Bioactive peptides and carbohydrates from seaweed for food applications: Natural occurrence, isolation, purification, and identification

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ABSTRACT
Macroalgae or seaweed are relative un-explored and promising sources of novel molecules for the food industry including peptides and carbohydrates for their use as functional foods and nutraceuticals. Several algal-derived bioactive compounds have shown a wide range of biological activities both in vitro and in vivo, i.e. antihypertensive and antioxidant, that are strongly associated with the chemical structure of the peptides or carbohydrates. Multiple improvements in the purification and analytical tools to characterize these compounds have been reported in recent years, aiming to gain further insight into the complexity of different molecular structures of bioactive peptides and carbohydrates. This paper discusses the variable composition of algae and the opportunities of the use of this biomass to obtain novel functional bioactive peptides and carbohydrates for functional food applications. The main biological activities of the discovered bioactive peptides and carbohydrates together with the analytical procedures used to purify and characterize multiple compounds are also discussed.

1. Introduction

Traditional food ingredients have gained increased scientific attention for their potential to generate functional foods and nutraceuticals. Functional foods are defined as foods that may impart a health benefit to the consumer that goes above and beyond basic human nutrition [1]. Several successful examples in the market include the use of fortified products with omega-3 polysaturated fatty acids of algal origin [1] and beverages containing bioactive peptides such as Evolus® or Calpis® [2].

Algae are a rich source of multiple valuable macro- and micro-nutrients, including proteins, carbohydrates, phenols, vitamins, and minerals [1,3]. These compounds were traditionally exploited by the food and animal feed industries due to their nutritional value, i.e. as a source of proteins rich in essential amino acids [4] or the use of non-digestible carbohydrates from seaweed as a source of dietary fibre [5]. This rich and variable composition of algae is being explored for its potential to obtain functional ingredients including bioactive peptides and carbohydrates [1,5–8]. Bioactive peptides are sequences of 2 to 30 amino acids in length that display hormone-like beneficial properties when released from their parent protein [9,10]. Biological effects described to date include antihypertensive, antioxidant, antithrombotic, antimicrobial, and immunomodulatory properties [1,5]. Currently there are a number of products in the market containing bioactive peptides, including a peptide soup containing the peptides LKPNM obtained from dried bonito and a sardine-derived product rich in the di-peptide VY [1]. Moreover, algal carbohydrates such as alginates or carrageenan are industrially commercialized as thickening and gelling agents with multiple applications in the food, textile, biotechnological, and biomedical industries [11]. These marine carbohydrates have recently come under the spotlight as functional food ingredients [5]. Seaweed carbohydrates such as alginites, carrageenan, and fucoidan showed a wide range of biological activities including anti-inflammatory, anticoagulant, antioxidant, antiproliferative, and immunostimulatory activities both "in vitro" and/or "in vivo" [5]. Furthermore, algal polysaccharides such as laminarin are non-digested in the upper gastrointestinal tract and are considered as dietary fibre [5]. The consumption of dietary fibre has a positive influence on human health with beneficial impacts including a reduced risk of suffering from colon cancer and constipation, but also reduced hypercholesterolemia, obesity, and diabetes [12].

The biological effects displayed by these marine bioactive peptides and carbohydrates depend on the chemical structure of these molecules [1,13]. These differences could be attributed to biological factors affecting the seaweed biomass (i.e. season and location of collection), but also to structural modifications of the molecules during the processes of
extraction and purification [5,14]. The increased interest in developing functional bioactive peptides and polysaccharides represents a challenge for analytical scientist trying to identify the relationship between the chemical structure of these compounds and their biological activities. Multiple analytical approaches have been used to date to purify and analyse the chemical structure of bioactive peptides and carbohydrates [5,15].

This paper discusses the variable composition of algae and the opportunities of the use macroalgae to obtain novel functional bioactive peptides and carbohydrates for functional food applications. The main biological activities of the discovered bioactive peptides and carbohydrates together with the analytical procedures used to purify and characterize multiple compounds are also discussed.

2. Chemistry and biochemistry of macroalgae

Macroalgae or seaweed comprise a heterogeneous group of approximately 10,000 species [16], being only few of them used for food applications, mainly as food additives or flavouring materials, especially in Asian countries. Indeed, seaweed is served in approximately 21% of meals in Japan [17] and some eastern varieties are increasingly consumed in western countries since oriental food became more popular.

Algae can be divided into three main groups or phyla: brown (Phaeophyceae), red (Rhodophyceae), and green (Chlorophyceae). Moisture content of fresh marine algae is very high and can account for up to 94% of the biomass [18]. In addition, algae have a highly variable composition, with large differences in their final content in minerals (including calcium, phosphorus, and potassium), vitamins, proteins, lipids, and fibre [8]. Seaweed composition, i.e. protein and carbohydrate contents, depends not only on the species, but also on the time of collection and habitat, and on external conditions such as temperature, light intensity, and nutrient concentration in water [16].

2.1. Protein and amino acid contents in macroalgae

2.1.1. Protein content

As mentioned previously, the protein content of seaweed differs depending on different factors. In addition, comparison of the protein content among algae is difficult because of methodological differences, especially during protein extraction, and the large number of species identified to date [19]. Although brown seaweed usually contains a low protein content when compared to that of green or red seaweed, Lourenço, Barbarino, De-Paula, Pereira and Marquez [19] reported a relatively high protein content ranging between 10 and 15% for the species Chnoospora minima, Dictyota menestrailis, Padina gymnospora, and Sargassum vulgare. Common brown seaweed includes Laminaria digitata, Ascophyllum nodosum, Fucus vesiculosus, and Himanthalia elongata. The average protein content of Laminaria digitata was recently calculated as 6.8%, with highest and lowest protein levels in the first and third quarter of the year, respectively [20]. This value was similar to that obtained by Peinado, Girón, Koutsidis and Ames [21], who calculated the protein content of Laminaria digitata as 5.8%. Comparable results were observed in this study for other brown algae species such as Laminaaria hyperborea, Saccharina latissima, and Alaria esculenta with average protein levels ranging between 6.8 and 11.0% of the total dry weight (DW) [20] and Ascophyllum nodosum, Pelvetia canaliculata, Fucus vesiculosus, and Fucus spiralis which presented an average protein content of 5.2, 7.3, 5.8, and 5.9%, respectively [21].

Although previous studies highlighted that in some green seaweed, such as those species belonging to the genus Ulva, the protein content can range between 10 and 26% of the total DW [22], a more recent study reported that the protein content of Ulva lactuca, harvested between May and June, was below 8.6% [23]. Møhre, Malde, Eilertsen and Ellevoll [23] obtained similar results from other green algae such as Cladophora rupestris and Enteromorpha intestinalis, which were also harvested between May and June in Scotland, and had a total protein content of 3.4 and 11.3%, respectively. In addition, Lourenço, Barbarino, De-Paula, Pereira and Marquez [19] calculated the protein content of the green algae Caulerpa fastigiata, Caulerpa racemosa, Codium decorticatum, Codium spongiosum, Codium taylorii, and Ulva fasciata, collected in Brazil in autumn, and results suggested that the protein content varied between 11 and 20%, depending on the species.

Higher protein concentrations were reported in red seaweed, such as Phoryphyra tenera and Palmaria palmata, which can be up to 47% [22]. However, in a more recent study, Møhre, Malde, Eilertsen and Ellevoll [23] calculated the protein content of Palmaria palmata and Vertebrata lanosa, harvested between May and June in Scotland, as 12.2 and 11.5%, respectively. Results correlate well with those obtained by Galland-Irmoli, Fluereuse, Lamghari, Luçon, Rouxel, Barbaroux, Bronovicki, Villauime and Guéant [24], who showed that the protein content of Palmaria palmata significantly varied depending on the season, with the highest protein content (21%) at the end of the winter season and the lowest (11%) in early autumn. The total protein content of the species Acanthophora spicifera, Aglaosthannion uruguayense, Cryptomonena seminervis, Gracilariopsis tenuifrons, Laurencia flagellifera, Pilocaum brasiliense, Petrocladiella capilacea, and Porphyra acanthophora was determined by Lourenço, Barbarino, De-Paula, Pereira and Marquez [19], who reported a variable protein content ranging between 12% (Acanthophora spicifera) and 27% (Aglaosthannion uruguayense) on a DW basis.

Numerous seaweed-derived proteins have been identified, and these can be used for the generation of biologically active peptides with health-promoting properties. However, among the different classes of proteins identified, it is important to emphasize lectins and phycobiliproteins. Lectins are glycoproteins with carbohydrate-binding properties which allows them to agglutinate microbes, yeasts, tumour cells, and erythrocytes [25]. Lectins are very interesting for a diversity of applications in immunological and histochemical studies [26] as well as in agricultural and medical applications due to their antimicrobial, antitumor, and antiviral activities [27]. In addition, phycobiliproteins are brilliantly coloured and highly fluorescent components which are currently used in numerous fluorescence-based techniques such as immunofluorescence, fluorescence-activated cell sorting, and fluorometric microplate assays [28].

2.1.2. Amino acid composition

The amino acid composition of seaweed proteins has been repeatedly studied and compared to that of other foods. Most seaweed contain all the essential amino acids, and aspartic and glutamic acids were suggested to constitute a large part of their amino acid fraction [22]. Indeed, these two residues represent 22 and 44% of the total amino acids in certain brown seaweed [29]. Brown seaweed have been suggested as rich sources of threonine, valine, leucine, lysine, glycine, and alanine previously, and amino acids such as cysteine, methionine, histidine, tryptophan, and tyrosine were recorded at lower levels [18]. Lourenço, Barbarino, De-Paula, Pereira and Marquez [19] studied the amino acid profile of four brown seaweed species namely Chnooospora minima, Dictyota menestrailis, Padina gymnospora, and Sargassum vulgare. All samples were collected in Brazil in June and September, and showed a high content of aspartic and glutamic acid, and a relatively high (over 8%) content of leucine.

The green seaweed Ulva pertusa contains high levels of proline [30], and the amino acid profile of the green seaweed Ulva lactuca revealed that this specie contained all the essential amino acids, in levels which were comparable to the dietary recommendations proposed by the Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO) [31]. Lourenço, Barbarino, De-Paula, Pereira and Marquez [19] reported high concentrations of aspartic and glutamic acids in six species of green algae, namely Caulerpa fastigiata, Caulerpa racemosa, Codium decorticatum, Codium spongiosum, Codium taylorii, and Ulva fasciata. The authors also recorded a relatively
high content of the residues leucine and alanine for all the studied species. In addition, leucine, threonine, isoleucine, valine, and methionine are well represented in the essential amino acid fraction of *Palmaria palmata*, with similar leucine and valine concentrations to those generally reported for ovalbumin, and similar isoleucine and threonine contents to those reported for leguminous proteins [22].

The amino acid profile of the red seaweed *Hypnea charioide and Hypnea japonica* revealed that both species contained all the essential amino acids (excluding tryptophan), which accounted for approximately 42–48% of the total amino acid content [31]. Red seaweed proteins also contain high quantities of glutamic and aspartic acid. However, previous studies suggested that the quantities of glutamic and aspartic acid in red seaweed were significantly lower than those observed in brown and green seaweed [32]. Lourenço, Barbarino, De-Paula, Pereira and Marquez [19] reported a concentration of aspartic and glutamic acid ranging between 10 and 15% in numerous red algae species including *Acanthophora spicifera*, *Aglaothamnion uruguayense*, *Cryopteris seminervis*, *Gracilariapis tenuifrons*, *Laurencia flagellifera*, and *Porphyria acanthophora*. Relatively high concentrations (over 7%) of leucine, valine, and glycine were recorded for the red seaweed *Porphyria tenra* [30], and concentrations of approximately 10% of threonine, proline, and histidine were reported for *Acanthophora delilii* [33]. Similar results were obtained for the red algae *Hypnea musciformis*, *Sebaldia polyactyla*, and *Scinia indica*, which had an approximate proline concentration of 10% [33].

Overall, glutamic and aspartic acid were the most abundant amino acids in most species. Methionine content was reported to be low in most of the species, and the mean values for individual amino acids were, in the majority of the reviewed studies, similar in brown, green, and red algae.

### 2.2. Carbohydrate contents in macroalgae

Seaweed also contain large amounts of polysaccharides with important functions for the macroagal cells including structural and energy storage [5]. The total polysaccharide concentrations in macroalgae ranged from 4 to 76% of DW with the highest contents described in *Acanthophora*, *Porphyra* and *Palmaria*; although other green species such as *Ulva* showed contents of up to 65% on a DW basis [18]. Seaweed polysaccharides include relevant bioactive compounds such as alginate, carrageenan, fucoidan, and laminarin. Furthermore other polycoscoloids from macroalgae, i.e. agar, are commonly used in the food and animal feed industries as stabilisers, thickeners, and emulsifiers [13,18].

Alginites are linear unbranched polysaccharides containing β-3-linked mannuronic acid (M) and α-1,4-guluronic acid (G) units linked by 1–4 glycosidic bonds [35]. These monomers are mainly arranged in sequences of homopolymeric blocks (MM and GG blocks) and heteropolymeric blocks (MG or GM blocks) [34]. Alginites are currently produced from brown seaweed of the genus *Laminaria*, *Saccharina*, *Lessonia*, *Macroystis*, *Durvillaea*, *Ecklonia*, and *Acanthophora* [34], being *Durvillaea potatorum* and *Macroystis pyrifera* the species with the highest yields of alginites of up to 55 and 45% of DW, respectively [35,36].

Carrageenans (CRGs) are sulphated linear galactans, consisting of alternating β-1,4- and α-1,3-linked D-galacto-syl residues (D- and G-units). Carrageenans are characterized depending on the disaccharide repeating unit and the degree of sulphation of the molecule [37]. The three most industrially exploited types, namely Kappa, Iota, and Lambda are distinguished by the presence of one, two, and three estersulphate groups per repeating disaccharide unit respectively. However, the sulphate contents of the commercial carrageenans can vary depending on the seaweed species or batch [38]. *Chondrus crispus* and *Kappaphycus* sp. contain up to 71 and 88% of carrageenan, respectively [18].

Fucoidans are cell wall polysaccharides present in brown macroalgae which play a crucial role in the protection of macroalgae against environmental challenges [5]. Fucoidans are fucose containing sulphated polysaccharides formed of a backbone of (1 → 3)-linked α-L-fucopyranosyl or alternating (1 → 3)- and (1 → 4)-linked α-L-fucopyranosyl residues with variable degrees of sulphation [5]. These polysaccharides are a chemically diverse group with molecular weights ranging from 43 to 1600 kDa [39]. The content and composition of fucoidan varies depending on the season and seaweed species [5]. *Fucus vesiculosus* had fucoidan levels ranging from 16 to 20%, while *Undaria pinnatifida* contained approximately 1.5% [18]. Furthermore, recent studies also showed differences in the fucoidan content depending on the stage of growth of the seaweed. Cultured *Laminaria japonica* showed the highest contents of fucoidan in October, when the seaweed blades were matured rather than at younger stages of development [40].

Laminarins are a group of energy storage polysaccharides composed by 1,3-linked β-D-glucose monosaccharidic acids with variable branching at β-1,6 [5]. The laminarin structure may vary in the degree of branching and degree of polymerization depending on the seaweed species, season, and other parameters such as extraction and purification procedures [5]. Laminarin is present in high yields in *Laminaria* sp. (up to 32%, depending on the season) and *Saccharina* sp., but these molecules have also been described in *Acsophyllum*, *Fucus*, and *Undaria* sp. [18]. Seasonal studies on *Laminaria* sp. showed higher accumulation of storage carbohydrates during summer and autumn that will be used during the winter as an energy source for new tissue growth [21].

### 3. Seaweed-derived bioactive compounds

#### 3.1. Bioactive peptides derived from edible seaweed

As mentioned previously, algae are known to contain high concentrations of high quality polysaccharides, minerals, and vitamins, as well as bioactive bioactive compounds including proteins, lipids, and numerous polyphenols [18]. Bioactive peptides have been generated from a wide variety of natural sources including meat [10,41,42], milk [43,44], cereals [45,46], and fish [47,48]. Although there has been a growing demand to isolate bioactive peptides from marine algae [9,49], the number of biologically active peptides generated from seaweed is still limited. A number of functional foods containing seaweed-derived peptides are currently commercialized, mainly in Japan. The Japanese Ministry of Health and Welfare established a policy for approving some selected functional food products as Foods for Specified Health Uses (FOSHU) whose health claims are legally permitted [50]. Seaweed-derived peptide-containing products with FOSHU approved anti-hypertensive claims include Wakame peptide jelly (Riken Vitamin Co., Ltd., Tokyo, Japan) and Nori peptide S (Shirako Co., Ltd., Tokyo, Japan) [51].

#### 3.1.1. Antioxidant peptides

A free radical can be defined as a chemical substance capable of independent existence with one or more unpaired orbital electrons, and can be produced either from normal cell metabolism in situ or from external sources such as radiations or pollution [52]. When free radicals are produced in excess, and cannot gradually be destroyed, their accumulation in the body generates a phenomenon called oxidative stress [52]. The regulation of oxidative stress is an important factor in both, tumour development and responses to anticancer therapies [53]. Antioxidants can counteract oxidative stress, and these are either produced by the human body in situ or incorporated through diet.

Seaweed are a rich source of antioxidants. For example, the known antioxidant peptides carnosine and glutathione, which are generally present in animal muscle, were identified in seaweed species previously [54]. In addition, as mentioned previously, seaweed can contain relatively high protein content, and seaweed-derived proteins can be used for the generation of antioxidant hydrolysates and peptides. For example, Heo, Park, Lee and Jeon [55] generated a large amount of antioxidant hydrolysates of proteins isolated from *Ecklonia cava*, *Ishige*...
okamureae, Sargassum fulvellum, Sargassum hornerti, Sargassum coreanum, Sargassum thunbergii, and Scytosiphon lomentaria using the commercial enzymes Alcalase, Flavourzyme, Neutrase, Protamex, and Kojizyme. In this study, antioxidant activity was assessed using four different anti-

tioxidant scavenging assays and results demonstrated that the anti-

oxidant potential of the Alcalase hydrolysates of *Sargassum hornerti* were do

dependent and thermally stable. In a more recent study, Cian, Martínez-Augustín and Drago [56] obtained different enzymatic hy-

drolysates from co-products of *Porphyra columbina* using alcalase, trypsin, and combinations of both. In addition, Harnedy, O’Keeffe, and FitzGerald (2017) generated an enzymatic hydrolysate of *Palmaria palmata* using the food-grade enzyme Corolase PP. The generated hy-

drolysate was sequentially fractionated using solid phase extraction and

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Table 1: Seaweed-derived peptides identified using LC-MS/MS during the period 2013–2018.

| Matrix  | Peptide sequence | Activity | Sample purification | Mobile phase | Column | Flow rate | References |
|---------|-----------------|----------|---------------------|--------------|--------|-----------|------------|
| Palmaria palmata | IRLIVLMPILMA | Renin inhibitory, antihypertensive in SHRs | Removal of polyethylene glycols using titanium dioxide. | Solvent A: 0.1% (w/v) formic acid in water Solvent B: 0.1% (w/v) formic acid in acetonitrile | ACQUITY BEH C18 (100 μm × 200 mm, 5.0 μm) and Symmetry C18 (180 μm × 200 mm, 5.0 μm) | 5 μL/min | Fitzgerald, Aluko, Hossain, Rai and Hayes [7] |
| Palmaria palmata | IRLIIVLMPILMA | | | | | | |
| Porphyra spp. | VPGTPKNLDSPR and MPAPSCALPRSVVPPR | Antiproliferative | MWCO filtration using 3, 5, and 10 kDa membranes followed by SEC using a Sephadex G-15 column (2.6 × 400 mm) eluted with water at 0.35 mL/min | MALDI-TOF-MS. 5 mg/mL alpha-cyano-4-hydroxycinnamic acid dissolved in 60:40 acetonitrile:water containing 0.1% trifluoroacetic acid was used as a matrix. | | | – Fan, Bai, Mao and Zhang [123] |
| Porphyra haitanensis | QTDDNHSNVLWAGFSR | Antiproliferative | MWCO filtration using 3, 5, and 10 kDa membranes followed by SEC using a Sephadex G-15 column (2.6 × 400 mm) eluted with water at 0.35 mL/min | MALDI-TOF-MS. 5 mg/mL alpha-cyano-4-hydroxycinnamic acid dissolved in 60:40 acetonitrile:water containing 0.1% trifluoroacetic acid was used as a matrix. | | | – Mao, Bai, Fan and Zhang [123] |
| Palmaria palmata | SDITRPGGNM | Antioxidant | SPE using a Phenomenex Strata-X 5 g/60 mL C18 followed by RP-HPLC with 0.1% formic acid in water (Solvent A) and 80:20 acetonitrile:water containing 0.1% formic acid at 5 mL/min | | ACQUITY BEH C18 (2.1 × 50 mm, 1.7 μm) | 0.2 mL/min | Harnedy, O’Keeffe and Fitzgerald [74] |
| Palmaria palmata | NIGK | PAF-AH inhibitory | RP-HPLC using a Phenomenex C10 column (100 μm × 212 mm, 5 μm) eluted with 0.1% TFA in water at a flow rate of 1 mL/min | | | | – Fitzgerald, Gallagher, O’Connor, Prieto, Mora-Soler, Grealy and Hayes [74] |
| Porphyra spp. | GGSK and ELS | α-Amylase inhibitory | | | | | Admassu, Gasmalla, Yang and Zhao [126] |
| Palmaria palmata | VYRT, LDY, LRY, FEQDWAS | ACE-I inhibitory | Sequential filtration by Miller-GV (0.22 μm) and Millipore UV (0.20 μm). RP-HPLC using a Methylsil 35C6 column (4.6 × 150 mm) with a linear gradient of ACN (5–80%) in 0.1% formic acid at 1 mL/min. Peptides analysed by Edman degradation method using a Procise 492HT protein sequencer and MALDI-TOF/MS/MS | | | | Furuta, Miyabe, Yasui, Kinoshita and Kishimura [127] |
anticoagulant, antiviral, antiproliferative, and antitumour properties [5,13]. These biological activities could be attributed to the high content of sulphated polysaccharides of seaweed (i.e. carrageenans, and fucoidan), which cannot be found in terrestrial plants [13]. Furthermore, seaweed polysaccharides such as alginites, fucoidan, and laminarin, are non-digestible in the upper digestive track of the animals, being macroalgae considered as a rich source of dietary fibre [5,13]. Despite these beneficial properties, seaweed carbohydrates are being currently exploited industrially for their physicochemical properties, i.e. the ability of alginites and carrageenans to form gels, allowing their use as thickening, gelling, and protein-suspending agents [13,38].

Alginites from brown macroalgae showed a wide potential for its application in the biomedical and bioengineering fields, due to its gelling capacity, biocompatibility, biodegradability and lack of toxicity [75]. Nutritionally, alginites are dietary fibres and contribute to the gut’s health due to its water binding properties and thus its effects in faecal bulking, decreasing the colonic transit times which could be beneficial in preventing colonic cancer [76]. Furthermore, the Food and Drug Administration (FDA) named alginic acid and its salts as generally regarded as safe (GRAS) ingredients for oral administration [75].

Other macroalgal polysaccharides such as laminarin and fucoidan are also considered as dietary fibres [5]. Laminarin modulates the intestinal metabolism by affecting the biochemical and microbiology of the human gut microflora [77]. In vivo studies showed a down-regulation of pro- and antiinflammatory cytokines in post-weaning pigs supplemented with laminarin [78]. Laminarin and fucoidan supplementation to sows during the periods of pregnancy and lactation showed an increase on immunoglobulin concentrations in the colostrum and a decrease of E. coli in suckling [79] and weaned piglets [80].

Further biological properties described for fucoidan with potential biomedical applications include the antioxidant and antiinflammatory activities, anticancer, anticoagulant and antithrombotic activities related to the structure and the degree of sulphation of the polysaccharide [81,82]. Fucoidan from Fucus evanescens showed anticoagulant activities similar to other drugs such as heparin [50,51]. Fucoidan extracts followed by ESI-MS analysis was used to determine the monosaccharide composition of fucoidan extracts using a C-18 column (ZORBAX Eclipse XDB, 5 μm) following the acidic hydrolysis and derivatization of the compounds using 1-phenyl-3-methyl-5-pyrazolone (PMP) for its detection with UV at 250 nm. UPLC systems were also used to purify and characterize multiple carbohydrates in products of plant [92,93] and animal origin [94]. Recently, Adrien, Dufour, Baudouin, Maugard and Bridau [96] used UPLC coupled with MS to analyse the monosaccharide composition of seaweed extracts from Himanthalia elongata, Laminaria digitata, Ascophyllum nodosum, Fucus vesiculosus, Ulva lactuca, and Chondrus crispus with anticoagulant properties.

Nevertheless, the direct application of one LC method does not allow the detection of the investigated compounds, especially if they are present in the mixture in low quantities. Therefore, multidimensional approaches are generally used. Ion exchange chromatography (IEC) is one of the classic methods among multidimensional approaches. IEC achieves the separation of peptides and carbohydrates based on the adsorption of the charged analytes on to immobilized ion exchange groups of an opposite charge in the stationary phase of the column and its elution by changing the concentration or pH of the mobile phase [5]. Carbohydrates from seaweed are normally separated using IEC containing positively charged resins as stationary phase with affinity for negatively charged analytes (anion-exchange chromatography (AEC)). AEC is one of the most commonly used purification techniques used to separate alginates and sulphated polysaccharides such as fucoidan [5] and carrageenan [38]. The sulphate ester groups linked to the backbone of these seaweed polysaccharides exhibit high anionic charges, which facilitate the use of AEC following different experimental conditions described in detail in Table 2. The elution of the adsorbed carbohydrates from different resins was performed mainly by stepwise [97] or linear gradient [98–100] of NaCl or NaOH to achieved multiple polysaccharide fractions. In addition, IEC purification of fucoidan extracts followed by ESI-MS analysis was used to determine the monosaccharide composition of the different fucoidan fractions obtained from Sargassum fusiforme [97], while IEC followed by ESI-MS/MS was used to characterize fucoidan carbohydrates with anticancer activity from Padina boryana [101]. ESI-MS/MS was used to characterize the monosaccharide units of immunomodulatory and anti-HIV fucoidan fractions from Sargassum mucicure, Sargassum polycystum.
Table 2

| Compounds of interest | Analytical method | Experimental conditions |
|-----------------------|-------------------|--------------------------|
| Algionates            | SEC               | Shodex SEC Shodex OHpak SB-806 HQ (8 × 300 mm) column. Eluted with 0.1 M NaOAc (pH 6). Flow rate: 1 mL/min. Refractive index detector (RI) Rhein-Knudsen, Ale, Ajalloueian and Meyer [107]. |
| Algionates            | IEC               | CarboPac PA1 (4 × 250 mm) column. Eluted using 0.1 M NaOH increasing up to 0.16 M NaOH, followed with 0.19 M sodium acetate. Flow rate: 1.50 mL/min. Pulsed amperometric detector (PDA) Cong, Chen, Liao, Xiao, Wang, Qin, Dong and Ding [97]. |
| Fucoidan              | SEC               | Shodex Shodex Asahipak GS-520 HQ and GS-620 HQ (7.5 mm × 300 mm) at 50 °C with elution by 0.5 M ammonium bicarbonate (0.8 mL/min). Refractive index detector (RI) Usoltseva, Anastyuk, Ishina, Isakov, Zvyagintseva, Thinh, Sterner, Ribeiro, Gröndahl and Edlund [100]. |
| Fucoidan              | IEC               | Q-Sepharose (65 ml, 5.0 ml/mL solution). Eluted with linear gradient 0-2 M NaCl and NaCl, followed with 0.3 M NaOH. Flow rate: 0.5 M ammonium bicarbonate (0.8 mL/min). Carboxypack PA-100 (4 × 250 mm) column. Eluted with 0.1 M NaOH (pH 6). Flow rate: 1 mL/min. Refractive index detector (RI) Zadorozhny, Dmitrenok and Ermakova [101]. |
| Alginate              | IEC               | DEAE-cellulose (60 ml, 0.1 M NaCl) column. Eluted with linear gradient 0-2 M NaCl. Flow rate: 3 mL/min. Carboxypack PA-100 (4 × 250 mm) column. Eluted with 0.1 M NaOH (pH 6). Flow rate: 1 mL/min. Refractive index detector (RI) Imbs, Ermakova, Malyarenko, Isakov and Zvyagintseva [98]. |
| Alginate              | IEC               | DEAE-cellulose (60 ml, 0.1 M NaCl) column. Eluted with linear gradient 0-2 M NaCl. Flow rate: 3 mL/min. Carboxypack PA-100 (4 × 250 mm) column. Eluted with 0.1 M NaOH (pH 6). Flow rate: 1 mL/min. Refractive index detector (RI) Earn, Zadorozhny, Dmitrenok and Ermakova [101]. |
| Alginate              | IEC               | DEAE-cellulose (60 ml, 0.1 M NaCl) column. Eluted with linear gradient 0-2 M NaCl. Flow rate: 3 mL/min. Carboxypack PA-100 (4 × 250 mm) column. Eluted with 0.1 M NaOH (pH 6). Flow rate: 1 mL/min. Refractive index detector (RI) Earn, Zadorozhny, Dmitrenok and Ermakova [101]. |

* Abbreviations are as follows: exclusion chromatography (SEC) and ion-exchange chromatography (IEC).
with LC for molecular fractionation prior to MS analysis (LC/ESI-MS) or to MS in tandem (LC/ESI-MS/MS) are powerful methods recently used to analyze peptides and carbohydrates in a complex biological samples, including seaweed extracts [97,101]. The conversion of the analytes to a gaseous phase performed during ESI could be difficult to achieve when analysing complex proteins and carbohydrates due to their high polarity and mass [106]. In MALDI, the analytes are normally mixed with a matrix to form a solid-state mixture that will be later irradiated with UV laser pulses (337–355 nm), generating ions analysed based on its mass-to-charge ratio by MS. Choosing the appropriate matrix for an analyte is crucial for successful MALDI analysis [112,113].

MALDI continues to be a major technique for the analysis of carbohydrates [112]. MALDI-TOF mass spectra was used to analyse the structure of antiinflammatory ω-carrageenans from red algae *T Macha corpus crinitus* [114]. Furthermore, MALDI was recently used to analyse fucoidan fractions with multiple biological properties from seaweed, including *Saccharina chioroides* [115], *Saccharina gurjanovae* [116], *Sargassum muticum* [117] and *Alaria angusta* [118].

NMR spectroscopy is one of the most powerful analytical tools available to date to determine the structure of bioactive compounds [38,119,120]. NMR is based on the principle of nuclear spin or the fact that when a molecule is placed in a magnetic field, this momentum will be aligned either in the same or opposite direction to the field [119]. This difference in resonance frequency will depend on the chemical environment of the nucleus in a molecule and on the magnetic field strength in an effect known as chemical shift [119]. Most elements have at least one naturally occurring NMR active isotope with variable frequency of occurrence i.e. the natural abundance of $^{1}$H is almost 100% being easier to detect by NMR compared to $^{13}$C and $^{15}$N isotopes that appeared naturally in 1.1 and 0.4% respectively [119]. For characterization of carbohydrates, the resonances of $^{1}$H NMR in the anumeric region (4.4–5.5 ppm) and the $^{13}$C NMR spectra (95–110 ppm) provide useful information on the number of monosaccharide units of the carbohydrates [119]. $^{1}$H NMR resonances bellow 1 ppm indicate the presence of CH$_{2}$-groups and above 2 ppm reveal N-acetyl and/or O-acetyl groups [119]. NMR have been used extensively to characterize the structure of seaweed carbohydrates. Carrageenan can be qualitatively and quantitatively characterized by NMR techniques, allowing the determination of molar ratios and the presence monosaccharides present in the extracts. Both $^{1}$H and $^{13}$C NMR spectroscopy are applicable for these purposes, but $^{1}$H NMR has the advantage of a relatively high sensitivity and lower time of analysis [38,119]. $^{1}$H NMR was also used to identify and characterize the structural units of alginites (MM, GG, MG or GM blocks) from multiple seaweed species [34,100]. $^{1}$H NMR and $^{13}$C NMR spectra together with 2D NMR was also used to characterize a fucoidan fraction with antiangiogenic activity from macroalgae *Sargassum fusiforme* [97]. $^{13}$C NMR analysis was also performed to characterize fucoidan fractions with hepatoprotective activities from *Kjellmniella crassifolia* [91]. NMR spectra using $^{1}$H and $^{13}$C among other techniques were also used by Youssouf, Lallemend, Giraud, Soulé, Bhaw-Luximon, Meilhac, D’Hellencourt, Jhurry and Couprie [121] alginates extracted from *Sargassum binderi* and *Turbinaria ornata* and carrageenans from the red macroalgae *Kappaphycus alvarezii* and *Euchema denticulatum*. NMR was recently used for the characterization of a glycoprotein with 36.24% carbohydrate, composed of rhamnose, galactose, glucose, and mannose with a mole ratio of 38:30:26:6 [122]. NMR spectra proved that the above mentioned glycoprotein, isolated from the brown seaweed *Codium decorticum*, contained protein and carbohydrate portions with (1→4)-linked β-galactose residues and β-linked glucose residues. Senthilkumar and Jayanthi [122] demonstrated the anticancer properties of this compound by different cell lines.

5. Conclusions

Seaweed are relative un-explored and promising of novel molecules for its use as functional foods and nutraceuticals including bioactive peptides and carbohydrates. Several seaweed derived bioactive compounds have shown a wide range of biological activities both in vitro and in vivo. Among the biological activities reported by bioactive peptides and polysaccharides the most promising include antihypertensive, antioxidant, anticoagulant, antitumour, anti-inflammatory, and immunostimulatory activities. These biological activities have increased the scientific interest in developing functional ingredients and to study the relationship between the chemical structure of the compounds and the biological activities. Multiple analytical approaches have been used to date and with the chemical structure of bioactive peptides and carbohydrates. Recent improvements in the purification and characterization methodologies gradually increased the use of novel chromatographic techniques i.e. ultra-performance liquid chromatography (UPLC) and analytical techniques such as NMR spectroscopy ($^{1}$H and $^{13}$C NMR) for qualitative and quantitative analysis and mass spectrometry (ESI and MALDI) to gain further insight into the complexity of different molecular structures of bioactive peptides and carbohydrates.

Authors’ contribution

All authors contributed equally to the conception of the manuscript and to its drafting and approval.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] M. Garcia-Vaquero, M. Hayes, Red and green macroalgae for fish and animal feed and human functional food development, Food Reviews International 32 (2016) 15–45.

[2] M.L. Ragavan, N. Patail, R. Muniyasamy, A. Roy, L. Deo, N. Das, Biochemical characterization and enzymatic profiling of potential probiotic yeast strains, Research Journal of Pharmacy and Technology 12 (2019) 3941–3944.

[3] G. Rajauria, Optimization and validation of reverse phase HPLC method for qualitative and quantitative assessment of polysaccharides in seaweeds, J. Pharm. Biomed. Anal. 148 (2018) 230–237.

[4] M. Garcia-Vaquero, M. Lopez-Alonso, M. Hayes, Assessment of the functional properties of protein extracted from the brown seaweed *Himanthalia elongata* (Linnaeus) SF gray, Food Res. Int. 99 (2017) 971–978.

[5] M. Garcia-Vaquero, G. Rajauria, J.V. D’Ottobiery, T. Sweeney, Polysaccharides from macroalgae: recent advances, innovative technologies and challenges in extraction and purification, Food Res. Int. 99 (2017) 1011–1020.

[6] S. Bleakley, M. Hayes, Algal proteins: extraction, application, and challenges concerning production, Foods 6 (2017) 33.

[7] C. Fitzgerald, R.E. Akoko, M. Hossain, D.K. Rai, M. Hayes, Potential of a renin inhibitory peptide from the red seaweed *Palmaria palmata* as a functional food ingredient following confirmation and characterization of a hypotensive effect in spontaneously hypertensive rats. J. Agric. Food Chem. 62 (2014) 8352–8356.

[8] C. Fitzgerald, E. Gallagher, D. Tademir, M. Hayes, Heart health peptides from macroalgae and their potential use in functional foods, J. Agric. Food Chem. 59 (2011) 6829–6836.

[9] M. Garcia-Vaquero, L. Mora, M. Hayes, In vitro and in silico approaches to
