

Seaweeds biorefinery into pigments and carrageenans: Testing a sequential extractions approach

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ABSTRACT

This study aims to test a sequential extraction (SE) method for red seaweeds (*Chondrus crispus*, *Mastocarpus stellatus*, *Gigartina pistillata*). The tested SE is a preliminary screening step towards the extrusion-based biorefinery (E2B2) of carrageenophytes into various natural products, a concept that still needs to be established. Phyco-biliproteins, chlorophyll-a, carotenoids, and hybrid-carrageenans (HK) were isolated with the following sequence: cold-water extraction (CWE), ethanolic extraction (EE), hot water extraction (HWE), and hot alkaline extraction (HAE). HK chemical structures and gelling properties were systematically compared with those of carrageenans isolated from direct extractions (controls). Such comparison, which is critically missing in earlier SE studies, allows to assess the impact of prior extraction sequences on such HK properties. SE effectively separated algal components, facilitating the recovery of HK, particularly with *M. stellatus* and *G. pistillata*, with superior gel elasticity and viscosity compared to the controls. HWE yielded less sulphated HK with higher molecular masses and enhanced gelling properties. HAE extracts exhibited lower molecular masses and reduced gelation potential due to the prior HWE sequence. Prolonged extraction generally improved molecular masses and gels elasticity, although a distinct trend was found with *C. crispus*. The results from this preliminary screening suggest that *M. stellatus* is not a good candidate for testing in E2B2.

1. Introduction

Carrageenans are sulphated polysaccharides present in the cell walls of red algae (Rhodophyta) and are known for their thermo-reversible gelling properties in water. They are widely used as food additives for thickening, stabilizing, and gelling, in products like e.g. ice cream and sauces (Piculell, 2006; Villanueva et al., 2004). More recently, carrageenans showed promise for the pharmaceutical industry as their antiviral, antibacterial and antitumor attributes, among others, are very attractive for wound healing (Krishnan et al., 2024) and tissue regeneration applications (Das & Bal, 2024).

Current industrial extraction routes in the carrageenan sector have several weaknesses, such as high consumption of time, energy and water. Additionally, large amounts of chemical reagents and solvents are required to achieve optimal yield and quality, and the resulting effluents pose significant risks to health and environment, necessitating strict control measures (Abdul et al., 2018). Although too few reports are available in the scientific literature, a recent case study identified potassium hydroxide, energy of heating and solid wastes as the main sources of emissions causing environmental impact (Zuhria, 2022). In

particular, the alkaline treatment step is a relatively harsh and destructive process for the algae, causing the breakdown of the tissue matrix and the efficient solubilisation of nearly all the carrageenan, and thus is the major source of negative environmental impact (Zuhria, 2022). The residual algal biomass, still rich in organic components, is generally unrecoverable and wasted after this step, though strategies to further refine the solid waste into value-added bio products have been recently proposed (Bianchi et al., 2022). Consequently, greener extraction methods that use less energy, reduce water consumption, and employ more environmentally friendly solvents are emerging (Abdul et al., 2018; Krishnan et al., 2024) such as microwave-assisted extraction, ultrasound-assisted extraction, hydrothermal extraction, enzymatic-assisted extraction, or reactive extrusion. These methods are, however, still expensive or challenging to implement in the carrageenan industry (Abdul et al., 2018; Krishnan et al., 2024).

Sequential extraction as a biorefinery approach holds the promise for being a more sustainable and resource-efficient method, allowing for the recovery of multiple valuable products. This approach was recently tested with *Chondrus crispus*, a carrageenophyte from which pigments, proteins and hybrid-carrageenans (HK) were recovered using a

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three-step sequential ultrasound-assisted extraction (Maia et al., 2023). The third step allowed for the recovery of HK in yields comparable with those achieved when ultrasound-assisted extraction of carrageenan is applied alone. However, the authors did not document the HK gelling properties. KH show functional properties intermediate between kappa- and iota-carrageenans resulting from their random block copolymer architecture, where lengths and positions of alternating blocks of kappa-, iota-, mu-, and nu-carrageenan disaccharides depend on the extraction route (Souza et al., 2023). Similarly, Bahari et al. (2021) proposed a method to extract both phycoerythrin and HK from *C. crispus*, starting with the extraction of phycoerythrin under mild conditions. Their findings showed that phycoerythrin extraction is dependent on algal particle size, whereas carrageenan extraction relies more heavily on temperature. However, the carrageenan yield was not benchmarked with yields achieved when HK is extracted in a conventional single step, nor HK gelling properties were reported. A sequential extraction approach has also been tested with *Solieria filiformis* to obtain a nutrient-rich extract, polyunsaturated fatty acids, and pure iota-carrageenan, using enzymatic-assisted and microwave-assisted extractions (Penuela et al., 2018). This method demonstrated the potential for enhancing the yield of iota-carrageenan when compared to conventional single extraction methods, while generating a significant reduction in algal residues

compared to those generated through direct extraction of each product. However, the functional properties, and in particular the gelling behaviour, of the recovered iota-carrageenan were not studied.

Another biorefinery which shares similarities with the sequential extraction approach was introduced nearly a decade ago: the extractive extrusion-based biorefinery (E2B2) of seaweeds (Hilliou, 2016). The E2B2 concept, which still needs to be demonstrated, consists in feeding a biomass into an extruder along with different chemicals introduced at different locations along the barrel where the different fractions are to be refined continuously. This approach is schematized in Fig. 1, which also shows cases the first E2B2 trials carried out with a carrageenophyte (Hilliou, 2016) to extract a gelling HK.

Here, a four-steps sequential extraction approach reminiscent from the sequences occurring during the E2B2 of seaweeds is applied to three carrageenophytes, focusing on the production of HK while minimizing the hydrocolloid extraction waste and valorising the biomass by recovering pigments and proteins. The main objective is to screen for the potential transposition of the sequential extractions to the E2B2 of seaweeds. For this, the role of each extraction step on the properties of the extracted HK needs to be understood. Thus, a systematic comparison of HK chemical structures and gelling behaviors with those of HK isolated by conventional direct extraction is reported here, as such benchmark

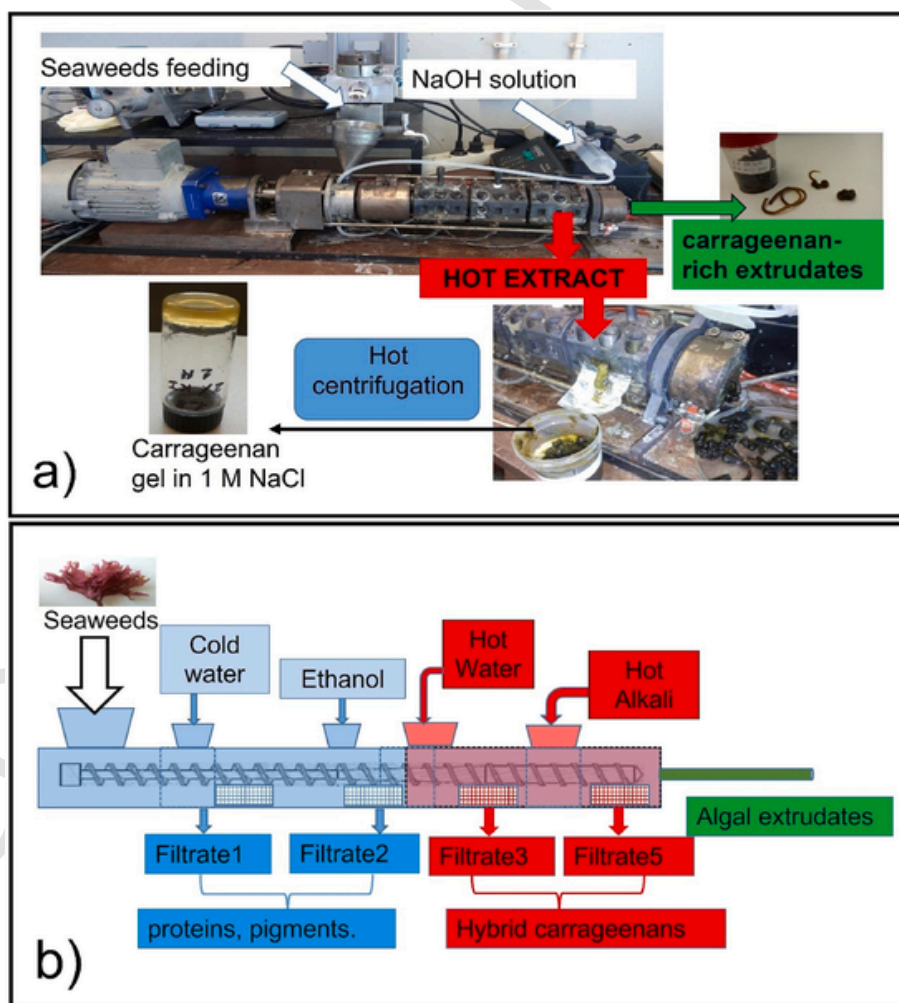


Fig. 1. the Extractive Extrusion-Based Biorefinery (E2B2) concept. a) extractive extrusion of a hybrid carrageenan from a powdered red seaweed using NaOH solution. b) Scheme of the E2B2 where a seaweed is refined into various fractions using the mechanical energy of the extruder's screw, different chemicals and in-line filtration in different zones of the extruder's barrel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

has not been sufficiently reported for carrageenophytes. The following four extractions are sequentially performed on a single biomass stream from step to step: a cold water extraction (CWE), an ethanolic extraction (EE), a hot water extraction (HWE) and eventually a hot alkaline extraction (HAE). In extrusion, a cooling step is a bad option due to heat transfer from heating blocks to the cooling blocks and to the material conveyed by the rotating screws. Rather, strong heating at the end of the barrel, after the cold extraction of other fractions, is more amenable. Thus, the four sequential extractions tested here start with cold extractions and finishes with hot extractions, whereas extraction times are short to target the short residence times typically found in the extrusion-assisted extraction of phycocolloids using laboratory scale extruders (Sugiono et al., 2019). In addition, the HK yields, chemical structures and gelling properties are systematically benchmarked with those of HK recovered by conventional single step extractions, in view to fill the gap of knowledge on the effects of previous extraction steps.

2. Experimental

2.1. Seaweeds and chemicals

Three commercial dried seaweeds were purchased from Algalplus (Ilhavo, Portugal). *Mastocarpus stellatus*, *C. crispus* and *Gigartina pistillata* were received in powdered form and used without further processing. Ethanol (96 %) was purchased from local supermarkets whereas sodium hydroxide and sodium chloride (both analytical grade) were sourced from Sigma-Aldrich Química SL (Sintra, Portugal).

2.2. Sequential extraction

Following the criteria set by the E2B2 concept, extraction steps 1 to 4 were sequenced as displayed in Fig. 2. This approach takes on board the outputs from the three-steps biorefinery of agarophytes which reported that agars produced through a sequential CWE, EE and HAE process showed improved gelling properties compared to hydrocolloids isolated from a single HAE (Gomez-dias et al., 2024). We conjecture that an equivalent sequential approach will have a similar positive im-

pact on the gelling properties of a parent sulphated polysaccharide, namely hybrid-carrageenans. Such hypothesis is to be worked out for the first time in the present study.

The extraction of the main product, hybrid carrageenan, was performed last, as it requires hot solvents for efficient extraction and holds the most significant commercial relevance. Carrageenans are economically more attractive when extracted in purer form, emphasizing the necessity of removing other fractions beforehand. The extraction times tested in steps 2 to 4 vary from 60 min to 10 min, in order to assess their impact on the characteristics of the recovered fractions and reproduce the short times typically found in extrusion. The amount of alkali used in step 4 for HAE results from earlier optimization studies for the extraction of HK from two seaweeds: *Mastocarpus stellatus* (Azevedo et al., 2013) and *Chondrus crispus* (Azevedo, Domingues, et al., 2015). A third seaweed, *Gigartina pistillata*, is also included in this study to assess the efficiency of the proposed sequential extraction process when applied to algae with varied contents of natural compounds targeted for refinement (Souza et al., 2023; Azevedo et al., 2013; Azevedo, Domingues, et al., 2015; Álvarez-Viñas et al., 2024). Each extraction sequence was initiated by mixing 2 g of seaweed powder with 60 mL of the different solvents (a sample:solvent ratio of 1:30). Extractions were carried out in triplicate and the extraction times studied (t) were: 1 h, 30 min and 10 min. In step 1, cold-water extraction (CWE) was performed by suspending the mixture in distilled water in 100 mL flasks, protected from light, at ambient temperature (25 °C) under orbital agitation (150 rpm) for approximately 16 h to recover phycobiliproteins. Then the samples were filtered, using a glass funnel and a metal mesh (Gomes-Dias et al., 2024). The filtered solid fraction was streamed to step 2, while the liquid fraction was centrifuged (Universal 320 Centrifuge, Hettich GmbH, Tuttlingen, Germany) at 8000 g for 10 min and the pellet was discarded. Ethanolic extraction (EE, step 2) was carried out by suspending the solid fraction from step 1 in a 96 % (v/v) ethanol solution placed on an orbital shaker (150 rpm), for the studied extraction time (t), at room temperature, protected from light. After EE, the samples were filtered as in step 1 and the solid fraction was streamed to step 3. The liquid fraction was centrifuged (8000 g for 10 min) and the pellet was discarded (Maia et al., 2023). Hot water extraction (HWE,

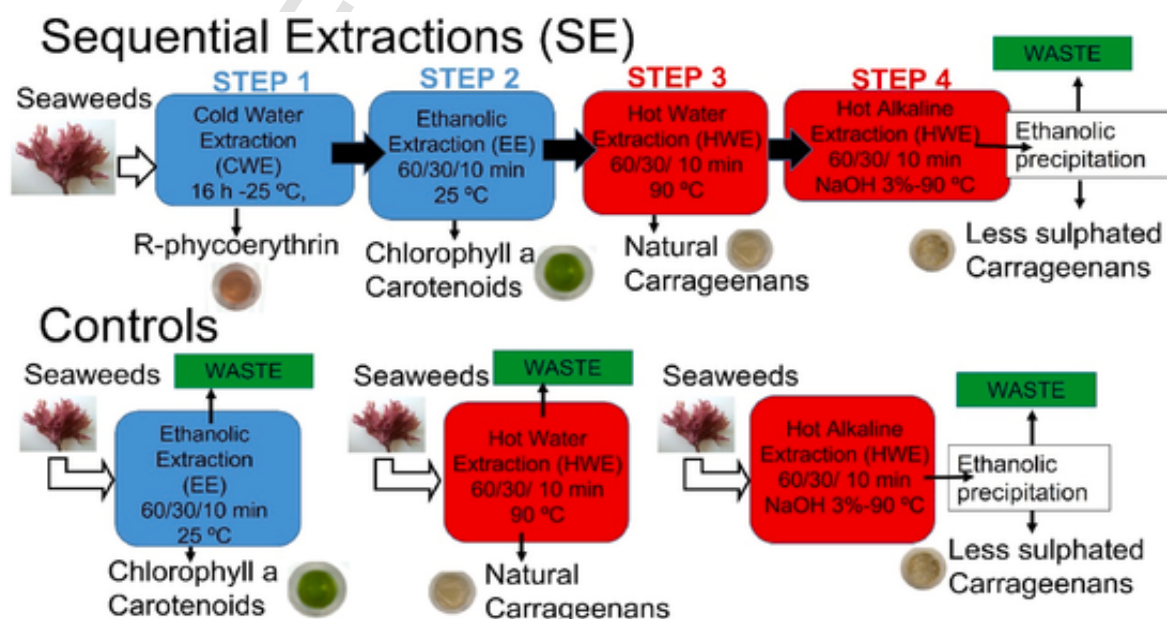


Fig. 2. Scheme of the four-steps sequential extraction approach used here (top) to biorefine proteins (from cold water extraction – CWE, step 1), pigments (from ethanolic extraction-EE, step 2), HK (from hot water extraction – HWE, step 3) and less sulphated HK (from hot alkaline extraction – HAE, step 4). The bottom row shows the direct extraction routes (controls) employed to assess the impact of sequential extractions on the yields and characteristics of isolated pigments and HK.

step 3) was performed by mixing the solids filtered in step 2 with distilled water in a 100 mL flask heated at 90 °C (Azevedo et al., 2013, 2015a) during the studied extraction time (t). The mixture was then centrifuged as above and the pellet was sent to the next step. The liquid fraction was cast into a plastic container and dried overnight in an air convection oven at 55 °C to form a hybrid-carrageenan film. Finally, the pellet recovered from step 3 was subjected to hot alkaline extraction (HAE, step 4) 4 by mixing with a 3 % (w/v) NaOH solution at 90 °C (Azevedo et al., 2013, 2015a) for the designated extraction time (t). After extraction, the solution was centrifuged as above, and the liquid fraction was cast into a plastic container and dried overnight at 55 °C under air convection. The next day, the resulting film sample containing both carrageenan and salt was weighted to determine the total weight after HAE, M_T , recovered from the triplicate extraction. Then, a portion of weight M_p (e.g., 5 g) of this total was dissolved in hot water (90 °C) to form a 2 % (w/v) carrageenan solution with satisfactory viscosity to maximize the ethanolic precipitation of the carrageenan as detailed elsewhere (Azevedo et al., 2013; Azevedo, Domingues, et al., 2015). This precipitate was transferred to a container and dried overnight, as in step 3.

2.3. Quantification of the recovered fractions

The R-phycoerythrin content (R-PE) in the phycobiliprotein-rich solutions isolated in step 1 was calculated based on the absorbances at 564 nm (A_{564}), 618 nm (A_{618}) and 730 nm (A_{730}) measured with a UV-VIS spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan), using the following equation (Sampath-Wiley & Neefus, 2007):

$$R - PE = 0.1247 [(A_{564} - A_{730}) - 0.4583 (A_{618} - A_{730})] \quad (1)$$

The chlorophyll-a (*Chloro-a*) and carotenoid (*Carot*) contents of the liquid samples recovered from step 2 were calculated based on the UV-VIS absorbances measured as described above at 480 nm (A_{480}), 632 nm (A_{632}), 652 nm (A_{652}), 665 nm (A_{665}) and 750 nm (A_{750}), using equations (2) and (3), respectively, (Stengel & Connan, 2015) which read

$$Chloro - a = -3.2416 (A_{632} - A_{750}) - 6.4151 (A_{652} - A_{750}) + 16.4351 (A_{665} - A_{750}) \quad (2)$$

$$Carot = 4 (A_{480} - A_{750}) \quad (3)$$

The yield of hybrid-carrageenan Y extracted in step 3 was computed from the ratio of the dried carrageenan film sample weight over the initial weight of powdered algae entering the SE process, whereas the yield YY of hybrid carrageenan recovered from HAE was computed using the following equation

$$YY = \frac{m_p M_T}{m_a M_p} \quad (4)$$

where m_p is the mass of dried carrageenan precipitated in ethanol, and m_a is the total mass of powdered algae used in the triplicate SE process.

2.4. Characterization of hybrid carrageenans

Fourier Transform Infrared Spectroscopy (FTIR) was used for the qualitative chemical characterization of the extracted HK. The FTIR analyses were performed with a Spectrum 100 FTIR spectrometer (PerkinElmer, Shelton, USA) equipped with an Attenuated Total Reflectance (ATR) accessory. Spectra were obtained in the range of 4000 to 600 cm^{-1} with 16 scans per sample and a resolution of 1 cm^{-1} .

^1H NMR spectroscopy was performed using a 400 MHz Avance III spectrometer (Bruker, Billerica, USA) at 70 °C to minimize the line broadening caused by the viscosity of the 1 % (w/w) carrageenan solu-

tions in D_2O . Indeed, sample solutions were ultrasonicated for several hours (using a DCG-300H bath, MCR Ltd.) to lower their viscosity prior to NMR characterization. Chemical shifts (ppm) were referenced to the D_2O lock signal (4.41 ppm) and further adjusted to match the chemical shift for kappa-carrageenan (5.09 ppm), as prescribed (van de Velde et al., 2004). The molar fractions (mol. %) of the carrageenan disaccharide repeating units (kappa - κ , iota - ι , mu - μ and nu - ν) were calculated as the integrated intensity of the corresponding ^1H NMR anomeric signal over the sum of the integrated intensities of all assigned carrageenan anomeric protons (van de Velde et al., 2004). Pyruvic acid acetals and floridean starch were also detected in the ^1H NMR spectra though their methyl proton resonances with chemical shifts of 1.44 ppm for the pyruvic acid and signals at 5.35 ppm and 4.98 ppm for floridean starch (van de Velde et al., 2004).

The molecular mass distribution was obtained by size exclusion chromatography and refractive index detection using a Waters 600 apparatus coupled to a Waters 2410 differential refractive index detector and a PolySep-GFC-P Linear column (Phenomenex, Alcobendas, Spain). This column has been calibrated with pullulan standards (Shodex, Munich, Germany) ranging from 6300 to 642,000 g/mol. Duplicate measurements were performed with 0.1 wt % HK solutions in 0.1 M NaCl at 40 °C.

All extracted HK samples were tested for their gelling or thickening ability. HK extracts 2 % (w/v) were dissolved in 1 M NaCl and stirred vigorously for 1 h at 80 °C. The concentration and ionic strength were specifically selected to produce gels with sufficient rigidity at 25 °C but without water release (Azevedo et al., 2014; Torres, Azevedo & Hilliou, 2016), while maintaining a single cation (Na^+) to promote gel formation, since HAE process was carried out with NaOH. After allowing the hot solutions to cool for 24 h, two possible outcomes were observed: gel formation or a liquid solution.

2.5. Rheological properties of HK gels and solutions

HK gelled samples were reheated to 85 °C and the resulting hot solutions were directly transferred into the pre-heated (80 °C) Couette accessory (gap of 0.5 mm) of a MCR300 rheometer (Anton Paar, Graz, Austria). The geometry surface was covered with dodecane to prevent water evaporation. Samples were then cooled to 25 °C within 5500 s. During cooling, small amplitude oscillatory shear was applied (with 0.1 % strain at a frequency of 1 Hz) to probe the temperature evolution of linear viscoelastic properties such as $\tan \delta = G''/G'$, where G' is the shear storage modulus and G'' is the shear loss modulus. The temperature at which $\tan \delta = 1$ was defined as the gel setting temperature T_g . Following cooling and gel setting, a frequency sweeps was performed at 25 °C with a 0.1 % dynamic strain, which ensured the measurement of equilibrated gels' mechanical spectra within the linear regime. An oscillatory shear strain sweep from 0.01 % to 1000 % with a frequency of 1 Hz was then made to characterize the gel behaviour under large deformation and to measure the linear elastic modulus G_0 of the gels from the linear viscoelastic regime of the sweep.

The HK solutions produced in section 2.4 were placed into the plate-plate geometry (40 mm diameter) of a stress-controlled rotational rheometer (MCR 302, Anton Paar, Graz, Austria) at 25 °C. Each sample was equilibrated during 300 s by applying an oscillatory strain of 10 % at 1 Hz and 25 °C. Then a sweep in steady shear rates from 1 s^{-1} to 1000 s^{-1} was performed and immediately followed by a sweep from 1000 s^{-1} down to 1 s^{-1} to measure the shear rate dependence of the apparent shear viscosity η (the flow curve), and to assess any possible thixotropic behaviour related with carrageenan aggregation with time. For this purpose, each steady shear rate was applied during 20 s and the shear viscosity η results from the average of the measurements performed during the last second of the step.

2.6. Statistical analysis

Statistical analysis was performed to identify significant differences among data sets using one-way ANOVA in Origin version 2024b (Learning Edition). Multiple comparisons were evaluated through Tukey's test at a 95 % confidence interval. For each seaweed species, analysis was conducted to assess whether the sequential extraction methods resulted in significant differences ($p < 0.05$) in the yield of extracted fractions, and all carrageenan characteristics. Results are presented as means $\pm$ standard deviation, with a minimum of three replicates conducted unless otherwise specified.

3. Results and discussion

To establish whether the SE of HK is a valid approach compared to their conventional batch extraction, the yields of the different fractions recovered in each step are first presented and discussed in light of the known compositions of the tested seaweeds. The chemical structures of the HK recovered in each step of the SE are then presented before the results from their rheological analysis.

3.1. Yields of proteins and pigments

The compound extracted during CWE is a known pigment of red algae, but also a protein, R-phycoerythrin (R-PE) (Gomes-Dias et al., 2024). CWE does not have a control, as it is step 1 of the SE and therefore the characteristics of this R-PE fraction are not influenced by any prior extraction. The R-PE yields computed from equation (1) are presented in Fig. 3 for each treated seaweed.

Comparing the R-PE yields shown in Fig. 3 with the R-PE contents of 1.99 mg/g d.w. and of 0.53 mg/g d.w. reported for *M. stellatus* (Nguyen, Moranchais, Fleurence, & Dumay, 2017) and *C. crispus* (Pina et al., 2014), respectively, it is evident that less than half of the R-PE reported in the literature is extracted from the algae studied here. As for *G. pistillata*, no reports were found to compare with the 0.307 ± 0.049 mg/g d.w. shown in Fig. 3. Furthermore, *C. crispus* shows comparatively lower R-PE yields than the other two algae. This is in qualitative agreement with results reported earlier for R-PE (Nguyen et al., 2017; Pina et al., 2014) and with the total protein contents found in HK isolated from the same species by hydrothermal extraction (Álvarez-Viñas, Domínguez & Torres, 2024). The low R-PE yield observed in Fig. 3 may be attributed to non-optimized extraction conditions. For instance, significantly higher yields were achieved for *M. stel-*

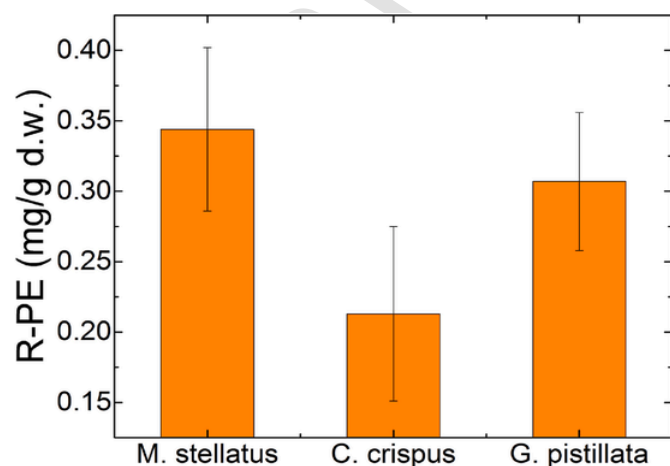


Fig. 3. Phycoerythrin (R-PE) yields from the CWE step of the sequential extraction, with standard deviations computed from triplicate CWE for the three carrageenophytes.

latus (Nguyen, Moranchais, Fleurence, & Dumay, 2017) by using a different method involving phosphate buffer (20 mM, pH 7.1) and xylanase to degrade the algal cell wall. It is possible that the CWE extract do not only contain R-PE, but also salts which leached from the seaweed, as well as sulphated carrageenans (e.g. lambda-carrageenans), as some studies reveal that it is possible to extract carrageenans from *C. crispus* at 25 °C for extended times period (Bahari et al., 2021). So, the liquid extracts from step 1 were cast into plastic molds and dried to check for the presence of HK. Indeed, films were obtained from the products of *C. crispus* and *G. pistillata* suggesting the recovery of polysaccharides which were then analyzed (see sections 3.2 and 3.3).

The extraction yields of chlorophyll-a obtained during the EE of step 2 and computed with equation (2) are presented in Fig. 4. For all seaweeds, the chlorophyll-a yield showed minimal or no statistical differences across extraction times (1 h–10 min), when each extraction type (sequential (SE) and control) was analyzed separately.

With *M. stellatus*, the 1 h SE with ethanol produced a lower yield than the 1 h control extraction, but for smaller times the yield was similar. Conversely, *C. crispus* exhibited a slight increase in chlorophyll-a yield during SE at 30 min of extraction, likely due to the initial step. Similarly, *G. pistillata* showed improved recovery with the 1 h SE compared to shorter control times (10–30 min), although the 1 h yields for both SE and control extractions were statistically similar due to high variability.

Yields for carotenoids computed with equation (3) are presented in Fig. 5. No attempt was made to separate the carotenoids from the ethanol, nor to recycle ethanol, as the main objective is to assess the impact of sequential extraction on the yields for caretonoids.

All extraction times and types (SE or control) produced similar yields in *G. pistillata* and *C. crispus*, indicating that SE does not significantly impact carotenoid recovery. An exception was observed for *M. stellatus*, where the 10 min control yielded less carotenoids than the SE, whereas the SE carried during 1 h yielded less product than the control. Thus, for longer extraction times, SE may actually reduce the carotenoids yield in this species. The large standard deviations, particularly for *G. pistillata* with 1 h extraction time, could be due to the sedimentation of pigments and suspended residual algal particles over time, with pigments potentially settling within minutes. Also, the light exposure in the moment before absorbance measurement could result in

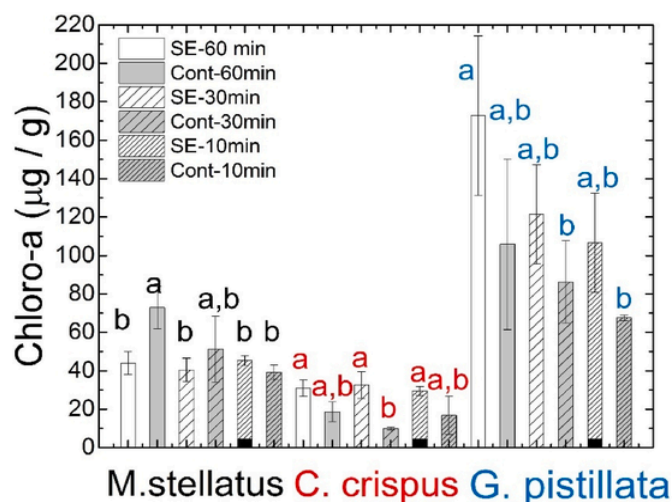


Fig. 4. Ethanol extraction yields of chlorophyll-a (Chloro-a) for the 3 seaweeds tested in step 2 of the sequential extraction (SE) and in the control extraction (Cont), with different extraction times. For each yield, bars (for standard deviations) with different letters indicate statistically significant differences ($p < 0.05$).

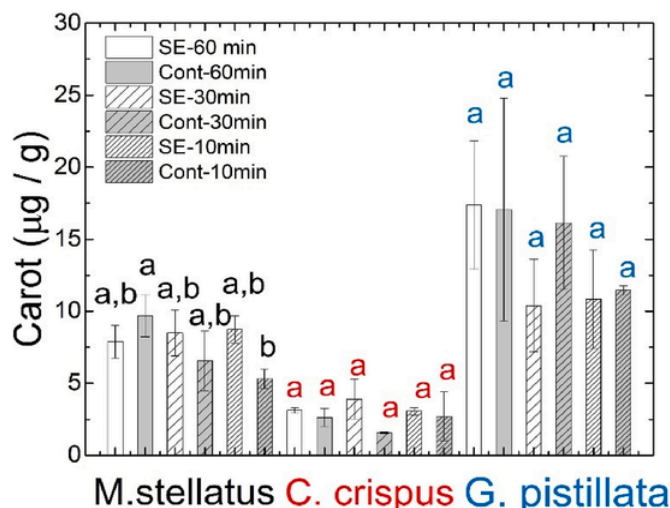


Fig. 5. Ethanol extraction yields of carotenoids (Carot) for the 3 seaweeds tested in step 2 of the sequential extraction (SE) and in the control extraction (Cont) with different extraction times. For each yield, bars (for standard deviations) with different letters indicate statistically significant differences ($p < 0.05$).

large experimental errors, since these pigments (chlorophylls and carotenoids) are prone to photo-oxidation. Degradation under light can result in inconsistent measured concentrations, causing fluctuations in pigment concentration (Cheloni & Slaveykova, 2018).

Comparing the three algae, *G. pistillata* shows the highest concentration of pigments (both chlorophyll-a and carotenoid), followed by *M. stellatus* and then *C. crispus*. These results are different from those reported elsewhere (Carpena et al., 2021) in which *M. stellatus* has the highest concentration of chlorophyll-a and *C. crispus* showed higher carotenoids levels. However in (Carpena et al., 2021) pigments were extracted by dissolving 1 g of alga in 20 mL of ethanol/water (50:50 v/v), stirring at 50 °C for 24 h, followed by re-extraction with fresh solvent under the same conditions. This method yielded significantly higher chlorophyll-a levels in the three seaweeds than those plotted in Fig. 4. Regarding carotenoids, less than 10 % of the full content was extracted in this work, compared to the data documented in (Carpena et al., 2021), likely due to the shorter extraction times used here, which contrast with durations of up to two days. Maia et al. (2023) also used extraction times shorter than 1 h and similar solvent (ethanol 95 % v/v), without any prior extraction or cleaning steps, for pigment extraction from *C. crispus*. However, these authors utilized ultrasound assisted extraction, resulting in concentrations ranging from 434.25 to 276.5 µg/g d.w. and from 63.2 to 38.1 µg/g d.w., for chlorophyll-a and total carotenoids, respectively. Overall, quantitative comparisons of yields are difficult as geographical and seasonal variations in the algae impact pigment concentrations, and ethanolic extractions reported in the literature are carried out under varying parameters. The fractions recovered in EE were not separated from ethanol, nor was the ethanol recovered, as the focus here is on the preliminary screening of carrageenophytes for their future testing in E2B2.

3.2. Yields of hybrid-carrageenans

In both HWE and HAE, the target is the recovery of carrageenans, and this section focuses on the carrageenan yields. In the HWE step 3, all the control extracts showed larger yields Y than the SE ones, see Fig. 6. The alga with the overall highest yield of HK in hot water (at 90 °C) was *C. crispus*, whereas *M. stellatus* showed the lowest yield. This is overall in harmony with the trends reported for these carrageenophytes

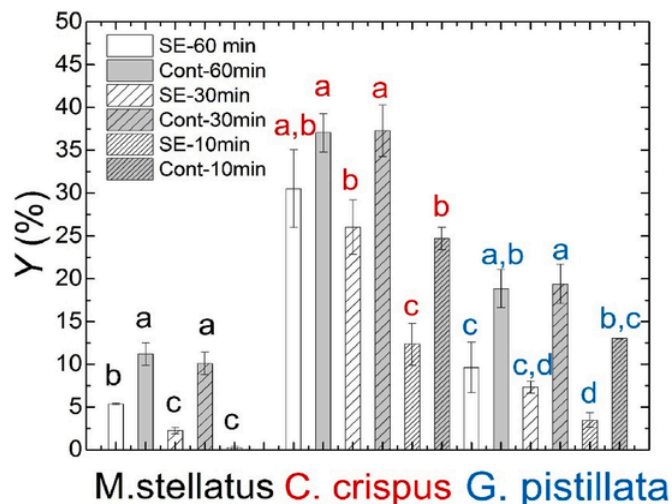


Fig. 6. Hot water extraction yields of carrageenans (Y) for the 3 seaweeds tested in step 3 of the sequential extraction (SE) and in the control extraction (Cont) with different extraction times. For each yield, bars (for standard deviations) with different letters indicate statistically significant differences ($p < 0.05$).

regardless of the extraction routes employed to estimate their carrageenan contents, their life stage, or geographical sourcing (Azevedo, Domingues, et al., 2015; Álvarez-Viñas, Domínguez & Torres, 2024; Azevedo, Torres, et al., 2015; Azevedo et al., 2013; Bahari et al., 2021; Hilliou et al., 2012; Pereira et al., 2009; Pereira & van de Velde, 2011).

Regarding extraction time, for the sequential samples, shorter processing times generally resulted in lower yields, as expected. These findings are consistent with studies showing that carrageenan extraction yield is largely time and temperature dependent, with longer durations and higher temperatures generally leading to better solubilisation and recovery of carrageenan (Bahari et al., 2021; Hilliou et al., 2006). For the controls, only extraction times below 30 min resulted in significantly lower carrageenans yields for all algae tested.

In the last extraction step (HAE), while alkaline extractions at lower temperatures may result in protein extraction (Gomes-Dias et al., 2024) the elevated temperature (90 °C) used in step 4 likely denatured any proteins which therefore were not accounted for here. The HAE yields YY computed from equation (4) are shown in Fig. 7.

As expected, the control samples showed higher yields than the controls in step 3. This is likely due to the ability of alkaline solutions to enhance solvent penetration into algae tissue cells (Azevedo et al., 2013, 2015a; Hilliou et al., 2006). The carrageenan yields for *C. crispus* processed in sequential extraction were notably lower than those of the control samples and the other algae. This outcome suggests that the previous HWE step may have already removed a substantial portion of the carrageenan content. Indeed, the carrageenan yields for the control experiments with 1h and 30 min extraction times reached up to 60 %, which is in good agreement with the carrageenan content reported elsewhere for *C. crispus* processed with NaOH (Pereira et al., 2009; Pereira & van de Velde, 2011). In contrast, larger carrageenan yields are achieved for *M. stellatus* and *G. pistillata* when compared with step 3, indicating that much of their HK remained in the alga after the HWE. The highest yield of *M. stellatus* (obtained from the 1 h SE extract) is within the range (40–50 %) obtained elsewhere (Azevedo et al., 2013) with a 3 h NaOH pre-treatment followed by a 1 h HWE at 90 °C. In other words, the SE method used in this study produced similar yields as those that used longer times of extraction. In the case of *G. pistillata*, the highest yield was achieved with the 30 min SE and the 1 h extracts (SE and control) were alike. As for the 10 min control extraction, no car-

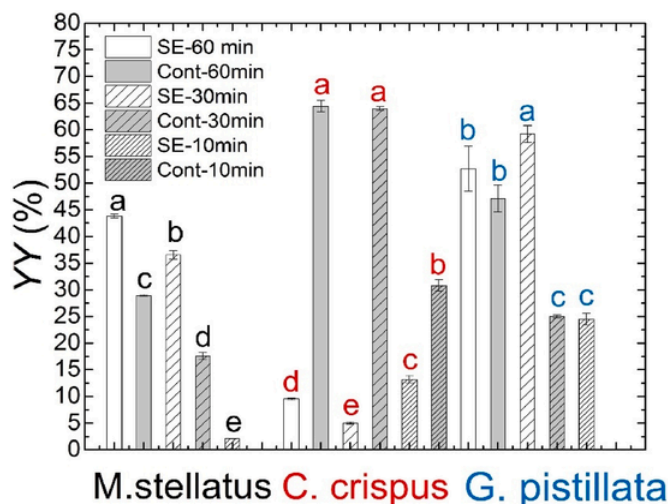


Fig. 7. Hot alkaline extraction yields of carrageenans (YY) for the 3 seaweeds tested in step 4 of the sequential extraction (SE) and in the control extraction (Cont) with different extraction times. For each yield, bars (for standard deviations) with different letters indicate statistically significant differences ($p < 0.05$).

rageenan was precipitated, but in SE the yield was more than 20 %. Thus, as for *M. stellatus*, SE enhances the extraction yield of alkali modified carrageenans, but the extraction time does not need to be more than 30 min. This is an important result since total extraction times of the order of 20 min to 1 h performed sequentially can potentially recover as much carrageenan as conventional longer extraction routes (Bahari et al., 2021; Souza et al., 2023). However, the composition and molecular characteristics of the extracted HK may differ.

3.3. Chemical characterization of hybrid-carrageenans

The molecular mass distributions and the contents in carrageenan disaccharide units are summarized in Tables 1–3 for all extracted HK, whereas representative FTIR spectra are displayed in Fig. 8. For *M. stellatus*, 10 min of extraction was a too short time to isolate any carrageenan. No film was formed after drying and the FTIR and ^1H NMR spectra were featureless in terms of carrageenan characteristic signals. Some Floridean starch was detected in the ^1H NMR spectra of the

30 min HAE control extracts from *G. pistillata* (see Fig. 9b) and of the 30 min SE and control HAE extracts from *C. crispus* (see Fig. 10).

This high starch content could interfere with the carrageenan yield, but it has been shown that, for the specific case of *C. crispus*, starch does not affect the carrageenan gel properties (Azevedo, Domingues, et al., 2015). Another contaminant, pyruvic acid, was detected in large amount in extracts from 60 to 30 min control HAE of *G. pistillata* and in smaller amounts in the 60 and 30 min control HAE and 30 min SE HWE extracts of *C. crispus* (see Fig. 10).

3.3.1. Cold water extracts

No detectable hybrid-carrageenan was found in the CWE product from *M. stellatus*, as the liquid failed to form a film upon drying. As such, the CWE step in *M. stellatus* has no impact on the recovery of carrageenans in the following HWE and HAE steps. Thus, the total carrageenan yield of nearly 50 % in both SE processes is in harmony with the carrageenan yields reported elsewhere for hours long alkali extraction routes (Azevedo et al., 2013), whereas it is almost twice the yield reported more recently for a HWE performed at 80–90 °C for a total of 3 h (Sanz et al., 2023).

FTIR and ^1H NMR analyses (Tables 2 and 3) of the liquid extracts obtained during the initial CWE with *C. crispus* and *G. pistillata* demonstrated the presence of non-gelling carrageenan. This is in agreement with Bahari et al. (2021) who reported approximately 5 wt% yield of highly sulphated carrageenan from *C. crispus* after 8 h extraction at 22 °C. In particular, the FTIR bands at 825–830 cm^{-1} for *G. pistillata* is indicative of the substantial presence of mu- or nu-carrageenan (Chopin & Whalen, 1993). The noisy ^1H NMR spectrum displayed in Fig. 9b indeed indicates this is rather nu-carrageenan.

Moreover, the characteristic bump at 5.54 ppm in the ^1H NMR spectra next to the nu-carrageenan peak confirmed the presence of lambda-carrageenans in these extracts (van de Velde et al., 2004), but this is not the major component as claimed elsewhere from FTIR spectra (Bahari et al., 2021). Essentially, CWE products from *G. pistillata* are more sulphated than the sequential and control HWE products. The preferential extraction of more soluble and highly sulphated carrageenans in the CWE may leave behind a carrageenan fraction richer in less sulphated hybrid carrageenans, which can thus be selectively extracted during the following sequences for recovering gelling fractions.

The molecular mass M_w of the carrageenans extracted through CWE was found to be comparable to those obtained from HAE for the same species, suggesting that the cold-water process is capable of recovering carrageenan chains of similar lengths, but needing a lot more time. However, the PDI of the CWE extracts from *C. crispus* was notably high, reflecting a broad distribution of molecular masses, likely influenced by

Table 1

Weight averaged molecular mass (M_w), polydispersity index (PDI), contents of carrageenan disaccharide units (κ , ι , μ , ν) and rheological properties (gel elastic modulus G_0 , gel setting temperature T_g and solution shear viscosity η_{500} measured during sweep up at 500 s^{-1} , of samples formed with 2 % w/v HK in 1 M NaCl) for all recovered HK from *M. stellatus* in step 1 (CWE), step 3 (HWE), step 4 (HAE) of the sequential (SE) and corresponding control extractions (Cont), performed with the indicated times in minutes or overnight (over). Different letters indicate statistically significant differences ($p < 0.05$).

Extraction step	type	time	M_w (10^5 g/mol)	PDI	Carrageenan composition (mol.%)				Rheology		
					κ	ι	μ	ν	G_0 (Pa)	T_g (°C)	η_{500} (mPa.s)
CWE	SE	over									
HWE	Cont	60	10.0 ± 2.5^b	2.00 ± 0.01^b	52	34	3	11			30.3
		30	7.55 ± 0.5^c	2.00 ± 0.01^b	79	17	3	1			2.9
		10									
	SE	60	11.9 ± 0.3^a	9.0 ± 0.3^a	51	38	7	4	376	26.4	
		30	8.7 ± 0.2^c	8.0 ± 0.7^a	44	38	12	6	168	35.9	
		10									
HAE	Cont	60	4.58 ± 0.01^b	4.00 ± 0.06^a	70	30					8.9
		30	3.60 ± 0.28^b	3.00 ± 0.41^a	54	32		14			1.6
		10									
	SE	60	6.64 ± 0.82^a	3.00 ± 0.61^a	66	34					6.6
		30	3.15 ± 0.25^b	4.00 ± 0.25^a	73	27					1.8
		10									

Table 2

Weight averaged molecular mass (M_w), polydispersity index (PDI), contents of carrageenan disaccharide units (κ , ι , μ , ν) and rheological properties (gel elastic modulus G_0 , gel setting temperature T_g and solution shear viscosity η_{500} measured during sweep up at 500 s^{-1} , of samples formed with 2 % w/v HK in 1 M NaCl) for all recovered HK from *C. crispus* in step 1 (CWE), step 3 (HWE), step 4 (HAE) of the sequential (SE) and corresponding control extractions (Cont) performed with the indicated times in minutes or overnight (over). Different letters indicate statistically significant differences ($p < 0.05$).

Extraction			Mw (10^5 g/mol)	PDI	Carrageenan composition (mol.%)				Rheology		
step	type	time			κ	ι	μ	ν	G_0 (Pa)	T_g ($^{\circ}\text{C}$)	η_{500} (mPa.s)
CWE	SE	over	2.66 ± 0.13	4.00 ± 0.04	59	27	5	9			4.9
HWE	Cont	60	7.74 ± 0.293^a	3.00 ± 0.05^a	60	29	6	5	50	>85	
		30	7.94 ± 0.06^a	4.00 ± 1.06^a	60	32	4	4			80
		10	4.66 ± 0.07^c	3.00 ± 0.01^a	56	30	7	7			58
		60	7.52 ± 0.04^a	4.00 ± 0.03^a	60	33	3	4	50	>85	
	SE	30	7.83 ± 0.05^a	3.00 ± 0.08^a	69	29	2		112	32.5	
		10	5.38 ± 0.01^b	3.00 ± 0.03^a	60	36	4				58
	HAE	60	4.07 ± 0.06^a	3.00 ± 0.04^a	59	36	5				
		30	$3.31 \pm 0.11^{a,b}$	3.00 ± 0.06^a	66	26	8				
HAE	Cont	10	$2.97 \pm 0.01^{b,c}$	3.00 ± 0.01^a	68	32					
		60	2.22 ± 0.18^c	4.00 ± 0.31^a	50	50					
		30	$2.43 \pm 0.43^{b,c}$	6.00 ± 2.62^a	55	45					
		10	$3.25 \pm 0.38^{a,b}$	4.00 ± 0.22^a	66	34					

Table 3

Weight averaged molecular mass (M_w), polydispersity index (PDI), contents of carrageenan disaccharide units (κ , ι , μ , ν) and rheological properties (gel elastic modulus G_0 , gel setting temperature T_g and solution shear viscosity η_{500} measured during sweep up at 500 s^{-1} , of samples formed with 2 % w/v HK in 1 M NaCl) for all recovered HK from *G. pistillata* in step 1 (CWE), step 3 (HWE), step 4 (HAE) of the sequential (SE) and corresponding control extractions (cont) performed with the indicated times in minutes or overnight (over). Different letters indicate statistically significant differences ($p < 0.05$).

Extraction			Mw (10^5 g/mol)	PDI	Carrageenan composition (mol.%)				Rheology		
step	type	time			κ	ι	μ	ν	G_0 (Pa)	T_g ($^{\circ}\text{C}$)	η_{500} (mPa.s)
CWE	SE	over	9.48 ± 0.09	2.00 ± 0.04	32	30	10	28			3.5
HWE	Cont	60	10.9 ± 0.03^b	2.00 ± 0.06^a	45	33	5	17			53
		30	11.5 ± 0.19^b	1.00 ± 0.02^c	45	37		18			11
		10	0.75 ± 0.56^c	2.00 ± 0.06^a	46	27		27			2.2
	SE	60	15.0 ± 0.05^a	2.00 ± 0.02^a	37	45		18	80	39	
		30	16.0 ± 0.24^a	2.00 ± 0.03^b	33	43		24	30	32.6	
		10	$15.2 \pm 0.07^{a,b}$	$2.0 \pm 0.06^{a,b}$	40	43		17			77
	HAE	60	$5.54 \pm 0.01^{a,b}$	4.00 ± 0.01^a	44	54		2			4.5
		30	$4.04 \pm 0.24^{a,b}$	4.00 ± 0.44^a	44	40		16			1.7
HAE	SE	10									
		60	6.70 ± 0.42^a	4.00 ± 0.08^a	55	45					11.4
		30	1.71 ± 0.54^b	3.00 ± 0.59^a	53	47					1.27
		10			41	59					

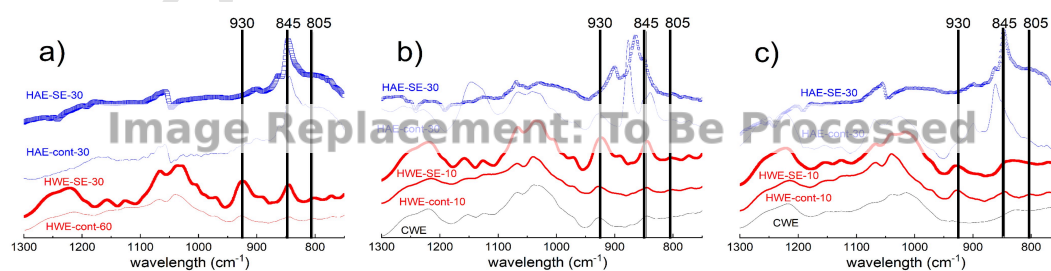


Fig. 8. FTIR spectra of HK extracted from *M. stellatus* (a), *C. crispus* (b) and *G. pistillata* (c) using different routes: cold water (CWE, black curves), hot water (HWE, red curves) or hot alkaline extraction (HAE, blue curves). The extraction process (sequential, SE, or control, cont) and duration in minutes are indicated for each curve. The three vertical lines indicate the bands assigned to specific chemical groups of galactose and 3,6-anhydrogalactose of specific carrageenan disaccharides (Chopin & Whalen, 1993). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the mild extraction conditions and the presence of precursors and lambda-carrageenans.

3.3.2. Hot extractions

Analysing the products from the HWE for *M. stellatus*, FTIR spectra from control experiments show bands in the $830\text{--}815 \text{ cm}^{-1}$ region (see

Fig. 8) assigned to nu- and mu-carrageenans (Chopin & Whalen, 1993), which is consistent with the content in mu- and nu-carrageenans computed from ^1H NMR, see Fig. 9a). Longer extraction times lead to the recovery of less sulphated HK and the SE approach produces larger chains with significantly increased polydispersity. In the subsequent HAE step, the extracts (both SE and controls) did not show precursors, aligning

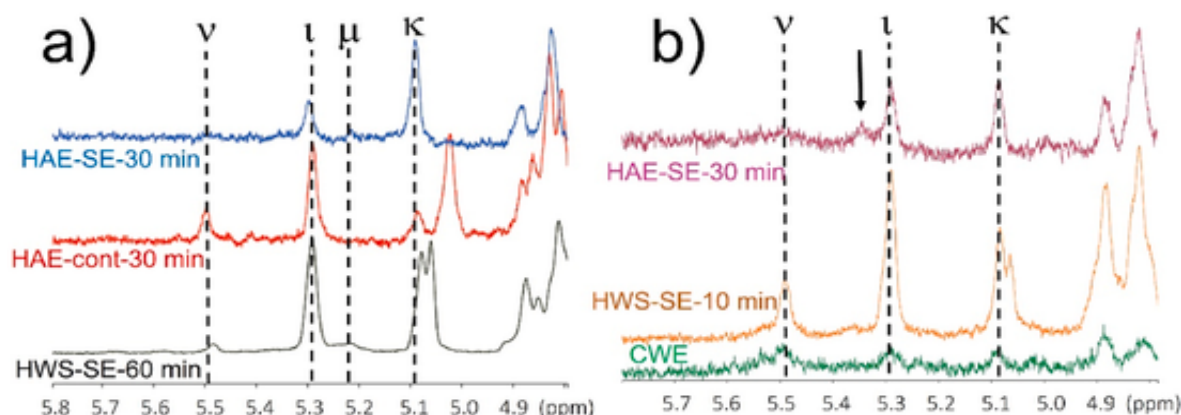


Fig. 9. ¹H NMR spectra of HK extracts from (see Table 1 for codes assigned to each type of extract) a) *M. stellatus*; b) *G. pistillata*. The arrow in b) indicates a signal assigned to starch.

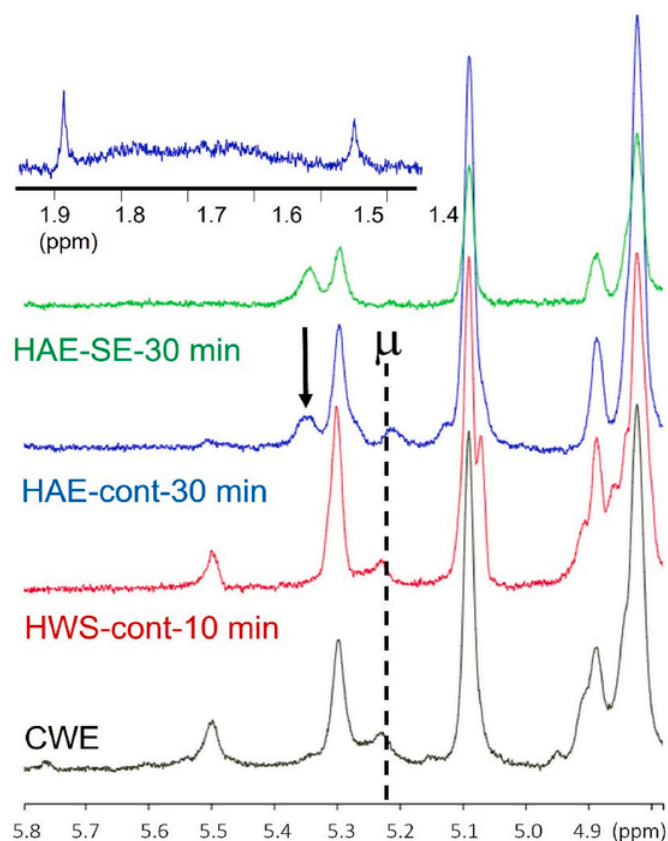


Fig. 10. ¹H NMR spectra of HK extracts from *C. crispus*. The arrow indicates a signal assigned to starch and the inset is a zoom for the chemical shift region pyruvic acid shows up next to 1.4 ppm for the HAE-cont 30 min extract.

with expectations from alkaline extractions. An exception was observed for the 30 min control alkaline extract, where ¹H NMR identified mu- and nu-carrageenans (see Fig. 9a), suggesting that the SE approach is an efficient route to remove these sulphated moieties during the HWE step. The sequential HAE extraction also favours the recovery of HK with larger *M_w*, a feature also seen with the hot water extracts.

For *C. crispus*, FTIR spectra showed minimal differences between HWE SE and control extracts. However, ¹H NMR revealed that HK from SE contained a slightly lower molar content of precursors than controls

in particular at shorter extraction times, which is consistent with the results found for *M. stellatus*. For HAE, differences were observed in FTIR signals at 930 cm⁻¹ and 845 cm⁻¹ between SE and controls extracts. However, since these signals are common across carrageenans, it is challenging to attribute them to specific chemical changes caused by SE. Notably, ¹H NMR showed stronger kappa-carrageenan signals in the HAE controls extracts. The latter also contained mu-carrageenans which were removed in the SE, see Fig. 10. Together with the computed yields, this again suggests that more sulphated HK were extracted during the HWE step. With respect to the molecular mass distribution, the sequential approach in HWE had no impact, except for 10 min where the SE produces larger chains. At longer times for HAE, the sequential extractions give always shorter chains than the controls, whereas the opposite is seen for 10 min. Overall, the benefits brought by the sequential extractions are in harmony with those achieved with *M. stellatus*.

For *G. pistillata*, SE products from HWE performed at shorter times contained fewer mu- and nu-carrageenans than the controls. For the HAE, SE and control extracts were largely similar. Interestingly, no FTIR or ¹H NMR signals were detected for the 10 min long alkaline control sample, indicating the absence of carrageenan in the recovered product. The HK from SE HWE consistently displayed larger *M_w* than the controls, in contrast to HAE products which showed similar molecular mass distribution across all samples.

Overall, SE generally contributes to the recovery of HK with larger *M_w* compared to controls, particularly during the HWE step. This effect was more pronounced in *M. stellatus* and *G. pistillata*. In addition, SE enhances the recovery of less sulphated carrageenans, especially with shorter extraction times, which can be explained by the leaching of more sulphated polysaccharides during the CWE and/or HWE steps. Finally, *M. stellatus* is not suited for extraction times as short as 10 min and assisted extractions should be specifically studied for this seaweed. All these findings point towards the improved gelling properties of HK recovered using the SE route.

3.4. Rheological properties of HK solutions and gels

Under the salt and polysaccharide conditions tested, all the 1 h and 30 min HWE SE samples gelled after 24 h at 25 °C, whereas the 10 min HWE SE samples remained liquid. The mechanical spectra of the 30 min HWE SE samples gelled in the rheometer are displayed in Fig. 11 together with their response to large amplitude oscillatory shear strains. In comparison, the control samples do not form gels, except for the 1 h HWE control from *C. crispus*, which form a gel with a similar elastic modulus *G'* to its respective HWE SE 1 h extract, see Table 2.

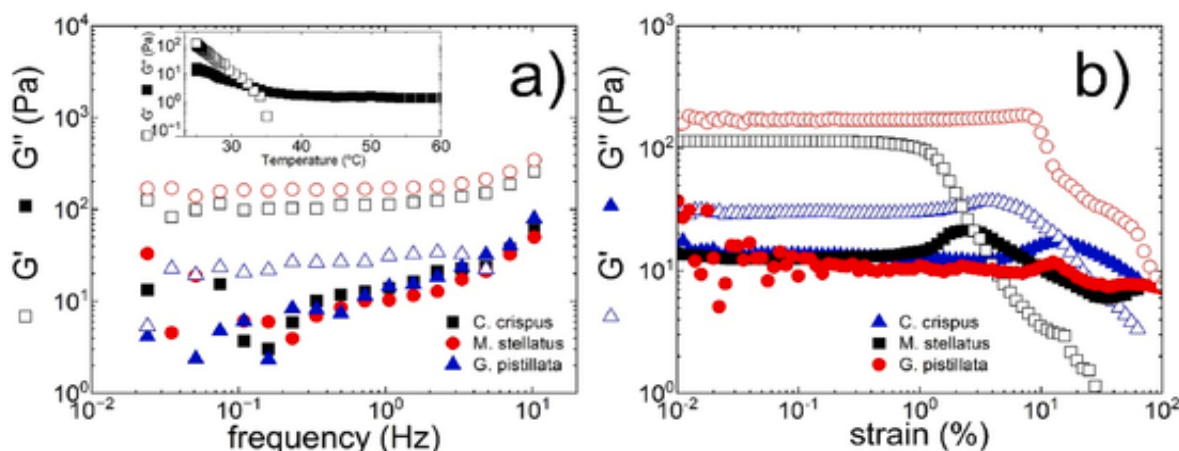


Fig. 11. Mechanical spectra (a) and response to large amplitude oscillatory strain sweep (b) of gels formed with HK isolated with HWE SE performed for 30 min. Gels were formed in the rheometer after cooling down to 25 °C hot solution of 2 % w/v of HK in 1M NaCl (see inset in a for the extract from *C. crispus*).

Overall, the data in Tables 1–3 suggest that the SE process contributes to improve the gel quality of extracts in hot water. HK made of only κ and ι blocks are known to exhibit gel elastic moduli G_0 which are an increasing function of the content in κ , whereas their gelling temperature T_g is reported to be constant around 25 °C (van de Velde, 2008). However, the relationship between carrageenan composition and G_0 is more complex for HK with a more heterogeneous composition in carrageenan disaccharides (Souza et al., 2023). Results recently reported for a set of HK extracted from commercial seaweeds with hot water, showed that, when the carrageenan composition is close to a balanced amount in ι and κ , no gel can be formed with 1 wt% in 1 M NaCl (Moraes & Hilliou, 2024). Note here that lower NaCl concentrations were shown to produce HK solutions for extracts from *M. stellatus* (Azevedo et al., 2014) and *C. chondrus* (Torres, Azevedo & Hilliou 2016) which justified the choice for the salt conditions used here. The non-gelling behaviour of the control samples is thus in agreement with the chemical characteristics presented in Tables 1–3. But this cannot explain the formation of gels in HWE SE samples with carrageenan compositions and molecular masses very similar to their respective controls, except for the extracts from *G. pistillata* which have larger Mw. In contrast, all HAE samples (both SE and control) remained liquid. Note here that the lack of rheological data for the HAE extracts of *C. crispus* is due to the insufficient amount of recovered HK during SE (see Fig. 7), which did not allow for a rheological characterization under the targeted HK concentrations. While the HWE samples with higher sulphate content were expected to exhibit weaker gels or lower viscosities due to the presence of carrageenan precursors acting as defects in the helical conformation (Piculell, 2006; Souza et al., 2023), their larger molecular mass compared to HAE extracts likely contributed to gel formation. This highlights the significance of molecular mass in gel setting, as smaller polysaccharides from the HAE route may struggle to aggregate or form helices. Indeed, all the Mw values reported in Tables 1–3 for gel forming HK are larger than the range of critical masses (from 1.3×10^5 to 5.7×10^5 g/mol) needed to gel HK at 10 g/L in 0.1 M KCl (Gonçalves et al., 2025). However, gelation in NaCl is notoriously different for kappa-carrageenans as their helices are more aggregated in the presence of K^+ (Piculell, 2006) and the specific ionic interactions that govern the HK helical aggregation in the presence of NaCl are not yet clarified (Souza et al., 2023). Considering the above discussion, the gel elasticity G_0 of HK in NaCl depends on a complex interplay between the lengths of blocks of κ and ι , interrupted or not by μ and ν , and the molecular mass. Nevertheless, restricting the discussion to HWE SE gels, the data suggest that larger G_0 values are achieved when the total amount of non-gelling μ and ν is minimal. There is no such clear corre-

lation for the gel setting temperatures T_g , as already reported elsewhere for sets of HK with very heterogeneous carrageenan compositions (Azevedo et al., 2013, 2015a).

Other ingredients can be extracted together with the carrageenans, which will affect the gel properties. For instance, electrostatic interactions and hydrogen bonding between carrageenans and proteins were recently found to enhance the gel properties (Lu et al., 2023). FTIR bands assigned to proteins found in carrageenan extracts usually show up at 1690 cm^{-1} for amide I, 1640 cm^{-1} for amide II and 1420 cm^{-1} for amide III (Chopin & Whalen, 1993). No such bands were found in the carrageenans from the three studied seaweeds. However, NMR signals between 7 and 10 ppm were found in the gelling HWE extracts of *M. stellatus*. Such signals were absent from the NMR spectra of the HAE products. This suggests that, in contrast to the alkali extraction, proteins streamed during the hot water extraction of *M. stellatus* reinforced the carrageenan gel structure. Thus, for the specific case of *M. stellatus*, rather than lower Mw, this is the absence of proteins that might well explain the lack of gelling behaviour of the corresponding carrageenan extracts.

The nonlinear viscoelastic properties of the produced gels show either strain hardening (G' increases with increasing strain before eventually dropping) or strain softening (G' decreases for larger strains), see Fig. 11b. Strain hardening is only observed with the softer gels within the extracts from the same seaweeds, which is in harmony with the hardening behaviour found in networks of semi flexible polymers (Broedersz & MacKintosh, 2014; Hilliou, 2021), with the filamentous structure reported for carrageenan gels (Hilliou, 2021; Piculell, 2006) and the reversible strain hardening found more recently with a set of carrageenans under the same salt conditions (Moraes & Hilliou, 2024). Although strain hardening in carrageenan gels should be the topic of future studies (Hilliou, 2021; Souza et al., 2023; van de Velde, 2008), the transition from the entropic to the enthalpic regime of filament elasticity, with the increase of strain which eventually stretches the filaments with finite extensibility, might be called here to explain the strain hardening (Broedersz & MacKintosh, 2014; Hilliou, 2021). The nonlinear viscoelastic behaviour of all the liquids made with the HK varies from Newtonian behaviour to strong shear thinning within the tested range of shear rates. This is illustrated for selected samples in Fig. 12, which also shows some hysteresis in the low shear rate range of the flow curves of specific samples. Indeed, the viscosity measured by sweeping up the shear rates is larger than the viscosity measured by sweeping down the shear rates immediately after the sweeping up (compare empty and solid squares in Fig. 12). As such, and for comparative purpose, the shear viscosity measured at 500 s^{-1} , η_{500} , is reported in Tables

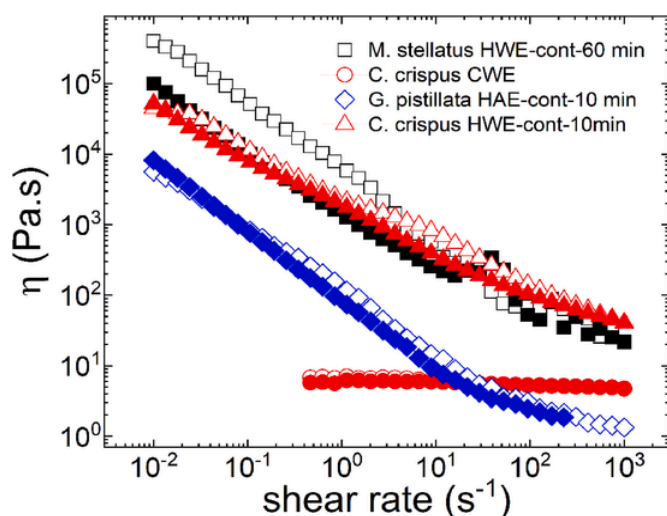


Fig. 12. Flow curves (ramping up the shear rate: empty symbols; ramping down the shear rate: empty symbols) of solutions prepared from carrageenans of *M. stellatus* (squares), of *C. crispus* (circles and triangles), and of *G. pistillata* (diamonds).

1–3, as these values do not depend on the sweeping mode of the shear rates. Starting with the CWE samples from *C. crispus* and *G. pistillata*, both produced essentially Newtonian HK solutions (see Fig. 11 for *C. crispus*) with a viscosity only a few times larger than the solvent. This stems from their high contents in both ν and μ for *G. pistillata* and from the rather low Mw of *C. crispus*. In contrast to this, the HWE products show strong shear thinning and significant flow hysteresis, in particular for extracts from *G. pistillata* and *M. stellatus* (see an example for the latter in Fig. 12).

This flow hysteresis indicates that, under low shear rates and within the experimental time frame, the viscosity did not recover its initial value exhibited during the first ramping up. This suggests the occurrence of flow-induced structural changes at larger shear rates likely resulting from the breakdown or rearrangement of carrageenan aggregates. Such behaviour thus shows up for HK having a greater propensity for self-aggregation into large structures, large enough to be affected by shear. Flow hysteresis is thus more evident in HWE samples from *M. stellatus* and *G. pistillata* compared with the corresponding HAE samples which show much smaller Mw. Fig. 12 also illustrates the effect of the HK chemical structure on the solution viscosity for extracts with nearly similar Mw. The HWE control extract with 10 min of *C. crispus* has larger amounts of κ and ι than the HAE control extract with 30 min of *G. pistillata*. Thus, the latter HK will form fewer helical conformers, resulting in less viscous solutions. Overall, when comparing the values for η_{500} in Tables 1–3, the SE leads to the recovery of HK exhibiting larger solution viscosity in 1 M NaCl. There are two exceptions within the set of six flow curves, with the 30 min HAE extracts from *G. pistillata* being the most striking. The HK produced with SE gives a Newtonian solution, whereas the control extract yields a far more viscous solution with strong shear thinning. Finally, longer extraction times across all samples were associated with larger viscosities, all attributable to the recovery of larger molecular mass carrageenans.

4. Conclusions

Sequential extraction tested on three seaweeds allowed to separate phycobiliproteins, chlorophyll-a, carotenoids, and carrageenans. When comparing the efficiency and quality of SE products with the controls, SE consistently facilitates the recovery of high molecular mass carrageenans during hot water extraction, particularly from *M. stellatus*

and *G. pistillata*. Overall, SE extracts demonstrate superior rheological properties under the studied conditions, including enhanced gel elasticity and comparable or higher viscosities in 1 M NaCl. This is attributed to the previous steps, such as cold-water extraction and ethanolic extraction, which removed more sulphated HK, yielding less sulphated chains in subsequent steps. Extraction times above 10 min typically result in the recovery of HK with larger molecular mass. Thus, the gel elasticity is enhanced by a factor two when the extraction time is doubled. Interestingly, *C. crispus* displayed an opposite behaviour, as doubling the hot water extraction time reduces the carrageenan gels elasticities by half. This highlights the necessity for seaweed-specific optimization of the sequential extraction parameters.

Overall, this study underscores the feasibility of tailoring sequential extraction protocols to deliver specific natural products from carrageenophytes. It pinpoints the critical influence of extraction order, duration, and solvent selection on HK gelling quality. The demonstration of the E2B2 as a new biorefinery concept is the true focus of the current preliminary work. But the main result of the SE approach presented here points towards the feasibility of an extractive extrusion-based biorefinery of *C. crispus* and *G. pistillata*, whereas *M. stellatus* is not to be considered in future E2B2 demonstrations.

Uncited references

Torres et al., 2016.

CRediT authorship contribution statement

Gabriela Gonçalves: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Izabel Cristina Freitas Moraes:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Bruno Faria:** Writing – review & editing, Validation. **Loïc Hilliou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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