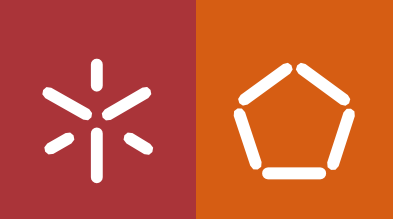




Hybrid Carrageenans as Alternatives to Commercial Blends:
Rheological Properties and Performance in a Food Application

Maria Alice Freitas Monteiro

UMinho | 2025



University of Minho
School of Engineering

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and Performance in a Food application**

Master's Dissertation
Master's in Chemical and Biological Engineering

Dissertation supervised by
Prof. Dr. Bruno Faria and
Prof. Dr. Loïc Hilliou

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STATEMENT OF INTEGRITY

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

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University of Minho, Braga, January 2025

Maria Alice Freitas Monteiro

RESUMO

Carrageninas Híbridas como Alternativas a Misturas Comerciais: Propriedades Reológicas e Desempenho numa Aplicação Alimentar

O principal objetivo deste projeto foi comparar as propriedades reológicas e os mecanismos de gelificação de carrageninas híbridas (KI) e misturas comerciais (K+I), com composição equivalente de κ , sob diferentes concentrações de sal e de polissacarídeo. A caracterização das carrageninas extraídas incluiu ^1H -RMN, FTIR e análise da distribuição de peso molecular. Os perfis de gelificação foram avaliados com diagramas de fase, enquanto os mecanismos de gelificação e o comportamento viscoelástico foram estudados reologicamente. O desempenho das carrageninas híbridas numa formulação de gomas vegan também foi investigado, comparando-as com misturas comerciais e avaliando a influência de precursores biológicos, bem como a incorporação de um aditivo natural.

A gelificação das KI foi mais dependente da concentração de polissacarídeo e da força iônica, enquanto as K+I gelificaram sob a maioria das condições. Em situações específicas, como altas concentrações de polissacarídeo em amostras ricas em ι ou equilíbrio κ/ι , e forças iônicas baixas em amostras ricas em κ , observou-se uma convergência nos perfis de gelificação. As K+I produziram géis mais rígidos e fortes, mas as KI apresentaram elasticidade superior em algumas condições, destacando o seu potencial em aplicações específicas. Contudo, maior elasticidade implicou menor resistência dos géis à deformação, resultando em maior fragilidade. Na formulação de gomas, as KI mostraram potencial como substitutos das K+I em termos de desempenho térmico, mas as misturas produziram géis mais fortes. O tratamento alcalino das KI aumentou a rigidez e a resistência à deformação dos géis, enquanto a incorporação do material biohíbrido melhorou a rigidez, mas reduziu a resistência, indicando a necessidade de otimização para equilibrar o desempenho técnico e a percepção do consumidor.

Estes resultados evidenciam o potencial das carrageninas híbridas como alternativas a misturas comerciais, com aplicações promissoras em alimentos funcionais. Em última análise, a viabilidade das carrageninas híbridas como substitutos de misturas tradicionais depende do equilíbrio desejado entre força, elasticidade e resistência à deformação do gel.

Palavras-chave: Carragenina híbrida, Misturas de carrageninas, Reologia, Carragenina K-2, Indústria alimentar

ABSTRACT

Hybrid Carrageenans as Alternatives to Commercial Blends: Rheological Properties and Performance in a Food Application

This study provides a comprehensive comparison between the thermal-rheological properties of hybrid carrageenans (KI) and commercial K/I carrageenan blends (K+I) with equivalent κ content, focusing on hydrogels formed under varying salt and carrageenan concentrations. $^1\text{H-NMR}$, FTIR, and molecular weight distribution analyses were employed to characterize hybrid carrageenans, while phase diagrams and rheological tests (temperature, frequency, and strain sweeps) evaluated gel formation profiles, gelling mechanisms, and viscoelastic properties. Additionally, the study investigated the performance of hybrid carrageenans in vegan gummy candy formulations, comparing them to commercial blends and assessing the influence of biosynthetic precursors and the incorporation of a natural additive.

KI showed greater dependence on polysaccharide concentration and ionic strength for gel formation compared to K+I, which gelled more readily under most conditions. KI demonstrated comparable performance under specific conditions, such as higher polysaccharide concentrations for ι -rich and balanced κ/ι samples or lower ionic strengths for κ -rich samples. While K+I generally produced stronger and stiffer gels, KI surpassed them in elasticity under certain ionic conditions, highlighting its potential for targeted applications despite trade-offs such as increased brittleness.

In gummy candy formulations, KI successfully produced gel-like textures and demonstrated potential as a replacement for K+I in terms of thermal performance. While K+I produced stiffer and stronger gels, the alkaline treatment of hybrids generally enhanced their stiffness and strain resistance. The incorporation of the biohybrid material improved stiffness but reduced strain resistance, underscoring the need for formulation-specific optimization to balance technical performance and consumer appeal.

These findings highlight the potential of hybrid carrageenans as clean-label, sustainable alternatives to commercial blends, with promising applications in functional foods. Ultimately, the viability of hybrid carrageenans as substitutes for commercial blends depends on the desired balance between gel strength, elasticity, and deformation resistance.

Keywords: Hybrid carrageenan, Carrageenan blends, Rheology, K-2 carrageenan, Food industry

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LIST OF ABBREVIATIONS

ACN	Anthocyanin
AFM	Atomic Force Microscopy
AG	Anhydrogalactose
ATR	Attenuated Total Reflectance
BH	Biohybrid Material
CG	Clear Gel
CGS	Clear Gel with Syneresis
CS	Clear Solution
DRIFT	Diffuse Reflectance Infrared Fourier Transform Spectroscopy
DS	Degree of Substitution
DSC	Differential Scanning Calorimetry
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gummy Candy
GRAS	Generally Regarded as Safe
IR	Infrared
KI	Hybrid Carrageenan
K+I	Commercial Carrageenan Blends
LAOS	Large Amplitude Oscillatory Shear
LVE	Linear Viscoelastic (region)
NMR	Nuclear Magnetic Resonance
PDI	Polydispersity Index
PSS	Phase-Separated (settled) Suspension
PSD	Particle Size Distribution
SAOS	Small Amplitude Oscillatory Shear
SAXS	Small-Angle X-ray Scattering
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SS	Stable Suspension

TG	Turbid Gel
TGS	Turbid Gel with Syneresis
TPA	Texture Profile Analysis
TS	Turbid Solution
T-KI	Treated Hybrid Carrageenans
UT-KI	Untreated Hybrid Carrageenans
XAN	Xanthan Gum
ZP	Zeta Potential

LIST OF SYMBOLS

κ	Kappa-carrageenan disaccharide moiety
ι	Iota-carrageenan disaccharide moiety
λ	Lambda-carrageenan disaccharide moiety
μ	Mu-carrageenan disaccharide moiety
ν	Nu-carrageenan disaccharide moiety
K	Kappa-carrageenan polymer
I	Iota-carrageenan polymer
L	Lambda-carrageenan polymer
$K-2$	Kappa-2 carrageenan, kappa/iota hybrid or weak-gelling kappa-carrageenan polymer
G'	Storage or elastic modulus (Pa)
G''	Loss or viscous modulus (Pa)
G_0	Elastic modulus in the linear viscoelastic region (Pa)
M_w	Weight-average molecular mass (g/mol)
γ_L	Limiting strain (%)
γ_F	Flow strain (%)
T_g	Gelation temperature (°C)
T_{start}	Gelation onset temperature (°C)

1. INTRODUCTION

1.1 Context and Motivation

Carrageenans are a family of sulfated polysaccharides, extracted from red seaweeds, which have long been integral to the food industry due to their unique gelling, thickening, and stabilizing properties (Blakemore, 2016; Souza et al., 2023). They play a crucial role in the formulation of a wide range of products, including dairy goods, meat products, and plant-based alternatives (Blakemore, 2016; Hotchkiss et al., 2016). The versatility and functionality of carrageenans make them indispensable in food technology, contributing to texture, mouthfeel, and product stability.

The focus of most commercial applications has been on the use of K (kappa) and I (iota)-carrageenans, either in pure forms or as blends, to achieve desired textural properties. Extensive research has been conducted on these carrageenans, particularly in terms of their chemical structure, gelling mechanisms, and interactions with other food ingredients (de Vries, 2002; Souza et al., 2023). However, the increasing demand for K and I carrageenans in both food and non-food industries has recently exerted significant pressure on carrageenan production and seaweed farming (Azevedo et al., 2014; Bixler & Porse, 2011), making it urgent to look for underexploited and sustainable resources.

In this context, K/I-hybrid carrageenans, which consist of a random block copolymer of κ -carrageenan and ι -carrageenan sequences, have emerged as promising alternatives to traditional commercial blends (Bixler et al., 2001; Villanueva et al., 2004). Industrially known as weak kappa or K-2, these hybrids form thermoreversible hydrogels with intermediate elasticity in comparison to those of K and I, offering unique structural and performance advantages in certain applications (Bixler et al., 2001; Hilliou, 2014; Hotchkiss et al., 2016; Villanueva et al., 2004). Moreover, they are predominantly sourced from wild seaweeds in cold waters, unlike K and I carrageenans, which are mainly obtained from intense farming of warm-water species, with associated production issues (Ward et al., 2020). This makes hybrid carrageenans not only a more sustainable option but also potentially more cost-effective, by eliminating the need for blending different types of carrageenans (Souza et al., 2023).

Despite their promise, hybrid carrageenans present several ongoing research challenges. Their chemical structure is influenced by various factors, such as the species of algae, the time and place of harvest, and the parameters and methods used during extraction (Ficko-Blean et al., 2015). While these

variables affect the mechanical and structural characteristics of the resulting hydrogel, the relationship between the gelation mechanism and the structure of hybrid carrageenans remains complex and poorly understood (Souza et al., 2023; van de Velde, 2008). Additionally, while the properties of commercial K and I carrageenans are relatively well-documented, this is not the case for their blends for which controversial models are proposed (Bhattacharyya et al., 2024; Geonzon et al., 2023).

Furthermore, comprehensive studies comparing the gelling mechanisms, phase diagrams, and rheological properties of hybrids with traditional blends are still lacking (Azevedo et al., 2014; Souza et al., 2023). Understanding the viscoelastic properties of hybrid carrageenan gels under large deformation regimes is also crucial, as these properties directly impact their functionality in industrial processes. Continued research in this area will help to define the conditions under which hybrid carrageenans can be most effectively used, potentially leading to new applications and improved product formulations.

This research is driven by both the scientific need to fill these gaps and the practical need to address challenges within the food industry. By exploring hybrid carrageenans and systematically comparing them with traditional commercial blends, this study aims to determine whether they can offer distinct structural and functional advantages, such as enhanced stability, improved texture, or greater processing efficiency. Moreover, these findings potentially contribute to a deeper understanding of the relationships between chemical structure, gelling behavior and viscoelastic properties of hybrid carrageenans, under small and large deformation regimes. Additionally, by evaluating their performance in a specific food application, this research seeks to highlight whether hybrid carrageenans can provide concrete benefits over conventional blends. These insights could help shape future research directions, guide industry practices, and contribute to the development of innovative food products that meet evolving consumer demands.

1.2 Research Objectives

The primary goal of this research is to perform a comprehensive comparison between the thermal-rheological properties of hybrid carrageenans and commercial K and I carrageenan blends—ensuring equivalent κ content –, focusing on the hydrogels produced. By evaluating these two carrageenan systems, the study seeks to determine the similarities and differences in how they behave under different salt and carrageenan compositions. This will provide insights into how these systems form gels, their elasticity, viscosity, and overall performance in conditions that mimic real-world applications.

The study also aims to compare the performance of hybrid carrageenans in a specific food application – vegan gummy candies – with traditional commercial blends, critically assessing potential benefits and limitations of using hybrid carrageenans as a viable alternative. Additionally, hybrid carrageenans without alkaline treatment were also tested in the gummy formulations, to investigate the effects of biosynthetic precursors on its overall performance. Furthermore, the effects of incorporating a natural additive into the formulation was also evaluated across all carrageenan systems studied.

1.3 Document Outline

This dissertation is structured as follows:

Chapter 1: Introduction provides an overview of the research context, motivation, and main objectives, emphasizing its relevance to both the field and market demands.

Chapter 2: State of the Art reviews the current state of research related to this study, covering key concepts and major contributions to the field, while also addressing ongoing challenges and knowledge gaps. More specifically, it provides insights into distinct types of carrageenans, their industrial applications, common extraction and characterization methods, gelling mechanisms, and rheological properties. Additionally, this chapter also provides an overview of their use in functional food formulations.

Chapter 3: Methodology outlines the research design and methods used in this study. It covers material selection, hybrid carrageenan extraction and characterization, preparation of commercial blends, and the methods used to evaluate their gelling mechanisms and rheological properties. The chapter also details the formulation of carrageenan-based gummies, and their assessment protocol.

Chapter 4: Results and Discussions present the study's findings and a thorough analysis of their significance. In the first part, the characterization of the hybrid carrageenans extracted is discussed, followed by a systematic comparison of their rheological properties, and phase diagrams, with those of the corresponding commercial blends. In the second part, the overall performance of hybrid carrageenans and traditional commercial blends in the formulation of vegan gummy candies is assessed, highlighting potential advantages or disadvantages of using hybrid carrageenans in food industry applications. Additionally, the effects of biological carrageenan precursors in the hybrid chemical structure, and the impact of incorporating a natural additive in the formulation are also evaluated.

Chapter 5: Conclusions and Future Work summarizes key insights, study limitations, and recommendations for future research.

2. STATE OF THE ART

This chapter provides a comprehensive review of the existing literature and current advancements relevant to this study.

2.1 Carrageenans

Carrageenans are a type of marine biopolymers synthesized by specific red algae from the class Rhodophyta (Hotchkiss et al., 2016; Souza et al., 2023), comprising up to 75% of the dry weight of the algae (Pacheco-Quito et al., 2020; Pereira, Critchley, et al., 2009). Their main function, within the cell wall of the marine algae, includes providing resistive action to oceanic stresses, such as osmotic pressure and desiccation during low tides (Ficko-Blean et al., 2015; Souza et al., 2023).

These hydrophilic polysaccharides have been well recognized for the ability of their solutions to form thermoreversible hydrogels, thereby making them useful for various industries as gelling, thickening, and stabilizing agents (Azevedo, Torres, et al., 2015; Necas & Bartosikova, 2013). Besides their functional properties, their bioactive attributes, affordability and relative abundance, make carrageenans suitable for food, cosmetic and pharmaceutical applications (Ahmed et al., 2014; De Jesus Raposo et al., 2015; Pacheco-Quito et al., 2020; Udo et al., 2023), and more recently bioremediation applications (Alshahrani et al., 2021; Hao et al., 2023; Hossain et al., 2024; G. Sharma et al., 2022).

2.1.1 Chemical Structure

Carrageenans are natural complex polysaccharides classified as sulfated galactans (Ficko-Blean et al., 2015; Pacheco-Quito et al., 2020), due to the presence of 15% to 40% of ester-sulphate within their molecular structure (Ciancia et al., 2020; Necas & Bartosikova, 2013; Pacheco-Quito et al., 2020; Véliz et al., 2017). This sulfur content substantiates their acidic and anionic nature. The structure of these polysaccharides is characterized by linear backbones of alternating D-galactose and 3,6-anhydrogalactose (3,6-AG) units, linked by specific glycosidic bonds (α -1,3 and β -1,4). This forms a repeating sugar unit called carrabiose moiety (Ciancia et al., 2020; Ficko-Blean et al., 2015; Necas & Bartosikova, 2013; van de Velde, 2008). Carrageenophytes differ from agar-producing algae (agarophytes) because of the specific stereochemical arrangement of the bicyclic α -3,6-AG unit in the galactan chain, which is in the L-configuration in agar and in the D-configuration in carrageenans (Ciancia et al., 2020; Ficko-Blean et al., 2015). While the chemistry of sulfated galactans from red seaweeds is well-documented, the biosynthetic

pathways involved remain the focus of ongoing research (Ciancia et al., 2020; Ficko-Blean et al., 2015; Souza et al., 2023).

The types and quantities of carrageenans biosynthesized by seaweeds depend on geographical and seasonal variations (*e.g.* environmental and oceanographic factors), as well as the species and the reproductive and vegetative life stages of the algae (Azevedo et al., 2022; Barahona et al., 2021; Ficko-Blean et al., 2015; Hilliou et al., 2012; Pereira & Van De Velde, 2011; Sanz et al., 2023; Tasende et al., 2012, 2013; Véliz et al., 2017). Additionally, the extraction methods and processing conditions employed can influence factors such as yield, molecular structure, sulfate content, and functional properties like gelling ability and viscoelasticity of the final product (Azevedo, Torres, et al., 2015; Hilliou, Larotonda, et al., 2006; Jouanneau et al., 2011).

Carrageenans are classified according to the number and position of sulfate groups on the repeating carrabiose moieties, as well as the content of 3,6-AG in the polymeric 4-linked residues (Blakemore, 2016; Campo et al., 2009; Necas & Bartosikova, 2013; van de Velde, 2008). Some carrageenans may exhibit more complex substitution patterns, including methyl or pyruvate groups, as well as higher degrees of branching, which contribute to the increased diversity of carrabiose structures (Campo et al., 2009; Ciancia et al., 2020; Jouanneau et al., 2011; van de Velde, 2008). This chemical variation among carrageenan types determines their distinct physicochemical, biological, and rheological properties, thereby influencing their performance and suitability for various industrial applications.

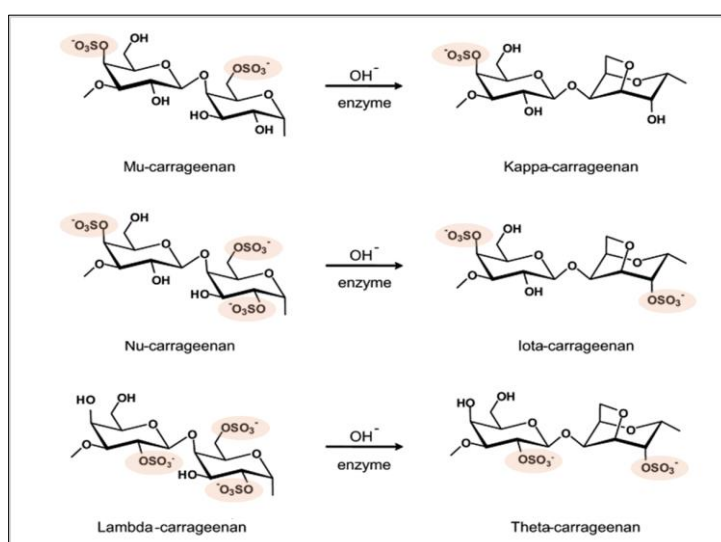


Figure 1: Chemical structures of main carrabiose units present in carrageenans highlighting respective sulfate substitution. Adapted from Pereira & Van De Velde (2011).

The three most exploited carrageenans are called κ (kappa)-, ι (iota)- and λ (lambda)-carrageenan, with one, two and three sulfate groups per carrabiose unit, respectively (see Figure 1) (Souza et al., 2023; van de Velde, 2008). Other two types called μ (mu)- and ν (nu)-carrageenan, commonly found in commercial carrageenans, with two and three sulfate groups, respectively, (Figure 1) are considered biosynthetic precursors of κ - and ι -carrageenan, in that order (Ficko-Blean et al., 2015; van de Velde, 2008).

In general, higher content of 3,6-AG and lower sulfate ester content result in greater gelling potential (BeMiller, 2019; Hotchkiss et al., 2016). K-carrageenan generally has a higher 3,6-AG content compared to I-carrageenan (Hotchkiss et al., 2016). Therefore, K-carrageenan, extracted from *Kappaphycus* species, particularly *K. alvarezii* (trade name *Eucheuma cottonii*) and *K. striatum*, produces stronger and more rigid thermo-reversible gels, while I-carrageenan, extracted mainly from *Eucheuma* species, such as *E. denticulatum* (trade name *Eucheuma spinosum*), *E. perplexum* and *E. isiforme*, yields soft and elastic gels (Blakemore, 2016; Hotchkiss et al., 2016; Souza et al., 2023; van de Velde, 2008). The transformation of the biological precursors into less sulfated κ - and ι -carrabioses is an industrial practice used to enhance gelling potential, achieved mainly through alkaline treatment of marine algae. This process can also be carried enzymatically *in vivo* using sulfatase, as illustrated in Figure 1 (Ahmed et al., 2014; Souza et al., 2023).

In contrast, L (lambda)-carrageenan does not exhibit gelling properties due to its high sulfate content (ranging between 30% and 40% (BeMiller, 2019) and the absence of the necessary 3,6-anhydro bridges; however, it forms viscous solutions, used as thickening and stabilizing agents (Blakemore, 2016; Hotchkiss et al., 2016; Rioux & Turgeon, 2015; Souza et al., 2023).

Commercial K- and I-carrageenan obtained from the extraction of tropical seaweeds are considered to be nearly pure, containing less than 10% ι -carrageenan and less than 15% κ -carrageenan, respectively (Bixler et al., 2001). Although an idealized disaccharide structure with repeating units has been proposed, carrageenans cannot be categorized as homopolymers, as they do not consist of only one type of monomeric unit repeated throughout their structure (Figure 2) (Martín-del-Campo et al., 2021; van de Velde, 2008). In fact, marine algae produce carrageenans with varying heterogenous structures, comprising a mixture of various sulfated oligomers, distinct polymers, and hybrid polymer types (van de Velde, 2008). In this context, different hybrid carrageenans have been extensively studied (Ciancia et al., 2020; van de Velde, 2008).

For example, hybrid K/I-carrageenans, also known as K-2 or weak-gelling K-carrageenans, are described as random block copolymers of κ -monomers and ι -monomers (Figure 2), with the proportions and arrangements specific to each macroalga and dependent on its biological cycle (Azevedo, Domingues, et al., 2015; Bixler et al., 2001; Hilliou, 2021; Hilliou, Larotonda, et al., 2006; Jouanneau et al., 2011; Martín-del-Campo et al., 2021). For industrial purposes, K-2 is arbitrarily set as containing between 20 and 50 mol.% of ι -monomers (Bixler et al., 2001; Villanueva et al., 2004) (Figure 2). One of the most robust methods for demonstrating the hybrid nature of K/I hybrids is through fractionation using KCl solubilization, combined with Nuclear Magnetic Resonance (NMR) spectroscopy (Souza et al., 2023; van de Velde, 2008). This method applies saline conditions, where only K-carrageenan gels in the presence of K^+ ions, enabling its separation from the remaining carrageenan fraction, predominantly I, and its subsequent quantification (Hotchkiss et al., 2016; Souza et al., 2023; van de Velde, 2008). This separation demonstrates that the carrageenan is not a homogeneous polymer but consists of sequences of κ and other monomers.

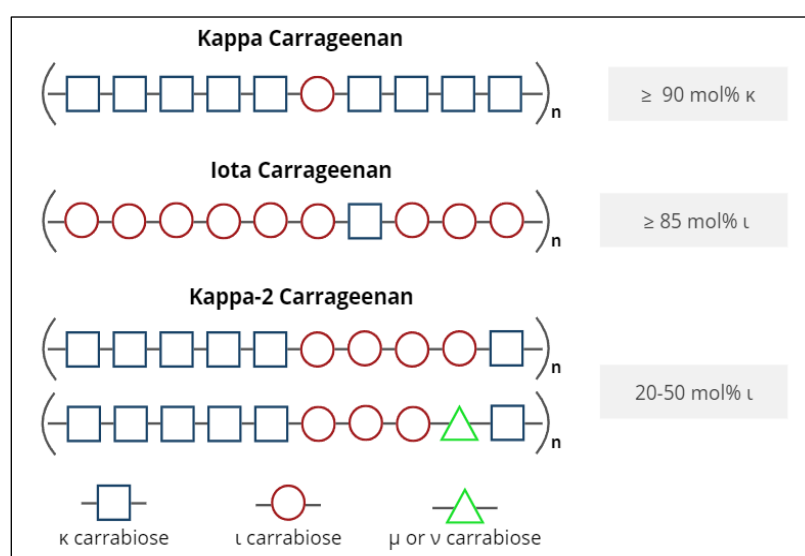


Figure 2: Chemical structures of the industrially relevant gelling carrageenans: K, I and K-2. Adapted from Hilliou (2021).

2.1.2 K-2 Carrageenans

More specifically, K-2 carrageenan chains are complex structures, containing K-carrageenan blocks, I-carrageenan blocks and random or alternating K/I-copolymers, (Guibet et al., 2008; Souza et al., 2023; van de Velde, 2008; Van De Velde et al., 2001). While the distribution of block lengths along the K-2 chain is specific to each alga, it may vary according to the extraction process. Therefore, K-2 are classified

as block copolymers with statistical distribution and polydispersity in both chain lengths and block lengths (Souza et al., 2023).

The designation as a copolymer arises from the fact that neither K nor I chains can be recovered separately, and thus hybrid carrageenan cannot be considered a simple mixture of K+I. These observations have been confirmed by analyzing K-2 products resulting from Smith degradation (de Vries, 2002), acid hydrolysis (A. O. Kravchenko et al., 2020; P. Wang et al., 2012), enzymatic degradation (Guibet et al., 2008), KCl fractioning (Arias et al., 2016; Hilliou, Larontonda, et al., 2006; A. O. Kravchenko et al., 2020; Perez Recalde et al., 2016; Rochas et al., 1989; Van De Velde et al., 2001), electrophoresis (de Vries, 2002), and by comparing phase diagrams and viscoelastic profiles of various K-2 and K+I with equivalent molar compositions of κ and ι (Azevedo et al., 2014; Stancioff, 1981; Torres, Azevedo, et al., 2016).

Indeed, differences in physical properties and performance of K-2 extracts compared with physical mixtures of K and I extracts have been reported. In general, K-2 forms a thermoreversible gel with intermediate elasticity and thermal hysteresis between K and I gels, at similar salt and polysaccharide concentrations, as the ι component reduces the strength and rigidity of the κ component (Azevedo et al., 2014; Bixler et al., 2001; Hilliou, 2021; Hotchkiss et al., 2016). Although the mechanical properties of these gels are weaker than those of K-carrageenan, hybrid carrageenans not only maintain reasonably high reactivity with milk but also present specific textural, viscoelastic and performance advantages, providing unique attributes for dairy product and niche applications within the food industry (Bixler et al., 2001; Hotchkiss et al., 2016; Villanueva et al., 2004).

The increasing attention from the scientific community is also largely driven by the growing demand for gelling additives for both food and non-food applications, which has placed significant pressure on the cultivation of marine algae that produce K- and I-carrageenans (Azevedo et al., 2014; Hilliou, 2014). Additionally, the intensive industrial cultivation of *Kappaphycus alvarezii* and *Eucheuma denticulatum* has recently experienced a decline partly due to disease and pest issues (Azevedo et al., 2022). This has intensified the search for alternative algal sources for carrageenan production. Moreover, while K- and I-carrageenans are almost exclusively sourced from these over farmed warm-water species, K-2 carrageenans are extracted from wild seaweeds found in cold waters (Bixler & Porse, 2011), primarily from the family Gigartinaceae, including genera such as *Chondracanthus*, *Chondrus*, *Gigartina*, *Iridaea*, and *Sarcothalia* (Azevedo et al., 2014; Hilliou, 2014; van de Velde, 2008). As a result, hybrid carrageenans represent a promising alternative for the market.

2.2 Extraction of Carrageenans

Traditional production of hybrid carrageenans from macroalgae generally involves several stages, including pre-treatment, extraction, filtration, concentration, and dehydration. However, variations in the process may occur based on the type of algae used and the desired characteristics of the final product.

After cultivation, macroalgae are typically cleaned manually to remove visible foreign materials, followed by thorough washing to eliminate excess salts and smaller debris. Subsequent drying and milling is standard industry practice prior to extraction. Several studies have suggested potential links between drying temperatures and the chemical structure and molecular weight of the resulting hybrid carrageenan, as well as the role of milling in extraction yield (Bahari et al., 2021; Moreira et al., 2016; Torres, Chenlo, et al., 2016). However, further research is required to definitively establish these relationships, as most of them are secured by industrial confidentiality.

Hybrid carrageenan extraction primarily relies on hot water, leveraging the solubility of various carrageenan polysaccharide fractions and their dispersion in aqueous solutions. Different carrageenan types (K, I, L, K-2) exhibit distinct solubility profiles in water at specific temperatures, requiring adjustments to optimize extraction conditions (Campo et al., 2009; Hilliou et al., 2006; Pacheco-Quito et al., 2020). Most carrageenan classes dissolve in hot water at temperatures above 60 °C, although solubility is also influenced by the solution composition and the presence of other solutes (Udo et al., 2023). Besides temperature, other critical factors – such as the type of cations present in the seaweed, pH, and extraction time – also play significant roles in determining hybrid carrageenan solubility (Bahari et al., 2021; Pacheco-Quito et al., 2020; Souza et al., 2023).

In some cases, alkaline pre-treatment is applied, where algal biomass is dissolved in an alkaline solution, such as sodium, calcium, or potassium hydroxide (Udo et al., 2023). This process promotes the conversion of precursor carrabioses μ and ν into less sulfated forms, κ and ι carrabioses, and facilitates the release of carrageenan polysaccharides, with better gel properties (Azevedo et al., 2013; Hotchkiss et al., 2016; Song et al., 2022). The extent of precursor conversion is influenced by variables such as alkaline concentration and treatment duration, determining residual μ - and ν -carrabiose levels in the final product (Azevedo, Torres, et al., 2015; Hilliou et al., 2009).

Prolonged treatment times and higher alkaline content have been found to yield hybrid carrageenans with fewer biological precursors μ and ν (Azevedo et al., 2013; Azevedo, Torres, et al., 2015; Souza et al., 2023). Some studies showed NaOH to be more efficient than KOH in converting these precursors, with shorter processing times needed to reduce sulfated disaccharides (Souza et al., 2023). However,

excessive alkaline concentrations and prolonged treatment may result in carrageenan extracts with smaller molecular mass and depressed gel properties (Azevedo, Torres, et al., 2015). Therefore, optimizing alkaline solution type and concentration, temperature, and treatment time is crucial based on the algae species and desired product quality.

In a study by Kailas et al. (2006), aqueous and alkaline extractions (using KOH and NaOH) of red algae were compared. While water extraction yielded more carrageenan, the resulting gels were weaker. In contrast, extended alkaline extraction, although leading to lower yields due to carrageenan degradation (especially with NaOH), produced stronger gels. The highest gel strength (350 g/cm²) was achieved by boiling algae in 0.05 M KOH for 3–4 hours, yielding 3.3 times stronger gels than those of water extraction. A similar trend was observed for a mixture of K and I carrageenans, where alkaline extraction resulted in stronger gels. Additionally, it was concluded that alkaline extraction increased the 3,6-AG content, which enhanced gel strength, although this was still lower in hybrid carrageenans compared to commercial K+I blends.

Despite its widespread use, alkaline pre-treatment is commonly associated with the production of polluting effluents (Song et al., 2022). A more environmentally friendly approach was tested on *Chondrus crispus*, involving the cultivation of carrageenophyte algae under dark condition, as described in Villanueva et al. (2009), or cultivation in low nutrient conditions (Villanueva & Montaña, 2014). This method led to increased 3,6-AG content in recovered K-2 carrageenans, alongside reduced sulfate levels, which enhanced gel strength to levels comparable to alkaline treatment.

Looking ahead, several green extraction technologies for hybrid carrageenan look promising. For example, microwave and ultrasound-assisted methods have shown higher extraction efficiency, improved gelation properties, or reduced processing times, when compared to traditional methods (Álvarez-Viñas et al., 2023; Martín-del-Campo et al., 2021; Ponthier et al., 2020; Souza et al., 2023; Torres et al., 2021). Additionally, subcritical water extraction (Álvarez-Viñas et al., 2022, 2024; Gereniu et al., 2018) or high-pressure treatments (Rudke et al., 2022) also represent interesting and more eco-friendly alternatives. Exploration of biorefinery approaches may also help recover hybrid carrageenans and bioactive compounds from industrial seaweed waste (Bianchi et al., 2022). Although enzymatic extraction remains costly for large-scale production, ongoing developments may eventually make it a viable alternative to alkaline modification processes for carrageenan and other seaweed hydrocolloids (Porse & Rudolph, 2017).

After extraction, solid algal residues are separated from the K-2-rich solution through filtration or centrifugation. The resulting hybrid carrageenan is typically concentrated by precipitation with alcohol or KCl, followed by pressing to remove excess liquid (Hotchkiss et al., 2016; Souza et al., 2023). Precipitation with KCl is often preferred for recovering K-2 with enhanced gelling properties (a higher κ fraction) or for partially removing non-carrageenan components from certain carrageenophytes (Barahona et al., 2021; A. Kravchenko et al., 2023; A. O. Kravchenko et al., 2020; Perez Recalde et al., 2016). Alternatively, hybrid carrageenan can be recovered as a film or powder through water evaporation or freeze-drying of the K-2-rich solution (Souza et al., 2023). The purity of the final carrageenan depends on the separation and recovery methods employed.

Based on earlier extraction studies on a set of hybrid carrageenophytes (Azevedo et al., 2013; Azevedo, Torres, et al., 2015; Hilliou, Larotonda, et al., 2006), extraction parameters, namely, algal content in extraction solvent, extraction duration and temperature, alga-carrageenan sol separation, and alkaline modification procedure were employed as described in Section 3.

2.3 Carrageenans Characterization Techniques

The chemical structure of carrageenans can be assessed through several analytical techniques, with NMR spectroscopy and FTIR (Fourier Transform Infrared Spectroscopy) being particularly common (Pereira, Amado, et al., 2009; Souza et al., 2023; van de Velde, 2008).

Vibrational spectroscopy methods like FTIR stand out as practical tools for qualitatively assessing the molecular structure and composition of materials (Pereira et al., 2003). FTIR is a fast and non-destructive technique, which measures the absorption of infrared radiation by a sample, requiring only a small amount of material for accurate measurements. The resulting spectrum gives detailed information about the molecular bonds and chemical groups present, as these correspond to specific wavelengths (Gómez-Ordóñez & Rupérez, 2011; Pereira, Amado, et al., 2009; Pereira et al., 2003). Recent research combining Fourier transform algorithms with attenuated total reflectance (ATR) techniques has further enhanced traditional infrared spectroscopy, by increasing the resolution of spectral data, simplifying sample preparation, and broadening the range of materials that can be analyzed (Gómez-Ordóñez & Rupérez, 2011; Pereira, Amado, et al., 2009).

When analyzing the structure of carbohydrates, five key frequency regions are typically identified in IR spectra ($4000\text{--}650\text{ cm}^{-1}$), as described by Mathlouthi & Koenig (1987): (1) $3600\text{--}2800\text{ cm}^{-1}$: OH and CH

stretching vibrations; (2) 1500–1200 cm⁻¹: local symmetry region; (3) 1200–950 cm⁻¹: CO stretching vibrations; (4) 950–700 cm⁻¹: the anomeric region; and (5) below 700 cm⁻¹: the skeletal region. For carrageenans, relevant FTIR absorption bands are observed around 2920, 1370, 1240, 930, 845, 820 and 805 cm⁻¹, as registered in Table 1. These bands provide insights into their structure, on major carrageenan types but also minor components, such as μ and ν (Chopin et al., 1999; Gómez-Ordóñez & Rupérez, 2011; Pereira, Amado, et al., 2009). However, while FTIR is a powerful tool, it does have limitations. It offers only a semi-quantitative analysis and may struggle to clearly identify the composition of complex or heterogeneous carrageenan structures. This is because multiple chemical groups can contribute to the same absorption band, complicating interpretation (Chopin & Whalen, 1993; Rochas et al., 1986).

Table 1: FTIR band assignment for each carrageenan disaccharide ^a

Wavelength (cm ⁻¹)	Bond(s)/group(s) Assignment	Found in Disaccharides
2920	C-H of total sugar content	κ , ι , μ , ν
1370, 1240–1260	S=O of sulphate esters	κ , ι , μ , ν
930, 1070 (shoulder)	C–O of 3,6-anhydrogalactose	κ , ι
840–850	C–O–SO ₃ on C4 of galactose	κ , ι , μ , ν
820, 825 (shoulder)	C–O–SO ₃ on C2 of galactose	ν
815–820, 867 (shoulder)	C–O–SO ₃ on C6 of galactose	μ , ν
800–805, 905 (shoulder)	C–O–SO ₃ on C2 of 3,6-anhydrogalactose	ι

^a Adapted from Gómez-Ordóñez & Rupérez (2011), Azevedo et al. (2013) and Pereira, Amado, et al. (2009)

NMR spectroscopy, which relies on the resulting signals from the interaction between atomic nuclei and an external magnetic field, provides insights into the chemical environment, structure, and composition of samples (Dais & Spyros, 2012). Specifically, ¹³C-NMR and ¹H-NMR target carbon-13 and proton atoms (hydrogen), respectively (Souza et al., 2023). Although solid-state ¹³C-NMR spectroscopy has shown potential for qualitative evaluation of carrageenans in seaweed, it remains underutilized in this context (Pereira, Amado, et al., 2009; Pereira et al., 2003; Souza et al., 2023).

¹H-NMR spectroscopy allows for the quantification of various carrageenan types by analyzing the position and intensity of the α -anomeric hydrogen resonances from the repeating disaccharide units, typically found between 5.1 and 5.7 ppm (Campo et al., 2009). The ¹H-NMR chemical shifts (ppm) of the α -anomeric protons of carrageenan disaccharides κ , ι , μ , and ν are registered in Table 2. More

importantly, proton NMR enables a quantitative assessment of the carrageenan chemical structures, as the peaks intensities relate to the molar content of the corresponding nuclei.

Table 2: ¹H-NMR chemical shifts (ppm) of the α -anomeric protons of carrageenan disaccharides ^b

Carrageenan disaccharide	Chemical shifts (ppm)
κ	5.09
ι	5.29
μ	5.24
ν	5.50

^bAdapted from Moraes & Hilliou (2024) and Van De Velde et al. (2004)

2.4 Gelling Mechanism – Coil-to-Helix Ion-Specific Transition

The gelling mechanism of carrageenans is widely recognized in literature as a two-step process: a coil-to-helix transition upon cooling in the presence of salt, followed by the self-assembly of helices, into a three-dimensional network (Campo et al., 2009; Hilliou, 2014, 2021; van de Velde, 2008). The exact nature of the helices and superstructures, and their relation to gel elasticity has long been debated, and several models have been proposed (Beaumont et al., 2021; Geonzon et al., 2020; Hilliou, 2021; Schefer et al., 2015). However, further studies are required to fully understand structure-elasticity correlations.

Atomic force microscopy (AFM) has recently provided detailed images of primary coils, secondary helices, and larger aggregated network structures for K- and I-carrageenans in the presence of sodium, calcium and potassium salts (Diener et al., 2019; Schefer et al., 2015). As salt concentration increases, electrostatic repulsion between negatively charged sulfate groups is reduced, promoting the formation of intramolecular helical structures. Expectedly, K-carrageenan undergoes this transition at lower salt concentrations compared to I-carrageenan. In NaCl and CaCl₂ environments, a progressive transition of polymer chains from random coils to helical conformations is observed, with K-carrageenan forming rod-like, stiffer helices and I-carrageenan forming curlier, more flexible helices. In contrast, KCl triggers further self-assembly of these helical polymer strands into supramolecular structures of higher complexity at high salt concentrations.

In the presence of potassium, Schefer et al. (2015) suggested that K-carrageenan forms rigid, twisted dimers that aggregate into stiff superstrands, contributing to brittle gel networks. Meanwhile, I-

carrageenan forms flexible, supercoiled multi-filament strands, contributing to more elastic gels. Diener et al. (2019) imaged quaternary structures of stacked super-stranded helices connected by helical branching in K-carrageenan with K^+ , while I-carrageenan appeared as loosely intertwined single helices, similar to its behavior in NaCl. Thus, a critical reading of these results suggests that both strand rigidity and connectivity in the network are responsible for the different elastic properties of K- and I-carrageenan gels.

The screening power of the divalent cation Ca^{2+} is higher, when compared to the monovalent Na^+ , for both K- and I-carrageenan, resulting in conformational transition at relative lower salt concentrations. In general, the order of effectiveness for inducing coil-to-helix transition for K-carrageenan is $K^+ \approx Ca^{2+} > Na^+$, whereas for I-carrageenan $Ca^{2+} > K^+ > Na^+$ is followed (Schefer et al., 2015).

AFM images of K-2 carrageenans helical assembly are mostly like K-carrageenan, though displaying more branching and a less rod-like morphology (Sokolova et al., 2013; Souza et al., 2023). The coil-to-helix transitions in K-2 are distinct for its K and I blocks, similar to the behavior seen in K+I mixtures, where both components undergo independent coil-to-helix transitions, followed by self-assembly into helical structures. These observations were confirmed in differential scanning calorimetry (DSC) studies performed on K-2 solutions in the presence of different salts (van de Velde et al., 2005) and in NaCl with different ionic strengths (Souza et al., 2011). Rheological data on K-2 solutions cooled in NaCl also showed a temperature dependence of two steps in both the shear storage (G') and loss modulus (G''), suggesting the aggregation of two types of helical assemblies (Hillieu et al., 2014; Hillieu & Gonçalves, 2007; Souza et al., 2011; Torres et al., 2017). However, carrageenan concentration, counter ion type and ionic strength highly influence the two steps gelling mechanism of K-2 and its thermal hysteresis (greater in the presence of K^+ (Chanvrier et al., 2004)).

In general, Na^+ promotes two-step gelation in K-2 solutions and favors the independent coil-to-helix transitions of I and K blocks, followed by the self-assembly of the helical structures. Conversely, in KCl or $CaCl_2$ environments, a more straightforward single-step gelation is usually seen in the rheological profiles (Bahari et al., 2022; Cosenza et al., 2014; Hillieu, Larontonda, et al., 2006; Hillieu & Gonçalves, 2007; Hughes et al., 2023; Torres et al., 2018).

In K+I mixtures, elasticity may arise either from microphase separation or interpenetrating networks of ι - and κ -rich domains (Brenner et al., 2014; Du et al., 2016; Geonzon et al., 2019; Hu et al., 2016; Parker et al., 1993) or even from co-aggregation of K and I helices into complex superstructures, which could explain the larger gel elasticity of K+I compared to the sum of individual gel elasticities (Bui et al.,

2019). Although the gelling process in K-2 resembles that of K+I mixtures, the nature of its self-assembly and super helical structures, and the origin of gel elasticity, remain poorly understood.

Further comparative investigations on K-2 and K+I blends under varying carrageenan concentrations, salt types, and ionic strengths are essential as studies on blends nearly exclusively focused on equal contents of κ and ι . These studies should include in-situ structural characterization to better understand the hierarchical structures and sources of elasticity in these gels (Souza et al., 2023). Alternatively, a more comprehensive comparative study of phase diagrams and gel properties of K-2 and K+I blends, covering a wider range of κ and ι compositions than in Azevedo et al. (2014) and Torres, Azevedo, et al. (2016), could be performed, as proposed here.

2.5 Viscoelastic Properties of Hybrid Carrageenans

Despite of K-2's reported intermediate elastic properties between K and I carrageenan gels, their linear viscoelastic properties are far less studied in literature. In general, the mechanical spectra of K-2, measured by Small Amplitude Oscillatory Shear (SAOS), show qualitative similarities with K or I gels (Souza et al., 2023). K-2 gels exhibit near-frequency-independent elastic modulus (G') with no significant water syneresis (Azevedo et al., 2013, 2014; Azevedo, Torres, et al., 2015; Ponthier et al., 2020; Souza et al., 2011; Torres, Azevedo, et al., 2016; Torres, Chenlo, et al., 2016; Torres et al., 2017, 2018), contrasting with K gels, which tend to release water during liquid-to-gel transition, particularly in the presence of K^+ (Souza et al., 2023).

However, the linear viscoelastic properties of K-2 gels are also influenced by various factors, such as chemical composition, polysaccharide concentration, salt type and ionic strength.

2.5.1 Effect of K-2 Chemical Composition and Molecular Mass

The elastic modulus of K-2 is significantly influenced by its chemical composition, especially the molar fraction of κ . Higher κ content correlates with stronger gels, especially in KCl solutions (Hughes et al., 2023; van de Velde et al., 2005). However, few studies focus on the relationship between K-2's composition and its viscoelastic properties (Souza et al., 2023). In contrast, no clear correlation is observed in NaCl, likely due to variations in molecular mass (M_w). The size of the K-2 chain influences the elasticity of gels formed in NaCl, with longer chains leading to gels with lower G' (Hilliou, Larontonda, et al., 2006; Souza et al., 2011; Torres, Chenlo, et al., 2016), while no clear trend is seen in KCl (van de

Velde et al., 2005). Therefore, more studies are needed to understand the impact of K-2's chemical composition and molecular mass on the gel elastic properties, in NaCl and KCl.

2.5.2 Effect of Salt Type and Ionic Strength

As already reported in Section 2.4, the type of salt and its ionic strength significantly impact the gelation behavior. The elastic modulus increases with ionic strength, and gels formed in KCl and CaCl_2 are typically stronger than those in NaCl, with K-carrageenan evidencing greater cation specificity. Overall, K-2 exhibits cation-specific aggregation, especially for K-helices (Souza et al., 2023).

A study by Torres et al. (2018) showed that stronger K-2 gels were obtained in CaCl_2 , while NaCl produced the weakest. However, the range of polysaccharide concentrations and ionic strength for which a gel is produced is very different for each salt. This finding is evident in studies comparing phase diagrams for different hybrid carrageenans concentrations in different salt types and concentrations, such as Azevedo et al. (2014), Torres, Azevedo, et al. (2016) and Torres et al. (2018). These studies have also highlighted how those parameters affect the formation of clear or turbid gels. Turbid gels are often associated with the formation of particulate suspensions for K-2 having more than 50 mol.% κ , along with an increase in ionic strength.

2.5.3 Effect of Polysaccharide Concentration

Similarly to what is reported in rheological properties of K and I gels, the elastic modulus of K-2 increases with polysaccharide concentration, following an exponential trend (Torres, Chenlo, et al., 2016), or a power law (Hilliou, 2021). Power laws with diverse exponent values have been suggested, due to the wide range of K-2 compositions and salt conditions used to prepare the gels (Hilliou, 2021; Hilliou & Gonçalves, 2007; Torres, Azevedo, et al., 2016). However, it is possible to infer an increase of power law exponent values (n) with higher ionic strength, suggesting a shift from worm-like to stiffer rod-like filament structures (Carrillo et al., 2013; Doi & Kuzuu, 1980; Jones & Marques, 1990; Meng & Terentjev, 2016). Gel turbidity also correlates with higher salt or κ content, leading to coarser structures in K-2 gels and larger n exponent values (Souza et al., 2023).

2.5.4 Viscoelastic Properties under Large Deformation

The study of the properties of gels under large deformations are of great industrial relevance and can contribute valuable insights to understand the gel's structure. In the linear viscoelastic regime, small

deformations result in a proportional response where properties like the shear modulus (which quantifies stiffness) remain constant. However, under large deformations, carrageenan gels deviate from predictable, linear responses, displaying instead complex nonlinear behaviors – its shear modulus changes, often hardening or softening depending on the filamentous network structure (Souza et al., 2023). Rotational rheometry applies simple shear (as opposed to compressive or indenting forces) to study how the material behaves under deformation. Simple shear ensures symmetric deformation, making it easier to measure and interpret stress - strain relationships (Souza et al., 2023).

Theoretically, filamentous networks show strain hardening, which means the gel becomes stiffer when subjected to larger strain before it eventually ruptures, due to finite extensibility of the filaments – *i.e.* they are stretched between crosslinks in the network, and this stretching causes them to stiffen as they approach their breaking point (Carrillo et al., 2013; Doi & Kuzuu, 1980; Hilliou, 2021; Meng & Terentjev, 2016). Such stretching also supposes some degree of freedom between crosslinks, that is, pre-tension of filaments by the network is not that big (Meng & Terentjev, 2016).

Experimental data from Large Amplitude Oscillatory Shear (LAOS) shows that K-2 gels, in KCl, undergo strain hardening, showing a relatively soft elasticity, with shear elastic modulus typically below 1 kPa (Azevedo et al., 2014; Bahari et al., 2022; Hilliou, 2021; Hilliou et al., 2009), when compared to K gels, which have much stiffer properties (up to 10 kPa). Strain softening, in which gels become less stiff under increased strain, was reported for certain K (Diener et al., 2019; Flores et al., 2017; Hilliou, 2021), I (Diener et al., 2019; Flores et al., 2017), and K+I blends (Flores et al., 2017), though the mechanisms behind this behavior remain unclear (Souza et al., 2023).

2.6 Hybrid Carrageenans – Market Trends and Application in Functional Foods

Carrageenan is currently the leading seaweed-derived food hydrocolloid and accounts for the sixth largest share of the global hydrocolloids market (in terms of value), after guar gum, gelatin, xanthan gum, cellulose gum, and arabic gum (Hotchkiss et al., 2016; Zhang et al., 2023). The global carrageenan market was worth \$850 million in 2022 and is expected to grow at an average yearly growth rate of 6.2%, reaching \$1.55 billion by 2032 (Pulidindi & Ahuja, 2023).

Carrageenans, with a molecular mass above 100 kDa (Necas & Bartosikova, 2013), are generally recognized as safe (GRAS) for consumption by both the FDA (Food and Drug Administration) and EFSA

(European Food Safety Authority), where they are classified as food additives E407 (refined) and E407a (semi-refined) (Rioux & Turgeon, 2015; Udo et al., 2023). Although toxicological assessments have confirmed that food-grade carrageenan has minimal physiological impacts, some studies suggest that degraded carrageenans may cause gastrointestinal health problems (Gotteland et al., 2020; Han et al., 2019; O'Sullivan et al., 2010; Shang et al., 2017; Sun et al., 2019), raising concerns about its safety (Bixler, 2017; Necas & Bartosikova, 2013). These controversies significantly influenced the carrageenan market, driving consumer demand towards perceived safer alternatives like agar, xanthan gum, and guar gum (Pulidindi & Ahuja, 2023).

Beyond their functional applications as texturizing, stabilizing and gelling agents, hybrid carrageenans also exhibit various bioactive properties and unique attributes, making them valuable across a broad range of fields besides the food industry. These include applications in cosmetics and personal care products (Dubey & Dubey, 2020; Kim et al., 2018; Shafie et al., 2022), the pharmaceutical and biomedical industries (Calvo et al., 2019; De Jesus Raposo et al., 2015; Ha et al., 2022; Mehvari et al., 2024; Pacheco-Quito et al., 2020; Padhi et al., 2016; Pradhan & Ki, 2023), and also bioremediation techniques (Alshahrani et al., 2021; Hao et al., 2023; G. Sharma et al., 2022).

2.6.1 Bioactive, Nutritional, and Health-Promoting Properties

Regarding functional food applications, carrageenans have been shown to promote health and prevent diseases by exhibiting antiviral, antibacterial, antioxidant, antitumoral, anti-inflammatory and immune-boosting effects (Bhuyan et al., 2023; Boulho et al., 2017; Dousip et al., 2014; Pradhan et al., 2022; Premarathna et al., 2024; Valado et al., 2019; W. Wang et al., 2011). Carrageenans also help improve gut health by acting as prebiotics, stimulating the growth of beneficial gut microbiota and increasing the production of short-chain fatty acids, such as acetate, propionate, and butyrate (Cherry et al., 2019; Kumari et al., 2023; Li et al., 2017; Liu et al., 2015). Moreover, the high level of dietary fiber in these biopolymers also adds to their nutritional value, helping with weight management and reducing the risk of cardiovascular disease due to their cholesterol-lowering effects (Dousip et al., 2014; Valado et al., 2019).

In addition, carrageenans represent important plant-based alternatives to animal-derived ingredients like gelatin, dairy, and processed meats, aligning with current market trends (Blakemore, 2016; Hotchkiss et al., 2016; Karim & Bhat, 2008; Necas & Bartosikova, 2013; Palanisamy et al., 2018; Pereira, Critchley, et al., 2009; Rashmi & Mona, 2023; Russo Spena et al., 2024; Song et al., 2022; Udo et al., 2023).

In general, the latter described bioactive and functional properties of carrageenans (synthesized in Figure 3) make them excellent candidates for inclusion in functional foods and nutraceuticals (Kumari et al., 2023; Prasetyaningrum et al., 2019), providing texture while also promoting health benefits beyond basic nutrition.

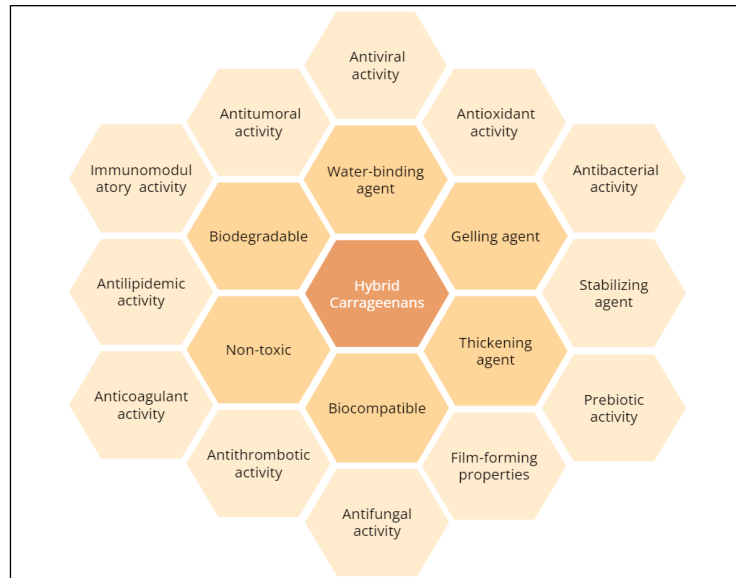


Figure 3: Functional and bioactive properties of hybrid carrageenans. Adapted from Pacheco-Quito et al. (2020) and Pradhan & Ki (2023).

2.6.2 Interaction with other Hydrocolloids and Ingredients

Potential synergistic interactions between carrageenans and other hydrocolloids have been studied to modify the texture, stability, and gelation properties of food products (Hotchkiss et al., 2016; Song et al., 2022; Tunieva et al., 2021). In particular, when combined with xanthan gum, K-carrageenans form gels with improved viscoelastic properties, optimized texture, enhanced stability and reduced water syneresis (liquid separation from gels), allowing for the creation of high-quality vegan formulations that closely replicate the sensory properties of traditional gelatin-based products (Avallone et al., 2023; Balasubramanian et al., 2018; Brenner et al., 2015; Tunieva & Spiridonov, 2020; Xu et al., 2022).

Traditional gummy candies (GC) have gel-like structures and are made using a combination of sugars, water, gelling agents (typically gelatin), and may also incorporate fruits, acids, aromas, and food colorants (Cappa et al., 2015; Ge et al., 2021; Mutlu et al., 2018; Teixeira-Lemos et al., 2021). Besides choosing vegan alternatives to animal-based gelatin, some other ways to align GC to current market trends and consumer demands, may comprise utilizing natural colorants, incorporating plant extracts, vitamins, or fruit derivatives, with bioactive and functional properties, and substituting sugar with other sweeteners,

such as honey or stevia (Mutlu et al., 2018; Palachum et al., 2023; Roudbari et al., 2024; Tarahi et al., 2023). These innovations aim to enhance the nutritional profile of a traditionally non-nutritious product, transforming it into a healthier option. This is particularly significant given the widespread consumption of jellies and gummies, especially among children and adolescents under 17 years old, which has been associated with a high incidence of obesity, tooth decay, and hyperglycemia (Grand View Research, 2024; Khawaja et al., 2019; Rippe & Angelopoulos, 2016; Tarahi et al., 2023). Carrageenan-based vegan gummy supplements have also been successfully used as a medium for delivering pharmaceuticals and/or nutritional bioactives, especially for children (Cheng et al., 2022; Qiu et al., 2024; Rashmi & Mona, 2023; Tarahi et al., 2023b).

In that context, bioactive compounds such as anthocyanins (ACNs) have gained great attention as a natural food colorant and functional ingredient (Lourenço et al., 2019; Rodriguez-Amaya, 2019). These compounds are responsible for the red, purple and blue hues observed in various fruits, vegetables, flowers and grains and exhibit potent antioxidant, anti-inflammatory, anti-allergic, anti-diabetic, and anti-carcinogenic activities (Rodriguez-Amaya, 2019; Shipp & Abdel-Aal, 2010). However, ACN activity is highly dependent on external factors such as light, pH, oxygen, temperature, and interactions with other ingredients, which limits their use in certain applications (Albuquerque et al., 2021; Cortez et al., 2017; Rodriguez-Amaya, 2019). To mitigate these limitations, stabilization techniques are commonly employed, including copigmentation, exclusion of O₂, encapsulation/microencapsulation within biopolymers and adsorption onto nanoclays (Cavalcanti et al., 2011; Coelho Leandro et al., 2021; Cortez et al., 2017; Koop et al., 2024; Mahdavi et al., 2014; Rodriguez-Amaya, 2019).

Thus, this work aimed to extract and characterize K/I hybrid carrageenans sourced from a diverse range of seaweed, focusing on their viscoelastic properties and their gelling behavior, under varying salt and carrageenan concentrations. These findings were systematically compared with commercial carrageenan blends of equivalent κ and ι composition.

In response to current market trends for plant-based functional products, a hybrid carrageenan-based vegan gummy candy was developed and characterized. In this context, the hybrid carrageenan's performance in this specific food application was compared to that of commercial carrageenan blends, of identical compositions. Moreover, the effects of including biosynthetic carrageenan precursors and the incorporation of an anthocyanin-based natural additive were determined by measuring the viscoelastic properties and overall stability of the formulation.

3. METHODOLOGY

3.1 Raw Materials

Commercial Red Algae:

- Twelve different commercial red algae samples (samples A to L) were used in this study. These samples were generously provided by Cargill France SAS (Baupre, France) in a dried, unprocessed state. The selection of commercial samples aimed to cover a diverse range of chemical structures of carrageenans, providing a broad basis for comparison with carrageenan blends.

Non-Commercial Red Algae:

- Three non-commercial red algae samples were also included in the study, as they contain hybrid carrageenans which permit a broader range of chemical structures to be covered (Azevedo et al., 2013, 2022; Azevedo, Torres, et al., 2015). In addition, they offer unique properties and potential to offer insights into underexplored carrageenan sources. These samples, which were obtained from ALGAplus (Ílhavo, Portugal), belonged to the specific families: *Mastocarpus stellatus* (sample M), *Chondrus crispus* (sample N), and *Gigartina pistillata* (sample O).

All supplies were stored in a dry, dark environment, protected from direct sunlight, and kept at room temperature.

3.2 Production of Hybrid Carrageenans

The procedure used to extract hybrid carrageenans was inspired by Azevedo et al. (2013) with some minor adaptations.

3.2.1 Hot Water Extraction

Dried seaweed samples (1.5 g) were soaked in 50 mL of distilled water, at 80 °C for 3 hours, with stirring every 30 minutes to ensure thorough extraction (Figures 4A and 4B). After the initial extraction period, 100 mL of distilled water was added to the algal suspension, which was then homogenized in a blender to enhance extraction efficiency (Figure 4C). For samples I, J, K and L the protocol was adjusted by increasing the water volume to 150 mL to reduce the viscosity of the algal suspension and facilitate

further centrifugation. The extraction process was then continued at 90 °C for an additional hour. Following this, the solution was centrifuged using a Hettich Lab Universal 320 centrifuge at 8000 rpm for 10 minutes, to recover the liquid phase, which was subsequently dried in an oven at 55 °C, overnight, to form carrageenan films (Figure 4D).

Extractions were performed in triplicate to produce the necessary amount of carrageenan for following characterizations.

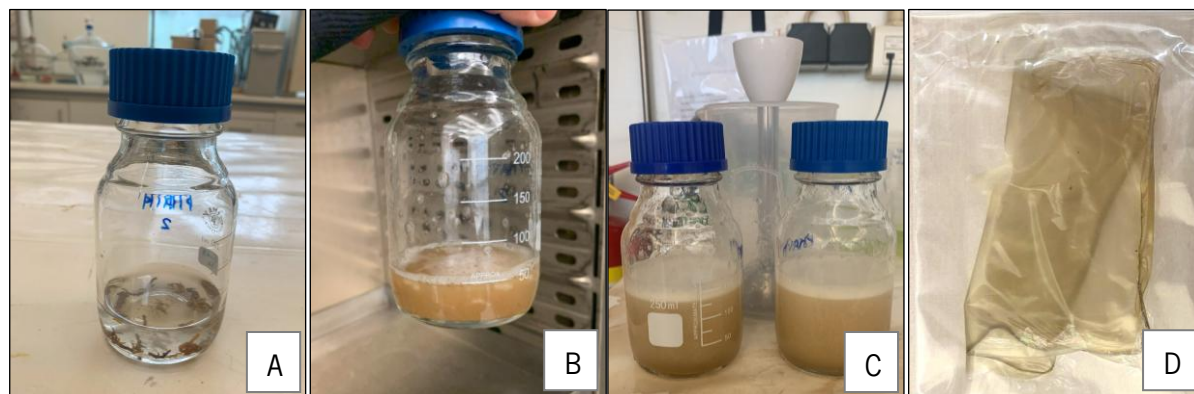


Figure 4: Hot water extraction procedure: sample of alga soaking in 50 mL of distilled water at **A)** $t=0$ h and $T=20$ °C; and **B)** $t=3$ h and $T=80$ °C; **C)** sample after the addition of 100 mL of distilled water and homogenization; **D)** Resulting carrageenan film.

3.2.2 Alkaline Treatment

The extracted carrageenans were treated with two different alkaline solutions: 3% (w/w) sodium hydroxide and 3% (w/w) potassium hydroxide (both from Sigma-Aldrich, St. Louis, MO, USA). These solutions were specifically selected to study the gelling behavior of carrageenans in NaCl and KCl environments, respectively. This approach also enabled the evaluation of how effectively these alkaline treatments converted the precursor carrageenan units, μ and ν , into the more desirable κ and ι types, while improving the quality of the gels produced.

To carry out the treatment, carrageenan films obtained from the initial extraction process were dissolved to create 2% (w/w) solutions in each alkaline solution. These mixtures were then heated under reflux in a water bath at 85 °C for 3 hours.

3.2.3 Isolation of Carrageenan

After the 3-hour reflux, an ethanolic precipitation was carried out using 96% (v/v) ethanol (Sigma-Aldrich, St. Louis, MO, USA), at a ratio of 1:2.5 (*i.e.*, 250 mL of ethanol per 100 mL of carrageenan solution).

The heated, treated carrageenan solution was slowly added to cold ethanol using a dropping funnel, with continuous stirring and refrigeration to facilitate precipitation (Figure 5A). The resulting precipitate was collected, filtered through a fine mesh net (Figure 5B), and then dried overnight at 55 °C to complete the process.

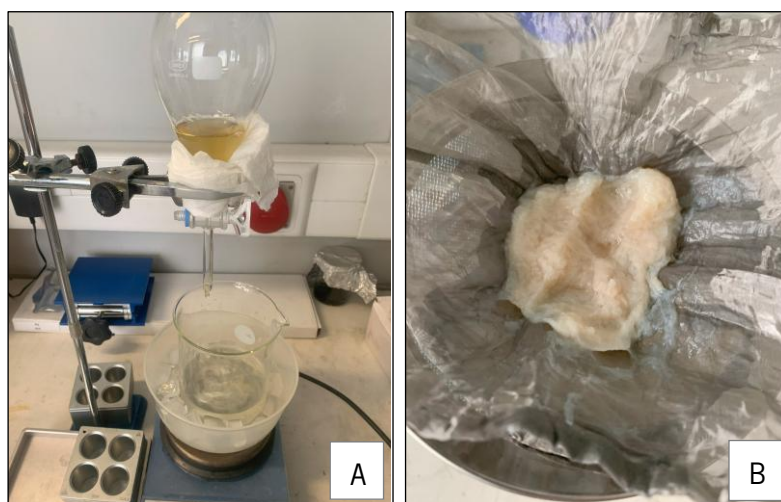


Figure 5: A) Setup for the ethanolic precipitation process; **B)** Filtration of the precipitate through the mesh net.

3.3 Carrageenan Characterization

3.3.1 FTIR-ATR spectra acquisition

For the FTIR assessment, 2% (w/w) carrageenan solutions were prepared, by dissolving each extract in water, heated under reflux, at 80 °C. The solutions were then cast in plastic molds and dried overnight, with the purpose of producing biofilms with constant thickness. The FTIR spectra of the dried carrageenan biofilms were recorded using the Spectrum 100 Optica FT-IR Spectrometer (PerkinElmer, Waltham, MA, USA), equipped with a Golden Gate ATR accessory (Specac Ltd, Orpington, UK), featuring a diamond crystal. The software used to acquire an average of 16 scans was Spectrum (version 6.3.5). The spectra range for the samples was 4000 to 600 cm^{-1} . The spectral resolution of the scans was 4 cm^{-1} . A background spectrum of air was recorded under the same instrumental conditions before each series of measurements.

3.3.2 Proton Nuclear Magnetic Resonance (^1H -NMR)

^1H -NMR of the extracted carrageenans was performed with a Bruker Avance III spectrometer (Billerica, MA, USA), at the Department of Chemistry from the University of Minho. Carrageenan solutions in D_2O

(Sigma-Aldrich, St. Louis, MO, USA) (≈ 1 wt.%) were ultra sonicated with a DCG-300H bath (MCR Ltd., Holon, Israel) during several hours to lower the viscosity before NMR spectroscopy. D_2O peak was used as reference signal and the operation conditions were 70 °C and 400 MHz NMR spectra were visualized and analyzed using NMRium® online software (DEMO version).

The molar fractions (mol.%) of the carrageenan disaccharide repeating units (κ , ι , μ , and ν) were calculated based on the integrated intensity of the respective 1H -NMR peaks, given in Table 2 (Section 2.3) divided by the sum of the integrated intensities of all assigned carrageenan anomeric protons. The presence of pyruvic acid and floridean starch can also be evaluated, through the detection of the signals at 1.45 ppm (Chiovitti et al., 1997), and simultaneously 5.37 ppm and 4.98 ppm (Knutsen & Grasdalen, 1987; Van De Velde et al., 2002), respectively.

3.3.3 Molecular Mass Distribution

The molecular mass distribution was determined using size exclusion chromatography (SEC) (Waters 600 system with a Waters 2410 differential refractive index detector, Waters Portugal, Lisboa, Portugal). The system was equipped with a PolySep-GFC-P Linear column (Phenomenex, Alcobendas, Spain) and calibrated using pullulan standards (Shodex, Munich, Germany) with molecular weights ranging from 6300 to 642,000 g/mol. Measurements were conducted in duplicate, with 0.1 wt.% carrageenan solutions prepared in 0.1 M NaCl at 40 °C.

3.4 NaCl and KCl Phase Diagrams

3.4.1 Samples Selection Criteria

The selection of samples for the NaCl and KCl phase diagrams was based on specific criteria designed to capture a wide range of chemical compositions and eliminate potential interferences from other components. The criteria are outlined as follows:

- **Broad range of chemical compositions:** Samples were chosen to cover a wide spectrum of κ - and ι -carrageenan mol.%, allowing a deeper exploration of how different mol.% values, particularly of ι -carrageenan, impact the gelling behavior.
- **Biosynthetic precursor content:** Only samples containing up to 5 mol.% of μ - and ν -carrabiose were included, as this margin was considered within the error range of 1H -NMR

measurements. This is because μ - and ν -carrabiose are known to act as defects in the hydrogel structure (Souza et al., 2023) and thus will jeopardize a fair comparison with blends of commercial carrageenans, which are μ - and ν -carrabiose free.

- **Absence of interfering components:** Samples with significant amounts of other substances, such as pyruvic acid or floridean starch, were mostly excluded to ensure that the focus remained solely on the carrageenan-specific interactions.

3.4.2 Preparation of Phase Diagrams

The strategy developed in Azevedo et al. (2014) was followed, with minor adaptations. Solutions of NaCl and KCl with four different ionic strengths (0.01, 0.1, 0.5 and 1 mol/L) were prepared by dissolving the appropriate amounts of salt in distilled water. The NaCl and KCl salts were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany), respectively. Solutions of extracted K/I hybrid carrageenans (KI) and commercial blends (K+I), with equivalent chemical composition to the KI, were prepared with 3 different total carrageenan concentrations (0.5, 1 and 2 wt.%) by dissolving, with continuous magnetic stirring at 80 °C for 1-2 h, appropriate amounts of carrageenan in 5 mL of salt solution with the four different ionic strengths. For the preparation of carrageenan blends, commercial grade K- (22048-100G-F, Sigma-Aldrich, St. Louis, MO, USA) and I-carrageenans (C1138-100G Sigma-Aldrich, St. Louis, MO, USA) were used and the solutions were produced as for the hybrid solutions.

In the preparation of KI samples, solutions of NaCl and KCl were used in samples treated with NaOH and KOH, respectively, to obtain a single cation type, Na^+ or K^+ .

After preparation, all flasks were stored at room temperature (25 °C) for 24 h in a stand still position. After that time, flasks were gently tilted to visually inspect the phase state of the carrageenan samples and to assess their turbidity, under calibrated illumination. Samples were thus sorted into clear/turbid solutions (CS/TS); stable suspensions (SS); phase separated (settled) suspensions (PSS); clear/turbid gels (CG/TG) and clear/turbid gels with syneresis (CGS/TGS), as represented in Figure 6.

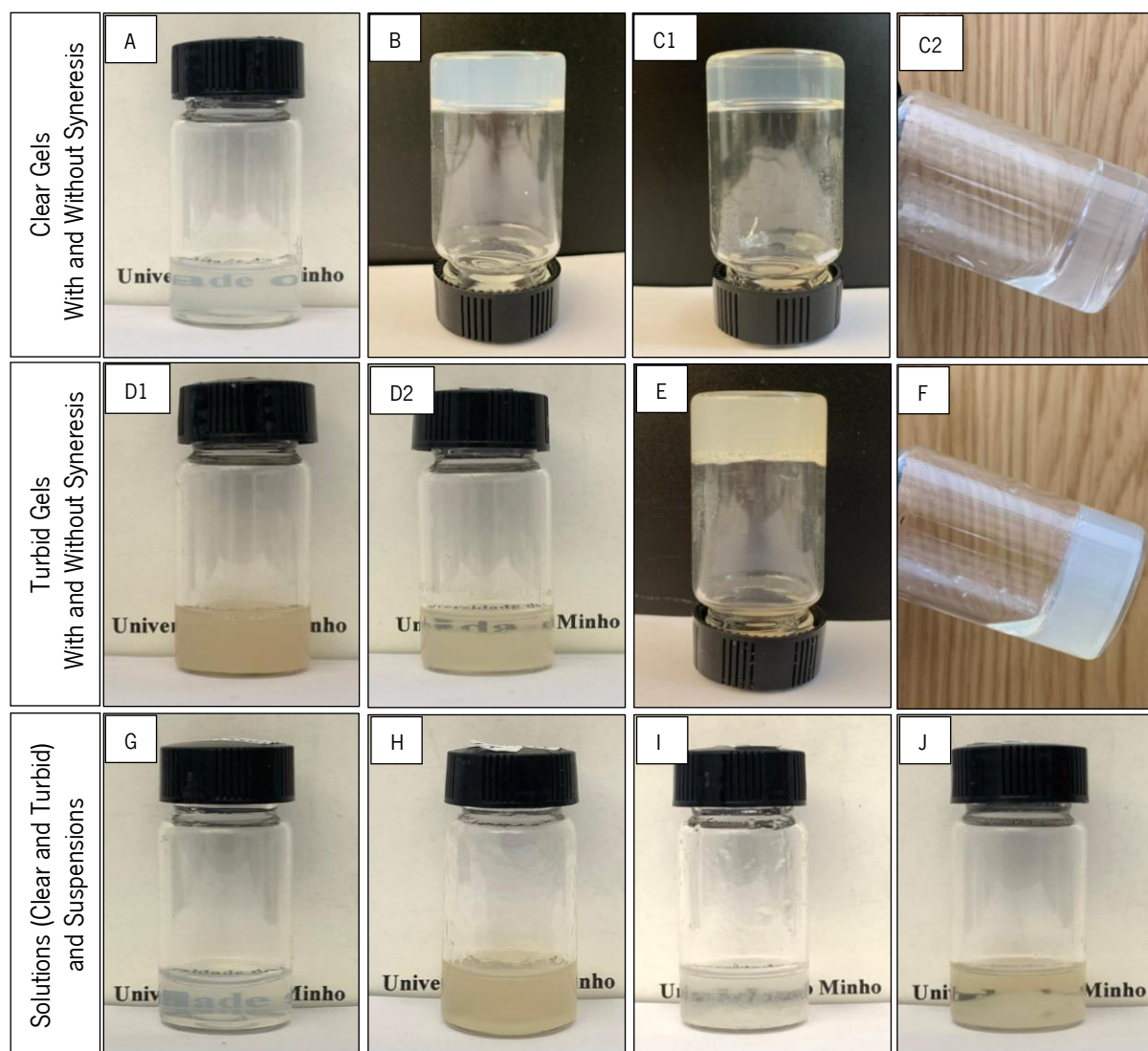


Figure 6: Representative images of the different states of samples: **A)** clear gel; **B)** clear gel without syneresis (CG); **C1, C2)** clear gel with water syneresis (CGS); **D1, D2)** turbid gels (gels with precipitated particles - D2 - were categorized as turbid for simplicity); **E)** turbid gel without syneresis (TG); **F)** turbid gel with water syneresis (TGS); **G)** clear solution (CS); **H)** turbid solution (TS); **I)** stable suspension (SS); and **J)** phase-separated (settled) suspension (PSS).

3.5 Rheological Characterization of Gels

3.5.1 Samples Selection Criteria

For the rheological assessment, only samples that had formed a gel after 24 hours of aging were considered. To investigate how varying mol.% values, particularly of ι -carrageenan, influence the viscoelastic properties of carrageenan gels, samples with significantly different κ/ι ratios were selected. To comply with the timeline established in the research plan, only three samples—C, E, and K—from the previously obtained phase diagrams in KCl and NaCl were chosen for rheological characterization. These

samples were chosen based on their distinct κ/ι ratios and gelling profiles, as they demonstrated gel formation across a broader range of salt and polysaccharide concentrations. For each sample, three different polysaccharide concentrations and ionic strengths were selected to provide a comprehensive understanding of their viscoelastic properties.

3.5.2 Rotational Rheometry Procedure

The viscoelastic properties were measured using a stress-controlled rotational AR-G2 rheometer (TA Instruments Ltd, New Castle, DE, USA), equipped with a 25-mm parallel-plate geometry and a gap of 500 μm , at 85 °C. To prevent water evaporation during testing, dodecane (Sigma-Aldrich, St. Louis, MO, USA) was applied around the sample rim. Data acquisition was carried out using Rheology Advantage software (TA Instruments Ltd, New Castle, DE, USA).

A time sweep was performed, where hot solutions were cooled from 85 °C to 25 °C, over 1.5 hours to assess the carrageenan sol-gel transition. The strain amplitude and frequency were set to 0.5% and 1.0 Hz, respectively, essentially to avoid any strain-induced change in the gel-setting behavior. During cooling, the rheometer's gap was adjusted to account for the thermal expansion of the plates, while the normal force was maintained at 0 ± 1 N to compensate for potential volume changes during the sol-to-gel transition.

Subsequently, the mechanical spectrum of the hydrogels at 25 °C was measured using a frequency sweep from 100 to 0.01 Hz, with a strain amplitude of 0.5%, essentially to keep the strain within the linear regime of viscoelasticity. Lastly, the linear and large deformation viscoelastic behavior of the hydrogels was analyzed at 25 °C by performing a strain amplitude sweep between 0.1% and 1000%, with a fixed frequency of 1 Hz.

3.6 Vegan Gummy Candy Formulation and Preparation

3.6.1 Raw materials and Formulation

Inspired by a study conducted by Song et al. (2022), the vegan gummy candies (GC) formula included a mixture of carrageenans (KI or K+I), distilled water, xanthan gum (XAN, food grade), rosemary honey, and a natural additive (Table 3). Xanthan gum was used due to its documented synergistic effects when mixed

with K-carrageenan (Avallone et al., 2023; Balasubramanian et al., 2018; Brenner et al., 2015; Tunieva & Spiridonov, 2020; Xu et al., 2022), assuming similar behavior occurs with K/I hybrids.

Rosemary honey served as a nutritious sweetener and texturizer, chosen for its therapeutic and preventive effects against inflammation-mediated chronic diseases (Ranneh et al., 2021). Rich in distinctive phenolic and flavonoid compounds, honey is reported to have health benefits, including antioxidant (Ranneh et al., 2018), anti-proliferative (Jaganathan et al., 2014), and anti-bacterial activity (Khan et al., 2018).

The natural additive was a biohybrid material (BH) composed of anthocyanins (ACNs) extracted from jambolan fruit (*Syzygium cumini*), which were stabilized by adsorption onto bentonite. The BH was kindly provided by Professor Germán Valencia, of the Department of Chemical and Food Engineering of the Federal University of Santa Catarina, Campus João David Ferreira Lima (Florianópolis, Brazil). It was used due to its antioxidant and antimicrobial activities, attributed to anthocyanins, as well as its increased stability (Koop et al., 2024).

For the formulation of the vegan gummy candies, three different alkali (KOH) treated hybrid carrageenans (T-KI) with significantly varying κ/ι ratios (samples C, F, and J) were evaluated, along with their respective commercial blends of similar chemical composition. Additionally, to investigate the potential effects of the carrageenan precursors μ and ν on the overall performance of the gummies, untreated hybrid carrageenans (UT-KI) extracted from the same sources (carrageenans C, F, and J) were also assessed.

In addition, each GC formulation was replicated without the BH material to better understand its impact on the stability, texture, and structural profile of the final product.

Table 3: Formula of the gummy samples, expressed as weight percentage (% w/w)

Ingredients	GC Samples with BH (% w/w)	GC Samples without BH (% w/w)
Water	86,5	87
Honey	10	10
Carrageenan (UT-KI, T-KI, or K+I)	2	2
Xanthan gum	1	1
Biohybrid	0,5	—

Therefore, a total of 18 GC samples were produced, considering three different κ/ι ratios tested in three different systems (UT-KI, T-KI, and K+I), each assessed with and without the biohybrid material, as shown in Table 4.

Table 4: Synthesized description of the comparative systems studied, and labelling of vegan gummy candies produced

	Untreated Hybrid Carrageenans (UT-KI)	Alkali-treated Hybrid Carrageenans (T-KI)	Commercial Carrageenan Blends (K+I)
GC Samples with BH	UT-C + BH	T-C + BH	10 κ +90 ι + BH
	UT-F + BH	T-F + BH	50 κ +50 ι + BH
	UT-J + BH	T-J + BH	90 κ +10 ι + BH
GC Samples without BH	UT-C	T-C	10 κ +90 ι
	UT-F	T-F	50 κ +50 ι
	UT-J	T-J	90 κ +10 ι

3.6.2 Gummy Candies Preparation Procedure

The preparation procedure of the gummies was inspired on Song et al. (2022). To prepare 10 g of GC samples, 0.2 g of carrageenan and 0.1 g of xanthan gum were weighed and gradually dispersed into a portion of distilled water, stirring vigorously with a spatula to prevent clumping. The solution was then heated to 80 °C with continuous magnetic stirring for 30 minutes.

Meanwhile, the remaining ingredients (the rest of the water, honey, and the biohybrid, or just water and honey for the control groups) were weighed and mixed at room temperature until a homogenous solution was obtained. This mixture was added to the carrageenan and xanthan gum solution after the initial 30-minute heating period of the latter, and the resulting preparation was then heated and stirred for an additional 30 minutes.

Finally, the samples were left to rest at room temperature overnight to promote gelation. After visually inspecting the phase state and coloration of the GC samples, they were stored in the fridge for preservation and taken out only for testing purposes.

3.6.3 Particle Size Distribution and Zeta Potential of the Biohybrid

The particle size distribution (PSD) and zeta potential (ZP) of the biohybrid material dispersed in water were analyzed to evaluate the magnitude of the electrostatic charge on the particle surfaces and their size. These factors are crucial for understanding potential interactions with other ingredients in the formulation, as well as their impact on the product's overall stability and consistency (Parupudi et al., 2022).

PSD was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK) based on laser-light scattering principles. Due to equipment database limitations, the sample was approximated as silica (SiO_2), with a refractive index of 1.457 and an absorption index of 0.010. Water was used as the dispersant, with a refractive index of 1.330.

The ZP was measured using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK), which uses Laser Doppler Velocimetry to determine electrophoretic mobility. Measurements were conducted in triplicate at 25 °C.

3.7 Gummy Candy Characterization

3.7.1 Optical Microscopic Imaging

Optical microscopic imaging was used to inspect the quality of the dispersion of biohybrid particles along the formulation matrix, using an optical transmission microscopy (Olympus BH2, 20x) coupled to a LEICA DFC 280 digital camera. Melted samples (≈ 85 °C) were placed on microscope slabs and gently squeezed with a glass cover.

3.7.2 pH Measurements

The pH of all samples was measured using a HI 2210 pH Meter (Hanna Instruments, Limena, Italy).

3.7.3 Rheological Characterization

All GC samples were rheological characterized following the same methodology as described in Section 3.5.2.

3.7.4 FTIR-ATR Spectra Acquisition

The FTIR-ATR analysis was conducted to understand whether the addition of the biohybrid material causes chemical changes within the matrix. For this assessment, a small sample of each gummy was collected, heated under reflux, at 80 °C, and finally dried overnight, to form a film. The FTIR-ATR spectra acquisition procedure used was the same as described in Section 3.3.1.

4. RESULTS AND DISCUSSION

4.1 Chemical Structure of Hybrid Carrageenans

Insights on the chemical characterization of the hybrid carrageenans produced were mainly obtained by FTIR-ATR, $^1\text{H-NMR}$ and SEC, to identify the material's molecular bonds and chemical groups, chemical composition and molecular mass distribution, respectively.

4.1.1 FTIR-ATR Spectra

All carrageenan samples, both KOH- and NaOH-treated, were analyzed using FTIR-ATR. However, since they exhibited similar spectral behaviors, only the spectra obtained from the KOH-treated samples are presented and discussed here. Thus, Figure 7 shows the FTIR-ATR spectra of the KOH-treated carrageenan samples within the range of $1400\text{--}600\text{ cm}^{-1}$. The FTIR-ATR spectra of the NaOH-treated carrageenan samples are registered in Figure A.1, in Appendix A.1 for further consultation.

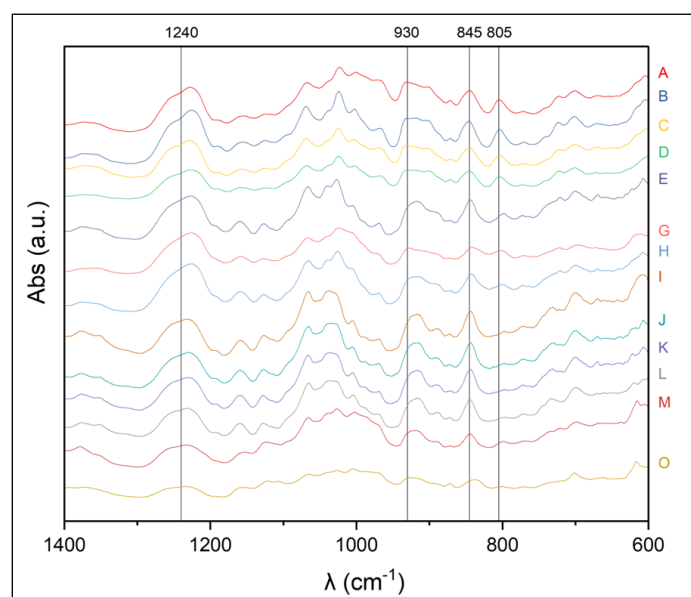


Figure 7: FTIR-ATR spectra of hybrid carrageenan samples treated with KOH, highlighting the most characteristic bands of carrageenans at 1240 , 930 , 845 , and 805 cm^{-1} . *Note: The FTIR-ATR spectra for samples F and N, in KOH, are not shown due to poor resolution.*

The FTIR spectra of the sample films revealed distinct carrageenan profiles. In particular, samples A, B, C and D displayed a more pronounced band at 805 cm^{-1} and a shoulder around 905 cm^{-1} , both associated to 3,6-anhydro-D-galactose-2-sulphate, characteristic of ι -carrageenan disaccharide units.

Conversely, samples I, J, K, L, M and O exhibited a prominent band near 930 cm^{-1} , alongside a shoulder at 1070 cm^{-1} , which are assigned to 3,6-anhydro-D-galactose moieties typically found in ι , but also κ -carrageenan units.

Samples E, G and H presented a spectrum with broader and less well-defined peaks around key wavelengths (*e.g.*, 930 cm^{-1} and 805 cm^{-1}), implying weaker structural contrasts. This profile may correlate with a more balanced or homogeneous distribution of κ - and ι -carrageenan fractions, leading to an overall blending of vibrational modes, in which the distinctive features of pure κ - or ι -carrageenan would be less pronounced. In addition, the FTIR-ATR spectra for samples F and N, in KOH, were not presented due to poor resolution, likely caused by residues or impurities in the film which may have hindered an accurate assessment.

Common to all carrageenan samples, one band appeared at approximately 2920 cm^{-1} on the FTIR spectra (data not shown) assigned to C–H stretching vibrations, corresponding to the total sugar content. Moreover, all samples showed bands at approximately 845 and 1240 cm^{-1} , corresponding to d-galactose-4-sulphate and sulphate ester groups, respectively, which are found across all carrabiose types (κ , ι , μ and ν). However, bands associated with μ - and ν -carrageenan, typically near 820 cm^{-1} , were not identified, suggesting that these types are either present in lower concentrations or their structural features are less detectable under the current conditions.

Ratio of Absorbances at 1370/2920 cm^{-1} – Sulfate-to-Sugar Index

The peak absorbances associated with total sulfate groups at 1240 cm^{-1} and 1370 cm^{-1} can be normalized using the peak corresponding to C-H stretching vibrations at 2920 cm^{-1} , which represents the total sugar content. The resulting ratios, A_{1240}/A_{2920} and A_{1370}/A_{2920} , serve as semi-quantitative indicators of the sulfate content, expressed as the degree of substitution (DS)—the average number of sulfate groups (in moles) per disaccharide unit in carrageenan (Azevedo et al., 2013; Rochas et al., 1986). According to Rochas et al. (1986), the accuracy of sulfate content estimation using these ratios is reliable for polymers with a DS of less than 0.8 for A_{1240}/A_{2920} and more than 0.4 for A_{1370}/A_{2920} , making the latter appropriate for determining sulfate content across a broader range of sulfated galactans. The acuity of the DS determination with the A_{1370}/A_{2920} ratio was recently verified with the FTIR analysis of films made of 4 blends of κ and ι with different ratios, spanning theoretical DS from 1 to 2 whereas the band ratio ranged from 1.3 to 1.9 (de Oliveira et al., 2020).

Theoretically, κ -carrageenan contains, on average, one sulfate group per disaccharide unit, while ι - and μ -carrageenans each contain two sulfate groups, and ν -carrageenan carries three sulfate groups per disaccharide (Rochas et al., 1986; van de Velde, 2008). Assuming that the hybrid carrageenans produced consist only of these four components, each contributing varying sulfate groups, it is expected that the DS sulfate values would ideally be above 1. Therefore, the ratio A1370/A2920 was used as a semi-quantitative index relating total sulfate to sugar content, as represented in Figure 8, for samples treated with KOH and NaOH. As all ratios in Figure 8 are above 2, a DS cannot be extracted from the data using the calibration carried out elsewhere (de Oliveira et al., 2020). Thus, the present analysis of the sulphate content is only qualitative.

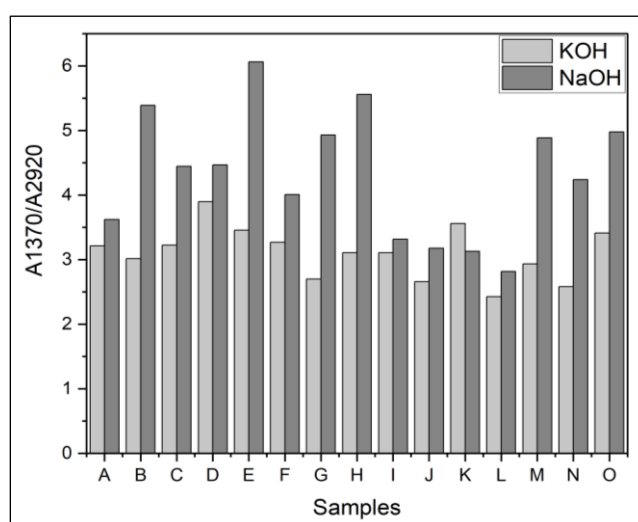


Figure 8: Ratio of absorbance at 1370/2920 cm^{-1} (A1370/A2920), indicative of the total sulfate content in carrageenan samples treated with KOH (light grey) and NaOH (dark grey). *Note: the standard deviation for each ratio falls within the range of 10^{-3} to 10^{-4} . While it has been included in the bar chart, it is not prominently visible due to its relatively small magnitude.*

According to Figure 8, the samples exhibited varying degrees of sulfation, with NaOH-treated samples generally showing higher sulfate-to-sugar ratios compared to KOH-treated ones, especially for samples B, E, G, H, M, N, and O. This suggests that NaOH-treated samples tend to preserve sulfation better than KOH-treated samples, which could impact their gelation, viscosity, and overall performance. Some samples (A, I, J, K, and L) had similar ratios between treatments, indicating less difference in sulfate retention. Samples I, J, K, and L showed consistently lower A1370/A2920 ratios, possibly due to a higher content of less sulfated moieties, such as κ -carrageenan.

Several factors beyond the actual presence of sulfated carrageenan fractions can contribute to higher sulfate content in hybrid carrageenan extracts. Co-extracted materials such as floridean starch, proteins,

or residual sulfate salts (due to the absence of pre-washing) may lead to misleading estimations of sulfate levels (Moses et al., 2015). Additionally, ion pair formation between sulfate groups and counter-ions (Na^+ or K^+) can enhance hydration, alter the vibrational energy, and increase absorbance at 1370 cm^{-1} (Rochas et al., 1986). This interaction could result in a higher A_{1370}/A_{2920} ratio, reflecting ionic influences on FTIR readings.

Ratio of Absorbances at $805/845\text{ cm}^{-1}$ - Relative Amount of ι -carrageenan

Additionally, the absorbance of the 805 cm^{-1} peak (assigned to 3,6-anhydro-D-galactose-2-sulfate) was normalized with respect to the 845 cm^{-1} peak (assigned to D-galactose-4-sulfate), with the A_{805}/A_{845} ratio reflecting the relative amount of ι -carrageenan (Azevedo et al., 2013). The A_{805}/A_{845} ratio is presented in Figure 9, for both KOH and NaOH-treated samples.

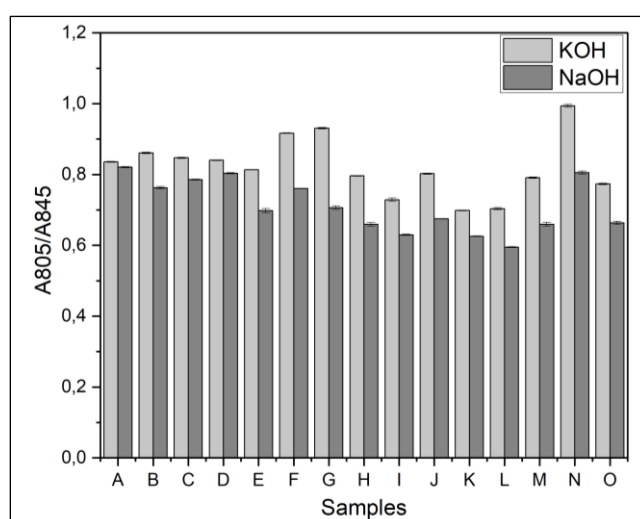


Figure 9: Ratio of absorbance at $805/845\text{ cm}^{-1}$ (A_{805}/A_{845}), indicative of the relative amount of ι -carrageenan in samples treated with KOH (light grey) and NaOH (dark grey). *Note: the standard deviation for each ratio falls within the range of 10^{-3} . While it has been included in the bar chart, it is not prominently visible due to its relatively small magnitude.*

The ratio A_{805}/A_{845} suggests that the NaOH and KOH treatments yielded chemically distinct carrageenans, with KOH-treated carrageenans (light grey) consistently showing a slightly higher relative ι -carrageenan content, especially in samples F, G, H, J, M, N, and O. In both scenarios, sample N presented the highest A_{805}/A_{845} ratio.

A possible explanation to the higher A_{805}/A_{845} ratios observed in KOH-treated samples and, particularly, in sample N could be the presence of higher concentrations of precursor units in the carrageenan chain. The absorption bands between 815 and 825 cm^{-1} , assigned to the sulfate groups on

C₂ and C₆ of galactose found in carrageenans μ and ν , could affect the intensity of the band at 805 cm⁻¹ attributed to the sulfate group on C₂ of 3,6-anhydrogalactose, leading to an overestimation of the relative ι -carrageenan content.

According to Figure 9, in NaOH, samples A, B, C, D, and N exhibited a higher A805/A845 ratio, indicative of greater ι -carrageenan content, followed by samples E, F, G, H, O, and M. Samples I, J, K, and L showed the lowest relative ι -carrageenan content in both alkaline solutions.

4.1.2 ¹H-NMR

FTIR spectroscopy data were confirmed by NMR spectroscopy analysis, as the chemical composition of all samples were analyzed using ¹H-NMR. Figure 10 presents the ¹H-NMR spectra of three chemically representative samples, processed with NMRium® software, along with the chemical shifts corresponding to the anomeric protons of the primary carrabiose units (κ , ι , μ , and ν). The molar fractions (mol.%) of each carrageenan disaccharide repeating unit are shown in Figures 11A and 11B for KOH- and NaOH-treated samples, respectively.

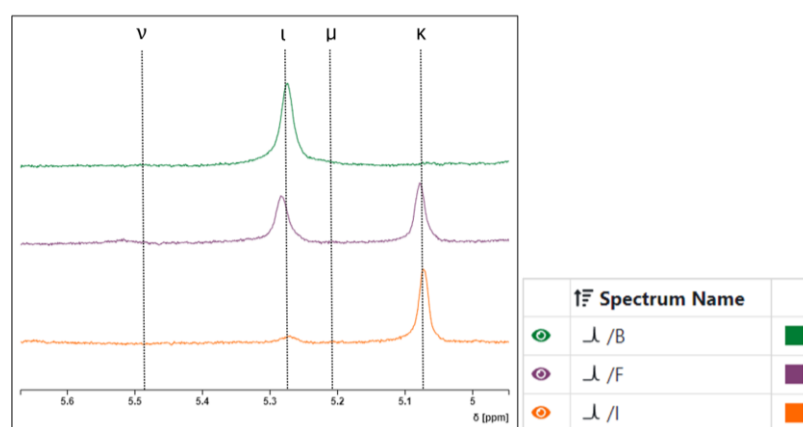


Figure 10: ¹H-NMR spectra of three significantly different KOH-treated samples, in terms of chemical composition, obtained via NMRium® software: sample B, rich in ι -carrageenan; sample F, with a more balanced κ/ι ratio, and sample I, rich in κ -carrageenan. Vertical dotted lines indicate the peaks of the assigned disaccharide units showing up at chemical shifts referenced in Van De Velde et al. (2004).

The ¹H-NMR spectra revealed slight deviations from the predicted chemical shifts for each anomeric proton of the κ , ι , μ , and ν carrabiose units (Table 2), with κ -carrageenan peaks appearing between 5.07 and 5.11 ppm, ι -carrageenan around 5.27 to 5.31 ppm, μ -carrageenan between 5.19 and 5.21 ppm, and ν -carrageenan from 5.46 to 5.51 ppm.

These deviations may be attributed to variations in the chemical environment surrounding the anomeric protons, including proximity to sulfate or carboxyl groups, and interactions between different disaccharide units within the hybrid carrageenan structure (Jouanneau et al., 2010). The slight perturbations in the local magnetic field experienced by these protons can result from such neighboring conditions. Additionally, the presence of residual salts or sample impurities may further contribute to these shifts, differing slightly from pure, commercial carrageenans (Azevedo, Torres, et al., 2015). The solvent (deuterium oxide) and measurement conditions could also play a role in these variations.

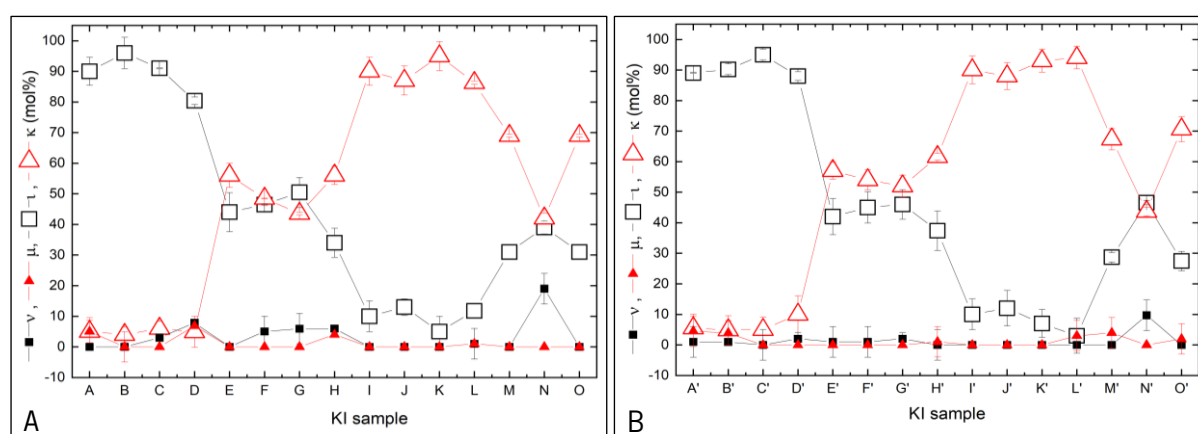


Figure 11: Chemical composition of carrageenan samples treated with **A)** KOH and **B)** NaOH, in terms of molar fraction (mol.%) of each carrageenan disaccharide repeating unit (κ , ι , μ , and ν).

In KOH-treated samples (Figure 11A), A, B, and C had a predominantly ι -carrageenan composition, exceeding 90 mol.% with minimal κ , μ , and ν fractions. Sample D also showed high ι -carrageenan content but contained roughly 15 mol.% of precursors (μ - and ν -carrageenan). In contrast, samples E, F, G, H, M, N, and O exhibited more balanced compositions, with approximately 30–50 mol.% ι -carrageenan, alongside varying levels of precursors, except for samples E, M, and O, which showed negligible precursor fractions. Samples I through L showed a distinct κ -carrageenan dominance, each containing over 87 mol.%, with minimal amounts of ι -carrageenan and no detectable μ - and ν -fractions. In general, the NaOH-treated hybrid carrageenans (Figure 11B) displayed similar compositions to those treated with KOH, but with slightly fewer precursor units (*e.g.* samples D', G' and H').

Sample N (extracted from *Gigartina*) showed significant levels of ν -carrageenan, with 19.0 mol.% for KOH and 9.7 mol.% for NaOH treatments. Additionally, samples D, G, and H in KOH, and sample A' in NaOH, also did not meet the maximum allowed content of precursor units (5 mol.%). On the other hand,

the alkali treatment proved to be particularly effective in samples with high κ -carrageenan content, for both KOH and NaOH solutions.

Comparison of ^1H -NMR and FTIR Results

The ^1H -NMR results showed some notable differences from the A805/A845 ratios obtained via FTIR. While both analyses indicated the chemical diversity of the extracted carrageenans, ^1H -NMR data revealed similar ι -carrageenan compositions for both NaOH and KOH treatments, which contrasts with the FTIR results for some samples. For example, samples F, G, H, M, N, and O, which showed higher A805/A845 ratios in KOH-treated samples (Figure 9), exhibited similar mol.% of ι -carrageenan in both treatments according to ^1H -NMR. Notably, sample N even had a higher ι -carrageenan content in NaOH (46.6 mol.%) compared to KOH (39.0 mol.%), despite the FTIR indicating a higher A805/A845 ratio in KOH.

In addition, while the A1370/A2920 ratio suggested that NaOH-treated had higher sulfate content, the ^1H -NMR analysis revealed that this treatment produced carrageenans with similar or even fewer sulfated precursor units than the KOH-treated samples.

These differences highlight the semi-quantitative nature of FTIR in estimating the composition of complex carrageenans and reinforces its use as a qualitative rather than quantitative tool for assessing κ - to ι -carrageenan ratios in algal extracts (Rochas et al., 1986). The reliability of assessing the relative ι -content using the A805/A845 ratio appears to be limited to samples with either very high or very low mol.% of ι and, crucially, without the presence of precursor units, which can further influence the accuracy of the FTIR analysis.

However, future studies focusing on identifying potential correlations between the relative ι -content assessed through the A805/A845 ratio and the mol.% of ι determined by ^1H -NMR could be highly valuable. This also applies to other ratios, such as A1240/A2920 and/or A1370/A2920 to determine the sulfate content (DS). Since FTIR is a much faster and more practical method for chemical analysis compared to ^1H -NMR, establishing a reliable calibration between these techniques could maximize the utility of FTIR. Such a calibration would provide a quicker insight into the chemical composition of carrageenan samples, addressing FTIR's current limitation in accurately estimating the composition of complex carrageenans on its own.

Comparison with Untreated Carrageenan Samples

A prior study conducted within the E2B2-PHACAR project aimed to characterize the chemical composition of samples A–L, extracted using the same procedure and processing conditions (as described in Section 3.2.1), but without any alkali treatment. The chemical composition, determined via ^1H -NMR using the method detailed in Section 3.3.2, is provided in Figure B.1, Annex B.1. These untreated samples displayed a substantial presence of precursor units, particularly in samples B, C, D, F, G, and H, with ν -carrageenan reaching 19.27 mol.% in sample H. Additionally, all samples exhibited varying amounts of μ -carrageenan, ranging from 3.26 to 11.37 mol.%.

Comparing the untreated samples composition with alkali treated ones, it is possible to conclude a significant decrease in precursor units. In almost all NaOH-treated samples, ν -carrageenan content was reduced to 0 mol.% or near 0 mol.% (*e.g.*, samples C, D, E, etc.). Similarly, μ -carrageenan content was also reduced in most samples to around 5 mol.% or below, with many samples showing no detectable amounts of μ -carrageenan. KOH treatment also reduced precursor content, but not as effectively as NaOH: some samples, such as D, G and H, still retain small amounts of ν -carrageenan (up to 7.84 mol.% in sample D) though this is lower compared to untreated samples. While μ -carrageenan is largely reduced, traces remain in a few samples, notably sample D with 6.86 mol.%.

In general, the alkaline treatment resulted in carrageenans with lower concentrations of the biological precursors μ and ν compared to the respective untreated samples. The removal of these precursor units is directly related to the higher κ - and ι -carrageenan contents observed in the treated samples. The reduction of precursors allows for a purer chemical composition, improving the functional properties of the carrageenan, such as gelling behavior. Furthermore, under the conditions studied ($T = 85\text{ }^\circ\text{C}$, $t = 3\text{ h}$, alkali concentration = 3% w/w), NaOH proved to be slightly more effective than KOH in promoting this conversion, with most samples exhibiting precursor levels close to 0 mol.%.

Potential Interfering Components: Starch and Pyruvic Acid

Floridean starch was detected in significant quantities (10–16 mol.%) in samples E, G, O (from *Chondrus*) and N (from *Gigartina*), across both KOH and NaOH treatments (data not shown). This contaminant, a branched (1–4,1–6)- α -D-glucan, is a water-soluble reserve polysaccharide found in red algae, which can co-extract with carrageenans during the extraction and precipitation processes (Fournet et al., 1999; Van De Velde et al., 2002). In agarophytes, high levels of starch have been linked to lower-quality extracts, as they increase the gravimetric yield and interfere with the gel's mechanical properties

(Chiles et al., 1989; Rincones et al., 1993; Rotem et al., 1986). While the impact of floridean starch on carrageenan yield and gel quality is less documented, it could be reasonable to assume a similar effect due to the comparable nature of the extraction processes. To remove this starch, enzymatic treatments such as amylase digestion or separation techniques targeting acidic and neutral polysaccharides are often employed (Azevedo, Torres, et al., 2015; Song et al., 2022; Van De Velde et al., 2002).

In this study, the presence of starch was generally considered undesirable. However, samples E' and H', treated with NaOH and having similar chemical compositions (60 mol.% κ + 40 mol.% ι ; Figure 11B), were compared in a phase diagram (see Table 6, Section 4.2.1) to assess potential differences in gelling behavior due to starch presence (in sample E'). Although this sampling is not representative, it highlights the need for further studies investigating the potential impact of floridean starch on hybrid carrageenan quality. A study by Azevedo, Torres, et al. (2015) found that starch removal had no significant effect on the gel elasticity of NaOH- and KOH-treated hybrid carrageenans from *C. crispus* and *A. devoniensis*. Future research should examine other hybrid carrageenan systems treated with NaOH and KOH, such as sample O (from *Chondrus*, containing starch) and sample M (from *Mastocarpus*), with 30 mol.% κ + 60 mol.% ι compositions, or samples F and G (with starch) with approximately 50 mol.% κ + 50 mol.% ι compositions.

Conversely, pyruvic acid (with signals showing up at 1.45 ppm), which is a common component of many complex carrageenans, was not detected in any of the samples.

4.1.3 Molecular Mass Distribution

Understanding the molecular weight (Mw) of carrageenans and its distribution provides valuable insights into their macromolecular chain length, which plays a crucial role in determining their gelling properties (Campo et al., 2009). For instance, alkali pre-treatment, when excessive—whether through higher concentrations or prolonged durations—can reduce the molecular mass of hybrid carrageenans, leading to a decline in gel strength (Azevedo et al., 2013; Azevedo, Torres, et al., 2015). It is therefore crucial to assess the molecular mass of hybrid carrageenans extracted to rule out any potential influence on their gelling behavior, ensuring that observed differences in gel strength are not due to variations in molecular mass but rather to other compositional factors such as the content in κ .

For pure K-carrageenan, mechanical properties of gels are highly affected for polysaccharide molecular masses below 3×10^4 g/mol (critical molecular mass, $M_{c\kappa}$). However, gel elastic properties

are independent of Mw, for values above 2×10^5 g/mol (200 kDa) (Rochas et al., 1990; Smidsrød & Grasdalen, 1984; Souza et al., 2011).

The K/I hybrids extracted from the seaweed samples yielded not only varying ι- and κ-carrageenan monomer compositions but also different molecular weights, which also varied according to the alkali treatment employed, as can be seen in Figures 12A and 12B.

The polydispersity index (PDI) serves as a measure of the breadth of the molecular mass distribution; a higher PDI indicates a broader distribution. For example, a monodisperse polymer, where all polymeric chains have the same length, has a PDI of 1 (Shrivastava, 2018). In this study, the PDI of hybrid carrageenan samples was calculated as the ratio of the weight average molecular weight (Mw) to the number average molecular weight (Mn).

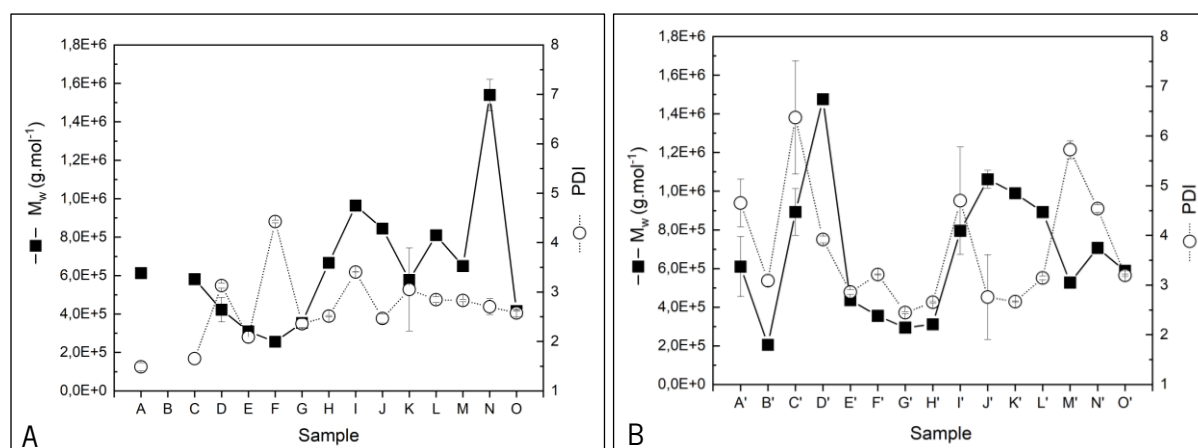


Figure 12: Molecular mass (Mw) and polydispersity index (PDI) of each sample, in **A)** KOH; and **B)** NaOH. Note: Mw and PDI of sample B, in KOH, was not assessed.

For both alkali treatments, the samples exhibited molecular masses exceeding 2.0×10^5 g/mol. Given the lack of literature on the impact of molecular mass in K/I hybrids and drawing from the behavior observed in pure K-carrageenan systems, the influence of molecular mass on the gelling properties of all samples was considered negligible.

Regarding polydispersity, the PDI values were consistently higher and more variable in NaOH-treated samples, implying that NaOH induced a broader distribution of molecular chain lengths compared to KOH. The KOH-treated samples, on the other hand, displayed more consistent and lower PDI values, suggesting that this treatment yields a more uniform polysaccharide chain distribution.

The molecular mass distribution was notably heterogeneous across all samples, showing no clear or linear correlation with either the chemical composition of the hybrid carrageenan (*e.g.*, increasing mol.%

of ι - or κ -carrageenan) or the type of alkali treatment. Even so, some samples, such as C, D, H, K and N, demonstrated higher sensitivity to the type of alkali solution employed, displaying larger variations in molecular mass. However, it is difficult to directly compare the molecular mass between samples with significantly different PDI values (*e.g.*, samples C and N). Even so, it can be observed that samples E, F and G, with balanced κ/ι ratios, consistently presented low Mw values across both treatments.

Comparison with Untreated Carrageenan Samples

The molecular mass distribution of samples A–L, produced without alkali pre-treatment (as previously mentioned in Section 4.1.2), as well as that of commercial I- and K-carrageenans, was also assessed in the aforementioned study, following the same procedure described in Section 3.3.3. The molecular mass (Mw) distribution and polydispersity index (PDI) of the untreated samples are provided in Figure B.2, Annex B.2.

Untreated samples also displayed a heterogeneous distribution, with values ranging from 2.34×10^5 g/mol to 1.45×10^6 g/mol. Across the set, ι -rich (A, B, C and D) and κ -rich (I, J, K and L) samples presented relatively higher molecular masses, closer to the Mw exhibited by commercial carrageenans I and K, of 9.06×10^5 and 1.14×10^6 g/mol, respectively. Conversely, samples E, F, G and H, which contain more balanced κ/ι ratios, presented comparatively lower Mw values. These observations could be linked to the higher content of precursor units (μ - and ν -carrageenans), in carrageenans with a more balanced κ/ι composition (Figure B.1, Annex B.1), which could interrupt the regular polymer chain structure, also leading to shorter polysaccharide chains during alkali treatment (Figure 12). However, this factor alone does not fully explain why untreated samples A–D and I–L, which also contain significant amounts of precursors, still show molecular masses similar to commercial carrageenans.

One plausible explanation could be related to the chain conformation in balanced κ/ι samples. The interaction between κ and ι units, combined with the presence of precursors, may result in weaker interactions between chains or looser network structures, leading to smaller aggregates or shorter polymer chains overall, thus reducing molecular mass. In contrast, κ - and ι -rich systems may form more regular and compact structures, which maintain a higher molecular mass, despite the presence of precursors. This would allow them to retain molecular weights closer to commercial K- or I-carrageenans.

However, this remains a hypothesis, as there is limited literature supporting these observations or exploring the relationship between molecular mass and the chemical composition of hybrid carrageenans. Future studies should aim to investigate this further, including the proposed hypothesis that precursors

in balanced κ/ι systems might weaken inter-chain interactions and loosen network structures. Additionally, other potential factors influencing molecular mass and chemical composition relationships should be explored to provide a more comprehensive understanding of these mechanisms.

Upon comparing the molecular mass data of the untreated samples with those from Figures 12A and 12B, it is evident that alkaline treatment generally led to a reduction in molecular mass, with a few exceptions—specifically, samples G and L in KOH, and C', D', G', and L' in NaOH. Alkaline treatment, particularly at elevated temperatures, can cleave the glycosidic bonds within carrageenan chains. This process, known as alkaline hydrolysis, breaks the polymer into smaller fragments, resulting in lower molecular mass values (Fuchs et al., 2024). The extent of this effect depends heavily on treatment conditions (temperature, pH, and duration), with harsher conditions inducing more significant chain scission, especially in NaOH solutions (Azevedo et al., 2013; Azevedo, Torres, et al., 2015). However, the decrease in molecular mass was more prominent in most KOH-treated samples, with exceptions such as samples H and I, which exhibited lower Mw values in NaOH, and sample A, which showed a similar decrease in both alkaline solutions.

4.2 Hybrid Carrageenans vs Commercial Blends

Selected hybrid carrageenans produced were systematically compared with commercial carrageenan blends of equivalent chemical composition through phase diagrams, in NaCl and KCl, for samples treated with NaOH and KOH, respectively. Subsequently, the rheological properties of selected gels were evaluated using rotational rheometry under small and large deformation.

4.2.1 Phase Diagrams of KI vs K+I in NaCl and KCl

The phase diagrams of the selected hybrid carrageenans (KI), alongside the chemically equivalent commercial blends (K+I) at comparable salt and polysaccharide concentrations, are presented in Table 5 for KCl and in Table 6 for NaCl. These samples were selected based on the criteria outlined in Section 3.4.1, ensuring a broad range of chemical compositions with minimal to no biological precursor content or interfering components, such as floridean starch. As an exception, sample E' (NaOH-treated), was intentionally included to assess potential gelling behavior differences due to starch content, as previously mentioned in Section 4.1.2.

The influence of salt type on the gelling behavior of K- and I-carrageenans is well documented: K-carrageenan is more salt-specific, showing higher gelling efficiency in the presence of K^+ ions. In contrast, I-carrageenan can also gel with Na^+ ions, though the process is slower, producing softer, more elastic gels. Overall, K^+ promotes faster and firmer gelation in K-carrageenan, while Na^+ induces softer, more flexible gels, especially for I-carrageenan (Diener et al., 2019; Piculell, 2006; Schefer et al., 2015). In contrast, the salt dependency of K-2 carrageenan is not as well understood as pure K- or I-carrageenan. Overall, K-2 exhibits intermediate gelling behavior between the two types of carrageenans, showing cation-specific aggregation, especially for K-helices (Souza et al., 2023). However, the range of polysaccharide concentrations and ionic strength for which a gel is produced is very different for each salt (Azevedo et al., 2014; Torres, Azevedo, et al., 2016; Torres et al., 2018).

Therefore, the main purpose of this section is to directly compare the gelling behavior of hybrid carrageenans with their commercial blend counterparts, along a wide range of compositions, in order to determine similarities and differences in how they behave under different salt and carrageenan compositions. Secondly, the comparison between NaOH and KOH systems mainly intends to evaluate how K-2 hybrid carrageenans behave in the presence of different salts: K^+ and Na^+ , since there is still a lack in the literature on this topic.

Overall, the comparison of phase diagrams in the right and left columns confirmed notable differences between K/I-hybrid carrageenans and the commercial K- and I-carrageenan blends (Azevedo et al., 2014; Villanueva et al., 2004). Gel formation (in green) in the presence of K^+ occurred more readily in commercial K+I blends than in KI carrageenans, happening across almost all chemical compositions (in terms of κ and ι content), polysaccharide concentrations, and ionic strengths, whereas KI carrageenans showed higher sensitivity to those parameters.

For ι -rich samples (B and C), gelation was more dependent on the polysaccharide concentration, occurring only at $KI \geq 1$ wt.%, indicating a threshold for forming a stable network. Below this concentration, KI samples formed stable suspensions, suggesting that while the polysaccharide chains are present, they are insufficient to form a fully connected gel. Instead, they may create microdomains or partial networks that do not completely immobilize the solvent, resulting in a suspension state rather than a solid gel. Given the high charge density of ι -carrageenan, this behavior is expected under low K^+ concentrations, where monovalent salts like KCl provide enough ionic shielding but not enough for stable gel network formation (Piculell, 2006). In contrast, all K+I samples formed gels across similar conditions.

Table 5: Phase diagrams of KI (left) and of K+I blends (right), in KCl

Phase Diagram in KCl														
KI Sample (%.mol κ + %.mol ι)	KI (wt%)	[K ⁺] (mol/L)					K+I Sample (%.mol κ + %.mol ι)	K+I (wt%)	[K ⁺] (mol/L)					
		0.01	0.1	0.5	1				0.01	0.1	0.5	1		
B (4 κ + 96 ι)	0.5	SS	SS	SS	SS		10 κ + 90 ι	0.5	CG	CG	CG	TG		
	1	SS	TG	TG	TG			1	CG	CG	TG	TG		
	2	TG	TG	TG	TG			2	CG	CG	TG	TG		
C (6 κ + 91 ι)	0.5	SS	SS	SS	SS				50 κ + 50 ι	0.5	CG	CGS	CG	TS
	1	SS	TG	SS	TG					1	CG	CGS	TG	TG
	2	TG	TG	TG	TG					2	CG	CG	TG	TG
F (48 κ + 47 ι)	0.5	PSS	SS	SS	SS		60 κ + 40 ι	0.5	CG	CGS	TS	TS		
	1	PSS	SS	PSS	TS			1	CG	CGS	TG	TG		
	2	TS	TGS	TGS	TS			2	CG	CGS	TG	TG		
E (56 κ + 44 ι)	0.5	CS	TG	TGS	TS		70 κ + 30 ι	0.5	CG	CGS	TS	TS		
	1	PSS	TGS	TGS	TS			1	CG	CGS	TG	TG		
	2	TGS	TGS	TGS	TS			2	CG	CGS	TG	TG		
M (69 κ + 31 ι)	0.5	SS	TG	CG	TS		90 κ + 10 ι	0.5	CG	CGS	TG	TG		
	1	TGS	TGS	TG	TS			1	CGS	CGS	TG	TG		
	2	TGS	TG	TGS	SS			2	CG	CGS	TGS	TG		
I (90 κ + 10 ι)	0.5	CGS	CGS	SS	SS		90 κ + 10 ι	0.5	CG	CGS	TGS	TS		
	1	TGS	TGS	TGS	SS			1	CGS	CGS	TG	TS		
	2	TGS	TGS	SS	PSS			2	CGS	CG	TG	TG		
J (87 κ + 13 ι)	0.5	CGS	CGS	TS	PSS				90 κ + 10 ι	0.5	CG	CGS	TGS	TS
	1	TGS	TGS	SS	PSS					1	CGS	CGS	TG	TS
	2	TGS	TG	SS	TS					2	CGS	CG	TG	TG
K (95 κ + 5 ι)	0.5	CGS	TGS	TGS	SS				90 κ + 10 ι	0.5	CG	CGS	TGS	TS
	1	TGS	TGS	TS	PSS					1	CGS	CGS	TG	TS
	2	TGS	TGS	TG	PSS					2	CGS	CG	TG	TG
CS	Clear Solution	SS	Stable Suspension	CG	Clear Gel	CGS	Clear Gel with Syneresis							
TS	Turbid Solution	PSS	Phase Separated (settled) Suspension	TG	Turbid Gel	TGS	Turbid Gel with Syneresis							

κ -rich KI samples, particularly sample K (95 mol.% κ), behaved comparably to their commercial counterparts. Generally, for samples with a high κ composition, ionic strength played a more significant role, with gel formation particularly challenged at elevated K⁺ concentrations (≥ 0.5 mol/L for samples I, J, and K, and 1 mol/L for the K+I blend). While potassium ions more effectively induce the helical and aggregated states of K-carrageenan, above the optimal salt concentration solvation and/or salt-out effects may lead to precipitation, compromising gel formation.

Similar high salt sensitivity was observed in samples E and M (60-70 mol.% κ) at K⁺ concentrations of 1 mol/L. These results align with other studies, which reported the formation of stable particle suspensions across all polymer concentrations at [K⁺] = 1 mol/L for KI samples with 75 mol.% κ (Torres, Azevedo, et al., 2016) and 70 mol.% κ (Azevedo et al., 2014). However, samples E and M gelation were also hindered by low ionic strengths and low polysaccharide concentrations. This observation contrasts with the previously mentioned studies, where samples formed clear or turbid gels under similar conditions (Azevedo et al., 2014; Torres, Azevedo, et al., 2016) in harmony with the trend found for κ -rich KI samples.

Sample F (K-2 carrageenan with ≈ 50 mol.% κ and 50 mol.% ι) exhibited the most difficulty in forming gels, which only occurred at high polysaccharide concentrations (2 wt% KI) and ionic strengths of 0.1-0.5 mol/L, in evident contrast to its K+I counterpart.

Interestingly, while sample F (48 mol.% κ + 47 mol.% ι) differs from sample E (56 mol.% κ + 44 mol.% ι) in chemical composition, the extent of this difference is comparable to that between samples E and M (69 mol.% κ + 31 mol.% ι), yet the latter two exhibit similar gelling behaviors. This suggests that factors beyond κ and ι composition are influencing gelation. One significant factor could be the high polydispersity index (PDI) of sample F, coupled with its average molecular mass being close to the lower limit (Figure 12A), potentially resulting in a substantial proportion of chains too short to form gels. This could also account for the differences observed with its commercial blend counterpart, as commercial K- and I-carrageenans have significantly higher molecular masses (Figure B.2 – Annex B.2). Additionally, the relatively higher content of precursor units (≈ 5 mol.%) in sample F (Figure 11A) may hinder its ability to establish the robust network structures necessary for gelation under broader salt and polysaccharide conditions.

Torres, Azevedo, et al. (2016) reported a K-2 carrageenan with a similar chemical composition to sample F (53 mol.% κ and 47 mol.% ι), extracted from *A. devoniensis*, that formed gels without syneresis for all conditions, except for 0.5-1 KI wt.%, at 0.01 mol/ [K⁺] which yielded turbid solutions. These differences suggest that intrinsic species-specific factors, such as K-2 chain length distributions and block length variability, also influence the overall gelling performance of extracted KI carrageenans, as the molecular masses of sample F and extract from *A. devoniensis* are both in the lower limit.

This inherent variability, common to natural products, complicates straightforward correlations between K-2 hybrids' gelling properties and their κ or ι fractions. Exploring diverse algae sources could help clarify this variability and establish a clearer κ content threshold, above which KI hybrids behave more like commercial mixtures in terms of gelling ability. However, it is worth noting that both commercial carrageenans exhibit higher molecular masses than sample F, further complicating direct comparisons.

Syneresis (depicted in light and dark fluorescent green) increased with higher κ -content (unnoticed for $\kappa < 50$ mol.%), especially in KI samples, where water separation occurred in a great part of gels. In commercial blends, syneresis was more common at 0.1 mol/L K⁺ for samples with ≥ 50 mol.% κ . These findings did not entirely align with a study by Azevedo et al. (2014), who observed no syneresis in KI gels with 70 mol.% κ in KCl, while K+I gels exhibited water release at higher K⁺ concentrations. In this study, syneresis occurred in both KI and K+I gels at [K⁺] < 1 mol/L. Both studies agree on the difficulty of gel

formation in κ -rich KI systems at 1 mol/L KCl. The discrepancies may result from differences in seaweed sampling (location and harvest time) and treatment methodology, as Azevedo et al. (2014) applied alkali treatment directly to seaweed pre-extraction, whereas this study treated the extracted carrageenan. This suggests that possible different Mw distributions, as well as κ and ι distributions along the polysaccharide chain, give different gel syneresis, whereas the chemical structure is essentially identical in terms of κ and ι contents. Note also that differences in the phase diagrams of K+I gels also point to possible differences in the experimental protocols employed (in particular the thermal history during cooling), in the assessment of phases (subjected to experimentalists criteria) or the K and I used, as commercial samples were sourced from the same provider.

Clear gels (with or without syneresis) were more frequently observed in the commercial blend systems, particularly at low ionic strengths ($[K^+] \leq 0.1$ mol/L). This observation aligns with findings by Azevedo et al. (2014), who linked the transition from clear to turbid gels to changes in gel structure and a shift in the concentration dependence of critical strain for gel break up. In contrast, nearly all KI samples showed some degree of turbidity, with clear solutions appearing only occasionally at lower polysaccharide concentrations. The inherent tendency of KI to exhibit turbidity likely results from several factors: KI samples were extracted from unprocessed and unwashed algae, allowing for possible co-extraction of soluble and insoluble impurities that contribute to turbidity, whereas the commercial carrageenans in K+I blends were obtained in a highly processed, purified state. Leaving aside the optical role of impurities, the turbidity suggests that KI samples tend to yield more heterogeneous structures, potentially forming larger, irregular microdomains compared to commercial blends. This structural heterogeneity increases light scattering and turbidity, especially at higher polymer concentrations.

Similarly to what was seen in KCl, in the presence of Na^+ hybrid carrageenans also demonstrated a more complex and variable gelling behavior compared to the commercial blends (Table 6). In NaCl, gel formation for K+I samples was mostly conditioned by low polysaccharide concentrations, not showing much dependence on the ionic strength (contrarily to what is seen in KCl). Notably, commercial blends in NaCl produced clearer gels and solutions compared to KCl.

As expected, due to the non-specific ion response of ι -carrageenan, ι -rich samples (C' and D') demonstrated consistent gelling behaviors in both Na^+ and K^+ . In contrast, κ -rich samples (I', J' and K') required higher polysaccharide concentrations to form gels in NaCl than in KCl. Notably, for K-2 carrageenans (samples F', E', H' and M') with ≈ 50 -70 mol.% κ , no gels formed under any condition,

contrasting starkly with K+I counterparts. The study by Torres, Azevedo, et al. (2016), though aligning with the notable gelling difficulty of hybrid carrageenans with 50-50 mol.% and 75-25 mol.% of κ - ι , stated the formation of gels at 1-2 wt.% of polysaccharide and 0.5-1 mol/L $[\text{Na}^+]$. Similarly, Azevedo et al. (2014) documented these results for 70-30 mol.% κ - ι hybrids.

Table 6: Phase diagrams of KI (left) and of K+I blends (right), in NaCl

Phase Diagram in NaCl																	
KI Sample (%.mol κ + %.mol ι)	KI (wt%)	[Na ⁺] (mol/L)					K+I Sample (%.mol κ + %.mol ι)	K+I (wt%)	[Na ⁺] (mol/L)								
		0.01	0.1	0.5	1				0.01	0.1	0.5	1					
C' (5 κ + 95 ι)	0.5	SS	CS	PSS	SS	10 κ + 90 ι	0.5	CG	CG	CG	CG						
	1	PSS	TG	TG	TG			1	CG	CG	CG	TG					
	2	TG	TG	TG	TG				2	CG	CG	CG	CG				
D' (10 κ + 88 ι)	0.5	SS	SS	SS	SS	50 κ + 50 ι	0.5			CS	CS	CS	CS				
	1	PSS	PSS	SS	SS			1		CG	CG	CG	CG				
	2	PSS	PSS	TS	TS				2	CG	CG	CG	TG				
E' (57 κ + 42 ι)	0.5	PSS	PSS	PSS	PSS	60 κ + 40 ι	0.5			CS	CS	CG	CS				
	1	PSS	PSS	PSS	TS			1		CG	CG	CG	CG				
	2	PSS	PSS	PSS	TS				2	CG	CG	CG	TG				
H' (62 κ + 37 ι)	0.5	CS	SS	PSS	TS	70 κ + 30 ι	0.5			CS	CGS	CG	CGS				
	1	SS	SS	PSS	TS			1		CG	CGS	CGS	TGS				
	2	SS	SS	TS	TS				2	CG	CGS	CG	TGS				
I' (90 κ + 10 ι)	0.5	CS	PSS	TS	TS	90 κ + 10 ι	0.5			CS	CS	CS	CS				
	1	SS	TG	TGS	TS			1		CG	CG	CG	TG				
	2	TG	TG	TGS	TGS				2	CG	CG	CG	TG				
J' (88 κ + 12 ι)	0.5	PSS	SS	PSS	SS	93 κ + 7 ι	0.5			CS	CS	CS	CS				
	1	PSS	SS	PSS	PSS			1		CG	CG	CG	TG				
	2	TG	TG	TG	TGS				2	CG	CG	CG	TG				
K' (93 κ + 7 ι)	0.5	PSS	PSS	PSS	PSS					0.5	CS	CS	CS	CS			
	1	PSS	TG	TGS	PSS			1							CG	CG	CG
	2	TG	TG	TG	TGS				2								
CS	Clear Solution	SS	Stable Suspension	CG	Clear Gel	CGS	Clear Gel with Syneresis										
TS	Turbid Solution	PSS	Phase Separated (settled) Suspension	TG	Turbid Gel	TGS	Turbid Gel with Syneresis										

For samples E' (containing starch) and H', both with similar κ and ι fractions, the phase diagram analysis revealed no significant difference, suggesting that starch presence did not have a noticeable impact. However, due to the inherent gelling difficulty of K-2 hybrids in NaCl, it is challenging to definitively conclude that starch has no effect on gelling properties.

Interestingly, some κ -rich gels registered the occurrence of syneresis in NaCl, which is not commonly reported in the literature. This happened specifically in the K+I blend with 70 %.mol κ , and for samples I', J' and K', at higher polymer and Na^+ concentrations. Although potassium ions are known to encourage syneresis in K-carrageenan gels due to their strong binding affinity, sodium ions generally have weaker interactions, so syneresis is less likely. Notably, in the case of the K+I blend with 70 mol.% κ , the results

are even more unusual, as other studies using the same experimental protocol and source of commercial carrageenans did not report any syneresis (Azevedo et al., 2014; Torres, Azevedo, et al., 2016). This further supports the possibility of user-related experimental errors, although none could be specifically identified. One potential explanation could be that, during cooling, water vapor from the gel or solution may condense on the cooler walls of the flask, forming a liquid film that could be mistakenly identified as syneresis.

To summarize this section, phase diagrams highlighted key differences between K/I hybrid carrageenans (KI) and commercial blends (K+I). KI carrageenans showed greater dependency on polysaccharide concentration and ionic strength for gel formation compared to K+I. Nonetheless, hybrid carrageenans exhibited gelation behaviors similar to commercial blends under specific conditions. These included higher polysaccharide concentrations for ι -rich samples in both KCl and NaCl, balanced κ/ι samples in KCl, and κ -rich samples in NaCl, as well as lower ionic strengths for κ -rich samples in KCl. However, hybrid carrageenans consistently formed more turbid gels. In comparing KI samples, KCl facilitated more consistent gel formation across concentrations, while NaCl resulted in diverse gelling behaviors, including more suspensions and phase separations.

Samples rich in κ -carrageenan showed more specificity towards KCl, whereas ι -rich samples presented similar phase diagrams in NaCl and KCl. K-2 carrageenans displayed salt-specific responses, forming gels in KCl but not in NaCl, underscoring their limited gelling potential in Na^+ environments, consistent with Azevedo et al. (2014).

In conclusion, KI carrageenans may serve as viable alternatives to K+I under specific polysaccharide concentrations and ionic strengths conducive to gel formation in both. However, rheological testing is essential to evaluate their comparative elasticity, modulus, and yield stress for optimal application. Additionally, KI may find unique value as a stabilizer in non-gelling applications, such as sauces, dressings, or emulsions, where gel-like networks aren't essential but water-binding and stable suspension properties are advantageous.

4.2.2 Rheological Characterization of Hydrogels

In the rheological assessment, specific samples were selected to provide insight into gel behavior across varying conditions, given limited time for analysis. Gels from samples C, E, and K in KOH and C' and K' in NaOH (sample E' did not form gels) were chosen, as these differed notably in κ - and ι -carrageenan

content, allowing the investigation of rheological responses tied to ι -content. This selection also facilitated the evaluation of how polysaccharide concentration and ionic strength influence rheology since these samples formed gels under a broader range of conditions, enabling direct comparisons within each sample. Therefore, three different polysaccharide concentration-ionic strength conditions were chosen for each sample, to allow for this comparison.

Since sample M's rheology has been extensively studied under similar conditions, as a carrageenan derived from *Mastocarpus* (Azevedo et al., 2014, 2022; Moreira et al., 2016; Torres, Chenlo, et al., 2016), the rheological characterization of sample E was preferred here. It gives a unique opportunity to explore less-documented carrageenan types, adding depth to the rheological findings, in particular in a composition of κ and ι close to 50 mol.%, which has been much studied in blends (Bui et al., 2019; Ridout et al., 1996).

Thermal-rheological Properties of Samples in KCl and NaCl Solutions

Each sample was subjected to a time sweep as hot solutions cooled from 85 °C to 25 °C to monitor the sol-gel transition and assess gelation behavior. This approach allows a detailed examination of the evolving viscoelastic properties (G' and G'') during cooling, highlighting gelation temperature (T_g) as the point where G' (storage modulus) crosses G'' (loss modulus), marking the shift from a viscous to a gel-like state. The temperature-dependence graphs of the viscoelastic moduli (G' and G'') for selected samples are presented in Figure 13, whereas the cooling curves for all other samples in KCl and NaCl are provided in Figures A.2 and A.3 of Appendix A.2.

Notably, different behaviors were observed among the samples. For instance, in the 1 wt.% K+I (10 κ 90 ι) sample in 1M KCl (Figure 13A), G' and G'' intersected, allowing for the identification of T_g . In contrast, other samples, such as 2 wt.% KI (sample E) in 0.5M KCl (Figure 13B), maintained a gel state ($G' > G''$) throughout the cooling process, suggesting that T_g occurs above 85 °C. In all cases, the start of gelation (T_{start}), marked by a steep increase in G' , was recorded. This approach enables comparison between samples, even when there is no clear crossover point.

A third behavior, involving a two-step gelation mechanism was also observed in samples, such as the 2 wt.% KI (sample C) in 1M KCl (Figure 13C). The latter is described as a two-step increase in the viscoelastic moduli, corresponding to independent coil-to-helix transitions of the K and I blocks at distinct temperatures (T_{start1} and T_{start2}). This type of gelling behavior has been reported for K+I in the presence of various salts and ionic strengths (Brenner et al., 2014; Bui et al., 2019; Du et al., 2016; Geonzon et al.,

2019; Hu et al., 2016; Parker et al., 1993; Piculell et al., 1992), but also for K-2 hybrid carrageenans, particularly in the presence of Na^+ salts (Hilliou et al., 2014; Hilliou & Gonçalves, 2007; Souza et al., 2011; Torres et al., 2017; van de Velde et al., 2005). Some studies also reported that the step-increase temperatures in moduli for K-2 hybrid carrageenans (Souza et al., 2011, 2023), as well as the temperatures for the coil-to-helix transitions of each blocks (Souza et al., 2011; van de Velde et al., 2005) match those of K+I blends with similar molar compositions, as well as isolated K or I solutions at equivalent concentrations, suggesting that conformational transitions and helical aggregation remain unaffected by the presence of the other component in the blend (Bui et al., 2019; Du et al., 2016; Parker et al., 1993; Souza et al., 2023; van de Velde et al., 2005).

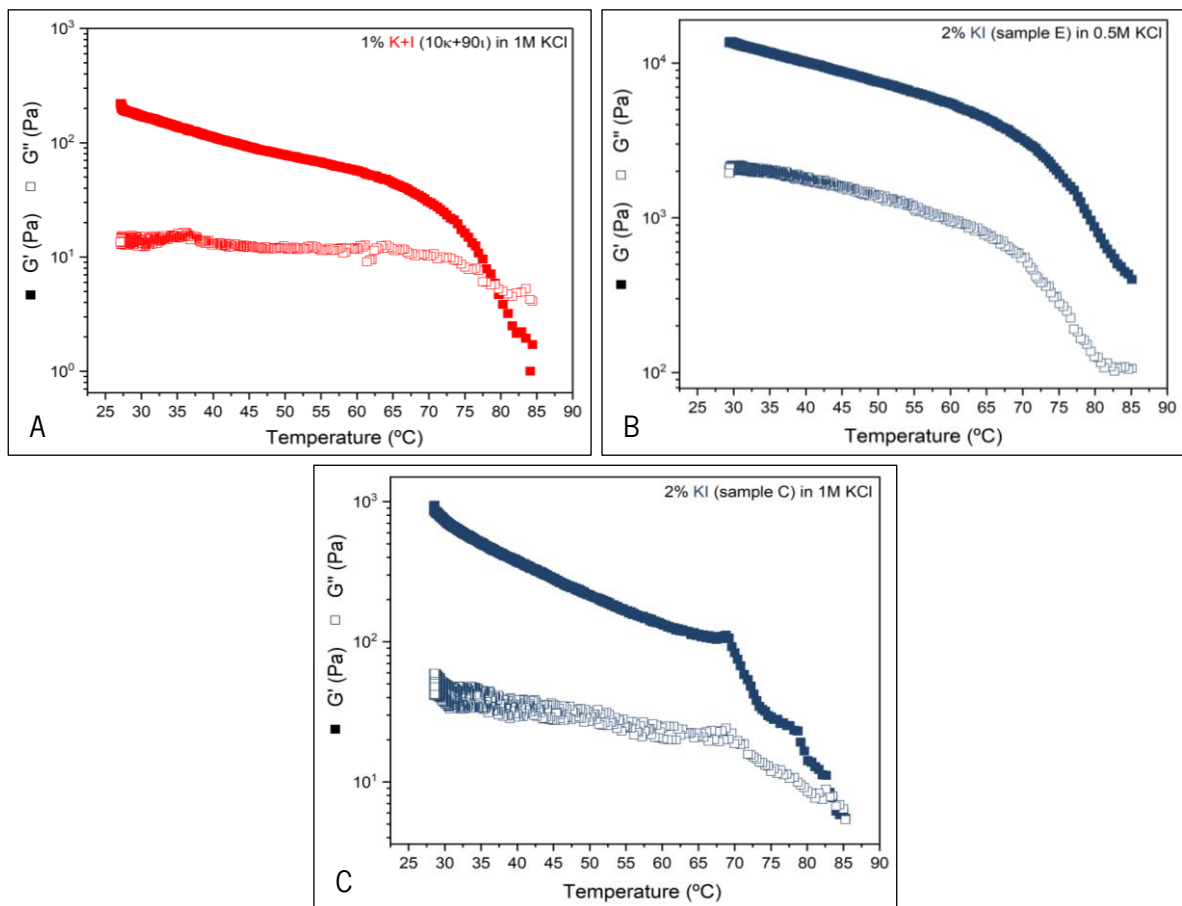


Figure 13: Temperature-dependent viscoelastic moduli (G' and G'') of selected carrageenan samples illustrating different gelation behaviors: **(A)** Gelation temperature (T_g) in 1 wt.% K+I (10 κ 90 ι) in 1 M KCl, where G' and G'' intersect; **(B)** Absence of T_g for 2 wt.% KI (sample E) in 0.5 M KCl, indicating a gel state maintained throughout cooling; **(C)** Two-step gelling mechanism observed in 2 wt.% KI (sample C) in 1 M KCl, associated with sequential coil-to-helix transitions in K and I blocks.

The values of T_g , T_{start1} and T_{start2} , along with their associated errors for each sample, are presented in Table 7 for both KCl and NaCl, as well as a characterization of the gelation mechanism (single-step or two-step process).

Table 7: Values of T_g , T_{start1} and T_{start2} , along with their associated errors for each sample, for both KCl and NaCl, as well as a characterization of the gelation mechanism (single-step or two-step process)

	Experiment	Sample	T _g (°C)	T _{start1} (°C)	T _{start2} (°C)	Gelling Mechanism
Samples in KCl	2 wt% in 0.01 M KCl	Sample C	43.6 ± 0.2	51.0 ± 1.0	31.7 ± 0.1	Two-step
		10κ90ι	47.6 ± 0.3	61.0 ± 2.0	34.0 ± 0.5	Two-step
	1 wt% in 1 M KCl	Sample C	-*	74 ± 1.0	60.0 ± 2.0	Two-step
		10κ90ι	79.4 ± 0.8	81.0 ± 1.0	46.0 ± 3.0	Two-step
	2 wt% in 1 M KCl	Sample C	85.4 ± 4.3	> 85	74.0 ± 0.5	Two-step
		10κ90ι	> 85	> 85	-	Single-step
	2 wt% in 0.01 M KCl	Sample E	66.1 ± 0.2	67.0 ± 0.3	54.0 ± 1.0	Two-step
		60κ40ι	47.5 ± 0.4	53.0 ± 1.0	39.5 ± 0.5	Two-step
	1 wt% in 0.5 M KCl	Sample E	> 85	> 85	-	Single-step
		60κ40ι	> 85	> 85	-	Single-step
	2 wt% in 0.5 M KCl	Sample E	> 85	> 85	-	Single-step
		60κ40ι	> 85	> 85	-	Single-step
	0.5 wt% in 0.01 M KCl	Sample K	-*	43.5 ± 1.5	-	Single-step
		90κ10ι	-*	32.0 ± 1.0	-	Single-step
	0.5 wt% in 0.5 M KCl	Sample K	> 85	> 85	-	Single-step
		90κ10ι	> 85	> 85	-	Single-step
2 wt% in 0.5 M KCl	Sample K	> 85	> 85	-	Single-step	
	90κ10ι	> 85	> 85	-	Single-step	
Samples in NaCl	2 wt% in 0.01 M NaCl	Sample C'	30.7 ± 0.5	38.0 ± 0.5	29.3 ± 0.1	Two-step
		10κ90ι	52.0 ± 1.5	55.5 ± 1.0	30.0 ± 1.0	Two-step
	1 wt% in 1 M NaCl	Sample C'	83.9 ± 3.4	> 85	-	Single-step
		10κ90ι	55.6 ± 0.3	58.3 ± 0.3	-	Single-step
	2 wt% in 1 M NaCl	Sample C'	< 25	25.0 ± 1.0	-	Single-step
		10κ90ι	> 85	> 85	62.0 ± 3.0	Two-step
	2 wt% in 0.01 M NaCl	Sample K'	39.4 ± 0.4	40.5 ± 0.5	-	Single-step
		90κ10ι	-*	44.5 ± 0.5	-	Single-step
	1 wt% in 0.5 M NaCl	Sample K'	-*	53.0 ± 1.0	-	Single-step
		90κ10ι	-*	46.0 ± 0.3	-	Single-step
	2 wt% in 0.5 M NaCl	Sample K'	-*	50.0 ± 1.0	-	Single-step
		90κ10ι	-*	54.7 ± 0.3	-	Single-step

*For these samples, the crossover point T_g was not detectable due to the equipment's limited sensitivity at the deformation level used in the measurement. While this small deformation setting was insufficient to identify T_g , it was chosen to prevent sample disruption and avoid interference with the gelation process. As a result, only the initial gelation temperature T_{start1} is presented for these samples.

Hybrid carrageenans and their chemically equivalent commercial blends exhibited broadly similar rheological cooling curves under identical polysaccharide concentrations and ionic conditions, particularly in KCl. However, significant variations in gelling or onset temperatures were observed, with discrepancies exceeding 20 °C in some cases (*e.g.*, ι -rich samples in NaCl). Such differences could be critical for specific applications requiring precise gelation control. Nonetheless, in certain systems, experimental limitations—such as strain sensitivity and restricted time or temperature ranges—may have obscured subtle transition and conditioned a detailed and reliable comparison in some systems. Future studies could employ advanced rheological techniques, extended cooling protocols, and complementary in-rheometer methods such as SAXS (Small-Angle X-ray Scattering), to further elucidate gelation mechanisms, particularly in mixed κ/ι systems.

These specific findings align with broader trends observed across the study, which highlight the key roles of molecular composition and ionic environment in determining gelation behavior. In general, higher ι -carrageenan content, lower ionic strengths, and the presence of K^+ ions were identified as key factors contributing to the formation of two-step gelling mechanisms. In contrast, higher ionic strengths and higher κ -content resulted in single-step gelation, either due to co-aggregation of K- and I-helices or because the second component of gelation could not be measured, as its corresponding elasticity was too weak compared to the first. Higher ionic strengths also promoted an earlier start of gelation (at higher temperatures), particularly in KCl. These behaviors underscore the interplay of molecular composition and salt conditions in shaping gelation kinetics.

To deepen the understanding of the gelation mechanisms and kinetics observed in this study, a detailed discussion of each set of samples—grouped by their chemical composition—was conducted, focusing on the interplay between carrageenan composition, ionic environment, and thermal gelation behavior. It is important to note that I-carrageenan typically has a higher T_g than K-carrageenan (Hilliou & Gonçalves, 2007), though the difference in T_g depends on the salt type and concentration (Rinaudo & Rochas, 1981). At higher salt concentrations, this difference in T_g is reduced. Rheologically, the formation of helices and subsequent aggregation can only be observed if they significantly increase elasticity or viscosity. Otherwise, the elasticity of K-carrageenan may dominate, obscuring the contribution from I-carrageenan helix formation.

Additionally, the literature on commercial carrageenan mixtures remains inconclusive, with some studies suggesting independent and separate gelation of the K- and I-fractions, while others propose co-

aggregation of helices. This lack of consensus further underscores the complexity of interpreting gelation behavior in these systems.

In KCl solutions, both ι -rich hybrid carrageenan samples (sample C) and their corresponding commercial blends (10 κ 90 ι) displayed two-step gelling mechanisms, with each carrageenan fraction (K and I) gelling independently at different temperatures. Despite their high ι -content ($\approx$ 90 mol.%), their gelling behavior deviated significantly from the monotonic increase in G' and G'' typically observed in pure I-carrageenan (Bui et al., 2019). This suggests that even small κ fractions ($\approx$ 10 mol.%) can form microdomains or secondary aggregates that reinforce the gel network and contribute to a second gelation step. Previous studies (Parker et al., 1993; Ridout et al., 1996) have reported the formation of phase-separated and/or interpenetrating networks in mixtures of pure K- and I-carrageenans, highlighting that κ fractions as low as 2.5% can significantly influence their rheological behavior. Additionally, residual κ content in commercial I-carrageenan ($\approx$ 10%) likely amplifies its contribution to network formation in the 10 κ 90 ι blend, raising the actual κ content to $\approx$ 18.5% (Piculell et al., 1992). This higher effective κ fraction may explain the formation of a secondary gelation phase in these ι -rich systems.

In this set of samples, increasing the salt concentration (from 0.01 to 1 M) resulted in a substantial rise in both the gelling and onset temperatures, exceeding 35 °C. Although higher polysaccharide concentrations also contributed to elevated temperatures, their impact appeared less pronounced and was more difficult to evaluate due to the limited temperature range. However, while ι -rich hybrid samples and commercial blends presented similar thermal-rheological parameters and gelling mechanisms, through Figures A.2 (A, B and C) from Appendix A.2.1, it is possible to observe clear differences between their cooling kinetics.

In NaCl, however, two-step gelation in ι -rich samples was only observed at low ionic strengths, transitioning to a single-step mechanism at 1 M NaCl. Particularly in these set of samples, the cooling of hybrids and commercial blends differed significantly (Figure A.3, Appendix A.2). At 0.01 M and 2 wt.% carrageenan, the commercial blend 10 κ 90 ι exhibited a more gradual and well-defined two-step process, while sample C' displayed rapid network formation, exhibiting two sharp increases in G' between 40 - 29 °C, going from $\approx$ 0.1 Pa to nearly 10^3 Pa, within this narrow temperature range. At 1 M and 1 wt.%, sample C' presented a single step gelling mechanism characterized by a slow and minimal increase in G' , while the commercial blend presented a sharp and distinct gelation transition, achieving a significantly higher gel elasticity. Interestingly, at 1 M NaCl and 2 wt.% polysaccharide, sample C' failed to gel within the

defined temperature range despite displaying gel-like behavior in the phase diagram (Table 6), which points towards the role of the cooling history on the resulting gel structure and elasticity.

In samples with balanced κ/ι ratios (*e.g.*, Sample E and commercial 60 κ 40 ι blends), the gelation process transitioned from a two-step mechanism at low K^+ concentrations to a single-step process at higher salt concentrations. Bui et al. (2019) suggested that the shift to a single gelation process at higher ionic strength relates to the easier co-aggregation of K- and I-helices, promoting an integrated network that forms at temperatures typically associated with I-gelation (Bui et al., 2019; Parker et al., 1993). Notably, the copolymeric nature of hybrid carrageenans may encourage simultaneous K/I helix aggregation, resulting in mixed network formation. However, one cannot exclude the separate association of K and I blocks in physically connected networks. At lower K^+ concentrations, the addition of the two elasticities is measured during cooling as the different blocks begin to aggregate at very different temperatures. However, at higher K^+ concentrations, the greater elasticity of the K-block network smears out the contributions of the I network as it starts to form at temperatures closer to those of the I network (Piculell, 2006; Rinaudo & Rochas, 1981).

However, these findings deviate from the expected behavior for commercial blends, where independent gelation of K and I fractions was anticipated (Bui et al., 2019; Rochas et al., 1989; Souza et al., 2023). This discrepancy could indicate co-aggregation of helices in commercial mixtures, resulting in a single network where the formation of helices occurs simultaneously or is thermodynamically coupled, eliminating separate gelation events. Alternatively, experimental limitations, such as strain sensitivity, instrument resolution, or restricted temperature ranges, may obscure distinct two-step gelation signatures (*e.g.*, by overlapping thermal transitions under certain conditions). Future studies using extended time frames, higher temperature ranges ($> 90\text{ }^{\circ}\text{C}$), and optimized rheometer settings could help clarify these transitions in mixed κ/ι systems.

All κ -rich samples, in both KCl and NaCl solutions, exhibited single-step gelation mechanisms, with commercial blends achieving higher G' values overall. The absence of a clear two-step mechanism in κ -rich systems, opposed to what was observed in ι -rich ones, may reflect the dominance of K-helices in forming a unified gel network. Furthermore, in NaCl, gelation onset appeared nearly independent of ionic strength and occurred at relatively lower temperatures, between $40\text{ }^{\circ}\text{C}$ and $55\text{ }^{\circ}\text{C}$.

Mechanical Spectra of Samples in KCl and NaCl Solutions

In evaluating the mechanical spectra of gels, a frequency range devoid of experimental artifacts was selected. At higher frequencies, increased nonlinearity of the equipment and sample inertia effects appeared to influence the results, so this range was excluded to ensure accuracy. Furthermore, my discussion of the frequency sweep will remain qualitative, as some gels were still forming their structures at lower frequencies (evidenced by a slight increase in G' with decreasing frequency), potentially impacting the accuracy of quantitative analysis. The slow gel forming kinetics was also noted in the temperature sweep of some samples, showing increasing G' values at lower temperatures (see, for example, Figure 13A and 13C, and Figures A.2 and A.3, in Appendix A.2). Therefore, quantitative assessment of the (linear) elastic modulus (G_0) of the gels will only be presented in the strain sweep discussion, as the extended timeframe of the preceding sweeps (temperature sweep: ≈ 1.5 hours; frequency sweep: ≈ 20 minutes) allowed the samples sufficient time to develop fully structured, three-dimensional networks.

The mechanical spectra yield valuable insights into the structural characterization of materials. In gel-like systems, G' is typically greater than G'' over a wide frequency range. Analyzing the gap between G' and G'' can indicate the stability of the network structure: wider gaps between the moduli are indicative of a strong and stable network structure with primarily elastic properties (if $G' > G''$), whereas closer values show elastically weaker structural characteristics. In some cases, G' and G'' might intersect at a particular frequency. This cross-over point can signify a relaxation process in the material's structural dynamics, such as the reptation of polymer chains from the elastic network of neighboring chains. Beyond this point, if G' becomes larger than G'' , the material becomes predominantly solid-like; if G'' becomes larger, it indicates predominantly liquid-like behavior (Castro, 2004; Mezger, 2006). Thus, indicating the viscoelastic character of the gel.

In KCl, all KI samples and respective K+I counterparts presented a solid-like mechanical spectra, with $G' > G''$, throughout all the considered range of frequency. For simplification, only three mechanical spectra are shown (one for each sample vs. blend system), in Figure 14. All the remaining mechanical spectra were registered in Figure A.4 – Appendix A.3, for further consultation.

In some samples, such as experiment 2 wt.% KI (sample K) in 0.5 M KCl (Figure 14C) and experiment 2 wt.% K+I (blend 10κ90t) in 0.01M KCl (Figure A.4, Appendix A.3), it was possible to notice a slight increase of G'' approaching G' , particularly at higher frequencies, suggesting that the material's elastic network begins to exhibit a relaxation process under rapid oscillation, transitioning towards a viscous or fluid-like behavior. This is typical in weaker or more loosely crosslinked gels, with less stable network

structures, showing a reduced capacity to store elastic energy (Castro, 2004; Mezger, 2006). However, it is difficult to ascertain whether this behavior genuinely reflects weaker network structures or if it may be influenced by experimental artifacts related to the equipment's limitations at higher frequencies.

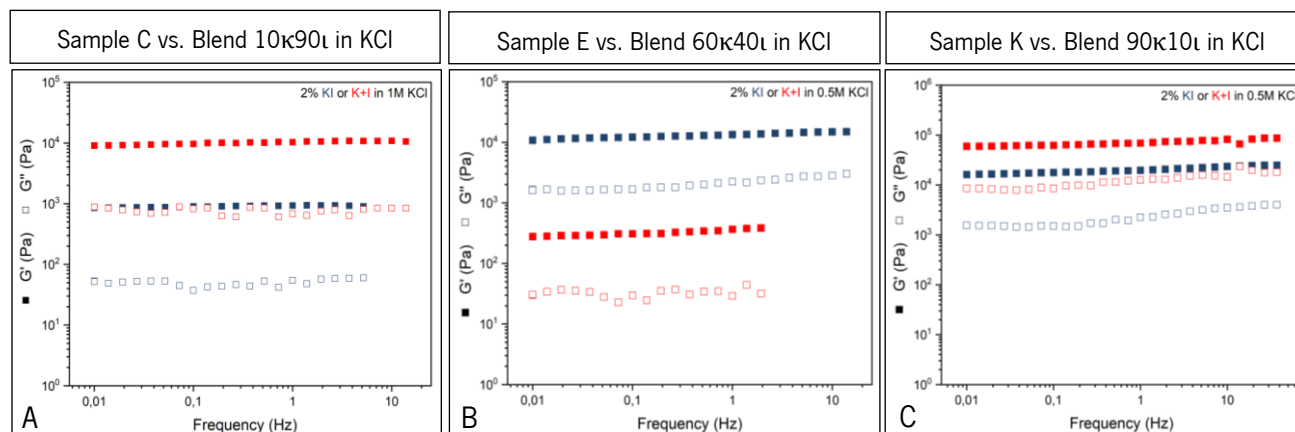


Figure 14: Mechanical spectra (storage modulus G' , full squares; loss modulus G'' , open squares) of hybrid carrageenan (KI) samples (in blue) compared to their respective commercial K+I carrageenan blends (in red) at different polymer concentrations and ionic strengths in KCl solutions: **A)** Experiment 2 wt.% KI or K+I in 1 M KCl, for Sample C and Blend 10κ90ι; **B)** Experiment 2 wt.% KI or K+I in 0.5 M KCl, for Sample E and Blend 60κ40ι; and **C)** Experiment 2 wt.% KI or K+I in 0.5 M KCl, for Sample K and Blend 90κ10ι.

All hybrid carrageenans and commercial blends exhibited increased elasticity with higher polysaccharide concentrations, as evidenced by the larger difference between the storage and loss moduli. This indicates that higher polysaccharide concentrations promoted stronger and more robust networks, which store more energy elastically rather than dissipating it. This effect was particularly pronounced in ι-rich and κ-rich samples.

The effect of increasing salt concentration, however, varied across samples. For hybrid samples C and K, as well as the commercial blend 60κ40ι, higher K^+ concentrations compromised network stability, as shown by a decrease in G' dominance, with the most significant effect observed in the 60κ40ι blend. Conversely, their counterparts (sample E and blends 10κ90ι and 90κ10ι) benefited from higher salt concentrations, showing wider gaps between G' and G'' , indicative of more stable and elastic structures.

These findings highlight fundamental structural differences between hybrid carrageenans and commercial blends, underscoring how variations in chemical composition influence the mechanical properties and gelation behavior of carrageenan systems.

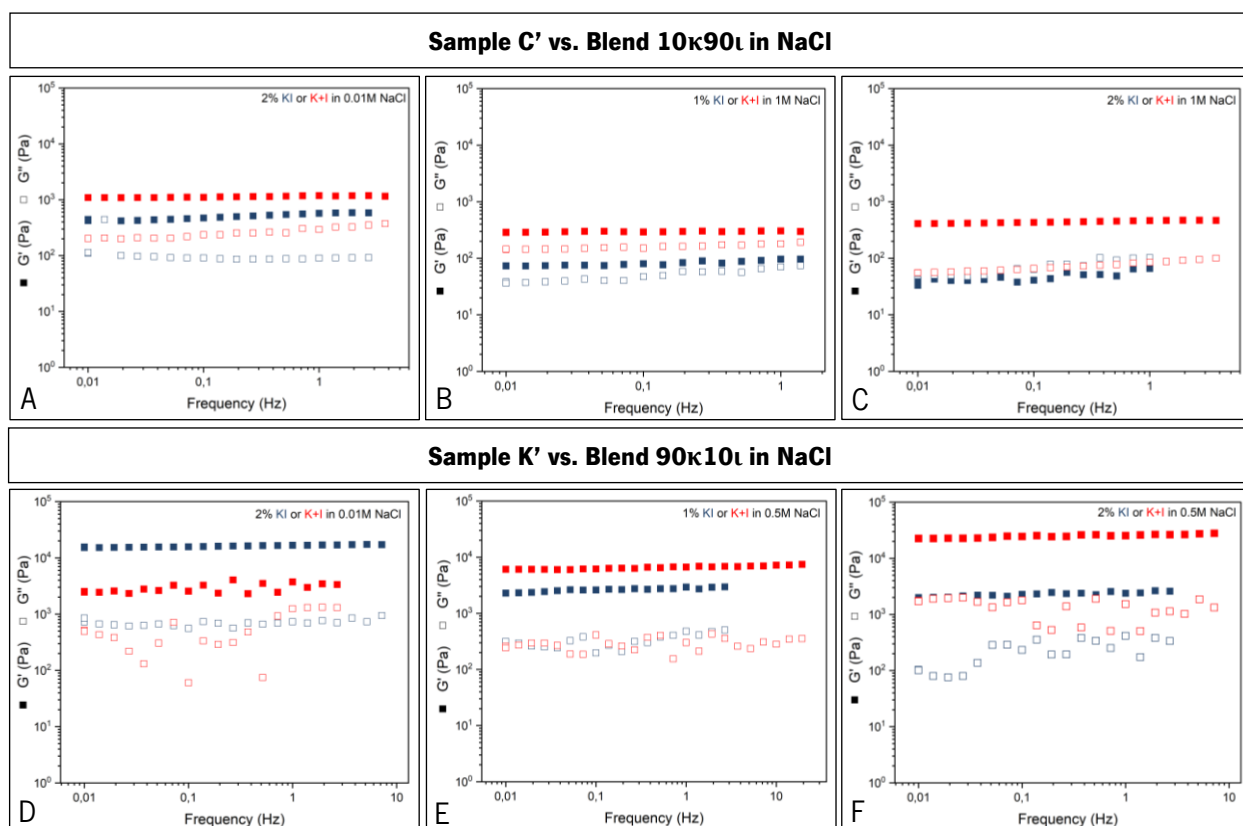


Figure 15: Mechanical spectra (storage modulus G' , full squares; loss modulus G'' , open squares) of hybrid carrageenan (KI) samples (in blue) compared to their respective commercial K+I carrageenan blends (in red) at various polymer concentrations and ionic strengths, in NaCl solutions. Each row represents a different hybrid carrageenan and blend pair: (top) sample C' vs. 10κ90ι, at **A**) 2 wt.% in 0.01 M NaCl; **B**) 1 wt.% in 1 M NaCl; **C**) 2 wt.% in 1 M NaCl; and (bottom) sample K' vs. 90κ10ι, at **D**) 2 wt.% in 0.01 M NaCl; **E**) 1 wt.% in 0.5 M NaCl; **F**) 2 wt.% in 0.5 M NaCl.

As for NaCl samples, all experiments using sample K' and the commercial blend 90κ10ι exhibited solid-like behavior, presenting $G' > G''$ across the entire frequency range (Figure 15D-15F). Notably, G' was significantly higher than G'' and displayed minimal frequency dependence, particularly for G' , confirming the formation of strong, elastic gel networks. However, under certain conditions (*e.g.*, Figures 15D and 15E), irregular and scattered G'' values were observed, likely due to equipment sensitivity limitations, which hindered the accurate analysis of these results.

In contrast, the mechanical spectra for experiments involving sample C' and its commercial blend counterpart showed strong dependence on both the ionic environment and polysaccharide concentration. In 0.01 M NaCl at 2 wt.%, both the hybrid carrageenan and the commercial blend demonstrated solid-like characteristics, forming relatively stable and strong networks (Figure 15A). Conversely, in 1 M NaCl at 1 wt.%, both systems exhibited weak gel-like behavior (showing narrower gaps between G' and G''), with viscoelasticity becoming more pronounced at higher frequencies (Figure 15B). At 2 wt.% in 1 M NaCl, the commercial blend formed a robust elastic gel network, while the hybrid sample remained

predominantly viscoelastic, characterized by $G'' > G'$ (Figure 15C). These observations align with the temperature sweep analysis previously conducted for the 2 wt.% sample C' in 1 M NaCl, which revealed that no gel had formed by the end of the test ($T=25\text{ }^{\circ}\text{C}$).

In the presence of NaCl, while increasing polysaccharide concentration led to an increase in elasticity (wider gap between G' and G'') for commercial blends, it did not exert significant differences for hybrid carrageenans C' and K'. On the other hand, except for commercial blend 90κ10ι, greater salt concentration typically resulted in the loss of elasticity and network stability. However, κ-rich samples consistently resulted in more stable and elastic gels in comparison to ι-rich samples.

Gel Properties under Large Deformation

In rheological studies of gels, several critical parameters provide insight into their mechanical behavior and structural integrity. The gel elasticity in the linear viscoelastic (LVE) range, represented by G_0 (elastic modulus), reflects the gel's stiffness and ability to resist deformation under small applied strains, where the structure remains undamaged. The limiting strain (γ_L) marks the boundary of this linear range, beyond which the gel structure begins to deform irreversibly or transition into a nonlinear viscoelastic response. To calculate γ_L , the tolerated deviation from the gel elasticity in the LVE range was defined as 5%, *i.e.*, all the G' values below 95% (in strain softening behaviors) or above 105% (in strain hardening behaviors) of the plateau value were considered to be outside of the LVE range. Another crucial point is the flow point (γ_F), defined by the crossover of storage (G') and loss moduli (G''). At this point, the gel transitions from a predominantly solid-like behavior ($G' > G''$) to a liquid-like behavior ($G'' > G'$), representing the onset of flow (Mezger, 2006). A strain sweep test illustrating these parameters is shown in Figure 16A.

Additionally, under large deformation, gels can exhibit strain hardening or strain softening behavior, depending on the strand-like structure of carrageenan gels, modulated by the bending rigidity of the filaments and the topology of the network (Carrillo et al., 2013; Meng & Terentjev, 2016; Piculell, 2006). Strain hardening (Figure 16B) refers to an increase in stiffness as strain increases, before rupturing, often indicating strong stretching of filaments. Conversely, strain softening (Figure 16A) reflects a decrease in stiffness with increasing strain, typically associated with structural breakdown or rearrangement (Hilliou, 2021; Mezger, 2006). Practically, these behaviors influence the gel's resilience and functionality in real-world applications, as strain-hardening gels present higher rupture resistance under stress, while strain-softening gels may fail more easily under high strain conditions. A distinctive behavior where samples presented a strain softening followed by a strain hardening response was also observed in some cases

(Figure 16C). Together, these parameters, registered in Table 8, provide a comprehensive understanding of the gel's mechanical properties.

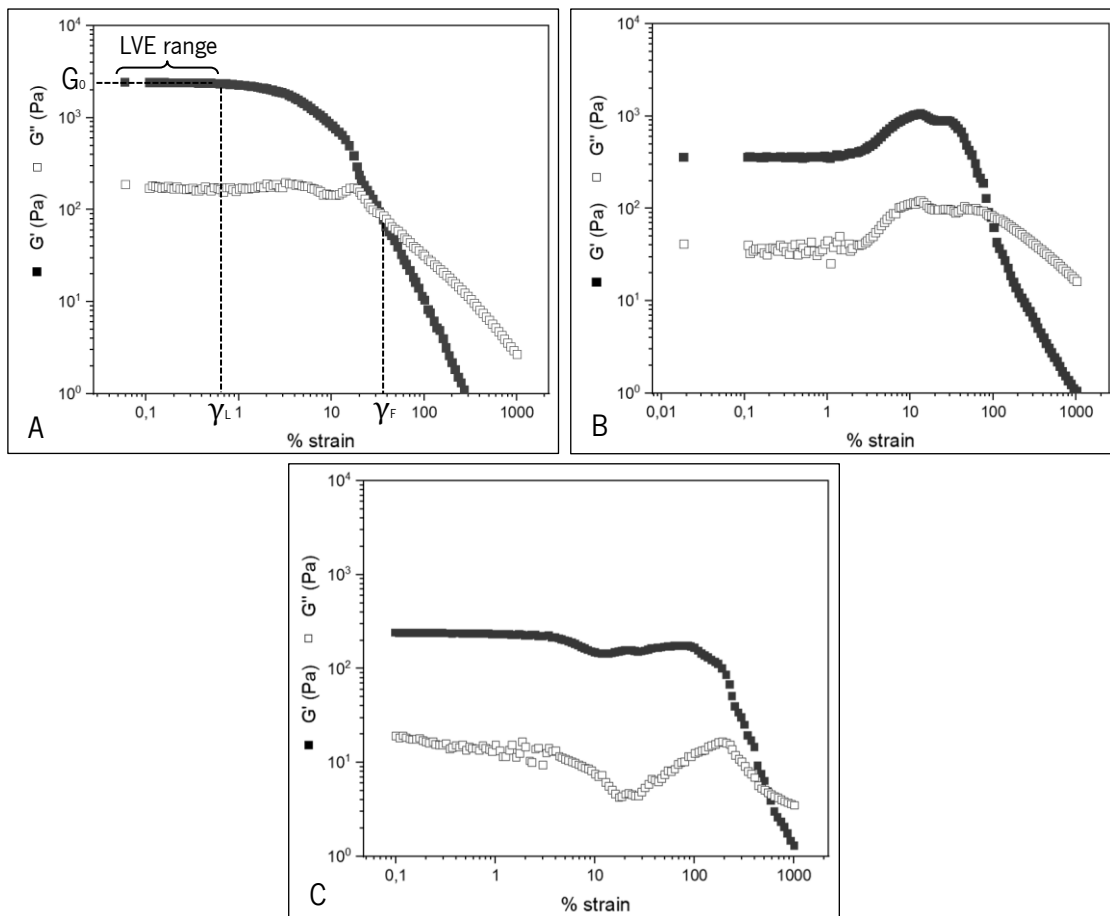


Figure 16: Representation of **A)** strain softening, **B)** strain hardening, and **C)** strain softening followed by strain hardening behaviors. The strain sweep example in **A** represents a gel-like sample, showing the elastic modulus, (G_0), the limiting strain (γ_L) at the limit of the LVE range, and the flow point (γ_F) where $G' = G''$.

Hybrid carrageenans and commercial blends under the same ionic environment and polysaccharide concentration, yielded different mechanical properties, such as strength and deformability. In the LVE region, commercial carrageenans generally produced stronger and stiffer gels (higher G_0 values). However, sample E, a K-2 hybrid carrageenan, significantly surpassed the elasticity of its commercial counterpart, under high ionic strengths. The same was also seen occasionally in low ionic strengths (*e.g.*, sample C vs. 10κ90 ι , in KCl, and sample K' vs. 90κ10 ι , in NaCl). Nonetheless, in most cases, greater elasticity came at the cost of increased gel brittleness (*e.g.*, sample E and sample 90κ10 ι , in KCl, and sample 10κ90 ι , in NaCl). In addition, increasing κ-content, especially in KCl, and increasing polysaccharide concentration usually enhanced gel elasticity.

Table 8: Rheological properties of hybrid carrageenans and commercial blends gelled in KCl and NaCl under varying polysaccharide and ionic conditions. The table includes gel elasticity in the LVE range (G_0), limiting strain (γ_L), and flow strain (γ_f), as well as strain hardening and softening behaviors under large amplitude oscillatory shear (LAOS). Cases where strain softening is followed by strain hardening are indicated as “No then Yes”

	Experiment	Sample	G_0 (Pa)	γ_L (%)	γ_f (%)	Strain Hardening?
Samples in KCl	2 wt% in 0.01M KCl	Sample C	2180 ± 17	1.40 ± 0.15	377 ± 30	No
		10κ90ι	1022 ± 20	0.69 ± 0.04	118 ± 13	No then Yes
	1 wt% in 1M KCl	Sample C	48.7 ± 0.3	1.50 ± 0.10	331 ± 14	No
		10κ90ι	237 ± 2	1.96 ± 0.13	556 ± 34	No then Yes
	2 wt% in 1M KCl	Sample C	985 ± 6	3.08 ± 0.18	404 ± 30	No then Yes
		10κ90ι	11281 ± 238	0.61 ± 0.03	388 ± 30	No
	2 wt% in 0.01M KCl	Sample E	7831 ± 23	0.93 ± 0.08	37 ± 5	No
		60κ40ι	15351 ± 46	2.04 ± 0.14	60 ± 7	No then Yes
	1 wt% in 0.5M KCl	Sample E	2391 ± 14	0.94 ± 0.06	35 ± 4	No
		60κ40ι	157 ± 1	0.95 ± 0.07	104 ± 10	No
	2 wt% in 0.5M KCl	Sample E	14860 ± 134	0.30 ± 0.02	11 ± 1	No
		60κ40ι	357 ± 2	1.58 ± 0.05	92 ± 4	Yes
	0.5 wt% in 0.01M KCl	Sample K	490 ± 4	1.38 ± 0.11	123 ± 6	Yes
		90κ10ι	535 ± 5	0.96 ± 0.04	136 ± 7	Yes
Samples in NaCl	2 wt% in 0.01M NaCl	Sample C'	738 ± 3	0.81 ± 0.05	181 ± 5	No
		10κ90ι	$1400 \pm 128^*$	0.31 ± 0.02	155 ± 13	No then yes
	1 wt% in 1M NaCl	Sample C'	$100 \pm 60^*$	$<100^*$	391 ± 47	No
		10κ90ι	$800 \pm 400^*$	$<1^*$	25 ± 3	No
	2 wt% in 1M NaCl	Sample C'	$80 \pm 20^*$	$-^*$	355 ± 30	No
		10κ90ι	$410 \pm 50^*$	4.57 ± 0.23	315 ± 38	No then yes
	2 wt% in 0.01M NaCl	Sample K'	18226 ± 111	1.17 ± 0.11	36 ± 5	No
		90κ10ι	$3832 \pm 140^*$	1.38 ± 0.02	100 ± 5	Yes
	1 wt% in 0.5M NaCl	Sample K'	3384 ± 82	0.64 ± 0.02	41 ± 2	Yes
		90κ10ι	6797 ± 45	1.21 ± 0.17	23 ± 1	No
	2 wt% in 0.5M NaCl	Sample K'	3118 ± 96	0.35 ± 0.04	11 ± 4	Yes
		90κ10ι	25535 ± 311	3.50 ± 0.16	49 ± 7	Yes

*The strain sweep analysis of these samples in NaCl revealed certain inconsistencies or experimental artifacts, as evidenced by the absence of a well-defined linear viscoelastic (LVE) region (see Figure A.6, in Appendix A.4). This limitation hindered the accurate determination of key parameters such as the linear elastic modulus (G_0) and the limiting strain (γ_L). The lack of a clear LVE region suggests potential deviations in the gel structure or external factors, such as instrument sensitivity, sample heterogeneity, or ionic interactions specific to NaCl systems.

In ι-rich samples in KCl, both salt and polysaccharide concentrations significantly influenced the gels' elastic and rheological properties. At low ionic strength (0.01 M KCl), the hybrid carrageenan (sample C) exhibited higher elasticity and greater resistance to deformation than the commercial blend 10κ90ι.

Conversely, at high ionic strength (1 M KCl), the commercial blend consistently displayed greater elasticity. Interestingly, in this ionic environment, the commercial blend at 1 wt.% polysaccharide and sample C at 2 wt.% polysaccharide exhibited strain-hardening behaviors, which coincided with the conditions under which each sample demonstrated their highest resistance to deformation.

Despite potential experimental artifacts and inconsistencies in ι -rich samples in NaCl, the commercial blends generally formed stronger gels than the hybrid carrageenans across all conditions studied as shown in Figure A.6, Appendix A.4). At low ionic strength (0.01 M NaCl), the commercial blend also withstood greater strain before permanently losing its linear viscoelasticity. However, at higher ionic strengths, deformation resistance was difficult to evaluate due to irregular and experimentally inaccurate strain sweep graphs. Except for the 1 wt.% polysaccharide experiment at 1 M NaCl, commercial blends and hybrid carrageenans exhibited similar yield points under comparable conditions. To clarify whether these irregularities were due to sample-specific factors or equipment limitations, further analysis under optimized and controlled conditions, using different shearing and/or gelling conditions, is required to ensure reliable and reproducible results and check for possible slip or nonlinear stress response.

In the case of sample E and commercial blend 60 κ 40 ι , at low ionic strength, the commercial blend exhibited superior gel elasticity and resistance to mechanical stress compared to the hybrid carrageenan. However, at high ionic strength, sample E showed greater elasticity but endured less deformation, losing structural integrity more easily.

Notably, while the gel elasticity of hybrid carrageenan E consistently increased with rising salt concentration, the linear elasticity of the commercial blend 60 κ 40 ι decreased under high ionic strength. This observation aligns with findings by Azevedo et al. (2014), who reported that the linear elasticity (G_0) of carrageenan blends increases with salt concentration up to critical point, beyond which the elasticity begins to decline, reflecting a weakening of the gel network. In contrast, the elasticity of hybrid carrageenans followed a different trend, increasing steadily with salt concentration until reaching a saturation point. Beyond this, G_0 either became independent of further salt addition or the critical salt concentration laid outside the studied range (0.01–1 M Na⁺ and K⁺).

In systems with higher κ -contents, both in KCl and NaCl, the commercial blends consistently exhibited greater elasticity than hybrid carrageenans under equivalent conditions. In KCl, this higher elasticity often came at the cost of increased brittleness. In contrast, in NaCl, commercial blends produced gels that were not only stronger but also softer, demonstrating a higher tolerance for deformation before reaching the irreversible damage limit (γ_c) and the final breakdown point (γ_r).

Notably, higher κ -contents were also correlated with enhanced gel elasticity when comparing certain commercial blends with lower mol.% κ (*e.g.*, 2 wt.% sample 60 κ 40 ι in 0.5 M KCl). These findings align with previous studies documenting the increase in the elastic modulus of mixed gels as κ -carrageenan content rises (Brenner et al., 2014; Rochas et al., 1989; van de Velde et al., 2005), which is further amplified by the presence of potassium ions (Chanvrier et al., 2004; Parker et al., 1993). However, while increasing κ -content significantly increased gel brittleness in commercial blends (consistent with Brenner et al. (2014)), this trend was not observed in hybrid carrageenans (*e.g.*, comparing experiments 2 wt.% in 0.5 M KCl, for samples E and K, with 60 and 90 mol.% κ , respectively).

These observations suggest fundamental structural differences between the two systems. While hybrid carrageenans displayed lower elasticity, they exhibited an intrinsically higher capacity to resist strain. However, further comparative studies are required to confirm this, particularly involving hybrid carrageenans and commercial blends with varying chemical compositions tested under identical polysaccharide and ionic conditions. Interestingly, both hybrid carrageenans and commercial blends rich in κ exhibited a strain-hardening response at low ionic strengths and a strain-softening response at high ionic strengths, highlighting a shared mechanical behavior despite structural differences.

In conclusion, in terms of viscoelastic properties hybrid carrageenans and commercial blends may offer specific advantages depending on the application. While stronger and stiffer gels produced by K+I make them better suited for applications requiring robust structural integrity and minimal deformation, the studied K-2 hybrid carrageenan, for example, showcased its potential for applications where gelling ability resistance to large amount of salt is desirable. However, since increased elasticity often came at the expense of reduced resistance to deformation, leading to greater gel brittleness, it is important to align the choice of carrageenan system with the specific functional requirements of the target application.

4.3 Food Application

Given that the food industry is one of the largest markets for carrageenan, and considering the sector's emerging trends, the formulation of functional and vegetarian gummies based on hybrid carrageenans was developed and tested. The significance of simulating a real-world application with hybrid carrageenans lies in the fact that their applicability is not solely dependent on their behavior in the presence of varying ionic strengths, salts, and polysaccharide concentrations, but also on their interactions and synergistic effects with other ingredients, particularly other biopolymers such as xanthan gum.

This specific food application was chosen because I had the opportunity to collaborate with Professor Germán Valencia from the Federal University of Santa Catarina (Florianópolis, Brazil) on an ongoing project led by him and his team. In particular, this work aligns with one of their project's goals: studying the application of a natural biohybrid ingredient, based on anthocyanins extracted from jambolan fruit and adsorbed onto bentonite, in carrageenan hydrogels. The formulation in their work, based on a study by (Song et al., 2022), was adapted to meet the specific objectives of my research and the resources available. Additionally, I recognized that this application aligns with current market trends, serving as a vegetarian alternative to animal-based (gelatin) gummies while tapping into the growing nutraceutical segment focused on functional or fortified gummies (Mahat et al., 2020). Given the global confectionery sector's position as one of the fastest-growing industries (Rawat et al., 2024), I personally saw significant potential and interest in exploring this specific application.

The primary goal of this section was to comparatively evaluate the performance of alkaline-treated hybrid carrageenans against both commercial carrageenan blends and untreated hybrid carrageenans. On the one hand, comparing alkali-treated hybrid carrageenans (*i.e.*, with biological precursors removed) to commercial blends aimed to assess whether, in a practical application, their performance differs significantly or if they could serve as viable substitutes. On the other hand, we looked into whether the presence of biological precursors in the composition of hybrid carrageenans resulted in a significant difference in gummy performance. This consideration holds industrial interest, as using untreated hybrid carrageenans could reduce processing costs and time. While it is known that alkaline treatment significantly enhances the gelation potential of hybrid carrageenans, when evaluated within a formulation containing other ingredients and biopolymers, these differences may or may not be critical to the final product's quality.

The hybrid carrageenan samples for this application were selected based on results from the phase diagram in KCl (Table 5, Section 4.2.1), as rheological tests were not yet completed at that time. Additionally, time and sample quantity constraints further influenced the selection of samples C, F, and J, limiting the assessment of other hybrid carrageenans. Nevertheless, these three samples, with significantly different κ/ι ratios, provide a general idea of the influence of ι -content on gummy performance when incorporated into a formulation with other biopolymers and functional ingredients. Only samples treated with KOH were tested, as this alkaline solution yielded a more comprehensive gelation profile compared to NaOH (Tables 5 and 6, Section 4.2.1).

4.3.1 Particle Size Distribution and Zeta Potential of the Biohybrid

The particle size distribution (PSD) and zeta potential (ZP) of the biohybrid material (BH) were assessed to evaluate their implications for stability and dispersion quality in the gummy candy matrix. The particle size distribution of the BH dispersed in water is shown in Figure 17.

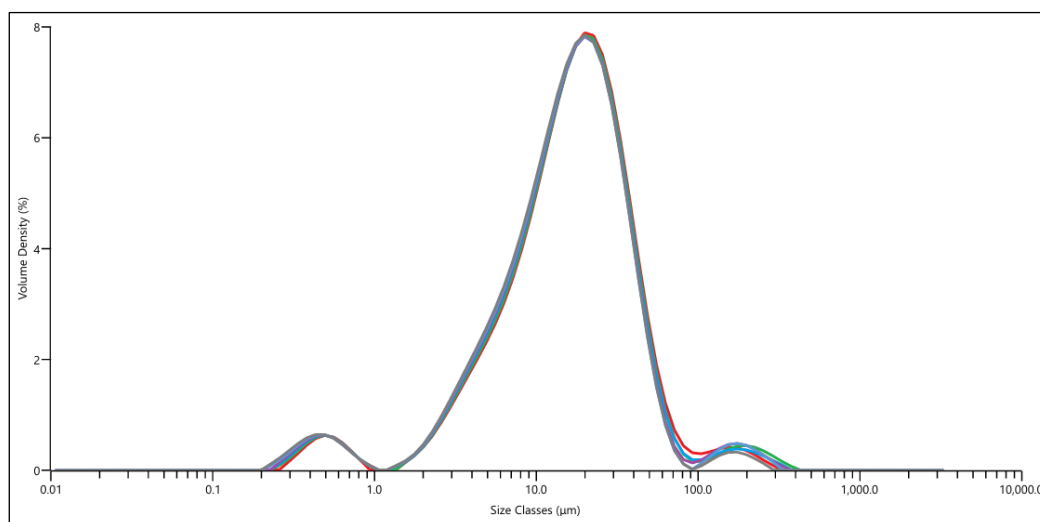


Figure 17: Particle size distribution of the biohybrid material dispersed in an aqueous solution.

The particle size distribution of the biohybrid material (bentonite with adsorbed anthocyanins) showed a trimodal distribution, reflecting its complex aggregation behavior. The first peak, around 0.5 μm , likely represents small clusters of bentonite particles. The second dominant peak, at 16-17 μm , corresponds to larger aggregates formed as anthocyanins adsorb onto the clay surface, likely promoting further clustering of particles. The third peak at 140 μm suggests the presence of larger, more loosely associated aggregates, potentially formed during the mixing or drying process.

This observed trimodal distribution aligns with behaviors reported in the literature, where bentonite-based materials commonly exhibit a broad particle size range due to hydration, swelling, and aggregation processes (Buntin et al., 2022; Magzoub et al., 2020).

Powdered bentonite, rich in montmorillonite, shows a finer and more uniform distribution, with a particle size distribution between 0.5 and 200 μm (Andrade et al., 2021; Vryzas et al., 2016). However, when bentonite is dispersed in water, its particles hydrate and swell, causing significant changes in the size distribution, especially since the clay particles can expand several times their dry size (Magzoub et al., 2020). The larger particle sizes in the biohybrid material could be attributed to the aggregation of montmorillonite layers, especially since anthocyanins adsorb onto the surface, which might bridge the

clay particles and cause clustering. This could also be due to the drying method or other preparation steps, which tend to encourage aggregation.

The zeta potential (ZP) of any particle is defined as the number of charges it carries, and, along with particle size distribution, its assessment is a very important parameter for inferring the stability of colloidal dispersions (Parupudi et al., 2022; M. Sharma, 2019; Singhvi et al., 2018). Generally, particles with zeta potential values ranging within -10 to $+10$ mV are considered to have neutral surfaces while those with larger absolute values of 30 mV ($+/-$) are considered to be strongly cationic or anionic. Therefore, high surface charge on particle surface leads to repulsion and thus prevents aggregation (Mekhamer, 2010; Patravale et al., 2012; Singhvi et al., 2018). The zeta potential of the biohybrid/water system was -19.4 mV, suggesting a moderate level of colloidal stability, with particles likely tending to aggregate over time. This ZP value allows for weak interactions between particles, leading to the formation of larger clusters, particularly in the micron and submicron ranges

For a more homogeneous distribution in future applications, improving electrostatic stabilization (*e.g.*, by adjusting pH or surface modification (Magzoub et al., 2020; Mekhamer, 2010) could reduce aggregation and result in a more uniform particle size distribution, which is crucial for enhancing the material's performance in the matrix.

4.3.2 Visual Characterization of Gummy Candy Samples

The visual appearance of all GC samples prepared was registered in Figures 18 A-C, for all systems in study, using samples C, F and J, respectively.

The carrageenan matrices used enabled gel formation for all GC samples except UT-F (Figure 18B). This outcome is consistent with the balanced composition in ι and κ of the KI, together with the higher content of precursor units (see Figure B.1 in Annex B.1), negatively influencing the gelling behavior. However, it could also be attributed to less careful handling during the physical phase assessment or slight temperature variations, as its counterpart (UT-F + BH) gelled without issue. Alternatively, BH could shift the gel formation conditions as less water is available. In any case, the rheological assessment, conducted under more controlled cooling conditions, will provide further insights into the true gelling behavior of this UT-F sample.

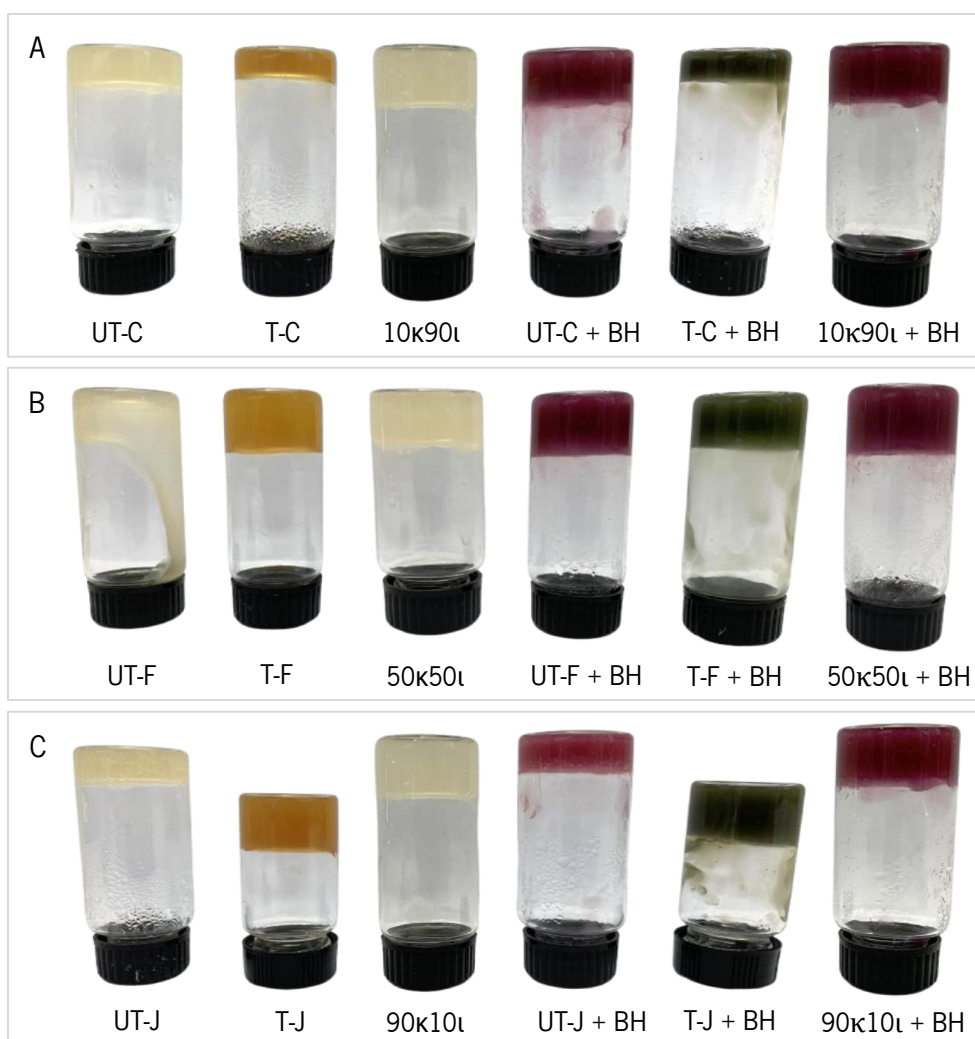


Figure 18: Images of vegan gummy candies prepared with untreated KI samples (UT-), treated KI samples (T-), and the respective commercial carrageenan blends (mol.% κ ; mol.% ι), both with and without the biohybrid material (BH). **A)** Sample C and Blend 10 κ 90 ι , rich in ι -carrageenan; **B)** Sample F and Blend 50 κ 50 ι , with a balanced κ/ι ratio; and **C)** Sample J and Blend 90 κ 10 ι , rich in κ -carrageenan.

Examining the images of the gummy candies, a noticeable color variation can be observed. Specifically, samples without the biohybrid material (BH) exhibited a more yellowish hue, while its addition resulted in a red/pink tone in the untreated KI and commercial K+I samples, and a green tone in the alkaline-treated KI samples. This color variation is related to the BH's response to different pH environments, due to the incorporation of pH-sensitive anthocyanins (ACNs) (Koop et al., 2024; Merz et al., 2020). The detailed data on the pH values of each gummy candy sample, as well as those of the rosemary honey and biohybrid material used, are gathered in Table A.1, in Appendix A.5.

In the presence of the BH, untreated KI and commercial K+I samples showed pH values between 3.6 and 3.9, at which the anthocyanins exhibit a pink hue. In contrast, alkali-treated samples presented higher pH values (around 7.8), where ACNs are oxidized, leading to a blue coloration (Koop et al., 2024). When

combined with the yellow matrix of the gummy candies, this blue coloration gave rise to a green color in the alkaline-treated KI samples. The increased pH in the alkali-treated KI samples can be attributed to residual KOH from the treatment process, leaving behind hydroxide ions ($[\text{OH}^-]$), which raise the pH of the medium, creating a more basic environment.

Microscopic images were captured for all GC samples to assess the dispersion quality of the biohybrid material (BH). Since the dispersion behavior was consistent across the three systems (C, F, and J), a detailed discussion will focus on the microscopic images of sample C (Figure 19). These images illustrate differences in the distribution and presence of BH within the matrices.

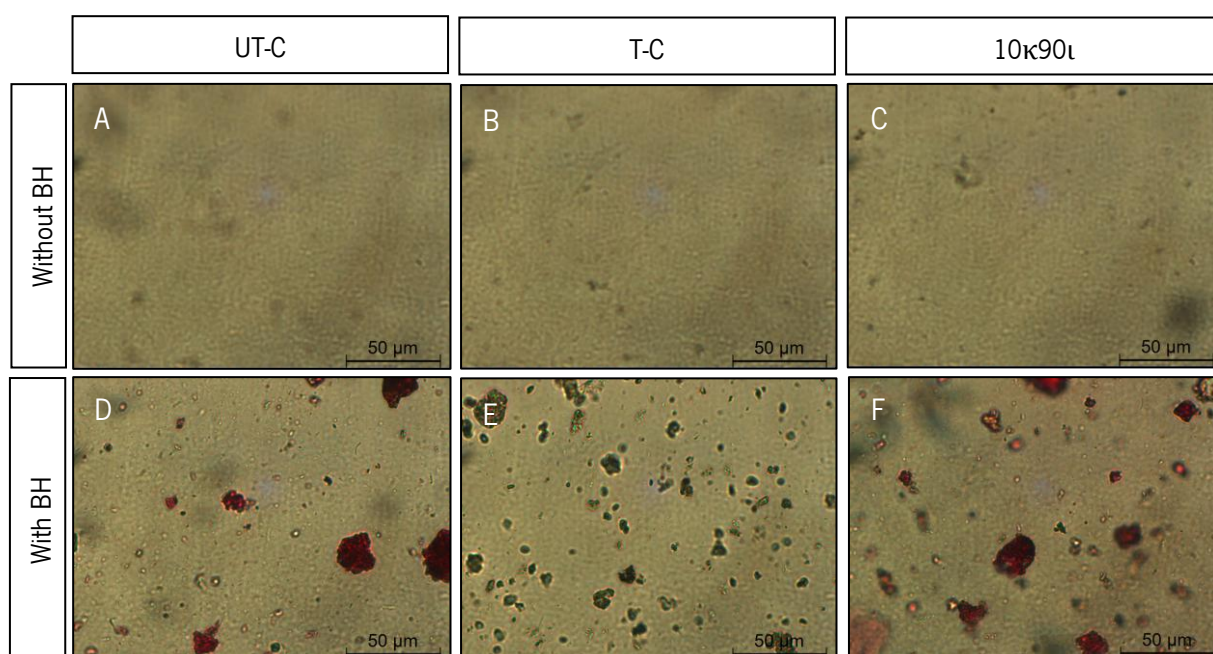


Figure 19: Microscopic images of gummy candy samples using untreated carrageenan C (UT-C) (**A, D**), alkali-treated carrageenan C (T-C) (**B, E**), and commercial blend 10κ90ι (**C, F**), without (**A-C**) and with (**D-F**) the addition of a biohybrid material (BH). The biohybrid, composed of anthocyanins adsorbed onto bentonite, appears as red or green particles (**D-F**).

In Figures 19 D, E and F the biohybrid material, colored red or green, appears as distinct aggregates within the matrix, indicating a non-homogeneous dispersion. These aggregates vary in size, with larger clumps and smaller particles dispersed throughout the matrix, in line with the previously discussed particle size distribution of the biohybrid and its zeta potential of -19.4 mV, which reflects moderate colloidal stability. The dispersion, though evident, is incomplete, with particles tending to cluster rather than distribute evenly.

This behavior likely results from insufficient compatibility between the biohybrid material and the matrix components, influenced by factors such as viscosity, the polarity of ingredients, and the processing conditions (*e.g.*, mixing duration and temperature). To improve dispersion, further optimization is necessary. Techniques such as enhancing mechanical shear forces during mixing, modifying the solubility parameters of the matrix to increase compatibility, or applying surface treatments to the biohybrid material (*e.g.*, adjusting the pH or ionic strength) to reduce aggregation may provide more uniform distribution (Cano-Sarmiento et al., 2015; Nile et al., 2020).

4.3.3 Rheological Characterization

In food product development, particularly for gummy candies, rheological properties such as elasticity, strain resistance, and flow behavior are critical for achieving the desired texture, structural stability, and sensory experience. These parameters determine how the product handles mechanical stresses during processing, packaging, and consumption, while also influencing its chewiness, firmness, and mouthfeel. A well-optimized gel structure ensures that the candy can withstand mechanical stresses during handling, packaging, transportation, and consumption, while maintaining its integrity and delivering a consistent sensory experience. This balance is pivotal for aligning the product with consumer preferences, which can vary widely—from soft, elastic, and viscoelastic textures that characterize traditional jelly gummies to drier, firmer, or even brittle profiles tailored to specific markets or applications. Ultimately, these rheological parameters provide manufacturers with the tools to design candies that meet both practical and sensory requirements, enhancing overall product performance and acceptance (Endress & Mattes, 2003).

The rheological analysis of the gummy candy samples was conducted using the same protocol as the hybrid carrageenan *vs* commercial blend comparison (Section 4.2.2.), as described in Section 3.5.2. Time, frequency, and strain sweep tests were performed to evaluate the thermal, mechanical, and viscoelastic properties of the GC formulations under small and large amplitude oscillatory shear. This section addresses three key objectives.

First, it explores potential differences or similarities between KOH-treated hybrid carrageenans and commercial blends when applied in a practical formulation, aiming to assess whether hybrid carrageenans can offer advantages over commercial blends. This analysis also focuses on the influence of polysaccharide's chemical composition, particularly the ι -content, and highlights the performance of K-2 hybrid carrageenan (sample F, 50 mol.% κ + 50 mol.% ι), which is less documented. Indeed, similar

comparative studies between blends and hybrids only targeted dairy applications (Bixler et al., 2001; Villanueva et al., 2004). The present study thus presents some novelty.

Second, the study investigates the effects of biological precursors (μ - and ν -carrageenans) by comparing untreated hybrid-carrageenans to their alkali-treated counterparts. This comparison evaluates whether the removal of precursors improves the viscoelastic performance of GCs and assesses whether that additional alkali treatment step is justified from an industrial perspective.

Finally, the impact of incorporating the biohybrid material is analyzed to evaluate whether the functional ingredient alters the viscoelastic properties of GC formulations. This objective is essential for determining whether the addition of such ingredients results in predictable or beneficial modifications, meeting industrial expectations for performance stability in the base formulation.

Thermal Behavior of Gummy Candy Samples

By analyzing the time sweep tests (see Figure A.7 of Appendix A.6), it can be observed that the addition of the BH did not result in significant or relevant differences in the thermal behavior of the GC samples. Notably, all GC samples assessed in this study exhibited a gelation temperature (T_g) above 65 °C and most likely below 100 °C. While a broader temperature range ($T > 90$ °C) would be required to definitively confirm the upper limit for all formulations, the T_g values observed offer both advantages and challenges for industrial applications. This balance highlights the importance of understanding the thermal properties of these gels when considering their practical use in food production.

This range of gelation temperatures is generally feasible for industrial processing, as it avoids the need for excessively high temperatures that could compromise the functionality of other ingredients. However, it is crucial to define the processing temperature appropriately during gummy candy production. For most studied cases, temperatures near or above 100 °C are recommended to ensure that the candy mixtures remain in a liquid state during the initial production stages. This prevents premature gelation, which could otherwise lead to clogged equipment or uneven textures in the final product. That said, maintaining the formulation at such elevated temperatures to avoid early gelation could increase energy costs and necessitate specialized equipment capable of providing uniform heating without scorching or overheating the ingredients. Additionally, at temperatures close to the upper limit of the gelation range, improper handling might result in phase separation or uneven gel formation, causing undesirable textural or visual defects in the final product.

On the positive side, a higher T_g enhances the thermal stability of the final product, reducing the risk of melting or structural failure during storage or transportation at room temperature or even under warmer conditions. This stability is especially beneficial for shelf-stable confectionery products, which must maintain their structural integrity across varying environmental conditions over extended periods.

However, the elevated temperatures required during processing may present challenges for incorporating heat-sensitive ingredients, such as the anthocyanin-based biohybrid additive. Prolonged exposure to high heat could lead to the degradation of anthocyanins, potentially compromising their functional properties, including antioxidant activity and vibrant coloration (Oancea, 2021). That said, the biohybrid material, by stabilizing the anthocyanins, may enhance their thermal stability at pH values above 3.5 and under prolonged heat exposure, similar to other heat-stabilization techniques for anthocyanins, such as micro-encapsulation or the addition of sugars or metallic ions (Oancea, 2021). However, no studies have been conducted on this specific stabilization method and for anthocyanins sourced from jambolan fruit, as the thermal stability of anthocyanins can also vary significantly depending on their source (type of fruit or vegetable). From an industrial perspective, process optimization could involve introducing the biohybrid additive during the cooling stage or minimizing its exposure to high temperatures to better preserve its functional properties.

General considerations regarding the thermal behavior of GC samples led to the conclusion that formulations containing higher proportions of ι -carrageenan displayed higher gelation onset temperatures, as expected. Additionally, across all cooling curves, no significant differences were observed between KI and K+I, suggesting that hybrids can effectively replace blends when thermal performance is the primary concern. Furthermore, the viscoelastic moduli at high temperature, *i.e.*, in the liquid state above 70 °C, were significantly smaller in GC formulated with KI or K+I containing higher κ content. While this characteristic may reduce processing energy costs, it could negatively affect the dispersion of the BH material.

The presence of biosynthetic precursors notably affected thermal properties in specific cases. For instance, in the untreated hybrid carrageenan UT-J, the thermal behavior resembled that of the commercial blend. However, when the hybrid is alkali-treated (T-J), the gelation temperature shifted significantly higher, from below 60 °C to over 70 °C. Similarly, the impact of precursors on viscosity at elevated temperatures was evident in UT-C, which demonstrated higher viscosity compared to its treated counterpart, albeit still less than the commercial mixture. This viscosifying effect likely stems from the larger molecular mass of the untreated hybrid (comparing Figure B.2 – Annex B.2 with Figure 12), rather

than its sulfate content, as the treated hybrid (T-C) has fewer sulfate groups yet shows reduced viscosity compared to the untreated version.

In summary, the observed T_g range strikes a promising balance between processing flexibility and product stability. Nonetheless, careful attention must be given to optimizing production conditions to manage energy costs, protect heat-sensitive ingredients, and ensure the desired texture and structural integrity of the final product. By addressing these considerations, the advantages of a higher T_g can be effectively leveraged while minimizing potential challenges.

Mechanical Properties of Gummy Candy Samples under Small and Large Amplitude Oscillatory Shear

In the frequency sweep tests, all GC samples exhibited solid-like behavior, with G' consistently exceeding G'' across the entire frequency range, indicating gel-like properties (Figure A.8, in Appendix A.7). During the visual characterization, it was initially observed that sample UT-F did not form a gel. However, during rheological testing, the sample successfully gelled, although it yielded a relatively weak gel, similar to its counterpart UT-F + BH. This reinforces the observation that, despite initial visual inconsistencies, the gelling potential of sample UT-F was not compromised and aligns with the behavior of its hybrid counterpart. In other words, the UT-F forms a weak gel, whose strength cannot support a tube tilting test (see G_0 in Table 9).

The rheological properties of the gummy candy samples, derived from strain sweep analyses (Figure A.9, in Appendix A.8), are summarized in Table 9. The data encompasses the elastic modulus (G_0), limiting strain (γ_L), and flow point (γ_F), which together characterize the gel's mechanical performance under deformation. Additionally, strain hardening or softening responses at large amplitude oscillatory shear (LAOS) were recorded, revealing potential nonlinear elastic effects and network rearrangements at higher strains. This table enables a comparative evaluation of the influence of the carrageenan source (commercial blends, treated, or untreated hybrid carrageenans), κ/ι ratios, and the incorporation of the biohybrid material (+BH) on the structural and mechanical properties of the gels.

Overall, commercial blends consistently exhibited higher G_0 , indicating stiffer gels, compared to treated and untreated hybrid carrageenans. This comes as no surprise as the rheological evaluation of the gels produced for the phase diagrams showed a similar tendency for more elastic commercial gels. Alkali treatment generally enhanced gel stiffness and strain resistance, though exceptions were noted in high ι -content samples, where untreated carrageenans outperformed their treated counterparts. Increasing κ -

content was associated with greater gel stiffness but reduced strain tolerance, reflecting a trade-off between elasticity and brittleness. The addition of the BH typically increased stiffness but often compromised strain resistance, with variability depending on chemical composition and treatment. These results underscore the complex interplay of factors influencing gel performance and the need for formulation-specific optimization.

Table 9: Elastic and rheological properties of gummy candy samples, prepared using commercial carrageenan blends (mol.% κ ; mol.% ι), KOH-treated (T-) or untreated (UT-) hybrid-carrageenans, with (+ BH) or without the biohybrid material. The table presents the gel elasticity in the LVE range (G_0), limiting strain (γ_L), and flow point (γ_f), as well as strain hardening and softening behaviors under large amplitude oscillatory shear (LAOS). Cases where strain softening is followed by strain hardening are indicated as “No then Yes”

	Sample	G_0 (Pa)	γ_L (%)	γ_f (%)	Strain Hardening?
Group 1	10 κ 90 ι	2254 $\pm$ 10	3.20 $\pm$ 0.24	162 $\pm$ 8	No then Yes
	10 κ 90 ι + BH*	2284 $\pm$ 278	0.49 $\pm$ 0.08	109 $\pm$ 5	No then Yes
	T-C*	227 $\pm$ 18	0.46 $\pm$ 0.03	352 $\pm$ 26	No then Yes
	T-C + BH	256 $\pm$ 3	2.14 $\pm$ 0.18	339 $\pm$ 13	No then Yes
	UT-C	419 $\pm$ 2	1.92 $\pm$ 0.08	302 $\pm$ 24	No then Yes
	UT-C + BH*	364 $\pm$ 34	0.34 $\pm$ 0.03	310 $\pm$ 17	No then Yes
Group 2	50 κ 50 ι	9967 $\pm$ 63	2.60 $\pm$ 0.09	41 $\pm$ 4	No
	50 κ 50 ι + BH	12196 $\pm$ 155	1.48 $\pm$ 0.13	47 $\pm$ 2	No
	T-F	1442 $\pm$ 22	0.74 $\pm$ 0.06	15 $\pm$ 1	No
	T-F + BH	341 $\pm$ 10	1.66 $\pm$ 0.08	35 $\pm$ 3	Yes
	UT-F	190 $\pm$ 2	1.44 $\pm$ 0.08	167 $\pm$ 7	No
	UT-F + BH	405 $\pm$ 15	0.93 $\pm$ 0.08	151 $\pm$ 12	No
Group 3	90 κ 10 ι	6707 $\pm$ 670	2.64 $\pm$ 0.16	127 $\pm$ 8	Yes
	90 κ 10 ι + BH	28329 $\pm$ 581	1.30 $\pm$ 0.04	28 $\pm$ 1	No
	T-J	13369 $\pm$ 333	4.34 $\pm$ 0.34	8 $\pm$ 1	No
	T-J + BH	23945 $\pm$ 875	0.49 $\pm$ 0.02	6.9 $\pm$ 0.3	No
	UT-J	9046 $\pm$ 158	1.10 $\pm$ 0.14	116 $\pm$ 9	No then Yes
	UT-J + BH	9503 $\pm$ 135	5.50 $\pm$ 0.27	63 $\pm$ 11	Yes

*The strain sweep analysis of these samples revealed certain inconsistencies or experimental artifacts, evidenced by the lack of a clear LVE region (see Figures A.9 A and B, in Appendix A.8). This limitation can affect the accurate determination of key parameters such as the linear elastic modulus (G_0) and the limiting strain (γ_L).

Impact of the Biohybrid Material

The addition of the biohybrid material to GC samples exhibited varying effects on their rheological properties, primarily influenced by the carrageenan's chemical composition, particularly its ι - or κ -content, and the source of the polysaccharide (commercial blend, treated, or untreated hybrid carrageenan).

In Group 1 (high ι -content samples), the incorporation of the biohybrid material had minimal impact in the elastic modulus (G_0) and flow point (γ_F). However, it notably influenced the limiting strain (γ_L) values. The BH reduced γ_L in commercial blends and untreated hybrid carrageenans, while increasing it in alkali-treated hybrid carrageenans. The lower γ_L values coincided with cases where mechanical spectra exhibited disturbances at low strains, lacking a well-defined linear viscoelastic region (Figure A.9, Appendix A.8), and likely compromising the accuracy of measurements. This suggests that these reductions are more attributable to experimental artifacts or equipment limitations than actual changes in the mechanical properties.

In Groups 2 (balanced κ/ι ratios) and 3 (high κ -content), the addition of the BH generally led to increased G_0 , particularly in commercial blends and κ -rich samples. For example, in the 90 κ 10 ι commercial blend, G_0 increased significantly from 6707 ± 670 Pa to 28329 ± 581 Pa with the BH addition. However, this often came at the cost of reduced structural resilience, with gels experiencing irreversible damage at lower strains. This trade-off highlights the BH's potential to enhance stiffness while diminishing resistance to deformation.

However, exceptions to these trends were observed. Sample T-F exhibited a pronounced decrease in G_0 (from 1442 ± 22 Pa to 341 ± 10 Pa), but demonstrated increasing resistance to deformation at higher strains attributed to a clear strain-hardening response. Another exception was sample UT-J, where the addition of the biohybrid caused a marked increase in γ_L , from 1.10 ± 0.14 % to 5.50 ± 0.27 %, suggesting an improvement in strain tolerance along with stronger gel structures. These exceptions underscore the variability of BH interactions with different formulations and the need for careful optimization.

Regarding the flow point, the addition of the biohybrid did not significantly alter this parameter for Group 2 samples or sample T-J from Group 3. However, samples 90 κ 10 ι and UT-J experienced an earlier transition from solid-like to liquid-like behavior, indicating reduced resistance to flow in these cases.

Impact of the Chemical Composition

Increasing the κ -content in GC samples generally resulted in stiffer gels (higher G_0), but also more brittle behavior, as indicated by lower flow points. However, the limiting strain did not exhibit straightforward correlations with κ -content.

For commercial blends, γ_L was highest in ι -rich samples but appeared largely independent of chemical composition for κ -contents exceeding 50 mol.%. For hybrid carrageenans, the response to strain varied more intricately with κ -content and BH presence. Treated samples typically maintained elasticity over a

broader range of strains as κ -content increased, whereas untreated samples tended to lose structural integrity more quickly. Interestingly, the BH reversed these trends: it reduced γ_L in treated samples but increased it in untreated samples as κ -content rose. These observations highlight the nuanced interplay between chemical composition, BH presence, and gel network behavior.

An intriguing strain-hardening response to increasing strain was observed in some samples, particularly those from Group 1 (high ι -content), where samples consistently exhibited softening beyond their LVE region, followed by a transient phase of strain hardening, forming an apparent plateau. This response likely reflects the initial disruption of weaker physical interactions within the gel network, followed by temporary reorganization or alignment of polysaccharide chains into a more stress-resistant configuration, until the eventual collapse of the structure's integrity. These mechanisms highlight the complexity of carrageenan gel networks, where nonlinear elastic effects and microstructural rearrangements can momentarily counteract deformation before the network ultimately breaks down (Hilliou, 2021; Moraes & Hilliou, 2024).

Impact of Carrageenan Source

The source of polysaccharide—whether a commercial blend, untreated hybrid carrageenan, or alkali-treated hybrid carrageenan—exerts a notable influence on the rheological properties of GC samples, particularly their elastic modulus and resistance to deformation.

Commercial blends consistently exhibited higher G_0 values compared to hybrid carrageenan samples, highlighting their ability to form stiffer gels. Among the hybrid samples, alkali-treated carrageenans generally promoted stronger gels relative to untreated samples. However, exceptions to this trend were observed, particularly in Group 1 (high ι -content). In this group, untreated hybrid carrageenan samples (*e.g.*, UT-C, with or without the BH) surpassed the elastic modulus of their treated counterparts (*e.g.*, T-C). Conversely, in Group 2 (balanced κ/ι ratios), the addition of the BH introduced additional complexity, as it altered the expected trends in G_0 .

The variability in the resistance to deformation (γ_L) further complicates conclusions regarding the impact of the polysaccharide source. The strain resistance of gels appeared to be highly dependent on the chemical composition, including κ/ι ratios, as well as the incorporation of additives such as the BH. This variability underscores the complexity of these systems and the challenge of generalizing the effect of polysaccharide sources on strain resistance.

Overall, the choice between untreated and treated hybrid carrageenans should be guided by the specific rheological properties desired for the final formulation. For example, in formulations with higher κ -content, it may not be necessary to use treated hybrid carrageenans to achieve higher G_0 values, as untreated samples can sometimes yield comparable or even superior stiffness. However, the decision must also consider the desired strain resistance and other viscoelastic properties, as these are equally influenced by the chemical composition, the presence of biosynthetic precursors, and the inclusion of additives.

General Conclusion

The interplay between polysaccharide source, chemical composition, and additives must be carefully considered to tailor gel properties to specific applications. Whether the goal is to maximize stiffness, enhance strain resistance, or achieve a balanced combination, each factor must align with the intended functionality of the final product. For gummy formulations, decisions regarding polysaccharide source and treatment are highly context-dependent, requiring a clear understanding of desired textural and mechanical properties.

However, while rheological measurements provide essential insights into gel behavior under stress, they capture only part of the story. Additional tests, such as texture profile analysis (TPA), shelf-life studies, microstructural analysis (*e.g.*, scanning electron microscopy, SEM) and techniques to assess thermal stability (*e.g.*, differential scanning calorimetry, DSC), are needed to fully understand the complexity of texture and stability in food applications (Mahat et al., 2020; Peleg, 2019; V. Sharma & Bhardwaj, 2019). Sensory testing with trained panels or consumer surveys could also provide valuable qualitative feedback on the texture, flavor, and overall satisfaction with the product. Integrating rheological data with these broader testing methods would offer a more comprehensive assessment of gummy performance, ensuring the final product meets both technical requirements and consumer expectations.

4.3.4 Chemical Interactions (FTIR-ATR)

The Fourier transform-infrared (FTIR) spectrum is a well-known method for detecting similarities and differences in chemical structures. Understanding whether the addition of a biohybrid material causes structural and chemical changes within the matrix is crucial for industrial applications, as it directly impacts the product's physical properties, stability, and functionality. It also ensures consistency in product quality and aids in predicting scalability challenges. Therefore, the GC samples were characterized using FTIR-ATR to infer possible chemical interactions between the ingredients.

This analysis was conducted for all the conditions studied: untreated-KI, alkali treated-KI, and respective commercial K+I blend, with and without the addition of the BH, using KI samples C, F and J. Since the general FTIR spectra was similar for the three systems: C, F and J, only sample J is discussed in detail (Figure 20). The spectra for systems using samples C and F are shown in Figures A.10 and A.11, respectively, in Appendix A.9.

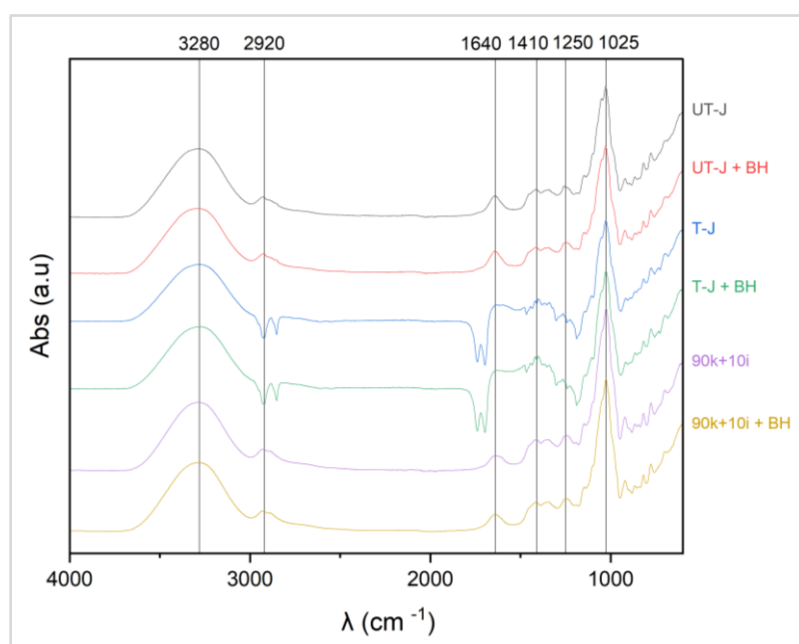


Figure 20: FTIR-ATR spectra of gummy candies prepared, using untreated carrageenan (UT-J), alkali treated carrageenan (T-J) and the chemical equivalent commercial blend (90k10i), with and without the biohybrid material (BH), for sample J.

The absorbance peaks found in the FTIR spectra (Figure 20) were interpreted considering the ingredients of the formulations: water, carrageenans, xanthan gum, honey and the biohybrid material, composed of bentonite and anthocyanins. Firstly, no differences were reported between samples with and without the BH, confirming that its addition did not cause significant chemical alterations within the matrix. This was an expected outcome due to the low concentration of BH used (only 0.5 wt.%), pointing towards a possible limit of sensitivity of FTIR to detect such chemical interactions.

The broad band at 3280 cm^{-1} is attributed to O-H stretching vibrations, which reflects the presence of water (Ilyas et al., 2016; Kędzierska-Matysek et al., 2018) and hydroxyl groups from carbohydrates (honey, xanthan, and carrageenans) (Damto et al., 2023; Kędzierska-Matysek et al., 2018; Said et al., 2021). The smaller peak found between 2880 and 2920 cm^{-1} arises from C-H stretching vibrations, indicating the presence of aliphatic chains from sugars in honey and polysaccharides like xanthan gum

and carrageenans (Kędzierska-Matysek et al., 2018; Pereira, Amado, et al., 2009; Rochas et al., 1986; Said et al., 2021).

The prominent peak at 1640 cm^{-1} corresponds to the O-H bending mode of water (Ilyas et al., 2016; Kędzierska-Matysek et al., 2018). The bands registered between 1350 and 1450 cm^{-1} are characteristic for bending vibration of O-CH and C-C-H in the carbohydrate structure or bending vibration coming from OH in the C-OH grouping, whereas the band at 1250 cm^{-1} is, in turn, a vibration characteristic for the stretching vibration of the C-H or CO in carbohydrates (Kędzierska-Matysek et al., 2018; Damto et al., 2023). Another possibility is that these FTIR bands, specifically at 1370 cm^{-1} and in the 1240 – 1260 cm^{-1} , range are related to the presence of sulfate esters in carrageenans and other sulfated polysaccharides, arising from vibrational modes of the S=O bond (Pereira, Amado, et al., 2009; Rochas et al., 1986).

The visible elbow at approximately 1050 cm^{-1} (next to the characteristic band at 1025 cm^{-1}) may be due to stretching vibration of C-O-C esters (present in honey and anthocyanins, while the band at 1025 cm^{-1} is associated with C-O stretching vibrations (Kędzierska-Matysek et al., 2018; Merz et al., 2020; Swer et al., 2018). In the region between 950 and 750 cm^{-1} , the peaks are likely due to C-O-S and C-O stretching from the 3,6-anhydrogalactose units and sulfate groups in carrageenans, as well as saccharide ring vibrations and C-H deformation, characteristic to the anomeric region of carbohydrates (Damto et al., 2023; Gómez-Ordóñez & Rupérez, 2011; Kędzierska-Matysek et al., 2018; Pereira, Amado, et al., 2009).

Formulations using alkali-treated carrageenans (T-J and T-J + BH) exhibited slight deviations compared to the other two systems under study. Notably, a minor shift was observed in the peak assigned to the C-H stretching vibration associated with the total sugar content, with a more pronounced band appearing around 2880 cm^{-1} . Additionally, a new peak emerged approximately at 1700 cm^{-1} , and instead of a single peak at 1250 cm^{-1} , the alkali-treated samples displayed two distinct peaks (at around 1200 cm^{-1} and 1300 cm^{-1}). These observations are consistent with the conversion of hydroxyl groups into carbonyl or ester groups, likely induced by the alkaline environment. The appearance of the carbonyl stretching band ($\approx 1700\text{ cm}^{-1}$) and the splitting of the C-O stretching region (≈ 1200 – 1300 cm^{-1}) are characteristic of esterification or structural rearrangements in polysaccharides (Mobaraki & Hemmateenejad, 2011; Smith, 2018).

In conclusion, while the incorporation of the biohybrid material imposed some changes in the rheological behavior of GC formulations, there were no visible differences in the FTIR spectra, indicating that no new chemical bonds were formed.

5. CONCLUSIONS AND FUTURE WORK

Selected hybrid carrageenans (KI), along with their chemically equivalent commercial blends (K+I), were evaluated through phase diagrams under comparable salt and polysaccharide concentrations. Gel formation occurred more readily in K+I than in KI carrageenans, across almost all chemical compositions (in terms of κ and ι content), polysaccharide concentrations, and ionic strengths, whereas KI carrageenans showed a higher dependency with those parameters. KI carrageenans demonstrated gelation behavior comparable to K+I under specific conditions, such as higher polysaccharide concentrations for ι -rich samples and κ/ι -balanced samples in KCl and NaCl, and lower ionic strengths for κ -rich samples in KCl. K-2 carrageenans displayed salt-specificity, forming gels in KCl but not in NaCl, highlighting their limited gelling potential in Na^+ environments.

KI and K+I exhibited comparable cooling rheological profiles under identical conditions, particularly in KCl at large ionic strengths. Higher ι -carrageenan content and lower ionic strengths favored two-step gelation mechanisms, while higher κ -content and ionic strengths resulted in single-step gelation. Experimental limitations, such as restricted temperature ranges and strain sensitivity, likely obscured subtle transitions in some systems. Future research should employ advanced rheological techniques with improved control of gelation kinetics for diverse applications.

Under Large Amplitude Oscillatory Shear (LAOS) testing KI and K+I yielded distinct mechanical properties under identical ionic environments and polysaccharide concentrations. K+I generally produced stronger and stiffer gels than KI. However, under high ionic strengths, the K-2 hybrid carrageenan significantly outperformed its commercial counterpart in gel elasticity. Similarly, at low ionic strengths, certain hybrids exhibited comparable or even superior elasticity. Increased elasticity often came at the expense of reduced resistance to deformation, leading to greater gel brittleness. This trade-off underscores the importance of aligning the choice of carrageenan system with the specific functional requirements of the target application. Ultimately, the viability of hybrid carrageenans as substitutes for commercial blends depends on the desired balance between gel strength, elasticity, and deformation resistance.

In gummy candy formulations, both KI and K+I carrageenans successfully produced gel-like textures across all systems. KI demonstrated potential as effective replacements for K+I, regarding thermal performance, offering a T_g range (65 °C–100 °C) that balances processing flexibility and product stability. In terms of mechanical properties, K+I produced stiffer gels (G_0) with superior strength and elasticity than

KI, consistent with prior findings. Alkaline treatment generally enhanced the stiffness and strain resistance of hybrid carrageenans, except for high ι-content samples, where untreated carrageenans outperformed their treated counterparts. This suggests that while precursor removal is often beneficial, the additional alkali treatment step may be unnecessary for specific applications. The incorporation of the biohybrid material often increased stiffness but reduced strain resistance, particularly in treated hybrids and commercial samples with greater κ-content.

These findings underscore the intricate interplay between carrageenan source, chemical composition, and additives in shaping gel performance, emphasizing the need for formulation-specific optimization. Hybrid carrageenans show significant potential as clean-label, vegan, and functional food ingredients, combining structural and health-promoting benefits. Refining formulations and processing conditions, alongside further evaluations of texture, thermal stability, and sensory properties, will be key to ensure the final product meets both technical and consumer requirements.

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A. APPENDIXES

A.1 FTIR-ATR Spectra of NaOH-treated Samples

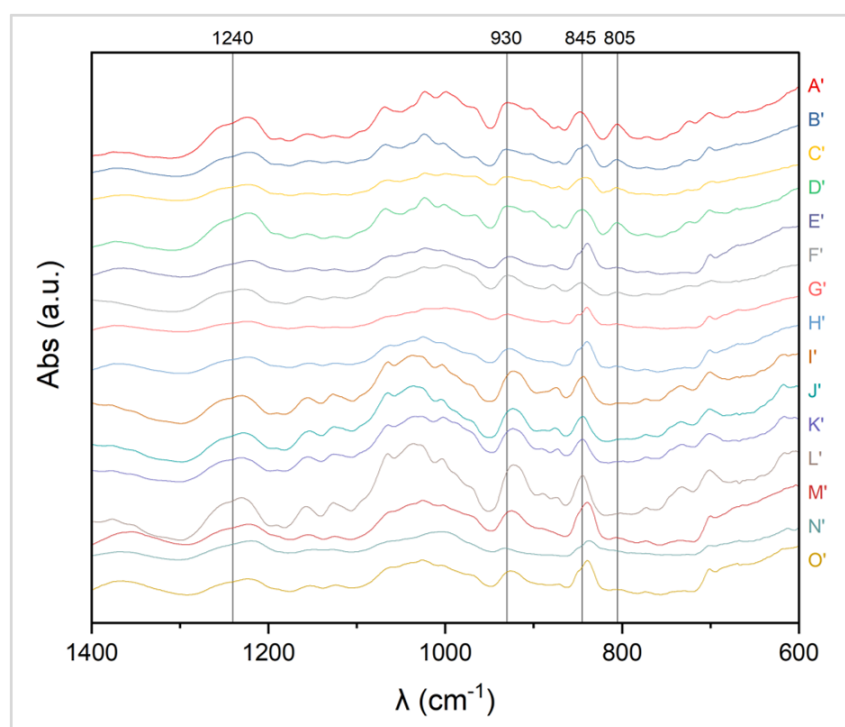


Figure A.1: FTIR-ATR spectra of hybrid carrageenan samples treated with NaOH, as well as most characteristic bands of carrageenans: 1240, 930, 845 and 805 cm^{-1} .

A.2 Temperature Dependence of the Storage (G') and Loss (G'') Moduli

A.2.1 Temperature Dependence of the storage (G') and loss (G'') moduli, for samples in KCl

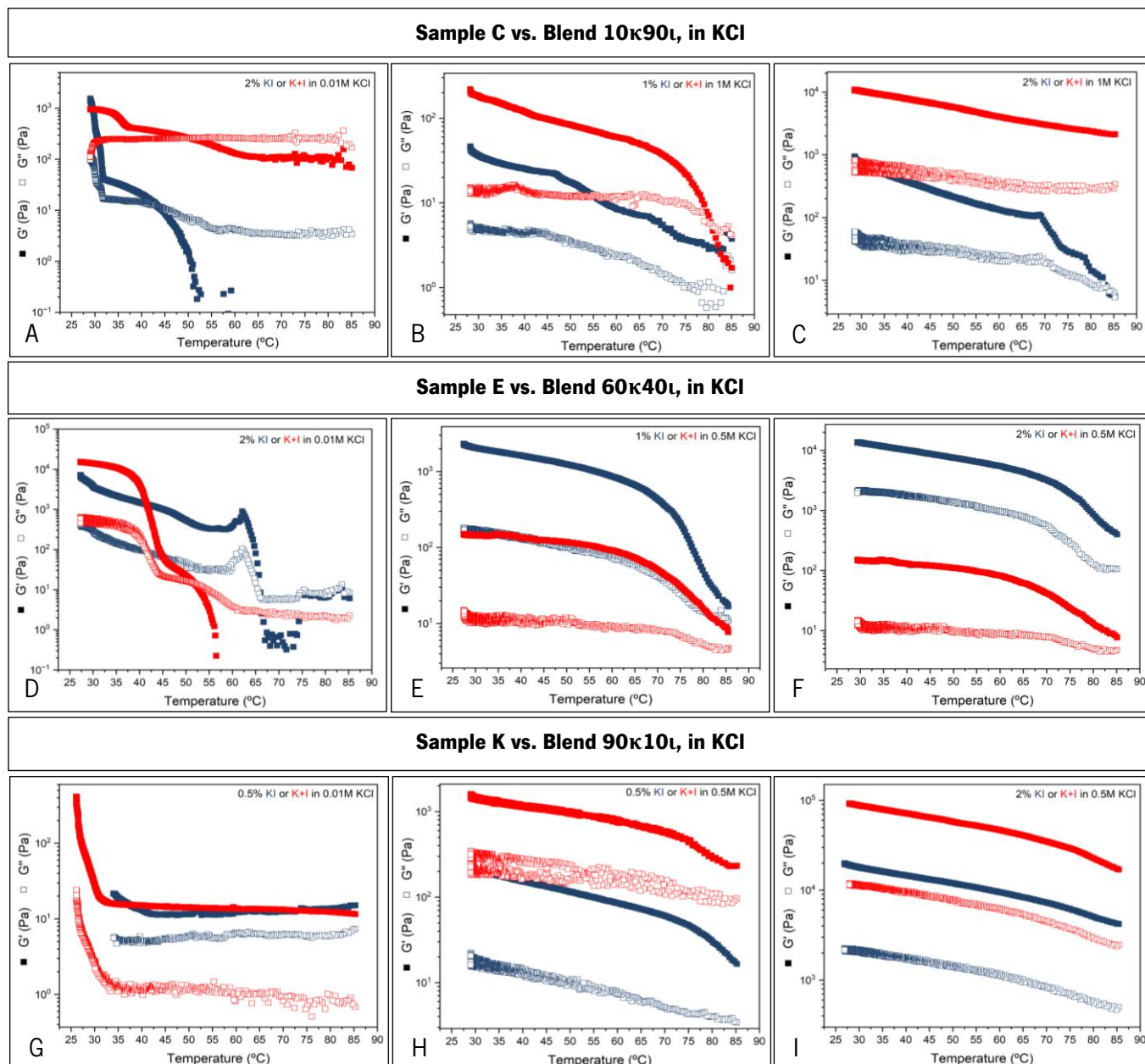


Figure A.2: Temperature dependence of the storage modulus - G' (full squares) and loss modulus - G'' (open squares) of hybrid carrageenan (KI) samples (in blue) compared to their respective commercial K+I carrageenan blends (in red) at various polymer concentrations and ionic strengths in KCl solutions. Each row represents a different hybrid carrageenan and blend pair, while each column shows results at different concentrations and ionic strengths. **Top row** - sample C vs. 10κ90ι at **A)** 2 wt.% KI or K+I in 0.01 M KCl; **B)** 1 wt.% KI or K+I in 1 M KCl; **C)** 2 wt.% KI or K+I in 1 M KCl; **middle row** - sample E vs. 60κ40ι at **D)** 2 wt.% KI or K+I in 0.01 M KCl; **E)** 1 wt.% KI or K+I in 0.5 M KCl; **F)** 2 wt.% KI or K+I in 0.5 M KCl; and **bottom row** - sample K vs. 90κ10ι at **G)** 0.5 wt.% KI or K+I in 0.01 M KCl; **H)** 0.5 wt.% KI or K+I in 0.5 M KCl; **I)** 2 wt.% KI or K+I in 0.5 M KCl.

A.2.2 Temperature Dependence of the storage (G') and loss (G'') moduli, for samples in NaCl

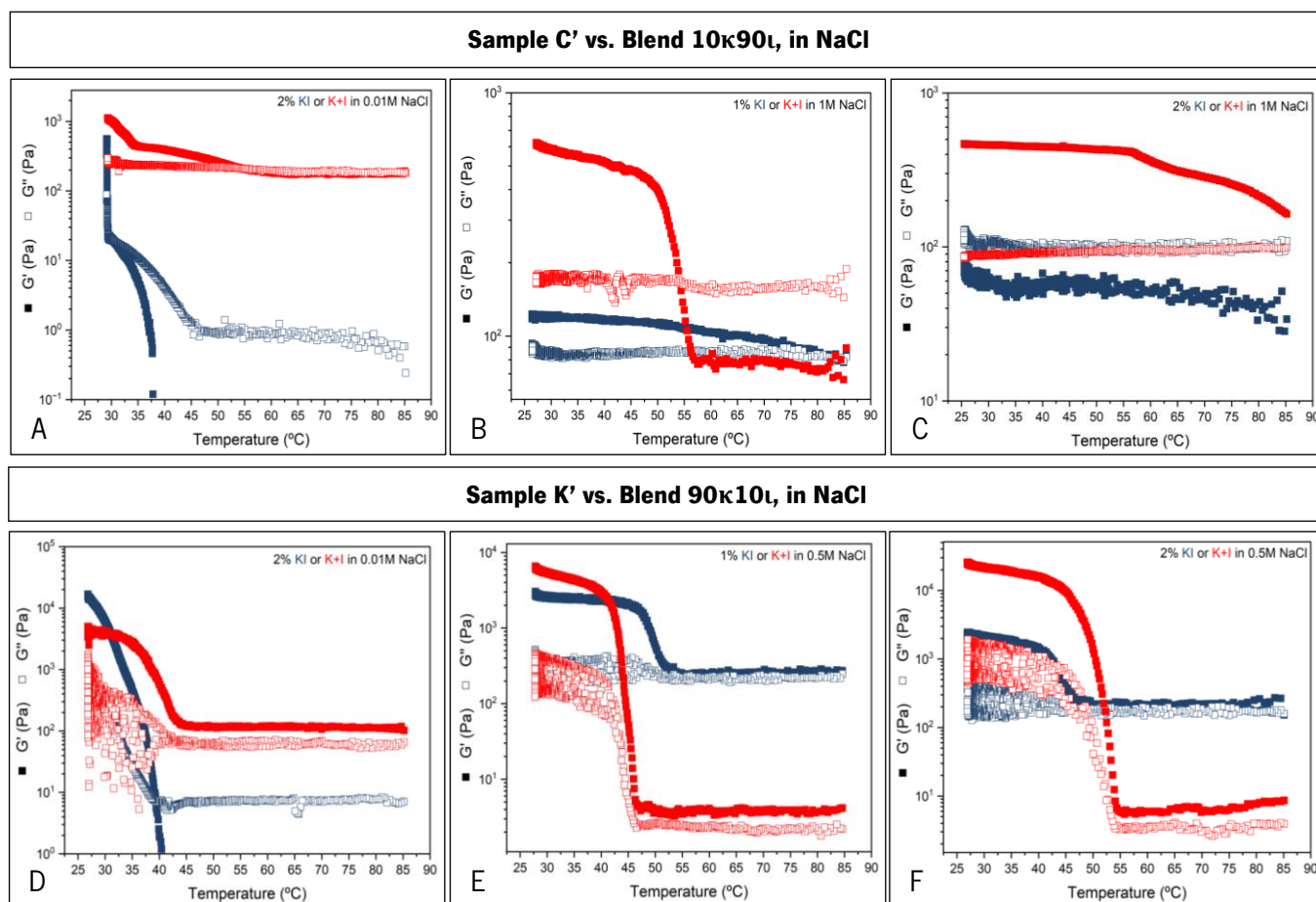


Figure A.3: Temperature dependence of the storage modulus - G' (full squares) and loss modulus - G'' (open squares) of hybrid carrageenan (KI) samples (in blue) compared to their respective commercial K+I carrageenan blends (in red) at various polymer concentrations and ionic strengths in NaCl solutions. Each row represents a different hybrid carrageenan and blend pair, while each column shows results at different concentrations and ionic strengths. **Top row** - sample C' vs. 10κ90ι at **A)** 2 wt.% KI or K+I in 0.01 M KCl; **B)** 1 wt.% KI or K+I in 1 M KCl; **C)** 2 wt.% KI or K+I in 1 M KCl; and **bottom row** - sample K' vs. 90κ10ι at **D)** 2 wt.% KI or K+I in 0.01 M KCl; **E)** 1 wt.% KI or K+I in 0.5 M KCl; **F)** 2 wt.% KI or K+I in 0.5 M KCl.

A.3 Mechanical Spectra of Samples in KCl

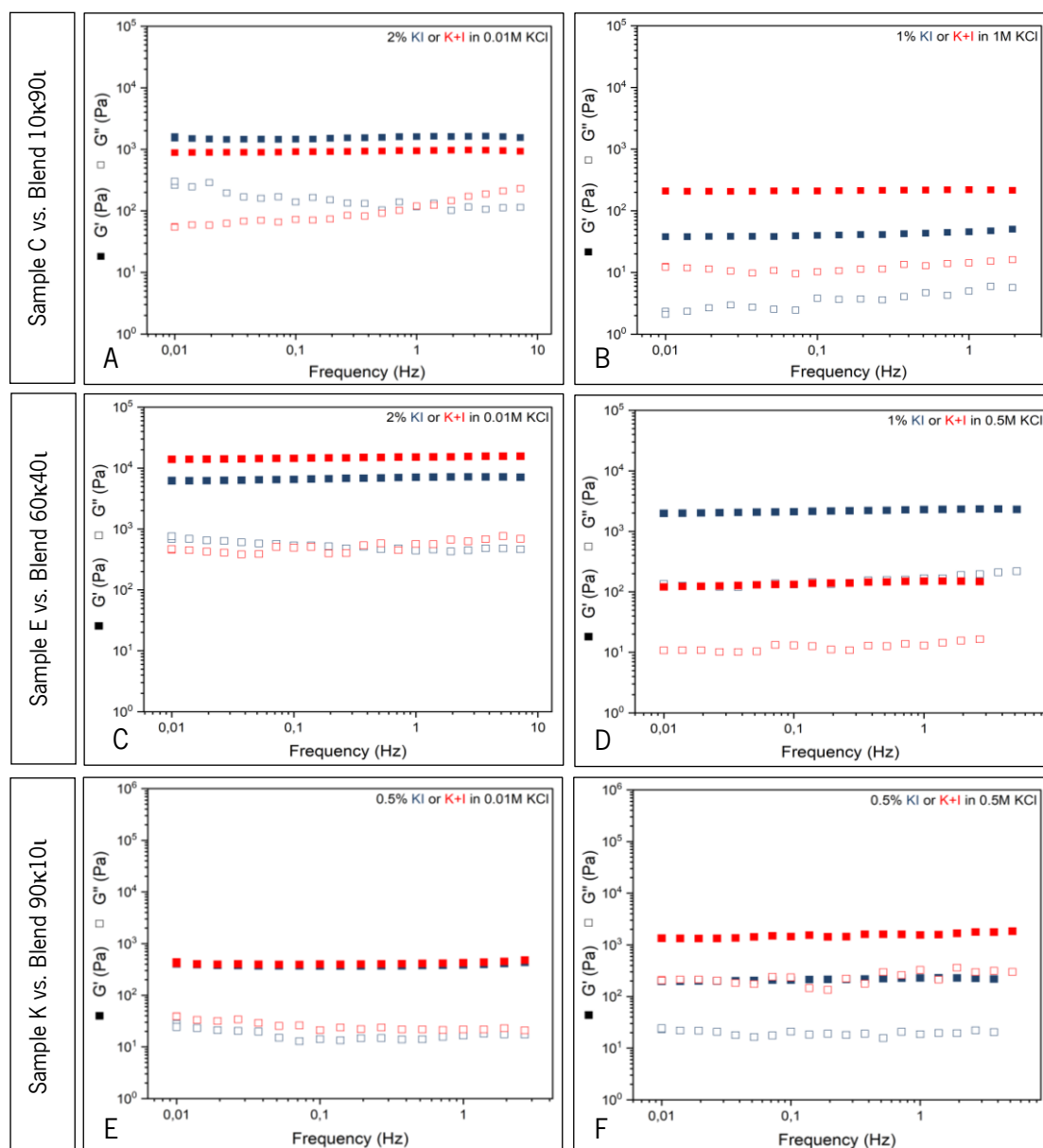


Figure A.4: Mechanical spectra (storage modulus G' , full squares; loss modulus G'' , open squares) of hybrid carrageenan (KI) samples (in blue) compared to their respective commercial K+I carrageenan blends (in red) at various polymer concentrations and ionic strengths in KCl solutions. Each row represents a different hybrid carrageenan and blend pair, and each column shows results at different concentrations and ionic strengths. **Top row** - sample C vs. 10κ90ι at **A)** 2 wt.% KI or K+I in 0.01 M KCl; **B)** 1 wt.% KI or K+I in 1 M KCl; **middle row** - sample E vs. 60κ40ι at **C)** 2 wt.% KI or K+I in 0.01 M KCl; **D)** 1 wt.% KI or K+I in 0.5 M KCl; and **bottom row** - sample K vs. 90κ10ι at **E)** 0.5 wt.% KI or K+I in 0.01 M KCl; **F)** 0.5 wt.% KI or K+I in 0.5 M KCl.

A.4 Large Amplitude Oscillatory Shear Tests

A.4.1 Large Amplitude Oscillatory Shear Tests of Samples in KCl

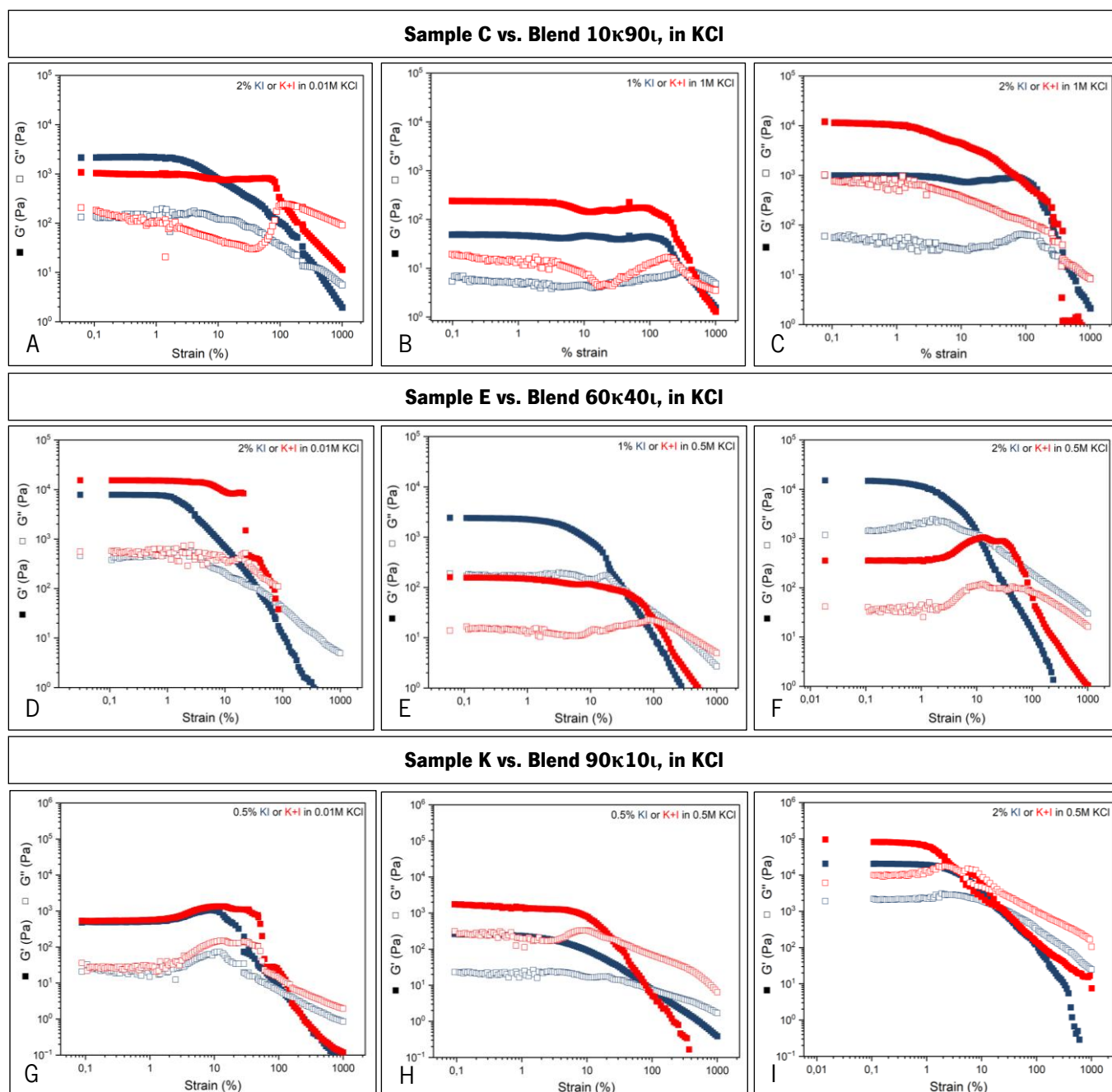


Figure A.5: Large amplitude oscillatory shear tests (storage modulus G' , full squares; loss modulus G'' , open squares, as a function of the applied strain) of hybrid carrageenan (KI) samples (in blue) compared to their respective commercial K+I carrageenan blends (in red) at various polymer concentrations and ionic strengths in KCl solutions. Each row represents a different hybrid carrageenan and blend pair, while each column shows results at different concentrations and ionic strengths. **Top row** - sample C vs. 10κ90_l at **A**) 2 wt.% KI or K+I in 0.01 M KCl; **B**) 1 wt.% KI or K+I in 1 M KCl; **C**) 2 wt.% KI or K+I in 1 M KCl; **middle row** - sample E vs. 60κ40_l at **D**) 2 wt.% KI or K+I in 0.01 M KCl; **E**) 1 wt.% KI or K+I in 0.5 M KCl; **F**) 2 wt.% KI or K+I in 0.5 M KCl; and **bottom row** - sample K vs. 90κ10_l at **G**) 0.5 wt.% KI or K+I in 0.01 M KCl; **H**) 0.5 wt.% KI or K+I in 0.5 M KCl; **I**) 2 wt.% KI or K+I in 0.5 M KCl.

A.4.2 Large Amplitude Oscillatory Shear Tests of Samples in NaCl

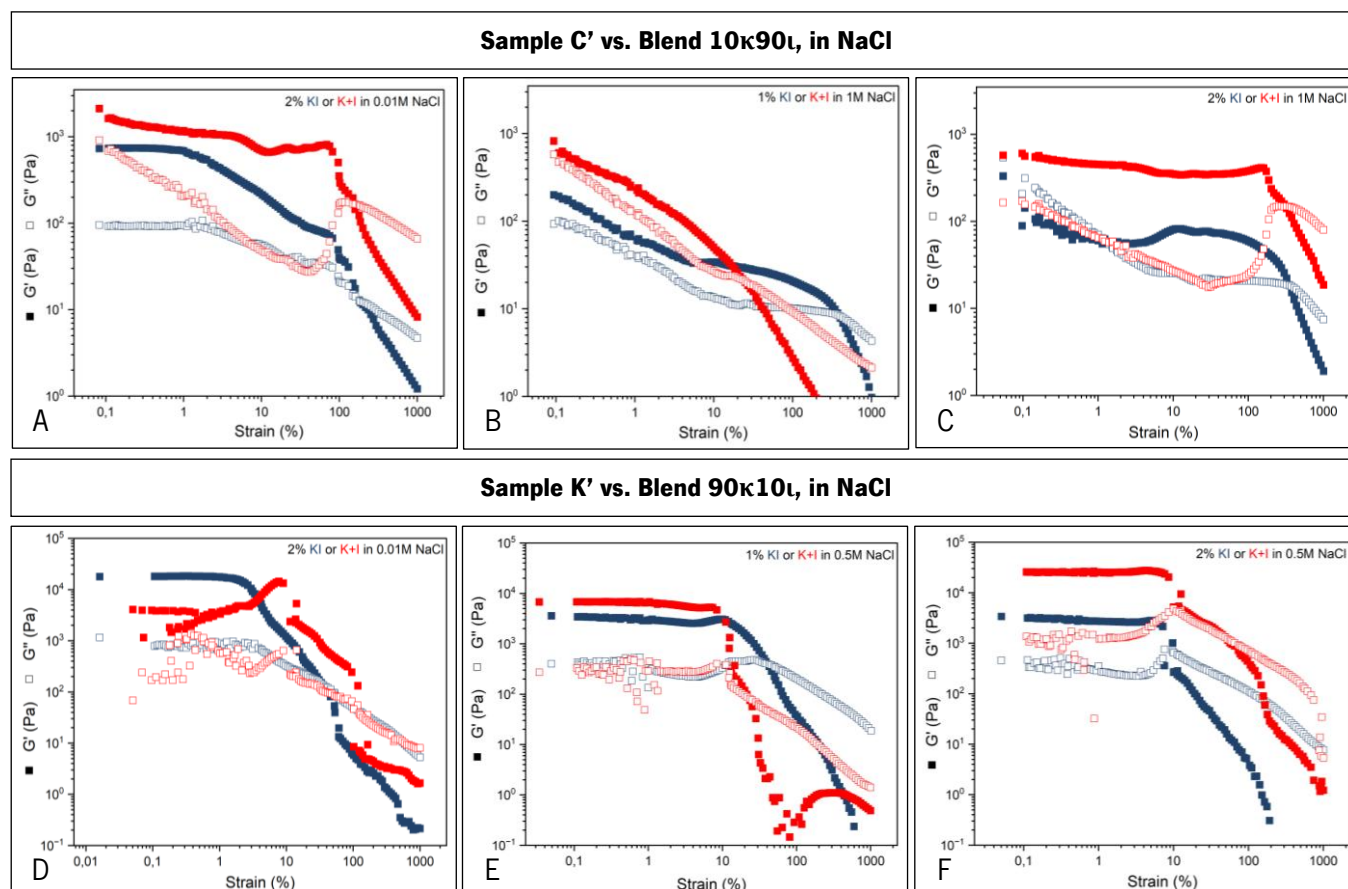


Figure A.6: Large amplitude oscillatory shear tests (storage modulus G' , full squares; loss modulus G'' , open squares, as a function of the applied strain) of hybrid carrageenan (KI) samples (in blue) compared to their respective commercial K+I carrageenan blends (in red) at various polymer concentrations and ionic strengths in NaCl solutions. Each row represents a different hybrid carrageenan and blend pair, while each column shows results at different concentrations and ionic strengths. **Top row** - sample C' vs. 10κ90ι at **A**) 2 wt.% KI or K+I in 0.01 M KCl; **B**) 1 wt.% KI or K+I in 1 M KCl; **C**) 2 wt.% KI or K+I in 1 M KCl; and **bottom row** - sample K' vs. 90κ10ι at **D**) 2 wt.% KI or K+I in 0.01 M KCl; **E**) 1 wt.% KI or K+I in 0.5 M KCl; **F**) 2 wt.% KI or K+I in 0.5 M KCl.

A.5 pH Values of Gummy Candies

Table A.1: pH values of gummy candies prepared with and without the biohybrid material (BH), as well as the pH of rosemary honey and the biohybrid used in the preparations

Sample			pH (T = 22 °C)
C	Untreated	UT-C	4.02
		UT-C + BH	3.48
	Alkali-Treated	T-C	7.99
		T-C + BH	7.90
	Blend	10κ90ι	4.45
		10κ90ι + BH	3.84
F	Untreated	UT-F	4.25
		UT-F + BH	3.65
	Alkali-Treated	T-F	7.58
		T-F + BH	7.79
	Blend	50κ50ι	4.38
		50κ50ι + BH	3.81
J	Untreated	UT-J	4.32
		UT-J + BH	3.71
	Alkali-Treated	T-J	7.75
		T-J + BH	7.64
	Blend	90κ10ι	4.73
		90κ10ι + BH	4.01
Rosemary Honey			3.54
BH + Distilled Water			3.88

A.6 Temperature Dependence of the Storage (G') and Loss (G'') Moduli in Gummy Candies

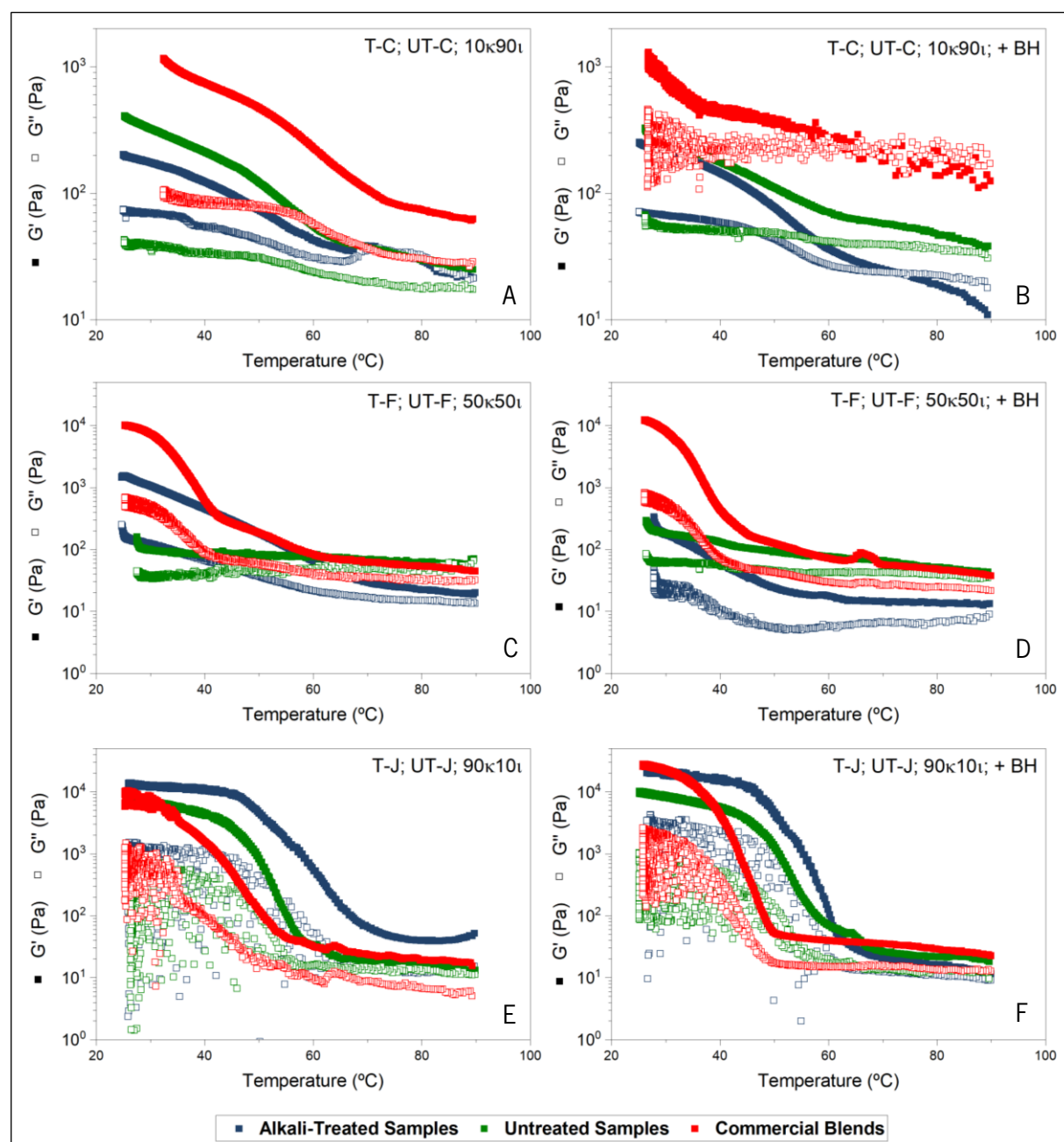


Figure A.7: Temperature dependence of the storage modulus (G' , full squares) and loss modulus (G'' , open squares) of gummy candy samples. Results are presented for commercial blends (in red), alkali-treated (T-) hybrid carrageenans (in blue) and untreated (UT-) hybrid carrageenans (in green). Each row represents a different set of samples based on their chemical composition: **top row (A, B)** 10κ90ι, T-C and UT-C; **middle row (C, D)** 50κ50ι, T-F and UT-F; **bottom row (E, F)** 90κ10ι, T-J and UT-J. Graphs in the right column (B, C, D) show the effects of incorporating the biohybrid material (+BH).

A.7 Mechanical Spectra of Gummy Candies

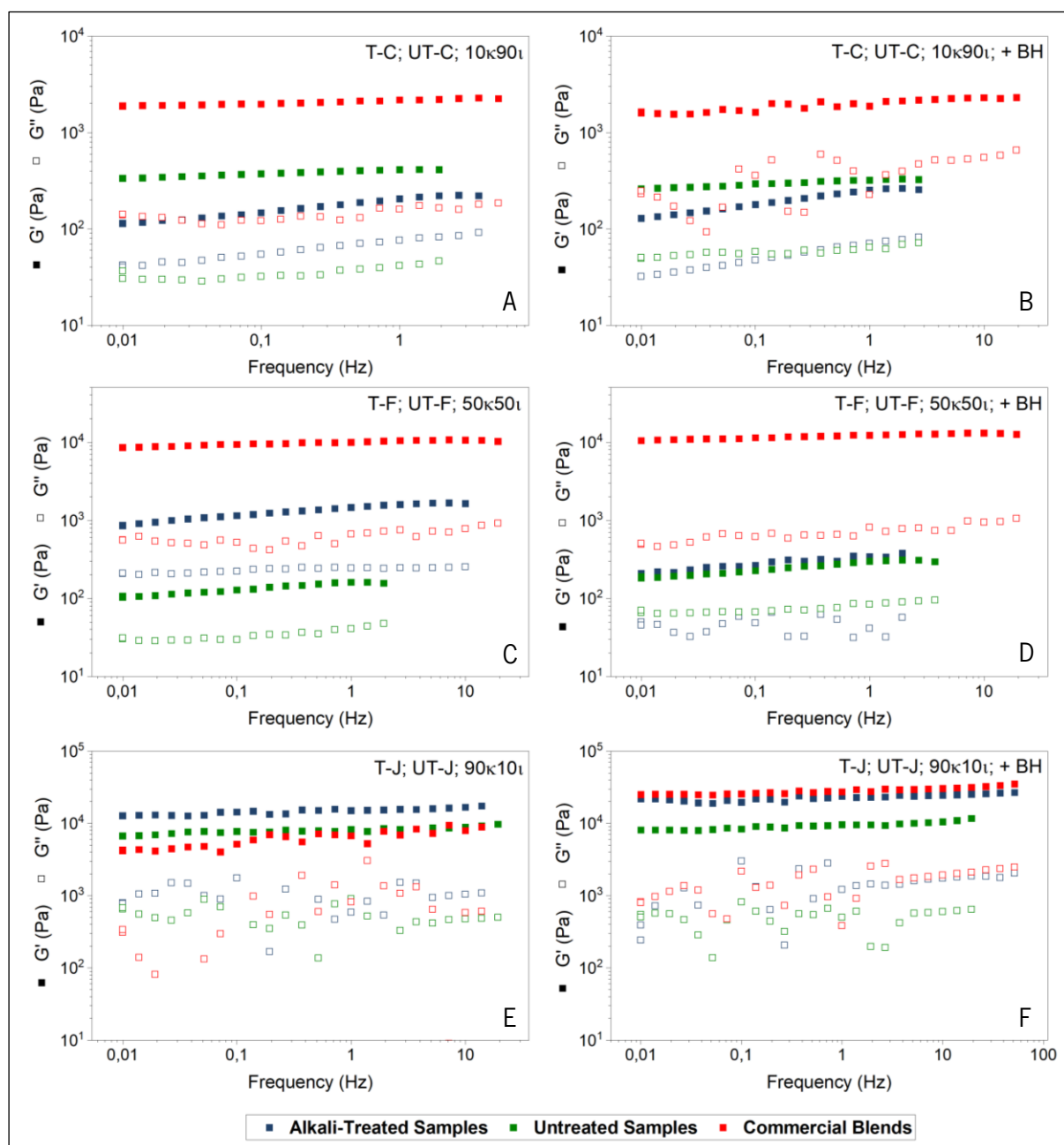


Figure A.8: Mechanical spectra of gummy candy samples, showing the storage modulus (G' , full squares) and loss modulus (G'' , open squares) as a function of frequency variation. Results are presented for commercial blends (in red), alkali-treated (T-) hybrid carrageenans (in blue) and untreated (UT-) hybrid carrageenans (in green). Each row represents a different set of samples based on their chemical composition: **top row (A, B)** 10κ90ι, T-C and UT-C; **middle row (C, D)** 50κ50ι, T-F and UT-F; **bottom row (E, F)** 90κ10ι, T-J and UT-J. Graphs in the right column (**B, C, D**) show the effects of incorporating the biohybrid material (+BH).

A.8 Large Amplitude Oscillatory Shear Tests of Gummy Candies

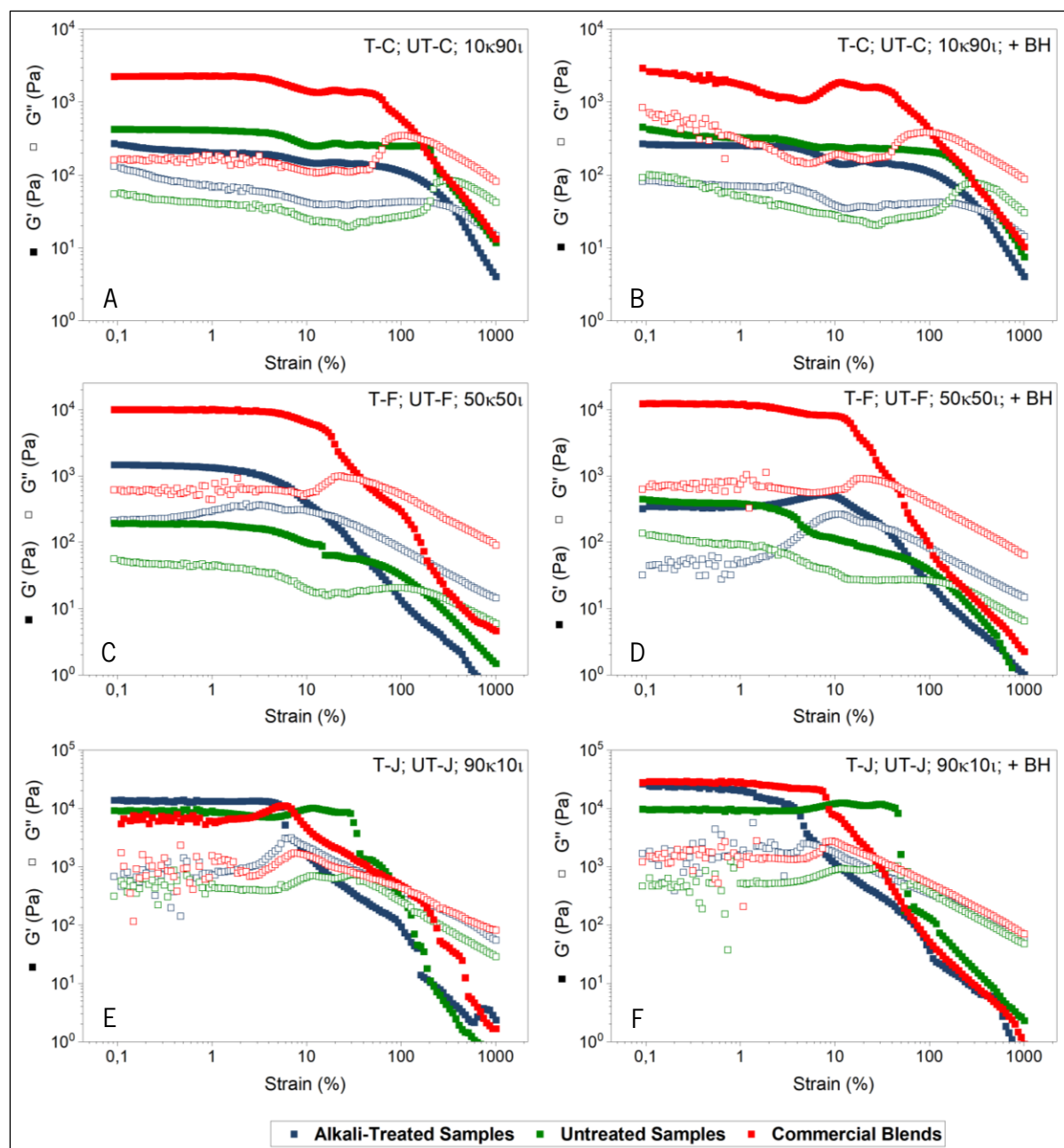


Figure A.9: Large amplitude oscillatory shear (LAOS) tests showing the storage modulus (G' , full squares) and loss modulus (G'' , open squares) as a function of applied strain for gummy candy samples. Results are presented for commercial blends (in red), alkali-treated (T-) hybrid carrageenans (in blue) and untreated (UT-) hybrid carrageenans (in green). Each row represents a different set of samples based on their chemical composition: **top row (A, B)** 10κ90ι, T-C and UT-C; **middle row (C, D)** 50κ50ι, T-F and UT-F; **bottom row (E, F)** 90κ10ι, T-J and UT-J. Graphs in the right column (**B, C, D**) show the effects of incorporating the biohybrid material (+BH).

A.9 FTIR-ATR Spectra of Gummy Candies

A.9.1 FTIR-ATR Spectra of Gummy Candies, for systems using Sample C and Commercial Blend 10κ90ι

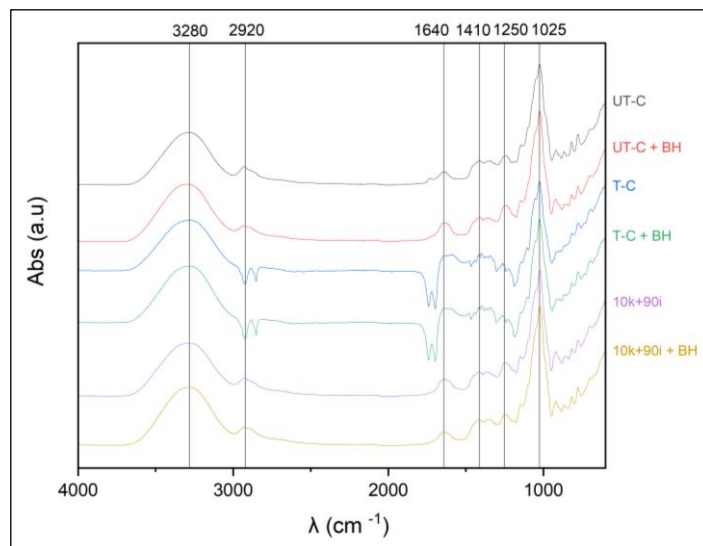


Figure A.10: FTIR-ATR spectra of gummy candies prepared, using untreated carrageenan (UT-C), alkali treated carrageenan (T-C) and the chemical equivalent commercial blend (10κ90ι), with and without the biohybrid material (BH), for sample C.

A.9.2 FTIR-ATR Spectra of Gummy Candies, for systems using Sample F and Commercial Blend 50κ50ι

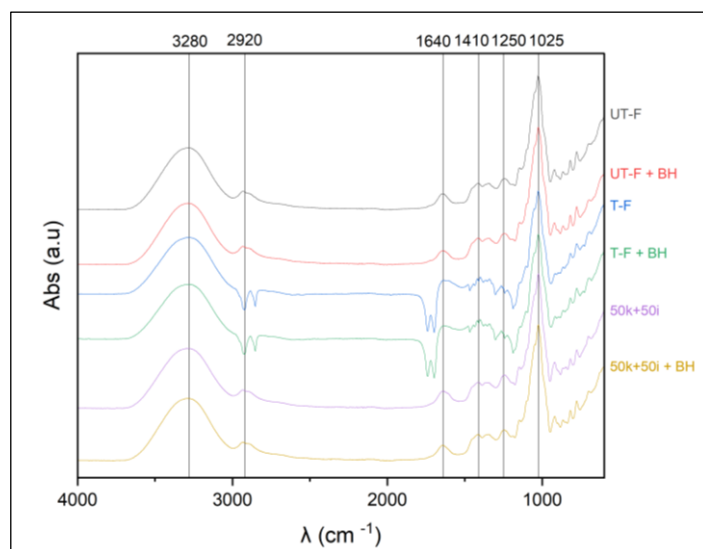


Figure A.11: FTIR-ATR spectra of gummy candies prepared, using untreated carrageenan (UT-F), alkali treated carrageenan (T-F) and the chemical equivalent commercial blend (50κ50ι), with and without the biohybrid material (BH), for sample F.

B. ANNEXES

B.1 Chemical Composition of Untreated Hybrid Carrageenans

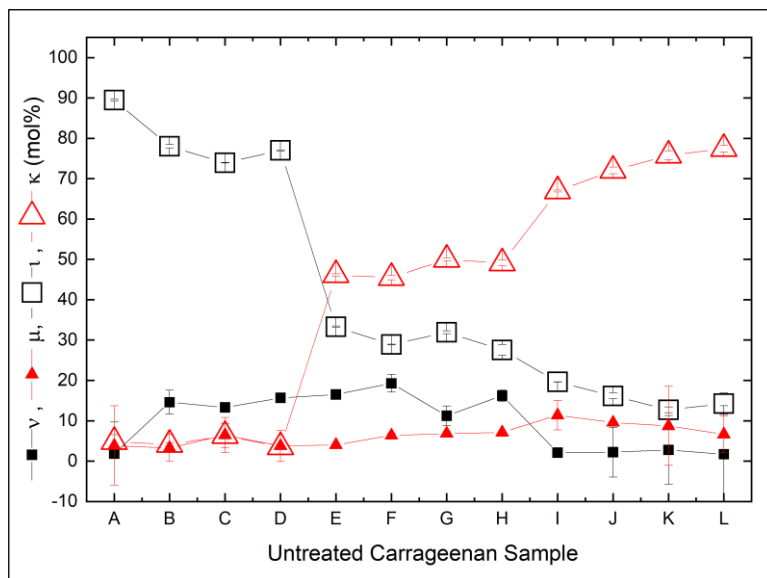


Figure B.1: Chemical composition of untreated hybrid carrageenan samples.

B.2 Molecular Mass Distribution of Untreated Hybrid Carrageenans and Commercial Pure K- and I-carrageenans

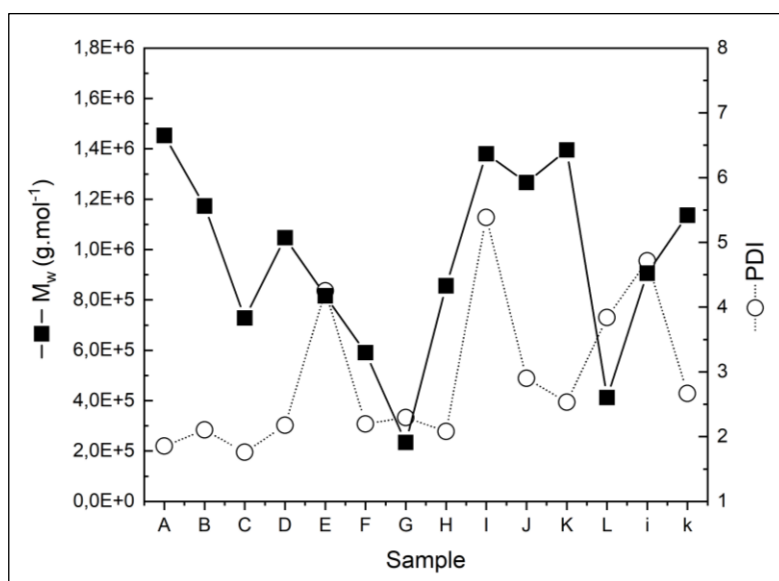


Figure B.2: Molecular mass distribution (M_w) and polydispersity index (PDI) of untreated samples (A–L), as well as commercial pure K- and I-carrageenans.