Extraction, characterization and seasonal variation of bioactive compounds (polyphenols, carotenoids and polysaccharides) from Irish origin macroalgae with potential for inclusion in functional food products.

By

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A Thesis Presented in Fulfilment of the Requirement for the Degree of
Doctor of Philosophy

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Abstract
Seaweeds contain polyphenols, carotenoids and polysaccharides with potential for use in functional foods. This study investigated these compounds using conventional and novel recovery techniques from seaweeds (*Fucus serratus*, *Laminaria digitata*, *Gracilaria gracilis*, *Codium fragile*, *Fucus vesiculosus*, *Himanthalia elongata* and *Cystoseira nodicaulis*) harvested from the Irish coast. The effect of seasonality on compound concentration, structure and bioactivity was assessed.

Solid liquid extraction (SLE) and pressurised liquid extraction (PLE) were investigated for the optimal recovery of antioxidant compounds. Methods were evaluated based on extraction yield and antioxidant properties. SLE extracts possessed higher antioxidant activity compared to PLE extracts. Enriched fractions from SLE extracts were generated using membrane dialysis. An increase in activity compared to the crude extracts was achieved. Initial weak activity in the < 3.5 kDa fraction of *F. serratus* was further enhanced using reversed-phase flash chromatography separation. Quadrupole time-of-flight mass spectrometry (Q-ToF-MS) confirmed the presence of a high abundance of low molecular phlorotannins. Phlorotannins characterisation with ultra-performance liquid chromatography tandem mass spectrometry from brown macroalgae demonstrated isomeric complexity. The level of isomerisation differed substantially within the species studied.

The seasonal variation on macro-composition of four seaweed over four seasons was investigated. Protein content was highest (28.70 %) in *G. gracilis* winter samples. Lipid content was low and ash content high in all species. Carbohydrate levels were highest in the brown seaweed (75-80 %). Insoluble dietary fibre ranged between 30-60% and was highest in spring. Soluble dietary fibre was highest in the spring/summer periods. Levels of phenolics in *F. serratus* were highest during the summer period and lowest in the autumn. This study demonstrated that seaweed composition is affected by seasonality and environmental factors.

Brown seaweeds contain carotenoids (fucoxanthin and xanthophyll) known to possess antioxidant activity. The efficiency of three extraction techniques (conventional SLE, supercritical CO$_2$ (SFE) extraction and supercritical CO$_2$ with co-solvent) and seasonal variation was examined for these carotenoids. SLE gave a higher yield while SFE gave a higher purity of extract. The winter/spring period gave the highest purity of carotenoids from *L. digitata*, however, the summer was the peak period for *F. serratus*.

The seasonal variation on structure and bioactivity of phlorotannins was also investigated in the brown algae *F. serratus*. Low molecular weight phlorotannins predominantly had a degree of polymerisation (DP) of phloroglucinol between 6-14 across all seasons/harvest sites. Winter samples exhibited the highest level of isomerisation (427 isomers) while summer samples had the lowest level of isomerisation (121 isomers). High levels of isomerisation were associated with higher bioactivity in the in-vitro tests.

The seasonal variation laminarin from *Laminaria digitata* was investigated in relation to molecular weight, content and chain length. ESI-Q-TOF-MS analysis showed that summer was the optimum for harvest time for extraction of higher content of laminarin, no laminarin was detected in the winter. Variation in the molecular weight based in the number of glucose monomers was observed.

This research highlighted the optimum extraction technologies for extraction of bioactive compounds (polyphenols, carotenoids and polysaccharides) from Irish macroalgae with potential for inclusion in functional foods. It has also demonstrated the seasonal variation of these compounds and its effect on structure and bioactivity.
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Chapter 1.

Literature Review
1.0 Marine Algae (Seaweed)

In recent years there has been influx of interest into the health boosting role of certain foods apart from their nutritional value. This has lead to research efforts to identify bioactive components from natural sources including the marine environment. As a result of this two groups of products have been developed “functional foods” and “nutraceuticals”. Functional foods are foods that are enriched with functional components which can reduce the risk of chronic diseases, beyond their basic nutritional function. Nutraceuticals on the other hand are bioactive compounds that are isolated or purified from a food (Shahidi et al., 2009). The role of a functional foods is to boost the general condition of the body and to reduce the risk of illness and disease occurring (Siró et al., 2008).

Marine nutrients and bioactives offer potential as functional food ingredients due to the many advantageous physiological effects and health benefits they display such as anticancer, antioxidant and anti-inflammatory activity. Irish marine resources particularly macro-algae are to this point largely unexplored as a source of bioactive molecules for inclusion in functional foods. However, several studies have shown that they have excellent nutritional properties. For example seaweeds are high in dietary fibre, proteins, polysaccharides, antioxidants, pigments and have a low content of saturated fats (Dawczynski et al., 2007). Marine organisms live in very complex habitats and are exposed to extreme conditions, and in order for them to withstand and adapt to these conditions they produce a variety of secondary metabolites that are biologically active which are not found in other organisms.

Macroalgae are categorized according to their pigmentation and are split into four groups, brown (Phaeophyta), red (Rhodophyta), green (Chlorophyta) and Blue-green (Cyanphyceae). Marine
algae thrive in well lit, shallow sea areas where they can photosynthesize and absorb nutrients and minerals. The growth rate and composition of a seaweed are dependent on environmental factors.

1.1 Utilization of marine algae

Macroalgae are harvested and used globally for many different applications. They currently have considerable ecological and economic importance arising from their use as emulsifying agents in foods (Dhargalkar and Pereira, 2005), as ingredients in cosmetic formulations (Dhargalkar and Verlecer, 2009) and as whole foods.

1.1.1 Commercial applications

Seaweeds are a plentiful source of polysaccharides that are used as bulking commodities in the food and pharmaceutical industries. Three significant commercial phycocolloids are extracted from seaweeds (agar and carrageenan from red seaweeds such as *Gracilaria*, *Chondrus* and *Hypnea*, and alginate from brown seaweeds like *Ascophyllum*, *Laminaria* and *Sargassum*). The technological functionality of these compounds is mostly attributable to their ability to hold significant amounts of water, their capacity to form gels and their metal chelating ability. Species such as *Chondrus crispus* and *Saccharina japonica* have commercial applications which are due to their phycocollid or non-fibrillar wall carbohydrates, which include agar, alginates and carrageenan (Dhargalkar and Pereira, 2005). In the food industry alginates are used as emulsifiers (Brownlee *et al.*, 2009), in biotechnology they function as immobilisers of biological catalysts (Dhargalkar and Pereira, 2005) and in cosmetics where alginate and carrageenan are utilised as thickening agents.

The seaweed industry produces products that have an estimated total annual production of US$5.5-6 billion (FAO, 2004), of this the human consumption of raw seaweed and products
containing seaweed contribute to about US$5 billion. The remaining billion dollars is made up by the hydrocolloid sector and the use in fertilizers and animal feed additives. The seaweed industry uses 7.5-8 million tonnes of wet seaweed annually (FAO, 2004). These are harvested from either naturally growing seaweed or from cultivated crops (FAO, 2004). Ireland’s seaweed and biotechnology industry is worth up to €18 million per annum (Morrissey et al., 2011), it processes 36,000 tonnes of seaweed (wild product), mainly comprising of Ascophyllum for alginate production (Morrissey et al., 2011). The source of seaweed presently restricted to wild seaweeds and product range is limited in the main to high volume, low value products such as animal feeds, plant supplements, specialist fertilisers, phycocolloids and agricultural products.

1.1.2 Human food
Seaweed has been widely used as a staple food in many Eastern countries such as Japan, Korea and China since pre-historic times. There are over twenty-one species that are used in everyday cookery in Japan and are also utilized in the manufacture of many seaweed products like jams, cheese, wine, tea, soup and noodles (Arasaki and Arasaki, 1983; Teas et al., 2004). Macroalgae species, such as “nori” (Porphyra sp.) (Kitmura et al., 2002) are used in sushi preparation, “habinori” (Petalonia binghamiae) which is eaten dried and lightly roasted (Kuda et al., 2006). “Hitjiki” (Hizikia fusiforme) is consumed in soups, salads and vegetable dishes (Wondimu et al., 2007), “Kombu (Laminaria sp.) is used in “dashi” soup, and “wakame” (Undaria pinnatifida) which is also added to salads and soups (Dawczynski et al., 2007) are traditionally used in the diets of these Eastern cultures.
1.1.3 Dietary supplementation

Seaweeds are utilized on a daily basis in processed food products such as meats, dairy and fruit and also in household commodities such as toothpastes, cosmetics and paint products (Dhargalkar and Pereira, 2005). Macroalgae are a good source of vitamins A, B1, B12, C, D and E, riboflavin, niacin, pantothenic acid and folic acid (Nisizawa, 1988) as well as minerals such as Ca, P, Na, K. Macroalgae contain many essential amino acids and over 54 trace elements required in the bodies physiological function. They often contain these in much higher quantities that of vegetables and other terrestrial plants (Dhargalkar and Pereira, 2005).

1.1.4 Food supplement for animals

Seaweeds are often used as a supplement in meals for cattle, poultry and other farm animals. Some studies have suggested that seaweed supplements in animal feed can increase fertility and birth rates of animals and also improves yolk colour in eggs (Chapman and Chapman, 1980). Seaweeds from the genus *Gracilaria*, *Hypnea*, *Gelidiella* and *Sargassum* are added to feed, to produce a mixture enriched with minerals, amino acids and carbohydrates (Kaladharan *et al.*, 1998) for fish and prawn cultures. The feed can also help to maintain the water quality in the aquaculture industry. Recent studies in Ireland have focused on using seaweed extracts as supplementation in animal feed to promote a biological effect. Sweeney *et al.* (2011) exhibited the antimicrobial properties of seaweed derived polysaccharides (fucoidan and laminarin) in pigs. Laminarin and fucoidan was included in their diet to help fight against post-weaning infection, they showed antimicrobial effects against *Salmonella Typhimurium* in the gastrointestinal. Leonard *et al.* (2010) also investigated the effects of maternal dietary supplementation with seaweed extract and fish oil from day 109 of gestation until weaning (24 days) on piglets. They determined that seaweed
supplementation demonstrated an influence on the piglets gut health and post weaning performance. Piglets had lower *Escherichia-coli* levels in the caecum and seaweed supplementation induced an increase in ileal TNF-α expression.

### 1.1.5 Organic manure

Seaweeds have been used for centuries as fertilizers. Seaweeds contain potassium, nitrogen, plant growth promoting hormones, micronutrients and humic acid which makes it an excellent fertilizer. Genus such as *Fucus, Laminaria, Ascophyllum* and *Sargassum* can be administered to the soil as mulch or via addition to a compost heap. Unlike chemical fertilizers, seaweeds are non-polluting, biodegradable and non-hazardous to humans, animals and birds. Farmers throughout the world are switching to organic fertilizers. The alginates in seaweed play an important role in soil by clumping the particles together which in turn holds the moisture near the root. There are currently many liquid seaweed fertilizers such as Maxicrop (UK), Kelpak66 (South Africa), Seagrow (New Zealand), Algifert (Norway), Plantozyme (India), Shaktizyme (India) which are available on the market. Recently, seaweed extracts from *Ascophyllum nodosum* were shown to increase the amount of phytochemicals and the nutritional quality of broccoli when applied as a fertilizer (Lola-Luz *et al.*, 2014).

### 1.1.6 Seaweed as a renewable energy source

The remaining unused seaweed biomass from the commercial industry can be employed in the production of biogas through anaerobic digestion to methane (Morand *et al.*, 1991). This is a common practice in developing countries such as China and India. BioMara an Irish and UK project aimed to demonstrate the feasibility and viability of producing biofuels from marine
biomass (microalgae and macroalgae). Vanegas and Bartlett (2013) investigated the feasibility of exploiting Irish origin seaweed for the production of biofuels and found that a range of seaweed species (*Saccorhiza polyschides, Ulva sp. Laminaria digitata, Fucus serratus* and *Saccharina latissimi*) investigated are good feedstock candidates as a source of energy such as biogas and methane. The methane and CO$_2$ production from these species ranged between 50-72 % and 10-45 %.

1.1.7 Cosmetics

At present seaweeds are used in many cosmetic products such as soaps, shampoos, creams, sprays and powders. Cosmetic companies in France and the UK are now producing skincare products that have incorporated seaweed extracts (Dhargalkar and Verlecer, 2009). These cosmetics are marketed as naturally revitalizing, moisturizing and containing amino acids, minerals and vitamins that nutrify the skin.

1.1.8 Traditional medicinal uses of seaweed

Traditionally seaweeds have been used as anaesthetics, vermifuges and ointments as well as treatment for wounds, gout, coughs and venereal diseases in maritime countries for many years. Seaweeds perform a range of functions such as anti-oxidant (Shitbata *et al.*, 2008; Tierney *et al.*, 2013a; Tierney *et al.*, 2013b), anti-allergic (Li *et al.*, 2008), anti-mutagenic (Okai *et al.*, 1996), anti-coagulant (Cumashi *et al.*, 2007), anti-tumour (Riou *et al.*, 1996) etc. Seaweeds also contain sterols and related compounds that have the ability to lower serum cholesterol levels (Guillon and Champ, 2000). They also play a role in the lipid metabolism (Jeon *et al.*, 2010). High intake of calcium and potassium are associated with lower mean systolic blood pressure and a lower risk of
hypertension. The use of seaweed in medicine is not as common as it once was, however, the use of seaweed polymer extracts in pharmacy, medicine and biochemistry is well established.

1.2 Marine algal components

The composition of marine macroalgae is an important determinant of their nutritional value to marine invertebrate (Hawkins and Hartnoll, 1983) and for their assessment as potential sources of carbohydrates, proteins and lipids for commercial use (Chapman and Chapman, 1980) or for human consumption (Abbott, 1988). The nutrient composition of seaweeds is dependent on many factors such as species, habitat, stage of maturity and environmental conditions (Ito and Hori, 1989). Seasonal variation in macro and micronutrients has also been reported in marine macroalgae from Hong Kong (Kaehler and Kennish, 1996), coastal India (Kumar, 1993) and Ireland (Mercer et al., 1993; Schmid et al., 2014).

1.2.1 Protein/Nitrogen

The protein nitrogen content of macroalgae is important for both their application in human nutrition and as a source of fertilizer (Stewart, 1974). The protein content of dried marine algae is typically 15-25 % in green seaweed, 5-15 % in brown seaweed and 15-30 % in red seaweed (Kim, 2012) (Table 1.1). The protein content of marine algae is relatively high when compared to plants (Souchet, 2004; Fleurence, 1999).

Marine algae synthesize protein through fixation of nitrogen, therefore protein content varies with harvesting period. Environmental factors such as light, temperature and salinity all influence protein synthesis (Stewart, 1974; Barsanti and Gualtieri, 2006). Both Chapman and Craigie (1977) and Souchet (2004) reported a correlation between protein and water nitrogen content in seawater,
consequently when nitrogen levels fixation reduced and the marine algae protein content was also
seen to be reduced. In addition, higher temperatures also increase energy consumption due to algal
respiration (Anderson et al., 1981). This energy demand exceeds the rate of energy capture by
photosynthesis and results in the diversion of fixed and stored carbon away from nitrogen
assimilation (Graham and Wilcox, 2000; Anderson et al., 1981). Other reports (Germann et al.,
1987; Chapman and Craigie, 1977) confirm the protein content is relatively low in summer and
higher in winter.

Table 1.1 Protein content (% dry mass) in various marine algae used in the food Industrya.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Content (% of dry mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmaria palmata</td>
<td>8-35</td>
</tr>
<tr>
<td>Porphyra tenera</td>
<td>33-47</td>
</tr>
<tr>
<td>Ulva lactuca</td>
<td>10-21</td>
</tr>
<tr>
<td>Ulva pertusa</td>
<td>20-26</td>
</tr>
<tr>
<td>Laminaria digitata</td>
<td>8-15</td>
</tr>
<tr>
<td>Fucus species</td>
<td>3-11</td>
</tr>
<tr>
<td>Ascophyllum nodosum</td>
<td>3-15</td>
</tr>
</tbody>
</table>

(a taken from; Fleurence, 1999)

1.2.2 Lipids

The lipid content of marine macroalgae is generally low, less than 4% of the dried mass, although
the brown algae Sargassum kjellmaniamum contains more than 6% (Sánchez-Machado et al.,
2004). In brown algae, the fatty acids include primarily C16, C18 and C20 forms, with palmitic
acid (C16) making up to 10-15% and considered as the major fatty acid. Unsaturated fatty acid
levels are found to be higher in brown algae (Sánchez-Macado et al., 2004) in comparison to green
and red species. Recent studies (Schmid et al., 2014) reported that the total fatty acid contents of
seaweed was estimated to be between 0.8-6.4 %. Consumption of macroalgal lipids has been linked
to decreased risk of heart disease, thrombosis, atherosclerosis and to an antiviral activity (Sánchez-
Machado et al., 2004). Due to the potential for the unsaturated lipids that macroalgae contain to undergo oxidation marine algae contain antioxidants such as α-tocopherol which can be used in cosmetics or health products (Norziah and Chi, 2000; Dawczynski et al., 2007).

1.2.3 Minerals

Mineral content varies with algal species, growing region, season and environmental conditions. Algae absorb and store several minerals from seawater. Minerals generally represent 10-35 % of the dry mass of seaweed (Ito and Hori, 1989) as compared to 5-10 % of the dry mass of land vegetation (Rupérez, 2002). Brown algae are abundant in minerals such as calcium, magnesium, potassium, sodium, phosphorus, sulphur, iodine and iron (Table 1.2 and 1.3). The iodine content of brown algae is especially high and the concentration varies depending on seaweed species (Küpper et al., 1998; Van Netten et al., 2000; Hou and Yan, 1998; Chance et al., 2009). Mineral rich products made from marine algae are marketed as natural health enhancing products. Rí Na Mara and Irish Seaweed Cosmetics produces bath salts from seaweed harvested off the Irish coast that are rich in minerals and vitamins.
Table 1.2 Ash content of a selection of edible brown and red algae genus.a.

<table>
<thead>
<tr>
<th>Type</th>
<th>Genus</th>
<th>Ash* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown</td>
<td>Fucus</td>
<td>30.10 ± 0.20</td>
</tr>
<tr>
<td>Brown</td>
<td>Laminaria</td>
<td>35.59 ± 0.40</td>
</tr>
<tr>
<td>Brown</td>
<td>Wakame</td>
<td>29.26 ± 0.24</td>
</tr>
<tr>
<td>Red</td>
<td>Chondrus</td>
<td>21.08 ± 0.12</td>
</tr>
<tr>
<td>Red</td>
<td>Nori</td>
<td>20.59 ± 0.16</td>
</tr>
</tbody>
</table>

*a Analysis condition: 550°C for 16 h (*taken from; Rupérez, 2002).

Table 1.3 Major mineral and trace elements (mg/100g dry weight) determined using atomic adsorption spectrophotometry in edible brown and red seaweeds.a.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Brown seaweeds</th>
<th>Red seaweeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fucus</td>
<td>Laminaria</td>
</tr>
<tr>
<td>Na</td>
<td>5469 ± 60</td>
<td>3818 ± 43</td>
</tr>
<tr>
<td>K</td>
<td>4322 ± 46</td>
<td>11,576 ± 128</td>
</tr>
<tr>
<td>Ca</td>
<td>938 ± 7</td>
<td>1005 ± 5</td>
</tr>
<tr>
<td>Mg</td>
<td>994 ± 13</td>
<td>659 ± 6</td>
</tr>
<tr>
<td>Fe</td>
<td>4.20 ± 0.17</td>
<td>3.29 ± 0.54</td>
</tr>
<tr>
<td>Zn</td>
<td>3.71 ± 0.37</td>
<td>1.77 ± 0.44</td>
</tr>
<tr>
<td>Mn</td>
<td>5.50 ± 0.11</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Cu</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Total cations</td>
<td>11,736 ± 127</td>
<td>17,060 ± 183</td>
</tr>
</tbody>
</table>

*a Mean values of triplicate determinations standard deviation (*taken from; Rupérez, 2002).

1.2.4 Vitamins

Like many vegetables, marine algae contain several vitamins. Marine algae contain vitamin B (cyanocobalamin), which is rare in vegetables. Vitamin B protein factor was found in marine seaweeds (Park et al., 1997 and 2000). The vitamin B12 in marine algae is similar to that formed in animal intestines and may be used to replace animal sources of this vitamin. Marine algae are also sources of vitamin E (α-tocopherol) and generally contain this vitamin at concentrations ranging from 0.7-8.0 mg per 100 g. However, the vitamin E content varies according to season. In
the case of vitamin C, the period from spring to early summer yields algae with the highest content of this vitamin (Park et al., 1997).

1.2.5 Polysaccharides

Large amounts of seaweed derived polysaccharides (agars and carrageenans) are utilised in foods, pharmaceuticals and other products for human consumption. Functional polysaccharides such as fucoidan and alginic acid derivatives which are produced by brown seaweeds are known to have many biological properties including anticoagulant, anti-inflammatory, anti-viral and anti-tumoral activities (Boisson-Vidal et al., 1995; Costa et al., 2012; Athukorala et al., 2008). In recent years, the sulphated polysaccharides including fucoidans have been isolated from different brown algal species such as, *Sargassum filipendula, Caulerpa profilera, Ecklonia cava, Ascophyllum nodosum* and *Undaria pinnatifida* (Costa et al., 2010; Athukorala et al., 2006; Matou et al., 2002). Sulfated polysaccharides are the most abundant and broadly investigated polysaccharides from non-animal origin. Marine algae contain large amounts of non-starch polysaccharides that cannot be digested completely by the human digestive system and therefore have potential as sources of dietary fibre, prebiotics and other functional ingredients (Lahaye, 1991; Mabeau and Fleurence, 1993).

1.2.5.1 Polysaccharides in green marine algae

Similar to vascular plants, green seaweed contains cellulose as the principal structural polysaccharide (Yaich et al., 2011). Although some contain other structural polysaccharides such as *Codium*, which contains β-1-4 linked mannan as a structural polysaccharide (Percival and McDowell, 1967) and Ulvan which is a water soluble polysaccharide found in the cell wall and generally makes up to 8-29% of the algae dry weight (Lahaye, 2007).
1.2.5.2. Polysaccharides in red marine algae

Most red algae contain cellulose as the main structural polysaccharide (Park et al., 2000), although the Rhodeminea species contains β-1-3 and β-1-4 linked xylans (Park et al., 1997). The mucilage polysaccharides of red algae contained α-1-3 and β-1-4-linked sulphated galactan. Other major polysaccharides in red algae are agar, carrageenan and porphyran (Park et al., 2000). Carageenan is a linear sulphated food grade polysaccharide obtained from red macroalgae. Carageenan is the main constituent of the seaweed and can be found in the cell wall and intracellular matrix of the plant tissue. Commercially harvested seaweed contains carrageenan levels ranging between 30-80 % of dry weight. Carageenans have the ability to form various gels (rigid or compliant, tender or tough and with high or low melting points). Carageenans are commonly used as food additives due to their gelling and emulsifying properties. It is an approved food additive and is used worldwide to enhance the textures of ice-creams, cheeses, chocolate milks, custards, meat and protein drinks. Carageenans have been utilised in antimicrobial films for food products (Campos et al., 2010) and have shown anticoagulant (Carlucci et al., 1997) and antiviral properties (Ono et al., 2003) proved to be an effective carrier for anti-retroviral drugs in HIV prevention and treatment (Witvrouw and Clercq, 1997).

1.2.5.3 Polysaccharides in brown marine algae

Cellulose is also present in brown macroalgae as one of the structural polysaccharides. Its content ranges from 5.7-14 % (Park et al., 2000). The mucilage polysaccharides are alginate, fucoidan and laminarin.

Fucoidan is a well-known mucilage heteropolysaccharide in brown algae (Park et al., 1997; Park et al., 2000; Percival and McDowell, 1967). It is a type of complex sulphated polysaccharide
mainly found in the cell wall matrix of various brown seaweed species (Kim et al., 2010; O’Connell et al., 2008; Teruya et al., 2007). It is synthesized in the Golgi apparatus inside cells and is found in the intracellular spaces throughout the algal tissue (Park et al., 1997). The principal role of fucoidan is to prevent drying out of the plant exposed to the air during tidal changes (Percival and McDowell, 1967). It is composed of L-Fucose with sulphate ester groups (Jiang et al., 2010; Li et al., 2008; Matou et al., 2002) and other less abundant monosaccharaiides such as D-galactose, D-xylose, D-glucose, D-mannose and D-uronic acid. Fucoidan is a complex heterogeneous polymer and no defined regularity has been observed (Kusaykin et al., 2008). Fucose is a hexose deoxy sugar and has the chemical formula C₆H₁₂O₅ and is the fundamental sub unit of the fucoidan polysaccharide. Fucoidan has been extensively studied in the past decade due to its wide range of biological activities. The bio-functional properties of fucoidans from brown seaweeds could contribute to the development of pharmaceuticals, cosmeceuticals and functional foods.

Several fucoidan structures have been isolated and many of their bioactivities such as antiocoagulant, antithrombotic, antitumor and immunomodulatory have been elucidated (Li et al., 2008). Previous research has shown that the composition and structural complexity of fucoidans can vary between brown seaweeds (O’ Connell et al., 2008) and is dependent on growing region and season (Park et al., 2000).

Laminarin is a low molecular mass polysaccharide of about 5 kDa, with a degree of polymerization (DP) ranging from 20 to 25 (Chizhov et al., 1998; Nelson and Lewis, 1974). Initially, laminarin was originally reported as a polymer of exclusively D-glucose linked via β (1, 3) bonds (Barry, 1939) however linkage via β (1, 6) bonds has also been observed (Peat et al., 1958). Some chains are terminated by D-mannitol residues (Nelson and Lewis, 1974). Laminarin
has several biological activities (Kim et al., 2006), past studies that have concentrated on the effect of laminarin on the gastrointestinal tract (Devillé et al., 2004, 2007; Michel et al., 1996), or the anticoagulant activity of a derivative, sulphated laminaran (Ito and Hori, 1989). Kuda et al. (2005) examined the effect of laminarin to inhibit putrefactive compound formation in faecal cultures of humans and in rat caecum. The results suggested that due to the fermentation of laminarin by intestinal bacteria, putative risk markers for colon cancer such as indole compounds and ammonia were suppressed. Recently, Ji et al. (2012) also reported that laminarin proved to be an effective anti-tumour agent by inducing apoptosis in human colon cancer LOVO cells through a mitochondrial pathway. Ji and Ji (2014) also reported the anti-tumour effect of laminarin in LOVO cells, apoptosis was induced through the TRAIL/DR pathway. Laminarin has also shown immunomodulatory effects (Kim et al., 2006) in mouse thermocytes where suppression of apoptotic death (2-3 fold) and extended cell life of 20-30% was observed. Research by Choi et al. (2012) observed that the antioxidant activity of laminarin increased after irradiation. They determined that this effect on the antioxidant activity was linked to low molecular weight reducing sugars compared to non-irradiated laminarin (Choi et al., 2011). Laminarin sulphate has shown anti-coagulant activity of about 30% of heparin and was found to be an effective therapeutic treatment of cerebrovascular diseases (Miao et al., 1995). Research carried out by Lee et al. (2012) reported that laminarin exhibited inflammatory activity. They examined the ability of laminarin to increase the release of inflammatory mediators such as hydrogen peroxide, nitric oxide, calcium, monocyte chemotactic protein-1, vascular endothelial growth factor, leukaemia inhibitory factor and granulocyte-colony-stimulating factor and the effect of boosting the expression of signal transducer and activator of transcription 1 (STAT1), STAT3, c-Jun, c-Fos and cyclooxygenase-2
mRNA in RAW 264.7 mouse macrophage cells. This showed great potential for laminarin to be as used as a therapeutic agent with immunostimulatory and anti-inflammatory properties.

The structure of laminarin varies considerably among species. Its solubility is dependent on chain length (CL). For soluble laminarin CL=7-11 and DP=26-31, while for insoluble laminarin CL=15-19 and DP= 16-24 (Souchet, 2004; Nelson and Lewis, 1974). Different types of laminarin can be classified as soluble and insoluble in cold water (20°C) and the insoluble laminarin fraction can be solubilised in water at 60-70°C (Souchet, 2004; Park et al., 2000).

The extraction of these compounds is influenced by the chemical nature of the components, the extraction method employed and the presence of any interfering substances (Chirinos et al., 2007). The polysaccharide content of seaweeds varies according to the species and the structure and biological activities of laminarin and fucoidan are thought to be influenced by environmental factors, such as water temperature, salinity, waves, sea current, and depth of immersion (Black, 1954; Black and Dewar, 1949; Lobban and Harrison, 1997; Percival and McDowell, 1967). The extraction method and the time of harvest are other important factors (Black et al., 1952; Haug and Jensen, 1956). The availability of nutritive salt, mostly nitrate, influences seaweed growth (Harlin and Craigie, 1978).

For laminarin, some researchers have shown the impact of environmental factors on laminarin production. Anderson et al. (1981) found that the maximum laminarin content was observed in June while the minimum content of laminarin was observed between December to February, when ice cover limited light levels. Laminarin content levels were determined using an enzymatic method and compared to an internal standard. They found that nutrient availability allowed the seaweed to take advantage of summer light and temperature conditions to grow rapidly. Chapman
and Craigie (1978) observed a similar trend with laminarin content at a maximum in late summer and minimum in February when the plant growth rate is increasing rapidly.

For fucoidan, studies have shown that harvest period has an impact on fucoidan content in seaweeds (Black, 1954), the exposure of the seaweed to air (Percival and McDowell, 1967) and frond age (Zvyagintseva et al., 2003) have been shown to affect the level of fucose in samples.

1.2.6 Polyphenols

Polyphenols or phenolic compounds are abundantly distributed in the plant kingdom (Urquiaga and Leighton, 2000). Polyphenol compounds are found in many commonly consumed fruits and vegetables such as peppers (Chuah et al., 2008), apples (Cao et al., 2009), red cabbage (McDougall et al., 2007) and grapes (Guerrero et al., 2008). Polyphenols are structurally characterised by the presence of several hydroxyl groups on aromatic rings (Manach et al., 2004), they are biosynthesized through either the shikimate/phenylpropanoid or the polyketide acetate/malonate pathway, or a combination of the two pathways (Vincenzo et al., 2009). Phenolic compounds are products of secondary metabolism of plants and have functions in pigmentation, reproduction, growth and protection against pathogens (Zern and Fernandez, 2005). Polyphenols have antioxidant properties and can protect cell components from oxidative damage and therefore may limit the risk of various degenerative diseases, such as cancers, cardiovascular diseases, osteoporosis and other diseases associated with oxidative stress (Scalbert et al., 2005). Polyphenols can be classified into different groups according to the number of phenol rings that they contain and the structural elements that bind them. Distinctions are thus made between phenolic acids, flavonoids, stilbenes and lignans (Figure 1.1). There are two main groups of polyphenols i.e., the flavonoid group (e.g., flavanol, isoflavanones and anthcyanins) and the non-flavanoid group (e.g.,
phenolic acids, stilbenes, hydrolysable tannins and simple phenols). Flavanoids consist of 2 aromatic rings that are bound together by 3 carbon atoms that form an oxygenated hetercycle (Manach et al., 2004). A common seaweed polyphenol is phlorotannin which is a tannin that is formed by the polymerisation of phloroglucinol (1, 3, 5-trihydroxybenzene) monomer units and biosynthesized through the acetate–malonate.

Brown seaweeds in particular are a rich source of phlorotannins, in which they can comprise up to 25-30 % of dry weight (Targett et al., 1995). Phlorotannins are known to protect macroalgae against UV radiation and also act as a chemical defence against herbivores (Boettcher and Targett, 1993; Pavia et al., 1997). Many of the useful properties of these compounds are related to their antioxidant ability. The radical scavenging ability of phlorotannins against reactive oxygen species (ROS) (Kim et al., 2009; Shibata et al., 2009) have been extensively investigated. For example,

Figure 1.1 Chemical structures of Polyphenols
Shibata et al. (2008) investigated the antioxidant activity of crude phlorotannins from Japanese Laminariaceae. They determined that their antioxidant properties were due to multiple sites at which free radicals can be resonance stabilised. There have been numerous studies that have investigated the antioxidant activity of phlorotannins from a range of seaweed species (Wang et al., 2012; Shibata et al., 2008; Connan et al., 2006) and some studies have even highlighted the fact that the molecular weight of phlorotannins and the level of isomerisation present has an impact on the bioactivity of the compounds (Tierney et al., 2013b; Tierney et al., 2014). Within each phlorotannin it is possible to have differences in linkage positions between monomers. Monomeric units in phlorotannins are connected through two bonds aryl–aryl bonds and diaryl bonds. These form different subgroups of phlorotannins (Glombitza et al., 2003). Fucols result from linkage of aromatic ring with only aryl–aryl bonds fucols (Figure 1.2) whereas aryl ether bonds form Phlorethols (Figure 1.2). Fuhalols are formed when phloroglucinol units are linked with para- and ortho-arranged ether bridges that contain one additional OH-group on every third ring (Figure 1.2). When at least one three-ring moiety with a dibenzodioxin elements is substituted by a phenoxyl group at C-4 occurs the group is named eckols (Figure 1.2). Carmalols are a derivative of phlorethols that contain a dibenzodioxin moiety. Endofucophlorethols and isofuhalols (Figure 1.2) are small and specialized groups (Glombitza et al., 2003). This leads to the formation of many structural isomers. This structural diversity, along with bioactivity are also affected by extrinsic factors such as seasonality and environmental factors such as light intensity, nutrient availability, predation and temperature. Due to this structural diversity, these polyphenolic compounds have exhibited an array of biological activities. In addition to their potent antioxidant activity phlorotannins have also shown anti-microbial activity (Nagayama et al., 2002), anti-diabetic properties (Eom et al., 2012), cytotoxic activity (Li et al., 2011) anti-hypertensive activity.
(Wijesinghe et al., 2011) and hyaluronidase inhibition (Ferreres et al., 2012). These activities have made phlorotannins favourable for use in functional food products, cosmetics and pharmaceuticals.

**Figure 1.2** Chemical structures of some common phlorotannins.

### 1.2.7 Carotenoids

Carotenoids are organic pigments that are found in the chloroplasts and chromoplasts of plants and photosynthetic organisms such as algae, some bacteria, and some fungi. Structurally carotenoids can be categorized into xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons, and contain no oxygen) (Sousa et al., 2006). Carotenoids are synthesised from eight isoprene molecules and contain forty carbon atoms in the basic skeleton. Carotenoids fulfil two key roles in plants and algae; they absorb light energy for use in photosynthesis, and they protect chlorophyll from photodamage. Fucoxanthin is a brown pigment which colours brown algae. Fucoxanthin is one of the most plentiful carotenoids contributing to approximately 10 % of the
estimated total production of carotenoids in nature (Matsuno, 2001). The structure of fucoxanthin is unique as it has an unusual allenic bond and a 5, 6-epoxide bond (Figure. 1.3). For different brown algal strains, the composition and profile of fucoxanthins were found to be different (Terasaki et al., 2009).

![Figure 1.3 Structure of fucoxanthin.](image)

Carotenoids not only function as colourings, they also contribute to the antioxidant activity of marine algae. Various classes of carotenoids like fucoxanthin, chlorophyll \(a\), phycoerythrobilin and their derivatives have shown to contribute to the antioxidants activity of selected seaweeds.

The antioxidant activity of carotenoids is dependent on their structure such as porphyrin ring, phythyl chain and extended system conjugated double bonds. Endo et al. (1985a, 1985b) reported that chlorophyll \(a\) exhibits antioxidant activity linked to the presence of a porphyrin ring. Normura et al. (1997) reported that some carotenoids such as \(\beta\)-carotene, zeaxanthin and lutein did not exhibit 2, 2,- Diphenyl -1-picryl-hydrayl (DPPH) scavenging activity, however, Yan et al. (1999) did show that fucoxanthin had strong radical scavenging ability. Fucoxanthin radical scavenging may be linked to the presence of an allenic bonds at C-7’ position. These findings were confirmed by Sachindra et al. (2007) who isolated fucoxanthin from *Undaria pinnatifida* and developed two
fucoxanthin metabolites (fucoxanthinol and halocynthiaxanthin). The three isolated carotenoids were subjected to antioxidant analysis using the DPPH assay. They were ranked in the following order of decreasing antioxidant activity: Fucoxanthin > fucoxanthinol > halocynthiaxanthin (Sachindra et al., 2007). With both fucoxanthin and fucoxanthinol containing an allenic bond it was suggested that the allenic bond is responsible for the higher scavenging activity observed in fucoxanthin and fucoxanthinol.

1.3 The health promoting properties of macroalgal derived antioxidants.

An antioxidant is any compound with the ability of delaying or preventing oxidation of other molecules. Oxidation is a chemical reaction which involves the transfer of electrons from a substance to an oxidizing agent. Free radicals are chemically active atoms with an unpaired electron, e.g., the superoxide anion, hydroxyl radical, nitric oxide, ozone and transition metals such as iron and copper (Valko et al., 2007). In vivo, some of these reactive oxygen species (ROS) have a positive effect in cell physiology; however they can also cause damage to cell membranes and DNA by inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, and DNA mutations which leads to cancer and other degenerative diseases (Finkel and Holbrook, 2000). Oxidants that develop from the processes within the body, form as a result of normal aerobic respiration, metabolism and inflammation. Antioxidants are molecules that interact with free radicals and terminate the chain reaction before any vital molecules are damaged by oxidation. Antioxidants are most often reducing agents like polyphenols and thiols.

Although many oxidation reactions are important in life some can be very damaging. Animals and plants have very complex systems of multiple types of antioxidants for example glutathione, vitamin C and vitamin E, as well as many enzymes such as catalyse, superoxide dismutase and various peroxidases. However, humans lack these complex systems and oxidative stress is a big
contributor to many human diseases. The use of antioxidants in the treatment of some diseases such as stroke and neurodegenerative diseases e.g., Alzheimer’s and Parkinson’s disease is being studied intensively (Moosmann and Behl, 2002; Uttara et al., 2009). In recent times there has been a surge in research toward finding natural antioxidant components that can offer protection against ROS. Components that demonstrate antioxidant capacity \textit{in-vitro} include vitamins C and E, carotenoids and polyphenols. Marine macroalgae have therefore gained much attention due to their untapped resource of both carotenoids and polyphenols for this purpose. Marine polyphenols known as phlorotannins have been reported to display strong radical scavenging capabilities (Kim et al., 2009; Shibata et al., 2009). Marine carotenoids have also gained attention due to their biological activity, in particular the carotenoid fucoxanthin which is unique to brown macroalgae. The fact that these compounds can be sourced naturally has added to the attractiveness of the possibility for their use as functional ingredients in food products, pharmaceuticals or cosmetics to promote a health effect.

1.4 Seaweeds under investigation in the present study

Seaweed harvesting has long been a traditional activity in Ireland. Historically in Ireland, seaweed was predominantly used to fertilise potatoes, and it was also burnt in kelp kilns for the extraction of soda and potash to make glass and soap. More recently, the alginate industry was established with the brown seaweed \textit{Ascophyllum nodosum} being the species most frequently harvested for this purpose.
Table 1.4. Estimated annual Irish national seaweed harvest.

<table>
<thead>
<tr>
<th>Species</th>
<th>Annual Harvest (tonnes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascophyllum nodosum</td>
<td>25,000</td>
</tr>
<tr>
<td>Fucus serratus</td>
<td>200</td>
</tr>
<tr>
<td>Palmaria palmata</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Chondrus crispus</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Laminaria digitata</td>
<td>&lt;150</td>
</tr>
<tr>
<td>Himanthalia elongata</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Saccharina lattisma</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Laminaria hyperborea</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Ulva rigida</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Porphyra spp</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

*a* taken from; Board Iascaigh Mhara, Irish Seaweed Fisheries Board, 2007-2013, Part1.

The Irish seaweed industry produces a range of products for which agricultural and horticultural uses are the most important by volume and value. The bulk of this production goes into plant supplements, animal feeds, specialist fertilizers and general agricultural products. A small amount of the national production goes into various cosmetics and therapies (Bord Iasaigh Mhara, 2007).

At present *Ascophyllum nodosum, Fucus serratus* and *Laminaria digitata* are the most extensively harvested seaweeds in the industry (FAO, 2004). However, these species and the vast resource of genus available off the coast of Ireland are in general underutilised in terms of potential. In recent years investigation of seaweeds has become extremely popular as they have been highlighted to be an excellent source of bioactive compounds for use as functional food ingredients to promote a health benefit (Lordan *et al.*, 2011).

### 1.4.1 Laminaria digitata

*Laminaria digitata* is a brown macroalgae and belongs to the division Heterokontophyta, the genus *Laminaria* and is commonly known as kelp or oarweed (Fig. 1.4). *Laminaria digitata* grows on lower littoral exposed sites where it can be the dominant algae. It grow to approximately 2 metres
in length has a claw like holdfast and a smooth flexible blade. This blade is split into finger like segments. *Laminaria digitata* has a high growth rate and an approximate carrying capacity of 40 kg wet weight/square metre. *Laminaria digitata* is highly liable to grazing by sea urchins and other animals (Guiry, 2015). Its dispersal is also restricted by extrinsic factors such as salinity, wave exposure, temperature, desiccation and stress (Bolton and Lüning, 1982).

![Image of Laminaria digitata](taken from; Guiry, 2015)

**Figure 1.4** Image of *Laminaria digitata*

In a study by Le Tutour *et al.* (1998) the ability *Laminaria digitata* to act as a radical scavenger was examined using kinetic studies. Seasonal variation of *L. digitata* was observed, with summer observed to be effective synergists to enhance the antioxidant activity of vitamin E and Butylhydroyl Toluene (BHT), while samples from winter displaying a negative synergism. *Laminaria digitata* has been reported to have antimicrobial properties. *Laminaria digitata* extracts containing laminarin and fucoidan have also been incorporated into the diets of pigs to improve meat quality. A statistically significant reduction in lipid oxidation was observed in the meat from 75% of pigs fed with laminarin and fucoidan wet form (Moroney *et al.*, 2012). Murphy *et al.* (2011) investigated the bacterial community shifts in the gastrointestinal tract of pigs fed diets
supplemented with β-glucan from *Laminaria digitata*, *Laminaria hyperborea* and *Saccharomyces cerevisiae*. They determined that the *L. hyperborea* β-glucan had the most significant effect on reducing coliform counts in ileum and proximal colon in pigs, with the β-glucan from *L. digitata* and *S. cerevisiae* also showing an effect but just to a lesser extent.

1.4.2 *Fucus serratus*

*Fucus serratus* is also a brown macroalga and belongs to the division Heterokontophyta, and the genus *Fucus* and is commonly known as toothed wrack (Fig. 1.5). It grows abundantly on exposed rocks or in rook pools of slow draining shores. It is olive brown in colour, similar to *Fucus vesiculosus* and *Fucus spiralis* which belong to the same division. It has flat fronds (2 cm wide) and 1 metre long, with short stipe’s. The blade has a distinct midrib that can easily be distinguished by its serrated edges. It does not have air vesicles, such as are found in *Fucus vesiculosus*, nor is it spirally twisted like *Fucus spiralis* (Guiry, 2015). *Fucus serratus* is commonly found along the Atlantic coast of Europe, in the Canary Islands and off the shores of North-East America. *Fucus serratus* is used in Ireland and France for the production of cosmetics and thalassotherapy.

*Figure 1.5 Image of Fucus serratus*
*taken from; Guiry, 2015*
Extracts from *Fucus serratus* has been reported to exhibited good radical scavenging ability (Le Tutour, 1998; Zubia, 2009; O’ Sullivan et al., 2011). In the study by Le Tutour (1998) *F. serratus* showed good inhibition of oxidation of methyl linoleate. It also showed activity in the study by Zubia (2009) where it had an EC$_{50}$ value of 5.51 as determined using the DPPH assay. This activity has been attributed to phlorotannins (Le Tutour, 1998; Connan, 2004).

### 1.4.3 *Gracilaria gracilis*

*Gracilaria gracilis* is a red algae and belongs to the division Rhodophyta, and the genus *Gracilaria* (Fig 1.6). It is known for its industry uses as an agarphyte (a seaweed from which agar can be extracted), as well as its use as a food for humans and various species of shellfish (Guiry, 2015). *Gracilaria bursa-pastoris silva* and *Gracilaria multipartite* (clemente) Harvey have long been established as agarphytes in southern England and north-western France (Ramadhani, 2013). *Gracilaria* is used as a food in Japan, Hawaii and the Filipines and is known as agonori, ogo and gulaman.

![Image of *Gracilaria gracilis*](image)

*Figure 1.6* Image of *Gracilaria gracilis*  
*taken from; Guiry, 2015*
Gracilaria gracilis has been reported to contain antibacterial effects. Cavallo et al. (2012) reported that extracts from Gracilaria gracilis exhibited antibacterial effects against fish pathogenic Vibrio species. Extracts from G. gracilis also exhibited cholinesterase inhibition in a study conducted by Natarajan et al. (2009).

1.4.4 Codium fragile

Codium fragile is a green algae and belongs to the division Chlorophyta and the genus Codium (Fig. 1.7). There are approximately 50 species of Codium worldwide. It has thalli of two forms, erect and prostate. The vertical plants are dichotomously branched to 40 centimetre long branches and form a compact spongy structure (Guiry, 2015). Codium adhaerens has been recorded from a number of sites on the west coast and from Troy island on the north coast in county Donegal. Codium fragile has been recorded on the West coast in Finnavarra Co. Clare (Fish and Fish, 2011). It is generally found in deep pools along rocky coasts. Codium will settle on just about any kind of hard surface including ship hulls, navigation markers and shellfish.

Figure 1.7 Image of Codium fragile
*taken from; Guiry, 2015*
*Codium fragile* has been reported to exhibit anticoagulant and antiviral activity. These activities have been linked to the presence of a high molecular weight sulphated galactan and also a high molecular weight sulphated proteoglycan. Work conducted by Ohta *et al.* (2009) found that a sulphated galactan isolated from *Codium fragile* demonstrated antiviral potency and inhibited the replication of the herpes simplex virus type 2 (HSV-2). The mechanism of inhibition was suggested to be associated with interference in the early steps such as virus adsorption and penetration into host cells. Studies by Kim *et al.* (1997) and Hudson *et al.* (1999) also reported that extracts from *C. fragile* showed antiviral activity when tested against the herpes simplex virus, sindbis virus and poliovirus. Reports by Jurd *et al.* (1995) found that a high molecular weight sulphated proteoglycan obtained from *Codium fragile* exhibited anticoalgulant activity. This action was anti-thrombotic in nature due to potentiation of heparin cofactor II and antithrombin III activity. Athukorala (2007) also isolated a high molecular weight proteoglycan from *Codium fragile* that showed high activated partial thromboplastin time (APTT). *C. fragile* methanol extracts have also exhibited anti-inflammatory effects in lipopolysaccharide stimulated Raw 264.7 cells (Kang *et al.*, 2012). It is believed that proteins were responsible for this bioactivity.

1.4.5 *Cystoseira nodicaulis*

*Cystoseira nodicaulis* is a brown macroalgae of the fucales family (Fig. 1.8). It can distinguished by its highly differentiated basal and apical regions and the presence of air-vesicles. The air vesicles keep the organism erect, by causing it to float in strong currents *Cystoseira* plants have an elongated main the lower parts are flattened into ‘foliar expansions’ or basal leaves (Guiry, 2015).
Cystoseira nodicaulis has previously been reported to display a range of biological activities. Ferreres et al. (2012) investigated the antioxidant activity and the hyaluronidase (HAase) inhibitory capacity of phlorotannin extracts isolated from the brown seaweed C. nodicaulis. It showed to be the most efficient superoxide radical scavenger in the study and also exhibited HAase inhibition. Lopes et al. (2012) also investigated the activities associated with phlorotannins isolated from C. nodicaulis. They reported that C. nodicaulis showed good anti-inflammatory potential by inhibiting nitric oxide (NO) production by lipopolysaccharide to stimulate RAW 254.7 macrophage cells. They also examined the antibacterial effect of these phlorotannin extracts and found they were effective against Gram-positive bacteria in particular Staphylococcus epidermis.

1.4.6 Fucus vesiculosus

Fucus vesiculosus is a brown macroalgae of the fucales family (Fig. 1.9). It is more commonly known as bladder wrack. It can be located on the coasts of the North Sea, the Western Baltic Sea, the Atlantic and Pacific Oceans. It is distinguished by its paired bladders on either side of a prominent midrib. Fucus vesiculosus is attached to rocks by a small, strongly attached disc which gives rise to a short stipe (Guiry, 2015).
*Fucus vesiculosus* has been shown to exhibit a range of biological activities. It has previously been reported to exhibited good antioxidant activity. Le Tutour *et al.* (1998) investigated the ability of *Fucus vesiculosus* to scavenge peroxyl radicals in kinetic studies in a model system. *Fucus vesiculosus* exhibited a good synergetic effect against free radicals. Wang *et al.* (2012) also investigated the antioxidant capacity of phlorotannins extracted from *Fucus vesiculosus* and determined that the ethyl acetate fraction exhibited the highest DPPH scavenging activity and reducing power. A study by Sandsalen *et al.* (2003) reported the isolation of a compound from *F. vesiculosus* which exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria tested. Research by Dürig *et al.* (1997) extracted low molecular weight fucoidan from *F. vesiculosus* which exhibited potent anticoagulant and fibrinolytic properties. Polysaccharides and polyphenols isolated from *F. vesiculosus* have also shown anti-HIV activity. These fractions were assessed for inhibition of both HIV-induced syncytium formation and HIV reverse transcriptase enzyme activity (Edmundo *et al.*, 1999).
1.4.7 Himanthalia elongata

Himanthalia elongata is a brown alga in the order Fucales and is commonly also known as sea spaghetti (Fig. 1.10). It is located in the North East Atlantic Ocean and in the North Sea. It has thallus that contain button shaped vegetative thalli about 30 mm wide and 25 mm high, and has a long narrow yellow-brown strap like reproductive receptacle which is approximately 2 metre in length (Guiry, 2015).

![Image of Himanthalia elongata](image_url) *taken from; Guiry, 2015

Himanthalia elongata has been reported to contain many biological activities. Le Tutour et al. (1998) investigated the ability of Himanthalia elongata to scavenge peroxyl radical by kinetic studies. H. elongata exhibited a synergistic effect on vitamin E and butylhydroxyl toluene (BHT) by scavenging peroxyl radicals. Rajauria et al. (2011) also reported that a methanol extract from H. elongata showed the highest reducing power and antioxidant capacity against DPPH radicals, metal ions, lipid peroxides and hydrogen peroxide radicals. Rajauria et al. (2011) also reported that the same methanol extracts exhibited 60 % inhibition against various food spoilage and pathogenic bacteria. Research has also highlighted that H. elongata contain polysaccharides with antiviral activity. Santoyo et al. (2010) acquired a polysaccharide rich fraction by pressurized
liquid extraction with ethanol and water. They examined the potential of these extracts against the herpes simplex virus type 1 (HSV-1) during different stages of viral infection. A protein extract isolated from *H. elongata* was studied in the central nervous system of mice to explore for myorelaxant, anti-convulsant and analgesic activity and for its effects on spontaneous locomotor activity, amphetamine-induced hypermotility, exploratory behaviour, barbiturate-induced sleep and body temperature. A significant reduction in spontaneous motor activity, hypermotility and exploratory behaviour were observed. The protein extract extended barbiturate induced sleep and postponed pentylemetetrazal-induced death. Weak myorelaxant, hypothermic and analgesic effects were also seen, showing that *H. elongata* can depress the central nervous system (Anca et al., 1993).

### 1.5 Extraction of antioxidant compounds from macroalgae

Many compounds found in macroalgae are tightly bound within the matrix of the macroalgae. Fucoidan and some phlorotannins are usually bound within the cell wall (Yang and Zhang, 2009), while carotenoids are membrane bound in the chromoplast and chloroplasts (Gunning and Steer, 1996). In order to accurately quantify these compounds before their incorporation into food products or use in other applications they need to be extracted efficiently from the macroalgae. A range of simple and advanced techniques exist for the extraction of these compounds from algae.

The most commonly used extraction technique is solvent extraction. The extraction solvent employed is dependent on the solubility of the desired compounds. For polar compounds such as phlorotannins, highly polar solvents such as water, acetone and alcohols are ideal. For non-polar/lipophilic compounds such as carotenoids and vitamin E which are insoluble in water, non-polar solvents such as hexane and chloroform are more suitable. Another factor that influences the extraction technique is the required purity of the target compound. Simple solvent methods like
maceration, infusion and more advanced techniques such as Soxhlet extraction, counter-current extraction, microwave assisted extraction (MAE), hydrodistillation, supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) have previously been used to extract compounds from plants (Tandan and Rane, 2008). Maceration (Lopez et al., 2011), Soxhlet extraction (Duan et al., 2006) and percolation (Ananthi et al., 2011) are the most commonly used methods for extraction of antioxidants from algae. More recent studies by Tierney et al. (2013a), Rajauria et al. (2011) and Ravikuman et al. (2011) have continued to use simple solvent extraction to extract phenolics from seaweeds, while Badrinathan et al. (2011) used Soxhlet with a fractionation step to obtain phenolic enriched fraction from Sargassum siliquastrum and Sargassum myriocystrum. These methods remain popular due to them being simple and do not require complex expensive equipment, however, they have some limitations such as long extraction times, high solvent usage, possible thermal degradation of the desired compound and the potential toxicity of some solvents such as methanol and hexane (Wang and Weller, 2006). Techniques such as supercritical fluid extraction (SFE), Accelerated solvent extraction (ASE) and subcritical water extraction (SWE) are being developed to overcome these limitations associated with these more established methods (Mendiola, 2008).

1.5.1 Solid-liquid extraction (SLE)

Solid-liquid extraction is a process that involves removing soluble components from a solid sample matrix using an extraction solvent with specific polarity for the target compound. The desired compounds are removed through dissolution in a dissolving solvent.

A typical extraction occurs as follows; (i) The extraction solvent diffuses into the solid sample matrix (ii) the solvent dissolves the target molecules (i.e. transfer the solute into the liquid fraction) (iii) solvent recovery is undertaken to separate the liquid fraction from the solid sample fraction.
This is usually done by filtration. (iv) solvent removal can be done to provide a pure dry extract of desired compound. This can be achieved by freeze-drying or solvent evaporation using a nitrogen stream or a rotary evaporator, this is dependent on the extractant solvent used.

Solvents such as methanol, ethanol, acetone, ethyl acetate and propanol and their combinations have previously been employed for the solid/liquid extraction of phenolic compounds, often with different proportions of water (Luthria and Mukhopadhyay, 2006; Naczk and Shahidi, 2006; Zadernawski et al., 2005). Extraction time also influences the recovery of polyphenols from sample matrices. Reported extraction periods vary from 1 min to 24 h (Price and Butler, 1977).

Pigments and carotenoids (fucoxanthin and chlorophyll) need to be extracted before analysis, and sometimes from a complex mixture. Complete extraction of pigments is difficult and may require several steps and may involve the use of a mixture of solvents. Chlorophylls and carotenoids have previously been extracted with acetone and the use of a homogeniser (Schoefs, 2002). While a combination of several organic solvents such as hexane, acetone and ethanol have also been used (Thompson et al., 2000).

Polysaccharides can be extracted using a variety of solvent systems. At present the most utilised extraction is the Black (1951) method which involves the use of a mild acid for extraction. Other used methods involve the use of water and ethanol. Procedures adapted from Mian and Percival (1973) and Souchet (2004) are commonly used. Extraction is generally carried out at room temperature and the solution is agitated by shaking or stirring. It is then heated to 60-70 °C to remove proteins and pigments which are denatured by the heat and precipitate out of solution. The residual seaweed is then treated with 2 % (w/v) CaCl₂ at 60-70 °C to precipitate alginates as well as to extract laminarin. Fucoidan is extracted from the residual seaweeds with 0.01 M HCl, pH 2 at 60-70 °C. Dialysis of these polysaccharide fractions is then carried out to fractionate according
to molecular size. Table 1.5 adapted from Kadam et al. (2015) reviews the various approaches used in the extraction of laminarin. Laminarin can be extracted readily in water with the use of high temperatures. A study by Zha et al. (2012) examined the influence of temperature of the extraction of laminarin. They found that the extraction yield increased with temperature until it reached 60 °C, beyond this point a negative effect was observed on yield. This was due to degradation of the polysaccharide into low molecular weight carbohydrates at high temperatures. Most methods use mild acid with the use of heat for extraction, followed by precipitation with ethanol. Sulphuric acid and hydrochloric acid are the most commonly used acids in the extraction of laminarin at a range between 0.09-0.1 M. Deville et al. (2004) examined four extraction conditions on laminarin. They determined laminarin content by measuring the amount of reducing sugars released after enzymatic hydrolysis. Their results indicated a yield decrease in the following sequence; hot HCl extract > cold HCl extract > Hot H₂SO₄ extract > cold H₂SO₃ extract (Deville et al., 2004). Rioux et al. (2010) extracted laminarin and fucoidan from species Saccharina longicruris using a 1 % (w/v) CaCl₂ solution at 85 °C for 4 h.

The most commonly used method of extraction of fucoidan (Black et al., 1952) involves the use of dilute acid at ambient or slightly elevated temperatures. Table 1.5 summarises the development of fucoidan extraction over the years and the influence on the composition between species and extraction technique. Hemmingson et al. (2006) extracted fucoidan from the species Undaria pinnatifida with 1 % H₂SO₄ at room temperature for 6 h and neutralised with 10 % NaOH. Cumashi et al. (2007) pre-treated the algal biomass to prior to extraction with MeOH-CHCl₃-H₂O (4:2:1) to remove lipid components. They extracted fucoidan using 2 % CaCl₂ for 5 h at 85 °C. The use of acid or base and the differences in extraction time and temperature during extraction and
purification of fucoidan have been shown to generate diverse compositions and structures of the compound (Table 1.6).
Table 1.5 Reported methods for extraction of laminarin\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Seaweed</th>
<th>Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminaria saccharina</td>
<td>Extracted with 0.09 M HCl at 4 °C for 2 h, after four successive extractions supernatant was precipitated by addition of absolute ethanol</td>
<td>Black et al. (1951)</td>
</tr>
<tr>
<td>Laminaria saccharina</td>
<td>Extracted with 0.09 M HCl at 4 °C for 2 h, after four successive extractions supernatant was neutralised with 1 M NaOH</td>
<td>Black et al. (1951)</td>
</tr>
<tr>
<td>Sargassum linifolium</td>
<td>Extracted with HCl or oxalic acid solutions, neutralised with saturated sodium carbonate solution</td>
<td>Abdel-Fattah &amp; Hussein (1973)</td>
</tr>
<tr>
<td>Laminaria saccharina, Laminaria digitata</td>
<td>Extracted with 0.3% H\textsubscript{2}SO\textsubscript{4} at 50 °C for 1 h</td>
<td>Voronova et al. (1991)</td>
</tr>
<tr>
<td>L. saccharina</td>
<td>Extracted with 0.09 M H\textsubscript{2}SO\textsubscript{4} (1:14 w/v) at 70 °C for 2.5 h and precipitation by absolute ethanol</td>
<td>Yvin et al. (1999)</td>
</tr>
<tr>
<td>L. saccharina</td>
<td>Extracted with 0.09 M HCl (1:14 w/v) at 70 °C for 2.5 h and precipitation by absolute ethanol</td>
<td>Deville et al. (2004)</td>
</tr>
<tr>
<td>Saccharina longicruris</td>
<td>Extracted with 1% CaCl\textsubscript{2} 85 °C for 4 h, further filtrate mixed with 2% NaCl and 95% ethanol at 1:2 ratio and dialysis using a 15 kDa cut-off membrane for 48 h</td>
<td>Rioux et al. (2010)</td>
</tr>
<tr>
<td>Laminaria sp.</td>
<td>Pretreated with ethanol, extraction with water at 50 °C for 2 h and precipitation using ethanol</td>
<td>Cheng et al. (2011)</td>
</tr>
<tr>
<td>Laminaria japonica</td>
<td>Extracted with dried seaweed to water ratio of 1:50 at 60 °C for 1 h</td>
<td>Zha et al. (2012)</td>
</tr>
<tr>
<td>Eisenia bicyclis</td>
<td>Defatted algal fronds extracted twice with 0.1 M HCl at 60 °C for 2 h and precipitated with four volumes of 96% ethanol</td>
<td>Ermakova et al. (2013)</td>
</tr>
<tr>
<td>Sargassum fusiforme</td>
<td>Extracted twice with 0.1 N HCl at room temperature for 2 h</td>
<td>Jin et al. (2014)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}taken from; Kadam et al. (2015)
Table 1.6 The development of fucoidan extraction methodology over the years (1913-1950’s) and its influences on fucoidan polysaccharide composition from different brown seaweed species.

<table>
<thead>
<tr>
<th>Year</th>
<th>Brown Seaweed Species</th>
<th>Reported FCSP’S Composition</th>
<th>Extraction method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1913</td>
<td><em>Laminaria and Fucus</em></td>
<td>Fucoidan contaminated with mannitol, alginate and laminaran</td>
<td>Dilute acetic acid extraction</td>
<td>Kylin (1913)</td>
</tr>
<tr>
<td>1915</td>
<td><em>Laminaria digitata</em></td>
<td>Fucoidan contained methylpentose, L-fucose, and some pentoses</td>
<td>Dilute acetic acid extraction</td>
<td>Kylin (1913)</td>
</tr>
<tr>
<td>1915</td>
<td><em>Macrocystis pyrifera</em></td>
<td>Dominantly alginic acid, with fucose-sulfate</td>
<td>Seaweed was soaked in 2% Na$_2$CO$_3$ for 24 h, filtered, HCl was added, the precipitate filtered, and then resolved in 2% Na$_2$CO$_3$</td>
<td>Hoagland and Lieb (1915)</td>
</tr>
<tr>
<td>1931</td>
<td><em>Laminaria digitata</em></td>
<td>Substantial amounts of calcium sulfate and uronic acid</td>
<td>Soaking of seaweed in water, precipitation of crude sulfate by ethanol</td>
<td>Bird and Haas (1931)</td>
</tr>
<tr>
<td>1931</td>
<td><em>Macrocystis pyrifera</em></td>
<td>Methylpentose monosulphate polymer with mainly fucose and alginate contaminants</td>
<td>Repeated extraction with 2% HCl at room temperature for 48 h, precipitated with 90% EtOH</td>
<td>Nelson and Cretcher (1931)</td>
</tr>
<tr>
<td>1937</td>
<td><em>Laminaria digitata</em></td>
<td>Sulfate residue must be substituted by fucose or another sugar residue</td>
<td>Prepared by precipitating the droplets exuded from seaweed in boiling ethanol</td>
<td>Lunde <em>et al.</em> (1937)</td>
</tr>
<tr>
<td>1950</td>
<td><em>Fucus vesiculosus, Fucus spirales, Himanthalia lorea, Laminaria clustoni</em></td>
<td>Substantial amount of fucose and sulfate; and small amounts of uronic acid, galactose and xylose; ash was mainly calcium sulfate</td>
<td>Acid pH 2–2.5, 70 °C for 1 h, 3-times; or aqueous at 100 °C for 24 h, lead acetate treatment, barium hydroxide addition</td>
<td>Percival and Ross (1950)</td>
</tr>
<tr>
<td>1952</td>
<td><em>Fucus vesiculosus</em></td>
<td>Fucose, ash, sulfate</td>
<td>pH 2–2.5, 70 °C for 1 h, 3-times</td>
<td>Black <em>et al.</em> (1952)</td>
</tr>
</tbody>
</table>

*taken from; Ale and Meyer, (2013)*
1.5.2 Pressurised liquid extraction (PLE)

Pressurised liquid extraction (PLE) sometimes referred to as accelerated solvent extraction (ASE©) or Pressurized Solvent Extraction (PSE) is an extraction technique used for the extraction of compounds from both solid and semi-solid sample matrices. It uses common solvents at elevated temperature and pressures. The use of high temperatures improves the extraction kinetics, while the high pressure prevents the solvent from boiling. PLE is becoming increasing popular as an extraction technique due to the fact that it uses shorter extraction times, has a low solvent use, higher extraction yields and it is a completely automated system (Huie, 2002). Extractions that would usually take hours can now be done in a matter of minutes using PLE (Mustafa and Turner, 2011). The many steps involved in extraction can now be automated with the use of PLE flow through technology, however, sample preparation is still labour intensive. Solid samples can be easily filtered and cleaned as part of the solvent extraction process in a single step.

![Figure 1.11 Schematic diagram of pressurised liquid extraction (taken from; Sticher, 2008)](image)

For a successful extraction, the solvent being utilised must have the ability to solubilise the analytes of interest, while leaving the sample matrix intact. The extraction solvent should be of a similar
polarity to that of the target compound. It is possible to extract a wide range of compounds by mixing solvents of different polarities. If a solvent extracts well using conventional extraction then it will generally work well in PLE also. Temperature is the main variable parameter that is used in PLE. The temperature and viscosity are inversely proportional, as the temperature is increased the viscosity of the solvent is reduced. Temperature increases the solvents ability to wet the sample and to solubilise the compounds targeted for extraction. Pressure also plays a vital role in the PLE system. Pressure operates to keep the solvent in liquid state while above their atmospheric boiling points, and also helps to move fluids through the system rapidly. Changing the pressure in the system has little impact on analyte recovery and thus is not considered a critical experimental parameter. Most PLE extractions are performed under pressures of 1000 psi (7 MPa) and 2000 psi (14 MPa), with 1500 psi (10 MPa) being the standard operating pressure. A PLE extraction procedure is as follows: (i) the sample in powered form is loaded into the cell; (ii) the cell chamber is filled with the extraction solvent and the pressure is increased; (iii) the initial heat-up step is applied; (iv) a static extraction step with all system values closed is performed; (v) the cell is rinsed (with 60 % cell volume using extraction solvent) to ensure complete extraction; (vi) the solvent is purged from the cell using N₂ gas and (vii) the cell is depressurized (Herrero et al., 2009). Between extractions a rinse of the complete system using extraction solvent is employed to avoid any extract carry over.

PLE has been used to optimise the extraction of antioxidants from certain species of microalgae, e.g., *Spirulina platensis* and *Haematococcus pluvialis* (Tanaka et al., 2004; Herrero et al., 2005; Jaime et al., 2010) for use in functional foods to provide physiological benefits for human health. PLE has been shown to provide extracts with good antioxidant properties, however, PLE does not
show comparable extraction yields to that of conventional extraction (Tierney et al., 2013a; Denery et al., 2004).

1.5.3 Supercritical fluid extraction (SFE)

A supercritical fluid can be defined as any substance at a temperature and pressure above its critical point (Sapkale et al., 2010). Supercritical fluids have the capacity to diffuse through solids, like a gas and dissolve substances like a liquid. Generally the extraction is performed on a solid sample matrix, but it can also be used on liquid samples. SFE offers many uses such as sample preparation for analytical purposes or large scale for the removal of unwanted substances from a product, e.g., decaffeination, or to collect a desired product, e.g., essential oils (Sapkale et al., 2010). SFE is currently applied in the commercial industry for the production of flavourings, cosmetics, and pharmaceutical and food products. An example of a typical; SFE system can be seen in Figure 1.12. Some examples of its use are decaffeinating coffee, hop extraction, extraction of turmeric essential oils and ginger flavouring. SFE offers many advantages over other extraction techniques like solvent extraction such as; (i) Environmental improvement and reduced product contamination, SFE offers an alternative to liquid extractions involving solvents such as hexane and dichloromethane. During liquid extractions there is often residual solvent left in the extract and matrix, and also the use of these such solvents causes environmental contamination due to their disposal. Carbon dioxide can be easily removed from the sample by simply reducing the pressure, leaving almost no trace in the extractant or the matrix. The use of SFE and CO₂ is widely accepted for the extraction of organic compounds in the food industry and is FDA approved (Rozzi and Singh, 2010). (ii) Speed of extraction, the extraction process is based on diffusion, where solvent is required to diffuse into the matrix, and the material of interest must diffuse out of the matrix into the solvent. Diffusion is much more rapid in supercritical fluids than in other liquids.
which means extraction can occur more rapidly. Reduced surface tension and viscosities are much lower than in solvents therefore the solvent can diffuse into small pores within the matrix, which would normally be inaccessible to liquids at ambient pressures. (iii) Selectivity of extraction. Temperature and pressure are critical variable parameters in the supercritical fluid extraction process. The nature of the supercritical fluid can be altered by simply varying these parameters which allows for selective extraction, e.g., volatile oils can be extracted from a plants using low pressures (100 bar), whereas liquid extraction would remove lipids. Lipids can be removed from samples using pure CO$_2$ at higher pressures, and phospholipids can be removed by the addition of small amounts of ethanol to the solvent (Lang and Wei, 2001).

Carbon dioxide is the main supercritical fluid used in SFE as it has many desirable properties such as being non-corrosive, non-toxic, non-flammable and non-explosive. It is also chemically stable. It is easily removed from the product and eliminates any problem related to toxic solvent residue. It is readily available and is inexpensive to buy. It has a low critical temperature and pressure (Tc= 31.1°C, Pc= 7.4 MPa) and can also be utilised in an energy saving process by recycling CO$_2$. CO$_2$ is not always suitable for extraction, and is a poor solvent for polar compounds. For some applications a small volume of a polar co-solvent/modifier suffices to improve the extraction of targeted components from a natural product matrix, such as water, ethanol, methanol, acetic acid, and ethylene glycol (Sovilj et al., 2010).
Figure 1.12 Schematic diagram of supercritical fluid extraction (taken from; Sticher, 2008).

SFE has previously been reported to be an efficient technique for the extraction of compounds from seaweed with bioactive potential such as carotenoids, pigments and lipids. Careri et al. (2001) and Macías-Sánchez et al. (2005) used SFE to extract carotenoids and pigments from microalgae. Macías-Sánchez et al. (2007) successfully extracted pigments from the microalgae Nannochloropisis gaditana. They found that higher pressures and temperatures produced a higher yield. This was also seen in the study by Mendes et al. (2003) where they investigated the effects of a range of temperatures and pressures on the pigment yield from the microalgae Chlorella vulgaris. Roh et al. (2008) successfully extracted fucoxanthin from the Undaria pinnatifida using SFE-CO₂, again higher temperature and pressure produced the best yield of extract. In addition to extraction of pigments and carotenoids SFE has also proven to be successful for the extraction of other valuable compounds. Choi et al. (1987) reported the use of SFE for the extraction and characterisation of lipids from Scenedesmus obliquus.
1.6 Fractionation and purification of bioactive compounds

Organic compounds are isolated from natural sources or from reaction mixtures. Generally these compounds are not in a pure form and are usually contaminated with other similar compounds which are have similar properties and can exist together in a mixture. For characterization of these compounds it is necessary to purify them. Purification of compounds is of great importance in chemistry and in the development of a functional food ingredient. Separations can be achieved using many techniques. Physical characteristics such as differences in boiling point or by chemical means, wherein differences in physical properties are enhanced by chemical reactions. Techniques readily used for the fractionation and purification of complex mixtures are dialysis, crystallization, sublimation, distillation, solid-phase extraction and chromatography. When applying a technique for a target compound it is important to keep in mind limitations such as the purity required and achievable by that method, the cost of the technique, the ease of scaling it up and the food friendliness of the technique if the final product is to be incorporated into a food (Sarker et al., 2005; Roberts, 1977). These techniques can prove useful for bioactivity guided fractionation to determine what fraction of the sample contains the active component (Figure 1.13).
Figure 1.13 Schematic diagram of typical procedures for bioactivity guided fractionation and purification of bioactive compounds.

1.6.1 Dialysis

Dialysis is a technique used for fractionation. It involves the transfer of a solute across a semi-permeable membrane. Dialysis is often used to separate components of a solution based on molecular weight. Dialysis is performed by placing a liquid solution within a semi-permeable membrane tubing of specific pore size. This is then submerged in a reservoir of the same fluid as the solution. The particles that are less than the molecular weight of the membrane will diffuse across into the reservoir solution. Particles that are greater than this molecular weight are retained within the membrane. By continuously or periodically refreshing the surrounding solution with
fresh solution generally all the smaller molecular weight particles will be transferred through the membrane (Thermo Scientific, 2015). Use of molecular weight cut-off (MWCO) dialysis has shown to successfully generate fractions of low molecular weight (< 3.5 kDa) and of high (3-100 kDa and > 100 kDa) from cold water, hot water and aqueous ethanolic solid-liquid extracts from brown macroalgae species (Tierney et al., 2013b).

### 1.6.2 Flash Chromatography

Flash chromatography, also known as medium pressure chromatography is different from the conventional technique in two ways, it uses a smaller silica gel particle size (250-400 mesh) and it is operated under pressurized gas (ca. 10-15psi) which is used to pump the solvent through the stationary phase which has restricted flow of solvent due to small gel particles. This results in a more rapid chromatographic process (Ayare et al., 2014). Flash chromatography has previously been shown to be a successful technique for the fractionation of macroalgal extracts from the brown macroalgae *Pelvetia canaliculata*, *Ascophyllum nodosum* and *Fucus spiralis* to produce phlorotannin enriched fractions (Tierney et al., 2013b). Flash chromatography has also previously been applied to generate fucoxanthin rich fractions from brown macroalgae (Urikura et al., 2011).

### 1.6.3 Solid-phase extraction (SPE)

Solid phase extraction is a favoured technique used in sample preparation, it is a rapid, inexpensive and selective method. The separation process involves dissolving compounds in a liquid mixture and the compounds are partitioned from other components in the mixture based on their physical and chemical properties. SPE can be used in concentration and purification of samples for analysis. It can be applied to a variety of matrices such as blood, urine, soil, beverages and animal tissues (Simpson, 2000; Žwir-Ferenc and Biziuk, 2006).
There are many stationary phases available (packed syringe-shaped cartridges, 96 well plates, a 47- or 90-mm flat disk). These SPE systems allow for multiple samples to be processed at once, they can hold several SPE cartridges in place which allows for a number of samples to pass through simultaneously. A vacuum can be applied to the system to speed up the process by extracting the liquid sample through the stationary phase. The analytes (target compounds) are collected in sample reservoirs below the cartridges. SPE cartridges and disks are available with a variety of stationary phases, which can separate analytes according to different chemical properties. SPE stationary phases are based on silica with a specific functional group bonded to it such as hydrocarbon chains of variable length (reverse phase SPE), quaternary ammonium or amino groups (anion exchange) and sulfonic acid or carboxyl groups (cation exchange) (Żwir-Ferenc and Biziuk, 2006). Solid phase extraction has previously been performed on flash chromatography fractions of phlorotannin enriched extracts using normal phase cartridges to provide a purified sample for UPLC-MS analysis (Tierney et al., 2013b).

![Solid-phase extraction](image)

**Figure 1.14** Solid-phase extraction
1.7 Measurement of in-vitro antioxidant activity

Reactive oxygen species (ROS) like superoxide anion (O$_2^-$), hydroxyl (-OH), peroxyl (ROO$^-$), and alkoxy radicals (RO$^-$), hydrogen peroxide (H$_2$O$_2$), and singlet oxygen (O$_2^1\Delta g$) can cause damage to proteins, lipids and DNA within the body which results in cell aging and diseases related to oxidative stress (e.g., cardiovascular and neurodegenerative diseases) and cancer (Repetto, 2012).

An antioxidant can be defined as “any substance that when present in relatively low concentrations, compared with those of the oxidisable substrate significantly delays or inhibits oxidation of that substrate” (Gutteridge and Halliwell, 1990). Antioxidants can be divided into two classes, chain breaking antioxidants or preventative antioxidants.

Chain breaking mechanism;

(Initiation) $L\cdot + AH \rightarrow LH + A\cdot$

(Propagation) $LO\cdot + AH \rightarrow LOH + A\cdot$

(Termination) $LOO\cdot + AH \rightarrow LOOH + A\cdot$

Radical initiation (reacting with a lipid radical) or propagation (reacting with peroxyl or alkoxy radicals) steps are inhibited. Preventative antioxidants operate by delaying the rate of oxidation e.g., transition-metal ion chelators may inhibit Fenton-type reactions that produce hydroxyl radicals therefore blocking the formation of a hydroxy radical. This hydroxy radical has the ability to initiate a chain reaction as illustrated below.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdotOH + OH^-$
It is often difficult to separate and quantify antioxidants in food/biological matrices due to their chemical diversity. Generally their combined effect is measured as the total antioxidant capacity of activity, it cannot be measure directly but can be indirectly measured by the effects of the antioxidant in controlling the extent of oxidation (Apak et al., 2013). There are a range of in-vitro antioxidant capacity methods using different substrates and mechanisms of action have been used (Heinonen et al., 1998). When determining antioxidant activity, the source of reactive oxygen species and the target substrate must be considered. Auroma et al. (1997) used several measures of antioxidant activity for evaluating antioxidant efficacy. The methods most commonly used to determine the total in vitro antioxidant capacity fall into two major groups, assays involving biological substrate and assays without a biological substrate. Assays without biological substrate can be further classified based on the type of reaction;

(i) hydrogen atom transfer (HAT)-based assays.

HAT-based assays are used to determine the ability of an antioxidant to stop free radicals by H-atom donation. The HAT mechanism of an antioxidant in which the hydrogen atom (H) of a phenol (ArOH) is transferred to a ROO· radical.

\[
\text{ROO}^\cdot + \text{AH/ArOH} \rightarrow \text{ROOH} + \text{A}^\cdot/\text{ArO}^\cdot
\]

Some HAT-based assays include; oxygen radical absorbance capacity (ORAC) assay, 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995; Bondet et al., 1997),
TRAP assay using R-phycoerythrin as a fluorescent probe, and the crocin bleaching assay using 2, 2’-azobis (2-amidinopropane) hydrochloride (AAPH).

(ii) electron transfer (ET)-based assays.

In ET-based assays the antioxidant activity is replicated with a suitable redox-potential probe that reacts with the fluorescent or coloured probe (oxidising agent) instead of peroxyl radicals. ET-based spectrophotometric assays measure the ability of an antioxidant in the reduction of an oxidant, which produces a colour change when reduced. The degree of colour change corresponds to the concentration of antioxidants in the sample. The 2, 2’-Azinobis-(3-ethylbenzothiazaline-6-sulfonic acid) (ABTS)/Trolox-equivalent antioxidant capacity (TEAC) (Sanchez-Moreno et al., 1998) is a decolourisation assay while the Folin-Ciocalteu total phenol (Singleton et al., 1999) and ferric reducing antioxidant power (FRAP) assays (Straitil, 1999; Benzie and Strain, 1996; Benzie and Szeto, 1999) give an increase in absorbance at a pre-specified wavelength.

1.7.1 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay is a rapid, simple and inexpensive in-vitro assay used to measure the antioxidant capacity of an extract. It involves the use of the free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH). DPPH which is used to assess the ability of compounds to act as free radical scavengers/hydrogen donors and to determine the antioxidant activity of foodstuffs. It is also commonly used to quantify antioxidant content in complex biological systems. The DPPH method is suitable for both solid and liquid samples. The method is not specific to any antioxidant compounds in particular but just relates to an overall antioxidant capacity but applies to the overall antioxidant capacity (Kedare and Singh, 2011).
This assay works by DPPH· accepting hydrogen from an antioxidant. The colour turns from purple to yellow following the formation of DPPH· upon absorption of hydrogen from an antioxidant. DPPH· shows a strong absorption maximum at 517 nm. The reaction is stoichiometric with respect to the number of hydrogen atoms absorbed, therefore, the antioxidant activity can be determined by following the decrease of UV absorption at 517 nm. Trolox is generally used as a standard and results are expressed as Trolox equivalents (TE). The antioxidant activities of many seaweeds has been assessed using the DPPH radical scavenging assay (Tierney et al., 2013a; O’ Sullivan et al., 2011; Ismail and Hong, 2002; Jiménez-Escrig et al., 2001; Matsukawa et al., 1997).

1.7.2 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is based on the reaction with electron-donating or hydrogen radicals (H·) producing compounds/antioxidants according to the reaction R· + Aox-H → RH + Aox-. Electron and hydrogen atom transfer reactions can often be difficult to differentiate. Hydrogen atom transfer reactions can be the result of proton-coupled electron transfer (Chanda and Dave, 2009).

The FRAP method is based on the ability of an antioxidant to reduce (electron transfer) Fe³⁺ to Fe²⁺ ions. When this occurs in the presence of TPTZ (2, 4, 6-Tri(2-pyridyl)-s-triazine), the reduction is accompanied by the formation of a Fe²⁺ - TPTZ complex which is blue in colour. This has an absorption maximum at 593 nm. The intensity of light absorption at that wavelength is proportional to the antioxidant activity. Standard curves are created using FeSO₄ and Trolox and the radical scavenging level in the samples are determined from this and are expressed as either Trolox equivalents or FeSO₄ equivalents. The antioxidant potential of common seaweeds has previously been assessed using the FRAP assay (Tierney et al., 2013a; O’ Sullivan et al., 2011; Rajauria, 2011; Matanjun et al., 2008; Rupérez et al., 2002).
1.7.3 Food compositional bioassays

1.7.3.1 Total phenolic content (TPC)

Phenolic antioxidants, a specific group of secondary metabolites, play a major part in protecting organisms against the harmful effects of oxygen radicals and other highly reactive oxygen species. TPC is determined based on chemical reduction of the Folin-Ciocalteu reagent. This reagent is a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction are blue in colour. This has an absorption maximum at 765 nm. The intensity of light absorption at the specific wavelength is proportional to the concentration of phenols in the sample (Singleton et al., 1999). A calibration curve is created using the standard gallic acid and the levels of phenols in the sample are determined from this and expressed as gallic acid equivalents (GAE). The total phenolic content has previously been determined using the Folin-Ciocalteu method (Tierney et al., 2013a; Keyrouz et al., 2011; Wang et al., 2009).
1.8 Compositional determination of algal biomass

1.8.1 Protein determination

Protein determination is very important and commonly practiced in general biochemistry research and routine laboratory practices (Olutucu et al., 2007). There has been an increase in the number of methods used for determination of protein in the last 20 years which has resulted in increased accuracy and sensitivity.

A number of colorimetric tests to measure the protein concentration are available based on UV-visible spectroscopy (Biuret, Lowry and Bradford methods). The basic principle in all of these are similar, the major difference in these tests are the chemical groups which are absorbing or scattering radiation such as the peptide bonds, aromatic side groups, basic groups and aggregated proteins. These methods are relatively simple to carry out and are quiet sensitive at determining protein levels as low as 0.001 wt % (McClements, 2003), however extensive sample preparation is required.

Other methods such as the Kjeldahl and Dumas which are officially recognised methods determine the protein content based on nitrogen present in the sample. The Kjeldahl method involves digestion of the sample in sulfuric acid with a catalyst. This results in the conversion of nitrogen to ammonia. Distillation of the ammonia into a trapping solution is then carried out and then a titration step to quantify the ammonia content against a standard solution. Due to the fact that the Kjeldahl method does not measure the protein content directly and conversion factor is used to convert the nitrogen content to protein content (Jung et al., 2003). This technique is labour intensive and generally takes upto 2 hours to complete the digestion and titration. The dumas method involves the sample to be well homogenised. The samples are heated in a furnace where combustion takes place rapidly at over 1000 °C in the presence of pure oxygen. This produces a
gaseous mixture containing CO$_2$, nitrogen, water and several oxides. This gaseous mixture is passed through a reduction chamber containing copper heated to 650 °C. This converts the nitrogen oxides into elemental nitrogen and collect the excess oxygen. The total nitrogen is measured by a thermal conductivity detector (Jung et al., 2003). The modern dumas system is fully automated and has the ability to measure the protein content of a sample rapidly (< 5 min). In general the Kjeldahl and Dumas methods are the commonly used techniques for determination of protein content. Shipgel et al. (1999) used the Kjeldahl method to determine the protein content in the seaweed *Ulva lactuca* L. While Lourenco et al. (2002) used the Dumas method to determine the protein content in 19 tropical seaweeds.

### 1.8.2 Carbohydrate determination

Carbohydrates are one of the most fundamental components that make up food and natural products. Carbohydrates can be single isolated compounds or they can be physically or chemically bound to other molecules. They can be classified based on the number of monomers they contain (monosaccharides, oligosaccharides or polysaccharides). Carbohydrate content can be determined using a range of techniques both analytical (Chromatographic and electrophoretic) and chemical (titrations, colorimetric assay or gravimetrically) or can be determined by calculating the percent remaining after all the other components have been determined: % carbohydrate = 100 - % moisture - % protein - % lipid - % mineral (McClements, 2003).

Chromatographic techniques such as thin layer chromatography (TLC), Gas chromatography (GC) and High performance liquid chromatography (HPLC) are very commonly used for the determination of the type and concentration of monosaccharides and oligosaccharides in a sample. Carbohydrates can be separated based on their partition coefficients, polarities and size.
Carbohydrates can also be separated using electrophoresis. They are required to be derivatized first to make them electrically charged. They are then applied to a gel and a voltage is applied across it. The carbohydrates are separated according to their size, smaller molecules move faster in an electrical field.

A number of chemical methods can also be used to determine monosaccharide and oligosaccharide. This is based on the fact that they are reducing agents and react with other components to form precipitates and coloured complexes which can be quantified gravimetrically (Munson and Walker method), spectrophotometrically (Dubois-Phenol-Sulfuric method) or by titration (Lane-Eynon method) (McClements, 2003). Manivannan et al. (2008) determined the total carbohydrate content of seaweeds from Mandapam Coastal region using the Dubois phenol-sulfuric method. Rupérez et al. (2002) investigated sulphated polysaccharides from the brown algae *Fucus vesiculosus* and used molecular exclusion high performance liquid chromatography to determine the average molecular weight of polysaccharide fractions and then \(^1\text{H}\) for structural confirmation. Rioux et al. (2007) used high performance size exclusion chromatography-multiangle laser light scattering (HPSEC-MALLS) to determine average molecular weight and high performance anion exchange chromatography (HPAEC) for identification of polysaccharides isolated from the brown macroalgae *Ascophyllum nodosum, Fucus vesiculosus* and *Saccharina longicruris*.

**1.8.3 Ash and mineral determination**

Ash is the residue remaining once all the water and organic matter have been removed. This can be achieved by heating the sample in the presence of oxidizing agents, which leaves a measure of the total amount of minerals in a sample. Commonly used techniques are based on the principle
that minerals have a low volatility and are not affected by high temperatures. Dry ashing, wet ashing and low temperature plasma dry ashing operate based on this principle. Dry ashing involves the uses of high temperatures such as those that can be achieved using a muffle furnace (500 and 600 °C). Water and volatile compounds are vaporized off and organic matter is burned in the presence of the oxygen. Oxides, sulfates, phosphates, chlorides or silicates remain. The sample is weighed before and after ashing to determine the percentage of ash. The dry ashing procedure is a simple technique, it is low cost, is not labour intensive and it allows many samples to be analysed simultaneously. However, it is time consuming and some volatile mineral components may be lost at the high temperatures. Wet ashing is a quicker technique and causes less loss of volatile minerals due to the lower temperatures applied (McClements, 2003).

Specific minerals can be determined using gravimetric methods, colorimetric methods and titrations. More commonly atomic spectroscopy is used to determine the mineral type and concentration. It is more sensitive, specific, and rapid method than traditional chemical methods. Atomic absorption spectroscopy (AAS) is based on the principle that atoms absorb radiation at particular wavelength. Samples to be analyzed needs to be ashed prior to analysis and then resuspended in an aqueous solution. This solution is then applied to the AAS where it is heated to vaporize and atomize the minerals. A beam of radiation is passed through the atomized sample. The absorption of radiation is measured at a specific wavelength which corresponds to specific minerals. Rupérez (2002) determined the mineral content of several edible seaweed brown (Fucus vesiculosus, Laminaria digitata and Undarai pinnatifida) and red (Chondrus crispus and Porphyra tenera) using the dry ashing method followed by mineral determination using atomic absorption spectrometry.
1.8.4 Lipid determination

Lipids are major constituents of food products and many natural products. They are soluble in organic solvents (ether, hexane and chloroform) but are insoluble in water. Lipid compounds can consist of tri-, di- and monoacylglycerols, free fatty acids, phospholipids, sterols and carotenoids. Triacylglycerols are esters of glycerol and three fatty acid molecules, however, the fatty acids can have varying chain lengths, branching, unsaturation and positions on the glycerol molecule. There are a number chemical tests that can be used to provide information about the type of lipids present in a sample which provide details of the lipid components present, such as the average molecular weight, degree of unsaturation or amount of acids present. The iodine content (IV) gives the measure of the average degree of unsaturation in a lipid. The higher the iodine content the more C=C double bonds present. The saponification test measures the average molecular weight of triacylglycerols in a sample and the acid value is an indication of the amount of free acids present in sample. These tests are relatively simple to perform and are inexpensive. They are widely used in industry and research. Chromatographic techniques (Thin layer chromatography (TLC), Gas chromatography (GC) and high pressure liquid chromatography (HPLC)) are more commonly used to analyze lipids components.

Sánchez-Machado et al. (2004) determined the fatty acids and total lipid content of edible seaweeds using gas chromatography. Gressler et al. (2010) determined the total lipid content of two red seaweeds from the Brazilian coast using the acid hydrolysis method and then gravimetric analysis, they then determined the fatty acid composition of the extracts using gas chromatography mass spectrometry (GC-MS).
1.8.5 Moisture determination

There are a number of ways to determine the moisture content. Evaporation methods using vacuum ovens, microwave ovens, infrared lamps and convection and forced draft ovens. Evaporation methods are most commonly used due to the simplicity and inexpense of the techniques, however, consideration needs to be taken to ensure these methods suit the sample type as decomposition and volatilization of sample components can occur at these drying tempertures. Another method used is distillation, which is a direct measurement of the amount of water removed from a sample, unlike vaporation which is an indirect measurement. Distillation involves boiling the sample in an inorganic solvent that is immiscible with water. The water evaporates into a graduated glass chamber where its mass is calculated. This method is time consuming, involves the use of flammable solvent and is only suitable for samples with low moisture and volatile components. A number of chemical methods can also be used to determine the moisture in samples. Reagents that react specifically with water to produce a measureable change (e.g., mass, volume, pressure, pH, colour, conductivity). These changes can be correlated to the moisture content. Two commonly used chemical methods are the Karl-Fisher titration and gas production methods.

Moisture analysis has been carried out on seaweed using a variety of methods, Wong et al. (2000) determined the moisture content of some sub-tropical seaweeds using an infrared moisture analyser at 120 °C, while Rupérez (2002) oven dried several brown seaweeds at 105 °C to determine the moisture content before mineral analysis. Marinho-Soriano et al. (2006) also used the oven drying method at 105 °C for moisture determination in two tropical seaweeds.
1.9 Structural elucidation and analysis of bioactive compounds from macroalgae

Structural elucidation of compounds plays an important role in modern life sciences and bio-analytical approaches. It is required in order to link biological activity to a specific compound or type of compound and also provides essential information on the dose required to produce a biological effect. Structural elucidation of bioactive compounds is now required in European Food Safety Authority (EFSA) claims in order to produce a functional ingredient. In marine algae most work on antioxidant compounds (phlorotannins and carotenoids) to date have concentrated on crude extracts. Few examples of the structural determination of individual phlorotannin compounds can be found to date due to their very complex nature. The polymeric nature of phlorotannins allows for the formation of a large number of isomers which are difficult to separate chromatographically and have thus far confounded the ability of researchers to structurally elucidate them. Steevenez et al. (2012) used liquid chromatography high resolution mass spectrometry (LC-MS) for the profiling of low molecular weight phlorotannins, however due to the lack of sensitivity of this method a full phlorotannin profile for these species of brown macroalgae was not successfully achieved. However, with the disposal of chromatography and mass spectrometry techniques a more thorough study of the isomeric complexity of phlorotannins is now attainable. Ultra-performance liquid chromatography (UPLC) with quadrupole tandem mass spectrometry combines the enhanced resolution power of diminished particle size UPLC columns (< 2 µm) with the increased sensitivity and greater scanning speeds of quadrupole mass spectrometry. UPLC-MS has previously been successfully used for the analysis of phlorotannins from the brown macroalgae species (*Fucus vesiculosus*, *Ascophyllum nodosum* and *Pelvetia canaliculata*) (Tierney et al., 2014). The most commonly used techniques for the structural determination of carotenoids and pigments are by chromatographic (HPLC, TLC) and
spectroscopic (Visible, EIMS, FABMS, FABMSMS, 2D $^1$H NMR) techniques. The carotenoid fucoxanthin was structurally elucidated by 13C NMR spectroscopy from the common edible seaweed *Hijikia fusiformis* (Yan *et al.*, 1999).

1.9.1 **HPLC analysis of antioxidant compounds**

An alternative method for determining antioxidant compounds is by application of high performance liquid chromatography (HPLC). HPLC is a chromatographic technique that is used to separate components in a mixture and to identify and quantify each component (Wang *et al.*, 2000; Edenharder *et al.*, 2001). It works by pumping a pressurized liquid/sample mixture through a column that is filled with a sorbent material, leading to separation of the sample components. Generally the method involves a liquid sample being passed over a solid adsorbent material packed into a column using a flow of liquid solvent. Each analyte in the sample has a certain affinity and interacts differently with the adsorbent material. This retards the flow of the analytes. If the affinity between the analyte and adsorbent is weak the analyte will elute off the column in a short amount of time. If the affinity is strong, then the elution time is longer. HPLC offers high accuracy, good reproducibility and a relative short analysis time (Kupiec, 2004). The most frequently utilized HPLC methods are based on a separation on a reverse phase (RP) column. There is no universal detector that can monitor all compounds, therefore the type of detector used is dependent on the analyte being detected. The most commonly used detectors include ultra violet (UV), visible (Vis) and photodiode array detectors (PDA).

The quantity of individual pigments can be determined with the use of HPLC and an external standard. Breithaupt (2004) demonstrated that HPLC can be used to analyse carotenoids coupled with a PDA. HPLC coupled with UV-Vis and fluorescence detectors have previously been successful in the identification of pigments from edible brown seaweed (Rodríguez-Bernaldo de
Separation of carotenoids can be carried out by using a C30 column. Carotenoids from supercritical fluid extracts of *Spirulina pacifica* algae were determined using HPLC. Separation was performed on a Spherisorb ODS2 column (150 x 4.6mm, 3μm) using an isocratic mobile phase (Careri *et al.*, 2001). RP-HPLC of phenolic compounds from *Ascophyllum nodosum* has also been successfully achieved with the use of a PDA detector (Audibert *et al.*, 2009).

### 1.9.2 UPLC-MS of antioxidant compounds

UPLC-MS/MS is an analytical technique that combines the physical separation ability of liquid chromatography with the mass analysis capabilities of a mass spectrometry. UPLC-MS/MS is a powerful technique that is utilised for many applications which require very high sensitivity and selectivity. Generally its application is aimed towards the detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). UPLC has been developed to endure much higher system back pressures than in conventional HPLC, allowing the use of columns with much smaller particle size (sub 2 μm), therefore, improving the chromatographic speed, sensitivity and resolution (Novakova *et al.*, 2006). UPLC-MS using high resolution mass spectrometry was employed to analyse phlorotannins from various species of brown algae (Steevensz *et al.*, 2012). Recent studies (Tierney *et al.*, 2014; Heffernan *et al.*, 2015) employed the use of UPLC-MS, using tandem mass spectrometry (multiple reaction monitoring (MRM) mode) to overcome the problems with chromatographic resolution of different molecular weight phlorotannins and their isomers, observed in other studies (Wang *et al.*, 2012) in conjunction with scanning speeds and sensitivity of a tandem quadrupole mass spectrometer (Tierney *et al.*, 2014).
1.9.3 Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) of algal sugars

MALDI has become a widespread analytical tool used in the MS analysis of biomolecules (lipids, carbohydrates, proteins, peptides and oligonucleotides). MALDI is a soft ionization technique which provides for the non-destructive vaporization of both large and small biomolecules. The analyte is first co-crystallized in a matrix compound, usually a UV-absorbing weak organic acid. The matrix material heavily absorbs the UV light, leading to the ablation of the upper matrix material and thus vaporization of the matrix/analyte mixture. The matrix therefore plays a key role by strongly absorbing the laser light energy and causing, indirectly, the analyte to vaporize (Lewis et al., 2000).

Previous reports have shown the efficacy of MALDI to structurally analyse sugars from macroalgae samples. Chizhov et al. (1998) reported the use of MALDI and Fast Atom Bombardment (FAB) in the structural analysis of laminarins. They determined the existence of both M-chains and G-chains (chains terminating at the reducing end by glucose or mannitol are known as G- or M- chains) in six of the eight samples analysed. Anastyuk et al. (2009) also reported the use of MALDI-TOF and tandem ESI mass spectrometry in the structural investigation of fucoidan from Fucus evanescens.
1.10 Factors affecting the antioxidant content of macroalgae

Due to the unpredictable nature of the marine environment, seaweed is subjected to a number of abiotic stresses. Marine algae contain six principal elements namely carbon, oxygen, hydrogen, nitrogen, phosphorus and sulphur (Park et al., 1997, Park et al., 2000), and many minor elements. Smaller quantities of calcium, potassium, sodium chloride, magnesium, iron and silicate are required for the metabolism of seaweed. Environmental conditions (light and temperature) and nutrient availability (nitrogen, minerals, silicate and phosphorus, etc) have an effect on the growth rate of the seaweed. Water salinity and maturity of the plant all affect the composition of seaweeds. Genetics may also play a role in the composition of the seaweed (Jormalainen et al., 2003; Jormalainen and Honkanen, 2004).

Algae utilize nitrogen in water to synthesize protein, phosphorus is needed for the production of nucleic acids and ATP for energetic functions. Reports by Barsanti and Gualtieri (2006) stated the importance of phosphorus for the growth of algae and plants. If all phosphorus is consumed, autotroph growth will cease, no matter how much nitrogen is available.

Macroalgae contain specific components not found in terrestrial plants, this is due to the severe conditions of their environment. Algae can be completely submerged in water or exposed to air and tides on the surface, sometimes the extremity of the algae floats on the water surface and is dried by sunlight and dry air. In order for algae to survive in these extreme conditions, algae contain some unique components not found in plants and these components differ according to algal species (Amer et al., 1997). For example, fucoidan is detected only in brown algae but not in red and green algae.
1.10.1 Salinity

The salinity/salt content, measured as salt in parts per thousand (ppt) of different waters varies from < 0.5 ppt salt in fresh waters to 35 ppt salt in seawaters. Carotenoids and polyphenol levels in algae have shown a positive correlation with increases in salinity (Chakraborty et al., 2010). Frazeli et al. (2006) observed that carotenoid and chlorophyll levels in *Dunaliella tertiolecta* increased in response to saline-induced stress. Connan and Stengel (2011) also observed a similar positive correlation between salinity levels and the polyphenol content in algae.

1.10.2 Temperature

The antioxidant profiles of algae can also be affected by air and water temperatures. When conditions are cold and freezing the light dependent stage of photosynthesis (Calvin cycle) which uses energy to convert CO$_2$ and water to organic compounds is reduced dramatically which results in the formation of reactive oxygen species (ROS) which cause cell damage (Yordanova and Popova, 2007). Işık et al. (2006) observed that vitamin C levels in *Spirulina platensis* increased during winter months when the temperature averaged 18.6 °C, while the vitamin E content was highest during the summer months when temperatures average 33.9 °C. They hypothesized that increases in vitamin C levels were due to a protection mechanism in the algae to tolerate the freezing conditions.

Stengel et al. (1998) quantified the level of carotenoids in the brown algae *Ascophyllum nodosum* and observed that levels increased during the colder months (Autumn-Winter) and decreased by up to 50 % in the summer period. Robledo (2005) observed similar results with levels reaching maximum during the cold seasons. Dhargalkar (2009) observed a similar trend in
seaweed harvested from the Antarctic, chlorophyll a levels increased when the cultured algae were transferred from a lower temperature range of -4 to 0°C to a higher temperature range of 0 to 20°C.

This was also the case for polyphenols in macroalgae. Ragan and Jensen (1978) found that levels of polyphenols in *Ascophyllum nodosum* and *Fucus vesiculosus* were higher during the warmer summer months and decreased significantly during the cold winter months.

### 1.10.3 Nutrient availability

There are a number of studies that suggest that antioxidant activity of macroalgae may be influenced by nutrient availability and the carbon/nutrients balance. Van Alstyne and Pelletreau (2000) hypothesized that macroalgae use carbon for growth when nutrients such as nitrogen are present in abundance, however, they also use it to produce carbon based compounds such as phlorotannins. Studies by Pavia and Brock (2000) and Arnold *et al.* (1995) support these findings. They observed a negative relationship between tissue nitrogen content and phlorotannin content of the species *Fucus vesiculosus* and *Lobophora variegata*. As nitrogen levels decreased the total phenolic content (TPC) increased but as the nitrogen levels increased the TPC levels in the macroalgae decreased. Van Alstyne and Pelletreau (2000) also found that the phlorotannin content in the embryos of *Fucus gardneri* decreased significantly when treated with phosphorus, nitrogen and iron.

### 1.10.4 UV exposure

A number of studies have highlighted the influence of UV light intensity on the phenol content of macroalgae. Solar radiation is essential for photosynthesis, however, high energy UV light cause damage to DNA and other organelles (Bischof *et al.*, 2006). Seaweeds have developed a protective mechanism against UV damage which uses cellular antioxidant systems and antioxidant
compounds such as polyphenols and carotenoids. In a study by Flodin et al. (1999) levels of bromophenols and bromoperoxidases were observed to be high in the summer months in Australia. In contrast, Chung et al. (2003) observed that level of bromophenol in the species Padina arborescens, Sargassum siliquastrum and Lobophora variegata were significantly higher in the winter months in the northern hemisphere. Other studies observed a similar trend with levels of phlorotannins and polyphenols increasing in response to UV exposure during the summer months in a range of species investigated (Abdala-Díaz et al., 2006; Pavia and Brock, 2000; Kamiya et al., 2010). Therefore, many studies concluded that the polyphenol levels were at a maximum during the summer and autumn months when UV radiation was at its highest.

UV exposure also has an impact on the carotenoid and chlorophyll levels in seaweed. Research conducted by Pinto et al. (2011) treated the seaweed Gracilaria tenusitipitata to high intensity UV light (1000 µmol photons/m²/s) and observed that the levels of several carotenoids and chlorophyll a increased. Honya et al. (1994) observed that tocopherol and β-carotene levels in Laminaria japonica were lower in the winter period and maximal in the summer period.

1.10.5 Dehydration

Dehydration is another important factor that influences the antioxidant properties of seaweed. Seaweeds in the intertidal zone are generally subjected to immersion during low tide and are exposed to high levels of dehydration, more so at times of prolonged sunlight or drought. This can lead to water losses in excess of 90 % leading to the production of ROS (Contreras-Porcia et al., 2010). Sampath-Wiley et al. (2008) suggested that increased levels of carotenoids and chlorophylls in Porphyra umbilicalis when immersed protected the seaweed from oxidative stress allowing it
to cope in the harsh marine environment. Similar trends were observed in dehydrated *Gracilaria corica* (Kumar *et al.*, 2011) where carotenoid and enzyme levels were elevated.

### 1.10.6 Maturity

Maturity of the plant also impacts on the antioxidant properties of a seaweed. Variations in phlorotannins have been reported in different tissues from the same plant (Connan *et al.*, 2006). Research by Pavia *et al.* (2003) found that the adult tissue of *Ascophyllum nodosum* contained a higher content of phlorotannins than the juvenile tissue. This is possibly due to the juvenile plant investing resources into growth rather than the production of phlorotannin compounds. Koivikko (2008) found that the phlorotannin concentrations were higher in the physodes than in the cell wall tissue of the brown algae *Fucus vesiculosus*. Maturity also affects the polysaccharide concentrations in the plant, fucoidan levels in *Undaria pinnatifida* and *Costaria costata* were higher in the mature stages of the plant in comparison to early growth periods (Skriptsova *et al.*, 2010; Imbs *et al.*, 2009).
Objective of thesis

Hypothesis: Seaweed extracts and pure compounds derived from seaweed have demonstrated antioxidant activity by scavenging ROS, which indicates that the inclusion of seaweed in the diet may be of benefit to health. Therefore, the objectives of this thesis was to investigate the *in vitro* antioxidant activity of extracts from a range of native Irish seaweed, to identify and characterise some of the compounds potentially responsible for this observed activity and to investigate the effect of season on seaweed composition on these compounds.

The objective of chapter 2 was to investigate two extraction techniques (a conventional method solid-liquid extraction and a modern technology known as pressurised liquid extraction) for the extraction of polyphenolic compounds from a range of Irish macroalgae species. The optimal extraction technique was decided based on antioxidant activity of extracts obtained and also the yield of extract obtained.

The objective of chapter 3 was to pinpoint the molecular weight region that contains these bioactive polyphenolic compounds and by generating fractions of varying molecular weight using molecular weight cut-off dialysis tubing and thus possibly enhancing the antioxidant activity of species that previously had shown poor antioxidant potential. This chapter also looked at developing a method for isolating and purifying these phlorotannins in low molecular weight fractions from the species *Fucus serratus* and subsequently profiling them using chromatographic techniques coupled with mass spectrometry.
The objective of chapter 4 was to utilise the previously developed method to investigate the profile of phlorotannins isolated from a range of brown macroalgae species using UPLC-MS/MS, and to investigate the bioactivity in relation to the profile obtained to determine the effects of isomerisation and abundance have on the activity of the phlorotannin fraction.

The objective of chapter 5 was to investigate the macro composition (protein, fat, fibre, phenolic content, ash and carbohydrate) of four macroalgae harvested from the west coast of Ireland over the period of a year and harvested from two locations. This study would determine the optimum time for harvesting for a particular compound group of interest.

The objective of chapter 6 was to compare conventional extraction (solid-liquid extraction) with a modern green method (Supercritical CO₂ extraction and Supercritical CO₂ with co-solvent) for the extraction of carotenoids in particular fucoxanthin and xanthophylls from Irish macroalgae. The seasonal variation of these compounds was also assessed to determine the influence of extrinsic factors (light intensity, temperature, nutrient availability and plant maturity) may have on the concentration of these valuable bioactive components.

The objective for chapter 7 was the use the methods from chapters 2 and 3 to extract, isolate, purify and characterise phlorotannins from seasonal samples of *Fucus serratus* to determine the effect seasonal changes may have upon the abundance and level of isomerisation, and to relate these changes to observed bioactivity.
The objective for chapter 8 was to extract laminarin a storage polysaccharide commonly found in brown macroalgae from ten samples of *Laminaria digitata* and investigate the seasonal variation in relation to content, molecular mass and number of glucose monomers.

Chapter 9 outlines the main findings of the thesis and highlight, summarizes scientific evidence arising from research undertaken.
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Chapter 2

Comparison of pressurized liquid extraction and solid-liquid extraction in the recovery of antioxidant compounds from four Irish origin macroalgae.

Based on:

Abstract

The efficiency of solid-liquid extraction (SLE) and pressurised liquid extraction (PLE) in the recovery of antioxidant and polyphenol compounds from the Irish macroalgae, *Fucus serratus*, *Laminaria digitata*, *Gracilaria gracilis* and *Codium fragile*, was assessed using the 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays and the Folin-Ciocalteu total phenol content (TPC) assay. Respectively, *Fucus serratus* had TPC and antioxidant activities thirty times higher than the other species. Solid-liquid extraction using cold water (CW\_SLE) resulted in the highest TPC (81.17 µg GAE mg\(^{-1}\) sample) derived from *F. serratus*, compared with a TPC of 61.12 µg GAE mg\(^{-1}\) sample for the corresponding PLE extract. For both SLE and PLE extracts, low TPC levels were observed in *L. digitata*, *G. gracilis* and *C. fragile*. The majority of the SLE extracts possessed higher FRAP and DPPH activities compared with their PLE counterparts. This study indicated that the high temperatures and pressures used in PLE did not improve the antioxidant activities extracted relative to conventional SLE.

2.0 Introduction

Macroalgae are an abundant and potentially renewable resource that are currently being explored as novel and sustainable sources of compounds for both pharmaceutical and nutraceutical applications (Park *et al.*, 2004; Duan *et al.*, 2006; Harnedy and FitzGerald, 2011). Ireland has a small but expanding macroalgae harvesting industry dominated largely by brown species of which only a small portion (4 %) is further processed after harvest. While there are different applications of macroalgae and their components such as their use in biopolymers, agriculture/horticulture, cosmetics, thalassotherapy and for human consumption, many species are utilised unmodified for agricultural purposes and relatively small quantities are used for secondary processing or in the cosmetics industry (Werner and Kraan, 2004). Currently, the largest amount of seaweed species
harvested in Ireland is *Ascophyllum nodosum* (Werner and Kraan, 2004), however, significant quantities of the species targeted in this study (*Laminaria digitata*, *Fucus serratus*, *Gracilaria gracilis* and *Codium fragile*) are also harvested and sustainable levels in excess of those currently targeted are likely to be available. Approximately 500 wet tonnes of *Laminaria digitata* is produced annually with 4 % used in primary processing, 10 wet tonnes in the case of *Fucus vesiculosus* and *Fucus spiralis* is produced annually with 4 % being used in primary processing. Finally, 300 wet tonnes of *Fucus serratus* is produced annually in Ireland with 100 % being used in primary processing (Nutramara, 2006).

Aside from their present uses, some of the potential commercial applications of macroalgae may arise from the relatively high levels of antioxidants present in certain species. Macroalgae are exposed to harsh conditions and harmful free radicals as a result of their intertidal habitat (Sampath-Wiley *et al.*, 2008). Thus, it is believed that they produce antioxidant species to scavenge and neutralise these radicals (Chew *et al.*, 2008). A number of potent antioxidant compounds have been isolated and identified from seaweeds, including phlorotannins (Ragan and Glombitza, 1986), sulphated polysaccharides (Rupérez *et al.*, 2002), carotenoid pigments, such as fucoxanthin (Haugan and Synn, 1994; Miyashita and Hosokawa, 2008), astaxanthin, ascorbic acid and tocopherols (Ferraces-Casais *et al.*, 2012; Lage-Yusty *et al.*, 2014), sterols, catechins and mycosporine-like amino acids (Yuan *et al.*, 2009). Brown macroalgal species contain one major class of antioxidants: phlorotannins, which are essentially oligomers of phloroglucinol (1, 3, 5-trihydroxybenzene), (Hupel *et al.*, 2011).

As a result of their condensed structure, phlorotannins possess multiple sites with the ability to scavenge free radicals, which can cause oxidative stress (Shibata *et al.*, 2002). Oxidative stress has been implicated in a wide variety of diseases, certain cancers (Reaven and Witzum, 1996), diabetes
(Wagener et al., 2009), rheumatoid arthritis (Ostrakhovitch and Afans’ev, 2001) and a number of neurodegenerative diseases (Emerit et al., 2004). In addition to their potential role in reducing the risk of some diseases, naturally derived antioxidants may find use in the control of oxidative processes, which may lead to losses in the quality of processed foods. For example, oxidation of unsaturated lipids leads to the production of rancid flavours and odours while also reducing the shelf life, nutritional quality and safety of food products (Zainol et al., 2003). Synthetic antioxidants, such as butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA), have been added to many foods to reduce oxidative deterioration. However, due to potential safety issues relating to toxicity (Witschi and Lock, 1978), consumers are beginning to show preferences for naturally derived antioxidants.

Despite their potential, there have been few examples of commercial uses of seaweed-derived phlorotannins. This is most likely because phlorotannins are difficult to extract, separate, purify and characterise due to their large size, structural similarity, polarity and reactivity with other compounds (Stern et al., 1996). Therefore, in order to realise their commercial potential in foods, these antioxidants will most likely be recovered as crude antioxidant-enriched fractions using inexpensive, environmentally and food-friendly extraction techniques. A variety of extraction methods, such as supercritical- and subcritical-fluid extraction, micro-wave-assisted extraction (MAE) and pressurised liquid extraction (PLE), have been developed in an attempt to overcome the drawbacks of conventional methods such as high solvent consumption, low extraction efficiency and selectivity and lack of automation (Huie, 2002).

PLE is a technique which involves extraction using liquid solvents at temperatures above their boiling points. The use of higher temperatures is reported to increase mass transfer and extraction rates (Huie, 2002). Generally, PLE is thought to result in reduced extraction times and higher
yields of target compound(s) while utilising significantly lower amounts of solvent (Herrero et al., 2006) compared with extraction at room temperature and atmospheric pressure (Reaven and Witzum, 1996). The use of PLE on an analytical scale has become more common, principally because of it extraction efficiency and ease of automation. Up-scaling of the technique is, however, problematic as it is expensive in terms of capital and running costs. In contrast SLE at room temperature and atmospheric pressure is relatively inexpensive and requires less state of the art equipment, knowledge and optimisation compared to PLE.

In this study, the capacity of PLE and SLE to extract antioxidant-enriched fractions from the four species of Irish macroalgae harvested from the west coast of Ireland was assessed. Two brown species *F. serratus* (Fucales) and *L. digitata* (Laminariales), one green species *C. fragile* (Codiaceae) and one red species *G. gracilis* (Gracilariaceae) were selected and the effectiveness of these techniques to extract antioxidant species on a range of macroalgae currently harvested for commercial exploitation was explored. The ultimate aim of this research was to provide an insight into the best extraction techniques to produce antioxidant-rich crude extracts from these species, which may prove to be useful for development of macroalgal-based functional foods and/or ingredients to inhibit oxidative deterioration in foods.

### 2.1 Materials and methods

#### 2.1.1 Chemicals

All chemicals used were reagent grade, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ferrous chloride, ferrozine, 2, 4, 6-tris(2-pyridyl)-s-triazine(TPTZ), 6-hydroxy-2, 5, 7, 8-tetramwthylchroman-2-carboxylic acid (Trolox) (Sigma, St. Louis, MO USA), and silica gel are
obtained from Sigma-Aldrich Chemical Co. (Arklow, Wicklow, Ireland). Diatomaceous earth was obtained from Dionex Corporation (Ballycoolin, Dublin, Ireland).

2.1.2 Macroalgal material

The macroalgae samples *F. serratus* (Fucaceae), *L. digitata* (Laminariaceae), *C. fragile* (Codiaceae) and *G. gracilis* (Gracilariaceae) used in this study were harvested from the west coast of Ireland in spring 2010. A random selection of different plants were taken from the shore, to allow for natural variability, washed thoroughly with fresh water and stored at -20 °C. The identity of each macroalgal specimen was verified by a trained phycologist. The macroalgal samples were subsequently freeze-dried (A12-60 Freeze Dryer; Frozen in Time Ltd., York, England), ground to a powder using a Waring® blender (New Hartford, CT, USA) and stored in vacuum-packed bags at -80 °C prior to extraction.

2.1.3 Solid-liquid extraction (SLE)

SLE was employed to extract antioxidants using four different solvent systems: cold water (CW), hot water (HW), ethanol/water (80:20, EW) and methanol/water (70:30, MW). Crude extracts were prepared by placing 150 g of the seaweed powder in a conical flask and adding the extraction solvent. For the methanol- and ethanol-based extracts the extraction solvent was added at a ratio of 10:1 (v/w). For the aqueous-based extracts, extraction solvent was added at a ratio of 20:1 (v/w). The mixture was then placed into a shaker (Thermo Scientific MaxQ6000, Dublin, Ireland) at room temperature for 24 h, in the case of the HW extract, the temperature used was 60 °C. The HW_{SLE} extraction was a sequential extraction carried out on the residue from a cold-water extract at 60 °C. The EW_{SLE} and MW_{SLE} extracts were filtered three times over a 24 h period through a Buchner
funnel, whereas the CW_{SLE} and HW_{SLE} were filtered twice over a 24 h period through glass wool, and the solvent refreshed each time. Alcohol was removed from EW_{SLE} and MW_{SLE} extracts using a large-scale rotary evaporator (BuchiRotavapor R-200 with a V710 vacuum pump, Flawil, Switzerland) with the water-bath set at 50 °C. The remaining aqueous portions of the alcohol based extracts were frozen and freeze-dried. The water-based extract was filtered through glass wool combined with cotton wool. These were then concentrated under reduced pressure using the large-scale rotary evaporator, frozen at -20 °C and freeze dried. All extracts were subsequently ground to a fine powder using a mortar and pestle prior to bioactivity testing and stored at -80 °C until further analysis.

2.1.4 Pressurised liquid extraction (PLE)

Pressurised liquid extraction was employed to extract antioxidants from freeze-dried powders of the macroalgae under investigation using an accelerated solvent extractor 200 (ASE®200; Dionex Corp., Dublin, Ireland), equipped with a solvent controller unit. The solvent combinations employed for PLE were 100% water (HW), the ethanol/water (80:20, EW) and methanol/water (70:30, MW). Prior to extraction, 2.5 g of macroalgal powder was mixed with silica, at a sample/silica ratio of 1:2 (w/w) and then loaded into 22 mL stainless steel extraction cells packed with diatomaceous earth. Hot-water PLE extracts (HW_{PLE}) were generated at 120 °C and at 1500 psi (Di et al., 2003). Ethanol/water PLE extracts (EW_{PLE}) were prepared at 100 °C and 1000 psi (Luthria and Mukhopadhyay, 2006). Extraction time for each cell was approximately 25 min total. Organic solvent was removed from extracts using a Labconco® centrifugal vacuum concentrator (Labconco Corp., Kansas City, MO, USA) at 40 °C. Extraction yield was expressed as a percentage
of the initial dried macroalgae material. Extracts were freeze-dried to remove remaining water. Dried extracts were stored at -80 °C until further analysis.

2.1.5 Total phenolic content (TPC)

The total phenol content of the extracts was quantified according to the method of Singleton et al. (1999). Samples were diluted in methanol and tested at 1 mg mL⁻¹. Briefly, a 100 µL aliquot of sample was mixed with a 100µL Folin-Ciocalteu phenol reagent, 100 µL methanol and 700 µL 20 % Na₂CO₃. The reaction mixture was mixed thoroughly using a vortex and allowed to stand for 20 min at room temperature in the dark. Samples were then centrifuged at 13,000 rpm for 3 min, and the absorbance of all samples was measured at 735 nm using a Hitachi U-2900 spectrophotometer. Gallic acid (GA) was used as the external standard for the quantification of the phenolic content in the macroalgal extracts (Heo et al., 2005), standards ranging from 10-200 µg mL⁻¹ were used to construct a standard curve. Methanol was used for the standard dilution and was also used as a blank. The TPC was expressed in terms of microgram Gallic acid equivalents per millgram of dry weight sample (µg GAE mg⁻¹ sample). All fractions were tested in triplicate.

2.1.6 In vitro antioxidant activity

2.1.6.1 Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed according to Straitil et al. (2006) with minor modifications (Tierney et al., 2013). The FRAP reagent contained 10 mL of 10 mmol L⁻¹ TPTZ solution in 40 mmol L⁻¹ of HCl along with 10 mL of 20 mmol L⁻¹ FeCl₃.6H₂O and 100 mL of 0.3 mol L⁻¹ acetate buffer, pH 3.6. A 2-mM Trolox stock solution was prepared and diluted with methanol to give concentrations ranging from 0.1-0.4 mM. In brief, 180 µL of freshly prepared FRAP reagent at 37
°C was pipette into a 96-well micro titre plate with either a 20μL test sample or standard (or methanol for the blank). Samples were tested at 1 mg mL⁻¹. Samples were incubated at 37 °C for 40 min, and then the absorbance was measured at 595 nm using a plate reader (BMG LabtechFLUOstar Omega, Germany). Trolox was used as a standard, and FRAP values were expressed as microgram trolox equivalents per milligram dry weight sample (µg TE mg⁻¹ DW sample). All fractions were tested in triplicate.

2.1.6.2 2, 2'-Diphenyl-1-picryl-hydrazyl (DPPH) scavenging activity

The free-radical scavenging capacity of the crude and the fractionated seaweed extracts were analysed using a modification of the (DPPH) assay according to Goupy et al. (1999) and (Kenny et al., 2013). A 2mM Trolox stock solution was prepared and diluted with methanol to give concentrations ranging from 0.1-0.4 mM. This standard curve was used to determine the IC₅₀ value for Trolox to ensure method and solutions were accurate and reproducible. One hundred millilitre of methanol was added to each well apart from well 1. Two hundred microlitre of each sample was pipette into well 1 of the plate. Serial dilutions of the seaweed samples starting at 2 mg mL⁻¹ in well 1 were prepared across a 96-well micro titre plate. One hundred microlitre of a 1 in 5 dilution of the DPPH/methanol (0.238 mg of DPPH per millilitre of methanol prepared daily) working solution was pipette into each well. The plate was then placed in the dark at room temperature for 30 min. The absorbance was measured at 515 nm using a plate reader. The decrease in absorbance of the sample extract was calculated by comparison to control (100 μL sample extraction solvent and 100 μL DPPH⁻¹). The relative decrease in absorbance (PI (% inhibition)) was calculated using Eq. 1 below;
\[ \text{PI}(\%) = \left[ 1 - \frac{\text{Ae}}{\text{Ab}} \right] \times 100, \]

Where \( \text{Ae} \)= absorbance of sample extract and \( \text{Ab} \)= absorbance of control. PI's were used to calculate the relative antioxidant activity according to the method of Ollanketo et al. (2002) i.e. where PI1 (superior) and PI2 (inferior) were used to estimate the concentration of extract required to result in a 50 % decrease of DPPH absorbance. Antioxidant activity was expressed as antiradical power (ARP), which is the reciprocal of the IC\(_{50}\) (mg mL\(^{-1}\)) used to define the concentration of a sample extract that produces a 50 % reduction of the DPPH radical absorbance (Ollanketo et al., 2002). High ARP values indicate a strong radical scavenging ability (RSA) of a sample (Brand-Williams et al., 1995). All fractions were tested in triplicate.

### 2.1.7 Statistical analysis

All extracts were analysed in triplicate (n=3). Values are presented as means ± standard deviation. One-way analysis of variance (ANOVA), followed by the Tukey post hoc comparison test, was carried out to test for significant differences in antioxidant activity and phenolic content between macroalgal extracts using the statistical program Minitab® Release 15 for Windows (Minitab, State College, PA, USA). A probability value of \( p< 0.05 \) was considered statistically significant.
2.2 Results and Discussion

2.2.1 Extraction yield

The extraction yields (defined as a % of the initial DW of freeze-dried powder for the four macroalgae species obtained using PLE and SLE utilising different solvents were examined as an indication of the overall extraction efficiency of the techniques and are presented in Table 2.1. Considerable variation in extraction yield was observed between the different seaweed species. The highest extraction yield was recorded for the CW SLE of C. fragile (48.2 %) the green algae, whereas the lowest yield was observed in the HW SLE for G. gracilis (5.5 %), the red algae.

In general, for most species, the SLE extracts for both the MW and EW extracts had a higher yield than their PLE counterparts except for F. serratus where the PLE extracts for both MW and EW had higher yields than SLE extracts for the same solvent combinations. The solvent used for extraction also had an impact on yield. For most species investigated, the extraction yields of the CW SLE extracts were highest. This was not unexpected as macroalgae are known to contain significant quantities of polysaccharides, such as fucoidan (Bilan et al., 2006), laminarin (Read et al., 1996), agarose (Rodriguez et al., 2009) and galactans (Ciancia et al., 2007). In all cases, the sum of the CW SLE and HW SLE yields were higher than that of the HW PLE extracts. However, it should be noted that the extraction time for the sum of the water extracts (48 h) was much longer than that of a single PLE extraction which was approximately 15-25 min. The extended extraction time for SLE is offset by the large quantities that can be easily extracted using this method (kilogram quantities) compared with PLE.
2.2.2 Total phenolic content

There is increasing interest in studying the antioxidant actions of plant and seaweed phenolic compounds due to evidence that consumption of these compounds contributes to protection from a number of ailments. Research by Lam et al. (2007) found that phenolic compounds from dietary plant sources, namely barbaloin, 6-gingerol and rhapontin, have potential applications in the management of pro-oxidant state-related cardiovascular disorders. Frankel et al. (1995) also determined that phenolic compounds in red wines inhibited the copper-catalysing oxidation of human low-density lipoproteins (LDL), which contribute to the onset of diseases such as atherosclerosis and coronary heart disease.

The level of phenols in the crude extracts of the four macroalgae species prepared using PLE and SLE with different solvents are presented in Table 2.2. For both SLE and PLE extracts, low TPC levels are observed in *L. digitata*, *G. gracilis* and *C. fragile* in comparison with *F. serratus*. A significant difference (*p < 0.05*) in the TPC was observed among species for the PLE and SLE extracts as shown in Table 2.2, and this ranged from 0.37 ± 0.189 to 81.17 ± 1.890 µg GAE mg⁻¹ of sample. The SLE and PLE extracts from *F. serratus* had higher TPC content in comparison with the other macroalgal species. The highest TPC content was observed in the CW<sup>SLE</sup> extract of *F. serratus* (81.17 µg GAE mg⁻¹), and a high TPC content was also observed in the MW<sup>SLE</sup> for the same species (80.70 µg GAE mg⁻¹). A significant difference (*p < 0.05*) was observed between PLE and SLE extracts for *F. serratus*. For example, the MW<sup>SLE</sup> for *F. serratus* has a TPC content of 80.70 µg GAE mg⁻¹, while its counterpart PLE extract has a TPC content of 61.14 µg GAE mg⁻¹. Similar to this, the EW<sup>SLE</sup> exhibits a greater TPC content of 75.96 µg GAE mg⁻¹ when compared to the EW<sup>PLE</sup> extract, which has a TPC content of 56.68 µg GAE mg⁻¹. Wang et al. (2009) have also reported high levels of TPC in extracts of *F. serratus* of 24.0 g PGE (Phloroglucinol
equivalents) 100 g\textsuperscript{-1} for a 70\% acetone extract and 16.9 g PGE 100 g\textsuperscript{-1} for a water extract of \textit{F. serratus} (results expressed as phloroglucinol equivalents (PGE)). Compared with PLE, the SLE method was consistently more effective for extraction of phenolics from the four seaweeds (Table 2.1). For example, a significant difference (\(p < 0.05\)) between the hot-water SLE and PLE extracts was observed in \textit{L. digitata} with SLE extracts exhibiting the highest TPC content (5.06 \(\mu\)g GAE mg\textsuperscript{-1}), for this species, while its counterpart PLE extract had a TPC content of 1.37 \(\mu\)g GAE mg\textsuperscript{-1}. Similar results were observed for \textit{C. fragile}. However, the phenolic content of the EW\textsubscript{SLE} for \textit{L. digitata} was lower than its PLE counterpart for the same species. The lower recovery of phenolic compounds in the PLE extracts for \textit{F. serratus} relative to the SLE extracts may be due to the loss of thermally liable antioxidant compounds as these extractions were carried out at much higher temperatures (Akowuah \textit{et al.}, 2009). It has been reported in other investigations that brown macroalgae generally have higher TPC than green and red macroalgae (Chew \textit{et al.}, 2008; Cox \textit{et al.}, 2010). This is thought to be because of the presence of phlorotannins in the brown macroalgae (Ragan and Glombitza, 1986) which are not known to be present in the other macroalgae groups.
Table 2.1 Extraction yields, expressed as a percentage of initial dry weight of freeze-dried powder, for four macroalgae species extracted using a variety of solvent combinations and extraction methods.

<table>
<thead>
<tr>
<th>Macroalgal species</th>
<th>Extract</th>
<th>SLE %</th>
<th>PLE %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fucus serratus</em></td>
<td>EW</td>
<td>24.9</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>26.3</td>
<td>29.2</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>6.2</td>
<td>33.4</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>35.9</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td>EW</td>
<td>35.2</td>
<td>24.8</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>36.5</td>
<td>28.2</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>7.9</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>39.5</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Codium fragile</em></td>
<td>EW</td>
<td>46.1</td>
<td>26.9</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>34.0</td>
<td>24.6</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>8.8</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>48.2</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Gracilaria gracilis</em></td>
<td>EW</td>
<td>25.8</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>29.2</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>5.5</td>
<td>26.9</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>25.9</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Extraction yield expressed as a percentage (%) of initial dried raw material. EW, ethanol/water; MW, methanol/water; HW, hot water; CW, cold water extract, N/D; not determined.
Table 2.2 Phenolic contents, expressed as microgram (µg) gallic acid equivalents (GAE) of extracts, for four macroalgae species extracted using a solid liquid and pressurised liquid extraction techniques and a variety of solvent combinations

<table>
<thead>
<tr>
<th>Macroalgal species</th>
<th>Extract</th>
<th>SLE</th>
<th>PLE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fucus serratus</em></td>
<td>EW</td>
<td>75.96 ± 10.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.68 ± 4.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>80.70 ± 1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.11 ± 5.39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>79.49 ± 1.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.32 ± 3.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>81.17 ± 1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td>EW</td>
<td>1.39 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.20 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>2.93 ± 0.77&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.18 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>5.06 ± 0.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>2.24 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Codium fragile</em></td>
<td>EW</td>
<td>2.40 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.31 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>0.93 ± 0.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.37 ± 0.19&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>7.89 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ± 0.45&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>3.18 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Gracilaria gracilis</em></td>
<td>EW</td>
<td>4.76 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.79 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>5.36 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.44 ± 0.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>3.49 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.50 ± 0.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>4.91 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
</tbody>
</table>

All values are means ± S.D. (n=3). Column values with different superscript letters (a, b, c & d) indicate significant difference (P <0.05). EW; ethanol/water, MW; methanol/water and HW; hot water, N/D; not determined.
Table 2.3 Radical scavenging ability (RSA), expressed as antiradical power (ARP) of extracts, for four macroalgae species extracted using a variety of solvent combinations and extraction methods

<table>
<thead>
<tr>
<th>Macroalgal species</th>
<th>Extract</th>
<th>SLE</th>
<th>PLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucus serratus</td>
<td>EW</td>
<td>18.55 ± 0.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.44 ± 1.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>7.88 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.66 ± 1.85&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>12.62 ± 0.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.94 ± 0.48&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>9.60 ± 0.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td>Laminaria digitata</td>
<td>EW</td>
<td>0.20 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.30 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>0.42 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>0.23 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>0.72 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td>Codium fragile</td>
<td>EW</td>
<td>0.17 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.15 ± 0.03&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>0.16 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.14 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>0.38 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>0.56 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td>Gracilaria gracilis</td>
<td>EW</td>
<td>0.16 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.15 ± 0.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>0.14 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.13 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>0.22 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>0.24 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
</tbody>
</table>

ARP is the reciprocal of an extracts IC<sub>50</sub> against the DPPH radical. Values are means ± SD (n=3). Column values with different superscript letters (a, b, c, & d) indicate significant difference (P <0.05). EW; ethanol/water, MW; methanol/water and HW; hot water, N/D; not determined.
Table 2.4 Ferric reducing antioxidant power (FRAP), expressed as µg trolox equivalents (TE) of extracts, for four species of macroalgae extracted using solid liquid and pressurised liquid extraction techniques and a variety of solvent combinations.

<table>
<thead>
<tr>
<th>Macroalgal species</th>
<th>Extract</th>
<th>SLE</th>
<th>PLE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fucus serratus</em></td>
<td>EW</td>
<td>78.30 ± 15.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.40 ± 1.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>75.63 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.57 ± 3.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>89.27 ± 0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.33 ± 10.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>84.37 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td>EW</td>
<td>8.51 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.80 ± 1.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>3.99 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.73 ± 0.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>0.49 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.83 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>8.17 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Codium fragile</em></td>
<td>EW</td>
<td>6.01 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.67 ± 1.82&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>3.44 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.37 ± 0.82&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>0.94 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.27 ± 0.57&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>2.77 ± 0.12&lt;sup&gt;bc,c&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
<tr>
<td><em>Gracilaria gracilis</em></td>
<td>EW</td>
<td>4.76 ± 0.16&lt;sup&gt;de&lt;/sup&gt;</td>
<td>2.58 ± 1.62&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>6.26 ± 0.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.68 ± 0.54&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>7.30 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02 ± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>10.91 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/D</td>
</tr>
</tbody>
</table>

All values are means ± SD (n=3). Column values with different superscript letters (a, b, c & d) indicate significant difference (P <0.05). EW; ethanol/water, MW; methanol/water and HW; hot water, N/D; not determined.

2.2.3 *In vitro* antioxidant activity

Plants synthesise antioxidants to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Work conducted by Vinson and Hontz, (1995) determined that plant polyphenols exhibited lipoprotein-bound antioxidant activity using an *in-vitro* oxidation model for heart disease.

The DPPH radical scavenging activity of the crude extracts derived from PLE and SLE methods with different solvent combinations are presented in Table 2.3. The radical scavenging activity (RSA) for the macroalgae extracts is expressed as the antiradical power (ARP) which is the reciprocal of the IC<sub>50</sub> (expressed as mg mL<sup>-1</sup>), the concentration of extract required to scavenge 50 % of the DPPH radical and therefore positively correlated with antiradical power. A significant difference (p < 0.05) in RSA was observed among species for the PLE and SLE extracts ranging
from ARP values of 18.63 in the EW\textsubscript{SLE} of \textit{F. serratus} to 0.13 in the MW\textsubscript{PLE} of \textit{G. gracilis}. Similar to the behaviour observed for phenolic content, \textit{F. serratus} extracts were the most effective scavengers of DPPH radicals, in particular the EW\textsubscript{SLE} which had the highest ARP value of 18.55 with the HW\textsubscript{SLE} extract having the next highest ARP value of 12.62. The SLE extracts in general showed much higher radical scavenging ability than the PLE extracts. For example, EW\textsubscript{SLE} has an RSA value of 18.55, whereas its corresponding PLE extract had and RSA value of 4.44. Similar results were observed in both the MW and HW extracts with SLE having a higher RSA value when compared to its counterpart PLE extract. The lowest ARP value was observed in the MW\textsubscript{PLE} extract of \textit{G. gracilis}, which was approximately 141-fold lower than that of \textit{F. serratus}. Similar to the trend of results obtained for the TPC assay, the majority of SLE extracts had higher RSA’s than the PLE extracts for the same species. ARP values for \textit{L. digitata}, \textit{G. gracilis} and \textit{C. fragile} extracts were low when compared to the brown macroalgae \textit{F. serratus}. Not surprisingly, extracts containing high levels of TPC were also potent DPPH radical scavengers as it has been shown that polyphenols such as phlorotannins are an important contributor to the radical scavenging ability of other brown species (Tierney \textit{et al.}, 2013).

FRAP values obtained were broadly in line with those previously discussed for RSA’s measured using the DPPH assay (Table 2.4). The reducing powers for the four macroalgae species ranged from 89.27 to 0.49 $\mu$g TE mg$^{-1}$ sample of extract. \textit{Fucus serratus} CW\textsubscript{SLE} and HW\textsubscript{SLE} exhibited the highest FRAP values of 89.27 and 84.37 $\mu$g TE mg$^{-1}$ sample, respectively, Table 2.3. The SLE extracts exhibited much higher FRAP activity in comparison with their PLE counterparts. The HW\textsubscript{SLE} had the highest FRAP activity of 89.27 $\mu$g TE mg$^{-1}$ sample, while the HW\textsubscript{PLE} had a FRAP activity of 32.33 $\mu$g TE mg$^{-1}$ sample. Similar results were also seen for the EW\textsubscript{SLE} and MW\textsubscript{SLE} extracts for \textit{F. serratus} having a higher FRAP activity when compared to their corresponding PLE
extracts. Again using the SLE method, *F. serratus* had a significantly higher FRAP activity compared with the other three macroalgae species, *L. digitata*, *C. fragile* and *G. gracilis* showed a slight increase in activity when compared to their PLE counterparts. In agreement with previous studies by O’ Sullivan *et al.* (2011) and Jiménez-Escrig *et al.* (2001), the *Fucus* species exhibited the highest FRAP activity.

While in the present case, it would appear that based on extraction yield, phenolic content and antioxidant activity, SLE should be the method of choice for seaweed processors, it should be stated that PLE offers some advantages for particular target compounds and matrices. For example, PLE extraction takes place in an anaerobic and light-free environment, which is beneficial when working with compounds that are liable to photo and oxidative damage. PLE is subject to shorter extraction times (approximately 25 min) and utilises less solvent than conventional SLE methods. In addition, by increasing the temperature and pressure, the solvent polarity is reduced (Ju and Howard, 2005), therefore, compounds of low polarity may be extracted using water in the PLE system, in turn reducing the need for organic solvent (Herrero *et al.*, 2010). The major practical disadvantages of PLE include the labour involved in packing of the cells and the necessity to avoid densely packed cells which can cause clumping thus reducing contact between solvent and target material resulting in lower extraction yields. PLE also uses elevated temperatures up to 100 °C, which could result in the loss of thermally labile target molecules, as indicated in this study. In addition, the use of PLE to extract bioactive compounds from macroalgae would require a significant capital investment, and an industrial scale version of the extraction is currently unavailable.
2.3 Conclusion

The present study investigated the phenolic content and antioxidant capacity of food-friendly extracts from four currently underexploited seaweed species, which can be found off the coast of Ireland and which are indigenous to the Northern Atlantic region. The main aim of the study was to provide recommendation to potential processors on the most efficient method for recovery of valuable antioxidant compounds and extracts. The antioxidant activity and TPC for the SLE extracts were generally higher than the equivalent PLE for the macroalgae extracts generated using the same solvents. As the equipment required to conduct SLE in comparison with PLE is relatively inexpensive, readily available, and easy to operate and is already in use by industry for large-scale extraction, it would appear that SLE is the method of choice for extraction of antioxidants from the selected macroalgal species investigated in this study. Solid-liquid extraction was also the preferred technique of choice for extraction of antioxidant compounds from macroalgae based on the yield and activity exhibited required. In fact, it would appear that in the present study, the use of high extraction temperatures and pressures as used in PLE were unwarranted for the production of food-friendly extracts with high phenolic and antioxidant contents.

The study presented may prove useful for the development of macroalgae-based products such as functional food and cosmetics where consideration is given to the most efficient extraction technique, extraction solvent and macroalgae species to be employed. However, based on the results obtained from of the four species investigated, extraction of *F. serratus* using SLE represents the best choice for extraction yields and warrants further investigation.
References


Chapter 3

Antioxidant activities and Phenolic contents of enriched fractions from four selected Irish macroalgae species (*Laminaria digitata*, *Fucus serratus*, *Gracilaria gracilis* and *Codium fragile*)

Based on:
Abstract

The total phenolic content (TPC) and antioxidant activity of crude and enriched aqueous, ethanol/water and methanol/water extracts from four species of Irish macroalgae Laminaria digitata, Fucus serratus, Gracilaria gracilis and Codium fragile were assessed. The antioxidant activity and TPC of crude and enriched extracts were assessed using the DPPH, FRAP and Folin-Ciocalteu assays. Antioxidant activities and phenolic contents of F. serratus were significantly higher than those of the other three species under investigation. Further enrichment based on liquid extraction and molecular weight cut-off (MWCO) dialysis generated fractions of low and high molecular weights, (< 3.5 kDa, 3.5-100 kDa, >100 kDa) and hydrophobic fraction with a significant higher antioxidant activities and TPC ($p<0.05$) compared to the crude extracts for all species. The initial low activity of TPC and antioxidant activity in the <3.5 kDa fractions of F. serratus was increased following reverse-phase flash chromatography fractionation. Analysis by quadrupole time-of-flight mass spectrometry (Q-Tof-MS) suggests that the <3.5 kDa fractions for F. serratus contained a high abundance of low-molecular weight phlorotannins. This study highlights techniques suitable for use to further enrich fractions with potential for further exploitation, in particular F. serratus.

3.0 Introduction

Ireland has a small but expanding macroalgae harvesting industry dominated largely by brown species of which only a small portion (4 %) is further processes after harvest (Werner and Kraan, 2004). In general, the harvested species are used mainly for food or agricultural purposes with minor quantities used for secondary processing or in the cosmetics industry. It is likely however that sustainable quantities of macroalgae in excess of those currently harvested are available if new markets and uses could be found. For example, Hesson et al. (1998) estimated that the total
sustainable yield of *Ascophyllum nodosum* was 74,845 t per annum; at the time of the report, the actual harvest was 35,850 or 48% of the potential yield. Werner and Kraan, (2004) estimated that there was 3,000,000 t of *Laminaria hypoborea/Laminaria digitata* along the coastline of Ireland. Currently, the largest amount of seaweed harvested is of *A. nodosum*, however, significant quantities of the species targeted in the present study (*Laminaria digitata, Fucus serratus, Gracilaria gracilis* and *Codium fragile*) are also harvested, and sustainable levels in excess of those currently targeted are likely to be available. Therefore, macroalgae could represent an abundant and renewable resource and are therefore being explored as novel sources of compounds with the potential for delaying the onset of certain disease such as chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Park *et al.*, 2004). Many inter-tidal seaweed species have well-developed antioxidant systems as a defence against reactive oxygen species (ROS) formed as a consequence of the dynamic environmental conditions of their habitat. These ROS are also formed in human cells by endogenous factors, and if they are not neutralised by *in-vivo* antioxidant defences they can cause oxidative damage which may contribute to the development of a variety of chronic disease states including coronary heart disease, cancer (Reaven and Witzum, 1996), diabetes, rheumatoid arthritis (Baverova and Bezek, 1999), chronic inflammatory disease of the gastrointestinal tract, Alzheimer’s disease (Chauhan and Chauhan, 2006) and other neurological disorders associated with the ageing processes (Temple, 2000). In addition to their potential role in reducing the risk of some diseases, naturally derived antioxidants could be used to control oxidative processes which lead to losses in the quality of processed foods. For example, oxidation of unsaturated lipids leads to the production of rancid off-flavours thus reducing the shelf-life, nutritional quality and safety of food products (Zainol *et al.*, 2003). Synthetic antioxidants such as butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole
(BHA) have been added to many foods to reduce oxidative deterioration. However, due to potential safety issues related to toxicity (Witschi and Lock, 1978), consumers are beginning to show preferences for naturally derived antioxidants.

Whilst a number of previous studies have assessed the antioxidant activity of crude macroalgae extracts from the species under investigation in the present study (Le Tutour, 1990; Le Tutour et al., 1998; O'Sullivan et al., 2011), relatively few have attempted to enrich the antioxidant components in these macroalgae species. In their crude form, extracts from macroalgae do not usually contain concentrations of antioxidants at levels comparable to those found in other well-known terrestrial sources of antioxidants such as herbs and spices (Hossain et al., 2008) and some berries and fruits (Heinonen and Meyer, 2002). Therefore, in order for macroalgae to realise their full commercial potential, relatively simple and inexpensive techniques to increase antioxidant levels in crude extracts are essential. Several types of antioxidants are found in macroalgae, however phlorotannins, a type of polyphenol unique to seaweeds, have thus far garnered the most attention. Phlorotannins result from the oligomeric dehydrogenative coupling of phloroglucinol (Quideau et al., 2011) and contain both multiple resonance stabilising sites and a range of condensed units which together have the potential to enhance their antioxidant activity. The range of sizes in which phlorotannins can occur varies greatly, and therefore, fractionation techniques which use molecular size as a basis for separation could have applications for enriching the antioxidant potential of crude macroalgal extracts. Recently, Tierney et al. (2013) demonstrated that the enrichment of macroalgal phlorotannins using solid-liquid extraction (SLE) of three brown macroalgae (Fucus spiralis, Pelvetia canaliculata and Ascophyllum nodosum) was possible using MWCO dialysis. The present study examined the use of polarity separation and MWCO dialysis as a means of enriching crude extracts from a further four species of macroalgae (L. digitata, F.
serratus, G. gracilis and C. fragile) based on phenolic content and activity in two in vitro antioxidant assays. Three of these species, L. digitata, G. gracilis and C. fragile, are known to have relatively low levels of antioxidant activity in crude extracts (Zhang et al., 2007: Wang et al., 2009), and it was envisaged that this fractionation approach could enrich the extracts from these species with antioxidant compounds. This would both increase the commercial potential of underutilised species whilst also providing vital insights into the molecular weight distribution of the antioxidant species therein.

3.1 Materials and methods

3.1.1 Chemicals

All solvents used were HPLC (high performance liquid chromatography) grade. 2, 2-diphenyl-1-picrylhydrazyl (DPPH·), ferrous chloride, ferrozine, 2,4,6-tris(2-pyridal)–s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium acetate anhydrous, Gallic acid, Folin-Ciocalteu’s phenol reagent and sodium carbonate were obtained from Sigma-Aldrich, (Wicklow, Ireland). BioDesignDialysisTubing™ with a 3.5 kDa cut-off was acquired from Fisher Scientific (Wicklow, Ireland). Spectra/Por® Biotech cellulose ester dialysis tubing with a 100 kDa cut-off was obtained from Apex Scientific (Kildare, Ireland).

3.1.2 Macroalgal materials

The four seaweed species investigated in this study, i.e., F. serratus, G. gracilis, C. fragile and L. digitata were collected from the West Coast of Ireland in Co. Clare in spring 2011. A random selection of different plants were taken from the shore to allow for natural variability, packed in cool boxes and transported immediately to the laboratory. Samples were washed with distilled
water to remove sand and epiphytes and were then stored at -18 °C. The taxonomy of all samples was verified by a trained phycologist, and a dried reference sample for each species was stored in National University of Ireland (NUI) Galway as part of the Marine Functional Foods (Nutramara) Research Initiative. Samples were freeze-dried then ground into a powder using a Waring blender and stored in vacuum-packed bags at -80 °C prior to extraction.

3.1.3 Preparation of crude seaweed extracts

A range of food-grade solvents were employed for extraction, however, methanol was also used to prepare crude extracts as to date it has been the solvent of choice for extracting polyphenols and thus served as a useful comparison to solvents such as water and ethanol. These solvents were previously shown to be effective solvents for the enrichment of antioxidants from macroalgae (Wang et al., 2009; Ye et al., 2009). Solid-liquid extraction using four different solvent systems, namely cold water (CW), hot water (HW), ethanol/water (80:20; EW) and methanol/water (70:30; MW) was used to prepare the crude extracts. Specifically, extracts were prepared by placing 150 g of the seaweed powder in a conical flask and adding the extraction solvent at a ratio of 10:1 (v/w) for the methanol- and ethanol-based extracts and a ratio of 20:1 (v/w) for the cold and hot water extracts. The mixture was then shaken at 150 rpm at room temperature for 2 h; in the case of the hot water extract, the temperature used was 60 °C. The HW extraction was a sequential extraction carried out on the residue from CW extraction at 60 °C to ensure that exhaustive extraction of the seaweed occurred. The EW and MW extractions were filtered three times over a 24 h period through a Buchner funnel, while the CW and HW were filtered twice over a 24 h period through glass wool, and the solvent refreshed each time. Alcohol was removed from EW and MW extracts using a large-scale rotary evaporator (BüchiRotavapor R-200 with a V710 vacuum pump, Flawil,
Switzerland) with the water-bath set at 50 °C. The remaining aqueous portions of the alcohol-based extracts were frozen and freeze-dried. The water-based extracts were filtered through glass wool combined with cotton wool to remove particles that were difficult to remove due to their viscous nature. These were then concentrated under reduced pressure using the large-scale rotary evaporator, frozen at -20 °C and freeze-dried. All extracts were subsequently ground to a fine powder using a mortar and pestle prior to bioactivity testing.

3.1.4 Preparation of molecular weight cut-off and hydrophobic fractions
Prior to dialysis the crude EW and MW extracts were subjected to exhaustive partitioning using water to create a hydrophobic fraction (HPF) (remaining residue) and the hydrophilic fraction (water soluble portion). The crude CW and HW solid liquid extraction (SLE) extracts and the hydrophilic fraction partitioned from the EW and MW extracts were dissolved in minimal volume of deionised water and decanted into 3.5 kDa dialysis tubing clamped at one end. The tubing was then clamped at the other end, immersed in a reservoir of deionised water and shaken moderately (50 rpm) at room temperature for 72 h. The reservoir of water was refreshed periodically until no further colour was visible in the dialysate. Both the high molecular weight (HMW) retentate (>3.5 kDa) and low-molecular weight (LMW) dialysate (<3.5 kDa) fractions were freeze-dried. The HMW retentate from the 3.5 kDa dialysis was dissolved in water, placed in 100 kDa dialysis tubing, and dialysis was carried out as described previously. Both the retentate (>100 kDa) and dialysate (3.5-100 kDa) fractions were freeze-dried. All MWCO fractions and the hydrophobic fractions were then assayed in vitro for total phenolic content (TPC), DPPH radical scavenging activity (RSA), and ferric reducing antioxidant power (FRAP) activity.
3.1.5 Reserve-phase (RP) flash chromatography

The lower molecular weight (<3.5 kDa) dialysates of the ethanol/water (EW) and cold water (CW) extracts obtained from *F. serratus* were further fractionated using a two-step reverse-phase (RP) flash chromatography method. RP flash chromatography was carried out on a Varian Intelliflash 310 (Dublin, Ireland) system using a TELOS ™ C18 with a sorbent mass of 375 g and mean particle size of 40-60 µm. Two grams of the < 3.5 kDa fraction material dissolved in water was loaded onto the column. A two-step elution gradient was employed. The mobile phase consisted of the primary eluent of HPLC grade water (0-20 min) and the secondary eluent of 100 % methanol (20-40 min). The flow rate was 50 mL min⁻¹. Flash fractions were collected from 0 to 20 min (flash fr. 1) and from 20 – 40 min (flash fr. 2). UV detection was performed at 210, 225 and 250 nm. All flash fractions were assayed for TPC, DPPH and FRAP activity.

3.1.6 Assays for antioxidant activities

3.1.6.1 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to Straitil *et al.* (2006) with minor modifications (Tierney *et al.*, 2013) as previously described in Chapter 2 section 2.1.6.1.

3.1.6.2 DPPH (2, 2-Diphenyl-1-picryl-hydrazyl) scavenging activity

The free-radical scavenging capacity of the crude and the fractionated seaweed extracts were analysed using a modified method of the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay according to Goupy *et al.* (1999) with minor modifications (Kenny *et al.*, 2013) as previously described in Chapter 2 section 2.1.6.2.
3.1.6.3 Determination of total phenolic content

The total phenol content of the extract was quantified according to the method of Singleton et al. (1999) as previously described in Chapter 2 section 2.1.5.

3.1.7 Quadrupole time-of-flight mass spectrometry (Q-Tof-MS)

Mass spectrometry analysis of the LMW polyphenol-enriched flash samples was performed using a Q-Tof Premier mass spectrometer (Waters Corporation, Micromass MA Technologies, UK) by direct infusion into the electrospray ionisation source. Mass spectral data was obtained in the negative ion mode for a mass range of 100 to 3,000 m/z. Capillary and cone voltages were set at 3 and 45 kV, respectively. The desolvation gas was set at 800 L h\(^{-1}\) while the cone gas was set at 50 L h\(^{-1}\). Samples were dissolved in methanol, filtered and infused at 10 µL min\(^{-1}\) for 2 min.

3.1.8 Statistical analysis

All analyses were performed in triplicate. Measurement values are presented as mean values ± S.D. One way analysis of variance (ANOVA), followed by the Tukey post hoc comparison test was carried out to test for significant differences between macroalgal species using the statistical program Minitab ® Release 15 for Windows. A probability value of \( p < 0.05 \) was considered statistically significant.
3.2 Results

3.2.1 Total phenolic content (TPC) of MWCO fractions

Tables 3.1-3.4 present the antioxidant activity and phenolic contents of crude and enriched extracts. The levels of phenols in the crude algae extracts varied considerably ranging from 0.99 to 81.93 µg GAE mg\(^{-1}\) sample in CW extracts from the brown seaweed *F. serratus* and the MW extract in the green seaweed *C. Fragile*. A greater than twofold increase was observed in the 30-100 kDa fraction of the methanol/water (MW) extracts (185.13 µg GAE mg\(^{-1}\) sample) in *F. serratus* in comparison to the corresponding crude extract (80.70 µg GAE mg\(^{-1}\) sample). However, the 100 kDa hot water fraction had a lower phenolic content than the crude extracts along with the 3.5 kDa fractions for all *F. serratus* extracts. In comparison to *F. serratus*, much lower levels of phenols were observed for *L. digitata* (MW crude= 2.93 µg GAE mg\(^{-1}\) sample), *C fragile* (MW crude= 0.99 µg GAE mg\(^{-1}\) sample) and *G. gracilis* (MW crude= 5.36 µg GAE mg\(^{-1}\) sample). On average, a 5-6 fold increase in the phenolic content of MWCO fractions was observed compared to the crude fractions. The methanol/water and ethanol/water hydrophobic fractions had the highest phenolic content. For example, for *L. digitata*, the hydrophobic fractions (HPF methanol/water= 28.13 µg GAE mg\(^{-1}\) sample and HPF ethanol/water= 37.00 µg GAE mg\(^{-1}\) sample) exhibited a fourfold increase in phenolics in comparison to their corresponding crude extracts.

3.2.2 DPPH activity

The CW and MW crude extracts of *L. digitata* had a low DPPH scavenging ability with an ARP value of 0.65 and 0.42 respectively. Low scavenging ability was also observed in the crude CW and HW extracts of *C. fragile* with an ARP values of 0.56 and 0.37, respectively. A very low scavenging ability was observed in all crude extracts of *G. gracilis* (MW = 0.4± 0.01; CW = 0.24±
Similar to the observation for phenolic contents, a higher activity was observed in the MWCO enriched fractions in comparison to the crude extracts. For example, a significant enrichment in MWCO fractions as observed in the 3.5 – 100 kDa (ARP = 20.448), >100 kDa (ARP = 24.12), the MW, 3.5 – 100 kDa (ARP = 48.128) and the > 100 kDa (ARP = 45.033) (EW) fractions from *F. serratus*. In particular, the 3.5 – 100 kDa EW fraction had an ARP value circa 2.5 times greater than the crude extract, and the >100 kDa MW fraction had an ARP 3 times greater than the crude fraction. Lower radical scavenging activity was observed in the <3.5 kDa fraction and the hydrophobic fractions for the same extracts. With respect to the aqueous extracts of *F. serratus*, a significantly higher activity (*p*<0.05) in comparison to crude extracts was observed for the >100 kDa fraction of the hot water (HW) and cold water (CW) extracts. In fact, the antioxidant activity in the <3.5 kDa and 3.5 – 100 kDa fractions was lower than that observed for these extracts in comparison to the crude extract. This implies that, for aqueous extracts, higher molecular weight compounds are the principal components responsible for the radical scavenging ability of *F. serratus*. For *L. digitata*, a twofold increase in activity was observed in the 3.5 – 100 kDa (ARP = 0.96) and a 3.5 fold increase in the >100 kDa (ARP = 1.53) fractions of the MW in comparison to crude extracts (MW crude APR = 0.42), a slight increase was also observed in the hydrophobic fraction but a decrease in radical scavenging activity was obtained for the <3.5 kDa fraction of the MW extract in comparison to the crude extract.
<table>
<thead>
<tr>
<th>Exports</th>
<th>Fraction (kDa)</th>
<th>TPC (µg GAE mg⁻¹ sample)</th>
<th>FRAP (µg TR equivalents mg⁻¹ sample)</th>
<th>DPPH (ARP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>Crude</td>
<td>80.70 ± 1.34e</td>
<td>75.55 ± 0.78g,h</td>
<td>7.82 ± 0.17g</td>
</tr>
<tr>
<td>MW</td>
<td>&lt;3.5</td>
<td>26.87 ± 0.35h</td>
<td>17.79 ± 0.18g</td>
<td>0.65 ± 0.03j</td>
</tr>
<tr>
<td>MW</td>
<td>3.5-100</td>
<td>185.13 ± 1.32a</td>
<td>152.85 ± 1.12d</td>
<td>20.45 ± 0.65d</td>
</tr>
<tr>
<td>MW</td>
<td>&gt;100</td>
<td>131.70 ± 0.40b</td>
<td>217.80 ± 5.44c</td>
<td>24.12 ± 0.96c</td>
</tr>
<tr>
<td>MW</td>
<td>HPF</td>
<td>91.40 ± 0.56d</td>
<td>42.01 ± 0.01m</td>
<td>2.93 ± 0.06i</td>
</tr>
<tr>
<td>EW</td>
<td>Crude</td>
<td>75.96 ± 10.11c,f</td>
<td>69.30 ± 0.85i</td>
<td>18.63 ± 1.46d</td>
</tr>
<tr>
<td>EW</td>
<td>&lt;3.5</td>
<td>27.40 ± 0.00h</td>
<td>10.91 ± 0.65p</td>
<td>0.63 ± 0.05j</td>
</tr>
<tr>
<td>EW</td>
<td>3.5-100</td>
<td>131.37 ± 0.40b</td>
<td>270.86 ± 1.28b</td>
<td>48.13 ± 1.79a</td>
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<tr>
<td>EW</td>
<td>&gt;100</td>
<td>130.27 ± 0.61b</td>
<td>313.53 ± 1.42a</td>
<td>45.03 ± 0.73b</td>
</tr>
<tr>
<td>EW</td>
<td>HPF</td>
<td>117.37 ± 0.45c</td>
<td>44.40 ± 0.00l,m</td>
<td>5.34 ± 0.15h</td>
</tr>
<tr>
<td>HW</td>
<td>Crude</td>
<td>79.49 ± 1.09e</td>
<td>89.27 ± 0.80e</td>
<td>12.46 ± 0.53f</td>
</tr>
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<td>HW</td>
<td>&lt;3.5</td>
<td>24.27 ± 0.25h</td>
<td>48.86 ± 0.59k,l</td>
<td>7.85 ± 0.30g</td>
</tr>
<tr>
<td>HW</td>
<td>3.5-100</td>
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<td>71.67 ± 0.33h,i</td>
<td>19.54 ± 0.77d</td>
</tr>
<tr>
<td>HW</td>
<td>&gt;100</td>
<td>39.13 ± 0.35g</td>
<td>24.97 ± 0.29n</td>
<td>11.50 ± 0.09f</td>
</tr>
<tr>
<td>CW</td>
<td>Crude</td>
<td>81.93 ± 1.93e</td>
<td>84.37 ± 0.38f</td>
<td>12.72 ± 0.62e,f</td>
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<td>CW</td>
<td>&lt;3.5</td>
<td>14.93 ± 0.12i</td>
<td>29.50 ± 0.40f</td>
<td>5.98 ± 0.46g,h</td>
</tr>
<tr>
<td>CW</td>
<td>3.5-100</td>
<td>68.50 ± 0.20f</td>
<td>50.22 ± 0.32k</td>
<td>14.94 ± 0.69e</td>
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<td>CW</td>
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<td>96.60 ± 0.66d</td>
<td>55.03 ± 1.52l</td>
<td>10.68 ± 0.72f</td>
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</table>

EW, ethanol/water extract, MW, methanol/water extract, HW, hot water extract and CW, cold water extract, HPF, hydrophobic fraction, GAE; Gallic Acid Equivalents, TR; Trolox, ARP; Antiradical power. Values are means ± S.D. (n=3). Column-wise values with different superscript letters (a, b, c etc.) indicate significant difference (p<0.05).
Table 3.2 Total phenol content (TPC), ferric reducing antioxidant power assay (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH·) scavenging assay of crude and enriched fractions from *Laminaria digitata*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Fraction (kDA)</th>
<th>TPC (µg GAE mg⁻¹ sample)</th>
<th>FRAP (µg TR equivalents mg⁻¹ sample)</th>
<th>DPPH (ARP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>Crude</td>
<td>2.93 ± 0.77k</td>
<td>3.99 ± 0.30h</td>
<td>0.42 ± 0.06e,f</td>
</tr>
<tr>
<td>MW</td>
<td>&lt;3.5</td>
<td>13.10 ± 0.00fg,h</td>
<td>25.90 ± 0.19c</td>
<td>0.27 ± 0.01g,h,i</td>
</tr>
<tr>
<td>MW</td>
<td>3.5-100</td>
<td>12.88 ± 0.06gh</td>
<td>8.04 ± 0.14g</td>
<td>0.96 ± 0.04b</td>
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<tr>
<td>MW</td>
<td>&gt;100</td>
<td>18.60 ± 0.17d</td>
<td>37.02 ± 0.29a</td>
<td>1.53 ± 0.09a</td>
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<tr>
<td>MW</td>
<td>HPF</td>
<td>28.13 ± 0.25b</td>
<td>4.72 ± 0.00h</td>
<td>0.60 ± 0.01d</td>
</tr>
<tr>
<td>EW</td>
<td>Crude</td>
<td>1.39 ± 0.24l</td>
<td>8.54 ± 0.27fg</td>
<td>0.21 ± 0.02ij</td>
</tr>
<tr>
<td>EW</td>
<td>&lt;3.5</td>
<td>12.87 ± 0.06gh</td>
<td>22.62 ± 0.31d</td>
<td>0.23 ± 0.01ij</td>
</tr>
<tr>
<td>EW</td>
<td>3.5-100</td>
<td>13.67 ± 0.29fg</td>
<td>13.15 ± 0.37e</td>
<td>0.62 ± 0.00d</td>
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<tr>
<td>EW</td>
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<td>16.10 ± 0.20e</td>
<td>21.15 ± 0.35d</td>
<td>0.14 ± 0.01j</td>
</tr>
<tr>
<td>EW</td>
<td>HPF</td>
<td>37.00 ± 0.20a</td>
<td>12.23 ± 0.00e</td>
<td>0.67 ± 0.00d</td>
</tr>
<tr>
<td>HW</td>
<td>Crude</td>
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<td>0.49 ± 0.15i</td>
<td>0.25 ± 0.03ki</td>
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<td>HW</td>
<td>&lt;3.5</td>
<td>11.80 ± 0.00i</td>
<td>9.10 ± 0.06fg</td>
<td>0.33 ± 0.01fg,h</td>
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<td>HW</td>
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<td>12.54 ± 1.69e</td>
<td>0.33 ± 0.01fg,h</td>
</tr>
<tr>
<td>CW</td>
<td>Crude</td>
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<td>8.17 ± 0.59g</td>
<td>0.65 ± 0.01d</td>
</tr>
<tr>
<td>CW</td>
<td>&lt;3.5</td>
<td>12.60 ± 0.00hi</td>
<td>9.86 ± 0.46f</td>
<td>0.43 ± 0.01e</td>
</tr>
<tr>
<td>CW</td>
<td>3.5-100</td>
<td>13.94 ± 0.06f</td>
<td>25.94 ± 0.43c</td>
<td>0.79 ± 0.02c</td>
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<tr>
<td>CW</td>
<td>&gt;100</td>
<td>14.07 ± 0.06f</td>
<td>22.43 ± 0.21d</td>
<td>0.41 ± 0.01cf</td>
</tr>
</tbody>
</table>

EW, ethanol/water extract, MW, methanol/water extract, HW, hot water extract and CW, cold water extract, HPF, hydrophobic fraction, GAE; Gallic Acid Equivalents, TR; Trolox, ARP; Antiradical power. Values are means ± S.D. (n=3). Column-wise values with different superscript letters (a, b, c etc.) indicate significant difference (*p*<0.05).
Table 3.3 Total phenol content (TPC), ferric reducing antioxidant power assay (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH·) scavenging assay of crude and enriched fractions from *Gracilaria gracilis*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Fraction (kDA)</th>
<th>TPC µg GAE mg⁻¹ sample</th>
<th>FRAP µg Tr equivalents mg⁻¹ sample</th>
<th>DPPH ARP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>Crude</td>
<td>5.36 ± 0.29&lt;sup&gt;i&lt;/sup&gt;</td>
<td>6.26 ± 0.31&lt;sup&gt;m&lt;/sup&gt;</td>
<td>0.14 ± 0.01&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW</td>
<td>&lt;3.5</td>
<td>11.97 ± 0.06&lt;sup&gt;h&lt;/sup&gt;</td>
<td>29.60 ± 0.37&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.49 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW</td>
<td>3.5-100</td>
<td>15.13 ± 0.06&lt;sup&gt;e&lt;/sup&gt;</td>
<td>30.40 ± 0.36&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.41 ± 0.01&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW</td>
<td>&gt;100</td>
<td>12.00 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.62 ± 0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15 ± 0.02&lt;sup&gt;h,i&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW</td>
<td>HPF</td>
<td>21.70 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.83 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.22 ± 0.01&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>EW</td>
<td>Crude</td>
<td>4.76 ± 0.17&lt;sup&gt;i&lt;/sup&gt;</td>
<td>4.76 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16 ± 0.00&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>EW</td>
<td>&lt;3.5</td>
<td>15.00 ± 0.10&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>24.43 ± 0.20&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.42 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>EW</td>
<td>3.5-100</td>
<td>16.00 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.43 ± 0.78&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.46 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EW</td>
<td>&gt;100</td>
<td>13.57 ± 0.12&lt;sup&gt;g&lt;/sup&gt;</td>
<td>35.17 ± 1.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.00&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>EW</td>
<td>HPF</td>
<td>34.30 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.57 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.00&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>HW</td>
<td>Crude</td>
<td>3.49 ± 0.30&lt;sup&gt;i&lt;/sup&gt;</td>
<td>7.30 ± 0.08&lt;sup&gt;k,l,m&lt;/sup&gt;</td>
<td>0.22 ± 0.01&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
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<td>17.80 ± 0.10&lt;sup&gt;o&lt;/sup&gt;</td>
<td>9.47 ± 0.06&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.48 ± 0.01&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HW</td>
<td>3.5-100</td>
<td>18.17 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.04 ± 0.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.41 ± 0.00&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>HW</td>
<td>&gt;100</td>
<td>14.53 ± 0.12&lt;sup&gt;c,f&lt;/sup&gt;</td>
<td>25.77 ± 0.55&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.15 ± 0.01&lt;sup&gt;h,i&lt;/sup&gt;</td>
</tr>
<tr>
<td>CW</td>
<td>Crude</td>
<td>4.91 ± 0.27&lt;sup&gt;i&lt;/sup&gt;</td>
<td>10.91 ± 0.19&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.24 ± 0.01&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>CW</td>
<td>&lt;3.5</td>
<td>14.43 ± 0.15&lt;sup&gt;c,f&lt;/sup&gt;</td>
<td>6.47 ± 0.18&lt;sup&gt;l,m&lt;/sup&gt;</td>
<td>0.27 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>CW</td>
<td>3.5-100</td>
<td>16.70 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.60 ± 0.32&lt;sup&gt;k,l&lt;/sup&gt;</td>
<td>0.39 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CW</td>
<td>&gt;100</td>
<td>14.30 ± 0.27&lt;sup&gt;f,g&lt;/sup&gt;</td>
<td>8.19 ± 0.48&lt;sup&gt;l,k&lt;/sup&gt;</td>
<td>0.34 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

EW, ethanol/water extract, MW, methanol/water extract, HW, hot water extract and CW, cold water extract, HPF, hydrophobic fraction, GAE; Gallic Acid Equivalents, TR; Trolox, ARP; Antiradical power. Values are means ± S.D. (n=3). Column-wise values with different superscript letters (a, b, c etc) indicate significant difference \( p<0.05 \).
Table 3.4 Total phenol content (TPC), ferric reducing antioxidant potential assay (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH·) scavenging assay of crude and enriched fractions from *Codium fragile*.

| Extracts | Fraction (kDa) | TPC µg GAE mg⁻¹ sample | FRAP µg Tr equivalents mg⁻¹ sample | DPPH ARP | MW Crude | 0.99 ± 0.11|h | 3.44 ± 0.29|h | 0.16 ± 0.01|h,i | MW <3.5 | 12.70 ± 0.10|f | 19.29 ± 0.26|c | 0.52 ± 0.01|a | MW 3.5-100 | 12.57 ± 0.06|f | 4.79 ± 0.15|g | 0.39 ± 0.01|b,c | MW >100 | 12.90 ± 0.00|c,f | 13.63 ± 0.28|a | 0.21 ± 0.02|f,g | MW HPF | 30.70 ± 0.20|b | 13.24 ± 0.00|c | 0.12 ± 0.02|b,i | EW Crude | 2.40 ± 0.50|h | 6.01 ± 0.29|g | 0.17 ± 0.01|g,h,i | EW <3.5 | 12.70 ± 0.00|f | 10.40 ± 0.19|f | 0.35 ± 0.01|c,d | EW 3.5-100 | 12.33 ± 0.06|f | 5.84 ± 0.27|g | 0.27 ± 0.00|f | EW >100 | 16.57 ± 0.06|c | 19.89 ± 0.38|e | 0.13 ± 0.01|f | EW HPF | 34.87 ± 0.06|a | 10.47 ± 0.00|f | 0.10 ± 0.05|f | HW Crude | 7.89 ± 0.14|g | 0.94 ± 0.03|i | 0.37 ± 0.03|c,d | HW <3.5 | 15.47 ± 0.84|d | 32.37 ± 0.25|a | 0.21 ± 0.02|g | HW 3.5-100 | 13.63 ± 0.06|e | 17.63 ± 0.19|d | 0.33 ± 0.00|d | HW >100 | 15.70 ± 0.10|d | 32.70 ± 0.10|a | 0.24 ± 0.01|c,f | CW Crude | 3.23 ± 0.12|h | 2.77 ± 0.12|h | 0.56 ± 0.01|a | CW <3.5 | 12.33 ± 0.12|f | 22.53 ± 0.25|b | 0.18 ± 0.01|g,h | CW 3.5-100 | 13.67 ± 0.06|e | 17.97 ± 0.23|d | 0.42 ± 0.01|h | CW >100 | 17.27 ± 0.06|c | 32.19 ± 1.40|a | 0.17 ± 0.01|g,h,i | EW, ethanol/water extract, MW, methanol/water extract, HW, hot water extract and CW, cold water extract, HPF, hydrophobic fraction, GAE; Gallic Acid Equivalents, TR; Trolox, ARP; Antiradical power. Values are means ± S.D. (n=3). Column-wise values with different superscript letters (a, b, c etc) indicate significant difference (p<0.05).

3.2.3 Ferric reducing antioxidant power (FRAP)

The level of FRAP activity differed quite significantly between species. A significant increase (p<0.05), in particular, was observed in the 3.5 – 100 kDa and >100 kDa methanol and ethanol extracts of *F. serratus* in comparison to crude extracts (MW crude= 75.55 µg TE mg⁻¹ sample and EW crude = 69.30 µg TE mg⁻¹ sample). However, a lower FRAP activity was observed in the hot and cold water extracts of *F. serratus*. This is also seen in the cold water extracts (<3.5 kDa =
6.47, 3.5 – 100kDa = 7.60 and >100 kDa = 8.19) of *G. gracilis*. Dialysis increased FRAP levels comparable with species with a good antioxidant activity. For example, in the MW >100kDa fraction of *G. gracilis* (65.62 µg TE mg⁻¹ sample) which exhibits a similar FRAP activity to the species *A. nodosum* ethanol extract (66.08 µg TE mg⁻¹ sample) from the study by Tierney *et al.* (2013).

### 3.2.4 Phlorotannin enrichment in low-molecular weight fractions of *Fucus serratus*

The flash chromatograms for both the CW and EW extracts showed a peak, eluting between 4 – 10 min which is likely to contain polar compounds, most probably simple sugars and sugar alcohols (Fig 3.1). A second peak was detected between 25 – 35 min in both chromatograms which has been reported by other authors (Tierney *et al.*, 2013) to correspond to the elution of the predominant polyphenolic species i.e., phlorotannins.
Figure 3.1 Reverse phase flash chromatogram of the reconstituted low molecular weight dialysate (<3.5 kDa) of a *Fucus serratus* cold water extract.

3.2.5 Total phenolic content (TPC) and in vitro antioxidant activity of phlorotannin enriched RP flash fractions.

More than a threefold increase in the TPC was observed in flash fr.2 for the ethanol/water fraction (FS-EW) (105.38 µg GAE mg⁻¹ sample) relative to the less than 3.5kDa fraction of the extracts (27.4 µg GAE mg⁻¹ sample) (Fig. 3.2) this is presumably due to the removal of low molecular weight compounds such as sugars from this fraction. A decrease in TPC activity was seen in flash fr.1 for the cold water fraction (FS-CW), a six fold increase was seen in the enriched flash fr.2 (89.79 µg GAE mg⁻¹ sample) compared to the crude <3.5 kDa fraction (14.93 µg GAE mg⁻¹ sample). The highest TPC activity of 105.38 µg GAE mg⁻¹ sample was observed in flash fr. 2 for the ethanol/water (FS-EW) extract. A decrease in activity was seen in flash fr.1 for both the CW
and the ethanol/water (EW) extracts in comparison to the 3.5 – 100 kDa crude fraction. It is most probable that the presence of large amounts of sugar based compounds in this fraction may have been responsible for diluting the concentration of phlorotannins in the crude <3.5 kDa fraction.

Similar to the TPC results, flash fr.2 for the ethanol/water extract (FS-EW) exhibited the highest ARP value of 51.984 a significantly higher activity in comparison to the >3.5 kDa fraction (ARP = 5.975). This fraction also had the highest FRAP activity (69.01 µg GAE mg\(^{-1}\) sample). An increase in activity was also seen in the cold water (FS-CW) flash fr.2 in both the DPPH and FRAP assays in comparison to the <3.5 kDa crude fraction.
Figure 3.2 Total phenolic content (TPC) expressed as microgram gallic acid equivalents (GAE) of less than 3.5 kDa molecular weight cut-off fraction derived from either cold water (CW) or ethanol water (EW) crude extracts and two flash chromatography fractions collected from the corresponding MWCO fractions from the seaweed *Fucus serratus*. Values are means ± S.D and are expressed as GAE; Gallic acid equivalents.
Figure 3.3 Ferric reducing antioxidant power (FRAP) expressed as microgram trolox equivalents (TE) of less than 3.5 kDa molecular weight cut-off fraction derived from either cold water (CW) or ethanol water (EW) crude extracts and two flash chromatography fractions collected from the corresponding MWCO fractions from the seaweed *Fucus serratus*. Values are means ± S.D. (n=3)
Figure 3.4 DPPH radical scavenging ability (RSA) expressed as antiradical power (ARP) of less than 3.5 kDa molecular weight cut-off fraction derived from either cold water (CW) or ethanol water (EW) crude extracts and two flash chromatography fractions collected from the corresponding MWCO fractions from the seaweed *Fucus serratus*. Values are means ± S.D. (n=3)
During separation moderately polar solvents are usually the solvent of choice when the target compounds are polar antioxidants such as polyphenols and tannins (Cho et al., 2007). In the present study, methanol (70 %), ethanol (80 %), cold water and hot water were used to extract antioxidant compounds from four macroalgae species harvested from Irish shores. Therefore, in the present study, a range of polar solvent systems were selected to generate initial crude extracts. In general, food-grade solvents were used; however, methanol was also used to prepare crude extracts as to date it has been the solvent of choice for extracting polyphenols and thus served as a useful comparison. However, the main objective of the study was to maximise the potential of seaweed species that generally would not be considered for bioactive compounds by identifying molecular weight fractions within the seaweed extracts with the potential to exploit. A profiling method was also developed to explore these bioactive compounds and to examine the feasibility.

**Figure 3.5** ESI-QToF-MS spectrum showing the distribution of molecular weights of molecules in *Fucus serratus* ethanol/water (FS-EW) phlorotannin enriched fraction.
of using simple and low cost enrichment technologies based on polarity partitioning and MWCO dialysis to enrich the crude fractions in the target species.

3.3 Discussion

Tables 3.1-3.4 present the antioxidant activity and phenolic contents of crude and enriched extracts of the four macroalgal species, and the proceeding sections will discuss the success of the enrichment approach in relation to antioxidant activity (FRAP and DPPH activity) and phenolic content for each of the selected macroalgae species.

The TPC of crude and enriched fractions from the four seaweed species could be ranked in the following order of decreasing total phenolic content, *F. serratus, L. digitata, G. gracilis* and *C. fragile*. This variation in part is a natural consequence of the type of seaweed under investigation. In particular, brown macroalgae are reported to generally contain higher amounts of polyphenols than red and green algae (Zubia *et al.*, 2007). Wang *et al.* (2009) have also reported high levels of TPC in extracts of *F. serratus*, a high TPC was observed in the 70 % acetone (24.0 g PGE (100 g)$^{-1}$) and water (16.9 g PGE (100 g)$^{-1}$) extracts of this species. MWCO dialysis of the crude extracts resulted in a significant increase ($p < 0.05$) in phenolic content in nearly all 3.5 – 100 kDa, >100 kDa and hydrophobic fractions from *F. serratus* in comparison to the crude extracts (Table 3.1). Following fractionation, a significant increase ($p < 0.05$) in phenolic content was observed in all MWCO dialysis fractions of *L. digitata, G. gracilis* and *C. fragile* in comparison to the crude extracts for the same species. The enrichment process using MWCO enhanced the activity of sample fractions to levels comparable with crude extracts from other macroalgae species that are considered to have good activity. For example, with *C. fragile*, the MW-HPF and the EW-HPF
fractions exhibited a TPC activity as high as the ethanol extract of *Ulva intestinalis* (41.40 µg PE mg\(^{-1}\) sample) as reported in the study of Tierney *et al.* (2013).

The DPPH scavenging activity was expressed as the antiradical power (ARP) which is the reciprocal of the IC\(_{50}\) (mg mL\(^{-1}\)) and defined as the concentration of sample extract that produces a 50 % reduction of the DPPH radical absorbance (Ollanketo *et al.*, 2002). All the *F. serratus* crude extracts showed a greater radical scavenging ability in comparison to the other species. This outcome is similar to the results observed by Jiménez-Escrig *et al.* (2001) who reported that the brown seaweeds (*Fucus vesiculosus, Laminaria ochroleuca* and *Undaria pinnatifida*) generally exhibited a better DPPH scavenging capacity than the red seaweeds *Chondrus crispus* and *Porphyra umbilicaalis*. The authors also reported that the highest scavenging activity was observed in *Fucus* species, and no activity was detected for *C. crispus*. Wang, (2009) also reported a high scavenging activity (ARP = 90.8) in the acetone extract of the species *F. serratus*, while *L. digitata* had a low scavenging activity for both the acetone and water extracts. Yilmax-Koz *et al.* (2009) also reported that *C. fragile* exhibited no antioxidant activity when assessed using the DPPH assay. *G. gracilis* also had low scavenging capability (3.62 %) when assessed by Zhang (2007). Similar to the observation for phenolic contents, higher activity was observed in the MWCO-enriched fractions in comparison to the crude extracts. In the EW, CW and HW extracts of *L. digitata*, a moderate increase was observed in the 3.5-100 kDa fractions in comparison to the crude extract. Increased radical scavenging activity was observed in most of the MWCO fractions of both *C. fragile* and *G. gracilis* in comparison to their crude extracts.

The level of FRAP activity differed quite significantly between species. *F. serratus* was the only seaweed that exhibited appreciable FRAP activity, the other three species e.g., *L. digitata, G. gracilis* and *C. fragile* had a low activity. However, similar to the effect outlined for the DPPH
assay above, a significant increase \( p < 0.05 \) was observed in the higher molecular weight (HMW) fractions of each extract. In agreement with other indices of antioxidant activity and TPC content, enrichment enhanced some sample fractions to levels as comparable to crude extracts of other macroalgae species with a good antioxidant activity. In agreement with previous studies by O’Sullivan, (2011) and Jiménez-Escrig et al. (2001), Fucus exhibited the highest FRAP activity. Again, it appears that this activity may be attributed to their phlorotannin content. Little work has been performed on F. serratus to fractionate and identify the molecular regions responsible for the observed activity; however, Tierney et al. (2013) and Wang et al. (2012) investigated the antioxidant activities of MWCO fractions derived from crude extracts of other brown macroalgae including A. nodosum, F. spiralis, P. canaliculata and F. vesiculosus. The authors reported similar results to those presented here, i.e., a significant increase in activity was observed in the HMW fractions (3.5 – 100 kDa and 100 kDa) with F. spiralis EW 3.5 – 100 kDa reported to have a very high FRAP activity of 559.96 µg TE mg\(^{-1}\) sample.

In summary, the results indicated that MWCO fractionation of the seaweed extracts using dialysis tubing of MWCO 3.5 and 100 kDa was an effective tool for significantly increasing the level of antioxidant activity in comparison to crude extracts from the macroalgae species examined in the present study. In particular, for species that would generally be considered to have low levels of antioxidant activity, enrichment enhanced the activity to levels comparable with crude extracts from other macroalgae species. In effect, these enrichment methods resulted in fractions with high antioxidant activity and/or phenolic content being obtained from species with low activity that are comparable to crude extracts from macroalgae with known high antioxidant content, this was best demonstrated in the C. fragile hydrophobic fraction in the TPC assay and the MW > 100 kDa fraction of G. gracilis in the FRAP assay. Kuda et al. (2006) also recently showed that the >5 kDa
fraction from *F. serratus* exhibited the highest DPPH scavenging activity and the highest phenolic content. This study and the results by Tierney *et al.* (2013) are in agreement with the antioxidant activities and phenolic contents reported in Tables 3.1, 3.2, 3.3, and 3.4 where an increase in activity is observed in mainly the HMW fractions. Previous reports by Wang *et al.* (2009) and Tierney *et al.* (2013) have also observed that the extracts containing high levels of TPC were also potent DPPH radical scavengers, thus suggesting that algal phlorotannins may be the principal constituents responsible for the antiradical properties of the extracts. Thus the high antioxidant and phenolic activities observed in *F. serratus* may be due to the presence of high phlorotannins contents that are found in various brown algae species. Studies reported by Tierney *et al.* (2013) and Wang *et al.* (2012) have highlighted high antioxidant activities and high phenolic contents in *Fucus* species in particular *F. spiralis* and *F. vesiculosus* and have attributed these high activities to the presence of phlorotannins.

Whilst TPC, DPPH scavenging and FRAP activities of the <3.5 kDa fractions were considerably lower than those from the 3.5 – 100 kDa and > 100 kDa for all seaweed species, only the brown macroalga *F. serratus* exhibited substantial total phenolic content and antioxidant activity. Therefore, further enrichment of the < 3.5 kDa was only pursued for this species. Further purification of the EW and CW fractions from *F. serratus* was carried out using flash chromatography to enrich the phenolic compounds in the < 3.5 kDa fractions. Water and methanol were used in a two-step gradient to produce two fractions, a polar fraction (presumably rich in low molecular weight sugars) and a phlorotannin-enriched fraction. This enrichment process, it is not suitable for use in food products (uses methanol- and silica-based stationary phase), however, it can be applied to enrich fractions for pharmaceutical use. Use of RP flash chromatography resulted in a threefold enhancement in the TPC in the flash fr.2 for the ethanol/water fraction (FS-EW)
relative to the 3.5 kDa fraction of the extracts. This is presumably due to separation of antioxidant species from removal of low-molecular weight compounds such as sugar from this fraction. A decrease in activity was seen in flash fr.1 for both the CW and EW extracts in comparison to the 3.5 kDa crude fraction. Based on the solvent conditions and the early elution time, this fraction would presumably contain a high proportion of LMW saccharides which could then serve to dilute the phenolic content of this fraction.

Similar results to those obtained for the TPC values of the enriched flash fraction were also noted in the DPPH and FRAP data (Figs. 3.3 and 3.4). Presumably for the reason outlined above there was considerable decrease in activity seen in flash fr.1 for both extracts. Comparable results were reported by Tierney et al. (2013), an increase in TPC, DPPH and FRAP activities was seen in the second flash fractions derived from the <3.5 kDa crude extract due to the presence of low-molecular weight phlorotannins in three brown seaweed, A. nodosum, P. canaliculata and F. spiralis.

Direct infusion of flash fraction 2 into a Q-Tof Premier mass spectrometer was employed to investigate the presence of phlorotannins in the <3.5 kDa fractions of F. serratus cold water (CW) and ethanol/water (EW) extracts. The mass spectrum for FS-EW (fr.2) in the negative ion mode can be seen in Fig. 3.5. The peak signals have been numbered, these numbers correspond to the degree of polymerization for each phlorotannin peak. The degree of phloroglucinol polymerization ranges 6 to 23 phloroglucinol units (PGU) with the most abundant phlorotannins containing between 8 and 13 phloroglucinol units (PGU) (m/z 993.2 to 1613.3). To the best of our knowledge, this is the first study confirming the presence and degree of polymerization of phlorotannins in F. serratus. A number of studies have previously reported the profiling of phlorotannins from other Fucus species such as F. vesiculosus (Steevensz et al., 2011) and F.
spiralis (Tierney et al., 2013). The profile of *F. serratus* reported here is somewhat different to *F. spiralis* where the main abundant phlorotannins were in the region of 5 to 8 phloroglucinol units (m/z 621.1 to 993.2) compared to 8 to 13 PGU for *F. serratus*. This study has provided a valuable insight into the phenolic content and antioxidant activities of both low-molecular weight and high-molecular weight fractions of this species, the MS data indicating that phlorotannins are the main component for this observed activity from this brown macroalgae.

### 3.4 Conclusion

In conclusion, in particular for *F. serratus* whose crude extracts have the highest activity in all assays, 3.5-100 kDa of the ethanol extract (EW) had the highest phenolic content of all food-grade fractions. In addition, the >100 kDa fraction of the ethanol extract (EE) of *F. serratus* exhibited the highest FRAP activity, and the 3.5-100 kDa fraction of the ethanol extract of *F. serratus* had the highest DPPH activity. These observations highlight the significant enrichment achievable for extracts from *F. serratus*, which, considering its appreciable antioxidant activity and phenolic content, could have implications with respect to their use as an antioxidant-rich ingredients. Other species examined in the present study initially exhibited very low levels of antioxidant at the crude extract level. MWCO dialysis was a useful low-cost tool for enriching the antioxidant activity and phenolic content of crude extracts from macroalgae with many of the fractions showing greater than threefold enrichment increases following fractionation. In general, higher molecular cut-off fractions had higher antioxidants activities and phenolic content indicating that the main species responsible for antioxidant activity were most likely to be high-molecular weight phlorotannins. In the case of *F. serratus*, further enrichment of the <3.5kDa fraction using RP flash chromatography enhanced the antioxidant activity and TPC of the extract by removing highly polar
compounds, most likely carbohydrates. The employment of Q-ToF-MS supported the hypothesis that phlorotannins were present in the sample, and the high activity observed in the DPPH, FRAP and TPC assay were due to this group of compounds. The activity seen in the high-molecular weight fractions are presumably due to HMW phlorotannins, which warrants further investigation. The study provides an interesting insight into the possibility of further utilisation of seaweed species that are generally underutilised and the simplicity and low cost of MWCO dialysis to increase the antioxidant activity and phenolic content of crude extracts from macroalgae. These techniques can also be scaled up and require a relatively low capital investment. The development of this profiling method will make it possible to conduct further more detailed analyses of spatial and temporal variabilities of phlorotannins in macroalgae. This analytical methodology will allow for a better understanding of the linkages between phenolic compositions, this along with the bioactivity will provide invaluable information for future targeted applications of seaweed phenolics.
References


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Chapter 4

Profiling of the molecular weight and structural isomer abundance of macroalgae derived phlorotannins

Based on:

Marine Drugs, 13(1), 509-528.
Abstract

Phlorotannins are a group of complex polymers of phloroglucinol (1, 3, 5-trihydroxybenzene) unique to macroalgae. These phenolic compounds are integral structural components of the cell wall in brown algae, but also play many secondary ecological roles such as protection from UV radiation and defense against grazing (Schoenwaelder and Clayton, 1998; Mazid et al., 2011). This study employed ultra-performance liquid chromatography (UPLC) with tandem mass spectrometry to investigate isomeric complexity and observed differences in phlorotannins derived from macroalgae harvested off the Irish coast (Fucus serratus, Fucus vesiculosus, Himanthalia elongata and Cystoseira nodicaulis). Antioxidant activity and total phenolic content assays were used as an index for producing phlorotannin fractions, enriched using molecular weight cut-off (MWCO) dialysis with subsequent flash chromatography to profile phlorotannin isomers in these macroalgae. Using UPLC-MS with multiple reaction monitoring (MRM) the level of isomerization for specific molecular weight phlorotannins between 3 and 16 monomers were determined. The majority of the low molecular weight (LMW) phlorotannins were found to fall within the molecular weight range equivalent to 4-12 monomers of phloroglucinol. The level of isomerization within the individual macroalgal species differed, resulting in substantially different numbers of phlorotannin isomers for particular molecular weights. F. vesiculosus had the highest number of isomers (61) at one specific molecular mass, corresponding to 12 phloroglucinol units (PGU’s). These results highlight the complexity of these extracts and emphasize the challenges involved in structural elucidation of these compounds.
**4.0 Introduction**

Brown macroalgae are a well-known source of structurally unique polyphenols known as phlorotannins derived from the oligomerisation and decoupling of the monomer phloroglucinol (1, 3, 5-trihydroxybenzene) with molecular weights ranging from 126 Da to 100 kDa range (Sailler and Glombitza, 1999; Boettcher and Targett, 1993; McClintock and Baker, 2001). Research suggests that these compounds act as defence compounds within macroalgae against herbivores (Steinberg, 1984; Targett and Arnold, 2001), microbes (Waterman and Mole, 1994; Pavia and Toth, 2000), and the detrimental effects of ultraviolet (UV) radiation (Pavia et al., 1997). Phlorotannins also have allelopathic activity against epibionts (Davis et al., 1989), and are important for cell wall development at early phases of zygote growth in the Fucaceae family (Schoenwaelder 2002; Arnold and Targett, 2003). The relative abundance of phlorotannins (between 5-30 % of the dry weight of the algae) in particular macroalgal species and their known biological activity has stimulated considerable research into their potential uses in a range of therapeutics. Reported bioactivities and beneficial health effects of phlorotannins include antioxidant properties (Fujii et al., 2013; Shibata et al., 2008; Tierney et al., 2013a; Tierney et al., 2013b), anti-allergic effects (Li et al., 2008), anti-inflammatory activity (Kim et al., 2009), anti-HIV-1 activity (Artan et al., 2008), anti-carcinogenic activity (Kong et al., 2009), anti-diabetic activity (Okada et al., 2004), acting as chemopreventative agents (Hwang et al., 2006), anti-plasmin (Fukuyama et al., 1989; Lordan et al., 2013) and HAase inhibitors (Ferreres et al., 2012; Shibata et al., 2002). However, relatively limited characterization of macroalgae derived phlorotannins has been carried out. This is possibly a consequence of the structural complexity which can arise from the polymeric nature of this group of compounds resulting in variation in both the number of monomers present and the positions at which they are linked. Predominantly,
only low molecular weight phlorotannins of 2-8 monomeric units have been characterized in the species *F. vesiculosus* (Fukuyama *et al.*, 1989) and *Ecklonia cava* (Shibata *et al.*, 2002). This represents only a small proportion of the phlorotannins present in *F. vesiculosus* according to reports from Wang *et al.* (2012) who reported that polyphenols in *F. vesiculosus* were found to consist mainly of high molecular weight phlorotannin polymers. Therefore, to realise the full potential of algal phlorotannins a deeper understanding of their structural complexity within individual macroalgal species is required and the availability of advanced chromatographic and mass spectrometric techniques opens up the possibility of more in-depth studies of this isomerically complex group of compounds. In particular the use of ultra-performance liquid chromatography (UPLC) with triple quadrupole tandem mass spectrometry (UPLC-QQQ-MS) couples the improved resolution power of reduced particle size UPLC columns (<2 μm) with the scanning speeds and sensitivity of a triple quadrupole mass spectrometer. UPLC-MS applications for the analysis of phlorotannins from various species of brown algae (*F. vesiculosus*, *Ascophyllum nodosum*, *Pelvetia canaliculata*) have been previously reported in the literature (Fujii *et al.*, 2013; Ferreres *et al.*, 2012). In addition Steevensz *et al.* (2012) reported the level of isomerisation for specific molecular weight phlorotannins between 3 and 16 monomers for six species of seaweed harvested off the Irish coast.

Therefore, the main objective of the present study was to use UPLC with tandem mass spectrometry in multiple reaction monitoring (MRM) mode as a tool to investigate the isomeric complexity of enriched phlorotannins extracts derived from a selection of sustainable macroalgal species harvested off the Irish coast (*F. serratus*, *F. vesiculosus*, *H. elongata* and *C. nodicaulis*). Information of this nature is not only important with regards to describing the isomeric complexity of phlorotannins but to highlight the differences between different macroalgal species which may
present significant stumbling blocks to their possible structural characterisation and use as therapeutic agents.

4.1 Materials and methods

4.1.1 Standards and reagents

All chemicals used were reagent grade, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ferrous chloride, Ferrozine, 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ), and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich Chemical Co. (Arklow, Wicklow, Ireland). All solvents used were HPLC grade. BioDesignDialysisTubing™ with 3.5 kDa cut-off was acquired from Fisher Scientific (Dublin 1, Ireland). Agilent SuperFlash™ SF25-55G C18,50µm were obtained from Apex scientific (Maynooth, Ireland).

4.1.2 Macroalgal samples

The brown macroalgae samples used in this study were identified by a trained phycologist and harvested off the west coast of Ireland. *Fucus serratus* (Fucaceae) was harvested form Finnavarra, Co. Clare in the summer of 2011, *Fucus vesiculosus* (Fucaceae) was harvested from Spiddal, Co. Galway in the autumn of 2010, *Himanthalia elongata* (Himanthaliaceae) was harvested from Finnavarra, Co. Clare in spring 2010 and *Cystoseira nodicaulis* (Fucaceae) was harvested from Finnavarra in the summer of 2012. A random selection of a large number of different plants were taken from the shore, to allow for natural variability, these were packed in cool boxes and transported immediately to the laboratory. Samples were washed thoroughly with fresh water to remove sand and epiphytes and were then stored in the freezer at -20 °C. A freeze dried sample of each was retained for reference at the Irish Seaweed Centre at NUIG. The macroalgal samples
were subsequently freeze dried, ground to a powder using a Waring® blender (New Hartford, CT, USA) and stored in vacuum packed bags at -80 °C prior to extraction.

4.1.3 Solid-liquid extraction (SLE)

Solid-liquid extraction was employed to extract the phlorotannins from the macroalgae under investigation using ethanol/water (80:20) as this solvent system has previously been shown to be effective for extracting phlorotannin compounds from macroalgae (Audibert et al., 2010; Heffernan et al., 2014a; Heffernan et al., 2014b). Method previously described in Chapter 3 section 3.1.3.

4.1.4 Partitioning and molecular weight cut-off (MWCO) dialysis

A hydrophilic fraction of the freeze dried powder was prepared by exhaustively extracting the powder with HPLC-grade water (20 mL), method previously described in Chapter 3 section 3.1.4.

4.1.5 Reversed-phase flash chromatography

The lower molecular weight (<3.5 kDa) fraction of each species was further fractionated using a two-step reverse phase (RP) flash chromatography method as described in Chapter 3 section 3.1.5 and Tierney et al. (2013).
4.1.6 Total phenolic content (TPC)

TPC of fraction 2 was quantified according to the method of Singleton et al. (1999) as previously described in Chapter 2 section 2.1.5.

4.1.7 In-vitro antioxidant activity

4.1.7.1. Ferric reducing antioxidant power (FRAP)

FRAP of the low molecular weight phlorotannin fractions were assessed according to (Stratil et al., 2006) with slight modifications (Tierney et al., 2013b) as previously described in Chapter 2 section 2.1.6.1.

4.1.7.2. DPPH (2, 2-diphenyl-1-picryl-hydrazyl) scavenging activity

The free-radical scavenging capacity of the phlorotannins fractions were analyzed using the DPPH assay according to Goupy et al. (1999) with slight modifications (Kenny et al., 2013) as previously described in Chapter 2 section 2.1.6.2.

4.1.8 Statistical analysis

All phlorotannin fractions were analysed in triplicate. Measurement values are presented in means ± standard deviation. One way analysis of variance (ANOVA), followed by the Tukey post hoc comparison test, was carried out to test for significant differences in antioxidant activity and phenolic content between macroalgal species using the statistical program Minitab® Release 15 for Windows. A probability value of $p < 0.05$ was considered statistically significant.
4.1.9 **UPLC-ESI-MS of phlorotannin enriched fractions**

UPLC-MS analysis of *F. serratus*, *F. vesiculosus*, *C. nodicaulis* and *H. elongata* ethanol/water phlorotannin enriched fractions (prepared as described in sections 3.1.3-3.1.5) was undertaken using an Acquity™ UPLC® System (Waters Corporation, Micromass MS Technologies, Manchester, UK) (Tierney et al., 2013). The system comprised of a binary pump solvent manager with the ability of generating pressures up to 15,000 psi, coupled with an Acquity™ TQD-MS (Waters Corp.) It was run in electrospray negative ion mode with multiple reactions monitoring (MRM) which was developed and optimized with a phlorotannin enriched sample using the IntelliStart™ software (Waters Corp.) according to molecular masses of phlorotannins containing 3-16 (maximum permitted with TQD) units of phloroglucinol. For separation a Waters Aquity™ HSS PFP column (100Å, 1.8 µm particle size, 2.1 mm x 100 mm) was used and maintained at 40 °C. The mobile phase consisted of 0.1 % formic acid in an aqueous solution (A) and acetonitrile with 0.1 % formic acid (B). The flow rate was set at 0.5 mL/min and injection volume was 5 µL. Elution was performed as follows: 0.5 % B from 0 to 10 min, 0.5-30 % B from 10 to 26 min, 30-90 % B from 26 to 28 min, and 0.5 % B from 28 to 30 min. The parameters from the MS were as follows: capillary voltage, 2.8 kV; source temperature, 350 °C; desolvation temperature, 50 °C; desolvation gas, 800 L/h (Ferreres et al., 2012).

4.2 Results and Discussion

4.2.1. **Total phenolic content (TPC) and in-vitro antioxidant activities**

Ethanolic extracts were partitioned exhaustively using HPLC grade water to obtain a hydrophilic fraction, which was freeze dried. This hydrophilic fraction was dissolved in a minimal volume of water and fractionated by molecular weight cut-off (MWCO) dialysis using a 3.5 kDa membrane.
Dialysis was used to fractionate the extracts in order to isolate the low molecular weight regions that could be responsible for activity and also to remove unwanted polysaccharides from the extracts. Phlorotannins were isolated from the low molecular weight (< 3.5 kDa) fraction using a reversed–phase (RP) flash chromatography system (Tierney et al., 2013b). The TPC and antioxidant activity of the enriched phlorotannin fraction from each species were tested to investigate if the activity observed is due to the high abundance of low molecular weight phlorotannins in the extracts. The level of phenols in the flash chromatography enriched fraction from the four seaweed species (F. vesiculosus, F. serratus, H. elongata and C. nodicaulis) is presented in Table 4.1. The macroalgae with the highest TPC was F. vesiculosus (231.95 ± 8.97 μg PGE/mg sample), followed by F. vesiculosus, H. elongata, F. serratus and C. nodicaulis, however there was no significant difference observed between these species. Wang et al. (2009) also reported high levels of TPC in 70% acetone crude extracts of F. serratus (24.0 g PGE/100g) and F. vesiculosus (24.4 g PGE/100g).

Similar trends were observed for DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and FRAP (Ferric reducing antioxidant power) enriched fractions with F. vesiculosus having the highest FRAP (307.27 ± 1.22 μg Trolox Equivalent (TE)/mg sample) and DPPH (IC\textsubscript{50} value of 4.00 ± 0.01 μg/mL). H. elongata had the next highest FRAP activity (130.89 ± 0.45 μg TE/mg sample) with both C. nodicaulis and F. serratus having slightly lower levels of 101.35 ± 0.36 μg TE/mg sample and 110.94 ± 0.65 μg TE/mg sample, respectively.

Wang et al. (2009) also found that F. vesiculosus had the highest radical scavenging ability in 70 % acetone extracts (EC\textsubscript{50}= 10.7 x 10\textsuperscript{-3} mg/ml) when compared to 11 other macroalgae species (Wang et al., 2009). Overall F. vesiculosus had significantly higher (p<0.05) TPC, DPPH and FRAP activities when compared to the other species investigated. Previous results have shown that
higher molecular weight fractions (3.5-100 kDa) exhibited greater antioxidant and TPC potential than lower molecular weight fractions (3.5 kDa) (Heffernan et al., 2014a). However using reverse phase flash chromatography on the lower molecular weight fraction (< 3.5 kDa) a phlorotannin enriched fraction could be obtained which had increased TPC and antioxidant activity.

Table 4.1 Total phenolic content (TPC) expressed as μg phloroglucinol equivalents (PE), Ferric reducing antioxidant power (FRAP) expressed as μg trolox equivalents (TE) and 2,2-diphenyl-1-picryl-hydrazl(DPPH) radical scavenging ability (RSA) expressed as IC\textsubscript{50} mg/ml of ethanol/water extracts of four macroalgae samples.

<table>
<thead>
<tr>
<th>Seaweed Species</th>
<th>TPC (μg PE/mg sample)</th>
<th>FRAP (μg TE/mg sample)</th>
<th>DPPH (IC\textsubscript{50} µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fucus vesiculosus</em></td>
<td>231.95 ± 8.97\textsuperscript{a}</td>
<td>307.27 ± 1.22\textsuperscript{a}</td>
<td>4.00 ± 0.01\textsuperscript{b}</td>
</tr>
<tr>
<td><em>Himanthalia elongata</em></td>
<td>198.28 ± 9.17\textsuperscript{b}</td>
<td>130.89 ± 0.45\textsuperscript{b}</td>
<td>14.00 ± 0.04\textsuperscript{a}</td>
</tr>
<tr>
<td><em>Cystoseira nodicaulis</em></td>
<td>89.14 ± 2.57\textsuperscript{c}</td>
<td>101.35 ± 0.36\textsuperscript{b}</td>
<td>28.00 ± 0.01\textsuperscript{a}</td>
</tr>
<tr>
<td><em>Fucus serratus</em></td>
<td>180.55 ± 16.98\textsuperscript{b}</td>
<td>110.94 ± 0.65\textsuperscript{b}</td>
<td>19.00 ± 0.03\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Values represented as mean ± standard deviation of three assays, performed in triplicate. Different superscript letters (a, b, c, etc) column wise indicate significant difference Tukey’s HSD test.

4.2.2 UPLC-QQQ-MS profiling of phlorotannin enriched fractions

Figure 4.1 shows a total ion chromatogram (TIC) of reversed phase (RP) flash chromatography enriched fractions of the four species under investigation, illustrating the structural complexity of fraction. The characterisation of phlorotannins, which in theory could be generated by indiscriminate coupling during biosynthesis, is taxing due to the difficulty in separating complex isomeric mixtures with identical molecular weights and similar structures into individual compounds (Wang et al., 2009). Due to the presence of large numbers of isomers present at each level of DP present in particular species it is unlikely that the isolation and subsequent characterisation of their structure will be attainable in the near future. Profiling of the isomeric
mixtures alone has been limited to date and is a more feasible aim for these species (Koivikko et al., 2007), particularly when individual structural elucidation of each phlorotannin would not be possible with current chromatographic techniques.

**Figure 4.1** Total Ion chromatogram (TIC) of Ethanol/water reverse phase (RP) flash chromatography enriched fractions of a *Fucus serratus* b *Fucus vesiculosus* c *Himanthalia elongata* d *Cystoseira nodicaulis*.

As illustrated in Figure 4.1a the majority of the species present in *F. serratus* enriched flash fractions had molecular weights between 745.2- 1689.5 m/z most likely corresponding to 6-12 phloroglucinol units. Similar to *F. serratus*, the TIC of *H. elongata* (Figure 4.1c) shows that the majority of the phlorotannins had molecular weights broadly in this range also. The most
predominant peak is observed in this species occurred at m/z 1,117 (DP of 9), with other noticeable peaks being observed at m/z 1,241 (DP of 10), 1,365 (DP of 11) and 1,490 (DP of 12). *F. vesiculosus* (Figure 4.1b) displays a very different profile to that observed for *F. serratus* and *H. elongata* with the most prominent peaks observed in this enriched fraction at m/z 497 (DP of 4), 621 (DP of 5), 745 (DP of 6) and 869 (DP of 7) i.e. a lower molecular weight range than that of the other two species previously discussed. The profile for *C. nodicaulis* Figure 4.1d is also different to the other species in this study displaying both a range of very low molecular weight phlorotannin polymers along with higher molecular weight phlorotannins. For example the most prominent peaks are m/z 993 (DP of 8), 1,117.40 (DP of 9), 1241 (DP of 10), 1365.40 (DP of 11). MS analysis has been performed previously on some of these species investigated in this study, i.e., *F. vesiculosus* and *C. nodicaulis* (Wang et al., 2012; Ferreres et al., 2012), however none have demonstrated successful separation of polymeric phlorotannins with greater than ten phloroglucinol units. Previous reports have suggested that the molecular weight of phlorotannins influence the antioxidant activity they possess. For example Hagerman et al. (1998) reported that high molecular weight condensed (plant) tannins exhibited antioxidant activities 15-30 times greater than simple phenolics and Trolox. They hypothesized that this activity was due to the presence of many aromatic rings and hydroxyl groups which improve the free radical scavenging ability of these compounds. Fan et al. (1999) also investigated the antioxidative properties of high molecular weight polyphenols isolated from the brown seaweeds *Sargassum kjellmanianum*. The results of their study indicated that hydroxyls in phlorotannins are hydrogen donors and the vicinal trihydroxyl is a more active hydrogen donor than meta-trihydroxyl. They thus concluded that the hydroxyl radical scavenging capacity is closely related to the number of sites in phlorotannin benzene rings available for the addition of hydrogen radicals. Wei et al. (2003) also investigated
the antioxidant activity of high molecular weight phlorotannins from the brown seaweed *Sargassum kjellmanianum* as well as *Sargassum thunbeergiikutze* and found them to show strong scavenging ability. However in the present study samples containing predominantly lower DP exhibited the greatest activity. For example *F. vesiculosus* contained mostly DP 4-7 and had a significantly greater antioxidant activity than the other three species investigated composed of DP 9-12. However, this is most likely due simply to the greater abundance of phenolic species in this extract as illustrated in Table 4.1 rather than a higher relative activity of low molecular phlorotannins.

Whilst there is little clarity as to the reason behind the different degrees of polymerisation exhibited by macroalgae derived phlorotannins it may be related to the greater number of resonance stabilisation sites present in higher molecular weight compounds thus giving them stronger antioxidative properties. Therefore the degree of polymerisation and the resulting number of isomers could therefore arise from *in-situ* production in response to stresses such as UV radiation, herbivore predation and changes in salinity for intertidal species. However further investigation would be necessary to confirm this hypothesis.

The UPLC-QQQ-MS method employed in this study resulted in the detection of phlorotannins in the range of 374 to 1,986 Daltons, using a tandem quadrupole MS in MRM mode for increased sensitivity and for isomer detection. By individually tuning the theoretical molecular weights of phlorotannins while infusing a complex phlorotannin mixture using the Intellistart™ software, combined with the manual tuning, suitable MRM fragments for each phlorotannin up to 16 phloroglucinol units in negative ionization mode could be determined. It was apparent that the level of phlorotannin isomerisation across species differed to some degree. This difference may be due to possible structural features of the phlorotannins present and the species specific biosynthetic
mechanisms. The monomeric units in phlorotannins are linked through aryl–aryl bonds and diaryl bonds, forming different subgroups of phlorotannins (Glombitza et al., 2003). When aromatic rings are connected purely by aryl–aryl bonds, a group of fucols is formed (Figure 4.2). Phlorethols are formed solely by aryl ether bonds (Figure 4.2). Fuhalols are constructed by phloroglucinol units that are connected with para- and ortho-arranged ether bridges containing one additional OH-group in every third ring (Figure 4.2). When there exists at least one three-ring moiety with a dibenzodioxin elements substituted by a phenoxy group at C-4, the group is named eckols (Figure 4.2). Carmalols are further derivatives of phlorethols containing a dibenzodioxin moiety. Endofucophlorethols and isofuhalols (Figure 4.2) are small, distinct, specialized groups. Figure 4.3 displays the molecular ion chromatograms for the MRM transitions of phlorotannins from F. serratus, highlighting the abundance of isomers present for individual deprotonated molecules. A high number of isomers were detected for individual molecular ions in the species F. serratus with 178 isomers in total being detected between DP 6 and DP 14 with DP 12 having the highest isomer number of 42. This would suggest a significant variation in branching positions is occurring between the monomeric units. Figure 4.4 presents a more detailed view of F. serratus MRM corresponding to DP of 12 (MS/MS 1,489.5-229.1 transition) to show distinctly the complexity of isomerisation occurring. Similar to what was observed for F. serratus, H. elongata has a large number of isomers between DP 7 and 12 units, 96 isomers in total were detected in this range with DP 8 having the highest isomer content of 32. Unlike F. serratus and H. elongata, F. vesiculousus phlorotannins eluted at early and the most prominent peaks were observed in the lower molecular range at DP 4 to 9. There were still a significant number of isomers observed in this range with 144 isomers detected in total. C. nodicaulis was similar to F. vesiculousus as in the phlorotannins eluted in low percentages of organic solvent. The most prominent peaks are in the range DP 8-11.
A lower number of isomers were observed for each deprotonated molecules [M-H]−. A total of 48 isomers were detected between the range DP 4-11. Due to the low level of isomers detected it may be assumed that there is less variation in branching between monomeric units.

The peak intensities and also the number of isomers for each species can be viewed in Figure 4.5. *F. serratus* and *C. nodicaulis* shows similar results with 90% of the phlorotannins being observed between 3-12 and 3-10 PGU’s, respectively. In the species *F. vesiculosus* the percentage peak intensities observed highlight that the majority of the phlorotannins are of lower molecular weight and upto 90% of the phlorotannins are found to be between 3-8 PGU’s. In *H. elongata* up to 80% of the phlorotannins found were between 7-11 PGU’s. Previous research to date on the isolation and characterisation of individual phlorotannin compounds from some of these species has been limited (Shibata *et al.*, 2002; Fujii *et al.*, 2013; Wang *et al.* 2009), this study is the first to highlight the complex nature of these compounds in relation to the number of isomers present even in these low molecular weight fractions. This outcome also draws attention to the considerable amount of research that would be required for complete characterization of some of the more complex species such as *F. serratus* and *H. elongata*.

To date only partial analysis of phlorotannin, in a lower molecular weight range, has been conducted on the species *F. vesiculosus, C. nodicaulis* and *H. elongata*, while no phlorotannins have been characterized from the species *F. serratus*. Ferreres *et al.* (2012) used HPLC-DAD-ESI-MS to analyse phlorotannins from the species *C. nodicaulis* with phlorotannin structures ranging from 499-743 m/z (4-6 PGU’s) being detected. Koivikko *et al.* (2007) detected a phlorotannin tetramer (497 m/z or 4 PGU’s) in the species *F. vesiculosus* using normal phase separation. Grosse-Damhues *et al.* (1983) isolated and characterised an eight ring phlorotannin from the brown species *H. elongata* using field desorption mass spectrometry. The present study has identified
phlorotannins at much higher degrees of polymerization i.e., up to 16 PGU’s along with observations with regard to the number of isomers for each particular molecular ion illustrating the complex nature of several of the species investigated. At present only a small number of phlorotannin compounds have been isolated and chemically characterised from these four macroalgae species. In the species *H. elongata* just one compound difucophlorethol has been isolated and the structure elucidated at the molecular mass 498.399 (PGU’s 4) using a combination of both MS and NMR (Grosse-Damhues *et al.* 1983). Only two compounds fucotetraphlorethol A (Glombitza and Grobe-Damhues, 1985) (Figure 4.5) and fucotetraphlorethol B have been reported at the molecular mass 746.591 (PGU’s 6) in the species *H. elongata*. However, for this molecular weight 22 isomers were identified which would suggest that there are still a substantial number of unreported structures requiring isolation and identification for this particular mass in this species. At the molecular mass 622.49 (PGU’s 6) just one compound has been identified fucotriphloroethol C (Glombitza and Grobe-Damhues, 1985) in the species *H. elongata*. In the species *F. vesiculosus* a greater number of phlorotannin compounds have been reported. For example Pary *et al.* (2010) isolated two phloroglucinol derivatives, belonging to the class of fucophlorethols along with the previously known fucotriphlorethol A from the ethanolic extracts of the brown macoralgae *F. vesiculosus* and investigated the chemopreventative potential of these phlorotannin compounds. Two compounds, fucodiphlorethol A and fucodiphlorethol E (Koivikko, 2008) have been isolated and structurally elucidated using MS and NMR with the molecular mass 498.399 (PGU’s 4) and five compounds have been characterized at the molecular mass 746.591 (PGU’s 6), fucotetraphlorethol C, D, E, F and G (Liu and Cu, 2012). This study has identified 13 isomers for this particular molecular weight range demonstrating the limited amount of structural elucidation that has occurred for these marcoalgal species. To date limited research has been carried out on
the identification of phlorotannin compounds from the species *F. serratus* without details on the level of isomers, while studies on *C. nodicaulis* have not been reported. This is most probably due to the complex nature of these species and also the level of isomerisation observed. Previous reports on phlorotannin compounds have merely suggested that they are responsible for observed activities (Wang *et al.*, 2012; Li *et al.*, 2011; Pavia and Toth, 2000; Arnold and Targett, 2003). Previous work done by this group (Heffernan *et al.*, 2014a) generated tannin enriched fractions using reversed phase flash chromatography and using LC-QTof-MS identified a high abundance of low molecular weight phlorotannins in the <3.5 kDa fractions from the species *F. serratus*. The only reports of work on the species *C. nodicaulis* was carried out by Ferreres *et al.* (2012) where eight compounds in the *m/z* range of 497-743 (Fucophlorethol, tridiphloroethol, fucotriphloroethol, 7-phloroekol, phlorofucofuroeckol and bieckol/dieckol) were detected in a range of brown algae species including *C. nodicaulis*. They observed similar results to the present report, three isomers were detected for the molecular ion 499 (PGU’s 4), compared to 2 isomers in this study. However, this study detected phlorotannin compounds in this species between the range of 3-16 PGU’s thus providing full profiling of the low molecular weight phlorotannins present in this species.
Figure 4.2 Chemical structures of a selection of phlorotannins.
Figure 4.3 UPLC-MS/MS MRM ion chromatograms of reversed-phase (RP) flash chromatography enriched fraction of *Fucus serratus* for deprotonated molecules [M-H] of 745.3, 869.3, 993.4, 1117.4, 1242.4, 1365.4 and 1489.4 m/z which correspond to phlorotannins of 6, 7, 8, 9, 10, 11 and 12 monomers in size respectively.
Figure 4.4 UPLC-MS/MS MRM extracted ion chromatogram of *Fucus serratus* phlorotannin isomers at the selected deprotonated molecules [M-H]$^-$ of 1490 $m/z$ which corresponds to a phlorotannin consisting of 12 PGUs in size.
Figure 4.5 UPLC-MS percentage peak intensity for individual molecular ions corresponding to phlorotannins of between 3 and 16 phloroglucinol units (PGUs) for phlorotannin enriched fraction from four species of macroalgae. Isomers for selected phlorotannins detected from each brown algal species and extracts are highlighted with bold numbers. *Fucus serratus* (FS), *Fucus vesiculosus* (FV), *Cystoseira nodicaulis* (CN), *Himanthalia elongata* (HE).
Figure 4.6 Chemical structure of a range of isomers (fucotetraphlorethol A-G), with degrees of polymerisation of 6.

4.3 Conclusion

This present study profiled the phlorotannin metabolite composition in macroalgae derived phlorotannins and determined the degree of isomerisation of compounds between 3-16 phloroglucinol monomers in four species (*F. serratus*, *F. vesiculosus*, *H. elongata* and *C. nodicaulis*). In general a significantly higher number of isomers were detected corresponding to phlorotannins of much higher degree of polymerization (up to 16 monomer units) in comparison to previous reports. The majority of the LMW phlorotannins found in *F. serratus*, *F. vesiculosus*, *C. nodicaulis* and *H. elongata* had molecular weight ranges from 6-12, 4-8, 7-12 and 4-11 monomers, respectively. Purification of extracts using MWCO dialysis and reversed phase flash chromatography for the removal of carbohydrates and combined with the use of a PFP column
during UPLC-MS analysis allowed for distinctive metabolite profiles to be elucidated and polymeric phlorotannin isomers to be detected. This allowed for a more complete picture of the phlorotannin composition in individual macroalgae species. The work has demonstrated that even in relatively LMW fractions, these molecules are highly complex and this complexity poses a real challenge in terms of further purification and chemical characterisation of individual compounds.

Separation of longer condensed tannins is a difficult task and is complicated by the increase in the number of isomers with increasing polymer length. At present, there are no reports suggesting branching may influence biological activity of phlorotannins. However, the results presented here have suggested that due the complex level of isomerization demonstrated within these species, investigation to link biological activity to a particular isomer may be a difficult task. In addition the biological rationale behind this structural complexity has yet to be elucidated if there is one. As well as molecular weight, seasonal variation may also affect the profile and biological activity of phlorotannin profiles. However, variations in phlorotannin concentrations among the development stages of brown algae have been reported (Koivikko, 2008). Further study may also highlight particular environmental factors that may affect the phlorotannin metabolite profile in individual macroalgal species. The present study has highlighted that UPLC-MS/MS can serve as a valuable tool for investigating factors that influence the range of molecular weights and isomeric complexity present in macroalgae derived phlorotannins.

This information also serves to highlight that although research in this area has been carried out for several decades there are still significant efforts required to gain greater understanding of these complex molecules in relation to their structure, bioactivity and exploitation.
References


Chapter 5

Seasonal and geographical variation of the nutritional composition and polyphenol content of four Irish macroalgal species
Abstract

Four seaweeds *Codium fragile* (green), *Laminaria digitata*, *Fucus serratus* (both brown) and *Gracilaria gracilis* (red) were collected from the west coast of Ireland over four seasons (2010-2012) and their macro composition, protein, lipid, ash and soluble and insoluble dietary fibre contents were determined. The mean protein content over the four seasons was highest in the winter sample of the red seaweed *G. gracilis* (28.70% Dry Weight Content (DWC)). The lowest protein content was present in the autumn sample of the brown seaweed *F. serratus* (4.91% DWC). In all cases the protein content is highest during winter. The lipid content of the macroalgae ranged from 0.72% DWC in the brown seaweed *L. digitata* to 2.81% DWC in the green seaweed *C. fragile*. Lipid content showed no apparent trend in seasonal variation. The ash content of all macroalgae species was relatively high ranging from 14.35% DWC in the summer sample of *F. serratus* to 44.70% DWC in the winter sample of *C. fragile*. Ash content was highest in winter for both brown macroalgal species (*L. digitata* and *F. serratus*). For *C. fragile* the maximum ash content was observed in autumn while for the red species *G. gracilis* was observed to have the highest ash content in spring. The brown macroalgae *F. serratus* and *L. digitata* had the highest carbohydrate content of 75-80 %, with the majority of species reaching maximum carbohydrate content at autumn and spring periods. Insoluble dietary fibre (IDF) was high in all seaweed species ranging from 17.25 % in *C. fragile* to 55.55 % in *L. digitata*. *F. serratus and L. digitata* contained the highest levels during spring. Overall the soluble dietary fibre (SDF) was higher in the brown macroalgae compared to both the green and red macroalgae and at its highest during the spring and summer seasons. The total phenolic content (TPC) content in the species *F. serratus* was analysed and was found to be highest during the summer period and lowest in the autumn. This may be contributed to by factors such as this period being the growth period for the plant. In
general the variation in composition observed in the macroalgae investigated was related to environmental factors, such as light intensity, temperature, salinity and nutrient availability.

5.0 Introduction

Marine macroalgae are a primitive group of plants, found growing abundantly in shallow waters of the sea, estuaries and backwaters. They can be classified into three groups: green, brown and red based on pigmentation. In the Far East and Asian Pacific, there is a long tradition of consuming seaweeds (Dawaczynski et al., 2007), whilst seaweed consumption in Western countries is increasing. From a commercial perspective the principal use of seaweeds is as a source of phycocolloids and thickening and gelling agents in foods (Abbott, 1996; Darcy-Vrillon, 1993; Mabeau and Fleurence, 1993). A lot of current interest has concentrated on seaweeds as novel sources of compounds with the potential for retarding the onset of certain diseases like chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Park et al., 2004). In addition to their disease retarding potential seaweeds are recognised as an excellent nutritional source as they contain high levels of proteins, essential amino acids, minerals and vitamins (Ortiz et al., 2006, Pereira, 2011). However, if seaweeds are to be harvested or cultured as a source of these nutrients, knowledge of the temporal variation in their macro composition is essential for the assessment of their nutritional value (Hawkins and Hartnoll, 1983), and for the evaluation as potential sources of proteins, carbohydrates and lipids for commercial use (Chapman and Chapman, 1980).

Seaweeds often reside in hostile intertidal habitats and therefore must continuously adapt to changes in salinity, sunlight and temperature by adjusting the proportions of nutrients they contain. For example, the chemical composition and abundance of carbohydrates vary among seaweed species, habitat, maturity and environmental conditions (Ito and Hori, 1989). Seasonal
variation in macro composition has been reported in common marine macroalgae from Hong Kong (Kaehler and Kennish, 1996), coastal India (Kumar, 1993) and Ireland (Mercer et al., 1993). The composition of edible seaweeds from some regions of the world has been well documented but no reports are available on the nutritive value of Irish macroalgal species including those under investigation in the present study (L. digitata, F. serratus, C. fragile and G. gracilis). Thus, the aim of the present study was to monitor the level of ash, protein, total lipids, carbohydrate, moisture and polyphenols of these species over a period of 2 years and at 2 locations to determine the best time for harvesting seaweed for the extraction of specific components.

5.1 Materials and methods

5.1.1 Chemicals

Ethanol, acetone, Hydrochloric acid, ethanol, petroleum spirits, ethyl ether, sodium azide, MES (2-(N-Morpholino) ethanesulfonic acid), TRIS (Tris (hydroxymethyl) aminomethane) and diatomaceous earth were obtained from Sigma Aldrich Ireland Ltd. (Co. Wicklow, Ireland). Fibre filter bags (DF-S, DF-FT) were obtained from ANKOM Technology (Macedon, New York, USA). The enzymes α-amylase, protease and amyloglucosidase were obtained from Megazyme International Ireland (Co. Wicklow, Ireland).

5.1.2 Sampling, collection and storage of macroalgae

The four seaweed species investigated in this study were F. serratus, G. gracilis, C. fragile and L. digitata. Several kilograms of whole seaweed plants of F. serratus and L. digitata were collected to reduce plant to plant variation from the west coast of Ireland in Finnavarra, Co. Clare (53° 9' 5" North, 9° 6' 2" West) and Spiddal, Co. Galway (53° 14' 48" North, 9° 18' 10" West) over a period
of a year from 2011-2012. Similarly *C. fragile* and *G. gracilis* were collected as whole plants from Finnavarra, Co. Clare over a period of one year from 2010-2012. For both brown algae *F. serratus* and *L. digitata* four samples were collected at each location (Table 5.1). For the red algae *G. gracilis* and the green algae *C. fragile* five samples were collected at 1 location. A random selection of different plants were taken from the shore, to allow for natural variability, packed in cool boxes, and transported immediately to the laboratory. Samples were washed with water to remove sand and epiphytes and were then stored at -18 °C. The taxonomy of all samples was verified by a trained phycologist and a dried reference sample for each species was stored in NUI Galway. Samples were freeze dried, then ground into a powder using a Waring ® blender and stored in vacuum packed bags at -80 °C prior to extraction.
Table 5.1  Dates and locations of sampling of four Irish seaweeds off the coast of Galway, Ireland.

<table>
<thead>
<tr>
<th>Seaweed</th>
<th>Location</th>
<th>Season</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Laminaria digitata &amp; Fucus serratus</em></td>
<td>Spiddal</td>
<td>Autumn</td>
<td>28&lt;sup&gt;th&lt;/sup&gt; September 2011</td>
</tr>
<tr>
<td></td>
<td>Finnavarra</td>
<td>Autumn</td>
<td>28&lt;sup&gt;th&lt;/sup&gt; September 2011</td>
</tr>
<tr>
<td></td>
<td>Spiddal</td>
<td>Winter</td>
<td>24&lt;sup&gt;th&lt;/sup&gt; January 2012</td>
</tr>
<tr>
<td></td>
<td>Finnavarra</td>
<td>Winter</td>
<td>24&lt;sup&gt;th&lt;/sup&gt; January 2012</td>
</tr>
<tr>
<td></td>
<td>Spiddal</td>
<td>Spring</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; May 2012</td>
</tr>
<tr>
<td></td>
<td>Finnavarra</td>
<td>Spring</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; May 2012</td>
</tr>
<tr>
<td></td>
<td>Spiddal</td>
<td>Summer</td>
<td>19&lt;sup&gt;th&lt;/sup&gt; July 2012</td>
</tr>
<tr>
<td></td>
<td>Finnavarra</td>
<td>Summer</td>
<td>20&lt;sup&gt;th&lt;/sup&gt; July 2012</td>
</tr>
<tr>
<td><em>Gracilaria gracilis &amp; Codium fragile</em></td>
<td>Finnavarra</td>
<td>Autumn</td>
<td>8&lt;sup&gt;th&lt;/sup&gt; October 2010</td>
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<td></td>
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<td>Winter</td>
<td>23&lt;sup&gt;rd&lt;/sup&gt; November 2010</td>
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<td>Finnavarra</td>
<td>Spring</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; February 2011</td>
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<td>Autumn</td>
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<td></td>
<td>Finnavarra</td>
<td>Winter</td>
<td>23&lt;sup&gt;rd&lt;/sup&gt; January 2012</td>
</tr>
</tbody>
</table>

5.1.3 Analytical methods

5.1.3.1 Ash content

Freeze dried samples in a pre-weighed crucibles were placed into a muffle furnace at 550 °C for more than 4 hrs. After ashing was complete the samples were removed from the furnace and placed in a desiccator to cool to room temperature. All samples were carried out in duplicate. The crucible was then reweighed and the ash content (% of dry weight) was determined using the equation (1) below;

\[
\text{% Ash} = \frac{M_2 - M_0}{M_1 - M_0} \times 100
\]

Where

\( M_0 \) = mass of dish (g)

\( M_1 \) = mass of dish and test sample (g)

\( M_2 \) = mass of dish and ash (g)
5.1.3.2 Total lipid content

The total lipid content was determined according to the AOAC method 922.06 (AOAC, 1990) with slight modifications. The freeze dried powdered seaweed sample (2g) was weighed into a 100 mL graduated cylinder. Ethanol (2 mL) and concentrated HCl (37 % v/v) (10 mL) was added and the solution was mixed. A stopper was placed on the cylinders and the cylinders were placed into a water bath at 80 ºC for approximately 10-12 hr. Cylinders were removed and allowed to cool. Ethyl ether (25 mL) was added and the cylinders were gently shaken; 25 mL of petroleum spirits was then added to the mixture followed by gentle shaking. Pre-weighed evaporating dishes were placed onto a boiling water bath and the ether layer was transferred from the graduated cylinder into the evaporating dish using a 10 mL pipette. A further 25 mL of ethyl ether was added to the remaining sample and the mixture was agitated and then allowed to separate. The ether layer was again transferred into the evaporating dish, this step was repeated 3 times. The petroleum layer was discarded. When the entire ether layer had evaporated the dishes were placed in an oven (105 ºC) to dry for 1h. The dishes were then placed in a desiccator to cool and when cool were weighted and the fat content calculated. All samples were carried out in duplicate.

5.1.3.3 Total protein content

Protein content was determined by the combustion method based on the Dumas principle (Dumas, 1826) using a FP-328 Leco nitrogen analyser (Leco Corporation, St Joseph, Michigan, USA). A standard compound ethylenediamine tetraacetic acid (9.57% N), was also run during analysis. Approximately 0.2 g of the freeze dried powdered sample was weighed and loaded into the loading head of the Leco. Combustion of the samples took place in a sealed furnace at 1,150 ºC. Whilst flushing with oxygen to ensure rapid combustion. All gas products in the ballast chamber were
allowed to become a homogenous mixture at a pressure of approximately 975 mm and a constant
temperature of 1,150 °C. A 10 cc aliquot of the sample mix was collected through the Lecosorb ®
and Anhydrone ® to remove water and CO₂. The remaining gas product nitrogen was carried by
helium to a thermal conductivity cell where it was quantified. Protein content was expressed as
weigh percentage of the total freeze dried sample. All samples were carried out in duplicate.

5.1.3.4 Carbohydrate content
Total carbohydrate content was determined as the remaining element once the protein, fat,
moisture and ash content have been summed.

5.1.3.5 Fibre analysis (soluble and insoluble)
Soluble (SDF) and insoluble dietary fibre (IDF) were determined using the ANKOM® Dietary
Fibre Analyzer (Macedon, New York, USA) according to AOAC method 991.43 (AOAC, 1995).
Pre-weighed bags were clamped into position and 1 g of diatomaceous earth was added to the
soluble dietary fibre bags and 1.5 g of crude seaweed sample was added to the insoluble dietary
fibre bags. Once the automated IDF and SDF process was complete the residue was rinsed with
acetone through the filter at the bottom of each bag. The ash content and protein content of the
residue of each sample was determined as described above. All samples were carried out in
duplicate. When the protein and ash determinations were complete, the % IDF and % SDF of each
sample was determined using equations (2) and (3) below
\[
\frac{\left(\frac{(fB_{F1} - fB_{S1}) + (fB_{F2} - fB_{S2})}{2}\right) - P_B - A_B}{(M_1 + M_2)/2} \times 100 \]

\[
\frac{\left(\frac{(R_1 + R_2)}{2} - (P - A_B)\right)}{(M_1 + M_2)/2} \times 100
\]

M_1, M_2 = Original wt for duplicate samples adjusted for pre-treatment fat and sugar losses (g).
R_1, R_2 = Residue for duplicate samples (g).
\(f_f\) = Final filter bag (g)
\(F_s\) = Initial filter bag (g)
P = protein of residue and bag (g)
A = Ash of residue and bag (g)
B = Blank (g)
\(B_{R1}, B_{R2}\) = Residue for duplicate blanks (g)
\(f_{Bf}\) = Final Blank Filter Bag (g)
\(f_{BS}\) = Initial Blank Filter Bag (g)
P_B = Protein of Blank filter bag (g)
A_B = Ash of Blank filter bag (g).
*\(D\) = Original wt of Diatomaceous Earth (g).
*\(DB\) = Original wt of Diatomaceous Earth in blank filter bag (g)

*Only apply to soluble dietary fibre.
5.1.4 Solid-liquid extraction (SLE) of phenolic compounds

Solid-liquid extraction was employed to extract polyphenols from the macroalgae under investigation using ethanol/water (80:20). Crude extracts were prepared by placing 50g of the seaweed powder into a conical flask and adding the extraction solvent at a ratio of 10:1 (v/w). The mixture was then placed into a shaker (Thermo Scientific MaxQ6000) at 150 rpm and room temperature for 24 h. Extracts were filtered three times over a 24 h period through a Buchner funnel and after each filtration the residue was replenished with fresh solvent. The extracts were pooled together and the ethanol was removed using a rotary evaporator (Büchi Rotavapor R-200 with a V710 vacuum pump, Switzerland) with the water bath set at 50 °C. The remaining aqueous portions of the extract were frozen and freeze dried. Extracts were subsequently ground to a fine powder using a mortar and pestle prior to use.

5.1.5 Determination of total phenolic content

The total phenol content of the extract was quantified according to the method of Singleton et al. (1999) as previously described in Chapter 2 section 2.1.5. All samples were tested in duplicate.

5.1.6 Statistical analysis

All analyses were performed in duplicate. Measurements are presented as mean ± SD (Standard deviation). One was analysis of variance (ANOVA) followed by the Tukey post hoc comparison test was carried out to test for significant differences between macroalgal species. This was done using statistical program Minitab® Release 17. A probability value of \( p<0.05 \) was considered statistically significant.
5.2 Results and discussion

Protein, carbohydrate and lipid are the most abundant macronutrients in macroalgae and the levels obtained in the four different seaweed species studied herein are presented in Tables 5.2-5.5. Generally, seaweeds are a rich source of non-starch polysaccharides, vitamins and minerals (Mabeau and Fleurence, 1993). In addition, they are regarded as potentially valuable sources of dietary fibre and food ingredients (Lahaye, 1991; Lahaye and Jeou, 1993; Ito and Hori, 1989; Fleurence, 1999).
Table 5.2 Levels of ash, lipid, protein and carbohydrate (soluble and insoluble) as a % of dry weight of *Laminaria digitata* collected at two locations off the Irish west coast on four separate occasions.

<table>
<thead>
<tr>
<th>Location</th>
<th>Season</th>
<th>Ash</th>
<th>Lipid</th>
<th>Protein</th>
<th>Carbohydrate (CHO)</th>
<th>Soluble CHO</th>
<th>Insoluble CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiddal</td>
<td>Spring</td>
<td>25.55 ± 1.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72 ± 0.04&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>12.40 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>61.33 ± 1.29&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.20 ± 1.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.60 ± 7.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>17.45 ± 0.50&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.79 ± 0.21&lt;sup&gt;ed&lt;/sup&gt;</td>
<td>5.66 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>76.10 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.90 ± 6.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.70 ± 5.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Autumn</td>
<td>16.40 ± 0.28&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.85 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.81 ± 0.08&lt;sup&gt;f&lt;/sup&gt;</td>
<td>75.94 ± 0.21&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.95 ± 1.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.55 ± 10.54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>31.60 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.87 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.10 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.43 ± 1.10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>9.20 ± 3.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.50 ± 14.85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Finnavarra</td>
<td>Spring</td>
<td>19.50 ± 0.42&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1.02 ± 0.18&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>10.20 ± 0.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>69.28 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.85 ± 0.071&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.64 ± 14.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>20.80 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.82 ±0.24&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.03 ± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
<td>71.35 ± 0.24&lt;sup&gt;ed&lt;/sup&gt;</td>
<td>12.75 ± 4.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.35 ± 10.82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Autumn</td>
<td>20.05 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.07 ± 0.04&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>5.62 ± 0.04&lt;sup&gt;f&lt;/sup&gt;</td>
<td>73.26 ± 0.65&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.25 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.75 ± 1.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>33.65 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ± 0.49&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>20.00 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.10 ± 0.46&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4.80 ± 1.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.25 ± 3.75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean values ± S.D. (n=2). Values are expressed on a dry weight basis. Different superscript letters (a, b, c, etc.) column wise indicate significant difference (*p* < 0.05).
Table 5.3 Levels of ash, lipid, protein and carbohydrate (soluble and insoluble) as a % of dry weight of *Fucus serratus* collected at two locations off the Irish west coast on four separate occasions.

<table>
<thead>
<tr>
<th>Location</th>
<th>Season</th>
<th>Ash</th>
<th>Lipid</th>
<th>Protein</th>
<th>Carbohydrate (CHO)</th>
<th>Soluble CHO</th>
<th>Insoluble CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>17.20 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.20 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.04 ±0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>70.56 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.70 ± 5.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.35 ± 7.85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spiddal</td>
<td>Spring</td>
<td>18.15 ± 0.07&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.57 ± 1.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.29 ± 0.03&lt;sup&gt;f&lt;/sup&gt;</td>
<td>72.99 ± 0.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.80 ± 5.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.15 ± 4.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>20.60 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.86 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.43 ± 7.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.11 ± 1.21&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13.25 ± 3.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.70 ± 2.97&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Autumn</td>
<td>22.50 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.23 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.33 ± 0.04&lt;sup&gt;e&lt;/sup&gt;</td>
<td>67.94 ± 0.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.10 ± 3.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.75 ± 3.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>17.90 ± 0.00&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.99 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.37 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67.74 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.90 ± 1.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.55 ± 13.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Finnavarra</td>
<td>Spring</td>
<td>14.35 ± 0.50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.05 ± 0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.95± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>79.11 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.80 ± 4.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.95 ± 2.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>19.20 ± 3.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.06 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.91 ±0.09&lt;sup&gt;e&lt;/sup&gt;</td>
<td>74.83 ± 2.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.10 ± 6.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.75 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Autumn</td>
<td>18.05 ± 0.07&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.25 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.96 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.74 ± 0.77&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.85 ± 4.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.55 ± 7.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean values ± S.D. (n=2). Values are expressed on a dry weight basis. Different superscript letters (a, b, c, etc.) column wise indicate significant difference ($p < 0.05$).
Table 5.4 Levels of ash, lipid, protein and carbohydrate (soluble and insoluble) as a % of dry weight of *Codium fragile* collected at one location off the Irish west coast on five separate occasions.

<table>
<thead>
<tr>
<th>Season</th>
<th>Ash</th>
<th>Lipid</th>
<th>Protein</th>
<th>Carbohydrates (CHO)</th>
<th>Soluble CHO</th>
<th>Insoluble CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>44.40 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.26 ± 0.21&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.38 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>40.96 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.05 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.30 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Winter</td>
<td>44.70 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.23 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.63 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.44 ± 0.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.50 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.45 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spring</td>
<td>39.80 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.81 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.81 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.58 ± 0.97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.90 ± 1.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.15 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Autumn</td>
<td>43.40 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.08 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.06 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>44.46 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.20 ± 3.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.45 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Winter</td>
<td>40.75 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.43 ± 0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>14.36 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.46 ± 0.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.20 ± 1.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.55 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean values ± S.D. (n=2). Values are expressed on a dry weight basis. Different superscript letters (a, b, c, etc.) column wise indicate significant difference (p < 0.05).
Table 5.5 Levels of ash, lipid, protein and carbohydrate (soluble and insoluble) as a % of dry weight of *Gracilaria gracilis* collected at one location off the Irish west coast on five separate occasions.

<table>
<thead>
<tr>
<th>Season</th>
<th>Ash</th>
<th>Lipid</th>
<th>Protein</th>
<th>Carbohydrates (CHO)</th>
<th>Soluble CHO</th>
<th>Insoluble CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>19.85 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.95 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.35 ± 0.41&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54.85 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.90 ± 1.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.65 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Winter</td>
<td>18.65 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.75 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.98 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.62 ± 0.30&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.20 ± 1.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.20 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spring</td>
<td>24.85 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.76 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.70 ± 0.27&lt;sup&gt;d&lt;/sup&gt;</td>
<td>50.69 ± 0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.45 ± 2.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.55 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Autumn</td>
<td>19.40 ± 0.57&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.39 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.50 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>54.71 ± 0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.95 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.85 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Winter</td>
<td>21.40 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.47 ± 1.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.70 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.43 ± 1.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.40 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.25 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean values ± S.D. (n=2). Values are expressed on a dry weight basis. Different superscript letters (a, b, c, etc.) column wise indicate significant difference (*p* < 0.05).
5.2.1 Protein composition

Across the four species, locations and times of collection crude protein content varied from 4.91-28.90 % dry weight extract (DWC). *G. gracilis* the red seaweed has the highest protein content of 28.70% DWE (autumn 2011) and the brown seaweed *F. serratus* gave the lowest protein content of 4.91 % (autumn 2011). This is expected as previous reports suggest that red seaweed have higher protein content than both green and brown varieties (Darcy-Vrillon, 1993; Fleurence, 1999; Mabeau and Fleurence, 1993). It has been reported that protein contents of seaweeds exhibit significant species and seasonal variation (Fleurence, 1999). Amongst the two brown species studied, *L. digitata* collected in winter exhibited the highest protein content and this was the case at both sampling locations (Spiddal (17.10%) and Finnavarra (20.00%)). For the species *F. serratus*, the highest protein content for the location Spiddal was observed in autumn (14.43%). However, a high content was also observed in winter and spring. The highest protein content for Finnavarra was observed in winter (16.96%) but spring also had a high content (13.37%). The highest protein content in *C. fragile* is seen in the spring (14.81%). There is a significant (*p*<0.05) difference observed in all the other seasons investigated. In most cases it was observed that the protein content was lowest during the summer and autumn months. Marinho-Soriano *et al.* (2006) also monitored seasonal protein variation in two seaweeds, the red seaweed *Gracilaria cervicornis* and the brown seaweed *Sargassum vulgare*. They observed lower protein content in summer which they and other authors have attributed to decreased nitrogen content in water resulting in the catabolism of amino acids for energy production and high temperatures during summer (Chapman *et al.*, 1977; Souchet, 2004; Percival and McDowell, 1967; Graham and Wilcox, 2002; Anderson, 1981).
5.2.2 Ash content

The ash content of the algae species tested was found to be between 14-45 % of the dry weight. The ash content was lowest in the brown seaweed *F. serratus* (14.35 % DWE; summer 2011, Finnavarra) and highest in the green seaweed *C. fragile* (44.70 % DWE; winter 2010, Finnavarra). Both brown algae had the highest ash content in autumn and winter. This was observed in both harvest locations with *L. digitata* harvested from Spiddal having an ash content of 31.60 %, while *L. digitata* harvested from Finnavarra had an ash content of 33.65 %. *F. serratus* harvested from Spiddal, winter had the highest ash content of 22.50 % whereas the autumn sample had the highest ash content in the samples harvested from Finnavarra with 19.20 %. The ash content in the green algae *C. fragile* was also highest in autumn and winter with winter containing 44.70 %. A significant (*p*<0.05) decrease in content was observed in spring (39.80 %). In contrast *G. gracilis* did not follow the same trend observed in both the brown and green macroalgae. The highest ash content was found in spring (24.85 %), with winter exhibiting the next highest with 21.40% and 19.85%. All seaweeds had ash contents between 14-45 % dry weight extract (DWC) which were consistent with previous results by Mabeau and Fleurence, (1993) and Ortega-Calvo *et al.* (1993) which reported ash contents of 8-40 % DWC. Macroalgae generally show great variation in mineral contents, which is related to several environmental factors such as water, temperature, salinity, light and nutrients. Marinho-Soriano *et al.* (2006) and Perfeto, (1998) found a negative correlation between mineral content and carbohydrate content. This is also observed in this study, when mineral content was high, the carbohydrate content was low, which could also suggest that the actual quantity of minerals does not change significantly but rather the proportion present is higher due to depletion of carbohydrate content. Changes in ecological conditions can stimulate or inhibit the biosynthesis of several nutrients (Marinho-Soriano *et al.*, 2006) According to their
results, protein, carbohydrate, lipid, fibre, ash and nitrogen content were influenced by environmental parameters. This was confirmed by the correlation found among these variables, indicating that environmental conditions act on biosynthetic pathways.

5.2.3 Lipid content

In general, macroalgae have relatively low lipid contents of less than 3 % (Parekh et al., 1977; Dawes et al., 1993; Sánchez-Machado et al., 2004; Schmid et al., 2013). In this study the lipid content observed in the four macroalgal species investigated was between 0.75-2.81 % DWC. In the brown algae *L. digitata* the highest lipid content was found in autumn and winter while lower levels were observed in spring and summer. The winter sample had the highest lipid content for this species (1.87 %; winter 2011, Spiddal) along with the winter sample collected at Finnavarra which also had a high lipid content (1.25 %). For the other brown species *F. serratus*, the highest lipid content was observed in summer at Spiddal (2.57 %), however autumn and winter had similar levels although there was no significant (*p > 0.05*) difference across the seasons. Schmid et al (2014) also observed similar results, where the lipid content in the species *L. digitata* was higher in the winter period (November) and lower in the summer time (June). They also observed that the lipid content of the algae *F. serratus* was higher in the summer months (4-5 % DW) and was lower in the winter period with (less than 3 % DW total lipid content). These results are in agreement with our findings. In the green seaweed *C. fragile*, the highest lipid content was observed in spring (2.81%) with similar levels also observed in autumn and winter. For the red algae *G. gracilis* the highest lipid content was obtained in autumn with a lipid content of 1.95 %, no significant (*p > 0.05*) difference was observed across seasons. It has been reported that many species of seaweeds such as *S. polyschides, H. elongata, L. orhroleuca, U. pinnatifida, Palmaria* and *Porphyra* species
contain a lipid content of less than 2 % (Sánchez-Machado et al., 2004; Park et al., 1997). It is not known why seaweeds have such low lipid contents, however, their polyunsaturated fatty acid percentage in the lipid portion can be as high as those of terrestrial vegetables (Darcy-Vrillon, 1993). Nelson et al. (2002) found that lipids were highest in winter and spring with the green algae Ulva lobata reported to have the highest lipid content (20-29 mg g⁻¹ dry mass) with the red algae Chondracanthus canaliculatus having the lowest lipid content (2-3 mg g⁻¹ dry mass) in their study. Our study also observed that the green algae C. fragile had the highest lipid content (2.43 % DWC) from the four species investigated and the red algae G. gracilis had the lowest lipid content (0.75 DWC). Variations in fatty acid contents are attributable to both environmental and genetic difference (Nelson et al., 2002). In the three species L. digitata, F. serratus and C. fragile the lipid content is highest in the winter, therefore the optimum harvest time for lipid rich seaweed samples is in the winter months. Smith and Harwood, (1984) examined the fatty acid composition of the major lipids in Fucus serratus. Most showed rather distinctive fatty acid contents. For example, diacylgalactosylglycerol was enriched in n-3 polyunsaturated fatty acids while phosphatidylcholine and phosphatidylethanolamine had very high levels of arachidonic acid, and Phosphatidylglycerol contained the unusual trans-Δ3-hexadecanoic acid. Schmid et al. (2014) also examined the fatty acid composition of F. serratus and found that it contained PUFA levels of 28.2 % and the most commonly distributed PUFA in the brown algae was 20:4 n6 (ARA). Other abundant PUFA’s included 20:5 n-3 (EPA) and stearidonic acid (SDA, 18:4 n-3) and two essential fatty acids linoleic (LA, 18:2 n-6) and alpha-linoleic (ALA, 18:3 n-3) acid. Research by Goeke et al. (2010) studied the fatty composition of C. fragile with Palmitic acid shown to be the main fatty acid, comprising of 28-54 %, followed by oleic and linoleic acids. The fatty acid hexadecatrienoic acid (C16:3) was also confirmed and is a characteristic fatty acids for the Codium genus. Limited
research has been reported to date on the fatty acid composition of the red seaweed *Gracilaria gracilis*, however studies carried out on other species in this genus showed them to contain C12:0, C16:0, C20:4 ω6 and C22:5 ω3 (Tabarsa et al., 2012).

### 5.2.4 Carbohydrate and fibre content

In the brown macroalgal species *L. digitata* harvested from Spiddal a slight difference (*p*<0.05) in carbohydrate content was observed in the autumn and Summer (76.10 % and 75.94 %), there was a drop of carbohydrate levels during the spring months (61.33 %) with the winter having the lowest levels (49.43 %). The same trend was observed for this species harvested from the second location Finnavarra. *F. serratus* exhibited a similar trend to that observed in *L. digitata* with summer having the highest content. For the green macroalgae *C. fragile* a significant difference in carbohydrate content is observed across the seasons with autumn 2011 having the highest value (44.46 %) while the autumn 2010 sample had the lowest content (40.96 %). In the red macroalgae *G. gracilis* a significant difference was observed across seasons. The winter 2011 sample had the lowest carbohydrate content of 48.43 % whereas the highest carbohydrate content was observed in the autumn 2010 (54.85 %). The type and abundance of carbohydrates vary strongly between algae species which are known to contain high polysaccharide content (Lahaye, 1991). Renaud and Luong-Van, (2006) also investigated the seasonal variation in the chemical composition of tropical Australian marine macroalgae and reported that the soluble carbohydrate content varied between species with Chlorophytes having between 2.5-25.8 %, Phaeophytes between 8.4-22.2 % and Rhodophytes between 1.7-39 %, with a significantly higher content observed in the Rhodophyte in the winter season. The high content in macroalgae is due to polysaccharides being a principle
energy reserve. Marcoalgae also contain large amounts of dietary fibre and are particularly rich in the soluble fraction (Darcy-Vrillon, 1993; Lahaye, 1991; Mabeau and Fleurence, 1993).

Insoluble dietary fibre (IDF) is high among all seaweed species ranging from 17-55 % and highest in the two brown macroalgae species *L. digitata* and *F. serratus*. This is expected as insoluble dietary fibre makes up the structural components of the macroalgae in the form of cell wall polysaccharides such as cellulose and alginates and insoluble lignins in brown macroalgae. *F. serratus* had the highest level of IDF (55.55 %; spring, Finnavarra), while the red algae had the lowest IDF level of 19.20 %; Winter 2010. Rupérez, (2001) observed similar IDF levels in a range of seaweeds with the species *Fucus vesiculosus* containing levels of 40 % IDF, similar to IDF levels seen in this study (*F. serratus*, 40-55 % IDF), they also reported 27 % IDF in the Laminaria species, whereas, this study observed IDF levels of 39-46 %. in *L. digitata*. For the red and green species they tested they observed levels of 12-26 %. Levels were lower in red and green algae due to the fact that proteins play a much greater structural role in these species than in brown algae. Again, similar results were observed in the green (*C. fragile*) and red species (*G. gracilis*) tested in this study (17-23 % IDF). There was no significant difference in IDF content observed across the seasons in all seaweeds tested.

Soluble dietary fibre (SDF) levels were much lower than IDF levels among the seaweeds with levels ranging from 3-20 %. Brown macroalgae had a much higher SDF content than the red or green macroalgae. *L. digitata* had the highest SDF content observed in the spring sample collected at Spiddal (16.90 %) with the summer Finnavarra (12.75 %). Much lower levels were observed during the autumn and winter seasons. Similar results are observed in the species *F. serratus*, with both spring and summer having higher SDF contents with a drop in content occurring in the autumn and winter months. This is most probably due to seasonal variation of storage glycan’s such as
laminarin and fucoidan which are predominantly found in brown macroalgae. Previous reports by Black et al. (1950) found that laminarin content was highest in *Laminaria digitata* during the spring and summer periods and then dropped off in the winter months. Similar results have also been reported for fucoidan with the level of fucoidan tending to increase from Spring to Autumn (Kim, 2012). Souchet, (2004) also reported that the fucoidan content of *Laminaria longicruris* in Quebec was high in the summer months. Rosenberg and Ramus, (1982) related the carbohydrate synthesis to periods of maximum growth, increased photosynthetic activity and a reduction in protein content. Increased temperatures, salinity and sunlight intensity appear to have a positive effect on carbohydrate synthesis (Munda and Kremer, 1977; Perfeto, 1998).

**Figure 5.1** Total phenolic content (TPC) of crude extracts of *Fucus serratus* from two locations off the west coast and from four separate occasions. Mean values ± S.D. Values are expressed as µg Gallic acid equivalents/mg dry weight sample. Different superscript letters (a, b, c etc) indicate significant ($p < 0.05$) difference.
5.2.5 Total phenolic content of Fucus serratus samples

Based on the high level of phenolics in Fucus serratus as reported in a previous study (Heffernan et al., 2014) it was the only species examined for seasonal variations. The other three species L. digitata, C. fragile and G. gracilis had levels of phenols below what would be considered to be economically viable to recover. The TPC of crude F. serratus were assessed and the results are presented in Figure 5.1. Levels of polyphenols were low in spring and then increased during the summer period, after which they dropped again in the autumn and increased slightly again in the winter months. The highest phenolic content was observed during the summer time at Finnavarra (181.73 μg gallic acid equivalents (GAE)/mg sample). The lowest phenolic content was observed in the autumn period at Spiddal (87.20 μg GAE/mg sample). The content of polyphenols in seaweed is affected by a range of factors including the age of the seaweed. For example, Pederson, (1984) reported that polyphenol content increased with the age of tissue in Ascophyllum nodosum (Pederson, 1984). Ragan and Jensen, (1978) also found similar results in the brown algae Ascophyllum nodosum and Fucus vesiculosus. They reported significant temporal correlation between polyphenol content and the reproductive stage of the algae. Polyphenol content was minimum during the period of fruit body shedding in the summer (8-10 %) and reached a maximum during the winter season (12-14 %). Similar results were observed in the present study with phenol levels ranging from 8.72- 18.17 % dry weigh extract. It has also been reported that large quantities of heavy metals contribute to the accumulation of the phenolics (physodes) in the brown macroalgae Fucus vesiculosus (Forsberg et al., 1998). These heavy metals also display seasonal variation, which is caused by dilution during the period of maximum growth and are more concentrated during periods of slow growth. Gorham, (1984) also observed that in the brown macroalgae Sargassum muticum, maximum polyphenol content occurred in early summer months,
when the plant was undergoing rapid growth. Abdala-Díaz et al. (2006) found that solar radiation had an effect on the phenolic content in the brown seaweed *Cystoseira tamariscifolia* with results indicating that higher quantities of phenolic compounds were produced during the summer months to serve as a photoprotective mechanism against higher irradiances that may be experienced. However, other studies have described maximum levels for fucales in winter (Ragan and Jensen, 1978) or from late summer to the middle winter (Ragan and Glombitza, 1986) depending on the species. Both light intensity and temperature variation may be invoked explaining seasonal variation in phenolic contents (Ragan and Glombitza, 1986). Overall, summer was considered the optimum seaweed harvest period for phenolic compound extraction.

### 5.3 Conclusion

Ash, lipid and protein content were not significantly affected by seasonal/geographical variation. Ash content for both brown algae species was maximum during winter, while for *C. fragile* the highest ash was seen in autumn and for *G. gracilis* the highest was spring. Protein content for three of the species (*L. digitata*, *F. serratus* and *G. gracilis*) was maximum in winter with *C. fragile* protein content highest in spring. Therefore, the best harvest time for high protein content would be during winter and spring. The lipid content showed variation across species with *C. fragile* having the highest lipid content (2.71 %) and *G. gracilis* the lowest lipid content (0.75 %). The carbohydrate content was found to be highest in the brown macroalgae *F. serratus* and *L. digitata*, particular in the summer period. Insoluble dietary fibre was high in all seaweeds, with spring having the highest content for both brown species (*L. digitata* and *F. serratus*). Soluble dietary fibre was observed at a much lower level with the maximum content generally observed during the spring and summer periods for the species *F. serratus*, *L. digitata* and *G. gracilis*, with the
maximum being observed in the autumn period in the green algae *C. fragile*. The level of phenolics in the species *F. serratus* was highest during the summer period, and lowest in autumn. It appears that polyphenols were at a maximum level during the rapid growth phase of the plant. This study has proved useful to determine the optimal harvest time for each of these species according to nutritional components. In view of the protein, mineral, fibre content combined with low fat content these seaweeds have been shown to have good nutritional properties which makes them a desirable food option for humans and animals. The data presented in this study could assist in the identification of the most suitable harvesting seasons for these nutritional components.
References


Chapter 6

Comparison of extraction methods for selected carotenoids from macroalgae and the assessment of their seasonal/spatial variation.
Abstract

Natural bioactive compounds provide an excellent source of molecules for the production of nutraceuticals, functional foods and food additives. Particular groups of these compounds can be found in high abundances in seaweed such as polyphenols (Heffernan et al., 2015), while others may only be present at relatively low concentrations such as carotenoids. In order to obtain sufficient amounts of the carotenoid components, optimization of extraction technologies to aid the recovery of these valuable compounds for use as nutraceutical or as ingredients in functional food products is required. In this study, carotenoid and xanthophyll extraction procedures using supercritical CO$_2$, conventional solvent extraction and supercritical CO$_2$ with ethanol as a co-solvent were explored to determine the optimum technique for obtaining carotenoid extracts of high purity and yield. This was carried out using dry biomass from two brown macroalgae, *Fucus serratus* and *Laminaria digitata*. Supercritical CO$_2$ (SCO$_2$) gave the greatest yield of carotenoid rich extract was at 50 °C, 300Atm (standard atmosphere) with an extraction time of 60 min. A higher yield of fucoxanthin and xanthophyll was observed in the SLE extracts compared to those prepared using SCO$_2$, however SCO$_2$ gave a higher purity for fucoxanthin, while SLE gave a higher purity of xanthophylls. As well as determining the optimal extraction technique of the selected carotenoids from these two species, seasonal/spatial variation based on the purity and yield of these compounds were subsequently investigated using the same extraction techniques. Seaweeds are exposed to seasonal and geographical variations that influence their metabolic responses (photosynthesis and growth rate). This study also investigated the seasonal variation in carotenoids based on the concentration and purity of these compounds present providing valuable information for optimal harvest time for these specific target molecules. The winter and spring period gave the highest purity of both fucoxanthin and xanthophyll for the species *L. digitata*. 
(P<0.05), while the summer period was the peak period for \textit{F. serratus}. There was no geographical variation observed between the two sites investigated (p<0.05).

\textbf{6.0 Introduction}

Carotenoids are highly conjugated polyprenoid compounds that contain two terminal ring systems. Carotenoids composed entirely of carbon and hydrogen are known as carotenes which are orange in colour, while those that contain oxygen are known as xanthophylls and are yellow in colour. Carotenoids are widely distributed in plants where they perform a role in a variety of processes like photosynthesis, light transmission and free radical scavenging (Yasushi, 1991; Sies and Stahl, 1995). Fucoxanthin is a xanthophyll compound which is commonly found in nature, specifically found in brown macroalgae (Peng \textit{et al.}, 2011). Fucoxanthin has a characteristic molecular structure in that it contains an allene group, this is relatively rare in natural carotenoids (Straub, 1987). Generally they are polygenic carotenoids with linear conjugated double bonds as observed for β-carotene (Figure 6.1). Fucoxanthin is purported to have antioxidant (Sachindra \textit{et al.}, 2007; Airanthi \textit{et al.}, 2011), anti-inflammatory (Heo \textit{et al.}, 2008; Kim \textit{et al.}, 2010), anti-cancer (Miki, 1991; Miyashita \textit{et al.}, 2011), anti-obesity (Maeda \textit{et al.}, 2005), anti-diabetic (Maeda \textit{et al.}, 2007; Hosokawa \textit{et al.}, 2010; Nishikawa \textit{et al.}, 2012), hepatoprotective (Woo \textit{et al.}, 2010) and skin-protective effects (Heo and Jeon, 2009). It therefore could be exploited for potential uses in both the pharmaceutical and food industries. Carotenoid content in macroalgae is known to vary due to seasonality and differs in brown algae collected in different locations (Normura \textit{et al.}, 2013). Considering any marine hydrobionts as a source of carotenoids or functional food components, determining the optimal period of harvesting algae with maximal content of these valuable substances is required.
At present the most used extraction technique for these compounds in research is liquid solvent extraction using non-polar solvents like toluene, hexane or petroleum ether. However, this method can be time-consuming due to multiple extraction steps and it requires large amounts of organic solvents which can be expensive and potentially harmful to the environment (Foster *et al.*, 1993). In fact there is an increase in public awareness of the health, environmental and safety hazards connected with the use of organic solvents in food processes and the possibility for solvent residue contamination of the final products. Currently, European regulations relating to extraction solvents for use in foodstuffs provide detailed guidelines that have to be strictly adhered to for the protection of human health, economic and technical needs (Anon, 2009). In addition the high expense of organic solvents and the strict environmental regulations in the food industry for ultra-pure and high added value products have expanded the need for the development of cleaner, simpler, rapid and more efficient technologies for the extraction of carotenoid compounds from natural sources (Mohamed and Mansoori, 2002). Supercritical fluid extraction (SFE) is an alternative extraction technique used in the food industry due to the fact that a product without solvent residues can be produced. Extracts from SFE contain fewer polar impurities than current organic liquid extracts.
therefore making subsequent purification steps less complicated (Noh et al., 1995). Supercritical fluid uses the capacity of particular gases/solvents to become more effective solvating agents for selected compounds under specific temperatures and pressures. A solvent becomes supercritical when its temperature and pressure is elevated above its critical point. Carbon dioxide is the most commonly utilised supercritical fluid, its critical point is at 31.06 °C and 7.386 MPa and is favourable due to it being GRAS approved (Generally Regarded As Safe), non-flammable, noncorrosive and inexpensive (Rizvi et al., 1994). The low critical temperature of CO$_2$ can prevent thermal degradation of bioactive compounds during the extraction. In addition, SC$_2$O is especially useful for targeting compounds with a low polarity like carotenoids due to the hydrophobic nature of supercritical carbon dioxide. Supercritical fluids offer many advantages such as a higher diffusion coefficient and lower viscosity than fluids, while the reduced surface tension in SFE allows for their faster penetration of the supercritical fluid into the pores of heterogeneous matrices thus enhancing extraction efficiencies (Eggers and Lack, 2012). The selectivity during extraction can be controlled by varying the temperature and pressure conditions, which can affect the solubility of the some components in the supercritical fluid. Supercritical fluids like carbon dioxide do not leave a chemical residue and can be recycled and used again as part of the unit operation (Rizvi et al., 1994). A limitation of supercritical CO$_2$ is that it can fail in the quantitative extraction of polar analytes from solid matrices due to the solvating ability and the insufficient interaction between CO$_2$ and the matrix. The use of an organic modifier has shown to greatly improve the extraction efficiency (Hamburger et al., 2004) by increasing the solubility of the analytes and by reducing their interaction with the sample matrix or by inducing modification of the sample matrix resulting in the release of the analytes from the matrix being greatly improved.
This study has two main aims: [1] investigate the various extraction techniques for the extraction of the common carotenoids, fucoxanthin and xanthophyll found in macroalgae and [2] to apply these methods to investigate the seasonal/spatial variation of these compounds over the period of a year.

6.1 Materials and Methods

6.1.1 Chemicals

CO$_2$ (N-38 quality) was obtained from AirLiquideEspaña S. A. (Madrid, Spain). Ethanol was purchased from Sigma Aldrich (Madrid, Spain), Laboratory grade sea sand was purchased from Fluka (Madrid, Spain). HPLC grade methanol and water and the internal standards fucoxanthin and xanthophyll were purchased from Sigma Aldrich (Dublin, Ireland).

6.1.2 Samples

The brown macroalgae samples used in this study were identified and harvested off the west coast of Ireland. *Fucus serratus* and *Laminaria digitata* was harvested from two locations Finnavarra, Co. Clare (53° 9ʹ5ʺ North, 9°6ʹ2ʺ West) and Spiddal Co. Galway (53°14ʹ48ʺ North, 9°18ʹ10ʺ West) over the period of a year from 2011-2012. A random selection of different plants were taken from the shore, to allow for natural variability, these were packed in cool boxes and transported immediately to the laboratory. Samples were washed thoroughly with fresh water to remove sand and epiphytes and were then stored in the freezer at -20 °C. The identity of each macroalgal specimen was verified and a freeze dried sample of each was retained for reference at the Irish Seaweed Centre at NUIG. The macroalgal samples were subsequently freeze dried and ground to a powder using a Waring® blender (New Hartford, CT, USA) and stored in vacuum packed bags at -80 °C prior to extraction.
6.1.3 Solid-Liquid extraction (SLE)

Solid liquid extraction was employed to extract the carotenoids from the macroalgae under investigation using hexane/acetone (70:30) as this solvent system has previously been shown to be effective for extracting pigments from plant materials (AOAC, 1984; Torres et al., 2014). Crude extracts were prepared by placing 10 g of the seaweed powder in a conical flask and adding the extraction solvent hexane/acetone (70:30) at a ratio of 10:1 (v/w). The mixture was then placed into a shaker (Thermo Scientific MaxQ6000) at room temperature for 24 hours. These extracts were filtered three times over a 24 h period through a Buchner funnel. The combined extracts were concentrated to remove all solvent using a rotary evaporator (BüchiRotavapour R-200 with a V710 vacuum pump) with the water bath set at 50 °C.

6.1.4 Optimization of Supercritical Carbon dioxide extraction

An Applied Separations Spe-ed SFE Helix extractor was used for the supercritical CO₂ extraction. Prior to starting extractions, optimization of the extraction time and conditions was determined. The extractions were performed in a semi-continuous flow type apparatus. Carbon dioxide pre-cooled to -20 °C prior to the pump inlet was pumped to the extractor by a positive displacement controlled volume metering pump. Pressure in the extractor was controlled by a back pressure regulator and the extraction vessel was heated to optimal temperature in an oven. 2 g of sample and 4 g of sea sand were loaded into a 20 mL stainless steel extraction cell with the cell fitted with glass wool at the inlet and outlet. To determine extraction conditions, extractions were performed at three different extraction temperatures (30, 40 and 50 °C) and at three different pressures (150, 225 and 300 Atm) for each temperature. Optimal conditions were chosen based on the extract yield obtained for each test parameter (fucoxanthin and xanthophyll). To optimize extraction time,
time versus yield curve was constructed at 50 °C and 300 Atm. The extract yield was measured in grams at 15 min intervals to determine when the extract was exhaustively extracted and thus allowing for optimum carotenoid extraction.

6.1.5 Supercritical CO$_2$ Extraction (SFE)

Extraction of carotenoids for seasonal and geographical studies was carried out using the optimal extraction conditions as described in section 6.1.4 (50 °C, 300 Atm and an extraction time of 60 min, CO$_2$ flow rate 1 mL/min). Extracts were prepared using 30 g of freeze dried seaweed powder mixed with 90 g of laboratory grade sea sand in a 5 litre stainless steel extraction cell. The extraction cell was packed with glass wool at the inlet and outlet. Extracts were collected in a sterile vessel and cooled by ice. The extracts were dried under a stream of nitrogen and stored at -20 °C.

6.1.6 Supercritical CO$_2$ Extraction (SFE) with ethanol as co-solvent

A SuprexPrepMaster (Suprex, Pittsburgh, PA) was used for Supercritical fluid extraction studies using ethanol as co-solvent. The extractor was equipped with a dual piston pump for CO$_2$. 2 g of seaweed sample was mixed with 4.0 g of laboratory grade sea sand and the mixture was loaded into a 20 mL stainless steel extraction cell. The extraction cell was fitted with glass wool at the inlet and outlet. Ethanol was pumped at 0.1 mL/min using a Jasco PU2080 HPLC pump (Jasco Inc., Easton, PA) and mixed at high pressure with supercritical CO$_2$ (SC-CO$_2$) which was pumped at 1 mL/min. Extraction conditions were as described in section 6.1.4 (50 °C, 300 Atm and 60 min extraction time). To ensure all ethanol was purged from the system and there was no carry over between extractions for the final 15 minutes only CO$_2$ was applied. Extracts were collected in a
sterile vessel and cooled on ice. To avoid sample degradation, the extracts were stored at -20 °C and protected from light until the drying step. Extracts were subsequently dried under a stream of nitrogen.

6.1.7 Analysis of Carotenoids by HPLC-DAD

The carotenoid extracts were analysed using HPLC (Waters Alliance 2695-Separations Module with Empower Pro Software 2002) equipped with a diode array detector (DAD) (Waters 996) with an absorbance range between 190 and 650 nm. Separation was carried out using a Zorbax C8 column (4.6mm ID x 250mm, 5μm) (Agilent Technology, Dublin 18). The mobile phase was a mixture of solvent A (methanol) and solvent B (water) at 1 mL/min according to a step gradient, lasting 30 min, which started from 85 % B, changing to 100 % B over 15 min and kept at 100 % B until 28.5 min followed by change to 85% B at 29.0 mins and held for one minute. Samples were prepared at a concentration of 5 mg/ml and a 20 μL injection volume was used throughout. A fucoxanthin calibration curve ($R^2= 0.9921$) was obtained by injecting fucoxanthin standard at concentrations ranging from 0.005 to 0.05mg/mL. A xanthophyll calibration curve ($R^2= 0.9933$) was obtained by injecting xanthophyll standard at concentrations ranging from 0.0005 to 0.050 mg/mL. All of the calibration points were injected in duplicate. The purity of both fucoxanthin and xanthophyll were determined from their standard curves respectively, and were expressed as milligram Fucoxanthin equivalents per milligram extract (mg FE/mg extract) and milligram Xanthophyll equivalents per milligram extract (mg XE/mg extract). The total content of both Fucoxanthin and Xanthophyll were determined by multiplying the calculated purity by the total extract yield, these were expressed at the Total Fucoxanthin content (TFC) in milligrams per gram.
dry weight extract (mg TFC/g DWE) and Total Xanthophyll content (TXC) in milligrams per gram dry weight extract (mg TXC/g DWE).

6.1.8 Statistical analysis
All extracts were analysed in duplicate. Measurement values are presented in means ± standard deviation. One way analysis of variance (ANOVA), followed by the Tukey post hoc comparison test, was carried out to test for significant differences using the statistical program Minitab® Release 15 for Windows. A probability value of $p < 0.05$ was considered statistically significant.

6.2 Results and Discussion

6.2.1 Optimization of SFE extraction
*Fucus serratus* samples were tested at three different temperatures (30, 40 and 50 °C) and three different pressures (150, 225 and 300 Atm) to determine the conditions resulting in the highest yield of carotenoid extracts (Figure 6.2). Results are presented as a percentage of the total extract yield. It was found that the highest temperature tested of 50 °C and also the highest pressure tested of 300 Atm gave the highest extract yield of 0.49 %. Macías-Sánchez *et al.* (2005) also found that a higher temperature and pressure of 50 °C and 300 bar gave the greatest yield of pigments (carotenoids and chlorophylls) from the microalgae *Nannochloropsis gaditana*. Similarly, Mendes *et al.* (2005) tested a range of temperatures (40-55 °C) and pressures and determined that the higher temperatures and pressures gave the greatest yield of carotenoids from the microalgae *Chlorella vulgaris*. Roh *et al.* (2008) also utilised SCO$_2$ for the extraction of fucoxanthin from the macroalgae *Undaria pinnatifida* and found that the higher temperatures and pressures of 200 bar
and 323 K (50 °C) along with 250 bar and 333 K (60 °C) gave the highest yield of fucoxanthin and polyphenols.

![Figure 6.2](image)

**Figure 6.2.** Yield of carotenoid expressed as percentage of the total extract yield following SFE extraction at three temperatures (40, 50 and 60 °C) and three pressures (150, 225 and 300 Atm) from the species *Fucus serratus*.

To ensure sufficient extraction time required to exhaustively extract the compound of interest from the sample, *Fucus serratus* was extracted over 105 mins to determine at what time the highest extract yield was obtained. At 15 minute intervals the collection vessel was weighed and a new vessel added for the next time interval. A plot of time versus cumulative yield was constructed to demonstrate the optimal extraction time (Figure 6.3). It can be seen that from 60 min to 105 min similar yields are obtained ranging from 8.2-8.9 mg/g extract (0.20-0.22% extract yield). Based on these results, and the cost involved in harvesting and preparing the seaweed an extraction time of 60 min was chosen to ensure that maximum extraction of the target compounds would be achieved with minimal waste.
Figure 6.3. Yield of carotenoid extract expressed as mg/g extract following SFE extraction over a 105 min time period at a temperature of 50°C, a pressure of 300 Atm and a flow rate of 1 ml/min.

6.2.2 Analysis of fucoxanthin and Xanthophyll Content using different extraction methods

Table 6.1 presents data from the optimised extraction of xanthophyll and fucoxanthin from *F. serratus* using SC0₂, solvent extraction and SC0₂ with ethanol as co-solvent. The purity of both fucoxanthin and xanthophyll were determined from their standard curves respectively using HPLC, and were expressed as milligram Fucoxanthin equivalents per milligram extract (mg FE/mg extract) and milligram Xanthophyll equivalents per milligram extract (mg XE/mg extract). SLE resulted in the highest total extract yield of 27.32 mg/g DWE (i.e., DW of all components extracted) with SC0₂ resulting in the lowest extract yield of 3.4 mg/g DWE. As well as producing the greatest yield, the SLE technique also produced extracts with the highest total fucoxanthin content (TFC) of 3.57 mg TFC/g DWE and also the highest total xanthophyll content (TXE) of 0.137 mg TXC/g DWE. The purity of both fucoxanthin and xanthophyll were greater for the SC0₂
samples, with the purity of fucoxanthin being significantly \((p > 0.05)\) lower in the SLE extracts. \(\text{SCO}_2\) provided the highest purity of Fucoxanthin (0.151 mg FE/mg extract) and also the highest purity of Xanthophyll (0.008 mg XE/mg extract). Roh et al. (2008) found that \(\text{SCO}_2\) gave a low yield of fucoxanthin of 0.00753 μg/g in freeze dried Undaria pinnatifida. Macías-Sánchez et al. (2007) stated that the yield depends on a complex balance in relation to the decrease in the supercritical carbon dioxide density and the increase in vapour pressure of the pigments as the temperature increases, which essentially represents the solubility of the pigment in the solvent.

Fucoxanthin is present at a much higher quantity than xanthophyll which is not surprising as it has been previously reported to be the main carotenoid found in brown macroalgae (Peng et al., 2011). Fucoxanthin is present in both L. digitata and F. serratus, however for investigation of optimal extraction only F. serratus was used. The purity of fucoxanthin following extraction is tenfold higher than that of xanthophyll. Purity of each compound was determined against a standard curve. This can be clearly observed the HPLC chromatogram in Figure 6.4 (SFE extraction) where the fucoxanthin peak is by far the most abundant with a fucoxanthin purity of 0.151 mg FE/mg extract, while the xanthophylls peak is almost 20 times smaller with a xanthophyll purity of 0.008 mg XE/mg extract. In the HPLC chromatogram for SFE and co-solvent extraction (Figure 6.5), fucoxanthin is less prominent and a lower purity of fucoxanthin was observed (0.136 mg FE/mg extract). The use of the modifier increased the extraction of xanthophyll to a level three fold higher than SFE, however the purity of the xanthophyll was reduced also in comparison to SFE (0.007 mg XE/mg extract). A number of other impurities are also present in the chromatogram in comparison to the SFE extraction. In Figure 6.6 (SLE extraction) the levels of both compounds are much higher with the level of fucoxanthin almost tripling and xanthophyll increasing to over three times more than observed in SFE with co-solvent. However, both the purity of fucoxanthin
and xanthophyll were again reduced when compared to the previously discussed extraction methods (0.131 mg FE/mg extract and 0.005 mg XE/mg extract).

In general, SLE delivers the highest total yield of the target compound while SCO$_2$ resulted in the best purity in relation to fucoxanthin. This outcome contrasts with a previous study by Marsili and Callahan (1993) that compared liquid solvent extraction and supercritical fluid extraction for the determination of carotenoids in vegetable. They observed that the SFE gave a higher yield of the carotenoid beta-carotene than the conventional solvent extraction but this could be due to the different matrix effect of the macroalgae.

A similar trend was also observed in the seasonal samples when these extraction methods were applied to both _Laminaria digitata_ and _Fucus serratus_. The highest fucoxanthin content in _Laminaria digitata_ was observed in the SLE extract (1.403 mg TFC/g DWE, Spiddal-Winter), however, the highest purity of fucoxanthin was observed in the SCO$_2$ (0.196 mg FE/mg extract, Spiddal-Spring) (Table 6.2). The same was observed for the xanthophylls in the species _Laminaria digitata_, with SLE resulting in higher quantity than SCO$_2$ but the higher purity of xanthophylls also seen in the SLE (Table 6.2). The highest Fucoxanthin content in _Fucus serratus_ was also seen in the SLE extracts (5.198 mg TFC/g DWE, Finnavarra-Summer), while, the highest purity of fucoxanthin was again observed in the SCO$_2$ extracts (Table 6.3). Xanthophyll quantities appeared to be greater in SLE extracts similar to trend observed for _Laminaria digitata_. It is clear from this study that SFE provides extracts with higher purity of Fucoxanthin, while SLE provides extracts with a greater yield and higher fucoxanthin content.
Table 6.1. Extract yield (mg/g dry weight extract (DW)), Fucoxanthin purity (mg fucoxanthin equivalents (FE)/mg extract), xanthophyll purity (mg xanthophylls equivalents (XE)/mg extract), Total Fucoxanthin content (TFC) (mg TFC/g dry weight extract (DWE)) and Total Xanthophyll content (TXC) (mg TXE/g dry weight extract (DWE)) from optimised extraction conditions on *Fucus serratus*, using supercritical fluid extraction (SCO$_2$), solid-liquid extraction (SLE) and SCO$_2$ with ethanol as co-solvent (SCO$_2$/EtOH).

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Total extract yield (mg/g DWE)</th>
<th>Fucoxanthin purity (mg FE/mg extract)</th>
<th>Xanthophyll purity (mg XE/mg extract)</th>
<th>Total Fucoxanthin content (mg TFC/g DW)</th>
<th>Total Xanthophyll content (mg TXC/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>27.32</td>
<td>0.131 ± 0.003$^b$</td>
<td>0.005 ± 0.001$^b$</td>
<td>3.57</td>
<td>0.137</td>
</tr>
<tr>
<td>SCO$_2$</td>
<td>16.00</td>
<td>0.136 ± 0.002$^b$</td>
<td>0.007 ± 0.003$^b$</td>
<td>2.18</td>
<td>0.112</td>
</tr>
<tr>
<td>SCO$_2$/EtOH</td>
<td>3.20</td>
<td>0.151 ± 0.006$^a$</td>
<td>0.008 ± 0.006$^a$</td>
<td>0.51</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Mean values ± S. D. Different superscript letters (a,b,c etc) column wise indicate significant difference.
Table 6.2. Fucoxanthin purity (mg fucoxanthin equivalents (FE)/mg extract) and Xanthophyll purity (mg xanthophylls equivalents (XE)/mg extract), and total extract yield (mg/g dry weight extract (DWE)) of solid-liquids extracts (SLE), supercritical fluid extracts (SFE) and supercritical fluid extracts with ethanol as co-solvent, Total Fucoxanthin content (TFC) (mg TFC/g dry weight extract (DWE)) and Total Xanthophyll content (TXC) (mg TXE/g dry weight extract (DWE)) of *Laminaria digitata* over four seasons and from two harvest locations.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Location</th>
<th>Season</th>
<th>Fucoxanthin purity (mg FE/mg extract)</th>
<th>Xanthophyll purity (mg XE/mg extract)</th>
<th>Extract yield (mg/g DWE)</th>
<th>Total Fucoxanthin content (mg TFC/g DWE) in <em>Laminaria digitata</em></th>
<th>Total Xanthophyll Content (mg TXC/g DWE) in <em>Laminaria digitata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Spring</td>
<td>0.180 ± 0.003abc</td>
<td>0.015 ± 0.003a</td>
<td>4.69</td>
<td>0.844</td>
<td>0.070</td>
</tr>
<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Spring</td>
<td>0.190 ± 0.006a</td>
<td>0.014 ± 0.006a</td>
<td>3.91</td>
<td>0.743</td>
<td>0.055</td>
</tr>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Summer</td>
<td>0.154 ± 0.001defg</td>
<td>0.008 ± 0.001b</td>
<td>3.19</td>
<td>0.491</td>
<td>0.026</td>
</tr>
<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Summer</td>
<td>0.159 ± 0.000ef</td>
<td>0.009 ± 0.000b</td>
<td>4.60</td>
<td>0.731</td>
<td>0.041</td>
</tr>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Autumn</td>
<td>0.130 ± 0.013d</td>
<td>0.002 ± 0.013d</td>
<td>4.28</td>
<td>0.556</td>
<td>0.009</td>
</tr>
<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Autumn</td>
<td>0.134 ± 0.003f</td>
<td>0.002 ± 0.010d</td>
<td>6.41</td>
<td>0.859</td>
<td>0.013</td>
</tr>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Winter</td>
<td>0.142 ± 0.004ghi</td>
<td>0.005 ± 0.004c</td>
<td>3.19</td>
<td>0.45</td>
<td>0.016</td>
</tr>
<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Winter</td>
<td>0.170 ± 0.004bcd</td>
<td>0.008 ± 0.005b</td>
<td>8.25</td>
<td>1.403</td>
<td>0.066</td>
</tr>
<tr>
<td>SCO₂/EtOH</td>
<td>Finnavarra</td>
<td>Spring</td>
<td>0.138 ± 0.001ghi</td>
<td>0.003 ± 0.001a</td>
<td>7.00</td>
<td>0.946</td>
<td>0.021</td>
</tr>
<tr>
<td>SCO₂/EtOH</td>
<td>Spiddal</td>
<td>Spring</td>
<td>0.140 ± 0.004ghi</td>
<td>0.004 ± 0.004a</td>
<td>8.00</td>
<td>1.123</td>
<td>0.032</td>
</tr>
<tr>
<td>SCO₂/EtOH</td>
<td>Finnavarra</td>
<td>Summer</td>
<td>0.139 ± 0.000ghi</td>
<td>0.003 ± 0.002a</td>
<td>7.00</td>
<td>0.973</td>
<td>0.021</td>
</tr>
<tr>
<td>SCO₂/EtOH</td>
<td>Spiddal</td>
<td>Summer</td>
<td>0.133 ± 0.004f</td>
<td>0.002 ± 0.004a</td>
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<td>0.798</td>
<td>0.012</td>
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<tr>
<td>SCO₂/EtOH</td>
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<td>0.003 ± 0.003a</td>
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<td>0.417</td>
<td>0.009</td>
</tr>
<tr>
<td>SCO₂/EtOH</td>
<td>Spiddal</td>
<td>Autumn</td>
<td>0.147 ± 0.008ghi</td>
<td>0.004 ± 0.006a</td>
<td>7.00</td>
<td>1.029</td>
<td>0.028</td>
</tr>
<tr>
<td>SCO₂/EtOH</td>
<td>Finnavarra</td>
<td>Winter</td>
<td>0.144 ± 0.002ghi</td>
<td>0.003 ± 0.004a</td>
<td>3.00</td>
<td>0.432</td>
<td>0.009</td>
</tr>
<tr>
<td>SCO₂/EtOH</td>
<td>Spiddal</td>
<td>Winter</td>
<td>0.157 ± 0.001defg</td>
<td>0.005 ± 0.001a</td>
<td>3.00</td>
<td>0.471</td>
<td>0.015</td>
</tr>
<tr>
<td>SCO₂</td>
<td>Finnavarra</td>
<td>Spring</td>
<td>0.188 ± 0.002ab</td>
<td>0.009 ± 0.003a</td>
<td>2.40</td>
<td>0.451</td>
<td>0.022</td>
</tr>
<tr>
<td>SCO₂</td>
<td>Spiddal</td>
<td>Spring</td>
<td>0.196 ± 0.003a</td>
<td>0.007 ± 0.002a</td>
<td>2.30</td>
<td>0.451</td>
<td>0.016</td>
</tr>
<tr>
<td>SCO₂</td>
<td>Finnavarra</td>
<td>Summer</td>
<td>0.154 ± 0.004deghi</td>
<td>0.003 ± 0.003b</td>
<td>2.30</td>
<td>0.354</td>
<td>0.007</td>
</tr>
<tr>
<td>SCO₂</td>
<td>Spiddal</td>
<td>Summer</td>
<td>0.136 ± 0.001hi</td>
<td>0.003 ± 0.001b</td>
<td>2.90</td>
<td>0.394</td>
<td>0.009</td>
</tr>
<tr>
<td>SCO₂</td>
<td>Finnavarra</td>
<td>Autumn</td>
<td>0.156 ± 0.004deghi</td>
<td>0.002 ± 0.006b</td>
<td>5.20</td>
<td>0.811</td>
<td>0.010</td>
</tr>
<tr>
<td>SCO₂</td>
<td>Spiddal</td>
<td>Autumn</td>
<td>0.159 ± 0.000ef</td>
<td>0.002 ± 0.000b</td>
<td>5.30</td>
<td>0.843</td>
<td>0.011</td>
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<tr>
<td>SCO₂</td>
<td>Finnavarra</td>
<td>Winter</td>
<td>0.194 ± 0.006ab</td>
<td>0.004 ± 0.008b</td>
<td>6.50</td>
<td>1.251</td>
<td>0.026</td>
</tr>
<tr>
<td>SCO₂</td>
<td>Spiddal</td>
<td>Winter</td>
<td>0.164 ± 0.010de</td>
<td>0.003 ± 0.000b</td>
<td>5.50</td>
<td>0.902</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Mean values ± S. D. Different superscript letters (a, b, c etc) column wise indicate significant difference.
Table 6.3. Fucoxanthin purity (mg fucoxanthin equivalents (FE)/mg extract), Xanthophyll purity (mg xanthophyll equivalents (XE)/mg extract) and total extract yield (mg/g dry weight extract (DWE)) of solid-liquids extracts (SLE), supercritical fluid extracts (SFE) and supercritical fluid extracts with ethanol as co-solvent, Total Fucoxanthin content (TFC) (mg TFC/g dry weight extract (DWE)) and Total Xanthophyll content (TXC) mg TXE/g dry weight extract (DWE)) of *Fucus serratus* over four seasons and from two harvest locations.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Location</th>
<th>Season</th>
<th>Fucoxanthin purity (mg FE/mg extract) <em>Fucus serratus</em></th>
<th>Xanthophyll purity (mg XE/mg extract) <em>Fucus serratus</em></th>
<th>Extract yield (mg/g DWE)</th>
<th>Total Fucoxanthin content (mg TFC/g DWE) in <em>Fucus serratus</em></th>
<th>Total Xanthophyll Content (mg TXC/g DWE) in <em>Fucus serratus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Spring</td>
<td>0.139 ± 0.006&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.003 ± 0.004&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.50</td>
<td>2.989</td>
<td>0.065</td>
</tr>
<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Spring</td>
<td>0.128 ± 0.003&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>0.002 ± 0.003&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.40</td>
<td>1.587</td>
<td>0.025</td>
</tr>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Summer</td>
<td>0.165 ± 0.007&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.008 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.50</td>
<td>5.198</td>
<td>0.252</td>
</tr>
<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Summer</td>
<td>0.167 ± 0.007&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.016 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.30</td>
<td>1.720</td>
<td>0.165</td>
</tr>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Autumn</td>
<td>0.167 ± 0.001&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.013 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.90</td>
<td>3.198</td>
<td>0.272</td>
</tr>
<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Autumn</td>
<td>0.153 ± 0.000&lt;sup&gt;bede&lt;/sup&gt;</td>
<td>0.005 ± 0.003&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>25.80</td>
<td>3.535</td>
<td>0.129</td>
</tr>
<tr>
<td>SLE</td>
<td>Finnavarra</td>
<td>Winter</td>
<td>0.137 ± 0.001&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>0.003 ± 0.004&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.50</td>
<td>3.570</td>
<td>0.077</td>
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<tr>
<td>SLE</td>
<td>Spiddal</td>
<td>Winter</td>
<td>0.129 ± 0.001&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>0.002 ± 0.005&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27.00</td>
<td>3.483</td>
<td>0.054</td>
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<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Finnavarra</td>
<td>Spring</td>
<td>0.149 ± 0.000&lt;sup&gt;edef&lt;/sup&gt;</td>
<td>0.005 ± 0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.00</td>
<td>4.619</td>
<td>0.155</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Spiddal</td>
<td>Spring</td>
<td>0.133 ± 0.004&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>0.003 ± 0.002&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.00</td>
<td>1.995</td>
<td>0.045</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Finnavarra</td>
<td>Summer</td>
<td>0.144 ± 0.003&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>0.007 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.00</td>
<td>3.312</td>
<td>0.161</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Spiddal</td>
<td>Summer</td>
<td>0.149 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004 ± 0.002&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.00</td>
<td>1.639</td>
<td>0.044</td>
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<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Finnavarra</td>
<td>Autumn</td>
<td>0.119 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.011 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.00</td>
<td>1.547</td>
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</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Spiddal</td>
<td>Autumn</td>
<td>0.146 ± 0.004&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.008 ± 0.005&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.00</td>
<td>2.190</td>
<td>0.120</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Finnavarra</td>
<td>Winter</td>
<td>0.164 ± 0.006&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.004 ± 0.006&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.00</td>
<td>4.428</td>
<td>0.108</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;/EtOH</td>
<td>Spiddal</td>
<td>Winter</td>
<td>0.147 ± 0.010&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.004 ± 0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.00</td>
<td>1.911</td>
<td>0.052</td>
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<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Finnavarra</td>
<td>Spring</td>
<td>0.135 ± 0.001&lt;sup&gt;fg&lt;/sup&gt;</td>
<td>0.002 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.80</td>
<td>0.918</td>
<td>0.014</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Spiddal</td>
<td>Spring</td>
<td>0.147 ± 0.001&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.004 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.90</td>
<td>0.720</td>
<td>0.020</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Finnavarra</td>
<td>Summer</td>
<td>0.173 ± 0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001 ± 0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.00</td>
<td>1.038</td>
<td>0.006</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Spiddal</td>
<td>Summer</td>
<td>0.179 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.005 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.20</td>
<td>0.931</td>
<td>0.026</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Finnavarra</td>
<td>Autumn</td>
<td>0.139 ± 0.001&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>0.003 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.60</td>
<td>0.778</td>
<td>0.017</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Spiddal</td>
<td>Autumn</td>
<td>0.155 ± 0.010&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.003 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.20</td>
<td>0.806</td>
<td>0.016</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Finnavarra</td>
<td>Winter</td>
<td>0.139 ± 0.001&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>0.001 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.60</td>
<td>0.635</td>
<td>0.005</td>
</tr>
<tr>
<td>SCO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Spiddal</td>
<td>Winter</td>
<td>0.137 ± 0.003&lt;sup&gt;efg&lt;/sup&gt;</td>
<td>0.002 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.60</td>
<td>0.602</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Mean values ± S. D. Different superscript letters (a,b, c etc) column wise indicate significant difference.
Figure 6.4. HPLC chromatogram (450nm) showing the optimization of the SFE extraction from *Fucus serratus*. Peak assignment; (a) fucoxanthin, (b) xanthophyll.
Figure 6.5 HPLC chromatogram (450nm) showing the optimization of the SFE and co-solvent (ethanol) extraction from *Fucus serratus*. Peak assignment; (a) fucoxanthin, (b) xanthophyll
Figure 6.6. HPLC chromatogram (450nm) showing the optimization of the SLE extraction from *Fucus serratus*. Peak assignment; (a) fucoxanthin, (b) xanthophyll.
6.2.3 Seasonal and geographical variation of Fucoxanthin and Xanthophyll

The two main carotenoids found in macroalgae are fucoxanthin and xanthophyll, therefore for this study these two components were investigated in the species *F. serratus* and *L. digitata*. The levels of these over the period of a year from two harvest sites are presented in Tables 6.2 and 6.3. To date several studies on the content of photosynthetic pigments of algae have been carried out (Aguilera *et al.*, 2002; Gudrum, 2005; Sarojini, 2009; Schmidt *et al.*, 2010), however limited information is available on the seasonal changes that can occur.

As observed in section 6.2.2 SLE resulted in highest total yield of fucoxanthin and xanthophyll from the species *F. serratus*, levels in these extracts have been used as the best index for total carotenoid content when discussing seasonal/geographical variation. In the species *L. digitata* the highest total fucoxanthin content in SLE extracts was observed in winter Spiddal extract (1.403 mg TFC/g DWE), with appreciable levels also observed in the spring period. Levels were significantly \( p < 0.05 \) lower in the summer period (0.491 mg TFC/g DWE). The xanthophyll content in the species *L. digitata* had the highest levels in the spring and the lowest levels in the autumn. This corresponds with the purity levels observed, with the highest purity of xanthophyll observed in spring and the lowest purity in autumn. For the species *F. serratus* the highest total fucoxanthin content was observed in the summer Finnavarra sample (5.198 mg TFC/g DWE), the lowest level was seen in the spring Spiddal (1.587 mg TFC/g DWE), this is also the case for the purity of fucoxanthin with the highest purity observed in the summer Spiddal sample (0.167 mg FE/mg extract) and the lowest purity in the spring Spiddal (0.128 mg FE/mg extract). A similar trend is observed for the xanthophyll with the greatest total xanthophyll content seen in summer and the lowest level in spring. In terms of geographical variation in *L. digitata*, no significant \( p > 0.05 \) variation in the TFC was observed between locations in the spring extracts, however...
variation is evident between harvest locations in summer, autumn and winter with Finnavarra having the consistently higher content. Significant \((p < 0.05)\) variation in TXC is observed between both harvest sites. For the species \(F. \textit{serratus}\) no significant variation is seen between harvest locations in autumn and winter, but variation is seen in spring and summer with Finnavarra having the higher TFC. Again variation in TXC is observed between harvest sites.

Whilst it is most useful to discuss the effect of seasonal and geographical variation in terms of the total yield of the target compounds in SLE extracts it is also interesting to note which extraction resulted in the highest purity extracts and at what time of the year. In the species \(L. \textit{digitata}\) the winter and spring periods appear to be the optimal season for obtaining fucoxanthin and xanthophyll in higher purity from all extracts, SLE, \(\text{SCO}_2/\text{EtOH}\) and \(\text{SCO}_2\). The lowest levels of fucoxanthin and xanthophyll purity were observed during the summer and autumn period in \(L. \textit{digitata}\). In general there was no significant \((p < 0.05)\) difference in relation to purity observed between locations in spring, summer and autumn, however, for several of the winter samples \((L. \textit{digitata} \text{SLE} \& \text{SCO}_2/\text{EtOH} \text{and} \text{Fucus serratus} \text{SCO}_2)\) a difference was observed in the purity of the compounds extracted between harvest locations. In the \(L. \textit{digitata}\) SLE winter extracts Spiddal gave a higher fucoxanthin purity \((0.170 \text{ mg FE/mg extract})\) than the Finnavarra sample \((0.142 \text{ mg FE/mg extract})\), this is also the case for the xanthophyll purity for the same samples. Similar results are observed in the \(L. \textit{digitata}\) \(\text{SCO}_2/\text{EtOH}\) winter samples from Spiddal which both a higher purity of fucoxanthin \((0.157 \text{ mg FE/mg extract})\) and xanthophyll \((0.005 \text{ mg XE/mg extract})\) in comparison to the Finnavarra sample for the same season. In the \(F. \textit{serratus}\) \(\text{SCO}_2\) winter samples the sample harvested from Finnavarra exhibits a higher fucoxanthin purity \((0.139 \text{ mg FE/mg extract})\) to the Spiddal sample but the Spiddal sample has a greater xanthophyll purity \((0.002 \text{ mg XE/mg extract})\).
The results observed for *L. digitata* are in agreement with reports from previous studies by Stengel and Dring (1998) which investigated carotenoid levels in the brown macroalgae *Ascophyllum nodosum*, they observed that levels increased during the autumn and winter months but were lower by 50% in the summer period. Similar results were also observed by Robledo (2005) where maximum carotenoid levels occurred during the cold seasons where higher precipitation occurred. Pereira (2012) also found that a higher carotenoid concentration occurred in months with less sunlight and greater nitrogen availability. Sampath-Wiley (2008) stated that carotenoid levels were affected mainly by exposure to sun. Shaded blades maintained high concentrations of carotenoids compared to sun exposed thalli. For the other species *F. serratus* the summer period produced the higher content and purity of fucoxanthin. Predominantly winter and spring periods produced the lowest content and purity of both fucoxanthin and xanthophyll with little difference observed in the different harvest sites. Stengel and Dring (1998) also observed that fucoxanthin peaked during the summer period in the brown seaweed *Ascophyllum nodosum*, and suggested that the variation in pigments was due to differences in water depth, self-shading and enhanced nutrient limitation in the summer period. Ramus *et al.* (1976) also observed that environmental variation in light, temperature and nutrients had an influence and brought about changes in the concentration of pigments in the thallus of the seaweed investigated.

### 6.3 Conclusion

Optimal conditions for producing the greatest yield of carotenoid rich extracts from *Fucus serratus* using SC$_2$O were at 50 °C and 300 Atm for an extraction time of 60 min. This study also examined the efficiency of this method in comparison to the more traditional solvent extraction method and also compared it to the SC$_2$O using ethanol as a modifier. It was found that SC$_2$O produced extracts
with a high purity of fucoxanthin while SLE provided a greater yield of fucoxanthin in the extract. It was found that SLE produced extracts with both a high purity and extract quantity of xanthophylls. Seasonal variation of these carotenoids within these two macroalgae (*Fucus serratus* and *Laminaria digitata*) was also investigated for SLE extracts. Winter and spring gave the highest purity and content of fucoxanthin and xanthophyll in the species *Laminaria digitata*, while summer had the greatest purity and content of these compounds for the species *Fucus serratus*. The seasonal variation observed in the two carotenoids investigated is mostly likely due to the plant being exposed to diverse environmental parameters such as light, temperature and nutrients, which have influenced and brought about change in the concentration of carotenoids in the samples tested.

As pigments from seaweeds are very much commercially valuable in terms of food colourings, functional ingredients and also in the cosmetic industry this information may prove to be useful in the selection of harvest times for particular compounds of interest and also in determining the optimal extraction technique depending on sample purity or quantity.
References


Kim, K.W., Heo, S.J., Yoon, W.J., Kang, S. M., Ahn, G. Y. T. H. and Jeon, Y. J. (2010). Fucoxanthin inhibits the inflammatory response by supressing the activation of NF-κB and


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Chapter 7

Seasonal and geographical investigation of antioxidant polyphenolic phlorotannins in *Fucus serratus*.
Abstract

Phlorotannins are a group of complex polyphenolic polymers found in brown macroalgae. They are composed solely of the monomer phloroglucinol (1, 3, 5-trihydroxybenzene). Previous reports have shown variations in the concentration of polyphenolic compounds using colorimetric assays. However, no study to date has investigated the effects of both seasonal and geographical variation on the structure and composition of phlorotannins the main polyphenolic found in macroalgae and the influence this has on their bioactivity. Ultra-performance liquid chromatography (UPLC) with tandem mass spectrometry was employed to investigate detailed molecular weight and isomeric differences present in the brown macroalgae *Fucus serratus* harvested from two locations in Ireland (Finnavarra, Co. Clare and Spiddal Co. Galway) over the period of a year. Antioxidant and total phenolic content assays, the current methodologies employed in the literature, were used as an index for comparison of crude extracts. Phlorotannin enriched fractions were profiled using UPLC-MS with multiple reaction monitoring (MRM) and the level of isomerisation for specific molecular weight phlorotannins between 3 and 16 monomers were profiled. The majority of low molecular weight phlorotannins were found to have a DP of 6-14 across all seasons and harvest sites. However, the level of isomerisation across seasons varied greatly with winter having the highest number of isomers (427 different phlorotannins structures), while isomeric variation was much lower in the summer samples (121 different phlorotannins). Samples with high levels of isomerisation also had the greatest antioxidant activity. The summer samples which had lower levels of isomerisation exhibited the lowest antioxidant potential and total phenolic contents. This may imply a positive correlation between high levels of isomerisation and *in-vitro* activity. These results highlight the complexity of these compounds and show that these molecules are influenced by extrinsic factors such as the environment and seasons.
7.0 Introduction

Phlorotannins are secondary metabolites composed of phloroglucinol units (1, 3, 5-trihydroxybenzene) found predominately in brown macroalgae (Hay and Fenical, 1988; Hay, 1996; Steinberg, 1992; Ragan and Glombitza, 1986). They are thought to act as a defence mechanism for macroalgae against grazers and bacterial colonisation (Amsler et al., 1998; Targett and Arnold, 1998; Pavia and Brock, 2000; Connan et al., 2004; Stiger et al., 2004). Similar to other phenolic compounds, phlorotannins have been reported to exhibit potential beneficial biological properties such as antioxidant (Shibata et al., 2002; Shibata et al., 2008, Tierney et al., 2013a), anti-inflammatory (Kim et al., 2009), anti-diabetic (Lordan et al., 2013), anti-allergic (Li et al., 2008), anti-HIV (Artan et al., 2008), chemo preventative agents (Hwang et al., 2006; Kong et al., 2009) and anti-plasmin (Fukuyama et al., 1989) and HAase inhibitors (Okada et al., 2004). This broad range of biological activity has generated considerable research into these marine compounds and their potential use in therapeutics or functional food products.

Relatively limited research has been reported on the characterisation of these compounds due to the high level of structural complexity exhibited in particular species as a result of their polymeric nature, as previously shown (Heffernan et al., 2015). This in turn permits significant variation in both the number of monomers present in each structure and the positions at which they are linked. Since in macroalgae phlorotannins fulfil an allelochemical role, variations in their structure and size may also be due to extrinsic factors such as climate or environment. Macroalgal polyphenolic contents have been shown to exhibit spatio temporal variations (Rönnberg and Ruokolahti, 1986; Targett et al., 1992; Steinberg, 1995 and Van Alstyne et al., 1999) which are related to factors such as the types of habitat (Hay and Fenical, 1988; Hay, 1996; Stiger et al., 2004), the quality and intensity of light and the exposure to UV radiation (Pavia et al., 1997), salinity (Pederson, 1984),
emersion depth, (Martinez, 1996) and nutrient concentrations (Yates and Peckol, 1993; Hemmi et al., 2004). Other studies have also examined phenolic content in relation to climate and geographical locations of macroalgal species (Targett et al., 1995). However, these studies only report on the total phenolic contents with no information reported on the influence of these factors on the chemical composition of the polyphenolic and in turn the effect of these variations on the antioxidant activity of these molecules. Recently it has demonstrated that ultra-performance liquid chromatography (UPLC) with tandem mass spectrometry was an effective method for the investigation of variations in degrees of polymerisation and isomeric in phlorotannins derived from macroalgae harvested off the Irish coast (Tierney et al., 2013b; Heffernan et al., 2015).

Therefore the main objective of the present study was to use the aforementioned technique to investigate seasonal and geographical variation of phlorotannins present in the brown macroalgae Fucus serratus. UPLC-MS/MS was used to determine the isomeric complexity and relative abundance of each degree of polymerization from enriched phlorotannin extracts. This study serves to highlight the differences in these compounds in relation to season and geographical variation and will give a better understanding of these complex molecules with regard to their antioxidant activity, structure and potential exploitation.
7.1 Materials and methods

7.1.1 Standards and Reagents

All chemicals used were reagent grade, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ferrous chloride, ferrozine, 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) and silica gel were obtained from Sigma-Aldrich Chemical Co. (Arklow, Wicklow, Ireland). All solvents used were HPLC grade. BioDesignDialysisTubing™ with 3.5 kDa cut-off was acquired from Fisher Scientific (Dublin 1, Ireland). Agilent SuperFlash™ SF25-55G C18 Si50, and SF10-8 g, 14.2 x 136mm, 50µm were obtained from Apex scientific (Maynooth, Ireland).

7.1.2 Samples

The brown macroalgae samples used in this study were identified by a trained phycologist and harvested off the west coast of Ireland. *Fucus serratus* (Fucaceae) was harvested from two locations, Finnavarra, Co. Clare (53°8’58.8°N- 9°8’7.76°W) and Spiddal Co. Galway (53.244184°N-9.30586°W) from Autumn 2011 to Summer 2012, samples were collected for each season starting with Autumn 2011, Winter 2011, Spring 2012 and Summer 2012. A random selection of a large number of different plants were taken from the shore, to allow for natural variability, these were packed in cool boxes and transported immediately to the laboratory. Samples were washed thoroughly with fresh water to remove sand and epiphytes, frozen at -20 ºC and freeze dried (A12-60 Freeze Dryer; Frozen in Time Ltd., York, England). A freeze dried sample of each was retained for reference at the Irish Seaweed Centre at NUIG. The macroalgae samples were ground to a powder using a Waring® blender (New Hartford, CT, USA) and stored in vacuum packed bags at -80 ºC prior to extraction.
7.1.3 Solid-Liquid extraction (SLE)

Solid liquid extraction was employed to extract the phlorotannins from the macroalgae under investigation using ethanol/water (80:20) as this solvent system has previously been shown to be effective for extracting antioxidants from macroalgae (Tierney et al., 2013a; Heffernan et al., 2014a). The method used has previously been described in Heffernan et al. (2014a; 2014b).

7.1.4 Partitioning and Molecular weight cut-off (MWCO) dialysis

Partitioning and molecular weight cut-off (MWCO) dialysis were employed to produce both hydrophilic and hydrophobic fractions, the hydrophilic fraction was then further fractionated using MWCO dialysis to produce MW’s of < 3.5 KDa and > 3.5 KDa which were frozen and freeze dried. This method has previously been described in Heffernan et al. (2014b).

7.1.5 Reverse-phase Flash Chromatography

The lower molecular weight (< 3.5 kDa) fraction of each species was further fractionated using a two-step reverse phase (RP) flash chromatography method (Tierney et al., 2013b). RP-flash chromatography was carried out on a Analogix Intelliflash 310 system (Varian Inc.) using a Septa C18 column with a sorbent mass of 55 g and a mean particle size of 40-60 µm. One gram of the < 3.5 kDa fraction material was dissolved in water and loaded onto the column. A two-step elution gradient was employed. The mobile phase consisted of the primary eluent of HPLC grade water (0-20 min) and the secondary eluent of 100 % methanol (20-40 min). The flow rate was 50 ml/min. Flash fractions were collected from 0 to 20 min (flash fr. 1) and from 20-40 min (flash fr. 2). UV detection was observed at 210, 225 and 250 nm. A phlorotannin polymer fraction was collected from 20 to 40 min.
7.1.6 Total phenolic content (TPC) and in-vitro antioxidant capacity

The total phenol content of the crude extract was quantified according to a previously described method (Heffernan et al., 2014b). Gallic acid was used as an internal standard and results were expressed in terms of microgram gallic acid equivalents per milligram of dry weight sample (µg GAE mg⁻¹ sample). All crude extracts were tested in triplicate. The Ferric reducing antioxidant power (FRAP) was assessed according Kenny et al. (2013). Trolox was used as an internal standard and results were expressed as microgram Trolox Equivalents per milligram dry weight sample (µg TE/mg DW sample). The free radical scavenging capacity of the crude extracts was analysed using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay as described elsewhere (Heffernan et al., 2014b). Trolox was used as an internal standards and results were expressed as antiradical power (ARP), which is the reciprocal of the IC₅₀ (mg ml⁻¹) used to define the concentration of sample extract that produces a 50 % reduction of the DPPH⁻ radical absorbance (Brand-Williams et al., 1995).

7.1.7 UPLC-MS/MS of Phlorotannin enriched fractions

UPLC-MS analysis of Fucus serratus ethanol/water phlorotannin reverse-phase (RP) flash chromatography enriched fractions (prepared as described in sections 7.1.2-7.1.6 was conducted using an Acquity™ UPLC® System (Waters Corporation, Micromass MS Technologies, Manchester, UK) on the phlorotannin enriched fraction from the <3.5. This method has previously been described previously (Heffernan et al., 2015).
7.1.8 Statistical analysis

All extracts were analysed in triplicate. Measurement values are presented in means ± standard deviation. One way analysis of variance (ANOVA), followed by the Tukey post hoc comparison test, was carried out to test for significant differences in antioxidant activity and phenolic content between macroalgal species using the statistical program Minitab® Release 17 for Windows. A probability value of $p < 0.05$ was considered statistically significant.

7.2 Results

7.2.1 Total phenolic content (TPC) and in-vitro antioxidant activities.

Figures 7.1 – 7.3 illustrate the phenolic content and in-vitro antioxidant activity (as measured using the DPPH and FRAP methods) of crude extracts from *Fucus serratus* harvested from Spiddal Co. Galway and Finnavarra Co. Clare in 2011-2012. These results are in line with previous results on this species (Heffernan *et al.*, 2014a). The FRAP results previously reported were 69.30µg TE/mg sample (Heffernan *et al.*, 2014a), the seasons summer, autumn and winter are similar to these observed results and fall between 57-98 µg TE/mg sample, spring however exhibits lower levels of 52.10 µg TE/mg sample. Seasonal TPC are slightly greater than previous values reported for this species (75.96 µg GAE/mg sample). Summer, autumn and winter values range between 87-182 µg GAE/mg sample, with again spring showing having the lowest level of activity of 87.20 µg GAE/mg sample. The DPPH assay results observed are much higher than this previous report (Heffernan *et al.*, 2014a) with values ranging between 68-175 ARP, while spring again was observed to have the lowest level of 68 ARP. With regard to time of harvest, phenolic contents of *Fucus serratus* crude extracts were significantly higher in the winter sample harvested from Spiddal, however, significantly ($p < 0.05$) lower levels were observed in the winter sample from Spiddal.
Finnavarra. Autumn samples from both locations also showed high phenolic contents and would be a good harvest time also. Ragan and Jensen (1978) observed that levels of polyphenols in two brown macroalgae (*Ascophyllum nodosum* and *Fucus vesiculosus*) were lower in spring and then increased during the summer months. Pederson (1984) reported that the polyphenol content in the seaweed *Ascophyllum nodosum* increased with the age of tissue as would have been the case for samples harvested in summer in the present study. More recent studies by Connan *et al.* (2004) investigated the interspecific and temporal variation in phlorotannin levels in an assemblage of brown algae. They observed that summer had the maximum phenolic content for fucales while winter was the maximum for a member of the Laminariales. Plouguerné *et al.* (2006) observed that phenolic content was highest in the species *Sargassum muticum* in the summer months when the plant was in maturity. The high levels of phenolics observed in summer support the idea of a photo-protective role for phenolic accumulation. While the high levels in the winter sample are in agreement with a previous report by Ragan and Glombitza (1986) who also observed a high phenolic content in the winter period. This phenolic accumulation may be linked to other environmental stresses (decrease in environment temperature, irradiance levels and defence related factors such as grazing) (Yate and Peckol, 1993). Stiger *et al.* (2004) determined that phenolic compounds were higher in nutrient rich sites and also in mature plants.

A similar trend was also observed in both the DPPH and FRAP results (Figure 7.1 and 7.2). The winter sample harvested from Spiddal exhibited the highest activity in the DPPH assay (ARP = 175.65), with the two autumn samples having the next highest (ARP= 133.04 Spiddal and 130.45 Finnavarra). Spring Finnavarra sample was observed to have the lowest activity (ARP = 68.54). Limited research has been completed on the investigation of the seasonality of these species in relation to their antioxidant activity. There was no significant (*p* < 0.05) difference between harvest
sites in autumn in the DPPH assay, however there was significant variation between harvest sites for spring, summer and winter.

Again the winter sample harvested from Spiddal yielded the highest antioxidant activity in the FRAP assay (98.13 μg TE/mg sample) with the spring Finnavarra sample having the lowest activity (52.10 μg TE/mg sample). There was a significant ($p < 0.05$) variation observed between harvest locations for all seasons.

**Figure 7.1.** 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) expressed as antiradical power (ARP) of crude extracts from *Fucus serratus* harvested at two locations over the period of a year 2011-2012. Values represented as mean ± standard deviation performed in triplicate. Different superscript letters (a, b, c etc) indicate significant difference. Tukey’s HSD test.
Figure 7.2 Ferric reducing antioxidant power (FRAP) expressed as Trolox equivalents of crude extracts from *Fucus serratus* harvested at two locations over the period of a year 2011-2012. Values represented as mean ± standard deviation performed in triplicate. Different superscript letters (a, b, c etc) indicate significant difference. Tukey’s HSD test.

![FRAP graph](image)

Figure 7.3. Total phenolic content (TPC) expressed as μg gallic acid equivalents (GAE), of crude extracts from *Fucus serratus* harvested at two locations over the period of a year 2011-2012. Values represented as mean ± standard deviation, performed in triplicate. Different superscript letters (a, b, c etc) indicate significant difference. Tukey’s HSD test.

![TPC graph](image)
7.2.2 UPLC-ESI-MS profiling of phlorotannin enriched fractions

Figure 7.4 presents a total ion chromatogram (TIC) of the reversed phase (RP) flash chromatography enriched fractions from the brown algae *Fucus serratus* harvested from Spiddal Co. Galway over the period of a year. It serves to illustrate the structural complexity of the isolated phlorotannins and the variation in relation to season. It can be seen in Figure 7.4 that the majority of the phlorotannins present in *Fucus serratus* have molecular weights ranging between 6 and 14 phloroglucinol units. There is clear variation in the phlorotannin profile observed between the seasons. Figures 7.5 and 7.6 show a more in-depth picture of the seasonal variation and displays MRM ion chromatograms of the reversed phase (RP) flash chromatography enriched fractions of *Fucus serratus* (Spiddal and Finnavarra) for deprotonated molecules [M-H]- of 745.3 and 1242.4 \( m/z \) (DP 6 and 10). Variation is seen in the abundance of isomers (Figure 7.7 and 7.8) for each particular molecular weight. At \( m/z \) 745.3 (DP 6) spring, autumn and winter have a relatively similar number of isomers (Spring 11, Autumn 11 and Winter 9) however there is a drop in the isomer number for the Summer sample (4 isomers). Similar results are seen at the \( m/z \) 1241.4 (DP 10) with summer having a much lower number of isomers of just 22 (13-19% peak intensity) in comparison to spring (34 isomer; 10-18% peak intensity), autumn (54 isomers; 12-20% peak intensity) and winter (40 isomers; 18-20% peak intensity).

Reversed phase flash chromatography enriched fractions from *Fucus serratus* Spring samples harvested from Spiddal and Finnavarra exhibited seasonal and geographical The most prominent peak observed in the spring sample from Spiddal is at \( m/z \) 1117 (DP of 9), while the most prominent in the Finnavarra sample is \( m/z \) 1,365 (DP of 11). The most abundant peak in the summer sample from Spiddal is at \( m/z \) 745 (DP of 6), while at the Finnavarra site the most noticeable peak is at a higher \( m/z \) of 1241 (DP of 10). Both the autumn and winter samples a
prominent peak at 1,117 (DP of 9) in the Spiddal samples and a prominent peak at 1,241 (DP of 10) in the Finnavarra samples were observed. Other noticeable peaks are observed in all seasons and locations at \( m/z \) 1,365 (DP of 11) and 1,490 (DP of 12).

There have been many reports that have suggested that the molecular weight of hydrolysable tannins influence the antioxidant activity they possess. Hagerman et al. (1998) previously reported that high molecular weight condensed (plant) tannins exhibited antioxidant activity up to 15-30 times more than that of simple phenolics and trolox. They purported that this activity was due to the high molecular weight of these molecules and the proximity of many aromatic rings and hydroxyl groups which increase the free radical scavenging ability of these compounds. In general samples that contained a low DP exhibited lower activity. The summer sample harvested from Spiddal had the broadest DP of 6-12 (~73% peak intensity), while most samples had DP of 9-13 (55-75% peak intensity). This sample also exhibited relatively lower activity across the in-vitro tests to the other samples.

There is little known as to the reason behind the varying degree of polymerisation seen in the macroalgae phlorotannins. These differences may be due to the in-situ response to stresses from the environment such as UV radiation, herbivore predation and changes in salinity. The level of phlorotannin isomerisation across seasons varied to some degree, however little difference was seen between the harvest sites. There have been many studies that have looked at the effects of seasonal and geographical variations on the concentration of phenolics in macroalgae, however, none to date have investigated the effects of these on the structural changes of these compounds and whether these changes influence their bioactivity. Stiger et al. (2004) looked at the effects of interspecific, ontogenetic and spatio-temporal variations of two tropical brown macroalgae and determined that phenolic compounds were higher in nutrient rich sites, during the summer months...
and in mature plants. Yate and Peckol (1993) suggested that within species variation in polyphenolics is due to a complex interaction of environmental (nutrient availability, irradiance levels) and defence related (grazing activity) factors.

Figure 7.4 displays the molecular ion chromatograms for the MRM transitions of phlorotannins from each season from the Spiddal harvest site and highlights the abundance of phlorotannins present. Figure 7.7 and 7.8 represents the number of isomers present for each particular DP and the total number of isomers present respectively. A high number of isomers were detected across all seasons for *Fucus serratus*. The winter Spiddal sample had the highest number of isomers detected, 427 in total, with the majority of these detected between DP 8-14 (~87% peak intensity). This would suggest significant variation in branching positions is occurring between monomeric units. The summer Spiddal sample had the lowest number of isomers of 121, this summer Spiddal also exhibited the lowest *in-vitro* activity. This would suggest that the level of isomerisation occurring within the samples is having a beneficial influence on the *in-vitro* activities, with samples with a greater level of isomerization also having a higher antioxidant activity. Samples with a higher DP and significant isomerisation occurring have higher antioxidant activity and TPC. A high abundance of isomers were detected in all samples between 6-14 PGUs (~87-94% peak intensity) (Figure 7.7). There is slight variation observed across the seasons in relation to the isomeric count at particular molecular ions. In general the isomer count decreases from spring to summer with summer having the lowest number and then gradually increases over autumn with the winter sample having the highest isomer content. This trend is seen through the individual molecular ions also. The peak intensities and also the number of isomers for each sample harvested can be seen in Figures 7.7 and 7.8. At least 80% of the phlorotannins in all samples are being observed between 6-14 PGUs. The high abundance of isomers is generally seen between 8-12
PGUs (~58-86% peak intensity). Limited research has been carried out on the identification of phlorotannins from the species *Fucus serratus*. This is most likely due to the complex nature of these molecules and the level of isomerisation and as seen in this study the varying isomerisation across seasons. Previous research by this group (Heffernan *et al.*, 2014) generated tannin rich fractions using reverse phase flash chromatography and LC-Q-TOF-MS identified a high abundance of low molecular weight phlorotannins in the < 3.5 kDa fraction of the species *F. serratus* and detected phlorotannin compounds in this species between the range of 3-16 PGUs. The previous study found that *Fucus serratus* had highest phlorotannin compound abundance in the range of 6-13 PGU’s, account for 90% peak intensity. However this present study provides a further insight into these compounds and the complexity of them in relation to changes in structure and isomerisation due to extrinsic factors. It has provided the ground work for further research into determining crucial information for better understanding and exploitation of these compounds.
Figure 7.4. Total Ion chromatogram (TIC) of reverse phase (RP) flash chromatography enriched fractions of *Fucus serratus* harvested from Spiddal Co.Galway in (a) Spring (b) Summer (c) Autumn (d) Winter.
Figure 7.5. UPLC-MS/MS MRM ion chromatograms of reversed phase (RP) flash chromatography enriched fractions for *Fucus serratus* for deprotonated molecules [M-H]$^-$ of 745.3 which corresponds to phlorotannins containing 6 phloroglucinol units. (a) Spring, Spiddal (b) Spring, Finnavarra (c) Summer, Spiddal (d) Summer, Finnavarra (e) Autumn, Spiddal (f) Autumn, Finnavarra (g) Winter, Spiddal (h) Winter, Finnavarra.
Figure 7.6. UPLC-MS/MS MRM ion chromatograms of reversed phase (RP) flash chromatography enriched fractions for *Fucus serratus* for deprotonated molecules [M-H]^- of 1242.4 m/z which corresponds to phlorotannins containing 10 phloroglucinol units. (a) Spring, Spiddal (b) Spring, Finnavarra (c) Summer, Spiddal (d) Summer, Finnavarra (e) Autumn, Spiddal (f) Autumn, Finnavarra (g) Winter, Spiddal (h) Winter, Finnavarra.
**Figure 7.7.** UPLC-MS percentage peak intensity for individual molecular ions corresponding to phlorotannins of between 3 and 16 phloroglucinol units (PGU’S) for phlorotannin enriched fractions from the species of *Fucus serratus* collected from two locations and over the period of a year. Number of isomers detected for each DP are highlighted with bold numbers.
7.3 Conclusion

This present study reports the profiling of phlorotannins from the macroalgae *Fucus serratus* and determined the degree of isomerisation between 3-16 phloroglucinol monomers in the species *Fucus serratus* harvested from two locations over four seasons.

It was observed that the majority of low molecular weight phlorotannins found in this species had molecular weight ranges of 6-14 monomers. This was seen across the seasons and also in the two locations. It was seen that seasons (autumn and winter) with a high molecular weight (DP 9 to DP 11) of phlorotannins contained much greater levels of isomers for particular molecular ions. This was evident in the winter and autumn samples in particular which showed higher numbers of isomers. For the summer and spring samples the molecular weight was much lower especially in the summer spiddal sample (DP of 6-14) (~80-85% peak intensity), the level of isomerisation is also much lower in this sample (121 total).
This study has highlighted that these compounds are liable to seasonal and spatial variation in relation to abundance and isomerisation occurring, and these intrinsic variations in turn influence the bioactivity of the samples. This information proves useful for industry to determine the optimum harvest time of this seaweed to gain maximum levels of these potentially useful compounds but also maximum bioactivity
References


Chapter 8

Investigation of the seasonal variation of Laminarin in the brown macroalgae Laminaria digitata
Abstract

With a view to evaluating its potential as an ingredient in functional foods, variations in the level and nature of the naturally occurring bioactive polysaccharide laminarin in the brown macroalgae *Laminaria digitata* was investigated. Laminarin was extracted from samples of the algae harvested from Irish coast (Finnavarra Co. Clare) over the period from 2012-2013. The extracts were fractionated using molecular weight cut-off dialysis with molecular weights and chain length of laminarin in the fractions analysed using ESI-QTOF-MS. Results indicated that samples harvested in the autumn/summer exhibited a higher content of laminarin (July 2012, August 2012, October 2012 and July 2013). Laminarin was not detected during the winter/spring period (December 2012, January 2013, March 2013 and May 2013). Variation in the molecular weight based on the number of glucose monomers in each sample was also observed. The sample in July 2013 exhibited the highest average molecular weight of 4393 (27 glucose units). In general the summer period showed high average MW with the samples from July-October 2012 having an average molecular weight of 4168 (25 glucose units). The autumn and spring were observed to have a much lower molecular weight (November 2012 – 2706 (16 glucose units) and April 2013 -3096 (19 glucose units)).

This study provided a good insight into the seasonal variation occurring in the laminarin content in the species *L. digitata* and how this also has an impact on the structure of the laminarin obtained. Through this research, during this time period, it was found that to obtain *L. digitata* with optimum levels of laminarin, samples should be harvested during the summer/autumn period. A more detailed study investigating the various environmental factors that could influence laminarin production would provide further insights into the seasonal variation of this polysaccharide.
8.0 Introduction

In the Western world seaweed has commonly used as a source of compounds for the pharmaceutical, cosmetic and food industry (Weijesinghe and Jeon, 2012). In Europe, the main seaweeds exploited for industry as a source of alginates are *Laminaria hyperborea, Laminaria digitata* and *Ascophyllum nodosum* (Netalga, 2014). Many seaweeds possess an abundance of compounds such as polysaccharides, peptides, omega-3 fatty acids, carotenoids, phenolics, vitamins and minerals, which could have the potential for improving human health. Therefore with an ever growing demand for ingredients of natural origin, seaweeds are often investigated as a potential source of these bioactive functional compounds. Seaweeds contain an array of different polysaccharides groups many of which are currently exploited such as alginate and carrageenan. Seaweeds contain polysaccharides in their cell walls that allow them to adapt to the water movements by providing structural support in the harsh environment in which they grow (Rinaudo, 2007). The concentration of polysaccharides in seaweeds ranges between 4-76 % dry weight, with Ascophyllum, Porphyra and Palmaria species recorded to contain the highest concentrations (Kraan, 2012).

Laminarin is a polysaccharide found in seaweed which some researchers have suggested has anti-tumour (Kuda *et al.*, 2005), anti-apoptotic (Kim *et al.*, 2006), anti-inflammatory (Neyrinck *et al.*, 2007), anticoagulant (Miao *et al.*, 1995) and antioxidant (Choi *et al.*, 2012) properties. For example Kuda *et al.* (2005) examined the inhibitory effects of laminarin on rancid compound formation in both human faecal culture and rat caecum. They reported that the fermentation of laminarin by intestinal bacteria suppressed the formation of these rancid risk markers like indole compounds and ammonia for colon cancer. Kim *et al.* (2006) showed immunomodulatory effects of laminarin in mouse thermocytes with a laminarin oligosaccharide and laminarin polysaccharides.
showing a suppression of apoptotic death of 2 to 3 fold and extended cell survival in culture at a rate of 30-20%. Neyrinck et al. (2007) investigated the hypothesis that laminarin can modulate systemic inflammation. Male Wistar rats were fed a standard diet (control) and a supplemented diet which contained laminarin for 25 days. The authors concluded that there is lower recruitment of inflammatory cells inside the liver tissue and lower secretion of inflammatory mediators due to a direct effect of β-glucan on immune cells, or to an indirect effect through their dietary fibre properties. Miao et al. (1995) showed that laminarin sulphate exhibited about 30% of the anticoagulant activity of heparin (in-vivo) and was effective therapeutically in the prevention and treatment of cerebrovascular diseases. Choi et al. (2012) observed an increase in antioxidant activity when laminarin was irradiated. The authors reported that irradiation breaks the glycosidic linkage of laminarin, generating radicals that are free to be hydroxylated.

In macroalgae, laminarin functions primarily as a food reserve found in the frond and consists of (1, 3)-β-D-glucopyranose residues with some 6-O-branching in the main chain and β-(1, 6)-intra chain links. The molecular weight of Laminarin is approximately 3-5 kDa and is dependent upon the degree of polymerization which is in the range of 20-25 glucose moieties (Nelson and Lewis, 1974; Alderkamp et al., 2007). Two types of laminarin chains can be found (Figure 8.1, M and G chains). M chains end with 1-O-substituted D-mannitol, while G chains end with glucose (Chizhov et al., 1998).
Figure 8.1 M and G chains of Laminarin

Water, temperature, salinity, waves, sea current and depth of immersion have all been shown to affect the composition of laminarin (Black, 1949; Bak, 2014). It has been reported that laminarin content is lowest in the period of fast growth in spring, but in autumn and winter it may account for up to 37 % of dry weight of fronds (Rinaudo, 2007). This variation presents particular
challenges to seaweed processors in the production of consistent seaweed products. Similar to most naturally occurring polysaccharides the biological activities of laminarin is a function of its molecular structure especially molecular mass, degree of branching and length of branches (Oi et al., 2005; Rioux et al., 2010). For example, Kim et al. (2006) reported that low molecular weight laminarin induces the expression of genes coding for immune response proteins and reduces apoptotic cell death. Therefore given its importance with regard to possible health imparting properties of this polysaccharide it is surprising to note that there is relatively little known with regard to how seasonality affects its abundance and molecular mass. This information is useful for particular industries that wish to exploit laminarin and will provide an insight into the optimum harvest time.

8.1 Materials and methods

8.1.1 Chemicals and equipment

All chemicals used were reagent grade, concentrated HCl (37%), Water and acetonitrile were obtained from Sigma-Aldrich Chemical Co. (Arklow, Wicklow, Ireland). BioDesign Dialysis Tubing™ with a 1 kDa and 8 kDa cut-off was acquired from Fisher Scientific (Wicklow, Ireland).

8.1.2 Macroalgal material

Ten samples Laminaria digitata (Laminariaceae) used in this study were harvested from the west coast of Ireland over the period of a year from July 2012-July 2013. Sampling was carried out by randomly selecting samples, to allow for natural variability. Samples were then washed thoroughly with fresh water and stored at -20°C and the identity of each macroalgal specimen was verified by a trained phycologist. The macroalgal samples were subsequently freeze-dried (A12-60 Freeze
Dryer; Frozen in Time Ltd., York, England), ground to a powder using a Waring® blender (New Hartford, CT, USA) and stored in vacuum-packed bags at -80°C prior to extraction.

8.1.3 Extraction of laminarin

Laminarin was extracted from freeze dried Laminaria digitata powders using the Black (1951) method with slight modifications. 10 g of the seaweed powder in a conical flask and adding 0.1 M HCl at a ratio of 10:1 (v/w). The mixture was then placed into a shaker (Thermo Scientific MaxQ6000, Dublin, Ireland) at 60 °C for 24 h at an rpm of 170. Extracts were exhaustively extracted by replenishing the extraction solvent over the 24 h period, this was done following filtration through a Buchner funnel. Extracts were neutralized to pH 7 using 0.1M NaOH, frozen at -20°C and freeze dried (A12-60 Freeze Dryer; Frozen in Time Ltd., York, England). All extracts were subsequently ground to a fine powder using a mortar and pestle prior to further analysis.

8.1.4 Molecular weight cut-off (MWCO) dialysis

Extracts (1 0 g) were dissolved in a minimal volume of deionised water and decanted into 1 kDa dialysis tubing clamped at one end. The tubing was then clamped at the other end, immersed in a reservoir of deionised water and shaken moderately (50 rpm) at room temperature for 72 h. The reservoir of water was refreshed periodically until no further colour was visible in the dialysate. Fractions (>1 kDa and <1 kDa) were frozen at -20 °C freeze dried (A12-60 Freeze Dryer; Frozen in Time Ltd., York, England) to obtain a powder. The > 1 kDa fraction was then subjected to further fractionation using the same process but with 8 kDa dialysis tubing. The 1-8 kDa fraction was then subjected to analysis using HPLC-ESI-QTOF-MS.
8.1.5 HPLC-ESI-QTOF-MS analysis of laminarin samples

LC-MS analysis of the laminarin samples was performed using a Q-TOF Premier mass spectrometer (Waters Corporation, Micromass MA Technologies, Manchester, UK) with electrospray ionisation and column-less injections using a Waters Alliance 2695 HPLC. Mass spectral data was obtained in the negative ion mode for a mass range of 100 to 3000 m/z. Capillary voltage and cone voltage were set at 2.6 kV and 30 kV, respectively. The desolvation gas was set at 800 l/h while the cone gas was set at 50 l/h. Samples were dissolved in HPLC water and centrifuged to remove insoluble material. HPLC was carried out using an isocratic mobile phase at 0.2 ml/ml consisting of 80:20 (water: acetonitrile) and an injection volume of 5 µL and a 2 minute runtime. Due to the presence of doubly and triply charged ions present under these conditions, spectral deconvolution was carried out using the Mass Lynx software.

8.2 Results and Discussion

ElectroSpray Ionisation Quadrupole-Time of Flight Mass Spectrometry (ESI-QTOF-MS) was employed in the analysis of laminarin from 10 seasonal samples of *Laminaria digitata* harvested from Finnavarra Co. Clare from July 2012-July 2013. A standard sample of laminarin was used as a reference for the mass spectrometric data. Laminarin is a β-glucan polysaccharide, like all polysaccharide, it does not have a single molecular weight but is usually described based on its average molecular weight and polydispersity (the range of the number of units of the monomer present). The presence of a series of ions with +162 between each ion correlates to an increase of one monomer of glucose in the polysaccharide chain. The presence of this series of ion was used to indicate the presence of laminarin in the samples. Due to the mass range of QTOF-MS, ions of
laminarin that were greater than 2 KDa appear as double charged ions and therefore a series of ions of +81 are present in these cases. The ESI-QTOF-MS spectrums of these laminarin extracts showed a complex spectrum of double and triple charged states with the possibility of a fourth charged state. Read et al. (1996) also observed double and triple charged sodium adducts \([M + 2Na]^2+\) and \([M + 3Na]^3+\) in permethylated oxidized reduced laminarin extracts. Harvey (2005) also observed that neutral glycans gave both single- and double-charged ions with larger glycans preferring the formation of doubly charged ions.

Seasonal samples collected between July-November 2012 clearly showed that they contain laminarin based on the mass spectrometric profile (Table 8.1). However, no laminarin was detected in the samples harvested over the winter and spring period (December, January and March) (Table 8.1). This was based on the low yield, the limited sample ion abundance under mass spectrometric conditions and the absence of +162 ion series or +81 ion series for doubly charged ions. A low level of laminarin content was again observed in the April and May sample and a high content of laminarin seen again in the July 2013 sample (Table 8.1).

Examination of these seasonal samples reveals extensive variation in the average/most abundance molecular weight and molecular weight range and distribution of laminarin. The standard sample supplied by Sigma-Aldrich which was derived from *Laminaria digitata* had an average molecular weight of 3844 based on the doubly charged ions present corresponding to 23 units of glucose present in the laminarin structure with a distribution of the polymer range from 20-30 units of glucose (Figure 8.2). On analysis of the QTOF data it was also observed that a significant number of triply charged ions were present within the data, although relatively low in abundance compared to the doubly charged ions. In these cases deconvolution was carried out to determine the full MW range for the polysaccharide. Deconvolution showed that the standard
sample, while most abundant between 20-30 units of glucose, had a distribution of between 11-61 (Figure 8.3) units of glucose, much higher than previously reported for laminarin. July to October 2013 extracts of laminarin had an average molecular weight of 4168 based on doubly charged ions present, corresponding to 25 units of glucose and a polymer distribution range from 15-32 glucose units (Table 8.1; Figure 8.4). Deconvolution of these extracts showed that polymers of between 15-32 glucose units were most abundant and a complete distribution of between 15-65 glucose units (Figure 8.5). The November extract of laminarin had much lower average molecular weight of 2708 (Figure 8.6) which corresponds to 16 units of glucose and polymer distribution range of 7-19 (1.5-3.2 kDa) units of glucose (Table 8.1). Samples from December, January and March contained no laminarin based on the low yield obtained, the low ion abundance and the absence of a +162 ion series and a +81 ion series (Figure 8.7). The sample harvested in April 2013 had an average molecular weight of 3096 based on doubly charged ion present, corresponding to 19 glucose units and a polymer distribution between 16-28 (Table 8.1). Deconvolution revealed the highest abundance was present between 16-28 glucose units and a distribution of 17-48 (2.8-7.8 kDa) glucose units (Table 8.1). The sample harvested in June showed a slight drop again in the average to 2657, corresponding to 16 glucose units, and a polymer range of 7-37 in deconvolution state (Figure 8.8). The July 2013 sample had an average molecular weight of 4393 based on doubly charged ions present, corresponding to 27 units of glucose and a polymer range of 20-30 (Figure 8.9). After deconvolution it showed a much greater DP range of 10-70 glucose units. The highest of all samples investigated (Figure 8.10).
Figure 8.2 ESI-QTOF-MS mass spectrum of laminarin standard (1 mg/ml dH₂O) from Laminaria digitata. Glucose units numbered and the average ion is highlighted. Data presented in raw state.
Figure 8.3 ESI-QTOF-MS mass spectrum of laminarin standard (1 mg/ml dH₂O) from *Laminaria digitata*. Glucose units numbered and the average/abundant ion is highlighted. Data presented in deconvoluted state.
Figure 8.4 ESI-QTOF-MS mass spectrum of laminarin extracted from *Laminaria digitata* harvested on the 3rd of July 2012. Glucose units numbered and the average ion highlighted. Data presented in raw state.
Figure 8.5 ESI-QTOF-MS mass spectrum of laminarin extracted from *Laminaria digitata* harvested on the 3rd of July 2012. Glucose units numbered and the average/abundant ion highlighted in red text. Data presented in deconvoluted state.
Figure 8.6 ESI-QTOF-MS mass spectrum of laminarin extracted from *Laminaria digitata* harvested on the 11\textsuperscript{th} of November 2012. Glucose units numbered and the average/abundant ion highlighted in red text. Data presented in deconvoluted state.
**Figure 8.7** ESI-QTOF-MS of extract from *Laminaria digitata* harvested in 13\textsuperscript{th} December 2012. Data presented in raw state.
Figure 8.8 ESI-QTOF-MS mass spectrum of laminarin extracted from *Laminaria digitata* harvested from the 10th of June 2013. Glucose units numbered and the average/abundant ion highlighted. Data presented in deconvoluted state.
Figure 8.9 ESI-QTOF-MS mass spectrum of laminarin extracted from *Laminaria digitata* harvested on the 27th of July 2013. Glucose units numbered and the average ion highlighted. Data presented in raw state.
Figure 8.10 ESI-QTOF-MS mass spectrum of laminarin extracted from Laminaria digitata harvested on the 27th of July 2013. Glucose units numbered and the average/abundant ion highlighted in red text. Data presented in deconvoluted state.
Table 8.1 Sample harvest period, molecular weight range, prominent ion \((m/z)\) and number of glucose monomers in laminarin extracted from *Laminaria digitata* harvested from July 2012 to July 2013 (laminarin standard also included for reference).

<table>
<thead>
<tr>
<th>Sample harvest period</th>
<th>Average/most abundant</th>
<th>Polydispersity in terms of glucose units (MW)</th>
<th>Deconvolution Range in terms of glucose units (MW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd July 2012</td>
<td>4168 (25 units)</td>
<td>15-32 (2.5-5.3Kda)</td>
<td>15-63 (2.5-10.2Kda)</td>
</tr>
<tr>
<td>20th August 2012</td>
<td>4168 (25 units)</td>
<td>15-32 (2.5-5.3Kda)</td>
<td>15-63 (2.5-10.2Kda)</td>
</tr>
<tr>
<td>2nd October 2012</td>
<td>4168 (25 units)</td>
<td>15-32 (2.5-5.3Kda)</td>
<td>15-65 (2.5-10.5Kda)</td>
</tr>
<tr>
<td>14th November 2012</td>
<td>2708 (16 units)</td>
<td>7-19 (1.5-3.2Kda)</td>
<td>NA</td>
</tr>
<tr>
<td>13th December 2012</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>29th January 2013</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>11th March 2013</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>28th April 2013</td>
<td>3096 (19 units)</td>
<td>16-28 (2.6-4.5Kda)</td>
<td>17-48 (2.7-7.8Kda)</td>
</tr>
<tr>
<td>10th June 2013</td>
<td>2657 (16 units)</td>
<td>ND*</td>
<td>7-37 (1.2-5.9Kda)</td>
</tr>
<tr>
<td>27th July 2013</td>
<td>4393 (27 units)</td>
<td>16-30 (2.6-4.9Kda)</td>
<td>10-70 (1.6-11.3Kda)</td>
</tr>
<tr>
<td>Standard</td>
<td>3844 (23 units)</td>
<td>20-30 (3.1-4.8Kda)</td>
<td>11-61 (1.8-9.9Kda)</td>
</tr>
</tbody>
</table>

*Interference of impurities limited visualisation of data and therefore deconvolution reported in this case

To our knowledge no other studies have investigated the molecular weight changes or changes in distribution in laminarin from *Laminaria digitata* in relation to season. Almost all studies measuring laminarin from algal material have used hydrolysis conditions. Quantification is usually undertaken either indirectly after hydrolysis by measuring the cleaved glucose units with enzymatic assays (Lloyd and Whelan, 1969; Gómez and Wiencke, 1998), or with the anthrone reagent methods (Yemm and Willis, 1954), both calibrated against a glucose standard. These techniques only provide information on the content of laminarin present, unlike this study which has investigated the changes in molecular weight. Graiff *et al.* (2015) looked at the chemical characterisation and quantification of laminarin from a range of algae’s using liquid chromatography mass spectrometry (LC-MS), *L. digitata* included. They determined that laminarin was present in 9 of the 12 algae’s investigated and had a typical molar mass distribution of between 2000-7000 kDa and showed variation in chain length between species. Based on the
comparison of the retention times and distinctive mass spectra of the standard they determined that laminarin from *L. hyperborea* consisted of the longest β-glucan chain, while laminarin isolated from *L. digitata* had the shortest glucose chain. Laminarin concentrations within the algae also varied from 0.03 to 0.86 % dry weight (DW). Rioux *et al.* (2009) also investigated the variation in composition of laminarin isolated from the brown macroalgae *Saccharina longicruci* using HPSEC (High performance size exclusion chromatography). To date research has focused mainly around the investigation of effect of seasonality on the content of laminarin in *L. digitata* rather than investigation into the structural changes.

Adams *et al.* (2011) investigated the content of laminarin from fermented extracts carried out on *L. digitata*. Using an enzymatic reaction with laminarinase and HPLC to determine the glucose concentration the laminarin content was determined. They observed that laminarin content in *Laminaria digitata* was low until June, peaked at 24 % dry weight in July and gradually decreased through the remainder of the year. These results are in agreement with the findings of this study where laminarin content was maximum in July and decreased over the year with no laminarin observed in the winter period. Schiener *et al.* (2012) also investigated the seasonal variation of laminarin from a range of brown macroalgae (*Laminaria digitata, Laminaria hyperborea, Saccharina latissima* and *Alaria esculenta*) using HPLC methods after acid hydrolysis. They observed a similar trend, with the laminarin content lowest in the winter months and highest in the summer months, they reported that the yields of laminarin coincided with low yields of ash, protein, moisture and polyphenols. Laminarin content in other brown macroalgae species such as *Ecklonia cava* using an enzymatic reaction reports that laminarin was only present in the summer samples when the plant was reaching maturity (Iwao *et al.*, 2008).
The large variation in average molecular weight observed for laminarin is most probably due to the seasonal changes in composition. Laminarin is a storage glycan (Rioux et al., 2007) that is produced during the summer periods when the plant growth period is at its peak (Rinaudo et al., 2007). There have been many studies on the changes in the content of photosynthates such as laminarin with regard to life cycle regulation and the seasonality (Black 1948 a, b, c; Black 1950; Stewart et al., 1961). Stewart et al. (1961) investigated seasonal variation in alginic acid, mannitol, laminarin and fucoidan in Ecklonia radiata (C. Agardh) J. Agard in Point Lonsdale, Australia. The authors observed that laminarin content peaked during the summer and autumn and decreases to zero in midwinter. They purported that this rapid decrease might be associated partly with the shedding of the bladelets as well as the use of reserve carbohydrates, as a source of energy for winter growth and/or winter survival. Black et al. (1950) reported that in spring, mannitol was at a low concentration and laminarin was absent, while alginic acids and ash were at a high concentration. Laminarin concentration was highest during the summer and mannitol increased also. According to previous reports (Lobban and Harrison, 1997; Percival and McDowell, 1967; Black et al., 1954 and Black and Dewar, 1949) laminarin structure and content are influenced by environmental factors such as water temperature, salinity, waves, sea current and depth of immersion. Black et al. (1951) investigated the variation in content of laminarin in a range of brown macroalgae including L. digitata and also found it to be the highest in the summer months where rapid photosynthesis is occurring. The availability of nutritive salts particularly nitrates also affect the growth of the seaweed, and in turn the production of laminarin (Harlin and Craigie, 1979). Anderson et al. (1981) found that the maximum production of laminarin was observed in the summer months when UV exposure was more intense than the winter period. As well as these extrinsic factors the growth cycle of the plant affects the production of laminarin. Chapman and
Craigie, (1978) observed that laminarin content was highest in the summer months and minimum during February when the plant growth rate is increasing rapid.

This study is one of the first of its kind that has examined the effect of season on the molecular weight range and the average value. It provides a good insight into the abundance of laminarin available in the species *Laminaria digitata* during its growth cycle, it also provides vital information of the changes occurring within the molecular weight distribution of this polysaccharide over the seasons. These structural variations occurring within laminarin are likely to effect the biological activity of the compound. It is known that the biological activities of laminarin can be enhanced or modified using various techniques including irradiation, sulphation, reduction and oxidation. Previous research by Choi *et al.* (2012) determined that the biological activities of laminarin extracted from *Cystoseira barbata* and *Cystoseira crinita* was enhanced when laminarin was degraded by gamma radiation. Further research is required to link these structural changes with the biological role of this compound. This information is crucial to industries which aim to exploit laminarin for inclusion in food products or cosmetics to promote a biological effect.

### 8.3 Conclusion

This present study has provided information on the average molecular weight and polysaccharide MW distribution and the effects of seasonal variation on laminarin extracted from *Laminaria digitata* from the west coast of Ireland. Results indicated that the summer/autumn period was the optimum harvest time for extracts of higher content of laminarin. The winter period showed no laminarin content which correlates well with previous reports where laminarin levels are maximum.
in summer and minimum in winter months. There was a variation observed in the average molecular weight and the polymer distribution of glucose monomers in each sample. The July 2013 having the greatest average molecular weight of 4393 (27 glucose units) and a polymer distribution of 10-70 (Deconvolution state) glucose units. In general, extracts showed a higher average molecular weight in the summer period with a decrease in molecular weight and polymer distribution in the autumn and spring periods. Although it not addressed in this research it is likely that these structural changes are likely to cause an effect on the biological activities of laminarin. Further research on linking the changes in molecular weight and distribution with specific biological activities is required. This study had however provided crucial information on the suitable harvest time to many industries such as processing, food and cosmetic that wish to harvest laminarin rich samples for use in products to promote a biological effect.
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Chapter 9

General Discussion
Macroalgae are a rich underexploited source of bioactive compounds such as polyphenols, carotenoids and polysaccharides. Their use to date has focused mainly on high volume low value products such as animal feeds, plant supplementation, specialist fertilisers and agricultural products. The aim of this study was to gain more from this industry by exploiting the less utilised marine resources for bioactive components by examining their potential as ingredients in functional foods.

In order to assess the functional food potential of Irish macroalgal resources the present study aimed to investigate various extraction techniques for the preparation of bioactive enriched extracts from a range of seaweeds harvested from the Irish coast, measure the bioactivity related to these compounds and determine the effects of seasonal/spatial variation on the structure and activity of these compounds.

Initially the efficiency of two extraction techniques (Chapter 2), SLE and PLE using a range of solvent combinations (80% ethanol, 60% methanol, cold water and hot water) were compared for the extraction of compounds with antioxidant activity, namely polyphenols. These techniques were tested on a range of seaweed species, *Laminaria digitata* (brown), *Fucus serratus* (brown), *Gracilaria gracilis* (red) and *Codium fragile* (green). Based on the extraction yield obtained and the total phenolic content and antioxidant activity exhibited, the application of SLE over a 24 h period using water at room temperature was the preferred technique for extraction of antioxidant compounds from macroalgae. Over all SLE extracts displayed significantly higher (approximately two-fold greater) antioxidant (DPPH and FRAP) and TPC than PLE. The species *L. digitata, G. gracilis* and *C. fragile* had low antioxidant properties with *F. serratus* the only species to exhibit considerably activity. Therefore given the low capital cost of SLE equipment in comparison to
PLE and the lack of scaled-up PLE equipment, SLE would appear to the method of choice for seaweed processors.

The ability of molecular weight cut-off (MWCO) dialysis to enrich crude extracts was investigated. Following dialysis, filtrates and rententates were reassessed using the TPC and antioxidant assays (FRAP and DPPH) to determine if this technique could increase the activity of extracts initially observed as being low (Chapter 3). MWCO dialysis proved to be a useful, low cost and low-tech tool for enriching the antioxidant activity and phenolic content of crude extracts from macroalgae with many of the fractions exhibiting a greater than threefold enrichment after fractionation. *F. serratus* exhibited high antioxidant activity in the crude extracts in all assays. The higher molecular weight fractions showed the highest phenolic and antioxidant activities indicating the main compounds responsible for antioxidant activity were more probably high molecular weight phlorotannins. Limited activity observed in the < 3.5 kDa fraction was further improved using reverse-phase flash chromatography. Flash chromatography produced a phlorotannin enriched fraction. This phlorotannin enriched fraction was analysed by quadrupole time-of flight mass spectrometry (Q-ToF-MS), which showed that the, < 3.5 kDa fraction of *F. serratus* contained a high abundance of low molecular weight phlorotannins with high antioxidant activity.

Whilst the economic feasibility of the enrichment protocol was not evaluated the study demonstrates the possibility of further utilization of seaweed species that are generally underutilized and the simplicity and low cost of MWCO dialysis to increase the antioxidant activity and phenolic content of crude extracts. Since the majority of seaweeds harvested in Ireland are utilised for polysaccharide recovery using aqueous extraction methods leaving the mildly polar phlorotannins behind these enrichment methods reported in Chapter 2 could be used to isolate bioactive compounds from the by-products produced by this industry.
Whilst the research in Chapters 2 and 3 demonstrated the potential of macroalgae as a source of antioxidant compounds it was felt that further characterisation of the enriched fraction was required to investigate which components were likely to be responsible for the antioxidant activity demonstrated. This was also important given the limited knowledge available for *F. serratus* and the possibilities of highly complex isomeric mixture that could arise from phloroglucinol polymers (Chapter 4). To this end characterisation of phlorotannin enriched fractions was carried out using (UPLC) with tandem mass spectrometry for four Irish origin brown macroalgae (*Fucus serratus, Fucus vesiculosus, Cystoseria nodicaulis* and *Himanthalia elongata*). A large number of isomers were detected corresponding to phlorotannins of much higher degree of polymerization (up to 16 monomer units) in comparison to previous reports. The majority of the low molecular weight (LMW) phlorotannins found in *F. serratus, F. vesiculosus, C. nodicaulis* and *H. elongata* had monomer numbers from 6-12, 4-8, 7-12 and 4-11 monomers, respectively. Purification using MWCO and reversed phase flash chromatography for removal of carbohydrates that mask the tannins and UPLC-MS/MS analysis allowed for distinctive metabolite profiles to be elucidated and polymeric isomers to be detected. This resulted in a more complete picture of the phlorotannin composition in individual macroalgae being reported for the first time and has indicated that even these LMW fractions are extremely complex and pose an almost impossible task in terms of further purification and chemical characterization of individual compounds. The possibility of using seaweeds as a source of compounds for inclusion in functional foods is contingent on them serving as a uniform source of the active compounds regardless of season or site of harvest. As well as changes in the structure and/or molecular weight, phlorotannins may also be affected by seasonal variation. Reports by Koivikko (2008) found that phlorotannin concentrations varied among development stages of brown algae. Environmental factors such as water temperature, salinity and
exposure the UV radiation may affect the metabolite profile in macroalgae. Studies into the environmental conditions of the harvested seaweed prior to this investigation could have provided vital information in linking these structural and bioactivity variations to a particular extrinsic factor. Research in Chapter 7 investigated this theory in relation to the possibility of seasonal variation of phlorotannins. To date studies have shown variations in the concentration of polyphenolic compounds using only simple colorimetric methods, however no literature has investigated the effects of seasonal/geographical variation on the structure and composition of the phlorotannins and the effects this has on their bioactivity. Again this study employed the techniques employed in Chapter 3 (UPLC-MS/MS) to investigate the molecular weight and isomeric difference in brown macroalgae. *F. serratus* harvested from two locations (Finnavarra Co. Clare and Spiddal Co. Galway) over the period of a year. *Fucus serratus* was the seaweed investigated in this study as it had been previously demonstrated to be a good source of phenolic compounds (Chapter 2) and thus far there has been little research carried out on the phlorotannin content in this brown macroalgae species. Antioxidant and TPC assays used as an index for producing phlorotannin enriched fractions. It was observed that the majority of low molecular weight phlorotannins found in this species had molecular weight ranges of 6-14 monomers. This was seen across all seasons and in the two locations. Seaweeds harvested in seasons with a higher abundance (DP 9 to 11) of phlorotannins also exhibited greater levels of isomerization. This was apparent in the winter and autumn samples, which showed a high abundance of phorotannins in the ion chromatograms presented, and also had the higher numbers of isomers. In the summer and spring samples the abundance was much lower especially in the summer Spiddal sample (DP of 6), the level of isomerisation was also much lower in this sample (121 total). Therefore in the present study seasonal and spatial variation in relation to abundance and isomerization was
detected for phlorotannins. However a much more extensive study over a longer period (circa 5 years) and including more locations would be required to confirm this observation. In addition to elucidate the underlying biological impetus behind the variation would require a lot more detailed information about the macroalgae themselves as a large number of factors have been shown to influence phlorotannin contents in macroalgae. For example previous studies have determined that herbivore grazing lead to increased phlorotannin levels in *Ascophyllum nodosum* (Svensson *et al.*, 2007), grazing pressure tends to be highest in the summer which correlates with phlorotannin maxima in this study. Increased phlorotannin content may also be due to exposure to ambient UV-B radiation (Pavia *et al.*, 1997; Pavia and Brock, 2000). Differences in habitats (Jormalainen and Honkonen, 2004), such as salinity, temperature, light intensity and ambient nutrients (Pavia and Toth, 2000, Jormalainen and Honkonen, 2004) also play a key role. These variations in turn influence the bioactivity of the samples and due to the complexity of these polymers the possibility of their use in functional food products in the near future would be limited as it is near to impossible to link the biological activity observed to any one characterizable species. This research has however provided an approach which could be adopted for further research in this area. Although these compounds at present aren’t suitable for inclusion in functional food products they may serve purpose as additives to foods for the prevention of lipid oxidation.

Apart from phlorotannin content it was clear from the literature that there was very limited information available on the how the macro-composition (protein, lipid, ash and soluble and insoluble dietary fibre) of Irish macroalgae (*Laminaria digitata*, *Fucus serratus*, *Gracilaria gracilis* and *Codium fragile*) was affected by seasonal variation therefore this was investigated in Chapter 5. The study demonstrated that the highest protein content was found in the winter sample of the red macroalgae *Gracilaria gracilis* (28.70% DWC), while the brown algae *Fucus serratus*
had the lowest protein content in Autumn (4.91% DWC). Protein content in all species tested was highest in winter. The lipid content was low in all species and ranged between 0.72% DWC in L. digitata to 2.81% in C. fragile. No significant seasonal variation was seen in lipid content. The ash content was high in all species with F. serratus having the lowest and highest observed values (summer 14.35% DWC- winter 44.70% DWC). Ash content was highest in winter for the two brown algae tested (L. digitata and F. serratus). The green algae C. fragile had the highest ash content observed in autumn, while the red algae G.gracilis had the highest ash content in spring. The carbohydrate content was highest in the brown algae with content between 75-80%. Most species were observed to have the highest carbohydrate between the autumn and spring periods. The insoluble dietary fibre (IDF) was high in all seaweeds (30.77% in C. fragile- 59.51% in L. digitata). The brown algae’s were observed to have highest levels in spring. The soluble dietary fibre was higher in the brown algae compared to both the green and red algae. The total phenolic content in the species F. serratus was analysed and was found to be at maximum levels during the summer months and lowest in autumn. Carbohydrate content peaked in autumn and spring periods with IDF and SDF maximum in spring/summer time. The TPC content of the brown algeae Fucus serratus was highest in the summer months. The biological reasons for the seasonal variation in macronutrients as demonstrated was not investigated and as detailed above would require a more extensive and long term investigation. However it may be related to changes in the environment such as water temperature, salinity, UV exposure (Banerjee et al., 2009; Perfecto, 1998; Munda and Kremer, 1977; Rosemberg and Ramus, 1982; Abdela-Diaz et al., 2006) plant immersion, nutrient availability (Marinho-Soriano et al., 2006) and plant growth period (Pederson, 1984; Gorham, 1984). For example when Abdala-Diaz et al (2006) assessed the biological activity of seaweeds in relation to seasonality. They found high levels of phenolic compounds were observed
in the summer period when irradiance levels were high, they determined that these phenolic compounds served as a photoprotective mechanism against these irradiances.

In recognition of the fact the phlorotannins are not be the only antioxidant species likely to be present in macroalgae, Chapter 6 detailed an investigation into the extraction of fucoxanthin and xanthophyll from the brown macroalgae *Fucus serratus* and *Laminaria digitata*. *Fucus serratus* and *Laminaria digitata*, two brown species were selected for this investigation as the main carotenoid found in macroalgae is fucoxanthin and this is predominantly abundant in brown seaweed. Optimization of extraction technologies to aid the recovery of these valuable components was investigated using solid-liquid extraction (SLE), supercritical CO$_2$ (SCO$_2$) and supercritical SCO$_2$ with ethanol as co-solvent (SCO$_2$/EtOH). The most optimal technique was determined based on the purity and yield of carotenoids obtained. SCO$_2$ gave the greatest yield of carotenoid extract at $50 \, ^\circ$C, 300 Atm with an extraction time of 60 min. This study determined that SLE extracts gave a higher yield of fucoxanthin and xanthophyll compared to SCO$_2$ and SCO$_2$/EtOH, however, SCO$_2$ gave a higher purity for fucoxanthin and SLE gave a higher purity for xanthophyll. It is likely that SCO$_2$ provided extracts of a higher purity and lower yield due to the selectively of the process, by adjusting the temperature and pressure it is possible to adjust the density, allowing the properties of the supercritical fluid to be fined tuned for the compound of interest, thus resulting in a higher purity extract but a lower yield. Selectivity of this nature cannot be achieved in SLE, minimal selectively can achieved by selecting a solvent of similar polarity to the target species, however inevitably this will produce extracts with a complex mixture of compounds due to a range of compounds having similar solubility in the solvents, thus, resulting in a lower purity but a greater yield of extract. Seasonal variations of these compounds from *F. serratus* and *L. digitata* were subsequently investigated based on SLE extracts which provided the maximum amount of
carotenoids. The winter and spring period gave the highest purity of fucoxanthin and xanthophyll in *L. digitata*, while the summer gave the greatest purity of these compounds in *F. serratus*. Again as aforementioned a more extensive study over a longer time and including a larger sample range would provide a clearer insight into this variation. Pigments are very much commercially valuable components as they are used as food colourings, functional ingredients and in the cosmetic industry. Nowadays there is a big emphasis on using compounds of natural origin and this study has provided useful information on the optimum extraction technique in terms of yield and purity for specific compounds and also in terms of the best harvest times for maximum carotenoid content.

Due to the observed variation in the macro composition in particular the carbohydrate variation, the seasonal variation of laminarin a common storage glycan found in brown algae was investigated. Samples of *Laminaria digitata* were harvested from Finnavarra Co. Clare over the period of 2012-2013 (Chapter 8). Laminarin was extracted using mild acid (0.1 M HCl) at 60°C. Molecular weight fractionation was carried out using MWCO dialysis tubing. Samples were analysed using ESI-Q-TOF-MS to investigate the difference in laminarin content, molecular weights and glucose monomer numbers in relation to seasonal variation. Results showed that samples harvested in the summer/autumn exhibited the highest content of laminarin. Laminarin was not detected in the winter/spring period. Variation in the molecular weight based on the number of glucose monomers in each sample was also noticed. The sample from July 2013 showed the highest average molecular weight of 4393 (27 glucose units) polymer distribution 10-70 (deconvolution state) glucose units. In general the summer period showed highest average MW with the samples from July-October 2012 having an average molecular weight of 4168 (25 glucose units). The autumn and spring were observed to have a much lower molecular weight (November
2012 – 2706 (16 glucose units) and April 2013 -3096 (19 glucose units)). This study gave an insight into the seasonal variation of laminarin in the species *L. digitata* and how this impacts on the structure of the laminarin obtained but again more extensive study would be required to confirm these findings.

In summary, the present thesis applied a range of extraction technologies for the optimal extraction of phlorotannins, fucoxanthin, xanthophyll and the polysaccharide laminarin to assess their potential for use as functional ingredients in food products. This study also investigated the seasonal/spatial variation of these compounds, in particular the variation in the structural complexity of the phlorotannins and laminarin and also the variation in the content and purity of the compounds. At present it is estimated that the global functional foods and beverages markets is set to exceed $130 billion by the end of 2015 (Freeman, 2014). A study conducted by Jaspers and Polmer (2013) found that seaweed enriched products marketed globally are predominantly seaweed enriched table salts and prebiotic drinks, indicating the enormous potential of Irish seaweed as functional food ingredients. A number of seaweed-derived ingredients have also exhibited prebiotic and cholesterol-reducing properties, as well as antioxidant and anti-diabetic properties, therefore seaweed extracts could be potentially used to enrich a wide variety of everyday food products.

The present study highlighted the complexity of these compounds in respect to the isomerization occurring in even the low molecular weight fractions which would limit their application as single pure compounds. As well as this there are also constraints to what constitutes as a functional food by EFSA regulations (EFSA, 2006). According to the European Food Information Council a “functional food should be in the form of a normal food and they must demonstrate their effects in amounts that can normally be expected to be consumed” (EUFIC, 2006). At present there are no
claims associated with any of the compounds investigated in the present study. Therefore, until this is the case they cannot realistically be used as components in functional foods. Therefore there is a substantial amount of work still to be undertaken to incorporate these compounds into food products, and also to investigate whether they uphold their biological effect once in the food matrix and if they influence any negative effect on the organoleptic properties of the food. They do however have the potential for use as technological ingredients to control lipid oxidation or as natural colorings.

This research did however highlight the potential of new analytical technologies for the extraction, purification and structural characterization of these compounds. In particular UPLC-Q-ToF-MS and HPLC-ESI-QTOF-MS were useful tools for highlighting the isomeric complexity of phlorotannins. These findings have emphasized the need for a much longer and wider range study to make strong conclusions with regards to seasonal and geographical variations, but with the use of these research the tools and methodologies to investigate these factors are now available and may be considered useful in the progression of the research in this field.
References


European Food Safety Authority (EFSA). Regulation 1924/2006 on nutrition and health claims made on foods.


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Appendix 1

Publications from the Thesis
