Universidade de Aveiro

2015

Departamento de Química

RICARDO FERREIRA JORGE

Isolamento e caracterização estrutural de fucoidanas da alga castanha *Fucus vesiculosus*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Doutora Cláudia Sofia Cordeiro Nunes, Pós-Doutorada do Departamento de Química da Universidade de Aveiro, e do Professor Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro.

Dedico este texto aos meus pais e amigos…

O júri / The jury

Presidente/ President

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Professor catedrático do Departamento de Química da Universidade de Aveiro

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palavras-chave

Algas castanhas, *Fucus vesiculosus*, fucoidana, polissacarídeos sulfatados

resumo

As algas castanhas fazem parte de um grupo de organismos cuja utilização tem vindo a crescer nas áreas da alimentação, agricultura, farmacêutica, cosmética e bioenergia. Estas algas são constituídas por diversos compostos com atividades biológicas destacando-se as fucoidanas. Estas são polissacarídeos compostos maioritariamente por fucose e ésteres de sulfato, apresentando várias atividades biológicas, tais como anticoagulante, anti trombótica, anti-inflamatória, anti-tumoral, antivírica e antioxidante.

Neste trabalho foram isoladas e caracterizadas as fucoidanas da alga castanha, *Fucus vesiculosus*, da Ria de Aveiro de forma a potenciar este recurso natural da região.

Os polissacarídeos constituintes da alga foram extraídos com água quente e fracionados por precipitação em etanol e sais de cloreto de cálcio. Os polissacarídeos foram purificados utilizando uma cromatografia de troca aniónica, permitindo separar os polímeros neutros (laminaranas) dos carregados negativamente (fucoidanas sulfatadas e alginatos).

Os polissacarídeos purificados eram constituídos por fucose (41 mol%) e sulfatos (50 mol%), contendo também galactose (6% mol), comprovando a presença de apenas fucoidanas sulfatadas. A análise das ligações glicosídicas, mostrou a presença de fucose terminal (25%) e (1→3,4)-Fuc (26%), demonstrando que as fucoidanas eram muito ramificadas. Outras ligações presentes são (1→2-)-Fuc (15%) e (1→3)-Fuc.

Neste trabalho foi também testada uma tecnologia de extração alternativa, o sistema de micro-ondas de hidro-difusão e gravidade, onde foi possível extrair alguns açúcares, embora com rendimentos reduzidos. Esta metodologia permitiu extrair os polissacarídeos, constituídos por fucose e ácidos urónicos, e também manitol sem necessidade de adicionar qualquer solvente e permitindo obter a alga seca no final da extração.

Este trabalho permitiu caracterizar estruturalmente as fucoidanas isoladas do *F. vesiculosus* da Ria de Aveiro. A elevada quantidade de ésteres de sulfato e o elevado grau de ramificação sugere que estes polissacarídeos têm potencial para serem estudados em relação às suas atividades biológicas para serem usados em aplicações biomédicas.

keywords Brown seaweed, *Fucus vesiculosus*, fucoidan, sulfated polysaccharides

abstract

Brown seaweeds are part of a group of organisms that has been used for food, agricultural, pharmaceutical, cosmetic or bioenergy applications. They contain bioactive compounds, namely, polysaccharides Fucoidan. These polysaccharides are mainly constituted by fucose residues and sulfate esters, and have been reported to possess a broad variety of bioactivities, such as anticoagulant, anti-thrombotic, anti-inflammatory, anti-tumor, antiviral and antioxidant.

In this work, the fucoidans from brown seaweed *Fucus vesiculosus* from "Ria de Aveiro" were isolated and characterized in order to add value to this natural resource of the region.

The polysaccharides from the algae were extracted with hot water and fractioned by ethanol precipitation and calcium chloride salts. They were further purified by using anion-exchange chromatography, allowing to separate the neutral polysaccharides (laminaranas) from those negatively charged (sulfated fucoidans and alginate).

The purified polysaccharides showed high content of fucose (41 mol%) and sulfates (50 mol%), having also galactose residues (6 mol%), which confirm the presence of only sulfated fucoidans. Glycosidic linkages analysis show the presence of high amounts of terminal fucose (25%) and (1→3,4)-Fuc (26%), allowing to infer that the fucoidans were highly branched. These fucoidans are composed also by (1→2)-Fuc (14%) and (1→3)-Fuc linkages (10-16%).

In this work it was also tested an alternative extraction technology, the microwave hydrodiffusion and gravity system, where it was possible to extract sugars, although in low yields. However, this methodology allowed to extract polysaccharides, constituted mainly by fucose and uronic acids, as well as mannitol, without the need to add any solvent, obtaining at the end the dry alga.

The current work allowed to characterize the structure of the fucoidans isolated from "Ria de Aveiro" *F. vesiculosus*. The presence of high content of sulfate residues and the high branch degree of the purified fucoidans allow to infer that these polysaccharides could have potential to be studied for biomedical applications, according to their biological activities.

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List of abbreviatures

ARA	Arachidonic acid
DW	Dry weight
EPA	Eicosapentaenoic acid
FCSP	Fucose containing sulfated polysaccharides
GI	Gastrointestinal tract
HIV	Human immunodeficiency virus
HS	Heparan sulfate
HSV	Herpes simplex viruses
LPS	Lipopolysaccharide
LTs	Leukotrienes
MAE	Microwave assisted extraction
NK	Natural killer cells
PGs	Prostaglandins
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SAR	Structure-activity relationship
SPs	Sulfated polysaccharides
TFPI	Tissue factor pathway inhibitor
TXs	Thromboxanes

1 Introduction

1.1 General aspects

More than 70% of world's surface is covered by oceans. The wide diversity of marine organisms offer a rich and variable source of natural and functional products, such as polyunsaturated fatty acids (PUFAs), polysaccharides, minerals and vitamins, enzymes and peptides (Cardozo *et al.,* 2007; Wijesekara *et al.,* 2011; Wijesinghe and Jeon, 2012). Macroalgae are some of the marine organisms under investigation for numerous commercial food, agricultural, pharmaceutical, cosmetic, and bioenergy applications (Borowitzka, 2013; El Gamal, 2010; Stengel *et al.,* 2011).

Marine macroalgae, commonly named seaweeds, are autotrophic and aerobic organisms that contain chlorophyll and photosynthesize as plant-like organisms (Barsanti and Gualtieri, 2006). There is no easily definable classification system acceptable for algae, because taxonomy is under constant and rapid revision at all levels following the new genetic and ultrastructural evidence. The fact is that ‘algae’ are an extremely diverse group of organisms that encompass (mostly) photosynthetic organisms within Eukaryotes and Bacteria domains. Algal diversity can be described based on taxonomic or phylogenetic relationships, life stage, morphological types, habitats occupied by different groups, or their chemical diversity. Currently, for convenient purposes, the seaweed in general are commonly identified by color as brown (*Phaeophyceae*), red (*Rhodophyta*) and green (*Chlorophyta*) algae (Stengel *et al.,* 2011; Barsanti and Gualtieri, 2006).

In Asia, seaweed are mainly used as food source (El Gamal, 2010). In Europe and North America seaweeds are mostly used as raw materials for other purposes, such as fertilizers, cosmetics, or food additives (Kraan, 2012). Seaweeds are a source of structurally diverse bioactive compounds with a broad spectrum of activities, including antioxidant activity, anticancer activity, antimicrobial activity, and bioremediation potentials (Cardozo *et al.,* 2007; El Gamal, 2010; Shalaby, 2014). These compounds include carotenoids, phenolic compounds, pigments, unsaturated fatty acids, and polysaccharides.

1.1.1 Marine algae (sulfated) polysaccharides

Polysaccharides are the major components of marine algae representing over 60% of dry weight (Rioux *et al.,* 2007). The specific characteristics of each polysaccharide (molecular mass, degree of polymerization, degree of branching, monosaccharide units and types of linkage) are variable in different species of algae. Furthermore, some monosaccharides can be substituted with sulfate groups which have an important effect on functionality and bioactivities.

Sulfated polysaccharides (SPs) comprise a complex group of macromolecules with a wide range of important biological properties (Berteau and Mulloy, 2003; Pomin and Mourao, 2008). Marine algae are the most important source of non-animal SPs and the chemical structure of these polymers are variable according to the algal species: I) ulvans from the green alg*ae (Chlorophy*ceae) (Lahaye and Robic, 2007); II) fucoidans and alginates from brown alg*ae (Phaeophy*ceae) (Cardozo *et al.,* 2007; Wijesinghe and Jeon 2012); and III) agar and carragenans isolated from red al*gae (Rhodophy*ceae) (Gómez-Ordóñez *et al.,* 2012); whose major structural features are represented in figure 1. The relationship between structure and bioactivity is not clearly established (Costa *et al.,* 2010; Li *et al.,* 2008).

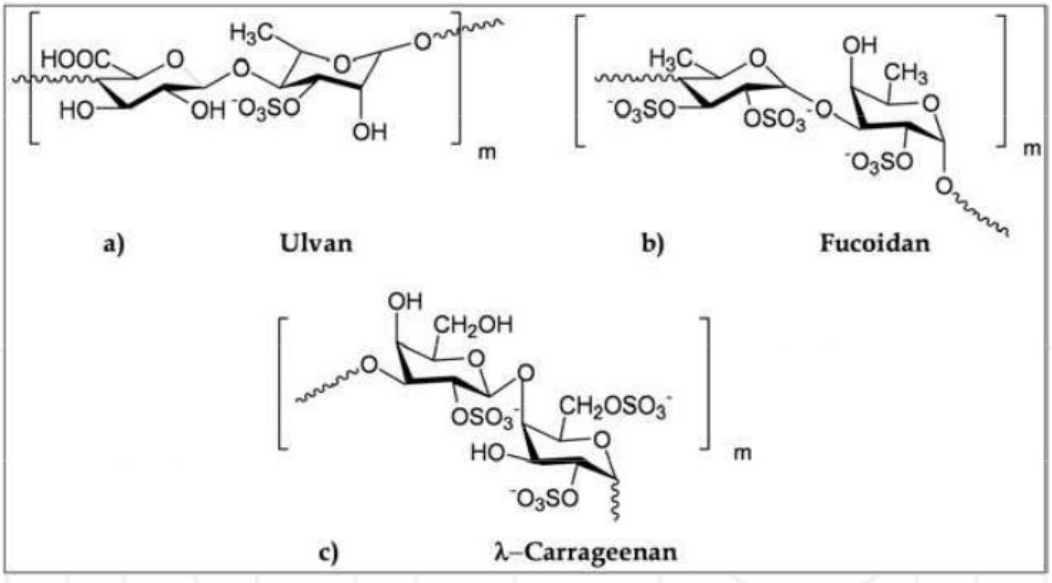

Figure 1 - Chemical structure of the dimeric repeating unit of sulfated polysaccharides. a) Ulvan, b) Fucoidan, c) λ-Carrageenan (Stengel *et al.,* 2011).

Ulvans show great complexity and variability in composition, but rhamnose, xylose, glucoronic and iduronic acids and sulfate groups have been identified as the main constituents of these polysaccharides. The oligosaccharide repeating units identified are 4-linked β-D-glucuronic acid and α-L-rhamnose-3-sulfate residues (figure 1a), or α-L-rhamnose-3-sulfate and 4-linked β-D-xylose, or 4-linked α-L-iduronic acid and α-L-rhamnose-3-sulfate residues can be commonly found (Lahaye and Robic, 2007; Jiao *et al.,* 2011). This polysaccharide have several physiochemical and biological features of potential interest for food, pharmaceutical, agricultural and chemical applications (Lahaye and Robic, 2007).

Fucoidans are polysaccharides containing mainly fucose with sulfate substitutions, (1→3)-linked α-L-fucopyranosyl residues or alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues (figure 1b). These polysaccharides have been investigated in recent years to develop novel drugs and functional foods due to their broad bioactivities (Jiao *et al.,* 2011; Li *et al.,* 2008; Wijesekara *et al.,* 2011).

Carrageenans are hydrophilic sulfated galactans, which consist mainly of 3-linked α-D-galactose and 4-linked α-L-galacto-6-sulfate (figure 1c) or 4-linked 3,6-anhydro-α-L-galactose units, isolated from marine red algae. These polymers are widely used as food additives, such as emulsifiers, stabilizers, or thickeners (Campo *et al.,* 2009; Jiao *et al.,* 2011).

1.2 Brown seaweed composition

Brown seaweed contains large amounts of non-starch polysaccharides that cannot be digested completely by the human digestive system, therefore having potential as new sources of dietary fiber, prebiotics or other functional ingredients. Similar to plants fiber, seaweed fiber is interesting because its consumption has been associated with a significant reduction of chronic diseases such as diabetes, obesity, heart diseases, and cancer (Kim, 2012).

Brown seaweeds have a high content of polysaccharide per weight. Other components of brown seaweeds, such as minerals, proteins, lipids, pigments, phenolic compounds, are worth being talked about because these compounds possess bioactive proprieties that can be beneficial to human health. These

compounds allow the brown seaweeds to have an added value and can be used in other applications as raw material for the extraction and purification of these compounds. In the table 1, can be seen the composition in the various constituents of brown seaweeds.

Table 1 - Overall composition of Brown seaweed from Northwest Europe (Adaptation from Holdt & Kraan, 2011)

Brown algae	**Laminaria Saccharina**	**Fucus**	**Ascophyllum**	**Undaria**	**Sargassum**
Moisture (%)	73 – 94	68 - 87	67 – 87	88	61
Polysaccharides (%)	38 – 61	45 – 70	42 – 70	35 – 46	< 68
Protein	3 – 21	1.4 – 17	1.2 – 12	11 – 24	9 – 20
Lipids (%)	0.3 – 2.9	0,5 – 3,1	1.2 – 4.8	1 – 4.5	0.5 – 3.9
Ash (%)	15 – 39	19 – 30	18 – 27	27 – 40	14 – 44
Phenolic content (%) (phlorotannin)	0.2 – 5.3	8 – 13	0.5 – 14 (5 – 13%)	< 0,4	2 – 6
Iodine (mg/100 g)	23 – 1,200	50 – 170	70 – 125	6 - 35	3 – 300

The composition of the marine algae is highly dependent of numerous parameters like wave-exposure, CO_2, salinity, temperature, pH levels factors and environmental conditions (light and temperature), salinity, and nutrient availability (nitrogen, phosphorus, and minerals), then the structures of compounds present variations between species and locations (Li *et al.,* 2008; Jiao *et al.,* 2011).

1.2.1 Polysaccharides in brown algae

Laminaran is a β-glucan, β(1→3)-linked polymer of D-glucose with some β(1→6) linkage with β(1→3):β(1→6) ratio being around 3:1 (figure 2i). It is the main storage polysaccharide in brown algae and may reach up to a maximum of 32 % dry weight. The biological activities of laminaran include stimulation of the immune system and cytotoxic effects on tumor cells (Kim, 2012; Kraan, 2012). The detailed structure of laminaran varies among species and its solubility depends on the amount of β(1→6)- branching. Highly branched laminaran is soluble in cold water whereas lower levels of ramification induce solubility only in warm water.

Cellulose is present in brown seaweed as structural polysaccharide of β(1→4) linked D-glucose units which content may reach from 6 to 14% dry weight. The mucilage polysaccharides are alginate, fucoidan, and laminaran.

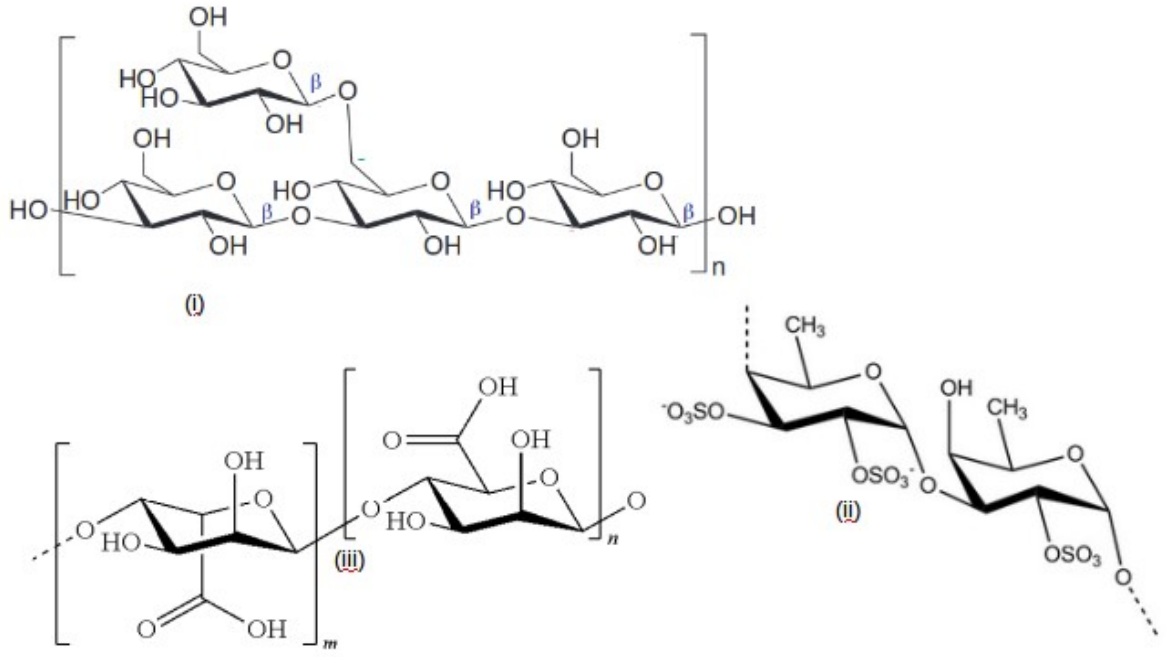

Figure 2 - Structure of most common polysaccharides in brown algae. (i) laminaran; (ii) representative structure of fucoidan polysaccharide; (iii) Alginic Acid structure

Fucoidan is a well-known mucilage heteropolysaccharide in brown algae composed essentially of fucose residues and sulfate esters (figure 2 ii). It is synthesized in the Golgi apparatus inside cells and is found in the intercellular spaces throughout the algal tissue. Although the function of fucoidan for the algae itself has not been thoroughly investigated, there are several theories on the subject. As the fucoidan content differs between the inter-tidal zone (high amounts of fucoidan) and the zone under the low water line (less amounts of fucoidan) as are the *Laminaria* species, conservation against dehydration can be assumed (Kim, 2012; Holtkamp, 2009). Another suggestion is the enhancement of cell wall stability, made to support the algae to the water salinity or as a protection agent against UV light exposure. This last proposed function could also be supported by the discovery that the sugar content of the algae gradually increases from April to September, during which time the algae are exposed to higher amounts of sunlight (Holtkamp, 2009). These hypothesis applies in studies of inter-tidal seaweeds

Fucus vesiculosus and *Ascophyllum nodosum,* as the function for submerged seweed may differ.

Alginate generally makes up to 10-30% of the dry mass of marine algae. Alginate is a β(1→4) linked polymer of D-mannuronic acid (ManA) and L-guluronic acid (GulA) (figure 2iii). Its properties, including solubility, depend on the ManA/GulA ratio and on the molecular mass, which ranges from 150 to 1700 kDa, depending on the source and the extraction method. Alginate have interesting proprieties, as gelling, thickening, emulsifying, and stabilizing properties which make it highly appreciated in the food industry (Rioux *et al.,* 2009; Kim, 2012).

1.2.2 Minerals

The mineral content in brown algae is generally high, with ash ranging between 5 and 40% dry weight, being dependent on the habitat conditions. Algae adsorb and store several minerals from seawater. Brown algae are rich in nutrients such as bromine, calcium, magnesium, potassium, sodium, phosphorus, sulfur, iodine, iron, and to a lesser extent, copper, chromium, chlorine, zinc, manganese, silicium, and selenium. The iodine content of brown algae is especially high, around 10-100 times higher than traditional vegetables and may reach alone up to 1.2% (Holdt and Kraan 2011).

1.2.3 Lipids and derivatives

Brown seaweeds may contain up to 1–5% of total lipids. On the other hand, a recent study reported that contents of lipids and omega-3 polyunsaturated fatty acids (PUFAs) of seaweeds vary seasonally, indicating that the lipids of some *Sargassaceae* brown seaweeds could reach 15 % in the winter (Miyashita *et al.,* 2013; Nomura *et al.,* 2012). A quantitative lipid analysis revealed that the lipid content of a major brown seaweed family, *Sargassaceae*, was higher in subarctic zones (approximately 5 %) than tropical zones (0.9–1.8 %), as so, cold temperatures play an important role in algae lipid content.

Brown seaweeds grown in temperate or subarctic areas can accumulate omega-3 and omega-6 polyunsaturated fatty acids (PUFAs). The major omega-3 PUFAs are eicosapentaenoic acid (EPA, 20:5n-3), stearidonic acid (18:4n-3) and α-linolenic acid (18:3n-3), while arachidonic acid (ARA, 20:4n-6) is the major

omega-6 PUFA. Some of the mentioned omega-3 PUFAs have been reported to demonstrate reduction of cardiovascular disease and cardio-protective effects (Miyashita *et al.,* 2013). PUFAs are important in the development and functioning of brain, retina, and reproductive tissues and can also be used in the treatment of various diseases, including a variety of cancers, and inflammatory disease (Nomura *et al.,* 2012; Miyashita *et al.,* 2013). These compounds, e.g. ARA, also play an important role in biological systems serving as precursors for hormone-like regulatory molecules, prostaglandins (PGs) and thromboxanes (TXs), and the leukotrienes (LTs) (Sayanova and Napier 2004; Miyashita *et al.,* 2013).

1.2.4 Pigments

Pigments are chemical compounds which have the ability to absorb certain wavelengths. Because they interact with light to absorb only certain wavelengths, pigments are useful to algae and other autotrophs organisms which make photosynthesis. In algae pigments are the means by which the energy of sunlight is captured for photosynthesis. However, since each pigment reacts with only a narrow range of the spectrum, there is usually a need to produce several kinds of pigments, each of a different color, to harvest most of the sun's light energy. Light harvesting processes of all photoautotrophic organism involves chlorophylls (all groups possess chlorophyll *a*) and various groups of organisms have accessory pigments which are specific to their class, e.g. different types of chlorophyll *c* and the fucoxanthin carotenoid are found in the brown-colored algae (Stengel *et al.,* 2011; Holdt and Kraan 2011).

Because brown seaweeds have these photosynthetic pigments (chlorophyll *c* and fucoxanthin)(figure 3), they can utilize more wavelengths for photosynthesis when compared with terrestrial plants or green algae, this allows the brown algae to grow in particular conditions (different depths and light intensities) (Makarov, 2012).

Besides the advantages to the brown alga both chlorophylls and fucoxanthin have been reported to have interesting biological effects. Chlorophyll is known to be converted into pheophytin, pyropheophytin and pheophorbide in processed vegetable food and these derivatives show antimutagenic effects and

may play a significant role in cancer prevention (Holdt and Kraan 2011). On the other hand the fucoxanthin exhibits characteristic biological activity, including anti-obesity, anti-diabetic, antioxidant, and anticancer effects (Miyashita *et al.,* 2011; Miyashita *et al.,* 2013).

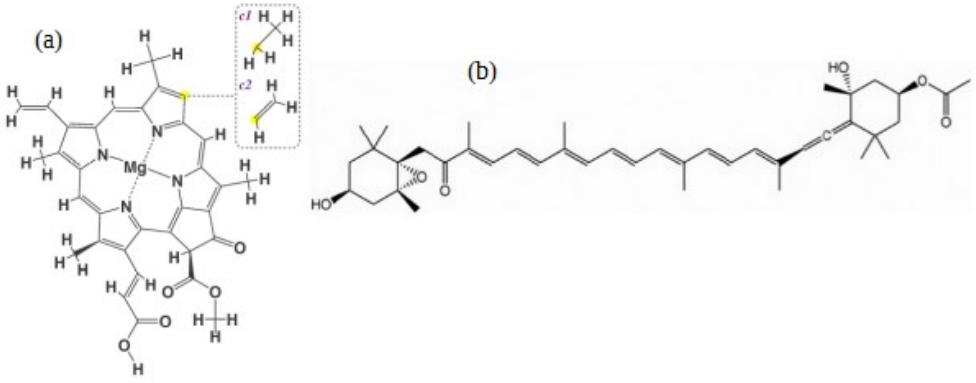

Figure 3 -Structure of characteristic pigments of brown seaweed, (a) Chlorophyll *c*; (b) Fucoxanthin

1.2.5 Phenol and phlorotannins

Phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (–OH) bonded directly to an aromatic hydrocarbon ring. Phlorotannins are a group of phenolic compounds found in brown seaweed. Usually phenolic content varies from <1% to 15% of dry seaweed biomass, with *Ascophyllum* and *Fucus* being the species with the highest content (14% and 12%, respectively) (Holdt and Kraan, 2011).

Phlorotannins are composed of several phloroglucinol units linked to each other in different ways. Constitute an extremely heterogeneous group of molecules (structure and polymerization degree) providing a wide range of potential biological activities. The chemical structures of few brown seaweed phlorotannins are shown in Figure 4 (Koivikko 2008; Thomas and Kim, 2011).

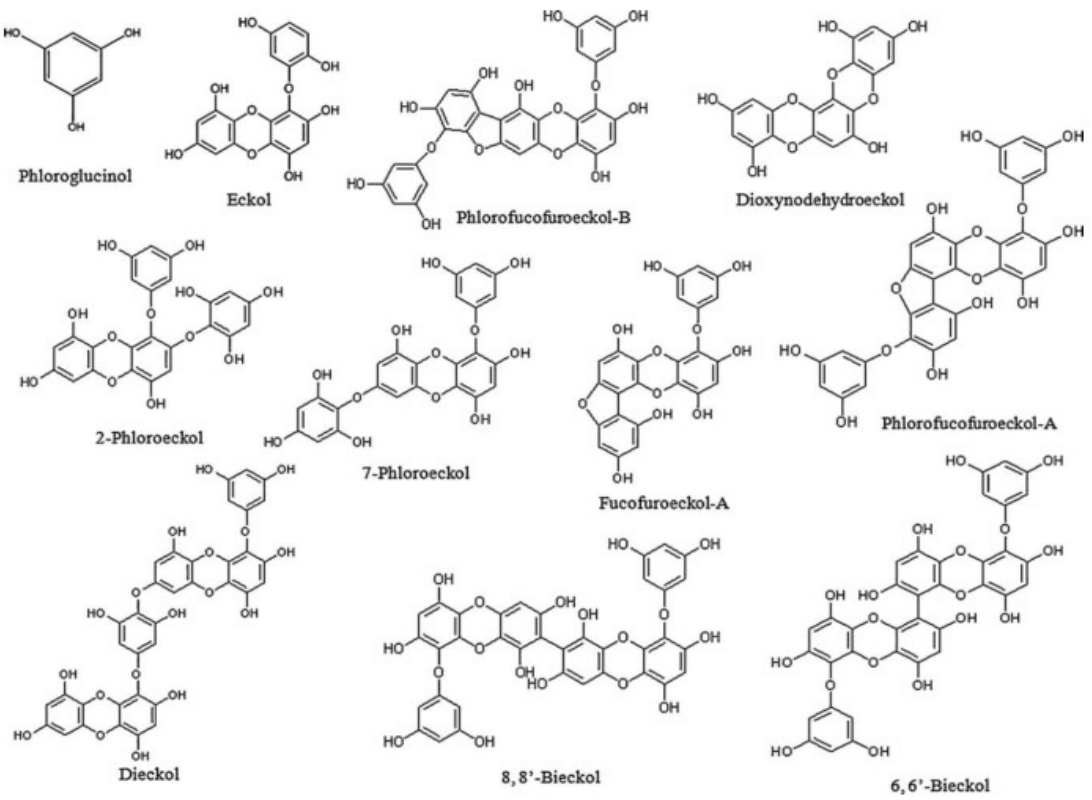

Figure 4 - Chemical structure of phlorotannins from brown algae (Myers *et al.,* 2011).

These phenolic compounds are integral structural components of cell walls in brown algae, but they also seem to play many other secondary activities, which make them candidate compounds for numerous applications, such as functional foods, pharmaceuticals, nutraceuticals and cosmeceuticals (Thomas and Kim 2011; Wijesinghe and Jeon 2011).

1.2.6 Proteins

Marine algae synthesize protein through fixation of Nitrate and ammonium in the waters, although Nitrate accumulation cost are high(~41 ATP equivalents) Nitrate vacuolar accumulation has also been proposed for some intertidal macroalgae, *Fucus* species, for instance, a nitrate internal concentration more than 10-fold higher than in the environment can be found in the winter, when growth is slower; (Giordano and Raven, 2014). The protein content varies with harvesting period, since environmental factors such as light, temperature and salinity influence protein synthesis (Fleurence 2004; Barsanti and Gualtieri, 2006).

The protein fraction of seaweed varies with the species, but is generally lower in brown seaweed, typically 3-15% (Fleurence 2004; Samarakoon and Jeon, 2012).

The brown species of seaweeds contain all the essential amino acids and are a rich source of the acidic amino acids, aspartic acid and glutamic acid. For example, in *Fucus sp.* aspartic acid and glutamic acid can represent between 19 to 41% of the protein fraction (Fleurence 2004). However, due to their low protein content brown seaweeds are rarely promoted for their nutritional value.

1.3 *Fucus vesiculosus*

Fucus vesiculosus is a marine algae assorted to the class of *Phaeophyceae* and order of *Fucales*. They are brown seaweeds that grow on rocky shores in areas with cold and temperate climate. Mostly at North American and Western European shores of the North Atlantic and the Pacific Ocean, measuring from 20 to 100 cm length, their flat thallus branches dichotomously (in two) and oval membranous structures filled with air make the seaweed float vertically (Figure 5). They usually live completely submerged as an intertidal marine alga or have long periods of exposition to the air (Kim, 2012).

Figure 5 - *Fucus vesiculosus* seaweed.

The common or commercial name of *Fucus vesiculosus* is bladderwrack, a type of seaweed that grows with cold and temperate temperature (European Medicines Agency 2013) commonly in the chores of Atlantic and Pacific oceans. It is a common food in Japan and is used as an additive or flavoring agent in various

food products in Europe. These species are useful as sources of bioactive elements (Guiry 2014; Kim, 2012).

*Fucus vesiculos*us have been administered either orally or topically. Oral uses include auxiliary measure for weight loss, treatment of gastritis, reflux oesophagitis and hiatus hernia, the prevention of atherosclerosis, viscous blood and hypercholesterolemia, the management of constipation, colitis, asthenia, fatigue, mineral deficit, anaemia, hair loss and leg cramps, an adjuvant for menopausal complaints, fibrocystic breasts, prostate complaints, growth deprivation, arthritis, arthrosis, gout, and lymphedema. External uses are treatment of wounds, an adjuvant in the therapy for cellulites and obesity and an aid for rheumatism and arthritis (European Medicines Agency 2013).

In vitro studies have been made with extracts of *F. vesiculosus* and its different components, mainly fucoidans and phlorotannins displayed significant activities. However the question remains whether the extract can reach the action site at a sufficient dose *in vivo* (effects on single parameters as hormones or blood glucose) and if the models are representative for *in vivo* effects. Just a low number of clinical studies were made in humans (effects on skin and menstrual cycle) (European Medicines Agency 2013).

Recently, as search for new drugs and natural products increased, fucoidans (extracted from *Fucus vesiculosus)* gained interest due to its biological activities and potential medical applications and low cytotoxicity (Holdt and Kraan 2011; Li *et al.,* 2008).

1.3.1 Polysaccharides composition of *Fucus vesiculosus*

The composition of the marine algae is dependent of numerous factors, as environmental conditions, nutrient and time of harvesting. As result, the same species have been reported to possess slightly different compositions, for example, an average content in polysaccharides in dried seaweed collected in Quebec (Canada) of 62,6% and 65,7% dry weight, respectively, was reported (Kim, 2012 and Rioux *et al.,* 2009), whereas seaweeds (Europe) on Barent's sea showed a total polysaccharide composition reaching the 45% (dry weight). Obluchinskaya (2008)

The content of the fucoidan is the highest in the representative genus of *Fucus* algae, among them, the *F. vesiculosus* is the species that is the most used in industry. According to the reported information this species' fucoidan reaches 9-22% of dry weight (Ale *et al.,* 2011b; Obluchinskaya 2008) and alginate content may reach from 8% to more than 30% dry weight. Such variability may be resultant from differences in the algae habitats and times of harvesting, as it was previously said, and may also have influence on both analytical methods and extraction procedures used. According to these studies, a decrease in the fucoidan contend of 15% in summer (August) and 10% in winter was observed. The same happened for alginate/alginic acid which shown values of 24% in summer (August) but a little over 8% in spring (April) (Obluchinskaya *et al.,* 2002; Obluchinskaya 2008).

1.4 Fucoidans

The term “fucans” was used to designate all polysaccharides rich in L-fucose, but as separation and analytical methods improved and a better knowledge about the composition and branching of this polysaccharide was attained, the nomenclature lead to some confusions. The “fucans” or “sulfated fucans” is used to refer to the sulfated polysaccharide from marine invertebrates that contain L-fucose and less than 10% of other monosaccharide units, while the name “fucoidan” is used for the ones with origin in seaweeds.

Fucoidans are sulfated water-soluble polysaccharides constituted essentially of sulfated α-L-fucopyranose residues, or may have a more complex and heterogeneous composition that, apart from α-L-fucose and sulfates, may contain other monosaccharides (galactose, xylose, mannose, rhamnose, glucose and/or uronic acids) and may even have acetylated groups (Morya *et al.,* 2012; Kusaykin *et al.,* 2008; Ale *et al.,* 2011c)

Controversial data can be found in literature even for the most studied fucoidan from *F. vesiculosus* (Li *et al.,* 2008). This might be in part because many studies used fucoidans from sources grew in different conditions. The fucoidan not always appear as a pure polysaccharide possibly having multiple forms –

differences in molecular weight, charge, branching and substitutions – and may have other constituents from the source.

1.4.1 Structure and composition of fucoidans

The simplest fucoidans, have a backbone chain of α-L-fucopyranose residues which can be assorted to two different types as show in Figure 6, a type (I) chain that is organized in repeating units of (1→3) linked α-L-fucopyranose residues, and a type (II) chain containing alternating (1→3) and (1→4)-linked residues (Cumashi *et al.,* 2007). The type (I) backbone chains of (1→3) α-L-fucopyranosyl are found in fucoidans from *Laminaria* or *Cladosiphon* species (Cumashi *et al.,* 2007).The type (II) backbone chains, with the alternating (1→3) and (1→4) linkage are found in algae of *fucales* order, like the brown seaweeds from *Fucus* and *Ascophyllum* genus (Kusaykin *et al.,* 2008; Cumashi *et al.,* 2007). Regarding the *Fucus* genus' fucoidans *(F.distichus, F.serratus* and *F.evanescens*), it was found that besides the existence of alternating (1→3) and (1→4) glycosidic linkages in fucoidan, there is a predominance in the (1→3) linkages relatively to the (1→4) linkages (Anastyuk *et al.,* 2009; Anastyuk *et al.,* 2014; Bilan *et al.,* 2002; Bilan *et al.,* 2004; Bilan *et al.,* 2006).

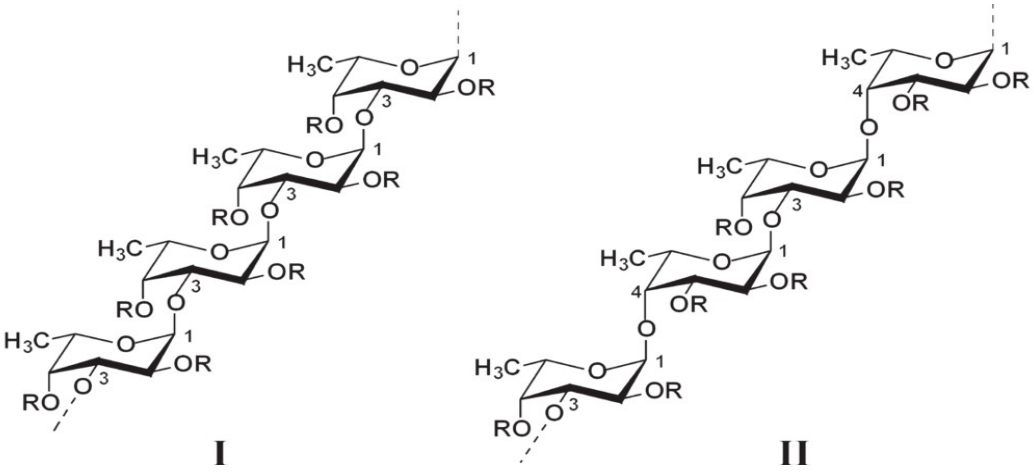

Figure 6 - Two types of homofucose backbone chains in brown seaweed fucoidans. Chain (I) are repeating (1→3)-linked α-L-fucopyranose residues whereas chains (II) contain alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues. R depicts the places of potential attachement of substituents (Cumashi *et al.,* 2007).

Algal fucoidans may be branched in different positions and have branches every 2-3 residues (Jiao *et al.,* 2011). The positions of substituents within the backbone happen at C2, C3 and/or C4 (Bilan *et al.,* 2010; Cumashi *et al.,* 2007; Jiao *et al.,* 2011). The most common substituents are sulfate groups, D-glucuronic acid (GlcA) or L-fucopyranosyl residues, which can also have branching by other monosaccharides such as galactose (Gal), mannose (Man), xylose (Xyl), glucose (Glc), or even acetyl goups (Jiao *et al.,* 2011; Cumashi *et al.,* 2007; Ale *et al.,* 2011c).

The fucoidans from *Fucus* seaweeds had sulfate groups mainly on C-2 from both the (1→3) and (1→4) linked α-L-fucopyranose residues, whereas an additional sulfate may occur in the C-4 position in some 3-linked fucose residues and acetylation is low and occurs randomly (Bilan *et al.,* 2002; Bilan *et al.,* 2004).Not all fucose residues have sulfate groups linked (Anastyuk *et al.,* 2009). In general, the sulfate content of fucoidan from brown algae is 35-40% of the dry weight of the fucoidan (Kim, 2012). Different pattern of substitution present in the fucoidans are reported, C 2-sulfated, C 2,3-disulfated, and C 2,4-disulfated (Ale *et al.,* 2011c) for different algae species(Figure 7).

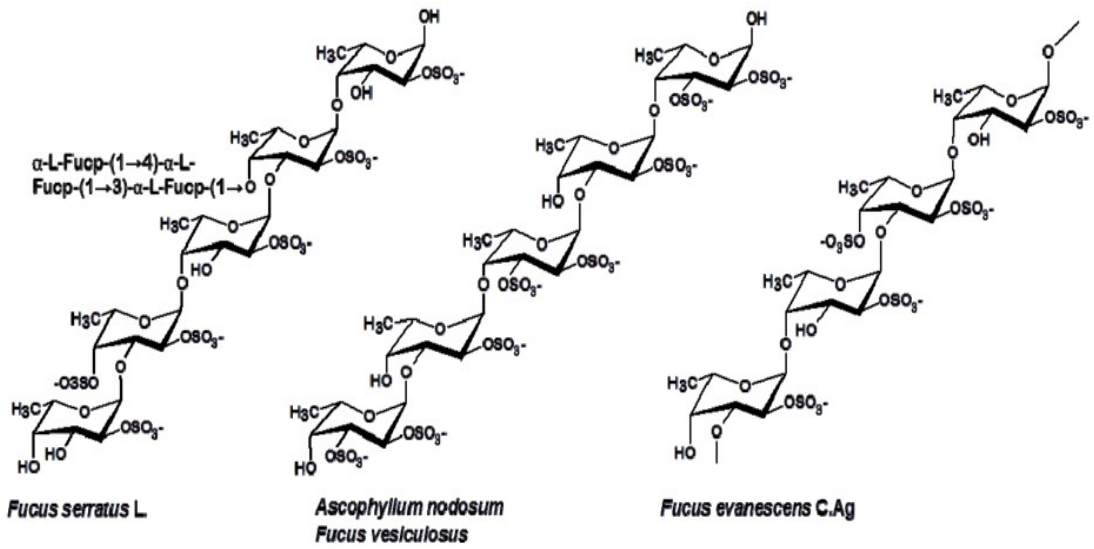

Figure 7 - Representation of the fucoidans from *Fucus* and *Ascophyllum* genus. (Ale *et al.,* 2011c).

Branched structures were proposed for *F.evanescens* in which (1→3) fucose oligosaccharides (1 to 4 units) were linked at the C-4 at near half of the 3-linked fucose residues of the backbone (Bilan *et al.,* 2006; Anastyuk *et al.,* 2009),

also minor components of xylose and galactose residues linked to fucose in (1→4) linkages are present. Glucuronic acid was also found as a part of a non-sulfated oligosaccharide with fucose (Anastyuk *et al.,* 2009).

Fucus vesiculosus fucoidans have a relatively simple structure, when compared to structures observed in fucose containing sulfated polysaccharides (FCSPs) fractions obtained from other seaweed, like *Sargassum fusiforme* (Fucales). These FCSPs from *S. fusiforme* had fucose, mannose, galactose, uronic acid and sulfate in comparable amounts. The analysis on this FCSP showed that the core of these FCSPs was mainly composed of alternating units of 2-linked α-D-mannose and 4-linked β- glucuronic acid residues, with a minor portion of β(1→4)-D-Gal units, that was branched with fucose oligosaccharides at C-3 of the 2-linked mannose (Figure 8) (Li *et al.,* 2006; Li *et al.,* 2008).

In *Sargassum stenophyllum* similar fucoidans with (1→2)-β-D-mannose and (1→6)-β-D-galactose units with branched chains of 'fucans', formed essentially by (1→3) and/or (1→4)-β-L-fucose, some (1→4)-α-D-glucuronic acid, β-D-xylose terminal and, sometimes, (1→4)-α-D-glucose may also be present (Duarte *et al.,* 2001; Ale *et al.,* 2011c). Structural analysis indicated that the sulfate groups might be found in any position on the galactose/mannose backbone or on the fucose units (Li *et al.,* 2008).

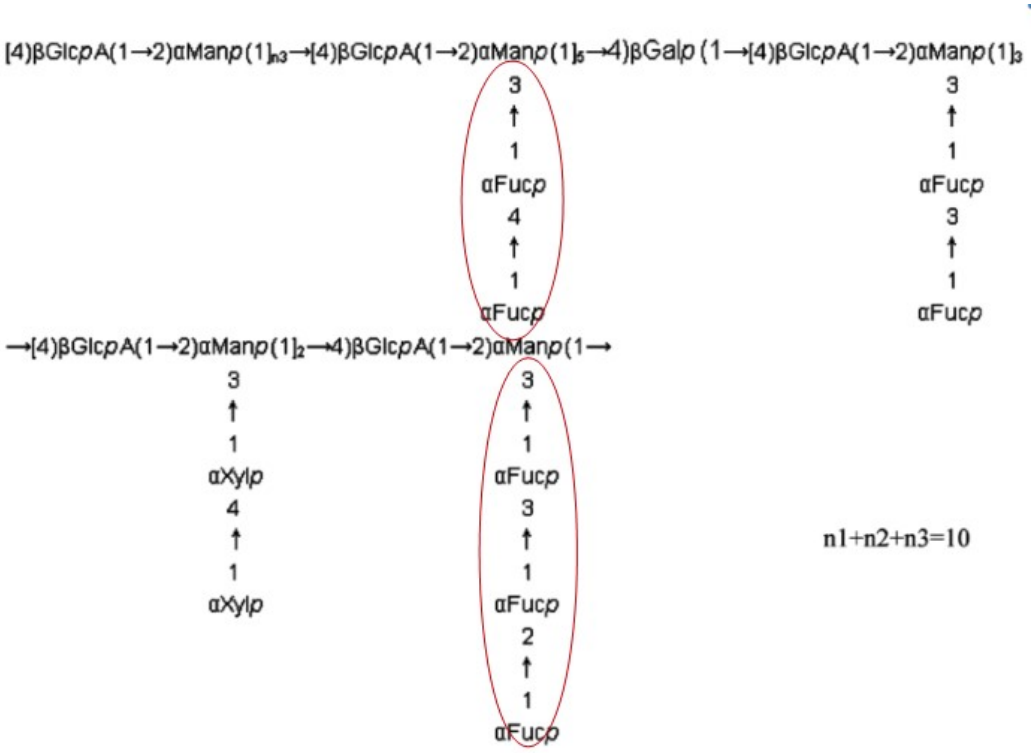

Figure 8 - Proposed structure of a FCSP isolated from *Sargassum fusiforme*. Core units of α-Mannose, β-Glucuronic acid with some β-Galactose, branched with fucoidans chains (Li *et al.,* 2006; Fitton 2011)

Despite the numerous studies in the past years about the many aspects of the promising biological activities of fucoidans that have been elucidated and the structural features that have been solved, the structure-activity relationship (SAR) is not clearly established due to many difficulties connected with the determination of the fine structure of the polysaccharide (Croci *et al.,* 2011; Bilan *et al.,* 2002; Cumashi *et al.,* 2007).

1.5 Properties of fucoidans from marine algae

Sulfated polysaccharides in seaweed contains diverse biological activities with potential medicinal value, such as anticoagulant, anti-thrombotic, anti-inflammatory, anti-tumor, contraceptive, antiviral, cardioprotective, antilipidemic immunomodulatory and antioxidant (Mayakrishnan *et al.,* 2013; Myers *et al.,* 2011; Senthilkumar *et al.,* 2013; Holdt and Kraan 2011; Cumashi *et al.,* 2007; Ale *et al.,* 2011a; Ale *et al.,* 2011c). The biological activities of the fucoidans are most likely to dependent be structural features, especially sulfate content (charge density) and position (Wang *et al.,* 2008; Ale *et al.,* 2011c; Gómez-Ordóñez *et al.,* 2012).

1.5.1 Anticoagulant, anti-thrombotic, and procoagulant activities

Anticoagulant activity is among the most widely studied properties of sulfated polysaccharides. Currently heparins are the only sulfated polysaccharides used as anticoagulant drugs. However these compounds have several side effects such as bleeding and thrombocytopaenia (refers to a relative decrease of platelets in blood). This increases the necessity to uncover new sources of anticoagulant agents, such as algae containing fucose sulfated polysaccharides (FCSPs) which have been described to possess an anticoagulant activity like heparin (Silva *et al.,* 2005; Costa *et al.,* 2010; Cumashi *et al.,* 2007). However, some fucoidans showed an activity below 10 folds compared to heparin, which was hypothesized to be due to presence of glucuronic acids in the branches together with its low sulfate content (Cumashi *et al.,* 2007).

Anticoagulation activity of fucans was positively correlated with sulfate content and only fucans with a sulfate total sugar residue ratio greater than one possessed significant activity (Jiao *et al.,* 2011). In addition, importance of sulfate group location on the sugar residues for anticoagulant activity was shown, since the presence of 2-*O*-sulfated and 2,3-*O*-disulfated fucose residues is required, whereas sulfation at the O-4 position did not appear necessary (Silva *et al.,* 2005; Jiao *et al.,* 2011). However, the presence of non-sulfated monosaccharide units at the end of nonreducing ends may abolish the anticoagulant effect of the polysaccharide (Costa *et al.,* 2010; Li *et al.,* 2008).

Although the basis for these activities is not completely understood various mechanisms of action have been suggested, which include both direct and indirect inhibition of thrombin. Fucoidans may inhibit thrombin activity by directly acting on the enzyme or through the activation of thrombin inhibitors, like anti-thrombin III or anti-thrombin III and heparin cofactor II (some fucoidans only activate antithrombin) (Cumashi *et al.,* 2007; Jiao *et al.,* 2011).

A possible relationship between anticoagulant activities of fucoidan and its physical, chemical, and structural proprieties still remain to be firmly established. The uncertainties rise due to the structural variations among algae species, whose different structural compositions and molecular weights give discordant results regarding the mechanisms of anticoagulant activities. However, the anticoagulant

effect of sulfated polysaccharides has been clearly shown that to be stereo-specific and not merely a consequence of their charge density or sulfate content (Ale *et al.,* 2011c; Costa *et al.,* 2010).

Among all the sulfated polysaccharides, the fucoidans, extracted from brown seaweeds, depending of the concentration, have been shown to both stimulate and inhibit blood coagulation *in vitro* (Zhang *et al.,* 2014). This propriety can be useful, modulating coagulation, in future treatments of hemophilia A and B, caused by deficient clotting factors FVIII and FIX, respectively (Lillicrap 2013). As fucoidans exert procoagulant activity by modulating the activity of tissue factor pathway inhibitor (TFPI) the major physiological inhibitor of the extrinsic coagulation pathway (Zhang *et al.,* 2014), still, despite being shown that fucoidan is part responsible by the benefits in treatments, the efficacy of fucoidan very likely involves more than just TFPI inhibition (Lillicrap, 2013).

Specific structural properties depend on algae species, harvest time, plant parts, location, and extraction procedures influence fucoidans' procoagulant and anticoagulant activities (Ale *et al.,* 2011b; Zhang *et al.,* 2014).

1.5.2 Anticancer and antitumural activities

Some sulfated polysaccharides have been used in traditional chinese medicinal therapies for nearly 2000 years for treatment of various diseases, including cancer, as is the case of *Sargassum sp*. fucoidan (Zhu *et al.,* 2013). Although the underlying anticancer and antitumural effects of fucoidan are largely unknown, it can directly induce cytotoxicity and apoptosis in cancer cells, (Kwak 2014; Senthilkumar *et al.,* 2013), likely by up-regulation or down-regulation of multiple signaling pathways (Senthilkumar *et al.,* 2013; Zhu *et al.,* 2013). Several studies have also indicated that sulfate groups of fucoidan play a major role in the suppression of cancer cell growth by binding with cationic proteins on the cell surface (Pielesz *et al.,* 2011).

Fucoidan can also affect cancer cells indirectly e.g., as an antiangiogenic agent and anti-metastasis (Senthilkumar *et al.,* 2013). Furthermore, fucoidan has immune-stimulating effects on dendritic cells (DCs) (Yang *et al.,* 2008) and natural killer (NK) cells (Ale *et al.,* 2011ac; Kwak 2014). Thus, fucoidan can enhance

anticancer immunity through immune cell activation and influx and stimulation of the production of anticancer cytokines (Kwak 2014).

1.5.3 Anti-inflammatory activity

A growing body of experimental evidence indicates that fucoidan, display a wide variety of pharmacological anti-inflammatory activities, being fucoidan able to have protective activities in gastrointestinal (GI) tract (Iraha *et al.,* 2013).

Various authors have evidenced the fucose sulfated polysaccharide to act as immunomodulatory mediator, since those polysaccharides have capacity to regulate the “expression” of some pro-inflammatory molecules and pathways (Do *et al.,* 2010).

1.5.4 Antiviral anti-HIV

Many species of marine algae contain significant quantities of complex structural SPs that have been shown to inhibit the replication of several encapsulated viruses including HIV (AIDS) and HSV (herpes simplex viruses) (Damonte *et al.,* 2004; Queiroz *et al.,* 2008; Wijesekara *et al.,* 2011).

The herpes simplex viruses attach to cells by an interaction between the envelope glycoprotein C and the cell surface heparan sulfate (HS). The virus-cell complex is formed by both ionic interactions between the anionic (mainly sulfate) groups in the polysaccharide and basic amino acids of the glycoprotein, and non-ionic ones depending on hydrophobic amino acids interspersed between the basic ones in the glycoprotein-binding zone. The antiviral activity of the sulfated seaweed polysaccharides is said to be based on the formation of similar complexes that block the interaction of the viruses with the cells(Damonte *et al.,* 2004).

In anti-HIV activity studies, sulfated fucans could inhibit reverse transcriptase activity, but with desulfation this activity is lost, which shows the importance of sulfated groups in these polysaccharides activities (Queiroz *et al.,* 2008; Wijesekara *et al.,* 2011).

1.5.5 Antioxidant activities

Antioxidants may have a positive effect on human health as they have protective properties against damage caused by reactive oxygen species (ROS), which attack macromolecules such as DNA, proteins and membrane lipids, leading to many health disorders such as cancer, diabetes mellitus, neurodegenerative and inflammatory diseases with severe tissue injuries. Lipid oxidation by ROS such as superoxide anion ($\bullet O^{2-}$), hydroxyl radicals(•OH) and hydrogen peroxide (H_2O_2) also causes a decrease in nutritional value of lipid foods, and affect their safety and appearance (Rocha de Souza *et al.,* 2007; Wang *et al.,* 2008; Wijesekara *et al.,* 2011).

In recent years, polysaccharides isolated from seaweeds have been demonstrated to be potential ROS scavengers. It has been demonstrated that SPs have potential antioxidant activity, having found that the effect was affected by molecular weight and sulfate content, with a positive correlation between sulfate content and antioxidant activity (Wang *et al.,* 2008; Rocha de Souza *et al.,* 2007). Superoxide scavenging ability was related to sulfate content and hydroxyl radical scavenging activity was related to sulfate/fucose ratio (Wang *et al.,* 2008).

1.5.6 Other activities

Besides this activities previously described, it has been reported some other activities for sulfated polysaccharides extracted from *F. vesiculosus*, such as: antilipidemic agent in the reduction of total cholesterol and serum tryglycerides (in rats) (Kraan, 2012). Furthermore the anti-hypertriglyceridemia activity of fucoidan signifies that the myocardial membrane was protected against damage (cardioprotective effect of fucoidans) (Mayakrishnan *et al.,* 2013) and potential hypoglycaemic agent in reduction of diabetes Type II (Kim, 2012). Also, it has been reported fucoidan effects against various renal, hepatic and uropathic disorders (Kraan, 2012; Jiao *et al.,* 2011).

1.5.7 Non Toxicity

Even positive functional material and compound might cause a side-effect and toxicity when it would be over-dosed or inappropriately used. Li *et al.*(2005) reported no toxicological effects for an oral intake of a fucoidan, from *Laminaria japonica,* below 300 mg/kg/day in Wistar rats. Hematological and serum biochemical parameters were evaluated but only placket clotting time showed significant differences for doses of 900 mg/kg/day and 2500 mg/kg/day, even after a recovery period (Mayakrishnan *et al.,* 2013).

Phase I and II studies on humans, have proven orally taken seaweed containing fucoidan extracts were safe over a period of 4 weeks, and still demonstrating potential as an immune modulator (Myers *et al.,* 2011). Those results suggest that fucoidan generally has low toxicity and is well tolerated (Kwak 2014). Sulfated polysaccharides at concentrations higher than the ones they exhibit biological activity show no toxicity (Croci *et al.,* 2011; Do *et al.,* 2010; Mayakrishnan *et al.,* 2013).

1.6 – Extraction methods

1.6.1 – Classic extraction procedures

Classical extraction procedures of fucose containing polysaccharides from brown seaweeds typically involved extended, multistep, hot, acid or $CaCl_2$ treatments, each one for several hours, followed by precipitation with organic solvents, calcium salts or surfactants.

More recently, studies for optimization of the process showed the effects of various variables in the extraction yields and polysaccharides content. Temperature increase, up to 90ºC, lead to an higher yield on polysaccharides as well as higher extraction times time (until 4h of extraction). Acid concentration has a negative effect in the fucose content within the polysaccharides, as acid causes degradation of the fucoidan and its structural stability (Ale *et al.*,2011b;Yu and Chao, 2013).

1.6.2 NEOS-GR microwave hydrodiffusion and gravity system

Microwave assisted extraction (MAE) is a simple technique that provides a novel way of extracting soluble products into a fluid, from a wide range of materials, helped by microwave energy. It offers a rapid delivery of energy to a total volume of solvent and solid plant matrix with subsequent heating of the solvent and solid matrix, efficiently and homogeneously (Tsukui and Rezende 2014). NEOS-GR (Milestone) is a solvent free system consisting in a simultaneous hydrodiffusion and gravity methodology assisted by microwave. The microwave energy is delivered to the hydrated raw material . When the water within the matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates extraction of the compounds from the matrix (Tsukui and Rezende 2014; Kaufmann *et al.,* 2001). The polar compounds can be extracted into the released water and collected by gravity.

1.7 Aim of the work

As described the brown seaweed, *Fucus vesiculosus,* has a broad range of uses and applications. The fucoidans from *F. vesiculosus* from “Ria de Aveiro” were never characterized. Then, the aim of this work was to isolate and structurally characterize the fucoidans of *F. vesiculosus,* from “Ria de Aveiro”, in order to find new applications, adding value to this endogenous resource.

2 Material and methods

2.1.1 Sample and pretreatment

The brown seaweed *F. vesiculosus* was harvested in winter of 2013/2014 (months of December to February) and previously dried in “AlgaPlus” company (Ílhavo, Aveiro, Portugal). The seaweed was grounded in a miller (Retsch ZM1000) with a sieve of 3.0 mm.

The milled seaweed (10 g) were treated with 100 mL of a mixture of MeOH:$CHCl_3$:H_2O (4:2:1) (1 g_{algae}:10 $mL_{solvent}$) and washed with acetone, then dried at room temperate, in order to remove colored matter and some other extractable compounds (Bilan *et al.,* 2004).

2.1 Extraction

2.1.2 Hot water extraction

The polysaccharide extraction was made using mild extraction procedure with hot water (Yu and Chao, 2013; Ale *et al.,* 2011b). To the pretreated alga (3 g) was added 70 mL of distilled water at 90ºC and stirred for 1 or 4 hours. The supernatant was collected and to the residue was added again water and stirred during 15 min in the same conditions. The supernatants were collected and dialyzed with a 12-14 000 Da dialysis membrane to remove low molecular material, and then the samples were lyophilized.

2.1.2 *Microwave hydrodiffusion and gravity extraction*

Microwave hydrodiffusion and gravity system (Milestone's NEOS-GR) was used for extracting the polysaccharides from the brown algae.

The dried algae were rehydrated by immersion for a day in water. The rehydrated alga (300 g) was put in the oven compartment and irradiated with 300 watts or 900 watts, until the internal sensor of the device gives signal for burning point. Fractions of 50 mL were collected. The material was removed from the oven and 100 mL of water was added and also 100 mL of ethanol. The supernatants were collected. The algae were submitted to a new irradiation with 300 watts or 900 watts collecting two more fractions.

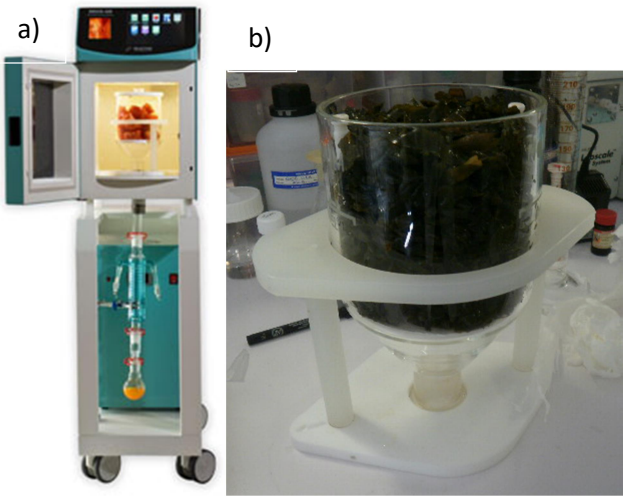

Figure 9 -a) NEOS-GR Microwave Hydrodiffusion and Gravity (MHG) System. b) Module with the re-hydrated algae.

2.2 Precipitation

2.2.1 Ethanol precipitation

The lyophilized water extracts were dissolved in distilled water and, then, ethanol was added in sequential increasing concentrations, 30%, 50% and 80% (v/v). The precipitate obtain in each ethanol concentration was separated by centrifugation, at 4ºC at 15000 rpm for 15 min. The supernatant obtained after 80% ethanol was evaporated in a rotary evaporator. All samples were lyophilized.

The procedure was later modified, at a concentration of 50% ethanol was added a solution with calcium chloride salts (400 mg/20 mL), the precipitate obtained was separated, and sequential increase to ethanol 80% was added to solution with calcium chloride salts.

2.2.2 Anionic exchange chromatography

Due to the differently negatively charged types of polysaccharides present in the brown seaweeds – alginate (uronic acids) and fucoidans (sulfated polysaccharides) – anionic exchange chromatography was used for further purification.

The resin DEAE (diethylaminoethanol)-Trisacryl M (Sigma) was used, as it had the desired characteristics of being a weakly basic exchange resin, (bead size 40-80µm).

The fractions precipitated in ethanol, rich in fucose and sulfate esters were further purified by anionic exchange chromatography. A portion of the sample was dissolved in Tris-HCl buffer 0.05 M, pH 7.4, (~1 mg/mL) and eluted in the column at a flow of 0.370 mL/min with increasing concentrations of NaCl of 0.5 M, 1 M, and 2 M in Tris-HCl buffer, in a column of 40 mL. Each fraction had 3.7 mL. The fractions containing sugars were collected, dialyzed and freeze dried for further characterization.

2.3 Sugar analysis

2.3.1 Phenol-sulfuric acid method

This method was used in order to detect the presence of sugars in the chromatographic fractions. To 80 µL of the eluted fraction were added 160 µL of phenol 5% (v/v) and 1 mL of concentrated sulfuric acid. The tubes were manually stirred and incubated at 100ºC in a water bath for 5 min. The tubes were cooled and the absorbance was measured at 490 nm.

2.3.2 Monosaccharides analysis

The analysis the neutral sugar composition of the polysaccharides was performed using the alditol acetates method. It consists in a polysaccharide hydrolysis to monosaccharides followed by a reduction and acetylation into alditol acetates and analysis by gas chromatography equiped with a flame ionization detector (GC-FID).

Hydrolysis: 1-2 mg of sample was weighted into a culture tube (~10 mL) then 200 µL of H_2SO_4 72% was added and incubated at room temperature for 3 h to dissolve the sample. Distilled water (2.2 mL) was added to the culture tube (final concentration of H_2SO_4 1 M) and hydrolyzed at 100ºC in a heating block for 2.5 h (after 1 h cool the sample and remove 0.5 mL to be used in uronic acid analysis, proceed with the hydrolysis for another 1.5 h). Cool the tubes in an ice bath.

Reduction and acetylation: 200 µL of an internal standard (2-deoxy-D-glucose 1 mg/mL) were added to each culture tube. A volume of the hydrolyzed sample of 1.0 mL was transferred to new culture tubes and then neutralized with

200 µL of NH_3 25%. The reducing agent, $NaBH_4$ 15% (w/v) in NH_3 3 M (100 µL), was added and incubated in the heating block at 30ºC for 1 h. The tubes were cooled in an ice bath and the excess of BH_4^- was eliminated by addition of 2 volumes of 50 µL of glacial acetic acid.

A volume of 300 µL was transferred to Sovirel tubes with Teflon caps, to which were added, in an ice bath, 450 µL of 1-methylimidazole and 3 mL of acetic anhydride, and incubated in the heating block at 30ºC for 30 min. In an ice bath, 3.0 mL of distilled water (to decompose the excess of acetic anhydride) and 2.5 mL of dichloromethane were added, stirred vigorously to extract alditol acetates, then centrifuged at 3000 rpm for 30 s. The aqueous phase was removed by suction. Again, 3.0 mL of distilled water and 2.5 mL of dichloromethane were added, stirred and centrifuged and aqueous phase removed as described previously. The organic phase was washed with 3 mL of distilled water, stirred, centrifuged at 3000 rpm for 30 s and then the aqueous phase was removed. This last step of washing with water was repeated once more.

The dichloromethane was evaporated in a centrifuge evaporator at reduced pressure. After the dichloromethane evaporation, 1 mL of anhydrous acetone was added and evaporated as previously described. This step was repeated once more.

GC-FID analysis: the alditol acetates were dissolved in 50 µL of anhydrous acetone and analyzed by GC-FID using a capillary column DB-225 (30 m long, 0.25 mm of diameter and 0.15 µm thick) in a Perkin Elmer – Clarus 400 chromatograph. The carrier gas was hydrogen (H_2) with a flow rate of 1.7 ml/min and a pressure of 17 psi. The injector temperature was at 220°C and the detector at 230°C. The injection was carried out in "split" mode. The temperature program used was as follows: initial temperature 200°C, increasing to 220°C at 40°C/min, keeping at this temperature for 7 min, followed by an increase to 230°C at 20°C/min, leaving at this temperature for 1 min.

The analysis of neutral sugars was carried out in duplicate. The sugars were identified by retention time in comparison with standards.

2.3.3 Uronic acids determination

The hydrolysis for uronic acids was made in simultaneous with the neutral sugars (described in section 2.3.2). The hydrolyzed samples (0.5 mL) were diluted with 3 mL of distilled water.

Galacturonic acid standard (0.5 mL) of concentration ranged from 0 to 80 µg/mL and 0.5 mL of the sample were added, in triplicate, to test-tube and, in an ice bath, it was added to each tube 3 mL of sodium borate 50 mM in concentrated H_2SO_4. After mixing, the samples were incubated at 100ºC for 10 min.

In the dark, 100 µL of MFF (m-phenylphenol 0.15% (w/v) in 0.5% (w/v) NaOH) were added to the sample/standard tube (not the blanks). The tubes were shaken and stored in the dark for 30 min. The absorbance was read at 520nm. The samples concentration was calculated by comparison with the calibration curve.

2.3.4 Sulfate esters determination

Sulfate esters were analyzed based on a modified (Dodgson and Price 1962) method for turbidimetric quantification of polysaccharide sulfate esters.

A solution of gelatin was prepared by dissolution of 2 g of gelatin in 400 mL H_2O (60-70ºC), followed by a rest time fat least 6 h in 4ºC. Barium chloride-gelatin reagent was made by dissolution of 1 g of barium chloride in the 200 mL of the gelatin solution, and used after 2-3 h rest at 4ºC.

The (sulfated) polysaccharide, usually 2-4 mg, was dissolved in 1 mL of 1 N hydrochloric acid, in order to give a final concentration of SO_4^{2-} ion between 40 and 90 µg/0.2 mL. The dissolved polysaccharide was then hydrolyzed in a temperature between 105-110ºC during 5 h. The tubes were cooled and 0.2 mL of sample was transferred to a tube containing 3.8 mL of trichloroacetic acid 3% (m/v), in duplicate or triplicate, to which were added 1 mL of barium chloride-gelatin reagent, and left under stirring for 20 min at room temperature. The absorbance of the resulting solutions was measured at 360 nm, using as a blank a solution where the 0.2 mL of sample was substituted by distilled water.

A second 0.2 mL portion of the hydrolysate was added to 3.8 ml of trichloroacetic acid, as described above, then 1 mL of gelatin solution (i.e. containing no barium chloride) was added. This 'control' solution was then measured at 360 nm against a reagent blank. This control gave a measure of the ultraviolet absorbing materials produced during hydrolysis and the reading obtained was subtracted. The resultant value is compared to a calibration curve of SO_4^{2-} made with concentrations of K_2SO_4 ranging from 0.1 – 1.0 mg, [SO_4^{2-}] from 11-110 µg/0.2 mL.

2.4 Methylation analysis

2.4.1 Methylation of polysaccharide

The lyophilized polysaccharides samples were weighted (1-2 mg) into a tube with septum and 1 mL of DMSO (dimethyl sulfoxide) was added and left overnight with stirring for a complete dissolution of the sample. Grinded NaOH (40 mg) were added to the solution and left under stirring for 30 min at room temperature. After this time, 80 µL of CH_3I were added to the solution and left reacting for 20 min under stirring (3 times). The sample was dissolved with 3 mL of $CHCl_3$:MeOH (1:1 v/v) and dialyzed against a solution of 50% ethanol:water (three times) and then concentrated to dryness. This methylation procedure was repeated.

The methylated polysaccharides were hydrolyzed by adding 500 µL of TFA 2 M (Tri-fluoracetic acid) and incubated at 121ºC for 1 h, followed by evaporation of the acid in the centrifuged evaporator. The hydrolyzed sugars were reduced and acetylated as described for monosaccharide analysis (section 2.3.2)

The partially methylated alditol acetates were dissolved in 50 µL of anhydrous acetone and analyzed by GC-MS (SHIMADZU GCMS-QP2010 Ultra) using a capillary column DB-1 (30 m long, 0.25 mm of diameter and 0.15 µm thick).

The carrier as was helium, at 1.84 L/min. The injector temperature was 220ºC and the detector was 250ºC.the oven temperature start at 80ºC, increasing 6ºC/min until reach the 140ºC, which is kept constant for 5 min following an

increase of 0.2ºC/min until reaching 150ºC, and an increase of 60ºC/min until reach 250ºC and keep on hold for 2 min.

3 Results/discussion

3.1 *Fucus vesiculosus* polysaccharides composition

The polysaccharides content of brown seaweed *F. vesiculosus* biomass is 55% (in dry mass), which is in accordance with the bibliography that report 45-70% of total carbohydrates (Holdt & Kraan, 2011). The biomass is mainly constituted by uronic acids (119.5 $\mu g/mg_{algae}$), fucose (111.8 $\mu g/mg_{algae}$), glucose (89.4 $\mu g/mg_{algae}$), and mannose (69.3 $\mu g/mg_{algae}$), showing also the presence of sulfate esters (141.0 $\mu g/mg_{algae}$) (Table 2). This composition is comparable to the ones reported for the brown seaweed due to the presence of alginate, fucoidan and cellulose, as well as, laminaran (Rioux *et al.,* 2007; Obluchinskaya 2008).

Table 2 – Monosaccharide composition w/w ($mg/g_{extract}$) and (% molar) and sulfate content of *Fucus vesiculosus* biomass and hot water extracts.

	Sample		Fuc	Rha	Rib	Xyl	Man	Gal	Glc	HexA	SO_4^{2-}	Total (mg/g)	Yield (% of dry matter)
	F. vesiculosus		111.8 (19.2)				69.3 (10.7)	16.0 (2.5)	89.4 (13.8)	119.5 (17.0)	141.0 (36.8)	547.0	
1 h extraction	E1	1h	199.9 (27.3)	2.5 (0.3)	0.1 (0.0)	12.4 (1.9)	37.0 (4.6)	41.2 (5.1)	93.4 (11.5)	175.6 (19.9)	140.9 (29.3)	703.1	5.35%
	E1b	+15 min	160.4 (25.9)	2.5 (0.4)	0.5 (0.1)	9.2 (1.6)	30.1 (4.4)	35.5 (5.2)	71.9 (10.5)	187.0 (25.0)	110.1 (27.0)	607.4	2.99%
	$R1_{res}$	Residue 1h	57.0 (16.5)	6.0 (1.8)	1.3 (0.4)	14.7 (4.7)	18.6 (4.9)	10.0 (2.6)	41.0 (10.7)	124.0 (29.8)	65.2 (28.7)	337.9	65.1%
4 h extraction	E4	4 h	231.9 (29.4)	8.2 (0.8)	2.9 (0.8)	22.3 (3.3)	15.4 (1.8)	43.5 (4.4)	67.1 (7.7)	98.1 (18.7)	173.9 (33.2)	663.3	11.2%
	E4b	+15 min	188.4 (16.5)	5.1 (1.8)	4.7 (0.4)	19.0 (4.7)	12.8 (4.9)	31.4 (2.6)	54.5 (10.7)	144.6 (29.8)	140.0 (28.7)	600.5	3.10%
	$R4_{res}$	Residue 4h	90.9 (23.8)	4.9 (1.3)	2.4 (0.7)	10.8 (3.1)	16.1 (3.8)	10.1 (2.4)	23.2 (5.5)	134.3 (29.3)	75.4 (30.1)	368.1	53.8%
Pretreatments using $MeOH:CHCl_3:H_2O$	E4n	Non-treated extract	210.0 (28.0)	2.4 (0.3)	2.5 (0.4)	16.5 (2.4)	11.5 (1.4)	35.4 (4.3)	70.4 (8.5)	171.3 (19.0)	176.4 (35.8)	696.4	8.1%
	E4p	Pretreated extract	215.6 (26.7)	2.8 (0.3)	2.9 (0.4)	18.5 (2.5)	13.8 (1.5)	38.1 (4.3)	64.5 (7.2)	206.6 (21.2)	190.3 (35.8)	753.1	9.7%
	$E4n_{res}$	Non-treated Reside	82.2 (16.9)	1.2 (0.2)	1.0 (0.2)	8.6 (1.9)	29.2 (5.4)	15.1 (2.8)	59.6 (11.0)	233.8 (39.8)	69.5 (21.7)	500.2	56.6%
	$E4p_{res}$	Pretreated Reside	78.1 (15.5)	1.3 (0.3)	0.0 (0.0)	8.1 (1.8)	22.9 (4.1)	13.7 (2.5)	64.7 (11.6)	252.8 (41.7)	60.8 (22.6)	502.3	60.4%

Classical extraction of fucose-containing sulfated polysaccharides (FCSP's) from brown seaweed species typically involves extended hot acid, and/or $CaCl_2$ treatments, with steps that may last several hours. Many extraction factors, like temperature, time of extraction, pH and ratio of algae/solvent have significant effects on the FCSP/fucoidans yield**.** Usually higher temperatures (80~90ºC) and time of extraction (up to 4 h) lead to higher amounts of polysaccharides extracted (Yu and Chao 2013; Ale *et al.,* 2011b). Acid extraction speeds the process of extraction, but high extraction times does not lead to better yield of polysaccharides and temperature, since may cause degradation of fucoidan (Yu and Chao 2013; Ale *et al.,* 2011b). The structural integrity of fucoidan should be better conserved using low acid treatment (Rioux *et al.,* 2007; Obluchinskaya 2008). In many reports it is common to find a pretreatment step, where the dry seaweed is pretreated with a mixture of solvents, usually chloroform/methanol/water (Bilan *et al.,* 2002; Bilan *et al.,* 2006; Ale *et al.,* 2011b).

In order to preserve the best structural characteristics possible of the *F. vesiculosus* polysaccharides, with special focus in fucoidans, it was used a mild hot water (90ºC) extraction. . The extraction on the pretreated algae was made in distilled water at 90ºC, for a period of 1 h (sample E1) or 4 h (sample E4) the supernatants were collected. Each residue was subjected to more 15 min of extraction at the same conditions (samples E1b and E4b, respectively). The effect of the use of a pretreatment using methanol/chloroform/water (4:2:1) was also accessed (extract E4p). The results of the monosaccharides analysis and yields of extracts and residues are shown in Table 2.

The E1 extract gave 5.35% yield, while the E4 extract yielded 11.2%. The 4 h extract (E4) presented a higher amount of fucose (232 mg/g) and sulfate esters (174 mg/g) than the 1 hour extract (E1), 200 mg_{Fuc}/g_{sample} and 141 $mg_{SO_4^{2-}}/g_{sample}$, respectively. These results confirm that time of extraction displays a positive role in the extraction yields. It could be concluded that the 4 h extraction gave better results. The E1b and E4b yielded near 3% for both initial times of extraction. The composition of both extracts (E1b and E4b) were identical, being constituted by mainly fucose (17-26 mol%), uronic acids (25-30 mol%) and glucose (11 mol%), as well as, sulfate esters (27-29 mol%).

The residues $E1_{res}$ and $E4_{res}$ have different yields, the $E1_{res}$ 65 % while $E4_{res}$ have 54%. This difference is due to the higher extraction of compounds in the 4 h extraction, remaining less quantity in the residue. Both resides (E1res and E4res) presented a low content in carbohydrates (34 - 37%) with lower content of fucose and sulfates than the water extracts (Table 2), which means that fucoidan was successfully extracted to the aqueous phase. Both extracts had significantly lower amounts of glucose when compared to biomass composition and water extracts, since a branched laminaran could have been extracted, which is highly soluble (Rioux *et al.,* 2010).

A pretreatment with MeOH:$CHCl_3$:H_2O (4:2:1) was performed to verify if had effect on polysaccharides yield and extracts composition. Two extractions using pre-treated and non-treated algae were made using 90ºC distilled water extraction for 4 h. The pretreatment removed some of the pigments, lipids, and other small compounds, in a total of 20% weight loss. The pretreatment allowed to obtain a more limpid extract when compared to the non-treated one (Figure 10).

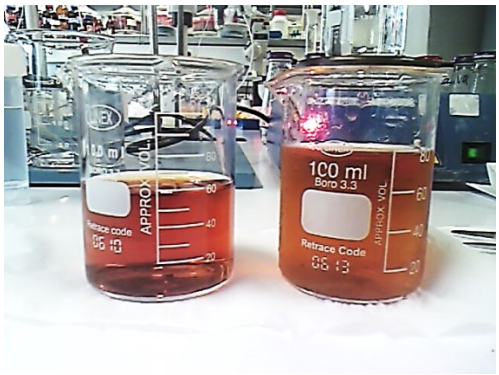

Figure 10 - Aqueous extracts of pretreated algae (left) and non-treated algae (right).

The pretreated extract (E4p) yielded slightly more mass (10%w/w) and total sugars (75 %) than the not-treated extract (E4n, 10% yield and 70% total sugars). The composition of these extracts show almost no difference to the previous 4 h extract (E4) that yielded 11 %w/w (table 2), being the major constituents sulfate (~35mol%), fucose (29%), and uronic acids (~20%) meaning that pretreatment did not affect the composition of the polysaccharides extracted with hot water. The pretreatment allowed to obtain a water extract richer in sugars per dry weight but

this pretreatment step can be omitted as it is not relevant for the polysaccharide extraction.

3.2 Purification of *Fucus vesiculosus* polysaccharides

3.2.1 Precipitation

In the isolation of polysaccharides from brown seaweed and other marine algae it is common to have a step of precipitation, namely sequential ethanol and calcium chloride precipitation, and/or ionic exchange chromatography (Bilan *et al.,* 2008; Suárez *et al.,* 2010; Mak 2012; Wijesinghe and Jeon 2012).

The extracts E1 and E4, which had higher content of polysaccharides, were precipitated by sequential addition of ethanol at increasing concentrations of 30%, 50%, and 80% v/v. The E4 extract did not show precipitate of polymeric material using 30% of ethanol. The increase of the ethanol concentrations to 50% caused the precipitation of low quantity of polymeric material (5%) for E4 (E4Et50), whereas for E1 extract the 50% ethanol extraction (E1Et50) yielded (17%) (Table 3).

Table 3- Yield and composition (mg/g extract) and (%molar) of the precipitates resulted from ethanol precipitation.

	Sample	Descrition	Fuc	Rha	Rib	Xyl	Man	Gal	Glc	HexA	SO_4^{2-}	Total (mg/g)	yield
E1 extract	E1Et50	Ethanol 50 %	48.0 (9.7)	(0.0)	17.0 (3.8)	11.4 (2.5)	44.6 (8.1)	9.6 (1.7)	28.0 (5.1)	262.1 (43.7)	83.1 (25.4)	503.9	17%
	E1Et80	Ethanol 80 %	124.1 (15.4)	(0.0)	3.0 (0.4)	15.3 (2.1)	21.6 (2.4)	22.5 (2.5)	62.0 (6.9)	342.0 (35.2)	185.4 (35.0)	775.8	13%
	E1Sn	Supernatant	234.0 (35.3)	(0.0)	(0.0)	23.7 (4.0)	24.2 (3.3)	63.0 (8.6)	101.3 (13.8)	243.1 (30.4)	20.7 (4.8)	710.1	60%
E4 extract	E4Et50	Ethanol 50 %	61.3 (7.0)	(0.0)	3.9 (0.5)	8.0 (1.0)	212.1 (21.7)	145.7 (14.9)	24.9 (2.5)	384.3 (36.2)	93.8 (16.2)	934.0	5%
	E4tap-water	Ethanol 50%+ tap water	31.1 (7.3)	(0.0)	3.2 (0.8)	7.1 (1.8)	5.4 (1.1)	6.1 (1.3)	8.5 (1.8)	372.2 (72.0)	39.3 (13.9)	472.9	22%
	E4Et80	Ethanol 80 %	286.2 (32.4)	(0.0)	(0.0)	18.1 (2.3)	9.8 (1.0)	47.8 (4.9)	53.1 (5.4)	109.3 (10.3)	254.2 (43.8)	778.6	46%
	E4Sn	Supernatant	19.3 (32.3)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	35.1 (53.1)	1.1 (1.5)	5.1 (13.1)	60.6	-
$E4_{p+n}$ extract	pEt50	Ethanol 50 %	109.9 (18.6)	3.0 (0.5)	9.7 (1.8)	23.2 (4.3)	15.9 (2.4)	11.1 (1.7)	20.1 (3.1)	316.4 (44.4)	73.4 (23.2)	582.7	37%
	$pEtCa^{2+}$	Ethanol + $CaCl_2$	156.3 (22.9)	2.2 (0.3)	3.6 (0.6)	21.2 (3.4)	9.8 (1.3)	19.4 (2.6)	27.5 (3.6)	260.0 (31.6)	122.5 (33.6)	622.4	18%
	pEt80	Ethanol 80 %	341.4 (29.0)	4.4 (0.4)	0.8 (0.1)	18.0 (1.7)	10.9 (0.8)	69.5 (5.3)	139.9 (10.7)	57.6 (4.1)	302.0 (48.0)	944.7	37%
	pEtSn	Supernatant	4.5 (15.6)	(0.0)	0.2 (0.8)	0.2 (1.0)	0.7 (2.1)	0.0 (0.0)	21.5 (66.9)	3.1 (9.0)	0.7 (4.6)	31.0	-

The E1Et50 and E4Et50 have similar compositions with high quantity of uronic acids, 262 and 384 mg/g respectively, and low amounts of fucose (<61 mg/g) and sulfate esters (<94 mg/g).

The fraction from the precipitation with 80% of E1 extract (E1Et80) yielded 13%, and the supernatant (E1Sn) yielded 61%, showing that most of the polysaccharides were not precipitated using ethanol, remaining soluble. The E1Et80 precipitate showed the presence of higher quantity of uronic acids (342 mg/g) and sulfate esters (185 mg/g) than the E1Sn (243 mg/g and 21 mg/g, respectively), whereas the fuc (124 mg/g for E1Et80 and 234 mg/g for E1Sn) and Glc (62 mg/g for E1Et80 and 101 mg/g for E1Sn) content were lower (table 3). The presence of 60% of the initial material in supernatant of ethanol precipitation implies that ethanol precipitation alone might not be enough to separate the

fucoidans from the remaining polysaccharides. However, the sulphated polysaccharides were mainly obtained with 80% ethanol.

The fractions from the precipitation of the 4 hour extract (E4) were different from E1. The E4tap-water precipitate (22 %w/w) lead to the precipitation of high amounts of uronic acids, being the main constituents uronic acids (72 mol%). The proposed explication was that the ions within the tap water, calcium ions in particular (that are known to form complexes with alginate in solution), were responsible for the precipitation of the uronic acids, probably alginate.

The precipitate E4Et80 and the supernatant E4Sn had different compositions comparatively to the 1 h analogues (E1Et80 and E1Sn). E4Et80 showed the presence of fucose and sulfate esters as the main constituents (32.4 and 43.8 mol%, respectively), whereas the uronic acids were present in lower amounts (10 mol%). The E4Sn (supernatant) had low yield in sugars, being the glucose the main sugar (35 mg/g) most of the mass in supernatant should be due to the salts.

For precipitation of soluble polysaccharides, such as fucoidans and alginates that are negatively charged, in a aqueous solution, due to the high dielectric constant of the water, molecules with charged groups are shielded, because charged groups are surrounded by hydration shells. The addition of ethanol, which was a lower dielectric constant, causes precipitation of polysaccharides, either by decreasing the polarity of the solution or by promoting the sulfate esters and carboxylic groups from the acidic polysaccharides (fucoidan and alginates) to form ionic bond with positive ions resulting in bigger complexes and thereafter, precipitation (Hahn *et al.,* 2012). In the precipitations with tap water (or cations) the cations enabled the formation of ionic bridges between the negatively charged groups, allowing the formation of structures of higher molecular weight that precipitate under the polarity decrease caused by the ethanol. (Smidsrød and Haug 1967; Hahn *et al.,* 2012)

The precipitation of acidic polysaccharides by salts addition in ethanol-water mixtures has positive results in the separation and precipitation of non-charged, carboxylated and sulfated polysaccharides in mixtures from brown seaweed. In ethanol-water mixtures polysaccharides could be precipitated by inorganic salt

addition (Smidsrød and Haug, 1967).

Since both aqueous extracts E4p andE4n, pretreated and not-treated, had the same composition, they were combined into $E4_{p+n}$ extract, and precipitated using Ethanol precipitation and $CaCl_2$ precipitation in ethanol-water mixture – to mimic the effect of the ions of the tap water, and separate the acidic polysaccharides according to previous findings and according the knowledge described above.

The precipitation using ethanol at 50%v/v, pEt50, yielded a total of 37% with a high amount of uronic acids (316.4 mg/g – 46.4 mol%). With the exception of the fucose (19.4%) and sulfate esters (19.7%) all the other monosaccharides were present in less than 5% molar.

The $pEtCa^{2+}$ fraction showed the presence of higher amounts of uronic acids (260.0 mg/g) but could be seen that some fucoidans were precipitated within this fraction, as the amount of fucose and sulfates was 156.3 mg/g (22 mol%) and 122.5 mg/g (36 mol%), respectively. As described by Smidsrød and Haug (1967), the addition of salt to ethanol water mixture causes the alginates to precipitate. However, if the concentration of cations is too high it may also cause the precipitation of fucoidans to some extent. In addition the complexes formed between calcium and alginates could entrapped some fucoidan that is found within this fraction.

The precipitate at ethanol concentrations of 80%v/v, sample pEt80, was the richest in fucose (341.4 mg/g) and sulfate esters (302.0 mg/g) content, more than twice compared to $pEtCa^{2+}$ and higher than any other precipitation, being the material characterized as sulfated polysaccharides (fucoidans). The presence of glucose (11.8 mol%) infer the existence of laminaran polysaccharides that are very soluble (Rupérez *et al.,* 2002; Rioux *et al.,* 2007). The uronic acids present only compromises to 4.5% of the molar composition of this extract. The precipitation procedure was effective in separating the uronic acids (alginates), $pEtCa^{2+}$, from the fucose containing sulfated polysaccharides, pEt80, extracted with hot water. This fraction has also some amounts of other sugar resides usually present within the fucoidans, as the xylose and galactose (Cumashi *et al.,* 2007). The fucoidan in

these two fractions ($pEtCa^{2+}$ and pEt80) could be similar, as the ratio SO_4^{2-}/Fuc are the same, respectively, 1.35 and 1.36.

The ethanol precipitation of polysaccharides with the help of calcium chloride salts allowed to separate the alginates and fucoidans from the brown algae. In the pEtSn (supernatant) fraction only a very low amount of sugars were present, up to 31.0 $mg/g_{fraction}$, from which 67.5% are Glc residues, which should be due to the presence of highly soluble laminaran (Rioux *et al.,* 2007; Rioux *et al.,* 2010).

The precipitation in ethanol-water using calcium chloride salts caused similar effects as the addition of tap-water salts to precipitation, but since the Ca^{2+} cation are more specific to form complexes with alginate, these effects were more expressive. The uronic acids content was lower in the fractions collected after the $pEtCa^{2+}$, and the pEt80 fraction was rich in sulfates and fucose (fucoidan). Neutral polysaccharides did not seem to be much affected by the presence of cations in the ethanol-water mixture as the yield increased in fractions precipitated at higher concentrations of ethanol. This result is in accordance to Smidsrød and Haug (1967) since precipitation with ethanol and inorganic salts may be a useful tool for the separation of the components of a polysaccharide mixture.

3.2.2 Anionic exchange chromatography

Since the fucose containing sulfated polysaccharides and alginates charged groups have different charges and dielectric constants they could be easily separated using an anionic exchange chromatography, with a weak anion exchanger diethylaminoethanol (DEAE) based resin (Rioux *et al.,* 2009; Yu and Sun 2014).

The samples E4Et80, E4tap-water and pEt80 were applied in anionic exchange column and the elution chromatograms were displayed in Figure 11.

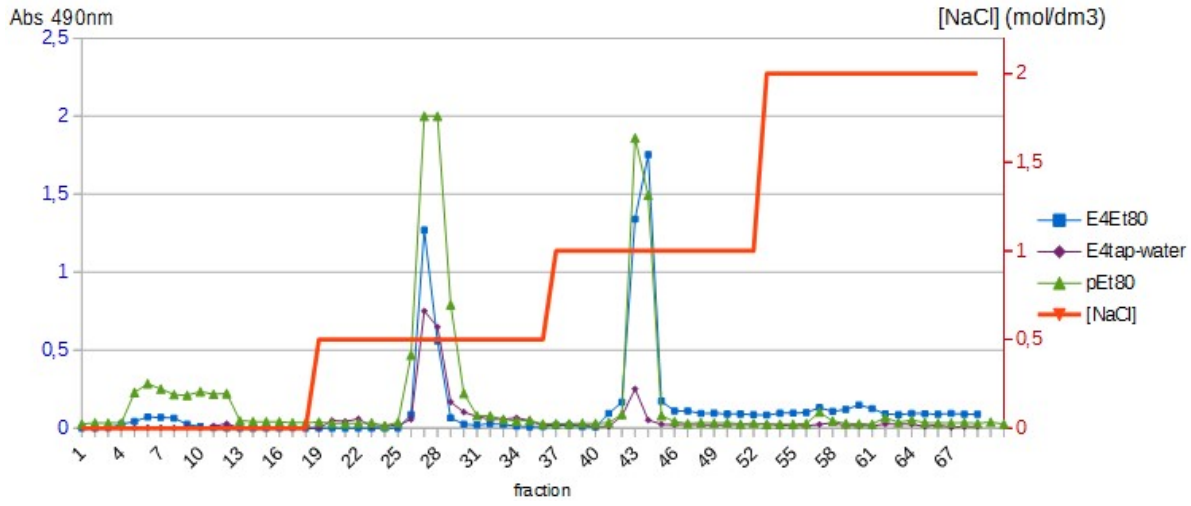

Figure 11- Anion exchange cromatogram of E4Et80, E4tap-water and pEt80 by stepwise elution with different concentrations of NaCl.

The chromatograms displayed (Figure 11) clearly show a separation in two main peaks in all the samples (fractions 0.5M and 1M [NaCl]). Both the yield and composition of the eluted fractions is showed in the Table 4.

Table 4- Monosaccharides composition (mg/g) and (%molar) and sulfates of the fractions of the anionic exchange chromatography.

	fraction	Fuc	Xyl	Man	Gal	Glc	HexA	SO_4^{2-}	sum	Yield (%w/w)
E4tap-water	CaF0.5	9.8 (0.0)	0.0	2.1	0.0	0.0	445.7 (1.0)	n.a		51
E4Et80	Et80F0.5	154.8 (0.3)	38.1 (0.1)	37.1 (0.1)	48.3 (0.1)	19.3 (0.0)	195.7 (0.4)	n.a		24
	Et80F1.0	516.8 (0.4)	10.1 (0.0)	0.7 (0.0)	85.3 (0.1)	2.4 (0.0)	2.1 (0.0)	429.1 (0.5)	1046.6	38.5-45.1
pEt80	pF0	0.0 (0.0)	0.0 (0.0)	25.1 (2.5)	0.0 (0.0)	772.1 (77.8)	50.5 (4.7)	87.9 (15.0)	935.6	12
	pF0.5	340.2 (33.0)	27.8 (3.1)	11.3 (1.0)	96.1 (8.4)	26.8 (2.3)	88.7 (7.1)	333.7 (44.8)	924.5	40
	pF1.0	401.4 (46.9)	2.1 (0.3)	0.0 (0.0)	22.0 (2.3)	7.1 (0.8)	10.0 (1.0)	274.1 (48.8)	716.7	22
	pF2.0	89.5 (58.9)	2.8 (2.0)	0.0 (0.0)	13.2 (7.9)	25.6 (15.2)	9.5 (5.2)	10.8 (10.8)	151.4	3

The E4tap-water only originated one fraction with significant amount of mass (51%w/w), named CaF0.5, this fraction showed almost only the presence of uronic acids (97%), 445.7 mg/g (Table 4).

The eluted samples E4Et80 and pEt80, are both precipitates from 4 hour extracts using ethanol 80% (v/v) after addition of a solution with salts. Two fractions were collected, one with 0.5M NaCl (Et80F0.5) that had more uronic acids (196 mg/g) and less fucose (155 mg/g), than the fraction eluted with 1 M NaCl (Et80F1.0) that presented low uronic acids content. . The Et80F1.0 presented mostly fucose (41 mol%, 517mg/g), sulfate esters (52 mol%, 429mg/g) and galatose (6 mol%, 85mg/g). These galactose residues might be part of the structure of this fucoidan as suggested by Bilan *et al. (*2006). It was expected that the fractions eluted with 0.5 M NaCl, are rich in alginates, while the fractions eluted with 1M NaCl, must be richer in sulfated polysaccharides. Sulfated polysaccharides are more electronegative, therefore should be adsorbed more strongly to the resin than carboxylated polysaccharides (uronic acids). The fraction Et80F0.5 have the presence of fucose (155 mg/g), which means that fucoidans could be co-eluted with alginates

The pF0 fraction (12%w/w), containing the neutral polymers, was able to be collected and was very rich in glucose (78%), 772 mg/g (table 4), due to the presence of neutral polysaccharides, such as laminaran.

In pF0.5, the highest yield was obtained (40%) with high amounts of fucose (340 mg/g) and sulfate (334 mg/g), confirming that Et80F0.5 fraction eluted fucoidan, as well as some alginates due to the presence of uronic acids (89 mg/g). Fraction pF1.0 presented a high content of fucose (401 mg/g) and sulfate esters (274 mg/g). Xylose and galactose residues were found to be present in very low quantities.

The results show that the anion exchange chromatography was a good method in separation of fucose containing sulfated polysaccharides from other polymers precipitated using 80%ethanol . Fractions eluted with 0 M NaCl are rich in glucose, main constituent of the laminaran polysaccharide. The fractions eluted with 0.5 M NaCl present high amounts of uronic acids but also fucose and uronic acids. The fractions eluted with 1M NaCl presented high quantities of fucose and

sulfates, inferring the presence of fucoidans.

3.3 Linkage analysis

To determinate the type of glycosidic bonds, linkage analysis was performed in the pF0.5 and pF1.0 fractions. The relative abundance of the sugar derivates is displayed in the table 5.

Fraction pF0.5, composed by 33% fucose, 8.4% galactose, and 3.0% xylose and 45% sulfates, showed a high abundance of terminal fucose (25%) and (1→3,4) Fuc (26%) which implies that fucoidan is highly branched. It were found also (1→3)-Fuc (17%), (1→2)-Fuc (15%), and (1→4)-Fuc (5%), which are the linkages from the main chains of not sulfated and not branched residues. Residues of (1→2,3)-Fuc (6%) and (1→2,3,4)-Fuc (8%) represent the fucose residues that are either branched or sulfated. Besides fucose derivatives, only a small amount of terminal Xyl (2%) and (1→ 4)-Xyl (3%) could be detected, in the fraction eluted with 0.5 M NaCl.

Table 5 - Relative abundance of the partially methylated alditol acetates present in the pF0.5 and pF1.0 fractions.

		pF0.5		pF1.0	
methyl derivative	Sugar derivative	% area total	% of the monosaccharide	%area	% of the monosaccharide
xylose			% of Xyl		% of Xyl
2,3,4-Me3-Xyl	t-xyl	2%	44%	4%	32%
2,4-Me2-Xyl	1,3-Xyl			1%	9%
2,3-Me2-Xyl	1,4-Xyl	3%	56%	7%	59%
Total xylose		5%		12%	
fucose			% of Fuc		% of Fuc
2,3,4-Me3-Fuc	t-Fuc	24%	25%	27%	32%
2,3-Me2-Fuc	1,4-Fuc	3%	3%	7%	9%
2,4-Me2-Fuc	1,3-Fuc	16%	17%	10%	12%
3,4-Me2-Fuc	1,2-Fuc	14%	15%	14%	16%
2-Me-Fuc	1,3,4-Fuc	25%	26%	14%	17%
4-Me-Fuc	1,2,3-Fuc	5%	6%	5%	6%
Fuc	1,2,3,4-Fuc	8%	8%	7%	8%
Total fucose		95%		85%	
galactose					% of Gal
2,3,4,6-Me4-Gal	t-gal			1%	31%
2,4,6-Me3-Gal	1,3-gal			2%	69%
Total galactose		-		3%	

Concerning the fraction F1.0, it could be seen that there was a higher amount of terminal fucose (32%), showing the presence of a fucoidan with low molecular weight, since the content of (1→3,4)-Fuc (17%) was lower. This sample

had high amounts of (1→2)-Fuc (16%), like the pF0.5 but a lower abundance of (1→3)-Fuc (10%) and a higher amount of (1→4)-Fuc (7%). Residues of (1→2,3)-Fucose (6%) and (1→2,3,4)-Fucose (8%) represent the fucose residues that are either branched or sulfated. In this fraction it could be found the presence of terminal xylose and (1→ 4)-Xyl with higher relative abundance than pF0.5, but minor amounts of galactose residues were found, namely terminal galactose (1%) and (1→3)-Gal (2%), that could not be found in methylation analysis of the pF0.5.

Both pF0.5 and pF1.0 revealed the presence of (1→2)-Fuc residues, despite most of the bibliography refer that fucoidans have α (1→ 3) and α (1→ 3)/α(1→ 4)-Fuc linkages (Kusaykin *et al.,* 2008). However, Conchie and Percival (1950) reported (1→2)-Fuc linkages in *F. vesiculosus* fucoidans and highly branched polymers. However, Patankar *et al. (*1993) later reported that the main linkages are the (1→3) type and (1→3) branched at C-4, being the reason to this difference due to the different methods of extraction.

These structural characteristics are somehow representative of the *fucales* order seaweeds, reported to have highly sulfated and highly branched fucoidans, rather than fucoidan from some brown seaweed species of the order Laminariales and Chordariales, reported to have a more linear structure with less sulfates and substitutions of a single fucose residue at C-2 (Ale *et al., 2011c)*.

The high branching of the fucoidan and the sulfate content are indicators of bioactivity, although, the fucoidans extracted from *F. vesiculosus* of “Ria de Aveiro” presented 1→2 linkages. These structural differences may be important for bioactivities of fucoidan, but more experiments need to be done to evaluate this hypothesis.

3.5 Microwave Hydrodiffusion and Gravity Extraction

The alga (300 g) was treated with 300 W and 900 W microwave irradiation. During the extraction at 300 W (W300) two fractions were collected, W300_1 and W300_2, same with the irradiation with 900 W.

After microwave irradiation at 300 or 900 W, the alga was hydrated with 200 mL of cold distilled water leading to the rupture of the algae bladders, W300_3 was collected (84 mL). Ethanol (100 mL) was further added to the algae a green fraction W300_4 was obtained (~78 mL). The alga was irradiated with 300 or 900 W again leading to the fractions W300_5 and W300_6.

The results of the total content in sugars are presented in the Figure 12 andare representative of the yields of extraction (see Annex 1A). After irradiation with 300 W and 900 W weighted 78.7g and 82.7g, respectively, and were completely dry.

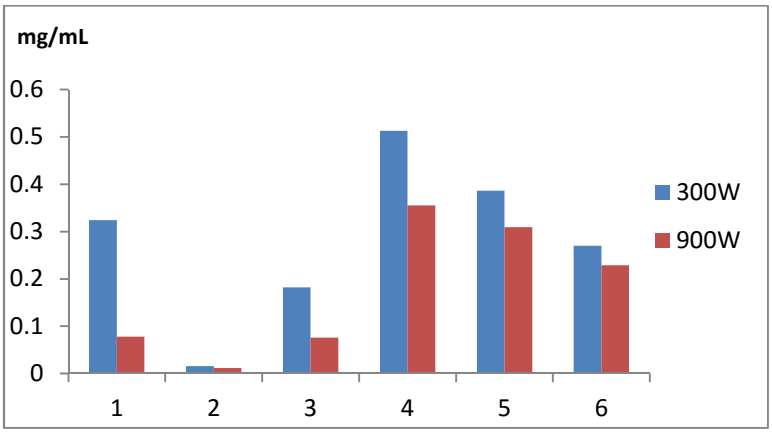

Figure 12 - Sugar content in the fractions collected by microwave extraction.

The results showed that at 300 W the first fractions had more carbohydrates and mass than at 900 W. The majority of the material is collected within the initial 50 mL and the fractions collected after the burst of *F. versiculosus* bladders and with ethanol addition.

Figure 12 shows the sugars composition of the fractions (more detailed information annex 1B). The fractions obtained with 300 W had a significantly higher abundance of mannose than the 900 W, near 10 times more. This high content of mannose could be due to the presence of mannitol, since this compound is reported to be present in brown algae. The 900 W fractions have

higher content of uronic acids, probably due to the presence of alginates. In the three last fractions (W900-4, 5, and 6) the content of fucose and sulfate is higher than the content of uronic acids, inferring that these fractions are richer in sulfated fucoidans.

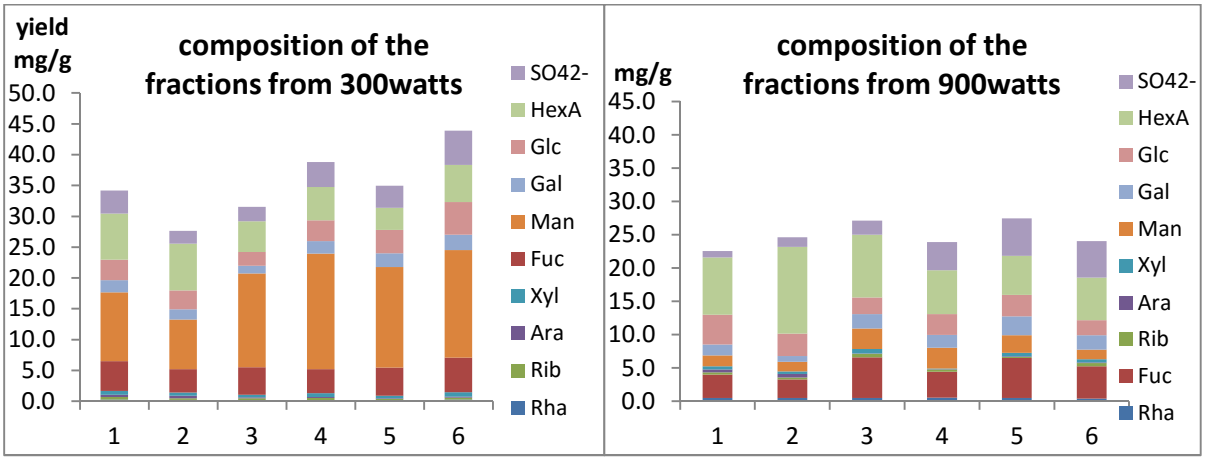

Figure 13 - composition (mg/g) of the microwave fractions collected with NEOS-GR: 300 W (left) and 900 Wt(right).

The probable reason for the different composition of the fractions obtained with 300 and 900 W was the higher energy that is converted into the water molecules. Since the matrix was not very compact water can flow in the chamber of the microwave system and condensate in the walls of the module containing the matrix. At 900 W the speed of the extraction and water evaporation is faster than at 300 W, being the rate at which the water is collected. At 300Watts this process happens at a slower rate allowing the water to carry small polar molecules, such as mannitol. Cell wall constituents like the alginates and fucoidans can only be collected when the irradiation is capable of separate them from the matrix and the water from hydrodiffusion is able to carry them.

After bladder burst with water and ethanol, the material that could not be extracted from the inner parts of the algae, can be easier collected, leading to a higher yield in the last fractions.

Although the process of extraction using NEOS-GR was made using dry algae that were rehydrated and not fresh ones, the results show that the technology may be useful as a drying process and while doing so, there exist the possibility of collect some molecules that could have value, such as alginate and fucoidans.

4 Conclusions

In this work it was studied a method to extract and isolate a sulfated polysaccharide of a brown seaweed from “Ria de Aveiro”. Also, the purified polysaccharides were structurally characterized in order to know their potential for commercialization.

The analysis of the 4 h aqueous extract showed a great abundance of Fuc(up to 232 mg/g) and sulfate 174 mg/g) and uronic acids (up to 98mg/g). This composition is due to the presence of the two main negatively charged polysaccharides that exist in brown seaweeds, sulfated fucoidan and alginate. The glucose that appears in small quantities (67 mg/g) is due to the presence of laminaran.

The ethanol precipitation with addition of $CaCl_2$ allowed to precipitate mostly alginates and the further precipitation using 80% ethanol to obtain fucoidans and laminarans. An anionic exchange chromatography was able to separate the different polysaccharides. A neutral fraction was obtained constituted mainly by glucose (laminaran), rising to 0.5 M NaCl a fraction with higher quantities of uronic acids (alginate) and some fucoidan is obtained and lastly a fucoidan with high amounts of sulfate esters can be purified by incrementing the ionic strength of the buffer to 1M NaCl.

The purified fucoidans showed to be constituted mainly by terminal fucose (25%) and (1→3,4) Fuc (26%) which infer that these fucoidans were highly branched. The presence of high contents of (1→3)-Fuc was observed in both samples inferring the presence of fucoidans with this linkage in the backbone. Besides, it could be observed reasonable amounts of (1→2)-Fuc in both pF0.5 and pF1.0 fractions, a type of linkage that is not usually reported for fucoidans,.

Microwave Hydrodiffusion and Gravity System was not effective to extract high quantities of sulfated polysaccharides from the brown seaweed *F. vesiculosus*. However, it could be a fast and clean method for drying the seaweed while collecting some small polar compounds as mannitol, as well as, fucoidans and alginate using the higher potency (900 W).

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6 Annexes

Annex 1A– Dubois results and yields of the microwave fractions.

300 Watts					900 Watts				
fraction	Dubois mg/mL	Volume (mL)	Mass lyophilized	Yield (%w/w)	fraction	Dubois mg/mL	Volume (mL)	Mass lyophilized	Yield (%w/w)
W300_1	0.32	50	57.7 mg	0.019%	**W900_1**	0.078	50	11.9 mg	0.004%
W300_2	0.02	50	3.6 mg	0.001%	**W900_2**	0.012	50	2.1 mg	0.001%
W300_3	0.18	84	62.5 mg	0.021%	**W900_3**	0.076	98	35.7 mg	0.012%
W300_4	0.51	78	265 mg	0.088%	**W900_4**	0.355	100	214 mg	0.071%
W300_5	0.39	50	117.5 mg	0.039%	**W900_5**	0.309	50	97.5 mg	0.033%
W300_6	0.27	50	80.2 mg	0.027%	**W900_6**	0.229	50	126 mg	0.042%
	Total	362 mL	586.2 mg	0.20%		Total	398 mL	487.5 mg	0.16%

Annex 1B – composition of the fractions from NEOS-GR

Table with the composition of the fractions from *Fucus vesiculosus,* collected with NEOS-GR, and irradiated with 300W and 900W (mg/g)

	Rha	Fuc	Rib	Ara	Xyl	Man	Gal	Glc	AU	SO_4^{2-}
W300_1	2.8	48.3	4.2	3.7	6.4	111.4	19.5	33.5	74.9	37.2
W300_2	2.0	37.8	3.0	4.0	5.1	80.6	16.5	30.6	75.9	20.6
W300_3	1.5	44.8	3.6	1.5	4.0	152.3	12.3	22.5	49.4	23.8
W300_4	1.7	38.7	3.8	1.4	6.4	187.6	20.1	33.7	54.1	40.5
W300_5	1.8	45.8	1.9	1.4	3.9	163.0	22.3	38.0	35.7	35.8
W300_6	2.6	56.0	3.5	1.6	7.1	174.7	24.9	52.7	60.4	55.6
W900_1	5.2	35.0	3.3	4.4	4.6	16.5	16.0	44.9	85.8	10.0
W900_2	5.0	27.7	3.0	5.3	3.9	14.5	8.7	33.3	130.1	14.6
W900_3	5.0	61.0	5.5	0.5	6.4	30.9	21.7	24.8	94.1	21.4
W900_4	5.3	39.1	3.3	0.7	0.7	31.1	19.4	31.3	66.0	42.2
W900_5	5.2	59.6	1.7	1.0	5.1	26.8	28.0	32.3	58.9	56.1
W900_6	4.1	48.5	4.7	1.0	5.0	14.2	21.6	22.4	64.2	54.7