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Master's Dissertation
Masters in Biological and Chemical Engineering

Dissertation supervised by
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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, Braga, December 2024

Maria Gabriela Afonso Gonçalves

RESUMO

Extração sequencial de produtos naturais de três carragenófitas: foco em carrageninas híbridas e o impacto da massa molecular nas suas propriedades de gelificação

As carrageninas são polissacarídeos sulfatados conhecidas pelas suas propriedades de gelificação termorreversíveis, amplamente utilizadas nas indústrias alimentar, farmacêutica e cosmética. Recentemente, as carrageninas híbridas (K2) têm despertado interesse devido às suas propriedades de gelificação intermediárias. Este estudo tem como objetivo desenvolver um método de extração sequencial (SE) para algas vermelhas (*Chondrus crispus*, *Mastocarpus stellatus*, *Gigartina pistillata*), alinhado ao conceito de biorrefinaria, por extrusão extrativa. Os principais objetivos deste trabalho incluem a extração e caracterização de K2-carrageninas, avaliando o impacto de extrações prévias e o tempo de extração. Adicionalmente, pretende-se estudar o efeito da redução de massa molecular (Mw) e determinar uma massa molecular crítica (Mc) para a gelificação da K2-carragenina. Para isolar ficobiliproteínas, clorofila-a, carotenoides e carrageninas das algas, foi utilizada a seguinte sequência: extração com água fria (CWE), extração etanólica (EE), extração com água quente (HWE) e extração alcalina quente (HAE). O rendimento foi determinado para cada fração, e os extratos de carrageninas foram analisados quanto à Mw, aos níveis de sulfatação (FTIR, ^{1}H-NMR) e às propriedades reológicas dos seus géis e líquidos resultantes. A redução da Mw foi alcançada através de ultrassonicação, com os produtos degradados sujeitos a análises semelhantes às anteriores. Os resultados indicam que a SE separa eficazmente os componentes das algas, facilitando a recuperação de carrageninas com alta Mw, particularmente em *M. stellatus* e *G. pistillata*, com elasticidade de gel (G') e viscosidade superiores em comparação com os controlos. A HWE produz carrageninas de alto Mw e com propriedades reológicas melhoradas, enquanto os extratos HAE apresentam menor Mw e potencial de gelificação reduzido. Em geral, extrações prolongadas melhoram a Mw e a G', embora *C. crispus* tenha mostrado uma tendência distinta. Os resultados da Mw destacam que o ultrassom leva à diminuição e dessulfatação das carrageninas ao longo do tempo de processamento. Ademais, a Mc depende da composição química, sugerindo uma Mc de ~$1{\cdot}10^5$ g/mol para híbridas ricas em κ e ~$3{\cdot}10^5$ g/mol para híbridas ricas em ι, além das quais a degradação adicional tem impacto mínimo na gelificação. Em suma, este estudo demonstra o potencial de adaptação de protocolos de extração para atender às necessidades industriais, contribuindo para a valorização sustentável das algas vermelhas através de métodos de biorrefinaria.

Palavras-chaves: Carrageninas híbridas; extração sequencial; massa molecular; reologia.

ABSTRACT

Sequential extraction of natural products from three carrageenophytes: focus on hybrid carrageenans and the impact of molecular mass on their gel properties

Carrageenans are sulphated polysaccharides known for their thermo-reversible gelling properties, widely used in the food, pharmaceutical, and cosmetic industries. Hybrid carrageenans (K2) have recently gained interest for their intermediate gelling properties. This study aims to develop a sequential extraction (SE) method for red seaweeds (*Chondrus crispus, Mastocarpus stellatus, Gigartina pistillata*), aligning with the concept of extractive extrusion biorefinery. The primary objectives of this study include extracting and chemically characterizing K2-carrageenans, evaluating the impact of prior extractions and time of extraction. Additionally, this work seeks to assess the influence of molecular mass (Mw) reduction on gelling properties and to determine a critical molecular mass (Mc) for gelation. To isolate phycobiliproteins, chlorophyll-a, carotenoids, and carrageenans from the algae, the following sequence was used: cold-water extraction (CWE), ethanolic extraction (EE), hot water extraction (HWE), and hot alkaline extraction (HAE). Each fraction's yield was determined, with carrageenan extracts analysed for Mw, sulphation levels (trough FTIR, ^{1}H-NMR), and their rheological properties in gels and liquids. Molecular mass reduction was achieved through ultrasonication, and the degraded products were characterized in a similar manner as before. Results indicate that SE effectively separates algal components, facilitating the recovery of high-Mw carrageenans, particularly in *M. stellatus* and *G. pistillata*, with superior gel elasticity (G') and viscosity compared to the controls. HWE yields high-Mw carrageenans with enhanced rheological properties, whereas HAE extracts exhibit lower Mw and reduced gelation potential. Prolonged extraction generally improves Mw and G', although *C. crispus* showed a distinct trend. The Mw results highlight that ultrasonication leads to the degradation and desulphation of carrageenans over processing time. Furthermore, the Mc depends on the chemical composition, suggesting an Mc of ~$1{\cdot}10^5$ g/mol for κ-rich hybrids and ~$3{\cdot}10^5$ g /mol for ι-rich hybrids, beyond which further degradation has minimal impact on gelation. In summary, this study demonstrates the potential for adapting extraction protocols to meet industrial demands, contributing to the sustainable valorisation of red algae through biorefinery methods.

Keywords: Hybrid carrageenan; molecular mass; rheology; sequential extraction.

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ACRONYMS

^{1}H-NMR	Proton Nuclear Magnetic Resonance
AFM	Atomic Force Microscopy
ATR	Attenuated Transmission Reflectance
CAGR	Compound Annual Growth Rate
Carot	Carotenoid
Chloro-a	Chlorophyll-a
Cont	Control
CWE	Cold Water Extraction
D-unit	α-D-galactopyranose
d.w.	Dry Weight
DA-units	3,6-anhydrogalactose
E2B2-PHACAR	Extractive Extrusion-Based Biorefinery of polyhydroxyalkanoates and carrageenans
EE	Ethanolic Extraction
FTIR	Fourier Transform Infrared Spectroscopy
G-unit	β-D-galactopyranose
G'	Dynamic/elastic moduli
G''	Loss moduli
G_0	G' at 1 Hz at 25°C of gels
G_1	G' at 1Hz and 25 °C of liquids
γ	Shear strain
HAE	Hot Alkaline Extraction
HWE	Hot Water Extraction
I	Iota (a type of carrageenan)
K	Kappa (a type of carrageenan)
K2	Hybrid K/I (a type of carrageenan)

L	Lambda
MAE	Microwave-Assisted Extraction
Mc	Critical Molecular Mass
Mn	Number-average Molecular Mass
Mu	G4S-D6S
Mw	Molecular Mass
Mwgel	Mw required for gel formation
Nu	G4S-D2S,6S
PDI	Polydispersity Index
PUFAs	Omega-3 and Omega-6 polyunsaturated fatty acids
R-PE	R-phycoerythrin
RTD	Residence Time Distribution
SE	Sequential Extraction
t	Time
T	Temperature
Tg	Gelation Temperature
Tm	Melting Temperature
TON	Onset Temperature
UAE	Ultrasound-Assisted Extraction
wt.%	% Total Weight
η	Apparent Shear Viscosity
ι	G4S-DA2S
κ	G4S-DA
ω	Frequency

LIST DE SYMBOLS

h	Hours
min	Minutes
°C	Degrees Celsius

1. INTRODUCTION

1.1 Context

1.1.1 Carrageenans

Carrageenans are sulphated polysaccharides, present in the cell walls of red algae (*Rhodophyta*) (Abdul et al., 2018; Campo et al., 2009; Shukla et al., 2016). They are commercially important due to their thermo-reversible gelling properties (Hilliou, 2021), and widely used as food additives (E407) for thickening, stabilizing, and gelling, in products like ice cream, sauces, meat, and as a vegan gelatine alternative (Souza et al., 2023; Stephen et al., 2006). The unique characteristics of carrageenans arise from variations in their molecular structure, including different sulphate groups and molecular mass. Understanding these properties is essential for tailoring their application in various fields, especially given the increasing demand for natural and sustainable bio-based products.

1.1.2 Project E2B2 – PHACAR

This study is part of the E2B2-PHACAR project, which aims to develop a novel biorefinery concept called the Extractive Extrusion-Based Biorefinery (E2B2). The E2B2 approach focuses on the continuous and sequential extraction of a range of valuable products, including polyhydroxyalkanoates (PHA), natural compounds, and gelling hybrid carrageenans (CAR). So far, commercial extruders have been employed for pre-treatment of very few biomasses (Vauchel et al., 2008) or for the extraction of single products (Sugiono et al., 2019). The E2B2 project seeks to expand these applications by feeding biomass into an extruder, to extract multiple products in a cascade manner, as seen in Figure 1. Within this framework, fermentation broths of PHA from mixed microbial culture and seaweeds containing hybrid carrageenans are being studied. The research team has confirmed the block copolymer structure of K2-carrageenans (Souza et al., 2023) and identified complex interactions between their chemical composition and the mechanical and structural properties of the resulting gels (Hilliou, 2021). However, the exact mechanism and structural characteristics of K2-carrageenan gels remain unresolved. Addressing these challenges is the second key objective of the E2B2-PHACAR project.

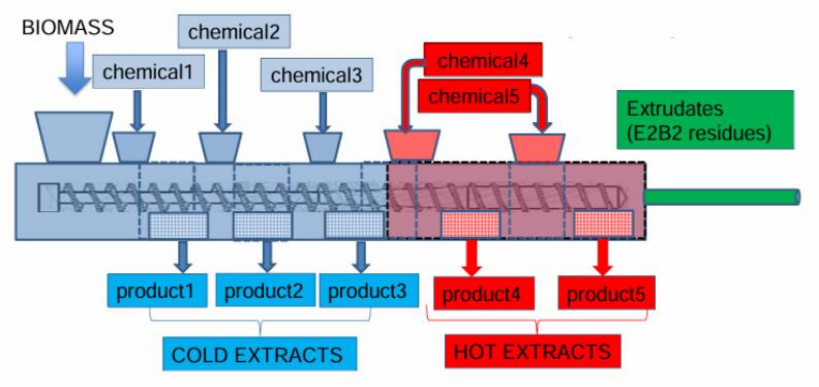


Figure 1. Model of the extractive-extrusion concept of the E2B2-PHACAR project.

1.2 Motivation

1.2.1 Biorefinery concept

Seaweeds possess unique bioactive components not found in terrestrial biomass, positioning them as an exceptional and sustainable resource. Their potential is particularly evident in seaweed-based biorefineries, which align with the principles of circular economy and sustainability by promoting the efficient use of resources, reducing environmental impact, and contributing to job creation and economic growth. This is also in line with key Sustainable Development Goals outlined by the United Nations, such as clean energy, economic growth, resource-use efficiency, responsible consumption and production, and climate action. (Álvarez-Viñas et al., 2019; Balina et al., 2017; Torres, Kraan, et al., 2019).

Sequential extraction as a biorefinery approach holds promise by allowing for the recovery of multiple valuable products. However, as said before, commercial extruders have traditionally been limited to delivering a single extract and an extrudate. Establishing a continuous biorefinery concept capable of refining biomass into a cascade of products directly along the extruder is a goal that remains unexplored (see Figure 1).

In the current state, K2-carrageenans are batch-extracted through a resource-intensive process, involving alkali pre-treatment followed by hot water extraction (Bianchi et al., 2022; Souza et al., 2023). Such methods require substantial chemical inputs and generate significant waste. The E2B2 project addresses these limitations by investigating the feasibility of sequential extraction within an extruder, where seaweed is processed with water and alkali, allowing for multiple products to be recovered with

minimal environmental footprint compared to conventional methods. Although E2B2's application has so far focused on K2-carrageenans, red seaweeds also contain other valuable components, such as proteins and pigments, which could be explored in future developments.

1.2.2 Impact of Mw in the rheological properties

The molecular mass (Mw) of carrageenans plays a pivotal role in determining their gelling properties, including elasticity and mechanical strength (Souza et al, 2023). In the context of the E2B2-PHACAR project, understanding how Mw variations affect the gels mechanical properties is crucial, as the project aims to refine and expand the sequential extraction process, and the extraction conditions directly influence the Mw of carrageenans. However, despite its importance, the literature lacks comprehensive studies specifically on the Mw-dependent gelling behaviour of K2-carrageenans. As part of the second key objective of the E2B2-PHACAR project, this study aims to address these knowledge gaps by exploring the relationship between Mw and gel formation, focusing on K2-carrageenans extracted through this novel biorefinery concept.

1.3 Goals

The primary goal of this thesis is to design a preliminary procedure for the sequential extraction of K2-carrageenans, to be used later with an extruder under the extractive-extrusion technique, in alignment with the objectives of the E2B2-PHACAR project. The aim is to extract various products from algal biomass using different solvents and extraction conditions, and to chemically and rheological characterize the K2-carrageenans obtained, assessing the impact of prior extractions on sequential products. Furthermore, this study seeks to establish relationships between extraction duration and gel elasticity.

The secondary goal of this thesis is to evaluate the effect of molecular mass reduction on the gelling properties of K2-carrageenans, using ultrasonication. Additionally, it seeks to determine whether a critical molecular mass (Mc) for K2-carrageenan gelation exists and, if so, to identify this threshold.

1.4 Structure of the dissertation

This document is organized as follows:

- **2. State of the Art:** This chapter provides a comprehensive overview of the key topics relevant to this dissertation. It begins by discussing the composition of red algae and exploring the different types and chemical structures of carrageenans, with a particular focus on K2-carrageenans. It also covers current extraction methods and the existing knowledge on the influence of molecular mass on gel properties. Additionally, it introduces the concept of sequential extraction and the extractive-extrusion technique, situating these approaches within the broader context of sustainable and efficient biorefinery practices.
- **3. Materials and Methods**. This chapter details the materials and methodologies employed to carry out the proposed tasks in this study. It includes a thorough description of the sequential extraction procedure, and the characterization techniques used to analyse the extracted and ultrasonicated K2-carrageenans.
- **4. Results and Discussion.** This section presents the results obtained throughout the study, accompanied by an in-depth analysis and discussion. It begins with the yield of extracted products, followed by the rheological and chemical properties of the various carrageenan extracts. Then, the next part addresses the impact of ultrasonication on K2-carrageenans, examining molecular mass and rheological behaviour to provide a holistic understanding of the effects of molecular breakdown.
- **5. Conclusions and future work.** This concluding chapter summarizes the key findings of the study, emphasizing the main contributions and insights gained. It also outlines potential avenues for future research, focusing on unresolved challenges and opportunities for advancing the sequential extraction process and optimizing carrageenan gel properties.

2. STATE OF ART

Marine macroalgae, commonly known as seaweed, are complex photosynthetic organisms present in various aquatic environments. They have been common since ancient times for alimentary and medicinal purposes, especially in Asian countries (Barbot et al., 2016). Advancements in scientific research and novel methodologies have facilitated the identification of compounds derived from seaweed. These compounds have been extensively studied for their potential therapeutic effects on the human body, including their antioxidant, antimicrobial, and antitumoral properties (Lomartire et al., 2021). Moreover, seaweeds might be also employed in several industrial applications, such as the production of fertilizers (Balina et al., 2017; Torres, Kraan, et al., 2019), aquaculture feed, biofuels, in wastewater treatments or to produce innovative, and ecologic materials to replace plastic equivalents (Kim et al., 2017; Raikova et al., 2019), contributing towards a sustainable solution to protect the environment from the discharge of non-biodegradable plastic. Also, marine macroalgae present economic and environmental advantages over terrestrial biomass, including rapid growth rate, no competition for agricultural land and the depolymerisation of seaweeds before the biological conversion into biofuels is facilitated due to the high carbohydrate and low lignin content, among other advantages (Álvarez-Viñas et al., 2019). So, seaweeds are an interesting option for the biorefinery concept.

Seaweeds are classified into three main groups according to their colour, which is determined by the presence of certain pigments. Brown seaweeds (phylum *Ochrophyta*, class *Phaeophyceae*) have an abundance of pigments that vary from yellow to dark brown (Seely et al., 1972). Red seaweeds (phylum *Rhodophyta*) are characterized by their high amounts of carotenoids, chlorophyll-a, phycoerythrin, phycocyanin, and allophycocyanin (Denis et al., 2009). Green seaweeds (phylum *Chlorophyta*) possess mainly the pigment chlorophyll, which has a crucial role in photosynthesis and gives the characteristic green colour to plants, algae, and cyanobacteria (Aryee et al., 2018).

2.1 Red Algae

Red seaweeds are of economic importance, they represent 61 % of global seaweed production (Penuela et al., 2018), and are primarily used for extracting agar and carrageenan. Moreover, they also contain other compounds of interest, in amounts varying qualitatively and quantitatively among different

species, especially since the extraction method is not generally standardized and for the fact that various abiotic and biotic factors, such as the growth stage, harvest location and period, depth, nutrient quantity and quality, temperature, salinity, light exposure, may also differ (Generalić Mekinić et al., 2023).

To provide a comprehensive understanding of the red seaweed, the following section will first explore the general composition of red algae, before focusing on specific compounds relevant to this study (pigments and carrageenans). These compounds will be studied in three distinct red algal species: *Chondrus crispus*, *Gigartina pistillata*, and *Mastocarpus stellatus.* Providing a valuable opportunity to explore their potential within the framework of a biorefinery approach.

2.1.1 Composition

It is fundamental to have some knowledge about the composition of the red algae and their most studied biological properties, to pave the way for the utilization of this resource. So, in the next lines the general composition of the red algae is discussed.

Carbohydrates

According to their prevalence in algal sources, agar and carrageenan, both sulphated polysaccharides, known as phycocolloids, are the most relevant carbohydrates in red seaweeds, accounting for up to 40–50 % of their dry weight (d.w.) (Torres, Flórez-Fernández, et al., 2019) followed by other polysaccharides found in significantly lower amounts, such as xylans, sulphated galactans and porphyrans (Øverland et al., 2019).

Proteins

Among the different algae classes found in marine ecosystems, red seaweeds exhibit the highest content of proteins (Belghit et al., 2017; Øverland et al., 2019). Generally, the protein content of algae ranges between 5 % and 20 %, although red algae may achieve greater proportions, with maximum values reaching 47 % of total d.w. (Cian et al., 2012; Praveen et al., 2019; Rudtanatip et al., 2018). Nevertheless, proteins depict a species-dependent occurrence, considering that some species, such as those from *Gracilaria* genus present a low protein content (below 5 % d.w.) whereas others like *Pyropia tenera* show a protein content of 37 % d.w. (Holdt & Kraan, 2011). Furthermore, in terms of total proteins, phycobiliproteins are the predominant proteins in red seaweeds, constituting up to 50 % of the total

protein content and contributing to the reddish coloration characteristic of these algae (Niu et al., 2010). Regarding the amino acid composition of red algae proteins, a high concentration of essential amino acids has been documented, with aspartic acid and glutamic acid being the most abundant residues, comprising 22–44 % of the total amino acids found in red algae proteins (Cian et al., 2012).

Lipids

Despite low lipid content, red algae are rich in nutritionally important PUFAs (Omega-3 and Omega-6 polyunsaturated fatty acids). Their omega-3:omega-6 ratio is higher than in terrestrial sources and could be used as nutraceuticals for their anti-hypercholesterolemic, antioxidant, anticancer, antidiabetic, antihypertensive, and anti-inflammatory activities (Chan & Matanjun, 2017; Kumari et al., 2013).

Vitamins and Minerals

Rhodophyceae species contain important macrominerals, like potassium, sodium, calcium and magnesium (6,1–21,9 g K/100 g d.w., 1,8–8,1 g Na/100 g, 0,2–0,9 g Ca/100 g d.w. and 0,2–0,5 g Mg/100 g d.w.) (Baghel et al., 2014; Kumar et al., 2011; Matanjun et al., 2009). In the group of trace minerals, iron was found to be the most abundant, followed by zinc, copper, and selenium. They could be potential ingredients for functional foods, providing a supplementation in deficitary elements in diets (Nunes et al., 2018). Red seaweeds also contain vitamins, with 2,1–2,7 mg pro-vitamin A/g, 0,05–1,54 mg vitamin B2/g, 3,8–4,8 mg vitamin B6/g, 0,4–1,0 mg vitamin B9/g, 2,5–501 mg vitamin C/ 100 d.w., 1,34 mg vitamin E/g d.w. and 4,61 µg α-tocopherol/g d.w.(Álvarez-Viñas et al., 2019; Chan & Matanjun, 2017). Indeed, vitamins from marine sources have been already used for the enrichment of functional foods (Šimat et al., 2020).

Phenolic Compounds

Red seaweeds contain a variety of phenolic compounds, including natural phenolics like phenolic acids and flavonoids, as well as specific marine-derived compounds, such as phlorotannin and bromophenol. These compounds are known for their powerful antioxidant properties. Thus, phenolic acids reported in red algae are coumaric acid, caffeic acid, salicylic acid, hypogallic acid, and chlorogenic acid (Carpena et al., 2023).

The composition of the compounds found in the red algae selected in this study (*Mastocarpus stellatus*, *Gigartina pistillata*, and *Chondrus crispus*) is detailed in Table 1.

Table 1. Average composition of widely used red seaweeds (*Mastocarpus stellatus*, *Gigartina pistillata* and *Chondrus crispus)*, expressed in % d.w. (with 5,3–12,1% moisture)

Red algae	*Mastocarpus stellatus*	*Gigartina pistillata*	*Chondrus crispus*	Reference(s)
Carbohydrates	60,43±0,59	53,72±0,75	53,43±0,01	(Carpena et al., 2021)
Protein	18,62±0,04	14,35±0,26	17,00±0,26	
Lipids	0,14±0,03	0,11±0,04	0,12±0,01	
Phenolics[a]	1,218	0,911	1,036	
Minerals	20,25±4,65	34,56	21,3±1,5	(Álvarez-Viñas et al., 2019)

Note: [a], gallic acid equivalents % d.w.

2.1.2 Pigments of Red Algae

Red seaweeds synthesise pigments compounds (i.e., chlorophyll-a, phycobiliproteins and carotenoids) and mycosporine-like amino acids, for their photo-protection against UV (Álvarez-Viñas et al., 2019). These pigments have garnered significant interest in the food and beverages industries, as well as in animal feed, cosmetics, and pharmaceutical products. The food colours market was valued at USD 3,02 billion in 2022 and it is estimated to reach USD 8,32 billion by 2031, growing at a CAGR (Compound Annual Growth Rate) of 11,9 % during the forecast period (2023–2031), and natural food colours will form the greatest share of this projection (*Food Colors Market Size, Share & Trends Analysis Report*, 2023). The increasing popularity of natural food colours, particularly those with added health benefits, is driven by consumer demand for food additives that are safe, non-synthetic, and available in a wide range of colours/shades. Among the natural sources, red seaweeds stand out for their potential to provide

valuable pigments and, meeting this demand requires the design of bioprocesses that can efficiently extract and purify these pigments and formulate them into forms that are stable and convenient. The most key value pigments of red seaweeds are the following:

i) **Phycobiliprotein** is a water soluble and fluorescence protein (Ghosh et al., 2021) present in red macroalgae in the range of <1–125 mg/g d.w. (Bahari, Moelants, Kloeck, et al., 2021), being R-phycoerythrin (R-PE) the major phycobiliprotein in marine red algae. Contents in the range 0,8–7 mg R-PE/g d.w. have been reported. They have a great economic potential and can be used in food as natural colourants or in cosmetics, and pharmaceutical industries, as antioxidants and antibacterial agents (Stadnichuk & Tropin, 2017). Studies also shown that they can enhance the activity of conventional anticancer medicines (Gantar et al., 2012).

ii) **Chlorophyll-a** is the principal type of chlorophyll in red seaweed. It has been reported in the range 0,35–9,8 mg/g d.w. (Álvarez-Viñas et al., 2019). These greenish pigments can be found in applications in pharmaceutical, cosmetic and food products for their antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic properties, as well as colorants (Jorge et al., 2024). Recent reports suggest that chlorophyll can also be used as a bio-mordant to enhance the dyeing process of textile products, but also as a textile dye with antimicrobial properties (Guesmi et al., 2013).

iii) **Carotenoids** in red seaweeds account for 0,2–1,8 mg/g d.w. (Chan & Matanjun, 2017), the major being α- and β-carotene, zeaxanthin, lutein and antheraxanthin (Shahidi et al., 1998). Carotenoids are natural antioxidants that help reduce obesity, inflammation and oxidative stress, potentially preventing metabolic diseases, as diabetes and hypertension (Ojulari et al., 2020). They have shown potential in food packaging film formulation, enhancing nutrient content and prolonging shelf life, and in food packaging to prevent oxidation and preserve quality (Andrade et al., 2021). Additionally, carotenoids are used in cosmetics to protect against UV radiation, delay skin aging, and improve skin health through anti-inflammatory and antioxidative effects (Davinelli et al., 2018). Currently, commercially available carotenoids are usually produced by chemical synthesis, which involves high economic costs and has a negative impact on the environment (Generalić Mekinić et al., 2023).

Table 2 gives a more detailed version of the quantification of these pigments (phycoerythrin, chlorophyll-a, carotenoids) on the raw material to be studied. It should be noted that these values are

highly variable depending on the collection area, the harvesting season and the method of extraction employed.

Table 2. Average composition of pigments: phycoerythrin, chlorophyll-a and carotenoids, in *Mastocarpus stellatus*, *Gigartina pistillata* and *Chondrus crispus, with extraction method used*

Red algae	*Mastocarpus stellatus*	*Gigartina pistillata*	*Chondrus crispus*	Extraction method
Phycoerythrin (mg/ g dw)	1,99 [1]	n.d.	0,53 [2]	1) Phosphate buffer, maceration, liquid nitrogen grinding, and xylanase-assisted hydrolysis under optimized conditions (12 °C, pH 6.45, xylanase/dry seaweed ratio 13.18 mg/g, 6 h in the dark). (Nguyen et al., 2017) 2) Grinding 100 mg of sample with phosphate buffer, vortexing for 15 min, extracting at 4 °C for 24 h (Pina et al., 2014)
Chlorophyll-a (µg/g dw)	618,94	402,43	136,08	Pigments were extracted using ethanol/water, stirred at 50 °C, re-extracted, centrifuged, evaporated, and reconstituted before filtration and analysis. (Carpena et al., 2021)
Carotenoids (mg/g dw)[a]	1,71	0,38	2,152	

Note: [a], µg β-carotene/g raw seaweed dry weight; n.d., no data.

2.2 Carrageenan

As previously mentioned, carrageenan can constitute nearly half of the composition of red algae, as seen in Table 3. Its economic and scientific value is substantial, making it a highly sought-after compound. The following sections will explore its various aspects, emphasizing its importance in both commercial applications and scientific research.

Table 3. Ranges of carrageenan (% d.w.) in *Mastocarpus stellatus, Gigartina pistillata* and *Chondrus crispus* from: (Pereira, 2013), with extraction method used

Red algae	*Mastocarpus stellatus*	*Chondrus crispus*	*Gigartina pistillata*	*Extraction Method*
Carrageenan (% d.w.)	39,2-43,0	34,2-60,8	54,2-61,1	The seaweed samples were placed in distilled water (50mL/g), pH 7 at 85 °C for 3h. For an alkaline extraction, the samples were placed in a solution (150mL/g) of NaOH (1M) at 80-85 °C for 3- 4 h and neutralised to pH 6–8 with HCl (0,3M).

2.2.1 Relevance

The commercial relevance of carrageenans is largely due to their thermo-reversible gelling capabilities (Hilliou, 2021). Carrageenans are widely used in the food and dairy industries, mainly as food additives (E407), for texturizing formulations due to their thickening, stabilizing and gelling properties (Souza et al., 2023; Stephen et al., 2006). These are used in the production of, for example, ice cream, sauces, meat products, as a vegan alternative to animal gelatine, among others (Souza et al., 2023; Stephen et al., 2006). Recently, carrageenans have entered the cosmetic and pharmaceutical markets, as excipients for capsules and tablets, for cell immobilization in the production of medicines, and as thickeners for toothpaste and cosmetics (Abdul et al., 2018; Souza et al., 2023). Due to their antiviral (e.g. as an inhibitor of the herpes virus), antioxidant, antitumor, immunomodulatory, antihyperlipidemic

and anticoagulant activities, carrageenans are of great interest to the pharmaceutical industry (Abdul et al., 2018; Shukla et al., 2016). In addition, these polysaccharides also have good application prospects in industrial suspensions and paints (Stephen et al., 2006).

The carrageenan industry is projected to grow from USD 0,952 Billion in 2024 to USD 1,494 billion by 2032, exhibiting a CAGR of 5,80 % during the forecast period (2024 - 2032). The increased use of carrageenan in supplements and various industries, and its application in pharmaceuticals are the key market drivers (Gorade, 2024).

2.2.2 Chemical structure of carrageenans

Carrageenans are sulphated polysaccharides present in the cell wall of red algae (*Rhodophyta*) (Abdul et al., 2018; Campo et al., 2009; Shukla et al., 2016). One of the most prominent characteristics of carrageenans is perhaps their diversity, depending on the origin of the algae and the method of preparation, non-gelling or gelling samples can be obtained, the latter having a wide variety of properties. The variations in carrageenan types stem primarily from differences in their chemical structure, particularly the number and position of sulphate groups in their repeating units (Stephen et al., 2006).

Carrageenans are traditionally categorized into eight types: (I)-, (K)-, (L)-, (μ)-, (υ)-, (θ)-, (ß)- and (ξ)-carrageenans (Campo et al., 2009; Hilliou, 2021). This classification is largely structural, as the presence of specific chemical substitutions results in distinct physicochemical properties that contribute to the differential features and applicability, associated with their derivative products (Carpena et al., 2023). Furthermore, specific distributions of carrageenans have been attributed to individual algal species. For instance, *C. crispus* presents a mixture of both K- and L-carrageenans that cannot be separated during their large-scale extraction procedure. Indeed, to produce individual compounds, different algal sources are employed. For example, K-carrageenan is usually extracted from *Kappaphycus alvarezii*, whereas L-carrageenan is isolated from different species from the genus *Gigartina* (Cunha & Grenha, 2016; Torres, Flórez-Fernández, et al., 2019).

The most commercially used are: i) K-carrageenan, which forms strong gels; ii) I-carrageenan, that forms weaker gels, and iii) L-carrageenan, which produces viscous solutions (Souza et al., 2023). In the last 20 years, another class has emerged - hybrid carrageenans - also known as kappa-2, K2, or weak K, that have gained great scientific attention and a progressively greater industrial impulse. K2 replaces mixtures of K+I in niche applications, such as dairy foods, where intermediate gelling properties are

required (Hilliou, 2021). Thus, its use avoids an additional, costly, mixing process after extraction of the K- and I-carrageenan mixtures (Souza et al., 2023).

Carrageenans are linear, partially hydrophilic, sulphated galactans formed by repeating disaccharide units (diads) of β-D-galactopyranose (labelled as **G**-units) and α-D-galactopyranose (**D**-units) or 3,6-anhydrogalactose (**DA**-units). The diads are linked together by the carbons 3 of **G,** and 4 of **D** (or **DA**). The letter codes assigned to the residues correspond to the simplified denomination developed by Knutsen et al., (1994), sulphated galactans are classified based on the presence of the 3,6-anhydro-bridge on the 4-linked galactose residue, as well as the position and number of sulphate groups. For example, I-carrageenan is made, essentially, of diads of β-D-galactopyranose sulphated at the fourth carbon (**G4S**) (linked by carbon 3 to the previous diad) and of 3,6-anhydrogalactose-D-galactopyranose sulphated at the second carbon (**DA2S**) (linked to the following diad by carbon 1). Thus, the I-carrageenan diad **G4S-DA2S** is annotated as ι and, **G4S-DA** as κ, and **G4S-D2S,6S** and **G4S-D6S**, as its biological precursors nu and mu, respectively, sulphated at the sixth carbon (Souza et al., 2023). The **G4S** group is common to all these disaccharides (κ-, ι-, nu-, and mu-carrageenans) and, therefore, characterizes this family of K-carrageenans (Campo et al., 2009).The ideal chemical structures of the repeating disaccharide units found in gelling carrageenans and its nomenclature, are given in Figure 2.

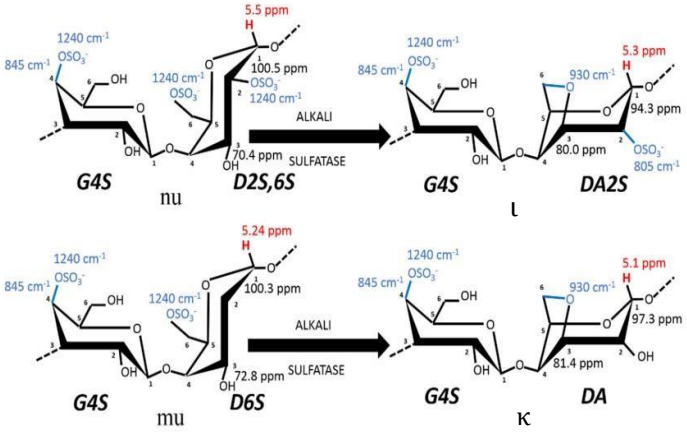


Figure 2. Chemical structure of nu-, ι-, mu-, κ-carrageenans. The blue numbers refer to the FTIR (Fourier Transform Infrared Spectroscopy) absorption bands, which characterize the corresponding chemical bonds highlighted, also in blue. While the red numbers correspond to the NMR (Nuclear magnetic resonance) chemical shifts of the corresponding anomeric proton. The numbers close to C1 and C3 correspond to the C-13 NMR chemical shifts of the corresponding carbon. The transformation of (**D**) to (**DA**) can be carried out in industry by alkaline (ALKALI) treatment or occurs enzymatically (SULFATSE) in vivo, to transform the biological precursors nu- and mu-

carrageenan disaccharides into less sulphated carrageenans (Souza et al., 2023). Figure retrieved from: Souza et al., (2023).

I-carrageenan is essentially a homopolymer of **G4S-DA2S** disaccharide units, containing a very low amount, of the order of 5 mol %, of **G4S-DA** disaccharide units. K-carrageenan is somewhat more heterogeneous, although still practically a homopolymer, with up to 10 mol % of **G4S-DA2S** disaccharide units, discontinuous with blocks of disaccharide units, **G4S-DA** (Hilliou, 2021; Souza et al., 2023). More simply, these carrageenans consist of polysaccharide chains containing both κ and ι units, as represented in Figure 3, ranging from nearly pure I-carrageenan to nearly pure K-carrageenan (Campo et al., 2009). Within the more heterogeneous carrageenans, the hybrids, K2-carrageenan has a polymeric structure (van de Velde, 2008), with sequences of **G4S-DA** (which make up 45-80 mol % of the hybrid chain) and **G4S-DA2S** (which represent 20-50 mol % of the chain) (Hilliou, 2021), hence its definition in the industry as kappa-2 or weak kappa (van de Velde, 2008). So, hybrid K2-carrageenan are copolymers made of blocks of κ and ι with various lengths and random distribution in the chain (Guibet et al., 2008). The block size along the K2 chain, varies from chain to chain, and its distribution in the chain is random (statistic copolymer) and specific to the seaweed biology that produces it, as well as the extraction process. So, K2-carrageenans are statistic block copolymers, with polydispersity both in the chain and in block size (Campo et al., 2009; Souza et al., 2023).

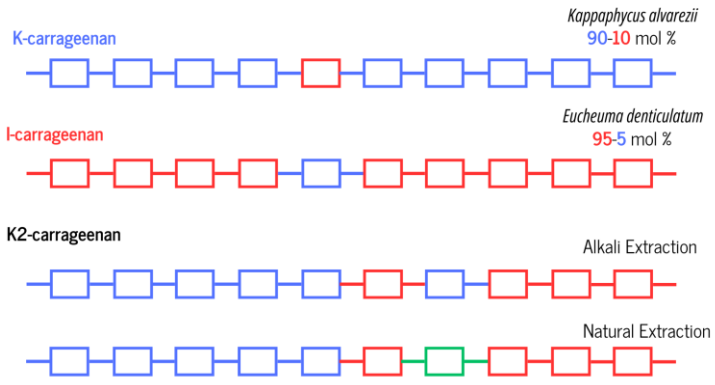


Figure 3. Schematic representation of the structure of carrageenans (K, I and hybrids). The κ-units are represented in blue, the ι-units in red and the biological precursors (nu/mu) in green.

2.2.3 Extraction

Carrageenans are traditionally extracted from *C. crispus* wild populations in Canada, Ireland, Portugal, Spain and France, and from *Gigartina* collected in South America and Southern Europe. However, the growing demands of carrageenans motivated the establishment of macroalgae farming systems, with *Eucheuma sp*. in Philippines (Carpena et al., 2023; Hedberg et al., 2018) becoming the major producer worldwide and spreading macroalgae cultivation along other Asian countries, promoting the production of *Porphyra sp.* (nori), *Kappaphycus alvarezii* (rich in K) and *Eucheuma denticulatum* (rich in I) (Carpena et al., 2023).

The current extraction processes are slow and complicated, involving several steps. Furthermore, it's quite difficult to optimize and control the production of carrageenan, due to its unique intrinsic properties, which are highly dependent on the origin of the seaweed, the geographical location and the harvesting season (Abdul et al., 2018). Normally, carrageenan industrial extraction processes are divided into drying and milling, alkaline pre-treatment, hot water extraction, precipitation and drying (Souza et al, 2023).

Native extracts, without alkaline treatment, generally exhibit poor or absent gelling properties. In contrast, an alkaline treatment at higher concentrations and extended durations results in carrageenan with significantly enhanced gel quality (Azevedo et al., 2015). So, an alkaline treatment is usually performed prior to extraction, but can also be done after, to reduce the number of sulphated groups (or precursors) in K2-carrageenan. This alkaline process can be adjusted to each seaweed (Abdul et al., 2018; Souza et al., 2023). Azevedo et al., (2015) when studying the extraction of K2-carrageenan from two algae, *C. crispus* and *Ahnfeltiopsis devoniensis*, observed that excessive KOH concentration promotes the depolymerization of K2 and, consequently, the elasticity of the gel is reduced (see section 2.2.5). The previous study also perceives that after three hours of the alkaline pre-treatment and one hour of natural extraction at 90 °C, yields for *C. crispus* achieved with NaOH are larger than those achieved with KOH (45-70 % against 35-60 %), whereas a reverse trend is observed for *A. devoniensis* (10-20% against 15-30%) (Azevedo et al., 2015). Using the same protocol Azevedo et al. (2013) shown that for *M. stellatus* yields obtained with NaOH were larger than those achieved with KOH (40 - 50% against 30 - 43%), in agreement with results obtained with *C. crispus*. So, for this two algae NaOH requires lower concentrations and shorter pre-treatment durations compared to KOH to isolate K2 with optimal gel properties, indicating that NaOH is more effective in converting the precursors into κ and ι-carrageenan (Azevedo et al., 2015).

The K2 extraction process usually begins by dispersing the algae in hot water (Abdul et al., 2018) It has recently been demonstrated that the solubility of hybrid carrageenans in water is strongly dependent

on the type of cations present in the algae after being harvested, the time of contact with the water and the temperature (T) used during extraction (Azevedo et al., 2015). In order to achieve optimal yields, industrial carrageenan extractions are known to be performed at 80 °C for 6 h. Additionally, it has been reported that the composition and rheological behaviour of carrageenan are different when extracted at different temperatures, for example between 60 °C and 90 °C (Bahari, Moelants, Wallecan, et al., 2021). As a rule, a higher T produces a less sulphated K2, however, there are exceptions, e.g. for *Gymnogongrus tenuis*, a less sulphated carrageenan was obtained in cold water than in water at 80 °C (Perez Recalde et al., 2016)

After extraction, the solid residues (salts formed from cations of the alkaline treatment and from the seaweeds) are separated from the K2-rich solution by filtration or centrifugation. K2 is then recovered from the solution, usually by precipitation with alcohol or with KCl (Abdul et al., 2018). Alternatively, K2-carrageenan not subjected to alkaline treatment can be recovered in film or powder form by evaporating water from solutions with high K2 concentrations or by freeze-drying. However, this method is suitable only for K2 with high κ content and sufficient salts to form a strong gel. Finally, purification of K2 can be achieved using enzymatic treatments, among other methods (Souza et al., 2023).

2.2.4 Gel Properties

The capacity to form gels is unique to carrageenans that have the ability to form ordered helical structures, being gelation one of the several possible consequences of helix formation (Stephen et al., 2006).

Gel formation mechanism

The mechanism of gel formation involves the coil-to-helix transition, obtained by cooling hot carrageenan aqueous solutions, followed by the aggregation of carrageenan helices, resulting in a three-dimensional network. This mechanism is widely recognized in literature, but this was recently challenged by Westberry et al., (2024) who proposed that the gel formation process is more intricate, involving cooperative interactions and the presence of pre-existing helical structures in the disordered state. This new understanding could prompt a reconsideration of the mechanisms behind carrageenan gelation and related polysaccharides. As for the structure of the network that permeates the volume and provides elasticity is a topic on which there is little agreement among authors. There are studies that report the

formation of single helices and others that support the formation of the double helix (Hilliou, 2021). Thus, several models have been proposed trying to describe the structure of the network. Recently, it was possible to visualize by atomic force microscopy (AFM) the primary (coils), secondary (helices) and super helical structures of solutions and gels. For K gels, rod-shaped helices were visualized, while more curved structures were reported for, I gels (Hilliou, 2021). Furthermore, in the presence of K^+, it was possible to observe quaternary structures of stacked helices linked by helical branching to other domains in K gels. However, for I gels, independently of the type of salt present, the single helix mesh was always observed. Also, the formation of networks at the super helical level, for K gels results in hard gels, in contrast to I gels, which are softer (Hilliou, 2021; Souza et al., 2023; Stephen et al., 2006).

The study of K2 gels is still under way. However, the few publications with AFM images suggest that the helices and structure of K2 are similar to those of K, with slightly more branching and a morphology less similar to a "rod". According to Souza et al. (2023) the mechanism of formation of K2 gel resembles that of K+I mixtures, essentially with coil-helix transitions, independent of each type of block and its aggregation. Nevertheless, it's indicated that these similarities do not help in the identification of the nature of the aggregation/structuring and even less in understanding the origin of the elasticity of K2 gels (Souza et al. 2023). As K2 gels are different from K+I mixtures, even with a similar G4S-DA (κ) molar content, under similar gelling conditions (total carrageenan concentration and ionic strength), in K+I mixtures, I can be separated from K under specific saline conditions, whereas in K2 this separation is not possible. K2 is capable of adopting helical conformations independently of its κ molar fraction, but there is still a long way to go until a complete understanding of the interaction between such macromolecular structures and the properties of the K2 gel (Souza et al., 2023; Stephen et al., 2006).

Additionally, there is no water syneresis in K2 gels, at least in the literature reviewed so far. Thus, the studied K2 gels may be a valuable alternative to the reported mixtures of commercial K and I gels, where water syneresis occurs during gel formation for systems with ionic strengths above 0,1 M. Namely, in applications where an ionic strength as high as 1 M is required, K2 will successfully replace commercial K+I mixtures as syneresis-free gels with similar elasticity are formed (Torres, Azevedo, et al., 2016).

Presence of salts

Since carrageenans are polyelectrolytes, they are highly sensitive to the composition of the salts present in the aqueous medium (Stephen et al., 2006). So, it is known that it is possible to adjust some gel properties, such as elasticity, yield stress and thermal stability, by controlling the amount and type of

salt added (Stephen et al., 2006). K-carrageenan is cation-specific, meaning its gel stiffness and brittleness depend on the presence of specific cations, such as K^+ or Ca^{2+}, which stabilize its helical structure (Hilliou et al., 2006). In solutions with KCl or $CaCl_2$, K-carrageenan forms strong, stiff, and brittle gels, whereas I-carrageenan, which lacks this specificity, produces much weaker gels regardless of the cation. Thus, the contribution from ι blocks to the gel structure and elasticity in K2 is actually masked by the contributions from κ blocks, when in the presence of the previous cations (K^+/Ca^{2+}). However, in the presence of NaCl, K forms weaker gels, and thus it is easier to access its contribution in K2 gels. As expected, similar results were obtained for the K2 gel, in which the weakest (and most stable) gels were obtained with NaCl, and the strongest for $CaCl_2$. When no salt is used, K2 can show a gel behaviour, but large concentrations and low temperatures are necessary (Torres et al., 2018).

Rheology

Studies of rheological behaviours, which focus on the dynamic storage/elastic and shear loss moduli (G' and G'', respectively) and the yield stress, are relevant for the industrial applications of carrageenans, but also to better understand the gelation mechanism (Souza et al., 2023). According to Van de Velde (2008), in a mixed K^+/Ca^{2+} solution, the gel elastic modulus (G') gradually decreased with the decreasing fraction of κ units, indicating that G' for K2 gels does not reach a maximum at any specific K^+ concentration. This reflects the intermediate nature of K2 hybrids, characterized by a continuous transition in gel stiffness rather than a distinct peak. However, the gelation temperature (Tg) of K2 hybrids with more than 50 mol% κ units remains independent of composition in the presence of KCl but becomes highly sensitive in NaCl solutions, increasing with higher κ content and decreasing with lower κ content (Souza et al., 2023). Additionally, there is also a strong dependence of the gel's melting temperature (Tm) on both the concentrations of K-carrageenan in K2 and NaCl (Torres, Azevedo, et al., 2016; Torres, Chenlo, et al., 2016).

2.2.5 Molecular Mass, Mw

The molecular mass (Mw) of K2 carrageenans is a complex characteristic influenced by extraction methods, temperature and the specific chemical structure of the carrageenan itself. The effect of molecular mass on the yield stress and G' of the gel was determined, for K-carrageenan, with different values of polymer concentrations and salt concentrations by Rochas et al., (1990), that concluded that

the yield stress is directly proportional to the molecular mass, but not very sensitive to the salt concentration. In contrast, the G' increased steadily with Mw, up to a critical value (Mc) of $1{,}8 \cdot 10^5$ g/mol, independently of the polymer concentration and ionic content. Beyond this limit, the G' remained constant and independent of the Mw, but very sensitive to the salt concentration (Rochas et al., 1990), as seen in Figure 4. For K2-carrageenan, there is still insufficient information to understand the effect of varying Mw and whether Mc depends on the chemical composition in κ and ι of K2.

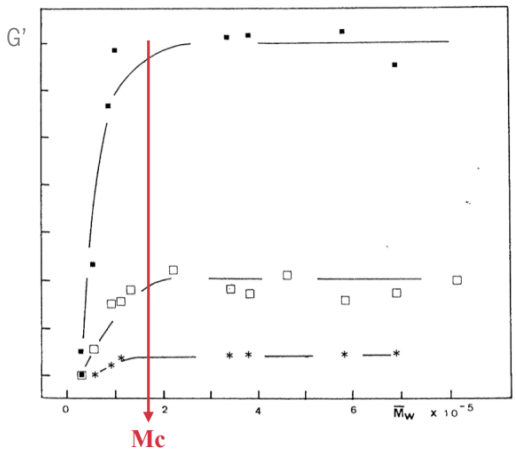


Figure 4. Illustration of the variation of the elastic modulus (G') with molecular mass (Mw) for K-carrageenan. It stabilizes from Mc, regardless of the 3 different polymer concentrations (5, 10, 20 g/L). Adapted from: Rochas et al., (1990).

Impact of Extraction Parameters

The molecular mass of isolated K2 samples is significantly influenced by the milling process prior to biopolymer extraction. Grinding seaweeds into smaller particles increases the yield of K2-carrageenans during water extraction, resulting in compounds with higher molecular masses. Consequently, larger molecular masses are obtained from powders with smaller particle sizes, while smaller molecular masses are associated with larger particle sizes. Furthermore, the polydispersity index (PDI), represented by the Mw/Mn ratio (where Mn is the number-average molecular weight), tends to increase with Mw (Torres et al., 2016). In the previous reference it was observed that differences in K2 molecular mass were likely due to larger K2 molecules experiencing greater diffusion resistance through the seaweed particles during extraction. In contrast, powders with smaller particle sizes resulted in a higher proportion of large K2

molecules, likely due to increased surface area and enhanced accessibility of precursors to the solvent. These findings suggest that particle size distribution and diffusion characteristics significantly impact the yield and molecular composition of the extracted fractions (Torres, Chenlo, et al., 2016).

Molecular mass is also highly influenced by the extraction parameters used during the recovery of carrageenan. It is well known that stronger and longer alkaline pre-treatments tend to reduce the chain size of K2, resulting in a lower Mw (Souza et al., 2023; Van De Velde et al., 2005). On K2-carrageenans studies showed that NaOH is faster than KOH in converting the biological precursors (mu e nu) into the disaccharide units κ and ι (Azevedo et al., 2013, 2015). So, this efficiency is reflected in the Mw of the resulting K2, as shorter alkaline treatment times are required to achieve the desired levels of sulphated disaccharide units (Velde et al., 2005; Souza et al., 2023).

The temperature variations can also lead to changes in Mw, resulting in different types of gels. During the drying step, in the case of *M. stellatus*, higher drying temperatures led to a lower Mw of K2-carrageenan (Moreira et al., 2016). But for K2 extracted, from *Gymnogongrus tenuis,* using cold water, in the extraction step, resulted in a different Mw profile compared to a more sulphated hybrid carrageenan recovered at 80 °C, which does not qualify as K2-carrageenan. Although, higher temperatures generally increase yield, excessive heat can lead to hydrolysis, causing degradation. Therefore, it is important to carefully control the extraction temperature to prevent excessive breakdown of the carrageenan (Bahari, Moelants, Wallecan, et al., 2021; Moreira et al., 2016; Souza et al., 2023).

Carrageenan hydrolysis can also occur in low pH conditions (Gustaw & Mleko, 2003) or under high salinity conditions, such as in the presence of NaCl or KCl. High salt concentrations can disrupt the intermolecular interactions that maintain carrageenan structure, making it more susceptible to hydrolysis, particularly when combined with high temperatures or acidic conditions. However, studies on the effects of salts on carrageenan extraction remain surprisingly limited (Bahari, Moelants, Wallecan, et al., 2021).

Toxicity

Understanding the Mw of K2-carrageenans is essential for its industrial applications, particularly in food and cosmetic industries where textural properties are critical. The ability to manipulate Mw through extraction methods can lead to tailored products that meet specific consumer needs, with the desired textures and stability in products. For example, food-grade carrageenan typically has a molecular mass greater than 100 kDa, with commercial products ranging from 200–800 kDa (Torres, Flórez-Fernández, et al., 2019). Notably, carrageenan is neither degraded nor absorbed in the gastrointestinal tract, but it

should not contain low molecular mass fractions, as these can have toxic effects, including gastrointestinal irritation and potential carcinogenic properties at high doses (Torres, Kraan, et al., 2019; Weiner, 2016). Further studies are necessary to explore its impact on digestive processes, the colon microbiome, and inflammation, especially in predisposed populations (Wu et al., 2016). To despite the previews claims in a review article, Bixler, (2017) emphasize that carrageenan is approved for food use by major regulatory agencies worldwide, including the example of its recent inclusion in liquid infant formula, that highlights its safety in sensitive applications, and that there is no *in vivo* evidence to support the previous claims. Thus, while regulatory bodies affirm its safety, public scepticism fuelled by misinformation remains a significant hurdle for the industry.

In resume, there is a recognized need for more studies focusing on the optimization of extraction parameters to better understand how they affect the Mw and chemical structures of K2-carrageenans. In particular, there is no published results (such as those illustrated in Figure 4) on the effects of Mw on the gel properties of K2-carrageenans, and this could lead to improved applications and functionalities of K2-carrageenans in various industrial contexts.

2.3 Sequential Extraction

The current industrial extraction routes in the carrageenan sector have several weaknesses, primarily due to their high consumption of time, energy and water. Additionally, large amounts of chemical reagents and solvents are required to achieve optimal yield and quality. The pollutants and effluents generated from chemicals used during extraction and alkaline treatment, such as KCl, KOH, and NaOCl, pose significant risks to health and the environment, necessitating strict control measures (Abdul et al., 2018). In addition, the alkaline treatment step, in particular, is a relatively harsh and destructive process for the algae, causing the breakdown of the tissue matrix and the efficient solubilization of nearly all the carrageenan. The residual algal biomass, still rich in organic components, is generally unrecoverable and wasted after this step. So, shortening the time of the alkaline step, while still producing carrageenans of high rheological quality would undoubtedly be of interest to the industry (Vauchel et al., 2008). Consequently, greener extraction methods that use less energy, reduce water consumption, and employ more environmentally friendly solvents are emerging. Microwave-assisted extraction (MAE) (Álvarez-Viñas et al., 2023) and Ultrasound-Assisted Extraction (UAE) (Maia et al., 2023) have shown the best results in terms of extraction yields and K2 gel properties, with a shorter extraction time and ecological impact (Kes

et al., 2017). However, MAE and UAE are difficult to apply in industries and expensive (Sugiono et al., 2019).

Another hypothesis involves using a twin-screw extruder-assisted extraction, which offers several advantages: it requires shorter processing times, reduces the amount of solvent and reactants needed, generates minimal waste, and is safe and suitable for industrial applications (Vauchel et al., 2008). Additionally, the current batch industrial process for carrageenan extraction is limited to producing only one polymer per cycle, leaving many valuable compounds unrecovered and wasted, so the full potential of the algal biomass is not being utilized. The global carrageenan industries process 202 500 dry tons of carragenophytes annually to produce 65 000 tons of carrageenan (Penuela et al., 2018) and the remainder is lost as waste. With the increase of carrageenan production expected in the coming years, the valorisation of the waste fractions becomes necessary.

2.3.1 Biorefinery Strategies for the Valorisation of Red Seaweeds

A succession of extraction steps can lead to the sequential separation of products of interest, from the same biomass, maximizing its value and generating fewer residues, while maintaining the focus on the production of phycocolloid. For carragenophytes, a biorefinery approach should prioritize carrageenan extraction as the primary objective, given its well-established and industrialized production process (Álvarez-Viñas et al., 2019; Balina et al., 2017; Torres, Kraan, et al., 2019). Efforts to valorise sides streams and utilize the entire red algae biomass through a biorefinery approach are increasingly being discussed. Various models have been proposed for seaweeds to produce a wide range of products, typically concluding with the extraction of phycocolloids, after all other valuable components have been removed.

In fact, biorefinery concepts have already been applied to *M. stellatus* industrial waste from conventional extraction and have demonstrated that K2-carrageenan and antioxidant compounds can be further extracted from the waste (Bianchi et al., 2022). Maia et al. (2023) developed a 3-step sequential extraction using UAE to recover pigments, proteins, and carrageenans from *Chondrus crispus*. The study reports the presence of chlorophyll-a and carotenoids with mean values varying from 289,2 and 432,2 µg/g extract d.w., to 39,9 and 59,9 µg/g d.w., respectively. Other fractions obtained through UAE contained proteins and carrageenans yields in the ranges of 3,6–41 g/100 g and 29,7–36,1 g/100 g, respectively. Moreover, Bahari et al. (2021) proposed a method to extract both phycoerythrin and hybrid

carrageenan from the same species, beginning with the extraction of phycoerythrin under mild conditions. Their findings showed that at an extraction temperature of 22 °C, reducing particle size significantly enhanced phycoerythrin concentration (from 0,26 mg/g in >2000 µm particles to 2,30 mg/g in <50 µm fractions). In contrast, carrageenan yield only slightly increased (from no recovery in >2000 µm particles to 2,1 g/kg in <50 µm), remaining much lower than the yields obtained at higher temperatures of 45 °C (5,4 g/kg) and 90 °C (9,9 g/kg). These results suggest that phycoerythrin extraction is surface-area dependent, whereas carrageenan extraction relies more heavily on temperature. A sequential extraction approach has also been tested with *Solieria filiformis* to obtain a nutrient-rich extract, PUFA-rich lipids, and pure I-carrageenan, using MAE. This method demonstrated the potential for maximizing yields while generating only 6,3–10,4 % of residues from the initial biomass, a significant reduction compared to the residues generated through direct extraction of each product, which were 15 times higher (Penuela et al., 2018).

In a study by Bahari et al. (2021), the extraction of K2-carrageenan from *C. crispus* was evaluated using a sequential approach under different times and temperatures, without the use of alkaline pre-treatment. This elimination aimed to explore a cleaner extraction process, though it allowed precursor units (mu and nu) to remain in the carrageenan, reducing its gelling properties. Besides, it has been shown that carrageenan's composition and rheological behaviour can vary significantly depending on the extraction temperature and duration. Knowing that the conventional industrial processes, carrageenan is typically extracted at 80°C for 6 hours, the previous study investigated two types of extraction procedures: the first was a two-step process, beginning with an initial wash (0,5 hours) and extraction of the seaweed at room temperature for 8 hours, followed by a second (sequential) extraction at a specific temperature (ranging from 22 to 90°C) and duration (from 2 to 8 hours); and the second method involved a direct extraction without an initial wash, conducted at a specific temperature and duration. Results indicated a linear correlation between the yield of carrageenan-rich precipitate (CRP) and the combination of temperature and time, suggesting that carrageenan solubility and leaching increase gradually with rising energy input. Additionally, the study found that removing endogenous salts improved the yield of hybrid carrageenan at lower extraction temperatures.

The effects of sequential extraction methods on the functional properties of agars and porphyrans derived from red seaweeds, with the aim of improving extraction yields and valorising by-products, was investigated by (Gomes-Dias et al., 2024). The study evaluated three extraction techniques: cold-water extraction (CWE), ethanolic extraction (EE), and alkaline extraction (HAE) — both individually and in combination, to determine their impact on the quality and yield of the extracted

hydrocolloids. The findings suggest that for *Porphyra dioica*, combining CWE and EE was most effective, leading to a significant increase in melting temperature and a reduction in sulphate content, which enhances the seaweed's functional properties. For *Gracilaria vermiculophylla*, sequential extraction enabled the recovery of additional bioactive fractions without compromising agar yield or quality. In *Gelidium corneum*, the sequential use of CWE, EE, and HAE produced the best results, significantly enhancing agar's texturizing capacity while maintaining high purity and yield. This research is particularly important because it demonstrates that sequential extraction methods can optimize the recovery and functionality of valuable compounds from red seaweeds, while potentially lowering processing costs and environmental impacts. The authors also highlight a significant gap in the existing literature regarding how different extraction methods affect the properties and functionality of the extracted fractions, underscoring the need for further research. Additionally, a techno-economic analysis on an industrial scale is essential to ensure that these processes are profitable, especially if further purification of the fractions is required (Gomes-Dias et al., 2024). This study lays the groundwork for developing more sustainable and cost-effective biorefinery approaches for red seaweeds, promoting their integral valorisation.

A clear understanding of the role of each extraction parameter and the sequence of extraction is crucial, as these factors significantly influence the properties of the extracted carrageenan. The results from this thesis will be the steering wheel for the demonstration of the extractive extrusion-based biorefinery concept (E2B2) of carragenophytes. As the twin-screw extruder-assisted extraction can be adapted to this concept, if the design of machine allows multiple outlets and inlets, the use of different solvents can lead to the extraction of different products along the screw, as will be discussed next.

2.3.2 Extractive – Extrusion

A promising ecological approach in chemical processing is the use of extruders as extractive reactors. However, there is no literature on the application of this method to carrageenan extraction and the cost of the equipment remains high. Despite these challenges, reactive extrusion, where extruders are used as chemical reactors in place of batch reactors, offers significant advantages as a continuous process, notably reducing water consumption and processing time (Abdul Khalil et al., 2018). Demonstrating the industrial feasibility of extruders for extracting K2-carrageenan, as well as other seaweed-derived extracts, is crucial.

The extractive-extrusion method has been widely applied to other areas, such as oil extraction from sunflower seeds and lignocellulose pre-treatment, for bioethanol production (Konan et al., 2022). The use of twin-screw extruders, to assist the alginate extraction process from brown algae has also been reported by (Baron et al., 2010), who focused on the effect of screw speed on the residence time distribution (RTD) of algae in the screw channel, and on the benefits of this extrusion-based pre-treatment for the following alginate extraction. Similarly, previous research has highlighted the importance of pre-treatment effects on the RTD and physicochemical properties of alginate (Sugiono et al., 2019). The twin-screw extruder has shown promise in extracting alginate from the brown alga *S. cristaefolium* at an industrial scale, with a residence time distribution (RTD) of 6,80 ± 0,089 minutes, a yield of 34,96 ± 0,09 %, an intrinsic viscosity of 447,39 ± 18,15 mL/g, and a molecular mass of 211,93 ± 8,74 kDa. Moreover, in another brown algae the use of the extruder, as a pre-treatment, before batch extraction, has been found to be more efficient than the single batch process for the extraction of alginates from *Laminaria digitata* in several key ways: time is reduced from about an hour to only a few minutes, water and reactant requirements are more than halved, and the extraction yield is 15 % higher (Vauchel et al., 2008). Additionally, the rheological properties of the product are enhanced, reactive extrusion yields alginates with chains that are almost three times longer than those produced using batch process. This may partly explain the enhanced extraction yield, as precipitation is more efficient with longer molecular chains, making reactive extrusion an attractive alternative for producing high-quality alginates in the alginate industry.

However, the concept of extractive extrusion of products, such as carrageenans, from seaweeds, is still to be demonstrated. Then, the effects of extractive extrusion parameters, such as the ratio of red algae to solution, feed rate, and pH, on the physicochemical properties (yield and molecular mass) and rheological characteristics (e.g., elastic storage) of carrageenan have yet to be thoroughly investigated. Also, the design of various inlets and outlets, specific screw modules and filtering blocks, all aiming at biorefining red algae into a set of high-value products, has never been explored to convert twin-screw extruders into extractive extruders.

3. MATERIALS AND METHODS

3.1 Study of the Sequential Extraction (SE)

3.1.1 Raw Material

The seaweeds used in this study were: *Mastocarpus stellatus*, *Chondrus crispus* and *Gigartina pistillata*. They were supplied in dry and powder form by AlgaPlus® (Ílhavo, Portugal) in 2024, and stored in a dry place, avoiding exposure to light.

3.1.2 Reagents

The reagents used during this work are described on Table 4, such as the companies that provided those reagents.

Table 4. Reagents used during this sequential extraction

Reagents	Company
Ethanol 96% (v/v)	Continente (Portugal), Auchan (Portugal)
Sodium hydroxide	Sigma Aldrich
Sodium chloride	Sigma Aldrich
Dodecane	Sigma Aldrich

3.1.3 Sequential Extraction (SE)

Following the criteria outlined in Figure 1, products from cold solvent extractions should be prioritized to avoid the need for additional energy, materials or time to cool the biomass. Consequently, the extraction of the main product, carrageenan, should occur last, given that it requires hot solvents for efficient extraction, but also since among all seaweed fractions, hydrocolloids hold the most significant commercial relevance, so it's more relevant that it's extracted purer, emphasizing the necessity of removing other fractions beforehand.

As highlighted in the State of the Art, pigments like R-PE, chlorophylls and carotenoids have substantial economic interest, warranting their extraction first. This study draws inspiration from the work

of Gomes-Dias et al. (2024), which employed a sequential extraction approach targeting agar, a similar sulphated polysaccharide, as the primary product. Phycobiliproteins were initially extracted overnight (about 16 h) at ambient temperature (25 °C), followed by ethanolic extraction for chlorophyll recovery. Similarly, Maia et al. (2023) extracted chlorophyll-a and carotenoids using 95% (v/v) ethanol as the solvent, with UAE methods, using an ultrasonic probe or bath for varying periods of 20 to 40 minutes, before extracting proteins and carrageenans from *C. Crispus*, as previously discussed in Section 2.3 on Sequential Extraction. However, unlike these studies, the present work employs alkaline extraction to target a less sulphated carrageenan rather than proteins, as the primary interest here lies in the recovery of phycocolloids. Additionally, it is known that at high temperatures, proteins may denature, whereas applying an alkaline treatment leads to the isolation of carrageenan with fewer sulphate groups and thus with better gelling properties (Souza et al, 2023). Thus, the following procedure was applied to align with the primary goal of extracting K2-carrageenan while considering the economic relevance of other components.

Each extraction sequence was initiated by mixing 2 g of seaweed (on a dry weight basis) with 60 mL of the different solvents (a sample: solvent ratio of 1:30). The mixture was subjected to cold water extraction (CWE), then ethanolic extraction (EE), then hot water extraction (HWE), and finally hot alkaline extraction (HAE). The extractions were carried out in triplicate and the extraction times studied (t) were: 1 h, 30 min and 10 min. When the time study was, e.g. 1 h, all the extractions had the duration of that time (1 h), except the CWE, that was performed overnight in all essays. The investigation of progressively shorter times was conducted to simulate shorter residence times which are at play within the screw channel of an extruder. In essence, this approach was designed to assess the effect of screw speed on the residence time distribution (RTD) of algae as they move from the feeding zone to the tip of the screw. However, the batch tests cannot precisely replicate the conditions within the screw, as factors such as pressure and mechanical energy are not accounted for in this setup.

Control experiments were performed for each sequential extraction step, excluding the initial one which is similar to a non-sequential extraction. In these controls, the seaweed biomass was subjected to an individual extraction processes to evaluate the impact of prior extraction steps on the final products obtained during the SE (sequential extraction). The extraction sequence, controls, and targeted products in each phase are depicted in Figure 5.

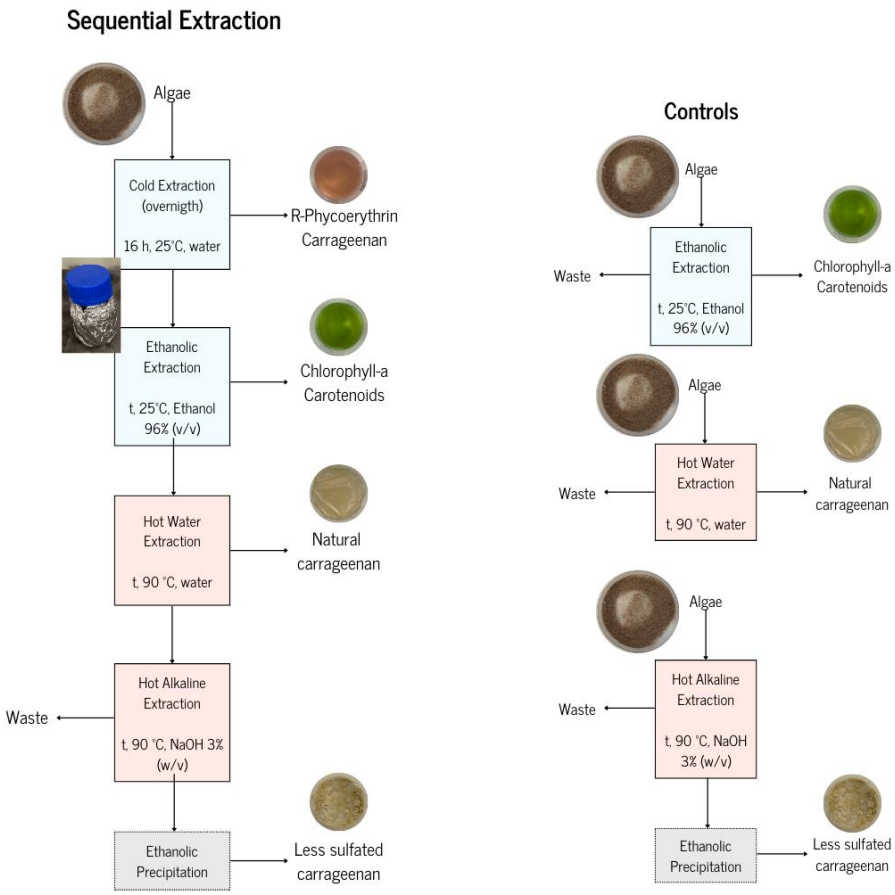


Figure 5. Scheme of sequential extraction and its controls.

3.1.4 Cold water extraction (phycoerythrin recovery)

The seaweed biomass was suspended in distilled water at room temperature (between 20 °C and 25 °C), overnight (about 16 h), under orbital agitation (150 rpm), protected from light. Then the samples were filtered, with a glass funnel and a metal mesh (Gomes-Dias et al., 2024). The filtered solid fraction was subjected to further processing (another extraction sequence), while the liquid fraction was centrifuged (Hettich Universal 320 Centrifuge) at 8 000 g for 10 min and the pellet discarded. The R-PE (R-phycoerythrin) content of the selected samples was calculated based on the absorbances at 564 nm, 618 nm and 730 nm (measured with a UV-VIS spectrophotometer – UV-2401PC, Shimadzu), using Equation (1) (Sampath-Wiley & Neefus, 2007).

$$\text{R-PE (mg/mL)} = 0{,}1247 \cdot [(A_{564} - A_{730}) - 0{,}4583 \cdot (A_{618} - A_{730})] \quad (1)$$

3.1.5 Ethanolic extraction (chlorophyll-a and carotenoid recovery)

The solid fraction recovered in 3.1.4 was suspended in a 96% (v/v) ethanol solution and placed on an orbital shaker (150 rpm), for the studied time (t), at room temperature, protected from light. Then, the samples were filtered, and the solid fraction underwent additional processing (see 3.1.6). The liquid fraction was centrifuged (8 000 g for 10 min) and the pellet discarded (Maia et al., 2023). The chlorophyll-a (Chloro-a) and carotenoid (Carot) contents of the samples were calculated based on the absorbance at 480 nm, 632 nm, 652 nm, 665 nm and 750 nm (measured with the same UV-VIS spectrophotometer as in 3.1.4.), using Equation (2) and (3), respectively (Stengel & Conan, 2015).

$$\text{Chloro-a } (\mu g/mL) = -3{,}2416 \cdot (A_{632} - A_{750}) - 6{,}4151 \cdot (A_{652} - A_{750}) + 16{,}4351 \cdot (A_{665} - A_{750}) \quad (2)$$

$$\text{Carot } (\mu g/mL) = 4 \cdot (A_{480} - A_{750}) \quad (3)$$

3.1.6 Hot water extraction (natural K2-carrageenan recovery)

This extraction consisted in heating a solution of distilled water plus the solid fraction from the previous stage, in an oven at 90 °C, for the studied time (t) (Azevedo et al., 2015). Then, the sample was centrifuged (8 000 g for 10 min) and the pellet was sent to the next step. The liquid fraction was placed in a plastic container and dried in an oven, with air convection, at 55°C, overnight (circa 16h). In the next day, the resulting dried carrageenan film was weighed to obtain the yield, Y, using Equation (4).

$$Y = \frac{\text{(sample mass)}}{\text{(algae mass)}} \quad (4)$$

3.1.7 Hot alkaline extraction (less sulphated K2-carrageenan recovery)

In this step, the pellet from the previous extraction was resuspended in a 3 % (w/v) NaOH solution for the designated extraction time (t) in an oven at 90 °C (Azevedo, 2015). After the extraction, the solution

was centrifuged at 8 000 g for 10 minutes, and the liquid fraction, containing carrageenan and salts, was transferred to a plastic container and dried at 55 °C overnight.

The next day, the dried sample (of the three replicas) was weighed to determine the total mass after HAE. Then, from this total, a portion (e.g., 5 grams) (mass after HAE used for the precipitation), was dissolved in water to form a 2 % (w/v) carrageenan solution. This solution was then subjected to ethanolic precipitation to remove excess salt and isolate the carrageenan (Azevedo et al., 2013). The precipitated carrageenan was transferred to a container and dried again in an oven at 55°C overnight. The following day, the dried precipitate was weighed (mass precipitated) to obtain the yield, YY, Equation (5).

$$YY = \frac{(\text{mass precipitated} \cdot \text{total mass after HAE})}{(\text{total algae mass} \cdot \text{mass after HAE used for the precipitation})} \quad (5)$$

3.1.8 Characterization of hybrid carrageenans

Gels preparation and viscoelastic characterization

K2 extracts 2% (w/v) were dissolved in solution with 1 M NaCl and stirred vigorously for 1 h at 80 °C. The concentration and ionic strength were specifically selected to obtain gels with sufficient rigidity at 25 °C, without water release, and to maintain a single cation (Na^+) to facilitate gel formation, since the alkaline extraction was carried out with NaOH (Azevedo et al., 2014). After allowing the solutions to cool for 24 h, two possible outcomes were observed: a) gel formation or b) no gel formation.

a. Gel formation) The samples that gelled were reheated to 85 °C and the resulting hot K2 solutions were directly transferred into the pre-heated (80 °C) Couette accessory (CC10) of a Paar Physica MCR300 rheometer (Anton Paar, Austria). The geometry surface was covered with dodecane oil to prevent water evaporation. The samples were then cooled to 25 °C within 5 500 seconds (≈1,5 h). During the cooling of the solution a small amplitude oscillatory shear was applied (with 0,1 % strain at a ω=1 Hz, the ω being the frequency) to probe the temperature evolution of linear viscoelastic properties such as tan δ=G''/G'. The temperature where tan δ =1 was defined as the gel setting temperature, Tg, as seen in Figure 6 a). Following the cooling and gel setting, at a 25 °C a frequency

sweeps was performed with a 0,1 % strain, which ensured the measurement of equilibrated gel's mechanical spectra in the linear regime and the determination of the G' at 1 Hz, label as G_0 (Figure 6 b)). A shear strain (ɣ) sweep from 0,01 to 1000 % with a frequency of 1 Hz was then made to verify that the produced frequency sweep and G_0 were acquired within the linear regime of elasticity (Souza et al., 2011) and to characterize the gel behaviour under large deformation, as shown in Figure 6 c).

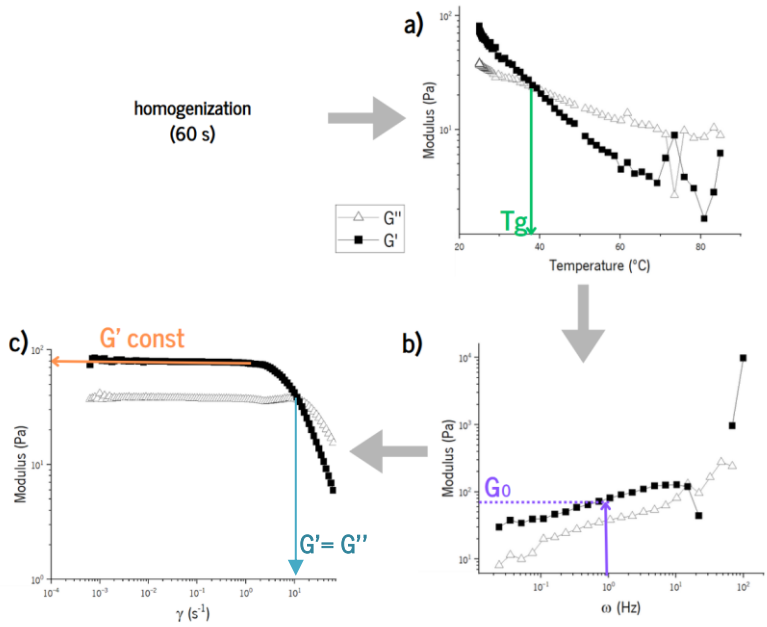


Figure 6. Example of the experimental protocol followed for the rheological characterization of the gels, in the Paar Physica MCR300 rheometer (Anton Paar, Austria).

b. No gel formation) The solutions were placed into a plate-plate geometry (40 mm diameter) of a stress-controlled rotational rheometer (MCR 302, Anton Paar) at 25 °C. Each sample was equilibrated during 300 seconds by applying an oscillatory strain of 10 % at 1 Hz and 25 °C. This step was followed by a ω sweep from 100 to 0,01 Hz, performed with a strain of 50,3 %, in view to record the mechanical spectrum of the solution and collect the G' at 1 Hz, label as G_1 (as seen in Figure 7 a)). Then, a shear rate (ɣ) sweep ranging from 1000 s^{-1} to 1 s^{-1}, immediately followed by sweep from 1 s^{-1} to 1000 s^{-1}, were performed to measure the shear rate dependence of the apparent shear viscosity, η, to measure the flow curve of the solution and assess any possible thixotropic behaviour related with carrageenan aggregation with time, shown in Figure 7 (b) and c)).

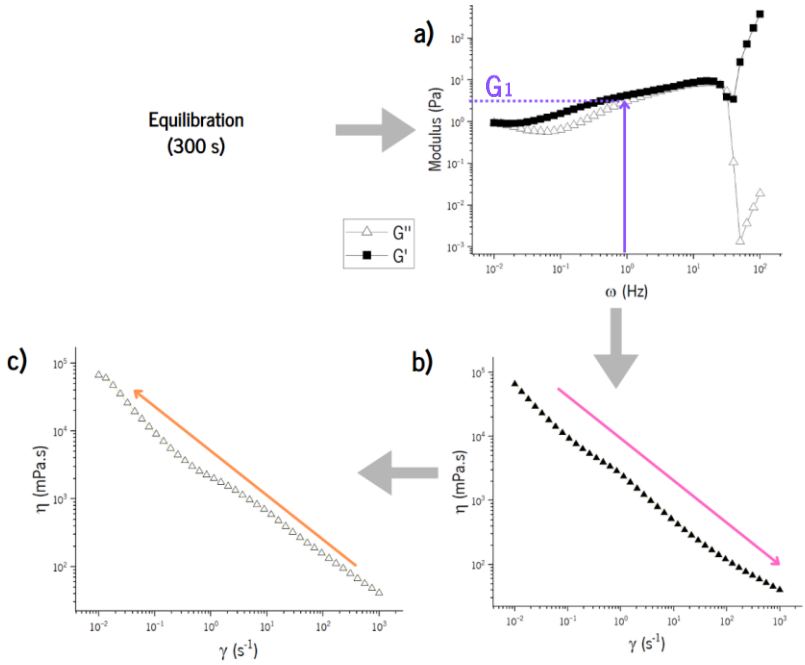


Figure 7. Example of the experimental protocol followed for the rheological characterization of the liquids, in stress-controlled rotational rheometer (MCR 302, Anton Paar).

Spectroscopic analyses

The Fourier Transform Infrared Spectroscopy (FTIR) method was used for the qualitative chemical characterization of the extracted K2. The FTIR analyses were performed on a Perkin-Elmer Spectrum 100 FTIR spectrometer equipped with an Attenuated Transmission Reflectance (ATR) accessory. The spectra were obtained in a range of 4000 to 600 cm^{-1} with 16 scans per sample and a resolution of 1 cm^{-1}. The assignments of the FTIR spectra were mostly based on the previous work of (Van De Velde et al., 2004) (see Table 5).

Nuclear Magnetic Resonance (NMR) spectroscopy was performed using a 400 MHz Avance III spectrometer (Bruker) at 70 °C to limit the line broadening caused by the viscosity of the 1 wt.% carrageenan solutions in D_2O. Indeed, the sample solutions were ultra sonicated during hours (using a DCG-300H bath, MCR Ltd.) to lower their viscosity prior to NMR characterization. Chemical shifts (ppm) were referenced to the D_2O lock signal (4,41 ppm) and further adjusted to match the chemical shift for κ (5,09 ppm), as prescribed by van De Velde et al., (2004). The molar fractions (mol %) of the carrageenan repeating units are calculated as the integrated intensity of the corresponding ^{1}H-NMR peak (see Table 6) over the sum of the integrated intensities of all assigned carrageenan anomeric protons (Van de Velde et al.,2004). The pyruvic acid acetals and floridean starch are also detected in the ^{1}H-NMR spectra by the methyl proton resonances with chemical shifts, relative to DSS (an NMR standard), of 1,44 ppm for the

pyruvic acid and 5,35 ppm and 4,98 ppm for floridean starch (Van De Velde et al., 2004). The NMR spectra were produced by the chemical analyses service at the department of chemistry of the University of Minho.

Table 5. Identification of carrageenan types by infrared spectroscopy (adapted from (Chopin & Whalen, 1993)). The intensity of the observed peaks is indicated by symbols: '+' (weak), '++' (moderate), '+++' (strong) and 's' (shoulder peak)

Wavenumbers (cm^{-1})	Bond(s)/group(s)	Letter Code	Type of carrageenan				
			κ	mu	ι	nu	L
1240-1260	S=O of sulphate esters	-	+	++	++	+++	+++
970-975	Galactose	D	+	s	+	s	-
930	C–O of 3,6-anhydrogalactose	DA	+	-	+	-	-
845	C–O–SO_3 on C4 of galactose	G4S	+	+	+	+	-
825-830	C–O–SO_3 on C2 of galactose	D2S	-	-	-	+	+
815-820	C–O–SO_3 on C6 of galactose	D6S	-	+	-	+	+
805	C–O–SO_3 on C2 of 3,6-anhydrogalactose	DA2S	-	-	+	-	-

Table 6. Chemical shifts (ppm) of the alfa-anomeric protons of carrageenans referred to DSS as internal standard at 0 ppm (adapted from: Van de Velde et al.,2004)

Types of carrageenan	Monosaccharide	Chemical shift (ppm)
κ	DA	5,093
Mu	D6S	5,238
ι	DA2S	5,292
Nu	D2S,6S	5,501

Molecular mass determination

The molecular mass distribution was obtained by size exclusion chromatography and refractive index detection from a service purchased to the Analise Laboratory of the LAQV-REQUIMTE Associate Laboratory, NOVA University of Lisbon. A Waters 600 apparatus coupled to a Waters 2410 differential refractive index detector was used, whereas the size exclusion chromatograph was a PolySep-GFC-P Linear column (Phenomenex, Alcobendas, Spain). This column has been calibrated with pullulan standards (Shodex, Munich, Germany) ranging from 6 300 to 642 000 g/mol. Duplicated measurements of Mw and PDI were performed with 0,1 wt % carrageenan solutions in 0,1 M NaCl at 40 °C.

3.2 Study of the Molecular Mass

3.2.1 Raw Material

The K2-carrageenans utilized in this study were hot-water extracted and then subjected to a 3% (w/v) KOH alkaline treatment by another research group, following earlier results reported by (Azevedo et al., 2013). The extractions were conducted with five commercially sourced algae, generously provided by AlgaPlus® and Cargill®. The resulting hybrid carrageenans were assigned specific codes and had varying chemical compositions to assess the impact of molecular structure on the breakdown of molecular mass: B-87 (4 mol % κ and 96 mol % ι); Gigartina (43 mol % κ, 40 mol % ι, and 17 mol % nu); Mastocarpus (70 mol % κ and 30 mol % ι); BAT 18-09 (56 mol % κ, 36 mol % ι, 4 mol % mu, and 4 mol % nu); and C-25 (90 mol % κ and 10 mol % ι).

3.2.2 Reagents

The reagents used during this work are described on Table 7, such as the companies that provided those reagents.

Table 7. Reagents used during the ultrasonication of K2-carrageenans

Reagents	Company
Potassium chloride	Sigma Aldrich
Dodecane	Sigma Aldrich

3.2.3 Ultrasonication of carrageenans and gel test

K2-carrageenan samples were dissolved in distilled water with vigorous stirring at 80 °C for 1 h to ensure complete dissolution, at a concentration of 0,5% (w/v), once is know that at lower concentrations the Mw breakdown of the polymer is faster (Rochas et al, 1990). Following this, the carrageenan solutions underwent ultrasonication for various durations in an ultrasonic bath (DCG-300H, MCR Ltd.), operated at 40 kHz and an ultrasonic power of 400 W in pulsed mode at 80 °C to mechanically reduce their molecular mass by cavitation, rather than by hydrolysis route which could affect the hybrid carrageenan composition. Ultrasonication was applied for various durations (1, 2, 3, 4, 12, 14, 16, and 20 h), generating distinct samples at each time interval. A gelation test was performed on each sample post-ultrasonication by preparing 1 % (w/v) carrageenan solutions in 0,1 M KCl. The solutions 0,5 % (w/v) were first evaporated to reach the prior concentration, followed by the addition of KCl in its salt form. These were heated to 80 °C for 1 h and then cooled to room temperature for 24 h to induce gel formation (Azevedo et al., 2015). As a control, a non-sonicated sample (0 h) was included, and all the carrageenan samples successfully formed gels at 0 h. The gelling properties of the samples following ultrasonication were compared, revealing that some samples lost their ability to gel after 4 hours of treatment, while others remained gelled until 20 hours. The ultrasonication duration of a carrageenan solution stopped when an aliquot sample remained liquid after the gel test.

3.2.4 Rheology

After the gelation test, the K2-carrageenan samples subjected to different ultrasonication times, as well as the non-sonicated control sample, were characterized by rotational rheometry to compare the gelling behaviour across the samples. The liquids were characterized as described in section 3.1.8 (Gels preparation and viscoelastic characterization). As for the gel, a new geometry and rheometer was used – to accommodate for the small number of available samples. A different plate-plate geometry (40 mm diameter) of a stress-controlled rotational rheometer (MCR 302, Anton Paar) was used, but the experimental protocol (Figure 8) closely resembles the previous one (Figure 6). It differs in the control of gel thickness, with the normal force set to 0 N during cooling. A new characteristic is evaluated: TON. This temperature is measured with more precision than the inflection point, Tg (see Figure 8.a.1)). TON can be identified from the Gap (mm) reduction, which indicates that the sample is transitioning from a liquid (where the temperature dependence of the gap is linear due to the thermal expansion of the shearing geometry) to a gel state (with the contraction of the sample's volume), as illustrated in Figure

8.a.2). The mechanical spectra (Figure 8 b)) and the shear strain test (Figure 8 c)) were performed as described before (in section 3.1.8).

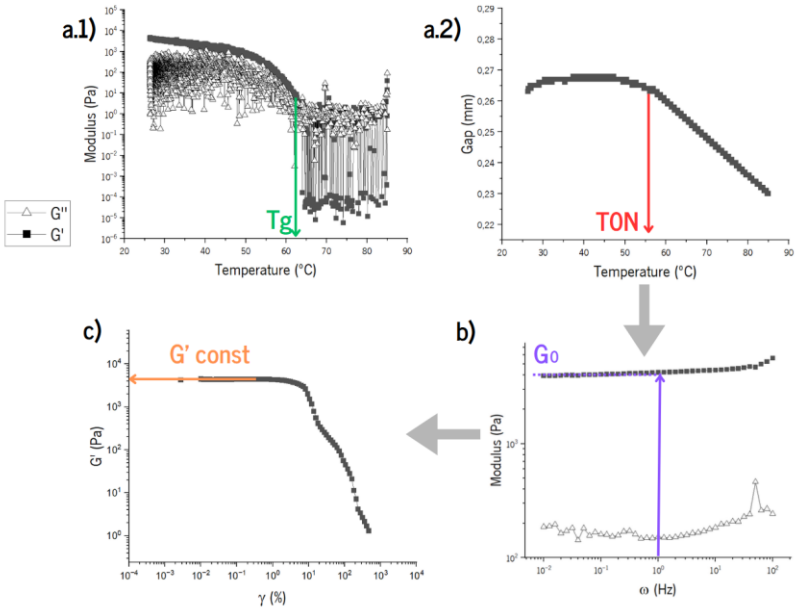


Figure 8. Example of the experimental protocol followed for the rheological characterization of the gels, in the stress-controlled rotational rheometer (MCR 302, Anton Paar).

3.2.5 Spectroscopic analyses

The FTIR method was employed for the qualitative characterization of the chemical structure of K2-carrageenan, comparing the non-sonicated sample (0 h) with the sample subjected to the longest ultrasonication time. This analysis aimed to verify whether any modifications occurred in the chemical structure of the carrageenan after the ultrasound. The experimental protocol followed is detailed in section 3.1.8 (Spectroscopic Analyses).

3.2.6 Molecular mass determination

To assess the molecular breakdown, the molecular mass of the carrageenans prior to and during ultrasonication was determined. The experimental protocol followed is detailed in section 3.1.8 (Molecular mass determination).

3.3 Statistical analysis

Statistical analysis was performed to identify significant differences using one-way ANOVA in Origin version 2024b (Learning Edition). Multiple comparisons were evaluated through Tukey's test at a 95 % confidence interval. For each seaweed species and carrageenan sample, an analysis was conducted to assess whether the sequential extraction and ultrasound methods produced significant differences ($p < 0,05$) in the yield of extracted fractions, the Mw and the PDI. Results are presented as means ± standard deviation, with a minimum of two replicates conducted unless otherwise specified.

4. RESULTS AND DISCUSSION

4.1 Sequential Extraction

The sequential extraction (SE) process enables the fractionation of pigments and K2-carrageenans over time, isolating fractions with distinct chemical and rheological properties. By analysing yields, chemical composition, and rheological behaviour across extraction stages, this study assesses the impact of SE and extraction time on the functional properties of K2-carrageenans. These findings are crucial for optimizing extraction time to screen for the potential of extractive extrusion, and tailoring carrageenans for specific industrial applications.

First, to validate SE as a viable alternative to conventional batch extraction for K2-carrageenans, the yields of fractions recovered at each step are analysed in relation to the known compositions of the studied seaweeds. Subsequently, the chemical structures of the carrageenans obtained through SE are discussed, followed by an in-depth assessment of their rheological properties.

4.1.1 Cold Water extraction (CWE)

The first extraction consists of an overnight cold water extraction. The compound extracted is a known pigment of the red algae, but also a protein, R-phycoerythrin (R-PE) (Bahari, Moelants, Kloeck, et al., 2021). This extraction does not have a control, as it is the first extraction and therefore not influenced by any prior extraction. The absorbance values at specific wavenumbers of the various liquid extracts (triplicated samples) can be consulted Appendix A.

Comparing the R-PE yields shown in Figure 9, specifically, 0,343 ± 0,058 mg R-PE/g d.w. for *M. stellatus*, and 0,214 ± 0,062 mg R-PE/g d.w. for *C. crispus* , with literature values of 1,99 mg R-PE/g d.w. for *M. stellatus* and 0,53 mg R-PE/g d.w. for *C. crispus* (Nguyen et al., 2017; Pina et al., 2014), it is evident that less than half of the R-PE reported in the literature is extracted from the algae studied here. As for *G. pistillata* no values are available in the literature to compare with the 0,307 ± 0,049 mg R-PE/g d.w. shown in Figure 9. Furthermore, *C. crispus* shows comparatively lower R-PE yields than the other two algae in this study and in the literature.

The low R-PE yield observed in this study may be attributed to non-optimized extraction conditions. For instance, Nguyen et al. (2017) achieved significantly higher yields for *M. stellatus* by using a different method involving phosphate buffer (20 mM, pH 7,1) and xylanase to degrade the algal cell

wall, yielding up to 1,99 mg R-PE/g d.w. Further studies should consider varying the algae used within *M. stellatus* and the other species, to assess seasonal and geographical impacts on R-PE yield, as R-PE content may differ between winter and summer.

It is possible that this extract does not only contain R-PE, but also salts from the seaweed, as well as sulphated carrageenans (e.g. L-carrageenans), as some studies reveal that it is possible to extract carrageenans at 25 °C, at extended times (Bahari et al, 2021). So, the liquid extracts were analysed further and discussed in section 4.1.5. Influence of the sequential steps.

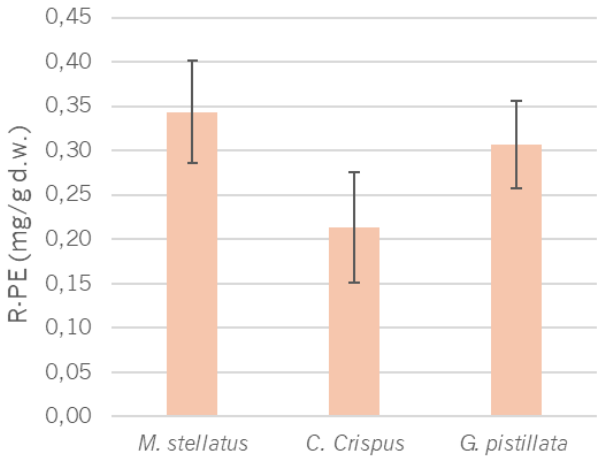


Figure 9. Extraction yield (mg/g d.w.) of the phycoerythrin, R-PE, with standard deviation error bars, for the three different algae (*Mastocarpus stellatus, Chondrus Crispus* and *Gigartina pistillata*), from the overnight (16h) cold extraction, first step of the sequential extraction.

4.1.2 Ethanolic Extraction (EE)

The second extraction, based on ethanol, showed varied outcomes for the algae, the extraction yield of chlorophyll-a (chloro-a) and carotenoids (carot), are shown in Figure 10 and Figure 11, respectively. Details on the values of each triplicated sample and absorbance is given in Appendix B.

For *M. stellatus*, *C. crispus*, and *G. pistillata*, the chlorophyll-a yield showed an increase or statistical similarity yield across extraction times (10 min to 1 h), when analysing separately both extraction types (sequential and control). In *M. stellatus*, the 1 h SE extract produced a lower yield than the 1h control, but for smaller times (10–30 min) the yield was similar. Conversely, *C. crispus* exhibited a slight increase in chlorophyll-a yield in SE extractions, likely due to the initial CWE enhancing pigment diffusion. Similarly, *G. pistillata* showed improved recovery with the 1 h SE extraction compared to shorter

control times (10–30 min), although the 1 h yields for both SE and control extractions were statistically similar due to high variability.

For carotenoids, all extraction times and types (SE or control) produced similar yields in *G. pistillata* and *C. crispus*, indicating that SE does not significantly impact carotenoid recovery (even though the maximum value of carotenoids for *C. crispus* was obtained at 30 min in the SE). An exception was observed for *M. stellatus*, where the 10 min control yielded less carotenoids than the 1 h control but was comparable to the 1 h SE extract.

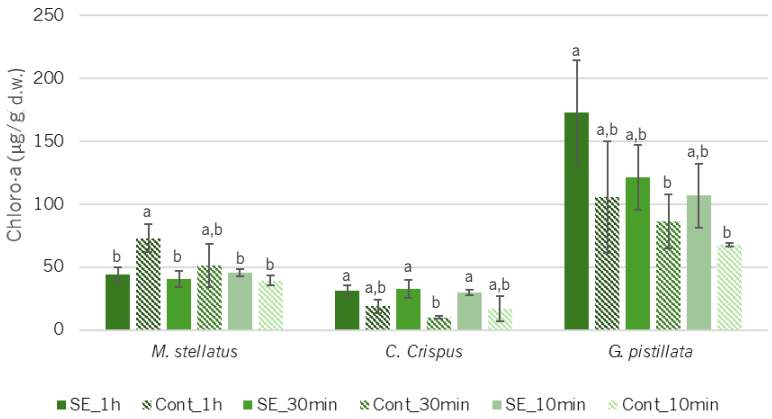


Figure 10. Extraction yield (µg/g d.w.) of chlorophyll-a, of the three different algae (*Mastocarpus stellatus, Chondrus Crispus* and *Gigartina pistillata*), from the ethanolic extraction (sequential – SE - and the control - Cont), with different extraction times (1 h, 30 min and 10 min). The different letters indicate statistically significant differences between bars from the same alga ($p < 0,05$).

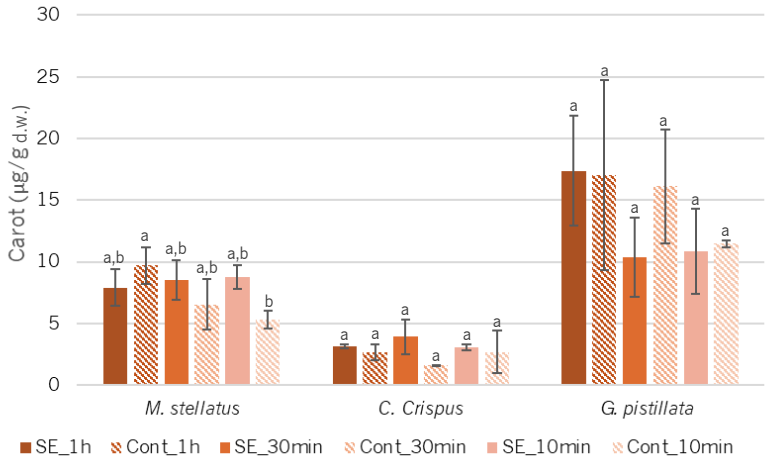


Figure 11. Extraction yield (µg/g d.w.) of carotenoids, of the three different algae (*Mastocarpus stellatus, Chondrus Crispus* and *Gigartina pistillata*), from the ethanolic extraction (sequential – SE - and the control - Cont), with different extraction times (1 h, 30min and 10min). The different letters indicate statistically significant differences between bars from the same alga ($p < 0,05$).

Although the SE and control samples are statistically similar in most cases, the prior water extraction of the SE biomass makes it less rigid and cleaner (less sands and salts) before reaching the EE step, possible resulting in a material with reduced structural integrity and more damp compared to the control samples. The large standard deviations in the previous figures, particularly for *G. pistillata* and 1 h extraction time, could be due to sedimentation of pigments and suspended algal particles over time, with pigments potentially settling within minutes. Furthermore, light exposure immediately before absorbance readings might introduce significant experimental error, as pigments like chlorophylls and carotenoids are highly susceptible to photo-oxidation. While this degradation under light and pigment sedimentation could act as systematic errors affecting all samples uniformly, they alone may not fully explain the high standard deviations observed. Additional factors, such as different time of light exposure or differences in pigment concentration within the tube samples, could contribute to the observed measurement inconsistencies (Cheloni & Slaveykova, 2018).

Comparing the three algae, *G. pistillata* shows the larger concentration of pigments (both chlorophyll-a and carotenoid), followed by *M. stellatus* and then the *C. crispus*. These results are different from those reported in the literature, in which *M. stellatus* has the highest concentration of chloro-a and *C. crispus* is the algae with more carotenoids (Carpena et al., 2021). In the reference work of Carpena et al. (2021), pigments were extracted by dissolving 1 g of alga in 20 mL of 50 % (v/v) ethanol solution, and stirred at 50 °C for 24 h, followed by re-extraction with fresh solvent under the same conditions. They obtained significantly higher chlorophyll-a levels than those plotted in Figure 10: 12x, 3x, and 2x more from *M. stellatus, C. crispus,* and *G. pistillata*, respectively. Regarding carotenoids, less than 10% of the full content was extracted in this work, compared to previous study (Carpena et al., 2021), this was likely due to the shorter extraction times used here, as the literature reports extraction durations of up to two days. Nevertheless, in a study of Maia et al. (2023), extraction times shorter than 1 h (20/40 minutes) and similar solvent (ethanol 95% (v/v)) were used, without any prior extraction or cleaning steps, for pigment extraction from *C. crispus*. However, these authors utilized an ultrasonic probe or bath, resulting in chlorophyll-a and total carotenoid concentrations of 434,25 – 276,5 µg chloro-a/g d.w. and 63,2 – 38,1 µg carot/g d.w., respectively, that were significantly higher than those displayed in Figures 10 and 11. Overall, quantitative comparisons of yields are difficult since geographical and seasonal variations in the algae have impact on the concentration of pigments, and ultrasonication significantly enhance the extraction efficiency. Also, in both referenced studies, ethanolic extraction was carried out at temperatures above 25 °C, which may have helped soften the algal cell wall matrix, facilitating pigment extraction.

4.1.3 Hot Water Extraction (HWE) & Hot Alkaline Extraction (HAE)

In both HWE and HAE, the target is the recovery of carrageenans, and this section focuses on the carrageenan yields. In the HWE step, all control extracts showed higher yields compared to the SE ones (see Figure 12), except for the 1 h extracts in *C. crispus*, which were similar. The alga with the overall highest sequential yield of K2-carrageenan in hot water (at 90 °C) was *C. crispus*, whereas *M. stellatus* showed the lowest yields, with no detectable carrageenan after 10 minutes of extraction (see Appendix C for detail).

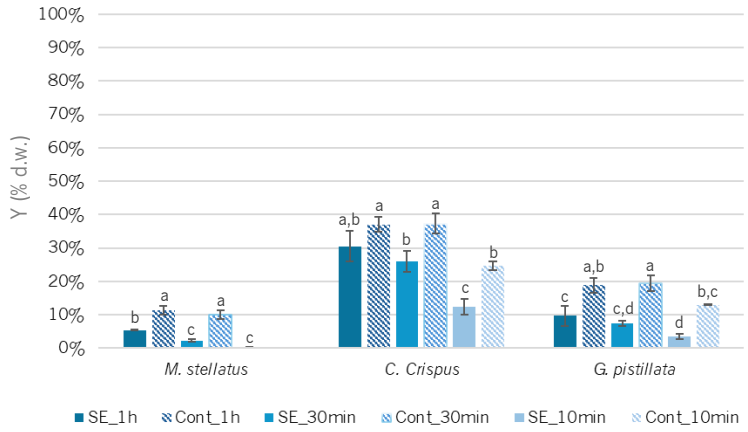


Figure 12. Yield of K2-carrageenans (Y), from the three different algae (*M. stellatus, C. Crispus* and *G. pistillata*), for the hot water extraction at 90 °C (sequential, SE, and the control, Cont), with different extraction times (1 h, 30 min and 10 min), for each seaweed and time point. The different letters indicate statistically significant differences between bars from the same alga ($p < 0,05$).

Regarding extraction time, for the sequential samples, shorter processing times generally resulted in lower yields, as expected. These findings are consistent with studies showing that carrageenan extraction yield is largely time and temperature dependent, with longer durations generally resulting in better solubilization and recovery of carrageenan (Bahari, Moelants, Wallecan, et al., 2021; Hilliou, Larotonda, Sereno, et al., 2006). In a study by Bahari et al. (2021), *C. crispus* underwent an initial extraction of 8 h at 22 °C, similar to the CWE step performed in this thesis, followed by a second extraction of 2 h at 90 °C, yielding approximately 30% d.w. of carrageenan. Notably, this yield is comparable to the results obtained in this work with 1 h and 30 min SE HWE extracts, despite the extraction time in the previous study being twice as long. For the controls, only an extraction time below 30 minutes yields significantly less carrageenans for all algae tested.

In the last extraction step (HAE), an alkaline (3% (w/v) NaOH) extraction was carried out at 90 °C. The yields achieved in the HAE step are shown in Figure 13 (see a more detail data and statistics in Appendix D). As expected, the HAE control samples showed higher yields than the samples from the HWE step (control and SE), likely due to the ability of the alkaline solutions to enhance solvent penetration into algae tissue cells (Azevedo et al., 2013; Hilliou et al., 2006).

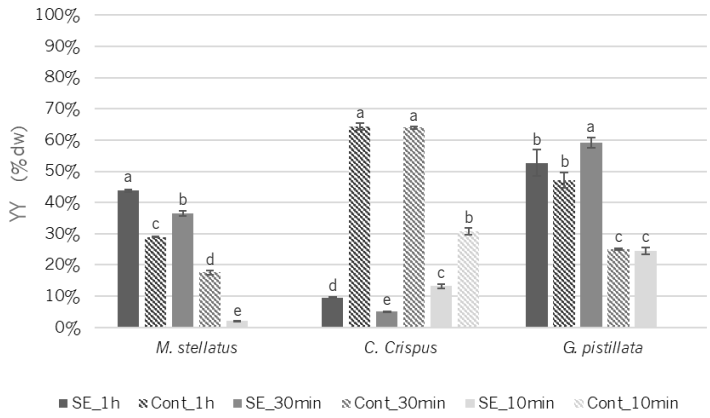


Figure 13. Yield of K2-carrageenans (YY), after precipitation, of the three different algae (*Mastocarpus stellatus, Chondrus Crispus* and *Gigartina pistillata*), from the alkaline water extraction (sequential – SE - and the control), with different extraction times (1h, 30min and 10min). The different letters indicate statistically significant differences between bars from the same alga ($p < 0,05$).

The carrageenan yields for *C. crispus* processed in the alkaline sequential extraction were notably lower than those of the control samples and other algae. This outcome suggests that the previous HWE sequence may have already removed a substantial portion of the carrageenan content (see Figure 12). And indeed, the carrageenan yields for the control experiments with 1 h and 30 min extraction times are up to 60 % d.w., which is in good agreement with the carrageenan content reported elsewhere for *C. crispus* processed with NaOH (Pereira, 2013). In contrast, larger carrageenan yields are achieved in the HAE *M. stellatus* and *G. pistillata* than in the HWE, indicating that much of their K2-carrageenan remained in the alga during the previous step.

The highest yield of *M. stellatus* (obtained from the 1 h SE extract) is within the range (40-50% d.w.) of the yield obtained by Azevedo et al. (2015), in a 3 h alkaline pre-treatment (with NaOH) followed by a 1 h HWE at 90 °C. In other words, the sequential extraction method used in this study produced similar yields as those that used longer times of extraction.

In the case of the *G. pistillata*, the highest yield was achieved at the 30 min SE extraction and the 1 h extracts (SE and control) were alike (statically). As for the 10 min control extraction, no carrageenan

was precipitated, but in SE the yield was larger than 20 % d.w. Thus, as for *M. stellatus,* SE enhances the extraction yield of alkali modified K2-carrageenans from *G. pistillata*, but the extraction times does not need to be more than 30 min. This is an important result since total extraction times of the order of 30 min to 1 h performed sequentially can potentially lead to the recovery of as much carrageenan as in conventional longer extraction routes. However, the composition and molecular characteristics of the extracts may differ.

4.1.4 Characterization of K2-Carrageenans

The sum of the results of the chemical and rheological characterization are shown in Table 8, respectively, for the three red algae, after CWE, HWE, and HAE, under the 2 conditions (SE and control) and at three different extraction times. The observation of the peaks of the FTIR-ATR are displayed in Table 9 and the spectra can be consulted in Appendix E. For the *M. stellatus,* it wasn't possible to get a clear ^{1}H-NMR nor FTIR spectra from the 10 min alkaline SE extracts, since there was a lot of noise, and the product didn't form a film after drying. As such, it can be assumed that there is no K2-carrageenan, and the 10 minutes alkaline extracts will be no further studied for this alga, similar to the 10 min control alkaline extracts from *G. pistillata*. As for the 10 minutes water extracted from the *M. stellatus* , it wasn't possible to extract any K2-carrageenan. Accordingly, no carrageenan characteristics are available in Table 8. The lack of data of the HAE extracts of the *C. crispus* and the 10 min SE HAE extract from *G. pistillata* is due to insufficient mass extracted to perform the rheological tests and molecular mass determination, respectively, and such characteristics were not determined.

Some floridean starch was detected in the ^{1}H-NMR spectra of the 30 min SE HAE extracts from *C. crispus* (more details available in Appendix F). This high content of starch could interfere with the carrageenan yield and the functional properties. However, Azevedo et al. (2015) showed that in particular for *C. crispus* starch did not affect the carrageenan gel elasticity. Starch was also detected in the 10 min control HWE extract in *G. pistillata.* And another contaminant, pyruvic acid, was detected in large amount in extracts from 60 min and 30 min control HAE of *G. pistillata* and in smaller amounts in the 60/30 min control HAE and 30 min SE HWE extracts of the same alga, making these extracts less pure.

Table 8. Summary of analytical data for K2-carrageenan extracts: Molecular Mass (Mw) and Polydispersity Index (PDI) values, along with standard deviations (SD); the Mn values are presented in Appendix G. Molar percentages (mol. %) of the carrageenan structure in algal extracts. For the carrageenans solutions (2% (w/v) car + 1M NaCl), is still presented the gel setting temperature (Tg); the elastic modulus (G') at 1 Hz, 25°C, 0,1% strain (for gels, G_0) and 50,3 % strain (for liquids, G_1), and the gel's yield strain (**ɣ**), when G'=G''. Viscosity measured at 500 s^{-1} and hysteresis are shown only for liquid samples, while gel-forming samples are marked as "Gel" and colour grey. The "nd" means not detected. And for each alga and time point, in a given extraction, values of Mw and PDI with different letters indicate statistically significant differences ($p < 0,05$)

Algae	Extract	Type	time	Extracted K2-carrageenans											
				Mw (g/mol)	PDI	κ (mol %)	ι (mol %)	mu (mol %)	nu (mol %)	G_0;G_1 (Pa)	Tg (°C)	ɣ (s⁻¹) (G'=G")	η500 (mPa/s)	Hyst?
Mastocarpus Stellatus	**CWE**	Sequential	16h	. . .	. .	.				.	.	.	.	.
	HWE	Control	1 h	1,00E+06 ± 2,55E+04 ^{b}	2 ± 0,001 ^{b}	52,01	34,35	2,92	10,72	0,00657	.	.	30,3	yes
			30 min	7,55E+05 . ^{c}	2 . ^{b}	79,28	16,88	2,6	1,24	3,61	.	.	2,91	yes
			10 min	. . .	. . .	.	.	.	.	.	.	.	.	.
		Sequential	1 h	1,19E+06 ± 2,71E+04 ^{a}	9 ± 0,266 ^{a}	58,65	9,22	17,09	15,04	376,32	26,43	22,8	Gel	.
			30 min	8,73E+05 ± 2,06E+04 ^{c}	8 ± 0,664 ^{a}	54,16	45,84	.	.	168,49	35,91	5,9	Gel	.
			10 min	. . .	. . .	.				.	.	.	.	.
	HAE	Control	1 h	4,58E+05 ± 1,12E+03 ^{b}	4 ± 0,062 ^{a}	70,07	29,93	.	.	3,92	.	.	8,9	yes
			30 min	3,60E+05 ± 2,85E+04 ^{b}	3 ± 0,406 ^{a}	63,83	3,76	18,87	13,53	0,0375	.	.	1,64	yes
			10 min	. . .	. .	nd				.	.	.	.	.
		Sequential	1 h	6,64E+05 ± 8,24E+04 ^{a}	3 ± 0,611 ^{a}	65,89	34,02	.	.	0,31	.	.	6,59	yes
			30 min	3,15E+05 ± 2,54E+04 ^{b}	4 ± 0,255 ^{a}	72,56	27,44	.	.	0,00658	.	.	1,8	yes
			10 min	. . .	. . .	.				.	.	.	.	.
Chondrus Crispus	**CWE**	Sequential	16 h	2,66E+05 ± 1,28E+04	4 ± 0,038	59,37	26,54	5,49	8,6	0,000776	.	.	3,54	yes
	HWE	Control	1 h	7,74E+05 ± 2,95E+04 ^{a}	3 ± 0,047 ^{a}	60,47	28,75	5,48	5,3	49,83	>85	4,7	Gel	.
			30 min	7,94E+05 ± 6,50E+03 ^{a}	4 ± 1,06 ^{a}	56,25	39,58	5,9	8,27	9,27	.	.	79,71	yes
			10 min	4,66E+05 ± 6,68E+03 ^{c}	3 ± 0,013 ^{a}	55,28	38,71	8,21	7,79	2,16	.	.	57,95	no
		Sequential	1 h	7,52E+05 ± 4,12E+03 ^{a}	4 ± 0,034 ^{a}	59,79	32,56	3,55	4,11	50,07	>85	4,17	Gel	.
			30 min	7,83E+05 ± 4,70E+03 ^{a}	3 ± 0,08 ^{a}	68,66	28,61	2,73	.	111,81	32,51	2,72	Gel	.
			10 min	5,38E+05 ± 1,22E+03 ^{b}	3 ± 0,026 ^{a}	59,95	35,87	4,17	.	4,44	.	.	57,75	no
	HAE	Control	1 h	4,07E+05 ± 6,11E+03 ^{a}	3 ± 0,036 ^{a}	59,2	35,84	4,95	.	no information				
			30 min	3,31E+05 ± 1,10E+04 ^{a,b}	3 ± 0,057 ^{a}	66,27	25,84	7,88	.					
			10 min	2,97E+05 ± 1,51E+02 ^{b,c}	3 ± 0,005 ^{a}	67,72	32,28	.	.					
		Sequential	1 h	2,22E+05 ± 1,82E+04 ^{c}	4 ± 0,308 ^{a}	49,64	50,36	.	.					
			30 min	2,43E+05 ± 4,26E+04 ^{b,c}	6 ± 2,618 ^{a}	44,69	55,31	.	.					
			10 min	3,25E+05 ± 3,82E+04 ^{a,b}	4 ± 0,217 ^{a}	65,97	30,43	.	.					
Gigartina pistillata	**CWE**	Sequential	16 h	9,48E+05 ± 6,81E+03	2 ± 0,042	31,93	30,16	10,18	27,73	0,0041	.	.	4,95	yes
	HWE	Control	1 h	1,09E+06 ± 3,03E+04 ^{b}	2 ± 0,058 ^{a}	52,14	35,78	4,56	7,52	1,94	.	.	53,03	yes
			30 min	1,15E+06 ± 1,89E+04 ^{b}	1 ± 0,018 ^{c}	45,22	37,45	.	17,32	0,00482	.	.	10,78	yes
			10 min	7,53E+05 ± 5,59E+04 ^{c}	2 ± 0,056 ^{a}	52,15	29,31	4,13	14,41	**nd**	.	.	2,23	yes
		Sequential	1 h	1,50E+06 ± 4,74E+03 ^{a}	2 ± 0,017 ^{a}	63,8	10,68	16,57	8,95	79,74	38,99	11,69	Gel	.
			30 min	1,60E+06 ± 2,40E+04 ^{a}	2 ± 0,026 ^{b}	40,32	57,91	.	1,77	30,28	32,61	13,18	Gel	.
			10 min	1,52E+06 ± 7,10E+03 ^{a}	2 ± 0,058 ^{a,b}	44,2	55,8	.	.	6,27	.	.	76,81	yes
	HAE	Control	1 h	5,54E+05 . ^{a,b}	4 . ^{a}	38,22	61,78	.	.	0,249	.	.	4,47	no
			30 min	4,04E+05 ± 2,40E+04 ^{a,b}	4 ± 0,444 ^{a}	41,29	58,71	.	.	0,0579	.	.	1,7	yes
			10 min	. . .	. . .	.	.	.	.	.	.	.	.	.
		Sequential	1 h	6,70E+05 ± 4,24E+04 ^{a}	4 ± 0,081 ^{a}	47,53	48,37	.	4,1	0,111	.	.	11,41	no
			30 min	1,71E+05 ± 5,41E+04 ^{b}	4 ± 0,587 ^{a}	47,31	52,69	.	.	0,00125	.	.	1,27	no
			10 min	no information		40,74	59,26	.	.	0,0382	.	.	**nd**	no

Table 9. Peaks associated with carrageenan in the infrared spectra of different alga across specific wavelength regions. The intensity of the observed peaks is indicated by symbols: "-" (no peak), "+" (weak), "++" (moderate), and "+++" (strong), with "no data" representing samples that were not subjected to FTIR analysis. The yellow columns are the ones related to κ or/and ι-carrageenan, and the white columns relate to mu and/or nu-carrageenan

Algae	Extract	Type	time	Extracted K2-carrageenans						
				Peaks at the wavelengths (cm^{-1})						
				1240-1260	970-975	930	845	825-830	815-820	805
Mastocarpus Stellatus	**CWE**	Sequential	16h	-	-	-	-	-	-	-
	HWE	Control	1 h	+	+	++	++	+	+	+
			30 min	+	-	++	++	+	-	+
			10 min	no data						
		Sequential	1 h	+	+	++	++	-	-	+
			30 min	+	+	++	++	-	-	+
			10 min	no data						
	HAE	Control	1 h	++	+	-	+	-	-	+
			30 min	-	+	+	++	-	+	+
			10 min	no data						
		Sequential	1 h	+	+	++	+++	-	-	+
			30 min	+	-	-	++	-	+	+
			10 min	no data						
Chondrus Crispus	**CWE**	-	16 h	+	-	++	++	-	-	+
	HWE	Control	1 h	+++	+	+++	+++	-	-	++
			30 min	++	+	+++	+++	-	-	++
			10 min	+++	+	+++	+++	-	-	++
		Sequential	1 h	+++	+	+++	+++	-	-	++
			30 min	+++	+	+++	+++	-	-	++
			10 min	+++	+	+++	+++	-	-	++
	HAE	Control	1 h	+	+	++	++	-	-	+
			30 min	+	+	++	++	-	-	+
			10 min	+	+	++	++	-	-	+
		Sequential	1 h	+	-	-	+	-	-	+
			30 min	+	-	+	+++	-	-	+
			10 min	-	+	+	+++	-	-	+
Gigartina pistillata	**CWE**	-	16 h	+	-	++	-	+	-	+
	HWE	Control	1 h	+	-	++	+	-	-	+
			30 min	+	+	++	+	-	-	+
			10 min	+	+	++	+	-	-	+
		Sequential	1 h	-	-	++	+	+	+	-
			30 min	-	+	++	+	+	+	+
			10 min	-	+	++	+	+	-	-
	HAE	Control	1 h	+	+	+	++	+	-	+
			30 min	+	-	-	-	-	-	+
			10 min	no data						
		Sequential	1 h	+	+	+	-	-	-	+
			30 min	+	+	+	++	-	+	+
			10 min	no data						

4.1.5 Influence of the sequential steps

The liquid extracts obtained during the initial cold water extraction (CWE) with *C. crispus* and *G. pistillata* were analysed to determine carrageenan content, and the presence of non-gelling carrageenan in these extracts was observed in the FTIR and NMR spectra. This is in agreement with Bahari et al.

(2021), who reported approximately 5 % yield of carrageenan, from *C. crispus,* following an 8 h extraction at 22°C, plus an additional 2 h extraction at the same temperature. The previous study confirmed that a small fraction of carrageenan, particularly non-gelling, L-carrageenans, as determined from FTIR, can be recovered under room-temperature conditions.

No detectable K2-carrageenan were found in the extract from *M. stellatus*, as the liquid failed to form a film upon drying. This result strongly suggests that the CWE in *M. stellatus* has no impact on the recovery of K2-carrageenans. Thus, the total carrageenan yield of nearly 50 % of the previous algae in both SE processes (HWE and HAE) is in harmony with the carrageenan yields reported elsewhere for hours long alkali extraction routes (Azevedo et al., 2013), whereas it is almost twice the yield reported more recently for a HWE performed at 80-90 °C for a total of 3 h (Sanz et al., 2023).

In the case of *G. pistillata* and *C. crispus*, both κ- and ι-carrageenans were identified alongside their precursors, mu- and nu-carrageenans. The FTIR band at 825–830 cm^{-1} for *G. pistillata* is indicative of the substantial presence of nu-carrageenan, corroborated by the ^{1}H-NMR spectrum. Moreover, the characteristic peak at 5,54 ppm in the ^{1}H-NMR spectra confirmed the presence of L-carrageenans in these extracts but is not the major component as claimed elsewhere from FTIR spectra (Bahari et al., 2021). Essentially, CWE products from *G. pistillata* are more sulphated than the sequential and control HWE products.

The Mw of the carrageenans extracted through CWE was found to be comparable to those obtained from HAE for the same species, suggesting that the cold-water process is capable of recovering carrageenan chains of similar lengths, but it needs a longer time. However, PDI of the CWE extracts from *C. crispus* was notably high (4 ± 0,038), reflecting a broad distribution of molecular masses, likely influenced by the mild extraction conditions and the presence of precursors and L-carrageenans.

An important observation is that the removal of carrageenan during the CWE could be a contributing factor to the reduced sulphation levels observed in subsequent SE steps. The preferential extraction of more soluble and highly sulphated carrageenans in the CWE may leave behind a carrageenan fraction richer in less sulphated hybrid carrageenans. This hypothesis aligns with the findings of lower sulphate content and enhanced rheological properties in SE extracts. So, the removal of precursors during CWE not only shifts the composition of subsequent extracts but also impacts their molecular characteristics, favouring the recovery of less sulphated carrageenans with improved gel-forming abilities.

Analysing the HWE results of *M. stellatus*, FTIR spectra revealed that SE carrageenans shared similar peaks with the controls. However, in the NMR spectra the 1 h SE extract exhibited a higher sulphate content than the control, at 30 min it was the opposite. In the subsequent HAE, alkali extracts (both SE and controls) didn't show precursors, aligning with expectations since the alkaline medium favours the extraction less sulphated carrageenans. An exception was observed for the 30 min control alkaline extract, where ^{1}H-NMR identified mu- and nu-carrageenans, suggesting that prior steps in the 30 min SE alkaline extract may have facilitated the recovery of less sulphated carrageenan in this step. Regarding the impact of the SE on Mw and PDI, water-based *M. stellatus* SE extracts displayed increased PDI, likely due to prior steps broadening the chain size distribution. The Mw of the 1 h SE water and alkaline extract was higher than its control, while for the 30 min, it was similar.

For *C. crispus*, FTIR spectra showed minimal differences between water-based SE and control extracts. However, ^{1}H-NMR revealed that all the water extracts contained precursors (less than 16 mol% in total), with the SE extracts having a slightly lower molar content of precursors than controls. Accordingly, gels are obtained with SE HWE extracts in contrast to the liquids obtained with extracts from control experiments, as will be discussed in section 4.1.8 Texturizing behaviour. In the alkaline extraction step, differences were observed in FTIR signals at 930 cm^{-1} and 845 cm^{-1} between SE and controls. However, since these signals are common across carrageenans, it's challenging to attribute them to specific chemical changes caused by SE. Notably, ^{1}H-NMR showed stronger κ-carrageenan signals in controls, which also contained mu-carrageenans absent in SE extracts. Together with the computed yields, this again suggests that more sulphated carrageenans were extracted during the HWE step, whereas parts of such chains still resist the alkali treatment and are thus extracted during the control experiments. With respect to the molecular mass distribution, the sequential approach in both HWE and HAE has no impact, except for shorter times with HWE and longer times for HAE, where sequential extractions gave longer chains than the control in the HWE and vice-versa in the HAE. The PDI values remained insensitive to the extraction protocols for *C. crispus*.

For *Gigartina pistillata*, SE products from HWE performed at shorter times contained fewer precursors than the controls. In the alkaline step, SE and control extracts were largely similar. In this alga, SE water extracts consistently displayed higher Mw than controls, suggesting that the SE sequence positively impacted Mw recovery. In alkaline extractions, the Mw of SE and control samples were quite similar.

In summary, Mw trends suggest that SE generally contributes to a more consistent recovery of high Mw carrageenans compared to controls, particularly during the HWE step. This effect was more pronounced in *M. stellatus* and *G. pistillata*, while *C. crispus* exhibited more stable Mw across conditions. Combining these observations with FTIR and ^{1}H-NMR data, it is evident that SE enhances the recovery of less sulphated carrageenans, especially in shorter extraction times. This suggests that the CWE step likely facilitated the leaching of more sulphated polymers which are not found in the subsequent extractions. Furthermore, SE extracts demonstrated superior rheological properties, with higher G' in gels and higher (or similar) viscosity in liquid states compared to their respective controls. These findings highlight the benefits of incorporating sequential extraction for improved carrageenan quality, particularly in optimizing Mw, sulphation levels, and rheological performance.

4.1.6 Effect of time

For this thesis, three extraction times were study: 1 h, 30 min and 10 min. This parameter affects the yield of the extraction as discussed before, but also the chemical composition of the type of carrageenans extracted.

For the HWE *M. stellatus* results, the ^{1}H-NMR reveals that the 30 min water SE K2-carrageenan, had no precursors (mu and nu), whereas 1 h had a 32,13% molar content of precursors. A similar trend is observed in the control samples, where the 1h extraction results in the removal of more precursors than the 30 min extraction (13,64 % molar and 3,84 % molar, respectively). Finally, *M. stellatus* is not suited for extraction times as short as 10 minutes, once the yield was very low and the extracts that were evaluated didn't show K2-carrageenan. As for the *C. crispus*, ^{1}H-NMR reveal that at shorter times (30 min and 10 min) of extraction the SE water extracts had less precursors, which is reminiscent from the results found for *M. stellatus*. However, the opposite is found for the controls. In the alkaline step, no precursors were detected for the two last algae. And it seems that for *C. crispus* the 10 min extraction delivers a κ-richer K2-carragenan than the 1 h, in the SE and controls. For *G. pistillata*, smaller SE HWE extraction times led to lower precursor contents (see FTIR with less pronounced bands symptomatic of sulphated groups and NMR results), in concordance with the other two algae. In the alkaline step, the chemical composition of K2-carragenans does not seem to be affected by the time, with the exception of a small molar percentage (4%) of nu in the 1h SE alkaline extracts.

So, the general trend is that with more time, more carrageenan (see Figure 12) with more sulphate is leached out from the seaweeds during the HWE. Practically, and in view the optimized

texturing properties of carrageenans, more extraction time brings benefits when alkaline is used as more carrageenan (see Figure 13) with less precursors are recovered.

As for the impact of time in the molecular mass, for *M. stellatus*, longer extraction times consistently leads to the isolation of longer or similar hybrid carrageenan chains with identical PDI. The same trend is found with *C. crispus,* except for K2-carrageenans obtained from the sequential HAE where smaller chains are recovered at longer times. Note that this may explain the corresponding small carrageenan yield as smaller chains are more difficult to precipitate. In *G. pistillata*, SE water extractions had a similar Mw across the time. As for the alkaline SE extracts there is an increase Mw over time, while the PDI maintains consistent. So, the overall trend is that the lower Mw polysaccharides are solubilized first at earlier times. These results also reveal that the time of extraction, besides affecting the yield and chemical structure of the K2-carrageenan, also significantly influence Mw. And consequently, the G' and the η was higher for the extracts with longer extraction times, comparing to the samples with similar conditions.

4.1.7 Natural carrageenans (HWE) vs less sulphated carrageenans (HAE)

It is well-established in the literature that K2-carrageenans extracted with alkaline solvents are less sulphated, while those obtained through water extraction, reflect their natural, more sulphated state (Souza et al., 2023). Comparing natural (from the HWE) and less sulphated K2-carrageenans (from the HAE) across the three algae species revealed that the main distinction in chemical composition is that HAE extracts are consistently less sulphated than HWE extracts, as expected.

The Mw of carrageenans within the same species was significantly higher (2–5 times) in HWE extracts compared to HAE extracts (see Table 8). This reduction in Mw, indicates that alkaline conditions promote carrageenan degradation, leading to lower molecular masses (Van De Velde et al., 2005; Souza et al., 2023). The broader PDI observed in some HAE extracts, highlights the degradation caused by alkaline conditions, which likely fragment carrageenan chains into molecules of varying lengths. This aligns with the expectation that alkaline treatments disrupt the structural integrity of natural carrageenans, leading to less uniform products (Van De Velde et al., 2005; Souza et al., 2023).

The comparison between natural and less sulphated K2-carrageenans underscores the complementary roles of water and alkaline extractions in modulating carrageenan properties. Water extractions retain the native, more sulphated form with higher Mw, while alkaline extractions favour desulphation and lower Mw. These findings suggest that strategic use of SE and extraction conditions can optimize the carrageenan recovery and tailor its properties for specific applications, offering valuable

insights into the biorefinery potential of red algae. For example, food-grade carrageenan typically has a molecular mass greater than 100 kDa, with commercial products ranging from 200–800 kDa (Torres, Flórez-Fernández, et al., 2019). So, from the same algae at different extraction times and with different solvents, different carrageenans can be obtained to satisfy various types of products.

4.1.8 Texturizing behaviour

The texturizing capacity of the carrageenan is a key factor in their commercial application and value. Quantitative results regarding the texturizing behaviour (gel elasticity, viscosity and gelling temperature) can be observed in Table 8.

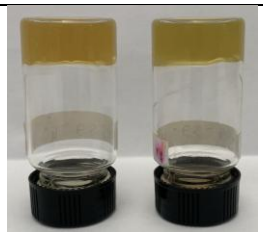
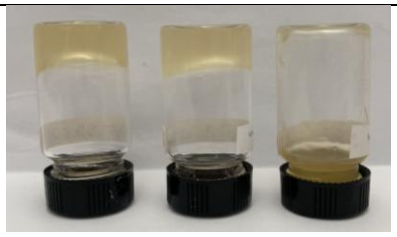
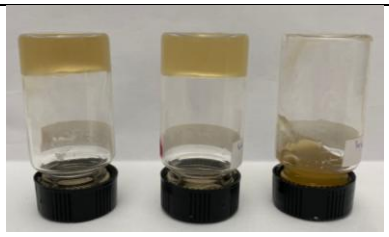
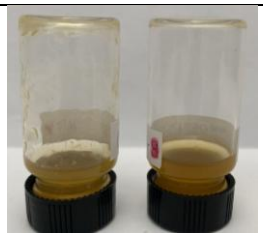
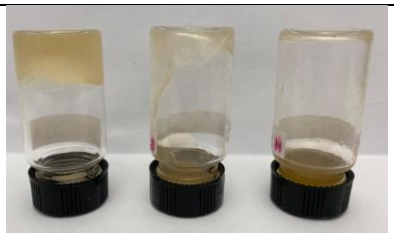
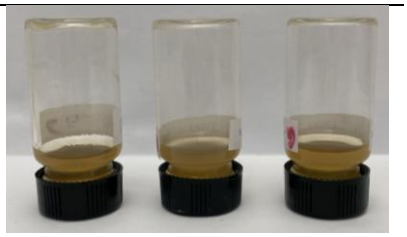
Table 10 shows that when 1 M NaCl was added to a 2% (w/v) carrageenan solution, all the samples of 1 h and 30 min hot water SE gelled after 24 h at 25 °C, in all species, whereas the 10 min hot water SE extraction samples remained liquid. In comparison, the control samples did not form gels, except for the 1 h hot water control from *C. crispus*, which exhibited a similar elastic modulus G_0 to its respective sequential extract (as showed in Table 8). So, overall, the "natural" K2-carrageenans obtained from the sequential HWE had better elasticity than the corresponding controls, suggesting that the sequential extraction process contributed to improve the gel quality. This is probably due to the lower amount in precursors for the SE extracts, as many of the carrageenan samples imaged in Table 10 show similar Mw but different gelling behaviour.

Results recently reported for a set of hybrid carrageenans extracted from commercial seaweeds with hot water, showed that when the hybrid carrageenan composition is close to a balanced amount in I- and K-carrageenans, no gel can be formed with 1 wt.% in 1 M NaCl (Moraes & Hilliou, 2024). This observation aligns with some of the non-gelling behaviour of the control samples, as suggested by the chemical characteristics presented in Table 8. If a threshold of at least 55 mol% of either I- or K-carrageenan is here hypothesized for gel formation in NaCl, the lack of gelation in the controls can be attributed to their composition, independently of the Mw effects. But it cannot explain the formation of gels in HWE SE samples, when they show a very close carrageenan composition and molecular masses to the respective controls.

In contrast, all HAE samples (both SE and control) remained liquid. While the HWE samples, with higher sulphate content (Table 8), were expected to exhibit weaker gels or lower viscosities due to the presence of carrageenan precursors incapable of gelling, the larger molecular mass of water-extracted K2-carrageenans likely contributed to gel formation. This highlights the significance of molecular mass in gel setting, as smaller polysaccharides from the alkaline extracts may struggle to aggregate or form

helices. Considering the above discussion for the HWE, the gel setting of hybrid carrageenans in NaCl depends on a complex interplay between the chemical structure, the length of κ and ι blocks and the molecular mass. As such, the gelling properties of these carrageenans (from the HWE and HAE) are better studied in the presence of KCl (Moraes & Hilliou, 2024) or even in mixtures of salts that favour the gelling of the K-carrageenan blocks (Van de Velde et al., 2005). Nevertheless, gelling in the presence of a single cation (Na^+, from NaCl and from the alkali chosen in extraction) was preferred here, in part to highlight the role of ι blocks in the hybrid carrageenan structure.

Table 10. Comparison of gels/liquids of solutions of carrageenan (2%(w/v) car + 1M NaCl), after cooling for 24 h at 25°C, from *Mastocarpus stellatus, Chondrus crispus,* and *Gigartina pistillata*, after different extraction times (1h, 30min, 10min) using HWE, at 90°C. The first row shows results from the sequential extraction (SE), while the second row displays the controls

Algae	*M. stellatus*		*C. crispus*			*G. pistillata*		
Time	1h	30min	1h	30min	10min	1h	30min	10min
SE								
Control								

Gels

The gelling temperature (Tg) is reported here as the temperature for the gel point, where the formation of helices and junction zones upon cooling leads to the development of a three-dimensional elastic network. For *C. Crispus*, for both the control and sequential 1 h hot water extracts, the graphs did not show a clear crossover point where G' equals G'', being the G' always higher than the G'', in the study temperature range (25 °C – 85 °C). In other words, the samples are never truly liquid, making it impossible to determine Tg accurately. So, it is assumed that Tg is higher than 85 °C (see Table 8), see Appendix H for more detailed information about the rheological behaviour of the samples.

When extraction time doubled from 30 min to 1 h, the Tg for *M. stellatus* decreased, this coincided with the presence of precursors (more than 30 mol %) in the 1 h K2-carrageenan extract, whereas no precursors were detected in the 30 min extract. In contrast, for *G. pistillata* and *C. crispus*, Tg increased with extended extraction time, even though the 1 h K2-carrageenan extracts also exhibited higher precursor contents, similar to *M. stellatus*. Prior research suggests that for K2-carrageenans containing only κ- and ι-carrageenan, Tg remains independent of carrageenan composition (van de Velde, 2005). Similarly, studies by Hilliou et al. (2006) and Azevedo et al. (2013, 2015) found no correlation between Tg and the chemical structures of hybrid carrageenans from *M. stellatus*, *C. crispus*, and *Ahnfeltiopsis devoniensis*, which displayed compositions similar to those shown in Table 8. Also, Hilliou et al., (2006) concluded that the time of extraction (30 min – 6 h) didn't seem to affect the Tg. Thus, the lack of a clear relationship between Tg and chemical structure here is consistent with previous findings. Even so, the measured Tg listed in Table 8, ranging from 26 - 39°C, are similar to the Tg reported in the literature for similar gelling conditions (Souza et al., 2011) and such values are suited to a wide range of food and non-food applications (Torres et al., 2018). Also, K2-carrageenans with Tg above 85 °C can find applications in various industries due to their stability and functional properties under heat (Chen et al., 2022).

Among the samples that formed gels, the 1 h hot water SE extract of *M. stellatus* produced the most elastic K2-carrageenan, with a G_0 of 376,32 Pa, while the 30 min hot water SE extract of *G. pistillata* yielded the least elastic gel, with a G_0 of 30,28 Pa. These results highlight the impact of extraction conditions and algae on the structural and rheological properties of carrageenan, particularly its gel elasticity. In *M. stellatus* and *G. pistillata*, increasing the extraction time from 30 min to 1 h significantly improved gel elasticity, with G_0 increasing approximately 2,43-fold. However, in *C. crispus*, the opposite trend was observed: the 30 min SE extract exhibited higher G_0 than the 1 h extract, with the latter showing a reduction of more than half in gel elasticity. This divergence is consistent with prior studies, such as those by Van de Velde et al. (2005), which demonstrate a direct correlation between κ-carrageenan content and gel elasticity. The 30 min SE extract of *C. crispus* contained fewer precursors (2,73 mol % mu-carrageenan) and a higher κ content (68,66 mol %) than the 1 h extracts (SE and control), which showed higher precursor levels (7,66–10,78 mol %) and lower κ content (approximately 60 mol %) (show in Table 8). This chemical composition explains the superior elasticity of the shorter extraction time sample, as the increased precursor content in the 1 h extract likely hindered the formation of robust helices. The same G_0 enhancement with the larger content in κ-carrageenan is found within gelling

extracts from *M. stellatus* and *G. pistillata.* To note that although the 1h SE water extract from *G. pistillata* has a higher κ content and Mw compared to the same extract from *M. stellatus*, the PDI of the latter is more than four times higher, suggesting that the higher G_0 observed in *M. stellatus* could be attributed to its broader Mw distribution, indicating the presence of carrageenan with longer chains.

As seen before the rheological properties of K2-carrageenan gels vary significantly between algae and extraction conditions, these differences directly influence their potential applications. The highly elastic gel from *M. stellatus* (376,2 Pa) is ideal for firm food products like jellies and puddings, as well as durable non-food uses such as wound dressings and cosmetic hydrogels. In contrast, the softer gel from *G. pistillata* (30,28 Pa) suits spreads, drinkable gels, and flexible films. Also, the gel's yield strain, when G'=G'' (see Table 8) reflects its resistance to deformation before fluidization and is a key parameter for tuning texture and performance in various applications (Franck, n.d.). So, by optimizing extraction conditions, these gels can be tailored to meet specific industrial needs (Mugdha Bhat et al., 2020; Russo Spena et al., 2024).

Liquids

To further evaluate the rheological behaviour of the liquid carrageenan samples, viscosity (η) as a function of shear rate (γ̇) was analysed. This included both the ramp-up (0 to 1000 s^{-1}) and ramp-down (1000 to 0 s^{-1}) in shear rates, allowing for evidencing any flow hysteresis (see Table 11). The *C. crispus*, doesn't have any data for the alkaline samples, since there wasn't enough mass extracted to do the tests, as said before. As for the 10 minutes hot alkaline SE from the *G. pistillata* the rheometer was unable to detect the viscosity, as it was close to the instrument's sensitivity limit and similar to the viscosity of water.

For comparative purpose, the viscosity values at a shear rate of 500 s^{-1} are reported in Table 8, since the solutions show no flow hysteresis when the shear rate is higher. Additionally, the elastic moduli (G') at 1 Hz and 25°C, G_1, was extracted providing insight into their structural rigidity, and as expected the G' of the liquid samples are very low and close to the sensitivity limit of the rheometer, which may not fully represent the true elastic moduli. The rheological plots are given in Appendix H.

Analysing the viscosity ramps for hot water extracts, distinct behaviours were observed among the K2-carrageenan solutions from different species. For *M. stellatus*, both samples exhibited non-Newtonian behaviour, and clear hysteresis was observed. This hysteresis indicates that, under low shear rates, the viscosity did not recover its initial value within the experimental time frame, suggesting

structural changes under shear stress. These changes likely result from the breakdown or rearrangement of carrageenan aggregates at higher shear rates, a phenomenon consistent with the polymer's high molecular mass and aggregation propensity.

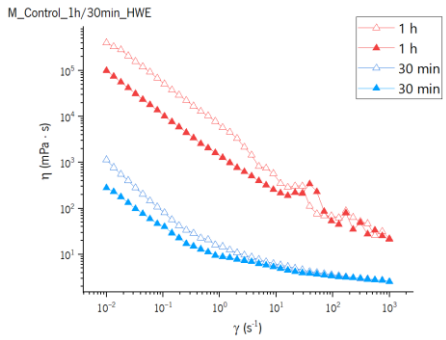
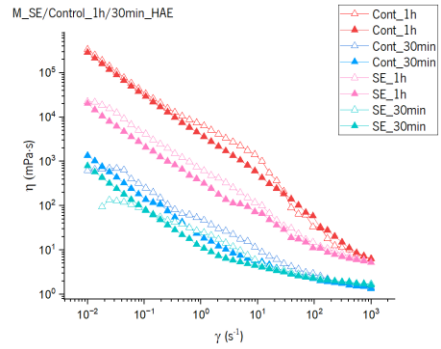
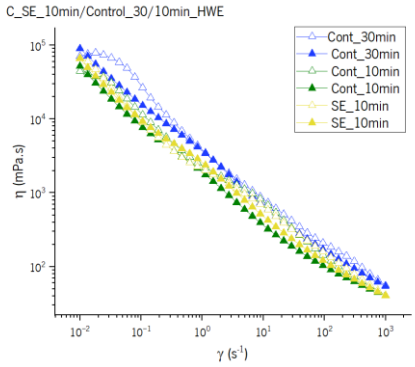
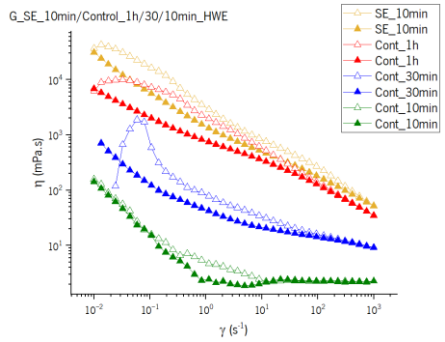
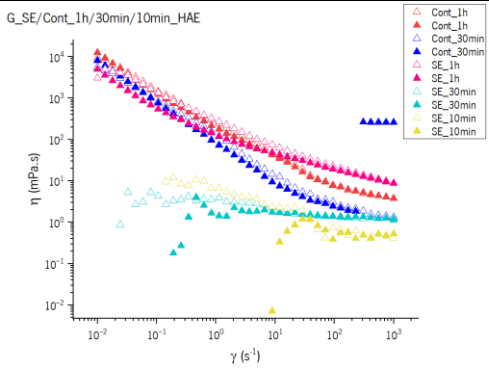
Conversely, for the same extraction (HWE), *C. crispus* K2-carrageenan solutions, while also non-Newtonian, exhibited limited hysteresis. For the 30 min control sample, the behaviour suggests less pronounced aggregation or smaller aggregate sizes that remain relatively unaffected within the applied shear rate range. This is likely due to the smaller molecular mass, or fewer aggregate-forming interactions compared to *M. stellatus* and *G. pistillata.* Indeed, for the *G. pistillata*, hysteresis was prominent in all HWE samples. The 30 min control sample exhibited a significant mismatch between flow curves near 0,01 s^{-1} shear rate, indicating more fragile carrageenan aggregates or a higher degree of breakdown under shear stress, limiting viscosity recovery. Interestingly, the 10 min control extract showed a viscosity approaching that of water (~1 mPa·s) (see Table 8), behaving as a Newtonian fluid at shear rates above 1 s^{-1}. At lower shear rates, the rheometer's torque sensitivity limited the measurements, resulting in curves with slopes approaching -1, for the previous sample. This behaviour highlights the influence of molecular structure and aggregation state on the observed viscosity profiles. Additionally, the hysteresis (Table 11) observed in the *M. stellatus* and *G. pistillata* extracts suggests that their carrageenans had a higher tendency to initially aggregate compared to *C. crispus*, that likely indicates that the higher molecular mass favours loose aggregation between chains, which are breaking down under shear stress, and need more time than the experimental time to re-aggregate at smaller shear rates.

For the HAE, *M. stellatus* sequential K2-carrageenan extracts and control samples also exhibited hysteresis. And, despite having similar molecular masses, differences in viscosity could be attributed to compositional variations, such as precursor content. In *G. pistillata*, the 30 min and 10 min SE extract behaved essentially as a Newtonian fluid , whereas data scattering at lower shear rates impedes a clear conclusion about flow hysteresis or limit of torque sensitivity.

Overall, when comparing liquid samples (Table 8), sequential extracts generally exhibited higher or comparable viscosities at 500 s^{-1} compared to controls. There is an exception for *M. stellatus*, the 1 h control extract showed slightly higher viscosity than its sequential counterpart, possibly due to a marginally higher κ-carrageenan molar content (~ 4% higher). Longer extraction times across all samples were associated with higher viscosities, attributable to the recovery of higher molecular mass carrageenans. Shorter extraction times yielded carrageenans with shorter chain lengths, which dissolved more readily but formed weaker structures. This aligns with studies showing that higher molecular mass

carrageenans contribute significantly to increased viscosity and elasticity (Campo et al., 2009; Van De Velde et al., 2005).

Table 11. Viscosity (η) as a function of shear rate (ɣ), showing possible hysteresis loops between a rise (0,01 s^{-1} to 1000 s^{-1} - empty symbols) and a drop (1000 s^{-1} to 0,01 s^{-1} - solid symbols) in shear rates, at 25 °C for K2-carrageenans 2 wt.% in 1 M NaCl solution, at different times of extraction and solvents, from three red algae

Algae	HWE	HAE
Mastocarpus stellatus		
Chondrus crispus		No information
Gigartina pistillata		

Carrageenans extracted overnight (CWE) remained in liquid form, exhibiting very low G' (Table 8) suggesting their inability to form gels under the study conditions. This behaviour may be due to higher sulphate content, lower molecular mass, and the presence of non-gelling L-carrageenans. Both polysaccharides displayed Newtonian-like fluid behaviour (Figure 14), with low shear rate data reflecting

the rheometer's torque limitations. These findings align with the understanding that molecular structure, including sulphation patterns and molecular mass distribution, plays a critical role in carrageenan functionality.

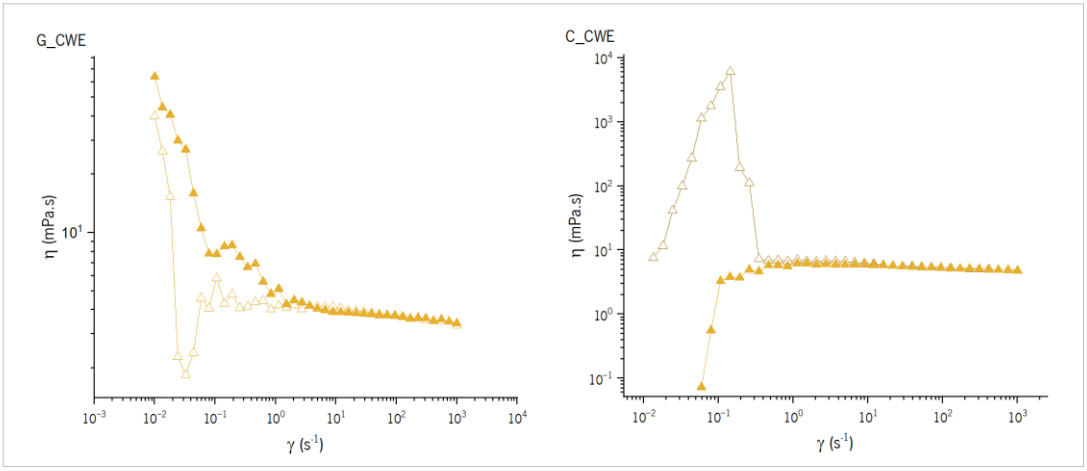


Figure 14. Viscosity (η) as a function of shear rate (γ), showing a transition from shear-thickening behaviour (0,01 s^{-1} to 1000 s^{-1}, empty symbols) to shear-thinning behaviour (1000 s^{-1} to 0,01 s^{-1}, solid symbols), at 25 °C for carrageenan (of the 16h overnight cold water extraction) from *Gigartina pistillata* (left) and *Chondrus Crispus* (right), in 2 wt.% in 1 M NaCl solution.

4.2 Impact of the Molecular Mass on the elasticity (G')

As discussed in the previous chapter, the molecular mass, Mw, seems to play a significant role in affecting the elasticity of the gel (indicated by G'). However, there are no specific studies focusing on the impact of the Mw of the K2-carrageenans on the elasticity of the gel, and various factors of extraction, such as the time of extraction, can have an impact of the molecular mass, as seen before. The point conclude from the previous discussion is that lower Mw seems to lower the G' and η, with the chemical structure of the carrageenans also playing a part. So, in addition to this work, five different K2-carrageenan samples were extracted during 4 h in natural (water) extraction (3 h at 80 °C + 1 h at 90 °C), followed by an alkaline treatment (3% (w/v) KOH for 3 h at 85 °C). This extraction route leads to the recovery of carrageenan with a cation K^+ as counter ion, which allows for the gel setting in the presence of KCl. Thus, a single cation which favours the gel elasticity of the K-carrageenan blocks can be used. The chemical composition of these carrageenans, as well as the code names of the algae from which they were extracted, are provided in Table 12.

Table 12. Chemical composition (in mol. %) of the K2-carrageenans extracted from: B-87, *Gigartina, Mastocarpus*, BAT 18-09 and C-25

Algae (Code)	κ (% molar)	ι (% molar)	mu (% molar)	nu (% molar)
B-87	4 ± 2,5	96 ± 5	-	-
Gigartina	42 ± 1,8	39 ± 2,1	-	19 ± 5
Mastocarpus	69 ± 0,4	31 ± 2,2	-	-
BAT 18-09	56 ± 2,9	34 ± 4,7	4 ± 1	6 ± 1
C-25	90 ± 4,5	10 ± 5	-	-

These initial hybrid carrageenan samples from different algae were subjected to ultrasonication with the aim of breaking down the molecular chains. The results of this process are discussed below.

4.2.1 Molecular mass (Mw) and Polydispersity (PDI) of ultrasonicated K2-carrageenans

In Figure 15, the molecular mass and polydispersity index are displayed after varying times of ultrasonication. For more detailed information, including the number-average molecular mass (Mn), refer to Appendix I. Generally, the results indicate that Mw decreases or remains steady over the course of ultrasonication, with a few anomalies observed at 2 and 4 h for B-87, 16 h for *Mastocarpus*, and 14 h for BAT 18-09, where the Mw seems to increase slightly. However, these data seem to be outliers, as Mw continues to decrease at longer times, except for B-87 where Mw remains essentially at $2{,}5 \times 10^5$ g/mol. These outliers may result from temporary re-aggregation of carrageenan fragments, during the sample preparation for Mw determination by size exclusion chromatography, where broken chains bond or entangle due to secondary interactions such as hydrogen bonding (Levy-Ontman et al., 2023), causing measurement inconsistencies. Indeed, these five hybrid carrageenans samples, originating from separate flasks and subjected to prolonged ultrasonication times, were not processed continuously due to the lengthy treatment duration. This discontinuity may have favoured chain re-aggregation during rest periods, potentially affecting the subsequent ultrasonication and thus Mw measurements.

As for the initial, non-ultrasonicated, molecular mass (Mw at 0h) of the K2-carrageenan from algae B-87, no data is yet available due to issues in the serviced experiments, and so no data is shown in Figure 15. But after an 1 h of ultrasound treatment, the Mw of the ultrasonicated K2-carrageenans from B-87 appears to stabilize over time. This trend is also observed in *Gigartina* samples, where Mw seems to reach a plateau after 1 h, after a “big drop” of more than a 60% reduction of the Mw, but after

4 h it seems to reduce again. As indicated in Table 12, the K2-carrageenans from these two algae (B-87 and *Gigartina*) contain the highest ι-carrageenan content from the study group, suggesting that ι-fraction is less resistant to ultrasonication when compared to the κ-fraction. However, such conclusion is pending the Mw data of the non-ultrasonicated B87 sample, to confirm that ι-rich hybrid carrageenans can be depolymerized within 1 h of ultrasonication. In contrast, the remaining K2-carrageenans, from three other algae with higher κ-carrageenan content (more than 50 mol%), show a different response. For *Mastocarpus*, BAT 18-09 and C-25, Mw decreases (in general) over time, in Figure 15.

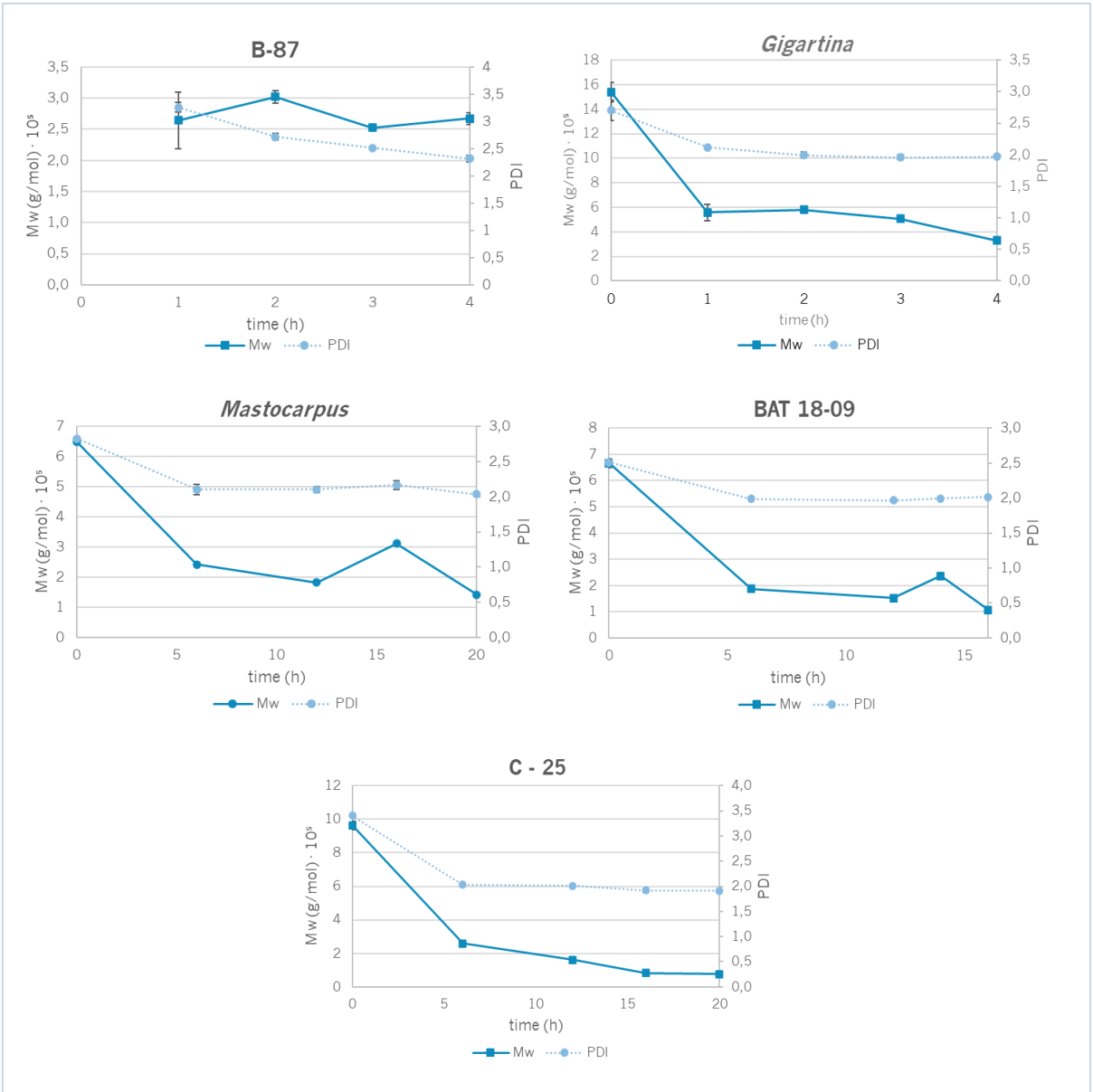


Figure 15. Molecular Mass (Mw) and polydispersity index (PDI) in function of the ultrasonication time, for the K2-carrageenan from the algae: B-87; *Gigartina; Mastocarpus;* BAT 18-09 and C-25.

After some time of ultrasound, a limit value of Mw for carrageenans with differing molar compositions appears to be reached. For algae B-87 and *Gigartina*, the Mw plateaus near $3 \cdot 10^5$ g/mol, while for *Mastocarpus*, BAT 18-09, and C-25 (richer in κ-carrageenan), it tends to be around $1 \cdot 10^5$ g/mol, a comparatively lower value (see Figure 15). Additionally, the presence of the nu precursor in *Gigartina* does not appear to significantly impact ι-fraction resistance. This may be due to the increased viscosity provided by highly sulphated precursors in aqueous solutions. Literature suggests that higher viscosity plays a role in the efficiency in ultrasonication, which could explain why nu-rich (19±5 mol%) samples like *Gigartina* retain higher Mw over time (Azizi & Farahnaky, 2016; Taghizadeh & Abdollahi, 2015). As for the PDI, after the initial drop within the first hours of ultrasonication, it stabilizes over time, staying close to 2 for all K2-carrageenan tested. This indicates that the distribution of molecular mass is uniform as ultrasonication progresses, suggesting a consistent reduction in larger chains without generating excessively small fragments due to the mechanical break-up of chain. Thus, the carrageenan chemical structure is not at play in the final PDI which reaches 2 for all samples.

4.2.2 The influence of the chemical structure

FTIR analysis of K-carrageenan ultrasonicated for 3 h (with a different equipment and at different temperature) by Tecson et al. (2021) suggests that, although the functional groups and molecular framework are retained, a decrease in the intensity at 843 cm^{-1} points to the partial cleavage of sulphate groups. The band at 843 cm^{-1} is assigned to the sulphate group of the G4S moieties, which are present in all carrageenans from the K family. Thus, this reduction could similarly affect the K2 structure, accompanying the gradual decrease in Mw with ultrasonication. Furthermore, NMR analysis from the previous study supports the hypothesis that ultrasonication predominantly cleaves O-glycosidic linkages while preserving the primary structural units of pure K-carrageenan (Tecson et al., 2021). This hypothesis suggests similar potential conditions influencing the degradation in K2-carrageenans during ultrasonication.

To investigate potential chemical changes during the mechanical breakdown, FTIR-ATR analysis was conducted on the K2-carrageenan samples at the initial time point (0 h) and after the final hour of ultrasonication, as shown in Figure 16, which highlights the spectral changes observed in the samples due to the process.

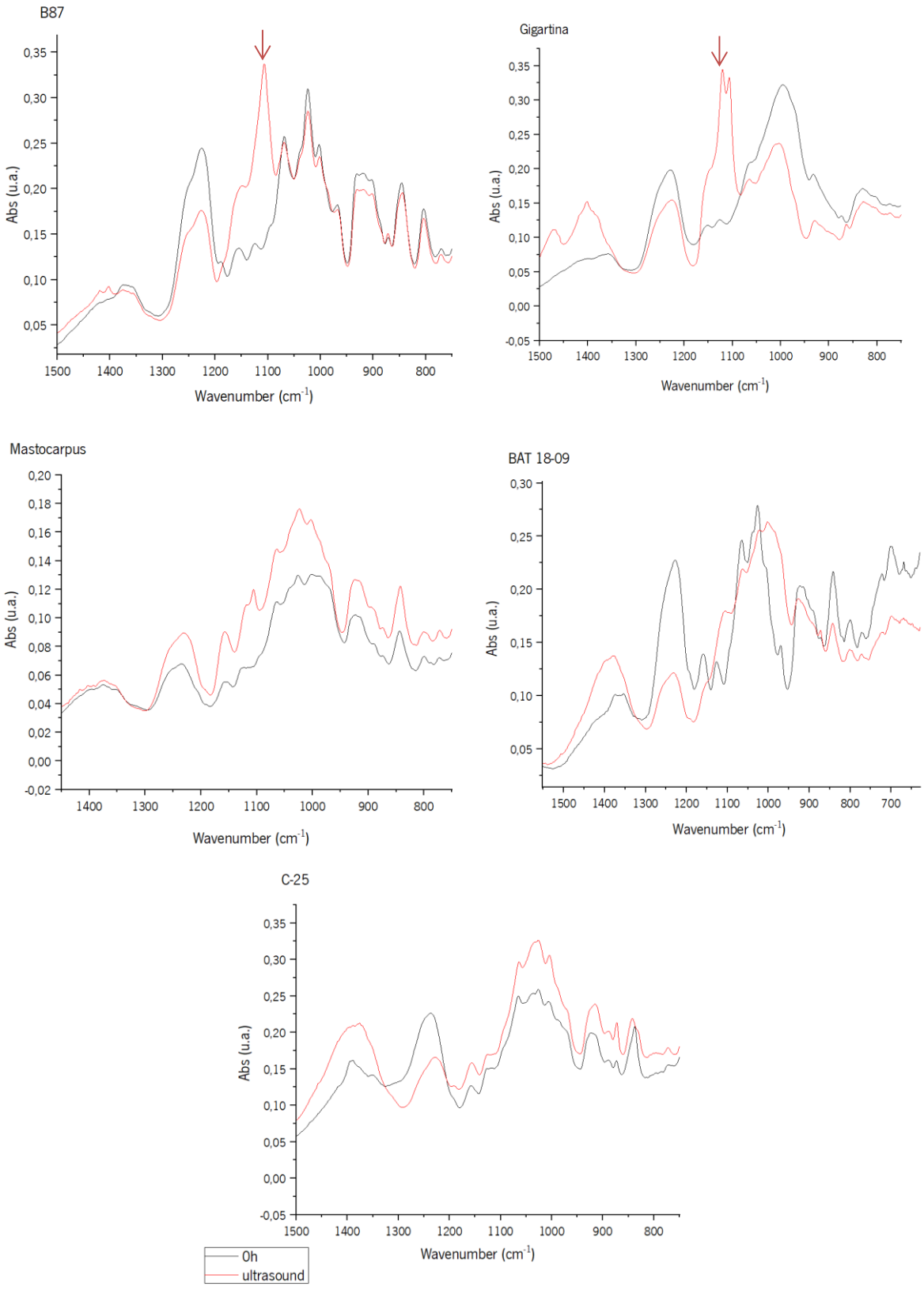


Figure 16. FTIR spectra of the non-ultrasonicated (black line) and ultrasonicated (red line) K2-Carrageenan from the algae B-87, *Gigartina*, *Mastocarpus*, BAT 18-09 and C-25.The red line in the first two spectra indicates the peak at 1100 cm^{-1}.

The results revealed notable alterations in the chemical composition, more pronounced in samples B-87 and *Gigartina*, where the appearance of a prominent absorption band around 1110 cm^{-1} was observed, after ultrasound. This band corresponds to O-H deformation, C-O stretching, and ring vibrations of polysaccharides, such as carrageenans (Masaki et al., 2017), that may result from the break-up of the glycosidic bonds. However, the modification in the 1110 cm^{-1} band was not observed in K2 with higher κ-carrageenan content. Additionally, evidence of desulphation was detected aligning with findings from the referenced study (Tecson et al., 2021). So, the structural modifications caused by the mechanical breakdown of the Mw encompasses the molecular structure of ultrasonicated hybrid carrageenans and thus impact their gelling properties (Souza et al., 2011). The stability and gelation performance of ultrasonicated hybrid carrageenans, therefore, appear to depend on both energy input and the different starting macromolecular chemical composition, as there is a distinctive sulphate composition provided by the κ and ι units in the K2-carrageenans, being that the κ-carrageenan has only one sulphate group and the ι-carrageenan has two sulphate groups (Souza et al., 2023). Although, the distinction in numbers of sulphate groups alone does not provide conclusive evidence for significant differences in their resistance to mechanical breakdown. Further chemical analyses are recommended to confirm these findings and provide deeper insight into the structural changes at each time point.

4.2.3 The impact of the Mw on the Rheology

Some carrageenans required no more than 2 h of ultrasonication to lose their gelling capability at 1 wt.% in 0,1 M KCl, as shown in Table 13, while others needed over 16 h to reach that state. This was expected, given their different initial chemical compositions and Mw. The table lacks some data due to some reasons: the B-87 samples were the only ones measured in a rheometer where the control of the gel thickness was different and didn't allow to extract Ton; for the *Gigartina* sample at 1 h, it was not possible to determine Ton, as the solution continued to increase in thickness throughout the tested temperature range (25 °C–80 °C). The corresponding rheological graphs from the different samples of each algae can be found in Appendix J

Table 13. Summary of κ-content (mol%), molecular mass (Mw), polydispersity index (PDI), phase behaviour post-gel test for (0,1% (w/v) carrageenan + 0,1 M KCl), elastic modulus G' (at 1 Hz, 25 °C, under 0,1% or 50,3% strain for gels (G_0) and liquids (G_1), respectively), gelation temperature (Tg), onset temperature (Ton) of the gels, and viscosity (η) at high shear strain for the liquids samples of the different K2-carrageenans ultrasonicated at various times. With "nd" representing no data and grey lines representing the gel samples. For each carrageenan and time point, values of Mw and PDI with different letters indicate statistically significant differences ($p < 0,05$)

Hybrid Carrageenans		κ (mol %)	Mw (g/mol) · 10^5	PDI	Phase (1%(w/v) car + 0,1 M KCl)	G_0/G_1(Pa)	Tg (°C)	TON (°C)	η_s (mPa/s) at 500 s^{-1}
Algae	Time (h)								
B-87	0	4 ± 2,5	nd	nd	Gel	20,76	>85	nd	-
	1		2,64 ± 0,46 ^{a}	3,26 ± 0,09 ^{a}	Gel	25,05	>85	nd	-
	2		3,02 ± 0,10 ^{a}	2,72 ± 0,06 ^{a}	Liquid	0,0322	-	-	10,09
	3		2,52 ± 0,01 ^{a}	2,51 ± 0,00 ^{a}	Liquid	0,0466	-	-	9,65
	4		2,67 ± 0,10 ^{a}	2,32 ± 0,06 ^{a}	Liquid	0,856	-	-	12,06
Gigartina	0	42 ± 1,8	15,40 ± 0,81 ^{a}	2,70 ± 0,16 ^{a}	Gel	638,19	60,03	53,27	-
	1		5,57 ± 0,67 ^{b}	2,12 ± 0,00 ^{a}	Gel	112,96	55	nd	-
	2		5,79 ± 0,04 ^{b}	2,00 ± 0,05 ^{a}	Liquid	1,64	-	-	26,97
	3		5,07 ± 0,03 ^{b,c}	1,96 ± 0,03 ^{a}	Liquid	0,976	-	-	24,37
	4		3,29 ± 0,01 ^{c}	1,97 ± 0,01 ^{a}	Liquid	0,655	-	-	17,03
Mastocarpus	0	69 ± 0,4	6,49 ± 0,06 ^{a}	2,83 ± 0,03 ^{a}	Gel	3518,53	57,68	55	-
	6		2,42 ± 0,01 ^{c}	2,11 ± 0,07 ^{a}	Gel	4232,02	62,07	56,56	-
	12		1,83 ± 0,01 ^{d}	2,11 ± 0,04 ^{a}	Gel	3542,98	65,61	52,96	-
	16		3,12 ± 0,03 ^{b}	2,17 ± 0,06 ^{a}	Gel	2189,33	62,92	51,07	-
	20		1,42 ± 0,01 ^{e}	2,04 ± 0,02 ^{a}	Liquid	4,85	-	-	32,79
BAT 18-09	0	56 ± 2,9	6,66 ± 0,17 ^{a}	2,51 ± 0,01 ^{a}	Gel	4235,96	62,76	54,68	-
	6		1,87 ± 0,06 ^{c}	1,99 ± 0,01 ^{a}	Gel	809,77	58,36	43,7	-
	12		1,53 ± 0,02 ^{d}	1,97 ± 0,00 ^{a}	Gel	4460,8	68,96	56,56	-
	14		2,37 ± 0,04 ^{b}	2,00 ± 0,01 ^{a}	Gel	1188,12	66,78	50,91	-
	16		1,08 ± 0,00 ^{e}	2,01 ± 0,03 ^{a}	Liquid	0,00466	-	-	7,08
C-25	0	90 ± 4,5	9,64 ± 0,26 ^{a}	3,41 ± 0,00 ^{a}	Gel	5543,03	63,33	54,52	-
	6		2,59 ± 0,03 ^{b}	2,03 ± 0,01 ^{a}	Gel	3603,74	67,52	54,84	-
	12		1,61 ± 0,02 ^{c}	2,01 ± 0,01 ^{a}	Liquid	1,38	-	-	14,16
	16		0,83 ± 0,01 ^{d}	1,92 ± 0,03 ^{a}	Liquid	0,401	-	-	5,4
	20		0,77 ± 0,01 ^{d}	1,91 ± 0,03 ^{a}	Liquid	0,55	-	-	10,51

4.2.4 Storage Modulus, G'

The data in Table 13 reveals a clear correlation between molecular mass and the storage modulus (G'), representing the elasticity of each sample: as the Mw reduces, the lower the G'. The molecular mass dependence of G' is qualitatively in concordance with the single literature which concluded that the elasticity of samples of 1 wt.% K-carrageenan in 0,1 M KCl increases with larger Mw (Rochas et al., 1990). Such correlation is less evident for sample B-87 since the ultrasonication did not produce carrageenans with very different molecular masses.

These results (in Table 13) contradict the conclusions of the study of Souza et al. (2011) performed with hybrid carrageenans dissolved in a different salt, 0,1 M NaCl. Under such gelling conditions, the highest elasticity is obtained with lowest molecular mass. However, G' is found to decrease with the increase of the ι-content in hybrid carrageenans, having similar Mw (Souza et al., 2011). Here,

only two samples offer such a comparison but in KCl since *Mastocarpus* and BAT 18-09 show similar initial Mw. However, the hybrid carrageenan chemical composition does not show sufficient difference in the ι-content to draw a clear conclusion.

The 6h BAT 18-09 sample appears to be an outlier, in terms of the G_0, as although the Mw decreased, as expected, its G' value is notably lower, compared to other samples from the same alga with similar Mw. This suggests a possible experimental error, during the rheology phase, potentially related to the addition of the salt (0,1 M KCl). Given the fact that only a very small amount of KCl (0,0373 g) was required, there may have been inaccuracies in weighing, which could have affected the sample's rheological properties. Repeating this sample measurement would be necessary to verify if the result was an anomaly due to sample preparation or an actual material property change.

The differences in the behaviour of K2-carrageenan, from different algae, when exposed to ultrasonication may also be attributed to variations in helix aggregation within their carrageenan structures, where ι-helices might exhibit greater resistance to mechanical stress compared to κ-helices. This difference aligns with observations of I gels displaying more curved helix structures, in contrast to the rod-like helices and more complex, branched aggregation patterns seen in K gels (Hilliou, 2021). The single-helix mesh in I gels results in softer textures, whereas the super helical network in K gels, especially in the presence of K^+, produces firmer gels (Souza et al., 2023; Stephen et al., 2006). For K2-carrageenans, early studies suggest a structure resembling with K-carrageenan, but with additional branching and unique aggregation behaviour that complicates understanding its mechanical properties (Souza et al., 2023). But there is still a long way to go until a complete understanding of the interaction between such macromolecular structures and the properties of the K2 gel, so the discussion cannot go further from the helix perspective.

4.2.5 Critical Molecular Mass, Mc

According to Rochas et al. 1990, the storage modulus (G') increases steadily with Mw until reaching a critical value, Mc, where it plateaus for a given salt concentration. For pure K-carrageenan the Mc given was $1{,}8 \cdot 10^5$ g/mol. However, in this work it was not possible to obtain clear constant G' values for gels, making it difficult to find a Mc, as shown in Figure 17. However, Rochas et al. 1990 extrapolated from their rheological data on gels that for Mw below $0{,}4 \cdot 10^5$ g/mol, no K-carrageenan gels can be formed for 1 wt.% carrageenan in 0,1 M KCl. This critical mass is much below the range of Mw (between $1{,}6 \cdot 10^5$ g/mol and $2{,}6 \cdot 10^5$ g/mol, see Table 13 and Figure 17) that separates liquids from gels with sample C-

25 (the κ-richer K2-carrageenan). This suggests that the extrapolation proposed is not valid, or that the drop in the sulphate content in C-25 that accompanies the decrease in Mw impedes a fair conclusion on such critical mass.

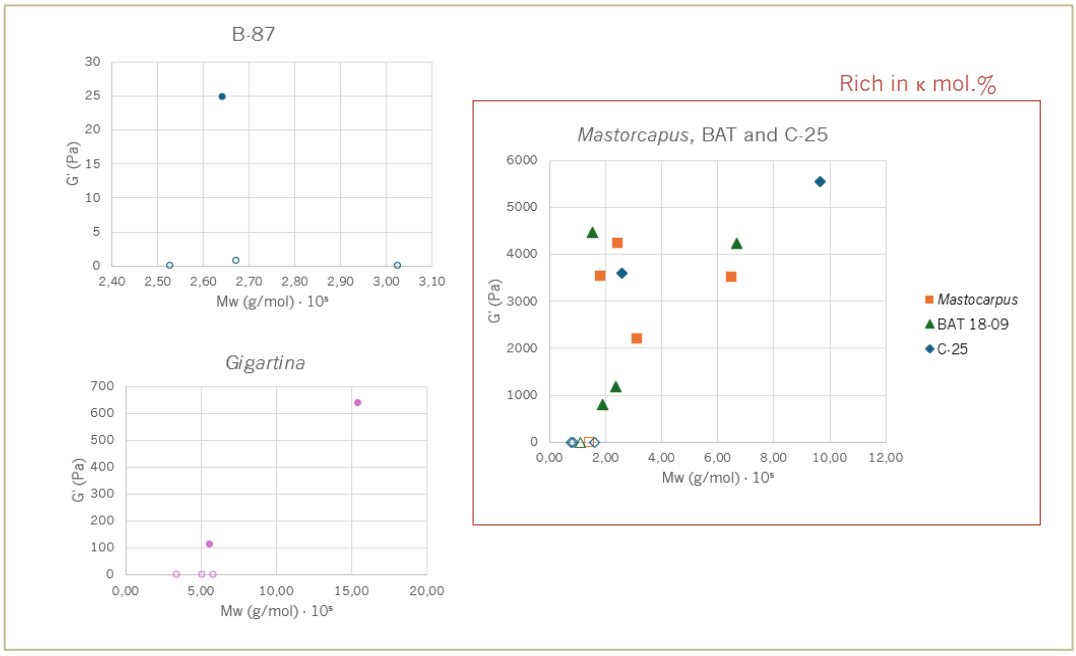


Figure 17. Storage modulus, G', as function of the molecular mass, Mw, of the different products before and after ultrasound (1 % (w/v) K2-carragenan + 0,1 M KCl) of the five different algae. The hybrid carrageenans with the initial higher molar content of κ-carrageenan, *Mastocarpus*, BAT 18-09 and C-25, had similar initial Mw and kind of similar behaviour, so it was possible to combine them in a graph, in the orange square. The gel samples are represented by the full symbols and the liquid sample as the empty symbols.

There is a possible secondary threshold Mc2, where (a very low) Mw stabilizes with further ultrasonication, showing little additional change over time, as seen in Figure 15, and discussed at the beginning of section 4.2.1, it seems that κ-rich samples (like Mastocarpus and C-25) had a Mc2 close to $1{\cdot}10^5$ g/mol, while more ι-rich hybrids, such as Gigartina and B-87, may have Mc2 close to $3{\cdot}10^5$ g/mol. The existence of an ultrasound resistant Mc2 may be possible, since the bonds that remain to be broken in carrageenans are very stable (e.g., C-C). Furthermore, more information is required to understand the rheological meaning of the value of Mc and its possible relation to the mesh size of the gel.

For κ-rich K2-carrageenans, the minimum Mw required for gel formation (hereafter referred to as Mwgel) at the study conditions, 1% wt. of K2carrageenan with 0,1 M KCl, appears to be very close to the critical molecular mass, Mc2. In contrast, B-87 K2-carrageenan, which is predominantly ι-

carrageenan (96 mol%), displays weak gelling properties from the outset. For this type, the liquid samples exhibit G' values that are relatively insensitive to Mw, likely because their Mw values (3,02–2,52·10^5 g/mol), which result in minimal elasticity, are near Mc2 (3·10^5 g/mol). However, a sample of B-87 forms a gel at Mw 2,64·10^5 g/mol, and when examining the PDI it is evident that liquid samples (PDI ≈ 2) have a narrower molecular size distribution compared to the gel-forming B-87 sample (PDI = 3,3), even though statically they are similar. This suggests that, even when the average Mw is near Mwgel, only a fraction of molecules with sufficiently high Mw may contribute to gelation. For K2-carrageenan derived from *Gigartina*, the transition from gel to liquid phase occurs at a Mw breakpoint near 5,5·10^5 g/mol (Mwgel), which is above Mc2 (3·10^5 g/mol). This discrepancy may stem from the presence of precursor molecules, specifically 19 ± 5 mol% nu-carrageenan. While nu-carrageenan does not directly affect Mc2, it may influence the rigidity of the polymer chains, thereby reducing the gelation ability of the sample.

4.2.6 Thermal behaviour of the gels

The Tg of *Gigartina* carrageenan samples decreases with a reduction in Mw, as seen in Table 13. However, for the more κ-rich K2-carrageenans (from *Mastocarpus*, BAT 18-09, and C-25), Tg tends to increase as Mw decreases, with BAT 18-09 6h standing out as an outlier in this group, as discussed before. A study by Van de Velde et al. (2005) on K2-carrageenans from *Mastocarpus*, with various chemical structures, reported that gelling temperature is independent of biopolymer chemistry in polysaccharides with more than 50 mol% κ-carrageenan disaccharide units. This suggests that Tg dependency in these carrageenans may be more influenced by Mw. Additionally, the gradual Tg variation of approximately 2 - 10 °C likely indicates shifts in the helical structure.

Ultrasonication time is also expected to affect TON by reducing Mw over time, yet the relationship between ultrasonication duration and TON appears inconsistent across samples. This indicates that the thermal properties of carrageenans may be sensitive to distribution and density of the polymer network rather than merely the average Mw value.

4.2.7 Viscosity of the liquids

The viscosity of the liquid solutions after varying durations of ultrasonication is shown in Table 13. In general, as molecular mass declines, viscosity also decreases due to the smaller size and lower sulphation (confirmed by the FTIR) of the K2-carrageenan molecules, which reduces resistance to flow, as the proportion of sulphate fractions and the equilibrium of cations in the water solution determine the

viscosity of solutions or strength of gels formed by carrageenans (Campos et al, 2009). For B-87, since the Mw remains relatively constant (close to $3 \cdot 10^5$ g/mol), viscosity also stays approximately the same.

The C-25 16h sample presents an unexpected result: its viscosity is less than half of the 20h sample, despite having a similar Mw and comparable elasticity (G_1) (as seen in Table 13). This discrepancy could be attributed to minor inconsistencies during sample preparation, such as slight variations in sample hydration, concentration, or solution heterogeneity. Additionally, this testing was done in the summer season, and the higher temperature of the cooling system may have lowered the viscosity. Repeating viscosity measurements and doing a chemical structure analysis for this sample could help determine whether this observation is due to experimental variability or a unique characteristic of the K2-carrageenan at this ultrasonication stage.

5. CONCLUSIONS AND FUTURE WORKS

In conclusion, the sequential extraction (SE) procedure and subsequent ultrasonication experiments collectively advance the understanding of optimizing K2-carrageenan extraction and molecular tailoring from carrageenophytes. In some algae, SE demonstrated yields of K2-carrageenans comparable to those obtained via conventional extraction methods with longer processing times, further supporting its industrial relevance.

The SE protocol proved highly effective in fractionating phycobiliproteins, chlorophyll-a, carotenoids, and carrageenans (natural and less sulphated) into distinct fractions, showcasing its potential for sustainable industrial applications. Its design aligns with biorefinery principles, promoting streamlined and efficient processes. While chlorophyll-a and carotenoids are co-extracted in the same solution, implementing advanced separation techniques like High-Performance Liquid Chromatography (HPLC) could provide deeper insights into their specific composition and concentrations. When comparing the efficiency and quality of SE to the control, of the carrageenans extracted, SE consistently facilitates the recovery of high molecular mass (Mw) K2-carrageenans during hot water extraction (HWE), particularly from *M. stellatus* and *G. pistillata.* Moreover, overall, SE extracts demonstrate superior rheological properties under the studied conditions, including enhanced gel elasticity and comparable or higher viscosities in liquid states. This is attributed to the pre-treatments, such as cold-water extraction (CWE), which yield less sulphated carrageenans in subsequent steps (in most cases), thereby improving gelling properties. Besides, K2-carrageenans extracted under alkaline conditions (HAE) are less sulphated but exhibit significantly lower Mw and lower rheological properties, compared to those obtained via HWE.

Ultrasonication effectively reduces molecular mass, a process strongly influenced by the chemical composition of K2-carrageenans, further elucidating the relationship between Mw and the elastic properties of gels. The reduction in Mw correlates with a decrease in gel elasticity, stabilizing near Mc2 (~$1 \cdot 10^5$ g/mol for κ-rich and ~$3 \cdot 10^5$ g/mol for ι-rich hybrids), where further degradation has minimal impact. Although a critical molecular mass (Mc) for gelation was not clearly defined, degraded K2-carrageenans exhibit reduced viscosity and gel elasticity, underscoring the essential role of molecular mass in determining functional properties.

This interplay between chemical composition and Mw highlights the complexity of optimizing extraction parameters to enhance both efficiency and product functionality. Extended extraction times typically result in higher molecular mass carrageenans with enhanced gel elasticity, though they also lead to higher sulphation levels, an apparent contradiction. Interestingly, *C. crispus* displayed an inverse

behaviour in the HWE. Additionally, shorter extraction times occasionally produced comparable yields, particularly in pigment extraction, underscoring the necessity for species-specific optimization. Achieving a balance between energy efficiency and extraction outcomes is essential.

The findings from the SE process and the ultrasonication study complement each other, offering a comprehensive understanding of how to optimize K2-carrageenan extraction and tailor its properties. This work underscores the critical influence of extraction sequence, duration, and solvent selection on product quality. By addressing key gaps in the literature, such as the impact of extraction pre-treatments on carrageenan sulphation and the relationship between Mw and gel functionality, it lays the groundwork for future innovations in carrageenophytes valorisation. Additionally, it contributes to the development of sustainable and cost-effective biorefinery methods, enhancing the valorisation of red seaweed through the extractive-extrusion approach.

Future Research

For future research, evaluating the gel strength of K2-carrageenan extracts in the presence of different cations (e.g., KCl) is also suggested to enhance their practical applications in various industries. Investigating the sequential extraction protocol's order, varying extraction times, and temperature ranges is also recommended to optimize yield and product quality. Additionally, comprehensive analyses of phenolic compounds and soluble proteins within the extracts should be performed, accompanied by bioactivity scans Assessing residual fractions, particularly the waste fraction, through weighing and compositional analysis could reveal new opportunities for valorisation, including the identification of bioactive compounds that support a holistic utilization of biomass resources. Furthermore, studies on alkaline extracts of *C. crispus* should be completed, particularly in areas where current data, limited by insufficient mass, does not allow for clear conclusions.

In alignment with the project under which this work is framed, future efforts are foreseen to adapt the sequential extraction protocol for use in an extruder. This adaptation could streamline the process, enhancing scalability and efficiency while maintaining eco-friendly practices. To achieve this, it will be necessary to investigate the operational parameters of extrusion and their impact on product quality and yield, refining the protocol to ensure its applicability to industrial processes.

For molecular mass studies focusing on the elastic properties of K2-carrageenan, it is essential to include pH monitoring throughout the process and perform chemical analyses on all samples to improve the accuracy of degradation assessments. Exploring additional conditions, such as varying the

types of salts and carrageenan concentrations, may provide a more comprehensive understanding of their effects on gel elasticity (G') and other rheological behaviours. Furthermore, evaluating additional time points during ultrasonication is recommended to better capture the progression of molecular degradation and its impact on functional properties. This approach would allow for a more detailed understanding of the degradation process. Finally, complementary studies on parameters like helix formation in K2-carrageenans could provide valuable insights into the complex interactions and structural dynamics of these polysaccharides. Such investigations would broaden the scope of research, offering a deeper understanding and expanding the potential applications of carrageenan-based materials.

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APPENDIX

Appendix A – Absorbance of the liquid extracts from the CWE

Calculation example of yield of the R-PE of the different extracts:

Sample: Test 1 of 60 min – *Mastocarpus stellatus*

$$R\text{-}PE\ (mg/mL) = 0{,}1247 \cdot [(A_{564} - A_{730}) - 0{,}4583 \cdot (A_{618} - A_{730})] \quad (1)$$

$$R\text{-}PE = 0{,}1247 \cdot [(0{,}0940 - 0{,}0170) - 0{,}4583 \cdot (0{,}0460 - 0{,}0170)] = 0{,}0079\ mg/mL$$

(0,0079 mg/mL) / (2,004 g/60 mL) = 0,2365 mg/g of R-PE.

Table A.1. Absorbance, yield and Standard Deviation (SD) of the R-PE extract from the CWE for the sequential extraction (60, 30 and 10 minutes) of algae *M. stellatus*

Algae		*Mastocarpus stellatus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,004	2,007	2,0015	2,0076	2,0083	2,001	2,0322	2,0168	2,0175
Cold Water Extraction (16h)	Abs 564	0,0940	0,1240	0,1340	0,2250	0,1840	0,1950	0,2020	0,2470	0,2100
	Abs 618	0,0460	0,0720	0,0740	0,1530	0,1140	0,1250	0,1370	0,1710	0,1410
	Abs 730	0,0170	0,0310	0,0320	0,0920	0,0610	0,0700	0,0830	0,1030	0,0840
	R-PE (mg/mL)	0,0079	0,0093	0,0103	0,0131	0,0123	0,0124	0,0118	0,0141	0,0125
	R-PE (mg/g)	0,2379	0,2767	0,3093	0,3915	0,3677	0,3731	0,3470	0,4186	0,3704
	Average R-PE (mg/g)	0,3436								
	SD R-PE (mg/g)	0,0580								

Table A.2. Absorbance, yield and Standard Deviation (SD) of the R-PE extract from the CWE for the sequential extraction (60, 30 and 10 minutes) of algae *C. Crispus*

Algae		*Chondrus crispus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0101	2,018	2,0267	2,0076	2,0083	2,001	2,0052	2,0044	2,0166
Cold Water Extraction (16h)	Abs 564	0,0870	0,1540	0,0940	0,2290	0,2120	0,2210	0,1150	0,2170	0,1780
	Abs 618	0,0670	0,1200	0,0720	0,1840	0,1700	0,1780	0,0880	0,1730	0,1410
	Abs 730	0,0440	0,0800	0,0480	0,1270	0,1190	0,1270	0,0570	0,1200	0,0980
	R-PE (mg/mL)	0,0040	0,0069	0,0044	0,0095	0,0087	0,0088	0,0055	0,0091	0,0075
	R-PE (mg/g)	0,1208	0,2064	0,1292	0,2828	0,2594	0,2641	0,1634	0,2714	0,2237
	Average R-PE (mg/g)	0,2135								
	SD R-PE (mg/g)	0,0624								

Table A.3. Absorbance, yield and Standard Deviation (SD) of the R-PE extract from the CWE for the sequential extraction (60, 30 and 10 minutes) of algae *G. pistillata*

Algae		*Gigartina pistillata*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0043	2,031	2,0618	2,0157	2,0045	2,0031	2,0019	2,0115	2,0123
Cold Water Extraction (16h)	Abs 564	0,1400	0,1420	0,1470	0,1680	0,1550	0,2000	0,1690	0,2050	0,2030
	Abs 618	0,0900	0,0920	0,0940	0,1120	0,0990	0,1260	0,1030	0,1340	0,1360
	Abs 730	0,0570	0,0590	0,0590	0,0710	0,0600	0,0770	0,0610	0,0820	0,0900
	R-PE (mg/mL)	0,0085	0,0085	0,0090	0,0098	0,0096	0,0125	0,0111	0,0124	0,0115
	R-PE (mg/g)	0,2534	0,2500	0,2611	0,2903	0,2879	0,3756	0,3317	0,3689	0,3418
	Average R-PE (mg/g)	0,3067								
	SD R-PE (mg/g)	0,0490								

Appendix B - Absorbance of the liquid extracts from the EE

For an example of the calculation see Appendix A, for more information.

1.Sequential Extraction

Table B.1.1. Absorbance, yield and Standard Deviation (SD) of the Chlorophyll-a and Carotenoid extract from the EE for the sequential extraction (60, 30 and 10 minutes) of algae *M. stellatus*

Algae		*Mastocarpus stellatus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,004	2,007	2,0015	2,0076	2,0083	2,001	2,0322	2,0168	2,0175
Ethanolic Extraction 95% (v/v)	Abs 480	0,056	0,068	0,086	0,066	0,103	0,081	0,084	0,073	0,069
	Abs 632	0,027	0,031	0,042	0,031	0,054	0,041	0,036	0,033	0,031
	Abs 652	0,06	0,067	0,086	0,063	0,094	0,076	0,083	0,073	0,069
	Abs 665	0,111	0,117	0,148	0,104	0,15	0,124	0,138	0,13	0,121
	Abs 750	0,002	0,003	0,007	0,006	0,017	0,014	0,001	0,002	0,002
	Chloro-a (µg /mL)	1,3383	1,3723	1,6971	1,1639	1,5720	1,3226	1,6121	1,5477	1,4320
	Chloro-a (µg /g)	40,0692	41,0245	50,8748	34,7860	46,9641	39,6582	47,5971	46,0452	42,5861
	Average Chloro-a (µg /g)	43,9895			40,4694			45,4095		
	SD Chloro-a (µg /g)	5,9820			6,1294			2,5653		
	Carot (µg/mL)	0,216	0,26	0,316	0,24	0,344	0,268	0,332	0,284	0,268
	Carot (µg /g)	6,4671	7,7728	9,4729	7,1727	10,2773	8,0360	9,8022	8,4490	7,9703
	Average Carot (µg /g)	7,9043			8,4954			8,7405		
	SD Carot (µg /g)	1,5072			1,6025			0,9501		

Table B.1.2. Absorbance, yield and Standard Deviation (SD) of the Chlorophyll-a and Carotenoid extract from the EE for the sequential extraction (60, 30 and 10 minutes) of algae *C. crispus*

Algae		*Chondrus crispus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0101	2,018	2,0267	2,0076	2,0083	2,001	2,0052	2,0044	2,0166
Ethanolic Extraction 95% (v/v)	Abs 480	0,029	0,028	0,025	0,047	0,027	0,029	0,035	0,032	0,032
	Abs 632	0,021	0,021	0,016	0,03	0,018	0,02	0,026	0,025	0,024
	Abs 652	0,043	0,045	0,035	0,057	0,038	0,042	0,048	0,046	0,043
	Abs 665	0,084	0,094	0,072	0,111	0,075	0,082	0,091	0,087	0,08
	Abs 750	0,003	0	0	0,001	0,002	0,002	0,007	0,007	0,008
	Chloro-a (µg /mL)	1,0163	1,1881	0,9069	1,3546	0,9170	0,9999	1,0559	1,0063	0,9069
	Chloro-a (µg /g)	30,3355	35,3265	26,8496	40,4844	27,3949	29,9807	31,5960	30,1218	26,9840
	Average Chloro-a (µg /g)	30,8372			32,6200			29,5673		
	SD Chloro-a (µg /g)	4,2607			6,9324			2,3555		
	Carot (µg/mL)	0,104	0,112	0,1	0,184	0,1	0,108	0,112	0,1	0,096
	Carot (µg /g)	3,1043	3,3300	2,9605	5,4991	2,9876	3,2384	3,3513	2,9934	2,8563
	Average Carot (µg /g)	3,1316			3,9084			3,0670		
	SD Carot (µg /g)	0,1863			1,3833			0,2556		

Table B.1.3. Absorbance, yield and Standard Deviation (SD) of the Chlorophyll-a and Carotenoid extract from the EE for the sequential extraction (60, 30 and 10 minutes) of algae *G. pistillata*

Algae		*Gigartina pistillata*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0043	2,031	2,0618	2,0157	2,0045	2,0031	2,0019	2,0115	2,0123
Ethanolic Extraction 95% (v/v)	Abs 480	0,158	0,108	0,185	0,12	0,083	0,072	0,098	0,12	0,064
	Abs 632	0,108	0,074	0,131	0,087	0,061	0,06	0,064	0,079	0,047
	Abs 652	0,251	0,174	0,295	0,199	0,139	0,135	0,148	0,185	0,11
	Abs 665	0,491	0,346	0,579	0,405	0,286	0,282	0,288	0,359	0,219
	Abs 750	0,004	0,002	0,003	0,003	0,003	0,008	0,003	0,003	0,004
	Chloro-a (µg /mL)	6,0822	4,3169	7,1785	5,0773	3,5907	3,5199	3,5561	4,4370	2,7142
	Chloro-a (µg /g)	182,0757	127,5297	208,8995	151,1313	107,4782	105,4347	106,5810	132,3486	80,9270
	Average Chloro-a (µg /g)	172,8350			121,3481			106,6189		
	SD Chloro-a (µg /g)	41,4645			25,8133			25,7108		
	Carot (µg/mL)	0,616	0,424	0,728	0,468	0,32	0,256	0,38	0,468	0,24
	Carot (µg /g)	18,4404	12,5258	21,1854	13,9306	9,5784	7,6681	11,3892	13,9597	7,1560
	Average Carot (µg /g)	17,3839			10,3924			10,8350		
	SD Carot (µg /g)	4,4254			3,2096			3,4356		

2. Controls

Table B.2.1. Absorbance, yield and Standard Deviation (SD) of the Chlorophyll-a and Carotenoid extract from the EE for the control extraction (60, 30 and 10 minutes) of algae *M. stellatus*

Algae		*Mastocarpus stellatus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Ethanolic Extraction 95% (v/v)	Algae mass (g)	2,0685	2,0323	2,0160	1,9999	2,0026	2,0100	2,0062	2,0011	2,0030
	Abs 480	0,0950	0,1020	0,0940	0,1280	0,1940	0,1020	0,0570	0,2510	0,1520
	Abs 632	0,0620	0,0640	0,0660	0,1120	0,1640	0,0830	0,0410	0,2330	0,1370
	Abs 652	0,1220	0,1390	0,1220	0,1420	0,2220	0,1220	0,0710	0,2670	0,1720
	Abs 665	0,2090	0,2460	0,2030	0,1880	0,3110	0,1820	0,1150	0,3150	0,2240
	Abs 750	0,0160	0,0060	0,0220	0,0870	0,1200	0,0530	0,0190	0,2020	0,1060
	Chloro-a (µg /mL)	2,3429	2,9032	2,1906	1,2261	2,3421	1,5802	1,1729	1,3397	1,4155
	Chloro-a (µg /g)	67,9582	85,7118	65,1968	36,7841	70,1728	47,1713	35,0773	40,1688	42,4001
	Average Chloro-a (µg /g)	72,9556			51,3760			39,2154		
	SD Chloro-a (µg /g)	11,1332			17,0869			3,7533		
	Carot (µg/mL)	0,316	0,384	0,288	0,164	0,296	0,196	0,152	0,196	0,184
	Carot (µg /g)	9,16606	11,3369	8,57143	4,92025	8,86847	5,85075	4,54591	5,87677	5,51173
	Average Carot (µg /g)	9,6915			6,5465			5,3115		
	SD Carot (µg /g)	1,4557			2,0640			0,6877		

Table B.2.2. Absorbance, yield and Standard Deviation (SD) of the Chlorophyll-a and Carotenoid extract from the EE for the control extraction (60, 30 and 10 minutes) of algae *C. crispus*

Algae		*Chondrus crispus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Ethanolic Extraction 95% (v/v)	Algae mass (g)	2,0078	2,0014	2,0010	2,0060	2,0240	2,0277	2,0108	2,0131	2,0250
	Abs 480	0,0400	0,0340	0,0440	0,0760	0,1040	0,0990	0,0470	0,0530	0,0940
	Abs 632	0,0290	0,0280	0,0360	0,0720	0,1000	0,0950	0,0440	0,0480	0,0790
	Abs 652	0,0470	0,0390	0,0480	0,0780	0,1060	0,1020	0,0510	0,0570	0,0990
	Abs 665	0,0790	0,0580	0,0690	0,0900	0,1180	0,1160	0,0630	0,0720	0,1350
	Abs 750	0,0120	0,0160	0,0240	0,0630	0,0910	0,0850	0,0350	0,0360	0,0550
	Chloro-a (µg /mL)	0,8215	0,5038	0,5467	0,3183	0,3183	0,3680	0,3284	0,4180	0,9547
	Chloro-a (µg /g)	24,5497	15,1043	16,3933	9,5218	9,4372	10,8896	9,7981	12,4598	28,2887
	Average Chloro-a (µg /g)	18,6824			9,9495			16,8489		
	SD Chloro-a (µg /g)	5,1219			0,8152			9,9962		
	Carot (µg/mL)	0,112	0,072	0,08	0,052	0,052	0,056	0,048	0,068	0,156
	Carot (µg /g)	3,34695	2,15849	2,3988	1,55533	1,5415	1,65705	1,43227	2,02672	4,62222
	Average Carot (µg /g)	2,6347			1,5846			2,6937		
	SD Carot (µg /g)	0,6284			0,0631			1,6964		

Table B.2.3. Absorbance, yield and Standard Deviation (SD) of the Chlorophyll-a and Carotenoid extract from the EE for the control extraction (60, 30 and 10 minutes) of algae *G. pistillata*

Algae		*Gigartina pistillata*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Ethanolic Extraction 95% (v/v)	Algae mass (g)	2,0510	2,0268	2,0000	2,0356	2,0421	2,0051	2,0038	2,0015	2,0039
	Abs 480	0,2280	0,0960	0,1360	0,1660	0,1740	0,3870	0,1000	0,0970	0,0980
	Abs 632	0,1160	0,0520	0,0720	0,1100	0,1110	0,2930	0,0510	0,0460	0,0450
	Abs 652	0,2550	0,1130	0,1580	0,1780	0,1870	0,3960	0,1100	0,1080	0,1080
	Abs 665	0,4480	0,1970	0,2720	0,2590	0,2790	0,5230	0,1900	0,1890	0,1910
	Abs 750	0,0100	0,0080	0,0090	0,0570	0,0540	0,2080	0,0070	0,0010	0,0000
	Chloro-a (µg /mL)	5,2833	2,2900	3,1624	2,3719	2,6599	3,6955	2,2042	2,2575	2,3004
	Chloro-a (µg /g)	154,5567	67,7921	94,8708	69,9113	78,1524	110,5825	66,0017	67,6746	68,8777
	Average Chloro-a (µg /g)	105,7399			86,2154			67,5180		
	SD Chloro-a (µg /g)	44,3917			21,5010			1,4444		
	Carot (µg/mL)	0,872	0,352	0,508	0,436	0,48	0,716	0,372	0,384	0,392
	Carot (µg /g)	25,509508	10,4204	15,24	12,8512	14,1031	21,425365	11,1388	11,5114	11,7371
	Average Carot (µg /g)	17,0566			16,1266			11,4624		
	SD Carot (µg /g)	7,7069			4,6314			0,3021		

Appendix C - Yield of the "natural" K2-carrageenan from the HWE (SE and Control)

Calculation example of yield:

Sample: Test 1 of 60 min – *Mastocarpus stellatus*

Y=(sample mass)/(algae mass) (4)

Y= 0,1049/2,004 · 100 = 5,284 % d.w.

1.Sequential extraction

Table C.1.1. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HWE for the sequential extraction (60, 30 and 10 minutes) of alga *M. stellatus*

Algae		*Mastocarpus stellatus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,004	2,007	2,0015	2,0076	2,0083	2,001	2,0322	2,0168	2,0175
Hot Water Extraction (90 °C)	Carrageenan (g)	0,1059	0,1076	0,1092	0,0370	0,0507	0,0488	0,0093	0,0025	0,0035
	Average Carrageenan (g)	0,1076			0,0455			0,0051		
	SD Carrageenan (g)	0,0017			0,0074			0,0037		
	Yield (% dw)	5,284%	5,361%	5,456%	1,843%	2,525%	2,439%	0,458%	0,124%	0,173%
	Average Yield (% dw)	**5,367%**			**2,269%**			**0,252%**		
	SD Yield (% dw)	0,086%			0,371%			0,180%		

Note: Due to the insufficient mass, it was not possible further chemical testing and due the fact that it does not form a film, it is **not** possible to confirm that it is **carrageenan** after **10 minutes** of extraction.

Table C.1.2. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HWE for the sequential extraction (60, 30 and 10 minutes) of alga *C. crispus*

Algae		*Chondrus crispus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0101	2,018	2,0267	2,0076	2,0083	2,001	2,0052	2,0044	2,0166
Hot Water Extraction (90 °C)	Carrageenan (g)	0,6157	0,5230	0,7098	0,4674	0,5088	0,5903	0,2623	0,1927	0,2900
	Average Carrageenan (g)	0,6162			0,5222			0,2483		
	SD Carrageenan (g)	0,0934			0,0625			0,0501		
	Yield (% dw)	30,630%	25,917%	35,022%	23,282%	25,335%	29,500%	13,081%	9,614%	14,381%
	Average Yield (% dw)	**30,523%**			**26,039%**			**12,358%**		
	SD Yield (% dw)	4,554%			3,169%			2,464%		

Table C.1.3. Yield and Standard Deviation (SD) K2-carrageenan extract from the HWE for the sequential extraction (60, 30 and 10 minutes) of alga *G. pistillata*

Algae		*Gigartina pistillata*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0043	2,031	2,0618	2,0157	2,0045	2,0031	2,0019	2,0115	2,0123
Hot Water Extraction (90 °C)	Carrageenan (g)	0,1253	0,2245	0,2400	0,1646	0,1389	0,1391	0,0854	0,0530	0,0732
	Average Carrageenan (g)	0,1966			0,1475			0,0705		
	SD Carrageenan (g)	0,0622			0,0148			0,0164		
	Yield (% dw)	6,252%	11,054%	11,640%	8,166%	6,929%	6,944%	4,266%	2,635%	3,638%
	Average Yield (% dw)	**9,649%**			**7,347%**			**3,513%**		
	SD Yield (% dw)	2,956%			0,710%			0,823%		

2.Controls

Table C.2.1. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HWE for the control extraction (60, 30 and 10 minutes) of alga *M. stellatus*

Algae		*Mastocarpus stellatus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Hot Water Extraction (90 °C)	Algae mass (g)	2,0173	2,0159	2,0042	2,0098	2,0161	2,0046	2,0082	2,0037	2,0157
	Carrageenan (g)	0,1976	0,2493	0,2301	0,1868	0,1897	0,2333	0,0000	0,0000	0,0000
	Average Carrageenan (g)	0,2257			0,2033			0,0000		
	SD Carrageenan (g)	0,0261			0,0261			0,0000		
	Yield (% dw)	9,795%	12,367%	11,481%	9,294%	9,409%	11,638%	0,000%	0,000%	0,000%
	Average Yield (% dw)	**11,214%**			**10,114%**			**0,000%**		
	SD Yield (% dw)	1,306%			1,321%			0,000%		

Table C.2.2. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HWE for the control extraction (60, 30 and 10 minutes) of alga *C. Crispus*

Algae		*Chondrus crispus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Hot Water Extraction (90 °C)	Algae mass (g)	2,0159	2,0134	2,0021	2,0148	2,0021	2,0039	2,0073	2,0023	2,0049
	Carrageenan (g)	0,6950	0,7773	0,7618	0,8027	0,7612	0,6814	0,5078	0,4638	0,5135
	Average Carrageenan (g)	0,7447			0,7484			0,4950		
	SD Carrageenan (g)	0,0437			0,0616			0,0272		
	Yield (% dw)	34,476%	38,606%	38,050%	39,840%	38,020%	34,004%	25,298%	23,163%	25,612%
	Average Yield (% dw)	**37,044%**			**37,288%**			**24,691%**		
	SD Yield (% dw)	2,241%			2,986%			1,332%		

Table C.2.3. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HWE for the control extraction (60, 30 and 10 minutes) of alga *G. pistillata*

Algae		*Gigartina pistillata*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Hot Water Extraction (90 °C)	Algae mass (g)	2,0043	2,0012	2,0192	2,0265	2,0118	2,0046	2,0176	2,0293	2,0158
	Carrageenan (g)	0,3265	0,4004	0,4081	0,3988	0,4329	0,3401	0,2619	-	0,2632
	Average Carrageenan (g)	0,3783			0,3906			0,2626		
	SD Carrageenan (g)	0,0451			0,0469			0,0009		
	Yield (% dw)	16,290%	20,008%	20,211%	19,679%	21,518%	16,966%	12,981%	-	13,057%
	Average Yield (% dw)	**18,836%**			**19,388%**			**13,019%**		
	SD Yield (% dw)	2,208%			2,290%			0,054%		

Note: For the sample test 2 at 10 minutes, the polysaccharide clued to the recipient, and it was not possible to remove in a way that didn't compromise the weight.

Appendix D - Yield of the "less sulphated" K2-carrageenan from HAE (SE and Control)

Calculation example of yield:

Sample: Test 1 of 60 min – *Mastocarpus stellatus*

YY = (mass precipitated · total mass after HAE) / (total mass of algae · mass after HAE used for the precipitation) (5)

YY = (1,7953 · (2,4899 + 2,4895 + 2,3826))/((2,004 + 2,007 + 2,0015) · 5,0166)) · 100 = 43,82 % d.w.

Using the formula for the propagation of error:

SD YY=YY·((SD mass precipitated / mass precipitated)^2+ (SD total mass after HAE / total mass after HAE)^2 + (SD total mass of algae / total mass of algae)^2 + (SD mass after HAE used for the precipitation / mass after HAE used for the precipitation)^2)$^{1/2}$

And, knowing that the mass precipitated and the mass after HAE used for precipitation didn't have a standard deviation error, the fraction equals zero, the equation looks like this:

SD YY = 43,82 ·((0)^2+ (0,06183 / (2,4899 + 2,4895 + 2,3826))^2 + (0,00275/ (2,004 + 2,007 + 2,0015))^2 + (0)^2)$^{1/2}$ = 0,369 % d.w.

1.Sequential Extraction

Table D.1.1. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HAE for the sequential extraction (60, 30 and 10 minutes) of alga *M. stellatus*

Algae		*Mastocarpus stellatus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,004	2,007	2,0015	2,0076	2,0083	2,001	2,0322	2,0168	2,0175
Hot Alkaline Extraction NaOH 3% (w/v) (90 °C)	Mass after HAE (g)	2,4899	2,4895	2,3826	2,8862	2,6986	2,5324	1,7948	1,6606	1,7625
	Precipitation									
	Total mass (g)	5,0166			5,0202			5,0551		
	Carrageenan precipitate (g)	1,7953			1,3604			0,1216		
	Yield (% dw)	**43,820%**			**36,557%**			**2,069%**		
	SD mass after HAE (g)	0,06183			0,17701			0,07004		
	SD alga mass (g)	0,00275			0,00403			0,00870		
	SD yield (% dw)	0,369%			0,798%			0,0279%		

Note: The chemical test didn't show any carrageenans on the 10 min extract, so the yield doesn't correspond to carrageenan.

Table D.1.2. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HAE for the sequential extraction (60, 30 and 10 minutes) of alga *C. crispus*

Algae		*Chondrus crispus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0101	2,018	2,0267	2,0076	2,0083	2,001	2,0052	2,0044	2,0166
Hot Alkaline Extraction NaOH 3% (w/v) (90 °C)	Mass after HAE (g)	2,8926	2,6062	2,7683	3,0070	2,8799	2,4134	2,5872	2,0315	2,8086
	Precipitation									
	Total mass (g)	2,0073			2,0001			2,0019		
	Carrageenan precipitate (g)	0,1402			0,0723			0,2142		
	Yield (% dw)	**9,537%**			**4,987%**			**13,188%**		
	SD mass after HAE (g)	0,14362			0,31255			0,40036		
	SD alga mass (g)	0,00830			0,00403			0,00682		
	SD yield (% dw)	0,166%			0,188%			0,711%		

Table D.1.3. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HAE for the sequential extraction (60, 30 and 10 minutes) of alga *G. pistillata*

Algae		*Gigartina pistillata*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Initial dry algae mass (g)		2,0043	2,031	2,0618	2,0157	2,0045	2,0031	2,0019	2,0115	2,0123
Hot Alkaline Extraction NaOH 3% (w/v) (90 °C)	Mass after HAE (g)	1,8314	1,7286	1,1391	1,8422	1,9988	2,1694	1,6494	1,7386	2,0993
	Precipitation									
	Total mass (g)	4,5518			4,3688			5,0166		
	Carrageenan precipitate (g)	3,1126			2,5928			1,3496		
	Yield (% dw)	**52,703%**			**59,221%**			**24,499%**		
	SD mass after HAE (g)	0,37358			0,16365			0,23821		
	SD alga mass (g)	0,02877			0,00691			0,00579		
	SD yield (% dw)	4,20%			1,61%			1,06%		

2.Control

Table D.2.1. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HAE for the control extraction (60, 30 and 10 minutes) of alga *M. stellatus*

Algae		*Mastocarpus stellatus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Hot Alkaline Extraction NaOH 3% (w/v) (90 ºC)	Algae mass (g)	2,0111	2,0180	2,0008	2,0314	2,0234	2,0048	2,0069	2,0169	2,0055
	Mass (g)	1,7373	1,7409	1,6992	2,9664	2,4320	2,9625	1,9413	2,1652	1,0088
	Precipitation									
	Initial mass (g) (1)	2			2,0019			2,0034		
	Carrageenan precipitate (g) (1)	0,6741			0,0800			0,6453		
	Initial mass (g) (2)				4,3606					
	Carrageenan precipitate (g) (2)				0,7315					
	TOTAL Initial mass (g)	2			6,3625			2,0034		
	TOTAL Carrageenan precipitate (g)	0,6741			0,8115			0,6453		
	Yield (% dw)	**28,940%**			**17,598%**			**27,327%**		
	SD mass after HAE (g)	0,02311			0,30742			0,61332		
	SD alga mass (g)	0,00866			0,01365			0,00622		
	SD Yield (% dw)	0,136%			0,648%			3,28%		

Note: The chemical test didn't show any carrageenans on the 10 min extract, so the yield doesn't correspond to carrageenan.

Table D.2.2. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HAE for the control extraction (60, 30 and 10 minutes) of alga *C. crispus*

Algae		*Chondrus crispus*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Hot Alkaline Extraction NaOH 3% (w/v) (90 ºC)	Algae mass (g)	2,0095	2,0086	2,0094	2,0167	2,0013	2,0280	2,0194	2,0194	2,0056
	Mass (g)	3,1357	2,9100	2,8512	2,7631	2,7377	2,6870	2,5981	3,0231	3,2390
	Precipitation									
	Initial mass (g) (1)	2,0077			2			2,0023		
	Carrageenan precipitate (g) (1)	0,8764			0,5205			0,4210		
	Initial mass (g) (2)				5,0081					
	Carrageenan precipitate (g) (2)				2,7897					
	TOTAL Initial mass (g)	2,0077			7,0081			2,0023		
	TOTAL Carrageenan precipitate (g)	0,8764			3,3102			0,4210		
	Yield (% dw)	**64,433%**			**63,967%**			**30,821%**		
	SD mass after HAE (g)	0,15019			0,03874			0,32609		
	SD alga mass (g)	0,00049			0,01340			0,00797		
	SD Yield (% dw)	1,09%			0,334%			1,14%		

Table D.2.3. Yield and Standard Deviation (SD) of the K2-carrageenan extract from the HAE for the control extraction (60, 30 and 10 minutes) of alga *G. pistillata*

Algae		*Gigartina pistillata*								
Time		60 min			30 min			10 min		
Test		1	2	3	1	2	3	1	2	3
Hot Alkaline Extraction NaOH 3% (w/v) (90 °C)	Algae mass (g)	2,0120	2,0487	2,0204	1,9915	2,0097	2,0340	2,0016	2,0014	2,0011
	Mass (g)	1,6485	1,2648	1,7254	1,5231	1,6041	1,5887	1,9611	2,1966	2,1707
	Precipitation									
	Initial mass (g) (1)	2			2,0011			2		
	Carrageenan precipitate (g) (1)	1,2345			0,5787			0,4189		
	Initial mass (g) (2)				2,0500					
	Carrageenan precipitate (g) (2)				0,7171					
	TOTAL Initial mass (g)	2			4,0511			2		
	TOTAL Carrageenan precipitate (g)	1,2345			1,2958			0,4189		
	Yield (% dw)	**47,084%**			**24,994%**			**22,076%**		
	SD mass after HAE (g)	0,24674			0,04301			0,12914		
	SD alga mass (g)	0,01923			0,02132			0,00025		
	SD Yield (% dw)	2,51%			0,244%			0,451%		

Note: The chemical test didn't show any carrageenans on the 10 min extract, so the yield doesn't correspond to carrageenan.

Appendix E – FTIR spectra of the extracts from CWE, HWE and HAE

E.1.Extracts from the CWE

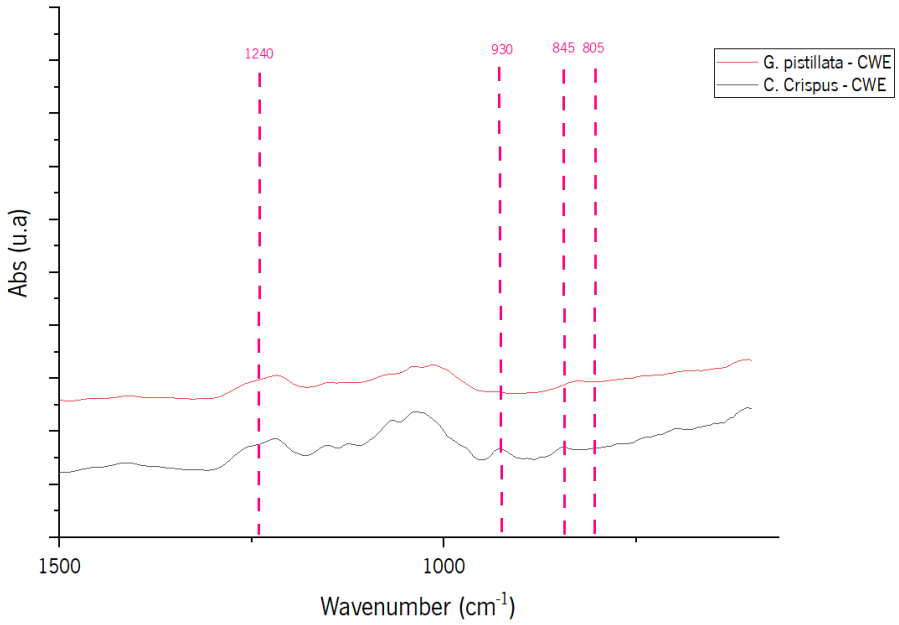

Figure E.1.1. FTIR spectra from the CWE K2-carrageenan extracts.

E.2.Extracts from the HWE

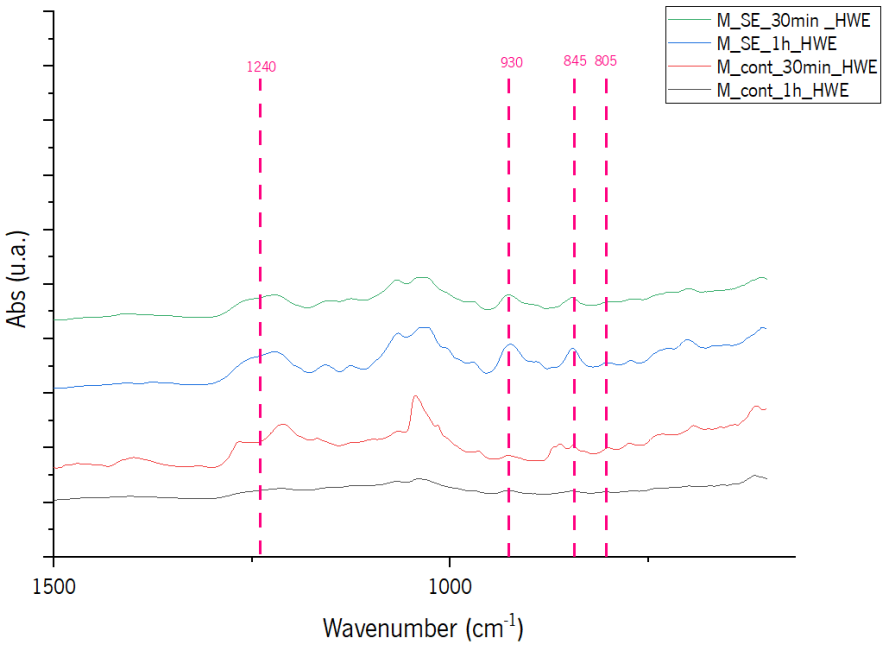

Figure E.2.1. FTIR spectra from the *M. stellatus* water K2-carrageenan extracts.

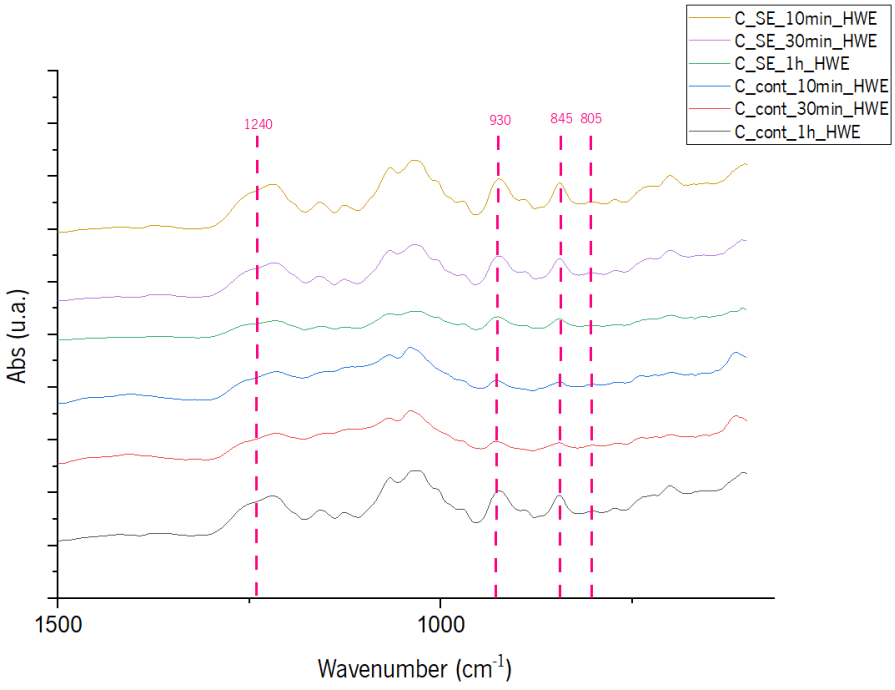

Figure E.2.2. FTIR spectra from the *C. Crispus* water K2-carrageenan extracts.

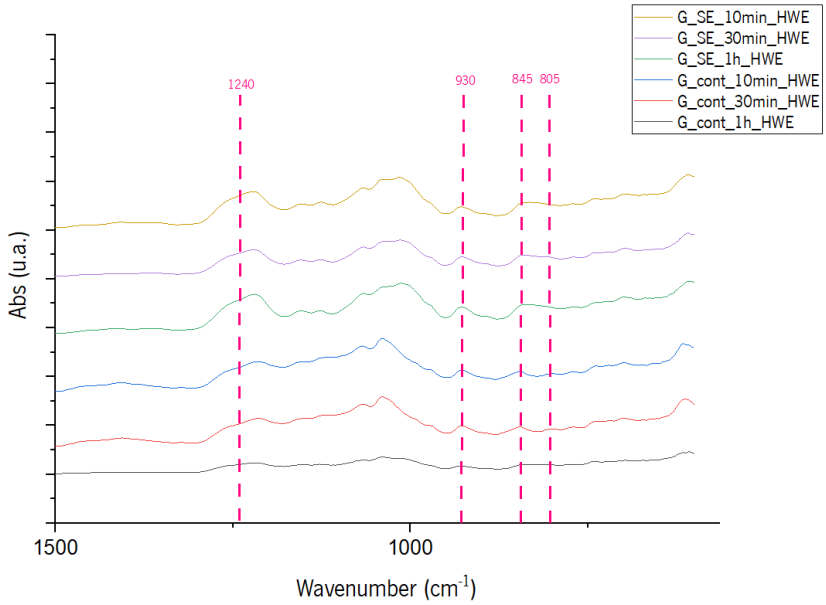

Figure E.2.3. FTIR spectra from the *G. pistillata* water K2 carrageenan extracts.

E.3.Extracts from the HAE

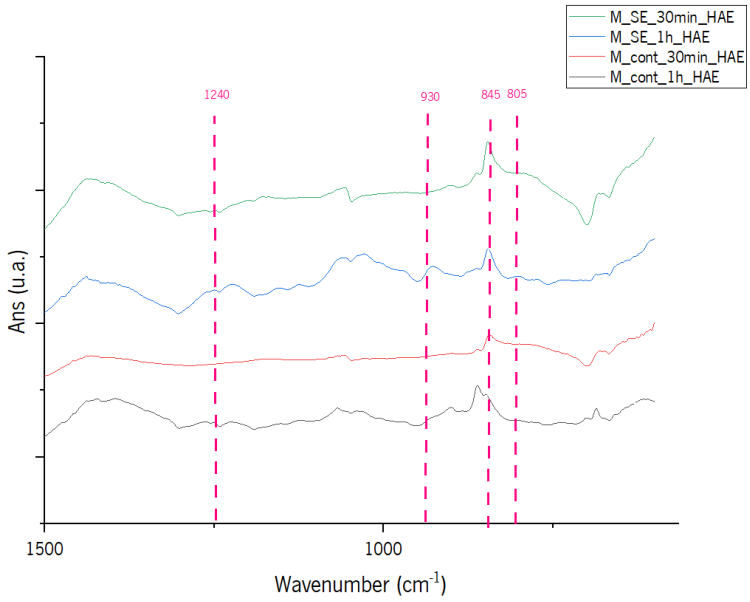

Figure E.3.1. FTIR spectra from the *M. stellatus* alkaline K2-carrageenan extracts.

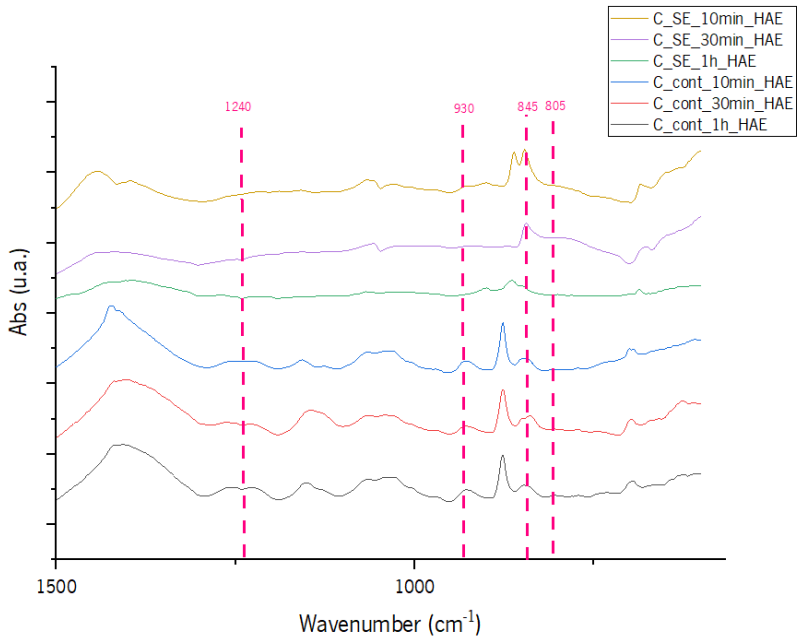

Figure E.3.2. FTIR spectra from the *C. crispus* alkaline K2-carrageenan extracts.

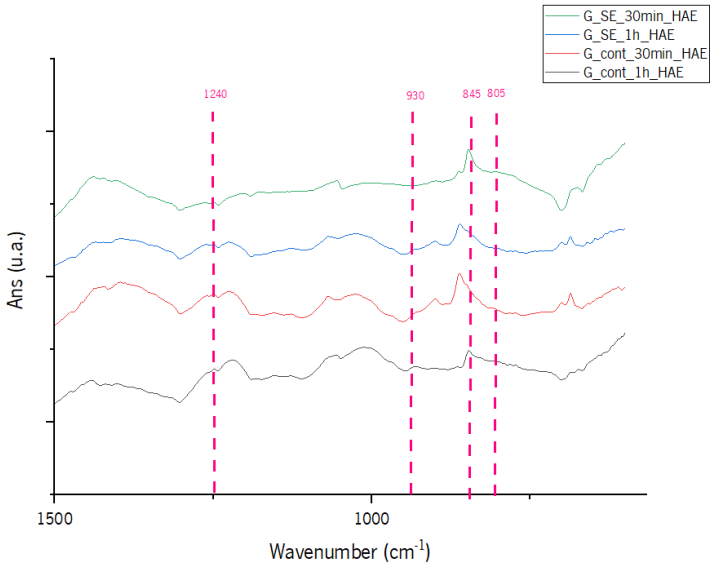

Figure E.3.3. FTIR spectra from the *G. pistillata* alkaline K2-carrageenan extracts.

Appendix F – Contaminants ^{1}H-NMR

Table F.1. The intensity of the observed peaks of the contaminants (starch and pyruvic acid, AP) is indicated by symbols: "-" (no peak), "+" (weak), "++" (moderate), and "+++" (strong), with "nd" (no data) representing samples that were not subjected to ^{1}H-NMR analysis, of the K2-carrageenans from the CWE, HWE, and HAE.

Algae	Extraction	Type	time	Starch		AP
Mastocarpus Stellatus	**CWE**	Sequential	16h	-		
	HWE	Control	1 h	-	-	-
			30 min	-	-	+
			10 min	**nd**	**nd**	**nd**
		Sequential	1 h	-	-	-
			30 min	-	-	-
			10 min	-	-	-
	HAE	Control	1 h	-	-	-
			30 min	-	-	-
			10 min	-	-	-
		Sequential	1 h	-	-	-
			30 min	-	-	-
			10 min	**nd**	**nd**	**nd**
Chondrus Crispus	**CWE**	Sequential	16 h	-	-	-
	HWE	Control	1 h	-	-	-
			30 min	-	-	+
			10 min	-	-	+
		Sequential	1 h	-	-	-
			30 min	-	-	-
			10 min	-	-	-
	HAE	Control	1 h	-	-	+
			30 min	-	-	+
			10 min	-	-	+
		Sequential	1 h	-	-	-
			30 min	+++	+++	+
			10 min	-	-	+
Gigartina pistillata	**CWE**	Sequential	16 h	+	+	-
	HWE	Control	1 h	-	-	+++
			30 min	-	-	+++
			10 min	+	+	+
		Sequential	1 h	-	-	-
			30 min	-	-	++
			10 min	-	-	-
	HAE	Control	1 h	-	-	++
			30 min	-	-	++
			10 min	**nd**	**nd**	**nd**
		Sequential	1 h	-	-	+
			30 min	-	-	-
			10 min	-	-	-

Appendix G – Weight-average molecular mass (Mw), number-average molecular mass (Mn) and polydispersity index (PDI) of various carrageenan extracts under different extraction conditions

Table G.1. Mw, Mn and PDI of K2-carrageenans, with their respective standard deviations (SD) for samples subjected to cold water extraction (CWE), hot water extraction (HWE), and hot alkaline extraction (HAE) (3% w/v NaOH), and 16-hour water extraction. The samples are labelled based on the extraction method (SE and control) and time of extraction.

Samples		Mw 1	Mw 2	Molecular Mass	SD	Mn 1	Mn 2	PDI 1	PDI 2	PDI	SD
		g/mol	g/mol	g/mol		g/mol	g/mol				
CWE	M_16h										
	G_16h	942862	952493	9,48E+05	6,81E+03	486588	507075	1,9377	1,8784	1,91	4,19E-02
	C_16h	256933	275004	2,66E+05	1,28E+04	61586	66783	4,17194	4,1179	4,14	3,82E-02
HWE	M_con_1h	984732	1020768	1,00E+06	2,55E+04	573902	594332	1,71585	1,7175	1,72	1,17E-03
	M_cont_30min	755245	·	7,55E+05	·	450169	·	1,67769	·	1,68	·
	G_cont_1h	1073022	1115883	1,09E+06	3,03E+04	711137	701222	1,50888	1,5913	1,55	5,83E-02
	G_cont_30min	1167424	1140702	1,15E+06	1,89E+04	788418	757477	1,48072	1,5059	1,49	1,78E-02
	C_cont_1h	753327	795035	7,74E+05	2,95E+04	294849	303273	2,55496	2,6215	2,59	4,71E-02
	C_cont_30min	789709	798900	7,94E+05	6,50E+03	185052	288569	4,2675	2,7685	3,52	1,06
	G_cont_10min	713107	792114	7,53E+05	5,59E+04	372894	397763	1,91236	1,9914	1,95	5,59E-02
	C_cont_10min	470571	461122	4,66E+05	6,68E+03	183522	178547	2,56411	2,5826	2,57	1,31E-02
	C_SE_1h	755038	749218	7,52E+05	4,12E+03	210975	206544	3,5788	3,6274	3,60	3,44E-02
	C_SE_30min	779546	786199	7,83E+05	4,70E+03	256127	249089	3,04359	3,1563	3,10	7,97E-02
	G_SE_1h	1504194	1497490	1,50E+06	4,74E+03	763542	769284	1,97002	1,9466	1,96	1,66E-02
	C_SE_10min	536852	538580	5,38E+05	1,22E+03	169362	167964	3,16985	3,2065	3,19	2,59E-02
	M_SE_1h	1169360	1207676	1,19E+06	2,71E+04	125694	135293	9,30323	8,9264	9,11	2,66E-01
	M_SE_30min	887614	858540	8,73E+05	2,06E+04	102761	111526	8,63765	7,6981	8,17	6,64E-01
	G_SE_30min	1583123	1616998	1,60E+06	2,40E+04	881395	919332	1,79616	1,7589	1,78	2,64E-02
	G_SE_10min	1527200	1517164	1,52E+06	7,10E+03	768886	796838	1,98625	1,904	1,95	5,82E-02
HAE	G_SE_1h	639686	699646	6,70E+05	4,24E+04	161815	182294	3,95319	3,838	3,90	8,14E-02
	M_cont_1h	456982	458567	4,58E+05	1,12E+03	127576	131250	3,58204	3,4938	3,54	6,24E-02
	C_SE_30min	212938	273249	2,43E+05	4,26E+04	26269	62053	8,10606	4,4035	6,25	2,62
	M_cont_10min	·	·	·	·	·	·	·	·	·	·
	M_cont_30min	340289	380652	3,60E+05	2,85E+04	116454	108892	2,92209	3,4957	3,21	4,06E-01
	C_cont_30min	322839	338352	3,31E+05	1,10E+04	110654	119250	2,91755	2,8373	2,88	5,67E-02
	G_cont_1h	554009	·	5,54E+05	·	150537	·	3,68022	·	3,68	·
	G_cont_30min	386991	420958	4,04E+05	2,40E+04	95333	89822	4,05936	4,6866	4,37	4,44E-01
	G_cont_10min	436066	631175	5,34E+05	1,38E+05	107462	132469	4,05786	4,7647	4,41	5,00E-01
	C_cont_1h	402475	411117	4,07E+05	6,11E+03	117802	118568	3,41654	3,4674	3,44	3,59E-02
	C_cont_10min	297319	297533	2,97E+05	1,51E+02	103008	103322	2,88637	2,8797	2,88	4,74E-03
	M_SE_1h	721746	605265	6,64E+05	8,24E+04	239482	281486	3,01378	2,1502	2,58	6,11E-01
	M_SE_30min	296890	332793	3,15E+05	2,54E+04	85025	86394	3,4918	3,852	3,67	2,55E-01
	G_SE_30min	209396	132898	1,71E+05	5,41E+04	62652	31852	3,34221	4,1724	3,76	5,87E-01
	G_SE_10min	·	·	·	·	·	·	·	·	·	·
	C_SE_1h	235224	209522	2,22E+05	1,82E+04	59543	59609	3,95049	3,5149	3,73	3,08E-01
	C_SE_10min	351884	297836	3,25E+05	3,82E+04	81816	64628	4,30092	4,6085	4,45	2,17E-01

Appendix H – Graphs from the rheological tests

H.1.*Mastocarpus stellatus*

1.1.Gels

Hot Water Extraction

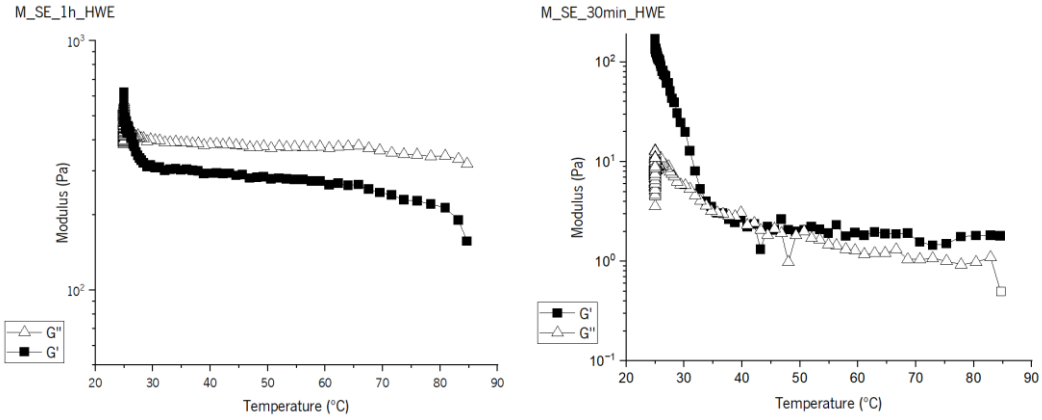

Figure H.1.1.1 Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 85 °C to 25 °C of K2-carrageenan samples (1 hour and 30 min sequential water extraction from *Mastocarpus stellatus*), in a 2 wt.% + 1 M NaCl solution.

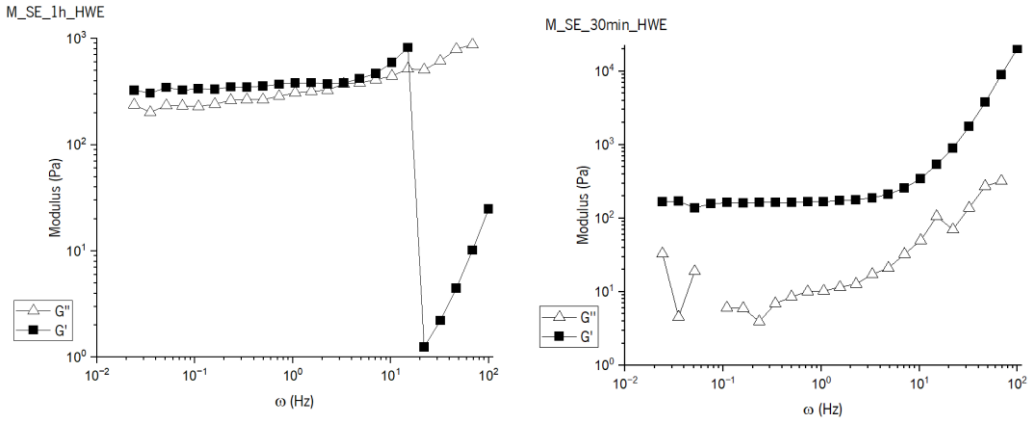

Figure H.1.1.2. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (1 hour and 30 min sequential water extraction from *Mastocarpus stellatus*), in a 2 wt.% + 1 M NaCl solution.

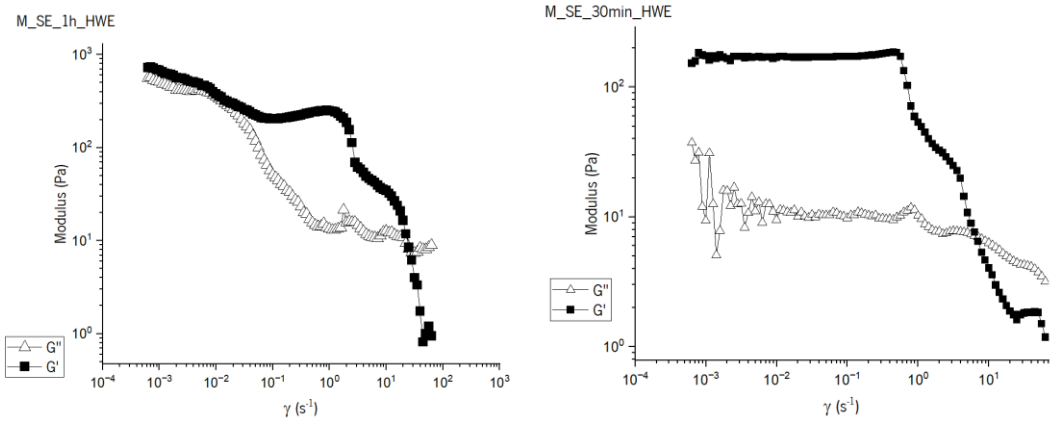

Figure H.1.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear rate (γ), at a 25 °C and 1 Hz, of K2-carrageenan samples (1 hour and 30 min sequential water extraction from *Mastocarpus stellatus)*, in a 2 wt.% + 1 M NaCl solution.

1.2. Liquids

Cold Water Extraction

The extract didn´t have K2-carrageenans.

Hot Water Extraction

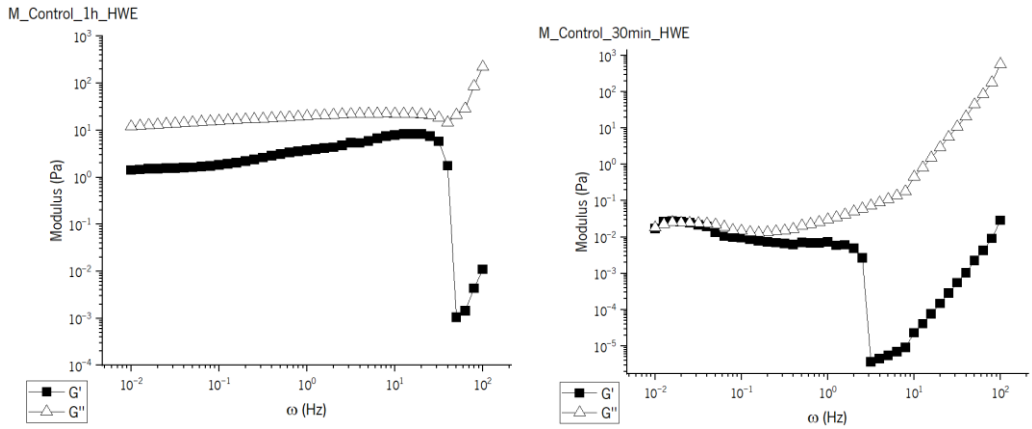

Figure H.1.2.4. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (1 hour and 30 min control water extraction from *Mastocarpus stellatus)*, in a 2 wt.% + 1 M NaCl solution.

Alkaline Extraction

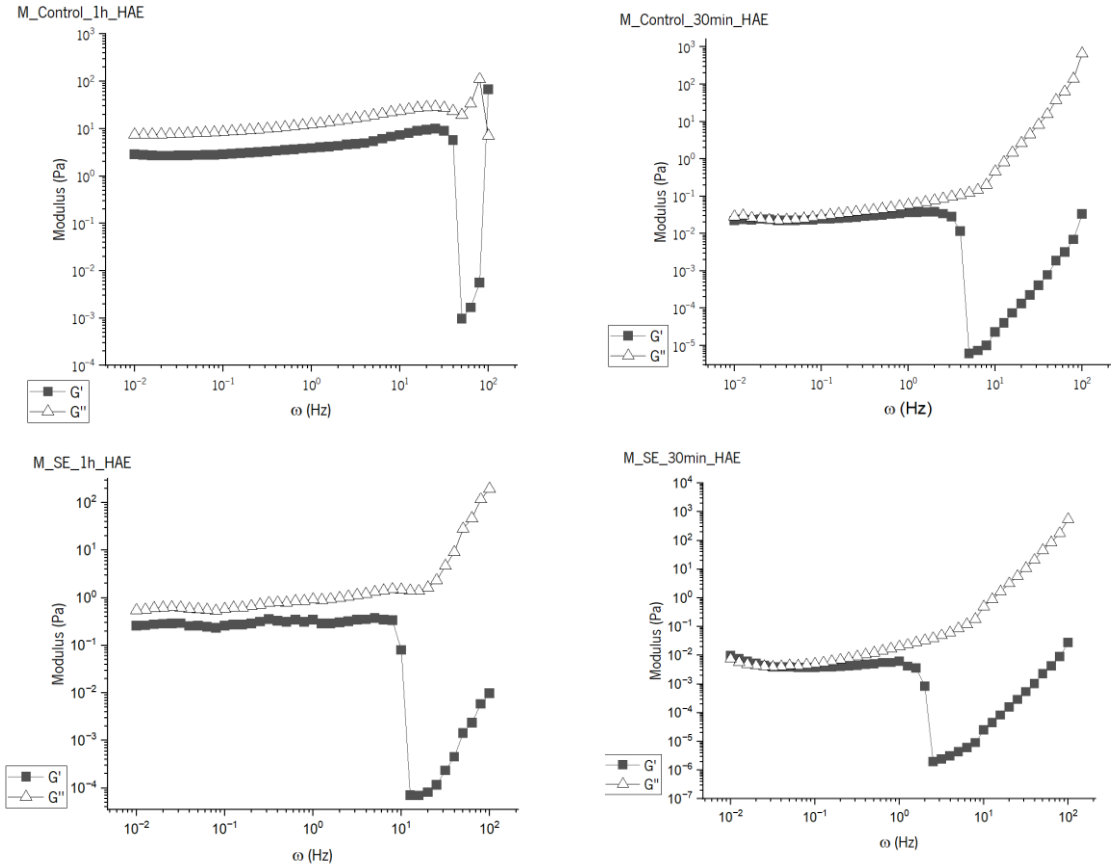

Figure H.1.2.5. Storage modulus (G') (solid symbols) and loss modulus (G'') (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (1 hour and 30 min control alkaline extraction (top line); 1 hour and 30 min sequential alkaline extraction (bottom line) from *Mastocarpus stellatus)*, in a 2 wt.% + 1 M NaCl solution.

H.2.*Chondrus crispus*

2.1.Gels

Hot Water Extraction

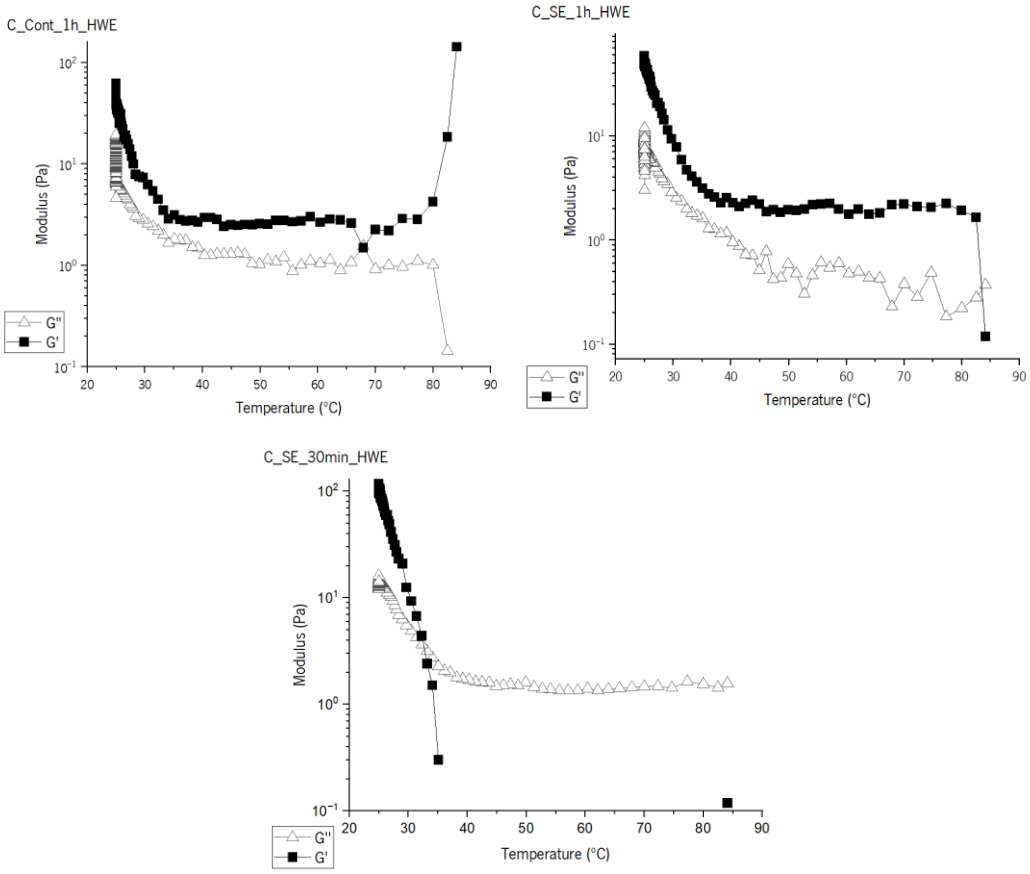

Figure H.2.1.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 85 °C to 25 °C of K2-carrageenan samples (1 hour and 30 min sequential and 30 min control water extraction from *Chondrus crispus*), in a 2 wt.% + 1 M NaCl solution.

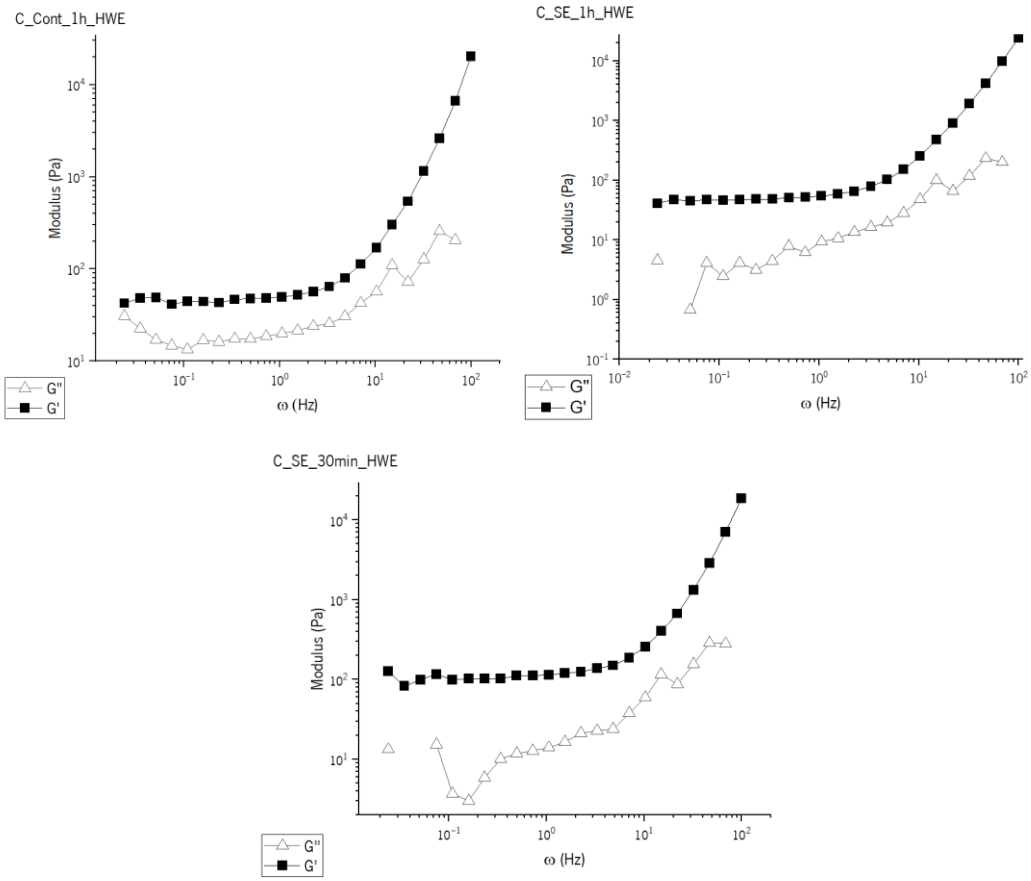

Figure H.2.1.2. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (1 hour and 30 min sequential and 30 min control water extraction from *Chondrus crispus*), in a 2 wt.% + 1 M NaCl solution.

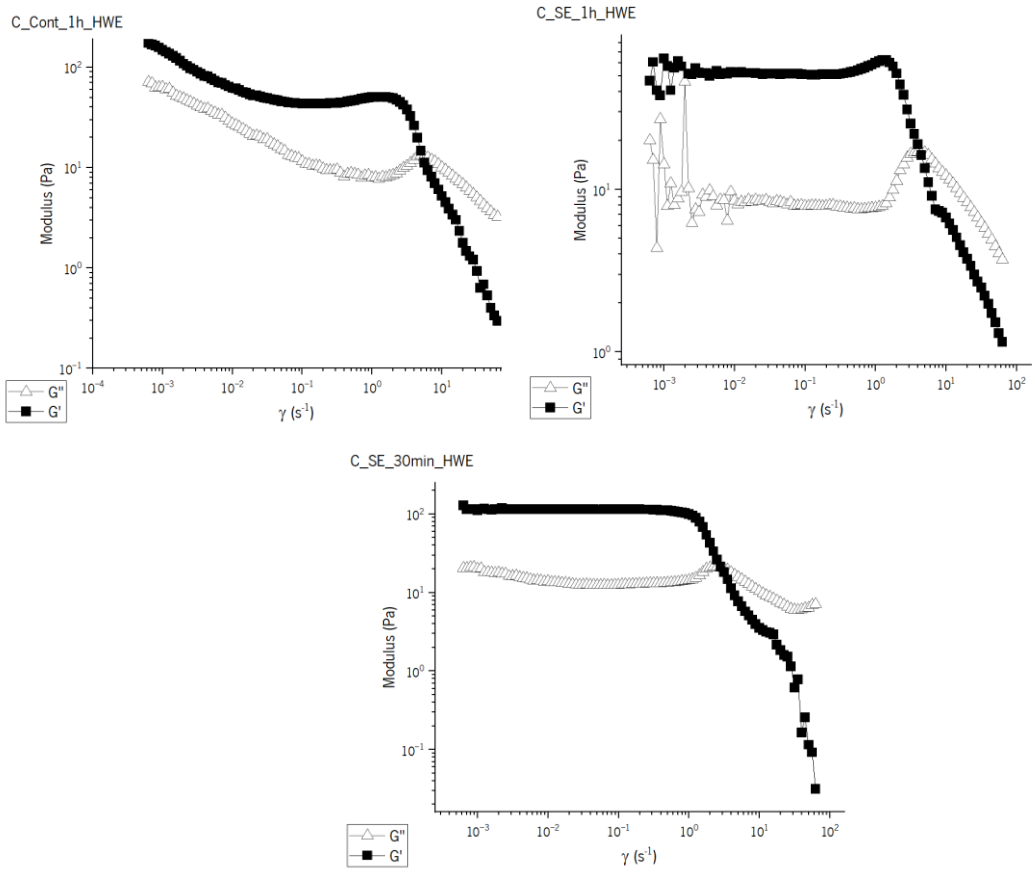

Figure H.2.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear rate (γ), at a 25 °C and 1 Hz, of K2-carrageenan (1 hour and 30 min sequential and 30 min control water extraction from *Chondrus crispus*), in a 2 wt.% + 1 M NaCl solution.

2.2.Liquids

Cold Water Extraction

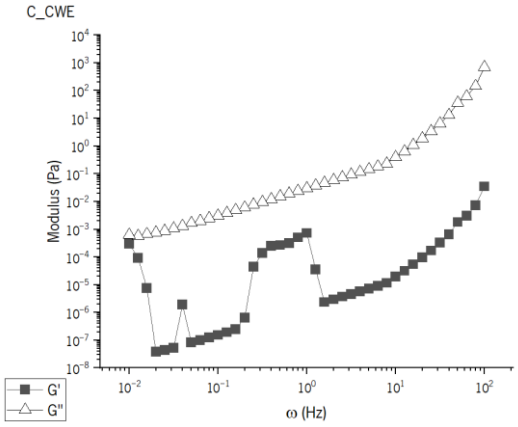

Figure H.2.2.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (16h overnight cold water extraction from *Chondrus crispus*), in a 2 wt.% + 1 M NaCl solution.

Hot Water Extraction

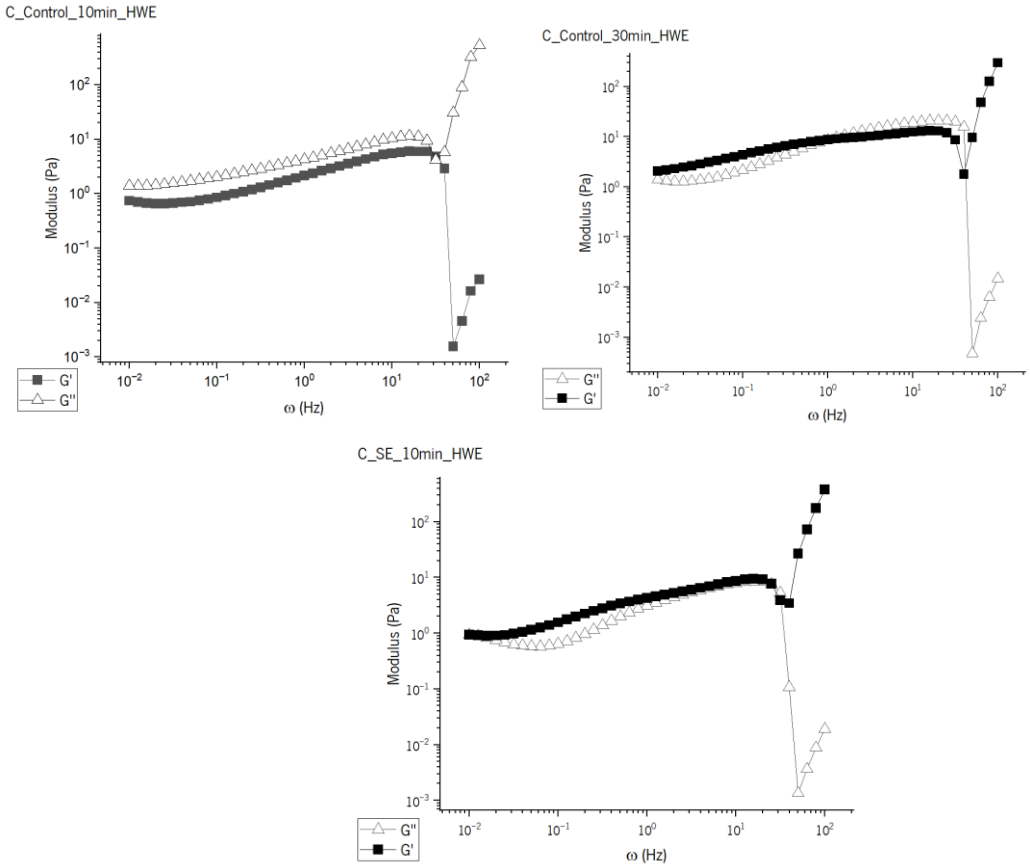

Figure H.2.2.2. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (10 min and 30 min control and 10 min sequential water extraction from *Chondrus crispus)*, in a 2 wt.% + 1 M NaCl solution.

Alkaline Extraction

There is no data, once the mass extracted wasn't enough for these tests.

H.3. *Gigartina pistillata*

3.1.Gel

Water Extraction

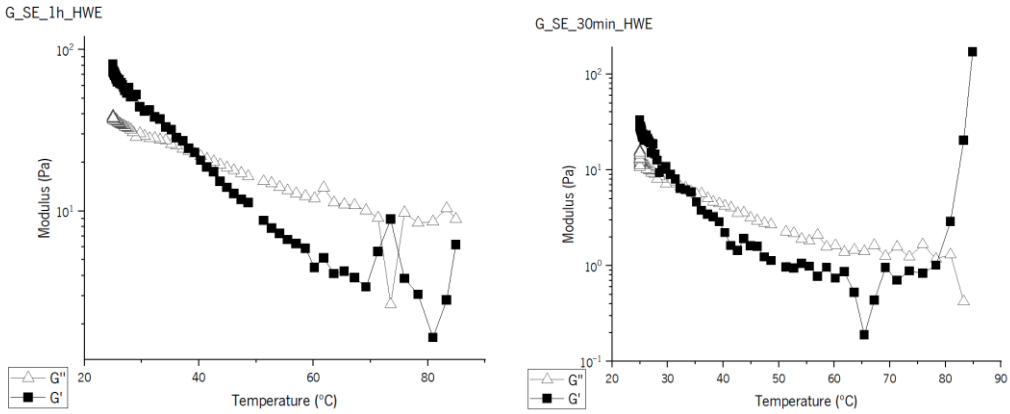

Figure H.3.1.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 80 °C to 25 °C of K2-carrageenan samples (1 hour and 30 min sequential water extraction from *Gigartina pistillata*), in a 2 wt.% + 1 M NaCl solution.

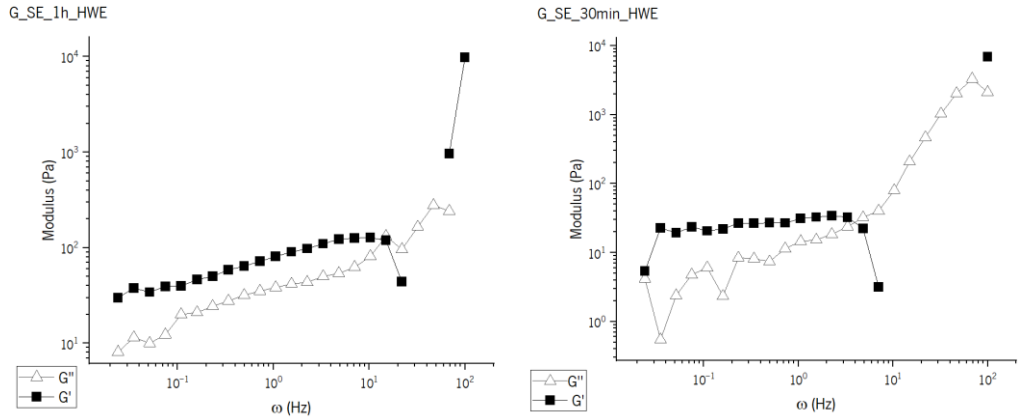

Figure H.3.1.2. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (1 hour and 30 min sequential and 30 min control water extraction from *Gigartina pistillata*), in a 2 wt.% + 1 M NaCl solution.

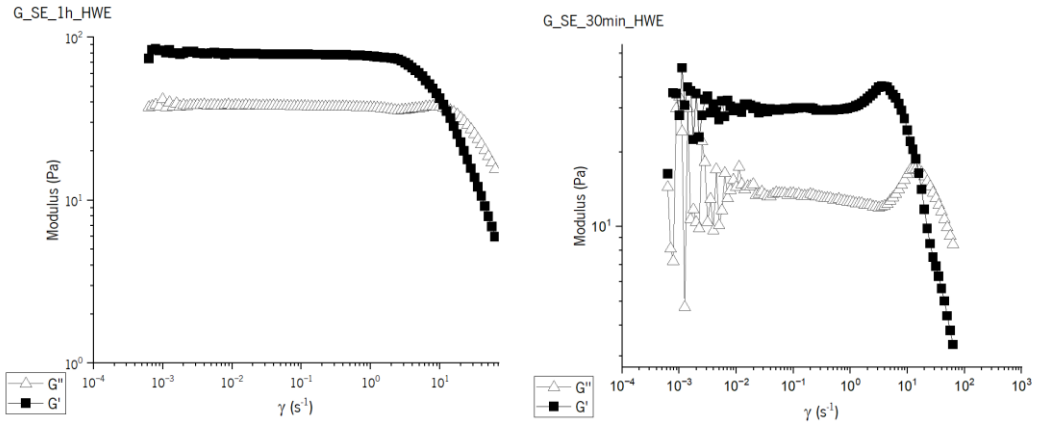

Figure H.3.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear rate (ɣ), at a 25 °C and 1 Hz, of K2-carrageenan (1 hour and 30 min sequential and 30 min control water extraction from *Gigartina pistillata*), in a 2 wt% + 1 M NaCl solution.

3.2.Liquids

Cold Water Extraction

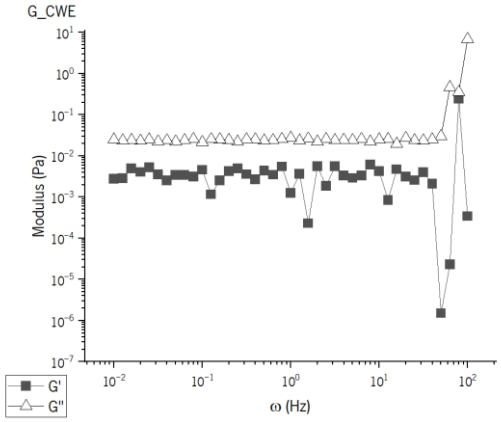

Figure H.3.2.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (16h overnight cold water extraction from *Gigartina pistillata)*, in a 2 wt.% + 1 M NaCl solution.

Hot Water Extraction

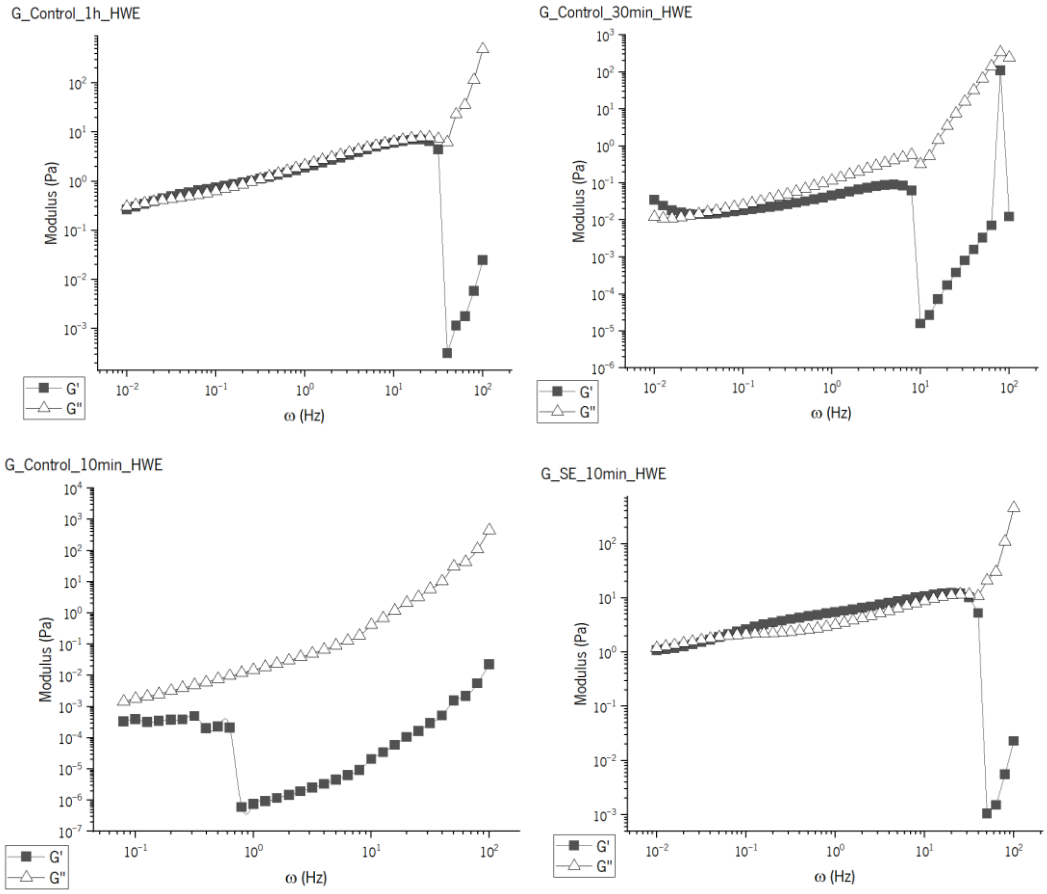

Figure H.3.2.2. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (1 hour, 30 min and 10 min control and 10 min sequential water extraction from *Gigartina pistillata)*, in a 2 wt.% + 1 M NaCl solution.

Hot Alkaline Extraction

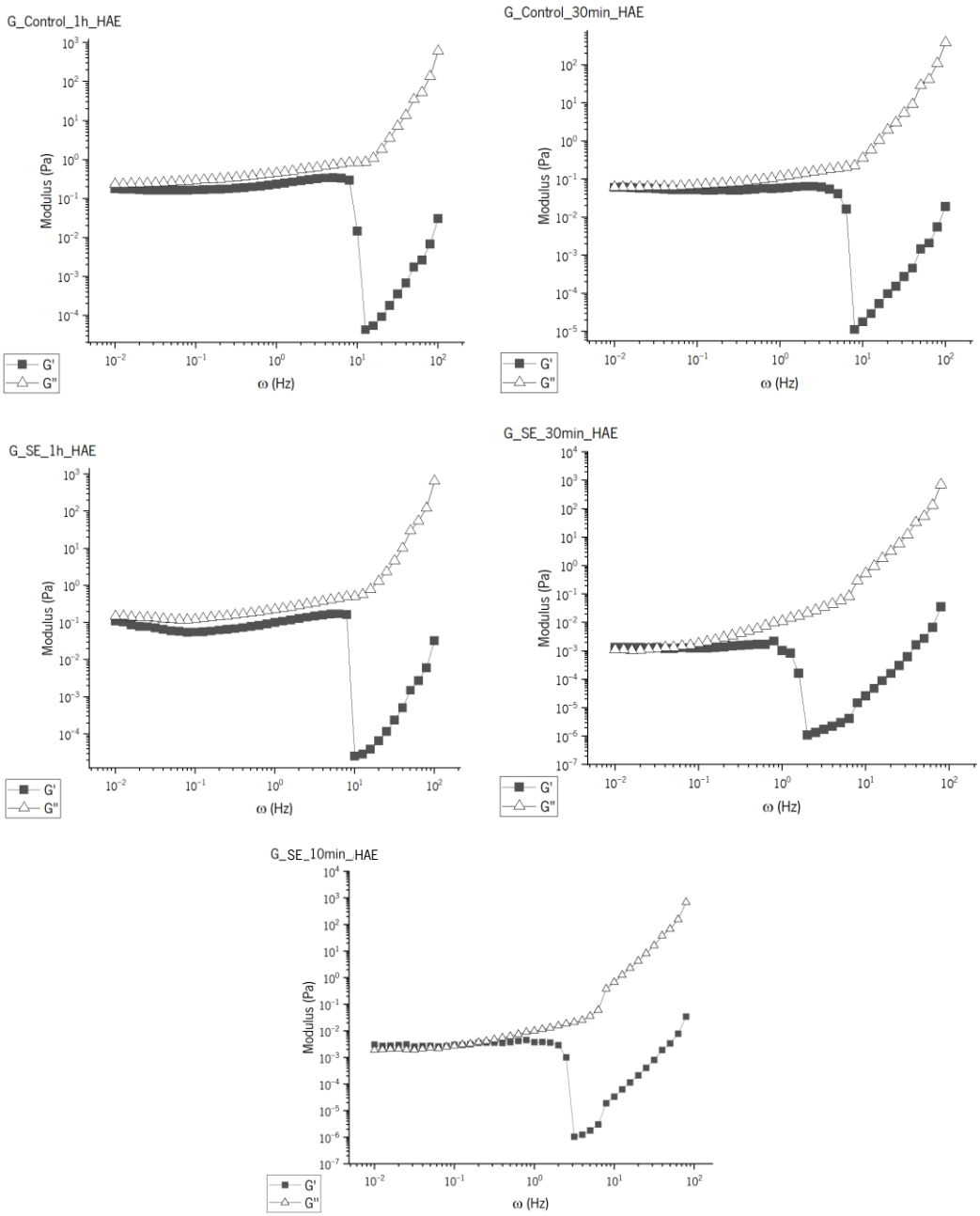

Figure H.3.2.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples (1 hour and 30 min control extraction; 1 hour, 30 min and 10 min sequential alkaline extraction) from *Gigartina pistillata)*, in a 2 wt.% + 1 M NaCl solution.

Appendix I - Weight-average molecular mass (Mw), number-average molecular mass (Mn) and polydispersity index (PDI) of various K2-carrageenan extracts under different times of ultrasonication

Table I.1. Mw, Mn and PDI of the K2-carrageenans, with their respective standard deviations (SD) for samples subjected to different times of ultrasonication, of five different algae

Algae	Time (h)	K2-carrageenan									
		Mw 1 (g/mol)	Mw 2 (g/mol)	**Mw** (g/mol)·10^5	SD	Mn 1 (g/mol)	Mn 2 (g/mol)	PDI 1	PDI 2	**PDI**	SD
B-87	0										
	1	231757	296517	2,64	0,46	69733	92814	3,32	3,19	3,26	9,10E-02
	2	309432	295223	3,02	0,10	112102	110083	2,76	2,68	2,72	5,55E-02
	3	251601	253381	2,52	0,01	100154	100832	2,51	2,51	2,51	5,38E-04
	4	274038	260185	2,67	0,10	116088	114463	2,36	2,27	2,32	6,19E-02
Gigartina	0	1596853	1482294	15,40	0,81	616689	526220	2,59	2,82	2,70	1,61E-01
	1	509941	604716	5,57	0,67	240414	285420	2,12	2,12	2,12	1,70E-03
	2	576233	582544	5,79	0,04	284091	296863	2,03	1,96	2,00	4,67E-02
	3	508711	504361	5,07	0,03	256473	260787	1,98	1,93	1,96	3,50E-02
	4	329460	328572	3,29	0,01	166888	167480	1,97	1,96	1,97	8,68E-03
Mastocarpus	0	653943	644873	6,49	0,06	229751	229574	2,85	2,81	2,83	2,64E-02
	6	242448	241094	2,42	0,01	112412	117380	2,16	2,05	2,11	7,27E-02
	12	183094	182349	1,83	0,01	88135	85473	2,08	2,13	2,11	3,96E-02
	16	314510	309780	3,12	0,03	148045	140094	2,12	2,21	2,17	6,14E-02
	20	140950	142610	1,42	0,01	68637	70540	2,05	2,02	2,04	2,25E-02
BAT 18-09	0	677772	654321	6,66	0,17	271124	259568	2,50	2,52	2,51	1,48E-02
	6	182986	191941	1,87	0,06	91718	96767	2,00	1,98	1,99	8,17E-03
	12	151374	153958	1,53	0,02	76949	78396	1,97	1,96	1,97	2,37E-03
	14	239886	234516	2,37	0,04	120815	116952	1,99	2,01	2,00	1,39E-02
	16	107675	107358	1,08	0,00	54012	52822	1,99	2,03	2,01	2,75E-02
C-25	0	982924	945485	9,64	0,26	288610	277650	3,41	3,41	3,41	2,86E-04
	6	260921	256116	2,59	0,03	128735	125868	2,03	2,03	2,03	5,65E-03
	12	162093	159513	1,61	0,02	80709	78920	2,01	2,02	2,01	9,08E-03
	16	81592	83624	0,83	0,01	42065	44126	1,94	1,90	1,92	3,15E-02
	20	76487	78031	0,77	0,01	39492	41309	1,94	1,89	1,91	3,38E-02

Appendix J – Rheology graphs of the ultrasonicated K2-carrageenans

J.1. Alga B-87

1.1.Gel

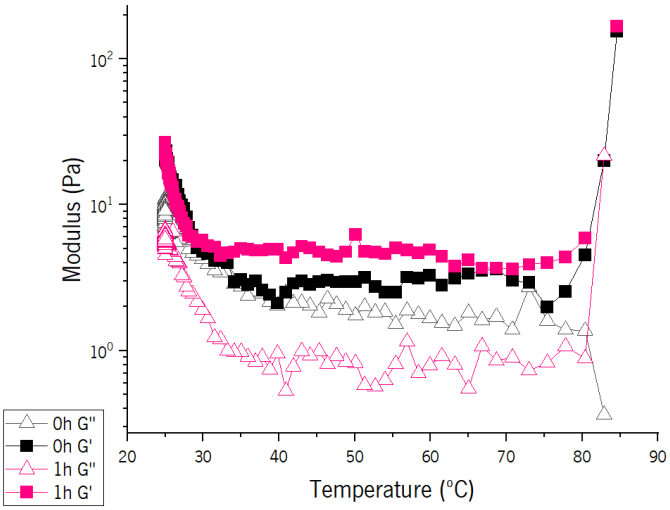

Figure J.1.1.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 85 °C to 25 °C of K2-carrageenan samples, from B-87, with 0h and 1h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

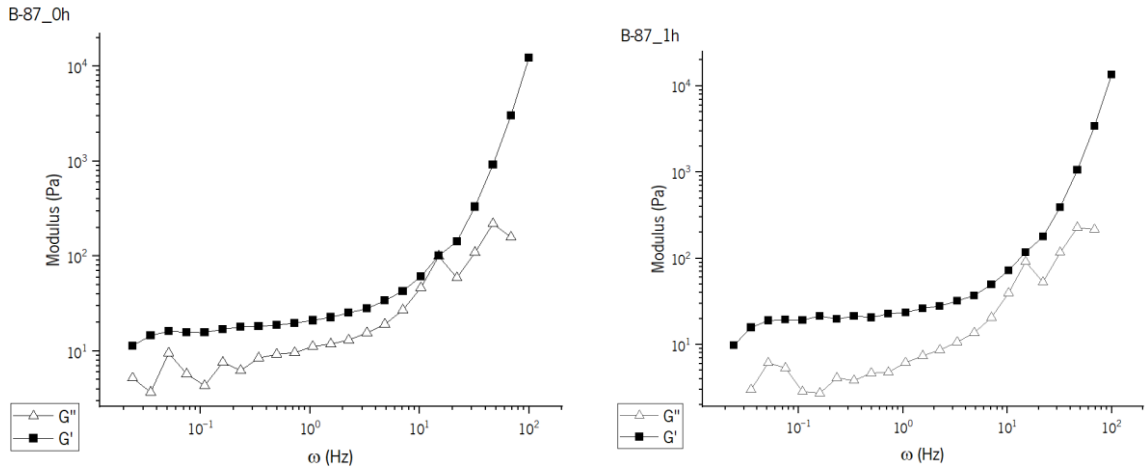

Figure J.1.1.2. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples, from B-87, with 0h and 1h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

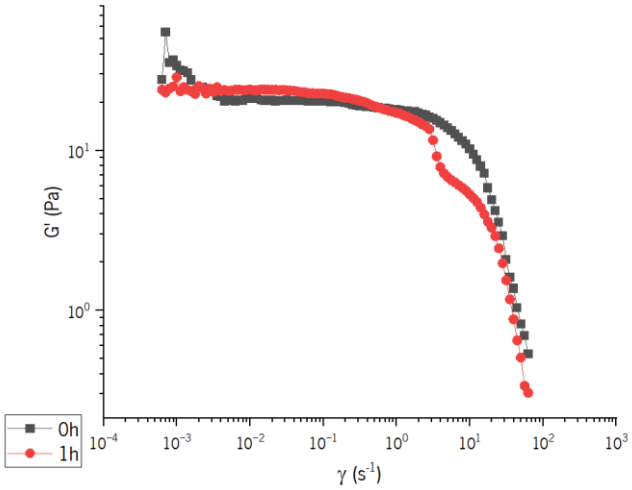

Figure J.1.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear rate (ɣ), at a 25 °C and 1 Hz, of K2-carrageenan from B-87, with 0h and 1h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

1.2.Liquid

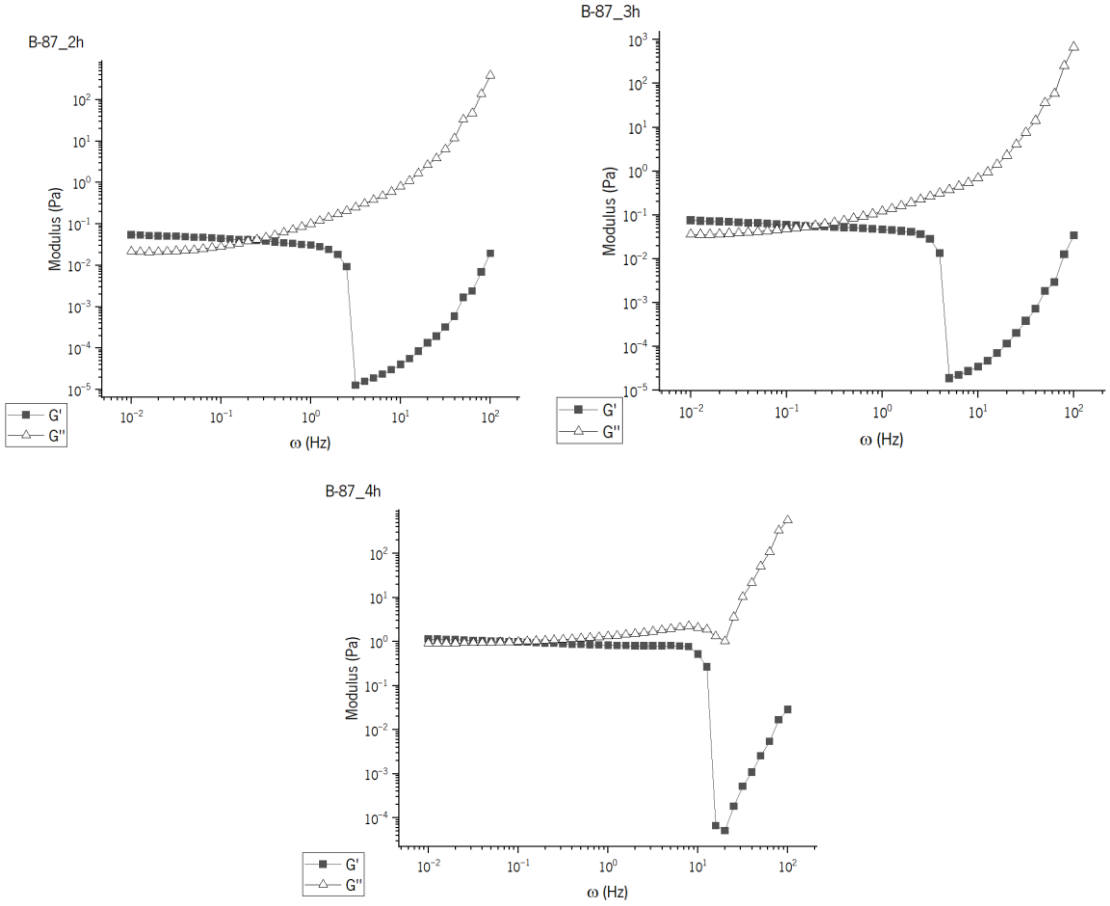

Figure J.1.2.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 50,3 % strain, of K2-carrageenan samples from B-87, with 2h,3h and 4h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

J.2. Alga *Gigartina*

2.1.Gel

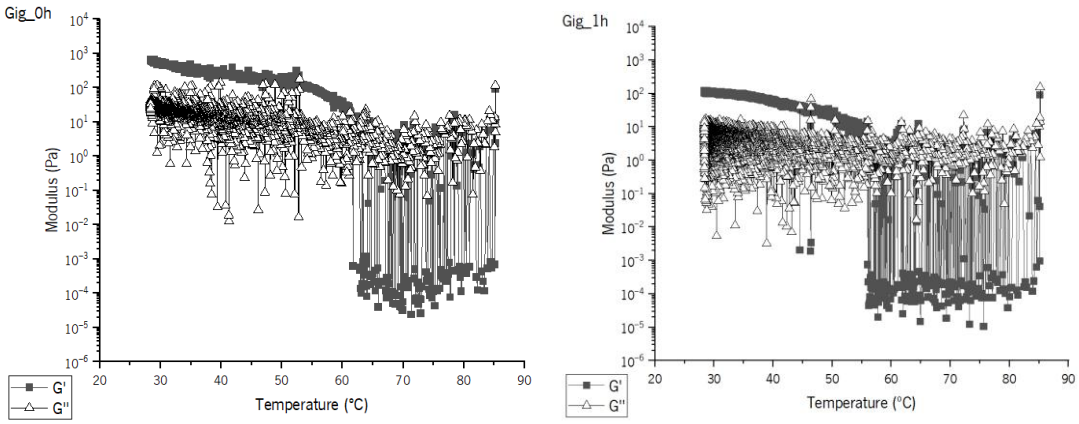

Figure J.2.1.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 85 °C to 25 °C of K2-carrageenan samples, from *Gigartina*, with 0h and 1h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

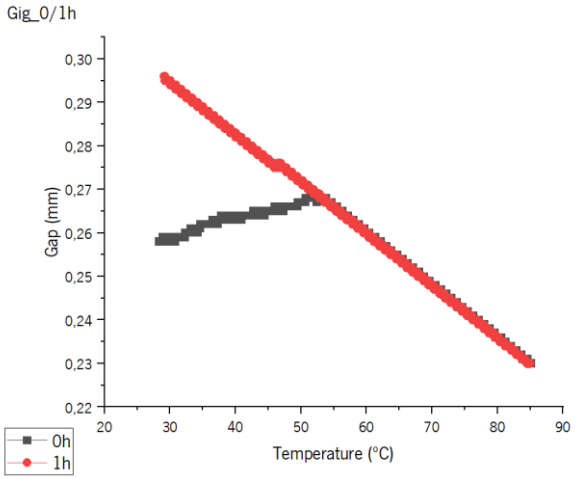

Figure J.2.1.2. Temperature dependence of the thickness (mm) of K2-carrageenan samples, from *Gigartina*, with 0h and 1h of ultrasound, in a 1 wt.% + 0,1 M KCl solution, during rheological testing.

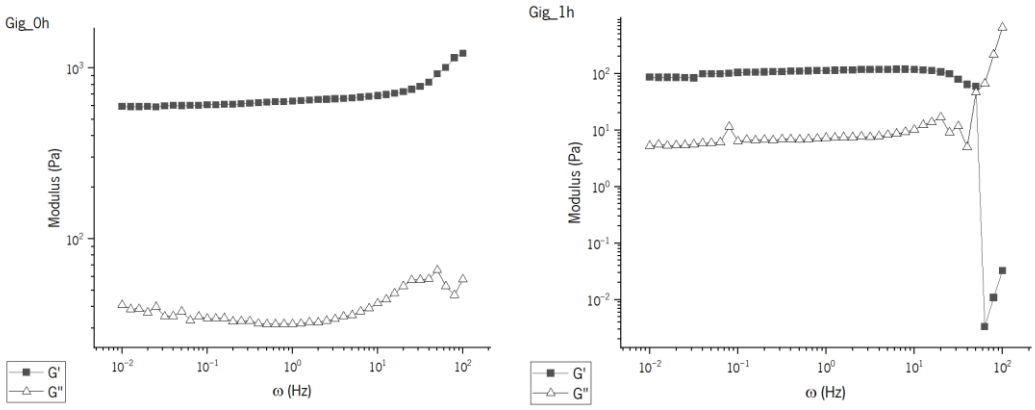

Figure J.2.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples, from *Gigartina*, with 0h and 1h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

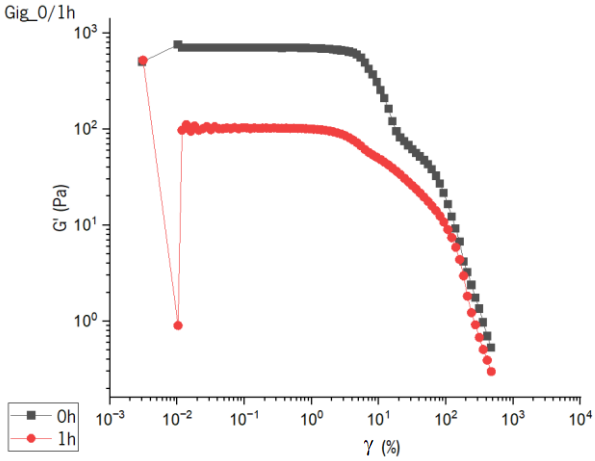

Figure J.2.1.4. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear strain (%), at a 25 °C and 1 Hz, of K2-carrageenan from *Gigartina*, with 0h and 1h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

2.2.Liquid

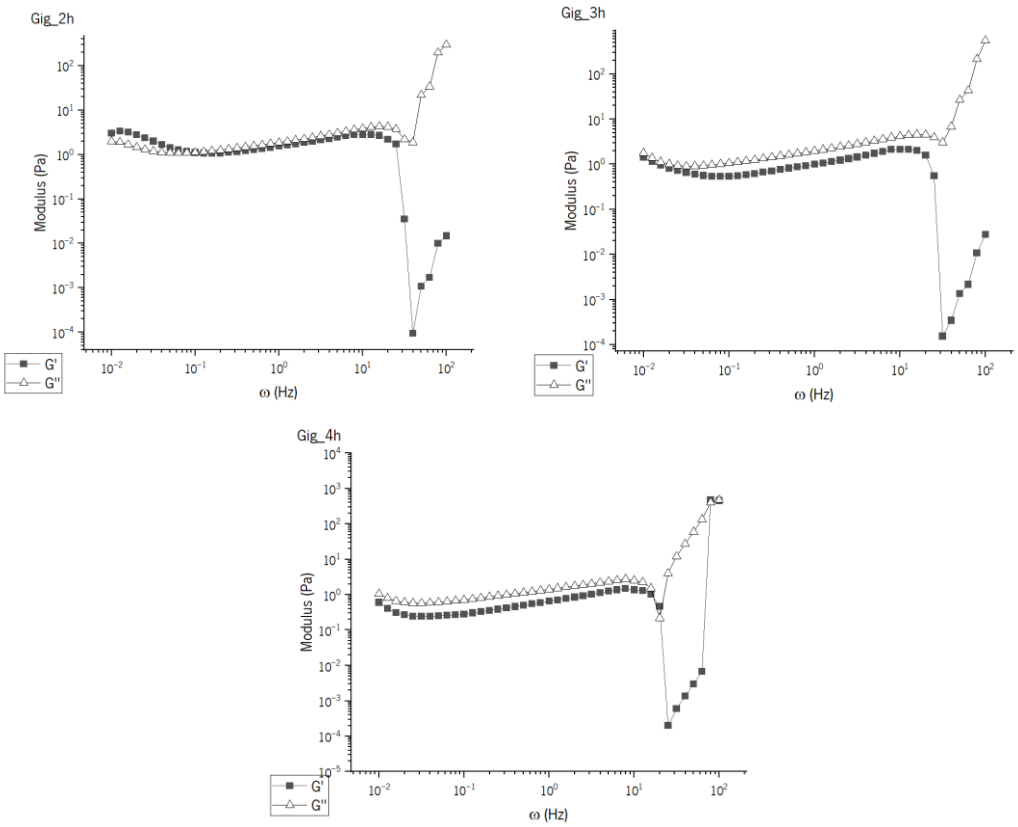

Figure J.2.2.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 50,3 % strain, of K2-carrageenan samples from *Gigartina* with 2h,3h and 4h of ultrasound, in a 1 wt.% + 0,1 M KCl solution.

J.3. Alga *Mastocarpus*

3.1.Gel

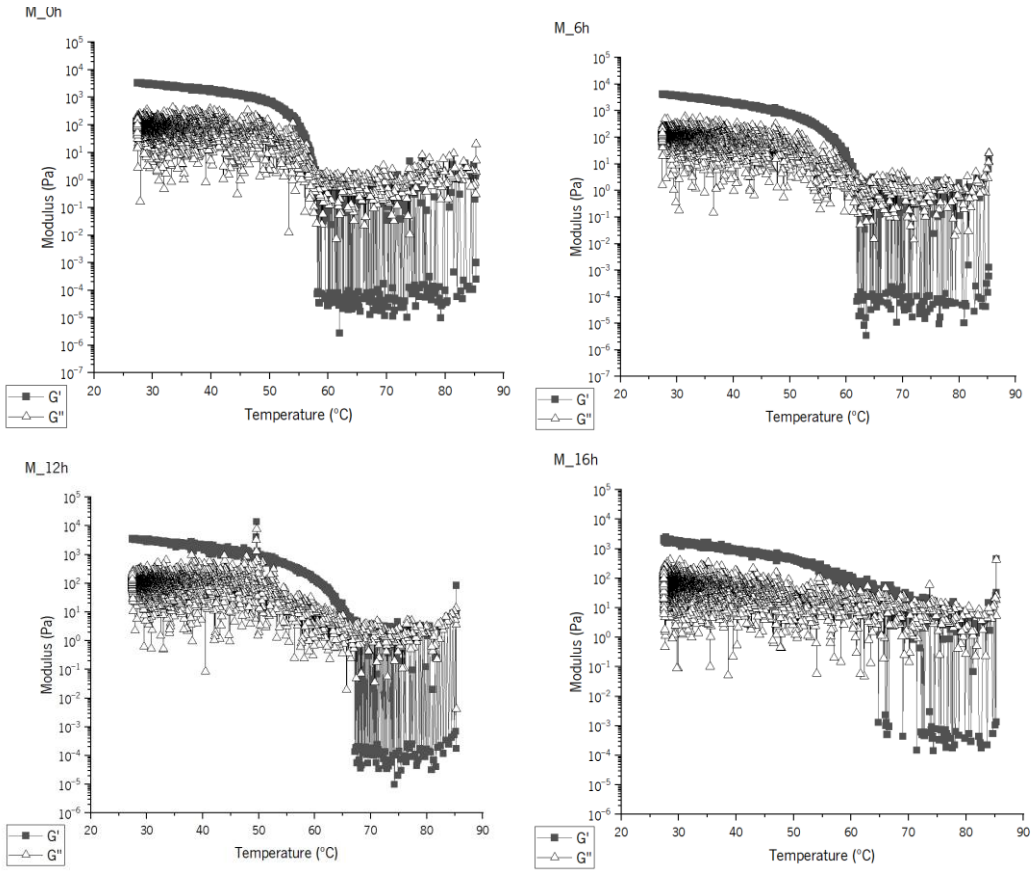

Figure J.3.1.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 85 °C to 25 °C of K2-carrageenan samples, from *Mastocarpus*, with 0h, 6h, 12h and 16h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

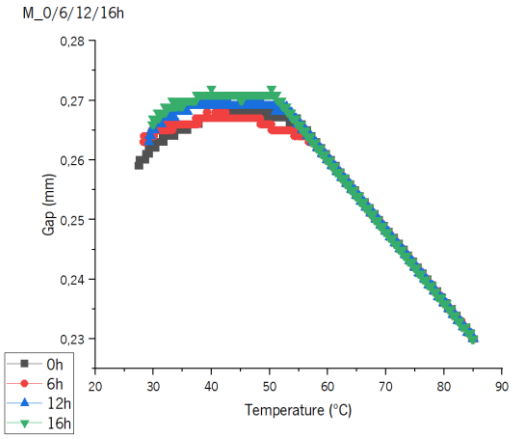

Figure J.3.1.2. Temperature dependence of the thickness (mm) of K2-carrageenan samples, from *Mastocarpus*, with 0h, 6h,12h and 16h of ultrasound, in a 1 wt.% + 0,1 M KCl solution, during rheological testing.

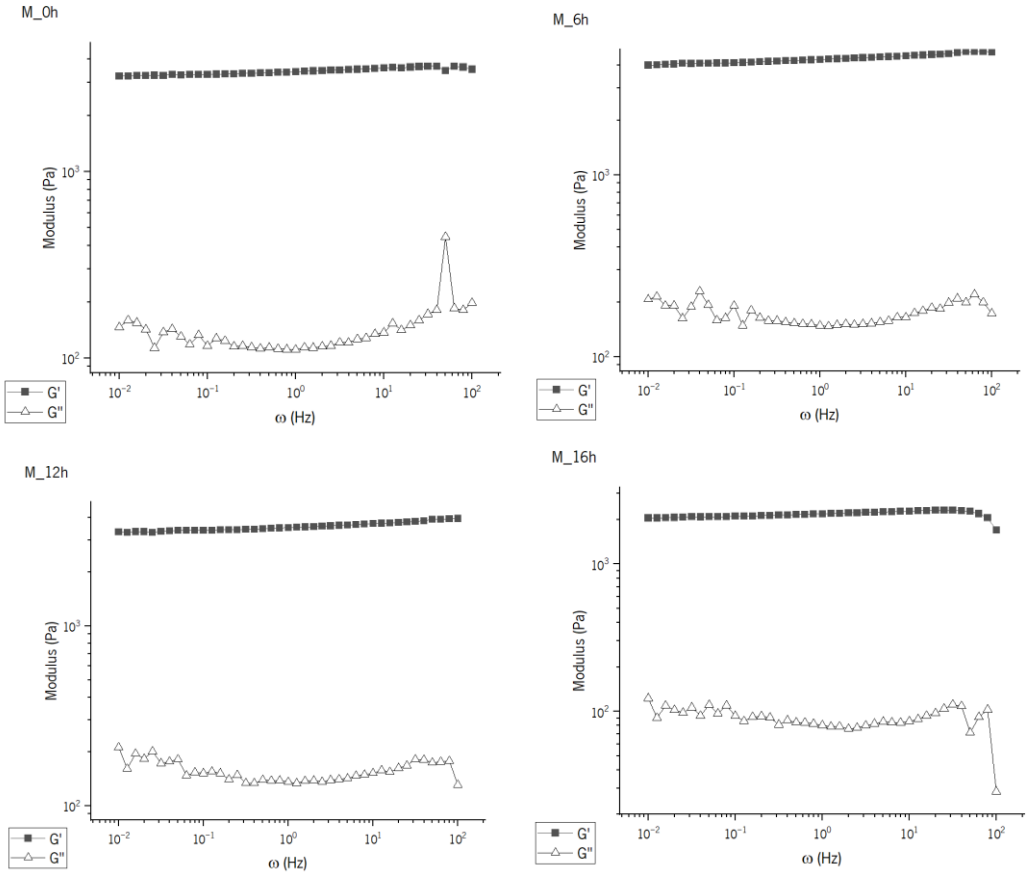

Figure J.3.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples, from *Mastocarpus*, with 0h, 6h, 12h and 16h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

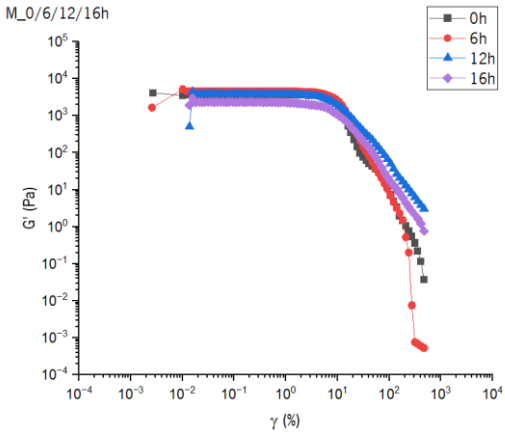

Figure J.3.1.4. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear strain (%), at a 25 °C and 1 Hz, of K2-carrageenan from *Mastocarpus*, with 0h, 6h, 12h and 16h of ultrasound, in a 1 wt.% + 0,1 M KCl solution.

3.2.Liquid

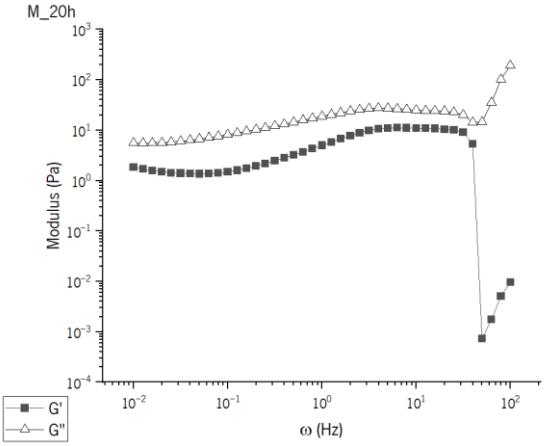

Figure J.3.2.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 50,3 % strain, of K2-carrageenan samples from *Mastorcapus* with 20h of ultrasound, in a 1 wt% + 0,1 M KCl solution.

J.4. Alga BAT 18-09

4.1.Gel

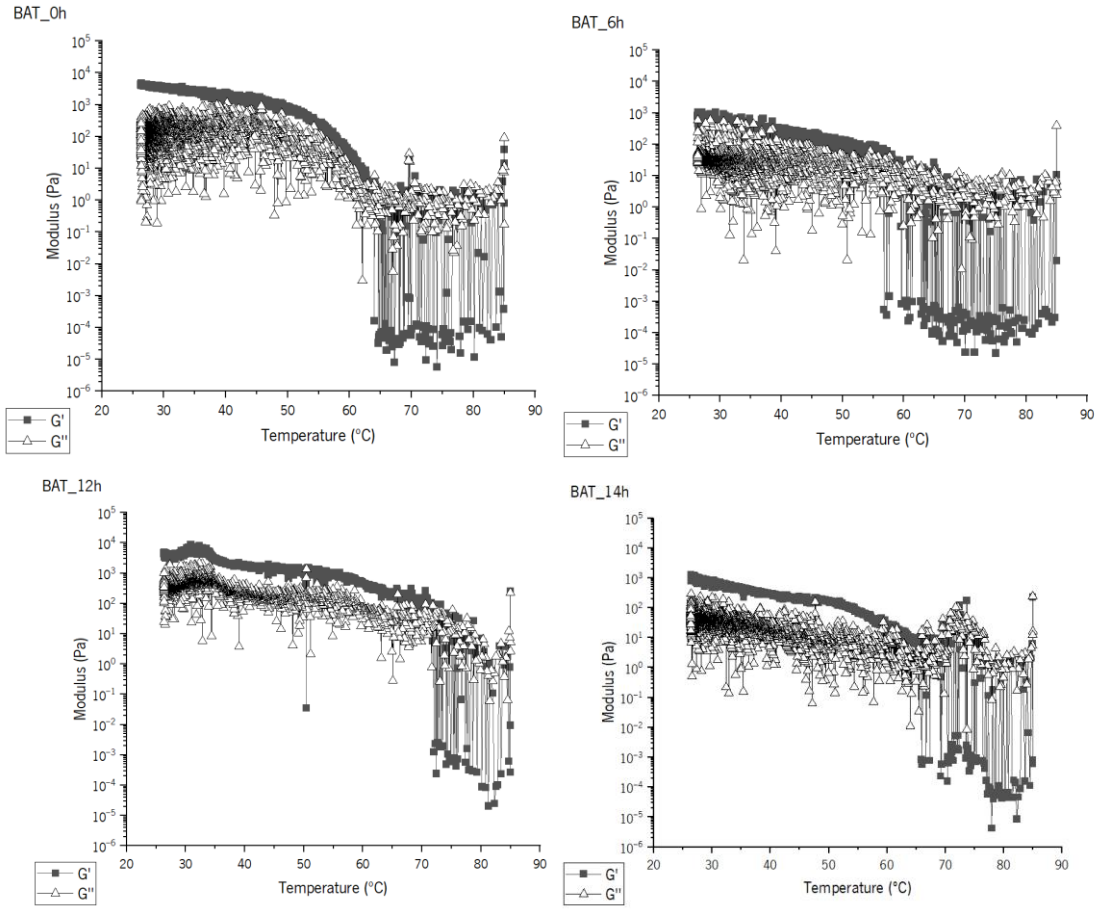

Figure J.4.1.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 85 °C to 25 °C of K2-carrageenan samples, from BAT 18-09, with 0h, 6h, 12h and 14h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

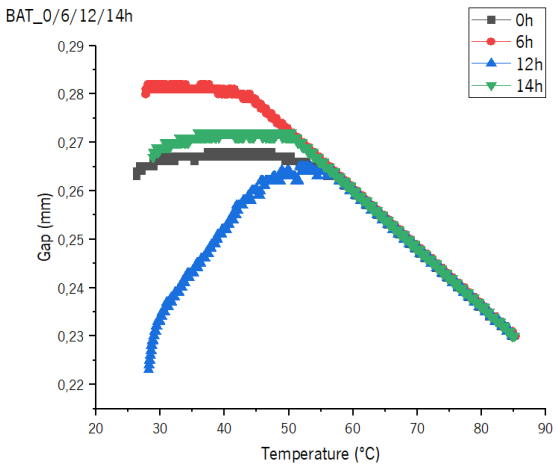

Figure J.4.1.2. Temperature dependence of the thickness (mm) of K2-carrageenan samples, from BAT, with 0h, 6h,12h and 14h of ultrasound, in a 1 wt.% + 0,1 M KCl solution, during rheological testing.

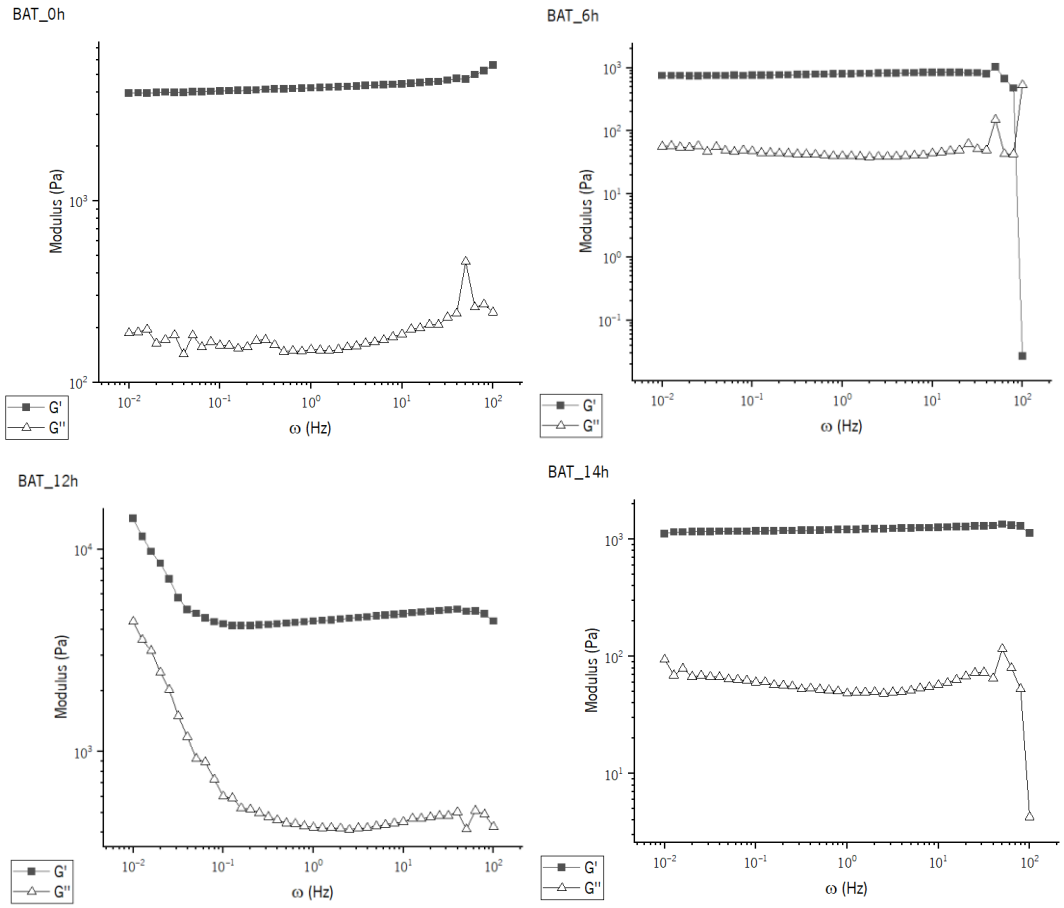

Figure J.4.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples, from BAT 18-09, with 0h, 6h, 12h and 14h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

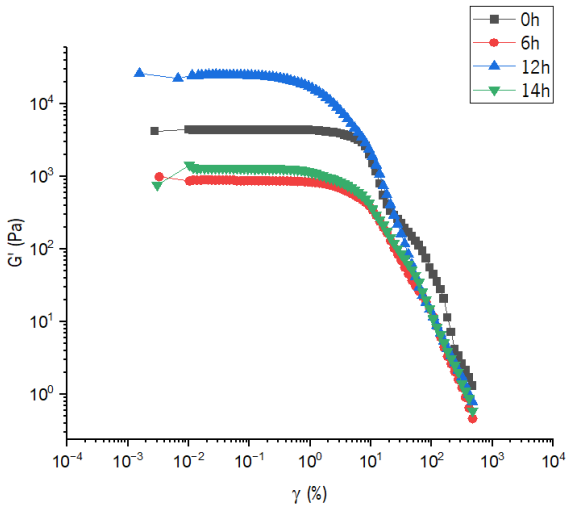

Figure J.4.1.4. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear strain (%), at a 25 °C and 1 Hz, of K2-carrageenan from BAT 18-09, with 0h, 6h, 12h and 14h of ultrasound, in a 1 wt.% + 0,1 M KCl solution.

4.2.Liquid

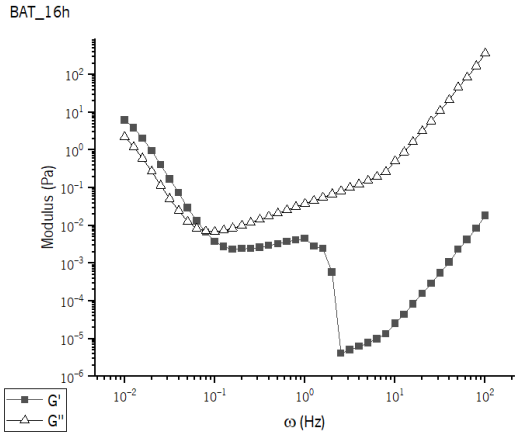

Figure K.4.2.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 50,3 % strain, of K2-carrageenan samples from BAT 18-09 with 16h of ultrasound, in a 1 wt.% + 0,1 M KCl solution.

J.5. Alga C-25

5.1.Gel

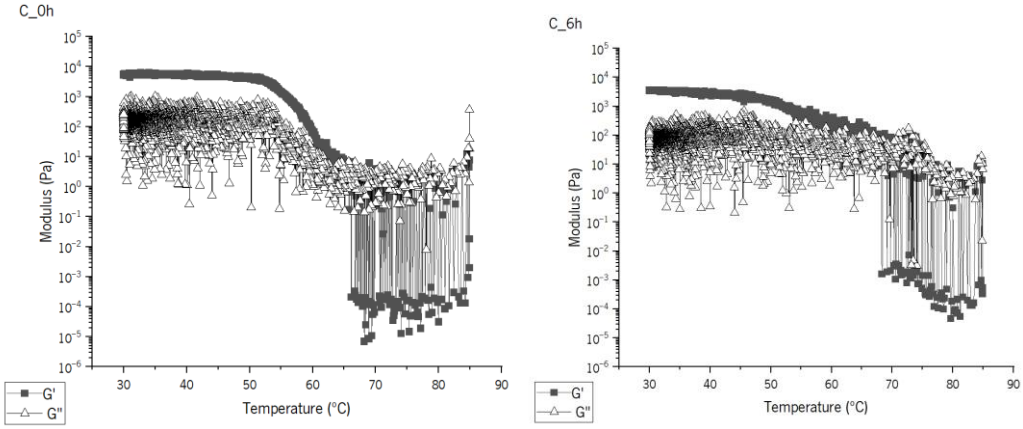

Figure J.5.1.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) during cooling from 85 °C to 25 °C of K2-carrageenan samples, from C-25, with 0h and 6h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

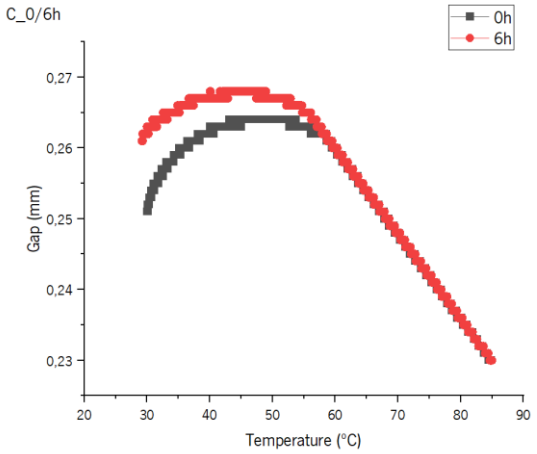

Figure J.5.1.2. Temperature dependence of the thickness (mm) of K2-carrageenan samples, from C-25, with 0h and 6h of ultrasound, in a 1 wt.% + 0,1 M KCl solution, during rheological testing.

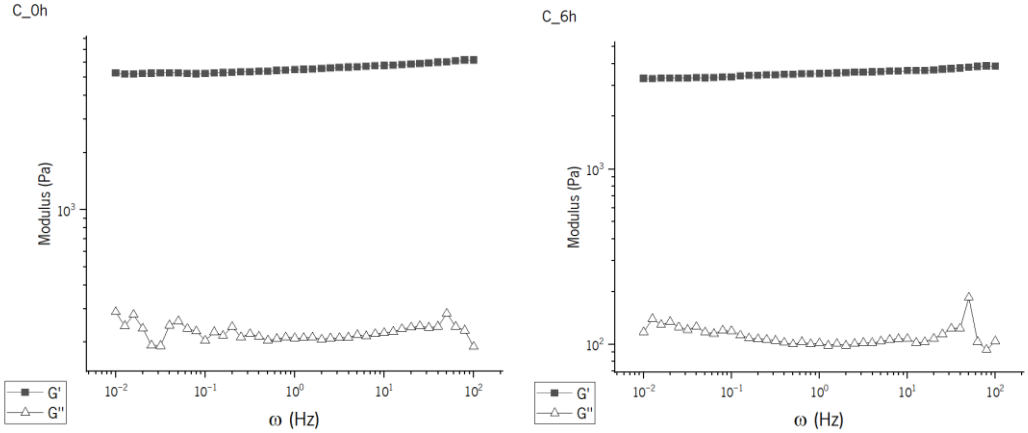

Figure J.5.1.3. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 0,1 % strain, of K2-carrageenan samples, from C-25, with 0h and 6h ultrasound, in a 1 wt.% + 0,1 M KCl solution.

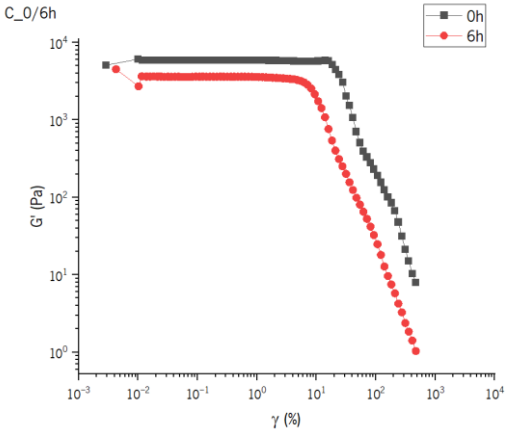

Figure J.5.1.4. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the shear strain (%), at a 25 °C and 1 Hz, of K2-carrageenan from C-25, with 0h and 6h of ultrasound, in a 1 wt.% + 0,1 M KCl solution.

5.2.Liquid

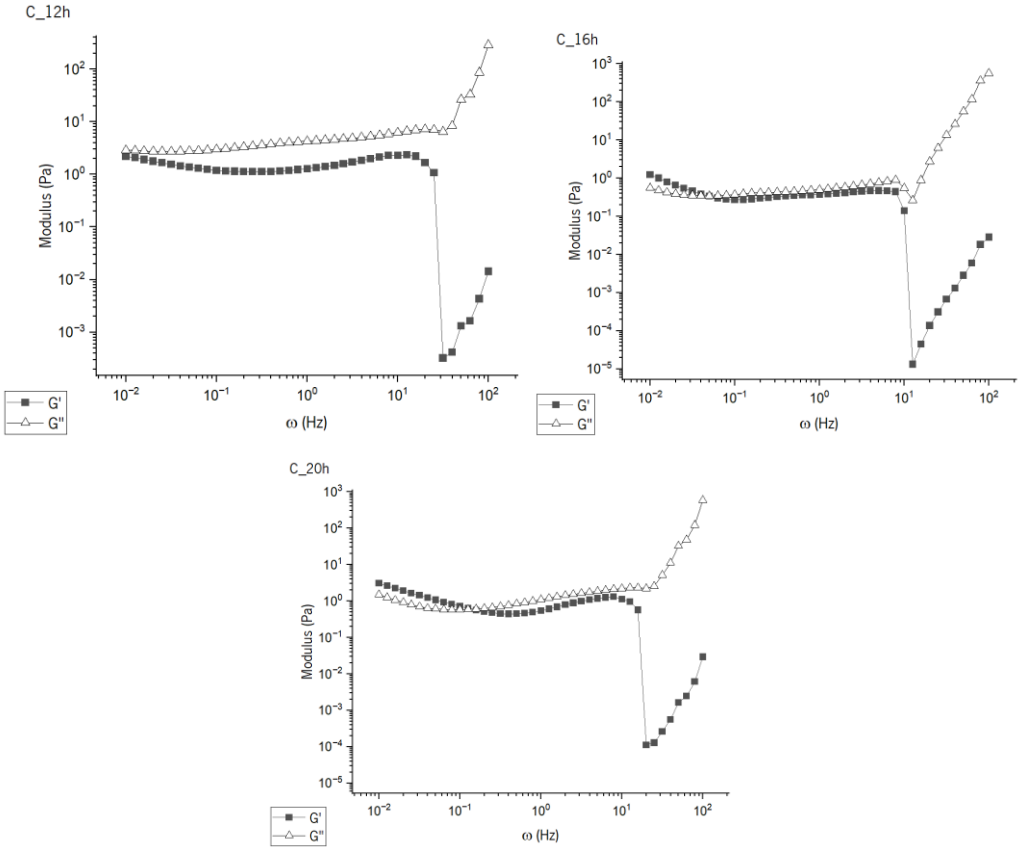

Figure J.5.2.1. Storage modulus (G′) (solid symbols) and loss modulus (G″) (empty symbols) as a function of the oscillation frequencies (ω), at a 25 °C with a 50,3 % strain, of K2-carrageenan samples from C-25 with 12h, 16h and 20h of ultrasound, in a 1 wt.% + 0,1 M KCl solution.