Extraction, Separation and Purification of Polyphenols, Polysaccharides and Pigments from British Seaweed for High-Value Applications

Henry Irvine Spurr

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Abstract

Seaweed represents a valuable, underused sustainable resource. Current investment in seaweed farming will provide large quantities of biomaterial for a range of applications. The major focus of this thesis is on a class of polyphenols known as phlorotannin, of interest for their antioxidant and biomedical applications.

Seaweed polyphenols have strong radical scavenging properties. Whilst there are many reported extraction methods for phlorotannin, it was not clear how different solvent systems affect the antioxidant capacity of the phlorotannin extracted. This work represents the first comparable study of its kind in which phlorotannin was isolated from seaweed using several extraction solvents and the antioxidant capacity evaluated using the DPPH radical scavenging assay. The structural profile and radical scavenging capacity of the phlorotannin did not change between the extracts. Water and ethanol-water mixtures were found to be the most suitable solvents for large-scale extraction of phlorotannin.

The first study on the seasonal variation in phenolic content of industrially farmed seaweed grown on ropes was performed. The phenolic content of the two species studied, *Fucus vesiculosus* and *Ascophyllum nodosum* varied between 4-9 % dry mass and showed variation that could be attributed to the growth cycle of the seaweed. This demonstrates that the phenolic content is high enough all year round to allow viable extraction of phlorotannin. The radical scavenging capacity of *Fucus vesiculosus* was found to be 12% greater than *Ascophyllum Nodosum*.

Given that the majority of farmed seaweed is destined for biofuel production, it was of interest to consider whether phlorotannin could be extracted during biofuel processing. A potential pre-treatment method proposed for processing seaweed for biofuel is the use of superheated water extraction. Phlorotannin was then extracted from superheated water, without impacting the radical scavenging capacity, demonstrating the potential for it to be integrated into future processing techniques.

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BADC	British Atmospheric Data Centre
Da	Dalton
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DPPH	α,α-diphenyl-β-picrylhydrazyl
EtOH	Ethanol
FAME	Fatty acid methyl ester
FC	Folin Ciocalteu
FCSP	Fucose containing sulphated polysaccharide
GCMS	Gas chromatography mass spectrometry
HPLC	High performance liquid chromatography
LCA	Life cycle assessment
LCMS	Liquid chromatography mass spectrometry
MAA	Mycosporine like amino acid
NMR	Nuclear magnetic resonance
PGE	Phloroglucinol equivalent
PT	Phlorotannin
PTFE	Polytetrafluoroethylene
SEC	Size exclusion chromatography
SPE	Solid phase extraction

1 Introduction

1.1 Seaweed as a Renewable Resource

There is a growing need to develop alternative sources of material feedstocks to those derived from petrochemicals.[1] The oceans make up 70% of the world's surface, bounded by extensive coastlines, providing an attractive opportunity for their exploitation as a sustainable source of biomaterial to meet this demand. Seaweed (macroalgae) has huge potential in this regard, reflected in the recent investment in the seaweed farming industry.[2] Since 2009 several hundred million dollars has been invested by the energy sector into developing new technology for farming seaweed to produce it as a feedstock to be processed as a source of biofuels. Investment comes from both governments as well as from companies such as DuPont. [2] Within the UK, £5 million has been invested by the Scottish Energy Ministry and the EU's INTERREG IVA research programme for the investigation of commercial scale production.[2] It can be harvested for its lipid content and also as a source of carbohydrates that can then be fermented to produce bioethanol.

1.2 Seaweed Farming

The cultivation of seaweed dates back thousands of years for use as a source of food and medicine. In more recent times, the range of applications has expanded to include use in fertilizers, cosmetics, animal feeds, food additives as well as a source of various salts, vitamins and minerals.[3-5] Seaweed is processed as a source of raw material for production of agar, carrageenan and alginates. Up until about 1980 most seaweed production came from the harvest of wild stocks. Around this time demand for seaweed started to outstrip supply and cultivation was viewed as a way to boost production, creating a drive in the development of farming technology.[6] It was soon recognised that seaweed farming was a profitable business, which further accelerated expansion in the industry. The Philippines began to supply guide books to farmers to spread knowledge on farming practices.[7] Further support also came from international development agencies.[8] The earliest culture methods were on shallow, flat bottom sand, usually about one meter below low tide. Seedlings were fixed to thin rope and tied between posts (Figure 1.1). This is known as the ‘off-bottom’ method and remains one of the most common methods still in use today.

Figure 1.1. The ‘off bottom’ method of growing seaweed attached to lines between posts. Photo by Moongateclimber[9]

1.2.1 UK Seaweed Farming

Britain has over 11,000 miles of coastline, supporting a range of seaweed species. The clean water surrounding Scotland provides an ideal environment for farming seaweed. The topography of the land in the Hebrides provides many sheltered bays for farming. This project collaborated with a seaweed farm, Böd Ayre Products Ltd (60.421146 N, 1.102152 W), located on Shetland, about 100 miles north-east of Scotland. A limitation of the ‘off bottom’ method is that it is restricted to shallow waters. As such, it is not suitable for use in countries such as the UK, where coastal waters are generally deeper than in Asia. An alternative method currently under commercial development within the Scottish seaweed industry is the cultivation of seaweeds on submerged ropes attached to buoys.[8] Seaweed spores are first nurtured in tanks, before being attached to the ropes, which are then laid out in the sea to mature for several months before harvesting. Figure 1.2 shows part of the seaweed farm at Böd Ayre. Ropes are held between floating orange buoys. The use of other materials such as textiles as supports is currently being investigated by the Scottish Association for Marine Science.[10] However, early trials have encountered problems with the chemicals used to treat the textiles, which appear to be slowing down algal growth.

The cost of labour is an important factor than can influence which method is most profitable. In China, the relatively low cost of labour means that seaweed farming can be done by hand with no mechanisation. In the UK however, the higher cost of labour means that farming will most likely have to be mechanised in order to be economically viable.[11]

Figure 1.2. The seaweed farm at Böd Ayre, Shetland, Scotland.

1.3 Seaweed as a Sustainable Source of Biomaterial

Seaweed is a good sustainable natural biomaterial for several reasons; it is a renewable material, making it a desirable alternative to materials sourced from non-renewable petrochemicals and as it is grown at sea it does not compete with agricultural land and food use. Unlike many land based bio-crops it does not require the use of pesticides, fertilizers or irrigation. This brings down the cost of production, as well as avoiding the potentially harmful effects of releasing pesticides and fertilizers into the environment. Fresh water is a valuable resource and the lack of any need for irrigation is an important advantage over land crops; something that may become more important if demand for water increases.

Seaweed can be grown with greater yields per square meter than land crops. Many species can be grown with yields of 13 kg m^{-1} over a 7-month growing period, compared to terrestrial plants, which typically achieve just 0.4-4.4 kg over an entire year.[12] This is for two reasons. Firstly, algae have a greater photosynthetic efficiency than land crops, meaning it is better at harvesting the sun’s energy, allowing faster growth than land crops.[1] Secondly, the sea supports the weight of the seaweed, allowing it to grow throughout the water column - provided it has sufficient light. Waste streams generated from industrial processing of seaweeds are used for other purposes such as animal feeds and fertilisers. Another important feature of seaweed is that it does not contain lignin, a polymer found in many land plants that can make mechanical processing more difficult.[1]

1.4 Seaweed Physiology

Figure 1.3 shows the key structural features of seaweed. Many of these features resemble those found in the plant kingdom; however, there are some key differences. Seaweeds do not possess a vascular system (xylem and phloem) and, whereas plants contain a root system to obtain nutrients, seaweeds instead obtain their nutrients from the surrounding water and usually have a holdfast which provides a point of anchorage to the seabed or shoreline.[13] There are, however, a few species of seaweeds that do not have holdfasts and instead float freely in the water. Seaweeds reproduce by releasing spores and hence do not have any flowering or seed producing parts to them.[4] Seaweeds mainly grow in salt water although can sometimes be found in fresh or brackish water. The depth they can grow down to varies and is dependent on light intensity. They typically grow down to depths of 8-40 m, though in clearer waters such as the Caribbean and the Mediterranean they can grow down to depths of 250 m. [4]

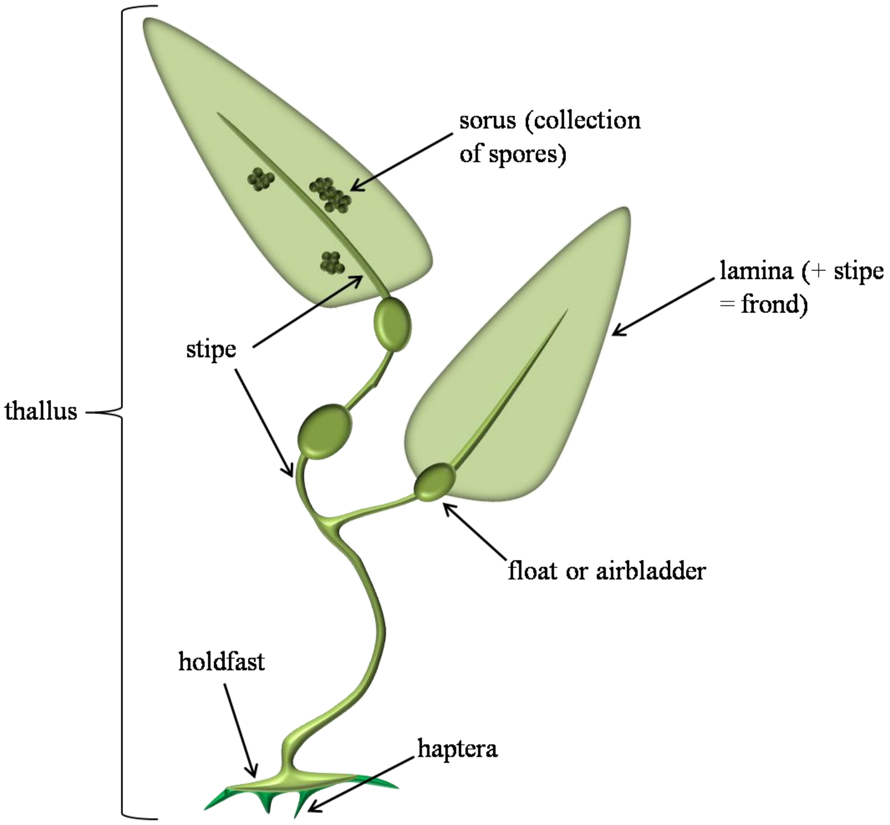


Figure 1.3. Diagram showing the key structural features of seaweed.[13]

Seaweeds contain a range of different classes of compounds that have been demonstrated to have a variety of applications. The following section details the current scientific literature on each of the major classes of compounds, including their extraction, characterisation and applications.

1.5 Pigments

Seaweeds contain a variety of different pigments. The types of pigments vary between species, giving them different colours. Seaweed species can be subdivided into three distinct types based on their colour; Chlorophyta (green), Rhodophyta (red), Phaeophyceae (brown). Table 1.1 shows the major pigments found in each of the three types of seaweed.[14] Of the ~12,000 species identified to date, approximately 4000 of these are Chlorophyta, 6000 are Rhodophyta, and 1800 are of Phaeophyceae.[15]

Table 1.1. The major pigments found in each type of seaweed.

Chlorophyta (green)	Phaeophyceae (brown)	Rhodophyta (red)
Chlorophyll *a*	Chlorophyll *a*	Chlorophyll *a*
Chlorophyll *b*	Chlorophyll c_1	Mycosporine like amino acids (MAAs)
Lutein	Chlorophyll c_2	α and β-carotene
α, β and γ-carotene	β-carotene	c-Phycoerythrin
Siphonoxanthin	Fucoxanthin	r-Phycocyanin
Siphonein		Allophycocyanin

1.5.1 Chlorophyll

There are several types of chlorophyll found in seaweed, which vary slightly in structure (Figure 1.4). They are responsible for the green colour in seaweeds, however in Phaeophyceae (brown) and in Rhodophyta (red) the green colour is often masked by the presence of other pigments, causing them to appear brown and red.[16] There are four types of chlorophyll in seaweed, *a*, *b*, c_1 and c_2. Chlorophyll *a* is present in all three types of seaweed. Chlorophyll *b* is found only in Chlorophyta and chlorophyll c_1 and c_2 are only found in Phaeophyceae.[16]

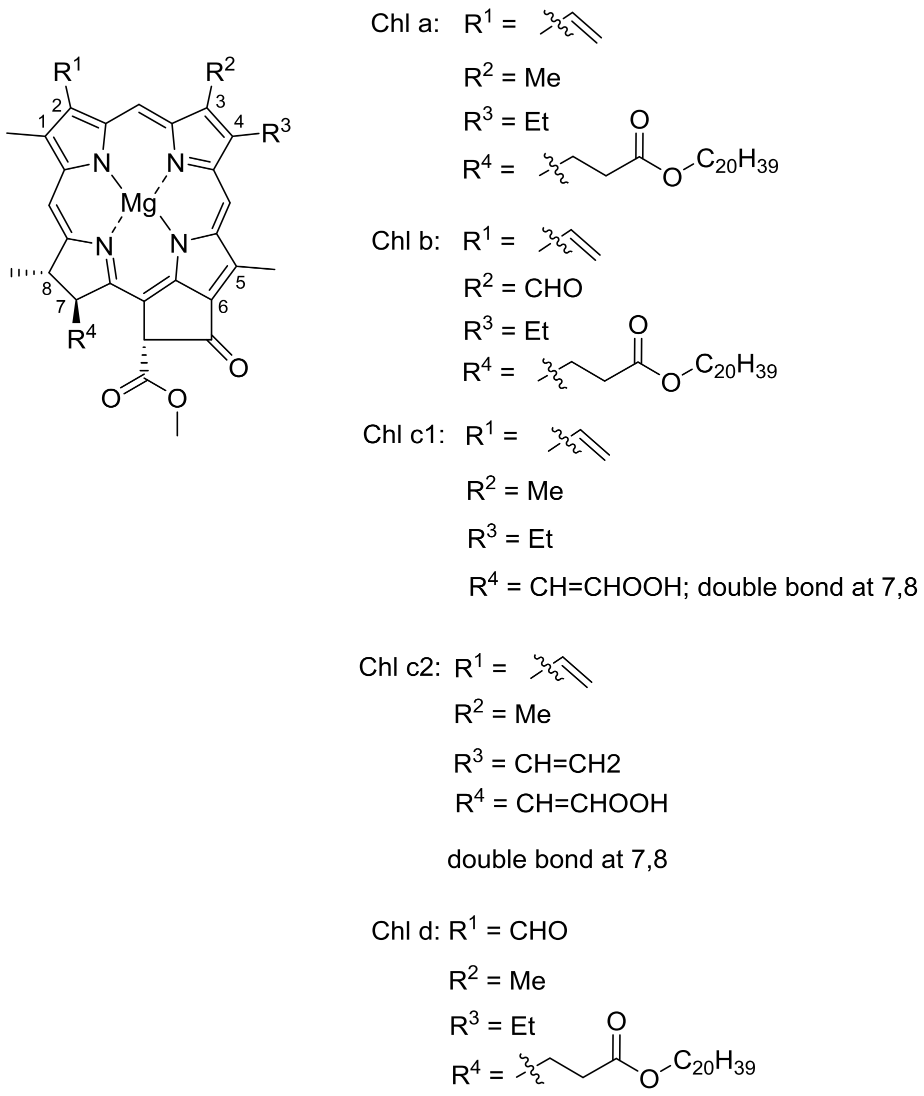


Figure 1.4. The different types of chlorophyll present in seaweed.

The strongly coloured nature of pigments was made them of interest for dyeing applications. Chemical modification of chlorophylls has been reported: acidic removal of the magnesium ion at the centre of chlorophyll *a* makes a pheophytin (Figure 1.5), which alters the colour of the seaweed tissue, giving it a brown colour.[17] [18] Hydrolysis and decarboxylation of the C10 carboxymethyl group of pheophytin gives pyropheophytin, again changing the colour of the tissue, to an olive-green. Colour changes have also been observed by varying the metal ion at the centre of the pigment.[17] [18]

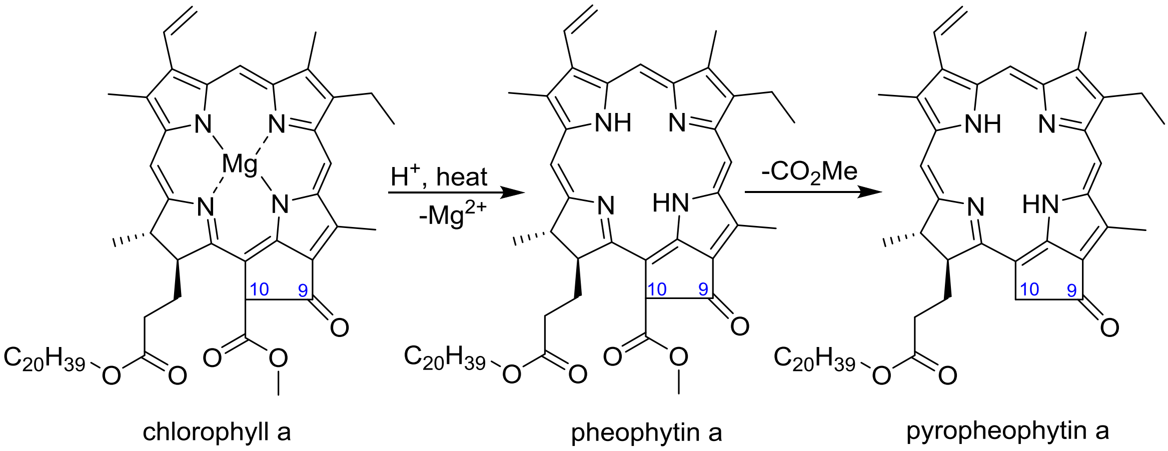


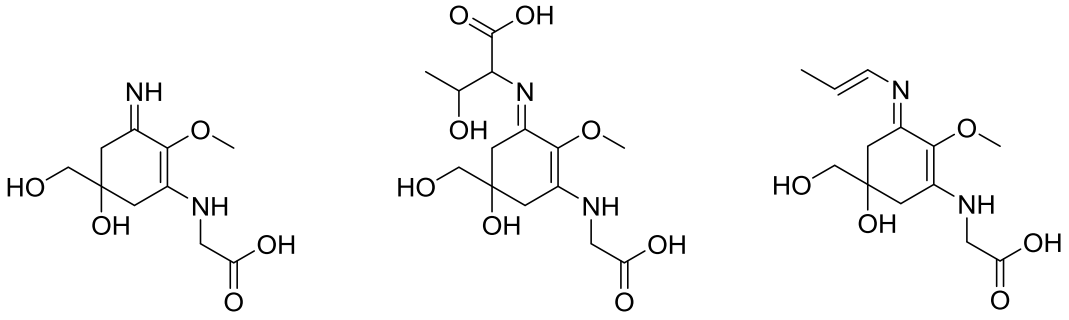
Figure 1.5 Pheophytin and pyropheophytin.

1.5.2 Accessory Pigments

In addition to chlorophylls, many species also contain other pigments, known as accessory pigments such as carotenes and xanthophylls. These pigments are responsible for the yellow, orange and red colours seen in seaweed. The composition of these accessory pigments differs between intertidal and subtidal seaweed species. Many of the accessory pigments have anti-oxidant and ultraviolet (UV) absorption properties, which serve to protect the chlorophyll centre against damage during times when it is under stress: *e.g.* at low tides or when there are high levels of UV during the summer months. They also alter the wavelength range at which the chlorophylls absorb light, allowing the absorption of longer wavelengths. Blue light does not penetrate through water as deeply as red light, and the presence of these accessory pigments allows the seaweed to live at deeper depths than it would normally be able to.[19] In this way, the brown and red seaweeds are able to avoid competition with green seaweed.[19] The different major types of accessory pigments are discussed below.

1.5.2.1 Mycosporine like Amino Acids (MAAs)

Mycosporine like amino acids (MAAs) are only found in Rhodophyta (Figure 1.6). A study of the seaweed *Palmaria palmata* found that MAAs extracted showed strong absorbance in the ranges of UV-A and UV-B, suggesting they may protect the chlorophyll centre from oxidative damage.[20] [21]



palythine porphyra-334 palythene

Figure 1.6. Palythine, Porphyra and Palythene - key MAAs extractable from the red algae *Palmaria palmata.*

1.5.2.2 Carotenoids

Another important class of pigments are the carotenoids, which have been found in a wide range of both micro and macro algae.[22-27] Figure 1.7 contains a selection of the carotenoids found in seaweed. Astaxanthin (Figure 1.7) is a strong anti-oxidant, extracted for use as a nutritional supplement and also for use as a food colorant.[28] Another carotenoid is the yellow pigment fucoxanthin (Figure 1.7), found only in Phaeophyceae, sometimes giving it its slight brown colour. Fucoxanthin allows chlorophylls to absorb at different wavelengths, as discussed above. It also displays good anti-oxidant capacity and strong UV absorption. Research has shown that environmental stress can increase the levels of pigments in seaweed.[29, 30] Diadinoxanthin, diatoxanthin and fucoxanthin have all been patented for their use in sunscreens due to their strong absorbance in the UV-A region.[31]

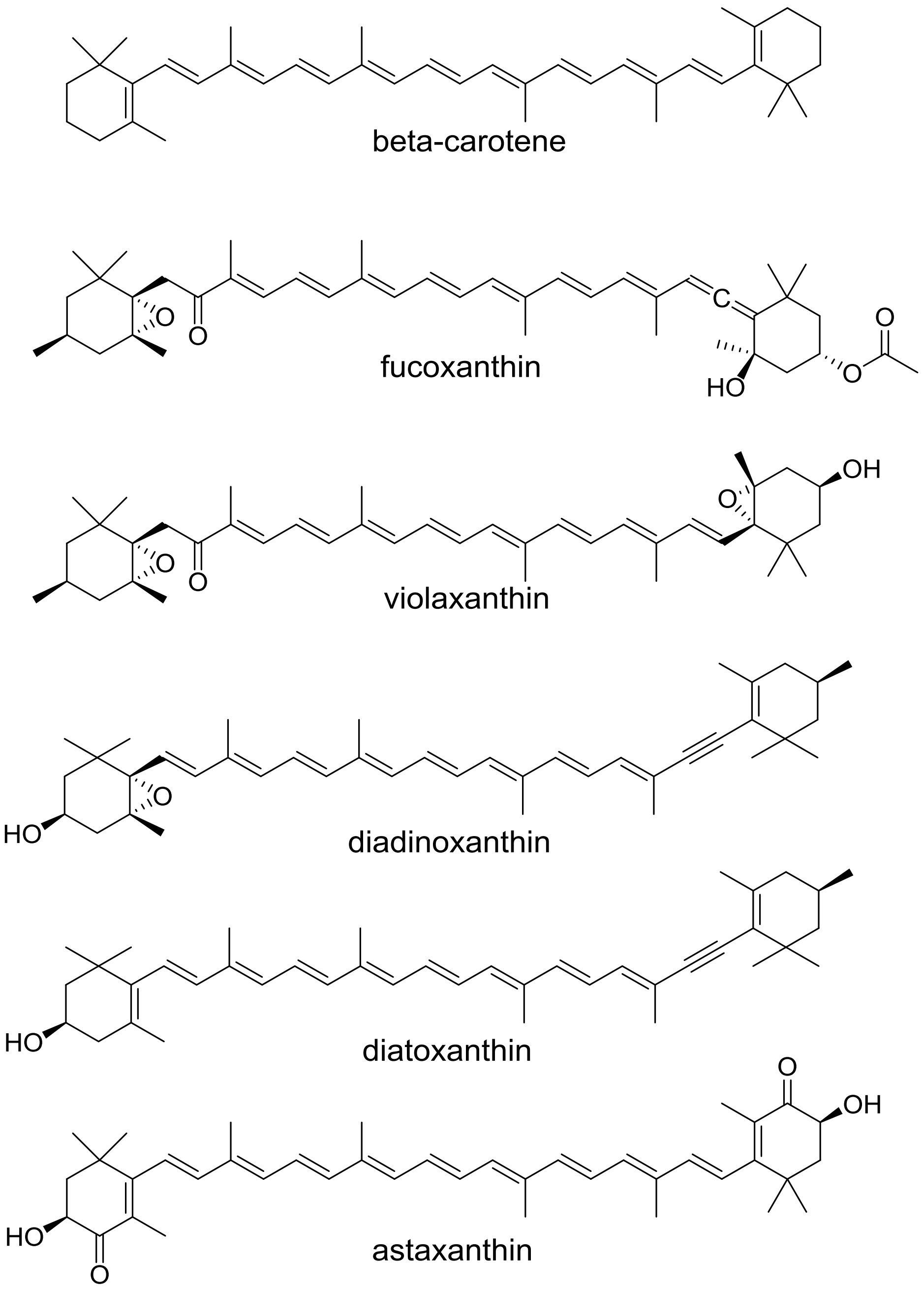


Figure 1.7. A selection of some of the carotenoids pigments found in seaweed.

1.5.2.3 Phycobilins

Phycobilins are a class of accessory pigments found only in Rhodophyta. They have a tetrapyrrole chromophore structure and are found within the phycobiliprotein, phycoerythrin.[32] The two major types are phycoerythrobilin and phycourobilin, given in Figure 1.8.

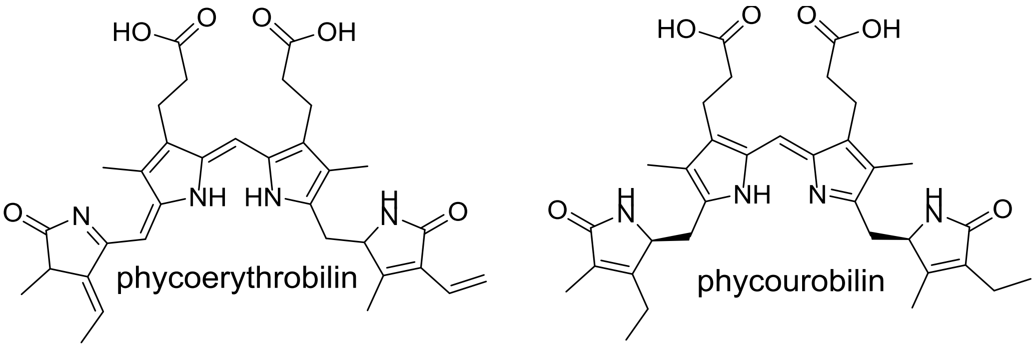


Figure 1.8. The structure of two phycobilins, phycoerythrobilin and phycourobilin.

The phycobiliprotein serves as an accessory pigment to chlorophyll, absorbing at wavelengths of 620-650 nm, which extends the range of UV absorption allowing Rhodophyta to absorb more light than Phaeophyceae and Chlorophyta, allowing it to grow at greater depths and so avoid competition.[33, 34]

1.5.3 Extraction Methods

Extraction of lipid-soluble pigments has been reported for a range of organic solvents, including acetone, dimethyl sulfoxide (DMSO), methanol and ethanol.[16, 35] A screen of solvents for extracting pigments from *Laminaria japonica* found that acetone was the most effective.[36] A 2008 patent by Zhang *et al*. reports a method for extracting astaxanthin in yields of > 8 % dry wt. by culturing green algae in such a way that increases its levels of astaxanthin from its normal level of 3.1 %wt.[37] Extracted mixtures of different carotenoids can be separated using solid phase extraction techniques.[38] Characterisation of isolated pigments can be made using HPLC-MS[22-24, 39-42] and by UV spectroscopy.[22, 27, 28]

1.6 Fatty Acids

Fatty acids are another class of compounds found in seaweed. Figure 1.9 shows a typical fatty acid found in seaweed in the form of a triglyceride. Fatty acids of seaweeds generally have chain lengths of between 14-20 carbon atoms with an even number of carbon atoms and usually contain one or two double bonds.[43, 44]

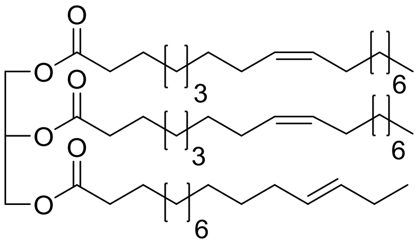


Figure 1.9. A typical fatty acid triglyceride found in seaweed.

In the commonly used system of nomenclature for fatty acids, the position of the double bond is counted from the terminal end of the chains, denoted by the Greek symbol omega (ω), as shown in Figure 1.10. Hence, the term 'omega-3', written as ω3.

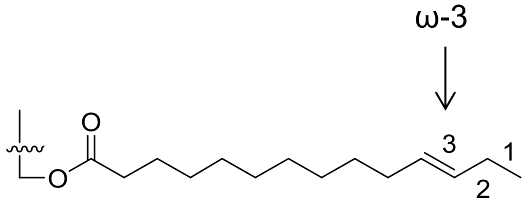


Figure 1.10. The numbering system for describing the position of the double bond, showing in this instance an ω3.

Seaweeds have been found to contain high proportions of ω3 and as a result are of value as a nutritional supplement.[45] The total lipid content of seaweeds is generally 1-5 % dry wt.[46] However, some studies have reported considerably higher values, such as the study by Nomura *et al.* which found two species, *S. Horneri* and *C.hakodatensis,* to have a total lipid content of 15 % dry wt.[47] The seaweed lipid content has been found to vary by species, geographical location, season, temperature, salinity and light intensity.[48] Tropical species have significantly lower lipid contents than cold-water species.[45] This may be because the cold water species have greater need for a higher lipid content, which serves an energy reserve throughout the colder months. There is increasing interest in the potential for seaweed lipids to be used as a source of biofuel, which is driving investment in seaweed farming technology.[1]

Seaweeds have been found to contain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (Figure 1.11).[49] The double bonds are cis and are separated by a methylene (CH_2) group unless stated otherwise. This can be written as C20:5ω3, which means a chain of 20 carbon atoms, with 5 double bonds, with the first double bond at the ω3 position. Biofuels made from saturated lipids typically have greater oxidative stability that those made form unsaturated lipids.[50]

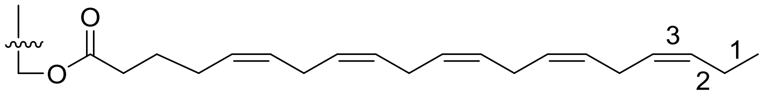


Figure 1.11. An example of a PUFA, eicosapentaenoic acid (C20:5ω3), containing 5 double bonds, with the first at ω3.

These PUFA are essential for human and animal nutrition. Seaweeds have a higher proportion of PUFA than terrestrial plants.[51] The two classes of PUFA (ω3 and ω6) have opposing physiological functions and their balance is important for normal growth and development.[51] In a healthy human diet the ratio of ω6/ω3 should not exceed 10:1. Brown algae typically have a ω6/ω3 ratio of 0.6–5.1:1.[51]

As well as nutritional value, fatty acids of certain seaweeds have also been reported to have antiviral properties.[52] The most common method for quantitative determination of fatty acids is by extraction with a polar solvent system such as 2:1 chloroform/methanol followed by analysis with GC-MS.[45] To aid in analysis they are first transesterified using methanolic HCl to form fatty acid methyl esters (FAME). Proton and carbon nuclear magnetic resonance can also assist in characterisation, especially with regards to identifying double bond configuration.[53, 54]

1.7 Phlorotannin

1.7.1 Structure

Phlorotannins are a class of polyphenols found only in brown algae and typically make up 1-10 % dry wt. of the thallus.[55, 56] They are secondary metabolites derived from phloroglucinol subunits to give a large, structurally varying polymer with a weight ranging from 126 Da to over 650 kDa, although they are more commonly observed between 10 and 100 kDa.[57] There are several different types of linkages found in phlorotannins, as illustrated by the examples in Figure 1.12 of simple phlorotannins isolated from between the rings, as in trifucol. Phlorethols are another type on linkage which contain diaryl ether bonds (Ph-O-Ph). A range of more complex structures have also been observed such as the eckol structural component as in fucofuroeckol B.[58, 59]

Around 150 algal polyphenolic structures have been isolated and characterised to date, with the Glombitza group achieving a large portion of this work.[60] The high structural diversity has limited the separation and characterisation to just the lower molecular weight species.

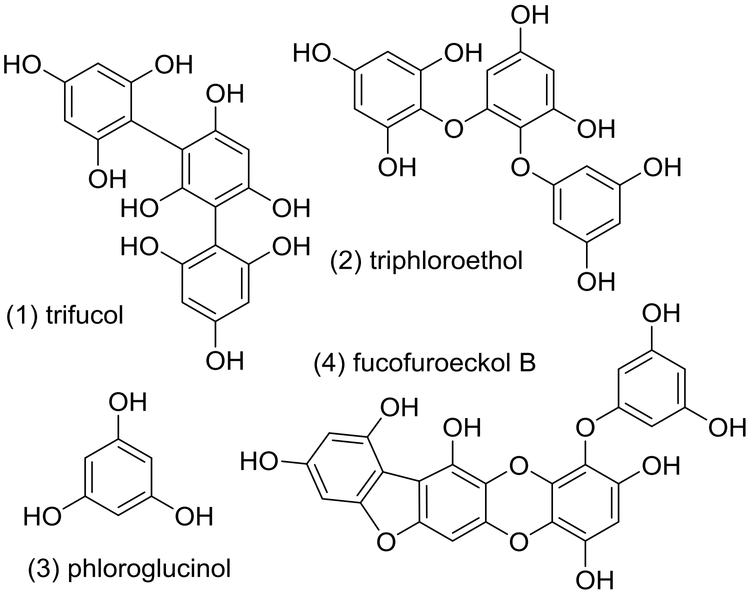


Figure 1.12. A selection of simple phlorotannins extracted from brown algae feature its key structural linkages.[61]

1.7.2 Biosynthetic Pathway

The biosynthetic pathway that produces phlorotannins is only partly understood. The formation of the phloroglucinol monomer is known to happen via the acetate-malonate pathway and involves a type III polyketide synthase enzyme.[62] The subsequent oxidative polymerisation processes by which the monomers are coupled are still unknown, although radical mechanisms have been proposed.[61, 62] There are reported examples elsewhere in nature of enzymes that couple phenols, such as the cytochrome P450 enzymes found in some bacteria, which facilitate both the Ar-Ar and the Ar-O-Ar linkages in the biosynthesis of the important antibiotic natural product vancomycin (Figure 1.13).[63] The existence of these enzymes elsewhere in nature suggests the possibility that seaweeds may also possess enzymes for the coupling of polyphenols, however, as yet, this is unconfirmed.

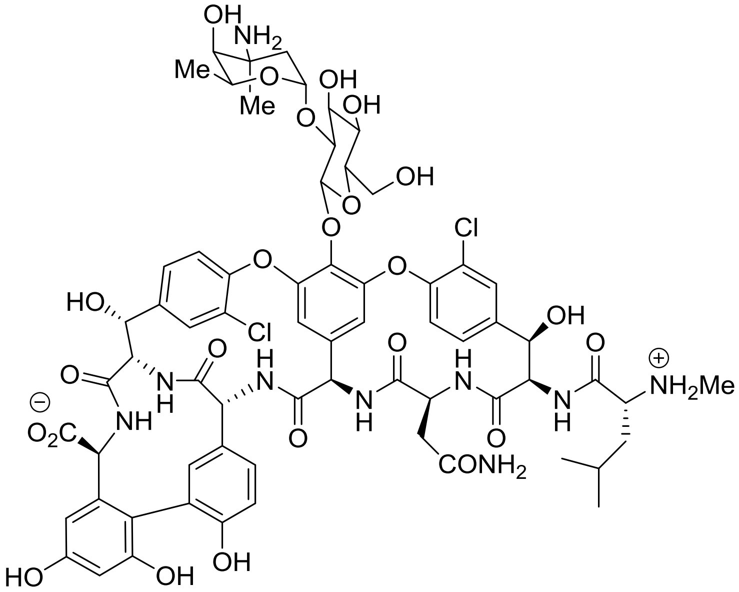


Figure 1.13. The natural product vancomycin, isolated from bacteria that features both Ar-Ar and Ar-O-Ar linkages that are coupled by P450 enzymes.

1.7.3 Properties and Physiological Functions

During the last few decades there have been many studies into the physiological roles of phlorotannin in seaweed. A range of purposes have been suggested.

The primary function of the phlorotannins is believed to be as a feature of the cell wall, in which it helps regulate osmotic pressure.[64-66] Phlorotannins have a strong UV absorbance between 190-240 nm leading to the theory that they serve as way of protecting the seaweed from potentially damaging UV solar radiation.

One of the most interesting and celebrated properties of polyphenolics is their high antioxidant capacity.[51] In vitro tests of phlorotannins have found them to be strong antioxidants and radical scavengers due to their hydroxyl groups and conjugated aromatic systems.[51] The antioxidant properties of phlorotannin and *in vitro* methods for their quantification are discussed in more detail in the materials and methods section. It has been proposed that these antioxidant properties may help protect intertidal species against oxidative damage, such as during periods of low tides when the tissue may experience desiccation. Sub tidal species were found to have greater phenolic contents than intertidal species, which may be due to the increased need of intertidal species to protect against oxidative damage.[67] *In vitro* tests have shown phlorotannins to have antibacterial properties.[68] Phlorotannins have been shown to be good at binding to metals and proteins.[69] Cheung and Wong have showed that protein extraction yield was inversely proportional to the total phenolic content, suggesting the protein affinity of phlorotannin to be responsible.[70]

A study has found that seaweeds which were subjected to heavier amounts of grazing from snails produced higher levels of phlorotannin, leading to the suggestion that it might serve as a deterrent against herbivorous predation.[71, 72] It has been suggested that the strong protein-binding nature of polyphenols may be key to this.[73]

1.7.4 Applications of Phlorotannin

The properties of phlorotannin discussed above have inspired research into a range of commercial applications. Consumer demand for cosmetic products containing only natural ingredients has led to a market drive to source natural extracts as ingredients. The strong antioxidant and UV absorbing properties of phlorotannin has led to them being used as active ingredients in cosmetic products for the purpose of UV protection, anti-aging and skin whitening.[74-76] While there is continued debate into whether these claims can be substantiated, the consumer demand for natural ingredients will likely to lead to phlorotannin and other seaweed extracts being increasingly used by the cosmetics industry.

Within the food industry, there is also growing consumer concern over the safety of synthetic additives such as preservatives and flavourings. This has driven research into the use of natural plant extracts as functional food additives, for which phlorotannin has attracted interest due to its strong antioxidant and antimicrobial properties.

The antioxidant properties of phlorotannin make it useful as a preservative, offering protection against lipid peroxidation, a major cause of food degradation which produces undesirable effects in food, including rancid odours and flavours, changes in colour and texture and a decrease in nutritional value.[77] Studies have found that phlorotannin can be as effective against lipid peroxidation as some widely used synthetic food antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroxyquinone (TBHQ) and propylgallate.[77] Additionally, phlorotannin has antimicrobial properties and has been shown to be effective against food borne bacteria.[68]

Phlorotannin has also been used in medical textiles, where they have been incorporated into scaffolds used for tissue regeneration. Work by Yeo *et al.* found that when phlorotannin was incorporated into the electrospun fabric, it increased the rate of cell growth on the scaffold.[78, 79]

Natural products and extracts are routinely screened for pharmaceutical applications. Phlorotannin has been found to have anti-inflammatory and antiallergenic properties.[80, 81]

1.7.5 Distribution and Variation in Phenolic Content

1.7.5.1 Distribution of Phlorotannin

The majority of phlorotannin in seaweed is packed within intracellular compartments called physodes, located in the outer cortical layer of the thalli, near to the surface of the seaweed.[58] Additionally, phlorotannins are known to bind to algal cell walls via an interaction with alginic acid.[65] The high strength of the alginate-phlorotannin interaction suggests that the mode of binding is via ester covalent bonds, though this is unconfirmed.[82]

1.7.5.2 Variation

Variation in phenolic content has been observed between species of brown algae as well as between different populations within each species.[64] It is also known to vary in different parts of the thallus.[83] A study of *L. digitata* and *L. hyperborean* were found to have elevated levels in the older sections of the thallus (holdfast and base of fronds). However, other studies on *A. nodosum* and *Sargassum muticum* found constant levels of phenolics throughout the thallus (5-6 % dry wt. phlorotannins).[64]

1.7.5.3 Seasonal Variation

Table 1.2 gives a summary of the studies that have looked at the seasonal variation in the levels of phlorotannins. All of these studies found the levels of phlorotannin to vary throughout the year. Many factors have been suggested as influencing the phenolic content in seaweed and the variation observed is likely to be a combination of many of these factors.

Table 1.2. Seasonal variation studies on polyphenols in seaweed

Author	Year	Location	Species	Phlorotannin variation
Ragen, and Jenson[84]	Oct 1976 - Oct 1977	Trondheimsfjord at Flak, Norway	*Ascophyllum nodosum*	6-14 % dry wt.
			Fucus vesiculosus	7-13 % dry wt.
Rönnberg and Ruokolahti[85]	1984	Aland, Sweden	*Fucus vesiculosus*	4.9-8.3 % dry wt. (shallower site) 5.3-6.6 % dry wt. (deeper site)
Peckol, Krane and Yates[86]	Mar-Nov, 1995	Rhode Island, USA	*Fucus vesiculosus*	2-8 % dry wt. (from high tide site) and 1-4.5 % (from low tide site)
Steinberg[87]	1998	NSW, Australia	*Ecklonia radiata*	3.2-9.4 % dry wt.
Parys, Kehraus, Pete, Kupper, Glombitza and Konig[88]	Jan-Dec 2005	Oban, Scotland	*Ascophyllum nodosum*	0.3-1 % fresh wt.
Kamiya, Nishino, Yokoyama, Yatsuya, Nishigaki, Yoshikawa and Ohki[83]	Jul 2006 - Jun 2007	Shikimi, Wakasa, Fukui, Japan	*Myagropsis myagroides*	1-6 % dry wt.
			Sargassum hemiphyllum	1-14 % dry wt.
			S. confusum	4-12 % dry wt.
			S. patens	3-12 % dry wt.
			S. siliquastrum	3-12 % dry wt.
Schiener, Black and Stanley[89]	Aug 2010 – Sep 2011	Isle of Seil, Scotland	*Laminaria digitata*	0.09-0.18 % dry wt.
			Laminaria hyperborea	0.10-0.23 % dry wt.
			Saccharina latissima	0.23-0.68 % dry wt.

A trend seen in two of the studies in Table 1.2 was that there were two yearly maxima in phenolic content; one in winter and one in summer.[84, 88] Arnold and Targett proposed a model of “Growth-defence trade off theory” to explain the optimal accumulation of phlorotannin based on trade-offs between growth and defence.[65, 90] This theory proposes that the seaweed allocates resources based on the competing need for either growth or defence and that the phlorotannin content reflects this.

Rönnberg and Ruokolahti studied the differences in seasonal trends between two sites of different depths. The first ‘sheltered’ site was shallower and occasionally exposed by low tide in spring.[85] The second ‘exposed’ site was in deeper water. Seasonal variation was experienced at both sites, but was much more pronounced in the sheltered site. An explanation was offered based on the light conditions to explain why the sheltered sight experienced more variation in light over the year.

In addition to the influences discussed above, there are various other factors that have been reported to contribute to this variation, including temperature, light intensity, increased UV radiation, salinity, nutrient availability, herbivorous predation and exposure to the elements.[76, 77, 91]

Glombitza also noted in his study that the composition of the extracted phenolics varied throughout the season; HPLC analysis showed that there were higher quantities of lower molecular weight phlorotannins found in March to June compared to the rest of the year.[88]

1.7.6 Methods for the Extraction of Phlorotannin

There are many different extraction systems reported in the literature for the extraction of phlorotannin from seaweed. When developing extraction methods for phenolics, it is very important to consider the interactions that phlorotannins have with the polysaccharides and proteins bound to the cell wall. The most common solvents reported for extraction of phenolics are aqueous acetone or ethanol.[55] It has been suggested that these solvents inhibit the interactions with polysaccharides and proteins, freeing the phlorotannin so that it can dissolve into the extraction liquor.[92, 93]

A key problem with handling phlorotannins is their sensitivity to auto-oxidative degradation.[94] Adding antioxidants such as ascorbic acid during the extraction has been shown to reduce this oxidation.[95, 96] Alternatively, a common practice used in the isolation of phlorotannins is to acetylate them immediately after extraction. Protection of the hydroxyl groups gives them greater stability during isolation, handing and characterisation.[97]

1.7.7 Methods in the Analysis of Phlorotannins

^{1}H and ^{13}C nuclear magnetic spectroscopy (NMR) provides a very effective way of characterising low weight (<3000 Da) phlorotannins isolated from extracts. Around 150 of these phlorotannins have been isolated and reported to date, with the majority of this work achieved by Glombitza *et al.*[97]

Spectra can be fully assigned using 2D NMR experiments such as HMQC and HMBC, however this becomes increasingly more complicated with increasing molecular weight. Sharper spectra are generally obtained using CD_3OD compared to other solvents. $CDCl_3$ is most commonly used for acetylated phlorotannins.

NMR spectra of mixtures of phlorotannins are more complex, due the large number of different bond types they contain. 2D NMR spectra cannot be used with mixtures because there is not enough of each of the molecules to give strong couplings. However, as discussed later, the proton spectra can be used to obtain some information about the relative proportions of the different types of linkage in the phlorotannin extract.

1.7.8 Determination of Total Phenolic Content

The most commonly used method for the determination of phenolic content is the Folin Ciocalteu (FC) method.[98] This is discussed in detail in the materials and methods section. Other methods have been assessed for their effectiveness in determining phenolic content; Parys *et al.* reported the use of ^{1}H NMR spectroscopy analysis, but concluded that the FC method was a more reliable assay due to poor reproducibility of the ^{1}H NMR spectroscopic method.[88]

1.7.9 Fractionation and Molecular Weight Profiling

This section describes the different methods reported in the literature for the fractionation of phlorotannin extracts.

1.7.9.1 Ultrafiltration

Wang *et al.* reported a method for fractionating phlorotannin using ultrafiltration, in which phlorotannin was centrifuged through a series of filters of decreasing pore sizes to give five molecular weight fractions in the range of >100 kDa, 30-100 kDa, 10-30 kDa, 5-10 kDa.[99] Boettcher and Targett used ultrafiltration to prepare fractions of different molecular weight phlorotannins for a biological study on the role of polyphenolic molecular size on the reduction of assimilation efficiency in marine herbivores.[72] This gave them five fractions between 1000 Da to 5000 Da. Ultrafiltration was also used by Audibert *et al.,* followed by dialysis to isolate low molecular weight phlorotannin (< 2kDa).[100]

1.7.9.2 Resin Affinity

Wang *et al.* reported the use of a size exclusion chromatography (SEC) resin to fractionate low weight phlorotannin (<1100 Da) by eluting with a series of solvents of decreasing polarity to obtain different fractions.[99] They claimed that the separation was based mainly on hydrogen bonding interactions between the phenolic hydroxyl groups on the phlorotannin and the ether bonds on the cross-linked chain of the resin.

1.7.9.3 Preparative HPLC

HPLC has been used to analyse phlorotannin extracts as well as to isolate them on a preparatory scale,[101] however it does have its limitations; resolution of peaks is typically only possible up to about 6,000 Da. Also, preparatory HPLC is usually limited to less than a gram of material per run, making it difficult to process large quantities of material.

1.7.10 Acetylation and Precipitation

As discussed earlier, a method used extensively in the literature to increase the stability of phlorotannin extracts is to derivatise them by acetylating the hydroxyl groups. Glombitza *et al.* reported a technique for the fractionation of acetylated phlorotannin based on its solubility in different solvent systems.[102] Larger phlorotannins (accounting for about 90 %) were precipitated from chloroform by addition of petroleum ether, leaving the smaller molecular portion in solution. This method then simplifies the subsequent finer separation techniques of the smaller phlorotannins. When separation is finished, the phlorotannins can be de-acetylated if needed. This technique was used by Glombitza *et al.* to isolate and characterise many small phlorotannins.[97]

1.7.11 Molecular Weight Profiling

Despite several decades of research on phlorotannin there has been no method reported in the literature for determining the complete molecular weight distribution of a phlorotannin extract. The development of such a quick and easy technique would be a useful tool in phlorotannin research, as it would open the door for a range of studies that could explore the relationship between molecular weight and other properties (*e.g.* anti-oxidant capacity, skin penetration *etc.*). Several techniques have been used that have achieved partial success for molecular weight separation of phlorotannin, however, the limitations of these techniques mean that they are incapable of providing a complete molecular weight profile of a phlorotannin extract.

Steevensz *et al.* reported a method for profiling the molecular weight distribution of phlorotannin using HPLC using a hydrophilic column coupled to high resolution mass spectrometry allowing the identification of phlorotannins up to 6 kDa.[103] The use of other mass spectrometry methods such as matrix-assisted laser desorption/ionization time of flight (MALDI TOF) has been explored by others.[104] It has been successfully used in analysis of small, isolated phlorotannins; however it was found to be unsuitable for use with phlorotannin fractions containing mixed weight fractions.

As discussed earlier, ultrafiltration has been used to fractionate phlorotannin. This can be used to obtain fractions of phlorotannin of different molecular weight and in theory could be used to profile the molecular weight range of a sample; however, there are some limitations with this approach. Strictly speaking, this method will separate molecules based on their physical size and shape, which may not necessarily correlate to molecular weight. The sample can easily be lost on the filters, leading to error in the results. The molecular weight profile obtained is limited to categories of molecular weight ranges. In theory, this could be improved by using a greater number of different size filters; however, this would increase loss of material on the filters, as well as increasing time and cost.

Lann *et al.* used dialysis to profile the molecular weight range of phlorotannin samples.[105] They used successive dialysis of decreasing molecular weight cut off and measured the total phenolic content at each stage in order to produce a profile of molecular weight distribution. They used cellulose bags in their study. As discussed earlier, phlorotannin has a strong affinity for polysaccharides. No mention was given to whether the total amount of phlorotannin measured equalled that of the starting sample. It is quite possible that a significant amount of material may have been lost on the bags due to interactions with the cellulose, which would cause inaccuracy in the results.

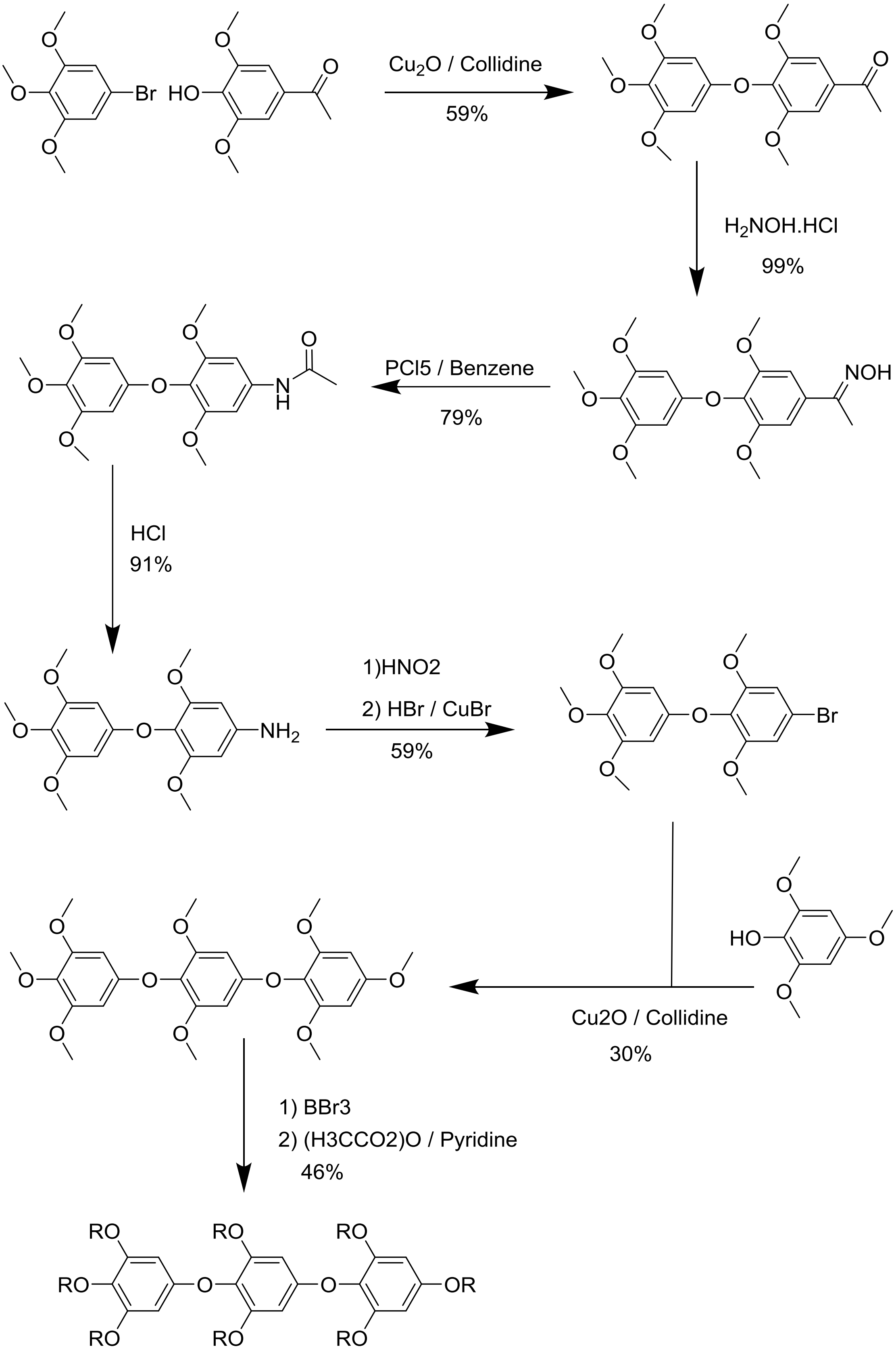
1.7.12 Synthesis of Phlorotannin

There are two reasons why it is desirable to synthesise natural products. Firstly, it can confirm the correct characterisation of a compound that has been isolated from an extract. Secondly, it can provide a large amount of an extract for research purposes if it cannot be obtained in sufficient quantities by extraction.

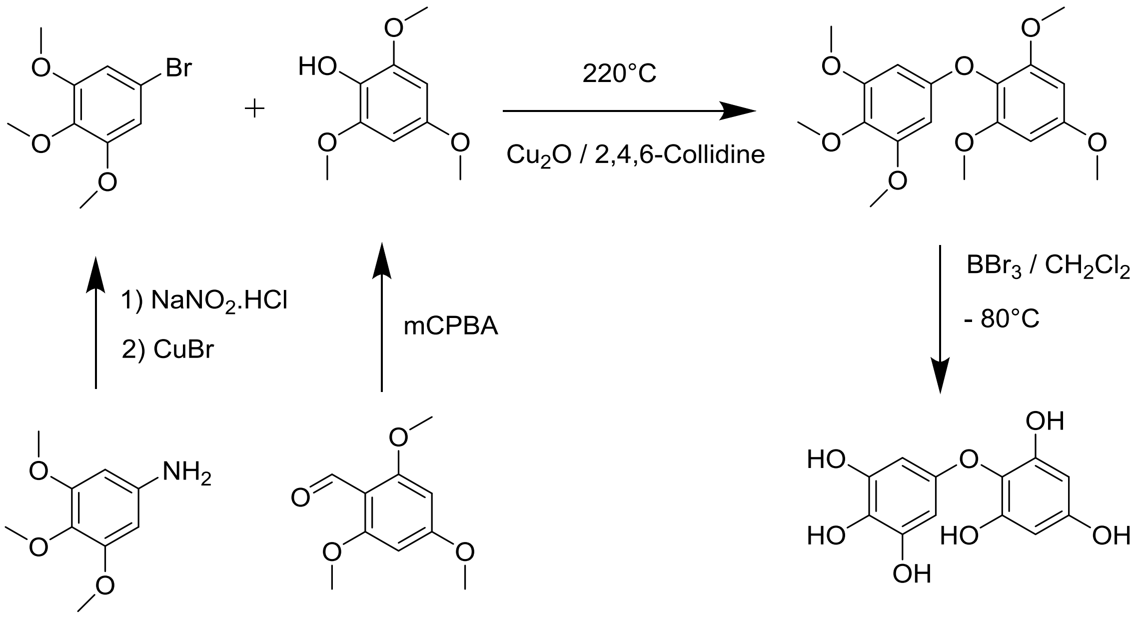
In the case of the synthesis of phlorotannin, their polymeric nature necessitates the use of protecting group strategies in order to achieve substitution at specific locations. A problem with this method is that yields decrease rapidly with each unit added, especially since these types of strategies often involve several steps for each unit added.

Scheme 1.1 shows a synthetic strategy reported by Glombitza *et al.* for the synthesis of phlorotannins, in which they coupled trimethoxybromobenzene to a phenol.[106] The phenol included a keto-group which could be transformed to a bromo-group from which the process could be repeated to add further units. Glombitza *et al.* only reported the synthesis of a trimer, but in theory any larger oligomer could be made.

Retey *et al.* have also reported the synthesis of a phlorethol linked dimer using a similar method (Scheme 1.2).[107] There may be scope for this technique to be developed using more protecting groups to allow coupling of different monomers together to form a specific sequence.



Scheme 1.1. The synthetic strategy for synthesis of polyphenols reported by Glombitza.[106]



Scheme 1.2. The synthetic strategy employed by Retey *et al*. to synthesise phlorethols.[107]

1.8 Polysaccharides

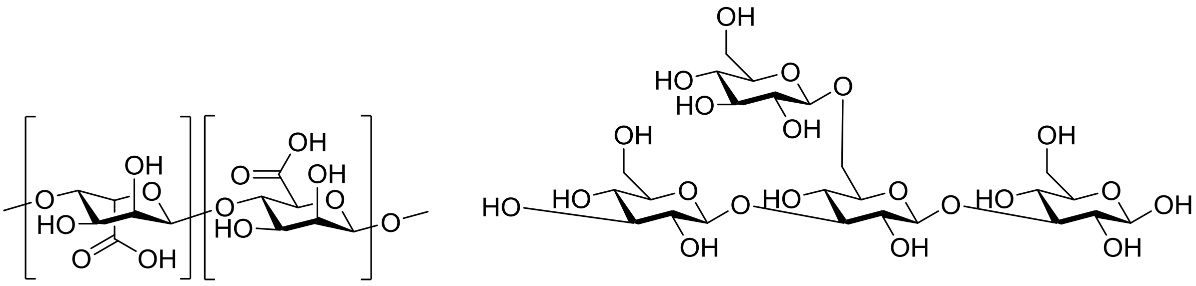
1.8.1 Types and Structure

Monomeric and polymeric saccharides typically account for > 50 % dry wt. of the total mass of seaweed. Like the pigments, the polysaccharides vary between the three classes of seaweeds, as shown in Table 1.3.

Table 1.3. The major saccharides and polysaccharides found in each of the three types of seaweed

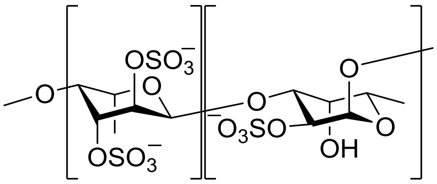
Chlorophyta (green)	Phaeophyceae (brown)	Rhodophyta (red)
Cellulose	Cellulose	Cellulose
Starch	Laminarin starch	Floridean starch
Mannan or galactan	Mannitol (monomer)	Agar
Heteroglycan	Alginic acid	Carrageenan
Ulvan	Fucose-containing-sulfated-polysaccharides (FCSP)	Xylan and galactan

The different saccharides and polysaccharides vary in the types of functional groups attached to them, which gives them a wide range of different solubilities and interactions with other species. Each of the three types of seaweed uses a different polysaccharide for energy storage; starch (in chlorophyta), laminarin (in Phaeophyceae) and floridean starch (in Rhodophyta). The polysaccharide research in this thesis was performed on the brown seaweed, *Fucus vesiculosus*. As such, the literature reviewed herein is limited to just those polysaccharides found in brown seaweed, namely laminarin, alginic acid and the fucose containing sulphated polysaccharides (FCSP). The structures of these polysaccharides are given in Figure 1.14. It should be noted that the structure of FCSP varies. The structure given in Figure 1.14 is simply an example to illustrate the key structural features.



alginic acid

laminarin starch



fucose containing sulfated polysaccharide FCSP (structure varies)

Figure 1.14. Examples of key macro-algae polysaccharides with varying functional groups.[108]

1.8.2 Fucose Containing Sulfated Polysaccharides (FCSP)

An important class of polysaccharides found in seaweed are the fucose containing sulphated polysaccharides (FCSP), sometimes referred to as fucoidan. They are only found in brown algae, as well as in some marine invertebrates such as sea urchins and sea cucumbers.[109, 110] The physiological role of FCSP in seaweed is thought to be concerned with osmotic regulation of the seaweed tissue.[111]

Various names and terminology have been given to refer to this group of polysaccharides over the last few decades. It is called ‘fucoidan’ under IUPAC rules, but is also known as fucan and fucosan. Researchers have also attempted to assign terms with structural definitions to subclasses of these polysaccharides. However, the structurally varying nature and difficulty in characterisation has led to much confusion, debate and no doubt some miscommunication. As a result, caution should be exercised when using these terms. The key features of this class of polysaccharides are that they predominantly consist of fucose as the main sugar type and that they are sulphated. As such, a safer approach is to refer to them as FCSP, as this is a ‘catch all’ term that avoids potential confusion.

FCSP were first described in the literature in the 1950s by O’Neill and Conchie, who proposed that the main unit of FCSP to be 1,2-α-fucose in which the sulphur groups mainly occur at the fucose C-4 position.[111] Since then many other structures have been described. In 1970, Anno *et al.* claimed to have isolated L-fucose 4-sulphate from hydrolysis of the polysaccharide.[112] In 1993, Patankar *et al.* published research showing that the core region of the FCSP was mostly a polymer of α-(1→3) linked fucose, with some of the C-4 position substituted with sulphur groups, as shown in Figure 1.15.[113] The chain was also branched roughly every 2-3 units.

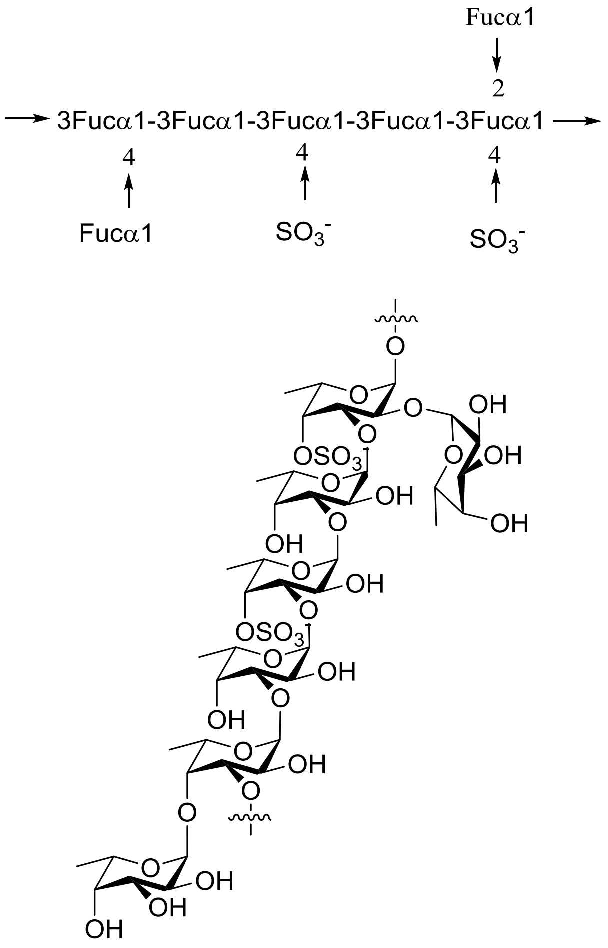


Figure 1.15. Patankar model for the average structure of FCSP shown in both shorthand notation (top) and drawn in full (bottom).[113]

The structure described by Patankar *et al.* is still accepted as being one of the more common structures of FCSP. However, it should be noted that many different structural motifs have been reported for FCSP. The degree of complexity is known to vary between species. *Fucus vesiculosus* is known to have relatively more simple structures, which contain mainly fucose, with few other sugar types. Others species are more complex featuring additional monosaccharides units (mannose, galactose, glucose, xylose, *etc.*). Interestingly, there are also reports of other types of FCSP isolated containing uronic acids, acetyl groups and even some proteins attached.

Many studies with FCSP have produced evidence for a range of various interesting biological activities. Extensive reviews have been published, notably by Fitton,[111] Li *et al.*[114] and by Jiao *et al.*[115] It is the presence of the sulphate groups on FCSP that is believed to be responsible for this bioactivity.

As early as 1958, the antiviral properties of seaweed extracts were reported when a study showed that they could protect chicken embryos from influenza.[116] Since then, sulfated polysaccharides extracted from seaweeds have exhibited both *in vitro* and *in vivo* antiviral activity.[114] They are of particular interest because of their low cytotoxicity compared to other antiviral drugs currently in use.[111] The mode of action is not yet fully understood. FCSP has not been shown to have any direct inactivating effect on virons in virocidal assays. A proposed mechanism for antiviral activity of FCSP is that they inhibit viral sorption so as to inhibit viral-induced syncytium formation.[117] The degree of sulfation of FCSP was found to be important for antiviral activity, supporting the claim that it is the sulphate groups that give FCSP its bioactivity.[118, 119] FCSP has been shown to have antiviral activity against viruses such as herpes,[118-123] human cytomegalovirus,[118] tobacco mosaic virus[124], influenza,[125, 126] dengue virus type II,[127] and HIV.[128, 129] FCSP have also been shown to have additional properties, such as anticoagulant, antithrombotic, antitumor, immunomodulatory and anti-Inflammatory.[111] FCSP have also found applications in skin care. Evidence suggests that FCSP can be used as a skin whitening and anti-aging agent, as well as offering UV-protection.[130-132] FCSP extracts have been incorporated into electrospun fabrics for medical applications.[133, 134]

1.8.3 Alginate

By contrast to FSCP, alginate is simple in structure. It is a copolymer consisting of just two saccharides; β-D-mannuronic acid and α-L-guluronic acid, often labelled M and G respectively (Figure 1.16).

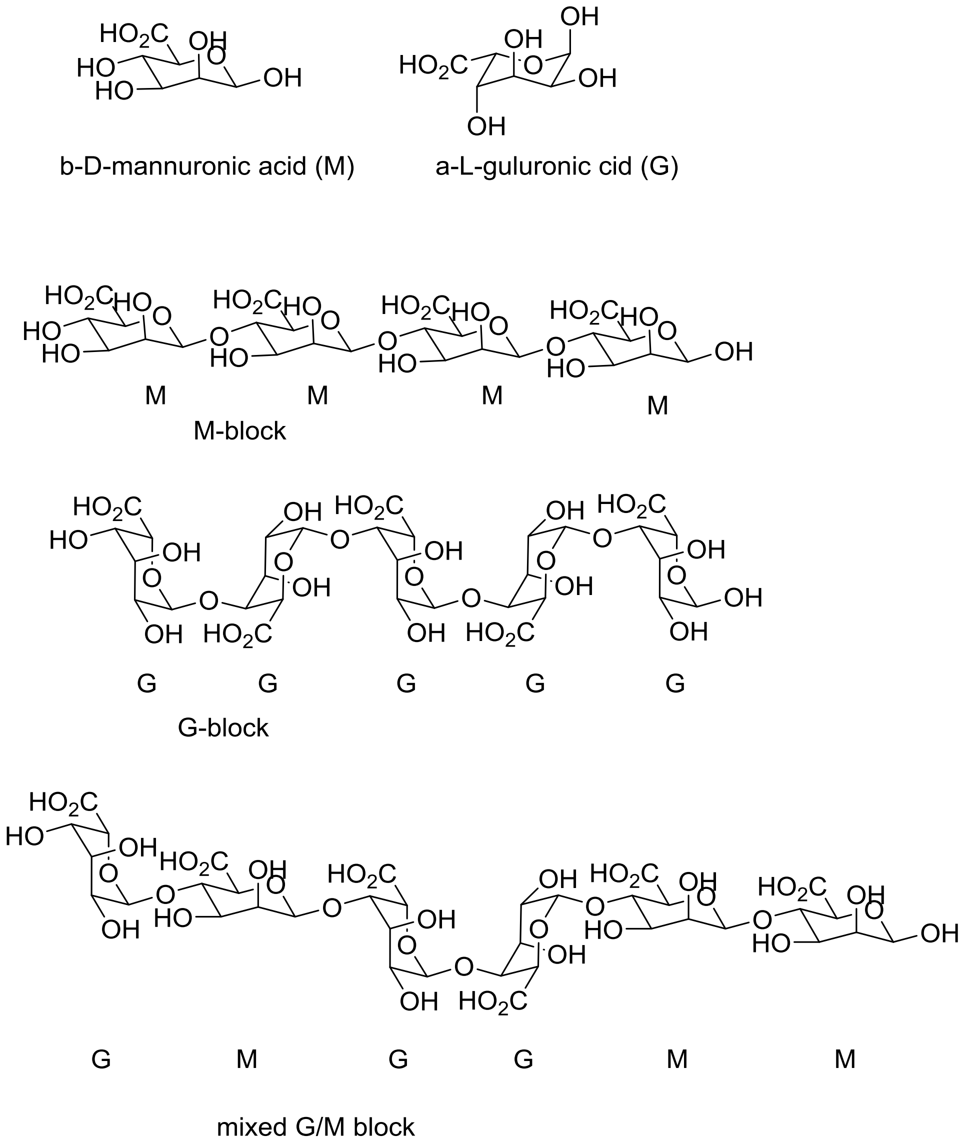

b-D-mannuronic acid (M)
a-L-guluronic cid (G)
M-block
G-block
mixed G/M block

Figure 1.16. The monomers β-D-mannuronic acid (M) and α-L-guluronic acid (G) together with an M block, a G-block and a mixed block.[135]

Sections of the polymers consisting of either only M or G units are referred to as M blocks and G blocks respectively. There is an important difference between the structure of M and G blocks. M blocks are straight and linear in shape, whereas G blocks form crinkled chains. G blocks also have the ability to complex metal ions in the gaps formed by their crinkled shape.

This allows the chains to aggregate together and form gel networks. The greater the proportion of G blocks in alginate, the more this cross linking occurs and the greater the strength of the polymer. The properties of the polymer are dependant on the molecular weight of the polymer and the concentration of those metal ions that bind to the alginate. The relative amount of M and G can be expressed numerically as the F_G number, which is the fraction of G units in the polymer. An additional descriptive parameter often used is the N_G value; this is the number-average of consecutive guluronic units in the G-blocks. This is important as it is only the consecutive G units that are able to chelate to metal ions and form gel networks. The alginate fractions may have the same F_G value, but different N_G values.

Alginates are found in the cell wall and intercellular matrix of seaweeds, providing the strength and rigidity necessary to support the seaweed in the water. By controlling the ratio of M and G units, seaweeds can tune the properties of alginic acid to suit their needs. In parts of the seaweed near the holdfast, where high rigidity in needed, the seaweed produces alginate with high F_G and N_G values. Conversely, in the growing tips of the seaweed, greater flexibility is required to allow the seaweed to move freely with the waves. Otherwise, high rigidity in these parts of the seaweed would lead to high levels of stress and tearing of the tissue. As a result the alginate in the tips of seaweeds has much lower F_G and N_G values than the holdfasts.

Alginate was first described in the literature by Edward Stanford in 1883.[136] The commercial potential for its use as a thickening agent was soon recognised. Commercial production started in 1927 and has now expanded to over 50,000 tonnes per year worldwide.[136] Around 30 % of this is used in the food industry and the remainder used in industrial, pharmaceutical and medical applications.[114, 137]

1.8.4 Laminarin

The third type of polysaccharide found in brown algae is laminarin. This polysaccharide consists predominantly of β-D-glucose, linked by either β(1→3) or β(1→6) linkages (Figure 1.17).

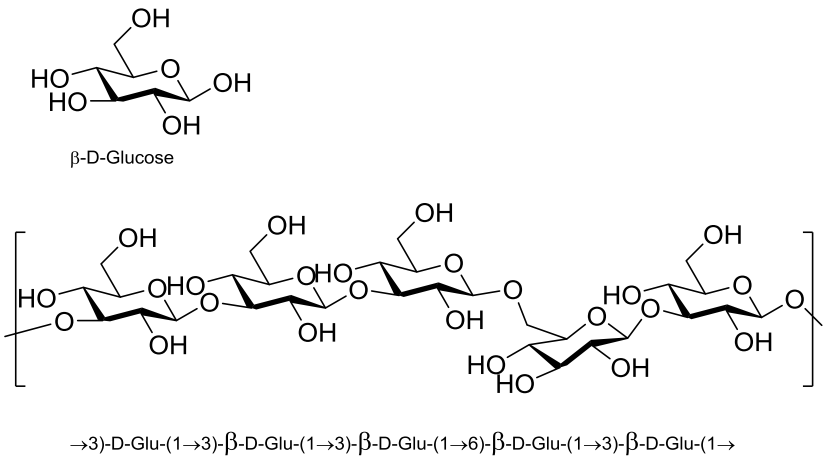


Figure 1.17. β-D-Glucose, the monomer of laminarin (top) and an example of the laminarin backbone (bottom), showing the two types of linkage: β(1→3) and β(1→6).[138]

Laminarin does not show the same degree of bioactivity as FCSP, possibly because of the lack of sulphate groups on it. As such, it has not been studied to the same degree as FCSP. It does, however, show antioxidant properties. Lee *et al.* used gamma radiation to degrade laminarin to produce lower molecular weight chains and then demonstrated an increased antioxidant capacity, which they attributed to the formation of carbonyl groups during degradation.[139]

1.8.5 Methods for the Extraction of Polysaccharides

This section discusses the current literature on the methods used for the extraction, separation and purification of polysaccharides. There is now a variety of well-established methods for the extraction and separation of algal polysaccharides. As already discussed above, alginate is extracted industrially on a huge scale. The comparatively less complex structure of alginate makes it easier to isolate from the other polysaccharides. Methods for extraction of FSCP are less well studied. However, the growing research demonstrating the potential applications of FCSP has driven the development of methods for their extraction, fractionation and purification.

1.8.5.1 Pre-extraction ‘Defatting’

A common practice when targeting polysaccharides is to first perform a series of extractions on the seaweed with organic solvents to remove compounds other than the polysaccharides such as pigments, lipids and polyphenolics, a process sometimes referred to as ‘defatting’. The removal of these species from the algal material simplifies subsequent extractions of polysaccharides.

A variety of aqueous extractions can then be performed on the defatted seaweed to obtain polysaccharide extracts. These crude polysaccharide extracts contain a mixture of the different types of polysaccharides (*e.g.* alginate, FCSP, laminarin *etc.*). Distinct classes of polysaccharides such as alginate can be separated and isolated. However, the structurally varying nature of FCSP and laminarin means that they cannot be ‘isolated’ *per se*. Instead, they are fractionated to obtain extracts containing mixtures of similar structures.

Fractionation of polysaccharides is achieved by exploiting differences in their physical properties, such as solubility, molecular weight, chelation abilities *etc.* In selecting a method for fractionation of polysaccharides it is important to know the structural features of polysaccharides present in the extract and to have an understanding of their respective physical properties and behaviour under various conditions such as temperature, solubility at pH *etc.*

1.8.5.2 Solvent System

Alginate, FCSP and laminarin are all water soluble, and so aqueous extraction systems are used to target them. Various physical parameters of the extraction such as temperature and pH can be adjusted depending on the polysaccharides being targeted. Early extractions often used strong acidic conditions *e.g.* that by Kylin and Black *et al.*[140, 141] Later extractions used milder techniques so as to avoid the possibility of altering the chemical structure of the polysaccharides under acid conditions. Such alterations could include hydrolysis of the polysaccharide backbone and cleavage of augmenting groups attached to it.

1.8.5.3 Separation of Polysaccharides

The techniques for the separation of polysaccharides are discussed below. They are based on exploiting the differences in physical properties of solubility, molecular weight and charge affinity. The presence of different augmenting groups in varying proportions affects the solubility of the polysaccharides. The molecular weight and degree of branching will also have an impact on solubility. These differences can be exploited as a way of separating mixtures by varying physical parameters such as solvent, pH, temperature and concentration. Additives such as metal ions can also be added.

Sequential precipitation can be achieved by gradual addition of an anti-solvent such as ethanol.[142-144] Alginates tend to precipitate out at lower ethanol concentrations than laminarin and FCSP, thus offering a way of separating alginate from the FCSP and laminarin. A limitation of this technique of course is that it can require large amounts of anti-solvent to reach higher ratios. The large volumes of solvent can then complicate subsequent recovery of the polysaccharides that remain in solution.

A recently patented method for the extraction of polysaccharides involves an initial extraction with alcohol to remove low molecular weight species followed by a second extraction on the remaining residue with aqueous HCl in a pH range of 2—2.5.[145] Addition of ethanol then allowed the sequential precipitation of FCSP and laminarin.

Addition of metal ions has been reported to selectivity precipitate polysaccharides capable of chelating them. These are the carboxylate groups on alginate and the sulphate groups on FCSP. Perhaps the most useful application of this technique is in the control of the solubility of alginate with calcium ions, which can be selectively precipitated out, leaving other polysaccharides in solution. $CaCl_2$ can also be added during the initial aqueous extraction as a way of precipitating it from solution. However, this can turn the liquor into a viscous gel that can become difficult to handle.

Organic salts have also been used; Cumashi *et al*. reported the use of the quaternary ammonium salt, Cetavlon^{TM} to precipitate acidic polysaccharides out from solution.[146] The ionic strength of the solution affects solubility of polysaccharides. Adding salts such as NaCl and $CaCl_2$ can cause precipitation, a process known as ‘salting out’.

Other metal ion species have been used. Percival *et al*. reported an extraction for FCSP involving the use of lead acetate to remove protein and alginate from solution.[147] The solution was then made just alkaline with barium hydroxide which precipitated out a lead hydroxide FCSP complex.[144] The toxicity of barium hydroxide and lead acetate would mean that they are not suitable for large scale extractions, particularly for extracts destined for food or cosmetic applications.

The solubility of polysaccharides is affected by pH. This is particularly the case for polysaccharides containing acidic groups such as FCSP and alginate. The pK_a of these groups is an important factor, as protonation can cause precipitation. The pK_a of alginic acid has been studied by Fukashima *et al.* [148] who found it to vary between 2-4. They also found that the pK_a drops when the ionic strength of the solution is increased. Alginates can also be precipitated by lowering the pH. Here, the range of precipitation depends on the molecular weight of the chain. The N_G Value is also important; blocks favour precipitation as they are more crystalline. No studies on the pK_a of FCSP could be found in the literature; however, it can be estimated from the pK_a of other sulphate compounds. The pK_a of $MeSO_3H$ is reported by Evans *et al.* as -2.6.[149] The pK_a of the sulphate groups on FCSP are likely to be similar to this value. The deprotonated anions may be able to hydrogen bond to the hydroxyl groups on the ring which would stabilise the anion and would lower the pK_a below -2.6. This means that the sulphate groups would only be protonated at extremely low pH and can therefore be considered to be always deprotonated. It is likely then, that the pK_a of alginate is much higher than that of FCSP by about 4-6 units. This difference may allow alginate to be selectively precipitated, leaving FCSP remaining dissolved in solution.

The different charge affinities of the polysaccharides can be exploited in order to separate them. Sulphate groups and carboxylic acid groups both have strong affinities towards positive charges when they are deprotonated. Laminarin does not possess these groups and so does not have a strong affinity for positive charges. This means that ion exchange resins can be used in the separation of polysaccharides. Turgeon *et al*. reported a method using ion exchange chromatography for the separation of laminarin, FCSP and alginate.[150] They also reported the fractionation of other polysaccharides, such as galactofucans using DEAE Sepharose ion exchange chromatography, eluting with increasing concentrations of NaCl.[151] Ion exchange chromatography was also used by Nishino *et al*. to fractionate a commercial sample of FCSP.[152]

A summary of various extraction procedures reported in the literature that target FCSP is given in Table 1.4. These range from the early extractions reported by Kylin in 1913, through to more recent reports by Mak *et al.* in 2012. They feature a range of different extraction and purification methods discussed above.

Sigma Aldrich currently offer a commercially available FCSP sold as ‘fucoidan’ (product ID: F5631). They describe the extraction method for this product as being prepared by a modified method of that reported by Black in 1952.[141] However, no details are given as to exactly how the method was modified, but it is likely that it still uses an acidic extraction method reported by Black in which seaweed was extracted using 0.1 M HCl at 70°C for 1 hr.

Table 1.4. The different methods reported for the extraction and purification of FCSP.

Year	Author	Species	Main extraction	Separation	Reported Composition
1913	Kylin *et al.*[140]	*Fucus vesiculosus*	Dilute acetic acid extraction	Dilute acetic acid extraction	Fucose, that contains mannitol, alginate and laminarin
1915	Kylin *et al.*[153]	*Laminaria digitata*	Dilute acetic acid extraction	Dilute acetic acid extraction	Fucoidan contains L-fructose and other pentoses
1915	Hoagland and Lieb[154]	*Macrocystis pyrifera*	Soaked in 2% Na_2CO_3 for 24 hr.	HCl addition, recovery of precipitate by filtration redissolution in 2% Na_2CO_3	Mainly alginic acid, with some fucose-sulfate
1931	Bird and Hass[155]	*Laminaria digitata*	Seaweed soaked in water	Precipitation of crude sulfated polysaccharides by ethanol	Substantial amounts of calcium sulfate and uronic acid
1931	Nelson and Cretcher[156]	*Macrocystis pyrifera*	Repeated extraction with 2% HCl at room temperature for 48 hr.	Precipitation with ethanol	Methyl pentose monosulphate polymer with fructose and alginate contaminants
1937	Lunde *et al.*[157]	*Laminaria digitata*	Precipitation of droplets exuded from seaweed in boiling ethanol	None	Proposed (R-R'-O-SO_2-OM)n with R=fucose, R' as unknown M being Na, K, $Ca_{0.5}$ or $Mg_{0.5}$
1950	Perdival and Ross[147]	*Fucus vesiculosus, Fucus spirales, Himanthalia lorea, Laminaria clustoni*	Aqueous extraction at 100°C for 24 hr.	Extract heated with lead acetate to precipitate alginate and proteins, solution treated with $Ba(OH)_2$ to precipitate a 'hydroxide fucoidan complex'	Substantial amounts of fucose and sulfate; small amounts of uronic acid, galactic acid, galactose and xylose; metals and ash were also detected, ash was mainly calcium sulphate
1952	Black *et al.*[141]	*Fucus vesiculosus*	Extracted three times with 0.1 M HCl at pH 2-2.5 and 70 °C for 1 hr.	Fractional precipitation with ethanol	Fucose, ash sulfate

Year	Author	Species	Main extraction	Separation	Reported Composition
1968	Percival *et al.*[158]	*Ascophyllum nodosum*	Extracted with hot water and dilute alkali, formaldehyde treatment then extracted with ammonium oxalate-oxalic acid for 6 hr. at 80 °C	None	Fucose, xylose, uronic acid, sulfate
1974	Abel-Fattah *et al.*[159]	*Sargussum linifolium*	Extracted with water at pH 1 (HCl) for 3 hr. at 80°C	None	Mannose, galactose, xylose, uronic acid and fucose residues
1984	Maruyama and Yamamoto[160]	*Laminaria religiosa*	Extracted in boiling water for 4 hr, fucoidan fraction was obtained by using 0.09 HCl at 4°C for 2hr	Precipitation with 85% EtOH and dried	Fucose, xylose, mannose, glucose, rhamnose, uronic acid and sulfate
1999	Nagaoka *et al.*[161]	*Cladosiphon okamuranus*	Seaweed was treated with 30% HCl (pH 3) at 100°C for 15 min. Supernatant was neutralised with NaOH, precipitated with $CaCl_2$ and EtOH for 20 h at 4 °C, precipitate was dissolved with H_2O then dried	Supernatant was neutralised with NaOH, precipitated with $CaCl_2$ and EtOH for 20 hr. at 4 °C, precipitate was dissolved with H_2O then dried	Fucose, glucose, uronic acid and sulfate
1999	Zvyagintseva *et al.*[162]	*Laminaria cichorioides, Laminaria japonica, Laminaria evanescens*	Extracted either with 0.4 % HCl or with hot water (60°C)	Separating using a hydrophobic Teflon column, eluting with various solvents	Fucose, galactose, mannose, xylose, glucuronic acid and rhamnose
1999	Chizhov *et al.*[109]	*Chorda filum*	A defatting step with $CHCl_3$-MeOH-H_2O (2:4:1). 80% EtOH, then extracted successively with 2% $CaCl_2$ at 20 and 70°C then with HCl (pH2) and 3% Na_2CO_3	Separation with anion exchange chromatography (DEAE Sephadex A-25)	Fucose, xylose, mannose, glucose, galactose, uronic acid
2001	Marais and Joseleau *et al.*[163]	*Ascophyllum nodosum*	Extracted at RT then at 70 °C with 0.01 NaCl containing 1% $CaCl_2$	Precipitation with ethanol or Cetavlon	Fucose, xylose, galactose sulfate

Year	Author	Species	Main extraction	Separation	Reported Composition
2006	Li *et al.*[164]	*Hizkia fusiforme*	Extracted with H_2O at 70 °C	Precipitated with EtOH and $CaCl_2$	Fucose, mannose galactose, xylose, glucose, rhamnose, arabinose, uronic acid and sulfate
2006	Hemmingson *et al.*[119]	*Undaria pinnatifida*	Extracted for 6 hr. with 1% H_2SO_4, neutralised with 10% NaOH, lyophilised	DEAE Sepharose CL-6B column	Fucose, mannose, xylose, rhamnose, galactose, glucose and sulfate
2006	Descamps *et al.*[165]	*Adenocystis utricularis*	Extracted initially with ethanol-formaldehyde-water (80:5:15). The extract was then extracted for 3 hr. at 70°C with a 0.01 N HCl solution containing 4% $CaCl_2$	Precipitation with ethanol	Mostly fucose
2007	Cumashi *et al.* [146]	*Laminaria saccharina, Laminaria digitata, Fucus vesiculosus, Fucus spiralis, Ascophyllum nodosum*	Extracted with 2% $CaCl_2$ for 5 hr. at 85°C,	Precipitated with Cetavlon, transformation of Cetavlonic salts into Ca salts and an alkaline treatment to remove acetyl groups and to transform fucoidan into sodium salts	Fucose, xylose, mannose, galactose, glucose, uronic acid and sulfate
2011	Ale *et al.*[166]	*Sargassum spiralis*	Extracted with 0.03% M HCl at 90 °C for 4 hr. single step	None	Fucose, rhamnose, galactose, glucose, mannose, xylose, uronic acid, sulfate

1.8.6 Characterisation of Polysaccharides

This section discusses the analytical methods used in the characterisation of polysaccharides. The structural elucidation of the polysaccharides found in seaweed is crucial to understanding their biological activity.[111] The complexity in the structure of the polysaccharides means that it is impossible to completely characterise the structural profile for an extracted fraction. However, a significant amount of structural information can be obtained by applying the range of modern analytical methods now available. Many reviews are available on the subject, such as that by Li *et al*. on the structural determination of FCSP.[114]

The characterisation of polysaccharides is challenging as they contain many structural features. The sugar composition that makes up the backbone varies greatly between polysaccharides. They contain a number of different sugar units and in different proportions. The mode of linkage is complicated by the fact that the sugars can link together via different hydroxyl groups and different anomeric configurations. Various different augmenting groups are known to be present on the ring *e.g.* sulfates and acetyl groups.[111] Polysaccharides vary in molecular weight and can be both linear or branched.[111] Some of the important methods used in the characterisation of polysaccharides are discussed below.

1.8.6.1 Hydrolysis

Breaking down the polysaccharides by hydrolysis simplifies characterisation. Hydrolysis can be achieved either enzymatically or under acidic conditions.[152] Hydrolysis followed by mass spectrometry is often reported as the preferred method for structural characterisation.[167, 168] Tissot *et al.* showed that isomers of sulphated fucose varying in the position of the sulphate position could be differentiated by MS-MS.[169] The molecular weight and degree of branching can be determined by gel permeation chromatography (GPC).[170]

IR can be a useful tool in characterising polysaccharides, providing a way of easily identifying the presence of specific functional groups.[171, 172] In the case of the sulphated polysaccharides, the C-O-S vibrations can be used to determine sulphate position as axial or equatorial.[162] A limitation of this is that C-H bending vibrations can obscure C-O-S vibrations. Ross *et al*. used pyrolysis coupled to GC-MS to identify different polysaccharides from seaweed fractions.[173] They compared the pyrolysis profiles of the extracts to those from reference polysaccharide samples.

The large number of different sugar units means that ^{1}H NMR spectra in the CH region are often too crowded to give any useful information, however the methyl group on fucose can easily be identified as it has a chemical shift of about 1.2 – 1.4 ppm, which is separate from the CH groups at about 3.5 – 4 ppm. If FCSP were the only polysaccharide that contained sugars with methyl groups then this could be a useful method of identifying FCSP in mixtures of polysaccharides. However, FCSP has varying amounts of fucose present and so this approach is not able to quantitatively determine the proportion of FCSP in a mixture of polysaccharides. Grachev *et al.* used NMR to investigate how the position of the sulphate groups influences the conformational behaviour of FCSP backbones.[174]

1.8.7 Antibodies as a Characterisation Method

The methods for characterisation described thus far are all able to provide a degree of structural characterisation of polysaccharides. However, the complex, structurally varying nature of these polysaccharides demands that the development of new methods is needed in order to achieve better characterisation. Of particular importance to this research is the development of characterisation methods that are of use in the evaluation of different extraction procedures for separating polysaccharides. Ideally, such methods should be quick, cheap and where possible, suitable for the analysis of solutions of polysaccharides, without the need to obtain purified dried extracts.

This research is set out to evaluate the potential of monoclonal antibodies as an analytical tool for this purpose. Monoclonal antibodies have been raised against many types of polysaccharides before.[175] The following section explains how monoclonal antibodies are raised against their target compounds, their applications in polysaccharide detection assays and discusses the advantages and limitations of their use.

Monoclonal antibodies are made by exploiting the immune response system in mammals. The target antigen is injected into the host (usually a rat or mouse) which triggers an immune response that creates proteins (antibodies) that have an affinity (referred to as reactivity) towards the antigen.[175] The cells that produce these antibodies are located in the spleen of the animal. Individual spleen cells are then isolated from the host and the cell lines are cultivated to provide a source of the monoclonal antibodies. A key problem when drawing conclusions from assays using antibodies is the problem of cross reactivity. This is when an antibody has reactivity towards a species other than the target species, giving a false-positive result.

This problem can be mitigated to a large extent by conducting detailed screening studies in which the reactivity of a newly developed antibody is characterised by screening it against a variety of structurally diverse species. This is discussed further later.

1.8.7.1 Applications of monoclonal antibodies

Several examples were found in the literature where monoclonal antibodies have been used for the purpose of detecting FCSP. Mizuni *et al.* reported the use of monoclonal antibodies in a method to quantify the amount of FCSP in an extraction sample by comparison to a calibration curve of FCSP concentration against assay response.[176] They compared the yields of FCSP from different species using a previously reported extraction technique by Zvyagintseva,[162] but did not report the use of the assay to compare extraction techniques.

There are examples of antibodies being used to identify FCSP in blood and urine. Irhimeh *et al.* reported the use of a competitive ELISA assay to measure FCSP levels in blood plasma and then used it to study the uptake of FCSP after ingestion in humans.[177] Nagamine *et al.* developed a binding assay to study the levels of FCSP in urine after ingestion as part of their research into how the body metabolised FCSP.[178] A Japanese company, Takara Shuzo Co., Ltd. has patented a FCSP antibody for the purpose of structural determination.[179]

Another application for monoclonal antibodies is their use to quantitatively map the distribution of different types of polysaccharides in algal tissues by soaking the tissues in a solution containing the monoclonal antibodies coupled to fluorescence tags. Cross sections of the tissue are prepared and then fluorescence images are taken under a microscope. Studies have used this technique to determine where the highest amounts of sulphated polysaccharides are located in seaweed tissues as well as how this changes throughout its lifecycle.[180] [181]

1.9 Antioxidants: Properties and Assays

As discussed earlier, there is growing interest in the antioxidant properties of molecules for a range of applications in a number of different fields.[182] This section discusses the chemistry of antioxidants and the assays used to analyse them.

1.9.1 Definition of Antioxidant

A simple definition of an antioxidant is that it is “a molecule capable of slowing or preventing the oxidation of other molecules, and can therefore be considered as a reductant.”[182] In the context of biological systems, more specific definitions have been offered, such as “any substance that when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidations of that substrate.” [183] Conversely, pro-oxidants are molecules that do the opposite and promote the oxidation. Some molecules have the potential to behave as both anti- and pro- oxidants depending on the conditions and concentrations they are in. Antioxidants can be classified as either primary or secondary antioxidants. Primary antioxidants are those that actively inhibit oxidation reactions. Secondary antioxidants are those that inhibit oxidation indirectly, by mechanisms such as oxygen scavenging or by binding to pro-oxidants.

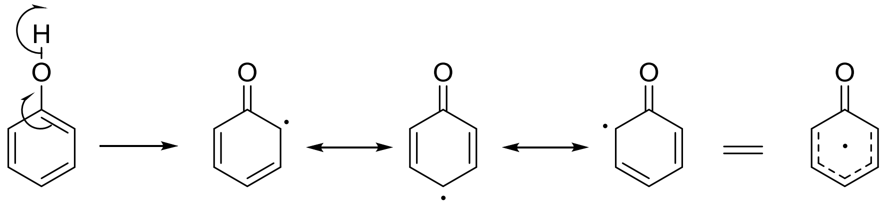
1.9.2 Mechanisms of Action of Antioxidants

The scavenging mechanisms of antioxidants are well documented.[184] There are two main types of mechanism by which the primary antioxidants react; hydrogen-atom transfer (HAT) and single-electron transfer (SET). Both of these mechanisms are relevant to phenolic compounds. In the HAT mechanism the antioxidant quenches a free radical by donating a hydrogen atom. Scheme 1.3 illustrates a reaction involving the HAT mechanism.

$$RO_2^{\bullet} + ArOH \longrightarrow ROOH + ArO^{\bullet}$$

Scheme 1.3. An example of a reaction involving the HAT mechanism.

In this example the hydroxyl group donates an H atom to an unstable free radical. This produces a more stable radical species in which the radical electron is delocalised over this ring (Figure 1.18).



Stable phenoxyl radical

Figure 1.18. The mechanism of formation of phenoxy radical and its delocalisation over the ring.

The weaker the reactive hydrogen atom is held to the hydroxyl group on the antioxidant the more likely it will participate in the HAT mechanism i.e. the greater the bond dissociation energy (B.D.E.) the less active it will be. The nature of other substituents on the ring has been shown to affect the activity. HAT reactions increase with the presence of bulky *t*-butyl groups at the 2- and 6- positions or with methoxy constituents at the 4- position.[185] These groups stabilise the resonance of the radical by donation of electron density.

In the SET mechanism, an antioxidant transfers a single electron to the radical, reducing it to form an anion. Scheme 1.4 illustrates an example of a SET mechanism. The resulting cationic radical is then deprotonated through reaction with water.

$$RO_2\bullet + ArOH \rightarrow RO_2^- + [ArOH]^{\bullet+}$$

$$[ArOH]^{\bullet+} + H_2O \rightarrow ArO\bullet + H_3O^+$$

Scheme 1.4. An example of a reaction involving the SET mechanism.

The SET mechanism involves the formation of an anionic species. The greater the ionisation energy of the antioxidant, the less likely it is to donate an electron. As a result, the ionisation potential can be a good indicator in predicting the ability of an antioxidant in scavenging free radicals via the SET mechanism. The ionisation energy decreases with increasing pH, meaning that such reactions are favoured in alkaline environments.

Mechanistic studies show that antioxidants often react via both HAT and SET mechanisms.[186] Migliavacca *et al.* assert that α-tocopherol undergoes HAT and SET reactions simultaneously.[187] The most prevalent mechanism will depend on the structures of the antioxidant and the species that it is reacting with, the solvent and other conditions, such as temperature, pH *etc.*[185] As in nearly all reactions, the solvent can have an influence on the mechanism. If antioxidant reactions are performed in hydrogen bond accepting environments, this can reduce the efficiency of the HAT reaction.[188]

Secondary antioxidants; metals such as iron and copper can accelerate the rate of oxidation in a system. Phenolic compounds have strong metal chelating properties.[185] They can chelate these metals and sequester them, preventing them from partaking in oxidation reactions. This is secondary antioxidative action, because the phenolics are indirectly inhibiting oxidation of the species.

1.9.3 Measuring Antioxidant Capacity

Many assays have been developed for the purpose of measuring antioxidant properties of chemicals and natural extracts. It is critical to select an assay that is suitable for the chemical that is to be studied. Of interest to this study is the radical scavenging capacity of phlorotannin to be extracted from seaweed. For this purpose, the DPPH (α,α-diphenyl-β-picrylhydrazyl) assay was selected. It is a well-established assay for measuring the radical scavenging capacity of compounds.[189] It is routinely used for phenolic compounds, including phenolics extracted from seaweeds.[77] Further details of the assay are given in Section 2.3.

1.10 Summary of Literature Review and Research Objectives

Seaweed has been used for thousands of years for use as both food and traditional medicine. There is now a rapidly expanding research field exploring the potential for seaweed extracts in a broad range of applications. This section discusses several key challenges in the field that will be addressed in this thesis.

Antioxidant Studies

The antioxidant and radical scavenging capacities of phlorotannin are well documented due to their potential applications for use as preservatives in foods and as skin actives in cosmetics.[74-76] Whilst much research has focused on demonstrating the application of phlorotannin extracts, little work has focused on the effect that extraction conditions have on the antioxidant capacity of the extract obtained. There are numerous reported methods for the extraction of polyphenols, together with evaluations of antioxidant capacity of these extracts.[100, 190, 191] However, the different methods used for the extractions, together with the varying assay methods means that it is not possible to make direct comparison with regards to evaluating the effect of extraction conditions on the yield and quality of the phlorotannin extracts. To date, no single study has been conducted that provides a comprehensive comparison of these procedures or any optimisation of the conditions. Section 3 details the work done to address this with the development of an extraction procedure for phlorotannin from seaweeds to achieve maximum antioxidant capacity by comparing commonly used solvents and work up procedures.

Molecular Weight Profiling

Conventional techniques for determining the molecular weight distribution of polymers have been found to be either unsuitable or of limited use for analysis of mixed weight polyphenolic extracts from seaweed. The development of such a method would allow for the effect of molecular weight to be assessed as an additional parameter when studying polyphenols. This would be of particular importance for applications where molecular weight is significant to the application; *e.g.* for use as a cosmetic ingredient, where molecular weight would influence permeation through the skin. Section 0 details the work performed towards the development of a method that gives the complete molecular weight distribution of a phlorotannin extract.

Seasonal Variation in Farmed Seaweed

The huge investment in seaweed farming technology will hopefully result in improved farming methods producing huge quantities of this valuable biomaterial, which in turn will provide a new supply of natural extracts. There are still many aspects of this that need to be addressed. Among these, a key consideration is how the new methods of farming seaweed will affect the chemical composition. The growing environment is known to influence the levels of many chemicals in seaweed and it is not yet clear how farmed seaweed will differ to naturally grown seaweed. Section 4 details work performed studying farmed seaweed, investigating how the levels of polyphenols vary throughout the year.

Analysis of polysaccharides

Sulphated polysaccharides, unique to brown algae have been found to possess many potential health benefits. The mixed structural nature of these polysaccharides greatly complicates the fractionation and analyses of these extracts. The analytical techniques currently used in the characterisation of these polysaccharides are often time consuming and give limited structural information. The development of additional analytical techniques in this field is needed in order to better understand the structure-activity relationships of FCSP in biological systems. The use of monoclonal antibodies can provide a fast, reliable method for the detection of polysaccharides, but as of yet, has been barely used in studying sulphated polysaccharides. Section 5 details the work performed to assess the use of monoclonal antibodies as an additional technique evaluating extraction procedures for sulphated polysaccharides.

Sequential Extraction from Seaweed

With a variety of compounds able to be extracted from seaweed it would be desirable to develop methods that allow for multiple compounds to be extracted from the seaweed to make full use of this valuable natural resource. Section 7 discusses different approaches to sequential extraction methods to target both polyphenols and polysaccharides. This section also discusses how phlorotannin extraction might be integrated into a biorefinery process aimed at biofuels and details a pilot scale extraction with superheated water.

Research Objectives

Five research objectives were set based on the challenges in the field discussed above.

1. To perform a comparative study of different extraction procedures to determine the optimal method for extraction of phlorotannin to achieve highest yield and antioxidant capacity.
2. To develop of an analytical method to measure the complete molecular weight distribution of a phlorotannin extract.
3. To perform a seasonal variation study on species of farmed seaweed to determine the seasonal variation in levels of phenolic content and the composition and antioxidant capacity of the extract obtained.
4. To evaluate the potential of monoclonal antibodies as an additional technique in evaluating extraction procedures for sulphated polysaccharides.
5. To evaluate potential options for the sequential extraction of multiple components from seaweed at a biorefinery.

2 Materials and Assay Methods

This section gives details of the materials and assay methods used in this work, including the assays used for the determination of phenolic content and for the measurement of the radical scavenging capacity of phlorotannin. Details of extraction procedures are described in the chapters and given in the Experimental (Section 9).

2.1 Seaweed Sourcing

2.1.1 Bod Ayre seaweed

As discussed in Section 1.2.1, this project collaborated with a seaweed farm, Böd Ayre Products Ltd (60.421146 N, 1.102152 W), located on Shetland, about 100 miles north-east of Scotland. The seaweed farm at Böd Ayre grew 10 different species of seaweed. They are listed in Table 2.1 and photos are given in Figure 2.1.

Table 2.1. Showing the species, class and phylum of 10 native Shetland seaweeds grown at the seaweed farm by Böd Ayre Products Ltd.

Genus	Species	Class	Phylum	Synonym
Alaria	*esculenta*	Phaeophyceae	Heterokontophyta	Dabberlocks
Ascophyllum	*nodosum*	Phaeophyceae	Heterokontophyta	Egg wrack
Fucus	*serratus*	Phaeophyceae	Heterokontophyta	Toothed wrack
Fucus	*spiralis*	Phaeophyceae	Heterokontophyta	Spiral wrack
Fucus	*vesiculosus*	Phaeophyceae	Heterokontophyta	Bladder wrack
Laminaria	*digitata*	Phaeophyceae	Heterokontophyta	Oarweed
Laminaria	*saccharina*	Phaeophyceae	Heterokontophyta	Sea belt
Pelvetia	*canaliculata*	Phaeophyceae	Heterokontophyta	Channelled wrack
Palmaria	*palmata*	Rhodymenio-phycidae	Rhodophyta	Dulse
Ulva	*lactuca*	Ulvophyceae	Chlorophyta	Sea lettuce

Ulva lactuca

Alaria esculenta

Laminaria saccharina

Ascophyllum nodosum

Laminaria digitata

Pelvetia canaliculata

Palmaria palmata

Fucus spiralis

Fucus serratus

Fucus vesiculosus

Figure 2.1. The species of seaweed grown at the seaweed farm.

2.1.2 Sourcing of a large batch of *Fucus vesiculosus*

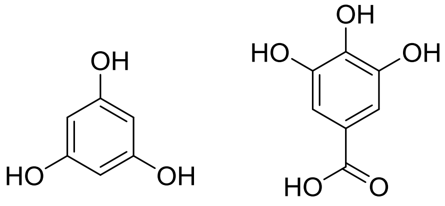
As discussed in Section 1.7.5, the composition of the seaweed varies between species, between populations, between individual plants and within the different parts of the plants themselves. For this study, this issue was addressed by obtaining a large batch of seaweed from the seaweed farm, which was then dried, powdered and mixed together to produce a single, homogenous batch. *Fucus vesiculosus* was selected as the study species because preliminary research performed by Thomas Farmer, a postdoctoral researcher in the group, found this to have the highest phenolic content of the species shown in Table 2.1. Several large batches of fresh *Fucus vesiculosus* were received from the seaweed farm during March 2013, which were dried down at 40 °C in an oven and then milled using a coffee blender and mixed thoroughly. Further experimental details are given in Section 9.1. This yielded 15 kg of dried blended seaweed. All extractions performed on dried seaweed were done using this batch. In this way, it has eliminated the issue of varying seaweed composition and has allowed for reliable comparisons to be drawn from the different extractions performed.

2.2 Assay for determining phenolic content (FC method)

The Folin-Ciocalteu method (FC method) is a colorimetric assay for the quantification of polyphenols. It was first developed by Folin and Dennis in 1912.[192] A revised method was later reported by Folin and Ciocalteu in 1927.[193] Since then various modifications have been reported, with the most commonly used method based on that by Glombitza *et al*.[98] It is the preferred method for the determination of total phenolic content, due to its simplicity, low cost, low detection limits, good reproducibility and relatively low interference.[98, 194]

The assay is carried out using a pre-prepared commercially available reagent, commonly referred to as FC reagent (Sigma 9252). The FC reagent is added to a solution of the phenolic compounds or extract being studied, followed by the addition of a base. The colour changes for this reaction are shown in Figure 2.3. The sensitivity of the assay requires the phenolic samples to be highly diluted such that they are usually almost completely colourless. The FC reagent is yellow in colour. It is added to the sample, turning it a pale yellow colour. The samples are then left to incubate, before addition of an alkaline solution, usually sodium carbonate. This turns the sample a dark blue colour.

The underlying chemistry of this reaction is not fully understood, however the blue chromophore produced is believed to be a phosphotungstic phosphomolybdic complex, possibly $[PMoW_{11}O_{40}]^{4-}$.[195, 196] The complex has an absorbance around 760 nm, proportional to the phenolic content of the sample. The phenolic content of the sample is then expressed in terms of the equivalent amount of a reference sample that gives the same response in the assay. This allows comparison to published data. Various reference samples can be used for this purpose. Usually a simple phenol is chosen that is similar in structure to the phenolics being studied, such as those shown in Figure 2.2. By far the most common standard reported in the literature is gallic acid. However, phloroglucinol is used in the analysis of seaweed extracts, as it is the monomer from which they are derived. A potential limitation of the assay is that interference can occur from the sample if it also absorbs in this region. However, the sensitivity of the assay means that samples are typically diluted by a factor of 1000 and so this is usually not an issue. In the case of phlorotannin, this effect is negligible as it does not absorb in this region.



Phloroglucinol Gallic acid

Figure 2.2. The structure of gallic acid and phloroglucinol used as standards in the FC assay.

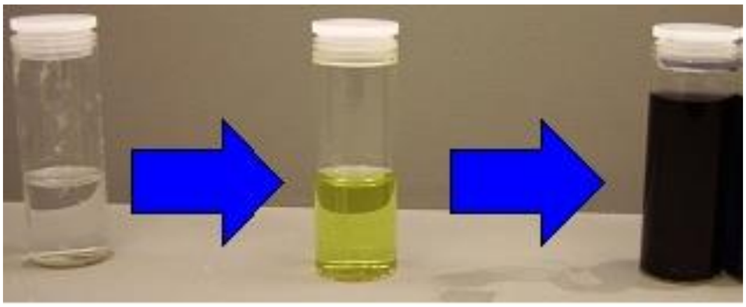


Figure 2.3. The colour changes observed during the Folin Ciocalteu assay, showing the almost colourless dilute phenolic sample (left), the sample following addition of FC reagent (centre) and then the dark blue colour formed upon the addition of a base (right).

The method used in this study is that reported by Glombitza *et al.*, but with slight modifications.[98] The method was scaled down so that samples could be prepared directly in cuvettes in order reduce cost and waste. A calibration was made using phloroglucinol as the standard. Samples were prepared as described in Section 9.2 of the experimental. All samples were analysed in triplicate. Figure 2.4 shows the calibration graph for phloroglucinol. There was a strong linear relationship between the concentration of phloroglucinol and assay response (the R^2 value was 0.9998).

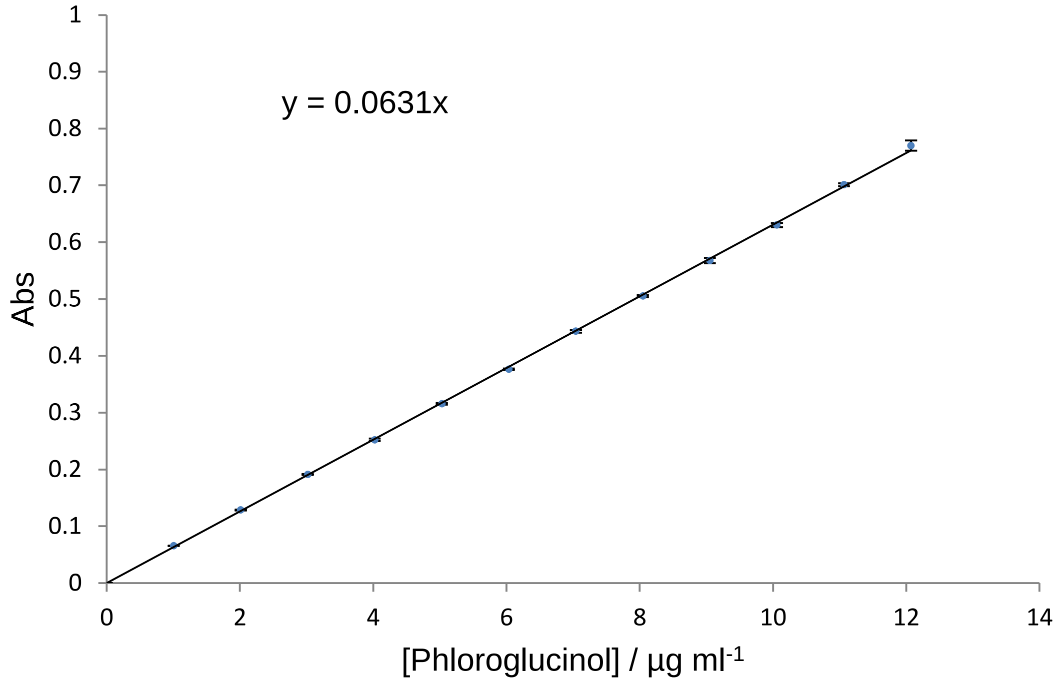


Figure 2.4. The standard curve for phloroglucinol in the FC assay. Points are the average of triplicate measurements.

When testing extracts for total phenolic content, the same method was used as for the calibration, but replacing the phloroglucinol for the extract. The extracts were diluted as necessary so as to give an assay response in the same range of the calibration graph. Total phenolic content was then expressed in terms of phloroglucinol equivalents (PGE).

2.3 DPPH assay of antioxidant capacity

2.3.1 Introduction to the DPPH assay

The DPPH assay is a well-established assay for measuring the radical scavenging capacity of compounds.[189] It is routinely used for phenolic compounds, including phenolics extracted from seaweeds.[77] The assay measures the radical scavenging capacity of the antioxidants towards the DPPH radical (Figure 2.5). DPPH is a stable free radical that has an absorbance around 515 nm in ethanol, giving a deep purple colour. When a solution a DPPH is mixed with a substance that can donate a hydrogen atom it is converted to its reduced form. The reaction can be followed by monitoring the concentration of the radical from the absorbance at 515 nm. The high stability of the DPPH radical comes from the steric bulk of the phenyl groups, which prevents two DPPH molecules from self-quenching. This means the DPPH radical is stable enough during storage, but still reactive towards antioxidant species in assays.

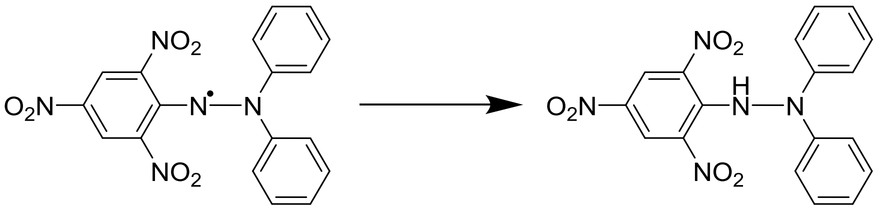


Figure 2.5. α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical (left), and its reduced form (right).

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Kinetics of the reaction

The rate of reaction varies widely between substrates. Small molecules often react faster and their reaction with Trolox is usually observed to go to completion in around 5 minutes.[197] Other molecules, including many polymers, react more slowly and can often take several hours to reach equilibrium. The best practice is to follow the reaction to completion, where possible. There are many reported studies that have used the DPPH radical test in which they measured the absorbance after just 30 minutes. If the reaction is slow, as is the case for many antioxidants then this will lead to an underestimation of the true antioxidant capacity. This was highlighted by Brand-Williams *et al*. in 1994 when studying several fruit extracts.[197] They found some extracts took up to 6 hours to reach the steady state and that their values for antioxidant capacity were higher than those previously reported by groups who measured after just 30 minutes.

Stoichiometry of the reaction

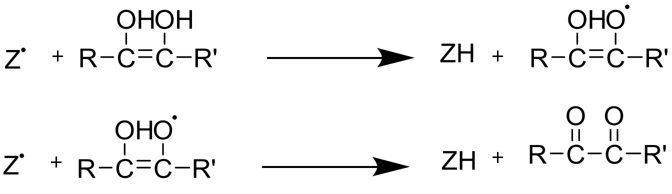
It is important to note that antioxidants can react with DPPH with different stoichiometric ratios. This is illustrated below in the difference between cysteine (1:1 ratio) and ascorbic acid (1:2 ratio). Cysteine reacts with one molecule of DPPH radical (Z•) (**Scheme 2.1**), producing a cysteine radical (RS•). The RS• species is then quenched by reacting with another molecule of RS•, leading to 1:1 stoichiometry.

$$Z^{\bullet} + RSH \longrightarrow ZH + RS^{\bullet}$$

$$2RS^{\bullet} \longrightarrow RS\text{-}SR$$

Scheme 2.1. The reaction of cysteine with DPPH.

However, molecules that have two adjacent sites for hydrogen donation (e.g. ascorbic acid) can react with two DPPH radicals and then quench intramolecularly, giving rise to a 1:2 stoichiometry (Scheme 2.2).



$$Z^{\bullet} + R-\overset{OH}{\overset{|}{C}}=\overset{OH}{\overset{|}{C}}-R' \longrightarrow ZH + R-\overset{OH}{\overset{|}{C}}=\overset{O^{\bullet}}{\overset{|}{C}}-R'$$

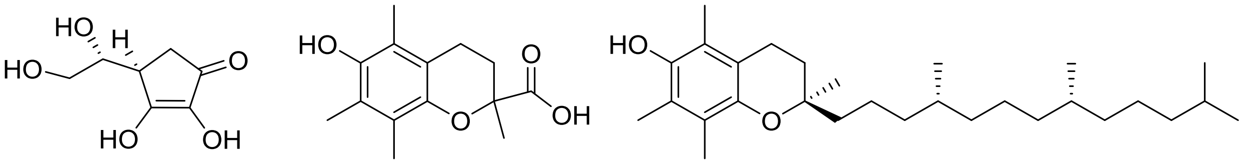
$$Z^{\bullet} + R-\overset{OH}{\overset{|}{C}}=\overset{O^{\bullet}}{\overset{|}{C}}-R' \longrightarrow ZH + R-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-R'$$

Scheme 2.2. The reaction of ascorbic acid with DPPH.

Expressing antioxidant capacity

There are several different ways to express the radical scavenging capacity of an antioxidant in its reaction with DPPH. Several commonly used methods are discussed below. The simplest method is just to calculate the percentage of

DPPH radical that has reacted. This is termed as the radical scavenging activity (%RSA). The %RSA is usually reported for a range of different concentrations of the antioxidant, measured after a fixed time. The antioxidant capacity of an antioxidant is often expressed as an EC_{50} value, which is the concentration of antioxidant needed to react with half of the radical. These values are then compared to a reference standard. This is the equivalent amount of the standard that gives the same radical scavenging as the extract. Commonly used standards include ascorbic acid, Trolox and α-tocopherol, shown in Figure 2.6.



Ascorbic acid Trolox α-tocopherol

Figure 2.6. The structures of some commonly used standards for the DPPH assay.

When working with single compounds, the concentration of the antioxidant is reported in moles. However, when working with extracts containing mixtures of compounds this is not possible, so capacity is reported per gram of material. The EC_{50} value is sometimes reported as its reciprocal, referred to as the antiradical power (ARP). This can be used to make the numbers easier to compare. Equation 1 gives the formula for calculating ARP.

$$ARP = 1/ EC_{50} \qquad \text{Equation 1}$$

Important Considerations

As with all assays, care must be given to consider factors that can cause interference. Although more stable than most radicals, DPPH can still react with strong light. For this reason the assay is performed in the dark. The pH is another factor that can influence the rate of reaction. For this reason it is necessary to ensure that extracts are of neutral pH by neutralising if necessary.

2.3.2 Choice of scale of the assay

The assay can be performed at different scales. One option is to prepare samples directly in cuvettes on a 2 ml scale. It can also be prepared on a scaled down version on 96-well microplates on a 200 µL scale and measured using a plate reader. In this work, it was decided that the assay would be carried out using microplates for several reasons. The advantage of using microplates over the cuvettes is that sample preparation time is much shorter. Less solvent is used and analysis is quicker because the entire plate can be measured in one go in less than 5 seconds. This quicker analysis time allows for greater precision over reaction time, ensuring more accurate results. Samples were measured on a Pelkin Elmer 2013 plate reader. A custom-made filter was purchased for the plate reader to allow absorbance to be measured at 515 nm.

A suitable concentration of DPPH was then determined by measuring the absorbance over a range of concentrations. The results are given in Figure 2.7. A concentration of 180 µM was selected as this gave an absorbance of just under 1. It can also be seen that there is a strong linear relationship between DPPH concentration and absorbance at 515 nm. This is necessary to ensure absorbance measurements can be used to quantify DPPH concentration.

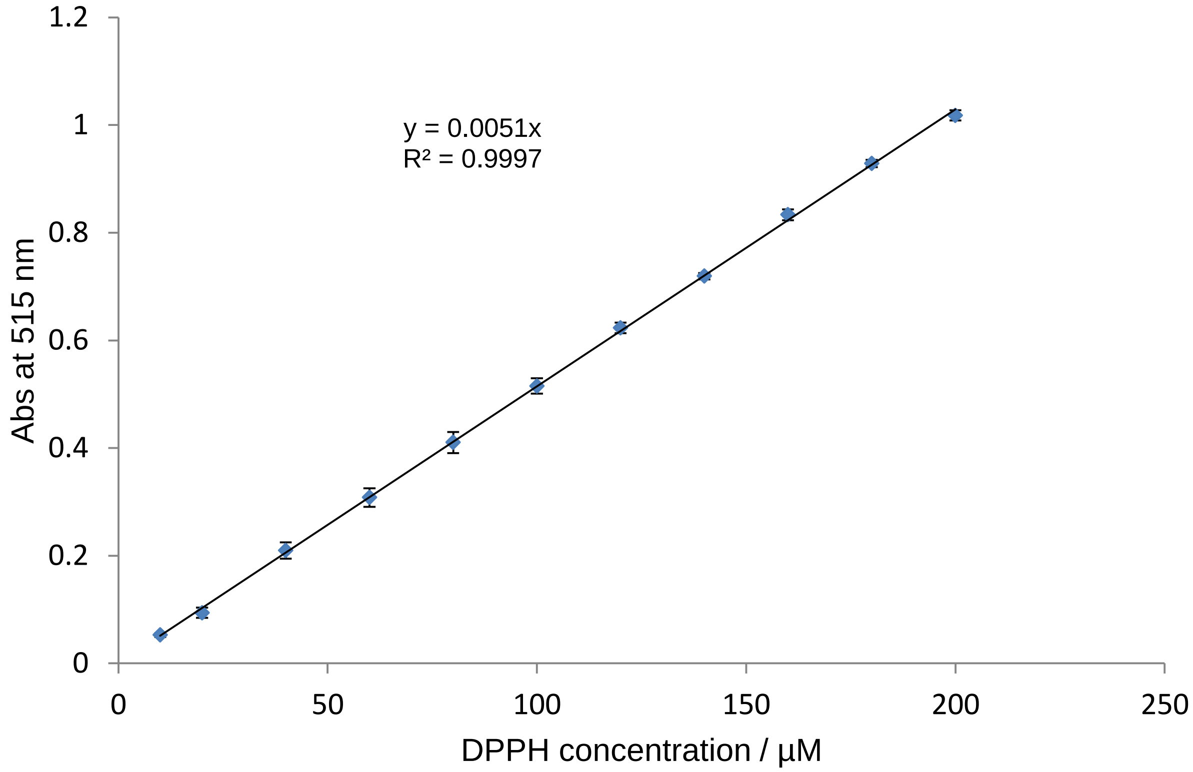


Figure 2.7. The range of concentrations tested in order to determine the suitable concentration of DPPH to be used in the assay.

2.3.3 Method of the Assay

The method was based on that reported by Sánchez-Moreno *et al*. with some modifications.[198] The method is briefly described below and further details are given in Section 9.3 of the Experimental. Figure 2.8 shows a diagram of a 96-well microplate. The outer wells of the plate were not used as it is known that they can be affected by interference. The remaining 60 wells were used to perform 20 experiments in triplicate.

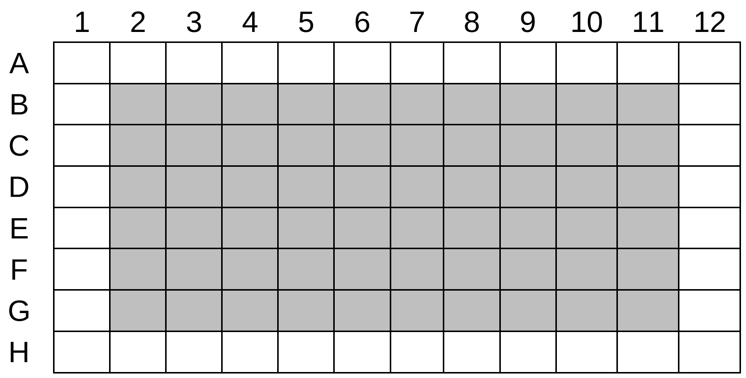


Figure 2.8. The 96 well microplate used for the DPPH assay. Only the cells highlighted in the shaded area were used.

The different combinations of tests required by the assay are given in Table 2.2. As well as the extract, a negative control containing just DPPH was required. The tests were performed with and without DPPH so that a blank could be subtracted. Trolox (Figure 2.6) was used as the positive control.

Table 2.2. The different tests performed as part of the DPPH assay.

	EtOH	Trolox	Extract	DPPH
Negative control	x			x
Positive control		x		x
Extract			x	x
Negative control (blank)	x			
Positive control (blank)		x		
Extract (blank)			x	

For the extract, the DPPH samples were prepared in the following order. Firstly, a solution of the extract in ethanol (200 µL at desired concentration) was added to the well. Secondly, a solution of DPPH (360 µM, 200 µL) was added to the wells by use of a multichannel pipette. The microplate was then agitated for 5 seconds using a Thermo Multidrop Combi to mix the samples and the absorbance measured immediately on the plate reader. The plates were left in the dark and then the absorbance was read again exactly 90 minutes after the addition of the DPPH. Further discussion on the choice of using 90 minutes for the measurement time is given in Section 2.3.4. Negative control experiments were performed by replacing the extract solution with ethanol. Blank samples were made, by replacing the DPPH with ethanol. The %RSA (as defined in Section 2.3.1) was then calculated using the formula in Equation 2:

$$\%RSA = \frac{\left[\left(A^{i}_{DPPH} - A^{i}_{DPPH\ blank}\right) - \left(A^{90}_{sample} - A^{90}_{sample\ blank}\right)\right]}{\left(A^{i}_{DPPH} - A^{i}_{DPPH\ blank}\right)} \times 100$$

Equation 2

where A^{i}_{DPPH} is the initial absorbance of the negative control, $A^{i}_{DPPH\ blank}$ is the initial absorbance of the negative control blank, A^{90}_{sample} is the absorbance of the sample after 90 minutes and $A^{90}_{sample\ blank}$ is the absorbance of the sample blank after 90 minutes.

The assay was calibrated using Trolox as the reference standard. The same method was used as above, but replacing the extract with a solution of Trolox. Figure 2.9 shows the standard curve for Trolox. When testing extracts in this work, every microplate included a test for Trolox at 50 µM so as to serve as a positive control.

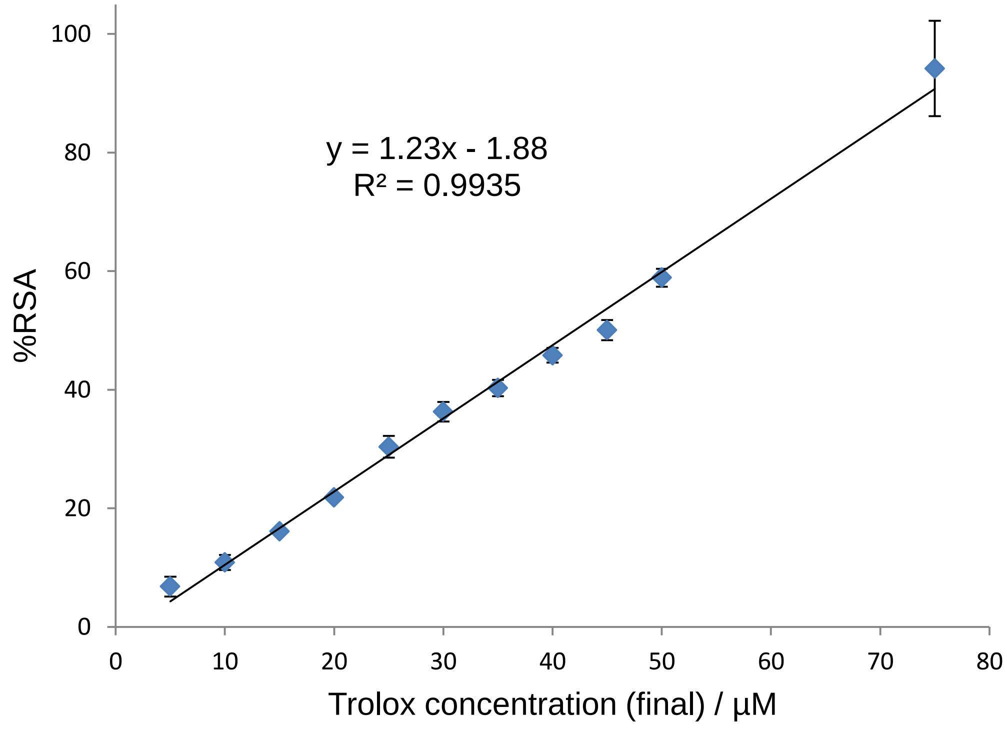


Figure 2.9. The calibration curve for Trolox.

2.3.4 Time of measurement

This section discusses why a measurement time of 90 minutes was chosen. As discussed above in Section 2.3.1, the rate of reaction of DPPH with different antioxidants can vary greatly. It is important to ensure that the reaction is allowed to reach equilibrium, as otherwise, if the sample is measured prematurely then a lower antioxidant capacity will be recorded, which underestimates the true radical scavenging capacity of the chemical or extract being tested. Samples are usually measured after 30 and 60 minutes and then again after 90 minutes to confirm that the reaction has reached equilibrium.

The assay was carried out with the intention of measuring periodically until the reaction reached equilibrium. It can be seen from the results in Figure 2.10 that even after 600 minutes the %RSA was still increasing relative to the negative control, indicating that the reaction was still progressing. The %RSA of the negative control increased slowly, indicating that the DPPH was not stable over these long time periods. As such, even if equilibrium was reached the drift in the negative control would have caused a large error in any measurements at this time.

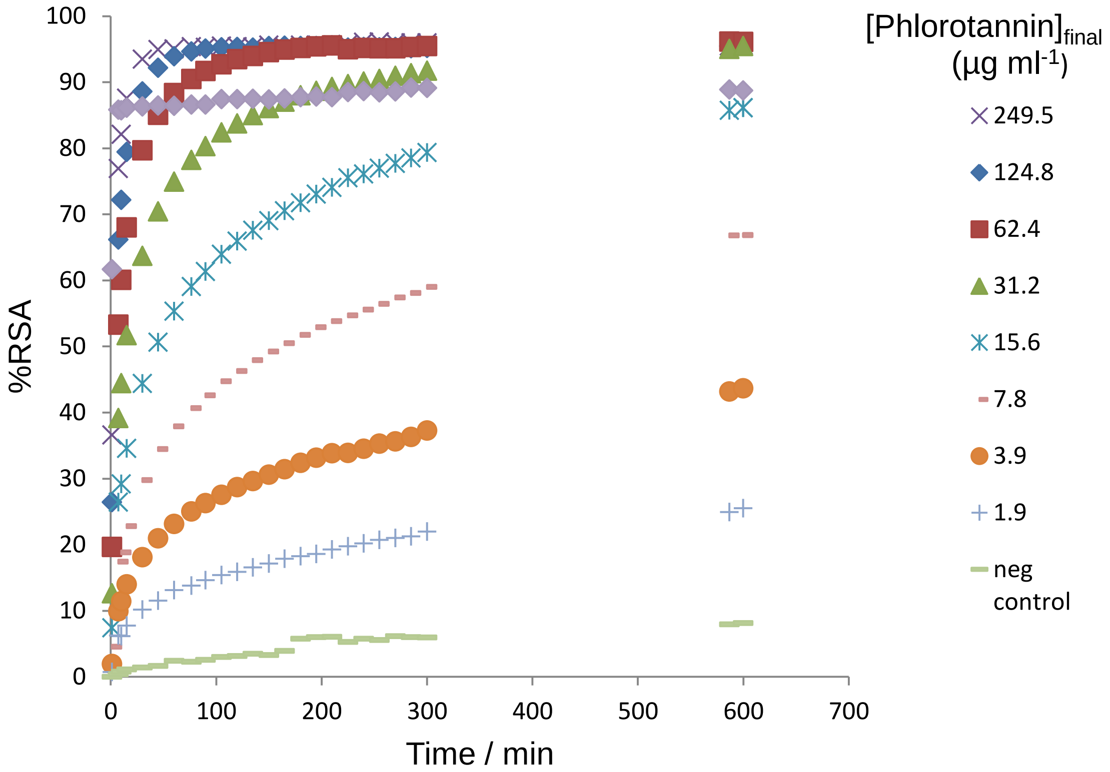


Figure 2.10. The analysis of phlorotannin extracts in the DPPH assay. Measurements were made every 15 minutes for the first 300 minutes and then again at 585 and 600 minutes.

In previous studies in the literature where the DPPH assay has been used on phlorotannin extracts, the absorbance was simply measured after a fixed time (usually, 30, 60 or 90 minutes).[97, 186, 187] No mention was given to the slow reaction times of phlorotannin or any justification as to why such a short reaction time was selected. This suggests that this issue was either not considered or ignored. The issue of reaching equilibrium is of importance when comparing extracts that differ greatly in their radical scavenging capacity. As a result, it means that all of the data reported in these studies will not be comparable where different measurement times have been used.

Given that equilibrium could not be reached, it was therefore necessary to measure after a set time. There were several factors that influenced the choice of measurement time. It can be seen from Figure 2.10 that the %RSA curve becomes flatter over time. The greater the measurement time, the flatter the curve and the smaller the error. However, given that the control was increasing steadily over time, a shorter measurement time was preferred.

Measurements at shorter times also minimise the potential for side reactions and degradation of the sample. A measurement time of 90 minutes was selected as a compromise.

This study was only concerned with measuring the relative differences in the %RSA of phlorotannin obtained via different extraction methods. Measurement at 90 minutes did not allow for the measurement of the complete RSA potential, but still served to allow comparison of phlorotannin extracts within this study and thus a comparison of the methods used to extract them. It is important to note that in adopting this approach an assumption was made that the extracts do not differ greatly in their kinetics.

The slower reaction of phlorotannin with DPPH radical compared to other antioxidants may be due to its larger polymeric structure. This may be advantageous for applications where a slower radical scavenging reaction is required.

2.3.5 Reproducibility tests

Three separate assays were performed on a phlorotannin extract to test the reproducibility of this method. Figure 2.11 shows the average of the three tests together with the standard deviation. From this it was concluded that there was excellent reproducibility in the assay.

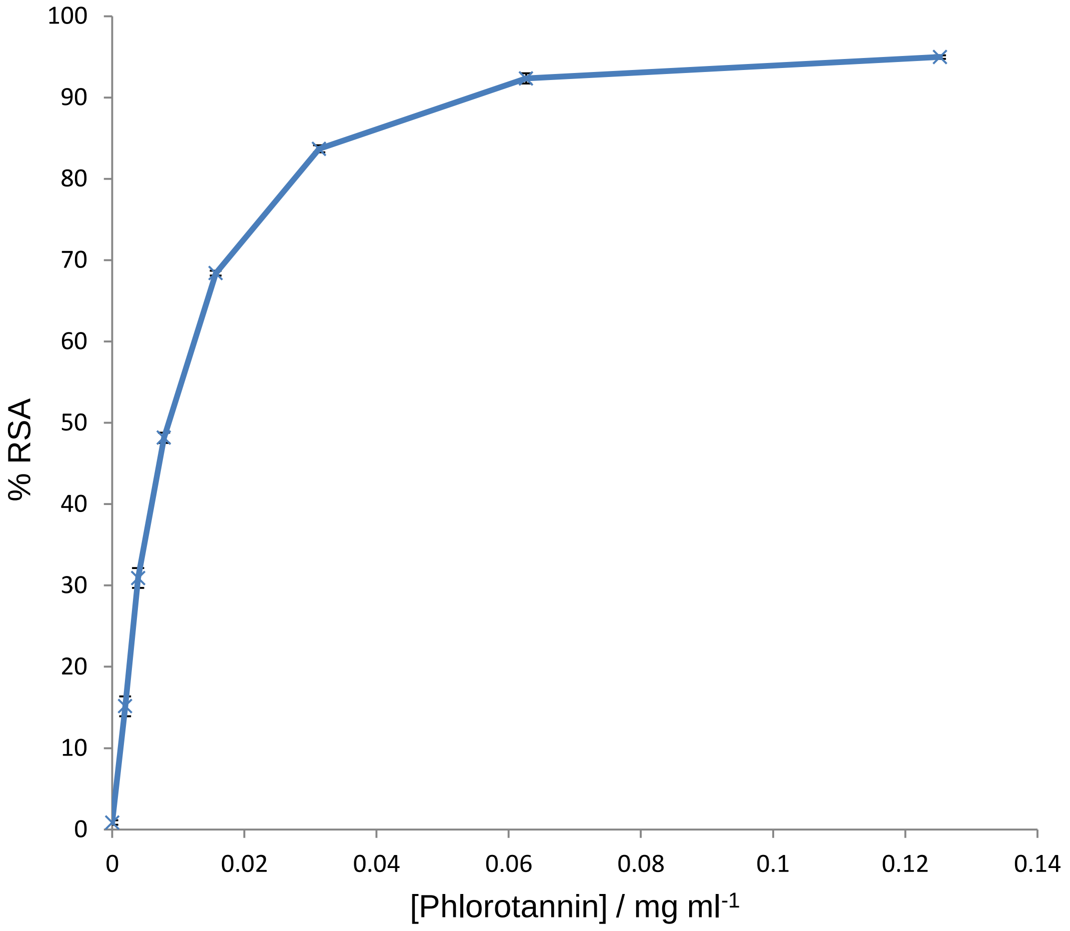


Figure 2.11. The test to confirm good reproducibility of the assay, showing the average of three separate measurements of a phlorotannin extract.

There are many reported studies in which DPPH assays have been used to measure the radical scavenging capacity of phlorotannin extracts.[100, 190, 191] The EC_{50} values of the phlorotannin extracts reported in these studies vary greatly between 10 – 100 µg ml^{-1}. Care must be taken when drawing comparisons between these studies as the DPPH assays were carried out using different methods, which will almost certainly give different results. The studies sourced their seaweed from different locations. As discussed previously in section 1.7.5, it has been shown that phlorotannin extracts vary between locations. As a result, it is not possible to make comparisons about how the different extraction methods affect the radical scavenging capacity of the phlorotannin extract obtained.

2.3.6 Use of robotic system for sample preparation

A robotic pipetting system was available in the department and potentially offered an attractive method for the preparation of the microplates (Figure 2.12). Using the robot instead of preparing the plates by hand allows for a faster preparation time. It also eliminates the chance of human error that could occur from hand pipetting.

Figure 2.12. The Hamilton Microlab Star robot for sample preparation.

When the assay was trialled with the robot, it was found to give very poor reproducibility. Closer inspection of the cause of the problem revealed that the robot was not pipetting accurately. With aqueous solutions the robot often achieves a high level of precision, usually higher than pipetting with hand held pipettes. Although technically capable of handling different solvents, this high accuracy cannot always be achieved. When using volatile solvents such as ethanol in this case, an increase in vapour pressure can occur inside the pipette. The robot uses pressure sensors inside the pipettes to detect these pressure changes and automatically adjusts the pipette filler to compensate, preventing the liquid being forced out of the pipette. The robot contains parameters for specific solvents to aid in this. Despite using the built in parameters for ethanol, the robot was not achieving accurate pipetting. Indeed, sometimes the pipettes were even observed to be dripping. Various combinations of parameters were trialled, but this did not resolve the issue. In an attempt to reduce the volatility of the solvent, a 50-50 ethanol-water mix was tried instead, however, the problem persisted. The use of lower concentrations of ethanol was precluded by the limited solubility of DPPH in water. As a result, the use of the robot was unfortunately not an option, meaning that the microplates needed to be prepared by hand pipetting.

3 Phlorotannin Extraction and Analysis

The antioxidant and radical scavenging capacities of phlorotannin are well documented due to their potential applications for use as preservatives in foods and as skin actives in cosmetics (Section 1.7).[51, 74-76] Whilst much research has focused on demonstrating the application of phlorotannin extracts, little work has focused on the effect that extraction conditions have on the antioxidant capacity of the extract obtained. There are numerous reported methods for the extraction of polyphenols, together with evaluations of antioxidant capacity of extracts.[51,58, 199-201, 93, 187188, 193-195,202.]

However, the different methods used for the extractions, together with the varying assay methods means that it is not possible to make a direct comparison to evaluate the effect of extraction conditions on the yield and quality of the phlorotannin extracts. To date, no single study has been conducted that provides a comprehensive comparison of these procedures or any optimisation of the conditions. This section details the work carried out to develop an extraction procedure for phlorotannin from seaweeds to achieve maximum antioxidant capacity by comparing commonly used solvents.

3.1.1 Comparison of solvents

A study was carried out to compare the use of several commonly used solvents reported in the literature in order to determine which was most suitable for the extraction of phlorotannin. By far the most commonly reported solvents in the literature for the extraction of phlorotannin from seaweed are ethanol, methanol, acetone and water as well as various combinations of organic-water mixtures.[51, 55, 203]

A key consideration of this work was to develop extraction methods that are suitable for the large scale extraction of phlorotannin from seaweed. Methanol was not included in this study as its use in the industrial processing of products destined for food and cosmetic applications is not favoured due to its high toxicity.

A study was devised to determine which solvents extracted the most phlorotannin from seaweed. Extractions were performed from dried *Fucus vesiculosus* using several different solvent systems and then the phenolic content of the extraction liquors was followed over time by taking samples from the extraction liquor and analysing them using the FC method, as described in Section 2.2. In order to ensure that the results were comparable, all extractions were performed on the same batch of dried seaweed (see Chapter 2 for further details).

3.1.2 Sampling Stability

The large number of aliquots that needed to be taken from the extraction process meant that it was more convenient to analyse them in batches. Because of the known instability of phlorotannin, it was first necessary to confirm that the aliquots could remain stable for a few hours while refrigerated.[94] During a preliminary extraction with ethanol-water (70-30 % V/V), the extraction liquor was sampled after 14 hours, taking three aliquots of the liquor. The aliquots were stored at 4 °C and analysed separately after 2, 8 and 26 hours to compare the response from the FC method. It can be seen from Table 3.1 that there was very little difference in the response from the FC method for each of the aliquots (<0.031 %), meaning that there is no decomposition occurring over time. Hence the aliquots could be refrigerated, and analysed after a few hours in batches to save time.

Table 3.1. The FC response of aliquots measured after varying periods of time stored in the fridge.

Time stored before measurement	Assay response (Absorbance 760 nm)				Standard deviation	% Change
(hr)	Replicates			Average		(%)
2	0.7805	0.7786	0.7843	0.7811	0.0029	0.000
8	0.7824	0.7756	0.7861	0.7814	0.0053	0.031
26	0.7838	0.7899	0.7694	0.7810	0.0105	-0.012

3.1.3 Solvent comparison for phenolic content

Extractions were then performed on dried *Fucus vesiculosus* using five different solvent systems (water, ethanol, 70-30 % ethanol-water, acetone and 70-30 % acetone water). The extraction liquor was sampled at various times over 48 hours, from which the phenolic content of the extraction liquor was determined using the FC assay. The results are given in Figure 3.1.

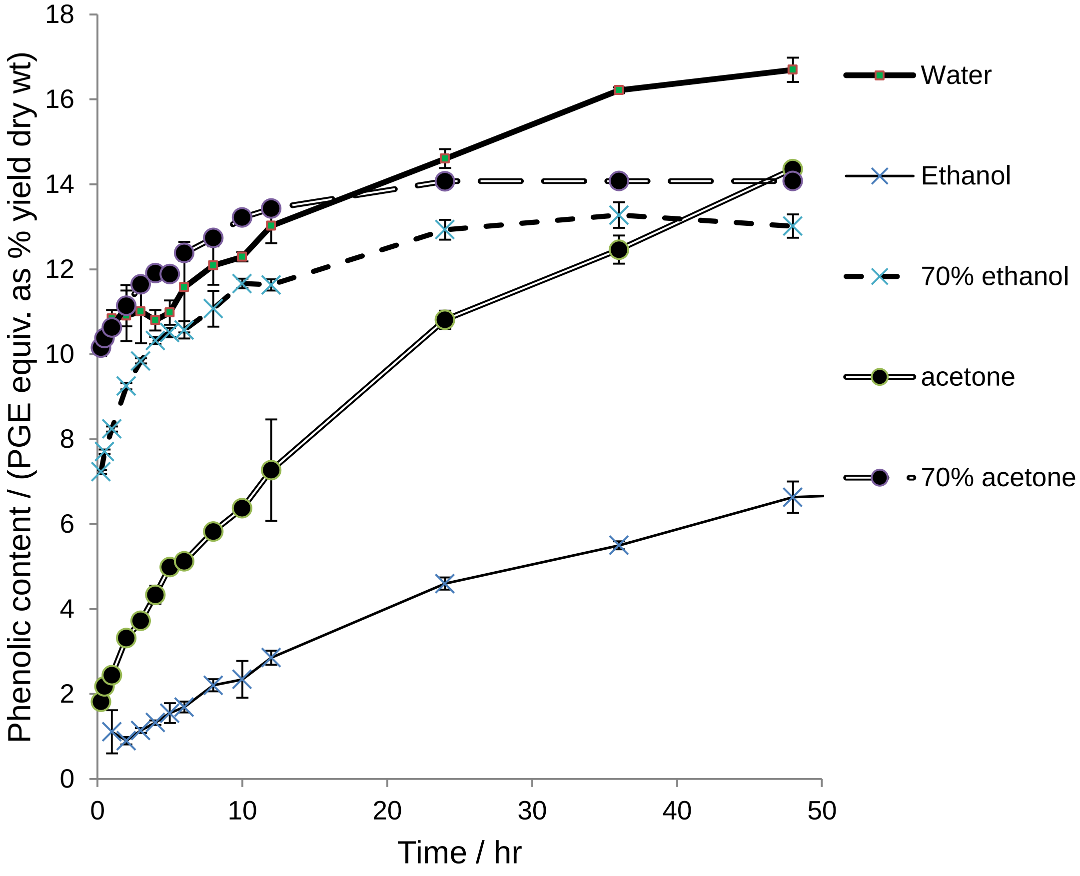


Figure 3.1. The total phenolic content of the extraction liquors for each solvent as measured over time by the FC method. Extractions were at 22 °C.

It can be seen from Figure 3.1 that phlorotannin was extracted by all of the solvent systems and that there were differences in the amount of phlorotannin that was extracted. The aqueous solvent systems caused a significantly larger initial release of phlorotannin compared to ethanol and acetone. A possible explanation for this might be because the cells hydrated in the presence of water and burst.[204] This would not have happened with ethanol and acetone as they did not contain any water.

After 48 hours the phenolic content of the extraction liquors was in the order: water > acetone ≈ 70 % acetone > 70 % ethanol > ethanol. The rate of increase in acetone was faster than in ethanol, meaning it was better at extracting phlorotannin. In each case, the water-organic mixtures gave higher yields than just the respective organic solvent.

It has been reported in the literature that the yield of phlorotannin from extraction generally increases with solvent polarity and it has been proposed that this is because polar solvents are better able to disrupt the hydrogen bonding between the phlorotannin and the cell wall.[92, 93] An attempt was therefore made to rationalise the results based on the polarity of the solvents. Solvent polarity data was obtained from the Reichardt and Weltonsolvent polarity table.[205] The values are given in Table 3.2. No data could be found for the ethanol-water and acetone-water mixtures; however, it is reasonable to assume that they will have higher polarities than just ethanol and acetone respectively.

Table 3.2. The solvent polarities as reported by Reichardt and Welton.[205]

Solvent	**Relative polarity[205]**
Water	1.00
Ethanol	0.654
Acetone	0.355

The differences in the polarity of the solvents partly match the order of the yields. Water is the most polar solvent and gave the highest yield. The aqueous acetone and ethanol extractions both gave higher yields than just acetone and ethanol respectively. However, the trend does not always follow; the yields for the acetone extractions were higher than their respective ethanol extractions, despite having a lower polarity. This suggests that extraction yield is only partly governed by solvent polarity and that there are other influencing factors.

Acetone may be better at solvating phlorotannin than ethanol due to the hydrogen bonding between its carbonyl groups and the phenolic groups on phlorotannin. Indeed, solubility tests conducted later on isolated phlorotannin extracts found them to be more readily soluble in acetone compared to ethanol, which would support this theory.

3.1.4 Analysis of the liquor

The liquors were reduced to dryness and analysed by ^{1}H NMR spectroscopy to determine what compounds were present in order to work out how the liquors could be purified to isolate phlorotannin. Several ^{1}H NMR solvents were used, as the dried liquor samples would not completely redissolve in any one solvent and required filtration before analysis. Analysis of the extracts by ^{1}H NMR is shown in Figure 3.2 for DMSO, Figure 3.3 for CD_3OD, Figure 3.4 for D_2O and Figure 3.5 for $CDCl_3$.

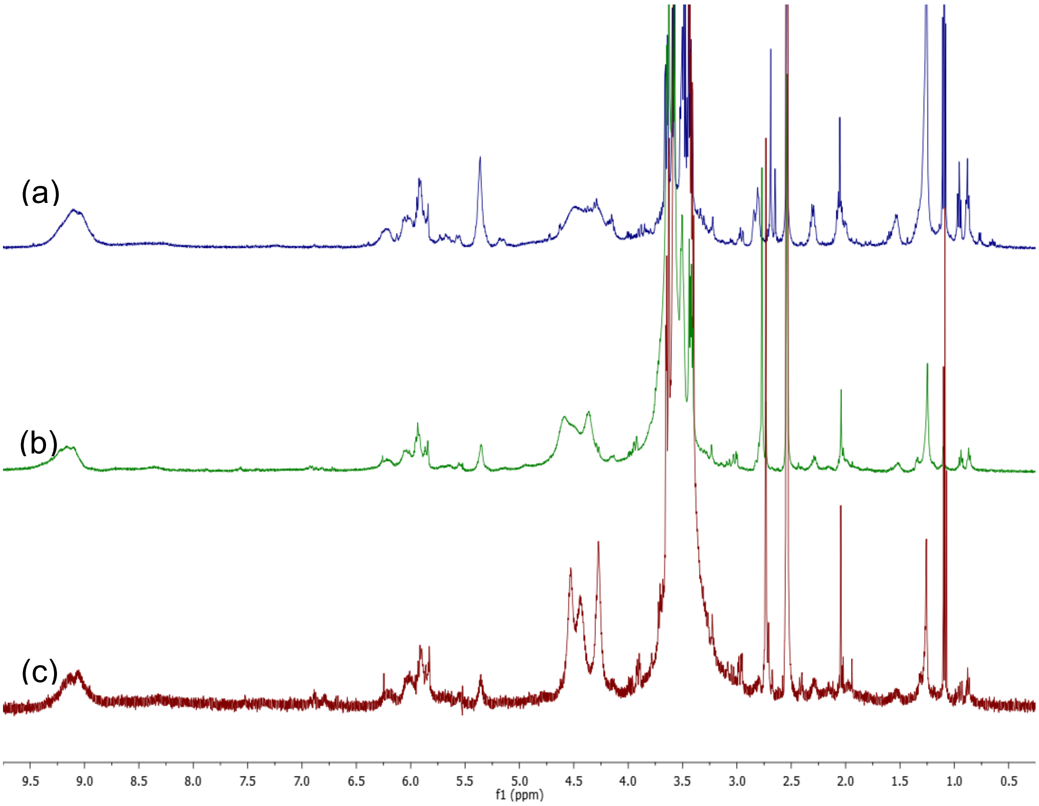


Figure 3.2. ^{1}H NMR spectra in DMSO of the reduced liquors, as extracted by (a) ethanol, (b) 70% ethanol, (c) water.

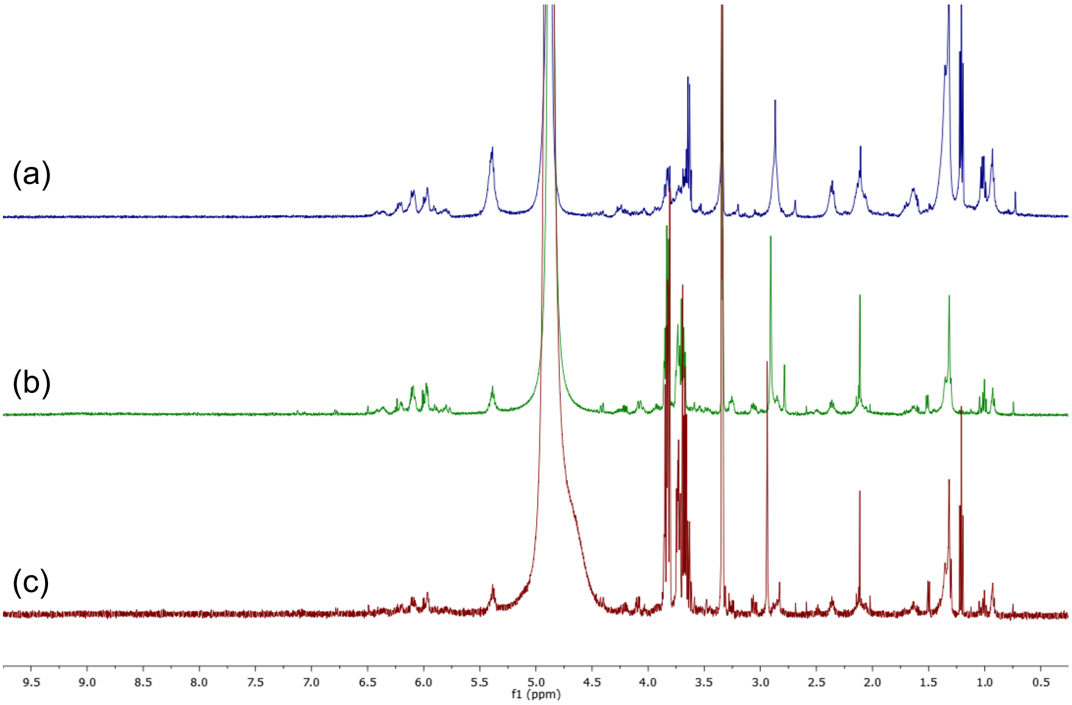


Figure 3.3. 1H NMR spectra in CD3OD of the reduced liquors, extracted by (a) ethanol, (b) 70% ethanol, (c) water.

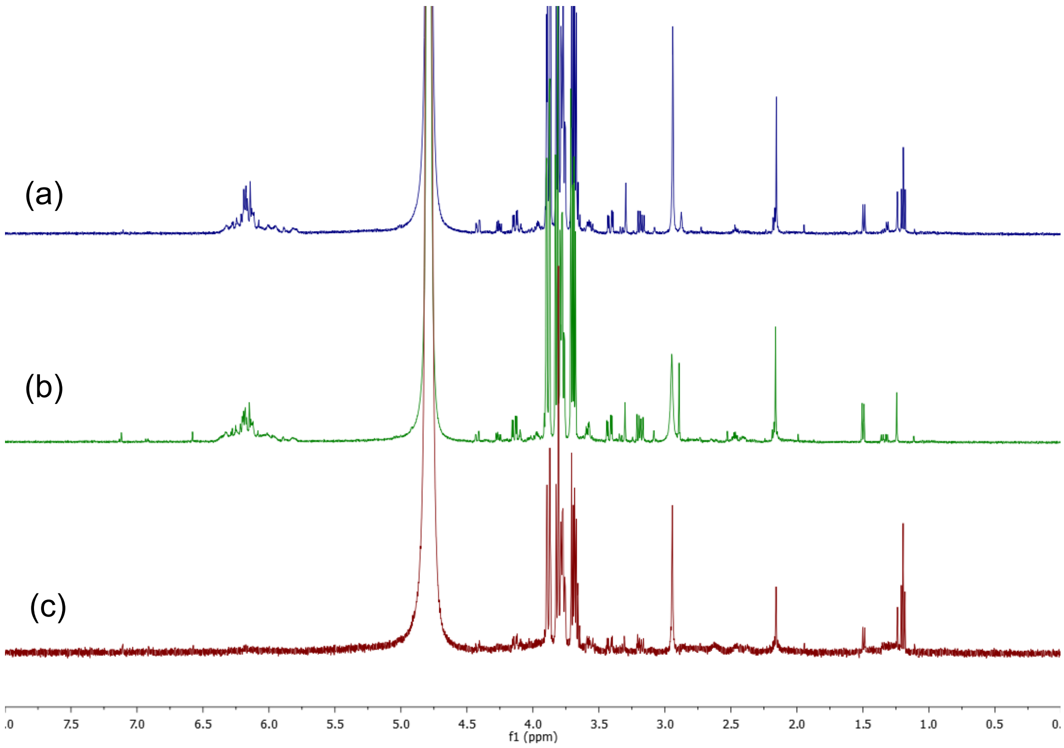


Figure 3.4. 1H NMR spectra in D2O of the reduced liquors, extracted by (a) ethanol, (b) 70% ethanol, (c) water.

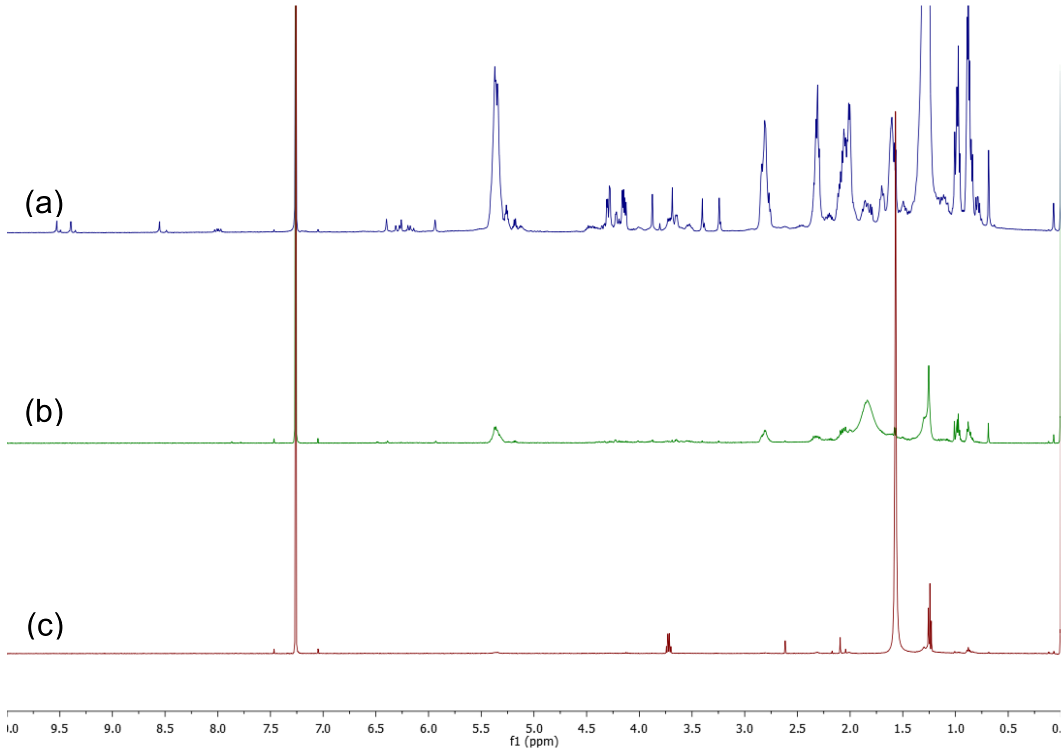


Figure 3.5. 1H NMR data in CDCl3 of the reduced liquors, extracted by (a) ethanol, (b) 70% ethanol, (c) water.

The ^{1}H NMR analysis of the reduced liquors contained a range of peaks, indicating that the solvents extracted a broad range of compounds. An assignment of these peaks is made below.

It can be seen from the ^{1}H NMR spectra in Figure 3.2 and Figure 3.3 that the extracts of all three solvents contained aromatic material (6.45 – 5.75 ppm).[206] No phlorotannin could be seen in the D_2O NMR spectra. This may be due to deuterium exchange occurring between D_2O and the phenol rings via keto-enol tautomerism. No phlorotannin could be seen in the $CDCl_3$ NMR spectra, although later solubility tests carried out on isolated phlorotannin found that it was not soluble in chloroform. Although these shifts are quite low compared to most aromatic molecules, they are typical of where the aromatic peaks on phlorotannin would be expected.[195, 200] The shifts are lower because the rings are very electron rich, caused by inductive effects from the surrounding oxygen atoms. This is strongly indicative that phlorotannin has been extracted, although further confirmation could not be made until the extract was isolated and analysed.

The wide variety of compounds in the extracts meant that they did not completely dissolve in any one NMR solvent. Additionally, pH could also have potentially had an effect on solubility. Because the extracts did not fully re-dissolve in the NMR solvents, it was not possible to make a direct reliable comparison between the integrations of the peaks in order to quantify the amount of material extracted. The phenolic content measurements in Figure 3.1 would suggest that water extracted the most polyphenols, however, the ^{1}H NMR analysis would appear to show that water extracted less aromatic material than ethanol or 70-30 % ethanol-water. There are several possible reasons why this might be. Firstly, the crude extract obtained from the water extraction did not fully re-dissolve in the NMR solvents (even in D_2O, despite being water soluble before). It may be that phlorotannin was trapped within alginate networks and not able to redissolve. The water liquor had to be heated to 50 °C for a prolonged period of time in order to reduce it to dryness, which may have degraded the phlorotannin or caused it to react with the other species present. This may offer a possible explanation as to why the ^{1}H NMRs showed little or no peaks in the aromatic region. It can also be seen that the

D_2O spectra generally has sharper peaks, which suggests that it contains lower molecular weight species.

The ^1H NMR spectra of the ethanol and 70-30 % EtOH-H_2O extraction liquors contained many more peaks compared to the water extraction, indicating that these solvents extracted more compounds from the seaweed. This difference is important when considering which solvent is most suitable for the extraction of phlorotannin as it is preferable to extract as few other species as possible in order to simplify the isolation from the phlorotannin. An assignment of these peaks is made below.

The ^1H NMR in D_2O of all three liquors contained a mass of peaks between 3.82-3.56, which is characteristic of mono- and polysaccharides. As discussed in the Introduction (Table 1.1), it is known from the literature that brown algae contains the monosaccharide, mannitol, together with the polysaccharides, alginate, FCSP and laminarin and so it is likely that these are present in the liquors.[207] A section of the ^1H NMR from Figure 3.4c is shown in Figure 3.7. Peaks corresponding to the monomeric sugar mannitol (Figure 3.6) could be seen identified at shifts of 3.85 (2H, dd, J = 11.7, 2.8 H_a), 3.78 (2H, d, J = 8.7 H_d), 3.74 (2H, ddd, J = 8.6, 6.0, 2.7 H_c), 3.66 (2H, dd, J = 11.5, 5.8 H_b).[208]

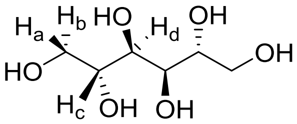


Figure 3.6. The structure of mannitol, found in brown algae.

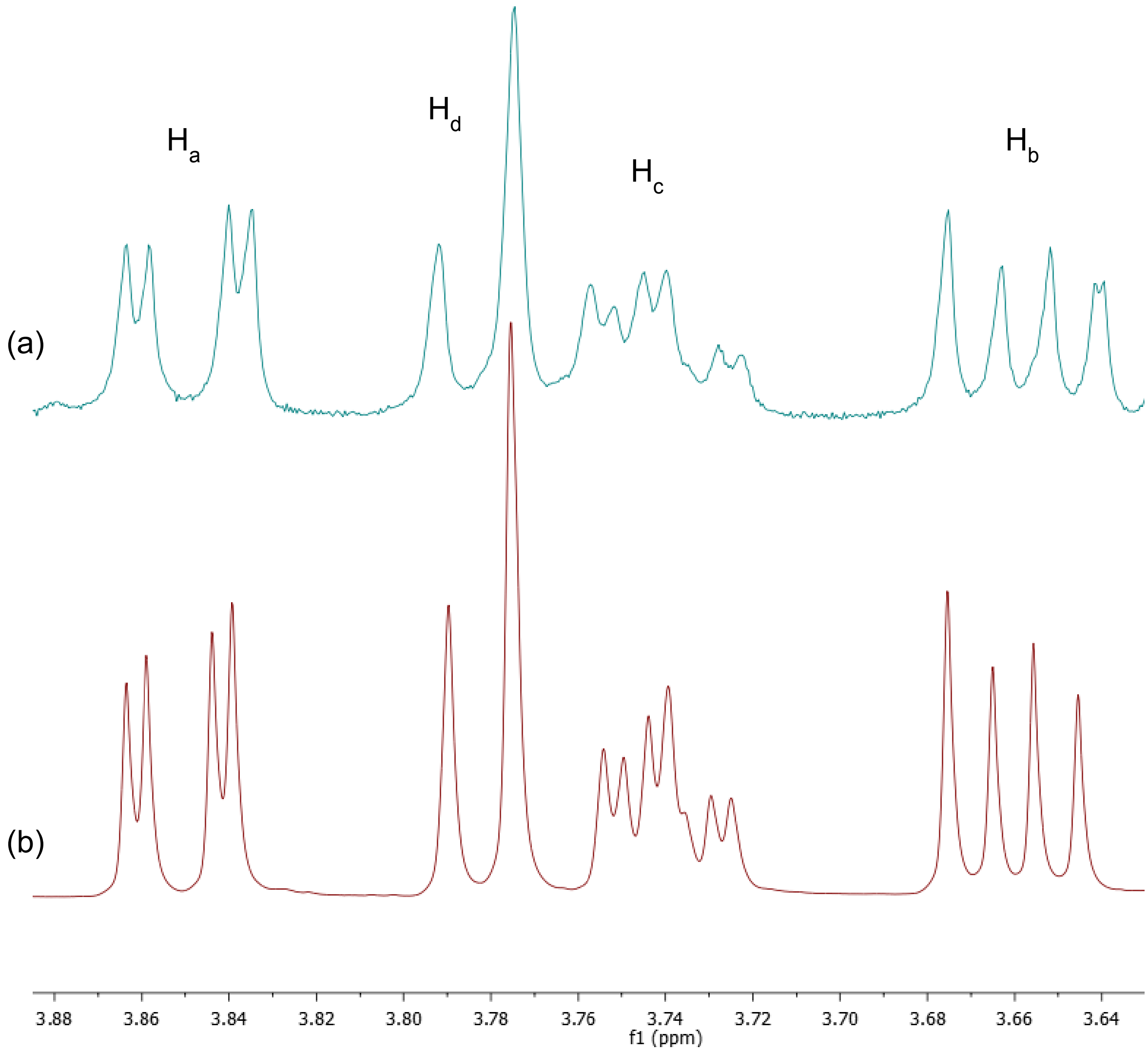


Figure 3.7. (a) The portion of the ^{1}H NMR spectra in D_2O in Figure 3.4c, showing the spectra of the sugar mannitol as extracted by water and (b) the literature spectra of this sugar.[208]

The ^{1}H NMR spectra in $CDCl_3$ indicated that ethanol extracted lipids from the seaweed (Figure 3.5a). Peaks could be seen in the spectra that could be assigned to lipids, known to be found in seaweed (see section 1.6); (5.43-5.29 (C-$\underline{H}$C=C), 2.85-2.76 (bisallylic), 2.36-3.28, 2.12-1.98 (allylic), 2.74-1.59 (C_3), 1.4 – 1.2 ($C\underline{H}_2$) 1.01 – 0.84 ppm ($-CH_2-CH_2-C\underline{H}_3$). The analysis of lipids is discussed further in Section 6. These peaks were not observed in the ethanol-water or water extracts, which is expected given the high polarity of water.

In all of the NMR solvents, the ethanol liquor contained many more peaks than the 70-30 % ethanol-water and water liquors. In addition to the aromatic region, the sugar region and the peaks corresponding to fatty acids, there were a number of other peaks that do not correspond to these types of compounds. An attempt was then made to match these peaks to NMR data from the literature of the other known compounds present in brown algae such as carotenoids, pigments and accessory pigments (see section 0).[16, 20, 21-27]

The $CDCl_3$ spectra of the ethanol liquor (Figure 3.5a) contained peaks at 6.40, 6.31, 6.28, 6.26, 6.20, 6.17, 6.14, 5.94 ppm, which are typical of the unsaturated alkene chains of the carotene compounds (Figure 1.7), however they did not match either of the two carotenoids known to be present in brown seaweed, fucoxanthin and β-carotene.[209-211]

As discussed in the Introduction in Table 1.1 brown seaweed is known to contain three types of chlorophyll, Chlorophyll *a*, Chlorophyll c_1 and Chlorophyll c_2. The structures are given in Figure 1.4. Several peaks corresponding to Chlorophyll *a* could be assigned from literature data (Figure 3.8).[212] The peaks that could be assigned were at 9.53 (1H, s H-β), 9.39 (1H, s, H-α), 8.55 (1H, s, H-δ), 7.99 (1H, dd, J=17.9, 11.2, vinyl $C\underline{H}$ at C_2), 3.71 (m, 4-CH_2), 3.88 (3H, s, 10-CO_2-Me), 3.69 (3H, s, 5-Me), 3.40 (3H, s, 1-Me), 3.24 (3H, s, 3-Me). The remaining peaks could not be assigned as they overlapped with peaks from fatty acids.

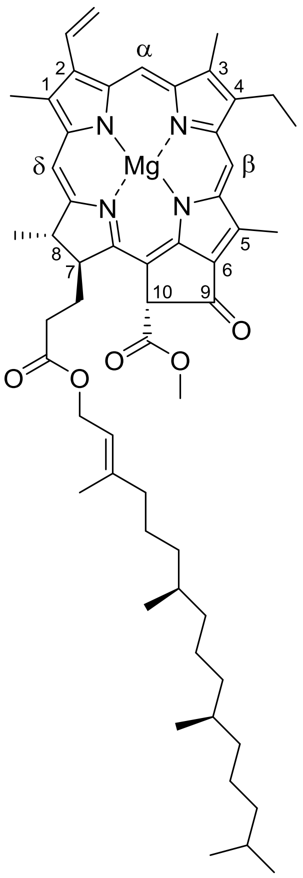


Figure 3.8.The structure of Chlorophyll *a*.

3.1.5 Purification Strategies

This section discusses strategies for the purification and isolation of phlorotannin from the extraction liquors of ethanol, 70 % ethanol and water. ^{1}H NMR analysis of the liquors found that they contained a range of compounds including phlorotannin, sugars and pigments and lipids. Purification processes for the isolation of phlorotannin from each of the extraction liquors from these species were then developed based on general experience within the research group and from reference texts on the subject.[213, 214]

A key aim of this research was to develop processing methods that were suitable for large scale processing of the seaweed. As such it was desirable to use only sustainable solvents and to minimise their use as much as possible.

A common initial purification step used after performing an alcohol-water extraction on plants is to remove the alcohol under reduced pressure, which causes the precipitation of non-water-soluble species as the alcohol evaporates.[214] In the case of these liquors, this approach represented a convenient way of removing the pigments and lipids, leaving phlorotannin in the aqueous layer together with the sugars as the only major impurity present.

Further consideration was then given to how the phlorotannin might be separated from the sugars present. An effective technique used within the research group for the separation of phenolic compounds from sugars is the use of affinity resins such as the Amberlite range (DOW Chemicals). One resin in particular, Amberlite XAD 7 HP, was found to be particularly effective for this purpose. Amberlite XAD 7 HP is an industrial grade polymeric adsorbent, designed for the removal, immobilization and recovery of peptides, proteins and low molecular compounds from aqueous solutions. It is an aliphatic cross-linked acrylamide structure, as shown in Figure 3.9.

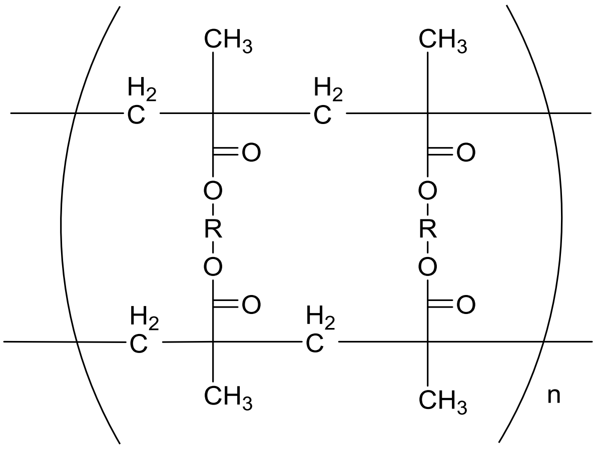
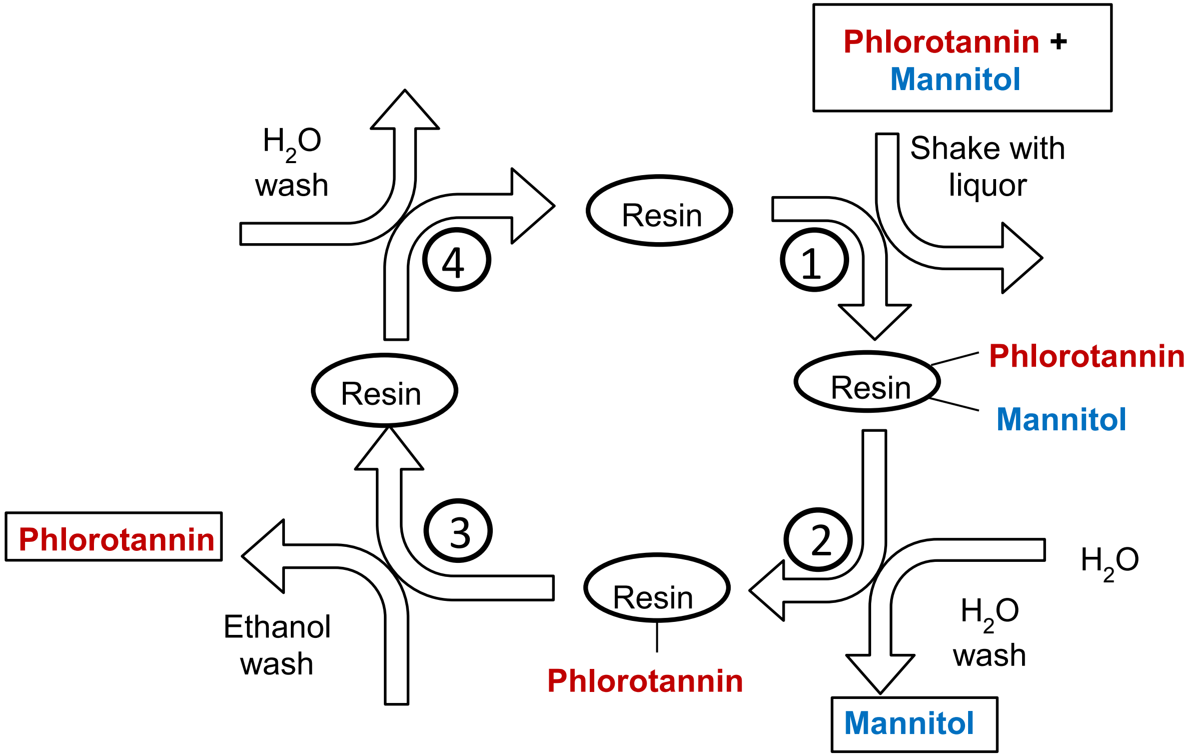


Figure 3.9. The structure of the Amberlite XAD-7HP supplied by Acros.

The general procedure for the solid phase extraction process is given in Scheme 3.1. The resin has a strong affinity for phlorotannin molecules, which form hydrogen bonding interactions with the carbonyl groups on the resin. When the resin is shaken with the extraction liquor, phlorotannin selectively adsorbs to the resin, allowing separation from the sugars (step1). The resin is then washed with water to remove any traces of sugars (step 2). Finally, the phenolic compounds are recovered from the resin by eluting with ethanol (step 3). The resin is then washed with water to remove ethanol (step 4) so that it can be reused. Further details are given in Section 9.6 of the experimental.



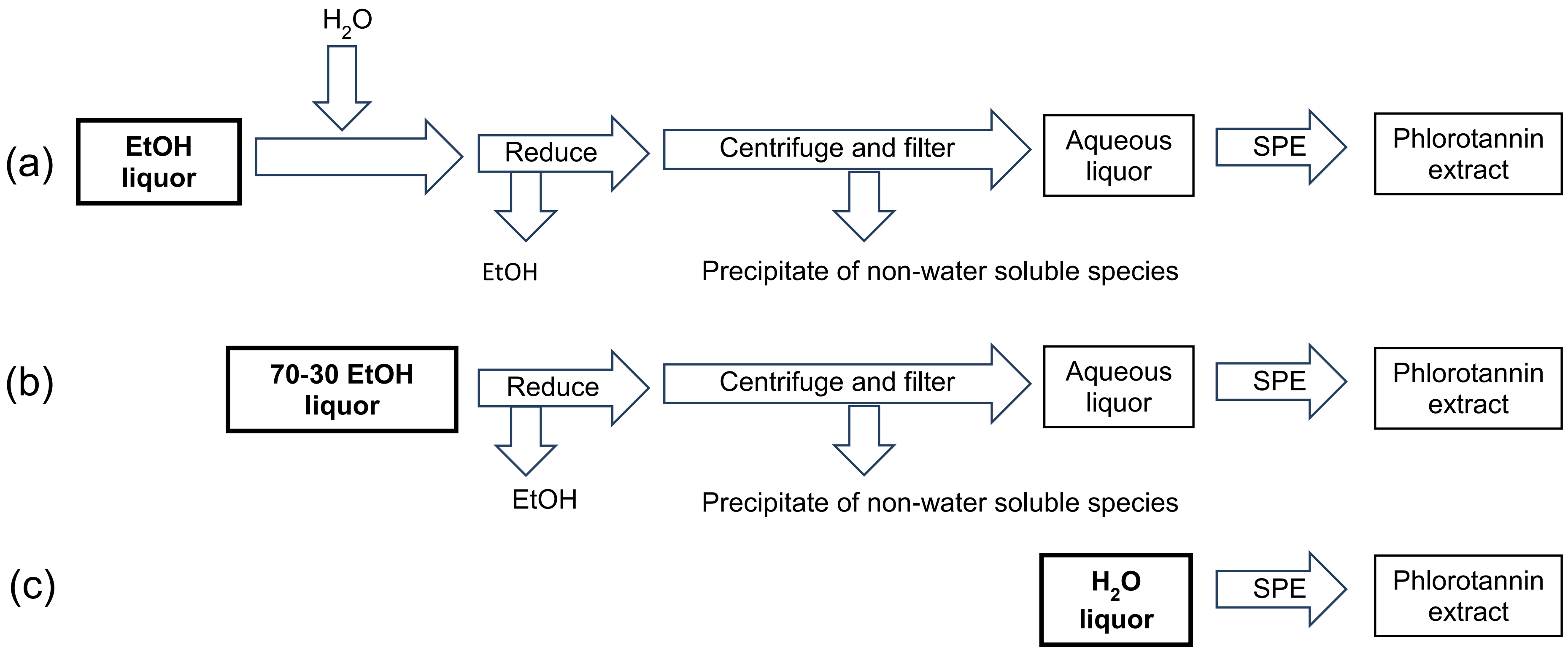
Scheme 3.1. The solid phase extraction (SPE) procedure for the purification of phenolic compounds from sugars using the Amberlite XAD 7 HP resin. Step 1: the resin is shaken with the liquor and filtered. Step 2: the resin is eluted with water to remove sugars. Step 2 the resin is eluted with ethanol to recover phlorotannin. Step 3: the resin is eluted with water to remove ethanol before it can be used again.

3.1.6 Isolation of phlorotannin

Further extractions were then performed with the aim of isolating phlorotannin by applying the purification methods described above. Extractions were performed on dried *Fucus vesiculosus* for both 1 and 24 hours, in order to determine if there was a difference in yield and to determine if there was a difference in the radical scavenging capacity of the phlorotannin extracts as measured by the DPPH assay.

The strategy described earlier in which liquors are reduced to remove alcohol in order to precipitate non water soluble species was then applied to the extractions with some slight modifications as shown in Scheme 3.2. In the case of the ethanol extraction, because the extraction system did not contain water, it was necessary to modify this procedure slightly by adding water to the liquor after the extraction so that it could then be reduced to remove the ethanol and leave the phlorotannin in the aqueous phase. In the case of the aqueous extraction system, no evaporation is necessary as there was no ethanol to remove. No modification was required for the 70-30 % ethanol-water.

Whilst performing the extraction and work up procedures, it was noted that the water liquor had a higher viscosity than both the ethanol or ethanol-water liquors. This is probably due to the polysaccharides present in the seaweed, such as alginate, that can form gel networks. As discussed in Section 1.8.3 these thickening properties of alginate are utilised for medical and industrial purposes. [114, 137] The higher viscosity of the water liquor meant that the filtration steps were considerably slower compared to the other liquors. This was still manageable at this small scale, but may cause complications if performed on a larger scale.



Scheme 3.2. The three different purification methods used to isolate phlorotannin after extraction from (a) ethanol, (b) 70-30 ethanol-water and (c) water.

The yields of the extracts obtained from the SPE process are given in Figure 3.10, calculated as weight % yield from the dried seaweed. To allow convenient comparison of the phenolic content measured in the previous study, selected data was taken from Figure 3.1 for the phenolic content of the liquor after 1 and 24 hours and is given in Figure 3.11.

It can be seen from Figure 3.10 that the yields obtained from SPE are about half of what would have been expected based on the phenolic content measured previously using the FC assay. This suggests that not all of the phlorotannin was recovered during the purification process. There are several possible stages where the phlorotannin could have been lost. Firstly, it is possible that phlorotannin was lost during the precipitation stages, where it may have precipitated out with the pigments and oils. It was also possible that not all of the phlorotannin was recovered from the resin during the SPE stage, which would have reduced the yields.

The isolated fractions were then analysed by ^{1}H NMR to determine how successful the SPE purification process was at isolating phlorotannin from its impurities. CD_3OD was used as the ^{1}H NMR solvent for two reasons; firstly, CD_3OD also dissolves many of the other species present, allowing confirmation of purity. Secondly, as discussed in the introduction, CD_3OD gives better resolution of the phlorotannin peaks, which allows comparison of the aromatic region.

The spectra are given in Figure 3.12. Despite drying under high vacuum for 24 hours, there was still some water present in the extracts. This was also observed by others in the research group working with polymeric extracts, and was probably caused by the strong hydrogen bonding properties of the phlorotannin which trapped solvent as it was reduced.[215] Comparison of these spectra to the spectra of the liquors before purification, confirms that the SPE process is highly efficient at isolating the phlorotannin from the other species present in the liquor, characterised by a large amount of aromatic material present (6.4 – 5.8 ppm) and very little else. The only exception was the 1 hour ethanol extract, which contained other peaks. The extract was green in colour, suggesting that it contained pigments. Disregarding this result, the typical purity of the other phlorotannin extracts was estimated from integration from the NMR spectra to be > 95%. The precipitate formed after reduction of the liquor was also analysed, which revealed it consisted mostly of lipids. It was a dark green in colour, suggesting it also contained traces of other pigments such as chlorophyll.

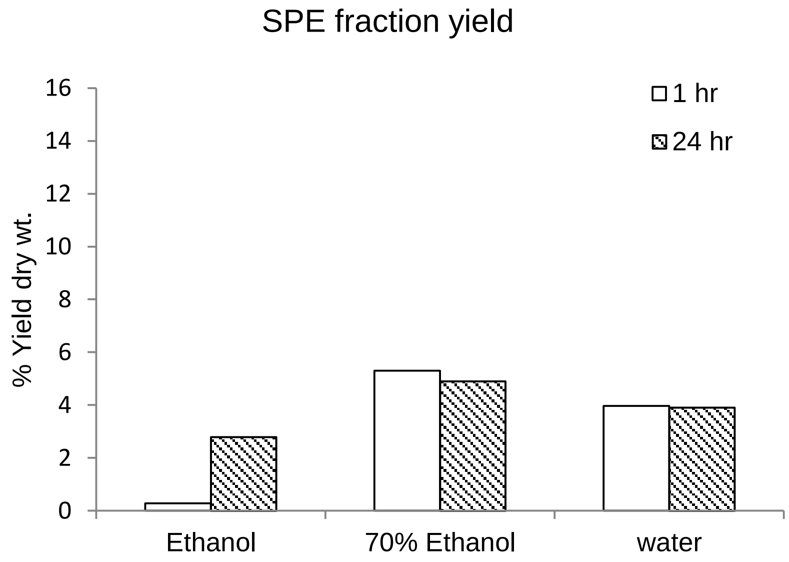


Figure 3.10. The yields of the SPE extraction expressed as %yield from dried seaweed.

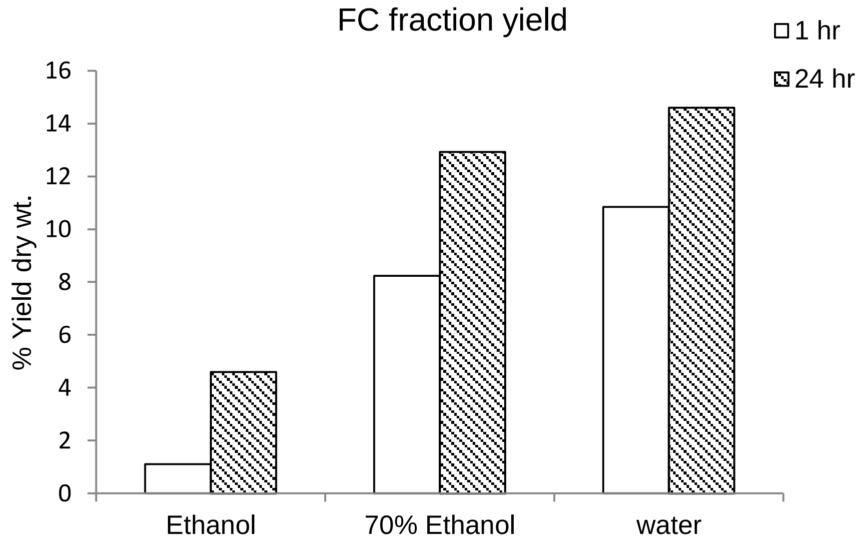


Figure 3.11. Selected data taken from Figure 3.1, showing the phenolic content of the liquor as determined by the FC assay at 1 hour and at 24 hours.

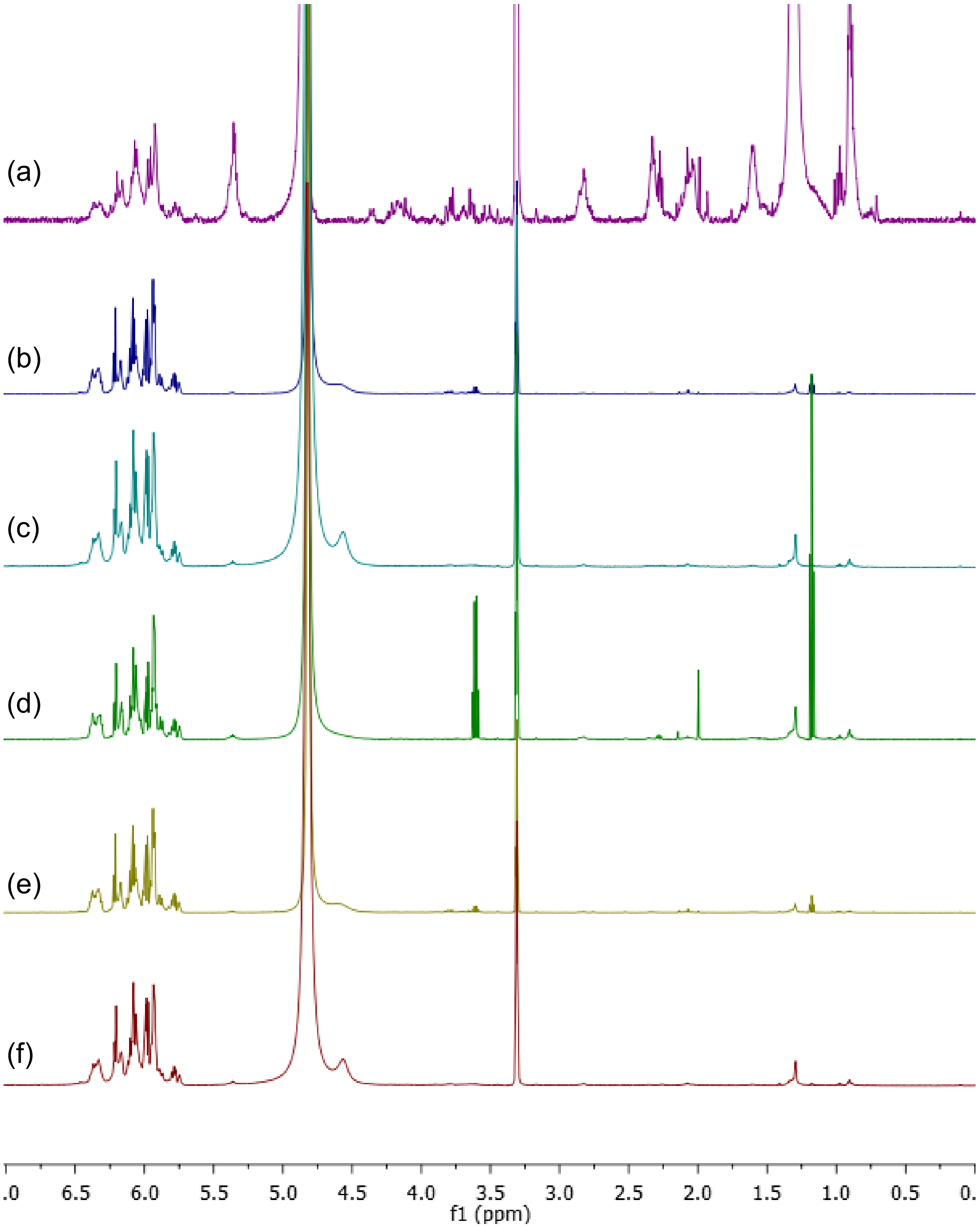


Figure 3.12 The ^1H NMR spectra of the isolated phlorotannin extracts after SPE as extracted by (a) 1 hr ethanol, (b) 1hr 70% ethanol, (c) 1 hr water, (d) 24 hr ethanol, (e) 24 hr 70% ethanol, (f) 24 hr water.

It can be seen from Figure 3.13 that the aromatic regions of these spectra have very similar profiles. From this it can be concluded that the solvent systems (H_2O, EtOH and 70-30 EtOH-H_2O) extracted phlorotannin with similar structural features.

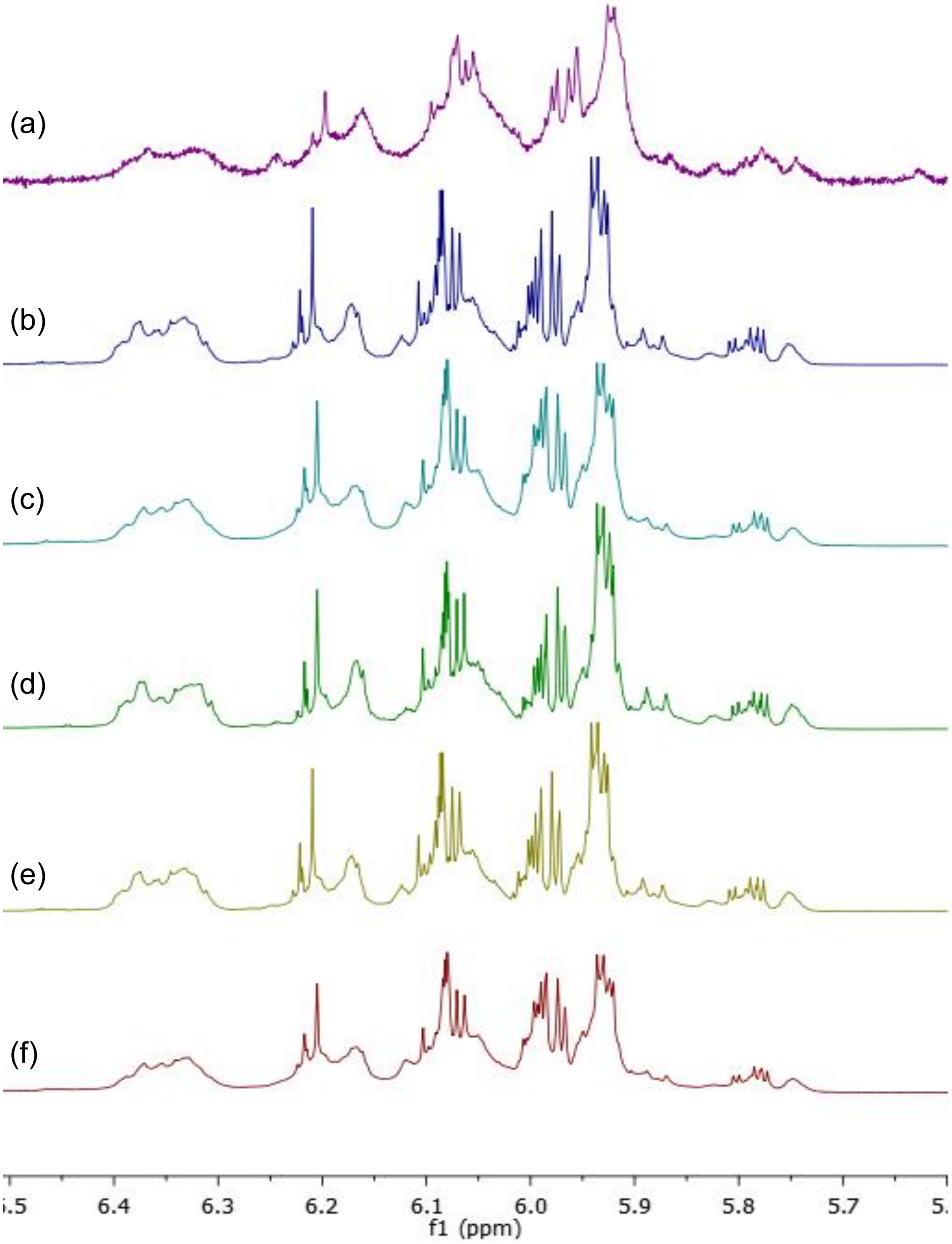


Figure 3.13. The ^{1}H NMR aromatic region of the spectra of the isolated phlorotannin extracts after SPE as extracted by (a) 1 hr ethanol, (b) 1hr 70% ethanol, (c) 1 hr water, (d) 24 hr ethanol, (e) 24 hr 70% ethanol, (f) 24 hr water.

The LC-MS trace of the phlorotannin extract contained peaks corresponding to the masses that would be expected from small oligomers of coupled phloroglucinol, which is strong evidence that the extract is of phlorotannin. This is discussed further in Section 3.2.1. The detection limit of the LC-MS was 2000 m/z and so it was not possible to detect larger oligomers.

3.1.7 Further ^{1}H NMR analysis from acetylation of the phlorotannin extract

The large structural diversity of phlorotannins give rise to ^{1}H NMR spectra with a complex mass of peaks in the aromatic region. As a result, the full structural assignment of phlorotannins is limited to just individually isolated molecules of low molecular weight (< 2000 Da). Although it was not possible to fully characterise mixtures of phlorotannins by ^{1}H NMR, the aim was to determine to what extent the ^{1}H NMR spectra could give some structural information about the types and proportions of the different linkages present.

About 150 different individual phlorotannins have been isolated and characterised to date.[60] The vast majority of these have been isolated as their acetates, using $CDCl_3$ as the NMR solvent as this gives better solubilisation, greater resolution and a larger chemical shift range. The ^{1}H NMR data on the most recent of these publications was compiled to produce a catalogue of the various different ring types and their chemical shifts in the aromatic region.[94, 196] In a novel approach, this catalogue was then used give a tentative characterisation of the phlorotannin samples.

The catalogue of the different ring types and their chemical shifts is given in Appendix A. The different ring types were classified by the number of other rings attached to them (*i.e.* mono-substituted, di-substituted or tri-substituted). Acetate groups were not counted as substituted. From these, 10 distinct types of ring were identified, summarised in Table 3.3 and Figure 3.15. The numbering system is shown in Figure 3.14 below.

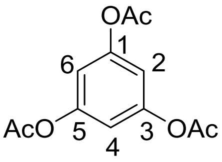


Figure 3.14. The numbering system for the phlorotannin rings.

Table 3.3. Summary of the different ring types reported in Appendix A, together with their chemical shifts in the aromatic region.

Ring type			Shift range
	Substitution (no. of rings attached)	Description	ppm
A	3	1,2,3, triether	6.26 - 6.35
B	2 (ortho)	1,2 di-ether	6.54
C	1	1-ether	6.56 - 6.59
D	3	1,4-diether, 2-Ar	6.56
E	2 (para)	1, 4 di ether	6.64 - 6.71
F	2 (ortho)	1,2 di-ether	6.75
G	1	4-ether	6.92 - 6.95
H	1	4-Ar	6.98 - 7.01
I	2 (meta)	2-ether, 4-Ar	7.12
J	2 (meta)	2,4 di Ar	7.16

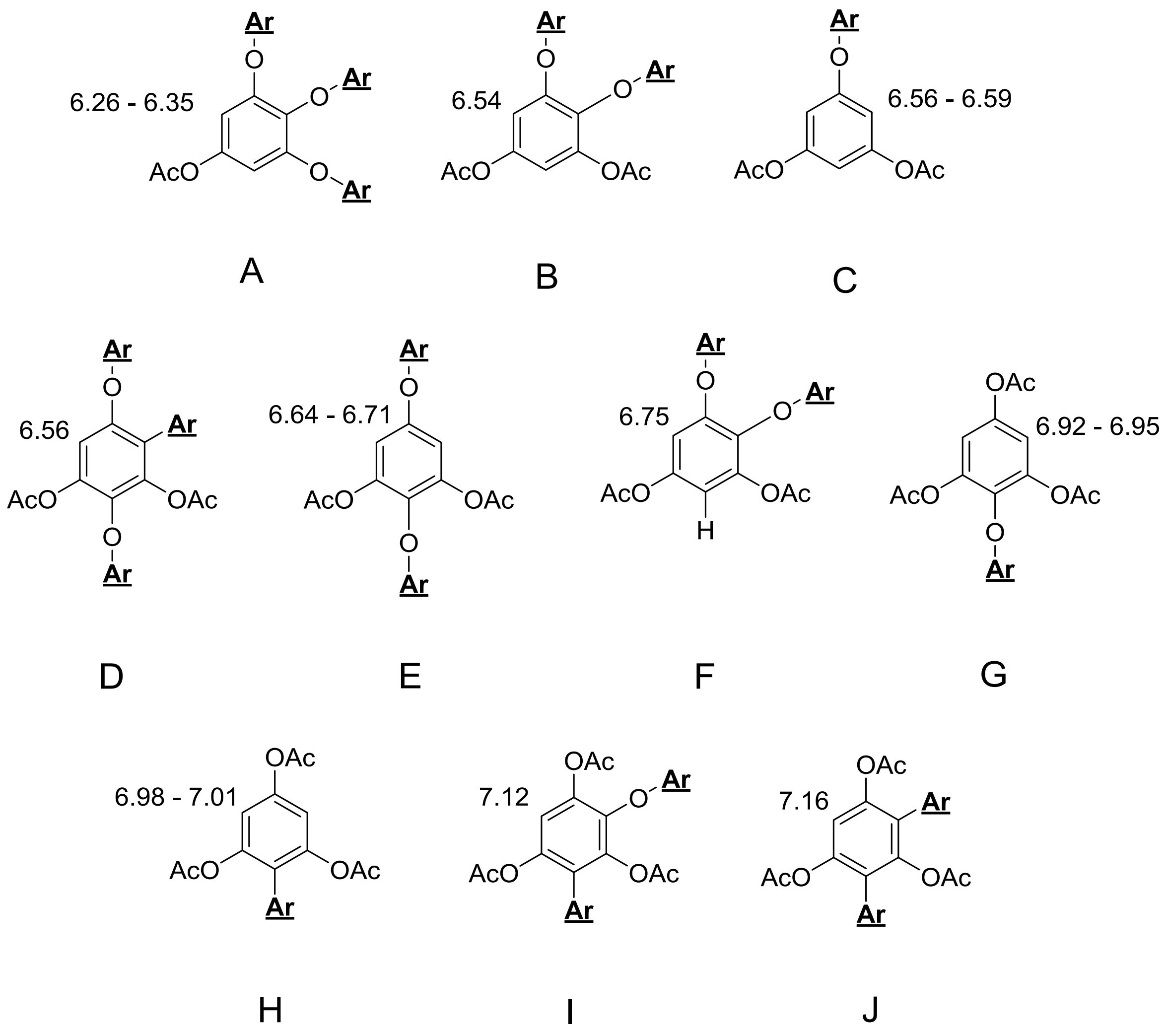
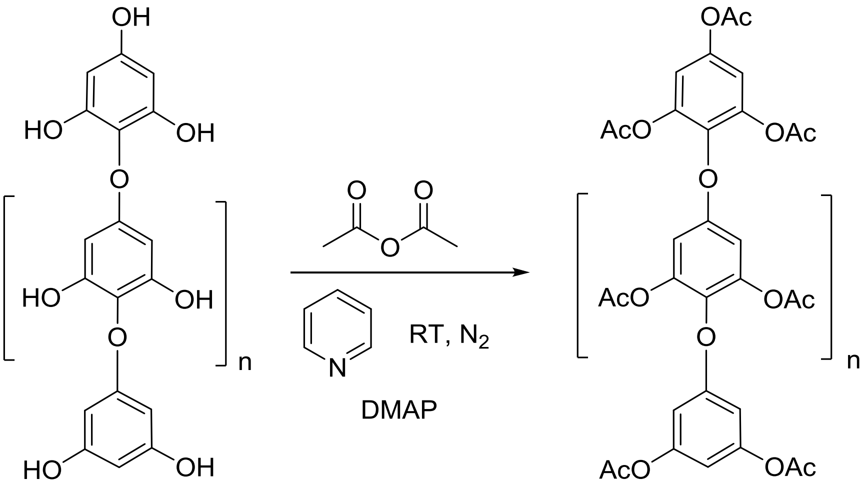


Figure 3.15. The 10 different ring types found in isolated phlorotannins reported in the literature.[94,196] Neighbouring rings are abbreviated to Ar.

The chemical shifts in Table 3.3 vary between 7.16 – 6.25 ppm. This is due to the differences in electron density and resonance affects that arise from the different substitution patterns. The two different linkage types (Ar-O-Ar and Ar-Ar) have opposite effects on the ring; the ether linkages donate electron density to the ring, shielding the ortho and para positions, whilst the Ar-Ar bonds are electron withdrawing, which has a deshielding effect and increases the chemical shift. Accordingly, it generally follows that the more oxygen substituents on the ring, the lower the chemical shift and the more Ar-Ar linkages the higher the chemical shift. Generally, the chemical shifts of the acetylated phlorotannin are higher than un-acetylated phlorotannin. This is because the acetate groups donate less electron density to the ring compared with hydroxyl groups, meaning the rings are less electron rich and consequently have higher chemical shifts.

This catalogue was then used to assign the ^{1}H NMR spectra of an acetylated phlorotannin extract. Phlorotannin was obtained via extraction of dried *Fucus vesiculosus* with 70-30 % ethanol-water which was then acetylated with acetic acid in pyridine, catalysed by DMAP, as shown in Scheme 3.3.



Scheme 3.3. The acetylation of phlorotannin extract, depicting a simple trimer as an example of how the hydroxyl groups are modified.

The ^{1}H spectra are given in Figure 3.16. There are two distinct regions in the spectra; there are about 30 peaks in the aromatic region between 7.12 to 6.20 ppm, which are grouped into clusters. Additionally, there is also a collection of singlet peaks between 2.28 - 1.60 ppm due to the methyl groups on the acetates.

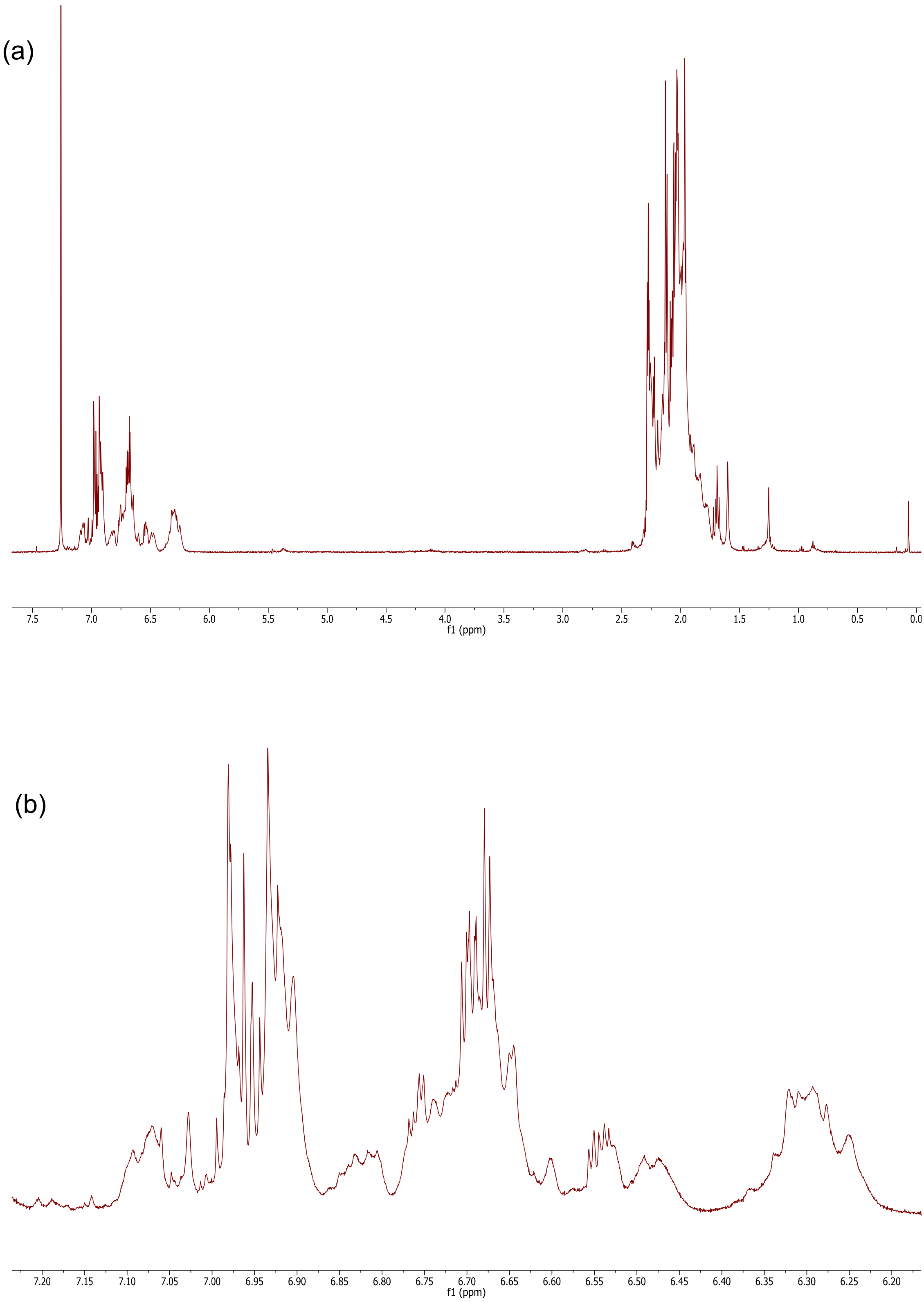


Figure 3.16. 1H NMR of acetylated phlorotannin sample in $CDCl_3$ showing (a) full spectrum and (b) the aromatic region.

Tentative assignments of the aromatic peaks in Figure 3.16 were then made based on the catalogue data as shown in Table 3.4 and plotted in Figure 3.17. The integrals of each region were calculated as a percentage of the total aromatic region. Generally, the observed peaks aligned well with literature values. Some slight variation in literature shifts would be expected due variations in the temperature, pH, concentration and presence of salts.[138]

Table 3.4. ^{1}H NMR shifts of acetylated phlorotannin extract and tentative assignment based on reported values given in Table 3.3.

Observed range (ppm)	Tentative assignment		Typical range (ppm)	% Integration of aromatic region
	Ring type	Description		
6.2 - 6.42	A	1,2,3 triether	6.26-6.35	15.78
6.42 - 6.51	unknown			3.91
6.51 - 6.56	B	1,2 diether	6.54	4.06
6.58 - 6.62	C	1- ether	6.56-6.59	2.66
6.62 - 6.71	E	1,4 diether	6.64-6.71	21.56
6.71 - 6.78	F	1,2 diether	6.75	9.84
6.78 - 6.87	unknown			5.16
6.87 - 6.95	G	4-ether	6.92-6.95	18.44
6.95 - 7.01	H	4-Ar	6.98-7.01	11.41
7.01 - 7.04	unknown			1.88
7.05 - 7.11	I	2-ether, 4-Ar	7.12	5.31

An estimation was made from the data in Table 3.4 of the ratio of Ar-O-Ar to Ar-Ar linkage types which was found to be 7.7:1. There are several possible reasons as to why there are more Ar-O-Ar than Ar-Ar linkages. The formation of Ar-Ar bonds requires the rings to come closer together, which would cause greater steric hindrance, therefore giving a slower reaction. Additionally, the coupling of two electron rich rings to form Ar-Ar bonds may have a high energy barrier. Section 1.7.2 discussed the possibility that enzymes might be responsible for these couplings. If specific enzymes are required for each coupling type, then the relative amounts and rates of reactions of these enzymes would also affect the ratio of the two linkage types in phlorotannin.

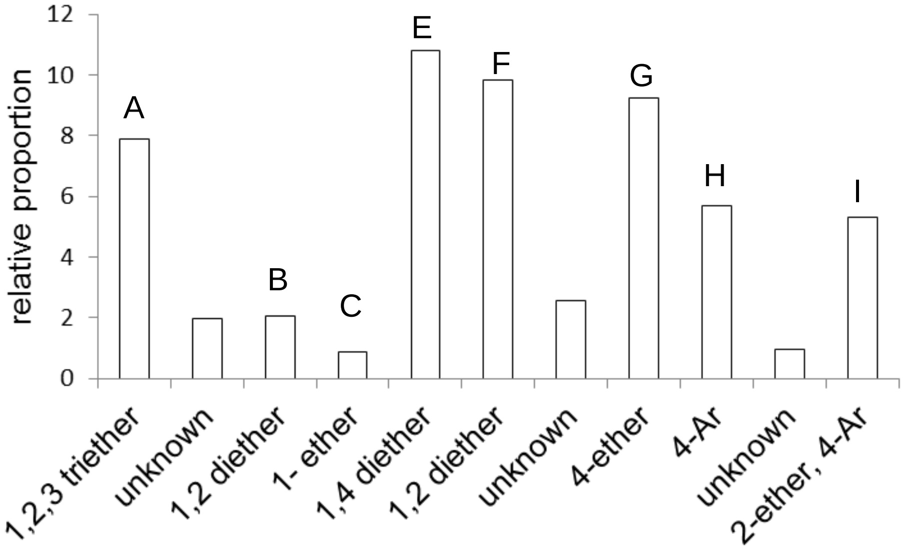


Figure 3.17. The proportions of the different ring types plotted from the data in Table 3.4. Values are weighted based on number of protons on the ring.

The relative proportions in the degree of coupling was then compared and plotted in Figure 3.18. Mono-coupled rings are defined as rings that are coupled to one other ring, such as ring type C in Figure 3.15. Similarly, di-coupled and tri-coupled rings are coupled to two or three other rings respectively. It can be seen that mono-coupled and di-coupled are more common than tri-coupled rings. This is in agreement with the structures reported in the literature and can be rationalised based on the decreasing reactivity of the aromatic ring the more substituted it becomes.[94, 196] This is also consistent with predominantly long chain structures of linked phloroglucinol units.

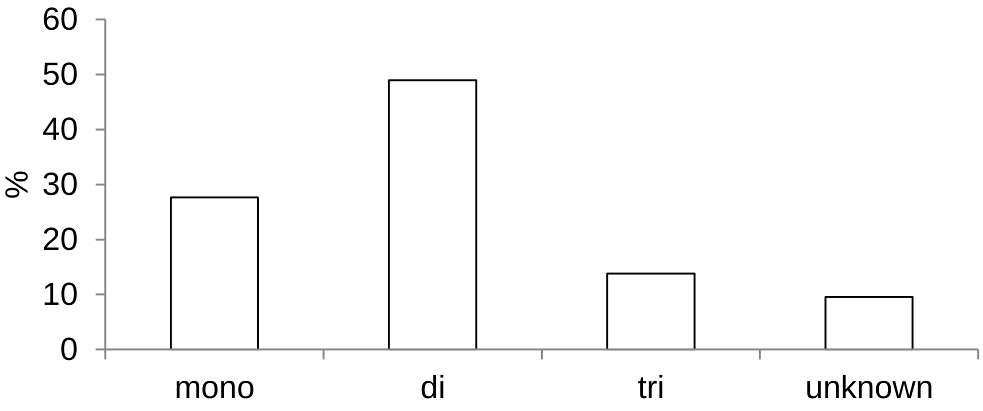


Figure 3.18. The relative proportions of the ring types by degree of coupling.

An estimation of the amount of hydroxyl groups present on the phlorotannin was made from the ratio of the total integrals between the acetyl groups and the aromatic protons, which was found to be 1.36:1. This means that there was an average of 1.36 hydroxy groups for every aromatic proton on the rings. This is consistent with the types of structures in Figure 3.15.

It is important to note that there are several potential limitations of this approach to assigning with NMR. Firstly, it assumes that all of the ring types present in the acetylated extract were also present in the catalogue. Whilst nearly all of the peaks observed could be tentatively characterised, there were three peak clusters that did not match any ring types in the catalogue (6.42 - 6.51, 6.78 - 6.87, 7.01 - 7.04 ppm). These peak clusters only account for 11 % of the total integration of aromatic region. Secondly, there is the possibility that phlorotannin might have been modified during the acetylation process, giving rise to different ring types. It is also important to note that there will be a limit to the size of the phlorotannins that are detectable by NMR. It is not possible to say what this limit would be. For proteins this is typically about 25 kDa for conventional NMR experiments. If it is similar for phlorotannins then it is possible that if there were phlorotannins present that were larger than this limit then they might not have been detected by NMR.

Unfortunately, the lack of ^{1}H NMR data in the literature on non-acetylated phlorotannin meant that it was not possible to compile a table of phlorotannin data for non-acetylated phlorotannin. Nor was it possible to correlate the peaks from the acetylated phlorotannin spectra to assign the MeOH NMR spectra of the unacetylated phlorotannin, which would have allowed a way of characterising the extracts without the need to acetylate them.

3.1.8 Effect of extraction solvent on antioxidant capacity

The antioxidant capacity of the phlorotannin samples isolated after SPE was then measured using the DPPH assay. It can be seen from the EC_{50} values in Figure 3.19 that the phlorotannin extracts have similar antioxidant capacities; around 8 µg ml^{-1}, with the exception of the 1 hour ethanol extract. As discussed earlier, the NMR analysis of this extract found that it contained other peaks, most likely pigments. The poor EC_{50} value measured was not representative of pure phlorotannin and was therefore ignored when comparing the other values. The similarity of the EC_{50} values is what would be expected given the similar structural nature of the phlorotannin extracts found by NMR in Figure 3.18.

There was also only a small reduction in the antioxidant capacity between the 1 hr and 24 hr samples, suggesting that no degradation of the phlorotannin occurred during the longer extraction. This has an important implication for any large scale processing operation as it demonstrates that extractions can be performed for longer periods of time to achieve higher yields without significantly affecting the radical scavenging capacity of the polyphenolic extracts obtained.

The values of EC_{50} for phlorotannin extracts reported in the literature varied between 10-100 µg ml^{-1}.[93, 187, 188] It is difficult to make a direct comparison between the results herein and those in the literature because the measurements were made using different variations of the DPPH assay, however it is encouraging that these values are measured below the range reported in the literature.

Two conclusions can therefore be drawn from this; firstly, that there is no advantage in extracting with a particular solvent to get a higher antioxidant capacity. Secondly, longer extractions can be performed to achieve higher yields, without impacting significantly on the antioxidant capacity of the extract.

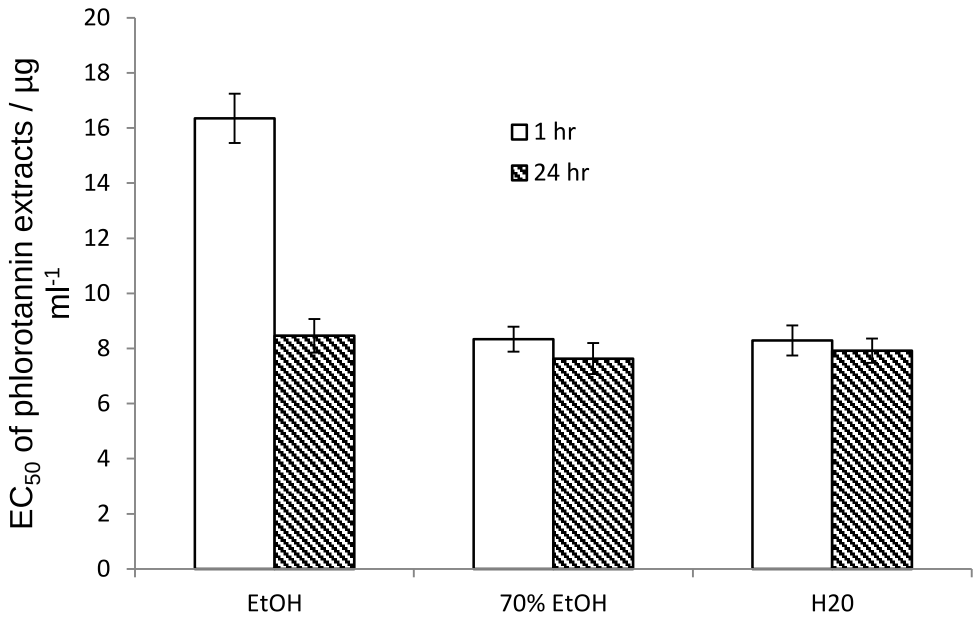


Figure 3.19. The radical scavenging capacity of the SPE purified phlorotannin extracts obtained from different solvents, extracted after either 1 or 24 hours.

3.1.9 Conclusions

The aim of the research in this chapter was to determine how different extraction procedures affected the radical scavenging properties of phlorotannin. This is the first comparative study of its kind into how different extraction solvents affect the radical scavenging capacity of phlorotannin extract obtained and represents an original contribution to knowledge.

Phlorotannin was extracted from *Fucus vesiculosus* using several solvents commonly reported in the literature. FC analysis of the liquors found that higher yields were generally obtained with more polar solvents, supporting the theory in the literature that more polar solvents disrupt the hydrogen bonds between the phlorotannin and the cell wall in the seaweed.[92, 93]

^{1}H NMR analysis of these solvent liquors found that all the solvents extracted phlorotannin and sugars. Ethanol also extracted lipids and pigments. Based on these findings, purification strategies were devised for isolating phlorotannin from each of the different solvent liquors using a solid phase extraction resin. The process was found to be highly efficient at isolating phlorotannin from the liquors with typical yields estimated from NMR to be >95 %. The aromatic region of the ^{1}H NMR spectra of the phlorotannin extract was found to be the same for each of the extracts. From this it can be concluded that each of the solvents tested extracted phlorotannin with the same structural composition.

An attempt was made to determine the types and proportions of the different ring types in the phlorotannin structure from the ^{1}H NMR of the acetylated phlorotannin sample. Tentative characterisation was made based upon comparison to literature data of low molecular weight phlorotannins that had been isolated, which found the phlorotannin extract to contain several of the key structural ring types in phlorotannin. The average ratio of hydroxyl groups to aromatic protons on the phlorotannin rings was found to be 1.36:1. Ar-O-Ar linkages were found to be more common than Ar-Ar by a ratio of 7.7:1. Rings that were coupled to two other rings were found to be more numerous than those coupled to either one or three, which could be rationalised based on the decreasing reactivity of the ring to further coupling as it becomes more substituted. The ratios are also consistent with the types of structures reported in Figure 3.15.

The radical scavenging capacity of phlorotannin extracts was measured with the DPPH assay, which found them to all have similar EC_{50} values of around 8 µg ml^{-1}. The similar EC_{50} values are what would be expected given the similar nature of the phlorotannin structures found from ^{1}H NMR. From this it can be concluded that there is no advantage in extracting using different solvents in order to obtain a phlorotannin extract of higher radical scavenging capacity. Additionally, the EC_{50} values did not decrease between 1 hr and 24 hour, meaning that seaweed can be extracted for longer to achieve higher yields without degrading the phlorotannin.

As such, the choice of solvent for a large scale processing operation is influenced by the yield from extraction and the suitability for scale up. Water and organic-water mixtures gave the highest yields with fewest other species extracted. These would therefore be preferred in a large scale operation. The use of water as an extraction solvent has several advantages over ethanol-water mixtures for three reasons; firstly, it is cheaper to use just water compared to ethanol. Secondly, water is a more sustainable solvent than ethanol. Thirdly, the purification procedure for isolating phlorotannin is simpler for water extraction; this is because ethanol-water mixtures also extracted pigments from the seaweed, which need to be removed. The only drawback of using water was the high viscosity of the liquor, which slowed the filtration steps considerably, which may cause problems during a large scale operation.

3.2 Molecular Weight Profiling

This section discusses the work performed to fractionate the phlorotannin extract and the development towards a method to determine the molecular weight profile of an extract. As discussed in Section 1.7.11, conventional techniques for determining the molecular weight distribution of polymers have been found to be either unsuitable or of limited use for analysis of mixed weight polyphenolic extracts from seaweed.[72, 99, 100, 103, 105] The development of such a method would allow for the effect of molecular weight to be assessed as an additional parameter when studying polyphenols. This would be of particular importance for applications where molecular weight is significant to the application; *e.g.* for use as a cosmetic ingredient for instance, where molecular weight could influence permeation through the skin.

3.2.1 LC-MS analysis of phlorotannin

LC-MS analysis was performed on the phlorotannin extract obtained from the ethanol-water extraction described in Section 3.1.6 and is shown in Figure 3.20. The annotated peak numbers represent peaks measured between 500 - 1740 m/z. It can be seen that phlorotannin showed separation up to the detection limit of the machine at 2000 Da. It can also be seen from this trace that the vast majority of phlorotannin in the UV trace eluted after the detection limit, suggesting that the majority of phlorotannin is greater than 2000 Da.

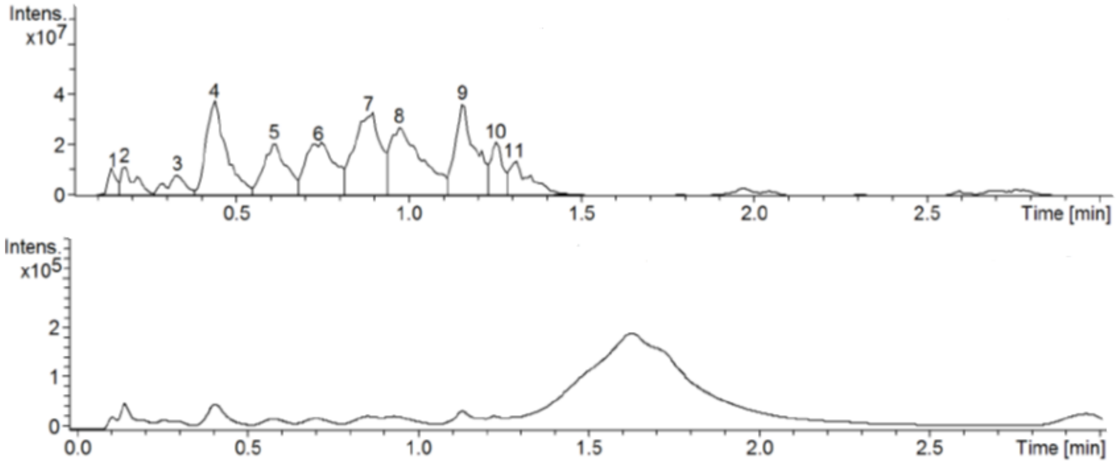


Figure 3.20. LC-MS analysis of the phlorotannin, showing the mass intensity (top) and the UV chromatogram (bottom). Ion source: ESI, positive mode, range: 200 – 2000 m/z, UV 190-650 nm.

3.2.2 HPLC analysis of phlorotannin

The phlorotannin extract was then analysed by HPLC in order to determine if greater resolution could be achieved. The UV chromatogram (Figure 3.21) of an initial run with a small injection (5 mg), showed clear separation of different molecular weight phlorotannins, indicating that fractionation was possible.

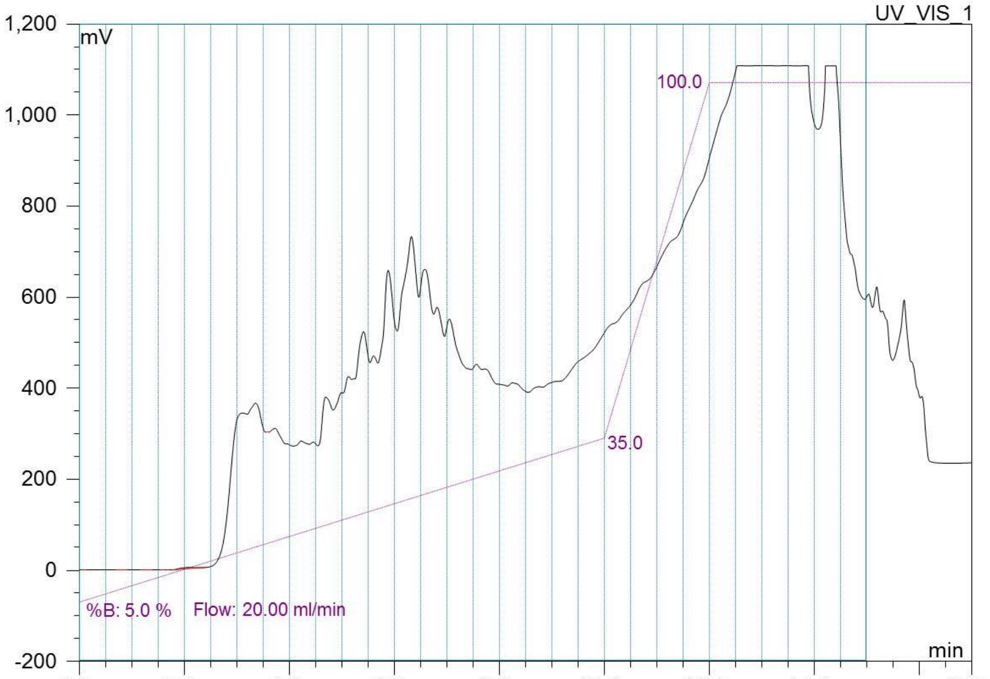


Figure 3.21. The HPLC trace of phlorotannin.

3.2.3 Prep HPLC

Based on the HPLC trace in Figure 3.21, the size-separation of the mixed weight phlorotannin obtained from extraction was attempted using prep-HPLC. The sample injection was increased from 5 mg to 380 mg. A total of 30 fractions were collected over 15 minutes (each fraction being 30 seconds as shown by the lines in Figure 3.21). LC-MS analysis of the fractions showed a clear difference between the fractions. A selection of these is shown Figure 3.22 below.

LC-MS analysis of the fractions confirmed that they contained oligomers. In order to obtain a large quantity of each of these fractions, a further 9 runs were performed. Each of the fractions was reduced to dryness to give a white powder. The yields obtained were very low; each fraction typically contained < 10 mg.

^{1}H NMR in D_2O analysis was performed on the fractions. The aromatic region of the spectrum was much weaker than would have been expected, despite running 512 scans on the NMR. This indicated that there was little phenolic material in the sample, though enough to have shown on the UV chromatogram. This suggests that there may be other inorganic material present, such as salts.

Two conclusions were drawn from the LCMS and HPLC analysis; firstly, that the proportion of phlorotannin under 2000 Da makes up only a small proportion of the extract and that the vast majority of the extract consists of larger molecular weight species. Secondly, HPLC is only capable of achieving fractionation of lower molecular weight species. The resolution that can be achieved decreases with increasing molecular weight. This is consistent with previous analysis of phlorotannin in the literature using HPLC, which reported that resolution of molecules could only be achieved up to 6000 Da.[101]

In theory, this limit could be extended slightly by increasing the run time; however, it would not be able to achieve full fractionation of the phlorotannin extract. As such, HPLC is only of limited used in assessing the molecular weight profile of phlorotannin extracts.

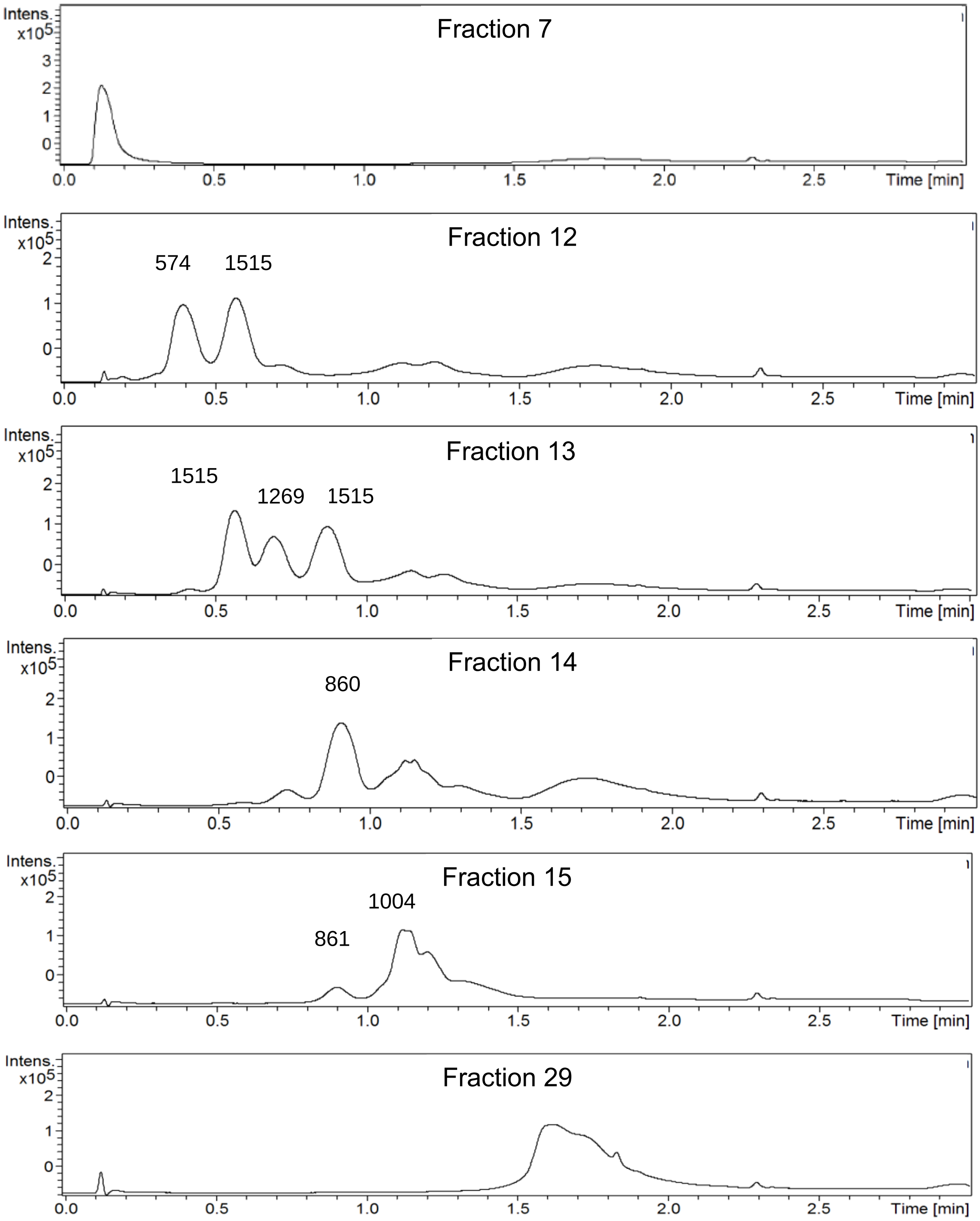


Figure 3.22 – UV Chromatograms from the LC-MS analysis of the fractions obtained from preparative-HPLC of phlorotannin showing a degree of fractionation. Ion source: ESI, positive mode, range: 200 – 2000 m/z, UV 190-650 nm.

3.2.4 Other approaches to molecular weight profiling

As discussed in the introduction in Section 1.7.11, there are methods reported that allow for partial determination of the molecular weight profile of phlorotannin, however each of these methods has its limitations and does not give the complete molecular weight profile. There are many techniques used in polymer science for determining molecular weight ranges. The suitability of some of these commonly used techniques for use with phlorotannin is discussed below.

Dynamic light scattering (DLS) is routinely used to measure the molecular weight distribution of polymers.[216] DLS was an attractive option as it is a relatively quick analytical process. However, this technique is likely to be unsuitable for phlorotannins as they would probably form clusters in solution, greatly complicating the calculations. Without knowing the size of the clusters it would be impossible to perform any meaningful calculations to determine the molecular weight range of the phlorotannin sample. Consequently, it was decided not to pursue this technique further.

3.2.5 Gel Permeation Chromatography

Another routinely used technique in the analysis of polymers is gel permeation chromatography (GPC), which separates molecules based on their size as they pass through a gel filtration medium packed in a column.[217] A key difference from ion exchange chromatography or affinity chromatography is that molecules do not bind to the medium. It is mainly used as a method of purifying proteins, polysaccharides, nucleic acids, but can also be used to fractionate mixed weight molecules to provide a molecular weight distribution analysis. Resin media consist of spherical beads with pores that allow molecules to diffuse into the beads, as shown in Figure 3.23. Small molecules can easily diffuse into the pores, however the larger the molecule the less it diffuses into the pores (it becomes more excluded). There is a cut off limit at which the molecule is too large to diffuse into the pores (at this stage it is said to be excluded).

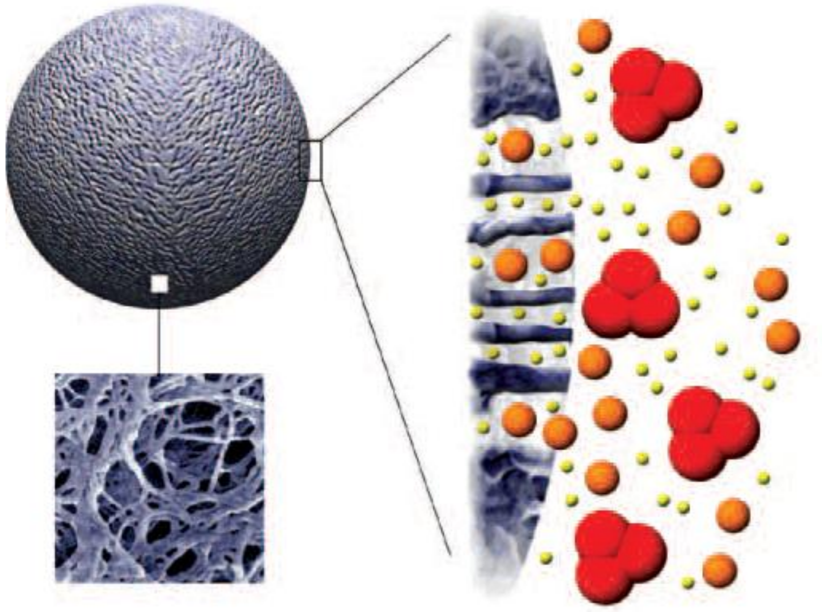


Figure 3.23. The structure of GPC media, showing the porous resin beads and their interaction with molecules based on size. Figure taken from GE Healthcare manual.[218]

A plot of elution volume of molecules against their molecular weight contains a region with a linear relationship, known as the *selectivity range* of the resin, as shown in Figure 3.24 This allows for the determination of molecular weight when compared against a set of standards.

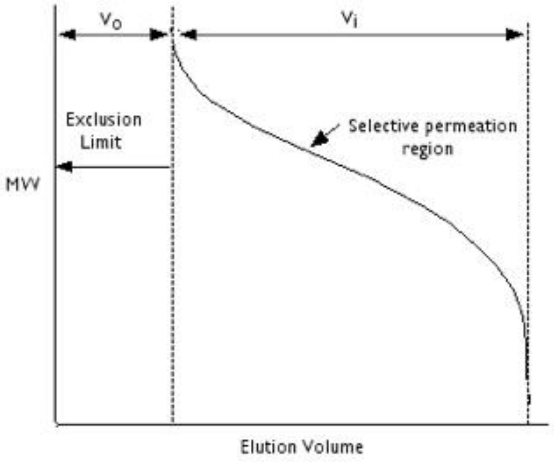


Figure 3.24. Relationship between molecular weight and elution volume during gel filtration, in which there is a linear region that can be used for determining the molecular weight distribution analysis.

3.2.6 Resin selection

Commercially available GPC resins are available with a range of different molecular weight selectivity ranges to suit the molecule being studied. In the case of phlorotannin the upper limit of the molecular weight distribution is unknown. As a result, Sephacryl-500 was selected for its large selectivity range (40kDa – 20,000kDa). The media is a copolymer of allyldextran and N,N'-methylene bisacrylamide (Figure 3.25) with a mean particle size of 50 µm.

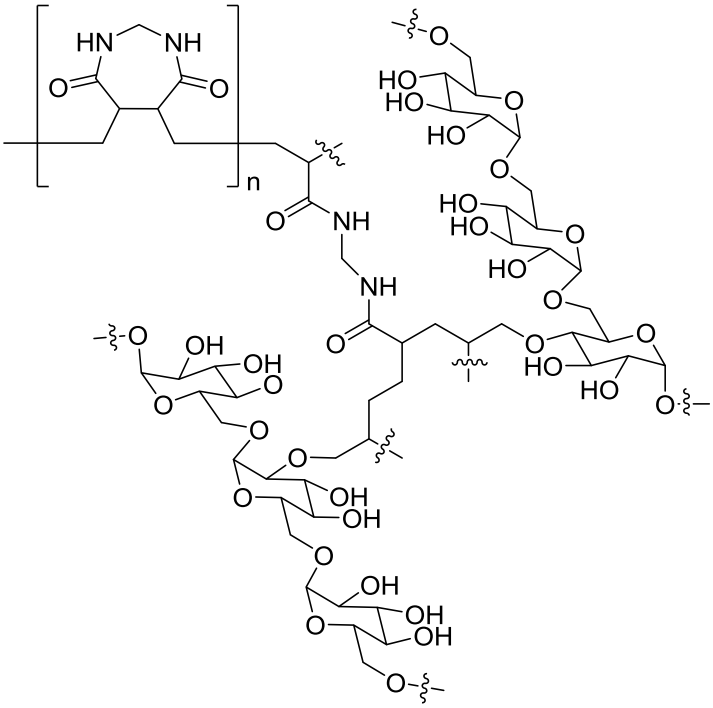


Figure 3.25. Structure of Sephacryl-500 media: a copolymer of allyldextran and N,N'-methylene bisacrylamide.[218]

3.2.7 Selection of solvent system

As mentioned above, a key difference to HPLC is that in GPC, there is no interaction between the resin and the molecules. The molecules diffuse into the pores but do not interact with the resin polymer. Importantly, in order to achieve a strong linear relationship between molecular weight and elution volume it is essential to eliminate non-specific interactions between the sample and the resin. Any interactions, such as hydrogen-bonding and van der Waals forces cause a retardation of the molecules on the resin, increasing elution time and ultimately resulting in an underestimation of molecular weight. As such, it is important to select a solvent system that eliminates these interactions. High ionic strength buffers are used to avoid these non-specific interactions. Additionally, organic solvents can also be added to disrupt any pi-pi stacking interactions. The suitability of different solvent systems was tested using small plugs of Sephacryl 500 resin and applying a solution of phlorotannin extract dissolved in the solvent and then eluting with the solvent. The solvents tested are listed below in Table 3.5.

Table 3.5. The different solvents tested for use with phlorotannin on the Sephacryl 500 GPC resin.

Aqueous salt buffers
Water
Sodium acetate buffer (pH5, 100 mM)
Sodium phosphate (50 mM, 150 mM)
0.5 M acetic acid
Organic solvents
30-70% ethanol-water
50-50 % ethanol-water
Ethanol
Ethanol with 0.5 M acetic acid added
Organic salt buffers
Ammonium acetate (500 mM)
Ammonium formate (500 mM)
Buffer Ammonium acetate / acetic acid (pH4, 100 mM)
Buffer Ammonium formate / formic acid buffer (pH 5, 100 mM)

All of the solvent systems tested left the plugs visibly stained brown, indicating that the phlorotannin had remained on the resin and was not eluting. This was probably due to the strong affinity of the hydroxyl groups attached on the phlorotannin, which formed hydrogen bonding interactions with the sugar groups on the resin.

The only solvent system that appeared to partially elute the phlorotannin was 50% acetone; however, acetone is not suitable for use with Sephacryl-500 as it can degrade the resin with prolonged use. Additionally acetone was also not compatible with the AKTA chromatography system that was intended for use with the column.

The lack of a suitable solvent system for phlorotannin led to alternative strategies being considered. One such strategy involved functionalization of the hydroxyl groups with protecting groups in order to prevent the hydrogen bonding interactions. There are some limitations to this approach. The increase in mass from adding a protecting group would need to be determined in order to determine molecular weight. In the event that fractionations were performed on a preparatory scale then a deprotection step would have been required. Several factors influence the choice of protecting group for this study. Smaller mass groups would be preferred, as they would not alter the properties as much. The protecting groups should be easy to add and remove from the phlorotannin using mild conditions that are less likely to modify the polymer. Additionally, the protecting group should not cause any additional interactions with the resin.

Acetylated phlorotannin was already available from research in earlier work and so provided a convenient starting point for acetylated phlorotannin. An added benefit of the protection would be that it might also provide increased chemical stability, as they are less prone to oxidative degradation. Glombitza *et al.* claimed that acetylation of OH groups helped improve their stability whilst handling them.[102]

Acetylated phlorotannin is insoluble in water and so aqueous buffers could not be used for the solvent system, meaning that an organic solvent system was required. Acetonitrile is a commonly used organic solvent that is compatible with the resin. Acetylated phlorotannin was found to be readily soluble in acetonitrile. The use of a water-acetonitrile mixture would be preferable to using straight acetonitrile as it is best to minimise exposure of the resin to organic solvents, however, precipitates formed when only low levels of water were added.

A small plug of the Sephacryl resin was used to test whether acetonitrile was suitable as a solvent system. The plug was eluted with 0.5 ml of acetonitrile at a time, collecting each fraction. TLC of the fractions showed that the acetylated phlorotannin was still being eluted after 10 column volumes. This indicated that the sample was still being retained on the resin and not eluting. In order for GPC to be used to accurately determine the molecular weight profile of a sample it is essential there is no interaction between the sample and the resin. In this case, it is clear that the use of acetylated samples with acetonitrile is not suitable for this purpose.

3.3 Conclusions

The aim of this section was to develop a method to determine the molecular weight profile of a phlorotannin sample. Several commonly used methods for determining the molecular weight of molecules were explored, however none were found to be suitable for this purpose.

LCMS analysis of the phlorotannin revealed that the majority of the sample had a molecular weight greater than 2000 Da. Preparative HPLC was found to only be capable of achieving resolution of low molecular weight molecules.

Attempts at using a gel permeation chromatography resin found that it was unsuitable for use with phlorotannin due to the strong interactions that phlorotannin formed with the resin. An attempt was made to overcome this problem by using acetylated phlorotannin; however, the phlorotannin still showed a strong affinity to the resin. Other types of GPC resin are available, such as hydrophobic resins, which may eliminate the interactions between the phlorotannin and the resin. However, further research with these resins was not pursued due to their prohibitively high cost.

4 Seasonal Variation of Phlorotannin in Farmed Seaweed

4.1 Introduction

This chapter discusses the work carried out to investigate the seasonal variation in the content of polyphenols in farmed seaweed. Section 1.1 discussed the growth of the seaweed farming industry and its potential for producing a range of biomaterials, including phlorotannin.[1, 2] An important consideration when assessing the viability of farming seaweed as a source of polyphenols is the variation in the levels of polyphenols throughout the year. Developing a good understanding of this seasonal variation is important for commercial activities; for example, it makes it possible to determine the optimal time of year to harvest the seaweed in order to obtain the highest yields of phlorotannin. Additionally, it is also desirable to gain an understanding of the mechanisms behind the factors that influence this variation, allowing for improved farming methods to obtain optimal polyphenol yields.

The previous studies listed in Table 1.2 in the Introduction found that the levels of polyphenols vary throughout the year, though these have all been conducted on seaweed growing naturally, sampled from intertidal regions. This work represents the first study into the seasonal variation in levels of polyphenols in farmed seaweed where phlorotannin was isolated and therefore has relevance for the commercial production of seaweed in giving a more consistent product supply. The growing conditions of farmed seaweed are different to seaweed that grows naturally on the intertidal seabed; for example, there are differences in water currents, which could influence nutrient availability and the spread of herbivorous predation. Farmed seaweed is grown on ropes anchored below floating buoys meaning that the seaweed is held at a constant depth below the surface. By contrast, naturally growing seaweed experiences changes in depth with the tides, which will result in different levels of light intensity and tidal exposure. Previous research has found that these factors influence levels of polyphenols in seaweeds.[76, 77, 91] It is therefore reasonable to expect that these differences in growing conditions could mean that farmed seaweed grown on ropes would experience different variation in the levels of phenolics compared to naturally grown seaweed.

Two species of brown seaweed, *Ascophyllum nodosum* and *Fucus vesiculosus* were selected to be studied, as preliminary work in the group showed them to contain the highest levels of polyphenols. It should be noted that these are intertidal species, which usually experience periodic desiccation due to tidal exposure. It is known that periodic desiccation reduces growth of epiphytic algae and invertebrates.[219] As such, a limitation of growing seaweed on ropes is that the lack of periodic desiccation can lead to them becoming overgrown with epiphytes, leading to increased fouling.

It was desirable to choose an extraction method for this study that was suitable for large-scale processing of seaweed; this allowed for a realistic assessment of the typical yields that could be expected from a large scale commercial operation. The method used is described in Section 2.3.6 in which phlorotannin is extracted using ethanol-water (70-30 V/V) and purified with a solid phase extraction resin. This extraction procedure is both sustainable and scalable, using only ethanol and water as extraction solvents without the need for other chemicals. The phlorotannin extracted is typically > 95% pure as estimated by NMR. All waste streams from processing can either be recycled or reused, *e.g*. for other applications such as in animal feedstock. A large scale processing operation would likely use fresh seaweed and so for this reason the extractions were performed on seaweed that had not been previously dried.

Samples of *Ascophyllum nodosum* and *Fucus vesiculosus* were collected by Böd Ayre from the ropes at their seaweed farm at the end of every month between July 2010 and June 2012 (with the exception of September 2011, which was not sampled). Care was taken to collect several plants to give a representative sampling of the seaweed. Samples were immediately frozen and stored at -20 °C until analysis. The phenolic content of these samples was determined in two ways: firstly from the yield of phenolic material recovered using the extraction method and secondly, a measurement was made from analysis of the extraction liquor using the Folin Ciocalteu method (Section 2.2).[98] All extractions were performed in duplicate. Most of the other seasonal variation studies reported in the literature were performed on dry seaweed. In order to allow for comparison to other studies, the dry weight of the seaweed was determined from additional drying experiments on the seaweed. Samples of the seaweed (3 x 10 g portions) were dried at 60 °C for 48 hours and the % dry mass determined from the mass measured before and after drying.

4.2 Results

The dry mass of the seaweed samples is given in Figure 4.1. It can be seen from Figure 4.1 that both species have a water content ranging between 30 – 55 % mass. The average water content is given in Table 4.1. *Ascophyllum nodosum* had a higher average dry mass than F*ucus vesiculosus* (48.0 % compared to 39.6 %), which was consistent with the appearance of the seaweed; *Fucus vesiculosus* had a more fleshy texture to it, while *Ascophyllum nodosum* was slightly tougher and more rigid. The lower water content of *Ascophyllum nodosum* meant that when the results are calculated on a dry weight basis there was a greater proportional increase in yields over *Fucus vesiculosus*.

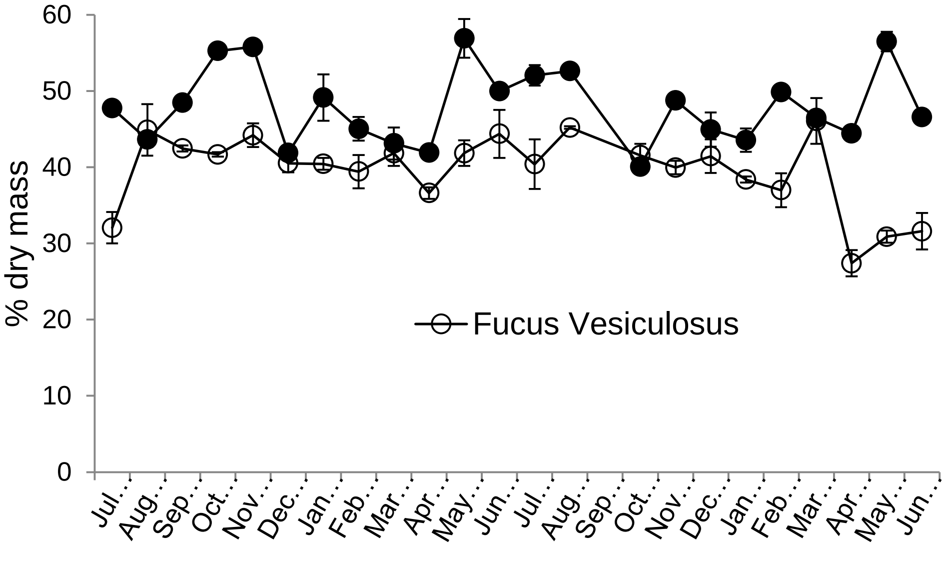


Figure 4.1. The variation in % dry mass of Fucus vesiculosus and Ascophyllum nodosum as determined from drying analysis.

Table 4.1. The average % dry mass of each of the seaweed species.

Species	Average	SD
Fucus vesiculosus	39.6	5.0
Ascophyllum nodosum	48.0	5.0

The fresh weight yields obtained from the extraction of phlorotannin are given in Figure 4.2 for *Fucus vesiculosus* and Figure 4.3 for *Ascophyllum nodosum*.

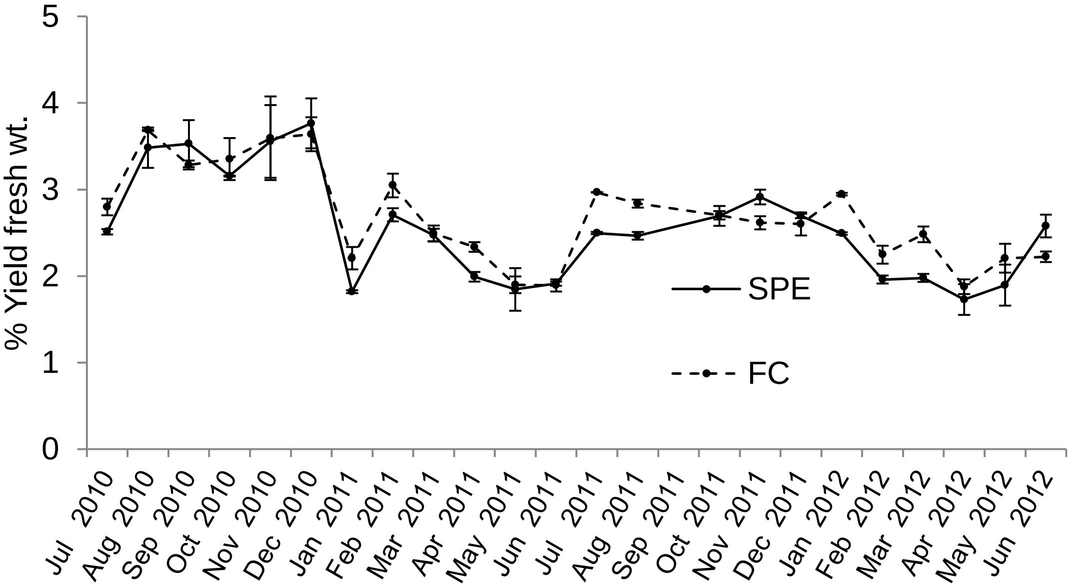


Figure 4.2. The phenolic content of *Fucus vesiculosus* (fresh weight) as determined by both the yield of phenolic material obtained from extraction (solid line) and by analysis of the extraction liquor using the FC method (dash line). Seasonal variation from July 2010 to Jun 2012 shown.

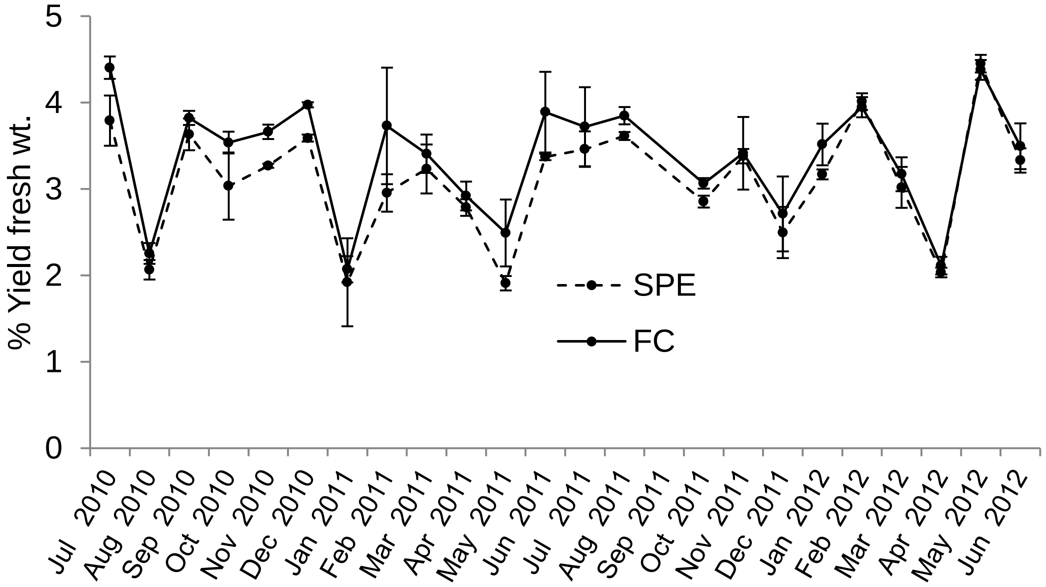
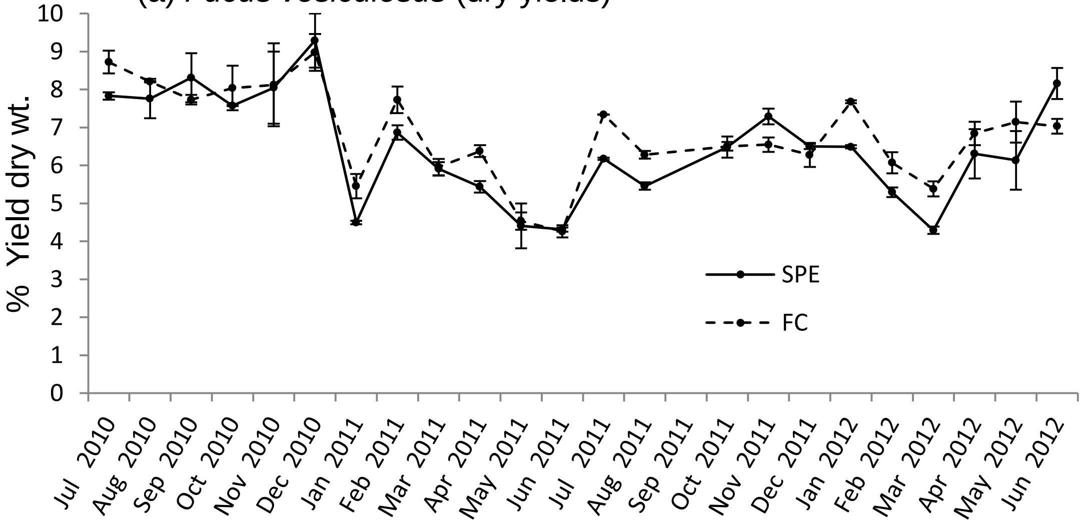


Figure 4.3. The phenolic content of *Ascophyllum nodosum* (fresh weight) as determined by both the yield of phenolic material obtained from extraction (solid line) and by analysis of the extraction liquor using the FC method (dash line). Seasonal variation from July 2010 to Jun 2012 shown.

It can be seen from the graphs in Figure 4.2 and Figure 4.3 that there was seasonal variation in the levels of phenolics of both species studied. As discussed above, in order to allow comparison to other studies, these results were then converted into dry mass yields using the results from the drying mass experiments. These were calculated using the individual monthly dry masses and are shown in Figure 4.4.

(a) *Fucus vesiculosus* (dry yields)



(b) *Ascophyllum nodosum* (dry yields)

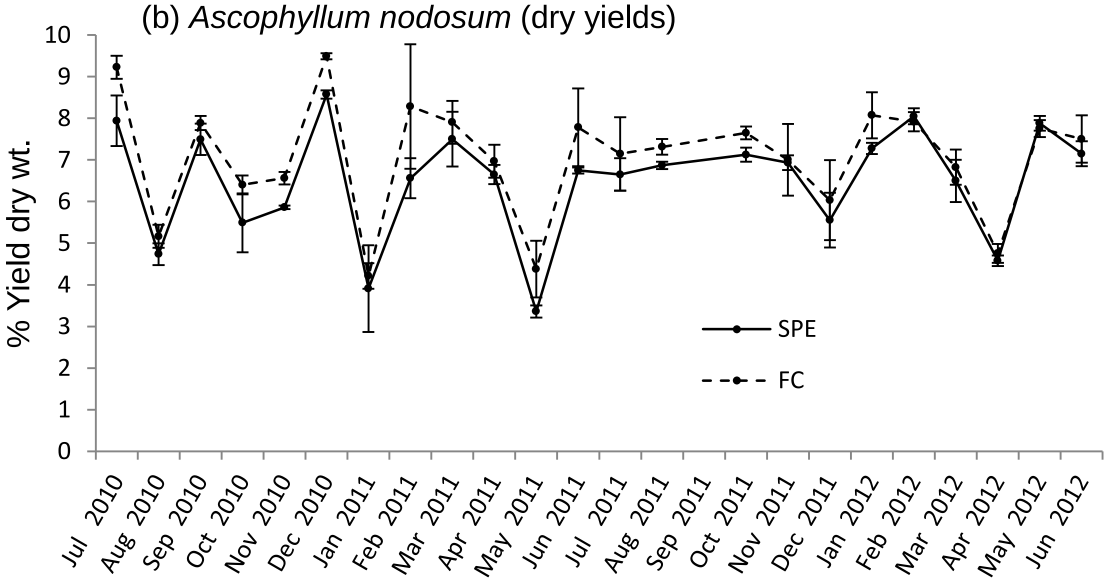
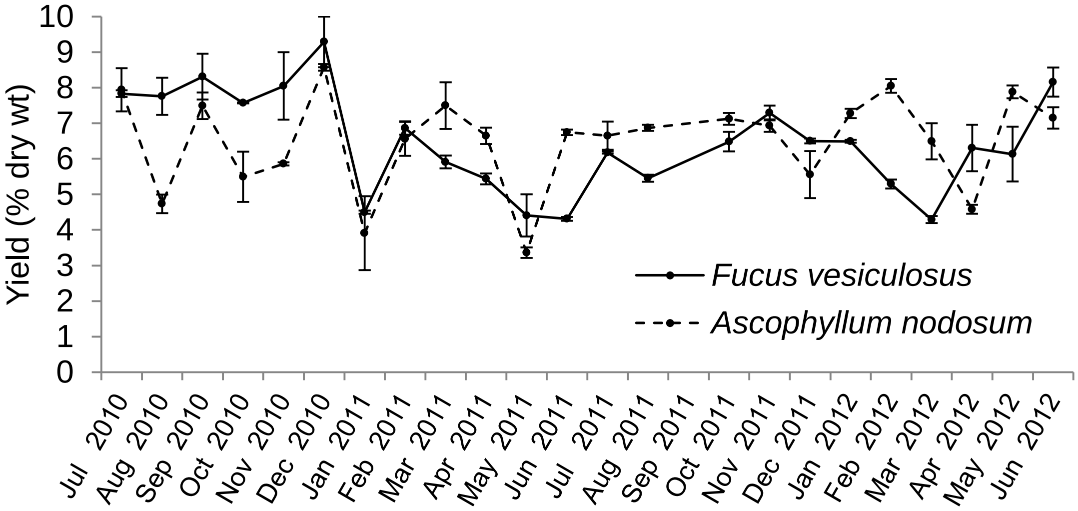


Figure 4.4. The seasonal variation in phenolic content of (a) *Fucus vesiculosus* and (b) *Ascophyllum nodosum* as calculated on a dry weight basis determined by both the FC and SPE methods.

To allow additional comparison, the graphs are also plotted comparing the test methods for each species (Figure 4.5).

(a) Dry yields (SPE) for both species



(b) Dry yields (FC) for both species

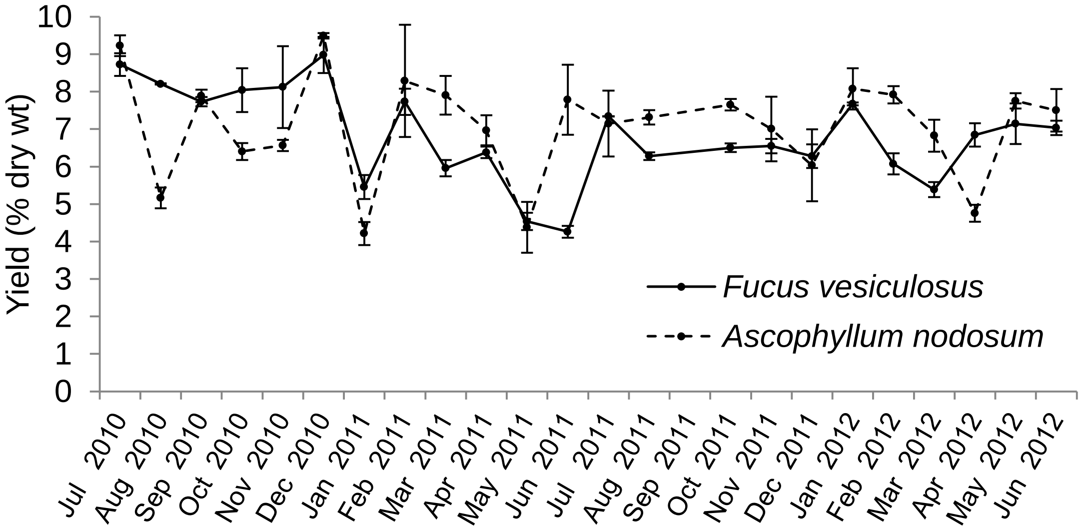


Figure 4.5. The seasonal variation in phenolic content of *Fucus vesiculosus* and *Ascophyllum nodosum* as determined on a dry weight basis by (a) SPE and (b) FC method.

4.2.1 Comparison between species

It can be seen from Figure 4.4 and Figure 4.5 that both species experienced variation in phenolic content. The phenolic content for *Fucus vesiculosus* ranged between 1.9 – 3.7 % fresh wt. and 4.3 - 9.0 % dry wt. The phenolic content of *Ascophyllum nodosum* ranged between 2.1 - 4.4 % fresh wt. and 4.2 – 9.2 dry wt. A comparison of the average yields is given in Table 4.2 and represented graphically in Figure 4.6. When compared on a fresh weight basis, the average yield of *Ascophyllum nodosum* was higher than *Fucus vesiculosus* by about 0.55 % as measured by SPE and 0.68 % as measured by FC.

When the yields are compared on a dry weight basis, the different in yields between the two species becomes smaller; just 0.02 % as measured by SPE and 0.21 % as measured by FC). The cause of this is due to the higher water content of *Fucus vesiculosus*.

Table 4.2. Comparison of the average yields obtained from each species using SPE and FC expressed as both fresh and dry weight.

Analysis method		Species			
		Fucus vesiculosus		*Ascophyllum nodosum*	
		Average yield (wt. %)	SD	Average yield (wt. %)	SD
Fresh	SPE	2.55	0.62	3.10	0.67
	FC	2.69	0.56	3.37	0.67
Dry	SPE	6.47	1.41	6.49	1.35
	FC	6.84	1.25	7.05	1.40

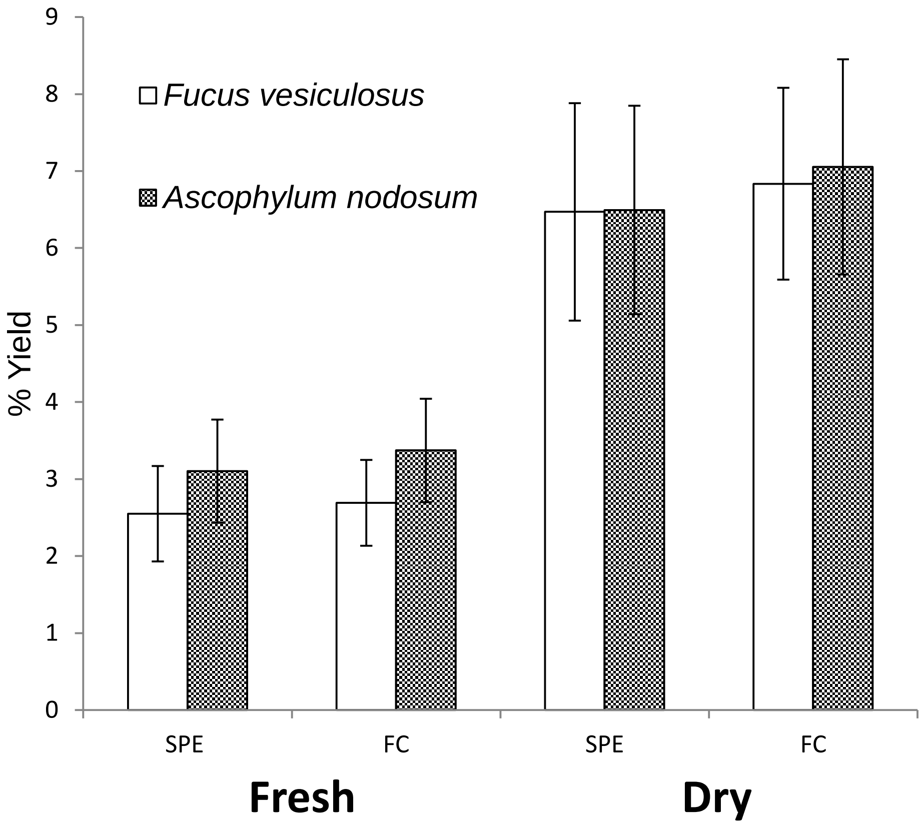


Figure 4.6. Comparison of the average yields obtained from each species using SPE and FC expressed as both fresh and dry weight.

It can be seen from Figure 4.4 and Figure 4.5 that both species follow a similar trend, roughly mirroring each other in their variation in phenolic content with corresponding points of maxima and minima at several points in the year. This suggests that they are both affected to a similar degree by the various environmental factors that influence phenolic content, which are discussed in more detail later. The degree of correlation was examined by plotting the yields from each month of each species against each other (Figure 4.7). The degree of correlation was then quantified using the Pearson's r test (Table 4.3), showing a moderate level of correlation; 0.35 for SPE and 0.41 for FC.[220]

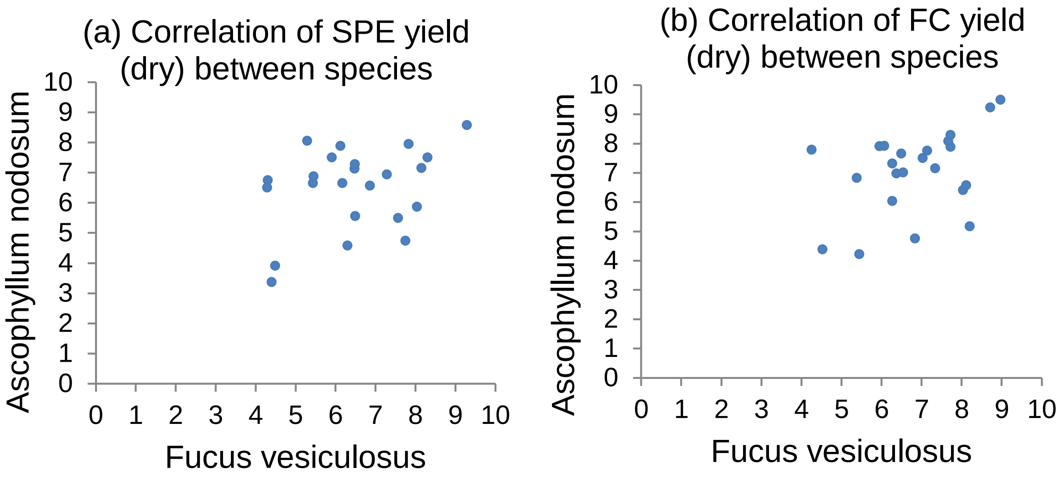


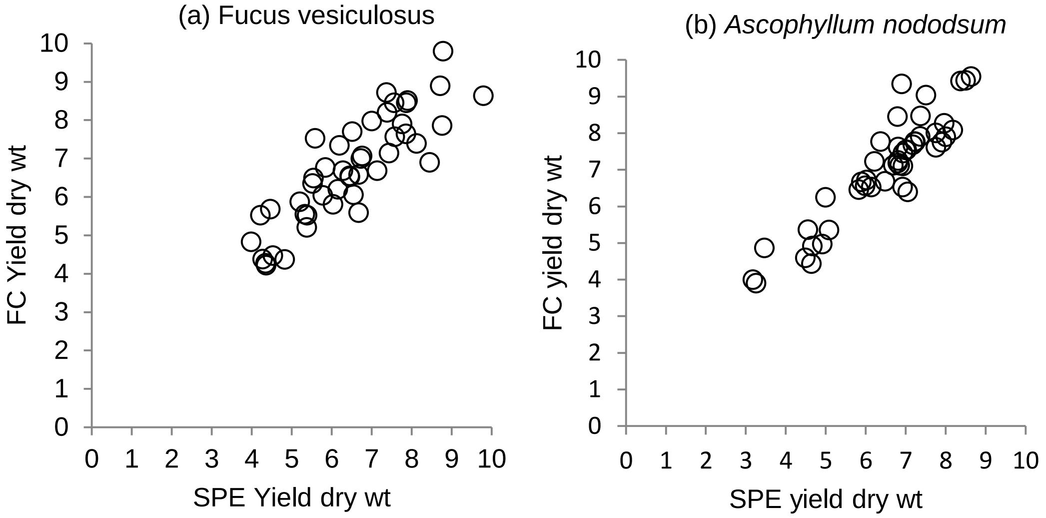
Figure 4.7. The correlation between the phenolic content of each of the species as measured by SPE and FC.

Table 4.3 The Pearson's r values of the correlation between species as measured as both SPE and FC method.

Method	**Correlation with Pearson's r**
SPE	0.35
FC	0.41

4.2.2 Comparison of test methods

There was good agreement between the two methods used to measure phenolic content as shown in Figure 4.8, in which the yields from SPE are plotted against those from FC on a dry weight basis. The degree of correlation was again quantified using Pearson's r test and found to be 0.88 for *Fucus vesiculosus* and 0.95 for *Ascophyllum nodosum* (Table 4.4), indicating a high level of correlation.



(a) Fucus vesiculosus

(b) *Ascophyllum nododsum*

(c) *Fucus vesiculosus* and *Ascophyllum nodosum*

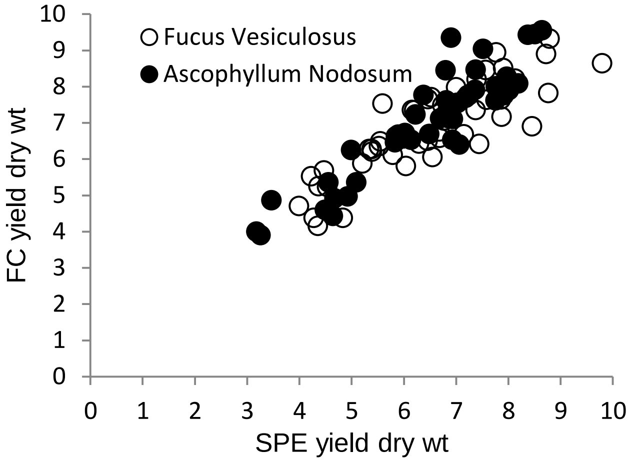


Figure 4.8. The correlation between the FC method and the SPE analysis for (a) *Fucus vesiculosus*, (b) *Ascophyllum nodosum* and (c) both species combined.

Table 4.4. The Pearson's r values for the correlation between SPE and FC yields for each species.

Species	**Correlation with Pearson's r**
Fucus vesiculosus	0.88
Ascophyllum nodosum	0.95

4.2.3 Characterisation of the extract

The ^{1}H NMR spectra of the phlorotannin extracts were compared to try to determine if any information on the structural features of the phlorotannin could be gleaned. Several extracts were chosen from months in which there were a maxima or minima in phenolic content. CD_3OD was used as the ^{1}H NMR solvent because this gave the sharpest resolution of the peaks, allowing better comparison. The spectra of several of the extracts are given in Figure 4.9, where it can be seen that there is a clear difference between the two species. There are many peaks common to both species; however, *Fucus vesiculosus* has some extra peaks present that *Ascophyllum nodosum* does not contain, including peaks at δ 6.42-6.37, 6.22 and 6.08 ppm. The spectra for each species showed no significant change throughout the year.

Two things can be concluded from this; firstly that the two species produce phlorotannin with different types and proportions of the ring types shown in Figure 3.15 in section 3.1.7. Secondly, it can be concluded that the structure of the phlorotannin in each species is consistent throughout the year, as far as can be discerned using this technique. An important implication of this is that seaweed can be farmed to produce a consistent phlorotannin extract all year round.

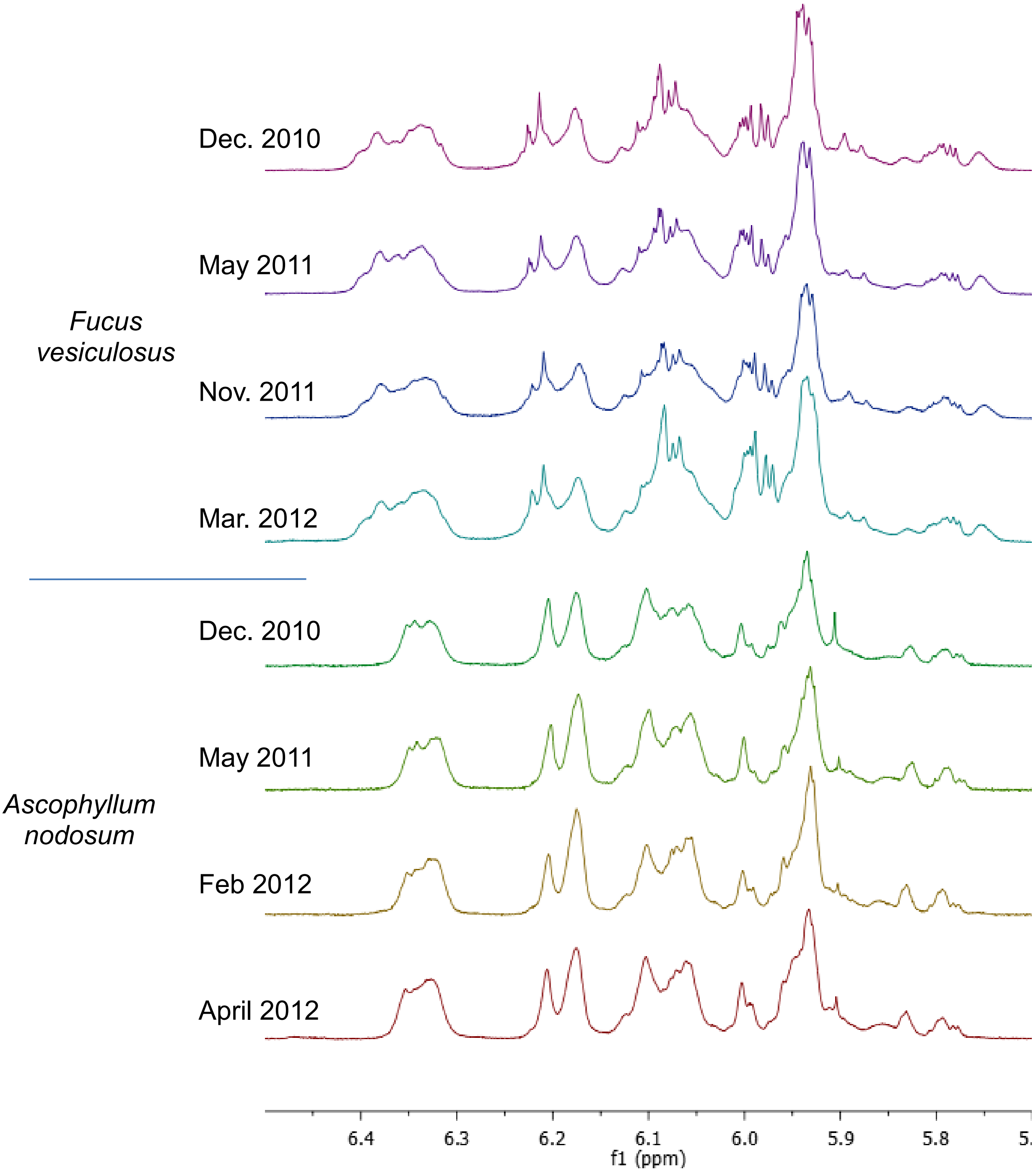


Figure 4.9. The ^{1}H NMR analysis of phlorotannin extracts from the months in which there were maxima and minima of phenolic content.

4.2.4 Acetylation

In order to further understand the differences in structure of the phlorotannin extract between the two species, four ^{1}H NMR phlorotannin extracts were then acetylated to allow comparison with the catalogue of ^{1}H NMR data from the literature, as described in section 3.1.7. The acetylation process was carried out as described in General Procedure B (See Section 9.10 of the Experimental).

The ^{1}H NMR spectra of the acetates are given in Figure 4.10. As with the NMRs of the unacetylated phlorotannin in Figure 4.9, a difference can be seen between the two species. A tentative assignment of the peaks in the spectra in Figure 4.10 was then made by comparison to the catalogue of literature data in section 3.1.7. The assignment is given in Table 4.5 and plotted as a bar chart in Figure 4.11.

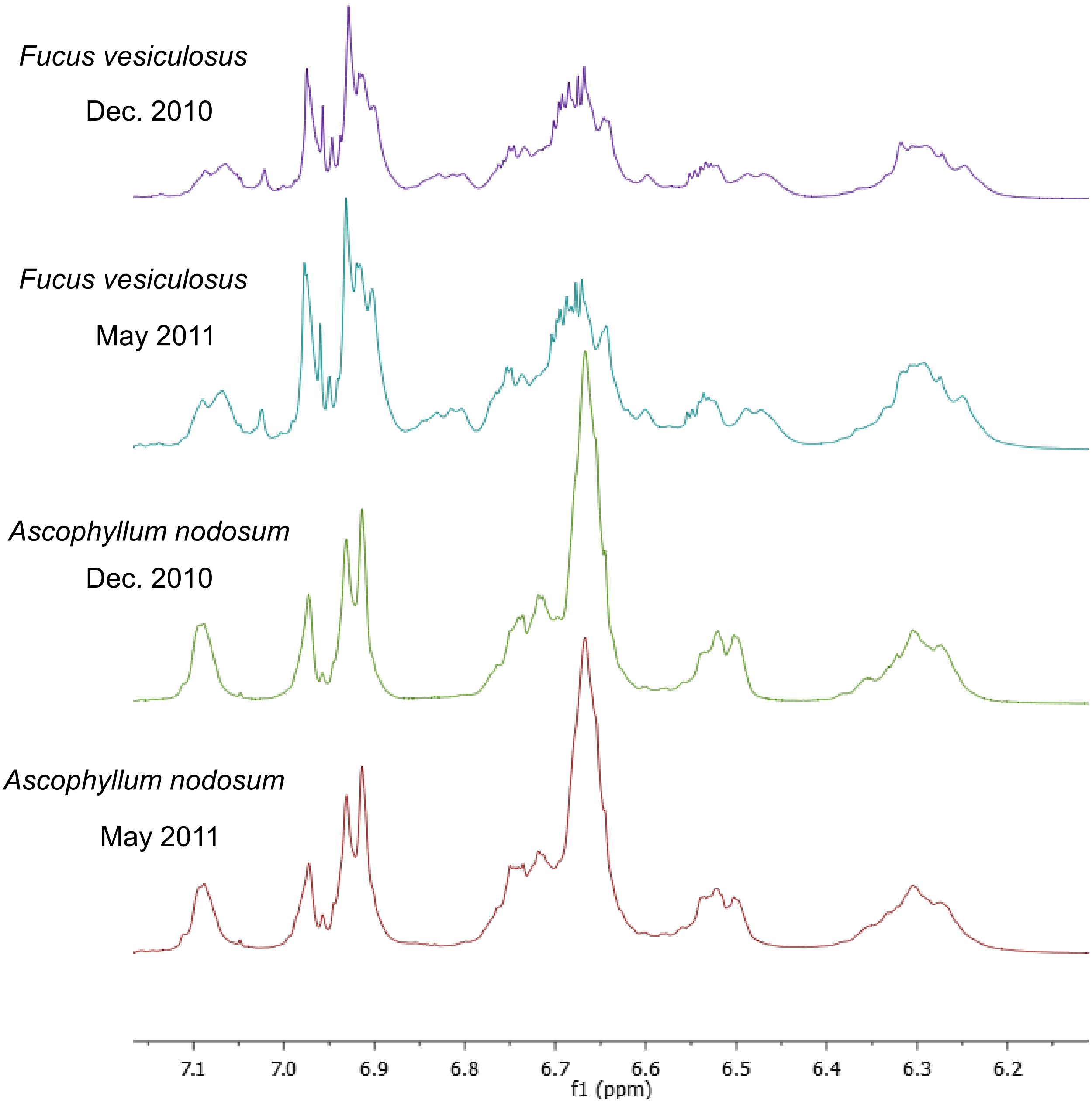


Figure 4.10. The aromatic region of ^{1}H NMR spectra of the acetylated phlorotannin extracts during selected months in $CDCl_3$.

Table 4.5. The tentative assignment of the spectra in Figure 4.10 based on the catalogue of literature data given in Table 3.3.

				% integration of aromatic region			
	Tentative assignment			*Fucus vesiculosus*		*Ascophyllum nodosum*	
Observed range (ppm)	Ring type	Description	Typical range (ppm)	Dec. 2010	May 2011	Dec. 2010	May 2011
6.2 - 6.42	A	1,2,3 triether	6.26-6.35	16.31	17.15	14.39	15.77
6.42 - 6.51	unknown			4.73	4.80	4.03	4.79
6.51 - 6.56	B	1,2 diether	6.54	4.24	4.29	5.61	5.72
6.58 - 6.62	C	mono ether	6.56-6.59	2.28	2.74	1.58	2.47
6.62 - 6.71	E	1,4 diether	6.64-6.71	22.35	20.41	33.81	30.45
6.71 - 6.78	F	1,2 diether	6.75	8.97	9.26	11.37	10.82
6.78 - 6.87	unknown			5.22	5.66	1.73	2.63
6.87 - 6.95	G	4-ether	6.92-6.95	19.41	18.01	13.38	13.29
6.95 - 7.01	H	4-Ar	6.98-7.01	7.99	8.06	5.47	4.79
7.01 - 7.04	unknown			1.47	1.37	0.43	0.46
7.05 - 7.11	I	2-ether, 4-Ar	7.12	4.57	5.15	6.04	4.95

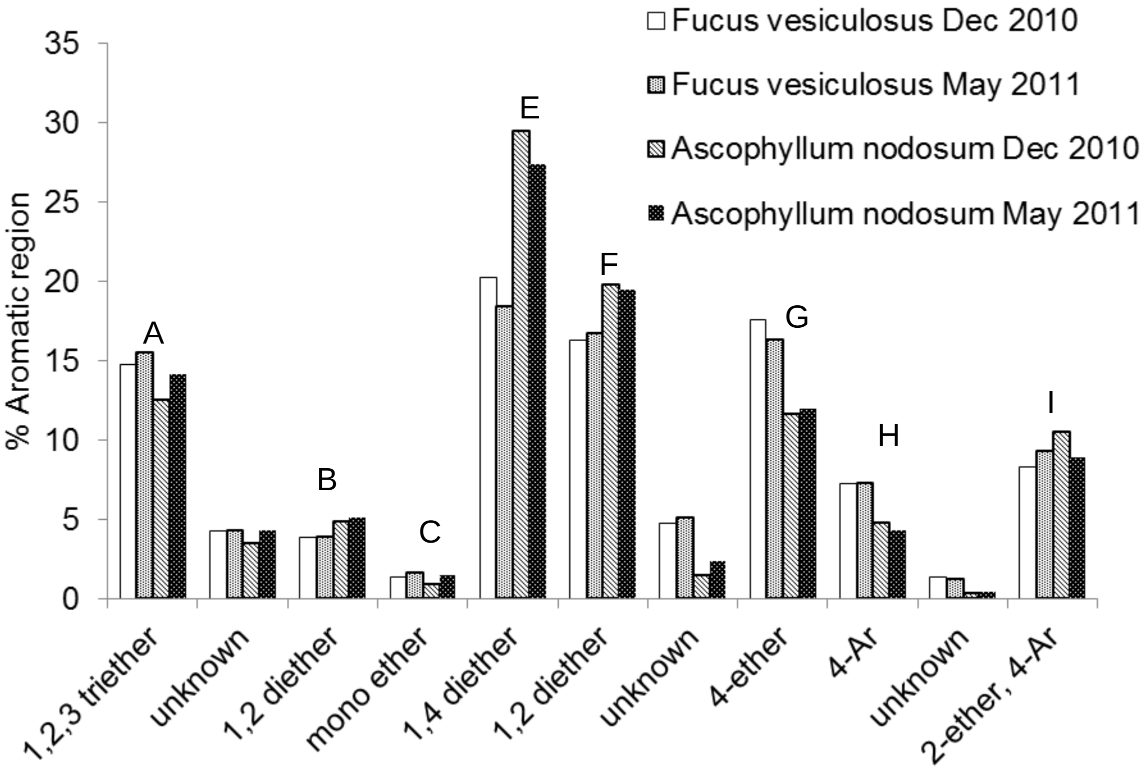


Figure 4.11. The proportions of the different ring types plotted from the data in Table 4.5. Values are weighted based on number of protons on the ring.

It can be seen from the data in Table 4.5 and Figure 4.11 that there are some differences in the proportions of the ring types (see Figure 3.15 for structures). There were two ring types in particular that showed a large difference; *Ascophyllum nodosum* contained almost 1.5 times the amount of ring type E (1,4-diether) as *Fucus vesiculosus*. *Fucus vesiculosus* contained about 1.25 times more of ring type G (4-ether) than *Ascophyllum nodosum*. An estimation was then made from the data in Figure 4.11 of the ratio of Ar-O-Ar to Ar-Ar linkage types (Table 4.6), which found *Ascophyllum nodosum* generally to have a higher amount of Ar-O-Ar linkages to Ar-Ar than *Fucus vesiculosus*.

An estimation of the amount of hydroxyl groups present on the phlorotannin was made by taking the ratio of the total integrals between the acetyl groups and the aromatic protons, which was found to be 1.39:1 in every sample. This means that there was an average of 1.39 hydroxy groups for every aromatic proton on the rings for both species.

Table 4.6. The ratio of Ar-O-Ar to Ar-Ar bond linkage types in the samples.

Species	Month	ratio of Ar-O-Ar to Ar-Ar
Fucus vesiculosus	December 2010	9.81
	May 2011	9.16
Ascophyllum nodosum	December 2010	11.05
	May 2011	12.79

4.2.5 Radical scavenging capacity

The high antioxidant capacity of the phlorotannin extracts makes it of interest for applications in the cosmetic and food industry.[50, 73-75] It was therefore of interest to determine if there was seasonal variation in the antioxidant capacity of the phlorotannin extracts. The seasonal variation in antioxidant capacity of phlorotannin extracts has not previously been reported and so this study represents the first of its kind. The radical scavenging capacity of several of the samples was measured using the DPPH assay in order to determine if there was a difference between phlorotannin extracts at different times of the year. The samples measured were the same as analysed by NMR in Figure 4.9.

The EC_{50} values are given below in Table 4.7 and also plotted as a bar chart in Figure 4.12. The EC_{50} values ranged between 6.56 – 7.46 for *Fucus vesiculosus* and 7.29 – 8.14 for *Ascophyllum nodosum*. The average EC_{50} value of *Fucus vesiculosus* was 0.94 µg ml^{-1} lower than *Ascophyllum nodosum*, meaning that on average, *Fucus vesiculosus* has a greater radical scavenging capacity than *Ascophyllum nodosum.*

An attempt was then made to rationalise the results of the DPPH assay using the differences in structure from the NMR analysis. If one species had had a higher proportion of hydroxyl groups then it would be expected to have had a higher antioxidant capacity. Since no difference was found, a comparison was then made based on the types of ring linkage

Table 4.7. The radical scavenging capacity of several of the samples, expressed as EC_{50} values.

Month	*Fucus vesiculosus*		*Ascophyllum nodosum*	
	EC_{50} ($\mu g\ ml^{-1}$)	SD	EC_{50} ($\mu g\ ml^{-1}$)	SD
December 2010	6.81	0.44	7.87	0.25
May 2011	6.68	0.29	8.14	0.18
November 2011	7.46	0.34	7.29	0.32
March 2012	6.56	0.40	8.00	0.08
average	6.88	0.40	7.82	0.37

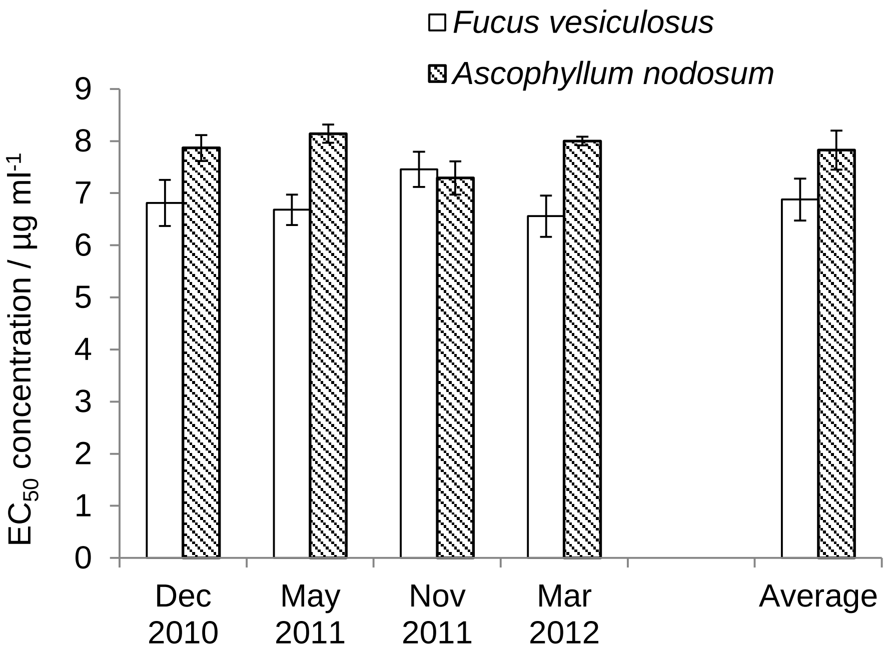


Figure 4.12. The radical scavenging capacity of several of the samples, expressed as EC_{50} values.

The EC_{50} values were then plotted against the ratio of Ar-O-Ar to Ar-Ar (Figure 4.13). The greater the radical scavenging capacity, the lower the EC_{50}. The graph would suggest that EC_{50} increases when the proportion of Ar-O-Ar increases, however more data points would be needed to confirm this.

This relationship could be rationalised based on the fact that Ar-Ar linked rings would be conjugated and therefore better able to stabilise the radicals. *Fucus vesiculosus* had more Ar-Ar linkages than *Ascophyllum nodosum* which would explain its higher antioxidant capacity (characterised by a lower EC_{50}).

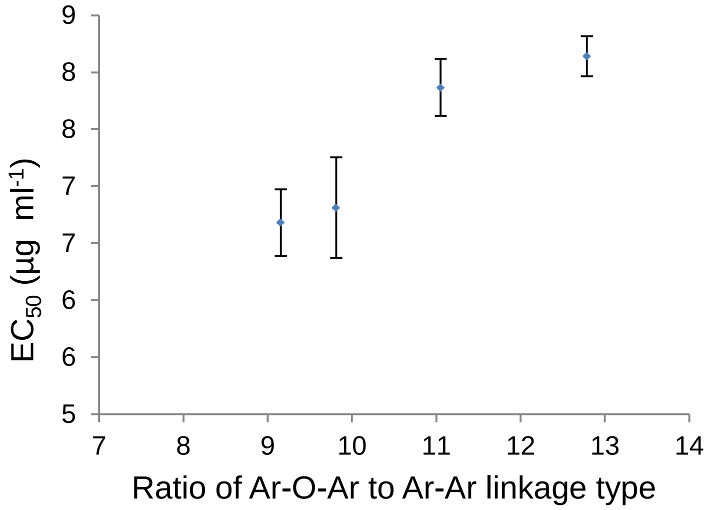


Figure 4.13. The relationship between EC_{50} and the ratio of Ar-O-Ar to Ar-Ar linkage type.

The large number of different ring types means that it is not possible to conclusively attribute the difference in radical scavenging capacity of the two species to specific ring types. However, a possible explanation for why *Fucus vesiculosus* has a higher radical scavenging capacity could be that it has a higher proportion of biaryl couplings rather than biaryl ethers, which means that more of the rings are conjugated together and are better able to stabilise the radicals formed.

4.2.6 Understanding seasonal variation

There are a many factors reported to influence the phenolic content of the seaweed, which complicates the process of understanding how these factors affect the seasonal variation.[76, 77, 91] Some of the key factors known to influence phenolic content are discussed below.

4.2.6.1 Correlations with reproductive cycle

Phenolic levels are known to be heavily influenced by the three main stages of yearly growth and development of seaweed:[221] a ‘summer’ season (typically around mid-June to Aug-Sep) characterised by vegetative growth and production of fruit, a ‘winter’ season (Oct to March), followed by a ‘fruiting’ season (April to June) when gametes are released and before the fruit is finally shed. There is often a steady decline in phenolic content over the fruiting seasonal, followed by a sharp rise towards the end when the fruit is finally shed. Other studies have cited this growth and development to explain observed variation in their results, including Parys *et al.* who reported a seasonal study in which there was a sharp rise around Jun-July, which they also attributed to this growth cycle.[88]

In this study, variation was seen that could be attributed to this development cycle. Figure 4.14 shows the variation in phenolic content, highlighting the fruiting season in the shaded region during 2011 and 2012. During this period, the levels of phenolics were at a maxima in the “winter” stage (Jan-Feb), followed by a drop over the next few months before a sharp drop in the end of the fruiting season coinciding at the point when fruit was being shed.

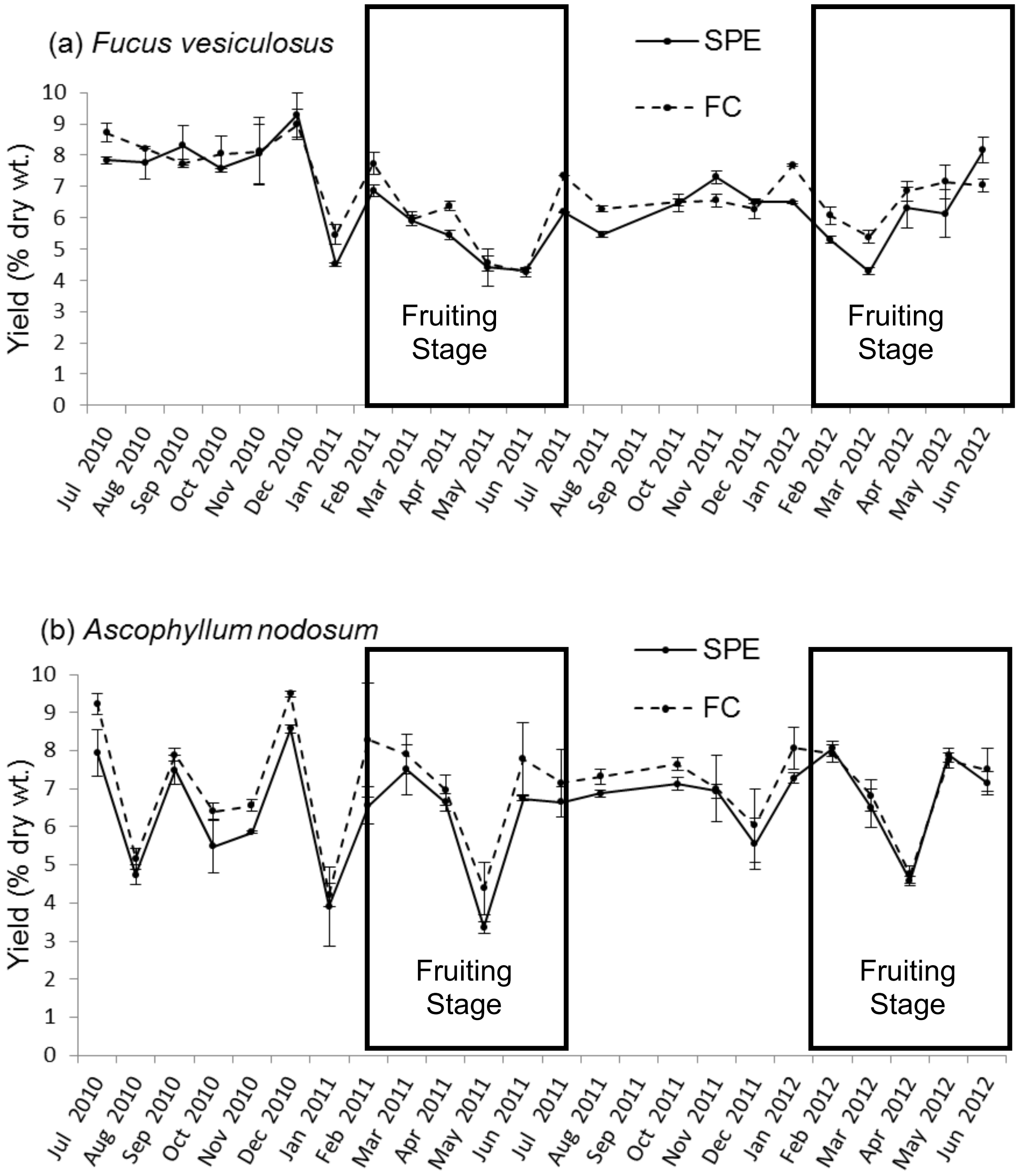


Figure 4.14. The phenolic content (a) *Fucus vesiculosus* and (b) *Ascophyllum nodosum*, highlighting the variation during the fruiting stage in the shaded region between February to June.

4.2.6.2 Sea temperature

The effect of sea temperature at the study site was examined in order to determine if there was an influence on the phenolic content of the seaweed.

Figure 4.15 shows the mean average sea surface temperatures at the study site measured by satellite. The data for the location of the seaweed farm was extracted from an online database after gaining special access, kindly granted by the British Atmospheric Data Centre (BADC).[222]

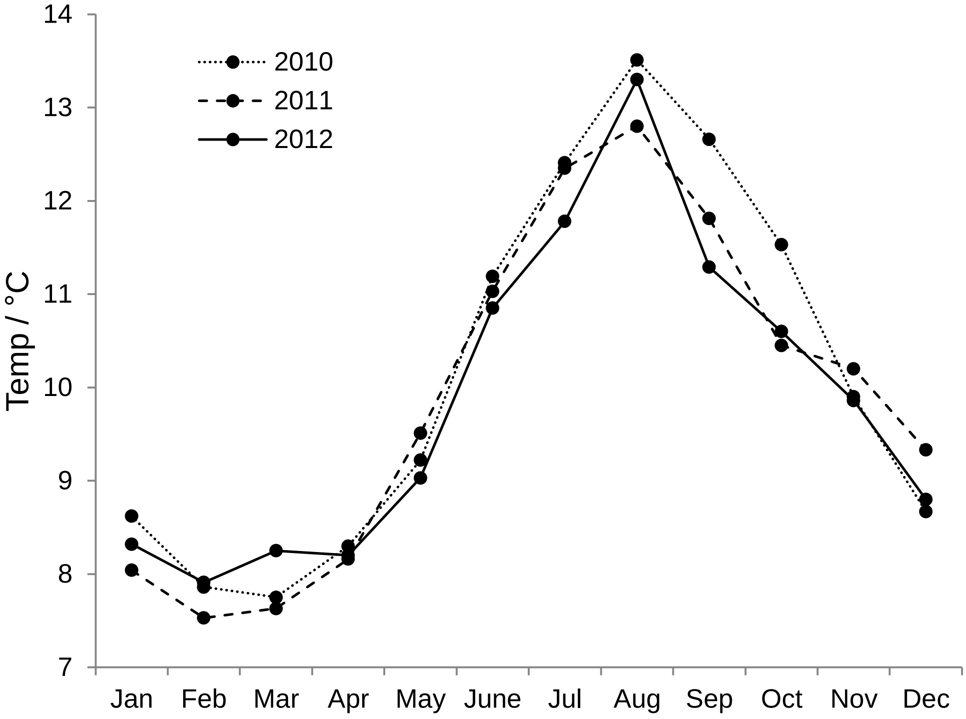


Figure 4.15. The mean average sea surface temperature at the study site at Böd Ayre obtained from satellite data provided by BADC.[222]

It can be seen from Figure 4.15 that the sea temperature varies between about 7.5 °C at its coldest point in February-March to around 13 °C in August. The sea temperature data was examined to look for relationships with phenolic content. It can be seen from Figure 4.14 that the trend observed in the fruiting stage for both species between January to June discussed above happens about a month earlier in 2012 compared to 2011. A close examination of the sea temperatures for these years found that they were approximately 0.5 °C higher during the six months leading up to this period (from November 2011 to April 2012) compared to the same period the previous year. This increase in temperature may have caused the growth cycle to occur earlier in the season, just as happens in land-based plants.

The phenolic content at the spring peak after the fruiting stage is higher for both species in 2012 compared to 2011, suggesting the possibility that the warmer sea temperatures may have led to higher levels of phenolics. The data therefore suggests a possible link between sea temperature and phenolic content, though it is not possible to convincingly argue these links from just two years of data. An extended study would need to be performed over many more years in order to better investigate the relationship that temperature has on phenolic content.

4.2.6.3 Fluctuation range

The range in fluctuation in phenolic content is of particular interest to this study as it could influence the choice of harvest time during a large scale processing operation. The range of fluctuation was compared to previously reported studies on these species.[84-86, 88] The comparison was made based on phenolic content as measured by the FC method because this was also the method used in the previous reported studies. The phenolic content observed in this study ranged between 4.3–9.0 % dry mass for *Fucus vesiculosus* and 4.2–9.2 % for *Ascophyllum nodosum*. This is represented as a histogram in Figure 4.16, from which is can be seen that the range is similar for both species.

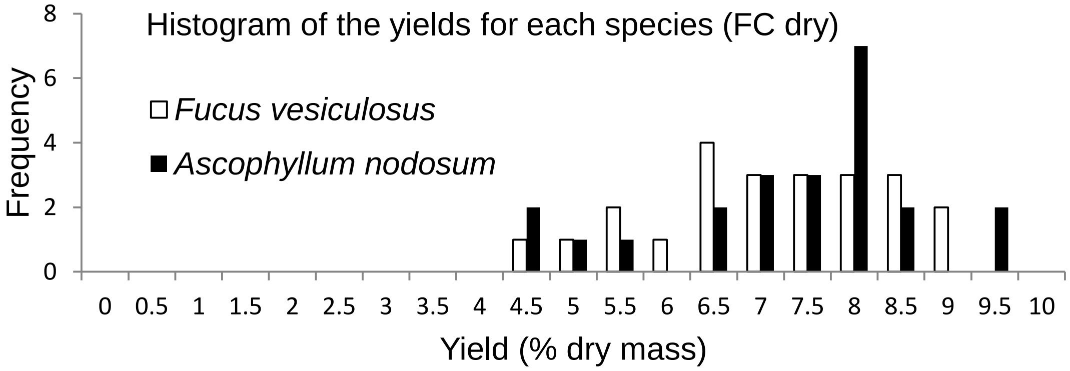


Figure 4.16. Histogram of the yields for each species (FC % dry wt. yield).

Table 4.8 summarises the results for this study along with other studies carried out on these species. The proportional variation in phenolic content for both species in this study is either similar or smaller when compared to other studies on these species.[82-84, 86] It can be seen from the values in Table 4.8 that the phenolic content in some of these studies varied by as much as a factor of 4.5 (Rhode Island in 1995).

Table 4.8. The phenolic content found in other studies performed on *Fucus vesiculosus* and *Ascophyllum nodosum*.

Species	Study Site	Study	Phenolic content (dry % mass)	Year	Ref
Fucus vesiculosus	Shetland, Scotland	Present work	4.3 – 9.0	2010 -2012	
	Rhode island, USA	Peckol	2 – 8 (high tide) 1 – 4.5 (low tide)	1995	[86]
	Aland, Sweden	Ronnberg	4.9-8.3 (shallower site) 5.3 – 6.6 (deeper site)	1984	[85]
	Flak, Norway	Ragen	7 – 14	1976	[84]
Ascophyllum nodosum	Shetland, Scotland	Present work	4.2 -9.2	2010 -2012	
	Oban, Scotland	Parys	0.3 -1	2005	[88]
	Flak, Norway	Ragen	9 – 14.5	1976	[84]

The differences in the magnitude of the fluctuation ranges are likely to be the result of the many factors reported to affect phenolic content.[65, 90,76,77,87,91] The lack of data on these factors means that they cannot be compared between the study sites. However, one study did identify a link between fluctuation range and the level of exposure; when Ronnberg *et al*. studied the difference in phenolic content of seaweed sampled at upper and lower intertidal regions they found that increased tidal exposure gave greater fluctuation in phenolic levels (4.9 – 8.3 % at the shallower site compared to 5.3

– 6.6 % at the deeper site).[85] The shallower site was at a depth of 0.5 – 3 m on a stony gravel and sandy bottom and subject to exposure from low tides during spring. The deeper site was at 3-10 m, experiencing less exposure to tides, but stronger currents. Ronnberg *et al.* offered an explanation based on the light conditions (with the shallower site experiencing more variation in light over the year) and production of phlorotannin as a response to stress factor to protect against oxidative stress from UV radiation.[85] Peckol *et al*. also found similar results in their study (Table 4.8), in which seaweed sampled from higher intertidal regions was also found to have greater fluctuation than seaweed sampled at lower intertidal regions.[86]

This relationship between exposure and fluctuation in phenolic content was then considered in the context of farmed seaweed. The method of growing seaweed on floating ropes meant that it remained at a constant depth, while seaweed growing naturally in and around intertidal zones will have experienced continuous changes of depth relative to the surface. As a result, naturally growing seaweed would have experienced greater variation in levels of exposure. This difference may account for why the fluctuation in levels of phenolics observed in this study was either similar or smaller compared to other studies. Further work is needed to test this hypothesis in which farmed seaweed is studied together with naturally growing seaweed at the same location at different depths.

4.2.6.4 Hours of daylight

Light intensity was suggested by Ronnberg *et al*. as a factor that might affect phenolic content.[85] The different latitudes of the study sites in Table 4.8 meant that they would have received different hours of light. The average monthly hours of daylight at each of the study sites was obtained and is given in Figure 4.17. Rhode Island has a latitude (41 °N)[86] that is lower than the European study sites (56 – 64 °N)[84, 85, 88] and experienced less fluctuation in daylight hours. It also has relatively small variation in phenolic content. Conversely, the study site at Flak, Norway (64 °N),[84] has the highest variation in daylight hours and the highest variation in phenolic content. This suggests that the hours of daylight may influence the level of fluctuation in phenolic content.

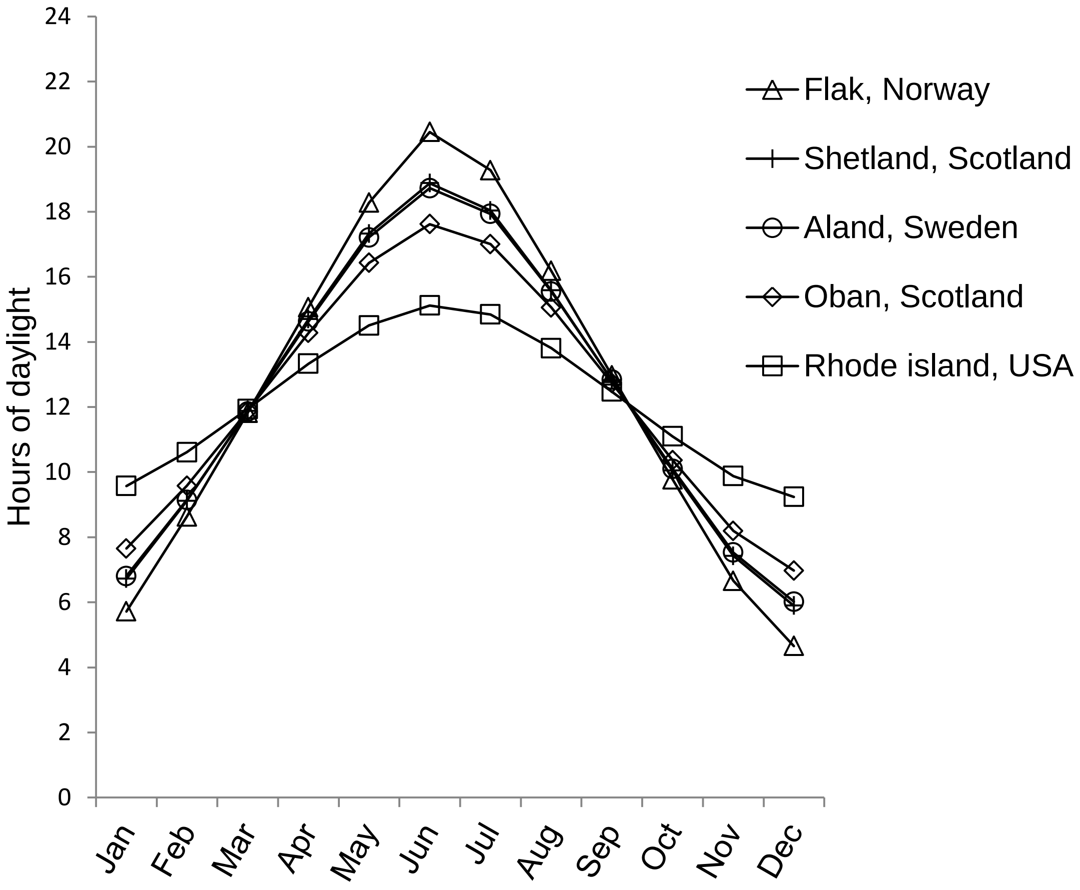


Figure 4.17. Mean monthly hours of daylight at each of the study sites.

4.2.6.5 Solar radiation

Another way of measuring light is to measure the total solar radiation energy. This data was only available for the chosen study site. The mean average monthly solar radiation falling at the study site as measured by a nearby weather station at Lerwick is given in Figure 4.18. The data was analysed to look for any relationship with phenolic content, however, no obvious relationship could be seen.

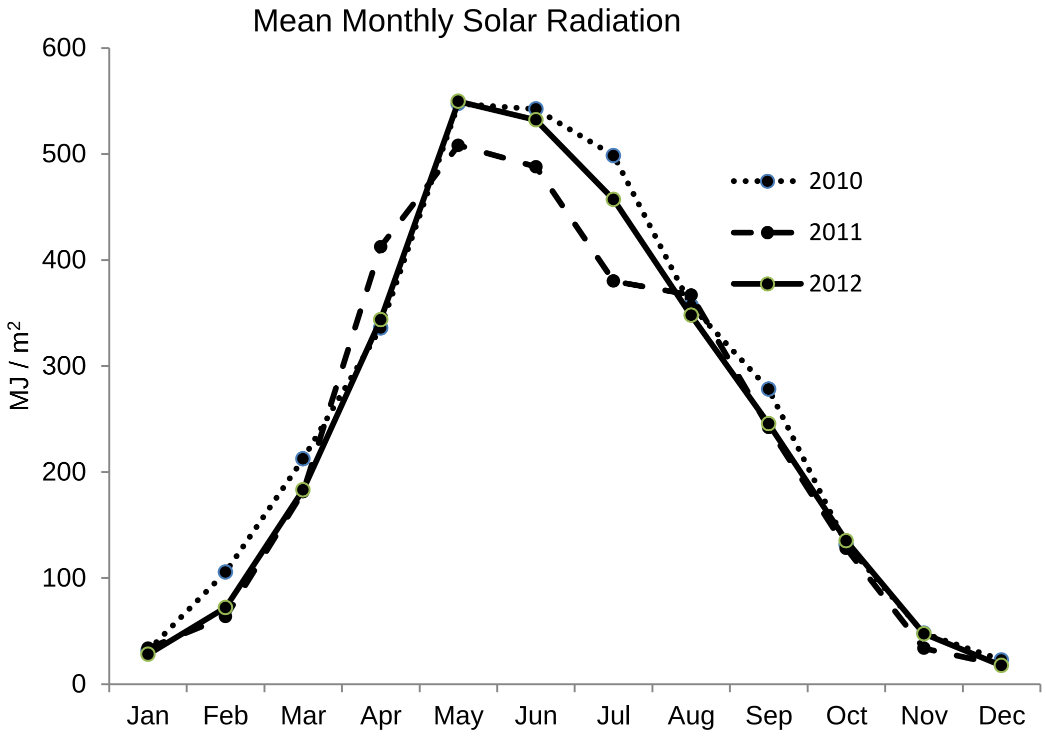


Figure 4.18. The mean monthly solar radiation measured at a nearby weather station in Lerwick. Data provided by BADC.[222]

4.3 Conclusions and implications for seaweed farming

Seaweed farming has huge potential to provide a large quantity of biomaterial for a range of applications. This work represents the first study carried out on the seasonal variation in phenolic content of farmed seaweed, where phlorotannin has been isolated and analysed.

Several conclusions were drawn from this study that have important implications for seaweed farming; firstly, the study confirms that the sustainable, scalable extraction method used is capable of extracting and isolating phlorotannin from the sugars, lipids and other species present to produce a highly pure extract (>95 % as estimated from ^{1}H NMR).

Secondly, the phenolic content of both species was found to vary between 4-9 % (dry mass), which has demonstrated that the phenolic content of these two species of farmed seaweed is sufficient to make it viable to be farmed at any time of the year. And, crucially, the fluctuation of phenolic content was less than other studies, meaning a more reliable harvest can be expected from seaweed farmed in this way.

Thirdly, the analysis of the extracts showed that the structural nature of the phlorotannin extracts and its antioxidant capacity showed little variation throughout the year, allowing for a consistent phlorotannin extract to be obtained from harvest all year round. There were, however, differences in the structural composition of the two species studied, which found that on average the EC_{50} of the phlorotannin from *Fucus vesiculosus* was 0.94 µg ml^{-1} lower than *Ascophyllum nodosum*; a difference corresponding to about 12 %.

The results of the study were compared to weather data, including sea temperature and daylight hours in order to look for correlations. There are many influencing factors on the phenolic content of seaweeds, which complicates an understanding of the mechanisms behind each of them. However, the study did lead to the formation of two hypotheses which has created scope for further research to test them. Firstly, the results have led us to hypothesise that warmer seasonal sea temperatures bring about a larger seasonal spike in phenolic content, as discussed in Section 4.2.6.2. Further work is needed to explore this relationship with temperature, which may have important future implications for choosing the location of seaweed farms based on differences in regional sea temperatures.

Secondly, the results have led us to hypothesise that the unique growing environment of farmed seaweed is responsible for the reduced fluctuation of the phenolic content. Further work is needed to test this hypothesis in which farmed seaweed is studied together with naturally growing seaweed at the same location at different depths. This could be advantageous in farming as it would provide a more consistent yield of phlorotannin. However, it may also be possible to increase the exposure levels in order to stress the seaweed into producing more phlorotannin. In addition to the factors discussed above, there are others that also influence phenolic content.

These further studies could also include the measurement of other parameters such as light intensity, herbivorous predation, desiccation rainfall, salinity *etc.* in order to better understand their influence on phenolic content in seaweed.[223] [224]

5 Polysaccharide Extraction and Analysis

Section 1.8 of the Introduction discussed the different types of polysaccharides found in seaweed. Of all of these polysaccharides, it is the sulphated polysaccharides, found in brown seaweed, that display the most impressive range of bioactivity.[111, 114-116, 118-129] It is important to better understand the structure of these polysaccharides in order to better understand their structure-activity relationships.[108] The mixed structural nature of these polysaccharides greatly complicates their fractionation and characterisation. The analytical techniques currently used in the characterisation of these polysaccharides are often time consuming and give limited structural information.[111, 114] Development of new techniques in the analysis of polysaccharides is therefore needed in order to be able to better characterise them and improve understanding of structure-activity relationships.

This section details the work performed to assess the potential for the use of monoclonal antibodies as an additional technique in the evaluation of extraction procedures for sulphated polysaccharides. For example, antibody techniques may have application during the development stages of extraction methods as a way of quickly screening an extract to confirm its FCSP content.

5.1 Monoclonal antibodies

Section 1.8.7 of the Introduction discussed the wide range of applications for monoclonal antibodies, including their use as a method for the detection of specific compounds and macromolecules.[175] They have been raised before for sulphated polysaccharides, but have not been used as an analytical tool for evaluating extraction procedures.[162, 176-179] This work represents the first study in which antibodies have been used for the quantification of FCSP to evaluate an extraction procedure.

This work was performed as part of a collaboration with another PhD student, Thomas Torode, whose PhD specialised in the development and use of monoclonal antibodies. The major part of Thomas Torode's work was the characterisation the monoclonal antibodies raised towards FCSP. In a collaborative project, these antibodies were then used to analyse the extracts isolated in this work. All of the extraction work was performed by myself and all of the antibody assays presented in this thesis were carried out by Thomas Torode. Details of Thomas Torode's work, including the raising and characterisation of the antibodies will be available from his PhD thesis, due for publication by the University of Leeds in 2014 / 2015.

5.1.1 Generation of the antibodies

Section 1.8.7 discussed the methods by which monoclonal antibodies are made. The antibodies used in this work are listed in Table 5.1, which showed reactivity towards the three main types of polysaccharide present in brown seaweed: laminarin, FCSP and alginate (see Section 1.8.1 for structures). Thomas Torode raised six novel fucan-binding rat monoclonal antibodies (Fu2 - Fu7). All were generated subsequent to immunization with commercially available fucoidan from *Fucus vesiculosus* (Sigma-Aldrich, F5631) coupled to Bovine Serum Albumin (BSA) to generate an immunogen.

Additionally, the group also possessed two other antibodies, LM7 and LM23, which were found to bind to algal cell wall polysaccharides. LM7 was originally raised against pectin homogalacturonan, but was also found to bind to alginate.[225] LM23 was originally raised against xylosyl residues in various glycans and was found to also bind to brown algal cell wall materials.[226, 227] GIN1 and GIN2 are antibodies that bind to alginate.

Table 5.1. Details of the monoclonal antibodies raised, including the polysaccharide source used and a description of the known reactivity.

Antibody name	Reactivity
LM7	laminarin, alginate
LM23	unknown
FUC2	sulfated polysaccharides
FUC3	
FUC4	
FUC5	
FUC6	
FUC7	
GIN1	alginate
GIN2	alginate

5.1.2 Understanding the reactivity

As part of his work on generating the monoclonal antibodies, Thomas Torode needed to characterise their activity in order to understand the nature of the epitopes they bound to. The antibodies were screened against the commercially available polysaccharide used to raise them as well as other polysaccharides in order to understand their reactivity.

FUC2 to FUC7 all showed selective reactivity to sulfated fucans. In order to demonstrate that the antibodies were binding to sulfate groups a sulfated polysaccharide sample was desulfated and then tested against the antibodies again. The antibodies then showed a marked reduction in reactivity towards to desulfated fucans. This demonstrated that the sulphate groups were necessary for the binding of the antibodies.

A potential limitation of using antibodies for this purpose is the issue of cross reactivity. This is where the antibodies show reactivity towards other structures in addition to the ones they are intended for. The FCSP antibodies were screened against each of the other polysaccharides found in seaweed.

No reactivity was found, confirming that the antibodies reacted only with sulfated polysaccharides.

5.2 Extractions

In order to demonstrate the potential of these monoclonal antibodies for use as an analytical tool in the characterisation of polysaccharides, they were used to analyse the extracts obtained from a commonly reported extraction method for FCSP reported by Black *et al*. in 1952.[141] Although this simple method is now largely outdated and superseded by improved methods, it is still of interest here as it is the method used by Sigma Aldrich to obtain the extract they offer for sale as ‘fucoidan’ (Sigma 5631). The crude extract was then further fractionated using a method from the literature using ethanol as an antisolvent.[142-144]

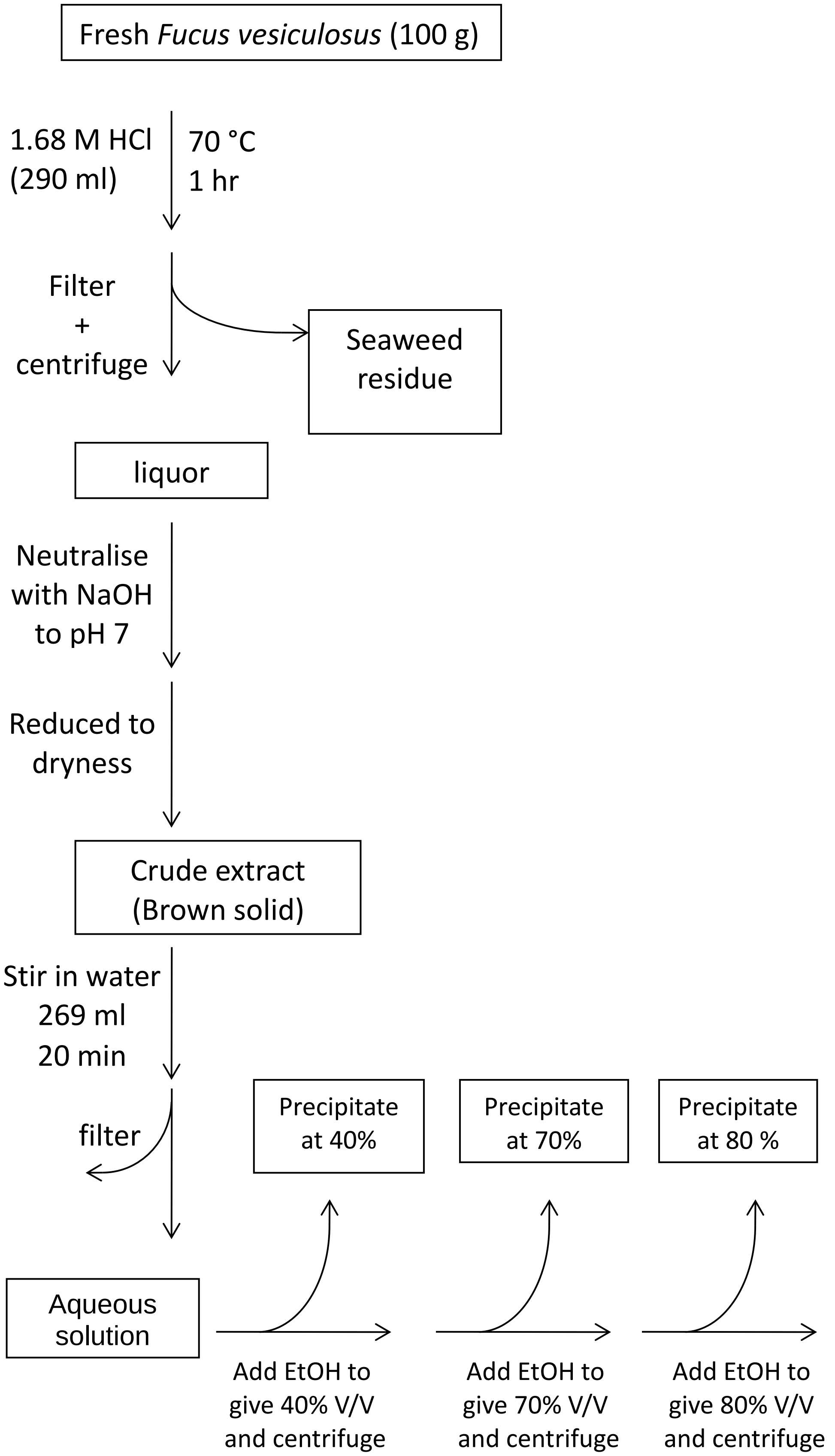
5.3 Polysaccharide extraction

A schematic of the extraction procedure is shown in Scheme 5.1 in which a fresh *Fucus vesiculosus* was extracted with acidified water (HCl, pH 2) for 1 hour at 70 °C to yield a brown solid (30.5 g, 30.5 % wt. fresh). This is typical of the crude yields reported for polysaccharide extractions.[111]

The crude polysaccharide extract was then fractionated using ethanol precipitation. 30 g of the crude extract was redissolved by stirring in deionised water (270 ml) for 20 minutes to form a brown suspension, which was then filtered (Whatman paper) to remove a soft brown residue. Ethanol (30 ml) was added to the filtrate, so as to give a 10 % V/V EtOH solution. No precipitation was observed and so further portions of EtOH were added in the same way so as to increase the concentration by 10% EtOH increments. The solution remained clear until 40% EtOH, at which point a cloudy suspension was observed. This was isolated by centrifugation. Further fractions were obtained in the same way at 70% and 80% ethanol. Further additions of ethanol were not attempted due to the very large quantities of ethanol that would have been required. The yields are shown in Table 5.2. The total amount of material precipitated only corresponds to 6 % of crude extract, meaning the vast majority had remained in solution.

Table 5.2. The yields obtained from the fractionation of the crude polysaccharide extract by precipitation using ethanol.

Description	Yield /g	Yield / % (from 30 g)
40 % fraction	1.01	3.36
70 % fraction	0.583	1.94
80 % fraction	0.357	1.19
Total	1.95	6.49



Scheme 5.1. The procedure for extraction of polysaccharide as reported by Black *et al*.[141] and the fractionation of the extract using ethanol.

The three fractions obtained from ethanol precipitation were then screened by Thomas Torode against the range of antibodies. The results are given in Figure 5.1 below, which shows the binding affinity of the antibodies towards the extracts.

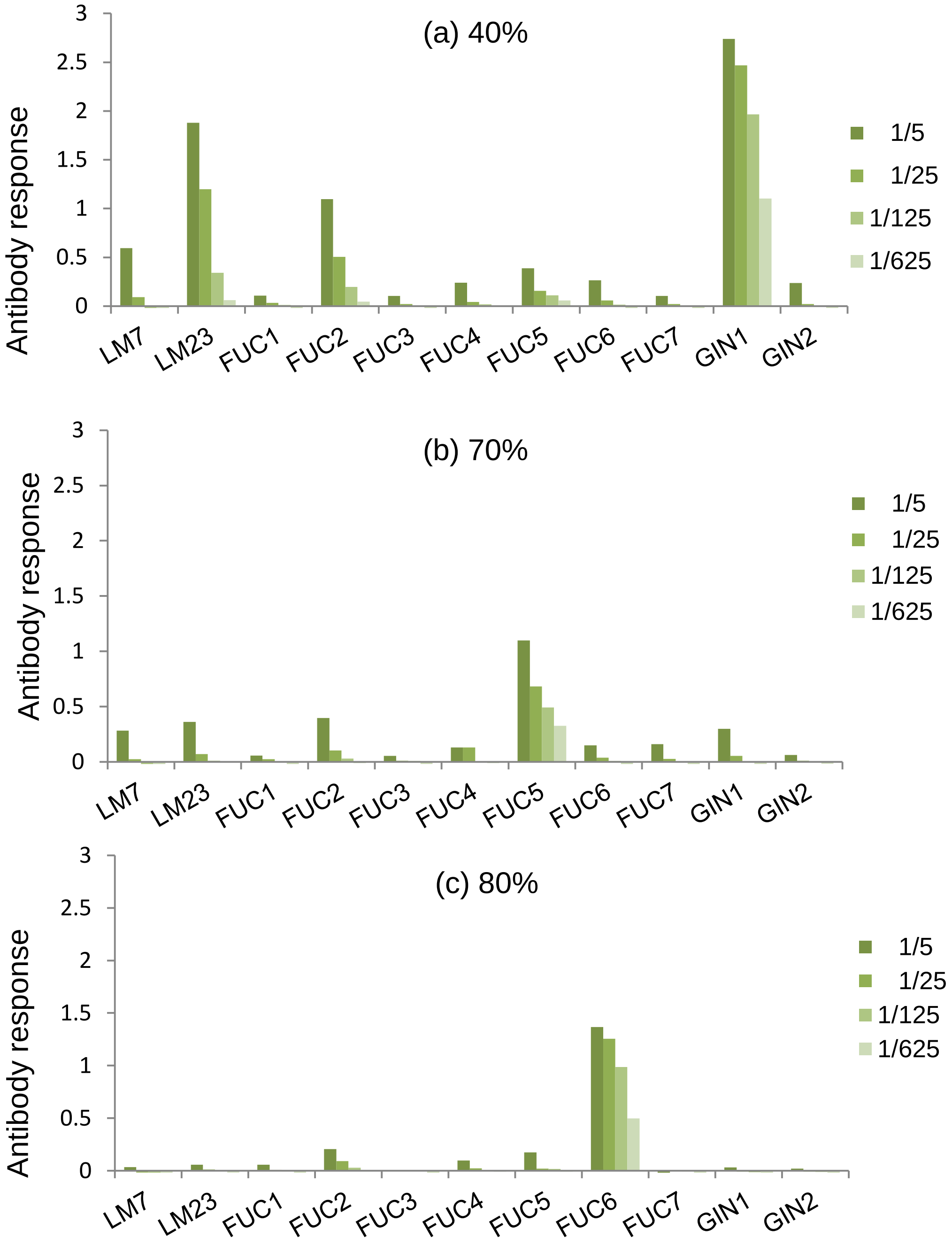


Figure 5.1. The response from the antibody analysis to the fractions obtained from ethanol precipitation. Samples were run at several dilutions as shown.

It can be seen from Figure 5.1 (a) that the 40% fraction showed strong reactivity with antibodies representing each of the major polysaccharides known to be present in brown seaweed; laminarin, fucoidan and alginate (Figure 1.14). There was a strong response against alginate and laminarin and a lesser response against FCSP. In the 70% fraction there was a dramatic drop off in activity against alginate and laminarin, suggesting that it had mostly precipitated in the first fraction. The response for antibody FUC5 was much stronger than the 40% fraction, indicating that this fraction contained more FCSP.

The 80% fraction showed stronger activity almost solely against FUC6, suggesting that it contained a FCSP fraction that has been largely purified from alginate and laminarin. These findings were in agreement with previously reported work that has used ethanol precipitation, which reported that alginate precipitated at lower concentrations than FCSP and laminarin.[142-144]

5.3.1 ^{1}H NMR analysis

Figure 5.2 shows the ^{1}H NMR spectra of the precipitated fractions, which were examined for the presence of methyl groups, which have would have indicated the presence of fucose. Fucose is the only known sugar found in seaweed to contain methyl groups and is present in FCSP, but not in alginate or laminarin. See Figure 1.14 for the structures of the polysaccharides found in seaweed. The triplet peak at 1.17 ppm and the quartet at 3.65 are due to traces of ethanol that remained despite drying under high vacuum for 24 hours. It is likely that the polymeric structure trapped solvent molecules as it dried, such that they were not removed whilst drying under vacuum. All of the fractions contained a broad signal between 1.42-1.19 ppm, which is consistent with where the methyl group on fucose has been reported.[228, 229] The broad signal is due to the varying structure of the fucose rings, giving rise to slightly different shifts. See Section 1.8.2. for the varying structure of FCSP.

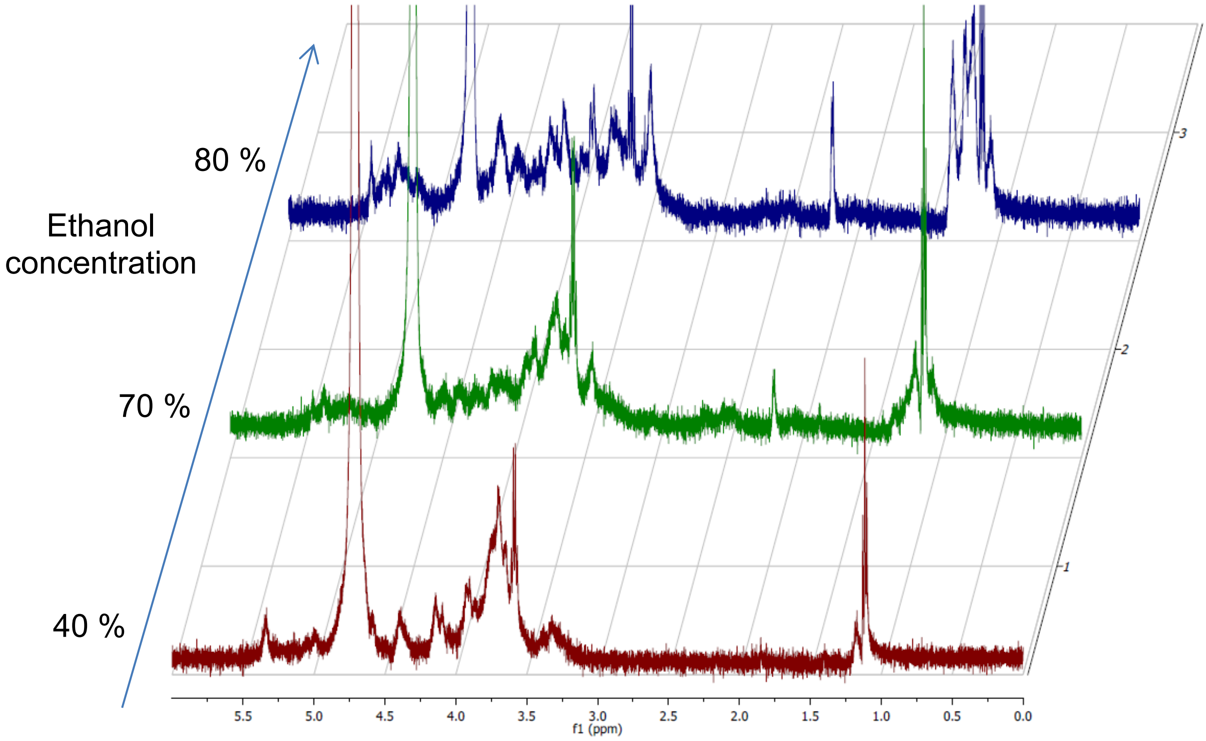


Figure 5.2. ^{1}H NMR analysis in D_2O of the precipitated polysaccharide fractions obtained from increasing the concentration of ethanol.

The NMR spectra were then used to make a rough estimation of the proportion of FCSP in the precipitated polysaccharide extracts. This was done by determining the integral of the methyl peaks as a percentage of the whole spectra between 4.50-1.00 ppm, as shown in Figure 5.3. The 40% EtOH precipitate has a lower fucose content that the crude and is likely to be predominately alginate. The fucose content then increases with increasing ethanol concentration up to 80%. This is in agreement with the results of the antibody assays, in which the FCSP antibodies gave little response for the 40% fraction and then a higher response for the 70% and 80% fractions.

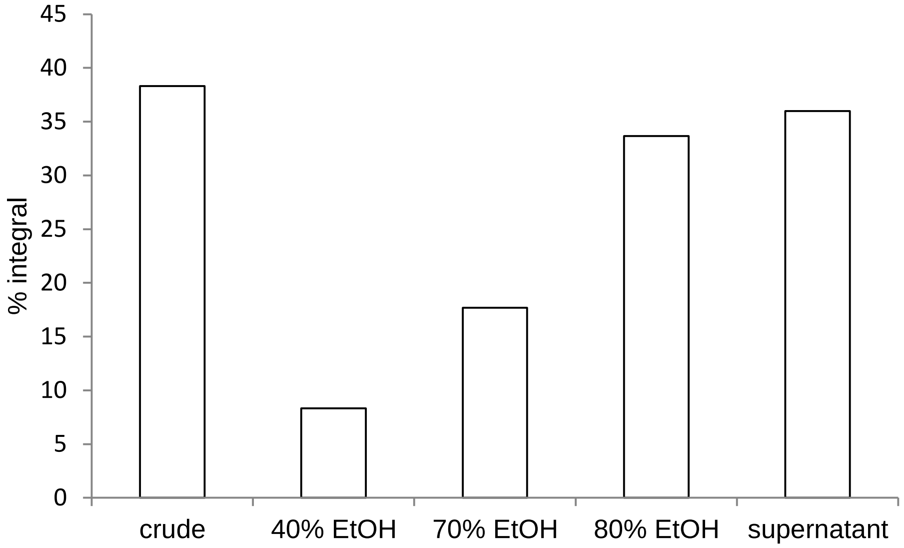


Figure 5.3. The integration of the methyl peak from the polysaccharide extracts as a fraction of the total spectra between 4.50 – 1.00 ppm.

5.3.2 Elemental analysis

The sulphur content of the extracts was then determined by elemental analysis so as to allow comparison with the antibody and ^{1}H NMR data. The results are given in Table 5.3.

Table 5.3. Elemental analysis of the polysaccharide extracts.

Sample	% Elemental analysis			
	C	H	N	S
Crude	11.20	3.05	0.00	1.30
Precipitate at 40 % EtOH	21.00	3.20	0.00	1.15
Precipitate at 70 % EtOH	14.70	2.60	0.20	9.10
Precipitate at 80 % EtOH	18.20	3.20	0.15	10.40
Supernatant	5.50	1.30	0.00	0.90

It can be seen from Table 5.3 that the sulphur content of the precipitated extracts increased in the order precipitation. This is in agreement with both the antibody and NMR analysis above.

5.4 Conclusions

The aim of the work in this section was to evaluate the potential of monoclonal antibodies as an analytical method for the analysis of polysaccharide fractions to quantify FCSP. A commonly used commercial extraction procedure for FCSP was performed and then the crude polysaccharide was fractionated using ethanol. The extracts were screened against a range of antibodies known to have binding affinity to the three major classes of algal polysaccharides, alginate, laminarin and FCSP. The binding response from the FCSP antibodies correlated with the observed results from the ^{1}H NMR and elemental analysis. This work has demonstrated that monoclonal antibodies do have potential in this regard and represents the first time that antibodies have been used for this purpose.

From a practical perspective, there are several advantages and disadvantages of using monoclonal antibodies for this purpose. In terms of cost, it compares favourably with existing methods for quantification of FCSP, such as elemental analysis, which is often expensive and time consuming. By contrast, monoclonal antibodies offer a cheaper method of analysis. Although the production of monoclonal antibodies can be expensive and labour intensive, once produced, they can be cultured to produce a limitless supply at relatively low cost. The assays can be performed on solutions without the need to first obtain dry samples and are extremely sensitive, capable of detecting very low substrate concentrations. They can also be performed in large batches on multi-well microplates allowing many samples to be rapidly screened. It should be noted that a major ethical disadvantage to using antibodies is that an animal is required to generate them, however, as discussed above, once cultured, this then produces a limitless supply of antibodies.

A further application for this approach is envisaged in which an extract is fractionated and screened for bioactivity. The fraction with the highest activity could then be used to raise monoclonal antibodies, which could then be used to optimise an extraction procedure for yield and purity of the desired extract.

6 Pigments and Lipids

Ethyl acetate is another sustainably sourced solvent that could be considered for use in a scalable extraction system. An exploratory extraction from dried *Fucus vesiculosus* with ethyl acetate was performed to determine what could be extracted with this solvent. Experimental details are given in Section 9.19. A dark, green oil was obtained (2.42 % yield dry weight). Analysis by ^{1}H NMR spectroscopy revealed it to predominantly consist of a mixture of triglycerides (as reported by Jin *et al*.[206]). Interestingly, the ^{1}H NMR spectrum showed very little in the way of impurities. Triglycerides of this type are normally colourless and so the dark green colour of the extract was most likely caused by the presence of traces of pigments such as chlorophyll (see Section 1.5.1). Integration of the ^{1}H NMR signals estimated the average chain lengths to be 20 carbons in length and an average of 4.5 double bonds for each molecule.

As discussed in the Introduction in Section 1.1, the lipid content of seaweeds makes them an attractive renewable resource for biofuel production.[2] However, the extraction of lipids with ethyl acetate in this way is unlikely to be a viable extraction method for lipids due to the use of an organic solvent. As discussed in Section 1.6, biofuels made from saturated lipids typically have greater oxidative stability that those made form unsaturated lipids.[50] Consequently, the high level of unsaturation estimated from the NMR analysis could mean that the lipid extracted would be unsuitable for biofuel.

As discussed in Section 1.6 in the Introduction, seaweed lipids are also used as nutritional supplements due to their high content of omega-3 fatty acids, such as that shown in Figure 1.10.

6.1.1 GC-MS analysis

The lipid extract was then further characterised by GCMS so that it could be compared to previously reported lipid extracts. The extract was trans-esterified with methanol to convert the triglycerides into their corresponding fatty acid methyl esters (FAME). The FAME was then purified by silica chromatography to remove traces of green pigments present to obtain a clear, colourless oil, which was then pure by ^{1}H NMR. GC-MS analysis of the purified oil was then performed to identify the carbon chain length distribution. Carbon chain lengths were then determined by comparison to a selection of reference standards, listed in Table 6.1.

Table 6.1. FAME standards used for calibration of GCMS analysis of fatty acids.

Peak	Retention Time (min)	C Chain length	FAME Standard
1	14.90	8	Octanoic acid methyl ester
2	20.05	10	Decanoic acid methyl ester
3	25.31	12	Dodecanoic acid methyl ester
4	30.62	14	Tetradecanoic acid methyl ester
5	35.51	16	(Z)-9-Hexadecenoic acid methyl ester
6	35.75	16	Hexadecanoic acid methyl ester
7	40.21	18	8-Octadecenoic acid methyl ester
8	40.35	18	(Z,Z)-9,12-Octadecadienoic acid methyl ester
9	40.54	18	Octadecanoic acid methyl ester
10	40.77	18	(Z,Z,Z)-9,12,15-Octadecatrienoic acid methyl ester
11	44.97	20	Eicosanoic acid methyl ester
12	48.83	22	(Z)-13-Docosenoic acid methyl ester
13	49.07	22	Docosanoic acid methyl ester
14	53.31	24	Tetracosanoic acid methyl ester

Figure 6.1 shows the GC trace of the extract with the retention times of the standards superimposed as dots (and plotted against their chain lengths on the left axis). The trace of the extract showed that it mostly consisted of even numbered carbon chains between C14 – C20 in length. There were also traces of C15, C17, C19, C22 and C24 present in smaller quantities. This

proportion of the different chain lengths is typical of what is reported in the literature.[43]

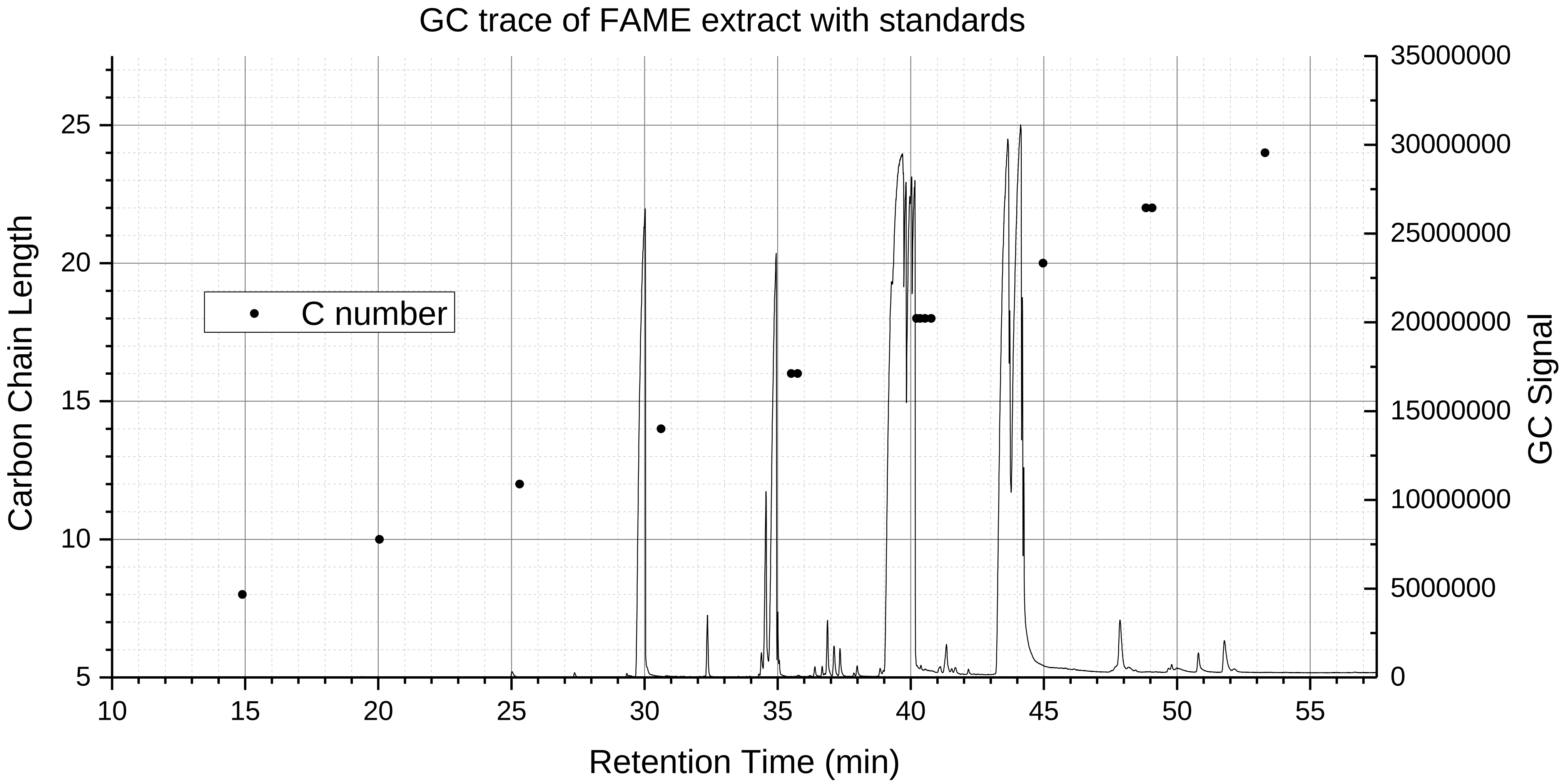


Figure 6.1. GC analysis of the FAME extract from ethyl acetate extraction. The chain lengths of the reference standards are plotted against their retention times (dots) and the trace of the FAME extract.

The GC trace in Figure 6.1 showed that there are multiple peaks corresponding for each carbon chain length, with the exception of C14, which has just one peak. As would be expected, the mass spectra of the carbon chains with multiple GC peaks contained a complex mixture of mass peaks. By contrast, the mass spectra of the peak for C14 was more simple (Figure 6.2) and corresponded to what would be expected from a saturated C14 FAME. A parent ion peak was seen at 243.2 corresponding to [MH]+.

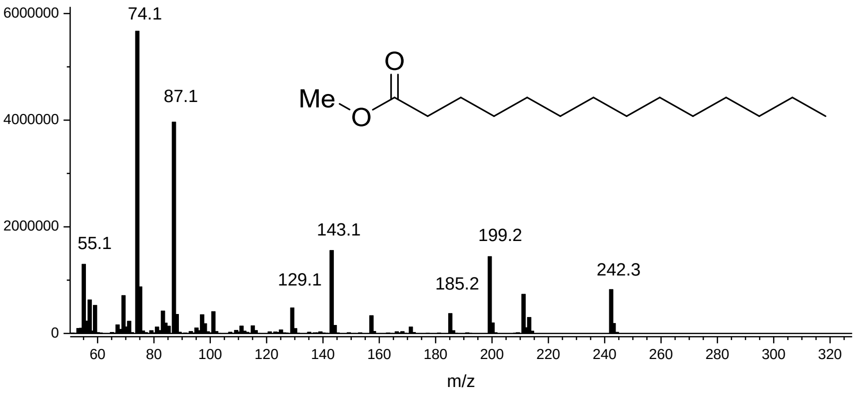


Figure 6.2. The MS data for the GC peak at 30 minutes.

6.1.2 NMR Analysis

The ^{1}H NMR spectra of the FAME extract was examined to confirm that the extract contained omega-3 oils (see section 1.6 for discussion on nomenclature). An example of such a fatty acid is given in Figure 6.3. The ^{1}H NMR spectrum is shown in Figure 6.4 and is consistent with previously published work.[230] See Section 9.20 for full characterisation.

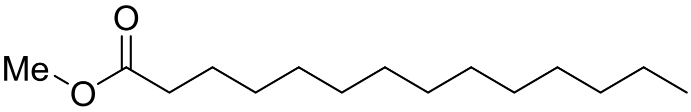


Figure 6.3. An example of an omega-3 FAME.

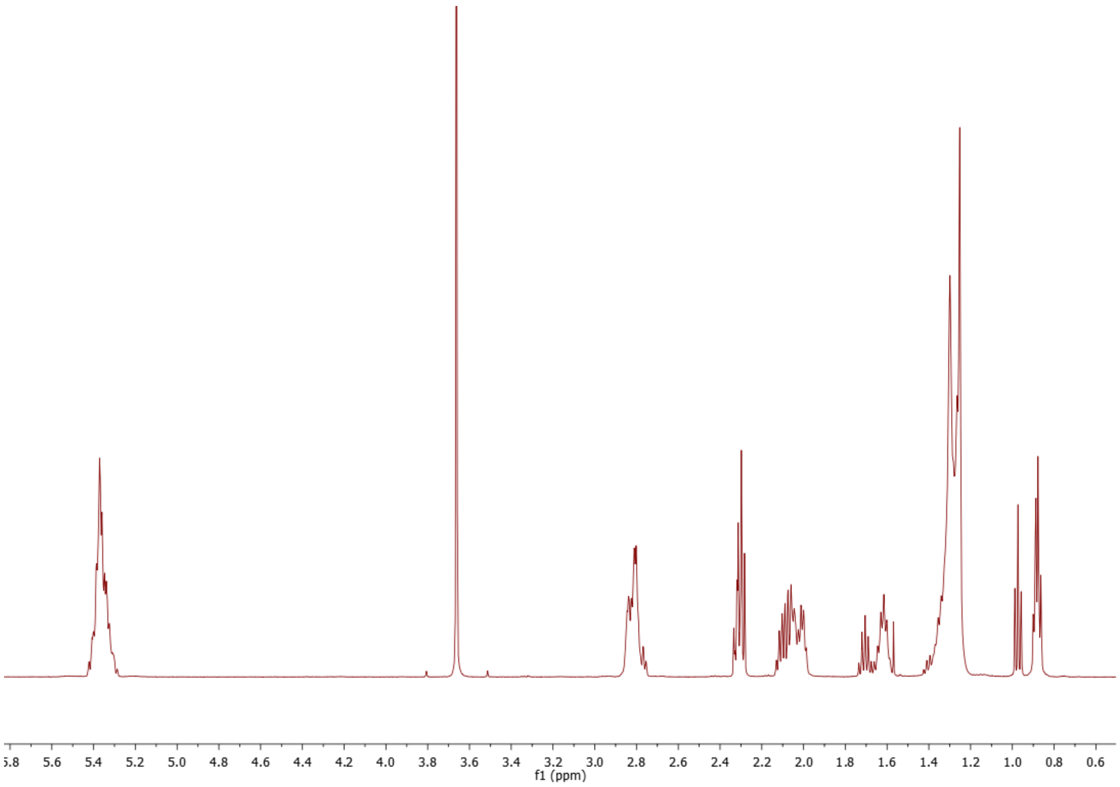


Figure 6.4. The ^{1}H NMR spectrum of the purified FAME mixture.

The COSY spectrum of the FAME mixture is given in Figure 6.5. It can be seen that a terminal methyl group at δ 0.98 is coupled to an allylic CH_2 group, confirming the presence of omega-3 fatty acids in the extract.

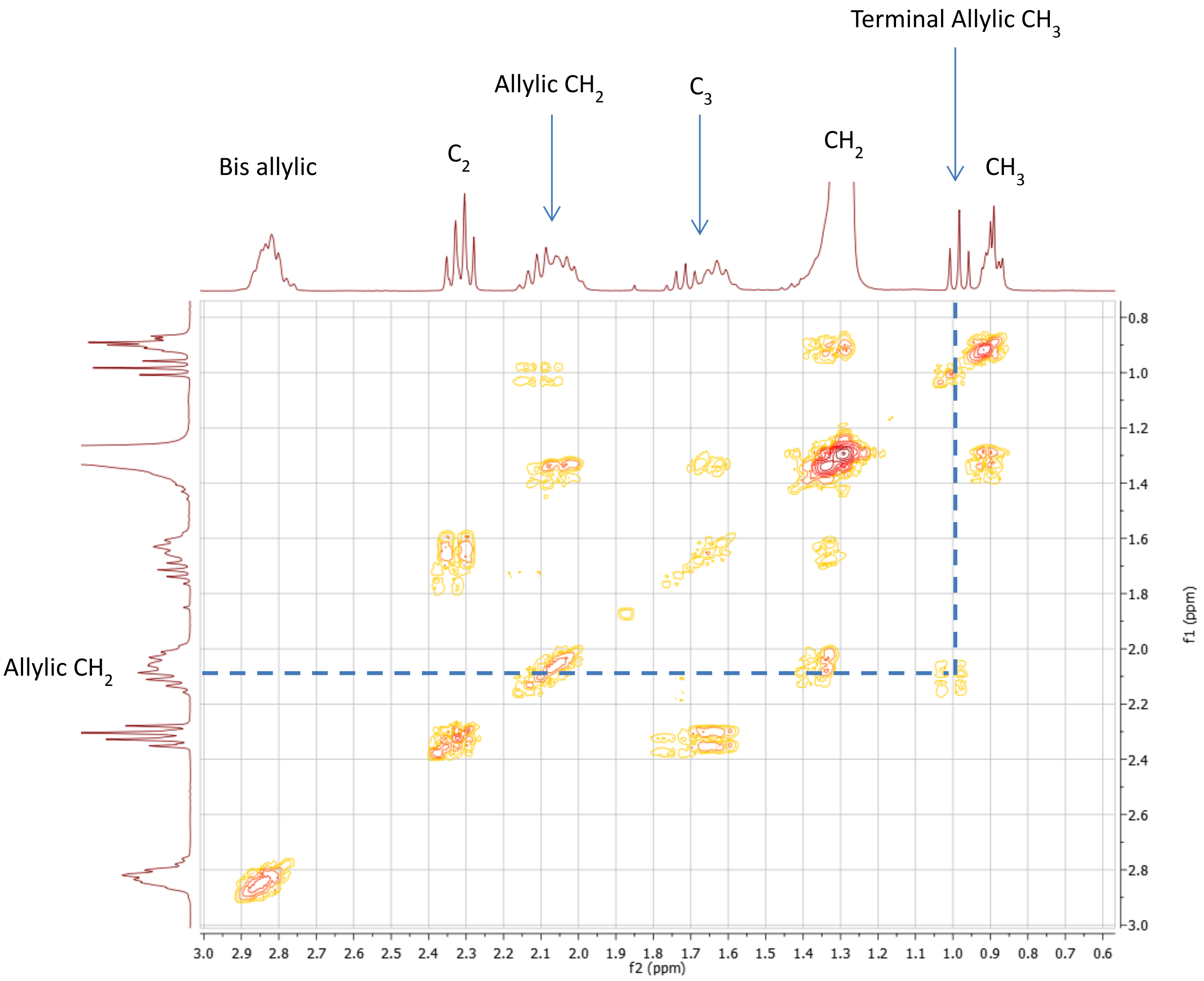


Figure 6.5. The COSY spectra of the purified FAME fraction.

This confirmed that the seaweed contained nutritional value. The methyl group at δ 0.98 accounted for 29 % of the total integral of the methyl groups, allowing an approximation of the quantity of omega-3 fatty acids present.

7 Large Scale Extraction from Seaweed

This section discusses the approaches that could be taken to sequentially extract polyphenols, polysaccharides and pigments from seaweed that would be suitable for on-site processing at a seaweed farm. Ideally, such a method should be a simple, energy efficient process that does not compromise the yield and quality compared to what has been achieved from individual extractions. Work in the previous chapters featured extraction methods targeting specifically either polyphenols or polysaccharides. Table 7.1 shows to what degree each of the solvents used in this work extracted each of the components present in seaweed.

Table 7.1. Summary of which compounds are extracted using different solvents. ✓ denotes an extracted species. (✓) denotes a partially extracted species. X denotes no extraction.

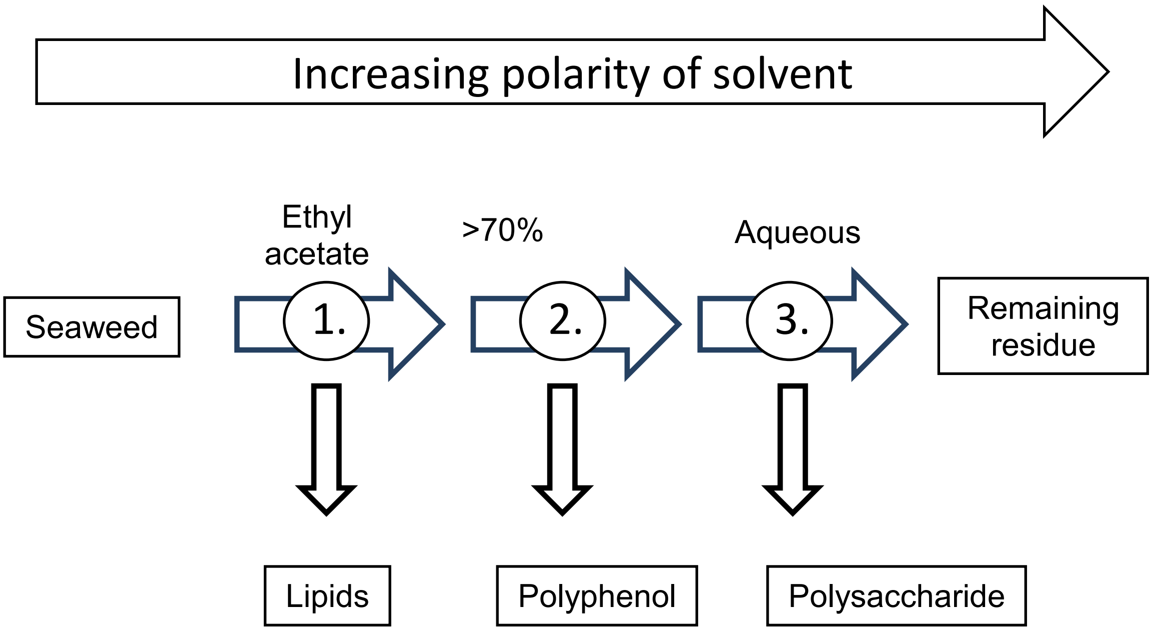
Solvent	Phlorotannin	Polysaccharides	Mannitol	Lipids and pigments
Ethanol	✓	x	✓	(✓)
Chloroform	x	x	x	✓
Ethyl acetate	x	x	x	✓
Water	✓	✓	✓	x
70% Ethanol	✓	✓	✓	✓
70% acetone	✓	x	✓	✓

Of the solvents studied, ethyl acetate, ethanol and water are favoured over acetone and chloroform as they are greener solvents.[231] Based on the knowledge in Table 7.1, several possible approaches to target multiple extractions were considered that use either ethyl acetate, water or ethanol. These are discussed below.

7.1 Sequential Polarity Approach

By applying the knowledge gained in Table 7.1, an obvious approach is to perform several sequential extractions using solvents of increasing polarity, as illustrated in Scheme 7.1. In this approach the lipids and pigments are removed first with ethyl acetate. Phlorotannin could then be removed next using an ethanolic solution greater than 70% (so as to prohibit the extraction of polysaccharides). Finally, aqueous extractions can be performed to obtain polysaccharides.

The lipids and pigments that would normally have been extracted using the 70% ethanol extraction are instead removed during the initial ethyl acetate extraction. As a consequence of this, the phlorotannin extraction is simplified because there is no need for the filtration step to remove these pigments after ethanol is evaporated.



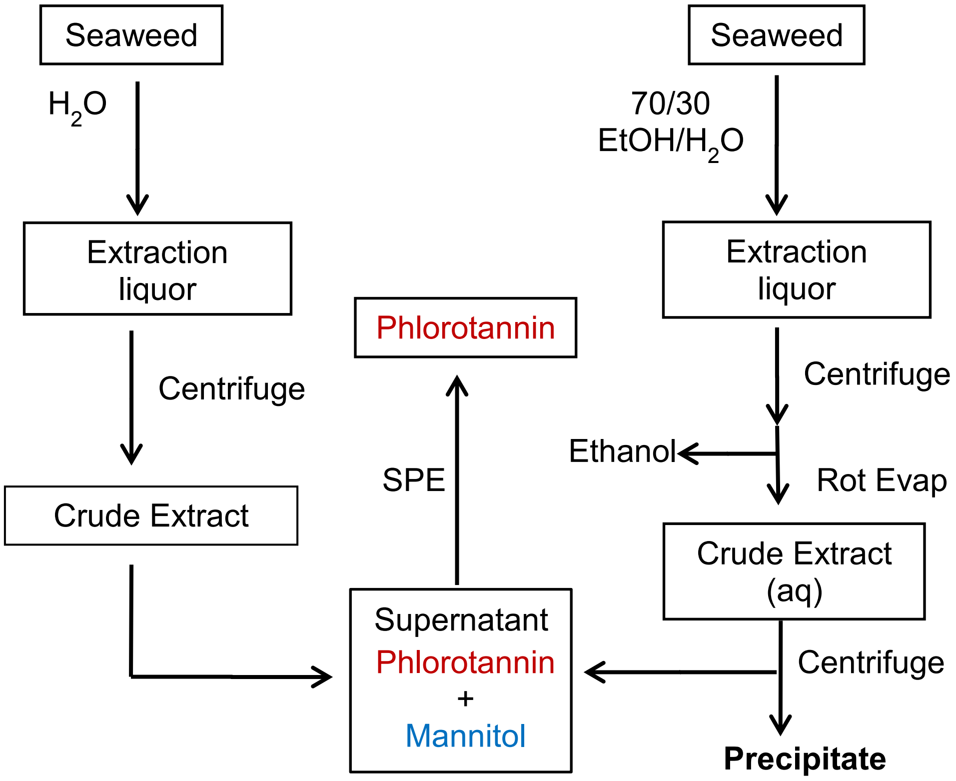
Scheme 7.1. The sequential extraction approach using solvents of increasing polarity to obtain lipids, polyphenols and polysaccharides.

7.2 Single Aqueous Extraction

In this approach, a single solvent is used to extract multiple target compounds, which are then separated from the liquor. Water will extract both polysaccharides and polyphenols. Pigments and lipids will also be released from the seaweed, but are not soluble in the water and so are present in a suspension. Phlorotannin can then be separated from polysaccharides using SPE.

A problem found with water extractions was that the polysaccharides caused the liquor to become highly viscous, which slows down filtration steps. This may be able to be overcome by using larger volumes of water. Using higher volumes of liquor may increase the time needed for SPE processes and may also complicate the separation of polysaccharides.

There are three big advantages of using just water as the solvent system. Firstly, it is cheaper to just use water. Secondly, there are legal restrictions associated with using ethanol; it is a restricted substance and its use is controlled by strict rules relating to handling and storage, which require licences. Thirdly, the extraction procedure for obtaining phlorotannin from the liquor is simpler when extracted by water, as illustrated in Scheme 7.2; the ethanol must be removed by rotary evaporation and then the resulting non-water-soluble precipitate that forms must then be removed by centrifugation and filtration. This is not necessary for water-only extraction.



Scheme 7.2. The purification processes used for processing seaweed extracted using either water or water-ethanol, which illustrates the simplified extraction route of using just water.

7.3 Biorefinery Integration

The large investment in seaweed farming technology from the biofuels sector is likely to mean that the vast majority of the seaweed farmed in the future may be processed for lipids or carbohydrates.[2, 232-234]

This represents an opportunity to utilise this source of seaweed to obtain the other valuable compounds it contains, such as phlorotannin. There would need to be no negative impact on the existing biofuels processing operation (e.g. safety, quality of the lipids, complications with processing).

There are several proposed methods for processing seaweed for biofuels. These include the use of hot, pressurized water, commonly referred to as hydrothermal liquefaction. [232, 234] Other methods include the production of ethanol via fermentation to produce ethanol, and anaerobic digestion to produce methane.[235, 236]

There are benefits to removing the ash from seaweed before processing as it can cause corrosion problems. One such method to remove ash could be with superheated water at 120°C. This process might also provide a convenient opportunity with which to extract phlorotannin. The removal of phlorotannin may also be advantageous for any subsequent anaerobic digestion process, given its known antibiotic properties.[68]

In order to assess whether this pre-treatment process was also suitable for the extraction of phlorotannin, a pilot scale superheated water extraction was commissioned to an external company, Critical Processes Ltd. *Fucus vesiculosus* was selected as it has been used throughout this study. It should be noted however, that *Fucus vesiculosus* is not regarded as a main contender for biofuel production. Other species such as kelps are preferred as they can be grown in larger yields.[237] An extraction from 6.13 kg of fresh *Fucus vesiculosus* with superheated water at 125 °C was performed by Harold Vandenburg and Professor Tony Clifford at Critical Processes Limited. An SPE extraction was then performed to obtain an ethanolic solution of the phlorotannin extract. This was then transported to the University of Leeds where it was reduced to dryness to obtain a phlorotannin extract. The yield was 99.9 g, equating to a 1.63 % fresh weight. The analysis of the extract is discussed below.

7.3.1 ^{1}H NMR Analysis of the Extract

The superheated water extract was acetylated and analysed by ^{1}H NMR in the same way as described previously in section 3.1.7. The results are given below in Table 7.2. The structures of the rings types are given in Figure 3.15. Figure 7.1 shows the results were then plotted along with the results from Section 3.1.7 and the seasonal variation study in Section 4.2.4.

Table 7.2. The relative proportion of the ^{1}H NMR shifts of the acetylated phlorotannin extract.

Observed range (ppm)	Tentative assignment		Typical range (ppm)	% integration of aromatic region
	Ring type	description		
6.2 - 6.42	A	1,2,3 triether	6.26-6.35	12.83
6.42 - 6.51	unknown	unknown		4.11
6.51 - 6.56	B	1,2 diether	6.54	4.23
6.58 - 6.62	C	1- ether	6.56-6.59	1.97
6.62 - 6.71	E	1,4 diether	6.64-6.71	16.94
6.71 - 6.78	F	1,2 diether	6.75	17.96
6.78 - 6.87	unknown	unknown		5.00
6.87 - 6.95	G	4-ether	6.92-6.95	14.11
6.95 - 7.01	H	4-Ar	6.98-7.01	11.93
7.01 - 7.04	unknown	unknown		2.44
7.05 - 7.11	I	2-ether, 4-Ar	7.12	8.47

It can be seen from the graph in Figure 7.1 that the phlorotannin extract obtained from superheated water extraction is very similar in structural composition to the phlorotannin samples isolated in Section 3.1.7 and Section 4.2.4.

The radical scavenging capacity of the extract was measured using the DPPH assay. The EC_{50} value was determined as 8.39 ± 0.59 $\mu g\ ml^{-1}$, which is very similar to the phlorotannin samples isolated in section 3.1.7. From this it can be concluded that there is no significant loss in radical scavenging capacity of the phlorotannin extract when it is extracted at elevated temperatures in superheated water. This demonstrates that a method typical of that used in the extraction of biofuels is also suitable for the extraction of phlorotannin and therefore might allow a way for phlorotannin to be extracted during the biofuel processing of seaweed.[232]

It is important to note there are still some technological and economic challenges to overcome before the methods for processing become energetically and financially viable.[234] The overall profitability of a biorefinery process can be maximised by extracting other high-value products seaweed, such as phlorotannin. The huge potential of seaweed as a natural resource, together with the increasing investment will hopefully drive research to overcome these challenges, thus resulting in a large supply of phlorotannin for use in a variety of industrial applications.[74-77, 234]

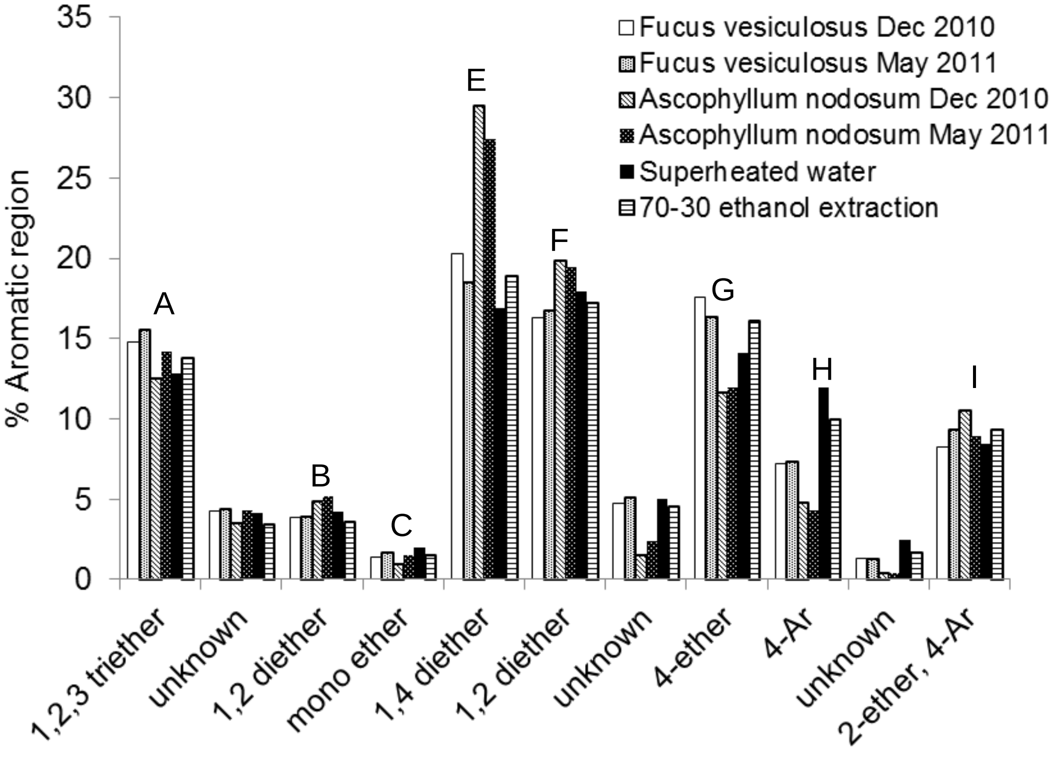


Figure 7.1. The proportions of the different ring types plotted from the data in Table 7.2.

8 Final Conclusions and Suggestions for Further Work

Seaweed represents a large, underused sustainable resource. There is now a rapidly expanding research field exploring the potential for seaweed extracts in a broad range of applications. Recent investment in seaweed farming technology is set to produce a large quantity of seaweed for commercial use. There were several key challenges relating to the utilisation of this seaweed that were addressed in this thesis. The work herein includes several novel contributions to research and has created scope for further research based on the findings.

Phlorotannin Extraction

Phlorotannin is a valuable secondary metabolite produced in brown seaweed. The vast majority of work on phlorotannin has been on the isolation of individual low molecular weight phlorotannins. Whilst the radical scavenging capacity of many of these lower molecular weight polyphenols has been studied, relatively fewer studies have looked at the radical scavenging capacity of whole phlorotannin extracts.

This work represented the first comparative study on the effect of extraction conditions on the radical scavenging capacity of phlorotannin extract obtained and represents an original contribution to knowledge. A comparison of different solvents has demonstrated for the first time that solvent choice does not affect the radical scavenging capacity of phlorotannin extracts obtained, which found them to all have similar EC_{50} values of around 8 µg ml^{-1}. Additionally, the EC_{50} values were not significantly different between 1 hr and 24 hour extractions, meaning that seaweed can be extracted for longer to achieve higher yields without loss of radical scavenging capacity of the phlorotannin.

^1H NMR analysis found the extracts to be similar in structure. A tentative characterisation was made based upon comparison to literature data of low molecular weight phlorotannins that had been isolated, which found the phlorotannin extract to contain several of the key structural ring types in phlorotannin. The ratio of OH to CH on the rings was found to be 1.36:1. Ar-O-Ar linkages were found to be more common than Ar-Ar by a ratio of 11:1. Rings that were coupled to two other rings were found to be more numerous than those coupled to either one or three, which could be rationalised based on the decreasing reactivity of the ring to further coupling as it becomes more substituted. This is also consistent with predominantly long chain structures of linked phloroglucinol units.

The similarity of the EC_{50} values in the radical scavenging assays is what would be expected given the similar nature of the phlorotannin structures found from ^1H NMR. From this it can be concluded that there is no advantage in extracting using any of the solvents tested in order to obtain a phlorotannin extract of higher radical scavenging capacity.

As such, the choice of solvent for a large scale processing operation is influenced by the yield from extraction and the suitability for scale up. Yields generally increased with increasing solvent polarity, with water and organic-water mixtures giving the highest yields with fewest other species extracted. These would therefore be preferred in a large scale operation. The use of water as an extraction solvent has several advantages over ethanol-water mixtures; firstly, it is cheaper to use just water compared to ethanol. Secondly, water is a more sustainable solvent than ethanol. Thirdly, the purification procedure for isolating phlorotannin is simpler for water extraction; this is because ethanol-water mixtures also extracted pigments from the seaweed, which need to be removed. The only drawback of using water was the high viscosity of the liquor, which slowed the filtration steps considerably, which may cause problems during a large scale operation.

There is potential for further research to build on the findings of this chapter; it is not yet fully understood how the different structures on the phlorotannin affect the radical scavenging capacity of the molecule. Further work could study these structure-activity relationships and then apply that understanding in order to fractionate the phlorotannin extract to obtain ‘enriched’ fractions with a higher radical scavenging capacity.

Seasonal variation study

A key consideration for seaweed farming is the seasonal variation in the extracts. This study was the first such study to be performed on farmed seaweed and thus represents an original contribution to knowledge. This was also the first seasonal study in which the phlorotannin was isolated, which allowed for further analysis of the phlorotannin extracts for the first time.

Several conclusions were drawn from this study that have important implications for seaweed farming; firstly, the study confirms that the sustainable, scalable extraction method used is capable of extracting and isolating phlorotannin from the seaweed with high purity (typically >95 % as estimated from ^{1}H NMR)

Secondly, the phenolic content of both species was found to vary between 4-9 % (dry mass), which has demonstrated that the phenolic content of these two species of farmed seaweed is sufficient to make it viable to be farmed at any time of the year. And, crucially, the fluctuation of phenolic content was less than other studies, meaning a more reliable harvest can be expected from seaweed farmed in this way.

Thirdly, ^{1}H NMR analysis found that phlorotannin did not vary significantly in structure throughout the year. This was reflected in the fairly consistent EC_{50} values of the phlorotannin samples measured and demonstrates that a consistent phlorotannin extract can to be obtained from harvest all year round.

There were, however, differences in the structural composition of the two species studied, which found that on average the EC_{50} of the phlorotannin from *Fucus vesiculosus* was 0.94 µg ml^{-1} lower than *Ascophyllum nodosum*; a difference corresponding to about 12 %.

The results of the study led to the formation of two hypotheses which has created scope for further research to test them. Firstly, the results have led to the hypothesis that warmer sea temperatures bring about a larger seasonal spike in phenolic content. Further work is needed to explore this relationship with temperature, which may have important future implications for choosing the location of seaweed farms based on differences in regional sea temperatures.

Secondly, the results have led us to hypothesise that the growing environment of farmed seaweed is responsible for the reduced fluctuation of the phenolic content. Further work is needed to test this hypothesis in which farmed seaweed is studied together with naturally growing seaweed at the same location at different depths. This could be advantageous in farming as it would provide a more consistent yield of phlorotannin. However, it may also be possible to increase the exposure levels in order to stress the seaweed into producing more phlorotannin. In addition to the factors discussed above, there are others that also influence phenolic content.

These further studies could also be expanded to include other species and as well as the measurement of other parameters such as light intensity, herbivorous predation, desiccation rainfall, salinity *etc.* in order to better understand their influence on phenolic content in seaweed.[223] [224]

Sulfated polysaccharides

A study using monoclonal antibodies found that they can be a useful tool to complement existing methods of analysis of polysaccharide extracts for the purpose of evaluating an extraction procedure. The binding response from the FCSP antibodies correlated with the observed results from the ^{1}H NMR and elemental analysis, demonstrating that antibodies can be used as a qualitative method for detection of FCSP.

Future research could develop this work further; there is still work to done in understanding the exact nature of the binding action of the antibodies to the sulfated polysaccharides. Additionally, the sulfated polysaccharide extracts could be fractionated and screened for bioactivity. The fractions with the highest activity could then be used to raise further monoclonal antibodies, which could then be used to optimise an extraction procedure for yield and purity of the desired extract.

Sequential extraction and biorefinery integration

Several possible extraction routes were proposed to target phlorotannin, polysaccharides and lipids from the seaweed using sustainable solvents, ethyl acetate, ethanol and water. Given that the vast majority of seaweed farmed in the future may be destined for biofuels, it made sense to consider whether it could be possible to also extract polyphenols as part of a biofuel extraction process. A process commonly reported for biofuels processing is the use of superheated water. A pilot scale operation was carried out on seaweed using superheated water in order to assess its suitability for extracting phlorotannin. The isolated phlorotannin was found to be similar in structure to that isolated from cold temperature extractions. The EC_{50} value was determined as 8.39 ± 0.59 µg ml^{-1}, which is not significantly different to the phlorotannin samples isolated during cold water extraction. From this it can be concluded that there is no significant loss in radical scavenging capacity of the phlorotannin extract when it is extracted at elevated temperatures in superheated water. This demonstrates that a method typical of that used in the extraction of biofuels is also suitable for the extraction of phlorotannin and therefore might allow a way for phlorotannin to be extracted during the biofuel processing of seaweed.[232] The increasing interest and investment in the use of seaweed by the biofuels sector could result in a large supply of phlorotannin for use in a variety of industrial applications.[74-77]

As discussed in Section 7.3.1, there are still some technological and economic challenges to be overcome before the methods for industrial processing become energetically and financially viable.[234] As new biorefinery processes are proposed to overcome these obstacles, there will be scope for further research to determine if these methods are also suitable for the extraction of phlorotannin.

9 Experimental

Nuclear magnetic resonance (NMR) spectra were recorded for ^{1}H at 500 MHz on a Bruker 500 Ultrashield. Chemical shifts are expressed at parts per million (ppm) downfield of tetramethylsilane as internal standard. The coupling constants are given in Hz, the multiplicities are as follows; s singlet, d doublet, t triplet, q, quartet). LC-MS was performed on an Agilent 2 Series fitted with a Phenomenex Luna C18 5µm x 50mm x 20 mm coupled to a Bruker HCT Ultra mass spectrometer. Elemental analysis was performed on a Thermo Flash EA 1112 Series Analyser. GC-MS analysis was performed on an Agilent 5975B using the RTX 1701 60 m capillary column, 0.25 i.d., 0.25 µm film thickness. The program started at 40 °C and was held for 2 min, then ramped to 280 °C at 60 °C min^{-1} and held for 10 min. The column head pressure was 30 psi at 40 °C. UV measurements for the FC assay were performed on a Jasco V630 Spectrophotometer. UV measurements for the DPPH assay were performed on a Pelkin Elmer 2013 plate reader. Infrared spectra were recorded on a Bruker Alpha Platinum ATR.

9.1 Procedure for drying large batch of *Fucus vesiculosus*

Several large batches of fresh *Fucus vesiculosus* were received from the seaweed farm, Böd Ayre Products Ltd (60.421146 N, 1.102152 W) during March 2013. The seaweed was washed with deionised water and any epiphytes removed and then the surface lightly dried to remove surface water. The seaweed was then dried at 40 °C in an oven and then milled using a coffee blender to a particle size of <500µm. and mixed thoroughly. This yielded 15 kg of dried blended seaweed. All extractions performed on this batch of dried seaweed, unless otherwise stated.

9.2 The FC Assay (determination of total phenolic content)

The method used is that reported by Glombitza *et al.*, but with slight modifications.[98] The method was scaled down so that samples could be prepared directly in 3 ml disposable polystyrene cuvettes (manufactured by Brand GMBH & Co CEL1012). A general background to the assay is discussed in Section 2.2. The method was as follows; a calibration was made using phloroglucinol as the standard. Calibration samples were prepared by adding 1600 µL of phloroglucinol solution to a cuvette, then 100 µL of FC reagent (Sigma). The samples were then mixed and left for 5 minutes before addition of 300 µL of sodium carbonate solution (20 % m/V). The samples were mixed again and left for 40 minutes in the dark. The absorbance at 760 nm was then measured on a Jasco V630 Spectrophotometer. All samples were analysed in triplicate. Figure 2.4 shows the calibration graph for phloroglucinol. When testing extracts for total phenolic content, the same method was used as for the calibration, but replacing the phloroglucinol solution with a diluted solution of the extract. The extracts were diluted as necessary so as to give an assay response in the same range of the calibration graph. Total phenolic content was then expressed in terms of phloroglucinol equivalents (PGE).

9.3 DPPH assay measurements

The method was based on that reported by Sánchez-Moreno *et al*. with some modifications.[198] The background to the assay is discussed in Section 2.3. The method is described below.

Samples were prepared directly in 96-well clear flat-bottomed microplates. Each test was performed in triplicate. Firstly, a solution of the extract in ethanol (200 µL at desired concentration) was added to the well. Secondly, a solution of DPPH (Sigma) in ethanol (360 µM, 200 µL) was added to the wells by use of a multichannel pipette. The microplate was then agitated for 5 seconds using a Thermo Multidrop Combi to mix the samples and the absorbance measured immediately on the plate reader (Pelkin Elmer 2013). The plates were then covered with a lid and left in the dark and then the absorbance was read again exactly 90 minutes after the addition of the DPPH. Negative control experiments were performed by replacing the extract solution with ethanol. Blank samples were made, by replacing the DPPH with ethanol. The %RSA was then calculated using the formula in Equation 2 (p. 64)

9.4 Experiment to test stability of FC sample aliquots

Dried *Fucus vesiculosus* (20 g) was extracted with 100 ml of 70-30 ethanol-water for 14 hours at 22 °C under nitrogen stirred on a hot plate stirrer. The liquor was centrifuged and filtered (Whatman), topped up to 100 ml with ethanol and then sampled (3 x 1 ml aliquots). Samples were stored at 4 °C until analysis by the FC method.

9.5 Comparison of solvent on phenolic content

Dried *Fucus vesiculosus* (20 g) was extracted separately with either ethanol, ethanol-water (70-30 % V/V) or water (200 ml) at 22 °C under nitrogen. 0.5 ml aliquots were extracted from the extraction liquors and stored at 4 °C until analysis using the FC method. The results are given in Table 9.1. Portions of each of the liquors (50 ml) were reduced to dryness on a rotary evaporator and then further dried under high vacuum and the ^{1}H NMR spectra recorded in DMSO, CD_3OD, D_2O and $CDCl_3$.

Table 9.1. The phenolic content of the extraction liquors as measured over time by the FC method. Results are the average of triplicate FC measurements.

Phenolic content of the extraction liquor as measured by the FC method										
Time (hr)	Water		Ethanol		Acetone		Acetone-water (70-30)		Ethanol-water (70-30)	
		SD		SD		SD		SD		SD
0.25					1.82	0.02	10.15	0.19	7.23	0.04
0.5					2.18	0.02	10.38	0.08	7.71	0.05
1	10.84	0.20	1.11	0.51	2.45	0.02	10.63	0.08	8.24	0.06
2	10.91	0.60	0.90	0.09	3.32	0.11	11.14	0.48	9.25	0.07
3	11.01	0.75	1.14	0.06	3.72	0.10	11.65	0.03	9.84	0.06
4	10.80	0.24	1.33	0.05	4.33	0.10	11.91	0.11	10.32	0.08
5	10.98	0.29	1.55	0.23	4.99	0.21	11.89	0.11	10.51	0.11
6	11.58	1.06	1.69	0.13	5.13	0.04	12.38	0.18	10.57	0.20
8	12.09	0.45	2.21	0.14	5.83	0.17	12.74	0.14	11.07	0.42
10	12.29	0.11	2.35	0.44	6.37	0.07	13.22	0.16	11.66	0.11
12	13.02	0.41	2.85	0.17	7.27	0.04	13.43	0.15	11.63	0.13
24	14.61	0.22	4.60	0.14	10.81	1.19	14.07	0.09	12.93	0.23
36	16.21	0.07	5.50	0.09	12.46	0.22	14.08	0.04	13.28	0.30
48	16.70	0.29	6.63	0.37	14.36	0.33	14.08	0.10	13.02	0.28

The yield from the ethanol extraction was 0.906 g (9.06 % dry wt.). δ_H (500 MHz, DMSO) 9.32-8.89 (m), 6.31-5.84 (m), 5.73-5.55 (m), 5.41-5.30 (m), 5.19-5.13 (m), 5.75-5.12 (m), 3.65 (1H, dd, 10.8, 3.4 Hz), 3.59 (1H, d, 8.4 Hz), 3.52-3.41 (m), 2.99-2.94 (m), 2.84-2.80, 2.69 (s), 2.65 (s), 2.33-2.27 (m), 2.33-2.27), 2.08-1.99 (m), 1.61-1.69, 1.37-1.20 (m), 1.09 (t, 7.1 Hz), 0.95 (t, 7.5 Hz), 0.88 (t, 6.8 Hz).

δ_H (500 MHz, CD_3OD) 6.44-5.77 (m), 5.48-5.31 (m), 3.86-3.81 (m), 3.74-3.62 (m), 3.35-3.33 (m), 3.22-3.19 (m), 2.92-2.79 (m), 2.71-2.67 (m), 2.41-2.32 (m), 2.17-2.05 (m), 1.74-1.59 (m), 1.42-1.30 (m), 1.21 (t, 7.1 Hz), 1.01 (m), 0.96-0.91 (m), 0.73 (s).

δ_H (500 MHz, D_2O) 6.13-5.77 (m), 4.42 (dd, 11.7, 2.5 Hz), 4.26 (11.7, 5.6 Hz), 4.13 (13.1 Hz, 3.9 Hz), 4.09 (t, 3.3 Hz), 3.98-3.94 (m), 3.88 (dd, 11.9, 2.7 Hz), 3.82-3.75 (m), 3.69 (dd, 11.8, 6.1 Hz), 3.59-3.55 (m), 3.41 (dd, 14.9, 3.7 Hz), 3.29 (s), 3.18 (dd, 14.8, 7.7 Hz), 2.94 (s), 2.88 (s), 2.16 (s), 1.95 (s), 1.49 (d, 7.3 Hz), 1.24 (s), 1.19 (t, 7.1 Hz).

δ_H (500 MHz, $CDCl_3$) 9.53 (s), 9.49 (s), 9.39 (s), 9.35 (s), 8.55 (s), 8.49 (s), 8.00 (dd, 17.9, 11.2 Hz), 6.40 (s), 6.31 (s), 6.28 (s), 6.26 (s), 6.17 (m), 5.94 (s), 5.43-5.31 (m), 5.27-5.23 (m), 5.18 (q, 10 Hz), 4.51-4.38 (m), 4.34 (dd, 11.5, 3.5 Hz), 3.29 (dd, 11.5, 4.5), 4.22 (m), 4.14 (dd, 11.5, 5.5), 4.01 (m), 3.88 (s), 3.81 (s), 3.71 (m), 3.69 (s), 3.65 (m), 3.55-3.49 (m), 3.40 (s), 3.38 (s), 3.24 (s), 3.23 (s), 2.84-2.76 (m), 2.36-2.26 (m), 2.20 (m), 2.13-2.96 (m), 1.88-1.79 (m), 1.71-1.68 (m) 1.61-1.57 (m), 1.51-1.47 (m), 1.35-1.23 (m), 1.00-1.96 (m), 0.89-0.84 (m) 0.79 (m) 0.69 (s).

The yield from the ethanol-water extract was 2.08 g (20.8 % dry wt.). δ_H (500 MHz, DMSO) 9.30-9.00 (m), 6.30-6.16 (m), 6.08-5.84 (m), 5.75-5.62 (m), 5.57-5.52 (m), 5.39-5.32 (m), 4.70-4.26 (m), 4.17-4.11 (m), 3.64-3.56 (m), 3.52-3.47 (m), 3.44-3.40 (m), 3.24 (s), 2.79 (m), 2.73 (s), 2.04 (s), 1.24 (m), 1.10 (s), 0.94 (t, 7.5 Hz), 0.87 (t, 6.8 Hz).

δ_H (500 MHz, CD_3OD) 6.42-5.77 (m), 5.54-5.33 (m), 3.85-3.81 (m), 3.75-3.67 (m), 3.28-3.22 (m), 3.08-3.03 (m), 2.91 (s), 2.78 (s), 2.38-2.33 (m), 2.11 (s), 1.66-1.59 (m), 1.51 (d, 7.3 Hz), 1.34-1.30 (m), 1.04 (m), 1.03 (m), 1.01 (m), 1.01 (m), 0.99 (m), 0.95-0.91 (m), 0.75 (m).

δ_H (500 MHz, D_2O) 6.37-5.94 (m), 4.42 (dd, 11.9, 2.6 Hz), 4.26 (dd, 11.8, 5.6 Hz), 4.14 (dd, 13.0, 3.5 Hz), 4.10 (t, 3.3 Hz), 4.04-3.95 (m), 3.89 (dd, 11.8, 7.2 Hz), 3.82 (d, 8.6 Hz), 3.78 (ddd, 8.6, 6.1, 2.8 Hz), 3.69 (dd, 11.8, 6.1 Hz), 3.60-3.66 (m), 3.42 (dd, 11.8, 6.1 Hz), 3.35 (s), 3.33 (s), 3.30 (s), 3.19 (dd, 14.9, 7.8 Hz), 3.08 (s), 2.95 (s), 2.89 (s), 2.53 (s), 2.49-2.45 (m), 2.16 (s), 1.99 (s), 1.50 (d, 7.3 Hz), 1.34 (dd, 17.3, 6.6 Hz), 1.24 (s), 1.11 (s).

δ_H (500 MHz, $CDCl_3$) 5.41-5.29 (m), 4.40-3.50 (m), 3.40 (s), 3.25 (s), 2.85-2.76 (m), 2.36-2.35 (m), 2.10-2.04 (m), 1.94-1.70 (m), 1.57 (d, 6.5 Hz), 1.30 (broad), 1.25 (broad), 1.16-1.06 (m), 1.01 (s), 1.00-0.96 (m), 0.90-0.84 (m), 0.69 (s), 0.69 (s).

The yield from the water extract was 3.54 g (35.4 % dry wt.). δ_H (500 MHz, DMSO) 9.27-8.92 (m), 6.28-5.82 (m), 5.39-5.32 (m), 4.57-4.59 (m), 4.46-4.39 (m), 4.30-4.23 (m), 3.65-3.39 (m), 2.73 (s), 2.04 (s), 1.26 (s), 1.09 (t, 7 Hz).

δ_H (500 MHz, CD_3OD) 6.26-5.93 (m), 5.42-5.343 (m), 3.86-3.81 (m), 3.74-3.71 (m), 3.69-3.61 (m), 3.28-3.24 (m), 3.08-3.03 (m), 2.94 (m), 2.89-2.82 (m), 2.11

(s), 1.67-1.60 (m), 1.50 (d, 7.2 Hz), 1.38-1.29 (m), 1.21 (t, 7.0 Hz), 1.05-0.98 (m), 0.94-0.91 (m).

δ_H (500 MHz, D_2O), 3.85 (2H, dd, J = 11.7, 2.8 mannitol), 3.78 (2H, d, J = 8.7 mannitol), 3.74 (2H, ddd, J = 8.6, 6.0, 2.7 mannitol), 3.66 (2H, dd, J = 11.5, 5.8 mannitol), 2.94 (s), 2.16 (s), 2.16 (s), 1.49 (d, 7.2 Hz), 1.24 (s), 1.20 (t, 7.1 Hz).

δ_H (500 MHz, $CDCl_3$) 5.41-5.32 (m), 4.16-4.11 (m), 2.85-2.73 (m), 2.62 (s), 2.36-2.29 (m), 2.17 (s), 2.09 (s), 2.04 (s).

9.6 General procedure A - SPE extraction

The general procedure for the solid phase extraction (SPE) of phlorotannin from an aqueous extraction liquor (as shown in Scheme 3.1) was as follows; the Amberlite XAD 7 HP resin (purchased from Acros) was first washed to remove the preservation salts by shaking on an orbital shaker for 30 minutes with deionised water (10 column volumes (C.V.)). The resin was then filtered and further washed with ethanol (3 C.V.) and again with water (10 C.V.). Solid phase extraction was then performed by adding 40 g of the cleaned resin to the extraction liquor and shaking on an orbital shaker for 30 minutes (Step 1 in Scheme 3.1). The mixture was then filtered to remove the liquor. The resin was then washed with 10 C.V. of water to remove sugars (Step 2). The phlorotannin was then recovered by eluting with ethanol until it ran colourless (step 3). The resin was then washed with 10 C.V. of water to remove any ethanol and then used to perform further extractions on the liquor in the same way until no more phlorotannin could be extracted (Step 4). The combined ethanol fractions (clear brown) were reduced to dryness on a rotary evaporator and then further dried under high vacuum to yield the phlorotannin extract as a dark brown powder.

9.7 Isolation of phlorotannin from ethanol extraction

Dried *Fucus vesiculosus* (10 g) was extracted for either 1 hr or 24 hr in ethanol (100 ml). The liquor was centrifuged and filtered (Whatman) and then reduced to a volume of 20 ml and then diluted with to a volume of 80 ml with water and then further reduced down to 50 ml to remove any remaining ethanol. The liquor was then diluted to a volume of 100 ml with water and then centrifuged and filtered (Whatman). Phlorotannin was isolated using General Procedure A for SPE extraction. The yield for 1 hr was 28.0 mg (0.28 % dry wt. %). δ_H (500 MHz, CD_3OD) 6.44-5.77 (multiplets), 5.41-5.31 (m), 4.38-3.32 (m), 4.25-4.06 (m), 3.82-3.61 (m), 2.88-2.76 (m), 2.38-2.25 (m), 2.14-2.01 (m), 1.99 (s), 1.93 (s), 1.65-1.55 (m) 1.40-1.19 (m), 1.01 (s), 1.98 (t, 7.5 Hz), 0.90 (m). The yield from 24 hour extraction was 279 mg (2.79 % dry wt. %). δ_H (500 MHz, CD_3OD) 6.44-5.77 (multiplets). v_{max}/cm^{-1} 3214 (broad), 1605, 1500, 1462, 1261, 1142, 1032, 997, 814.

9.8 Isolation of phlorotannin from ethanol-water extraction

Dried *Fucus vesiculosus* (10 g) was extracted for either 1 hr or 24 hr in 70-30 ethanol-water (100 ml). The liquor was centrifuged and filtered (Whatman) and then reduced to a volume of 20 ml. The liquor was diluted to a volume of 100 ml with water and then centrifuged and filtered (Whatman). Phlorotannin was isolated using the General Procedure A for SPE extraction. The yield for 1 hr was 530 mg (5.3 % dry wt. %). δ_H (500 MHz, CD_3OD) 6.44-5.77 (multiplets). v_{max}/cm^{-1} 3183 (broad), 1604, 1500, 1462, 1258, 1144, 1032, 999, 815. The yield from 24 hour extraction was 490 mg (4.90 % dry wt. %). δ_H (500 MHz, CD_3OD) 6.44-5.77 (multiplets). v_{max}/cm^{-1} 3177 (broad), 1606, 1500, 1460, 1260, 1144, 1032, 998, 815

9.9 Isolation of phlorotannin from water extraction

Dried *Fucus vesiculosus* (10 g) was extracted for either 1 hr or 24 hr in 70-30 ethanol-water (100 ml). The liquor was centrifuged and filtered (Whatman). Phlorotannin was isolated using the General Procedure A for SPE extraction.

The yield for 1 hr was 397 mg (3.97 dry wt. %). δ_H (500 MHz, CD_3OD) 6.44-5.77 (multiplets). v_{max}/cm^{-1} 3179 (broad), 1601, 1500, 1460, 1260, 1142, 1032, 999, 811. The yield from 24 hour extraction was 391 mg (3.91 dry wt. %). δ_H (500 MHz, CD_3OD) 6.44-5.77 (multiplets). v_{max}/cm^{-1} 3188 (broad), 1604, 1500, 1459, 1260, 1143, 1032, 998, 813. The radical scavenging capacity of the extracts was measured using the DPPH assay is given below in Table 9.2.

Table 9.2. The EC_{50} radical scavenging capacities of the phlorotannin extracts isolated from *Fucus vesiculosus* using different extraction solvents.

Solvent	Time (hr)	EC_{50} ($\mu g\ ml^{-1}$)	SD
Ethanol	1	16.35	0.90
	24	8.46	0.61
Ethanol-water (70-30)	1	8.34	0.45
	24	7.63	0.56
Water	1	8.29	0.55
	24	7.92	0.44

9.10 General procedure B - The acetylation of phlorotannin extracts

The general procedure for the acetylation of phlorotannin was as follows; phlorotannin extract (200mg) was dissolved in pyridine (2 ml) with DMAP (5.0 mg, 0.041 mmol) under nitrogen. Acetic anhydride (2 ml, 21.2 mmol) was added slowly over 5 minutes. The solution was stirred for 18 hours under nitrogen and then poured into a well-stirred ice slurry (40 ml). The brown precipitate was collected at the pump and washed with cold water (5 ml). The precipitate was redissolved in chloroform (3 ml), washed with 5% HCl (2 x 3 ml), sat. Na_2CO_3 (1 x 3 ml) and brine (2 x 3 ml) dried, ($MgSO_4$), reduced to dryness on a rotary evaporator and then further dried under high vacuum to yield a light brown amorphous solid.

9.11 Acetylation of phlorotannin sample in Section 3.1.7

Acetylation was performed as described in General Procedure B using phlorotannin isolated from the ethanol-water extraction. The yield was 279 mg. δ_H (500 MHz, $CDCl_3$) 7.13 – 6.20 (multiplet), 2.28 – 1.76 (multiplet). ν_{max}/cm^{-1} 1768, 1612, 1482, 1430, 1367, 1169, 1113, 1037, 1017, 881, 734.

9.12 Preparative HPLC separation of phlorotannin

A solution was prepared by dissolving phlorotannin (1.01 g) in HPLC-grade H_2O (4 ml). 1500 µL of the solution was injected into a Gilson prep-HPLC fitted with a phenomenex hyperclone C18 prep column (250 mm x 21.1 mm x 5 µm), flow rate= 20 ml min^{-1}, UV = 250 nm,. Solvent was H_2O ramping to 35% acetonitrile for 0-10 min, then ramping up to 100 % acetonitrile at 12 min and maintaining at 100% acetonitrile. Fractions were collected every 30 sec. A Total of 9 runs were performed. Fractions were analysed by LC-MS then reduced to dryness on a GeneVac (HT-4 model) at 1700 rpm, 14 mbar. Fraction 4; ν_{max}/cm^{-1} 3300 (broad), 1606, 1436, 1272, 1151, 1045, 1002, 820. Fraction 12; 3231 (broad), 1604, 1438, 1268, 1151, 1045, 1001, 820. Fraction 15; 3246 (broad), 1603, 1437, 1266, 1149, 1045, 1001, 817.

9.13 General Procedure C - Seasonal Variation Study

The general procedure for the analysis of seaweed samples in the seasonal study in Section 4 was as follows; samples of *Ascophyllum nodosum* and *Fucus vesiculosus* were collected by Böd Ayre from the ropes at their seaweed farm at the end of every month between July 2010 and June 2012 (with the exception of September 2011, which was not sampled). Care was taken to collect several plants to give a representative sampling of the seaweed. Samples were immediately frozen and stored at -20 °C until analysis.

Each sample of the frozen seaweed was defrosted by washing with deionised water and any epiphytes removed and then the surface lightly dried to remove surface water. The dry mass of the seaweed was determined after drying 3 x 10 g portions at 60 °C for 48 hrs in an oven. For extraction, 2 x 50 g portions of the freshly washed seaweed were blended with 100 ml of water in a food processor to a smooth slurry. Ethanol (233 ml) was added and the mixture and stirred under nitrogen for 24 hrs. The liquor was then centrifuged and filtered. A 1 ml sample of the liquor was retained for Folin-Ciocalteau analysis. The liquor was then reduced to a volume of 100 ml, causing the precipitation of non-water-soluble species, which were centrifuged and filtered. Phlorotannin was extracted from the supernatant using exhaustive extraction with Amberlyst XAD-7HP resin, as follows. The resin (10 g) was shaken with the supernatant for 20 minutes then isolated by filtration. The resin was washed with water (200 ml) to remove carbohydrates and then ethanol (200 ml) to recover the phlorotannin. The remaining liquor was extracted repeatedly in this way until no more phlorotannin was collected. The ethanol portions were combined and reduced to dryness on a rotary evaporator to yield the phlorotannin extract as a brown powder, which was further dried under high vacuum. Yields for all extractions are given below in Table 9.4 for *Fucus vesiculosus* and Table 9.6 for *Ascophyllum nodosum*.

Fucus vesiculosus (Dec 2010); ν_{max}/cm^{-1} 3291 (broad), 1603, 1498, 1459, 1251, 1138, 1028, 994, 871, 812. *Fucus vesiculosus* (May 2011); ν_{max}/cm^{-1} 3207 (broad), 1605, 1498, 1459, 1253, 1140, 1028, 995, 870, 813. *Ascophyllum nodosum* (Dec 2010); ν_{max}/cm^{-1} 3158 (broad), 1603, 1498, 1458, 1254, 1172, 1115, 1030, 994, 813. *Ascophyllum nodosum* (May 2011); ν_{max}/cm^{-1} 3167 (broad), 1606, 1497, 1455, 1253, 1172, 1116, 1030, 993, 814.

Table 9.3. The % dry mass of the seaweed as determined from the drying 3 x 10 g portions at 60 °C for 48 hrs in an oven. Results are given as the average of the triplicate measurements.

Determination of % dry mass of the seaweed samples				
Date	*Fucus vesiculosus*		*Ascophyllum nodosum*	
	Average	SD	Average	SD
Jul 2010	32.1	2.1	47.7	0.4
Aug 2010	44.9	3.4	43.6	1.0
Sep 2010	42.5	0.4	48.5	0.6
Oct 2010	41.7	0.3	55.3	0.3
Nov 2010	44.2	1.5	55.8	0.5
Dec 2010	40.5	1.2	41.9	0.3
Jan 2011	40.4	0.8	49.2	3.0
Feb 2011	39.4	2.2	45.0	1.5
Mar 2011	41.8	1.7	43.1	2.1
Apr 2011	36.6	0.7	41.9	0.4
May 2011	41.8	1.7	56.9	2.5
Jun 2011	44.4	3.2	50.0	0.4
Jul 2011	40.4	3.3	52.0	1.3
Aug 2011	45.2	0.2	52.6	0.9
Sep 2011	Not sampled		Not sampled	
Oct 2011	41.6	1.5	40.1	0.4
Nov 2011	40.0	0.9	48.7	0.6
Dec 2011	41.5	2.2	44.9	2.2
Jan 2012	38.4	0.4	43.5	1.5
Feb 2012	37.0	2.2	49.9	0.8
Mar 2012	46.1	3.0	46.5	0.9
Apr 2012	27.4	1.7	44.4	0.9
May 2012	30.9	0.8	56.5	1.3
Jun 2012	31.6	2.4	46.6	0.3

Table 9.4. Yields from the seasonal variation study for *Fucus vesiculosus* discussed in Section 4.2. Yields were determined by extraction (SPE) and FC assay. Both the wet and dry yields are quoted.

	% Phlorotannin yield for *Fucus vesiculosus*											
	1st replicate				2nd replicate				Average			
Date	SPE		FC		SPE		FC		SPE		FC	
	wet	dry	wet	dry	wet	dry	wet	dry	wet	dry	wet	dry
Jul 2010	2.49	7.76	2.87	8.94	2.53	7.90	2.73	8.51	2.51	7.83	2.80	8.72
Aug 2010	3.65	8.13	3.69	8.22	3.32	7.39	3.68	8.20	3.48	7.76	3.68	8.21
Sep 2010	3.72	8.77	3.32	7.82	3.34	7.86	3.25	7.64	3.53	8.31	3.28	7.73
Oct 2010	3.16	7.58	3.18	7.63	3.15	7.56	3.52	8.45	3.16	7.57	3.35	8.04
Nov 2010	3.26	7.37	3.25	7.35	3.85	8.72	3.93	8.90	3.56	8.05	3.59	8.12
Dec 2010	3.56	8.79	3.78	9.32	3.97	9.79	3.50	8.64	3.76	9.29	3.64	8.98
Jan 2011	1.83	4.53	2.11	5.23	1.81	4.47	2.30	5.68	1.82	4.50	2.21	5.45
Feb 2011	2.65	6.73	2.95	7.48	2.76	7.00	3.14	7.98	2.71	6.87	3.05	7.73
Mar 2011	2.42	5.78	2.56	6.11	2.53	6.04	2.43	5.80	2.47	5.91	2.49	5.96
Apr 2011	1.95	5.33	2.30	6.27	2.03	5.55	2.37	6.49	1.99	5.44	2.33	6.38
May 2011	1.67	3.99	1.97	4.70	2.02	4.83	1.83	4.37	1.85	4.41	1.90	4.53
Jun 2011	1.93	4.35	1.84	4.15	1.90	4.27	1.94	4.37	1.91	4.31	1.89	4.26
Jul 2011	2.49	6.15	2.97	7.35	2.50	6.20	2.96	7.34	2.50	6.18	2.97	7.34
Aug 2011	2.44	5.39	2.81	6.21	2.50	5.52	2.87	6.35	2.47	5.45	2.84	6.28
Sep 2011	Not sampled											
Oct 2011	2.61	6.29	2.67	6.42	2.78	6.68	2.74	6.58	2.70	6.48	2.70	6.50
Nov 2011	2.97	7.44	2.56	6.41	2.85	7.14	2.67	6.68	2.91	7.29	2.62	6.55
Dec 2011	2.68	6.46	2.70	6.50	2.71	6.55	2.51	6.05	2.70	6.50	2.60	6.28
Jan 2012	2.48	6.46	2.93	7.65	2.50	6.52	2.96	7.70	2.49	6.49	2.95	7.67
Feb 2012	1.99	5.38	2.32	6.27	1.93	5.21	2.17	5.87	1.96	5.29	2.25	6.07
Mar 2012	2.01	4.36	2.42	5.24	1.95	4.22	2.55	5.52	1.98	4.29	2.48	5.38
Apr 2012	1.60	5.85	1.82	6.63	1.85	6.77	1.94	7.06	1.73	6.31	1.88	6.84
May 2012	2.06	6.68	2.09	6.76	1.73	5.59	2.32	7.52	1.89	6.13	2.21	7.14
Jun 2012	2.49	7.87	2.27	7.17	2.67	8.45	2.18	6.90	2.58	8.16	2.22	7.03

Table 9.5. The yields from the seasonal variation study for *Ascophyllum nodosum* discussed in 4.2.

	% Phlorotannin yield for *Ascophyllum nodosum*											
	1st replicate				2nd replicate				Average			
Date	SPE		FC		SPE		FC		SPE		FC	
	wet	dry	wet	dry	wet	dry	wet	dry	wet	dry	wet	dry
Jul 2010	3.59	7.51	4.31	9.03	4.00	8.37	4.50	9.42	3.79	7.94	4.40	9.23
Aug 2010	2.15	4.92	2.17	4.97	1.99	4.55	2.34	5.36	2.07	4.74	2.25	5.16
Sep 2010	3.50	7.23	3.77	7.77	3.76	7.76	3.88	8.00	3.63	7.49	3.82	7.89
Oct 2010	2.76	4.99	3.45	6.24	3.31	5.99	3.63	6.56	3.03	5.49	3.54	6.40
Nov 2010	3.25	5.83	3.60	6.46	3.29	5.89	3.72	6.67	3.27	5.86	3.66	6.56
Dec 2010	3.62	8.64	4.00	9.54	3.56	8.50	3.95	9.44	3.59	8.57	3.97	9.49
Jan 2011	2.28	4.64	2.18	4.43	1.56	3.17	1.96	3.99	1.92	3.91	2.07	4.21
Feb 2011	3.11	6.90	4.21	9.34	2.80	6.22	3.26	7.23	2.96	6.56	3.73	8.29
Mar 2011	3.43	7.96	3.56	8.27	3.03	7.03	3.25	7.54	3.23	7.50	3.41	7.90
Apr 2011	2.72	6.48	2.80	6.69	2.85	6.81	3.04	7.25	2.79	6.65	2.92	6.97
May 2011	1.97	3.46	2.77	4.86	1.85	3.25	2.22	3.90	1.91	3.36	2.49	4.38
Jun 2011	3.35	6.69	3.56	7.12	3.40	6.80	4.22	8.45	3.37	6.75	3.89	7.78
Jul 2011	3.61	6.93	3.40	6.53	3.32	6.37	4.04	7.77	3.46	6.65	3.72	7.15
Aug 2011	3.58	6.81	3.78	7.18	3.65	6.93	3.92	7.45	3.62	6.87	3.85	7.31
Sep 2011	Not sampled											
Oct 2011	2.90	7.24	3.11	7.76	2.81	7.00	3.02	7.54	2.85	7.12	3.06	7.65
Nov 2011	3.44	7.06	3.12	6.39	3.32	6.81	3.71	7.62	3.38	6.93	3.41	7.00
Dec 2011	2.71	6.02	3.02	6.72	2.29	5.09	2.41	5.35	2.50	5.55	2.71	6.03
Jan 2012	3.13	7.18	3.35	7.68	3.21	7.37	3.69	8.46	3.17	7.28	3.52	8.07
Feb 2012	3.95	7.91	3.87	7.75	4.08	8.18	4.03	8.08	4.01	8.05	3.95	7.92
Mar 2012	3.19	6.86	3.31	7.12	2.85	6.14	3.03	6.52	3.02	6.50	3.17	6.82
Apr 2012	1.99	4.49	2.04	4.59	2.07	4.67	2.18	4.92	2.03	4.58	2.11	4.75
May 2012	4.52	8.01	4.46	7.90	4.38	7.75	4.30	7.61	4.45	7.88	4.38	7.75
Jun 2012	3.23	6.94	3.31	7.10	3.43	7.36	3.68	7.90	3.33	7.15	3.50	7.50

9.14 Acetylation of phlorotannin monthly sample *Fucus vesiculosus* from December 2010

Acetylation was performed as described in General Method B. The yield was 283 mg. δ_H (500 MHz, $CDCl_3$) 7.13 – 6.20 (m, Ar-H), 2.28 – 1.67 (m, acetate CH_3). ν_{max}/cm^{-1} 1767, 1612, 1487, 1432, 1367, 1170, 1118, 1037, 1018, 889, 834.

9.15 Acetylation of phlorotannin monthly sample *Fucus vesiculosus* from May 2011

Acetylation was performed as described in General Method B. The yield was 269 mg. δ_H (500 MHz, $CDCl_3$) 7.13 – 6.20 (m, Ar-$\underline{H}$), 2.28 – 1.67 (m, acetate $C\underline{H_3}$). ν_{max}/cm^{-1} 1767, 1612, 1487, 1432, 1367, 1170, 1117, 1037, 1018, 889, 833.

9.16 Acetylation of phlorotannin monthly sample *Ascophyllum nodosum* from December 2010

Acetylation was performed as described in General Method B. The yield was 273 mg. δ_H (500 MHz, $CDCl_3$) 7.13 – 6.20 (m, Ar-$\underline{H}$), 2.28 – 1.76 (m, acetate $C\underline{H_3}$). ν_{max}/cm^{-1} 1768, 1612, 1484, 1431, 1367, 1169, 1113, 1037, 1017, 880, 734.

9.17 Acetylation of phlorotannin monthly sample *Ascophyllum nodosum* from May 2011

Acetylation was performed as described in General Method B. The yield was 259 mg. δ_H (500 MHz, $CDCl_3$) 7.13 – 6.20 (m, Ar-$\underline{H}$), 2.28 – 1.76 (m, acetate $C\underline{H_3}$); ν_{max}/cm^{-1} 1768, 1614, 1485, 1432, 1367, 1170, 1114, 1037, 1018, 880, 834.

9.18 Extraction of polysaccharide

Fucus vesiculosus (frozen, harvested at Bod Ayre 2/7/2012) was defrosted by washing with deionised water and any epiphytes removed and then the surface lightly dried to remove surface water. 100 g of the seaweed was blended in a food processor with deionised water (250 ml) and then the slurry was acidified to pH 2 with c. HCl (40 ml) and heated with stirring to 70 °C over 20 minutes and then maintained at this temperature for a further 1 hr, left to cool for 30 minutes then centrifuged (1000 g, 5 min) and then the supernatant neutralised with NaOH (0.4 g ml^{-1}).

The solution was reduced to dryness by rotary evaporation at 50 °C and then further dried under vacuum to yield a brown solid (30.5 g, 30.5 % wt. fresh). The extract was precipitated as follows; 30 g of the crude extract was redissolved by stirred in deionised water (270 ml) for 20 minutes to form a brown suspension, which was then filtered (Whatman paper) to remove a soft brown residue. EtOH (30 ml) was added to the filtrate, so as to give a 10 % V/V EtOH solution. No precipitation was observed and so further portions of EtOH were added in the same way so as to increase the concentration by 10% EtOH increments. The solution remained clear until 40% EtOH, at which point a cloudy suspension was observed. This was isolated by centrifugation. Further fractions were obtained in the same way at 70% and 80% ethanol. Further additions of ethanol were not attempted due to the very large quantities of ethanol that would have been required. The yields of the extracts are given in Table 9.6. The total amount of material precipitated only corresponds to 6 % of crude extract.

Table 9.6. The yields of each of the extracts obtained from fractionation of the crude extract.

Description	Yield /g	Yield / % (from 30 g)
40 % fraction	1.01	3.36
70 % fraction	0.583	1.94
80 % fraction	0.357	1.19
Total	1.95	6.49

9.19 Lipid extraction

Dried *Fucus vesiculosus* (large batch, 50 g) was extracted with ethyl acetate (500 ml) for 6 days under nitrogen. The mixture was then filtered and the filtrate reduced by rotary evaporation to yield a dark green oil (1.429 g from 50 g, 2.86 % dry wt.).

9.20 Transesterification of the lipid

The triglyceride extract (1.01 g) was refluxed in methanolic HCl (Fluka, 15 ml, 1.25 M) for 6 hours. Hexane (50 ml) was added and then it was washed with $NaHCO_3$ (50 ml), water (3 x 50 ml) and then brine (50 ml). The organic layer was dried with $MgSO_4$ and then further purified by silica chromatography (95-5 hexane-ethyl acetate) to remove traces of pigments to obtain a colourless oil (159 mg). δ_H (500 MHz, $CDCl_3$) 5.53-5.29 (m, C=C), 3.65 (s, 1H, $\underline{Me}O$), 2.85-2.76 (m, bisallylic), 2.34-2.29 (m, C_2), 2.13-1.99 (m, allylic), 1.74-1.59 (m, C_3), 1.57 (s, C_3), 1.42-1.25 (m, $C\underline{H}_2$), 0.98 (t, $-CHCHCH_2C\underline{H}_3$), 0.90-0.87 (m, $-CH_2-CH_2-C\underline{H}_3$). ν_{max}/cm^{-1} 3011, 2925, 2854, 1738, 1458, 1435, 1363, 1265, 1196, 1170. GC-MS analysis was performed on an Agilent 5975B using the RTX 1701 60 m capillary column, 0.25 i.d., 0.25 µm film thickness. The program started at 40 °C and was held for 2 min, then ramped to 280 °C at a rate of 60 °C min^{-1} and held for 10 min. The column head pressure was 30 psi at 40 °C.

9.21 Superheated water extraction

This extraction was performed by Critical Processes Ltd. The final stage of the work up was performed by Henry Spurr at the University of Leeds. Fresh *Fucus vesiculosus* (6.13 kg, harvested at Böd Ayre) was extracted in a single batch extraction with superheated water at 125°C in a 22 L vessel (solvent loading of 3.6 L kg^{-1}) at Critical Processes Ltd. The temperature was reached after 1 hr of ramping and held for 1 hr. 10 L of the initial extract was collected and kept and then the output was flowed through a column containing 5 L of XAD HP7 resin. The column was then washed with 10 L of water and the water remaining in the column was pushed out with compressed air. The material trapped on the column was then eluted with ethanol. The effluent from the column was initially rejected (displaced water) and then collected after it became coloured. The last part of the ethanolic extract was then pushed out with compressed air. The aqueous liquor extracted was then extracted a second time in the same way and both of the ethanolic solutions were combined.

The ethanolic solution was transported to the University of Leeds where it was reduced to dryness on a rotary evaporator to yield a brown amorphous solid. The overall yield for the extractions was 99.9 g, corresponding to 1.63 % fresh weight. δ_H (500 MHz, CD_3OD) 6.08-5.44 (muliplets). v_{max}/cm^{-1} 3238 (broad), 1602, 1499, 1449, 1252, 1142, 1031, 998, 872, 815. The EC_{50} radical scavenging capacity value was determined as 8.39 ± 0.59 µg ml^{-1} using the DPPH assay.

9.22 Acetylation of superheated water extract

Acetylation was performed as described in General Method B. The yield was 263 mg. δ_H (500 MHz, $CDCl_3$) 7.13 – 6.20 (m, Ar-$\underline{H}$), 2.28 – 1.76 (m, acetate $C\underline{H_3}$); v_{max}/cm^{-1} 1767, 1611, 1485, 1433, 1365, 1171, 1113, 1037, 1020, 880, 831.

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Appendix A – The ^{1}H NMR shifts of common ring types found in phlorotannin

This appendix contains a catalogue of the common ring types found in phlorotannin together with their chemical shifts. It was compiled from the reported literature on small molecular weight phlorotannins that have been isolated and characterised as their acetates.[94, 196] This catalogue was then used in Section 3.1.7, Section 4.2.6 and section 7.3.1 for the anaysis of phlorotannin extracts. The different ring types below are classified by the number of other rings attached to them (*i.e.* mono-substituted (Figure A.1), ortho- and meta-di-substituted (Figure A.2), para-di-substituted (Figure A.3) or tri-substituted (Figure A.4). Acetate groups were not counted as substituted. The rings are shown in red with neighbouring rings shown in black.

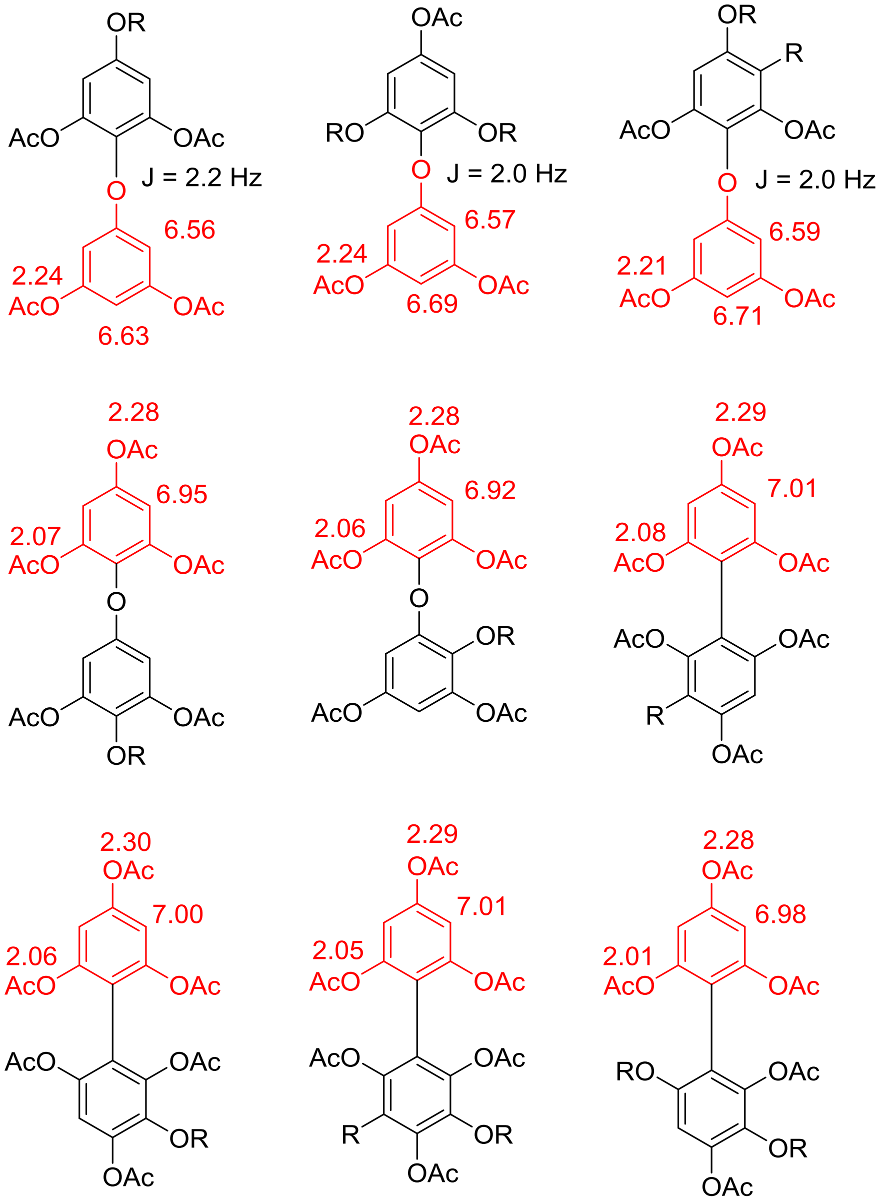

Figure A.1. CDCl3 ^{1}H NMR spectra of common acetylated monosubstituted ring types found in phlorotannin.

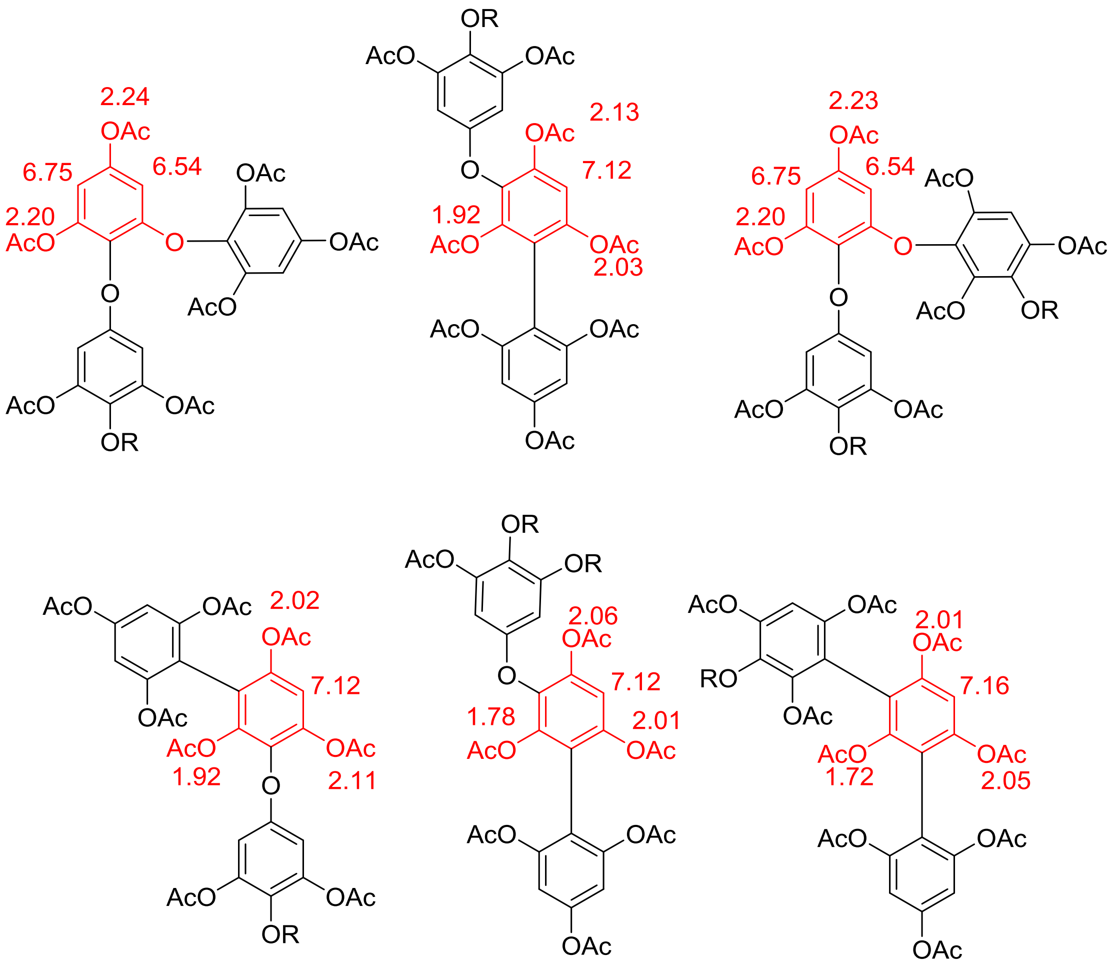

Figure A.2. CDCl$_3$ ^{1}H NMR spectra of common acetylated ortho- and meta-disubstituted ring types found in phlorotannin.

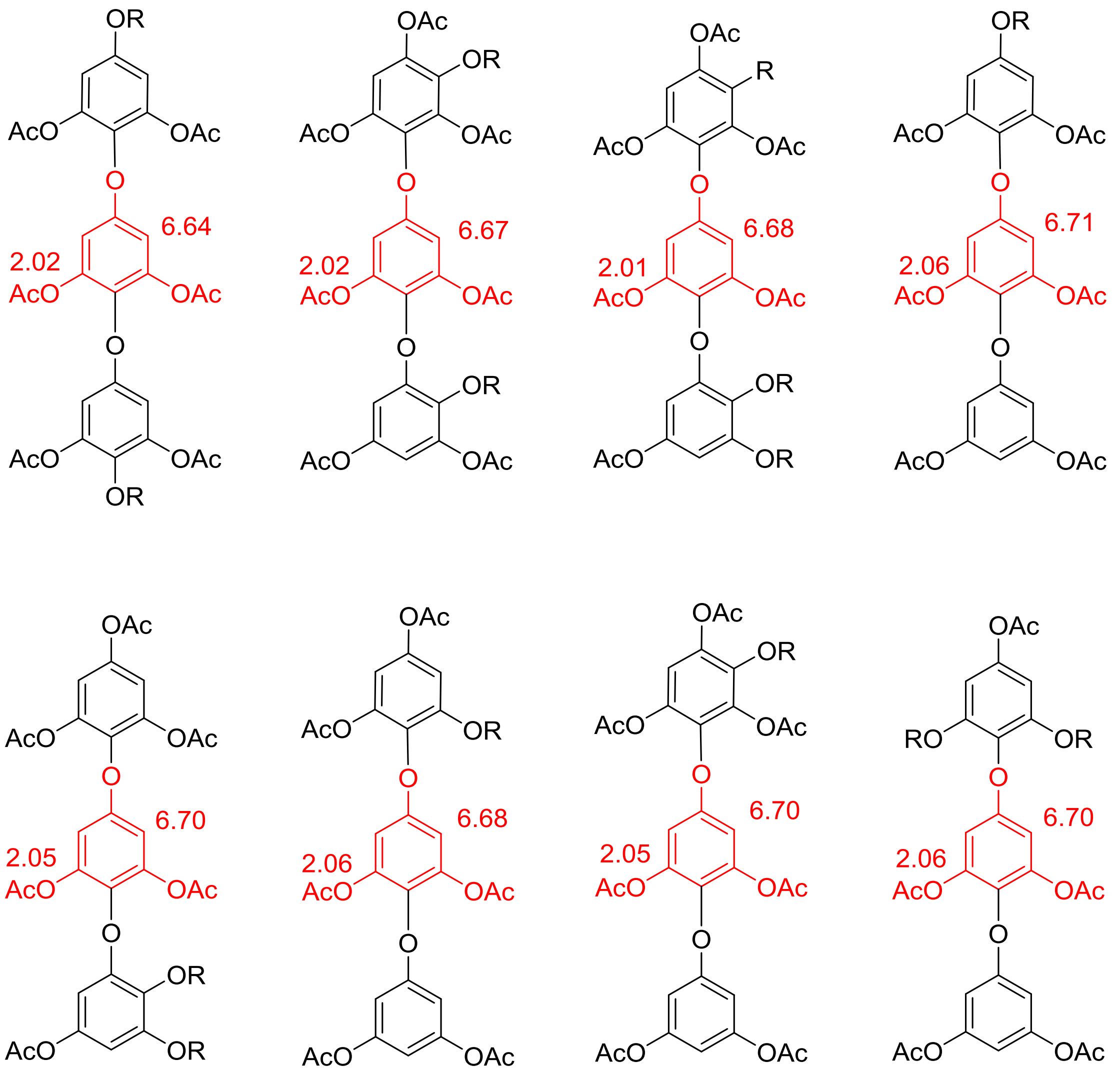

Figure A.3. CDCl$_3$ ^{1}H NMR spectra of common acetylated para-disubstituted ring types found in phlorotannin.

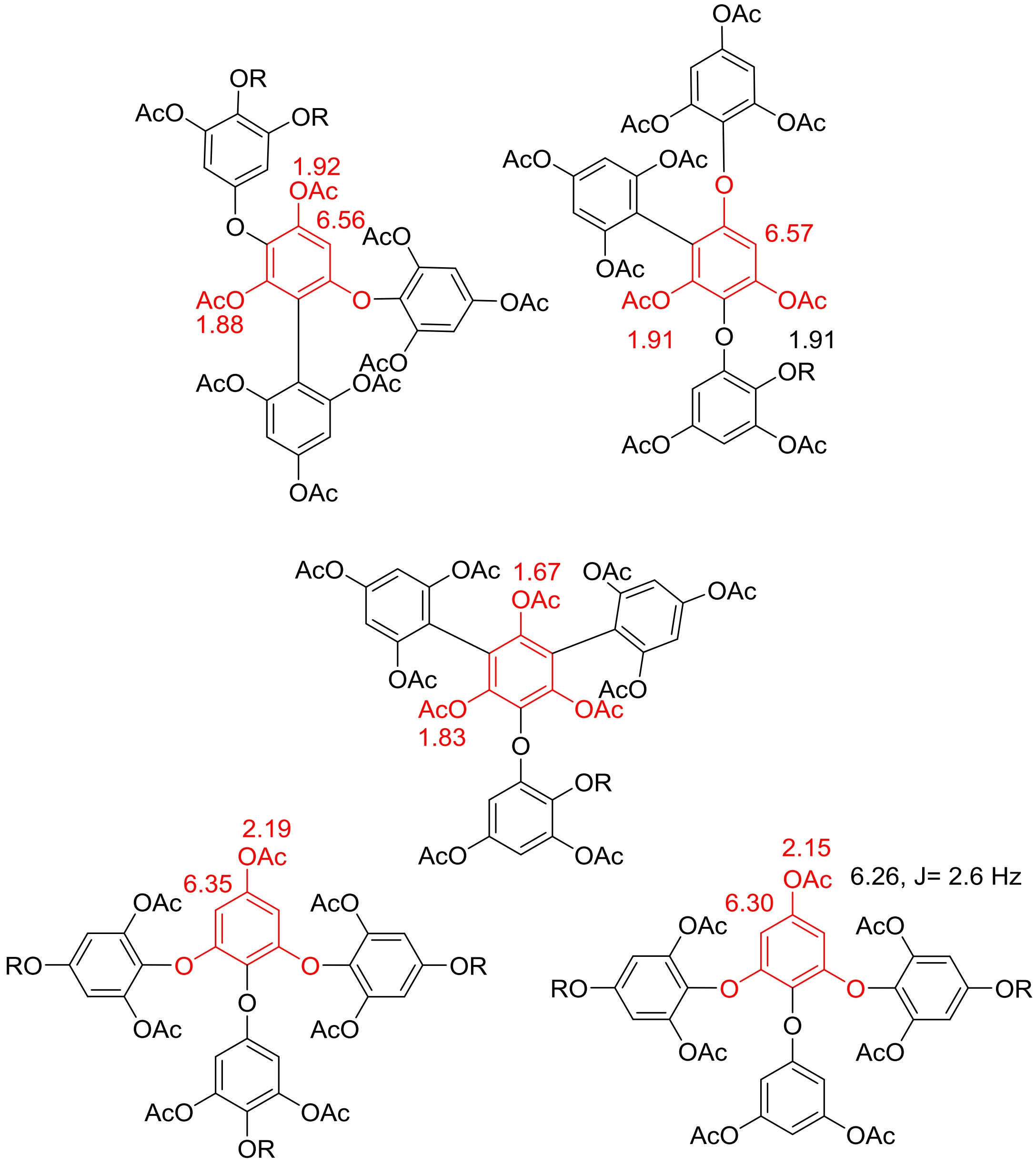

Figure A.4. CDCl$_3$ ^{1}H NMR spectra of common acetylated trisubstituted ring types found in phlorotannin.