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The impact of seaweed life phase and postharvest storage duration on the chemical and rheological properties of hybrid carrageenans isolated from Portuguese *Mastocarpus stellatus*

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ABSTRACT

Variations in chemical and gelling characteristics of hybrid carrageenan extracted from *Mastocarpus stellatus* seaweeds are studied in order to explore potential links between the seaweeds life phase, the seaweeds postharvest storage duration and the phycocolloids properties. Chemical structures of phycocolloids were assessed by Fourier Transform Infrared spectroscopy (FTIR) and ^{1}H NMR spectroscopy. Rheological properties of hybrid carrageenans, such as intrinsic viscosity ($[\eta]$), gel elasticity (G_0), gel setting temperature (T_g) and gel melting temperature (T_m), were measured with a stress rheometer. Seasonal variation in the degree of sulphates of native extracts and in their corresponding gelling properties is found. The minimum in gelling properties coincides with a minimum of fructified gametophytes in populations harvested during the cold season. Alkali treated extracts also show minimum gelling properties during the cold season but no correlation with variations in the chemical characteristics could be identified. The gel setting temperature is the only significant change in the properties of hybrid carrageenans extracted from dried seaweeds stored over 39 months in opaque and sealed plastic bags. These results point to non trivial relationships between the life stages of *M. stellatus* seaweeds, the chemical structure and gel properties of the alkali-extracted phycocolloids, and suggest a route towards the sustainable exploitation of the natural resource.

1. Introduction

Carrageenans are natural linear polysaccharides extracted from red seaweeds (*Gigartinales*, *Rhodophyta*) and are extensively used as thickeners, gelling, texturing, suspending or stabilising agents in various industrial applications ranging from food products to pharmaceutics (Piculell, 1995; Bixler, 1996; Lahaye, 2001). Carrageenans have been sorted in three industrially relevant classes of phycocolloids depending on their gel-forming or viscosity enhancement ability: these are namely lambda-carrageenans which lead to viscous solutions, iota-carrageenans which form weak gels but with high gel-setting temperatures and kappa-carrageenans which form strong gels that set at lower temperatures but with a tendency towards water syneresis.

A fourth class of carrageenans, namely hybrid carrageenans, has recently attracted an increased interest due to its peculiar gelling behaviour which is described as "weak gelling kappa" (Pereira & van de Velde, 2011; van de Velde, Peppelman, Rollema, & Tromp, 2001). Indeed, the gel properties depend on the complex chemical structure of this type of phycocolloids. Complexity arises first from the heterogeneity in the chemical structure of the monomers building up the polysaccharide. Hybrid carrageenans are now recognized (Guibet et al., 2008; van de Velde, 2008; van de Velde et al., 2001) as copolymers possessing a statistical distribution of sequences (blocks) of ideal sulphated disaccharide monomers of the carrageenan type, with length of sequences varying from a single disaccharide monomer to well above the critical amount of monomers needed to promote helix formation. Complexity also arises from the copolymer composition diversity: the statistical arrangement of block sequences may differ from chain to chain.

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For instance, many seaweeds of the *Gigartinaceae* (*Rhodophyta*) family produce mixtures of gelling and non gelling carrageenans which can be separated by fractionation in KCl salt (McCandless, West, & Guiry, 1983). Because carrageenans are one of the components building up the outer mucilageous layer of cell walls of certain red seaweeds, their complex biosynthesis naturally strongly depends on the environmental factors that affect the life cycle of the seaweeds. In Particular, the biochemical alternation of generations in *Gigartinaceae* is well established (Chopin, Kerin, & Mazerolle, 1999; Falshaw & Furneaux, 1998): gametophytes produce hybrid polysaccharides belonging to the kappa-carrageenan family, whereas tetrasporophytes essentially contain lambda-carrageenans (lambda, theta and xi-carrageenan) (Pereira, Amado, Critchley, van de Velde, & Ribeiro-claro, 2009; Pereira, Critchley, Amado, & Ribeiro-Claro, 2009; Pereira & Mesquita, 2003). As such, seasonal variation in the type of phycocolloid produced by carrageenophytes is one of the parameters taken into account for the industrial harvesting of this natural resource. Interestingly, the tetrasporophytic life stage of *Mastocarpus stellatus* (Stackhouse) Guiry is crustose (Guiry & West, 1983), which is strongly attached to intertidal rocks and impedes any industrial harvesting. Thus, *M. stellatus* can be harvested in the gametophytic life stage at all times of the year, as a source of phycocolloids belonging to the kappa/iota-hybrid carrageenan family (Bixler, 1996), the kappa/iota/mu/nu-hybrid carrageenan family (Chopin et al., 1999) and the kappa carrageenan family (Pereira & Mesquita, 2003). The gel properties of hybrid carrageenan extracted from *M. stellatus* in 0.05 M KCl range from those of a pure ι-carrageenan gel to those of a pure κ-carrageenan gel (Hilliou & Gonçalves, 2007). As a result, this biopolymer could be an interesting alternative for applications where blends of pure iota- and kappa-carrageenans are currently used (Villanueva, Mendoza, Rodrigueza, Romero, & Montaño, 2004) or in dairy applications (Bixler, Johndro, & Falshaw, 2001). An attempt to relate the gel mechanical and thermal properties to the biopolymer chemical structure and molecular mass distribution has also been presented (Hilliou, Larotonda, Sereno, & Gonçalves, 2006; Souza, Hilliou, Bastos, & Gonçalves, 2011). In the present study, we explore the potential correlations between the phycocolloid chemical structure, its gelling properties and the seaweed life phase as observed over a one year period. A recent study reported the absence of geographical variation in the chemical structure of the kappa/iota-hybrid carrageenan isolated from *M. stellatus* collected at different locations on the northern Portuguese coast (Pereira & van de Velde, 2011), but the gelling behaviour of extracted phycocolloids was not investigated. Reports on the seasonal variation of the biochemical properties of hybrid carrageenan extracted from *M. stellatus* are limited (Mathieson & Tveter, 1976) and essentially focused on phycocolloid yield and viscous properties. However, seasonal variation in phycocolloid yield were reported elsewhere for other hybrid carrageenophytes including *M. stellatus* (Pereira, Amado, et al., 2009; Pereira, Critchley, et al., 2009). To the best of our knowledge, a comprehensive study of biological–chemical–physical interrelations is not yet documented for *M. stellatus*, despite the fact that this seaweed has long been recognized for its industrial relevance in Portugal (Machado et al., 1966).

Industrial harvesting of carrageenophytes has a direct impact on the corresponding seaweeds populations. One full seaweed life cycle between harvesting periods is the minimum requirement to ensure the sustainability of the wild natural resource. Summer harvesting of *M. stellatus* was found to be the best in order to fully recover the biomass within a year, whereas 19 months were needed when seaweeds are harvested in December (Burns & Mathieson, 1972). It is thus of practical importance to evaluate the stability of hybrid carrageenan functional properties in postharvested *M. stellatus*. There are a limited number of comprehensive studies on the postharvest degradation of hybrid carrageenan in carrageenophytes. A literature survey suggests that a single report (Young & Goring, 1958) focusing on the specific case of *Chondrus crispus* points to a slight decrease in the sulphate degree and to a significant degradation in the gel strength after two years storage of dried seaweeds in sealed polyethylene bags at room temperature. The authors concluded that *C. crispus* seaweeds should not be stored more than a year. We propose here to study the effect of postharvest storage duration on the chemical structure and the gelling properties of hybrid carrageenans isolated from dried *M. stellatus*.

2. Experimental

2.1. Seaweed sampling and postharvest storage

M. stellatus seaweeds were collected between February 2004 and November 2004, on intertidal rocks and in tide pools located in Vila Praia de Ancora (41°48.93 N, 8°51.94 W). This region has a Mediterranean-like climate with two main seasons, namely a warm (temperatures ranging from 20 °C to 30 °C), dry and sunny summer from May to October, and a mild (temperatures ranging from 5 °C to 15 °C) and rainy winter from November to April. Also, the Koppen classification is temperate climate with rainy winter (Cs). Right after sampling, seaweeds were washed several times with tap water in order to remove epiphytes, salt, sand and other non algal materials. Clean seaweeds were then laid over a perforated tray and dried in an oven with forced air convection at 60 °C for 48 h, in order to reach a relative humidity lower than 5%. Dried seaweeds were then stored at room temperature in sealed and opaque plastic bags.

2.2. Life stage identification and quantitative sorting

The heteromorphic life history of *M. stellatus* belonging to the Southern Europe breeding group (Guiry & West, 1983) consists of a frondose gametophyte alternating with a crustose sporophyte (thin crust attached to a rock, previously known as *Petrocelis cruenta*), which cannot be easily exploited as crust removal from the rock substrate is a tough task. A 100 g (wet weight) algal material was sorted into two reproductive stages, namely gametophytic fronds and non fructified fronds (absence of cystocarps). Results from this quantitative biological sort out are summarized in Fig. 3, top left chart.

2.3. Carrageenan extraction

Dried seaweeds were ground to a powder and extraction (40 g dried alga in 4 L tap water) was conducted at 95 °C during 2 h at pH 7 (controlled by addition of either 0.01 M HCl or Na_2CO_3 dry powder) within a week after algal sampling. The neutral pH limits the alkaline conversion of carrageenan biological precursor monomers into less sulphated carrageenan monomers, without any significant loss in the extraction yield and biopolymer molecular mass. As a result, this extraction process is expected to have little effect on the chemical characteristics of isolated hybrid carrageenans. The hot suspensions were passed through metallic screens and further clarified with cotton clothes. The filtrates were concentrated by rotary-evaporation and precipitated in two volumes of ethanol (95%). The precipitates were further dehydrated with ethanol, dried at 60 °C under vacuum and ground. The resulting powders were then dissolved in hot distilled water by stirring for 1 h and a subsequent centrifugation at 14,000 × *g* and 38 °C during 40 min leads to the recovery of a supernatant which after drying at 60 °C under vacuum gave a purified native carrageenan sample. The latter was finally weighed to obtain the carrageenan yield

with respect to the initial dried algal weight. Extractions were performed in triplicates. In addition, phycocolloids were alkali-extracted in triplicates following an optimized procedure described elsewhere (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Hilliou, Larotonda, Sereno, et al., 2006) in order to enhance the gelling properties of the isolated phycocolloids in a way similar to the industrial extraction. Briefly, 200 g wet seaweeds were soaked at room temperature for 48 h in 8 L of 0.1 M Na_2CO_3, and then washed with tap water to lower the pH of the bath to 7 prior to extraction. Alkali-extracted hybrid carrageenans were essentially used for the postharvest study performed with seaweeds collected in November 2004 (see below), and limitation in algal material from this batch did impede the study of past-harvest effects longer than 39 months.

2.4. Chemical structure of carrageenans

Chemical structures of phycocolloids extracted for the study of seasonal variation were assessed by Fourier Transform Infrared spectroscopy (FTIR) and ^{1}H NMR spectroscopy, following the experimental protocols detailed elsewhere (Hilliou, Larotonda, Abreu, et al., 2006). Extracts used for the postharvest study were analyzed using a Bomem MB-series FTIR spectrometer (ABB Bomem, Inc., Quebec). Each spectrum is the average of 8 scans acquired at 2 cm^{-1} resolution on films cast from aqueous solutions. In the FTIR analysis, the absorbance of the following bands were recorded: 1240 cm^{-1} attributed to total sulphate, 930 cm^{-1} assigned to 3,6-anhydrogalactose, 845 cm^{-1} assigned to sulphate ester at 4-position, and 805 cm^{-1} corresponding to sulphate ester at 2-position of 3,6-anhydrogalactose residues (Chopin et al., 1999). The ratio of band intensities at 1240 and 930 cm^{-1} was then calculated as to give a semi quantitative estimate of the sulphate degree (Villanueva, Sousa, Gonçalves, Nilsson, & Hilliou, 2010). ^{1}H NMR spectra were recorded at 80 °C on a Bruker ARX 400 NMR spectrometer (400 MHz), using carrageenan solutions in D_2O and TMSPSA as internal standard.

2.5. Viscoelastic properties of hybrid carrageenan solutions

Phycocolloids were dissolved at various concentrations (0.01–1 wt%) in hot (80 °C) 0.1 M NaCl solutions during 1 h under strong stirring. The solutions were then directly loaded in the pre-heated (80 °C) cone and plate geometry (diameter 6 cm, angle 0.2 rad) of a stress rheometer (ARG2, TA Instruments Inc., New Castle, DE, USA) and the shearing geometry was covered with paraffin oil to prevent water loss. Solutions were then cooled (−5 °C min^{-1}) down to 25 °C while the dynamic loss modulus G'' measured at 1 Hz with a 10% shear strain amplitude allowed to monitor the monotonic increase of the solution viscosity and the achievement of sample equilibrium at 25 °C. Under such salt and temperature conditions, hybrid carrageenans are known to show no aggregation (van de Velde et al., 2001) as biopolymers remain in the coil conformation. The latter was confirmed by the absence of any step increase of the shear viscosity during cooling, which would indicate a coil-to-helix conformational transition (Souza et al., 2011). The flow curves at 25 °C were then obtained from steady stress sweep tests (shear rate measured over the last 10 s of a step shear stress with 60 s duration, and steady state defined within a 2% tolerance for shear rate variation) performed between 0.1 Pa and 100 Pa. These tests allowed the determination of the zero shear viscosity η_0 (Newtonian viscosity). For hybrid carrageenan solutions with viscosity values below the sensitivity of the stress rheometer (for concentration below roughly 0.1 wt%), a Cannon–Fenske capillary viscometer (COMECTA S.A., Barcelona, Spain) was used and intrinsic viscosities [η] were computed using the Huggins' equation.

2.6. Gel properties of kappa/iota-hybrid carrageenans

Small amplitude oscillatory shear experiments were performed with the stress rheometer in order to determine the gel properties of carrageenans extracted from *M. stellatus*. For the carrageenan extracts used in the study of the postharvest duration, a 1.5 wt% carrageenan solution in 0.05 M KCl was loaded in the pre-heated (90 °C) cone and plate geometry of the rheometer, whereas a concentration of 1 wt% and a serrated plate geometry (40 mm diameter) were used for the study of seasonal variation, due to the low amount of algal biomass (especially in February) and thus recovered polysaccharide. For both studies, the following experimental procedure was used. A temperature sweep from 90 °C to 20 °C performed at a rate of −2.5 °C min^{-1} with a 1 Hz excitation and 0.5% strain amplitude was first carried out to determine the gel setting temperature T_g. The latter is determined as the temperature where the tangent of the phase shift angle $\tan\delta = G''/G'$ equals 1 (see Fig. 4A). This test was followed by a time sweep performed at 20 °C, with a 1 Hz excitation and a strain with amplitude 0.5% in order to capture the gel structural build up and assess the gel elastic properties at equilibrium (see Fig. 4B). The latter were obtained from a subsequent frequency sweep performed at 20 °C with a 0.5% strain. The gel elasticity G_0 is defined as the value of the storage modulus G' recorded at 1 Hz (see Fig. 4C). Finally, a temperature sweep from 20 °C to 90 °C was performed at a rate of 2.5 °C min^{-1} with a 1 Hz excitation and 0.5% strain amplitude to obtain the gel melting temperature T_m again defined as the point where $\tan\delta = 1$ (see Fig. 4D).

2.7. Statistical analysis

A one-way analysis of variance (ANOVA) was performed using Microcal Origin software (Microcal Software, Inc., Northampton, MA) version 6.0, in order to test for significant (the significance level was 0.05) differences between monthly collected data and between data obtained as a function of storage duration. STATISTICA software (StatSoft Inc., Tulsa, OK) version 6.0 was used to compute Pearson correlations between carrageenan yields, viscoelastic properties, chemical parameters, and seaweeds biological variables.

3. Results and discussion

3.1. Seasonal variation in the chemical structure and gel properties of carrageenans

Fig. 1 presents the FTIR spectra obtained for phycocolloids extracted from *M. stellatus* collected from February to November 2004. Spectra in Fig. 1 are qualitatively similar to spectra presented elsewhere for alkali-extracted or native hybrid carrageenan from *Gigartinacean* gametophytes collected in the Portuguese western coast (Pereira & Mesquita, 2003; Hilliou, Larotonda, Abreu, et al., 2006; Hilliou, Larotonda, Sereno, et al., 2006) and to spectra obtained from ground *Gigartinacean* gametophytes (Pereira, Amado, et al., 2009) and ground *M. stellatus* seaweeds (Gómez-Ordóñez & Rupérez, 2011) collected in the same geographical area. Strong bands at 930 and 845 cm^{-1} are identified for all extracts, together with a weak band at 805 cm^{-1} which is a diagnostic band for iota-carrageenan. A qualitative difference is observed in the spectrum of the native extract from seaweeds collected in May as an additional band is resolved at 872 cm^{-1} (see vertical arrow in Fig. 1). This band is reminiscent from the 867 cm^{-1} band usually assigned to highly sulphated carrageenan biological precursors (Chopin et al., 1999), which are expected to occur when the seaweed is growing. As such, the disappearance of the 872 cm^{-1} band

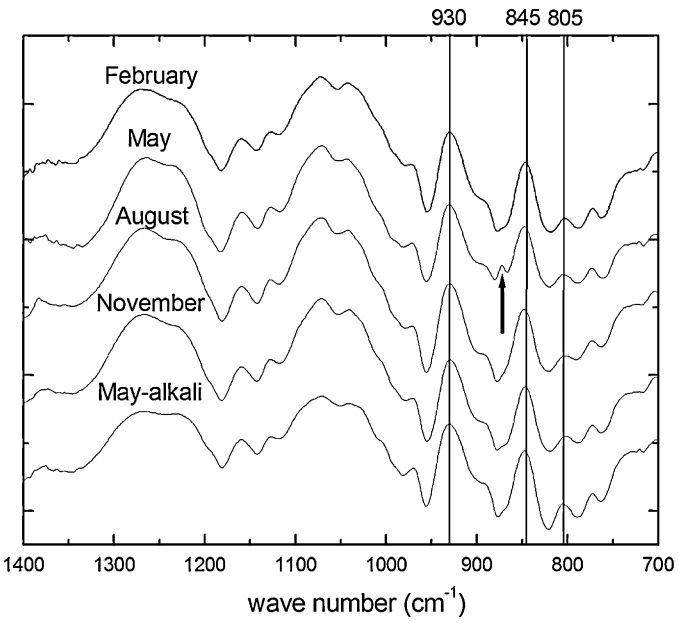

Fig. 1. FTIR spectra of the native phycocolloids extracted from seaweeds collected at different months during 2004. The FTIR spectrum at bottom corresponds to the alkali-modified phycocolloid extracted from seaweeds harvested in May. The arrow points to the specific absorption band at 867 cm^{-1} resolved in the spectrum of the native phycocolloid obtained from seaweeds collected in May.

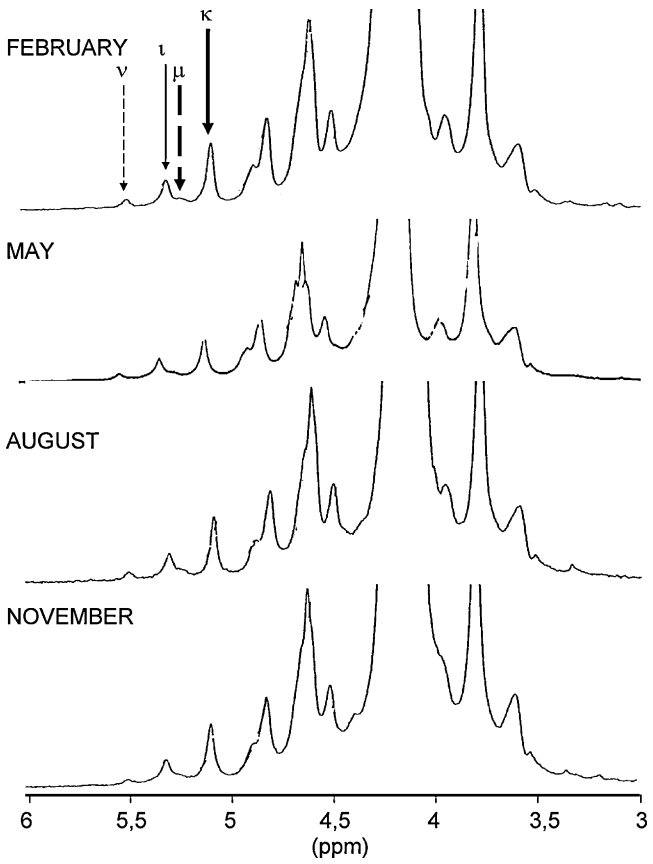

Fig. 2. ^{1}H NMR spectra of native phycocolloids extracted from seaweeds collected at different months during 2004. Thin solid and dashed arrows indicate peaks assigned respectively to iota- and nu-carrageenan, whereas the thick solid and dashed arrows indicate peaks assigned to kappa- and mu-carrageenan, respectively.

in the spectrum of the alkali-extracted biopolymer (see bottom FTIR spectrum) comes as no surprise and confirms the proposed assignment.

Seasonal variation in the chemical structure of native hybrid carrageenan extracts was also studied with ^{1}H NMR spectroscopy. Fig. 2 presents the spectra obtained for the native extracts. Spectra are essentially all similar with peaks assigned to nu-carrageenan (5.52 ppm), which is the biological precursor of iota-carrageenan (5.32 ppm), and kappa-carrageenan (5.11 ppm) (van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). In addition, a line broadening shows up at nearly 5.26 ppm which can be attributed to mu-carrageenan (the biological precursor of kappa-carrageenan), which is in agreement with earlier report (Hilliou, Larotonda, Abreu, et al., 2006). In contrast to FTIR data, Fig. 2 does not indicate any qualitative change in the chemical structure of carrageenans extracted from seaweeds collected in May. The lack of spectral resolution in the 5.32–5.26 ppm and the 5.6–5.4 regions which relate to other biological precursors (Pereira & van de Velde, 2011) could explain the lack of differences among the spectra. However, the main qualitative outcome of chemical analyses is that the native polysaccharide isolated from *M. stellatus* is essentially a kappa/iota/mu/nu-hybrid carrageenan, in agreement with a recent report (Gómez-Ordóñez & Rupérez, 2011), with noticeable amounts of additional biological precursors in May, as inferred from FTIR.

Results from the semi quantitative chemical analysis performed with the 1240/930 bands ratio in FTIR (sulphate degree) and with the relative intensities of peaks at 5.52 and 5.11 ppm in ^{1}H NMR are presented in Fig. 3 for all isolated hybrid carrageenans (native and alkali-extracted) together with results from the seaweed population study, the carrageenan yield and corresponding gel elastic properties. Table 1 summarizes the statistical analysis of all data for the native extracts. The sulphate degree is the only chemical property that shows a significant seasonal variation. Hybrid carrageenans with minimum sulphate degree are obtained from seaweeds collected in February (cold season) which coincides with a minimum population in fructified gametophytes. This result is expected as seaweeds show minimal growth during the cold season. Interestingly, the relative content in kappa-carrageenan does not show any significant seasonal variation. The relative content in nu-carrageenan shows large variation in the values but the ANOVA treatment indicates no statistical significance between seasons. This quantitative result suggests that the highly sulphated nu-carrageenan does not contribute to the qualitative variation detected with FTIR. We conjecture that other sulphated biological precursors which could not be resolved with ^{1}H NMR are responsible for the seasonal variation in the sulphate degree. The gel thermorheological properties are displayed in Fig. 4 for all native extracts. The corresponding statistical analysis reported in Table 1 shows a significant seasonal variation in G_0 which evidently correlates with the seasonal variation in both gelling and melting temperatures. Minimum gel properties are found in February.

The ^{1}H NMR spectra of alkali-extracted carrageenans show peaks at 5.52 and 5.26 ppm (results not shown) thus confirming that the present alkali treatment with Na_2CO_3 is not efficient in fully converting the precursor sulphated moieties in the galactan backbone into 3,6-anhydrogalactose (see for instance Fig. 2 in Hilliou, Larotonda, Sereno, et al., 2006). No qualitative difference could be seen in the FTIR and ^{1}H NMR spectra of alkali-extracted hybrid carrageenans. The statistical analysis of data displayed in Fig. 3 is reported in Table 2 and indicates that the relative contents in kappa- and nu-carrageenan show a significant seasonal variation, with minimum values found in August and November, respectively. The latter is in harmony with the minimum sulphate degree found in February for the native extracts as it corresponds to a life cycle with minimum seaweed growth and corresponding low amounts of biological carrageenan precursors. The seasonal

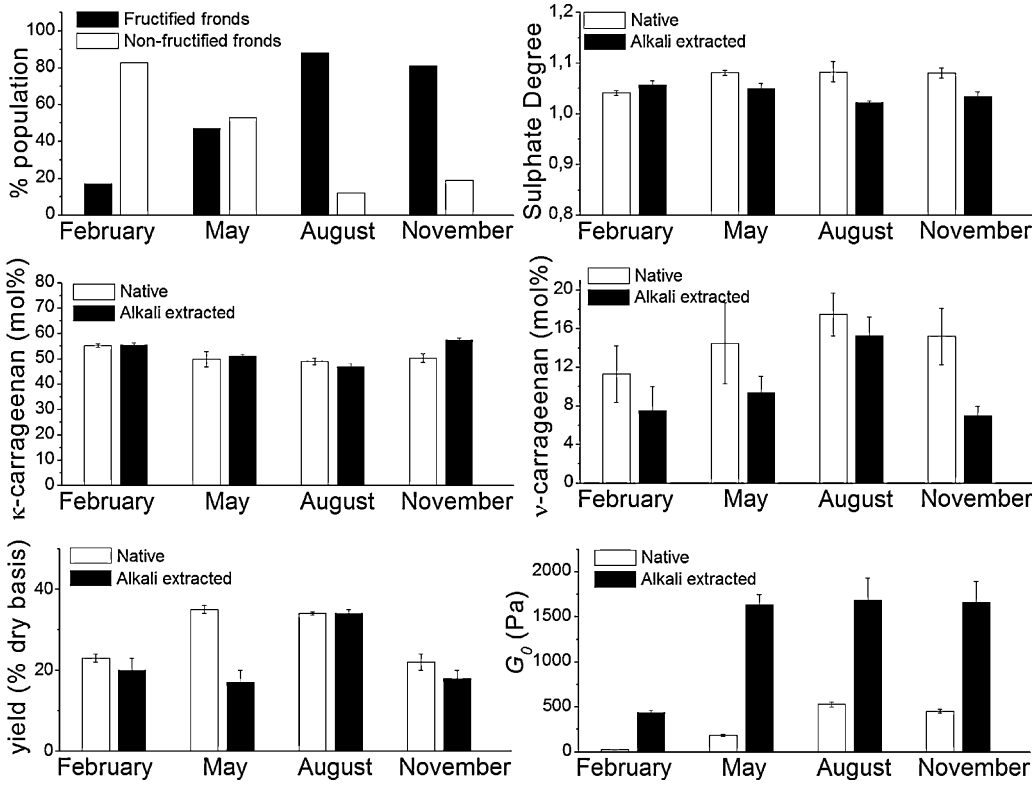

Fig. 3. Monthly variation in *M. stellatus* population, sulphate degree (determined by FTIR), and relative content in kappa- and nu-carrageenans (determined by ^{1}H NMR), hybrid carrageenan yield and gel elasticity G_0 for native (empty bars) and alkali treated (solid bars) extracts.

Table 1
One-way ANOVA on the effect of season on the chemical and thermorheological characteristics of native hybrid carrageenans, and on population structure of seaweeds.

Property	Range	*F*-ratio	Probability	Minimum
Yield (%, dry basis)	20–36	94.10	<0.001	November
Sulphate degree	1.036–1.103	9.04	0.006	February
Kappa carrageenan (mol%)	47–56	2.28	0.156[a]	May
Nu carrageenan (mol%)	8–20	0.67	0.594[a]	February
G_0 (Pa)[c]	24–552	541.6	<0.001	February
T_g (°C)[c]	20.3–36.5	1622.5	<0.001	February
T_m (°C)[c]	34.9–50	1207.9	<0.001	February
Fructified gametophytes (%)[b]	17–88	–	–	February
Non-fructified gametophytes (%)[b]	12–83	–	–	August

[a] Non significant variation at 0.05.
[b] Percentage of gametophytic and non-fructified fronds in 100 g (wet weight) sample.
[c] G_0: gel elasticity; T_g: gel-setting temperature; T_m: gel melting temperature.

variation in the carrageenan yield is of practical significance and data in Table 2 suggests that seaweeds should not be harvested during the fast growing period (May), as they contain a minimum amount of alkali-extractable hybrid carrageenan. A significant seasonal variation in the gel properties is also evident in Table 2 and in Fig. 3. Seaweeds collected in February are not industrially attractive as corresponding hybrid carrageenans give gels with the weakest elasticity, thus confirming the data obtained with native extracts (see empty squares in Fig. 4). From the data presented in Fig. 3, we can conclude that seaweeds collected in August are the best candidates for industrial application as the alkali-extracted hybrid carrageenan combines best gelling properties and highest yield.

Table 2
One-way ANOVA on the effect of season on the chemical and thermorheological characteristics of alkali-treated hybrid carrageenans.

Property	Range	*F*-ratio	Probability	Minimum
Yield (%, dry basis)	14–35	32.83	<0.001	May
Sulphate degree	1.019–1.059	4.27	0.062[a]	August
Kappa carrageenan (mol%)	45–57	15.56	0.006	August
Nu carrageenan (mol%)	7–19	5.30	0.032	November
G_0 (Pa)[b]	420–1932	11.82	0.003	February
T_g (°C)[b]	30–48.4	0.798	0.52[a]	February
T_m (°C)[b]	42–51.5	86.89	<0.001	February

[a] Non significant variation at probability <0.05.
[b] G_0: gel elasticity; T_g: gel-setting temperature; T_m: gel melting temperature.

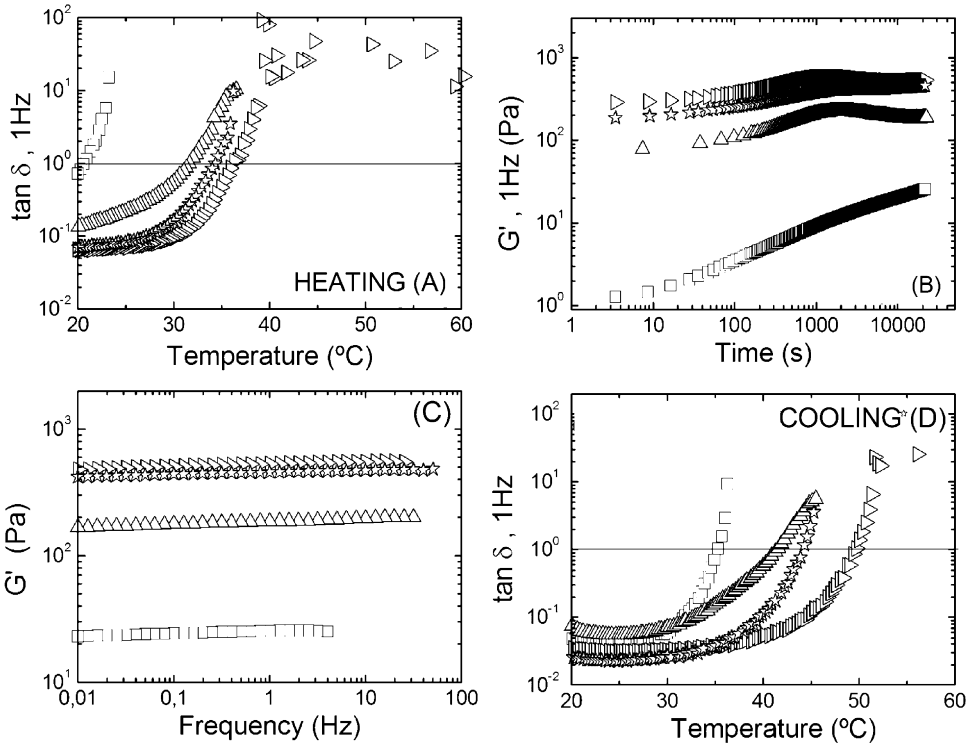

Fig. 4. Rheological properties of representative 1.5 wt% hybrid carrageenans solutions in 0.05 M KCl. Phycocolloids were extracted from seaweeds collected in February 2004 (□), May 2004 (△), August 2004 (▷) and November 2004 (☆). (A) Temperature dependence of the tangent of the phase shift angle δ recorded upon cooling. (B) Time dependence of the storage modulus G' at 20 °C. (C) Frequency dependence of the storage modulus G' at 20 °C. (D) Temperature dependence of the tangent of the phase shift angle δ recorded upon heating.

3.2. Relationships among seaweeds life stages, hybrid carrageenan chemistry and gel properties

Linear correlation coefficients between all parameters in Tables 1 and 2 that show significant seasonal variation are summarized in Table 3, in an attempt to find relationships between seaweeds biological properties (life stage), hybrid carrageenan chemical structure and gelling properties. No correlation is found between the carrageenan yield and all other properties, thus hampering any rationale for the maximum yield of biopolymer achieved during the hot season. Actually, maximum biomass in *M. stellatus* has been recorded during the hot season (Burns & Mathieson, 1972), whereas minimum carrageenan content was reported during the periods of maximum protein levels in the seaweeds (Mathieson & Tveter, 1976) corresponding to the fastest growth rates. As carrageenans are polysaccharides contained in the cell walls, higher carrageenan content is usually found when seaweeds present a minimal growth rate (hot season), in contrast to the protein content (Fogg, 1964; Freile-Pelegrín & Robledo, 2006).

No correlation is found between chemical properties and gelling properties of alkali-extracted hybrid carrageenans. This result is in harmony with the conclusion reached in an earlier study (Hilliou, Larotonda, Sereno, et al., 2006), and with the absence of correlation between gel strength and the relative content in sulphate found in seaweeds from the *Euchema* genus and the *Kappaphycus* genus (Wakibia, Bolton, Keats, & Raitt, 2006). The absence of simple relationship between chemical characteristics determined with ^{1}H NMR and for instance G_0 motivated a dedicated investigation where parameters such as the molecular mass or the relative content in iota-carrageenan were separately controlled in extracts from *M. stellatus* in order to selectively assess their effect on the gel properties in NaCl salt (Souza et al., 2011). The absence of rheological–chemical correlation for the alkali-extracted hybrid carrageenans in Table 3 is contrasting with the direct relationship between gel strength and relative fraction of kappa-carrageenan found for *C. crispus* Stackhouse (Fuller & Mathieson, 1972), a seaweed from a different family but which is claimed to be closely related to *M. stellatus*. However, it has been questioned whether the seasonal variation of the relative abundance of gametophytes and tetrasporophytes could have been interfering in such relationship (Jackson & McCandless, 1979). To that respect we note here that *M. stellatus* is a "model system" as such interference cannot occur: gametophytes (with fructified and non-fructified fronds) are solely harvested, since the tetrasporophytes are crustose.

Significant positive correlations are found between the sulphate degree and the gelling properties (gel elasticity, gelling and melting temperatures) of native hybrid extracts. This positive correlation is at odd with the negative correlation found for alkali-extracted phycocolloids in a separate study (Hilliou, Larotonda, Sereno, et al., 2006). Such discrepancies are hard to rationalize, as many parameters such as the polysaccharide molecular mass distribution or the relative content in iota-carrageenan, and the chemical structure (distribution of gelling blocks in the carrageenan copolymer) are known to affect G_0 (Piculell, 1995; Souza et al., 2011). In addition, the distribution of sulphated monomers within the polymer chain, which act as defects in the formation of helical conformers, has a strong influence on the gel structural build-up and elastic properties (van de Velde et al., 2001). Therefore, a similar sulphate degree could lead to dramatic differences in the resulting gel properties.

Weaker gels (lower elastic modulus G_0) with lower gel setting and gel melting temperatures are obtained when non-fructified gametophytes are accounting for most of collected seaweeds, as inferred from the negative coefficients reported in the last row of Table 3. This result is stiff as it does not depend on the extraction method used to isolate the hybrid carrageenan. The evidence for relationships between fructified gametophytes and optimum

Table 3
Correlation coefficients between yield, thermorheological properties (G_0, T_g, T_m) and chemical composition (sulphate degree: SD; relative contents in kappa-carrageenan, Kappa, and nu-carrageenan, Nu) of all extracted phycocolloids and occurrence of non fructified fronds (Non fruct.) of *M. stellatus*.

	Native						Alkali treated				
	SD[c]	Yield	G_0[b]	T_g[b]	T_m[b]		Kappa	Nu	Yield	G_0	T_m
Yield	0.52[a]	1				Nu	−0.78				
G_0[b]	0.67	0.20[a]	1			Yield	−0.01[a]	0.36[a]	1		
T_g[b]	0.86	0.46[a]	0.91	1		G_0[b]	−0.25[a]	0.30[a]	0.14[a]	1	
T_m[b]	0.76	0.49[a]	0.94	0.94	1	T_m[b]	−0.59[a]	0.65	0.40[a]	0.89	1
Non fruct.	−0.75	−0.24[a]	−0.99	−0.95	−0.95	Non fruct.	0.29[a]	−0.30[a]	−0.50[a]	−0.77	−0.89

[a] Non significant correlation ($P > 0.05$).
[b] G_0: gel elasticity; T_g: gel-setting temperature; T_m: gel melting temperature.
[c] SD: sulphate degree.

carrageenan gel elasticity in *Gigartinaceaes* is not widely reported in the literature. Indeed, a single report on *C. crispus* could be found where the seaweed reproduction showed no effect on the properties of the extracted carrageenans (Fuller & Mathieson, 1972).

3.3. Effect of postharvest storage duration

Fig. 5 shows the chemical pattern of two extracts obtained at month 0 and month 39. No additional bands or shoulders are observed after 39 months postharvest storage. This suggests that the hybrid carrageenan isolated from stored seaweeds essentially kept its chemical integrity. A semi quantitative analysis of the sulphate degree was performed by computing the bands ratio 1240/930 for all spectra recorded with phycocolloids extracted throughout 39 months. The statistical analysis of variance for the computed sulphate degrees is presented in Table 4. The sulphate degrees are larger than those reported in Tables 1 and 2. This difference is related to the use of different FTIR spectrophotometers, and underlines the semi quantitative information than can be extracted with such chemical analysis. Nevertheless, data in Table 4 indicate that the sulphate degree does not show any significant variation, thus confirming that the chemistry of hybrid carrageenans is unchanged within 39 months. A similar conclusion was drawn from chemical and FTIR analyses of sulphate content in an agarophyte seaweed stored in perforated plastic bags over a period of 31 months under tropical conditions (Romero, Villanueva, & Montano, 2008). Alternatively, Young and Goring (1958) reported a slight diminution in the relative content of sulphates which occurred after 20 months of storage of *C. crispus* in sealed polyethylene bags at 20 °C. However, seaweeds stored at lower temperatures did not present any measurable variation in the sulphate degree. Table 4 also presents the statistical analysis of all other parameters studied throughout the 39 months of seaweeds storage. Hybrid carrageenan yield is not affected by post harvest storage duration. A similar conclusion was reached by Young and Goring (1958), and constant agar yield is also reported for storage periods as long as 31 months (Romero et al., 2008). Both gel elasticity and gel melting temperature remained unchanged for 39 months. The preservation of gel properties over 39 month contrasts with the decreasing gel elasticity documented in the limited literature for carrageenan and agar (Freile-Pelegrín, 2000; Romero et al., 2008; Young & Goring, 1958). In these reports, the decrease in the molecular mass of the polysaccharide was identified as the main responsible for the loss of gel quality after 6 months. An indirect but cheap measure of the macromolecular size of extracted hybrid carrageenans is given by the intrinsic viscosity [η], which connects to an averaged molecular mass through the Mark–Houwink–Sakudara relationship, but also depends on the macromolecular conformation (Doi & Edwards, 1986, chaps. 4 & 8). Despite these limitations, and as far as postharvest storage effects are concerned, the intrinsic viscosity in Table 4 does not indicate any significant variation with storage duration, which is consistent with the preserved gel properties over 39 months. These results suggest that the low relative humidity achieved by convective drying of seaweeds together with postharvest storage in sealed and opaque plastic bags prevent any significant carrageenan degradation by enzymatic (carrageenase) or hydrolytic reactions which could negatively impact on the phycocolloid gelling properties.

The gel setting temperature is the only parameter in Table 4 that shows a small but significant decrease (nearly 4 °C) within 39 months. This decrease is hard to correlate with all other invariant parameters. Indeed, the gel mechanism and the interplay between gel structure and chemistry for hybrid carrageenan still needs to be elucidated (van de Velde, 2008). The viscoelastic properties of

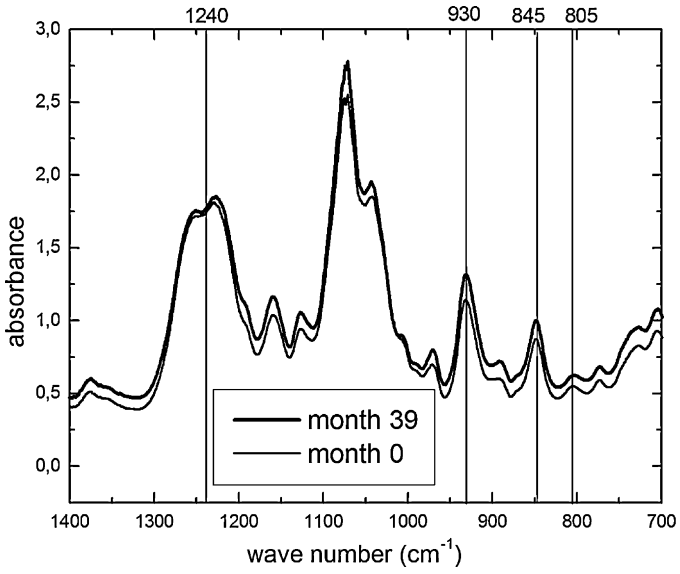

Fig. 5. FTIR spectra of alkali-treated phycocolloids extracts from seaweeds collected in November and obtained after 0 month (bottom and thin line) and 39 months (top and thick line) of post harvest storage.

Table 4
One-way ANOVA on the effect of dried seaweeds storage duration on the chemical and physical properties of alkali treated phycocolloids.

Property	Mean ± s.e.[a]	*F*-ratio	Probability
Sulphate degree	1.38 ± 0.06	13.7	0.07
Yield (%)	20 ± 3	4.1	0.07
G_0 (Pa)[c]	3521 ± 784	1.34	0.34
T_g (°C)[c]	38.9 ± 4.3	5.06	0.04[b]
T_m (°C)[c]	50.6 ± 2.1	2.37	0.17
[η] (dL/g)[d]	4.0 ± 1.1	1.95	0.22

[a] s.e. is standard error, $n = 12$.
[b] Significant variation at probability <0.05.
[c] G_0: gel elasticity; T_g: gel-setting temperature; T_m: gel melting temperature.
[d] [η]: intrinsic viscosity.

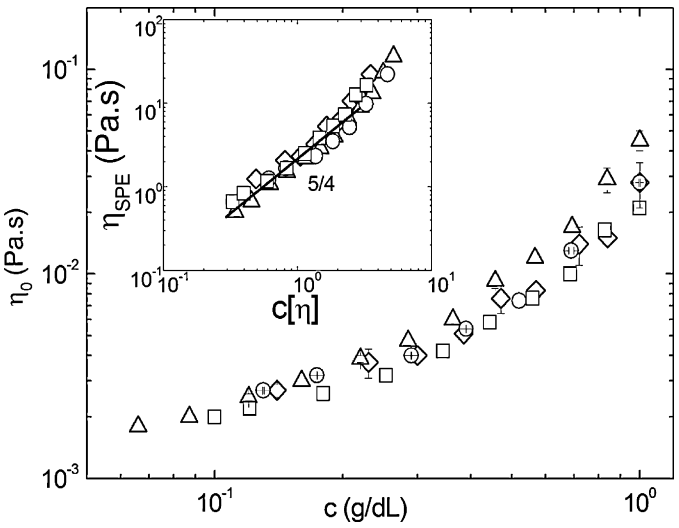

Fig. 6. Concentration dependence of the zero shear viscosity η_0 of hybrid carrageenan solutions in 0,1 M NaCl obtained with seaweeds harvested in November: (□) month 0, (○) month 24, (△) month 30, (◊) month 39. Inset: rescaling of the zero shear viscosities where specific viscosities η_{SPE} are plotted as a function of the overlap parameter.

semi-dilute polysaccharide solutions are very sensitive to macromolecular conformation, intermolecular interaction and tendency towards aggregation (Rinaudo, 2006) en route to gel formation. This gives the motivation for exploring the effect of postharvest storage duration on the zero shear viscosity η_0 of semi-dilute hybrid carrageenan solutions. The concentration dependence of η_0 of all hybrid carrageenan solutions obtained with phycocolloids extracted throughout 39 months is displayed in Fig. 6. Error bars correspond to triplicate extractions, i.e. for each storage duration, three phycocolloids are extracted and the zero shear viscosity of each extract was measured at the corresponding concentration to give the averaged viscosity and standard error. At lower concentrations, all viscosity data overlap, which is in agreement with the unaffected values of $[\eta]$ with storage duration, since $[\eta]$ is the major contribution to the solution viscosity in the dilute regime. At concentrations in excess of 0.7 g/dL, a larger scatter in zero shear viscosities is showing up, which underlines an effect of postharvest storage duration. Actually, the hybrid carrageenan extracted after 30 months storage gives significantly more viscous solutions. However, there is no clear trend on the effect of storage duration, since viscosities corresponding to "fresh" extracts (month 0) cannot be distinguished from viscosities measured with phycocolloids extracted at month 39. The inset in Fig. 6 provides an alternative representation of the viscoelastic properties of hybrid carrageenan solutions, where the specific viscosity $\eta_{SPE}=(\eta_0-\eta_S)/\eta_S$, with η_S the solvent viscosity, is plotted as a function of the overlap parameter $c[\eta]$. In this representation, all viscoelastic contributions from the molecular mass or the polysaccharide conformation (coil, semi flexible worm-like chain, or rigid rod) are cancelled. As a result, all viscosity data should fall on a single master curve if no additional viscoelastic contributions come into play (Clasen et al., 2006). At lower concentrations, experimental results follow this prediction and a power-law behaviour with exponent 5/4 is evidenced: this is the exponent expected for polyectrolytes solutions at high ionic strength, where neutral polymer behaviour in good solvent is retrieved (Hilliou et al., 2009). For larger concentrations, data show a departure from the power-law behaviour related to the onset of strong interactions between the polysaccharides. This is in this regime that specific viscosities do not fall on a single master curve, thus suggesting that physical interactions such as entanglements or aggregation between hybrid carrageenan chains are affected by the postharvest storage duration.

4. Conclusions

Seasonal variation in the chemical composition and gelling properties of kappa/iota/mu/nu-hybrid carrageenans extracted from *M. stellatus* seaweeds has been evidenced and correlated with the life stage of the seaweeds. Native extracts show seasonal variation in the sulphate degree and all gel properties studied. Correlations were found between gel properties and the occurrence of fructified gametophytes. For industrially attractive alkali-treated extracts, a seasonal variation is found in the relative contents in kappa- and nu-carrageenan. Gel elasticity and gel melting temperatures also show significant variation throughout the year. However, gel elasticity does not correlate with the chemical composition of alkali-treated extracts determined either with ^{1}H NMR or FTIR. Summer is the best season to harvest *M. stellatus* seaweeds since the alkali-extracted hybrid carrageenans show optimum properties for gelling application and extraction yield is maximal at this period of the year. In summer, *M. stellatus* population essentially consists of fructified gametophytes (over 80%), which is coincident with the best gel properties. However, the seaweed population does not correlate with the chemical structure of alkali-extracted phycocolloids. Postharvest storage of dried seaweeds (relative humidity below 5% dry basis) in sealed and opaque plastic bags allows the preservation of the gelling properties of the hybrid carrageenan upto 39 months. Within this storage duration, no significant change in the intrinsic viscosity, sulphate degree as semi-quantitatively determined with FTIR, and in the gel elasticity occurred. Results presented here suggest that a sustainable exploitation of wild *M. stellatus* for food and non food gelling additives is possible in Portugal since the functional properties of the phycocolloids can be preserved over a longer duration than the time frame needed to renew the natural resource. The price to pay for this eco-efficient exploitation is the cost of the drying and packaging of seaweeds.

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