VALORIZATION OF MARINE BIOMASS FOR THE DEVELOPMENT OF BIOPOLYMERIC MATERIALS AND ADDITIVES WITH FUNCTIONAL PROPERTIES

VALORIZACIÓN DE BIOMASA MARINA PARA EL DESARROLLO DE MATERIALES BIOPOLIMÉRICOS Y ADITIVOS CON PROPIEDADES FUNCIONALES

DOCTORAL THESIS

Vera Cebrián Lloret

Directed by:
Dra. Amparo López Rubio
Dra. Marta Martínez Sanz
Dr. Antonio Martínez Abad

February 2024

iata Instituto de Agroquímica y Tecnología de Alimentos
EXCELENCIA SEVERO OCHOA
UNIVERSITAT POLITÈCNICA DE VALÈNCIA

CSIC
Consejo Superior de Investigaciones Científicas

Universitat Politècnica de València

TESIS DOCTORAL

Doctorado en Ciencia, Tecnología y Gestión Alimentaria

Vera Cebrián Lloret

Valorization of marine biomass for the development of biopolymeric materials and additives with functional properties

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Noviembre, 2023

AGRADECIMIENTOS

Llegado a este punto, quiero expresar mi agradecimiento a todos los que tanto de forma directa como indirecta han formado parte de esta tesis. Este camino ha estado lleno de desafíos y aprendizajes, y no puedo más que agradecer a aquellos que han sido mi soporte constante. Esto no habría sido posible sin el apoyo de cada uno de vosotros.

En primer lugar, agradezco a Hispanagar, en especial a Jose y Javier, por brindarme la oportunidad de realizar esta tesis.

Agradezco enormemente a mis directores, ya que, sin duda, esto no habría sido posible sin su apoyo. Marta, no sé qué habría hecho sin ti; gracias por tu paciencia infinita y por quererme tanto y tan bien. Verdaderamente, no puedo expresar lo agradecida que estoy de haberte encontrado en mi camino. Amparo, ojalá hubiera más personas como tú. Eres maravillosa, gracias por la confianza que me has brindado a lo largo de estos años y por guiarme en este camino siempre con una sonrisa en la cara. Antonio, gracias por estar ahí en todo momento y por animarme siempre con tus palabras llenas de bondad.

Quiero agradecer a Carlos, mi compañero de vida y mi mayor apoyo. Gracias por impulsar mis alas, incluso en los momentos en los que yo misma pensaba que no podía ni caminar. Formamos un gran equipo, ahora y siempre. Te amo.

Zaida y Cynthia, creo fervientemente que la vida sería mucho menos bonita si no os hubiera conocido. Os merecéis todo lo bueno que os traiga la vida, y ojalá esté yo ahí para verlo. Os quiero con toda el alma.

Hylenne, comenzamos este viaje juntas y vamos a terminarlo juntas. ¡Vamos, que ya se ve el final! Muchísimas gracias por todo tu apoyo y cariño a lo largo de estos años. Isaac, has sido un grandísimo apoyo aquí. Aprecio muchísimo las tardes enteras que has dedicado a escucharme e intentar que estuviera bien. Gracias. Daniel, gracias a tu sentido del humor único, has logrado que los días sean más alegres. Os quiero mucho a los tres.

Josep, María, Santi, Mercedes, Carlos, Laura, Eugenia, Alcira, Agustín y Mahrokh, muchísimas gracias por vuestro apoyo y por todos los buenos momentos que hemos compartido juntos, ya

sea en el laboratorio o fuera de él. Espero poder seguir viéndoos evolucionar y crecer durante muchos años más. Os adoro, sois maravillosos.

MJ, irradias luz. Todo a tu alrededor se vuelve más sencillo. Gracias por tu cariño y apoyo constante.

A toda la gente del CIAL, ya os siento como mi segunda casa. Muchísimas gracias por haberme apoyado en todo, espero que nos veamos pronto.

Por último, pero no menos importante, a Enrique, Magdalena, Alejandro y María, mi familia, mis mayores fans. Muchísimas gracias por estar siempre ahí. Sois lo mejor que tengo, mi mayor tesoro. Os quiero.

ABSTRACT

This doctoral thesis focused on the valorization of different sources of marine biomass with the main purpose of developing biopolymeric materials and ingredients for the food industry. Thus, in the first part of the thesis, the valorization of biomass from different algae species was carried out to identify compounds of interest and assess their potential to produce biopolymeric materials and protein-rich food ingredients. Among these species was *Ruguloperyx okamurae*, an invasive brown seaweed that poses a threat to marine ecosystems. However, this species was shown to have significant potential as a sustainable source of bioactive compounds, transforming its role as a threat into a valuable opportunity.

Red seaweed biomass was also highlighted for its potential in a variety of applications, including the development of cost-effective and environmentally friendly materials for food applications obtained through minimal processing. In this context, the composition and structure of the algal cell walls played a crucial role in understanding how each species behaves during processing and in the properties of the resulting films. In addition to the production of biopolymeric materials, red seaweeds were also studied as a source of proteins for human consumption. Although native seaweeds had low digestibility, this was improved through processing. However, it is important to note that, despite the improvement in digestibility, processing could induce the degradation of more labile amino acids, having a negative impact on the nutritional quality of the proteins.

In the second part of this thesis, several species of red seaweeds were explored to extract agar more efficiently by simplified methods, and the suitability of the extracts obtained to develop biopolymeric materials and hydrogels for food applications was evaluated. It could be observed that the degree of agar purification had a significant impact on the final properties of both films and hydrogels. In the case of films, those made of pure agar exhibited superior properties; however, they underwent significant changes in their semicrystalline structure during storage. In contrast, those films derived from less purified agar extracts showed greater stability over time, suggesting the potential of less purified agars as additives to reduce costs and improve the stability of pure agar films. Regarding the hydrogels, agar purification led to the formation of hydrogels with significantly higher strength and hardness. On the other hand, the simplified

extraction protocol produced less purified agar fractions containing other compounds such as proteins, polyphenols, and minerals. Although this resulted in less rigid and resistant hydrogels, this feature could be advantageous for the production of hydrogels with bioactive properties.

In the third and last part of the thesis, the valorization of industrial wastes generated after alginate extraction was addressed, with the purpose of obtaining cellulosic fractions by simplified methods for their application in the production of biopolymeric materials. In this context, it was observed that the residues of *Alaria esculenta* and *Saccharina latissima* were suitable for the extraction of cellulosic fractions, while *Ascophyllum nodosum* might be more interesting for the production of fucoidan-rich extracts. Cellulosic fractions with higher purity produced films with more desirable mechanical properties and visual appearance. Conversely, less purified fractions presented a greater barrier to water vapor. According to the results, it was determined that the simple application of an alkaline treatment could produce cellulosic fractions that resulted in films with an optimal balance between functional properties and economic and environmental efficiency in biopolymeric material production.

RESUMEN

Esta tesis doctoral se centró en la valorización de diferentes fuentes de biomasa marina con el propósito principal de desarrollar materiales biopoliméricos e ingredientes destinados a la industria alimentaria. Así pues, en la primera parte de la tesis, se llevó a cabo la valorización de la biomasa de diversas especies de algas con el objetivo de identificar compuestos de interés y evaluar su potencial para la producción de materiales biopoliméricos e ingredientes alimentarios ricos en proteínas. Entre estas especies se encontraba *Ruguloperyx okamurae*, un alga parda invasora que representa una amenaza para los ecosistemas marinos. No obstante, se demostró que esta especie posee un potencial significativo como fuente sostenible de compuestos bioactivos, transformando su papel de amenaza en una valiosa oportunidad. La biomasa de las algas rojas también destacó por su potencial en diversas aplicaciones, incluyendo el desarrollo de materiales rentables y respetuosos con el medio ambiente para aplicaciones alimentarias obtenidos mediante de un procesado mínimo. En este contexto, la composición y la estructura de las paredes celulares de las algas desempeñaron un papel fundamental para entender cómo se comporta cada especie durante el procesado y en las propiedades de las películas resultantes. Además de la producción de materiales biopoliméricos, también se estudiaron las algas rojas como fuente de proteínas para el consumo humano. Aunque las algas nativas presentaron una baja digestibilidad, esta mejoró mediante su procesado. No obstante, es importante destacar que, a pesar de esta mejora en la digestibilidad, el procesado podía inducir la degradación de los aminoácidos más lábiles, lo que repercutía negativamente en la calidad nutricional de las proteínas.

En la segunda parte de esta tesis, se exploraron varias especies de algas rojas para extraer agar de forma más eficiente mediante métodos simplificados, y se evaluó la idoneidad de los extractos obtenidos para desarrollar materiales biopoliméricos e hidrogeles para aplicaciones alimentarias. Se pudo observar que el grado de purificación del agar tuvo un impacto significativo en las propiedades finales tanto de las películas como de los hidrogeles. En el caso de las películas, las fabricadas con agar puro presentaron propiedades superiores; sin embargo, sufrieron cambios significativos en su estructura semicristalina durante el almacenamiento. Por el contrario, las películas derivadas de extractos de agar menos purificados mostraron una

mayor estabilidad a lo largo del tiempo, sugiriendo su potencial como aditivo para reducir costes y mejorar la estabilidad de las películas de agar puro. En el caso de los hidrogeles, la purificación del agar condujo a la formación de hidrogeles con una resistencia y dureza significativamente altas. Por otro lado, el protocolo de extracción simplificado produjo fracciones de agar menos purificadas que contenían compuestos adicionales como proteínas, polifenoles y minerales. Aunque esto dio lugar a hidrogeles menos rígidos y resistentes, esta característica podría ser beneficiosa para la producción de hidrogeles con propiedades bioactivas.

En la tercera y última parte de la tesis, se abordó la valorización de los residuos industriales generados tras la extracción de alginato, con el propósito de obtener fracciones de celulosa mediante métodos simplificados para su aplicación en la producción de materiales biopoliméricos. En este contexto, se observó que los residuos de *Alaria esculenta* y *Saccharina latissima* resultaron adecuados para la extracción de fracciones celulósicas, mientras que *Ascophyllum nodosum* podría ser más interesante para la producción de extractos ricos en fucoidano. Las fracciones de celulosa con mayor pureza generaron películas con propiedades mecánicas y aspecto visual más deseables. En cambio, las fracciones menos purificadas presentaron una mayor barrera al vapor de agua. De acuerdo con los resultados, se determinó que la simple aplicación de un tratamiento alcalino puede producir fracciones de celulosa que dan lugar a películas con un equilibrio óptimo entre propiedades funcionales y eficiencia económica y medioambiental.

RESUM

Aquesta tesi doctoral es va centrar en la valorització de diferents fonts de biomassa marina amb el propòsit principal de desenvolupar materials biopolimèrics i ingredients destinats a la indústria alimentària. Així doncs, en la primera part de la tesi, es va dur a terme la valorització de la biomassa de diverses espècies d'algues amb l'objectiu d'identificar compostos d'interés i avaluar el seu potencial per a la producció de materials biopolimèrics i ingredients alimentaris rics en proteïnes. Entre aquestes espècies es trobava *Ruguloperyx okamurae*, una alga marró invasora que representa una amenaça per als ecosistemes marins. No obstant això, es va demostrar que aquesta espècie posseeix un potencial significatiu com a font sostenible de compostos bioactius, transformant el seu paper d'amenaça en una valuosa oportunitat. La biomassa de les algues roges també va destacar pel seu potencial en diverses aplicacions, incloent-hi el desenvolupament de materials rendibles i respectuosos amb el medi ambient per a aplicacions alimentàries obtinguts mitjançant d'un processament mínim. En aquest context, la composició i l'estructura de les parets cel·lulars de les algues van exercir un paper fonamental per a entendre com es comporta cada espècie durant el processament i en les propietats de les pel·lícules resultants. A més de la producció de materials biopolimèrics, les algues roges també es van estudiar com a font de proteïnes per al consum humà. Encara que les algues natives van presentar una baixa digestibilitat, aquesta va millorar mitjançant el seu processament. No obstant això, és important destacar que, malgrat aquesta millora en la digestibilitat, el processament podia induir la degradació dels aminoàcids més làbils, la qual cosa repercutia negativament en la qualitat nutricional de les proteïnes.

En la segona part d'aquesta tesi, es van explorar diverses espècies d'algues roges per a extraure agar de manera eficient mitjançant mètodes simplificats i es va avaluar la idoneïtat dels extractes obtinguts per a desenvolupar materials biopolimèrics i hidrogels per a aplicacions alimentàries. Es va poder observar que el grau de purificació de l'agar va tindre un impacte significatiu en les propietats finals tant de les pel·lícules com dels hidrogels. En el cas de les pel·lícules, les fabricades amb agar pur van presentar propietats superiors; no obstant això, van patir canvis significatius en la seua estructura semicristal·lina durant l'emmagatzematge. No obstant això, les pel·lícules derivades d'extractes d'agar menys purificats van mostrar una major

estabilitat al llarg del temps, suggerint el seu potencial com a additiu per a reduir costos i millorar l'estabilitat de les pel·lícules d'agar pur. En el cas dels hidrogels, la purificació de l'agar va conduir a la formació d'hidrogels amb una resistència i duresa significativament altes. D'altra banda, el protocol d'extracció simplificat va produir fraccions d'agar menys purificades que contenien compostos addicionals com a proteïnes, polifenols i minerals. Encara que això va donar lloc a hidrogels menys rígids i resistents, aquesta característica podria ser beneficiosa per a la producció d'hidrogels amb propietats bioactives.

En la tercera i última part de la tesi, es va abordar la valorització dels residus industrials generats després de l'extracció d'alginat, amb el propòsit d'obtindre fraccions de cel·lulosa mitjançant mètodes simplificats per a la seua aplicació en la producció de materials biopolimèrics. En aquest context, es va observar que els residus de *Alaria esculenta* i *Saccharina latissima* van resultar adequats per a l'extracció de fraccions cel·lulòsiques, mentre que *Ascophyllum nodosum* podria ser més interessant per a la producció d'extractes rics en fucoidans. Les fraccions de cel·lulosa amb major puresa van generar pel·lícules amb propietats mecàniques i aspecte visual més desitjables. En canvi, les fraccions menys purificades van presentar una major barrera al vapor d'aigua. D'acord amb els resultats, es va determinar que la simple aplicació d'un tractament alcalí pot produir fraccions de cel·lulosa que donen lloc a pel·lícules amb un equilibri òptim entre propietats funcionals i eficiència econòmica i mediambiental.

INDEX

I. INTRODUCTION

1.1 Valorization of marine biomass

Nowadays, society confronts a critical challenge imperilling the planet's sustainability: resource scarcity and climate change. The exponential growth of the global population and the overexploitation of finite resources have led to an unprecedented environmental crisis (Ghadge et al., 2020; Islam & Karim, 2019); pollution, food scarcity, and global warming are just a few of the many challenges that must be addressed to ensure a viable future for next generations (Abbass et al., 2022; Corwin, 2021).

Among the most pressing concerns emerging from this situation is the massive accumulation of plastics within natural ecosystems. It is noteworthy that the production of plastics has experienced a substantial increase in recent decades, currently amounting to approximately 350 million tons per year (Heidbreder et al., 2019). However, given the non-biodegradable character of conventional plastics, disposal of the generated residues has become a major issue. Currently, millions of tons of plastic waste end up in the oceans each year, impacting marine life and, ultimately, humans, by entering the food chain (Barnes, 2019; H. L. Chen et al., 2021; Peng et al., 2020). Efforts to recycle and reuse plastics have proven insufficient to address this problematic, forcing a rethinking of how materials are produced and consumed.

In this context, the concept of circular economy (Figure 1) emerges as a promising alternative to address these challenges. The main principle of this economic model is to reduce, reuse, recycle, and regenerate resources instead of merely extracting, using, and discarding them (Geisendorf & Pietrulla, 2018; Geng et al., 2019). The main aim is to minimize waste generation and pollution, while promoting efficiency in the use of natural resources.

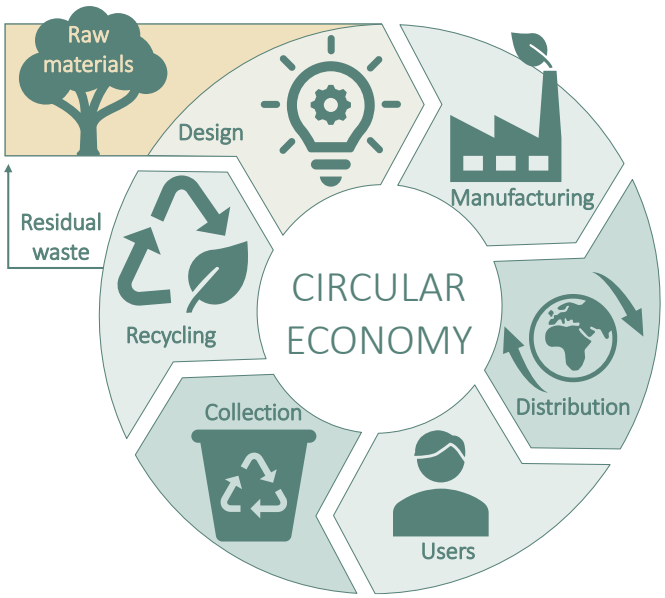

Figure 1. Circular economy model.

In this regard, one of the most promising solutions to address plastic pollution is the replacement of conventional plastic materials with biodegradable polymers (George et al., 2020). Within the biodegradable materials, the bio-based ones (e.g. starch, cellulose, etc.), which are typically obtained from renewable land-based sources such as corn, potatoes, or mushrooms have attracted a great interest, due to their renewable nature and their ability to decompose via exposure to naturally occurring microbes, thus preventing long-term pollution (Hazrol et al., 2021; Jagadeesan et al., 2020; Pérez-Bassart, Martínez-Abad, et al., 2023). Although they still face some technical and economic challenges to become a competitive alternative to conventional plastics (Benito-González et al., 2019), their progressive incorporation into the market can significantly contribute to reducing the carbon footprint and preserving the environment.

Along these lines, the trend towards reducing the processing of raw materials as much as possible to generate the final products (minimal processing) is also attracting attention in the search for sustainable production strategies (Pérez-Bassart, Reyes, et al., 2023). The central premise is to take advantage of resources in their most natural state and minimize the generation of by-products and waste, thus promoting a cleaner and more efficient production.

In addition to changing the way materials are produced, it is also crucial to explore novel food sources. Since agriculture is ultimately responsible for approximately 40% of all greenhouse gas emissions, thus significantly contributing to global warming (Abbass et al., 2022), the utilization of alternative sources and waste to generate novel foods has emerged as a burgeoning area of research and development. For instance, the use of insects as a valuable source of protein for both human and animal consumption has been explored over the last few years, which could alleviate pressure on conventional agriculture and reduce deforestation (Akhtar & Isman, 2018; Kim et al., 2019). In addition, there has been a notable growth in vegetarian and vegan markets. This shift in consumers' perspective reflects a culturally significant transformation in how dietary habits and their impact on the planet are perceived. This trend involves a greater willingness to adjust habits in response to climate change, opting for plant-based foods instead of animal-derived alternatives (Boukid et al., 2022; Bryant, 2022; de Boer & Aiking, 2021; Pam Ismail et al., 2020).

In the context of exploring alternative biomass sources, other than conventional crops, for the production of both biopolymeric materials and novel foods, marine resources such as algae have emerged as an interesting sustainable alternative to terrestrial biomass (Abdul Khalil et al., 2017; Rawiwan et al., 2022). This is mainly due to the sessile nature of most algae, which have evolved to survive in changing, extreme, and hostile environments, adapting to temperature variations, salinity, and to the presence of pollutants (Leandro et al., 2020). This adaptation has allowed them to generate a variety of compounds known as 'secondary metabolites', which include pigments, vitamins, phenolic compounds and steroids (Cotas et al., 2020; Rosa et al., 2019). Additionally, they produce proteins, fatty acids, and various types of polysaccharides which are essential for their physiological functions (Leandro et al., 2020; Olsson et al., 2020). Consequently, algae have gained remarkable commercial importance in recent years, being used worldwide for purposes ranging from industrial extraction of phycocolloids, to obtaining bioactive compounds (Baghel et al., 2021; Polat et al., 2023). However, oftentimes the yields for the industrial extraction of these compounds are not very high and, thus, substantial amounts of algae biomass waste are generated, which are generally disposed of as waste (Bharadwaj et al., 2022; Rudovica et al., 2021). Apart from the industrial waste, algae biomass can also

accumulate ashore, on coasts and beaches upon their natural developmental cycle due to marine currents. Although algae are natural and essential components of the marine ecosystem, their proliferation in certain circumstances can have negative effects on the environment and coastal communities, not only due to waste generation (Harb & Chow, 2022), but also due to excessive spreading of invasive species. Hence, the waste derived from algae biomass, whether natural waste or industrially-derived waste, emerges as a more environmentally sustainable option for producing both biopolymers and active compounds intended for food applications, aligning with the principles of circular economy.

1.2 Main compounds of interest in macroalgae

Macroalgae, also known as seaweeds, are categorized into three main groups: brown seaweeds (Phaeophyta), green seaweeds (Chlorophyta) and red seaweeds (Rhodophyta). Although both their composition and the structure of their cell walls vary significantly depending on the species, in general, many of them stand out for containing compounds of interest, such as phycocolloids, cellulose, and diverse bioactive compounds (Leandro et al., 2020; Ponthier et al., 2020).

1.2.1 Phycocolloids

Phycocolloids are a group of natural polysaccharides that are present in the cell walls of some marine seaweed species. They are known for their capacity to form gels and colloids upon water contact, making them valuable in a variety of food applications. The phycocolloids obtained from marine seaweeds with greater industrial relevance are alginate, carrageenan, and agar (Häder, 2021; Lomartire & Gonçalves, 2023).

1.2.1.1 Alginate

Alginic acid, also known as alginate, is the predominant polysaccharide in the cell walls of some brown seaweed species, constituting up to 40% of their dry weight (Bojorges et al., 2023). The presence of alginate in the cell walls confers them flexibility and resistance, protecting the seaweeds from the impact of strong seawater waves (Ahmad Raus et al., 2021).

This polysaccharide is configured as a linear polymer, composed of units of β-D-mannuronic acid (M) and α-L-guluronic acid (G), interconnected through 1,4 linkages (Figure 2). Moreover, its structure allows organization into M-blocks, G-blocks and MG-blocks (Bojorges et al., 2023; Lomartire & Gonçalves, 2023) whose disposition is influenced by the different seaweed's sources and growth conditions (Puscaselu et al., 2020).

Due to the ability of alginate to form hydrogels, it is widely known as a stabilizer, thickener, gelling and emulsifying agent (Ahmad Raus et al., 2021). Moreover, it has been observed that the physicochemical properties of alginate hydrogels are directly influenced by its structure, including factors such as molecular weight (Mw), degree of acetylation, M/G ratio, and arrangement of M and G residues (Cheng et al., 2020). In fact, various studies have demonstrated that alginate hydrogels with low M/G ratios tend to exhibit stiffness and brittleness, while those with high M/G ratios exhibit greater elasticity and flexibility (Ahmad Raus et al., 2021; Bojorges et al., 2023; Lomartire & Gonçalves, 2023). Additionally, it has been shown that as the molecular weight of alginate increases, its viscosity also rises, hence having a strong impact on hydrogel preparation (Ahmad Raus et al., 2021).

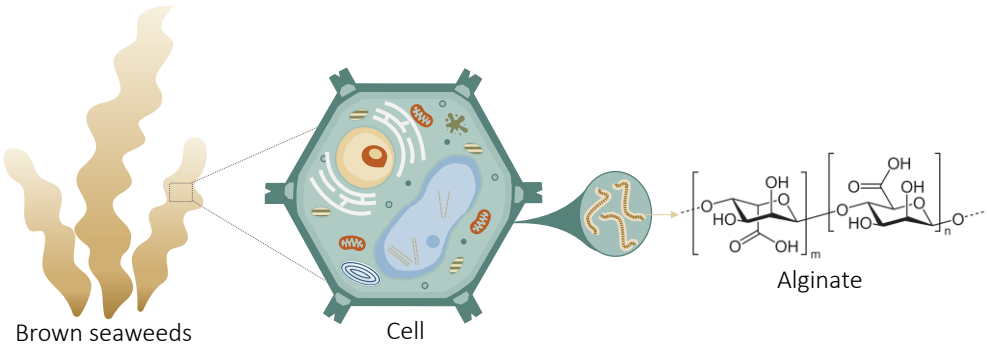

Figure 2. Alginate molecular structure.

1.2.1.2 Carrageenan

Carrageenans are sulphated polysaccharides found in the cell walls of certain red seaweed species, like *Eucheuma, Gigartina, Chondrus*, and *Hypnea* species, constituting between 30-75%

of their dry weight (Guo et al., 2022; Qureshi et al., 2019). These polysaccharides have gained significant importance in the food industry due to their remarkable gelling, thickening, and stabilizing properties (Fontes-Candia et al., 2020; Pacheco-Quito et al., 2020).

Carrageenans are linear polysaccharides consisting of galactose and 3, 6-anhydro-galactose units that are linked by α-1,3 and β-1,4 glycosidic bonds (Liu et al., 2020). They can be classified into six basic types depending on the amount and position of sulphate groups: *kappa* (κ-), *iota* (ι-), *lambda* (λ-), *mu* (μ-), *nu* (ν-), *beta* (β-), and *theta* (θ-) carrageenan, each one with unique functional properties (Pacheco-Quito et al., 2020). However, κ-, ι- and λ- carrageenans (containing one, two and three sulphate groups per repetitive disaccharide unit, respectively, cf. Figure 3) are the most commonly used in the food industry, since they are considered as generally recognized as safe (GRAS) (Fontes-Candia et al., 2020). These different types of carrageenans are usually obtained separately or as a well-defined mixture, as most seaweeds contain hybrid carrageenans (Pacheco-Quito et al., 2020).

κ-carrageenan, due to its limited content of sulphated groups, can form solid and resistant gels in the presence of cations, especially potassium. On the other hand, ι-carrageenans, in the presence of calcium, exhibit the ability to form flexible and elastic gels with remarkable stability during freezing and thawing processes, without showing syneresis. In contrast, λ-carrageenans, with a greater amount of sulphate groups, do not have the ability to form gels in the presence of cations, but they do have the ability to act as thickening and stabilizing agents (Fontes-Candia et al., 2020; Pacheco-Quito et al., 2020).

Although gel-forming ability is one of the most important properties of carrageenans, these compounds have also been found to exhibit anticoagulant and antithrombotic activity, antiviral properties, antitumor effects and antioxidant properties (Lomartire & Gonçalves, 2023)

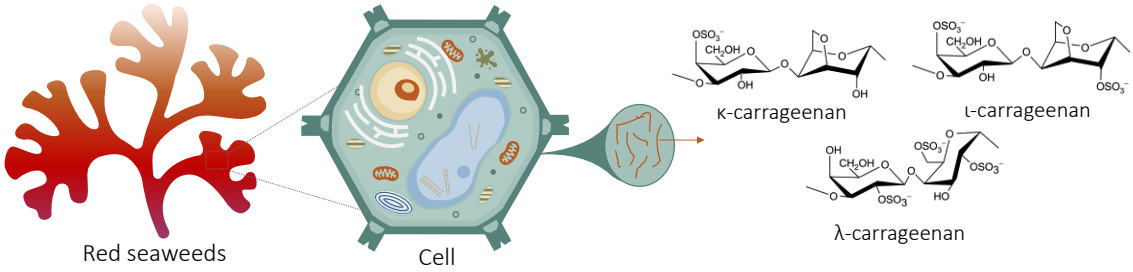

Figure 3. Repeating disaccharide units in the three most commercially relevant types of carrageenans.

1.2.1.3 Agar

Agar is a sulphated polysaccharide naturally found in the cell walls of certain red seaweed species (Martínez-Sanz, Gómez-Mascaraque, et al., 2019), where it plays a crucial role in protecting them against pathogens, maintaining cellular ionic balance, and safeguarding them from extreme conditions such as salinity, pH variations, temperature fluctuations, and desiccation (Lomartire & Gonçalves, 2023).

Agar is composed of two main fractions: agarose and agaropectin. Agarose constitutes the gel-forming fraction and consists of alternating units of β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl linked together by β-1,3 and α-1,4 glucosidic bonds (Figure 4). On the other hand, agaropectin has a similar structure to agarose, but it contains between 5-10% sulphate esters in addition to other residues such as methoxyl groups and pyruvic acid (Guerrero et al., 2014; Martínez-Sanz, Gómez-Mascaraque, et al., 2019).

In general, the physical properties and gelation capacity of agar are closely related to its chemical structure. Agar has the ability to form reversible gels when a previously heated aqueous solution is cooled below the gel point. At the gelled state, agar's molecular chains crosslink to form a three-dimensional network through mutual associations, adopting a double helix structure (Jayakody et al., 2022; Martínez-Sanz et al., 2020).

Typically extracted from the *Gracilariaceae* and *Gelidiaceae* families (Häder, 2021), in the food industry, agar is commonly used as a gelling and thickening agent. Its ability to form firm and stable gels at different temperatures makes it a valuable ingredient for improving the texture and quality of foods (Ferreira et al., 2019; Trigueros et al., 2021).

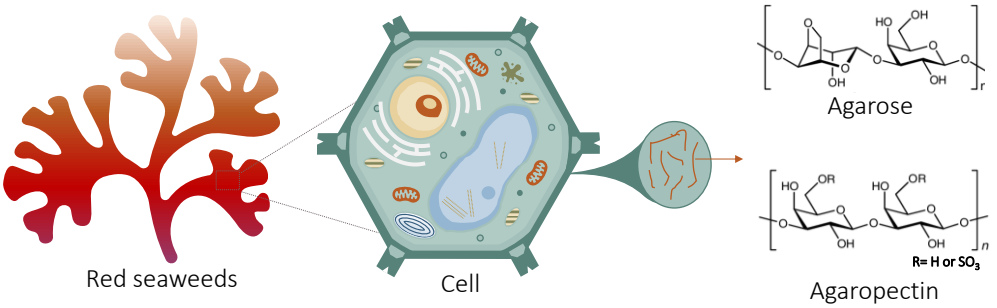

Figure 4. Chemical structure of the building blocks of agarose and agaropectin.

1.2.2 Cellulose

Cellulose is considered the most abundant natural polymer on Earth, where it exists as a structural component in the cell walls of plants and some species of seaweeds, as well as in biofilms secreted by bacteria (Liu et al., 2021, 2022). Regardless of its origin, it is a high molecular weight linear polymer formed by repeating units of D-glucose connected by β-1,4-glucosidic bonds (Liu et al., 2021; Rana et al., 2021). Each glucose unit has hydroxyl groups at carbons 2, 3, and 6. Thanks to the presence of these hydroxyl groups, these linear chains of glucose units are held together by van der Waals forces and intermolecular hydrogen bonds, forming elementary fibrils that pack into larger aggregates known as microfibrils. Cellulose fibrils consist of alternated highly ordered (crystalline) and disordered (amorphous) regions. In crystalline regions, cellulose chains are tightly and orderly packed, while in amorphous regions, cellulose chains are randomly arranged. This makes amorphous regions more labile to both physical and chemical treatments, compared to crystalline regions (Li et al., 2021; Liu et al., 2021; Nagarajan et al., 2021; Rana et al., 2021).

Furthermore, the extensive orientation of the hydrogen bond network and the flexibility in how glucose units can arrange themselves in different directions allow cellulose to take on different crystalline forms, known as allomorphs. Specifically, four types of crystalline allomorphs have been described: cellulose I, II, III, and IV (Rana et al., 2021). These allomorphs can be naturally found on different resources or they can be formed as a result of different chemical processes. Cellulose I is the typical crystalline form of native cellulose, and the most extensively studied. It is present in all plant cell walls and is composed of two metastable structures, namely triclinic (Iα) and monoclinic (Iβ), with the triclinic form (Iα) being the most abundant in seaweeds (Seddiqi et al., 2021). Cellulose II, which originates after the chemical conversion of cellulose I through solvation in certain solvents or treatments with some acids and bases, exhibits various possible antiparallel packing arrangements of the cellulose network linked by hydrogen bonds (Rana et al., 2021). Additionally, immersing cellulose I and II in liquid ammonia or organic amine at low temperatures leads to cellulose III, while cellulose IV can be derived by heating cellulose III in glycerol at 260°C (Rana et al., 2021; Wohlert et al., 2022).

As a natural biopolymer, cellulose possesses excellent physicochemical characteristics, including high chemical resistance, great strength, good durability, high thermal stability, good biocompatibility, and biodegradability (Liu et al., 2021), which has garnered significant attention worldwide as a biomass resource with immense potential for various applications (Liu et al., 2022).

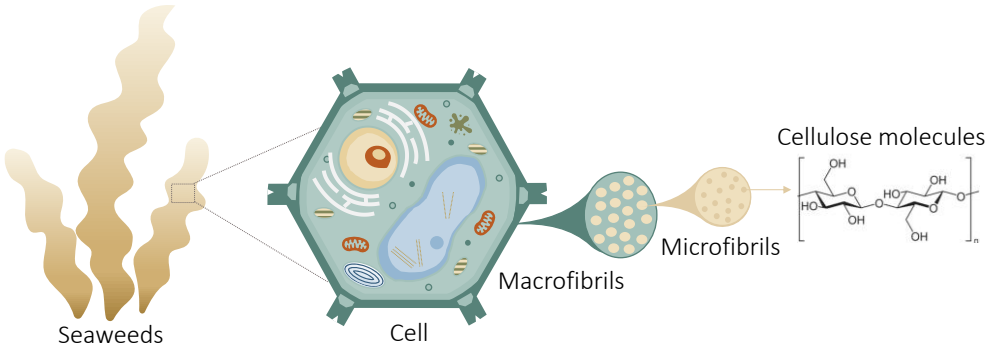

Figure 5. Cellulose hierarchical structure in seaweeds' cell walls.

1.2.3 Other bioactive compounds

Along with phycocolloids, seaweeds are also known to contain other bioactive secondary metabolites that can be utilized for developing functional products with applications across various industries. Examples of these include pigments, minerals, phenolic compounds, polyunsaturated fatty acids (PUFA), vitamins and peptides.

Pigments

One of the most notable compounds found in seaweeds are natural pigments, which can be classified into three main groups: chlorophylls, carotenoids, and phycobiliproteins (Lomartire & Gonçalves, 2022).

Chlorophyll, which is a natural pigment with green hues, plays a crucial role in photosynthetic organisms by participating in light absorption, energy transfer, and electron transport (Gomes et al., 2022). In the case of seaweeds, four types of chlorophyll have been identified, with chlorophyll a being the most predominant. Chlorophyll b is the second most abundant type and is exclusively found in green seaweeds. Finally, chlorophyll c is found in brown seaweeds, with two known variants: c1 and c2 (Gomes et al., 2022; Kalasariya et al., 2021). Chlorophylls are known to exhibit a wide range of beneficial effects, including antioxidant, antimutagenic, antigenotoxic, anticancer, and anti-obesogenic activities (Martins et al., 2023).

Carotenoids are also found in a wide range of seaweeds, primarily in brown seaweed, and their main function is to collect and transfer light energy to chlorophyll, as well as to provide photoprotection against photooxidative damage (Gomes et al., 2022). These are pigments ranging from yellow to orange-red and can be divided into three classes: carotenes, xanthophylls, and lutein (Lomartire & Gonçalves, 2022). Carotenoids play a significant role in human health by providing provitamin A and acting as photoprotection and anticancer agents and against cerebrovascular diseases (Lee et al., 2021).

Phycobiliproteins constitute a group of water-soluble pigments found in red seaweeds. They can be classified into three categories based on their protein structure: phycocyanins (blue pigment), allophycocyanins (light blue pigment), and phycoerythrins (red pigment), with the latter being the most abundant (Lomartire & Gonçalves, 2022). Currently, various physiological

activities have been attributed to phycobiliproteins, including antioxidant, anti-inflammatory, antitumor, regulation of body metabolic balance, and immunomodulation (Ma et al., 2022).

Minerals

Seaweeds acquire a significant wealth of mineral elements from their marine environment, which can represent up to 40% of their dry weight (Peñalver et al., 2020). Although the mineral composition of seaweeds can vary significantly depending on several factors such as taxonomic group, geographical variations, seasonal factors, and physiological aspects, in general, they contain a wide variety of essential minerals such as sodium, calcium, magnesium, potassium, chloride, sulphate and phosphorus (Peñalver et al., 2020; Polat et al., 2023). Furthermore, it is important to note that both their iron and copper content exceed the concentrations found in products like meat and spinach (El-Beltagi et al., 2022).

Another important aspect to consider is that marine seaweeds, especially brown seaweeds, are a good source of iodine, an essential element for thyroid hormone production, and playing an important role as an anticancer, antioxidant and key nutrient in metabolic regulation (El-Beltagi et al., 2022; Polat et al., 2023). However, brown seaweeds can contain very high levels of iodine, and both deficiency and excess of this mineral can increase the risk of negative health effects. In the European Union, there is currently no specific regulation to control the iodine content in seaweeds or in foods that contain them (Blikra et al., 2022). Nevertheless, a daily intake of 150 µg of iodine is recommended for adults (WHO et al., 2014).

Phenolic compounds

Phenolic compounds constitute a broad family of natural organic compounds found in seaweeds (Lee et al., 2021), particularly in brown seaweeds, where they can account for up to 14% of their dry weight (Peñalver et al., 2020). Among the polyphenols synthesized by seaweeds are bromophenols, flavonoids, phenolic terpenoids and phlorotannins, with the latter being the most prominent due to their beneficial effects, including neuroprotective, antidiabetic, anticancer, antioxidant, anti-inflammatory, and antimicrobial properties (Lee et al., 2021; Lomartire & Gonçalves, 2022; Peñalver et al., 2020).

Polyunsaturated fatty acids (PUFA)

Seaweeds are also an important source of polyunsaturated fatty acids (PUFA), which consist of long hydrocarbon chains ending with carboxyl groups (Lomartire & Gonçalves, 2022). PUFA are classified according to the position of the first carbon-carbon double bond into omega-3 or omega-6 and can account for up to 74% of the total lipid content (Polat et al., 2023).

Among the most important omega-3 fatty acids are alpha-linolenic acid (ALA, C18:3n3), eicosapentaenoic acid (EPA, C20:5n3), and docosahexaenoic acid (DHA, C22:6n3), all of which play a crucial role in human health. In particular, EPA and DHA are attracting significant attention due to their ability to provide benefits in conditions such as cardiovascular disorders, Alzheimer's disease, hypertension, coronary artery disease, arthritis, and cancer (Lomartire & Gonçalves, 2022; Rengasamy et al., 2020).

Vitamins

Marine seaweeds are generally rich in water-soluble vitamins, including vitamins A, C, E, D, as well as the B-vitamin complex, which encompasses B1 (thiamine), B2 (riboflavin), B6, B9 (folic acid), and even vitamin B12, which is otherwise only found in animal products. These components are particularly relevant for the immune system and also play a significant role in the health of the skin, hair, nails, and connective tissue (Y. Kumar et al., 2021; Peñalver et al., 2020; Polat et al., 2023).

Proteins

The protein content in seaweeds varies depending on the species, season, and geographical location, and it can represent up to 47% of the weight in some red seaweed species (El-Beltagi et al., 2022). This amount is comparable to the protein content of protein-rich foods, such as soy (43% protein) (Polat et al., 2023). In general, the most abundant amino acids in seaweeds are aspartic acid and glutamic acid, which can make up to 40% of the total amino acid content and are responsible for giving seaweeds their unique flavour and aroma known as "umami" (Astorga-España et al., 2016; Polat et al., 2023). Moreover, the protein found in marine seaweeds is also an excellent source of essential amino acids, accounting for nearly half of their total amino acids (Freitas et al., 2022). Therefore, from a nutritional perspective, seaweeds are particularly interesting and present an outstanding alternative to animal-derived proteins

(Freitas et al., 2022; Rawiwan et al., 2022). However, how these proteins are digested in the body is still unknown, and further research is needed to evaluate their nutritional quality.

1.3 Industrial applications

In recent years, due to their great potential, seaweeds have gradually made their way into various industrial sectors, serving as texture modifiers in food products, substrates for the production of bio-based packaging, or as potential ingredients for the development of new food products, among others.

1.3.1 Texture modifiers

The galactans found in red seaweeds are widely used for industrial applications due to their rheological properties, which confer thickening (λ-carrageenan) or gelling (agar, κ- and ι-carrageenan) capabilities (Cosenza et al., 2017). Specifically, in the food industry, carrageenans are employed in dairy production, as they interact with the positive charges present in milk proteins, thus affecting their functional properties. For instance, κ-carrageenan is used to prevent whey separation and keep cocoa in suspension in chocolate milk. On the other hand, λ-carrageenan enhances the stability and texture of various products (Cosenza et al., 2017). Carrageenans are also utilized in the clarification of beer and wine, leveraging their interaction with proteins (Tuvikene, 2021). Gel-forming carrageenans are also used as substitutes for gelatin or pectin in the preparation of low-calorie, vegetarian, or vegan desserts (Cosenza et al., 2017). Furthermore, in the meat industry, carrageenans are valuable for gel formation and moisture retention, which is especially attractive in low-fat meat products to try to mimic the texture of conventional meat (Rupert et al., 2022).

Agar is less frequently used in the food industry compared to carrageenan, partly due to its higher price. Nevertheless, owing to its high melting and gelling temperature, agar proves useful in preparing fillings and glazes for pastry products and in the production of gelled meats and fish (Cosenza et al., 2017). It also plays a significant role in the confectionery industry, improving the stability and texture of sorbets and ice creams, and it is also used in the production of cheeses and dairy products (Pandya et al., 2022). Additionally, in bakery applications, agar's water-retaining properties enhance the viscoelastic characteristics, texture, and moisture

content of dough and pasta (Cosenza et al., 2017). Agar is also employed as a clarifying agent in gels, broths, sauces, beer, and other liquids (Häder, 2021).

Alginates, due to their thickening, gelling, or encapsulation properties, are the most widely used brown seaweed polysaccharides in industrial applications (Cosenza et al., 2017; Häder, 2021; Qin et al., 2018). The thickening behaviour of alginates is applied to sauces, syrups, and ice cream toppings. They are also used to prevent separation in mayonnaise or settling in juices and chocolate milk (Cosenza et al., 2017; Qin et al., 2018). Additionally, they are employed to improve the structure of bakery products (Häder, 2021). As gelling agents, with the addition of salts, alginates form stable gels at high and low temperatures and low pH levels, making them a versatile choice for preparing jellies and desserts (Qin et al., 2018). Additionally, combinations of alginates and pectin generate synergistic thermoreversible gels under conditions where neither of them would form a gel on their own, making them ideal for producing low-calorie jellies (Cosenza et al., 2017). Furthermore, alginate-based jellies have the advantage of not melting, maintaining a distinct and firmer texture compared to gelatin-based jellies, which can soften and melt at body temperature (Qin et al., 2018). Alginate gels are also used in the manufacture of restructured foods such as nuggets or olive fillings due to their interaction with proteins (Cosenza et al., 2017).

1.3.2 Bio-based packaging

In the quest for sustainable alternatives to conventional plastics, seaweeds have emerged as a promising source of materials for the production of biodegradable packaging (Kartik et al., 2021). In recent years, various compounds found in macroalgae, that are suitable for this application, have been investigated, exploring both traditional approaches and innovative trends in sustainable packaging production (Lim et al., 2021; Martínez-Sanz, Martínez-Abad, et al., 2019).

Cellulose and its derivatives have become one of the most studied compounds for this purpose, thanks to their mechanical and barrier properties, low weight, minimal filler loading requirements, biodegradability, and widespread availability (Shaghaleh et al., 2018). Furthermore, cellulose has generated considerable interest as a reinforcing material in

biopolymeric composites, and its versatility is further expanded when combined with other hydrocolloids (Abdul Khalil et al., 2017).

Although less explored in this area, phycocolloids also show a great potential in the search for new biodegradable materials (Abdul Khalil et al., 2017). Typically, alginate films have been developed through a solution casting process, using a plasticizer to obtain thin films. However, pure alginate films exhibit a significantly weak water permeability barrier. To address this limitation, these films are often cross-linked with calcium to enhance their strength (Jumaidin et al., 2018). Carrageenan, on the other hand, also demonstrates excellent film-forming capacity. However, these films still face challenges regarding their water vapour permeability, owing to their inherently hydrophilic nature and structural fragility (Sedayu et al., 2019). Agar films are biologically inert, allowing them to readily interact with various bioactive substances and cover the surface of food products (Mostafavi & Zaeim, 2020). Additionally, they possess characteristics such as transparency, thermosealing capability, and biodegradability (Jumaidin et al., 2018). Nonetheless, compared to conventional plastic packaging materials, pure agar films tend to be relatively brittle, exhibit low elasticity, high water sensitivity, and elevated water vapour permeability (Mostafavi & Zaeim, 2020). In general, these drawbacks can limit the applicability of films produced from phycocolloids. However, by combining these compounds with other materials such as biopolymers, hydrophobic substances, plasticizers, nanoparticles, and antimicrobial agents, it is possible to enhance their properties, harness their beneficial characteristics and significantly broaden their range of applications (Abdul Khalil et al., 2017; Mostafavi & Zaeim, 2020).

1.3.3 Food products

In the search for sustainable and healthy alternatives in the food industry, marine seaweeds have emerged as a valuable resource (A. Kumar et al., 2023). Although commonly consumed in Asia, seaweeds have been largely underutilized in western countries (Blikra et al., 2021). However, in recent years, there has been a growing interest in their potential as a primary and functional ingredient in European food markets (Figueroa et al., 2023). This increasing interest has been driven by various key factors. Firstly, European consumers are increasingly interested in food products that combine health and nutrition (A. Kumar et al., 2023). Marine seaweeds

stand out for their nutritional value, as they provide an excellent source of high-quality proteins and offer a wide range of essential nutrients, vitamins, and minerals (Anusha Siddiqui et al., 2023; Rawiwan et al., 2022). Additionally, their cultivation is environmentally friendly (Nova et al., 2020) and, thus, offers a sustainable alternative to land-based crops. In response to this demand for healthier and more sustainable foods, the European food industry is exploring different approaches to incorporate marine seaweeds into conventional food products (Figueroa et al., 2023). This has led to a significant increase in the launch of new foods and beverages that include seaweeds in their formulations in recent years (Nova et al., 2020), including snacks, bread and soups, among others (Figueroa et al., 2023; Nova et al., 2020).

As the gelling process can be easily triggered by different factors, these phycocolloids also have a great potential as encapsulation agents. In the food industry, alginate encapsulation and immobilization technologies are used for various purposes, such as protecting reactive or volatile molecules like acidulants, fats, and flavourings (Qin et al., 2018), but other applications such as targeted release of bioactive compounds in the human gastrointestinal tract have also been widely explored over the last few years.

1.3.4 Other industrial applications

In recent years, the use of marine seaweeds has been expanding into different industries, such as the microbiological, pharmaceutical, cosmetic, and agricultural industries.

Microbiology and molecular biology

Agar, due to its gelling capacity, has been widely used in microbiology for the cultivation of bacteria and other microorganisms (Häder, 2021). Furthermore, agarose can form an inert matrix that can be employed in the separation and purification of nucleic acids, proteins, antibodies, and other macromolecules. Its applications extend beyond traditional electrophoresis and encompass techniques such as chromatography (gel, affinity, or ion exchange), immunodiffusion, support in biocatalysis, use in solid culture media, and in the growth of protein crystals (Park et al., 2020).

Pharmacological applications

In recent years, pharmaceutical companies have shown a growing interest in marine organisms, including seaweeds, aiming to develop new drug delivery systems from natural resources (Abdul Khalil et al., 2017). Seaweeds are particularly attractive due to the ability of phycocolloids to form hydrogels and their hypoallergenic nature, which expands their potential use in medical applications, especially in controlled drug administration (Polat et al., 2023).

Additionally, phycocolloids also have a great potential as encapsulation agents. In the food industry, alginate encapsulation and immobilization technologies are used for various purposes, such as protecting reactive or volatile molecules like acidulants, fats, and flavourings (Qin et al., 2018), but other applications such as targeted release of bioactive compounds in the human gastrointestinal tract have also been widely explored over the last few years. Furthermore, the controlled release of drugs through these biopolymers can contribute to reducing drug-associated toxicity (Das et al., 2023).

Compounds derived from seaweeds also hold promising applications in fields such as bone tissue engineering, wound dressing materials, pharmaceutical tablet dispersants and cell encapsulation (Abdul Khalil et al., 2017).

Throughout history, the use of natural products has been explored to prevent or treat various health conditions. Seaweeds emerge as ideal candidates for disease prevention or treatment due to their remarkable biological properties (Abdul Khalil et al., 2017; Polat et al., 2023).

Cosmetic and dermatological applications

The demand for skincare products aimed at delaying skin aging has experienced a notable increase. In this context, the use of metabolites from seaweed in cosmetic and dermatological applications has gained relevance in recent years (Polat et al., 2023). Seaweed extracts can play a crucial role, whether as excipients in formulations, acting as emulsifiers or stabilizers in cosmetic products, or as therapeutic agents due to their bioactive properties (X. Chen et al., 2021). The bioactive compounds present in seaweed are easily absorbed by the skin and the body, providing a range of benefits, including the reduction of imperfections and redness, hydration, re-mineralization, and skin firmness (Ashokkumar et al., 2022).

Agriculture applications

The growing consumer awareness of healthy foods has increased the significance of organic farming. Consequently, organic producers/farmers have turned to the use of natural stimulants to enhance crop yields. Seaweeds are highly regarded due to their rich content of bioactive compounds (Ashokkumar et al., 2022), which are effective in promoting seed formation and germination, plant growth productivity, resistance to biotic/abiotic stress, and post-harvest shelf life of plants (Polat et al., 2023).

In addition to their plant growth-promoting effect, marine seaweeds also bring benefits to the physical, chemical, and biological properties of the soil, as they stimulate the growth of beneficial microorganisms and enhance soil health by increasing its moisture retention capacity (Kaur, 2020; Polat et al., 2023).

1.4 Scientific and technological challenges

The seaweeds production market has undergone a significant evolution over the last years, becoming a high-value industry generating approximately 32 million tons annually (Harb & Chow, 2022). Most of the seaweed biomass is destined for human consumption, representing 70% of the total use. This high percentage is mainly attributed to the widespread consumption of seaweeds as a food source in Asia. The remainder of the biomass is employed in various applications, including the extraction of phycocolloids (Poblete-Castro et al., 2020). Despite the considerable growth of the seaweed markets, there are still several challenges that need to be addressed in order to widespread their use in different industrial sectors and further increase the value of this biomass source:

- The procedures for extracting phycocolloids are not usually highly energy-efficient. An example of this is the industrial agar extraction process, which involves the use of alkaline treatments, followed by extraction at high temperatures and pressures, multiple filtration stages, and freezing-thawing cycles to purify the product (V. Kumar & Fotedar, 2009). Since this is a time and energy-consuming process, efforts are needed to develop simplified extraction methods that are more energy-efficient or even consider the possibility of using seaweed biomass directly, thus avoiding the extraction processes.

- The extraction of phycocolloids often results in the generation of a significant amount of industrial waste. For instance, in the alginate extraction industry alone, approximately 39.000 tons of waste are produced globally each year (Z. peng Li et al., 2022; Salgado et al., 2021). Additionally, as mentioned before, there is the issue of waste resulting from the massive accumulation of seaweeds on beaches worldwide (Gibilisco et al., 2020). Around 70% of these solid wastes are disposed of in landfills, raising concerns related to air pollution, leachate contamination, and soil erosion (Z. peng Li et al., 2022). It is worth noting that these wastes represent a rich source of high-value bioactive compounds with applications across various industries. Therefore, in accordance with the principles of the circular economy, they present an excellent opportunity for valorization. However, this poses new challenges, as it requires the ability to extract, transform, or purify these compounds of interest, as well as an understanding of how these compounds impact the final product.
- Another aspect to consider is that in ocean depths, up to 180 meters deep, approximately 6.000 species of seaweeds can be found (Bharadwaj et al., 2022), of which, to date, only around 221 species have been commercially exploited (Harb & Chow, 2022). In the specific case of agar extraction, the most widely used species worldwide is *Gelidium corneum*. However, its wild harvesting, along with other factors such as climate change and pollution, has had a significant impact on the populations of this species, and, although its cultivation is still feasible, it does not yield sufficient returns to be economically profitable (Mouga & Fernandes, 2022). Therefore, it is essential to research new sources for obtaining high-quality agar with the goal of reducing dependence on *Gelidium corneum* and, at the same time, promoting its regeneration in the oceans.
- Despite seaweeds gaining ground in the European food industry, there are still significant challenges to address. An example of this is that, although several studies have highlighted the potential of seaweeds as a source of protein (Echave et al., 2021; Thiviya et al., 2022), there is a considerable knowledge gap regarding their structure and functional properties, their impact on health and their digestibility, even though these are essential parameters for assessing the nutritional quality of a protein source. Therefore, obtaining more

information on these topics would, among others, help establishing regulations and quality standards for food products made from seaweeds.

Therefore, it can be said that the inclusion of seaweeds in the industrial market is an exciting, yet complex challenge, which involves numerous scientific and technological hurdles, some of which will be tackled in this thesis.

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II. OBJECTIVES

Objectives

The general objective of this doctoral thesis was the valorization of marine biomass for the development of biopolymeric materials and ingredients for diverse food applications**.**

To achieve this general objective, several specific objectives were proposed:

- Valorize the biomass of various seaweed species **to identify compounds of interest** and evaluate their potential for producing **biopolymeric materials** and **protein-rich food ingredients**.
- Examine various seaweed species for **efficient agar extraction** using simplified methods and evaluate the suitability of the obtained agar-based extracts to **develop hydrogel-like structures and biofilms** for food applications.
- Valorize industrial waste derived from alginate processing to obtain **cellulose-based fractions using simplified methods** and evaluate their potential for biopolymeric material production.

These general objectives were materialized in 6 individual case studies, which have been structured in 3 chapters in this thesis.

Chapter 1. Valorization of whole macroalgae biomass for food-related applications.

1.1. Characterization of the invasive macroalgae *Rugulopteryx okamurae* for potential biomass valorization.
1.2. Sustainable bio-based materials from minimally processed red seaweeds: Effect of composition and cell wall structure.
1.3. *In vitro* digestibility of proteins from red seaweeds: Impact of cell wall structure and processing methods.

Chapter 2. Valorization of phycocolloids extracted from macroalgae biomass.

2.1. Agar-based packaging films produced by melt mixing: Study of their retrogradation upon storage.
2.2. Exploring alternative red seaweed species for the production of agar-based hydrogels for food applications.

Chapter 3. Valorization of industrial residues derived from phycocolloid extraction.

3.1. Valorization of alginate-extracted seaweed biomass for the development of cellulose-based packaging films.

III. RESULTS

This section includes the results from 6 individual case studies, which have been structured in 3 chapters:

Chapter 1. Valorization of whole macroalgae biomass for food-related applications.

1.1. Characterization of the invasive macroalgae *Rugulopteryx okamurae* for potential biomass valorization.

1.2. Sustainable bio-based materials from minimally processed red seaweeds: Effect of composition and cell wall structure.

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Chapter 2. Valorization of phycocolloids extracted from macroalgae biomass.

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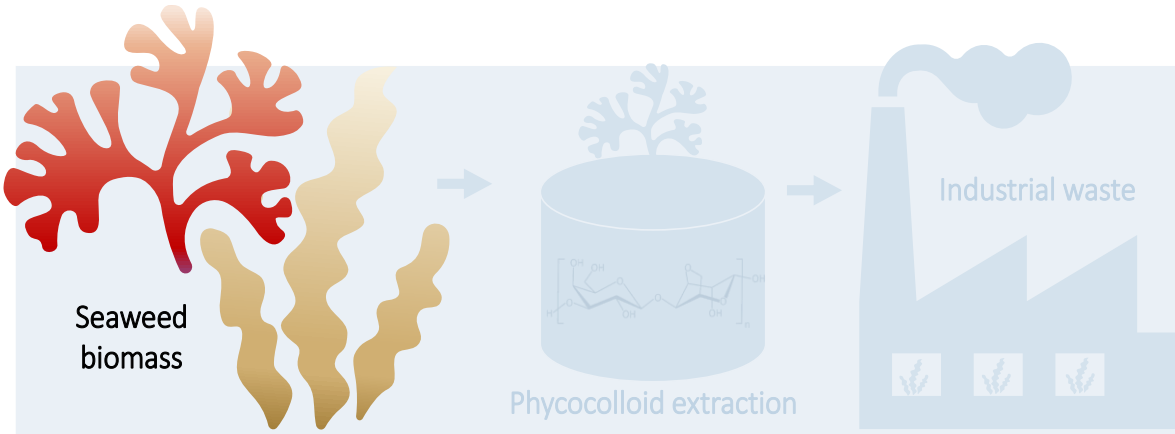
Chapter 3. Valorization of industrial residues derived from phycocolloid extraction.

3.1. Valorization of alginate-extracted seaweed biomass for the development of cellulose-based packaging films.

CHAPTER 1

VALORIZATION OF WHOLE MACROALGAE BIOMASS FOR FOOD-RELATED APPLICATIONS.



The oceans harbour an abundant variety of seaweed species, many of which are already exploited, but a significant proportion of them remains unstudied. Therefore, the exploration of new seaweed species for the purpose of obtaining added-value compounds is of great interest, as it would alleviate the pressure on species already industrially exploited, thus promoting their regeneration in the oceans. This is why the first study in this chapter reports on a comprehensive analysis of the biomass of the invasive macroalgae *R. okamurae*. The aim was to deeply understand its composition and properties, with the intent of identifying potential opportunities for valorization and applications. The results revealed that *R. okamurae* holds significant potential as a sustainable source of several compounds of interest, that can add value to various sectors, including the food industry, animal feed production, pharmaceuticals, and cosmetics.

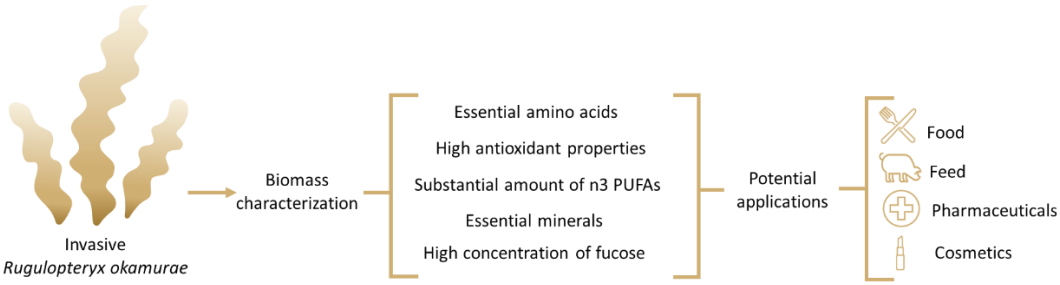
The industrial interest in seaweeds primarily stems from their natural abundance of polysaccharides such as alginate, carrageenan, and agarose, which are used in the food industry as thickeners and stabilizers, as well as in the production of biopolymeric materials. However, the conventional extraction process for these phycocolloids entails high energy consumption and low yields. Hence, an emerging trend is the direct utilization of the whole algal biomass instead of extracting specific compounds, hence simplifying the process and reducing its environmental footprint. This is why, in the second study within this chapter, the steps of phycocolloid extraction were omitted, and biopolymeric materials for packaging applications were produced more energy-efficiently directly from the biomass of four species of agar-producing red seaweeds (*Gelidium corneum, Agarophyton chilensis, Gracilaria tenuistipitata*, and *Gracilariopsis longissima*). The effect of the composition and structure of the cell walls of each seaweed species on the final performance of the films was also investigated. The results of this study demonstrated the potential of red algal biomass from alternative species to generate cost-effective and environmentally friendly food packaging materials.

Seaweeds not only contain polysaccharides but are also a rich source of vitamins, minerals, antioxidants, and high-quality proteins. This nutritional richness makes them ideal candidates for their use as nutritional supplements, functional foods or as ingredients in food products such as snacks. However, it is important to note that, to date, limited attention has been given to

exploring the true impact of these compounds on health. This is why, in the third study comprising this chapter, the nutritional quality and digestibility of proteins present in two of the red algal species studied in the previous works from this thesis, *Gelidium corneum* and *Gracilaripsis longissima,* were evaluated. Additionally, the impact on digestibility of the seaweeds' cell wall structure and composition was investigated, along with the effect of two different processing methods that could be used to produce seaweed-based snacks. The results obtained in this study serve as a foundation for developing strategies that enhance the protein quality of these seaweed species, enabling the production of high-quality foods with potential applications in the food industry.

3.1.1

CHARACTERIZATION OF THE INVASIVE MACROALGAE *RUGULOPTERYX OKAMURAE* FOR POTENTIAL BIOMASS VALORIZATION.



This section is an adapted version of the following published research article:

Cebrián-Lloret, V., Cartan-Moya, S., Martínez-Sanz, M., Gómez-Cortés, P., Calvo, M. V., López-Rubio A. & Martínez-Abad, A. (2024). Characterization of the invasive macroalgae *Rugulopteryx okamurae* for potential biomass valorization. *Food Chemistry,* 440,138241.

1. Abstract

This study aimed to examine the composition and properties of the invasive macroalgae *R. okamurae* and explore potential applications. The results showed that the seaweed biomass is mainly composed of structural carbohydrates, with alginate being the main constituent, accounting for 32% of its total composition and with a mannuronic and guluronic acid ratio (M/G) ratio of 0.93. It also has a relatively high concentration of fucose, related to the presence of fucoidans that have important biological functions. Among the mineral contents, a high magnesium and calcium (7107 and 5504mg/kg) concentration, and the presence of heavy metals above legislated thresholds, were notable. *R. okamurae* also contained a high lipid content of 17%, mainly composed of saturated fatty acids, but with a significant fraction of n3 polyunsaturated fatty acids (18%) resulting in a low n6/n3 ratio (0.31), that has health benefits. The protein content of *R. okamurae* was 12%, with high-quality proteins, as essential amino acids (mainly leucine, phenylalanine and valine) constitute 32% of the total amino acids. It also showed a high polyphenol content and outstanding antioxidant properties (106.88 mg TE/g). Based on these findings, *R. okamurae* has significant potential as a sustainable source of bioactive compounds that can add value to different sectors, including food, feed, pharmaceuticals and cosmetics.

2. Introduction

The introduction of invasive species is one of the biggest threats to biodiversity due to the huge ecological impact it causes. In this respect, seaweeds are considered to be one of the greatest problems due to their contribution to marine primary production and their role as habitat providers for many wildlife species (Navarro-Barranco et al., 2019). There are currently more than 400 species of invasive marine seaweeds worldwide, 50 of which are found in the Iberian Peninsula (Patón et al., 2023).

Rugulopteryx okamurae (*R. okamurae*) is a brown seaweed of the *Dictyotacea* family and native of the Japan, Taiwan, China, Korea and North America area. It was previously registered as *Dilophus okamurae* and it is also known as *Dictyota marginata* or *Dilophus marginatus*. Its presence in Europe was first reported in a French harbour (Thau Lagon) in 2002. Later, in 2015, it was first seen in the Strait of Gibraltar, in Ceuta, but it was not until 2016 when it appeared in

Tarifa (Cádiz, Spain). To this date, its presence has also been reported in the Alboran sea, the Provence (France) and in the Portugal coast also reaching the Azores Islands (Faria et al., 2022). This is a case of cryptic invasion, where the invasive species is so similar to the native one that it can be difficult to identify, in this particular case it may be mistaken for seaweeds from the Dictyota genera (Altamirano et al., 2019). *R. okamurae* is highly competitive, reaching a level of coverage close to 90% on rocky bottoms (García-Gómez et al., 2020), causing a displacement of the local biota and modifying the communities. It has also been reported to be dangerous for some species of gastropods, echinoderms or even some fish communities due to the production of toxic substances such as terpenoids and other secondary metabolites that prevent its predation (Bernal-Ibáñez et al., 2022). The current presence of this invasive macroalgae not only causes ecological problems, but also socio-economical ones, affecting fishing activity, beach management and tourism (Altamirano et al., 2019). In fact, to prepare for the tourist season, towards the end of spring, massive deposits of *R. okamurae* are usually removed from the coasts, which implies additional costs for the municipalities. For this reason, possible applications of this species are being studied as an alternative to its removal to landfills (García-Gómez et al., 2020).

Seaweeds are known to contain many interesting compounds, including polysaccharides, polyunsaturated fatty acids (PUFAs), bioactive peptides or alkaloids (Shepon et al., 2022). The multiple applications of these compounds to different industries has led to a rise on the market value. Specifically, in brown seaweeds the presence of fucoidans, alginates and phenolic compounds such as phlorotannins is remarkable (Bojorges et al., 2023; Olsson et al., 2020). These biomolecules are currently being used in the food, nutraceutical and pharmaceutical industry, having a great value due to their technological, functional and bioactive properties. Regarding *R. okamurae*, some studies have highlighted its potential for various applications such as bioplastic production or composting (Barcellos et al., 2023). However, to the best of our knowledge, none of them have focused on a detailed analysis of the actual composition of this particular species, as they typically rely on general information about brown seaweeds composition. Therefore, this study aims to undertake a comprehensive examination of the biomass of the invasive macroalgae *R. okamurae*, including amino acid, fatty acid, carbohydrate

and mineral with the overarching objective of acquiring an in-depth understanding of the seaweed's composition and properties and identifying potential added-value compounds. This is aimed at transforming a current threat and the waste generated into an opportunity, exploring potential valorization pathways or higher added value market applications of the species in different sectors.

3. Materials and methods

3.1. Raw materials

R. okamurae was collected from seaweed upwelling, which migrates to the beaches of Costa Tropical in Granada, specifically in the Alboran Sea. The seaweed biomass was washed to remove sea salts and sand and dried for 24 h at 60 ºC. The dried seaweed was then ground to a powder with a particle size of less than 250 µm and kept in a 0%RH chamber before processing.

3.2. Compositional analysis of *R. okamurae*

All analyses were performed at least in triplicate.

3.2.1. Carbohydrate profile

The carbohydrate content and composition of *R. okamurae* was estimated using acid methanolysis, which is a technique that cannot break down crystalline polysaccharides but has been found to be a good compromise between the lability of guluronic acid and complete scission of most glycosidic linkages (Bojorges et al., 2023). As brown seaweeds contain cellulose, a two-step sulfuric acid hydrolysis was also performed to determine the total glucose content, and the difference was attributed to the crystalline cellulose content. The sample was then subjected to analysis using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with an ICS-6000 system (Dionex) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex) at 30 °C and a flow rate of 1 mL min−1. Neutral sugars were eluted in water for 16 min with post-column addition of 0.5 mL min−1 of 300 mM sodium hydroxide after a preconditioning isocratic step with 260 mM sodium hydroxide and 68 mM sodium acetate (7 min) and 5 min equilibration time in water prior to injection. Uronic acids were eluted in a gradient of 100 to 200 mM sodium acetate in 10 mM sodium hydroxide

over 20 min. Mixtures of fucose, glucose, galactose, arabinose, xylose, mannose, mannitol, guluronic acid, mannuronic acid, and glucuronic acid were used. Commercial microcrystalline cellulose and sodium alginate were also used as reference controls.

3.2.2. Protein content

An Elemental Analyser Rapid N Exceed (Paralab S.L., Spain) was used to analyse the total nitrogen concentration of *R. okamurae***.** Around 100 mg of the sample powder was pressed forming a pellet and then analysed by using the Dumas method, which relies on the detection of the released N_2 after the combustion of the sample. The nitrogen content obtained was then multiplied by a factor of 5 to estimate the total protein content, following previous findings for other brown seaweeds.

3.2.3. Amino acid profile

The total amino acids were determined after acid hydrolysis with HCl 6 N at 110 °C for 24 h. The analysis was carried out in a Biochrom 30 series Amino Acid Analyser (Biochrom Ltd, Cambridge, UK) equipped with a cation-exchange column. The post-column derivatization was achieved by mixing the eluent column with ninhydrin and by passing this mixture through a high temperature reaction coil. Finally, absorption was measured at 440 and 570 nm.

3.2.4. Lipid extraction

Lipids were extracted by the Folch method with slight modifications. Briefly, 30 mg of sample resuspended in 200 µL of MilliQ water were placed into a 10 mL tube to which 1660 µL of methanol were added and stirred at 1400 rpm for 10 minutes. Next, 3320 µL of dichloromethane was introduced into the tube and stirred at 1400 rpm for 20 min. 1000 µL of 20 mM acetic acid were added and stirred at 1400 rpm for another 10 min. The tube was centrifuged, and the lower organic phase was transferred to another tube while the aqueous phase was washed with 1660 µL of dichloromethane and then, the centrifugation and phase separation process was repeated. After pooling of the organic phase, the contents of this tube were filtered with a glass syringe using a 0,45 m filter The organic solvent was evaporated, and the total lipid content was determined gravimetrically.

3.2.5. Fatty acid profile

Fatty acid methyl esters (FAME) were prepared using methanolic sodium methoxide, applying the direct transesterification method described by (Golay et al., 2007). Briefly, the internal standard mixture was prepared by dissolving equal amounts of tritridecanoin and methyl undecanoate (150 ± 0.1 mg) in n-hexane. Derivatization was carried out at room temperature for 3.5-4 min after the addition of 5 mL of the internal standard solution and 5 mL of 5% methanolic sodium methoxide to the sample, followed by continuous stirring for 10 s. The reaction was stopped by the addition of 2 mL of n-hexane and 10 mL of an aqueous solution of disodium hydrogencitrate (at a concentration of 0.1 g/ml) and sodium chloride (at a concentration of 0.15 g/ml). After phase separation, the supernatant was diluted prior to analysis.

An Autosystem chromatograph (PerkinElmer, Beaconsfield, UK) equipped with a flame ionization detector (GC-FID) was used to perform the FAME analysis, following the method described in (Calvo et al., 2020). Response factors for individual FAME were determined using anhydrous milk fat with a certified composition (BCR-164, European Community Bureau of Reference, Brussels, Belgium) as a reference, and tritridecanoine (Sigma, St. Louis, MO) was employed as internal standard.

3.2.6. Total phenolic content

The Folin-Ciocalteu technique was used to determine the total phenolic content of *R. okamurae*. For this purpose, the dried material was dissolved at a concentration of 5 mg/mL for this colorimetric assay. 200 µL of the sample was combined with 1000 µL of the Folin-Ciocalteau reagent (diluted 1:10 with distilled water). After adding 800 µL of sodium carbonate (75 mg/mL), the sample was heated for 30 minutes at 50 °C. The absorbance value was measured at 750 nm wavelength. Gallic acid was used as a reference to create the calibration curve. The total phenolic content was expressed as mg of gallic acid (GA)/g extract.

3.2.7. Dietary mineral and heavy metal composition

An Inductively Coupled Plasma Mass Spectrometry (ICP-MS) equipment model ICPMS7900 manufactured by Agilent Technologies was utilized to conduct a quantitative analysis of the

minerals and metals present in *R. okamurae.* Sample preparation consisted of a sample digestion process in a microwave oven.

3.2.8. Ash content

The mineral content of the dried seaweeds was determined using the TAPPI T211 om-07 method. To achieve this, around 0.25 g of dry substance was placed in a pre-weighed crucible, which was then weighed. The crucible was combusted in a muffle furnace at 550°C for 24 hours, and the resulting ash content was measured gravimetrically.

3.3. $ABTS^{\cdot+}$ radical cation scavenging activity

The dried seaweed $ABTS^{\cdot+}$ radical cation scavenging capacity was assessed according to (Re et al., 1999). In order to generate the $ABTS^{\cdot+}$ radical cation, 0.192 g of ABTS were first dissolved in 50 mL of PBS at a pH of 7.4 and then combined with 0.033 g of potassium persulfate overnight in the dark. The $ABTS^{\cdot+}$ was diluted with PBS for an initial absorbance of ~0.700 ± 0.02 at 734 nm at room temperature, before to use in the test. Free radical scavenging activity was evaluated by combining 20 µL of sample (dissolved in distilled water at a concentration of 5 mg/mL) with 230 µL of diluted $ABTS^{\cdot+}$ and observing the change in absorbance after 6 minutes. The calibration curve was developed by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). The antioxidant capacity was expressed as mg Trolox equivalents (TE)/g extract.

4. Results and discussion

4.1. Characterization of the seaweed biomass

To valorise the invasive alga *R. okamurae,* the gross composition of the dried biomass was analysed, since, to our knowledge, no previous work has reported on it, and the results are compiled in Table 1.

Table 1. Proximate composition and antioxidant capacity of the *R. okamurae* biomass (dry weight).

Lipids (%)	Proteins (%)	Carbohydrates (%)	Polyphenols (%)	Antioxidant capacity ABTS (mg TE/g sample)	Ashes (%)
17.3 ± 3.2	12.2 ± 0.2	60.4 ± 5.1	4.5 ± 0.3	106.88 ± 0.05	11.30 ± 0.08

4.1.1. Lipid composition of *R. okamurae*

The proximate composition of macroalgae in general is subjected to variations based on several factors, including the environmental conditions, water temperature, nutrient availability, and light intensity (Olsson et al., 2020). However, it is generally known that the lipid content is relatively low, typically ranging from 1% to 5% of the dry weight of the seaweed biomass, even though some species of the Dictyota gender may reach up to 20% (Sun et al., 2018). When compared to other seaweeds of the same order, it is noted that *R. okamurae* has a greater content (ca. 17%) than others species such as *Dictyopteris australis* (ca. 8.5%), *Dictyota bartayresiana* (ca. 12%) or *Stypopodium zonale* (ca. 11%) (Gosch et al., 2012). It is also well known that brown seaweeds exhibit significant quantities of terpenoids, primarily diterpenes, sesquiterpenoids, and meroditerpenes. *R. okamurae*, in particular, stands up for its elevated levels of sesquiterpenes, which have facilitated the spread of this invasive species as a result of their function as feeding deterrents against predators (Patón et al., 2023). Moreover, the presence of omega-3 polyunsaturated fatty acids (n3 PUFA) in brown algae has attracted significant interest due to sustainability issues related to the increasing demand of n3 PUFA for human consumption (Shepon et al., 2022). The high lipid content of *R. okamurae* makes it an excellent source for the extraction and isolation of these compounds with proven health benefits.

In this study, the fatty acid (FA) composition of *R. okamurae* was determined (Table 2), with the aim of gaining a comprehensive understanding of their potential benefits and applications across different industries. The results show that saturated fatty acids (SFA) were predominant (ca. 61%) in the lipid profile of *R. okamurae*, followed by PUFA (ca. 24%) and monounsaturated fatty acids (MUFA) (ca. 15%). The main FAs present in *R. okamurae* were palmitic (C16:0) and myristic (C14:0), accounting for 32% and 15% of the total FA content, respectively. Such C16:0 content, is consistent with previous findings in other species of brown seaweeds (Belattmania et al., 2018; Gosch et al., 2015). Regarding C14:0, it has also been detected in other brown seaweed species, but it typically constitutes less than 10% of total FAME (Belattmania et al., 2018). The consumption of these two SFA) has traditionally been linked to an elevated risk of cardiovascular disease, insulin resistance, and inflammation. Nonetheless, recent research has

investigated the potential of myristic and palmitic acids in reducing inflammation and protecting against oxidative stress. *R. okamurae* also possesses a significant amount (13%) of oleic acid (C18:1 cis-9), being the main MUFA. This is in agreement with oleic acid concentrations detected in other brown algal species, with values ranging from 7-24% of the total FA content (Belattmania et al., 2018).This is of particular interest since oleic acid is well-known to contribute to the promotion of a healthy blood lipid profile, mediate blood pressure, and have a favourable effect on insulin sensitivity and glycaemic control. Noteworthy is the high presence of hexadecatetraenoic acid (C16:4 n3), which makes up almost 8% of total fat. This n3 FA is known to be found in certain types of fish and marine organisms like krill (Yamada et al., 2017), however, to our knowledge, it has only been reported to be present in some macroalgae species like *Ulva spp*, in which it represents up to 12% of the total FA (Premarathna et al., 2022). Unlike other n3 FA like eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3), it has received less research attention. Studies have proposed that hexadecatetraenoic acid has anti-inflammatory properties and may help prevent chronic diseases like cardiovascular disease. Nonetheless, additional research is necessary to fully comprehend the potential health benefits of this FA. Although to a lesser extent, the n3 PUFAs alpha-linolenic acid (ALA) (C18:3 n3) and stearidonic acid (SDA, C18:4 n3) are also present at 4% and 3%, respectively. The role of these n3 FAs in improving cardiovascular functions, regulating systolic and diastolic blood pressure, and enhancing neurological disorders has been previously described, thus possessing antiarrhythmic and anti-inflammatory properties (Gogna et al., 2023). It is important to take note that the overall n6/n3 ratio stands at 0.31. This ratio is of great significance because it reflects the balance between n6 and n3 PUFA) which is known to have a great impact on health through the diet. A high ratio (15 and greater) has been linked to the development of chronic diseases like inflammatory and cardiovascular diseases, while a lower n6/n3 ratio can help preventing these diseases and provide anti-inflammatory benefits (Ferreira et al., 2022). This oil, with such a low n6/n3 ratio is not very common in nature and, therefore, the oil from *R. okamurae* would have high biological and commercial value. In this line, oils from certain wild berries, including crowberries, blackcurrants, and raspberries show low n6/n3 ratios, that has been related to their neuroprotective action, as well as anti-

inflammatory and antioxidant properties, which may affect various bodily systems, including the skin, cardiovascular, nervous, and pulmonary systems. Therefore, these results suggest that *R. okamurae* has the potential to be a highly effective sustainable source for the extraction of lipid compounds which may have important implications for a wide range of industries, including food, feed, pharmaceuticals and cosmetics.

Table 2. Fatty acid composition (% of total fatty acid methyl esters) of oils obtained from *R. okamurae.*

Fatty acid	
C4:0	2.79 ± 0.66
C5:0	1.50 ± 0.42
C8:0	0.58 ± 0.14
C13 *iso*	0.69 ± 0.09
C14:0 *iso*	0.27 ± 0.15
C14:0	15.15 ± 0.29
C15:0	1.48 ± 0.15
C16:0 *iso*	0.68 ± 0.20
C16:0	31.75 ± 0.39
C17:0 *aiso*	0.76 ± 0.29
C18:0	3.13 ± 0.22
C22:0	1.60 ± 0.14
C24:0	0.72 ± 0.14
∑ SFA	61.12 ± 1.59
C16:1 *trans*-9	<0.3
C16:1 *cis*-9	1.62 ± 0.07
C18:1 *cis*-9	12.64 ± 0.25
C18:1 *cis*-11	0.83 ± 0.08
∑ MUFA	15.26 ± 0.30
C16:4 n3	7.82 ± 0.14
C18:2 n6	1.68 ± 0.12
C18:3 n3	4.09 ± 0.91
C18:4 n3	3.17 ± 0.78
C20:2 n6	1.58 ± 0.18
C20:3 n6	<0.3
C20: n6	2.18 ± 0.10
C20:4 n3	0.90 ± 0.34
C20:5 n3	0.70 ± 0.13
C22:5 n3	1.30 ± 0.18
∑ PUFA	23.62 ± 1.70
∑ n6	5.65 ± 0.44
∑ n3	17.98 ± 1.32
n6/n3	0.31 ± 0.01

Data are presented as mean values of duplicate samples ± SD (n=2).

4.1.2. Protein profile of *R. okamurae*

The protein content of brown seaweeds exhibits significant variation, as reported in previous literature. Within the *Dictyotacea* family, protein content has been observed to range from 1.7% to 27.6% (Bogaert et al., 2020; Olsson et al., 2020). Therefore, the protein content value obtained for *R. okamurae,* 12.3%, falls within the observed range of variability. To assess the potential of this seaweed species as a food source and to determine the quality of its proteins, a comprehensive analysis of its total amino acid profile was conducted and is presented in Figure 1. As observed, essential amino acids make up 32% of the total amino acids, similar to that found in animal sources such as eggs (32%), casein (34%) or milk (39%), (Gorissen et al., 2018) which means that the proteins of this seaweed species can be considered to be of high quality. This is of particular interest since each of these amino acids plays a crucial role in various physiological processes, such as protein synthesis, muscle growth and repair, immune function, and hormone regulation. The most abundant essential amino acids in *R. okamurae* are leucine and phenylalanine, each comprising 6.8%, closely followed by valine (5.4%), threonine (4.5%), and to a lesser extent, isoleucine (3.3%), lysine (2%), methionine (1.6%), and histidine (1.4%). Depending on the species, different essential amino acid contents for brown seaweeds have been reported in the literature (Astorga-España et al., 2016). The amino acid profile does differ much from that of other previously reported species of the same family, such as *Dictyota dichotoma* (Bogaert et al., 2020). Among the non-essential amino acids, aspartic acid represents the most dominant one, constituting 12.9% of the total amino acid content. This is followed by glycine at 12.6%, alanine at 12%, and glutamic acid at 11.8%. Other amino acids present in significant amounts include serine at 7.7% and proline at 5.8%. On the other hand, the quantities of tyrosine, arginine, and cysteine are relatively low, with percentages of 2.4%, 2.2%, and 0.5% respectively. Seaweed species are typically characterized by the high content of aspartic acid and glutamic acid, which contribute to the unique flavour and aroma associated with seaweed and marine products (umami) (Astorga-España et al., 2016). Additionally, the presence of glycine and alanine also imparts a sweet taste (Ramu Ganesan et al., 2020). As in *R. okamurae,* in other species of brown seaweeds such as *Dictyota dichotoma* , cysteine is typically present in very small quantities that are almost undetectable, while tyrosine and arginine tend to be a little

more abundant (Bogaert et al., 2020). In fact, several investigations have reported that lysine, threonine, tryptophan, histidine and sulphur amino acids (cysteine and methionine) are considered the limiting amino acids in algal proteins, despite their elevated levels compared to those present in land-based plants (Fouda et al., 2019). Even so, the protein derived from this seaweed exhibits a nutritionally compelling profile of amino acids (AAs) and contains a higher content of essential amino acids (EAAs) compared to alternative non-animal sources. Consequently, *R.okamurae* could potentially contribute to minimizing the deleterious environmental ramifications that arise due to animal protein production. As such, algal proteins have garnered recognition as a feasible protein source for the coming times.

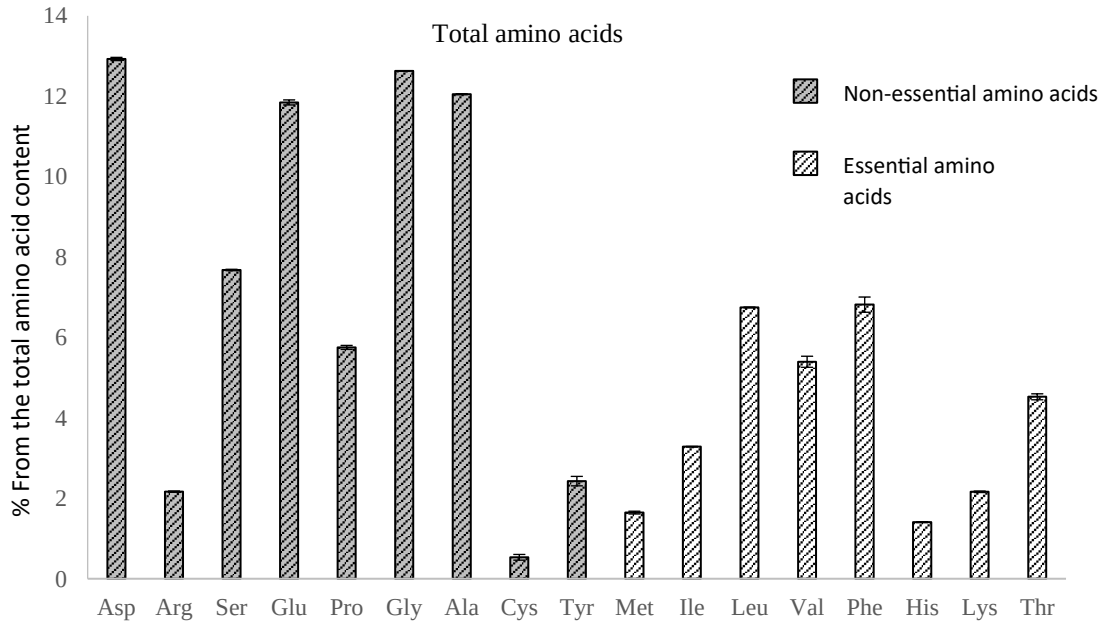


Figure 1. Amino acid profile of *R. okamurae* (expressed as % from the total amino acid content)

4.1.3. Carbohydrate profile of *R. okamurae*

Seaweeds are generally characterized by the presence of structural carbohydrates, which constitute the primary component of their cell walls, providing the necessary strength and rigidity to withstand environmental stresses such as mechanical pressure and wave action. In *R. okamurae,* carbohydrates constitute roughly 60% of its composition on a dry weight basis, with alginate being the principal constituent, making up approximately 32% of its total composition, which is similar to that previously reported for typical alginate sources such as the *Laminaria* (Schiener et al., 2015) and *Ascophyllum* (Cebrián-Lloret et al., 2022) genera, with a 40 and a 30 % of alginate respectively. Alginate is a linear polymer composed of two types of monomers, namely guluronic acid (G) and mannuronic acid (M). The proportion of these monomers, or M/G ratio, may vary according to the seaweed species, affecting the physical and chemical properties of alginate (Cebrián-Lloret et al., 2022). In the case of *R. okamurae*, the M/G ratio was determined to be 0.93, which is lower than the M/G ratios previously reported for alginates extracted from other brown seaweed species, such as *S. latissima* (M/G ratio 1.7) and *A. nodosum* (M/G ratio 1.6) (Bojorges, Martínez-Abad, et al., 2023). It is known that alginates with a lower M/G ratio result in stronger gel structures, as G-blocks have a higher affinity for calcium ions than M-blocks (Bojorges, Martínez-Abad, et al., 2023). Therefore, investigating the potential applications of alginate from *R. okamurae* would be of great interest. Moreover, the carbohydrate profile of *R. okamurae* exhibits a higher concentration of fucose (8%) compared to other brown seaweed species such as *Saccharina japonica* (3-4%), *Stephanocystis crassipes* (6%), and *Fucus vesiculosus* (4.5%) (Obluchinskaya et al., 2022). However, the fucose levels in *R. okamurae* are lower than those observed in other species belonging to the *Fucales* order, where fucose can make up more than 70% of total carbohydrates (Ponce & Stortz, 2020). The presence of fucose in *R. okamurae*, along with other sugars such as galactose and xylose, is of particular interest since it is linked to the presence of fucoidans (e.g. fucogalactans, galactofucans or fucoglucuronans), which have shown significant biological functions such as anticancer, antiviral, antiallergic, anticoagulant, antioxidant, anti-inflammatory, immunostimulant, cardioprotective, and hepatoprotective properties. The seaweed biomass was composed of 7% glucose arising from cellulose. This cellulose content is in line with that observed in other brown

seaweed species within the same order, such as *Dictyota bartayresiana* with 9.3% or *Dictyota dichotoma* with 9.5% (Siddhanta et al., 2013). Despite this, it has been reported that the cellulose content of brown seaweed biomass typically falls within the range of 2.2% to 10.2% (Baghel et al., 2021). Some studies demonstrate that cellulose could be economically extracted from phycocolloids industrial waste (Baghel et al., 2021; Cebrián-Lloret et al., 2022). Therefore, the possibility of valorising distinct added-value polysaccharides, such as fucoidans, alginate and cellulose in *R. okamurae,* portends great potential for this species in a variety of commercial applications.

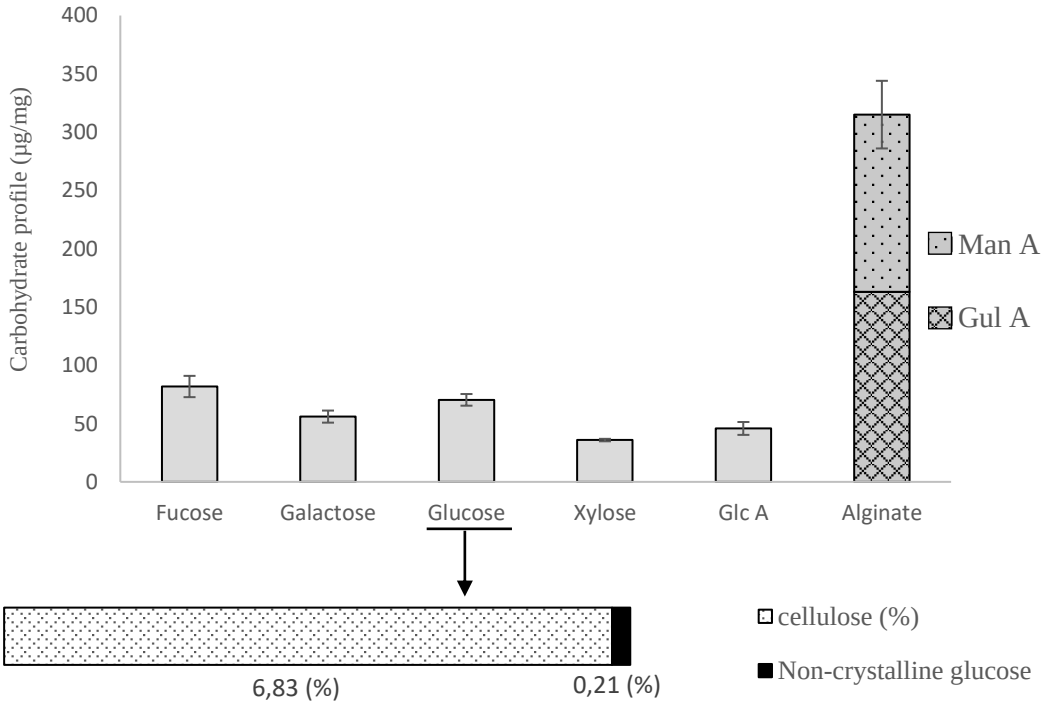


Figure. 2. Carbohydrate profile of *R. okamurae*.

4.1.4. Potential functional activity of *R. okamurae*

To obtain a better understanding of the potential properties of the seaweed biomass, a functional characterization was also conducted, which included an evaluation of the total phenolic content and antioxidant capacity. The results (shown in Table 1) evidenced that *R.*

okamurae presents a high polyphenol content (4.5%) compared to other brown seaweed species of the *Dictyotaceae* family, such as *Dictyota ciliolate*, which has a total polyphenol content of 0.08%, *Dictyopteris polypodioides* with 0.09%, and *Stypopodium zonale* with 1.22% (Mekinić et al., 2019), or even other seaweed species. The most significant type of polyphenols found in brown seaweeds are phlorotannins (Sathya et al., 2017). Several research studies have indicated that the phlorotannins obtained from various species of brown seaweeds exhibit robust antioxidant properties that may be related to their unique molecular structure (Ford et al., 2019; Sathya et al., 2017). The several antioxidant capabilities of polyphenols are closely linked to the existence of phenolic rings, which function as electron traps to neutralize harmful peroxy, superoxide, and hydroxyl radicals (Sathya et al., 2017). This, combined with the presence of sulphated polysaccharides, is consistent with a notoriously high antioxidant activity of *R. okamurae* (106.88mg TE/g), which is much higher than some commonly used organic antioxidants such as ginger (43.6 ± 0.86 mg TE/g) and turmeric (27.5 ± 0.40 mg TE/g) (Hossain et al., 2008). Phlorotannins have been shown to possess defensive properties against predators by suppressing digestive enzymes, which, together with a high terpenoid content, could explain the large proliferation of this invasive seaweed in recent years. Thus, *R. okamurae* has the potential to serve as a plant-based alternative to conventional sources of phenolic compounds and antioxidants for use in the food, pharmaceutical, and cosmetic sectors.

4.1.5. Mineral and heavy metal composition of *R. okamurae*

Since ash represented up to 11% of the overall weight of the seaweed biomass, a quantitative elemental analysis was conducted to identify the mineral composition of *R. okamurae* and the results are compiled in Table 3. This analysis mainly focuses on human consumption of seaweed, as the levels of certain elements may indicate both potential benefits and risks associated with its consumption. It is noteworthy that the consumption of seaweeds is a relatively new trend in Europe, thus, as of now, there are no regulations in place that control the maximum levels of all toxic elements found in seaweeds, but only for some specific metals such as cadmium, which is limited to a maximum of 3 mg/kg for food supplements composed predominantly or solely of dried seaweed. (EC, 2021). Consequently, *R. okamurae*, which contains a cadmium content of 0.38 mg/kg, complies with the established limits. With regards to mercury, Regulation (EC) No.

396/2005 of the European Parliament and of the Council currently imposes a default maximum residue limit (MRL) of 0.01 mg/kg for seaweeds and prokaryotic organisms (EC, 2017). So, *R. okamurae* with 0.03 mg/kg exceeds the established limits. The lead content of *R. okamurae*, which is 2.24 mg/kg, also exceeds the regulated maximum content of 0.1 mg/kg wet weight (ww) for vegetable consumption. (EC, 2006). Similarly, the arsenic level in *R. okamurae* is over established legislative limits, as it is 11.16 mg/kg, although its content is still lower than that reported for other brown seaweed species such as *Ascophyllum nodosum* with 21.5 mg/kg or *Saccharina latissima* with 54.4 mg/kg (Olsson et al., 2020), Also, the iodine contents (416 mg/kg) was relatively high, but certain members of the *Laminariales* order, such as *Saccharina latissima*, may have as much as 7200 mg/kg (Roleda et al., 2018). As a possible solution against the presence of heavy metals, a previous investigation conducted by Noriega-Fernandez et al. demonstrated that exposing the brown seaweed species *L. hyperborea* to a combination of ultrasound (US) and ethylenediaminetetraacetic acid (EDTA) at 50°C for 5 minutes resulted in a significant reduction of 32% for As, 52% for Cd, and 31% for iodine content (Noriega-Fernández et al., 2021). Consequently, to ensure the suitability of this seaweed species for food-related applications, the implementation of this metal elimination protocol would be necessary.

Seaweeds exhibit high variability in essential mineral content depending on the species. Typically, brown seaweeds contain substantial amounts of calcium, with *Laminaria digitata* reaching levels as high as 12400 mg/kg, and magnesium, with some species such as *Ascophyllum nodosum* containing values of up to 8700 mg/kg (Olsson et al., 2020). *R. okamurae* contains relatively high quantities of essential minerals, such as magnesium (7107 mg/kg), calcium (5504 mg/kg), potassium (2590 mg/kg), iron (686 mg/kg), and to a lesser extent, zinc (34.73 mg/kg), phosphorus (22.54 mg/kg), and manganese (12.71 mg/kg). This indicates that *R. okamurae* could potentially serve as a dietary supplement, fortifying or nutraceutical ingredient, leading to a potential market for such products.

Table 3. Minerals and elements present in *R. okamurae (*dry weight).

Element	Concentration (mg/Kg) dw	Element	Concentration (mg/Kg) dw
Na	2556.00	Cd	0.38
Mg	7107.00	Ti	40.11
Al	1020.00	P	22.54
Cl	1140.00	V	7.69
K	2590.00	Mn	12.71
Ca	5504.00	Co	0.40
Sr	1570.00	Rb	2.83
I	416.04	Ba	31.36
Br	513.45	La	1.70
Fe	686.01	Ce	1.99
Hg	0.03	W	2.96
Pb	2.24	B	41.63
Ni	5.98	Pt	0.02
Cr	4.24	Au	0.04
As	11.16	Ag	0.55
Cu	4.74	Y	0.72
Zn	34.73	Zr	1.40

4.2. Potential valorization strategies

The objective of this study was to conduct a comprehensive analysis of the biomass composition of the invasive macroalgae *R. okamurae*, with a focus on exploring potential valorization strategies for the different compounds. *R. okamurae* was mainly composed of structural carbohydrates (60%), with alginate being the main constituent, accounting for 32% of its total composition. Apart from being a rich source of alginate, comparable to other currently used brown algae sources, the alginate of *R. okamurae* showed a M/G ratio of 0.93 equal of even lower than common industrial alginates. *R. okamurae* also contains a relative high concentration of fucose (8%) which is related to the presence of fucoidans with important biological functions. The oil obtained from *R. okamurae* is characterized by a high content of n3 PUFA and extremely low n6/n3 ratio (0.31), indicating a good balance between n6 and n3 fats, which is truly

beneficial from a nutritional standpoint. The presence of hexadecatetraenoic acid in relevant amounts makes *R. okamurae* a sustainable source worth evaluating. The content of n3 PUFA found in *R. okamurae* (18%) is comparable to those in widely utilized microalgae species like Chlorella vulgaris (Maltsev et al., 2021). According to the previous literature, n3 PUFA exhibit potential applications in treating various diseases such as atherosclerosis, Parkinson's, and Alzheimer's. In addition, *R. okamurae* could also be used for biofuel production, to generate renewable biofuels that are considered third-generation fuels.

The seaweed biomass was found to contain 12.2% protein and the analysis of its amino acid profile indicated that the proteins present an interesting amino acid profile, with essential amino acids comprising 32% of the total amino acids, with leucine being the most abundant. Non-essential amino acids, such as aspartic acid and glutamic acid, are also present, contributing to the unique flavour and aroma of seaweeds and seafood products. Depending on the source, vegetable proteins may be deficient in some essential amino acids. Some legumes such as soybeans, beans or peas are often deficient in sulphur amino acids such as methionine, while cereals tend to have low levels of lysine (Otero et al., 2022). This is why *R. okamurae* could serve as an alternative to animal protein sources and could complement the amino acid profile of existing vegan products.

R. okamurae also shows a significant polyphenol content of 4.5%, and outstanding antioxidant properties. In fact, its antioxidant activity clearly surpasses that of commonly utilized organic antioxidants like ginger or turmeric, revealing extraordinary nutritional potential.

Last, the seaweed biomass also contains essential minerals such as magnesium, calcium, potassium, iron, zinc, phosphorus, or manganese, which exert beneficial effects on the human organism, allowing, among others, normal bone development, good muscle and immune system functioning or blood pressure regulation. While certain foods like peanuts are recognized for their elevated essential mineral, especially magnesium, content, this remains considerably lower than that found in *R. okamurae*. Consequently, *R. okamurae* emerges as a viable alternative for sourcing essential minerals to produce dietary supplements.

Based on the findings of this research, it can be asserted that the invasive macroalgae *R. okamurae* could be a sustainable source of functional, nutritional, technological or bioactive

compounds which exhibit promising potential for their utilisation in various industrial sectors, such as food, feed, pharmaceuticals and cosmetics, transforming the threat into an opportunity within the circular economy.

5. Conclusions

The aim of this study was to investigate the composition of the invasive macroalgae *R. okamurae,* so as to find potential applications for its complex matrix. The high quantities and low M/G ratio of alginate prompt further research on their technological properties and potential extraction strategies. It also contains a relatively high concentration of fucose, which is related to the presence of fucoidans with important biological functions. For a macroalgae, a relatively high lipid content of 17% was found, with significant amounts of n3 PUFA (13%) and low n6/n3 ratio, indicating a good balance between essential fatty acids. It also contained alternative vegan protein with good nutritional potential, including essential amino acids. Additionally, *R. okamurae* showed significant polyphenol content and outstanding antioxidant properties, surpassing the antioxidant activity of commonly used organic antioxidants. While the seaweed biomass contained essential minerals, such as magnesium, calcium, potassium, iron, zinc, phosphorus, and manganese, it also presented concerning levels of toxic elements like lead and arsenic that exceed regulated limits for human consumption and must be considered prior to marketing. Overall, this study suggests that *R. okamurae* could be a sustainable source of various functional, nutritional, technological, or bioactive compounds, with promising potential for removal of this invasive species and utilization in various industrial sectors.

6. Acknowledgements

This work was done within the framework of a collaboration with HISPANAGAR S.L (Burgos, Spain). The research was funded by the Spanish Ministry of Science, Innovation and Universities through projects PDI2020-114821RB-I00, PID2020-117744RJ-I00, as well as TED2021-129711B-I00 and CEX2021-001189-S, funded by MCIN/AEI/10.13039/501100011033. The authors also acknowledge funding through project CIRCALGAE (Horizon Europe) under grant agreement

101060607. In all projects, funding from “ERDF A way of making Europe”, the “European Union” or the “European Union NextGenerationEU/PRTR” is also acknowledged, as appropriate.

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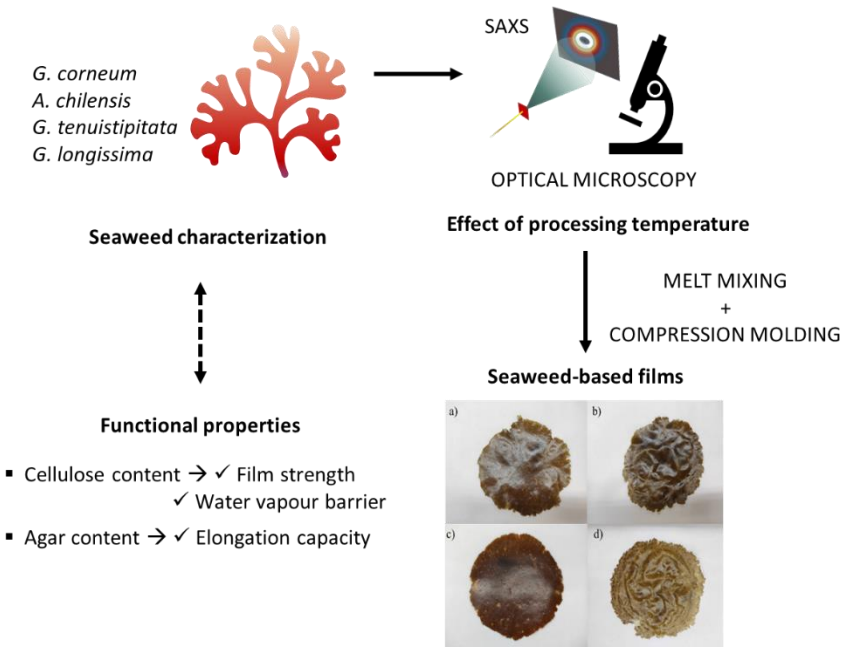
3.1.2

SUSTAINABLE BIO-BASED MATERIALS FROM MINIMALLY PROCESSED RED SEAWEEDS: EFFECT OF COMPOSITION AND CELL WALL STRUCTURE.



This section is an adapted version of the following published research article:

Cebrián-Lloret, V., Martínez-Abad, A., López-Rubio, A., & Martínez-Sanz, M. (2022). Sustainable bio-based materials from minimally processed red seaweeds: Effect of composition and cell wall structure. *Journal of Polymers and the Environment,* 31(3), 886-899.

1. Abstract

This study reports on the use of whole seaweed biomass to obtain bio-based films for food packaging applications. Specifically, four different species of agarophytes (*Gelidium corneum, Agarophyton chilensis, Gracilaria tenuistipitata* and *Gracilariopsis longissima*) were minimally processed by melt blending and compression molding, and the effect of their composition and cell wall structure on the final performance of the films was investigated. The seaweed biomass was mainly composed of carbohydrates (35-50%), but significant amounts of proteins and ashes were also detected. Temperature-resolved SAXS experiments and microscopy analyses evidenced that a higher temperature of 130 ºC is required to promote the release of agar from the tougher cell walls from *G. corneum* and *G. tenuistipitata*. The higher cellulose content of *G. corneum* (ca. 15%) resulted in films with higher mechanical resistance and water vapor barrier capacity, while the higher agar content of *A. chilensis* improved the elongation capacity of the films. The results from this work evidence the potential of red seaweed biomass to generate food packaging materials in a cost-effective and environmentally friendly way.

2. Introduction

Plastics are one of the materials most widely used due to their low cost, good processability and wide range of barrier and mechanical properties. However, conventional petroleum-based plastics are not biodegradable and therefore accumulate in natural ecosystems for up to several thousand years after disposal, causing serious environmental problems (Abdul Khalil et al., 2017; Doh et al., 2020; Otari & Jadhav, 2021). As a more sustainable alternative, biodegradable polymers obtained from renewable natural resources, i.e. biopolymers, are being studied (Cebrián-Lloret, Metz, et al., 2022; Fathiraja et al., 2021; Martínez-Sanz, Cebrián-Lloret, et al., 2020). However, the properties of biopolymers are not yet comparable to those of benchmark synthetic polymers, especially in terms of mechanical and barrier properties. Even though for certain applications the properties of some biopolymers may be acceptable, their high production costs are one of the main factors precluding their commercialization. Moreover, the raw materials generally used for biopolymer production come from land-based crops, competing with their traditional use, the food sector. This is why, as an alternative, aquatic biomass sources such as marine plants or seaweeds are being explored (Abdul Khalil et al., 2017;

Cebrián-Lloret, Metz, et al., 2022; Kartik et al., 2021; Martínez-Sanz, Pettolino, et al., 2017; Martínez-Sanz, Cebrián-Lloret, et al., 2020).

Macroalgae are classified into three groups: Ochrophyta, Phaeophyceae (brown seaweeds), Chlorophyta (green seaweeds) and Rhodophyta (red seaweeds) (Leandro et al., 2020; Øverland et al., 2018; Ponthier et al., 2020) and their composition and cell wall structure is strongly dependent on the species. In particular, red seaweeds are rich in minerals and vitamins, as well as some bioactive compounds such as proteins, carotenoids, phenols or lipids (Chan & Matanjun, 2017; Ponthier et al., 2020). Furthermore, their high polysaccharide content makes them an abundant source of biopolymers. Of particular interest is agar, which is the main structural component of the cell walls in some red seaweed species (Martínez-Sanz et al., 2019). It is composed of two main fractions: agarose, responsible for its gelling capacity, which consists of repeating units of alternating β- D -galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl groups; and agaropectin, which has a similar structure, but contains several substituent groups such as sulphates, methyl ethers and pyruvates (L. S. Ferreira et al., 2019; Martínez-Sanz et al., 2019). Due to its high gelling capacity, agar is widely used in the food and pharmaceutical industry (L. S. Ferreira et al., 2019; Trigueros et al., 2021). However, due to its excellent film-forming ability, in recent years its use in the production of food packaging materials has also been studied (da Rocha et al., 2018; Martínez-Sanz et al., 2019; Roy & Rhim, 2019; Wang et al., 2018). Although to a lesser extent, these seaweeds also have other polysaccharides of interest, such as cellulose, which also has a high potential for the development of bio-based food packaging (Cebrián-Lloret et al., 2022) since, due to its semi-crystalline structure, it has excellent mechanical and barrier properties. However, due to the limited processability of cellulose, it is generally used as a filler to improve the properties of other biopolymers (Benito-González et al., 2020; Cebrián-Lloret et al., 2022).

The industrial processes for the extraction of agar and cellulose are time and energy consuming (Kumar & Fotedar, 2009; Martínez-Sanz et al., 2019). To obtain biopolymeric materials for food packaging applications in a more energy efficient way, this study proposes to skip these extraction steps and produce packaging materials directly from seaweed biomass. This represents an innovative approach since, to the best of our knowledge, no previous works have

reported on the production of pure seaweed based films before.To this end, four different species of agar-producing red seaweeds (*Gelidium corneum, Agarophyton chilensis, Gracilaria tenuistipitata* and *Gracilariopsis longissima*) have been minimally processed using the melt blending technique combined with compression molding, to produce films. The effect of the composition and cell wall structure of each seaweed species on the final performance of the films has also been investigated as a strategy for the optimization of these materials.

3. Materials and methods

3.1. Raw materials

The four red seaweeds [*Gelidium corneum* (formerly *Gelidium sesquipedale*), *Agarophyton chilensis (*formerly *Gracilaria chilensis), Gracilaria tinuistipitata* and *Gracilariopsis longissima* (formerly *Gracilaria verrucosa*)] were kindly donated by Hispanagar S.A. (Burgos, Spain). The dried seaweeds were ground to powder of particle size < 250 µm before further processing. Glycerol was purchased from Panreac Quimica, S.A. (Castellar Del Vallés, Barcelona, Spain).

3.2. Compositional analysis and antioxidant properties

All the determinations were performed in triplicate, as follows.

3.2.1. Carbohydrate analysis

Carbohydrate composition was determined by reductive hydrolysis following the protocol established by Stevenson & Furneaux with slight modifications (Quemener & Lahaye, 1998; Stevenson & Furneaux, 1991). Briefly, 10 mg of dry sample was dissolved in a 5 mL aqueous rhamnose solution (0.5 mg/mL) for 40 min at 95 °C. Then, 0.5 mL of the solution was placed in Pyrex tubes and dried. A pre-hydrolysis step was conducted with 50 µL of 4-methylmorpholine-borane and 200 µL of 3 M trifluoroacetic acid were added to the tubes and placed in a heat block at 80 °C for 30 min. After cooling, 50 µL more 4-methylmorpholine-borane solution was added and the samples were dried. For the main hydrolysis, 200 µL of 2 M trifluoroacetic acid were added to the tubes and kept at 120 °C in the thermoblock for 1 h, followed by the addition of 100 µL of 4-methylmorpholine-borane and drying at 50 °C. After drying, the samples were resuspended in 1 mL H_2O, filtered through 0.45 µm syringe filters and transferred to chromatography vials. The monosaccharides were then analyzed using high-performance anion-

exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-6000 (Dionex, ThermoFisher Scientific, Sunnyvale, CA, USA). Control samples of known concentrations of mixtures of glucose, galactose, rhamnose, 3,6-anhydro-L-galactose and 6-0-methyl-D-galactose were reduced to their corresponding alditols and used for calibration.

3.2.2. Protein content

The protein content was estimated from the nitrogen content determined through the Dumas method, using an Elemental Analyser Rapid N Exceed (Paralab S.L., Spain), as described in (Cebrián-Lloret, Metz, et al., 2022). The nitrogen content was multiplied by a factor of 6.25.

3.2.3. Ash content

The mineral content of the dried seaweeds was determined by dry biomass calcination, according to the standard TAPPI T211 om-07 method, as described in (Cebrián-Lloret, Metz, et al., 2022).

3.2.4. Lipid content

The lipid content of the dried seaweeds was determined gravimetrically according to AOAC method 991.36 (AOAC, 1990) with some minor modifications, following the procedure described in (Cebrián-Lloret, Metz, et al., 2022).

3.2.5. Total phenolic content

The Folin-Ciocalteu technique was used to determine the total phenolic content of the dried seaweeds (Singleton et al., 1999), following the protocol described in (Cebrián-Lloret, Metz, et al., 2022).

3.2.6. $ABTS^{\cdot+}$ radical cation scavenging activity

The dried seaweeds' $ABTS^{\cdot+}$ radical cation scavenging capacity was assessed according to (Re et al., 1999), as detailed in (Cebrián-Lloret, Metz, et al., 2022).

3.3. Production of seaweed-based films

Films based on seaweed biomass were prepared by melt compounding, followed by compression molding, using formulations based on mixtures of seaweed powder and water, with the addition of glycerol as a plasticizer (30% w/w with respect to the amount of seaweed powder in the mixture). For *G. corneum, A. chilensis* and *G. tenuistipitata* films, the seaweed powder: water ratio used was 1:4 (w/w), while a lower ratio of 1:5 (w/w) was used for the *G. longissima* film. These ratios were chosen based on preliminary tests to maintain a suitable balance between the generated films' mechanical integrity and proper processability. The different mixtures were melt-mixed in a Brabender Plastograph (Brabender GmbH, Duisburg**,** Germany) internal mixer at a temperature of 130 °C and 60 rpm for 4 min. These conditions were selected based on preliminary trials. Subsequently, 4 g of the obtained blends were spread evenly on Teflon films and placed in a compression mold (Carver 4122, USA) at a pressure of 16 tons and 130 °C for 4 min to form one film. The films were then stored in cabinets equilibrated at a relative humidity of 53% and 25 °C for at least 7 days.

3.4. Temperature-resolved small angle X-ray scattering (SAXS) experiments

Small angle X-ray scattering (SAXS) experiments were carried out in the Non-Crystalline Diffraction beamline, BL-11, at ALBA synchrotron light source. The same formulations used to prepare the films were placed into sealed 2 mm quartz capillaries (Hilgenburg Gmbh, Germany) and analyzed. The energy of the incident photons was 12.4 keV or equivalently a wavelength, λ, of 1 Å. The SAXS diffraction patterns were collected by means of a Pilatus 1 M photon counting detector with an active area of 168.7 × 179.4 mm^2, an effective pixel size of 172 × 172 μm^2 and a dynamic range of 20 bits. The sample-to-detector distance was set to 7570 mm, resulting in a q range with a maximum value of q = 0.19 $Å^{-1}$. An exposure time of 1 s was selected based on preliminary trials. Samples were heated from 30 ºC to 130 ºC at a heating rate of 0.5 ºC/min and frames of 1 s, followed by a period of 239 s in which the samples were protected from the beam by a local shutter. Each data frame corresponds to a temperature increase of 2 ºC. The data reduction was treated by pyFAI python code (ESRF) (Kieffer & Wright, 2013), modified by ALBA beamline staff, to perform on-line azimuthal integrations from a previously calibrated file. The calibration files were created from a silver behenate standard. The radially averaged intensity

profiles were then represented as a function of q using the IRENA macro suite (Ilavsky & Jemian, 2009) within the Igor software package (Wavemetrics, Lake Oswego, Oregon).

3.5. Optical Microscopy

The seaweed aqueous solutions were analyzed by optical microscopy. Images were captured using a Nikon Eclipse 90i microscope with a 5-megapixel Nikon Digital Sight DS-5Mc cooled digital color microphotography camera (Nikon Corporation, Japan). Images of the materials were also captured using a fluorescent filter UV-2A (Excitation 330-380 nm, Dichroic Mirror 400, and LongPass 420 nm for emission). Nis-Elements Br 3.2 Software was used to analyze, and process acquired pictures (Nikon corporation, Japan).

3.6. X-ray diffraction (XRD)

XRD measurements were performed using a Bruker diffractometer model D5005. The instrument was equipped with a secondary monochromator and a Cu tube. The configuration of the equipment was θ–2θ, and the samples were examined over the angular range of 3°–60° with a step size of 0.02° and a count time of 200 s per step. Peak fitting was carried out using the Igor software package (Wavemetrics, Lake Oswego, Oregon), using the same protocol described in a previous work (Martínez-Sanz et al., 2019). The obtained values from the fitting coefficients are those that minimize the value of Chi-squared, which is defined as:

$$\chi^2 = \sum \left(\frac{y - y_i}{\sigma_i}\right)^2 \quad (1)$$

where y is a fitted value for a given point, y_i is the measured data value for the point and σ_i is an estimate of the standard deviation for y_i. The curve fitting operation is carried out iteratively and for each iteration, the fitting coefficients are refined to minimize χ^2. The crystallinity index was determined from the obtained fitting results by applying the following equation:

$$X_C(\%) = \frac{\sum A_{Crystal}}{A_{Total}} \times 100 \quad (2)$$

where A_{Total} is the sum of the areas under all the diffraction peaks and $\Sigma A_{Crystal}$ is the sum of the areas corresponding to the crystalline peaks.

3.7. Thermogravimetric analyses (TGA)

Thermogravimetric curves (TG) were recorded with a TA 550 (Waters- TA Instruments, New Castle, EEUU). Under an oxygen environment, the samples (5 mg) were heated at a rate of 10 °C/min from 30 to 800 °C. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

3.8. Scanning electron microscopy (SEM)

SEM experiments were carried out using a Hitachi S-4800 microscope with an accelerating voltage of 10 kV and a working distance of 8–16 mm. Before their morphology was evaluated, small sections of the seaweed films were sputtered with a gold-palladium combination under vacuum for 2 min.

3.9. Contact angle measurements

Contact angle measurements were carried out in a DSA25 equipment (Krüss, Hamburg, Germany) equipped with image analysis AD4021 software at ambient conditions. Using a precision syringe, a water droplet (3 µL) was placed on the film's surface. Contact angle values were obtained by analyzing the shape of the water drop after it had been placed over the film for 10 s. Results were obtained from an average of at least 5 measurements.

3.10. Water vapor permeability (WVP)

Direct permeability to water was determined from the slope of the weight gain versus time curves at 24 °C, following the same method described in (Cebrián-Lloret, Metz, et al., 2022). The tests were done at least in triplicate.

3.11. Water sorption

The water sorption capacity of the films was evaluated by registering the weight gain, using an analytical balance, when placing the samples in a cabinet equilibrated at 25 °C and 100% RH. Square samples with a total surface area of 6.25 cm^2 were cut from the films and their initial weight was registered. The assays were carried out at least in triplicate.

3.12. Mechanical properties

Tensile tests were performed at ambient conditions on a universal test Machine (Instron, USA), according to the method described in (Cebrián-Lloret, Metz, et al., 2022). The stress-strain curves were used to determine the elastic modulus (E), tensile strength (TS), and elongation at break (ε_B) of the films. At least, three specimens of each film were tested.

3.13. Statistics

The average ± standard deviation has been used to represent all the data. Different letters show significant differences in both graphs and tables. ($p \leq 0.05$). Analysis of variance (ANOVA) followed by a Tukey-test were used.

4. Results and discussion

4.1. Compositional characterization of the dried seaweeds

To understand the impact of the seaweed choice on the processability and functional properties of the films obtained by minimal processing, the composition and the antioxidant capacity of the different agarophytes were investigated.

As observed in Table 1, all seaweeds were mainly composed of structural carbohydrates (i.e., agar and cellulose), ashes and proteins. When comparing the four seaweeds, it could be observed that *G. corneum* presented the highest protein content (ca. 19%) and the lowest carbohydrate (ca. 35%) and lipid content (ca. 5%). It should be highlighted that despite its lowest carbohydrate content, the amount of cellulose was the highest in this seaweed (ca. 14%). Interestingly, although the agar content (ca. 21%) was the lowest compared to the other seaweed species, almost 97% of it consisted of agarose, i.e., the fraction that provides gelling capacity. Although a detailed characterization of the composition of *Gelidium corneum* is not available in the existing literature, other *Gelidium* species have been reported to have a similar composition (Álvarez-Viñas et al., 2019; Cavaco et al., 2021).

Despite belonging to the *Gracilaria* genera, the other three seaweed species showed significant differences in their composition. *Gracilariopsis longissima* presented the highest cellulose content (ca. 10%) and the lowest agar content (ca. 30%), with almost 26% of it constituted by agaropectin. *A. chilensis* and *G. tenuistipitata* exhibited very similar cellulose (ca. 6%) and

protein (ca. 19 and 16%, respectively) contents, but significantly differed in their agar concentration. In fact, A*. chilensis* was the species with the highest agar content (ca. 44%), although its agaropectin fraction was also quite high (ca. 23%). In contrast, *G. tenuistipitata,* showed a lower agar content (ca. 31%), but consisted of almost 93% agarose. Also noticeable was the high mineral content of all species, in agreement with previous values reported for red seaweeds, being significantly higher in *G. tenuistipitata* and *G. longissima* (ca. 23 and 28%, respectively). The lipid content, constituting up to ca. 9% of *A. chilensis* composition, was higher than the values previously reported for several *Gracilaria* species (Álvarez-Viñas et al., 2019). However, it should be noted that a great variability in the composition of the different species of the *Gracilaria* genera has been reported in the literature (Álvarez-Viñas et al., 2019; Lozano et al., 2016; Nunraksa et al., 2019; Siddhanta et al., 2009). This is also related to the fact that the chemical composition of seaweeds does not only vary according to the species, but also due to other aspects such as habitat, seasonality, maturity and environmental conditions (Banerjee et al., 2009; Chan & Matanjun, 2017; Naseri et al., 2019)Phenolic compounds, which are known to present antioxidant properties, have also been reported to be present in red seaweeds (up to ca. 20 mg GAE/g sample for extracts of various species of the *Gracilaria* genera (Chan et al., 2015; Ortiz-Viedma et al., 2021; Sanz-Pintos et al., 2017), or up to 14.1 mg GA/g sample for species of the *Gelidium* genera (de Oliveira et al., 2019), although it is difficult to find consistent values on their antioxidant capacity. Regarding polyphenol content, *G. corneum* showed the highest one (ca. 15 mg GAE/g sample), followed *by A. chilensis* and *G. tenuistipitata* with ca. 11.1 and 6 mg GAE/g sample respectively, while *G. longissima* presented the lowest content (ca. 1 mg GAE/g sample). However, although the antioxidant capacity of seaweeds is usually linked to the presence of polyphenols, our results evidence that the antioxidant capacity may be also linked to the presence of other bioactive

compounds such as sulphated polysaccharides (e.g., agaropectin) and lipids. In particular, *A. chilensis*, presenting the highest agar and lipid content from all the species, showed the highest antioxidant capacity (12.6 mg TE/g sample), while the lowest antioxidant capacity was obtained for *G. corneum*, the seaweed with the lowest agar and lipid content.

Table 1. Composition and antioxidant capacity of the dried seaweeds. Values within the same column with different letters are significantly different ($p \leq 0.05$).

	Protein (%)	Mineral (%)	Lipid (%)	Carbohydrate (%)		Polyphenol (mg GAE/g sample)	Antioxidant capacity ABTS (mg TE/g sample)
				Agar*	Cellulose		
G. corneum	19.4 ± 0.5^{d}	9.2 ± 0.05^{a}	4.7 ± 0.6^{a}	21 ± 0.5 [97]^{a}	14.2 ± 1.2^{c}	15 ± 0.9^{d}	3.8 ± 0.5^{a}
A. chilensis	18.6 ± 0.06^{c}	9.6 ± 0.1^{a}	9.1 ± 0.9^{c}	43.7 ± 6 [77]^{d}	5.9 ± 0.2^{a}	11.1 ± 0.3^{c}	12.6 ± 0.6^{c}
G. tenuistipitata	15.5 ± 0.09^{b}	23.3 ± 0.4^{b}	4.8 ± 1^{a}	30.7 ± 4 [92]^{b}	6.1 ± 0.02^{a}	6.3 ± 0.3^{b}	7.6 ± 0.6^{b}
G. longissima	8 ± 0.04^{a}	28 ± 1.3^{c}	6.9 ± 0.6^{b}	29.7 ± 3.7 [74]^{c}	9.5 ± 0.4^{b}	1 ± 0.2^{a}	9.4 ± 0.05^{d}

* The value in brackets corresponds to the percentage purity in agarose of each agar fraction as determined by HPAEC-PAD after reductive hydrolysis.

4.2. Structural effects of processing temperature

To determine the optimal processing conditions for the production of the films, preliminary tests were carried out in which aqueous dispersions of the four seaweeds were subjected to temperatures from 30 ºC up to 130 ºC and their nanostructure was characterized by means of SAXS. The scattering patterns obtained at selected temperatures were graphically represented in Kratky plots to enhance the visualization of structural features.

As observed in Figure 1, the temperature had a clear effect on all seaweeds, with the scattering intensity generally increasing as the temperature was raised. This may be explained by the diffusion of agar from the interior of the cell walls towards the liquid medium when increasing the temperature, hence increasing the scattering length density of the whole suspensions. In fact, a shoulder feature centered at q = 0.015 $Å^{-1}$ was detected in the *G. corneum* suspension heated at 130ºC. Similar features have been previously detected in the SAXS patterns from agars (Martínez-Sanz, Ström, et al., 2020) and although their origin is still uncertain, they have been hypothesized to be originated by a form factor effect associated with the length of the agarose double helices. Interestingly, in *A. chilensis* and *G. longissima*, the scattering intensity decreased

when raising the temperature from 110 ºC to 130 ºC. This may be originated by the disruption of the double helical structure in the agarose fraction when reaching high temperatures. The semi-crystalline structure of agarose double helices is thought to provide higher scattering contrast with the solvent than the amorphous agaropectin domains. It is reasonable to hypothesize that the smaller fraction of semi-crystalline agarose in these two seaweed species is disrupted more quickly when increasing the temperature, hence producing a decrease in the scattering intensity.

In addition, several small scattering peaks were detected within the high q region in all samples. In *G. corneum*, i.e., the seaweed with the highest cellulose content, a weak peak centered at ca. 0.14 $Å^{-1}$ (corresponding to a real distance of ca. 4.5 nm) was observed in the suspension at 30 ºC and shifted towards lower q values when heating above 90 ºC. The shape and position of this peak is very similar to that previously reported for other cellulose-rich sources, such as cotton, in the dry state (Martínez-Sanz, Pettolino, et al., 2017). This interference peak has been related to the center-to-center distance between the cellulose microfibrils, which in the dry state are closely packed together. The fact that this peak could be observed in the aqueous suspensions indicates a high degree of packing even in the hydrated state, in contrast to other cellulosic sources were water can easily penetrate the space between the microfibrils and increase their interspacing distance (Martínez-Sanz, Mikkelsen, et al., 2017; Martínez-Sanz, Pettolino, et al., 2017). This weak peak was also observed in *A. chilensis* and *G. tenuistipitata*, although its position was slightly different depending on the species. In the case of *G. tenuistipitata*, the peak appeared at 0.08 $Å^{-1}$ (corresponding to a real distance of ca. 8.1 nm) for the suspension at 30ºC, while it shifted towards higher q when raising the temperature (being centered at 0.09 $Å^{-1}$ when the sample reached 130 ºC).

In *A. chilensis*, two peaks were detected at temperatures up to 90 ºC; the first one was located at 0.10 $Å^{-1}$ (real distance of ca. 6.5 nm) at 30 ºC and shifted towards higher q when increasing the temperature, whereas the second peak was located at 0.14 $Å^{-1}$ (real distance of ca. 4.5 nm). In *G. longissima*, a sharper and more intense peak, located at 0.14 $Å^{-1}$, was detected for temperatures up to 110ºC. The sharper appearance of this peak in *G. longissima* may be explained by the relatively high cellulose content of this seaweed (as compared to the other

species) and its low agarose content, thus originating higher contrast between the crystalline cellulose and the amorphous agaropectin.

It should be noted that while an interfibrillar distance of 4.5 nm corresponded to the species with the highest cellulose content (i.e., *G. corneum* and *G. longissima*), which is very similar to the values previously reported for dry cotton and bacterial cellulose (Martínez-Sanz, Mikkelsen, et al., 2017; Martínez-Sanz, Pettolino, et al., 2017), greater distances were detected for the other two species, which was presumably due to the presence of other polysaccharides interacting with cellulose and increasing the distance between microfibrils. Furthermore, increasing the temperature in the suspensions may have promoted the penetration of water within the interfibrillar space and/or affected the conformation of non-cellulosic polysaccharides, hence modifying the distance between the microfibrils.

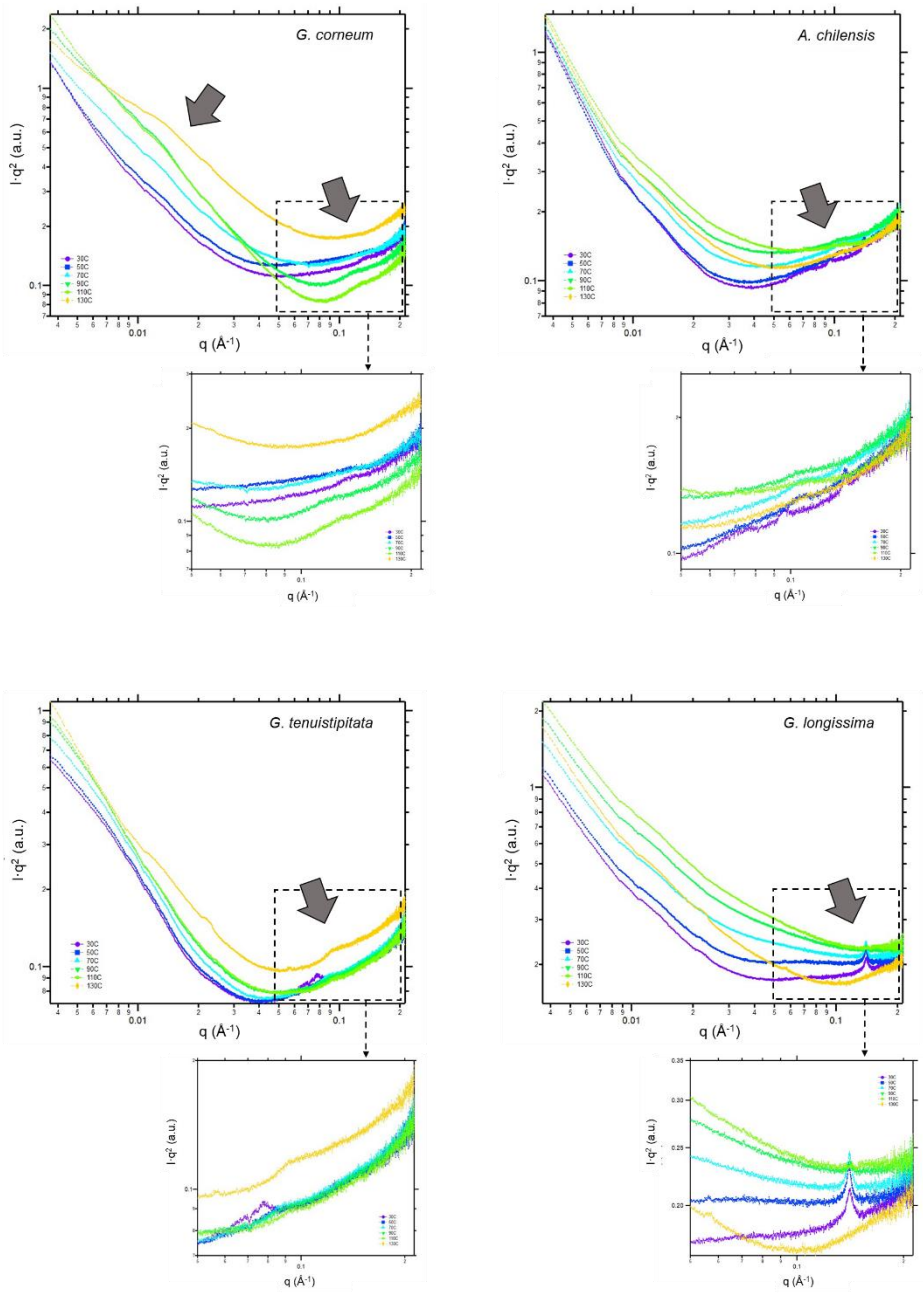


Figure 1. SAXS Kratky plots of seaweed aqueous dispersions processed at different temperatures from 30 to 130 ºC. The arrows point towards structural peaks, which are more clearly visualized in the insets.

The effect of temperature on the cell structure of the different seaweeds was also studied through optical microscopy. The seaweed aqueous suspensions were examined after reaching temperatures of 110 and 130 °C (above the melting temperature of agar). Figure 2 shows representative images taken with bright light and ultraviolet filters.

The images taken with bright light clearly show changes in the morphology of the seaweeds when the temperature increased. In particular, the rough edges of the seaweed cells became less defined and the cell size increased. This swelling effect was less noticeable in *G. corneum* while it was much more evident in *A. chilensis* and *G. longissima*. The increase in the temperature in the presence of moisture is expected to promote the melting of the agar contained within the seaweed cell walls, which may be able to diffuse towards the liquid medium. It seems that the high cellulose content in *G. corneum*, yielding tough cell walls, together with the high agarose/agaropectin ratio, did not favour the diffusion of agar. On the contrary, the lower agarose/agaropectin ratio in *A. chilensis* and *G. longissima* seemed to promote agar diffusion. This is further supported by the observation of the samples under the UV filter. While the images of the unprocessed native seaweeds were mostly dominated by the appearance of bright blue fluorescent regions, the images of the processed seaweeds showed very faint to no light blue fluorescence. According to compositional analyses, it may be hypothesised that the blue fluorescent areas correspond to agar-rich regions, which disappear almost completely after processing (especially at 130 °C) indicating the release of agar from the cell walls. In all cases it seems that increasing the temperature from 110 to 130 ºC produced a greater release of the agar, which would be, in principle, more optimum for the production of the seaweed-based films.

It should be noted that temperatures higher than 130 ºC were not suitable to process these seaweeds since some degradative processes (Maillard reactions, glycerol thermal degradation, polyphenol degradation, etc.) led to a substantial detriment in the mechanical properties of the obtained films.

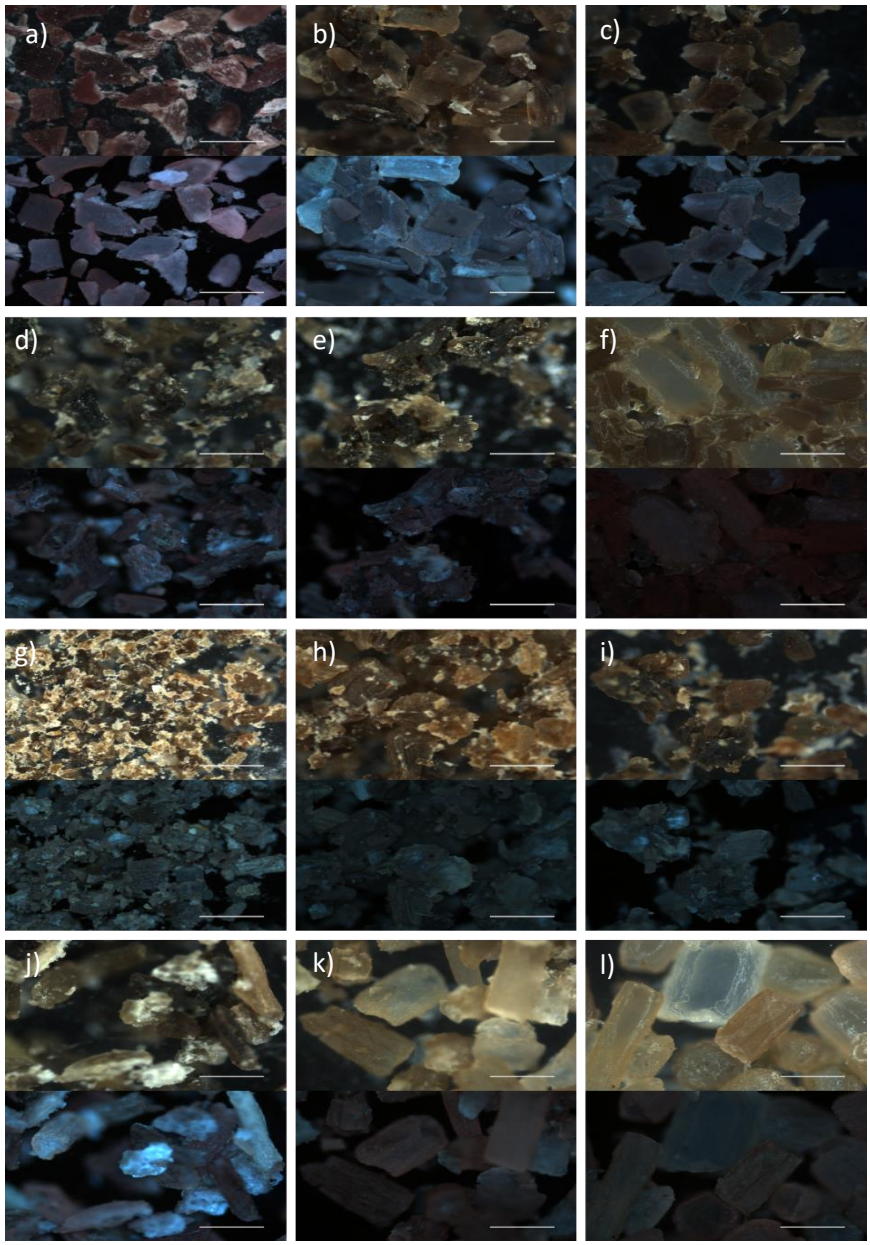


Figure 2. Optical microscopy images of seaweed aqueous suspensions in their native state (a, d, g, j) and after being processed at 110 ºC (b, e, h, k) and 130 ºC (c, f, i, l) for *G. corneum*, (a, b, c) *A. chilensis*, (d, e, f) *G. tenuistipitata,* (g, h, i) and *G. longissima (j, k, l)*. Scale bars correspond to 500 µm. Top images were taken with bright light while bottom images were taken using a fluorescent filter UV-2A.

4.3. Characterization of the seaweed-based films

Once the optimum processing temperature of the seaweeds was determined, the films were produced by melt mixing, followed by compression molding. Figure 3 shows the visual appearance of the films. As expected, due to the variety of compounds present in the seaweed biomass, all of them were opaque with a brownish coloration, similar to that previously observed in films produced from less purified agars extracted from *G. corneum* (Martínez-Sanz et al., 2019).

The films' surface morphology was analyzed by SEM and representative images are shown in Figure 3. Due to the addition of glycerol, small particles homogeneously distributed over the whole surface of the films were visible in all samples. However, a noticeable morphological difference was observed between the films made from *G. corneum* and the films based on *Gracilaria* species. While the *G. corneum* film exhibited a more homogeneous surface, where only the glycerol particles were visible, the films based on *Gracilaria* species showed significantly rougher surfaces, fact which could be related to the greater swelling of the cell walls from these species, generating larger particles.

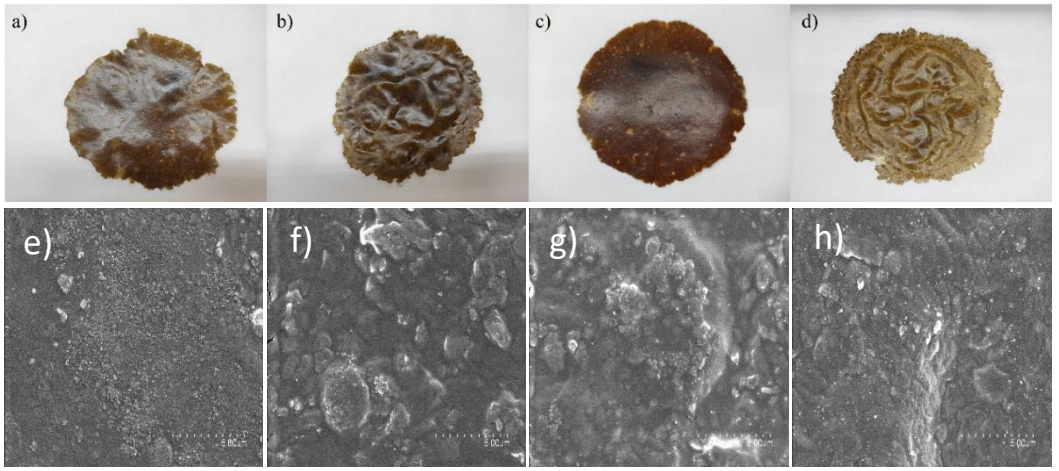


Figure 3. Visual appearance (a-d) and SEM images (e-h) of the seaweed-based films: *G. corneum* (a, e), *A. chilensis* (b, f), *G. tenuistipitata* (c, g), *G. longissima* (d, h).

TGA analyses were carried out to determine the thermal stability of the films. The derivative of the weight loss with temperature was plotted and the results are shown in Figure 4.

The films showed a multistep degradation mechanism, which was mostly dominated by a strong peak around 270-280 °C, attributed to agar degradation (Duman et al., 2020; Shankar et al., 2014). All the samples exhibited a small degradation step centered around 150 °C, which may be attributed to moisture loss and glycerol degradation (Duman et al., 2020; Raphael et al., 2010; Wang et al., 2018). The degradation steps centered around 230 °C, which may be attributed to the presence of proteins (Cebrián-Lloret et al., 2022), and at 330 °C attributed to cellulose (Cebrián-Lloret et al., 2022; Shankar & Rhim, 2016), although present in all samples, were more evident in the film made from *G. corneum.* This indicates that a multi-phase structure was attained in the films from *G. corneum*, which can be linked to the fact that this seaweed species is structurally less affected by the melt mixing temperature than the *Gracilaria* seaweeds, as evidenced by optical microscopy (cf. Figure 2).

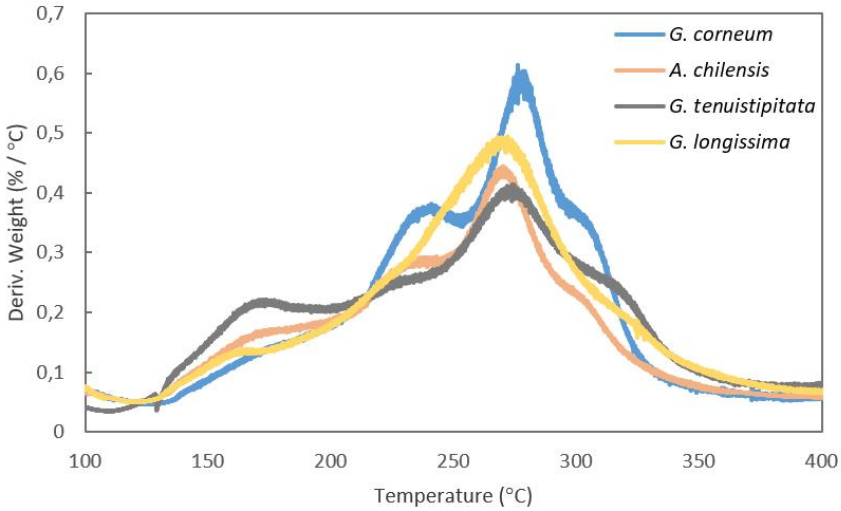


Figure 4. TGA derivative curves of the seaweed-based films.

XRD analyses were carried out to investigate the crystalline structure of the produced films and the obtained patterns are shown in Figure 6.

As observed, all the films presented a broad diffraction band within the range of 12-25º with a maximum at 19º, similar to the patterns reported for agar-based films (Guerrero et al., 2014; Martínez-Sanz et al., 2019). Furthermore, in the *G. corneum* films and also, more subtly, in the *G. longissima* films, a peak centered at 22.5º, related to the crystalline structure of cellulose I (Y. W. Chen et al., 2016) was also noticeable. This is consistent with the higher cellulose content present in these seaweed species. Several sharp and intense peaks could also be observed in the films from *G. tenuistipitata* and, to a lesser extent, from *A. chilensis*. These peaks may be attributed to the presence of mineral compounds, such as silica (SiO_2) and weddellite ($CaC_2O_4 \cdot 2H_2O$), which have been previously detected in the XRD spectra of some red seaweeds (Martínez-Sanz et al., 2019).

The crystallinity of the films was estimated by fitting the areas under the diffraction patterns and, as deduced from the values shown in Table 2, *G. tenuistipitata* and *G. corneum* films presented a more crystalline structure. However, it should be considered that, for the calculation of the overall crystallinity, other components such as cellulose and minerals are also contemplated and, therefore, the values are not indicative of the degree of crystallinity of the agar. Looking at the values calculated only considering the crystalline peak corresponding to agar, it is evident that those seaweeds with the greatest agarose/agaropectin ratio (i.e., *G. corneum* and *G. tenuistipitata*) presented a more crystalline agar structure. On the other hand, despite being the species with the greatest overall polysaccharide content, the film from *A. chilensis* presented the most amorphous behavior, which may be ascribed to the greater proportion of agaropectin in this seaweed.

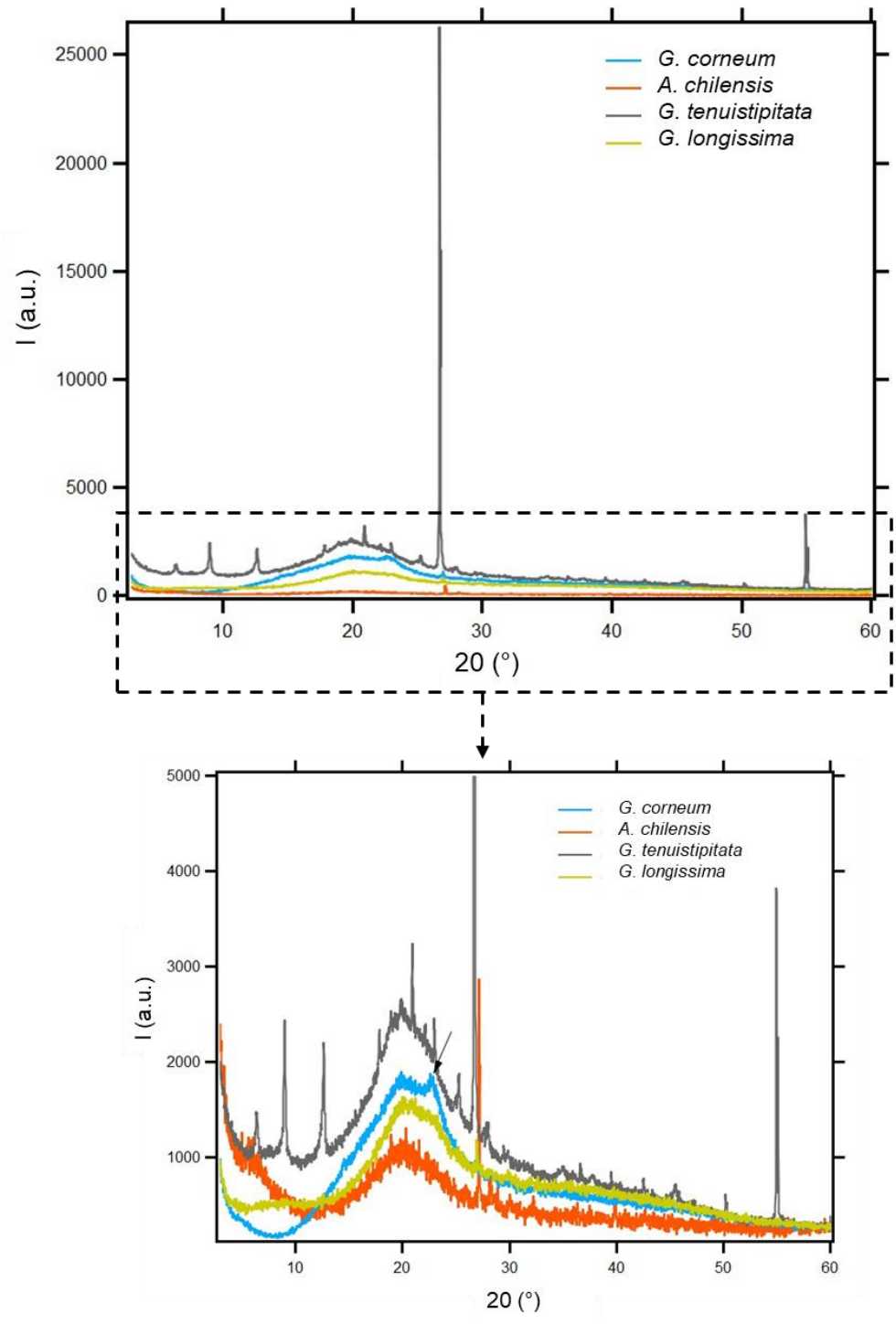


Figure 5. XRD patterns of the seaweed-based films.

Table 2. Crystallinity, mechanical and water barrier properties of the seaweed-based films. Values within the same column with different letters are significantly different ($p \leq 0.05$).

	Xc (%)*	E (MPa)	σ (MPa)	ε_b (%)	$P_{H2O} \cdot 10^{-13}$ (k g·m/s·m^2·Pa)	Water sorption (%)
G. corneum	50 [44]	1213 ± 95^c	14.1 ± 1.5^c	1.8 ± 0.17^a	1.6 ± 0.08^{ab}	130 ± 4^a
A. chilensis	27 [25]	581 ± 42^a	9.6 ± 0.4^a	4.9 ± 0.26^c	1.9 ± 0.17^b	---
G. tenuistipitata	62 [46]	748 ± 70^b	9.9 ± 1^a	2.9 ± 0.06^b	1.8 ± 0.05^b	---
G. longissima	40 [31]	749 ± 10^b	11.6 ± 0.7^b	2.5 ± 0.2^b	1.4 ± 0.02^a	152 ± 1^b

X_C: Crystallinity index; E: Young's modulus; σ: tensile strength; ε_b: elongation at break; P_{H2O}: water permeability.

* The crystallinity values estimated by considering only the agar characteristic peaks are shown between brackets.

To evaluate the potential of the films as food packaging materials, their performance properties were also characterized. The mechanical properties were assessed by means of tensile testing and the most representative parameters obtained from the stress-strain curves are summarized in Table 2.

As expected, due to its higher cellulose content, *G. corneum* resulted in stiffer (E ≈1213 MPa) and more resistant (σ ≈ 14.1 MPa) films with a lower elongation at break (εb ≈ 1.8%). In contrast, the higher agar and lower cellulose content of *A. chilensis* significantly decreased the elastic modulus (E ≈ 581 MPa) and tensile strength of the film (σ ≈ 9.6 MPa) but improved its ductility (εb ≈ 4.9%). *G. tenuistipitata* and *G. longissimaa*, showed non-significant differences between them in terms of stiffness and elongation at break. However, the higher cellulose content of *G. longissima* improved the tensile strength of the films. When comparing the results obtained for seaweed-based films with those reported for some reference biopolymers such as PLA (E =3290 MPa, σ=49.6 MPa, εb= 2.4%) (B.-K. Chen et al., 2010; Mathew et al., 2005) or thermoplastic starch (E =29.8 MPa, σ=3.1MPa, εb= 62.6%) (Cyras et al., 2008; Majdzadeh-Ardakani et al., 2010) it can be noted that the minimal processing method of seaweeds results in biofilms with an

intermediate behavior between that from PLA and thermoplastic starch in terms of mechanical properties. Furthermore, the mechanical properties are also comparable to those reported for agar films processed by casting (E =29-1600 MPa, σ=6-38 MPa, ε_B=15-26%) (Makwana et al., 2020; Martínez-Sanz, Martínez-Abad, et al., 2019).

The water vapor permeability (WVP) of the films was also characterized and the results, gathered in Table 2, suggest that the seaweeds with the highest cellulose content (i.e., *G. longissima* and *G. corneum*) produced films with a better barrier capacity. As seen in previous studies, this may be due, amongst other factors, to the reduced amount of free hydroxyl groups due to the interactions established between cellulose and agar (Cebrián-Lloret et al., 2022; Martínez-Sanz et al., 2019). As previously reported, both agar and cellulose have a high barrier capacity (Cebrián-Lloret et al., 2022; Martínez-Sanz et al., 2019; Rhim, 2011; Shankar & Rhim, 2016). Even so, it is surprising that these films, made from seaweed biomass without any purification process, presented WVP values comparable to those reported for pure cellulose films extracted from aquatic biomass sources such as *Arundo donax* (terrestrial angiosperm) (Martínez-Sanz et al., 2018) or *Posidonia oceanica* (marine angiosperm) (Benito-González et al., 2018), as well as films made from unpurified agar-based extracts (Martínez-Sanz et al., 2019). Interestingly, the cellulose content seemed to be the prevailing factor controlling the permeability, rather than the overall crystallinity of the films.

To evaluate the sensitivity of the films to moisture, their water sorption capacity was also measured. While the films from *G. corneum* and *G. longissima* maintained their structure throughout the experiment, the films made from *A. chilensis* and *G. tenuistipitata* disintegrated over time. Therefore, in line with the water vapor barrier capacity, this indicates that seaweeds with a higher cellulose content result in films with a higher structural stability to moisture.

Interestingly, the films made from *G. longissima* and *G. corneum*, exhibited a slightly more hydrophilic surface than the films made from *G. tenuistipitata* and *A. chilensis*, as evidenced by the contact angle measurements (cf. Figure 7). This may be due, amongst other factors, to the higher agar and protein content of the two latter species, since, as previously reported, protein/polysaccharide interactions can have a hydrophobising effect on the surface of the films (Guerrero et al., 2013). In general, seaweed-based films showed a similar behavior to that

reported for films based on some polysaccharides such as agar (50°) (Rhim, 2011) or cellulose nano whiskers (44°) (Martínez-Sanz et al., 2013).

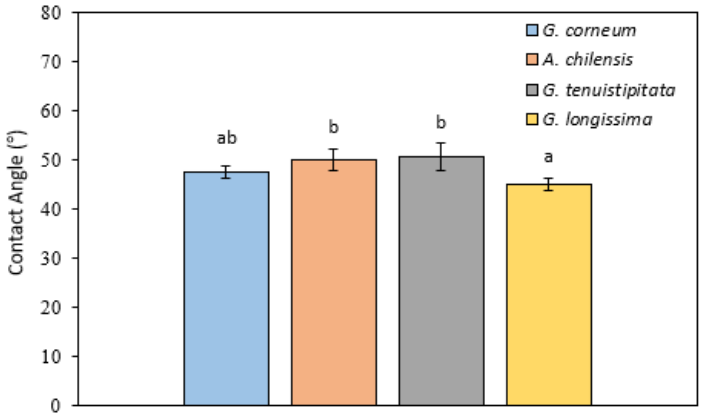


Figure 6. Water contact angle measurements of the seaweed-based films.

5. Conclusions

Four different agarophyte species were minimally processed by melt blending combined with compression molding and the effect of the composition and cell wall structure of the different species on film performance were investigated. The seaweed biomass was mainly composed of carbohydrates (35-50%), but significant amounts of protein and ashes were also detected. *G. corneum* showed the highest cellulose content (ca. 15%) and the lowest agar content (ca. 21%), although it contained the highest agarose ratio (97%). *G. tenuistipitata* also showed a relatively low agar content (ca. 31%) of which almost 93 % was agarose. In contrast, the agar fraction *in A. chilensis* and *G. longissima* contained a significant proportion of agaropectin, with the latter one having a relatively high amount of cellulose (ca. 10%). The greater cellulose content in *G. corneum* limited the diffusion of agar when seaweed aqueous suspensions were processed at high temperatures, while the more amorphous character of the agar in *A. chilensis* and *G. longissima* promoted its diffusion towards the liquid medium. In any case, increasing the temperature to 130 ºC seemed to promote a greater release of agar.

The cellulose content seemed to be the main factor controlling the mechanical and water barrier performance of the films. The higher cellulose content of *G. corneum* resulted in stronger films with high water vapor barrier capacity, while the higher agar content of A*. chilensis* improved its elongation capacity. The films made from *G. longissima* also showed interesting properties due to the relatively high cellulose content, thus offering an interesting alternative to produce biopolymeric films. The results from this work evidence the potential of red seaweed biomass to generate food packaging materials in a cost-effective and environmentally friendly way.

6. Acknowledgements

This work was financially supported by Hispanagar. Synchrotron experiments were performed at NCD beamline at ALBA Synchrotron with the collaboration of ALBA staff (2020024090 project

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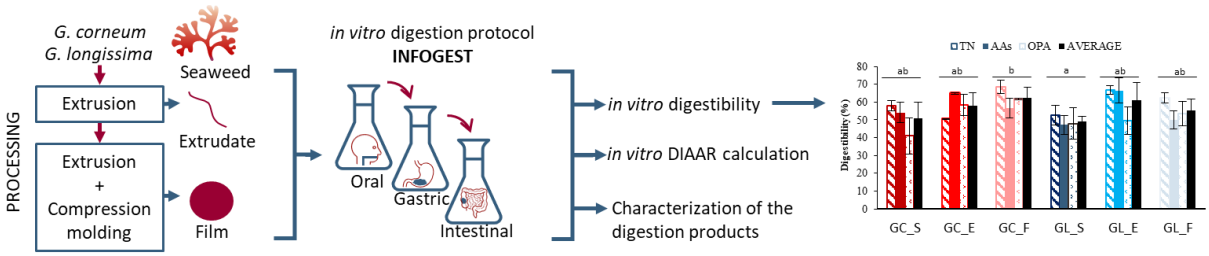
3.1.3

IN VITRO DIGESTIBILITY OF PROTEINS FROM RED SEAWEEDS: IMPACT OF CELL WALL STRUCTURE AND PROCESSING METHODS.



This section is an adapted version of the following published research article:

Cebrián-Lloret, V., Martínez-Abad, A., Recio, I., López-Rubio, A., & Martínez-Sanz, M. (2024). *In vitro* digestibility of proteins from red seaweeds: Impact of cell wall structure and processing methods. *Food Research International*, *178*, 113990.

Section 3.1.3

1. Abstract

This study aimed to assess the nutritional quality and digestibility of proteins in two red seaweed species, *Gelidium corneum* and *Gracilaropsis longissima*, through the application of *in vitro* gastrointestinal digestions, and evaluate the impact of two consecutive processing steps, extrusion and compression moulding, to produce food snacks. The protein content in both seaweeds was approximately 16%, being primarily located within the cell walls. Both species exhibited similar amino acid profiles, with aspartic and glutamic acid being most abundant. However, processing impacted their amino acid profiles, leading to a significant decrease in labile amino acids like lysine. Nevertheless, essential amino acids constituted 35-36% of the total in the native seaweeds and their processed products. Although the protein digestibility in both seaweed species was relatively low (<60%), processing, particularly extrusion, enhanced it by approximately 10%. Interestingly, the effect of the different processing steps on the digestibility varied between the two species. This difference was mainly attributed to compositional and structural differences. *G. corneum* exhibited increased digestibility with each processing step, while *G. longissima* reached maximum digestibility after extrusion. Notably, changes in the amino acid profiles of the processed products affected adversely the protein nutritional quality, with lysine becoming the limiting amino acid. These findings provide the basis for developing strategies to enhance protein quality in these seaweed species, thereby facilitating high-quality food production with potential applications in the food industry.

2. Introduction

The food industry is continuously seeking to develop a broad range of products to cover the diverse demands of consumers, which implies, among others, exploring novel alternative sources of protein production (Fuenzalida et al., 2016; Kaur et al., 2022). Although animal proteins continue to play an important role in the food industry (Pam Ismail et al., 2020), their large-scale production has negative consequences for biodiversity, climate change, and leads to the depletion of freshwater resources. Moreover, motivated by the growth of vegetarian and vegan markets, along with the rising preference for healthier and sustainable dietary options,

consumers increasingly opt for plant-based foods over animal-based alternatives, thus reducing their carbon footprint (He et al., 2021; Kumar et al., 2022; McClements & Grossmann, 2021; Williams & Phillips, 2021). Thus, in response to the social, environmental, and economic challenges, it has become crucial to explore new sources of proteins for sustainable food production (Kumar et al., 2022). In this regard, several recent studies have investigated algae as a potential source for obtaining proteins, as well as other bioactive compounds, such as carotenoids, phenols, or lipids (Cebrián-Lloret et al., 2022; Salehi et al., 2019; Yang et al., 2021). Traditionally, the use of algae in food has been closely associated with Asia, where they have been an essential part of the diet and culture for centuries (Mahadevan, 2015). In this region, algae are highly valued for their taste, nutritional value, and health benefits. However, in recent years, the interest in algae as a food source has transcended Asian borders and spread globally due to their interesting nutritional profile (good source of proteins, high dietary fibre content and abundance of vitamins and minerals) and sustainable potential as a food resource (Rawiwan et al., 2022; Rioux et al., 2017).

The term algae refers to a diverse group of eukaryotic, photosynthetic organisms which includes both microalgae (unicellular organisms) and macroalgae (or seaweeds), which are multicellular and macroscopic organisms that can reach several meters in length (Pereira, 2021). Depending on their pigments, seaweeds can be classified into three groups: Chlorophyta (green seaweeds), Phaeophyceae (brown seaweeds), and Rhodophyta (red seaweeds) (Cebrián-Lloret et al., 2022; Leandro et al., 2020; Ponthier et al., 2020). Red seaweeds exhibit a high content of polysaccharides, making them a valuable source of biopolymers (Cebrián-Lloret et al., 2022; Mostafavi & Zaeim, 2020). Amongst them, agar stands out as the main structural component of the cell walls of many red seaweed species (Martínez-Sanz et al., 2019). Agar consists of two major fractions: agarose, which is responsible for its gelation capacity and is composed of repetitive units of β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl groups, and agaropectin, which shares a similar structure but incorporates diverse substituent groups such as sulphates, methyl ethers, and pyruvates (Cebrián-Lloret et al., 2022; Ferreira et al., 2019; Jayakody et al., 2022). Due to its stabilizing, gelling, emulsifying and viscosity-enhancing properties, agar is widely used as additive in food products such as jams, jellies, ice creams and

other dairy products (Nishinari & Fang, 2017; Qin, 2018; A. M. M. Sousa et al., 2021). Although, to date, red seaweeds such as *Gelidium* and *Gracilaria* species have been mostly exploited in the food industry for the extraction of agar, they also represent an appealing source of alternative proteins, since they present high levels of usable protein (up to 47%) and a relatively high content of essential amino acids (EAAs) (de Souza Celente et al., 2023; Rawiwan et al., 2022). However, despite several studies investigating the potential of seaweeds as a protein source (Bjarnadóttir et al., 2018; Echave et al., 2021; Thiviya et al., 2022), there is limited research focusing on their digestibility, which is an essential parameter for evaluating the nutritional quality of a protein source (De Bhowmick & Hayes, 2022; Rawiwan et al., 2022). A previous study revealed that the digestibility of proteins present in red seaweeds is superior compared to proteins derived from green seaweeds (Wong & Cheung, 2001). However, as observed in the case of *C. crispus*, the *in vitro* digestibility of seaweeds is, in general, relatively low, not exceeding 45%, which has been attributed to the presence of mucilaginous polysaccharides. Another study also highlighted that phenolic compounds and quinones present in seaweeds can form non-specific complexes with proteins, thereby reducing their digestibility (Ramin et al., 2019). This is why, to enhance the protein quality, various authors have studied the impact of food processing on plant proteins, primarily through the use of thermal treatments (Reynolds et al., 2022). Some examples of processing techniques which have been reported to improve digestibility include cooking (Park et al., 2010), autoclave sterilization (Sun et al., 2012), microwave heating (Sun et al., 2012), and extrusion (Wu et al., 2015). In the particular case of red seaweeds, the distinct cell wall structure and integrity of different species has demonstrated to affect their behaviour when subjected to processing methods such as heating at high humidity conditions (Cebrián-Lloret et al., 2022). Thus, it is expected that different red seaweeds present distinct digestibility, which may also be differently affected by processing.

The aim of this study was, thus, to evaluate the nutritional quality and digestibility of proteins present in two red seaweeds, which are widely used in the food industry to extract agar, but could also represent a potential source of food proteins: *Gelidium corneum* (previously known as *Gelidium sesquipedale*) and *Gracilaripsis longissima* (previously known as *Gracilaria*

verrucosa). Additionally, the impact of the seaweeds cell wall structure and composition, as well as the effect of two different processing methods which could be used to generate seaweed-based snack foods: (1) extrusion alone, and (2) extrusion and subsequent compression moulding to obtain biofilms, on their digestibility, were investigated.

3. Materials and methods

3.1. Raw materials

Two seaweed species, *Gelidium corneum* and *Gracilariopsis longissima*, were kindly donated by Hispanagar S.A. (Burgos, Spain). Prior to further processing, the seaweed biomass was washed, dried by means of a Digitronic-TFT oven (J.P. SELECTA, Spain) for 24 h at 50 °C, and ground into a powder with a particle size of 250 µm using a ZM 200 Model Ultra-Centrifugal Mill (Retsch, Germany).

3.2. Production of extrudates and films from seaweed biomass

To produce the extruded products and films from seaweed biomass, a mixture of seaweed powder and water was utilized, along with glycerol as a plasticizer (at a concentration of 30% w/w relative to the seaweed powder content in the mixture). These proportions were determined based on initial tests to ensure a suitable balance between the physical integrity of the resulting products and their processability. The different mixtures were then processed using an Xplore Micro Compounder 15HT (Sittard, The Netherlands) at a temperature of 130°C and a speed of 100 rpm for 4 minutes. To produce the films, 4 g of the extrudates were uniformly applied onto Teflon films and placed within a compression mould (Carver 4122, USA) under a pressure of 16 tons and at 130°C for 4 minutes. The processing parameters to produce seaweed biomass-based extruded products and films were selected based on prior research (Cebrián-Lloret et al., 2022).

The samples were coded as follows: the *G.corneum* seaweed was coded as GC_S, and the processed materials obtained from this seaweed species were coded as GC_E for the extrudate and GC_F for the film. In the case of *G. longissima,* the seaweed was coded as GL_S, the extrudate as GL_E, and the film as GL_F. The visual appearance of the native seaweeds and their processed materials is shown in Figure S1.

3.3. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was employed to visualize the microstructure of seaweeds and their extruded products, as well as to determine the distribution of proteins. To achieve this, samples were completely soaked with Fast Green (0.1%) to stain the protein. Images were acquired using an FV 1000-IX81 microscope (CLSM, Olympus, Japan) with an excitation wavelength of 635 nm and an emission wavelength of 647 nm for Fast Green detection. The obtained images were analysed and processed using FV10-ASW Version 4.02.03.06 (Olympus Corporation, Tokyo, Japan).

3.4. Fourier transform infrared spectroscopy (FT-IR)

The native seaweeds and their processed products were analyzed by FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm^{-1} resolution in a wavelength range between 400 and 4000 cm^{-1} and averaging a minimum of 32 scans.

3.5. *In vitro* simulated gastrointestinal digestions

Both raw seaweeds and their respective processed products were digested according to a modification of the *in vitro* gastrointestinal digestion protocol INFOGEST (R. Sousa, Recio, et al., 2023), with some changes to ensure proper digestion of seaweed-based samples. Briefly, the required amount of sample was added to a tube to achieve a 2% protein concentration in the oral phase. To simulate the oral phase, 1 mL of human salivary fluid and 1 mL of milliQ water were added, and the mixture was incubated at 37°C in stirring (120 rpm) for 2 minutes. Subsequently, 1.6 mL of simulated gastric fluid (SGF) (pH = 3), containing porcine gastric mucosa pepsin (final concentration of 2000 U/mL), 1µL of calcium chloride and the necessary volume of milliQ water to reach a final volume of 4 mL were added. Samples were then incubated for 2 hours under the same conditions mentioned above. The gastric digestion was stopped by adjusting the pH to 7 using 1 M NaOH. To simulate the intestinal phase, the endpoint of the gastric phase was mixed with 1.7 mL of simulated intestinal fluid (SIF) (pH = 7), containing porcine pancreatic pancreatin (100 U trypsin activity per mL of final mixture), 0.5 mL of porcine bile extract, and 8 µL of calcium chloride. The total volume was adjusted to 8 mL with milliQ water, and samples were incubated for 2 additional hours under the same conditions. To terminate the intestinal digestion, 32 mL of pure methanol were added to the samples. After

being in the freezer for 1 hour, the digested samples were subjected to centrifugation at 2000 × g for 10 minutes at 4℃ to separate them into two fractions: the pellet (P, non-absorbable fraction) and the supernatant (S, absorbable fraction). Subsequently, the pellet was washed twice with 80% methanol. To do this, methanol was added to the pellet, and then it was centrifuged for 5 minutes at 2000 × g. After centrifugation, the methanol was carefully removed. Finally, both fractions were freeze-dried and stored for subsequent analysis.

Simultaneously, a protein-free control blank underwent the same digestion protocol and subsequent analyses were conducted on their resultant digestion products in the same way.

3.6. *In vitro* total digestibility and DIAAR calculations

The *in vitro* total digestibility of the different substrates was evaluated using three different analytical endpoints. This involved calculating the total amounts of nitrogen (N), total primary amines (R-NH_2), or amino acids (AAs) in the supernatants and pellets, according to the following equation, as previously described in (R. Sousa, Portmann, et al., 2023):

$$In\ vitro\ \text{digestibility}\ (\%) = \frac{(Fs - Cs)}{(\text{Fs} - \text{Cs}) + \max\ (0; Fp - Cp)}\ x\ 100 \qquad (1)$$

where Fs = Food supernatant, Cs = Protein-free control supernatant, Fp = Food pellet, Cp = Protein-free control pellet. The term (max(0;Fp-Cp)) indicates that the amount of AAs from the protein-free blank digest was set as a minimum and values below the enzyme background (resulting from analytical bias for highly digestible substrates) were set to zero.

The digestible indispensable amino acid ratio (DIAAR) was calculated for each indispensable amino acid (IAA) according to equation (2) by calculating the DIAA for each IAA:

In vitro DIAA = mg of IAA per g of food protein × *in vitro* digestibility of IAA

$$In\ vitro\ \text{DIAAR}(\%) = 100x\ \frac{mg\ of\ in\ vitro\ digestible\ dietary\ IAA\ in\ 1g\ of\ protein}{mg\ of\ the\ same\ dietary\ IAA\ in\ 1g\ of\ the\ reference\ protein} \qquad (2)$$

As reference protein, the amino acid scoring patterns recommended by the FAO (FAO, 2013) were used.

The DIAAR was calculated for all individual indispensable amino acids, and the DIAAS of a food is the lowest DIAAR, as defined by FAO (FAO, 2013).

3.7. Analysis of total nitrogen by Kjeldahl

The quantification of total nitrogen in the dried seaweed, extrudates and films was conducted using the Kjeldahl method, in accordance with ISO 8968-3:2007/IDF 20-3:2007 (ISO 8968-3, 2007). The same method was employed to measure total nitrogen in the pellet (P) and supernatant (S) of the digests. The nitrogen content obtained was then multiplied by a factor of 5 to estimate the total protein content, following previous findings for other red seaweed species (Angell et al., 2016).

3.8. Total amino acids determination

The total amino acid (TAA) content in the samples was determined by AOAC 2018.06. First, the samples were subjected to acid hydrolysis with 6 N HCl at 110 °C for 24 h. Then, an Amino Acid Analyzer Biochrom 30 (Biochrom Ltd, Cambridge, UK) with a cation-exchange column followed by ninhydrin derivatization was used for quantification and determination of the amino acid profile.

3.9. Determination of free amino acids

Protein from freeze-dried digests was precipitated using 4-sulphosalicylic acid (12.5 mg/mg of protein). The mixture was kept on ice for 1 hour to promote precipitation. Subsequently, centrifugation was performed at 15,000 × g and 4 °C for 15 minutes. The resulting supernatant was filtered through 0.45 µm membranes, and its pH was adjusted to 2.2 using 0.3 M NaOH. Amino acid analysis was carried out using a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd, Cambridge, UK).

3.10. Quantification of total amino groups ($R\text{-}NH_2$, OPA method)

The total amino groups ($R\text{-}NH_2$) in the supernatant and pellets after acid hydrolysis were measured using the o-phthalaldehyde (OPA) method. Before conducting the analyses, acid hydrolysis was performed using a 6 M HCl solution. For this purpose, approximately 4.40 mg of each digest was weighed. Then 260 µL of water, 120 µL of 3,3'-ditiodipropionic acid (DDP) / 1% NaOH solution (0.2 mol/L), 120 µL of HCl (0.2 mol/L), and 500 µL of HCl (37%) were added.

Subsequently, all samples were placed in an oven at 110 °C for 15 hours, with the samples being weighed before and after hydrolysis to ensure less than 10% evaporation. After hydrolysis, the samples were diluted 1/10 with perchloric-borate acid (0.15 mol/L). After derivatization with OPA in the presence of 2-mercaptoethanesulfonic acid, the resulting 1-alkylthio-2-alkylindole compounds were measured using UV/VIS spectrophotometry at 340 nm. The concentrations of primary amines were calculated based on a standard curve of glutamic acid. The protein-free blank control was used as a background measurement to subtract the enzyme contribution.

3.11. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted with minor modifications following the protocol described by (Santos-Hernández et al., 2018). Samples were dissolved in a sample buffer comprising Tris-HCl (0.05 M, pH 6.8), SDS (1.6% w/v), glycerol (8% v/v), β-mercaptoethanol (2% v/v), and bromophenol blue indicator (0.002% w/v) at protein concentrations of 2.0 mg/mL. Prior to this step, native seaweeds and their processed products were ground into powder. Furthermore, after adding the samples in the sample buffer they were homogenized with an Ultra-turrax and sonicated for 5 min to facilitate the solution of proteins. The samples were heated at 95°C for 5 minutes and kept warm until loaded onto 12% Bis-Tris polyacrylamide gels. Electrophoretic separations were performed at 150 V using XT-MES as running buffer in the Criterion cell. The gels were stained with Coomassie Blue.

3.12. ζ-Potential

The Zeta potential of both, the samples before digestion and the supernatants obtained after the intestinal phase were measured. For this purpose, 5 mL of the sample (diluted to 5 mg/mL in milliQ water or digestion medium) were added to a polycarbonate capillary cell with two gold laminated electrodes (Malvern Instruments, England) and analysed using a Nano ZS Dynamic Light Scattering Analyzer (Malvern, England).

3.13. Statistics

All data were represented using the average ± standard deviation. Significant differences in both graphs and tables were indicated by different letters, with a significance level of $p \leq 0.05$.

Section 3.1.3

Analysis of variance (ANOVA) was employed, followed by a Tukey test, using Statgraphics Centurin 18 software.

4. Results and discussion

4.1. Microstructure and characterization of the protein fraction in the raw seaweeds and their processed products

The total protein content of the dried seaweed, the extruded products and the films was determined by Kjeldahl, and the results are shown in Figure 1A. As observed, both seaweed species exhibited a very similar protein content, with 15.6% for *G. longissima* and 15.5% for *G. corneum*, which decreased to 11-13% in both cases after processing due to the addition of glycerol. This protein content is similar to that found in some conventional vegetal food sources, such as barley (12.5%), wheat (7-22%) or mung beans (16%) (Jimenez-Pulido et al., 2022; Senthilkumaran et al., 2022). In order to assess the potential of both seaweeds and their processed products as protein sources, an analysis of their total amino acid (AA) content and profile was also conducted. The processing of both seaweed species resulted in a noticeable decrease in the total AA content, similar to the decline observed in the nitrogen content. In the case of *G. longissima,* the AA content decreased from approximately 16 % to 12% in the films, while for *G. corneum*, it decreased from 16% to approximately 11% in the films. Interestingly, there were no significant differences in the AA content between the extrudate and the film for both seaweed species. The reduction in both the overall quantity of AAs and the protein content of the processed products can be attributed to the inclusion of glycerol in the composition of the extrudates and films, which led to a decreased proportion of seaweed biomass in the materials, consequently lowering the amount of protein present.

Regarding the AA profile, as observed in Figure 1B, both seaweed species exhibited a similar profile, with proportions of essential amino acids (EAAs) higher than 34% of the total AAs in both cases. *G. longissima* exhibited a proportion of 34.7% EAAs, whereas *G. corneum* showed 35.5%. It should be noted that this proportion was not affected by processing, as it remained consistent in both the extrudates and the films of both seaweed species. Thus, all samples exhibited a similar ratio of EAAs, comparable to those found in various animal sources, such as

eggs (32%), milk (39%), or casein (34%) (Gorissen et al., 2018), indicating that the proteins present in these two seaweed species and their processed products have potential for food applications.

Overall, after processing, a decrease in the relative contents of some labile AAs such as lysine, glutamic acid and aspartic acid was observed, leading to an increase in the relative contents of other AAs. In particular, lysine was found at a concentration of 5% in *G. longissima* and 7% in *G. corneum*, decreasing down to approximately 2% in the extrudates and films. These changes indicate that the AAs present in the native seaweeds might have undergone chemical reactions upon processing, such as Maillard and Strecker degradation reactions, similarly to what was previously reported for different types of substrates upon heating treatments (Hidalgo et al., 2013; Murata, 2021; Zhao et al., 2018). It should be noted that, as observed in a previous study (Cebrián-Lloret et al., 2022), the cell walls from both seaweed species can be disrupted upon processing, releasing not only proteins but also polysaccharides. At the high temperatures applied during the extrusion process, some of these polysaccharides may be degraded and the resulting sugars may promote the chemical modifications of labile AAs. This is of particular interest, as both lysine and threonine are considered limiting AAs in plant-based protein sources (Fouda et al., 2019; Pangestuti & Kim, 2015). Thus, a decrease in the lysine content after processing might be detrimental for the nutritional quality of seaweed proteins.

Among the most abundant EAAs present in these seaweed species, leucine stands out, constituting 7% of the total amino acids in *G. longissima* and 6% in *G. corneum*. The proportion of this EAA increased after processing, reaching 8% and 7% in the extrudates and films from the two seaweeds, respectively. Valine was detected as the second most abundant AA, being present in approximately 6% in both seaweed species. Another EAA notably present in these seaweeds was threonine, representing 6% of total AAs in *G. longissima* and 5% in *G. corneum*. In the case of *G. corneum*, its proportion slightly increased after processing, reaching 6%. Lastly, phenylalanine was also present at an approximate concentration of 5% in both seaweed species. Similar to other AAs, its relative content increased after processing, reaching 6% in the *G. corneum* extrudates and films.

Regarding non-essential AAs, seaweeds are typically characterized by their high content of aspartic acid and glutamic acid. Aspartic acid was present at 12% in *G. longissima* and *G. corneum*, slightly decreasing to around 11% upon processing. Glutamic acid followed the same trend, decreasing from 12% in both seaweeds, to 10% in the case of *G. longissima* and 9% in the case of *G. corneum*. Glycine and alanine, were also present in significant amounts in both seaweeds species, representing approximately 11-12% each, in the seaweeds and their processed products. Serine (approximately 8% in all samples) and proline (approximately 6% in all samples) were also present in reasonable amounts. Finally, although in smaller quantities, it is worth noting that the relative concentration of cysteine and especially tyrosine, increased after processing in both seaweed species. These results evidence that the proteins present in the two red seaweed species, as well as in their processed products, exhibit a nutritionally compelling profile of AAs, with a notably high content of EAAs. However, it should be noted that the chemical reactions undergone by some of the most labile AAs upon processing might have an impact on the digestibility of the extrudates and films. In particular, lysine seemed to be one of the AAs most notably affected by processing, in line with what has been reported in previous studies (Korus, 2012; Mesías et al., 2016).

A)

	GL_S	GL_E	GL_F	GC_S	GC_E	GC_F
Protein content (%)	15.6 ± 0.2^{c}	12.8 ± 0.1^{b}	10.6 ± 0.04^{a}	15.5 ± 0.5^{c}	12.7 ± 0.3^{b}	12.5 ± 0.07^{b}
TAA (%)	15.6 ± 1.4^{b}	11.2 ± 2.0^{a}	12.0 ± 0.2^{a}	15.9 ± 1.4^{b}	10.9 ± 0.9^{a}	11.1.± 0.2^{a}

B)

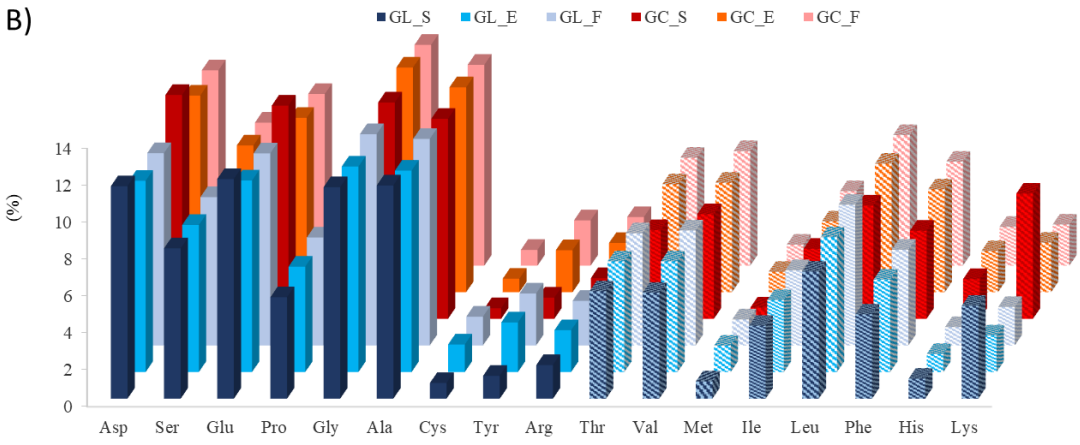


Figure 1. (A) Total protein and amino acid (AA) content and (B) amino acid profile (expressed as % from the total amino acid content) in *G. corneum, G longissima* and their processed products. Solid bars represent the non-essential AAs, patterned bars represent the essential AAs. GL_S refers to the seaweed *G. longissima*; the derived materials from this seaweed were coded as GL_S for the extrudate and GL_F for the film. Similarly, GC_S designates the seaweed *G. corneum*, with GC_E representing the extrudate and GC_F denoting the film. Values with different letters in the same roware significantly different ($p \leq 0.05$).

The effect of processing on the microstructure and the distribution of proteins in the two seaweeds was investigated by confocal laser scanning microscopy (CLSM), and representative images are shown in Figure 2. The differences in the microstructure of the two seaweed species are evident in the images, as *G. corneum* appears to be composed of smaller and denser cell structures as compared to *G. longissima*. However, once the seaweeds are processed by extrusion, the differences in cell structure are no longer discernible, as the cell walls appear distorted and with much less defined wall structures. The presence of well-defined cells in the images from the native seaweeds stained with Fast Green suggests that proteins are an integral part of the seaweed cell walls. In agreement with a previous study (Cebrián-Lloret et al., 2022), the application of mechanical processing in conjunction with high temperature can effectively permeabilize or partially disrupt the cell wall structures, resulting in the outward migration of proteins. As a result, proteins seem to be more homogeneously distributed throughout the whole structures of the extrudates and films. This was more evident in the case of *G. longissi*ma extrudates and films, and is in agreement with a previous study evidencing that the cell walls from this seaweed species are more labile than those from *G. corneum* (Cebrián-Lloret et al., 2022). This is expected to have an impact on the digestibility of the processed samples, as proteins are no longer entrapped within the cell walls, as it was the case in the native seaweeds and, thus, they might be more accessible to the digestive enzymes. *G. corneum* and its processed products exhibit a higher level of fluorescence compared to *G. longissi*ma and its processed products

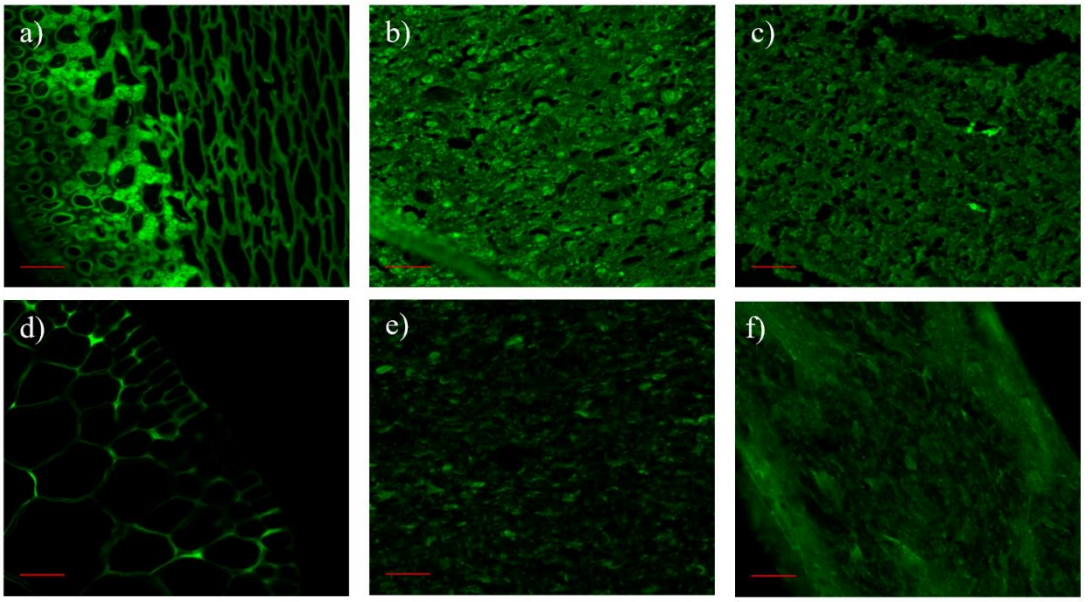


Figure 2. Confocal laser scanning microscopy images of the native seaweeds and their processed products stained with Fast Green: (**a**) GC_S (*G. corneum* seaweed) (**b**) GC_E (*G. corneum* extrudate*)* (**c**) GC_F (*G. corneum* film) (**d**) GL_S (*G. longissima* seaweed) (**e**) GL_E (*G. longissima* extrudate) (**f**) GL_F (*G. longissima* film). Scale bars correspond to 20 µm. Images with no staining, showing the inherent autoflorescence of the samples are shown in Figure S2.

The samples were also characterised by FT-IR and the obtained spectra (Figure S3) provide further evidence for structural changes taking place after processing the seaweeds. In particular, the relative intensity of the band located at 1030 cm^{-1}, which is common to all polysaccharides and is primarily attributed to the coupling of C-O or C-C stretching modes with C-OH bending modes (Cebrián-Lloret et al., 2024), was strongly affected by processing. This can be explained by structural changes taking place in the most abundant polysaccharide found in the two seaweeds, i.e. agar, which, as shown in previous work (Cebrián-Lloret et al., 2022), can be released from the seaweeds' cell walls upon processing. In the case of *G. corneum*, the most notable change in the relative intensity of this band occurred when the two processing steps

were applied for film formation. On the other hand, in *G. longissima*, the most noticeable change was observed after the extrusion step, with a very minor effect of the subsequent compression moulding step to produce films. This suggests that, in the case of *G. longissima*, extrusion alone is sufficient to induce structural changes in the cell walls, whereas in the case of *G. corneum*, an additional processing stage is required to alter it significantly. These structural changes are also reflected in bands associated with proteins, specifically in the amide I (1642 cm^{-1}) and amide II (1540 cm^{-1}) bands (Guerrero et al., 2014). In the case of *G. corneum*, the ratio of the amide I/amide II bands was seen to increase with each successive processing step, whereas in the case of *G. longissima*, the ratio between the two bands was increased after the extrusion step, but reduced again after the subsequent compression moulding process. This, in agreement with previous studies (Cebrián-Lloret et al., 2022), supports the fact that *G. corneum* has tougher cell walls, and proteins are released to a greater extent when applying subsequent processing steps. In contrast, *G. longissima* possesses more labille or permeable cell walls, and proteins and agar are more easily released upon the first extrusion step. Applying a subsequent processing step might lead to unwanted reactions occurring between the protein and the polysaccharide fractions, as pointed out by the changes observed in the AA profile of the films.

4.2. *In vitro* protein digestibility and calculation of DIAAR values

In vitro gastrointestinal digestions were performed on the native seaweeds and their extrudates and films, and the digestibility was evaluated using a previously established method (R. Sousa, Recio, et al., 2023) based on the harmonized INFOGEST protocol, with slight modifications. After completion of the gastrointestinal digestions, two distinct phases were obtained: (i) supernatant (absorbable fraction) and (ii) pellet (non-absorbable fraction). The digestibility of the different samples was then estimated using three different methods, and the results are presented in Figure 3. Overall, it is observed that both seaweed species exhibit a similar, rather low, digestibility in their native state (<58%). Previous studies have reported higher *in vitro* digestibility values for different red seaweed species, such as *P. columbina* or *P. palamata,* ranging around 75% (Cian et al., 2014, 2015), and reaching up to 89% in the case of some *Hypnea* species (Wong & Cheung, 2001). It must be noted that, as demonstrated in a previous work

(Cebrián-Lloret et al., 2022), even different species belonging to the same genus may show great differences in their cell wall structure and integrity. Also, the cell wall structure can vary greatly depending on growth stage or seasonal variations; thus, the digestibility values reported for other red seaweeds may only be used as a reference and not to establish direct comparisons. In general, and given the large variability in the digestibility values depending on the method used, the two species used in this work did not show significant differences in their digestibility. Compared to other plant-based protein sources, such as pea protein concentrate (80% digestibility) (Çabuk et al., 2018), *Moringa oleifera* seed protein (81%-89% digestibility) (Aderinola et al., 2020), pigeon peas (96% digestibility), black beans (82% digestibility) or peanuts (96% digestibility) (R. Sousa, Recio, et al., 2023), the seaweed species used in this work present a low digestibility and, thus, it would be advisable to develop strategies to increase the bioaccessibility of their proteins.

Looking at the effect of processing on the average digestibility values (i.e. average values from the three methods), no significant differences were found. However, a positive trend in improving the digestibility with processing, especially through extrusion, can be appreciated. Additionally, slight differences in the behaviour of both species can be observed. In the case of *G. corneum*, the digestibility increased with each successive processing step (ca. 14% increase after the extrusion step and 22% increase after extrusion and compression moulding), while for *G. longissima*, the digestibility reached its maximum value after extrusion (ca. 24% increase, as compared to 12% increase after extrusion and compression moulding). This disparity could be attributed to the distinct composition and cell wall structure of the two seaweed species, as already suggested by the FT-IR results. In particular, as seen in a previous study (Cebrián-Lloret et al., 2022), both seaweed species exhibit variations in the structure of their cell walls. *G. corneum* displays lower sensitivity to heat treatment in comparison to *G. longissima*. When *G. longissima* is subjected to heating, an easier release of agar from the cells takes place together with the formation of a "jelly-like" structure, indicating that its cell walls are more permeable. This enhanced permeability would also facilitate the release of proteins during the extrusion process. Consequently, *G. corneum* may require an additional processing step to disrupt or permeabilize, at least partially, the cell walls, thus enhancing the bioaccessibility of the proteins.

On the other hand, the cell walls from *G. longissima* appear to be more labile and the extrusion step is sufficient to disrupt the cell wall structure and promote the release of proteins, while adding a second processing step for film formation does not seem to provide additional benefits. In turn, the more exposed proteins released after the extrusion step might undergo structural changes, such as aggregation (as demonstrated latter in the SDS-PAGE profiles of the processed samples), upon further processing. Such structural changes could have a negative impact on the digestibility of the processed samples; thus, overprocessing of the samples should be avoided.

It should be noted that the variability in the digestibility values calculated by each method was quite high, which can be attributed to the great heterogeneity of the samples. Different modifications in the experimental protocol are currently being studied to minimize the potential sources of errors, making it more suitable for high fibre substrates such as seaweeds. From the three different methods used to calculate the digestibility, OPA seems to have larger associated standard deviations. Since it is a colorimetric method, it is not possible to discard that other components present in the samples, such as pigments, may interfere in the measurements.

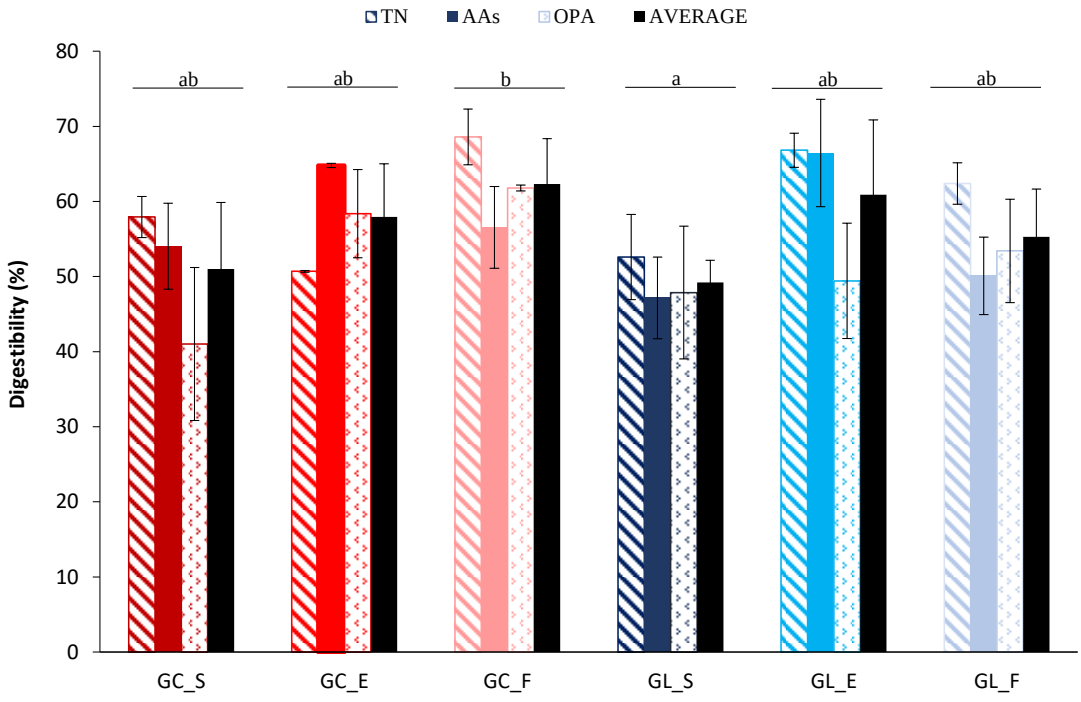


Figure 3. Protein digestibility results obtained from total N analysis (TN), quantification of total amino acids (AAs) and OPA analysis, and values calculated from the average of the three methods. Different letters indicate significant differences in average digestibilities considering the average values of the three methods. GC_S refers to the seaweed *G. corneum*; the derived materials from this seaweed were coded as GC_S for the extrudate and GC_F for the film. Similarly, GL_S designates the seaweed *G. longissima*, with GL_E representing the extrudate and GL_F denoting the film.

The *in vitro* DIAAR values were calculated based on the specific requirements for different population groups. As an example, the DIAAR values corresponding to older children, adolescents and adults (which are most likely the main target group for seaweed-based snacks) are shown in Figure 4. Likewise, those for infant (birth to 6 month) and preschool children (6 months to 3 years), are shown in Figures S4 and S5, respectively. It can be observed that the DIAAR values for *G. corneum* increased after each processing step for threonine and for the aromatic and sulphur-containing AAs (cysteine and methionine). On the contrary, the DIAAR values for lysine were significantly decreased in the processed products. The sulphur AAs were the limiting in the native seaweed, in line with what has been previously reported for other red seaweed species, such as *Chondrus crispus* or *Osmundea pinnatifida* (Černá, 2011; Vieira et al., 2018). However, lysine became the limiting AA after processing. As for *G. longissima*, histidine emerged as the limiting AA in the native seaweed, while lysine remained the limiting AA in the processed products. Notably, while the DIAAR values for the aromatic and sulphur-containing AAs increased after extrusion, they dropped after the subsequent compression moulding step. Similarly to *G. corneum*, lower DIAAR values with processing were noted for lysine, but also for other AAs such as valine and isoleucine.

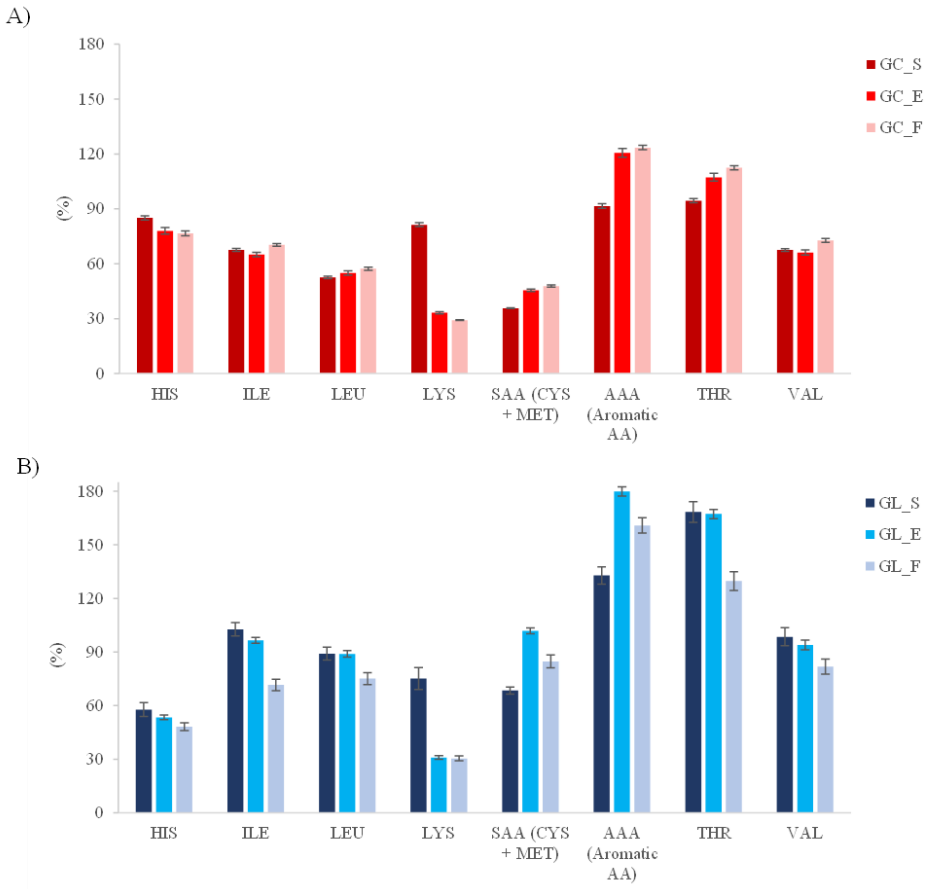


Figure 4. DIAAR values for older children, adolescents and adults based on *in vitro* digestibility of AA for A) *G. corneum*: seaweed (GC_S), extruded (GC_E), film (GC_F), and (B) *G. longissima*: seaweed (GL_S), extruded (GL_E), film (GL_F)

Table 1 shows the DIAAS values calculated for the native seaweeds and their processed products, for the three age groups established by FAO. As observed, *G. longissima* presented significantly higher DIAAS values than *G. corneum*, evidencing a higher protein nutritional quality for the former. As previously mentioned, processing had a notable effect on the more labile AA lysine, which had a notable negative impact on the protein nutritional quality from *G. longissima,* reducing its DIAAS values to levels similar to those of *G. corneum.* This means that even though the overall digestibility of the seaweed increases after processing due to improved

protein accessibility, the AA profile is negatively impacted from a nutritional perspective due to the degradation of labile AAs such as lysine. Lysine and sulphated amino acids also appear as limiting amino acids in other vegetable protein sources, such as peanuts or peas, while in animal proteins like whey protein, histidine emerges as a limiting amino acid (R. Sousa, Recio, et al., 2023), similarly to *G. longissima*. Thus, our results indicate that to increase the overall digestibility while preserving the nutritional quality of their proteins, seaweeds should be processed by other methods, such as mechanical treatments or milder thermal processing, which do not lead to degradation of labile AAs. Another possibility would be to improve the nutritional quality of the processed seaweed products, by complementing them with other protein sources richer in lysine, such as peas or beans (Dhull et al., 2022; Semba et al., 2021)

Table 1. Limiting AAs and corresponding DIAAS values for the three age groups established by FAO (FAO, 2013) for the seaweeds and their processed products.

	GC_S	GC_E	GC_F	GL_S	GL_E	GL_F
Infant (birth to 6 month)	SAA: 22.8	LYS: 23.6	LYS: 19.6	HIS: 44.0	LYS: 17.7	LYS: 22.6
Child (6 month to 3 year)	SAA: 27.9	LYS: 28.6	LYS: 23.8	HIS: 46.2	LYS: 21.4	LYS: 27.3
Older child, adolescent, adult	SAA: 32.8	LYS: 33.9	LYS: 28.2	HIS: 54.2	LYS: 25.5	LYS: 32.4

SAA (Sulfated amino acids): CYS+MET

4.3. Characterization of the digestion products

To analyse protein degradation during the digestion process and to evaluate the possible presence of proteins or peptide fragments of higher molecular weight after the intestinal phase, the digestion products were characterized by polyacrylamide gel electrophoresis (SDS-PAGE) and the results are presented in Figure 5A (*G. corneum*) and Figure 5B (*G. longissima*). A protein-free blank sample was also included, where bands corresponding to digestive enzymes such as trypsin (23.3 kDa), pepsin (34.5 kDa) and amylase (45 kDa) (Fontes-Candia et al., 2022; Rho et al., 2019; Sanchón et al., 2018) were identified in the pellet. In the supernatant, a stained region

below 10 kDa was observed, which may correspond to soluble peptides originated from a certain extent of enzyme autolysis. In the case of *G. corneum*, a band around 20 kDa was detected in the native seaweed (undigested). As indicated in a previous study (Pimentel et al., 2020), this band could be attributed to the presence of phycobiliproteins, a family of light-harvesting pigment protein complexes found in red seaweeds, with subunits presenting molecular weights between 18-21 kDa. The fact that only this protein band could be detected in the native seaweed is related to the low accessibility of most of the proteins, located within the cell walls, as evidenced by CLSM images (cf. Figure 2). Notably, the samples became even more insoluble after processing and the protein band detected in the seaweed could only be very faintly detected in the extrudate. Even though processing promoted the release of proteins from the seaweed cell walls, such proteins were most likely linked to polysaccharides or aggregated due to the applied thermal treatments, thus being highly insoluble in the sample buffer. Furthermore, agar is also released from the seaweed cell walls upon processing (Cebrián-Lloret et al., 2022), hence producing gelation of the samples upon the heating-cooling process undergone before injection in the gels. In the pellets from the digested samples, representing the non-absorbable fraction, the band corresponding to phycobiliproteins was not detected; however, stained broad bands were detected in the high molecular weight region, indicating that protein aggregates were present. This supports the fact that processing induces significant structural changes in the seaweed proteins, leading to the formation of aggregates which remain in the non-absorbable fraction. In addition, other bands at around 12 kDa and 15 kDa were observed, which showed greater intensity in the pellet of the unprocessed seaweed. These bands may correspond to partially hydrolysed proteins, or proteins that are insoluble in the native seaweed but became solubilized after being partially digested. In the supernatants, the same <10 kDa region observed in the blank was detected, which in this case may correspond to peptides generated as a result of protein hydrolysis. These smaller-sized peptides found in the supernatant may have nutritional and functional implications, as their reduced size could facilitate their absorption in the gastrointestinal tract and their availability to the organism (Dhaval et al., 2016; Suwanangul et al., 2022).

In the case of *G. longissima,* a band at 18-21 kDa, that could be associated with phycobiliproteins, was also detected in the native seaweed, together with additional bands at around 75 kDa and 50 kDa. These bands were not detected in the pellets of the digests; however, high molecular weight bands were noted, similarly to *G. corneum*, as well as broad bands in the low molecular weight region. In the case of the supernantants, the <10 kDa region attributed to peptides was also detected. This suggests that while part of the proteins were aggregated as a result of processing, another faction could be digested to form lower molecular weight peptides.

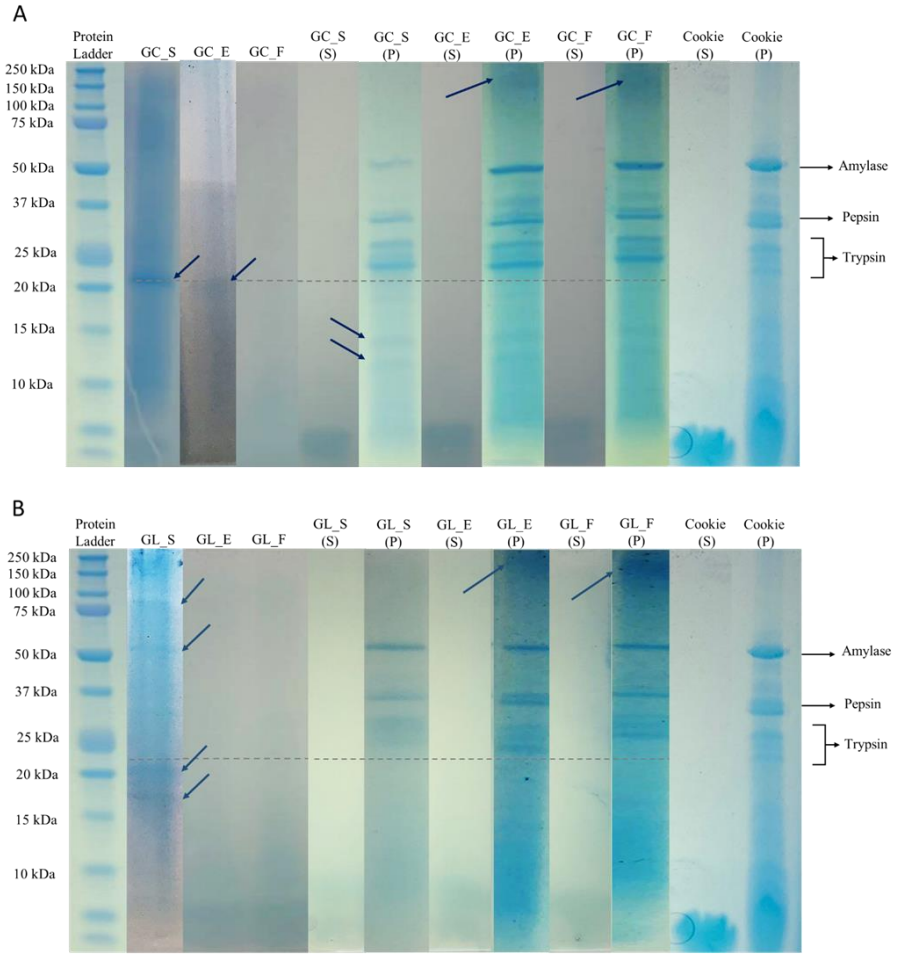


Figure 5. SDS-PAGE protein profiles of (A) *G. corneum*: seaweed (GC_S), extruded (GC_E), film (GC_F), and their digestion products, and (B) *G. longissima*: seaweed (GL_S), extruded (GL_E),

film (GL_F), and their digestion products. Digestion products are coded as (S) for supernatants and (P) for pellets.

The analysis of the total AA profiles in the digestion products (cf. Figure S6 revealed a noticeable change in the distribution of AAs within the supernatant and pellet fractions. The absorbable fraction exhibited AA profiles which closely resembled those from the original samples before digestion. Interestingly, in the pellet fractions (with the exception of the *G. longissima* extrudates and *G. corneum* films) the prevalence of a few AAs that have been previously reported as poorly digestible by the organism, such as tyrosine, methionine, and phenylalanine (Bosch et al., 2016; Puntigam et al., 2020), was noted.

In addition, both the content and profile of free AAs in the absorbable fraction (supernatant) were determined, since this fraction should be composed of smaller peptides and free AAs (R. Sousa, Recio, et al., 2023). As shown in Figure 6, the absorbable fraction obtained from the digestion of *G. corneum,* and its processed products exhibited a higher content of free AAs compared to the fractions obtained from the digestion of *G. longissima* and its processed products. This could be related to the higher agar content in *G. longissima* (Cebrián-Lloret et al., 2022), which may have led to the establishment of saccharide-peptide interactions, hence limiting the amount of free AAs formed upon digestion. Generally, lysine and leucine were found to be the most abundant essential AAs in the digests of both seaweed species, with levels of around 14-20 mg/g of protein in the freeze-dried digests. Among the non-essential AAs, serine followed by tyrosine and arginine were the most abundant across all samples, with concentrations of approximately 13-21 mg/g of protein in the freeze-dried digests. These results are consistent with findings reported for other intestinal digests derived from vegetable protein sources such as soybean or pea (Santos-Hernández et al., 2020).

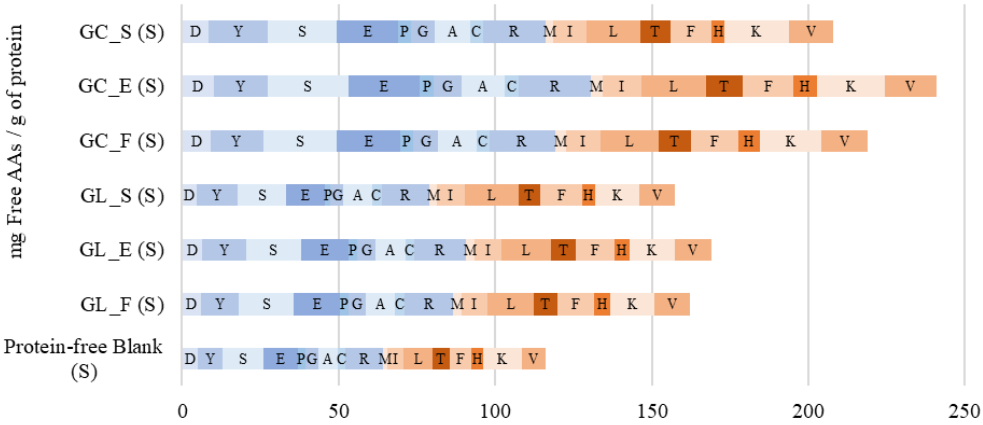


Figure 6. Composition of free AAs (mg/g of protein) in the absorbable fractions (supernatants) obtained after gastrointestinal digestion. Orange: essential AAs; blue: non-essential AAs. GC_S refers to the seaweed *G. corneum*; the derived materials from this seaweed specie were coded as GC_S for the extrudate and GC_F for the film. Similarly, GL_S designates the seaweed *G. longissima*, with GL_E representing the extrudate and GL_F denoting the film.

The ζ-potential measurements were employed to determine the surface charge of the digests' supernatants. Figure 7 illustrates the results obtained when comparing the surface charge values of the digests with their corresponding undigested samples (in water and in the gastrointestinal digestion medium, without enzymes). The digestion fluids contain a significant amount of salts, which were expected to have a noticeable effect on the surface charge of the samples. Both seaweeds and their processed products displayed negative ζ-potential values, ranging from -19 to -27. These results indicate that, without any pH adjustment, they present negative surface charges. These values are in line with those reported for proteins obtained from other vegetable sources, such as millet proteins (-33mV) (Nazari et al., 2018), or faba bean protein (-26mV) (Martínez-Velasco et al., 2018). When measuring the samples in the gastrointestinal digestion medium, the ζ-potential values became more negative, as expected, due to the basic pH of the medium. Additionally, all the digests exhibited more negative values compared to the undigested samples in the digestion medium. Notably, the ζ-potential values were more negative in the digests of the processed samples, particularly after the extrusion

step, in comparison to the digests of the unprocessed seaweeds. In particular, the percentage of decrease in the ζ-potential of the digests compared to the samples in the digestion medium was about 19% for *G. corneum* and about 23% in the extruded products and the films. In the case of *G. longissima*, this change was even more pronounced, with a decrease in ζ-potential of 3% in the seaweed, 32% in the extruded product and 51% in the film, after the gastrointestinal digestions. This increase in negative charge is linked to the protein hydrolysis, with peptides and negatively charged AAs such as glutamic or aspartic acid (Brinkerhoff et al., 2021; Tavafoghi & Cerruti, 2016), along with other neutral AAs that become negatively charged in a basic environment, such as tyrosine and glycine (both of them found in a significant proportion in the digests) (Attia et al., 2012; O'Connor et al., 2018), being released upon the digestion process. In fact, as observed in Figure S6, the proportion of tyrosine and arginine increased in the supernatants from the digests obtained from all the analysed samples.

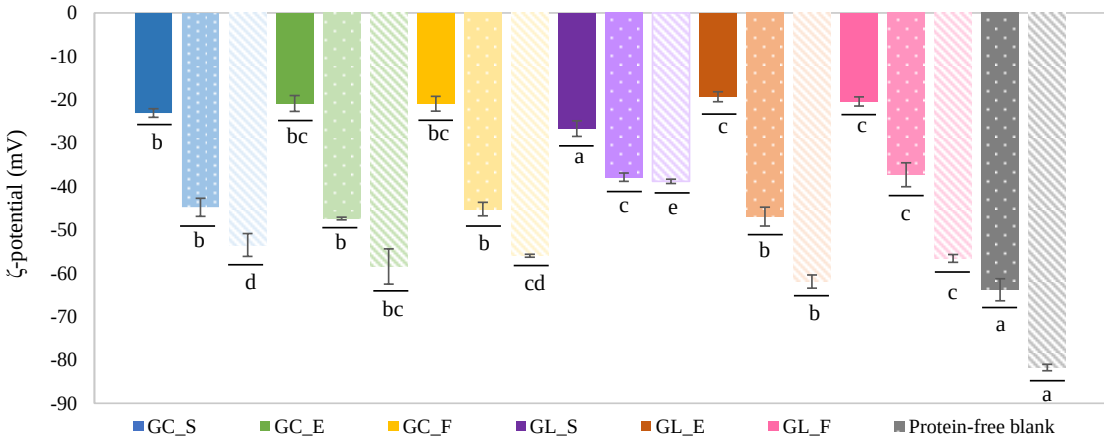


Figure 7. ζ-potential values. Comparison of samples before digestion dispersed in water (solid colour bars) and dispersed in the digestion medium (dotted bars), and the digestible fraction (S) obtained after *in vitro* gastrointestinal digestions (striped bars). GC_S refers to the seaweed *G. corneum*; the derived materials from this seaweed were coded as GC_S for the extrudate and GC_F for the film. Similarly, GL_S designates the seaweed *G. longissima*, with GL_E representing the extrudate and GL_F the film. Values with different letters in the same dispersion medium are significantly different ($p \leq 0.05$).

5. Conclusions

This study evaluated the nutritional quality and digestibility of proteins from two red seaweed species, *Gelidium corneum* and *Gracilaria longissima*, by applying a method based on the INFOGEST harmonized protocol, and evaluated the effect of processing methods to produce seaweed-based snack foods.

The initial protein content of both seaweed species was around 16%, predominantly entrapped within the seaweeds' cell walls. Similar amino acid profiles, with notable concentrations of aspartic acid, glutamic acid, glycine, and alanine, were observed in both species. Processing induced alterations in amino acid profiles, notably a significant decrease in labile amino acids, particularly lysine, indicating the occurrence of chemical reactions such as Maillard reactions, promoted by the high temperatures applied and the presence of polysaccharides. Despite processing-induced changes, essential amino acids constituted over 34% of total amino acids in both seaweeds and their processed products.

The complex cell wall structure resulted in low protein digestibility (around 49-51%), mitigated by extrusion processing, which facilitated the permeabilization of cell walls and improved overall protein digestibility. However, differences in cell wall structure manifested in distinct responses for both seaweeds species. In the case of *G. corneum*, digestibility increased with each subsequent processing step, reaching 62% for the films. In contrast, *G. longissima* exhibited maximum digestibility (61%) after extrusion, with compression molding showing a detrimental effect, likely to a greater release of agar, forming a gelling network.

Moreover, processed products displayed changes in amino acid profiles, impacting the nutritional quality of their proteins, with lysine becoming the limiting amino acid.

These results provide a basis for the development of strategies to improve the nutritional quality of proteins from *G. corneum* and *G. longissima* to produce high-quality food products, thereby opening the way for potential applications in the food industry.

6. Acknowledgements

This work was done within the framework of a collaboration with HISPANAGAR S.L (Burgos, Spain). This research work was financially supported by the grant TED2021-129711B-I00, funded by MCIN/AEI/10.13039/501100011033 and by the European Union NextGenerationEU/PRTR.

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8. Supplementary material

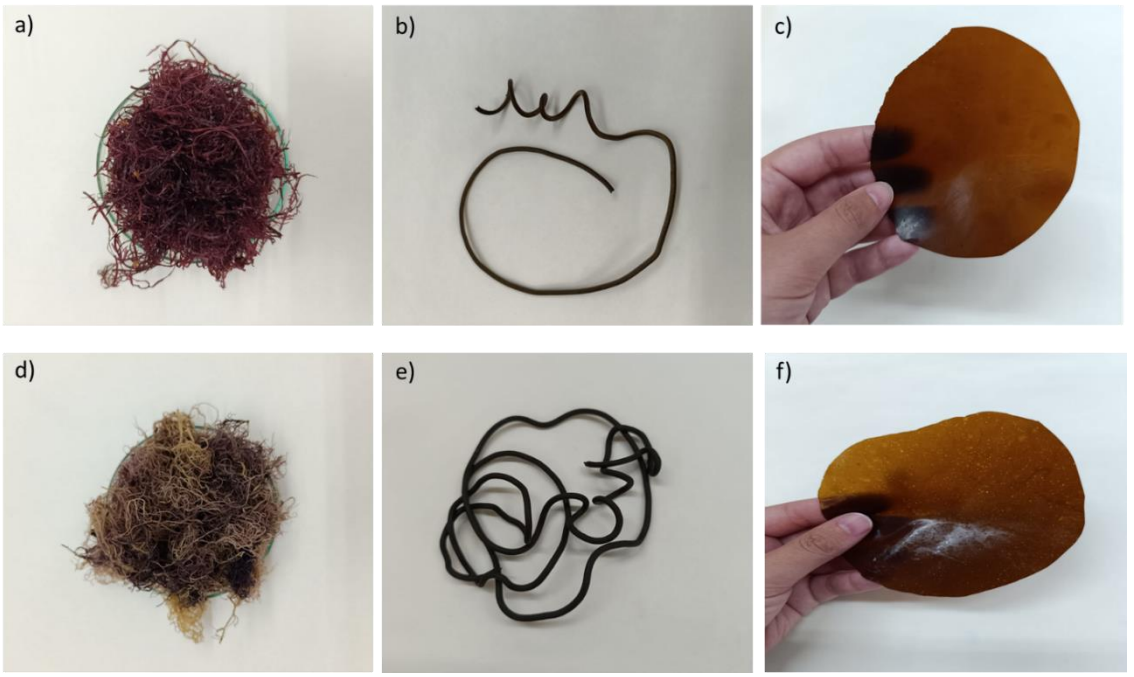


Figure S1. Visual appearance of the native seaweeds and their processed products: (**a**) GC_S (*G. corneum* seaweed) (**b**) GC_E (extrudate *from G. corneum)* (**c**) GC_F (film from *G. corneum*) (**d**) GL_S (*G. longissima* seaweed) (**e**) GL_E (extrudate from *G. longissima*) (**f**) GL_F (film from *G. longissima*).

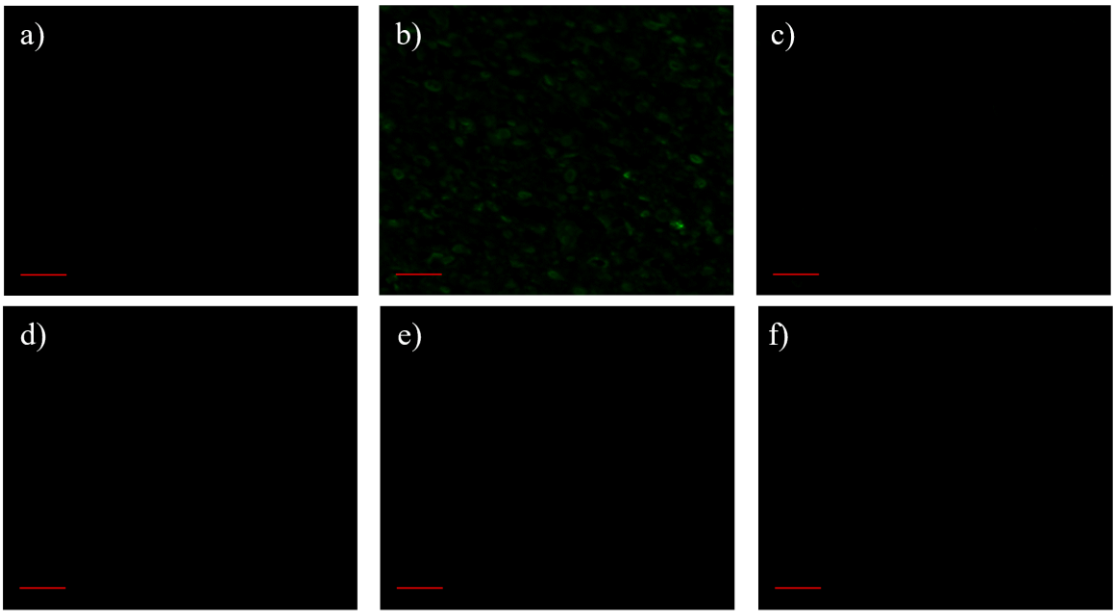


Figure S2. Confocal laser scanning microscopy images of the inherent autoflorescence of the native seaweeds and their processed products: (**a**) GC_S (*G. corneum* seaweed) (**b**) GC_E (extrudated *from G. corneum)* (**c**) GC_F (film from *G. corneum*) (**d**) GL_S (*G. longissima* seaweed) (**e**) GL_E (extrudated from *G. longissima*) (**f**) GL_F (film from *G. longissima*). Scale bars correspond to 20 µm.

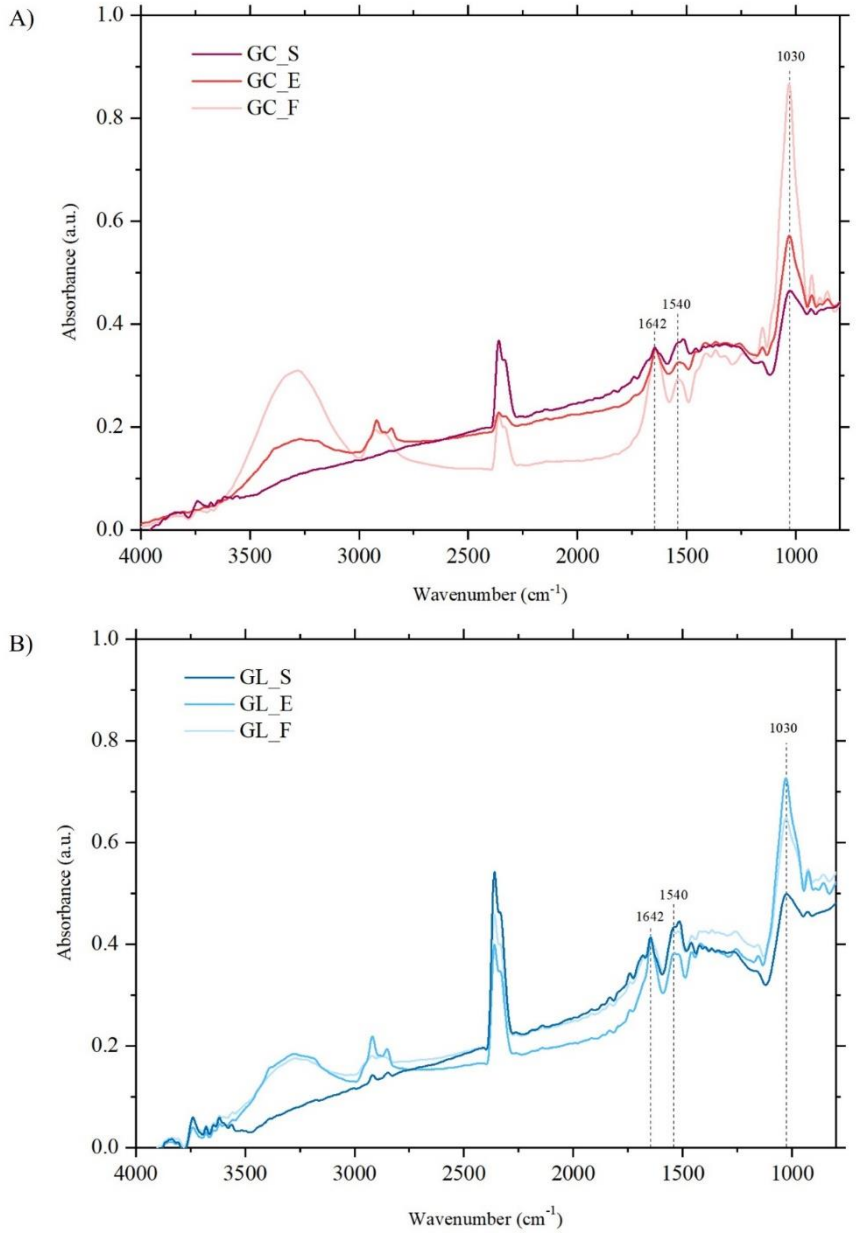


Figure S3. FT-IR spectra of the native seaweeds and their processed products: (A) GC_S (*G. corneum* seaweed), GC_E (extrudated from *G. corneum*) and GC_F (film from *G. corneum*). (B) GL_S (*G. longissima* seaweed), GL_E (extrudated from *G. longissima*) and GL_F (film from *G. longissima*).

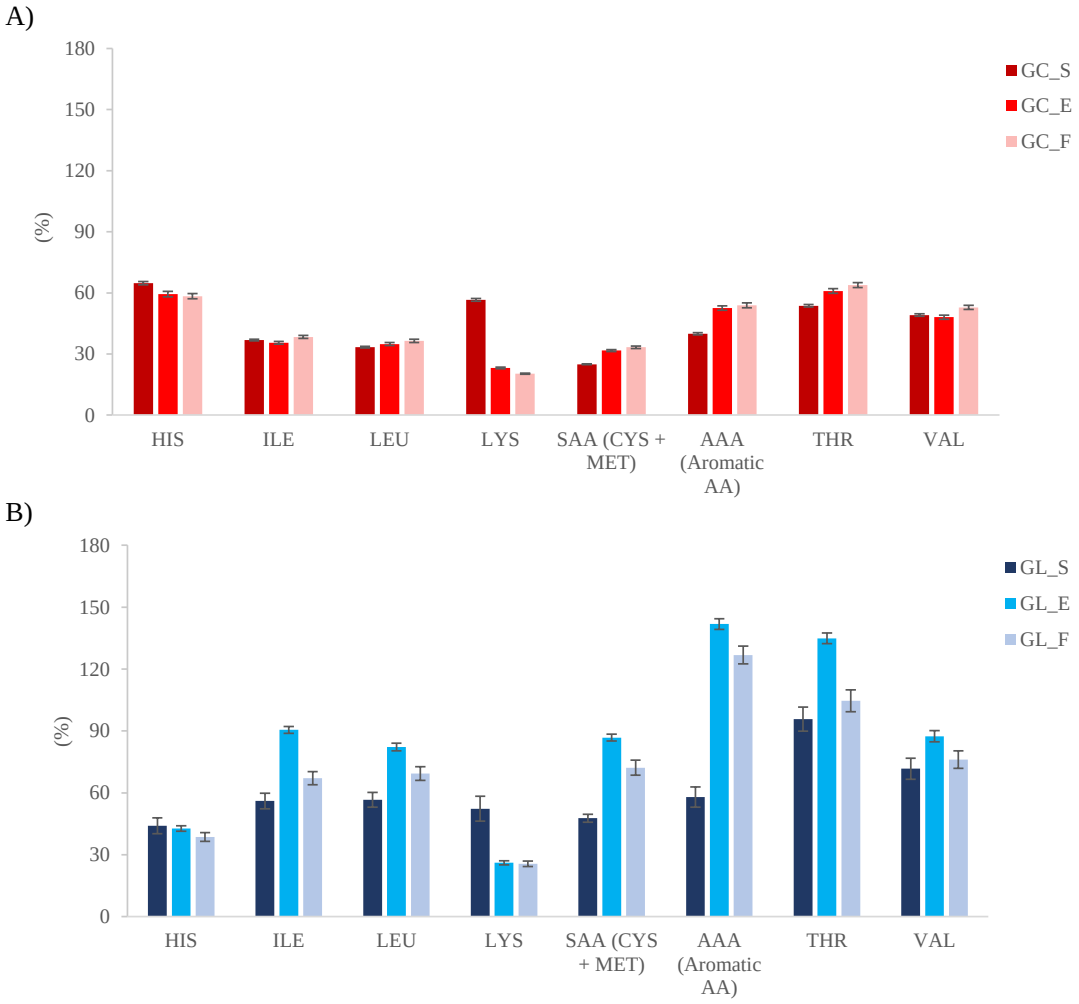


Figure S4. DIAAR values for infant (birth to 6 month) based on *in vitro* digestibility of AA for A) *G. corneum*: seaweed (GC_S), extruded (GC_E), film (GC_F), and (B) *G. longissima*: seaweed (GL_S), extruded (GL_E), film (GL_F).

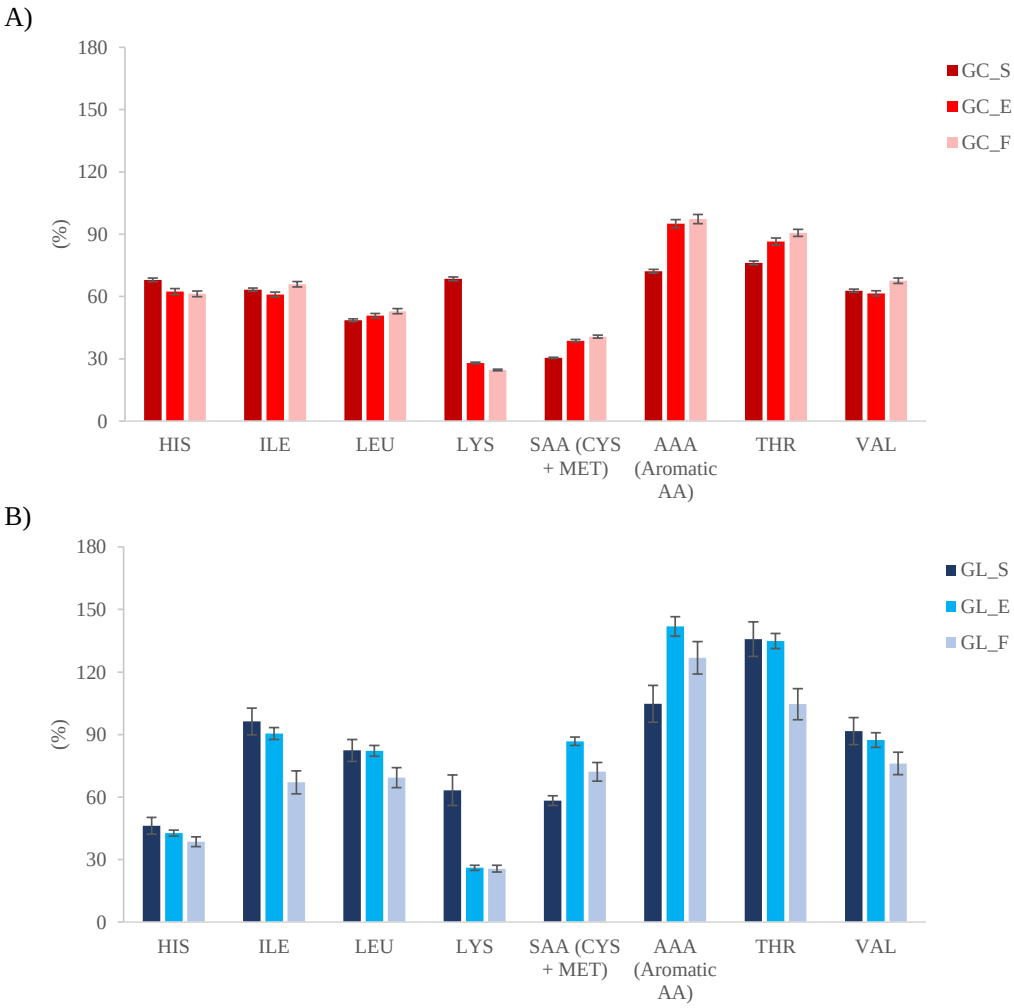


Figure S5. DIAAR values for preschool children (6 months to 3 years) based on *in vitro* digestibility of AA for A) *G. corneum*: seaweed (GC_S), extruded (GC_E), film (GC_F), and (B) *G. longissima*: seaweed (GL_S), extruded (GL_E), film (GL_F).

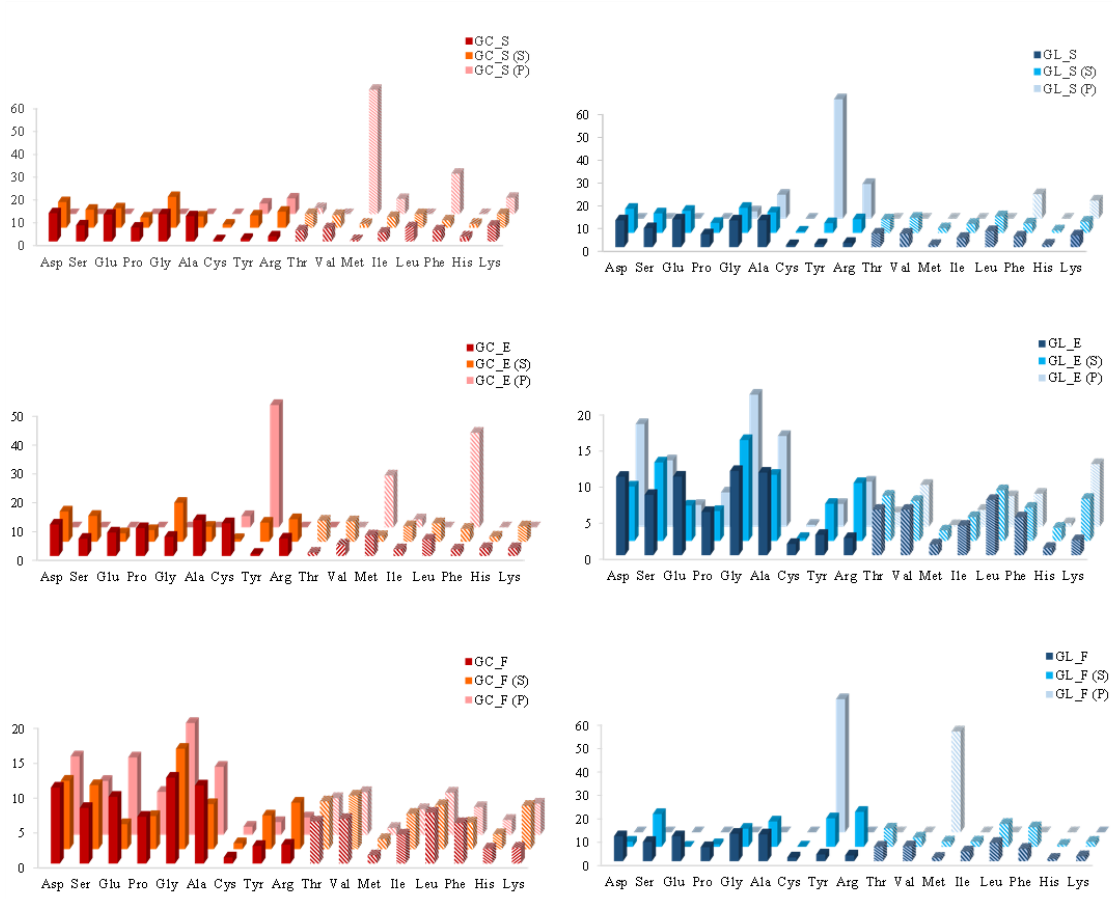


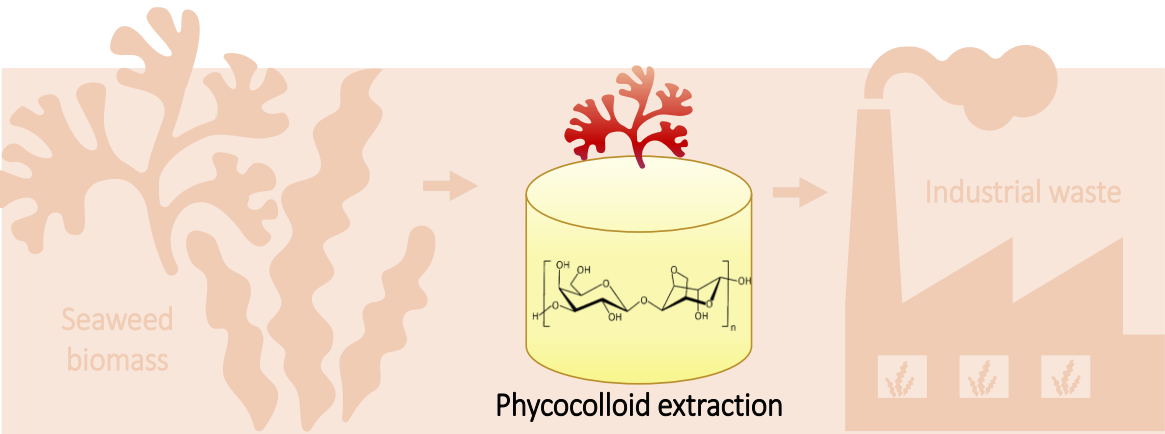
Figure S6. Distribution of total AAs in the supernatant (S) and pellet (P) fractions from the digestion products. The bars with lines represent the essential AAs.

CHAPTER 2

VALORIZATION OF PHYCOCOLLOIDS EXTRACTED FROM MACROALGAE BIOMASS

2.1. Agar-based packaging films produced by melt mixing: Study of their retrogradation upon storage.

2.2. Exploring alternative red seaweed species for the production of agar-based hydrogels for food applications.



In addition to its significance in marine ecology, macroalgae hold a prominent value in the industry, mostly owing to their phycocolloidal contents. Specifically, agar, a phycocolloid present in some red seaweed species, has emerged as a widely utilized resource in the food industry, where it plays a fundamental role as a gelling and thickening agent in a variety of products, ranging from desserts to canned foods. However, in recent years, agar has also gained recognition for its potential to produce biopolymeric materials, proving useful in the creation of edible films, sustainable packaging and biodegradable products.

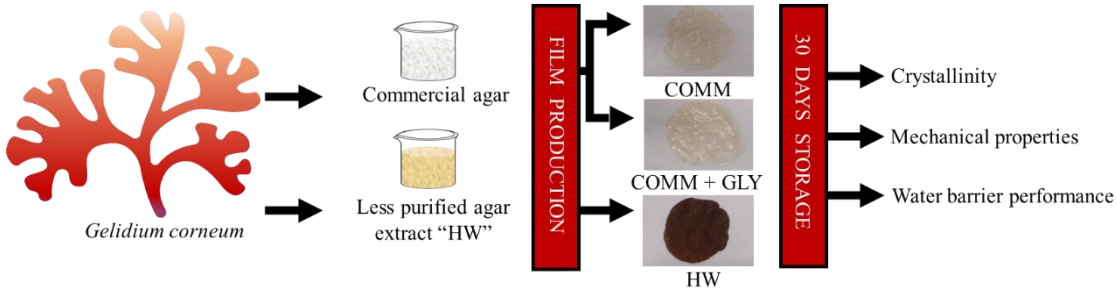
Nevertheless, the industrial extraction methods employed for the production of agar are often inefficient in terms of time and energy consumption. Hence, there is a pressing need for the development and adoption of more energy-efficient agar extraction protocols. Within this context, the first study of this chapter aimed to assess the processability of agars extracted from *Gelidium corneum* with varying degrees of purity, including a commercial high-purity grade and a less purified agar extract produced through a more energy-efficient simplified extraction method. The materials were processed by melt mixing and the structural and functional properties of the obtained films were examined. Furthermore, the structural stability of agar was evaluated by assessing the evolution of the functional properties during extended storage. The obtained results highlighted the potential application of less purified agar-based fractions as additives for biopolymeric materials, capable of reducing costs and enhancing the stability of pure agar films.

Another aspect to consider is that the overexploitation of seaweed species such as *Gelidium corneum* for the extraction of agar raises environmental concerns, as it has contributed to the decline in the populations of this species in the oceans. Consequently, to avoid unbalances in marine ecosystems and guarantee a sustainable agar production, it is essential to explore and study other varieties of red seaweeds that possess an agar content and quality suitable for industrial use. Therefore, the second study included in this chapter conducted a comparative analysis of the composition of various agar-based fractions produced from different red seaweed species, using two different methods: a conventional purification process similar to

that commonly employed in the industry and an alternative, simplified extraction protocol that yields less purified agar fractions. The obtained agar-based fractions were then used to develop hydrogels, whose textural and rheological properties were evaluated. The results revealed the potential of *G. longissima* as a viable alternative to the more widely used *G. corneum* in the production of agar hydrogels for food applications.

3.2.1

AGAR-BASED PACKAGING FILMS PRODUCED BY MELT MIXING: STUDY OF THEIR RETROGRADATION UPON STORAGE



This section is an adapted version of the following published research article:

Cebrián-Lloret, V., Göksen, G., Martínez-Abad, A., López-Rubio, A., & Martínez-Sanz, M. (2022). Agar-based packaging films produced by melt mixing: Study of their retrogradation upon storage. *Algal Research,* 66.

Section 3.2.1

1. Abstract

The ability of agar with different purification degrees to produce packaging films has been evaluated and the stability of the obtained materials after prolonged storage has been investigated. The less purified agar resulted in films with higher water vapour permeability and lower mechanical performance than pure commercial agar. However, the commercial agar film required the addition of a plasticiser to produce films that could be manipulated. It has also been observed that prolonged storage at 53% RH results in a change in the semi-crystalline structure of the agar and in water-polysaccharide interactions. As a result, pure agar films undergo a rigidizing effect resulting in unmanageable films after 7 days of storage. The presence of glycerol improved the stability of the films by limiting the structural changes up to 14 days of storage. In contrast, the films from the least purified agar extract, seemed to be less affected by moisture, showing a higher stability during storage. This points to the potential of the less purified extract to be used as an additive to reduce costs and improve the storage stability of pure agar films.

2. Introduction

The excessive production of petroleum-derived plastics has become a major problem in recent years. This is mainly due to the fact that these materials are not biodegradable; consequently, they accumulate in natural ecosystems for hundreds of years causing severe environmental problems (Emadian et al., 2017; Karan et al., 2019; Yang et al., 2014). Although recycling strategies have been promoted worldwide over the last decade, the replacement of conventional plastics with biodegradable polymers obtained from renewable natural resources, i.e. biopolymers, is being studied and considered as a more sustainable alternative to address this problem in the long term. However, the production costs of biopolymers are too high to compete against conventional petroleum-based polymers on the market nowadays. On top of that, their properties are not yet comparable to those of benchmark synthetic polymers, especially in terms of mechanical and barrier properties. Moreover, the raw materials commonly used for the production of biopolymers come from land-based crops and, thus, they compete with their main use as food sources. This is why, as an alternative, marine resources (e.g. aquatic plants or seaweeds) are being explored as a source for obtaining biopolymers

(Benito-González et al., 2019; Cebrián-Lloret et al., 2022; Kartik et al., 2021; Martínez-Sanz, Cebrián-Lloret, et al., 2020; S. Thakur et al., 2018). The cell walls of seaweeds are rich in polysaccharides, whose composition depends on the seaweed species, being cellulose, the most important structural component providing mechanical strength, while other polysaccharides are responsible for different functionalities. In particular, sulphated polysaccharides (i.e. carrageenan and agar) are highly relevant to the food industry, due to their extensive use as gelling agents, thickeners and stabilizers (Lahaye, 2001; Pangestuti & Kim, 2015). Amongst them, agar, which is typically found in the cell walls from some red seaweeds (Rhodophyceae) (Hii et al., 2016), has a great industrial relevance as gelling agent, not only for food applications, but also for microbiology. This polysaccharide contains two main components: agarose and agaropectin. Agarose constitutes the gelling fraction and consists of alternating units of β-D-galactopyranosyl and 3,6-anhydrous-α-L-galactopyranosyl. On the other hand, agaropectin presents a structure similar to agarose, but contains 5-10% sulphate esters in addition to other residues such as methoxyl groups and pyruvic acid (Guerrero et al., 2014; Martínez-Sanz, Gómez-Mascaraque, et al., 2019; Wang et al., 2015). The agar extraction protocol is very well established at industrial scale; it involves the application of alkaline pre-treatments, followed by high temperature and pressure extraction and several filtration processes and freeze-thawing cycles to purify the product (Kumar & Fotedar, 2009; Martínez-Sanz, Cebrián-Lloret, et al., 2020). Since this is a very time and energy consuming process, efforts are being made to develop more energy-efficient extraction protocols. For instance, previous studies have reported on alternative methods for obtaining less purified agar fractions with good antioxidant properties, reducing the total extraction time and the amount of extraction steps (Li et al., 2021; Martínez-Sanz, Gómez-Mascaraque, et al., 2019; Martínez-Sanz et al., 2021). Although the less purified agars produced by means of these simplified extraction protocols may not be suitable for applications where high purity is a requirement, they might be valuable for the development of bio-based packaging materials with a more sustainable character and reduced production costs. In fact, a recent work showed that the presence of other polysaccharides (mainly floridean starch) and proteins in less purified agar-based extracts had a positive effect on the mechanical and water barrier performance of the films produced by the casting methodology (Martínez-

Sanz, Gómez-Mascaraque, et al., 2019). This work showed that agar-based films have promising properties for the development of sustainable bio-based films for food packaging applications. However, the solvent casting methodology used lacks industrial applicability. Inspired by the existing works reporting on the processing of other commercial biopolymers, in this work we have developed a simple methodology to produce agar-based films by means of melt mixing and hot pressing.

Amongst the most popular plant-derived polysaccharides currently used for bio-based packaging production, starch is undoubtedly one of the most promising materials due to its abundance, cost-effectiveness and excellent film-forming capacity (R. Thakur et al., 2019). Although starch can be processed through different techniques, the basis for its processing lays in the gelatinization phenomenon: under adequate heat and moisture conditions, the semi-crystalline structure of starch is partially or completely destroyed (phenomenon known as cooperative melting), hence producing an amorphous material which can be easily processed. Interestingly, after processing, upon cooling and storage, the amylose and amylopectin chains in starch can re-associate to form a more ordered structure via hydrogen bonding (Eom et al., 2018; Soni et al., 2020). This process, referred to as retrogradation, leads to the modification of several properties such as opacity, mechanical performance and vapour barrier capacity of the films; thus, in food packaging applications the shelf life and quality of the packaged product can be strongly affected due to changes in starch structure upon storage (Fekete et al., 2019). Based on the behaviour of starch, we developed a methodology in which agar-based extracts are subjected to heat and high moisture conditions during processing in an internal mixer. This allows to dissolve the agar molecular chains, which then are able to re-associate upon cooling, similarly to the gelation process. Since the formation of bundles of agar double helices has been shown to result in the formation of semi-crystalline structures (Martínez-Sanz, Gómez-Mascaraque, et al., 2019), it is reasonable to hypothesize that the properties of agar-based materials may also be modified with storage time due to re-crystallization processes taking place. Thus, the aim of this study was to determine the processability of agars with different degrees of purity (one commercial grade with high purity and one less purified agar-based extract produced by a more energy-efficient extraction protocol) by means of the melt mixing

technique and evaluate the performance properties of the obtained films. Furthermore, the evolution of these properties upon prolonged storage has been assessed to determine the effect of possible re-crystallization processes in the produced films.

3. Materials and methods

3.1. Materials

The seaweed *Gelidium corneum* and the commercial grade agar PRONAGAR were kindly donated by Hispanagar (Burgos, Spain). The dried seaweed was ground to powder before further processing. Glycerol, used as plasticizer, was purchased from Panreac Quimica, S.A. (Castellar Del Vallés, Barcelona, Spain).

3.2. Production of the less purified agar-based extract

A less purified agar-based extract was produced from the raw seaweed by applying a hot water treatment, as previously described by Martinez-Sanz et al.(Martínez-Sanz et al., 2019). Briefly, 50 g of dried seaweed powder were immersed in 500 mL of distilled water and heated at 90 °C for 2 h. Then, the agar-based solution was separated from the solid residue by filtration using a muslin cloth when the solution was still hot. The filtrate was allowed to form a gel upon cooling and it was subsequently frozen overnight at -21 °C. The material was then subjected to two freeze-thaw cycles (-21°C/25°C) to remove water-soluble impurities. Finally, the obtained gel was freeze-dried (Martínez-Sanz et al., 2019). The obtained agar-based extract, coded as HW, has been previously characterized, showing a total carbohydrate content of ca. 39-42% (from which galactose represented 74%), ca. 11-14% proteins, ca. 35% ash and ca. 3% polyphenols (Martínez-Sanz et al., 2019; Martínez-Sanz et al., 2020).

3.3. Preparation of agar-based films

Agar-based films were prepared by melt compounding, followed by compression molding, using formulations based on mixtures of agar and water, with and without the addition of a plasticizer. In the case of the pure commercial agar, the agar:water ratio used was 1:3 (w/w), while a higher ratio of 1:0.5 (w/w) was used in the case of the less purified agar extract HW, since the agar content in that sample was lower. These ratios were selected on the basis of preliminary trials, to ensure a good balance between proper processability (i.e. enough water to aid the

cooperative melting of agar) and good mechanical integrity of the obtained films (since too high water contents led to sticky materials, while too low water contents led to heterogeneous films). In the case of the pure commercial agar the obtained films showed a rigid behaviour and, therefore, we decided to explore the effect of adding a plasticizer on the final properties of the films. To do so, an additional formulation containing glycerol (30% with respect to the amount of agar in the mixture) was also prepared by adding the plasticizer to the commercial agar to form the final paste with water before the melt mixing step. The addition of plasticizer was not necessary in the case of the less purified agar films, which showed a much less rigid behaviour. It was hypothesized that the presence of other compounds in the extract could exert a plasticizing effect. The obtained pastes were then melt-mixed in a Brabender Plastograph (Germany) internal mixer at a temperature of 110 °C and 60 rpm for 2 min. Subsequently, 4 g of the obtained blends were spread evenly on Teflon films and placed in a compression mould (Carver 4122, USA) at a pressure of 16 tons and 110 °C for 4 min to form one film. The films were then stored in cabinets equilibrated at a relative humidity of 53% and 25°C for the 30 days of the study. The samples were coded as follows: COMM (commercial agar), COMM+GLY (commercial agar with glycerol as plasticizer) and HW (less purified agar-based extract). Samples were taken for further analyses right after being processed (t=0) and after different storage periods (t=3, 7, 14 and 30 days).

3.4. Moisture content

The variability in the moisture content of the films over time was calculated from the difference between the weight after drying and the initial weight of the samples, before placing them in an oven at 60 °C for 24 hours.

3.5. Fourier transform infrared spectroscopy (FT-IR)

The films were analyzed by FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm^{-1} resolution in a wavelength range between 400-4000 cm^{-1} and averaging a minimum of 32 scans.

3.6. X-ray diffraction (XRD)

XRD measurements were carried out on a D5005 Bruker diffractometer. The instrument was equipped with a Cu tube and a secondary monochromator. The configuration of the equipment was θ–2θ, and the samples were examined over the angular range of 3°–60° with a step size of 0.02° and a count time of 200 s per step. Peak fitting was carried out using the Igor software package (Wavemetrics, Lake Oswego, Oregon), using the same protocol described in a previous work (Martínez-Sanz et al., 2019) The obtained values from the fitting coefficients are those that minimize the value of Chi-squared, which is defined as:

$$\chi^2 = \Sigma\left(\frac{y-y_i}{\sigma_i}\right)^2 \quad (1)$$

where y is a fitted value for a given point, y_i is the measured data value for the point and σ_i is an estimate of the standard deviation for y_i. The curve fitting operation is carried out iteratively and for each iteration, the fitting coefficients are refined to minimize χ^2. The crystallinity index was determined from the obtained fitting results by applying the following equation:

$$X_C(\%) = \frac{\sum A_{Crystal}}{A_{Total}} \times 100 \quad (2)$$

where A_{Total} is the sum of the areas under all the diffraction peaks and $\Sigma A_{Crystal}$ is the sum of the areas corresponding to the crystalline peaks.

3.7. Scanning electron microscopy (SEM)

SEM was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8-16 mm. Small pieces of the agar films were sputtered with a gold–palladium mixture under vacuum during 2 min before their morphology was examined.

3.8. Water vapor permeability (WVP)

Direct permeability to water was determined from the slope of the weight gain versus time curves at 24ºC. The films were sandwiched between the aluminum top (open O-ring) and bottom (deposit for the silica) parts of a specifically designed permeability cell with screws. A Viton rubber O-ring was placed between the film and bottom part of the cell to enhance sealability. These permeability cells containing silica were then placed in an equilibrated relative humidity cabinet at 75% RH and 25°C. The weight gain through a film area of 10 cm^2 was

monitored and plotted as a function of time. Cells with aluminum films (with thickness of ca. 11 µm) were used as control samples to estimate weight gain through the sealing. The WVP was calculated according to the following equation:

$$WVP = \frac{\mathrm{WVTR\ x\ L}}{\Delta \mathrm{P}} \quad (3)$$

Where WVTR is the water vapor transmission rate (kg/s·m2) (calculated from the slope of the linear region of the weight gain vs. time, divided by the exposed film area), L is the mean film thickness (m), and ΔP is the difference of vapor pressure between the two sides of the film (Pa). The tests were done at least in triplicate.

3.9. Mechanical properties

Tensile tests were carried out at ambient conditions of typically 24ºC and 50%RH on a Mecmesin MultiTest 1-i (1 kN) machine (Virginia, USA) with the Emperor™ software. Pre-conditioned rectangular-shaped specimens with initial gauge length of 8 cm and 1 cm in width were cut directly from the films. A fixed crosshead rate of 25 mm/min was utilized in all cases. The elastic modulus (E), tensile strength (TS), and elongation at break (ε_B) were determined from the stress-strain curves, estimated from force–distance data obtained for the different films. At least, three specimens of each film were tensile tested as to obtain statistically meaningful results.

3.10. Statistical analysis

All data have been represented as the average ± standard deviation. Different letters show significant differences both in tables and graphs ($p \leq 0.05$). Analysis of variance (ANOVA) followed by a Tukey-test were used.

4. Results and discussion

In this work commercial agar and a less purified agar-based extract (described in section 2.2) were processed by melt mixing and compression moulding to prepare films for food packaging applications. As commented in section 2.3, adjusting the water content in the formulations was crucial to obtain homogeneous materials with a good mechanical integrity. Figure 1A shows the visual appearance of the films. As observed, the colour and transparency of the films were significantly affected by the agar purification. While the films prepared from pure commercial

agar were highly transparent, the HW films presented a brownish coloration and were more opaque. This is most likely due to the presence of other components such as proteins and polyphenols in the agar-based extract. The films' surface morphology was analysed by SEM and representative images are shown in Figures 1B-G. A noticeable morphological difference was observed between the films right after being processed (t=0). Furthermore, the morphology of the films evolved differently with storage time depending on the formulation. As it can be observed, the COMM film showed a very smooth and homogeneous morphology, which was maintained over storage time. In contrast, with the addition of glycerol the surface of the COMM+GLY film became rougher and more heterogeneous, with small particles, probably corresponding to glycerol, homogeneously distributed through the whole film surface. Interestingly, these particles were no longer visible in the film after 30 days of storage and the surface of the film was characterized by the appearance of large cracks, which may be due to glycerol migration through the film and/or dehydration of the material. In fact, previous studies have demonstrated that glycerol undergoes migration in other polysaccharide-based materials, such as starch films (López et al., 2013). The HW film presented a very different microstructure, with significantly rougher surfaces and large particles distributed along the surface, probably due to the presence of components other than agar in these samples. Given the appearance of these particles, which resembled crystalline clusters, and the high ash content previously reported for this type of agar (Martínez-Sanz, Gómez-Mascaraque, et al., 2019), it is suspected that they corresponded to minerals such as silica (SiO_2) and weddellite ($CaC_2O_4{\cdot}2H_2O$), which have been previously reported to remain in the agar-based extract (Martínez-Sanz, Gómez-Mascaraque, et al., 2019). The appearance of these particles changed after 30 days of storage, becoming more diffuse and with a less defined structure, which may be attributed to the hydration of the salts upon storage at a constant relative humidity of 53%.

These results evidence that water may be playing an important role in the structural variation of the films upon storage. Thus, the moisture content of the films was monitored during the 30 days of the study (cf. Figure S1) in order to better understand the changes in the properties of the films during storage. In the case of the COMM films, the moisture content increased during the first 14 days of storage (from 16% w/w to 178% w/w) and after that, the moisture content

of the film was stabilised. As expected, the presence of a highly hydrophilic plasticizer such as glycerol led to a higher moisture content in the films at t= 0 (68% w/w). In that case, the moisture also increased during the first 14 days of storage (up to 676% w/w), and after that, the films dehydrated significantly (reaching a moisture content of ca. 246% w/w). In contrast, the HW films showed a less pronounced increase in the moisture content during the first 7 days of storage (from 110% w/w to 170% w/w) and after that, the moisture content slightly decreased and then remained quite stable until the end of the study (reaching a final value of ca. 100% w/w). This hydration-dehydration phenomenon may be related to a structural re-organization of the agar and changes in the type of interactions being established with water. These structural changes seemed to be less evident in the HW films, which is reasonable given the lower agar content in this material. It should also be noted that for the as-prepared films, the moisture content was the highest in the HW films, even though the amount of water added to process the formulations in the internal mixer was lower in that case (cf. section 2.3). This may be related to the more amorphous structure of agar (as suggested by the XRD results presented later) and explain why these materials did not require the addition of any plasticizer to obtain flexible and easy-to-handle films, since bulk water seemed to act as a plasticizer.

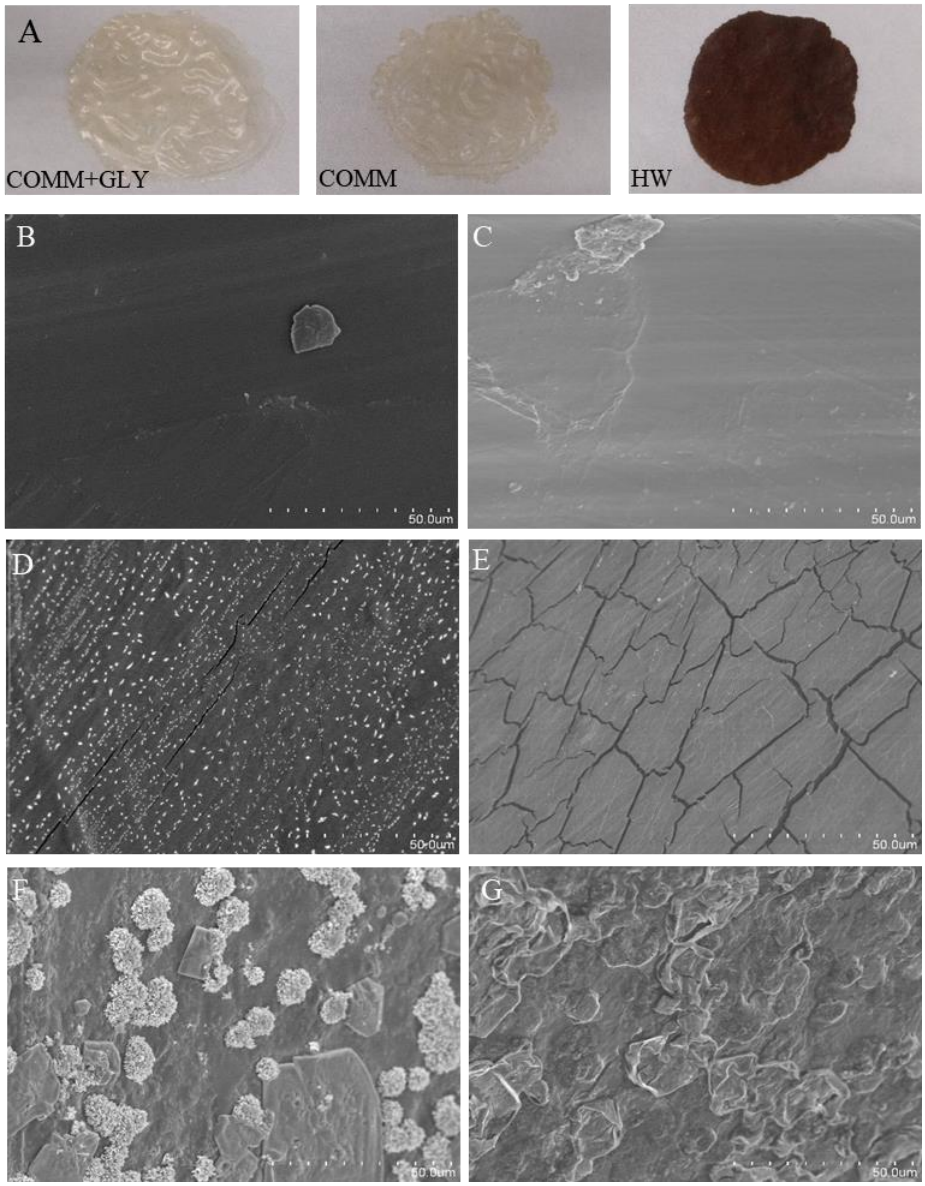
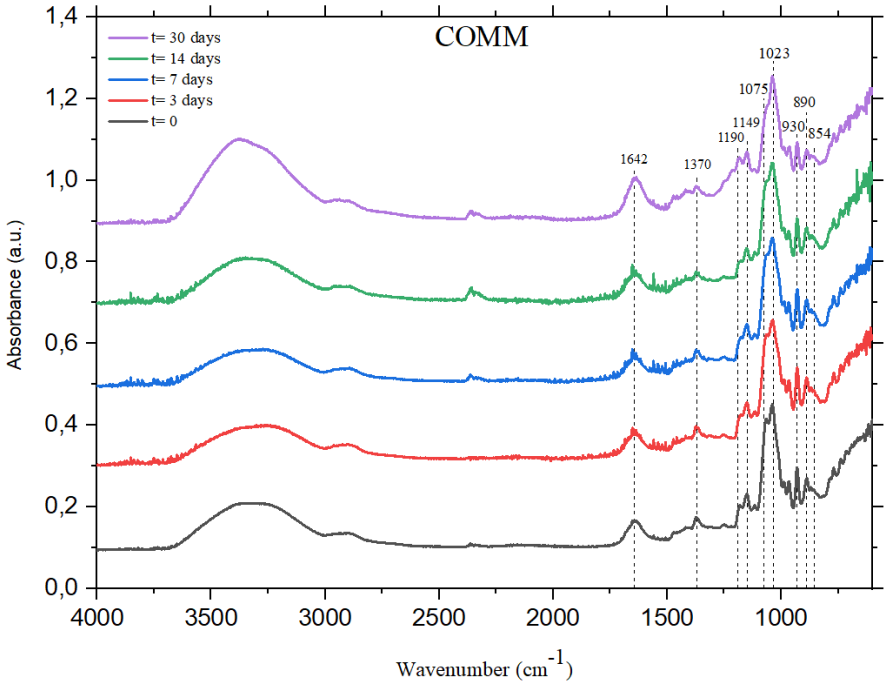
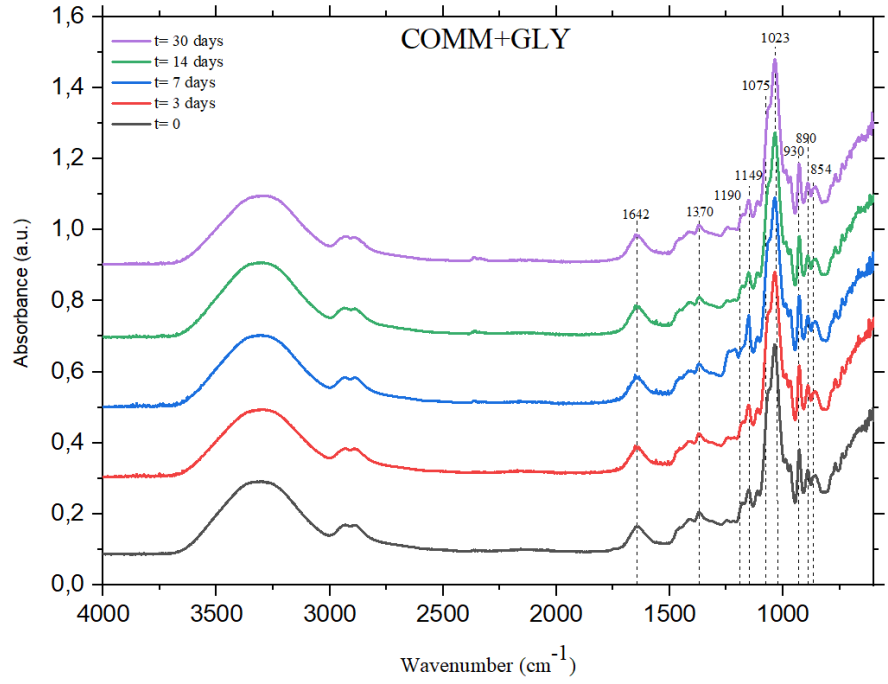


Figure 1. (A) Visual appearance of the agar-based films after being processed (t=0). (B-G) SEM images of the surface of agar films: (B) COMM t= 0; (C) COMM t= 30 days; (D) COMM+GLY t= 0; (E) COMM+GLY t= 30 days; (F) HW t=0 and (G) HW t= 30 days. Scale bars correspond to 50 µm.

FT-IR characterization of the obtained films was carried out to identify changes in the molecular structure of the films over time. Figure 2 shows the spectra of the different freshly prepared films (t=0) and after different storage periods. As observed, the three films presented the most characteristic agar bands, located at 890 cm^{-1}, assigned to the C-H bending at the anomeric carbon in β-galactopyranosyl residues, and at 930 cm^{-1}, associated with the vibration of the C-O-C bridge of the 3,6-anhydro-galactose (Freile-Pelegrín et al., 2007; Guerrero et al., 2014; Martínez-Sanz et al., 2019). As expected, the relative intensity of these bands was stronger for the films made from pure commercial agar, while they were much less intense in the HW film, which is reasonable given the lower agar content in the less purified agar-based extract. Other agar-characteristic bands, related to the sulfation degree, are those located at 1370 cm^{-1}, 1243 cm^{-1} and 1190 cm^{-1}, which are linked to the vibration mode of the sulphate groups, the band at 1149 cm^{-1}, which is mainly associated to the vibration mode of the ester-sulphate bond, and the small signal at 854 cm^{-1}, which is assigned to the sulphate at C-4 of galactose (Guerrero et al., 2014; Kanmani & Rhim, 2014; Martínez-Sanz et al., 2019). Despite the lower agar content in the HW extract, the relative intensity of most of these bands was quite high, which is due to the greater degree of sulphation in this type of extracts, as previously reported (Martínez-Sanz et al., 2019; Martínez-Sanz et al., 2021). The broad band at 1642 cm^{-1} confirmed the presence of significant amounts of bound water in all samples, which is not surprising due to the hydrophilic character of agar. In the particular case of the HW film, this band overlaps with the amide I band, due to the presence of considerable amounts of proteins in this sample (Kanmani & Rhim, 2014; Martínez-Sanz et al., 2019). Interestingly, while the spectra from the COMM+GLY and HW samples did not extensively change upon storage, some evident changes were detected in the case of the COMM film. The most notable changes occurred in the relative intensity of the bands located at 1190 cm^{-1}, 1075 cm^{-1} and 1023 cm^{-1}, which are associated to C−C, C−O, C−H stretching and COH bending modes. Although it is difficult to assign bands from this region to specific structural features, since they are present in many different polysaccharides, it is interesting to note that the band at 1075 cm^{-1} has been previously related to vibrational modes within the amorphous phase of starch (Lopez-Rubio et al., 2008). The fact that the relative intensity of this band (with respect to the one at 1023 cm^{-1}) changed along the storage time may be indicative

of marked structural modifications taking place in the semi-crystalline fraction from agar. In addition, the shape and relative intensity of the broad band at 3030-3350 cm^{-1}, corresponding to hydrogen-bonded OH stretching, was significantly affected, with the relative intensity being lower at t=3-14 days and then increasing at t=30 days. It should also be noted that the shape of the bands within the region 3000-3700 cm^{-1} changed significantly from a broad band without any shoulders, which is characteristic of the presence of liquid bulk water (Guan et al., 2011), to a sharper band with different shoulders after 30 days of storage. Moreover, the relative intensity of the bound water, located at 1642 cm^{-1}, also increased after 30 days of storage. This may seem counterintuitive if compared to the evolution of the moisture content in the films. However, it should be considered that the gravimetrically determined moisture content is indicative of the overall amount of water, i.e. bulk and bound to the polysaccharide. Thus, while the overall water content in the films increased upon storage, the FT-IR results suggest that, due to structural re-organization of the agar molecular chains, the nature of interactions between the polysaccharide and water changed along the storage period. This phenomenon is expected to be linked to major changes in the semi-crystalline structure of agar (as demonstrated later by XRD). Despite the fact that the overall moisture content in the COMM+GLY films also varied upon storage, the relative intensity of the OH vibration and bound water bands (with respect to the band at 1023 cm^{-1}) remained quite constant during the whole experiment. This suggests that structural changes in the semi-crystalline structure of agar are limited due to the presence of glycerol. In the case of the HW films, changes occurred mostly in the relative intensity of the bands at 1190 cm^{-1}, 1075 cm^{-1} and 1023 cm^{-1}, suggesting that small structural changes took place upon storage, while the relative intensity of the bands associated to bound water and OH vibration were slightly affected during storage.


COMM
t= 30 days
t= 14 days
t= 7 days
t= 3 days
t= 0
1642
1370
1190
1149
1075
1023
930
890
854
Absorbance (a.u.)
Wavenumber (cm-1)


COMM+GLY
t= 30 days
t= 14 days
t= 7 days
t= 3 days
t= 0
1642
1370
1190
1149
1075
1023
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Absorbance (a.u.)
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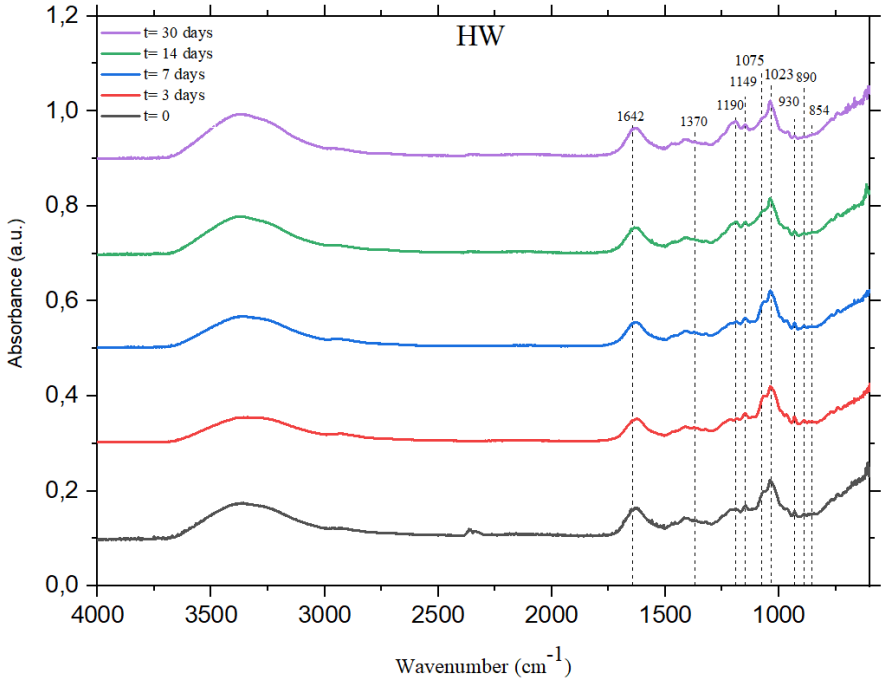

Figure 2. FT-IR spectra from the agar-based films after being processed (t=0) and after different storage times. All the spectra were normalized to the intensity of the band at 1023 cm^{-1}.

Previous characterization of the commercial agar and the less purified agar-based extract used in this work to produce the films showed that indeed these agars present a semi-crystalline structure (Martínez-Sanz, Gómez-Mascaraque, et al., 2019). Thus, to analyse the changes in the semi-crystalline structure of the agar-based films upon storage, they were characterized by XRD and the obtained patterns are shown in Figure 3. The XRD patterns from the three films at t=0 showed the same features previously reported for agars with semi-crystalline structure, with a well-defined peak at 19.0° and a shoulder at 13.9° (Freile-Pelegrín et al., 2007; Kanmani & Rhim, 2014b) suggesting that these films presented a certain degree of order in their structure. Furthermore, two broad shoulders located at ca. 27° and 40° were clearly detected in all the samples but were less evident in the HW films. Such shoulders have been previously noted in the XRD patterns from other polysaccharides such as cellulose (Martínez-Sanz et al., 2016) and chitosan/glucomannan blends (Lopez-Rubio et al., 2016) and correspond to water molecules

bound to the surface of the semi-crystalline polysaccharide. Thus, it seems that even though the amount of moisture in the HW films at t=0 was the highest, this was mainly bulk water, which was not strongly interacting with the polysaccharide. In contrast, in the case of the COMM and COMM+GLY films a certain fraction of water was strongly bound to the polysaccharide and adopting a partially ordered conformation. It should be noted that the agar crystalline peaks were more intense in the COMM+GLY than in the COMM sample at t=0, suggesting that the presence of glycerol induced the formation of a more crystalline structure after processing the material. This was indeed, reflected on the estimated crystallinity values (cf. Table 1), which were higher in the freshly prepared films containing glycerol. This increase in the crystallinity due to the plasticizer addition has been previously observed in other polysaccharides such as chitosan (Epure et al., 2011) and thermoplastic starch (Xie et al., 2014). On the contrary, due to its lower agar content, the agar characteristic peaks were much weaker in the HW film which, in turn, showed multiple intense and sharp peaks that were absent in the commercial agar films and also contributed to the overall crystallinity of the material. These peaks have been reported to appear in the XRD patterns from less purified agar-based extracts and were attributed to the presence of minerals such as silica and weddellite (Martínez-Sanz et al., 2020). Interestingly, the semi-crystalline structure of the films evolved differently upon storage time. In the case of the COMM sample, it was clearly observed that the relative intensity of the crystalline peaks changed with storage time, suggesting a structural re-organization of the agar chains into different semi-crystalline conformations. Moreover, the contribution from the water shoulders seemed to be reduced after 14 days of storage. The agar crystallinity index reached a maximum of 27% at 7 days of storage and remained constant at 10% during the rest of the experiment. These results confirm that the structure of agar undergoes significant changes during the storage period, being water essential for these structural changes. In contrast, in the case of the in the COMM+GLY sample, the relative intensity of the agar-characteristic peaks was not strongly modified and the crystallinity index remained fairly constant throughout storage up to 14 days. Interestingly, contrarily to the COMM films, the contribution of the shoulders assigned to water slightly increased along storage. It should be noted that these two samples could not be measured at the end of the storage experiment (t=30d) since it was not possible to obtain

completely flat film surfaces on the XRD sample holder due to an excessive rigidity of the films, hence preventing a correct measurement of the specimens. In the case of the HW film, the overall crystallinity increased after the first 3 days of storage and then remained fairly constant. The crystallinity corresponding to the agar fraction, representing only 15-30% of the overall crystallinity, slightly increased with respect to the film at t=0, but the values were still very low, indicating the existence of a more amorphous agar. These results show that, in the absence of other components, the semi-crystalline structure of agar undergoes significant changes upon storage, which are most likely driven by variations in the moisture content and water re-organization within the film structure. These structural changes are minimized or delayed when glycerol is added as plasticizer into the film formulation or by the presence of other components in the HW film, hence providing materials with a better stability upon prolonged storage.

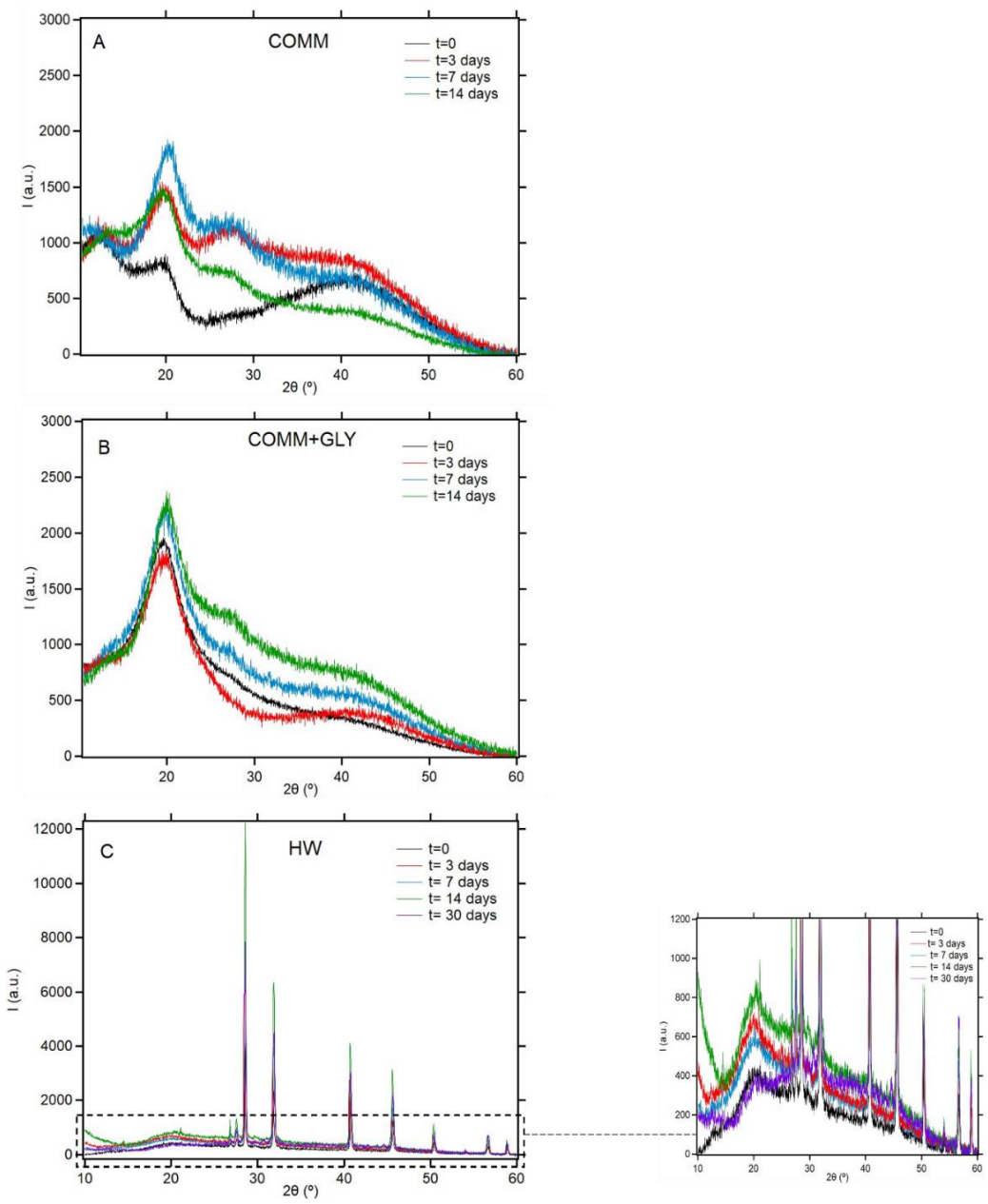

Figure 3. XRD patterns of the agar-based films after being processed (t=0) and after different periods of storage. The patterns from the COMM films are shown in (A), while (B) corresponds to the COMM+ GLY films and (C) to the HW films.

Table 1. Crystallinity index determined from the XRD patterns from the agar-based films after different storage times. In the case of the HW films, the crystallinity values estimated by considering only the agar characteristic peaks are additionally shown between brackets.

	Xc (%)				
	t=0	t=3 days	t=7 days	t=14 days	t=30 days
COMM	10	10	27	10	---
COMM+GLY	17	15	12	18	---
HW	12 [2]	21 [6]	23 [5]	20 [3]	18 [4]

The mechanical properties of the agar-based films are highly relevant to determine their suitability to be used as packaging materials. Therefore, they were evaluated by tensile tests and the most representative parameters obtained from the stress-strain curves are shown in Figure 4. It is evident that there was a large difference between the mechanical performance of the pure commercial agar films and those obtained from the less purified agar-based extract. For the freshly processed films, the COMM sample presented the highest elastic modulus (E≈1500 MPa) and tensile strength (σ≈34 MPa) values. The addition of glycerol had a clear plasticization effect, reducing both the elastic modulus (E≈640 MPa) and tensile strength (σ≈15 MPa), while increasing the elongation at break (from 10% for COMM to 32% for COMM+GLY). As expected, due to the lower agar concentration in the HW films, they presented poorer mechanical resistance, with low elastic modulus (E≈60 MPa), tensile strength (E≈3 MPa) and elongation at break (ε_B≈6%). Interestingly, the mechanical performance of the COMM films was drastically affected upon storage, with the three measured parameters significantly decreasing over time. This was also directly reflected in the appearance of the films since, after two weeks of storage, the material became extremely rigid and brittle, impeding a proper characterization of its mechanical properties. This trend in worsening the mechanical properties upon storage is in line with the results reported by Freile-Pelegrin et al. in their study on the biodegradability of agar films in a humid tropical climate, attributing this effect to a reduction in the molecular weight of agar (Freile-Pelegrín et al., 2007). Our results do not show any signs of agar hydrolysis

upon storage, since the crystallinity of the films was not strongly modified. Instead, a re-organization of the semi-crystalline structure of agar was observed and the proportion of water tightly bound to the polysaccharide and forming part of its semi-crystalline structure was reduced after 14 days of storage. Thus, it seems that the loss of water within the agar semi-crystalline structure (even though the overall amount of moisture in the films increased) was the main driver for the rigidizing effect induced by storage. In contrast, the mechanical properties of the more ductile COMM+GLY films varied erratically for the first 14 days of storage, which may be a consequence of glycerol migration within the film structure and/or slight variations in the amount of water tightly bound to the polysaccharide (as suggested by XRD). This was followed by a rigidizing effect after 30 days of storage. At this point, the elongation at break decayed to approximately 5% and the elastic modulus increased up to 1100 MPa. Note that, at this storage time, the total amount of moisture in the films also experienced a sharp decrease (cf. Figure S1). Such phenomenon may be explained by an excessive migration of glycerol from the structure of the film, thus promoting dehydration and rigidization. On the other hand, the mechanical properties of the HW films remained quite stable throughout the storage time. In fact, the elongation at break was even slightly improved, reaching a similar value to that obtained for the COMM+GLY film ($\varepsilon \approx 7\%$) after 30 days of storage. This slight improvement may have been originated by structural changes taking place in the salts present in the agar-based extract, as evidenced by the SEM images (cf. Figure 1). An important implication is that the mechanical properties of the agar films processed by melt mixing are superior to those from other reference biopolymers such as thermoplastic starch ($E = 29.8$ MPa, $\sigma = 3.1$MPa, $\varepsilon_B = 62.6\%$) (Cyras et al., 2008; Majdzadeh-Ardakani et al., 2010) and comparable to the values reported for agar-based films processed by casting ($E = 29\text{-}1600$ MPa, $\sigma = 6\text{-}38$ MPa, $\varepsilon_B = 15\text{-}26$ %) (Arham et al., 2016; Makwana et al., 2020; Martínez-Sanz et al., 2019). Although the pure agar presents much better mechanical performance than that from the less purified agar-based extract, the addition of plasticizer in the former is required to obtain films with a certain stability upon storage (up to ca. 14 days), while that is not needed in the case of the HW films. Thus, while the HW extract may not be competitive for the development of packaging

films on its own, it may be an interesting choice as additive to reduce costs and improve the stability upon storage of pure agar films.

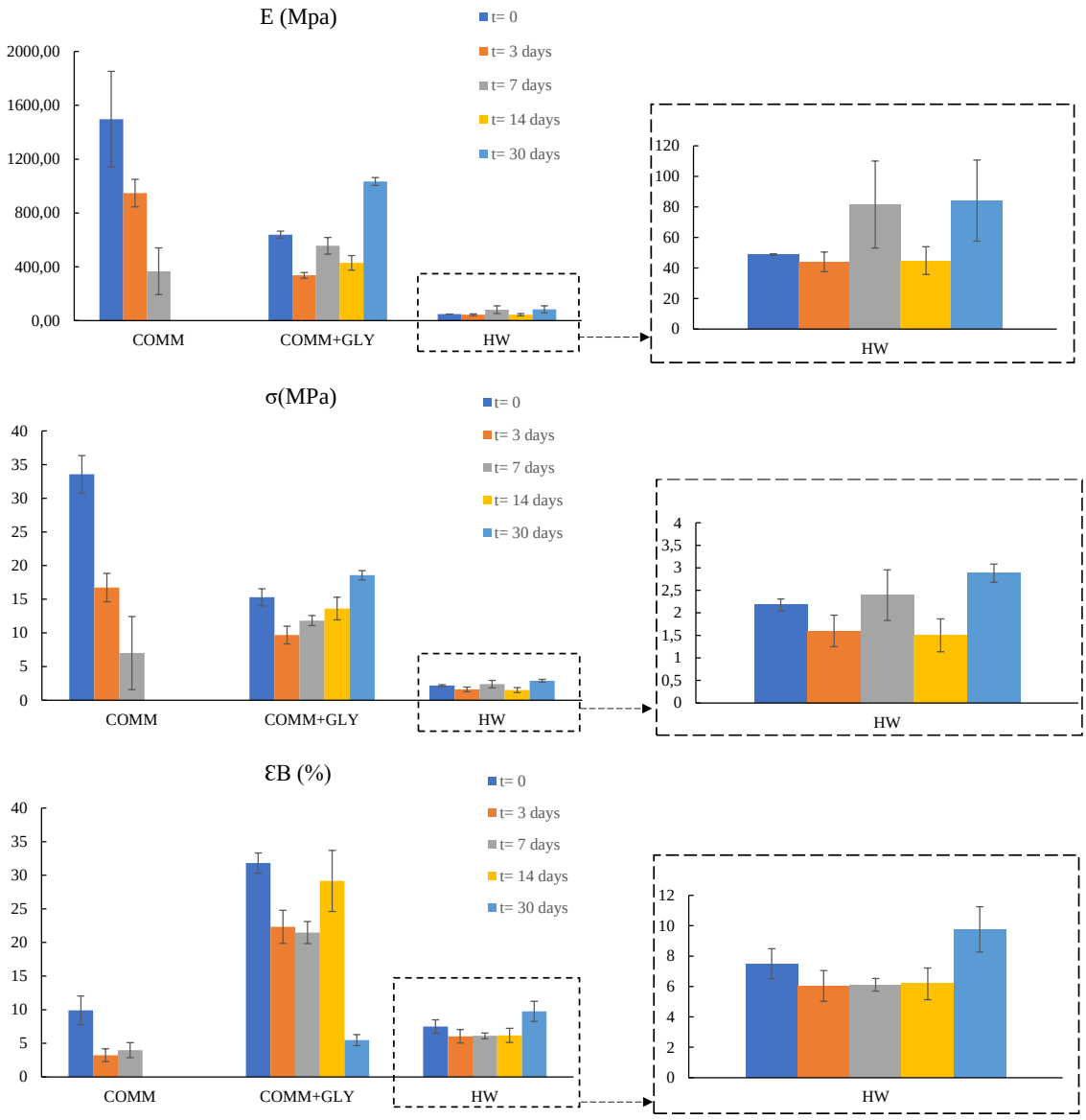

Figure 4. Mechanical properties of the agar-based films after different storage times. E: Elastic modulus, σ: tensile strength and $Ɛ_B$: elongation at break. Data correspond to the mean calculated values, n=3.

The water vapor permeability (WVP) of the films was also characterized and the obtained results are shown in Figure 5. As observed, the freshly made COMM film exhibited the lowest water permeability ($8.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa), which decreased slightly during the two following weeks. As previously mentioned, this film could not be measured for the entire duration of the experiment due to its physical deterioration. It should also be noted that the presence of glycerol in the COMM+GLY film had a slightly negative impact on the barrier capacity. This was mainly noticeable during the first two weeks of storage, in which the permeability values remained roughly stable (~$9.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa). The detrimental effect of hydrophilic plasticizers on the WVP of other polysaccharide-based films has been reported before and may be attributed to a distortion in the network of the hydrogen bound hydroxyl groups from the polysaccharide (Martínez-Sanz et al., 2013). Finally, after 30 days of storage the permeability decreased to a minimum of $6.1 \cdot 10^{-14}$ Kg·m/s·m^2·Pa. This is in line with the rigidizing effect observed in the mechanical properties and would be consistent with the migration of a significant amount of the highly hydrophilic glycerol plasticizer from the film structure. On the other hand, the presence of other components in the HW extract resulted in a higher water permeability in the freshly made film ($1.3 \cdot 10^{-13}$ Kg·m/s·m^2·Pa). Surprisingly, the barrier capacity improved over time, reaching a permeability value comparable to that of the commercial agar films after 30 days of storage ($7.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa). Previous studies have reported that the improvement in the permeability of the less purified extract can be explained by the formation of partially intertwined three-dimensional networks at the molecular scale between the agar and the proteins contained in these materials (Donato et al., 2005; Martínez-Sanz. Another possible explanation may be related to the more homogeneous integration of the salt particles in the film structure, as evidenced by the SEM characterization. It is also worth noting that the WVP values of the three films obtained in this study were lower than those previously reported by Rhim et al. for casting-processed agar films ($2.2 \cdot 10^{-14}$ Kg·m/s·m^2·Pa) (Rhim et al., 2011) and even for reference biopolymers such as thermoplastic starch ($2.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa) (Benito-González et al., 2018), thus highlighting the potential of these materials to be used in packaging applications requiring high barrier properties. Once again, the results suggest that the use of the HW agar-based extract as additive in more purified agar films might be interesting to reduce

costs, while improving the stability of the films upon storage in terms of crystallinity and mechanical performance and having a positive effect on the water barrier capacity.

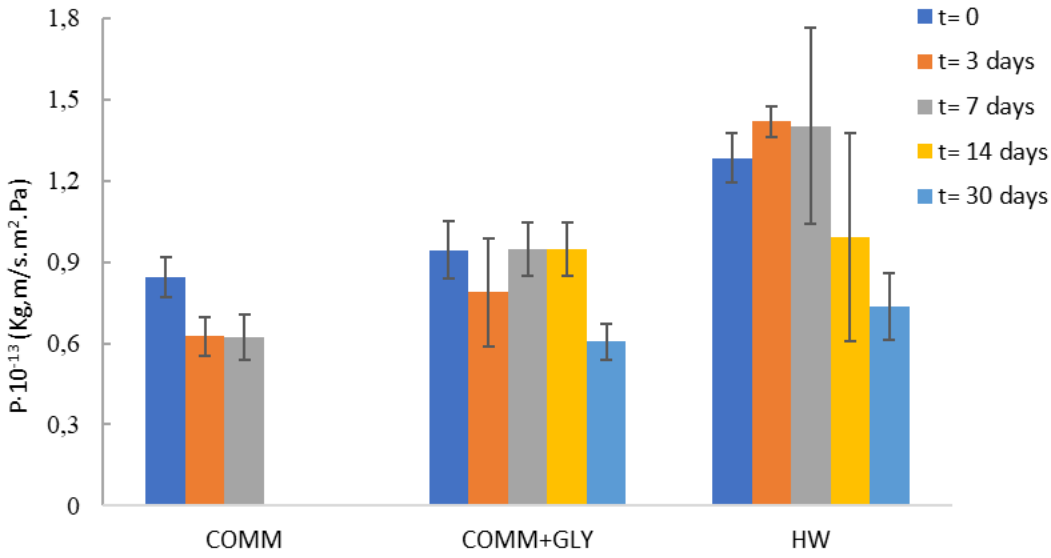

Figure 5. Water Vapor Permeability (WVP) of the agar-based films after different storage times. Data correspond to the mean calculated values, n=3.

5. Conclusions

The capacity of agars with different degrees of purity to form packaging films by means of the melt mixing technique has been evaluated and the stability of the obtained materials upon prolonged storage has been investigated. The degree of purification of the agar had a major impact in the properties of the films. In particular, the less purified agar-based extract yielded more opaque films with a brownish coloration, lower mechanical performance and higher water vapour permeability than the commercial pure agar. On the other hand, the extremely rigid behaviour of the commercial agar film required the addition of a plasticizer to produce films which could be manipulated without causing breakage. Indeed, the addition of glycerol produced more ductile films but reduced their mechanical resistance. Interestingly, this study

has demonstrated that when the films are stored at 53%RH after preparation, the semi-crystalline structure of agar undergoes significant changes and the proportion of tightly bound water is modified. As a result, the pure agar films undergo a rigidizing effect, which is also reflected in a reduction of the water permeability, making the films unmanageable after 7 days of storage. The presence of glycerol prevented these changes in the semi-crystalline structure of agar, improving the stability of the films up to 14 days of storage. However, the films were rigidized after 30 days of storage, most likely due to glycerol migration. Notably, the water-polysaccharide interactions seemed to be more limited in the films from the less purified agar-based extract, hence showing a greater stability upon storage. These results show the potential of the less purified agar-based extract, produced by a more energy efficient extraction protocol, to be used as additive to reduce costs and improve storage stability of pure agar films.

6. Acknowledgements

This work was financially supported by Hispanagar.

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8. Supplementary Material

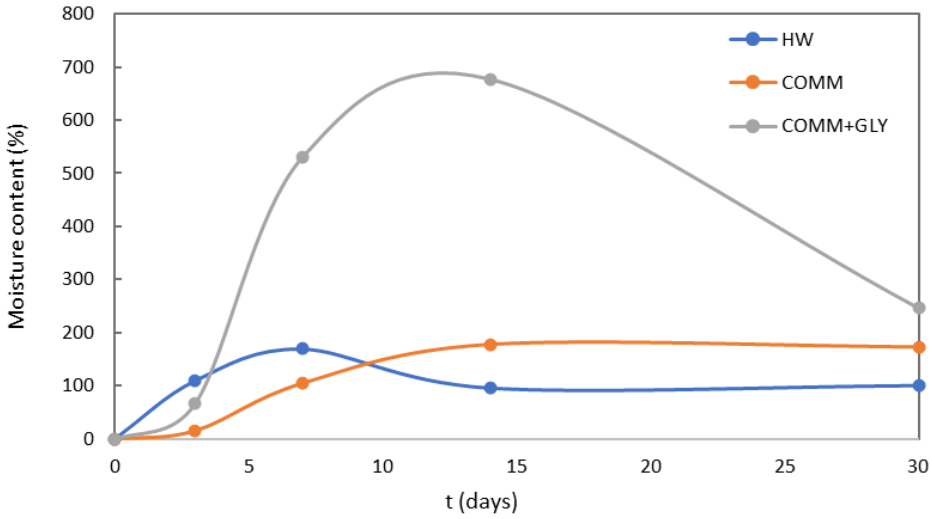
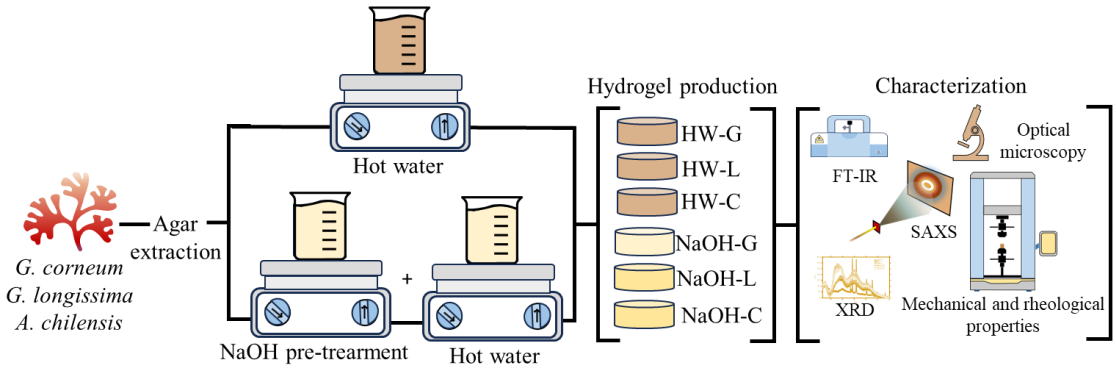


Fig S1. Moisture content determined in the agar-based films at different storage times.

3.2.2

EXPLORING ALTERNATIVE RED SEAWEED SPECIES FOR THE PRODUCTION OF AGAR-BASED HYDROGELS FOR FOOD APPLICATIONS.



This section is an adapted version of the following published research article:

Cebrián-Lloret, V., Martínez-Abad, A., López-Rubio, A., & Martínez-Sanz, M. (2024). Exploring alternative red seaweed species for the production of agar-based hydrogels for food applications. *Food Hydrocolloids*, 146.

1. Abstract

Three different red seaweed species, *Gelidium corneum* and two *Gracilaria* species (*Agarophyton chilensis,* and *Gracilariopsis longissima*), were used to produce agar-based fractions through conventional and simplified extraction methods and their composition and gel-forming properties were evaluated. The use of an alkaline pre-treatment was effective in removing impurities such as proteins, lipids, and ash, while the agarose/agaropectin ratio in agar was not affected. This led to the formation of hydrogels with higher stiffness and strength. Surprisingly, the presence of semi-crystalline agaropectin in the agar fractions from the two *Gracilaria* species, especially *G. longissima*, promoted the formation of more densely packed and stronger hydrogel networks, with higher gelling temperatures and superior mechanical properties. Thus, these results suggest that *G. longissima* has the potential to be used as an alternative to the more widespread use of *G. corneum* for the production of agar hydrogels for food applications.

2. Introduction

Thickening and gelling agents play a critical role in the food industry, as they are widely employed to modify the texture and consistency of food products (Qin et al., 2018). These gelling agents are typically hydrocolloids that have the unique ability to bind with water molecules and produce gel-like structures, imparting a desirable texture to the final product (Fathima et al., 2022; Williams & Phillips, 2021). The properties of the resulting gels, such as firmness, elasticity, and stability, depend on the type and concentration of the gelling agent used (Cong et al., 2022), as well as the gelling mechanism and parameters affecting the process, such as the temperature, presence of salts, etc. Traditionally, thickening and gelling agents derived from animals, such as gelatine derived from animal bones and skin, have been extensively utilized in the food industry (Williams & Phillips, 2021). However, due to the heightened consciousness of animal welfare, the rapid growth of the vegetarian and vegan markets, and consumers' inclination towards healthy and eco-friendly food options, individuals are increasingly willing to modify their habits and assume accountability for climate change by selecting plant-based foods over animal-based alternatives, thereby reducing their carbon footprint (He et al., 2021; McClements & Grossmann, 2021; Williams & Phillips, 2021). In this

context, marine sources, such as seaweeds or aquatic plants, are being investigated as a potential source of biopolymers with gelling properties (Kartik et al., 2021; Martínez-Sanz et al., 2020; Özçimen et al., 2017). In particular, algal cell walls contain a variety of polysaccharides with different functionalities which can be exploited as gelling agents (Kartik et al., 2021). Sulphated polysaccharides, such as carrageenan and agar, are of particular interest to the food industry as they can be used as gelling, thickening, and stabilizing agents (Cebrián-Lloret et al., 2022; Jayakody et al., 2022; Pangestuti & Kim, 2015). Agar, found in the cell walls of some red seaweed species (Rhodophyta), is composed of two main fractions: agarose, the gelling fraction, which consists of alternating units of β-D-galactopyranose and 3,6-anhydro-α-L-galactopyranose, and agaropectin, which presents a structure similar to agarose but may also contain sulphate esters and other residues, such as methoxyl groups and pyruvic acid. The presence of a greater proportion of sulphate groups in agar is frequently related to a reduced gelling ability and it influences gelling temperature and melting behaviour (Cebrián-Lloret et al., 2022; Lee et al., 2017; Martínez-Sanz et al., 2020; Yarnpakdee et al., 2015). For the extraction of agar, *Gelidium corneum* (formerly known as *Gelidium sesquipedale*) is the most commonly utilized seaweed species in Spain and Morocco, as it yields agars with higher gelling capacity compared to those obtained from other species, such as *Gracilaria spp.* (Martínez-Sanz et al., 2019; Rocha et al., 2019; Wang et al., 2017). The industrial-scale extraction of agar involves alkaline pre-treatments, high-temperature and pressure extraction, filtration processes, and freeze-thaw cycles. However, due to the time and energy requirements of this process, efforts are being made to develop more sustainable and energy-efficient extraction protocols. In line with this, simplified methods, reducing the total extraction time and the number of extraction steps, have been proposed to obtain less purified agar fractions, which have shown good antioxidant properties (Cebrián-Lloret et al., 2022; Martínez-Sanz et al., 2021; Martínez-Sanz et al., 2019). Even if the less purified agar-based fractions may not meet the requirements of high-purity applications (e.g. microbiology applications), they can still be beneficial for other purposes, such as food additives with bioactive properties or food packaging materials (Cebrián-Lloret et al., 2022). Furthermore, for food applications in which high agarose purity is not a

requirement, it may also be interesting to explore other seaweed species with more competitive prices than *Gelidium corneum*.

Consequently, three different species of red seaweeds were employed for this study: *Gelidium corneum* (formerly *Gelidium sesquipedale*) and *Agarophyton chilensis* (formerly *Gracilaria chilensis*), which are traditionally used for agar extraction, as well as *Gracilariopsis longissima* (formerly *Gracilaria verrucosa)* which is a species that is not commonly utilized for agar extraction but is cost-effective to cultivate (Bermejo et al., 2020; Mouga & Fernandes, 2022).

The aim of this study was, therefore, to conduct a comparative analysis of the composition of various agar-based fractions produced using two distinct methods: (i) a conventional agar purification process similar to the one commonly used in the industry (including a harsh alkaline pre-treatment followed by extraction with boiling water), and (ii) an alternative simplified extraction protocol that yields less purified agar fractions (omitting the pre-treatment step). In addition, the properties of the gels formed by each of the agar-based fractions have been evaluated to determine their suitability for application in the food industry. This will allow evaluating the potential of producing high-quality agar-based additives through more sustainable processes and using more cost-effective sources.

3. Materials and methods

3.1. Materials

Hispanagar S.A. (Burgos, Spain) kindly provided the three red seaweed species, *Gelidium corneum*, *Agarophyton chilensis,* and *Gracilariopsis longissima.* Prior to the extraction of agar, the seaweeds were washed with tap water to remove sand and other impurities, dried by means of a Digitronic-TFT oven (J.P. SELECTA, Spain) for 24 hours at 50 °C, and ground into fine powder with a particle size of less than 250 µm using a ZM 200 Model Ultra-Centrifugal Mill (Retsch, Germany).

3.2. Production of the agar-based extracts

Agar was extracted from the biomass of the three seaweed species by applying a hot water extraction procedure, as well as a pre-treatment purification step, as previously described by Martinez-Sanz et al. (Martínez-Sanz et al, 2019). The extraction procedure involved immersing 50g of dry seaweed powder in 500mL of distilled water, heating to 90°C for 2 hours, and then

quickly separating the hot agar-based solution from the solid residue by filtration using a muslin cloth at room temperature. The resulting filtrate was gelled and frozen overnight at -21°C, followed by two freeze-thaw cycles to improve the elastic properties of the gels, and finally the resulting gel was freeze-dried using a Lyobeta 6PL freeze dryer (Telstar, Japan). The so obtained agar-based fractions from *G. corneum, A. chilensis*, and *G. longissima* were coded as HW-G, HW-C, and HW-L respectively. In addition, a pre-treatment step was evaluated prior to the extraction process, which involved soaking 50g of dry seaweed powder in 500mL of 10% (w/v) NaOH solution, heating to 90°C for 2 hours, filtering using a muslin cloth and washing the solid material with distilled water. The obtained solid was then subjected to the hot water treatment described above. The resulting purified agar fractions were coded as NaOH-G, NaOH-C, and NaOH-L, respectively.

3.3. Production of the agar-based dispersions and hydrogels

Freeze-dried agar-based extracts were dispersed in distilled water at a concentration of 2.5% (w/w) by heating at 95°C for at least 45 minutes. The resulting hot solutions were then used to fill the rheometer plate or SAXS capillaries. For compression tests, the hot solutions were poured into 18 mm diameter methacrylate moulds and allowed to cool overnight at 25°C to form disk-like hydrogel specimens.

3.4. Compositional analysis

All the determinations were carried out at least in triplicate, as described below.

3.4.1. Carbohydrate analysis

To determine the carbohydrate composition, the methodology established by Stevenson & Furneaux (Quemener & Lahaye, 1998; Stevenson & Furneaux, 1991), with slight modifications, was employed. In brief, 10 mg of dry sample were dissolved in a 5 mL aqueous rhamnose solution (0.5 mg/mL, internal standard) and incubated at 95 °C for 40 min. Subsequently, 0.5 mL of the solution was dried in Pyrex tubes, followed by a pre-hydrolysis step involving the addition of 50 µL of 4-methylmorpholine-borane (MMB) and 200 µL of 3 M trifluoroacetic acid to the tubes, which were then placed in a heat block at 80 °C for 30 min. After cooling, 50 µL of MMB solution were added, and the samples were dried. For the main hydrolysis, 200 µL of 2 M

trifluoroacetic acid were added to the tubes, which were then kept at 120 °C in the thermoblock for 1 h. Following this, 100 µL of MMB solution was added, and the samples were dried at 50 °C. After drying, the samples were resuspended in 1 mL H_2O, filtered through 0.45 µm syringe filters, and transferred to chromatography vials. The monosaccharides were then analysed via high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-6000 (Dionex, ThermoFisher Scientific, Sunnyvale, CA, USA). Control samples containing known concentrations of mixtures of glucose, galactose, rhamnose, 3,6-anhydro-L-galactose, and 6-0-methyl-D-galactose were reduced to their corresponding alditols and used for calibration.

3.4.2. Ash content

To determine the mineral content of the agar-based fractions, the ash content was measured through dry biomass calcination using the standard TAPPI T211 om-07 method. About 0.25 g of dried material was placed into a pre-weighed crucible, which was then weighed. The combustion process was carried out in a muffle furnace at 550 °C for 24 hours, after which the ash content was determined through gravimetric quantification.

3.4.3. Lipid content

The Folch method, with slight modifications, (Löfgren et al., 2012) was employed to estimate the total lipid content of the samples. In brief, 30 mg of the sample was suspended in 200 µL of MilliQ water and transferred to a 10 ml tube. Subsequently, 1660 µL of methanol was added and mixed at 1400 rpm for 10 minutes. Next, 3320 µL of dichloromethane was added, and the mixture was stirred at 1400 rpm for 20 minutes. A solution of 1000 µL of 20mM acetic acid was added to the tube and stirred at 1400 rpm for another 10 minutes. After centrifugation, the lower organic phase was transferred to a new tube, and the aqueous phase was washed with 1660 µL of dichloromethane. This process of centrifugation and phase separation was repeated, and the organic phase was pooled. The content of this tube was then filtered using a glass syringe and a 0.45 nm filter. The organic solvent was evaporated, and the total lipid content was determined gravimetrically.

3.4.4. Protein content

The total nitrogen content of the samples was determined by employing an Elemental Analyser Rapid N Exceed (Paralab S.L., Spain). To accomplish this, approximately 100 mg of each powdered sample was pressed to form a pellet and subjected to analysis using the Dumas method. This technique is based on the complete and instantaneous oxidation of the sample by combustion with oxygen at a temperature of approximately 1020°C. The combustion products are transported by the carrier gas to a chromatographic column where the separation takes place. A thermal conductivity detector provides the signal for N_2 which is translated into percentage content. (Wiles et al., 1998). The total protein content was then estimated by multiplying the nitrogen content by a factor of 6.25.

3.4.5. Total phenolic content

To determine the total phenolic content of the agar-based fractions, the Folin-Ciocalteu method (Singleton et al., 1999) was employed. The dry samples were dissolved in water at a concentration of 5 mg/mL. A colorimetric assay was performed by mixing 125 µL of a 1:10 dilution of Folin-Ciocalteau reagent with 20 µL of the sample. Next, 100 µL of sodium carbonate (75 mg/mL) were added and the samples were heated at 40 °C for 10 min. Absorbance values were measured at a wavelength of 750 nm, and a calibration curve was constructed using gallic acid as a standard. The total phenolic content was expressed as mg of gallic acid (GA)/g extract.

3.4.6. Sulphate content

The sulphur content of the samples was determined using a Flash Smart™ Elemental Analyzer (Thermofisher, Massachusetts, USA). To this purpose, the samples underwent a complete and instantaneous oxidation through combustion with oxygen at an approximate temperature of 1020°C.

3.5. $ABTS^{\cdot+}$ radical cation scavenging activity

The agar-based extracts were evaluated for their ability to scavenge the $ABTS^{\cdot+}$ radical cation, as per the methodology described by Re et al. (Re et al., 1999). In summary, 0.192 g of ABTS was dissolved in 50 mL of PBS at pH 7.4 and combined with 0.033 g of potassium persulphate to generate the $ABTS^{\cdot+}$ radical cation, which was left to incubate in the dark overnight. Prior to the

assay, the $ABTS^{\cdot+}$ solution was diluted with PBS to obtain an initial absorbance of ~0.700 ± 0.02 at 734 nm and room temperature. The radical scavenging activity of the agar-based extracts was measured by mixing 230 µL of the diluted $ABTS^{\cdot+}$ solution with 20 µL of each sample and observing the change in absorbance after 6 minutes. The antioxidant capacity of the extracts was calculated in mg Trolox equivalents (TE)/g extract, using a calibration curve obtained with 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox).

3.6. Fourier transform infrared spectroscopy (FT-IR)

The agar-based fractions were analysed using FT-IR in attenuated total reflectance (ATR) mode, utilizing a Jasco FT/IR-4100 (Easton, USA) instrument. Spectra were recorded with a resolution of 4 cm^{-1}, covering a wavelength range from 400 to 4000 cm^{-1}, and an average of at least 32 scans was obtained for each measurement.

3.7. Small Angle X-ray Scattering (SAXS) experiments

Small Angle X-Ray Scattering (SAXS) experiments were conducted at the Non-Crystalline Diffraction beamline (BL-11) from the ALBA synchrotron light source. The different agar-based aqueous dispersions were placed into 2 mm quartz capillaries (Hilgenburg Gmbh, Germany) and left at room temperature for at least 24 h to form the gels inside the capillaries, which were then sealed for analysis. The incident photons had an energy of 12.4 keV or a wavelength of $\lambda = 1$ Å. The SAXS diffraction patterns were collected using a Pilatus 1 M photon counting detector, which had an active area of 168.7 x 179.4 mm^2, an effective pixel size of 172 x 172 μm^2, and a dynamic range of 20 bits. The sample-to-detector distance was set at 6570 mm, providing a q range with a maximum value of $q = 0.2$ $Å^{-1}$. Preliminary trials were conducted to determine an appropriate exposure time of 5 s. The pyFAI python code (ESRF) (Kieffer & Wright, 2013), modified by the ALBA beamline staff, was used for online azimuthal integrations from a previously calibrated file, with the calibration files created from a silver behenate standard. The radially averaged intensity profiles were then plotted as a function of q using the IRENA macro suite (Ilavsky & Jemian, 2009) within the Igor software package (Wavemetrics, Lake Oswego, Oregon).

The experimental data were fitted using either a correlation length model or a two-level unified model. The correlation length model contains a first term, described by a power-law function, which accounts for the scattering from large clusters in the low q region and a second term,

consisting of a Lorentzian function, which describes scattering from polymer chains in the high q region:

$$I(q) = \frac{A}{q^n} + \frac{C}{1+(q\xi_L)^m} + bkg \quad (1)$$

where n is the power-law exponent, A is the power-law coefficient, m is the Lorentzian exponent, C is the Lorentzian coefficient and ξ_L is the correlation length for the polymer chains (which gives an indication of the gel's mesh size).

The unified model considers that, for each individual level, the scattering intensity is the sum of a Guinier term and a power-law function:

$$I(q) = \sum_{i=1}^{N} G_i \exp\left(-q^2 \cdot \frac{R_{g,i}^2}{3}\right) + \frac{B_i\left[\mathrm{erf}\left(qR_{g,i}/\sqrt{6}\right)\right]^{3P_i}}{q^{P_i}} + bkg \quad (2)$$

$G_i = c_i V_i \Delta SLD_i{}^2$ is the exponential pre-factor (where V_i is the volume of the particle and ΔSLD_i is the scattering length density (SLD) contrast existing between the i^{th} structural feature and the surrounding solvent), $R_{g,i}$ is the radius of gyration describing the average size of the i^{th} level structural feature and B_i is a q-independent prefactor specific to the type of power-law scattering with power-law exponent, P_i.

The obtained values from the fitting coefficients are those that minimize the value of Chi-squared, which is defined as:

$$\chi^2 = \sum\left(\frac{y-y_i}{\sigma_i}\right)^2 \quad (3)$$

where y is a fitted value for a given point, y_i is the measured data value for the point and σ_i is an estimate of the standard deviation for y_i. The curve fitting operation is carried out iteratively and for each iteration, the fitting coefficients are refined to minimize χ^2.

3.8. X-ray Diffraction (XRD)

The X-ray diffraction (XRD) analysis was conducted using a Bruker diffractometer model D5005, which was equipped with a secondary monochromator and a Cu tube. The experimental setup employed a θ-2θ configuration, with the samples examined within the angular range of 3° to 60°. Each measurement step had a precision of 0.02°, and a count time of 200 seconds was

allocated per step. Peak fitting of the XRD data was performed utilizing the Igor software package (Wavemetrics, Lake Oswego, Oregon), following the same procedure outlined in a previous study (Martínez-Sanz et al., 2019). The obtained fitting coefficients were determined by minimizing the value of Chi-squared according to Equation (1):

$$\chi^2 = \Sigma \left(\frac{y - y_i}{\sigma_i}\right)^2 \quad (4)$$

In this equation, y represents the fitted value for a given point, y_i corresponds to the measured data value for that point, and σ_i is an estimate of the standard deviation for y_i.

The iterative curve fitting process involved refining the fitting coefficients in each iteration to minimize χ^2. From the obtained fitting results, the crystallinity index was determined using Equation (2):

$$X_C(\%) = \frac{\sum A_{Crystal}}{A_{Total}} \times 100 \quad (5)$$

Where A_{Total} represents the sum of the areas under all the diffraction peaks, and $\Sigma A_{Crystal}$ corresponds to the sum of the areas corresponding to the crystalline peaks.

3.9. Texture profile analysis (TPA)

The textural properties of agar-based hydrogels were assessed at ambient conditions using a universal test Machine (Instron, USA) equipped with a cylindrical aluminium plunger (diameter: 3.6 cm) and a 30 N load cell. Gel disk samples were compressed twice with a 50% deformation ratio, while pre-test, test, and post-test speeds were maintained at 1 mm/s. The tiger force, tiger distance, and time interval between bites were set at 5 g, 2 mm, and 50 s, respectively. Each formulation underwent at least three independent measurements. The TPA curves provided information on the mechanical parameters, including hardness (N), which indicates the maximum force required to compress the sample for the first time, and cohesiveness, which is the ratio of positive force during the second compression cycle to that of the first.

3.10. Oscillatory rheological measurements

Rheological measurements were carried out using a HR-20 rheometer from TA Instruments (Crawley, England). The cone-plate geometry employed had a diameter of 4 cm, 2° angle, and 53 µm gap. To regulate the temperature, a Peltier plate was used, and the cone was equipped with a solvent trap and an evaporation blocker from TA Instruments. Additionally, the samples were coated with a layer of paraffin oil. The hot solutions were freshly prepared and loaded onto the pre-heated rheometer plate at 90°C. Following a 3-min equilibration period, the temperature was gradually reduced from 90°C to 20°C at a constant rate of 2°C/min, then held at 20°C for 5 min, and finally increased back to 90°C. The storage (G') and loss (G") moduli were measured as a function of temperature at a strain of 0.1% and a frequency of 6.28 rad/s, within the linear viscoelastic region.

3.11. Uniaxial compression

An Instron universal testing machine (Instron, USA), equipped with a cylindrical aluminium plunger with a diameter of 3.6 cm and a 30 N load cell, was utilized to carry out uniaxial compression tests under ambient conditions. Gel disk specimens were compressed to 90% of their original height at a crosshead speed of 1 mm/s. True stress (σT) and true strain (εT) were determined from the force (N) and distance (mm) using Eqs. (1) and (2), respectively:

$$\sigma_T = \frac{F(t)(h_0 - \Delta h(t)}{\pi r^2 h_0} \tag{6}$$

$$\varepsilon_T = ln \frac{h_0}{h_0 - \Delta h(t)} \tag{7}$$

where F(t) is the force, Δh(t) is the change in the height during compression, h_0 is the initial height of the sample and r is the initial radius of the sample.

Compression moduli were calculated from the slopes of the initial linear zone of the true stress vs. true strain curves. The measurements were performed at least in triplicate.

3.12. Optical Microscopy

The agar-based hydrogels were subjected to analysis using optical microscopy. Images were captured employing a Nikon Eclipse 90i microscope equipped with a 5-megapixel Nikon Digital Sight DS-5Mc cooled digital colour microphotography camera (Nikon Corporation, Japan). Additionally, images of the materials were obtained using a fluorescent filter UV-2A (Excitation 330–380 nm, Dichroic Mirror 400, and LongPass 420 nm for emission). The acquired pictures were subsequently analysed and processed using Nis-Elements Br 3.2 Software (Nikon Corporation, Japan).

3.13 Statistics

All data were represented using the average ± standard deviation. Significant differences in both graphs and tables were denoted by different letters ($p \leq 0.05$). The analysis of variance (ANOVA) was conducted followed by a Tukey-test.

4. Results and discussion

4.1. Composition of the agar-based extracts

Agar-based fractions from three different agarophytes, namely *Gelidium corneum, Agarophyton chilensis*, and *Gracilariopsis longissima*, were obtained using two different extraction protocols. A simplified hot water extraction protocol was applied to obtain less purified agar-based fractions. Furthermore, the impact of an initial alkaline pre-treatment step to remove impurities from the raw seaweeds, similar to that used in the agar extraction industry, was evaluated. According to the results presented in Table 1, the extraction yields for HW-G, HW-L, HW-C, NaOH-G, NaOH-L, and NaOH-C were 10.9 ± 0.3%, 25.6 ± 4.1%, 26.0 ± 1.1%, 3.5 ± 0.3%, 3.9 ± 0.3%, and 6.8 ± 0.7%, respectively. This indicates that, as expected, the purification process negatively affected the extraction yields, which decreased from 3- to 7-fold. This observation is consistent with a previous study, which also involved the extraction of agar fractions from *G. corneum*, in which the application of an alkaline pre-treatment led to a 4-fold decrease in the extraction yield (Martínez-Sanz et al., 2019). It is also noteworthy that the extraction yields significantly increased for the less purified agar-based fractions obtained from the *Gracilariales* as compared to that obtained from *G. corneum.* Owing to the extensive range of reported

extraction parameters, it is difficult to make direct comparisons with previous studies. Nonetheless, high extraction yields have been reported for *Gracilaria* species, with up to 55% yields reported for agars from *Gracilaria cliftonii* produced under conditions similar to those employed in this study (Kumar & Fotedar, 2009).

The composition of the agar-based extracts is compiled in Table 1. The first clear observation is that, as expected, the alkali pre-treatment yielded extracts with higher polysaccharide contents, while other components (especially proteins and ashes) were present in greater amounts in the corresponding extracts produced by omitting the pre-treatment step. In particular, the total carbohydrate content increased from 60-64% up to 77-81% when applying the alkaline pre-treatment. Despite their lower carbohydrate content, the HW-G, HW-L and HW-C extracts had a relatively high agar contribution of more than 84% of the total carbohydrates. Surprisingly, the agarose proportion in the agar fraction did not increase with the alkaline pre-treatment. This indicates that the pre-treatment was mainly effective in removing components other than polysaccharides, but did not modify the agarose/agaropectin proportion in the agar. It is also noteworthy that all samples contained glucose, which is likely due to the presence of floridean starch, a storage polysaccharide that may be co-extracted with agar (Carmona et al., 1998; Martínez-Sanz, Ström, et al., 2020). In particular, HW-L presented the highest amount of glucose, suggesting that a greater proportion of starch was co-extracted with agar. Although the antioxidant capacity of all agar-based fractions decreased with increasing purification levels, the NaOH-L extract did not exhibit this trend. All samples presented values that were within the range reported for agar fractions extracted from *G. corneum* under similar conditions (Martínez-Sanz et al., 2021; Martínez-Sanz et al., 2019). Typically, the antioxidant capacity of seaweed-derived compounds is attributed to the presence of phenolic compounds. However, in this case, other bioactive compounds, such as sulphated polysaccharides (e.g. agaropectin) and lipids, may also contribute to the antioxidant capacity, as previously reported (Cebrián-Lloret et al., 2022).

Interestingly, a clear difference was observed between the composition of the less purified fractions obtained from *G. corneum* and those from *Gracilariales*, since HW-G had the highest protein (18%), ash (24%) and polyphenol (1%) contents, while HW-C and HW-L had higher lipid

contents (>4.5%). The protein and ash contents of HW-G were slightly different to those previously reported for agar-based fractions obtained from *G. corneum* using a similar extraction protocol (Martínez-Sanz et al., 2021; Martínez-Sanz et al., 2020), which can be due to the impact of seasonal variation on the composition of the raw seaweeds. Another interesting observation is that the agar fractions extracted from *G. corneum* consisted of more than 94% agarose; in contrast, the fractions obtained from species of the *Gracilaria* genera contained less than 82% agarose. This indicates a higher agaropectin content in the fractions extracted from *Gracilariales*, which is expected to have an impact on their gelling behaviour. Surprisingly, despite their lower agarose proportion, the amount of sulphur in the HW-L and HW-C extracts was lower than in HW-G. Although sulphate groups in agar are typically associated to agaropectin, these results suggest that the agaropectin fractions from the *Gracilaria* and *Gelidium* species present different molecular structures, having the agaropectin from *Gracilaria* seaweeds lower degree of sulphate substitution.

It should also be noted that the mass balance was close to 100% for most of the samples, except for the NaOH-G extract, which accounted for a total of ca. 88%. This discrepancy could be attributed to the considerable agarose content present in this sample, which may be less susceptible to the hydrolysis process used to determine the carbohydrate content, leading to an underestimation of the total carbohydrate content.

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Table 1. Extraction yield, macronutrient composition, antioxidant capacity and sulphur content (S) of the agar-based fractions.

	Extraction yield (%)	*Carbohydrate (%)*		Protein (%)	Lipid (%)	Ash (%)	Polyphenol (mg GAE/g sample)	Antioxidant capacity ABTS (mg TE/g sample)	S (%)
		Agar* (%)	Glucose (%)						
HW-G	10.9 ± 0.3^{c}	56 ± 10.4 [97] ^{a}	3.5 ± 1^{b}	18.2 ± 1.4^{e}	0.3 ± 0.01^{a}	23.6 ± 1.1^{d}	8.9 ± 1.8^{d}	6.7 ± 0.07^{e}	3.2 ± 0.02^{e}
HW-L	25.6 ± 4.1^{d}	54.4 ± 1.5 [71]^{a}	9.7 ± 0.6^{e}	13.7 ± 0.04^{d}	4.7 ± 0.02^{c}	8.5 ± 1.5^{a}	6.2 ± 1.0^{c}	6.0 ± 0.06^{c}	2.1 ± 0.003^{d}
HW-C	26.0 ± 1.1^{d}	59.8 ± 3.7 [82]^{a}	2.4 ± 0.03^{a}	8.8 ± 0.1^{c}	4.5 ± 0.08^{c}	16.8 ± 0.2^{c}	4.5 ± 0.4^{b}	$6.4 \pm 0{,}07^{d}$	2.1 ± 0.014^{d}
NaOH-G	3.5 ± 0.3^{a}	72.0 ± 2.9 [94]^{b}	4.7 ± 0.6^{c}	2.2 ± 0.02^{a}	-	9.6 ± 0.9^{ab}	2.6 ± 0.2^{a}	3.9 ± 0.07^{b}	0.3 ± 0.05^{b}
NaOH-L	3.9 ± 0.3^{a}	75.1 ± 8.8 [75]^{b}	3.2 ± 0.7^{ab}	2.7 ± 0.2^{a}	3.7 ± 0.2^{b}	7.7 ± 0.4^{a}	2.7 ± 0.1^{a}	3.5 ± 0.2^{a}	0.2 ± 0.007^{a}
NaOH-C	6.8 ± 0.7^{b}	73.3 ± 2.7 [77]^{b}	4.9 ± 0.1^{b}	6.6 ± 0.08^{b}	3.7 ± 0.3^{b}	9.1 ± 0.6^{ab}	3.3 ± 0.06^{ab}	7.1 ± 0.09^{f}	0.4 ± 0.007^{c}

Values with different letters are significantly different ($p \leq 0.05$).

* The value in brackets corresponds to the percentage purity in agarose of each agar fraction as determined by HPAEC-PAD after reductive hydrolysis.

The FT-IR spectra of agar-based extracts were recorded to identify their main compositional differences. As observed, all samples exhibited a pronounced band at 1038 cm^{-1}, which is common to all polysaccharides and is primarily attributed to the coupling of C-O or C-C stretching modes with C-OH bending modes (Guerrero et al., 2014). The most characteristic agar bands, such as that located at 890 cm^{-1}, attributed to the C-H of residual β-galactose carbons (Guerrero et al., 2014; Martínez-Sanz et al., 2019; Martínez-Sanz et al., 2019) as well as those at 930 and 1070 cm^{-1},associated with the 3,6-anhydro-galactose bridges (Freile-Pelegrín et al., 2007; Guerrero et al., 2014), although present in all fractions, were more evident in the purified extracts, demonstrating the effectiveness of the purification process. Several peaks associated with the vibration of sulphate groups were also observed in the samples such us the peak centred at 1149 cm^{-1}, present in all samples, or the peak at 1370 cm^{-1}, which was more pronounced in the purified fractions. Also associated with the vibration of the sulphate groups are the band centred at 1250 cm^{-1}, which disappeared almost completely in the purified fractions, or the peak centred at 1190 cm^{-1} which was present in both the purified fractions and the less purified HW-G fraction. Generally, the sulphate group-associated peaks seemed to be more prominent in the fractions obtained from *G. corneum*, consistent with the results from the compositional characterization. This is of particular interest since the presence of 6-sulphate-α-L-galactose units has been reported to decrease the gel strength of agars (Freile-Pelegrín et al., 2007; Nishinari & Fang, 2017). Finally, the higher protein content in the HW-G fraction was consistent with the greater intensity of the amide I band at 1640 cm^{-1} (Guerrero et al., 2014) when compared to the other samples.

The crystallinity of the different agar-based fractions was calculated from the XRD patterns of the samples, which are shown in Figure 1B. As observed, all the samples presented diffraction patterns characteristic of semi-crystalline materials. HW-G displayed a pattern similar to that previously reported for agars extracted from *G. corneum* (Martínez-Sanz et al., 2019; Rhim et al., 2013, 2014), where two main diffraction peaks centred at ca. 13-14 º and 19.6º, corresponding to the crystalline fraction of agarose, were combined with multiple sharp peaks, arising from the presence of mineral compounds which are found in the raw seaweed, such as silica (SiO_2) and weddellite ($CaC_2O_4 \cdot 2H_2O$) (Y. W. Chen et al., 2016; Singh et al., 2017). These

peaks arising from minerals were not detected in HW-L and HW-C, which is probably due to a lower proportion of this type of compounds in the corresponding seaweeds (Cebrián-Lloret et al., 2022). After the alkaline pre-treatment, these peaks were also absent in NaOH-G, confirming the efficiency of the process to remove impurities. The crystallinity indexes were significantly increased from 13% for HW-G, 40% for HW-L and 36% for HW-C, to 78% for NaOH-G, 82% for NaOH-L and 72% for NaOH-C. This agrees with the increase in the polysaccharide content produced by applying the pre-treatment step, which removed more amorphous components such as proteins and non-crystalline ashes. Interestingly, the higher agarose proportion in the agar-based extracts from *G. corneum* did not have a significant impact on the crystallinity, hence suggesting that the agaropectin fraction present in the *Gracilariales* might also be arranged in an ordered conformation, giving rise to the formation of crystalline domains. This might be directly related to the lower degree of sulphate substitution in the agaropectin from these seaweeds.

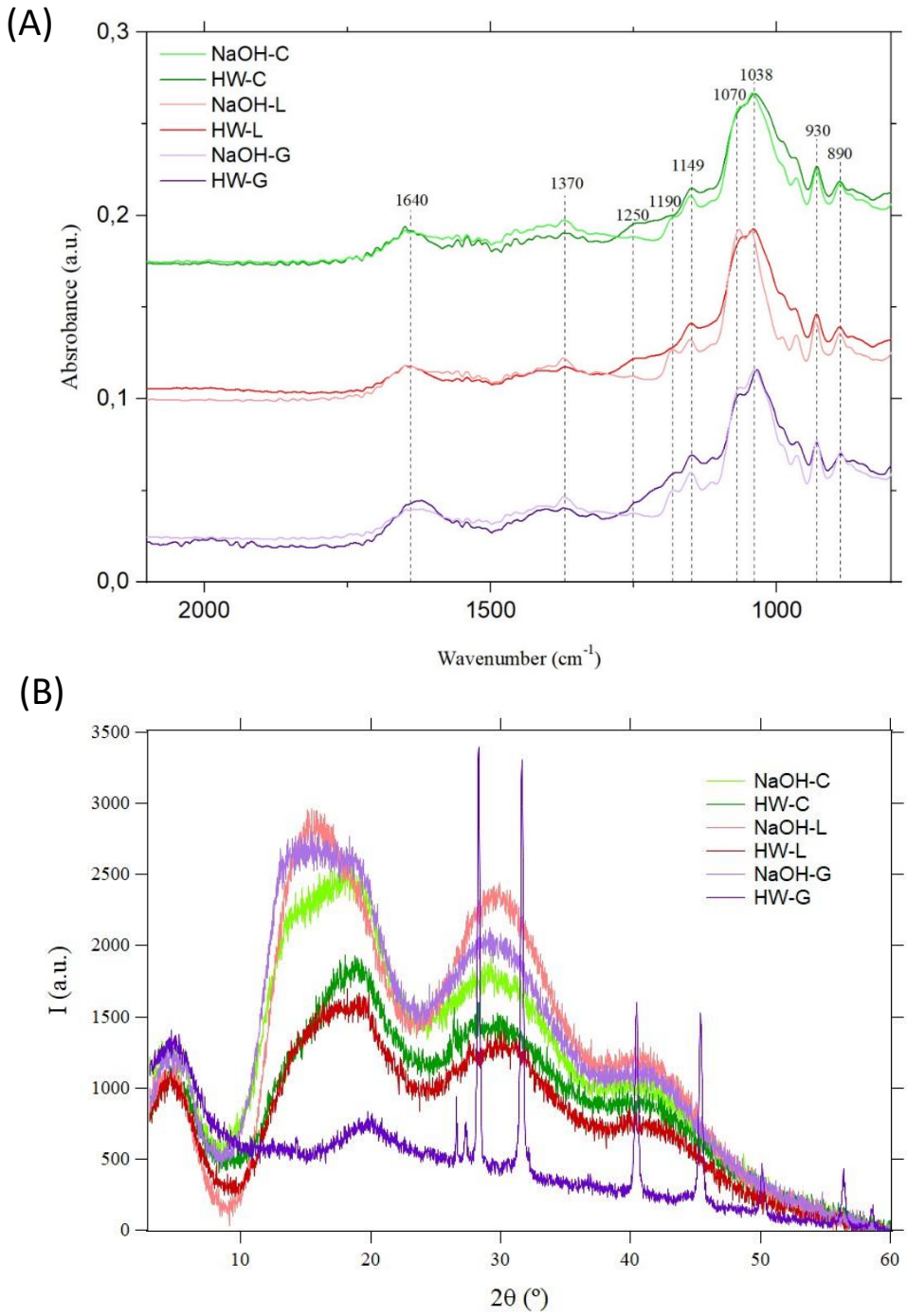

Figure 1. (A) FT-IR spectra and (B) XRD patterns from the agar-based fractions.

4.2. Structural and functional properties of the agar-based hydrogels

To assess the potential of the agar-based fractions as gelling agents in the food industry, hydrogels were generated, and their functional properties were characterized. The visual appearance of the resulting hydrogels is shown in Figure 2 (a). As expected, all the samples exhibited a brownish coloration, attributed to the diverse array of compounds present in the extracts. However, the more purified fractions, particularly NaOH-G, showed a lower degree of coloration compared to the other fractions probably due to its lower concentration of polyphenols and proteins. The hydrogels' microstructure was also investigated using optical microscopy and representative images captured in brightfield and under ultraviolet filter are presented in Figure 2 (b-g). The images obtained in brightfield revealed clear changes in the hydrogel structure, both among different seaweed species and with different degrees of purification. In hydrogels prepared from less purified agar fractions, the presence of impurities in the form of non-solubilised cellular tissues was primarily observed. However, these insoluble impurities were mostly removed in the alkali-treated fractions. For the purified agar fractions, the hydrogels clearly showed distinct microstructures depending on the seaweed species of origin. Specifically, the hydrogels from *G. corneum* were characterized by the presence of fibrillar structures, while those from *Gracilariales* species exhibited globular structures. When observing the images captured under the UV filter, it is noticeable that both the cellular structures found in hydrogels prepared from less purified fractions and the fibres and globular structures observed in hydrogels from pure fractions exhibited a bright blue fluorescence. As previously indicated (Cebrián-Lloret et al., 2022), these fluorescent regions are likely indicative of agar-rich areas. The greater proportion of agarose in the NaOH-G extract, which is known to form double helices which then aggregate into larger bundles(Dai & Matsukawa, 2013; Descallar & Matsukawa, 2020), may be responsible for the observed fibrillar structures. In contrast, the presence of semi-crystalline agaropectin in the *Gracilariales* may have given rise to the formation of globular structures.

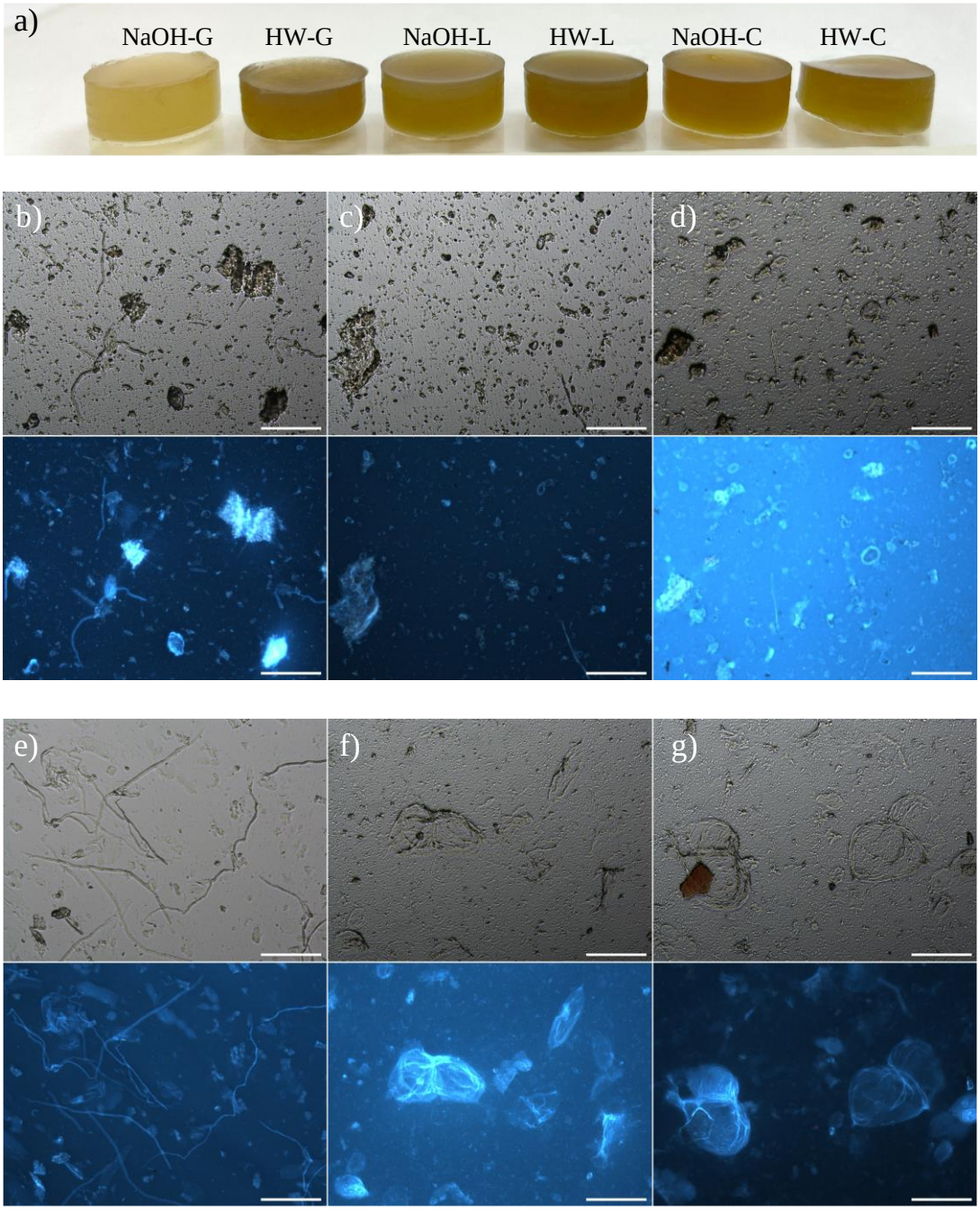

Figure 2. Visual appearance **(a)** and optical microscopy images **(b-g)** of the agar-based hydrogels from HW-G (**b**), HW-L (**c**), HW-C (**d**), NaOH-G (**e**), NaOH-L (**f**), NaOH-C (**g**). Scale bars correspond to 100 µm. Top images were taken with bright light while bottom images were taken using a fluorescent filter UV-2A.

The mechanical properties of the agar-based hydrogels were assessed by means of compression tests and the most representative parameters, i.e. Young's modulus (E) and maximum stress (σ_{max}), obtained from the stress–strain curves (cf. Fig. S1 from the Supplementary Material) are summarized in Table 2. As expected, the presence of other compounds in the less purified fractions resulted in hydrogels that showed less stiff behaviour within the linear elastic region and less resistance to fracture during compression. Notably, amongst the less purified fractions, HW-G yielded stiffer and stronger gels compared to the other two fractions. This could be attributed to the higher agarose proportion in the agar from this extract, since several studies have reported that agarose is the main fraction responsible for the formation of strong hydrogel networks (Martínez-Sanz et al., 2020; Nishinari & Fang, 2017; Zarrintaj et al., 2018). Nonetheless, the higher protein content of HW-G could also be playing an important role on its mechanical performance, as previous studies have shown that agar can interact via hydrogen bonds with proteins to form a network with enhanced physicochemical properties (Garrido et al., 2016; Gupta & Nayak, 2015). In the case of the hydrogels produced from the purified fractions, those obtained from the *Gracilariales* exhibited the highest Young's modulus and tensile strength, with the NaOH-L fraction demonstrating slightly superior stiffness of 27 kPa and a maximum strain of 4.7 kPa. These values fall within the range previously reported for commercial agar hydrogels (Martínez-Sanz et al., 2020; Ross et al., 2006; Sharma & Bhattacharya, 2014), demonstrating that *Gracilariopsis longissima* is a cost-effective alternative to traditional agar sources for obtaining high-stiffness hydrogels. The slightly superior mechanical performance of the hydrogels from *Gracilariales* might be attributed to (i) the presence of a semi-crystalline agaropectin network, capable of strengthening the agarose gelling network and/or (ii) a beneficial effect of small amounts of other components such as proteins, lipids and polyphenols, capable of interacting with agar and contributing to the formation of stronger networks.

TPA analyses were also performed on the agar-based hydrogels, and the curves provided information on relevant mechanical parameters, including hardness (N) and cohesiveness. The results obtained are compiled in Table 2. As anticipated, the more purified agar samples exhibited higher hardness, which is related to the gel strength. Specifically, the NaOH-L hydrogel

had the highest hardness (ca. 29 N), followed by NaOH-G (ca. 22 N) and NaOH-C (ca. 13 N), in accordance with the results obtained from the compression tests. In contrast, the presence of other compounds in the less purified agar samples led to gels with lower toughness, yet, in the case of the HW-L and HW-C hydrogels, with higher cohesiveness. This latter parameter is a measure of a gel's ability to withstand external damage while retaining its structural integrity, and it is thus indicative of internal binding strength (H. Chen et al., 2021; Fontes-Candia et al., 2022). This higher cohesiveness may be partly attributed to the higher lipid content in these agar-based fractions, as previous studies have reported that an increase in fat content can directly affect the increase of this textural parameter (Badar et al., 2021; Fontes-Candia et al., 2023). Other factors, such as degree of sulphation or the different composition of the extracts may also influence the cohesiveness values.

Table 2. Mechanical properties of the agar-based hydrogels.

	E (kPa)	σ_{max} (kPa)	Hardness (N)	Cohesiveness
HW-G	11.2 ± 0.9^{b}	2.0 ± 0.07^{c}	1.1 ± 0.1^{a}	2.9 ± 0.4^{a}
HW-L	5.9 ± 0.3^{a}	1.0 ± 0.02^{b}	6.4 ± 0.3^{c}	7.6 ± 2.5^{b}
HW-C	4.1 ± 1.7^{a}	0.3 ± 0.02^{a}	3.6 ± 0.2^{b}	8.8 ± 1.8^{b}
NaOH-G	22.3 ± 1.5^{c}	3.3 ± 0.3^{d}	21.8 ± 2.2^{e}	3.5 ± 0.2^{a}
NaOH-L	27.5 ± 1.3^{d}	4.7 ± 0.1^{f}	29.2 ± 1.6^{f}	3.6 ± 0.1^{a}
NaOH-C	26.7 ± 0.9^{d}	4.1 ± 0.04^{e}	13.0 ± 1.1^{d}	3.8 ± 0.4^{a}

Values within the same column with different letters are significantly different ($p \leq 0.05$).

In order to investigate the influence of the composition on the gelling mechanism of the agar-based extracts, oscillatory rheological tests were conducted. The elastic (G') and viscous (G") moduli were recorded during cooling and heating ramps, and the results are presented in Figure 3. During the cooling phase, all samples exhibited similar behaviour, showing an initial stage in which G' and G" remained relatively constant, followed by a sharp increase in both moduli as the temperature decreased below a certain value, and a final stage in which both moduli reached a plateau or continued to increase slowly. The gelation temperature of agars has been

frequently estimated from the crossover point of G' and G" during cooling ramps. However, except for samples HW-L and HW-C, for which the crossover point was detected at 42.2°C and 39.4°C respectively, none of the other samples exhibited true solution behaviour (G">G'). Instead, they showed a behaviour typical of solid-like entangled networks at the initial temperature of 75 °C, similarly to that previously observed for agar fractions obtained from *G. corneum* (Martínez-Sanz et al., 2020). Since no true crossover between G">G' was observed, in line with other previous studies (Alehosseini et al., 2018; Martínez-Sanz et al., 2020), the apparent gelation temperature was defined by determining the point at which there was an abrupt increase in G' and G". As shown in Table 3, this transition occurred in the 37-42°C range for all the less purified extracts, as well as the purified NaOH-G extract, which is consistent with the range of gelation temperatures commonly reported in the literature for agar samples (40-10 °C) (Martínez-Sanz et al., 2020; Rocha et al., 2019). This may suggest that the presence of other components, such as ashes or proteins, in the less purified extract from *G. corneum*, did not have a strong impact on the temperature at which agarose helices aggregated to form hydrogels, in line with previous studies (Martínez-Sanz et al., 2020). In contrast, the more purified extracts obtained from *Gracilariales* showed higher gelation temperatures (ca. 48°C) than those of their corresponding less purified extracts. This is consistent with previous findings showing that high gelation temperatures are common in agars obtained from *Gracilaria spp.,* particularly if they are highly methylated (Rocha et al., 2019) and might indicate that, when reducing the amount of other components, the agaropectin present in these seaweeds is capable of forming a strong gelling network along with the agarose. The elastic modulus (G') is a measure of the solid nature of a sample and is expected to be positively correlated with gel strength. In fact, the values for G' at 20°C followed a similar trend to those from the gel strength, being the highest in the more purified agars, particularly in the NaOH-L hydrogel (ca. 11 kPa), followed by NaOH-G (ca. 9 kPa) and NaOH-C (ca. 5 kPa). Furthermore, the presence of other compounds in the less purified fractions negatively affected the elastic modulus of the hydrogels, consistent with the results obtained during mechanical testing. However, several authors have also reported values similar to those obtained in this study for agars obtained from different agarophyte species (Bertasa et al., 2020; Rocha et al., 2019). In contrast, tan δ values

(tan δ = G"/G') were similar for all the samples (approximately 0.03-0.11), indicating a comparable amount of elastic response.

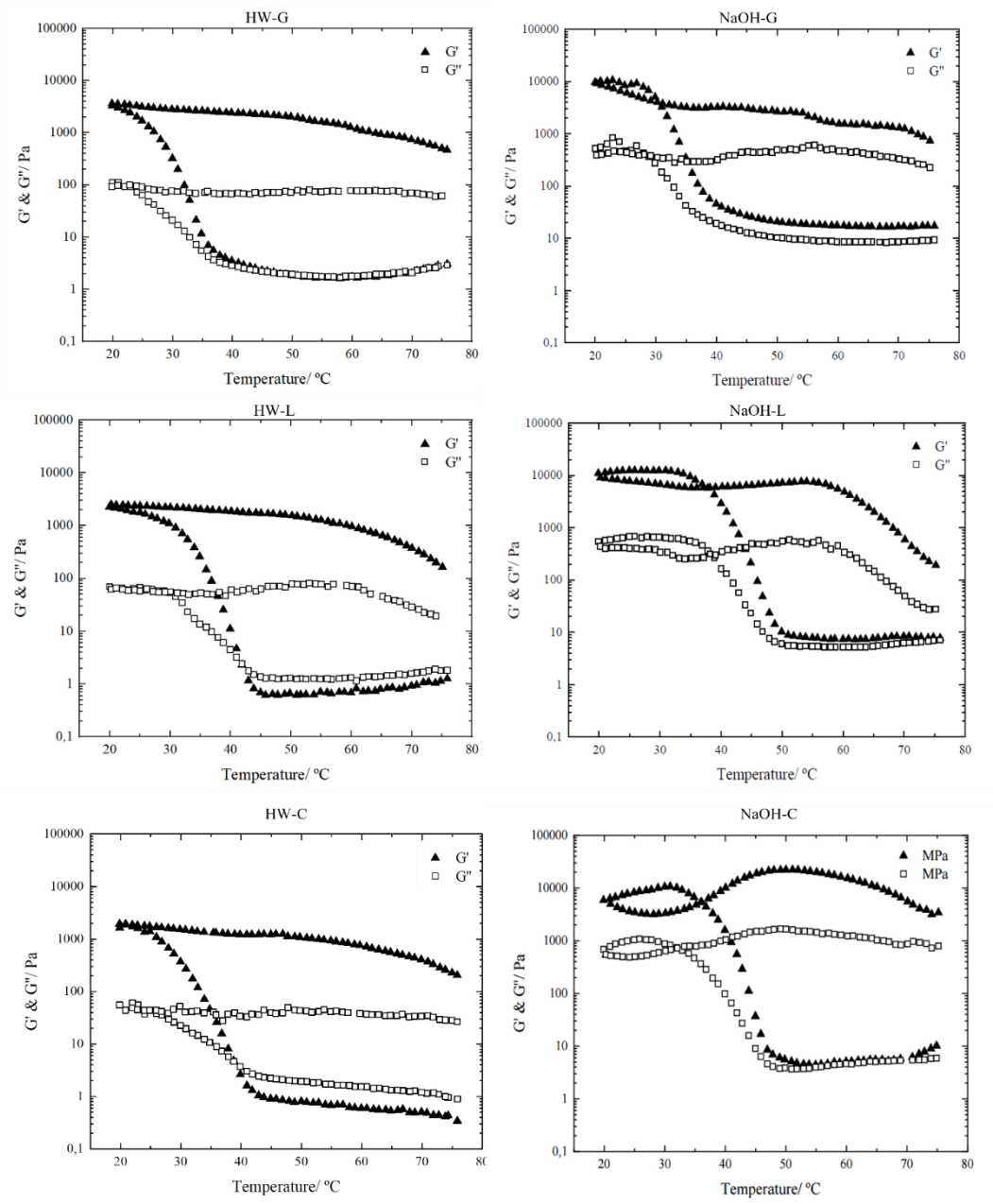

Figure 3. Temperature dependence of G' (filled triangles) and G" (open squares) moduli of agar-based extracts during cooling and heating ramps.

Table 3. Rheological and mechanical properties of the different agar-based extracts: Apparent gelation temperature (Tg'), elastic modulus ($G'_{20ºC}$), viscous modulus ($G''_{20ºC}$) and tan δ measured at 20 °C.

	Tg' (°C)	$G'_{20°C}$ (kPa)	$G''_{20°C}$ (kPa)	tan δ
HW-G	37.8 ± 0.07^{a}	3.6 ± 0.4^{b}	0.12 ± 0.02^{b}	0.03 ± 0.002^{a}
HW-L	42.2 ± 0.1^{d}	2.3 ± 0.006^{a}	0.08 ± 0.02^{ab}	0.04 ± 0.006^{a}
HW-C	39.4 ± 0.03^{c}	1.5 ± 0.1^{a}	0.05 ± 0.01^{a}	0.04 ± 0.01^{a}
NaOH-G	38.5 ± 0.1^{b}	8.6 ± 0.7^{d}	0.5 ± 0.01^{c}	0.06 ± 0.008^{a}
NaOH-L	48.4 ± 0.4^{e}	10.5 ± 0.3^{e}	0.6± 0.08^{d}	0.05 ± 0.008^{a}
NaOH-C	48.2 ± 0.07^{e}	5.1 ± 1.1^{c}	0.7 ± 0.003^{d}	0.11 ± 0.04^{b}

Values within the same column with different letters are significantly different ($p \leq 0.05$).

The nanostructure of the agar-based gels was investigated by means of SAXS and the obtained scattering patterns are shown in Figure 4. The scattering data from most of the samples could be fitted using a simple correlation length model, while in the case of the NaOH-L hydrogel, given the appearance of two obvious shoulder-like features (which are more clearly visualized in the Kratky plot, Fig. 4B), a unified model with two structural levels produced much better fit to the experimental data. For the less purified agars, the power-law exponents were close to 3, indicating the existence of clustered networks formed by the association of the agarose double helices to form the gelling networks, while the Lorentzian exponents, close to 2, were indicative of polymeric chains with a random coil conformation. The correlation length, which can be related to the size of the agarose double helices, was ca. 5 nm for HW-L and HW-C, while it was ca. 12 nm for HW-G. This is in agreement with the higher agarose content in HW-G, hence facilitating the formation of larger aggregates upon gelation, in line with previous work (Martínez-Sanz et al., 2020). The alkaline pre-treatment led to the formation of larger agarose bundles, as evidenced by the greater correlation lengths for NaOH-G (ca. 26 nm) and NaOH-C (ca. 53 nm). In the particular case of the NaOH-L hydrogel, the applied unified model suggested

the existence of two different structural levels. One of them might be related to the individual agarose double helices (ca. 4 nm) and the other one might be attributed to the formation of larger aggregates or bundles of double helices (ca. 53 nm). The existence of thicker agarose bundles or aggregates in alkali-treated agar hydrogels has been previously noted and has been related to the greater agar purity obtained after applying the pre-treatment (Martínez-Sanz et al., 2020). The fact that larger aggregates seemed to be present in the NaOH-L and NaOH-C can explain the superior mechanical and rheological properties of these hydrogels as compared with NaOH-G and might be explained by the semi-crystalline character of the less sulphated agaropectins present in the *Gracilariales* species, which could be interacting with agarose to form thicker bundles. It should also be noted that the NaOH-L and NaOH-C hydrogels presented a small sharp peak at 0.14 $Å^{-1}$, corresponding to a real distance of ca. 4.5 nm. A similar peak has been reported to appear in cellulose-based hydrogels (Martínez-Sanz et al., 2015), corresponding to the centre-to-centre distance from the tightly packed cellulose microfibrils. The fact that this peak was visible in these agar-based hydrogels indicates the existence of highly ordered structures, most likely formed by tightly packed agarose/agaropectin double helices. Once again, this provides evidence for the formation of more densely packed hydrogel networks in the case of NaOH-L and NaOH-C and, thus, confirms that these two *Gracilariales* could be used to produce agars with superior mechanical performance to that from *G. corneum,* provided that an alkaline pre-treatment is applied.

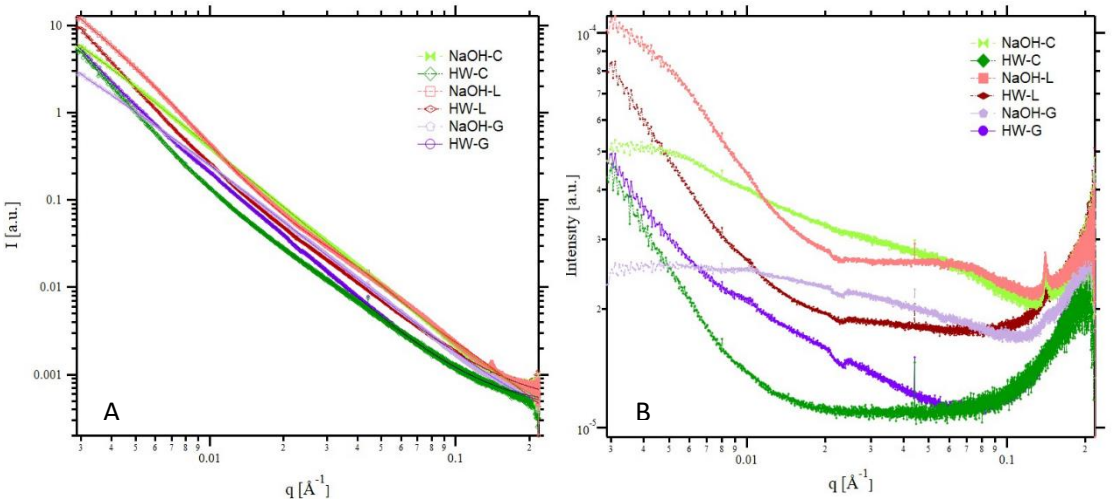

Figure 4. (A) SAXS patterns and (B) corresponding Kratky plots from the agar-based hydrogels. In (A) markers represent the experimental data and solid lines show the fits obtained using the corresponding fitting models.

5. Conclusions

The aim of this work was to compare the composition and gelling behaviour of agar fractions obtained from different red seaweed species, using both conventional and alternative extraction methods, hence assessing their appropriateness for use in the food industry. The different seaweeds used comprised two species commonly used for agar extraction (*Gelidium corneum* and *Agarophyton chilensis*) and a more inexpensive species that is not typically employed for this purpose (*Gracilariopsis longissima*). The results of this study indicate that the conventional alkaline purification process had a detrimental impact on the extraction yield, which decreased by up to four-fold, but produced extracts with a greater carbohydrate content, by removing other components such as proteins, lipids, and ashes. In turn, the agarose/agaropectin ratio in the agar fraction was not affected by the pre-treatment. Despite the presence of these compounds in the less purified agar fractions, the gelation temperature was not affected, but the hydrogels presented a less rigid behaviour and lower fracture toughness than their more purified counterparts. Surprisingly, the purified fractions from the *Gracilariales*, particularly NaOH-L, exhibited greater gel strength and toughness, and displayed a higher gelation

temperature (48°C), than the purified fraction from *G. corneum*. This was attributed mainly to the presence of agaropectin with a lower degree of sulphation in the *Gracilariales*, which presented a semi-crystalline structure and seemed to interact with the agarose fraction to form thicker bundles of double helices, hence strengthening the hydrogel network.

The more energy-efficient alternative extraction protocol yields less purified agar fractions, containing additional compounds such as proteins, polyphenols, and minerals. This might be advantageous for the production of hydrogels with bioactive properties that may be suited for particular applications in the food industry, such as texture modifiers or thickening agents. Furthermore, this study highlights the potential of *Gracilariopsis longissima* as a cost-effective alternative to traditional agar sources for obtaining high stiffness hydrogels. It should be noted that due to the lack of specific regulations for the human consumption of seaweed-based products, there are still many open questions with regards to the requirements of these type of products for food applications. However, given the emergence of seaweeds as novel food sources, the situation is expected to change over the next few years.

6. Acknowledgements

This work was financially supported by Hispanagar. Synchrotron experiments were performed at NCD beamline at ALBA Synchrotron with the collaboration of ALBA staff (proposal 2022025569). The authors also acknowledge funding from the Spanish ministry of Science and Innovation through project PID2020-117744RJ-I00 and grant CEX2021-001189-S as well as the European Commission through project CIRCALGAE (Horizon Europe) under grant agreement 101060607.

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8. Supplementary material

Table S1. Parameters obtained from the fits of the SAXS data unsing the correlation length model (*n*: power-law exponent, *m*: Lorentzian exponent, ξ: correlation length, d: real distance corresponding to the Gaussian peak) or the unified model (P_i: power-law exponent for the structural level i, Rg_i: radius of gyration for the structural level i).

	n	m	ξ (nm)	Rg_1 (nm)	P_1	Rg_2 (nm)	P_2
HW-G	3.1	2.3	11.6	---	---	---	---
HW-L	3.1	2.2	5.0	---	---	---	---
HW-C	3.1	2.2	5.3	---	---	---	---
NaOH-G	2.6	2.1	26.1	---	---	---	---
NaOH-L	---	---	---	75.3	3.1	4.3	2.1
NaOH-C	2.8	2.2	52.6	---	---	---	---

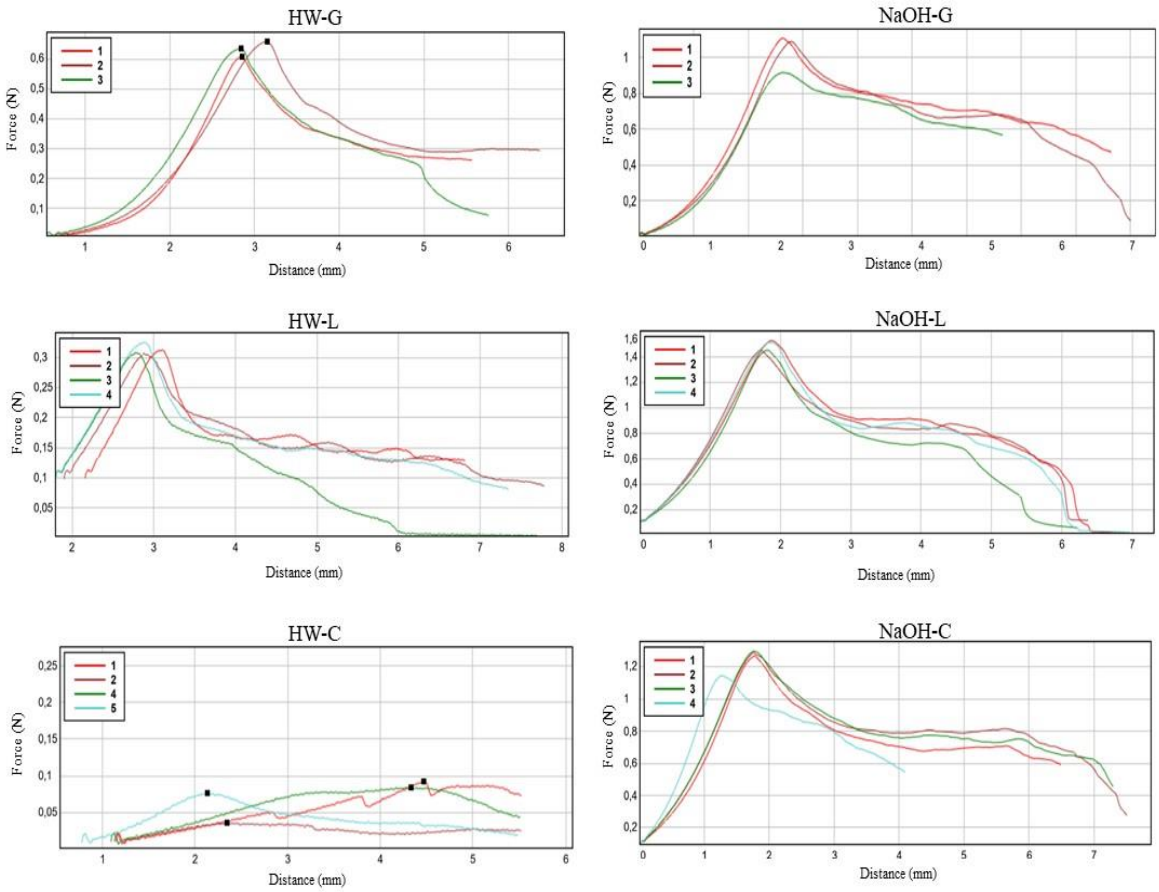
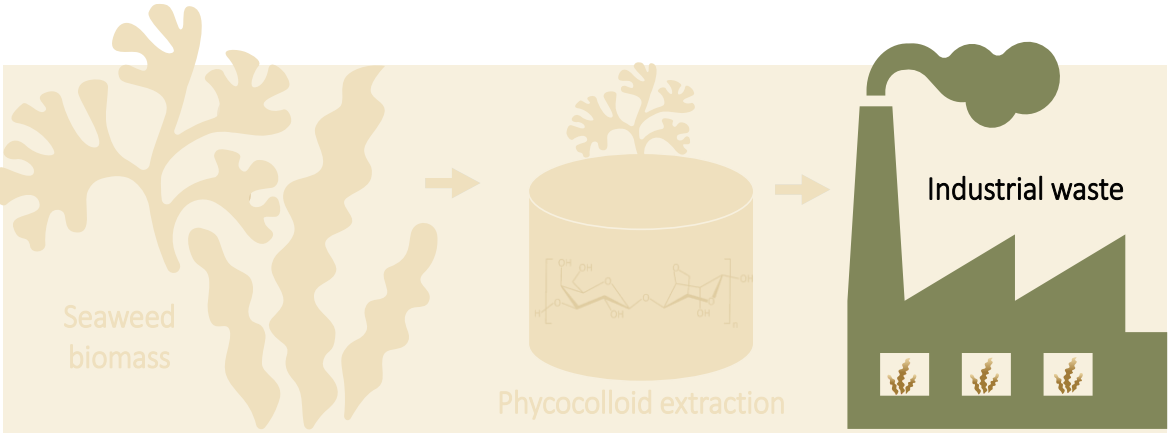


Figure S2. Stress–strain curves from the agar-based hydrogels obtained during uniaxial compression tests.

CHAPTER 3

VALORIZATION OF INDUSTRIAL RESIDUES DERIVED FROM PHYCOCOLLOID EXTRACTION

3.1. Valorization of alginate-extracted seaweed biomass for the development of cellulose-based packaging films.



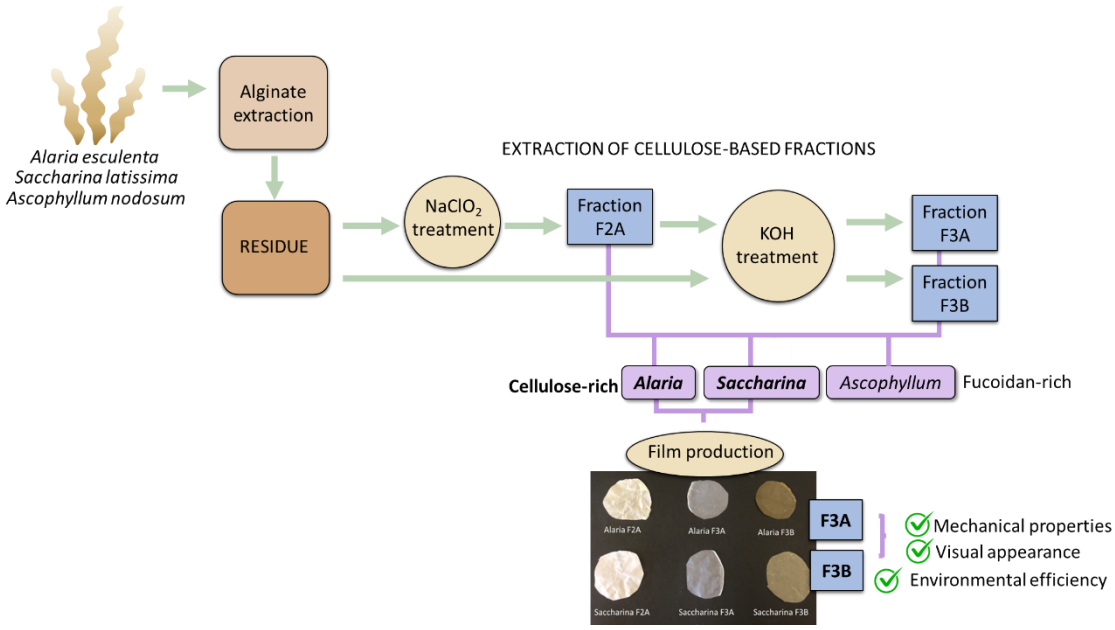
INTRODUCTION TO CHAPTER 3

As mentioned earlier, macroalgae are widely employed in the food industry due to their richness in phycocolloids. However, since the extraction yields are typically not very high, the industrial production of these phycocolloids results in a significant amount of waste biomass, which is not currently valorised. However, this biomass contains biopolymers of interest (e.g. cellulose) and bioactive compounds of high value, with potential application in various industries. Therefore, they represent an outstanding opportunity for valorization.

Hence, this study focused on the valorization of waste generated after the extraction of alginate from three different brown seaweed species. These residues were utilized for the extraction of cellulose-based fractions using more sustainable simplified procedures. Furthermore, the feasibility of using these fractions for the production of biopolymeric films intended for food packaging applications was assessed, examining how the composition of the different fractions influenced the films' performance. The results highlighted the potential of a simple alkaline extraction method to produce less purified cellulosic fractions which were suitable to generate films from seaweed waste, achieving an optimal balance between functional properties, economic efficiency and environmental sustainability.

3.3.1

VALORIZATION OF ALGINATE-EXTRACTED SEAWEED BIOMASS FOR THE DEVELOPMENT OF CELLULOSE-BASED PACKAGING FILMS.



This section is an adapted version of the following published research article:

Cebrián-Lloret, V., Metz, M., Martínez-Abad, A., Knutsen, S.H., Ballance, S., López-Rubio, A., & Martínez-Sanz, M. (2022). Valorization of alginate-extracted seaweed biomass for the development of cellulose-based packaging films. *Algal Research, 61.*

1. Abstract

Seaweed residues from *Alaria esculenta, Saccharina latissima* and *Ascophyllum nodosum* after alginate extraction have been valorized to produce cellulose-based fractions with different purification degrees. The residues were mainly composed of carbohydrates (35-57%) and proteins (12-37%), *Alaria* and *Saccharina* being richer in cellulose and *Ascophyllum* richer in fucoidan. The lower cellulose content in the latter made it unsuitable for the extraction of cellulosic fractions.

Self-supporting films were obtained from the cellulosic fractions from *Saccharina* and *Alaria* residues. While the higher cellulose purity films presented more desirable characteristics in terms of mechanical properties (with elastic moduli of ca. 5-7 GPa and elongation values of ca. 3-5%) and visual appearance, the presence of non-cellulosic components in the films from less purified fractions reduced their water sensitivity and promoted greater water barrier (with water permeability values of ca. 4-6 $Kg \cdot m/s \cdot m^2 \cdot Pa$). These results point towards the potential of a simple alkaline extraction to generate cellulose-based films from seaweed residuals with the best compromise between functional properties and economical and environmental efficiency.

2. Introduction

Since synthetic polymers began replacing natural materials more than half a century ago, the use of plastics has grown exponentially and is now an indispensable part of our lives (Shah et al., 2008). The massive use of synthetic plastics is mostly due to their easy manufacturability, good insulating properties, low cost, high mechanical strength and durability (Thompson et al., 2009; Turner, 2018). However, it is precisely their extremely long durability that has led to the persistence of plastic waste, resulting in the accumulation of more than 25 million tons of plastic waste in the environment each year (Lee & Liew, 2019). In this regard, the replacement of conventional plastics with biodegradable polymers made from renewable natural resources, i.e. biopolymers, are being considered lately as a more sustainable alternatives. However, the properties of biopolymers are still not comparable to those of reference synthetic polymers, especially in terms of barrier and mechanical properties, and their production costs are too high to compete in the market. Moreover, raw materials generally used for the production of biopolymers come from land-based crops and therefore compete with their traditional use: the

food and feed industries. In this context, aquatic biomass sources, such as seaweeds and aquatic plants, rich in cellulose, are being explored as an efficient alternative to land-based biomass for the production of biopolymers. Of special interest may be the use of seaweed industrial waste streams, such as those generated during alginate production. Alginate is an anionic polysaccharide found in brown seaweeds (*Phaeophyta*), widely used in the food industry, mainly as stabilizing, emulsifying, gelling, and thickening agent (Draget & Taylor, 2011; Fuenzalida et al., 2016). The alginate-producing industry typically extracts this phycocolloid from brown seaweeds by applying the following main steps: dilute formaldehyde treatment, dilute acid treatment, alkaline extraction, solid-liquid separation, precipitation and drying. During alkaline extraction the acidified seaweeds are immersed in a sodium carbonate or sodium hydroxide solution to convert the insoluble alginic acid into soluble sodium alginate (Abraham et al., 2018; Vauchel et al., 2008). Depending on the seaweed species, the season and extraction parameters used, the extraction yields of alginate have been reported to range from 17% to 40% (Chee et al., 2011; Davis et al., 2004; Gomez et al., 2009). Therefore, large amounts of seaweed residues are generated at industrial scale after its extraction, which are generally discarded as waste. However, part of the components present in the native seaweeds, which include cellulose, laminarin, fucoidans and to some extent alginate depending on extraction efficiency and starting materials, may remain in the residue (Bertagnolli et al., 2014; Cardoso et al., 2016) depending on the procedure. In particular, cellulose is expected to remain unaffected by the alginate extraction treatments; thus, its exploitation for the production of cellulosic materials would be an opportunity to add value to this industrial waste stream.

Cellulose presents a high potential for the development of bio-based food packaging and it has been widely used as a filler to improve the properties of other biopolymers (Ramamoorthy et al., 2015; Trache et al., 2016) due to its high resistance and rigidity, among other aspects (Khalil et al., 2017; Wahlström et al., 2020). Although it is traditionally obtained from terrestrial biomass, the extraction of cellulose from marine biomass is of particular interest due to the abundance, great compositional diversity and interesting functional properties of marine resources. In fact, several studies have already reported on the possibility of developing bio-based food packaging materials based on cellulosic fractions extracted from marine biomass

(Benito-González et al., 2019; Fontes-Candia et al., 2019; Hasan et al., 2019). Although most of the available studies focus on the production of pure cellulose (Siddhanta et al., 2009; Singh et al., 2017), a recent study has reported on the application of simplified extraction protocols yielding less purified cellulosic fractions with better mechanical and barrier performance (Benito-González et al., 2019). Additionally, the presence of bioactive components may confer these cellulosic fractions interesting functionalities, such as antioxidant capacity (Martínez-Sanz et al., 2020). Thus, the application of simplified extraction protocols for the production of cellulose-based fractions can be beneficial not only from an environmental and economic perspective, but also in terms of material properties.

Therefore, the aim of this work was the valorization of the waste generated after a typical extraction of alginate from three different brown seaweed species (*Alaria esculenta*, *Saccharina latissima* and *Ascophyllum nodosum*) for the extraction of less purified cellulose-based fractions by means of simplified extraction protocols. Furthermore, the suitability of these fractions to produce biopolymeric films for food packaging applications is evaluated, investigating the effect of the distinct fractions' composition on the performance of the films.

3. Materials and methods

3.1. Materials

3.1.1. Raw materials

The seaweeds *Alaria esculenta* and *Saccharina latissima* (referred to as *Alaria* and *Saccharina*) were cultivated at the site of Seaweed Energy Solutions AS, Frøya, Norway harvested, rinsed in seawater and dried in May 2017. *Ascophyllum nodosum* (referred to as *Ascophyllum*) was collected in Kerry, Ireland in November 2018. The dry seaweeds and residues were ground into powder and stored at 0%RH cabinets until further use. The materials represent typical samples of common dried brown seaweeds available in Europe. All chemical reagents were obtained from Sigma-Aldrich (Spain).

3.1.2. Alginate extraction and recovery of residuals

All steps regarding the alginate extraction aiming to generate non-extractable seaweed residuals were performed in a 1L centrifugation bottle at ambient temperature. Sodium alginate was extracted from the raw seaweeds according to the following procedure 20 g dried seaweed

(previously powdered in a Retch hammer mill (< 0,5 mm)) was suspended in 500 mL 0.2M HCl and subjected to gentle shaking for 12 h in an ion exchange procedure to replace all carboxylic counter ions with protons. After one centrifugation cycle (15 min at 3500 rpm), the supernatant was discharged and the residual was again homogeneously suspended in water, shaken for 1 h and subjected to a new centrifugation cycle. The supernatant was discharged and the residual, now containing alginic acid, was re-suspended in 900 mL 0,1M $NaHCO_3$ to facilitate solubilization of sodium alginate. pH was adjusted to 8 with NaOH (pH paper) and the mixture was vigorously shaken for 2 h. After a new centrifugation step (45 min at 4000 rpm), the now viscous supernatant was removed and subjected to alginate recovery (see below), otherwise this extract was discharged. The residual fraction was again suspended in water at pH > 7 and subjected to shaking for another 2 h. The centrifugation step was then repeated, the viscous supernatant was discharged/recovered and the insoluble residual was re-extracted. In short, this extraction procedure in water (pH >7,5) was repeated 3 times, producing 4 supernatants containing sodium alginate and one non-extractable residual. These, being the target product of the current experiments, were frozen and freeze-dried. This extract production was done 8 times for each seaweed. When alginate was to be recovered, NaCl(s) was suspended in a small volume of water and added to the 4 individual extracts assuring 0.2% (w/v), and equal volumes of isopropanol was added by slowly stirring with a glass rod, leaving the mixture to precipitate. The fibrous alginate formed was recovered by a weak centrifugation cycle (2000 rpm 10 minutes). Then, the fibrous alginate precipitate was re-suspended in 250 mL 50% isopropanol by homogenisation to a completely homogeneous paste and subjected to centrifugation. Washing and recovery was repeated with another portion of 50% isopropanol and finally pure isopropanol. The homogenized light-yellow alginate was drained on a filter paper to remove excess isopropanol and subjected to drying at 65°C overnight. The recovery and washings of alginate from each extraction procedure required the consumption of 4 L of isopropanol for each series and were therefore not executed for all 24 extractions.

3.1.3. Preparation of cellulosic fractions

Three different extraction protocols were carried out to obtain cellulosic fractions with different levels of purification, using the residues generated after extraction of alginate from *Alaria*,

Saccharina and *Ascophyllum* seaweeds. For the production of the fractions coded as F2A, a treatment which is aimed to remove lignin, pigments and some non-cellulosic carbohydrates (Benito-González et al., 2018) was applied. Briefly, 8 g of dry residue were added under stirring to 700 mL of 1.4% $NaClO_2$ solution, having the pH adjusted to 3 with acetic acid. The extraction took place at 70°C for 5 h and after that, the excess liquid was decanted. The solid fraction was collected and repeatedly washed with distilled water until neutral pH was reached. To obtain cellulosic fractions with a greater degree of purity, coded as F3A, a subsequent alkaline treatment step was applied to remove non-cellulosic carbohydrates. In that case, 8g of sample (dry basis) were added to 400 mL of 5% KOH solution and the material was stirred at room temperature for 24 h. Afterwards, the mixture was heated up to 90°C for 2 h. The resulting solid fraction was separated by filtration and thoroughly washed with distilled water until reaching neutral pH. As an alternative to the two-step purification protocol, the fractions coded as F3B were produced by subjecting the seaweed residues directly to the alkaline treatment. All the obtained fractions, in the form of a partially hydrated material, were stored in the fridge until further use. A small amount of each fraction was subjected to freeze-drying for further analyses.

3.1.4. Production of cellulosic films

Cellulosic films were prepared by dispersing 0.25 g of the extracted fractions (dry weight) in 50 mL of distilled water. Homogenization was achieved through stirring with an ultra-turrax for approximately 1 min. The dispersions were then vacuum filtered using PTFE filters with 0.2 µm pore size to remove water. The solid material remaining in the filter was then dried at room temperature overnight. The formed films were peeled off the filters and stored in a desiccator at 0%RH. The thickness of the films, which was measured using a Palmer electronic digital micrometer, was within the range of 25-60 µm.

3.2. Compositional analysis

3.2.1. Carbohydrate analysis

The carbohydrate composition and amount in all samples were estimated after acid methanolysis, a technique which although not being able to cleave crystalline polysaccharides, has been reported as a good compromise between the lability of guluronic acid and scission of most glycosidic linkages (Diepenmaat-Wolters et al., 1997) . As cellulose is present in the tested

brown algae, a two-step sulphuric acid hydrolysis was also performed (Saeman, 1945) as to determine the total glucose and the difference was ascribed to the crystalline cellulose content (Willför et al., 2009). The samples were then analysed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a ICS-3000 system (Dionex) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex)at 30 °C and a flow rate of 1 mL min^{-1}. Neutral sugars were eluted in water for 16 min with post-column addition of 0.5 mL min^{-1} of 300mM sodium hydroxide after a preconditioning isocratic step with 260mM sodium hydroxide and 68mM sodium acetate (7 min) and 5 min equilibration time in water prior to injection. Uronic acids were eluted in a gradient of 100 to 200mM sodium acetate in 10mM sodium hydroxide over 20 min. Fucose, glucose, galactose, arabinose, xylose, mannose, mannitol (Merck), guluronic acid, mannuronic acid (Carbosynth, UK) and glucuronic acid were used for calibration and commercial microcrystalline cellulose and sodium alginate were used as positive controls. All experiments were carried out in triplicate.

3.2.2. Protein content

Samples were analysed for total nitrogen content using an Elemental Analyser Rapid N Exceed (Paralab S.L., Spain). About 100 mg of each of the powdered samples were pressed to form a pellet which was then analysed using the Dumas method, which is based on the combustion of the sample and subsequent detection of the released N_2 (Wiles et al., 1998). The total protein content was estimated from the nitrogen content multiplied by a factor of 6.25. This multiplication factor was verified by estimation of protein from total amino acid content (not including tryptophan or cysteine) following hydrolysis of the samples in 6 M HCl, 110 °C, 24 h, and subsequent analysis via HPLC quantification (Cohen & Michaud, 1993).

3.2.3. Ash content

The ash content (measure of mineral content) was determined by dry biomass calcination, according to the standard TAPPI T211 om-07 method. Approximately 0.25 g of dried material were added to a pre-weighed crucible and weighed. Combustion took place at 550°C for 24 h in a muffle furnace. The ash content was gravimetrically quantified after combustion.

2.2.4. Lipid content

The lipid content was estimated using a Soxhlet extractor according to AOAC method 933.06 with slight modifications. Approximately 4g of dry sample was extracted using a Soxhlet apparatus with 200 mL of hexane over 6 h. The lipid content was then gravimetrically quantified (AOAC, 1990).

3.2.5. Lignin analysis

The Klason lignin content in the seaweed residues was determined according to the TAPPI T222 om-06 method, with slight modifications according to (Paz et al., 2018; Sameni et al., 2016). Approximately 300 mg of dry sample were weighed into pressure tubes and 3mL of 72% H_2SO_4 were added. The sample was mixed thoroughly and transferred to a water bath at 30°C for 1 h. The tube was stirred every 10 min and afterwards neutralized with 84 mL water. The mixed tubes were placed in an autoclave for 1h at 121°C and then cooled down to room temperature. The resulting material was filtered, washed and dried overnight. The lignin content was determined gravimetrically.

3.2.6. Total phenolic content

The total phenolic content of the dried seaweeds and the residues was determined by the Folin-Ciocalteu method (Singleton et al., 1999). This colorimetric assay was carried out by dissolving the dry samples in water (for the residues) and ethanol (for the raw seaweed) at a concentration of 5 mg/mL. The Folin-Ciocalteau reagent was diluted 1:10 with distilled water and 125 μL of the final dilution was mixed with 20 μL of sample. Finally, 100 μL of sodium carbonate (75 mg/mL) were added and the samples were heated up to 40ºC during 10 min. Absorbance values were read at 750 nm wavelength. The calibration curve was built using gallic acid as a standard. The total phenolic content was expressed as mg of gallic acid (GA)/g extract. Determinations were carried out in triplicate.

3.3. $ABTS^{\cdot+}$ radical cation scavenging activity

The $ABTS^{\cdot+}$ radical cation scavenging activity of the seaweeds and the residues was determined according to (Re et al., 1999). Briefly, 0.192 g of ABTS were dissolved in 50 mL of PBS at pH 7.4 and mixed with 0.033 g of potassium persulfate overnight in the dark to yield the $ABTS^{\cdot+}$ radical

cation. Prior to use in the assay, the $ABTS^{\cdot+}$ was diluted with PBS for an initial absorbance of ~0.700 ± 0.02 (1:50 ratio) at 734 nm, at room temperature. Free radical scavenging activity was assessed by mixing 1.0 mL diluted $ABTS^{\cdot+}$ with 10 µL of sample (5 mg/mL of seaweeds in ethanol or 5 mg/mL of residues in water) and monitoring the change in absorbance at 6 min. A calibration curve was developed by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox).

The $ABTS^{\cdot+}$ radical scavenging activity of the samples was expressed as a percentage of inhibition as follows:

$$\%\ Inhibition = \frac{(Acontrol - Asample)}{Acontrol} x\ 100 \quad (1)$$

Where the Acotrol is the absorbance of the control ($ABTS^{\cdot+}$ without sample), the Asample is the absorbance of the test sample (the sample test and $ABTS^{\cdot+}$ solution)

On the basis of a trolox calibration curve, results were then expressed as mg Trolox equivalents (TE)/g extract. All determinations were carried out in triplicate.

3.4. Fourier Transform Infrared Spectroscopy (FT-IR)

The dry seaweeds, residues and the extracted cellulosic fractions were analyzed by FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm^{-1} resolutions in a wavelength range between 400-4000 cm^{-1} and averaging a minimum of 32 scans.

3.5. Thermogravimetric analyses (TGA)

Thermogravimetric curves (TG) were recorded with a Setaram TG/DTA92 (SETARAM Instrumentation, France). The samples (ca. 10 mg) were heated from 30 to 800ºC with a heating rate of 10ºC/min under argon atmosphere. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

3.6. Scanning electron microscopy (SEM)

SEM was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8-16 mm. Small pieces of the cellulosic films were sputtered with a gold–palladium mixture under vacuum before their morphology was examined.

3.7. Optical Microscopy

Dispersions of the native seaweeds (5 mg/mL in ethanol) and the residues (5 mg/mL in water), as well as the extracted cellulosic fractions (in their partially hydrated form) were analysed by optical microscopy. Digital images were taken using an Eclipse 90i Nikon microscope (Nikon corporation, Japan) equipped with 5-megapixels cooled digital colour microphotography camera Nikon Digital Sight DS-5Mc. A fluorescent filter UV-2A (Excitation 330-380 nm, Dichroic Mirror 400, LongPass 420 nm for emission) was additionally used to acquire images from the samples. Acquired images were analysed and processed by using Nis-Elements Br 3.2 Software (Nikon corporation, Japan).

3.8. X-ray diffraction (XRD)

XRD measurements were carried out on a D5005 Bruker diffractometer. The instrument was equipped with a Cu tube and a secondary monochromator. The configuration of the equipment was θ–2θ, and the samples were examined over the angular range of 3°–60° with a step size of 0.02° and a count time of 200 s per step. Peak fitting was carried out using the Igor software package (Wavemetrics, Lake Oswego, Oregon) as described in a previous work (Martínez-Sanz et al., 2015). The crystallinity index was determined by the method reported by Wang et al. (Wang et al., 2007)

$$X_C(\%) = \frac{\sum A_{Crystal}}{A_{Total}} \times 100 \quad (2)$$

Where A_{Total} is the sum of the areas under all the diffraction peaks and $\Sigma A_{Crystal}$ is the sum of the areas corresponding to the three crystalline peaks from cellulose I.

3.9. Water vapor permeability (WVP)

Direct permeability to water was determined from the slope of the weight gain versus time curves at 24ºC. The films were sandwiched between the aluminum top (open O-ring) and bottom (deposit for the silica) parts of a specifically designed permeability cell with screws. A Viton rubber O-ring was placed between the film and bottom part of the cell to enhance sealability. These permeability cells containing silica were then placed in an equilibrated relative

humidity cabinet at 75% RH and 25°C. The weight gain through a film area of 10 cm^2 was monitored and plotted as a function of time. Cells with aluminum films (with thickness of ca. 11 µm) were used as control samples to estimate weight gain through the sealing. The WVP was calculated according to the following equation:

$$WVP\ (\mathrm{kg\ m/Pa\ s\ m2}) = \frac{\mathrm{WVTR\ x\ L}}{\Delta \mathrm{P}} \quad (3)$$

Where WVTR is the water vapor transmission rate (kg/s·m2) (calculated from the slope of the linear region of the weight gain vs. time, divided by the exposed film area), L is the mean film thickness (m), and ΔP is the difference of vapor pressure between the two sides of the film (Pa). The tests were done at least in triplicate.

3.10. Water uptake

The water swelling capacity of the cellulosic films was evaluated by soaking samples in sealed containers with 15 mL of distilled water at 25ºC. Square specimens with a total surface area of 6.25 cm^2 were cut from the films and their initial weight as well as the weight gain after equilibration (24 h) were registered using a Precisa Gravimetrics AG SERIES 320XB analytical balance (Dietikon, Switzerland). Water uptake was calculated according to the following equation:

$$Water\ uptake = \frac{\mathrm{m2-m1}}{\mathrm{m2}}\ x \quad (4)$$

Where, m1 is the weight of the film, and m2 is the equilibrium weight of the film after immersion in water. The assays were carried out at least in triplicate.

3.11. Mechanical properties

Tensile tests were carried out at ambient conditions of typically 24ºC and 50%RH on a Mecmesin MultiTest 1-i (1 kN) machine (Virginia, USA) with the Emperor™ software. Pre-conditioned rectangular-shaped specimens with initial gauge length of 8 cm and 1 cm in width were cut directly from the films. A fixed crosshead rate of 25 mm/min was utilized in all cases. The elastic modulus (E), tensile strength (TS), and elongation at break (ε_B) were determined from the stress-strain curves, estimated from force–distance data obtained for the different films. At least, two specimens of each film were tensile tested as to obtain statistically meaningful results.

3.12. UV and visible transmittance

Spectral transmittance of film samples was recorded on n 8453 Agilent UV–Vis spectrophotometer. A suitable size of rectangle film sample was directly inserted in a quartz cuvette and scanned in the UV-visible range 200–700 nm with an empty cuvette as a reference. UV and visible transmittance factor T_F (transmittance of a film sample per unit thickness) is defined as the following expression:

$$T_F = \frac{T_{300}}{x}, \frac{T_{350}}{x}, \frac{T_{450}}{x} \quad (5)$$

Where T_{300}, T_{350} or T_{450} is the value of transmittance at 300 nm (UVB), 350 nm (UVA) or 450 nm (visible), and x is the film thickness (in µm).

3.13. Statistics

All data have been represented as the average ± standard deviation. Different letters show significant differences both in tables and graphs ($p \leq 0.05$). Analysis of variance (ANOVA) followed by a Tukey-test were used.

4. Results and discussion

4.1. Chemical composition of the raw seaweeds and the alginate-extracted residues

Compositional and structural analyses were firstly carried out on the residues generated after alginate extraction to evaluate their potential for the production of cellulose-based fractions, since, to the best of our knowledge, no previous works have reported on this. Moreover, to get a better understanding on the compositional and structural differences, the native dried seaweeds were also characterized. Table 1 summarizes the results from the compositional analysis.

Table 1. (A) Macronutrient composition and (B) antioxidant capacity of the native dry seaweeds and the residues generated after alginate extraction.

A

	Alaria		*Saccharina*		*Ascophyllum*	
	Seaweed	Residue	Seaweed	Residue	Seaweed	Residue
Carbohydrate (%)*	46.2 ± 13.7 ^{a}	35.5 ± 2.2 ^{a}	34.1 ± 2.8 ^{a}	48.7 ± 7.4 ^{a}	55.2 ± 4.9 ^{a}	57.2 ± 6.4 ^{a}
Protein (%)	10.2 ± 0.2 ^{ab}	29.9 ± 2.9 ^{c}	13.5 ± 0.1 ^{b}	37.5 ± 0.8 ^{d}	7.1 ± 0.3 ^{a}	12.1 ±1.5 ^{ab}
Minerals (%)	37.6 ± 1.0 ^{d}	2.9 ± 1.5 ^{a}	47.6 ± 0.6 ^{e}	4.2 ± 1.6 ^{a}	19.8 ± 1.0 ^{c}	9.87 ± 0.8 ^{b}
Lipid (%)	1.94 ± 0.5 ^{a}	6.5 ± 3.4 ^{ab}	2.2 ±0.1 ^{a}	7.5 ± 3.6 ^{b}	4.6 ± 1.5 ^{ab}	4.4 ± 0.04 ^{ab}
Sum (%)	96	75	97	98	87	84

B

	Alaria		*Saccharina*		*Ascophyllum*	
	Seaweed	Residue	Seaweed	Residue	Seaweed	Residue
Polyphenols (mg GAE/g sample)	21.7 ± 1.9 ^{b}	39.4 ± 2.2 ^{c}	5.6 ± 1.9 ^{a}	19.5 ± 3.6 ^{b}	31.9 ± 4.6 ^{c}	51.6 ±1.0 ^{d}
Antioxidant capacity ABTS (mmol TE/g sample)	248.8 ± 8.7 ^{c}	309.4 ± 3.1 ^{d}	40.6 ± 3.0 ^{a}	72.0 ± 4.5 ^{b}	328.5 ± 4.9 ^{e}	377.5 ± 3.0 ^{f}

Data shown as mean +/-SD, n=3. Values within the same row with different letters are significantly different ($p \leq 0.05$).

*Total carbohydrate content calculated as the sum of all monosaccharide units analyzed by HPAEC.

When comparing the three dried seaweed preparations, it was clearly seen that they were mainly composed of carbohydrates (representing ca. 34-55% of the total dry mass), as recently reported for brown seaweeds (Sharma et al., 2018), but they also contained significant amounts of proteins (ca. 7-14%) and minerals (ca. 19-47%). The macronutrient composition of *Alaria* and *Saccharina* (both belonging to the *Laminariales* order) was very similar, while *Ascophyllum* (belonging to the *Fucales* order) slightly differed, presenting the greatest amount of carbohydrates and the lowest protein content. When considering the seasonal variation of constituents, the estimated carbohydrate contents are within the estimated range, consistent with previous studies (Blanco-Pascual et al., 2014; Rioux et al., 2007; Schiener et al., 2015; Sharma et al., 2018). The protein content for brown seaweeds has been reported to be generally low (3-15%) compared to red and green seaweeds (10-47%) (Kumar & Sahoo, 2017). Thus, the

determined values are in good agreement with the literature. As seen in Table 1, *Saccharina* contained slightly more protein than the other two seaweeds. The protein content is not only determined by the seaweed species, but it is also seasonal dependent, being the highest during the winter season (with values around 10% for brown seaweeds) and the lowest during the summer season (around 6%) (Fleurence et al., 2018; Vilg et al., 2015). This is mirroring the huge seasonal variation in the storage polysaccharide laminaran with a peak in early autumn and accumulation of minerals in the spring (Manns et al., 2014; Schiener et al., 2015). The values reported in the literature for the mineral content in brown seaweeds are highly variable due to seasonal variations, but minerals can make up to 50% of the dry weight (Monteiro et al., 2021). For instance, mineral contents ranging between 14-46% for *Saccharina latissima* (Manns et al., 2017; Monteiro et al., 2021), 25-32% for *Alaria esculenta* (Schiener et al., 2015) and up to 26% for *Ascophyllum nodosum* (Black, 1948) have been reported. The mineral contents determined in this study were reasonable given the large variability in the literature values. It should be considered that since this work focuses on the valorization of the residuals, the seaweeds were processed as received, i.e. no further washing in freshwater was carried out; thus, any residual sea salt remaining in the samples would have been concentrated in the seaweed biomass during drying and would have significantly contributed to the mineral content, although these residual salts were expected to be removed in the liquid effluents generated during the alginate extraction process. The low lipid content in the native seaweeds (2-5%), is in agreement with values previously reported for *Alaria, Saccharina* and *Ascophyllum*, ranging from 1% to 4% (Foseid et al., 2020; Lorenzo et al., 2017; Marinho et al., 2013).

As deduced from the results in Table 1, after alginate extraction the protein content increased significantly in the three residues, while the mineral content was reduced, as expected. Furthermore, the amount of lipids increased in *Alaria* and *Saccharina*. On the other hand, the total amount of carbohydrates was not significantly affected. These results suggest that most proteins and lipids were unaffected by the alginate extraction process, while a significant proportion of minerals were washed off or degraded during the different steps applied for alginate extraction. The alginate extraction yields were estimated to be around 10% for *Alaria*, 15% for *Saccharina* and 27% for *Ascophyllum*. On the other hand, the amount of residue

generated after the extraction of alginate was ca. 35% for *Alaria*, 31% for *Saccharina* and 45% for *Ascophyllum*, which means that approximately 50% of the water soluble low molecular weight constituents were removed within the liquid effluents generated during the extraction process from *Alaria* and *Saccharina*, while ca. 30% from *Ascophyllum* was lost upon extraction. As deduced from the results summarized in Table 1, the mass balance of macronutrient content was greater than 84% for most of the samples, which is within an acceptable margin of experimental error, thus confirming the reliability of the obtained values. The lower mass balance obtained for the residue from *Alaria* is most likely due to an underestimation of the amount of carbohydrates and indicates that the hydrolysis protocol applied for the monosaccharide analyses should be optimized for this type of samples in the future.

It should be noted that, as expected, due to the inherent low lignin content in brown seaweeds (Schiener et al., 2015), very minor amounts were detected in the residues (ca. 1-2%). This confirms that the first step in the typical cellulose extraction protocol, which consists of a $NaClO_2$ treatment and aims to remove mainly lignin, pigments and polyphenols, may be skipped in this case.

Brown seaweeds are generally rich in phenolic compounds, which have been reported to have antioxidant properties (Gupta & Abu-Ghannam, 2011). While some data about the phenolic content of brown seaweeds are available in the literature (around 3% for *Alaria* (Schiener et al., 2015; Stévant et al., 2017), 1% for *Saccharina* (Schiener et al., 2015; Sharma et al., 2018; Stévant et al., 2017; Vilg et al., 2015) and 2.5% for *Ascophyllum* (Tabassum et al., 2016)), it is hard to obtain reliable and consistent values for the antioxidant capacity. The polyphenol content and the antioxidant capacity of the three seaweeds and corresponding residues were determined using a simplified crude colorimetric approach and the results are shown in Table 1. Regarding the polyphenol content, *Ascophyllum* seaweed showed the highest one (ca. 32 mg GAE/g sample), while *Saccharina* showed the lowest content (ca. 6 mg GAE/g sample). For the three seaweeds, the amount of polyphenols increased in the residues after alginate extraction, reaching values of around 44 mg GAE/g sample for *Alaria* residue, 20 mg GAE/g sample for *Saccharina* residue and 52 mg GAE/g sample for *Ascophyllum* residue. Even though the alginate extraction treatment produced the breakage of the seaweed cell wall, the polyphenols did not

seem to be released into the liquid effluents generated during the extraction process, suggesting that they were still linked to the polysaccharides remaining in the solid residues, such as cellulose and fucoidans (cf. Figure 1). This is typical for alginate extractions at around neutral pH (Davis et al., 2004) as used in our study. Values of ca. 30-150 mg GAE/g sample, 44-95 mg GAE/g sample and 9-59 mg GAE/g sample have been reported for various extracts obtained from *Ascophyllum* (Audibert et al., 2010), *Alaria* and *Saccharina* extracts (Afonso et al., 2020), respectively. Thus, the polyphenol contents obtained in this study are within reasonable ranges. The fact that the polyphenols remain in the residues is the main reason we chose not to use a conventional formaldehyde pre-treatment prior to alginate extraction, since for food applications the residuals should be free of formaldehyde that would otherwise react with and cross-link the polyphenols. Formaldehyde is principally used in alginate production to improve alginate quality as a preservative and by reducing polyphenol catalysed depolymerisation and decolouration especially if using an alkali-based extraction (Davis et al., 2004).

In line with the results from the polyphenol content, the antioxidant capacity of the residues was greater than for the native seaweeds, being the highest in both the *Ascophyllum* seaweed and residue. The values increased from 329 μmol Trolox/g sample to 378 μmol Trolox/g sample for *Ascophyllum*, from 249 μmol Trolox/g sample to 309 μmol Trolox/g sample for *Alaria* and from 41 μmol Trolox/g sample to 72 μmol Trolox/g sample for *Saccharina*, after the alginate extraction. The high antioxidant capacity of *Ascophyllum* seaweed is not only linked to its greater polyphenol content, but also to its greater content in fucoidan (as later demonstrated by the monosaccharide analysis, cf. Table 2), a sulphated polysaccharide with attributed high antioxidant capacity (Lim et al., 2014). These results highlight the potential of the residues generated after the alginate extraction, in particular from *Ascophyllum* seaweed, as antioxidants. This may be of interest for the development of bioactive packaging films (Blanco-Pascual et al., 2014; Gupta & Abu-Ghannam, 2011), as well as for nutraceutical applications (Cunha & Grenha, 2016).

4.1.1. Carbohydrate composition of the raw seaweeds and the alginate-extracted residues

Since carbohydrates were the main component in both the native seaweeds and the residues, a more detailed analysis of the carbohydrate composition was carried out and the results are shown in Figure 1

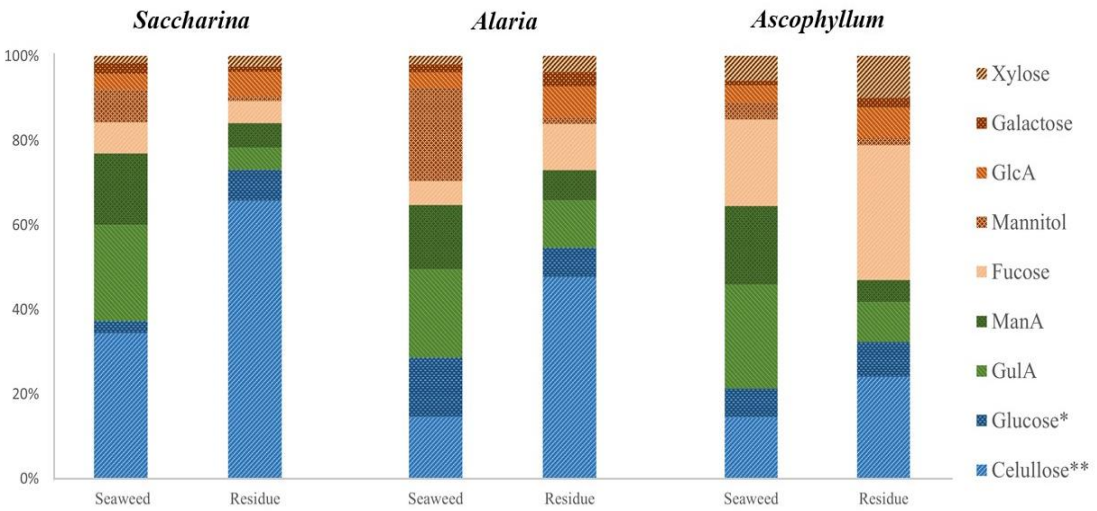


Figure 1. Relative carbohydrate composition of the native seaweeds and the residues generated after alginate extraction. The results from the sugar constituents are expressed as g polysaccharide per 100 g total carbohydrate. Data correspond to the mean calculated values, n=3.

** The crystalline cellulose content was determined as the difference between the typical Saeman sulphuric hydrolysis (Saeman,1945) and the non-crystalline glucose determined after acid methanolysis (Bertaud, 2002; Willför, 2009).

* Glucose contribution from the non-crystalline fraction.

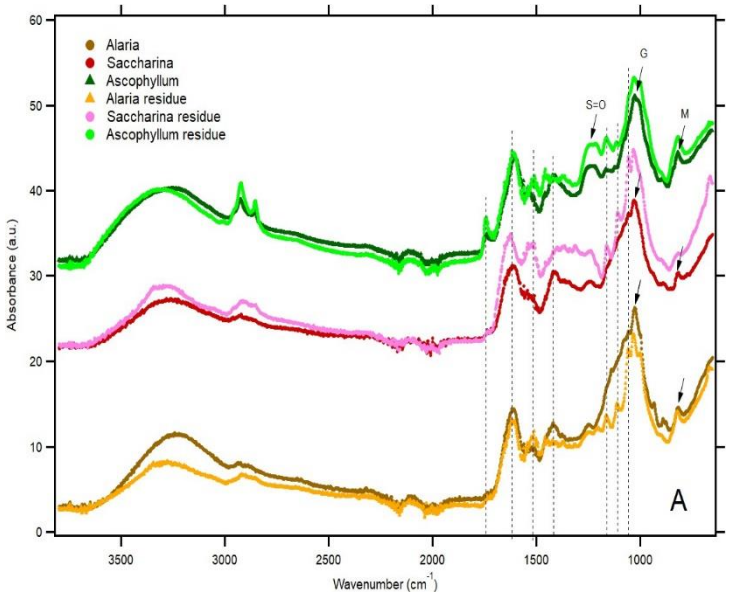
Mannose was detected in only very minor amounts.

Alginate is the main carbohydrate in brown seaweeds, accounting for up to 40% of the dry matter (Horn et al., 1999), and it consists of (1→4)-linked β-D-mannuronic acid and α-L-guluronic acid residues. Thus, the relative amount of alginate in the samples was estimated from

the mannuronic acid (ManA) and guluronic acid (GulA) contents (cf. Figure 1). As expected, the alginate content in the seaweeds was significantly reduced in the residues after the applied extraction treatments, decreasing from ca. 16% to 8% of the total dry weight for *Alaria*, from ca. 13% to 5% for *Saccharina* and from ca. 23% to 8% for *Ascophyllum.* The inability of conventional alginate extraction processes to quantitatively extract all the alginate present in brown seaweeds and consequently, the presence of significant quantities of alginate in the residual biomass might actually be positive, as this polysaccharide might complement the mechanical properties of cellulose and in the extracted fractions for the production of packaging films. Apart from mannuronic acid and guluronic acid, other constituent sugars such as glucose, mannitol and fucose were abundant in the seaweed biomass. Glucose can be mainly associated to the presence of cellulose. On the time when *Alaria* and *Saccharina* were harvested the content of laminarin is typically at its minimum, and if present in the dried and finely milled seaweed, the major part of laminaran will be efficiently removed during acid pre-extraction and the initial water washings. Glucose was more abundant in the *Saccharina* biomass, representing ca. 36% of the total carbohydrate fraction in the native seaweed and ca. 72% in the residue. Although the relative amount of glucose increased in the three seaweeds after alginate extraction, this was less obvious in the case of *Ascophyllum*, suggesting that the amount of crystalline cellulose, resistant to the extraction treatments, was lower in that case. Mannitol, which represented ca. 21% of the total carbohydrate fraction in *Alaria*, was almost completely removed upon alginate extraction, as expected (Kerner et al., 1991). On the other hand, fucose, mainly attributed to the presence of fucoidan, represented ca. 20% of the total carbohydrate in *Ascophyllum*, in line with previous work (Kim et al., 2014). Fucoidans have been reported to remain in the residue generated after alginate extraction, being subsequently extracted by acidic treatments (Yuan & Macquarrie, 2015); thus the increased amount of fucose in the residue, accounting for ca. 31% of the total carbohydrate, was not surprising. The greater fucoidan content in the *Ascophyllum* biomass can be in fact linked to its greater antioxidant capacity (cf. Table 1). These results indicate that while *Saccharina* residue might be the most appropriate for the extraction of cellulose, *Ascophyllum* residue may have potential for the extraction of bioactive fucoidan-rich fractions.

4.1.2. Structural characterization by FT-IR

To better understand the compositional differences between the native seaweeds and their residues, they were characterized by means FT-IR and the obtained spectra can be seen in Figure 2.



Wavenumber (cm^{-1})	Assignment
3600-3200	O-H and N-H
3000-2900	C-H
1730	C=O
1640	amide I
1620	COO-
1540	amide II
1410	C–OH
1300-1100	C-O-C
1200–1260	S = O
1160, 1105, 1060	C-C and C-O
1030-1080	Guluronic acid
787-808	Mannuronic acid

Figure 2. FT-IR spectra of the native seaweeds and the residues generated after alginate extraction. G: guluronic acid; M: mannuronic acid.

In general, all the samples presented spectra characteristic from materials rich in polysaccharides and proteins, showing the broad band at 3600-3200 cm^{-1} (characteristic from O-H and N-H stretching), the bands at 3000-2900 cm^{-1} (associated to C-H stretching), as well as several pronounced bands within the range of 1300-1100 cm^{-1} (corresponding to C-O-C stretching vibrations of polysaccharides). Two alginate-characteristic bands, located at ca. 787-808 cm^{-1} and ca. 1030-1080 cm^{-1}, assigned to mannuronic and guluronic acids, respectively (Chandía et al., 2001), were detected in all the samples. Additionally, the band at ca. 1620 cm^{-1},

which has been linked to the symmetric COO- stretching vibration in alginate (Gómez-Ordóñez & Rupérez, 2011), was also visible. The appearance of these bands in the residues confirms the presence of residual alginate, as suggested by the monosaccharide analyses. Moreover, the band at ca. 1200–1260 cm^{-1}, associated to S = O stretching vibration of sulphate groups (García-Ríos et al., 2012), was more evident in the Ascophyllum seaweed and residue and was correlated with the greater abundance of sulphated fucoidan in these seaweeds. It should be noted that the bands at ca. 1160, 1105 and 1060 cm^{-1}, which are typically linked to C-C stretching and C-O stretching from cellulose (Gómez-Ordóñez & Rupérez, 2011; Leal et al., 2008; Martínez-Sanz et al., 2020), became more intense in the residues, confirming the concentration of cellulose in the biomass generated after alginate extraction. The presence of proteins was also confirmed by the appearance of the bands located at 1640 cm^{-1} (amide I) (overlapped with the band at 1620 cm^{-1}) and 1540 cm^{-1} (amide II). The greater intensity of the 1510 cm^{-1} band in the residues confirms that the proteins were not affected by the alginate extraction and were more concentrated in the residues. Interestingly, the peak at ca. 1730 cm^{-1}, corresponding to C=O stretching, was much sharper and intense in Ascophyllum (both seaweed and residue). This band has been detected in fucoidans extracted from several seaweed species and has been suggested to arise from acetylation of glucuronic moieties (Blanco-Pascual et al., 2014; García-Ríos et al., 2012; Gómez-Ordóñez & Rupérez, 2011). According to the literature, the band at ca. 1410 cm^{-1}, which can only be seen in the raw seaweeds, may be related to C–OH deformation vibration with contribution of O–C–O symmetric stretching vibration of carboxylate group (Gómez-Ordóñez & Rupérez, 2011; Leal et al., 2008). This band has been previously attributed to the presence of soluble alginate (Blanco-Pascual et al., 2014),which was removed upon the applied extraction treatments.

4.1.3. Morphological characterization

The morphology of the different seaweeds and residues was studied by means of optical microscopy. Figure 3 shows representative images taken with bright light and ultraviolet filters, in which fluorescent characteristics of material components can be observed.

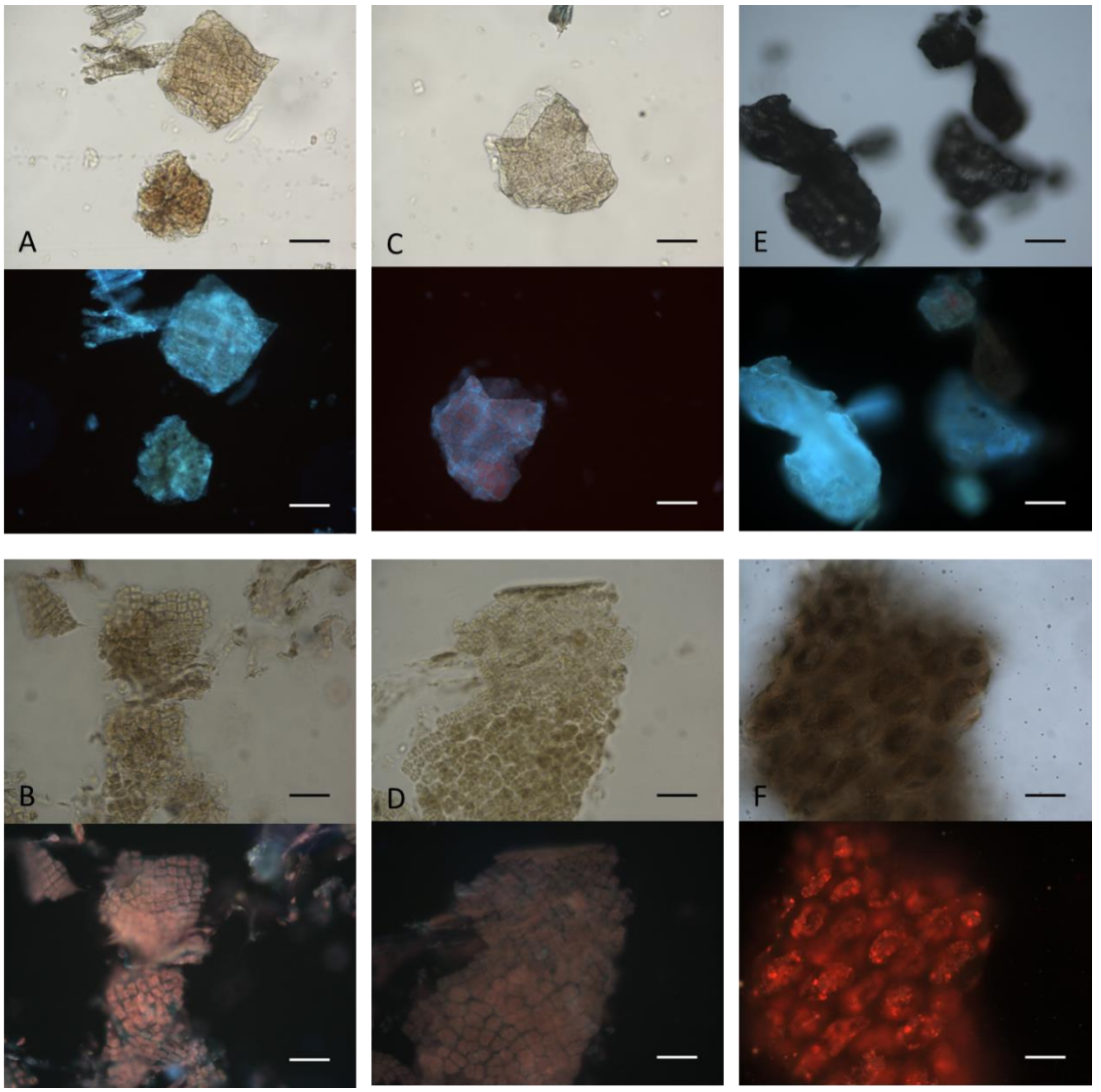


Figure 3. Optical microscopy images of the native seaweeds and the residues generated after alginate extraction. (A) *Alaria* seaweed, (B) *Alaria* residue, (C) *Saccharina* seaweed, (D) *Saccharina* residue, (E) *Ascophyllum* seaweed and (F) *Ascophyllum* residue. Scale bars correspond to 50 microns. Top images were taken with bright light while bottom images were taken using a fluorescent filter UV-2A.

The images evidence a great difference in the cell structure of *Ascophyllum* as compared with *Alaria* and *Saccharina*. While *Alaria* and *Saccharina* showed well-defined rectangular-shaped cells, *Ascophyllum* presented cells with a less defined shape which appeared to be more round-shaped. This can be related to the lower cellulose content in *Ascophyllum*. After the extraction of alginate, the cell walls from the three seaweeds did not lose their integrity, which is reasonable since cellulose (one of the major structural components in cell walls (Deniaud-Bouët et al., 2014; Salmeán et al., 2017) was not affected by the extraction treatments. However, instead of being tightly packed, the individual cells were away from each other, indicating that the components removed upon the applied treatments were acting as gluing agents. Upon observation of the samples with the ultraviolet filter, it was evident that while the images from the native seaweeds were mostly dominated by the appearance of bright blue fluorescent regions, the images of the corresponding residues showed very faint to no light blue fluorescence and instead showed red fluorescent regions, which were much brighter and intense in the case of the *Ascophyllum* residue. According to the compositional analyses, it is likely that the blue fluorescent areas correspond to alginate-rich regions, which were almost completely removed after the applied treatments. On the other hand, based merely on the compositional analyses, the bright red areas may correspond to protein- or polyphenol-rich domains, mainly located intracellularly.

4.2. Characterization of the extracted cellulosic fractions

Different extraction protocols were applied to the residues to obtain cellulosic fractions with different levels of purification, being F3A the most purified one, while F2A and F3B were expected to contain other components other than cellulose. When using the residue from *Aschopyllum*, very low extraction yields (ca. 0.2-3%) were obtained. This supports the hypothesis of *Aschopyllum* having a lower cellulose content, as deduced from the monosaccharide analyses (cf. Figure 1). As expected, the extraction yield decreased with the purity of the fractions, going from 21 to 15% for *Alaria* residue and from 39 to 26% for *Saccharina* residue (cf. Table 2). The higher extraction yields for *Saccharina* residue are reasonable given its higher cellulose content (cf. Figure 1).

4.2.1. Carbohydrate composition

Monosaccharide analyses of the fractions extracted from *Alaria* and *Saccharina* were carried out to investigate their carbohydrate composition and the results are summarized in Table 2. It is worth noting that the residual alginate was not removed upon the bleaching treatment with $NaClO_2$. Thus, the F2A fractions were mainly composed of cellulose and residual alginate, being *Alaria* richer in alginate and *Saccharina* richer in cellulose. In contrast, the F3A and F3B fractions were almost pure cellulose (>95%).

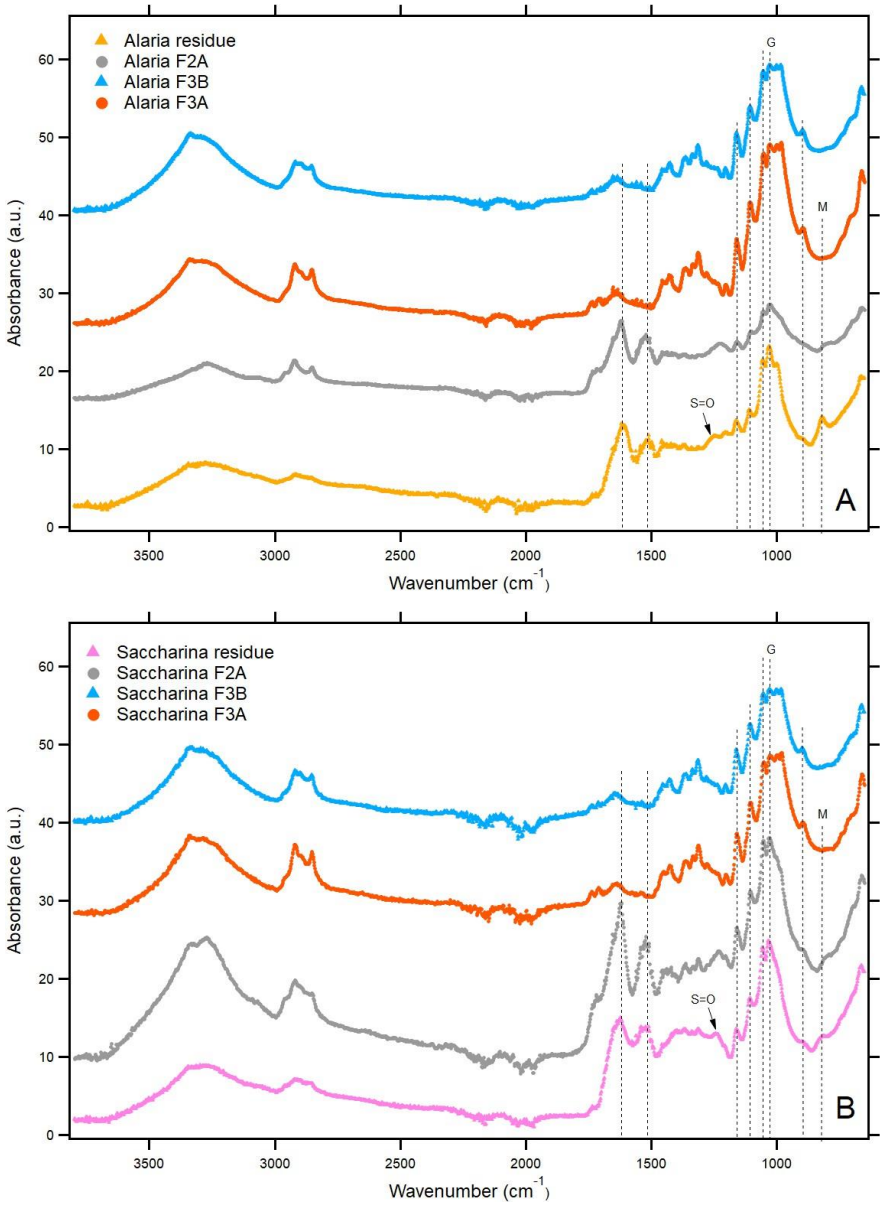
Table 2. Extraction yields and carbohydrate composition of the cellulosic fractions extracted from the residues of *Alaria* and *Saccharina*. The results from the sugar constituents are expressed as g polysaccharide per 100 g dry weight sample.

	Alaria			*Saccharina*		
	F2A	F3A	F3B	F2A	F3A	F3B
Extraction yield (%)	20.6 ± 0.4^{b}	14.6 ± 0.2^{a}	18.5 ± 0.3^{b}	38.6 ± 2.0^{d}	25.8 ± 0.5^{c}	37.5 ± 1.1^{d}
Fucose	3.0 ± 0.8^{b}	<0.5	0.7 ± 0.2^{a}	2.9 ± 0.3^{b}	<0.5	<0.5
Galactose	2.5 ± 0.4^{a}	<0.5	1.8 ± 0.2^{a}	2.2 ± 0.3^{a}	<0.5	<0.5
Glucose	34.1 ± 1.6^{a}	90.1 ± 9.8^{b}	83.6 ± 7.2^{b}	37.6 ± 10.0^{a}	85.9 ± 10.1^{b}	78.0 ± 7.4^{b}
Mannose	2.8 ± 0.6^{a}	tr	tr	4.2 ± 0.7^{a}	tr	tr
Xylose	1.0 ± 0.3^{a}	1.2 ± 0.4^{a}	0.8 ± 0.3^{a}	2.8 ± 1.0^{b}	<0.5	<0.5
GlcA	3.9 ± 0.4^{a}	<0.5	<0.5	6.1 ± 1.1^{a}	<0.5	<0.5
GulA	9.2 ± 0.4^{c}	<0.5	<0.5	6.7 ± 0.4^{b}	0.6 ± 0.3^{a}	<0.5
ManA	8.6 ± 0.2^{c}	<0.5	<0.5	6.5 ± 0.2^{b}	0.5 ± 0.3^{a}	<0.5

Data shown as mean +/-SD, n=3. Values within the same row with different letters are significantly different ($p \leq 0.05$).

4.2.2. Structural characterization by FT-IR

FT-IR characterization of the obtained fractions was carried out to identify changes in the composition and molecular structure of their main components after the different extraction procedures. Figure 4 shows the spectra for the different fractions extracted from the residues.



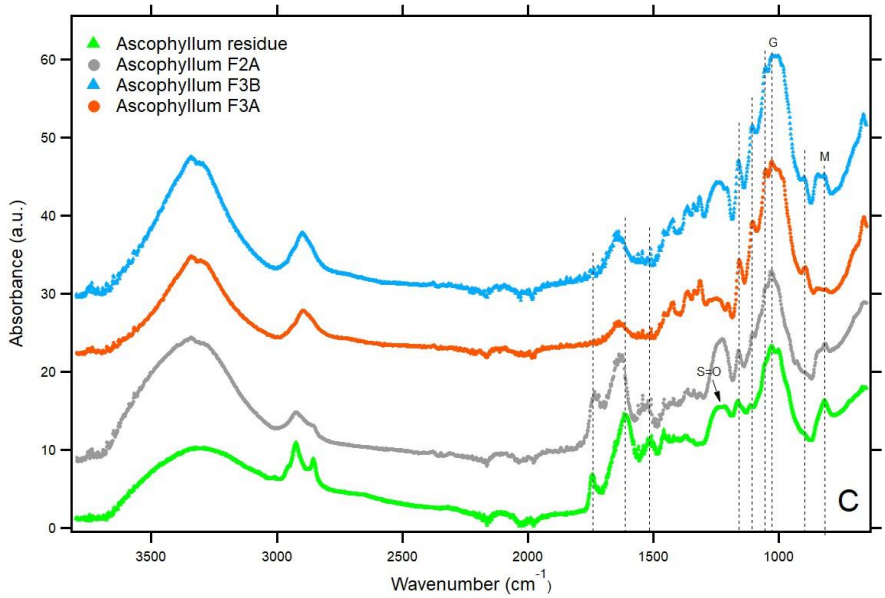


Figure 4. FT-IR spectra of the cellulosic fractions extracted from (A) Alaria residue, (B) Saccharina residue and (C) Ascophyllum residue.

As observed, the F3A and F3B fractions presented very similar spectra, while F2A fractions differed significantly, showing spectra more similar to those from the corresponding residues. The bands related to mannuronic and guluronic acid, located at ca. 787-808 cm^{-1} and ca. 1030-1080 cm^{-1} respectively, were only detected in the residues and in F2A fractions. Furthermore, these fractions showed a relatively intense band at 1620 cm^{-1}, also visible in the residue, which is associated to carbonyl groups of uronic acid (Gómez-Ordóñez & Rupérez, 2011; Yuan & Macquarrie, 2015). In the case of the F3A and F3B fractions this band was not visible and instead a weak band at 1630 cm^{-1} (corresponding to bound water) was detected. This indicates that the residual alginate remaining in the residue was only removed when the alkaline treatment was applied. The band located at 1200-1260 cm^{-1}, indicative of the presence of sulphate, which may arise from the presence of fucoidan (Gómez-Ordóñez & Rupérez, 2011), was detected in all the F2A fractions, but it appeared much more intense in the case of Ascophyllum. In that case, the band was visible for the three fractions, but it appeared much fainter in the case of F3A, confirming its greater degree of cellulose purity. Furthermore, the 1540 cm^{-1} band,

characteristic of proteins (amide II), was only visible in the F2A fractions. In fact, the protein content in the F2A fractions was determined to be approximately 31% for Alaria, 33% for Saccharina and 15% for Ascophyllum. On the other hand, protein contents lower than 2% were determined for the F3A and F3B fractions from the three seaweed residues. In agreement with the monosaccharide analyses, several of the cellulose characteristic bands, such as those located at a. 1160, 1105 and 895 cm^{-1} (Gómez-Ordóñez & Rupérez, 2011; Leal et al., 2008; Martínez-Sanz et al., 2020), showed higher relative intensity in the F3A and F3B fractions, confirming their greater amount of cellulose.

4.2.3. Morphological characterization

Similar to the raw seaweeds and residues, the extracted fractions were analysed by optical microscopy. The images, shown in Figure S1, illustrate the changes in the cell structure with the different extraction methods and purification levels. An obvious difference in the cell morphology of the fractions extracted from *Ascophyllum* residue can be observed. As mentioned before, the cell structure of *Ascophyllum* is very different from that of the two other seaweed species, which is reasonable given the fact that they belong to different seaweed families. Notably, unlike the cell clusters observed in the case of the residues, the individual cells could be clearly identified in the F2A fractions from the three seaweed species; however, the cell wall structure must have not been completely disrupted with the $NaClO_2$ treatment since the boundaries of the cells could still be seen. For the F3A and F3B fractions cell tissue could still be seen, but the cell walls appeared to be completely destroyed. Furthermore, the blue regions observed with the fluorescent filter, attributed to alginate-rich regions, were visible in the F2A fractions (composed of cellulose and alginate) while the F3A and F3B fractions (almost pure cellulose) showed no fluorescence. It should also be noted that F3A and F3B fractions showed almost identical morphologies, suggesting that the alkaline treatment alone was sufficient to disrupt the cell wall structure of the residues.

4.2.4. Thermal stability

TGA analyses were also carried out to determine the thermal stability of the extracted fractions and link their degradation profiles to their composition. The derivative of the weight loss with the temperature was plotted and the results are shown in Figure 5.

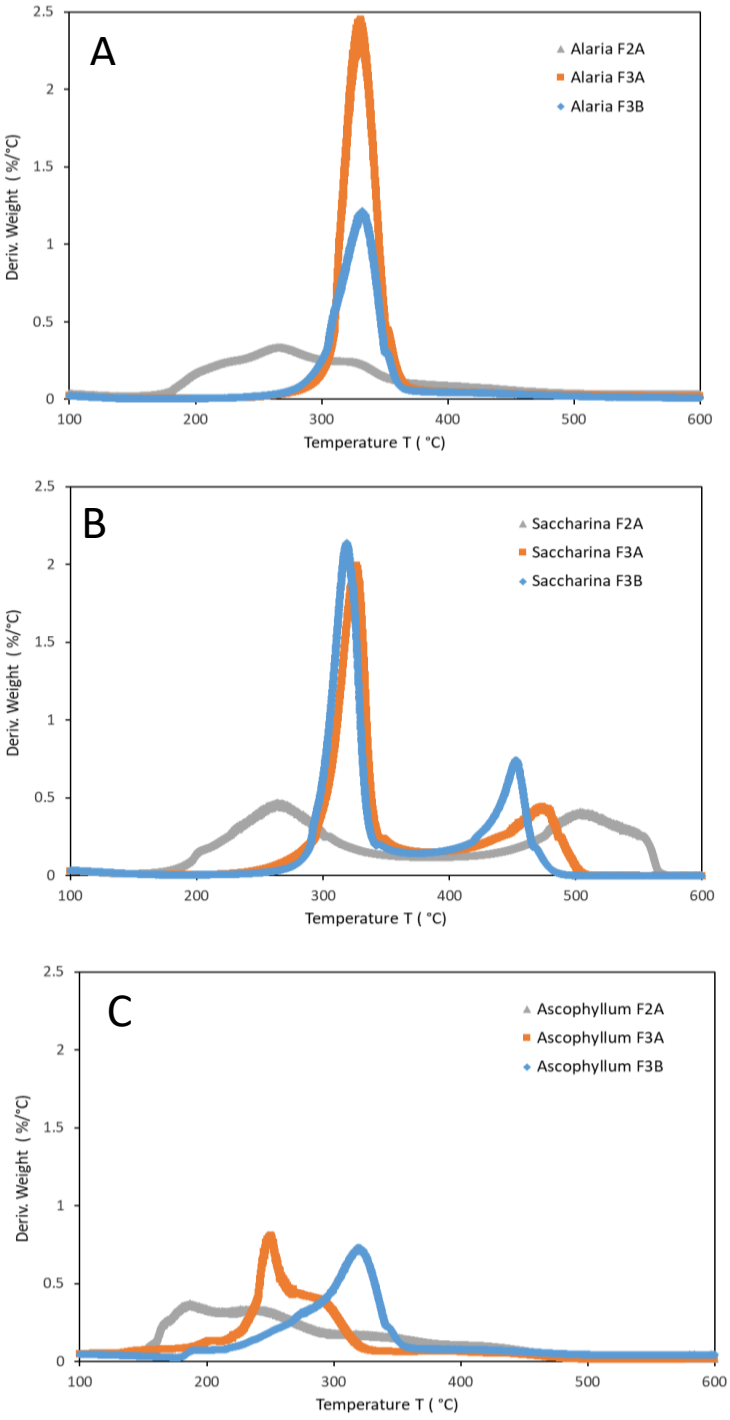


Figure 5. TGA derivative curves of the cellulosic fractions extracted from (A) *Alaria* residue, (B) *Saccharina* residue and (C) *Ascophyllum* residue.

The different levels of cellulose purification in the extracted fractions clearly influenced their degradation profiles. While the *Alaria* and *Saccharina* F3A and F3B fractions showed a degradation mechanism clearly dominated by one strong peak at around 300-330ºC, attributed to the degradation of cellulose (Das et al., 2010), the F2A fractions showed a multi-step degradation profile. The degradation step centred around 230°C for *Alaria* and *Ascophyllum* F2A can be attributed to the presence of proteins and other polysaccharides in these fractions. Notably, the fractions extracted from *Saccharina* showed an additional degradation peak at around 460°C, similarly to what has been reported for *Laminaria* seaweed (Membere & Sallis, 2018a); this peak may originate from the presence of secondary cellulose degradation products, such as cellulose esters or complexes formed with residual cations remaining in the material (Jandura et al., 2000; Membere & Sallis, 2018b). The different degradation behaviour of the fractions extracted from *Ascophyllum* could be explained by lower cellulose content in these samples, which in turn were expected to be richer in other polysaccharides such as fucoidan. In fact, the F2A fraction extracted from *Ascophyllum* residue presents a very similar degradation profile to that previously reported for pure fucoidan (Saravana et al., 2016).

4.3. Production and characterization of cellulosic films

The cellulosic fractions extracted from *Alaria* and *Saccharina* were used to produce films and their structural and functional properties were characterized to evaluate their potential as food packaging materials.

4.3.1. Transparency and microstructure of the films

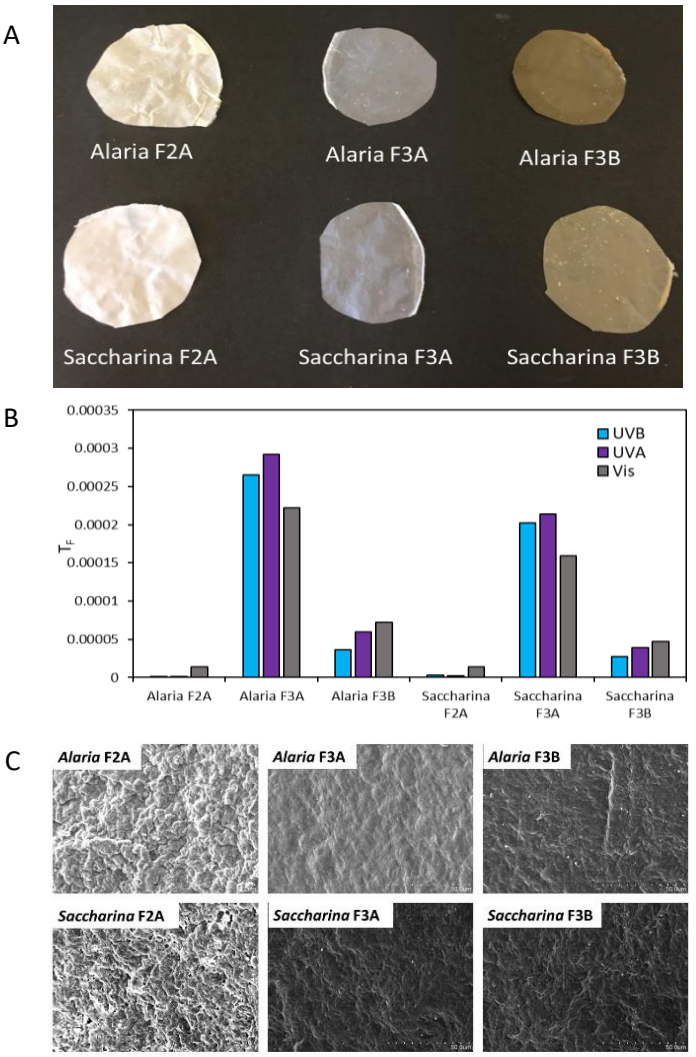


Figure 6. (A) Visual appearance and (B) transmittance factor of the cellulosic films obtained from the seaweed residues in the UVB, UVA and visible wavelength regions (C) SEM images from the surface of the cellulosic films obtained from the seaweed residues. Scale bars correspond to 50μm.

As seen in Figure 6A, the visual appearance, i.e. colour and transparency, of the films was significantly affected by the purification level. While the F3A and F3B films were translucent, the F2A films were completely opaque, which may be due to the presence of non-cellulosic carbohydrates. In fact, the transparency of the films was assessed by internal transmittance (Ti)

measurements and the results (cf. Figure 6B) evidenced that the F3A films, i.e. the ones with the greatest cellulose content, were the most transparent. It was also noted that while the fractions subjected to the bleaching treatment (F2A and F3A) yielded films with a white coloration, the F3B films showed a greenish colouration, which could arise from the presence of seaweed pigments. Considering that for food packaging applications transparent materials are preferable, the F3B and F3A films would be more interesting. It should also be noted that the appearance of small aggregates in all the developed films, which most likely arise from an insufficient dispersion of cellulose in the aqueous dispersions prepared prior to the vacuum filtration step, could be avoided by applying an additional homogeneization step such as sonication. A better dispersion of cellulose is expected to improve further the functional properties of the films and thus, this will be optimized in the future.

The morphology of the different films was studied by SEM and representative images are shown in Figure 6C. As observed, the more purified films (F3A and F3B) presented a more compact and uniform surface than the less purified F2A films. The greater heterogeneity of the F2A films can be attributed to the presence of amorphous components, such as proteins and non-cellulosic carbohydrates. The fibrillar structure characteristic from cellulose can be identified in the F3A and F3B films; however, instead of showing pure cellulose fibres, the images evidence the presence of cellulose embedded in an amorphous matrix. A similar surface morphology was reported for cellulosic films containing residual agar (Martínez-Sanz et al., 2020). It is suspected that the residual alginate remaining in the fractions, acted as an amorphous matrix in which the cellulose fibrils were embedded.

4.3.2. Characterization of the crystalline structure of the cellulosic films by XRD

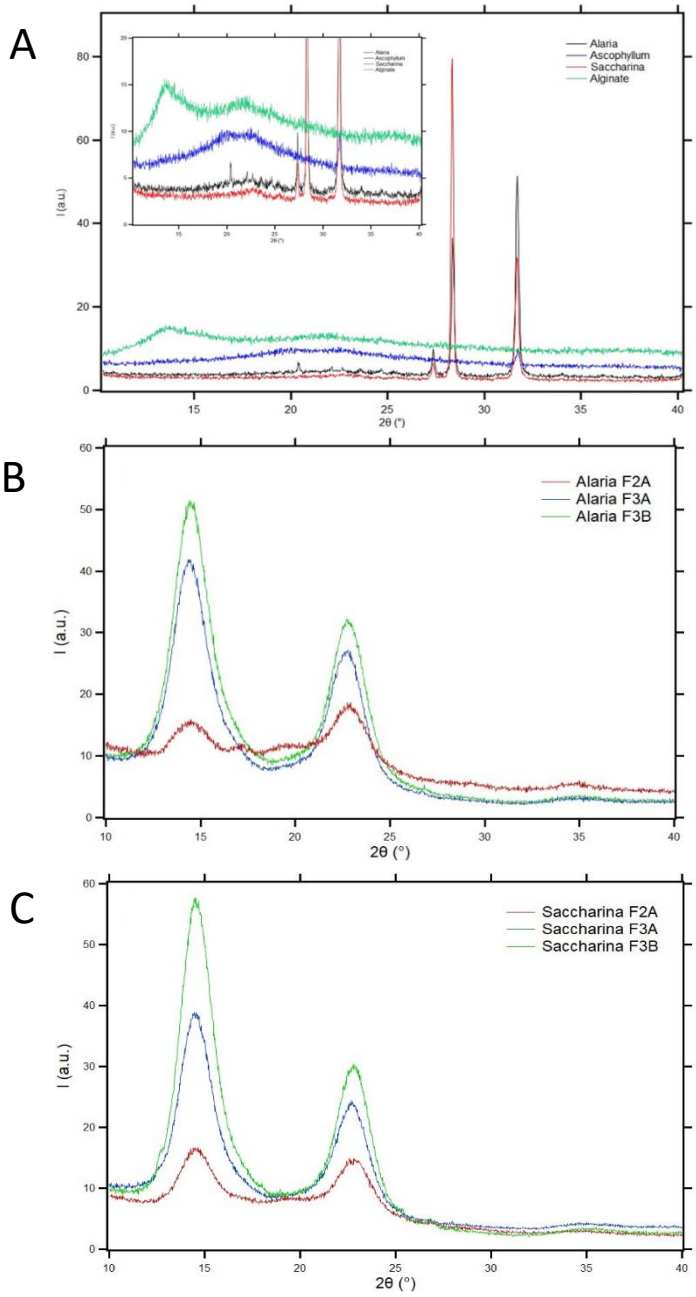


Figure 7. XRD patterns from (A) the native seaweeds and an alginate standard, (B) the cellulosic films obtained from the residues of *Alaria* and (C) the cellulosic films obtained from the residues of *Saccharina*.

XRD analyses were carried out to investigate the crystalline structure of cellulose in the produced films. For reference, the XRD patterns from the native seaweeds, as well as an alginate standard, were also acquired and the results are shown in Figure 7A. As expected, the alginate standard showed a spectrum with very broad and weak peaks, located at 13.6° and 21.6°, indicating its highly amorphous character. In the case of *Ascophyllum*, two overlapping weak shoulders at 20° and 22.5° (corresponding to the alginate), as well as one small peak at 31.7° were detected. In contrast, the patterns from the native *Alaria* and *Saccharina* seaweeds were dominated by several sharp and intense peaks at 27.4°, 28.3° and 31.7°, arising from crystalline components such as minerals and salts. None of these peaks were visible in the spectra from the cellulosic films, as shown in Figure 7B-C, suggesting that these components were removed upon the applied purification treatments. All the films presented three main diffraction peaks located at 14.5°, 17.0º (being those two peaks overlapped in the F3A and F3B films) and 22.7°, which can be assigned to crystalline cellulose I (Thomas et al., 2013). One additional broad shoulder at 19° was visible in the F2A films, being more evident in the case of *Alaria.* This shoulder may correspond to the residual alginate remaining in the F2A fractions, which was in fact more abundant in *Alaria* (cf. Table 2). Interestingly, the relative intensity of the cellulose crystalline peaks in the F3A and F3B was not as expected. A preferential orientation in the (200) crystalline plane has been typically reported for native cellulose regardless of its origin (Agarwal et al., 2018; Das et al., 2010); therefore the diffraction peak associated with this crystalline peak, appearing at 22.7º, is the most intense. In contrast, the relative intensity of peak at 14.5° (corresponding to the (1-10) crystalline plane) was much more intense in the F3A and F3B fractions. This suggests a preferential orientation of the (1-10) crystal plane parallel to the film surface. This effect has been previously reported to occur in regenerated cellulose films (Yamane et al., 2006) and thus, may be indicative of an alteration in the crystalline arrangement of cellulose as a result of the alkaline treatment applied for the production of F3A and F3B fractions. The crystallinity of the films was estimated by fitting the areas under the diffraction patterns, resulting in values of ca. 80% for *Alaria* F2A, 89% for *Alaria* F3A and 89% for *Alaria* F3B, 88% for *Saccharina* F2A, 91% for *Saccharina* F3A and 96% for *Saccharina* F3B. These results

confirm the high cellulose purity in the F3A and F3B fractions and evidence a higher amount of crystalline cellulose in the *Saccharina* biomass.

4.3.3. Mechanical properties and water barrier performance

Table 3. Crystallinity, mechanical and water barrier properties of the cellulosic films obtained from the residues of *Alaria* and *Saccharina*.

	X_C (%)	E (GPa)	TS (MPa)	e_b (%)	$P_{H2O}\cdot10^{-14}$ (Kg·m/s·m^2·Pa)	Maximum water swelling (%)
Alaria F2A	80	2.3 ± 1.1 ^{a}	39.2 ± 9.2 ^{a}	2.2 ± 1.2 ^{a}	5.6 ± 0.9 ^{ab}	335 ± 69 ^{a}
Alaria F3A	89	4.8 ± 1.3 ^{ab}	68.4 ± 20.7 ^{a}	4.6 ± 1.7 ^{a}	7.8 ± 0.6 ^{b}	627 ± 75 ^{c}
Alaria F3B	89	4.6 ± 1.1 ^{ab}	74.9 ± 25.2 ^{a}	4.6 ± 1.1 ^{a}	6.1 ± 0.1 ^{ab}	550 ± 53 ^{c}
Saccharina F2A	88	4.8 ± 0.6 ^{ab}	51.9 ± 13.2 ^{a}	4.2 ± 1.1 ^{a}	4.3 ± 0.6 ^{a}	350 ± 32 ^{a}
Saccharina F3A	91	5.1 ± 0.5 ^{ab}	64.0 ± 18.9 ^{a}	3.2 ± 1.2 ^{a}	10.7 ± 0.7 ^{c}	445 ± 7 ^{abc}
Saccharina F3B	96	6.8 ± 2.2 ^{b}	69.0 ± 42.3 ^{a}	2.9 ± 1.1 ^{a}	3.9 ± 0.6 ^{a}	362 ± 37 ^{a}

Data shown as mean +/-SD, n=3. Values within the same column with different letters are significantly different ($p \leq 0.05$).

X_C: Crystallinity index determined from the XRD patterns; E: Young's modulus; TS: tensile strength; ε_b: elongation at break; P_{H2O}: water permeability.

The mechanical properties of the cellulosic films were evaluated through tensile testing. The results, summarized in Table 3, evidence an improvement in the mechanical performance of the films with the purification of cellulose, which is in agreement with previous works (Benito-González et al., 2018). In fact, the F2A films had the least desirable mechanical properties, i.e. the lowest Young's moduli and tensile strength. This can be attributed to the presence of other

components such as proteins and amorphous non-cellulosic polysaccharides (Martínez-Sanz et al., 2018), making it difficult to obtain homogeneous aqueous suspensions and yielding heterogeneous structures, such as those observed by SEM (cf. Figure S2). In contrast, the F3B and F3A films presented higher rigidity and strength, while also showing a reasonably good ductility when compared to other cellulosic films (Benito-González et al., 2018, 2020; Benito-González et al., 2019). These films show superior mechanical performance in terms of strength than nanocomposite brown seaweed films (Doh et al., 2020). They perform even better than commercial biopolymers such as thermoplastic starch or poly(lactic) acid in terms of rigidity and strength (Benito-González et al., 2019; Bhasney et al., 2019). It should also be mentioned that the slightly greater degree of cellulose purity in F3A did not produce any additional advantage in terms of mechanical performance.

The water vapour permeability (WVP) of the films (cf. Table 3) was also affected by the purification degree of the fractions, with the most purified F3A films showing less barrier (i.e. higher permeability) than the F2A and F3B films. This might be contrary to what expected, considering the greater crystallinity of the F3A fractions and the higher permeability values reported for pure alginate films (Rhim, 2004); however, a previous study showed a similar trend for cellulose-based films containing residual agar and attributed this effect, amongst other factors, to the reduced amount of free hydroxyl groups due to the interactions established between cellulose and agar (Martínez-Sanz et al., 2020). Furthermore, the high protein content in the F2A films may have also contributed to reduce their WVP, since some studies have reported on the good water resistance for protein films (Park & Chinnan, 1995; Seung & Rhee, 2004). The water swelling capacity of the films was also measured to evaluate their moisture sensitivity. The results (cf. Table 3) indicate that, in general, *Alaria* films showed a higher water swelling capacity than *Saccharina* films. This is in line with the XRD results and could be explained by the greater amount of amorphous polysaccharides, such as residual aginate, in the fractions extractred from *Alaria* residue. Furthermore, maximum water swelling values were obtained for the F3A films. The greater swelling of the most purified fractions supports the hypothesis of a higher amount of free hydroxyl groups in F3A fractions after removal of other components, such as residual alginate and proteins. It is expected that the cellulose and the

residual alginate establish interactions through hydrogen bonding, thus reducing the amount of free hydroxyl groups able to bind to water.

5. Conclusions

Cellulose-based fractions with different levels of purification were extracted from the residues generated after alginate extraction from three brown seaweeds, namely *Alaria esculenta, Saccharina latissima* and *Ascophyllum nodosum*. The biomass was mainly composed of carbohydrates (34-55%), but significant amounts of proteins and minerals were also detected. After the alginate extraction, proteins and polyphenols were concentrated in the residues, increasing their antioxidant potential. Furthermore, although some residual alginate remained in the residues (5-8%), the carbohydrate fraction from *Alaria* and *Saccharina* residues was enriched in cellulose, while fucoidan concentration increased in *Ascophyllum*. The lower cellulose content in the latter resulted in very low extraction yields (0.2-3%) for the cellulose-based fractions as compared with *Alaria* (15-21%) and *Saccharina* (26-39%) residues. This indicates that while *Saccharina* and *Alaria* residues are suitable for the extraction of cellulosic fractions, *Ascophyllum* residue may have potential for the extraction of bioactive fucoidan-rich fractions.

The less purified F2A fractions from *Saccharina* and *Alaria* were mainly composed of cellulose and residual alginate, while the F3A and F3B fractions were almost pure cellulose (>95%). These fractions were used to produce films, which were subsequently characterized to evaluate their potential as food packaging materials. Overall, the films with higher cellulose purity (F3A and F3B), presented more desirable characteristics in terms of mechanical properties and visual appearance. On the other hand, the reduced amount of free hydroxyl groups in the F2A and F3B fractions as a consequence of interactions being established between cellulose and other components reduced their water sensitivity and permeability. The simple alkaline treatment applied for the extraction of F3B produces films with the best compromise between functional properties and economical and environmental efficiency. In particular, the greater content of crystalline cellulose in *Saccharina* residue, led to greater extraction yields and produced more rigid and less permeable films. These results confirm that the waste stream generated after

alginate extraction without a formaldehyde pre-treatment is a valuable source for the extraction of carbohydrates with interest in food packaging applications.

6. Acknowledgements

This work was financially supported by the "Agencia Estatal de Investigación" (PCI2018-092886 Grant) and co-funded by the European Union's Horizon 2020 research and innovation programme (ERA-Net SUSFOOD2).

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8. Supplementary material

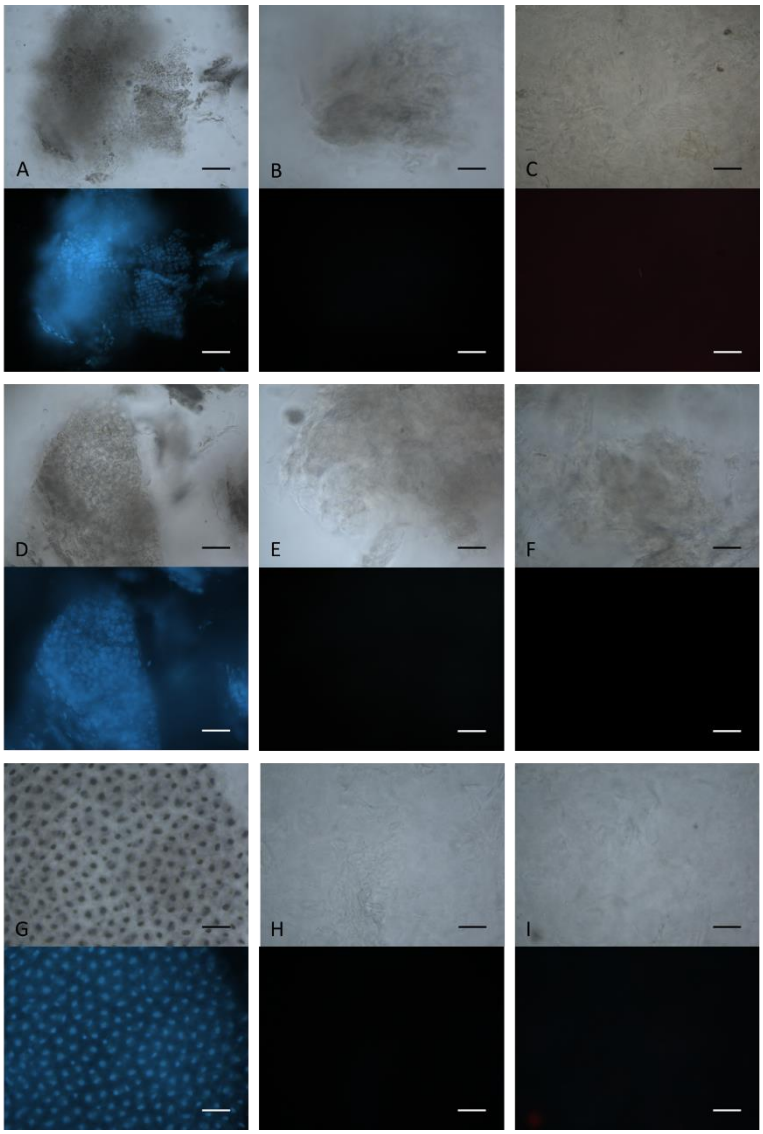


Figure S1. Optical microscopy images of t the cellulosic fractions extracted from the different seaweed residues. (A) *Alaria* F2A, (B) *Alaria* F3A, (C) *Alaria* F3B, (D) *Saccharina* F2A, (E) *Saccharina* F3A, (F) *Saccharina* F3B, (G) *Ascophyllum* F2A, (H) *Ascophyllum* F3A and (I) *Ascophyllum* F3B. Top images were taken with bright light while bottom images were taken using a fluorescent filter UV-2A. Scale bars correspond to 50 microns.

Table S2. Carbohydrate composition of the native seaweeds and the residues generated after alginate extraction. The results from the sugar constituents are expressed as g polysaccharide per 100 g dry weight sample.

	Alaria		*Saccharina*		*Ascophyllum*	
	Seaweed	Residue	Seaweed	Residue	Seaweed	Residue
Fucose	2.5 ± 0.4^{a}	3.8 ± 0.3^{a}	2.4 ± 0.0^{a}	2.5 ± 0.3^{a}	10.9 ± 0.7^{b}	17.8 ± 0.1^{c}
Galactose	0.8 ± 0.1^{ab}	1.2 ± 0.1^{c}	0.9 ± 0.0^{b}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	1.3 ± 0.0^{c}
Glucose*	6.23 ± 1.3^{e}	2.43 ± 0.1^{b}	0.9 ± 0.08^{a}	3.5 ± 0.2^{c}	3.63 ± 0.4^{cd}	4.6 ± 0.6^{d}
Cellulose**	6.51 ± 2.4^{a}	16.63 ± 0.6^{d}	11.2 ± 1.6^{abc}	31.5 ± 5.7^{e}	7.74 ± 0.7^{ab}	13.44 ± 4.4^{bc}
Xylose	0.9 ± 0.1^{a}	1.3 ± 0.3^{a}	<0.5	1.2 ± 0.3^{a}	3.1 ± 0.2^{b}	5.5 ± 0.1^{c}
GlcA	1.7 ± 0.5^{ab}	2.6 ± 0.2^{ab}	1.2 ± 0.1^{a}	2.8 ± 0.4^{b}	2.2 ± 0.1^{ab}	4.0 ± 0.1^{c}
GulA	9.3 ± 2.4^{cd}	3.9 ± 0.2^{ab}	7.4 ± 0.2^{bc}	2.5 ± 0.2^{a}	13.1 ± 1.5^{d}	5.3 ± 0.1^{abc}
ManA	6.8 ± 1.7^{bc}	2.5 ± 0.1^{a}	5.5 ± 0.4^{ab}	2.8 ± 0.1^{a}	9.9 ± 1.0^{c}	2.9 ± 0.0^{a}
Mannitol	9.8 ± 3.8^{b}	<0.5	2.5 ± 0.0^{ab}	<0.5	2.1 ± 0.0^{ab}	1.0 ± 0.2^{a}

Data shown as mean +/-SD, n=3. Values within the same row with different letters are significantly different ($p \leq 0.05$).

IV. GENERAL DISCUSSION

GENERAL DISCUSSION OF THE RESULTS

Throughout this doctoral thesis, consisting of 6 different scientific works distributed in 3 chapters, the valorization of various sources of seaweed biomass has been undertaken in an effort to address several of the challenges directly impacting the seaweed production and exploitation market. These challenges include (i) simplifying extraction processes to produce phycocolloids with lower purification degrees, (ii) valorizing the waste generated in these processes, (iii) exploring new seaweed sources for obtaining packaging films, phycocolloids and other high-value compounds, and (iv) assessing the nutritional properties of seaweeds as a source of proteins. The main results from each of the thesis chapters, relevant to the aforementioned challenges, are discussed as follows.

4.1. Valorization of whole macroalgae biomass for food-related applications

In this chapter, the valorization of biomass from various species of marine seaweeds was conducted to identify compounds of interest and evaluate their potential for producing biopolymeric materials and protein-rich food ingredients.

In the first study included in this chapter, a comprehensive analysis of the composition of the invasive macroalgae *R. okamurae* was conducted. The focus was on exploring potential strategies to valorize the different compounds present in it. This analysis showed that *R. okamurae* mainly consists of structural carbohydrates, representing 60% of the total content. Notably, among these carbohydrates, alginates stood out, constituting 32% of the macroalgae's total composition and exhibiting an M/G ratio of 0.93, which is equal to or even lower than that of common industrial alginates. This characteristic is of great interest, since alginates with a lower M/G ratio are associated to yield stronger gels. Furthermore, among these carbohydrates, its 8% fucose content was also highlighted, which is related to the presence of fucoidans that have potential bioactivities. This seaweed species also presented a relative high lipid content of 17%, characterized by its high content of omega-3 polyunsaturated fatty acids (PUFA n3), reaching 18% of the whole lipid content, a value comparable to that of some microalgae species such as *Chlorella vulgaris*. Among the n3 PUFAs, the uncommon presence of hexadecatetraenoic acid in remarkable amounts in *R. okamurae* is also noteworthy and deserves further analysis, given the potential utility of n3 PUFAs in the treatment of diseases such as atherosclerosis,

Parkinson or Alzheimer, among other potential applications in feed and aquaculture. Additionally, it is worth noting the remarkably low n6/n3 ratio of 0.31, indicating a favourable balance between n6 and n3 fatty acids, rendering it a nutritionally beneficial option. Furthermore, this seaweed species contained 12.2% of proteins with an interesting amino acid profile, where essential amino acids constitute 32% of the total protein fraction, comparable to what can be found in some animal protein sources, such as eggs. Additionally, it also contains non-essential amino acids, such as aspartic and glutamic acid, which contribute to the distinct flavour and aroma of seaweeds and marine products. Given that some plant proteins may lack certain essential amino acids, *R. okamurae* could offer an alternative to animal protein sources and improve the amino acid profile of existing vegan products. *R. okamurae* also exhibits exceptional antioxidant properties, clearly surpassing common antioxidant foods such as ginger or turmeric, highlighting its significant nutritional potential. Additionally, the algal biomass contains a variety of essential minerals, including magnesium, calcium, potassium, iron, zinc, phosphorus and manganese, which have beneficial effects on human health, supporting bone development, muscle function, the immune system and blood pressure regulation. Based on the results of this research, it can be asserted that the invasive macroalgae *R. okamurae* has significant potential as a sustainable source of nutritional and bioactive compounds that can add value to different sectors.

In the second study from this chapter, four different species of red seaweeds were minimally processed through a combination of melt blending and compression moulding, aiming to obtain packaging materials in a more energy efficient way. Additionally, the impact of the composition and cell wall structure of the different seaweed species on the final performance of the films was investigated. The analysis of their composition revealed that the seaweed biomass was mainly composed of carbohydrates, representing up to 50% of their composition, although significant amounts of proteins were also found, constituting up to 19% of their composition, together with ashes, that could represent up to 28% of the seaweed biomass. *G. corneum* presented the highest cellulose content, approximately 14%, and the lowest agar content, around 21%, although this agar stood out for containing 97% of agarose, i.e., the fraction that provides gelling capacity. Despite belonging to the *Gracilaria* genera, the other three seaweed

species showed significant differences in their composition. *G. tenuistipitata*, presented an agar content of around 31%, which was composed of almost 93% agarose. In contrast, the agar fractions in *A. chilensis* and G. *longissima* presented a significant proportion of agaropectin, which could reach up to 26% of their composition. Disparities in cellulose content were also noted; while in *A. chilensis* and *G. tenuistipata* it was around 6%, in the case of *G. longissima*, it increased to almost 10%. It was evidenced that the higher amount of cellulose, along with the high agarose/agaropectin ratio in *G. corneum*, limited the diffusion of agar when the aqueous seaweed suspensions were processed at high temperatures, whereas the more amorphous agar in *A. chilensis* and *G. longissima* promoted its diffusion in the liquid medium. However, in all cases, an increase in processing temperature up to 130 °C seemed to favour a greater agar release. The characterization of the films showed that the cellulose content present in the seaweeed biomass appeared to be the main factor influencing the mechanical performance and water barrier capacity of the resulting films. In fact, the higher cellulose content in *G. corneum* resulted in the formation of stiffer and more resistant films with high water vapour barrier capacity, while a higher agar and lower cellulose content in *A. chilensis* seemed to improve its elongation capacity. Films made from the less exploited *G. longissima* also exhibited interesting properties due to their relatively high cellulose content, offering an attractive alternative for the production of biopolymeric films. Therefore, the results of this study highlight the potential of red seaweed biomass to generate cost-effective and environmentally-friendly food packaging materials.

After studying the composition of these red seaweed species and noticing their relatively high protein content, the idea emerged that, in addition to the production of biopolymeric materials, they could be also used as food sources for human consumption. However, no studies are currently available reporting on the nutritional quality of the proteins from these seaweeds. Consequently, in the third study from this chapter the nutritional quality and digestibility of the proteins present in two of the previously studied red seaweed species, *G. corneum* and *G. longissima*, were evaluated through *in vitro* gastrointestinal digestions. The initial protein content of both seaweed species was around 16%, being most of them entrapped within the seaweeds' cell walls. Both species exhibited similar amino acid profiles, with aspartic acid,

glutamic acid, glycine and alanine being the most abundant. The amino acid profiles of the seaweeds were affected by processing, with a significant decrease in the proportion of more labile amino acids such as lysine, suggesting the development of chemical reactions, such as Maillard reactions, promoted by the high temperatures applied and by the presence of polysaccharides. Nonetheless, essential amino acids constituted more than 34% of the total amino acids in the seaweeds and their processed products. Due to the intricate cell wall structure of the seaweeds, both species presented rather low protein digestibility values of around 49-51%. Processing through extrusion (with or without an additional compression moulding step) permeabilized the cell walls and promoted the release of proteins, therefore enhancing the overall protein digestibility. Nevertheless, the differences in the composition and cell wall structure of the two seaweed species resulted in different behaviours. The more rigid and resistant cell walls from *G. corneum*, required a greater extent of processing to be partially distorted; accordingly, the digestibility appeared to increase with each subsequent processing step, up to 62% for the films. In contrast, the thinner cell walls from *G. longissima* were capable of releasing proteins and agar to a greater extent with the extrusion step and the digestibility reached its maximum value (61%). In that case, further processing by compression moulding seemed to be detrimental for the protein digestibility, which could be attributed to a greater release of agar, forming a gelling network. Also related to the greater amount of agar in *G. longissima*, the digests' absorbable fraction from this seaweed species contained a lower proportion of free amino acids than that from *G. corneum*, which is most likely due to saccharide-peptide interactions limiting the hydrolysis of the digestion products down to free amino acids. Interestingly, the changes produced in the amino acid profiles of the processed products were detrimental for the nutritional quality of their proteins, with lysine becoming the limiting amino acid and a significant reduction in the *in vitro* DIAAS values for *G. longissima*. Thus, to improve the protein digestibility while preserving the nutritional quality, other processing techniques which do not induce amino acid chemical reactions should be tested. Alternatively, the processed products could be complemented with other protein sources rich in lysine to improve their nutritional quality. These results provide a basis for the development of strategies to improve the nutritional quality of proteins from *G. corneum* and *G. longissima*

to produce high-quality food products, thereby opening the way for potential applications in the food industry.

4.2. Valorization of phycocolloids extracted from macroalgae biomass

In this chapter, various seaweed species were examined to efficiently extract agar through simplified methods and evaluate the suitability of the agar-based extracts for developing hydrogel-like structures and biofilms for food applications.

In the first study included in this chapter, the ability of two agar-based extracts with different degrees of purity obtained from *G. corneum* to form packaging films through a combination of melt blending and compression moulding, was evaluated. Additionally, the stability of these films during prolonged storage was investigated. It was observed that the properties of the films were significantly influenced by the degree of purification of the agar. In the case of the less purified agar extract, due to the presence of other compounds such as proteins and polyphenols, films with a brownish colouration, higher opacity, higher water vapour permeability and lower mechanical performance were generated compared to the films made from high purity commercial agar. On the other hand, the presence of these compounds had a plasticizing effect, thus eliminating the need to use additives for film formulation. In contrast, the commercial agar films exhibited an extremely rigid behaviour, requiring the addition of a plasticizer to produce films that could be properly handled. The addition of glycerol as plasticizing agent produced more ductile films but their mechanical resistance was impaired. This work also reported on a change in the semi-crystalline structure of agar and water-polysaccharide interactions after prolonged storage at a relative humidity of 53%. As a result, a stiffening effect occurred in pure agar films, making them unmanageable after 7 days of storage. The addition of glycerol in the film formulation appeared to improve film stability by limiting structural changes for up to 14 days of storage. However, probably due to glycerol migration through the film, these films eventually stiffened after 30 days of storage. This was not the case for the less purified agar films, whose structure and mechanical properties were more stable up to 30 days of storage. The results obtained in this study demonstrate the potential of the less purified agar extract, produced through a more energy-efficient extraction protocol, to be used as an additive to reduce costs and improve the storage stability of pure agar films.

In order to investigate new sources for obtaining high-quality agar, the second study in this chapter conducted a comparison of the composition and gel-forming behaviour of agar fractions obtained from different red seaweed species through two different methods. One of these methods was a conventional extraction process, similar to the one used in the industrial agar extraction, while the other was a more energy-efficient extraction protocol. These methods were applied to various species of red seaweeds, including *G. corneum* and two species from the *Gracilaria* genera (*A. chilensis* and *G. longissima*). Additionally, the suitability of these agar fractions for their use in the food industry was assessed. The results of this study showed that, despite a significant decrease in extraction yield, the application of a conventional alkaline purification pretreatment to remove impurities was effective in producing extracts with higher carbohydrate content without affecting the agarose/agaropectin ratio. Consequently, hydrogels with greater firmness and strength were generated. Surprisingly, the presence of agaropectin, with a semi-crystalline structure and lower sulfation degree, in the agar fractions of *Gracilaria* species, especially *G. longissima*, promoted the formation of more densely packed hydrogel networks with superior mechanical properties and higher gelation temperatures than those observed in the purified fraction of *G. corneum*. On the other hand, the more energy-efficient extraction protocol, produced less purified agar fractions that contained additional compounds such as proteins, polyphenols, and minerals. Despite the presence of these compounds, the gelation temperature remained unaffected. However, the hydrogels exhibited less rigid behaviour within the linear elastic region and lower resistance to fracture during compression compared to their more purified counterparts. Nevertheless, these less purified agar fractions could be valuable in the production of hydrogels with bioactive properties, used as texture modifiers in specific applications within the food industry. The study also highlights the potential of *G. longissima* as a cost-effective alternative to the more commonly used *G. corneum* for the production of agar hydrogels for food applications.

4.3. Valorization of industrial residues derived from phycocolloid extraction

The aim of this chapter was to valorize the industrial by-products generated after alginate extraction, to obtain cellulose fractions through simplified methods and assess their potential in the production of biopolymeric materials. To this purpose, the solid cake by-product from the

extraction of alginate from three brown seaweed species, specifically *A. esculenta*, *S. latissima*, and *A. nodosum*, was utilized. It was observed that the native seaweed biomass consisted of up to 55% carbohydrates, along with significant amounts of other compounds, such as proteins and minerals. After alginate extraction, a significant reduction in mineral content was observed, while both protein and polyphenol content were concentrated in the residues, increasing their antioxidant potential. Carbohydrate content, on the other hand, was not significantly affected. In this regard, a small amount of residual alginate, ranging from 5% to 8%, was identified in the residues. Furthermore, when delving into the carbohydrate composition of these residues, it was found that *Alaria* and *Saccharina* residues were notably enriched in cellulose, whereas *Ascophyllum* residues exhibited a higher concentration of fucoidan. Consequently, due to its lower cellulose content, extraction yields for cellulose fractions in *Ascophyllum* residues were significantly lower, ranging from 0.2% to 3%, contrasting to those of *Alaria* residues (with yields ranging from 15% to 21%) and Saccharina residues (with yields ranging from 26% to 39%). This suggests that while *Alaria* and *Saccharina* residues are suitable for obtaining cellulose fractions, *Ascophyllum* residue may hold promising potential for the extraction of fucoidan-rich fractions. A range of cellulosic fractions with different purification degrees were produced. Specifically, the F2A fractions (obtained by applying a $NaClO_2$ treatment to remove lignin, pigments and some non-cellulosic carbohydrates) were mainly composed of cellulose and residual alginate, with *Alaria* being richer in alginate and *Saccharina* richer in cellulose. Conversely, the F3A (obtained by applying, in addition to the treatment with $NaClO_2$, a subsequent alkaline treatment to remove non-cellulosic carbohydrates) and F3B (obtained by directly applying the alkaline treatment) fractions were almost pure cellulose, with a content exceeding 95%. These fractions were subsequently employed for film production and their structural and functional properties were characterized aiming to evaluate their suitability as materials for food packaging. The higher content of crystalline cellulose in *Saccharina* residue led to higher extraction yields and the production of stiffer and less permeable films. Overall, films with higher cellulose purity, specifically F3A and F3B, exhibited more desirable characteristics in terms of visual appearance and mechanical properties. However, possibly due to the reduction in the quantity of free hydroxyl groups resulting from interactions with other components, a

decrease in water sensitivity and permeability was observed in the F2A and F3B films. Therefore, the simple alkaline treatment applied in the extraction of the F3B fraction results in the production of cellulosic films from seaweed waste with an optimal balance between functional properties and considerations of economic and environmental efficiency.

V. CONCLUSIONS

The main conclusions from this thesis are summarised as follows:

- The invasive macroalgae ***R. okamurae*** emerges as a **new alternative source of sustainable biomass** for obtaining functional, nutritional, technological and bioactive compounds, which show **promising potential** for use in **various industrial sectors**, including food, feed, pharmaceuticals and cosmetics. In this way, it would transform its role from a threat into a valuable opportunity.

- **Cost-effective** and **environmentally-friendly materials** for food applications can be developed from **red seaweed biomass** through **minimal processing** approaches. In this sense, the **composition** and **structure of the seaweeds' cell walls** play a fundamental role in understanding how each species behaves during processing. Moreover, the composition of the seaweeds also influences the properties of the resulting films, with **cellulose content** being the main factor determining the **mechanical** and **water barrier performance** of the films.

- The evaluation of the nutritional quality and digestibility of proteins in two species of red seaweeds, using the INFOGEST harmonized *in vitro* protocol, revealed that the **native seaweeds** have **low digestibility**, which **improves after processing** though extrusion and compression moulding. However, this improvement is **influenced by** both the **composition** and the **structure of their cell walls**, as species with tougher cell walls and higher cellulose content require a higher degree of processing to enhance digestibility. Despite this improvement in digestibility, **processing negatively impacts the amino acid profile** and nutritional quality of the proteins. Nevertheless, these results provide a basis for the development of strategies to improve the nutritional quality of seaweed proteins for the production of high-quality food products.

- **Agar-based extracts** with different degrees of purity are able to form **films** through melt mixing and compression moulding. The **degree of purification** of agar has a significant

impact on the final properties of the films. In fact, the application of a simple treatment to obtain **less purified fractions** results in films with **lower mechanical performance** and **higher water vapour permeability** compared to pure commercial agar. Nevertheless, films derived from **less purified agar extracts** exhibit **greater storage stability without** the need for large quantities of **plasticizers,** suggesting their potential as an additive to reduce costs and enhance the storage stability of pure commercial agar films.

- The application of an **alkaline purification** process, similar to that used in the industrial production of agar, **reduces** the agar **extraction yield**. However, this results in extracts with a **higher carbohydrate content** by removing impurities. These extracts lead to the formation of **hydrogels** with significantly **higher strength** and **hardness**. On the other hand, the simplified extraction protocol produces **less purified agar fractions** that contain additional compounds such as proteins, polyphenols and minerals. While this results in hydrogels that are less rigid and resistant, this characteristic could be advantageous for the production of hydrogels with **bioactive properties**, which can be useful in specific applications in the food industry, such as texture modifiers or thickening agents.

- Due to its composition and cell wall structure, *G. longissima* emerges as a **cost-effective alternative** to traditional agar sources for the production of **highly rigid agar hydrogels**, as well as for the production of **biopolymeric materials** with interesting properties from minimally processed seaweed biomass.

- Cellulosic fractions with different levels of purification have been extracted from the **residues generated after the extraction of alginate** from three varieties of brown algae: *Alaria esculenta*, *Saccharina latissima*, and *Ascophyllum nodosum*. However, while the residues of ***Alaria*** and ***Saccharina*** are suitable for the extraction of **cellulosic fractions**, the residues of ***Ascophyllum***, with a lower cellulose content, show a greater potential for the extraction of fucoidan rich bioactive fractions. Cellulose fractions with **higher**

purity can generate films with **more desirable mechanical properties** and visual appearance, while the reduced amount of free hydroxyl groups in the **less purified fractions**, leads to **greater water vapour barrier**. Nonetheless, the application of a **simple alkaline treatment** is sufficient for the production of cellulose fractions that result in films with an **optimal balance between functional properties and economic and environmental efficiency**.

VI. ANNEXES

Annex A: List of publications included in this thesis

Food Chemistry 440 (2024) 138241

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterization of the invasive macroalgae *Rugulopteryx Okamurae* for potential biomass valorisation

Vera Cebrián-Lloret [a], Sara Cartan-Moya [a], Marta Martínez-Sanz [b], Pilar Gómez-Cortés [b], María Visitación Calvo [b], Amparo López-Rubio [a], Antonio Martínez-Abad [a,*]

[a] Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, Paterna 46980, Valencia, Spain
[b] Department of Bioactivity and Food Analysis, Institute of Food Science Research, CIAL (CSIC-UAM), Nicolás Cabrera, 9, 28049 Madrid, Spain

ARTICLE INFO

Keywords:
R. okamurae
Brown algae
Invasive species
Valorisation
Blue bioeconomy

ABSTRACT

This study aimed to examine the composition and properties of the invasive macroalgae *R. okamurae* and explore potential applications. The results showed that the seaweed biomass is mainly composed of structural carbohydrates, with alginate being the main constituent, accounting for 32 % of its total composition and with a mannuronic and guluronic acid ratio (M/G) ratio of 0.93. It also has a relatively high concentration of fucose, related to the presence of fucoidans that have important biological functions. Among the mineral contents, a high magnesium and calcium (7107 and 5504 mg/kg) concentration, and the presence of heavy metals above legislated thresholds, were notable. *R. okamurae* also contained a high lipid content of 17 %, mainly composed of saturated fatty acids, but with a significant fraction of n3 polyunsaturated fatty acids (18 %) resulting in a low n6/n3 ratio (0.31), that has health benefits. The protein content of *R. okamurae* was 12 %, with high-quality proteins, as essential amino acids (mainly leucine, phenylalanine and valine) constitute 32 % of the total amino acids. It also showed a high polyphenol content and outstanding antioxidant properties (106.88 mg TE/g). Based on these findings, *R. okamurae* has significant potential as a sustainable source of bioactive compounds that can add value to different sectors, including food, feed, pharmaceuticals and cosmetics.

1. Introduction

The introduction of invasive species is one of the biggest threats to biodiversity due to the huge ecological impact it causes. In this respect, seaweeds are considered to be one of the greatest problems due to their contribution to marine primary production and their role as habitat providers for many wildlife species (Navarro-Barranco et al., 2019). There are currently more than 400 species of invasive marine seaweeds worldwide, 50 of which are found in the Iberian Peninsula (Patón et al., 2023).

Rugulopteryx okamurae (*R. okamurae*) is a brown seaweed of the *Dictyotacea* family and native of the Japan, Taiwan, China, Korea and North America area. It was previously registered as *Dilophus okamurae* and it is also known as *Dictyota marginata* or *Dilophus marginatus*. Its presence in Europe was first reported in a French harbour (Thau Lagon) in 2002. Later, in 2015, it was first seen in the Strait of Gibraltar, in Ceuta, but it was not until 2016 when it appeared in Tarifa (Cádiz, Spain). To this date, its presence has also been reported in the Alboran sea, the Provence (France) and in the Portugal coast also reaching the Azores Islands (Faria et al., 2022).

This is a case of cryptic invasion, where the invasive species is so similar to the native one that it can be difficult to identify, in this particular case it may be mistaken for seaweeds from the Dictyota genera (Altamirano et al., 2019). *R. okamurae* is highly competitive, reaching a level of coverage close to 90 % on rocky bottoms (García-Gómez et al., 2020), causing a displacement of the local biota and modifying the communities. It has also been reported to be dangerous for some species of gastropods, echinoderms or even some fish communities due to the production of toxic substances such as terpenoids and other secondary metabolites that prevent its predation (Bernal-Ibáñez et al., 2022). The current presence of this invasive macroalgae not only causes ecological problems, but also socio-economical ones, affecting fishing activity, beach management and tourism (Altamirano et al., 2019). In fact, to prepare for the tourist season, towards the end of spring, massive deposits of *R. okamurae* are usually removed from the coasts, which implies additional costs for the municipalities. For this

* Corresponding author.
E-mail address: conaba@iata.csic.es (A. Martínez-Abad).

https://doi.org/10.1016/j.foodchem.2023.138241
Received 2 August 2023; Received in revised form 15 November 2023; Accepted 18 December 2023
Available online 22 December 2023

Journal of Polymers and the Environment (2023) 31:886–899
https://doi.org/10.1007/s10924-022-02648-2

ORIGINAL PAPER

Sustainable Bio-Based Materials from Minimally Processed Red Seaweeds: Effect of Composition and Cell Wall Structure

Vera Cebrián-Lloret[1] · Antonio Martínez-Abad[1] · Amparo López-Rubio[1] · Marta Martínez-Sanz[2]

Accepted: 17 October 2022 / Published online: 5 November 2022

Abstract
This study reports on the use of whole seaweed biomass to obtain bio-based films for food packaging applications. Specifically, four different species of agarophytes (*Gelidium corneum, Gracilaria chilensis, Gracilaria tenuistipitata* and *Gracilariopsis longissima*) were minimally processed by melt blending and compression molding, and the effect of their composition and cell wall structure on the final performance of the films was investigated. The seaweed biomass was mainly composed of carbohydrates (35–50%), but significant amounts of proteins and ashes were also detected. Temperature-resolved SAXS experiments and microscopy analyses evidenced that a higher temperature of 130 ℃ is required to promote the release of agar from the tougher cell walls from *G. corneum* and *G. tenuistipitata*. The higher cellulose content of *G. corneum* (ca. 15%) resulted in films with higher mechanical resistance and water vapor barrier capacity, while the higher agar content of *G. chilensis* improved the elongation capacity of the films. The results from this work evidence the potential of red seaweed biomass to generate food packaging materials in a cost-effective and environmentally friendly way.

Keywords Biopolymers · Agar · Cellulose · Films · Macroalgae

Introduction

Plastics are one of the materials most widely used due to their low cost, good processability and wide range of barrier and mechanical properties. However, conventional petroleum-based plastics are not biodegradable and therefore accumulate in natural ecosystems for up to several thousand years after disposal, causing serious environmental problems [1–3]. As a more sustainable alternative, biodegradable polymers obtained from renewable natural resources, i.e. biopolymers, are being studied [4–6]. However, the properties of biopolymers are not yet comparable to those of benchmark synthetic polymers, especially in terms of mechanical and barrier properties. Even though for certain applications the properties of some biopolymers may be acceptable, their high production costs are one of the main factors precluding their commercialization. Moreover, the raw materials generally used for biopolymer production come from land-based crops, competing with their traditional use, the food sector. This is why, as an alternative, aquatic biomass sources such as marine plants or seaweeds are being explored [2, 4, 6–8].

Macroalgae are classified into three groups: Ochrophyta, Phaeophyceae (brown seaweeds), Chlorophyta (green seaweeds) and Rhodophyta (red seaweeds) [9–11] and their composition and cell wall structure is strongly dependent on the species. In particular, red seaweeds are rich in minerals and vitamins, as well as some bioactive compounds such as proteins, carotenoids, phenols or lipids [11, 12]. Furthermore, their high polysaccharide content makes them an abundant source of biopolymers. Of particular interest is agar, which is the main structural component of the cell walls in some red seaweed species [13]. It is composed of two main fractions: agarose, responsible for its gelling capacity, which consists of repeating units of alternating β-D -galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl groups; and agaropectin, which has a similar structure, but contains several substituent groups such as sulphates, methyl ethers and pyruvates [13, 14]. Due to its high gelling capacity, agar is widely used in the food and pharmaceutical industry [14, 15]. However, due to its excellent film-forming

✉ Marta Martínez-Sanz
marta.martinez@csic.es

[1] Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

[2] Instituto de Investigación en Ciencias de La Alimentación (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain

Food Research International 178 (2024) 113990

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

In vitro digestibility of proteins from red seaweeds: Impact of cell wall structure and processing methods

Vera Cebrián-Lloret [a], Antonio Martínez-Abad [a], Isidra Recio [b], Amparo López-Rubio [a], Marta Martínez-Sanz [b,*]

[a] Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain
[b] Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain

ARTICLE INFO

Keywords:
Agarophytes
Alternative proteins
Infogest
DIAAS
Extrusion
Algae
Phycocolloids

ABSTRACT

This study aimed to assess the nutritional quality and digestibility of proteins in two red seaweed species, *Gelidium corneum* and *Gracilaropsis longissima*, through the application of *in vitro* gastrointestinal digestions, and evaluate the impact of two consecutive processing steps, extrusion and compression moulding, to produce food snacks. The protein content in both seaweeds was approximately 16 %, being primarily located within the cell walls. Both species exhibited similar amino acid profiles, with aspartic and glutamic acid being most abundant. However, processing impacted their amino acid profiles, leading to a significant decrease in labile amino acids like lysine. Nevertheless, essential amino acids constituted 35–36 % of the total in the native seaweeds and their processed products. Although the protein digestibility in both seaweed species was relatively low (<60 %), processing, particularly extrusion, enhanced it by approximately 10 %. Interestingly, the effect of the different processing steps on the digestibility varied between the two species. This difference was mainly attributed to compositional and structural differences. *G. corneum* exhibited increased digestibility with each processing step, while *G. longissima* reached maximum digestibility after extrusion. Notably, changes in the amino acid profiles of the processed products affected adversely the protein nutritional quality, with lysine becoming the limiting amino acid.

These findings provide the basis for developing strategies to enhance protein quality in these seaweed species, thereby facilitating high-quality food production with potential applications in the food industry.

1. Introduction

The food industry is continuously seeking to develop a broad range of products to cover the diverse demands of consumers, which implies, among others, exploring novel alternative sources of protein production (Fuenzalida et al., 2016; Kaur et al., 2022). Although animal proteins continue to play an important role in the food industry (Pam Ismail et al., 2020), their large-scale production has negative consequences for biodiversity, climate change, and leads to the depletion of freshwater resources. Moreover, motivated by the growth of vegetarian and vegan markets, along with the rising preference for healthier and sustainable dietary options, consumers increasingly opt for plant-based foods over animal-based alternatives, thus reducing their carbon footprint (He et al., 2021; Kumar et al., 2022; McClements & Grossmann, 2021; Williams & Phillips, 2021). Thus, in response to the social, environmental, and economic challenges, it has become crucial to explore new sources of proteins for sustainable food production (Kumar et al., 2022). In this regard, several recent studies have investigated algae as a potential source for obtaining proteins, as well as other bioactive compounds, such as carotenoids, phenols, or lipids (Cebrián-Lloret et al., 2022; Salehi et al., 2019; Yang et al., 2021). Traditionally, the use of algae in food has been closely associated with Asia, where they have been an essential part of the diet and culture for centuries (Mahadevan, 2015). In this region, algae are highly valued for their taste, nutritional value, and health benefits. However, in recent years, the interest in algae as a food source has transcended Asian borders and spread globally due to their interesting nutritional profile (good source of proteins, high dietary fibre content and abundance of vitamins and minerals) and sustainable potential as a food resource (Rawiwan et al., 2022; Rioux et al., 2017).

The term algae refers to a diverse group of eukaryotic, photosynthetic organisms which includes both microalgae (unicellular organisms) and macroalgae (or seaweeds), which are multicellular and

* Corresponding author.
E-mail address: marta.martinez@csic.es (M. Martínez-Sanz).

https://doi.org/10.1016/j.foodres.2024.113990
Received 13 September 2023; Received in revised form 2 January 2024; Accepted 5 January 2024
Available online 6 January 2024

Algal Research 66 (2022) 102802

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Agar-based packaging films produced by melt mixing: Study of their retrogradation upon storage

Vera Cebrián-Lloret [a], Gülden Göksen [b], Antonio Martínez-Abad [a,c], Amparo López-Rubio [a,c], Marta Martínez-Sanz [c,d,*]

[a] Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain
[b] Department of Food Technology, Vocational School of Technical Sciences at Mersin Tarsus Organised Industrial Zone, Tarsus University, 33100 Mersin, Turkey
[c] Interdisciplinary Platform for Sustainable Plastics Towards a Circular Economy-Spanish National Research Council (SusPlast-CSIC), Madrid, Spain
[d] Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain

ARTICLE INFO

Keywords:
Seaweed
Valorization
Retrogradation
Agar
Biopolymers
Packaging

ABSTRACT

The ability of agar with different purification degrees to produce packaging films has been evaluated and the stability of the obtained materials after prolonged storage has been investigated. The less purified agar resulted in films with higher water vapor permeability and lower mechanical performance than pure commercial agar. However, the commercial agar film required the addition of a plasticizer to produce films that could be manipulated. It has also been observed that prolonged storage at 53 % RH results in a change in the semi-crystalline structure of the agar and in water-polysaccharide interactions. As a result, pure agar films undergo a rigidizing effect resulting in unmanageable films after 7 days of storage. The presence of glycerol improved the stability of the films by limiting the structural changes up to 14 days of storage. In contrast, the films from the least purified agar extract, seemed to be less affected by moisture, showing a higher stability during storage. This points to the potential of the less purified extract to be used as an additive to reduce costs and improve the storage stability of pure agar films.

1. Introduction

The excessive production of petroleum-derived plastics has become a major problem in recent years. This is mainly due to the fact that these materials are not biodegradable; consequently, they accumulate in natural ecosystems for hundreds of years causing severe environmental problems [1–3]. Although recycling strategies have been promoted worldwide over the last decade, the replacement of conventional plastics with biodegradable polymers obtained from renewable natural resources, i.e. biopolymers, is being studied and considered as a more sustainable alternative to address this problem in the long term. However, the production costs of biopolymers are too high to compete against conventional petroleum-based polymers on the market nowadays. On top of that, their properties are not yet comparable to those of benchmark synthetic polymers, especially in terms of mechanical and barrier properties. Moreover, the raw materials commonly used for the production of biopolymers come from land-based crops and, thus, they compete with their main use as food sources. This is why, as an alternative, marine resources (e.g. aquatic plants or seaweeds) are being explored as a source for obtaining biopolymers [4–8]. The cell walls of seaweeds are rich in polysaccharides, whose composition depends on the seaweed species, being cellulose, the most important structural component providing mechanical strength, while other polysaccharides are responsible for different functionalities. In particular, sulphated polysaccharides (i.e. carrageenan and agar) are highly relevant to the food industry, due to their extensive use as gelling agents, thickeners and stabilizers [9,10]. Amongst them, agar, which is typically found in the cell walls from some red seaweeds (Rhodophyceae) [11], has a great industrial relevance as gelling agent, not only for food applications, but also for microbiology. This polysaccharide contains two main components: agarose and agaropectin. Agarose constitutes the gelling fraction and consists of alternating units of β-D-galactopyranosyl and 3,6-anhydrous-α-L-galactopyranosyl. On the other hand, agaropectin presents a structure similar to agarose, but contains 5–10 % sulphate esters in addition to other residues such as methoxyl groups and pyruvic acid [12–14]. The agar extraction protocol is very well established at industrial scale; it involves the application of alkaline pre-treatments, followed by high temperature and pressure extraction and several

* Corresponding author at: Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain.
E-mail address: marta.martinez@csic.es (M. Martínez-Sanz).

https://doi.org/10.1016/j.algal.2022.102802
Received 3 April 2022; Received in revised form 12 July 2022; Accepted 19 July 2022
Available online 29 July 2022

Food Hydrocolloids 146 (2024) 109177

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Exploring alternative red seaweed species for the production of agar-based hydrogels for food applications

Vera Cebrián-Lloret [a], Antonio Martínez-Abad [a], Amparo López-Rubio [a], Marta Martínez-Sanz [b,*]

[a] Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain
[b] Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM, CEI UAM + CSIC), Nicolás Cabrera 9, 28049, Madrid, Spain

ARTICLE INFO

Keywords:
Seaweeds
Agar
Hydrogels
Gelation
SAXS

ABSTRACT

Three different red seaweed species, *Gelidium corneum* and two *Gracilaria* species (*Agarophyton chilensis*, and *Gracilariopsis longissima*), were used to produce agar-based fractions through conventional and simplified extraction methods and their composition and gel-forming properties were evaluated. The use of an alkaline pre-treatment was effective in removing impurities such as proteins, lipids, and ash, while the agarose/agaropectin ratio in agar was not affected. This led to the formation of hydrogels with higher stiffness and strength. Surprisingly, the presence of semi-crystalline agaropectin in the agar fractions from the two *Gracilaria* species, especially *G. longissima*, promoted the formation of more densely packed and stronger hydrogel networks, with higher gelling temperatures and superior mechanical properties. Thus, these results suggest that *G. longissima* has the potential to be used as an alternative to the more widespread use of *G. corneum* for the production of agar hydrogels for food applications.

1. Introduction

Thickening and gelling agents play a critical role in the food industry, as they are widely employed to modify the texture and consistency of food products (Qin, Jiang, Zhao, Zhang, & Wang, 2018). These gelling agents are typically hydrocolloids that have the unique ability to bind with water molecules and produce gel-like structures, imparting a desirable texture to the final product (Fathima et al., 2022; Williams & Phillips, 2021, pp. 3–26). The properties of the resulting gels, such as firmness, elasticity, and stability, depend on the type and concentration of the gelling agent used (Cong et al., 2022), as well as the gelling mechanism and parameters affecting the process, such as the temperature, presence of salts, etc. Traditionally, thickening and gelling agents derived from animals, such as gelatine derived from animal bones and skin, have been extensively utilized in the food industry (Williams & Phillips, 2021, pp. 3–26). However, due to the heightened consciousness of animal welfare, the rapid growth of the vegetarian and vegan markets, and consumers' inclination towards healthy and eco-friendly food options, individuals are increasingly willing to modify their habits and assume accountability for climate change by selecting plant-based foods over animal-based alternatives, thereby reducing their carbon footprint (He, Meda, Reaney, & Mustafa, 2021; McClements & Grossmann, 2021; Williams & Phillips, 2021, pp. 3–26). In this context, marine sources, such as seaweeds or aquatic plants, are being investigated as a potential source of biopolymers with gelling properties (Kartik et al., 2021; Martínez-Sanz, Cebrián-Lloret, Mazarro-Ruiz, & López-Rubio, 2020; Özçimen, Benan, Morkoç, & Efe, 2017, pp. 7–14). In particular, algal cell walls contain a variety of polysaccharides with different functionalities which can be exploited as gelling agents (Kartik et al., 2021). Sulphated polysaccharides, such as carrageenan and agar, are of particular interest to the food industry as they can be used as gelling, thickening, and stabilizing agents (Cebrián-Lloret, Göksen, Martínez-Abad, López-Rubio, & Martínez-Sanz, 2022; Jayakody, Vanniarachchy, & Wijesekara, 2022; Pangestuti & Kim, 2015). Agar, found in the cell walls of some red seaweed species (Rhodophyta), is composed of two main fractions: agarose, the gelling fraction, which consists of alternating units of β-D-galactopyranose and 3,6-anhydro-α-L-galactopyranose, and agaropectin, which presents a structure similar to agarose but may also contain sulphate esters and other residues, such as methoxyl groups and pyruvic acid. The presence of a greater proportion of sulphate groups in agar is frequently related to a reduced gelling ability and it influences gelling temperature and melting behaviour (Cebrián-Lloret, Göksen, et al., 2022; Lee et al., 2017; Martínez-Sanz, Ström, et al., 2020; Yarnpakdee, Benjakul, & Kingwascharapong, 2015). For the extraction of

* Corresponding author
E-mail address: marta.martinez@csic.es (M. Martínez-Sanz).

https://doi.org/10.1016/j.foodhyd.2023.109177
Received 21 June 2023; Received in revised form 3 August 2023; Accepted 15 August 2023
Available online 18 August 2023

Algal Research 61 (2022) 102576

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Valorization of alginate-extracted seaweed biomass for the development of cellulose-based packaging films

Vera Cebrián-Lloret [a], Mandy Metz [a], Antonio Martínez-Abad [a,c], Svein Halvor Knutsen [b], Simon Ballance [b], Amparo López-Rubio [a,c], Marta Martínez-Sanz [a,c,*]

[a] Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain
[b] Nofima AS, Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås, Norway
[c] Interdisciplinary Platform for Sustainable Plastics Towards a Circular Economy-Spanish National Research Council (SusPlast-CSIC), Madrid, Spain

ARTICLE INFO

Keywords:
Seaweed
Valorization
Cellulose
Biopolymers
Packaging

ABSTRACT

Seaweed residues from *Alaria esculenta*, *Saccharina latissima* and *Ascophyllum nodosum* after alginate extraction have been valorized to produce cellulose-based fractions with different purification degrees. The residues were mainly composed of carbohydrates (35–57%) and proteins (12–37%), *Alaria* and *Saccharina* being richer in cellulose and *Ascophyllum* richer in fucoidan. The lower cellulose content in the latter made it unsuitable for the extraction of cellulosic fractions.

Self-supporting films were obtained from the cellulosic fractions from *Saccharina* and *Alaria* residues. While the higher cellulose purity films presented more desirable characteristics in terms of mechanical properties (with elastic moduli of ca. 5–7 GPa and elongation values of ca. 3–5%) and visual appearance, the presence of non-cellulosic components in the films from less purified fractions reduced their water sensitivity and promoted greater water barrier (with water permeability values of ca. 4–6 kg·m/s·m^2·Pa). These results point towards the potential of a simple alkaline extraction to generate cellulose-based films from seaweed residuals with the best compromise between functional properties and economical and environmental efficiency.

1. Introduction

Since synthetic polymers began replacing natural materials more than half a century ago, the use of plastics has grown exponentially and is now an indispensable part of our lives [1]. The massive use of synthetic plastics is mostly due to their easy manufacturability, good insulating properties, low cost, high mechanical strength and durability [2,3]. However, it is precisely their extremely long durability that has led to the persistence of plastic waste, resulting in the accumulation of more than 25 million tons of plastic waste in the environment each year [4]. In this regard, the replacement of conventional plastics with biodegradable polymers made from renewable natural resources, i.e. biopolymers, are being considered lately as a more sustainable alternatives. However, the properties of biopolymers are still not comparable to those of reference synthetic polymers, especially in terms of barrier and mechanical properties, and their production costs are too high to compete in the market. Moreover, raw materials generally used for the production of biopolymers come from land-based crops and therefore compete with their traditional use: the food and feed industries. In this context, aquatic biomass sources, such as seaweeds and aquatic plants, rich in cellulose, are being explored as an efficient alternative to land-based biomass for the production of biopolymers. Of special interest may be the use of seaweed industrial waste streams, such as those generated during alginate production. Alginate is an anionic polysaccharide found in brown seaweeds (*Phaeophyta*), widely used in the food industry, mainly as stabilizing, emulsifying, gelling, and thickening agent [5,6]. The alginate-producing industry typically extracts this phycocolloid from brown seaweeds by applying the following main steps: dilute formaldehyde treatment, dilute acid treatment, alkaline extraction, solid-liquid separation, precipitation and drying. During alkaline extraction the acidified seaweeds are immersed in a sodium carbonate or sodium hydroxide solution to convert the insoluble alginic acid into soluble sodium alginate [7,8]. Depending on the seaweed species, the season and extraction parameters used, the extraction yields of alginate have been reported to range from 17% to 40% [9–11]. Therefore, large amounts of seaweed residues are generated at industrial

* Corresponding author at: Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain.
E-mail address: mmartinez@iata.csic.es (M. Martínez-Sanz).

https://doi.org/10.1016/j.algal.2021.102576
Received 10 June 2021; Received in revised form 10 November 2021; Accepted 25 November 2021
Available online 11 December 2021

Annex B: List of additional publications

Carbohydrate Polymers 233 (2020) 115887

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Improved performance of less purified cellulosic films obtained from agar waste biomass

Marta Martínez-Sanz*, Vera Cebrián-Lloret, Jesús Mazarro-Ruiz, Amparo López-Rubio

Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

ARTICLE INFO

Keywords:
Seaweed
Gelidium
Valorization
Cellulose
Agar
Biopolymers
Packaging

ABSTRACT

The residues generated after the extraction of agar from *Gelidium sesquipedale* by means of a hot-water treatment, with (NaOH + HW residue) and without (HW residue) an alkali pre-treatment have been valorized to produce high performance cellulosic films. Both residues were mainly composed of structural carbohydrates (in particular, agar), ashes and lipids. The residual agar could only be completely removed by applying a two-step process based on bleaching and alkaline treatments.

The application of the alkaline pre-treatment for the extraction of agar did not significantly affect the properties of the films produced from the extracted fractions, hence making the HW residue more sustainable and economically viable. The agar remaining in the less purified fractions had a positive effect on the performance of the films, improving their transparency, mechanical properties and water vapour barrier, outperforming benchmark biopolymers; in addition, these materials presented antioxidant capacity inhibiting the degradation of β-carotene.

1. Introduction

The severe environmental issues associated to the use of synthetic plastics are currently one of the hot topics in the society. Synthetic plastics are petroleum-based, hence consuming large amounts of fossil fuels for their production; moreover, they are not biodegradable and, thus, after disposal they can accumulate in natural ecosystems up to several thousands of years. Given the large amount of plastics consumed nowadays, with almost 350 million tons produced worldwide in 2017 (Plastics - the Facts 2018, 2018), it is necessary to find a long-term solution to overcome these issues. The replacement of conventional plastics by biodegradable polymers obtained from renewable natural resources (i.e. biopolymers) is envisaged as the most sustainable and long-term solution. However, the properties of biopolymers are still far from those of benchmark synthetic polymers (especially in terms of barrier and mechanical performance) and their production costs are too high to compete in the market. Furthermore, the raw materials typically used for the production of biopolymers originate from land crops, whose primary use is the food sector. As an alternative, marine resources such as seaweeds and aquatic plants, given their abundance and interesting composition, are also being explored as a source for the production of biopolymers (Benito-González, López-Rubio, Gavara, & Martínez-Sanz, 2019; Cian, Salgado, Drago, González, & Mauri, 2014; Khalil et al., 2017; Martínez-Sanz, Erboz, Fontes, & López-Rubio, 2018).

Amongst the several biopolymers which are being currently explored for their utilization as packaging materials, cellulose is one of the most promising. Although cellulose has been typically extracted from land crops, it can also be obtained from aquatic plants and seaweeds. Interestingly, in line with circular economy policies, several recent works have reported on the valorization of low-cost aquatic biomass such as residues directly derived from seaweed/aquatic plants (Benito-González, López-Rubio, Gavara et al., 2019; de Oliveira et al., 2019; Fontes-Candia, Erboz, Martínez-Abad, López-Rubio, & Martínez-Sanz, 2019) and from industrial processes making use of algal biomass (Chen, Lee, Juan, & Phang, 2016; El Achaby, Kassab, Aboulkas, Gaillard, & Barakat, 2018; Singh, Gaikwad, Park, & Lee, 2017) for the extraction of cellulosic materials. Cellulose is typically isolated from the raw biomass by applying a sequential extraction protocol to remove other cell wall components such as lignin, non-cellulosic carbohydrates and other minor compounds (proteins, waxes, pigments, lipids, etc.) (Lu & Hsieh, 2012; Oksman, Etang, Mathew, & Jonoobi, 2011; Sun, Sun, Zhao, & Sun, 2004). While most of the available studies in the literature focus on the purification of cellulose, recent work has demonstrated that the presence of other components such as non-cellulosic carbohydrates and/or lipidic compounds in less purified fractions may enhance the mechanical and barrier properties of cellulosic films (Benito-

* Corresponding author.
E-mail address: mmartinez@iata.csic.es (M. Martínez-Sanz).

https://doi.org/10.1016/j.carbpol.2020.115887
Received 21 November 2019; Received in revised form 15 January 2020; Accepted 15 January 2020
Available online 16 January 2020

Artículos

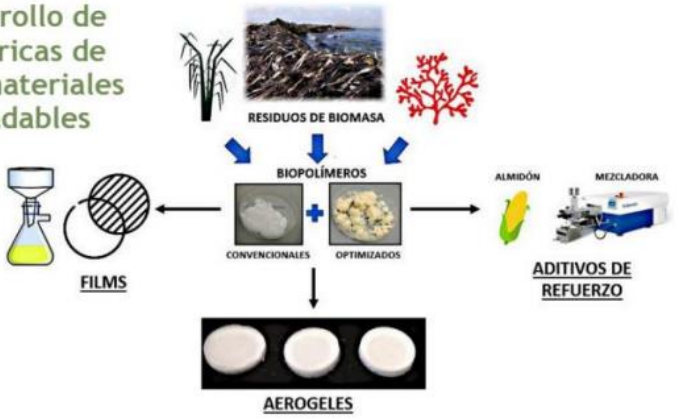
Valorización de biomasa acuática para el desarrollo de estructuras biopoliméricas de uso alimentario (1): materiales para envases biodegradables

Autores: Isaac Benito-González, Marta Martínez-Sanz, Cynthia Fontes-Candia, Vera Cebrian-Lloret, Antonio Martínez-Abad, Amparo López-Rubio*

Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

Interdisciplinary Platform for Sustainable Plastics towards a Circular Economy- Spanish National Research Council (SusPlast-CSIC), Madrid, Spain

*amparo.lopez@iata.csic.es



Resumen

La biomasa acuática representa un recurso altamente inexplorado y con un tremendo potencial para el desarrollo de estructuras biopoliméricas con aplicación en el área de envasado alimentario. En general, la celulosa es el biopolímero más ampliamente utilizado como aditivo en estructuras de envase alimentario, pero las hemicelulosas presentes en la biomasa vegetal en general y, más concretamente, en la biomasa acuática, pueden aportar propiedades muy interesantes a los materiales. Por ello, en este artículo se describen metodologías simplificadas para la obtención de distintas fracciones a partir de algas y plantas acuáticas y su utilización para el desarrollo de distintos tipos de estructuras y/o aditivos de envase biodegradables y sostenibles.

Palabras clave: algas; *Gelidium sesquipedale*; biomasa acuática; envases biodegradables; celulosa.

Abstract

Aquatic biomass represents a highly unexplored resource with a tremendous potential for the development of biopolymeric structures with application in the food packaging area. In general, cellulose is the most widely used biopolymer as additive in food packaging structures, but hemicelluloses present in vegetable biomass in general and, more particularly, in aquatic biomass, can provide very interesting properties to the materials. Therefore, in this manuscript we describe simplified methodologies to obtain different fractions from seaweed and aquatic plants and their use for developing different types of food packaging structures and/or additives which are biodegradable and sustainable.

Keywords: seaweed; *Gelidium sesquipedale*; aquatic biomass; biodegradable packaging; cellulose.

Artículos

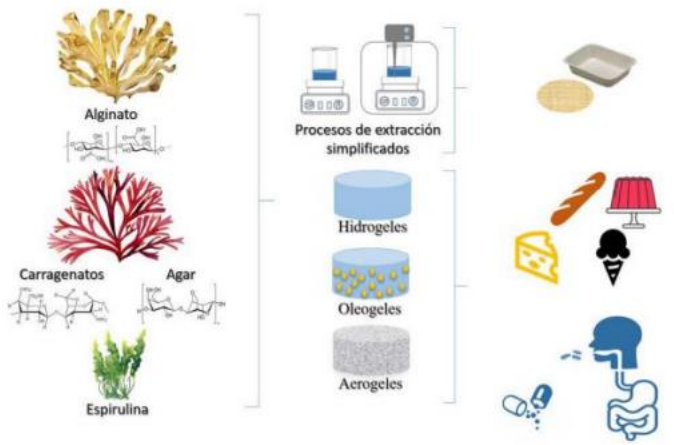
Valorización de biomasa acuática para el desarrollo de estructuras biopoliméricas de uso alimentario (2): aplicaciones como texturizantes de alimentos

Autores: Cynthia Fontes-Candia, Marta Martínez-Sanz, Isaac Benito-González, Vera Cebrian-Lloret, Antonio Martínez-Abad, Amparo López-Rubio*

Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

Interdisciplinary Platform for Sustainable Plastics towards a Circular Economy- Spanish National Research Council (SusPlast-CSIC), Madrid, Spain

*amparo.lopez@iata.csic.es



Resumen

Ficocoloides como agar, carragenatos o alginatos, extraídos a partir de algas rojas y marrones, se utilizan ampliamente en la industria alimentaria como texturizantes y pueden estructurarse para formar distintos tipos de geles (hidrogeles, oleogeles y aerogeles). Frente a los métodos tradicionales de extracción utilizados a nivel industrial, se están explorando métodos más eficientes que dan lugar a extractos menos purificados, pero con funcionalidades adicionales. Entender los procesos de gelificación y cómo la composición afecta a la estructuración de estos biopolímeros es clave para el correcto diseño en función de la aplicación final deseada. En este artículo se describen, por un lado, metodologías simplificadas para la extracción de estos ficocoloides y, por otro lado, el uso de los mismos como ingredientes texturizantes.

Palabras clave: algas; *Gelidium sesquipedale*; biomasa acuática; ficocoloides; capacidad antioxidante; geles.

Abstract

Phycocolloids like agar, carrageenan or alginates, extracted from red and brown seaweed, are broadly used in the food industry as texturizing ingredients and they can be structured giving rise to different types of gels (hydrogels, oleogels and aerogels). In order to make phycocolloids extraction processes more efficient industrially, alternative protocols are being established which give rise to less purified extracts, but with additional functionalities. Understanding the gelling processes and how composition affects biopolymer structuring is key for the rational design aiming for a certain application. In this manuscript we describe, on one hand, simplified methodologies to extract the phycocolloids from seaweeds and, on the other hand, the use of the extracted biopolymers as texturizing food ingredients.

Keywords: seaweed; *Gelidium sesquipedale*; aquatic biomass; phycocolloids; antioxidant capacity; gels.