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Microwave-assisted extraction of the Carrageenan from *Hypnea musciformis* (Cystocloniaceae, Rhodophyta)

E. Vázquez-Delfín · D. Robledo · Y. Freile-Pelegrín

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Abstract *Hypnea musciformis* (Wulfen in Jacqu.) J. V. Lamour. (Rhodophyta) was investigated for its carrageenan production. Traditionally, the desulfation process for carrageenans has been promoted by an alkaline treatment of up to 3 h by conventional heating during carrageenan extraction. New extraction techniques based on microwave irradiation may accelerate this reaction with the advantages of reduced consumption of solvents, energy, and extraction time, suggesting the feasibility of this method as a "Green" technology. In this study, aqueous- and alkali-treated carrageenans from *H. musciformis* collected along Quintana Roo coast of Yucatan Peninsula (Mexico) were extracted by conventional method and by microwave-assisted extraction (MAE). Microwave irradiation in closed vessels was used to carry out the alkaline modification. The influence of temperature (85, 95, and 105 °C) and extraction time (10 and 20 min) in MAE was investigated in terms of yield, sulfate, and 3,6-anhydrogalactose contents, and Fourier transformed infrared spectra. Although lower carrageenan yields were obtained during MAE extraction, the κappa/iota hybrid carrageenan obtained by this novel method is comparable to that extracted by conventional technique. At the maximum temperature used for MAE (105 °C), an increase of 3,6-anhydrogalactose as well as an increase of the κappa-proportion was observed indicating that MAE could be an adequate procedure for carrageenan extraction of *H. musciformis*; however, further extraction parameters should be tested to optimize extraction.

Keywords *Hypnea musciformis* · Rhodophyta · Alkaline treatment · Carrageenan · Microwave-assisted extraction

E. Vázquez-Delfín · D. Robledo · Y. Freile-Pelegrín (✉)
Marine Resources Department, CINVESTAV, Merida. km 6 Carretera Antigua a Progreso, Mérida, Yucatán, Mexico
e-mail: freile@mda.cinvestav.mx

Introduction

Carrageenans are economically important polysaccharides because of their use in the industry as thickening and stabilizing agents. They consist of linear chains of alternating 3-linked β-D-galactose (G) and 4-linked α-D-galactose (D) units, sometimes the last occurring as 4-linked 3,6-anhydrogalactose (DA) unit. Carrageenans are usually sorted into different types based on the presence or not of DA unit and the sulfation pattern: kappa (κ-; G4S-DA), iota (ι-; G4S-DA2S), and lambda (λ-; G2S-D2S,6S), represented by codes proposed by Knutsen et al. (1995), which represent ideal chemical structures with different industrial applicability associated with gelling or viscous enhancement properties. Minor types are mu (μ-) and nu (υ-), which are κ- and ι-precursors, respectively, that contain sulfate groups linked to C6 (D6S) that may be converted to the corresponding DA units by an alkaline treatment (Noseda and Cerezo 1995). This reaction is highly specific as no other sulfate group is affected and is widely used at industrial level to improve the gelling properties of carrageenans (Van de Velde and De Ruiter 2005; Campo et al. 2009).

The order Gigartinales (Rhodophyta) has the greatest number of commercially exploited carrageenophytes (Bixler 1996) and most of their members consistently display a predominance of either κ- and/or ι-carrageenan. Nowadays, farmed *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva and *Eucheuma denticulatum* (N.L.Burman) F.S.Collins & Hervey are almost exclusively the sources of κ- and ι-carrageenans, respectively. Other sources yielding κ-/ι-hybrids are obtained from Chilean *Gigartina* and *Sarcothalia* species after an alkali treatment.

The market of carrageenan continues to expand and is maintaining reasonably well despite the economic recession. Since 1999 however, the average carrageenan prices have increased in the last 10 years from US$7.25 to 14 kg^{-1}, as shown by Bixler and Porse (2011) in a recent and complete overview of the seaweed hydrocolloid industry. The authors argued that higher prices of carrageenans are linked to higher

energy cost during the extraction process, large volume of chemicals, and high raw material prices related to seaweed availability. In the current industrial practice of producing κ- and/or ι-carrageenans, large quantities of sodium or potassium hydroxide at elevated temperatures are used for several hours (Navarro and Stortz 2005). Carrageenans, as viscous materials, transfer energy poorly and large thermal gradients can result in sub-optimal conversions and loss of product. In this context, new extraction techniques have been tested in the last decade to accelerate the reaction based on microwave irradiation. Microwave-assisted extraction (MAE) uses microwave energy to heat solvents in contact with a sample (Tatke and Jaiswal 2011). Algal matrix is highly susceptible to microwave irradiation owing to its high natural moisture content. Rapid internal heating brings about effective cell rupture, releasing the analytes into the solvent (Mandal et al. 2007; Jain et al. 2009). The main advantages of the proposed procedure are the reduced consumption of solvents compared with those reached by traditional methods, using less energy and reduction of extraction time. Despite the benefits of this novel technique, its application for carrageenan extraction is new and scarce. MAE has been used for *E. denticulatum* and *K. alvarezii*, obtaining ι- and κ-carrageenans, respectively (Uy et al. 2005). Carrageenan from *Iridaea undulosa* Bory de Saint-Vincent, was also extracted using this technique (Navarro and Stortz 2005; Navarro et al. 2007). The red seaweed *Hypnea musciformis* (Wulfen in Jacqu.) J. V. Lamour mainly contains κ-carrageenan in the cell walls (Greer et al. 1984; Bi et al. 2006; Arman and Qader 2012) and has been suggested as a potential economic species because of its high yield, fast growth rate (20 % day^{-1}) and its tolerance to a great extent of environmental conditions (Guist et al. 1982; Wallner et al. 1992; Berchez et al. 1993; Mshigeni and Chapman 1994; Bravin and Yoneshigue-Valentin 2002; Reis et al. 2003; Ganesan et al. 2006; Reis et al. 2006). Although different methodologies have been employed for carrageenan extraction from *Hypnea* (Table 1), to our knowledge MAE has never been applied before. The aim of the present study was to compare carrageenan from *H. musciformis* obtained by conventional extraction methods (aqueous and alkali) against MAE in terms of yield, DA and sulfate contents, and Fourier transformed infrared (FTIR) spectra; at the same time, we evaluate the effect of temperature and extraction time during carrageenan extraction by the MAE technique.

Materials and methods

Hypnea musciformis grows abundantly as a perennial species on the intertidal rocky shores at Quintana Roo coast of the Yucatan Peninsula, Mexico. *H. musciformis* was collected during November 2012 as part of a seasonal biomass assessment study in the Quintana Roo coast (20°39′36.50″ N, 87°02′ 06.01″ W). Fresh seaweed samples were transported to the laboratory, cleaned with filtered seawater to eliminate epiphytes and then rapidly washed with tap water to eliminate salt residues, and dried at 60 °C in a conventional oven, powdered and stored under vacuum for further extraction.

Conventional extraction

Conventional extraction was performed using the method described by Freile-Pelegrín et al. (2006). Dry algae (5 g) were hydrated for 12 h in 500 mL distilled water for aqueous

Table 1 Carrageenan properties (%) of *H. musciformis* extracted with different methods

Extraction conditions	Yield	DA	Sulfate	Locality	Reference
Aqueous (C)	34	22	14	Israel	Friedlander and Zelikovitch (1984)
Aqueous, HCl pretreated (C)	0.4–25	–	–	Italy	Knutsen et al. (1995)
Alkali (0.1 M NaOH; C)	20–36	–	–	Tanzania	Mtolera and Buriyo (2004)
Aqueous (HCl pretreated; C)	34–44	20.9–27.6	23.6–35.4	Pakistan	Bi et al. (2006)
Alkali (C)	40	25.9	14.8	Pakistan	Bi et al. (2006)
Aqueous (C)	13.5–41	39	15–23	Morocco	Aziza et al. (2008)
Aqueous (C)	21–48	–	–	Brazil	Reis et al. (2008)
Aqueous (C)	30.2	27.6	17.4	Pakistan	Arman and Qader (2012)
Alkali (0.1 N NaOH; C)	39.8	21.8	44.1	Pakistan	Arman and Qader (2012)
Acid (0.1 N HCl; C)	4.3	19.9	41.3	Pakistan	Arman and Qader (2012)
Aqueous (C)	37.8	29.7	19.8	Mexico	This study
Aqueous (MAE)	21.5	24.7	16.7	Mexico	This study
Alkali (3 % KOH; C)	18.7	24.7	15.8	Mexico	This study
Alkali (3 % KOH; MAE)	16.6	31.6	19.4	Mexico	This study

C conventional method, *MAE* microwave-assisted extraction method at 105 °C (10 min)

treatment and KOH solution (3 %) for alkaline treatment. The weight ratio of sample to reagent (KOH) was performed at 1:3. The mixture was extracted at 85 °C for 3.5 h, mixed with diatomaceous earth (Celite), pressure filtered and the filtrate neutralized to pH 8.9 with 5 M HCl prior to the recovery of the carrageenan from the solution. For alkali treatment, the extract was washed with distilled water before the filtration in order to eliminate KOH residues. The filtrate was neutralized to pH 8.9 with 5 M HCl and precipitated with 250 mL of Cetavlon (hexadecyl-tri-methylammonium bromide) in 9:1 (*v*/*v*) distilled water/acetone (Craigie and Leigh 1978) and recovered over filter paper in vacuum. The fibrous carrageenan was carefully washed three times with 63 mL 95 % ethanol nearly saturated with sodium acetate to remove Cetavlon residues. Sodium acetate was removed in three final washings with 95 % ethanol. Carrageenan was dried at 60 °C for 24 h and powdered for further analysis. For each treatment, three replicate samples were processed.

Microwave-assisted extraction

Extractions were performed using a Microwave Accelerated Reaction System (MARS 800W, CEM, Matthews, NC, USA) at 100 % of full power and at a frequency of 2450 MHz. Preliminary extractions were performed to establish time and temperature parameters to be used during MAE. These results indicated that under extreme conditions (3 % KOH, 105 °C, 25 min) most of the carrageenan was degraded, therefore, MAE conditions were defined based on three temperatures (85, 95, and 105 °C) and two extraction times (10 and 20 min). For each treatment, samples of 1 g (n=3) were hydrated for 12 h in 50 mL distilled water (aqueous treatment) and in 3 % KOH (alkaline treatment). The weight ratio of sample to reagent (KOH) was 1:1.5. The samples were placed in a closed-vessel system (OMNI/XP-1500) designed to remain completely sealed during operation to prevent solvent and analyte loss. By using closed vessels, the extraction can be performed at elevated temperatures accelerating the mass transfer of target compounds from the sample matrix. The internal temperature and pressure conditions were monitored within one reference OMNI/XP-1500 vessel equipped with temperature and pressure probes for regulating extraction conditions. The vessels are designed to vent excess pressure. The maximal pressure during the extraction was approximately 159 kPa. After opening the vessels and recovering the extract the protocol was the same as described in the conventional extraction section above.

Carrageenan analysis

Conventional and microwave-extracted carrageenans (aqueous and alkali treated) were analyzed for DA and sulfate content following Matsuhiro and Zanlungo (1983) and Jackson and McCandless (1978) methods respectively. Sample analysis was performed for each treatment in triplicate. Standards of ι- and κ-carrageenans (SIGMA commercial grade) were included for comparisons.

Fourier transformed infrared spectra

For FTIR spectra, carrageenan films were prepared by evaporation of carrageenan in an aqueous solution (0.2 %). The spectra were recorded in FTIR-NIR Spectrometer (Perkin Elmer, Frontier). Standards of ι- and κ-carrageenans (SIGMA commercial grade) were included for comparisons.

Statistical analysis

For conventional and microwave-extracted carrageenan, differences in yield, DA content and sulfate content between

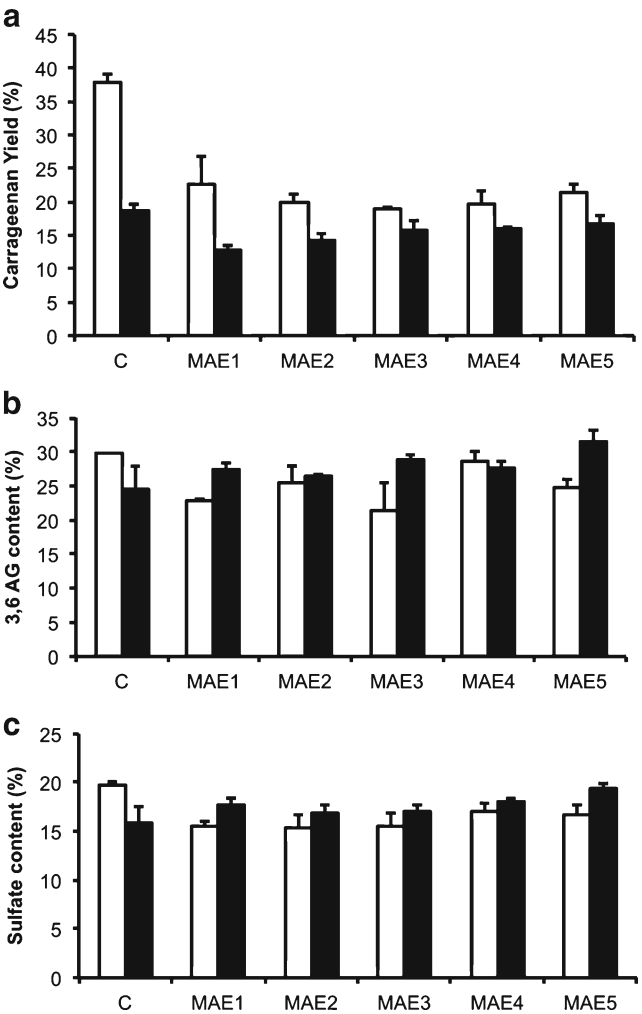

Fig. 1 Yield (**a**), DA content (**b**), and sulfate content (**c**) of *Hypnea musciformis* carrageenan extracted with conventional technique (**c**) and under different conditions of MAE (*MAE1* 85 °C (10 min), *MAE2* 85 °C (20 min), *MAE3* 95 °C (10 min), *MAE4* 95 °C (20 min), *MAE5* 105 °C (10 min)). *White bars* correspond to aqueous extracted carrageenan and *black bars* to those alkali treated

aqueous and alkali treatment were tested with a one-way analysis of variance (ANOVA). To compare between treatments a post hoc analysis using the Tukey's (honestly significant difference (HSD)) test was done when equal variance was assumed. When equal variance was not assumed Games–Howell test was performed. To test the effect of temperature and extraction time in MAE a two-way ANOVA was performed with a post hoc analysis using Tukey's (HSD) test to compare extraction conditions.

Results

H. musciformis from Quintana Roo (Mexico) exhibited yields and chemical properties that are in the range of those reported in the literature (Table 1). The yields of carrageenan extracted by the conventional method and by MAE at different conditions of time and temperature are shown in Fig. 1a. For aqueous treatment, highest yield was obtained by conventional extraction method (37.8 %) when compared with those obtained by MAE, which ranged between 18.9 and 22.7 % with no significant differences among all MAE conditions. In general, for both extraction methods and for all MAE conditions a decrease in the yield after alkali treatment was observed. The yields obtained after alkali treatment by both methods were comparable, slightly lower for MAE (18.7 % for conventional and 16.6 % for MAE at 105 °C at 10 min).

The DA content in the carrageenans extracted by the conventional method and MAE at different conditions of time and temperature are shown in Fig. 1b. For aqueous treatment the

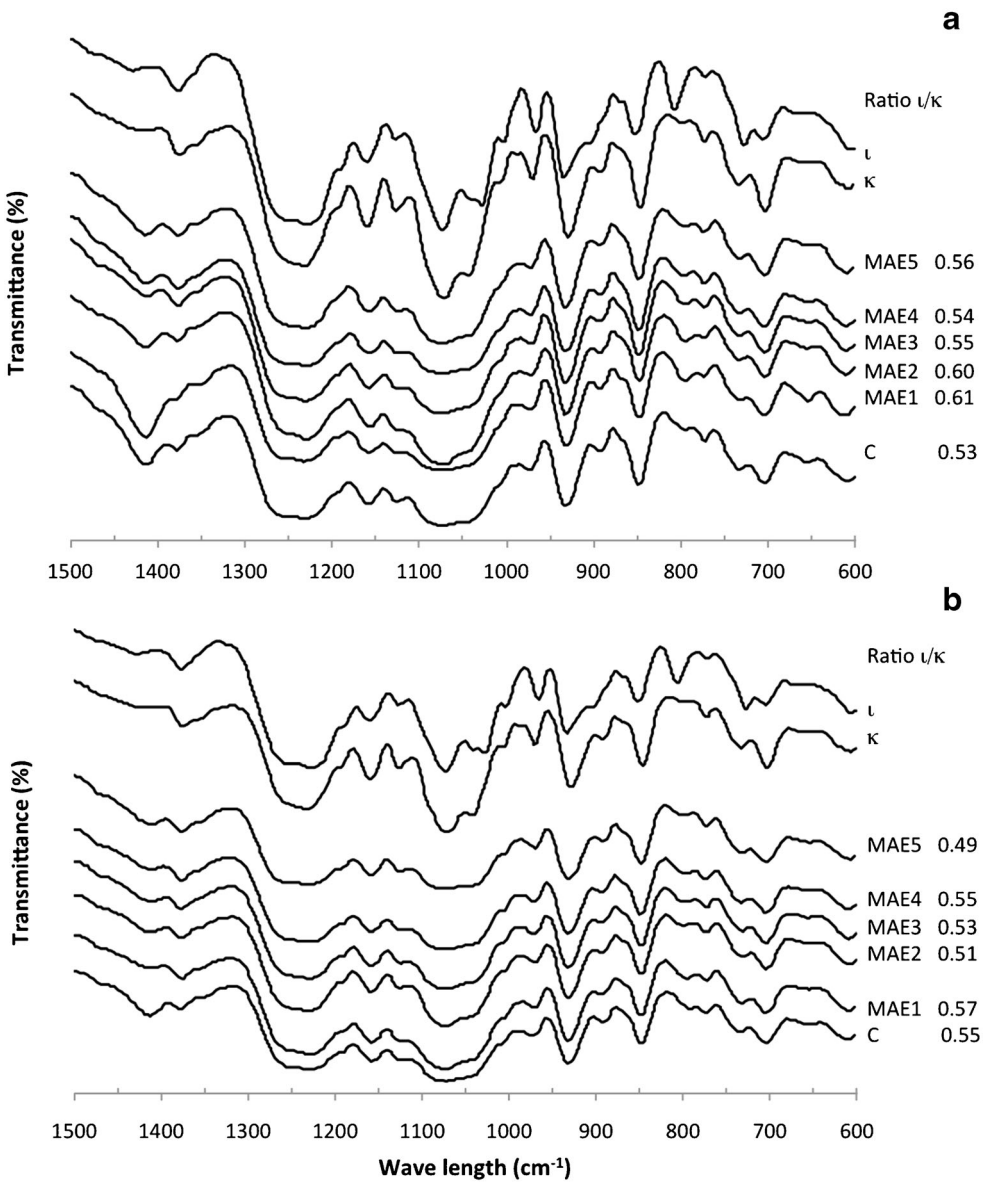

Fig. 2 FTIR spectra of aqueous- (**a**) and alkali-treated (**b**) carrageenans from *H. musciformis* extracted with conventional technique (**c**) and under different conditions of MAE (*MAE1* 85 °C (10 min), *MAE2* 85 °C (20 min), *MAE3* 95 °C (10 min), *MAE4* 95 °C (20 min), *MAE5* 105 °C (10 min)). Spectra of κ- and ι-commercial carrageenan are included

highest DA content was recorded for the conventional method (29.7 %) with no significant difference to that obtained for MAE at 95 °C for 20 min (28.7 %). After alkali treatment, the highest DA content was recorded for MAE at 105 °C at 10 min, increasing significantly ($p<0.05$) from 24.7 % (aqueous) to 31.6 % (alkali treated). In the MAE method, a significant effect of time was observed under aqueous treatment ($F=13.5$, $p<0.05$), showing an increase in DA content as time increased. By contrast, temperature had a significant effect in DA content under alkaline treatment ($F=15.8$, $p<0.05$).

The sulfate content in the carrageenans extracted by the conventional method and MAE at different conditions of time and temperature are shown in Fig. 1c. Sulfate content was significantly higher for aqueous conventional extraction (19.8 %) when compared with alkali conventional extraction (15.8 %). For aqueous treatment under MAE the sulfate content ranged from 15.4 to 17 %, whereas for alkali treatment sulfate contents ranged from 16.9 to 19.4 %. Although a slight increase of sulfate content was noted after alkali treatment under MAE conditions, no significant differences were found ($p>0.05$).

FTIR spectra

The FTIR spectra for aqueous- and alkali-treated carrageenans from *H. musciformis* extracted with conventional and MAE at different conditions of time and temperature are presented in Fig. 2. Aqueous and alkali treated carrageenan spectra were similar to κ- and ι-standards, independently of the extraction conditions used. All spectra showed absorption bands between 1,220 and 1,260 cm^{-1} corresponding to sulfate esters (Chopin et al. 1999). The presence of a strong band a 930 cm^{-1} in the FTIR spectra indicates the presence of DA in all samples (Chopin et al. 1999; Pereira et al. 2003). No increase of this band between the aqueous and alkali treated carrageenans was observed, implying either absence or undetectable levels of the precursor 1,4-linked galactose-6-sulfate (D6S). Moreover, bands at 825 and 867 cm^{-1}, corresponding to the existence of μ- and υ-precursors, were not evident.

All spectra also showed a band at 845 cm^{-1}, which is assigned to D-galactose-4-sulphate (G4S), and a band at 805 cm^{-1} attributed to the presence of 3,6-anhydro-D-galactose-2-sulphate (DA2S), which is specific to ι-arrageenan. These fingerprint signals corroborate the presence of a κ-/ι-hybrids. The ratio between 805 and 845 cm^{-1} absorption bands in FTIR spectra (indicated in Fig. 2) was calculated and used as a qualitative parameter to determine the degree of ι-/κ-hybridization, where an increase in the ratio indicates a decrease in κ-proportion (Rochas et al. 1986). In general, the ratio ranged between 0.49 and 0.61 indicating a high proportion of ι-carrageenan in all samples. Except for conventional method, the κ-proportion increased slightly after alkali treatment for all MAE conditions, reaching its maximum value at 105 °C for 10 min.

Discussion

Conventional extraction of *H. musciformis* from Quintana Roo, Mexico, exhibited carrageenan yields within the range of those reported previously for this species (Table 1). The highest yields were obtained by the conventional technique compared with those obtained with MAE under all conditions tested. Several factors are known to affect carrageenan yield, including extraction conditions. The hot alkaline extraction operations inevitably involve some degradation of the polysaccharide because of the rigors (heat and alkalinity) of processing (Stanley 1987). For this reason, an understandable decrease in carrageenan yield was observed for both conventional and MAE methods. Conversely, the lower content of carrageenan obtained in both aqueous and alkali extraction by MAE compared with conventional method could be explained by extreme conditions (such as elevated temperatures) by microwave heating which could favor the degradation, and therefore, loss of the polysaccharide. It must be taken into account that microwave irradiation is different from the conventional heating since thermal energy is delivered via conduction. Microwave energy is directly delivered to the materials and heat can be generated throughout their water content, resulting in rapid and uniform heating. The efficiency in which different solvents heat up under microwave depends on the dissipation factor, which is indeed the measure of the ability of the solvent to absorb microwave energy and pass it on as heat to the surrounding molecules. A list of dissipation factors for solvents commonly used in MAE was reported by Mandal et al. (2007): acetone, 20.7; acetonitrile, 37.5; ethanol, 24.3; hexane, 1.89; methanol, 32.6; 2-propanol, 19.9; and water, 78.3. Given the high microwave absorbing properties of water, high-moisture samples such as seaweed can reach higher temperatures than with conventional heating, causing a hydrolysis and therefore a decrease of the carrageenan yield. Some papers have shown that the heat by microwave radiation affects degradation of polymeric material (Zhou et al. 2006 and references therein). For this reason, further studies should be done considering the use of lower temperatures using MAE

Our results suggest that κ-/ι-hybrid carrageenans are extracted from *H. musciformis* from Quintana Roo whichever the extraction method (conventional or MAE). The hybrid nature of *H. musciformis* carrageenan has been reported before (Greer et al. 1984; Bi et al. 2006; Arman and Qader 2012), although with a smaller proportions of iota type (17 %). Nevertheless, seasonal variations have been found in carrageenans from *Ahnfeltiopsis devoniensis* (Greville) P.C. Silva and DeCew and *Gigartina pistillata* (S.G. Gmelin)

Stackhouse from Portugal, indicating that during autumn/winter this ratio was higher than that found during spring/summer, thus indicating a higher percentage of ι-type (Pereira and Mesquita 2003). In the present study, *H. musciformis* was collected during winter. Although no seasonal carrageenan variation has been studied on this specie from Quintana Roo, the above could be the reasons for different κ-/ι-proportions related to prevailing environmental conditions at different seasons.

In relation to sulfate content, no desulfation was observed after alkali treatment for MAE method at all conditions, probably because of the alkali-stable sulfate hemiester group and the lack of κ- and ι-precursors. The slight increase in sulfate content after alkali treatment could be attributed to depolymerization occurred in the polysaccharide at the MAE conditions performed, probably related to time, as observed Navarro et al. (2007). These authors tested microwave-assisted desulfation of carrageenans by heating their pyridinium salts dissolved in dimethyl sulfoxide, showing that long reaction times lead to a substantial degradation of the polysaccharides. Despite this, in our study it is worth noting that sulfate contents in both native and alkali treated carrageenan were below (<20 %) the range reported for κ-/ι-producing species (25–34 %, Imeson 2000; Campo et al. 2009), independently of extraction method (conventional or MAE). Conversely, low irradiances occurred in November as a result of cloudy days and high turbidity together with low seawater temperatures (data not shown) which could also explain the unusual pattern of sulfates and DA contents obtained.

In the FTIR spectra, there was no evidence of the μ- and υ-precursors however, the significant increase of DA after alkali treatment, and the increase of κ-proportion at 105 °C for 10 min during MAE, may suggest the existence of undetectable levels of the precursor μ-carrageenan. Moreover, DA value reached under this condition was comparable to that reported for κ-carrageenan producers (Imeson 2000; Campo et al. 2009). To corroborate the above, other complementary techniques such as FT-Raman and NMR spectroscopy must be used.

In conventional method, the nonconversion by alkali treatment could be related to KOH concentration used rather than with factors tested (temperature and time). On this regard, during conventional extraction in other close related species, *Hypnea bryoides* Børgesen, it has been reported that 6 % NaOH is more efficient than 3 % KOH treatment in achieving high levels of conversion to kappa carrageenan with an identical quality to that of commercial sigma carrageenan (Al-Alawi et al. 2011). It would then be necessary to evaluate the effect of hydroxide concentration and the type of ion (Na^+ or K^+) used in conventional and microwave extraction of *H. musciformis*. Moreover, other studies have remarked the importance of temperature, pressure and hydroxide concentration interaction during MAE of red seaweed galactans (Navarro and Stortz 2005; Sousa et al. 2010). The MAE method was found to be a useful tool for carrageenan extraction for this species when small amounts of algae samples are available, reducing time extraction and solvents used. Based on our results, microwave heating accelerated the rate of the reaction 22 times with respect to the usual conditions during conventional heating, using half of the reagent in the MAE method. On this regard, Navarro and Stortz (2005) reported that the rate of the reaction could be speeded up to 60 times.

In conclusion, despite the lower yields obtained with MAE the κ-/ι-hybrid carrageenans from *H. musciformis* are comparable to those extracted by conventional technique, as confirmed by DA, sulfate content and FTIR spectra. From these results, we can presume that there are not κ-/ι-precursors; however, at the maximum temperature used in MAE (105 °C) an increase in DA as well as an increase of the κ-proportion was observed indicating that MAE could be an adequate procedure for carrageenan extraction of this species. Further extraction parameters should be tested to optimize extraction with MAE taking into account environmental factors associated with the carrageenan content and quality of this species.

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