DOCTORAL THESIS

Biorefinery of the relevant brown algae *Saccharina latissima* and *Ascophyllum nodosum* towards novel, sustainable, and functional ingredients

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ABSTRACT

This doctoral thesis aims to provide fundamental and practical knowledge on the valorization of brown seaweeds. It has sought to understand the relationship between the structure and functionality of their polysaccharides and proteins, considering their functional, technological and bioactive properties. All this has been approached from a biorefinery perspective, focused on circular economy and sustainability.

First, an optimization for extracting valuable compounds from brown seaweeds was carried out using various innovative technologies. A high-pressure pre-treatment of brown seaweeds (*Ascophyllum nodosum* and *Saccharina latissima*) to extract alginate was investigated and compared with the conventional extraction method. The purpose was to understand how initial differences in algal cell wall composition and structure affected the extraction of this polysaccharide and its technological properties, such as its ability as emulsifier and gelling agent. Likewise, the extraction of protein-polysaccharide conjugates was examined by sequential application of several extraction methods, as well as their functional properties (water, oil retention activity and emulsifying capacity) and bioactive properties (antioxidant activity and antihypertensive capacity). In these studies, differences were observed in the cell wall architecture of the algae, belonging to different orders, such as Fucales and Laminariales, which directly influenced the extracted compounds and their structural and technological properties. However, despite these differences, the results showed the potential of the extracted compounds as ingredients in the food and pharmaceutical industries.

The second section of this thesis explored the valorization of by-products generated during alginate extraction. An exhaustive study of all the fractions and by-products produced in this process was carried out to identify the most promising fractions for valorization. The results showed that solid wastes had the most significant potential due to their physicochemical composition. Therefore, possible strategies to valorize these wastes were suggested, highlighting their high potential as bioactive, texturizing, or nutritional value-added ingredients for food, feed, cosmetic, and pharmaceutical applications. Besides, from this work, sequential and combined extraction by different methods was investigated to recover as much intracellular protein as possible from these solid wastes. It was found that the protein fraction of the extracts contained a high percentage of essential amino acids, adequate functional properties, and an interesting antihypertensive activity.

Finally, the third part of this thesis focused on generating knowledge through the structural elucidation of alginate. For this purpose, a rapid method was developed to determine the M/G ratio of alginate, which showed values close to the reference method (^{1}H NMR), demonstrating its accuracy. Overall, this PhD thesis represents a significant advance in generating knowledge and valorizing algal biomass and its residues into high-value compounds. Furthermore, these approaches are potentially applicable to other algal biomasses.

RESUMEN

Esta tesis doctoral pretende aportar conocimientos fundamentales y prácticos sobre la valorización de las algas pardas. Se ha tratado de comprender la relación entre la estructura y funcionalidad de sus polisacáridos y proteínas, considerando sus propiedades funcionales, tecnológicas y bioactivas. Todo ello se ha abordado desde una perspectiva de biorrefinería, enfocada a la economía circular y la sostenibilidad.

En primer lugar, se ha llevado a cabo una optimización para la extracción de compuestos valiosos a partir de algas pardas utilizando diversas tecnologías innovadoras. Se investigó un pretratamiento a alta presión de algas pardas (*A. nodosum y S. latissima*) para extraer alginato y se comparó con el método de extracción convencional. El objetivo era comprender cómo las diferencias iniciales en la composición y estructura de la pared celular de las algas afectaban a la extracción de este polisacárido y a sus propiedades tecnológicas, así como, su capacidad como emulsionante y gelificante. Asimismo, se examinó la extracción de conjugados proteína-polisacárido mediante la aplicación secuencial de varios métodos de extracción, así como sus propiedades funcionales (capacidad de retención de agua y aceite, emulsionante) y bioactivas (actividad antioxidante y capacidad antihipertensiva). En estos estudios se observaron diferencias en la arquitectura de la pared celular de las algas, pertenecientes a distintos órdenes, como Fucales y Laminariales, que influyeron directamente en los compuestos extraídos y en sus propiedades estructurales y tecnológicas. Sin embargo, a pesar de estas diferencias, los resultados mostraron el potencial de

los compuestos extraídos como ingredientes en la industria alimentaria y farmacéutica.

La segunda sección de esta tesis exploró la valorización de los subproductos generados durante la extracción del alginato. Se realizó un estudio exhaustivo de todas las fracciones y subproductos producidos en este proceso con el objetivo de identificar las fracciones más prometedoras para su valorización. Los resultados mostraron que los residuos sólidos tenían el potencial más significativo debido a su composición fisicoquímica. Por lo tanto, se sugirieron posibles estrategias para valorizar estos residuos, destacando su alto potencial como ingredientes bioactivos, texturizantes o de valor añadido nutricional para aplicaciones alimentarias, de piensos, cosméticos y farmacéuticos. Además, a partir de este trabajo, se investigó la extracción secuencial y combinada por diferentes métodos para recuperar la mayor cantidad posible de proteína intracelular de estos residuos sólidos. Se comprobó que la fracción proteica de los extractos contenía un alto porcentaje de aminoácidos esenciales, adecuadas propiedades funcionales y una interesante actividad antihipertensiva.

Finalmente, la tercera parte de esta tesis se centró en la generación de conocimiento a través de la elucidación estructural del alginato. Para ello, se desarrolló un método rápido para determinar la relación M/G del alginato, que mostró valores cercanos al método de referencia (1H NMR), demostrando su precisión. En conjunto, esta tesis doctoral representa un avance significativo en la generación de conocimiento y valorización de la biomasa algal y sus residuos en compuestos de alto valor. Además, estos enfoques son potencialmente aplicables a otras biomasas algales.

RESUM

Esta tesi doctoral pretén aportar coneixements fonamentals i pràctics sobre la valorització de les algues marrons. S'ha tractat de comprendre la relació entre l'estructura i funcionalitat dels seus polisacàrids i proteïnes, considerant les seues propietats funcionals, tecnològiques i bioactivas. Tot això s'ha abordat des d'una perspectiva de biorrefinería, enfocada a l'economia circular i la sostenibilitat.

En primer lloc, s'ha dut a terme una optimització per a l'extracció de compostos valuosos a partir d'algues marrons utilitzant diverses tecnologies innovadores. Es va investigar un pretractament a alta pressió d'algues marrons (*A. nodosum* i *S. latissima*) per a extraure alginat i es va comparar amb el mètode d'extracció convencional. L'objectiu era comprendre com les diferències inicials en la composició i estructura de la paret cel·lular de les algues afectaven l'extracció d'este polisacàrid i a les seues propietats tecnològiques, així com, la seua capacitat com a emulsionant i gelificant. Així mateix, es va examinar l'extracció de conjugats proteïna-polisacàrid mitjançant l'aplicació seqüencial de diversos mètodes d'extracció, així com les seues propietats funcionals (capacitat de retenció d'aigua i oli, emulsionant) i bioactivas (activitat antioxidant i capacitat antihipertensiva). En estos estudis es van observar diferències en l'arquitectura de la paret cel·lular de les algues, pertanyents a diferents ordes, com Fucales i Laminariales, que van influir directament en els compostos extrets i en les seues propietats estructurals i tecnològiques. No obstant això, malgrat estes diferències, els resultats van mostrar el potencial dels compostos extrets com a ingredients en la indústria alimentària i farmacèutica.

La segona secció d'esta tesi va explorar la valorització dels subproductes generats durant l'extracció de l'alginat. Es va realitzar un estudi exhaustiu de totes les fraccions i subproductes produïts en este procés amb l'objectiu d'identificar les fraccions més prometedores per a la seua valorització. Els resultats van mostrar que els residus sòlids tenien el potencial més significatiu a causa de la seua composició fisicoquímica. Per tant, es van suggerir possibles estratègies per a valorar estos residus, destacant el seu alt potencial com a ingredients *bioactivos, texturitzants o de valor afegit nutricional per a aplicacions alimentàries, de pinsos, cosmètics i farmacèutics. A més, a partir d'este treball, es va investigar l'extracció seqüencial i combinada per diferents mètodes per a recuperar la major quantitat possible de proteïna intracel·lular d'estos residus sòlids. Es va comprovar que la fracció proteica dels extractes contenia un alt percentatge d'aminoàcids essencials, adequades propietats funcionals i una interessant activitat antihipertensiva.

Finalment, la tercera part d'esta tesi es va centrar en la generació de coneixement a través de l'elucidació estructural de l'alginat. Per a això, es va desenvolupar un mètode ràpid per a determinar la relació M/G de l'alginat, que va mostrar valors pròxims al mètode de referència (1H NMR), demostrant la seua precisió. En conjunt, esta tesi doctoral representa un avanç significatiu en la generació de coneixement i valorització de la biomassa algal i els seus residus en compostos d'alt valor. A més, estos enfocaments són potencialment aplicables a altres biomasses algals.

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I. Introduction

"What we know is a drop, what we don't know is an ocean."
Isaac Newton

1. Valorization of brown seaweeds

Brown seaweeds are a macroalgae group comprising between 1500 and 2000 species classified in 250 genera belonging to the class Phaeophyceae and, in particular, to two orders, Laminariales and Fucales (Kouhgardi et al., 2023). These types of seaweeds are mainly found along rocky shores, in cold water habitats in the northern and southern hemispheres, where temperatures are below 20ºC (Sudhakar et al., 2018). Traditionally, seaweed harvesting has been performed by hand. However, growing interest and increased demand for seaweeds have led to the development and optimizing seaweed cultivation in several Asian, European, and American countries (Peteiro, 2018a).

The world production of brown seaweeds (including cultivation and wild harvesting) has increased an estimated 60-fold over the past 70 years, from 0.3 million wet tonnes in 1950 to 17.6 million wet tonnes in 2020, owing to its growth in cultivation (Figure 1a). In 2020, brown seaweed cultivation accounted for 47.7% of the whole production in 20 countries. The largest brown seaweed-producing countries are China, the Republic of Korea, the Democratic People's Republic of Korea, Japan, and Norway (Figure 1b). China is the leading country in the world's production of brown seaweed, with 77.5%, and the main species produced are *Laminaria japonica*, *Undaria pinnatifida*, and *Sargassum fusiforme* (FAO, 2022). The increase in the cultivation of brown seaweeds is mainly due to interest as a food product and as a raw material for extracting hydrocolloids (Araújo et al., 2021). Although brown algae have played a role in human nutrition throughout history, recent studies have shown that algal biomass has a promising content of other

nutritionally relevant macronutrients. These macronutrients are mainly polysaccharides, proteins, and phenolic compounds (Reboleira et al., 2019).

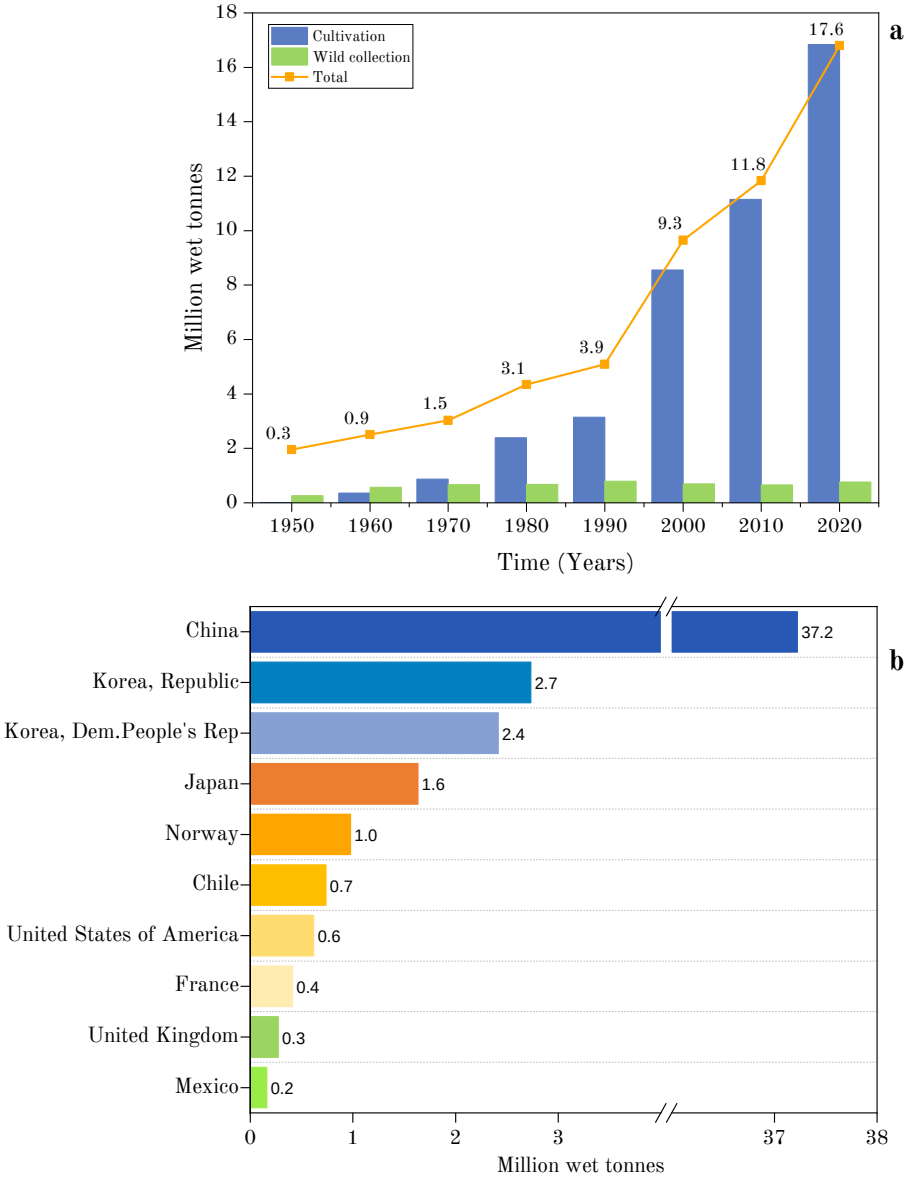

Figure 1. (a) Brown seaweed world production, 1950-2020, (b) Principal brown seaweed-producing countries (million wet tonnes). Data source: FAO 2022. Global Fishery and Aquaculture Production Statistics (FishStatJ)

Therefore, their food, biomedical and pharmaceutical applications are numerous. In fact, in 2019, the algae industry accounted for an estimated total annual value of US$ 2.65 billion, of which about US$ 1.7 billion came from the hydrocolloid industry (Cai et al., 2021).

Despite the use of algae as a commodity at the industrial level, marine biomass is highly underexploited and mostly unknown compared to plant terrestrial biomass. Thanks to global policies promoting a "blue bioeconomy," much industrial and research efforts have been devoted to understanding marine biomass and exploring potential applications to produce added-value bioproducts (Bojorges et al., 2022).

Currently, there is a growing interest in biorefineries harnessing as much biomass as possible for the simultaneous production of bioenergy and bioproducts (Wei et al., 2013). The biorefinery concept contemplates the cascade valorization approach to obtain products (including selective and sequenced extraction of added-value compounds) and produce biofuels, biostimulants, bioplastics, pigments, and other compounds of interest (Wei et al., 2013) (Figure 2).

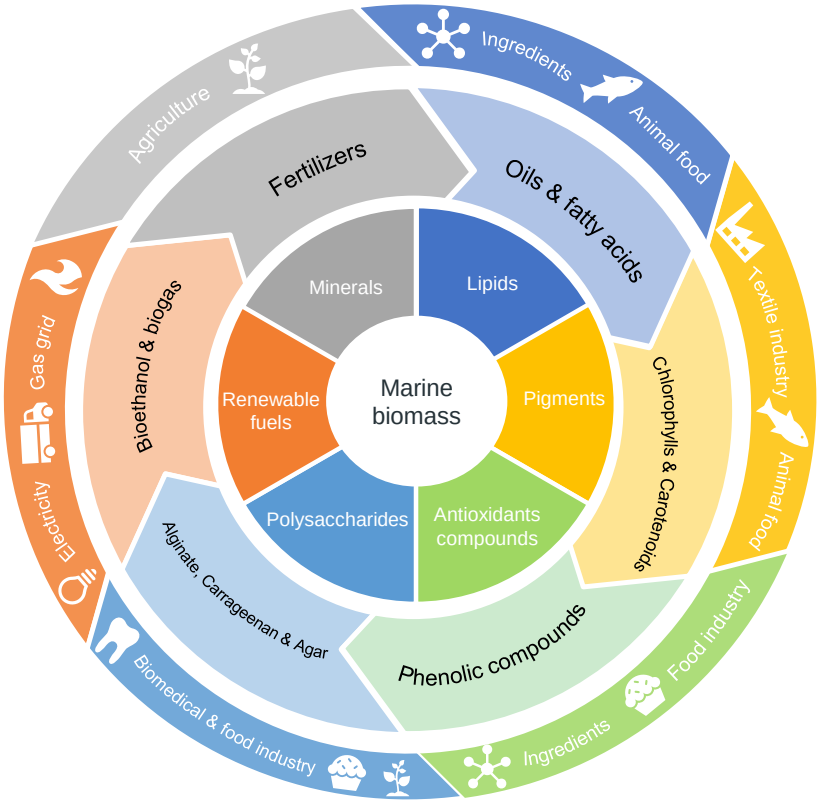

Figure 2. Diagram for using marine biomass as a renewable source of compounds of interest in the industry. Adapted from Pardilhó et al. (2022).

1.1 Composition

Brown seaweeds are considered an excellent potential source of highly bioactive compounds, such as phenolic compounds, carotenoids, proteins and, especially, polysaccharides (Silberfeld et al., 2010; Wijesinghe & Jeon, 2012). Compared to terrestrial plants, brown seaweeds have higher water, carbohydrate and protein content and lower lipid content. Table 1 shows the gross compositional range of brown seaweeds.

Table 1. Gross compositional range of brown seaweeds (Sudhakar et al., 2018).

Component	Composition
Water	90 % wet weight
Carbohydrates	30 – 50 % dry weight
Proteins	3 – 15 % dry weight
Minerals	7 - 38 % dry weight
Lipids	1 – 3 % dry weight

The main polysaccharides in greater proportion in the cell wall of brown algae are alginate (which represents approx. 40% of the dry matter of seaweed), fucoidan, and laminarin (Łabowska et al., 2019). However, the content of these polysaccharides in brown algae depend on the species, harvest period, habitat conditions and the part of the thallus (Chandia et al., 2001; Haug et al., 1974).

On the other hand, protein is another one of the main compounds present in brown seaweed. These protein fractions are composed of abundant essential amino acids and bioactive peptides, which could be used for human and animal nutrition purposes (Zhou et al., 2022) as it will be discussed below.

1.1.1. Alginate

Alginate is the main structural constituent of brown algae cell walls and intercellular matrix, which provides the mechanical strength and flexibility necessary to withstand the force of the sea (Łabowska et al., 2019).

Alginate is a linear copolymer constituted by monomeric units of 1→4 linked α-L-guluronic acid (G block) and β-D-mannuronic acid (M block) (Figure 3a) (Usov & Zelinsky, 2013). These uronic acids can form homogeneous blockchains of MM or GG units and chains with alternate blocks of mannuronic and guluronic acid (MG) (Figure 3b) (Haug et al., 1967). Therefore, the pattern of sequence, the G-block length, the M/G ratio, and the molecular weight are important factors that influence the physicochemical properties of alginate (Fertah et al., 2017).

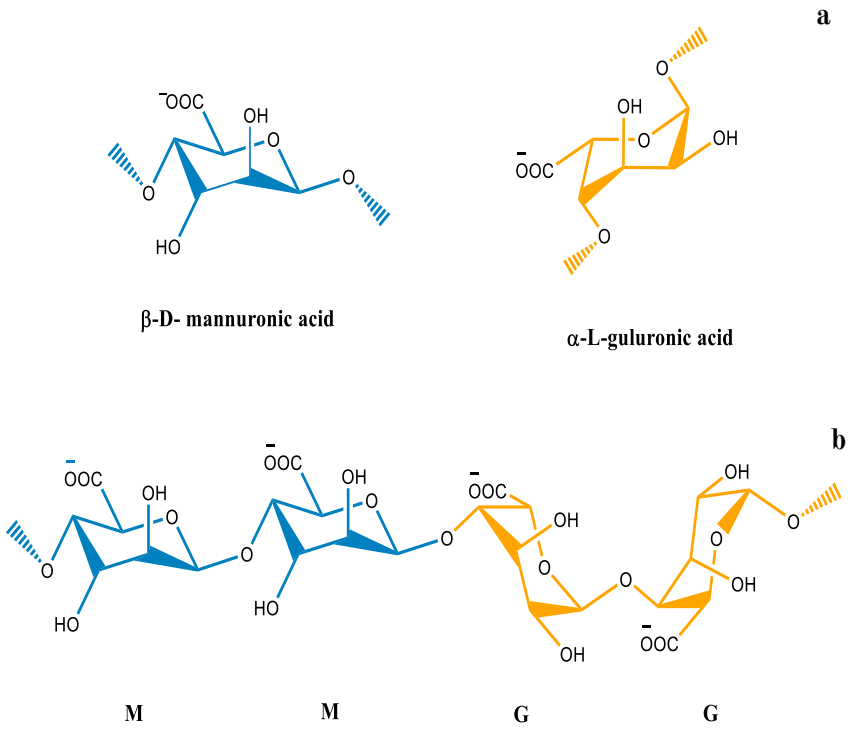

Figure 3. (a) Chemical structure of β-D-mannuronic acid and α-L-guluronic acid, (b) Stereochemical structures of M and G block conformation of alginate

Similar to other seaweed-derived polysaccharides, alginate has gel-forming capabilities. Nonetheless, the mannuronic and guluronic acid content of the alginate extracts determines the nature of the gels produced. In general, a high M/G ratio (>1) and a lower content of M blocks provide an elastic and soft gel, while a lower M/G ratio (<1) and a large proportion of G blocks produce a strong and rigid gel (Gómez-Ordóñez & Rupérez, 2011). However, the sequence and the content of the M and G blocks depend on many factors, such as seaweed species, plant age, environmental conditions, harvesting

season, part of the tissue and extraction methods (Deniaud-Bouët et al., 2017; Donati et al., 2003).

Thereby, the industrial utilization of alginate depends on its properties as well as its uronic acid composition. Currently, alginate is one of the most versatile and useful polymers widely used in the industry.

Alginate is classified as Generally Recognized as Safe (GRAS) for its application as a food ingredient and for drug delivery (Cattelan et al., 2020). Alginate is characterized by its biodegradability, biocompatibility, non-toxicity and high viscosity, as well as for its wide range of valuable biological and technological applications, including texturizing (thickening and gelling ability) (Benjamin et al., 2018), emulsifying (Yang et al., 2020), fat substitute (Kang et al., 2020) and tenderizer for meat (Shi et al., 2020), natural edible coatings (Tabassum & Khan, 2020), biodegradable films (Ehsani et al., 2020), hybrid biopolymeric nanofibers (Yilmaz et al., 2020) or even encapsulating structures for drug delivery systems (Rhein-Knudsen et al., 2017). Among its main properties, the one that stands out is its ability to form gels in the presence of certain divalent cations (e.g., Ca^{2+}) through a cross-linking reaction (Wüstenberg, 2014). According to this model, the gel network is formed by the intermolecular association of divalent cations that lodge into the cavities formed by two adjacent polymeric chains containing GG blocks, adopting helical conformations. This process produces a network of chains forming an "egg-box" structure, as shown in Figure 4 (Fertah et al., 2017; Plazinski, 2011).

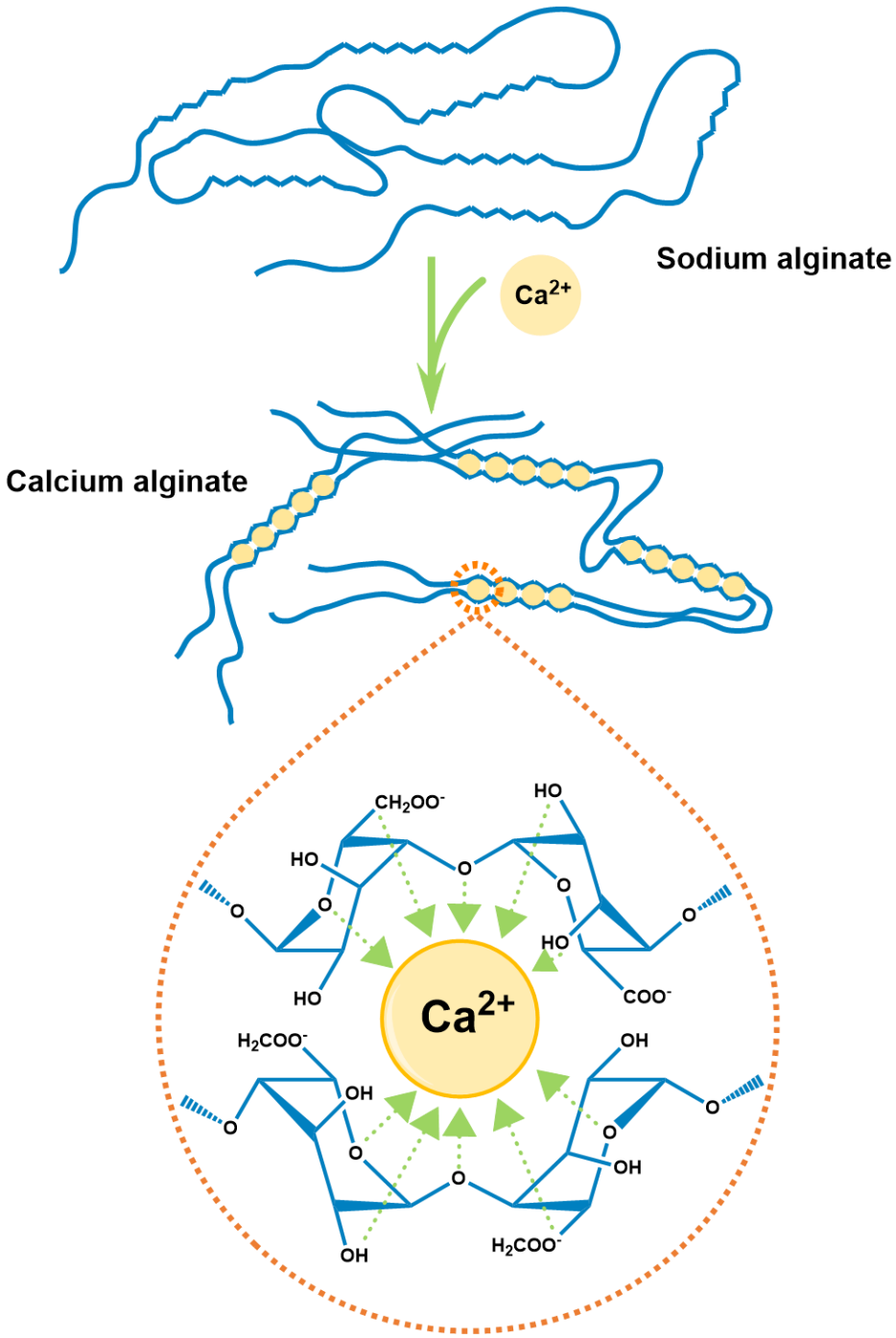

Figure 4. Graphical description of the egg-box model for alginate gelation

Gel formation depends on the composition (M and G blocks), sequence, and length of the alginate polymer chain, as well as the type and concentration of the cross-linking agents. Alginate has been found to exhibit different affinities for certain divalent ions, e.g., $Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+}$ (Hu et al., 2021; Y. J. Xu et al., 2021). However, calcium is the most commonly used cation to produce alginate gels. Figure 5 illustrates a schematic representation of three possible unions in alginate gels.

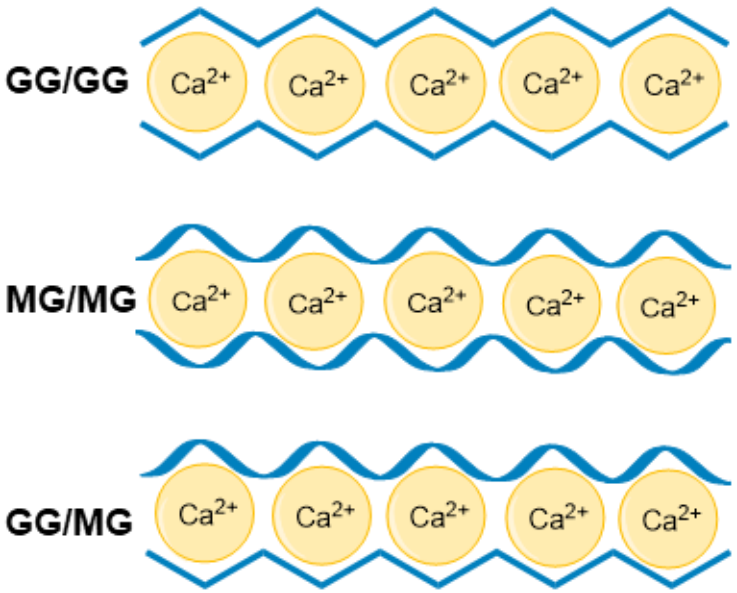

Figure 5. Illustrative scheme of possible junction points for alginate gelation. Straight and bending lines represent G and MG alginate regions, respectively (Adapted from Donati et al., 2005)

Calcium cross-linking in alginate can occur through two different techniques. The first technique is *"diffusion,"* where cross-linking ions diffuse into the alginate solution from an external reservoir. In the second technique,

known as *"internal setting,"* the ion source is already dispersed in the alginate solution and a controlled activator trigger releases the ions for cross-linking into the solution. These triggers can be the pH or solubility of the ion source. The difference between these two techniques is that gels produced by diffusion result in a concentration gradient of Ca^{+2} ions throughout the thickness, whereas gels produced by internal setting produce gels with uniform concentrations throughout the thickness (García-González et al., 2011; Pawar & Edgar, 2012). For example, diffusion gels are usually produced by dropping a sodium alginate solution into a $CaCl_2$ bath, while gels produced by internal setting, usually use an insoluble calcium salt as the calcium source (e.g., $CaCO_3$) and the pH change is induced by lactone hydrolysis (e.g., GDL), which results in the release of calcium ions inside and leads to gel formation (Pawar & Edgar, 2012).

Likewise, alginate with a higher G content has a higher affinity for these crosslinkers compared to alginates with a higher M content. It is well known that G-rich gels are strong and brittle, while gels with high M content or mixed sequences produce more flexible and weaker gels (Guo et al., 2020). Therefore, alginate gels' physical and chemical properties are determined by all these factors, which define their use in specific applications and, consequently, their commercial value.

1.1.2. Fucoidan

Fucoidan is a heterogeneous sulfated polysaccharide (Figure 6) mainly found in the cell wall of brown algae. Fucan structures consisting of α-(1→3) linked L-fucose units, partially sulfated at the O-4 or O-2 position, are the primary

component in fucoidans. Different fucoidan families vary in the proportions and branching degree of D-galactose, D-xylose, D-mannose, and uronic acids or a C3 sulfate ester group (Deniaud-Bouët et al., 2017). However, the structure of fucoidan is complex and it greatly varies depending on the algal species from which it is isolated (Pádua et al., 2015). Hence, its molecular weight reported varies from 100 kDa to 1600 kDa (Gupta & Abu-Ghannam, 2011). Fucoidan has been found only in brown seaweed, mainly in the orders of Fucales and Laminariales (Jiang et al., 2010). Fucoidan has also shown promising biological activities. Many research works have shown that fucoidan has antioxidant, anti-inflammatory, antiviral, anticoagulant, antitumor, antiviral, antiproliferative, antiapoptosis, and immunostimulatory properties (Cumashi et al., 2007).

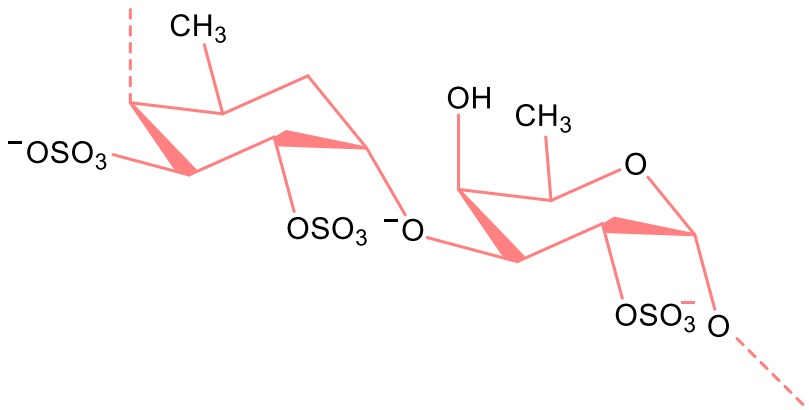

Figure 6. Typical structure of fucoidans. Adapted from Wijesinghe & Jeon (2012).

These biological activities may depend not only on the species originating the fucoidan, but also on the different structural fragments isolated from the fucoidan (Bilan et al., 2010). Currently, some applications of fucoidan have emerged as an additive in beverages, cosmetics, food, and pharmaceuticals (Pádua et al., 2015).

1.1.3. Algal proteins

Algae are recognized as valuable sources of protein, which contain all the essential amino acids and have abundant bioactivity and functional properties (Salido et al., 2024). The protein content of macroalgae differs according to season, species and geographical location. Hence, the variability in the protein content can fluctuate from 3 to 47 % (Kadam et al., 2017). Generally, the highest protein content of seaweed is found in the winter period and early spring, while the lowest content is found during the summer (Pangestuti & Kim, 2015). Brown algae have generally lower protein contents than the other seaweed groups (red > green > brown algae; Table 2). However, Kelp seaweed is the most cultivated species and accumulates large amounts of biomass, the protein of some kelp species being above other red or green algae (Table 2). Consequently, using a biorefinery approach, protein extraction from brown seaweed could be economically interesting (Abdollahi et al., 2019).

Macroalgae biomass comprises a variety of proteins, including glycoproteins (proteins covalently linked to glycans), lectins (proteins or glycoproteins that have at least one non-catalytic domain capable of reversibly binding to specific oligosaccharides or monosaccharides), peptides (protein fragments containing between 3 and 40 amino acids) and cell wall-attached proteins, such as

arabinogalactan proteins (highly glycosylated proteins found in some algae) (de Souza Celente et al., 2023). Given the diversity in the types of protein and their characteristics, extraction efficiency varies according to the seaweed species.

Table 2. Protein content reported of seaweed species.

	Phaeophyta		**Chlorophyta**		**Rhodophyta**	
Seaweeds species	*Ascophyllum nodosum*[a]	*Saccharina latissima*[b]	*Palmaria palmata*[c]	*Ulva pertusa*[d]	*Chondrus crispus*[e]	*Porphyra tenera*[f]
Protein content (% dw)	3 -15	26	8 - 35	17 -26	21	33 - 47

[a](Coulson, 1953), [b](Abdollahi et al., 2019), [c](Morgan et al., 1980), [d](Nisizawa et al., 1987), [e](Young & Smith, 1958), [f](Fujiwara-Arasaki et al., 1984).

Overall, seaweed proteins contain all amino acids, especially glycine, proline, alanine, arginine, aspartic, and glutamic acid (Table 3). Concerning essential amino acids, seaweeds contribute adequately to the FAO/WHO/UNU dietary protein requirements. However, relative to other protein-rich sources, seaweeds are limited in lysine, tryptophan, threonine, methionine, and cysteine (Pangestuti & Kim, 2015).

Interestingly, some studies have reported that brown algae have higher acidic amino acids than red and green algae or some plant sources such as soybeans. For example, some brown seaweed species had high valine and histidine levels (Ortiz et al., 2006). In another study, the aspartic and glutamic

acid content stood out, representing about 20 and 44% of the total aminoacids, respectively (Munda, 1977).

Table 3. The amino acid composition of some brown seaweeds and soy proteins (g amino acid/100g de protein) has also been included for comparative purposes.

Amino acids	Brown seaweed			*Soybeans*[d]
	Ascophyllum nodosum[a]	*Laminaria digitata*[b]	*Saccharina latissima*[c]	
Alanine (Ala)	6.1	14.4	7.7	4.5
Arginine (Arg)	4.7	0.3	4.7	7.3
Aspartic acid (Asp)	14.0	8.7	8.3	11.9
Cysteine (Cys)	0.4	1.7	3.3	1.5
Glutamic acid (Glu)	24.2	9.4	7.5	18.3
Glycine (Gly)	5.7	4.3	10.0	4.4
Histidine (His)	2.4	1.3	2.4	2.6
Isoleucine (Ile)	4.0	2.7	5.1	4.6
Leucine (Leu)	6.2	5.4	7.6	7.7
Lysine (Lys)	5.9	3.7	4.0	6.3
Methionine (Met)	-	1.6	1.3	1.3
Phenylalanine (Phe)	5.2	3.2	5.0	4.9
Proline (Pro)	-	3.7	4.9	5.5
Serine (Ser)	4.5	4.0	3.8	5.5
Taurine (Tau)	-	-	3.1	-
Threonine (Thr)	5.1	4.4	3.8	4.1
Tyrosine (Tyr)	2.8	1.5	-	3.6
Valine (Val)	5.9	4.2	5.6	4.7

[a](Kadam et al., 2017), [b](Augier & Santimoine, 1978), [c](Harrysson et al., 2018), [d](Van Der Heijden et al., 2023).

2. Extraction of valuable components from brown seaweeds

2.1 Alginate extraction

In general, extraction is a mass transfer process involving the diffusion of the solvent into the matrix to access and separate the compounds of interest (Ummat et al., 2021). Currently, strategies adopted to extract alginate and improve its performance are well documented and include the use of pre-treatments, cell disruption techniques, as well as conventional and novel extraction processes. Therefore, efforts have focused on eliminating or reducing toxic chemical solvents, reducing time and energy consumption, and improving the yield and quality of the alginate obtained. Figure 7 summarizes the most common procedures used for alginate extraction, which will be described later. Usually, these procedures are followed by extensive physicochemical characterization and biological activity assays to determine their potential application in the industry (Dobrinčić et al., 2020).

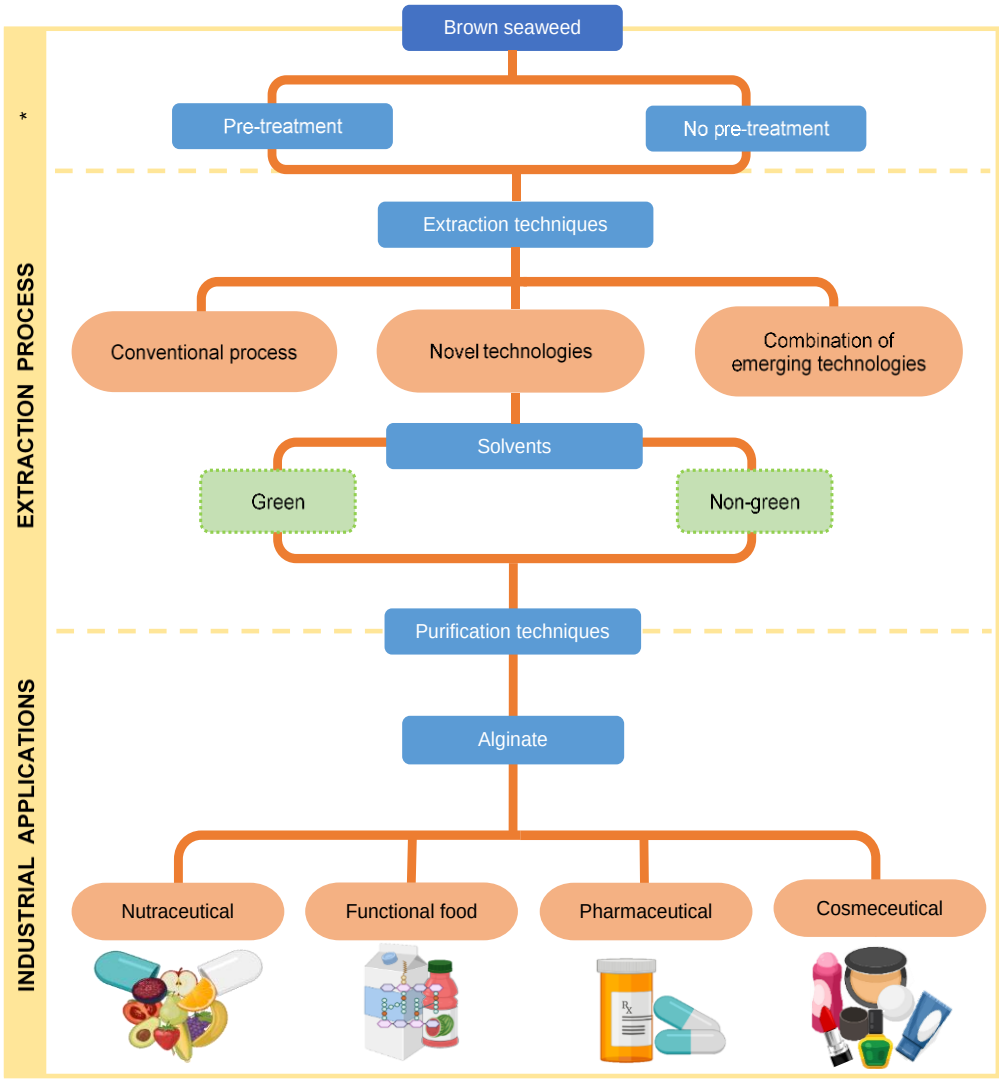

Figure 7. Overview of the alginate extraction process. *Preparation of macroalgae (Bojorges et al., 2023).

2.1.1 Conventional method

Conventional alginate extraction from brown algae is usually performed using the pre-treated macroalgae, mainly followed by solvent extraction, with substantial variability in terms of the conditions used in the literature. The most commonly used solvent is water (pre-extraction) and, generally, slightly acidic solutions (low molarity of HCl), followed by alkaline extraction (neutralization), solid/liquid separation, precipitation, drying and milling, have been reported (Hernández-Carmona et al., 2013; Peteiro, 2018b) (Figure 8).

2.1.1.1 Pre-treatment techniques of brown seaweeds

Alginate extraction from brown seaweeds requires a first stage of washing, preferably with distilled water, to remove salt residues, sand, impurities, and epiphytes. The washed algae can then be subjected to different types of pre-treatments, such as drying, crushing, or washing with other solvents (Garcia-Vaquero et al., 2017; Hahn et al., 2012). Pre-treatment of marine biomass is one of the most common unit operations performed to improve the extraction yield and make the process cost-effective (Gao et al., 2015). There are two types of pre-treatments; the first one aims to break the cell wall and improve mass transfer, while the second avoids co-extraction of bioactive compounds with similar solubility (Dobrinčić et al., 2020).

The first type of pre-treatment includes one of the most commonly performed, drying, which can be done with hot air, solar drying, or freeze-drying. Drying is used to modify cell membrane permeability and accelerate

mass transfer (Chan et al., 1997; Ummat et al., 2021). However, drying may affect the alginate's quality, requires significant energy, and could result in the loss of some phytochemical compounds, such as phenols and flavonoids (Gupta et al., 2011). The second type of pre-treatment focuses on removing compounds highly bound to the alginate. Consequently, it is common for the seaweed to be pre-treated with ethanolic or formaldehyde solutions to remove phenols, proteins, lipids, or mannitol compounds bound to the polysaccharide and, thus, favor the purification degree of the resulting alginate extract (Hahn et al., 2012).

2.1.1.2 Acid pre-extraction

The acidification can have several goals, (1) it is customarily carried out to eliminate salts, (2) residual organic solvents used in the pre-treatment (Hernández-Carmona et al., 2013), and (3) remove non-target compounds such as polyphenols, mannitol, and other easily degradable polysaccharides (e.g., laminarins, fucoidan), as well as other low molecular weight compounds. The use of acid solutions improves the extraction yield, favoring the disruption of the cell wall and, thus, the release of these substances (Dobrinčić et al., 2020). Moreover, acid treatment is necessary to convert alginate salts (Na^{+}, Ca^{2+}, K^{+}) into water-insoluble alginic acid (Hernández-Carmona et al., 1999).

2.1.1.3 Alkaline extraction

After the acid treatment of the algae, an alkaline extraction is performed. The solution is brought to a pH between 9 and 10, predominantly with sodium carbonate (Na_2CO_3). However, other salts can be used such as sodium

hydroxide (NaOH) (Davis et al., 2004), sodium bicarbonate ($NaHCO_3$) (Bojorges et al., 2022), or sodium chloride (NaCl) (Yan et al., 2020). In this step, the water-insoluble alginic acid present in the algae cell wall is converted into water-soluble salts (sodium alginate) and then precipitated, purified, and dried (Dobrinčić et al., 2020; Hernández-Carmona et al., 2013). The extraction efficiency depends on temperature, alkali concentration, time, pH, and the interactions between them.

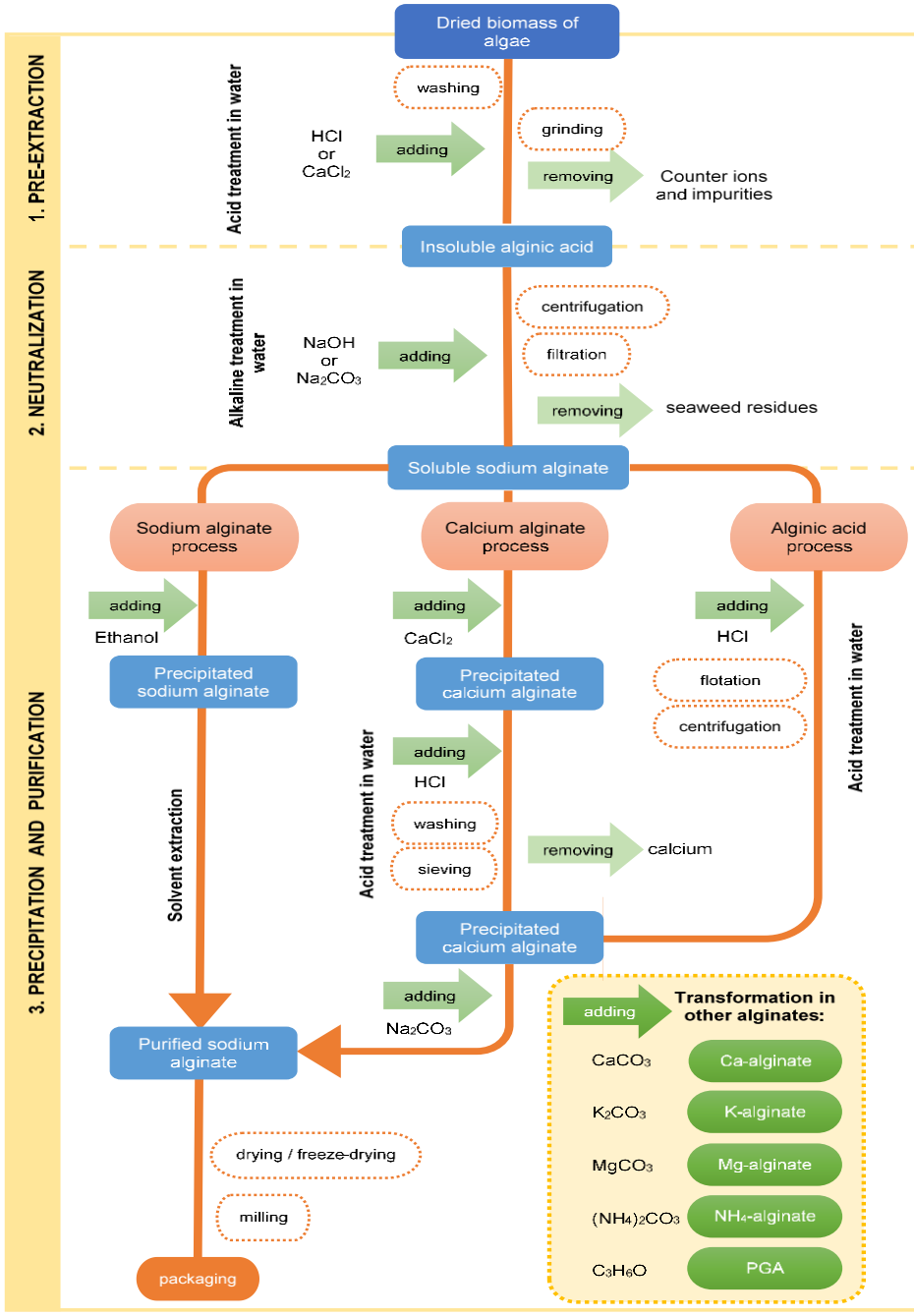

Figure 8. Scheme flow chart of sodium alginate extraction and other alginates from brown seaweed (Adapted from Peteiro, 2018).

2.2 Protein extraction

The food industry is currently experiencing a growing interest in recovering vegetable proteins, commonly called "greens". Although algae, in taxonomic terms, are not considered plants, they are of great industrial importance. The cultivation of marine algae is distinguished by its ability to do without agricultural land and freshwater, taking advantage mainly of the extensive unused oceanic spaces (Bleakley & Hayes, 2017a). In addition, some seaweed species outperform conventional plant protein sources in terms of productivity per unit area per year, exhibiting total protein levels comparable to those found in traditional sources of both animal origin, such as meat and eggs and from plant origin, such as soybeans (Gregersen et al., 2021).

In recent years, this trend has driven a significant increase in research on macroalgae as a raw material for protein extraction and studying their functional properties for application in the food industry (Rawiwan et al., 2022). However, protein extraction remains a major challenge due to the complex nature of the cell wall. Protein extraction highly depends on bioaccessibility since most proteins are produced intracellularly (Gordalina et al., 2021). In addition, the presence of polysaccharides and polyphenols in the cell wall and their binding to proteins reduces the protein extraction capacity, thus requiring additional treatments for fractionation and purification (Pliego-Cortés et al., 2020).

Some studies have shown that polysaccharides strongly induce electrostatic interactions with proteins (Weinbreck et al., 2004). Moreover, polyphenols can either oxidize to quinones to establish irreversible hydrogen

bonds with proteins or form reversible hydrogen bonds with proteins (Veide Vilg & Undeland, 2017). Therefore, combining different protein extraction and purification methods is necessary to improve the protein's extraction yield, purity, and digestibility. Consequently, extraction process efficiency and industrial scalability remain as important challenges (Rawiwan et al., 2022).

2.2.1 Conventional extraction

Generally, conventional protein extraction involves aqueous, acidic, and alkaline methods, followed by various centrifugation steps, fractionation and enrichment methods, such as ultrafiltration, precipitation, or chromatography (Bleakley & Hayes, 2017b). In order to protect the protein structure, various food-grade approaches with non-toxic reagents and mild extraction conditions have been employed (Gordalina et al., 2021).

The aqueous extraction is considered green and one of the simplest. This extraction allows osmotic shock and facilitates protein extraction. However, this only yields the quantity of water-soluble proteins in the algae; thus, the yield is generally low compared to other chemical extraction systems (Cermeño et al., 2020). In contrast, extractions with acid and alkaline solutions have effectively extracted proteins from *A. nodosum*, *L. digitata*, and *Ulva spp.* (Fleurence et al., 1995; Jordan & Vilter, 1991; Kadam et al., 2017). It has been shown that applying an acid treatment favors the release of polysaccharides and proteins in the cell wall matrix. Subsequent alkaline treatment contributes to protein solubilization and increases protein recovery (Kadam et al., 2017).

Protein precipitation is performed with organic solvents such as trichloroacetic acid or through pH shifting with hydrochloric, sodium hydroxide or acid citric. The aim is to solubilize and precipitate protein after the isolation and fractionation of other compounds, such as polysaccharides or phenolic compounds (Cermeño et al., 2020). It has been reported that precipitation combined with two-phase separation can provide high-purity proteins with bioactive properties (Mittal et al., 2019). In other cases, alkaline solubilization combined with isoelectric precipitation and freeze/thawing exhibited an increase in the protein extracted (Harrysson et al., 2018).

Other investigations have used alkaline solutions together with reducing agents to dissociate proteins from polysaccharides, which could improve the alkaline soluble protein yield. Although β-mercaptoethanol has been reported to increase protein extraction yield, it has limitations for food applications (Harnedy & FitzGerald, 2013).

2.3 Novel technologies and methods

There is a growing demand for developing new, more sustainable, and environmentally-friendly methods for the extraction of biopolymers and proteins. Therefore, simpler extraction processes and novel technologies, such as microwave-assisted extraction, ultrasound, high pressure, pressurized fluid extraction, and enzyme-assisted extraction (Figure 9), have been explored to improve sustainability in terms of process efficiency, environmental impact, and economic viability (Gullón et al., 2020; Kadam et al., 2013). However, because the differences between each method are multivariable, the extraction parameters must be optimized (Raja et al., 2022).

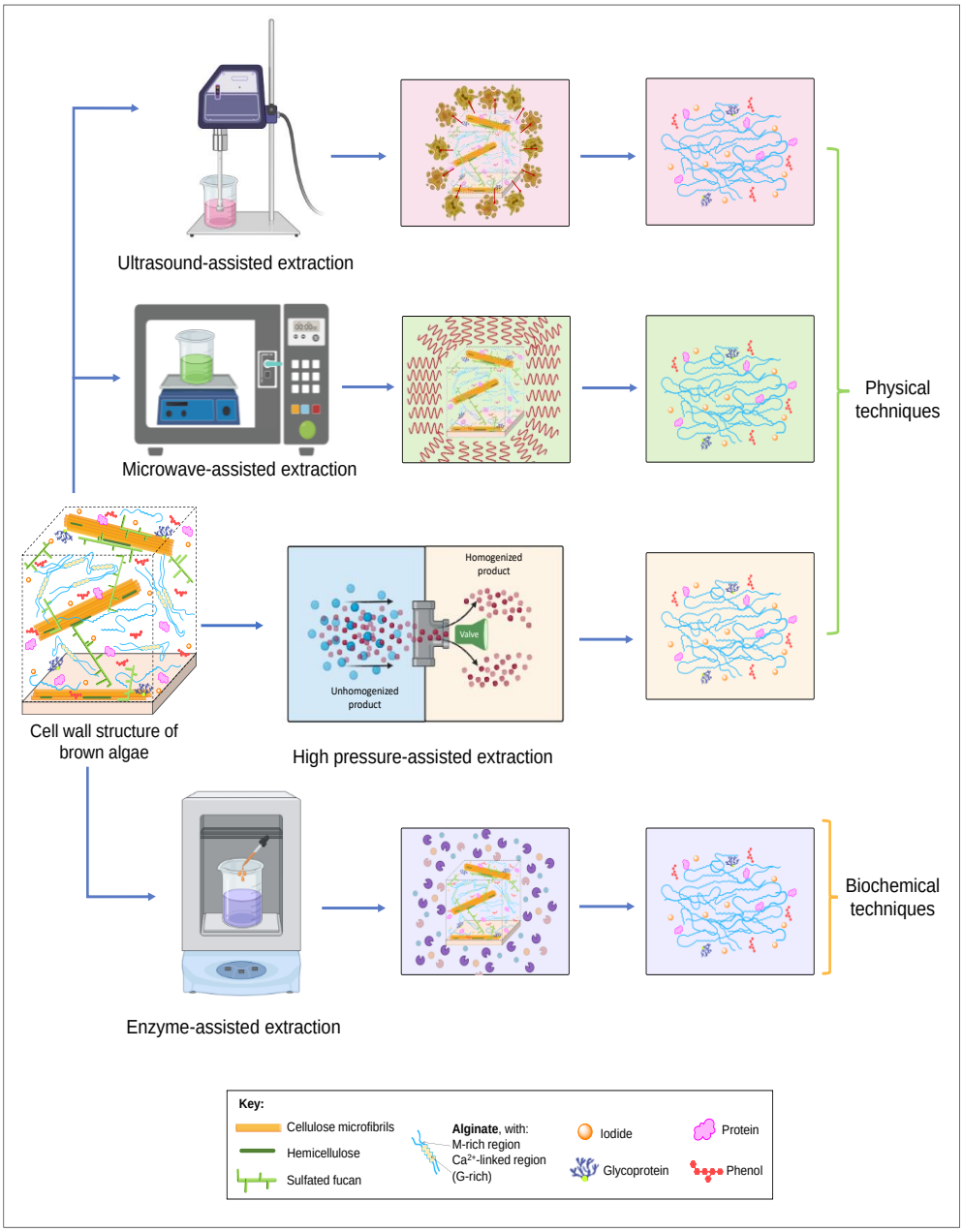

Figure 9. Scheme of classification of extraction treatments assisted by novel technologies applied to brown algae (adapted from Kumar et al., 2021).

2.3.1 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction is considered a novel, environmentally-friendly extraction technique because it requires low volumes of solvents, and allows for a fast extraction rate, easy handling, low cost, and low processing time (Rodrigues et al., 2015; Yao et al., 2020).

UAE is based on the utilization of sound frequencies above the audible range (20-100 kHz) and below microwave frequencies ($\leq$10 MHz), with high power that range from 80-200 W. Ultrasound waves are associated with the phenomenon of acoustic cavitation, which refers to bubble formation, growth and the collision of cavitation bubbles during the propagation of the ultrasound waves in the microparticles present in algae biomass and the extraction media (Gomez et al., 2020; Kentish & Ashokkumar, 2011).

The application of ultrasound is used to disrupt cell walls, cause a reduction in particle size, accelerate diffusion and increase mass transfer, allowing greater penetration of solvents and facilitating the release of desired compounds (Kadam et al., 2013; Mena-García et al., 2019; Praveen et al., 2019; Yao et al., 2020).

The UAE provides numerous advantages over the conventional extraction method. For example, the temperature range in which it operates is mild, thus preventing the degradation of thermolabile compounds (Gullón et al., 2020). Other advantages include the simplicity of the method, the increase of extraction yield and the fact that the operating costs are less expensive than other extraction technologies, making them a suitable method to be

implemented on an industrial scale (Kadam et al., 2013). In addition, UAE can be combined with other non-conventional extraction technologies, such as subcritical water extraction or microwave extraction (Alboofetileh et al., 2019).

The principal parameters that could influence the efficiency of the UAE process is the wavelength, amplitude, frequency, time, temperature, and solid:liquid ratio (Mena-García et al., 2019; Pingret et al., 2013). In terms of yield, usually the highest efficiency of the UAE can be reached by increasing the ultrasonic power to improve solvent-solid contact and optimizing the temperature to reduce the extraction time (Chemat et al., 2017). Despite the many advantages of UAE, there are few published studies, possibly due to the cost of additional equipment and scaling up of the process, so more research is needed to demonstrate the benefits.

Nevertheless, some studies indicate that the required high energy and pressure waves input could be detrimental to the structural and chemical composition of polysaccharides (Praveen et al., 2019) and thus, mild extraction conditions are highly recommended to obtain high extraction yields, without modifying the structural and functional properties of the alginate.

For proteins, ultrasonic extractions have been shown to have the ability to modify the molar mass distribution and improve their functional properties, such as solubility and foaming characteristics (Ma et al., 2024). Similar to polysaccharides, to obtain high extraction yields, it is necessary to optimize parameters such as extraction time, sample substrate ratio, ultrasound power, pH, and temperature (Youssouf et al., 2017), as mentioned above.

However, applying prolonged and more intense levels of sonication energy can decrease protein yields. This effect is exacerbated by the formation of disulfide bonds between cysteine residues, stimulated by hydroxyl free radicals generated during ultrasound exposure, leading to protein aggregation (Kumar et al., 2021) and denaturation of soluble protein fractions (Terme et al., 2020).

Besides, it is also essential to assess the impact of time and energy on performance individually, considering the costs associated with the installation and implementation of UAE. Furthermore, undesirable ultrasound-induced structural modifications represent an additional factor contributing to the challenges in their industrialization (de Souza Celente et al., 2023).

2.3.2 Microwave-assisted extraction (MAE)

Microwave-assisted extraction is considered one of the most efficient techniques compared to conventional extraction methods. It has been widely used to extract bioactive compounds from different biomasses (Dobrinčić et al., 2020).

MAE is based on applying non-ionizing electromagnetic waves at variable frequencies ranging from 300 MHz to 300 GHz and wavelengths (1 mm to 1m) to disrupt or generate changes in the structure of cells into the matrix (Gomez et al., 2020). Microwaves produce vibrations that cause an increase in the temperature of intracellular fluids. This heating is generated by the ionic conduction of dissolved ions and by the dipolar rotation of the

polar solvent. The internal heating induces a rupture in the effective cell wall that releases the target compounds into the solvent (Gomez et al., 2020; Mena-García et al., 2019). Thus, higher extraction yields can be obtained using less amount of solvent, time and energy, since this methodology enables a better heating distribution control as compared to the conventional extraction process (Gullón et al., 2020). Compared to ultrasound, MAE is a method that requires smaller amounts of solvents and can improve extraction yields (Garcia-Vaquero et al., 2017). In addition, this methodology is a simpler, faster and economically feasible option.

For the extraction of polysaccharides such as alginate, MAE is a suitable process considering the M/G ratio is not affected, which is relevant for understanding the gelation process and the characteristics of alginate gels (Gomez et al., 2020; Li et al., 2013). However, its application may be limited for the extraction of heat-sensitive bioactive compounds (Tiwari & Troy, 2015). For this reason, MAE method has received less attention in extracting protein compounds than carbohydrates and phenolic compounds, which have been more extensively studied (Cermeño et al., 2020). Nonetheless, in the protein field, several studies suggest that combining MAE-assisted extraction with other biochemical or physical techniques sequentially could be an effective method to improve extraction efficiency (Liu et al., 2017; Phongthai et al., 2016). In addition, MAE has been proposed to extract proteins from industrial waste or by-products, which are often rigid in structure or difficult to digest by enzymes and/or ultrasound. It has been shown that high-energy microwaves can effectively penetrate the cell walls of these challenging

materials, which contributes to improved protein recovery (Kumar et al., 2021).

Although MAE is an efficient method for extracting polysaccharides, its exclusive application in fractionating these compounds requires excellent selectivity. The choice of suitable solvents and their ratio represents a significant challenge in MAE (Ruiz-Aceituno et al., 2016). This process is mainly determined by the solvent's ability to absorb microwaves, its selectivity toward the desired analyte, and its interaction with the matrix. In addition, it is necessary to consider the risks associated with solvents, such as their toxicity, environmental impact and cost. Therefore, coupling with other methods or solvents is necessary to extract the target compound selectively (Kapoore et al., 2018).

In addition, although MAE has been mainly used in laboratory settings, some cases of its application on an industrial scale have been reported, especially in the extraction of soybean and rice bran oil (Terigar et al., 2011). In fact, its efficacy has been commercially demonstrated in producing biodiesel from used cooking oil (Tatke & Jaiswal, 2011). However, it is essential to consider both the initial cost of the equipment and the maintenance costs, which can be significantly high. For this reason, initiatives have been proposed to establish economically sustainable biorefinery platforms where priority is given to obtaining high-value products (Kapoore et al., 2018).

In summary, overcoming all these limitations represents a significant challenge that requires meticulous optimization to maximize the potential of MAE in extracting commercially-viable products from algae. It is, therefore,

essential to continue exploring various strategies to expand the industrial use of MAE to other high-value compounds.

2.3.3 High-pressure assisted extraction (HPP)

Non-thermal high-pressure assisted extraction emerges as a promising methodology for obtaining bioactive compounds, allowing both the reduction of extraction time and the more efficient use of organic solvents (Bai et al., 2024).

HPPis mainly composed of three stages. First, the product is mixed with the extraction media and introduced into the pressure vessel. Then, pressure quickly increases from the ambient level to the required value. Typically, the liquid pressure ranges from 100 to 1000 MPa (Hewage et al., 2022). As the pressure increases, the pressure difference between the inside of the plant cells and their surrounding environment increases, resulting in deformation and damage to the cell wall. As a result, the solvent penetrates through the damaged cell wall, thus facilitating the mass transfer of soluble compounds into the extraction medium. If the compression force does not exceed the deformation limit of the cells, the solvent permeates through the cell wall under pressure, resulting in rapid saturation of the cells. In this process, the bioactive components dissolve directly into the solvent. On the other hand, if the compression applied to the product exceeds the cell deformation limit, the cell wall is ruptured and the active compounds flow out of the cell and dissolve in the extraction medium (Kumar et al., 2021).

Subsequently, in the pressure maintenance stage, the pre-defined pressure is kept constant to balance the pressure outside and inside the cell. During this time, the solvent persists in its penetration process through the cell wall, dissolving the components. Extending this stage can lead to an increase in extraction yield (Huang et al., 2013). In the final stage of the process, as the pressure decreases, the pressure built up inside the cell drops to atmospheric pressure, causing expansion and deformation of the cell (Kumar et al., 2021). With a shorter duration of the pressure release process, more pores are generated in the cells, which increases the surface area of the feedstock and facilitates the diffusion of the active compounds, leading to high efficiency in the extraction process (Chen et al., 2009).

Recently, several studies reported the use of HPP for the extraction of some polysaccharides such as pectin or xyloglucans and, compared with other extraction methods, HPP showed the highest efficiency (Guo et al., 2012; Limsangouan et al., 2020; Ouyang et al., 2023). However, HPP can induce modifications in the structure of polysaccharides, which may involve molecular alterations such as folding, stretching, distortion, or expansion, which, in turn, can lead to variations in the size and molecular weight of polysaccharides (Bai et al., 2024). Additionally, some studies have observed that HPP-treated extracts exhibited enhanced anti-tumor, antioxidant, and hypoglycemic activities (F. Xu et al., 2016; Zheng et al., 2023; Z.-Y. Zhu et al., 2016).

Likewise, the efficacy of HPP in protein extraction has been investigated and it has been observed that HPP induces the destabilization of hydrogen bonds and the disruption of electrostatic interactions between

proteins (de Souza Celente et al., 2023). This effect leads to changes in protein structure, such as denaturation, aggregation, conformational changes or gelation (Laguna et al., 2017; Zhao et al., 2022) and influences secondary (Velazquez et al., 2021), tertiary and quaternary structure (Zhang et al., 2017). These structural changes expose previously hidden hydrophobic groups in the inner regions of the protein, resulting in increased protein surface hydrophobicity and improved protein digestibility (Mulla et al., 2022).

Nevertheless, there is a lack of comprehensive documentation regarding the utilization of HPP in seaweeds. O' Connor et al. (2020) investigated protein recovery from several seaweed species, including *A. esculenta* (15.0%), *C. crispus* (16.1%), *F. vesiculosus* (23.7%) and *P. palmata* (14.9%), by pre-treatment with HPP (600 MPa, 4 min). Their findings indicated that, although HPP failed to significantly improve protein extraction compared to other methods such as sonication, autoclave shock and freeze-thaw, it did improve the extraction of a glutamic acid-rich fraction for all algae, in contrast to the previously mentioned and more traditionally used methods.

Although HPP can achieve more efficient extraction in less time, with lower energy and solvent usage and avoid thermal degradation of thermolabile compounds (de Souza Celente et al., 2023), it has some considerable drawbacks. These include higher initial equipment costs and limited expandability, resulting in higher production costs. In addition, the use of HPP can trigger depolymerization of polysaccharides or fragmentation of their linear side chains (Bai et al., 2024), as well as induce protein denaturation

(Velazquez et al., 2021), which can result in alterations in the chemical composition, structure, as well as functional and bioactive properties (Hewage et al., 2022) of both polysaccharides and proteins.

2.3.4 Enzyme-assisted extraction (EAE)

EAE is currently a promising, environmentally-friendly procedure and an alternative to conventional methods due to its high efficiency and mild extraction conditions. The enzymes used are food grade, environmentally-friendly, non-toxic and could be applied in large-scale operations. Nonetheless, enzymes are limited by their availability and large-scale biotechnological production, so many enzymatic activities are not economically feasible yet for industrial applications (Garcia-Vaquero et al., 2017; Michalak & Chojnacka, 2014).

It has been extensively documented that certain enzymes such as cellulases, alginases, hemicellulases, xylanases, β-glucanases, and enzyme cocktails can modify algal cell walls (Bleakley & Hayes, 2017b). In this context, the main enzymes used in alginate extraction are cellulases and proteases, which act to alter the algal cell wall's integrity, thus improving extraction yield (Charoensiddhi et al., 2017a). On the other hand, in the case of protein extraction, cellulase cocktails have been observed to efficiently hydrolyze intracellular polysaccharides such as alginate, agar, and carrageenan, thus releasing intracellular protein (Vásquez et al., 2019). In addition, digestive enzymes such as chymotrypsin, pepsin, and trypsin have been used to release bioactive peptides from the original seaweed protein (Pliego-Cortés et al., 2020).

Enzyme selectivity and specificity are influenced by multiple factors such as solvent, temperature, pH, time, enzyme concentration and substrate-to-enzyme ratio, which should be considered to optimize the enzyme reaction and obtain the highest hydrolytic activity (Gomez et al., 2020; Grosso et al., 2015). EAE presents several advantages such as high selectivity, minimization of solvent usage, energy consumption and extraction time, which can allow high yields and a positive impact on the quality of the extracted polysaccharides or proteins compared to conventional techniques from diverse biomass (Charoensiddhi et al., 2016; Gomez et al., 2020; Hardouin et al., 2014; Terme et al., 2020).

Building on these advantages, a recent study by Okolie et al. (2020) reported that EAE produced the highest amount of alginate and did not significantly alter the M/G ratio compared to MAE and UAE. However, it has also been reported that EAE can extract alginate with a lower molecular weight and higher polyphenol content (Bojorges et al., 2023).

On the other hand, EAE is commonly applied to obtain proteins from red and green algae. However, its use in brown algae has been limited, mainly due to the complexity of their cell wall. The effectiveness of enzymes in breaking down the cell wall depends on their composition, which varies among species. For example, cellulases are effective in protein recovery in *P. palmata* (Joubert & Fleurence, 2008) but not in *U. lactuca* and *U. rigida*, where other enzymes are needed (Fleurence et al., 1995). Therefore, enzyme-assisted protein extraction should be evaluated for each seaweed, even within the same species, due to geographical and seasonal differences that may affect cell wall

polysaccharide content, influencing enzymatic performance in solubilizing proteins (de Souza Celente et al., 2023).

Furthermore, in most cases, especially in polysaccharide and protein extraction, a high enzyme:substrate ratio is required, which may not be economically feasible on an industrial scale (Harnedy & FitzGerald, 2013). Therefore, many research studies apply enzyme technology as a pre-treatment prior to extraction or in combination with other novel techniques including UAE, MAE, SFE, and SWE (Mena-García et al., 2019; Ummat et al., 2021).

2.4 Waste streams

The alginate industry generates considerable volumes of liquid and solid waste streams, representing approximately 80% of the original dry biomass currently being discarded (Bojorges et al., 2022). Although alternatives have been explored to reduce chemical use, such as cascade extraction of co-compounds, changing alginate industrial practices seems unlikely.

The lack of accurate statistics on the amount of waste generated by the various algae processors is mainly due to the complexity of the supply chain of algae-derived products. However, these by-products are estimated to represent at least 72,000 dry metric tons annually (Yun et al., 2023). Their end-use is usually soil amendment with little or no return to the algae processing industries or the society. They are, nonetheless, presumed to contain considerable concentrations of proteins and carbohydrates, thus offering ample potential as a feedstock for bioproducts (Bojorges et al., 2022; Salgado et al., 2021).

The valorization and sustainable management of macroalgae waste is proposed in response to this challenge. Extracting other compounds of interest, like proteins, is presented as an environmentally-friendly alternative for leveraging readily available raw materials. This strategy addresses waste management and promotes a circular bioeconomy and more sustainable practices within the alginate industry.

Therefore, a comprehensive characterization of the various waste streams generated during alginate extraction from brown algae is needed. This approach would provide evidence of possible valorization schemes that can improve the ecological sustainability of the current industrial process. Although there are several options for valorization, a schematic representation of some examples is presented in Figure 10. One particularly prominent option consists of obtaining protein concentrates from the solid residue. This protein could be recovered by conventional extraction and purification methods, separating it from the cellulose and fucoidan-rich residue. Although such protein, when extracted, could be partially denatured or degraded, it could be feasible to use it as an ingredient in protein-rich vegan foods or cosmetics. This would significantly reduce Europe's dependence on protein imports, in line with Horizon Europe's current research and development strategies (Bojorges et al., 2022).

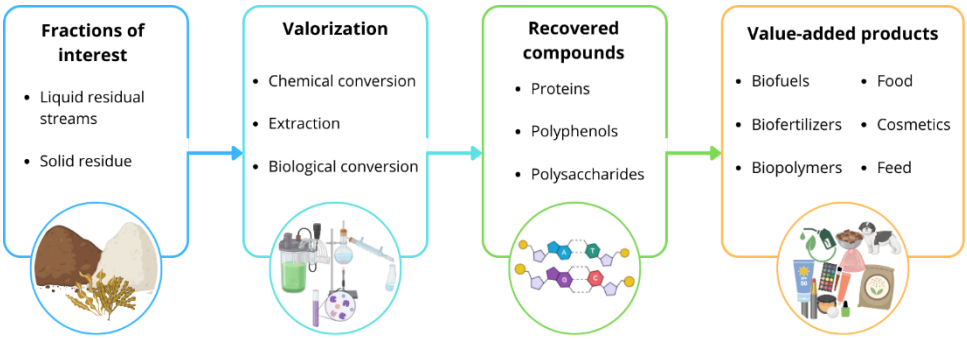

Figure 10. Potential utilization strategies for specific waste streams derived from alginate production in value-added products.

3. Applications and functionality in the food industry

3.1 Alginate

In recent years, polysaccharides extracted from brown seaweed have been reported to have a market value of $10.000 per ton of dried algae (Lenstra et al., 2011). Among the main polysaccharides of brown seaweed, we find alginate. As commented in section 1.1.1, alginate is a polysaccharide of industrial relevance due to its unique properties, such as its gelling power, high viscosity in aqueous solutions, biocompatibility, non-toxicity, biodegradability and, therefore, its use is widespread in many fields, such as the food, pharmaceutical, cosmetic and textile industries (Guo et al., 2020; B. Zhu et al., 2020).

In Japan, sodium alginate is considered a "food for health and longevity," but it is an important food additive in Western countries, as well

(Guo et al., 2020). According to FAO certification, alginate is considered one of the safest food additives in the world (Vijayalakshmi et al., 2017). Alginate as additive can improve the structure and physicochemical properties of foods. The addition of this polysaccharide in foods can have multiple goals, as it is used as gelling, setting, emulsifying and stabilizing agent and to prevent drying of foods (B. Zhu et al., 2020). Moreover, as a texturizing filler, it can help reduce production costs and improve the company's economic efficiency. For example, the addition of sodium alginate (<1 %) in bread making could prevent drying and aging of the finished product; or the incorporation of alginate in yogurt (<2 %) contributes to stabilizing and improving the curd shape, prevent viscosity decrease and extend its shelf life without altering the flavor (Guo et al., 2020).

In fresh foods, alginate has been used as a coating and edible film to maintain flavor, increase the water barrier, prevent microbial contamination, reduce lipid oxidation and, therefore, extend the shelf life of various foods such as fruits (Valero et al., 2013), vegetables (Ben-Fadhel et al., 2018), and meat (Bojorges et al., 2020).

Currently, there are different types and grades of alginates from marine algae on the market, classified according to their M-block and G-block distribution pattern, purity, molecular weight, and composition (Abka-khajouei et al., 2022). This variability provides a wide range of functional properties that determine their use in different specific applications and, therefore, also their commercial value (Peteiro, 2018b).

Besides food applications, alginate has also been explored in broader biomedical and pharmaceutical fields. Demand for this polymer has increased over the past few years, and this trend will likely continue. Recently, a study by Zhao et al., 2020 showed an upward trend in research on alginate and other seaweed polysaccharides in the last ten years due to the growing awareness of using sustainable materials to replace fossil resource-based products.

3.2 Algal proteins

The functional properties of proteins modify food systems' flavor, texture, color properties and improve their nutritional value. Besides, they contribute to forming and stabilizing specific mesostructures, such as those formed during gelling, emulsification and foaming (Foegeding, 2015; Rioux et al., 2017). Therefore, the protein should be soluble and maintained at an optimal pH to stabilize multiphase systems. Proteins should not co-precipitate with polysaccharides so that the functional properties of the protein are not compromised (Vásquez et al., 2019). Currently, there are limited studies on the functionalities of macroalgae proteins, so this is a research opportunity.

In addition, the use of proteins or protein fractions from macroalgae as ingredients in the food industry is limited (Rioux et al., 2017). However, human consumption of seaweeds in the East is a matter of tradition due to their high protein content and favorable essential amino acid profile (MacArtain et al., 2007). At the laboratory level, macroalgae have been incorporated as a functional ingredient in pasta and cereals. For example, *U. pinnatifida* (wakame) was used in pasta and found its antioxidant activity and

acceptable sensory attributes among its main characteristics at an incorporation level of 10 % (Prabhasankar et al., 2009). Whereas bread containing 4 % of *A. nodosum* significantly reduced post-meal energy intake (Hall et al., 2012).

Likewise, similar concentrations of renin inhibitory peptides from *P. palmata* protein hydrolysates were added to the bread, which provided acceptable sensory attributes and bioactive properties that persisted after baking (Fitzgerald et al., 2014). Another study indicated that crude protein from *P. yezoensis* could reduce muscle atrophy incited by dexamethasone *in-vivo* (Lee et al., 2018). Besides, it was shown that macroalgal polypeptides could improve endurance capacity in exercise (Zhi-mei, 2016). All these studies have evidenced and supported the use of macroalgae protein for incorporation into functional foods. However, it is required to optimize extraction methods to achieve a technological and nutritional functionality and maximize profitability at the industrial level (Rawiwan et al., 2022).

On the other hand, some studies have focused on using macroalgae protein as animal feed, including aquaculture, farm animals and pets. For example, *Porphyra spp.* was tested in the diet of red sea bream and it was found to increase animal weight gain and improve survival rate (Mustafa et al., 1995). However, because *Porphyra spp.* is consumed as human food, it is not economically viable and limits its use as feed in fish diets. Therefore, other marine macroalgae or residues generated by the polysaccharide industry from marine sources could be used in this context.

4. References

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II. Objetives

"Nothing in life is to be feared, it is only to be understood."

Marie Cuerie

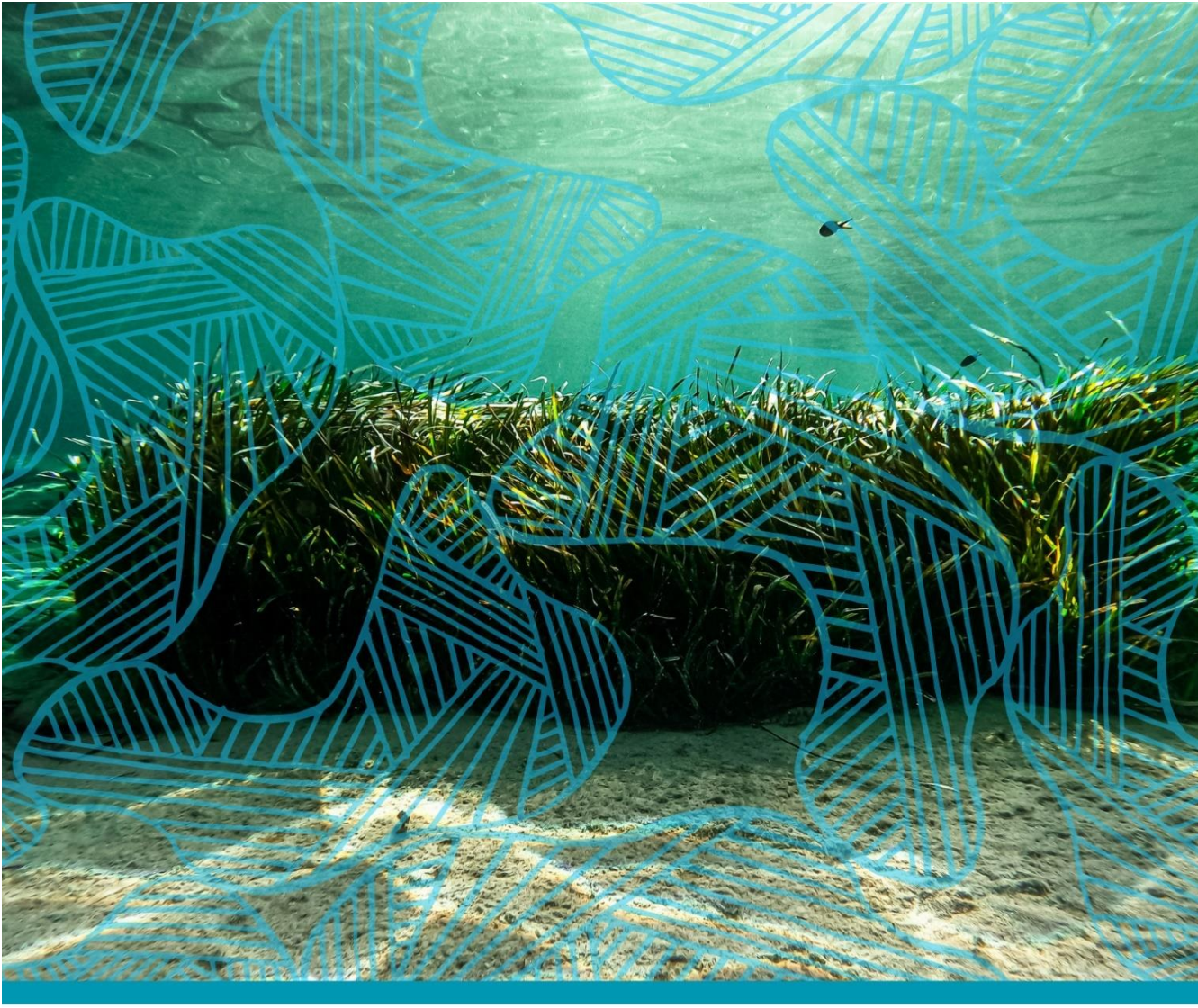
II. OBJECTIVES

The main objective of the present Ph.D. thesis was to develop an integrated strategy for valorizing brown algal biomass and its by-products to generate high-value compounds for use in food applications. For this purpose, several specific objectives were proposed:

1. Enhance the efficiency of extracting high-value compounds, such as alginate and proteins, from brown algae by implementing innovative technologies in the extraction process.

2. Valorize the by-products and effluents generated during polysaccharide extraction into added-value products.

3. Generate knowledge on brown algae composition and structure, by developing a robust method for the quantitative determination of uronic acids, aiming to elucidate the structural composition and enhance the understanding of the function-structure relationship of these compounds in various contexts.



III. Results

"Nothing in this world can take the place of persistence [...] persistence and determination alone are omnipotent."

Calvin Coolidge

III. RESULTS

Currently, brown algae play a crucial role as a source of biomass, with alginate as the main polysaccharide in the hydrocolloid industry. This scenario has led to a growing interest in optimizing this polysaccharide's extraction process, resulting in several strategies being implemented. These strategies include pre-treatments, cell disruption techniques and the adoption of innovative technologies. In addition, they focus on reducing or eliminating the use of chemical solvents and decreasing time and energy consumption to improve yield without compromising the quality of the alginate obtained. However, significant amounts of liquid and solid waste are generated during the extraction process, representing up to 80% of the original dry biomass and are discarded, thus contributing to environmental pollution.

In order to tackle the issue, the thesis focused on enhancing the process of extracting polysaccharides and proteins, as well as on the valorization of the generated by-products and residues. The outcomes of this research are demonstrated in five studies organized in three chapters (Table i).

Table i. Organizational structure of the research thesis

Chapter 1.	**Optimization of extraction of valuable compounds from brown seaweeds**
1.1	Structural and functional properties of alginate obtained by means of high hydrostatic pressure-assisted extraction
1.2	Study of protein extraction and its functionality from *Ascophyllum nodosum* and *Saccharina latissima*
Chapter 2.	**Trash to Treasure: Valorization of residues produced during polysaccharide extraction**
2.1	Alginate industrial waste streams as a promising source of value-added compounds valorization
2.2	Extraction of intracellular protein of alginate-extracted solid residues from *Ascophyllum nodosum* and *Saccharina latissima*
Chapter 3.	**Challenges in structural elucidation for knowledge generation**
3.1	Estimation of alginate purity and M/G ratio by methanolysis coupled with anion exchange chromatography

The first chapter optimized the alginate extraction process using two species from different families of brown algae (*Ascophyllum nodosum* and

Saccharina latissima). Innovative technologies were introduced, highlighting high pressures to adopt more sustainable and environmentally-friendly practices. This approach was thoroughly compared with the conventional extraction method, evaluating performance and structural properties as well as techno-functional properties.

Furthermore, since brown seaweed farming does not compete directly with food crops in terms of land and natural resources, the production of protein extracts from seaweed was explored. One of the main challenges in this process is the disruption of brown seaweed cell walls since many proteins are intracellularly located or embedded in these walls, which makes their extraction difficult. It is important to note that efforts have primarily concentrated on obtaining high-purity proteins. Nonetheless, the polysaccharides present in brown algae could also offer numerous health benefits and interesting functional properties. For this reason, the efficacy of sequential extraction was investigated by combining different methods, such as acid/alkaline, enzymatic, ultrasound and pH-shift processes, to achieve optimal extraction of protein-polysaccharide conjugates. The objective was to obtain extracts enriched in proteins and polysaccharides and to analyze their functional and bioactive properties.

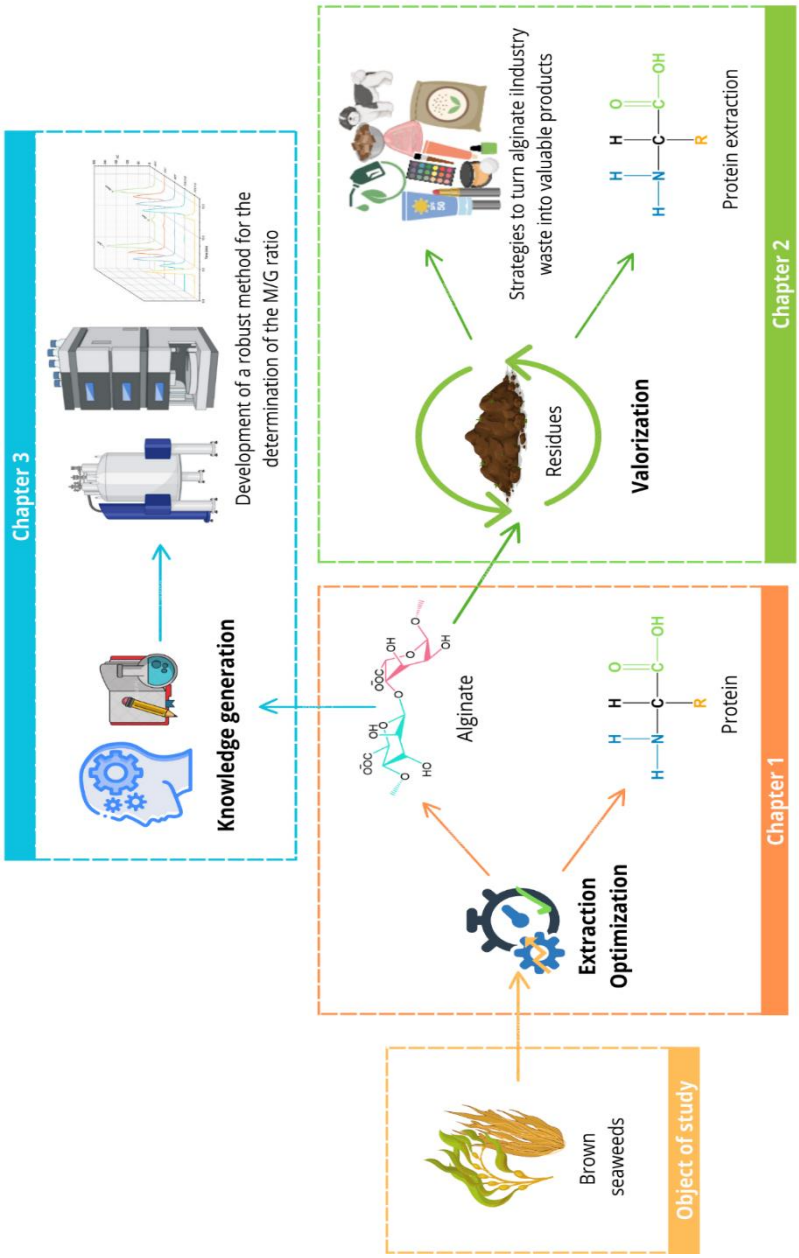
The second chapter analyzed the residues and effluents generated during extraction, seeking their use in developing value-added compounds. The main objective was to carry out an integral valorization of biomass to generate high-value ingredients and respond to the growing demand for natural bioactive compounds in the food industry, as well as promoting circular

economy practices. As a result of this effort, several valorization approaches were proposed for these wastes, highlighting their outstanding potential as bioactive ingredients, texturizers, or providing nutritional value in various fields such as food, cosmetics, feed, or pharmaceutical applications.

Additionally, we delved into extracting proteins from the residues obtained after the alginate extraction process to ensure industrial relevance and sustainability. This comprehensive analysis evaluated the efficacy of sequential extraction, which combined different extraction techniques, including chemical extraction, ultrasound, enzymatic treatments and precipitation by pH-shift process. Our study revealed the efficient release of intracellular proteins and offered a promising avenue for improved protein recovery. This finding is particularly significant as it opens up new possibilities for improving these proteins' functional and bioactive properties.

Finally, to contribute to the general methodological knowledge in brown algae research and provide a basis for future advances, a robust and accurate method for quantifying uronic acids was developed in the third chapter. This method allowed the determination of alginate's M/G ratio by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Compared to spectroscopic (Nuclear Magnetic Resonance and Fourier-transform infrared spectroscopy) and colorimetric methods, this method demonstrated accuracy under optimal conditions comparable to that of the reference method (NMR). This enables the analysis of alginate in a broad range of applications relevant to various disciplines.

THESIS FLOW CHART



Chapter 1

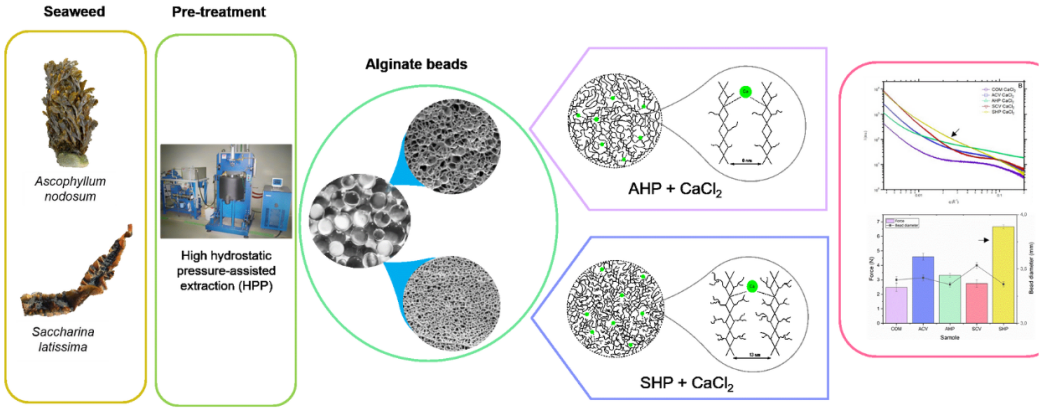
Optimization of extraction of valuable compounds from brown seaweed

1.1 Structural and functional properties of alginate obtained by means of high hydrostatic pressure-assisted extraction

1.2 Emulsifying and bioactive properties of protein-polysaccharide conjugates from brown algae: exploring novel functional ingredients

1.1

Structural and functional properties of alginate obtained by means of high hydrostatic pressure-assisted extraction



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

Bojorges, H., Martínez-Abad, A., Martínez-Sanz, M., Rodrigo, M. D., Vilaplana, F., López, A., & Fabra, M. J. (2023). Structural and functional properties of alginate obtained by means of high hydrostatic pressure-assisted extraction. Carbohydrate Polymers, 299, 120175. https://doi.org/10.1016/J.CARBPOL.2022.120175

ABSTRACT

The effects of the high hydrostatic pressure (HPP) pre-treatment on the alginate extraction were seen to greatly depend on the recalcitrant nature of two algae species. Alginates were deeply characterized in terms of composition, structure (HPAEC-PAD, FTIR, NMR, SEC-MALS), functional and technological properties. The pre-treatment significantly increased the alginate yield in the less recalcitrant *A. nodosum* (AHP) also favoring the extraction of sulphated fucoidan/fucan structures and polyphenols. Although the molecular weight was significantly lower in AHP samples, neither the M/G ratio nor the M and G sequences were modified. In contrast, a lower increase in alginate extraction yield was observed for the more recalcitrant *S. latissima* after the HPP pre-treatment (SHP), but it significantly affected the M/G values of the resulting extract. The gelling properties of the alginate extracts were also explored by external gelation in $CaCl_2$ solutions. The mechanical strength and nanostructure of the hydrogel beads prepared were determined using compression test, synchrotron small angle X-ray scattering (SAXS), and cryo-scanning electron microscopy (Cryo-SEM). Interestingly, the application of HPP significantly improved the gel strength of SHP, in agreement with the M/G values and the stiffer rod-like conformation obtained for the SHP samples.

1. Introduction

Macroalgae are one of the renewable resources of the marine environment, which have received rising attention due to their important role as a valuable

source of hydrocolloids and bioactive compounds (Abka Khajouei et al., 2021; Hentati et al., 2018). There are about 9000 species of macroalgae, which, depending on their photosynthetic pigments, can be classified into Chlorophyta (green), Phaeophyta (brown), and Rhodophyta (red) (Sanjeewa et al., 2018). Specifically, brown algae are rich in polysaccharides, phenolic compounds, carotenoids, proteins, vitamins, and minerals (Kadam & Prabhasankar, 2010). The main polysaccharide present in the cell wall of brown algae is alginate and can represent up to 40% of the dry matter of the seaweed (Łabowska et al., 2019). Alginate is a linear polymer constituted by two monomeric uronic acids, β-(1-4)-D-mannuronic acid (M block) and α-L-guluronic (G block) acid (Fertah et al., 2017). The arrangement of the monomers creates homogeneous MM and GG blocks only composed of one uronic acid species and heterogeneous MG (or GM) blocks (Khajouei et al., 2018). The biological and physicochemical properties of alginates depend on the M/G ratio and on the structure and distribution of acid blocks in the polysaccharidic backbone, which varies depending on the species, the type of tissues, age or harvesting seasons (Karaki et al., 2013). In this regard, stiff and brittle alginate gels are known to have low M/G ratios, while elastic and flexible gels are known to have high M/G ratios. Specifically, in the food industry, they are widely used ingredients due to their stabilizing, thickening and gelling properties (Brownlee et al., 2005), also having a role as dietary fiber. All these properties are highly affected not only by the molecular weight and M/G ratios of alginates but also by their molecular conformations (Zrid et al., 2016).

Generally, the alginate extraction process involves an acid pre-treatment to remove pigments, carbohydrates and low molecular weight compounds. Then, an alkaline extraction is performed, followed by solid/liquid separation, drying and milling, to produce high-quality alginate (Bojorges et al., 2022). However, some alternative and novel protocols have been proposed in the last decade (Gomez et al., 2020; Ummat et al., 2021; Yang et al., 2022), such as ultrasound-assisted extraction (Flórez-Fernández et al., 2019), enzyme-assisted extraction (Borazjani et al., 2017), microwave-assisted extraction (Okolie et al., 2020), subcritical water extraction (Saravana et al., 2018), and supercritical fluid extraction (Balboa et al., 2015) with the aim of causing microstructural modifications in the treated seaweeds, favoring their extractability (Abka Khajouei et al., 2021). High hydrostatic pressure processing (HPP) is one of the most economically viable non-thermal treatments (Rastogi et al., 2010). The application of HPP is generally used to inactivate pathogens for food preservation without negatively affecting their organoleptic and functional properties (Marin et al., 2020). Likewise, it has been found that the application of HPP in extraction processes may bring about structural changes in foods, such as rupture of the plant cell walls, improving the mass transfer rate, as well as enhance the solvent permeability in plant cells and secondary metabolite diffusion (Pinton et al., 2021; S. Zhao et al., 2014).

Recent studies have indicated that HPP may increase the extraction yield of bioactive compounds from plant sources and reduce the processing time (Malafronte et al., 2021; Schultz et al., 2004; Zuluaga et al., 2016). However, to the best of our knowledge, there is no existing literature on the

use of HPP for alginate extraction, and no data have been reported regarding the physicochemical properties of the so-obtained alginates. The main hypothesis of this work was that HPP could increase the yield, inducing changes in the extracts' composition and structure, which will have an impact on its functional and technological applications. Therefore, the possibility of using HPP as a pre-treatment in the alginate extraction protocol has been evaluated also studying the effect of this pre-treatment on the composition, structure, and antioxidant capacity of the generated alginate-based extracts. Moreover, a detailed structural characterization of the different alginate gels produced by external gelation with $CaCl_2$ and of their mechanical performance was carried out using small angle X-ray scattering (SAXS), cryo-scanning electron microscopy (Cryo-SEM), and texture analysis.

2. Materials and methods

2.1 Materials

The brown seaweed *Ascophyllum nodosum (A. nodosum)* and *Saccharina latissima* (*S. latissima*) were supplied by Porto-Muiños S.L. (Cerceda, A Coruña, Spain) as a dry powder ($<$ 2% water content). Folin-Ciocalteu's reagent, sodium bicarbonate, hydrochloric acid (37% (v/v)), gallic acid ($\geq$98.0% purity), Trolox (97% purity), sodium hydroxide (pharma grade), ABTS+· (HPLC grade), and commercial alginate (*COM*) (PHR1471) were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Alginate extraction

Seaweeds *A. nodosum* and *S. latissima* were pre-treated using HPP and alginate extracted using the method described by Bojorges et al., 2022 to yield AHP and SHP alginate samples, respectively. Briefly, samples were dispersed in water, individually packed in sterile polyethylene bags and heat-sealed using a liquid:solid ratio of 1:25 (wt.). Additionally, two overlapping bags were used to avoid any potential contact between the pressurizing fluid in the samples before being introduced into the High-Pressure Food Processor (EPSI NV, Belgium) vessel. HPP pre-treatment was carried out in a pilot-scale unit (2-liter vessel volume).

It is known that HPP can alter the cell permeability of fruits and vegetables. The degree of cell alteration depends not only on the level of pressure applied but also on the type of plant cell. It has been observed that the integrity of some vegetables at (400 MPa/30 min/5°C) affects the organization of parenchyma cells, leading to cavity formation and plant cell disintegration (Préstamo & Arroyo, 1998). Thus, similar phenomena can be expected to occur when seaweeds are subjected to HPP. Besides, it has been observed that 400 MP significantly affects proteolytic and aminopeptidase activity in brown algae (del Olmo et al., 2019), so it was decided to use conditions below 400 MP to avoid polysaccharide degradation and increase extraction yields. Therefore, the samples were pressurized at 350 MPa, for 5 min at room temperature (20-22 ºC). The pressure level, pressurization time, and temperature were automatically controlled. After completing the pre-treatment, the samples were removed from the container and then submitted

to the conventional extraction protocol, as previously described by Bojorges et al., 2022. Alginate extracts from *A. nodosum* and *S. latissima* were also obtained using the conventional protocol (without HPP pre-treatment; ACV and SCV, respectively), and a commercial sodium alginate (COM; Sigma-Aldrich, PHR1471) was further used for comparative purposes.

2.3 Characterization of alginate extracts

2.3.1 Proximate composition

The total nitrogen content was analyzed using an Elemental Analyzer Rapid N Exceed (Paralab S.L., Spain). Approximately 250 mg of alginate samples were pressed into pellets and then analyzed using the Dumas method, which is based on the combustion of the sample and subsequent detection of the released N2 (Wiles et al., 1998). The total protein content was determined from the nitrogen content multiplied by a factor of 5 (Angell et al., 2016). The determinations were carried out in triplicate.

The ash content was determined by dry biomass calcination of *ca.* 3 g of freeze-dried alginate and alginate-based fractions powder in a muffle furnace, according to the standard method (AOAC, 1990).

2.3.2 Determination of monosaccharide composition

The carbohydrate content and sugar composition of the fractions were determined in triplicate after acid methanolysis according to Bojorges et al., 2022, using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a Dionex ICS-6000

(ThermoFisher). In brief, the dry samples were incubated with 2M HCl in dry methanol (1 mg/mL) at 100ºC for 5h. Subsequently, samples were neutralized with pyridine, dried under a stream of air, and further hydrolyzed with 2 M TFA for 1h at 120 °C. The samples were air-dried again and dissolved in water. Finally, the sample (4 mg) was incubated with 150 µL of 72% H_2SO_4 for 3h at room temperature; the solution was diluted with 1250 µL of deionized water and then incubated at 100 °C for 3 hours. All experiments were carried out in triplicate.

2.3.3 ABTS·+ radical cation scavenging activity

The antioxidant capacity of the different alginate extracts was determined, in triplicate, using the Trolox equivalent antioxidant capacity, according to (Re et al., 1999) with some modifications described in Bojorges et al., 2022. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as antioxidant standard.

2.3.4 Phenolic content

The total phenolic content was determined, in triplicate, by the Folin-Ciocalteau reagent as described by Singleton, Orthofer and Lamuela-Raventós (1999) with a slight modification previously described in Bojorges et al., 2022. The calibration curve was generated by using gallic acid as the standard, and the total phenolic content was expressed as mg of gallic acid (GA)/g extract.

2.3.5 Fourier transform infrared spectroscopy (FT-IR)

Freeze-dried alginate powdered was analyzed by FT-IR (FTIR; Bruker, Vertex, USA) using the attenuated total reflectance (ATR) sampling method. The spectra were recorded at 4 cm^{-1} resolution in a wavelength range between 400 and 4000 cm^{-1} and averaging a minimum of 64 scans.

2.3.6 Size exclusion chromatography (SEC)

The molar mass distribution of the alginate was analyzed by size exclusion chromatography (SECcurity 1260, Polymer Standard Services, Mainz, Germany coupled to a multiple-angle laser light scattering detector (MALLS; BIC-MwA7000, Brookhaven Instrument Corp., New York), and a refractive index detector (SECcurity 1260, Polymer Standard Services, Mainz, Germany) thermostated at 45 °C. The alginate solutions (1 mg mL^{-1}) were dissolved in 100mM $NaNO_3$ with 5mM NaN_3 and were filtered through a nylon filter (0.2 μm) prior to the analysis. Sample injections of 100 μL were performed into a combined column set-up with a SUPREMA pre-column, a SUPREMA 1000Å, and two SUPREMA 3000Å analytical columns (PSS, Mainz, Germany) eluting with 1 mL min^{-1} of 100 mM $NaNO_3$ with 5 mM sodium azide as mobile phase at 40 °C. The detailed procedure, calibration and setting parameters are detailed in the Supplementary Material.

2.3.7 Nuclear Magnetic Resonance (NMR) Spectroscopy

^1H liquid-state NMR measurements were performed on a Bruker AV400 spectrometer operating at a frequency of 400 MHz for ^1H nuclei. The experiments were conducted with one single-pulse at 80ºC and the locked

solvent to D_2O, recorded over a spectral width of 15 ppm during 4 s acquisition time, and 128 scans were collected. The M/G ratio and the diade/triade sequences were calculated from the area of ^1H NMR signals, according to Jensen et al. (2015) and the equations are detailed in the Supplementary Material.

2.3.8 Steady-shear flow measurements of alginate-based solutions

The rheological behavior of the alginate-based solutions was studied using a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany). Briefly, alginate solutions (2.5%, w/v) were prepared, in triplicate, by dissolving the powered samples in deionized water. The shear stress (σ) was measured as a function of the shear rate ($\dot{\gamma}$) from 0 to 1000 s^{-1} at 25 $\pm$ 2 ºC using a cone-plate geometry with a diameter of 35 mm and a gap of 54 μm. The power law model (equation 1) was applied to determine consistency index (k) and flow behavior index (n). Apparent viscosities were determined at 500 s^{-1}.

$$\sigma = \kappa \cdot \dot{\gamma}^n \qquad (1)$$

2.4. Development and characterization of alginate beads

The alginate beads were prepared by the syringe extrusion method. One mL of each alginate solution was extruded into a gelling bath containing 50 mM $CaCl_2$ solution. The distance between the tip and the gelling bath was set at 8 cm. The extruded gel particles were allowed to stand for 30 min for hardening, before being collected and rinsed with milli-Q water until testing.

The cationic composition (Ca^{2+} and Na^{+}) of the alginate beads was determined by inductively coupled plasma mass spectrometry (ICP-MS). The samples were subjected to an acid digestion in an oven at 200 °C and analyzed using an Agilent 7900 instrument.

2.4.1. Texture analysis

For the textural measurements, the alginate beads underwent uniaxial compression using a TA-XT Plus Texture Analyzer (Stable Micro Systems, UK) equipped with a 50 mm diameter cylindrical probe operating at a speed of 1 $mm \cdot s^{-1}$ and 80 % strain at ambient temperature (22 °C) (Krop et al., 2019). Due to the size variation of the alginate beads, ten beads from each alginate extract were measured and replicated thrice (Ching et al., 2016).

2.4.2. Cryo-scanning electron microscopy

A JSM-5410 SEM microscope (JEOL, Tokyo, Japan) was used with a Cryo CT-1500C unit (Oxford Instruments, Witney, UK) for the Cryo-SEM observation. Alginate beads were placed in the holder and frozen in liquid nitrogen ($T \leq -210$ °C). Then, the samples were transferred to the cryostorage, fractured, etched at -90ºC for 15 min, and sputtered with a gold layer. Afterward, the samples were examined at -130ºC, 1.5 kV, and at a working distance of 15 mm in order to ensure high image resolution.

2.4.3. Small angle X-ray scattering (SAXS)

SAXS experiments were performed on the non-crystalline diffraction beamline, BL-11, at the ALBA synchrotron light source

(www.albasynchrotron.es). The nanostructure of alginate solutions, before and after the addition of $CaCl_2$, was characterized by means of SAXS. The experimental procedure as well as data analysis were based on previous works from the co-authors (Gómez-Mascaraque et al., 2019; Gómez-Mascaraque et al., 2021). Aliquots of alginate solutions (containing 2.5% alginate and 50 Mm $CaCl_2$) were placed in 2 mm sealed quartz capillaries (Hilgenberg Gmbh, Germany) and allowed to cool at 25 °C for 24 h to form gels prior to the experiments.

The energy of the incident photons was 12.4 keV or, equivalently, a wavelength, λ, of 1 Å. SAXS diffraction patterns were collected using a photon counting detector, Pilatus 1 M, 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-detector distance was set to 6425 mm, resulting in a q-range with a maximum value of q = 0.23 Å-1. An exposure time of 2 s was selected based on preliminary tests. The data reduction was treated with the pyFAI python (ESRF) code (Kieffer & Wright, 2013), modified by the ALBA beamline staff, to make online azimuthal integrations from a previously calibrated file. Calibration files were created from a silver behenate (AgBh) standard. 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). A scattering background (corresponding to a quartz capillary filled with distilled water) was subtracted from all samples.

The scattering data were fitted to a unified model with two or three levels, depending on the sample:

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

This model considers that, for each individual level, the scattering intensity is the sum of a Guinier term and a power-law function (Beaucage, 1995). $G_i = c_i V_i \Delta SLD_i{}^2$ is the exponential prefactor (where V_i is the volume of the particle and ΔSLD_i is the scattering length density (SLD) contrast existing between the ith structural feature and the surrounding solvent), $R_{g,i}$ is the radius of gyration describing the average size of the ith 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.

2.5 Statistical analysis

The statistical analysis of experimental data was conducted using IBM SPSS Statistics software (version 23, IBM Corp., USA) through one-way analysis of variance (ANOVA). Tukey's test was used at a significance level of $p<0.05$ for multiple comparison tests.

3. Results and discussion

3.1 Extraction yield and composition of the alginate extracts

In the first part of this work, the alginate extracts obtained by applying the HPP pre-treatment were characterized and compared with their counterparts

prepared by means of the conventional method. The yield and composition of the extracts obtained from the two species were investigated and the results are compiled in Table 1. As observed, the alginate extraction yield of HPP pre-treated samples was 23 and 14 % for *A. nodosum* and *S. latissima*, respectively (Table 1), being significantly higher than their counterparts prepared with the conventional method. It is worth mentioning that the yield increase after the pre-treatment was substantially greater for *A. nodosum* pointing out that the high pressure applied was more effective in this case for causing cell wall disruption, resulting in a significant increase in the alginate yield. Similarly, the application of HPP has been reported to improve the extraction yield of polysaccharides and proteins from *P. palmata* and *S. chordalis*, respectively (Suwal et al., 2019). Furthermore, other intracellular compounds such as proteins, polyphenols (Jurić et al., 2019), lipids (Imatoukene et al., 2020), and other bioactive compounds (Navarro-López et al., 2020) can be simultaneously extracted using HPP treatments, ascribed to the cell wall damage. In fact, the polyphenol content in HPP pre-treated samples significantly increased as compared to their counterparts prepared by means of the conventional methodology, evidencing that the cell wall disruption favors the co-extraction of phenolic compounds with alginate.

It should be highlighted that different seaweed species show very variable extraction yields and, not only the extraction conditions but also the harvest season and the environmental factors can significantly affect the yield (Okolie et al., 2020). In this work, the obtained alginate extraction yields using both extraction methodologies were among the highest reported in the literature, such as C. *barbata* (9.9%) (Sellimi et al., 2015), C. *sedoides* (11%)

(Hadj Ammar et al., 2016), *S. oligocystum* (18.9%) (Davis et al., 2004), *D. dichotoma* (20.94%) and *S. fluitans* (21.1%) (Parthiban et al., 2012), *S. angustifolium* (3.5%) (Borazjani et al., 2017), *C. compressa* (21.6%) (Hentati et al., 2018).

Table 1. Proximal composition of alginate samples

Composition (dry wt. %)	ACV	AHP	SCV	SHP	COM
Yield	14.1 ± 0.4^{b}	23.0 ± 0.2^{c}	11.2 ± 0.4^{a}	13.9 ± 0.2^{b}	n/a
Protein	$3.3 \pm 0.3^{a,b}$	2.8 ± 0.0^{a}	2.9 ± 0.3^{a}	3.6 ± 0.1^{b}	<0.1
Ash	19.4 ± 0.1^{b}	20.9 ± 0.1^{d}	21.3 ± 0.0^{e}	19.1 ± 0.1^{a}	20.0 ± 0.0^{c}
Carbohydrates[1]	$68.8 \pm 1.7^{a,b}$	$70.0 \pm 1.8^{a,b}$	68.0 ± 2.1^{a}	$69.3 \pm 1.7^{a,b}$	72.0 ± 2.8^{b}
of which[2]					
Fucose	$2.8 \pm 1.2^{b,c}$	4.3 ± 1.1^{c}	1.1 ± 0.7^{b}	1.1 ± 0.7^{b}	$<0.1^{a}$
Xylose	$1.9 \pm 0.7^{b,c}$	2.7 ± 0.8^{c}	0.2 ± 0.2^{a}	0.3 ± 0.3^{a}	$1.1 \pm 0.1^{a,b}$
GulA	22.9 ± 0.7^{a}	24.2 ± 1.6^{a}	24.2 ± 1.6^{a}	25.3 ± 1.5^{a}	22.9 ± 1.7^{a}
GlcA	1.5 ± 0.2^{a}	1.2 ± 0.3^{a}	0.7 ± 1.0^{a}	0.6 ± 0.4^{a}	0.7 ± 0.7^{a}
ManA	39.6 ± 1.4^{a}	38.1 ± 1.8^{a}	40.8 ± 1.3^{a}	41.0 ± 2.3^{a}	45.3 ± 2.5^{b}
Polyphenols (mg GAE/g sample)	60.0 ± 2.5^{d}	67.6 ± 2.4^{e}	45.6 ± 1.4^{b}	56.2 ± 2.1^{c}	11.2 ± 1.4^{a}

The values report means (n=3) ± standard deviation. For alginate samples, different letters in the same row indicate significant differences between the samples ($p<0.05$).
[1]Total carbohydrate content calculated as the sum of all monosaccharides detected by HPAEC-PAD
[2]Galactose, Glucose, Mannose and Mannitol were detected in negligible amounts (<1wt%) in all samples.

The proximate composition, ash and protein contents were also analyzed and the results are also shown in Table 1. The protein content did not significantly vary among samples. On the other hand, the ash content results agreed with available literature in which a ~30% ash was found in alginate extracts from C. *schiffneri* (Benslima et al., 2021). Polyphenols and

proteins (5.9 mg/g) are also present in minor quantities in commercial food-grade sodium alginates (Dusseault et al., 2006). As expected, all the alginate extracts presented high polysaccharide contents, alginate units (mannuronic acid -ManA- and guluronic acid -GulA) begin the main sugar constituents, followed by fucose, xylose and glucuronic acid (GlcA). The high alginate content in the extract was reflected by the sum of GulA and Man A units, which was around 62 wt.% and 65-66 wt% for *A. nodosum* and *S. latissima*, respectively, and is in line with the purity of typical commercial alginates (COM). The relatively high purity of HPP pre-treated samples evidences that the higher yields were not the result of higher extraction of untargeted compounds (Deniaud-Bouët et al., 2017).

Considering that the alginate content in the raw seaweeds was 21.5 wt% and 25.4 wt % for *A. nodosum* and *S. latissima*, respectively (Bojorges et al., 2022), the HPP pre-treatment greatly promoted the extraction of alginate with high purity from both seaweeds. This was more evident for *A. nodosum* in which 97.5% of the alginate present in the raw seaweeds was extracted together with other components such as polyphenols and sulphated polysaccharides. This effect can be ascribed to the differences in the cell wall structure of both species (Deniaud-Bouët et al., 2017), and it may be interesting for the production of functional extracts as it will be described below. It is also worth noting that the use of HPP promo ted the extraction of fucose and xylose units from *A. nodosum*, highlighting the presence of sulphated fucoidan/fucan structures in the extract, in agreement with their higher abundance in the cell wall architecture of this family (Deniaud-Bouët et al., 2017). The alginate extracts were also qualitatively

characterized by means of FT-IR, and the obtained spectra are shown in Figure 1. As observed, the spectra were not significantly different to those from the commercial alginate, thus confirming the purity of the samples. This was reflected in the range between 800 and 1700 cm^{-1} in which the characteristic alginate bands could be identified. Specifically, the band at around 1600 cm^{-1}, corresponding to the asymmetric carbonyl stretching in the carboxylate anion (COO-), the bands at 1100 and 1025 cm^{-1}, associated to the C-C and C-O stretching vibrations of the pyranose ring (Montes et al., 2021) and the fingerprint or anomeric region (750-950 cm^{-1}) (Flórez-Fernández et al., 2019; Mohd Fauziee et al., 2021) described by the band observed at 918 cm^{-1} related to the C-O stretching vibration of the uronic acid residue, the band at 850 cm^{-1} associated with the C1-H deformation vibration of β-mannuronic acid, and the weak bands from 780-815 cm^{-1} assigned to the vibrations of mannuronic acid residues (Gómez-Ordóñez & Rupérez, 2011).

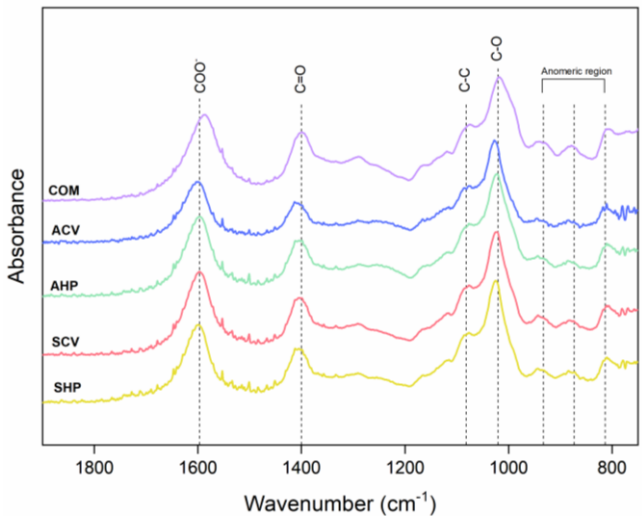


Figure 1. FTIR spectra of alginate samples.

The weight average molecular weight (Mw), the number average molecular weight (Mn), the dispersity (Đ = Mw/Mn), and radius of gyration (Rg) of the different alginate extracts and the reference (COM) were analyzed by means of SEC-MALLS, and the results are gathered in Table 2. Furthermore, Figure 2a depicts the relative molar mass distribution of all samples. A single peak was clearly observed in all samples, except in the commercial one. As observed, the M_w of the different alginate extracts ranged between 169 and 257 kDa (see Table 2), while the commercial alginate was significantly lower (around 68 kDa). It should be highlighted that the dispersity for all the samples was greater than 1, indicating high Mw dispersity, especially in the commercial one. According to the literature, the weight average molecular weight of commercial alginate from brown seaweeds ranges between 32 and 400 kDa (Rinaudo, 2007).

It is worth mentioning that the HPP pre-treatment significantly reduced the Mw values of the AHP samples as compared to its counterpart prepared with the conventional method. In contrast, this effect was not observed in SHP samples, which could be ascribed to the differences in chemical composition and cell wall structure and pointing out to a more recalcitrant nature of the alginate from *S. latissima*.

It is well known that SEC alone separates grounded on hydrodynamic volume rather than molecular weight, with light scattering also allowing the assessment of chain conformation as represented by the fractal dimension ν_g, which occurs as an exponent in the de Gennes scaling law concept: $R_g = K_g(Mw)^{\nu g}$ (Marczynski et al., 2021). For COM and SCV alginates, the ν_g adopts values of 0.63 and 0.64, respectively, corresponding to a random coil with a relatively flexible conformation. For the alginates extracted by the conventional method ACV and AHP, ν_g values of 0.66 and 0.67 were obtained, respectively, which would indicate a slightly stiffer conformation, while for SHP the ν_g was 0.73, which corresponded to an even stiffer conformation (Table 2, Figure 2b). A more rigid polymeric chain conformation may contribute to the formation of stronger SHP hydrogels, as it will be detailed below.

Table 2. Physicochemical properties of alginates extracts and COM.

Sample	M_w (kDa)	M_n (kDa)	Đ	V_g (-)
COM	68.34	19.24	3.55	0.63
ACV	241.90	157.40	1.54	0.66
AHP	179.70	115.20	1.56	0.67
SCV	169.40	74.74	2.27	0.64
SHP	257.30	148.60	1.73	0.73

The abbreviations Mw, Mn, and νg denote the molecular weight, number molecular weight, dispersity, and fractal coefficient, respectively.

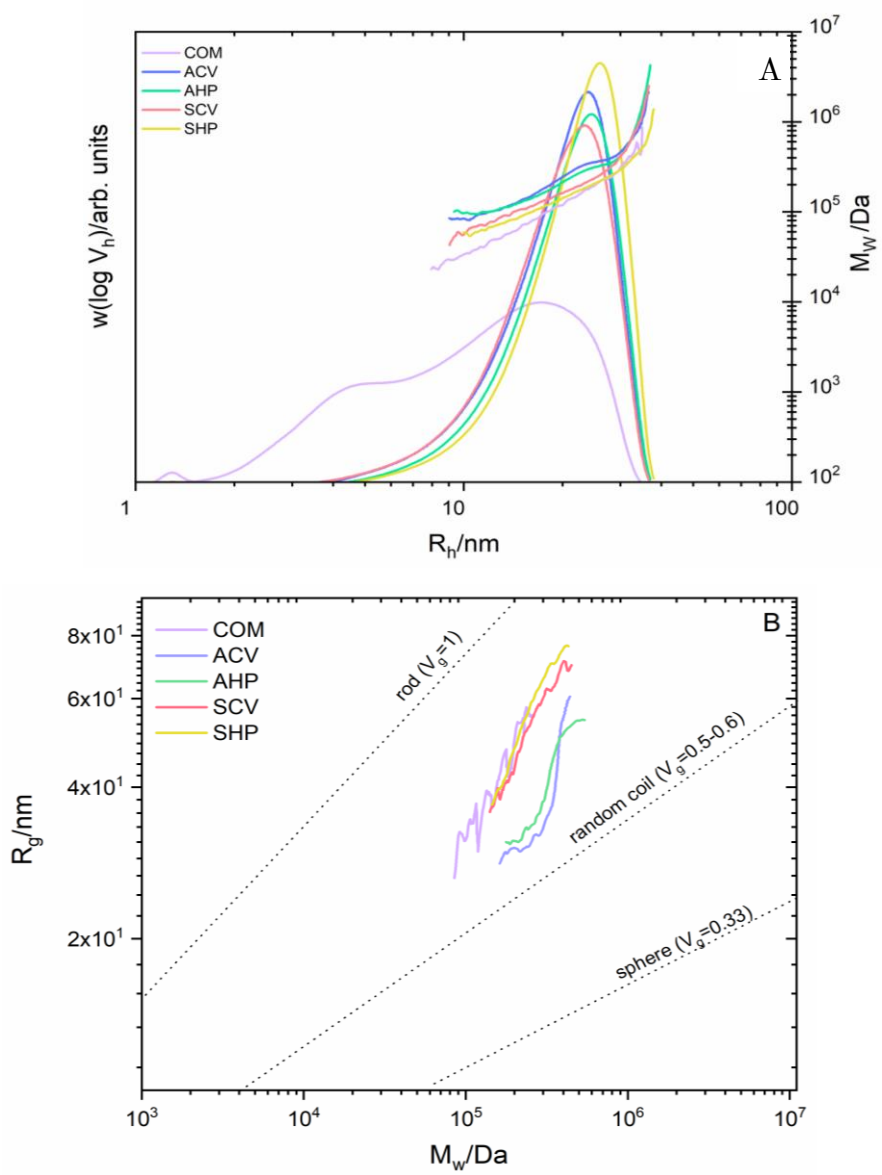


Figure 2. (A) SEC molecular weight [w (log V_h)] and weight average molecular weight [M_w (V_h)] distributions of different alginates extracted, as function of the hydrodynamic radius of these samples, (B) Fractal dimension ν_g allows for assessing the alginate conformation.

The physical properties, the ability to form gels and the strength of the alginate gels depend not only on the molecular weight distribution, but also on the uronic acid composition and the relative properties of the three types of blocks. In fact, it is well-known that stronger gels can be formed with alginates with high guluronic acid content since the egg-box conformation is preferentially formed among G-sequences forming junctions with divalent cations (Chan et al., 2011; Mørch et al., 2006). Therefore, the monomeric sequences of the alginate extracts, and reference samples were also compared by NMR. The calculated M/G ratios as well as the monads (M and G), diades (MM, GG, MG), and triades (MGM, GGM, GGG), shown in Table 3, revealed great differences between them.

Table 3. Composition data and sequence parameters of alginates extracts and COM.

	Frequency[a]				
	ACV	**AHP**	**SCV**	**SHP**	**COM**
M	0.56	0.58	0.60	0.54	0.55
G	0.44	0.42	0.40	0.46	0.45
MM	0.40	0.41	0.41	0.36	0.34
GG	0.28	0.24	0.21	0.28	0.24
MG	0.16	0.18	0.19	0.18	0.21
MGM	0.15	0.14	0.11	0.14	0.10
GGM	0.02	0.04	0.08	0.04	0.11
GGG	0.25	0.20	0.13	0.27	0.13
M/G	1.8	1.9	2.1	1.6	2.3
$\mathbf{N_{G>1}}$	14.50	7.00	3.63	7.00	8.00

[a] Expressed as molar fraction. M: β-D -mannuronic acid monomer; G: α- L-guluronic acid monomer. Mannuronate (M) and guluronate (G); $N_{G>1}$: average block length; M: M fraction; G: G fraction; MM: homopolymer fraction diads; GM: heteropolymer fraction diads; GGG and MMM homopolymer fraction triads; MGM and GGM: heteropolymer fraction triads.

In general, the amount of heteropolymeric diad blocks (MG) was lower (around 0.16-0.21) than MM homopolymeric blocks (0.40-0.46), followed by GG (0.34-0.41). The content of GGM triades ranged between 0.02 and 0.11, MGM values were around 0.10-0.15, and GGG values ranged between 0.13 and 0.27.

M/G ratios and G-sequences of alginate extracts obtained from *S. latissima* by means of the conventional method and the commercial one, SCV and COM were very similar whereas ACV presented lower M/G values and higher GG and GGG blocks, thus suggesting that ACV would give stronger gels, as it was later on confirmed. An important observation is that the HPP pre-treatment did not alter M/G values of the resulting AHP extracts and a slight reduction in the G-sequences was noted. For the SHP extract, a lower M/G ratio was observed, the homopolymeric content of MM was significantly lower (0.36), while the GGG content (0.27) was clearly higher than in the non-pretreated extract or the other alginate extracts. It has been described that Ca^{+2} cations bind both to G sequences and to alternating GM and GGM dimeric/trimeric blocks but not to M-blocks only (Mørch et al., 2006), both being highest in SHP extracts.

Therefore, the HPP pre-treatment had a different effect on the alginate extraction depending on the recalcitrant nature of the algae and significant differences were observed between the two species. Alginate yield was significantly higher in those obtained from *A. nodosum* and the molecular weight was greatly affected (lower Mw values) by the HPP pre-treatment, probably ascribed to a partial degradation of the polymer chains. However,

neither the M/G ratio nor the M and G sequences were greatly modified. In contrast, the more recalcitrant nature of *S. latissima* was patent on the lower increase in the extraction yield after the HPP pre-treatment (as compared to *A. nodosum*). In this case, the HPP pre-treatment resulted in an alginate with increased Mw and higher abundance of G-sequences, all of which will impact the molecular organization and technological performance.

3.2 Antioxidant capacity of the alginate extracts

Algae and seaweeds are known to contain bioactive compounds such as polyphenols and sulphated polysaccharides that possess antioxidant properties (Abdel-Latif et al., 2022). Therefore, the antioxidant capacity of the alginate-based extracts from both species was evaluated. As shown in Figure 3, the alginate extracts showed higher antioxidant activity than the commercial one (COM). This can be ascribed to their higher polyphenol and protein content (see Table 1). In fact, several authors have reported that the antioxidant activity of macroalgae is related not only to the polyphenol content (Hardouin et al., 2014; Suwal et al., 2019), but also to the protein content and the presence of polysaccharide degradation products (Siriwardhana et al., 2008). It is worth mentioning that the antioxidant capacity was significantly higher for the AHP. This was already anticipated due to the higher polyphenol content, the lower molecular weight of the alginate and the presence of sulphated polysaccharides in the alginate-based extracts obtained from *A. nodosum* (Abka Khajouei et al., 2021). In fact, some works have demonstrated that alginates with lower molecular weight showed higher antioxidant activity (Kelishomi et al., 2016; Khajouei et al., 2018).

Furthermore, other authors have shown that alternative extraction procedures (such as enzymatic and radiation methods) may provide antioxidant properties to the resulting alginate extract, ascribed to the alginate depolymerization (Falkeborg et al., 2014; Kelishomi et al., 2016; X. Zhao et al., 2012). Therefore, the lower molecular weight found in AHP sample could have also contributed to an increase on its antioxidant activity.

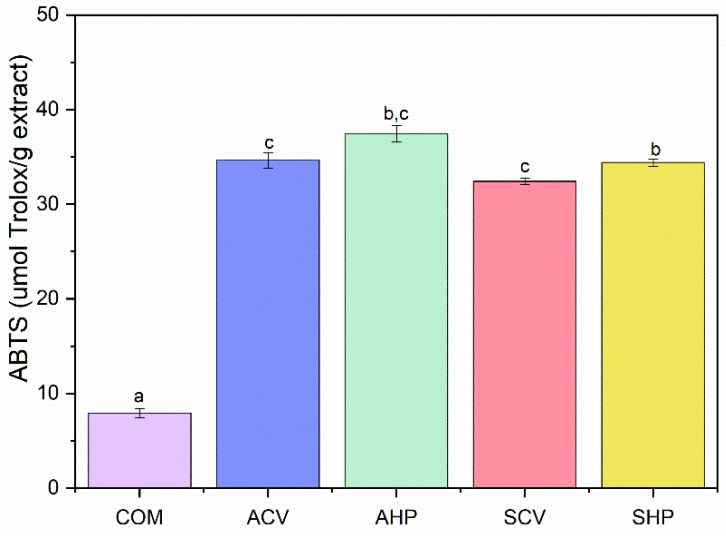


Figure 3. Antioxidant capacity of the alginates determined by ABTS radical scavenging activity. Different letters indicate significant differences ($p<0.05$).

3.3 Rheological behavior of the different alginate-based extracts.

Complete flow curves shown in Figure 4 indicate that all the solutions presented a shear thinning behavior (pseudoplastic), which was more accentuated when the HPP pre-treatment was applied. The rheological data of pseudoplastic solutions were fitted to Ostwald-de Waele model (Equation

1). Table S1 gathers the flow (n) and consistency indexes (κ), together with the apparent viscosity (η_{ap}) values at the shear rate of 500 s^{-1}. The values of the correlation coefficient were in all cases around 0.99, evidencing that the rheological behavior of the alginate-based solutions was well-described by the power-law model, in agreement with the results found in literature (Abka Khajouei et al., 2021; Cevoli et al., 2013; Hentati et al., 2020; Ma et al., 2014). In fact, the rheological parameters and the apparent viscosity at 500 s^{-1} of the alginate extracts obtained using the conventional extraction protocol (ACV and SCV) were in the order of those found by Ma et al. (2014). It was clearly observed that the HPP pre-treatment (AHP and SHP) promoted significant changes in the rheological patterns of the alginate extracts, increasing the apparent viscosity and consistency index (*k*) and decreasing the flow index (n). These results indicate that AHP and SHP made the fluid solutions more viscous and more shear thinning, probably due to the formation of different intermolecular entanglements (Montes et al., 2021), which is greatly influenced by the molecular weight of the biopolymers (higher M_w values for SHP) and the presence of other low molecular weight compounds (such as polyphenols).

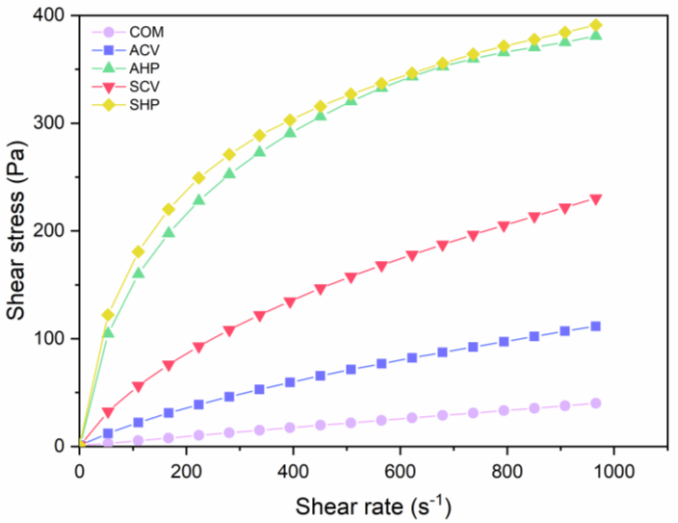


Figure 4. Experimental (symbols) and predicted (lines) flow curves obtained by Ostwald de Waele model.

3.4 Alginate-based hydrogels

The four alginate extracts and the commercial one were used to produce hydrogels with the addition of $CaCl_2$. Figure 5A shows representative cross-section micrographs of the corresponding beads. As observed, a less compact network was found in hydrogel capsules prepared with alginate extracts from *A. nodosum*, being the pore size greater than in their counterparts prepared with *S. latissima*. This effect could be ascribed to the presence of other components which could interfere in the gelation process of the alginate. Interestingly, the effect that the HPP pre-treatment had on the microstructure of the hydrogel capsules depended on the raw material used. It was clearly observed that in the alginate from *S. latissima* the pre-treatment promoted

the formation of a denser structure with a decrease in the average pore size, in agreement with the SAXS results (Figure 6).

The gel strength of the obtained hydrogel capsules from the different alginate extracts was evaluated by means of uniaxial compression tests. Figure 5B summarizes the calculated gel strength values for the alginate-based capsules. The first thing to highlight is that the application of HPP pre-treatment significantly improved the gel strength of SHP. This improvement can be related to a higher molecular weight, a slightly lower M/G ratio (Tables 1 and 3), especially, to a stronger presence of G blocks (GG and GGG; table 3). In contrast, the gel strength of AHP was significantly decreased as compared to ACV, fact which can be related to the lower molecular weight of the AHP, the presence of other low molecular weight compounds which could interfere in the gelation mechanism and the lower abundance of G blocks (table 3). Furthermore, these results agreed with the conformation results for alginate molecules already presented in Table 2. As it was anticipated, AHP alginates showed the most flexible linear conformation whereas SHP samples comprised a stiffer conformation.

To further investigate structural differences between the various hydrogels, the amount of Ca^{2+} in the developed capsules was also determined since the stiffness of alginate beads not only depends on alginate concentration and composition, M/G ratio, and the alternating M and G blocks, but is also affected by the degree of crosslinking. To confirm that, the cationic composition of all the alginate beads was determined by means of ICP-MS to quantify the amounts of Na^{+} and Ca^{2+}. The results, compiled in Table S2,

evidence Ca^{2+} as the most abundant cation in SHP and AHP hydrogels, its concentration increasing with HPP pre-treatment compared to SCV and ACV. However, an opposite tendency was observed with sodium, this increasing in AHP and decreasing in SHP. The higher Ca^{2+} is consistent with the structural properties of the alginate extracts. (Mørch *et al.*, 2006).

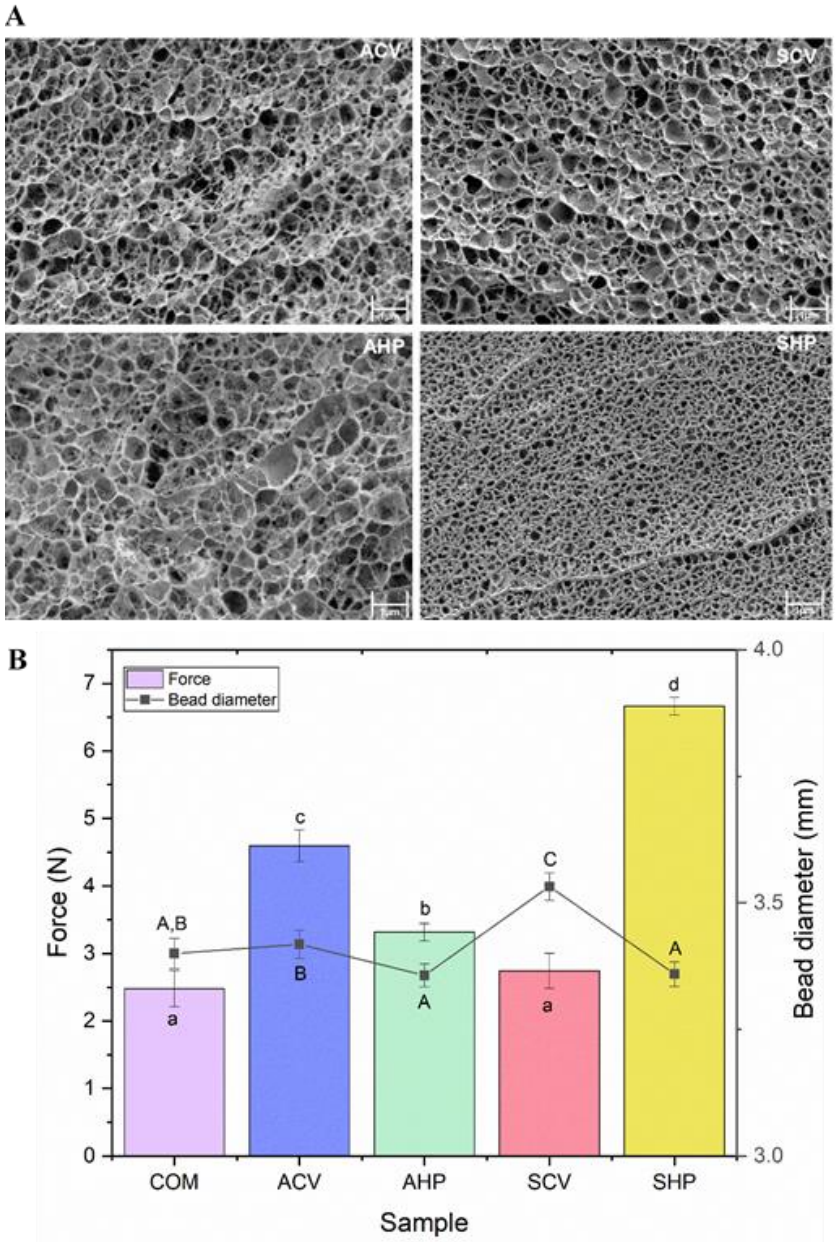


Figure 5. (A) Cryo-SEM micrographs of alginate beads (B) Comparison of maximum force and average alginate bead diameter. Different lowercase letters indicate significant differences ($p<0.05$) in the force of the alginate beads. Different uppercase letters indicate significant differences ($p<0.05$) in bead diameter.

3.5. SAXS characterization of the alginates and alginate hydrogels.

The nanostructure of the alginate aqueous solutions, before and after the addition of $CaCl_2$ to induce the association of alginate chains and promote the formation of gels, was investigated by means of small angle X-ray scattering (SAXS) and the obtained scattering patterns are shown in Figure 6. As observed, most of the samples were characterized by the appearance of a broad scattering peak within the high q region, being this feature more evident in the solution of the commercial alginate. On the contrary, this feature was not visible in the SHP alginate (i.e. the extract with the highest Mw). Interestingly, the intensity of this peak was reduced when $CaCl_2$ was added to the solutions, being the difference between the solutions before and after $CaCl_2$ addition more obvious in the commercial alginate and the *A. nodosum* alginates. Similar scattering peaks have been detected in the SAXS patterns from commercial carrageenan hydrogels; however, the peaks were no longer visible when KCl was incorporated into the formulations (Fontes-Candia et al., 2020).

Furthermore, this scattering peak has not been reported for alginate gels produced by the addition of $CaCl_2$ (Gómez-Mascaraque et al., 2019). Fitting models based on cylindrical form factors or their sum with a power-law model (to account for the scattering intensity within the low q region) did not provide satisfactory fits for the experimental data. Thus, it is not expected that this peak arises from the form factor of the alginate molecular chains. In the absence of salts, the repulsion between negative changes may lead to the formation of extended molecular chains with a rod-like conformation;

however, the neutralization of some of these negative charges would lead to ionic interactions, producing entangled networks. Accordingly, the scattering feature may correspond to an interference peak from the distance between adjacent polymeric chains within aggregates formed as a result of the presence of Na^+ cations neutralizing the negative charges in the alginate molecular chains.

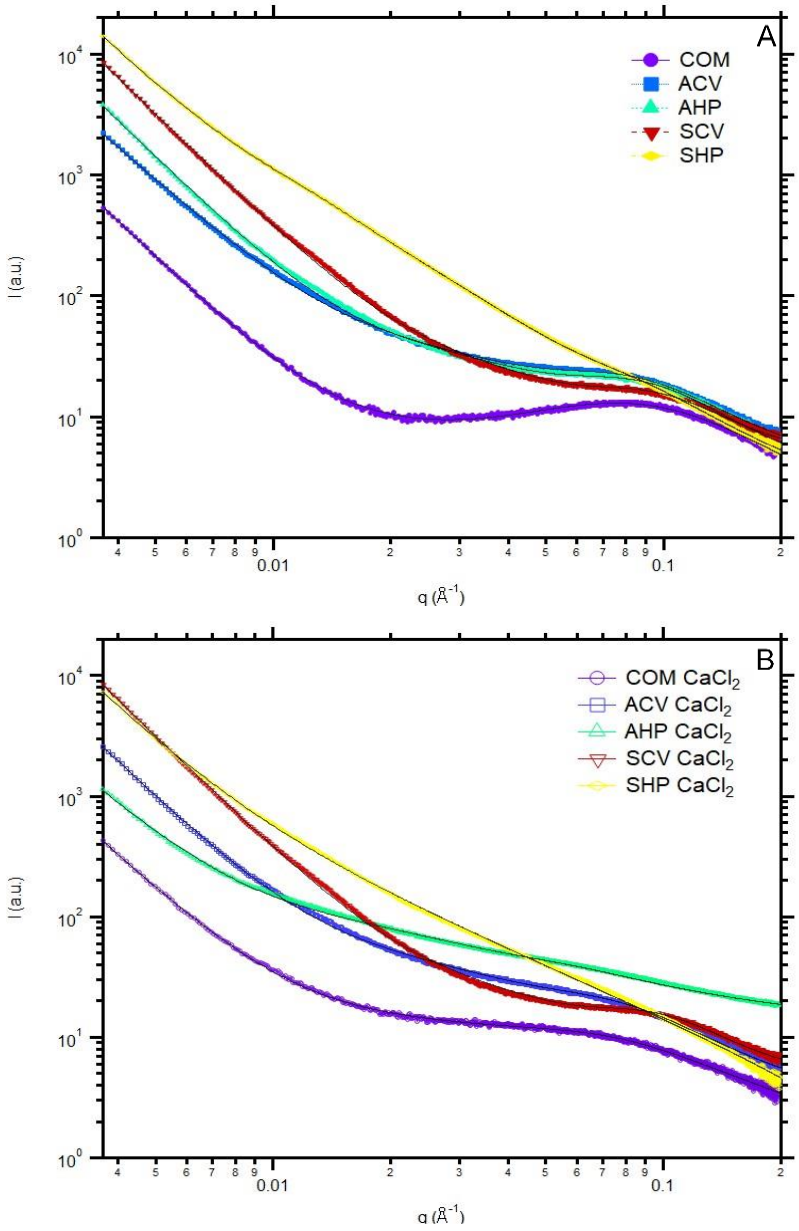


Figure 6. SAXS patterns from (A) the 2.5% w/v alginate solutions and (B) the same samples after the addition of 50 mM $CaCl_2$. Markers correspond to the experimental data and black solid lines represent the fitting curves

The experimental data were fitted using an empirical Beaucage model (also known as unified model), which is described by the following mathematical equation:

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

This model considers that, for each individual level (i), the scattering intensity is the sum of a Guinier term and a power-law function (Beaucage, 1995). $G_i = c_i V_i \Delta SLD_i{}^2$ is the exponential prefactor (where V_i is the volume of the particle and ΔSLD_i is the scattering length density (SLD) contrast existing between the ith structural feature and the surrounding solvent), $R_{g,i}$ is the radius of gyration describing the average size of the ith 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. In the specific case of this study, all the data could be fitted using two levels, except for the data corresponding to SHP, in which case an additional level was required to account for the appearance of a weak shoulder-like feature within the mid-q region (see Figure S2).

As deduced from the obtained fitting parameters, gathered in Table 4, the scattering intensity within the low q region could be described by a power-law function with exponents of ca. 2.9-3.1. This suggests the existence of highly clustered networks within the corresponding size range (170-70 nm). In the particular case of the alginate samples, the implication of this is that the alginate molecular chains are entangled forming highly branched networks

due to inter-/intramolecular interactions. Thus, a greater P_2 exponent indicates a greater clustering degree in the sample. From all the samples, only SHP presented a lower P_2 exponent, suggesting that the degree of chain association was more limited in that case.

The radius of gyration associated to the scattering peak, related to the distance between adjacent molecular chains within the clusters, corresponded to 3.3-12.6 nm and, in general, the size increased with the addition of $CaCl_2$. This suggests the existence of surface fractals within the corresponding size range (170-70 nm), i.e. highly branched networks. The radius of gyration associated to the scattering peak corresponded to 3.3-12.6 nm and, in general, the size increased with the addition of $CaCl_2$. This suggests that, as commented before, in the presence of a certain amount of Na^+ cations the polysaccharide chains are intertwined, with small distances of 3-5 nm between adjacent chains. The addition of Ca^{2+} cations displaces Na^+ cations and promotes the formation of different structures, where the polysaccharide chains are more extended in a rod-like conformation. In fact, the addition of $CaCl_2$ produced a decrease in the power-law exponent corresponding to the high q region (P_3), reaching values close to 1 (corresponding to rigid rods) in some of the samples. In contrast, the samples before the addition of $CaCl_2$ generally presented P_3 values of 2-3, corresponding to mass fractals, i.e. structures containing branching to form a 3D network. A greater P_3 exponent implies a greater deviation from the rigid rod-like behaviour and indicates that the alginate chains are less linear and more entangled. This deviation from the rigid rod-like behaviour was more notable in the SCV and AHP samples. It is worth noting that at the $CaCl_2$ concentration added in these experiments, it

looks like egg-box structures were not abundant in the samples, and thus an associated correlation peak was not detected.

Table 4. Parameters obtained from the fits of the SAXS data from the alginate solutions before and after the addition of $CaCl_2$ (Rg_i: radius of gyration for the structural level i; P_i: power-law exponent for the structural level i).

	P_1	Rg_2 (nm)	P_2	Rg_3 (nm)	P_3
COM	---	---	3.1	3.6	1.2
COM $CaCl_2$	---	---	2.9	4.8	1.2
ACV	---	---	2.9	3.8	2.3
ACV $CaCl_2$	---	---	3.0	4.3	2.0
AHP	---	---	3.1	3.6	2.7
AHP $CaCl_2$	---	---	2.9	8.1	1.0
SCV	---	---	3.1	3.3	3.1
SCV $CaCl_2$	---	---	3.0	3.4	2.6
SHP	3.6	37.1	2.3	4.5	2.1
SHP $CaCl_2$	---	---	3.0	12.6	1.4

4. Conclusions

HPP pre-treatment was applied for the production of alginate-based extracts from *S. latissima* and *A. nodosum* (SHP and AHP, respectively) and compared to those obtained using the conventional extraction protocol (SCV and ACV). The HPP pre-treatment was able to effectively increase alginate yields, especially in *A. nodosum*, without compromising the purity. The higher

polyphenol content in HPP pre-treated samples conferred them a relatively high antioxidant activity, being higher in AHP samples due to the presence of sulphated polysaccharides (fucoidan). The AHP extracts presented lower Mw than their ACV counterparts which could be ascribed to a partial depolymerization of the alginate during the extraction process. It also formed softer gels, owed to the lower Mw and less abundance of G-blocks. In contrast, *S. latissima*, the pre-treatment increased both the abundance of G-blocks and the Mw of the resulting polysaccharide. This explained the higher viscosity of the corresponding solutions and the stronger hydrogels formed in this case. Likewise, the nanostructure showed that the addition of Ca2+ promotes the formation of different structures, which tend to a rod-like conformation.

These results evidence the potential of the HPP pre-treatment as an efficient path towards a better utilization of marine sources, with a potential improvement on the technological or functional properties of the alginates obtained, depending on the source.

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6. Supplementary Material

Detailed description of the methodology used for size exclusion chromatography (SEC)

Universal calibration of the SEC set-up (equation 1) was performed using pullulan standards with known molar masses ranging from 342 to 708 000 Da (PSS, Germany) and their corresponding Mark-Houwink parameters in aqueous solutions at 40 °C estimated at $K = 1.0176 \times 10^{-3}$ dL g^{-1} and $a = 0.525$ (Sullivan et al. 2014). Here, V_h is the hydrodynamic volume; K and a are the Mark-Houwink parameters for pullulan; N_A is Avogadro's number; and M_n is the number-average molar mass. This procedure enables the conversion of the elution volume, which is dependent on the experimental conditions of the separation, into hydrodynamic volume (V_h) or in this case, hydrodynamic radius (R_h), with $V_h = \frac{4}{3} \cdot \pi \cdot R_h^3$.

$$V_h = \frac{2}{5} \cdot \frac{K \cdot M_n^{1+a}}{N_A} \qquad (1)$$

After universal calibration, the SEC weight distributions $w(\log V_h)$ for the alginate fractions were calculated from the refractive index signal, whereas the absolute weight-average molar mass $M_w(V_h)$ and the radius of gyration (Rg) were obtained from the MALLS data using the Zimm extrapolation (Zimm, 2004), following the procedure reported by Vilaplana and Gilbert (2010). A refractive index increments dndc of 0.14 mL g^{-1} for the alginates in the mobile phase solution was calculated injecting a series of

alginates with concentrations between 0.5 - 2 g L^{-1} and was used for the molar mass determinations (Figure S1).

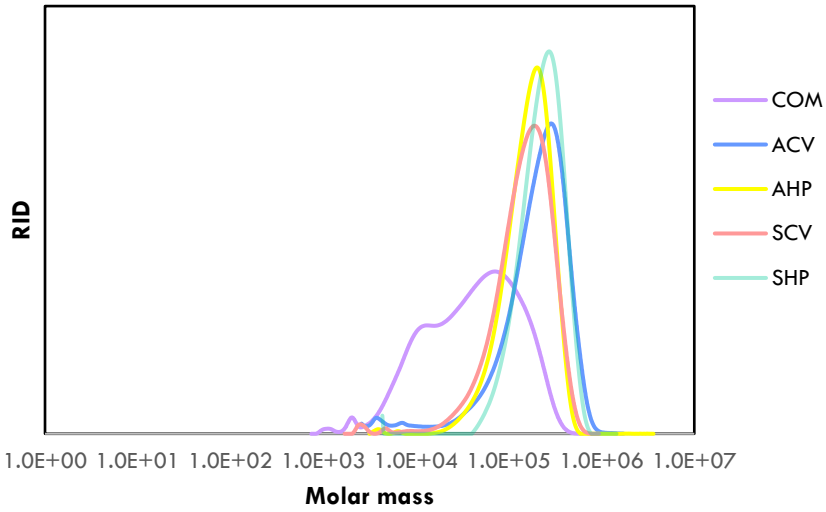

Fig S1. Molecular weight distributions as determined by MALLS at alginate concentration of 1.0 mg mL-1 with Light-Scattering detector.

Setting parameters and methodology used for Small angle X-ray scattering (SAXS)

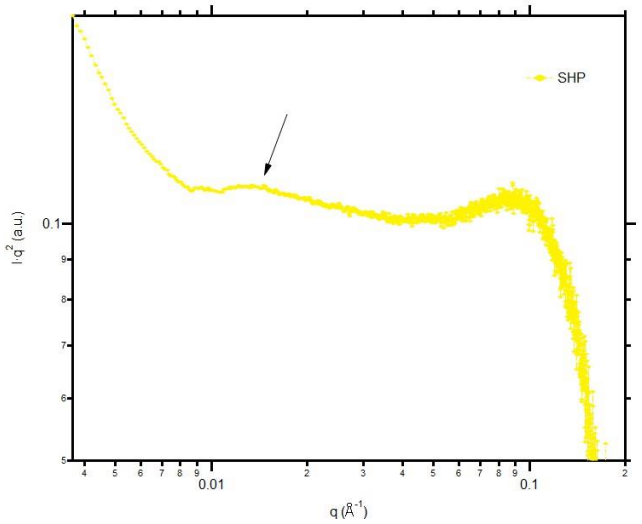

Figure S2. SAXS Kratky plot for the SHP alginate solution. The arrow points towards the shoulder feature detected in the mid-q region.

Nuclear Magnetic Resonance (NMR)

Fig. S3 shows a proton NMR spectrum allowing to determine the M/G ratio of sodium alginate by comparison of the relative areas of the three signals (I_A, I_B and I_C) using the method described by Jensen, Larsen and Engelsen (2015).

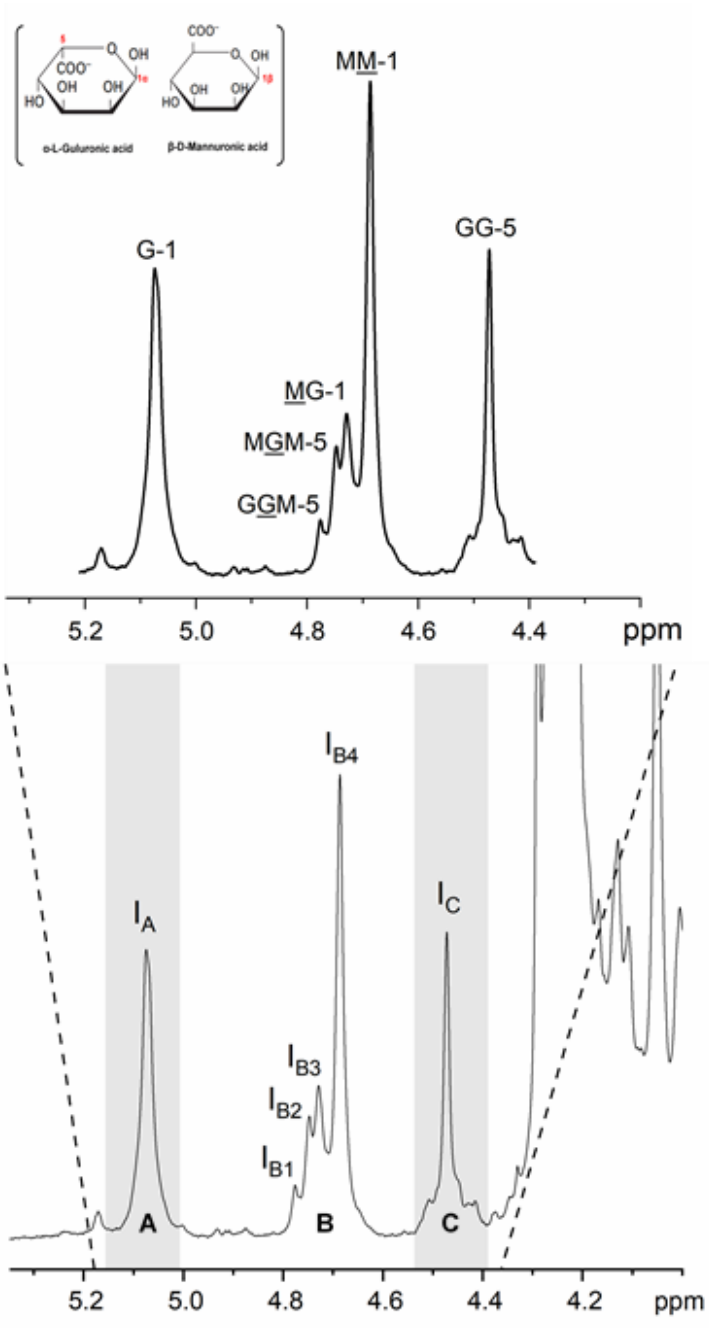

Fig. S3. Expanded regions of the ^{1}H NMR spectrum of the SHP alginate in D_2O and a schematic representation of diades.

A, and C were integrated regions to obtain the correspondent integrals: I_A and I_C, while the B (B1-B4) region was obtained by deconvolution to obtain integrals: I_{B1}, I_{B2}, I_{B3}, and I_{B4} (Fig. S3) used for M/G ratio calculation (Eq. 1, 2, and 3)

$$G = 0.5 * \left(I_A + I_C + 0.5 * (I_{B1} + I_{B2} + I_{B3})\right) \quad (1)$$

$$M = I_{B4} + 0.5 * (I_{B1} + I_{B2} + I_{B3}) \quad (2)$$

$$M/G\ ratio\ = M/G \quad (3)$$

Furthermore, the fraction of the diade and triade blocks (GG, MG, MM, GGM, MMG, and GGG) following the equations:

$$GG = 0.5 * (I_A + I_C - 0.5 * (I_{B1} + I_{B2} + I_{B3})) \quad (4)$$

$$MG = GM = 0.5 * (I_{B1} + I_{B2} + I_{B3}) \quad (5)$$

$$MM = I_{B4} \quad (6)$$

$$GGM = MGG = I_{B1} * 0.5 * (I_{B1} + I_{B2} + I_{B3})/(I_{B1} + I_{B2}) \quad (7)$$

$$MMG = I_{B2} * 0.5 * (I_{B1} + I_{B2} + I_{B3})/(I_{B1} + I_{B2}) \quad (8)$$

$$GGG = GG - GGM \quad (9)$$

Table S1. Ostwald-de Waele model fitting parameters of alginate solutions

Sample	Ostwald-de Waele fitting model parameters			
	n	κ	η_{ap} (cPs)	R^2
COM	0.70	0.33	0.04	0.99
ACV	0.82	0.79	0.35	0.99
AHP	0.51	13.71	0.46	0.99
SCV	0.69	3.16	0.31	0.99
SHP	0.36	45.43	0.64	0.99

Table S2. Cationic composition of the alginate beads samples (mg/g).

Sample	Na^+	Mg^+	Ca^{2+}	K^+
COM	96.5 ± 1.2	<0.01	93.3 ± 1.1	0.2 ± 0.0
ACV	86.8 ± 1.4	0.4 ± 0.0	80.0 ± 4.0	3.8 ± 0.5
AHP	94.3 ± 0.3	0.9 ± 0.1	103.0 ± 3.0	0.9 ± 0.1
SCV	98.3 ± 1.1	0.3 ± 0.0	74.3 ± 1.1	0.6 ± 0.1
SHP	88.6 ± 1.7	1.9 ± 0.5	107.0 ± 4.0	0.5 ± 0.0

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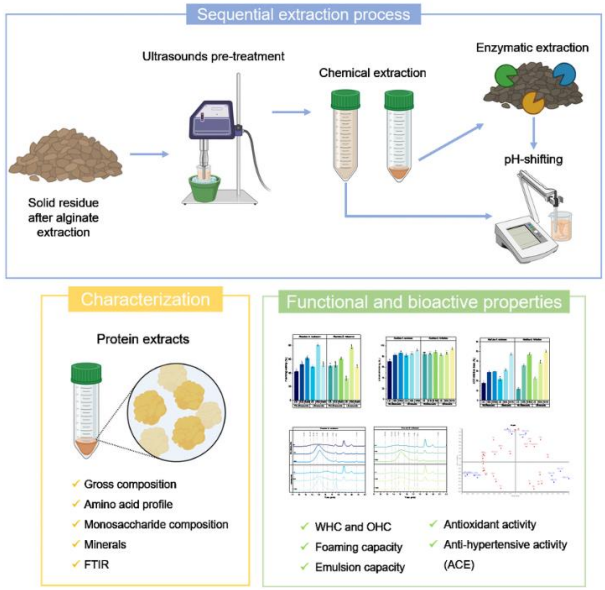
1.2

Emulsifying and bioactive properties of protein-polysaccharide conjugates from brown algae: exploring novel functional ingredients



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

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Emulsifying and bioactive properties of protein-polysaccharide conjugates from brown algae: exploring novel functional ingredients

Submitted to International Journal of Biological Macromolecules

ABSTRACT

The present work reports on the production of protein-polysaccharide extracts from brown algae, specifically from *A. nodosum* (A) and *S. latissima* (S) with potential application in the food industry. A sequential extraction method combining ultrasound, pH-shift and enzymatic (E) treatment was used to maximize protein extraction and, the obtained extracts were in-depth characterized in terms of composition, bioactive and emulsifying properties. Although the overall extraction yield of the extracts was significantly higher in those obtained from *A. nodosum*, the protein content in the extracts increased at least 3-fold after the enzymatic treatment, reaching the highest percentage in those obtained from *S. latissima*. The enzymatically-treated extracts (SE and AE) were mostly composed of low molecular weight proteins (17-25%) and carbohydrates (59-62%), alginate ad fucoidan being the major polysaccharides found in the extracts. These extracts exhibited significantly high emulsifying capacity (~ 99%) and stability during storage time, mainly ascribed to a combination of their relatively higher protein content (acting as the surface-active material), coupled with their lower molecular weight, which further contributed to stabilizing the oil droplets in the emulsions. This higher protein content was also positively correlated with angiotensin-converting enzyme (ACE) inhibitory activities. The antioxidant activity of the extracts was higher in those obtained from *A. nodosum* which was correlated with the extraction of sulfated fucoidan/fucan structures and polyphenols.

1. Introduction

Currently, global population growth, accompanied by improvements in living conditions, is expected to result in a doubling of food demand by 2050 (Steinbruch et al., 2023), with projected increases of 158% in global meat consumption patterns (de Souza Celente et al., 2023). However, this increasing demand for protein, particularly from animal sources, significantly contributes to carbon dioxide emissions and exacerbates climate change compared to other protein sources (Gregersen et al., 2021).

It is imperative to find alternative protein sources with a lower ecological impact than traditional agricultural systems to meet our needs. Seaweeds are a promising option to effectively address these challenges because they do not require arable land, freshwater, artificial fertilizers or pesticides and have high productivity (Wijers et al., 2020). Macroalgae are a rich source of nutritional compounds, including proteins, carbohydrates, polyphenols, vitamins, and minerals (Wells et al., 2017). The food and feed industry have widely used these compounds due to their high nutritional value, either as a source of proteins rich in essential amino acids or as dietary fiber through non-digestible carbohydrates (Lafarga et al., 2020).

The protein content in macroalgae depends on the species and the extraction method used. Brown algae, frequently described in the biorefinery context due to their high carbohydrate content, usually have lower protein content (Veide Vilg & Undeland, 2017). Despite this, their high productivity allows for a competitive yield per cultivation area compared to other species (Kraan, 2013).

Macroalgae proteins are stored in the cytoplasm or bound to the cell membrane. The rigid and resistant cell wall, mainly composed of polysaccharides such as cellulose and alginate, must first be broken down to extract these proteins. This implies an additional macrostructural obstacle for extracting intracellular compounds (Postma et al., 2018). Additionally, the ionic interactions between the cell wall components and proteins play a significant role in the extractability of the proteins (Bleakley & Hayes, 2017).

Current research efforts are focused on producing economically valuable ingredients, including bioactive peptides and carbohydrates, and exploring the application of macroalgae-derived proteins in human and animal food production (O'Brien et al., 2022). Bioactive peptides are small protein fragments, generally composed of between 2 and 20 amino acids, that help regulate various human processes, including the cardiovascular, nervous, and immune systems (Piovesana et al., 2018). These peptides have diverse biological effects and comprise diverse bioactivities, including antioxidant, antihypertensive, antimicrobial, and immunomodulatory properties (Lafarga et al., 2020).

Although there is increasing interest in proteins from macroalgae, extracting them poses great challenges. This has led to a lack of research on the quality and suitability of these proteins as functional ingredients and food supplements. Therefore, the main goal of this study was to evaluate a combined process that uses acid-alkali treatment and enzymatic treatment to extract proteins from *Ascophyllum nodosum* and *Saccharina latissima*. Furthermore, the functional and bioactive properties of the extracted proteins

were analyzed to determine their potential use in food formulations. It is important to emphasize that the potential success of any plant protein as a food ingredient is intrinsically linked to its functional properties and nutritional value.

2. Materials and methods

2.1 Materials and reagents

The brown algae S*accharina latissima* (S. *latissima*, designated as "S") and *Ascophyllum nodosum* (A. *nodosum*, designated as "A") were kindly provided by Porto-Muiños S. L. (Cerceda, Coruña, Spain) as a dry powder.

The commercial enzymatic preparations used in this study were two types of cellulases, Celluclast® (C2730, Sigma) and Cellic CTec2® (SAE0020, Sigma). Hippuryl-L-histidyl-leucine (HHL), quinoline, benzene sulfonyl chloride (BSC), commercial enzyme angiotensin-converting enzyme (ACE) from rabbit lung, ethanol, and hydrochloric acid (HCl), Folin-Ciocalteu reagent, ABTS™ Solution, fucose, arabinose, mannitol, galactose, glucose, xylose, and glucuronic acid, guluronic acid standards were purchased at Sigma-Aldrich (USA). Guluronic and mannuronic acid standards were from Biosynth Carbosynth Ltd. (Compton, UK).

2.2 Production of protein-polysaccharide extracts

2.2.1 Conventional extraction

Several extraction methods commonly reported in the literature were examined in this study (Fig. 1). Based on previous results carried out by Kadam et al. (2017) and those recently obtained in our group, the protein extraction was carried out using an ultrasound treatment to enhance the protein extraction yield in the extracts. Briefly, aqueous extraction was conducted by gently mixing algal biomass in distilled water (1:20 w/v) overnight at 4 °C. The suspensions were then ultrasonicated for 10 min with an ice bath and centrifuged at 350 rpm for 20 min. The supernatant was collected for posterior analysis. The pellets were suspended in 0.4 M HCl (1:15 w/v), stirred at 4ºC for 1 h, and centrifuged at 9000 rpm for 20 min. After the centrifugation, the pellet was resuspended in 0.4 M NaOH under the same conditions as the acid extraction. All supernatants (referred to as AA from *A. nodosum* and SA from *S. latissima*) were mixed and freeze-dried.

2.2.2 Enzymatic extraction

As part of the sequential extraction, the pellet obtained after the acid and alkali treatment was freeze-dried and used for an enzymatic-assisted extraction. The pellet was rehydrated with distilled water using a solid solvent ratio of 1:25; then, the mixture was sonicated for 10 min in an ice bath using ultrasound equipment (UP-400S, Hielcher GmbH, Germany) at 750 W. The suspension was kept in an ice-cold water bath to prevent overheating. Then, the enzymatic hydrolysis was conducted according to a Jamshidi et al. (2018),

with slight modifications. Briefly, 75 µL of each enzyme was added to the suspension, and it was performed using the optimum enzyme conditions (pH 4.5, at a temperature of 50 °C) for 12 h, under continuous agitation at 250 rpm using a thermo-shaker incubator (Labolan, Spain). Finally, the enzymes were inactivated by boiling the sample at 100 ºC for 5 min and immediately cooled in an ice bath. The resulting hydrolysates were centrifuged at 9000 rpm for 20 min at 4 ºC, collecting the supernatant, which was subsequently freeze-dried for further analysis and labeled AE and SE.

Control samples were prepared by subjecting the pellets obtained from the acid and alkaline treatment to the same conditions as the enzymatic process but without enzymes and the supernatants obtained were designated as AC and SC. Besides, a blank (enzyme in the buffer in the absence of dried algae) was included to subtract the added enzyme content from the protein content of the algae.

In order to clarify and facilitate understanding, samples´ nomenclature was “XY”, where "X" denotes the type of algae (“A” or “S” *Ascophyllum nodosum* and *Saccharina latissima*, respectively*)* and "Y" indicates the type of treatment (“A” to designate the acid/alkali treatment, “E” for the enzymatic treatment, and “C” for the control) to which the samples were subjected.

2.2.3 Precipitation of extracted proteins by a pH-shift process

All supernatants obtained were precipitated by a pH change process, following the method by Harrysson et al. (2018) with some modifications with the aim

of obtaining protein-rich extracts and remove other substances, such as polysaccharides, polyphenols, minerals, and other low molecular weight compounds. Briefly, the dried supernatants were suspended in distilled water (1:6 w/v) and incubated for 1h at 20 °C, then pH was adjusted to 12 with 1M NaOH, and the mixtures were centrifuged at 9000 rpm at 4 °C for 20 min. Afterwards, in order to determine the maximum protein precipitation, the supernatants were gathered, and an isoelectric precipitation was conducted by gradually adding HCl at various concentrations (ranging from 1 to 0.01 M), depending on the current pH level. This process was carried out to adjust the pH from 12 to 2, decreasing it by 1 pH unit at a time. Subsequently, the mixture was left undisturbed for 2 hours to enable the particles to aggregate. The highest precipitation was at pH 2 (data not shown). Then, the samples were frozen to improve the protein yield. After thawing, centrifugation was performed, and the resulting pellet was collected and subjected to freeze-drying for further analysis. The pellet and the supernatant were measured for protein content, as described in section 2.3.1. The precipitates were referred to as protein extracts. The protein yield was calculated as g protein extract/g brown seaweed on dry weight (dw).

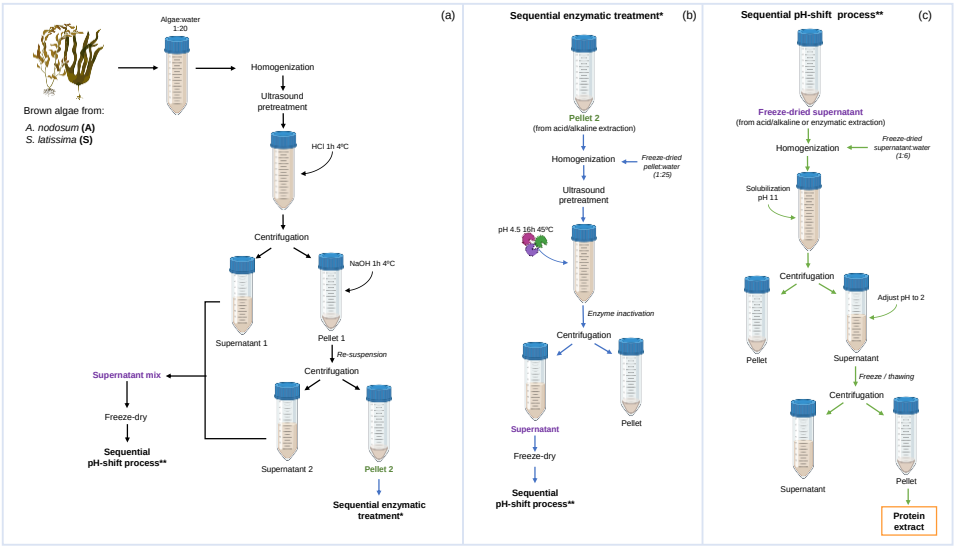

Figure 1. Scheme of the sequential protocols followed to produce protein-polysaccharide conjugate extracts from *A. nodosum* and *S. latissima*. (a) acid and alkaline extraction, (b) enzymatic treatment and (c) pH-shift process.

2.3 Composition analysis

2.3.1 Protein content

The protein content was assessed by quantifying the total nitrogen content utilizing an Elemental Analyzer Rapid N Exceed (Paralab S.L., Spain). Subsequently, the total nitrogen content was converted into protein using a multiplication factor of 6.25.

2.3.2 Determination of monosaccharide composition

The carbohydrate content and sugar composition of the fractions were analyzed following the methodology outlined by Bojorges et al. (2023).

Monosaccharides were determined using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Herisau, Switzerland). Control samples containing known concentrations of fucose, mannitol, arabinose, galactose, xylose, glucose, glucuronic acid, guluronic acid, and mannuronic acid were used for calibration. All experiments were conducted in triplicate.

2.3.3 Phenolic content

The phenolic content was evaluated utilizing the Folin-Ciocalteau assay method that Singleton et al. (1999) described with slight modifications. The sample extracts were dissolved in distilled water at a 5 mg/mL concentration. The Folin-Ciocalteau reagent was then diluted at 1:10 with distilled water, and 1 mL of the resulting dilution was combined with aliquots of 0.2 mL of the sample extract. The sample extracts were solubilized in distilled water at a 5 mg/mL concentration. Subsequently, the Folin-Ciocalteau reagent was diluted to a ratio of 1:10 with distilled water, and 1 mL of this solution was mixed with aliquots of 0.2 mL of the sample extract. Afterward, 0.8 mL of sodium carbonate solution (75 mg/mL) was added to the mixture. The solutions were incubated at 50°C for 30 minutes, followed by measurement of absorbance values at 750 nm. Calibration curves were generated using gallic acid as the standard. Total phenolic content was expressed as milligrams of gallic acid (GA) per gram of extract. All analyses were performed in triplicate.

2.4 High performance size exclusion chromatography (HP-SEC)

The extracts underwent dilution in a 0.01 M NaOH solution, filtered with a 0.22-micron membrane. Molecular weight distribution estimation was performed using a 10 by 30 cm CH HP-SEC ACQ Arc Sys Core system (Waters, USA), outfitted with a Waters 2414 RI detector with columns polysep 4000 and polysep 2000 (Phenomenex) in series at 60ºC and a flow rate of 0,8 mL/min. Pullulan solutions (PSS Polymer Standards Service GmbH, Germany) served as reference standards for comparative analysis.

2.5 Fourier transform infrared spectroscopy (FT-IR)

The dried extracts were analyzed using Fourier-transform infrared spectroscopy (FT-IR) with attenuated total reflectance (ATR) sampling method, conducted on a Bruker Vertex instrument. Spectra were recorded with a resolution of 4 cm^{-1} within a wavelength range spanning from 400 to 4000 cm^{-1}, with a minimum averaging of 64 scans.

2.6 Color measurement

The color of the samples was assessed using a colorimeter (CR-400, Konica Minolta Sensors, Japan) and registered as brightness (L*), chroma (C*), and hue (hº) in triplicate measurements.

2.7 Functional properties

2.7.1 Water-holding capacity (WHC)

The water-holding capacity was evaluated following the method described by Zhang et al. (2023), with minor modifications. Briefly, 10 mL of distilled water was mixed with protein extracts (0.5 g) in a Thermoshaker Incubator (MSC-100, UK). Subsequently, the samples were centrifuged at 5500 rpm for 30 minutes. After carefully decanting the supernatants, the remaining pellets were weighed. WHC was expressed as the amount of water retained per gram of protein and calculated using Equation 1.

$$WHC(gH_2O) = \frac{W_2 - W_1}{W_0} \quad (1)$$

Where W0 is the weight of the dry sample (g), W1 is the weight of the tube plus the dry sample (g), and W2 is the weight of the tube plus the pellet (g).

2.7.2 Oil-holding capacity (OHC)

The oil-holding capacity was determined using 0.5 g of the sample, which was transferred into a pre-weighed tube and thoroughly mixed with 10 mL of sunflower oil. Subsequently, the protein-oil mixture was centrifuged 30 min at 6000 rpm. After decanting the supernatants, the remaining pellets were weighed. OHC (grams of oil per gram of protein) was determined using the following Equation 2.

$$OHC(gOil) = \frac{F_2 - F_1}{F_0} \quad (2)$$

Where F_0 is the weight of the dry sample (g), F_1 is the weight of the tube plus the dry sample (g), and F_2 is the weight of the tube plus the pellet (g).

2.7.3 Emulsifying properties

The emulsifying activity was evaluated employing the protocol established by Nackz et al. (1985). Protein solutions were diluted in distilled water to a concentration of 1% w/v. Emulsions were prepared by incorporating sunflower oil into the aqueous phase, with an oil-to-protein ratio of 3:2. Afterward, this mixture underwent homogenization using an Ultraturrax at 9000 rpm for 1 minute. The volume of the emulsion layer was measured at various time intervals, and the emulsification activity was determined using the formula (Equation 3).

$$Emulsifyingactivity(\%) = \frac{Volumeoftheemulsionlayer}{Totalvolumeofthemixture} x100 \quad (3)$$

2.8 Bioactivity properties

2.8.1 ABTS·+ radical cation scavenging activity

The antioxidant capacity of the extracts was assessed following the methodology outlined by Re et al. (1999). Briefly, 20 µL of aqueous solutions containing the extracts (5mg/mL of sample in distilled water) were combined with 230 µL of the ABTS+ solution, and absorbance was measured at 734 nm after 6 minutes. A calibration curve was constructed using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as the antioxidant standard. The results were quantified and expressed as milligrams of Trolox

equivalents (TE) per gram of extract. Determination was conducted in triplicate.

2.8.2 Inhibition of angiotensin-converting enzyme (ACE)

The evaluation of potential antihypertensive properties was carried out in vitro, focusing on inhibiting the angiotensin-converting enzyme (ACE) employing rabbit lung as a model system by the protocol detailed by Li et al. (2005). In brief, a solution containing protein extract at a concentration of 1 mg/mL or a 0.1 μM captopril solution (10 μL) as an ACE inhibitor, together with 25 μL of 5 mM HHL in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl, was preincubated at 37 °C for 5 min. The enzymatic reaction was initiated by adding 12.5 μL of ACE solution (100 mU mL^{-1}). After incubation at 37 °C for 1 h, the reaction was terminated by adding 50 μL of 1 M HCl. Subsequently, 300 μL of quinoline, 100 μL of BSC, and sodium borate buffer were added to the reaction mixture and incubated for 30 min at 30 °C. Finally, 1.85 mL of ethanol was added to the reaction mixture, and the absorbance was measured spectrophotometrically at 490 nm. All measurements were performed in triplicate, and the ACE inhibitory activity (%) was calculated using the provided formula (Equation 4).

$$ACE\ inhibitory\ activity\ (\%) = \frac{B - A}{B - C}\ x\ 100 \qquad (4)$$

where A is the absorbance observed in the presence of sample, B the absorbance of the control (without an inhibitor) and C is the absorbance of the blank (HCl was added before ACE).

2.9 Statistical analysis

Statistical analysis was performed using SPSS (IBM Corp., USA). One-way analysis of variance (ANOVA) and Least Significant Difference (LSD) tests were used to analyze the significance differences ($p<0.05$) for multiple comparison tests.

3. Results

3.1 Chemical composition of the brown algae

The chemical composition of *A. nodosum* and *S. latissima* is shown in Table 1. It can be seen that carbohydrates are the main component of brown algae (represented approximately 60% of the total dry mass), as reported for brown algae (Blanco-Pascual et al., 2014; Sharma et al., 2018).

The monosaccharide composition exhibited a high content in alginate, evidenced by the combination of GulA and ManA units, which represented 21.7 % and 25.3 % of the total for *A. nodosum* and *S. latissima*, characteristic of alginophyte species. Fucose and glucose were identified as the most abundant monosaccharides in *A. nodosum* and *S. latissima*, respectively. This evidenced the main differences in cell wall architecture between the species. In particular, a predominance of sulfated structures, such as fucoidan, is expected in *A. nodosum* (Deniaud-Bouët et al., 2017). Conversely, the presence of glucose from cellulose in *S. latissima* underscores these species' contrasting cell wall composition.

A. nodosum and *S. latissima* exhibited significant amounts of protein, ranging from 9.8 % to 14.9 %, and ash contents of 20.1 % and 21.7 %, similar to those reported in the literature (Bikker et al., 2020). The slight differences in composition could be attributed to seasonal variations. Regarding lipid content, both algal species presented relatively low values. These results agree with those reported by Tagliapietra & Clerici (2023), who reported a lipid content in the range of 0.2-6.5 % in brown algae.

Generally, brown algae are abundant in phenolic compounds, which have been identified for their antioxidant properties (Priya & Khora, 2023). *A. nodosum* was observed to possess a significant level of phenolic compounds (25.6 mg GAE/g sample) and higher than other brown algal species (Konstantin et al., 2023).

Table 1. Chemical composition of the native dry brown seaweeds

Composition (dry wt. %)	*A. nodosum*	S. latissima
Proteins	9.8 ± 0.1^{a}	14.9 ± 0.2^{b}
Ashes	20.1 ± 0.3^{a}	21.7 ± 0.9^{a}
Lipids	3.1 ± 0.7^{a}	2.3 ± 0.6^{a}
Carbohydrates[1]	63.6 ± 1.5^{a}	60.9 ± 1.1^{a}
of which (µg/mg dry basis)		
Fucose	24.1 ± 0.5^{b}	3.8 ± 0.3^{a}
Galactose	1.5 ± 0.3^{a}	0.8 ± 0.2^{a}
Cellulose[2]	6.6 ± 0.2^{a}	22.7 ± 0.4^{b}
Glucose[3]	0.9 ± 0.1^{a}	1.4 ± 0.5^{b}
Xylose	3.5 ± 0.1^{b}	0.5 ± 0.1^{a}
GulA	7.7 ± 0.3^{a}	9.2 ± 0.1^{b}
GlcA	1.7 ± 0.2^{b}	0.7 ± 0.0^{a}
ManA	14.0 ± 0.9^{a}	16.1 ± 0.5^{a}
Mannitol	3.6 ± 0.1^{a}	5.7 ± 0.2^{b}
Polyphenols (mg GAE/g sample)	25.6 ± 1.6^{b}	5.1 ± 1.0^{a}

The values represent means ± SD (n=3). Values within the same row with same letters are not significantly different ($p>0.05$).
[1]Total carbohydrate content calculated as the sum of all monosaccharides detected by HPAEC-PAD
[2]The crystalline cellulose content was quantified as the difference between the Saeman sulfuric hydrolysis method (Saeman, 1945) and the non-crystalline glucose content obtained through acid methanolysis (Bertaud et al., 2002; Willför et al., 2009)
[3]Glucose contribution from the non-crystalline fraction.

3.2 Compositional analysis of the protein-polysaccharide extracts

The algal cell wall, a complex structure, is primarily composed of polysaccharides that interact through various intricate mechanisms, such as glycosylation, sulfation, and other post-translational modifications, posing a significant challenge to efficient protein extraction (Mazéas et al., 2023;

Ramazi & Zahiri, 2021). This study proposed a sequential protein extraction methodology comprising chemical techniques, such as acid and alkaline extraction, combined with ultrasounds. Subsequently, an enzymatic extraction was carried out on the residue resulting from the chemical extraction to recover the maximum percentage of proteins still present in the cell wall matrix of the residue.

Figure 1 presents the total yield of protein-polysaccharide extracts derived from the different extraction processes performed sequentially to achieve higher protein recovery. In terms of combined yields, it was observed that *A. nodosum* exhibited the highest overall yield compared to *S. latissima* (26.1 and 19.1 %, respectively). However, when considering the specific yields for the different types of treatment applied, it was evident that the acid/alkaline treatment (A) showed a higher extraction yield, with a higher trend in *A. nodosum* compared to *S. latissima* (19.6 $\pm$ 2.7 and 12.9 $\pm$ 1.1 %, respectively), which was probably due to differences in the cell wall architecture of both algae as previously reported (Bojorges et al., 2023). *A. nodosum* is from the order Fucales, with a relatively less rigid cell wall than *S. latissima* (Laminariales). Likewise, at this stage, most of the accessible components (non-recalcitrant proteins and carbohydrates) were obtained in this step (Table 2).

Subsequently, enzymatic treatment (E) resulted in a similar yield between the two species (between 6.2 and 6.5 %). The high activity and broad specificity of cellulases allowed the release of a recalcitrant protein tightly bound to the cell wall, significantly contributing to the increased protein

recovery. However, in the case of *A. nodosum*, no significant differences ($p \geq 0.05$) in yield were observed between the enzymatic treatment (AE) and the control (AC), which recorded values of 6.5 ± 0.3 % and 5.4 ± 0.1 %, respectively. This could be related to the lower cellulose content present in this type of algae, suggesting that the enzymatic action may not be as effective in breaking down the cell walls and releasing a higher content of protein-polysaccharide conjugates.

Nonetheless, although the enzymatic treatment yield was lower than the acid/alkaline treatment, it was noted that the protein content in this extract was significantly higher (see Table 2). This suggests that the unique cell wall composition of each species had a notable impact on enzyme efficiency and optimal conditions for extraction. In this context, it could be argued that, for *A. nodosum*, the presence of enzymes for cell wall lysis is unnecessary since a mild treatment (45 °C, pH 4.5, 12h) is sufficient to reach the maximum yield. Kadam et al. (2017) reported a similar effect on protein extraction from brown algae *Ascophyllum nodosum* by acid treatments and ultrasound application. This suggests a remarkable improvement in acid hydrolysis after ultrasound application, indicating that the cell wall could not be disrupted by acid treatment alone. Although the above recovery yields are lower than those obtained by sequential extraction with acid/alkaline treatment, the use of ultrasound may allow a decrease in extraction costs in this case, by not requiring enzymatic treatment.

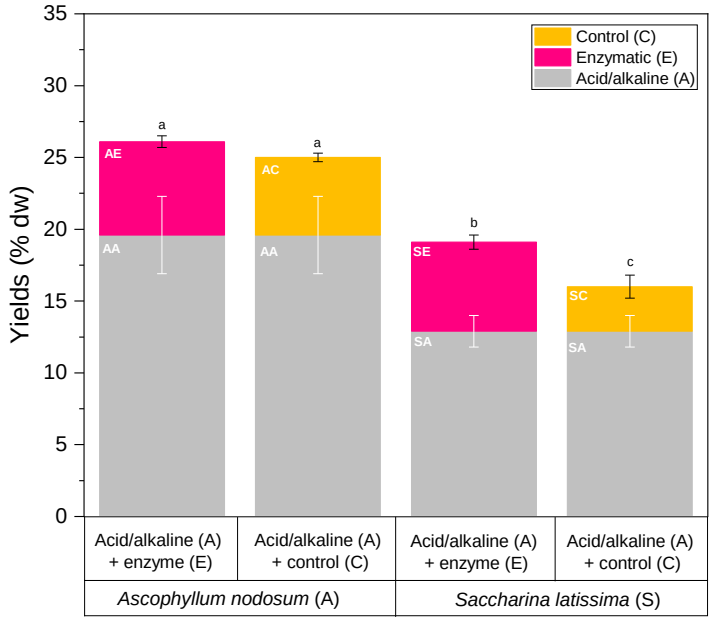

Figure 1. Total yields of the extracts obtained during the whole integrated sequential process from *A. nodosum* and *S. latissima*

Concerning the proximate composition of the extracts, the ash content was significantly lower than that of the raw seaweeds, ranging from 8% to 12% (Table 2), thus evidencing that most of the salts were removed during the purification process. The carbohydrate content was similar to that reported by Trigo et al. (2023), who stated a carbohydrate content of 65 and 78 % in protein extracts from *S. latissima*.

Table 2. Chemical composition and polyphenol content of the protein-polysaccharide extracts from *A. nodosum* and *S. latissima*.

Composition (dry wt%)	*A. nodosum*			*S. latissima*		
	AA	**AE**	**AC**	**SA**	**SE**	**SC**
Protein	4.5 ± 0.2^{a}	17.7 ± 0.4^{d}	12.9 ± 0.4^{c}	7.7 ± 0.1^{b}	25.4 ± 0.1^{f}	23.7 ± 0.1^{e}
Ash	12.7 ± 0.9^{b}	$9.2 \pm 1.8^{a,b}$	8.0 ± 1.6^{a}	12.4 ± 1.2^{b}	7.9 ± 1.4^{a}	8.1 ± 2.0^{a}
Carbohydrate	78.7 ± 2.1^{d}	59.2 ± 0.8^{a}	69.9 ± 1.2^{c}	76.7 ± 2.7^{d}	$62.0 \pm 0.9^{a,b}$	64.8 ± 2.5^{b}
Sulfate	1.9 ± 0.2^{b}	9.8 ± 0.1^{d}	8.6 ± 0.7^{c}	0.8 ± 0.3^{a}	2.2 ± 0.1^{b}	1.1 ± 0.2^{a}
Polyphenols (mg GAE/g sample)	12.2 ± 1.6^{c}	18.1 ± 1.3^{e}	15.8 ± 1.0^{d}	4.4 ± 0.7^{a}	7.6 ± 1.0^{b}	$5.7 \pm 1.0^{a,b}$

Values are shown represent means ± SD (n=3). Different letters within the same row denote significant differences ($p>0.05$).

The carbohydrate profile of the obtained extracts was also evaluated and the results are shown in Figure 2. The presence of uronic acids was evidenced by its high content in ManA and GalA units, followed by fucose and mannitol. Therefore, a high uronic acid content can be associated with alginate. Meanwhile, the presence of fucose is attributed to sulfated compounds, characteristic of fucal species, like *A. nodosum*. A significant amount of glucose was also detected, especially in cellulase-treated samples (AE and SE), which may be ascribed to digested/extracted cellulose mono- or oligomers (Bogolitsyn et al., 2020; K. G. Bogolitsyn et al., 2022). It is also interesting to observe a preferential extraction of alginate in the acid/alkaline stages, while the subsequent extraction is comparatively richer in fucoidan, for both tested species. It is feasible that during the application of the pH change and its adjustment to the protein isoelectric point (pI), the cationic segments of the protein established attractive interactions with the anionic groups of the polysaccharide, resulting in the generation of a weak electrostatic complex. Further reduction in pH brought about stronger protein-polysaccharide interactions, eventually leading to the precipitation of protein-polysaccharide complexes (Evans et al., 2013).

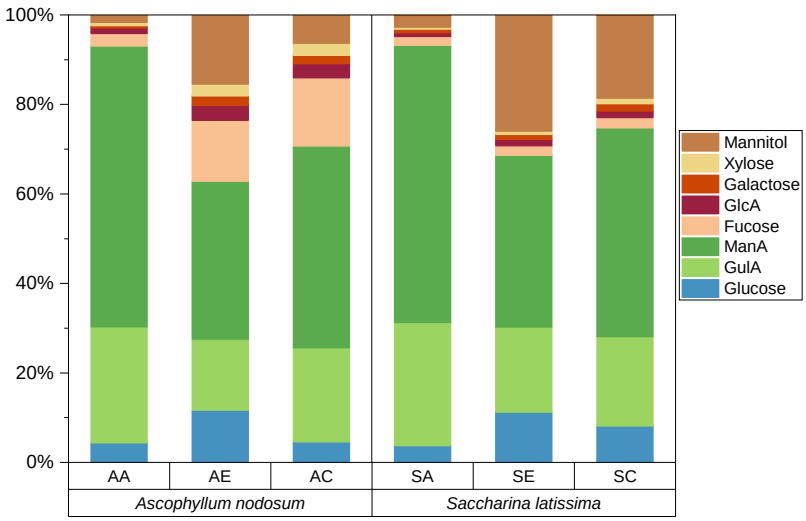

Figure 2. Relative monosaccharide composition of the protein-polysaccharide extracts. The results are expressed as micrograms of polysaccharides per milligram of dry weight.

As for the total polyphenol content of the extracts, also shown in Table 2, it was found to be significantly higher in *A. nodosum* extracts than in *S. latissima* ones. In particular, a high polyphenol content was observed in sample AE, which was subjected to enzymatic treatment, followed by AC and AA. This phenomenon is probably attributed to the different polyphenol content of the starting algae, as well as to the effect of hydrolysis during enzymatic treatment. During this process, intracellular proteins and polysaccharides interact with the phenolic compounds of the cell wall. These interactions can occur through non-covalent bonds, such as hydrophobic and hydrogen ions or covalent bonds (Lima et al., 2023). Therefore, the presence of a less resistant cell wall, such as *A. nodosum*, facilitated the release of protein-polyphenol complexes, increasing the polyphenol content of these

samples. As expected, these difference in the polyphenol content had a direct impact on visual appearance and colour parameters of the obtained extracts (see Supplementary Material), ascribed to the light selective absorption of polyphenols at low wavelengths that imparts a reddish color, evidenced by an increase in hue values for AE and AC samples.

Similarly, Liu et al. (2017) observed an increase in total polyphenol content in rice bran extracts after being subjected to different enzymatic treatments. Hwang & Do Thi (2014) also noted that enzymatic hydrolysis promoted the release of phenolic compounds into the medium, increasing the total phenolic content in protein extracts. This is interesting because the interaction between proteins and polyphenols can modify the structure of proteins, enhance their functional properties, and elevate the potential value of both components in the food industry (Wang et al., 2021).

3.2.1 Relative size distribution

The molecular weight of the protein-polysaccharide extracts was analyzed by HP-SEC. Figure 3 presents the chromatograms and the relative distribution of peak size to total peak area. Most samples were characterized by a relatively higher proportion of the 100-700 kDa fraction, followed by the 10-100 kDa fraction. Notably, samples subjected to acid/alkali treatment exhibited a higher proportion of the 100-700 kDa fraction in the AA and SA samples, accounting for 60% and 54%, respectively, compared to the enzymatic treatments, which can be attributed to the polysaccharides present in the extracts.

It is well known that enzymatic hydrolysis has a significant impact on molecular weight distribution, especially in the higher molecular weight fractions (García Arteaga et al., 2020). This process causes a reduction in protein structure, resulting in the extraction of smaller peptides. These results agree with those reported by Teixeira-Guedes et al. (2023), who indicated that the use of enzymes during the extraction of *G. vermiculophylla*, *F. vesiculosus*, *U. rigida*, and *P. dioica* led to the hydrolysis of proteins into peptides with reduced molecular weight.

It was observed that temperature-treated samples (45 °C, enzymatic treatment, and control) showed an increased presence of glycopeptides <10 kDa, representing between 16% and 3% of the total relative peak area.

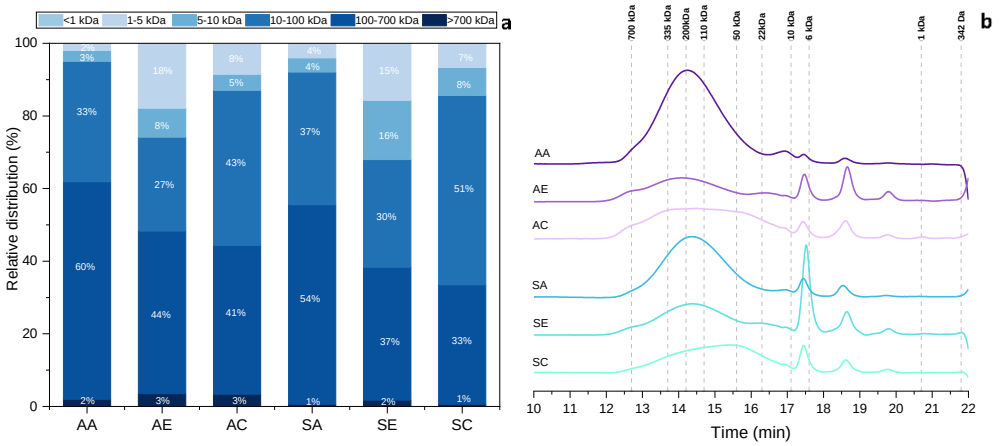

Figure 3. The molecular weight distribution of protein and polysaccharide conjugates extracts from *A. nodosum* and *S. latissima*. Results are presented as a relative percentage of the total peak area (a), and the HP-SEC chromatograms of protein extracts (b).

3.2.2 Structural characterization by FT-IR

FT-IR spectra from the extracts were analyzed to qualitatively determine the compositional differences amongst them. As shown in Figure 4, in all cases, a band at 3350 cm^{-1} was observed, possibly attributed to the O-H hydroxyl groups of the stretching vibrations, which would be present mainly on the N-H groups in the amide A region related to the polypeptide structure (Ji et al., 2020). The band observed at 2910 cm^-1 is attributed to C-H stretching vibrations of alkanes, possibly associated with polysaccharides or polyphenols (Vásquez et al., 2019). Notably, this band is more pronounced in *A. nodosum* extracts, especially in AE, suggesting and confirming a higher polyphenol content in these samples. Likewise, the main spectral bands confirming the presence of proteins were identified in the samples, located in the amide I at 1600 cm^{-1} and amide II at 1570 cm^{-1} regions, corresponding to the stretching vibrations of the peptide C=O bonds (Rawiwan et al., 2022). The samples also showed bands commonly associated to sulphated polysaccharides, such as those located at 1260 cm^{-1} (related to S=O vibration and characteristic of fucoidan) and at 1050 cm^{-1} (ascribed to the stretching vibrations of C-C and C-O bonds in the pyranose ring , Bojorges et al., 2022), thus confirming the co-precipitation of polysaccharides together with proteins, as it was confirmed in Table 2.

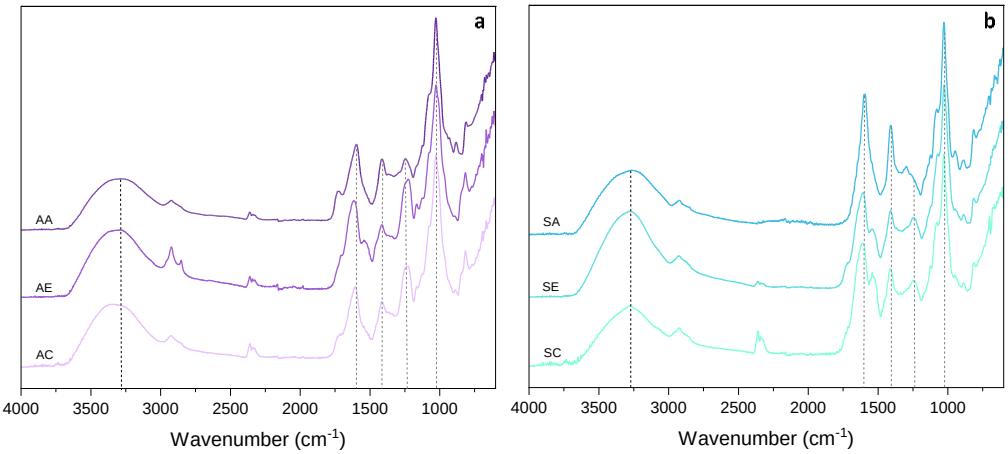

Figure 4. FT-IR spectra of the protein-polysaccharide extracts obtained from the (a) *A. nodosum* and (b) *S. latissima*.

4. Functional properties of protein-polysaccharide extracts

In the food industry, proteins are used according to their specific functional properties, which are closely linked to their molecular structure. Therefore, this study is not limited to analyzing the physicochemical properties of the obtained extracts but also investigates their functional properties in order to provide a better understanding of their potential applications.

4.1 Water- (WHC) and oil-holding capacity (OHC)

Figure 5 gathers the Water-Holding (WHC) and Oil-Holding (OHC) Capacity of the obtained extracts. WHC reflects the ability of a protein to bind water under limiting conditions, which makes it a property of great importance in the food industry, given that this property exerts a significant influence on the

flavor and texture characteristics of foods (Garcia-Vaquero et al., 2017). It is well-known that WHC values are related to protein-polysaccharide interactions based on electrostatic, hydrophobic, and hydrogen bonding forces and to the presence of proteins that bind to polysaccharides and to the higher content of neutral sugars present in the soluble dietary fibers in algae (El-Sayed & Ismail, 2022 , Yao et al., 2018). As shown, samples with the highest WHC were SA and AA (7.6 ± 0.2 and 7.2 ± 0.2, respectively), followed by AC and SC (7.2 ± 0.2 and 6.9 ± 0.3, respectively). In contrast, SE and AE (5.6 ± 0.3 and 5.9 ± 0.2, respectively) exhibited the lowest WHC values. High WHC values may be associated with the hydrophilic properties of the polysaccharides present in the extracts, such as alginate, which was significantly higher in SA and AA samples (see Table 2). Interestingly, enzymatically-treated samples (AE and SE) exhibited lower WHC values than their SC and AC counterparts. However, intrinsic factors affecting the WHC of dietary proteins include amino acid composition, protein conformation, and protein surface polarity or hydrophobicity (Kumar et al., 2014).

Oil absorption capacity (OHC) is another crucial parameter of ingredients in formulated foods. Maintaining high OHC levels is beneficial for preserving flavor and enhancing the palatability of products (Garcia-Vaquero et al., 2017). The OHC values of the extracts are shown in Figure 5. The first clear observation is that OHC was significantly lower compared to WHC. However, the samples that stood out for a higher OHC value were SC (6.6 ± 0.2 g oil/g), followed by AC and AA (6.3 ± 0.3 and 6.2 ± 0.2 g oil/g, respectively). These differences could be explained by the surface properties,

total charge density and hydrophobic nature of the samples' particles (Rezvani & Goli, 2023).

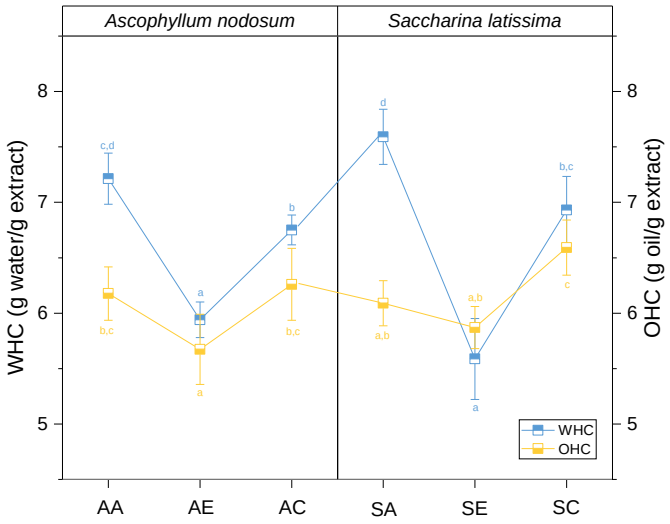

Figure 5. The water and oil holding capacity of the protein-polysaccharide extracts obtained from A. nodosum and S. latissima

4.2 Emulsion capacity and characterization.

The emulsifying properties of proteins play a crucial role in food formulation, as they are closely related to food products' texture and sensory characteristics (Zhang et al., 2023). These properties are evaluated by the ability of the protein to form and stabilize an emulsion. Stabilization refers to the ability of the protein to keep the emulsion intact, preventing it from changing its structure (e.g., coalescence, flocculation, creaming, or sedimentation) over a specific time (Suresh Kumar et al., 2014).

Figure 6 depicts the emulsifying activity of the obtained extracts. In general terms, an adequate emulsification capacity was observed, with the maximum rates reached in samples SE (98.8 ± 1.5) and AE (98.6 ± 1.1). The high emulsifying capacity observed in this study is similar to that reported for protein extracts of *H. elongata* with olive oil at pH 6 (95.7%) (Garcia-Vaquero et al., 2017) and that reported for protein extracts of K. alvarezzi with different oils (75.7-99.7%) (Kumar et al., 2014). While AA and SA samples (89.4 ± 1.0 and 86.1 ± 1.9, respectively) presented the lowest emulsification capacity. Differences in emulsification capacity may be influenced by several factors, including the surface charge of the extracts, variations in the hydrophilic-lipophilic balance, viscosity of the continuous phase and the presence of low molecular weight polypeptides, among others (Cui et al., 2020).

Some authors suggest that a higher degree of hydrolysis correlates positively with the emulsifying capacity of proteins (Gereniu et al., 2017). In this sense, it is plausible that the AE and SE samples, previously subjected to acid/alkaline treatment followed by enzymatic treatment, first underwent partial denaturation of some proteins or cross-linking with other components of the cell wall, such as polysaccharides, which would have hindered their complete release. Subsequently, upon enzymatic treatment with cellulases, these enzymes could have facilitated the release of low molecular weight protein-polysaccharide conjugates or glycopeptides embedded in the cell wall.

As a result, the protein-polysaccharide conjugates in these types of samples exhibited improved emulsifying ability. This improvement was

manifested in a more effective interaction at the interface between water and oil, leading to the formation of a viscoelastic film and a reduction in surface tension. This phenomenon favored the fragmentation of droplets into smaller sizes (Lima et al., 2023). In addition, the variation in the composition of the extracts, in terms of protein content (higher for samples SE, AE, and SC), as well as the aggregation of protein molecules, also influenced the emulsifying properties of the extracts (Zhang et al., 2022).

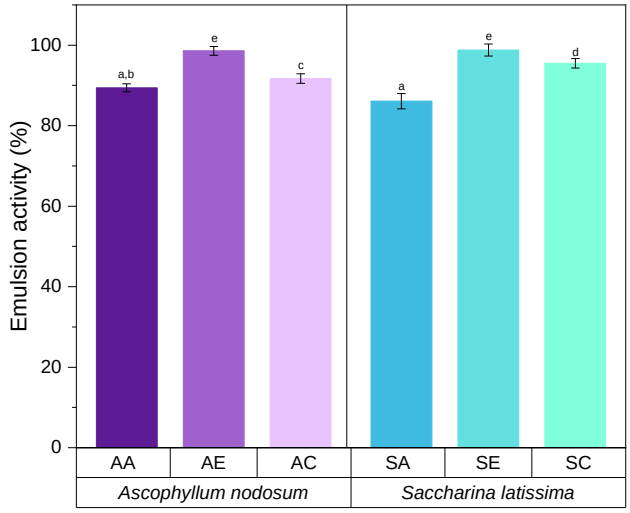

Figure 6. Emulsifying capacity of protein-polysaccharide extracts of *A. nodosum* and *S. latissima*.

The analysis of the size and distribution of oil droplets in emulsions is relevant due to its influence on long-term stability. Factors such as cream formation, coalescence and flocculation are affected by the gradual increase in droplet size. Therefore, droplet size is a crucial indicator for assessing emulsion stability. For this purpose, the parameter $D_{4,3}$ was determined, which represents the volume-weighted average and is particularly sensitive to

phenomena such as aggregation, disaggregation, or flocculation. The evolution of $D_{4,3}$ in the emulsions during a storage period of 7 days at 4 °C is detailed in Table 3 and, representative images analyzed by laser scanning confocal microscopy are compiled in Fig. 7, together with the size distribution of the oil droplets. Notably, the protein-polysaccharide extracts containing higher protein content (SE, SC and AE) led to a significantly decreased in particle size, which were kept after the storage time. This is most likely due to proteins were located at the O/W interphase, as observed in the magnified images of the corresponding emulsions, playing a major role in emulsion stabilization.

In contrast, it was observed that samples with a lower protein content (AA and SA) showed higher average oil droplet size and presented a broader particle size distribution, suggesting the formation of larger aggregates. Therefore, it is hypothesized that most of the proteins in these extracts are interacting with carbohydrates and they could not act as surface active compounds at the O/W interphase. In fact, although some proteins were detected at the O/W interface in the AA and SA samples, this layer was markedly diminished as compared to their enzymatically-treated counterparts (cf. Figure 7). As a result, the emulsifying properties are conditioned not only by the amount of protein but also by its potential interactions with other compounds, the configuration of the proteins with which they may interact, the accessibility of these proteins, and the structural complexity of the polysaccharides to which they are bound. Zang et al. (2019) found that the addition of anionic polysaccharides, such as alginate, to rice bran protein emulsions did not improve emulsion stability, promoting aggregation of

droplets in the emulsion. According to the authors, this could be due to the depletion mechanism, which refers to an excess of free polysaccharide in solution, which did not favor its adsorption and instead increased the osmotic forces of attraction between the oil droplets, promoting droplet coalescence (Qiu et al., 2015).

Although the presence of polyphenol compounds has been reported in the literature to improve emulsifying properties (Lima et al., 2023; Yan et al., 2022), this was not a determining factor for the extracts obtained in this study. For example, when comparing extracts of the same species, better emulsifying properties were observed in those with a higher concentration of polyphenols. However, upon comparing the extracts between species, it became evident that, although those obtained from *A. nodosum* had a higher polyphenolic content than those from *S. latissima*, they showed worse emulsifying properties. Therefore, the polyphenol content was not the decisive factor in this context.

Table 3. Average oil (SO) particle size of the emulsions measured at two different times (freshly prepared samples and after 7 days of storage at 4 °C).

Sample	Particle size $D_{4,3}$ (μm)	
	Day 0	**Day 7**
AA	$163.9 \pm 20.1^{e*}$	$266.0 \pm 35.7^{d*}$
AE	102.2 ± 4.0^{c}	105.9 ± 0.6^{b}
AC	$133.5 \pm 15.8^{d*}$	$162.5 \pm 31.1^{c*}$
SA	$150.7 \pm 3.8^{d,e*}$	$175.8 \pm 9.6^{c*}$
SE	39.6 ± 0.1^{a}	40.1 ± 0.6^{a}
SC	63.9 ± 1.2^{b}	64.8 ± 0.2^{a}

Values are mean ± SD (n=3). Different letters within the same column indicate significant differences ($p<0.05$). (*) denotes significant differences ($p < 0.05$) in the same row (related to storage time).

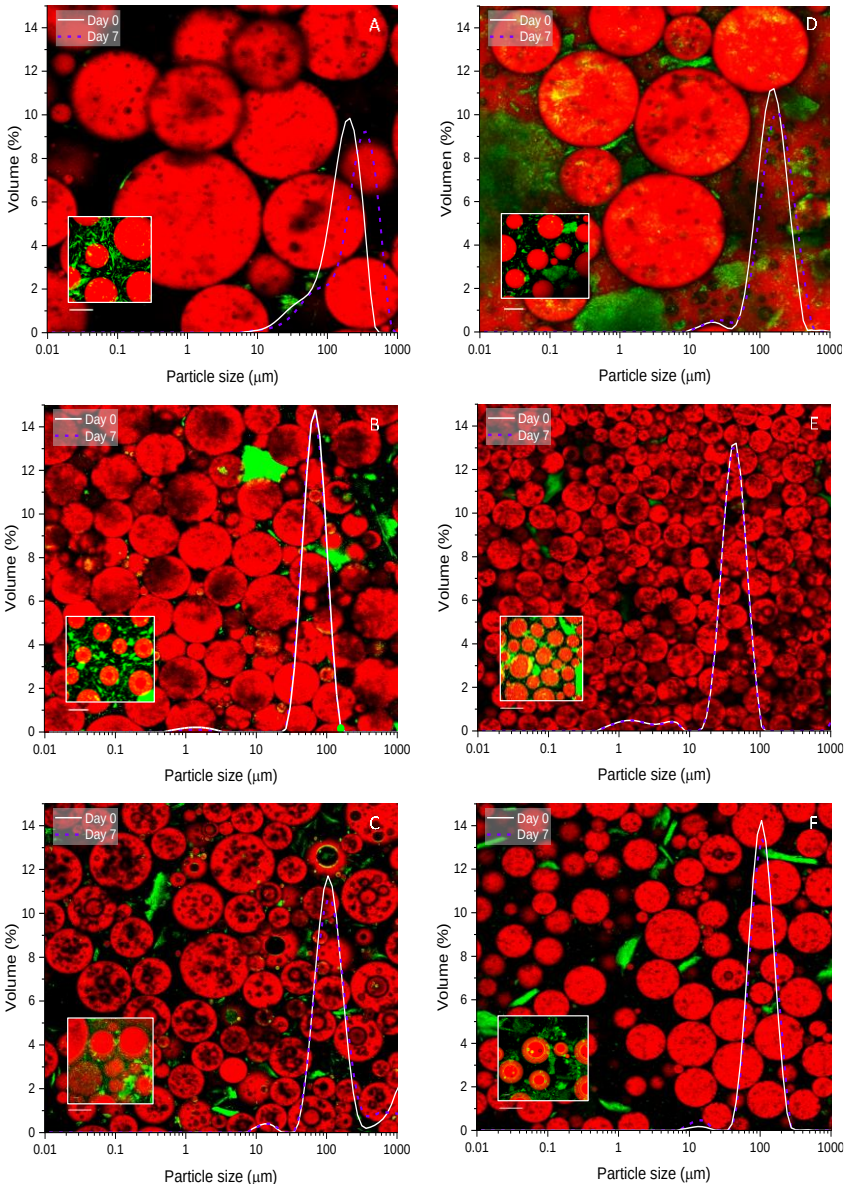

Figure 7. Confocal microscopy images of the emulsions and their particle size distribution were measured in the freshly prepared emulsions (solid line), (a) AA, (b) AE, (c) AC, (d) SA, (e) SE, and (f) SC. The particle size after 7 days of storage at 4 °C is the dashed line. All pictures were taken with the same light intensity and magnification (scale bar corresponds to 60 μm). The oil phase was stained with Nile Red and the protein phase with Fast green

5. Bioactivity properties

5.1 ABTS $^{\bullet+}$ assay

The antioxidant activity of the protein extracts was analyzed using the ABTS$^{\bullet+}$ radical scavenging method (Figure 8a). The results reflected a trend parallel to that observed for total phenolic content, highlighting that the protein extracts that presented the highest antioxidant capacity were those of *A. nodosum*. An antioxidant capacity relationship was established between the extracts: AE > AC > AA, suggesting a close association between phenolic content and antioxidant activity. In addition, the higher antioxidant activity can be explained by the increased concentration of sulfates in these samples, as shown in Table 2, suggesting the possible presence of low molecular weight sulfated compounds, such as fucoidans (Chen et al., 2021) and polyphenolic compounds such as phlorotannins, known for their remarkable antioxidant activity (Imbs & Ermakova, 2021). Wang et al. (2008) reported a positive association between the amount of sulfates and antioxidant activity, specifically in terms of superoxide radical scavenging capacity, as well as sulfate/fucose content as an indicator of metal ion chelation and hydroxyl radical scavenging. It was suggested that the overall antioxidant potential of glycoconjugates might be due to the synergistic action of phenolic compounds, proteins, and polysaccharides (Wang et al., 2016). Likewise, uronic acid content has been identified as another factor influencing the overall antioxidant activity of the extracts. The existence of electrophilic groups in polysaccharides increases their ability to donate hydrogen (Wang et al., 2016).

On the other hand, Rawiwan et al. (2022) pointed out that smaller peptides exhibit higher antioxidant activity than their higher molecular weight counterparts. This suggests that the higher antioxidant capacity of enzymatically treated samples (compared to their counterparts) can be linked to the presence of a higher number of glycopeptides.

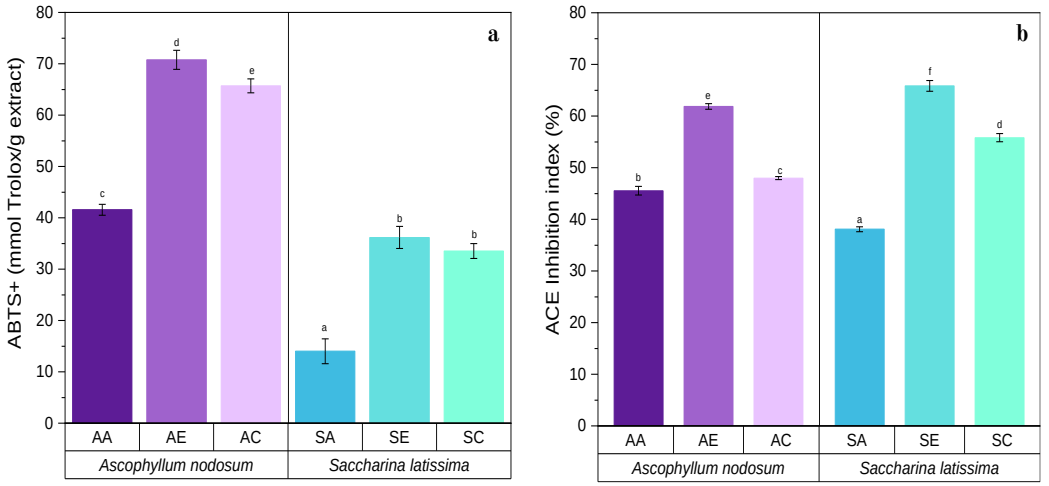

Figure 8. The antioxidant activity (a) and the ACE inhibition of the protein-polysaccharide extracts from *A. nodosum* and *S. latissima*.

5.2 Antihypertensive potential (ACE)

Hypertension, associated with increased risk of cardiovascular disease, renal disease, cognitive impairment, stroke, heart failure, and premature death, has become a growing global health concern (Lu, 2024). In treating this condition, commonly used strategies focus on angiotensin I-converting enzyme (ACE) inhibition. This enzyme plays a crucial role by transforming angiotensin I into angiotensin II, thus contributing to the development of hypertension.

Therefore, research on ACE inhibition provides insights into the antihypertensive properties of specific biomolecules (Habeebullah et al., 2023).

The ACE inhibitory activity of the algal protein extracts is shown in Figure 8b. These extracts exhibited outstanding antihypertensive activity, with the SE and AE samples being the most efficient, with ACE inhibition of 62.9 ± 0.3% and 59.7 ± 0.6%, respectively. This higher inhibition could be attributed to the higher protein content in AE, SE and SC samples (see table 2) as well as the release of small glycopeptides during enzymatic hydrolysis (higher values for AE and SE), playing a crucial role in the antihypertensive mechanism. Moreover, these values are similar than those reported by Cian et al. (2015) in protein hydrolysates of *P. columbina* (44.8 ± 2.4%). Numerous studies have pointed out that antihypertensive activity is associated with a total protein amount and lower molecular weight (Kim et al., 2024).

6. Correlation analysis and discussion

Pearson's correlation coefficient was used to analyze the relationship among composition and functional properties of the obtained extracts. All the correlation coefficients are displayed in Figure 9. According to the results, the water holding capacity was positively correlated with the carbohydrate content (r=0.88), ascribed to the well-known hydrophilic nature of alginate or fucoidans present in the extracts. However, there were significant negative correlations between WHC and emulsion properties (r=-0.94 and 0.99, respectively). High WHC values have been reported to be related to lower moisture loss in packaged bakery products and contribute to the preservation

of the fresh and moist feel of baked goods (Pourfarzad et al., 2013). A high WHC value in the extracts could prevent shrinkage and improve the texture and viscosity of certain foods. Likewise, WHC is a fundamental property of proteins in viscous textured foods, such as soups, custards, doughs, and baked goods. Its relevance lies in the ability of these proteins to absorb water without the proteins dissolving, which confers body, density, and viscosity to such foods (Suresh Kumar et al., 2014).

On the other hand, a positive correlation was observed between protein content and emulsion capacity (r=0.86). In contrast, a negative correlation was found between protein content and carbohydrate content, as well as with particle size, evidencing that the presence of more accessible proteins (acting as surface-active materials) induced the formation of a more homogeneous particle size distribution and smaller average oil droplet particle (as observed in Figure 7) with a positive impact on the emulsifying properties.

Therefore, the ability of the protein to interact with oil phase and to be retained and absorbed is essential to preserve the stability of emulsions, which plays a crucial role in the formulation of food products such as cake mixes, sausages, dressings, mayonnaises, meat substitutes and extenders (El-Sayed & Ismail, 2022).

Likewise, as mentioned previously, a strong positive correlation was observed between the variables antioxidant activity (ABTS), total polyphenol content (r=0.96), sulfate content (SO_4) (r=0.94), and hue (h^{o}_{ab}) (r=0.97). This suggests that the overall antioxidant capacity of glycoconjugates may be attributable to the combined interaction of phenolic compounds, proteins, and

sulfated polysaccharides. The presence of polyphenols positively related to hue values (see Supplementary Material). This effect can be explained by the light selective absorption of polyphenols present in the extracts at low wavelengths that imparts a reddish color, thus increasing hue values.

In contrast, angiotensin-converting enzyme (ACE) activity was positively correlated with protein content (r=0.85) in the obtained extracts and, as expected, it appeared to be negatively correlated with carbohydrate content (r=-0.91). This confirms the presence of bioactive peptides or peptide precursors, which could have counteracted ACE activity. This is consistent with the literature, as peptides, phenolic compounds, as well as phlorotannins (secondary metabolites belonging to the phenolic compound family) present in seaweeds have been reported to significantly inhibit ACE inhibitory capacity (Cian et al., 2015; Nagappan et al., 2017). However, although some authors have suggested that phlorotannins or phenolic compounds can inhibit ACE by capturing zinc ions (Zn^{2+}), an essential enzyme component (Nagappan et al., 2017), this trend was not clearly observed in the extracts obtained in the present work, attributing the ACE inhibition mainly to the size of the peptides released after enzymatic treatment.

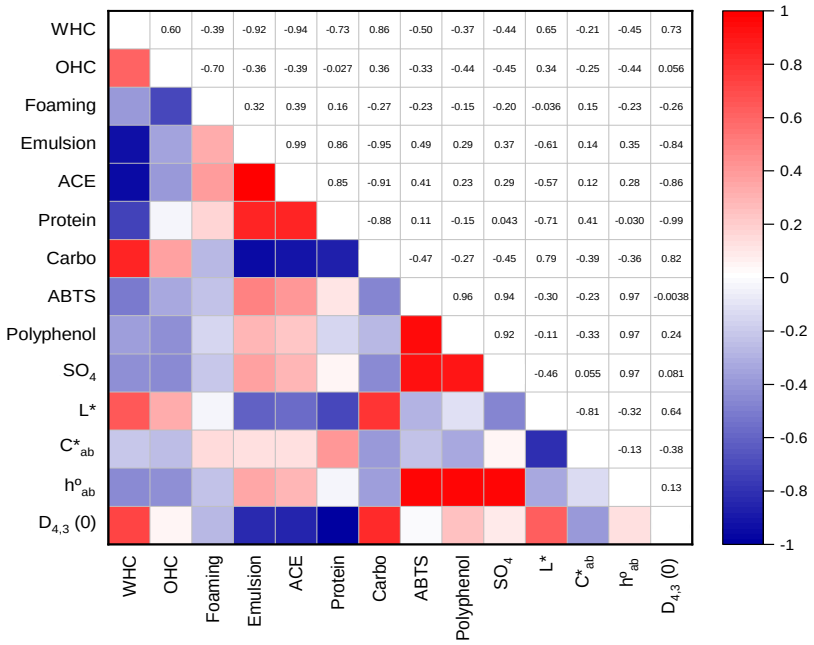

Figure 9. Heat map of the Pearson correlation analysis of the protein-polysaccharide extracts from *A. nodosum* and *S. latissima* (*$p < 0.05$).

5. Conclusions

The present work provides relevant information on the extraction of protein-polysaccharide extracts from brown algae *(A. nodosum* and *S. latissima*). The application of enzymatic treatments effectively increased the overall extraction yields and protein content of the extracts. Although all extracts showed good emulsifying properties, the higher protein content with significantly lower molecular weight in the enzymatically-treated samples conferred relatively superior emulsifying properties and better stabilization over time.

Regarding the bioactive properties of the obtained extracts, a strong positive correlation was observed between the antioxidant activity (ABTS) and the total polyphenol content and sulfate content, being higher for those obtained from *A. nodosum*, which was attributed to the extraction of sulphated fucoidan/fucan structures. The angiotensin-converting enzyme (ACE) activity correlated positively with protein content in the obtained extracts, being higher in the enzymatically-treated samples due to their higher protein content with a relatively lower molecular weight. These results evidence that the obtained extracts have excellent properties for incorporation as food ingredients.

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6. Supplementary Material

Optical properties

Color is one of the most important attributes of consumer acceptance (Cömert et al., 2020). The evaluation of this attribute can be beneficial given the close relationship between chromatic appearance and human taste perception. Thus, modifications in food color directly impact people's sensory judgment of taste (Garcia-Vaquero et al., 2017). The color evaluation of the extracts obtained from *A. nodosum* and *S. latissima* was determined from the lightness (L*), Chroma (C^*_{ab}), and Hue (h^o_{ab}) (Table 3). The AA extract was observed to have the highest lightness value, while the AE and SE samples exhibited the lowest lightness. This could be due to the enzymatic process having co-extracted polyphenols and other low molecular weight compounds along with the protein, which probably led to a decrease in color brightness in the sample, in contrast to those in which the enzymatic treatment was not applied. In addition, it was observed that AE and AC showed a tendency toward a browner color (higher h^*_{ab}), possibly due to a higher polyphenol content. On the other hand, SE and SA exhibited a more saturated reddish hue (higher C^*_{ab}).

Table S1. Comparison of the color of the different protein-polysaccharide extracts from *A. nodosum* and *S. latissima*.

Sample	L*	C^*_{ab}	h^o_{ab}
AA	70.93 ± 0.03^{d}	10.92 ± 0.01^{a}	74.66 ± 0.26^{b}
AE	40.57 ± 1.01^{a}	18.09 ± 0.18^{c}	77.39 ± 0.01^{c}
AC	43.04 ± 0.07^{b}	$17.57 \pm 0.02^{b,c}$	77.33 ± 0.03^{c}
SA	50.24 ± 0.05^{c}	21.09 ± 0.06^{e}	72.90 ± 0.41^{a}
SE	39.71 ± 0.19^{a}	19.33 ± 0.32^{d}	74.30 ± 0.12^{b}
SC	48.61 ± 1.92^{c}	17.16 ± 0.74^{b}	73.19 ± 0.01^{a}

Lightness (L*), chroma (C^*_{ab}), and Hue (h^o_{ab}). Values reported are their means (n=3) ± standard deviation. Different letters in the same column indicate significant differences between the samples ($p \leq 0.05$).

Chapter 2

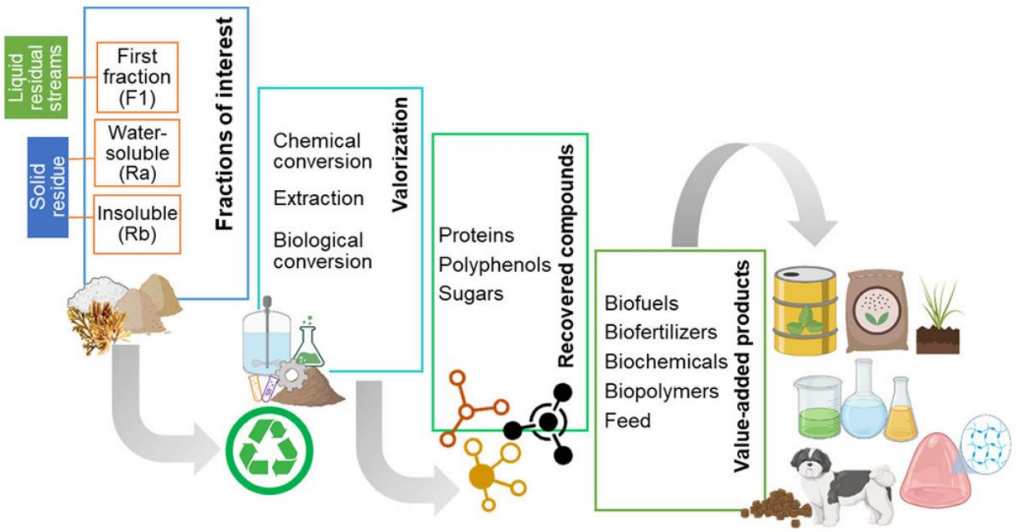
Trash to Treasure: Valorization of residues produced during polysaccharide extraction

2.1 Alginate industrial waste streams as a promising source of value-added compounds valorization

2.2 Protein extraction from the industrial solid residue of brown seaweed after alginate extraction

2.1

Alginate industrial waste streams as a promising source of value-added compounds valorizationn



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

Bojorges, H., Fabra, M. J., López-Rubio, A., & Martínez-Abad, A. (2022). Alginate industrial waste streams as a promising source of value-added compounds valorization. Science of The Total Environment, 838, 156394. https://doi.org/10.1016/J.SCITOTENV.2022.156394

ABSTRACT

The alginate industry processes more than hundred thousand tons per year of algae in Europe, discarding around 80% of the algae biomass as different solid/liquid residual streams. In this work, *Saccharina latissima* and *Ascophyllum nodosum*, their generated alginates and all residual fractions generated in the process were characterized in terms of lipid, ash, protein content, and the carbohydrate composition and antioxidant capacities analysed. The first fraction after acid treatment (ca. 50% of the initial dry biomass) was rich in phlorotannins (15 mg GAE/g) and bioactive fucoidans (15-70%), with a high sulfation degree in A. nodosum. Two fractions generated from the solid residue, one soluble and another insoluble (Ra and Rb, respectively), constituted 9% and 5-8% of the initial biomass and showed great potential as a source of soluble protein (30% for S. latissima), and cellulose (70%) or fucoidan, respectively. Valorization strategies are suggested for these waste streams, evidencing their high potential as bioactive, texturizing or nutritional added-value ingredients for cosmetic, food, feed or pharmaceutical applications.

1. Introduction

Marine biomass is highly underexploited and mostly unknown, compared to plant terrestrial biomass, and constitutes a vast renewable source for food ingredients, biomedical, cosmetic, specialty chemicals and materials in future biorefineries. Thanks to global policies fostering a “blue bioeconomy”, much research and industrial efforts have been devoted to understanding marine

biomass and explore potential applications. Brown algae are one of the most exploited and promising families of macroalgae, due to their high content in valuable compounds such as functional polysaccharides (e.g. fucoidan, laminarin, etc.), polyphenols (e.g. phlorotannins), protein, vitamins and minerals.

Fucoidans and fucans are a wide family of fucose-containing polysaccharides which has attracted much research attention in recent years due to abundant scientific evidence on bioactive properties, such as anticoagulant, anti-inflammatory, antioxidant, which in turn may alleviate many important chronic diseases (Deniaud-Bouët et al., 2017; Ponce and Stortz, 2020). Many of these bioactivities have been related to the degree of sulfation, and the ability of the sulfate groups to interact with other biological compounds or act as electron donors (Deniaud-Bouët et al., 2017; Yuan and Macquarrie, 2015). Phlorotannins are a family of polyphenols of varying molecular weight present in brown algae, with many similar ascribed bioactivities as fucoidans, linked again to their antioxidant, anti-inflammatory capacities which in turn modulate biochemical processes linked to chronic diseases (neurodegeneration, cancer, cardiovascular disease, diabetes, etc.)(Fernando et al., 2021; Shrestha et al., 2021). The interest in these compounds has prompted much research on the development of suitable extraction and purification methods towards a sustainable cascade valorization of several brown algae species (Ale et al., 2011; Allwood et al., 2020; Yuan and Macquarrie, 2015; Zhang et al., 2020).

Nevertheless, the vast heterogeneity and complexity of brown algae in terms of composition, recalcitrance, solubility and potential bioactivities is huge and poses important challenges to establish clear structure-activity relationships or create standardized processes to bring valuable products to the market (Deniaud-Bouët et al., 2017; Flórez-Fernández et al., 2018).

The main source of brown algae exploitation to date is the alginate industry, processing more than hundred thousand tons of either harvested or cultivated algae in Europe on a yearly basis (Araújo et al., 2021). Alginate is a linear heteropolysaccharide composed of 1,4-linked β-D-mannuronic acid (M) and 1,4 α-L-guluronic acid (G) and the main cell wall component of the cell wall in many brown seaweeds, representing 17-45% of the algae dry weight (Vera et al., 2011). The principal properties of alginates, including their gelling, emulsifying and film-forming abilities have made them broadly used ingredients in a number of fields such as in the pharmaceutical, cosmetic, textile, and food industries (Gomaa et al., 2018). Several species of the genus *Laminaria* (kelp) and the species *Ascophyllum nodosum* (rock kelp) are among the most heavily wild harvested species for alginate production in Europe, while *Saccharina latissima* is the most widely cultivated species (aquaculture) for direct consumption or alginate production (Araújo et al., 2021). Despite much policy efforts have been made to promote circular bioeconomy practices (Dutta et al., 2021; Khoshnevisan et al., 2021; Mak et al., 2020) and new ways of sustainable exploitation of the oceans, little has changed in the industrial processing techniques used to obtain alginate. Generally, the alginate extraction process at industrial level involves collection, transport and storage of the algae, with eventual addition of

formaldehyde to avoid chemical or enzymatic reactions which might decrease the quality of the alginate. An acid pre-treatment is applied at room temperature to remove pigments, other carbohydrates and low molecular weight compounds. Then, an alkaline extraction is perfomed, followed by solid/liquid separation, drying and milling, to produce high quality alginate (Fawzy et al., 2017; Pawar and Edgar, 2012). This multistep extraction process generates large amounts of liquid and solid waste streams, which constitute around 80% of the initial dry biomass and are currently discarded (Mohd Fauziee et al., 2021).

While research efforts have focused on alternative processes to minimize the use of chemicals or to target extraction of bioactive fucoidans or phlorotannins, it is unlikely that current industrial alginate production practices change in the short- or mid-term. In the mean-time, the valorization of these already available waste streams into -added-value products could be a step towards a more circular bioeconomy and more sustainable practices. In fact, some of these waste streams may well still contain some the afore-mentioned valuable compounds (e.g. bioactive polysaccharides or polyphenols), together with other nutritionally valuable protein, lipid or mineral fractions. A proper characterization is nevertheless needed to ascertain their potential, which, to the best of our knowledge, is lacking. The integration of valorization strategies for these waste streams into the industrial process without altering current alginate production, would minimize waste generation and achieve a higher degree of valorization for algae biomass, while creating new cost-efficient ingredients for a wider product portfolio of the alginate processing industries.

Therefore, the focus of this work was to perform a thorough characterization of the different waste streams generated during alginate extraction from two brown algae from different families, *Saccharina latissima* and *Ascophyllum nodosum*, to create evidence for potential cascade valorization schemes which would increase the eco-sustainability of the currently used industrial process. The rationale for selecting these species in the present work was based on several reasons. First of all, they are amongst the most heavily exploited species for alginate production, both in aquaculture (*S. latissima*) and through wild harvesting (*A. nodosum*) and with 376 and 82000 tons/year, respectively, in Europe only (Araújo et al., 2021). Moreover, alginate is produced from algal species in the Laminariales and Fucales families and, thus, at least one candidate from each of the families should be selected to have a broader insight into the potential residual streams derived from them. Another reason for selection was that these specific species are easily harvested with little contamination from other species, e.g. *A. nodosum* from the shoreline and *S. latissima* is cultivated in threads in controlled areas.

2. Materials and methods

2.1 Materials and reagents.

The brown seaweeds *Saccharina latissima* (*S. latissima*) and *Ascophyllum nodosum (A. nodosum)* were kindly supplied by Porto-Muiños S.L. (Cerceda, A Coruña, Spain) as a dry powder ($<$ 2% water content). The practices for harvesting in this company involved manual hand-picking, which ensured the removal of other contaminant algal species or impurities. All chemicals and

reagents were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) unless otherwise stated. Mannuronic and guluronic acid standards were purchased at Biosynth Carbosynth Ltd (Compton, UK).

2.1 Alginate extraction

The alginate extraction process was carried out mimicking conventional industrial practices according to information gathered through the BIOCARB-4-FOOD project (SUSFOOD2), in order to generate the different waste streams and residues for further analysis (Cebrián-Lloret et al., 2022). Addition of formaldehyde was avoided even though it is a common industrial practice as a preservative during transport. The alginate extraction process was divided into four steps: an acid treatment, alkali extraction, precipitation, and drying (Figure 1). Except the drying process, all processes were performed at room temperature. Briefly, the brown seaweeds were subjected to acid treatment (0.2M HCl) overnight at a solid/liquid ratio (S/L) of 1:25 (wt.) under mechanical stirring. The residue was recovered by centrifugation and re-suspended in 0.1 M $NaHCO_3$ (S/L 1:50) for 2 hours. The pH was then adjusted to 7.5-8 with $NaHCO_3$ /HCl and the solution incubated overnight with slow stirring. The suspension was centrifuged to separate the residual algae, which was freeze-dried and then washed with distilled water to separate the soluble and insoluble parts of this residue. 0.2% NaCl (w/v) was added to the solution and the sodium alginate was precipitated using different volumes of isopropanol in subsequent cycles. Finally, the last precipitate was filtered and dried overnight at 60 °C. Dried alginate samples were milled and stored

in a desiccator chamber (0% HR) until use. The extraction yield was calculated from the following equation:

$$Yield\ (\%) = \frac{Mass\ of\ alginate\ (g)}{Mass\ of\ brown\ seaweed\ (g)} x\ 100 \qquad (10)$$

All liquid and solid residual fractions generated through the process were dried and the yield calculated analogously as for alginate.

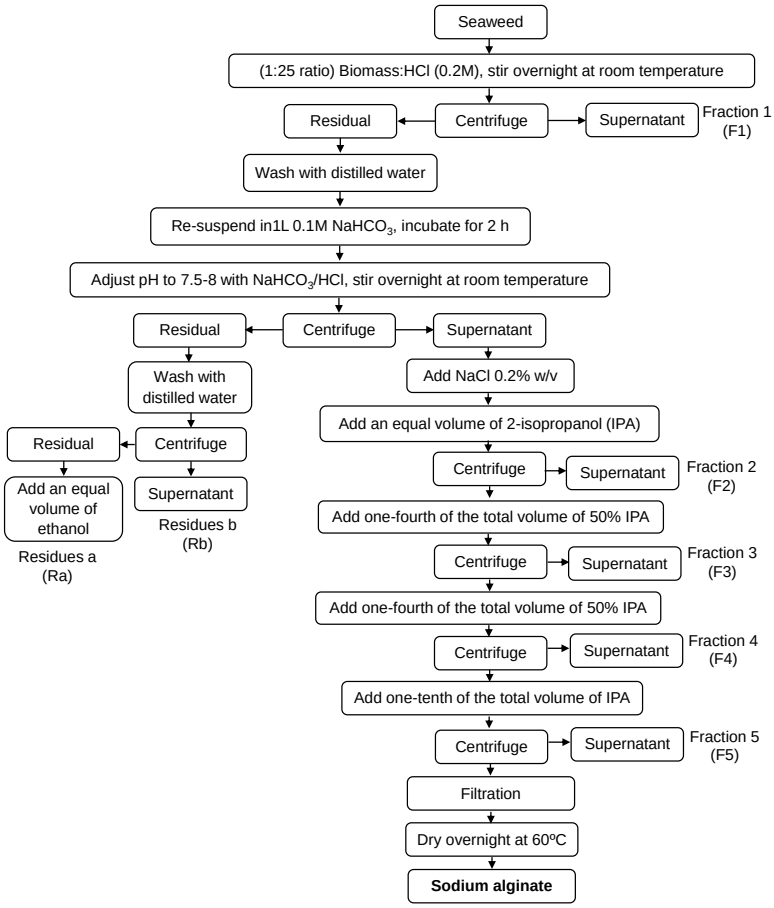


Figure 1. Schematic diagram of sodium alginate extraction process

2.3 Chemical composition

The total nitrogen content was determined using an Elemental Analyzer Rapid N Exceed (Paralab S.L., Spain). About 250 mg of each of the powdered samples were pressed to form a pellet which was then analyzed 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 lipid and ash content were determined by AOAC Official Methods 991.36 and 920.153, respectively (AOAC, 1990).

Sulfate content was calculated from the sulfur content through elemental analysis using a Thermofisher Flashsmart organic elemental analyzer. All tests were conducted in triplicate.

2.3.1 Determination of monosaccharide composition

The carbohydrate content and monosaccharide composition of the alginates and the fractions were determined after acid methanolysis as previously described Martínez-Abad et al. (2018). The monosaccharides were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-3000 (Dionex, Sunnyvale, CA, USA). All experiments were carried out in triplicate.

2.4. ABTS·+ radical cation scavenging activity

The antioxidant capacity of alginate was determined using the Trolox equivalent antioxidant capacity according to Re et al. (1999) with some

modifications. Briefly, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as antioxidant standard. Each sample was dissolved in distilled water at a concentration of 5 mg/mL and analyzed for ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity. To this end, 20 μL of aqueous solutions of the alginate-based extracts were added to 230 μL of the $ABTS^{+}$ solution, and absorbance at 734 nm was registered after 6 min. For the calibration curve, Trolox standards of distinct concentrations were prepared, and the same procedure was followed. The results were expressed as μmol Trolox equivalents (TE)/g extract. All determinations were carried out in triplicate.

2.5. Phenolic content

The total phenolic content was determined, in triplicate, by the Folin-Ciocalteau reagent as described by Singleton et al. (1999) with a slight modification. Briefly, Folin-Ciocalteau reagent was diluted 1:10 with distilled water and 1 mL mixed with 0.2 mL of each sample (5 mg/mL). 0.8 mL of Na_2CO_3 (75 mg/mL) were, then, added and the samples were heated up to 50 ºC for 30 min. Finally, the absorbance values were read at 750 nm. The calibration curve was generated by using gallic acid as the standard, and the total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g extract.

2.6. β-carotene–linoleic acid assay

The β-carotene–linoleic acid model system was assessed, in triplicate, as previously described by Koleva et al. (2002). A stock solution was prepared

with 0.5 mg of β-carotene in 1 mL chloroform, and it was added to 25 µL linolenic acid and 200 µL of Tween 40. The mixture was placed in a rotary evaporator under vacuum to remove the chloroform. Distilled water (100 mL) was added, and the resulting mixture was vigorously stirred. Aliquots (200 µL) of the β-carotene–linoleic acid emulsion were transferred into 50 mL of each of the fractions in triplicate at different concentrations and incubated at 50 ºC for 2 h. The absorbance was measured at 470 nm. The antioxidant activity was expressed as inhibition percentage according to the following equation:

$$\beta - carotene - bleaching\ inhibition\ (\%) = \left[1 - \frac{A_0 - A_t}{A'_0 - A'_t}\right] x\ 100 \quad (11)$$

Where A_0 and A'_0 are the absorbance of the sample and control, respectively, measured at time zero, and A_t and A'_t are the absorbance of the sample and the control, respectively, measured after 2 h.

2.7. Fourier transform infrared spectroscopy (FT-IR)

All samples were analyzed by FT-IR (FTIR; Bruker, Vertex, USA) using the attenuated total reflectance (ATR) mode. The spectra were recorded at 4 cm^{-1} resolution in a wavelength range between 400 and 4000 cm^{-1} and averaging a minimum of 64 scans.

2.8. Statistical analysis

The statistical analysis of experimental data was performed using IBM SPSS Statistics software (version 23, IBM Corp., USA). One-way analysis of

variance (ANOVA) was done to determine if differences between sample means were significant at a significance level of $p<0.05$. The mean tests were performed using Tukey's Test.

3. Results and Discussion

3.1. Characterization of the raw seaweeds and their alginate extracts

The proximate and carbohydrate composition of the *Saccharina latissima* and *Ascophyllum nodosum* raw seaweeds and their alginate extracts was first analyzed, and the results are summarized in Table 1. In general, the compositional values for the raw seaweeds are in agreement with available literature on the composition of *S. latissima* (Bikker et al., 2020) and *A. nodosum* (Rioux et al., 2007; Tabassum et al., 2016) and the slight compositional differences can be ascribed to the environmental factors and harvest season variations which have been widely reported to affect seaweed composition (Samarasinghe et al., 2021). The total lipid content in both algae species was relatively low (<4%) confirming these algae are not a good source of lipids. These results are in agreement with Susanto et al. (2016) and Zhang et al. (2020), who reported a lipid content in the range from 1.7–7 wt.% in brown seaweeds. The ash contents are quite high, indicating the presence of significant amounts of diverse mineral components in the seaweed biomass. As expected, the protein content varied between 9 and 11% depending on the species, being lower on average than that in red or green seaweeds (10-30% dry weight) (Burtin, 2003).

The carbohydrate contents of the seaweeds comprised 60–65% of the total dry weight, similarly to other reported values for brown seaweeds (Guangling et al., 2012; Morrissey et al., 2001; Rioux et al., 2007). The monosaccharide composition revealed fucose and glucose as the main carbohydrate units for *A. nodosum* and *S. latissima*, respectively. The high sulfur content in *A. nodosum* points towards sulfation of fucoidan structures (Deniaud-Bouët et al., 2017).This evidences the important differences in the cell wall architecture, with higher abundance of sulfated fucoidan/fucan structures or cellulose, depending on the species. The high alginate content in these species is reflected in the sum of GulA and ManA units, with 21.5 wt% and 25.4 wt% alginate in the raw seaweeds, respectively.

Table 1. Proximal composition of raw seaweed and alginates from *Saccharina latissima* and *Ascophyllum nodosum*

	Seaweed		Alginates		
Composition (dry wt. %)	*S. latissima*	*A. nodosum*	*S. latissima*	*A. nodosum*	Commercial*
Yield	n/a	n/a	11.2 ± 0.9^{a}	13.8 ± 0.9^{b}	n/a
Ash	25.9 ± 0.1	18.3 ± 0.0	24.2 ± 0.3^{c}	21.2 ± 0.2^{a}	22.45 ± 0.2^{b}
Lipid	2.6 ± 0.8	3.0 ± 0.3	n/a	n/a	n/a
Protein	7.3 ± 0.1	9.0 ± 0.9	3.6 ± 0.3^{a}	4.1 ± 0.3^{a}	<0.1
Sulfate	2.0 ± 0.0	5.1 ± 0.1	0.6 ± 0.2^{a}	1.8 ± 0.1^{b}	<0.1
Carbohydrates[1]	61.1 ± 2.0	64.2 ± 2.5	68.2 ± 2.1^{a}	68.9 ± 1.7^{a}	72.0 ± 2.8^{a}
of which (µg/mg dry basis)					
Fucose	3.8 ± 0.3	25.8 ± 0.6	1.1 ± 0.1^{a}	2.7 ± 0.2^{b}	<0.1
Galactose	0.8 ± 0.1	1.5 ± 0.1	0.5 ± 0.1^{b}	0.3 ± 0.1^{a}	0.9 ± 0.3^{c}
Cellulose[2]	23.1 ± 0.7	6.3 ± 2.0	<0.1	<0.1	<0.1
Glucose[3]	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1^{b}	0.2 ± 0.1^{a}	1.0 ± 0.2^{c}
Mannose	0.1 ± 0.0	1.4 ± 0.1	<0.1	<0.1	<0.1
Xylose	0.3 ± 0.0	3.2 ± 0.2	0.3 ± 0.1^{a}	1.8 ± 0.7^{b}	1.1 ± 0.1^{b}
GulA	9.2 ± 0.5	7.9 ± 0.2	23.7 ± 1.6^{a}	23.7 ± 0.7^{a}	23.0 ± 1.6^{a}
GlcA	0.8 ± 0.1	1.3 ± 0.0	0.7 ± 0.2^{a}	1.6 ± 0.2^{b}	0.7 ± 0.1^{a}
ManA	16.2 ± 0.3	13.6 ± 0.2	41.2 ± 1.3^{b}	38.6 ± 1.4^{a}	45.3 ± 2.5^{c}
Mannitol	5.9 ± 0.2	3.8 ± 0.2	<0.1	<0.1	<0.1

The values report means (n=3) ± standard deviation. For alginate samples, different letters in the same row indicate significant differences between the samples ($p<0.05$). GulA: guluronic acid. ManA: mannuronic acid. GlcA: glucuronic acid. *Commercial alginate from Sigma-Aldrich (PHR1471)

[1]Total carbohydrate content calculated as the sum of all monosaccharides detected by HPAEC-PAD

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

[3]Glucose contribution from the non-crystalline fraction.

The industrial production of alginate always follows an acid and alkaline treatment as described in Figure 1 and may differ in the recovery of the soluble sodium alginate after the alkaline treatment by different precipitation or flocculation methods. The most common method is the addition of sodium chloride and isopropanol in varying quantities to promote the flocculation of alginate to the upper phase or its precipitation. In this work, this common procedure was followed, and alginate was centrifuged and washed with 50% isopropanol and pure isopropanol twice to ensure recovery of alginate with high purity (Figure 1). Yield values of the alginate extracts (11-14%) were significantly higher ($p \leq 0.05$) for *A. nodosum* than for *S. latissima*, which can be ascribed to differences in the cell wall structure of both species (Deniaud-Bouët et al., 2017). In general, similar or only slightly higher alginate yields have been reported by other authors for some well-known worldwide alginophytes, including *A. nodosum* (18% dw) (Yuan and Macquarrie, 2015), *S. polyschides* (16% dw) (Jard et al., 2013), *F. vesiculosus* (16.2% dw)(Mohd Fauziee et al., 2021) and *P. pavonica* (17.5% dw) (Okolie et al., 2020) pointing out that slight differences in the washing/precipitation steps or salt/solvent concentrations used do not highly affect the yield. As expected, the main sugar constituents were ManA and GulA, with no significant differences between the two seaweeds, and the protein content was relatively low (3-5%), all of which evidences the relatively high purity of the alginate extract, in line with commercial alginate samples (Table 1). The mannuronic acid:guluronic acid (M/G) ratio was 1.7 and 1.6 for *S. latissima* and *A. nodosum*, respectively. These are within the range for alginate ratios previously reported from species of the same genus (Fertah et al., 2017; Torres et al., 2007; Zrid et al., 2016). These M/G ratio results

were also confirmed by NMR, as shown in Figure S1 from the Supplementary Material, evidencing HPAEC-PAD also as a useful tool for determining the M/G ratio. Considering the alginate content in the raw material as the sum of mannuronic and guluronic acid units, these results point out the relative inefficiency in the industrial process, which aims at the extraction of a high-quality alginate leaving a significant alginate content in the residual streams, besides other potentially valuable compounds.

3.2. Compositional analysis of the different waste stream fractions

Instead of collecting the waste streams from the industry, a common industrial alginate extraction process was mimicked in the lab and the waste streams produced in a controlled manner. This not only prevented deterioration of the fractions during manipulation and transport from the industries to the lab, but also guaranteed no other additives (such as formaldehyde) were present through the process. Waste streams generated through the sequential alginate extraction process for both seaweed species were quantified and characterized in terms of their composition in order to evaluate their potential for further valorization (see Figure 1). Two species from different families (Laminariales and Fucales) with distinct cell wall organization and recalcitrance (Deniaud-Bouët et al., 2017) were deliberately selected to evidence differential valorization possibilities. The yield, protein, ash, and sulfate content, as well as the carbohydrate composition of each fraction is shown in Table 2 and Figure 2, respectively.

Table 2. Chemical composition of alginate extracts, the different waste stream fractions obtained during the extraction process.

Algae	Sample	Yield* (%)	Protein (%)	Ash (%)	Carbohydrate (%)	Sulfate (%)
S. latissima	F1	45.1 ± 0.9^{i}	5.0 ± 0.7^{g}	42.5 ± 0.9^{e}	42.5 ± 1.6^{d}	$1.5 \pm 0.0^{c,d}$
	F2	19.9 ± 1.2^{g}	$3.9 \pm 0.6^{e,f}$	77.8 ± 0.6^{k}	$12.9 \pm 1.6^{a,b}$	0.2 ± 0.0^{a}
	F3	2.7 ± 0.5^{e}	1.3 ± 0.7^{a}	75.9 ± 0.2^{j}	$17.4 \pm 2.3^{a,b,c}$	$0.6 \pm 0.3^{a,b}$
	F4	$1.1 \pm 0.0^{a,b}$	$3.4 \pm 0.1^{d,e,f}$	66.9 ± 0.9^{h}	24.4 ± 2.6^{c}	$0.5 \pm 0.5^{a,b}$
	F5	0.3 ± 0.1^{a}	$2.4 \pm 0.5^{b,c}$	58.9 ± 0.5^{f}	27.5 ± 2.3^{c}	$1.4 \pm 0.3^{c,d}$
	Ra	9.1 ± 0.3^{f}	31.5 ± 0.3^{k}	14.6 ± 0.7^{b}	$40.2 \pm 1.1^{d,e}$	6.1 ± 0.1^{f}
	Rb	$8.2 \pm 0.2^{e,f}$	14.7 ± 0.2^{i}	12.6 ± 0.4^{a}	68.9 ± 2.2^{f}	$4.1 \pm 0.0^{e,f}$
A. nodosum	F1	44.9 ± 0.8^{i}	$4.4 \pm 0.1^{f,g}$	39.8 ± 0.8^{d}	$48.1 \pm 1.9^{d,e}$	7.5 ± 1.0^{h}
	F2	21.8 ± 0.2^{h}	$3.1 \pm 0.7^{c,d,e}$	80.2 ± 0.3^{l}	12.2 ± 1.6^{e}	$1.1 \pm 0.4^{b,c}$
	F3	2.6 ± 0.1^{e}	$1.5 \pm 0.1^{a,b}$	70.2 ± 0.2^{i}	$23.8 \pm 1.1^{b,c}$	5.3 ± 0.2^{g}
	F4	1.4 ± 0.2^{b}	$3.3 \pm 0.0^{c,d,e}$	70.1 ± 0.7^{i}	$23.7 \pm 1.0^{b,c}$	4.4 ± 0.0^{e}
	F5	1.3 ± 0.2^{b}	$2.7 \pm 0.1^{c,d}$	62.6 ± 1.0^{g}	25.2 ± 1.8^{c}	$1.5 \pm 0.3^{c,d}$
	Ra	8.0 ± 0.3^{e}	15.7 ± 0.6^{j}	19.5 ± 0.4^{c}	45.7 ± 1.6^{e}	7.4 ± 0.2^{h}
	Rb	5.6 ± 0.7^{d}	11.6 ± 0.3^{h}	15.8 ± 0.3^{b}	67.3 ± 2.0^{f}	7.7 ± 0.0^{h}

For a full description of the procedure to obtain the different fractions refer to Fig. 1. The values reported are their means ($n=3$) $\pm$ standard deviation. Different letters in the same column indicate significant differences between the samples ($p<0.05$).

* The yield expresses the mass fraction of the specific waste stream divided by the total algal biomass in dry weight.

In order to assess the potential of the waste stream fractions generated in the production of alginate, the solid yield is a crucial parameter and was calculated from the initial algae solid biomass, considering the addition of acid, alkali or sodium chloride and bicarbonate salts through the process. The fraction with highest yield was the acid treated first waste stream fraction (F1), representing ~50% of all solids for both species, followed by fraction F3 (20-25%). A significant part of the initial algae materials (13-20%) was also recovered from the solid precipitate, either as a water soluble (Ra) or insoluble (Rb) waste fraction. The rest of fractions were recovered in lesser quantities (<3%), which may already hamper their potential valorization.

F1, the fraction with highest solid yield in both seaweed species, was mainly composed by salts, carbohydrates and minor amounts of protein. Minerals and carbohydrates were the main constituents in this fraction with similar contribution. The significant amount of carbohydrates (43-48%) present in F1 highly differed depending on the algae species. Fucose containing polysaccharides were predominant in *A. nodosum* (Figure 2) typical of more soluble sulfated low-molecular weight (LMW) fucoidan fractions. A very high degree of sulfation was found in this polysaccharide rich fraction (Table 2), which is usually desirable as it provides hydrogen-donating ability to the polysaccharides, which in turn is ascribed to their antioxidant activities (Yuan and Macquarrie, 2015) as well as many other bioactivities commented in the introduction. However, a fraction of the sulfate content could arise from de-sulfation of fucoidan or other polysaccharide populations not extracted in this first step. *S. latissima*, on the other hand, showed additional significant amounts of glucose (Figure 2), while sulfate

content was drastically lower in comparison. The relative lower ash content in F1 from *S. latissima* compared to *A. nodosum* is probably attributed to the relative increase in glucose. This glucose can arise from the potential presence of laminarin, which highly depends on harvesting season (Manns et al., 2014; Schiener et al., 2015), from amorphous cellulose released during the acid treatment and from free glucose present in species from the Genus "*Saccharina*" (sugar), as previously reported by Ravanal et al. (2017).

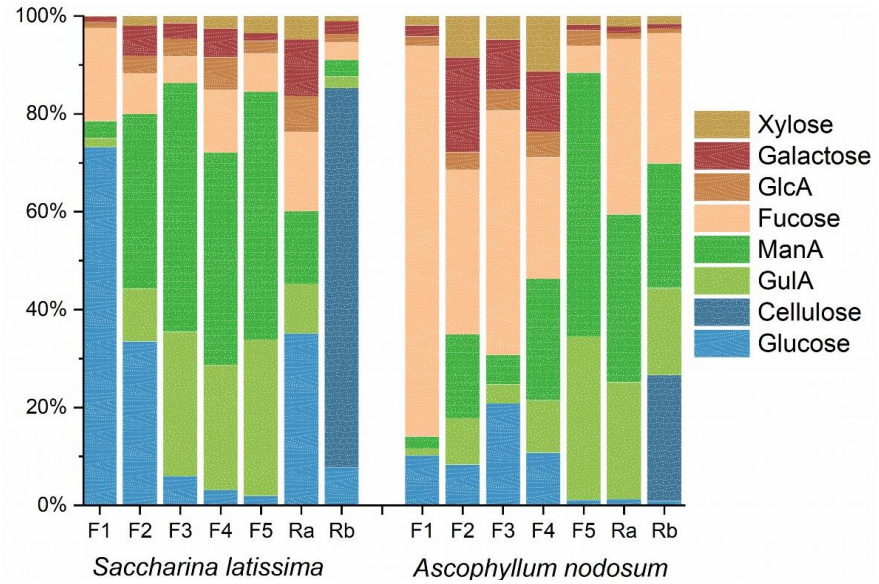


Figure 2. Relative monosaccharide composition of the carbohydrate fractions of all waste streams (refer to Figure 1 for nomenclature). Crystalline cellulose and glucose from non-crystalline polysaccharides were calculated as in Table1.

The second waste stream fraction in terms of solids content was F2 which was generated from the supernatant upon addition of a volume of 50% isopropanol. The proximate analysis revealed this fraction to be mainly salts

(75-80%) with minor quantities of carbohydrates or protein (Table 2). The addition of 50% isopropanol might explain the massive precipitation of salts and confirms the need for this step to remove them and purify alginate. In the case of *S. latisima*, these low amounts of carbohydrates mainly correspond to alginate, probably of a very low molecular weight and not contributing to the desired rheological properties of commercial alginate (Figure 2). With subsequent washings steps (F3-F5), overall solids and minerals recovered from these residual fractions gradually decreased while these very minor residual low molecular alginate is enriched. For *A. nodosum*, on the other hand, fucoidans with a high degree of galactose, xylose and glucuronic acid contribution predominate in F2-F4, but the proportion of alginate rises in the negligible last washing steps (F4-F5; Figure 2). Although removal of these residual mineral and low molecular polysaccharides contributes to further purify an alginate with excellent gelling properties, the low yields of F4 and F5 suggest that these steps may be omitted in the process.

As commented above, a significant amount of the initial biomass remained in the solid residue after alkaline treatment (fractions Ra and Rb). In this work, this solid residue was further washed with distilled water to separately investigate those compounds which were solubilized through the alkaline treatment (Ra), from those insoluble in water (Rb). Interestingly, the water-soluble fraction (Ra) showed higher yields and was found to be very rich in protein, values being twice higher in *S. latissima* than in *A. nodosum*. Apart from soluble protein, Ra fractions also contained a significant amount of salts (15-19%) and polysaccharides. As to the composition of this polysaccharide fraction, fucan and alginate were found in *A. nodosum*,

whereas fucoidan and alginate were predominant in Ra from *S. latissima* (Figure 2). Other low molecular weight compounds or degradation products might be present in this soluble fraction which are not accounted for in a proximate analysis (Table 2). The water insoluble solid waste stream fraction (Rb) consisted mainly of polysaccharides (67-69%) although significant amounts of both insoluble protein and salts were also found (Table 2). The main insoluble carbohydrate fraction was, as expected, arising from the cellulose present in the algae (Figure 2), which was not significantly extracted throughout the process and, thus, enriched in the Rb fractions. A much lower cellulose content has been reported for *A. nodosum* than for *S. latissima* (35% and 18% for the raw seaweeds (Cebrián-Lloret et al., 2022); which explains the lower yield of Rb in *A. nodosum* (Table 2) and a lower glucose content in the carbohydrate composition (Figure 2). Instead, a recalcitrant fucoidan population remains in Rb from *A. nodosum*, which was not found in *S. latissima*, evidencing big differences in the cell wall architecture of these two seaweed species from the Fucales and Laminariales families and encouraging future research on the elucidation of these distinct fucoidan populations in *A. nodosum*. It is also notable to observe a significant alginate fraction which still remained in this residual fraction (around 25-75% of Rb for both species; Figure 2). Unexpectedly, this residual alginate displayed a similar M/G ratio than the purified alginate. This may be positive for its potential valorization; as high G content is usually associated to better gelling properties (Hu et al., 2021).

FT-IR analyses were also carried out to assess the molecular organization and confirm compositional differences. Figure 3a depicts the

spectra from the alginates produced in this work as compared with a commercial one. The spectra from the liquid waste stream fractions F1-F5 for *S. latissima* and *A. nodosum* are displayed in Figure 3b and 3c, respectively, while waste streams generated from the solid residue are shown in Figure 3d and 3e. No notable differences are observed between all three alginates, again confirming the reproducibility of the mimicked industrial process and the purity of the samples (Figure 3a). For both seaweeds, fractions F3-F5 showed similar spectra as that from the alginate, confirming the presence of this residual alginate as main component in these low yield fractions. This is reflected in the bands at around 1615-1626 cm^{-1}, corresponding to the asymmetric carbonyl stretching in the carboxylate anion (COO^-), the bands at 1080 and 1000cm^{-1}, associated to the C-C and C-O stretching vibrations of the pyranose ring (Mateos-Aparicio et al., 2018), and the fingerprint or anomeric region (950-750 cm^{-1}) (Chandía et al., 2004; Gómez-Ordóñez and Rupérez, 2011). The absence of carbohydrates is patent in F2, with these bands having much less intensity, while a broad band around 1412 cm^{-1} has been associated to symmetrical stretching of the –C═O from inorganic carbonates (Leal et al., 2008). In F1, signals from the pyranose rings are present but not carboxylate signals, typical from neutral sugars, in agreement with the compositional results. In the fractions generated from the solid residue (Ra and Rb; Figure 3d and 3e), both C-C and C-O stretching vibrations of the pyranose ring and carbonyl from carboxylate anions denote the presence of neutral sugars and alginate in this carbohydrate components, especially in Rb fractions. In the case of Ra, amide I and amide II signals at 1650 $cm^{-1}$and 1525 cm^{-1}, respectively, evidence the presence of protein. In the case of Ra from *S. latissima*, these bands are especially predominant, in

agreement with the compositional results. The presence of sulfate was difficult to assign, as the S=O stretching band is partially overlapped by C-O stretching bands from sugars around 1200-1260 cm^{-1} (Gómez-Ordóñez and Rupérez, 2011; Palanisamy et al., 2017).

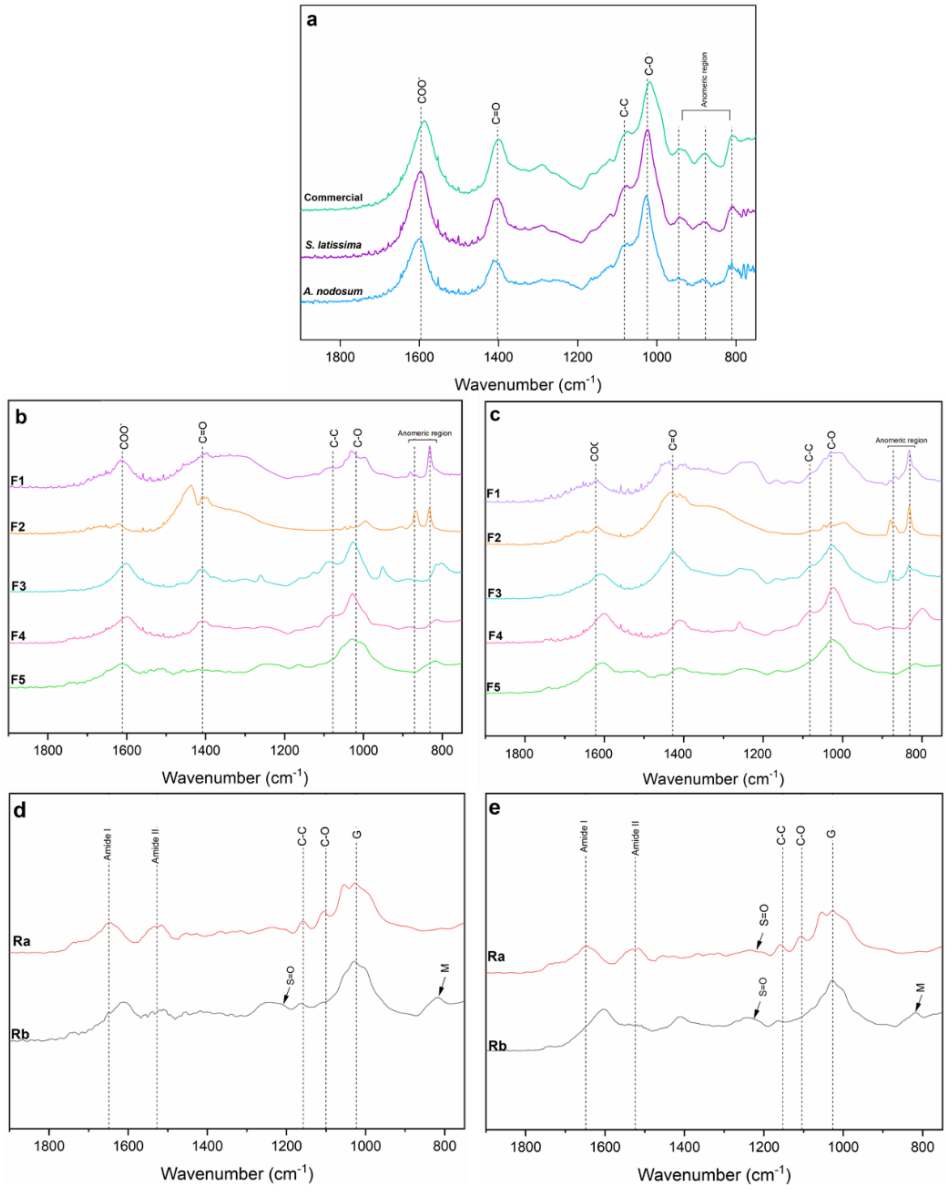


Figure 3. FT-IR spectra of alginates (a), liquid residual streams (b) and (c), and fractions obtained from the solid residue residues (d) and (e) from *S. latissima* and *A. nodosum*, respectively. For a full description of the procedure to obtain the different fractions and their nomenclature refer to Fig. 1. G: guluronic acid; M: mannuronic acid.

3.3. Polyphenol content and antioxidant properties of the different fractions and residues

Many bioactive properties in brown algae extracts are related to their capacity to interfere with several biochemical mechanisms that regulate oxidative stress and inflammation which, in turn, has been mostly ascribed to the antioxidant or radical scavenging capacity of either the phlorotannin or sulfated polysaccharide content (Araújo et al., 2021). The determination of the ABTS radical scavenging activity or beta-carotene bleaching assay can be complementary methods to indirectly evidence a potential bioactive capacity in unknown samples, as they cover both the antioxidant and the radical scavenging capacity of either insoluble or soluble compounds, respectively (Méndez et al., 2022). Figure 4 shows the polyphenol content, which complements previous compositional analyses, as well as the radical scavenging and antioxidant capacities of all waste streams from both seaweeds. This activity assays further back up and relate to the previous compositional results.

Interestingly, the highest polyphenol content was found in the waste streams from the solid residue after alkaline extraction, Ra and Rb, followed by F1, for both seaweeds (Figure 4a). The polyphenol content of fractions F2 to F5 and extracted alginate did not show significant differences for both seaweed species and were significantly lower ($p<0.05$) than the values obtained for F1, Ra or Rb. Higher total polyphenol content (TPC) values were found here for *A. nodosum*, which can be explained by a higher TPC content in the pristine algae compared to kelp species such as *S. latissima* or *A.*

esculenta (Li et al., 2020; Schiener et al., 2015). Phlorotannins have also been described to be linked to the fucoidan or fucan structures, much more abundant in *A. nodosum*. This would explain not only an overall higher abundance in *A. nodosum*, but also an evidently higher contents of polyphenols in Rb compared to *S. latissima*'s cellulose-rich Rb fraction (Figure 2). Another possible explanation is that the weaker cell wall of *A. nodosum* might have suffered greater cellular damage after the acid treatment, favoring the subsequent release of bound phenolic compounds (the Folin method mostly detects unbound ones). Apart from phlorotannins, previous studies have also reported the presence of some specific carotenoids (fucoxanthin, chlorophyll-*a*, or phycoxanthin) commonly forming complexes with protein in thylakoids and working as a light-harvesting system (Blanco-Pascual et al., 2014; Khajouei et al., 2018), which could be released upon acid/alkali treatments and further add up to the polyphenol and antioxidant activities (Sappati et al., 2019). In order to assess the antioxidant capacity of the different waste streams, both the ABTS method and β-carotene bleaching assay were performed and the results are displayed in Fig. 4b and 4c, respectively. Again, the results show that F1, Ra, and Rb had the highest antioxidant activity for both seaweeds, with significantly ($p<0.05$) better results for *A. nodosum* than for *S. latissima*. This is consistent with the results on polyphenol content (Figure 4a). The antioxidant capacity of fractions F1 was nevertheless higher than expected by the polyphenol content, suggesting this activity might also arise from the high content in highly sulfated fucoidan of this fraction (Figure 2). The antioxidant activity of polysaccharide components from brown seaweed may depend on different factors such as molecular weight, sulfation level, and sugar residue composition (Jiménez-Escrig et al., 2011). In general, similar

bioactive properties have been ascribed to both sulfated fucoidan and phlorotannins, e.g. preventing cardiovascular disease, obesity, neurodegeneration, cancer, used as food prebiotics or preservatives, etc (Jiang et al., 2021; Meng et al., 2021).

Some authors raised a controversy when ascribing functional properties to either sulfated fucoidan or phlorotannins (Imbs and Ermakova, 2021). Indeed, most studies focus in ascribing a specific functional activity to either a specific phlorotannin-rich or a sulfated fucoidan-rich extract, while neglecting the possible polyphenol-carbohydrate complexes and potential synergistic effects of both components. Further research is needed to accurately establish these structure-activity relationships. Regardless of the real source of the antioxidant or other bioactivity, this work evidences a high antioxidant potential, not only for the first acid effluents (F1), but also for the solid waste streams even after acid/alkaline treatment.

To confirm the antioxidant properties of the fractions and residues and to investigate how they are affected during the extraction process, ABTS+ radical scavenging and β-carotene-bleaching inhibition assays were carried out on all extracts. Antioxidant activities are in the range of 40-70 μmol TEAC, which are relatively high values, in line with food natural compounds widely recognized as good antioxidants, such as caffeic acid (13.3 μM TE/g) or ferulic acid (60.8 μM TE/g) (Rodrigues et al., 2019). Through the beta-carotene bleaching assay, antioxidant activities of F1 or Ra fractions were found to be slightly lower than that of butylhydroxytoluene (BHT), a typical food additive used as strong antioxidant. Differences in the antioxidant

response in Rb, or even F1, for both methods, might arise from the different solubility and availability of the antioxidant compounds when dispersed in an emulsion. These results pose both research and industrial challenges as to elucidate structure-activity and potential valorization strategies for these residues. As commented in section 2.2, formaldehyde is often added to the harvested seaweed through transport as a preservative and to improve the quality of the extracted polysaccharide. Apart from being toxic, allergenic and possibly carcinogenic, formaldehyde can also cross-link with polyphenols (Cebrián-Lloret et al., 2022; Davis et al., 2004). This should be taken into account when designing a valorization strategy in this direction and it was one of the reason it was not used in this study.

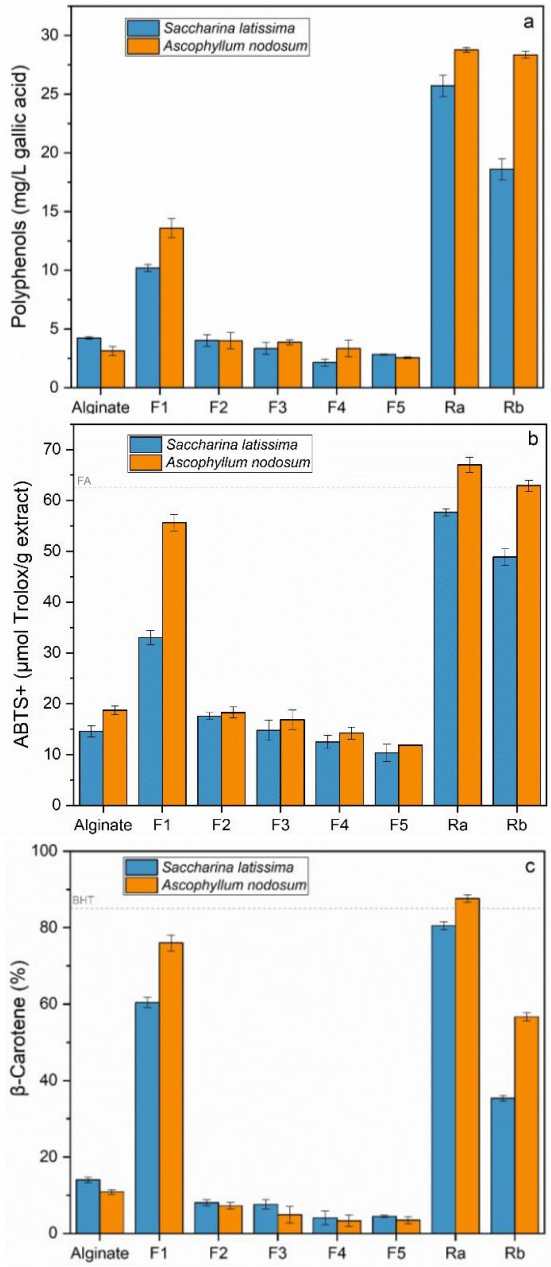


Figure 4. Polyphenol content (mg GAE/g) (a), ABTS+ antioxidant capacity (b), and β-carotene-bleaching inhibition (%) of alginates and waste streams from *S. latissima* and *A. nodosum* (see Figure 1). The antioxidant activity of pure ferulic acid (FA) and butylhydroxytoluene (BHT) is added in a dotted line as comparison.

3.4. Potential valorization strategies for the waste streams

The compositional results and antioxidant activity studies of some of the waste stream fractions (F1, Ra and Rb) pose great potential pathways for their valorization. These promising results encourage further research efforts for the evaluation of the nutritional value of potential vegan protein and soluble fiber ingredients or mineral components and an in-depth analysis of specific bioactive properties of the fucoidan/polyphenol rich waste streams. The high yields of the soluble F1 and Ra fractions could effectively ensure a constant feedstock for the production of hundreds of tons of these high value vegan nutritional or cosmetic ingredients. Although many other valorization pathways are possible, a schematic overview of some examples is depicted in Fig. 5 and discussed below. Although alginate producing species in the same family, such as kelp species in the Laminariales family or typical nearshore algae in the Fucales family, have similar cell wall architecture and thus similar results are expected, compositional analyses to the specific waste streams should be confirmed before designing future valorization schemes.

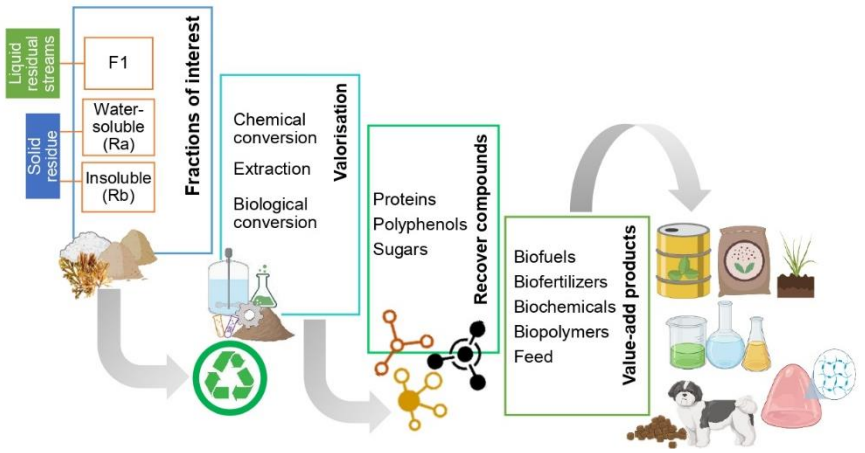


Figure 5. Possible valorization strategies for specific waste streams generated in the alginate industry into novel added-value products.

Fraction F1, representing around 50% of all biomass, is a mostly water-soluble fraction containing high amounts of bioactive carbohydrates and minerals. In the case of *A. nodosum*, the abundance of highly sulfated fucoidan and polyphenols, together with excellent antioxidant properties, suggest this stream a firm potential candidate for the development of bioactive ingredients. For certain skin care applications, which are one of the main current market applications for soluble algae extracts, the presence of naturally occurring sea minerals may actually be positive to the formulation. For other applications, such as food or pharmaceutical ingredients bound to be ingested, simple purification steps, such as ultrafiltration or solvent precipitation, might highly purify these high-value ingredients. In the case of F1 from *S. latissima*, the high amounts of soluble glucans and free glucose makes it an interesting candidate for fermentation into biofuels or other platform chemicals. Indeed, these brown algae have been explored as a carbon source for the fermentation

process into bioethanol (Albers et al., 2021; Sharma et al., 2018) or to produce terpenes, or methyl ethyl ketones (Peralta-Yahya et al., 2012; Scullin et al., 2015). Current exploitation of *A. nodosum* in Europe would allow a constant feedstock of thousands of tons/year of this up-cycled residual. Laminarin has also been ascribed a strong prebiotic effect (Nguyen et al., 2016), together with further antitumor or immunomodulating properties (Menshova et al., 2014) and has also been explored as a feed supplement (Walsh et al., 2012).

Fraction Ra, produced in this work by a simple water washing of the solid residue constitutes around 9% of all biomass, and could have as such a great potential as a food ingredient, owing to its high soluble protein content. In *S. latissima*, soluble protein in this fraction accounted for >30%. Given the high demand for soluble protein as texturizing/nutritional ingredients and the current trend towards removing animal-based texturizers, such as gelatin, from food ingredients, this fraction could be explored as a food additive in vegan or other food formulations.

On the other hand, a significant fraction of the insoluble protein is enriched in fractions Rb. This insoluble protein could be recovered by common protein extraction/purification methods and separated from the cellulose/fucoidan rich residue. This protein, even if partially denatured or degraded when extracted, might have use in the preparation of peptones for biotechnology purposes or used as a feed protein ingredient. This would help in decreasing Europe's dependence on protein import in line with current Horizon Europe R&D strategies. On the other hand, a substantial amount of alginate (nearly

half of its initial content in the raw algae) also remains in the residual Rb fraction (also in Ra for *A. nodosum*). Although this alginate may not be extracted as a high molecular weight alginate with excellent gelling properties, it could still be valorized as partially degraded insoluble fiber or lower value thickener, depending on its molecular weight and rheological properties. The prebiotic activity of low molecular weight alginate has already been demonstrated (Li et al., 2020; Okolie et al., 2020). After recovery of these protein and polysaccharide fractions, the cellulose enriched residue could then be valorized as absorbents or food packaging materials as already reported by (Cebrián-Lloret et al., 2022).

As commented above, fraction F2 is also a significant waste stream in the process, made up of mostly minerals. In this case, further analysis of the mineral composition should ascertain its suitability for soil amendment or any other supplements (González-López et al., 2012). It may also be possible that this fraction contains most of the bicarbonate and chloride salts added to the process, which would definitely hamper valorization.

Due to their low yield, the rest of fractions could have limited use, as they are composed mainly of minerals, with very low quantities of soluble carbohydrates or protein.

4. Conclusions

In this work, the common alginate extraction process used in the industry was mimicked for two brown algae with different cell wall architecture, *A. nodosum* and *S. latissima,* and the different solid and liquid waste streams

currently not valorized by the industry were characterized to explore potential valorization strategies. Produced alginates had similar composition or M/G ratio as commercial alginates, confirming the reproducibility of the industrial process. As for the waste streams, the first waste stream fraction after acid treatment (F1) showed a great potential as a bioactive ingredient, due to a high yield in sulfated fucoidan and polyphenols, which demonstrated a high antioxidant efficiency by both ABTS and beta-carotene bleaching assays. A simple water washing of the solid residue after alkaline treatment (Ra) produced relatively high yields of a fraction with good antioxidant properties and rich in soluble protein (30 %wt for *S. latissima*), with great potential as a texturizing vegan/functional ingredient. The solid residue (Rb) still contained a significant amount of insoluble protein and alginate, the valorization of which could be explored as feed supplement or, after further down-stream processing, as peptones or thickener ingredients, respectively. The distinct cell wall architecture of the algae was reflected in the composition of the fractions for both species. In the case of *S. latissima*, >30wt% glucose/glucan content was found in F1, higher protein content in Ra and higher cellulose content in Rb. Comparatively, for *A. nodosum*, significantly higher antioxidant capacities were observed in F1, Ra and Rb (50-70 µM TE/g) and higher yields (>45%wt%) of a highly sulphated fucoidan were recovered in F1. These results provide the basis for a potential valorization of residual waste streams from alginate production and open up the possibility of using these fractions for the extraction ofadded-value ingredients for different applications within the cosmetic, pharma, food or feed industry, such as alternative protein sources, bioactives or texturizers. Considering the huge quantities of algae processed by the alginate industry, the transformation of

these waste streams into added-value products would constitute a significant step towards a more circular and sustainable economy.

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6. Supplementary Material

^{1}H NMR spectra of the alginates are shown in Fig. S1. Alginates were partially digested and the M/G ratio calculated using the method according to Jensen et al. (2015). The distribution of M and G blocks could be studied based on signals from the anomeric region. The M/G ratio was 1.65 and 1.60 for *S. latissima* and *A. nodosum*, respectively.

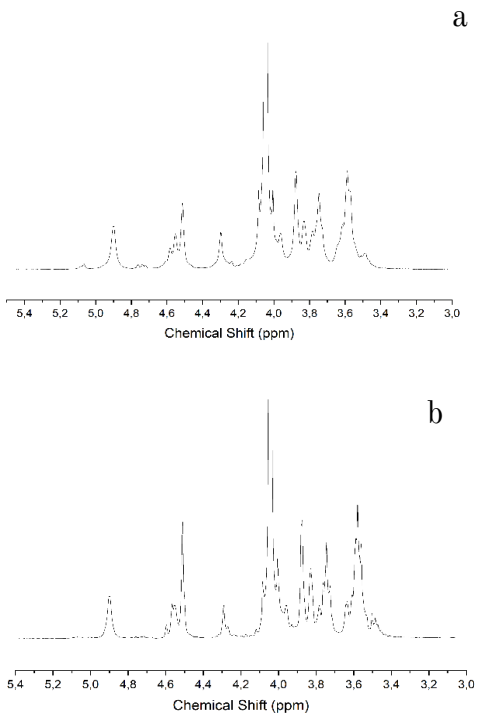
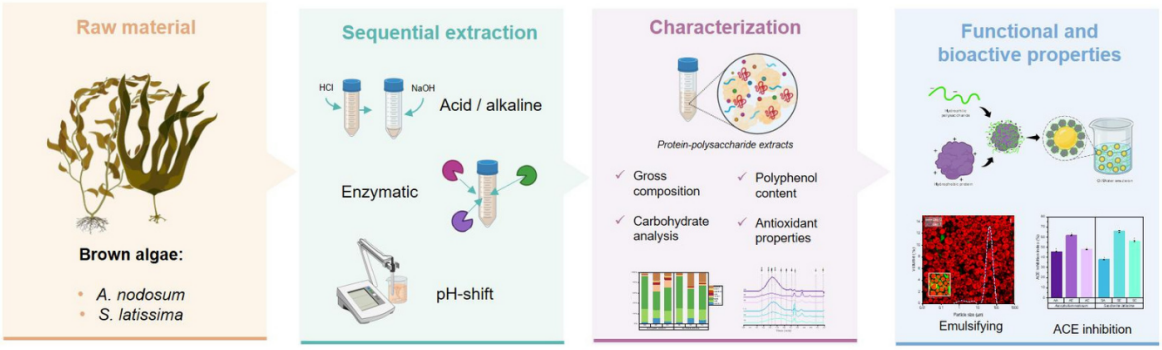


Figure S1. ^{1}H NMR Spectra of alginates extracted from (a) *Saccharina latissima* and (b) *Ascophyllum nodosum*. Spectra were recorded at 353 K of samples dissolved in D_2O and the residual water resonance at 4.32 ppm was suppressed using excitation sculpting with gradients.

2.2

Protein extraction from the industrial solid residue of brown seaweed after alginate extraction



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

Bojorges, H., Martínez-Abad, A., López-Rubio, A., & Fabra, M. J.
Protein extraction from the industrial solid residue of brown seaweed after alginate extraction

Submitted to Food Hydrocolloids

ABSTRACT

The growing interest in the "blue-bioeconomy" and the valorization of macroalgae has driven research and industrial efforts to develop valorization strategies for the by-products generated during alginate extraction. Sequential extraction methods, such as acid/alkali treatment, enzymatic, ultrasound, and pH-shift process, were investigated and compared in order to obtain protein extracts from the solid residues after alginate extraction from two brown seaweed species (*Ascophyllum nodosum* and *Saccharina latissima*). Applying a sequential extraction, combining several methods, allowed the recovery of more than 29.5 % of the total protein from the residues. The resulting extracts exhibited optimal functional properties in terms of oil and water retention and outstanding emulsifying and foaming capacity. In addition, the protein extracts showed considerable polyphenol content, remarkable antioxidant activity, and an enhanced ability to inhibit angiotensin-converting enzyme (ACE). This study demonstrated that the residues after alginate extraction are a sustainable and promising source of protein with potential applications in the food industry.

1. Introduction

The global population is estimated to increase to around 2.3 billion individuals by 2050. Due to this growth, it is forecasted that global food production will have to rise by 70% in the upcoming years (Bleakley & Hayes, 2017). However, protein, as one of the primary nutrients, is expected to become scarce for human food and animal feed. Currently, the production of animal

protein for human consumption is widely regarded as inefficient and is a major contributor to environmental disruption. This process negatively impacts carbon cycles, generates polluting emissions and significantly contributes to soil degradation and deforestation (Tang et al., 2023). Therefore, reducing animal protein consumption could beneficially mitigate the consequences of climate change. Furthermore, it is imperative to explore alternative protein sources in order to satisfy increasing consumer needs and anticipated protein demands in the future (Naseri et al., 2020).

While research efforts are being focused on protein production from plant sources, residues derived from food and other plant-related chains can likewise be used as protein sources. In this regard, a biorefinery strategy is appropriate because a number of valuable products can be obtained from a single feedstock, contributing to shifting the current economic model towards a circular economy one. Industrial algae by-products, especially those derived from alginate production, make them intriguing in this context. Alginate is a heteropolysaccharide, and the primary constituent of the cell wall is the main component of the cell wall in brown seaweed, extensively utilized in the food, cosmetic, and pharmaceutical industries as an emulsifying, thickening, stabilizing and gelling agent (Gomaa et al., 2018). In fact, after industrial alginate extraction, substantial amounts of seaweed by-products are produced, generally disposed of as waste (Bojorges, 2022). Nevertheless, some of the constituents found in native algae, such as polysaccharides, sulfated compounds, and proteins to a certain degree, can potentially persist in the solid residue based on the initial materials used and the efficiency of the extraction process after the dissolution and separation of alginate (Bojorges et al., 2022;

Cebrián-Lloret et al., 2022). However, there is a possibility that protein aggregates may have formed or undergone some type of hydrolysis, which could result in the generation of small peptides of industrial interest. For this reason, various extraction methods were investigated to efficiently obtain the protein that remained attached to the cell wall, intending to explore its potential use as an additional protein production source, representing an opportunity to valorize this industrial waste.

Therefore, this paper presents a study carried out to determine the feasibility to obtain protein extracts from an industrial brown algae waste produced after alginate extraction process from two different species (*Ascophyllum nodosum* and *Saccharina latissima*). Simplified extraction protocols were utilized to acquire less purified protein extracts, offering advantages not just in terms of economic and environmental approach but also in satisfying consumer and industry demands.

2. Materials and methods

2.1 Materials

The macroalgae *A. nodosum* and S. *latissima* were provided freeze-dried and ground by Porto-Muiños S. L. (Spain). The method described by Bojorges et al., 2022 was applied for alginate extraction and the solid dry residues were powdered and stored at a 0%RH desiccator until further use. Unless specified otherwise, all reagents and chemicals were sourced from Sigma-Aldrich Chemicals (Spain).

2.2 Protein extraction

The protein was extracted through a series of sequential extractions. The first treatment was a chemical extraction consisting of an acid and alkaline treatment (Fig. 1a), conducted according to the procedure described by Kadam et al. (2017) and with additional ultrasound and/or enzymatic treatments. Briefly, seaweed residues were suspended in distilled water (1:20 w/v) and stirred at 4 °C overnight (Figure 1). The suspension was centrifuged at 4 °C at 9000 rpm for 20 min, and the supernatant was collected. The pellet obtained was resuspended in 0.4 M HCl buffer (1:15 w/v), stirred at 4 °C for 1 h, and centrifuged at 4 °C for 20 min at 9000 rpm. After the centrifugation, the pellet was treated with 0.4 M NaOH buffer under the same conditions as the acid extraction. In addition to this methodology, an ultrasound-assisted pre-treatment was further performed in the rehydration step with 0.4 M HCl using the same solvent-solid ratio (1:15). The mixtures were sonicated for 10 min in an ice bath, using an ultrasound equipment UP-400s with a capacity of 750 W and a frequency of 24 kHz (Hielcher, Germany). Upon completion of the pre-treatment, the supernatant and pellet underwent treatment using the described protocol above. All supernatants were combined, mixed, freeze-dried and they were identified as sample CE with or without ultrasound.

In sequential order, protein extraction was carried out from the freeze-dried pellet, both in ultrasonicated and non-ultrasonicated samples, obtained after the acid/alkali extraction process using enzymatic pre-treatment (Fig. 1b). This procedure was performed following the method described by Nunes et al. (2024), with slight modifications. Briefly, the pellet was subjected to a

mixture (1:1) of cellulase mix (Cellic CTec2®; SAE0020, Sigma) and Celluclast® (C2730, Sigma) at two concentrations, pellet:enzyme ratio was 1:0.2 w/v (E20) and 1:10 w/v (E100). A blank in the absence of dried biomass was performed to subtract the protein contribution of the enzymes. The optimal enzymatic hydrolysis conditions were at pH 4.5 and 45 ºC overnight at 250 rpm. The enzyme was inactivated by subjecting the samples to heating at 100 ºC for 5 min, followed by immediate cooling in an ice bath. The extract was centrifuged at 4 °C at 9000 rpm for 20 min, and the resulting supernatant was freeze-dried.

Subsequently, all the supernatants from the previous processes were precipitated by a pH-shift process (Fig. 1c), according to the method described by Harrysson et al. (2018), with the aim of isolating the protein from other compounds (e.g. polysaccharides, minerals, polyphenols or other low molecular weight compounds). Specifically, the freeze-dried supernatant was resuspended in distilled water (1:6 w/v), adjusted to pH 11 using NaOH, and incubed in ice bath for 20 min, followed by centrifugation at 4 °C at 9000 rpm for 10 min. Then, the supernantant was adjusted to pH 2 using HCl, and samples were frozen shortly at -20ºC overnight to enhance the protein yield. After thawing, the samples were centrifuged, and the protein-rich pellet was collected and freeze-dried for further analysis.

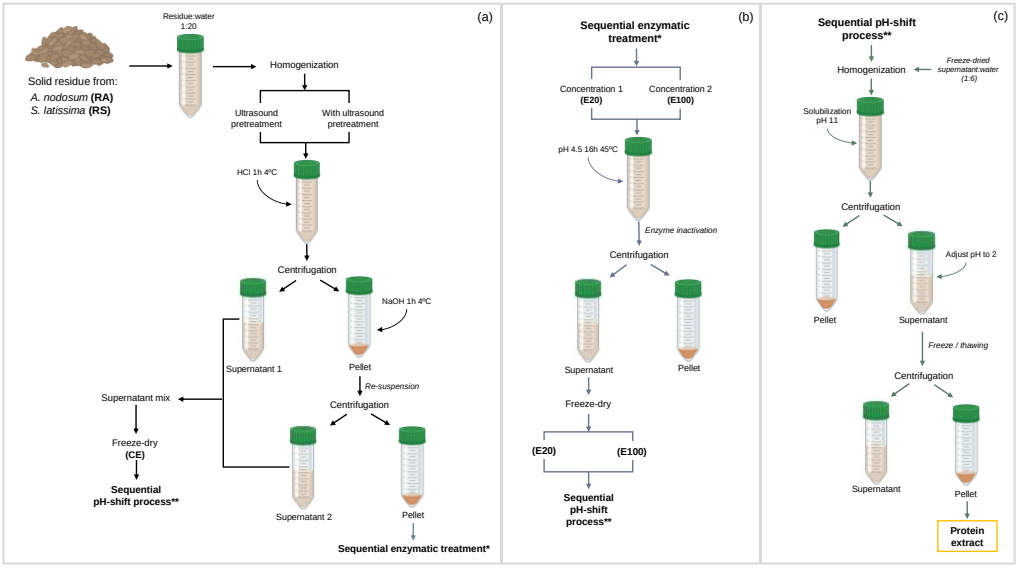


Figure 1. Sequentially of protocols for protein extraction from brown algae solid residue after alginate extraction. Schematic overview of (a) chemical treatment, (b) enzymatic treatment, and (c) pH-shift process. Asterisks (*) and (**) indicate that the process is continuing.

2.3 Composition analysis

2.3.1 Protein content

The protein content was assessed by quantifying the total nitrogen with an Analyzer Rapid N Exceed (Elementar, Germany). The protein content was obtained by multiplying the total nitrogen content by a conversion factor 6.25. This factor was validated through the calculation of protein based on the total amino acid content following hydrolysis of the samples with 6 M HCl for 24h at 110ºC and afterward quantification by HPLC (Cohen & Michaud, 1993).

2.3.2 Analysis of total amino acids

Total amino acid analyses were performed after hydrolyzing with 6 M HCl at 110 °C for 24 h to break down proteins and peptides, thereby releasing amino acids. The samples were then centrifuged at 15000 x *g* at 4 °C for 15 min. Amino acid analysis was conducted using a Biochrom Amino Acid Analyser 30 series (Biochrom, UK) fitted with a cation-exchange column. Then, ninhydrin was mixed with the column eluent through a high-temperature reaction coil to perform post-column derivatization. Subsequently, absorption was measured at 570 and 440 nm. Tryptophan, glutamine and asparagine were not determined under these analysis conditions.

2.3.3 Monosaccharide composition

The determination of the composition of monosaccharides and the content of carbohydrates in the samples was conducted according to Bojorges et al. (2022). The analysis was performed by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD, Switzerland). Calibration was performed using control samples containing predetermined concentrations of galactose, arabinose, mannitol, xylose, fucose, glucose, mannuronic acid, glucuronic acid, and guluronic acid mixtures.

2.3.4 Ash content

The ash content was carried out by igniting the samples of ca. 1 g of freeze-dried native algae or residues powder in a muffle furnace at 550 ºC for 24 h, according to the standard method (AOAC, 1990).

2.3.5 Phenolic content

The total phenolic content was analyzed from the method described by Singleton et al. (1999), with a slight modification. Briefly, the samples were dissolved in distilled water (5 mg/mL). Folin-Ciocalteau reagent was diluted with distilled water at a ratio of 1:10. Then, 1 mL of the resulting dilution was mixed with 0.2 mL aliquots of the sample, followed by the addition of 0.8 mL of Na_2CO_3 (75 mg/mL). Finally, the solutions were incubated at 50 ºC for 30 min. The standard used for preparing the calibration curve was gallic acid. The absorbance values were measured at 750 nm. The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g extract.

2.4 High performance size exclusion chromatography (HP-SEC)

Protein extracts were diluted in 0.01 M NaOH solution and filtered through syringe filters with a 0.22 membrane. Molecular weight estimation was carried out using a 10 by 30 cm CH HP-SEC ACQ Arc Sys Core system (Waters, USA) equipped with a Waters 2414 detector and a Waters 2998 PAD module. Solutions from pululan (PSS polymer Standards Service GmbH, Germany) were used as reference standards.

2.5 Fourier transform infrared spectroscopy (FTIR)

The dry protein extracts were subjected to analysis by FTIIR (Bruker, USA) employing attenuated total reflectance (ATR) sampling mode. The spectra were recorded in a wavelength range between 400 and 4000 cm^{-1} at 4 cm^{-1} resolution and averaged with 64 scans.

2.6 Color measurement

The color of samples was determined at regular intervals using a colorimeter CR-400 (Konica Minolta Sensors, Japan) and reported as the brightness (L*), chroma (C^*_{ab}) and hue (h^*_{ab}).

2.7 Functional properties

2.7.1 Water-holding capacity (WHC)

Water-holding capacity was determined according to Bencini (1986), with slight modifications. The protein extracts (0.5 g) were mixed with 10 mL of distilled water for 5 min in a Thermoshaker Incubator (MSC-100, UK) and centrifuged at 5500 rpm for 30 min. The supernatants were carefully removed, and the pellets were weighted. WHC was expressed as a gram of water held per gram of protein, and it was calculated with the following Equation 1.

$$WHC(g\ H_2O) = \frac{W_2 - W_1}{W_0}\ x\ 100 \qquad (1)$$

Where W_0 represents the grams of the dry sample, W_1 denotes the grams of the tube plus the dry sample, and W_2 indicates the grams of the tube plus the pellet.

2.7.2 Oil-holding capacity (OHC)

In order to determine the oil-holding capacity (sunflower oil/g protein extract), 0.5 g of the sample was transferred into a pre-weighed tube and mixed with 10 mL of sunflower oil. Then, the mixture was centrifuged at 5500 rpm for 30 min. The supernatants were decanted, and the pellets were weighted. OHC (gram of oil per gram of protein) was calculated as follows (Eq. 2)

$$OHC(g\ Oil) = \frac{F_2 - F_1}{F_0}\ x\ 100 \qquad (2)$$

Where F_0 indicates the grams of the dry sample, F_1 represents the grams of the tube plus the dry sample, and F_2 denotes the grams of the tube plus the pellet.

2.7.3 Foaming capacity and stability

The foaming capacity and stability of the samples were determined using the method the method described previously by Nath and Rao (1981). Protein suspensions (2 % w/v) were adjusted to pH 7 and mixed using an Ultraturrax at 10,000 rpm for 2 min. The volume increase was expressed as percent foaming capacity (Eq. 3).

$$Foaming\ Capacity\ (\%) = \frac{V_f - V_i}{V_i}\ x\ 100 \qquad (3)$$

Where V_f represents the volume of foam generated after homogenization, and V_i indicates the initial volume of protein solution before homogenization.

The stability of the foam was assessed by measuring the reduction in foam volume over time at intervals of 30, 60, 90, and 120 minutes (Eq. 4).

$$Foam\ stability\ (\%) = \frac{Volume\ after\ standing - Volume\ before\ whipping}{Volume\ before\ whipping}\ x\ 100 \qquad (4)$$

2.7.4 Emulsifying activity and emulsion stability

Emulsifying activity was determined using a described method of Nackz et al. (1985). Protein solutions were diluted in distilled water (1% w/v). To produce an emulsion, sunflower oil was mixed to the aqueous phase containing the protein extracted at oil:protein ratio was 3:2. This mixture was homogenized utilizing an Ultraturrax at 9000 rpm for 1 min. The volume of the emulsion layer was measured at different time intervals and the emulsification activity was determined by the formula (Eq. 5).

$$\text{Emulsifying activity (\%)} = \frac{Volume\ of\ the\ emulsion\ layer}{Total\ volume\ of\ the\ mixture}\ x\ 100 \qquad (5)$$

Emulsion stability was expressed as the percentage of decrease of the emulsion volume over time after 30, 60, 90 and 120 min.

2.8 Bioactive properties

2.8.1 Antioxidant activity (ABTS)

The antioxidant capacity of the protein extracts was calculated according to Re et al. (1999). Briefly, 20 µL of aqueous solutions of the protein extracts (5mg/mL of sample in distilled water) were added to 230 µL of the ABTS+ solution, and absorbance was read at 734 nm after 6 min. For the calibration curve, Trolox was used as the antioxidant standard. The results were expressed as µmol Trolox equivalents (TE)/g extract.

2.8.2 Inhibition of angiotensin-converting enzyme and its inhibitory mechanism (ACE)

The potential antihypertensive efficacy of the protein extracts was evaluated *in vitro* by assessing the inhibition of angiotensin-converting enzyme (ACE) from rabbit lung, according to the methodology described by Li et al. in 2005. Briefly, 25 µL of 5 mM HHL in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl was mixed with a solution of protein extract (1 mg/mL) or 10 µL of 0.1 µM captopril solution as ACE inhibitor, and then subjected to pre-incubation for 5 min at 37 °C. The reaction was initiated by adding 12.5 µL of RCT solution (100 mU/mL). After incubation for 1h at 37 °C, the reaction was stopped by adding 50 µL of 1 M HCl. Subsequently, 100 µL of BSC, 300 µL of quinoline, and sodium borate buffer were added to the reaction mixture and incubated for 30 min at 30 °C. Finally, 1.85 mL of ethanol was added to the reaction mixture, and the absorbance was measured

at 490 nm. ACE inhibitory activity (%) was calculated by the following formula (Eq. 6):

$$\text{ACE inhibitory activity (\%)} = \frac{B - A}{B - C}\, x\, 100 \qquad (6)$$

where B indicates the absorbance of the control (without an inhibitor), A represents the absorbance of the sample, and C denotes the absorbance of the blank (HCl was added prior to ACE).

2.9 Statistical analysis

All the experiments were conducted in triplicate. The SPSS software (IBM, USA) was used to carry out ANOVA followed by the least significant difference (LSD) test ($p<0.05$).

3. Results and discussion

3.1 Characterization of the raw brown seaweeds and seaweed residues after alginate extraction

The residues obtained after the alginate extraction were characterized to determine their suitability for protein extraction since, to our knowledge, no previous studies have addressed this issue. Furthermore, an analysis of native dried brown seaweeds was conducted to understand the compositional variances better. The proximate composition analysis is summarized in Table 1.

Table 1. Proximal composition of the native algae and the initial raw materials

Composition (dry wt. %)	*A. nodosum*		*S. latissima*	
	Seaweed	**Residue**	**Seaweed**	**Residue**
Protein	7.6 ± 0.4^{a}	27.3 ± 0.2^{e}	10.1 ± 0.1^{b}	39.4 ± 0.7^{d}
Ash	21.1 ± 0.3^{c}	12.8 ± 0.2^{b}	25.2 ± 0.2^{d}	9.3 ± 0.4^{a}
Carbohydrates[1]	63.3 ± 1.2^{c}	52.8 ± 4.0^{ab}	61.1 ± 2.8^{bc}	44.4 ± 4.0^{a}
of which (μg/mg dry basis)				
Fucose	22.7 ± 1.3^{d}	17.1 ± 0.3^{c}	2.1 ± 0.1^{a}	3.8 ± 0.1^{b}
Galactose	1.3 ± 0.0^{d}	1.1 ± 0.0^{c}	0.8 ± 0.1^{b}	0.4 ± 0.1^{a}
Cellulose[2]	6.4 ± 0.1^{a}	12.7 ± 0.1^{b}	23.3 ± 0.3^{c}	32.0 ± 5.9^{d}
Glucose[3]	0.9 ± 0.1^{a}	2.4 ± 0.1^{b}	3.1 ± 0.1^{c}	3.6 ± 0.1^{d}
Xylose	3.6 ± 0.3^{c}	6.1 ± 0.1^{d}	0.4 ± 0.2^{a}	1.1 ± 0.1^{b}
GulA	7.7 ± 0.5^{c}	3.5 ± 0.3^{b}	9.1 ± 0.3^{d}	2.6 ± 0.3^{a}
GlcA	1.4 ± 0.1^{b}	3.4 ± 0.2^{d}	0.7 ± 0.0^{a}	2.3 ± 0.1^{c}
ManA	13.8 ± 0.7^{c}	2.2 ± 0.2^{a}	15.9 ± 0.6^{d}	2.6 ± 0.1^{b}
Mannitol	3.4 ± 0.5^{b}	1.1 ± 0.2^{a}	5.7 ± 0.8^{c}	<0.5

The values report signifies (n=3) ± standard deviation. Distinct letters within the same row indicate statistically significant sample variations ($p<0.05$).

[1]Total carbohydrate content is the sum of all monosaccharides determined by HPAEC-PAD

[2]The crystalline cellulose content was quantified by subtracting the non-crystalline glucose content obtained through methanolysis (Bertaud et al., 2002; Willför et al., 2009) from the total glucose obtained through the standard Saeman sulfuric hydrolysis method (Saeman, 1945).

[3]Glucose contribution from the non-crystalline fraction.

The proximate composition analysis showed that the two seaweeds mainly contained carbohydrates (between 60-63 %), although the samples also contained significant amounts of minerals (21-25 %) and proteins (7-10

%), as recently reported for brown seaweed (Bojorges et al., 2022; Sharma et al., 2018). It is well known that the algal species and seasonal variations determine the chemical composition of algae. It has been reported that the ash content in brown algae is subject to significant variation due to seasonal conditions and can represent $\geq$ 50 % of the dry weight (Monteiro et al., 2021). The ash content ranges from 18-33 % for *A. nodosum* (Tabassum et al., 2016), whereas greater contents (47 %) have been observed for *S. latissima* (Cebrián-Lloret et al., 2022). Therefore, the ash content determined in this study was within the range of those reported in the literature.

Regarding the carbohydrate content, the main polysaccharide in brown seaweed is alginate, representing up to 40% of the dry matter (Łabowska et al., 2019), which was estimated from the sum of GulA and ManA. As expected, the alginate content decreased significantly in the post-extraction residues. Likewise, the algae and residues showed high cellulose, glucose, and fucose content. The high glucose content is mainly associated with cellulose and, as expected, increased in the residues after alginate extraction.

On the other hand, in Table 1, it can be observed that *S. latissima* presented higher protein content compared to *A. nodosum*, which may be ascribed to the fact that they belong to different orders (Laminariales and Fucales, respectively). In this case, the values obtained in this study are within the estimated range, in agreement with previous studies (Fleurence et al., 2018; Schiener et al., 2015; Sharma et al., 2018; Vilg et al., 2015).

It can also be observed that, after alginate extraction, the relative protein content increased considerably in *A. nodosum* and *S. latissima*

residues (27.3 % and 39.4%, respectively). On the contrary, as expected, the mineral content decreased, and the carbohydrate content slightly decreased. The results suggest that the proteins did not undergo alterations during extraction with alginate. In addition, a remarkable removal of minerals was observed throughout the different phases of the extraction process. These findings indicate that the residues from alginate extraction could be an excellent option for protein extraction.

3.2 Protein extraction yield and compositional analysis of the protein extracts

It is well known that algae proteins can be localized intracellularly or cross-linked within macromolecular assemblies to the cell wall via disulfide bonds to polysaccharides within these assemblies (Naseri et al., 2020). Some studies report that an acid treatment prior to alkali can contribute to release protein from polysaccharides or from the cell matrix of the cell wall, thus allowing the solubilization of protein molecules (Kadam et al., 2017).

Therefore, in the first experiment, the effect of chemical extraction (CE) (acid and alkaline extraction) with or without ultrasound was studied to determine the extraction efficiency. The yields obtained with the above extraction method (CE) are shown in Figure S1 of the supplementary material. The percentage of protein recovery ($<$2%) was lower than in the literature. Kadam et al. (2017) reported that the percentage of protein recovery was 59% for *A. nodosum* with chemical extraction. The difference in yields between our study and that reported by Kadam et al. (2017), following a similar procedure, is probably due to the fact that, in our study,

pH precipitation was performed after acid/alkali extraction, implying that the protein was isolated from other compounds, such as polysaccharides, minerals or polyphenols. Likewise, it is possible that protein is being lost in this last step.

In addition, it was observed that the highest protein content remained in the pellet even after chemical extraction. Something similar was reported by Aasen et al. (2022), who reported that the highest amount of protein (79%) remained in the insoluble residue, so, the strategy chosen was to maintain the insoluble fraction, which was rich in protein, as the main product, in order to increase the production of protein-enriched biomass.

Consequently, in the second experiment, an enzymatic treatment was performed with the objective of increasing extraction yields. Therefore, the insoluble residues obtained after the chemical extraction (CE) were subjected to an enzymatic treatment (with cellulases) to degrade the cellulose chains. The enzymes were tested at two different concentrations with or without ultrasounds and then precipitated at acid pH. Figure 1 shows the percentage of protein recovered, where CE represents exclusively the percentage of protein obtained by the acid/alkali treatment, while E20 and E100 indicate the total protein obtained in the enzymatically-treated samples. An increase in the total percentage of recovered protein is highlighted when applying the enzymatic treatment to the solid after chemical extraction, especially at higher concentrations. In this context, a higher yield was obtained and the resulting extract exhibited the highest protein content among all the samples analyzed (Table S1). The effect appears to be more pronounced in the *S. latissima*

residue, possibly due to differences in cell wall architecture that are distinctive for this type of algae (Deniaud-Bouët et al., 2017). The complex structure of this type of algae (*S. latissima*) contributes to the fact that their cell walls are more robust and rigid (Bojorges et al., 2023).

This possibly hindered the extraction of intracellular proteins from the in *S. latissima* residue compared to the *A. nodosum* residue, where protein release was more accessible and could have been lost at earlier stages, such as during alginate extraction. However, it is feasible that the enzymatic treatment was more effective in the *S. latissima* residue due to its higher cellulose content, possibly facilitating the release and recovery of more intracellular proteins. As a result, a higher percentage yield was observed in the *S. latissima* residue. Moreover, in most cases, especially in the *S. latissima* residue, this phenomenon was significantly enhanced by the application of ultrasound treatment, presumably due to bubble cavitation, which favors matrix disruption and facilitates subsequent protein release (Kumar et al., 2021), reaching protein fraction yields of 29% for the *S. latissima* residue and 17% for the *A. nodosum* residue. This indicates that up to 73.6 and 62.3%, respectively, of proteins were recovered from these residues.

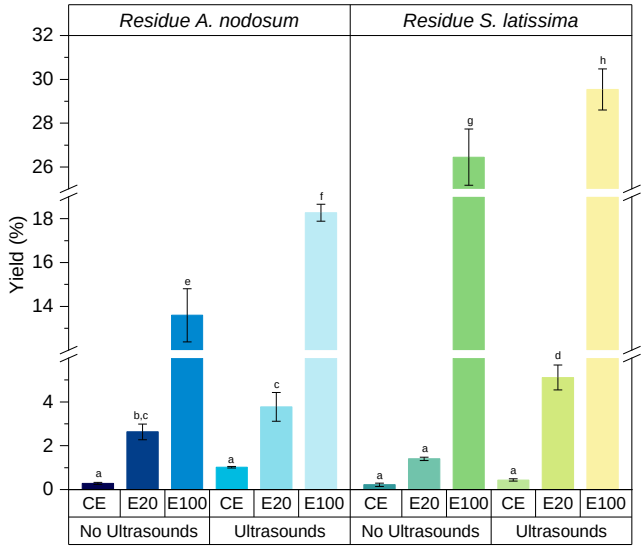


Figure 2. Protein yield extracted from the solid residue after the alginate extraction from *S. latissima* and *A. nodosum*. CE: chemical extraction, E20: Enzymatic treatment at low concentration and E100: Enzymatic treatment at high concentration. A blank was conducted to eliminate the protein contribution from the enzymes.

3.2.1 Amino acid profile of the protein extracts

To analyze the differences between extraction treatments, each protein extract was studied individually to identify and characterize its inherent variations. This detailed analysis is particularly relevant given the crucial importance of proteins in food systems, which exert a remarkable impact on the nutritional quality and functionality of food products. Accordingly, the composition of the essential amino acids (EAA) and the non-essential amino acids (non-EAA) were evaluated to determine the quality of the residue-extracted proteins after the alginate extraction process. The amino acid profile of the protein extracts is presented in Table 2, while the raw material amino acid profile is shown in

Table S2. As observed, the *A. nodosum* residue was characterized by a high proportion of glutamic acid (Glu), with an EAA proportion of 20.1%, while the *S. latissima* residue was mostly composed of Glu and Alanine (Ala), with an EAA proportion of 9.6%. The AA profile of the protein extracts was slightly modified depending on the extraction method. Specifically, the extracts obtained from both residues without the enzymatic treatment were richer in aspartic acid (Asp), Cysteine (Cys), Glu and phenylalanine (Phe), while the relative content of Glu and Ala was comparatively reduced in the extracts obtained with the enzymatic treatment. Interestingly, the percentage of EAAs increased up to 43-50% in the extracts from both residues, with ratios of EAA/non-EAA from 0.76 to 1.01. This range is comparable to that found in various animal sources, such as fish (43.8%), milk (43%), beef (42%), eggs (43.7%), insects (40.4%), as well as in plant sources such as common beans (41.8%) and spinach (44.6%) (Van Der Heijden et al., 2023). These findings suggest that the proteins present in the residues after alginate extraction have promising potential for applications in the food industry due to their interesting AA profiles.

It was observed that Glu and Asp were some of the most abundant AAs in all the samples evaluated, together representing up to 22% of the total AAs. Previous research indicates that the concentration of Glu and Asp in algae can reach up to 40% of the total (Raja et al., 2022). Likewise, the presence of Glu and Asp are prominent in seaweeds and associated with umami flavor, thus playing a crucial role in using seaweeds as flavor enhancers (Fernández-Segovia et al., 2018). In our case, being protein extracts from a by-product, the lower percentage suggests that these AAs persist as recalcitrant in the

algal cell wall, even after the alginate extraction process. These results suggest that protein extracts obtained from residues after alginate extraction possess the potential to be exploited as a source of protein, i.e., as a source of AAs.

Table 2. Amino acid composition of protein extracts from the residues *A. nodosum* and *S. latissima* (% of total amino acid content).

Amino acid (%)[1]	Residue *A. nodosum*						Residue *S. latissima*					
	No ultrasounds			Ultrasounds			No ultrasounds			Ultrasounds		
	CE	E20	E100	CE	E20	E100	CE	E20	E100	CE	E20	E100
Asp	14.3	16.2	13.3	13.8	15.9	13.7	12.6	15.3	13.6	12.4	15.9	13.2
Thr	1.2	3.7	9.5	2.3	3.5	9.3	1.7	3.7	9.4	1.3	3.6	9.1
Ser	2.4	6.4	8.6	3.5	6.0	8.4	2.7	5.9	8.5	2.8	5.7	8.2
Glu	11.6	9.2	9.6	11.2	8.9	9.8	11.4	9.4	9.8	10.0	8.9	9.9
Pro	4.2	4.8	5.9	4.9	4.9	5.9	5.9	5.1	5.9	5.7	4.6	6.0
Gly	7.5	9.1	7.1	7.5	9.1	7.1	6.7	9.5	7.1	7.1	8.4	7.1
Ala	11.1	8.9	6.2	10.3	9.0	6.2	11.0	9.7	6.2	11.8	9.5	6.2
Cys	6.6	3.4	2.8	3.3	3.2	2.8	6.9	1.9	2.8	9.6	3.6	2.6
Val	7.2	4.7	5.2	5.9	4.8	5.1	6.6	4.4	5.0	8.0	4.4	5.1
Met	1.6	1.6	1.5	2.4	1.7	1.5	1.0	1.6	1.5	0.7	1.6	1.5
Ile	5.2	3.6	3.8	4.6	3.9	3.8	4.5	3.1	3.8	3.2	3.1	3.9
Leu	9.9	6.6	6.9	9.3	7.0	7.0	9.9	6.5	7.0	9.0	6.6	7.2
Tyr	4.9	6.5	6.6	4.8	6.7	6.4	5.9	7.0	6.5	5.7	7.6	6.8
Phe	9.2	10.3	4.5	10.4	10.9	4.6	9.9	11.6	4.7	10.1	11.8	4.9
His	0.5	1.2	1.9	1.0	0.8	1.9	0.5	0.8	1.8	0.3	0.9	2.0
Lys	2.5	2.2	3.1	3.4	2.0	3.1	2.7	2.5	3.0	2.3	2.2	3.1
Arg	0.0	1.6	3.5	1.4	1.7	3.3	0.1	1.9	3.1	0.0	1.4	3.3
EAA	48.8	43.8	45.9	47.4	44.5	45.6	49.7	43.1	45.7	50.2	45.5	46.1
Non-EAA	51.2	56.2	54.1	52.6	55.5	54.4	50.3	56.9	54.3	49.8	54.5	53.9
EAA/Non-EAA	0.96	0.78	0.85	0.90	0.80	0.84	0.99	0.76	0.84	1.01	0.99	0.86

[1]The contribution of amino acids present in the enzyme cocktails was subtracted from the blank

3.2.2 Monosaccharide composition of the protein extracts

Unlike other studies, in the present work the composition of the obtained protein extracts was also analyzed concerning the carbohydrates. The objective was to verify the efficacy of precipitation at acid pH and to determine whether protein isolation from carbohydrates had been achieved and assess how protein precipitation also affects the carbohydrate fraction. Carbohydrates were the predominant fraction in all samples, representing a total monosaccharide content of 35-68 % (Table S1, Supplementary Material). Figure 2 shows the monosaccharide composition of the protein extracts. In general, it can be observed that the protein extracts obtained from *S. latissima* resiude presented a higher glucose content, while the ones obtained from *A. nodosum* residues showed a high fucose content. This increase in fucose, together with the glucuronic acid and xylose contribution, could be attributed to recalcitrant fucoidan structures co-precipitated with the protein. In addition, the presence of this compound could be partially responsible of the brownish appearance observed in the protein extracts (Table S1). This dark brown hue points out to the presence of brown marine pigments, such as fucoxanthin, violaxanthin, or chlorophyll a and c, which are trapped in the recalcitrant fucoidan (Saepudin et al., 2018). The presence of glucose CE samples, on the contrary, where only very low yields of protein-rich extracts could be recovered, are richer in alginate monomers, suggesting the presence of minor amounts of recalcitrant alginate recovered as oligomers. Likewise, this observation was confirmed by identifying characteristic bands

associated with proteins and polysaccharides in the FTIR spectra of the protein extracts (Figure S3).

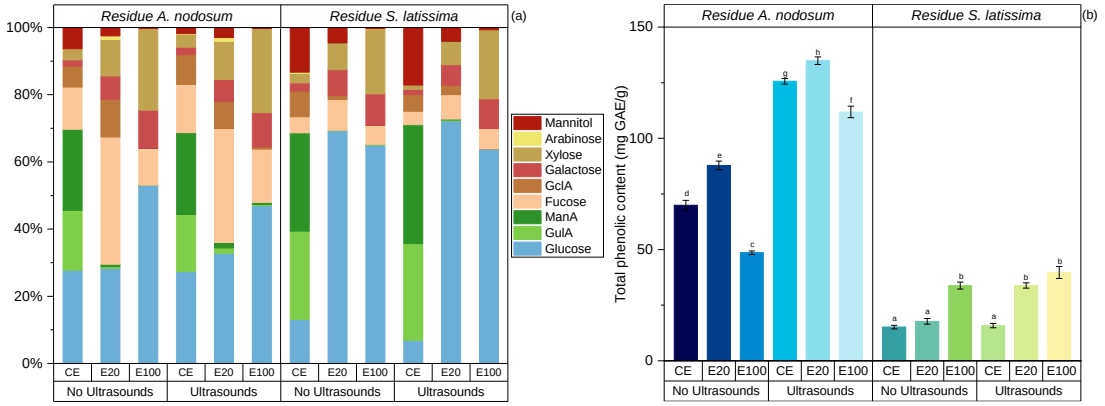


Figure 3. Monosacchride and polifenol content. (a) Relative monosaccharide composition of the protein extracts. The results are expressed as dry weight % from the total carbohydrate content (Table S1). (b) Total phenolic content of the protein extracts.

3.2.3 Polyphenol content

Polyphenols found in brown algae are of particular interest among the various biologically active compounds due to their antioxidant properties. The total phenolic content in the protein extracts was determined by the modified Folin-Ciocalteu method and is included in Figure 3. Higher polyphenol content was observed in the *A. nodosum* residues, specifically in the CE and E20 samples, being even higher when the ultrasound treatment was applied, indicating that polyphenols were co-extracted with the proteins. However, the polyphenol content decreased significantly in *A. nodosum* residues treated with the highest enzyme concentration (E100). An important observation is that,

although the polyphenol content was increased with the enzymatic pre-treatment in *S. latissima* residue, it remains significantly lower than their counterparts obtained from *A. nodosum* residue, and they were not significantly improved using the ultrasound pre-treatment, thus suggesting differences in the wall structure of the two algae residues (Deniaud-Bouët et al., 2017). It has been reported that in most cases, plant proteins contain non-protein residues, such as polyphenols, which is attributed to their propensity to bind to proteins by adsorption (Papadopoulou & Frazier, 2004). Generally, interactions between polyphenols and proteins are predominantly through covalent bonds; however, non-covalent interactions, such as hydrogen, ionic, and hydrophobic bonds, can also occur. However, the latter are usually weaker and reversible (Quan et al., 2019). At the industrial level, covalently-bound protein-polyphenol conjugates are preferred in food applications due to their stronger interactions and high stability (Liu et al., 2017). Among the main properties of protein-polyphenol conjugates is their ability to modify or improve protein functionality, such as gelation, emulsification, or foaming capacity (Lin et al., 2023).

Besides, considering that polyphenols are bioactive compounds widely recognized for their potential benefits to human health (Hoehnel et al., 2022), the presence of these compounds in protein extracts can provide them with "added value." This quality reinforces the use of algal biomass residues obtained after alginate extraction to produce environmentally-sustainable protein in a circular economy.

3.3 Protein relative size distribution

Molecular weight analysis of the protein extracts was performed by HP-SEC. Figure 4 shows the chromatograms of the apparent molecular weight. Three principal populations were identified: the first spanned a range from 350 kDa to 16 kDa, followed by a second population from 16 kDa to 5 kDa, and finally a third from 5 kDa to 1.3 kDa. Interestingly, in the first population, samples subjected to enzymatic treatment (RASC and RSAC) showed a much higher proportion of a broad population with a peak around 100 kDa fraction, providing evidence that the enzymatic treatment effectively released protein-rich fractions and that larger protein conjugates were formed. It is plausible that, the presence of residues of the enzyme used in the process may have also contributed to this fraction in the samples. Likewise, in a similar context, Trigo et al. (2023) reported the extraction of proteins from *S. latissima* with a high molecular weight ($>$ 670 kDa), suggesting the formation of protein aggregates, primarily because it encourages the formation of hydrophobic interactions among proteins. In contrast, samples subjected to acid/alkali treatment exhibited a higher proportion in the low molecular weight range (1-5 kDa). This finding is consistent with observations made on PAGE-SDS gels (Figure S2), where bands in the $<$10 kDa range were evident. These results align with those reported by Kadam et al. (2017), where it was indicated that the highest molecular weight was 3.8 kDa for proteins extracted by acid/alkaline treatment.

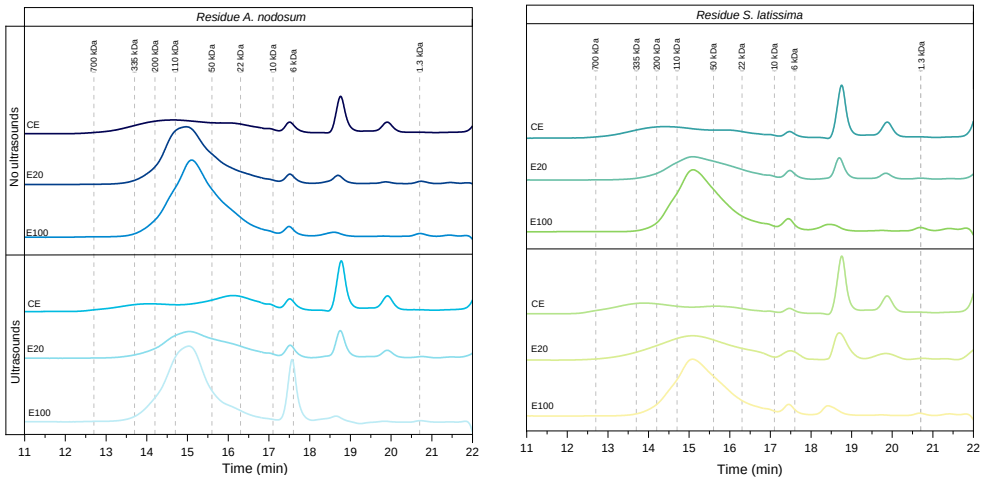


Figure 4. The HP-SEC chromatograms of apparent molecular weights of protein extracts derived from the solid residues generated after alginate extraction

3.4. Functional properties

In industry, proteins are widely used according to their specific functional properties. Differences in the functional properties of protein extracts are usually attributed to their structural properties. Therefore, this study is not only limited to analyzing the physicochemical properties of proteins but also investigates the functional properties of the protein extracts obtained.

3.4.1 Water- (WHC) and oil-holding capacity (OHC) of the protein extracts

Protein interactions with water and oil are relevant to the food industry due to their influence on flavor and texture (Suresh Kumar et al., 2014). Some intrinsic factors of proteins, such as conformation, amino acid composition, or hydrophobicity/polarity, can influence their WHC and OHC (Garcia-Vaquero

et al., 2017). The WHC of the protein extracts is depicted in Figure 5a. The highest were observed for ultrasound-treated CE samples obtained from both residues, being around 5.6 and 4.4 g water/g protein, respectively. Considering that WHC is the ability of proteins to associate with water under limiting conditions, the higher WHC values could be attributed not only to the influence of polar amino acids at the protein-water interface but also to the character of the water binding sites on the protein molecules, as well as to a higher content of total carbohydrates, especially uronic acids, in this type of samples. Furthermore, it has been reported that low molecular weight peptides exhibit a higher WHC than those of high molecular weight (Guo et al., 2022). This effect may be due to the exposure of hydrophilic amino acids on the surface of the smaller peptides, which causes an increase in the concentration of hydrogen ions. Consequently, the additional formation of hydrogen bonds with water increases WHC (Bing et al., 2023). These values were higher than those reported in previous studies, obtained from *K. alvarezii* (2.2 ± 0.1 g water/g), (Suresh Kumar et al., 2014), *E. tubulosa* (1.3 ± 0.1 g water/g), *E. compressa* (1.5 ± 0.1 g water/g protein) and *E. linza* (1.2 ± 0.1 g water/g) (Kandasamy et al., 2012), being even higher than that for soy (3.0 ± 0.7 g water/g) (Foh et al., 2012) or casein concentrates (2.5 ± 0.1 g water/g)(Chandi & Sogi, 2007).

High WHC values help preserve the freshness and moist mouthfeel of baked foods and decrease moisture loss in packaged bakery products (Suresh Kumar et al., 2014). Likewise, high WHC values are desirable in food products with high viscosity, such as custards, doughs, sausages, and baked goods, since they contribute to water retention without involving protein

dissolution, providing thickening, body, and viscosity (Garcia-Vaquero et al., 2017; Kandasamy et al., 2012).

On the other hand, oil holding capacity (OHC) is another property of relevance in the food industry, especially for food ingredients in formulated foods. The OHC values of the protein extracts are exhibited in Figure 5b. It can be observed that the highest values were for CE and E20 samples obtained with ultrasound for both residues, with a mean of 4.3 g oil/g protein for CE and E20 of *A. nodosum* residue, and as a mean 6.0 g oil/g protein for CE and E20 of *S. latissima* residue. It is hypothesized that the application of ultrasound increased exposure of hydrophobic groups, promoting protein denaturation and increasing OHC. Although a similar content of hydrophilic and hydrophobic amino acids was recorded in the samples (around 44-55%), the improvement in OHC could be attributed to the specific structure and conformation of the protein, as well as its molecular weight (Zhang et al., 2022). A broader molecular weight distribution (from $<$100 kDa to $>$ 1 kDa) was associated with increased OHC value. This finding aligns with that reported by Yolandani et al. (2023) in soy protein isolates.

Nevertheless, the results obtained were high compared to those reported for *K. alvarezzii* protein (1.3 $\pm$ 0.2 g oil/g) (Suresh Kumar et al., 2014), *E. compressa* (1.3 $\pm$ 0.1 g oil/g), *E. tubulosa* (1.1 $\pm$ 0.04 g oil/g), *E. linza* (1.1 $\pm$ 0.1 g oil/g) (Kandasamy et al., 2012), and similar to Kapparazii powder™ (5.1 $\pm$ 0.4 g oil/g) (Sjamsiah et al., 2014) and to rice bran protein concentrates (3.7-8.2 g oil/g) (Chandi & Sogi, 2007). Protein needs to have fat-binding capacity, so OHC is a critical parameter in flavor retention. Hence,

a high OHC value is necessary to formulate foods, such as sausages, salad dressings, cake mixes, and mayonnaise (Chandi & Sogi, 2007).

The protein extracts obtained in this study were shown to have water and oil retention capacity, which makes them valuable and ideal for various food applications, as well as texture and palatability enhancers, especially in viscous formulations.

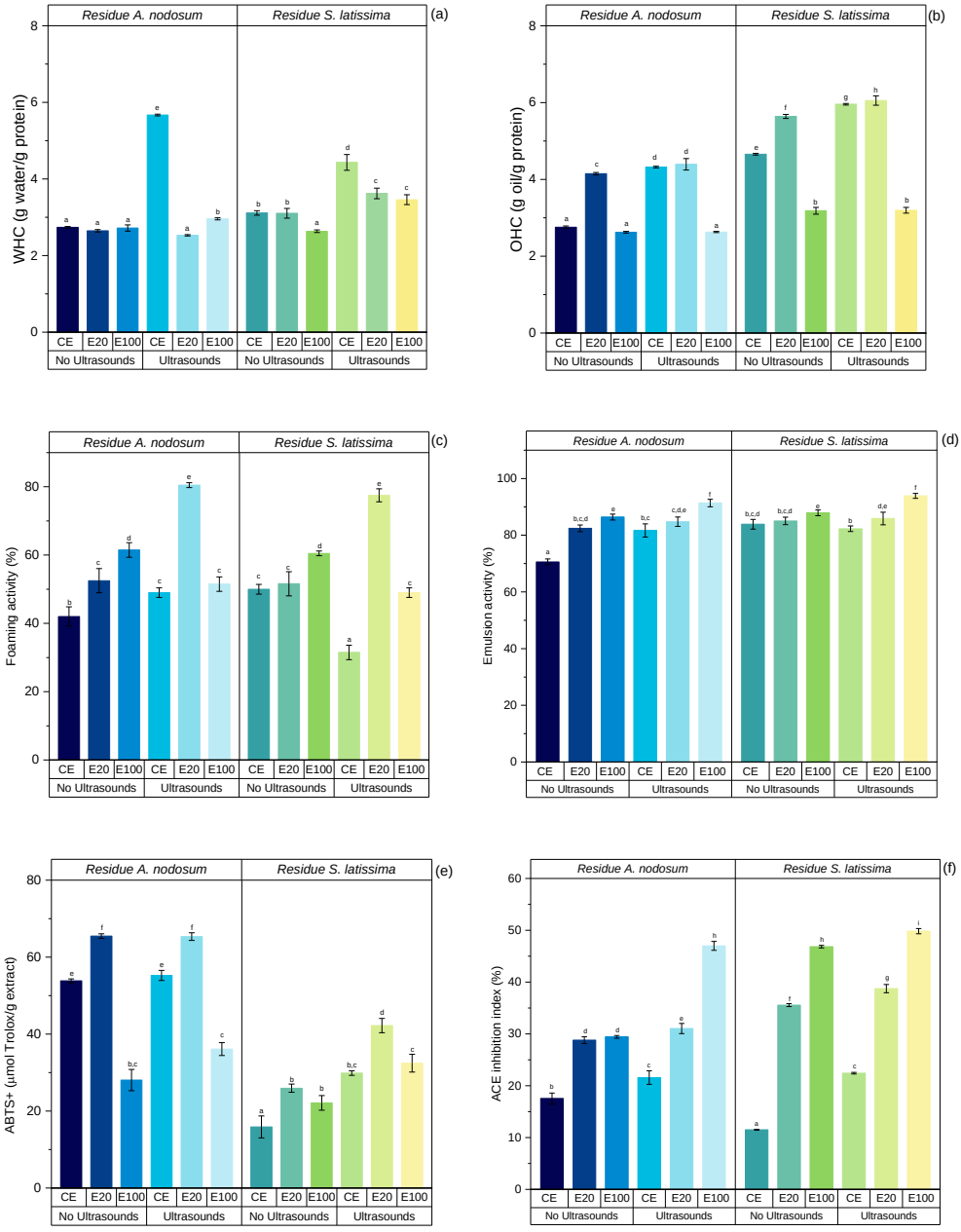


Figure 5. Functional and bioactive properties of the protein extracts from the *A. nodosum* and the *S. latissima* residues. (a) Water holding capacity and (b) oil holding capacity, (c) foaming and (d) emulsion capacity. (e) Antioxidant activity and (f) ACE inhibitory activity.

3.4.2 Foaming capacity

Foaming capacity is an interesting functionality of proteins in food processing; they improve lightness and softness, and facilitate flavor volatilization, improving food palatability (Garcia-Vaquero et al., 2017). Amphipathic molecules of proteins promote migration and adsorption at the air/water interface, unfolding to form droplets and reorganizing at the interface (Bergfreund et al., 2021). Besides, due to the interfacial properties of foam, protein is widely used as a foaming agent in foods such as mousse or bavarois, cakes, whipped cream, etc. (Qi et al., 2023).

In addition, it is well known that proteins tend to have higher foaming capacity at pHs close to neutrality. This could be because there are higher net charges on the protein, causing weakening of hydrophobic interactions and increasing protein flexibility, leading to faster protein diffusion at the air/water interface and encapsulation of air particles, thus enhancing foaming (Garcia-Vaquero et al., 2017). Therefore, the samples were stabilized and measured at pH 7 and compared with those reported in the literature at a similar pH (close to neutral). Figure 5c shows the foaming capacity of the protein extracts. The CE samples had the lower foaming capacity (31.5-49%) as compared to their enzymatically-treated counterparts, regardless of whether they were or not ultrasonicated. The samples with the highest foaming capacity were those enzymatically-treated, especially the E20 samples with ultrasound (52.5-80.5%). This could be ascribed to the fact that the combination of ultrasound and enzyme treatment could induce proteins hydrolysis, precipitating a high percentage of shorter peptides and increseased

adsorption at the air/water interface. Furthermore, protein extracts of *A. nodosum* and *S. latissima* residues had a higher foaming capacity than protein concentrates of *K. alvarezii* (38 ± 2%) (Suresh Kumar et al., 2014), *E. tubulosa* (45 ± 2.0%), *E. compressa* (40.9 ± 2.9%), *E. linza* (15.6 ± 0.9%) (Kandasamy et al., 2012), *H. elongata* (64.4 ± 2.22%) (Garcia-Vaquero et al., 2017). In general, the protein extracts exhibited considerable foaming capacity and foam stability (Figure S3) and could, therefore, be considered for developing food formulations.

3.4.4 Emulsifying capacity of protein extracts and their stability

The ability of proteins to form emulsions is one of the main functional properties for food formulation, such as dairy beverages and meat analogs (Garcia-Vaquero et al., 2017). The emulsification activity of protein extracts of *A. nodosum* and *S. latissima* residues was determined, and the results are shown in Figure 5d. The maximum emulsification rates were observed in samples E100 with ultrasonication for *S. latissima* and *A. nodosum* residues (91.8 ± 0.8 %, 91.3 ± 1.3 %, respectively), while CE samples with or without ultrasonication showed the lowest emulsification rate (between 70.5 ± 1.0 % and 82.3 ± 1.0 %). This can be explained by the relationship between the emulsifying activity and the dependence on the protein concentration and its adsorption kinetics. Thus, at low protein concentrations, the diffusion process plays a crucial role in controlling protein adsorption at the oil-water interface (Agyare et al., 2009).

On the other hand, those peptides with higher hydrophilicity or higher molecular weight can favorably contribute to emulsification properties by diffusing rapidly and adsorbing at the interface (Małecki et al., 2021).

For example, E100 samples, with or without ultrasound from both residues, showed a higher content of hydrophilic amino acids than the other protein extracts. Among these amino acids, Asp, Thr, Ser, His, and Arg stand out due to their higher individual presence in these samples compared to the others. This increase in the proportion of hydrophilic amino acids was accompanied by a higher relative molecular weight fraction, around 100 kDa. This may have positively favored the emulsification properties. In addition, the high emulsifying capacity could be linked to the denaturation and partial unfolding of proteins during the extraction process, providing rapid adsorption of the globular head of the hydrophobic protein with the non-polar lipid phase, thus contributing to the hydrophilic-lipophilic equilibrium (Abdollahi & Undeland, 2018). However, the emulsion stability was affected by time (Figure S3).

3.5 Bioactive properties

3.5.1 Antioxidant properties of the protein extracts

Figure 5e also compares the antioxidant capacity of the protein extracts obtained from both species. Interestingly, extracts from the *A. nodosum* residue exhibited the highest antioxidant activity, especially CE and E20. This can be ascribed to the higher sulfate content in these samples (Table S1l), which could indicate the presence of very low molecular weight sulfate

compounds such as fucoidans (Chen et al., 2021). These sulfated polysaccharides possess antioxidant potential, which increases in direct proportion to the sulfate content (Chen et al., 2021). According to Wang et al. (2008), there is a positive correlation between sulfate content and antioxidant activity (superoxide radical scavenging capacity), as well as the sulfate/fucose content as an indicator of metal ion chelation and hydroxyl radical scavenging.

Besides, it can be seen that the E20 samples (with or without ultrasound) showed the highest antioxidant activity, most likely because the enzymatic treatment effectively altered the cell wall of the *A. nodosum* residue, facilitating the joint release of protein and certain sulfated compounds such as fucoidan highly recalcitrant. This enhanced antioxidant activity can also be explained by the presence of amino acids with or aromatic side groups, such as Trp, Tyr, and Phe or sulfur-rich nucleophilic side groups, such as Met and Cys, (Steinbruch et al., 2023). Indeed, several authors have documented the presence of antioxidant capacity in macroalgae, including macroalgae extracts, is not only attributed to the presence of polyphenols but also to bioactive proteins, peptides, and free amino acids, which are considered influential factors (Kazir et al., 2019). Therefore, the non-protein fraction, such as sulfated polysaccharides and polyphenols found in these protein-rich extracts, could significantly contribute to the antioxidant activity.

3.5.2 Inhibition of angiotensin-converting enzyme and its inhibitory mechanism (ACE)

Hypertension is one of the leading causes of cardiovascular disease. Angiotensin-converting enzyme (ACE) inhibition is a crucial strategy to control and prevent hypertension, reducing risks such as stroke and myocardial infarction (Mousaie et al., 2022).

The study evaluated the ACE inhibitory activity of protein extracts obtained from RASC and *S. latissima* residue, as detailed in Figure 5f. The results indicate that the extraction method significantly influenced the level of ACE inhibition. Protein extracts that underwent a combination of ultrasound and enzymatic extraction exhibited a high level of ACE inhibitory activity. It was observed that the E100 protein extracts of *A. nodosum* and *S. latissima* residues showed maximum ACE inhibition, reaching 49.8 and 47%.

Some studies suggest that antihypertensive capacity is related to the total amount of protein present (Kim et al., 2024; Paiva et al., 2017). Pearson's correlation analysis revealed a highly significant correlation between antihypertensive activity and protein content, as detailed in Table S3.

On the other hand, the results obtained were higher than the reported value for the protein extract of *M. pyrifera* (38.8%), obtained through the action of α-amylases (Vásquez et al., 2019). Furthermore, in another study by Olivares-Molina & Fernández (2016), it was reported that extracts of *L. nigrescens* and *M. pyrifera*, obtained with cellulase, presented

an inhibitory activity of 45 and 8.4%, respectively. Meanwhile, Cian et al. (2015) indicated that the ACE inhibitory activity of hydrolyzed protein extracted from *P. columbina* reached 12.4%. In contrast, Mousaie et al. (2022) reported that the ACE inhibitory activity of hydrolyzed protein from *S. ilicifolium* was approximately 55%. Thus, it is evident that bioactive peptides from marine algae have a great capacity to inhibit ACE.

4.5 Principal Component Analysis

To better understand the influence of the extraction method, composition, and functional capacity of the protein extracts, a principal component analysis (PCA) was carried out (Figure 6). The two principal components explained 72% of the total variability, with the most significant principal component (PC-1) explaining 47% of the total variation.

Figure 6 shows an apparent clustering of protein extracts according to the extraction method. Specifically, low-concentration enzymatic extraction (E20) samples are predominantly found in the positive quadrant. In contrast, E100 samples are in the lower left quadrant, and CE samples, extracted by acid/alkali methods, are in the lower right quadrant. This arrangement highlights the remarkable differences between the extraction methods, which are reflected in the functional and bioactive properties of the protein extracts.

It can be observed how the variables related to antioxidant capacity (ABTS and polyphenol content) presented positive loads close to the samples obtained from E20 of *A. nodosum* residue, which highlights the notable difference with the E20 of *S. latissima* residue (located parallel to *A. nodosum*

residue), probably due to the presence of recalcitrant sulfated compounds such as fucoidan. In addition, significant proximity was observed between the amino acids Asp, Gly, and Phe and the E20 protein extracts, suggesting a higher content of these amino acids in this type of sample. Interestingly, the water holding capacity variable also showed a positive correlation, close to antioxidant capacity and Phe, a hydrophobic amino acid.

Likewise, it was observed that the yield and enzyme treatment variables showed negative loadings and proximity to the E100 samples of *A. nodosum* and *S. latissima* residues, indicating a possible positive correlation between yield and enzyme treatment. In addition, similar patterns were identified for other variables, such as ACE, total protein content, and emulsion, which showed negative loadings on the X-axis and positive loadings on the Y-axis. This finding suggests an association between protein content and its bioactive activity, demonstrated by ACE, and the remarkable proximity of specific amino acids, such as Ser, Arg, Thr, His, and Tyr, with ACE and emulsion variables. Although the relationship between peptide structure and ACE inhibitory activity has yet to be thoroughly explored, it is suggested that the inhibitory potential could be closely linked to the structural and compositional characteristics of the peptides. Finally, the proximity of specific amino acids, such as Cys, Ile, Val, Leu, and Glu, to the non-ultrasound CE protein extracts suggests a possible influence of the non-ultrasound extraction process. This method probably led to less denaturation and more effective preservation of the protein structure, particularly concerning these specific amino acids.

Therefore, this analysis provided insight into the relationship between extraction methods and functional properties, offering a more profound insight into the composition and functionality of the extracts. This valuable information enriches the existing knowledge base and is essential for guiding future research and practical applications.

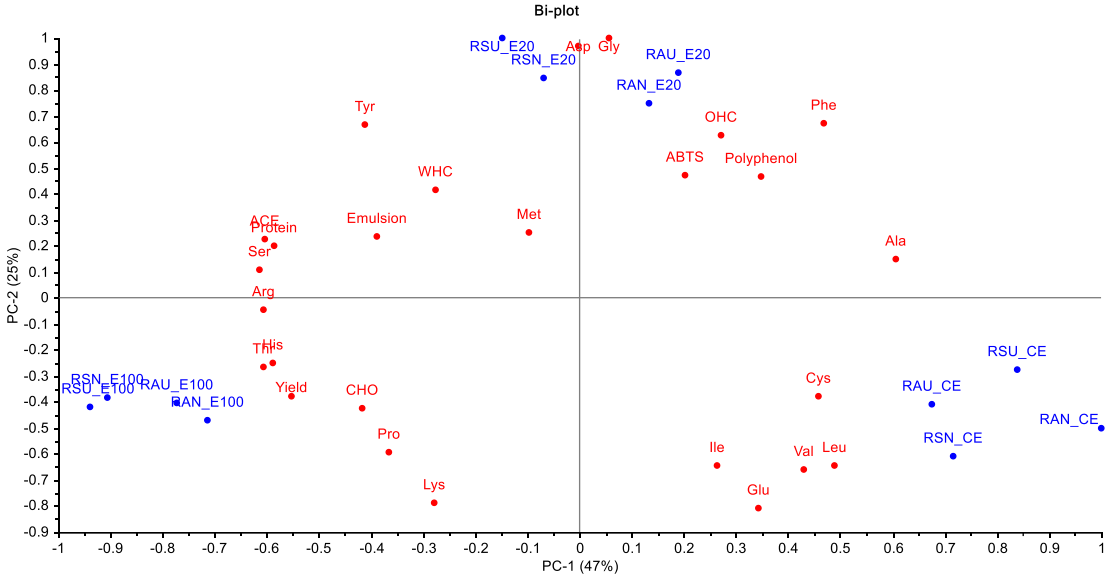


Figure 6. Principal components analysis (PCA) for the different protein extracts (RA: Residue of *A. nodosum*; RS: Residue of *S. latissima*), depending on the treatment (N: without ultrasounds; U: with ultrasounds; CE: chemial extraction; E20 and E100: enzymatic-assisted extraction), composition (P: protein content, yield and CHO: carbohydrate content), antioxidant activities (ABTS and polyphenol content), functional properties (WHC and OHC: water and oil holding capacity, respectively; emulsion capacity) and anti-hypertensive activity (ACE).

5. Conclusions

This study provides information on the sequential extraction of various technologies for maximized intracellular protein recovery. The findings reveal that protein extracts possess diverse functional and bioactive properties, which open the door to using them in food or nutraceutical systems that demand specific functional properties in their formulation. In economic terms, the proposed feasibility could be improved by integrating it as a secondary recovery after alginate extraction. Thus, this process could generate a higher net benefit compared to the costs inherent to the process. Furthermore, this approach could be extrapolated to other algal biomasses, increasing their value as raw biomass for recovering industrially valuable compounds.

Acknowledgements

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7. Supplementary Material

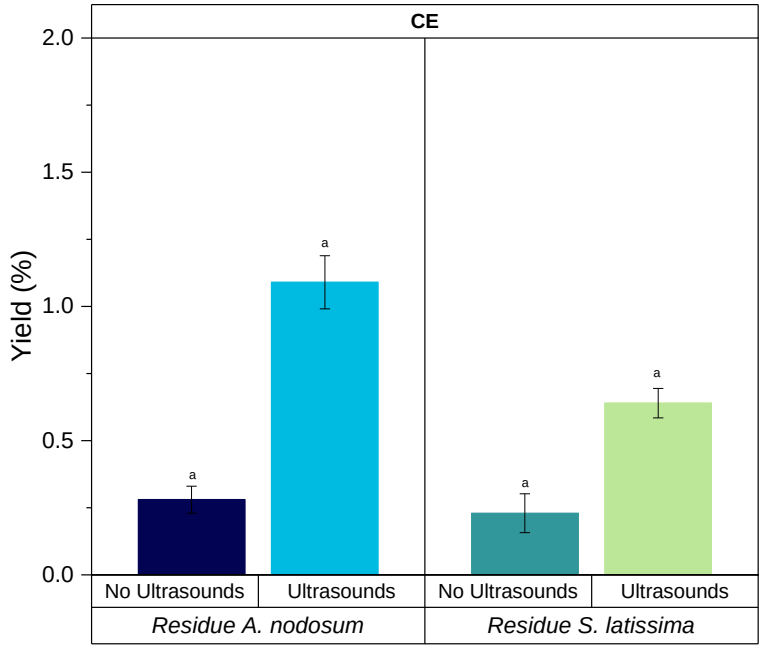


Fig S1. Protein yield extracted from the chemical extraction (CE) of *S. latissima and A. nodosum* residues.

Table S1. Characterization of protein extracts in terms of chemical composition and color properties

			Composition				Color properties			
Residue	Sample		Carbohydrate[1] (%)	Ash (%)	Sulfate (%)	Protein[2] (%)	L*	C*	h°	Example color
A. nodosum	No ultrasounds	CE	66.7 ± 1.6^{g}	$24.1 \pm 1.4^{d,e}$	$2.8 \pm 0.0^{d,e}$	3.6 ± 0.5^{a}	77.9 ± 0.0^{h}	10.7 ± 0.0^{f}	81.1 ± 0.0^{g}	
		E20	48.8 ± 1.6^{e}	$20.4 \pm 1.8^{a,b,c}$	$2.9 \pm 0.0^{e,f}$	26.3 ± 0.9^{c}	64.5 ± 0.1^{a}	12.4 ± 0.0^{h}	64.9 ± 0.1^{c}	
		E100	$32.8 \pm 1.9^{a,b,c}$	$21.9 \pm 1.7^{c,d}$	2.5 ± 0.0^{d}	$40.6 \pm 1.0^{e,f}$	66.2 ± 0.0^{c}	17.5 ± 0.0^{j}	63.3 ± 0.0^{b}	
	Ultrasounds	CE	58.8 ± 2.7^{f}	26.3 ± 1.9^{e}	3.4 ± 0.0^{g}	7.6 ± 0.8^{b}	78.2 ± 0.0^{i}	12.1 ± 0.0^{g}	81.6 ± 0.0^{h}	
		E20	43.1 ± 1.5^{d}	$18.6 \pm 1.0^{a,b}$	4.1 ± 0.3^{h}	31.2 ± 2.0^{d}	69.8 ± 0.2^{d}	10.3 ± 0.0^{e}	68.8 ± 0.0^{e}	
		E100	30.9 ± 2.0^{a}	$20.8 \pm 1.5^{a,b,c}$	$3.1 \pm 0.0^{f,g}$	39.5 ± 0.9^{e}	65.6 ± 0.0^{b}	17.2 ± 0.0^{i}	63.0 ± 0.0^{a}	
S. latissima	No ultrasounds	CE	61.2 ± 1.0^{f}	25.6 ± 1.1^{e}	$1.4 \pm 0.1^{b,c}$	4.0 ± 0.8^{a}	77.0 ± 0.1^{g}	9.6 ± 0.0^{c}	86.2 ± 0.0^{k}	
		E20	$34.6 \pm 1.9^{b,c}$	$18.7 \pm 1.5^{a,b,c}$	0.4 ± 0.1^{a}	$42.6 \pm 0.9^{f,g}$	82.6 ± 0.1^{l}	9.3 ± 0.0^{a}	83.2 ± 0.1^{j}	
		E100	$32.2 \pm 1.4^{a,b}$	$21.3 \pm 2.0^{b,c,d}$	1.1 ± 0.2^{b}	43.5 ± 0.8^{g}	70.1 ± 0.1^{e}	18.6 ± 0.0^{k}	68.3 ± 0.1^{d}	
	Ultrasounds	CE	68.6 ± 2.1^{g}	$24.3 \pm 1.2^{d,e}$	1.5 ± 0.3^{c}	3.6 ± 0.4^{a}	79.8 ± 0.0^{j}	9.8 ± 0.0^{d}	89.1 ± 0.0^{l}	
		E20	$32.8 \pm 2.3^{a,b,c}$	17.7 ± 0.5^{a}	0.6 ± 0.1^{a}	39.5 ± 1.2^{e}	80.5 ± 0.0^{k}	9.4 ± 0.0^{b}	82.9 ± 0.0^{i}	
		E100	35.9 ± 1.6^{c}	$19.5 \pm 1.1^{a,b,c}$	$1.2 \pm 0.0^{b,c}$	47.6 ± 0.9^{h}	71.0 ± 0.0^{f}	18.9 ± 0.0^{l}	69.3 ± 0.0^{f}	

The values report means (n=3) ± standard deviation. Different letters in the same column indicate significant differences between the samples ($p<0.05$).
[1]Total carbohydrate content calculated as the sum of all monosaccharides detected by HPAEC-PAD
[2]The contribution of the protein present in the enzyme cocktails was subtracted from the blank

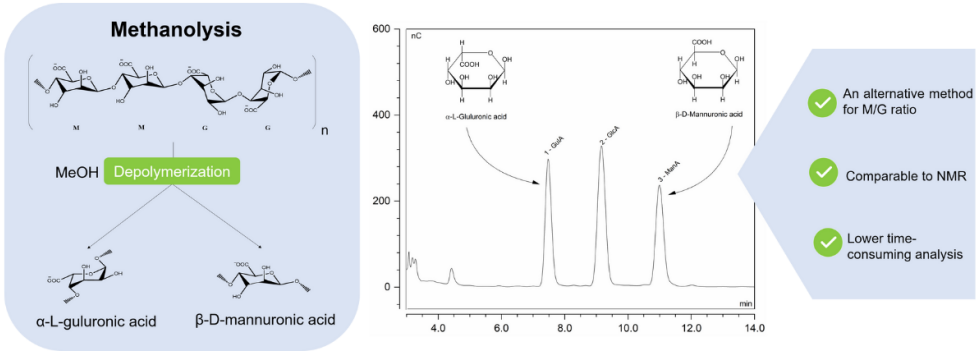
Chapter 3

Challenges in structural elucidation for knowledge generation

3.1 Estimation of alginate purity and M/G ratio by methanolysis coupled with anion exchange chromatography

3.1

Estimation of alginate purity and M/G ratio by methanolysis coupled with anion exchange chromatography



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

Bojorges, H., López-Rubio, A., Fabra, M. J., & Martínez-Abad, A. (2023). Estimation of alginate purity and M/G ratio by methanolysis coupled with anion exchange chromatography. Carbohydrate Polymers, 321, 121285. https://doi.org/10.1016/j.carbpol.2023.121285

ABSTRACT

Alginates are industrially relevant polysaccharides widely used in the food and biomedical industries for their excellent gelling properties. A growing focus towards the valorisation of the marine resources has evidenced the lack of straightforward methods for the rapid determination of both the alginate content and M/G ratio. The growing emphasis on the valorization of marine resources has revealed the need for more streamlined methods for the determination of both alginate content and the M/G ratio. This study describes the application of acid methanolysis and separation by anion exchange chromatography. Five samples, including alginates extracted from Saccharina latissima, Ascophyllum. nodosum, a certified standard, and two poly-uronates (Poly-M and Poly-G), were analysed for their M/G ratio and alginate content at different treatment conditions, and compared with other conventionally used or reference methods (NMR, FTIR, and colorimetric methods). Methanolysis conditions already used for uronic-rich plant polysaccharides (100°C, 5 h or 80°C, 16 h) yielded the highest alginate recovery for high molecular weight alginates, while less aggressive conditions (3-4 h at 100°C) were more favourable for low molecular weight samples. Quantitative estimation of alginate was reproducible and relatively accurate, as compared with other colorimetric methods, while M/G ratios were not significantly different from those with the reference method (^{1}H-NMR). The results evidence that methanolysis may be applied as a potential alternative to existing NMR or colorimetric methods for the purity and M/G ratio estimation of alginate-rich samples.

1. Introduction

Alginates are industrially relevant polysaccharides extracted from brown seaweed (Phaeophyceae) and widely used as thickeners, stabilisers and gelling agents in the food, pharma, or cosmetic industries. Alginates can be described as copolymers of (1– 4) linked β-D-mannuronic acid (ManA or M) and α-L-guluronic acid (GulA or G). These polysaccharides are typically described by their M/G ratio, molecular weight and distribution of M and G units, as M, MG or G blocks along the chain, as these parameters highly determine the biological and physical properties of alginate (Haug et al., 1967). The alginate contents, as well as its extractability or recalcitrance within the cell wall architecture, its molecular weight, and sequential structure, all greatly vary, depending on the brown algae species, season, growing stage, and geographic location (Haug et al., 1974; Indergaard et al., 1990). A growing focus towards a "blue bioeconomy" and the valorisation of ocean resources has prompted research and industrial efforts to deeper study the composition and potential applications of brown algae, evidencing the lack of straightforward methods for the rapid determination of both alginate content and M/G ratio.

The estimation of alginate content is usually based on colorimetric methods, among which many different possibilities have been proposed and modified through many years and are still used by different authors. These methods mostly involve the formation of a coloured complex in the presence of uronic acids with reagents such as resorcinol, carbazole, m-hydroxydiphenyl, etc. (Kumar & Kumar, 2017). The most widely used chemometric method for the quantitative determination of uronic acids is the

method developed by Blumenkrantz & Asboe-Hansen (1973). Although very suitable for pectin or other plant samples, the original method was not tested or developed for its application on alginates and may present certain limitations. A further modification of this method, involving the addition of sulfamate to m-hydroxydiphenyl, was described by Tullia, Fillissetti-Cozzi and Carpita, which aimed at reducing interference from neutral sugars, and was applied to both polygalacturonates as well as to alginic acid (Filisetti-Cozzi & Carpita, 1991). There is, however, no generally accepted or reference method for alginate analysis.

The reference method for the determination of the M/G ratio is liquid-state nuclear magnetic resonance (NMR) spectroscopy, based on extensive work performed in the late 70s and early 80s (ASTM F2259-10, 2012; Grasdalen, 1983; Grasdalen et al., 1979). NMR not only gives an estimate of the overall M/G ratio, but also information about the block distribution, which also plays a crucial role in the rheological behaviour. This analysis is, however, complex, due to the need of a pre-hydrolysis step to enhance solubility and reduce viscosity. NMR must also be done at increased temperature (80-90°C) for resolved peaks allowing integration.

In this approach, another technique is Solid-state ^{13}C NMR as a non-destructive method to determine the alginate's M/G ratio. However, this technique could exhibit lower signal sensitivity than liquid-state NMR, making it difficult to detect and quantify monomers accurately and obtain information about M- and G-blocks (Ishii et al., 2001; Salomonsen et al., 2009; Shimba et al., 2004). In addition, using this technique often requires establishing a

considerably large calibration model or prior knowledge of the M/G ratio in the calibration samples (Salomonsen et al., 2009). Therefore, it implies the need to use advanced analysis and processing techniques to calculate the M/G ratio, which can be complex and require specialized expertise, increasing analysis time and driving this technique less attractive in terms of efficiency.

Owed to the industrial need of estimating M/G ratio in a rapid and non-destructive manner, vibrational spectroscopy, e.g. infrared (IR), Raman and near infrared (NIR) spectroscopy have also been optimized in combination with chemometrics and proven useful for quantitative estimation of the M/G ratio of alginates (Jensen et al., 2015; Salomonsen et al., 2008). These approaches are, however, designed for relatively pure alginate samples, not giving a quantitative estimation of alginate content, and also other compounds might overlap with the signals of interest.

Other techniques explored for determining alginate's M/G ratio are circular dichroism (CD). However, CD has some disadvantages, such as lower sensitivity compared to other analytical methods, such as NMR, which could affect obtaining high-quality spectra and will depend on the purity and homogeneity of the alginate sample (Morris et al., 1980). Likewise, interpreting CD spectra can be challenging, especially when dealing with mixtures of different monomer compositions or conformational variations in the alginate structure (Donati et al., 2003, 2005).

Chromatographic methods allowing separation of the different monomeric units for their quantification would, in principle, allow for direct estimation of both alginate content and M/G ratio, regardless of the purity or

the presence of other compounds. The hydrolysis step to transform the polysaccharide into their monomers is challenging, because of the acid lability of alginate monomers, prone to degradation. Previous studies on alginate samples using trifluoracetic acid (TFA) or formic acid, for example, have evidenced the narrow balance between quantitative scission of all glycosidic bonds and degradation of the monomeric units (Chandía et al., 2004; Lu et al., 2015). Methanolysis is performed in the absence of water with anhydrous acidic methanol and has been applied to plant hemicelluloses presenting significant quantities of uronic acids, such as glucuronoxylans, as these conditions result in higher selectivity in the recovery of the uronic acids compared to TFA or sulphuric acid (Saeman) hydrolysis (Bertaud et al., 2002; Willför et al., 2009). All this raised a hypothesis about whether methanolysis could be an alternative method for carbohydrate analysis in alginate-rich samples and provide results comparable to those of current NMR or chemometric methods. Therefore, this study aimed at exploring the application of acid methanolysis followed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for the quantitative determination of the carbohydrate composition, alginate purity, and M/G ratio of alginate-rich samples. Different methanolysis conditions were applied based on previous works on plant polysaccharides and the results are critically discussed and compared to colorimetric methods, IR spectroscopy, or NMR.

2. Materials and methods

2.1. Alginate samples used in the study

Five alginate-rich samples were used in this study. A certified reference sodium alginate (Ref. PHR1471; Lot#: LRAB5524; referred to as REF) with a reported purity of 87.6 ± 0.2 % was purchased from Merck. Poly-guluronic acid (Poly-G) and poly-mannuronic acid (Poly-M) were supplied by Carbosynth, Biosynth (UK). Furthermore, two alginate samples were produced in the lab from the brown seaweeds Ascophyllum nodosum (ASC) and Saccharina latissima (SAC). Both seaweeds were harvested in February 2017 and September 2018, respectively, and kindly supplied as a dry powder by Porto-Muiños S.L. (Cerceda, A Coruña, Spain). The algae were submitted to the acid-alkaline treatment conventionally used to extract sodium alginate at industrial scale as described in Fig S1 of the suppl. material. D_2O (≥99.9 atom% D) were purchased from Sigma-Aldrich (Canada), TTHA (Triethylenetetramine-N,N,N′,N″,N‴,N‴-hexaacetic acid, T7633, Sigma, Japan), and 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium (TSP-d4, ≥98 atom% D).

2.2. Proximal composition

The total nitrogen content of the samples was measured using an Elemental Analyzer Rapid N Exceed (Paralab S.L., Spain). Around 250 mg of alginate samples were compressed into pellets and subjected to analysis using the Dumas method, which involves combusting the sample and detecting the resulting release of N_2 gas (Wiles et al., 1998). The total protein content was

calculated by multiplying a factor of 5 (Angell et al., 2016). These analyses were performed in triplicate.

The ash content was estimated by calcination in a muffle furnace, following the standard method (AOAC, 1990). Approximately 3 g of freeze-dried alginate and alginate-based fractions powder were calcined at 500 ºC.

The lipid content of the samples was quantified through a gravimetric analysis using the Soxhlet method (Method 920.39; AOAC, 1990). All data were expressed as a percentage of dry weight (DW).

2.3. Methanolysis and HPAEC-PAD analysis

The carbohydrate composition, including neutral sugars and uronic acids of the alginates and poly-uronates was determined after acid methanolysis and separation by HPAEC-PAD. Freeze-dried samples (2 mg) were incubated with 1 mL of 2 M hydrochloric acid (HCl) in dry methanol at different times at 80 °C or 100 °C. The samples were neutralized with pyridine, dried under a stream of air, and further hydrolysed with 2 M trifluoroacetic acid (TFA) at 120 °C for 40 min. The samples were again dried under a stream of air, re-dissolved in ultrapure water, and filtered (0.2 μm). The monosaccharides were analysed using HPAEC-PAD with an ICS-6000 system (Dionex) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex). Neutral monosaccharides were separated at 30 °C isocratically with 8 mM NaOH for 15 min after a conditioning step before each run of 7 min with 250 mM NaOH and 70 mM sodium acetate (NaAc) and 6 min equilibration time before injection. Uronic acids were separated at 30 °C with 30 mM NaOH and a

100-180 mM NaAc gradient over 20 min. The eluents were filtered and degassed with helium and kept under helium pressure during the analysis. Control samples of known concentrations of mixtures of fucose (Fuc), rhamnose (Rha), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man), galacturonic acid (GalA), glucuronic acid (GlcA), all purchased at Merck, as well as mannuronic acid (ManA) and guluronic acid (GulA), purchased at Carbosynth, Biosynth (UK), were used for calibration. A typical chromatogram of uronic acids from an alginate sample is shown in Figure S2. The M/G ratio was calculated by dividing the mannuronic acid concentration between the guluronic acid concentration. All determinations were carried out at least in triplicate.

2.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR analysis was carried out using a modified version of the method described by Jensen et al. (2015). ^{1}H liquid-state NMR measurements were performed on a Bruker AV400 spectrometer operating at a frequency of 400 MHz for ^{1}H nuclei using a BBI ATM 1H/BB inverse multinuclear probe (5 mm). Prior to analysis, the samples were depolymerized by a mild partial hydrolysis. A 0.1 % (w/v) sodium alginate solution and the pH was adjusted to 5.6 using 0.1M HCl and heated at 100 ºC for 1h. After cooling to room temperature, the pH was re-adjusted to 3.8 with 0.1M HCl and incubated at 100 ºC for 30 min. Subsequently, samples were neutralized (pH 7) with 0.1 M NaOH and freeze-dried overnight. The sample was dissolved in 5 mL of D_2O (99.9 %) and freeze-dried again in other to minimize the presence of non-deuterated water. 10 mg of the sample was then dissolved in 1 mL of D_2O

(99.9 %) and 700 µL of the solution was added into NMR tube along with 20 µL of TTHA solution. The TTHA was utilized as a chelating agent to prevent traces of divalent cations from interacting with the alginates. The NMR spectra were recorded at 80 ºC with a 30º pulse angle, relaxation delay 2 s, and acquisition time of 4.096 s. 128 scans were accumulated for signal averaging. The chemical shifts of the anomeric proton signals were A (guluronic acid anomeric proton) at around 5.07 ppm; B1 (H-5 proton of the central guluronic acid residue in a GGM triad) at 4.77 ppm; B2 (H-5 proton of the central guluronic acid residue in a MGM triad) at 4.74 ppm; B3 (anomeric proton of the mannuronic acid residue neighboring a mannuronic acid) at 4.71 ppm; B4 (anomeric proton of the mannuronic acid residue neighboring a guluronic acid) at 4.68 ppm and C (guluronic acid proton 5) at 4.49 ppm. The M/G ratio, and diads and triads of uronic blocks were determined according to Jensen et al. (2015) and the equations are described in the supplementary material. The obtained spectra were processed using Mnova NMR software (Mestrelab Research, Spain).

2.5. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra were collected on a Cary 630 FTIR spectrometer (Agilent, USA) using the attenuated total reflectance (ATR) sampling method. The IR spectra were recorded at 4 cm^{-1} resolution in a wavelength range between 400 and 4000 cm^{-1} and averaging a minimum of 64 scans. Spectra were analysed with Origin pro 2019 software (OriginLab Corporation, USA). All the samples were prepared in the form of KBr pellets and alginic acid was used as standard. Triplicated KBr pellets were employed for measurement.

Triplicated KBr pellets were employed to estimate the sample's M/G ratio, obtained by calculating the mean value of absorbance strength observed at the relevant bands. The M/G ratio of the brown algae was estimated according to the ratio of the intensities of the bands assigned specifically to mannuronic acid (M) and guluronic acid (G) at approximately 1030 cm^{-1} and 1080 cm^{-1}, respectively (Sakugawa et al., 2004). The baseline method was employed to determine the transmittance at the above wavenumbers in the average IR spectra (Rochas et al., 1986). Then, the M/G ratio was calculated by comparing the absorbance at the corresponding wavenumbers using the following equation:

$$A = \log T_b/T_p \quad (1)$$

Where T: transmittance (T), A: absorbance of the baseline (b) and peak (p).

2.6. Size exclusion chromatography (SEC-MALLS)

The molar mass distribution of the samples was determined (Bojorges et al. (2023) using size exclusion chromatography (SECcurity 1260, Polymer Standard Services, Mainz, Germany coupled to a multiple-angle laser light scattering detector -MALLS; BIC-MwA7000, Brookhaven Instrument Corp., New York), and a refractive index detector (SECcurity 1260, Polymer Standard Services, Mainz, Germany) thermostated at 45 °C. The eluent was 0.1 M $NaNO_3$ containing 5 mM NaN_3 at a 0.6 mL/min flow rate. Samples were dissolved in the eluent phase at 1 mg/mL concentration. Sample injections of 100 µL were injected into a combined column set-up with a SUPREMA pre-column, a SUPREMA 1000 Å, and two SUPREMA 3000 Å

analytical columns (PSS, Mainz, Germany). A comprehensive description of the procedure, calibration, and parameter settings can be found in the Supplementary Material.

2.7 Statistical analysis

The statistical analysis of experimental data was performed using IBM SPSS Statistics software (version 23, IBM Corp., USA). One-way analysis of variance (ANOVA) was done to determine if differences between sample means were significant at a significance level of $p<0.05$. The mean tests were performed using the Least Significant Difference (LSD) Test.

3. Results

3.1. Gross composition and molecular weight of the tested alginates

In order to indirectly assess the purity of the five alginate samples and the nature of other components other than alginate, the samples were first analysed on their protein and ash content (Table 1). The very low content of protein and lipids (not significantly present in any of the samples) reflects the high purity of the tested alginate samples. Nevertheless, as all samples were isolated as sodium alginate salts, significant ash content was expected to be found in all samples. This ranged between 20-25 %, with the partially digested poly-G and poly-M having significantly higher amounts. Alginates produced from A. nodosum and S. latissima following conventional alginate extraction methods presented minor amounts of protein, while the rest of commercially available purified samples only showed negligible or trace

amounts. The downstream purification processes or digestion steps to further purify these commercial samples (REF, POLY-M, and POLY-G) might also explain their significantly lower molecular weight, as compared to ASC and SAC samples. It should be emphasized that all samples' dispersity was>1, showing high Mw dispersity, particularly in the REF sample.

Table 1. Gross composition and molecular weight of the alginate samples

	ASC	SAC	REF	POLY-M	POLY-G
Protein (%)	$3.3 \pm 0.3^{b,c}$	2.9 ± 0.3^{b}	$<0.1^{a}$	$<0.1^{a}$	$<0.1^{a}$
Ash (%)	19.4 ± 0.1^{a}	21.3 ± 0.0^{c}	20.0 ± 0.0^{b}	22.6 ± 0.5^{d}	25.5 ± 0.3^{e}
Lipids (%)	<0.1	<0.1	<0.1	<0.1	<0.1
Mw (kDa)	241.90	169.40	68.34	4.08	3.79
Mn (kDa)	157.40	74.74	19.24	3.95	3.62
Đ	1.54	2.27	3.55	1.03	1.04

Superscripts with different letters in the same row denote significant differences between samples ($p<0.05$). The abbreviations Mw, Mn, and Đ denote the molecular weight, number molecular weight, and dispersity, respectively.

3.2. Methanolysis of alginates

In order to investigate the optimum conditions for the methanolysis of alginates into their monomeric units (M and G), samples were subjected to acid methanolysis at 100°C or 80°C, and aliquots were taken after 3, 4, 5 and 6 hours or after 14, 16 and 18 hours, respectively. The range of time and temperature conditions was selected based on conditions already optimized for uronic acid-rich plant polysaccharides (Bertaud et al., 2002; Martínez-Abad

et al., 2018, 2020; Willför et al., 2009). Figure 1 shows the total uronic acid content and M/G ratio of the tested alginates.

The range of aggressiveness in the treatments allowed observing two opposite tendencies with increasing methanolysis time. The increase in methanolysis time made it possible to observe the impact of the treatments on the samples, showing two contrasting trends: a delicate balance between achieving adequate hydrolysis and avoiding further degradation. First, a slight increase in the uronic acid yields in some samples may be ascribed to higher success in the cleavage of glycosidic bonds. First, a slight increase in uronic acid yields observed in some samples may be attributed to enhanced efficiency in the cleavage of glycosidic bonds. Then, a decrease in uronic acid yields and a drastic increase in the M/G ratio indicates degradation of alginate monomers, with preferential degradation of the more labile G units. Therefore, a decrease in uronic acid yields and a significant increase in the M/G ratio indicate degradation of the alginate monomers. This degradation exhibits a proclivity for degrading the more labile G units. As commented in the introduction, chromatographic determination of alginate monomers after acid treatment is challenging. First, a complete scission of all glycosidic linkages has to take place. Here, previous studies have ascertained a higher lability of G-M linkages (Holtan et al., 2006). Furthermore, acid conditions can degrade the alginate monomers, M and G, which would reduce their recovery through chromatographic separation. Previous studies have also noted reduced recovery of G units at acidic conditions, which clearly impacts the M/G ratio (Lu et al., 2015).

When methanolysis was performed at 100 °C, alginate samples obtained from A. nodosum and S. latissima following the conventional industrial extraction and purification, showed increasing uronic acid content from 3 h to its maximum at 5 h methanolysis, although differences were not significant, while 6 h methanolysis resulted in a decrease in uronic acid yield and drastic increase in the M/G ratio, pointing at degradation. although differences were not statistically significant, the treatment of 6 h methanolysis led to a reduction in uronic acid yield and a considerable increase in the M/G ratio, pointing at degradation.

The reference alginate showed the highest response in uronic acid content after 4 hours methanolysis at 100 °C, with values around 850 mg/g, in accordance with the certified specifications (see section 2.1). The higher degree of purification, involving a lower molecular weight (Table 1) and less recalcitrant nature, might explain slightly shorter times needed to achieve optimum yields. For the digested and purified Poly-M and Poly-G alginate fractions, with molecular weight <5 kDa, highest yields were observed with only 3 h methanolysis at 100 °C.

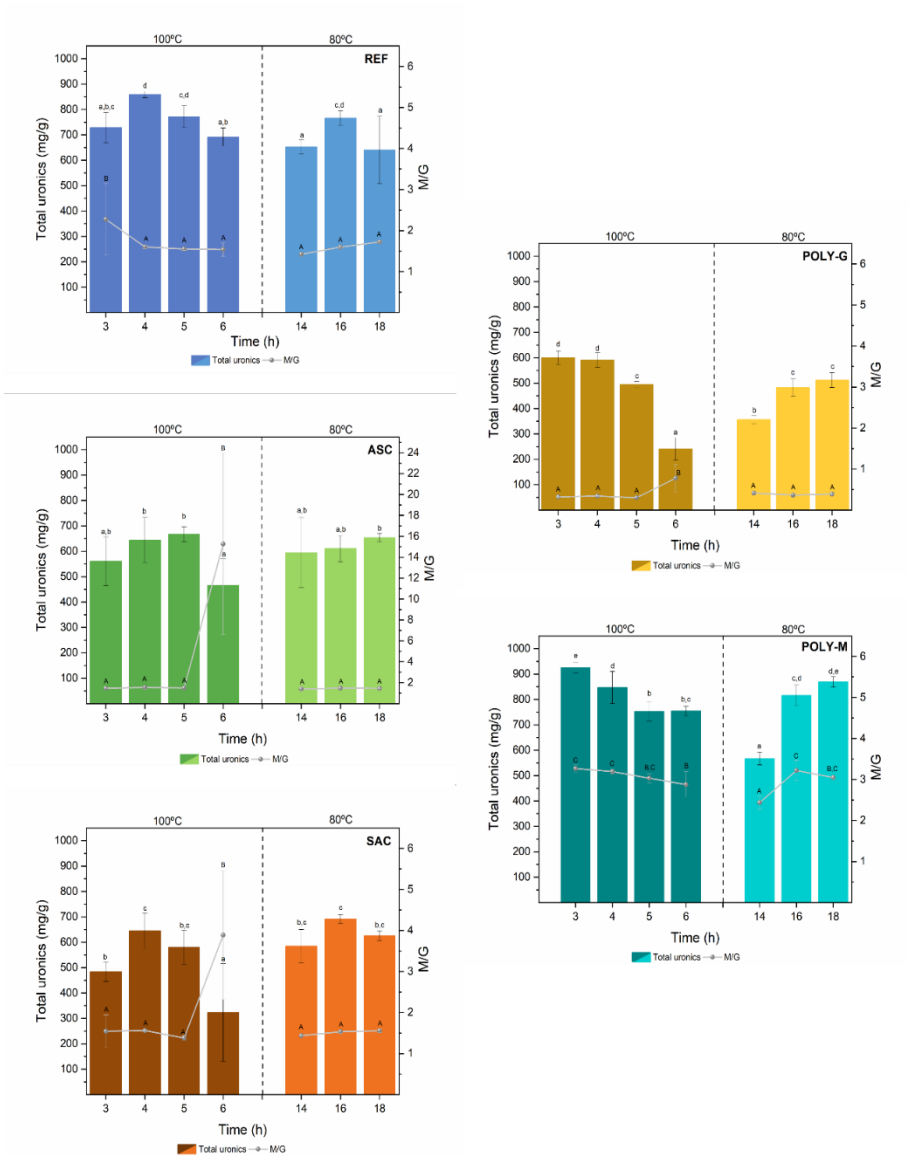


Figure 1. Total uronic acid content, displayed as the sum of guluronic, mannuronic, and glucuronic acids. Glucuronic acid was only present in minor amounts (<15 mg/g) and galacturonic acid was not detected in any of the samples tested. Different lowercase letters indicate significant differences ($p < 0.05$) in the total uronics content. Different uppercase letters indicate significant differences ($p < 0.05$) in M/G ratio.

When methanolysis was performed at 80 °C but longer times (14-18 h), maximum yields were observed for 16 h or 18 h, depending on the sample. For the higher molecular weight alginates (SAC and ASC) yields were comparable to the ones observed after 5 h at 100 °C, at all selected time points (14-18 h), where 16 h was the optimum treatment time at 80 °C for the more labile REF and Poly-uronate samples. These results illustrate the complexity of accurately determining alginate and M/G ratio depending on the origin and structural features of the samples tested, owed to a difficult balance between cleavage and degradation.

3.3. Carbohydrate composition

A chromatographic approach not only allows for the estimation of alginate monomers, but also enables identification and estimation of the whole carbohydrate composition, including other neutral sugars or polysaccharides present in the sample. This might be of special interest in less pure alginate samples or brown seaweed samples including other interesting polysaccharides, such as fucans or fucoidan, laminaran, etc. Although all neutral (Fuc, Ara, Glc, Gal, Rha, Man, Xyl, etc) and acid (GalA, GlcA, ManA, and GulA) monomers may be separated in a longer single run, we found it practical to create two separate gradients to check for either neutral sugars or uronic acids (section 2.2). Table 2 summarizes the carbohydrate composition of the five alginate samples. As partially digested or reference alginate standards may not be the target of routine analysis, the methanolysis conditions selected for this and later sections were 5 h at 100 °C giving a high response for the high molecular weight alginates (Figure 1). The carbohydrate

composition confirms the relatively high purity of all samples, consisting mainly of alginate GulA and ManA units, and only very minor amounts of Fuc and Xyl were found in SAC and ASC, probably arising from fucoidan.

Table 2. Carbohydrate composition and uronic acid content of the samples (dry wt.%)

	ASC	SAC	REF	POLYM	POLYG
Fucose[1]	$2.8 \pm 1.2^{b,c}$	1.1 ± 0.7^{b}	$<0.2^{a}$	$<0.2^{a}$	$<0.2^{a}$
Xylose[1]	$1.9 \pm 0.7^{b,c}$	0.2 ± 0.2^{a}	$1.1 \pm 0.1^{a,b}$	$<0.2^{a}$	$<0.2^{a}$
GulA[1]	$26.6 \pm 4.5^{b,c}$	24.2 ± 3.3^{b}	30.2 ± 2.2^{c}	18.6 ± 0.9^{a}	38.1 ± 1.0^{d}
GlcA[1]	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	$<0.2^{a}$	$<0.2^{a}$	$<0.2^{a}$
ManA[1]	39.7 ± 0.4^{b}	33.5 ± 5.0^{b}	46.9 ± 3.9^{c}	56.6 ± 4.0^{d}	11.5 ± 0.3^{a}
Uronic acids[1]	66.6 ± 4.9^{b}	57.7 ± 8.3^{b}	77.1 ± 6.1^{c}	75.2 ± 4.9^{c}	49.6 ± 1.0^{a}
Uronic acids[2]	20.0 ± 3.9^{b}	32.2 ± 9.9^{b}	41.5 ± 2.8^{c}	31.9 ± 10.2^{c}	23.3 ± 2.1^{a}
Uronic acids[3]	64.7 ± 2.8^{b}	85.7 ± 6.2^{b}	82.7 ± 1.1^{c}	75.6 ± 3.5^{c}	64.9 ± 5.0^{a}

Gal, Glc, Ara, Man, Rha, GalA were not detected or only in trace amounts (<2mg/g)
Values with different letters in the same line are significantly different ($p \leq 0.05$).
[1] As determined by methanolysis/HPAEC-PAD proposed
[2] As determined colorimetrically after Blumenkrantz and Asboe-Hansen (1973)
[3] As determined colorimetrically after Tullia, Filisseti-Cozzi and Carpita (1991)

In order to compare the results from methanolysis coupled with HPAEC-PAC with other typically used methods for the determination of alginate, their uronic acid content was further analyzed following the colorimetric method described by Blumenkrantz and Asboe-Hansen, later modified by Tullia, Filisetti-Cozzi and Carpita, using GalA as standard for the calibration curve. The former research study clearly showed a drastic difference in the colour response depending on the monosaccharide used for calibration, e.g. mannuronic acid compared to glucuronic or galacturonic acid (Blumenkrantz & Asboe-Hansen, 1973). We further confirmed that also GulA

gave a significantly lower response, following both colorimetric methods (Figure S4 and S5). The low colour response of M and G units may result in underestimation of alginate content in some cases. This was especially patent when applying the Blumentkrantz method (Blumenkrantz & Asboe-Hansen, 1973), which although used to determine uronic content in alginates, was not originally designed for alginate samples, thus showing significant discrepancies with the other tested methods (Table 2). However, if ManA or GulA were used for calibration, results were overestimated, most probably due to the higher lability of these monomers to concentrated sulfuric acid (Table S1). Overall, both the methanolysis/HPAEC-PAD and the colorimetric method described by Tullia, Filissetti-Cozzi, and Carpita gave comparable and plausible results, if the purity and ash of the samples is considered, with the exception of SAC and POLY-G samples. In general, the results obtained from both the methanolysis/HPAEC-PAD method and the colorimetric method proposed by Tullia, Filissetti-Cozzi, and Carpita demonstrate comparability and plausibility when considering the purity and ash content of the samples. However, it is worth noting that exceptions were observed in the case of the SAC and POLY-G samples. In this case, we might hypothesize that the HPAEC-PAD method underestimates high G content samples. However, it is important to note that the HPAEC-PAD method provides a more comprehensive mass balance, considering factors such as ash and protein content, and aligns with the reference M/G ratio obtained by NMR

3.4. M/G ratio

As outlined in the introduction, the estimation of the M/G ratio in alginate samples is of utmost importance, as this ratio has a strong influence in both their biological and technological properties. Table 3 summarizes the M/G ratio of the five tested samples resulting from methanolysis/HPAEC-PAD under the selected conditions (5 h at 100 °C), compared with the results from the reference method, liquid-state ^{1}H NMR, and with estimation by ATR-FTIR. The frequency of GulA and ManA, diads uronates and triads uronates, and the ^{1}H NMR spectrums are in section 1.3 of the supplementary material. For all methods, the hierarchical order is the same, with M/G ratio of REF > ASC > SAC for the alginate samples, while Poly-M and Poly-G had the highest and lowest M/G ratio, respectively. This indicates that either of the non-reference methods would be suitable to compare among samples. The M/G ratio values given by all three different methods were not significantly different for the reference and higher molecular weight alginates. However, significant differences were found for poly-uronate samples, with higher values of Poly-M with NMR and higher Poly-G values through FTIR analysis, as compared with the other two methods. Nevertheless, it seems logical that differences become more pronounced when the difference between M and G is greater or values are closer to threshold or baseline detection values. This withstanding, the present results show that methanolysis/HPAEC-PAD may be a suitable method to evaluate differences in the M/G ratios of different alginate samples, with quite comparable results to the reference or other spectrometric methods.

Table 4. Mannuronate (M)/guluronate (G) ratio of the alginate and poly-uronate samples tested in this study through different methods.

Sample	M/G ratio		
	NMR	HPAEC-PAD	ATR-FTIR
ASC	$1.5 \pm 0.1^{a,B,C}$	$1.5 \pm 0.1^{a,B}$	$1.7 \pm 0.1^{a,B}$
SAC	$1.4 \pm 0.0^{a,B}$	$1.4 \pm 0.1^{a,B}$	$1.5 \pm 0.1^{a,A}$
REF	$1.7 \pm 0.1^{a,C}$	$1.6 \pm 0.1^{a,B}$	$1.9 \pm 0.1^{a,C}$
POLYM	$4.0 \pm 0.0^{c,D}$	$3.0 \pm 0.2^{b,C}$	$2.5 \pm 0.1^{a,D}$
POLYG	$0.2 \pm 0.0^{a,A}$	$0.3 \pm 0.0^{b,A}$	$1.3 \pm 0.0^{c,A}$

Results are expressed as mean values plus standard deviation of n=6 from three independent analyses performed in duplicate. Different lowercase letters in the same row indicate significant differences ($p < 0.05$) for methods. Different uppercase letters in the same column indicate significant differences ($p < 0.05$) for samples.

4. Conclusions

Methanolysis followed by separation by HPAEC-PAD was investigated as a means to analyse the carbohydrate composition, alginate purity, and M/G of different alginate and poly-uronate samples. Five samples, including alginates extracted from S. latissima, A. nodosum, a certified standard and two poly-uronates were tested, as to encompass a relevant application range. The selectivity of methanolysis to cleave glycosidic bonds without incurring in major degradation, already used for uronic-rich plant polysaccharides, was successfully applied with optimal response at similar methanolysis conditions, although less aggressive conditions were more favourable for low molecular

weight samples. The methanolysis/HPAEC-PAD and the colorimetric method described by Filisetti-Cozzi and Carpita (1991) gave both comparable and plausible results, considering the lack of protein, lipids, and contribution of ashes in the samples. As for the M/G ratio, the same tendencies and differences were observed for all samples, following estimation by either NMR, methanolysis, or ATR-FTIR. M/G ratios were not significantly different in the alginate samples when estimated by methanolysis compared to liquid ^{1}H-NMR (reference method), except for low molecular weight poly-uronate samples. The method does not involve a pre-hydrolysis step or expensive equipment, such as with NMR, and offers the opportunity of estimating alginate content, M/G ratio, and carbohydrate composition in a single analysis. This method may therefore constitute a potential analytical alternative to NMR or other spectroscopic or colorimetric methods in alginate-rich samples of different purity.

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7. Supplementary Material

1. Materials and methods

1.1 Alginate extraction

Alginate samples (ASC and SAC from *A. nodosum* and *S. latissima*, respectively) were extracted as previously described by Bojorges et al. (2022) (Figure S1).

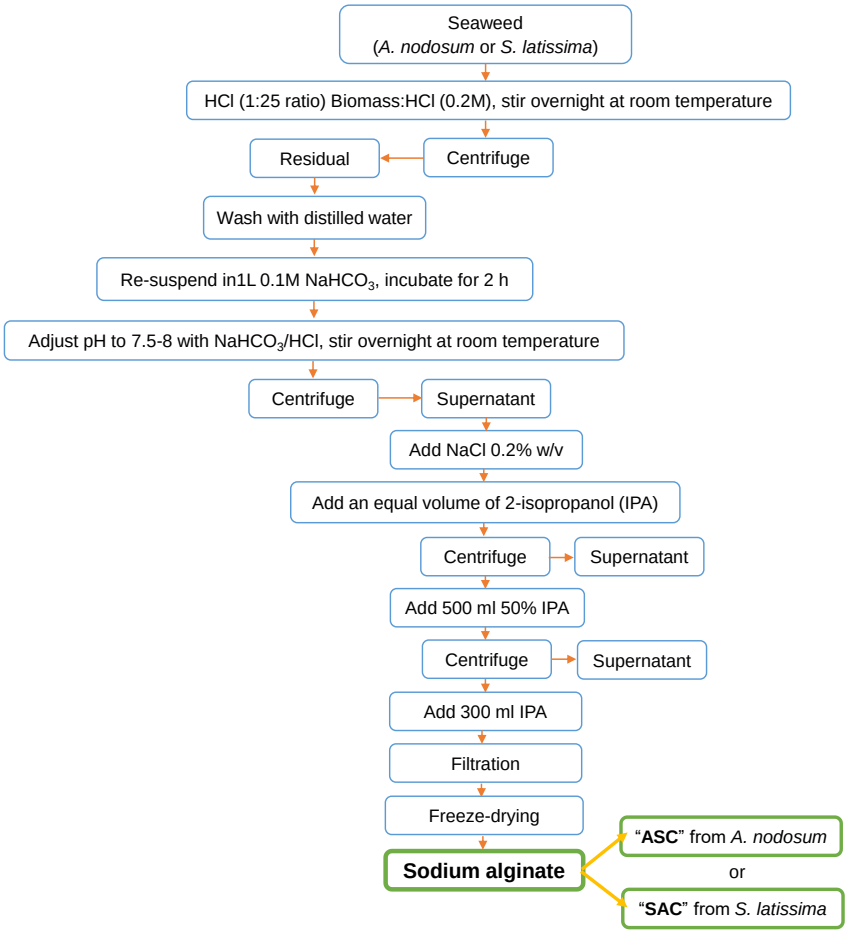


Figure S1. Schematic diagram of sodium alginate extraction from *A. nodosum* and *S. latissima*, adapted from (Bojorges et al., 2022).

1.2 HPAEC-PAD of uronic acids.

The use of a mild sodium acetate gradient starting at 200mM (see main text section 2.2) results in neutral sugars eluting at the beginning of the chromatogram, while uronic acids are separated along the range 8-20 min.

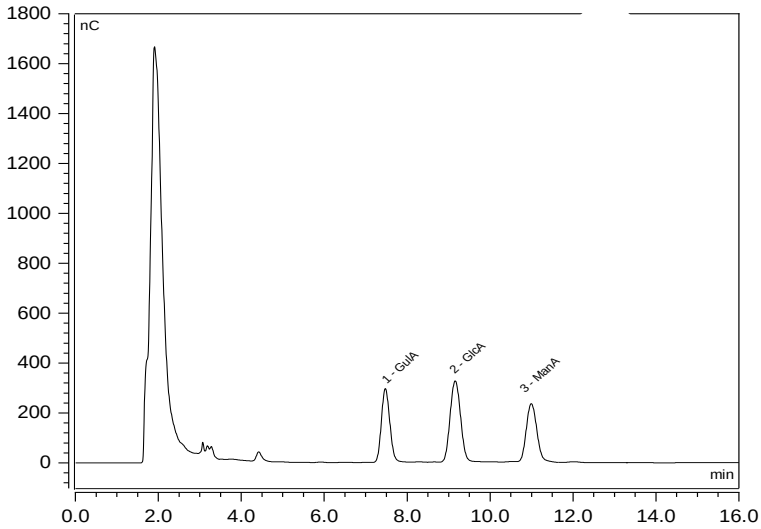


Figure S2. Example of high-performance anion-exchange chromatography (HPAEC) of standards of neutral sugars, eluting after the void volume, and uronic acids. Peaks corresponding to GulA, GlcA, and ManA are marked.

1.3 NMR analysis

Alginate composition information was determined according to Jensen et al. (2015). The ^{1}H NMR spectra was carefully assignment and integrating to the anomeric proton of G (A) due to H1 and H5 of the G residues, the anomeric proton of M and H5 of G residues adjacent to M (B), and H-5 of G-units adjacent to G (C) was shown in Fig. S3.

The M/G ratio and the uronic acid sequence was determined by utilizing the following equations:

$$G = 0.5 * \left(A + C + 0.5 * (B1 + B2 + B3)\right) \quad \text{(S12)}$$

$$M = B4 + 0.5 * (B1 + B2 + B3) \quad \text{(S13)}$$

$$M/G\ ratio\ = M/G \quad \text{(S14)}$$

$$GG = 0.5 * (A + C - 0.5 * (B1 + B2 + B3)) \quad \text{(S15)}$$

$$MG = GM = 0.5 * (B1 + B2 + B3) \quad \text{(S16)}$$

$$MM = B4 \quad \text{(S17)}$$

$$GGM = MGG = B1 * 0.5 * (B1 + B2 + B3)/(B1 + B2) \quad \text{(S18)}$$

$$MMG = B2 * 0.5 * (B1 + B2 + B3)/(B1 + B2) \quad \text{(S19)}$$

$$GGG = GG - GGM \quad \text{(S20)}$$

1.4. Size exclusion chromatography (SEC-MALLS)

The universal calibration of the SEC setup (Equation S10) was conducted using pullulan standards with known molar masses ranging from 342 to 708,000 Da (PSS, Germany) were utilized. The corresponding Mark-Houwink parameters for pullulan in aqueous solutions at 40 °C were estimated at $K = 1.0176 \times 10^{-3}$ dL g^{-1} and $a = 0.525$ (Sullivan et al., 2014). In equation S10, V_h represents the hydrodynamic volume, K and a denote the Mark-Houwink parameters for pullulan, N_A represents Avogadro's number, and M_n is the number-average molar mass. Through this procedure, it becomes

possible to convert the elution volume, which depends on the separation's experimental conditions, into the hydrodynamic volume (V_h), or in this specific case, the hydrodynamic radius (R_h), with $V_h = \frac{4}{3} \cdot \pi \cdot R_h^3$

$$V_h = \frac{2}{5} \cdot \frac{K \cdot M_n^{1+a}}{N_A} \qquad \text{(S10)}$$

Following the universal calibration, the alginate fractions' SEC weight distributions w(log V_h) were determined based on the refractive index signal. Meanwhile, the absolute weight-average molar mass M_w (V_h) and the radius of gyration (Rg) were obtained from the MALLS data using the Zimm extrapolation method (Zimm, 2004), following the procedure outlined by Vilaplana and Gilbert (2010). For the determination of molar mass, a refractive index increment (dndc) value of 0.14 mL g^{-1} for the alginates in the mobile phase solution was calculated. This calculation involved injecting a series of alginates with concentrations ranging between 0.5 to 2 g L^{-1}. Fig. S3 depicts the relative molar mass

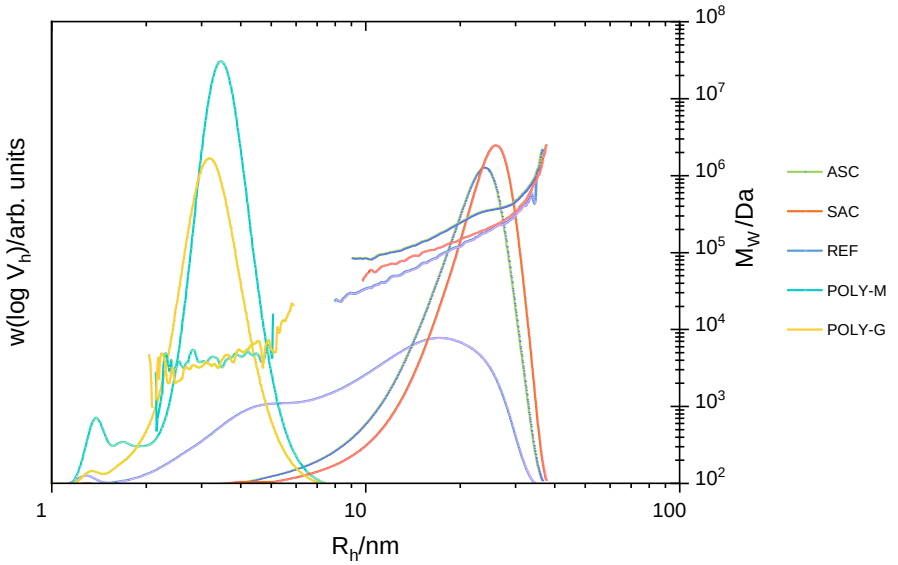


Figure S3. Molecular weight [w (log V_h)] and weight average molecular weight [M_w (V_h)] distributions of the samples, as function of the hydrodynamic radius of these samples.

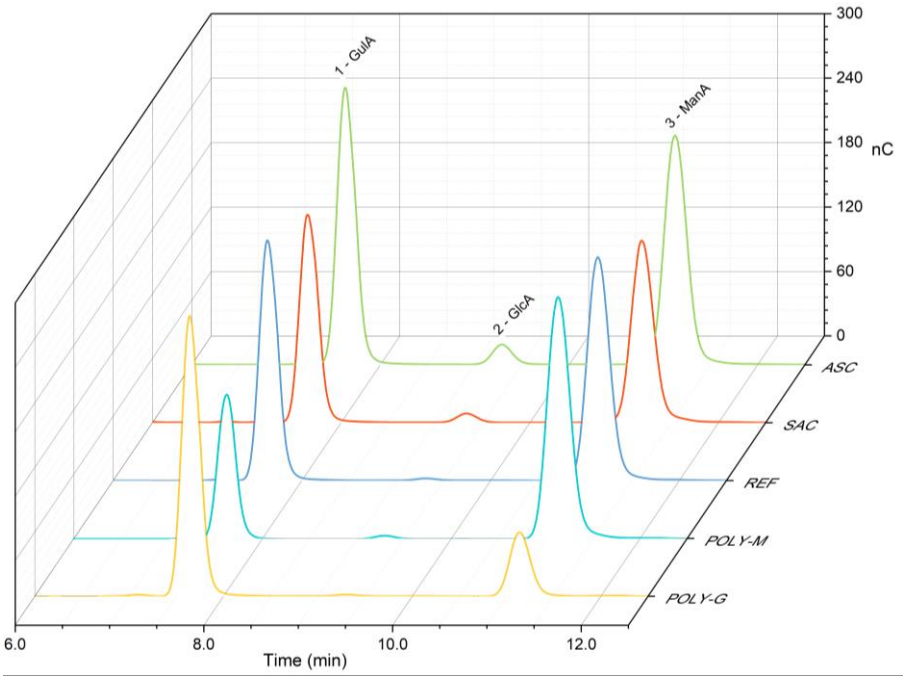


Figure S4. Typical chromatograms (HPAEC) of alginate samples (ASC, SAC and REF) and urinate samples (POLY-M and POLY-G). Peaks corresponding to GulA, GlcA, and ManA are marked.

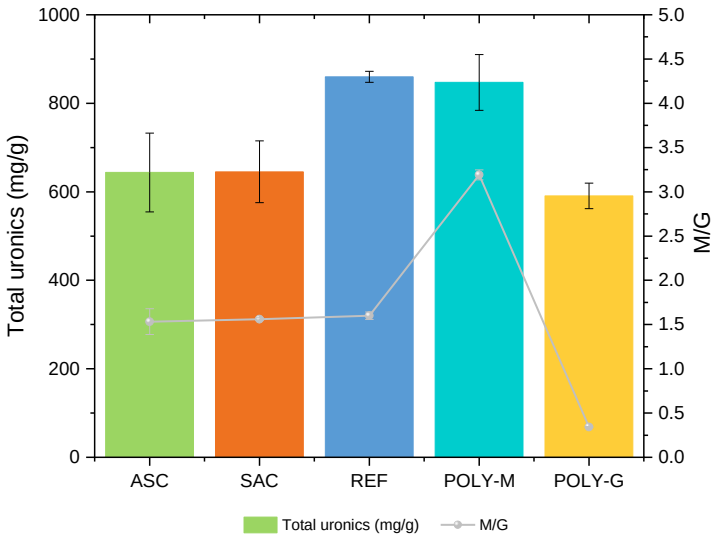


Figure S5. Total uronic acid content indicated as the sum of guluronic, mannuronic, and glucuronic acids (bars) and M/G ratio (points) for the 5 tested samples following methanolysis coupled with HPAEC-PAD at the selected conditions (100°C, 4h).

2.2. Determination of the uronic acid content following colorimetric methods.

For the total uronic acid content, the total uronic acid content results from methanolysis coupled with HPAEC-PAD were compared with the colorimetric method developed by Blumenkrantz and Asboe-Hansen, as well as with its later modification by Tullia, Filissetti-Cozzi and Carpita. Figure S4 and S5 show the colorimetric response of increasing amounts of different uronic acid monomers. For the former method (Figure S3), the lower response of ManA compared to GalA or GlcA is confirmed, in agreement with results from the original article. Additionally, GulA also shows a relatively low response. The low response of G units is again illustrated in Figure S5 when following the other method. This low response might be the result of

preferential degradation of tehse monomers over the formation of the coloured complex. This would explain why results are overestimated if M or G is used as standard (Table S1).

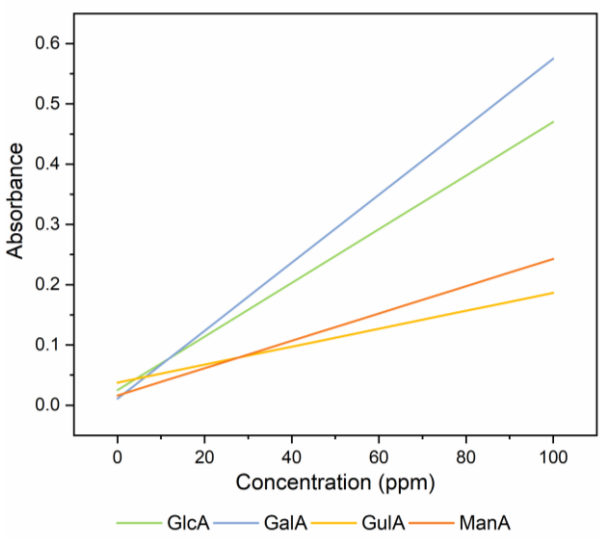


Figure S6. Fitted calibration curves (r>0.98) for the colorimetric response of different uronic acid monomers after the method described by Blumenkrantz and Asboe-Hansen. The response of ManA and GulA monomers is much lower than that of GlcA and GalA.

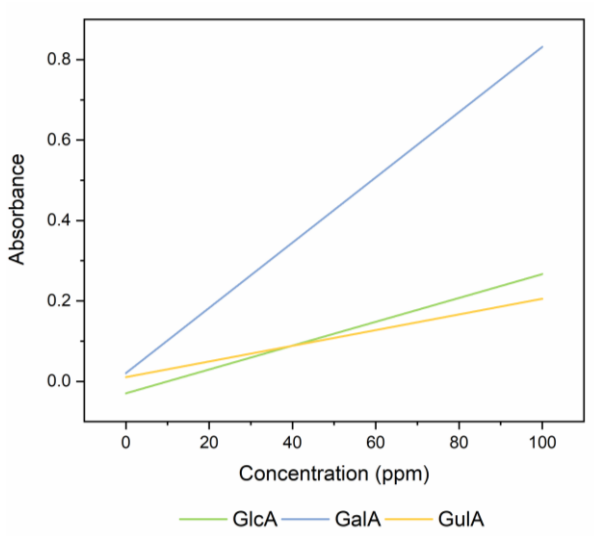


Figure S7. Fitted calibration curves ($r>0.98$) for the colorimetric response of different GalA, GulA and Glucose monomers after the method described by Tullia, Filisseti-Cozzi and Carpita. The response with GalA is much higher than that of glucose, in agreement with what is described in the original publication. However, GulA response is much lower and could not be used as standard, as it clearly overestimated alginate concentration (Table S2).

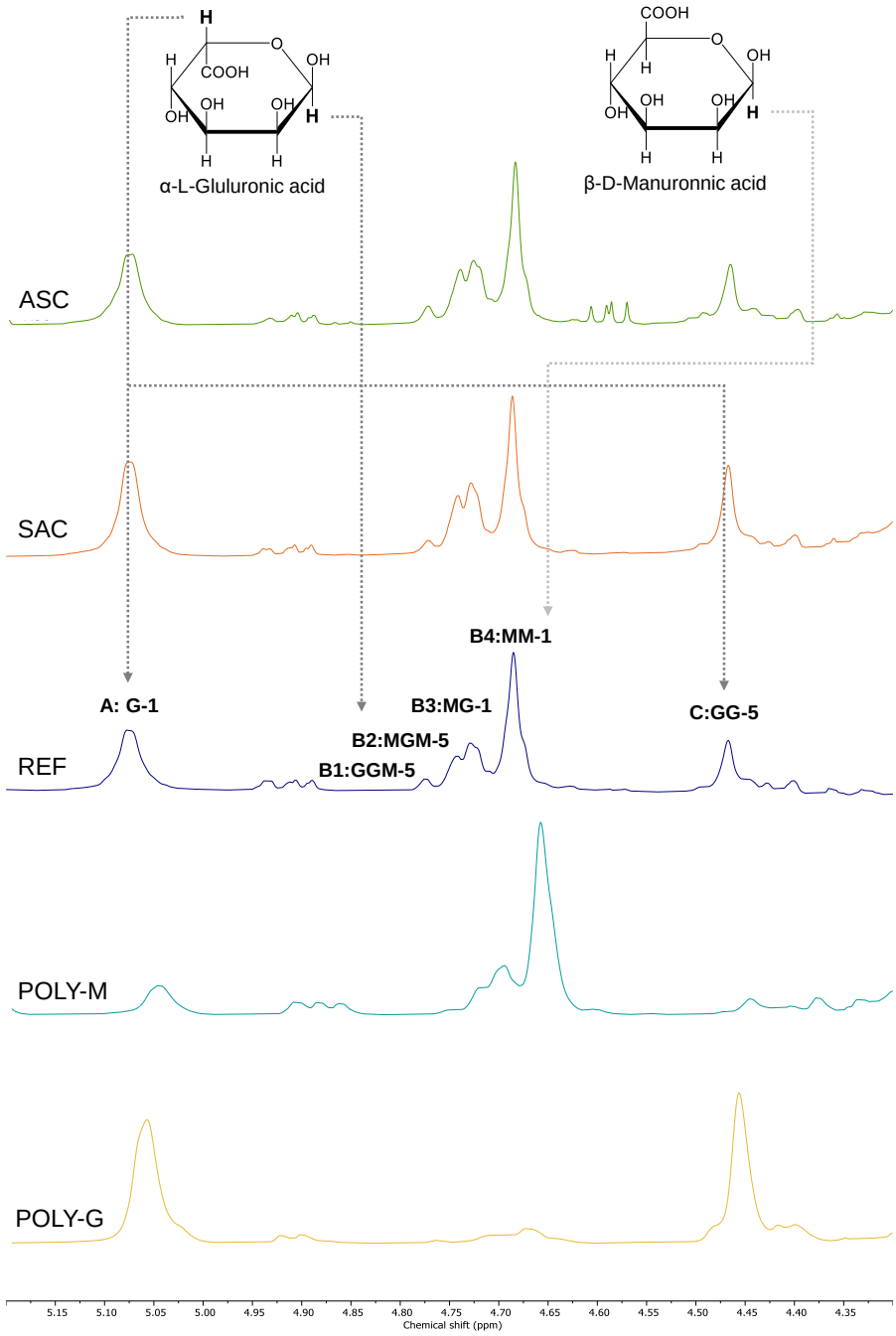


Figure S4. ^{1}H NMR spectrums of sodium alginates and Poly-M and Poly-G, add their specific positions in the monomeric structure of alginate: A-(G), B1-(GGM), B2-(MGM), B3-(MG), B4-(MM), and C-(GG).

Table S1. Frequency of GulA and ManA, diads uronates (F_{GG}, F_{MM}, F_{GM}, F_{MG}), and triads uronates (F_{GGG}, F_{MGM}, F_{GGM}, F_{MGG}).

	ASC	SAC	REF	POLY-M	POLY-G
F_G	0.43 ± 0.01^{c}	0.42 ± 0.01^{c}	0.37 ± 0.02^{b}	0.20 ± 0.01^{a}	0.83 ± 0.03^{d}
F_M	0.57 ± 0.02^{b}	0.58 ± 0.01^{b}	0.63 ± 0.02^{c}	0.80 ± 0.01^{d}	0.17 ± 0.02^{a}
F_{GG}	0.26 ± 0.01^{c}	0.26 ± 0.02^{c}	0.21 ± 0.01^{b}	0.08 ± 0.01^{a}	0.81 ± 0.02^{d}
F_{MM}	0.40 ± 0.02^{b}	0.42 ± 0.01^{b}	0.47 ± 0.02^{c}	0.67 ± 0.03^{d}	0.14 ± 0.01^{a}
F_{GM}	0.17 ± 0.01^{c}	0.16 ± 0.02^{c}	0.16 ± 0.01^{c}	0.12 ± 0.01^{b}	0.03 ± 0.01^{a}
F_{GGG}	0.24 ± 0.03^{c}	0.24 ± 0.02^{c}	0.16 ± 0.01^{b}	0.07 ± 0.01^{a}	0.80 ± 0.03^{d}
F_{MGM}	0.15 ± 0.02^{c}	0.14 ± 0.01^{c}	0.11 ± 0.02^{b}	0.12 ± 0.01^{b}	0.02 ± 0.01^{a}
F_{GGM}	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}	0.05 ± 0.02^{c}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
η	0.69 ± 0.02^{c}	0.66 ± 0.03^{c}	0.69 ± 0.01^{c}	0.75 ± 0.02^{c}	0.21 ± 0.01^{a}
$N_{G>1}$	14.00 ± 0.08^{c}	14.00 ± 0.07^{c}	5.20 ± 0.03^{a}	8.00 ± 0.07^{b}	81.00 ± 0.10^{d}

Different letters in the same row indicate significant differences between the samples ($p < 0.05$).
η: Sequence distribution parameter
$N_{G>1}$: Average number of G-block length lager than one

Table S2. Comparison of uronic acid content in the 5 alginate or poly-uronate samples, determined by both colorimetric methods and using different carbohydrate monomers as standard (dry wt.%)

Sample	Filissetti-Cozzi			Blumenkrantz			
	GulA	GalA	Glc	GlcA	GulA	ManA	GalA
ASC	118.7 ± 4.5	64.7 ± 2.8	71.2 ± 2.9	37.6 ± 1.6	110.7 ± 5.5	77.4 ± 3.4	20.0 ± 3.9
SAC	114.3 ± 9.1	85.7 ± 6.2	68.4 ± 5.8	63.8 ± 6.6	201.5 ± 23.0	133.6 ± 14.2	32.2 ± 9.9
REF	212.8 ± 17.3	82.7 ± 1.1	102.1 ± 11.0	47.3 ± 9.5	144.3 ± 32.9	98.2 ± 20.4	41. 5± 2.8
POLY-M	147.9 ± 15.4	75.6 ± 3.5	89.6 ± 9.8	38.8 ± 6.7	115.1 ± 23.0	80.1 ± 14.3	31.9 ± 10.2
POLY-G	123.6 ± 8.7	64.9 ± 5.0	74.3 ± 5.5	45.4 ± 11.2	137.9 ± 38.8	94.2 ± 24.0	23.3 ± 2.1

IV. General discussion

"To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge."

Nicolaus Copernicus

IV. GENERAL DISCUSSION

In the context of the present research thesis and throughout the different chapters, the optimization of the extraction of polysaccharides and proteins, the valorization of the by-products generated during these procedures and the structural elucidation through the implementation of innovative methodologies aimed at the generation of knowledge have been systematically addressed. These efforts converge with the central objective of developing a comprehensive strategy for valorizing brown algae biomass into high-value-added compounds.

The first chapter of the research addressed the use of the whole algae biomass in the biorefinery, with different novel treatments to valorize protein or enhance the extraction efficiency of polysaccharides. High hydrostatic pressure (HPP) was explored as an innovative technology to improve the efficiency of alginate extraction from brown algae. During this research, significant differences in cell wall structure were identified between the two orders: Fucales for *A. nodosum* and Laminariales for *S. latissima*. These variations were reflected in a significant increase in extraction yield from 14% to 23% for *A. nodosum*. This increase suggests that the application of HPP effectively facilitated cell wall rupture, thus generating an increase in alginate yield. Additionally, HPP pre-treatment was observed to influence the structural properties directly, affecting the rheological and gelation properties of the alginate, as well as increased antioxidant activity. In the case of *S. latissima* alginate, an increase in the abundance of G-blocks and in the molecular weight was observed. In contrast, in *A. nodosum* samples, an

opposite effect was observed. These findings underline the effectiveness of HPP pre-treatment as an efficient way to optimize the exploitation of marine sources, allowing improvements in the technological or functional properties of the alginates obtained, depending on the origin of these sources.

In addition, this chapter explored the extraction of protein-polysaccharide conjugates from brown algae by integrating innovative technologies, such as ultrasound, enzymatic extraction, and pH shifts. The main objective was to maximize the recovery of intracellular protein in the algae. These methods were applied sequentially and compared with the conventional extraction (acid/alkaline treatment) approach. The results of our study were promising. The sequential application and combination of these techniques significantly improved the protein extraction yield in these extracts.

A marked carbohydrate content (approximately 50%) was identified in the compositional analysis of the extracts obtained. The carbohydrate fraction was mainly composed of uronic acids, followed by mannitol and glucose. Nevertheless, these polysaccharides contributed to a higher emulsion stabilization and an outstanding antioxidant capacity. An example was the protein extracts of *A. nodosum* obtained by enzymatic treatment, which exhibited the highest antioxidant capacity due to their high content of recalcitrant sulfated compounds.

Regarding the functional properties of the extracts, a higher emulsifying capacity was observed in the extracts obtained by the enzymatic method for both algae, possibly due to the release of low molecular weight

peptides after partial hydrolysis. This could increase the exposure of hydrophobic residues and the dispersion of peptides, facilitating the interaction between water and oil interface, forming a viscoelastic film, reducing the tension at the interface, and avoiding coalescence. In addition, differences in the composition of the extracts, such as protein content and aggregation of protein molecules, also influenced their emulsifying properties.

In terms of bioactivity, it was observed that the protein extracts obtained by the enzymatic method for both algae exhibited outstanding antihypertensive activity, achieving ACE inhibition between 59% and 63%. This enhanced inhibition could be attributed to the release of small peptides during enzymatic hydrolysis, which played a crucial role in the antihypertensive mechanism. In this context, it is relevant to highlight that macroalgae emerge as a promising, sustainable and economically-viable source of dietary protein. Although their protein content may be subject to seasonal variability and the influence of pre-treatment and extraction methods, these algae exhibit functional and bioactive properties that support their potential in the food industry. These findings establish a solid basis for future research in this field.

In line with Horizon Europe's R&D guidelines, the **second chapter** examines strategies for waste valorization through two specific investigations. These investigations were especially dedicated to addressing the valorization of waste derived from the alginate extraction process.

In the first study, the conventional alginate extraction process used in the industry was replicated, focusing on two types of brown algae with

different cell wall architectures: *A. nodosum* and *S. latissima*. Detailed characterizations of the different solid and liquid waste streams, currently not utilized by the industry, were performed. The objective was to explore potential strategies for the valorization of these waste streams. During this study, a comprehensive analysis of all residual fractions (both liquid and solid) generated during the alginate extraction procedure was carried out. These residues were evaluated regarding their protein content, lipid content, carbohydrate composition, ash and antioxidant capacity. In the initial analysis of the waste streams, it was observed that the initial fraction after acid treatment, exhibited a remarkable abundance in polyphenols (15 mg GAE/g) and bioactive fucoidans (15-70%), with a marked degree of sulphation in *A. nodosum*. These findings suggest a considerable potential of this fraction as a bioactive ingredient, highlighting its high yield in sulfated fucoidan and polyphenols. Its antioxidant efficacy was confirmed by ABTS and beta-carotene bleaching assays, demonstrating significant levels of antioxidant activity. Two fractions were generated from the solid residue, the soluble fraction (Ra) showing relatively high yields, presenting antioxidant properties and a significant soluble protein content (30% by weight for *S. latissima*). This fraction exhibits promising potential as a vegan ingredient with functional texturizing properties. On the other hand, the insoluble fraction (Rb) was mainly constituted by cellulose (70%), with considerable amounts of insoluble protein and alginate. The valorization of this fraction could be explored, either as a food supplement or, after further processing, as peptones or thickening ingredients for various applications in the cosmetic, food, feed, or pharmaceutical sectors.

As expected, the differences in algal cell wall architecture was evident in the composition of the fractions of both species. In particular, in the case of *S. latissima*, it was highlighted by a glucose/glucan content that exceeded 30% by weight in the first fraction (F1), a higher protein concentration in the fraction of soluble residue and increased cellulose content in the fraction of insoluble residue. In contrast, *A. nodosum* exhibited significantly higher antioxidant capacities in F1, Ra, and Rb (50-70 µM TE/g), in addition to obtaining higher yields (>45 wt.%) of a highly sulfated fucoidan in F1. These observations highlight the distinct differences between the species, providing essential information for accurate raw material selection in specific applications. These findings also lay the groundwork for a possible optimization of the waste generated during alginate production, opening the door to using these fractions to extract valuable ingredients with various uses in the food, pharmaceutical, cosmetic, and animal feed industries. These could include alternative protein sources, bioactive compounds or texturizing agents. Given the enormous number of algae processed in the alginate industry, converting these wastes into high-value-added products represents a significant step towards a more sustainable and circular economy.

Therefore, in the second work of this chapter, the extraction of proteins from the residues generated during the process of obtaining alginate was addressed, focusing specifically on the solid residues of the brown algae *A. nodosum* and *S. latissima*. This approach was strategically conceived to optimize the valorization of these underutilized resources and generate high-value-added compounds. However, one of the inherent challenges in protein extraction from algae lies in the need to disrupt the cell walls to access, in this

case, the protein. This challenge originates from proteins' intracellular location and their embedding in the cell wall matrix. Consequently, the presence of proteins was anticipated to be recalcitrant in the cell wall structure of these residues, adding complexity to the extraction process.

In order to overcome this obstacle and efficiently extract the proteins in the algae, several extraction methods and their combination were implemented, such as ultrasound, acid and alkaline treatments, enzymatic and pH-shift processes. These strategies were sequentially applied in order to increase extraction yields. Thus, the insoluble residues obtained after protein extraction with the chemical extraction (CE) treatment were subjected to an enzymatic treatment (cellulases) at two concentrations, one low and one very high (E20 and E100, respectively), to degrade the cellulose chains. Subsequently, a pH-shift was applied to isolate the proteins from the polysaccharides and precipitate as much protein as possible.

The study stated that the most effective combination for protein extraction was the joint application of enzymatic treatment (E100) and ultrasound, achieving yields of 43% and 58% for *A. nodosum* and *S. latissima* residues, respectively. The resulting protein extracts were characterized by a predominant proportion in the 10-100 kDa range, followed by smaller peptides in the 1-5 kDa range. Interestingly, samples subjected to enzymatic treatment (E20 and E100) exhibited a higher proportion in the 100-700 kDa fraction, ranging from 49% to 74%. This evidenced that the enzymatic treatment effectively released fractions with high protein content and promoted the formation of larger protein conjugates. It is also possible that

the presence of residues of the enzyme used in the process may also have contributed to this fraction present in the samples.

Meanwhile, CE samples, especially from *S. latissima* residue, revealed a higher proportion of biomolecules in the 1-5 kDa range, registering 40% without ultrasound and 43% with ultrasound. This pattern suggests that the soluble and more accessible proteins presented a lower molecular weight, while those of higher molecular weight were more recalcitrant in the cell wall structure. In addition, these extracts were found to be rich in essential amino acids, representing between 43% and 50% of the total. This indicates that the appropriate ratio of essential to non-essential amino acids was between 0.76 and 1.01. This range is similar to the protein content found in different animal sources (42-43.8%), as well as plant sources like common beans (41.8%) or spinach (44.6%).

In the analysis of the functional properties of the protein extracts, adequate water and oil retention capacities were highlighted in all the protein extracts evaluated. Notably, protein extract E20, without the application of ultrasound, stood out for its significant foaming capacity in both *A. nodosum* and *S. latissima* residues. Under these conditions, foaming capacity ranging from 52.5% to 80.5% was observed for *A. nodosum* and *S. latissima* residues, respectively. In contrast, regardless of ultrasound application, the CE samples exhibited the lowest foaming capacity, ranging from 31.5% to 49%.

These results suggest that enzymatic treatment at a low concentration without ultrasound intervention led to protein extracts with superior foaming capacity compared to other treatment conditions. This finding may be of great

relevance when considering the applicability of these extracts in food applications and other areas of industrial interest.

On the other hand, the maximum emulsification rates were observed for samples E20 and E100 with ultrasonication in *S. latissima* residue (91.8 and 91.3 %, respectively). In comparison, CE samples with ultrasonication for both residues showed the lowest emulsification rate (70.5 and 82.3 %, respectively). This could be because the E20 and E100 protein extracts contained predominantly hydrophobic groups that favor proteins reaching the interface and having optimal contact of non-polar groups with the oil phase, thus contributing to the hydrophilic-lipophilic equilibrium.

In the context of bioactive properties, the protein extracts coming from *A. nodosum* residue exhibited the highest polyphenol content and high antioxidant activity, especially in CE and E20 samples, with no significant impact of ultrasound application. This phenomenon is possibly attributed to a close correlation with the high fucose levels in this sample, suggesting the possible existence of low molecular weight fucoidans, characteristic of Fucales species such as *A. nodosum*.

Regarding antihypertensive activity, the inhibition of angiotensin-converting enzyme (ACE) activity was investigated. It was observed that the protein extract E20 and CE of *S. latissima*, obtained using ultrasound at a concentration of 1 mg/mL, exhibited the maximum inhibition of ACE, reaching 50% and 47%, respectively. These results show the antihypertensive potential of these extracts.

The results indicate that the protein extracts derived from the by-products generated during the alginate extraction process exhibit significant potential as a source of protein, emerging as a valuable source of amino acids with functional and bioactive properties.

In the current scenario, where the significance of valorizing marine biomass and hydrocolloid industry by-products is emphasized, the need for innovation in developing alternative methods to determine alginate content and M/G ratio is evident. Implementing new methods could offer significant advantages compared to currently established methods. This approach is fully integrated with the **third chapter**, which tackles the challenge of elucidating alginate structure.

In that chapter, an innovative method was developed to estimate alginate purity and M/G ratio by acid methanolysis and separation by anion exchange chromatography. The samples analyzed included alginates extracted from *A. nodosum* and *S. latissima*, a certified standard alginate and two poly-uronates (Poly-G and Poly-M). The objective was to determine their M/G ratio and alginate content under different treatment conditions, comparing them with the reference (NMR) and other spectrophotometric (FTIR) and colorimetric methods.

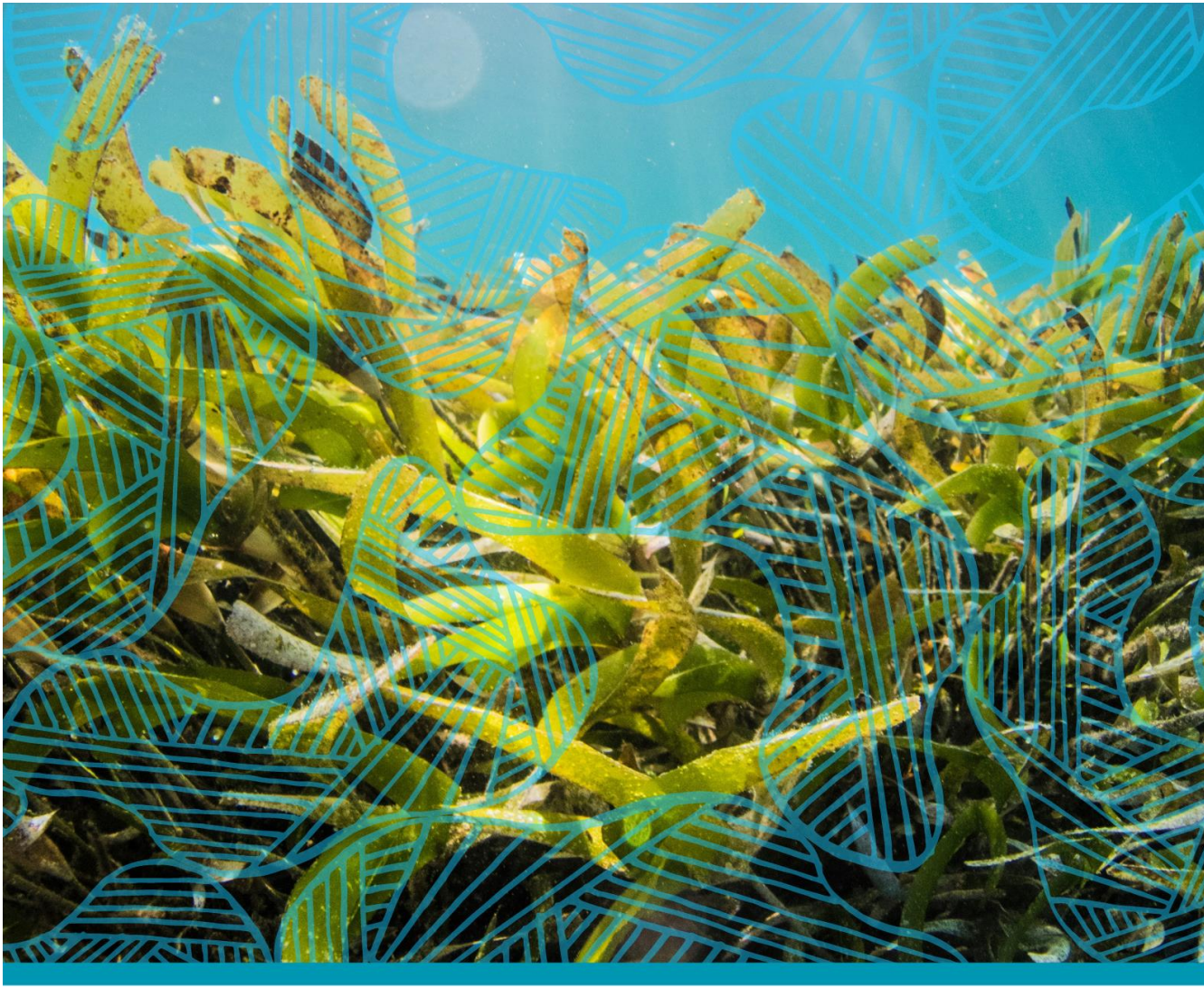
Significant challenges were faced during the investigation to determine the optimal conditions for acid methanolysis in the chromatography of alginate monomers. One consisted of the overriding need to achieve complete cleavage of the glycosidic bonds without causing degradation of the M and G monomers under acidic conditions. This requirement was crucial, as any degradation

would have a negative impact on the recovery by chromatographic separation, affecting the M/G ratio of the alginate.

However, two opposite trends were observed with methanolysis time. First, there was a slight increase in the yield of uronic acid in some samples, which was associated with a higher efficiency of glycosidic bond cleavage. However, at prolonged times, a decrease in uronic acid yields and a significant increase in the M/G ratio were evident, indicating degradation of alginate monomers, with a bias towards G units, clearly affecting the M/G ratio.

Consequently, it was determined that the optimum acid methanolysis conditions for accurate quantitative estimation of alginate's M/G ratio were 4 h at 100 °C. Under these conditions, the M/G ratio estimation showed no significant differences compared to those determined by the reference method (^{1}H NMR) or FTIR protocols. These results evidenced that methanolysis can be applied to simultaneously estimate the purity and M/G ratio of alginate-rich samples in a single analysis.

Nevertheless, it is essential to emphasize that the purpose of the proposed method is not to replace or refine the reference method but rather to investigate a possible alternative for the simultaneous estimation of M/G ratio, purity, and carbohydrate composition in alginate-rich samples. This perspective could prove relevant for samples with lower alginate purity, where information on total alginate and other polysaccharide components could be of greater utility than a thorough characterization of alginate. This approach contributes significantly to the detailed understanding of the composition and structure of such compounds.



V. Conclusions

"Every end is a new beginning"

Anonymous

V. CONCLUSIONS

This thesis was aimed at developing a biorefinery for the brown algae *Saccharina latissima* and *Ascophyllum nodosum* to obtain innovative, sustainable, and functional ingredients. The main conclusions from this research are summarized below:

- High Hydrostatic Pressure (HPP) has proven to be a highly effective strategy to improve alginate extraction from brown algae, significantly increasing the yield. The variation in cell wall structure among different algal families, together with HPP treatment, directly influences alginate's structural and functional properties, affecting its rheological properties, gelation and antioxidant activity.

- The conversion of by-products into high-value-added products is an important challenge toward a more sustainable and circular economy within the valorization strategies of effluents and solid waste generated during alginate extraction. The study of all fractions generated reveals key opportunities to extract valuable ingredients with various industrial applications.

- The sequential and combined application of various extraction methods, such as ultrasound, enzymatic extraction, and pH-shift processes, maximized the recovery of algal intracellular protein-polysaccharide conjugate extracts and proteins in the solid residues generated during the alginate extraction

process. These approaches significantly improved extraction yields compared to the chemical (acid/alkali) method.

- Seaweed extracts and residues stand out for their high carbohydrate and polyphenol content, which gives them antioxidant and emulsion-stabilizing properties. In addition, they possess remarkable bioactivity, acting as potent angiotensin-converting enzyme (ACE) inhibitors and demonstrating antihypertensive activity superior to that of other macroalgae extracts. This opens up new possibilities for industrial and medical applications.

- Brown macroalgae and their solid residues generated during the alginate extraction process emerge as a promising and sustainable option for obtaining extracts rich in proteins and polysaccharides. This fact highlights their potential application in the food industry, contributing to a sustainable valorization drive and promoting a circular economy. Thus, a solid basis for future research in this field is established.

- The innovative method based on acid methanolysis and anion exchange chromatography is an efficient alternative to evaluate alginate's purity and M/G ratio. It simplifies the analytical process and offers accuracy comparable to the reference method (liquid ^{1}H NMR). This method is a valuable complementary option, especially useful in samples of lower purity, providing detailed information on alginate and other polysaccharides without pretending to replace the reference method.

Finally, using marine biomass to recover high-value compounds from brown algae, such as hydrocolloids or proteins, offers considerable potential in biorefining. This strategy could lead to the production of currently under-exploited products, representing a significant advance in environmental and economic sustainability.

In addition, knowledge generation aimed at simplifying and developing accessible and inexpensive reference methods can be a valuable analytical tool for characterizing brown algal hydrocolloids, such as alginate. Together, these actions have the potential to contribute significantly to the development and efficiency of the seaweed-based biorefinery industry.

VI. Annexes

"Science is the great antidote to the poison of enthusiasm and superstition."
Adam Smith

VI. ANNEXES

Annex A. List of publications included in this thesis

Carbohydrate Polymers 299 (2023) 120175

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Structural and functional properties of alginate obtained by means of high hydrostatic pressure-assisted extraction

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ARTICLE INFO

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HPP
Molecular weight
Nanostructure
Brown seaweed
M/G ratio
Polysaccharide extraction

ABSTRACT

The effects of the high hydrostatic pressure (HPP) pre-treatment on the alginate extraction were seen to greatly depend on the recalcitrant nature of two algae species. Alginates were deeply characterized in terms of composition, structure (HPAEC-PAD, FTIR, NMR, SEC-MALS), functional and technological properties.

The pre-treatment significantly increased the alginate yield in the less recalcitrant *A. nodosum* (AHP) also favoring the extraction of sulphated fucoidan/fucan structures and polyphenols. Although the molecular weight was significantly lower in AHP samples, neither the M/G ratio nor the M and G sequences were modified. In contrast, a lower increase in alginate extraction yield was observed for the more recalcitrant *S. latissima* after the HPP pre-treatment (SHP), but it significantly affected the M/G values of the resulting extract. The gelling properties of the alginate extracts were also explored by external gelation in $CaCl_2$ solutions. The mechanical strength and nanostructure of the hydrogel beads prepared were determined using compression tests, synchrotron small angle X-ray scattering (SAXS), and cryo-scanning electron microscopy (Cryo-SEM). Interestingly, the application of HPP significantly improved the gel strength of SHP, in agreement with the lower M/G values and the stiffer rod-like conformation obtained for these samples.

1. Introduction

Macroalgae are one of the renewable resources of the marine environment, which have received rising attention due to their important role as a valuable source of hydrocolloids and bioactive compounds (Abka Khajouei et al., 2021; Hentati et al., 2018). There are about 9000 species of macroalgae, which, depending on their photosynthetic pigments, can be classified into Chlorophyta (green), Phaeophyta (brown), and Rhodophyta (red) (Sanjeewa et al., 2018). Specifically, brown algae are rich in polysaccharides, phenolic compounds, carotenoids, proteins, vitamins, and minerals (Kadam & Prabhasankar, 2010). The main polysaccharide present in the cell wall of brown algae is alginate and can represent up to 40 % of the dry matter of the seaweed (Łabowska, Michalak, & Detyna, 2019). Alginate is a linear polymer constituted by two monomeric uronic acids, β-(1-4)-D-mannuronic acid (M block) and α-L-guluronic (G block) acid (Fertah et al., 2017). The arrangement of the monomers creates homogeneous MM, and GG blocks only composed of one uronic acid species and heterogeneous MG (or GM) blocks (Khajouei et al., 2018). The biological and physicochemical properties of alginates depend on the M/G ratio and on the structure and distribution of acid blocks in the polysaccharide backbone, which varies depending on the species, the type of tissues, age, or harvesting seasons (Karaki et al., 2013). In this regard, stiff and brittle alginate gels are known to have low M/G ratios, while elastic and flexible gels are known to have high M/G ratios. Specifically, in the food industry, they are widely used ingredients due to their stabilizing, thickening, and gelling properties (Brownlee et al., 2005), also having a role as dietary fiber. All these properties are highly affected not only by the molecular weight and M/G ratios of alginates but also by their molecular conformations (Zrid et al., 2016).

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Alginate industrial waste streams as a promising source of value-added compounds valorization

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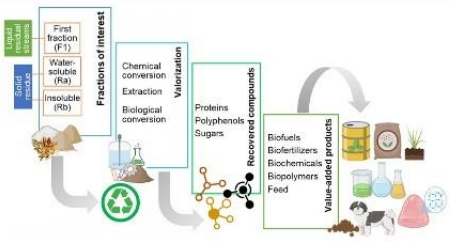
HIGHLIGHTS

- Alginate industry effluents composition varies depending on the algae species.
- Acid effluents contain significant quantities of sulfated fucoidan and polyphenols.
- Soluble protein-rich or alginate fractions recovered from the solid residue.
- Fucoidan, insoluble protein or cellulose predominates in the final residues.
- Valorisation towards bioactive, texturizing or nutritional added-value ingredients

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Daniel CW Tsang

Keywords:
S. latissima
A. nodosum
Antioxidant activity
Fucoidan
Waste valorization
Circular economy

ABSTRACT

The alginate industry processes more than hundred thousand tons per year of algae in Europe, discarding around 80% of the algae biomass as different solid/liquid residual streams. In this work, *Saccharina latissima* and *Ascophyllum nodosum*, their generated alginates and all residual fractions generated in the process were characterized in terms of lipid, ash, protein content, and the carbohydrate composition and antioxidant capacities analyzed. The first fraction after acid treatment (ca. 50% of the initial dry biomass) was rich in phlorotannins (15 mg GAE/g) and bioactive fucoidans (15–70%), with a high sulfation degree in *A. nodosum*. Two fractions generated from the solid residue, one soluble and another insoluble (Ra and Rb, respectively), constituted 9% and 5–8% of the initial biomass and showed great potential as a source of soluble protein (30% for *S. latissima*), and cellulose (70%) or fucoidan, respectively. Valorization strategies are suggested for these waste streams, evidencing their high potential as bioactive, texturizing or nutritional added-value ingredients for cosmetic, food, feed or pharmaceutical applications.

1. Introduction

Marine biomass is highly underexploited and mostly unknown, compared to plant terrestrial biomass, and constitutes a vast renewable source for food ingredients, biomedical, cosmetic, specialty chemicals and materials in future biorefineries. Thanks to global policies fostering a "blue bioeconomy", much research and industrial efforts have been devoted to understanding marine biomass and explore potential applications. Brown algae are one of the most exploited and promising families of macroalgae, due to their high content in valuable compounds such as functional polysaccharides (e.g. fucoidan, laminarin, etc.), polyphenols (e.g. phlorotannins), protein, vitamins and minerals.

Fucoidans and fucans are a wide family of fucose-containing polysaccharides which has attracted much research attention in recent years due to abundant scientific evidence on bioactive properties, such as anticoagulant, anti-inflammatory, antioxidant, which in turn may alleviate many

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Estimation of alginate purity and M/G ratio by methanolysis coupled with anion exchange chromatography

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ARTICLE INFO

Keywords:
Alginate
HPAEC-PAD
NMR spectroscopy
Uronic acid determination

ABSTRACT

Alginates are industrially relevant polysaccharides widely used in the food and biomedical industries for their excellent gelling properties. The growing emphasis on the valorization of marine resources has evidenced the need for alternative methods for the determination of both alginate content and the M/G ratio. This study describes the application of acid methanolysis and separation by anion exchange chromatography. Five samples, including alginates extracted from *Saccharina latissima*, *Ascophyllum nodosum*, a certified standard, and two polyuronates (Poly-M and Poly-G), were analysed for their M/G ratio and alginate content at different treatment conditions, and compared with other conventionally used or reference methods (NMR, FTIR, and colorimetric methods). Quantitative estimation of alginate was relatively accurate at optimum conditions (4 h at 100 °C), as compared with the certified standard or with other colorimetric methods. M/G ratios were not significantly different from those determined after the reference method (^{1}H NMR) or compared to FTIR protocols. The results evidence that methanolysis may be applied to simultaneously estimate the purity and M/G ratio of alginate-rich samples in a single analysis.

1. Introduction

Alginates are industrially relevant polysaccharides extracted from brown seaweed (Phaeophyceae) and widely used as thickeners, stabilisers and gelling agents in the food, pharma, or cosmetic industries. Alginates can be described as copolymers of (1–4) linked β-D-mannuronic acid (ManA or M) and α-L-guluronic acid (GulA or G). These polysaccharides are typically described by their M/G ratio, molecular weight and distribution of M and G units, as M, MG or G blocks along the chain, as these parameters highly determine the biological and physical properties of alginate (Haug, Myklestad, Larsen, & Smidsrød, 1967). The alginate contents, as well as its extractability or recalcitrance within the cell wall architecture, its molecular weight, and sequential structure, all greatly vary, depending on the brown algae species, season, growing stage, and geographic location (Haug, Larsen, & Smidsrød, 1974; Indergaard, Skjåk-Bræk, & Jensen, 1990). A growing focus towards a "blue bioeconomy" and the valorisation of ocean resources has prompted research and industrial efforts to deeper study the composition and potential applications of brown algae, evidencing the lack of straightforward methods for the determination of both alginate content and M/G ratio.

The estimation of alginate content is usually based on colorimetric methods, among which many different possibilities have been proposed and modified through many years and are still used by different authors. These methods mostly involve the formation of a coloured complex in the presence of uronic acids with reagents such as resorcinol, carbazole, *m*-hydroxydiphenyl, etc. (Kumar & Kumar, 2017). The most widely used chemometric method for the quantitative determination of uronic acids is the method developed by Blumenkrantz and Asboe-Hansen (1973). Although very suitable for pectin or other plant samples, the original method was not tested or developed for its application on alginates and may present certain limitations. A further modification of this method, involving the addition of sulfamate to *m*-hydroxydiphenyl, was described by Fillissetti-Cozzi and Carpita, which aimed at reducing interference from neutral sugars, and was applied to both polygalacturonates as well as to alginic acid (Filisetti-Cozzi & Carpita, 1991). There is, however, no generally accepted or reference method for alginate analysis.

The reference method for the determination of the M/G ratio is liquid-state nuclear magnetic resonance (NMR) spectroscopy, based on extensive work performed in the late 70s and early 80s (ASTM F2259-10, 2012; Grasdalen, 1983; Grasdalen, Larsen, & Smidsrød, 1979).

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Overview of alginate extraction processes: Impact on alginate molecular structure and techno-functional properties

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ARTICLE INFO

Handling editor: I Oey

Keywords:
Phaeophyceae
Biopolymers
brown seaweeds
Novel processing
Physicochemical properties

ABSTRACT

Background: Brown algae are a valuable source of various biopolymers, with alginate standing out as a phycocolloid of great industrial importance. Although the extraction process is well established on an industrial scale, several innovative and environmentally friendly extraction technologies have been explored to improve the efficiency of the process both in terms of yields and energy and solvent consumption.

Scope and approach: The review aims to describe and analyze different strategies used to extract alginate from brown algae, including different pre-treatment methods used before extraction. In addition, the impact of these extraction technologies, with or without pre-treatments, on alginate's structural and techno-functional properties is also discussed.

Key findings: The extraction process parameters play a key role and significantly influence the quality of alginate and the extraction yield. Innovative technologies have the potential to enhance extraction capacity, improve process efficiency, and promote environmental friendliness. Research efforts are focused on achieving sustainable alginate production and co-production of other value-added products. This review provides useful information to the alginate industry towards a more sustainable approach and foster the diversification of high-value products.

1. Introduction

Brown algae are the second most abundant group of marine multicellular algae, being considered an excellent potential source of highly valuable molecules, including phenolic compounds, carotenoids, proteins, and polysaccharides (Hakim & Patel, 2020). The main polysaccharides found in the cell wall of brown algae are alginate (representing around 40% of the dry matter of seaweed), fucoidan, and laminarin (Łabowska et al., 2019). Alginate is a linear copolymer constituted by (1 → 4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues randomly distributed along the carbohydrate chains (Fig. 1) (Usov & Zelinsky, 2013). These uronic acids can form homogeneous blockchains of MM (M blocks) or GG units (G blocks) and chains with alternate blocks of mannuronic acid and guluronic acid (MG blocks) (Makarova et al., 2023). Alginate structure, mainly defined by sequence pattern, G-block length, M/G ratio and molecular weight, is key in determining its physicochemical and technological properties (Fertah et al., 2017).

Alginate is characterized by its biodegradability, biocompatibility, non-toxicity, and high viscosity, as well as its wide range of valuable biological and technological properties. Alginates have been used as texturizing ingredients (having thickening and gelling ability) (Benjamin et al., 2018), emulsifiers (Yang et al., 2020), fat substitutes (Kang et al., 2020), tenderizers for meat (Shi et al., 2020), natural edible coatings (Tabassum & Khan, 2020), biodegradable films (Ehsani et al., 2020), hybrid biopolymeric nanofibers (Yilmaz et al., 2020), and even as encapsulating matrices for drug delivery applications (Rhein-Knudsen et al., 2017), membrane development (Kurkuri et al., 2002), as well as a polymeric system in various biomedical applications, such as tissue engineering, biosensing, wound healing, etc. (Hegde et al., 2022). Therefore, alginate has been the subject of research in various fields related to the exploitation of marine biological resources, and it has been found that the structure and composition of alginate significantly influence its biological activity (Li, Zhu, et al., 2023).

Commercially, alginate is mainly extracted from brown seaweed as soluble sodium alginate. Annual world production of brown algae in dry weight is estimated to be approximately 85 thousand tons, from which about 23 thousand tons of alginate is obtained (Bertagnolli, da Silva, &

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Annex B. List of additional publications

Polímeros naturales obtenidos de residuos agroindustriales y sus potenciales aplicaciones

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Resumen

En el contexto de la economía circular, en el que se busca un aprovechamiento integral de los recursos, la valorización de residuos generados en actividades agroindustriales para la obtención de materiales es un área de creciente interés. En concreto, la obtención de biopolímeros a partir de residuos es una opción muy atractiva, ya que permitiría, por un lado, abaratar el coste de materiales necesarios para que puedan competir con los plásticos convencionales, sobre todo en aplicaciones de menor coste añadido (como es el área del envasado alimentario) y, por otro lado, valorizar macromoléculas presentes en dichos residuos con potenciales aplicaciones en distintas áreas. En este artículo mostramos el potencial de diferentes residuos agroindustriales, concretamente los residuos del cultivo de setas, corteza de sandía y de algas marrones para la obtención de biopolímeros de interés industrial.

Palabras clave: pectinas, fucoidanos, beta-glucanos, alginatos, laminarina.

Abstract

In the context of circular economy, in which an integral exploitation of resources is sought, the valorisation of agro industrially generated residues for obtaining materials is an area of increased interest. Specifically, the extraction of biopolymers from residues is a very attractive option as it would allow, on one hand, to lower the price of materials needed to compete with conventional plastics, especially in low added-value applications (like in the case of food packaging) and, on the other hand, to valorise macromolecules present in these residues with potential applications in different areas. In this dissemination paper, we will show the potential of various residues, concretely residues from mushroom cultivation, watermelon rinds and residues from brown algae for obtaining biopolymers with industrial interest.

Keywords: pectins, fucoidans, beta-glucans, alginates, laminarin.

Biotintas de alginato para impresión 3D: efecto de la estructura en sus propiedades reológicas

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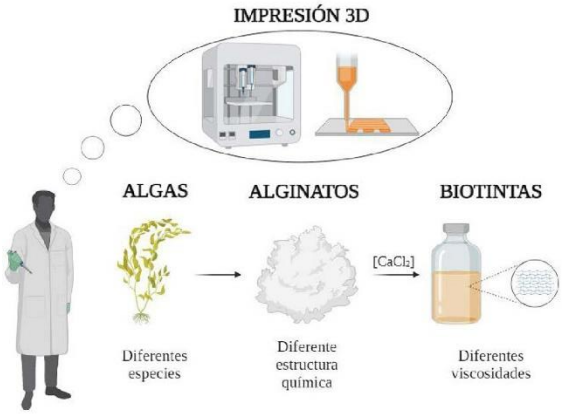

Resumen

El alginato es un polímero de origen natural que gelifica rápidamente a temperatura ambiente en presencia de cationes divalentes, ampliamente utilizado en impresión 3D por extrusión. En este trabajo se emplearon tres alginatos con diferentes características estructurales, uno comercial y dos extraídos en el laboratorio para la preparación de biotintas de alginato. Para la preparación de las biotintas, se entrecruzaron los alginatos con diferentes concentraciones de cloruro cálcico. Los resultados obtenidos mediante pruebas de vial invertido y medidas de flujo mostraron que el alginato con relación menor M/G dio lugar a biotintas con mayores valores de viscosidad a menores concentraciones de cloruro cálcico. Este trabajo pretende demostrar la gran influencia que la estructura del alginato tiene sobre la preparación de biotintas que puedan ser utilizadas para la producción de andamios por impresión 3D.

Palabras clave: alginato, estructura, biotinta, viscosidad, impresión 3D.

Abstract

Alginate is a natural polymer with a fast gelation process at room temperature in the presence of divalent cations, which is widely used in 3D printing by extrusion. In this work, three different alginates with different structural characteristics, one commercial and two extracted in the lab from algae were used to prepare alginate bioinks. These bioinks were crosslinked with different concentrations of $CaCl_2$. Results from the inverted vial and flow measurements allowed to conclude that alginate with the lowest M/G ratio gave rise to bioinks with higher viscosity at lower concentrations of $CaCl_2$. This work demonstrates the influence that the alginate structure possesses for the generation of bioinks that will be used to produce scaffolds by 3D printing technique.

Keywords: alginate, structure, bioink, viscosity, 3D printing.

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