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From the seaweeds' carrageenan composition to the hybrid carrageenans' hydrogel elasticity: Identification of a relationship based on the content in iota-carrageenan

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ABSTRACT

The carrageenan composition of 12 commercial seaweeds used in the production of hybrid carrageenans (HC) is estimated from the analysis of solid-state NMR spectra and is systematically compared with the chemical structure of HC extracted in hot water. ^{1}H NMR showed that the isolated HC contain from 5 to 80 mol% kappa-carrageenan, from 90 to 10 mol% iota-carrageenan, while from 8 to 23 mol% biological precursors are present in the copolymer's chain. A good correlation is found between the iota-carrageenan content in the seaweeds and in the extracted HC. The shear elastic modulus of 1 wt% HC gels in the presence of 0.1 M KCl is significantly weaker and gels form at lower temperatures when the iota-content in the seaweed is larger than the kappa-content. However, these weaker gels show strain hardening. A cut-off of 30 mol.% of iota-carrageenan in the polysaccharide is identified that separates strain softening stiffer gels from strain hardening softer gels. Overall, the set of data suggests that the carrageenan gel elasticity is ruled by the self-assembly of iota-carrageenan moieties in the HC, and HC gel elasticity can be directly linked to the iota-carrageenan composition of seaweeds.

1. Introduction

Various families of red seaweeds, belonging to the Gigartinales order, bio synthesize in their cell walls a family of sulphated linear polysaccharides known as carrageenans. These natural polymers find commercial applications as gelling agents, viscosity enhancers or formulation stabilizers mainly in the food industry (Piculell, 2006) and more recently in the pharmaceutical sector (Pacheco-Quito, Ruiz-Caro, & Veiga, 2020). The carrageenan industry classifies these polysaccharides by their viscoelastic properties in water: the kappa-carrageenans which form strong but brittle hydrogels, the iota-carrageenans which produces less elastic gels and the non-gelling lambda-carrageenans. The chemical structure of these linear polyelectrolytes is however more complex than this application-driven classification.

Carrageenans are better described as random block copolymers (Guibet et al., 2008; van de Velde et al., 2005; Hilliou, 2021) with blocks composed of different sequences of disaccharide units consisting of 3-linked β-D-galactopyranose (***G***-units) and 4-linked α-D-galactopyranose (***D***-units) or 4-linked 3,6-anhydro-α-D-galactopyranose (***DA***-units). The most naturally occurring disaccharide units are presented in Fig. 1, which takes on board the nomenclature introduced by Knutsen, Myslabodski, Larsen, & Usov, 1994. The variety of block types, lengths and distribution on the carrageenan chain stems from the natural complexity of their biosynthesis in the cell walls of the seaweeds. The carrageenan chemical structures span from homopolymers, such as kappa-carrageenans made of 100% to 93 mol.% of ***G4S-DA*** (here labelled as κ) (Rochas, Rinaudo, & Landry, 1989; van de Velde et al., 2005), to highly heterogeneous polymers, called hybrid carrageenans, made of blocks of κ, blocks of ***G4S-DA2S*** (here labelled as ι), blocks of ***G4S-D2S*** (μ) and blocks of ***G4S-D2S,6S*** (ν) all randomly distributed on the chain. Carrageenan chemical heterogeneity is specific to a fourth class of industrially relevant carrageenan, the kappa-2 or weak kappa, which are made of 20–50 mol.% of ι, the rest being κ (Bixler, 1996; Villanueva, Mendoza, Rodrigueza, Romero, & Montaño, 2004), endowing this carrageenan with gel properties intermediate between

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kappa- and iota-carrageenans.

The monotonic increase in the hydrogel elasticity with the **κ** mol.% content in hybrid carrageenans only made up of **κ** and **ι** blocks, is reported for a large set of carrageenans in a seminal study by van de Velde et al., 2005. Such relationship is yet to be established for the more heterogeneous hybrid carrageenans containing all disaccharide units presented in Fig. 1 (Souza et al., 2023, Kraiem, Ben Yahia, Aschi, & Hilliou 2023). Indeed, the macromolecular models relating the carrageenans hydrogel microstructures to their elastic properties have recently been questioned (Hilliou, 2021), as well as the coil-to-helix transition prerequisite to gel formation (Westberry, Rio, Waterland, & Williams, 2024).

The nature of the relationship between the carrageenans chemical structure and its gel elasticity is thus yet unresolved despite the extent of research on kappa- and iota-carrageenan homopolymers which supports their industrial use. We aim here to extend the scope of the relationship, with the possibility to find a link between the carrageenan's composition of commercial seaweeds and the gel properties of the polysaccharides from which these are extracted. The identification of such a link will boost the carrageenan industry which sources the biomass from cultivated and wild seaweeds specifically selected for the extraction of a single type of carrageenan (Bixler, 1996). The screening of algae for their polysaccharide composition can rely on well-established infrared spectroscopy techniques directly performed on dried samples (Chopin & Whalen, 1993; Gómez-Ordóñez & Rupérez, 2011; Pereira, Amado, Critchley, van de Velde, & Ribeiro-Claro, 2009). However, infrared spectroscopy does not provide quantitative estimates of e.g. **κ** or **ι** contents in the seaweeds. Solid-state NMR spectroscopy is more sensitive but has virtually not been used for a quantitative chemical analysis of algal materials (Ghassemi et al., 2021; Rochas & Lahaye, 1989). High-Resolution Cross-Polarization Magic Angle Spinning High Power Decoupling ^{13}C NMR spectroscopy (HR CP-MAS-HPD ^{13}C NMR) of grinded dried seaweeds was recently performed to quantitatively assess the carrageenan composition (Azevedo, Torres, Almeida, & Hilliou, 2022) and also to address the effects of aquaculture conditions on the carrageenan growth in the cultivated algal material (Azevedo et al., 2015). But no correlation was found between the seaweed's carrageenan composition and the chemical structures of the extracted carrageenans nor their gel properties. Two issues may have led to unresolved relationships. Firstly, the set of studied seaweeds produced carrageenans containing 70 to 40 mol.% **κ**. This is a too small window to identify a relationship with the gel elastic properties, as these are difficult to access by rotational rheometry, in particular in the range 50 to 40 **κ** mol.% (van de Velde et al., 2005). Secondly, an ethanol precipitation was used to recover the carrageenans, which might have resulted in a fractionation of the carrageenans actually contained in the seaweeds.

Here, the range of chemical composition of carrageenans is extended by studying 12 commercial seaweeds used in the industry to produce kappa-, iota- and various kappa-2-carrageenans. Whole carrageenan extracts were isolated from the seaweeds, thus no ethanol precipitation was used. The rheological characterization of carrageenan gels was extended to the large deformation regime which has been overlooked in the literature (Hilliou, 2021; Hilliou, Wilhelm, Yamanoi, & Gonçalves, 2009; van de Velde, 2008). Finally, taking on board the colloidal gels theories which showed that the elasticity is ruled by the mechanically weaker structural unit (Shih, Shih, Kim, Liu, & Aksay, 1990), we systematically laid down the gel rheological characteristics as a function of the **ι** mol.% content. The objective of such data treatment is to show that the **ι** mol.% content will be more effective than the **κ** mol.% content employed earlier (van de Velde et al., 2005) and will contribute to identify a valuable chemical structure-function relationship which is still critically missing for carrageenan gels (Chavda et al., 2024).

2. Experimental

2.1. Seaweeds

12 commercial dried seaweeds donated by Cargill were used as received for carrageenan extraction. These seaweeds are labelled from A to L and belong to the Spinosum (A to D) and Cottonii (I to L) genus (trade names) for the industrial production of the iota- and kappa-carrageenans, whereas seaweeds E to H belong to the Gigartinaceae family for the production of hybrid carrageenans. For HR CP-MAS-HPD ^{13}C NMR spectroscopy, seaweeds were crushed into powders using a coffee grinder, resulting in seaweed particles with sizes below 0.5 mm.

2.2. Carrageenan extraction

12 types of carrageenans were obtained from the 12 commercial seaweeds. The extraction was performed by soaking the dried whole thalli (1.5 g) in 100 mL distilled water at 80 °C for 2 h. Then the suspension was homogenized in a blender and the resulting slurries were

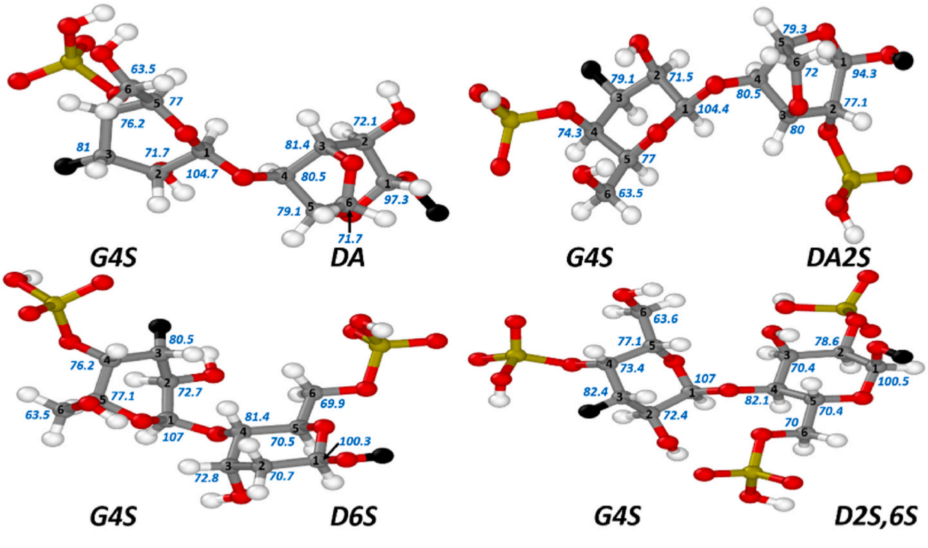

Fig. 1. Chemical structures of the disaccharides units mostly found in gelling carrageenans: **κ** (***G4S-DA***),**ι** (***G4S-DA2S***), **μ** (***G4S-D6S***) and **ν** (***G4S-D2S,6S***). Black atoms indicate those connected to other disaccharide units, whereas grey, red, white and yellow atoms indicate carbon, oxygen, hydrogen and sulphur atoms, respectively. Numbers close to the carbon atoms indicate the ^{13}C NMR chemical shifts in ppm (van de Velde et al., 2004) of the peaks assigned to the corresponding carbon.

further heated at 90 °C for 1 h. Solid/liquid separation was performed by centrifuging the hot slurries at 8000 rpm for 10 min. The resulting supernatant was recovered, cast into plastic cups and dried in an air ventilated oven at 50 °C overnight. The resulting film samples of carrageenans were finally stored in plastic bags in a fridge until further analysis within 1 month.

2.3. NMR spectroscopy

The experimental protocol used to perform High-Resolution Cross-Polarization Magic Angle Spinning High Power Decoupling ^{13}C NMR spectroscopy (HR CP-MAS-HPD ^{13}C NMR) has been detailed elsewhere (Azevedo et al., 2022). In brief, algal powders were loaded in CP-MAS tubes and spectra were recorded at room temperature and 500 MHz with a Bruker AVANCE III spectrometer. MAS was performed at 10 kHz, and 1000 scans were accumulated to record a spectrum. The contact time for cross-polarization was 1500 μs and a variable ramp amplitude pulse was used. A spinal composite pulse sequence was used for the high-power proton decoupling.

Proton NMR spectra were recorded on 1 wt% carrageenan solutions in D_2O. Prior to measurements, solutions prepared by stirring hot carrageenan suspensions at 80 °C for 1 h were ultra sonicated during hours using a DCG-300H bath (MCR Ltd., Holon, Israel), until the viscosity of solutions significantly dropped to avoid NMR line broadening. The solutions were then loaded in NMR tubes and spectra recorded with a Bruker Avance III spectrometer (Billerica, MA, USA) at 400 MHz and at 70 °C. D_2O signals were used as chemical shift reference, which likely causes slight variations with respect to the chemical shifts referenced elsewhere for carrageenans (van de Velde et al., 2004, Pereira, & Rollema, 2004).

2.4. Carrageenans' molecular mass and salt composition

Size exclusion chromatography (Waters 600 apparatus, with a Waters 2410 differential refractive index detector, Waters Portugal, Lisboa, Portugal) was performed with carrageenan solutions (0.1 wt%) prepared in 0.1 M NaCl at 90 °C. Hot solutions were injected in a PolySep-GFC-P Linear column (Phenomenex, Alcobendas, Spain), and measurements were performed at 40 °C. The column was previously calibrated with pullulan having molecular masses ranging from 6300 to 642 000 g/mol. All measurements were conducted in duplicate.

The contents in potassium, sodium and calcium of extracted carrageenans were measured by Inductively Coupled Plasma-Atomic Emission Spectroscopy (Ultima, Horiba Jobin-Yvon, France). Carrageenan film samples were dissolved in hot water (typically 0.1 g in 100 mL) at 80 °C, and solutions were loaded in the ICP-AES autosampler. The calibration was performed with solutions containing a single element, but with varying concentrations.

2.5. Gels preparation and rheological characterization

Carrageenan gels were prepared in 0.1 M KCl with a polysaccharide concentration of 1 wt%. Under such salt and ionic strength, gels with sufficient elasticity to be studied by rotational rheometry, are produced for all extracted polysaccharides, in contrast to 1 wt% carrageenan in 1 M NaCl (Moraes & Hilliou, 2024). Hot carrageenan solutions were loaded at 85 °C in the parallel plate geometry of a stress controlled rotational rheometer (MCR 302, Anton Paar, Graz, Austria). The plates were pre-heated at 85 °C, and the rim of the shearing geometry was covered with dodecane to limit water loss. A temperature quench from 85 °C to 25 °C was performed within 1 h while both elastic (G') and loss (G'') moduli were recorded by applying a small amplitude oscillatory shear with a frequency of 1 Hz and an amplitude of 0.01 %. The latter is too small to generate large enough stress when the samples are in the liquid phase at higher temperatures, but ensures that no strain-induced structure is setting during the liquid-to-solid transition. The normal force was maintained at 0 N during cooling by allowing the gap to accommodate for the volume change during the liquid-to-solid transition. This strategy avoids any artefact during the measurement of G' and G'' through the phase transition (Mao, Divoux, & Snabre, 2016). Finally, the gels were equilibrated during 30 min at 25 °C before performing a strain sweep from 0.01% to 500% at 1 Hz, to assess the large deformation behaviour of the gels.

3. Results and discussion

3.1. Carrageenan composition of seaweeds

The HR CP-MAS-HPD ^{13}C NMR spectra of representative seaweeds are displayed in Fig. 2. All other seaweeds showed qualitatively similar spectra including the broad peaks in the 175 ppm region and between 40 and 20 ppm, which are indicative of pyruvates, lipids and pigments present in seaweeds (Rochas & Lahaye, 1989), see Fig. S1 in Supplementary Information.

Focusing on the chemical shifts regions where signals assigned to carbons from the disaccharide units illustrated in Fig. 1 are expected, differences in the intensities and broadening of lines in the 110-90 ppm region are evident in Fig. 2. In this region, 3 NMR lines contribute to the spectra which are assigned to carbon number 1 (C1) of ***G4S*** (107-104 ppm), of ***D6S*** and ***D2S,6S*** (100 ppm), and of **DA** and ***DA2S*** (97-94 ppm), according to the chemical shifts listed for the carrageenans from the kappa family (van de Velde et al., 2004).

More differences are seen in the region 80-65 ppm, which is due to the contributions of all other carbons from all disaccharide units displayed in Fig. 1, resulting in 5 lines. In contrast to this, the peak at 60 ppm is nearly identical for all seaweeds as only C6 from ***G4S*** contributes to this signal. The fitting of each spectrum $y(x)$ with the following equation based on the addition of 9 Lorentzians L_i was shown to provide a satisfactory quantitative estimate of the disaccharide units $\boldsymbol{\kappa}$, $\boldsymbol{\iota}$, $\boldsymbol{\mu}$ and $\boldsymbol{\nu}$ contained in the seaweeds (Azevedo et al., 2015; Azevedo et al., 2022):

$$\begin{aligned} y = y_0 &+ (a+b+c+d)L_1(x_1) + (c+d)L_2(x_2) + (a+b)L_3(x_3) \\ &+ (2b+c+2d)L_4(x_4) + (4a+2b+c+d)L_5(x_5) + (2a+2b \\ &+ 2c+d)L_6(x_6) + (a+d)L_7(x_7) + (2a+3b+5c+4d)L_8(x_8) \\ &+ (a+b+c+d)L_9(x_9) \end{aligned} \tag{1}$$

In equation (1), the Lorentzians $L_i(x_i)$ are given by

$$L_i(x_i) = \frac{2}{\pi}\frac{w}{4(x-x_i)^2 + w^2} \tag{2}$$

where w is the width of the Lorentzians (w is taken as a constant

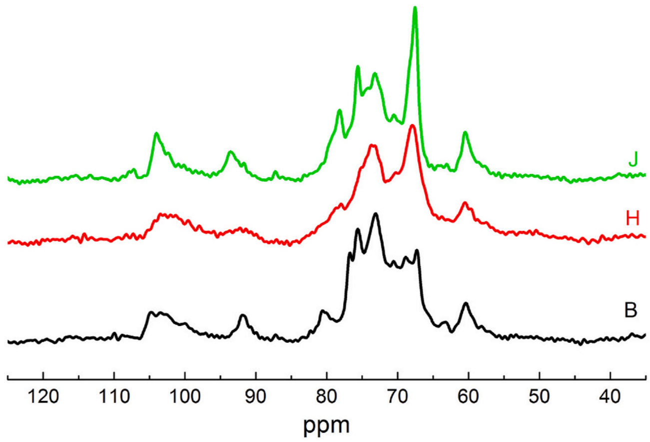

Fig. 2. HR CP-MAS-HPD ^{13}C NMR spectra of three grinded dried seaweeds labelled B, H and J. The intensities of NMR lines are vertically shifted to allow for the stacking of all spectra in the figure and facilitate the comparison.

assuming for simplicity that all carbons in the carrageenan disaccharide units show identical molecular dynamics and witness identical cross polarization effects of neighbouring protons), and x_i is the position (in the ppm scale x) of the Lorentzian line L_i. The factors multiplying the different L_i in equation (1) result from the relative contents a, b, c and d in the type of disaccharide units (κ, ι, μ and ν, respectively) multiplied by the number of carbons of that disaccharide unit that contribute to the line L_i. Thus, only 4 parameters (a, b, c and d) are fitted in equation (1) to each spectrum, whereas the positions x_i of the 9 Lorentzians L_i are fixed during the fitting but within the chosen range of ppm assigned for each line (see respective ranges in Tablet 2 of (Azevedo et al., 2022)). Fig. 3 illustrates the fitting of the spectrum measured with seaweed A to equation (1). Equation (1) entails a set of hypothesis detailed elsewhere (Azevedo et al., 2022). The main assumption is that the 9 lines are contributed by 4 carrageenan disaccharide units. Therefore, contributions from other types of polysaccharides or other non-carrageenan compounds, which could show up around 100 and 60 ppm, are not taken into account in equation (1). As such, the computed line in Fig. 3 does not reproduce some details of the experimental spectrum. Nevertheless, the fits did converge for all experimental spectra and returned R^2 values better than 0.9 in all cases, in spite of the noise inherent to the HR CP-MAS-HPD 13C NMR spectra.

The result from the fitting of equation (1) to all spectra recorded with the seaweed samples is presented in Fig. 4. It displays the carrageenan composition (in κ, ι, μ and ν) of all seaweeds obtained by the computation of parameters a, b, c and d from the fitting procedure. The quality of the fits is mirrored in the error bars computed from the fits for each disaccharide unit.

This carrageenan analysis indicates that the seaweeds contain all types of disaccharide units from the kappa family, but in different proportions, varying from seaweeds rich in ι to seaweeds rich in κ.

Seaweeds E to H show more heterogeneous compositions in disaccharide units and are thus used for the extraction of kappa-2 carrageenans, in contrast to other seaweeds industrially used for the extraction of iota- (seaweeds A to D) or kappa-carrageenans (seaweeds I to L). Interestingly, all seaweeds contain a significant proportion of carrageenan biological precursors μ andν. In particular, seaweed H is made of more than 50 mol.% of μ disaccharide units which are the biological precursors of κ disaccharide units (Chopin & Whalen, 1993; Piculell, 2006).

3.2. Characterization of extracted carrageenans

The chemical structure of the 12 carrageenans extracted from the seaweeds is presented in Fig. 5. The relative contents in κ, ι, μ and ν are computed from the ratios of the peak area of the signal assigned to the anomeric proton of a specific disaccharide units over the total area of the four peaks assigned to all anomeric protons from the kappa-carrageenan family. Examples of proton NMR spectra of selected carrageenans can be found in Fig. S2. NMR signals which are not assigned to carrageenans were found in some spectra. For instance, a peak at 5.28 ppm was found in all extracts from the Spinosum, with larger intensity for sample D (see Fig. S3), and to a lesser extent in all extracts from the Cottonii. Similarly, a peak at 5.02 was found for sample I, whereas peaks corresponding to pyruvic acids and other compounds were found up field in the spectra of all carrageenans but those from Cottonii (see inset to Fig. S3). These signals can be caused by low molecular weight compounds such as sugars or pigments and salts which are not removed by the extraction route employed here. As such, these compounds, which were not further analysed, can impact the gelling properties reported here, or at least affect the effective carrageenan concentrations used in the rheological study.

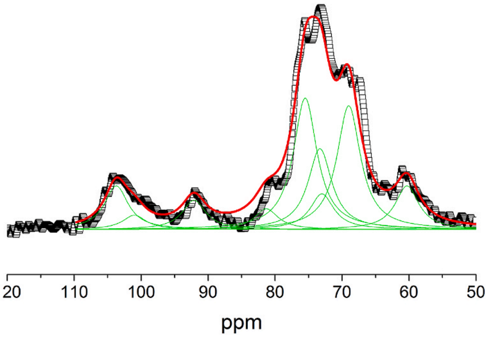

Fig. 3. HR CP-MAS-HPD ^{13}C NMR spectrum of seaweed A (symbols) and fit of equation (1) to the spectrum (red thick line) resulting for the addition of the 9 Lorentzian lines (thin green lines).

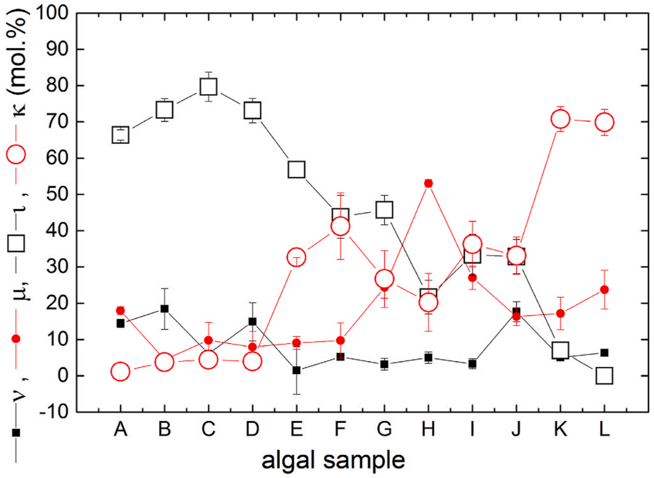

Fig. 4. Carrageenan composition (in mol.% of κ, ι, μ and ν) of all tested seaweeds.

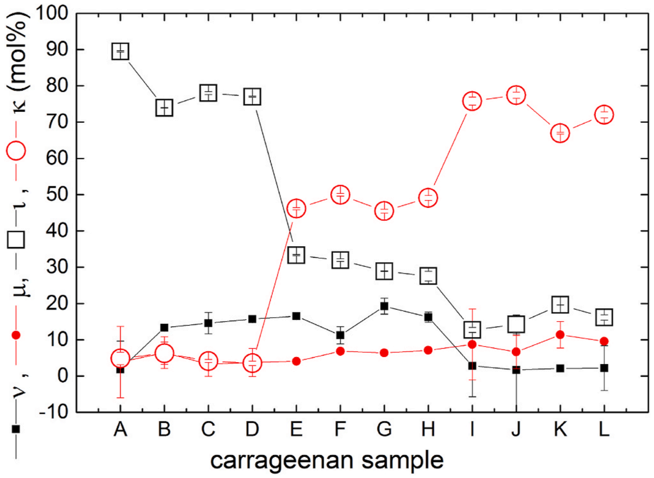

Fig. 5. Disaccharide units composition (in mol.%) of the 12 extracted carrageenans assessed from the quantitative analysis of their proton NMR spectra.

The chemical structures of all extracts are qualitatively reminiscent from the carrageenan composition of seaweeds, as is evidenced when comparing Figs. 4 and 5. Whereas carrageenan A is essentially a iota-carrageenan containing small amounts (below 10 mol.%) of μ and ν disaccharide units, all other carrageenans exhibit more heterogeneous structures, from polymer chains rich in ι, to more hybrid structures containing all κ, ι, μ and ν units and chains which are richer in κ. Nonetheless, 8 carrageenans (from E to L) contain 10–30 mol.% of ι as well as significant amounts of μ and ν. As such, none of these carrageenans qualify for the industrial definition of kappa-2 (Bixler, 1996;

Villanueva et al., 2004) which is based on the contents in **κ** and **ι**. Carrageenan precursors **μ** and **ν** are usually converted during the alkaline extraction of carrageenans, which was avoided in the present study to minimize the carrageenan physical and chemical changes occurring during extraction.

Table 1 summarizes the molecular mass distributions of all extracted carrageenans, as well as their composition in elements whose chlorine salts are known to facilitate the aggregation of carrageenan helices and their consecutive gel setting.

All carrageenan possess molecular masses between 200 kDa and 2 × 10^6 kDa, which is in agreement with the molecular masses reported for samples extracted from various carrageenophytes (Piculell, 2006; van de Velde et al., 2005). All M_w are above the critical molecular mass M_c identified by Rochas, Rinaudo, and Landry (1990) for kappa-carrageenans. Above M_c = 180 kDa, kappa-carrageenan gel properties do not depend on M_w. Note however that another study showed that two hybrid carrageenans with identical chemical structure but with different $M_w > M_c$ showed very different gel properties (Souza, Hilliou, Bastos, & Gonçalves, 2011). The molecular mass distributions PDI of the carrageenan listed in Table 1 are mostly around 2, but carrageenans E and K show much larger PDI, which suggests that some polysaccharides in these samples may show M_w below $M_{c.}$ Unfortunately, there is a lack of reports about the effect of molecular mass distributions, and particularly the role of smaller chains, on the gelling properties of carrageenans (Rochas et al., 1990; Souza et al., 2011). As such, and assuming that the value of M_c holds true for the carrageenans reported in Table 1, one can conjecture that the molecular masses of the carrageenan samples studied here will not affect the gelling properties.

The elemental compositions reported in Table 1 suggest that carrageenan samples do not present significant amounts of calcium. However, carrageenans rich in **κ** or in **ι** are extracted with more potassium and sodium than the more hybrid carrageenans. This higher content in cations cannot however be correlated with the total amount of more sulphated disaccharide units as carrageenans B to H show around 20 mol.% of **μ** and **ν** units. Since distilled water was used during extraction, these cations originate from the seaweeds. It has been recently showed that salts formed with the cations carried by *Chondrus crispus* seaweeds can affect the recovery of more or less sulphated carrageenans (Bahari et al., 2021). However, further extraction study will be needed to elucidate the origin of the leaching of more cations with less heterogeneous carrageenans.

The chemical characteristics displayed in Fig. 5 and Table 1 do not allow for detailing the variation in the carrageenan chemical compositions from chains to chains within each extract. Dedicated characterizations after fractionation or enzymatic hydrolysis as performed elsewhere would be required (Rochas et al., 1989; Guibet et al., 2008), which are out of the scope of the present study. Carrageenans with large PDI such as sample K could actually be mixtures of nearly homopolymers made of kappa-carrageenan or iota-carrageenan disaccharide units (Rochas et al., 1989), instead of more hybrid copolymers. However, it has been shown that carrageenans from the commercial seaweeds selected in the present study, especially from the Gigartinaceae, are rather copolymers as no separation of kappa- and iota-carrageenan homopolymers could be seen (van de Velde et al., 2005), whereas carrageenans from Cottonii and Spinosum are essentially homopolymers (van de Velde, 2008).

Table 1

Weight averaged molecular mass (M_w), polydispersity of the molecular mass distribution (PDI) and elemental composition in potassium (K), sodium (Na) and calcium (Ca) of all extracted carrageenans.

Carrageenan sample	Mw (10^6 g/mol)	PDI	K (wt.%)	Na (wt.%)	Ca (wt.%)
A	1.453	1.85	7.25	3.36	0.29
B	0.728	1.76	12.1	6.1	0.66
C	1.173	2.10	14.63	9.08	0.34
D	1.047	2.18	5.67	2.82	0.27
E	0.816	4.25	2.28	6.12	0.37
F	0.234	2.29	2.69	6.61	0.38
G	0.591	2.19	1.93	5.24	0.32
H	0.856	2.08	1.92	5.87	0.35
I	1.395	2.53	10.3	2.82	0.31
J	0.412	3.84	11.7	3.4	0.58
K	1.380	5.39	19	6.63	0.24
L	1.266	2.90	5.04	1.27	0.19

3.3. *Can we link the carrageenan composition of seaweeds with the chemical structure and gelling properties of extracted carrageenans?*

3.3.1. *Chemical analyses*

Fig. 6 compares the quantitative chemical information retrieved from the solid-state NMR spectra with those measured by proton NMR, focusing on disaccharide units **κ** and**ι**, which are responsible for the gelling capabilities of carrageenans from the kappa–carrageenan family, in opposition to **μ** and **ν** units which are viewed as defects (Hilliou, 2021; Piculell, 2006; van de Velde et al., 2005).

The figure shows that carrageenans rich in **κ** or **ι** are extracted from seaweeds that contain large amounts of corresponding disaccharide units. The correlation is less satisfactory for more hybrid carrageenans, since data drift away from the line that suggests a perfect linear relationship between the two sets of data. Pearson correlation analyses of the two sets of data return a Pearson's coefficient of 0.865 (*p*-value of 2.8 × 10^{-4}) for both **κ**- and **ι**-contents. However, if error bars are considered in the linear correlation analysis (using error bars as weights in a linear fit to the data), a better result is obtained for **ι** disaccharide units (coefficient of determination $R^2 = 0.91$) than for **κ** disaccharide units (coefficient of determination $R^2 = 0.79$). Such difference is naturally caused by the larger error bars associated with the solid-state NMR analysis of **κ**-contents in the seaweeds (see Fig. 6). Moreover, **ι**-contents span a wider range of values (from 0 to 90 mol.%) than **κ** contents (from 1 to 77 mol.%). As such, the content in **ι** disaccharide units works better in linking the carrageenan composition of seaweeds to the chemical structure of the extracted carrageenans.

No correlation was found between the contents in **μ** and**ν** units of the seaweeds and in respective carrageenans (see Fig. S4). This is inherent to the large errors associated with the quantification of these disaccharide, as their small amounts in both seaweeds and carrageenans are close to the limit of detection of NMR spectroscopy. Also, as mentioned above, other low molecular weight compounds extracted together with the carrageenans, and the presence of other compounds in the seaweeds contributing to signals overlapping those assigned to **μ** and**ν** in the solid

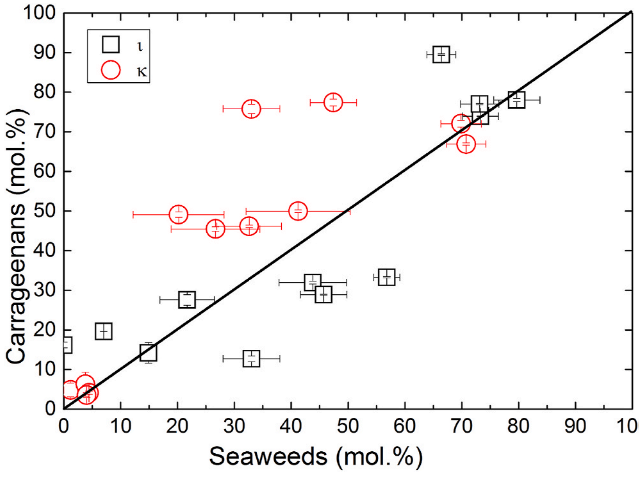

Fig. 6. Fractions of **κ** and **ι** contained in the extracted carrageenans versus the **κ** and **ι** contents in the seaweeds. The line is a guide to the eyes to evaluate a possible linear correlation between the two sets of data.

state NMR spectra, can altogether be responsible for the lack of correlation. In addition, μ andν units can be converted into κ and ι units during carrageenan extraction. As such, all carrageenan gelling properties will now be laid down as a function of the ι contents in the seaweeds.

3.3.2. Gelling properties

The setting of the carrageenan gels during the cooling of hot 1 wt% solutions in 0.1 M KCl is shown in Fig. 7. These salt conditions were chosen to screen any effect from the salts brought by the carrageenans (taking the sample with the largest amount of potassium K in Tables 1 and 1 g of this carrageenan in 100 mL of 0.1 M KCl will bring 0.24 g of salt, which is residual when compared with the nearly 4 g of K brought by KCl), while gels with sufficient elasticity could be formed in the rheometer. However, the temperature dependence of the storage modulus G' is rather noisy at higher temperatures and renders difficult a clear estimate of the temperature T_{on} for the onset of gel setting based of the rise in the elasticity G' of the cooling solution. Similar difficulties were reported for the rheological study of a set of hybrid carrageenans with κ fractions below 50 mol.%, where no temperature for the gel transition defined as the temperature where $G' = G''$ could be measured (van de Velde et al., 2005).

To bypass the low shear stress generated by the gelling under an oscillatory strain of 0.01 %, T_{on} is here inferred from the temperature dependence of the gap which is set by maintaining the normal force to 0 N. This is illustrated in Fig. 7b for selected carrageenan gels. At higher temperature, the gap grows linearly with the cooling, as expected from the thermal expansion of the shearing geometry. At the sol-gel transition, the sample shrinks (thus exerting a negative normal force on the plates) and the gap is decreased to maintain the normal force to 0 N, as well as a contact between the sample and the shearing plate. The temperature at which the thermal dependence of the gap is no more linear is taken here as T_{on}. Fig. 7b indicates that sample B does not form a gel within the tested temperature range since the gap only exhibits a linear thermal expansion. This can be attributed to the fact that this carrageenan presents the lowest amount of ι within the set of ι–rich samples (see Fig. 5). In addition, carrageenan B does not contain enough κ disaccharide units sensitive enough to KCl to promote the formation of a network with sufficient elasticity to generate a measurable stress under a 0.01 % strain (Piculell, Nilsson, & Muhrbeck, 1992). In contrast to this, sample A forms a turbid and weak gel, as the presence of only 5 mol.% of κ disaccharide units weakens the iota-carrageenan network as shown in Piculell et al., 1992.

Fig. 8 presents a selection of strain sweep tests performed on equilibrated gels. The LAOS behaviour of all tested carrageenan gels is shown in Fig. S5.

In addition to the measurement of the elasticity G_0 of the gels defined here as the value of G' measured in the linear regime, two types of large amplitude oscillatory shear (LAOS) behaviour can be inferred from the curves. The gel formed with carrageenan I shows a larger elasticity G_0 and an abrupt loss of elasticity at strains of the order of 10 %. In contrast to this, gelled carrageenans A and E sustain much larger strains as G' increases with the applied strain, passes through a maximum and eventually drops at larger strain values. The increase in elasticity, i.e. the strain hardening, was shown to be reversible when consecutive strain sweeps are applied within this regime of strains, in contrast to the irreversible drop of elasticity exhibited by sample I (Moraes & Hilliou, 2024). Strain hardening is the hallmark of fibrillar networks (Meng & Terentjev, 2017; Storm, MacKintosh, Lubensky, & Janney, 2005), including pectin (Carillo, MacKintosh, & Dobrynin, 2013) and agarose (Bertula et al., 2019) gels. Overall, the data reported in Fig. 8 are in qualitative agreement with theoretical predictions for networks made of fibre-like structures. Note here that colloidal gels described as a fractal network of clusters made of strings of particles show strain hardening when strings' elasticity is weaker than the elasticity of contacts between clusters (Gisler, Ball, & Weitz, 1999). Indeed, the strain hardening of gels formed with different concentrations of a hybrid carrageenan with a chemical structure equivalent to samples E to H, was successfully rationalized by the theory for fractal colloidal gels. The fractal dimension computed from the fitting of the theory to the strain dependent shear modulus nicely matched the fractal dimension measured from the analysis of microscopic images of the gels' structures (Hilliou et al., 2009). Another possibility for explaining the strain hardening comes from a rod and coils model devised for gelatin gels (Groot, Bot, & Agterof, 1996): loose carrageenan chains not involved in rod-like aggregates could stretch under deformation and give the reported strain hardening. This model has been indeed tested with some success for a hybrid carrageenan gel formed in NaCl (Hilliou, 2021).

3.3.3. Hybrid carrageenan gel properties relate with the content of iota–carrageenan in the seaweeds

Having all seaweeds' carrageenan compositions and the carrageenan gels properties at hand, it is natural to question about the existence of a relationship, which could underpin biomechanical differences in carrageenophytes' cell walls, but also predict the gel properties of the carrageenan without carrying out any extraction. An attempt to address this relation is proposed in Fig. 9a where all hybrid carrageenan gels' linear and nonlinear thermo-rheological properties are plotted as a function of the ι–content in the seaweeds.

Fig. 9a establishes a relationship between the linear elastic

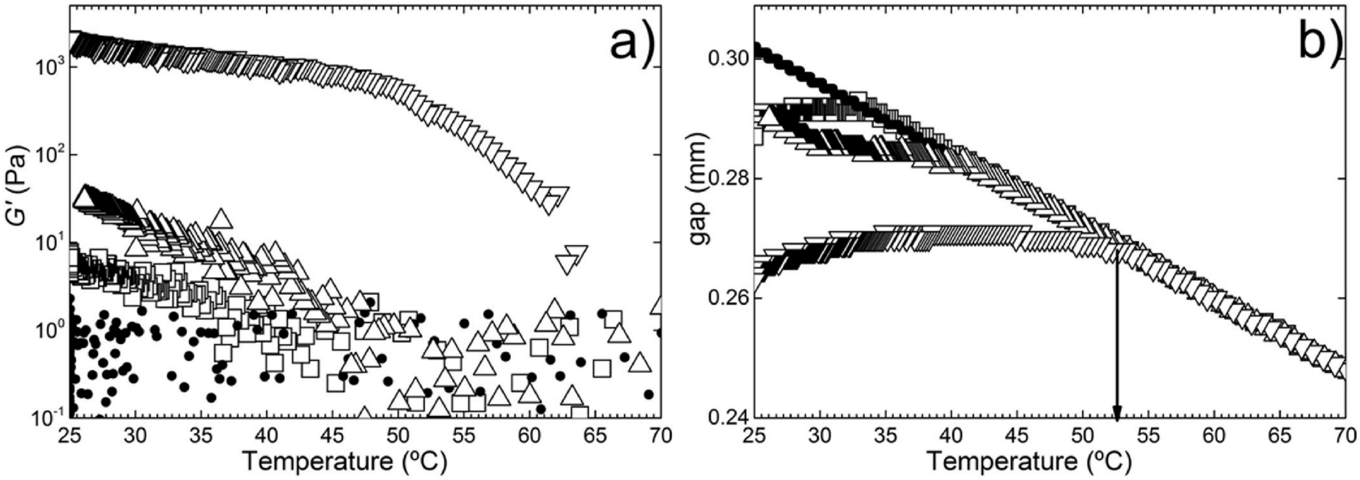

Fig. 7. Evolution of the storage modulus G' (a) and the gap of the rheometer (b) during the cooling of hot carrageenan solutions: sample A (squares), sample B (black dots), sample H (up triangles) and sample J (down triangles). The arrow in (b) indicates the temperature T_{on}.

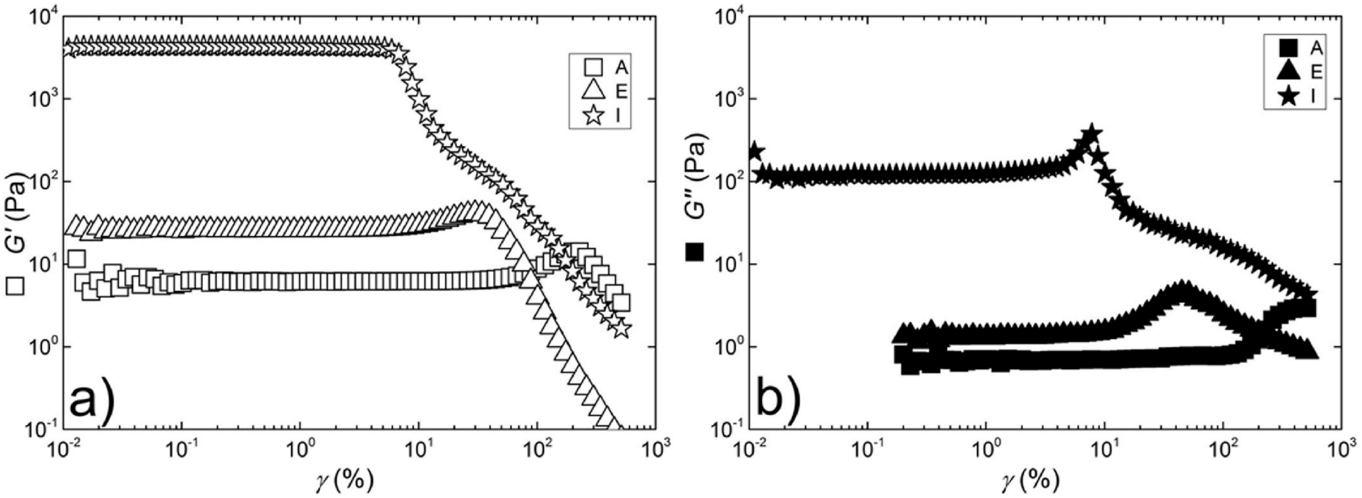

Fig. 8. Strain dependence of (a) the storage (G') and (b) the loss (G'') moduli of equilibrated carrageenan gels at 25 °C in 0.1 M KCl.

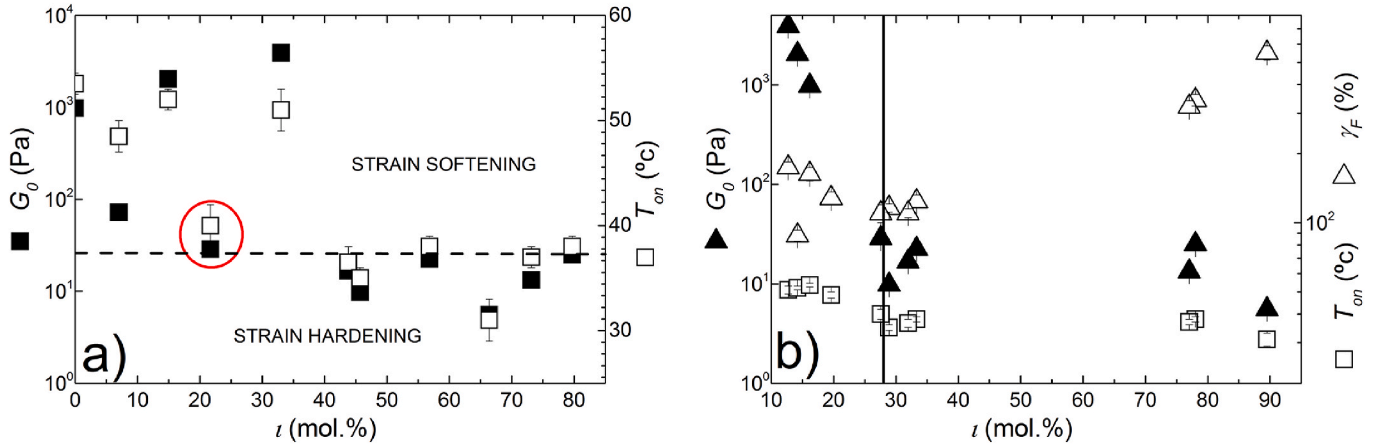

Fig. 9. Relationships between the gels linear elastic modulus G_0, the temperature T_{on} for onset of gelation, the strain γ_F for gel fluidization, and (a) the ι–content in carrageenophytes, (b) the ι–content in the extracted hybrid carrageenan. The horizontal dashed line in (a) indicates the frontier between strain hardening and strain softening hybrid carrageenan gels. The red circle in (a) highlight the data from seaweed H which show up as an outlier (see text for detail). The vertical line in (b) indicates the cut-off in the ι–content of hybrid carrageenans that delimits the strain hardening (for ι–content larger than the cut-off line) and strain softening behaviour of corresponding gels.

properties of the hybrid carrageenan gels and the ι–content in the seaweeds: seaweeds with larger ι–contents deliver less elastic gels, as expected for hybrid carrageenan gels (Bixler, 1996; Hilliou, 2021; Piculell, 2006; van de Velde, 2005) including those made from polysaccharides with significant amounts of biological precursors (Hughes, Leonardi, & Genovese, 2023). Note that such relationship is much less convincing if the comparison is carried out with the κ–content, as there are gaps along the κ–content scale and similar κ–content give gels with very different elasticities, see Fig. S6. The gel elasticmoduli G_0 of the ι–rich hybrid carrageenans in Fig. 9 are smaller than 116 Pa, the value measured with a commercial iota-carrageenan in similar gelling conditions (Moraes & Hilliou, 2024). The same holds true with κ–rich hybrid carrageenans which show moduli smaller than 21 kPa, the modulus measured with a commercial kappa-carrageenan (Moraes & Hilliou, 2024). The data also show that the temperature T_{on} for the onset of gelation follows the same trend, for the whole range of measured ι–content in the seaweeds. This is a rather novel result since an earlier study reported that the gelling temperature does not relate with the chemical structure of the hybrid carrageenans (van de Velde et al., 2005). However, both G_0 and T_{on} measured with the gels made from extracts of seaweeds H are off the trend shown by the remaining set of data, as G_0 and T_{on} are too small when compared with those from gels associated with equivalent low ι–content. Looking at Fig. 4, it is clear that such odd result is related to the large content in non-gelling $\boldsymbol{\mu}$ disaccharide units making up seaweed H. Indeed, when comparing both G_0 and T_{on} with the ι–content in the extracted hybrid carrageenan, see Fig. 9b, these outlier points vanish as the extraction process significantly reduced the amount in biological precursors, and theι–content is readily shifted to larger values. Evidently, more data in the range 40–70 mol.% of ι–content in the polysaccharide chain is needed to explore the chemical structure-gel elastic relationships for this class of hybrid carrageenan. However, the set of data reported in Fig. 9b is of industrial relevance as they show chemical structure-gelling temperature-elasticity relationships for low cost (no alkali modification) hybrid carrageenans (Bixler, 1996; Villanueva et al., 2004).

Interestingly, the qualitative analysis of the LAOS experiments performed on all carrageenan gels suggests that there is a cut-off in the ι–content of hybrid carrageenans, of the order of 30 mol.% (compare Fig. 9a and b), below which the strain hardening vanishes, and stronger gels show a strain softening behaviour. Based on theoretical arguments (Carillo et al., 2013; Meng & Terentjev, 2017; Storm et al., 2005, Pastore, MacKintosh, Lubensky, & Janney, 2005), one may conjecture from the LAOS behaviour that hybrid carrageenans containing more than 30 mol.% of ι are essentially fibrillar networks, whereas hybrid carrageenans with lower *ι*–content build gels with a denser or more crosslinked structure which does not allow for the strain-induced stretching of structural elements in the network. Alternatively, the bending rigidity of the filaments, or the compression accumulated on filaments during gel formation, would be responsible for differences in the LAOS behaviour whereas the network structure is essentially the same (Carillo et al., 2013; Meng & Terentjev, 2017). However, studies with hybrid carrageenans free of *μ* and *ν* should be carried out to validate this proposed gel structure-elasticity relationship as well as the 30 mol.% cut-off content in *ι*. Note here that a more quantitative analysis of the large deformation behaviour, for instance with the fluidization strain γ_F signalling the strain-induced gel to liquid transition where $G' = G''$ as shown in Fig. 9b, does not pick up the effect of *ι*–content on the carrageenan gel characteristics.

4. Conclusions

HR CP-MAS-HPD ^{13}C NMR spectroscopy directly performed on dried and grinded seaweeds gave access to the contents in *ι* disaccharide units in the seaweeds. This solid-state NMR experimental tool and the quantitative analysis presented here are of interest to screen for industrial carrageenophytes. From the study of 12 seaweeds and corresponding 12 hybrid carrageenan extracts, a good correlation between the *ι*–contents in the seaweeds and in the carrageenan was found, which resulted from both a better precision in the quantification of such disaccharide units in the seaweeds and a good span in the range of *ι*–contents in both carrageenans and seaweeds. The hypothesis that the *ι*–content in the seaweeds rules the hydrogel elastic properties of extracted carrageenan could thus be tested, going beyond the classical carrageenan structure-gel properties relationships often studied based on the *κ*-content, since kappa-carrageenans are known to build stiffer gels in water. Indeed, seaweeds rich in *ι*–content can be used to extract hybrid carrageenans with lower gel setting temperatures, of the order of 40 °C, with gel elastic moduli below 100 Pa for 1 wt% in 0.1 M KCl, and with large strain resistance characterized by strain hardening properties. A minimum amount of 30 mol.% of *ι* disaccharide units in the hybrid carrageenan chemical structure is needed to achieve such properties. On the basis of fibrillar network theories, beyond 30 mol.% of *ι* content, the network is denser, or the bending rigidity of filaments is no more ruled by the *ι* disaccharide units, resulting in stiffer and more brittle gels formed at higher temperatures. The rheological characterization adopted here, based on gel setting under zero normal force and on the systematic study of the gels LAOS behaviour, and the underlying theoretical framework, are promising for revisiting the chemical composition - gel structure - elastic properties relationships in kappa2-carrageenan systems, which are chemically simpler than the hybrid carrageenans studied here, and in mixtures of kappa- and iota-carrageenans. As for future works, it would be interesting to apply the solid-state NMR approach presented here to algal residues as to assess the efficiency of new extraction routes.

CRediT authorship contribution statement

Loic Hilliou: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Izabel Cristina Freitas Moraes:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Pedro Lúcio Almeida:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation.

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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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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.111007.

Data availability

Data will be made available on request.

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