UNIVERSIDADE D
COIMBRA

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NOVEL EXTRACTION METHODS, YIELD, STRUCTURAL AND RHEOLOGICAL PROPERTIES OF CARRAGEENAN FROM NOVEL *KAPPAPHYCUS ALVAREZII* STRAINS FROM THE PHILIPPINES

Tese de Mestrado em Biodiversidade e Biotecnologia, orientada pelo Professor Doutor Leonel Pereira e pela Doutora Ana Marta Gonçalves, e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Julho de 2023

The author is grateful for the support of the FCT—Foundation for Science and Technology, I.P., within the scope of the project LA/P/0069/2020 granted to the Associate Laboratory ARNET, UIDB/04292/2020 granted to MARE—Marine and Environmental Sciences Centre. This work has also funded by the INTEREG project NASPA (EAPA_451/2016).

This work was also financed by project MENU—Marine Macroalgae: Alternative recipes for a daily nutritional diet (FA_05_2017_011), funded by the Blue Fund under Public Notice No. 5—Blue Biotechnology which co-financed this research and project PORBIOTA, E-Infrastructure Portuguese Information and Research in Biodiversity (POCI-01-0145-FEDER-022127), supported by Competitiveness and Internationalization Operational Programme and Regional Operational Programme of Lisbon, through FEDER, and by the Portuguese Foundation for Science and Technology (FCT), through national funds (OE).

Acknowledgments/Agradecimentos

Ao meu orientador, Professor Doutor Leonel Pereira, pelo apoio, disponibilidade, rigor, conhecimento científico e compreensão. Pela confiança que depositou em mim e por me dar a oportunidade de trabalhar neste tema.

À minha co-orientadora, Doutora Ana Marta Gonçalves, pelos desafios lançados e possibilidade de complementar o meu trabalho. Pela ajuda, sugestões e contribuições.

Aos meus colegas e amigos do Laboratório de Algas Marinhas (LAM), pela amizade, camaradagem e partilha de conhecimentos. Em especial ao Mestre João Cotas, pelas sugestões dadas em grande parte deste trabalho, pelos ensinamentos, paciência, e motivação ao longo deste caminho.

À Mestre Irene Gutiérrez pelo apoio, acompanhamento e contribuição científica nas análises bioquímicas realizadas no laboratório Marefoz.

Agradeço ao Doutor Michael Y. Roleda (Marine Science Institute, University of the Philippines Diliman) e ao Doutor Alan T. Critchley (Verschuren Centre for Sustainability in Energy and Environment, Cape Breton University) pela gentileza na cedência das amostras de *Kappaphycus alvarezii*, pela confiança e entusiamo demonstrado no trabalho, disponibilidade e pela leitura crítica do manuscrito.

Aos meus amigos, pelo apoio nos momentos de inquietude e em que a vida pesa; por conseguirem sempre que volte a ver como sou capaz de dançar. Em especial, às minhas amigas-irmãs Carolina, Alice e Jegundo por serem inspiração, admiração e carinho na minha vida.

Agradeço por fim à minha família, em especial à minha mãe e à minha avó por me terem educado com amor, valores e sentido de responsabilidade. Por serem exemplo de persistência e resiliência. Obrigada pelos sacrifícios que fizeram, por acreditarem em mim e me fazerem voar. Tudo o que há em mim é um bocadinho vosso também.

Resumo

A carragenana é um hidrato de carbono economicamente importante (polissacarídeo) derivado de algas vermelhas (Rhodophyta) devido à sua utilização na indústria como agente gelificante, espessante e estabilizante. *Kappaphycus alvarezii* é a fonte mais comum de kappa-carragenana, cultivada principalmente em países asiáticos. A crescente procura de polissacáridos de algas marinhas levou à introdução de algas não nativas em muitas regiões tropicais. Estas introduções intencionais podem conduzir à sua propagação a partir das zonas agrícolas, o que pode estabelecer-se e alterar a dinâmica dos ecossistemas beneficiários. O aquecimento global representa uma grande ameaça para o cultivo de *K. alvarezii*. Mudanças abruptas na salinidade, temperatura oceânica e intensidade luminosa causam danos físicos ao *Kappaphycus* spp., afetando os processos eco-fisiológicos, reprodutivos e metabólicos das algas marinhas, tornando-as mais suscetíveis a doenças e diminuindo sua produtividade (por exemplo, "doença do gelo"). Isto cria a necessidade de produzir novas estirpes destas algas para garantir fontes de carragenana.

Convencionalmente, o processo de extração das carragenanas tem sido realizado por um tratamento alcalino de até três horas com aquecimento. A inovação de técnicas de extração aprimoradas é crucial para acelerar as técnicas de extração, reduzindo a pegada ecológica e os custos de solventes, energia e tempo. Este estudo tem como objetivo investigar oito novas estirpes de *K. alvarezii* das Filipinas e testar e desenvolver novos métodos de extração de carragenana recorrendo a diferentes tratamentos químicos: extração assistida por ultrassons (EAU) e extração por fluido supercrítico (EFS).

A espectroscopia FTIR-ATR foi utilizada para caracterizar a estrutura das diferentes frações de carragenana. A caracterização bioquímica foi utilizada para avaliar a eficiência das extrações, e a medição da viscosidade e as propriedades físico-químicas também foram determinadas. O potencial de absorvância UV também foi determinado por espectrofotometria UV-Vis. Assim, informações detalhadas sobre as propriedades e estrutura dos polissacarídeos presentes nos novos cultivares foram reveladas e o potencial industrial avaliado. Os resultados iniciais da extração de polissacarídeos permitiram identificar o cultivar com maior teor de carragenana e foi realizada uma caracterização profunda sobre este, de forma a recolher informação e otimizar os métodos de extração para cada um dos cultivares restantes.

Tanto o método de extração convencional quanto a assistida por ultrassom (EAU) usando meio alcalino NaOH (1 M) revelaram valores dentro da gama aceitável para a produção de carragenana refinada comercial. Além disso, a EAU usando meio alcalino KOH (8%) revelou um rendimento alto e semelhante ao método de extração convencional com KOH, aplicando metade do tempo de extração e melhorando a viscosidade, revelando fontes promissoras de carragenana semi-refinada comercial. A extração por fluido supercrítico (EFS) revelou um rendimento relativamente alto de carragenana, mas a viscosidade foi menor e o pH ácido.

Palavras-chave: Rhodophyta, polissacarídeo sulfatado, extração assistida por ultrassons, extração por fluido supercrítico.

Abstract

Carrageenan is an economically important carbohydrate (polysaccharide) derived from red seaweed (Rhodophyta) because of its use in industry as a gelling, thickening, and stabilizing agent. *Kappaphycus alvarezii* is the most common *kappa*-carrageenan source, primarily farmed in Asian countries. The increasing demand for seaweed polysaccharides has led to the introduction of non-native seaweeds in many tropical regions. Such intentional introductions can lead to the spread of these from farming areas, which can become established and alter the dynamics of the recipient ecosystems. Global warming poses a major threat to *K. alvarezii* cultivation. Abrupt changes in salinity, ocean temperature, and light intensity cause physical damage to *Kappaphycus* spp., affecting the eco-physiological, reproductive, and metabolic processes of the seaweed, making it more susceptible to diseases and declining its productivity (e.g., "ice-ice disease"). This creates the need to produce novel strains of this seaweed to guarantee carrageenan sources.

Conventionally, the extraction process for carrageenans has been performed by an alkaline treatment of up to three hours with heating. The innovation of enhanced extraction techniques is crucial to accelerate extraction techniques while reducing the ecological footprint and the costs of solvents, energy, and time. This study aims to investigate eight novel strains of *K. alvarezii* from the Philippines and test and develop novel carrageenan extraction methods using different chemical treatments: ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE).

FTIR-ATR spectroscopy was used to characterize the structure of the different carrageenan fractions. Biochemical characterization was used to assess extractions efficiency, and viscosity measurement and physio-chemical proprieties were also determined. UV-absorbance potential was also determined by UV-Vis spectrophotometry. Thus, detailed information regarding the properties and structure of the polysaccharides present in these novel strains was revealed and their industrial potential evaluated. The initial results of polysaccharide extraction allowed to identify the strain with the highest carrageenan content and a deep characterization was performed on this, in order to collect information and optimizing the extraction methods for each of the remaining strains.

Both the conventional extraction method and UAE using NaOH (1 M) revealed values within the acceptable range for commercial refined carrageenan. Furthermore, UAE using KOH (8%) showed a high and similar yield compared with the conventional extraction method under half of the extraction time and improved carrageenan viscosity, revealing promising sources of semi-refined carrageenan. SFE showed a relatively high yield of carrageenan, but the viscosity was found to be lower and the pH acidic.

Keywords: Rhodophyta, sulphated polysaccharide, ultrasound-assisted extraction, supercritical fluid extraction.

Table of contents

Figures index

Tables index

Abbreviation list

% - Percentage

°C - Degree Celsius

cm - Centimetre

DW - Dry weight

EC - Electrical conductivity

TDS - Total dissolved solids

FAO - Food and Agriculture Organization

FTIR-ATR - Fourier-Transform Infrared - Attenuated Total Reflectance

cP - Centipoise

μS - Microsiemens

h - Hour

min - Minute

ι - Iota

κ - Kappa

λ - Lambda

g - Gram

mg - Milligram

mL - Millilitre

rpm - Revolutions per minute

UV/VIS - Ultraviolet/ Visible

ANOVA - Analysis of Variance

A - 3,6-Anidrogalactose

D - a-D-galactose

FAO - Food and Agriculture Organization

G - b-D-Galactose

GLC - Gas Liquid Chromatography

HPLC - High Performance Liquid Chromatography

NE – Native extraction

CE – Conventional extraction

MAE - Microwave-assisted extraction

UAE - Ultrasound-assisted extraction

EAE - Enzymatic-assisted extraction

SWE - Subcritical water extraction

SFE - Supercritical fluid extraction

FDA - Food and Drug Administration

EC - European Parliament and Council Regulation

JECFA - Joint FAO/WHO Expert Committee on Food Additives

IUPAC - International Union of Pure and Applied Chemistry

kDa - Kilodalton

Kg - Kilogram

M - Molar

mPa - Millipascal

ppm - Parts per million

RC - Refined Carrageenan

SRC - Semi-Refined Carrageenan

List of papers

This thesis is based on the following papers:

Paper I (Review): Mendes, M., Cotas, J., Pacheco, D., Ihle, K., Hillinger, A., Cascais, M., Marques, J.C., Pereira, L., Gonçalves, A.M.M. (2023). Factors impacting red seaweed (Rhodophyta) polysaccharides yield and quality (manuscript)

Paper II: Mendes, M., Cotas, J., Gonçalves, A.M.M., Pereira, L., Critchley, A., Lourie Ann R. Hinaloc, and Roleda, M.Y. (2023). Novel extraction methods, yield, structural and rheological properties of k-carrageenan from a novel haploid *Kappaphycus alvarezii* strain from the Philippines (manuscript)

1. Introduction

1. Introduction

1.1. Seaweeds

Under the sea surface, providing a significant part of the world's oxygen and forming habitats teeming with life, seaweeds grow abundantly. Seaweeds are photoautotrophic multicellular organisms (mainly marine, with some species that live in freshwater) belonging to the domain Eukarya and the kingdoms Plantae (green and red algae) and Chromista (brown algae) and are divided into three phyla according to their pigment composition and chemical content (Pereira, 2021; Silva et al., 2019): green (Chlorophyta), brown (Ochrophyta, Phaeophyceae), and red (Rhodophyta). They are widely distributed geographically, from tropical to polar regions, with ecoregions ranging from intertidal to submerged zones that are still exposed to sunlight (Tirtawijaya et al., 2022). Seaweeds chemical content diversity results from this distribution, which translates into a diversity of environments. In response to harsh environmental stresses, seaweeds synthesize unique polysaccharides, long-chain polymers made up of simple sugars that are chained together with glycosidic bonds. These polysaccharides have several functions, such as protection against waves and desiccation, maintenance of ionic equilibrium, structure for the cell walls, and as food reserves (Lee, et al., 2017a; Percival, 1979).

Thousands of tons of seaweed are collected each year around the world, with China and Japan leading the way in consumption. Although a large part of the seaweed produced is still intended for direct human consumption, the continuously increasing demand for secondary metabolites, such as carrageenan (polysaccharide), has resulted in carrageenophyte seaweeds currently being the most cultivated (FAO 2022). Carrageenans are sulphated polysaccharides found in the cell wall of certain red algae that exist in three main isomers (*kappa-*, *iota-* and *lambda* carrageenan) with different properties and uses. The main produced types of carrageenan are iota and kappa, mainly obtained from eucheumatoid seaweeds *Eucheuma denticulatum* and *Kappaphycus alvarezii*, respectively (Bixler & Porse, 2011), with κ-carrageenan presenting higher demand globally, making *K. alvarezii*, a commercially highly valuable Rhodophyta (Lim et al., 2017).

1.2. Eucheumatoid seaweeds

Kappaphycus and *Eucheuma* genera, are the major sources of carrageenans and are collectively known as eucheumatoid seaweeds, belonging to the Eucheumatoideae tribe, Solieriaceae family, Gigartinales order, Rhodophyta phylum.

1.2.1. Cultivation of eucheumoid seaweeds

Eucheumatoids were first cultivated in Southeast Asia in the 1970s (Parker, 1974). The growing demand for *k*-carrageenan due to its wide range of uses has resulted in a rapid increase of *K. alvarezii* cultivation worldwide. In the last decade, algae production increased from 20 174.3 thousand tonnes (2010) to 35 077.6 thousand million tons (2020), of which eucheumatoid seaweeds (*Eucheuma* spp., *Kappaphycus alvarezii* and *Eucheuma denticulatum*) are currently responsible for 28.2% of the world production of major aquaculture species, with *K. alvarezii* contributing 4.6% (FAO 2022). Due to the effectiveness of eucheumatoid farming, cultivation was extended throughout the tropical and subtropical world, within and outside of the species native range. The low degree of technology and investment required, as well as its potential as an alternative livelihood in rural areas since it is naturally compatible with traditional fishing and other subsistence uses of the inshore environment, were the key reasons for the fast growth of eucheumatoid farming (Pickering, 2006). *K. alvarezii* is therefore primarily grown in Asian nations (such as the Philippines, Vietnam, Indonesia, and Malaysia) where seaweed cultivation contributes substantially to the sustainable development of the economic condition of coastal women by providing livelihood opportunities and ensuring financial solvency (Sultana et al., 2022; Li et al., 2016). Other producing countries also include Madagascar, India, Tanzania (Zanzibar), Central/South Pacific Islands (Kiribati, Fiji, and Solomon Islands), and East Timor (Kumar et al., 2020; Rupert et al., 2022). The methodologies used include the fixed, off-bottom line method, the floating raft method and basket method (Kim et al., 2017). Although it has a low degree of technology and investment required, mariculture of these seaweeds has some challenges, such as pests, diseases, and epiphytes (Kim et al., 2017).

1.2.2. Eucheumatoid seaweeds reproduction

Eucheumatoid seaweeds are capable of reproducing both sexually and asexually. In natural populations under native conditions, these seaweeds (Rhodophyta phylum) exhibit sexual reproduction, with *Polysiphonia*-type of life cycle triphasic, consisting of three stages: tetrasporophyte (2n), gametophyte (n), and carposporophyte (2n). The gametophyte is dioecious. The male thallus produces ameboid spermatia within spermatangia, while the female thallus produces many few-celled carpogonial branches in the cortex of the thallus, of which the tip acts as the carpogonium or receptor cell for the spermatia. Fertilization results in the carposporophyte (within the tissue of the female gametophyte) which produces carpospores (2n) mitotically that once released develop into the tetrasporophyte phase. Afterwards, the tetrasporophyte produces tetrasporangia, which undergo meiotic divisions resulting in the production of tetraspores (n). Upon their release, the tetraspores develop into male and female gametophytes, completing the life cycle (Pereira & Yarish, 2008; Pereira, 2021). However, eucheumatoid seaweeds sexual reproduction is associated with low frequency (Doty and Santos, 1978; Lluisma and Ragan, 1995; Bulboa et al., 2008) and low viability of spores.

As previously mentioned, the genus *Kappaphycu*s have been intentionally introduced for mariculture, especially *Kappaphycus alvarezii* (Doty ex P.C. Silva, 1996), native to the Philippines (Azanza-Corrales et al., 1992). This seaweed is widely cultivated in non-native locations through vegetative propagation (asexual reproduction) of the tetrasporophyte (2n) and is referred several times on the literature that some of the strains/varieties/cultivars only reproduce asexually, which in the majority of the times is true (40-45 days of cultivation through propagation) however, the possibility of sexual reproduction happen from different cultivars outside its native range cannot be excluded. One of the examples is in the Hawaii, where populations of *Kappaphycus* sp. are said to only reproduce vegetatively (Smith et al., 2002), yet under laboratory conditions sexual reproduction occurred (Chandrasekaran et al., 2008). Other examples are some areas of cultivation in the Philippines where it was believed that the biomass presented a variable ratio of kappa/iota due to mix cultivation of *Kappaphycus/Eucheuma*, however recent studies point to the possibility that in some areas of cultivation of *K. alvarezii* and *K. striatus* sexual reproduction can be present (carposporophyte and tetrasporophyte). Adult thallus, when they have no visible reproductive structures, are normally considered to be male gametophytes, but it is normal for them to be called "unfruited thallus" making the

report as male gametophytes rare; however, the finding of cystocarps is enough to affirm that sexual reproduction is occurring (Azanza-Corrales et al., 1992).

Regarding asexual reproduction, it has been shown that introduced eucheumatoids have become established outside farms in several areas (Azanza-Corrales et al., 1992) becoming pests in Hawaii, India, and Panama and environmental impact has been registered in several other cultivation sites. However, predictions about invasiveness are uncertain and security protocols approved by governments, including quarantine procedures as part of permanent monitoring of sites, are essential to prevent environmental risks (Azanza and Ask, 2017) but are often not correctly applied (Reis et al., 2009).

There is some irony to the fact that even after nearly six decades, the sex and ploidy of those *Kappaphycus* species that are commercially cultivated remain mostly unknown. Moreover, whether there are differences in growth rates, biochemistry, and rheological properties between vegetative and reproductive thalli and among different life history stages (i.e., male and female gametophytes and tetrasporophytes) are rarely even considered if they have significant economic impacts. Among other commercially important taxa, agar yield and quality in *Gelidiella acerosa* varies between vegetative and reproductive fronds (Roleda et al., 1997), whilst the carrageenan composition in *Chondrus crispus* varies significantly between the diploid tetrasporophyte (mainly lambda-carrageenan) and the haploid gametophyte (mainly kappa/iota-carrageenans) life stages (Lipinska et al., 2020).

1.2.3. *Kappaphycus alvarezii*

Kappaphycus alvarezii (commercially known as *"Cottonii"*) is the major industrial source of relatively pure κ-carrageenan (Gereniu et al., 2017; Rupert et al., 2022). Being a red seaweed, contain as photosynthetic pigments chlorophyll a, phycobilins (phycoerythrin and phycocyanin), and some carotenoids. The pigment phycoerythrin provides the seaweed's red pigmentation by reflecting red light and absorbing blue light waves. Depending on the phycoerythrin pigment concentration, it can be found in reddish, yellowish, brown, and green colors (Rudke et al., 2020). The *Kappaphycus* genus shows rapid growth rates, with an increase near 4.5% daily (Gereniu et al., 2017), harvest cycles of 45–75 days and high polysaccharide yields (de Góes & Reis, 2012; Rupert et al., 2022).

Depending on the cultivation conditions (water temperature, salinity, sunlight, light intensity, depth, waves power, among others) chemical constituents of *K. alvarezii* and concentrations are substantially variable. Besides high content of carbohydrates (average of 50.8%), *K. alvarezii* also contains in average 3.3% proteins, 3.3% lipids, 15.6% ash, 12.4% sulphated groups and 3.0% insoluble aromatics (Khalil et al., 2018; Rudke et al., 2020; Solorzano-Chavez et al., 2019). The lipidic fraction is mostly composed of saturated fatty acids (64.28%), in particular C16:0 (46.51%). *K. alvarezii* also contains essential amino acids, up to 43% of the total amino acid content, noteworthy phenylalanine, leucine and threonine (Naseri et al., 2019). In addition, it is a very important source of carotenoids, fibers, minerals, vitamins, and other compounds (Nagarani and Kumaraguru 2012).

1.3. Carrageenan

1.3.1. Historical background and current market

Carrageenan, polysaccharide first known as “carrageen”, was discovered by the British pharmacist Stanford discovered this polysaccharide in 1862, while extracted it from Irish Moss (*Chondrus crispus*). Carrageenan is a gel-forming and viscosifying polysaccharide, which is mainly obtained by the extraction from the cell walls of certain species from Rhodophyta phylum (Van De Velde et al, 2002) predominantly *Chondrus*, *Gigartina*, and various *Eucheuma/Kappaphycus* species (Necas & Bartosikova, 2013).

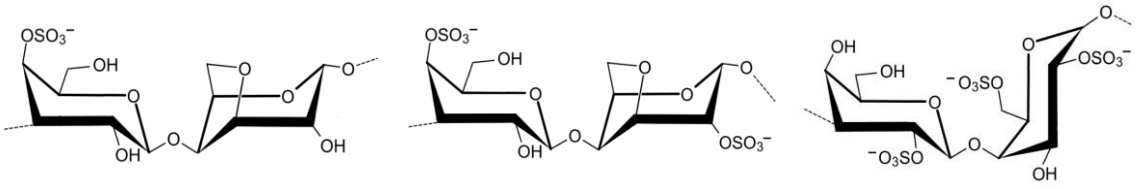
Carrageenan has been used in Ireland since 400 AD as a gelatine and as a home remedy to cure coughs and colds and in China around 600 before century (Necas & Bartosikova, 2013), being introduced to the industry in the early 1930s. Carrageenan has no nutritional value, being used in food industry for its gelling, thickening, stabilizing and emulsifying properties (Van de Velde et al. 2002). When used in food products, carrageenan has the EU additive E-number E407. The E-number E407a is attributed to another EU additive that contains a considerable amount of cellulose in comparison to E407, being commonly applied in animal feed sector.

Nowadays, the demand of carrageenan in the international market is increasing due to its applications. FAO statistics documents the major carrageenan seaweed farming countries being, as previously referred, Indonesia, the Philippines, the United Republic of Tanzania, Malaysia and China (in order of quantity produced). According to Global

Market Insights (GMI), carrageenan market size valued 850 million in 2022 and is anticipated to showcase over 6% compound annual growth rate (CAGR) from 2023 to 2032. Carrageenan market is dominated by kappa carrageenan, and it's expected to grow at 6.5% CAGR in Europe market through 2032, due to its increasing utilization as a gelling agent and stabilizer in the food sector, specifically in dairy and meat products.

1.3.2. Chemical structure

Carrageenan contains D-galactose and 3,6-anhydro-D-galactose and can reach up to 50-80% in species like *K. alvarezii*. The chemical structure of carrageenan is very heterogeneous and can be classified depending on the number and position of sulphate substitutions as well as the location of the 3,6-anhydro bridge in -1,4-linked galactose residues (Pereira et al., 2009). Most common types of carrageenan are traditionally identified by a Greek letter. Most important carrageenans at industrial level are kappa (κ-), iota (ι-) and lambda (λ-) type carrageenans (Fig. 1) according to IUPAC (International Union of Pure and Applied Chemistry) and according to the letter code (Knutsen et al., 1994) G4S-DA, G4S-DA2S, e G2S-D2S,6S, respectively (Table 1). Besides these mains carrageenan variants, other types like beta (β), xi (ξ), mu (μ), nu (ν), or theta (θ) are reported (Cunha & Grenha, 2016). Carrageenan's double helix arrangement offers anion-hosting sites for improved gel structure. The ions that the various fractions accept vary, with ι-carrageenan frequently accepting divalent ions (like Ca^{2+}) and κ-carrageenan typically receiving monovalent ions (like K^{+}).



kappa (κ) iota (ι) lambda (λ)

Figure 1. Chemical structure of kappa-, iota-, and lambda-carrageenan.

Commercial type carrageenan sulphate content usually varies between 22-38%. Besides galactose and sulphate, additional carbohydrate residues can be found in carrageenan compositions, such as glucose, xylose, mannose, arabinose, ribose, and uronic acids (Olasehinde et al., 2019; Rhein-Knudsen et al., 2017). The presence of 3,6-

anhydrogalactose units and the sulfation pattern of carrageenan are believed to contribute significantly to the variation in their structural composition, with ι- and κ-carrageenan being jellifying carrageenans, while lambda is a viscosifying/ thickener (Van De Velde et al., 2002).

Table 1. Most important carrageenans according to IUPAC (International Union of Pure and Applied Chemistry) and according to the letter code (Knutsen et al., 1994).

Carrageenan	G units β-D-galactose	D units α-D-galactose	Letter code
Kappa (κ)	4-sulphate	3,6-anydro	G4S DA
Iota (ι)	4-sulphate	3,6-anydro 2-sulphate	G4S DA2S
Lambda (λ)	2-sulphate	2,6-dissulphate	G2S D2S6S

1.4. Carrageenan extraction methods

Besides abiotic and abiotic factors, carrageenan extraction processes influence polysaccharide biochemical content. There are numerous techniques for extracting carrageenans from seaweed, and the processing conditions are frequently treated as trade secrets by manufacturers because they alter the final carrageenan qualities. This subtopic discusses the available approaches, benefits and drawbacks of each technique and novel extraction techniques.

1.4.1. Alkaline modification of carrageenan

Commercial carrageenans are normally divided, as previously referred, into three structural types: kappa, iota and lambda forms (see section 1.3.2.). The idealized disaccharide repeating units of these carrageenans are given in Fig. 1, however, seaweeds do not produce these idealized and pure carrageenans, but a range of hybrid structures. As previously referred, several other carrageenan repeating units exist: xi, theta, beta, mu and nu (Fig. 2). The precursors (mu and nu), when exposed to alkali conditions, are modified into kappa and iota, respectively, through formation of the 3,6-anhydro-galactose bridge.

The formation of 3,6-anhydrous-α-D-galactose (**DA**) units from α-D-galactose 6-sulphate (**D6S**) residues through alkaline treatment is a significant and well-known reaction (Pereira and Van de Velde, 2011).

The alkali modification of carrageenan in industrial carrageenan extraction is intended to improve polysaccharide extraction and accelerate 6-sulphate removal from the monomer form 3,6-anhydro-D-galactose, thereby increasing gel strength and product reactivity on the protein (Rupert et al., 2022). The cyclization reaction is carried out by the hydroxide ion (OH^-) as a catalyst, while *in vivo*, iota and kappa carrageenan are enzymatically formed from their precursors, by the action of a sulphohydrolase. The inverting of the chair conformation from $^1C_4$ to $^4C_1$ due to the presence of 3,6-anhydrogalactose is favorable to the gelation characteristics of carrageenans (Rhein-Knudsen et al., 2015) .

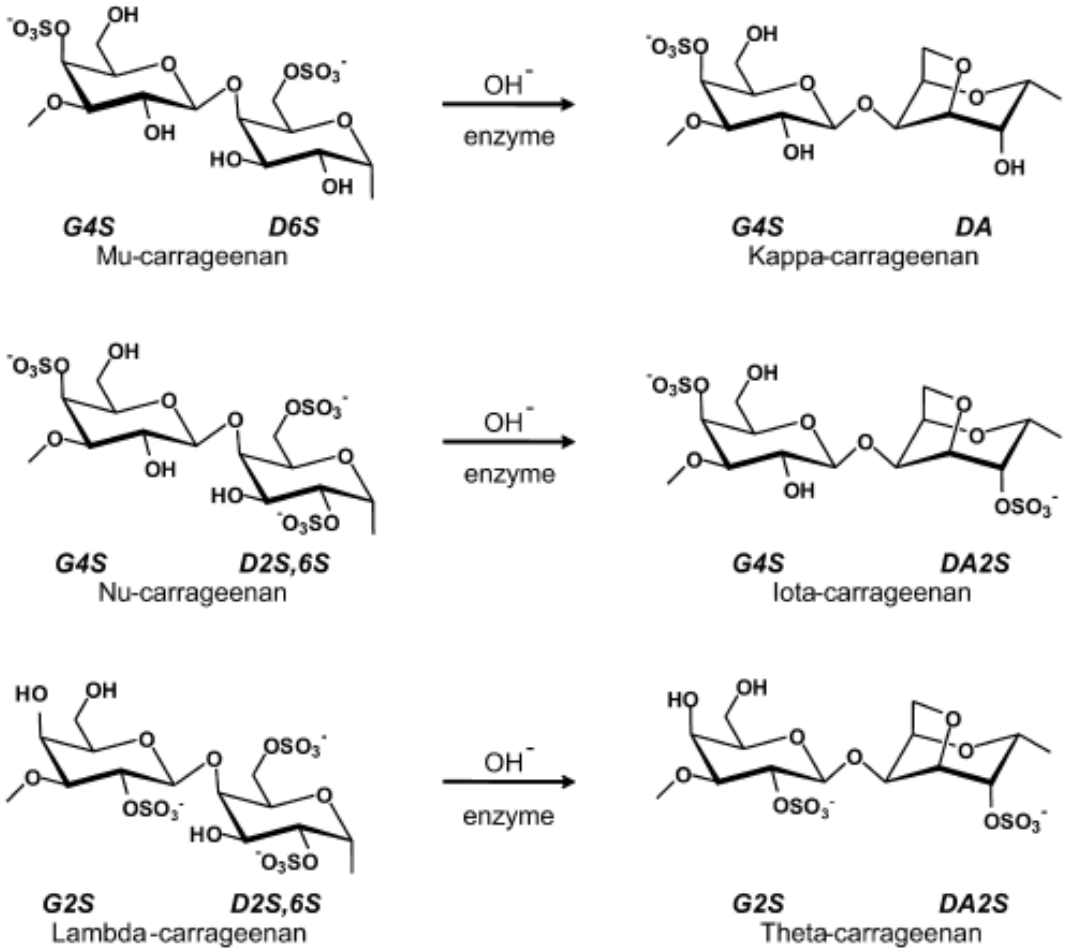


Figure 2. Idealized units of the main carrageenan types (Pereira and Van De Velde, 2011), adapted.

1.4.2. Conventional extraction techniques

There are two conventional alkali extraction procedures to extract refined carrageenan (RC) and semi-refined carrageenan (SRC) (Tarman et al., 2020; Rupert et al., 2022), which are assigned with EU additive E-numbers E407 and E407a, respectively.

For refined carrageenan production, carrageenan is solubilized in hot alkaline solutions, such as sodium hydroxide (NaOH), at temperatures ranging from 95-110 °C, and the remaining insoluble compounds are removed *via* filtration. The solution containing carrageenan is concentrated and carrageenan is then precipitated by alcohol-precipitation method, which is used to purify all types of carrageenan (Campbell & Hotchkiss, 2017). After this step, the biomass is dried and milled.

The gel press technique, which replaces the precipitation stage with a gelling step using potassium chloride, followed by a gel pressing step to dewater the gel and generate a high-solids-content carrageenan cake, is another typical extraction method for refined carrageenan. This method produces refined carrageenan with high gel strength and clarity, but with a slightly higher cellulose component and residual levels (1-2%) of potassium chloride (which means it will always gel on the addition of water, unlike alcohol-precipitated carrageenan, which requires the addition of ions to form a gel) (Rupert et al., 2022). Application of gel-pressed carrageenans mostly in jellies and confectionery, mainly in Asia, drives the market for gel-pressed carrageenan; however, for many applications, high clarity and gel strength are not as important as other functional properties, for example, water binding. Semi-refined carrageenan provides a cheaper alternative for use in such applications and, in some cases, can provide better technical functionality. It has since developed into the fastest-growing carrageenan technology and, in conjunction with gel pressing, has been the major cause of the decline in alcohol precipitation (Hotchkiss et al., 2016).

While in the refined process carrageenan is retrieve from the seaweed matrix, in the semi-refining process treats the seaweed matrix with aqueous potassium hydroxide (KOH) at 75-80 °C (temperature should never exceed the 80 °C in order to prevent the seaweed from dissolving and allow the conversion of the precursors within the seaweed) for 2 h in order to dissolve and remove soluble compounds other than carrageenans, such as salts, soluble sugars, and soluble sugars proteins (Bono et al., 2014; Heriyanto et al., 2018). The hydroxide component of the reagent inputs the seaweed and reduces the amount of sulphate in the carrageenan while simultaneously increasing the amount of 3,6-

anhydrogalactose. The reagent's potassium component reacts with the carrageenan in the seaweed to form a gel, which prevents it from dissolving in the hot solution. Soluble carbohydrate, protein, fats and salts are eliminated when the solution is drained, and the residue is rinsed several times. This washing step aims to remove the alkali and any other compounds that might dissolve in the water. The alkali-treated seaweed is then dried before being cut and ground into a powder as SRC, also designated as seaweed flour (Bono et al., 2014).

Cost wise SRC extraction method is less expensive than the RC process since carrageenan precipitation and solvent recovery costs are avoided. However, the SRC process extracted product present lower quality and overall unsuitable for human food application, although being used in processed meat products, to extend the meat and other sources of protein application. SRC is mainly fabricated for pet food production because of its slightly cloudy and colored looks, also often containing high bacterial population (Rhein-Knudsen et al., 2015). RC presents higher quality, clear look and is referred to as raw carrageenan (Rupert et al., 2022). Despite the benefits of alkali treatment, the procedure invariably results in some polysaccharide degradation due to extreme heat and high alkalinity concentration that can lead to carrageenan depolymerization (Rupert et al., 2022).

1.4.3. Novel carrageenan extraction techniques

Conventional polysaccharide extraction methods are labor-intensive, present medium to low extraction rates and require significant amounts of chemicals, water, and energy, generating waste as well throughout the entire process, making them less environmentally and economically inefficient (Abdul Khalil et al., 2018). Considering all of this, several green extraction methods are emerging in order to reduce the use of chemicals while improving the extraction yield and quality of seaweed-derived polymers. These new techniques include microwave-assisted extraction (MAE) (Rodriguez-Jasso et al., 2011; Topuz et al., 2016), ultrasound-assisted extraction (UAE) (Ummat et al., 2020), enzymatic-assisted extraction (EAE) (Wijesinghe & Jeon, 2012) as well as green solvent extraction, which comprises subcritical water extraction (SWE) and ionic liquid extraction (Habeebullah et al., 2021; Kadam et al., 2015; Wijesinghe & Jeon, 2012); supercritical fluid extraction (SFE) and others such as reactive extrusion and photo-bleaching methods. Due to the various unique approaches that are still in use, there is not

much information available. Currently, MAE and UAE techniques are low-cost and have been successfully applied in large-scale commercial sites (Dulanlebit, 2023; Vinatoru, 2001).

1.4.3.1. Ultrasound assisted extraction (UAE)

Applying sonic cavitation, ultrasound assisted extraction (UAE) reduces the size of the particles and increases the surface-contact between the solvent and the target substance by enhancing cell wall permeability (Rajbhar et al., 2015). Sound vibrations with a frequency of 20-2000 kHz travel through the substance and cause changes in pressure. Cellular stability and structure are disrupted by the growing and collapsing acoustic cavitations, which turn sound vibrations into mechanical energy (Hahn et al., 2012; Ying et al., 2011). UAE is occasionally employed prior to extraction, as a pre-treatment technique, by immersing the sample in an ultrasonic bath or using an ultrasound probe tool (Klejdus et al., 2010). This method is a straightforward, affordable option that can be applied in both small- and large-scale situations (Shirzad et al., 2017) and produce encouraging results (Dulanlebit, 2023). According to Wang et al. (2009), the disruption of cell walls, reduction in particle size, and increase in mass transfer to the cell contents may have contributed to the higher extraction yield from *Hypnea musciformis* (Rhodophyta) using the aqueous UAE method (500 W, 20 min.). Additionally, the intensity of the ultrasonic power increased the vibration of the sample molecules, which helped to recover the target compounds from the solid material to the liquid solvent phase (Wang et al. 2009). For example, in a study conducted by (Youssouf et al., 2017), UAE of *K. alvarezii* and *Eucheuma denticulatum* reached a 50–55% yield of carrageenans in 15 min with similar results in both red seaweeds species. Interestingly, a longer ultrasound treatment (30 min.) did not further improve the extraction yield. Another study was ultrasound assisted extraction of ι-carrageenan was also performed in *E. denticulatum*, demonstrated a yield of 57% (Krishnaiah et al., 2013). Sari et al., (2019) preformed a study regarding temperature influence in UAE and conclude that increased temperature extraction caused higher cavitation bubble that made the ultrasonic irradiation is more effective so the mass transfer of sulphate that removed is more and accelerate the formation of 3.6-anhydro-galactose during the extraction process. Increase in temperature can also lead to increased solvent solubility and enlarge the pores of solids so that the

solvent could enter through the pore solids and dissolves the solids component that trapped in the form of sulphate, allowing for the obtention of a high-quality carrageenan.

1.4.3.2. Supercritical fluid extraction (SFE)

In the supercritical extraction method (SFE), temperature and pressure are applied to swiftly permeate the substance and produce pure extraction results. Concerning extraction efficiency, the material's water content, fluid, solvent flow rate, temperature, pressure, and particle size must be considered (Dulanlebit, 2023). By adjusting the temperature, pressure, or addition of solvents that can stop the extract from degrading, this approach can be used to extract seaweed hydrocolloids such as agar and carrageenan (Dulanlebit, 2023). SFE has several benefits, including the use of environmentally friendly solvents, speedy extraction, and high-quality extract outputs. Currently this method was mainly tested for lipid extraction of PUFA's from *K. alvarezii* (SFE using CO_2 as solvent, at 500 psi, 55 ºC for 3 h) (Chen & Chou, 2002; Cheung, 1999)and little to non has been performed for carrageenan extraction (Machmudah et al., 2017).

1.5. Characterization techniques and assessment of carrageenan quality

Since polysaccharides present varied composition, which can be attributed to the extraction process or to abiotic and biotic factors, there is a need to use several techniques to characterize them and guarantee their quality.

1.5.1. Current applied techniques

1.5.1.1. Chromatography

Chromatography is a physical method of separation in which the components of a mixture are separated by their distribution between two phases; one of these phases in the form of a porous bed, bulk liquid, layer, or film is generally immobile (stationary phase), while the other is a fluid (mobile phase) that percolates through or over the stationary phase (Haddad, 2004). Chromatography follows a wide range of techniques that can be applied in a sequential way to isolate with excellent rate and high efficiency in the characterization of seaweed quality, however these techniques are often costly (Misra et

al., 2015). Nevertheless, there are other methods of chromatography, such as high-performance anion exchange chromatography (HPAEC) that can be used to quantify and characterize seaweed polysaccharide fraction (Nishino et al., 1989), which are the trickiest to analyze by liquid extracts due to their viscosity properties (Cotas et al., 2020). This method uses a strong anion exchange to separate the fractions by pH and the acidic nature of the seaweed polysaccharides (seaweed carbohydrates) (Lim et al., 2014).

1.5.1.2. Fourier transformed IR from attenuated total reflectance (FTIR-ATR)

Infrared (IR) spectroscopy is one of the most useful techniques for polysaccharide structure identification. This technique is based on the analysis of absorption peaks at certain wave numbers (cm^{-1}). FTIR-ATR (Fourier transformed IR from attenuated total reflectance) spectroscopy, the combination of Fourier transform algorithm with attenuated total reflectance (ATR) techniques has improved conventional IR spectroscopy with various and important advantages. FTIR-ATR is a direct and non-destructive technique, requires small amounts of dried material (few milligrams) and is a quick method (few min), not requiring extensive extractions and further sample preparation (Pereira & Mesquita, 2004). Therefore, phycocolloids present in seaweeds can be quickly identified by FTIR-spectroscopy directly on only a few mg of dried, ground seaweed material. Beside information on polysaccharide composition and structure, also protein or sulphate content, can be gained from seaweed infrared spectra. Thus, this technique allows to preliminary identify the main polysaccharides in an unknown seaweed sample.

For the structural analysis of carbohydrates, five main frequency regions can be distinguished in the spectra (4000-650 cm^{-1})(Gómez-Ordóñez & Rupérez, 2011; Rodrigues et al., 2015):

(1) region of O–H and C–H stretching vibrations at 3600–2800 cm^{-1};
(2) region of local symmetry at 1500-1200 cm^{-1};
(3) region of CO stretching vibration at 1200-950 cm^{-1};
(4) fingerprint or anomeric region at 950-700 cm^{-1};
(5) skeletal region below 700 cm^{-1}.

N–H stretching vibrations at 3700–2900 cm^{-1} as well as from amide I and amide II at 1700–1420 cm^{-1} can be related to proteins (Chopin et al., 1999).

In Table 2 are presented the main absorption bands used to obtain information on the structure of carrageenans according to letter code.

Table 2. FTIR main absorption bands for carrageenan characterization, letter code nomenclature and band identification.

Wave number (cm^{-1})	Bound	Letter code	Kappa (κ)	Mu (μ)	Iota (ι)	Nu (ν)	Lambda (λ)
1240	Sulphate ester (S=O)	**S**	+	+	+	+	+
930	3,6-anhydro-D-galactose	**DA**	+	-	+	-	-
845	D-galactose-4-sulphate	**G4S**	+	+	+	+	-
830	D-galactose-2-sulphate	**G2S**	-	-	-	+	+
820	D-galactose-2,6-disulphate	**D2S, D6S**	-	+	-	+	+
805	3,6-anhydro-D-galactose-2-sulphate	**DA2S**	-	-	+	-	-

+, presence -, absence

1.5.2. Future potential complementary techniques

There is currently an increasing demand for seaweed polysaccharides by the food industry. Also, a quick, reliable, and low-cost methods to assess the quality are needed. Information on protein or sulphate content prevision, can be gained from simple techniques such as the measurement of physico-chemical parameters and UV-VIS spectroscopy to preliminary identify these constitutions.

1.5.2.1. Physico-chemical measurements: viscosity and pH

The measurement of physico-chemical parameters such as viscosity can provide clues about carrageenan structure and content, in specific sulphate content. High viscosity is usually associated with high content in sulphate (Astuti et al., 2017; Montoro et al.,

2019). High sulphate can also be associated to lower the gel strength. The repulsion force between negative charges along the polymer chain of the sulphate group causes the chain of the molecules to tighten. Due to its hydrophilic nature, the polymer is covered by immobilized water molecules, thus causing the solution to become viscous (high viscosity) (Astuti et al., 2017).

1.5.2.2. UV-VIS spectroscopy

UV-Vis spectroscopy is an analytical technique that compares the amount of discrete wavelengths of UV or visible light absorbed or transmitted by a sample to a reference or blank sample. The sample composition influences this attribute, which may provide information about what is in the sample and at what concentration.

The analyses of carrageenan solutions through UV-Vis spectroscopy provide information regarding its UV-absorbance potential. It is likely to compare the antioxidant potential of the seaweeds based on the bioactive compounds analyzed by UV-vis absorption spectrum. Peaks between 200-270 nm possibly correspond to polysaccharide bound covalently with aromatic compounds, giving clues about purity of the extracted carrageenan regarding protein content at analyzed concentration (Saravana et al., 2016). Absorption peaks between 266-374 nm determines the presence of phenolic compounds (Ray et al., 2013) which are known for their potential role in both UV photoprotection and ROS scavenging (Zerrifi et al., 2018).

Red algae are also known to accumulate photoprotective compounds with ultraviolet radiation absorption capabilities such as mycosporine-like amino acids (MAAs), which absorb in UV-A region (320–400 nm) (Orfanoudaki et al., 2019; Pangestuti et al., 2018).

1.6. Food security: Carrageenan as food additive

Finding novel, effective an environmentally friendly extraction methods for commercial scale is very important. Currently availably carrageenan in market is dominated by companies such as DuPont, Ingredion Incorporated, Cargill, Inc., Ceamsa, Gelymar, Caldic B.V., W Hydrocolloids, however the carrageenan processed usually implies the low-efficient use of large quantities of chemicals, however there are novel and equally acceptable options to attain industry productivity demand. Another problem

is that current studies regarding bioactivities are mainly tested with commercial samples without characterizing them, and these aren't representative of novel options. For this reason, when studying novel strains and extraction methodologies for carrageenan obtention for food approval is important to study the identity and purity prior to its use in new studies to access its safety. Important identity characteristics to evaluate in carrageenan samples used in research studies include (Weiner, 2016):

(1) Composition: Carrageenan, moisture, inorganic salts, protein, acid insoluble matter, diluents, and standardizing agents.
(2) Purity: Microbial quality, heavy metals, volatiles.
(3) Carrageenan Type: Kappa, Iota, Lambda, cations, ester sulphate, ash.
(4) Carrageenan Quality: Viscosity, pH, Mw profile (Mw, Mn, PDI)

Also, when studying a new strain and evaluating its carrageenan content, for possible industry application, especially as food additive, is important to consider mandatory and regulatory status. Under the Food and Drug Administration (FDA) regulations in USA, the European Parliament and Council Regulation (EC), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), carrageenans are considered safe and authorized for consumption as food additives (Huang et al., 2020).

Both refined carrageenans (RC) and semi-refined carrageenans (SRC) are considered safe by the European Food Safety Authority (EFSA) with no evidence of adverse effects in humans (EFSA, 2018), when consumed in quantities that are required to provide the desired food texture. As previously shown, the three main forms, kappa (k), lambda (l) and iota (i) differ in the content of 3, 6-anhydrogalactose, ester sulphate and therefore in their functional properties (see Table 3) (Weiner, 2016).

According to the Commission Regulation (EU) the purity criteria have been defined for RC and SRC, present in table 4.

Regarding molecular weight, carrageenans are defined as having an average in the range of 200–800 kDa (EFSA, 2018). The polydispersity of the preparations is high and a small fraction of lower-molecular-weight polymeric chains (20–50 kDa) occurs naturally in all carrageenan samples, however, during extensive hydrolysis at low pH (< 1.3) and high temperature (> 80 °C) conditions for an extended period of time, a non-naturally occurring fraction of degraded carrageenans, named 'poligeenan', can be formed. This fraction has a weight average molecular weight of 10–20 kDa and does not

provide texturizing properties (McKim et al., 2019). Studies performed in rodents, demonstrated that the consumption of high amounts of poligeenan is associated with ulcerative colitis (Benard et al., 2010). Therefore the quantity of the low-molecular-weight polysaccharide chains in food-grade carrageenan is regulated (see Table 4).

Table 3. Functional properties of carrageenans.

Carrageenan type	Kappa (κ)	Iota (ι)	Lambda (λ)
Solubility			
Hot water (80 ºC)	Soluble	Soluble	Soluble
Cold Water (20 ºC)	Na^+ salt soluble, limited hydration and swelling of K^+ and Ca^{2+} salts	Na^+ salt soluble, Ca^{2+}salt particles hydrate and swell to thixotropic mix	Soluble
Hot Milk (80 ºC)	Soluble	Soluble	Soluble
Cold Milk (20 ºC)	Insoluble	Insoluble	Thickens
Gel formation			
Effect of cations	Gels with K^+	Gels with Ca^{2+}	Non-gelling
Gel texture	Brittle	Elastic	Non-gelling
Syneresis	Yes	No	No
Gel Conformation (<0.1%)	Random Coil	Random Coil	Random Coil
Gel Conformation (>0.1%)	Helical	Helical	Random Coil
Protein reactivity			
All proteins	Strong binding	Strong binding	Strong binding
Milk protein (<0.1%)	Stabilizes on cooling	Stabilizes on cooling	Thickens on cooling
Milk protein (>0.1%)	Gels on cooling	Gels on cooling	Thickens on cooling
pH Stability			
Above pH 6	Stable	Stable	Stable
Below pH 5	Hydrolysis in random coil conformation, gels in helical	Hydrolysis in random coil conformation, gels in helical	Hydrolysis
Hydrolysis	Increased rate of hydrolysis of glycosidic linkages with increased temperature and time and lower pH (below pH 3)		

Adapted from Weiner (2016).

Currently there is no validated testing method in practice for the quantification of these low-molecular-fraction in carrageenan samples, however viscosity measurements allow to distinguishing between carrageenans and poligeenans. The viscosity (1.5% solution at 75 °C) of 5 mPa.s corresponds to a sample with a molecular weight of approximately 100–150 kDa (Huang et al., 2020).

Table 4. Chemical and physical characteristics according to Commission Regulation (EU) for RC and SRC.

Chemical and physical characteristics of RC and SRC		
Appearance		Yellowish to colorless Fine powder Practically odorless
Viscosity (1.5% at 75 °C)		≥ 5 mPa.s
Sulphate content (as SO_4)		15-40%
Ash content (550 °C)		15-40%
pH (1%)		8–11
Moisture (105 °C, 4 h)		≤ 12%
Solubility	RC	Soluble in hot water < 2% acid-insoluble mater
	SRC	Cloudy viscous suspension in water 8%–15% acid-insoluble matter
Heavy metals/metalloids		Arsenic (As): ≤ 3 mg/kg Lead (Pb): ≤ 5 mg/kg Mercury (Hg): ≤ 1 mg/kg Cadmium (Cd): ≤ 2 mg/kg
Molecular weight		200–800 kDa Must not contain > 5% of Mw <50 kDa (poligeenan)

1.7. Objectives

The objectives of this study are to:

1) determine which "strain" has the best yield,

2) develop novel carrageenan extraction methods,

3) characterize the extracted polysaccharides to get a deeper understanding regarding its physical and biochemical characteristics and these are affected by each extraction protocols.

2. Material and Methods

2. Materials and Methods

This study involves novel eight strains of *Kappaphycus alvarezii* (Fig. 3), and an additional strain (tetrasporophyte) denominated as Doty ex P. C. Silva (1996), native to the Philippines and introduced into Brazil (see Annex II). This species, belonging to the Eucheumatoideae tribe, contain carrageenan imbedded in the cell wall and intracellular and are extremely morphologically plastic, which historically has led to systematic issues and can come in a variety of colors and shapes as it is the case of this strains (Fig. 3 and Annex II). The novel strains were clonally propagated in an outdoor hatchery in Bolinao, Pangasinan, Philippines. The categorically confirmed specific life history phase, sex and ploidy was derived from the progeny of wild fertile individuals, collected from Guiuan, Samar, Philippines (Hinaloc & Roleda, 2021; Roleda et al., 2021). In one of the strains (G-Q2), two samples were studied, one harvested in May and other in June to understand and see if there were differences in the type and carrageenan content.

In preliminary runs (Annex I), three treatments (conventional alkali extractions with NaOH and KOH, and native extraction as control) allowed to select the strain with relatively higher yields and industrial potential for a deeper characterization (fatty acids, carbohydrate, uronic acids and protein content) and evaluation of the efficacy of both conventional and novel methods (ultrasound-assisted extraction and supercritical fluid extraction) (section 3.1.). G-N7 was the selected strain, since revealed the highest result for native (control) and second highest values for both convention extraction with NaOH and KOH solutions (Annex I).

All these extraction conditions were later tested in all strains (section 2.2) in order to characterize the strains, compare and understand the impact of the extraction conditions.

Figure 3. *Kappaphycus alvarezii* novel strains: (1) G-N7, (2) G-N11, (3) G-N14, (4) G-Q2, (5) TR-C3.2, (6) TR-C4.2, (7) TR-C18 and (8) TR-C21.2.

2.1. Novel extraction methods, yield, structural and rheological properties of κ-carrageenan from a novel haploid *Kappaphycus alvarezii* strain from the Philippines

2.1.1. Seaweed harvest and cultivation

The new strain, G-N7, is a haploid female gametophyte clonally propagated in an outdoor hatchery in Bolinao, Pangasinan, Philippines. The specific life history phase, sex and ploidy were derived from the progeny of a wild fertile individual, a diploid tetrasporophyte KaTR-N, collected from Guiuan, Samar, Philippines (Hinaloc & Roleda, 2021). Based on the cox2-3 spacer sequence, this strain belongs to KALV-3, a predominant wild haplotype in the collection site that is different from the commercially cultivated haplotype 3 (Fig. 4; Roleda et al., 2021).

G-N7 was clonally propagated using branch cuttings in an aquarium (58 × 30 × 40 cm) with air and flow-through, nutrient-replete, sand-filtered seawater pumped from approximately 12 - 15 m depth of the Guiguiwanen Channel, near the land-based hatchery. The outdoor hatchery is covered by translucent roofing receiving seminatural solar radiation where the average noontime surface irradiance at the top of each aquarium was 506 µmol photons m^{-2} s^{-1}, measured using a cosine sensor coupled to a LI-COR light meter (LI-14000, LiCOR, Nebraska, USA). The mean daytime water temperature was 31.7 °C, measured using HOBO data loggers (UA-002–64, HOBO, Onset Computer

Corporation, Massachusetts, USA) (Narvarte et al., 2022). The average growth rate was approximately 2% day^{-1}. Biomass was harvested every 45–50 days or when the aquarium was filled to the brim with biomass. Harvested samples were sun and air dried down to an approximate 10% moisture content and packed in airtight plastic bags at room temperature until transport for further chemical analyses.

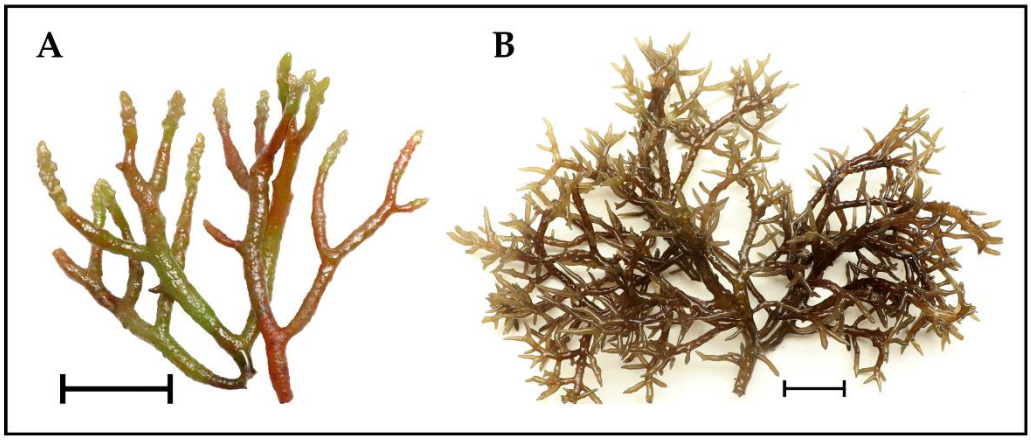


Figure 4. *Kappaphycus alvarezii* cultivars. A. Novel haploid female gametophyte G-N7 strain. B. Commercially cultivated *Tambalang* strain. Scale bar = 5 cm.

2.1.2. Sample preparation

The sun-dried biomass of the G-N7 *Kappaphycus alvarezii* strain as received in the Portuguese laboratories by courier from the Philippines was processed using standard protocols. The raw dried seaweed (RDS) was washed with distilled water to remove excess salt. Thereafter, the RDS was dried in a forced-air oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 °C for 48 h.

2.1.3. Carrageenan extraction

Carrageenan extraction was performed by either a conventional method or by applying ultrasound-assisted extraction (UAE) or supercritical fluid extraction (SFE) methods. For each treatment, three replicate samples were processed.

2.1.3.1. Native extraction (NE)

The “native” phycocolloid was extracted by placing the samples in distilled water (100 mL/g), pH 7, at 100 °C for 3 h (Pereira e Mesquita, 2004; Fig. 5). The solutions were hot filtered under vacuum through a cloth filter supported in a Buchner funnel, followed by a Goosh 2 silica funnel filtration. The extract was evaporated (rotary evaporator: 2600000, Witeg, Germany) under vacuum to one-third of the initial volume. Carrageenan was precipitated by adding twice its volume of 96% ethanol (José Manuel Gomes dos Santos, Portugal). The precipitate was washed and stored with 96% ethanol at 4 °C for 48 h before being dried in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 °C for 48 h (Pereira et al., 2003). Finally, the dried carrageenan was weighed to determine the extraction yield (% of dry weight) and milled into powder (particles <0.05 cm) with a commercial grinder (TitanMill 300 DuoClean, Cecotec, Valencia, Spain) for further analyses.

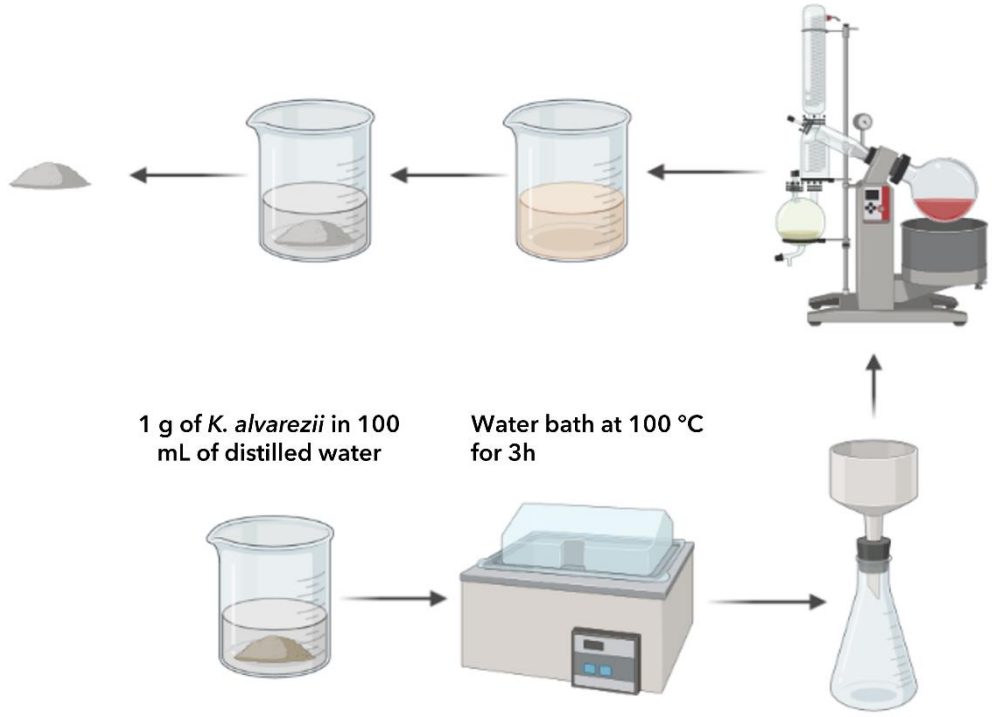


Figure 5. Workflow of the native extraction method performed for carrageenan extraction.

2.1.3.2. Conventional extraction (CE)

2.1.3.2.1. Refining process.

Dry seaweed samples (1 g) were placed in a solution (150 mL/g) of NaOH (1 M) at 90-100 °C for 3 h, according to the method described by Pereira and Van De Velde (2011) (Fig. 6). The solutions were hot filtered under vacuum through a cloth filter supported in a Buchner funnel. After that, the extract was again filtered under vacuum with a Goosh 2 silica funnel. The extract was evaporated under vacuum to one-third of the initial volume (50 mL). Carrageenan was precipitated by adding twice its volume of 96% ethanol (100 mL). Due to its gelling behavior, after precipitation, some samples were filtered using a cloth. After that, carrageenan retained in the cloth was washed and stored with 96% ethanol for 48 h at 4 °C before drying in an air force oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 °C for 48 h and then weighed to determine the carrageenan content (% of dry weight) and ground into a powder as refined carrageenan (RC) for further analyses.

2.1.3.2.2. Semi-refining process.

Dry seaweed samples (1 g) were placed in a solution (150 mL/g) of KOH (8%) at 75-80 °C for 2 h (Fig. 6). The solutions were hot filtered under vacuum through a cloth filter supported in a Buchner funnel. The residue, retained in the cloth, was rinsed several times to remove the alkali and anything else that might dissolve in the water during the rinsing process. The alkali-treated seaweed was washed with 96% ethanol for 48 h at 4 °C before drying in an air force oven (60 °C, 48 h) and then ground into a powder as semirefined carrageenan (SRC) for further analyses.

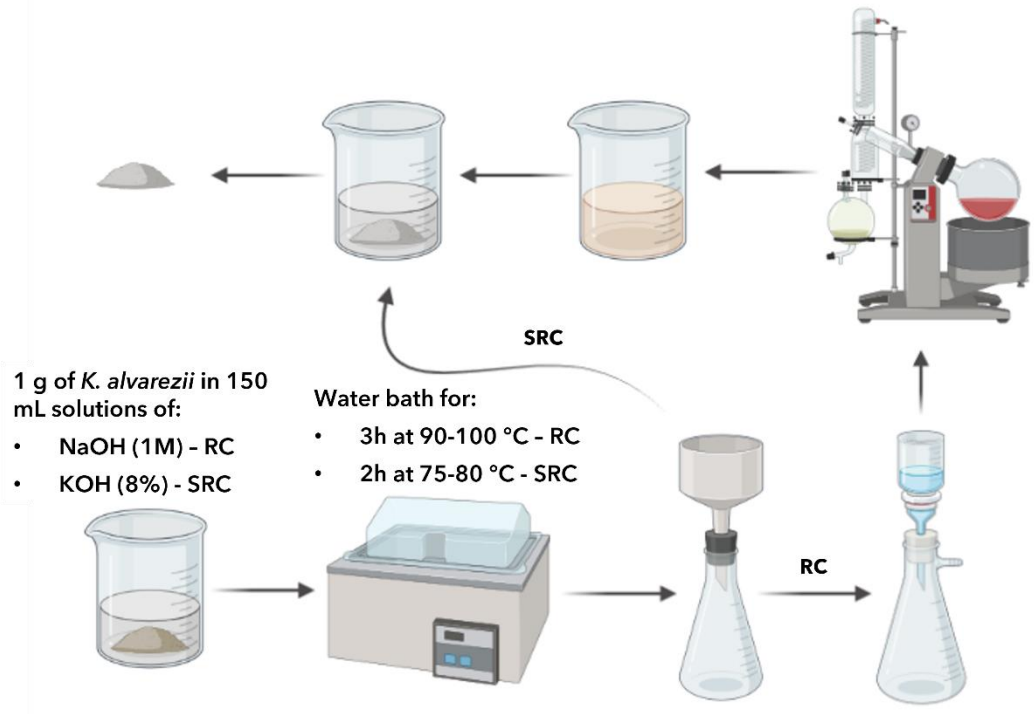


Figure 6. Workflow of the conventional extraction method performed for carrageenan extraction (RFE: refined extraction; and SRC: semi-refined extraction).

2.1.3.3. Ultrasound-assisted extraction method (UAE)

Extractions were performed using a heated ultrasonic bath (120 W).

2.1.3.3.1. Refining process.

Forced-air, oven dried samples (1 g) were placed in a solution (150 mL/g) of NaOH (1 M) and subjected to an ultrasound machine (ultrasonic cleaner ULTR-3L2-001, IBX instruments, Barcelona, Spain) at 70 °C for 60 min (Fig. 7). The solutions were hot filtered under vacuum through a cloth filter supported in a Buchner funnel. After that, the extract was again filtered under vacuum with a Goosh 2 silica funnel. The extract was evaporated under vacuum to one-third of the initial volume (50 mL). Carrageenan was precipitated by adding twice its volume of 96% ethanol (100 mL). Due to its gelling behaviour, after precipitation, some samples were filtered using a cloth. Precipitated carrageenan was washed with 96% ethanol for 48 h at 4 °C before drying in a forced-air oven (60 °C, 48 h).

2.1.3.3.2. Semi-refining process.

Dry seaweed samples (1 g) were placed in a solution (150 mL/g) of KOH (8%) and subjected to ultrasound at different temperatures (45 °C and 70 °C) for 60 min (Fig. 7). The solutions were hot filtered under vacuum through a cloth filter supported in a Buchner funnel. After that, carrageenan retained in the cloth was collected and washed with 96% ethanol for 48 h at 4 °C before drying in a forced-air oven (60 °C, 48 h).

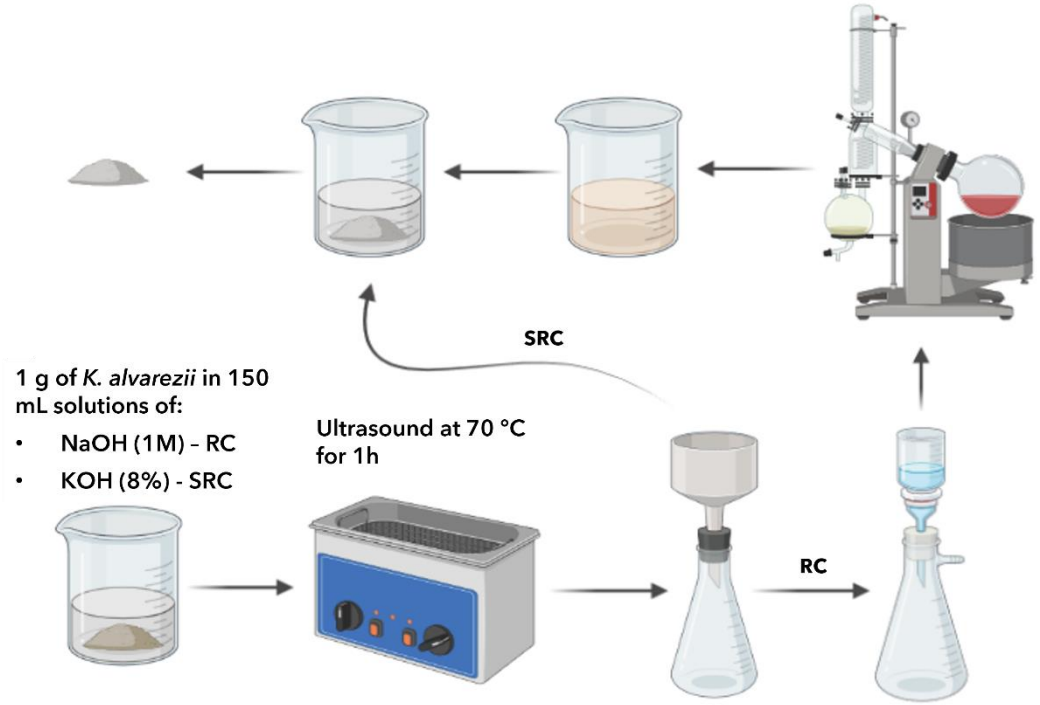


Figure 7. Workflow of the ultrasound-assisted extraction method performed for carrageenan extraction (RFE: refined extraction; and SRC: semi-refined extraction).

2.1.3.4. Supercritical fluid extraction (SFE)

Supercritical fluid extraction was performed by placing forced-air dried seaweed (6 g) and 600 mL distilled water into an electric pressure cooker (Aigostar 300008IAU, Aigostar, Madrid, Spain) at a temperature of 115 °C with an air pressure of 80 KPa for 2 h (Fig. 8). The solution was hot filtered under vacuum through a cloth filter supported in a Buchner funnel. Carrageenan was precipitated by adding twice its volume of 96% ethanol (100 mL). The precipitated carrageenan was washed with 96% ethanol for 48 h at 4 °C before drying in an air force oven (60 °C, 48 h).

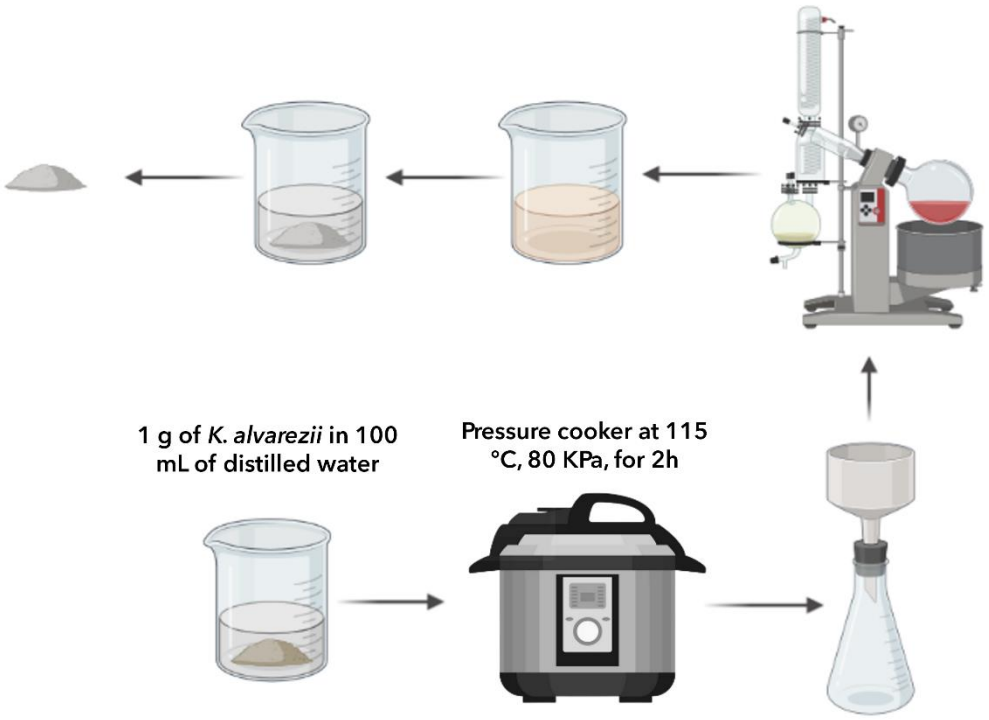


Figure 8. Workflow of the supercritical fluid extraction (SFE) method performed for carrageenan extraction.

2.1.4. Analyses of extracted carrageenan

2.1.4.1. Yield

The formula used to determine the carrageenan yield of each sample was:

$$Yield = \frac{We}{Wds} \times 100$$

where We is the extracted carrageenan weight (g) and Wds is the dried seaweed weight (g) used for extraction.

2.1.4.2. Content of Fatty Acids

Three replicates of each extraction method were processed to obtain the extracts. Lipid extraction was performed following the technique described by Gonçalves et al. (2012). Samples were incubated with methanol for the methylation of lipids. Then, n-hexane was added, and the samples were centrifuged at 2500 rpm (650×g) for 15 min. to extract the fatty acid methyl esters (FAMEs). The internal standard nonadecanoic acid

C19 was added to each sample to quantify the fatty acid (FA) and stored at -80 °C. FAMEs separation was carried out through gas chromatography–mass spectrometry (GC–MS) equipped with a 0.32 mm internal diameter, 0.25 μm film thickness and 30 m long TR-FFAP column. The sample (1.00 μL) was injected in spitless mode. The initial column temperature was programmed at 80ºC and held for 3 min., the first ramp (20ºC min^{-1}) increases the temperature up to 160ºC, a second ramp (2ºC min^{-1}), up to 190ºC and the last ramp (5ºC min^{-1}) reaches final temperature of 220 °C that holds for 10 min. Helium was the carrier gas, at a flow rate of 1.4 mL min^{-1}. The identification of each peak was performed by the retention time and mass spectrum of each FAME compared to the Supelco®37 component FAME mix (Sigma-Aldrich, Steinheim, Germany). Integration of the FAME peaks were carried out using the equipment's software. Quantification of the FAMEs was performed as previously described in Gonçalves et al. (2012).

2.1.4.3. Carbohydrate and uronic acid content.

Carbohydrate analysis was carried out after fatty acid extraction using the same biomass. Samples were submitted to hydrolysis in triplicate after Selvendran et al. (1996) for the extraction of neutral sugars and uronic acids, followed by reduction and acetylation described by Coimbra et al. (1994) to obtain the alditol acetates from each neutral sugar to be analysed by gas chromatography. Neutral sugars were run through a Thermo Scientific Trace 1310 chromatograph equipped with a flame ionization detector (GC-FID). A TG-WAXMS A (30 m length, 0.32 mm i.d., 0.25 μm film thickness) GC column was used. The initial temperature of the oven is 180 °C, held for 1 min before increasing at a rate of 15 °C min^{-1} up to 220 °C. The temperature is hold for 5 min before the last ramp (1°C min^{-1}) until reaching the final temperature of 230 °C, that is maintained for 5 min. Helium is used as carrier gas at a flow rate of 1.7 mL min^{-1}. Peaks are identified by retention time and quantification were conducted comparison with standards. Uronic acids were determined after Blumenkrantz and Asboe-Hansen, (1973) using a Biochrom EZ Read 2000 Microplate reader (Biochrom Ltd., Cambridge, United Kingdom), reading at an absorbance of 520 nm wavelength. Galacturonic acid (Merck KGaA, Darmstadt, Germany) was used to make the calibration curve for the measurements, and the colorimetric reagent used was 3-phenylphenol.

2.1.4.4. Protein content.

Carrageenan solutions (1 mg/mL) were prepared by dissolving the polysaccharide in distilled water. Protein content was measured by the Bradford method (Bradford, 1976), based on spectrophotometry, adapted to microplate. Samples were analysed at 595 nm using a Biochrom EZ Read 2000 Microplate reader (Biochrom Ltd., Cambridge, United Kingdom). Protein concentration was calculated by comparison using bovine serum albumin (Merck KGaA, Darmstadt, Germany) as a standard. Three replicates of each extraction method were performed.

2.1.4.5. Viscosity, pH, EC and TDS.

Carrageenan solutions (1% m/v) were prepared by dissolving the polysaccharide in distilled water using temperature and magnetic stirring. Then, the solutions were cooled to a temperature of 30 °C. Later, viscosity measurement was carried out using spindles SP2 and SP3 in a IKA Rotavisc Viscometer, with a speed of 100 rpm at room temperature for 1 min. A pH/Conductivity/TDS meter (Combo HI98129, HANNA instruments) was used to measure the pH and TDS values of the carrageenan solutions.

2.1.4.6. Spectrophotometric profiles of carrageenan solutions.

Carrageenan solutions (1% m/v) prepared for the viscosity analyses were diluted in distilled water (1:2), and UV–VIS absorption spectra were measured in the range of 200 to 800 nm using a UV-3100PC, UV/VIS Scanning Spectrophotometer (VWR® Radnor, PA, USA) with 1 cm quartz cuvettes.

2.1.4.7. FTIR-ATR analysis.

Attenuated total reflectance (ATR) Fourier transform infrared (FT-IR) spectroscopy was employed to characterize the structure of dried extracted carrageenans. The IR spectra (24 scans) were obtained at room temperature (referenced against air) in the wavenumber range of 400–4000 cm^{-1} (resolution of 4 cm^{-1}) using a Bruker Alpha II (Bruker, Ettlingen, Germany). Spectra were analysed with OPUS 7.2 software (Bruker, Ettlingen, Germany). Commercial standards of polysaccharides kappa- and iota

carrageenan were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The ratio between the 805 and 845 cm^{-1} absorption bands in the spectra was calculated (Correa-Diaz et al., 1990; Pereira & Mesquita, 2004) and used as a parameter to determine the degree of the iota/kappa hybridization.

2.1.5. Statistical analysis

All experiments were performed in triplicate, and data are presented as the means ± standard deviations. Analysis of variance (ANOVA) was performed, and comparisons of means were conducted using different multiple comparison tests using the Sigmaplot program (version 14.0). Values were considered to differ significantly if the *p* value was < 0.05.

For the exploratory analysis, a multilinear regression (singular value decomposition) was used to estimate the relationship between each sample and the standard commercial kappa-carrageenan and iota-carrageenan using the Spectragryph program (version 1.2.16) in the spectral range of 400–4000 cm^{-1}.

2.2. Carrageenan yield and quality of hatchery-cultivated novel strains of different ploidy of *Kappaphycus alvarezii*

2.2.1. Sample preparation

Seven novel strains, donated by Dr. Michael Roleda, were clonally propagated in an outdoor hatchery in Bolinao, Pangasinan, Philippines. The categorically confirmed specific life history phase, sex and ploidy was derived from the progeny of a wild fertile individuals, collected from Guiuan, Samar, Philippines (Hinaloc & Roleda, 2021; Roleda et al., 2021).

The raw dry seaweed material was washed with distilled water to remove salt excess and dried in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 °C for 48 h (Fig. 9). After that, samples were stored inside vacuum plastic bags (to prevent from air humidity), in the dark at room temperature until use. For Fourier transform infrared attenuated total reflection (FTIR-ATR) analysis the dried seaweeds

were finely ground (50 mesh) with a commercial grinder (TitanMill 300 DuoClean, Cecotec, Valencia, Spain).

Figure 9. Dry seaweed biomass, after distilled water washing, ready to be dried.

2.2.2. Carrageenan extraction

Carrageenan from all strains were extracted according to the methods previously described in the chapter 2.1.3, namely native extraction (NE), conventional extraction (CE), ultrasound assisted extraction (UAE) and supercritical fluid extraction (SFE).

2.2.3. Analyses of extracted carrageenan

2.2.3.1. Yield

The formula used to determine the carrageenan yield of each sample was:

$$Yield = \frac{We}{Wds} \times 100$$

where We is the extracted carrageenan weight (g) and Wds is the dried seaweed weight (g) used for extraction.

2.2.3.2. Viscosity, pH, EC and TDS.

Carrageenan solutions (1% m/v) were prepared by dissolving the polysaccharide in distilled water using temperature and magnetic stirring. Then, the solutions were cooled to a temperature of 30 °C. Later, viscosity measurement was carried out using spindles SP2 and SP3 in a IKA Rotavisc Viscometer, with a speed of 100 rpm at room temperature

for 1 min. A pH/Conductivity/TDS meter (Combo HI98129, HANNA instruments) was then used to measure the pH and TDS values of the carrageenan solutions.

2.2.3.3. Spectrophotometric profiles of carrageenan solutions.

Carrageenan solutions (1% m/v) prepared for the viscosity analyses were diluted in distilled water (1:2), and UV–VIS absorption spectra were measured in the range of 200 to 800 nm using a UV-3100PC, UV/VIS Scanning Spectrophotometer (VWR® Radnor, PA, USA) with 1 cm quartz cuvettes.

2.2.3.4. FTIR-ATR analysis.

Attenuated total reflectance (ATR) Fourier transform infrared (FT-IR) spectroscopy was employed to characterize the structure of dried extracted carrageenans. The IR spectra (24 scans) were obtained at room temperature (referenced against air) in the wavenumber range of 400 – 4000 cm^{-1} (resolution of 4 cm^{-1}) using a Bruker Alpha II (Bruker, Ettlingen, Germany). Spectra were analysed with OPUS 7.2 software (Bruker, Ettlingen, Germany). Commercial standards of polysaccharides kappa- and iota carrageenan were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The ratio between the 805 and 845 cm^{-1} absorption bands in the spectra was calculated (Correa-Diaz et al., 1990; Pereira & Mesquita, 2004) and used as a parameter to determine the degree of the iota/kappa hybridization.

2.2.4. Statistical analysis

All experiments were performed in triplicate, and data are presented as the means ± standard deviations. Analysis of variance (ANOVA) was performed, and comparisons of means were conducted using different multiple comparison tests using the Sigmaplot program (version 14.0). Values were considered to differ statistically significant if the *p* value was <0.05.

For the exploratory analysis, a multilinear regression (singular value decomposition) was used to estimate the relationship between each sample and the standard commercial kappa-carrageenan and iota-carrageenan using the Spectragryph program (version 1.2.16) in the spectral range of 400 – 4000 cm^{-1}.

3. Results

3. Results

3.1. Novel extraction methods, yield, structural and rheological properties of k-carrageenan from a novel haploid *Kappaphycus alvarezii* strain from the Philippines

3.1.1. Extraction yield and biochemical composition

The carrageenan extraction yields using different protocols ranged from means of 33.73 - 77.33% (Fig. 10, Table 5). The highest yield of carrageenan (77.33±2.49%) was obtained from the alkali (KOH)-treated conventional method, while the lowest yield (33.73±10.52%) was obtained from the alkali (NaOH)-treated UAE method. However, despite the 44% difference in the minimum and maximum carrageenan yields, the variation among the different extraction protocols was not statistically significant but can have economic implications.

In general, the yield under an alkaline (KOH) environment was higher for both conventional (CE; 90 °C, 2 h) and ultrasound-assisted (UAE; 120 W, 1 h) extraction methods. UAE (120 W, 1 h) revealed values close to the conventional carrageenan yield in a shorter time.

The protein content (Fig. 10, Table 5) in all treatments showed very low values, with UAE (NaOH) extraction exhibiting the highest content (0.04 ± 0.02%).

Uronic acid content analysis (Fig. 10, Table 5) showed significant differences, with NE, SFE and CE (NaOH) extraction (13.59 ± 1.97; 13.52 ± 1.37 and 13.95 ± 1.05%, respectively) standing out from the remaining extraction methods, exhibiting a considerably higher content of uronic acids. On the other hand, UAE (NaOH) extraction presented the lowest content of uronic acids.

Higher total FAMEs were observed in the KOH alkali-treated RC (Fig. 10, Table 5). FAME analyses allowed us to identify two saturated fatty acids (SFAs) (i.e., palmitic acid (C16:0) and stearic acid (C18:0)) and one monounsaturated fatty acid (MUFA) (oleic acid (C18:1)). Palmitic acid (C16:0) values revealed no statistically significant differences; however, all alkali treatments performed with KOH showed higher values. Stearic acid (C18:0) showed very similar values between extraction methods, and oleic acid (C18:1) was detected only in both conventional extraction methods [CE (NaOH) and CE (KOH)] and UAE (KOH45).

The total monosaccharide content (Table 5) showed higher values for the alkali treatments performed with KOH [CE (KOH), UAE (KOH45) and UAE (KOH)]. Carbohydrate analysis allowed us to identify four different molecules, in addition to galactose, namely, glucose, fucose, arabinose and xylose. The galactose content was the most abundant residue in all treatments. Glucose presented the second highest value, being only detected in all alkali extractions with KOH [CE (KOH), UAE (KOH45) and UAE (KOH)] and a very low value in UAE (NaOH).

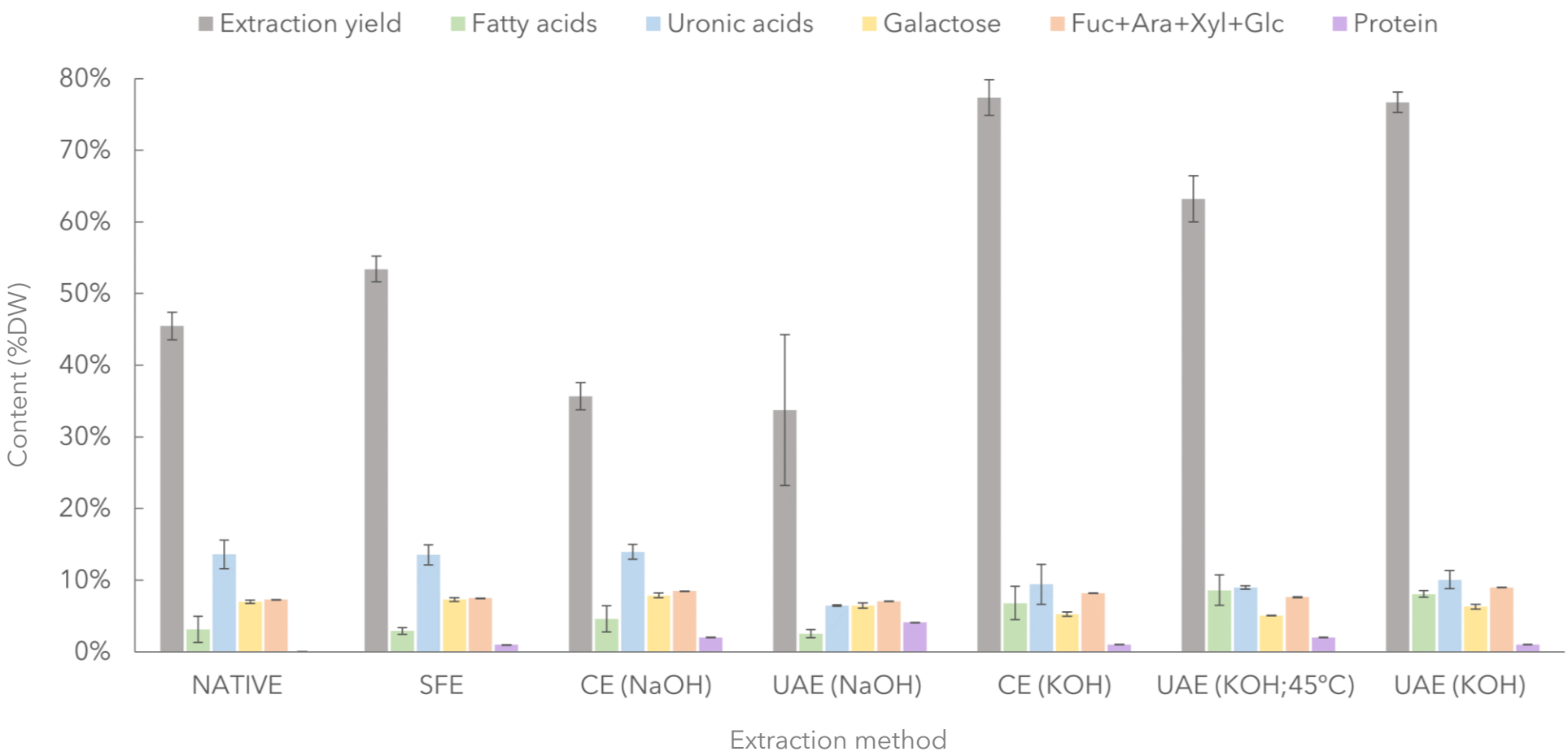


Figure 10. Extraction yield, total fatty acid (FAMEs), uronic acid, galactose, other monosaccharides (fucose, arabinose, xylose and galactose) and protein content of carrageenan extracted from the novel *Kappaphycus alvarezii* strain with native extraction (NE), supercritical fluid extraction (SFE), conventional extraction (CE: NaOH and CE: KOH) and ultrasound-assisted extractions (UAE: NaOH, 120 W, 70 °C, 1 h; UAE: KOH, 120 W, 45 °C, 1 h; UAE: KOH, 120 W, 70 °C, 1 h). Data are presented as the mean ± SD. Values were considered to differ statistical significantly if the *p* value was less than 0.05.

Table 5. Overview of carrageenan extraction methods *versus* yield, protein content, uronic acid content, relative and total composition of fatty acids and monosaccharides (wt%) present in carrageenan extracted using the different methods. Data are given as the means ± SDs. Values with the same letter are not significantly different ($p > 0.05$).

		Extraction method						
		NE	SFE	CE (NaOH)	CE (KOH)	UAE (NaOH)	UAE (KOH45)	UAE (KOH)
Extraction yield (%)		45.47±1.92^{a}	53.40±1.80^{a}	35.67±1.89^{a}	77.33±2.49^{a}	33.73±10.52^{a}	63.20±3.23^{a}	76.70±1.44^{a}
Protein (%)		0.00±0.01^{b}	0.01±0.01^{a,b}	0.02±0.01^{a,b}	0.01±0.01^{a,b}	0.04±0.02^{a}	0.02±0.01^{a,b}	0.01±0.01^{a,b}
Uronic acids (%)		13.59±1.97^{a}	13.52±1.37^{a}	13.95±1.05^{a}	9.43±2.78^{a,b}	6.43±0.11^{b}	8.95±0.23^{a,b}	10.06±1.28^{a,b}
Fatty Acids (%)	C16:0	0.02±0.01^{a}	0.01±0.00^{a}	0.02±0.01^{a}	0.05±0.02^{a}	0.01±0.00^{a}	0.07±0.02^{a}	0.07±0.00^{a}
	C18:0	0.01±0.01^{a}	0.01±0.00^{a}	0.02±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}
	C18:1	-	-	0.01±0.01^{a}	0.01±0.00^{a}	-	0.01±0.00^{a}	-
	Σ	0.03	0.03	0.05	0.07	0.03	0.09	0.08
Monosaccharides (%)	Galactose	6.98±0.23^{a,b}	7.28±0.29^{a,b}	7.87±0.36^{a}	5.23±0.30^{c}	6.45±0.37^{b}	5.06±0.05^{c}	6.29±0.34^{b}
	Glucose	-	-	-	1.92±0.08	0.04±0.06	1.87±0.19	2.20±0.03
	Fucose	0.21±0.03^{b,c}	0.15±0.02^{c}	0.36±0.09^{b,c}	0.68±0.02^{a}	0.31±0.12^{b,c}	0.44±0.11^{a,b}	0.34±0.11^{b,c}
	Arabinose	-	-	-	0.03±0.01^{a}	-	0.06±0.02^{a}	0.08±0.00^{a}
	Xylose	0.10±0.00^{b,c}	0.05±0.03^{c}	0.24±0.03^{a}	0.32±0.02^{a}	0.23±0.08^{a}	0.19±0.02^{a,b}	0.05±0.03^{c}
	Σ	7.29	7.49	8.48	10.10	7.06	9.49	11.16

- value below the detection level

3.1.2. Viscosity, pH, EC and TDS

The physico-chemical parameters measured are summarized in Table 6. The 1% solutions of all carrageenan extracts obtained through alkali [UAE (KOH)] treatment exhibited the presence of non-dissolvable particles, and all the samples showed an overall yellowish appearance except for the commercial samples, which showed a clear tone. Solutions prepared using commercial iota and kappa carrageenan exhibited the lowest EC and TDS values. Carrageenan extracted using the alkali [UAE (KOH)] method measured the highest viscosity (658.7 cP). The pH of all samples derived from different extraction methods was alkaline except for SFE, which had a slightly acidic pH (6.79), and NE, which had a neutral pH (7.34). The EC and TDS of alkaline [UAE (NaOH)] treated extracts were higher compared to the rest, wherein values were around the same range.

Table 6. Physico-chemical parameters of the novel *Kappahycus alvarezii* haploid strain solution (1%): viscosity, pH, electrical conductivity (EC) and total dissolved solids (TDS).

Extraction method	Viscosity (cP)	pH	EC (µS cm^{-1})	TDS (ppm's)
NE	16.8	7.34	2373	1158
SFE	7.8	6.79	2323	1170
CE (NaOH)	15.9	10.25	3156	1604
CE (KOH)	50.87	10.85	2733	1366
UAE (NaOH)	8.1	10.70	>3999	>2000
UAE (KOH45)	183.6	10.89	2817	1439
UAE (KOH)	658.7	9.30	2492	1286
Iota standard (ι)	134.7	9.69	2019	1012
Kappa standard (κ)	79.2	8.66	1891	949

3.1.3. UV–VIS absorption spectra of carrageenan solutions

The carrageenan obtained through the different methods differ in the spectral profiles, suggesting that the composition and the UV-absorbance potential vary depending on the applied extraction method (Fig. 11).

Only native carrageenan solution (NE) absorbed part of UV-A radiation (315 - 400 nm), exhibiting a peak at 321 nm.

The SFE, UAE (NaOH), UAE (KOH), UAE (NaOH) and UAE (KOH45) extracts showed peaks at 202, 204, 206 and 206 nm, respectively.

No pigments were detected in the carrageenan solutions.

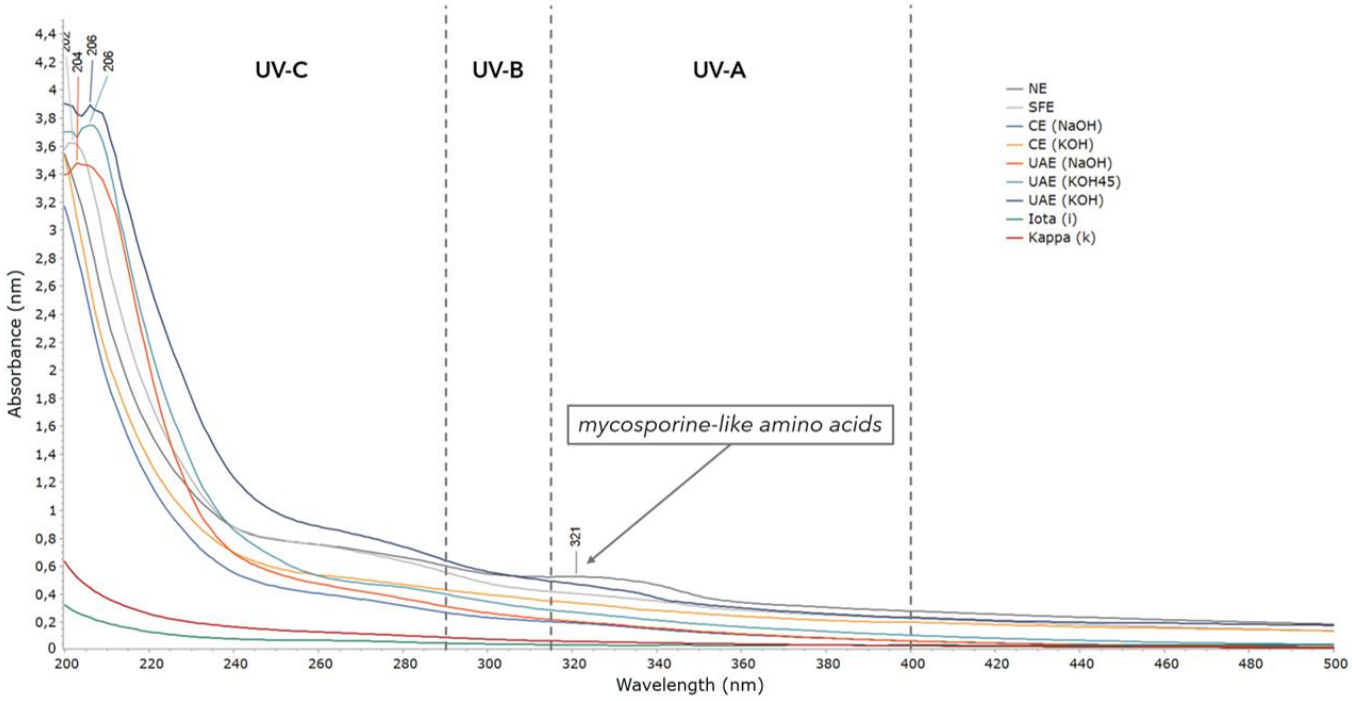


Figure 11. UV absorption spectra (λ = 200–600 nm) of carrageenan solutions extracted by native extraction (NE), conventional extraction (CE: NaOH and CE: KOH), ultrasound-assisted extractions (UAE: NaOH, 120 W, 70 °C, 1 h; UAE: KOH, 120 W, 45 °C, 1 h; UAE: KOH, 120 W, 70 °C, 1 h) and supercritical fluid extraction (SFE). UV-C range: 100 - 290 nm; UV-B range: 290 - 315 nm and UVA range: 315 - 400 nm.

3.1.4. FTIR-ATR analysis

The FTIR-ATR spectra of the commercial iota-carrageenan and kappa-carrageenan (Fig. 12a and 12b, respectively; Thermo Fisher Scientific, Waltham, MA, USA) were used as baseline in comparison to the spectral profile obtained from extracted carrageenan using different methods (Fig. 12c-i). FTIR-ATR band assignment, letter code nomenclature and band identification of extracted carrageenans are presented in Table 7.

The broad band (1220 cm^{-1}), present between 1210 and 1260 $cm^{-1,}$ is characteristic of sulphate esters in general, is common to all sulphated polysaccharides, and is a good total sulphate indicator (sulphate esters) (Pereira et al., 2003; Sekkal & Legrand, 1993). This band is verified to be strong in carrageenan standards, as shown in the spectra (Fig. 12a and 12b) (Gómez-Ordóñez & Rupérez, 2011). There are several

vibrational bands characteristic of carrageenans. The absorption band at 930 cm^{-1} indicates the presence of 3,6-anhydro-Dgalactose (**DA**), which is present in the spectra of the kappa and iota carrageenans. In addition, the band at 845 cm^{-1}, related to the presence of D-galactose-4-sulphate (**G4S**), is characteristic of the spectra of carrageenan kappa, mu, iota and nu. On the other hand, the band at 805 cm^{-1}, related to the presence of the sulphate ester at position 2 of anhydrous-D-galactose (**DA2S**) residues, is only observed in the spectra of iota-carrageenan.

The weak bands in the 770 cm^{-1} region are related, according to Matsuhiro (1996), to the skeleton bending of pyranose present in carrageenan. Additionally, the band at 1150 cm^{-1} may be assigned to C-O and C-C stretching vibrations of the pyranose ring common to all polysaccharides (Gómez-Ordóñez & Rupérez, 2011).

Going further on the spectra, the band at 1639 cm^{-1} is known to be an indicator of amide I, the presence of H_2O or proteins CO-NH/amide II from proteins (Marques and Pereira, 2016).

FTIR-ATR spectra of extracted carrageenan from the novel haploid *K. alvarezii* strain using various methods present absorption bands at the 930 cm^{-1} region and at the 845 cm^{-1} region, both typical and revealing the presence of kappa-carrageenan.

The conventionally extracted [CE (NaOH)] carrageenan (Fig. 12f) showed a slightly higher shoulder at 930 cm^{-1} in the spectra, and a decrease in the ratio 805/845 compared to native carrageenans was verified, meaning a decrease in the iota fraction relative to the kappa fraction (Table 8).

The FTIR-ATR spectra of conventionally extracted [CE (KOH)] carrageenan (Fig. 12c) is different from CE (NaOH), showing a slightly more visible band in the 805 cm^{-1} region (**DA2S**). An increase in the iota/kappa ratio is verified in comparison to native carrageenan and overall alkali extracted (NaOH) carrageenan, corresponding to an increase in the iota fraction relative to the kappa fraction.

Ultrasound-assisted extracted refined carrageenan [UAE (NaOH)] (Fig. 12g) is similar to conventionally extracted [CE (NaOH)], and although the peak at 845 cm^{-1} is less sharp, the ratio 805/845 is the same (Table 8).

Ultrasound-assisted extracted semi-refined carrageenan [UAE (KOH)] and UAE (KOH45) (Fig. 12e and 12d, respectively) revealed very similar spectra, with UAE (KOH) presenting a slightly higher peak in the 845 cm^{-1} region, which is visible in the lower ratio iota/kappa in comparison to UAE (KOH45) (Table 8).

Bands at 970-975 cm^{-1} regions relate to galactose (G) and are present in all samples, with CE(NaOH), NE and SFE extractions (Fig. 12f, 12h and 12i, respectively) showing more absorption in comparison to the rest of the samples.

Comparing the ratio iota/kappa (Table 8) of each extraction method with the iota-(0.82) and kappa-carrageenan (0.51) commercial samples, values ranged from 0.65 to 0.79.

In Table 9 is presented the absolute spectral similarity between carrageenan extracted from the various methods and each commercial sample. Commercial kappa-carrageenan samples revealed higher absolute spectral similarity than iota-carrageenan in all carrageenan samples obtained through the different extraction methods. However, iota-carrageenan still showed relatively high values. Carrageenan extracted through CE (KOH) shows the highest value of 64.66%.

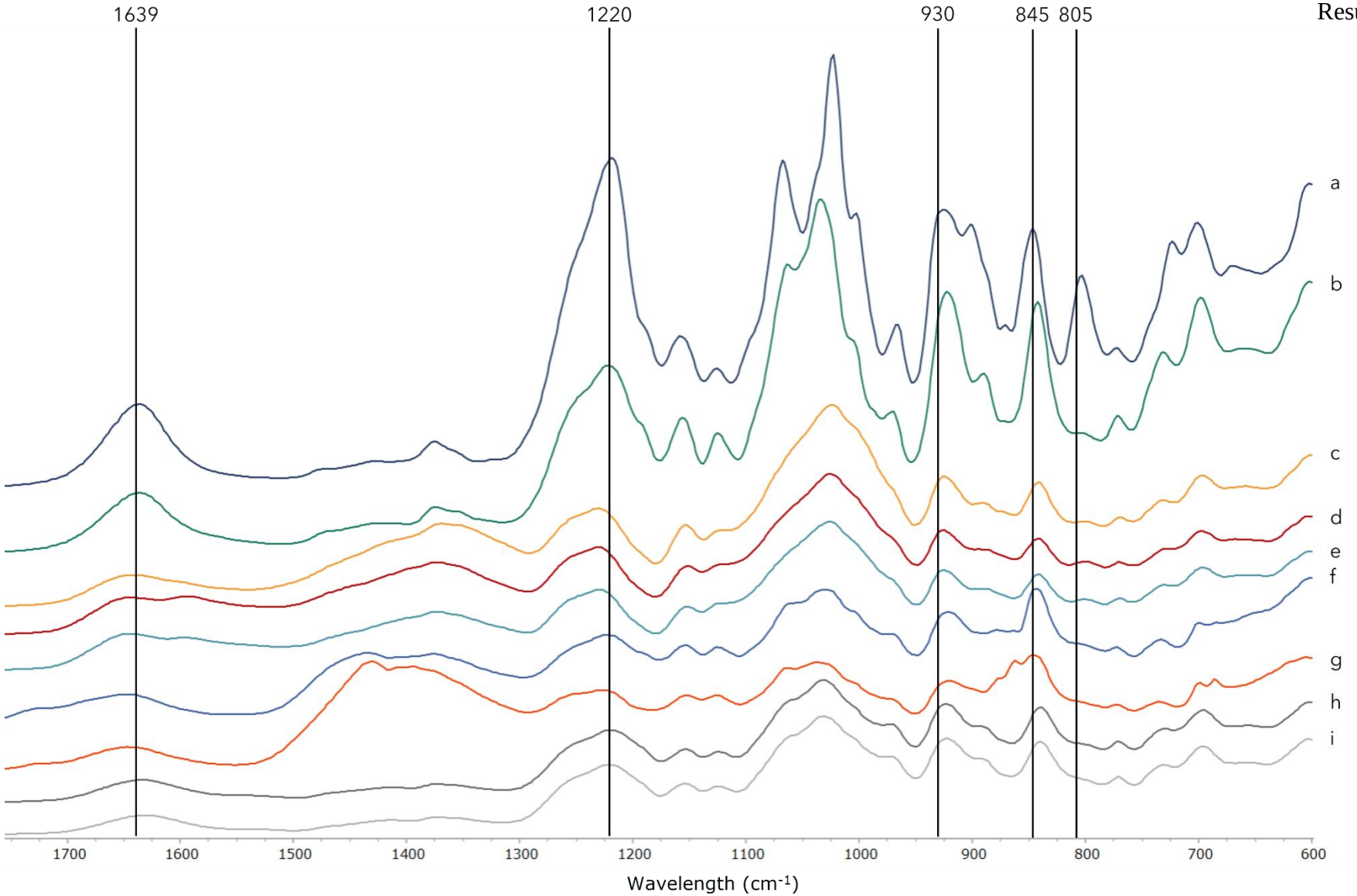


Figure 12. FTIR-ATR spectra of carrageenan standards: (a) iota-carrageenan; (b) kappa-carrageenan from Thermo Scientific and carrageenans from the novel strain of *K. alvarezii* obtained through the various extraction techniques: (c) conventional extraction (KOH); (d) ultrasound-assisted extraction UAE (KOH 45); (e) ultrasound-assisted extraction (KOH); (f) conventional extraction (NaOH); (g) ultrasound-assisted extraction (NaOH); (h) native extraction and (i) supercritical fluid extraction.

Table 7. FTIR band assignment, letter code nomenclature and band identification of carrageenans obtained through the various methods.

Wave number (cm^{-1})	Bound	Letter code	Iota (ι)	Kappa (κ)	NE	SFE	CE (NaOH)	CE (KOH)	UAE (NaOH)	UAE (KOH45)	UAE (KOH)
1210-1260	Sulphate ester (S=O)	**S**	1219	1222	1220	1221	1223	1231	1227	1231	1230
928-933, 1070 (shoulder)	3,6-anhydro-D-galactose	**DA**	925.2 (1067)	922.3 (1063)	923.1	923	921.5	924.6	920.9 (1063)	925.7	925.1
970-975	Galactose	**G/D**	967	971.3	972.8	972.8	-	-	-	-	-
890-900	β-D-galactose-de-sulphated	**G/D**	902.1	889.9	-	-	-	890.9	-	-	888.5
840-850	D-galactose-4-sulphate	**G4S**	846.4	842.2	839.7	839.7	842.4	841.5	846.2	841.6	841.4
830	D-galactose-2-sulphate	**G2S**	-	-	-	-	-	-	-	-	-
820, 825 (shoulder)	D-galactose-2,6-disulphate	**D2S, D6S**	-	-	-	-	-	-	-	-	-
810-820, 867 (shoulder)	D-galactose-6-sulphate	**G/D6S**	-	-	-	-	-	-	-	-	-
800-805, 905 (shoulder)	3,6-anhydro-D-galactose-2-sulphate	**DA2S**	803.4	-	-	-	-	800.2	-	800.7	800.9

- not detected

Table 8. Iota/Kappa ratio of carrageenan extracted from the various methods and each commercial sample.

	Iota (ι)	Kappa (κ)	NE	SFE	CE (NaOH)	CE (KOH)	UAE (NaOH)	UAE (KOH45)	UAE (KOH)
Iota/Kappa ratio	0.82	0.51	0.68	0.66	0.65	0.73	0.65	0.79	0.78

Table 9. Absolute spectral similarity between carrageenan extracted from the various methods and each commercial sample.

	Similarity (%)						
	NE	SFE	CE (NaOH)	CE (KOH)	UAE (NaOH)	UAE (KOH45)	UAE (KOH)
Iota (ι)	33.59	32.17	47.39	53.83	38.82	43.18	41.55
Kappa (κ)	40.10	38.35	56.83	64.66	46.92	51.98	49.93

3.2. Carrageenan yield and quality of hatchery-cultivated novel strains of different ploidy of *Kappaphycus alvarezii*

3.2.1. Yield

Yield of native (NE), conventional (CE), ultrasound-assisted (UAE) and supercritical fluid (SFE) extracted carrageenan from *K. alvarezii* strains are presented in Figure 13.

In native carrageenan extraction (NE), sample G-Q2M showed the highest yield (43.43±4.07%), although there were no statistically significant differences between the samples, and sample of *K. alvarezii* Doty (TR-B) the lowest (33.87±3.72%).

Conventional refined carrageenan (RC) extraction with NaOH (Fig. 13c) present overall between 37.33±2.49 and 17.67±3.86 % (G-N14 and TR-C18 samples, respectively).

Conventional semi-refined carrageenan (SRC) extraction with KOH (Fig. 13d) yield ranged from 84.67±0.47 and 39.60±1.65% (G-N11 and G-Q2M samples, respectively).

Regarding ultrasound-assisted methods (UAE) in refined carrageenan (RC) extraction (Fig. 13e) with NaOH [UAE (RC)], sample G-N14 revealed the highest yield (50.93±7.80%) and TR-B the lowest (6.70±0.91%). Compared with the conventional method, it reveals some samples with lower yield values (samples G-N11, TR-C3.2, TR-C21.2 and TR-B) and others with higher (samples G-N14, G-Q2M, G-Q2J, TR-C4.2 and TR-C18). Samples G-Q2M, G-Q2J, TR-C18, TR-C21.2 and TR-B revealed statistically significant differences between the methods.

On the other hand, in ultrasound-assisted method of semi-refined carrageenan extraction with KOH [UAE (SRC)] (Fig. 13g), sample G-Q2J revealed the higher yield of 73.53±2.29 % while sample G-N11 the lowest with a value of 54.57±0.49%. Compared with the conventional method, reveals overall higher values, with samples G-Q2M, G-Q2J, TR- C4.2, TR-C18 and TR-C21.2 showing statistically significant differences between these two methods.

The ultrasound-assisted method of semi-refined carrageenan extraction with KOH, performed at 45 ºC [UAE (SRC45)] (Fig. 13f), presents a higher yield with a value of 73.87±1.18% regarding sample G-Q2J and the lowest at 47.40±2.14% regarding samples G-Q2J. Comparing UAE (SRC), UAE (SRC45) yield was slightly lower, with samples G-Q2M and TR-C18 showing statistically significant differences between the method.

In supercritical fluid extraction (SFE) (Fig. 13b) sample G-Q2M showed the highest yield (43.12±2.29%), and sample G-Q2M the lowest (28.78±3.23%).

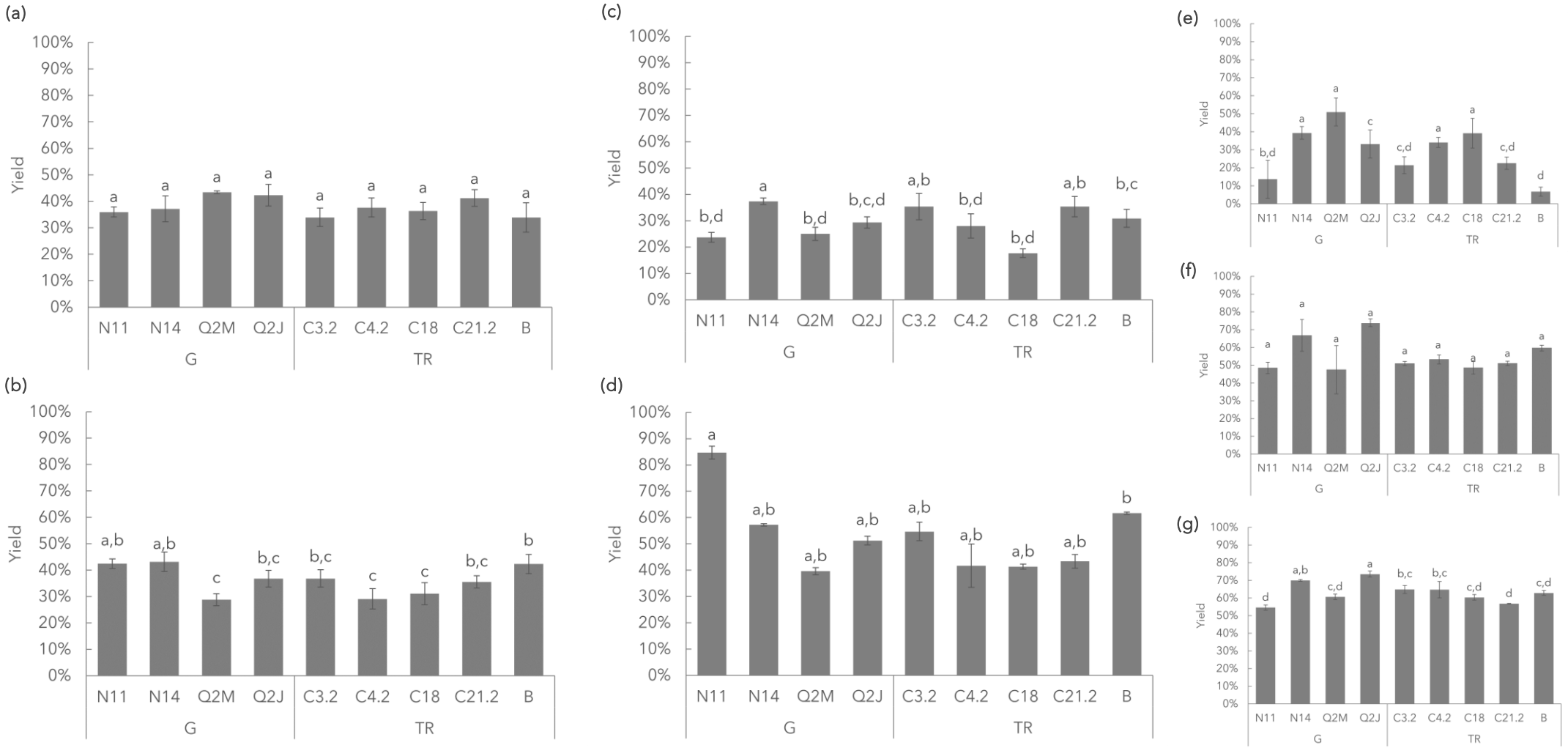


Figure 13. Extraction yields of carrageenan extracted from *K. alvarezii* novel strains through a) native extraction (NE), b) supercritical fluid extraction (SFE), conventional extraction: c) CE (Refined Carrageenan) and d) CE (Semi-Refined Carrageenan); and ultrasound-assisted extractions: e) UAE (Refined Carrageenan), f) UAE (Semi-Refined Carrageenan) at 45°C and g) UAE (Semi-Refined Carrageenan). The results are from separate experiments, and data is presented as mean±SD. Values were considered to differ significantly if the *p* value was <0.05 and are referred to each sample.

3.2.2. Viscosity, pH, EC and TDS

Viscosity, pH, EC and TDS of native (NE), conventional (CE), ultrasound-assisted (UAE) and supercritical fluid (SFE) extracted carrageenan solutions (1%) are presented in Table 10.

In native carrageenan extraction (NE), sample G-N11 presented the highest value (811.1 cP), followed by sample TR-B (628.7 cP), and sample TR-C18 the lowest value (10.8 cP).

Conventional refined carrageenan (RC) extraction with NaOH presented values ranging for 9.3 (TR-C21.2) to 28.2 cP (TR-C3.2).

Conventional semi-refined carrageenan (SRC) extraction with KOH viscosity values were higher than any other of the extraction methods, ranging from 1074 cP (G-N14) to 21.3 (TR-C4.2).

Ultrasound-assisted methods (UAE), refined carrageenan (RC) extraction with NaOH revealed overall lower values in comparison to refined carrageenan extraction, except for sample TR-B, which increase and shows the highest value (26.4 cP).

About ultrasound-assisted methods (UAE), the semi-refined carrageenan (SRC) extracted at 70 ºC show close and similar values (except for sample TR-B) to the ones extracted at 45 ºC. Comparing these with the conventional extraction (SRC) they were lower, except in sample G-N11.

Commercial carrageenans used as control in this study exhibited lower values, presenting 134.7 and 79.2 cP (ι- and κ-carrageenan, respectively).

Regarding pH, commercial carrageenans used (control) exhibited values of 9.69 and 8.66 for ι- and κ-carrageenan, respectively. Overall treatments showed alkali pH with values ranging from 9.3 to 10.89, except for NE and SFE that revealed acidic pH with values between 7.38 and 5.62 (Table 10).

Both TDS and EC showed values higher than the exhibited by commercial carrageenan samples. These were especially higher in the solutions from refined carrageenan extracted by ultrasound assisted method with values above the measurement range (Table 10).

Table 10. Physico-chemical parameters (viscosity at 1% and pH) of *K. alvarezii* novel strains for each extraction method.

		G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B	Iota (i)	Kappa (k)
NE	η (cP)	811.1	62.4	28.5	24.9	35.4	11.7	10.8	12.6	628.7	-	-
	pH	6.95	7.05	6.65	7.38	8.47	6.8	7.33	7.1	6.98	-	-
	EC (µS cm^{-1})	>3999	2782	3686	2323	2886	3146	3126	2123	2951	-	-
	TDS (ppm's)	>2000	1395	1837	1164	1445	1593	1579	1242	1477	-	-
SFE	η (cP)	9.6	24.9	5.7	12.9	7.8	6	5.7	5.1	225	-	-
	pH	5.63	6.12	5.9	6.01	5.62	5.82	5.82	5.9	6.43	-	-
	EC (µS cm^{-1})	2835	2766	2601	2304	2802	>3999	3512	2417	3059	-	-
	TDS (ppm's)	1427	1388	1306	1155	1403	>2000	1700	1206	1530	-	-
CE (RC)	η (cP)	19.8	23.4	12.3	18.0	28.2	13.2	10.8	9.3	22.8	134.7	79.2
	pH	10.43	10.43	10.19	10.43	10.26	10.18	10.71	10.43	10.68	9.69	8.66
	EC (µS cm^{-1})	3193	3697	2919	3348	3420	2819	>3999	3886	>3999	2019	1891
	TDS (ppm's)	1566	1813	1487	1682	1719	1426	>2000	1959	>2000	1012	949
CE (SRC)	η (cP)	18.3	1074	196.2	214.5	760.7	21.3	126.9	171.9	322.8	-	-
	pH	10.77	10.61	10.72	10.75	10.88	10.75	10.74	10.67	10.93	-	-
	EC (µS cm^{-1})	2603	2816	2769	2419	2902	2669	3499	3000	2827	-	-
	TDS (ppm's)	1300	1410	1389	1215	1453	1333	1730	1498	1412	-	-
UAE (RC)	η (cP)	9.6	10.5	6.9	11.7	7.2	6.3	6.9	8.4	26.4	-	-
	pH	10.53	10.74	10.28	10.35	10.85	10.49	10.77	10.88	11.02	-	-
	EC (µS cm^{-1})	>3999	>3999	>3999	>3999	>3999	>3999	>3999	>3999	3303	-	-
	TDS (ppm's)	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	1652	-	-
UAE (SRC45)	η (cP)	37.8	17.4	12.3	11.1	14.7	8.7	10.8	8.7	136.8	-	-
	pH	10.35	10.76	10.57	1229	1454	2722	1411	1443	10.32	-	-
	EC (µS cm^{-1})	2808	2852	3366	2427	2861	1367	2770	2847	2192	-	-
	TDS (ppm's)	1406	1419	1754	1229	1454	2722	1411	1443	1102	-	-
UAE (SRC)	η (cP)	33.9	19.2	9.9	15.3	23.7	9	15	19.8	26.4	-	-
	pH	10.7	10.7	10.69	10.65	10.67	10.54	10.44	10.47	11.2	-	-
	EC (µS cm^{-1})	2836	3084	3080	3186	2767	2908	3501	2898	3303	-	-
	TDS (ppm's)	1416	1537	1539	1633	1397	1459	1746	1456	1652	-	-

3.2.3. Spectrophotometric profiles of carrageenan solutions

Native carrageenan samples (Table 11; Annex III) G-Q2M and TR-C21.2 only showed peaks between 320-322 nm (UVA range 15–400nm), G-Q2J, TR-C3.2 and TR-C18 samples only exhibited peaks between 201-204 nm (UV-C range 100- 290nm), while G-N11, TR-C4.2 and TR-B revealed peaks in both UV-A range (between 320-325 nm) and UV-C range (between 201-203 nm). No peaks on the UV- B range were detected.

Table 11. Band assignment of spectrophotometric profile of commercial (iota and kappa) and native carrageenan

Spectrophotometric profile of native carrageenan												
Wavelength (nm)	UV band	ι	κ	G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B
100-290	UVC	-	-	201	-	-	203	201	203	204	-	202
290-315	UVB	-	-	-	-	-	-	-	-	-	-	-
315-400	UVA	-	-	325	-	324	-	-	320	-	322	324

Conventional alkali-extracted carrageenan both RC (Table 12; Annex III) and SRC (Table 13; Annex III) few samples showed UV absorption peaks and only in the UV-C range (between 201-203 nm). Amoun CE (RC) samples, G-N14, TR-C4.2, TR-C18 and TR-B reveled peaks ranging from 201 to 210 nm, while in CE (SRC) only TR-B reveled peak at 201 nm.

Table 12. Band assignment of spectrophotometric profile of conventional alkali-extracted carrageenan (RC)

Spectrophotometric profile of conventional alkali-extracted carrageenan (RC)										
Wavelength (nm)	UV band	G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B
100-280	UVC	-	201	-	-	-	202	206	-	210
280-315	UVB	-	-	-	-	-	-	-	-	-
315-400	UVA	-	-	-	-	-	-	-	-	-

Table 13. Band assignment of spectrophotometric profile of conventional alkali-extracted carrageenan (SRC)

Spectrophotometric profile of conventional alkali-extracted carrageenan (SRC)										
Wavelength (nm)	UV band	G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B
100-280	UVC	-	-	-	-	-	-	-	-	201
280-315	UVB	-	-	-	-	-	-	-	-	-
315-400	UVA	-	-	-	-	-	-	-	-	-

In ultrasound-assisted RC (Table 14; Annex III) all samples, except TR-C21.2, showed UV absorption peaks only in the UV-C range (between 201-203 nm). In UAE (SRC) (Table 15; Annex III), all samples showed peaks ranging from 207 to 211 nm (UV-C range 100- 290 nm), while G-N14 and TR-C18 also showed peaks at 342 and 397 nm (UVA range 15–400 nm), respectively.

Regarding UAE (SRC45) (Table 16; Annex III), samples G-N14, TR-C18 TR-B revealed peaks in both UV-A range (between 320-325 nm) and UV-C range (between 201-203 nm). Only carrageenan extracted from TR-B sample through this method showed absorption in all UV ranges.

Table 14. Band assignment of spectrophotometric profile of ultrasound-assisted extracted carrageenan (RC)

Spectrophotometric profile of ultrasound-assisted extracted carrageenan (RC)										
Wavelength (nm)	UV band	G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B
100-280	UVC	206	207	207	202	203	206	205	-	202
280-315	UVB	-	-	-	-	-	-	-	-	-
315-400	UVA	-	-	-	-	-	-	-	-	-

Table 15. Band assignment of spectrophotometric profile of ultrasound-assisted extracted carrageenan (SRC)

Spectrophotometric profile of ultrasound-assisted extracted carrageenan (SRC)										
Wavelength (nm)	UV band	G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B
100-280	UVC	208	210	207	210	209	209	211	207	205, 211
280-315	UVB	-	-	-	-	-	-	-	-	-
315-400	UVA	-	342	-	-	-	-	397	-	-

Table 16. Band assignment of spectrophotometric profile of ultrasound-assisted extracted carrageenan (SRC) at 45 ºC

Spectrophotometric profile of ultrasound-assisted extracted carrageenan (SRC) at 45 ºC										
Wavelength (nm)	UV band	G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B
100-280	UVC	204	207	202	208	207	216	211	206	210
280-315	UVB	-	-	-	-	-	-	-	-	274
315-400	UVA	-	342	-	-	-	-	399	-	401

All samples of supercritical fluid extracted carrageenan (Table 17; Annex III), showed UV absorption peaks in the UV-C range (between 201-203 nm). TR-B sample showed absorption peaks at 210 (UV-A range 15 – 400 nm), 274 (UV- B range 290–315 nm) and 401 nm (UV-C range 100 - 290 nm).

Table 17. Band assignment of spectrophotometric profile of supercritical fluid extracted carrageenan.

Spectrophotometric profile of supercritical fluid extracted carrageenan											
Wavelength (nm)	UV band	G-N7	G-N11	G-N14	G-Q2M	G-Q2J	TR-C3.2	TR-C4.2	TR-C18	TR-C21.2	TR-B
100-280	UVC	202	204	202	202	204	202	-	207	206	201
280-315	UVB	-	-	-	-	-	-	-	-	-	-
315-400	UVA	-	-	-	-	-	-	-	-	-	-

3.2.4. FTIR-ATR analysis

In each extraction method FTIR-ATR spectra, the novel strain G-N7, analyzed in section 3.1. is presented (dotted line) for comparison purposes.

3.2.4.1. FTIR-ATR spectra of standard commercial carrageenan samples

FTIR-ATR band assignment, letter code nomenclature and band identification of standard commercial carrageenans are present in Table 18.

In Fig. 14 are present two spectra relative to commercial iota and kappa-carrageenans from Thermo Fisher Scientific.

The broad band (1220 cm^{-1}), between 1210 and 1260 cm^{-1} is characteristic of sulphate esters in general, being common to all sulphated polysaccharides, and a good total sulphate indicator (sulphate esters) (Pereira et al., 2003; Sekkal & Legrand, 1993). This band is verified to be strong in carrageenan standards as shown in the spectra (Fig. 14C1 and 14C2) (Gómez-Ordóñez & Rupérez, 2011). There are several vibrational bands characteristic of carrageenans. The absorption band at 930 cm^{-1}, is one of them, and it is related to the vibrations of the 3,6-anhydrolactose bridges, being present in the spectra of the carrageenans kappa, iota and theta. In addition to this, the band at 845 cm^{-1}, associated with vibrations of C(4)-O-SO3 (fragment of sulphated galactose) is characteristic of the spectra of carrageenan kappa, mu, iota and nu. On the other hand, the band at 805 cm^{-1}, related to the vibrations around C(2)-O-SO3, a fragment of the sulphated 3,4-anhydrolactose, is only observed in the spectra of carrageenan iota and theta. Therefore, the relative intensity of the peaks 805 cm^{-1} and 845 cm^{-1} allows the determination of hybridization of carrageenans by calculating its Iota/Kappa ratio (ratio 805/845 cm^{-1}).

The FTIR-ATR of carrageenan kappa (Fig. 14C2) peaks at 845 cm^{-1}, related to the presence of D-galactose-4-sulphate (**G4S**) and a prominent peak around 930 cm^{-1}, which indicates the presence of 3,6-anhydro-Dgalactose (**DA**).

Figure 14C1 shows the spectrum of carrageenan iota. The spectrum of this carrageenan also exhibits peaks at 845 cm^{-1} and 930 cm^{-1} like kappa carrageenan, however a new peak appears at approximately 805 cm^{-1}, indicating the presence of the sulphate ester at position 2 of anhydrous-D-galactose (**DA2S**) residues, which is typical of iota carrageenan.

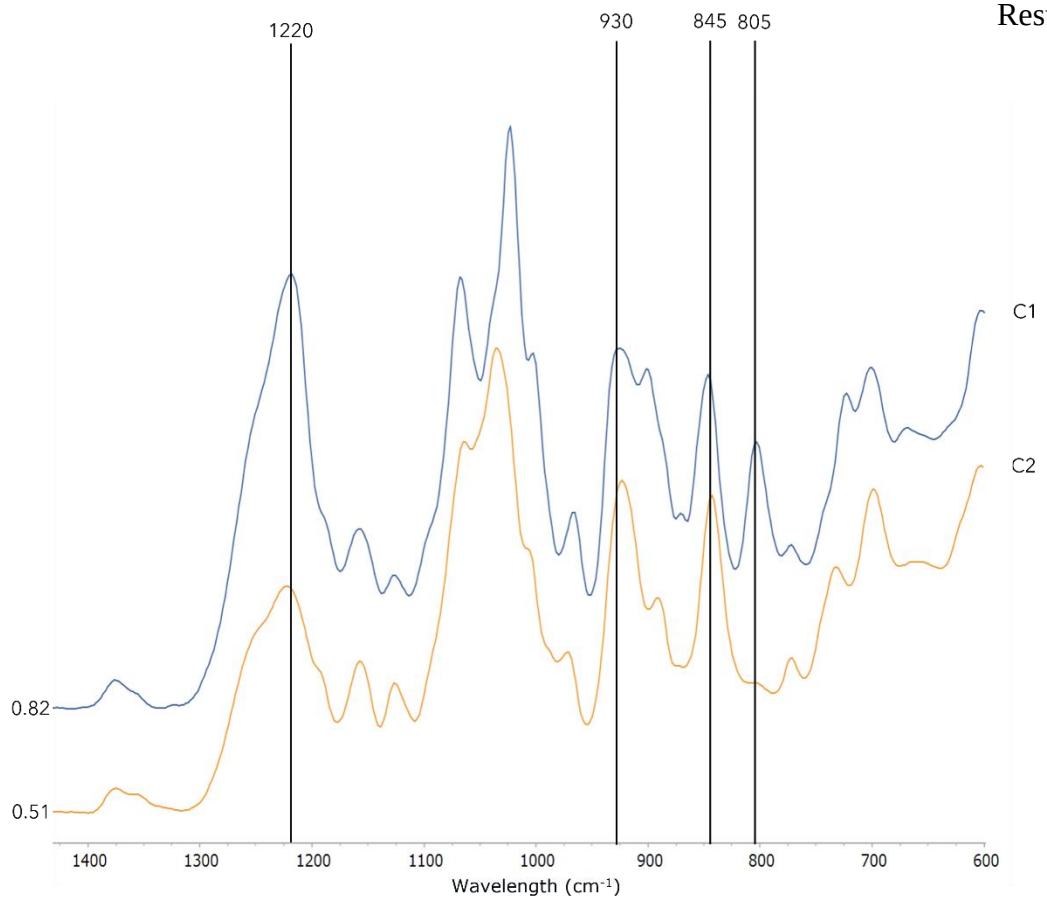


Figure 14. FTIR-ATR spectra of carrageenans from Thermo Fisher Scientific: (C1) iota-carrageenan and (C2) kappa-carrageenan.

Table 18. FTIR-ATR band assignment, letter code nomenclature and band identification of standard commercial carrageenans.

Wave number (cm^{-1})	Bound	Letter code	Iota (C1)	Kappa (C2)
1210-1260	Sulphate ester (S=O)	**S**	1219	1222
928-933; 1070 (shoulder)	3,6-anhydro-D-galactose	**DA**	925.2 (1067)	922.3 (1063)
970-975	Galactose	**G/D**	967	971.3
890-900	β-D-galactose-desulphated	**G/D**	902.1	889.9
840-850	D-galactose-4-sulphate	**G4S**	846.4	842.2
830	D-galactose-2-sulphate	**G2S**	-	-
820, 825 (shoulder)	D-galactose-2,6-disulphate	**D2S, D6S**	-	-
810-820; 867 (shoulder)	D-galactose-6-sulphate	**G/D6S**	-	-
800-805; 905 (shoulder)	3,6-anhydro-D-galactose-2-sulphate	**DA2S**	803.4	-

3.2.4.2. FTIR-ATR spectra of ground samples and native carrageenan

FTIR-ATR spectra of ground *K. alvarezii* samples (Fig. 15) and native carrageenan (Fig. 16) present absorption bands at 930 cm^{-1} (**DA**) and at 845 cm^{-1} (**G4S**) regions in all samples, both typical and reveal the presence of κ-carrageenan. These spectra also show some absorbance in the 805 cm^{-1} region (**DA2S**), typical of ι-carrageenan.

Ground seaweed presented a higher iota/kappa ratio (Fig. 15, Table 19), with values between from 0.74 (TR-B) and 0.93 (G-Q2J), than native carrageenan, witch ratio ranged from 0.63 (TR-B) to 0.69 (G-N14).

A broad band at 1220 cm^{-1} is present in all samples, and especially strong in carrageenan standards as shown in the spectra (Fig. 14C1 and 14C2). Additional peaks at 867 cm^{-1} (**G/D6S**), 825 cm^{-1} (**G/D2S**) and 820 cm^{-1} (**G/D6S**), with little intensity are also present. A weak band at 770 cm^{-1} and further at 1150 cm^{-1} region was also detected.

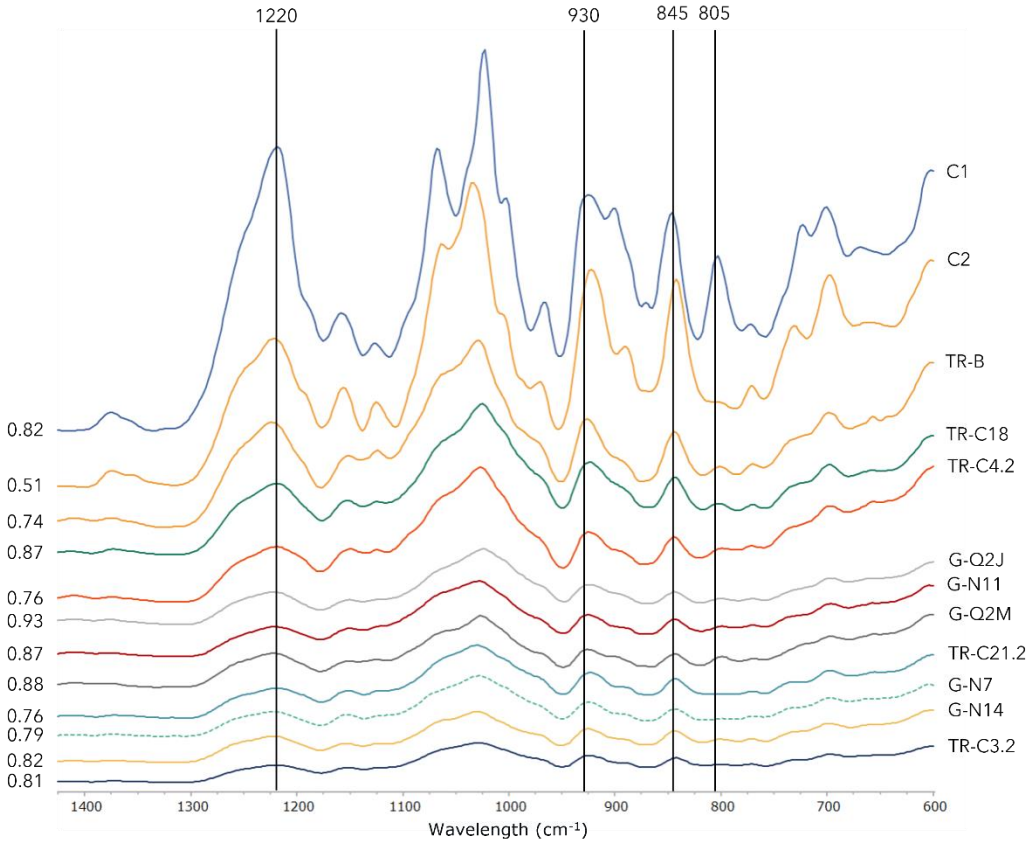


Figure 15. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan), and ground seaweed samples of *Kappaphycus alvarezii*.

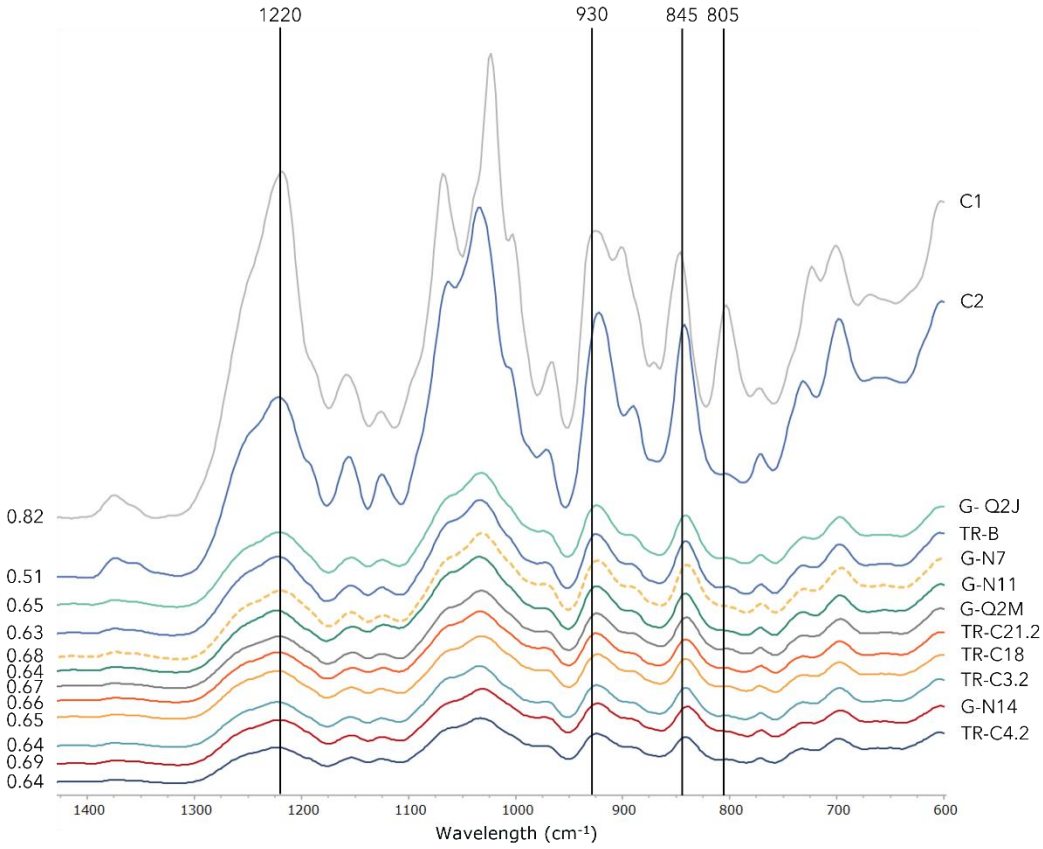


Figure 16. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan) and native carrageenan extracted from *Kappaphycus alvarezii* samples.

3.2.4.3. FTIR-ATR spectra of conventional alkali-extracted carrageenan (RC and SRC)

The alkali-extracted RC carrageenan (Fig. 17) showed slightly lower intensity of galactose (970-975 cm^{-1} region). A broad and slightly higher shoulder present at 930 cm^{-1} in the spectra is present. An overall increase in the ratio 805/845, although not very significant and not seen in all *K. alvarezii* strains (sample G-N14 show lower ratio and TR-C3.2 equal ratio) in the alkali-extracted carrageenan samples, compared to native carrageenans is verified, meaning an increase of the iota fraction relative to kappa fraction (Table 19).

The FTIR-ATR spectra of alkali-extracted SRC carrageenan (Fig. 18) are different from the alkali-extracted RC, showing slightly visible band on 805 cm^{-1} region (**DA2S**), broader bands between 950-1100 cm^{-1} and at 1220 cm^{-1} for sulphate esters.

An increment in the iota/kappa ratio was verified in comparison to native carrageenan and overall alkali extracted (NaOH) carrageenan, corresponding to an increment of the iota fraction relatively to kappa fraction.

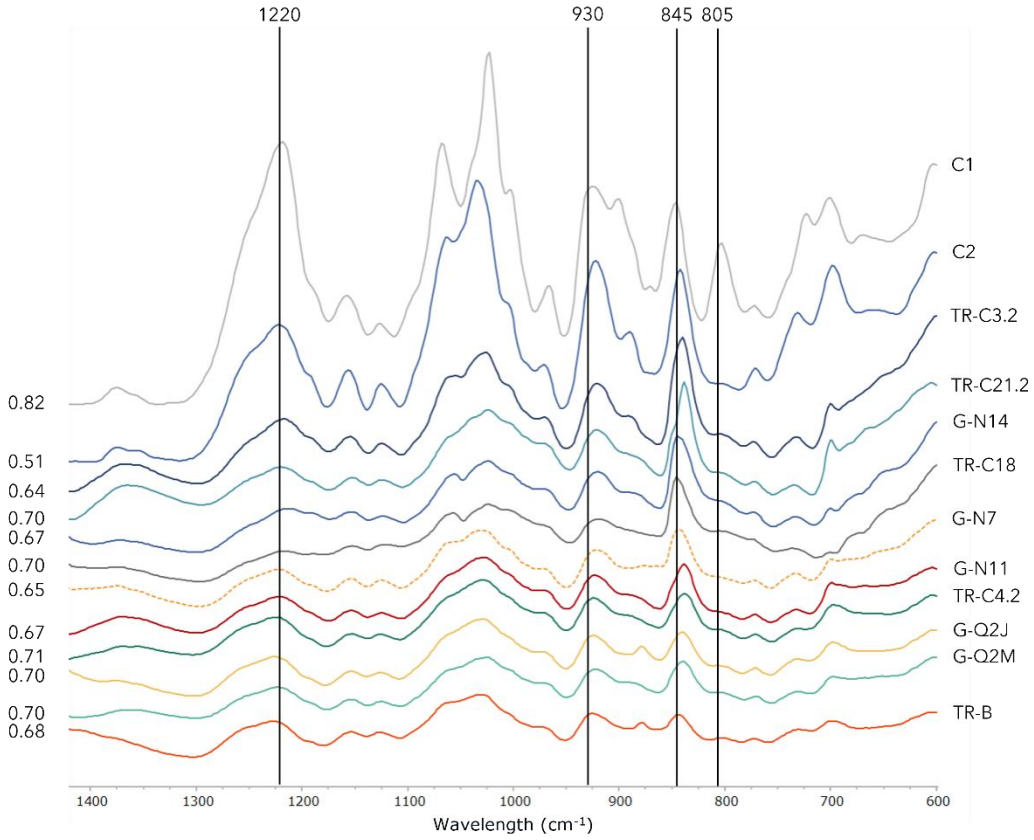


Figure 17. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan) and carrageenan extracted from *Kappaphycus alvarezii* samples by CE (RC).

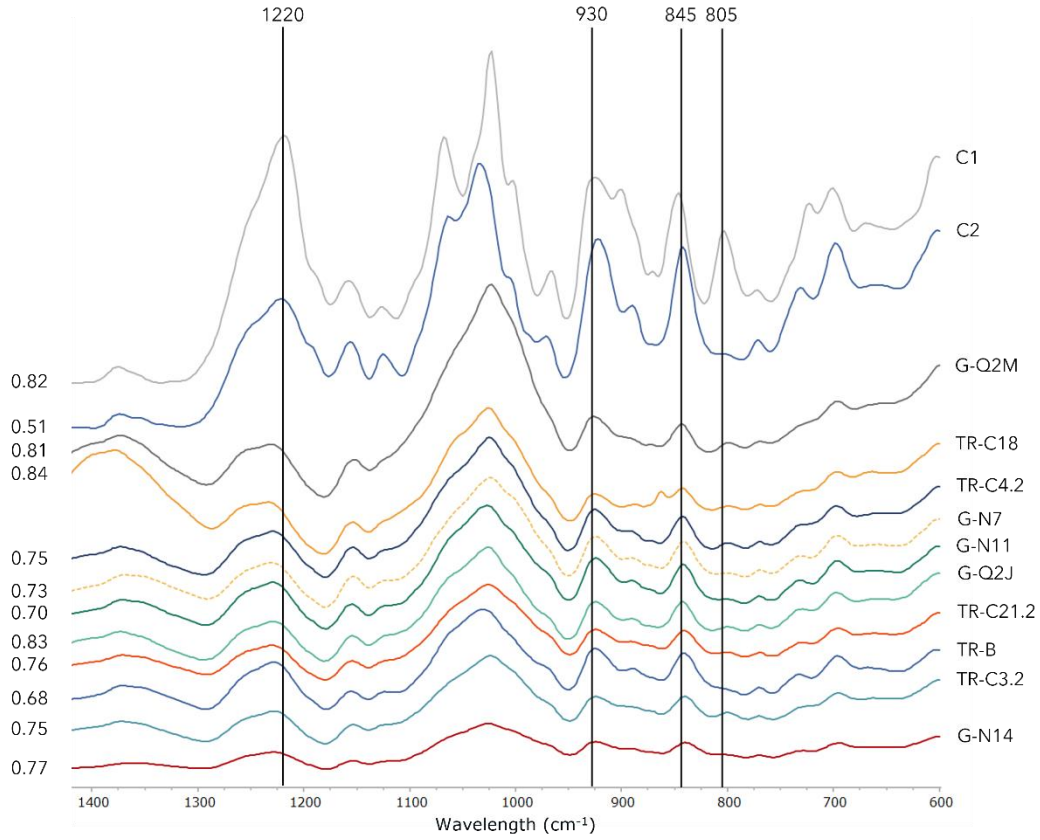


Figure 18. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan) and carrageenan extracted from *Kappaphycus alvarezii* samples by CE (SRC).

3.2.4.4. FTIR-ATR spectra of ultrasound-assisted extracted carrageenan (RC and SRC)

Ultrasound-assisted extracted refined carrageenan (RC) (Fig. 19) in the 1070 cm^{-1} region regarding 3,6-anhydro-D-galactose (**DA**) it was visible a lower intensity in comparison to conventionally extracted (Fig. 17). A much weaker bands at 845 cm^{-1} region were detected, being translated in overall decrease in the ratio 805/845, with some samples revealing equal ratios (G-Q2M and TR-B) compared to conventional extraction.

Ultrasound-assisted extracted semi-refined carrageenan (SRC) and (SRC45) (Fig. 20 and Fig. 21, respectively) revealed very similar spectra, with some samples of UAE (SRC) presenting higher peaks in the 845 cm^{-1} region, except for G-N14 and G-Q2J which is visible in the lower ratio iota/kappa.

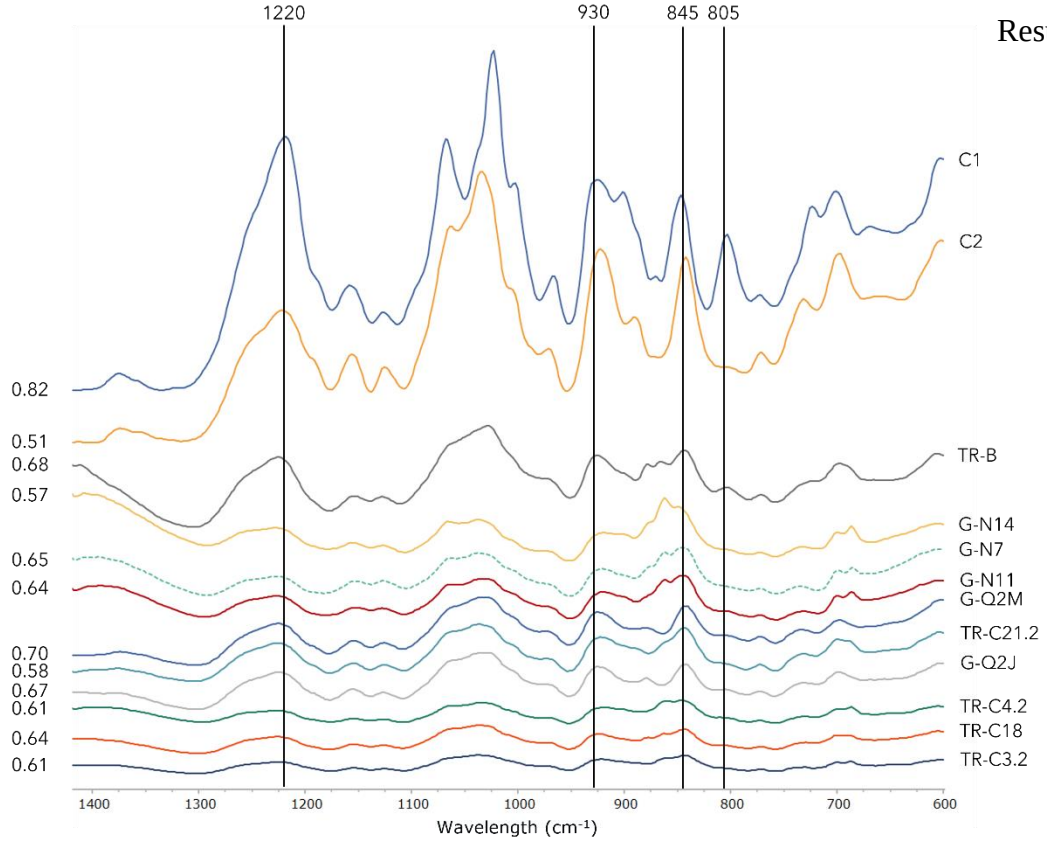


Figure 19. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan) and carrageenan extracted from *Kappaphycus alvarezii* samples by UAE (RC).

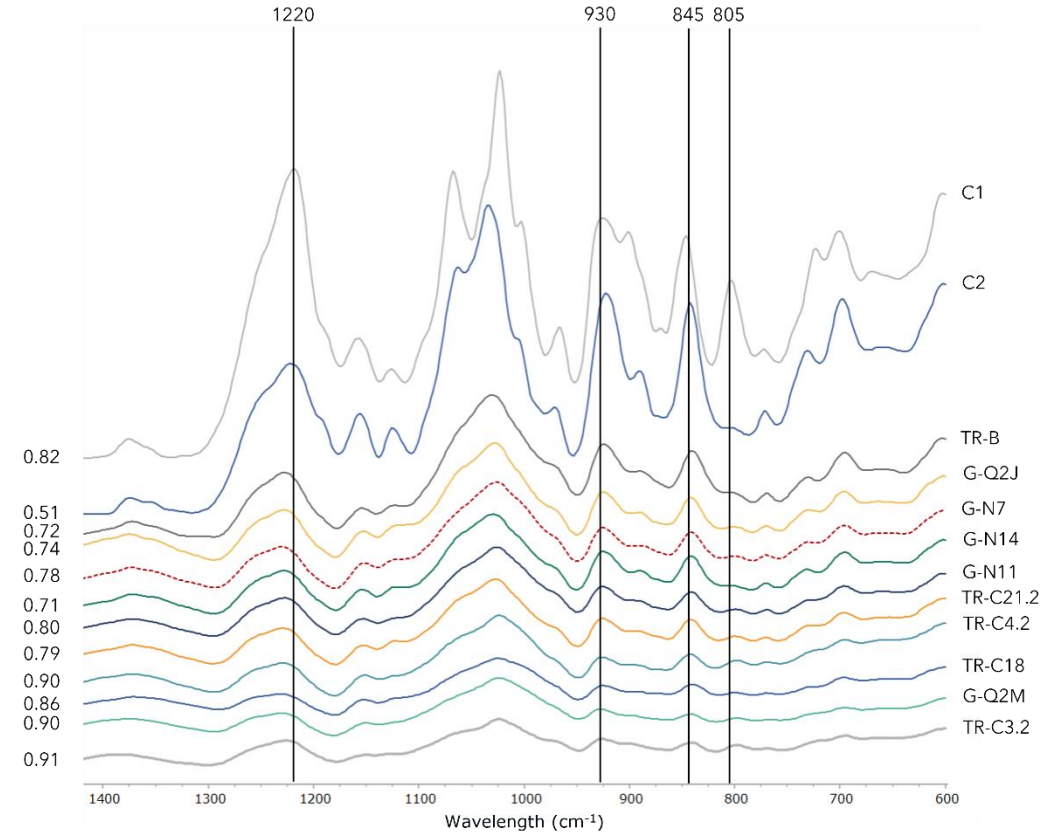


Figure 20. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan) and carrageenan extracted from *Kappaphycus alvarezii* samples by UAE (SRC).

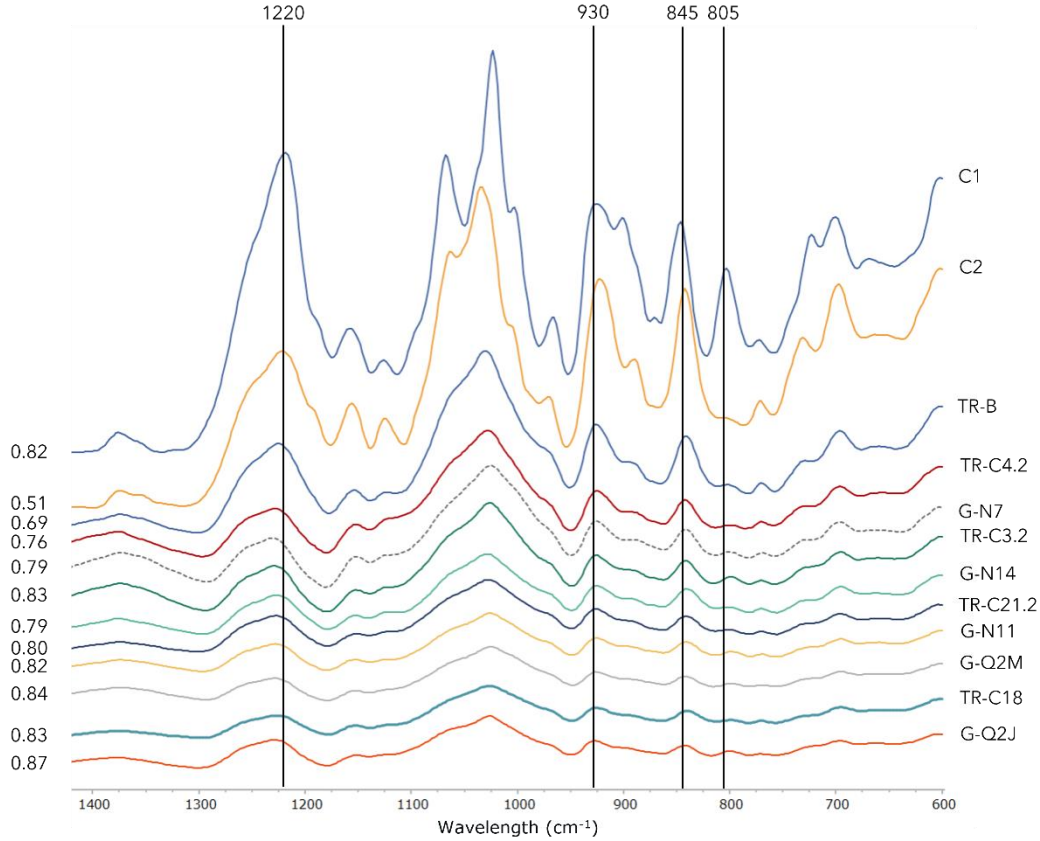


Figure 21. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan) and carrageenan extracted from *Kappaphycus alvarezii* samples by UAE (SRC45).

3.2.4.5. FTIR-ATR spectra of supercritical fluid extracted carrageenan.

FTIR-ATR spectra of supercritical fluid extracted carrageenan (Fig. 22) present similar absorption bands of native extracted (Fig. 16), at 930 cm^{-1} (**DA**) and at 845 cm^{-1} (**G4S**) regions in all samples, both typical and reveal the presence of κ-carrageenan These spectra also show some absorbance in the 805 cm^{-1} region (**DA2S**), typical of iota-carrageenan.

The iota/kappa ratio (Table 19) values are similar to native carrageenan, ranging from 0.64 (TR-C4.2) to 0.70 (G-Q2J)

A broad band at 1220 cm^{-1} is present in all samples and additional peaks more prominent at 1120 cm^{-1} and further at 1160 cm^{-1}.

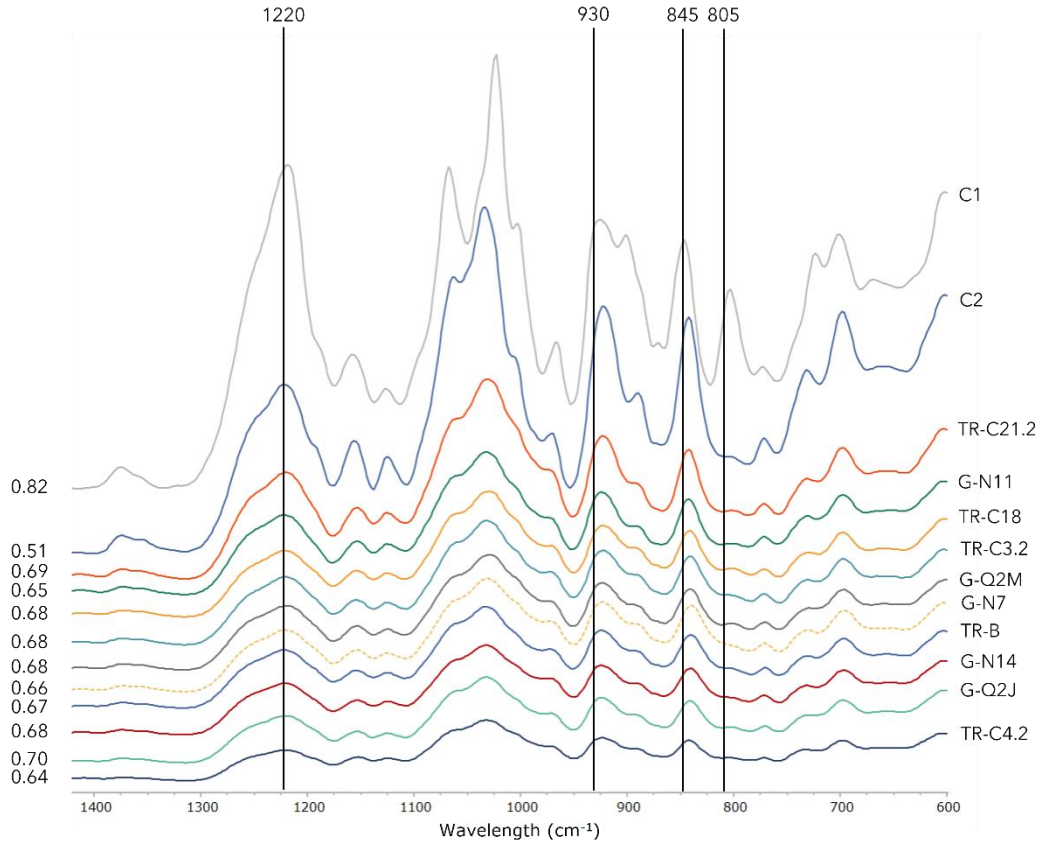


Figure 22. FTIR-ATR spectra of carrageenans from (C1) Thermo Fisher Scientific (ι-carrageenan), (C2) Thermo Fisher Scientific (κ-carrageenan) and carrageenan extracted from *Kappaphycus alvarezii* samples by SFE.

Table 19. Iota/kappa ratio of ground seaweed (GS) samples, native (NE), supercritical fluid (SFE), conventional (CE: RC and CE: SRC) and ultrasound-assisted (UAE: RC, 120 W, 70 °C, 1h; UAE: SRC, 120 W, 45 °C, 1h; UAE: SRC, 120 W, 70 °C, 1h) carrageenan extracted from *Kappaphycus alvarezii* samples.

Iota/kappa ratio								
	GS	NE	SFE	CE (RC)	CE (SRC)	UAE (RC)	UAE (SRC45)	UAE (SRC)
G-N11	0.87	0.64	0.65	0.67	0.70	0.64	0.82	0.80
G-N14	0.82	0.69	0.68	0.67	0.77	0.57	0.79	0.71
G-Q2M	0.88	0.67	0.68	0.70	0.81	0.70	0.84	0.90
G-Q2J	0.93	0.65	0.70	0.70	0.83	0.67	0.87	0.74
TR-C3.2	0.81	0.64	0.68	0.64	0.75	0.61	0.83	0.91
TR-C4.2	0.76	0.64	0.64	0.71	0.75	0.61	0.76	0.90
TR-C18	0.87	0.65	0.68	0.70	0.84	0.64	0.83	0.86
TR-21.2	0.76	0.66	0.69	0.70	0.76	0.58	0.80	0.79
TR-B	0.74	0.63	0.67	0.68	0.68	0.68	0.69	0.72
Iota (ι)					0.82			
Kappa (κ)					0.51			

3.2.4.6. FTIR-ATR spectra similarity to standard commercial carrageenan

Commercial kappa-carrageenan sample revealed in all samples higher similarity than iota-carrageenan (Table 20). However, iota-carrageenan still showed relatively high values. In ground seaweed samples, TR-B exhibited the highest similarity to both iota and kappa-carrageenan (62.83 and 74.66 %, respectively) and G-N14 the lowest (21.33 and 25.41%, respectively). In native extracted carrageenan, TR-C18 sample showed highest similarity (50.65 %) to iota-carrageenan and Q-Q2J sample to kappa-carrageenan (41.52 %). The lowest values for both iota and kappa-carrageenan (21.33 and 23.90 %, respectively) were present in sample G-N14.

In conventional refined carrageenan (RC) extraction with NaOH, sample TR-C3.2 exhibited the highest similarity to both iota and kappa-carrageenan (83.79 and 100.09 %, respectively) and TR-B the lowest (28.52 and 34.26 %, respectively). In conventional semi-refined carrageenan (SRC) extraction with KOH, sample G-Q2M exhibited the highest similarity to both iota and kappa-carrageenan (76.55 and 91.56 %, respectively) and G-N14 the lowest (21.33 and 25.55 %, respectively).

Looking at ultrasound-assisted methods (UAE), refined carrageenan (RC) extraction with NaOH, sample TR-B exhibited the highest similarity to both iota and kappa-carrageenan (44.73 and 53.54 %, respectively) and TR-C3.2 the lowest (10.81 and 13.00 %, respectively).

In ultrasound-assisted semi-refined carrageenan (SRC) extracted at 45 ºC, sample TR-B exhibited the highest similarity to both iota and kappa-carrageenan (58.81 and 70.22 %, respectively) and G-Q2J the lowest (19.39 and 23.23 %, respectively). In ultrasound-assisted semi-refined carrageenan (SRC) extracted at 70 ºC, sample TR-B exhibited the highest similarity to both iota and kappa-carrageenan (50.54 and 60.58 %, respectively) and TR-C3.2 the lowest (5.70 and 6.80 %, respectively).

Finally, in supercritical fluid extracted (SFE) carrageenan, sample TR-C21.2 exhibited the highest similarity to both iota and kappa-carrageenan (54.41 and 64.85 %, respectively) and TR-C4.2 the lowest (18.39 and 22.05 %, respectively).

Table 20. Similarity (%) of ground seaweed (GS) samples, native (NE), supercritical fluid (SFE), conventional (CE: RC and CE: SRC) and ultrasound-assisted (UAE: RC, 120 W, 70 °C, 1h; UAE: SRC, 120 W, 45 °C, 1 h; UAE: SRC, 120 W, 70 °C, 1 h) carrageenan extracted from *Kappaphycus alvarezii* samples.

Similarity (%)																
	DS		NE		SFE		CE (RC)		CE (SRC)		UAE (RC)		UAE(SRC45)		UAE (SRC)	
Sample	*iota*	*kappa*	*iota*	*kappa*	*iota*	*kappa*	*iota*	*kappa*	*iota*	*kappa*	*iota*	*kappa*	*iota*	*kappa*	*iota*	*kappa*
G-N11	29.87	35.65	29.87	35.70	41.07	49.01	40.96	49.13	51.11	61.44	34.55	41.75	22.47	26.92	35.47	42.54
G-N14	21.33	25.41	21.33	23.90	26.31	31.34	63.03	75.24	21.33	25.55	30.39	36.90	30.66	36.75	38.51	46.27
G-Q2M	30.09	36.11	30.09	31.00	33.51	39.94	33.65	40.20	76.55	91.56	36.45	43.50	21.50	25.79	22.09	26.57
G-Q2J	31.01	36.96	31.01	41.52	25.30	30.16	36.99	44.29	49.94	59.89	28.36	33.90	19.39	23.23	44.02	52.88
TR-C3.2	14.75	17.57	14.75	25.12	35.18	41.96	83.79	100.09	39.65	47.41	10.81	13.00	41.46	49.69	5.70	6.80
TR-C4.2	50.47	60.13	50.47	20.63	18.39	22.05	36.95	44.32	54.83	65.80	15.32	18.47	45.26	54.40	34.91	41.78
TR-C18	50.65	60.15	50.65	26.06	36.15	43.18	58.53	69.93	61.90	75.02	16.04	19.27	20.58	24.66	24.32	29.22
TR-C21.2	27.92	33.54	27.92	27.83	54.41	64.85	67.95	81.44	41.56	49.80	29.20	34.89	25.86	30.98	34.75	41.65
TR-B	62.83	74.66	33.85	40.32	27.93	33.33	28.52	34.26	43.74	52.69	44.73	53.54	58.81	70.22	50.54	60.58

4. Discussion

4. Discussion

4.1. Novel extraction methods, yield, structural and rheological properties of κ-carrageenan from a novel haploid *Kappaphycus alvarezii* strain from the Philippines

The obtained yield of native extraction is slightly higher compared to those reported in most published studies (Table 21) using different cultivars (Hilliou et al. 2006; Hayashi et al. 2007; Hung et al. 2009; Bui et al., 2019). Polysaccharide content and quality in seaweeds vary greatly with season and are influenced by many different biotic and abiotic factors (Lee et al., 2017b; Usman et al., 2017; Véliz et al., 2017), and although there are many reports regarding carrageenan yields of *K. alvarezii*, quantitative and qualitative comparisons are difficult since they vary depending on the extraction method and the strain or cultivar of the species. When comparable extraction conditions are performed, the observed higher yield may be due to the higher carrageenan content of the strain.

The CE (NaOH) extraction method presented close but lower values (35.67 ± 1.89%) than reported, and CE (KOH) had higher values (77.33 ± 2.49%). It is important to note that this higher yield measured in CE (KOH) treatment can be because residual cellulose from the cell walls remained after washing, which only eliminates residual minerals, proteins, and lipids.

According to a study conducted by Rafiquzzaman et al., (2016) on carrageenan extracted from *Hypnea musciformis*, the yield was higher using the aqueous UAE (500 W, 20 min.) method compared to the aqueous-extracted conventional method (native extraction). The present work achieved, comparing the conventional methods with the ultrasound-assisted extraction, CE (NaOH) (35.67 ± 1.89) vs. UAE (NaOH) (33.73 ± 10.52) and CE (KOH) (77.33 ± 2.49) *vs*. UAE (KOH) (76.70 ± 1.44), the ultrasound-assisted method showed slightly lower extraction yields than conventional extraction; however, these were performed in a shorter time (one-third of the time in NaOH solution and half in KOH) and at a lower temperature. Such results can be attributed to the effective disruption of cell walls, reduction in particle size and increase in the mass transfer to the cell contents (Rafiquzzaman et al., 2016). Looking at the two performed ultrasound-assisted extraction methods using KOH, UAE (KOH45) (63.20 ± 3.23), the yield was lower than UAE (KOH) (76.70 ± 1.44); however, it was used at almost half of

the temperature of extraction (45 °C). A less abrupt reduction in the yield may be due to lower possible degradation of the polysaccharide due to temperature.

SFE carrageenan extraction (53.40 ± 1.80%) was lower than that reported by Gereniu et al. (2018) but still valuable and higher than CE (NaOH) and UAE (NaOH).

Table 21. Yield (%) of carrageenan extracted from *Kappaphycus alvarezii* of different localities using different methods.

Extraction conditions	Yield (%)	Locality	Reference
Aqueous (Native)	40-50	Cam Ranh Bay, Vietnam	(Bui et al., 2019)
Aqueous (SFE)	71	Wagina, Solomon Islands	(Gereniu et al., 2018)
Alkali (NaOH)	48	Palk Bay, India	(Mishra et al., 2006)
Alkali (KOH)	52	Palk Bay, India	(Mishra et al., 2006)
Alkali (4% KOH)	53.2	Philippines (commercial sample)	(Ohnol et al., 1996)
Alkali (6% KOH)	54.6	Philippines (commercial sample)	(Ohnol et al., 1996)
Alkali (8% KOH)	53.7	Philippines (commercial sample)	(Ohnol et al., 1996)
Aqueous UAE	50-55	BIS Algoculture (Madagascar)	(Youssouf et al., 2017)

The biochemical analyses performed on extracted carrageenan differentiate its composition and quality from those of the whole seaweed biomass. There are several studies on the biochemical composition of dried biomass of different *K. alvarezii* strains; however, there are very few studies on the polysaccharide itself. The FAME composition and content of carrageenan extracted through the different methods were determined to understand to what extent the extraction method was able to remove these compounds. A total of three fatty acids were detected: two saturated fatty acids (SFAs) and one monounsaturated fatty acid (MUFA). These results, as expected, are lower than those reported for the raw biomass of *K. alvarezii* in previous studies but present the same proportion as previous results (Adharini et al., 2019; Illijas et al., 2023; Matanjun et al., 2010; Yong et al., 2015), with saturated fatty acids (SFAs) presenting higher contents, especially palmitic acid (C16:0) and stearic acid (C18:0), followed by monounsaturated fatty acid (MUFA) oleic acid (C18:1). From all extraction methods, CE (NaOH)

extraction presented the highest fatty acid content, followed by UAE (KOH), UAE (KOH45) and NE. These values were even lower if before extraction, the ground dry material was pretreated with a mixture of solvents (such as acetone and methanol); however, in the present work, this was not applied, and the values are still acceptable for food approval.

The present work is one of the few publications reporting the carbohydrate composition of carrageenan from *K. alvarezii*. The obtained results were compared to those of previous studies, such as Rhein-Knudsen et al., (2017), which evaluated the monosaccharide composition of native and alkali-extracted carrageenan of *K. alvarezii* from Nha Trang, Vietnam, by HPAEC-PAD analysis. The values obtained in their study presented higher contents of galactose (68-70%) and glucose (3-6%), and other monosaccharides were detected at lower contents (2%), namely, fucose, xylose and mannose. In another study conducted by Meinita et al. (2012), the total carbohydrate content of raw seaweed *K. alvarezii* was obtained from various places in Indonesia, and the values were even higher, ranging from 35 to 78% by weight. These variations seem to be related to the type of extraction method performed, geographic origin, strain/cultivar and harvest time (Usman et al., 2017; Véliz et al., 2017).

No previous studies determined the uronic acid content in carrageenan extracted from *K. alvarezii*; however, in comparison to analyses preformed in dried seaweed biomass of another red seaweed species, namely *Asparagopsis armata*, *Calliblepharis jubata*, *Chondracanthus teedei* var. l*usitanicus* and *Grateloupia turuturu*, the values are very low (Pacheco et al., 2021).

In general, the alkali extractions performed with KOH showed lower values in galactose, possibly due to the benefit of 3,6-anhydrogalactose. This corresponds to the conversion of the 4-linked galactose-6-sulphate in native samples to anhydro-galactose in the alkali-extracted carrageenans. Thus, the biological precursors mu and nu carrageenan were converted into kappa and iota-carrageenan, respectively (Pereira and Mesquita 2004).

The protein content in all samples presented very low values, similar to commercial carrageenans (Chan et al., 2013), meeting the Food and Agriculture Organization of the United Nations (FAO) quality criteria, which requires values lower than 2%.

Regarding measured physico-chemical parameters, in addition to protein content, carrageenan pH and viscosity are also monitored and regulated parameters used to assess

the quality of the produced polysaccharide, and it must be greater than 5 cP to meet FAO quality criteria. The results from the present work show that, regarding pH criteria, the extracted carrageenan from the NE and SFE methods are not suitable for food applications due to pH < 8. The viscosity varied greatly (7.8 to 658.7 cP). This can be due to the different independent variables, e.g., (1) extraction time, (2) alkali treatment, (3) type of alkali (NaOH or KOH), (4) extraction method (NE, UAE or SFE), and (5) temperature.

SFE showed the lowest viscosity value (7.8), possibly due to irregularities in the chain, as reported by Whistler and Be Miller (1997) and Rao (1999), caused by the high temperature and pressure, causing carrageenan to lose its properties. Higher viscosity values are usually attributed to carrageenan obtained by extraction methods using an alkali solution of KOH. Bono et al. (2014) investigated the influence of process conditions on the viscosity of conventional alkali KOH-extracted carrageenan from *K. alvarezii* and determined the optimal conditions to be 80 °C for 30 min and a 10% w/w KOH solution, which resulted in a gel viscosity of 1291.84 cP. In the present work, UAE (KOH) revealed the highest value of viscosity (658.7 cP at 1%). Comparing UAE (KOH) with UAE (KOH45), these higher values may be due to temperature, since it is the only parameter varying between them. The high viscosity value can also mean higher sulphate levels are present. According to Astuti et al., (2017), the viscosity of carrageenan can be influenced by levels and is directly proportional to the content of sulphate. A higher sulphate content resulted in a higher viscosity, which is due to the ability of the sulphate group in carrageenan to exert a repulsion force between negative charges along the polymer chain, and as a result, the molecular chain stiffens so that the viscosity increases (Montoro et al., 2019).

No literature regarding EC and TDS values from carrageenan solutions of *K. alvarezii* was found; however, in comparison to the commercial carrageenans used as standards, all values were higher, especially in UAE (NaOH), CE (NaOH), CE (KOH) and UAE (KOH45), possibly indicating high levels of charged ions and substances not soluble or precipitated.

Among the UV–VIS spectra of different carrageenan solutions, only native extracted carrageenan absorbed part of the UV-A radiation (320–400 nm). As previously reported, red seaweeds accumulate photoprotective compounds with ultraviolet radiation absorption capabilities, such as mycosporine-like amino acids (MAAs), which absorb in this specific UV region (Orfanoudaki et al., 2019). The UV absorption spectrum with prominent peaks between 320-340 nm is in accordance with the presence of MAAs

absorbing in this range (Castejón et al., 2021). This finding reveals a possible application of the novel strain in the pharmaceutical and cosmetic industries for UV screen protection. NE, CE (KOH), UAE (KOH; 45 °C), and UAE (NaOH) extracts at the preform dilution showed a peak between 200-270 nm, which corresponds to polysaccharides bound covalently with aromatic compounds (Jesumani et al., 2020). Additionally, these results partially correlate with the protein content. The lack of pigment detected in the UV–VIS supports the purity of the extracted carrageenan and reinforces the lack of a depigmentation step in the extraction processes of this seaweed strain.

The FTIR-ATR spectra of extracted carrageenan from the *K. alvarezii* strain using various methods revealed the presence of kappa-carrageenan and iota-carrageenan, especially in alkali-extracted CE (NaOH) and UAE (NaOH) (Fig. 8h, 8i, 8f and 8g, respectively), indicating that the novel strain presents a hybrid kappa/iota carrageenan, as checked in previous studies in several other *K. alvarezii* strains (Van De Velde et al., 2005). This is easily understood, especially in the iota/kappa ratio (Table 5), with CE (NaOH) and UAE (NaOH) presenting a lower value (0.65), meaning a lower content in iota-carrageenan, while UAE (KOH45) presented the highest (0.79), and there was a higher content in iota-carrageenan. Bands at 970 - 975 cm^{-1} regions identified as galactose (G), especially in CE (NaOH), NE and SFE extractions (Fig. 8f, 8h and 8i, respectively), and showing more absorption in comparison to the rest of the samples corroborate the results obtained in its quantification.

Similarity values provide valuable insights regarding carrageenan composition; however, it is important to consider that similarity is relative to all spectra. The fact that sample G-N7 presents hybrid carrageenan is enhanced, and from the values higher in kappa carrageenan we would assume higher content, however, being the similarity regarding all spectra this can be due to other peaks, less determinant, and iota carrageenan still shows relatively high values.

4.2. Carrageenan yield and quality of hatchery-cultivated novel strains of different ploidy of *Kappaphycus alvarezii*

There are many reports regarding carrageenan yields of *K. alvarezii*, however, the quantitative and qualitative comparisons are difficult, since polysaccharide content and composition in seaweeds vary greatly with seasons and are influenced by many different biotic and abiotic factors, resulting in different types of strain or cultivar. Besides these

factors, they also vary greatly depending on the extraction method and chemicals used. Concerning yield and associated carrageenan viscosity through each extraction method, in native carrageenan extraction (NE), sample G-Q2M showed the highest yield (43.43±4.07%), similar to the reported in the previous study in the novel haploid sample G-N7 (45.47±1.92%) (**section 3.1.**). These yields obtained from native extraction are similar to those reported in previous studies (35-42%) concerning different cultivars (Hilliou et al. 2006; Hayashi et al. 2007; Hung et al. 2009; Bui et al., 2019). Viscosity values measured at 30 ºC, ranged from 10.8 (TR-C18) to 811.1 cP (G-N11).

Carrageenan yield of alkali treated *Kappaphycus* commonly is > 30% (Hayashi et al., 2007; Luhan et al., 2022; Mishra et al., 2006; Wakibia et al., 2006). In conventional refined carrageenan (RC) extraction with NaOH (CE (RC)), the highest value was presented by sample G-N14, with an overall lower carrageenan yield than native carrageenan yields, in accordance to previously published literature (for example, Periyasamy et al., (2014), for cultivated *K. alvarezii* (Doty) on Tamil Nadu). This is to be expected due to the partial degradation of the polysaccharide with the alkali treatment. Viscosity values were also lower, ranging from 9.3 (TR-C21.2) to 28.2 cP (TR-C3.2).

Conventional semi-refined carrageenan (SRC) extraction with KOH (CE (SRC) yield values are higher than those reported also for *K. alvarezii* cultivars, with sample G-N11 presenting the highest yield (84.67±0.47%). Yields of 41.16% have been reported by (de Góes et al., 2012) in Brazil, 45% in Indonesia and 54.5% in the Philippines by Ohno et al., (1996), 57% by Hayashi et al., (2007) and 42.42 to 58.36% in India reported by Subba Rao et al., (2008), although extraction conditions in these studies vary. In comparison to conventional refined (RC) extraction of the present study, the values were also substantially higher. This can be because residual cellulose from the cell walls remains after washing process, which only eliminates residual minerals, proteins, and lipids. However, the washing step in this study can be considered efficient, since the previous study (**section 3.1.**) shown the presence of just residual values of lipids and protein in the extracted carrageenan from a novel strain G-N7 (gametophyte). Viscosity values were higher than any other of the extraction methods, however, viscosity of carrageenan extracted through this method could be as high as 1196 cP (Ohno et al., 1996) or as low as 23 cP (Hurtado-Ponce, 1995) in *K. alvarezii*. The viscosity of SRC carrageenan extracted from experimental tetrasporophyte (83.8 cP) produced in a study conducted by Luhan et al., (2022) was twice that of carrageenan from the gametophyte (45.3 cP) tissue, which in this case we can't say that there is a correlation of this variation

to the life cycle, since values vary randomly. These values can be due to higher sulphate levels present. According to Astuti et al., (2017) the viscosity of carrageenan can be influenced by sulphate levels and is directly proportional to the content of sulphate. A higher sulphate content resulted in a higher viscosity, which is due to the ability of the sulphate group in carrageenan to exert a repulsion force between negative charges along the polymer chain and as a result, the molecular chain stiffens so that the viscosity increases (Montoro et al., 2019).

UAE was performed for both alkali solutions (NaOH for RC and KOH for SRC) at a temperature of 70 °C for 1h. UAE (SRC) showed more promising results compared to UAE (RC), and because of that another UAE (SRC) extraction was performed at a lower temperature (45 °C) for 1h to evaluate its influence in the efficiency and type of extract obtained.

Regarding ultrasound-assisted methods (UAE), refined carrageenan (RC) extraction with NaOH [UAE (RC)], compared with the conventional method, revealed some samples with statistically significant higher yields (sample G-Q2M with the highest value). Considering that UAE used 1/3 (1 h) of the time and lower temperature (70 °C), these values can be attributed to disruption of cell walls, reduction in particle size and increase in the mass transfer to the cell contents (Wang et al. 2009).

On the other hand, ultrasound-assisted method of semi-refined carrageenan extraction with KOH [UAE (SRC)], compared with the conventional method SRC, reveals overall higher values, with half of the time used for CE (SRC) and lower temperature, however, is associated to lower viscosity. Sample G-N14 revealed the highest yield.

Comparing UAE (SRC45) to UAE (SRC), yield was only slightly lower, however it was used almost half of the temperature of extraction (45 °C). A not so abrupt reduction of the yield may be due to lower possible degradation of the polysaccharide since lower temperature was used. In UAE (SRC45) highest yield was obtained in sample G-Q2J.

In supercritical fluid extraction (SFE) sample G-Q2M showed yields similar to native extraction. On the other hand, the viscosity was low explaining that carrageenan exposed to high temperature and pressure loses its properties. This might be due to irregularities in the chain (Misra et al., 2015).

Regarding pH, commercial carrageenans used (control) and overall treatments showed alkali pH, except for NE and SFE that reveled acidic pH.

Comparing all results yield of carrageenan extracted from *K. alvarezii* samples don't seem affected by its life history phase, as observed by Hayashi et al., (2007) in Brazil and by Luhan et al., (2022) in Philippines, but by its extraction method. However, looking at Table 22 which gather the samples that exhibited the highest extraction methods in each method, and respective physicochemical properties, all of them are gametophytes.

According to European Food Safety Authority (EFSA) criteria regarding pH and viscosity for food approval, carrageenan native extracted and SFE, although showing viscosity values ≥ 5 cP, it does not meet the pH range values (8–11), limiting its applications. Samples obtained from the remaining preformed extractions reveal potential, especially CE (SRC) and UAE (SRC) due to high yields as previously shown in **section 3.1.**.

Table 22. *K. alvarezii* strains that exhibited the highest yield in each method, and respective physico-chemical properties.

Extraction method	Sample	Yield (%)	Viscosity (cP)	pH	EC (µS cm^{-1})	TDS (ppm's)
NE	G-Q2M	43.43±4.07	28.5	6.65	3686	1837
CE (RC)	G-N14	37.33±2.49	23.4	10.43	3697	1813
CE (SRC)	G-N11	84.67±0.47	18.3	10.77	2603	1300
UAE (RC)	G-Q2M	50.93±7.80	6.9	10.28	>3999	>2000
UAE (SRC45)	G-N14	66.70±13.58	17.4	10.76	2852	1419
UAE (SRC)	G-Q2J	73.53±2.29	15.3	10.65	3186	1633
SFE	G-N14	43.12±2.29	24.9	6.12	2766	1388

The studied strains differ in the spectral profiles suggesting, as expected, that the composition and the UV-absorbance potential vary between them, and that the type of extraction technique have a significant effect (Annex III). Most sunscreen products protect the skin by absorbing UV radiation before it reaches the skin. When human skin is overexposed to UV light (between 320-400 nm), also known as UVA and UVB, it results in suntan, wrinkles, and hyperpigmentation. The obtention and analyses of spectrophotometric profile of extracted carrageenans allowed to demonstrate the absorption ability of carrageenan solutions at the key wavelengths of UVA and UVB.

The peaks present between 200-270 nm possibly correspond to polysaccharide bound covalently with aromatic compounds, since same kind of result was observed with the polysaccharide from a brown seaweed (*Saccharina japonica*) (Saravana et al., 2016).

The absorption peaks at 342 between 266-374 nm determines the presence of phenolic compounds (Ray et al., 2013) which are known for their potential role in both UV photoprotection and ROS scavenging (Zerrifi et al., 2018).

Red algae are also known to accumulate photoprotective compounds with ultraviolet radiation absorption capabilities such as mycosporine-like amino acids (MAAs), which absorb in UV-A region (320 – 400 nm) (Orfanoudaki et al., 2019; Pangestuti et al., 2018). In a study conducted by Castejón et al., (2021) where water extraction was preformed to a red seaweed *Palmaria palmata*, the absorption maximum peaks were detected at 325 to 330 nm, and also in previous other studies (Nishida et al., 2020). Therefore, it is possible to assume that the peaks observed between 320 and 340 nm in native (samples G-N11, G-Q2M, TR-C4.2, TR-C21.2 and TR-B), UAE (SRC) (samples G-N14 and TR-C18), UAE (SRC45) (samples G-N14, TR-C18 and TR-B) and SFE (sample TR-B) extracted carrageenan may be due to the presence of MAAs.

The peaks present between 200-270 nm that possibly correspond to polysaccharide bound covalently with aromatic compounds, observed in all treatments, give clues about purity of the extracted carrageenan regarding protein content at analyzed concentration. Taking this in mind, conventional extraction may present lower protein content, however protein quantification is needed to affirm this. In section 3.1., where protein quantification of the extracted carrageenans from sample G-N7 was performed, protein values were overall low, with UAE (RC) presenting the higher content (0.04%).

In this study commercial standards of kappa- and iota carrageenan from Thermo Fisher Scientific (Waltham, MA, USA) were used as controls. Manufacturing method for this commercial carrageenan samples was not specified by the manufacturer and no previous studies recorded/reported this specific brand ATR-FITR spectra, with main studies using Sigma commercial samples. Thus, this work is one of the few providing information regarding its chemical structure.

As we have seen, the characterization of the natural polysaccharide composition can be made by direct analysis of dried seaweed powder by FTIR-ATR (Pereira and Mesquita, 2004) or by the analysis of the "total extracted", "aqueous extracted" or “native extracted” phycocolloids, taking into account that in these type of extractions there is no alkaline transformation of the biological precursors (mu and nu) of gelling carrageenans (kappa and iota) that contain one sulphate-ester group on the 6 position of the unit α-D-galactose 4, producing a type of structure that reduces the ability of carrageenan to form a gel.

FTIR-ATR spectra of ground *K. alvarezii* samples and native carrageenan present absorption bands at 930 cm^{-1} (**DA**), 845 cm^{-1} (**G4S**) and 805 cm^{-1} region (**DA2S**), typical of a hybrid kappa/iota-carrageenan. Since it is a spectrum of a native carrageenan, obtained by the method of total extraction, some additional peaks are visible at 820 cm^{-1} (G/D6S), 825 cm^{-1} (G/D2S) and 867 cm^{-1} (G/D6S), corresponding to the presence of the precursors of carrageenan kappa (carrageenan mu) and carrageenan iota (carrageenan nu). However, the peaks 825 cm^{-1} and 867 cm^{-1} are usually more visible in FT-Raman spectra than in FTIR-ATR.

Ground seaweed presented a higher iota/kappa ratio than native carrageenan. Native carrageenan ratio ranged from 0.63 (TR-B) to 0.69 (G-N14). These variations seem related to the duration of the seaweed cultivation and the amount of precursor (mu) present at the time of harvest of *K. alvarezii* (Pereira 2004); one of the examples that may corroborate this theory is *K. alvarezii* G-Q2 samples, one harvested in May (G-Q2M) and other in June (G-Q2J), showing differences in this ratio possibly due to this, however to confirm this, a detailed study of cultivation duration and presence of carrageenan forms is required. It was interesting to note that, except for ground seaweed, the iota fraction was higher in a longer cultivation period (sample G-Q2J). This tendency has been previously referred by Hayashi et al., (2007). As carrageenans serve a structural function analogous to that of cellulose in land plants one can hypothesize that the older tissues could produce more iota carrageenan, markedly elastic, which would preserve possible breaks of the thalli because of biomass increase.

Through alkali-treatment as explained in section 1.4.1 most of the 6-sulphated units are converted into the corresponding 3,6-anhydrous. The alkaline extraction methods (both conventional and ultrasound-assisted methods) enabled the extraction of carrageenans and promoted the cyclization of precursors. However, in some cases and samples, it is possible to find carrageenans extracted under these conditions, still with a small percentage of biological precursors.

The conventional extracted RC carrageenan showed slightly lower intensity of galactose, this can be due to the conversion of the 4-linked galactose-6-sulphate in native samples to anhydro-galactose in the alkali extracted carrageenans. The broad and slightly higher shoulder present at 930 cm^{-1} in the spectra is referred by many authors (Fournet et al., 1999; Pereira et al., 2003, 2009) to be a characteristic signal of the presence of 3,6-anhydrogalactose, by the existence of the C-O linkage from 3,6- anhydro-galactose molecule (reduced when precursors, and also contributed by galactose 4- sulphate). The

overall increase in the ratio 805/845, although not very significant and not seen in all *K. alvarezii* strains (sample G-N14 with lower ratio and TR-C3.2 with equal ratio) in the all the alkali-extracted carrageenan samples, compared to native carrageenans, corresponded to an increase of the iota fraction relative to kappa fraction. The somewhat lower extraction yields in conventional extracted RC are possibly due to alkaline destruction of galactans; also, the low-molecular products cannot be precipitated by alcohol (do not constitute yield).

The FTIR-ATR spectra of conventionally extracted SRC carrageenan is different from the conventionally extracted RC, showing slightly visible band on 805 cm^{-1} region (**DA2S**), broader bands between 950-1100 cm^{-1} and at 1240 cm^{-1} for sulphate esters. An increment in the iota/kappa ratio was verified in comparison to native carrageenan and alkali extracted (NaOH) carrageenan, corresponding to an increment of the iota fraction relatively to kappa fraction. This explains in part the observed high values of viscosity, since iota carrageenan presents more sulfation, and higher sulfation corresponds to higher viscosity (Astuti et al., 2017; Montoro et al., 2019).

Ultrasound-assisted extracted refined carrageenan (RC) compared to conventional extraction reveled overall less iota fraction (decrease in the ratio 805/845) except for samples G-Q2M and TR-B (equal ratios).

Ultrasound-assisted extracted semi-refined carrageenan (SRC) and (SRC45) revealed very similar spectra, with some samples of UAE (SRC) revealing higher kappa fraction (higher peaks in the 845 cm^{-1} region), except for G-N14 and G-Q2J (lower ratio iota/kappa).

Similarity values bring valuable insights regarding carrageenan composition, however, is important to consider that similarity is relative to all spectra. Commercial kappa-carrageenan sample revealed in all sample's higher similarity than iota-carrageenan. However, iota-carrageenan still showed relatively high values. In the table below are gathered the samples with highest similarity in each extraction method.

Table 23. *K. alvarezii* strains that exhibited the highest similarity to standard carrageenan in each method.

Extraction method	Sample	Iota (%)	Kappa (%)
GS	TR-B	62.83	74.66
NE	TR-C18	50.65	-
NE	G-Q2J	-	41.52
CE (RC)	TR-C3.2	83.79	100.09
CE (SRC)	G-Q2M	76.55	91.56
UAE (RC)	TR-B	44.73	53.54
UAE (SRC45)	TR-B	58.81	70.22
UAE (SRC)	TR-B	50.54	60.58
SFE	TR-C21.2	54.41	64.85

Looking at native carrageenan similarity to controls, it was as expected, lower, just like iota/kappa ratio than the other extraction methods (except from SFE). This can be explained by a higher amount of carrageenan precursors. The fact that all strains present hybrid carrageenan is enhanced, and from the values higher in kappa carrageenan we would assume higher purity, however, being the similarity regarding all spectra this can be due to other peaks, less determinant, and iota carrageenan still shows relatively high values.

5. Conclusion

5. Conclusion

In this study, novel strains of *K. alvarezii* were assessed for their potential as new source for carrageenan production. The assessments were based on comparison of yields, chemical composition, and characteristics of the extracted carrageenan with commercial carrageenan samples. We were able to address the impact off each extraction method and identify which is the best one for each strain.

Rheological characteristics were also compared with standard carrageenan. The novel extraction technology adopted in this study, namely ultrasound-assisted extraction using alkaline treatment with KOH (8%) shows very satisfactory yield for carrageenan (%) requires half of the time and lower temperature that the conventional extraction. Moreover, the composition analysis of the extracted carrageenan indicated successful modification during alkali-treatment, which was supported by FTIR analysis and oscillatory rheological measurements.

Is interesting to understand the impact that different extraction methods have in the type of extracted carrageenan. This puts in perspective on how efficient industries are, and of the current applying methods should be adapted according to the used biomass (carrageenan source).

Here, strain G-Q2J presents as a new, high yield source of hybrid iota/kappa semi-refined carrageenan (SRC) thought the novel ultrasound-assisted extraction method using alkali solution of KOH (8%).

Besides, the techniques used for the characterization, namely UV spectroscopy, revealed as potential future complementary techniques for carrageenan quality and safety assessment.

6. Future perspectives

6. Future perspectives

Although the general steps in the extraction process are known, the extraction variables do differ as seaweeds differ in their composition and conditions and stage of growth. Therefore, alkali treatment of each strain must be developed and variables like temperature, alkali concentration, and extraction time must be optimized to induce as much de-sulfation as possible, while still avoiding the yield losses due to degradation and leaching caused by the treatment.

This work offers a preliminary characterization and quality assessment of new potential source of carrageenan, however for further application, many other analyses are required to be performed regarding composition (moisture, inorganic salts, protein, ash, acid insoluble matter, diluents, and standardizing agents), purity (microbial quality, heavy metals, volatiles) and carrageenan Quality (gel strength, Mw profile [Mw, Mn, PDI]).

Further analyses for carrageenan quantification of the iota and kappa fractions through nuclear magnetic resonance (NMR), in specific proton nuclear magnetic resonance spectroscopy (^{1}HNMR) are also needed to understand the best application for each strain.

Moreover, the ultraviolet radiation absorption capabilities detected in native carrageenan of all strains by the presence mycosporine-like amino acids (MAAs), are worth further analyses for possible cosmetic and cosmeceutical formulations in the near future.

7. References

7. References

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8. Annexes

8. Annexes

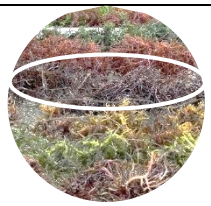
Annex I - Preliminary results

Preliminary results of native (NE) and conventional extractions (CE) preformed in all *K. alvarezii* strains samples.

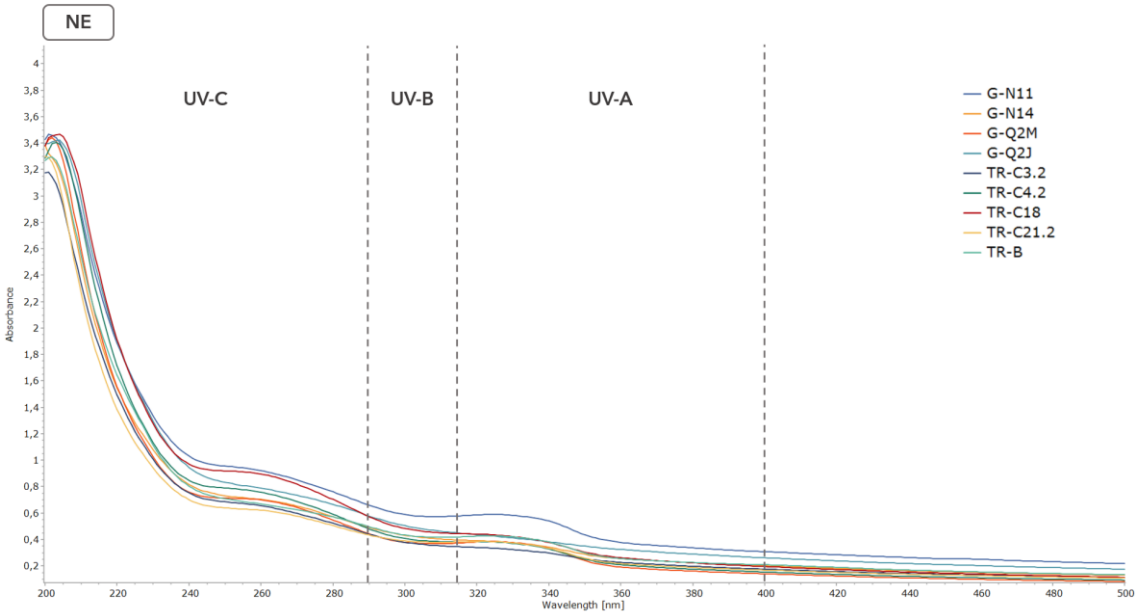
	Extraction method		
	NE	CE (RC)	CE (SRC)
Sample	Yield (%)	Yield (%)	Yield (%)
G-N7	48	37	77
G-N11	34	24	84
G-N14	37	38	56
G-Q2M	43	24	40
G-Q2J	43	28	49
TR-C3.2	35	34	55
TR-C4.2	38	28	42
TR-C18	37	16	39
TR-C21.1	42	34	43
TR-B	36	27	62

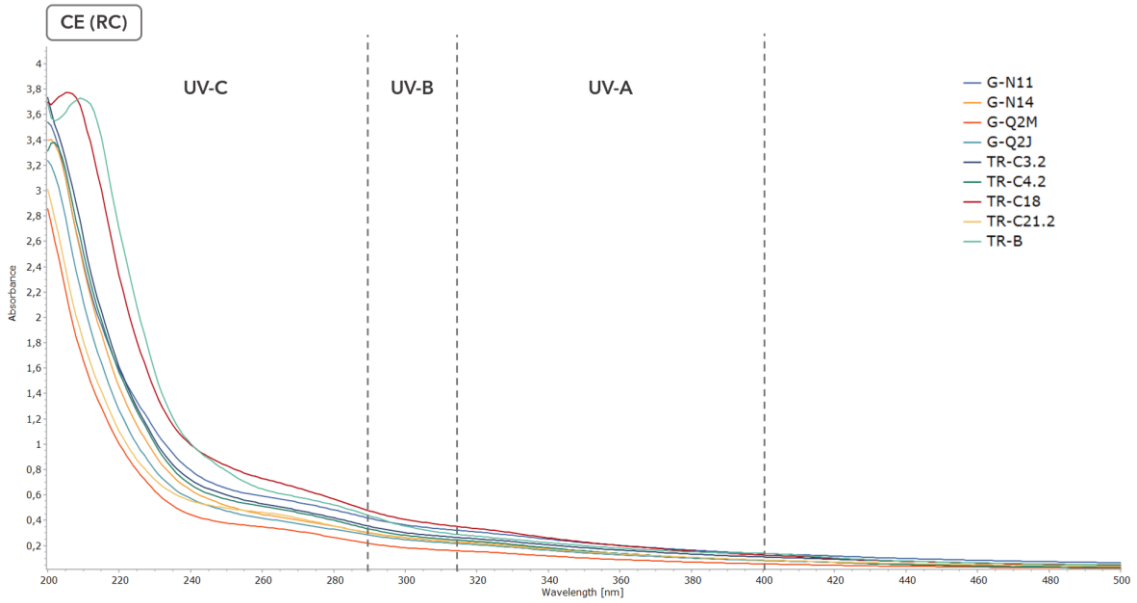
Annex II- *Kappaphycus alvarezii* novel strains information

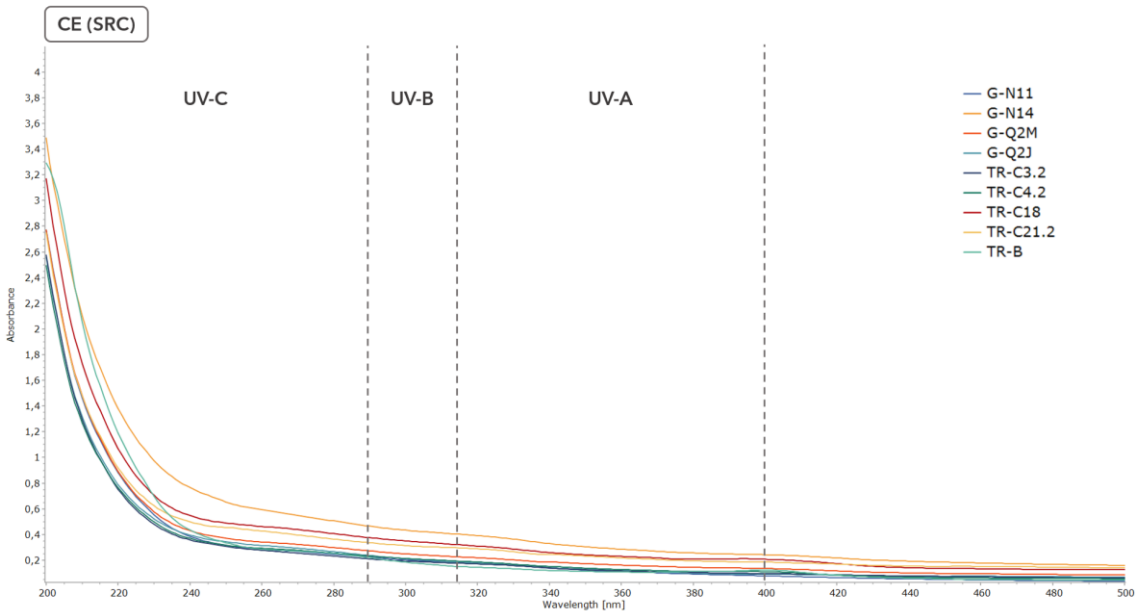
Sample	Harvest date	Image	Description
G-N7	27/08/2021		Parental plant: KaTR-N Gametophyte (n) Female
G-N11	13/08/2021		Parental plant: KaTR-N Gametophyte (n) Male
G-N14	13/07/2021		Parental plant: KaTR-N Gametophyte (n) Female
G-Q2	20/05/2021 15/06/2021		Parental plant: KaTR-Q Gametophyte (n) Male
TR-C3.2	13/05/2021		Parental plant: KaCR-C Tetrasporophyte (2n)
TR-C4.2	19/05/2021		Parental plant: KaCR-C Tetrasporophyte (2n)
TR-C18	13/03/2021		Parental plant: KaCR-C Tetrasporophyte (2n)
TR-C21.2	19/05/2021		Parental plant: KaCR-C Tetrasporophyte (2n)

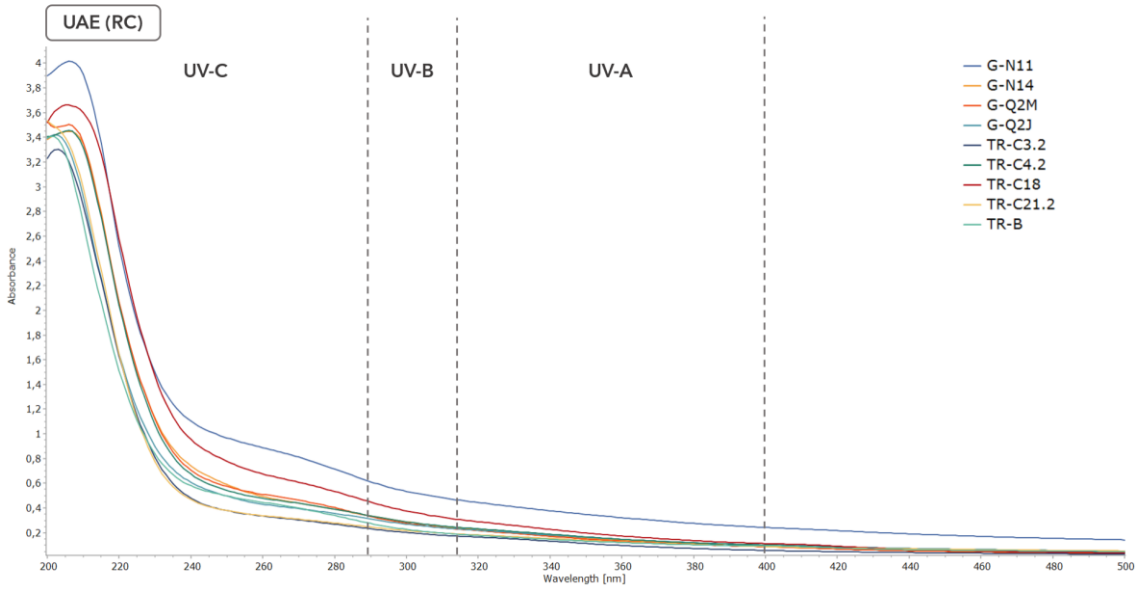
TR-B	-		Tetrasporophyte (2n) (Commercial sample from Brazil)

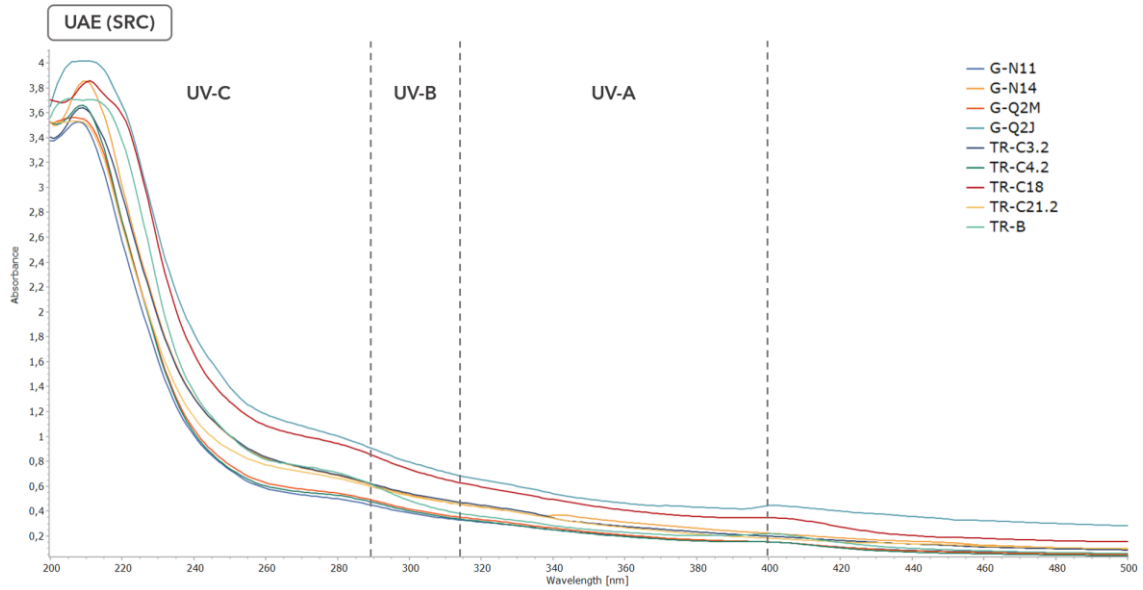
Annex III - UV absorption spectra of extracted carrageenan

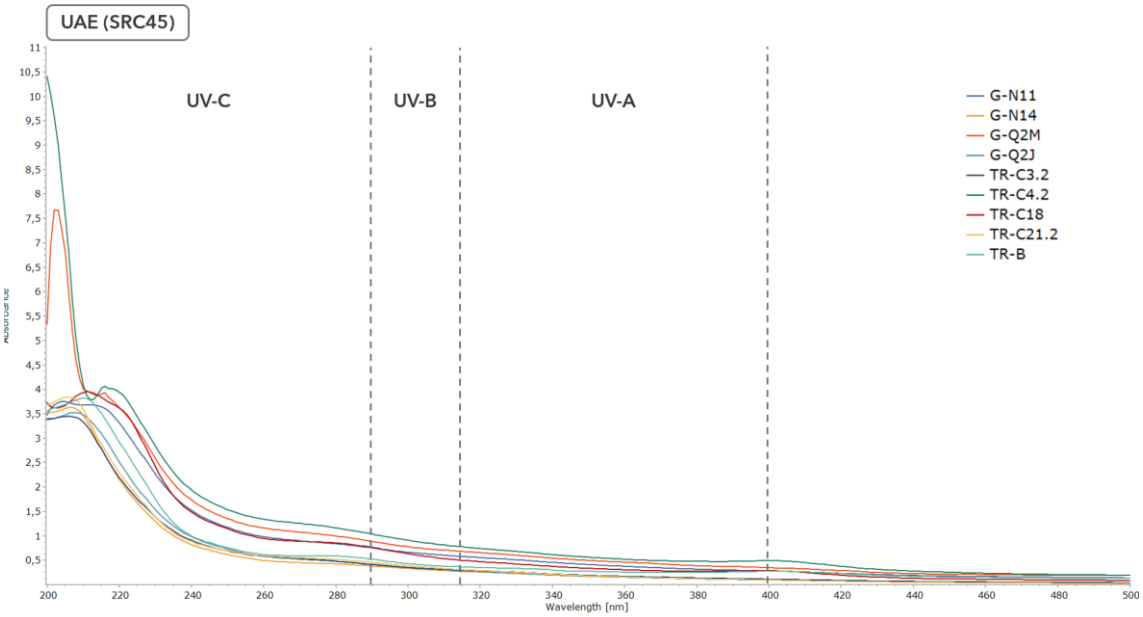


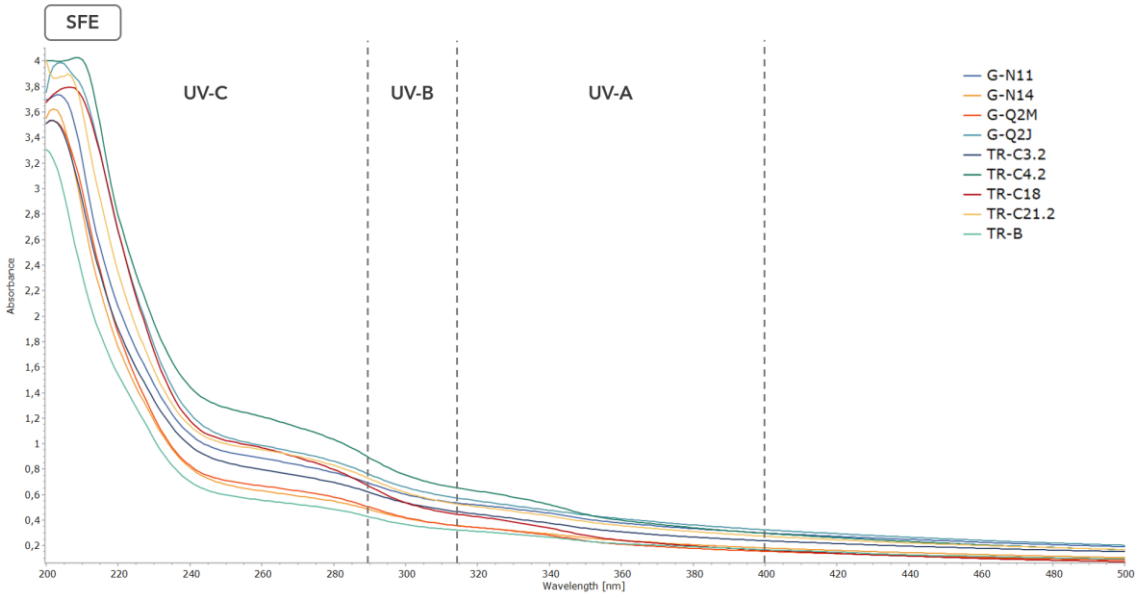



CE (SRC)
UV-C
UV-B
UV-A
G-N11
G-N14
G-Q2M
G-Q2J
TR-C3.2
TR-C4.2
TR-C18
TR-C21.2
TR-B
Absorbance
Wavelength [nm]


UAE (RC)
UV-C
UV-B
UV-A
G-N11
G-N14
G-Q2M
G-Q2J
TR-C3.2
TR-C4.2
TR-C18
TR-C21.2
TR-B
Absorbance
Wavelength [nm]


UAE (SRC)
UV-C
UV-B
UV-A
G-N11
G-N14
G-Q2M
G-Q2J
TR-C3.2
TR-C4.2
TR-C18
TR-C21.2
TR-B
Absorbance
Wavelength [nm]


UAE (SRC45)
UV-C
UV-B
UV-A
G-N11
G-N14
G-Q2M
G-Q2J
TR-C3.2
TR-C4.2
TR-C18
TR-C21.2
TR-B
Wavelength [nm]


SFE
UV-C
UV-B
UV-A
G-N11
G-N14
G-Q2M
G-Q2J
TR-C3.2
TR-C4.2
TR-C18
TR-C21.2
TR-B
Absorbance
Wavelength [nm]