



Title	Polysaccharides from macroalgae: Recent advances, innovative technologies and challenges in extraction and purification
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Publication date	2016-11
Publication information	García-Vaquero, Marco, Gaurav Rajauria, John V. O'Doherty, and Torres Sweeney. "Polysaccharides from Macroalgae: Recent Advances, Innovative Technologies and Challenges in Extraction and Purification." Elsevier, November 2016. https://doi.org/10.1016/j.foodres.2016.11.016 .
Publisher	Elsevier
Item record/more information	http://hdl.handle.net/10197/8191
Publisher's statement	This is the author's version of a work that was accepted for publication in Food Research International. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Food Research International (9, Part 3, (2017)) DOI:10.1016/j.foodres.2016.11.016
Publisher's version (DOI)	10.1016/j.foodres.2016.11.016

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1 **Polysaccharides from macroalgae: recent advances, novel technologies and challenges in**
2 **extraction and purification**

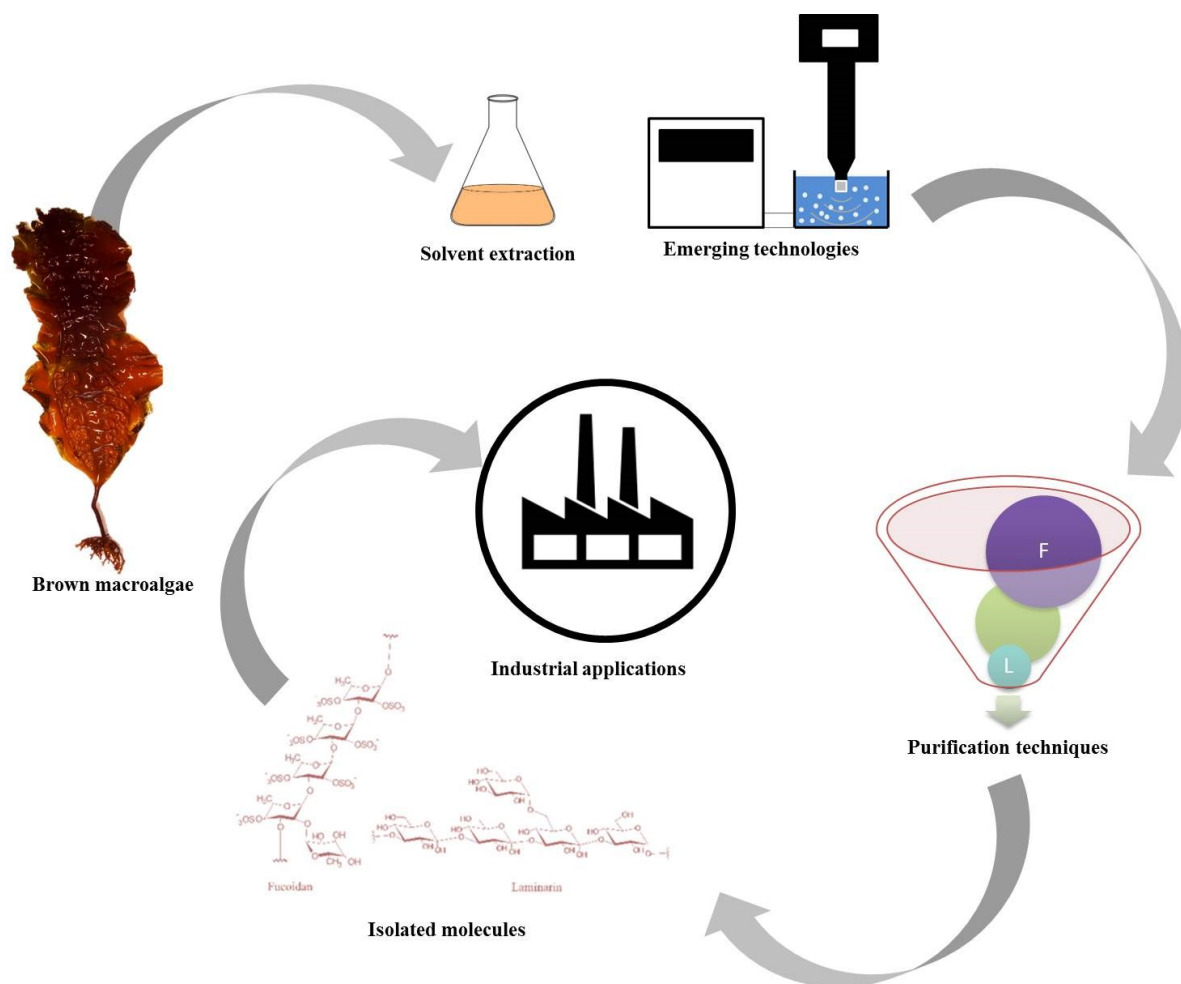
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15 ***Abstract***

16 Polysaccharides obtained from macroalgae have promising prospects both at research and
17 industrial level, and could contribute greatly to the future of marine based bio-economy.
18 Specifically, laminarin and fucoidan from brown macroalgae have a wide variety of potential
19 industrial applications including functional foods, nutraceuticals, pharmaceuticals and
20 cosmeceuticals due to their broad range of biological activities both *in vitro* and *in vivo*.
21 These beneficial biological activities are related to the chemical composition and structure of
22 the macroalgal polysaccharides. The molecular weight, monosaccharide composition and
23 sulphate content of these polysaccharides could be influenced by both macroalgal biology
24 (variations in polysaccharide composition due to macroalgae species and their biological
25 cycle) and different extraction/purification techniques employed to obtain polysaccharide
26 enriched products (i.e. de-sulphation or fragmentation of sulphated polysaccharides). This
27 review focuses on the extraction and purification methods for laminarin and fucoidan
28 macroalgal polysaccharides used in the recent literature. The application of innovative
29 extraction technologies (such as ultrasound, microwave and enzyme-assisted extractions), as
30 well as new purification techniques (i.e. membrane separation), are also discussed together
31 with the challenges concerning molecule structure-function relationship and macroalgae
32 variability.

33

34 **Keywords:** seaweed; bioactivity; fucoidan; laminarin; extraction; purification; innovative
35 technology

36

37 **1. Introduction**

38 Macroalgae are a large and diverse group of marine organisms with more than 10,000
39 different species described to date (Collins, Fitzgerald, Stanton, & Ross, 2016). Marine
40 macroalgae are able to adapt to the changing and extreme marine environmental conditions
41 i.e. salinity, temperature, nutrients, radiation and combination of light and oxygen
42 concentration by producing unique secondary metabolite compounds including
43 polysaccharides (Collins et al., 2016; Rajauria, Jaiswal, Abu-Ghannam, & Gupta, 2010).
44 These macroalgal polysaccharides, particularly fucoidan and laminarin, show a wide range of
45 biological activities such as antiinflammatory, antimicrobial, anticoagulant, antiadhesive,
46 antioxidant, antiviral, antipeptic, antitumour, antiapoptosis, antiproliferative and
47 immunostimulatory in *in vitro* and *in vivo* model systems (Cong et al., 2016; Jin, Zhang,
48 Wang, & Zhang, 2013; Kadam, Tiwari, & O'Donnell, 2015; Lim et al., 2014; Menshova et
49 al., 2015; Moroney, O'Grady, O'Doherty, & Kerry, 2012; Ngo, Wijesekara, Vo, Van Ta, &
50 Kim, 2011; Roohinejad et al., 2016; Smith et al., 2011)

51 A representation of the chemical structure of laminarin from *Laminaria digitata* proposed by
52 Adamo et al. (2011) is presented in Figure 1. Laminarin was described as 1,3-linked β -d-
53 glucose residues with different degrees of branching at β -(1,6) that influence the water
54 solubility of the molecules (Rioux, Turgeon, & Beaulieu, 2010). The structure composed of
55 (1,3)- β -D-glucopyranose residues consisting some 6-O-branching (in the main chain) and β -
56 (1,6) (intrachain links). The laminarin structure may vary in degree of branching, the degree
57 of polymerization and the ratio of (1,3)- and (1,6)-glycosidic bonds. They are uncharged
58 molecules at neutral pH which are stabilized by inter-chain hydrogen bonds, thus unable to
59 hydrolyse in the upper gastrointestinal tract and are classified as dietary fibers (O'Sullivan et
60 al., 2010). Laminarins are energy reserve polysaccharides present in reserve vacuoles inside

61 the macroalgal cell and may constitute up to 35% of the dried weight of the macroalgal
62 biomass (Kadam, Tiwari, et al., 2015).

63 The chemical structure of fucoidan from *Laminaria saccharina* proposed by Cumashi et al.
64 (2007) is shown in Figure 1. Fucoidan or fucose-containing sulphated polysaccharides have a
65 backbone of (1→3)-linked α -l-fucopyranosyl or alternating (1→3)- and (1→4)-linked α -l-
66 fucopyranosyl residues, but also include sulphated galactofucans with backbones built of
67 (1→6)- β -d-galacto- and/or (1→2)- β -d-mannopyranosyl units with fucose or fuco-
68 oligosaccharide branching, and/or glucuronic acid, xylose or glucose substitutions (Ale,
69 Mikkelsen, & Meyer, 2011b). The l-fucopyranose residues may be substituted with sulphate
70 on C-2, C-4 and rarely on C-3 positions (Ale, Mikkelsen, & Meyer, 2011a). Fucoidans are an
71 integral part of the cell walls and intercellular spaces in brown macroalgae, playing a crucial
72 role in the protection of the macroalgae against the effects of desiccation when the biomass is
73 exposed at low tide (Ale, Mikkelsen, et al., 2011b; Senthilkumar, Manivasagan, Venkatesan,
74 & Kim, 2013).

75 There is a marked relationship between the chemical structure of both macroalgal
76 polysaccharides (i.e. molecular weight, monosaccharide composition, sulphate content and
77 position) and their biological activity. These differences could be attributed to factors such as
78 macroalgae species, parts of the macroalgae sampled and differences in polysaccharide
79 content and composition depending on the season (Kim, 2012; Men'shova et al., 2012;
80 Skriptsova, Shevchenko, Tarbeeva, & Zvyagintseva, 2011). Furthermore, it was also
81 observed that structural modifications to these molecules (i.e. molecular weight, degree of
82 sulphation) can occur during the processes of extraction and purification; for instance, use of
83 different extraction solvents and experimental conditions (such as pH, time, temperature and
84 pressure) (Ale, Mikkelsen, et al., 2011a; Foley, Szegezdi, Mulloy, Samali, & Tuohy, 2011;
85 Hahn, Lang, Ulber, & Muffler, 2012; Lorbeer, Lahnstein, Bulone, Nguyen, & Zhang, 2015).

86 Therefore, for the extraction and purification of different fractions of laminarin and fucoidan,
87 details on the macroalgal biomass used as well as the methodology followed are extremely
88 important to achieve the bio-activities required as well as complying with good
89 manufacturing practices. The lack of standardized extraction methodologies has prevented
90 the approval of polysaccharides or their derived fractions to be officially approved for
91 pharmaceutical, dermatological, nutraceutical or other commercial applications to date (Ale
92 & Meyer, 2013).

93 Thus, the present review focuses on the extraction and purification techniques of laminarin
94 and fucoidan from macroalgae described in the recent literature. New processes, technologies
95 and optimized extraction and purification procedures are reported together with the
96 challenges concerning molecule structure-function relationship and macroalgal variability.

97 ***2. Extraction of polysaccharides from macroalgae***

98 The process of extraction of macroalgal polysaccharides could include several steps
99 summarized in Figure 2. These extraction steps include preparation of the macroalgal
100 biomass, pre-treatments of the macroalgae, extraction techniques (traditional solvent
101 extraction and innovative technologies) and purification techniques to obtain the
102 polysaccharides of interest and proceed to test its biological activity and potential industrial
103 uses.

104 **2.1 Preparation of macroalgal biomass**

105 The procedure for the extraction of polysaccharides from macroalgae involves cleaning of
106 macroalgae with either sea water or distilled water to remove sand and epiphytes and then
107 drying (oven-drying or freeze-drying). The dried biomass is then milled to obtain the highest

108 surface-to-volume ratio during the latter extraction procedures (Hahn et al., 2012; Imbs,
109 Ermakova, Malyarenko, Isakov, & Zvyagintseva, 2016).

110 As an alternative to drying processes, Hjelland, Andersen, and Yang (2012) patented the
111 exudate method to obtain laminarin and fucoidan at commercial amounts from live
112 macroalgal tissue. Using this technique, the fresh macroalgae was cut in pieces of ≥ 1 cm and
113 piled in a dark-humid place to obtain the exudate from the live macroalgae tissue. After this
114 step, the polysaccharides laminarin and fucoidan could be extracted and purified from the
115 exudates obtained.

116 **2.2. Pre-treatment of macroalgae**

117 Different pre-treatments applied to the dried biomass, together with the detailed description
118 of the extraction techniques described in the recent literature are presented in Table 1. The
119 most commonly performed pre-treatments in the literature were washing of the dried biomass
120 with a mixture of methanol, chloroform and water (4:2:1; v/v/v) (Ale, Maruyama, Tamauchi,
121 Mikkelsen, & Meyer, 2011; Lim et al., 2014), or with acetone alone (Dore et al., 2013).
122 However, a mixture of acetone and ethanol (Shan et al., 2016) or one or several ethanolic pre-
123 treatments at different temperatures have also been applied recently (Imbs et al., 2016; Yuan
124 & Macquarrie, 2015). These alcohol treatments were applied to remove lipids (defatted),
125 proteins (deproteinated) and phenols (dephenolated), but also mannitol and chlorophyll,
126 compounds that are highly bound to the polysaccharides, contaminating the target compounds
127 (Hahn et al., 2012). Other novel pre-treatments recently described in the literature include
128 compressional-puffing-hydrothermal process consisting of heating at atmospheric pressure
129 (140°C, 180°C and 220°C), followed by rapid reduction of pressure in a vessel containing
130 superheated water, which allows the modification of the cellular structure of the macroalgae
131 prior to extraction of fucoidan (Huang, Wu, Yang, Kuan, & Chen, 2016).

132 **2.3. Extraction techniques of polysaccharides**

133 **2.3.1. Solvent extraction**

134 The extraction of macroalgal polysaccharides is mainly carried out by solvent extraction,
135 with a wide variation in the conditions and methods used in the literature to date as seen in
136 Table 1. The extraction of macroalgal polysaccharides is typically performed using pre-
137 treated dried macroalgae or defatted biomass with different solvents at different temperatures
138 ranging from room temperature to 120°C, for several hours. The most commonly used
139 solvents are water (Cong et al., 2016; Cong, Xiao, Liao, Dong, & Ding, 2014; Shan et al.,
140 2016; Vijayabaskar, Vaseela, & Thirumaran, 2012; J. Wang, Wang, Yun, Zhang, & Zhang,
141 2012; P. Wang et al., 2012) and ethanol (Foley et al., 2011; Huang et al., 2016). Slightly
142 acidic solutions, i.e. low molarity solutions of HCl have also been described (Anastyuk,
143 Shevchenko, Dmitrenok, & Zvyagintseva, 2012; Dinesh et al., 2016; Imbs et al., 2016;
144 Lorbeer et al., 2015; Menshova et al., 2015).

145 Following the initial extraction, the solution containing mixed polysaccharides is treated to
146 obtain the specific compounds of interest. One of the main polysaccharide residues at this
147 stage are alginates. Alginates – composed of consecutive mannuronate residues (M-blocks),
148 consecutive guluronate residues (G-blocks), or alternate mannuronate and guluronate residues
149 (MG-blocks) – show gelation properties in the presence of Ca^{2+} or Mn^{2+} . The 2 ions interact
150 with the anionic polymers containing carboxylate groups through chemical binding, mainly to
151 the stiffer guluronate chains (Assifaoui et al., 2015). Therefore, CaCl_2 solutions are normally
152 used in the recent extraction protocols to eliminate the alginates from the mixture containing
153 polysaccharides (Cong et al., 2016; Dinesh et al., 2016; Yuan & Macquarrie, 2015). Other
154 authors described the use of CaCl_2 solutions at different concentrations and temperatures as
155 an alternative to the initial water or slow acidic conditions to obtain alginates, laminarin and

156 fucoidan in a single step and separate them further by applying several consecutive
157 purification steps (Lim et al., 2014).

158 After the initial solvent extraction, the application of organic solvents (i.e. ethanol) or tensids
159 (cetyltrimethylammonium bromide) are common practices to precipitate the polysaccharides
160 from the mixture (Hahn et al., 2012; Kadam, Tiwari, et al., 2015). The precipitated
161 polysaccharides can then be dialyzed or precipitated further with ethanol in one or several
162 steps to remove salts and other compounds from the enriched extract. Laminarin and fucoidan
163 were further purified from the extract using different techniques such as membrane filtration
164 or chromatographic procedures (Ale, Mikkelsen, et al., 2011a; Cong et al., 2016; Cong et al.,
165 2014).

166 **2.3.2. Innovative extraction technologies**

167 Innovative or novel technologies have been used to develop extraction processes more
168 efficient in terms of yield, time and cost and more environmentally friendly by lowering the
169 energy consumption (Kadam, Tiwari, & O'Donnell, 2013; Barba, Grimi, & Vorobiev, 2015).
170 Extraction technologies such as ultrasound-assisted extraction (UAE), microwave-assisted
171 extraction (MAE) and enzyme-assisted extraction (EAE) have been used to extract
172 macroalgal polysaccharides and other marine bioactive compounds.

173 ***Ultrasound-assisted extraction***

174 Ultrasound-assisted extraction has been utilized to extract a number of functional ingredients
175 from multiple plant sources (C Chen, You, Abbasi, Fu, & Liu, 2015; Roselló-Soto et al.,
176 2015; Xu, Zhang, Yang, Song, & Yu, 2015; Ying, Han, & Li, 2011), microalgae (Barba et al.,
177 2015; Parniakov, Apicella, et al., 2015; Parniakov, Barba, et al., 2015) and macroalgae such
178 as phycoerythrin (Le Guillard et al., 2015) and amino acids (Romarís-Hortas, Bermejo-

179 Barrera, & Moreda-Piñeiro, 2013). UAE was used to obtain polysaccharides from algae
180 (Wijesekara, Pangestuti, & Kim, 2011) and laminarin from *Laminaria hyperborea* and
181 *Ascophyllum nodosum* in combination with weak acid solutions (Kadam, Tiwari, et al.,
182 2015). The technology has also been used to breakdown already extracted polysaccharide
183 fractions. The small molecular weight molecules generated during this process showed
184 promising biological and physico-chemical properties than those described in its original
185 conformation in the algae (Sun, Wang, & Zhou, 2012).

186 UAE is a non-thermal extraction technique wherein sound waves migrating through a
187 medium, inducing pressure variations and cavitations that grow and collapse, transforming
188 the sound waves into mechanical energy, which disrupts the cell walls, reduce the particle
189 size and enhance the contact between the solvents and the targeted compounds (Michalak &
190 Chojnacka, 2014; Ying et al., 2011). Despite being an energy input extraction method, UAE
191 has certain advantages over conventional solvent extraction methods. UAE is cost-effective
192 in comparison with other novel extraction techniques and has high possibilities to scale up to
193 industry. Other advantages of UAE include low solvent consumption, high level of
194 automation, and possibilities to combine this technique with others – i.e. superfluid assisted
195 extraction or microwave-assisted extraction (Ibañez, Herrero, Mendiola, & Castro-Puyana,
196 2012; Michalak & Chojnacka, 2014).

197 ***Microwave-assisted extraction (MAE)***

198 Microwave-assisted extraction (MAE) has been used to extract multiple compounds of
199 interest from both plants (Mendes et al., 2016) and algae (Gilbert-López, Barranco, Herrero,
200 Cifuentes, & Ibañez; Michalak & Chojnacka, 2014). MAE technology was used to extract
201 fucoidan from macroalgae *Fucus vesiculosus* (Rodriguez-Jasso, Mussatto, Pastrana, Aguilar,
202 & Teixeira, 2011), *Undaria pinnatifida* (Quitain, Kai, Sasaki, & Goto, 2013) and

203 *Ascophyllum nodosum* (Yuan & Macquarrie, 2015) showing yields of fucoidan similar to
204 those obtained with conventional techniques (Rodriguez-Jasso et al., 2011; Yuan &
205 Macquarrie, 2015). However, effect of the microwaves on the structural properties and
206 bioactivity of fucoidan is still unknown. Some studies used MAE on previously extracted
207 fucoidans from sporophylls of *Undaria pinnatifida* to produce more valuable low-molecular-
208 weight products of around 5-30 kDa (You, Yang, Lee, & Lee, 2010).

209 This extraction technology, a thermal based approach, uses microwaves causing temperature
210 rise and water evaporation of intracellular fluids that leads to break down of the cell walls,
211 thus release the intracellular compounds into the medium (Michalak & Chojnacka, 2014).
212 Compared to the ultrasounds, MAE is an energy-assisted extraction method, uses lower
213 amounts of solvents and improved the extraction yields of certain intracellular compounds.
214 However, the heat generated during the extraction process might cause damages to heat-
215 sensitive compounds (Michalak & Chojnacka, 2014).

216 ***Enzyme-assisted extraction (EAE)***

217 Enzyme-assisted extraction is a promising alternative to the conventional solvent based
218 methods, due to its high catalytic efficiency, high specificity, and mild reactive conditions
219 (Kulshreshtha et al., 2015). Additionally, the enzymes used are eco-friendly, non-toxic, food
220 grade and could be used in large scale operations (Michalak & Chojnacka, 2014). However,
221 the usage of enzymes is limited due to their high price in industrial applications (Hahn et al.,
222 2012; Michalak & Chojnacka, 2014).

223 Enzyme-assisted extraction (EAE) was used in macroalgae to enhance the extraction of
224 several targeted compounds such as proteins and phenols (T. Wang et al., 2010), carotenoids
225 and lipids (Billakanti, Catchpole, Fenton, Mitchell, & MacKenzie, 2013). The use of protease
226 enzymes was described for the isolation of fucoidan with anticoagulant, antithrombotic,

227 antioxidant and antiinflammatory effects from *Sargassum vulgare* biomass followed by
228 solvent precipitation with acetone (Dore et al., 2013). Recently, the use of a combination of
229 carbohydrase and protease enzymes resulted in the extraction of potent antiviral compounds
230 from brown algae *Chondrus crispus* and *Codium fragile* (Kulshreshtha et al., 2015).

231 This innovative technology relies on the application of one or several enzymes that interact
232 with its substrates under particular conditions defined by each enzyme, which help the
233 extraction of particular compounds by degrading the cell wall. Macroalgae cells are
234 chemically and structurally more complex than the terrestrial plants. The application of a
235 well-defined enzyme mixture and the optimal conditions (mainly pH and temperature) for
236 enzymatic activity, are the main parameters to obtain a good recovery of the compounds of
237 interest (Jeon, Wijesinghe, & Kim, 2011). Jeon et al. (2011) has described the optimum
238 conditions of pH (ranging from 3.8 to 8) and temperature (from 40 to 60°C) for enzymes such
239 as amyloglucosidase (AMG), agarase, alcalase, carragenanase, cellucast, cellulose protamex,
240 kojizyme, neutrase, termamyl, ultraflo, umamizyme, xylanase and viscozyme which are most
241 commonly used in EAE.

242 **3. Purification techniques**

243 After the process of extraction, the polysaccharides are dissolved in a rich mixture (with
244 variable monosaccharide composition and sulphate contents), together with small amounts of
245 proteins and phenolic compounds that could also display several beneficial biological
246 activities both *in vitro* and *in vivo* (Ale, Mikkelsen, et al., 2011b). Before the bioactivity tests,
247 the polysaccharide mixture usually undertakes one or more processes of purification to
248 further enrich the extracts with the polysaccharides of interest (J in et al., 2013; P. Wang et
249 al., 2012). The most commonly used techniques in polysaccharide purification are ion-

250 exchange chromatography (IEC), size-exclusion chromatography (SEC), affinity
251 chromatography and membrane filtration.

252 **3.1. Ion-exchange chromatography (IEC)**

253 Ion-exchange chromatography (IEC) is an established technique used in the separation of
254 charged molecules across a breadth of applications and industries. The separation of the
255 compounds achieved with IEC is based on the adsorption of charged solute molecules on to
256 immobilized ion exchange groups of opposite charge and its elution by changing the
257 concentration or pH of the running buffer (Rieman & Walton, 2013). The high performance
258 and resolution showed by AEC, together with its relatively low average cost, large sample
259 handling capacity and automation made this technique one of the most widely used liquid
260 chromatography technique for purification (Acikara, 2013).

261 Positively charged ion exchange resin with affinity for molecules with net negative surface
262 charges (anion-exchange chromatography (AEC)) are commonly used in sulphated
263 polysaccharide purification. Fucoidans exhibit high anionic charges, even at low pH values,
264 due to the sulphate ester groups linked to the carbohydrate backbone. Thus, AEC is one of the
265 most common purification techniques used under different experimental conditions (Table 2).
266 The elution of the adsorbed fucoidan from different resins was performed by stepwise
267 (Anastyuk, Imbs, Shevchenko, Dmitrenok, & Zvyagintseva, 2012; Cong et al., 2016) or
268 linear (Dinesh et al., 2016; Imbs et al., 2016; Menshova et al., 2015; J. Wang et al., 2012)
269 NaCl salt gradient. Different fucoidan fractions could be further separated by AEC based on
270 the degree of sulphation of the molecules. In general, highly sulphated fucoidan fractions
271 showed stronger interactions with the resins, which require higher salt concentrations to elute
272 these compounds. Thus, AEC could be used to purify different fucoidan fractions from
273 macroalgae that exhibit distinct structural and chemical properties (Hahn et al., 2012).

274 **3.2. Size-exclusion chromatography (SEC)**

275 Size-exclusion chromatography (SEC) is used to separate the molecules based on their size as
276 they pass through a porous matrix of particles with chemical and physical stability and
277 inertness. Unlike IEC, molecules do not bind to the chromatography medium in SEC thus, the
278 buffer composition does not directly affect the degree of separation between the peaks.
279 Subsequently, a significant advantage of SEC is that it can be used directly after IEC or other
280 purification techniques using the buffer that better suits the type of sample in terms of
281 preservation or further purification (Mori & Barth, 2013). As seen in Tables 2 and 3, the
282 application of SEC after IEC is a common procedure in the purification of polysaccharides.
283 Different SEC conditions used for polysaccharide purification in the recent literature are
284 compiled in Table 3. During this purification step, different single columns such as Sepharose
285 CL-6B, PL aquagel-OH, Sephacryl S-300 or Superdex 200 (Cong et al., 2016; Huang et al.,
286 2016; P. Wang et al., 2012), or columns successively connected i.e. TSK G4000 SW-XL and
287 TSK G3000 SW-XL (Anastyuk, Imbs, et al., 2012; Foley et al., 2011; Imbs et al., 2010) were
288 used. The application of SEC allows fractionation of the laminarins and fucoidans according
289 to their molecular weights. Furthermore, it can also help in desalting the purified compounds
290 from the high salt concentrations, a major step during AEC purification (Hahn et al., 2012).

291 **3.3. Affinity chromatography**

292 Affinity chromatography is based on the reversible interaction between the molecules and a
293 specific ligand coupled to a chromatography matrix, enabling the purification of different
294 compounds on the basis of its biological function or individual chemical structure (Dunlap,
295 2013). For instance, the use of immobilized dyes (dye-affinity chromatography) to
296 specifically bind different proteins from other cellular crude extracts is a common practice in
297 the process of protein purification (Janson, 2012).

298 In the case of polysaccharides, fucose-binding lectins were used effectively to purify
299 fucoidan from crude solutions; however problems could arise if the sulphate content of the
300 polysaccharide blocks the fucose units that interact with the lectins (Hahn et al., 2016).
301 Strong interactions have been appreciated between the anionic sulphate esters of the
302 sulphated polysaccharides and different cationic dyes (i.e. toluidine and methylene blue),
303 forming a strong donor-acceptor complex (Hahn et al., 2016). Recently, Hahn et al. (2016)
304 developed a new dye-affinity chromatography method using modified amino-derivatized
305 sepabeads with toluidine blue, showing promising results in the purification of fucoidan from
306 brown macroalgae at higher purity levels than the commercially available purified fucoidan
307 from Sigma-Aldrich (95%).

308 **3.4. Membrane filtration**

309 Novel technologies, such as membrane filtration, are used to obtain polysaccharide fractions
310 based on the molecular weight of the molecules of interest. Authors in the recent literature
311 used one or several steps of filtration and/or dialysis of the polysaccharide mixture with
312 different molecular weight cut off (MWCO) membranes/filters mainly to remove salts from
313 the extracts (Anastyuk et al., 2010). The use of MWCO membranes to separate laminarin
314 from fucoidan and/or fractionation of the different polysaccharides from macroalgae is barely
315 described (Hjelland et al., 2012). However, membrane separation methods have been
316 successfully applied in other fields i.e. separation of milk components (Costa, Elias-Argote,
317 Jiménez-Flores, & Gigante, 2010), extraction and concentration of bacterial extra-cellular
318 polysaccharides (Wingender, Neu, & Flemming, 2012) and hydrosoluble polysaccharides
319 from terrestrial plants (Wan, Prudente, & Sathivel, 2012) and microalgae origin (CS Chen et
320 al., 2011; Marcati et al., 2014; Mezhoud et al., 2014; Patel et al., 2013).

321 Filtration techniques seem well suited for initial extraction and purification steps at industrial
322 scale as they can be automatized and allow the separation of large volumes of solutions (Patel
323 et al., 2013). However, their widespread applications have been hindered due to excessive
324 membrane fouling (which could result in reduced performance), high energy inputs and
325 frequent membrane cleaning or replacement (Marcati et al., 2014). Nevertheless, to improve
326 the efficiency of membranes in the separation of different compounds of interest and to avoid
327 fouling, both tangential flow filtration (diafiltration) (Patel et al., 2013) or a combination of
328 sequential ultrafiltration and diafiltration with membranes with decreasing MWCO (Marcati
329 et al., 2014) have been proposed.

330 ***4. Optimization of the methods***

331 As described in sections 2 and 3, there are many protocols proposed for the extraction of
332 polysaccharides, however, there is a need to optimize the different conditions of the
333 extraction processes to obtain the best possible response. Traditionally, the optimization in
334 analytical chemistry has been carried out by studying “one-variable-at-a-time”. In this
335 optimization strategy, the conditions of one parameter were changed while the others
336 remained constant (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). This optimization
337 technique is time- and cost-consuming and does not include the interactive effects among the
338 variables studied (Bezerra et al., 2008; Roselló-Soto et al., 2015).

339 To solve these problems, response surface methodology (RSM), a multivariate statistic
340 technique, has been considered the most relevant in analytical optimization. RSM is based on
341 the fit of a polynomial equation to the experimental data with the objective of making
342 statistical previsions. RSM could include first-order models (i.e. when the data does not
343 present curvature), and more commonly experimental designs such as three-level factorial,
344 Box-Behnken, central composite and Doehlert designs. Box-Behnken design is an efficient

345 and economical design when dealing with large number of variables (Bezerra et al., 2008;
346 Roselló-Soto et al., 2015) with the advantage that only 3 levels per factor are included in the
347 model. However, the confidence interval of this design is broader in comparison to central
348 composite designs (Hahn et al., 2012).

349 The yield and chemical structure of polysaccharides (i.e. molecular weight, sulphate content
350 etc) could be affected by different factors (such as pH, temperature, time, pressure and
351 sample to solvent ratio) used during extraction. Therefore, it is essential to optimize at least
352 some critical factors that could directly affect the extraction of polysaccharides during multi-
353 step extraction processes (Hahn et al., 2012). Studies on the optimization of one or more steps
354 of the extraction processes of polysaccharides from macroalgae mainly used Box-Behnken
355 and central composite designs to analyse at least 3 or more factors affecting the extraction
356 process (Ale, Mikkelsen, et al., 2011a; Hifney, Fawzy, Abdel-Gawad, & Gomaa, 2016;
357 Lorbeer et al., 2015).

358 Ale, Mikkelsen, et al. (2011a) optimized one step process of fucoidan extraction from
359 *Sargassum* sp. using a central composite design. The model took 3 factors (molarity,
360 temperature and time) into account when extracting fucoidan from the original dried-milled
361 biomass with HCl solution. The authors concluded that one single step of extraction with
362 0.03M HCl at 90°C for 4 hours showed optimum results in terms of high fucoidan yield
363 without disrupting the structural integrity of the polymer (Ale, Mikkelsen, et al., 2011a).
364 Recently, Box-Behnken design was used to optimize the pH, temperature and time of
365 application of HCl treatment to a defatted biomass of *Ecklonia radiata* to obtain a high yield
366 of both fucoidan and alginates and low degradation of alginates, measured by their molecular
367 weight characteristics of each extract obtained (Lorbeer et al., 2015). The same RSM design
368 was applied most recently to optimize the yield and degree of sulphation of fucoidan from

369 dried-milled *Sargassum* sp. using McIlvaine buffer solution at different temperatures, pH and
370 macroalgae to buffer ratios (Hifney et al., 2016).

371 **5. Biological activity and potential health applications of macroalgal polysaccharides**

372 The addition of macroalgae to food resulted in an increase of the health-related properties of
373 the resultant products against different diseases (i.e. obesity, dyslipidaemia, hypertension and
374 diabetes) (Roohinejad et al., 2016). Macroalgal polysaccharides display a wide range of
375 biological properties including antiinflammatory, immunostimulatory, antioxidant,
376 anticoagulant, antimicrobial, antiviral, antipeptic, antiadhesive, antiproliferative,
377 antiapoptosis and antitumour properties (Aspidou, Moschakis, Biliaderis, & Koutsoumanis,
378 2014; Dore et al., 2013; Huang et al., 2016; Kadam, Tiwari, et al., 2015; Roohinejad et al.,
379 2016; Sweeney et al., 2012; Wijesekara et al., 2011). Table 4 summarised antioxidant,
380 anticancer, anticoagulant and antiinflammatory activities which are among the most widely
381 studied properties of macroalgal derived fucoidan and laminarin in recent times (Cong et al.,
382 2016; Hifney et al., 2016; Wu, Shiu, Hsieh, & Tsai, 2016).

383 The *in vitro* antioxidant activity of polysaccharides have been determined mainly by 1,1-
384 diphenyl-2-picryl hydrazil (DPPH) radical scavenging, ABTS radical scavenging and ferric
385 reducing antioxidant power (FRAP) assays amongst others (Hifney et al., 2016; Huang et al.,
386 2016). There is a marked relationship between the chemical structure of the macroalgal
387 polysaccharides and their antioxidant activity *in vitro*. Low molecular weight sulphated-
388 polysaccharides showed higher antioxidant activity than high molecular weight
389 polysaccharides in macroalgae (Morya, Kim, & Kim, 2012). Fucoidan fractions containing
390 high levels of total sugars, fucose, and uronic acids showed increased radical scavenging
391 activity while molecules with high degree of sulphation showed high FRAP antioxidant
392 activity (Hifney et al., 2016).

393 Antiproliferative activity *in vitro* of macroalgal polysaccharides have been demonstrated in a
394 wide variety of cell line cultures. Low molecular weight fucoidan fraction from *Alaria*
395 *angusta* showed antiproliferative effects against HT 29 and T-47D cell lines, while laminarin
396 fraction showed antiproliferative effects against HT 29 cell lines (Menshova et al., 2015).
397 Fucoidan from *Coccophora langsdorfii* inhibited SK-MEL-5 and SK-MEL-28 melanoma
398 cells (Imbs et al., 2016). Also, fucoidan fractions from *Sargassum fusiforme* showed potent
399 antiangiogenic activities that could be useful in cancer therapy (Cong et al., 2016).

400 The anticoagulant and antithrombotic activities *in vitro* of macroalgal polysaccharides have
401 been predominantly related to the structure and the degree of sulphation of the
402 polysaccharide (Silva et al., 2010; Wijesinghe & Jeon, 2012). O-sulphated 3-linked α -
403 galactans and 3-O-sulphation at C-3 of 4- α -L-fucose-1 \rightarrow units (in fucoidan) is mainly
404 responsible for its anticoagulant activity (Dore et al., 2013; Jin et al., 2013; Wijesinghe &
405 Jeon, 2012). These sulphate linked sugar units increased the inhibition of factors Xa, IIa and
406 thrombin with the help of antithrombin and heparin cofactor II in blood coagulation intrinsic
407 pathway (Jin et al., 2013; Wijesekara et al., 2011).

408 Though both laminarin and fucoidan have shown multiple biological activities in *in vitro*
409 systems, their efficiency and bioavailability in *in vivo* model is not completely explored. *In*
410 *vivo* studies using β -glucans from *Laminaria digitata*, *Laminaria hyperborea* and
411 *Saccharomyces cerevisiae* in the diet of pigs showed a down-regulation of the expression of
412 inflammatory cytokines in the colon and liver of the animals (Sweeney et al., 2012) and
413 mucin gene expression in the ileum and colon (Ryan et al., 2010; Smith et al., 2010). A
414 down-regulation of pro- and antiinflammatory cytokines (IL-6, IL-17A, IL-1b and IL-10) was
415 reported in the colon of post-weaning pigs supplemented with laminarin (Walsh, Sweeney,
416 O'Shea, Doyle, & O'Doherty, 2013). The animals receiving fucoidan showed a reduction in
417 *Enterobacteriaceae* counts and improved faecal consistency due to the ability of fucoidan to

418 inhibit the attachment of certain bacterial species in the porcine gastrointestinal tract (Walsh
419 et al., 2013).

420 The role of the chemical structure of macroalgal polysaccharides and their biological
421 activities *in vitro* and *in vivo* is poorly understood to date. It is known that the chemical
422 structure of the macroalgal polysaccharides varied according to factors affecting the
423 macroalgal biomass (i.e. macroalgae species, environment and geographical location) as well
424 as conditions during the extraction and purification of the polysaccharides (i.e. temperature,
425 time). In general, macroalgae is considered a renewable biomass which can be utilized for
426 sustainable polysaccharide production. More research is required to improve extraction and
427 purification techniques of polysaccharides from macroalgae, as well as studies on the
428 chemical structure of the obtained polysaccharides and their promising biological activities
429 both *in vitro* and *in vivo* for its future use at industrial scale.

430 **6. Conclusions**

431 Laminarin and fucoidan showed a wide range of biological activities (i.e. anticoagulant,
432 antithrombotic, antiviral, antioxidant and antitumour activities amongst others), with potential
433 use in several markets such as the functional foods and nutraceutical, pharmaceutical and
434 cosmeceutical industries.

435 There are many extraction and purification techniques available to achieve laminarin and
436 fucoidan with different purity levels. There is a need to optimize the protocols of extraction
437 already available in the literature to improve the yields and to preserve the activities of the
438 extracted polysaccharides, while reducing the time, solvents and energy inputs applied during
439 the extraction processes. Novel extraction technologies such as UAE and EAE showed
440 promising industrial potential in terms of scalability and large sample treatment; however, the
441 high costs associated in terms of machinery, enzyme and energy input are important factors

442 when considering using these novel techniques at large scale to date. Recent use of dye
443 affinity chromatography techniques and widely used AEC and SEC showed important results
444 not only to achieve high purity levels of polysaccharides but also to isolate and collect
445 different fractions of either laminarin or fucoidan with different chemical structure and
446 biological properties.

447 The chemical structure of the polysaccharides could also be affected by parameters related
448 with the biology of macroalgae. Due to the fact that the majority of macroalgae currently
449 commercialized in the European market is wild harvested biomass, therefore there is a need
450 to study seasonal differences and its influence on the content and chemical structure of
451 polysaccharides to achieve a better understanding of these compounds and its future
452 exploitation from both wild and cultured harvested macroalgae. In addition, several other
453 challenges (i.e. sustainability of macroalgae supply, automatization/scalability of the
454 employed techniques) must be addressed by researchers to achieve its full potential in terms
455 of commercialization and future industrial applications.

456 *Acknowledgments*

457 This work was supported by the Science Foundation Ireland (SFI) [grant number:
458 14/IA/2548].

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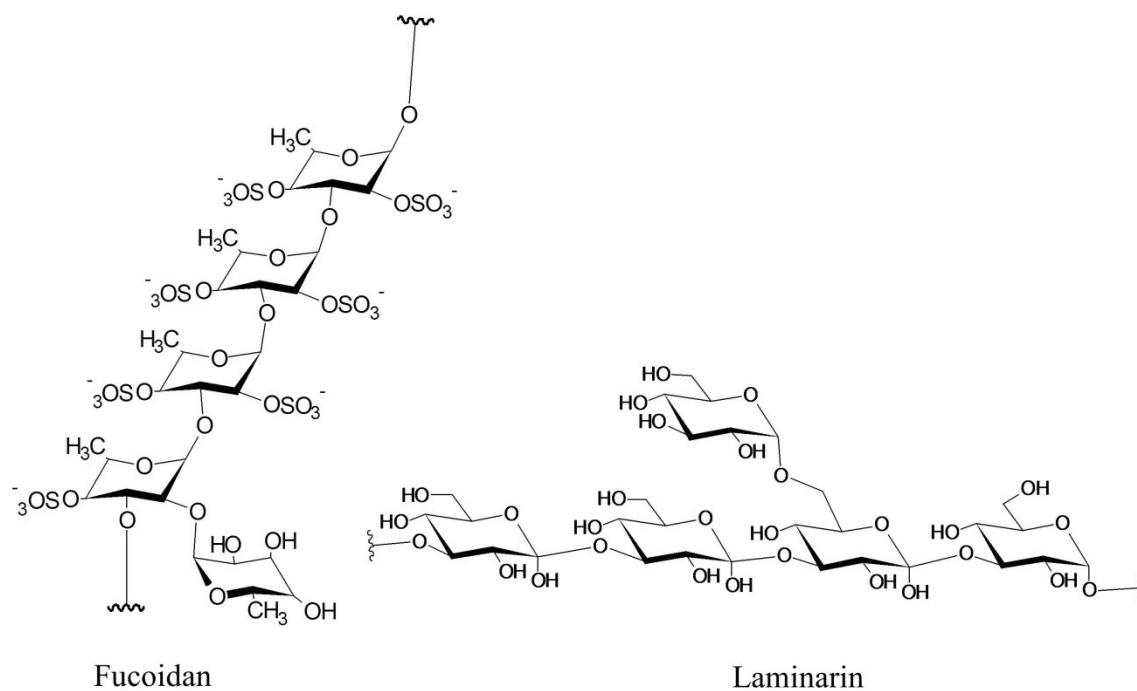
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753 **Figure 1.** Chemical structure of fucoidan from *Laminaria saccharina* and laminarin from
754 *Laminaria digitata* proposed by Cumashi et al. (2007) and Adamo et al. (2011) respectively.

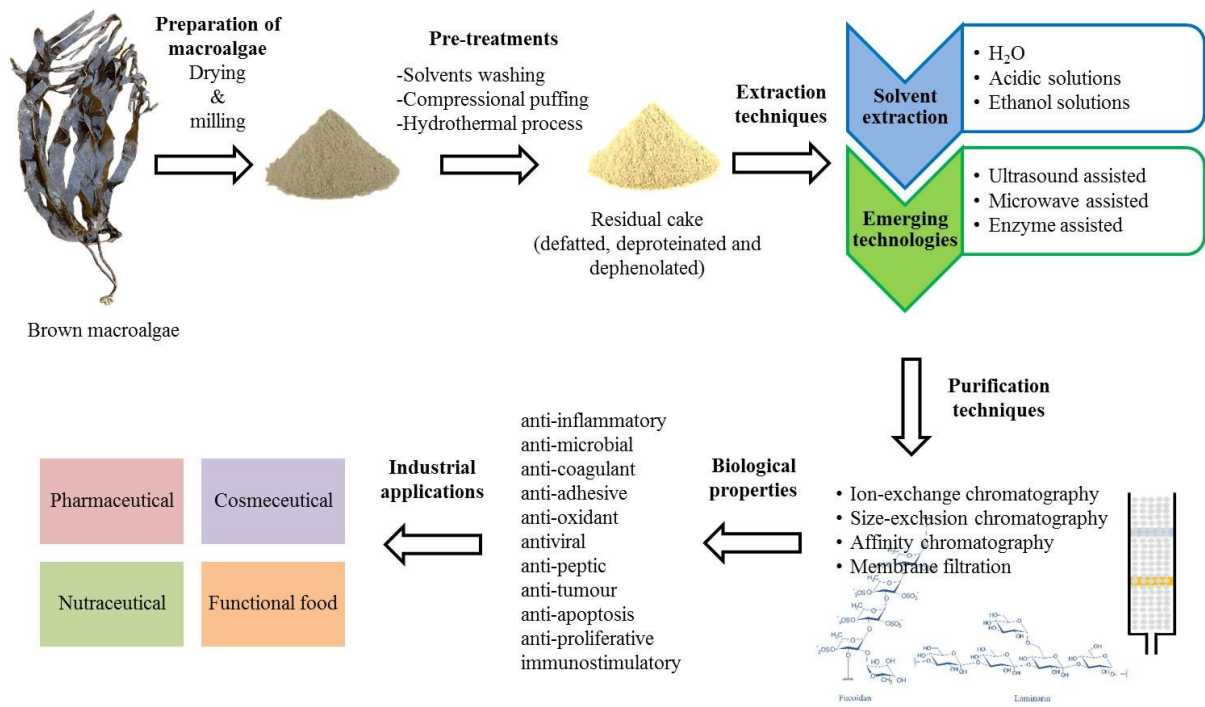


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758 **Figure 2.** Schematic diagram of polysaccharide extraction and purification from macroalgal
 759 biomass.



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Table 1. Extraction methods of polysaccharides from different macroalgae species.

Macroalgae sp.	Compound of interest	Pre-treatments	Extraction	References
<i>Silvetia babingtonii</i> , <i>Costaria costata</i> , <i>Fucus evanescens</i> , <i>Laminaria cichorioides</i>	Fucoidan	Ethanol (40°C, 3 h)	HCl (60°C, pH 2-3, 3 h, 2 times) followed by concentration, dialysis and freeze-dry.	Anastyuk, Imbs, et al. (2012); Anastyuk, Shevchenko, et al. (2012); Anastyuk et al. (2010); Imbs et al. (2016)
<i>Sargassum henslowian</i> , <i>Fucus vesiculosus</i>	Fucoidan	-	HCl (0.03 M, 90°C, 4 h) followed by filtration and ethanol (60%) precipitation. The final pellet was freeze-dried.	Ale, Mikkelsen, et al. (2011a)
<i>Ascophyllum nodosum</i>	Fucoidan	-	Ethanol (80%, at room temperature, 12 h) followed by the same treatment at 70°C. The pellet was treated twice at room temperature and 70°C with H ₂ O (7h). The pulled supernatants were treated with CaCl ₂ (2M, 5 h), centrifuged and the pellet dialyzed (1 kDa) and freeze-dried.	Foley et al. (2011)
<i>Ascophyllum nodosum</i> ; <i>Fucus vesiculosus</i>	Laminarin, galactofucans	-	CaCl ₂ (1%, 85°C, 4 h) followed by centrifugation (16887g, 20 min). The supernatant was precipitated with ethanol (1 h) and re-precipitated with ethanol (-20°C, 48 h). The pellets were dialyzed (15 kDa) and freeze-dried.	Kim (2012)
<i>Sargassum swartzii</i>	Sulphated polysaccharides	-	H ₂ O (90-95°C, 16 h) followed by filtration and concentration. The extract was precipitated with ethanol, centrifuged and the pellet dehydrated with dimethyl ether.	Vijayabaskar et al. (2012)
<i>Laminaria japonica</i>	Fucoidan	-	H ₂ O (120°C, 3 h) followed by filtration and addition of MgCl ₂ and ethanol (85%) and filtrate again. The new filtrate was dialyzed (3.6 kDa, 24 h), precipitated with ethanol (95%, 3 times). The pellet was dried (80°C).	J. Wang et al. (2012)

<i>Sargassum vulgare</i>	Fucoidan	Acetone	Proteolytic enzyme in NaCl (0.25 M , pH 8, 60°C, 24 h) followed by filtration, precipitation with acetone (0.3, 0.5, 1 and 1.5 volumes) and collect the different precipitates generated by the different volumes to freeze-dry.	Dore et al. (2013)
2 <i>Sargassum sp.</i> , 3 red macroalgae	Fucoidan	Methanol : chloroform : water (4:2:1) several times	CaCl ₂ (2%, 85°C, 24 h, 6 times). The combined supernatants were treated with 10% CH ₃ (CH ₂) ₁₅ N(Br)(CH ₃) ₃ , followed by centrifugation (3000g, 10 min) and the pellet washed several times with H ₂ O and ethanol (20%) and dialyzed (2kDa).	Lim et al. (2014)
<i>Laminaria hyperborea</i> , <i>Ascophyllum nodosum</i>	Laminarin	-	Sonication 15 min at room temperature with HCl 0.1 M (better yield) and water, followed by centrifugation and precipitation of the supernatant with ethanol (4°C overnight). The mixture was centrifuged and the pellet freeze-dried.	Kadam, O'Donnell, et al. (2015)
<i>Alaria angusta</i>	Galactofucan	Ethanol 70%, 10 days	HCl (0.1M, 60°C, 2 h, 2 times) followed by centrifugation, dialysis of supernatant and freeze-dry.	Menshova et al. (2015)
<i>Ascophyllum nodosum</i>	Fucoidan	Ethanol (80%, room temperature, 18 h) and repeat again at 70 °C (4 h)	Microwave treatment (120°C, 15 min) with biomass in HCl (0.1 M). The mixture was dried (80°C), re-dissolved CaCl ₂ (2%, 4°C, overnight) followed by centrifugation and ethanol precipitation of the supernatant. The new pellet collected after centrifugation was freeze-dried	Yuan and Macquarrie (2015)
<i>Sargassum fusiforme</i>	Fucoidan		H ₂ O (100°C) followed by concentration and centrifugation. The supernatant was precipitated with ethanol (95%) and the pellet treated with CaCl ₂ (2%) and freeze dried.	Cong et al. (2016)

<i>Sargassum swartzii</i>	Fucoidan	Soxhlet ethanol-acetone for 24 h	HCl (0.05 M, room temperature, 24 h, 2 times). Supernatants mixed with CaCl ₂ (4%, 4°C, overnight) followed by centrifugation and ethanol precipitation of supernatant and dialysis of the pellet.	Dinesh et al. (2016)
<i>Sargassum galaucescens</i>	Fucoidan	Compressional puffing (0-140-180-220°C) followed by ethanol (1:10, room temperature, 1 h)	H ₂ O (85°C, 1h) followed by centrifugation (3870g, 10 min). The supernatant was precipitated with ethanol (20% to precipitate alginic acid) and then 50% followed by centrifugation and drying (40°C).	Huang et al. (2016)
<i>Coccophora langsdorffii</i>	Laminarin, fucoidan and alginates	Ethanol (96%, 40°C, 24 h) and acetone washes	HCl (0.1 M, room temperature, 2 times) and the supernatant neutralized with NaHCO ₃ (3%) to pH 5.7-6.1, followed by concentration, dialysis and freeze-dry.	Imbs et al. (2016)
<i>Sargassum</i> sp., <i>Fucus vesiculosus</i> , <i>Ascophyllum nodosum</i> , <i>Lessonia nigrescence</i> , <i>Kjellmaniella crassifolia</i> , <i>Costaria costata</i> .	Fucoidan	Ethanol (95%, 80°C, 4 h, 2 times)	H ₂ O (80 °C, 3 h, 3 times), followed by centrifugation (5000 rpm, 10 min). Supernatants were precipitated with ethanol (80%) and the pellet washed in several steps with ethanol-acetone and centrifuged each time until final dialysis and freeze-dry.	Shan et al. (2016)

Table 2. Experimental conditions used for the purification of macroalgal polysaccharides with anion-exchange chromatography (AEC).

Compounds of interest	Experimental conditions	References
Laminarin and fucoidan	DEAE-cellulose column (3.5 × 22 cm, Cl ⁻ form) equilibrated with 0.05 M HCl. Laminarin eluted with 0.05 M HCl, and fucoidan fractions with 0.5, 1 and 2 M NaCl.	Anastyuk, Imbs, et al. (2012)
Fucoidan	Q-Sepharose Fast Flow column. Eluted with a gradient of 0–3 M NaCl. Flow rate: 3 mL/min.	P. Wang et al. (2012)
Fucoidan	Macro-Prep DEAE column (2.5 × 9 cm, Cl ⁻ form) equilibrated with 0.1 M NaCl. Eluted with a linear gradient of 0.1-2 M NaCl	Menshova et al. (2015)
Fucoidan	DEAE-cellulose (50 × 5 cm, Cl ⁻ form). Eluted stepwise with 0, 0.2, 0.4, 0.8 and 1.6 M NaCl, followed with 0.3 M NaOH	Cong et al. (2016)
Fucoidan	DEAE cellulose-52 (3 × 30 cm). Eluted with linear gradient 0–1 M NaCl and 0.05 M sodium phosphate buffer solution. Flow rate: 60 mL/h	Dinesh et al. (2016)
Laminarin and fucoidan	DEAE-cellulose (Fluca) column (3.0 × 14 cm). Eluted first with H ₂ O and the fractions obtained further eluted with a linear gradient 0-2 M NaCl	Imbs et al. (2016)

Table 3. Experimental conditions used for the purification of polysaccharides from macroalgae with size-exclusion chromatography (SEC).

Compounds of interest	Experimental conditions	References
Fucoidan	Shodex Asahipak GS-520 HQ and Shodex Asahipak GS-620 HQ (7.5 × 300 mm). Eluted with H ₂ O. Flow rate: 0.8 mL/min.	Imbs et al. (2010)
Fucoidan	TSK G4000 SW-XL (300 x 7.8 mm) and TSK G3000 SW-XL (300 x 7.8 mm). Eluted with 0.1M NH ₄ OAc acetate. Flow rate: 0.5 mL/min.	Foley et al. (2011)
Fucoidan	Shodex Asahipak GS-520 HQ and GS-620 HQ (7.5 × 300 mm). Eluted with H ₂ O. Flow rate: 0.8 mL/min.	Anastyuk, Imbs, et al. (2012)
Fucoidan	PL aquagel-OH column. Eluted with 0.2 M Na ₂ SO ₄ . Flow rate: 0.5 mL/min.	P. Wang et al. (2012)
Laminarin	TSKgel column G4000PWXL (7.8 x 300 mm) and G5000PWXL (7.8 x 300 mm). Eluted with H ₂ O. Flow rate: 0.5 mL/min.	Zha et al. (2012)
Fucoidan	Sepharose CL-4B (140 × 1.8 cm). Eluted with 0.2 M acetic acid.	Dore et al. (2013)
Fucoidan	Sephacryl S-300 column (100 × 2.6 cm). Equilibrated and eluted with 0.2 M NaCl.	Cong et al. (2016)
Fucoidan	Sepharose 6B column (90 × 1.0 cm). Eluted with 100 mM sodium phosphate buffer. Flow rate : 0.6 mL/min.	Dinesh et al. (2016)
Fucoidan	Superdex 200 (300 × 10 mm). Eluted with 0.2 M NaCl. Flow rate: 0.3 mL/min.	Huang et al. (2016)

Table 4. Bioactivities *in vitro* and *in vivo* of marine polysaccharides isolated from macroalgae.

Biological activity	Macroalgal polysaccharides	Method	References
Antioxidant <i>in vitro</i>	Fucoidan	Total antioxidant, ferric reducing and hydroxyl radical scavenging activities.	Hifney et al. (2016)
	Fucoidan	Ferrous ion-chelating activity, DPPH and ABTS radical scavenging.	Huang et al. (2016)
	Fucoidan	DPPH radical scavenging.	Dore et al. (2013)
Antitumour <i>in vitro</i>	Fucoidan	Sulforhodamine B assay.	You et al. (2010)
	Fucoidan	Inhibit growth of HT 29 and T-47D cell lines.	Menshova et al. (2015)
	Laminarin	HT 29 cell lines.	
	Fucoidan	SK-MEL-5 and SK-MEL-28 melanoma.	Imbs et al. (2016)
Anticoagulant <i>in vitro</i>	Fucoidan	Thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) coagulation assays.	Dore et al. (2013); Jin et al. (2013)
	Laminarin (laminarin sulphate)	Upon the structural modification (sulphation) with both O-sulphate and N-sulphate groups.	Kadam, Tiwari, et al. (2015)
Antiinflammatory <i>in vivo</i>	Laminarin	Decrease of inflammatory cytokines in colon and liver <i>in vivo</i> .	Sweeney et al. (2012)
	Laminarin	Downregulation of IL-6, IL-17A, IL-1b and IL-10 in the colon of post-weaning pigs <i>in vivo</i> .	Walsh et al. (2013)