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The biorefinery concept applied to the red macroalgae *Kappaphyccus alvarezii*: Green extraction technologies

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RESUMO

Kappaphycus alvarezii (KA) é uma macroalga amplamente difundida no mundo, e uma das principais fontes industriais de carragenana, polissacarídeo empregado em diferentes setores das indústrias alimentícia, farmacêutica e química. No entanto, além da carragenana, a biomassa KA contém diversas moléculas de alto valor agregado como compostos fenólicos e ácidos graxos que podem ser recuperados por diferentes métodos de extração, especialmente tecnologias de extração ambientalmente sustentáveis. Assim, este trabalho teve como objetivo a valorização desta macroalga por meio da aplicação do conceito de biorrefinaria usando técnicas de extração não convencionais. Nesse sentido, uma revisão de literatura foi elaborada com o objetivo de identificar as potencialidades da KA sob a luz do conceito de biorrefinaria. Em seguida, foram avaliadas algumas técnicas de extração realizadas de maneira direta, nomeadamente tais como avaliou-se a extração direta dos compostos: extração de ácidos graxos, compostos fenólicos, e carragenana por meio das técnicas: extração com fluido supercrítico do (inglês SFE), extração com líquido pressurizado (PLE), extração assistida por micro-ondas (MAE) e extração de carragenana a alta pressão (PWCE), realizadas individualmente. Estas técnicas foram comparadas com os métodos tradicionais de extração, mais especificamente a maceração e Soxhlet, em termos de rendimento, perfil químico dos extratos, obtido por cromatografia gasosa e líquida, conteúdo total de compostos fenólicos (TPC), atividade antioxidante pelos métodos de DPPH e FRAP, e também viscosidade e força de gel para a fração de carragenana. Além disso, foi avaliada a técnica de extração assistida por ultrassom (UAE), para a recuperação da carragenana, neste caso, foram avaliados os efeitos dos parâmetros de processo e pré-tratamentos no rendimento e na qualidade da carragenana (viscosidade e força do gel). Com base nas extrações individuais foi desenvolvida uma rota inédita de extração sequencial: (i) PLE a 40 ºC com etanol, seguido de (ii) PWCE a 80 ºC com água, selecionadas para recuperação de compostos fenólicos, carragenana, celulose e compostos de baixo peso molecular. Esta rota de extração sequencial a alta pressão foi comparada com a extração Soxhlet (etanol) seguida de maceração (água) para a obtenção das mesmas frações, representando uma rota tradicional. Além disso, a carragenana obtida pela rota sequencial foi utilizada para a produção de um aerogel, que foi caracterizado e comparado com o aerogel produzido com carragenana comercial. Os resultados obtidos demostraram que as amostras de PLE apresentaram maior potencial antioxidante e TPC, com perfil diversificado de compostos fenólicos. Além disso, a carragenana foi recuperada eficientemente por MAE e por PWCE, apresentando viscosidade e força de gel semelhantes às obtidas por meio de maceração em água. No ultrassom foi possível verificar como as variáveis de processo bem como os pré-tratamentos afetavam o rendimento e a qualidade da carragenana. Portanto, as técnicas alternativas de extração empregadas foram eficientes no fracionamento da biomassa KA, em comparação com técnicas tradicionais. A carragenana obtida pela extração sequencial apresentou valores de viscosidade e força do gel semelhantes aos da carragenana obtida por método convencional, e com composição química similar, exceto pela fração de glucana, encontrada apenas na carragenana comercial. O aerogel produzido com a carragenana obtida do processo sequencial a alta pressão apresentou também características próximas as da carragenana comercial. Neste sentido esta tese inovou trazendo o conceito de biorrefinaria utilizando técnicas verdes de extração para uma alga que até então seu principal foco sempre foi a carragenana. Esta pesquisa contribui para entender como uma técnica de extração afeta a etapa subsequente e, ainda, ela pode servir de base para auxiliar no desenvolvimento de plantas pilotos em solo brasileiro, que se for cultivada em mais estados pode abastecer o mercado interno com carragenana, bioestimulante, extrato fenólico, celulose e compostos de baixo peso molecular com atividade antioxidante.

Palavras-chave: Rhodophyta; economia circular; métodos de extração não convencionais; biopolímero; compostos bioativos.

ABSTRACT

Kappaphycus alvarezii (KA) is a macroalgae widespread throughout the world. It is one of the main industrial sources of carrageenan, a polysaccharide used in different food, pharmaceutical, and chemical sectors. However, besides carrageenan, KA biomass contains several high-value-added molecules, such as phenolic compounds and fatty acids, that can be recovered by different extraction methods, especially green extraction technologies. Thus, this work aimed to valorize this macroalgae through the concept of biorefinery using unconventional extraction techniques. To this end, a literature review was prepared to identify the potential of KA in light of the biorefinery concept. Then, the direct extraction of the compounds was evaluated: extraction of fatty acids, phenolic compounds, and carrageenan using the techniques supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and high-pressure carrageenan extraction (PWCE), carried out individually. These techniques were compared with traditional extraction methods, more specifically maceration, and Soxhlet, in terms of yield, the chemical profile of the extracts obtained by gas and liquid chromatography, total phenolic content (TPC), antioxidant activity by DPPH methods and FRAP, viscosity and gel strength of the carrageenan fraction. Furthermore, ultrasound was evaluated as a possibility for extracting carrageenan; in this case, process parameters and pre-treatments were evaluated, and how they affected the yield and quality of carrageenan (viscosity and gel strength). Based on individual extractions, an unprecedented sequential extraction route was developed: (i) PLE at 40 ºC with ethanol, followed by (ii) PWCE at 80 ºC with water, selected for the recovery of phenolic compounds, carrageenan, molecular weight, cellulose, and low-fat compounds. This high-pressure sequential extraction route was compared with Soxhlet (ethanol) and maceration (water) to obtain the same fractions, representing a traditional route. Furthermore, carrageenan from the sequential route was used to produce an aerogel, which was characterized and compared with the aerogel produced with commercial carrageenan. The results demonstrated that the PLE samples presented greater antioxidant potential and TPC, with a diverse profile of phenolic compounds. Furthermore, carrageenan was efficiently recovered by MAE and PWCE, presenting viscosity and gel strength similar to those obtained through maceration in water. Using ultrasound, it was possible to verify how process variables and pre-treatments affected the yield and quality of carrageenan. Therefore, the alternative extraction techniques used were more efficient in fractionating KA biomass than traditional techniques. Regarding sequential extraction, carrageenan presented viscosity and gel strength values similar to those obtained by conventional methods, and presented a similar chemical composition, except for the glucan fraction, found only at the commercial carrageenan. The aerogel produced with carrageenan obtained from the high-pressure sequential process also presented characteristics close to those of commercial carrageenan. In this sense, this thesis innovated by bringing the concept of biorefinery using green extraction techniques for algae that, until then, the focus had always been carrageenan. This research contributes to understanding how an extraction technique affects the subsequent stage. It can serve as a basis to assist in developing pilot plants on Brazilian lands, which, if cultivated in more states, can supply the domestic market with carrageenan, a biostimulant, phenolic extract, cellulose and low molecular weight compounds with antioxidant activity.

Keywords: Rhodophyta; circular economy; non-conventional extraction methods; biopolymer; bioactive compounds.

RESUMO EXPANDIDO

Introdução

A alga vermelha *Kappaphycus alvarezii* (KA), que apresenta rápido crescimento, tem sido cultivada em estados como São Paulo, Rio de Janeiro e Santa Catarina. Essa alga é a principal fonte de κ-carragenana, um polissacarídeo usado nas indústrias alimentícia, farmacêutica e cosmética. Além da carragenana, outros valiosos componentes podem ser extraídos desta alga, como compostos fenólicos, celulose, compostos de baixo peso molecular, bioestimulante e ácidos graxos. Métodos de extração não convencionais como extração com líquido pressurizado, extração assistida por micro-ondas, extração assistida por ultrassom e extração com fluido supercrítico têm sido foco de estudos recentes por frequentemente apresentarem alta seletividade na obtenção de compostos alvo. A carragenana, polissacarídeo encontrado na KA também tem sido amplamente investigado em formulações alimentícias, além de aplicações inovadoras, como em impressão 3D, biofilmes e cápsulas de medicamentos. Neste sentido, aerogéis à base de carragenana, por exemplo, são materiais leves, porosos e podem exibir alta capacidade de absorção de água e óleo.

Objetivo

Este trabalho teve por objetivo principal aplicar o conceito de biorrefinaria utilizando técnicas de extração verdes para recuperar diferentes frações da macroalga vermelha *Kappaphycus alvarezii* (KA).

Metodologia

A alga *Kappaphycus alvarezii* foi gentilmente cedida pela EPAGRI, lavada com água da torneira e seca com estufa de circulação de ar a 40 °C por 24 horas. Em seguida, moída e congelada para ser utilizada nas demais etapas do trabalho.

As extrações com fluido supercrítico foram realizadas em equipamento previamente descrito por Weinhold *et al.*, 2008), e conduzidas a 200 bar e 50 °C por 2 h a uma vazão de 1.2 kg/h usando 15 g de alga seca usando o procedimento descrito por Guindani *et al.*, (2016). A extração Soxhlet foi realizada conforme metodologia 920.39C da AOAC, (2005). Extração com líquido pressurizado foi realizado em equipamento descrito por Rodrigues et al., (2019) usando 5 g de amostra. Extração assistida por micro-ondas foi realizada seguindo os procedimentos de Mazzutti et al., (2017) com algumas modificações.

Os rendimentos de extração foram determinados considerando a razão entre a massa de extrato e a massa de matéria seca. TPC foi determinado como descrito por Koşar et al., (2005) com modificações, DPPH foi realizado conforme descrito por Brand-Williams et al., (1995). FRAP foi realizado como descrito por Benzie & Strain, (1996). As frações oleosas foram analisadas por cromatografia gasosa-espectrometria de massa conforme relatado por Rozas et al. (2019) o perfil de compostos fenólicos foi avaliado por HPLC usando a forma de preparação e a forma de identificação e quantificação conforme descrita por Schulz et al., (2015).

As extrações da carragenana foram realizadas conforme descrito por Boulho et al., (2017) para o método convencional, e conforme Mazzutti et al., (2017) para extração com micro-ondas, e de acordo com Gereniu, Saravana & Chun, (2018) para a extração a alta pressão sempre usando água destilada como solvente. As amostras de carragenana foram analisadas por FTIR para determinar o tipo de carragenana e comparar o perfil obtido pelas diferentes técnicas de extração, além de avaliar a viscosidade e força do gel conforme descrito por Bono, Anisuzzaman & Ding, (2014).

As variáveis de processo avaliadas para a técnica de extração assistida por ultrassom foram tempo, potência e temperatura, visando obter a melhor combinação de rendimento, viscosidade e força do gel da carragenana, determinados como citado anteriormente. A influência dos pré-tratamentos (secagem, uso de KOH e separação da fração “bioestimulante”) também foi observada para as mesmas respostas.

A biomassa seca de *Kappaphycus alvarezii* (KA) foi submetida a extrações sequenciais: Extrações com etanol para a recuperação de compostos fenólicos, realizadas por extração com líquido pressurizado (PLE) e Soxhlet (SOX). Os sólidos resultantes das extrações etanólicas foram empregados para a recuperação de carragenana utilizando água como solvente, pela técnica de alta-pressão (PWCE) e pelo método convencional (CCE). As frações recuperadas foram avaliadas conforme descrito anteriormente. Análises de cromatografia de permeação de gel foi empregada para avaliar a massa molar da carragenana, microscopia eletrônica de varredura para avaliar a estrutura da carragenana, e composição química para avaliar a separação das frações baseado no conteúdo de glucana e galactana.

As frações de carragenanas, avaliadas por viscosidade e cor, foram empregadas na produção de aerogéis/criogeis em formato de beads conforme descrito por Robitzer, Renzo & Quignard (2011). Os aerogéis/criogeis foram caracterizados com relação ao volume (LENTZ *et al.*, 2022), densidade (aparente e real) (TARASHI *et al.*, 2022), porosidade (TARASHI *et*

al., 2022), microscopia eletrônica de varredura, encolhimento volumétrico (LENTZ *et al.*, 2022), firmeza (DOGENSKI *et al.*, 2020), área específica da superfície, diâmetro do poro, volume do poro e cinética de absorção de água e óleo (DOGENSKI *et al.*, 2020).

Resultados e discussão

A fração oleosa, recuperada com CO_2 supercrítico e por Soxhlet com hexano apresentaram rendimentos de 0,43 e 0,75%, respectivamente. Na amostra recuperada com CO_2 foram identificados 24 compostos, dentre eles 13 ácidos graxos, enquanto que na amostra SOX somente 3 ácidos graxos foram encontrados. O ácido palmítico foi o principal ácido graxo encontrado.

As extrações etanólicas por PLE, MAE, SFE-Co e SOX possibilitaram a recuperação da fração fenólica da KA, onde o etanol foi selecionado devido à sua característica GRAS (geralmente reconhecido como seguro) e baixa solubilidade da carragenana, possibilitando a separação das frações (fenólicos e carragenana). O estudo das cinéticas de extração definiu o tempo total dos processos: 40 min para PLE e 15 min para MAE. O rendimento da fração fenólica variou de 1,14 a 1,98 % para MAE e de 1,55 a 2,37 % para PLE, sendo inferior ao método convencional (SOX, 2,95 %). PLE apresentou melhor desempenho na recuperação de fenólicos e antioxidantes, seguido de MAE e SOX. Compostos fenólicos, como ácido elágico e protocatecuico foram identificados e quantificados, destacando as propriedades antioxidantes da fração fenólica, com destaque para a maior eficiência do método PLE.

O rendimento de carragenana variou de 17.16 a 50.25 %. A viscosidade das amostras variou de 21 ± 1 a 57 ± 9 cP, e a força de gel de 25 a 131 $g.cm^{-2}$, com valores mais baixos do que o comercial. Embora os rendimentos sejam menores, MAE e PWCE são métodos promissores por fornecerem amostras com viscosidade e força de gel similares ou superiores ao método convencional, em menor tempo de extração.

As frações de carragenana da KA extraídas por ultrassom (UAE) em diferentes condições apresentaram rendimento de até 58,67 %, obtido a 70 °C, 500 W e 10 minutos, enquanto o menor rendimento (23,32 %) foi obtido nas mesmas condições de temperatura e tempo, mas com 100 W. O rendimento variou com as condições de extração, influenciado por fatores como a idade da alga e o clima. A viscosidade das amostras variou de 6 cP a 39 cP, aumentando com a potência e tempo de extração. A resistência do gel foi menor a 100 W, sem diferenças significativas nas potências mais altas. A condição ideal de UAE foi definida como 70 °C, 15 minutos e 300 W. Os efeitos de tratamento com KOH, remoção da fração aquosa e

secagem, e indicaram maior rendimento e viscosidade com o emprego de KOH e remoção da fração aquosa. A secagem em estufa foi mais eficaz que a secagem por congelamento.

Uma extração sequencial a alta pressão foi selecionada como rota alternativa para biorefino da KA devido à alta atividade antioxidante da fração fenólica e alto rendimento em carragenana (PLE seguido de PWCE), e foi comparada com uma rota convencional (SOX seguido de extração de carragenana pelo método convencional CCE), e avaliando o emprego de KOH. O rendimento de carragenana variou de 46,03% a 62,71%, onde o emprego de KOH reduziu a viscosidade da carragenana devido a sua degradação, com valores entre 22 e 81 cP. A força do gel variou de 35 a 56 g.cm², aumentando para amostras tratadas com KOH. A análise de FTIR confirmou que todas as amostras são do tipo κ-carragenana, similares à amostra comercial. As amostras de carragenana apresentaram alta pureza, com baixos teores de proteínas e lipídios e ausência de glucanas, sugerindo uma boa separação entre as frações de carragenana e celulose.

A viscosidade das amostras de caragenana reduziu com o aumento da temperatura variando de 40 a 724 cP para a amostra a alta pressão (HP) e de 125 a 1.524 cP a para a carragenana comercial (CC). Entre 20-30 ºC observou-se intensa redução na viscosidade, sugerindo uma transição gel-sol, causada pela desestruturação da rede de gel com o aquecimento. O estudo aponta que a viscosidade da CC é consistentemente maior que da amostra HP, o que pode estar relacionado ao maior peso molecular da CC. A solubilidade em água e a viscosidade da carragenana são fatores importantes na produção de aerogéis e criogéis.

As amostras CC e HP foram empregadas para a produção de aerogel (obtidos por secagem supercrítica), onde a amostra CC apresentou menor perda de massa durante a troca de solventes, sugerindo uma rede tridimensional mais forte. Os aerogéis apresentaram superfícies lisas, enquanto criogéis (por liofilização) apresentam superfícies rugosas, possivelmente devido à formação de cristais de gelo durante o congelamento. O encolhimento volumétrico foi maior para HP, e a porosidade foi maior para criogéis em comparação com aerogéis. A porosidade dos aerogéis foi menor do que a dos criogéis, que também apresentaram uma área superficial muito baixa devido a secagem por congelamento. A firmeza foi maior nos aerogéis comparada aos criogéis, especialmente para as amostras de HP, devido à menor porosidade.

Considerações finais

A *Kappaphycus alvarezii* é uma macroalga de alto potencial econômico e fonte de diversos produtos valiosos, como proteínas, compostos fenólicos, carragenana e glicose,

reforçando o conceito de biorrefinaria. Embora a KA seja bem estudada, há poucas pesquisas sobre o uso de métodos de extração não convencionais para fracionamento dos compostos de interesse presentes nesta biomassa. Este trabalho explorou as técnicas não convencionais para recuperar frações ricas em fenólicos e carragenana, com resultados promissores em termos de rendimento e qualidade. A pesquisa foi pioneira na aplicação de rotas sequenciais de alta pressão para fracionar a biomassa de KA e produzir aerogéis com propriedades comparáveis aos comerciais, contribuindo para os Objetivos de Desenvolvimento Sustentável da ONU, como vida aquática, produção sustentável e inovação tecnológica. Para futuros trabalhos sugerimos investigar os efeitos da extração aquosa antes da extração de compostos fenólicos, analisar a viabilidade de escalonar o processo de alta pressão para reduzir custos, estudar a cinética de despolimerização da carragenana em condições de alta pressão e avaliar o uso de aerogel/cryogel em sistemas complexos.

Palavras-chave: Rhodophyta; economia circular; métodos de extração não convencionais; biopolímero; compostos bioativos.

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Acronyms	
ABTS	*2 2'-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid).*
ASE	*Acelerated Solvent Extraction.*
AOAC	Association of Official Analytical Chemists
DPPH	*2 2-diphenyl-1-picrylhydrazyl.*
DMSO	*Dimethyl sulfoxide*
EC_{50}	*Effective concentration*.
EFSA	*European Food Safety authority*
EMATER	Instituto de Assistência Técnica e Extensão Rural (Institute of Technical Assistance and Rural Extension)
EMPARN	Empresa de Pesquisa Agropecuária do Rio Grande do Norte (Agricultural Research Corporation of Rio Grande do Norte)
EPA	*Eicosapentaenoic acid*
EPAGRI	Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Company of Agricultural Research and Rural Extension of Santa Catarina)
FAO	*Food and agriculture organization*
KA	*Kappaphycus alvarezii*
FDA	*Food and drug administration*
FRAP	*Ferric reducing antioxidant power.*
GC	*Gas chromatography*
GRAS	Generally recognized as safe
G30	Borosilicate glass flask (30 mL)
HMF	*Hydroxymethylfurfural*
HPLC	*High pressurized Liquid Chromatography.*
PWCE	*High pressure carrageenan extraction*
IBAMA	*Instituto brasileiro do meio ambiente e recursos naturais renováveis (*Brazilian Institute for the Environment and Renewable Natural Resources)
LATESC	Laboratório de Termodinâmica e Tecnologia Supercrítica.
LC-ESI-MS/MS	*Liquid Chromatography-Electrospray Ionization- Mass spectrometry.*
LOD	*Limit of Detection.*
LOQ	*Limit of Quantification.*
MAE	*Microwave Assisted Extraction.*
NMR	Nuclear magnetic resonance
ORAC	*Oxygen Radical Absorbance Capacity.*
pH	Potential of hydrogen.
PHSE	*Pressurized Hot-Solvent extraction.*
PLE	*Pressurized Liquid Extraction.*
PUFA	*Polyunsatured fatty acids*
RJ	Rio de Janeiro.
RN	Rio grande do Norte
SAPE	*Secretaria de estado da agricultura, da pecuária e da pesca (*State Department of Agriculture, Livestock and Fisheries)

SFE	*Supercritical fluid extraction*
SFME	*Solvent free microwave extraction.*
SP	São Paulo
SOX	Soxhlet.
SUDENE	Superintendência do desenvolvimento do Nordeste (Superintendence for Development of the Northeast)
SUDEPE	Superintendência do desenvolvimento da Pesca (Superintendence for Fisheries Development)
SWE	Subcritical water extraction
TPC	Total Phenolic compounds.
UAE	*Ultrasound assisted extraction*
UFRN	*Universidade Federal do Rio grande do Norte*
UFSC	*Universidade Federal de Santa Catarina*

Units	
kHz	KiloHertz.
MHz	MegaHertz.
GHz	GigaHertz.
µm	Micrometer.
mm	Milimeter.
cm	Centimeter.
m	Meter.
min	Minute.
h	Hour
mg	Miligram.
g	Gram.
kg	kilogram
µL	Microliter.
mL	Milliliter
L	Liter.
w/v	Weight/volume ratio.
W	Watt.
MPa	Megapascal.
GAE	Gallic acid equivalent.
mM	miliMolar.
TE	Trolox equivalent
%	Percentage
º C	Degree Celsius.
cp	centipoise
Tons	Tonnes

CONTENTS

THESIS STRUCTURE

This document is divided into 7 chapters, to make it easier to read. First, below is the conceptual diagram that provide an overview of thesis highlighting potential, gaps and opportunities.

Chapter 1 is composed of introduction and objectives.

C**hapter 2** lays on a brief review on red macroalgae in the world and Brazil, KA (composition and products that can be obtained from the seaweed), nonconventional extraction techniques, and the biorefinery concept applied to macroalgae.

Chapter 3 presents an empirical study of three non-conventional extraction techniques to obtain fatty acids, phenolic compounds, and carrageenan, which are compared to conventional extraction methods.

Chapter 4 describes an empirical data on the influence of the parameters of ultrasound, namely temperature, time, and power, and pre-treatments, namely drying conditions, aqueous fraction removal, and KOH pre-treatment in the *Kappaphycus Alvarezii* for recovery carrageenan and the impact of these variables in yield and the viscosity and gel strength.

Chapter 5 is a comparative study of the extraction routes for the KA biorefinery using high-pressure methods and conventional methods.

Chapter 6 presents an application of carrageenan (aerogel) obtained from the sequential route (high-pressure) of KA comparing with a commercial carrageenan.

Chapter 7 shows the conclusion and suggestions to future works.

CONCEPTUAL DIAGRAM

WHY?

- KA is one of the most cultivated macroalgae in the world.
- KA is an industrial source of carrageenan that can be used by different industries, including food, cosmetics and pharmaceuticals.
- The Brazilian cultivation of KA has been increasing. The *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais* (IBAMA), allowed the KA cultivation in the brazilian States - Santa Catarina, Rio de Janeiro, and São Paulo.
- KA biomass is composed of various remarkable molecules, including carrageenan, phenolic compounds, among others.
- Non-conventional extraction approaches can be used to obtain, eco-friendly, carrageenan and other compounds.
- The KA biorefinery using non-conventional extraction technologies is aligned, at least, with 3 united nations sustainable development goals (sustainable consumption and production, action against global climate change, life on water).
- Brazil has a very long marine coastline, making it possible to cultivate KA and obtain large quantities of carrageenan, allowing it to supply the domestic and foreign markets.

WHAT HAS ALREADY BEEN INVESTIGATED?

- There is a lack of information about the use of non-conventional technologies, particularly high-pressure methods for the recovery of valuable components from KA.
- Few works have reported the KA biorefinery using low-pressure extraction techniques.
- Few works have reported the impact of conditions of extraction and pre-treatments in carrageenan obtained by ultrasound.
- No reports were found in the literature carried out for KA biorefinery using high-pressure extraction technologies.
- There is no reports from production of aerogel and cryogel using carrageenan obtained by high-pressure method.

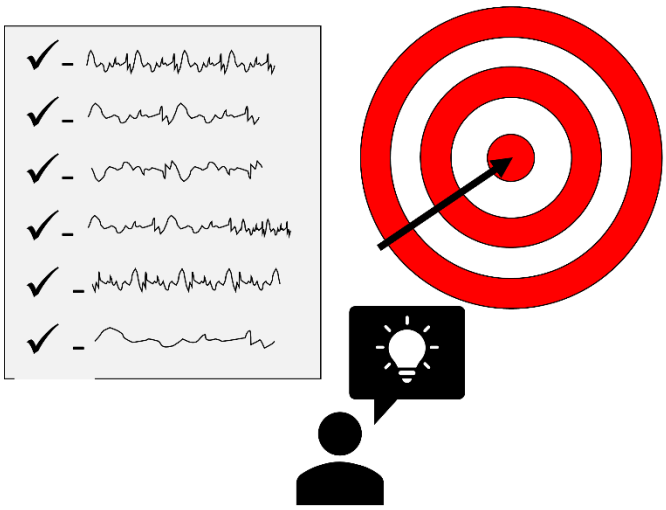
HYPOTHESIS

- It is possible to obtain different KA compounds using the high-pressure green extraction technologies.
- The KA biorefinery approach by using high-pressure extraction technologies allow high products yield similar to obtained by conventional methods.
- The carrageenan obtained by sequential route is technologically viable for aerogel production.

METHODOLOGIES

- To optimize the non-conventional technologies, performed individually, enable the selection of the best conditions for a sequential extraction, to evaluate the products obtained.

- To study different process routes of the KA biorefinery and characterize the biomass in different steps of the sequential process, and evaluate the products obtained to compare with those from individual extractions and conventional methods.
- To evaluate the carrageenan obtained from the sequential process and verify if it can be used for aerogel production, determining its characteristics such as mechanical properties, and compare with that from commercial carrageenan.



CHAPTER 1– INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

The oceans are remarkable sources of unexplored and valuable materials, in particular seaweed, since they contain unique molecules including phenols, pigments, polysaccharides, proteins, and bioactive peptides, among others (PANGESTUTI *et al.*, 2019; PANGESTUTI; SIAHAAN; KIM, 2018). Furthermore, it is worth mention that the seaweed cultivation is well aligned with the principles of sustainable production, since seaweed can mitigate around 20 tons of CO_2/hectare/year, there is no need of fresh water, and no competition for land, in relation to food production (HARGREAVES *et al.*, 2013).

Based on the photosynthetic pigments, seaweeds can be classified into three groups: green algae (Chlorophyta), brown algae (Heterokonthophyta) and, red algae (Rhodophyta). Among them, red algae represent more than 60% out of 30 thousand tons of algae cultivated around the world (FAO, 2018a). The main interest on red algae is related to their easy cultivation and high content of unique polysaccharides (40−50% of the dry weight), in particular carrageenan and agar (FAO, 2018b; KHAMBHATY *et al.*, 2012; SOLORZANO-CHAVEZ *et al.*, 2019a; TORRES; FLÓREZ-FERNÁNDEZ; DOMÍNGUEZ, 2019). In this sense, red seaweed species, KA, *Eucheuma denticulatum*, *Chondrus crispus*, and *Sarcothalia crispate*, are the main sources of carrageenan, at industrial scale (NASERI; HOLDT; JACOBSEN, 2019).

Besides, seaweed in general present several high-added value molecules such as minerals, polyphenols, lipids and proteins (peptides) which are mostly scientifically and industrially unexplored.

Brazil has a very long marine coastline, which is a valorous opportunity to explore more largely the algae cultivation, such as KA, and obtaining large quantities of polymers and other high-value biomolecules. The KA is a macroalgae of fast grow, already cultivated in Rio de Janeiro, São Paulo, and Santa Catarina, and can be explored in other places on the Brazilian coast, improving the carrageenan production for further supply to domestic and foreign markets.

The KA is a red alga with fast growth (≈4.5% daily) and the main source of κ-carrageenan a polysaccharide that can be used in food, pharmaceutical, and cosmetic industries. In general, treatments are carried out on the seaweed to improve this biopolymer recovery. However, other products can be obtained from this alga using the

biorefinery concept, components such as pigments, phenolic compounds, proteins, minerals, fatty acids, and other structural carbohydrates.

According to the green chemistry principles, the recovery of high added-value molecules by using environmentally friendly extraction methods should be inherently associated to the biorefinery concept, which aims to obtain, as extended as possible, a wide variety of products and energy (CHERUBINI, 2010; HERRERO; IBAÑEZ, 2018). In this context, a recent scientific approach is the green non-conventional extraction methods such as pressurized liquid extraction (PLE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE).

Carrageenan has been extensively investigated, into food formulations related to meat products, dairy products, sauces, soups, bakery, and beverage clarification. Currently, the scientific research addresses carrageenan as a wall material for well-controlled release (for the food and pharmaceutical sector), as a material for 3D printing, scaffold, biofilm, aerogel, oleogel, and material to produce drug capsules. Furthermore, carrageenan can be used in different sectors as a substitute for gelatin, since there is a gelatin restriction by vegan market. Among the recent studies of carrageenan applications, aerogels are materials that have low weight, low density, and thermal conductivity. In addition, they are highly porous materials (high surface area/volume) and high mechanical strength, which makes them materials studied to evaluate controlled release, gas and liquid absorption capacity, and active packaging.

1.2 OBJECTIVES

1.2.1 Main objective

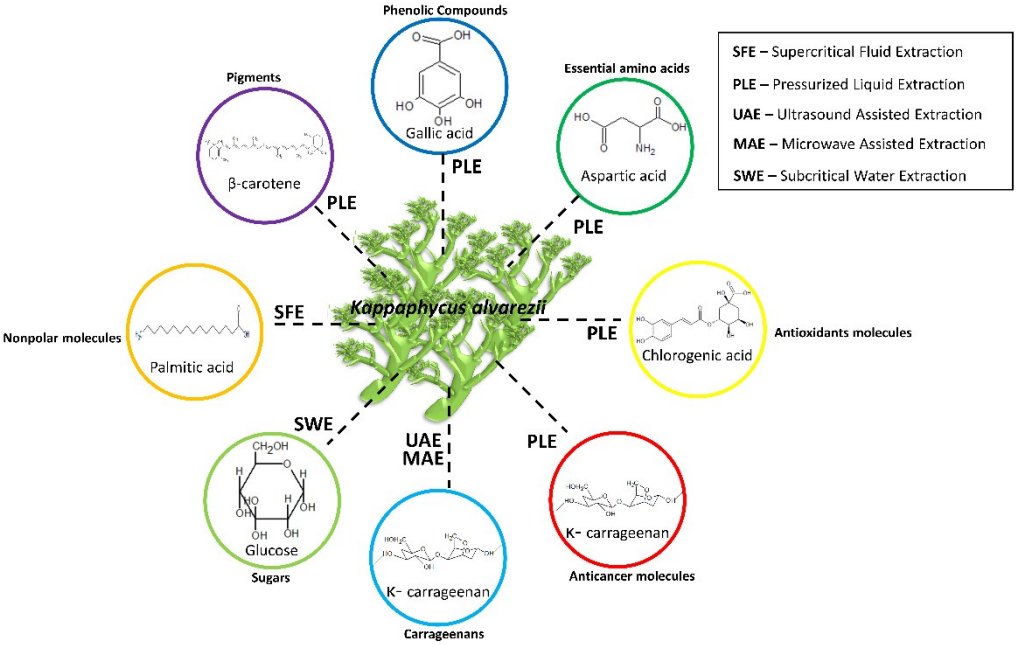
To apply the biorefinery concept using green extraction techniques for the recovery of different valuable fractions from the red macroalga *Kappaphyccus alvarezii* (KA).

1.2.2 Specific objectives

- Review the chemical composition of KA.
- Review the use of non-conventional extraction techniques applied in KA.

- Extract the oily fraction from *Kappaphycus alvarezii* (KA) using SFE and using Soxhlet-hexane.
- Compare the yield and fatty acid profile obtained with SFE and with Soxhlet-hexane.
- Define extraction time for obtaining KA extracts using PLE and MAE to enrich in phenolics.
- Evaluate the temperature influence on yield (40-80 ºC), total phenolic compounds, antioxidant activity (FRAP and DPPH) and profile of phenolic compounds of PLE and MAE extracts.
- Obtain KA extract using SFE and ethanol as co-solvent and Soxhlet-ethanol to obtain phenolics compounds.
- Compare extracts obtained with MAE, PLE, Soxhlet-ethanol and SFE using co-solvent.
- Define the extraction time for carrageenan extraction using MAE-water.
- Extract carrageenan using PWCE at the time defined in the MAE.
- Evaluate the temperature influence (60 to 100 ºC) on yield, gel strength and viscosity of carrageenan obtained using PWCE and MAE.
- Extract carrageenan using conventional method at 80ºC.
- Compare yield, FTIR spectra, gel strength and viscosity of carrageenan obtained by MAE, PWCE and by the conventional method.
- Evaluate the influence of ultrasound assisted extraction (UAE) conditions (time, temperature, and power) on the carrageenan yield and quality (viscosity and gel strength).

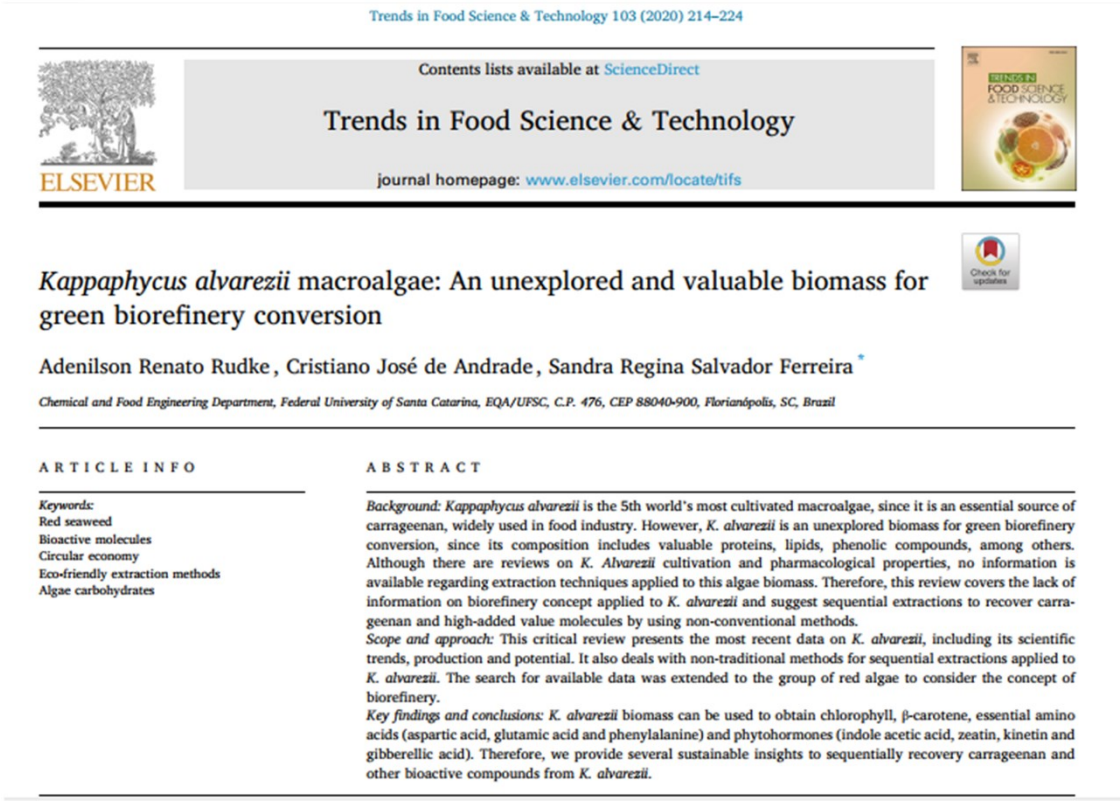
- Investigate the effect of KOH, the aqueous-phase separation and the drying method on the yield and quality of carrageenan.
- Characterize the KA biomass in some steps of biorefinery process.
- To do sequential process to obtain phenolic fraction, carrageenan, cellulose fraction, and low molecular fraction.
- Evaluate the effect of the sequential process in the carrageenan properties.
- Determine the molecular weight of the carrageenan samples from the sequential process.
- Measure the ethanol recovered from the phenolic compound extraction process and from carrageenan precipitation.
- Evaluate the composition of solid materials obtained after each step of sequential process.
- Calculate the mass balance related to the conventional route and the high-pressure route.
- Produce, characterize and compare the carrageenan aerogel produced with high pressure sequential extraction route and with commercial carrageenan.



CHAPTER 2 – LITERATURE REVIEW

In the previous chapter, we introduced the topic discussed in this thesis and presented the aims. In this chapter, we will review the topic of algae, focusing on KA, including green extraction techniques, the compounds that can be obtained from the alga, and the use of the biorefinery concept for this alga.

A part of this chapter has been published in Trends in Food Science and Technology.



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Kappaphycus alvarezii macroalgae: An unexplored and valuable biomass for green biorefinery conversion

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ABSTRACT

Background: Kappaphycus alvarezii is the 5th world's most cultivated macroalgae, since it is an essential source of carrageenan, widely used in food industry. However, *K. alvarezii* is an unexplored biomass for green biorefinery conversion, since its composition includes valuable proteins, lipids, phenolic compounds, among others. Although there are reviews on *K. Alvarezii* cultivation and pharmacological properties, no information is available regarding extraction techniques applied to this algae biomass. Therefore, this review covers the lack of information on biorefinery concept applied to *K. alvarezii* and suggest sequential extractions to recover carrageenan and high-added value molecules by using non-conventional methods.

Scope and approach: This critical review presents the most recent data on *K. alvarezii*, including its scientific trends, production and potential. It also deals with non-traditional methods for sequential extractions applied to *K. alvarezii*. The search for available data was extended to the group of red algae to consider the concept of biorefinery.

Key findings and conclusions: K. alvarezii biomass can be used to obtain chlorophyll, β-carotene, essential amino acids (aspartic acid, glutamic acid and phenylalanine) and phytohormones (indole acetic acid, zeatin, kinetin and gibberellic acid). Therefore, we provide several sustainable insights to sequentially recovery carrageenan and other bioactive compounds from *K. alvarezii*.

2.1 RED SEAWEED

Rhodophyta or red algae can be cultivated in temperate and sub- or tropical zones. The most important, industrially, red algae species are *Eucheuma denticulatum*, *Kappaphycus alvarezii*, *Chondrus crispus* and *Sarcothalia crispate*; since they are sources of carrageenan (NASERI; HOLDT; JACOBSEN, 2019).

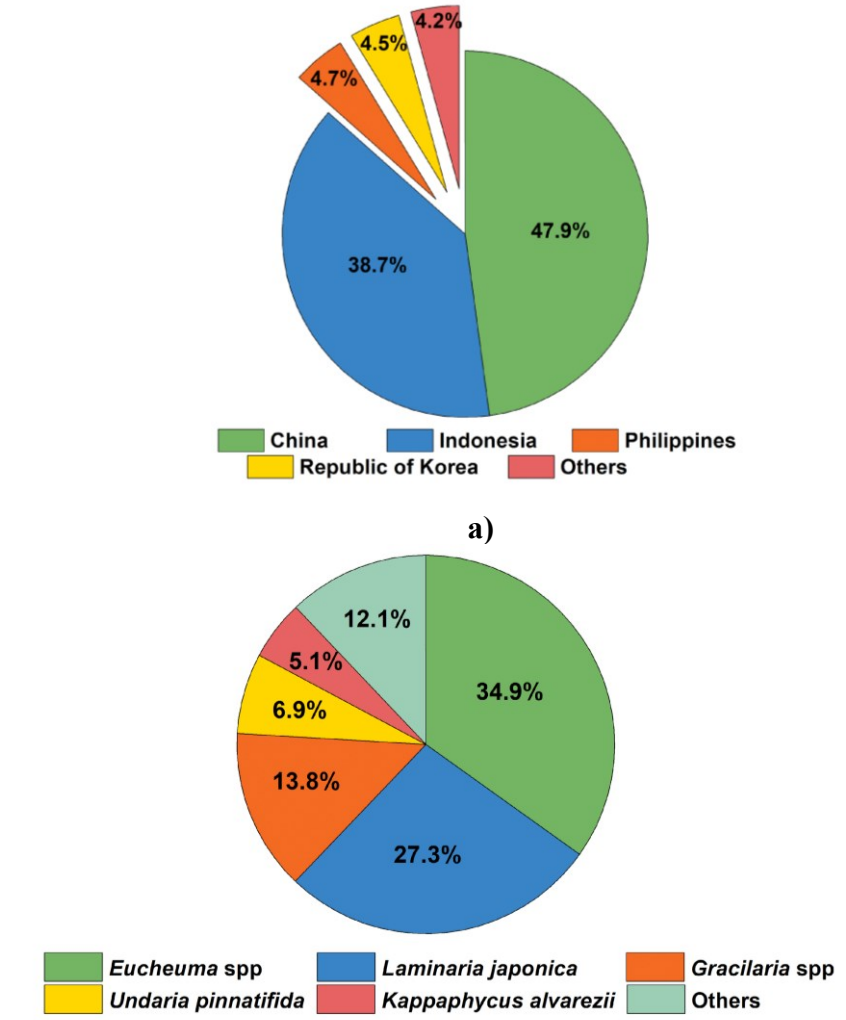
2.1.1 Red algae world production

According to Food and Agriculture Organization of the United Nations (FAO), in 2016 the production of aquatic plants (macroalgae and microalgae) was over 30 million

tons (FAO, 2018a). China, Indonesia, and the Philippines were the main producers (Figure 2-1a), in which red algae representing near 60% of the total production. The industrial interest on red seaweed is mainly associated with agar and carrageenan extraction, since they are widely used as additives for different industrial segments, especially in food industries. Carrageenan, for instance, is used by food industry to produce ice-cream, cheese, jam and bread, as well as for cosmetic industries as thickeners and stabilizers. Besides, the carrageenan applications depend on its types (ι, κ, and λ – section 2.2.1). In this sense, a quite novel approach is the application of hybrid carrageenan, which is a copolymer composed of different unit types (ι, κ, λ) in a single polymer chain. When compared to homo-polymeric chains, the hybrid carrageenan has wider range of applications, since the different properties from each carrageenan type can be complementary to one another (AZEVEDO *et al.*, 2015). Also, spreading the applications, antiviral activities were recently associated to alginates from brown macroalgae; and the microsphere and microcapsule properties of carrageenans from red macroalgae have been drawing increasing attention from pharmaceutical industry (RHEIN-KNUDSEN; ALE; MEYER, 2015).

According to FAO data, in 2016 the KA production was 1527 thousand tons, which corresponds to approximately 5.1% out of the total macroalgae production (Figure 2-1– Algae production in the World: (a) percentage of major farmed producers in 2016 (b) main algae produced in 2016.Figure 2-1b). It is noteworthy that the first five most produced macroalgae are *Eucheuma* spp*., Laminaria Japonica, Undaria pinnatifida*, *Gracilaria* spp., and KA, respectively (FAO, 2018a). KA is an endemic seaweed from Philippines. Until 2008, the Philippines was the largest supplier of *Kappaphycus* species. However, in 2008 they were overcome by Indonesia (HAYASHI *et al.*, 2017).

Figure 2-1– Algae production in the World: (a) percentage of major farmed producers in 2016 (b) main algae produced in 2016.



Source: Adapted from(FAO, 2018b).

2.1.2 Red algae in Brazil

The extractive exploitation of macroalgae means that there are no cultivations handled by humans. In Brazil, the "agar project" started in 1964 at Federal University of Rio Grande do Norte (UFRN) and detected that some marine algae species could be economically explored. In the following years, 3 companies started to explore two native red seaweeds: *Gracilaria* spp. and *Hypnea musciformis*. Nevertheless, the overexploitation and the lack of legislation led to resource depletion in less than 10 years (CÂMARA NETO, 1987). Later, the first attempts of macroalgae cultivation (*Gracilaria* ssp.) were implanted in Northeast Brazil, with studies carried out by a consortium of Brazilian institutions, composed by UFRN, Superintendence for Development of the

Northeast (SUDENE), International Foundation of Science (IFS) and the Superintendence for Fisheries Development (SUDEPE)(CÂMARA NETO, 1987). Since then, the cultivation of *Gracilaria birdiae* in the States of Ceará, Rio Grande do Norte and Paraíba (Northeastern Brazil) is an interesting alternative for local producers. Currently, *Gracilaria* species are still cultivated in the Northeast Region, and also in São Paulo (Southeastern Brazil) (ANDRADE *et al.*, 2020; HAYASHI *et al.*, 2014).

Regarding KA, it was introduced in Brazil in 1995 in São Paulo, mainly to supply the Brazilian carrageenan market (HAYASHI; REIS, 2012). Commercial cultivation began in 1998 in Ilha Grande Bay-RJ (Southeastern Brazil). Later, in 2003, a commercial farm was established in Sepetiba Bay-RJ. In 2008, after more than 10 years of research, the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA), a governmental agency, authorized the KA cultivation by the normative instruction nº 185, 2008, exclusively, in the area between Sepetiba Bay-RJ and Ilha Bela-SP. In 2008, the KA cultivation was implanted in Florianópolis-SC (Southern Brazil), a subtropical climate, by Federal University of Santa Catarina (UFSC) and the Company of Agricultural Research and Rural Extension of Santa Catarina (EPAGRI) (HAYASHI *et al.*, 2011; SIMIONI; HAYASHI; OLIVEIRA, 2019). Currently, the commercial cultivation of KA between Itapoá (SC) and Jaguaruna (SC) was authorized by the normative instruction nº 1, 2020, IBAMA. In Florianópolis, the mariculturists (~104) which are authorized for KA cultivation (four years of concession) must be located in aquaculture park number 5 (South Bay, the coasts of Caieira Barra do Sul, Ribeirão da Ilha, and Tapera) and accomplish environmental monitoring (annual report for IMA – Instituto do Meio ambiente) (G1, 2021). In the Northeast, more specifically in RN, studies are being carried out (environmental and commercial feasibility) for the implementation of KA cultivation by a group formed by members of Sape (State Department of Agriculture, Livestock and Fisheries), of the Agricultural Research Corporation of RN (Emparn), of the Institute of Technical Assistance and Rural Extension (Emater), of universities, syndicate, Association and the Federal Superintendence of Agriculture in RN (SAPE, 2022).

Brazil has remarkable potential as a seaweed biomass producer due to its enormous coastline (8500 km) (16th world's largest coastline) (MINISTÉRIO DO MEIO AMBIENTE, 2020), and different climate, tropical to subtropical (cultivation possibilities), with average sea temperatures of 27.8 ºC and 22.5C, respectively. However,

the industrial macroalgae cultivation is underexplored. According to FAO between 2012 and 2016, Brazil was among the 20th higher carrageenan importers, with up to 2731 tons of carrageenan per year. Furthermore, 1409.8 tons of seaweed per year were also imported.

2.2 KAPPAPHYCUS ALVAREZII

The alga *Kappaphycus Alvarezii* (Figure 2-2*)*, also known by its commercial name *cotonii*, is a class of Rhodophyceae. This type of alga is found in reddish, yellowish, brown and green colors depending on the phycoerythrin pigment concentration. It is easily cultivated and grows fast, with an increase near 4.5% daily (GERENIU *et al.*, 2017).

Figure 2-2– Green variant of *Kappaphycus alvarezii*.

Source: The author (2024).

KA is the major industrial source of κ-carrageenan (DAS; PRASAD, 2015; GERENIU *et al.*, 2017), since this polysaccharide represents up to 39 wt% of the algae, in dry base (BIXLER, 1996). Due to the physicochemical properties of κ-carrageenan such as gelling, thickening, emulsifying and stabilizing, it has a wide range of applications such as thickening agent for milk-based desserts (ZARZYCKI *et al.*, 2019) and sausages (ATASHKAR; HOJJATOLESLAMY; SEDAGHAT BOROUJENI, 2018; BARACCO; FURLÁN; CAMPDERRÓS, 2017), and also assisting the formation of drug delivery

systems (RASOOL *et al.*, 2020; VIJAYAKUMAR *et al.*, 2020), and skin lotions, tooth paste, shaving foams (AHSAN, 2019).

The chemical constituents and its concentrations present in KA are highly variable and depend of the cultivation conditions, such as water temperature, salinity, sunlight, climatic conditions, light intensity, depth, waves power and others. In general, the KA is composed on average by 50.8% carbohydrates, 3.3% proteins, 3.3% lipids, 15.6% ash, 12.4% sulphated groups, and 3.0% insoluble aromatics (MASARIN *et al.*, 2016; SOLORZANO-CHAVEZ *et al.*, 2019a). The lipid fraction presents the highest variation, associated with the cultivation conditions (light index, salts, etc.), and it is mostly composed of saturated fatty acids (64.28%), with C16:0 and C17:0 as the most relevants. In addition, KA presents essential amino acids, up to 43% of the total amino acid content, in particular phenylalanine, leucine and threonine (NASERI; HOLDT; JACOBSEN, 2019).

In general, KA is essentially cultivated for carrageenan extraction. However, KA contains high added-value molecules and other compounds that can be converted into a variety of products Table 2-1 that can be commercially exploited - simultaneously to the production of carrageenan – as detailed below.

Table 2-1 – Researches including products, biological activities and molecules from *Kappaphycus alvarezii*.

Activities	Products and molecules	References
Anticancer activities		(RAMAN; DOBLE, 2015) and (BAKAR *et al.*, 2017)
Antioxidant activities		(KUMAR; GANESAN; RAO, 2008), (GERENIU *et al.*, 2017) and (MAKKAR; CHAKRABORTY, 2017).
	Lipids	(ADHARINI *et al.*, 2019; HANS *et al.*, 2023; NASERI; HOLDT; JACOBSEN, 2019)
	Carrageenan	(UY *et al.*, 2005), (MONTOLALU *et al.*, 2008), (WEBBER; DE CARVALHO; BARRETO, 2012a), (VÁZQUEZ-DELFÍN; ROBLEDO; FREILE-PELEGRÍN, 2014), (RHEIN-KNUDSEN; ALE; MEYER, 2015), (DAS *et al.*, 2016), (MANUHARA; PRASEPTIANGGA; RIYANTO, 2016), (USULDIN *et al.*, 2017), (YOUSSOUF *et al.*, 2017a), (GERENIU; SARAVANA; CHUN, 2018) and (BUI *et al.*, 2019).
	Carragenan film	(GANESAN; MUNISAMY; BHAT, 2018).
	Pigments	(INDRIATMOKO *et al.*, 2015) and (BASKARARAJ *et al.*, 2019).
	Glucose and etanol	(KHAMBHATY *et al.*, 2012), (HARGREAVES *et al.*, 2013), (MASARIN *et al.*, 2016),(ROLDÁN *et al.*, 2017), (MEINITA *et al.*, 2019) and (SOLORZANO-CHAVEZ *et al.*, 2019a).
	Phytohormones	(MONDAL *et al.*, 2013), (DAS; PRASAD, 2015) and ((MONDAL *et al.*, 2015).
	Lectin homologous	(KAWAKUBO *et al.*, 1999).
	Protein concentrate	(KUMAR *et al.*, 2014).

Source: The author (2024).

2.2.1 Lipids

KA presents a lipid content less than < 9% on a dry basis, i.e., a seaweed not characterized as source of lipids, nevertheless, it is essential to correlated the lipid fraction with cultivation and harvest time (SOLORZANO-CHAVEZ et al., 2019). Concerning cultivation conditions, Siddiqui et al., (2022) detailed changes in the KA profile of lipids under hypo- (16 ppt of NaCl) and hyper-saline (48 ppt) cultivation compared with a control (32 ppt). Then, the authors evaluated the fatty acids profile, detecting an increase in linoleic acid content (an unsaturated fatty acid) at hypo-salinity conditions. Concerning the harvest time Solorzano-Chavez et al., (2019) found higher lipid content (~8.5%) in algae harvested between the months of September and December in Brazil.

Saturated fatty acids, correspond to the largest fraction of fatty acids from KA, mainly C16:0 and C17:0. The main unsaturated fatty acid is C18:4 (n-3) (NASERI; HOLDT; JACOBSEN, 2019).

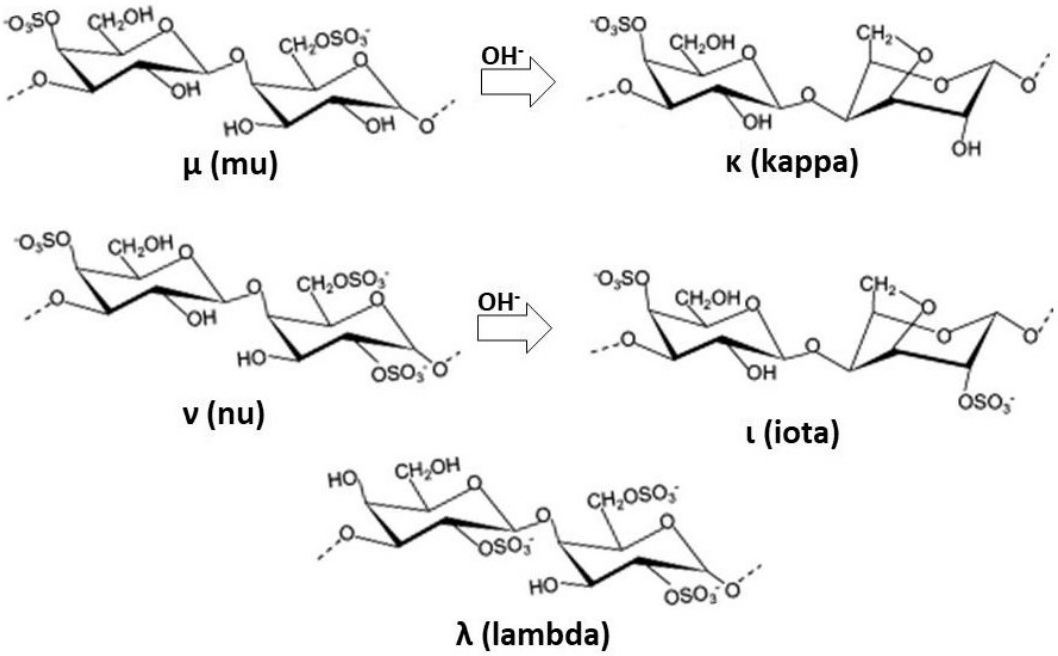
2.2.2 Carrageenan

Carrageenan is the major sulphated polysaccharide found in red seaweed, most particularly in high content at KA (PANGESTUTI; SIAHAAN; KIM, 2018). Carrageenan is located on the algal cell wall in amorphous form and hydro soluble (LECHAT *et al.*, 1997). The carrageenan is a linear molecule, formed by glycosidic linkages between D-galactopyranose and 3,6-anhydro-α-D-galactopyranose, and alternatively by α (1 → 3) and β (1 → 4) linkages (RAMAN; DOBLE, 2015). There are three mostly known carrageenan types, the κ- (kappa), ι- (iota), and λ- (lambda) carrageenans (DAS *et al.*, 2016), shown in Figure 2-3, together with the μ – (mu), ν – (nu) carrageenan precursors. These carrageenans differ depending the number and position of sulphate groups and the content of 3,6-anydro-D-galactose. However, the κ- and ι-are the most common carrageenan types applied to food products (BUI *et al.*, 2019). The applications of carrageenan are related to its type (ι, κ, λ) due to the variations in physico-chemical properties (GANESAN; MUNISAMY; BHAT, 2018). For instance, the number of sulphate groups affects the water solubility and gel strength. The λ-carrageenan has three sulphate groups, and these chemical groups enhance the water solubility (it is water soluble, even at room temperature). In addition, λ-carrageenan has poor gelling

properties, and it is in general most used as thickener. On the other hand, κ- and ι-carrageenans have one and two sulphate groups, respectively, conferring excellent gelling properties, and are also partly water soluble at 60 ºC, and totally water soluble at 80 ºC (CAMPO *et al.*, 2009).

Carrageenan extraction can be carried out by thermal alkali methods and by other methods. Some works related to the extraction of carrageenan from KA are presented in Table 2-1. The presence of alkali promotes the transformation of μ e ν-carrageenan precursors in κ- and ι-carrageenan Figure 2-3, respectively (RHEIN-KNUDSEN; ALE; MEYER, 2015). However, green alternative extractions have been developed, for instance, using red algae solution (distilled water) and the methods ultrasound (YOUSSOUF *et al.*, 2017a) and microwave, with different solvents (acetone, ethanol, methanol, 2-propanol and water) (UY *et al.*, 2005; VÁZQUEZ-DELFÍN; ROBLEDO; FREILE-PELEGRÍN, 2014), or with alternative solvents such as ionic liquids (GERENIU; SARAVANA; CHUN, 2018).

Figure 2-3 – Repetitives structures of dissacharides μ – (mu), ν – (nu), ι- (iota), κ- (kappa) e λ- (lambda) carrageenans.



Source: Adapted from (DYRBY *et al.*, 2004).

The carrageenan type extracted from red algae can be verified by vibrational spectroscopic techniques, this occurs because different functional groups will vibrate at different frequencies when in contact with an infrared source, these vibrations are plotted in an absorbance spectrum in relation to the wave number. These predictable vibrations of these functional groups can provide a region called “fingerprint” this region of the different types of carrageenan consists of the wave number that goes from 1300 to 800 to cm^{-1} and can be verified in the Table 2-2 (PEREIRA, 2017; RODRIGUEZ-SAONA; AYVAZ; WEHLING, 2019)

Table 2-2- Identification of carrageenan type by infrared spectroscopy taking into account the type of bonds and their respective wave number.

Wavenumber (cm^{-1})	**Bond(s)/group(s)**	**Carrageenan type**				
		Mu (m)	**Kappa (k)**	**Nu (ν)**	**Iota (ι)**	**Lambda (λ)**
1240-1260	S=O sulfate esters	++	+	+++	++	+++
1070	C-O of 3,6 anhydrogalactose	-	+	-	+	-
970-975	Galactose	s	+	s	+	-
930	C-O of 3,6 anhydrogalactose	-	+	-	+	-
905	C-O-SO_3 on C2 of 3,6 anhydrogalactose	-	-	-	+	-
867	C-O-SO_3 on C6 of galactose	+	-	+	-	+
845	C-O-SO_3 on C4 of galactose	+	+	+	+	-
825-830	C-O-SO_3 on C2 of galactose	-	-	+	-	+
815-820	C-O-SO_3 on C6 of galactose	+	-	+	-	+
805	C-O-SO_3 on C2 of 3,6 anhydrogalactose	-	-	-	+	-

-: absent; **+:** medium; **++:** Strong; **+++:** very Strong; **s**: shoulder peak.

Source: Adapted from Pereira et al., (2009).

The viscosity of carrageenans has been extensively studied in the literature (FARIA; HAYASHI; MONTEIRO, 2014; HAYASHI *et al.*, 2011; WEBBER; DE CARVALHO; BARRETO, 2012a) and it can be affected before extraction, during extraction (by the process conditions) and during its application. The viscosity of carrageenans is affected before their extraction by the cultivation conditions and harvest time (HAYASHI *et al.*, 2011). In extraction, it is affected by the binomial timextemperature, use of KOH as well as its concentration (BONO; ANISUZZAMAN; DING, 2014; MONTOLALU *et al.*, 2008) since, depending on these conditions, the extracted carrageenan may have higher or lower molecular weight. And during its application it will depend on the concentration of carrageenan in solution, the presence or absence of other solutes, the temperature of analysis and carrageenan type (CAMPO *et al.*, 2009; MONTOLALU *et al.*, 2008).

Another parameter of great industrial interest for carrageenan, in addition to viscosity, is its gel strength. Gel strength can be defined as a measure of the ability of a gelatinous solution to maintain its gel properties when using an applied external force. The measurement of gel strength depends on the gel preparation method (depends on the concentration of carrageenan in the solution, which can be prepared in water or milk and on the presence of salts), whether or not an alkaline method is used before extraction and type of carrageenan (kappa, iota, lambda or mixtures thereof) (HAYASHI, 2001; REY; LABUZA, 1981). According to a study by Bono et al., (2014) the temperature and the concentration of KOH used in the extraction significantly affect the strength of the gel while time exerts less influence. More specifically, about KA, (HAYASHI *et al.*, 2011) found that the gel strength will depend on the time of harvest, as well as its variant (green, red or brown).

2.2.3 Pigments

The concentration of the pigments present in each alga type depends on cultivation conditions such as solar incidence, medium composition, macroalgae specie, cultivation depth, among others (PANGESTUTI; SIAHAAN; KIM, 2018). For instance, higher KA cultivation depth results in algae with higher chlorophyll concentration (INDRIATMOKO *et al.*, 2015).

Pigments such as chlorophylls and carotenoids can be found in KA. According to Indriatmoko et al., (2015), the major components found in brown and green KA variants are chlorophyll and β-carotene, respectively. Although, other pigments such as phycobiliproteins, a protein-phycobilin complex, including allophycocyanin, phycocyanin, and phycoerythrin can be also found (SCHMIDT *et al.*, 2010; SHARMILA BANU *et al.*, 2017). R-phycoerythrin, is the main phycobiliproteins found in KA, and can be applied as fluorescent label in immunology and cell biology, and also as flow cytometry, natural food pigment, marker in gel electrophoresis, among others (COKROWATI *et al.*, 2023; ROSSANO *et al.*, 2003).

2.2.4 Protein

A study of the amino acids profile present in KA was conducted by Naseri et al., (2019). From this study, 15 amino acids were quantified:lysine, arginine, alanine, cysteine, methionine, leucine, phenylalanine, proline, threonine, tyrosine, aspartic acid, serine, valine, glutamic acid, histidine, tryptophan, isoleucine, and glycine. Thus, taking into account these 15 amino acids, 56.7% are non-essential and 43.3% are essential amino acids, then, KA is a potential source of essential amino acids. It is worth noting that aspartic acid (4.19 mg g^{-1}), glutamic acid (3.16 mg.g^{-1}) and phenylalanine (3.02 mg.g^{-1}), showed the highest amino acids concentrations.

The protein concentration in KA can reach up to 18 wt%. Kumar et al., (2014) obtained protein concentrate from KA (essentially alkaline extraction pH 12 with NaOH 1 M). Moreover, the authors tested emulsifying properties with different oils (until 72 h) and foaming capacity and stability over a range of pH 2−10. The protein concentrate showed remarkably stable emulsions with Jatropha oil. Regarding foaming properties, the foaming capacity of protein concentrate is pH dependent; in which the highest foam capacity was at pH 4.0.

2.2.5 Phenolic compounds

Red seaweeds present phenolic molecules. Baskararaj et al.,(2019), extracted phenolic compounds from KA biomass using microwave-assisted extraction with different solvents (acetone, n-propanol, ethyl acetate, n-hexane, methanol, ethanol and chloroform). The optimal extraction condition was methanol 80%, microwave power

20%, radiation induced temperature 45 ºC and radiation exposure time 12.5−14.5 min. Four phenolics compounds were identified: chlorogenic, cinnamic, gallic and 3-hydroxy-benzoic acids.

Kumar et al., (2008) tested different solvents for the recovery of extracts from KA and then evaluate the total phenolic compounds (TPC) from these extracts. The TPC was evaluated by Folin-Ciocalteu method and the extracts antioxidant activity was detected by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical method, Ferrous ion-chelating activity, reductive potential and also by linoleic acid system with ferrothiocyanate reagent. The authors used Soxhlet method to obtain the extracts, and the results indicate better performances in antioxidant activities provided by extracts recovered by ethanol and methanol as solvents.

Naseri et al., (2019) found higher phenolic content in extract obtained from KA with water as solvent, compared with ethanol and methanol. (Gereniu et al., (2017) used subcritical water and evaluated the phenolic content of two strains of KA, brown and red ones. The samples showed similar amount of phenolics, with the highest content obtained at 270 ºC and 8 MPa. In addition, they found that water acidified at 1% with formic acid led to higher phenolic content, compare to non-acidified water.

2.2.6 Glucose and galactose

The KA seaweed cell wall is composed of cellulose and galactan, polysaccharides formed by repeated units of glucose and galactose, respectively, which could be converted into fermentable sugars (glucose and galactose, respectively). Roldán et al., (2017) and (MEINITA *et al.*, 2019) studied the possibility of obtaining ethanol from the residue of carrageenan process. The waste, rich in glucan, can be hydrolyzed by enzymes, producing glucose that, after fermentation, generate ethanol. Roldán et al., (2017) found the glucan conversion to glucose was three times higher compared to sugarcane bagasse. For Meinita et al., (2019), more than 3000 tons of ethanol can be obtained per year, considering the production of 17,549 tons of carrageenan waste per year.

2.2.7 Anticancer and antioxidant activities

The soluble dietary fiber from KA could be a potential functional food, with properties that reduce or avoid colon carcinogenesis (RAMAN; DOBLE, 2015). The authors reported that low molecular weight carrageenan molecules may be associated with this anticancer activity. The study conducted by Bakar et al., (2017) detected, with the use of ethanolic extract from KA, the *in vivo* degeneration of breast cancer cells. Besides, KA has also been associated with various types of anticancer activity, as can be seen in a review recently published by Liu et al., (2019).

Some works presented in the literature also demonstrate the antioxidant potential of KA extracts (GERENIU *et al.*, 2017; KUMAR; GANESAN; RAO, 2008; MAKKAR; CHAKRABORTY, 2017). Kumar et al., (2008) and Makkar & Chakraborty, (2017) evaluated the antioxidant activity of KA extracts by DPPH free radical method. The KA extracts were recovered by Soxhlet with different solvents and by exhaustive extraction with ethyl acetate:methanol mixture (1:1 v/v), respectively. The antioxidant activity from the KA extracts (Soxhlet and exhaustive method) was similar to synthetic antioxidant butylated hydroxytoluene (BHT).

2.2.8 Phytohormones

The liquid solution obtained from the fresh KA, mechanically crushed, is known as K-sap. It is a potent plant stimulant. This biostimulating action seems to be linked to four plant growth hormones (indole acetic acid, zeatin, kinetin and gibberellic acid (GA_3), in addition to inorganic micronutrients and potassium (MONDAL *et al.*, 2015).

Das & Prasad, (2015) studied the extraction of K-sap using ionic liquid. The authors tested three different ionic liquids and found that the ionic liquid [Bmim] [PF_6] can be used to obtain plant growth regulators.

2.3 NON-CONVENTIONAL METHODS OF RED ALGAE EXTRACTION

The extraction of target compounds from red algae can be carried out by conventional or by non-conventional methods. Conventional procedures are well stablished methods, such as maceration, percolation, and Soxhlet, otherwise, PLE, SWE, SFE, MAE and UAE are within the non-conventional procedures. These alternative methods have several advantages over conventional ones such as high yield, restrictive

use of organic solvent, fast process, and low temperature, selectivity and cost, which result mostly in environmentally friendly processes (CIKOŠ *et al.*, 2018; KADAM; TIWARI; O'DONNELL, 2013). Therefore, most non-conventional methods are aligned with the biorefinery concept. Despite the clear advantages of non-conventional techniques, there are still a long way to spread worldwide their industrial applications, particularly when high pressures are associated, in methods such as SFE, SWE and PLE.

Regarding the red algae biorefinery, the biomass can provide products such as carrageenan, ethanol, biofertilizer, biogas, besides lipids-fertilizers-agar-bioethanol among others, as recently demonstrated by Álvarez-Viñas et al., (2019). The biorefinery concept applied to KA by means of using conventional extraction techniques was already investigated (GANESAN; MUNISAMY; BHAT, 2018; INGLE *et al.*, 2018; MASARIN *et al.*, 2016; MEINITA *et al.*, 2019; MONDAL; PRASAD, 2017; ROLDÁN *et al.*, 2017). However, no studies have been found related to KA biorefinery using non-conventional extraction methods, and therefore, should be investigated. Some works involving non-conventional methods are shown in Table 2-3.

Table 2-3 – Non-conventional extraction methods used in *Kappaphyccus alvarezii.*

Extraction method	Aim	Studied process conditions	References
SWE	Monosaccharides and bioactive compounds	**P:** 1 - 10 MPa; **R:** 150 rpm; **S:** water, formic acid (1%) and sodium hydroxide (1%); **S/L:** 1:40 (m/v); **T:** 150 – 300 ºC e **t**: 5 min.	(GERENIU *et al.*, 2017)
	Carrageenan extraction using ionic liquid	**P**: 5 MPa; **R**: 200 rpm; **S:** water and many diferente ionic liquids 1%. **S/L:** 1:80 (m/v); **T:** 60 – 180 ºC e **t**: 5 min.	(GERENIU; SARAVANA; CHUN, 2018)
MAE	Carrageenan extraction	**F:** 2450 MHz; **PW:** 300 W; **S:** water, potassium hydroxide 6%, sodium hydroxide 6%, methanol-water (45:55 m/m), ethanol-water (37-63 m/m), acetone-water (55-45 m/m) and 2-propanol-water (40-60 m/m); **t:** 30 min.	(UY *et al.*, 2005)
	Process conditions that maximize biomass yield, antioxidant, chlorophyll and β-carotene content.	**F:** 2450 MHz; **PW:** 106 – 192 W; **S:** Acetone, n-propanol, ethyl acetate, n-hexane, methanol, ethanol e chloroform (all in 70 % v/v in water). Besides, metanol 57.5 – 87.5 % v/v in water; **S/L:** 1:10 (m/v) **T:** 37.5 a 67.5 ºC. **t:** 5-15 min.	(BASKARARAJ *et al.*, 2019)
	Study of the effect of heating mechanism on the formation of products and their composition in Microwave-assisted pyrolysis	**PW:** 560 W; **T:** 500 ºC. **t:** 15 min.	(GAUTAM *et al.*, 2019)
	Determine minerals and trace elements using microwave assisted digestion	**S:** nitric acid supra pure metal 65%; **S/L:** 1:10 (m/v); **T:** 200 ºC. **t:** 15 min.	(YOGANANDHAM *et al.*, 2019)
	Produce bioactive oligosasaccharides from carrageenans	**T:** 140 – 160 ºC**; t:** 2- 30 min; **[]:** 1 and 5 mg.mL^{-1}; **S:** water	(BOUANATI *et al.*, 2020)
UAE	Carrageenan extraction	**pH:** 7.0; **PW**: 150 W; **S:** water; **S/L:** 1:100 (m/v); **T:** 90 ºC e **t:** 15 e 30 min.	(YOUSSOUF *et al.*, 2017a)

F: Frequency; **P:** Pressure; **PW**: Power; **R:** Rotation; **S**: Solvent; **S/L**: Solid-Liquid Ratio; **T**: Temperature; **t**: time; **[]:** concentration.

Source: The author (2024).

2.3.1 Supercritical fluid extraction

Discovered in 1822 by Baron Charles Cagniard, the supercritical fluid (SF) is a state of matter in which a given substance is above its critical temperature and critical pressure (CAGNIARD DE LA TOUR, 1822; KNEZ *et al.*, 2019). In this state, the substance has physico-chemical characteristics that are intermediate between liquid and gas (HEDRICK; MULCAHEY; TAYLOR, 1992). Among these properties we can mention diffusivity, density, viscosity and dielectric constant (SILVA *et al.*, 2016). In this case its density is close to that of a liquid, its viscosity is like gas and its diffusivity is intermediate between a liquid and a gas (ROSELLÓ-SOTO *et al.*, 2016). These characteristics make the SF a solvent with improved mass transfer as it penetrates in raw material with gas-characteristic and its near liquid density allows for a greater lug of the compounds present in the matrix.

Thus, the Supercritical Fluid Extraction (SFE) is a separation technique where compounds that are present in the matrix are extracted using a supercritical fluid. Among the solvents that are used for extraction the most frequently are CO_2 (BARBA *et al.*, 2016). Its use is due to some characteristics that CO_2 presents as: generally recognized as safe (GRAS) by FDA (Food and Drug Administration) and EFSA (European Food Safety Authority), non-toxic, non-flammable, cheap, non-explosive and with mild temperature and pressure critical conditions (MENDIOLA *et al.*, 2007; YOUSEFI *et al.*, 2019). The fact that this solvent has relatively low critical conditions and occurs in a closed system (in absence of light and oxygen) allows the extraction of thermolabile and highly prone to oxidation reaction compounds without any (or few) degradations (HERRERO *et al.*, 2010). Moreover, the fact that CO_2 is a gas at ambient conditions makes extraction with this substance advantageous since the extract is free of the solvent (SILVA *et al.*, 2016). Besides that, supercritical CO_2 has nonpolar characteristics, so this substance extracts nonpolar compounds such as hydrocarbons. So, in some cases to increase extraction yield or allow extraction of a wider range of molecules a co-solvent (or modifier) is added. The modifiers can be ethanol, methanol, water and others, and they are addicted in general between 1 and 10 % (HERRERO *et al.*, 2015a; WANG; WELLER, 2006).

The red seaweed group, which includes KA, contain valuable polyunsaturated fatty acids (PUFA's) (PEÑUELA *et al.*, 2018), which can be recovered from this type of

biomass. In this sense Chen & Chou, (2002) extracted PUFA's from 10 red algae species by SFE using CO_2 as solvent, and at 500 psi, 55 ºC for 3 h. The crude lipid fraction in dry mass base (% wt/wt) ranged from 11.2 (*Porphyra dentate*) to 21.5 (*Liagora boergesenii*). In general, 16:0, 20:4ω6 and 20:5 ω3 (eicosapentaenoic acid - EPA) represented over 70% out of the total fatty acids. It is worth noting that 22:6ω3 (docosahexaenoic acid - DHA) was not detected. *Liagora boergesenii* had the highest concentration of EPA (6.78 mg/100 mg crude lipid), which is higher when compared to fish oil. Thus, red algae, in particular *Liagora boergesenii*, have a great potential to be used as a source of EPA for SFE at an industrial scale.

Cheung, (1999) studied the SFE from the red algae *Hypnea charoides*, and evaluated the effect of temperature (from 40 to 50 ºC) and pressure (from 24.1 to 37.9 MPa) on the recovery of fatty acids. The maximum yield of lipids was 67.1 mg g^{-1} (on dry base of freeze-dried seaweed) at 37.9 MPa and 50 ºC. The highest extraction yield of saturated fatty acids was obtained at 24.1 MPa and 50 ºC (39.9% of total fatty acids). On the other side, regarding polyunsaturated fatty acids, the highest extraction yield was at 31.0 MPa and 40 ºC (51.7% of total fatty acids).

The red seaweed *Gracilaria mammilaris* was studied by Ospina et al., (2017), which used SFE at different conditions. The authors evaluated the effect of temperature, pressure, and co-solvent concentration to obtain the best antioxidant sample. The extract recovered by SFE at 30 MPa, 60 ºC and with 8% ethanol as co-solvent showed the highest total phenolic content of 3.791 mg GAE.g^{-1}, while the extract obtained at 30 MPa, 50 ºC and 5% ethanol as co-solvent provided the highest carotenoid content of 5.038 mg carotenes per gram of seaweed in dry basis.

Extract of red alga *Gloiopeltis Tenax* was obtained by SFE with CO_2 at 30 MPa and 45 ºC and ethanol as co-solvent. Antioxidant assays from the extract were carried out by DPPH, β-carotene/linoleic acid-coupled oxidation reaction and deoxyribose degradation by iron-dependent hydroxyl radical. From the recovered extracts 30 components were identified by gas chromatograph (GC) coupled with mass spectrometer, including six sesquiterpenes (14.39%), three ketones (5.02%), seven fatty acids and their esters (29.1%), two phenols (1.71%), and three sterols (12.81%). In addition, the extracts showed remarkable antioxidant activity (ZHENG *et al.*, 2012).

Although technically feasible, and analogous to the above-mentioned studies for different red algae, no literature data, related to the extraction of molecules of interest from KA using the non-conventional method SFE, were found.

2.3.2 Pressurized liquid extraction and subcritical water extraction

Pressurized Liquid Extraction (PLE) or Accelerated Solvent Extraction (ASE) is a technique that employs fluids in liquid state throughout the process with a wide temperature range (25-200 ºC) (HERRERO *et al.*, 2015b; MUSTAFA; TURNER, 2011). Pressurizing the system allows the solvent to be in its liquid state even though the temperature is above its atmospheric boiling point, and this implies improved solubility and mass transfer (Mustafa & Turner, 2011). Decreasing solvent viscosity and surface tension assists in the disruption of solute-matrix interactions and increases diffusion coefficients. In general, rising temperatures increase the solubilization of the compounds in the solvent, just as the pressure used favors the penetration of the solvent into the matrix facilitating mass transfer (CAMEL, 2001; HOSSAIN *et al.*, 2011).

These processes can be operated in static, dynamic mode or a combination of both. In dynamic mode, the solvent is continuously pumped into the extraction vessel at a constant flow rate, which causes a shift of the chemical equilibrium, thereby accelerating the mass transfer (HERRERO *et al.*, 2013; VAZQUEZ-ROIG; PICÓ, 2015). In static mode, the sample and solvent are under a certain time and predetermined temperature and pressure conditions. In this operation mode solvent saturation may occur, so is usually done the use of more than one extraction cycle with solvent renewal between the cycles (CARABIAS-MARTÍNEZ *et al.*, 2005; MUSTAFA; TURNER, 2011). The dynamic turns out to be more advantageous than the static mode. It is generally more efficient, and the static operates with a limited extraction volume making it impossible to fully obtain the compounds of interest (MUSTAFA; TURNER, 2011; VAZQUEZ-ROIG; PICÓ, 2015).

The extraction principle when water is used is the same. However, it is common to find subcritical water in the literature. This term is valid when the water is in conditions of temperature and pressure between 100 ºC and 0.10 MPa and 374 ºC and 22 MPa (critical point) to keep the water in the liquid state (generally, pressures between 3.5 and 20 MPa), but with physical and chemical properties being altered, such as a decrease in

viscosity, surface tension and dielectric constant and an increase in diffusivity, with the temperature being the factor with the most significant impact in extraction with subcritical water (CASTRO-PUYANA; MARINA; PLAZA, 2017; HERRERO *et al.*, 2013; PANGESTUTI; SIAHAAN; KIM, 2018; PLAZA; MARINA, 2019).

Klejdus et al., (2017) evaluated different extraction methods, individual or in combination, for the recovery of phenolic compounds from four seaweeds: *Sargassum muticum*, *Undaria pinnatifida* and *Cystoseira abies-marina* (brown seaweeds) and *Chondrus crispus* (red seaweed). The authors carried out preliminary tests only using *Cystoseira abies-marina*. The analysis of the results indicated that sequential extraction method composed of UAE followed by PLE led to the highest phenolics recovery when compared to other four methods: (I) passive leaching extraction, (II) ultrasound-assisted extraction followed by passive leaching extraction, (III) Ika Ultra-Turrax® Tube Drive and (IV) Ultrasound extraction followed by Ika Ultra-Turrax® tube. Then, the extraction method composed by UAE followed by PLE was applied to *Chondrus crispus* (red seaweed). The phenolic profile of the red seaweed *Chondrus crispus* was evaluated by rapid chromatography and MS/MS. The results demonstrated a presence of phenolic compounds such as vanillin (highest concentration), 3,4-dihydroxybenzaldehyde, *p*-hydroxybenzaldehyde, and, gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, salicylic, and sinapic acids.

Gereniu et al., (2017) evaluated subcritical water extraction for KA extracts. The authors used different solvents, pressures and temperatures (Table 2-3). The highest values of antioxidant activity, total phenolic, total flavonoids and protein contents were obtained at 270 ºC/8 MPa. On the other hand, higher concentrations of reducing sugar and total reducing sugar was obtained at 150 ºC/5 MPa. Whereas Pangestuti et al., (2019) evaluated the temperature and solid to liquid ratio influence on the hydrolization of red algae *Hypnea musciformis* by SWE. The authors founded that 210 ºC is the optimum temperature to obtain an extract with better antioxidant activity, within the studied range between 120 and 270 ºC. However, temperatures below 180 ºC were better to obtain extracts enriched in sugar compounds.

Gereniu et al., (2018) tested κ-carrageenan extraction using ionic liquids (1%) in a subcritical water apparatus (extractions conditions as shown in Table 2-3). The authors tested seven different ionic liquids and compared with water under subcritical conditions and with an extraction method using an autoclave. The highest extraction yield of

carrageenan was found for the ionic liquid 1-butyl-3-methylimidazolium acetate (BMIMAc) when compared with the other six tested solvents, in particular at higher temperatures from 120 to 180 ºC.

2.3.3 Microwave assisted extraction

Electromagnetic waves with frequencies from 300 MHz (1 m) to 300 GHz (1 mm) are denominated microwaves (MARIĆ *et al.*, 2018; PANJA, 2017). In microwave-assisted extraction, the analytes of interest are obtained by breaking the weak hydrogen bonds caused by the dipolar rotational motion (CIULU *et al.*, 2017). Microwave can operate in closed system (above atmospheric pressure) or open system (atmospheric pressure) (CHAN *et al.*, 2011). Two forms of microwave extraction are known and reported in the literature: the solvent extraction and solvent-free microwave extraction (SFME). The first is used for extracting non-volatile bioactive compounds and the second for volatile compounds like essential oils. In the second case, since the extraction is "solvent free" the matrix must have a certain humidity for extraction to occur, since the water present inside the plant cells will heat up under microwave irradiation (VINATORU; MASON; CALINESCU, 2017; ZHANG; LIN; YE, 2018).

Vázquez-Delfín et al., (2014) evaluated the influence of time and temperature on the content and type of carrageenan obtained from *Hypnea musciformis*. The authors found that, when compared to conventional method (hydrothermal), which is costly (KOH solution 3%, at 85 ºC for 3.5 h), MAE showed slightly lower carrageenan yield, 18.7% and 16.6%, respectively. However, MAE is dramatically faster (10 min).

Boulho et al., (2017) performed the extraction of carrageenan from the red algae *Solieria chordalis* by MAE and by the conventional method (KOH solution 1%, at 85 ◦C for 3.5 h). Then, the biological properties of both carrageenans were compared. The authors used animal cells - Vero cell lines (line no. ATCC CCL81) – to correlate the carrageenans with alterations in cell morphology, in particular swelling, shrinkage, granularity, and detachment, cytotoxicity, and also antiviral property against *Herpes simplex* virus type 1 (HSV-1; family Herpesviridae). Conventional and MAE carrageenans showed similar chemical properties (protein, total sugar, sulphate, Fourier transform infrared spectra and ^{13}C NMR spectra analysis). However, it should be emphasized that MAE has a clear advantage, it is faster, with 10–25 min, compared to 3.5

h from conventional method. In addition, MAE carrageenan showed higher antiviral activity against the HSV-1 virus (PEÑUELA *et al.*, 2018).

Levulinic acid is considered a chemical platform because it is technically feasible to generate, from it, a wide range of derivatives, and consequently different products can be synthesized, such as plasticizers, polymers, pharmaceuticals, herbicides and fuel additives. Levulinic acid is obtained from rehydration under acidic conditions of the 5-(hydroxymethyl)furfural (HMF). HMF is produced by the dehydration of hexose. Hexoses are present in red algae biomass. Based on this, Cao et al., (2019) applied microwave assisted low-temperature to carried out the saccharification of red algae *Gracilaria lemaneiformis*. The optimal condition (180 °C, 20 min, 0.2 M H_2SO_4 and 5% (w/v) of solid/liquid ratio) achieved 16.3 wt% of levulinic acid. It is worth noting that similar levulinic acid yields were obtained from wheat straw (CHANG; CEN; MA, 2007), although microwave assisted low-temperature is significantly faster, 20 min process, when compared to conventional heating (2 h).

Microwave extraction can be enhanced by enzymes, (LEE *et al.*, 2016) evaluated the production of polysaccharides with antioxidant properties from red algae *Pyropia yezoensi* by MAE with carbohydrate hydrolytic enzymes: Viscozyme, Ultraflo, amyloglucosidase (AMG), Termarmyl and Celluclast. The authors investigated different extraction times (10 min to 2 h) and enzyme concentrations (10:1 and 100:1) with microwave operating at 400 W. AMG (10:1 and 2 h) showed the highest degree of hydrolysis (25%). In addition, it was observed a synergic effect between microwave and AMG, in which it doubled the degree of hydrolysis of the polysaccharides when compared to microwave hydrolysis or enzymatic hydrolysis.

Uy et al., (2005) performed the extraction of carrageenan from KA using a continuous microwave method with aqueous mixtures (extraction conditions as shown in Table 2-3). The authors showed that carrageenan can be extracted in high purity, with lower solvent consumption and extraction time compared to the traditional hot water extraction.

Regarding other biomolecules instead carrageenan, (BASKARARAJ *et al.*, 2019) optimized the extraction yield of β-carotene and chlorophyll from KA. The authors evaluated the influence of the solvent concentration using methanol-water mixtures, and also the extraction time, temperature, and microwave power (extraction conditions as

shown in Table 2-3. The optimum condition for these responses was methanol-water concentration of 80%, 12.5−14.5 min of extraction time, 45 ºC and 170 W.

2.3.4 Ultrasound assisted extraction

The ultrasound assisted extraction operates between the audible range (> 20 kHz) and the microwave range (up to 10 MHz). The main phenomenon involved in ultrasound extraction is the cavitation, which promotes the break of cell wall (microjets are generated by the shock between bubbles produced by cavitation). The extraction using ultrasound can be occurs using a bath or an ultrasonic probe. Using a bath, the exist a barrier which reduces the reproducibility and ultrasound power, on the other hand the probe is inserted directly into the liquid used for promotes the extraction which causes higher yields (CHEMAT; ZILL-E-HUMA; KHAN, 2011; PANJA, 2017; PICÓ, 2013; TIWARI, 2015; VINATORU; MASON; CALINESCU, 2017).

The UAE has also been used to obtain pigments from red macroalgae. (LE GUILLARD *et al.*, 2015) used an ultrasonic flow reactor to extract the pigment R-phycoerythrin from *Grateloupia turuturu*. The seaweed was cut in small pieces (5−7 mm^2) and homogenized in tap water, with pH adjusted to 5.5. The UAE occurred assisted by different Enzymatic cocktails, Sumizyme TG, Sumizyme MC, Multifect® CX 15 L and Ultraflo® XL at 22 or 40 ºC, 200 W or 340 W for 6 h. The authors found that UAE at 22 ºC led to higher extraction (3.6 ± 0.3 $mg.g^{-1}$) of the thermosensitive pigment R-phycoerythrin.

Subsequently, the same research group proved that the UAE of carbohydrates and amino acids from *Grateloupia turuturu* Yamada was enhanced by an enzymatic cocktail composed of Sumizyme TG, Sumizyme MC, Multifect® CX 15 L and Ultraflo® XL, that is, when compared to UAE, the ultrasound-assisted enzymatic extraction showed promising results (Le Guillard et al., 2016). All experiments were performed at 40 ºC, pH 5.5 for 6 h with reaction mixture, composed of 20% wet and cut seaweed homogenized in tap water.

Similarly, Romarís-Hortas et al., (2013) applied ultrasound-assisted enzymatic hydrolysis to enhance the extraction of iodinated amino acids from red seaweed *Palmaria palmate* and *Porphyra umbilicalis*. Pancreatin was the most feasible enzyme. The ultrasound-assisted enzymatic hydrolysis was carried out at 45 kHz, pH 8.0, at 50 ºC for

12 h. It is worth noting that when compared to green and brown species, the authors found the lowest concentrations of iodine, 21 and 38 $g.g^{-1}$, for the seaweed *Palmaria palmate* and *Porphyra umbilicalis,* respectively.

Topuz et al., (2016) optimized the UAE of phenolic compounds and antioxidant molecules, detected by ABTS method, from a red seaweed, the *Laurencia obtuse*. The optimal conditions obtained were: 1:30 ($g.mL^{-1}$), 50 ºC and 42.8 min and 1:24 ($g.mL^{-1}$), 45 ºC and 58 min for phenolics and antioxidant activity, respectively.

The UAE has also been used for the carrageenan extraction from red algae species KA and *Euchema denticulatum.* The carrageenan yield for both algae, recovered by ultrasound, was similar 50–55% (extraction conditions as shown in Table 2-3), but they were superior to the conventional method (27%), while extraction times were 15 and 120 min, for UAE and conventional method, respectively (YOUSSOUF *et al.*, 2017a).

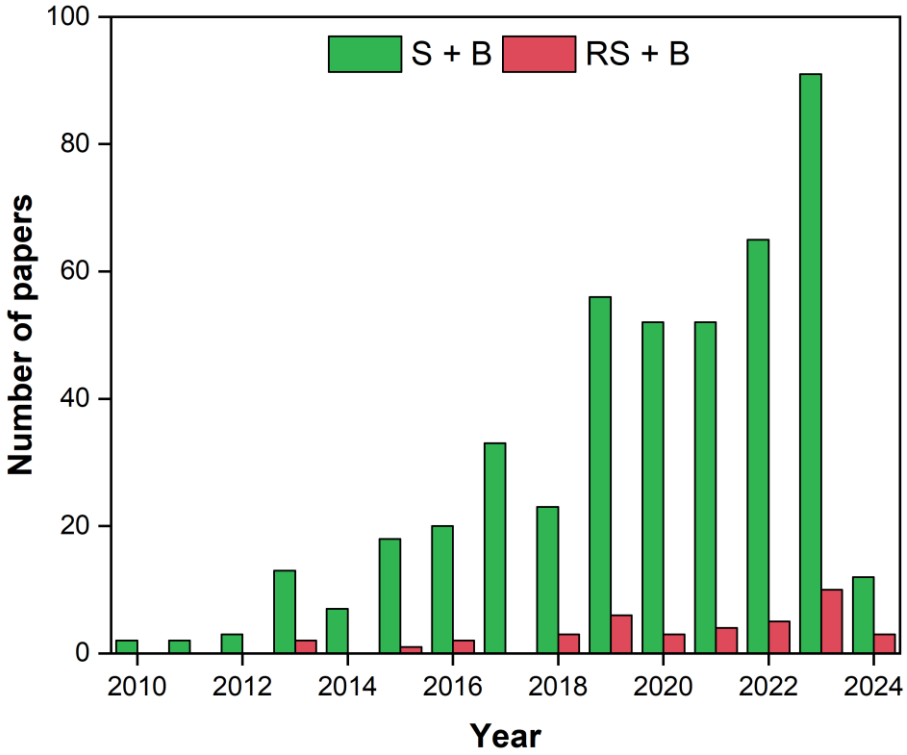
2.4 PROCESS INTEGRATION AND BIOREFINERY

Red algae have high content of polysaccharides, and also a considerable concentration of proteins, lipids, minerals, and phenolic compounds. Based on the variety of valuable compounds, red algae, in particular KA, are potential raw materials for biorefineries (ÁLVAREZ-VIÑAS *et al.*, 2019; TORRES; FLÓREZ-FERNÁNDEZ; DOMÍNGUEZ, 2019). Therefore, researches focused on macroalgae and combined with the biorefinery concept have been growing in recent years. The Scopus Database (www.scopus.com) was used to identify the scientific trends. The search was carried out on 3rd February, 2024 using the following keywords/booleans "macroalgae" OR "seaweed" (including all green, red and brown macroalgae) AND "biorefinery" OR "process combination" OR "cascade processing" OR "sequential process". The search parameters were title, abstract, keywords, document type, and publication data, from 2010 to 2024. As a result, 451 documents were obtained Figure 2-4. According to Figure 2-4, a significant increase in scientific documents was detected from 2013 to 2023, with 13 and 91, respectively. Regarding documents type, 279 (61.9%) are research articles, 88 (19.5%) review papers and 63 (14.0%) book chapters, whereas conference papers, conference reviews, notes, books, editorials and letters, represent 21 (4.60%). In order to narrow the subject, a second search was conducted including the term "red", i.e., "red macroalgae" OR "red seaweed" AND "biorefinery" OR "process combination" OR

"cascade processing" OR "sequential process". As result, only 39 documents were found between the years 2010–2024, 31 (79.5%) research articles, 6 (15.4%) review papers and others 2 (5.10%).

As already mentioned, KA is an unexplored and valuable biomass for green biorefinery conversion, due to the presence of pigments (2.2.3), proteins (2.2.4), phenolic compounds (2.2.5), glucose/galactose (2.2.6) anticancer and antioxidant molecules (2.2.7) and, fertilizers (Figure 2-5). However, KA is essentially cultivated as carrageenan source, thus, KA exploitation is underestimated, generating large amounts of residues from the carrageenan production. Therefore, the application of the biorefinery concept is an essential strategy to aggregate value to the production chain of KA carrageenan and should be deeply investigated.

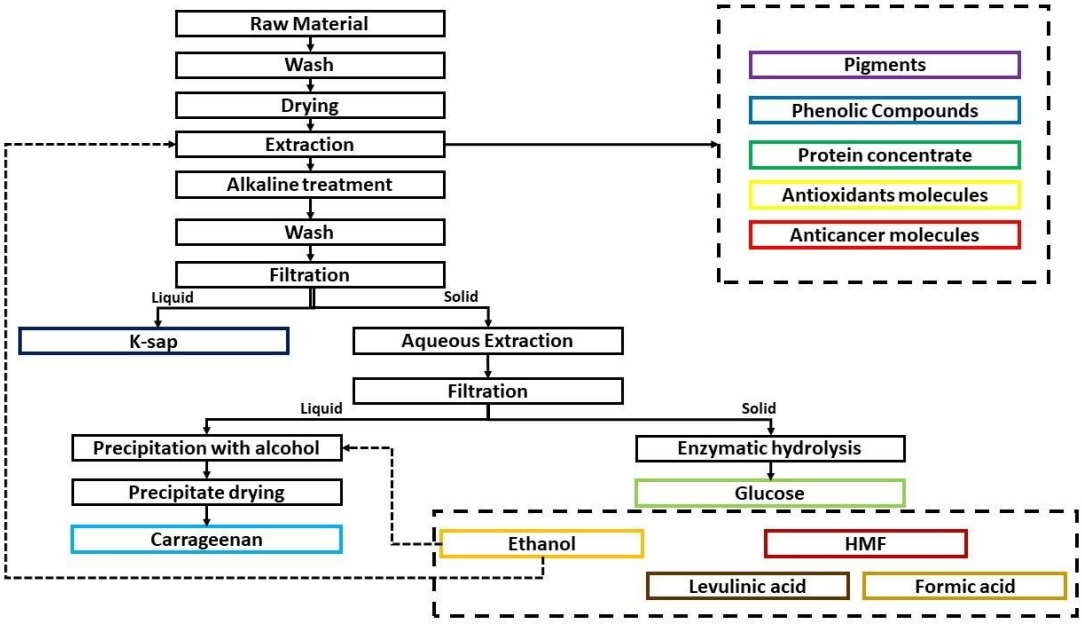
Figure 2-4 – Number of researches published from 2010 to 2024 (February 2024) according to the Scopus database platform. (S+B) refers to searches "Seaweed" OR "macroalgae" AND "biorefinery" OR "process combinations" OR "cascade processing" OR "sequential process". Otherwise, (RS +B) refers to the searches of "Red seaweed" OR "red macroalgae" AND "biorefinery" OR "process combinations" OR "cascade processing" OR "sequential process". The data show the results for all document types: Article, review, book chapters, among others.



Source: The author (2024).

Figure 2-5 presents the biorefinery concept applied in KA (flowchart and sequential processes), which was elaborated based on scientific literature (from previous sections) and our empirical research prospecting. This concept could be used to all red algae species with high content of carrageenan or agar, together with other compound(s) of interest, where only few fine adjustments in extraction methods and conditions should be considered in order to adapt for each biomass.

Figure 2-5– Process flowchart applied *Kappaphycus alvarezii* in a biorefinery concept.

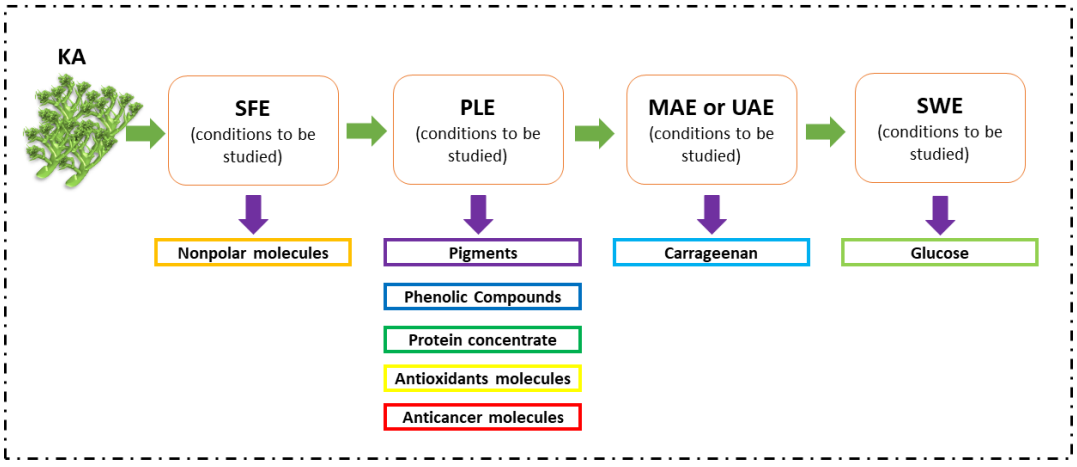


Source: The author (2024).

In this suggested biorefinery approach Figure 2-5, the seaweed biomass is first washed with tap water to remove salt, sand, and debris. Then the biomass is dried and grounded, in order to minimize reactions (e.g microbial degradation and oxidation) and increase extraction efficiency, respectively. The grounded biomass is then submitted to extraction procedures with non-aqueous solvents, since it avoids the loss of carbohydrates. However, the experimental conditions must preserve the adequate recovery of carrageenan, as well as the extraction of other valuable substances. In this case, sequential extractions with low nonpolar solvents such as hexane, and/or intermediate polar ones like ethanol could be performed to obtain fractions of this biomass. Then, in a sequence, an alkaline treatment could be used to increase the yield of carrageenan. In this step, the precursors μ- and ν-carrageenan are converted to κ- and ι-

carrageenan, respectively, as described in section 2.3.1 (RHEIN-KNUDSEN; ALE; MEYER, 2015). After that, the biomass is water washed to remove the excess of alkali, and then filtered, separating in a liquid stream, which could be used to obtain K-sap, a fertilizer, and a solid material, that could be used in a sequential process, for instance, to obtain carrageenan (HAYASHI *et al.*, 2014) and cellulose. Thus, the solid material is used in hydrothermal extraction (the classical approach), with water at 80 ºC, leading to the production of carrageenan (liquid phase) after precipitation with alcohol (HAYASHI *et al.*, 2014). It worth noting that carrageenan precipitates, while cellulose and galactan, from KA seaweed cell wall, remain in ethanol fraction (supernatant). The precipitate can be dried to obtain carrageenan, whereas the supernatant can be added to the hydrolyzed material to get glucose and galactose for bioethanol production (MEINITA *et al.*, 2019). Therefore, we hypothesize a complex sequential process for the extraction of valuable substances from the red algae and obtaining different products in a rational sequential extraction methodology, which may result in the recovery of lipids, pigments, carrageenan and glucose, as proposed in Figure 2-6.

Figure 2-6 – Sequential extraction process of carrageenan and other compounds from *K. alvarezii* using non-conventional extraction methods.



Source: The author (2024).

No studies were found involving the extraction of KA by SFE, and it could be applied, prior to carrageenan extraction. This subject should be deeply investigated to detect the influence of the conditions of temperature and pressure of the supercritical fluid that affect the recovered fractions from KA. Also, pre-treatment conditions such as drying

procedures, biomass gridding and others, prior to SFE, could affect the resulting product. It is worth mention that SFE was already applied to extract antioxidants and antimicrobial compounds from *Agaricus brasiliensis* (MAZZUTTI *et al.*, 2012), carotenoids from pink shrimp (*Penaeus brasiliensis*) (MEZZOMO *et al.*, 2011) (Mezzomo et al., 2011), antitumoral compounds from *Cordia verbenacea* (PARISOTTO *et al.*, 2012) (Parisotto et al., 2012), alkaloids such as piperine from *Piper nigrum* (Andrade et al., 2017), among others.

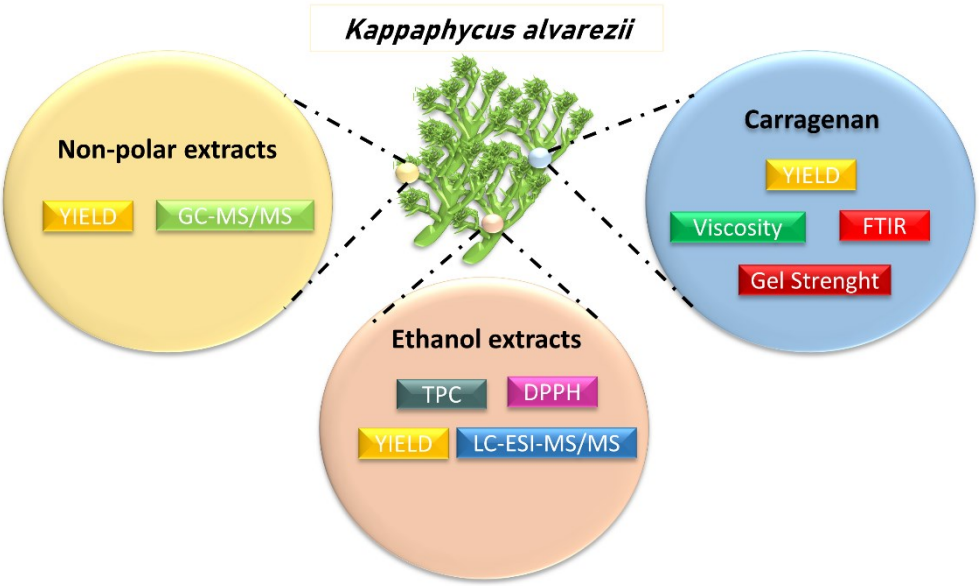
Following the proposed biorefinery concept, the biomass residue of SFE could be used for PLE in order to recover antioxidant molecules (GERENIU *et al.*, 2017) or carrageenan (GERENIU; SARAVANA; CHUN, 2018) as presented in section 2.3.2. In this sense, other green methods for carrageenan extraction can be used, for instance ultrasound with distilled water (YOUSSOUF *et al.*, 2017a); and microwave with ethanol and water (VÁZQUEZ-DELFÍN; ROBLEDO; FREILE-PELEGRÍN, 2014). Then, mostly, cellulose and, very likely, few types of molecules remain in the KA (insoluble). Cellulose can be hydrolyzed to obtain glucose by either subcritical water or cellulases.

Then, the glucose can be used by yeasts to produce ethanol. The produced ethanol can be used for carrageen extraction, for instance by microwave method (VÁZQUEZ-DELFÍN; ROBLEDO; FREILE-PELEGRÍN, 2014).

The interactions among the different recovered products (from the various process steps) could be also investigated. For instance, since carrageenan is an encapsulating agent, the product from SFE could be encapsulated (complex coacervation process) by the carrageenan recovered from MAE or UAE (BAKRY *et al.*, 2019; SOUZA *et al.*, 2020); or the KA extracts and the carrageen could be used for the production of active biofilms (biopolymer) (VIJAYAKUMAR *et al.*, 2020) or blended with other polymers, as chestnut starch, in order to improve the mechanical/drying properties of biofilms as detailed by Moreira et al., (2011).

Different extraction routes can be used to obtain high-value molecules from KA, such as the sequential recovery of bioactive compounds and carrageenan, in particular proteins/carrageenan or also K-sap/carrageenan, or pigments/proteins/carrageenan, or other combinations. In this sense, since there is no information about this strategy, the biorefinery concept involving KA should be investigated, and in fact it should be aligned to an economic feasibility study. Then, the best route can be scaled-up industrially. Similarly, the concept of circular economy could be also applied for it, since the wastes

from carrageenan production can be converted into ethanol. Then, the ethanol, in turn, can be used as solvent for the recovery of phenolic compounds, or antioxidant molecules prior to the carrageenan extraction or for the carrageenan precipitation.



CHAPTER 3 – GREEN EXTRACTION OF PHENOLIC COMPOUNDS AND CARRAGEENAN FROM THE RED ALGA *KAPPAPHYCUS ALVAREZII*

In the previous chapter, the review of literature was presented. In this chapter, the methods Supercritical Fluid Extraction, microwave assisted extraction, pressurized liquid extraction was compared with the conventional low-pressure extraction for the recovery of different fractions from KA biomass, in direct extraction methods.

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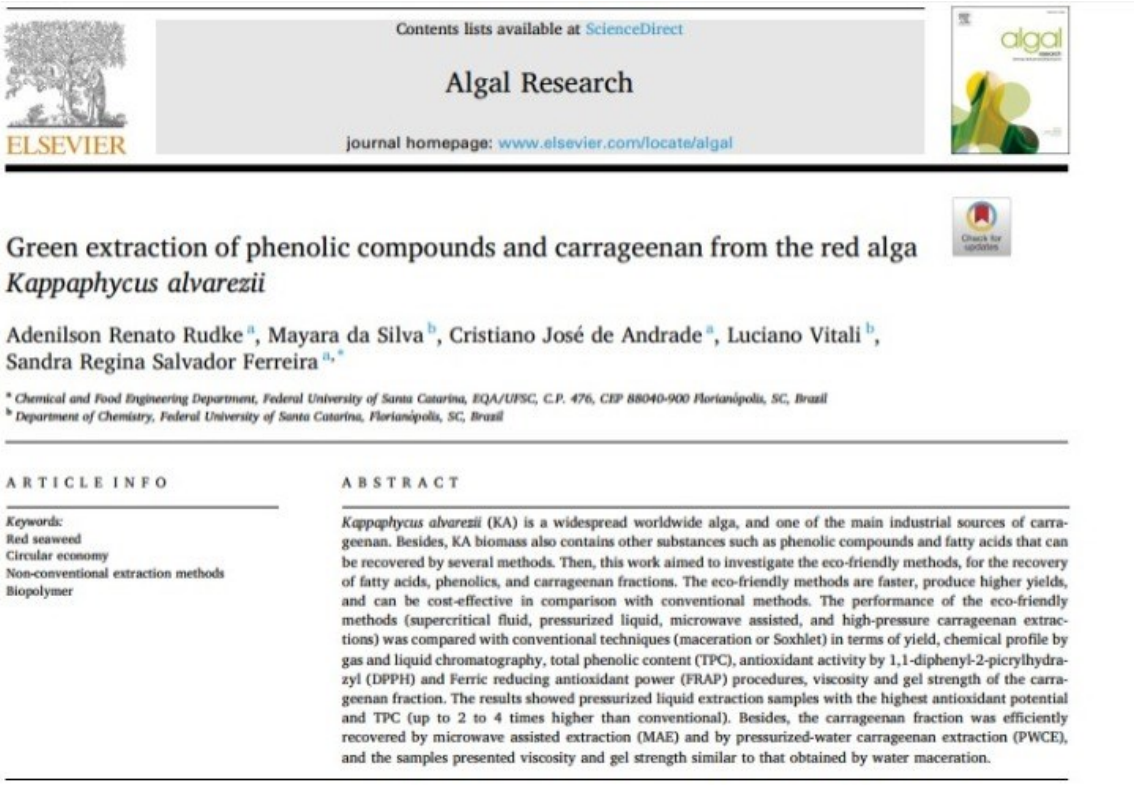

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Green extraction of phenolic compounds and carrageenan from the red alga *Kappaphycus alvarezii*

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ABSTRACT

Kappaphycus alvarezii (KA) is a widespread worldwide alga, and one of the main industrial sources of carrageenan. Besides, KA biomass also contains other substances such as phenolic compounds and fatty acids that can be recovered by several methods. Then, this work aimed to investigate the eco-friendly methods, for the recovery of fatty acids, phenolics, and carrageenan fractions. The eco-friendly methods are faster, produce higher yields, and can be cost-effective in comparison with conventional methods. The performance of the eco-friendly methods (supercritical fluid, pressurized liquid, microwave assisted, and high-pressure carrageenan extractions) was compared with conventional techniques (maceration or Soxhlet) in terms of yield, chemical profile by gas and liquid chromatography, total phenolic content (TPC), antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Ferric reducing antioxidant power (FRAP) procedures, viscosity and gel strength of the carrageenan fraction. The results showed pressurized liquid extraction samples with the highest antioxidant potential and TPC (up to 2 to 4 times higher than conventional). Besides, the carrageenan fraction was efficiently recovered by microwave assisted extraction (MAE) and by pressurized-water carrageenan extraction (PWCE), and the samples presented viscosity and gel strength similar to that obtained by water maceration.

3.1 INTRODUCTION

Kappaphycus alvarezii (KA), also known as *Eucheuma cottonii*, is the 5th most cultivated macroalgae worldwide, mainly due to its rapid growth rate (about 4.5 % daily) and its high carrageenan content, a polysaccharide from the algae cell walls, which is widely used in food (cheese, puddings, dairy desserts, hamburgers, and sausages, among others), pharmaceuticals (mainly as excipients for controlled release), and cosmetics

industries (toothpaste, skin lotions, and shaving foams)(CAMPO *et al.*, 2009; RUDKE; DE ANDRADE; FERREIRA, 2020a).

Carrageenan constitutes the largest mass fraction of KA (39%) (BIXLER, 1996). In addition, the seaweed also contains pigments (INDRIATMOKO *et al.*, 2015), phenolic compounds (BASKARARAJ *et al.*, 2019), proteins (NASERI; HOLDT; JACOBSEN, 2019), minerals, fatty acids (NASERI; HOLDT; JACOBSEN, 2019), and other carbohydrates (cellulose and some monosaccharides) (MEINITA *et al.*, 2019), which can be extracted by different methods. In general, conventional extraction techniques are time- and solvent-consuming, whereas eco-friendly techniques such as subcritical water extraction (SWE), pressurized liquid extraction (PLE), ultrasound assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), among others, can reach high extraction yield and quality (compounds).

There is data on the antioxidant activity of KA biomass (BHUYAR *et al.*, 2020; DIYANA; SHAHRUL HISHAM; CHAN, 2015; DOUSIP *et al.*, 2014; GERENIU *et al.*, 2017; LING *et al.*, 2015) nevertheless most of them are related to the antioxidant activity from aqueous extracts, which also solubilize proteins and carrageenan, along with the phenolic components. Besides, the literature discusses about extracts from KA obtained using solvents such as acetone, ethanol, methanol, hexane, ethyl acetate, which are more selective than water and aqueous solutions, and present low carrageenan solubility (ARAÚJO *et al.*, 2020; FAYAZ *et al.*, 2005; KUMAR; GANESAN; RAO, 2008; NAGARANI; KUMARAGURU, 2012; PAPITHA *et al.*, 2020). For instance, Uy et al., (2005) used MAE with aqueous solutions of different solvents (methanol, ethanol, acetone or 2-propanol) for the recovery of carrageenan. Similarly, Bouanati et al., (2020) evaluated MAE with water to provide the depolymerization of carrageenan. Gautam et al., (2019) used microwave (500 ºC, 15 min) to provide biomass pyrolysis and obtain the bio-oil from KA. Baskararaj et al., (2019) optimized the MAE conditions to obtain a phenolic-rich sample from KA using methanolic aqueous solution (57.5 to 87.5%). Gereniu et al., (2017), using PLE, evaluated the hydrolysate obtained from KA using water, 1% formic acid, and 1% sodium hydroxide. Gereniu et al., (2018) considered subcritical water modified with ionic liquids for carrageenan extraction from KA.

Therefore, unprecedentedly, the aim of this chapter was to evaluate the recovery of different bioactive/valuable fractions from KA using alternative procedures: (I) SFE with CO_2, to recover the oily (non-polar) fraction; (II) PLE and MAE using ethanol to obtain the phenolics fraction (intermediate polarity); and (III) MAE and high-pressure carrageenan extraction (PWCE), performed with water, to recover the carrageenan fraction. The effect of extraction temperature on yield, TPC, DPPH, FRAP, chromatographic profile, and carrageenan viscosity and gel strength were evaluated. These alternative methods were compared to conventional maceration for carrageenan recovery, and to Soxhlet, with hexane and ethanol for the oily and phenolics recovery, respectively.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals

The ethanol, methanol, sodium carbonate and hexane were purchased from Neon (Suzano, SP, Brazil), Folin-Ciacalteau reagent and Sodium hydroxide were from Êxodo Cientifica (Sumaré, SP, Brazil), DPPH reagent (>97%) was from TCI Chemicals (Tokyo, Japan), and 99.9% pure CO_2 from White Martins, Brazil. Commercial carrageenan was purchased from AGARGEL (São Paulo, SP, Brazil), Trolox (97%) from Sigma Aldrich (San Luis, MI, USA), diethyl ether from Dinamica Química Contemporânea (Indaiatuba, SP, Brazil) and, methanol (HPLC grade) from Supelco/Merck (Darmstadt, Germany).

3.2.2 Raw material

The KA biomass was kindly provided by EPAGRI (Agricultural Research and Rural Extension Company of Santa Catarina, Brazil) and consisted of a mixture of green and red algae strains farmed in Florianópolis, Santa Catarina, Brazil. The seaweed sample was washed with tap water to remove sand and epiphytes, then dried in an oven with air circulation at 40 ºC for 24 h. Finally, the dried material (12.81 ± 0.18 % of moisture content) was ground in a Willey knife mill (De Leo, Porto Alegre/RS, Brazil), reserved in polyethylene bags, and frozen at -18 ºC until the tests were carried out.

3.2.3 Extraction procedures

3.2.3.1 Supercritical fluid extraction

The SFE was performed in an unit previously described by (WEINHOLD *et al.*, 2008), and conducted at 200 bar and 50 °C for 2 h, solvent flow rate of 1.2 $kg.h^{-1}$, and using 15 g of dryer seaweed. The extractions follow the procedure described by (GUINDANI *et al.*, 2016). One assay was performed using CO_2 as solvent (SFE-CO_2) to recover the oily fraction from KA. Another SFE assay was conducted using a mixture of CO_2 and 8% ethanol as co-solvent (SFE-Co). The SFE conditions were based on Ospina et al., (2017) about the extraction from *Gracilaria mammilaris*. The recovered extracts were stored in amber flasks at – 18 ºC, until analysis. The oily sample recovered by SFE-CO_2 was analyzed by GC/MS/MS (section 3.2.7.1) and compared with the sample by SOX-hexane (section 3.2.3.2). In contrast, the SFE-CO_2 sample was analyzed by HPLC (section 3.2.7.2) and compared with samples recovered by other methods (PLE, MAE and SOX-ethanol).

3.2.3.2 Soxhlet (SOX)

The Soxhlet extractions were carried out according to the method 920.39C of the (AOAC, (2005), and performed as reference assays. Briefly, 5 g of dried seaweed sample filled a filter paper cartridge and inserted in the Soxhlet extractor. Then, 150 mL of the solvent, hexane (to recover the oily fraction) and ethanol (to recover the phenolics fraction), used separately, refluxed at solvent boiling temperature and recycled repeatedly for a total of 6 h. After the extraction, the solution was evaporated in a rotary evaporator at 40 °C (Fisatom, model 801, São Paulo, Brazil) to remove the solvent and the extract were stored in amber flasks in a domestic freezer at -18 ºC until further analysis. The extractions, named as SOX-hexane and SOX-ethanol, were performed in triplicate.

3.2.3.3 Pressurized Liquid Extraction (PLE)

PLE was performed using the equipment described by Rodrigues et al., 2019). Shortly, the jacket extractor was filled with 5 g of sample, mixed with 90 g of glass

spheres between cottons. Ethanol was used as solvent to recover the phenolic fraction from KA, using a flow rate of 3 mL.min^{-1} (fed at 10 mL.min^{-1} until 10 MPa) controlled by an HPLC pump (Waters, model 515, USA, volume flow rate 0.001 to 10 mL.min^{-1}), pressure was fixed at 10 MPa, and processing temperature controlled by electrical heating jacket system.

A kinetics study, conducted at 60 °C and 10 MPa and 3 mL.min^{-1}, aiding to evaluate the extraction time influence, was performed with samples collected at 20, 40, 60 and 80 min. The results were observed in terms of process yield, TPC values and antioxidant activity of the samples. Then, after defining the process time, the performance of PLE temperature was observed through assays conducted at 40, 60, and 80 ºC, maintaining the other process parameters constant. After the extractions, the solvent was evaporated in a rotary evaporator and the samples were stored in amber flasks in a domestic freezer at -18 ºC. All PLE assays were performed in triplicate.

3.2.3.4 Microwave-assisted extraction (MAE)

Microwave-assisted extraction was performed following the procedure described by Mazzutti et al., (2017) with minor modifications. The extractions were conducted in a microwave reactor (Monowave^{TM} 200, Anton Paar) using ethanol as solvent and maintaining a solid/liquid ratio of 1:30 (0.6 g in 18 mL). The extraction time was defined through to a kinetics study, carried out in assays at 60 ºC and times of 1, 2.5, 5, 10, 15, 20 and 30 min. Briefly, a magnetic stirring bar was maintained in a borosilicate glass flask (G30), together with the dried seaweed sample and the solvent (ethanol). The flask (G30) was then closed and placed at the MAE unit, which operates in automated cycles based on three steps: (1) heating the sample as fast as possible until reaching the working temperature, with 1,000 rpm stirring; (2) maintaining the reaction temperature and 1,000 rpm during the extraction time; and (3) cooling the system to 30 °C using compressed air.

After defining the process time, the extractions were performed at temperatures of 40, 60 and 80 ºC, while keeping constant the other process parameters. After all extractions, the solvent was evaporated in a rotary evaporator to remove the solvent, and

the extracts were stored in amber bottles in a domestic freezer at -18 ºC. All extractions were performed in triplicate.

3.2.4 Global extraction Yield

The global extraction yield was determined by the ratio between the mass of recovered extract and the dried seaweed mass multiplied by 100%. Results are expressed as mean value and standard deviation (expressed as percentage) of triplicate tests.

3.2.5 Total phenolics content (TPC)

The Total Phenolics Content (TPC) from the KA extracts was determined by the Folin-Ciocalteu method, according to Koşar et al., (2005) with modifications. First, dried extracts were diluted in dimethyl sulfoxide (DMSO) up to a concentration of 20 $mg.mL^{-1}$. In a microcentrifuge tube, 600 µL of distilled water, 10 µL of sample, and 50 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, EUA) were mixed. After 1 min, 150 µL of Na_2CO_3 20% (m/v) and 190 µL of distilled water were added. Then, 300 µL of each homogenized sample were transferred to a 96 well microplate. After 2 h, in darkness and at room temperature, the absorbance was measured at 760 nm in a microplate reader (Tecan Infinite M200). The analyses were performed at least in triplicate and the TPC values were calculated according to a standard curve prepared with gallic acid (125 to 1,000 $\mu g.mL^{-1}$). The results are expressed in mg of Gallic Acid Equivalent per g of extract (mg of $GAE.g^{-1}$).

3.2.6 Antioxidant capacity

3.2.6.1 DPPH assay

The KA extracts were evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, according to Brand-Williams et al., (1995). Briefly, 50 µL of extract sample were added to a microplate with 250 µL of a 0.125 mM DPPH methanolic solution. Samples were kept in the dark for 30 min at room temperature. Then, absorbance values were

measured at 517 nm in a microplate reader (Tecan Infinite M200). The analyzes were performed in triplicate and accompanied by a control (50 µL of solvent, 250 µL DPPH) and a blank for each sample (50 µL of sample, 250 µL of methanol). DPPH results are expressed in µmol of Trolox equivalent per g of dry extract (µmol TE g extract $^{-1}$), using a standard Trolox curve (50 to 500 µM).

3.2.6.2 FRAP assay

The ferric reducing antioxidant power (FRAP) of KA extracts was performed according to Benzie & Strain, (1996). Briefly, 10 µL of sample and 290 µL of FRAP reagent were added onto a microplate. This solution was kept in the dark at room temperature for 30 min and absorbance was measured at 593 nm in a microplate reader (Tecan Infinite M200). The analyzes were performed in triplicate with a blank for each sample (10 µL of solvent + 290 µL of FRAP). Trolox was used as a reference and values calculated from the standard curve (50 to 500 µM). The results are expressed as µmol of Trolox equivalent per g of dry extract (µmol TE g extract^{-1}).

3.2.7 Chromatographic analysis

3.2.7.1 GC-MS/MS

The oily fractions from KA, obtained by SFE with CO_2 and by SOX-hexane, were analyzed by gas chromatography–mass spectrometry (Shimadzu GCMS-TQ8040 NX), as reported by Rozas et al., (2019). First, the samples were dissolved in methanol (300 µL), shaken for 2 min and dried under vacuum. Then, 150 µL of a mixture containing bis-(trimethylsilyl) trifluoroacetamide, acetonitrile, dichloromethane, cyclohexane, and triethylamine (10:5:4:1 v/v) was added for derivatization. The solution was vortexed for 2 min, maintained at 60 °C (1 h) and centrifuged at 11.300 × *g* for 2 min at room temperature. The supernatant was collected and, transferred to a vial for GC-MS/MS analysis. The equipment consists of a 30 m long capillary column, 0.25 mm i.d. and film thickness of 0.25 µm. A temperature ramp was set to increase at a rate of 5 °C/min to 280 °C and held at this temperature for 10 min to cook the column. The sample inlet and

GC/MS/MS transfer line were maintained at 280 °C and 300 °C, respectively. Electron impact spectra in positive ionization mode were acquired between 40 and 600 m/z.

3.2.7.2 Extract preparation for LC-ESI-MS/MS

For liquid chromatographic analysis, the extract samples recovered from KA, using ethanol as solvent by different extraction methods, were prepared according to (SCHULZ *et al.*, 2015). Briefly, KA extract samples were defatted with hexane (0.1 g and 25 mL of hexane) in an ultrasonic bath (Eco-Sonics, Ultronique Q3.0/37 A, Brazil) for 15 min at room temperature and centrifuged (Quimis, Q222 T, Brazil) at 3400 rpm, 15 min, at room temperature to remove supernatant. The defatting procedure was repeated twice. Then, the samples were submitted to acid hydrolyses using 5 mL of methanol and 5 mL of hydrochloric acid 6 $mol.L^{-1}$ at 85 ºC (30 min), and the pH was adjusted to 2 with NaOH solutions (6 mol.L-^{1} and 1 $mol.L^{-1}$). Next, the samples were partitioned with 10 mL of diethyl ether shaken and centrifuged at 3400 rpm for 10 min, repeated three times. After that, they were evaporated at 40 ºC for solvent removal. Finally, samples were resuspended in 1 mL of HPLC grade methanol, diluted ten times with methanol/water (30/70 v/v), and injected into the liquid chromatography electrospray ionization mass spectrometric (LC-ESI-MS/MS) system.

Identification and quantification of phenolic compounds were performed according to Schulz et al., (2015) using a high-performance liquid chromatography (HPLC) apparatus (1200 Series, Agilent Technologies, Waldbronn-BW, Germany) coupled with a mass spectrometry system composed of a hybrid triple quadrupole/linear ion trap mass spectrometer (Q Trap 3200 Applied Biosystems/MDS Sciex, Canada). Mass spectrometer operated in negative electrospray (TurboIonSpray Applied Biosystems/MDS Sciex, Canada) ionization mode. MS/MS parameters were: capillary needle maintained at 4500 V; curtain gas at 10 psi; source temperature at 400 ºC; gas 1 and gas 2 at 45 psi; and CAD gas, medium. Separation was performed on a Synergi 4u Polar-RP column (4.0 μm, 150 × 2.0 mmi.d.; Phenomenex, USA). The mobile phases were composed of mixtures of methanol 95% and water 5% (v/v), channel A, and water and formic acid 0.1% (v/v), channel B. The separation was carried out at 30 ºC using a

segmented elution gradient of 10% A for 0-5 min, 10-90% for 5-7 min, 90% A for 7-10 min, and 10% A for 10–17 min. Between analyses the column was conditioned for 5 min with the initial proportion of the mobile phase. The flow rate was set at 250 μL min^{-1} with injection of 10 μL sample sizes. The software Analyst version 1.6.2 was used for the HPLC–ESI-MS/MS system control and data analysis.

Forty-nine phenolic compounds were monitored and the quantification was performed using calibration curves of standards. LC-ESI-MS/MS parameters for phenolic compounds identification and quantification of the KA extracts are available in Table 3-1. Compounds identities from samples were confirmed by matching two to three mass transitions for each analyte in the specific chromatographic retention time. These parameters were previously determined with the individual standard of each analyte in MS infusions and chromatographic separations using a mix of standards in LC-MS/MS.

Table 3-1– Parameters for High performance liquid Chromatography (HPLC) identification and quantification of phenolic compounds in KA extracts.

	PHENOLIC COMPOUNDS	[M-H] (m/z)	Quantitative ion (m/z)	Retention time (min)	Regression equation	R^2	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
Coumarin								
1	Scopoletin	190.9	176.0	10.93	$y= 5.8\ 10^5\ x + 11440$	0.995	0.002	0.010
2	Umbelliferone	160.9	133.1	10.76	$y= 7.8\ 10^5\ x + 32213$	0.989	0.001	0.010
Flavonoids								
3	Apigenin	268.9	117.1	12.47	$y = 2.2\ 10^5\ x + 7909$	0.998	0.002	0.006
4	Aromadendrin	287.0	151.0	11.65	$y= 3.0\ 10^5\ x + 3442$	0.999	0.010	0.030
5	Catechin	289.0	245.1	8.39	$y= 3.5\ 10^4\ x - 739$	0.996	0.030	0.090
6	Chrysin;	252.9	62.9	13.48	$y= 3.1\ 10^5\ x + 3102$	0.996	0.005	0.015
7	Epicatechin	288.9	109.0	9.14	$y= 2.1\ 10^5\ x - 5869$	0.992	0.003	0.010
8	Eriodictyol	286.9	151.0	11.66	$y= 4.5\ 10^5\ x + 15603$	0.995	0.040	0.100
9	Fustin	286.9	109.0	10.19	$y= 1.1\ 10^5\ x + 1060$	0.998	0.020	0.050
10	Galangin	268.9	117.0	12.47	$y= 5.3\ 10^2\ x + 664$	0.975	0.090	0.270
11	Hispidulin	298.9	284.0	12.57	$y= 8.9\ 10^5\ x + 4726$	0.996	0.003	0.010
12	Isoorientin	446.9	326.9	10.11	$y= 1.0\ 10^4\ x + 270$	0.998	0.007	0.020
13	Isoquercitrin	463.2	271.1	10.61	$y= 1.8\ 10^5\ x + 3876$	0.997	0.008	0.020
14	Isoxanthohumol	353.1	118.9	13.70	$y= 1.8\ 10^3\ x + 483$	0.969	0.160	0.500
15	Kaempferol	284.9	93.0	12.20	$y= 3.9\ 10^4\ x + 9788$	0.988	0.060	0.200
16	Myricetin	316.9	151.0	11.15	$y= 1.1\ 10^4\ x - 9342$	0.966	0.330	1.000
17	Naringenin	270.9	151.1	12.20	$y= 3.7\ 10^5\ x + 571$	0.996	0.004	0.010
18	Naringin	580.3	151.0	11.03	$y= 3.1\ 10^3 x + 114$	0.995	0.130	0.400
19	Pinocembrin	255.1	151.0	13.21	$y= 2.8\ 10^5\ x + 2265$	0.996	0.010	0.030
20	Quercetin	300.9	151.0	11.67	$y= 4.1\ 10^4\ x + 4118$	0.995	0.100	0.300
21	Rutin	609.2	300.1	10.61	$y= 2.6\ 10^5\ x + 11011$	0.997	0.010	0.030
22	Taxifolin	303.0	285.0	10.53	$y= 7.3\ 10^4\ x + 6622$	0.993	0.010	0.040
23	Vitexin	430.9	310.9	10.46	$y= 1.9\ 10^5\ x + 17085$	0.997	0.010	0.030
24	Xanthohumol	353.1	118.9	12.93	$y= 2.7\ 10^3\ x + 323$	0.991	0.015	0.046
Phenolic acids								

25	4-aminobenzoic	135.9	91.9	10.26	$y = 9.4\ 10^3\ x + 3101$	0.993	0.200	0.600
26	Caffeic acid	178.9	135.0	9.26	$y = 2.0\ 10^6\ x + 35690$	0.993	0.010	0.030
27	Cinnamic acid	146.9	102.9	11.65	$y = 6.3\ 10^4\ x + 13341$	0.992	0.240	0.730
28	Chlorogenic acid	353.2	191.0	9.05	$y = 2.4\ 10^5\ x + 5819$	0.997	0.010	0.030
29	Ellagic acid	300.9	145.0	11.71	$y = 1.1\ 10^4\ x - 502$	0.991	0.060	0.200
30	Ferulic acid	192.9	134.0	10.63	$y = 9.6\ 10^4\ x + 2560$	0.980	0.006	0.010
31	Gallic acid	168.9	125.0	3.73	$y = 3.4\ 10^5\ x - 23145$	0.997	0.030	0.100
32	4-Hydroxymethylbenzoic acid	150.9	136.0	10.26	$y = 2.7\ 10^5\ x + 18553$	0.997	0.120	0.360
33	Mandelic acid	150.9	107.0	6.84	$y = 1.1\ 10^5\ x - 23128$	0.993	0.160	0.480
34	Methoxyphenylacetic acid	164.9	‘121.1	10.17	$y = 5.5\ 10^4\ x + 13630$	0.993	0.170	0.500
35	*p*-Anisic acid	150.9	136.1	11.50	$y = 4.4\ 10^4\ x + 1697$	0.989	0.120	0.370
36	*p*-Coumaric acid	162.9	119.1	10.28	$y = 1.0\ 10^6\ x + 262.67$	0.998	0.003	0.010
37	Protocatechuic acid	152.9	109.0	6.21	$y = 2.6\ 10^5\ x + 13392$	0.998	0.050	0.160
38	Rosmarinic acid	359.1	161.0	10.71	$y = 2.5\ 10^5\ x + 3039$	0.998	0.002	0.010
39	Salicylic acid	136.9	93.0	10.67	$y = 2.0\ 10^6\ x + 39357$	0.995	0.010	0.020
40	Sinapic acid	223.0	164.0	10.83	$y = 7.6\ 10^4\ x + 869$	0.993	0.020	0.050
41	Syringic acid	196.9	121.1	9.87	$y = 7.4\ 10^4\ x + 2489$	0.998	0.010	0.040
42	Vanillic acid	166.9	152.0	9.38	$y = 3.8\ 10^4\ x - 2687$	0.995	0.060	0.190
Phenolic aldehydes								
43	Coniferaldehyde	177.0	162.0	11.17	$y = 4.8\ 10^5\ x + 3991$	0.994	0.005	0.010
44	Sinapaldehyde	207.0	192.1	11.33	$y = 2.8\ 10^6\ x + 92$	0.996	0.003	0.010
45	Syringaldehyde	180.9	166.0	10.66	$y = 4.8\ 10^4\ x + 58451$	0.986	0.004	0.010
46	Vanillin	150.9	136.0	10.26	$y = 1.4\ 10^5\ x + 25181$	0.991	0.020	0.070
Phenolic diterpene								
47	Carnosol	329.2	285.2	13.68	$y = 2.0\ 10^6\ x + 2628$	0.999	0.002	0.010
Stilbene								
48	Resveratrol	226.9	142.9	11.09	$y = 6.4\ 10^4\ x + 4235$	0.996	0.010	0.030

LOD – Limit of identification; LOQ – Limit of quantification, respectively calculated by signal-to-noise ratio of 3 and 10.

Source: The author (2024).

3.2.8 Carrageenan extractions

3.2.8.1 Conventional carrageenan extraction

A conventional carrageenan extraction from the KA was carried out using a Dubnoff bath (ethiktechnology, model 304-TPA) according to Boulho et al., (2017) with modifications. Briefly, 0.7 g of the seaweed sample and 70 mL of distilled water were shaken at 80 °C for 2 h. The obtained solutions were hot filtered using a vacuum pump (model 825T, Fisatom, Brazil), then rotaevaporated to half the initial volume and precipitated with cold ethanol at a ratio of 1:3. The precipitate was separated and dried in an oven at 60 ºC for 24 h. All extractions were performed in triplicate.

3.2.8.2 Microwave carrageenan extraction

Carrageenan extraction was conducted using the microwave reactor following the procedure described in section 3.2.3.4. The carrageenan fraction from KA was recovered using water as solvent (MAE-water), and the extraction was carried out maintaining a solid/liquid ratio of 1/100 (0.2 g in 20 mL), with seaweed samples mixed with water by a magnetic stirring bar in a borosilicate glass flask (G30). Briefly, the G30 bottle was closed and placed at the MAE unit, which operates in an automated three steps cycle: the first two steps were the same as mentioned in section 3.2.3.4, while the third step (cooling) was carried out to 70 °C using compressed air. The MAE-water processing time was defined by a kinetics assay, carried out at 80 ºC, and at different times of 5, 15 and 30 min. Then, after extraction time definition, MAE-water assays were conducted at 60, 80 and 100 ºC. The recovered extracts (water solutions) were hot filtered using a vacuum pump, then rotaevaporated to half the initial volume and precipitated with cold ethanol at a ratio of 1:3. The precipitate was separated and dried in an oven at 60 ºC for 24 h. All extractions were performed in triplicate.

3.2.8.3 High pressure carrageenan extraction (PWCE)

The high-pressure carrageenan extraction was performed according to (GERENIU; SARAVANA; CHUN, 2018), with modifications, using a jacketed stainless-

steel reactor with internal volume of 70 mL. The jacket extractor was kept at a temperature of 60 or 80 ºC by a thermostatic bath. Briefly, 0.7 g of seaweed sample were placed inside the reactor with a magnetic stirring bar, and distilled water was pumped (ConstaMetric® 3200, Thermo Separation Products) into the reactor until pressure of 50 bar (± 2 bar). This pressure was kept constant by intermit pumping of water into the reactor for 15 minutes. After the extractions the resulting solutions were hot filtered using a vacuum pump, then rotaevaporated to half the initial volume and precipitated with cold ethanol at a ratio of 1:3. The precipitate was separated and dried in an oven at 60 ºC for 24 h. All extractions were performed in triplicate.

3.2.8.4 Carrageenan yield

The carrageenan yield was calculated as the ratio between dry carrageenan mass and dried seaweed sample used for extraction multiplied by 100%. Results are expressed as mean value and standard deviation (expressed as percentage) of triplicate assays.

3.2.8.5 FTIR from carrageenan fraction samples

The Fourier transformed infrared (FTIR) analysis was conducted by the infrared equipment Cary 660 (Agilent Technologies, Santa Clara, CA, USA). Prior to analyses, carrageenan samples (extracted and commercial) were mixed with potassium bromide (KBr) to form disks, which were thereafter scanned from 400 to 4000 cm^{-1} at 2 cm^{-1} resolution.

3.2.8.6 Viscosity and gel strength

The analysis of viscosity and gel strength from the carrageenan samples were carried out according to procedure described by (BONO; ANISUZZAMAN; DING, 2014). First, the samples (0.45 g) were dissolved in 30 mL of distilled water (1.5% solution) and the solution was heated at 75 ºC until the complete solubilization (for about 30 min). The viscosity was determined using a ThermoHaake ViscoTester 6 L at 30 rpm, and 75 °C (controlled by solid Steel circulating bath), using the L1 spindle, and the measurement was taken after six complete revolutions, with three readings for each

sample. The assay was performed in duplicate and the results are expressed in centipoise (cp) (mean ± standard deviation).

Regarding gel strength, evaluated according to Bono et al., (2014), the solubilized solution was stored in an aluminum cylindrical container (50 mm diameter, 30 mm height) at room temperature for 24 h prior to analysis. The gel strength was evaluated using Texture Analyzer TA.HD. plus (Stable Micro Systems, Godalming, Surrey, England) and a cylindrical steel (11.5 mm diameter and 50 mm height). The penetration speed was 2 $mm.s^{-1}$ at a depth of 10 mm. A load cell of 50 kg was used. Five readings were performed for each sample and the analysis was performed in duplicate. Results are expressed in $g.cm^{-2}$ (mean ± standard deviation).

3.3 RESULTS AND DISCUSSION

3.3.1 Oily fraction

SFE-CO_2 was performed for the recovery of the oily fraction from the KA samples, defatting the raw material. The SFE-CO_2 results (process yield and gas-chromatographic profile of the extract) were compared with SOX-hexane, due to the non-polar characteristic of the solvents.

The SFE-CO_2 yield was 0.43%, while SOX-hexane was 0.75 ± 0.09 %. Probably the lower yield of SFE-CO_2, compared to SOX-hexane is associated with the higher temperature and extraction time, and solvent recycling, from SOX-hexane, which increases yield (Andrade et al., 2017). SFE-CO_2 was performed at 50 °C whereas SOX-hexane was conducted at temperature close to hexane boiling point (69°C). On the other hand, SFE-CO_2 was carried out for 2 h, whereas SOX-hexane for 6 h. In addition, supercritical CO_2 is a selective solvent, narrowing the components profile, compared to SOX-hexane (TEIXEIRA *et al.*, 2020). These results for SFE-CO_2 and SOX-hexane were similar to obtained by Araújo et al., (2020) and by Fayaz et al., (2005), which provided yields lower than 1% for KA extraction of non-polar fraction using SOX-hexane indicating the low oily fraction from KA.

The GC chemical profiles from SFE-CO_2 and SOX-hexane are presented in Table 3-2 and Table 3-3, respectively. As expected, many identified compounds were detected

from the SOX-hexane (59 components), compared to 24 compounds detected at SFE-CO_2. On the other hand, 13 fatty acids were identified from SFE-CO_2, while from SOX-hexane only hexadecanoic acid, octadecanoic acid and palmitelaidic acid were detected. The main fatty acids detected from SOX-hexane were also observed, at the highest relative area, from SFE-CO_2. The chromatogram peak area from hexadecanoic, octadecanoic and palmitelaidic acids, from SFE-CO_2, were, very likely, higher than that observed for SOX-hexane, indicating a reduction in fatty acid content at SOX temperatures (higher than SFE temperature). Similarly, Messyasz et al., (2018) also found lower fatty acids content from SOX-hexane, compared to SFE-CO_2, for assays conducted for *Cladophora glomerata.*

Table 3-2- Compounds identified using Gas Chromatography mass spectrometry GC-MS/MS in KA extract from Supercritical fluid extraction using CO_2 as solvent.

Retention Time (min)	**Compound**	**Peak Area (%)**
	Fatty acids	
26.50	Dodecanoic acid	0.16
29.82	Nonanedioic acid	0.71
33.04	n-pentadecanoic acid	0.2
34.87	Palmitelaidic acid	6.79
35.39	Hexadecanoic acid	23.12
36.98	Heptadecanoic acid	1.59
38.16	9,12-Octadecadienoic acid	0.41
38.34	Oleic acid	2.46
38.44	11-trans-Octadecenoic acid	0.76
38.98	Octadecanoic acid	8.48
40.17	11-cis-Octadecenoic acid	1.21
41.08	Arachidonic acid	0.69
45.46	Docosanoic acid	1.08
	Others	
10.68	Propanoic acid, 2-[(trimethylsilyl)oxy]-	0.16
22.87	Acetic acid, bis[(trimethylsilyl)oxyl]-	0.22
23.53	2,4-Disilapentane, 3-(tert-butoxyvinylidene)-2,2,4,4-tetramethyl-	0.77
23.64	2,4,6-Tri-t-butylbenzenethiol	1.28
27.58	1,2-Benzenedicarboxylic acid, ditridecyl ester	0.28
27.68	Nonadecane	1.16
31.01	L-alanine, N--(trifluoroacetyl)-, trimethylsilyl ester	1.11
32.69	Trimethylsilyl ether of Glucitol	2.32

52.75	Cholesterol trimethylsilyl ether	0.21
52.93	Octacosanol trimethylsilyl ether	0.89
53.35	Trimethylsilyl ether of glycerol	3.72

Source: The author (2024).

Table 3-3- Compounds identified using Gas Chromatography mass spectrometry GC-MS/MS in KA extract from Soxhlet using hexane as solvent..

Retention Time (min)	**Compound**	**Peak Area (%)**
	Fatty acids	
34.92	Palmitelaidic acid	0.63
35.25	Hexadecanoic acid	1.83
36.50	Octadecanoic acid	0.26
	Others	
8.04	Hexanedioic acid. 3-methyl-. bis(tert-butyldimethylsilyl) ester	0.43
9.56	Acetamide. 2.2.2-trifluoro-N-methyl-N-(trimethylsilyl)-	2.92
10.95	Tris(trimethylsilyl)borate	0.59
11.04	Silanamine. 1.1.1-trimethyl-N-(trimethylsilyl)-N-[2-[(trimethylsilyl)oxy]ethyl]-	1.15
11.61	Glyoxime. bis(trimethylsilyl)-	0.56
11.95	4-Pentenoic acid	1.15
12.72	Cyclopentanecarboxylic acid	4.58
12.80	Cyclohexene. 3-methyl-1-(trimethylsilyloxy)-	3.91
13.05	.beta. Hydroxybutyric acid di-TMS	0.68
13.22	2-Propen-1-ol. 2-(trimethylsilyl)methyl-. acetate	1.44
13.41	Butanoic acid. 3-[(trimethylsilyl)oxy]-	2.93
13.56	2-Hydroxy hexanoic acid	1.42
13.72	Pentanoic acid. 2-[(trimethylsilyl)oxy]-.	2.13
13.87	3-Methyl-1.2-bis(trimethylsilyl)butane	1.21
14.04	Hexanoic acid. 2-[(trimethylsilyl)oxy]-.	2.86
14.09	Butane. 2.3-bis(trimethylsiloxy)-	1.14
14.16	3.6-Dioxa-2.7-disilaoctane. 2.2.4.7.7-pentamethyl-	3.12
14.46	2-Butene. 3-methyl-2-[(tert-butyldimethylsilyl)oxy]-1-cyano-	1.56
14.75	2.4(1H.3H)-Pyrimidinedione. dihydro-1.3-bis(trimethylsilyl)-	1.39
14.84	3.10-Dioxa-2.11-disiladodeca-5.7-diene. 2.2.11.11-tetramethyl-	2.48
14.95	Acetic acid. iodo-	2.15
15.22	4.6-Dioxoheptanoic acid ditms	1.27
15.48	Hexanoic acid. 5-oxo-.	1.49
15.72	Sorbic acid.	1.68
16.04	Cyclopropane. 1-methylene-2-(1-trimethylsilyloxyvinyl)-	5.08
16.12	Cyclopenta[g]-2-benzopyran. 1.3.4.6.7.8-hexahydro-4.6.6.7.8.8-hexamethyl-	0.67
16.3	3.4-Dihydrocoumarin. 4.4.5.7.8-pentamethyl-6-cyano-	0.13

16.43	3.8-Dioxa-2.9-disiladecane. 2.2.9.9-tetramethyl-5.6-bis[[(trimethylsilyl)oxy]methyl]-	0.13
17.09	Pentenoic acid. 4-[(trimethylsilyl)oxy]-.	0.13
17.49	Butanedioic acid. bis(trimethylsilyl) ester	0.55
17.69	1H-Azepine. hexahydro-1-(1.2.3.4-tetrahydro-4.5.8-trimethyl-2-naphthyl)-	0.75
17.88	Cyclohexanamine. N.N-dimethyl-1-[2-thionaphthenyl]-	1.71
18.03	3-Butenoic acid. 3-(trimethylsiloxy)-. trimethylsilyl ester	3.86
18.31	2-Pentenoic acid. 3-methyl-2-[(trimethylsilyl)oxy]-. trimethylsilyl ester	1.01
18.39	Pyrrole-2-carboxylic acid. N-trimethylsilyl-. trimethylsilyl ester	1.26
18.46	Ferrocene. 1.2.3.4.5-pentamethyl-	1.13
18.71	Ethanone. 1-(3-chloro-4-methoxyphenyl)-	1.03
18.92	4.6-Dioxohept-2-enoic acid. tri-TMS	0.93
19.13	2-Pentenoic acid. 2-[(trimethylsilyl)oxy]-.	0.19
19.67	3.9-Dioxa-2.10-disilaundecane. 2.2.10.10-tetramethyl-5-[(trimethylsilyl)oxy]-	0.29
20.28	3-Trimethylsiloxy(trimethylsilyl)caproate	0.58
20.39	Pentanedioic acid. bis(trimethylsilyl) ester	1.01
20.57	1H-Indole-3-acetonitrile	1.23
20.99	Methylmaleic acid. bis(trimethylsilyl) ester	2.33
21.09	Cyclopentane. 2-methyl-1.4-bis[(trimethylsilyl)oxy]-3-(2-propenyl)-	1.61
21.49	2-Ethylacetoacetate. bis(trimethylsilyl)- deriv.	1.59
21.60	1.2-Bis(trimethylsiloxy)cyclohexene	0.39
21.99	(E)-4.6-Dioxohept-2-enoic acid. di-TMS	0.62
22.78	Methyl 2.3-bis-O-(2.4-dinitrophenyl)-.alpha.-d-glucopyranoside	0.17
23.24	L-Proline. 1-(tert-butyldimethylsilyl)-. tert-butyldimethylsilyl ester	0.84
23.63	Imidazolidin-4-one. 2-t-butyl-1.3-dimethyl-5-trimethylsilylmethyl-	0.26
23.71	2.4.6-Tri-t-butylbenzenethiol	0.37
24.44	Piperazine. 2.5-diketo-. bis(trimethylsilyl)-	0.1
25.24	Ethanesulfonic acid. 2-[(trimethylsilyl)oxy]-.	0.43
38.34	Pentanoic acid. 4-methyl-.	0.13
53.01	3.beta..5-Bis(trimethylsiloxy)-5.alpha.-cholestane	0.68

Source: The author (2024).

Considering the fatty acids results (Table 3-2 and Table 3-3), hexadecanoic acid (palmitic acid) was the main component, in relative area, detected from KA oily fractions (SFE-CO_2 and SOX-hexane), representing 48 and 67% of the total identified fatty acids for SFE-CO_2 and SOX-hexane, respectively. Hexadecanoic acid was also the main compound from KA identified by Jayasinghe et al., (2018) and by Naseri et al., 2019),

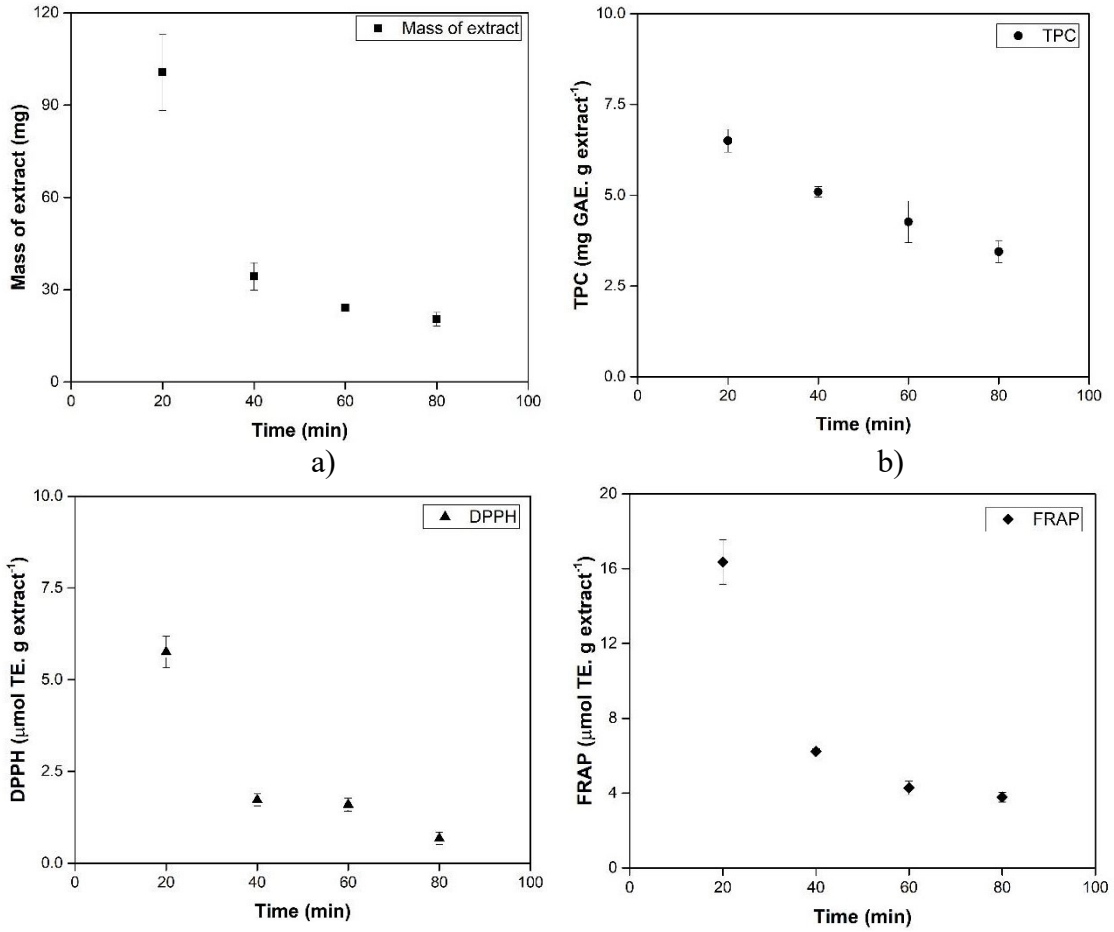
with 40 and 46% of the total fatty acids, respectively, from samples recovered using chloroform:methanol (2:1), which confirms hexadecanoic acid as the main fatty acid from KA. Then, although SFE-CO_2 provided lower yield compared to SOX-hexane, the recovered sample is richer in fatty acids than SOX-hexane.

3.3.2 Phenolics fraction

Ethanol was used as solvent by different methods (PLE, MAE, SFE-Co and SOX) for the recovery of the phenolics fraction from KA due to its GRAS (Generally recognized as Safe) characteristic, and low carrageenan solubility (YOUNES *et al.*, 2018). The low solubility of carrageenan in ethanol is desirable to enable the separation of the phenolic compounds from the carrageenan fractions. Besides, ethanol is a low-cost solvent, and easily available in high purity.

The PLE condition of 10 MPa was selected to keep the solvent in its liquid state, and because above 10 MPa the increase in pressure provides low effect on PLE performance (HERRERO *et al.*, 2013). The flow rate was defined (3 $mL.min^{-1}$) based on studies by Andrade et al., (2017); Rudke et al., 2019 and Santos et al., (2019). The processing time was defined based on the kinetics study, conducted at different extraction times (20, 40, 60, and 80 min), with the results presented in Figure 3-1, compared in terms of process yield (Fig. 1a), TPC (Fig 1. b), DPPH (Fig 1 c), and FRAP (Fig. 1d) values for the recovered extract samples.

Figure 3-1 – Kinetics assays for pressurized liquid extraction (PLE) from *Kappaphycus alvarezii* with ethanol as solvent, at 60 ºC, 10 MPa and 3 $mL.min^{-1}$, and samples recovered at 20, 40, 60 and 80 min of extraction: (a) mass of extract; (b) Total phenolic compounds (TPC) values; (c) DPPH values; (d) FRAP values.

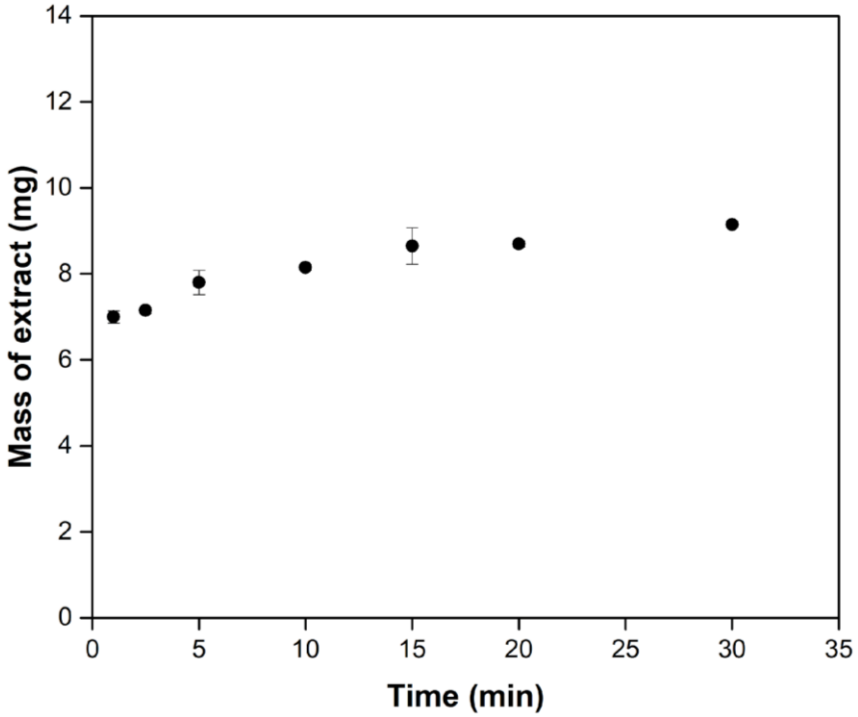


Source: The author (2024).

According to Figure 3-1, all evaluated parameters decrease with increasing the extraction time, with the lowest values obtained from 60 to 80 min. This result suggests that the phenolic compounds and other molecules with antioxidant potential are recovered in the beginning of the extraction. This behavior was explained by Mustafa & Turner, (2011), which defined two mass transfer steps: the initial, controlled by the solute(s) solubility, and the diffusion-controlled step. Therefore, in the beginning of the extraction, the compounds are easily recovered due to strong interaction with the solvent, and its extraction decreases with time progressing. Then, the compounds from inside the solid matrix, also soluble in the solvent at the processing conditions, are extracted with time progressing, and transported by diffusion to solid surface, reducing the extraction rate. This behavior is analogous to described for SFE (WEINHOLD *et al.*, 2008).

Then, the PLE time was based on the combined results from Figure 3-1 (process yield and quality attributes from the samples). According to the results, low difference was detected above 40 min extraction time, particularly for yield, DPPH and FRAP values (Fig. 1 a, c and d), therefore, 40 min was defined as PLE time. Similarly, the processing time for MAE assays was defined based on the kinetics extraction curve (Figure 3-2), which shows low yield variation above 15 min, with no significant difference (at 5% level) from 15 to 20 and 30 minutes, where the values were 8.7 ± 0.4; 8.7 ± 0.1 and 9.2 ± 0.1 mg of extract, respectively. This enables the definition of 15 min for MAE processing. Therefore, PLE and MAE results, for assays conducted with ethanol at 40 and 15 min, respectively, and different temperatures, are compared in Table 3-4 in terms of yield, TPC, DPPH and FRAP values. Results for SOX-ethanol are presented as reference for conventional method.

Figure 3-2- Kinetic extraction of *Kappaphycus alvarezii* using ethanol at 60 ºC in Microwave-assisted Extraction (MAE).



Source: The author (2024).

Table 3-4 – Global yield, total phenolics content, and antioxidant capacity by DPPH and FRAP methods from extract samples recovered by Microwave assisted extraction (MAE), Pressurized Liquid

Extraction (PLE) , and Soxhlet (SOX) using ethanol as solvent at different temperatures compared to SOX-ethanol.

Method	T (ºC)	Yield* (%)	TPC* mg GAE. g^{-1}	DPPH* µmol TE. g^{-1}	FRAP* µmol TE. g^{-1}
MAE	40	1.14 ± 0.08^{f}	6.14 ± 0.09^{c}	4.60 ± 0.08^{bc}	17.08 ± 0.27^{d}
PLE		1.55 ± 0.06^{de}	7.82 ± 0.17^{a}	8.06 ± 1.26^{a}	23.52 ± 0.27^{a}
MAE	60	1.32 ± 0.03^{ef}	5.56 ± 0.11^{d}	3.88 ± 0.22^{bc}	15.51 ± 0.27^{e}
PLE		1.88 ± 0.07^{cd}	8.06 ± 0.34^{a}	5.45 ± 1.31^{b}	21.05 ± 0.43^{b}
MAE	80	1.98 ± 0.15^{c}	4.66 ± 0.16^{e}	2.66 ± 0.22^{cd}	11.86 ± 0.13^{f}
PLE		2.37 ± 0.16^{b}	7.01 ± 0.11^{b}	4.64 ± 0.61^{bc}	18.47 ± 0.35^{c}
SOX	78.3	2.95 ± 0.12^{a}	3.42 ± 0.02^{f}	2.11 ± 0.17^{d}	5.67 ± 0.09^{g}

*The same letter in the same column indicates no significant difference at the level of 5% by the Tukey test ($p < 0.05$).

Source: The author (2024).

Yield values from phenolic fraction varied from 1.14 to 1.98% for MAE, and from 1.55 to 2.37% for PLE, while SOX-ethanol reached 2.95%. There was no significant difference at 5% level in yield from MAE and PLE between 40ºC and 60ºC, while from 60 to 80ºC a significant difference was observed. A positive influence of temperature on yield values, for MAE and PLE, was detected from 40 to 80 ºC and 60 to 80 ºC, respectively, probably due to an increase in solute-solvent interactions with increasing temperature, contributing to solute solubilization, as reported by Carpena et al., (2021), related to maceration of *Chondrus crispus*, *Mastocarpus stellatus*, and *Gigartina pistillata* (red seaweeds). The yield values from Table 3-4 are similar to obtained by Prasasty et al., (2019) for ethanolic maceration of KA at 50°C, which reached 2.2% yield. Otherwise, Araújo et al., (2020) obtained yields of 2.67% and 3.43% for methanolic maceration of different KA strains, at room temperature for 72 h (solvent change every 24 h). Bhuyar et al., (2020) used ethanol 70% as solvent and reached yields up to 15%, although, the use of aqueous solutions as solvent probably caused the recovery of carrageenan and protein, together with the phenolic fraction.

The yield differences for the methods PLE, MAE, and SOX-ethanol can be associated to variation in extraction time (40 min, 15 min, and 6 h, respectively), and also on intrinsic characteristics of the methods, such as high pressure (PLE), microwaves weakening molecules bonds (MAE) and solvent reflux (SOX).

The TPC, DPPH, and FRAP results from the ethanolic samples, recovered by different processes and temperature conditions, are also compared at Table 3-4. TPC values ranged from 3.42 to 8.06 mg GAE.g^{-1}, while DPPH and FRAP varied from 2.11 to 8.06 μmol TE.g^{-1}, and from 5.67 to 23.52 μmol TE.g^{-1}, respectively. PLE method provided better phenolic (TPC) and antioxidant (DPPH and FRAP) performances, followed by MAE and then SOX. The increase in temperature mostly reduced TPC and antioxidant results for PLE and MAE, and this suggested negative effect of temperature on TPC results was also observed by Afonso et al., (2021), which obtained higher TPC from ethanolic extraction from *Gracilaria gracilaris* (Red seaweed) at 40 ºC, compared to 70 °C. The temperature increase (MAE and PLE) also reduced FRAP values, probably due to the degradation of phenolic compounds and other antioxidant molecules such as chlorophyll, carotenoids and phycoerythrin (BASKARARAJ *et al.*, 2019; UJU *et al.*, 2020). The TPC results were higher than reported in the literature for KA extract. For instance, Araújo et al., (2020) found TPC from 0.408 to 0.585 mg GAE.g^{-1} for methanolic extracts from different KA strains; Ling et al., (2015), using methanol 80% as solvent, obtained 0.261 to 0.533 mg GAE.g^{-1} from KA samples at different drying conditions; Charles et al., (2020) detected TPC from 0.14 to 0.36 mg GAE.g^{-1} from ethanolic extracts from KA samples, also at different drying conditions Chew et al., (2008) using methanol as solvent, detected TPC of 0.28 mg GAE.g^{-1} for KA samples. Otherwise, Bhuyar et al., (2020) detected TPC of 20.25 mg GAE.g^{-1} for extracts recovered from KA by 70% ethanol. Despite the differences in KA samples conditions and strains, from all these authors, it is worth noting that aqueous solutions as solvents increases TPC and global yield, as provided by Chew et al., (2008) and by Diyana et al., (2015), which is probably due to higher solubilization of proteins, carrageenan and other compounds, increasing yield and affecting the TPC analysis (EVERETTE *et al.*, 2010; PRIOR; WU; SCHAICH, 2005).

In general, the antioxidant activity should be evaluated by more than one method, due to the different mechanisms detected by the methodologies. The antioxidant activity from KA extracts was evaluated by FRAP method, which detects single electron transfer (SET), and by DPPH, which is based on SET and HAT (hydrogen atom transfer) (PRIOR;

WU; SCHAICH, 2005). These are widely used methods due to their high reproducibility, and well applied for KA samples.

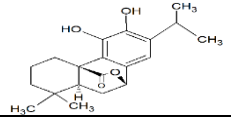
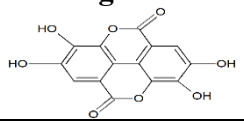
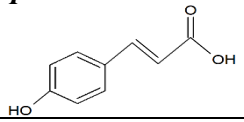
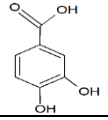
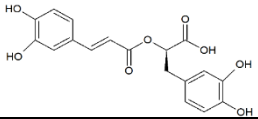
The antioxidant results (Table 3-4) were higher than reported by Mohamed & Abdullah, (2016) for samples recovered by acetone 50% from KA, with FRAP values from 0.100 to 0.169 μmol of TE.g^{-1}. Besides, the antioxidant activity depends on the drying pre-treatment, on solvent type and algae strain used (ARAÚJO *et al.*, 2020; KUMAR; GANESAN; RAO, 2008; LING *et al.*, 2015). The FRAP results were higher than obtained by DPPH, which was also observed by Araújo et al., (2020) for methanolic extracts from KA demonstrating a higher tendency of the KA compounds to be detected by SET antioxidant mechanism (PRIOR; WU; SCHAICH, 2005). In terms of TPC, DPPH, and FRAP values, best PLE performance was detected, followed by MAE, compared with SOX-ethanol, which is most likely due to the combination of short time, high pressure and solvent renewal.

3.3.2.1 Chemical profile of the phenolic fraction

The KA extract samples obtained by PLE (40, 60, and 80°C), MAE (40, 60, and 80°C), SOX-ethanol and SFE-Co (8% ethanol) were analyzed by LC-ESI-MS/MS (section 3.2.7.2). From the 49 standard phenolic components evaluated, 13 were detected from KA extract samples: apigenin, cinnamic acid, chlorogenic acid, hispidulin, isoorientin, isoquercitrin, kaempferol, methoxyphenylacetic acid, naringenin, salicylic acid, scopoletin, rutin, and xanthohumol. The main identified phenolic components were: chlorogenic acid and salicylic acid, which were detected at all samples; and also xanthohumol, apigenin, and isoorientin, present at one sample each, the PLE 40, PLE 80 and MAE 80, respectively. Besides the 13 compounds identified, five others were quantified, as presented at Table 3-5. The amount of the quantified phenolics ranged from 0.91 to 24.99 μg.g^{-1}, and there was no difference between the samples obtained with PLE at different temperatures. Furthermore, there were no differences between the samples obtained with SFE-Co and MAE at different temperatures. For SOX-ethanol, otherwise,

none of the phenolic components were quantified, corroboration with the low values of TPC, FRAP and DPPH previously described.

Table 3-5- Phenolic compounds quantified from ethanolic extracts obtained from Microwave assisted extraction (MAE), pressurized Liquid Extraction (PLE), Soxhlet with ethanol (SOX-ethanol) and Supercritical Fluid Extraction with co-solvent (SFE-Co) ($\mu g.g^{-1}$).

PHENOLIC COMPOUNDS	PLE 40 ºC*	PLE 60 ºC*	PLE 80 ºC*	MAE 40 ºC*	MAE 60 ºC*	MAE 80 ºC*	SOX-ethanol*	SFE-Co*
Carnosol 	0.17 ± 0.01[a]	0.15 ± 0.01[a]	0.15 ± 0.03[a]	0.15 ± 0.03[a]	0.15 ± 0.01[a]	0.15 ± 0.02[a]	<LOD	0.16 ± 0.04[a]
Ellagic acid 	22.19 ± 1.70[a]	18.89 ± 12.11[a]	13.96 ± 0.00[a]	<LOD	<LOD	<LOD	<LOD	<LOD
***p*-coumaric acid** 	1.15 ± 0.46[a]	<LOQ	0.98 ± 0.38[a]	0.30 ± 0.03[a]	0.38 ± 0.09[a]	0.75 ± 0.02[a]	<LOQ	0.47 ± 0.07[a]
Protocathecuic acid 	<LOD	<LOD	7.80 ± 2.06	<LOD	<LOD	<LOD	<LOD	<LOD
Rosmarinic acid 	1.48 ± 0.14[a]	1.42 ± 0.10[a]	1.24 ± 0.10[a]	1.28 ± 0.00[a]	1.23 ± 0.05[a]	<LOD	<LOD	1.38 ± 0.05[a]
Total quantifiable compounds	24.99 ± 1.77[a]	20.47 ± 12.11[a]	24.13 ± 2.10[a]	1.73 ± 0.04[b]	1.76 ± 0.10[b]	0.91 ± 0.03[b]	-	2.01 ± 0.10[b]

*The same letter in the same column indicates no significant difference at the level of 5% by the Tukey test ($p < 0.05$). limits of identification (LOD) and quantification (LOQ)

Source: The author (2024).

Then, PLE was more efficient in phenolics recovery compared to MAE and SOX. Ellagic acid was the quantified in the largest amount from PLE samples, from 57 to 92% of the total quantified phenolics. It is worth pointed out that protocatechuic acid was quantified only at PLE-80 ºC sample, demonstrating high thermal stability, compared to other antioxidant molecules. Likewise, Vo Dinh et al., (2018) reported the recovery of protocatechuic acid increased up to 225 ºC, from brown macroalgae (*Saccharina japonica*) biomass.

Baskararaj et al., 2019) and Namvar et al., (2012) also reported the presence of chlorogenic acid, cinnamic acid and rutin from KA samples. Apigenin, cinnamic acid, chlorogenic acid, rutin, *p*-coumaric acid, protocatechuic acid, rosmarinic acid, and scopoletin have also been reported from extracts from the red algae *Gracilariopsis persica*, *Chondrus crispus*, *Centroceras* sp., and *Grateulopia* sp.(ALKHALAF, 2021; POURAKBAR *et al.*, 2021a; ZHONG *et al.*, 2020). However, to the best of our knowledge, this is the first report with quantification of phenolic compounds from KA biomass, and this is the first detection of hispidulin, isoorientin, isoquercitrin, methoxyphenylacetic acid and xanthohumol from red seaweed.

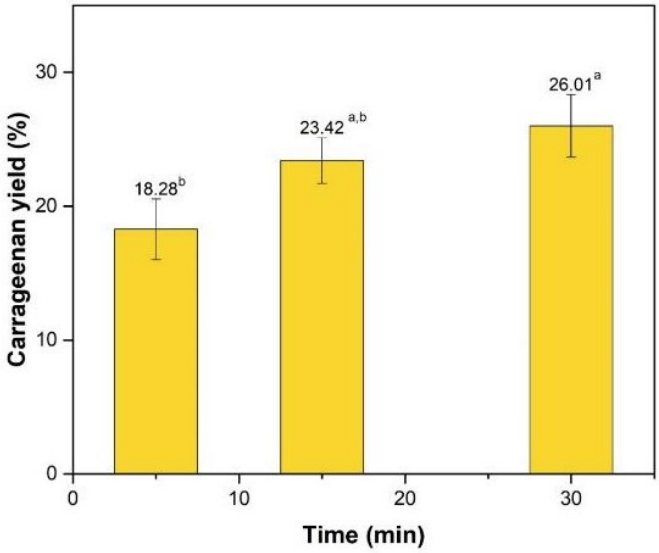
Considering the quantified compounds, carnosol is a diterpene phenol with neuroprotective activity and anticancer properties; *p*-coumaric acid is a hydroxylated derivative of cinnamic acid and a precursor of polyphenols, flavonoids and other phenolic acids, with neuroprotective effects and antineoplastic activities; rosmarinic acid is an ester of caffeic acid with antiviral and antimutagenic activities; protocatechuic acid is a phenolic acid with anticancer, antiulcer, antidiabetic, antiaging, antifibrotic, antiviral, analgesic, antiatherosclerotic, hepatoprotective, neurological and nephroprotective activities; ellagic acid is a polyphenol with anticarcinogenic, antiplasmodial, antiviral, hepatoprotective, antifibrotic, immunomodulatory and neuroprotective activities. Furthermore, these components have antioxidant, anti-inflammatory and antimicrobial activity (ALSAMRI *et al.*, 2019; FERREIRA *et al.*, 2019; GUPTA *et al.*, 2021; KAKKAR; BAIS, 2014; LUO *et al.*, 2020).

Thus, combining the results of phenolics recovery and quality, the PLE method showed better performance than MAE and SOX procedures.

3.3.3 Carrageenan fraction

Figure 3-3 shows the influence of microwave extraction time, up to 30 min extraction, for carrageenan recovery at 80 ºC with water. The carrageenan yield ranged from 18.28 to 26.01%, from 5 to 30 min MAE, increasing with progressing time, and with no significant difference at 5% level from 15 to 30 min. Therefore, 15 minutes was defined to carrageenan recovery by MAE, and to evaluate the temperature effect, for assays conducted at 60 ºC and 100 ºC. The same extraction time was used for the carrageenan recovery by PWCE.

Figure 3-3– Influence of extraction time for carrageenan recovery by Microwave assisted extraction (MAE) at 80 ºC using water as solvent.



Source: The author (2024).

The results for carrageenan yield, viscosity and gel strength are compared at Table 3-6, for samples obtained by MAE and PWCE at temperatures, and compared with the conventional carrageenan extraction. The carrageenan yield by MAE ranged from 17.16 to 23.42%, with no significant difference for assays at 80 ºC and 100 ºC, while for PWCE ranged from 29.00 to 34.12 %, with no significant difference for assays at 60 ºC and 80 ºC. Otherwise, the conventional carrageenan extraction provided yield of 50.25%, probably because of the long extraction time (2 h), compared to MAE and PWCE methods.

Table 3-6– Carrageenan yield, viscosity and gel strength of samples using Microwave assisted extraction (MAE) and Pressurized water carrageenan extraction (PWCE) in different temperatures and comparison with conventional carrageenan extraction method.

Extraction Technique	T (ºC)	Time (min)	Yield (%)	Viscosity (cp)	Gel Strength (g.cm^{-2})
MAE-water	60	15	17.16 ± 2.46^{d}	21 ± 1^{d}	26 ± 1^{c}
PWCE			29.00 ± 1.60^{bc}	60 ± 2^{b}	25 ± 1^{c}
MAE-water	80		23.42 ± 1.76^{c}	51 ± 2^{bc}	36 ± 4^{bc}
PWCE			34.12 ± 1.38^{b}	57 ± 9^{b}	36 ± 3^{b}
MAE-water	100		23.02 ± 2.94^{cd}	22 ± 3^{d}	32 ± 7^{bc}
Conventional	80	120	50.25 ± 2.65^{a}	46 ± 2^{c}	33 ± 2^{bc}
Commercial			-	135 ± 7^{a}	131 ± 13^{a}

*The same letter in the same column indicates no significant difference at the level of 5% by the Tukey test ($p < 0.05$).

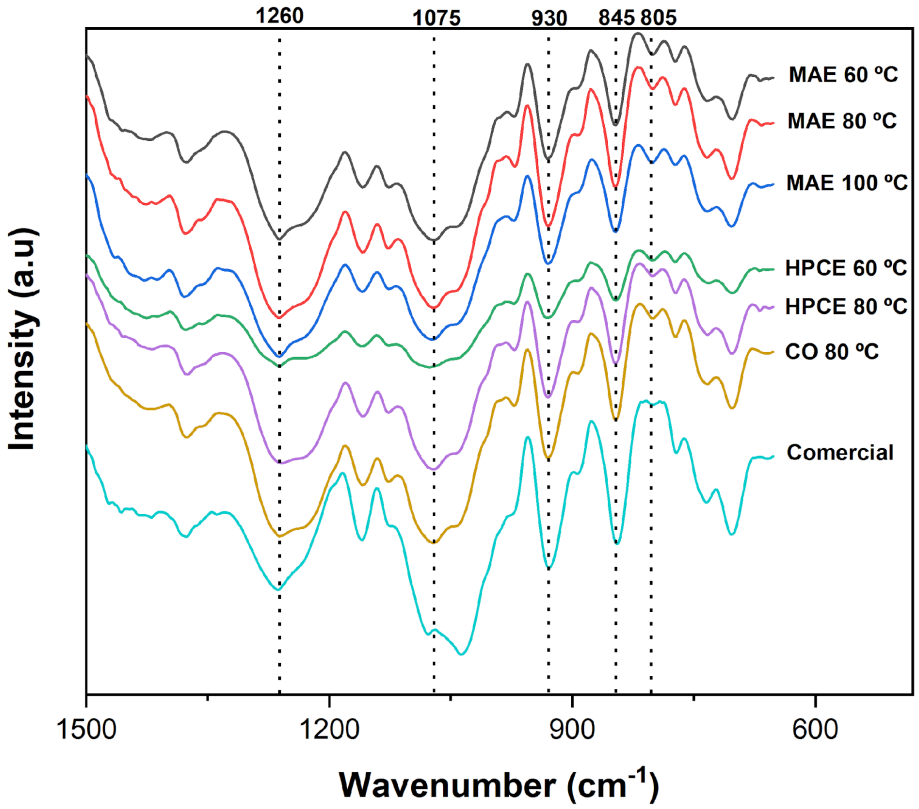
Source: The author (2024).

The carrageenan yield by MAE agrees with data by Boulho et al., (2017), which reached 14% to 30%, and by Vázquez-Delfín et al., (2014), from 17% to 25%, for MAE conducted at different times and temperatures (85 to 105 ºC and 10 to 25 min) for seaweed *Solieira Chordalis* and *Hypnea musciformis*, respectively. However, no carrageenan yield obtained from KA using MAE was found. Regarding PWCE, carrageenan yields from KA, reported by Gereniu et al., (2018) from 60 and 90 ºC (~55 and 60%) were higher than showed at Table 3-6.

The Fourier transform infrared (FTIR) spectra from carrageenan recovered by PWCE and MAE-water at 60, 80 and 100 ºC were compared with the conventional extraction at Figure 3-4. The carrageenan FTIR characteristics are evaluated according to Pereira et al., (2009) which describes the presence or absence of bands from different types of carrageenan. The band at 1260 cm^{-1} indicates the presence of sulfate ester (detected at all carrageenan types except the β-carrageenan), the band at 1070 cm^{-1} indicates the CO bond of 3,6 anhydrogalactose (κ-,ι- and θ-), while the band at 930 cm^{-1} indicates 3,6-anhydro-D-galactose (present at κ-, ι- and θ-), and at 845 cm $^{-1}$ indicates D-galactose-4-sulfate (κ-,ι-,). The spectra from all carrageenan samples were equivalent to commercial carrageenan, except for the presence of a band at 805 cm^{-1}, regardless the extraction method or temperature used. The low absorbance band at 805 cm^{-1} is attributed

to 3,6-anhydro-D-galactose-2-sulfate, indicating the presence of impurity (characteristic band of ι-carrageenan) (ŞEN; ERBOZ, 2010).

Figure 3-4– Fourier transform infrared (FTIR) spectra from commercial carrageenan compared to carrageenan samples recovered by Microwave assisted extraction (MAE) and pressurized water carrageenan extraction (PWCE) at different temperature conditions.



Source: The author (2024).

The viscosity of carrageenan obtained by MAE-water and PWCE ranged from 21 ± 1 to 57 ± 9 cP, for MAE-water at 60 ºC and for PWCE at 80°C, respectively (Table 3-6). The viscosity from samples PWCE 60 ºC, PWCE 80 ºC and MAE 80 ºC show no statistical differences at 5% significance, with the highest viscosity value, although lower than obtained from the commercial sample (135 ± 7 cP). Very likely, the first-step extraction (KOH), used to obtain the commercial carrageenan, does affect the chemical structure providing a sample with higher viscosity as reported by Rafiquzzaman et al., (2016) and by Freile-Pelegrín et al., (2006). The viscosity values for PWCE 60 ºC (60 ± 2 cP), MAE 80 ºC (51 ± 2 cP) and PWCE 80 ºC (57 ± 9 cP) were higher than reported by

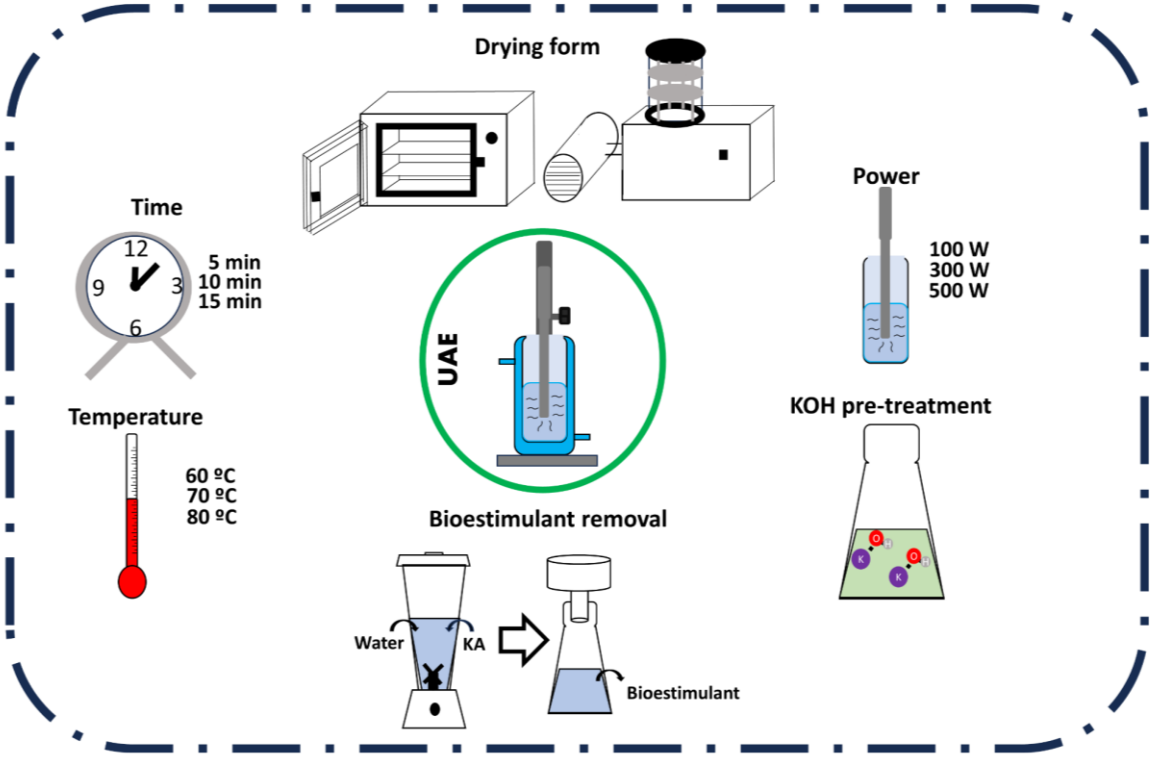
Rafiquzzaman et al., (2016) (~30-40 cP) and by Webber et al., (2012) (9 to 45 cP), which extracted carrageenan from *Hypnea musciformis* using ultrasound (with water) and from KA by conventional method with water, followed by spray drying, respectively.

The gel strength of the samples ranged from 25 to 131 $g.cm^{-2}$ for PWCE 60 ºC and the commercial sample, respectively. However, no significant difference was observed for samples obtained at 80 ºC (conventional, PWCE and MAE) and MAE at 100 ºC. The gel strength of the extracted carrageenan samples was lower than the commercial sample, probably due to KOH treatment, which leads to higher content of 3,6-anhydrogalactose to promote the formation of a double helix, assisting to reinforce the gel structure (BONO; ANISUZZAMAN; DING, 2014; FREILE-PELEGRÍN; ROBLEDO; AZAMAR, 2006). Then, the results of gel strength were similar to that from Bono et al., (2014) (3 to 114 $g.cm^{-2}$) and Freile-Pelegrín et al., (2006) (< 50 $g.cm^{-2}$). Therefore, despite the low carrageenan yields, compared to conventional method, the MAE and PWCE methods are promising alternatives since they provide samples with equal or higher viscosity and gel strength than conventional extraction method one, and recovered by a fast method (15 min from MAE and PWCE against 120 min from conventional extraction).

3.4 CONCLUSION

SFE from KA promoted a selective extraction, with the recovery of a fraction with a high fatty acid content, however, lower yield than SOX-hexane. Meanwhile, PLE method with ethanol was efficient for recovery of the phenolic compounds, a fraction with higher antioxidant activity, compared to SOX and MAE samples recovered with the same solvent. The extraction temperature, for PLE and MAE processes, showed a negative influence on TPC, DPPH, and FRAP performance of the recovered samples. The carrageenan extraction by MAE or PWCE methods was efficient to provide, in a very short time, a sample with physical properties similar to conventional carrageenan. The alternative extraction methods were suitable to provide two valuable fractions from KA biomass. Furthermore, this was the first work that quantified phenolic compounds from

KA, as far as we know. In addition, it is the first which compare the influence of green extraction techniques (MAE, PLE, and PWCE) in obtaining ethanolic extracts and carrageenan from KA.



CHAPTER 4 ULTRASSOUND APPLIED FOR THE RECOVERY OF CARRAGEENAN FROM *KAPPAPHYCUS ALVAREZII* – EFFECTS OF EXTRACTION PARAMETERS AND PRETREATMENT ON PROCESS YIELD AND QUALITY OF CARRAGEENAN

In the previous chapter, carrageenan was directly recovered from the KA biomass using microwaves and PWCE methods. However, ultrasound is also a fast and simple technique that offers great yields. Therefore, this chapter had the objective to observe the aspects of Ultrasound Assisted Extraction (UAE) affecting the carrageenan yield, and the viscosity, and gel strength of the carrageenan recovered at different conditions of time, temperature, and ultrasound power. Furthermore, at the optimized conditions of UAE, the influence of aqueous-phase removal, the drying method and the use of KOH, on the carrageenan extraction was also observed.

This chapter was developed with the aid of the undergraduate student Juliana Amaro, within her scientific initiation research project, from 2021 to 2023, thoughtfully accompanied and managed by my supervision (PhD student).

4.1 INTRODUCTION

Kappaphycus alvarezii (KA) is one of the most cultivated algae in the world due to its fast growth, easy cultivation, and high κ-carrageenan content (almost 40% in dry basis), a valuable biopolymer used in food, pharmaceutical, and other industries (RUDKE; DE ANDRADE; FERREIRA, 2020a). This edible biopolymer, mostly recover from red seaweed, can be obtained by different methods such as aqueous or alkali maceration, ultrasonication, homogenization, or with the aid of enzymes (HANS *et al.*, 2023). Ultrasound assisted extraction (UAE) can reach high yields, with low time- and energy- consume, compared to traditional maceration methods, and only few studies are available in the literature regarding the carrageenan recovery from KA using UAE (HANS *et al.*, 2023; YOUSSOUF *et al.*, 2017a). Although this research evaluated carrageenan recovery from KA by ultrasound extraction, no information was provided about the effects of ultrasound parameters and sample pre-treatment on yield and properties of the carrageenan.

Process parameters such as power, time, and temperature can significantly influence the yield and quality of carrageenan. Increase in ultrasound power is associated to increase in process efficiency caused by stronger shear forces (CARREIRA-CASAIS *et al.*, 2021). Temperature is a fundamental parameter directly associated with solvent viscosity and surface

tension and associated with increase in diffusion and break of chemical bonds, contributing to process efficiency. Nevertheless, excessive temperature can affect the integrity of the target compounds (CARREIRA-CASAIS *et al.*, 2021). Also, time consuming processes (long extractions) contribute to the power and temperature effects on carrageenan cell wall.

KA pretreatment can also affect the extraction process, for instance, drying the KA biomass may change the carrageenan chemical structure, modifying its gelling properties, characteristics still very poorly investigated (FARIA; HAYASHI; MONTEIRO, 2014; MOREIRA; CHENLO; TORRES, 2016). A pretreatment with cold-water can remove substances that could affect the subsequent carrageenan extraction.

The elementary carrageenan forms, based on the sulfate group number, are nu (ν), mu (μ), iota (ι) and kappa (κ), in crescent order, which affect the carrageenan gel strength and solubility (CAMPO *et al.*, 2009). The carrageenan from KA presents high-content of κ-carrageenan (Rupert et al., 2022), although, a KOH pre-treatment can change μ- and ν-carrageenan into κ- and ι-carrageenan, which present higher gel strength and viscosity properties (GANESAN; MUNISAMY; BHAT, 2018).

Therefore, the aim of this chapter was to investigate the UAE for the recovery of carrageenan from KA, evaluating the extraction conditions (time, temperature, and power) on the yield and quality of carrageenan, and the effects of KOH and drying pretreatments on carrageenan recovery.

4.2 MATERIAL AND METHODS

The KA biomass was kindly provided by EPAGRI (Agricultural Research and Rural Extension Company of Santa Catarina, Brazil) and consisted of a mixture of green and red algae strains farmed in Florianópolis/SC-Brazil. The seaweed sample was washed with tap water to remove sand and epiphytes, then dried in an oven with air circulation at 40 ºC for 24 h. Finally, the dried material was ground in a Willey knife mill (De Leo, Porto Alegre/RS-Brazil), reserved in polyethylene bags, and frozen at -18 ºC until further use.

4.2.1 Optimization of the Ultrasound Assisted Extraction (UAE)

The ultrasound extractions were carried out varying the following process parameters: time (5, 10, and 15 min), power (100, 300, and 500 W), and temperature (60, 70, and 80 ºC), where the parameter levels were selected based on the literature data (time), characteristics of the ultrasound equipment (power), and solubilization of carrageenan (temperature). These processing conditions were evaluated in terms of process yield and carrageenan properties, to optimize the UAE method. Briefly, 1 g of dried KA was mixture with 100 mL of distilled water. The solution was inserted into a jacketed glass reactor coupled to the bath (Microquímica, Palhoça/SC-Brazil) through hoses to maintain the temperature throughout the extraction. Then, the ultrasound titanium probe of 4 mm diameter (Eco - Sonics, Indaiatuba/SP-Brazil - Model QR500) was placed at 1.5 cm depth inside the liquid sample (system), the reactor was centralized, and time and power conditions adjusted. The extract solution was hot-filtered using a vacuum pump (Fisatom, Perdizes/SP-Brazil - Model 825T) and cotton filter. Finally, the carrageenan yield (ratio between dry carrageenan mass and dried seaweed multiplied by 100%) was obtained for each UAE condition (nine assays), and the results expressed as mean value and standard deviation (in percentage) from triplicate data.

4.2.1.1 Carrageenan fraction properties (viscosity, and gel strength)

The viscosity and gel strength of the carrageenan fractions recovered by UAE were carried out according to BONO; ANISUZZAMAN; DING, (2014). The samples (0.45 g) were dissolved in 30 mL of distilled water (1.5% solution) and the solution was heated at 75 ºC until complete solubilization (for about 30 min). A total of nine samples recovered by UAE at different conditions (carrageenan fraction) were evaluated. The viscosity was determined using a ThermoHaake ViscoTester 6 L at 30 rpm, and 75 °C (controlled by solid Steel circulating bath), using the L1 spindle, and the measurement was taken after six complete revolutions, with three readings for each sample. The assays were performed in duplicate and the results expressed in centipoise (cp) (mean ± standard deviation).

The gel strength analysis was also according to Bono et al. (2014), where the solubilized solution was stored in an aluminum cylindrical container (50 mm diameter, 30 mm height) at room temperature for 24 h prior analysis. The gel strength was evaluated using Texture

Analyzer TA.HD. plus (Stable Micro Systems, Godalming, Surrey, England) and a cylindrical steel (11.5 mm diameter and 50 mm height). The penetration speed was 2 $mm.s^{-1}$ at a depth of 10 mm. A load cell of 50 kg was used. Five readings were performed for each sample and the analysis was performed in duplicate. Results are expressed in $g.cm^{-2}$ (mean ± standard deviation).

4.2.2 Raw material pretreatments

4.2.2.1 Drying process and aqueous fraction removal before carrageenan extraction

Wet KA samples were separated in four groups for different drying/separation processes as can observed in Figure 4-1. Biomass 1 (B1): 0.5 kg of wet KA was freeze dried (Liotop, model LD101, Sao Paulo, Brazil) until completely dry (constant mass); Biomass 2 (B2): 0.5 kg of the wet KA, dried in an air circulation oven at 40 ºC for 24 h (WEBBER; DE CARVALHO; BARRETO, 2012). The other two groups were submitted to an additional treatment to remove the water-soluble components, with the remaining solid phase submitted to drying pretreatment, as follows: Biomass 3 (B3): 0.5 kg of the wet KA, blended with approximately 500 mL of water, and filtered to separate the phases (aqueous and solid fractions). Then, the solid phase was freeze dried, as described in B1. Finally, Biomass 4 (B4): 0.5 kg of the wet KA was blended with approximately 500 mL of water and then filtered to separate the liquid (aqueous fraction) from the solid, and the solid phase was dried at 40 ºC, as in B2. All dried materials (B1-B4) were particulate by a Wiley knife mill (De Leo, Porto Alegre/RS-Brazil), and the resulting powder was reserved in identified falcon tubes, and frozen at -18 ºC until further extraction of the carrageenan samples by UAE conducted at the optimized conditions (section 4.2.1).

4.2.2.2 KOH pre-treatment

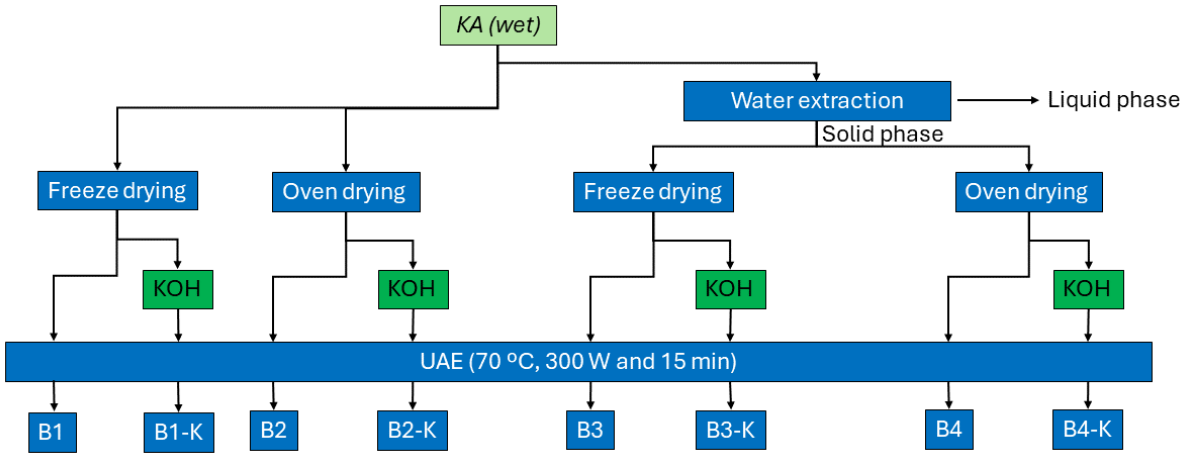
After drying processes, the biomass samples (B1-B4) were treated with KOH (alkaline treatment). Briefly, 2 g of samples (B1-B4) were mixture into 200 mL of 6% water KOH solution (w/v), then maintained for 2 h at 80 °C in the Dubnoff bath (Ethiktechnology, model 304-TPA), vacuum filtered and washed with water until neutralized. The samples were dried in

an oven at 40 ºC. Then, the dried samples were stored at -18 ºC for further carrageenan extractions. After KOH treatment, the four samples were identified as B#-K (B1-K to B4-K).

4.2.3 Influence of KA pretreatment on carrageenan extraction by UAE

The carrageenan extraction by ultrasound was conducted following the optimized conditions for UAE defined for the assays as described as section 4.2.1. Then, the effect of KA pretreatments was evaluated to the dried and/or alkaline samples (B1 to B4 and B1-K to B4-K) at the optimum UAE parameters (70 °C, 15 min, and 300 W: according to section 4.3.1). Figure 4-1 shows a schematic design of the UAE procedures applied to KA for the recovery of carrageenan rich fractions.

Figure 4-1 - Scheme of the pretreatments applied to *Kappaphycus Alvarezii* (KA) biomass (drying process, water soluble components removal; potassium hydroxide (KOH)) on carrageenan yield, and viscosity and strength of the carrageenan gel.



Source: the author (2024).

After the UAE conducted at optimum condition for all samples (triplicate), the recovered solutions (B# and B#-K) were hot-filtered and the filtrate was mixed with 300 mL of ice-cold ethanol and left to rest for 20 minutes, for the carrageenan-fraction precipitation. Subsequently, the solution was sieved to separate the ethanol and water from the carrageenan-rich samples, and then dried in an oven at 60 °C for 24 h and stored at -18 ºC for further evaluation. The yield values of the carrageenan fractions from all samples were calculated as described at section 4.2.1 for triplicate assays.

4.2.3.1 Characteristics of carrageenan fractions after pretreatments

The effect of KA pretreatments on carrageenan attributes (viscosity and gel strength) were evaluated for all samples (B# and B#-K). The viscosity of the samples was obtained from the solubilized samples (1% w/v), analyzed in rheometer (Anton Paar, MCR 72) with concentric cylinder geometry-PP27, with a shear rate of 50 rotations per second (rps) at 75 ° C. Several readings were taken for each sample, and the assay was performed in duplicate, with results (mean ± standard deviation) expressed in cP.

The gel strength analysis was performed according to Faria et al. (2014) with modifications. Briefly, 0.2 g of carrageenan was solubilized in 40 mL of 0.07% KCL solution and stored in domestic refrigerator overnight for gelatinization. The gel strength was evaluated using Texture Analyzer TA.HD. plus (Stable Micro Systems, Godalming, Surrey, England) and cylindrical steel (35 mm). The penetration speed was 2 $mm.s^{-1}$ at a depth of 10 mm (target). A load cell of 50 kg was used. Analyzes were mostly carried out in duplicate, according to the amount of sample recovered. Results are expressed in $g.cm^{-2}$ (mean ± standard deviation).

4.3 RESULTS AND DISCUSSION

4.3.1 UAE for the recovery of carrageenan fraction: process optimization

Carrageenan fractions from KA were recovered by UAE at different extraction conditions, as presented at section 4.2.1.The yield results for the UAE assays from KA biomass, conducted varying the process temperature, power, and time are presented at Table 4-1.The highest carrageenan yield values were obtained at 70 °C, 500 W and 10 min. (58.67%), while the lowest yield was obtained at 70 °C, 100 W and 10 min. (23.32%). The yield values are within the range presented by Youssouf et al. (2017) (50-55%) for the carrageenan recovery from KA using UAE, and by Torres et al. (2021) (15-35%) using UAE for recovery carrageenan from *Mastocarpus stellatus*. The differences in the carrageenan yield can be associated with variations in extraction conditions, seaweed, seaweed age, climate conditions and other factors for carrageenan evaluation.

Table 4-1 - Influence of time, temperature and power on the yield, viscosity and gel strength values obtained for carrageenan obtained using ultrasound.

UAE (Run)	Time (min)	Power (W)	Temperature (°C)	Yield (%)	Viscosity (cP)	Gel strength (g.cm-2)
1	5			44.01± 0.12^{d}	33 ±1^{b}	45 ± 1^{a}
2	10	300	70	48.85±0.09^{c}	36 ± 2^{ab}	35 ± 1^{cd}
3	15			55.89 ± 0.91^{b}	39 ± 1^{a}	44 ± 1^{ab}
4	10	100	70	23.32 ± 0.99^{e}	6 ± 0 c	33 ± 2^{d}
5		500		58.67 ± 0.52^{a}	36 ± 3^{ab}	39 ± 2^{bc}
6	10	300	60	43.50 ± 0.64^{d}	36 ± 1^{ab}	45 ± 3^{a}
7			80	47.23 ± 0.63^{c}	36 ± 1^{ab}	36 ± 1^{cd}

*The same letter in the same column indicates no significant difference at the level of 5% by the Tukey test ($p < 0.05$).

Source: The author (2024).

The time effect on yield, at constant temperature and power (70 ºC and 300 W, respectively) varying from 44.01 to 55.89%, show an increase with time and statistical difference at 5% level within the time intervals evaluated. The yield results from Table 4-1 increase with time, probably due to the impact of ultrasonic waves on the sample and cavitation intensification with time progression, which result in the collapse of the bubbles, increasing the cell wall disruption, enhancing yield. Youssouf et al. (2017) also observed the positive relation between time and extraction yield of carrageenan extracted from *Mastocarpus stellatus* using ultrasound. The results obtained in this work was similar as observed by Youssouf et al. (2017), which used UAE for carrageenan extraction from KA at 90 °C, 150 W, and time 15 min., with yields from almost to 55%, a result similar to that reported in this work 55.89% using 70 °C, 300 W for 15 min.

The effect of ultrasound power on extraction yield is also presented at Table 4-1 with values from 23.32 to 58.67%, from 100 to 500W, respectively (results statistically different at 5% level), for assays at 70 ºC for 10 min. From Table 4-1, varying the UAE power from 100 to 300W the yield increases 2.1 times, and from 300 to 500 W the yield increase was 1.2 times using water as solvent. The same behavior between yield and power was observed by Youssouf et al. (2017), for the power range between 75-150 W. The power increase provides zones of high-pressure and high temperature, which contribute to yield increase due to cell wall rupture (Chemat et al., 2011). Also, the increase in power generates cavitations that cause interfacial turbulence and cell rupture, increasing the solute diffusion (LIAO, 2022).

The temperature effect (Table 4-1), from 60 to 80 °C provides a discrete yield increase from 43.50 to 48.85%, with no significant difference between 70 and 80 ºC, for assays conducted at 300 W for 10 min. A positive influence of temperature on yield was observed by Youssouf et al. (2017) for alginate (an algae-based biopolymer) extraction. This behavior was also verified by Webber et al. (2012) for carrageenan extraction from KA, for temperature from 32 to 88 ºC, with the yield of 32% at 88 °C. This temperature influence is explained by the increase in carrageenan solubility and the mass transfer rate with increase in temperature (WEBBER *et al.*, 2012). However, a combination of high-power and high-temperature, for long periods can also promoted the carrageenan degradation, therefore the UAE conditions must be defined considering the process yield, but also que properties of the recovered fraction, such as carrageenan.

4.3.1.1 Carrageenan viscosity

Table 4-1 shows the influence of the UAE conditions of time, power, and temperature in the carrageenan viscosity. As observed in yield, the lowest viscosity (6 cP) was obtained for the sample recovered at 100 W, 70 ºC, and 10 min. Very likely, this result is associated to lower carrageenan yield. On the other hand, the highest viscosity (39 cP) was obtained for the sample recovered at 70 ºC, and 300 W and 15 min. However, except for the condition of 100 W, 70 ºC, and 10 min all carrageenan viscosity values were similar (33-39 cP).

The influence of extraction time on viscosity (Table 4-1) increased with time, with significant difference between 5 and 15 min. WEBBER et al. (2012) observed carrageenan viscosity increasing with progression of extraction time, for long processes (between 2 and 6 h), for extraction conducted by maceration. They found higher viscosity with 5.4 h of extraction (37.78 cP), a value similar to presented at Table 4-1 for UAE using only 15 min (39 cP). LEVY-ONTMAN; ABU-GALIYUN; HULEIHEL, (2023) did not observe large differences in viscosity between 5 and 10 min., and between 5 and 15 min. However, they noticed a large difference between 15 and 30 min., indicating that ultrasound used for long periods can significantly affect the viscosity of carrageenan.

The power influence on carrageenan viscosity was detected comparing only the lower level (100 W) (Table 4-1), for assays conducted at 70 ºC and 10 min. No significant difference

in viscosity was observed between 300 and 500 W at the 5% level, a result also presented by RAFIQUZZAMAN et al., (2016), which found no difference in carrageenan viscosity from *Hypnea musciformis* for ultrasound power from 400 to 500 W, with 36 cP.

Similarly, no influence of temperature on viscosity (Table 4-1) was detected, within the process conditions studied, for assays conducted at 300 W and 10 min. For a wide temperature range and long extraction times, Webber et al. (2012) verified that higher carrageenan viscosity was obtained at 60 ºC and 5.4 h, a value (37 cP) close to found in this work (36 cP), obtained at 60ºC for 10 min, indicating the efficiency of the present work.

4.3.1.2 Carrageenan gel strength

Table 4-1 shows the influence of UAE time, power, and temperature on the strength of carrageenan gel. Similar to the yield and viscosity results, the lowest gel strength value (33 $g.cm^{-2}$) was obtained at 100 W, 70 ºC and 10 min; however, it did not differ statistically from the sample obtained at 300 W, 80 °C and 10 min. On the other hand, the highest values were found from samples from runs 1, 3 and 6, with no significant differences. These results suggest little influence of the studied ultrasound conditions on the gel strength of the carrageenan samples. Considering only the extraction time, and at fixed 70 ºC and 300 W, the gel strength ranges from 35 to 45 $g.cm^{-2}$, with no significant difference between 5 and 15 min, and the lower value observed in 10 min (35 $g.cm^{-2}$). This result may be due to protein extraction at 5 min, its degradation up to 10 min, and then the increase in carrageenan extraction up to 15 min, respectively. Some proteins, with considerable gel strength, can be degraded by ultrasound, depending on the conditions used (FLORES-JIMÉNEZ *et al.*, 2019).

The variation in ultrasound power provided gel strength ranging from 33 to 39 $g.cm^{-2}$, for UAE at 70 ºC and 10 min, with higher gel strength with higher power used. There was no significant difference between 100 and 300 W, and between 300 W and 500 W at 5% level. Otherwise, higher gel strength was observed at lower temperature, comparing assays 2, 6 and 7, with values ranging from 35 to 45 $g.cm^{-2}$. There was no significant difference between 70 ºC and 80 ºC at the 5% level. The higher gel strength at 60 ºC can be associated with the increased in carrageenan structure degradation at higher temperatures (WEBBER *et al.*, 2012), or to the protein extraction, contributing to gel strength (FLORES-JIMÉNEZ *et al.*, 2019).

Therefore, based on the results obtained for carrageenan yield, and viscosity, and gel strength, the best condition selected for UAE was 70 °C, 15 min, and 300 W. This UAE condition was applied to study the effects of different pre-treatments on the yield, viscosity, and strength of the carrageenan gel as can be seen in section 4.3.2.

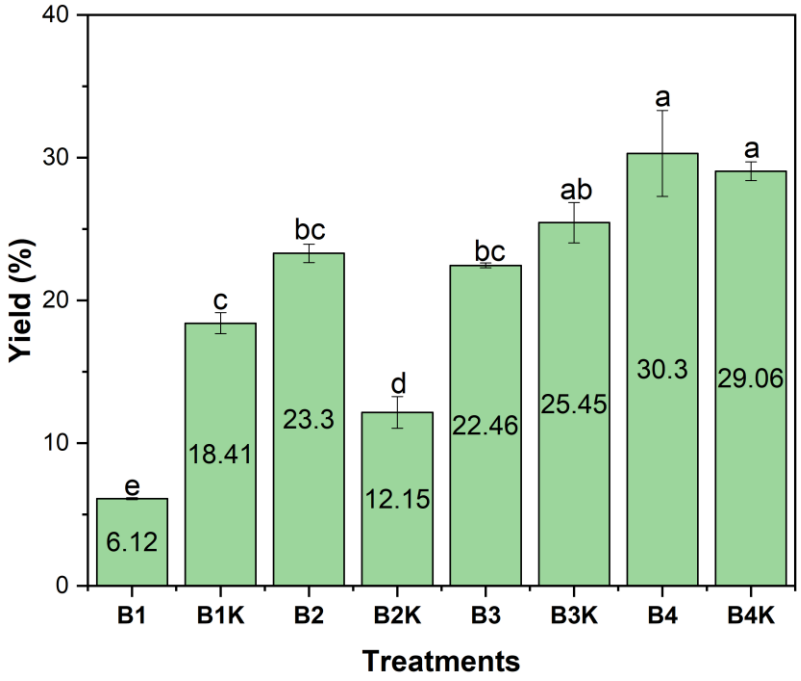
4.3.2 Influence of KA biomass pretreatments

4.3.2.1 Carrageenan yield

Figure 4-2 presents the results of carrageenan yield recovered from KA using UAE at the best condition described in previous section and comparing the use of algae pre-treatments (drying, aqueous fraction removal, and KOH), represented by the assays B1 to B4 and B1-K to B4-K. The yield values ranged from 6.12 to 30.3%, with highest yields obtained for assays B3, B4 and B4-K, with no significant differences. On the other hand, the lowest yield was provided by assay B1 (6.12%), a value significantly different than all others. The results obtained in this section were lower than reported in Table 4-1, and the results obtained by Hans et al. (2023) (55%), and by Youssouf et al. (2017) (50-55%) for carrageenan extraction from KA, with differences mainly associated with pre-treatments, and carrageenan separation use of KCL to coagulate the sample, for example.

The alkaline pretreatment (KOH) had a positive influence on carrageenan yield for the freeze-dried samples obtained by B1, compared to B1-K with the sample obtained by alkaline treatment (B1-K) providing yield three times higher than B1. Otherwise, a yield reduction was observed for the oven dried samples obtained by B2, compared to B2-K, with almost 2 times yield reduction. The literature is also not clear about the influence of the alkaline pretreatment on ultrasound yield of carrageenan extraction. For instance, Rafiquzzaman et al. (2016) observed a negative effect of KOH on yield as observed in B2 and B2-K, while Hans et al., (2023) detected a slight increase in yield with KOH treatment, as detected for B1 and B1-K samples. The KOH contribute to remove interferents for the conversion of *mu* and *nu* carrageenan's into *kappa* and *iota*, which precipitate in the presence of alcohol and increase the yield. Also, high KOH concentrations for long periods can contribute to carrageenan degradation, reducing yield (FREILE-PELEGRÍN; ROBLEDO; AZAMAR, 2006).

Figure 4-2 - Carrageenan yield from *Kappaphycus Alvarezii* (KA) using ultrasound (70 ºC, 15 min, and 300 W) in different batches (B1- dried seaweed in the freeze dryer, B2 - dried seaweed in the circulation oven, B3 - Removal of the aqueous fraction and drying of the resulting material for carrageenan extraction in the freeze dryer, and B4- Removal of the aqueous fraction and drying of the resulting material for carrageenan extraction in the circulation oven. The suffix K after the numbers of Batch indicates the pre-treatment with potassium hydroxide (KOH).



Source: The author (2024).

The influence of aqueous fraction removal on yield, comparing B1 *vs.* B3; B1-K *vs.* B3-K; B2 *vs.* B4; and B-2K *vs.* B4-K, provided an increase in carrageenan extraction yield, probably due to the breaking bonds of molecules that affect carrageenan recovery due to act as interferents. Additionally, the aqueous phase removal, previous to carrageenan extraction from the solid phase, provide an additional product for the algae industries, possibly with high market value due to the presence of water-soluble components which, according to TRIVEDI et al., (2023), contribute to the growth of several cultivars, a relevant attribute for the agricultural sector.

The influence of drying process on carrageenan yield (B1 *vs.* B2, B1-K *vs.* B2-K, B3 *vs.* B4) suggest mostly the best performance of oven drying compared to freeze drying. The yield results obtained in this work (12.15-30.3%) were close to reported by Faria; Hayashi & Monteiro, (2014) for carrageenan from KA, and close to the carrageenan yields from freeze dried *Chondrus crispus* reported by Torres, López-Hortas, & Domínguez, (2023). Moreira,

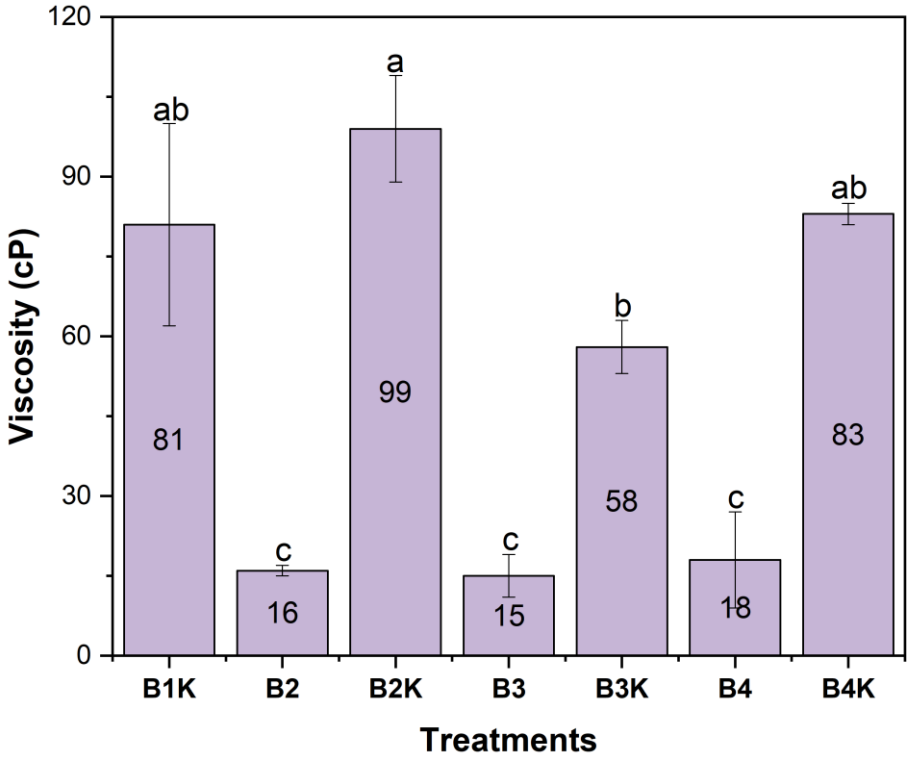
Chenlo, & Torres, (2016) and Faria, Hayashi, and Monteiro, (2014) tested different drying temperatures, observing an increase in carrageenan yield with the increase in drying temperature. According to Moreira, Chenlo, & Torres, (2016), this increase in yield is related to the fact that drying temperatures favor the carrageenan diffusion through the algae cell walls, facilitating the extraction.

Comparing the results from Figure 4-2 with data from Table 4-1, a reduction in yield is observed. While in Table 4-1 for conditions of 70 ºC, 300W, and 15 minutes the observed yield was between 55.89%, in this section the effect of pre-treatments provided yield from 6 to 30%, probably due to: I) carrageenan extraction conditions, involving different pre-treatments (KOH, drying method, presence of aqueous-phase, and precipitation of carrageenan with alcohol). The aqueous-phase removal, the use of KOH, and the alcohol precipitation reduces the yield due to reduction in impurities, compared to results from Table 4-1, which samples may contain other molecules (proteins, sugars, pigments, among others) in addition to carrageenan; II) the use of two different baches of KA, which may have influence in carrageenan yield, as demonstrated by other authors (SHANMUGAM; SETH, 2018).

4.3.2.2 Carrageenan viscosity

Figure 4-3 presents the viscosity of carrageenan samples recovered from the assays using different pretreatments (drying conditions, removal of aqueous phase, and use of KOH). The viscosity ranged from 15 to 99 cP, with the lower values, significantly, for the samples without KOH treatment, while the highest values were obtained for KOH treated samples, with no significant differences. The other treatments had no influence on carrageenan viscosity. The same behavior was observed by Rafiquzzaman et al., (2016), with viscosity values between 30-40 cP for sample without KOH, and from 60-80 cP for treated samples, with this increase in viscosity associated with a reduction in the number of sulfate groups due to the conversion of *mu* and *nu* into *kappa* and *iota* carrageenan types (FREILE-PELEGRÍN; ROBLEDO; AZAMAR, 2006)

Figure 4-3 - Carrageenan viscosity from *Kappaphycus Alvarezii* (KA) using ultrasound (70 ºC, 15 min, and 300 W) in different batches (B1- dried seaweed in the freeze dryer, B2 - dried seaweed in the circulation oven, B3 - Removal of the aqueous fraction and drying of the resulting material for carrageenan extraction in the freeze dryer, and B4- Removal of the aqueous fraction and drying of the resulting material for carrageenan extraction in the circulation oven. The suffix K after the numbers of Batch indicates the pre-treatment with potassium hydroxide (KOH).



Source: The author (2024).

Also, no influence in viscosity was detected with aqueous phase removal, nor with drying process, within the extraction conditions used. Regarding the freeze-drying, no studies were found in the literature that evaluated its impact on carrageenan viscosity, although, one should consider the cost involved in the pretreatments applied. On the other hand, the effect of drying temperature on carrageenan viscosity was studied by Faria, Hayashi & Monteiro, (2014) and Moreira, Chenlo, & Torres (2016), detecting a reduction in viscosity with the increase in drying temperature from 40 to 90 ºC, probably due to carrageenan depolymerization.

It is not possible to compare the process parameters influence on viscosity (Table 4-1), with the pre-treatments effects (Figure 4-3), due the use of different concentrations (1% and

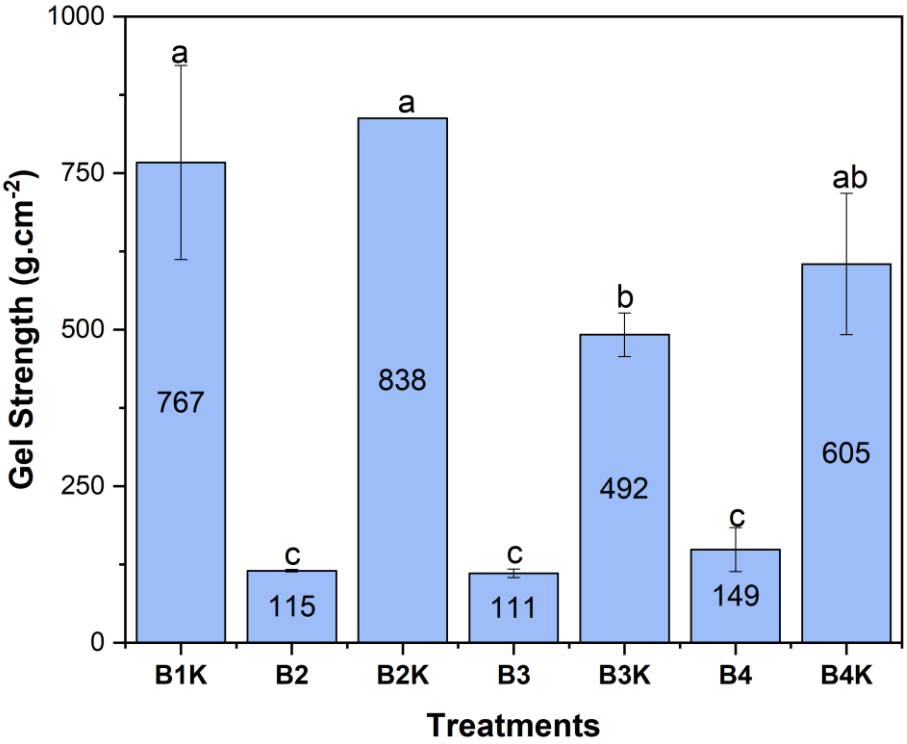
1.5%), different equipment (Anton Paar rheometer and ThermoHaake ViscoTester 6 L viscometer) and shear rate (50 and 30 rpm).

4.3.2.3 Carrageenan gel strength

The gel strength values for carrageenan samples obtained using different pretreatments are presented at Figure 4-4. The gel strength ranged from 111 to 838 $g.cm^{-2}$, with the lower values to samples without KOH, while the highest values were obtained for B1-K, B2-K and B4-K, with no significant difference. The KOH treatment had a positive effect on gel strength, probably associated with removing 6-sulfate from the galactose units, and producing 3,6-anhydrogalactose, which increases the strength of the carrageenan gel. This behavior was verified by different authors, as stated by Abdul Khalil et al., (2018) in their review.

The aqueous phase removal provided lower gel strength, with statistical differences detected by comparing B1-K with B3-K, 767 and 492 $g.cm^{-2}$, respectively. A reduction in gel strength for samples treated with KOH when the aqueous fraction was removed was also verified by Shanmugam & Seth, (2018), from 330 $g.cm^{-2}$ to 185$g.cm^{-2}$ for samples before and after aqueous fraction removal, respectively.

Figure 4-4 - Carrageenan gel strength from Kappaphycus Alvarezii *(KA)* using ultrasound (70 ºC, 15 min, and 300 W) in different batches (B1- dried seaweed in the freeze dryer, B2 - dried seaweed in the circulation oven, B3 - Removal of the aqueous fraction and drying of the resulting material for carrageenan extraction in the freeze dryer, and B4- Removal of the aqueous fraction and drying of the resulting material for carrageenan extraction in the circulation oven. The suffix K after the numbers of Batch indicates the pre-treatment with potassium hydroxide (KOH).



Source: The author (2024).

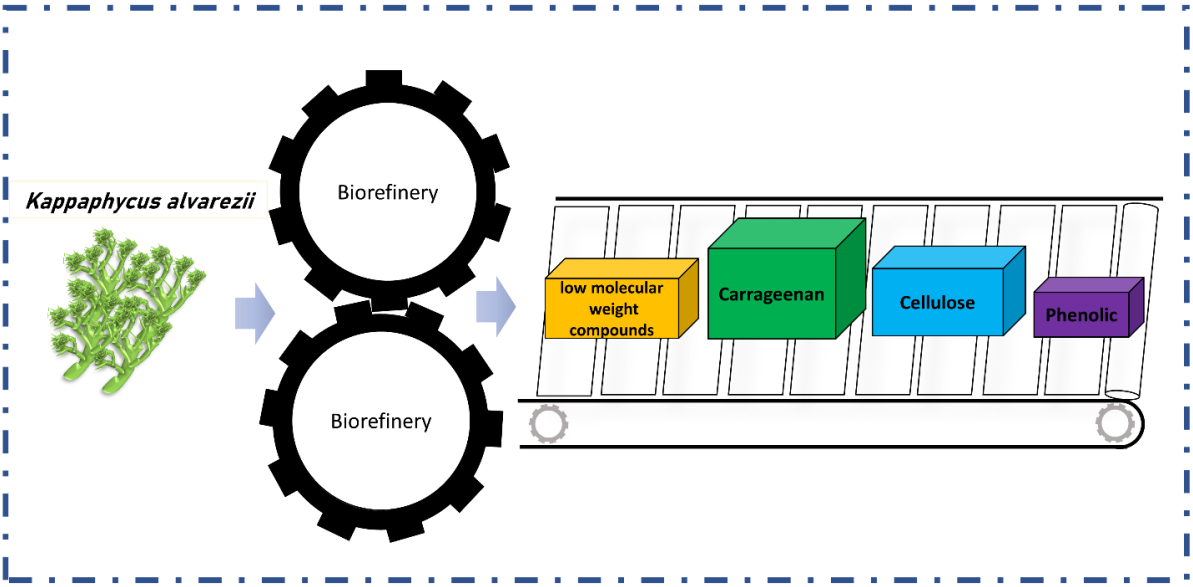
Finally, the drying conditions had no influence on the carrageenan gel strength, under the conditions studied. This same observation was verified by FARIA; HAYASHI; MONTEIRO, (2014).

It is not possible to compare the process parameters influence on gel strength (Table 4-1), with the pre-treatments effect (Figure 4-4), due the use of different concentrations (0.5% with 0.07% KCL and 1.5%), and different probes (35 and 11.5 mm cylindrical steel).

4.4 CONCLUSION

The ultrasound assisted extraction applied to KA biomass was thoroughly evaluated under different process conditions, revealing the effectiveness of ultrasound as a method for recovering carrageenan. The analysis of the process parameters highlighted the influence of temperature, power, and extraction time, and pre-treatments on the yield and properties of the

carrageenans obtained. The results of parameters influence indicate that the ideal conditions for carrageenan recovery were achieved at 15 minutes, 300 W, and 70 ºC, and using these conditions, the results for extraction yield, and viscosity and gel strength of carrageenan were 55.89%, 39 cP, 44 $g.cm^{-2}$, respectively. In addition, pre-treatments such as the use of KOH, the aqueous-phase removal, and the drying method, were evaluated and indicated that samples B4-K (treated with KOH, dried in oven, and with the aqueous-phase) resulted as the most efficient conditions, providing an excellent combination of extraction yield and carrageenan quality.



CHAPTER 5 DECONSTRUCTION OF *KAPPAPHYCUS ALVAREZII* BIOMASS BY PRESSURIZED SOLVENTS TO INCREASE THE CARRAGEENAN PURITY

In the previous chapter, the main factors that influence the characteristics of carrageenan were elucidated, including the use of KOH and the cascading impact of previous steps on the yield and subsequent properties of the recovered material. In this chapter, we will deal with the sequential extraction of KA using high-pressure methods to obtain different compounds and compare them with the conventional method. The use of KOH and the effect of the previous step on the next step will be considered.

This chapter has been accepted and is published in Food Hydrocolloids

5.1 INTRODUCTION

Red algae are remarkable sources of biopolymers such as carrageenan, a hydrocolloid that can be used in different food formulations such as jam, marshmallow, ice-cream, pudding, cooked ham, hamburger, cake glaze, salad dressings, among others (GEADA *et al.*, 2021; NASERI; HOLDT; JACOBSEN, 2019; ROLDÁN *et al.*, 2017; SIMATUPANG *et al.*, 2021). Carrageenan is commercialized around 5.50 to 9.00 dollars per kg, depending on its purity: semi-refined or refined (KUMAR *et al.*, 2020), respectively. Within seaweeds, *Kappaphycus alvarezii* (KA) stands out for its fast growth (100-120 days) and high content of κ-carrageenan, one of the basic carrageenan forms (μ, ν, κ and ι) with adequate properties for food, pharmaceutical, and cosmetic products, acting as gelling, thickening, and stabilizing agent (ALI; CRITCHLEY; HURTADO, 2020; KUMAR *et al.*, 2020; RUPERT *et al.*, 2022).

In addition to carrageenan, KA contains fatty acids, pigments, phenolic compounds, proteins, and cellulose that can be recovered through sequential extractions (RUDKE; DE ANDRADE; FERREIRA, 2020b). The sequential extraction approach can lead to different fractions – aligned with the biorefinery concept (Torres, Kraan, Domínguez, 2019), providing different products and maximizing the biomass value (INGLE *et al.*, 2018). However, the main challenges associated with algae biorefinery are the development and control of the extraction processes, ensuring high yields while maintaining the quality of the recovered fractions. Therefore, considering the high number of process variables involved, it is essential to develop a careful and well-detailed sequential extraction applied to algae biomass, in particular KA. To the best of our knowledge, it has been little investigated.

The sequential extraction of KA was conducted to obtain a polar fraction (extract) with antioxidant activity and the carrageenan fraction, focusing on maximizing the antioxidant

extractions without affecting the physical properties of the carrageenan. The design of sequential approach used was based on Gilbert-López et al. (2017), Gilbert-López et al., (2015), and Rebelatto et al. (2020), i.e., the sequential processes were conducted by increasing the solvent polarity and process temperature, providing different fractions with diverse characteristics.

Low-pressure sequential extractions were applied to KA biomass in order to recover different components, such as biostimulant and semi-refined carrageenan (SHANMUGAM; SETH, 2018), carrageenan and ethanol (MASARIN *et al.*, 2016; MEINITA *et al.*, 2019; ROLDÁN *et al.*, 2017), and carrageenan, biofertilizer, ethanol, and biogas (INGLE *et al.*, 2018). Rudke et al. (2022) previously detailed the effect of low-pressure extraction methods on the quality of the recovered fractions (phenolics and carrageenan) conducted as individual extraction processes. On the other hand, no data related to KA sequential extractions by low- or high-pressure methods is available. Therefore, the novelty of this chapter is the investigation of high-pressure KA deconstruction, with the recovery of fractions rich in phenolics, carrageenan, cellulose, and low molecular weight compounds. The effects of each extraction step in the sequential extraction, in terms of process efficiency and carrageenan quality, were also evaluated, comparing high- and low-pressure extraction routes and the carrageenan quality confronted with the commercial carrageenan.

5.2 MATERIAL AND METHODS

5.2.1 Chemicals

Ethanol and sulfuric acid were purchased from Neon (Suzano, SP, Brazil), 2,2-diphenyl-1-Picrylhydrazil (DPPH) (>97%) from TCI chemicals, Trolox (97%) from Sigma Aldrich (San Luis, MI, Germany). Carrageenan was acquired from Agargel (João Pessoa, PB, Brazil), Dinitrosalycilic acid and Potassium hydroxide from Exodo Cientifica (Sumaré, SP, Brazil), and Sodium hydroxide from MH_3 Industrial (Jaraguá do Sul, SC, Brazil). Other reagents used were of HPLC grade.

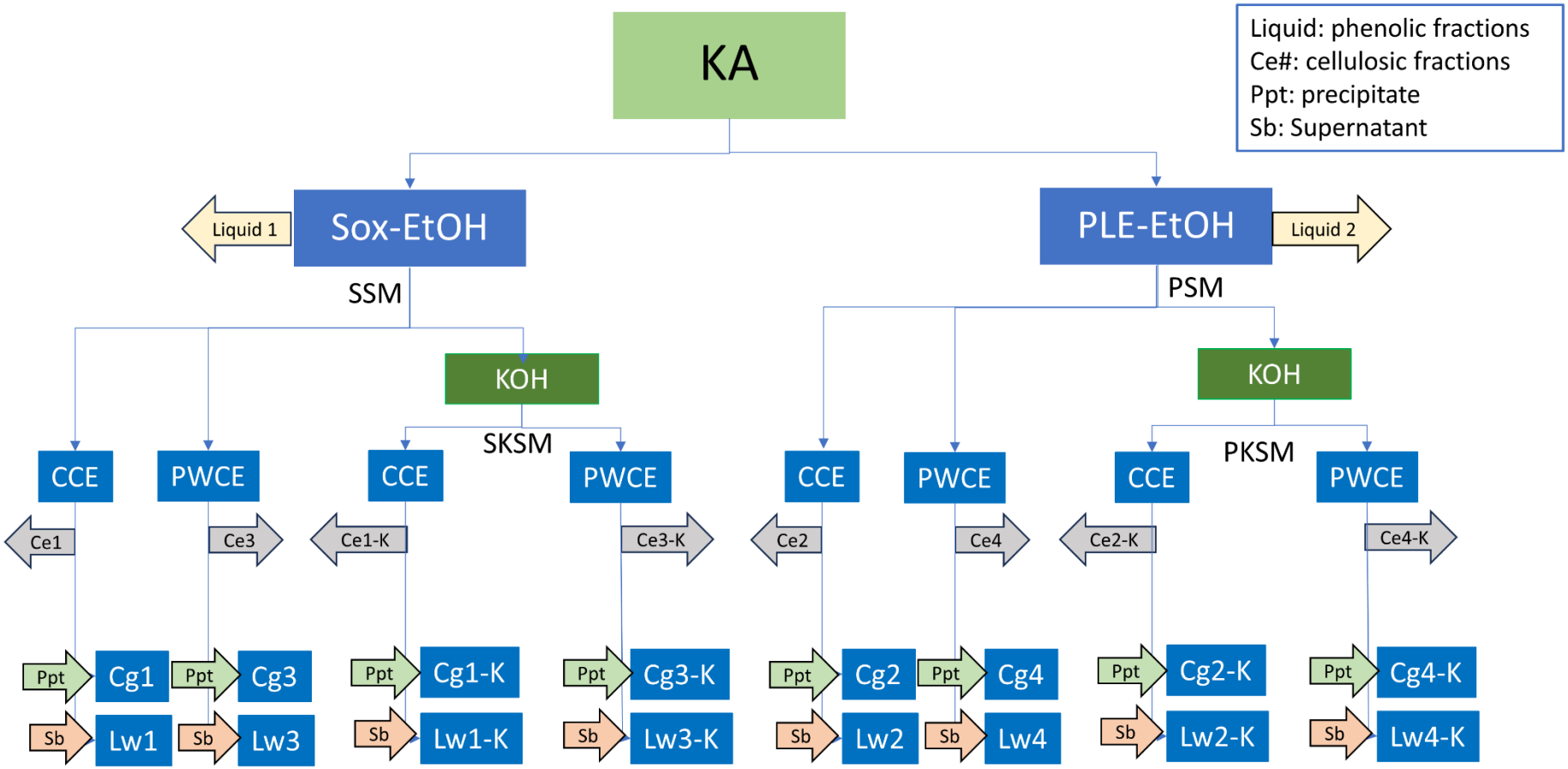
5.2.2 Kappaphycus alvarezii

The KA biomass was acquired and treated as described in section 3.2.2. The KA biomass was kindly provided by EPAGRI (Agricultural Research and Rural Extension Company of Santa Catarina, Brazil) and consisted of a mixture of green and red algae strains farmed in Florianópolis, Santa Catarina, Brazil. The seaweed sample was washed with tap water to remove sand and epiphytes, then dried in an oven with air circulation at 40 ºC for 24 h. Finally, the dried material was ground in a Willey knife mill (De Leo, Porto Alegre/RS, Brazil), reserved in polyethylene bags, and frozen at -18 ºC until the tests were carried out.

5.2.3 Sequential extractions

Dried KA biomass was submitted to sequential extractions according to Figure 5-1: Ethanolic extractions for phenolics recovery were conducted by pressurized liquid extraction (PLE) and Soxhlet (SOX) (section 5.2.3.1). The resulting solid materials (after extractions) were divided into two groups: treated with KOH and non-treated (section 0). The carrageenan extraction was conducted using water as solvent by high-pressure techniques (PWCE) and by conventional carrageenan extraction (CCE) (sections 5.2.4.1 and 0, respectively).

Figure 5-1 - Biorefinery flow chart used for the recovery of the fractions: carrageenan, phenolic, cellulose, and low molecular weight samples, obtained from *Kappaphycus alvarezii* (KA). Where: SSM: Soxhlet Solid Material, SKSM: Soxhlet KOH Solid Material; PSM: PLE Solid Material, PKSM: PLE KOH Solid material, CCE (conventional carrageenan extraction) and PWCE (Pressurized water carrageenan extraction) are the processes for carrageenan recovery; Ce# are the cellulose samples (fractions); Cg# are the carrageenan samples obtained from the different processing steps; Lw# are the low molecular weight carrageenan fractions, obtained by different processing steps.



Source: The author (2024).

5.2.3.1 Recovery of the phenolics fraction

Soxhlet (SOX) method: Briefly, 5 g of sample and 150 mL of ethanol were used in a Soxhlet apparatus for 6 h, close to the solvent boiling temperature, with recycle (one cycle every 20 min). After extraction, the residual solid was coded as SSM (Soxhlet Solid Material), dried for 24 h at 60 ºC, and stored in a domestic freezer for use in the sequential process.

Pressurized Liquid Extraction (PLE): Pressurized extraction was performed according to Gonçalves Rodrigues et al. (2019) and Rudke et al. (2022), using ethanol at 40 ºC and 10 MPa for 40 min, flow rate of 3 $mL.min^{-1}$ and 5 g of sample mixed with 90 g of glass sphere. The residual solid, coded as PSM (PLE solid material), was dried for 24 h at 60 ºC and stored in a domestic freezer for use in the sequential process.

5.2.3.2 Ethanol removal from SOX and PLE samples

The ethanolic extracts were evaporated in a rotary evaporator (Fisatom, model 801, São Paulo, Brazil) for solvent removal, and the phenolic fractions (by SOX and PLE) were stored in amber flasks at -18 ºC in a domestic freezer. The amount of ethanol recovered (%) from the samples (by SOX and PLE) was determined by volume difference.

5.2.3.3 Potassium hydroxide (KOH) treatment

The KOH treatment was performed on SSM and PSM samples (Figure 5-1) (solids after ethanolic extractions), as described by Roldán et al. (2017) with modifications. Briefly, 2.0 g of dry sample and 200 mL of a KOH solution 6% ($m.v^{-1}$) were mixed, shaken in a Dubnoff bath for 24 h at 30 °C ("cold" alkali transformation), and filtered. The retained solids were washed with water to remove excess KOH until the water pH reached 7.0 and then dried at 60 °C for 24 h. The solids were coded as SKSM (Soxhlet-KOH-solid material) and PKSM (PLE-KOH-Solid material), from SSM and PSM samples.

5.2.4 Carrageenan extractions

The carrageenan samples were obtained, by low- and high-pressure method, from the solid co-products of the ethanolic extractions (SOX and PLE), as represented by the schematic assays diagram for carrageenan recovery from KA biomass (Figure 5-1), as described in the sequence.

5.2.4.1 Conventional carrageenan extraction (CCE):

Conventional carrageenan extraction, a maceration method, was performed according to Boulho et al. (2017), with modifications. Shortly, 0.7 grams of dried solid residues (SSM, SKSM, PSM, and PKSM) and 70 mL of distilled water were shaken in a Dubnoff bath (Ethiktechnology, model 304-TPA) at 80 °C for 2 h.

5.2.4.2 Pressurized water carrageenan extraction (PWCE):

High-pressure carrageenan extraction was carried out at 80 ºC, 50 bar (± 2 bar), for 15 min, with 0.7 g of dried solid samples (SSM, SKSM, PSM, and PKSM), and water as solvent, as described by Rudke et al. (2022).

5.2.4.3 Carrageenan Precipitation

The solutions obtained by CCE and PWCE were hot filtered (near 60 °C) to avoid gel formation using a vacuum pump. The cellulose fraction was retained on the filter. At the same time, the liquid phase was rotaevaporated to half the initial volume and precipitated with cold ethanol at 1:3 ($v.v^{-1}$), resulting in carrageenan fraction, identified as *Cg1* to *Cg4* Figure 5-1) dried at 60 °C for 24 h. The supernatant (low-molecular-weight fraction - *Lw*) evaporated and weighed.

5.2.5 Carrageenan properties

5.2.5.1 Viscosity and gel strength

The viscosity and the gel strength of the carrageenan samples (Figure 5-1: *Cg#* and *Cg#-K*, where # varies from 1 to 4) were determined according to Bono et al. (2014) with modifications. ThermoHaake ViscoTester 6 L was used for viscosity determination

using a carrageenan water solution of 1.5% ($w.v^{-1}$), moved by L1 spindle at 30 rpm, and maintained at 75 ºC. The measurement was taken after 6 spindle revolutions, with three readings for each sample. The gel strength was evaluated and measured using a Texture Analyzer TA.HD. plus (Stable Micro System, Godalming, Surrey, England) and a cylindrical aluminum container (50 mm diameter, 30 mm height). The penetration speed was 2 $mm.s^{-1}$, with a load cell of 50 kg and a depth of 1 cm. Five readings in different parts of gel were performed, and the analysis was conducted in duplicate. Results were performed for each sample, expressed in $g.cm^{-2}$ (mean ± standard deviation). The viscosity and gel strength of the carrageenan samples were compared with the result obtained for a commercial carrageenan (*CCg*), presented by Rudke et al. (2022).

5.2.5.2 Fourier transformed infrared spectroscopy (FTIR)

The carrageenan samples, analyzed by FTIR (Agilent Technologies – Cary 660 FTIR), were mixed with KBr and scanned from 400 to 4000 cm^{-1} (2 cm^{-1} resolution). The FTIR profile of the samples was compared with a commercial carrageenan (*CCg*) (Rudke et al., 2022).

5.2.5.3 Antioxidant potential by DPPH method

The antioxidant activity of the supernatant (Figure 5-1: samples *Lw#* and *Lw#-K*, where # varies from 1 to 4) was measured by the 2,2-diphenyl-1-Picrylhydrazil (DPPH) method (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). Briefly, 20 mg of the sample were diluted in 1.5 mL of distilled water. 50 µl of this solution was mixed with 250 µl 0.125 mM DPPH ethanolic solution. Samples were left in the dark for 30 min at room temperature, and the absorbance was measured at 517 nm using a microplate reader (Epoch, BioTek, USA). The analysis, in triplicate, was compared to a control (50 µL of water, 250 µL DPPH solution) and a blank for each sample (50 µL of sample, 250 µL of ethanol). DPPH results are expressed in µmol of Trolox equivalent per g of sample (µmol TE.g $sample^{-1}$), using a standard Trolox curve (50 to 350 µM).

5.2.5.4 Gel permeation Chromatography (GPC)

The GPC analysis was performed on a Viscotek size exclusion chromatograph consisting of a GPCmax VE-2001 module (pump, degasser, and autosampler) and a refractive index detector. The molecular weight of the carrageenan samples was expressed as number-average molecular weight (M_n), weight-average molecular weight (M_w), and z-average molecular weight (M_Z). The selected carrageenan samples (Figure 5-1) were the low-pressure route (*Cg1* and *Cg1-K*) and the high-pressure route (*Cg4* and *Cg4-K*). Carrageenan samples (5 $mg.mL^{-1}$) were diluted in 0.1 M $NaNO_3$ and filtered (0.22 μm nylon filters), with the column at 35 ºC, the flow rate of 0.5 $mL.min^{-1}$, and injection volume of 100 μL, with increments of 8.3 $\mu L.min^{-1}$. The results expressed in kDa were compared with the commercial carrageenan sample (*CCg*).

5.2.6 Proximate composition

Proximal composition (lipids, protein, ash, and carbohydrate) of the solid samples (before and after extractions) followed the procedures by AOAC, (2012). The **lipids** content (method 920.39) was determined by Soxhlet with hexane for 6 h. P**rotein** content (method 954.01) was quantified by a micro Kjeldahl digester to determine the total nitrogen, and a conversion factor of 5.0 was used (Angell et al., 2016). **Ash** content (method 923.03, AOAC) was determined at 575 ºC in a muffle furnace for 3 h, and **carbohydrate** content was determined by DNS (3,5-dinitrosalicylic acid) and by UFLC (Ultra Fast Liquid Chromatography) methods, according to Miller, (1959) and Masarin et al. (2016), respectively. Previous to DNS and UFLC analysis, the samples were hydrolyzed by H_2SO_4, thermo-pressurized, and then filtrated (carbohydrate is in the permeate, whereas the retentate contains the non-hydrolyzed biomass). For DNS analysis, the permeate was mixed (1:1) with an alkaline solution, vortexed, thermally treated, cooled, and absorbance read at 540 nm (Epoch, BioTek, USA) (Miller, 1959)*, with the* results *based on a* glucose *calibration curve* ($y=0.6116x + 0.0412$, $R^2=0.9985$). UFLC coupled with refractive index was conducted with the filtered permeate, injected in HPX-87H column using the analytical standards of glucose, galactose, and formic acid. The injected sample (10 μL) was eluted (acid solution) isocratically at 0.5 $mL.min^{-1}$ and 35 ºC for 30 min (Monteiro et al., 2021). The hydrolysis correction factors were galactose to

anhydrogalactose degradation of 1:1.27 and glucose or galactose to anhydromonomers of 0.9 (Masarin et al., 2016).

5.2.7 SEM analysis

SEM analysis was performed using a microscope JEOL JSM 6390 LV (Musashino, Akishima, Japan) for the biomass solid samples (before and after extraction procedures) to investigate the effects of the extraction process in the solid materials and the separation of the cellulose and carrageenan fractions. The samples were fixed in stubs and covered with a thin layer of gold (Baltec SCD 0005).

5.2.8 Mass balance of the combined process applied to KA biomass

After the treatments, the fractions were dried at 60 ºC for 24 h and weighed. Then, the mass balance (1 ton of KA - calculation basis) was calculated, taking into account the content of the fractions rich in carrageenan, phenolics, and cellulose to account for the process losses.

5.2.9 Statistical analysis

All analyses were carried out in triplicate, except for viscosity and gel strength (n=2), GPC (n=1), and DNS (n=2). The results were expressed as mean ± standard deviation and statistically analyzed by one-way analysis of variance (ANOVA) using the Statistica software. Significant differences ($p<0.05$) were analyzed using the Tukey test.

5.3 RESULTS AND DISCUSSION

5.3.1 Ethanolic extractions by SOX and PLE methods

The yield values obtained by SOX and PLE were 3.09 ± 0.09 and 1.54 ± 0.10%, respectively, representing the phenolics-rich fractions (liquid phase) (RUDKE *et al.*, 2022). The yield results were similar to those reported by Araújo et al. (2020) (2.67-3.43%) and Prasasty et al. (2019) (2.2%) using methanol and ethanol as solvents, respectively. The higher SOX yield compared to PLE is probably due to the extraction

time (6 h for SOX and 40 min for PLE), solvent reflux (mass transfer), and the higher temperature used.

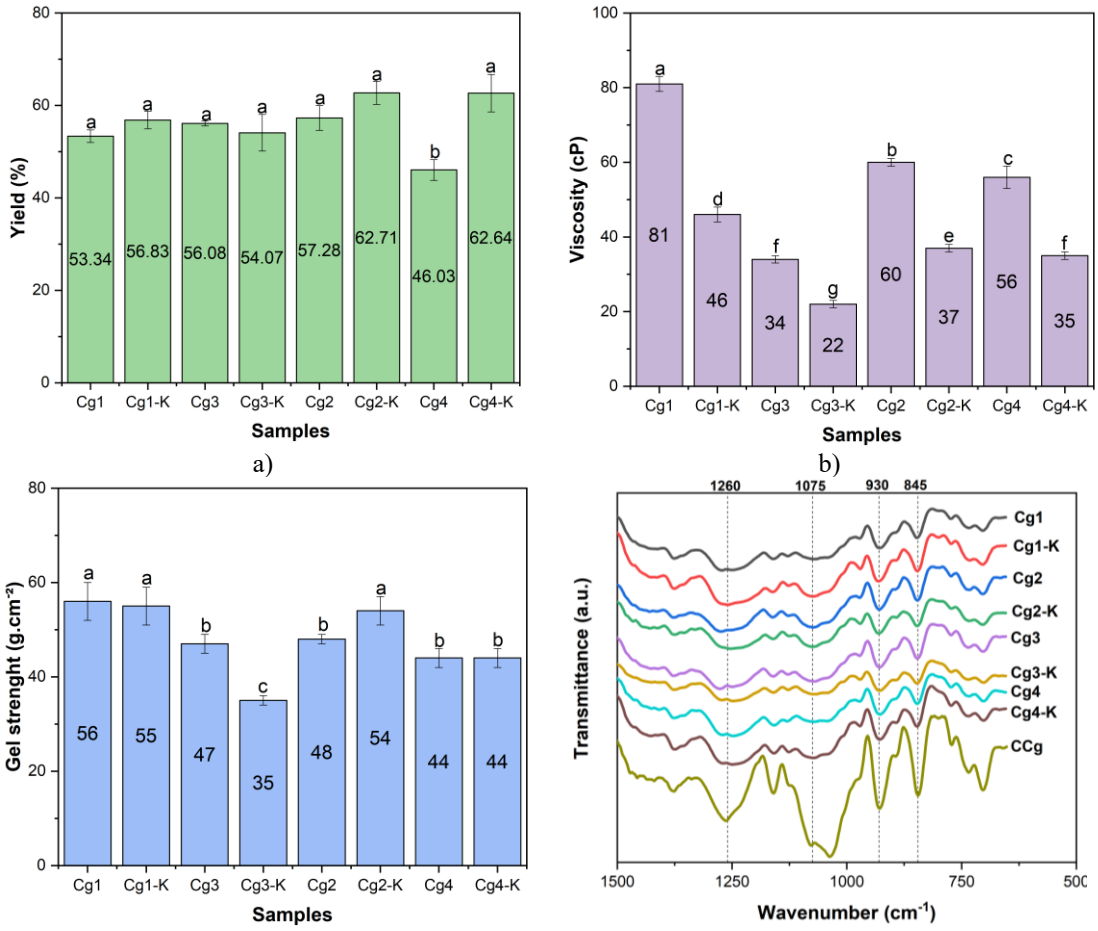
The phenolic fractions from KA (by SOX and PLE), characterized by Rudke et al. (2022), show that the PLE sample presented higher amounts of the quantified compounds (carnosol, ellagic acid, *p*-anisic acid, and rosmarinic acid), and higher antioxidant capacity (DPPH and FRAP) and TPC, compared to SOX sample. The presence of carnosol, ellagic acid, *p*-anisic acid, and rosmarinic acid was reported for the seaweeds *Gracilariopsis persica, Codium* sp., *Caulerpa* sp., and *Ecklonia* sp. (POURAKBAR *et al.*, 2021b; ZHONG *et al.*, 2020). Therefore, although PLE provided lower yield compared to SOX, the better quality of the PLE phenolics fraction (higher antioxidant potential and relevant quantified components), the high-pressure method can be considered as a suitable alternative for the phenolic recovery from KA biomass.

The remaining solid materials (co-product) from SOX and PLE were submitted to further treatments to obtain the carrageenan fractions (Figure 5-1), and the ethanol used as solvent was recovered by rotary evaporation, reaching 80% recovery from both samples (SOX and PLE). It is worth mentioning the importance of ethanol recovery from the phenolic fractions, which can be used for carrageenan precipitation or in phenolic extraction of a new KA inlet (Figure 5-1) in a circular processing chain.

5.3.2 Carrageenan extraction

The solid co-products (from SOX and PLE extracted with ethanol) were used for the carrageenan extractions without KOH pre-treatment, and with KOH pre-treatment (Figure 5-1). The results of carrageenan yield, viscosity, gel strength, and FTIR spectra from the carrageenan samples are presented in Figure 5-2.

Figure 5-2 Yield (a), Viscosity (b), gel strength (c), and FTIR spectra (d) from carrageenan samples recovered by Convention carrageenan extraction (CCE) and by pressurized water carrageenan extraction (PWCE). Where Cg# are the carrageenan samples obtained from the different processing steps according to Figure 5-1.



Source: The author (2024).

5.3.2.1 Carrageenan yield

The carrageenan yield values (Figure 5-2-a) varied from 46.03 to 62.71%, with the lower values provided by sample *Cg4*, which is statistically different than other samples, lowering the relevance of route *Cg4* (combines PLE at 40 ºC/40 min with PWCE at 80 ºC/15 min) for the carrageenan recovery, within the parameters studied. Nevertheless, the yield values (Figure 5-2-a) were higher than obtained by Ganesan et al. (2018), with data from 28 to 37.5%, and by Rudke et al. (2022), from 17 to 50%. Data from Figure 5-2-a were similar than provided by Hans et al. (2023) and by (Solorzano-Chavez et al., 2019), with yield varying from 30 to 55% and 53 to 69%, respectively. It is also essential to consider that pre-treatments, such as phenolic extraction and/or KOH treatment, may lead to carrageenan loss due to hydrolysis, reducing the extraction yield (Rupert et al., 2022).

Carrageenan yield values are dependent on factors such as raw material characteristics (algal species or abiotic factors associated with environmental conditions), extraction method (ultrasound, microwave, high-pressure, enzymatic, or maceration), and pre-treatments like phenolics extraction and use of KOH.

5.3.2.2 Carrageenan viscosity

The use of KOH as pre-treatment, followed by the carrageenan extraction method (CCE or PWCE), affected the carrageenan viscosity, as presented in Figure 5-2-b, with the viscosity values ranging from 22 cP to 81 cP. These values present statistically significant differences among the treatments.

The KOH treatment reduced the carrageenan viscosity for all treatments (comparing *Cg# vs. Cg#-K* from Figure 5-2-b). Freile-Pelegrín et al. (2006) also observed a viscosity reduction using treatment with KOH 7% ($w.v^{-1}$), where the viscosity of the treated sample reduced by half compared to control, probably due to carrageenan degradation with high KOH concentration (> 3%).

The use of high-pressure for carrageenan extraction (PWCE) compared to low-pressure (CCE) also reduces the carrageenan viscosity, as observed comparing *Cg1 vs. Cg3* and *Cg2 vs. Cg4* (Figure 5-2-b), probably due to the pressure effect (PWCE) on carrageenan characteristics, providing chemical modifications or purity changes in the carrageenan samples.

The method for phenolics recovery (SOX or PLE), previous to carrageenan extraction (CCE or PWCE), had a more complex influence on carrageenan viscosity. Comparing routes *Cg1 vs. Cg2* the viscosity reduces probably due to higher PLE influence (*Cg2*) on carrageenan properties than SOX (*Cg1*). Otherwise, comparing routes *Cg3 vs. Cg4* the viscosity increases probably because route *Cg3* combines high-temperature/6 h and high-pressure (SOX and PWCE, respectively), which may have intensified the viscosity reduction (carrageenan hydrolysis) (Montolalu et al., 2008).

The viscosity values (Figure 5-2-b) agree with Shanmugam & Seth, (2018) data, ranging from 35.5 to 87.70 cP; with Ali et al. (2020) (30 and 70 cP), and with Rudke et al. (2022) (21 to 60 cP). On the other hand, the values were lower than obtained by Kumar et al. (2020) and by Shalvina et al. (2022). There is a high variation in the viscosity of

algae carrageenan, very likely due to the cultivation conditions and the different extraction methods and treatments, which may induce to carrageenan degradation, as observed by Gereniu et al. (2018).

5.3.2.3 Carrageenan gel strength

The gel strength of the carrageenan samples ranged from 35 to 56 g.cm-^{2} (Figure 5-2-c), with the highest values for *Cg1*, *Cg1-K*, and *Cg2-K* samples, with no statistical differences. These results were followed by the samples *Cg2*, *Cg3*, *Cg4*, and *Cg4-K* (statistically similar values). The lowest gel strength was obtained by *Cg3-K* sample.

It was not possible to verify a clear influence of the phenolics recovery step (SOX or PLE) on gel strength (*Cg1* and *Cg3* compared with *Cg2* and *Cg4,* respectively, with or without KOH*).* Otherwise, the pressure effect for carrageenan recovery mainly reduced the gel strength, by comparing PWCE with CCE (*Cg1* compared with *Cg3,* and *Cg2* compared with *Cg4,* with or without KOH*)*, which is probably due to high-pressure effect on carrageenan characteristics, similar to viscosity, where the high-pressure contribute to carrageenan depolymerization (GERENIU; SARAVANA; CHUN, 2018).

Additionally, the KOH influence on gel strength was not clearly observed. An increase in gel strength was only detected by comparing *Cg2* with *Cg2-K*. Nevertheless, within the process conditions studied, no difference in gel strength was detected comparing *Cg1* with *Cg1-K* and *Cg4* with *Cg4-K* (Figure 5-2). In theory, KOH treatment increases the gel strength due to the reduction of sulfate groups, which affects the basic forms of carrageenan, i.e., increasing the conversion of μ- and ν-carrageenan into κ- and ι-carrageenans, respectively, which have higher gel strength compared to μ and ν forms (Rupert et al., 2022; Shalvina et al., 2022). Therefore, the gel strength is correlated to carrageenan forms, as presented by FTIR results in section 5.3.2.4.

The gel strength of the carrageenan samples (Figure 5-2-c) was within the range reported by Bono et al. (2014) (3.27 to 114.40), by Ferdiansyah et al. (2023) (12.78 to 137.93), and by Freile-Pelegrín et al. (2006) (<50 g.cm^{-2}). Besides, the present results were mostly higher than obtained by Rudke et al. (2022) (25 to 36 g.cm^{-2}), probably due to the phenolics extraction conducted as pre-treatment, compared to the literature. Ali et al. (2020) and Faria et al. (2014) evaluated the gel strength of carrageenan from KA, with

results from 1,487 to 1,695 g.cm^{-2} and from 1,325 to 1,727 g.cm^{-2}, respectively. These values are above data from Figure 5-2-c, probably because the authors used KCl for carrageenan precipitation, which remains in the carrageenan sample, increasing the gel strength.

Considering viscosity and gel strength of carrageenan, the lowest values were obtained from *Cg3-K* sample (22 cP and 35 g.cm^{-2}, respectively), which combines Soxhlet (SOX), KOH treatment, and high-pressure carrageenan extraction (PWCE), where the SOX conditions (high-temperature/6 h) mainly contribute to carrageenan depolymerization. According to Montolalu et al., (2008), 90 ºC maceration for 1 h provides carrageenan of 2,490 kDa molecular weight, whereas longer period (90 ºC for 5h) reduces to 380 kDa. Therefore, the conditions of *Cg3-K* route, of high-temperature/6h (SOX) and high-pressure (PWCE), may have contributed to carrageenan depolymerization (ÁLVAREZ-VIÑAS *et al.*, 2024; GERENIU; SARAVANA; CHUN, 2018), reducing the viscosity and the gel strength of the recovered product.

5.3.2.4 Carrageenan FTIR spectra

Figure 5-2-d presents the FTIR spectra of the carrageenan samples. The FTIR analysis was carried out to verify the type of carrageenan (basic forms) samples obtained from each extraction route in comparison with the commercial carrageenan (*CCg*).

The types of carrageenan are represented (Figure 5-2-d) by the bands 1,260, 1,070, 930, and 845 cm^{-1}, referred to the sulfate ester, the CO bond of 3,6 anhydrogalactose, the 3,6-anhydro-$_D$-galactose, and the $_D$-galactose-4-sulfate, respectively, which are present in κ-carrageenan (Pereira et al., 2009), and detected in all samples. Therefore, the FTIR analysis suggests that all routes applied for carrageenan recovery provided samples within the same type, the κ-carrageenan, compatible with the commercial sample (*CCg*) and the literature data (Pereira et al., 2009). Thus, the lack of bands at 815 and 867 cm^{-1} (vibration of C-O-SO_3 on C_6 of galactose) and bands at 825-830 cm^{-1} (vibration C-O-SO_3 on C_2 of galactose) indicates the absence of μ- and ν-carrageenan, respectively (PEREIRA *et al.*, 2009). Then, all samples are mostly κ-carrageenan, which explains the non-effect of KOH on gel strength (section 5.3.2.3) since the KOH contributes to the conversion of other basic forms into κ-carrageenan.

5.3.2.5 Carrageenan molecular weight

The molecular weight of the samples, evaluated through GPC analysis, is expressed in terms of number-average molecular weight (M_n), weight-average molecular weight (M_w), z-average molecular weight (M_Z), and polydispersity index (M_w/M_n). These analyses were conducted to compare the low-pressure route (*Cg1*) with the high-pressure route (*Cg4*). The results obtained from the precipitate (*Cg1* and *Cg4*) were compared with the values from the commercial carrageenan (*CCg*) in Table 5-1.

Table 5-1 – Molecular weight averages (n=1) (M_n, M_w, and M_Z) and polydispersity index (M_w/M_n) from samples of carrageenan from *Kappaphycus Alvarezii obtained by routes 1 an*d 4 (low and high-pressure methods, respectively) and the commercial carrageenan (CCg).

Samples	M_n (KDa)	M_w (KDa)	M_z (KDa)	M_w/M_n
Cg1	135.93	653.25	1056	4.81
Cg1-K	16.23	118.586	326.89	7.31
Cg4	84.87	376.92	714.09	4.44
Cg4-K	94.79	475.03	821.76	5.01
CCg	2,344.00	35,240.00	24,5000.00	15.03

Where: Cg# are the carrageenan samples obtained from the different processing steps according to Figure 5-1.

Source: The author (2024).

According to the European Food Safety Authority (EFSA), the molar weight of carrageenan ranges from 30 kDa to 5,000 kDa, with an average molecular weight (Mw) between 200 and 800 kDa (EFSA *et al.*, 2018). The results from the carrageenan samples obtained from routes 1 and 4 (Table 5-1) are within the EFSA values. Otherwise, the *CCg* sample presented an M_w value (Table 5-1) significantly higher (35,240 kDa) than the EFSA range, probably because *CCg* also contains other polymers such as cellulose (based on the molecular weight of glucose, as the cellulose base unit).

The results from the carrageenan samples suggest that the KOH treatment reduces M_w by 5.5 times for the low-pressure route (from *Cg1* to *Cg1-K*), while KOH increases 1.26 times the M_w value for the high-pressure route (from *Cg4* to *Cg4-K*). For the low-pressure route, the KOH treatment *was applied to the SOX sample* (high-temperature/6 h) which contributed to carrageenan hydrolyzed (reducing Mw), compared to the high-pressure route*, where the* KOH treatment was applied to PLE sample (40 ºC/40 min). Additionally, the polydispersity index (Mw/Mn) increased with KOH for all samples, probably due to the combined influence of KOH in hydrolyzing the carrageenan (reducing

size), and also binding the polymer chains (increasing carrageenan 3-dimensional structure), as discussed by Hayashi et al., (2007); Heriyanto et al., (2018); Rhein-Knudsen et al., (2015).

The molar weight values (M_w) obtained in this work are comparable with literature data, which present a wide range of values. For instance, Ferdiansyah et al. (2023) obtained M_w from 7.59 kDa to 289.90 kDa, Montolalu et al. (2008) from 220 kDa to 8,540 kDa, Webber et al. (2012) of 259 kDa, Hayashi et al. (2007) from 1100 to 2,700 kDa, and Gereniu et al. (2018) with values lower than 60 kDa. This wide range is probably associated with the carrageenan source (cultivation, climate, and other factors), the applied methods (extraction, pre-treatments, process conditions), and the carrageenan purity.

5.3.3 Proximal composition and mass balance

5.3.3.1 Proximal composition

The proximal composition in macronutrients for the solid samples, representing routes 1 (low-pressure) and 4 (high-pressure), were conducted according to section 2.5.5. The results, showing the values of protein, ash, total reducing sugar, glucan, and galactan, are presented in Table 5-2, and compared with the commercial carrageenan (*CCg*).

Table 5-2 – Content of protein, ash, total reducing sugars (TRS), glucan, and galactan from the *Kappaphycus alvarezzi* fractions, and for the commercial carrageenan.

Samples*	Protein	Ash	TRS g/100g	Glucan	Galactan
KA	3.35 ± 0.01^{c}	23.32 ± 0.69^{bc}	22.50 ± 0.48^{hi}	3.41 ± 0.11^{d}	25.14 ± 1.89^{ef}
SSM	3.70 ± 0.47^{c}	29.59 ± 0.39^{a}	25.43 ± 0.17^{fg}	4.29 ± 0.12^{d}	29.16 ± 1.97^{e}
PSM	3.35 ± 0.02^{c}	29.15 ± 0.24^{a}	28.02 ± 0.25^{de}	4.66 ± 0.41^{d}	32.79 ± 1.31^{bcde}
SKSM	1.35 ± 0.00^{e}	16.60 ± 0.07^{de}	33.40 ± 0.65^{c}	7.11 ± 0.55^{c}	43.87 ± 2.39^{a}
PKSM	1.34 ± 0.00^{e}	16.35 ± 0.16^{de}	28.84 ± 0.53^{d}	5.29 ± 0.00^{cd}	32.18 ± 0.00^{cde}
Cg1	0.67 ± 0.00^{f}	23.82 ± 0.51^{bc}	24.14 ± 0.23^{gh}	nd	39.27 ± 4.54^{abc}
Cg4	1.34 ± 0.01^{e}	25.16 ± 0.71^{b}	24.05 ± 0.23^{gh}	nd	38.63 ± 3.15^{abcd}
Cg1-K	0.67 ± 0.00^{f}	17.70 ± 0.73^{d}	26.83 ± 0.02^{ef}	nd	41.58 ± 0.30^{ab}
Cg4-K	0.67 ± 0.00^{f}	17.92 ± 0.12^{d}	33.27 ± 0.89^{c}	nd	44.81 ± 4.85^{a}
CCg	0.69 ± 0.02^{f}	21.43 ± 1.63^{c}	21.22 ± 0.32^{i}	13.35 ± 0.03^{b}	29.29 ± 1.49^{e}
Ce1	10.00 ± 0.01^{a}	13.59 ± 0.07^{ef}	34.24 ± 0.65^{c}	25.27 ± 0.66^{a}	16.83 ± 1.84^{fg}
Ce4	7.43 ± 0.04^{b}	12.32 ± 1.67^{fg}	34.62 ± 0.23^{c}	15.18 ± 0.31^{b}	26.57 ± 0.87^{e}
Ce1-K	2.03 ± 0.00^{d}	9.80 ± 0.72^{gh}	42.81 ± 0.91^{b}	25.67 ± 1.05^{a}	15.80 ± 0.27^{g}
Ce4-K	2.02 ± 0.01^{d}	8.12 ± 1.10^{h}	47.74 ± 0.28^{a}	24.71 ± 1.22^{a}	29.55 ± 0.75^{de}

* Where: KA: *Kappaphycus alvarezii* biomass; SSM (SOX solid material); PSM (PLE solid material); SKSM (SOX Solid material treated with KOH); PKSM (PLE Solid material treated with KOH); *Cg1* (SOX-Carrageenan); *Cg1-K* (SOX-Carrageenan with KOH); *Ce1* (SC-Cellulose); *Ce1-K* (SOX-Cellulose with KOH) and; *Cg4* (PLE-Carrageenan); *Cg4-K* (PLE Carrageenan with KOH); *Ce4* (PLE Cellulose); *Ce4-K* (PLE Cellulose with KOH).

Source: the author (2024).

The lipids content from the raw material (KA) was 0.75 g.100 g^{-1}, and lower than 0.2% for solid samples after PLE or SOX (SSM and PSM respectively), indicating the low relevance of lipids compared to other constituents, for all solid samples evaluated. These results agree with Roldán et al., (2017), which obtained 0.8% of lipids in the raw material and neglectable concentrations in other solid samples after treatments.

The protein content from the raw material (KA) was 3.35 g.100 g^{-1}, while the values from carrageenan samples from routes 1 and 4 varied from 0.67 to 1.34 g.100 g^{-1}, similar to the commercial sample (*CCg*) of 0.69 g.100 g^{-1}. This behavior suggests that routes 1 and 4 are adequate for carrageenan recovery instead of protein, contributing to its purity. Besides, the protein values from SSM and PSM (samples after phenolics recovery with ethanol by SOX and PLE, respectively) were similar to KA due to the low protein solubility in ethanol (Pace et al., 2004; Sawada et al., 2014). However, the decrease in protein content with KOH (from 3.70 to 1.35, and from 3.35 to 1.33 g.100 g^{-1} for SKSM and PKSM samples, respectively) was probably due to high algal protein solubility at alkaline medium (Geada et al., 2021; Harrysson et al., 2018), which is removed from the solid phase with the leaching step after KOH treatment. The same behavior was detected for the cellulosic fraction (*Ce1* and *Ce4*), as reported by Masarin et al., (2016), Roldán et al., (2017), and Solorzano-Chavez et al., (2019).

The ash content (Table 5-2) from KA was 23.32 g.100 g^{-1}, whereas from SSM and PSM samples (after ethanolic extractions) were 29.59 and 29.15 g.100 g^{-1}, respectively. KOH treatment reduced ash and protein contents (all samples), unlike observed by Masarin et al. (2016), Roldán et al., (2017), and Solorzano-Chavez et al. (2019). The authors suggested a mineral enrichment due to KOH deposited on the sample surface, probably due to inefficient KOH washing. Otherwise, pH evaluation (until neutrality) confirmed an efficient KOH washing in the present work, warranting complete KOH leaching. Also, lower ash content was verified from the cellulose fraction compared to carrageenan fraction, probably due to the sulfate groups from the carrageenan structure which is aligned with Masarin et al. (2016) and Roldán et al. (2017). The ash content from carrageenan fractions is within the value reported as standard by EFSA (15 – 40%) (EFSA *et al.*, 2018).

Related to the carbohydrate fraction from carrageenan samples, the KOH treatment increased the TRS values from routes 1 and 4 (*Cg1* and *Cg4* compared to *Cg1-*

K and *Cg4-K*, respectively), with values in the same range as obtained by the commercial carrageenan (*CCg*). The quality of the carbohydrate was evaluated by the content of glucan and galactan for all samples (also in Table 5-2). The glucan content was based on the amount of glucose, while galactan content was based on formic acid, galactose, and anhydrogalactose (Table 5-3). Samples from routes 1 and 4 presented only galactan, producing carrageenan fractions free of glucan (cellulose). High galactan content (samples *Cg1*, *Cg4*, *Cg1-K*, and *Cg4-K*) was expected since carrageenan is composed of galactose linked with anhydrogalactose by glycosidic bonds. This behavior indicates good separation from the cellulose fractions (*Ce1* and *Ce1-K*, and *Ce4* and *Ce4-K*). Besides, the cellulose fractions with high glucan content suggest good separation of cellulose from the carrageenan samples. Surprisingly, significant amounts of glucan were detected from the commercial carrageenan (*CCg*), probably indicating the presence of cellulose in this sample, which may suggest low purity to the commercial carrageenan. Masarin et al. (2016), Roldán et al. (2017), and Solorzano-Chavez et al. (2019) also detected high amounts of glucan in the cellulose fractions and high amounts of galactan in the carrageenan fractions, which may be associated to the good separation of the carbohydrate fraction, in accordance with our findings.

Therefore, KA processing enabled the components fractionation, i.e., the carrageenan samples presented no glucans (cellulose components) and negligible amounts of protein and lipids (< 1%), suggesting carrageenan products with high purity.

Table 5-3 - Glucose, galactose, formic acid, and anhydro-galactose quantified in different fractions of *Kappaphycus alvarezzi* and commercial carrageenan.

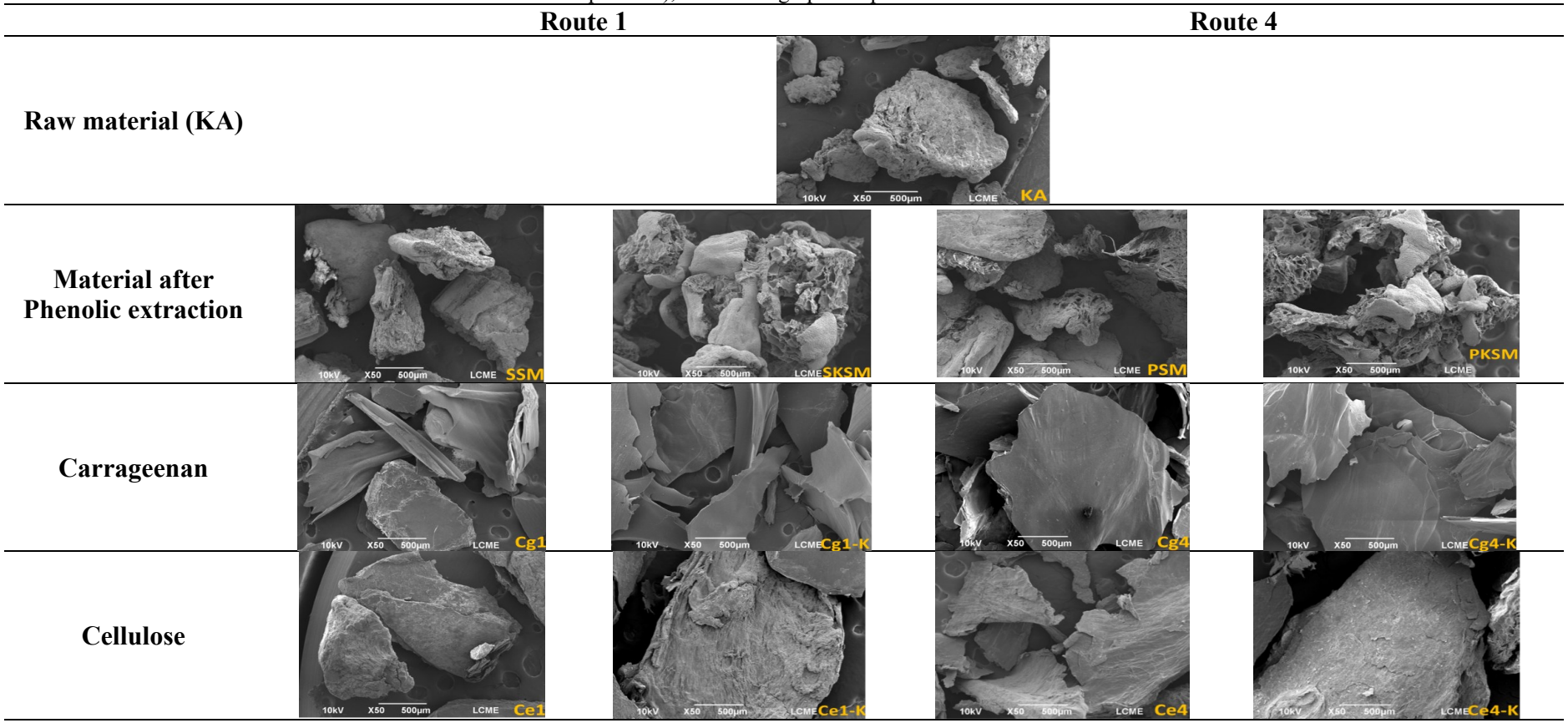
Sample	Glucose	Galactose	Formic acid	Anhydro-galactose
			g/100g	
KA	$3.79^{d} \pm 0.13$	$12.31^{ef} \pm 0.93$	nd	$15.63^{ef} \pm 1.18$
SSM	$4.76^{d} \pm 0.13$	$14.27^{e} \pm 0.96$	nd	$18.13^{e} \pm 1.22$
PSM	$5.18^{d} \pm 0.46$	$16.05^{bcde} \pm 0.64$	nd	$20.38^{bcde} \pm 0.82$
SKSM	$7.90^{c} \pm 0.61$	$21.39^{a} \pm 1.17$	$4.65^{a} \pm 0.03$	$27.17^{a} \pm 1.49$
PKSM	$5.88^{cd} \pm 0.00$	$15.68^{cde} \pm 0.00$	$3.92^{b} \pm 0.00$	$19.92^{cde} \pm 0.00$
Cg1	nd	$19.22^{abc} \pm 2.22$	nd	$24.41^{abc} \pm 2.82$
Cg4	nd	$18.91^{abcd} \pm 1.54$	nd	$24.02^{abcd} \pm 1.96$
Cg1-K	nd	$20.29^{ab} \pm 0.14$	$3.78^{b} \pm 0.15$	$25.76^{ab} \pm 0.18$
Cg4-K	nd	$21.86^{a} \pm 2.36$	$3.88^{b} \pm 0.05$	$27.76^{a} \pm 2.99$
CCg	$14.83^{b} \pm 0.03$	$14.34^{e} \pm 0.73$	nd	$18.21^{e} \pm 0.93$
Ce1	$28.08^{a} \pm 0.73$	$8.24^{fg} \pm 0.89$	nd	$10.46^{fg} \pm 1.14$
Ce4	$16.86^{b} \pm 0.35$	$13.01^{e} \pm 0.42$	nd	$16.52^{e} \pm 0.54$
Ce1-K	$28.53^{a} \pm 1.17$	$7.73^{g} \pm 0.13$	nd	$9.83^{g} \pm 0.17$
Ce4-K	$27.45^{a} \pm 1.35$	$14.46^{de} \pm 0.36$	nd	$18.37^{de} \pm 0.46$

Source: The author (2024).

5.3.3.2 SEM analysis

Figure 5-3 shows the SEM images of samples from routes 1 and 4 (different steps). The analysis of the SEM images suggest that KA has intact and rough surface, with little change in the structure due to phenolics extraction (SSM and PSM), as observed by Hans et al. (2023). However, when the material is treated with KOH (SKSM and PKSM), a significant opening in the surface structure occurs, which becomes more evident at 5,000X magnification (Figure 5-4). The carrageenan samples (*Cg#*) have a granular shape and smooth surface with evident fibrils at 5,000X (Figure 5-4), compared to the rough cellulose samples (*Ce#*). Also, no significant variation between images from routes 1 and 4 was observed. Nevertheless, the cellulose fractions still present galactan (carrageenan basic unit), suggesting carrageenan fragments at cellulose surface (increasing rugosity), similar to the commercial sample (*CCg*).

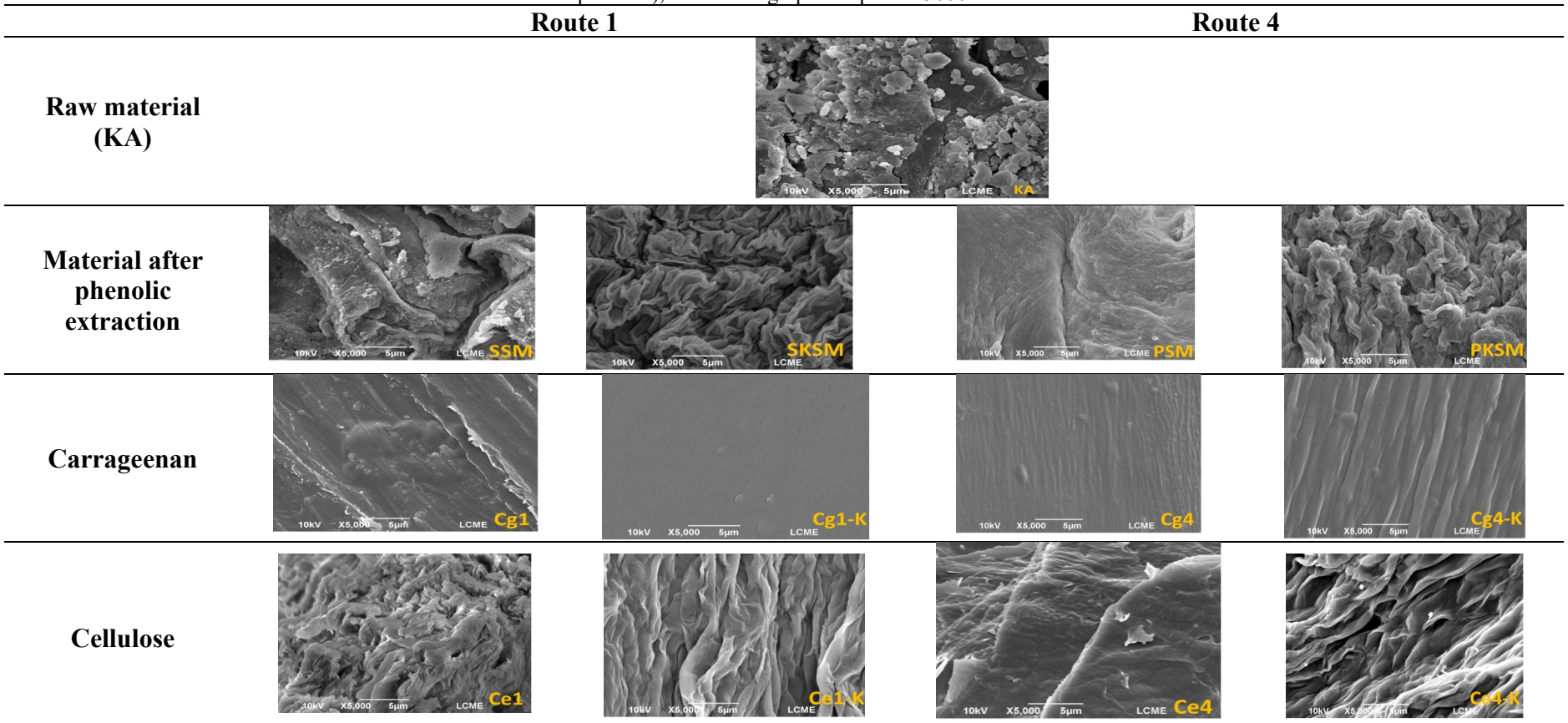
Figure 5-3 – Scanning Electron Microscopy (SEM) of the raw material (*Kappaphycus alvarezii*) and solid samples from Route #1 (low-pressure) and Route #4 (high-pressure), with micrographs amplified 50X.



KA: Raw material; SSM (SOX solid material); SKSM (SOX Solid material treated with KOH); Cg1 (SOX-Carrageenan); Cg1-K (SOX-Carrageenan with KOH); Ce1 (SC-Cellulose); Ce1-K (SOX-Cellulose with KOH) and PSM (PLE solid material); PKSM (PLE Solid material treated with KOH); Cg4 (PLE-Carrageenan); Cg4-K (PLE Carrageenan with KOH); Ce4 (PLE Cellulose); Ce4-K (PLE Cellulose with KOH).

Source: the author (2024).

Figure 5-4 – Scanning Electron Microscopy (SEM) of the raw material (*Kappaphycus alvarezii*) and solid samples from Route #1 (conventional route) and Route #4 (high-pressure), with micrographs amplified 5000X.



KA: Raw material; SSM (SOX solid material); SKSM (SOX Solid material treated with KOH); Cg1 (SOX-Carrageenan); Cg1-K (SOX-Carrageenan with KOH); Ce1 (SOX-Cellulose); Ce1-K (SOX-Cellulose with KOH); PSM (PLE solid material); PKSM (PLE Solid material treated with KOH); Cg4 (PLE-Carrageenan); Cg4-K (PLE Carrageenan with KOH); Ce4 (PLE Cellulose); and Ce4-K (PLE Cellulose with KOH).

Source: the author (2024).

The visual evaluation of SEM images (Figure 5-3) shows that samples changed from green (KA, SSM, and PSM) to pale yellow (SKSM and PKSM) and to white/transparent after KOH treatment (*Cg1-K* and *Cg4-K*). Cellulose samples without KOH treatment (*Ce1* and *Ce4*) presented an intense green color, whereas samples treated with KOH appeared yellow (*Ce1-K* and *Ce4-K*). These color changes were due to chlorophyll saponification in alkaline media, producing water-soluble chlorophyllins removed by washing (LI *et al.*, 2016; PARK *et al.*, 2020). Similarly, Masarin et al. (2016) reported that KOH treatment yellowed the samples; in addition, the carrageenan was smooth and translucent; on the other hand, cellulose samples presented an opaque appearance.

5.3.3.3 Mass balance

Considering the KA processing from Figure 5-1, routes 1 and 4 were selected to compare low- and high-pressure methods. The mass balances of the two routes are compared in Table 5-4 for KA as raw material. Thereby, 3% and 2% of phenolic components were recovered from KA by SOX (routes 1) and PLE (route 4), respectively (RUDKE *et al.*, 2022). The solid residues from both extractions (SSM and PSM) provided carrageenan yields of 47% (*Cg1*) and 40% (*Cg4*), where the high-pressure route (*Cg4*) lasted 55 min (40 min from PLE plus 15 from PWCE), while the low-pressure route (*Cg1*) was conducted during 8 h (6 h from SOX plus 2 h from CCE). Besides, the quality of both carrageenan samples was very similar, as presented in section 5.3.2

Table 5-4 - Mass balance of *Kappaphycus Alvarezii* (KA) biorefinery considering route 1 (low-pressure) and route 4 (high-pressure) to obtain different fractions.

Route 1*			Route 4*		
Fractions	**Yield (%)**	**Loss (KA) (%)**	**Fractions**	**Yield (%)**	**Loss (KA) (%)**
KA	100	0	**KA**	100	0
Liquid 1(1)	3	9	**Liquid 2(1)**	2	11
SSM	88		**PSM**	87	
Cg1	47		**Cg4**	40	
Lw1	11	23	**Lw4**	12	30
Ce1	19		**Ce4**	18	
SKSM	60	40	**PKSM**	57	43
Cg1-K	34		**Cg4-K**	36	
Lw1-K	5	47	**Lw4-K**	4	49

Ce1-K	14	**Ce4-K**	11

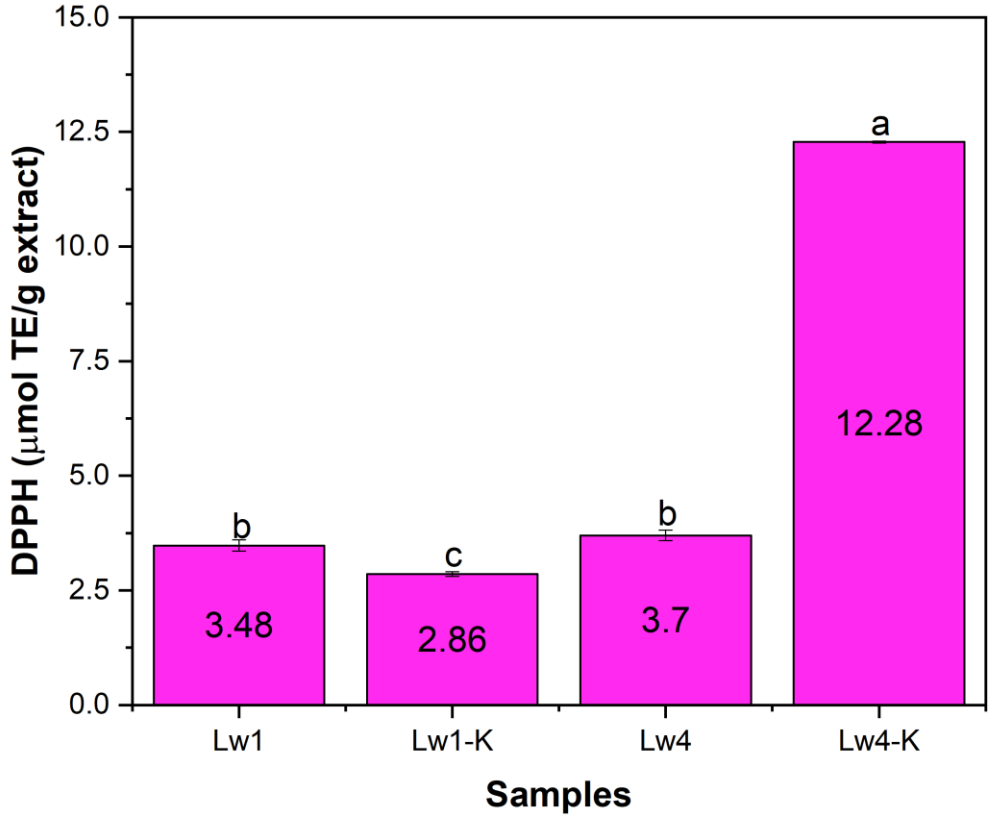
(1) (RUDKE *et al.*, 2022).(*) The loss value, as well as the step yields, are a function of the input material (KA): Raw material; Liquid 1: phenolic fraction of SOX; Liquid 2: phenolic fraction of PLE; SSM: solid material after SOX; PSM solid material after PLE; Cg1: Carrageenan SOX; Cg4: Carrageenan PLE; Lw1: low molecular weight fraction from SOX; Lw4: low molecular weight fraction from PLE; Ce1: Cellulosic SOX; Ce4: Cellulosic PLE; SKSM: Solid material of SOX treated with KOH; PKSM: Solid material of PLE treated with KOH; Cg1-K Carrageenan SOX after KOH; Cg4-K Carrageenan PLE after KOH; Lw1-K: low molecular weight fraction from SOX treated with KOH; Lw4: low molecular weight fraction from PLE treated with KOH; Ce1-K Cellulosic SOX after KOH; Ce4-K Cellulosic PLE after KOH.

Source: the author (2024).

Table 5-4 also indicates 40% loss with KOH treatment of SKSM sample, and 43% for PKSM sample due to washing and filtering, since water-leaching removes soluble compounds such as minerals and proteins/peptides (values reduction from Table 5-2). The KOH conventional process (*Cg1-K*) reduced the carrageenan yield compared to the high-pressure method (*Cg4-K*) by 34 and 36%, respectively.

The non-precipitated liquid fractions presented yields of 11% (*Lw1*) and 12% (*Lw4*), without KOH pretreatment, and with 5% (*Lw1-K*) and 4% (*Lw4-K*) with KOH. The lower yields obtained from KOH treated samples are probably due to the water-leaching, increasing losses. Also, the KOH treatment may increase the molecular weight of the recovered fraction, as observed by Hayashi et al., (2007), with higher precipitation in ethanol. According to Torres, et al. (2019), low molecular weight carrageenan (*Lw*) presents antioxidant potential. Then, *Lw* samples were evaluated by DPPH to detect their antioxidant potential, and the results (Figure 5-5) ranged from 2.86 to 12.21 μmol TE.g extract^{-1}. The highest value was obtained by *Lw4-K* and the lowest by *Lw1-K*. The KOH treatment increased the antioxidant potential of the PLE sample (*Lw4*) while decreased for the SOX sample (*Lw1*), suggesting antioxidant degradation by SOX method (6 h), as demonstrated by Rudke et al. (2022). Also, PWCE sample had higher antioxidant potential than CCE sample, suggesting that the high-pressure process may preserve the antioxidant molecules.

Figure 5-5 - Antioxidant activities by DPPH method from Lw fractions for route 1 from Route #1 (conventional route) and Route #4 (high-pressure).



Where: Lw# are the low molecular weight carrageenan fractions, obtained by different processing steps according to Figure 1.

Source: the author (2024).

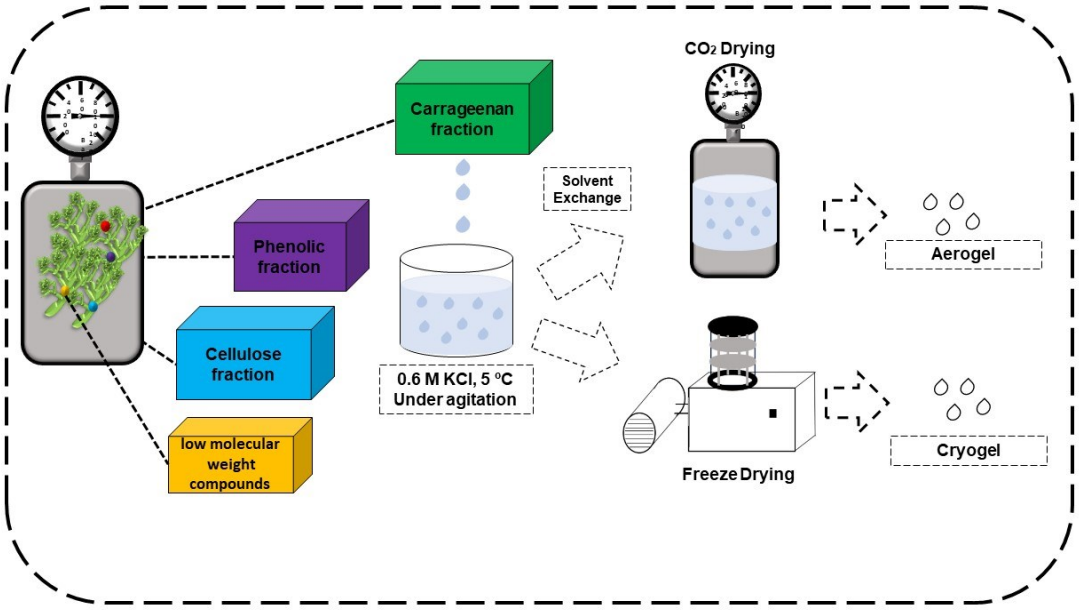
The yield behavior of the cellulose fractions was similar to *Lw*, with lower values from KOH treated samples, compared to non-treated (19 and 18% for *Ce1* and *Ce4* respectively, and 14 and 11% for *Ce1-K* and *Ce4-K*, respectively), also due to water-leaching. The *Ce#*-K yields (KOH treatment) were lower than the 23.5% obtained by Roldán et al. (2017), a difference probably associated with variations in raw material (KA) or processing losses.

For biorefinery and circular economy approaches, the cellulose fraction is also important. According to Ingle et al. (2018), cellulose can be converted into ethanol with conversion factor between 0.41 and 0.68 kg ethanol/kg cellulose (*S. cerevisiae* and *E. coli*, or one followed by the other). Then, from Table 5-4, 1,000 kg of seaweed (dry KA) can produce between 50 and 150 kg of ethanol, and this ethanol can be used for phenolics or carrageenan recovery, contributing to the Sustainable Development Goals (SGD) from United Nations.

Therefore, 1,000 kg of dry KA produced 30 and 20 kg of phenolic compounds by routes 1 and 4, respectively (Figure 5-1 - Biorefinery flow chart used for the recovery of the fractions: carrageenan, phenolic, cellulose, and low molecular weight samples, obtained from *Kappaphycus alvarezii* (KA). Where: SSM: Soxhlet Solid Material, SKSM: Soxhlet KOH Solid Material; PSM: PLE Solid Material, PKSM: PLE KOH Solid material, CCE (conventional carrageenan extraction) and PWCE (Pressurized water carrageenan extraction) are the processes for carrageenan recovery; Ce# are the cellulose samples (fractions); Cg# are the carrageenan samples obtained from the different processing steps; Lw# are the low molecular weight carrageenan fractions, obtained by different processing steps.). Additionally, 470 or 340 kg of carrageenan were produced from *Cg1* or *Cg1-K*, from route 1 (low-pressure), while route 4 (high-pressure) provided 360 or 400 kg of carrageenan for *Cg4* or *Cg4-K*, respectively. Nevertheless, the fractions quality and the time- and energy-consumption must be considered, with results favoring route 4 as previously discussed, which suggests high-pressure methods as greener sequential extractions.

5.4 CONCLUSION

The KA biomass was deconstructed by sequential extractions comparing low- and high-pressure routes, enabling the recovery of different fractions: phenolics, cellulosic, carrageenan, and low molecular weight. The results of the two routes were similar, mainly in quantity. However, the high-pressure assays (route 4) are significantly faster, representing lower energy consumption than low-pressure methods (route 1). Route 4 also produces fractions with improved/changed quality attributes, such as higher antioxidant potential from phenolic fraction and lower carrageenan viscosity with smooth surface (higher purity), compared to products from route 1 and commercial carrageenan. The results also indicated that KOH treatment affect the carrageenan properties, such as TRS, gel strength and viscosity, and should be considered for carrageenan applications. In addition, the recovery of the cellulosic fraction, with relevant antioxidant potential, contributes to the novelty approach for KA biorefinery.



CHAPTER 6 – HIGH PURITY κ-CARRAGEENAN FROM *KAPPAPHYCUS ALVAREZII* ALGAE FOR AEROGEL PRODUCTION BY SUPERCRITICAL CO_2 DRYING

In the previous chapter, the sequential extraction of KA at high pressure was carried out, and different compounds, including carrageenan, were obtained. In this chapter, the carrageenan obtained from the high-pressure sequential process will be used to produce an aerogel/cryogel, and these materials will be compared with an aerogel/cryogel produced with commercial carrageenan.

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6.1 INTRODUCTION

Aerogels are extensively used by cosmetic, biotechnological, food, and pharmaceutical industries. These high-porous materials have low weight, density, and thermal conductivity, while present high surface area/volume ratio, water absorption capacity, and mechanical strength. Other functional properties of the aerogels, such as oil absorption capacity, depend on the biopolymer characteristics (size, shape, purity), and the drying method used for aerogel production (CUCE *et al.*, 2014; EL-NAGGAR *et al.*, 2020; GARCÍA-GONZÁLEZ; ALNAIEF; SMIRNOVA, 2011; MIKKONEN *et al.*, 2013; WOIGNIER *et al.*, 2015). Due to the variety of the base-biopolymers used to produce the aerogels, these versatile materials present several functionalities, such as absorbers for water, oil or gas components, molecule carriers, and active agents (packaging), among others. Mostly, the aerogels used in food industry are based on biopolymers such as cellulose, starch, alginate, chitosan, agar, and carrageenan (AGOSTINHO *et al.*, 2020; MIKKONEN *et al.*, 2013).

Carrageenan is a sulfated biopolymer from red seaweed such as *Kappaphycus alvarezii* (KA) and, because it represents almost 40% of the algae, KA is the main industrial source of this biopolymer. The carrageenan is a linear polysaccharide composed of glycosidic linkages between $_D$-galactose and 3,6-anhydro-galactose joined by α (1 → 3) and β (1 → 4) (RAMAN; DOBLE, 2015). It can be classified in the basic forms of: iota (ι), kappa (κ), lambda (λ), mu (μ), nu (ν), and theta (θ). The dimeric units of carrageenan present the following contents of sulfate groups: λ and ν (3); μ, ι, and θ (2); and κ (1). The carrageenan applications are associated to their basic forms, and the

increase in the number of sulfate groups also increases its water solubility (CAMPO *et al.*, 2009).

The most common type of carrageenan from KA is the κ-carrageenan (RUPERT *et al.*, 2022), widely used due to properties such as gelling, thickening, emulsifying, and stabilizing (RUDKE *et al.*, 2022; RUDKE; DE ANDRADE; FERREIRA, 2020b). Different methods and solvents can be used for the recovery of carrageenan from KA biomass, affecting the characteristics and purity of the biopolymer. Besides, since KA also contains other valuable components, such as fatty acids, pigments, phenolic compounds, proteins, and cellulose, the presence of these fractions also affect the carrageenan purity and properties. For instance, low-pressure sequential extractions were applied by Shanmugam & Seth (SHANMUGAM; SETH, 2018) to recover biostimulant and semi-refined carrageenan, while Meinita et al., (MEINITA *et al.*, 2019) produced carrageenan and ethanol. Otherwise, Rudke et al, (RUDKE *et al.*, 2024) used a high-pressure sequential route, involving Pressurized Liquid Extraction (PLE) with different solvents, to produce a carrageenan fraction with higher purity compared to a commercial carrageenan.

Carrageenan-based aerogels can be produced by solvent separation from gel solution by drying methods. For instance, freeze-drying from the hydrogel solution, or supercritical CO_2 drying from the alcogel solution, with the attention to preserve the functional and physical-chemical properties of the produced porous material (DOGENSKI *et al.*, 2020). Besides, the aerogels can be formed in different sizes and shapes, affecting their uses or applications, such as beads (LENTZ *et al.*, 2022; ROBITZER; RENZO; QUIGNARD, 2011) microparticles (ALNAIEF; OBAIDAT; MASHAQBEH, 2019), and others (disk, nanocomposite, monolite, and rectangle) (AGOSTINHO *et al.*, 2020; DOGENSKI *et al.*, 2020; MANZOCCO *et al.*, 2017; PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019; ZAMORA-SEQUEIRA *et al.*, 2018).

Considering that the extraction method affects the carrageenan purity and properties, such as viscosity, gel strength, and molecular weight (GERENIU; SARAVANA; CHUN, 2018; HILLIOU *et al.*, 2006; MONTOLALU *et al.*, 2008), which also affects the aerogel structure, the aim of this chapter was to compare the properties of

aerogels obtained from a commercial carrageenan, and compare with the one from a pure carrageenan recovered by high-pressure sequential extractions by Rudke et al., (RUDKE *et al.*, 2024) a novel precursor for aerogel production. The drying step during the aerogel production also affects the material structure and was evaluated comparing freeze-drying with supercritical CO_2 drying.

6.2 MATERIAL AND METHODS

Aerogel and cryogel beads were produced from carrageenan powders of two sources: a commercial carrageenan (CC), purchased from AGARGEL (São Paulo, SP, Brazil), and an alternative carrageenan of high-purity, produced by high-pressure sequential extractions as described by Rudke et al (RUDKE *et al.*, 2024).

The high-pressure carrageenan (HP) was obtained from the sequential extractions, according to the following conditions [14]: PLE with ethanol at 100 bar, to remove the phenolic fraction from KA biomass, followed by pressurized water extraction (50 bar/15 min) applied to the biomass treated with KOH (24 h at 30 °C). The resulting extract was hot-filtered, concentrated, and precipitated with ethanol (1:3 v/v ratio). The powder HP sample resulted from the drying (60 °C for 24 h) of the ethanol precipitate. Both carrageenan samples (CC and HP) were characterized in terms of viscosity profile and color (section 2.1) and used for aerogel and cryogel production (section 2.2).

6.2.1 Viscosity and color of carrageenan samples

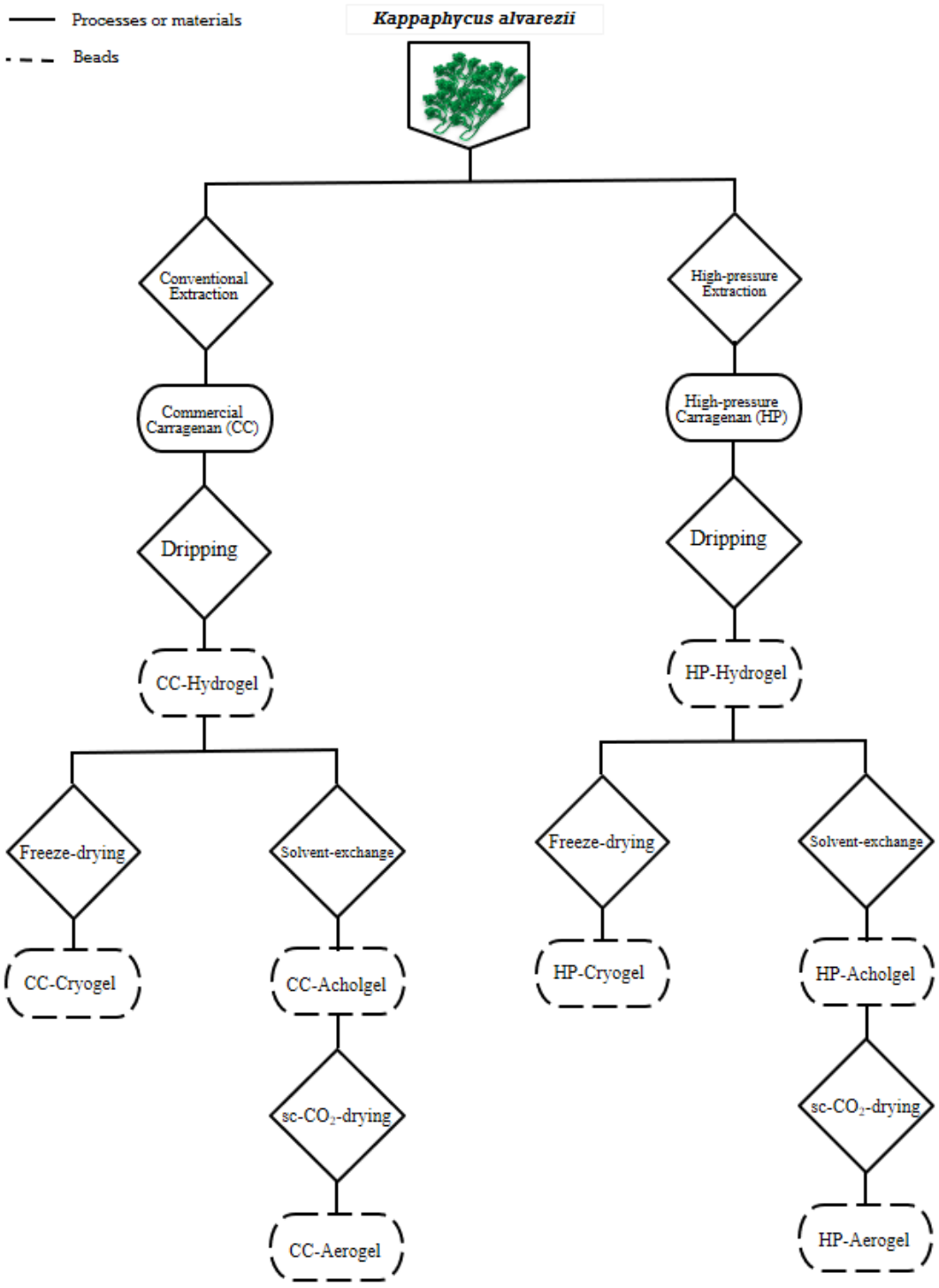
The carrageenan samples (CC and HP) were characterized considering the viscosity profile at different temperatures and the color attributes. The viscosity was evaluated using the MCR 72 rheometer from Anton Paar. Briefly, 0.45 g of carrageenan samples were solubilized into 30 mL of distilled water (1.5% w/v) at 80 ºC for 30 min (volume and concentration of sample solutions were kept constant by adding water to replace the evaporated amount). Then, 15 mL of each carrageenan-water solutions (CC or HP) were placed at the rheometer, operating with concentric cylinders. The equipment operated with a fixed shear rate of 50 (s^{-1}), and the viscosity was recorded for the temperature range of (75 to 15 ºC) for each carrageenan sample. The analysis was performed in duplicate, and the average of each point is presented in mPa.s.

The color parameters of the carrageenan samples (CC and HP) were measured using a colorimeter (Delta Vista, model 450 G SN 7012003357, São Leopoldo-RS, Brazil). The parameters L* (+= light, – = dark), a*(+= red, – = green), b* (+= yellow, – = blue), C* (chromaticity), and h° (hue angle) were recorded. The analysis was performed for both carrageenan powder samples (CC and HP) in duplicate.

6.2.2 Aerogel and cryogel production

The aerogel beads of carrageenan were prepared following different steps for each sample (CC and HP), which includes hydrogel preparation, cryogel formation by freeze-drying, hydrogel to alcogel conversion by solvent replacement, and alcogel to aerogel conversion by supercritical drying. The preparation steps are presented at Figure *6-1* and described as follow:

Figure 6-1- Flowchart for the production of cryogels and aerogels using commercial (CC) and high-pressure (HP) carrageenan.



Source: the author (2024).

***Hydrogel preparation*:** The hydrogel samples (by CC and HP) were prepared according to Robitzer, Renzo & Quignard (ROBITZER; RENZO; QUIGNARD, 2011), with modifications, using a drop dipping apparatus. The self-assembled apparatus consists of a thermostatic bath (Microquímica, Palhoça, SC, Brazil) connected to a jacked glass ware of 500 mL, coupled to a control gauge (1,000 μL tip). Briefly, 30 mL of 1.5% (w/v) carrageenan-water solutions (80 ºC) were dripped into a Beaker with 50 mL of 0.6 M KCl solution at 5 ºC, under mechanical stirring to form the carrageenan beads (CC or HP). The formed beads were stored overnight at 5 ºC for gel stabilization, and the excess of KCl was removed by washing with 50 mL of water at 5 ºC, producing the hydrogel samples obtained from different biopolymers, CC and HP.

***Cryogel production (Hydrogel to aerogel by freeze-drying)*:** the hydrogel samples (CC or HP) were frozen at -20 ºC for 24 h, and then freeze-dried (Liotop, model LD101, Sao Paulo, SP, Brazil) for 48 h. The resulting samples, in beads form, were called cryogel from each sample: Cryogel-CC, from the commercial carrageenan, and Cryogel-HP from the high-pressure carrageenan. The process was made in triplicate.

***Hydrogel to alcogel conversion*:** The water from the hydrogel beads (CC and HP) was replaced with ethanol (solvent exchange step) by successive immersions (1 h) of the hydrogel beads in 50 mL ethanol solutions at different concentrations (10, 30, 50, 70, 90, 100, and 100%) (ROBITZER; RENZO; QUIGNARD, 2011). After each immersion, the alcogel beads (CC and HP) were filtered (steel sieved), and the excess of ethanol was removed by filter paper, and the samples were weighted to calculate the mass loss during solvent exchange. The process was made in triplicate.

***Alcogel to aerogel conversion (by supercritical drying)*:** The alcogel beads (CC and HP) were dried for 1 h using supercritical carbon dioxide (sc-CO_2) in a high-pressure unit previously described by Andrade et al., (ANDRADE *et al.*, 2012). Shortly, an amount of 10 g of carrageenan alcogel beads (for CC and HP samples) and 20 mL of ethanol were placed in the stainless-steel jacketed column (2 cm inner diameter, 32 cm long, and 100 cm^3 capacity). Then, the sc-CO_2, used as ethanol remover, upflow the column through the packed beds formed by alcogel beads (CC of HP). The conditions for the supercritical drying were set at 1 kg/h (CO_2 flow rate), 120 bar and 40 ºC for 1 h, as previously defined in the literature (DOGENSKI *et al.*, 2020; LENTZ *et al.*, 2022). The dried beads, after

ethanol removal, resulted in the aerogel samples: aerogel-CC (from commercial carrageenan) and aerogel-HP (from high-pressure carrageenan). The process was made in triplicate.

6.2.3 Aerogels and cryogels characterization

6.2.3.1 Scanning Electron Microscopy (SEM)

The SEM analysis was used to evaluate the external structure of the beads (aerogel and cryogel), produced using the commercial and the high-pressure carrageenan samples (CC and HP). The samples were fixed in stubs using double-sided tape and recovered with a thin layer of metalized gold (Baltec SCD 0005). The analysis was performed at the Central Electron Microscopy Laboratory from the Federal University of Santa Catarina (Florianópolis, SC, Brazil) using a microscope JEOL JSM 6390 LV (Musashino, Akishima, Japan).

6.2.3.2 Volume of beads

The average beads volume (V_b) and diameter (d_b) were calculated as described by Lentz et al. (LENTZ *et al.*, 2022) using the software ImageJ 1.8 (Bethesda, MD, USA). These determinations were provided for hydrogel, alcogel, aerogel, and cryogel samples, produced from CC and HP. Briefly, photos were taken (Redmi 9 cell phone) at a distance of 20 cm using at least 50 beads for each sample, and the photos, calibrated with a ruler, were processed by the ImageJ software. Due to the transparency of the hydrogel and alcogel samples, these beads were darkened by image editor, for software recognition. The analysis expresses the beads area (square pixels) and their sphericity, resulting in the V_b and d_b determinations according to the equations:

$$V_b = \frac{(\phi \times A_b)^{\frac{3}{2}}}{6 \times \pi^{\frac{1}{2}}} \quad (1)$$

$$d_b = \left(\frac{6 \times V_b}{\pi}\right)^{\frac{1}{3}} \quad (2)$$

Where V_b is the beads average volume, A_b is the beads average area, ϕ the beads sphericity, and d_b is the average beads diameter.

6.2.3.3 Density and porosity of the CC and HP beads

The bulk density (ρ_{bulk}) was calculated by the weight of a given quantity of beads (CC and HP) and their occupied volume measured by graduated cylinder. Skeletal density (ρ_{skel}) was obtained by gas pycnometer (Micromeritics AccuPyc II 1340, USA) using helium at room temperature. The porosity (ε) was calculated by equation (3) (TARASHI *et al.*, 2022):

$$\varepsilon\,(\%) = 1 - \left(\frac{\rho_{bulk}}{\rho_{skel}}\right) \times 100\% \quad (3)$$

6.2.3.4 Volume shrinkage

The volume shrinkage (*Vs*) was evaluated for different process steps: from hydrogel to alcogel, from hydrogel to aerogel, and from hydrogel to cryogel. The values were obtained through equation (4) (LENTZ *et al.*, 2022):

$$Vs_{hyd \to n}\,(\%) = \left(\frac{V_{hyd} - V_n}{V_{hyd}}\right) \times 100\% \quad (4)$$

Where *Vs* is the volume shrinkage, *V* is the average volume of beads, and the subscripts *hyd* refers to hydrogel, and *n* refers to alcogel, aerogel, and cryogel, depending on the shrinkage volume calculated.

6.2.3.5 Firmness

The firmness of aerogel and cryogel beads was determined according to Dogenski et al. (DOGENSKI *et al.*, 2020), with modifications. The firmness was evaluated under uniaxial compression tests on a texture analyzer (TA.HD. plus Texture Analyzer, Stable Micro System). At least 10 beads were compressed until 70% strain using a 50 kg load cell and a cylindrical steel probe (35 mm diameter) with a test speed of 0.1 mm/s. The force-strain curves were obtained from the compression tests, and firmness was taken as the maximum force (N) required to strain 70% of the sample. The analyses were repeated at least 3 times for each sample. The results were expressed as mean ± standard deviation.

6.2.3.6 BET and BJH

Brunauer–Emmett–Teller (BET) method was performed by the nitrogen adsorption-desorption analysis. The specific surface area (A_{BET}) of the aerogel and cryogel samples (CC and HP) was measured by Autosorb 1 (Quantachrome Instruments, Germany). The Berrett-Joyner- Halenda (BJH) method was used to determine the total pore volume V_p (determined at a relative pressure (p/p0) of 0.9994) and the average pore diameter d_p of the aerogel and cryogel samples (CC and HP).

6.2.3.7 Water and oil absorption kinetics

For water and oil absorption tests, 0.1 g of dry aerogel or cryogel samples was immersed into 50 mL of water or oil as reported by Dogenski et al. (DOGENSKI *et al.*, 2020). The water or oil mixtures were placed in Beakers and maintained in water bath at 25 ºC for kinetics determination. Excess water or oil was gently removed with paper towel, and the mass of wet aerogels or cryogels was recorded at different time intervals. The water and oil absorption capacity values were calculated by the ratio between the mass of water or oil absorbed and the initial mass of the beads (aerogel or cryogel). The analysis was carried out in triplicate and the results are expressed in g water/g sample or g oil/g sample.

6.3 RESULTS AND DISCUSSION

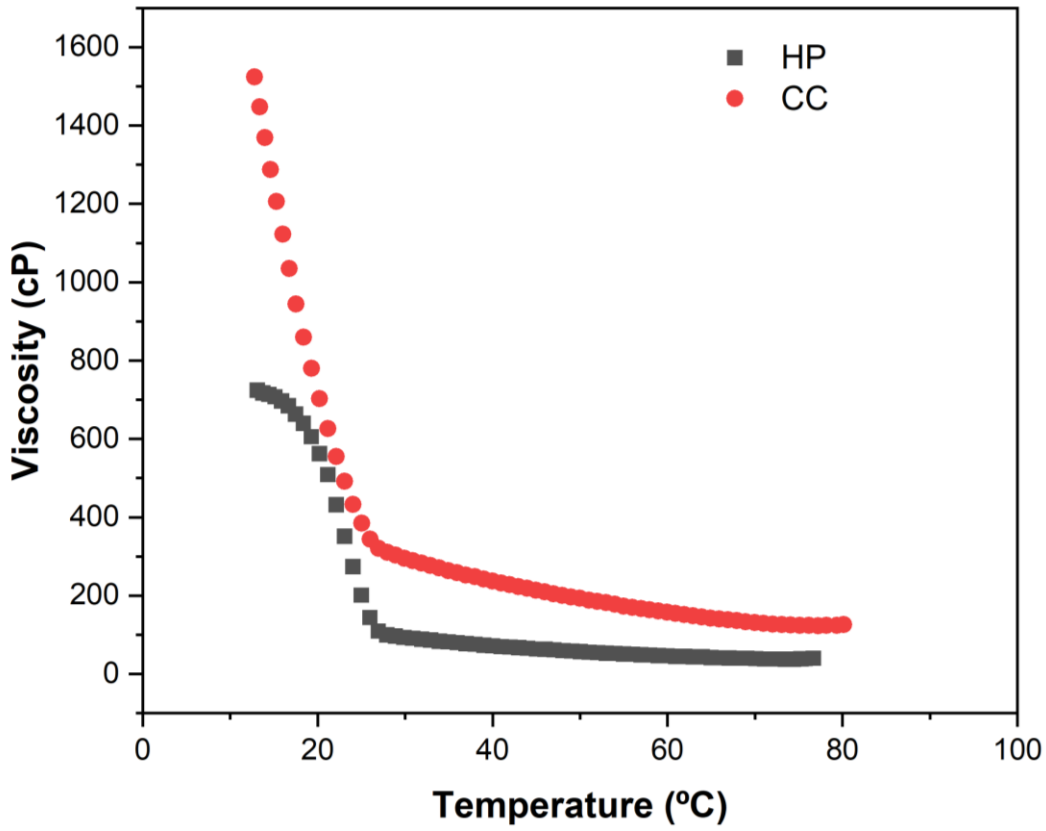
6.3.1 Carrageenan characteristics

6.3.1.1 Viscosity

The viscosity-temperature profiles of the carrageenan samples (CC and HP) are presented at Figure 6-2. The viscosity decreased with the increase in temperature for both samples, ranging from 40 to 724 cP and from 125 to 1,524 cP, for the HP and CC, respectively. Intense reductions in viscosity, for both samples, were detected between 20-30 ºC, indicating a gel-sol transition (MONTOLALU *et al.*, 2008) which may occur due to changes in gel network with heating, reducing the viscosity (RHEIN-KNUDSEN; ALE; MEYER, 2015). The viscosity of the CC sample was also previously evaluated by

Rudke et al. (RUDKE *et al.*, 2022) at a fixed temperature of 75 ºC, with result of 135 ± 7 cP, which is within the values found for CC in the present work, considering the temperature range from 10 to 80 ºC.

Figure 6-2- Viscosity profile of the commercial and high-pressure carrageenan samples (CC and HP) at different temperatures.



Source: the author (2024).

The water solubility and the viscosity of carrageenan affect the aerogel or cryogel production. For instance, the drop dipping of the carrageenan solutions (section 2.2) was set at 80 ºC because at this temperature the κ-carrageenan is completely solubilized in water (ROBITZER; RENZO; QUIGNARD, 2011), with random coil structures due to electrostatic repulsions between adjacent polymer chains (RHEIN-KNUDSEN; ALE; MEYER, 2015). Also, low viscosity values of the carrageenan solutions (CC and HP) were detected above 20 °C (Figure 6-2), contributing to the drop dipping for beads formation. Figure *6-2* also shows high viscosity values below 20 °C, for CC and HP samples, justifying the use of KCl medium at 5 °C, where the carrageenan solutions drips, allowing precipitation and beads formation with the increase in viscosity. The dripping

of a hot carrageenan solution (random coil structures) into a cold KCl medium promotes the formation of helix structures due to the approximation of the polymer chains. The presence of cations, such as K+, aggregates the helical dimers, forming a stable three-dimensional network due to intermolecular interactions between carrageenan chains, which significantly increases the viscosity (RHEIN-KNUDSEN; ALE; MEYER, 2015)

Higher viscosity values were observed for CC compared to HP, for all temperatures (Figure 6-2), suggesting that the carrageenan samples present different characteristics such as molecular weight. According to Blanco-Díaz., (BLANCO-DÍAZ *et al.*, 2018), the viscosity is sensitive to average molecular weight (Mw), this occurs because greater molar mass increases the chances of molecules colliding with each other, generating greater friction, and consequently increasing the force necessary to promote the movement of these molecules. For instance, CC and HP samples used in the present study were characterized by Rudke (RUDKE *et al.*, 2024), which observed Mw values of 475.05 kDa (HP) and 35240.00 kDa (CC), confirming the relation between Mw and viscosity, which justifies the higher viscosity values for CC, compared to HP.

6.3.1.2 Color

The color parameters obtained for the carrageenan samples are presented in Table 6-1. These data are relevant to establish the carrageenan applications, as the color attributes affect significantly on the consumer's decision. The data from Table 6-1 indicate statistical differences in the parameters L*, b*, h° and C* between CC and HP samples.

Table 6-1 - Colorimetric data based on the CIELab scale (L*, a*, b*), chromaticity (C*), hue angle (h°), of the commercial (CC) and high-pressure (HP) carrageenan.

	L*	**a***	**b***	**h°**	**C***
CC	97.3 ± 0.1^{a}	1.9 ± 0.0^{a}	9.5 ± 0.1^{b}	78.9 ± 0.3^{b}	9.7 ± 0.0^{b}
HP	82.5 ± 0.4^{b}	1.9 ± 0.1^{a}	14.7 ± 0.1^{a}	82.6 ± 0.4^{a}	14.8 ± 0.1^{a}

Different letters in the same column indicate statistical difference at the 5% level using the Tukey test.

Source: the author (2024).

The L* refers to luminosity, ranging from 0 (black) to 100 (white), with lower value for HP, indicating the commercial samples as whiter than HP sample. Nevertheless, the L* for the HP (82.5) is within the range reported by Sjamsiah et al., (SJAMSIAH *et*

al., 2014), between 82 and 89, for KA carrageenan samples. The b* parameter varies from positive (yellowish) to negative (bluish), and the results indicate the HP sample (b*=14.7) is more yellowish that CC sample (b*=9.5). The b* for HP sample agrees with the carrageenan value reported by Martín-Del-Campo et al., (MARTÍN-DEL-CAMPO *et al.*, 2021) and by Norhazariah et al., (NORHAZARIAH *et al.*, 2018) , obtained from *Chondracantus canaliculatus* (between 11.84 and 17.10 varying with extraction condition), and from KA (close to 15).

No differences were found for a* values (Table 6-1), representing from green (negative) to red (positive). The results for both samples (1.9) show a discrete tendency towards red, but close to neutrality. The Chromaticity (C*), indicating the color intensity, was higher for HP compared to CC, 14.7 and 9.7 respectively. The HP value was similar to reported by Martín-Del-Campo et al., (MARTÍN-DEL-CAMPO *et al.*, 2021) (15.83) for carrageenan from *C. canaliculatus* recovered using water-based ultrasound extraction.

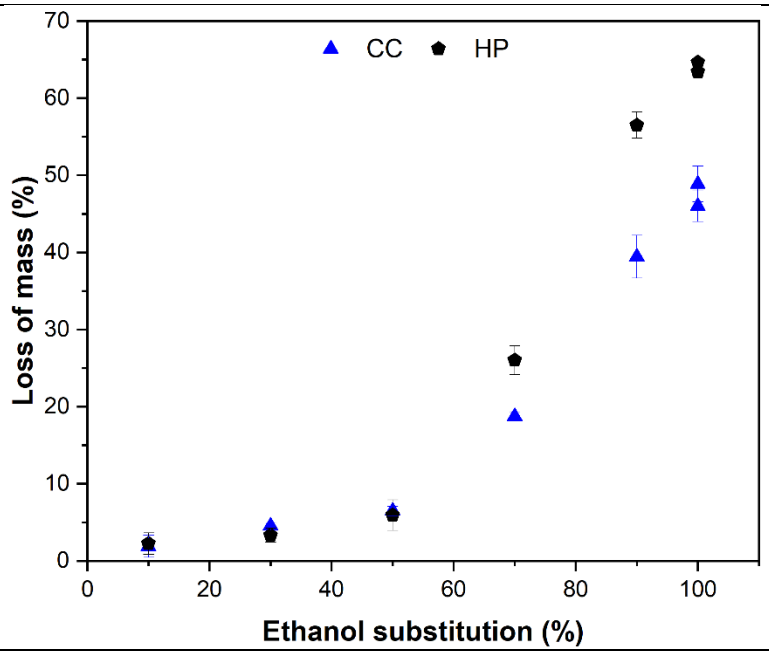
The hue angle (h^o) indicates the visual sensation, and the values were 78.9 and 82.6, for CC and HP respectively. This parameter, related to Consumer's perception, suggests that the yellowish aspect of the HP sample is more significant than the CC color. Similar h^o value was obtained for carrageenan from *C. canaliculatus*, of 86.60 (MARTÍN-DEL-CAMPO *et al.*, 2021).

The differences for the parameters L*, a*, b*, h^o, and C*, comparing CC, HP and literature data, are mainly due to the algae species and extraction/purification methods. However, the color characteristics of CC and HP samples agree with the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which indicates carrageenan as yellowish, tan, or white (EFSA *et al.*, 2018).

6.3.2 Solvent exchange mass loss

Figure 6-3 shows the hydrogel mass loss during the solvent exchange step (replacement of water by ethanol for alcogel production). An increase in mass loss was detected above 70% of ethanol replacement, for both samples (CC and HP) because water has higher density than ethanol (alcogel are lighter than hydrogel), increasing mass losses with water removal.

Figure 6-3- Mass loss of commercial and high-pressure carrageenan beads (CC and HP samples) during conversion from hydrogel to alcogel.



Source: the author (2024).

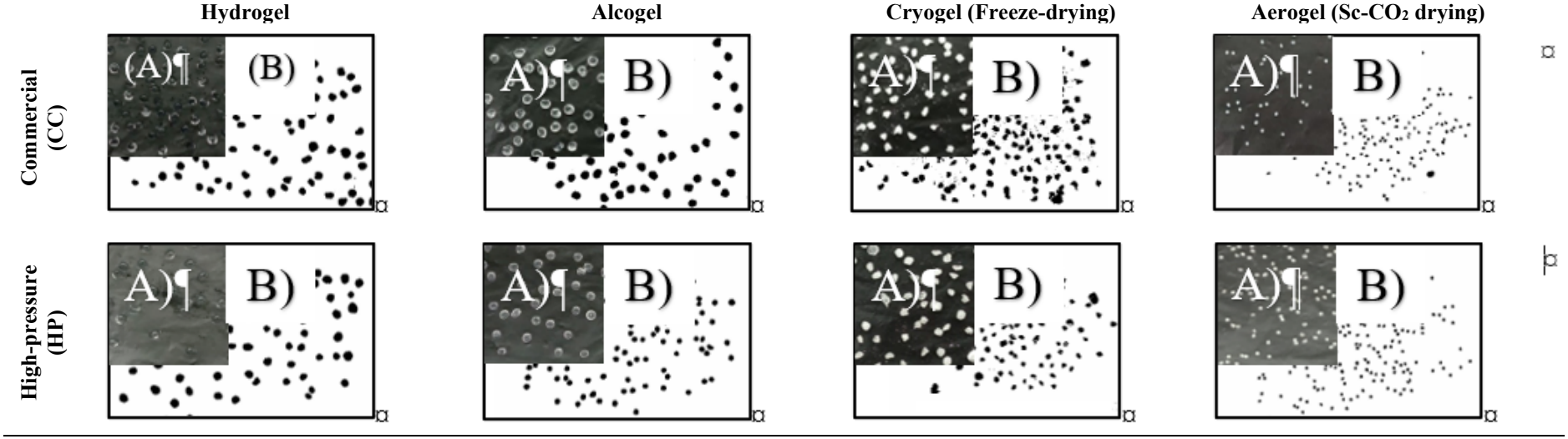
The mass loss during solvent exchange depends on the carrageenan interactions with water or with ethanol. Since carrageenan has very low solubility in ethanol, the water interactions must be stronger. Then, comparing CC and HP, the mass loss differences increased at ethanol concentration above 70%, with HP presenting higher mass loss than CC. This difference suggests that HP has weaker water interactions than CC, i.e., HP has lower gel strength (hydrogel) than CC, facilitating the removal of water molecules. The gel strength of the CC and HP samples was evaluated by Rudke et al (RUDKE *et al.*, 2024), and the results were 131 and 44 $g.cm^{-2}$, respectively, indicating the three-dimensional network formed by CC maintained higher water amount inside, compared to the network formed with HP (PEREDA *et al.*, 2005).

6.3.3 Characterization of the CC and HP beads

Images of the carrageenan beads produced by CC and HP, from different processing steps (hydrogel, alcogel, aerogel and cryogel), are compared at Figure *6-4*. The samples for each step are presented as: Figure *6-4*A (pictures of the produced beads) and

Figure *6-4*B (processed pictures by software Image J: section 2.3.2), used for volume determination.

Figure 6-4- Beads obtained from the commercial (CC) and high-pressure (HP) carrageenan at different processing steps (hydrogel, alcogel, cryogel and aerogel): (A) Photos of the produced beads; and (B) processed photos of the beads.

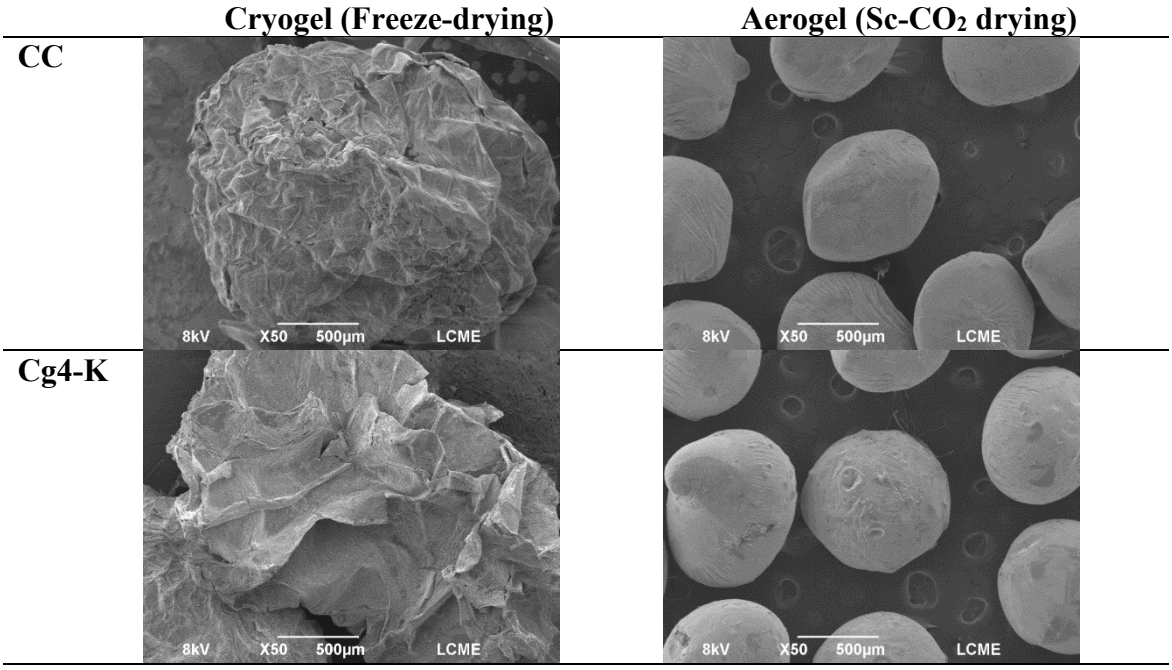


Source: the author (2024).

Hydrogel and alcogel samples are transparent (Figure *6-4*), changing to opaque white with drying (aerogel or cryogel). A relevant shrinkage was detected from alcogel to aerogel, while no significant shrinkage was visible between hydrogel and cryogel, probably due to the solvent exchange step (aerogel production), and to the drying method.

A closer look of the produced materials is provided by SEM images at 50X amplification (Figure 6-5), where aerogel and cryogel samples are compared. The aerogel samples (CC and HP) are sphere particles with sleek surface, while cryogel samples are rougher. The more spherical aerogel structure compared to cryogel is probably due to ice crystals formation during freeze-drying (cryogel), which must have disrupted the carrageenan circular structure. The characteristics of the samples can be better elucidated by geometric parameters (Table 6-2) and physical-chemical parameters (Table 6-3).

Figure 6-5 - Scanning Electron Microscopy (SEM) images of cryogel and aerogel samples from commercial (CC) and high-pressure (Cg4-K) carrageenan, amplified 50X.



Source: the author (2024).

Table 6-2 – Physical characteristics (volume, diameter and volumetric shrinkage) of hydrogels, alcogels, aerogels and cryogels obtained from carrageenan obtained at high pressure (HP) and commercial carrageenan (CC).

	Hydrogel		**Alcogel**		**Aerogel**		**Cryogel**	
	Volume (mm^3)	**Diameter (mm)**	**Volume (mm^3)**	**Diameter (mm)**	**Volume (mm^3)**	**Diameter (mm)**	**Volume (mm^3)**	**Diameter (mm)**
CC	1.61 ± 0.13^a	1.45 ± 0.04^a	1.51 ± 0.09^a	1.43 ± 0.03^a	0.06 ± 0.00^a	0.50 ± 0.01^a	0.58 ± 0.02^a	1.04 ± 0.01^a
HP	1.42 ± 0.06^a	1.39 ± 0.02^a	0.77 ± 0.04^b	1.14 ± 0.02^b	0.05 ± 0.00^b	0.45 ± 0.00^b	0.41 ± 0.06^b	0.92 ± 0.04^b
				Volume shrinkage (%)				
CC	-		3.19 ± 0.06^b		95.99 ± 0.13^b		63.65 ± 1.18^b	
HP	-		45.72 ± 2.97^a		96.55 ± 0.08^a		71.38 ± 3.98^a	

Different letters in the same column indicate statistical difference at the 5% level using the Tukey test.

Source: the author (2024).

Table 6-3 – Physical-chemical characteristics of aerogels and cryogels, from commercial (CC) and high-pressure (HP) κ-carrageenan, represented by bulk and sketetal densities (ρ_{bulk}, ρ_{skel}), porosity (ε), and the specific surface area (A_{BET}), and total pore volume and average pore diameter (V_P and d_p).

	ρ_{bulk} ($g.cm^{-3}$)	**ρ_{skel} ($g.cm^{-3}$)**	**ε (%)**	**A_{BET} ($m^2.g^{-1}$)**	**$V_{P,\ BJH}$ ($cm^3.g^{-1}$)**	**$d_{p,\ BJH}$ (nm)**
			AEROGEL			
CC	0.37 ± 0.02^b	1.77 ± 0.00^a	78.26 ± 0.83^b	88.90	0.31	13.94
HP	0.46 ± 0.02^a	1.67 ± 0.00^b	72.99 ± 0.19^c	105.40	0.49	18.89
			CRYOGEL			
CC	0.06 ± 0.00^d	1.61 ± 0.01^c	95.96 ± 0.34^a	n.d.	n.d.	n.d.
HP	0.10 ± 0.01^c	1.51 ± 0.00^d	93.53 ± 0.82^a	n.d.	n.d.	n.d.

Different letters in the same column indicate statistical difference at the 5% level using the Tukey test. n.d. not detected.

Source: the author (2024).

The geometric characteristics (volume, diameter and volumetric shrinkage) of the CC and HP samples (hydrogel, alcogel, aerogel and cryogel) are compared at Table 6-2. The diameter and volume of alcogel, aerogel and cryogel were higher for CC compared to HP, while no significant differences were detected for hydrogel samples. Besides, the volume and diameter of the beads reduced with drying, for CC and HP samples, observed by comparing alcogel with aerogel (sc-CO_2 drying), and hydrogel with cryogel (freeze-drying). The hydrogen bonds between κ-carrageenan and water (cryogel) or ethanol (aerogel) are reduced during drying, favoring the interactions between individual polymeric chains, which increases the density, lowering the diameter and volume of the beads (PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019).

The volume shrinkage (Table 6-2), obtained from processed images (Figure *6-4*B), shows smaller values for CC compared to HP. The solvent exchange step (hydrogel to alcogel) provided the highest shrinkage difference between CC and HP (3.19% for CC and 45.72% for HP). Also, higher volume shrinkage was observed for aerogels (sc-CO_2 drying) compared to cryogels (freeze-drying), for CC and HP. The aerogel shrinkage results agree with literature data for carrageenan aerogels (AGOSTINHO *et al.*, 2020; PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019; QUIGNARD; VALENTIN; DI RENZO, 2008; ROBITZER; RENZO; QUIGNARD, 2011) of about 95%. The higher shrinkage values for aerogel compared to cryogel (CC and HP) is probably due to the solvent exchange step, which increases the salt (KCl) leaching compared to cryogel. Higher salt concentration (cryogel) reduces the solvated water molecules around sulfate ions, decreasing repulsive forces of carrageenan fibers, increasing shrinkage (GANESAN; RATKE, 2014).

The volumetric shrinkage from the aerogel samples are within the wide range of literature data, from 7.4 to 91% (Plazzotta, Calligaris and Manzocco (PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019) and Tarashi et al. (TARASHI *et al.*, 2022), respectively). However, regarding the cryogel samples, the variation in volumetric shrinkage compared to literature can be associated to the freezing method, i.e., Plazzotta, Calligaris and Manzocco (PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019) submitted the hydrogel samples to -80 ºC, previous to freeze-drying, while in the present study, the samples were frozen at -20 ºC, prior freeze-drying. At -80 ºC smaller spherical

ice crystals are produced, while larger prickly ice crystals are formed at -20 ºC, increasing gel rupture and shrinkage (GANESAN *et al.*, 2018; OKAY; LOZINSKY, 2014).

The volume shrinkage can be reduced by the use of hybrid aerogels, such as carrageenan added with polymers (BOISSIÈRE *et al.*, 2006; TARASHI *et al.*, 2022; ZAMORA-SEQUEIRA *et al.*, 2018) or by increasing the concentration of carrageenan (CHANDRASEKARAN *et al.*, 2021; GANESAN; RATKE, 2014; MANZOCCO *et al.*, 2017). As explained by Boissière et al. (BOISSIÈRE *et al.*, 2006), carrageenan fibrils are more flexible than other polysaccharides due to stronger adhesion forces, and fibrils are closer, which increases the volume shrinkage.

6.3.3.1 Aerogel and cryogel physical-chemical characteristics

The bulk density (ρ_{bulk}) varied from 0.06 to 0.46 g/cm³, and the skeleton density (ρ_{skel}) from 1.51 to 1.77 g/cm³ (Table 6-3), with higher values for aerogel compared to cryogel. The bulk density values agree with literature data (ALNAIEF; OBAIDAT; MASHAQBEH, 2018; CHANDRASEKARAN *et al.*, 2021; MANZOCCO *et al.*, 2017; OBAIDAT; ALNAIEF; MASHAQBEH, 2018; PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019), as well as the skeleton densities (CHANDRASEKARAN *et al.*, 2021; GANESAN; RATKE, 2014; ROBITZER; RENZO; QUIGNARD, 2011). The density differences between CC and HP can be associated with the biopolymer (carrageenan) purity, where CC presents about 13% (w/w) cellulose, not detected at the HP samples, and also to the polydispersity index (Mw/Mn), of 15 and 5, respectively, as presented by Rudke et al. (RUDKE *et al.*, 2024).

The porosity values (ε) follow the density results (higher bulk density represents lower porosity), expressing lower values for aerogel porosity (78.26 and 72.99% for CC and HP respectively) compared to cryogel porosity (95.96 and 93.53% for CC and HP respectively), and also lower porosity for HP compared to CC samples. This behavior is explained by the higher volume shrinkage from aerogel compared to cryogel, due to the proximity of the gel fibers, reducing porosity. Besides, the ice crystals formed during cryogel production contribute to the rupture of the polymeric matrix, increasing the size and number of pores, compared to aerogel. The differences between CC and HP may be

caused by the closer fibrils (HP-pure carrageenan compared to CC: Rudke et al, (RUDKE *et al.*, 2024)), resulting in lower porosity.

The porosity values for the carrageenan aerogels are within the wide range of literature data, from 65 to 95.9% (CHANDRASEKARAN *et al.*, 2021; LI *et al.*, 2022; PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019; ROBITZER; RENZO; QUIGNARD, 2011), which varies with the process for aerogel production, and the carrageenan concentration, among other factors. The results presented at Table 6-3 are within these values, with the cryogel porosity higher than obtained by Tarashi *et al.* (TARASHI *et al.*, 2022), of 84 to 86%. The porosity variation from literature data is probably due to differences in carrageenan concentration and quality (purity, molecular size and polydispersity index), as discussed by Rudke et al, (2024). Nevertheless, the aerogels/cryogels applications are related to physical-chemical characteristics, affecting the functionalities and resistance of the materials.

The aerogel surface area was 88.90 m^2/g for CC and 105.40 m^2/g for HP, while the surface area for cryogel samples (CC and HP) were not detected by BET analysis, probably due to: (I) the freeze-drying effect, which may have induced the formation of pores smaller than the nitrogen molecule (gas used in BET analysis), compromising the measurement; (II) the pore blocked the gas penetration in the cryogel sample; (III) the nitrogen condensation, which could also contract the pores; (IV) the carrageenan aerogels could simply be compressed under high nitrogen pressure (GANESAN *et al.*, 2018), and/or (V) the dispersion of κ-carrageenan chains by the ice crystals (size and forms) (PLAZZOTTA; CALLIGARIS; MANZOCCO, 2019). The formation of small pores for cryogel samples is corroborated by the porosity values determined by helium pycnometer (Table 6-3), with higher values for cryogel compared with aerogel samples. The surface area for a commercial κ-carrageenan aerogel beads, obtained with Sc-CO_2 drying by Alnaief et al (ALNAIEF; OBAIDAT; MASHAQBEH, 2018), were 97 m^2/g, similar to the aerogel values obtained in from CC and HP.

Baudron et al (BAUDRON *et al.*, 2019) also detected higher surface area for aerogel samples compared to cryogel, produced from starch, with values of 183-197 m^2/g for aerogel, and from 0.6 to 7.7 m^2/g for cryogel. These authors were also unable to determine the surface area using BET analysis for the cryogel samples from starch.

Boissieire et al (BOISSIÈRE *et al.*, 2006) found low surface area values (1.5 m^2/g) for κ-carrageenan xerogel (air-based drying) compared to aerogel (114 m^2/g), indicating that surface area is related to the drying method, corroborating with the different behavior of cryogel (freeze-drying) compared to aerogel (Sc-CO_2 drying) for the surface area evaluation. Besides, literature data show low surface area values for cryogels formed by κ-carrageenan with carboxymethylcellulose, with results varying from 1.553 to 4.220 m^2/g (LI *et al.*, 2022). Because cryogel samples were not adequately evaluated by the BET approach, its characterization is not feasible based on this method (CIUFFARIN *et al.*, 2023). The surface area evaluation using nitrogen presents challenges that arise from the pore types of the material, including accessible (open), partially accessible (restricted pores), and closed pores. The quadrupole moment of the molecule of nitrogen, its size and slow diffusion kinetics also obscure the characterization of aerogels/cryogels samples (BEDA; VAULOT; GHIMBEU, 2021).

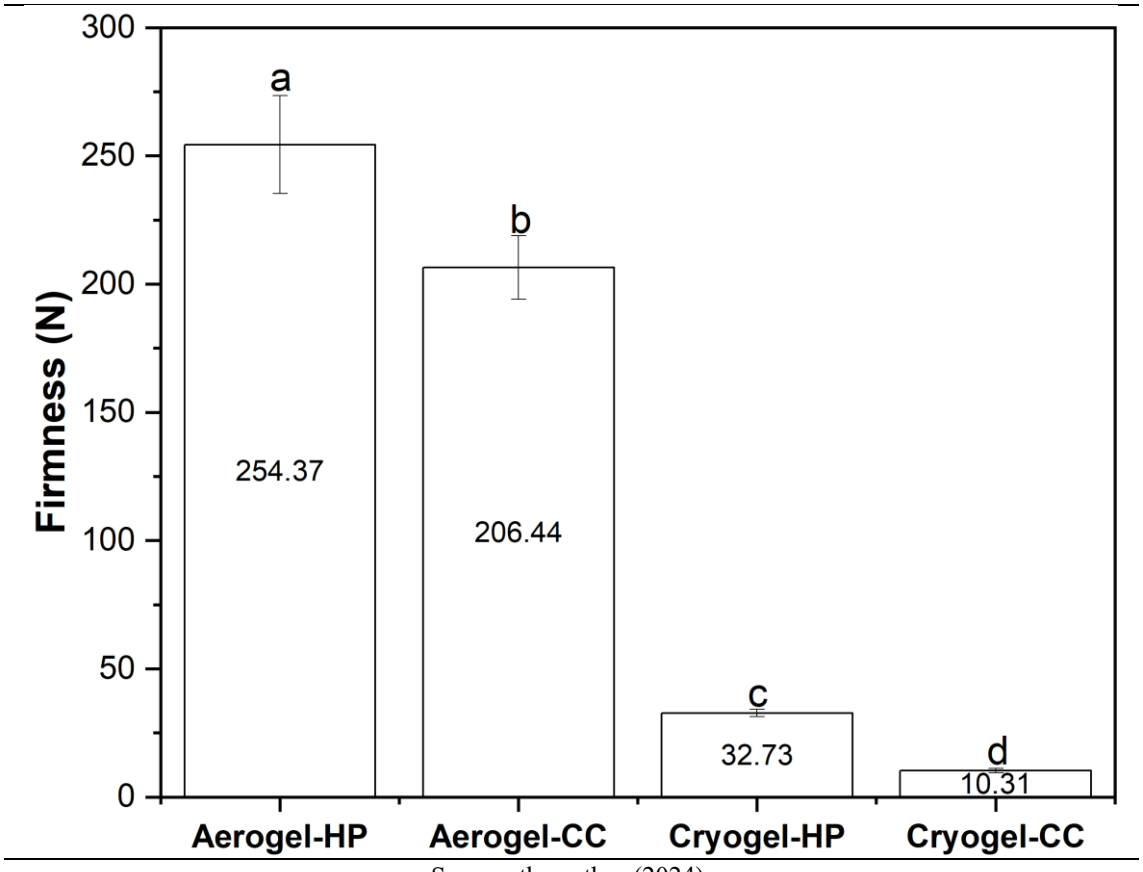
The pore volume for the aerogel samples were 0.31 cm^3/g (CC) and 0.49 cm^3/g (HP), evaluated through BET analysis, and estimated using BJH, while the values for cryogel were non detected by this analysis, probably due to the freeze-drying process, as discussed for surface area results. According to Agostinho et al. (2020), the pore volume of aerogel varied from 0.32 to 0.52 cm^3/g using different dissolution and crosslinking media for the aerogel formation. Alnaief, Obaidat & Mashaqbeh, (2018) found pore volume ranging from 0.10 to 0.45 cm^3/g for aerogel from different carrageenan types, concentration, crosslinking media, and temperature.

The aerogel pore diameters of CC and HP were 13.94 nm and 18.89 nm, respectively, whereas regarding the cryogel pore diameters of CC and HP, the values were also non detectable by BET analysis, probably due to the freeze-drying process, as discussed for surface area results. The pore diameter values for aerogel samples are comparable to obtained for κ-carrageenan aerogels produced by Alnaief et al, Obaidat et al., and Boissiere et al. (ALNAIEF; OBAIDAT; MASHAQBEH, 2018; BOISSIÈRE *et al.*, 2006; OBAIDAT; ALNAIEF; MASHAQBEH, 2018), which ranged from 8.5 to 16.5 nm. Lv, Li & Wang, [46] found average pore diameter of 2.21 nm for k-carrageenan cryogel using BJH method.

6.3.3.2 Firmness

The firmness of aerogel and cryogel samples, presented at Figure *6-6*, ranged from 10.31 to 254.37 N, with higher values for aerogel compared to cryogel (up to 20 times), a behavior also reported by Plazzotta, Calligaris & Manzocco, (2019) (47 and 1.26 for aerogel and cryogel, respectively). Besides, HP samples presented better firmness performance compared to CC, due to the lower porosity from HP samples (Table 6-3). The firmness values were higher than obtained by Manzocco et al. (2017) for aerogel (114 to 165 N), and for cryogels compared to Plazzotta, Calligaris & Manzocco (2019) (0.31-0.35 N). Also, firmness is directly related to volume shrinkage because higher shrinkage increases the density of the polymer-3D structure, increasing its compression resistance, an important characteristic for absorbent selection.

Figure 6-6- Firmness of aerogel and cryogel beads using carrageenan commercial and obtained by high pressure method. Aerogel-CC, Aerogel-HP, Cryogel-CC, and Cryogel- HP.

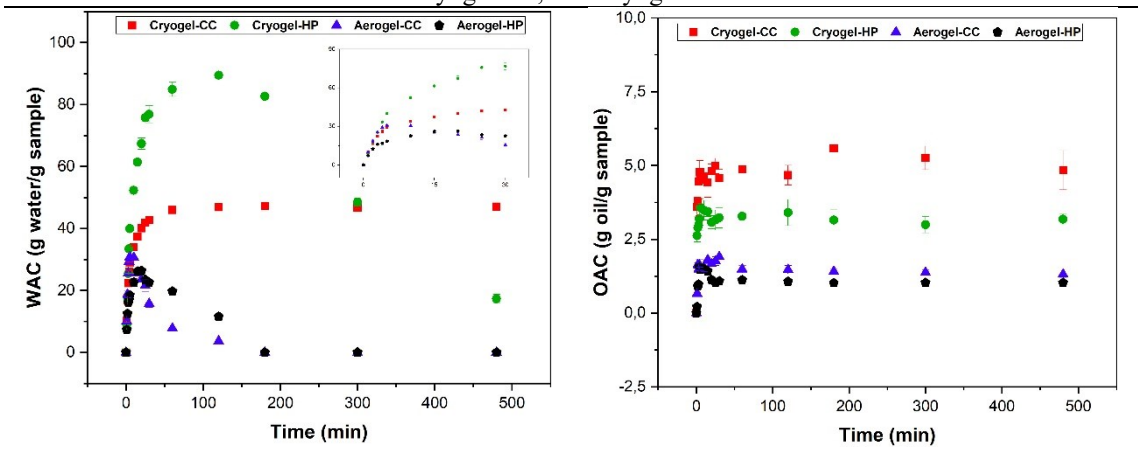


Source: the author (2024).

6.3.3.3 Water and oil absorption capacity

The kinetics of water and oil absorption capacities for aerogel and cryogel are illustrated in Figure *6-7*. Cryogel samples exhibited highest water and oil absorption capacities, up to 500 min, compared to aerogel samples, for CC and HP materials. The HP samples (aerogel and cryogel) provided better water absorption capacity than CC samples, while for oil absorption the CC samples provided higher values. The oil absorption capacity values agree with literature (MANZOCCO *et al.*, 2017) for materials with concentrations of k-carrageenan from 0.4 to 2%.

Figure 6-7 – Water absorption capacity (a) and oil absorption capacity (b) of aerogel and cryogel beads using carrageenan commercial and obtained by high pressure method. Aerogel-CC, Aerogel-HP, Cryogel CC, and Cryogel-HP.



Source: the author (2024)

The absorption capacities for aerogel or cryogel can be associated with the preparation steps: hydration, exposure, equilibrium, solubilization. During hydration, water is absorbed providing polymer structure expansion, varying with the polymer used. From the expanded polymer, the exposure is the contact between solvent and hydrophobic or hydrophilic structures, maintaining the structure and elasticity of the material (aerogel or cryogel). Then, absorption and retention forces are balanced at equilibrium, reaching maximum absorption capacity. The solubilization occurs when the interaction between solvent and polymer is stronger than the retention capacity (DHUA; GUPTA; MISHRA, 2022). For WAC (Figure *6-7*-A), only cryogel-CC was stable, with no reduction in WAC with time. Aerogels/cryogels with a compact structure and small pores tend to completely

disintegrate in contact with water over time (SELVASEKARAN; CHIDAMBARAM, 2022).

From Figure *6-7*-B, highest OAC values, for aerogel and cryogel, were achieved by CC sample, compared to HP, although, with very lower difference for aerogel samples. The OAC results for aerogels and cryogels were lower than reported by Plazzotta; Calligaris & Manzocco, (2019), which found values of 3.41 g oil/g sample for aerogel and 29.99 g oil/g sample for the cryogel, produced with 0.4% carrageenan solution. Also, OAC from cryogel were higher that aerogel samples. The water or oil absorption capacities by aerogel/cryogel is affected by the volumetric shrinkage and architecture of the material, with factors like pores size, and distribution, tortuosity, and internal surface (MANZOCCO *et al.*, 2017). Therefore, higher volumetric shrinkage corresponded to lower liquid absorption.

6.4 CONCLUSION

The purity and the molecular weight of the carrageenan samples (HP and CC) interfere in the viscosity profile, with samples presenting a reduction in viscosity with increasing temperature, and higher viscosity values for CC compared to HP. Aerogels and cryogels produced with HP showed higher firmness, WAC, volume shrinkage, and bulk density, compared to CC samples. The higher bulk density and lower porosity of the aerogel and cryogel samples produced by HP, compared to CC carrageenan may be associated to higher purity of HP polymer, due to the 13% of cellulose from CC (not detected at HP). Aerogel samples, with smooth and spherical structure, presented much higher firmness values, and lower WAC and OAC, compared to cryogels. These important attributes suggest valuable applications for the HP aerogel, as scaffolds, packaging material or delivery systems.

CHAPTER 7 - GENERAL CONCLUDING REMARKS AND SUGGESTIONS FOR FURTHER STUDIES

7.1 CONCLUSION

Considering the worldwide productions and high economical potential of KA, it has been extensively demonstrated the potential of this macroalgae as a source of different products such as proteins, phenolic compounds, fertilizers, pigments, and glucose (as a base for ethanol production or other products such as formic acid and levulinic acid), among other valuable components like carrageenan, which reinforce the biorefinery concept applied for KA. Although this alga is well studied, it was highlighted that there is few information on the use of non-conventional methods (high-pressure procedures) for the extraction of valuable molecules from this remarkable raw material, the KA. The microwave is the most actual technique used to obtain compounds of interest from KA and other red algae. Although, high-pressure methods such as PLE, SWE, and SFE have been little explored for the fractionation of KA biomass. In general, ultrasound is used as a pre-treatment for this biomass. Then, considering the various different classes of relevant compounds present in KA biomass, the proposal of the present work was to study the newest biorefinery concept to obtain different products from this red macroalgae. With the KA valorization, it is expected that the extraction of carrageenan from KA should be expanded to obtain ethanol, fertilizers, pigments, concentrates proteins, among other products that can be obtained from this biomass.

In chapter 3, the SFE from KA promoted a selective extraction, with the recovery of a fraction with a high fatty acid content, however, lower yield than SOX-hexane. Meanwhile, PLE method with ethanol was efficient for recovery of the phenolic compounds, a fraction with higher antioxidant activity, compared to SOX and MAE samples recovered with the same solvent. The extraction temperature, for PLE and MAE processes, showed a negative influence on TPC, DPPH, and FRAP performance of the recovered samples. The carrageenan extraction by MAE or PWCE methods was efficient to provide, in a very short time, a sample with physical properties similar to conventional carrageenan. The alternative extraction methods were suitable to provide two valuable fractions from KA biomass. Furthermore, and to the best of our knowledge, this was the first work that quantified phenolic compounds from KA. In addition, the novelty of the present work was the evaluation of the influence of green extraction techniques (MAE, PLE, and PWCE) in obtaining ethanolic extracts and carrageenan fraction from KA.

In chapter 4, the ideal conditions for carrageenan recovery by UAE were achieved at 15 minutes, 300 W, and 70 ºC, where the pre-treatments provide an excellent combination between extraction yield and carrageenan quality. The freeze-drying method did not present significant advantages on the recovering carrageenan, in addition to being a more costly process.

In chapter 5, it was proved that it is possible to obtain carrageenan through a high-pressure sequential route, providing the recovery of a phenolics-rich fraction, a carrageenan fraction, a cellulose fraction, and a low-molecular weight fraction.

In chapter 6, the carrageenan obtained by the high-pressure route was adequately used to produce beads of aerogel/cryogel with properties similar the samples obtained using a commercial carrageenan.

This work was among the pioneers in relation to the use of sequential high-pressure methods for the fractionation of KA and valorization the quality of the carrageenan-rich fraction recovered. Therefore, this work contributes to some of the UN's sustainable development objectives, in particular stimulating life in the water (SDG 14), due to stimulate the cultivation of algae to obtain polymers; the sustainable production and consumption (SDG 12), since the sequential extraction is the most feasible in terms of sustainability; the innovation and infrastructure (SDG 9), as high-pressure technology for extracting KA using less time is not yet used industrially.

7.2 SUGGESTIONS FOR FURTHER STUDIES

- Apply the aerogel/cryogel obtained in a more complex system such as food or live organism and evaluate how it behaves.
- Evaluate the influence of aqueous extraction (used as a biostimulant) prior to the phenolic extraction and its effects on the next steps.
- Evaluate the possibility of scaling up the high-pressure process to verify the possibility of cost reduction.
- To investigate (mechanism) the depolymerization kinetics of carrageenan (and other biopolymers) at high-pressure method.

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