricornutum extracts as structuring agents for food applications: physicochemical and functional properties, Food Hydrocoll. 124 (2022), 107276, https://doi.org/10.1016/j.foodhyd.2021.107276.
[47] M. Lahaye, C. Rochas, Chemical structure and physico-chemical properties of agar, Hydrobiologia 221 (1991) 137–148, https://doi.org/10.1007/BF00028370.
[48] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[49] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–355.
[50] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76, https://doi.org/10.1006/abio.1996.0292.
[51] N.J. Miller, C.A. Rice-Evans, Spectrophotometric determination of antioxidant activity, Redox Rep. 2 (1996) 161–171, https://doi.org/10.1080/13510002.1996.11747044.
[52] M.S. Blois, Antioxidant determinations by the use of a stable free radical, Nature 181 (1958) 1199–1200, https://doi.org/10.1038/1811199a0.
[53] C.I. Teixeira-Guedes, D. Oppolzer, A.I. Barros, C. Pereira-Wilson, Phenolic rich extracts from cowpea sprouts decrease cell proliferation and enhance 5-fluoro-uracil effect in human colorectal cancer cell lines, J. Funct. Foods 60 (2019), https://doi.org/10.1016/j.jff.2019.103452.
[54] P. Cherry, C. O'hara, P.J. Magee, E.M. Mcsorley, P.J. Allsopp, Risks and benefits of consuming edible seaweeds, Nutr. Rev. 77 (2019) 307–329, https://doi.org/10.1093/nutrit/nuy066.
[55] A. Bahari, K. Moelants, M. Kloeck, J. Wallecan, G. Mangiante, J. Mazoyer, M. Hendrickx, T. Grauwet, Mechanical disintegration and particle size sieving of Chondrus crispus (Irish moss) gametophytes and their effect on carrageenan and phycoerythrin extraction, Foods 10 (2021), https://doi.org/10.3390/foods10122928.
[56] V. Ummat, B.K. Tiwari, A.K. Jaiswal, K. Condon, M. Garcia-Vaquero, J. O'Doherty, C. O'Donnell, G. Rajauria, Optimisation of ultrasound frequency, extraction time and solvent for the recovery of polyphenols, phlorotannins and associated antioxidant activity from brown seaweeds, Mar. Drugs 18 (2020), https://doi.org/10.3390/md18050250.
[57] Q. Xiao, X. Wang, J. Zhang, Y. Zhang, J. Chen, F. Chen, A. Xiao, Pretreatment techniques and green extraction technologies for agar from gracilaria lemaneiformis, Mar. Drugs 19 (2021), https://doi.org/10.3390/md19110617.
[58] M. Ge, J. Shen, C. Liu, W. Xia, Y. Xu, Effect of acidification and thermal treatment on quality characteristics of high-moisture laver (Porphyra spp.), J. Food Process. Preserv. 46 (2022), https://doi.org/10.1111/jfpp.16762.
[59] A.M.M. Sousa, S. Morais, M.H. Abreu, R. Pereira, I. Sousa-Pinto, E.J. Cabrita, C. Delerue-Matos, M.P. Gonçalves, Structural, physical, and chemical modifications induced by microwave heating on native agar-like galactans, J. Agric. Food Chem. 60 (2012) 4977–4985, https://doi.org/10.1021/jf2053542.
[60] Y. Freile-Pelegrín, E. Murano, Agars from three species of Gracilaria (Rhodophyta) from Yucatán peninsula, Bioresour. Technol. 96 (2005) 295–302, https://doi.org/10.1016/j.biortech.2004.04.010.
[61] S. Yarnpakdee, S. Benjakul, P. Kingwascharapong, Physico-chemical and gel properties of agar from Gracilaria tenuistipitata from the lake of Songkhla, Thailand, Food Hydrocoll. 51 (2015) 217–226, https://doi.org/10.1016/j.foodhyd.2015.05.004.
[62] C. Zhang, D. An, Q. Xiao, H. Weng, Y. Zhang, Q. Yang, A. Xiao, Preparation, characterization, and modification mechanism of agar treated with hydrogen peroxide at different temperatures, Food Hydrocoll. 101 (2020), https://doi.org/10.1016/j.foodhyd.2019.105527.
[63] M.-F. Lai, C.-Y. Lii, Rheological and Thermal Characteristics of Gel Structures from Various Agar Fractions, 1997.
[64] S.G. Pereira, J.S. Gomes-Dias, R.N. Pereira, J.A. Teixeira, C.M.R. Rocha, Innovative processing technology in agar recovery: combination of subcritical water extraction and moderate electric fields, Innov. Food Sci. Emerg. Technol. 84 (2023), https://doi.org/10.1016/j.ifset.2023.103306.
[65] W.K. Lee, Y.Y. Lim, A.T.C. Leow, P. Namasivayam, J.O. Abdullah, C.L. Ho, Factors affecting yield and gelling properties of agar, J Appl Phycol. 29 (2017) 1527–1540, https://doi.org/10.1007/s10811-016-1009-y.
[66] C.I.K. Diop, S. Beltran, I. Jaime, M.-T. Sanz, Adjustable gel texture of recovered crude agar induced by pressurized hot water treatment of Gelidium sesquipedale industry waste stream: An RSM analysis, Foods 11 (2022) 2081, https://doi.org/10.3390/foods11142081.
[67] M. Martínez-Sanz, A. Ström, P. Lopez-Sanchez, S.H. Knutsen, S. Ballance, H. K. Zobel, A. Sokolova, E.P. Gilbert, A. López-Rubio, Advanced structural characterisation of agar-based hydrogels: rheological and small angle scattering studies, Carbohydr. Polym. 236 (2020), https://doi.org/10.1016/j.carbpol.2019.115655.
[68] M.D.N. Meinita, D. Harwanto, M.A. Amron, G.T. Hannan, I.S. Jeong, J.S. Choi Moon, A concise review of the potential utilization based on bioactivity and pharmacological properties of the genus Gelidium (Gelidiales, Rhodophyta), J. Appl. Phycol. (2023), https://doi.org/10.1007/s10811-023-02956-7.
[69] P. Rawiwan, Y. Peng, I.G.P.B. Paramayuda, S.Y. Quek, Red seaweed: a promising alternative protein source for global food sustainability, Trends Food Sci Technol. 123 (2022) 37–56, https://doi.org/10.1016/j.tifs.2022.03.003.
[70] T. Bito, F. Teng, F. Watanabe, Bioactive compounds of edible purple laver Porphyra sp. (nori), J. Agric. Food Chem. 65 (2017) 10685–10692, https://doi.org/10.1021/acs.jafc.7b04688.
[71] K.D. González-Gloria, R.M. Rodríguez-Jasso, E. Shiva, M.L. Chávez Aparicio, E. T. González, H.A. Ruiz Kostas, Macroalgal biomass in terms of third-generation biorefinery concept: current status and techno-economic analysis – a review, Bioresour Technol Rep. 16 (2021), 100863, https://doi.org/10.1016/j.biteb.2021.100863.

[72] D. Seisun, N. Zalesny, Strides in food texture and hydrocolloids, Food Hydrocoll. 117 (2021), 106575, https://doi.org/10.1016/j.foodhyd.2020.106575.
[73] J. Park, H. Lee, T.B. Dinh, S. Choi, J. De Saeger, S. Depuydt, M.T. Brown, T. Han, Commercial potential of the cyanobacterium Arthrospira maxima: physiological and biochemical traits and the purification of Phycocyanin, Biology (Basel). 11 (2022), https://doi.org/10.3390/biology11050628.
[74] K.V.K. Boodhoo, M.C. Flickinger, J.M. Woodley, E.A.C. Emanuelsson, Bioprocess intensification: a route to efficient and sustainable biocatalytic transformations for the future, Chemical Engineering and Processing - Process Intensification. 172 (2022), 108793, https://doi.org/10.1016/j.cep.2022.108793.