Microwave Assisted Hydrothermal Extraction of Carbohydrates from Macroalgae and the Impact of Seasonal Variation.

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Following jointly authored papers are part of this thesis:

1. Fletcher, H.R., Biller, P., Ross, A.B. and Adams, J.M.M, (In Press), The Seasonal Variation of Fucoidan within three Species of Brown Macroalgae, Algal Research
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Details of contributions from the candidate and co-authors are listed below:

1. The candidate performed all of the experimental work, analysis and write-up. Samples were collected and prepared by Dr. Adams. Dr. Ross and Dr. Biller contributed with comments, guidance and proof reading.
2. The candidate performed the majority of the experimental work, analysis and write up. Dr. Adams, Dr. Winters and Bogaerts contributed the polyphenol analysis and write-up. Dr. Ross and Dr. Biller contributed with comments, guidance and proof reading.

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Abstract

Macroalgae represent a diverse and abundant resource, containing an array of unique chemicals with useful properties. These chemicals include: alginate, a long chain carbohydrate with gelling properties; laminarin, a carbohydrate consisting of glucose residues, which is readily fermented; mannitol a sugar alcohol that can be used as an artificial sweetener and fucoidan, a sulphated polysaccharide famed for its biomedical properties. Their current use in industry is minor, with the main focus being as a food source and for alginate extraction. However, there is great potential for this feedstock in chemical and fuel production, especially for biorefinery development, which makes use of the whole resource by providing multiple products from one feedstock. Brown macroalgae offer the most promising option in Northern Europe, being the largest and most fast growing of the seaweed species, as well as being plentiful around the coast of the UK with the potential for cultivation alongside harvesting from wild stock.

A potential barrier to the use of seaweed in industry is their seasonal variation in chemical content. In order to fully understand this, a study detailing the variations in carbohydrates, protein and ash, as well as a detailed study into the variation in composition and structure of fucoidan, identified as the most valuable of the potential extraction products due to its interest in the pharmaceuticals market, have been conducted. Three species of brown macroalgae, *Fucus serratus* (FS), *Fucus vesiculosus* (FV) and *Ascophyllum nodosum* (AN), have been analysed over a 12 month period. The results indicate that mannitol, laminarin and fucoidan are all highest at the end of the growing season in late summer and that ash, alginate and protein are highest during the winter months. The composition and structure of fucoidan is also seen to vary over the year, with FS having the highest sulphate content and results indicating a consistently more branched structure than was seen for FV and AN.

In order to make the best use of the macroalgal feedstock, a three step hydrothermal microwave assisted biorefinery is proposed, with utilisation of the waste as a feedstock for fuel production or as a fertiliser being considered. For this, a sample of FS, identified in the seasonal variation study to have the best potential for chemical extraction, has been used. A low temperature step at 50°C in water firstly removes mannitol and a portion of the salts, followed by processing at 120°C in water to extract fucoidan and alginate. Alginate is precipitated from the extract with calcium carbonate and fucoidan with ethanol. The final step is processed at 120°C with sodium chloride to extract the remaining alginate from the residue. A mass balance of the proposed biorefinery shows that 90% of mannitol, 79% of fucoidan and 79% of alginate have been extracted during processing. A study into the quality of the fucoidan extracted by microwave heating is comparable to that extracted from the raw biomass by conventional means. A comparison of microwave and conventional heating shows the benefits in using microwaves, with decreased extraction temperature and a full energy balance of the system significant energy reductions associated with microwave heating on a laboratory scale.

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Introduction

It is widely accepted that anthropogenic greenhouse gas (GHG) emissions are a significant factor causing change in climate conditions, with levels of carbon dioxide (CO_2) already reaching 391 ppm, more than 100ppm higher than pre industrial levels [1]. In an attempt to curb further emissions and changes in climate, the UK government introduced the Climate Change Act [2], which legislates that the UK must reduce its GHG emissions by 80%, from a 1990 baseline, by 2050. In order to meet these targets, utilisation of sustainable feedstocks, such as biomass, for the production of green chemicals and fuel is important, replacing fossil fuel derived alternatives. To date, there are 4 main classifications of biofuel feedstock, which also largely correspond to green chemical production, classified on the type of biomass being utilised. They are: first generation biofuels, derived from simple materials (such as mono-/disaccharides, starch etc…) found in traditional food crops, including corn, wheat and sugar cane [3]; second generation biofuels, derived from lignocellulosic, non-food crops such as wood, organic waste, food crop waste and grasses [4]; third generation biofuels, derived from higher yielding biomass which have the capacity to produce more fuel from a smaller area such as micro- and macroalgae , and finally, fourth generation biofuels which utilises carbon capture and storage (CCS) along with any of the above feedstocks to create an overall carbon negative process (e.g. BECCS).

The initial inspiration for this thesis was to extract a high value chemical from seaweed prior to biofuel production, in order to improve the economics and produce a competitively priced alternative to conventional fossil based fuels or to first and second generation biofuels, from terrestrial crops. A significant portion of GHG emissions come from the transport sector: in 2011, 21.5% of the UK's total GHG emissions are attributed to transport [5], this is an important area for reductions in terms of meeting government targets. One way in which to achieve this is through the replacement, either in part or fully, of fossil based fuels with those derived from organic matter, otherwise known as biofuels. Current UK policy mandates the blend of 4.75% biofuel with both petrol and diesel under the Renewable Transport Fuel Obligation (RTFO) [6]. This policy applies to suppliers producing more than 450,000 litres of fuel per year and uses a complicated certificate and buyout scheme to regulate it. Current engine technology allows a 5% v/v blend of bioethanol with petrol without any engine modification and up to an 85% v/v blend in flex-fuel engines [7]. While heat and electricity can be produced by a range of renewable sources (such as wind, solar etc…), the need for high energy density, low volume energy storage required by the transport sector make biomass derived fuels likely to be the only viable alternative to traditional fossil based fuels under the current transport infrastructure [4]. There are many benefits to using biofuels over the obvious reduction in GHG emissions. These include: improved air quality; waste reduction; vehicle performance and additional agriculture markets [8]. Currently, the majority of this mandate in the UK is fulfilled with imported, food crop based fuels, such as corn or sugarcane, which in some cases have a questionable carbon footprint [9]. Through a shift to UK based feedstocks for biofuel production, greater GHG savings could be achieved, while also making use of an plentiful resource.

Seaweed is a third generation feedstock and common around the coast of the UK and offers a good alternative to land-based biomass for these applications, being an abundant resource, accounting for 30-50 million tonnes of biomass around the coast of Scotland alone [10]. Considering only around 25 million tonnes per year worldwide [11], is used currently for industrial purposes there is clearly room for considerable expansion of the seaweed industries. Table 0.1 gives a breakdown of the recent and total potential harvest from regions around the world for brown macroalgae, and highlights the disparity between current usage and total production worldwide. Furthermore, seaweed, being a water-based plant, doesn’t compete with food crops for land space, thereby overcoming the “food vs fuel” issue of terrestrial plants and are able to grow in three dimensions, meaning that more biomass is produced per unit area than terrestrial plants. Coupled with their fast growth rate, approximately two times higher than the productivity of sugarcane and five times higher than corn [12], high photosynthetic efficiency, reportedly 6 to 8% compared to 1.8 to 2.2% for terrestrial sources [13] and wide range of unique chemicals and applications, seaweed makes a very attractive feedstock for chemical and fuel production. These chemicals include the storage carbohydrates laminarin and mannitol, both of which have been shown to be possible feedstocks for bioethanol production [14], but could also be used as a building block chemicals for other green chemicals. Alginate, a carbohydrate composed of uronic acids, is already widely extracted from seaweed for use as a thickener in the food industry, due to its gelling properties [15]. Finally, fucoidan, a sulphated polysaccharide, is of interest due to its biomedical properties, including anti-cancer [16] and anti-viral behaviour [17]. The specific properties it displays are largely dependent on its structure, which vary with species, season, harvest location and plant maturity [18].

Table 0.1: Total harvest and potential output of brown seaweed from different locations around the world. (Adapted from [19])

Area	Brown Algae (in '000 metric tonnes)	
	Recent Harvests	**Potential Output**
Arctic	-	-
Northwest Atlantic	6	500
Northeast Atlantic	223	2 000
West Central Atlantic	1	1 000
East Central Atlantic	1	150
Mediterranean and Black Sea	1	50
Southwest Atlantic	75	2 000
Southeast Atlantic	13	100
West Indian Ocean	5	150
East Indian Ocean	10	500
Northwest Pacific	825	1 500
Northeast Pacific	-	1 500
West Central Pacific	1	50
East Central Pacific	153	3 500
Southwest Pacific	1	100
Southeast Pacific	1	1 500
Antarctic	-	-
Total (approx.)	1 315	14 600

The shift towards the development of biorefineries, defined by the IEA as “the sustainable processing of biomass into a spectrum of marketable products and energy” [20], as a means to increase production while minimising feedstock and energy requirements is becoming more prevalent as a means to reducing GHG emissions. In order to make full use of macroalgae as a feedstock, the development of a biorefinery is important. There are many schematics possible, depending on the desired end product, but the ability to extract useful and high value chemicals and use the remaining “waste” biomass for fertiliser or fuel production ensures the resource is used to its full potential, gaining maximum value for minimum cost. Furthermore, by integrating several extraction processes together, it is possible to incur further energy savings by heat integration and solvent recycling; an activity which is not possible if different chemicals are extracted in different places. Although there is a lot of literature instilling the need for macroalgal biorefineries, there is currently little research into actual schematics, extraction efficiencies and the best set-up for chemicals of interest. Furthermore, of the work so far presented, none attempt to further reduce the energy requirements by considering the impact of chemical use and alternative heating sources for extraction. The ability to reduce or eliminate chemicals, while still gaining good extraction yields is important for a low carbon process, as there are processing and energy requirements to produce those chemicals. Being able to process in water alone would significantly reduce the environmental impact of a process, as well as reducing operating costs. Furthermore, the use of alternative heating sources, such as microwaves, would also reduce energy requirements, both reducing costs and environmental impact. Microwaves are famed for their reduced energy requirements, due to the way in which the biomass is heated evenly throughout as the microwaves penetrate to the centre of the material and heat via the friction of rotating polar molecules, rather than from the outside in as is the case in conventional, convection heating. Furthermore, they are reported to give more even heating, higher reproducibility, fast and selective heating, ease of automation and increased safety. Furthermore, the high levels of polar salts present in seaweed make it an ideal candidate for microwave heating, which uses these polar molecules to heat and will, therefore, require less energy for the same heating with increasing polarity of the material.

The chemical content of seaweed is known to vary seasonally, with harvest location, weather conditions and plant maturity [21]. These differences in the chemical content would have a marked impact on an industrial process, where consistent production is key and, therefore, detailed documentation of these variations is important. Currently, there is little published research documenting the seasonal variation of brown seaweeds common the UK coastline, the majority of which dates from the 1950's [21-24]. Of these studies, only one focuses on *Fucoids*, a class of brown macroalgae common to the UK coastline and is of interest due to their high fucoidan and relatively low ash content [25]. With fucoidan being a high value chemical in seaweed and likely to be of interest in a biorefinery setting, making use of these high fucoidan species appears to be an advantageous option. With advances in analytical techniques as well as potential variation in seaweed biomass due to climate change and increased ocean acidification [26], up to date studies into the seasonal variation of brown macroalgae is important. Furthermore, the biomedical properties of fucoidan have been shown to vary with structure , which has also been reported to vary seasonally [18], although there appears to be very little literature to support this at present. Therefore, a detailed study into the seasonal variation of both the quantity and quality of fucoidan from *Fucoids* would provide important information for industry about both the best harvest times and possible bioactivity of the extracted carbohydrate.

This thesis aims to address the research gaps highlighted here by developing a low energy, low solvent macroalgal biorefinery using microwave heating. The main chemicals of interest are extracted sequentially in water at varying temperatures, with consideration of the waste biomass as a fuel or fertiliser. This gives a final schematic which makes full use of the biomass resource to produce a range of bulk and high value chemicals, as well as the potential for fuel. In order to appreciate fully how seasonal variation of seaweed impacts an industrial process, the seasonal variation of components, including alginate, mannitol, laminarin, fucoidan, ash and protein, of three species of *Fucoid*, *Fucus serratus*, *Fucus vesiculosus* and *Ascophyllum nodosum*. The trends throughout the year will be highlighted, as well as the potential impact these have on industrial applications, including the effect on the microwave biorefinery developed in this thesis. The seasonal variation of fucoidan from these three species will be studied in more detail, due to its high value potential for use in the pharmaceutical industry. The fucose and sulphate content have been analysed, as well as attempting to gain some more in depth insight into the structural variations via the use of size exclusion chromatography and liquid chromatography-mass spectrometry.

Nomenclature

Abbreviation	**Full**
AN	Ascophyllum nodosum
BMP	Bo-methane potential
d.a.f.	Dry ash free
DDGS	Distillers dried grain with solubles
FS	Fucus serratus
FV	Fucus vesiculosus
GHG	Greenhouse gas
HPLC	High performance liquid chromatography
HTC	Hydrothermal carbonisation
LC-MS	Liquid chromatography mass spectrometry
MAE	Microwave assisted extraction
MW	Molecular weight
NMR	Nuclear magnetic resonance
PDA	Photo diode array
RDA	Recommended daily allowance
RFS	Renewable fuel standard
RUI	Relative unit of intensity
S:S	Seaweed to solvent ratio
SEC	Size exclusion chromatography
TGA	Thermogravimetric analysis

Aims and Objectives

The general aim of this PhD research is to assess the potential of macroalgae as a feedstock for an algal biorefinery, extracting high value chemicals followed by utilising the remaining biomass for bioenergy applications, such as bioethanol production. In order to realise this fully, the seasonal variation in chemical content needs to be analysed and taken into account. In order to achieve this, the main aims are:

1. Identify the best seaweed species for a biorefinery, taking into account their chemical content and abundance around the UK
2. Assess the seasonal variation in the chemical content of these species, including carbohydrates, protein and metals.
3. Undertake detailed analysis of the seasonal variation of the main high value carbohydrate, fucoidan.
4. Assess the potential for a macroalgal biorefinery using microwaves from the species which presents the best option from the details seasonal analysis and compare this to a conventional heating equivalent.
5. Fully develop a microwave biorefinery based on sequential extraction of chemicals.
6. Explore the possibilities for upgrading of the residue to a fuel or fertiliser and assess the need for waste management.

In more detail, these will include:

1. An literature review on the chemical content of macroalgal species common to the UK coastline, taking into account the abundance of key chemicals, such as fucoidan and carbohydrate content. Drawbacks to a particular species, such as high ash content, which can lead to processing

problems, also needs to be taken into account. Finally, the abundance of the species, leading to the potential for upscaling of the final process, needs to be considered.

2. The seasonal variation in the chemical content has the potential to cause a undesirable impact on industrial processes, meaning its quantification and trends need to be thoroughly understood in order to make the full use of the biomass. This includes the impact on harvest time on the potential applications of the seaweed and also the variation in which year round harvesting could have on the outputs from an industrial process.
3. Fucoidan, a carbohydrate unique to seaweed which displays a range on biomedical properties, is a particularly important chemical in terms of extraction. This is due to its potentially high value to the pharmaceutical market. As the structure and chemical make-up of this polysaccharide is known to vary over the year, it is important that a detailed understanding of this is completed. This includes both the chemical make-up and some insight into how it's structure varies over the course of the year. The difference in structure could have an impact on its functionality, and knowledge of this is important for harvest time.

   An initial feasibility study into a microwave assisted macroalgal biorefinery needs to be undertaken, varying the temperature, seaweed to solvent ratio and microwave residence time of the microwave and assessing the chemical content of the extract and residue. Comparison to the equivalent extractions using conventional heating should be assessed, in order to ascertain the differences between the two heating methods, and whether there are benefits to microwave heating over the reduced energy savings they present. Furthermore, reduction in the use of chemicals will be achieved by processing in water alone.

4. Using the data from the feasibility study, fully develop a microwave macroalgal biorefinery using a stepwise extraction of chemicals based on temperature, including assessing the optimum extraction time, solvent to seaweed ratio and temperature. This data will then be used to create a full mass and energy balance for the process, based on the laboratory scale process. The potential seasonal variation of the process, based on the ratio of chemicals extracted and the seasonal variation data collected, will be calculated in order to evaluate its impact on the process and what this means for industrial applications.
5. Finally, the utilisation of the process wastes will be considered, calculating the potential yields if it were to be used for bioethanol or anaerobic digestion and gauging its potential for use as a fertiliser by assessing nitrogen, phosphorus and potassium content. Again, the seasonal variation of these factors will be calculated and assessed from an industrial viewpoint. The need to waste management, such as the removal of metals from waste water, will also be considered.

Thesis Plan

Chapter 1 includes a literature review, covering the background information on seaweeds, including their classification, habitat and economic status. The chemical content of brown macroalgae common to the coast of the UK has been reviewed, including the structure and functionality of the main carbohydrates present. The literature on their seasonal variation has also be reviewed and appraised and speculation on how the chemical content and its variations may be affected by a changing climate have also been assessed. In light of the development of a macroalgal biorefinery, literature presented on this topic has been reviewed, as well as literature pertaining to different methods of chemical extraction and biofuel production. The use of microwaves for chemical extraction has also been reviewed, as well as background into microwave theory and why they are useful for a low energy, sustainable biorefinery.

Chapter 2 details the methods used in the chemical analysis of seaweed samples in order to determine their chemical content. Conventional and microwave heating methods used as a comparison for the development of a macroalgal biorefinery are detailed, alongside the methods used to analyse the extracts and residues obtained from these processes.

Chapter 3 details the seasonal variation in chemical content of three species of brown macroalgae; the fucoids *Fucus serratus*, *Fucus vesiculosus* and *Ascophyllum nodosum* over a year period. Monthly samples have been analysed for the four main carbohydrates laminarin, mannitol, alginate and fucoidan, as well as protein, ash and the proximate and ultimate content. In all cases, the impact of the trends seen on industry are evaluated, suggesting the best harvest months for different uses of the macroalgae, including for biofuel, fucoidan extraction and as a food source for either animals or humans.

Chapter 4 contains the seasonal variation of fucoidan for the same three species of *Fucoid*, including its fucose and sulphate content, as well as the amount in the raw biomass, has been evaluated. Furthermore, liquid chromatography has been used in order to gain some insight into the variation in structure of the polysaccharide over the year. From this and the results in chapter 3, the species with the most potential for a macroalgal biorefinery have been found. The development of a rapid determination of fucoidan content using a spectrophotometric method, with a comparison between the results from conventional extraction for validation has also been undertaken.

Chapter 5 contains the initial steps and feasibility towards the development of a seaweed biorefinery using microwave heating, based on sequential extraction of chemicals at increasing processing temperature. *Fucus serratus* was chosen to be the best species, based on the results in chapters 3 and 4, so this was carried forward for biorefinery development. Comparison of the chemical extraction of raw biomass by conventional and microwave heating is assessed. Three parameters: temperature, seaweed to solvent ratio and residence time, have been varied to fully appreciate the differences between the two heating methods. The extracts have been analysed for their polysaccharide content by SEC and for their monomeric sugar content, in order to perceive any carbohydrate breakdown.

Chapter 6 contains the full development of a sequential extraction of chemicals using microwave heating. The final schematic includes a three step process, sequentially extracting mannitol, fucoidan and alginate. A full mass and energy balance for the process has been calculated, with a comparison of energy use between microwave and conventional heating highlighting the energy savings offered by microwave extraction. The residue from the process is characterised and assessed for its potential use as a feedstock for fermentation of bioethanol, anaerobic digestion to bio-methane and for use as a fertiliser. Using the seasonal variation data in chapters 3 and 4, the impact of this on the process year round, including the potential use of the residues is calculated, giving some understanding into how this phenomenon may affect an industrial process. Finally, waste water treatment for the high heavy metal content is considered, appraising the literature and giving suggestions for the best methods for clean-up of the waste water.

1 Literature Review

1.1 Macroalgae

Macroalgae are photosynthetic organisms which vary widely in size, the smallest being only a few centimetres and the largest reaching as much as 60m in length [27]. Generally they are found growing on rocky ground, forming established, multi-layered, perennial vegetation [14]. They require a stable seabed in order to be able to anchor themselves, while still being close enough to the surface to absorb sufficient light. They are, therefore, most commonly found on the continental sea shelf [27], forming dense forests, under which almost no light penetrates [28]. On the whole, macroalgae tends to grow zoned by species, with little integration, meaning that different areas of the seabed will be dominated by one species.

The distribution around the UK coast of the three species of fucus in this study, *Fucus serratus* (FS), *Fucus vesiculosus* (FV) and *Ascophyllum nodosum* (AN) are given in Figure 1.1. All three species are widely distributed, covering a large area of coastline. The species are more prevalent on the west coasts of the UK and Ireland, although they can still be found on east coast locations.

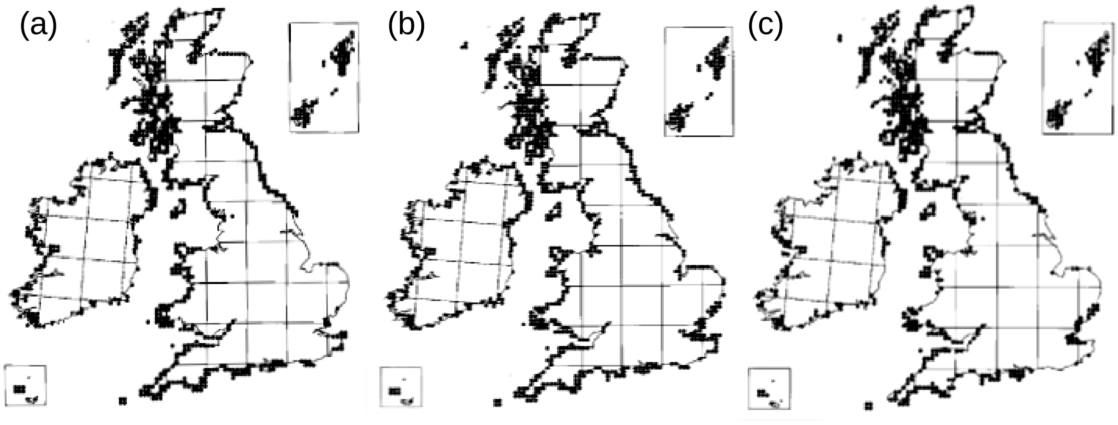


Figure 1.1: Distribution of (a) Fucus serratus*, (b)* Fucus vesiculosus *and (c)* Ascophyllum nodosum *around the coast of the UK [29].*

1.1.1 Classification

There are estimated to be around 20,000 different species of algae worldwide. On the whole, they fall under three main categories: green; red and brown, colours which derive from natural pigments and chlorophylls [30]. For the most part, algae consist of a holdfast, which attaches to the seabed and holds the algae in place; a stipe, which is similar to a terrestrial plant's stem and a frond or blade, not dissimilar to a leaf [31]. An example seaweed structure and how it corresponds to a terrestrial plant is given in Figure 1.2. Some also contain air bubbles or "bladders" to help them to float, allowing them to be able to capture more sunlight.

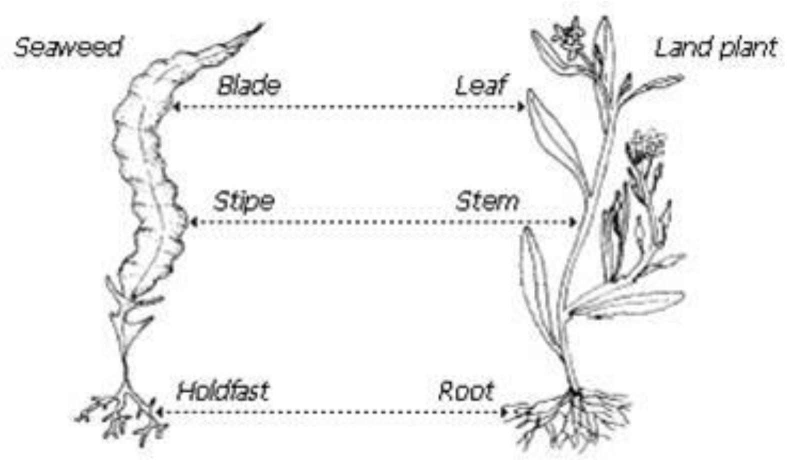


Figure 1.2: Diagram of a typical seaweed structure and how it relates to terrestrial plants [32].

Green algae or *Chlorophyta* are found in both fresh and seawater habitats, although freshwater species are more prevalent (around 85%) [33]. Within this classification, there are currently 4,548 known species, although it is thought there are many more than this as yet undiscovered [34]. They also show similar evolutionary and biochemical traits with higher, terrestrial plants [35]. This includes a very similar chemical make-up, with the presence of cellulose and chlorophyll [36]. Due to their need for abundant amounts of sunlight for photosynthesis, they are most commonly found in shallow waters, such as bays, estuaries or intertidal pools [33].

Red algae or *Rhodophyta* mainly prefer to grow in deeper water, away from tidal fluctuations (>10m deep) [37]. There are known to be at least 6,131 species of red algae, predominantly found in the marine environment [34]. Generally they consist of cellulose, glucan and galactan, with the cell wall containing agar and carrageenan, both of which have gel-forming abilities [33].

Brown algae or *Pharophyta* includes over 1,792 known species, which habit both marine- and freshwater environments [34]. They are predominately found below the tide level, at depths of between 10 and 20m [37]. They can grow to as large as 100m in length at rates up to 50cm/day [33]. They generally contain up to 55% dry weight of carbohydrates, including laminarin and mannitol [33]. Brown macroalgae are the most abundant group found around the shores of the UK, as well as the majority of the rest of Northern Europe, and have the best potential for cultivation. As a consequence, the use of this classification will be the primary focus of the presented studies and literature review. On the whole, brown algae have the highest photosynthetic rate of the seaweeds, although it is dependent on species, and are reported to be between 124 and 561 µmol CO_2/ hr g dry biomass, compared to 30 to 468 µmol CO_2/ hr g dry biomass for green macroalgae and 21 to 174 µmol CO_2/ hr g dry biomass for red [38].

1.1.2 Cultivation and Harvesting

Under the present production requirements, harvesting seaweed from natural stocks is a very viable option. Indeed, some sources state that cultivation in Western Europe many not even be necessary [39]. There are vast areas of algal growth which remain untapped, with only 1% of available seaweed currently being utilised [40]. As long as harvesting is carried out with ecological impact in mind, it could be a very sustainable and cheap source of biomass. However, as this reserve would not be sufficient to meet world demand and with current restrictions on harvesting natural resources in the UK [39], cultivation is an important factor, which needs to be explored. At present, the majority of harvesting from these natural stocks is carried out by hand, a method that, while very environmentally friendly, is not viable for the high volume production which would be required for industrial usage. The use of mechanical means, however, is strictly regulated through most of Europe due to the adverse effect it can have on the marine environment [39], although there is evidence to suggest it could be undertaken sustainably, as long as the correct regulation is in place and the process is closely monitored.

Of the approximate 200 species of seaweed harvested for use worldwide, currently around 10 of them are cultivated intensively [39], mainly in Eastern Asia. The majority of current production, however, is harvested from wild sources or from cultivation techniques based on traditional methods [41]. While this is effective at the scale of the current market, it has some significant problems and short fallings. If large scale chemical extraction from seaweed sources is going to be a viable option, more research in mass cultivation and harvesting techniques will be required. There are a wide range of options being proposed, including cultivation in tanks; on ropes, which could be suspended between posts/wind turbines or grids of rope suspended from buoys and integration with fish farming.

From the sea-based methods, the rope configuration, more commonly referred to as “long line”, appears to be the most promising. It consists of a 16-18mm thick rope anchored by concrete blocks, suspended between wind turbines or bound into ring or grid shapes and can be deployed both in- and offshore. The ropes are seeded with seaweed spores and supply an anchor to which they attach themselves to grow [42]. Harvest form this type of configuration is achievable with small boats, although optimisation of the process has yet to be performed. Wegeberg and Felby [42] report that, for a ring structure, 300kg (wet weight) of seaweed was successfully cultivated on 84m of rope in one growing season.

1.1.3 Current Economic Status of the Seaweed Industry

Currently, seaweeds are farmed for use as a food source, fertiliser, stabilising agents and for high value, low volume products such as amino acids [43]. Table 1.1 gives an overview of the main products currently being manufactured and the relative production quantities and value of the markets. While not insignificant, seaweed production is a relatively small industry, clearly with room to expand. Jung et al. [30] estimate that total macroalgae production is around 15.7 million wet metric tonnes per year, which equates to roughly 2% of current corn production, adding further weight to this point. Taking biofuel production as an example industry for seaweed production to expand into: under the Renewable Fuel Standard (RFS) in the US, 36 billion gallons of renewable fuel is required per year to be blended with transport fuel by 2022 [44]. Using the optimum bioethanol production of 19,000l/ha/year quoted by Wargacki et al [45], to meet this target purely from macroalgae would require 87,000 km^2 in production. While this is clearly not going to be achieved in time to meet this target, considering the US has an Exclusive Economic Zone (an area of costal water a certain distance from the shore, over which a country can claim exclusive rights to economic activities) of 11.66 million km^2 [35], the area required for seaweed cultivation to meet this target is an insignificant portion, less than 1% of available US waters, and clearly plausible at some point in the future.

An addition to use as biofuels, there is an abundance of research being carried out into the uses of chemicals, unique to seaweed, which have a high value and beneficial properties. Carrageenan (currently used in toothpaste production), fucoidan and phycarine are just a few of such chemicals which have been found to have a range of medicinal properties, including anti-fungal and anti-cancer [43]. If these chemicals could be extracted simultaneously with the production of biofuels, it could offer a significant benefit to the economic feasibility of seaweed as a potential for mass biofuel production.

Table 1.1: Commercial market value and production quantities of some seaweed products (adapted from [40]).

Industry	**Market Value/$**	**Quantity/tonnes**
Food (human)	6 billion	6.4 million
Hydrocolloids (inc. medical uses)	0.702 billion	~0.9 million
Agar	255.6 million	55,650
Alginate	158.4 million	126,500
Carrageenan	288 million	33,000
Feed (animal)	6 million	50,000
Fertiliser	6 million	10,000
Total	**6.6-7.2 billion**	**7.5-8 million**

1.1.4 Chemical Content

Seaweeds contain an abundance of different chemicals, many of which are unique to the marine plant environment. Table 1.2 outlines the main polysaccharides of the 3 classifications (red, brown and green), as well as those found in lignocellulosic biomass for comparison. It is interesting to note that green macroalgae most closely corresponds to the lignocellulosic biomass, whereas the brown and red macroalgae have very little in common. Furthermore, brown and green macroalgae contain no lignin, as this is used for support in terrestrial plants and is not required in seaweed, which gets support from the surrounding water [36]. This is advantageous when considering their conversion to fuel, as the removal of lignin is a complex and expensive process and one of the main drawbacks/challenges in the use of lignocellulosic biomass as a fuel feedstock source.

Table 1.2: Carbohydrates found in red, green and brown macroalgae compared with lignocellulosic biomass (adapted from [36]).

Green	**Red**	**Brown**	**Lignocellulosic**
	Polysaccharide		
Mannan	Carrageenan	Laminarin	Cellulose
Ulvan	Agar	Mannitol	Hemicellulose
Starch	Cellulose	Alginate	Lignin
Cellulose	Lignin	Fucoidan	
		Cellulose	

As the main focus of this study is brown macroalgae, the following sections will describe, in more detail, the structures and properties of the main chemicals found within this classification.

1.1.4.1 Laminarin

Laminarin typically makes up 0-30% of the dry weight of brown macroalgae, with the concentration being dependent on the season [7] and is the main storage carbohydrate. It is a carbohydrate which is typically made up of β-1,3-linked glucose residues with small amounts of β-1,6-linkages and has a polymer chain of around 25 units [7], the basic structure for which is given in Figure 1.3. Its molecular weight is approximately 500 Da, although varies depending on the degree of polymerisation and also seems to be dependent on the month in which the sample is collected [46, 47]. It takes two different forms, which are: M chains, ending in a mannitol residue and G chains, ending in a glucose residue [46]. Its solubility in water is dependent on the amount of branching and commonly both soluble and insoluble forms can be found within brown macroalgae, with quantities of each depending on the species [48]. The structure of laminarin is noted to differ, including the M:G ratio, the degree of branching, the number of sugar residues per chain and the ratio of (1→3)- and (1→6)- glycosidic bonds [49]. Laminarin isolated from *Laminaria digitata* by Read et al. [50] has been shown to contain a small amount of G-laminarin, containing 22-28 glucosyl residues and a more abundant fraction of M-laminarin, containing 20-30 glucosyl residues. Chizhov et al. [49] have shown a variation in laminarin between species, with varying M:G ratio and also variation in peak chain length, with *Laminaria cichorioides* and Laminaria hyperborea having peak chain length of 26 residues, *Cystoseira crinita* of 23 residues and *Cystoseira filium* only 12 residues. This clearly shows the variation in laminarin structure between species.

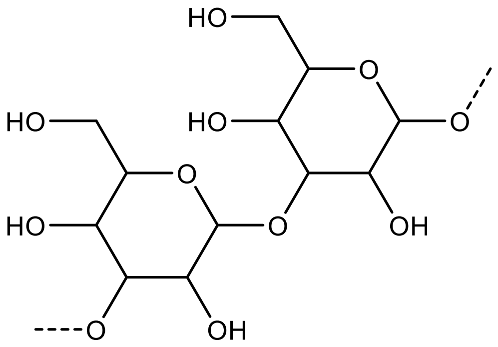


Figure 1.3: Monomer structure of Laminarin

1.1.4.2 Mannitol

Mannitol is a sugar alcohol [7] which typically makes up 4-25% [51] of the dry weight of brown macroalgae, again depending on the season. Its structure can be seen in Figure 1.4. Mannitol has significant commercial viability as a product, as it is a commonly used low calorie sweetener. Current interest in mannitol in the literature mainly corresponds to its potential for conversion to bioethanol [52-54]. Due to its linear structure, traditional yeasts are unable to ferment mannitol directly, so either an enzymatic process to convert it to a fermentable sugar or direct conversion to ethanol via a novel yeast or bacterium needs to be carried out. Other conversion routes for mannitol have also been described, including that of Xia et al [55], who proposed its conversion to hydrogen via the use of a mix of anaerobic fermentative bacteria. They report good yields, dominated by hydrogen (17%), butyric acid (38%) and ethanol (34%), all of which are useful fuels and building block chemicals. Aside from fuel uses, mannitol has also been shown to be a possible feedstock for the production of ridged polyurethane foams [56] and converted into intermediates for the production of detergents, polymers, fuel additives and plasticisers [57].

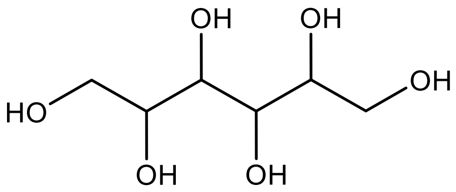


Figure 1.4: Structure of Mannitol

1.1.4.3 Alginate

Alginate is a linear block copolymer consisting of 2 uronic acids, β—D-mannuronate (M) and α-L-guluronate (G), arranged in varying sequences [46], the structures of which are shown in Figure 1.5. These units are arranged in a non-regular, block wise order along the chain. Gels of alginate are formed by ionic interactions between guluronic acid resides of 2 or more alginate chains [58]. The gelling ability of the alginate, therefore, is dependent on the ratio of G:M, a low ratio accounting for weak gelling properties [15]. Functional and physical properties such as mechanical strength, porosity and gel uniformity also depend on the G:M ratio [58]. Alginate can account for up to 40% of the dry weight of the macroalgae, again depending on the season [36]. They are generally soluble in water, although their solubility depends on the pH, concentration, ions in solution and the presence of divalent ions and ionic force [45]. Alginate is associated with a cation, rendering both soluble and insoluble forms, depending on whether it is associated with a Ca (insoluble) or a Na (soluble) ion [59]. This is an important consideration for the extraction of alginate, where two different techniques or an initial pretreatment step to convert all alginate to the same form may be necessary to remove both the soluble and insoluble polymers. The extraction of alginate from macroalgae is already widely performed in industry, with alginic acid being commonly used in the food and pharmaceutical industries as a thickener, in textile printing, paper coating [15], and in cosmetics such as shampoos and lotions [60]. They have also been investigated for use as a low-cost sorbent [61] and have been shown to have metal binding capabilities for a range of heavy metals proportional to the total carboxyl group content [62].

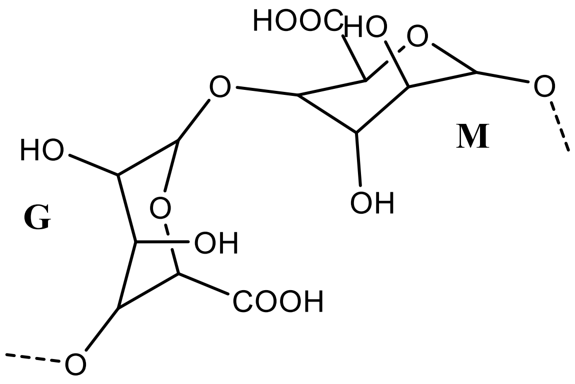


Figure 1.5: Monomer structure of Alginate

1.1.4.4 Fucoidan

Fucoidan typically makes up 5-20% of the algal dry weight [63-65]. It is a sulphated polysaccharide, supported by a sugar backbone, which mainly consists of α-1,3 and α-1,4 linked units of fucose [66] as well as small quantities uronic acids, galactose, xylose and other sugars [48]. A simplified structure, showing the linkages between the backbone residues, is given in Figure 1.6. However, the overall structure of fucoidan is dependent on many different factors, including the species of seaweed, the season and location from which it is harvested and the maturity of the specimen [18].

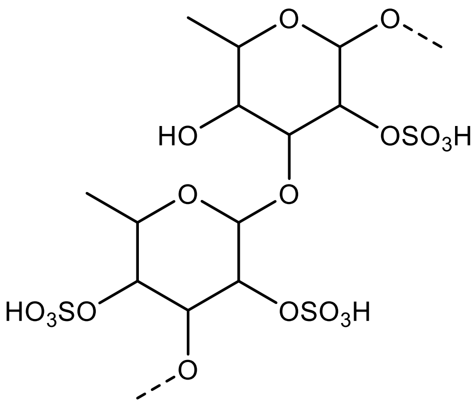


Figure 1.6: Monomer structure of fucoidan backbone

Full structures of fucoidan have been determined by Bilan et al., notably *Fucus distichus* [67], *Fucus serratus [68]* and *Fucus evanescens [69],* all collected during the Summer months. The fucose: sulphate: acetate ratio is 1:1.21:0.8, 2:1:0.1 and 1:1.23:0.36 for *F. distichus, F. serratus* and *F. evanescens* respectively, highlighting the difference in chemical make-up between species for fucoidan harvested at the same time of year. Furthermore, the studies highlighted structural differences between the fucoidans, analysed via nuclear magnetic resonance (NMR). *F. distichus* was noted to have a regular structure consisting of repeating disaccharide units, with *F. evanescens* having a similar linear backbone, but with additional sulphate groups. *F. serratus* was shown to have a branched structure, while also containing small amounts of xylose and galactose.

In a study by Mak et al. [18], the sulphate content was shown to more than double between July and September for the same species of macroalgae and it is well known that the chemical composition of macroalgae varies with season [70]. Additionally, it's reported molecular weight ranges between 43 and 1600 kDa [46, 48]. This makes the specific determination of fucoidan from a particular species difficult and general analytical methods, such as colourimetric tests, size exclusion chromatography and methylene blue staining are employed in order to confirm its presence [66].

The extraction of fucoidan is of particular interest to researchers due to its many and varied biomedical properties. These include anti-tumour [16], anticoagulant [71], antivirus [17] and antioxidant activities [72] among others. The differences in bioactivity depend upon several factors, which include its molecular weight, monosaccharide composition, sulphate content and the position of the sulphate ester groups within the ring structure [66]. The effect of the degree of sulphation on the biomedical properties of fucoidan has been assessed by Haroun-Bouhedja et al [73], who showed that a sulphate content below 20% leads to a complete loss of two biomedical properties studied: anti-proliferative and anticoagulant activity, and that an increase in sulphate leads to an increase in the effect. Due to this, it is key to the extraction procedure that the structure of the fucoidan isn't compromised and thus techniques employing mild conditions are favoured.

1.1.4.5 Protein

Protein is also an important constituent of seaweed, having a wide range of functional food, nutraceutical and cosmetic applications [74]. Seaweeds contain a wide range of proteins, which are reported to contain all the amino acids and have levels of essential amino acids comparable to those required for dietary proteins [74]. The proteins present in brown seaweed tend to include higher levels of acidic amino acids compared to their red and green counterparts [75]. The protein content of FV has been reported to be between 3 and 14 dry wt%, with other fucoid species lying within this range [76]. Algal proteins are currently of interest in the literature due to their inclusion of bioactive peptides [77], including antioxidant and antihypertensive properties [78], making them an attractive extract.

1.1.4.6 Ash and Alkali Metals

The ash content of seaweeds is also typically high, between 14 and 30% [76] for Fucoids and up to 50% in Kelps [21], due to the high salt level in seawaters. It has been shown that the ash content is dominated by the alkali metals sodium and potassium, as well as high levels of calcium and magnesium [79]. These levels are commonly between 0.5-11 dry wt%, compared to 1-1.5% for terrestrial biomass [80].

A high ash content is an important consideration for industrial use of seaweed, due to the associated slagging, fouling and corrosion of equipment in its presence [81]. Furthermore, it can also lead to interference in any chemical reactions required for processing. Ross et al. [82] noted the effect these higher levels have on combustion and flash pyrolysis. It is concluded that thorough washing of the biomass is beneficial for maintaining equipment components and recommends processes which are tolerant of the high ash and metal content found in seaweeds.

In contrast to this, the food industry views the high ash content of seaweeds as a benefit, as they contain all the essential minerals and trace elements needed for human health [83]. For example, the levels of iron in 8g of dried seaweed is equivalent to that of 100g of steak [84] and contains up to 3% dry weight of calcium, an essential mineral for bone health, especially important to expectant mother, adolescents and the elderly [83]. However, the high heavy metal content must be kept in mind when considering seaweed use for food purposes. Cadmium, lead, mercury, copper, zinc and arsenic are all reported to be high in seaweeds [85] and high intake of these metals can lead to health problems. A study by Besada et al [86] into the heavy metal content of 11 species of macroalgae showed that, on the whole, the level of these metals falls within EU limits, with only cadmium persistently exceeding maximum levels. However, the levels are still high when compared to other foods for all heavy metals, meaning eating large quantities is not advisable.

1.1.4.7 Others

As well as the most abundant chemicals previously discussed, there are many others, present in small amounts. These include: lipids and host of metabolites, such as phlorotannins, diterpenes and fucoxanthin [87]. The function of most of these metabolites remains speculative, but includes defensive compounds designed to deter consumption and antimicrobial compounds. Pigments and vitamins are also an important part of the chemical make-up of seaweeds. In the case of brown seaweeds, pigments include the carotenoids β-catotene, fucoxanthin, violaxanthin and zeaxanthin, chlorophylls and tetrapyrrotes, as well as water soluble vitamins B1, B2, B12 and C and the fat soluble vitamins A, E, D and K [83]. Commercially, pigments are used as food colourants and in nutritional supplements, but have been shown to have a host of other health benefits, including antioxidant and anti-cancer [83]. Research into the use of these pigments is beginning to become more prevalent, with Calogero et al. [88] extracting chlorophyll based dyes from seaweed to use as an alternative to pyridil-based Ru complexes for dye sensitised solar cells.

1.1.5 Seasonal Variation

The seasonal variation in the chemical content of macroalgae is a well-known phenomenon. The chemical content of seaweed was first analysed over 150 years ago, with mannitol being identified by Stenhouse in 1844 [89]; the beginning of extensive chemical analysis spanning the next 75 years. Despite the relatively large size of the seaweed industry at the time, it largely focussed on alkali production for potash and the first seasonal variation studies of seaweed were not carried out until 1919 by Lapicque [90]. After this point, many studies were undertaken, most notably by Black [21], who fully developed the methods of extracting and quantifying the main chemical components in seaweed and created the foundation for future seasonal variation studies. He characterised the seasonal variation in a wide range of species common to the coastline of the UK, including: *Ascophyllum nodosum* [22]; *Laminaria cloustoni* [23]; *Fucus serratus, Fucus vesiculosus, Fucus spiralis Pelvetia canaliculata* [24] and *Laminaria digitata* and *Saccharina latissima* [21]. His studies included changes in laminarin, alginates, mannitol and ash and in the 1940’s, he completed the only other study the authors could find detailing the seasonal variation of fucoids [22, 24]. Over a two year period, Black studied the variation of FS, FV [24] and AN [22] and found that, in general, mannitol and laminarin peak in late summer/early autumn and are lowest in Feb/March. Ash, protein and alginate peak in the early part of the year (March/April) and are lowest in July/August, although it was noted that these maximums and minimums could vary by up to two months, depending on the weather for the particular year. Figure 1.7 details the maximum and minimum levels of each of the biochemical components for Fucoids and Kelps and clearly shows the two distinct regions of high and low values in January to April and June to November in both cases.

Recent reports of seasonal variation include those of Adams et al. and Schiener et al. [79, 81], who focus on *Laminaria digitata* and 4 species of kelp respectively. While their overall findings echo those of Blacks earlier studies into the same species, additional information, such as polyphenol content and characterisation of the ash has been included. A study by Rosell and Srivastave [91] into the variation in ash content of the brown algae, *Macrocystis intergrifolia* and *Nereocycstis luetkeana* harvested from the coast of Canada, found the variation is largely due to changes in potassium and phosphate, which are high in the winter and low in the summer. All other metal constituents remained relatively constant over the year. Other than these studies, there is little recent literature on the seasonal variation of macroalgae. Due to advances in analytical techniques since the 1950’s, as well as anthropogenic changes to the marine environment, including ocean acidification and sea temperatures, updated studies on seasonal variation are key for the industrial utilisation of macroalgae.

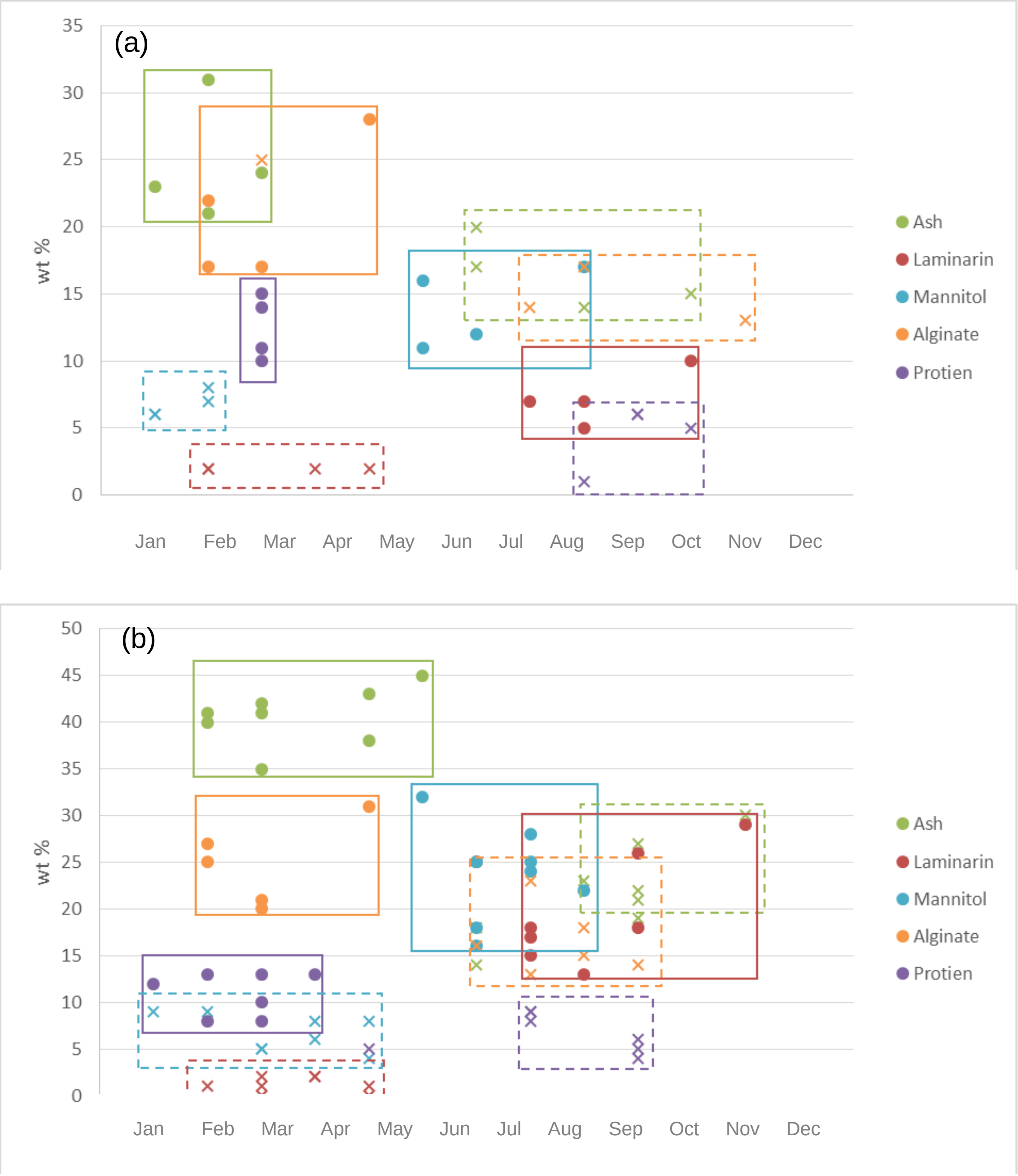


Figure 1.7: High and low values and the month in which they occur for the main chemicals found in (a) Fucoids and (b) kelps. Values taken from [21-24, 79, 81]. Solid border denotes high values and dashed boarder low values.

1.1.5.1 The Seasonal Variation of Fucoidan

The seasonal variation of fucoidan is often mentioned in the literature, although there is very little published data on the subject at present and the few references cover only a few months of the year. Rioux et al. [92] have investigated the bioactive polysaccharides of 4 samples of *Saccharina longicruris*, from March, April, November 2005 and June 2006. The galactofucans (a type of fucoidan containing roughly equal proportions of fucose and galactose) extracted were seen to have an increase in sulphate content of 1.6% between March and November 2005, while decreasing by 7.2% between November 2005 and June 2006. A similar study by Mak et al [93] investigated the variation in fucoidan between July and October for *Undaria pinnatifida*. They found that the fucoidan content almost quadrupled between July and September (3.6-13.7 wt%) and only dropped slightly in October. A similar trend was observed in the sulphate content of the fucoidan. The fucose content decreased significantly between July and September. Again, the need for detailed seasonal variation, including the chemical make-up and changes in structure throughout the year is important for industrial extraction, as well as for guaranteeing the bioactivity of the polysaccharide.

1.1.6 The Effect of Climate Change on Seaweeds

Climate change is having an appreciable impact on the levels of CO_2 present in our oceans, as well as increased sea and sea surface temperatures. The effect this could have on our seaweed abundance, as well as the ecosystems they form, is an important factor for consideration for a viable, large scale seaweed industry in the UK. While it has been noted that the UK may have some tolerance to climate change in terms of species zonation, due to being in the centre of the NE Atlantic distribution for the majority of large brown species [94], there have been several reports noting the loss of species [95], depleted genetic diversity [96] and the increase in abundance of some species [97] in various locations. The effect of climate change on the abundance and location of seaweed species has been explored by Yesson et al [98], who show that, on the whole, there is a decrease in abundance for kelps in the South of the UK, while Central and Northern regions see an increase in both wracks and kelps. The variation in abundance has also been shown to correlate with summer and winter sea surface temperatures, indicating changes in temperature due to climate change are having an effect on the abundance and distribution of seaweed around the UK coastline.

Ocean acidification, caused by increased CO_2 levels in sea water, is also likely to have an effect on seaweed growth and abundance. Oceans absorb over 25 million tonnes of CO_2 produced by humans every day, causing detrimental effects to ocean chemistry [99]. Clements et al. [26] hypothesise that an increased CO_2 level in seawater will have a positive effect on seaweed growth and species will be more competitive in a high CO_2 environment, although a negative impact may be seen on germination for some species, which require a certain pH for growth. The effect of ocean acidification on FV has been explored by Gutow et al. [100] and, surprisingly, has been shown to negatively affect the growth rate and C:N ratio of the seaweed; an opposition to the expected result. The nutritional value, however, was shown to remain unchanged. A study by Porzio et al [99] also shows a decrease in algal growth: on average 5% for ocean acidity levels predicted for 2100. Although their data forecasts that many species will be tolerant of long-term increases in ocean CO_2 levels, they predict that macroalgal habitats will be significantly altered.

1.1.7 Biofuel production from Macroalgae

The way in which biofuels are made and their feedstock's has evolved over time and there are now regarded to be 4 classifications. First generation biofuels are derived from simple materials (such as mono-/disaccharides, starch etc.…) found in traditional food crops, including corn, wheat and sugar cane [3]. Second generation biofuels are derived from lignocellulosic, non-food crops such as wood, organic waste, food crop waste and grasses [4]. Third generation biofuels from higher energy yielding feedstocks, such as micro- and macroalgae, which have the capacity to produce more fuel from a smaller area. Finally, fourth generation utilises carbon capture and storage (CCS) along with any of the above feed stocks to create an overall carbon negative process. Although the ability to ferment first generation feed stocks is well known and relatively simple, as they use what are traditionally food crops, there is debate over “food versus fuel”, and whether the required amounts of biofuels can be generated without conflict with world food supplies [101]. In addition, even if conflicting food supplies were not an issue, if all US corn and soybean production was converted to fuel, it would only meet 12% of petrol and 6% of diesel demand [102].

While second generation feed stocks offer advantages over first generation: a better energy balance [101]; non-food crops and the ability to utilise non-arable land, there are still drawbacks in their use. Changes in land use, biodiversity and the use of water and fertilisers all call into question the energy and carbon balances of the system [39]. Additionally, ethanol production from cellulose costs twice as much as from corn starch [103].

Due to these short fallings, there is increased interest in algal based biofuel solutions. This falls into two categories: microalgae, eukaryotic organisms including phytoplankton and blue-green algae [104]; and macroalgae, larger marine plants such as seaweed and kelp. Their energy balance is reported to be greater than for their first generation counterparts: approximately two times higher than the productivity from sugarcane and five times higher than corn [45]. Microalgae offer significant research potential, with high energy gains over a small area [105]. They are the fastest growing plants in the world and can survive a wide range of conditions and habitats [106]. In addition, over the last 30 years the amount of naturally occurring biomass has increased due to eutrophication caused by increasing CO_2 levels [107]. Their main drawback is their cost of production, especially when considered on a large scale [108]. Macroalgae also offer a promising alternative, having a photosynthetic efficiency of around 6 to 8%, compared to 1.8 to 2.2% for terrestrial sources [13] and their lack of lignin makes enzymatic hydrolysis of algal cellulose much simpler [106].

There are many possible conversion routes for macroalgae, depending on the end product being created. Many involve the use of heat and/or pressure to break open the cell wall, releasing the chemicals found inside before some other process, such as fermentation, is performed to convert the compounds found into usable fuel. Figure 1.8 provides an overview of all the possible fuels from algae and their conversion routes. However, as bioethanol and bio-methane are the most viable fuel sources from a biorefinery perspective, they will be the focus of the literature review in this section.

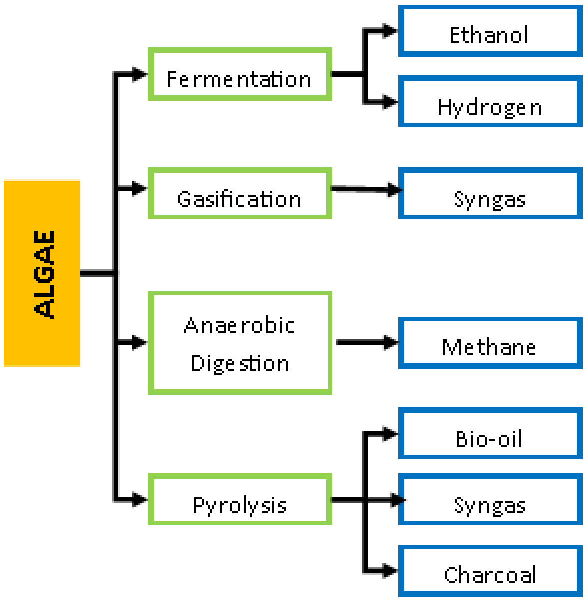


Figure 1.8: Possible fuels from alga. Adapted from [109].

1.1.7.1 Bioethanol Production

Ethanol from macroalgae is produced via a fermentation process, similar to that used in beer or wine making. The main steps of the process for first generation feedstocks is outlined in Figure 1.9. While the process for macroalgae will be largely the same, there are likely to be more pretreatments steps to release the hydrolysable carbohydrates and the enzymes and fermentation yeast/bacteria are likely to be different, due to the unusual carbohydrates found in seaweed biomass. The feedstock is first milled to break down the outer casing of the feedstock and release carbohydrate. The saccharification process involves adding water and enzymes to breakdown carbohydrates into their monomer sugar units, required for fermentation. Traditional fermentations uses the yeast strain *Saccharomyces cerevisiae* to convert the sugars into ethanol and CO_2. Distillation then separates the solid and liquid fraction. The liquid fraction, containing the ethanol, is further purified to its azeotrope at 95% in a distillation column, before the rectifier purifies the mixture further to remove dissolved ions. Finally a dehydration step takes the purity up to 99.7vol% ethanol. The solids are dried and pelleted to produces distiller's dried grains with solubles (DDGS), which is pelleted for use as an animal feed [110].

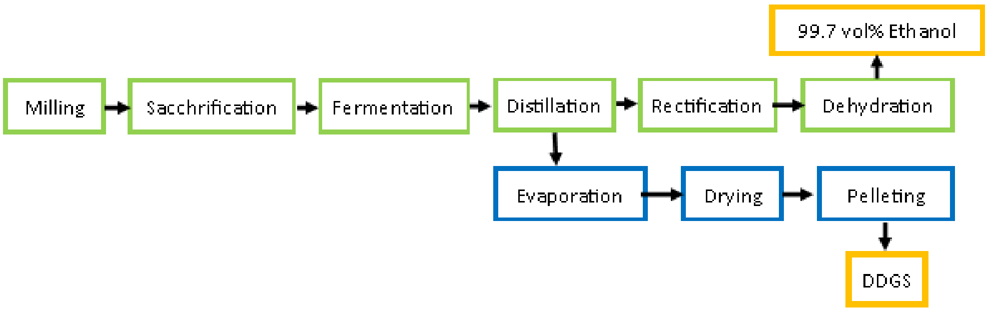


Figure 1.9: Steps involved in the fermentation and purifying of carbohydrate based feedstocks to high purity bioethanol

Researchers are particularly interested in bioethanol production from macroalgae due to their abundant hydrolysable carbohydrate content [111]. Mannitol and laminarin are the main carbohydrates which can be utilised in a fermentation process [14]. They are found in fairly large quantities, with Adams et al. [112] reporting up to 55% of the dry weight being made up of laminarin and mannitol, meaning it is possible to achieve relative high concentrations of ethanol within the final products (up to 15%). Although laminarin is readily broken down into glucose via enzyme hydrolysis using β-1-3-glucanases [113], mannitol is not so readily utilised. In order for mannitol to be fermented, it first has to be oxidised to fructose using the enzyme mannitol dehydrogenase, a reaction which produces NADH. Regeneration of NAD^+ requires either oxygen or transhydrogenase [106]. Therefore most microorganisms cannot ferment mannitol anaerobically. Red algae are reported to produce the most energy of any biomass source [14], although, as shown in Table 1.3, macroalgae as a whole have the potential to produce significantly larger volumes of ethanol per unit area than any of their terrestrial counterparts.

Table 1.3: A comparison between the major bioethanol crops and macroalgae (adapted from [112]).

Feed stock	**Wheat**	**Maize**	**Sugar beet**	**Sugar cane**	**Macroalgae**
Average world yield (kg/hr yr)	2,800	4,815	47,070	68,260	730,000
Hydrolysable carbohydrates (kg/ha yr dry)	1,560	3,100	8,825	11,600	40,150
Potential volume of ethanol (L/ha yr)	1,010	2,010	5,150	6,756	23,400

Due to the unique nature of carbohydrates present in macroalgae, achieving sufficient yields of ethanol can be a challenge and considerable research is being conducted to try to overcome this. Although laminarin can be readily broken down to glucose by a wide range of already commercially available microorganisms, as discussed before, mannitol is less readily utilised. For the fermentation of laminarin, *Saccharomyces cervisiae,* a strain of yeast, is most commonly used [30, 112, 114]. It is the most widely used, commercially available yeast strain [30] and is already widely applied to baking, wine-making and brewing applications. Its kinetics, optimum fermentation conditions and general practise for use are very well known and understood by industry. Although this would make an excellent and cheap source for bioethanol production from seaweeds, as it is only able to utilise the laminarin present, ethanol yields are low, which means the economics of the process are unfavourable [112].

To overcome this, research into microorganisms which can ferment mannitol is being undertaken. Horn et al. [115] have shown that bacteria *Zymobacter palmae* will readily convert mannitol to ethanol and, although laminarin could not be used directly, it was capable of converting glucose, showing that, if pretreatment was carried out to break down the laminarin, this bacteria could be a useful route to ethanol production from seaweed. However, as discussed earlier, the way in which mannitol is broken down requires oxygen and it was shown that mannitol is not utilised by *Z. palmae* unless the mixture is oxygenated.

In another paper, Horn et al. [113] explore fermentation of seaweed using 4 different microorganisms: *P. angophorae; Kluyvermyces marxianus* and *Pacchysolen tannophilus*, all yeast strains and the bacteria *Z. Palmae*. As described before, *Z. Palmae* was able to utilise mannitol, but had no effect on the total sugars concentration (which in this experiment is mostly made up of laminarin). *P. tannophilus* and *K. marxianus* were both able to utilise the sugars, but not the mannitol. However, what is most interesting from this experiment is that *P. angophorae* was able to ferment both mannitol and sugars, making it a viable candidate for effective bioethanol production from seaweeds.

1.1.7.2 Bio-methane Production

Anaerobic digestion (AD) is the process by which organic matter is decomposed by microbes in the absence of oxygen to produce biogas [116]. This involves 4 main process steps, detailed in Figure 1.10, which occur simultaneously in the same space [117] and can convert carbohydrates, fats and protein to bio-methane. The first step, hydrolysis, uses enzymes, excreted by hydrolytic microorganisms, to decompose carbohydrates, proteins and fats into their monomer units. Acidogenesis, the second step, uses fermentative bacteria to produce volatile fatty acids (VFA's), alcohols, hydrogen, CO_2 and ammonia. The VFA's and alcohols are converted into acetic acid, CO_2 and hydrogen, which, in turn, are converted into methane and CO_2 by methanogenic bacteria. Depending on the feedstock being used, there are three possible options for AD, which differ in their process temperature and retention time: psychrophilic has the longest retention time at 70-80 days and lowest temperature of less than 20°C; mesophilic is in the middle, with temperatures between 30 and 42°C and retention times of 30-40 days and finally thermophilic, having the highest temperature at 43 to 55°C and shortest retention time of 15-20 days. The main benefit of this process is its ability to utilise many of the component parts in the feedstock and not only the carbohydrates, which is the case in fermentation. This means a waste product, such as that from a biorefinery process, would be able to be more fully utilised to form a fuel than if fermentation were to be used, where a portion of the carbohydrates will have been extracted prior to AD.

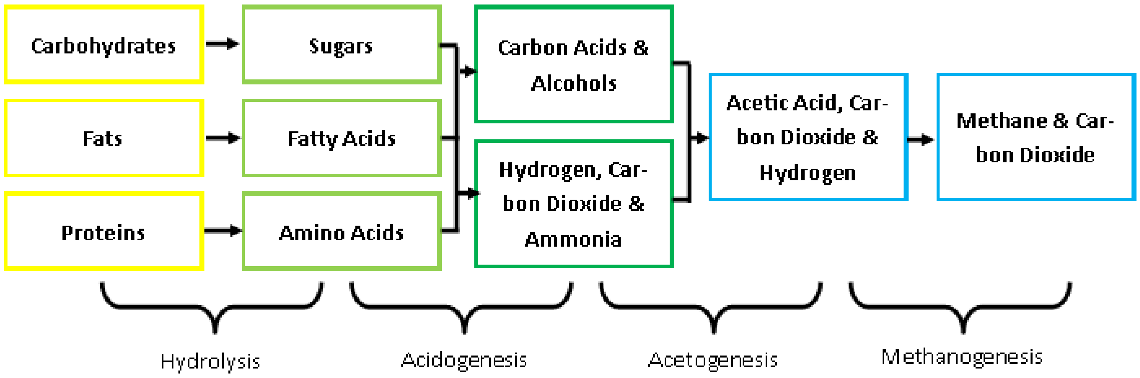


Figure 1.10: Main process steps in anaerobic digestion.

The quantity of bio-methane produced, termed its biochemical methane potential (BMP), is very dependent on the particular feedstock being used. Allen at al. [118] analysed 84 different feedstocks from first, second and third generation sources, available in Ireland, for their theoretical and actual BMP. A summary of their results are given in Table 1.4. The average methane produced from the BMP assays shows that first and second generation feedstocks give very similar production, while third generation is significantly lower at 213 L CH_4/kg VS compared to 350 CH_4/kg VS, despite their theoretical BMP being relatively similar to first and second generation. This is likely due to the low C:N value, which is optimum at between 25:1 and 30:1 [117] and with a low ratio being quoted to have the potential for inhibition due to the formation of high levels of ammonia [119]. The theoretical seaweed BMP, especially for the fucoid species, is relatively high, at 532 L CH_4/kg VS for *F. serratus* and 488 L CH_4/kg VS for *A. nodosum*. This indicates that either blending with another feedstock or pre-extraction of protein to reduce the protein content would improve the actual yields of bio-methane on processing. The needs for pretreatment of biomass is advantageous for a biorefinery system, where prior extraction of high value chemicals will likely improve the C:N ratio of the biomass, improving the possible bio-methane yield.

Table 1.4: Average bio-methane production for first, second and third generation biofuels and data for brown seaweeds. Adapted from [118].

Substrate	C:N	Theoretical BMP L CH_4 kg−1VS	BMP assay L CH_4 kg−1VS
All average ± S.D.			
First Generation	44 ± 22	496 ± 118	353 ± 89
Second Generation	17 ± 9	571 ± 137	350 ± 166
Third Generation	25 ± 17	453 ± 89	213 ± 64
Brown Seaweeds			
H. elongate	22	334	261
L. digitata	23	479	218
F. spiralis	17	540	235
S. latissima	24	422	341
A. nodosum	25	488	167
F. serratus	16	532	102
F. vesiculous	17	249	126
S. polyschides	23	386	263
A. esculenta	16	474	226

Other studies into the AD of seaweed include that of Edward et al. [120] who note a BMP of 141 L CH_4/kg VS and 113.3 L CH_4/kg VS for dried *Laminaria digitata* and *Laminaria hyperborea* respectively. They noted that pre-treating the biomass by washing and drying and pre-incubation of the inoculum both increased the biogas yield and aided in the faster degradation of the biomass. Tabassum et al [121] have investigated blending *Laminaria digitata* and *Saccharina latissima* with dairy slurry. The results indicate that mixing these two feedstocks together decreases the total biogas produced, with the reported values for 100% and 33.3% Lamininaria digitata being 288 L CH_4/kg VS and 166 L CH_4/kg VS respectively and slightly negatively effecting the comparison of theoretical and actual values compared to digestion of the feedstocks alone. While bending with a different feedstock still may lead to a higher conversion rate, it is clear that there is no synergistic effects to the co-digestion of seaweed and dairy slurry.

The seasonal variation in the bio-methane production from seaweed has been investigated by several authors. Tabassum et al [122] detail the seasonal variation of *Laminaria digitata* harvested off the coast of Southern Ireland. Their findings show a lowest BMP of 203 L CH_4/kg VS in April and a highest BMP of 327 L CH_4/kg VS in August, corresponding to between 44 and 72% conversion when compared to the theoretical yields. These high values are accredited to the high C:N ratio and low ash: volatiles (A:V) ratios present in the biomass harvested. The effect of high salinity was noted to adversely affect the AD process, so high ash containing seaweeds are likely to produce a lower biogas yield when compared to their theoretical yields. Similar results were seen in a study by Adams et al [51] for samples of Laminaria digitata collected off the West coast of Wales. Lowest BMP yields were recorded in March at 196 L CH_4/kg VS, with highest in July at 254 L CH_4/kg VS, accounting for between 55 and 61% of the theoretical yield. A study into the potential for AD from *Ascophyllum nodosum* has also been conducted by Tabassum et al [123]. Despite having higher theoretical yields than for the kelp species: averaging 567 L CH_4/kg VS compared to 456 L CH_4/kg VS and 381 L CH_4/kg VS respectively for the preceding two studies, the actual BMP yields are lower, with a highest value of 215 L CH_4/kg VS in October and lowest of 95 L CH_4/kg VS in December. This significantly lower conversion, between 16 and 46% of the theoretical values, has been shown to correlate with the polyphenol content, with a higher polyphenol content corresponding to a lower conversion. They therefore conclude that polyphenols play an inhibitory part in the AD process.

1.1.8 Macroalgal Biorefineries

The concept behind a biorefinery is to integrate biomass conversion processes and equipment to produce a sequence of products, which can include fuel, chemicals and electricity [102]. This approach is advantageous, as it allows for the integration of heat and power across a system, giving the lowest possible energy usage while creating the most value out of the feedstock by using as much of the biomass as possible and, therefore, maximising profits. At present, most chemicals produced from biomass sources are made in singular and any residues from the process, which often contain useful and extractable chemicals, are either wasted or processed at a different site [4]. An example of this is in the alginate industry, where they extract acid soluble chemicals, such as mannitol, laminarin and fucans, with dilute sulphuric acid, which is then treated for disposal [124]. The general set up of a biorefinery is envisioned to produce a small number of high-value, low-volume chemicals and a low-value, high-volume transport fuel, with the hope that there will be enough heat and power produced on site to meet the needs of the process [102]. If this were the case, then the whole process could be seen as carbon neutral, especially if transportation of feedstock and energy demands were run by the biofuel the plant produces.

There are many possible routes for a macroalgal biorefinery, depending on the desired end products. These have been thoroughly discussed in review papers, including those of Trivedi et al [125], Jung et al [38] and Suganya et al [126], but there appear to be few fully researched and valorised in the literature. Included in the work the author could find is the results of Kumar et al [127], who present a possible biorefinery approach for macroalgae through the use the red algae, *Gracilaria verrucosa* to produce agar and ethanol via enzyme hydrolysis followed by fermentation. They report that from 1000kg of biomass, 280kg of agar and

38kg of ethanol could be made. While the yield of agar seems reasonable, the ethanol yield is very low and could certainly be improved upon by utilisation of better fermentation techniques. There are also other components, such as lipids and proteins, which remain unused and further value could be added if these were extracted in the process.

Marinho et al [128] detail a biorefinery for the brown seaweed Saccharina latissima. The schematic first involves enzyme hydrolysis of the seaweed biomass to release fermentable sugars. The hydrolysate is fermented with a strain of *A. succinogenes* to produce succinic acid to a maximum concentration of 36.8g/L. The solid residue from enzyme hydrolysis is then used for phenol extraction and fertiliser production.

A dark fermentation of seaweed to biohydrogen is suggested as a possible option by Sambusiti et al [129]. This process involves the production of hydrogen using a mixture of bacteria in the absence of light, with the solid residue being suggested to be used as a feedstock for anaerobic digestion (AD), gasification or pyrolysis.

Overall, a biorefinery concept looks to be a very good way of producing sustainable fuel and chemicals, making the most out of the biomass feedstock and reducing costs through lower energy and transportation requirements. Additionally, by producing high value products alongside fuels, it is possible that the cost of biofuels could compete with fossil fuel equivalents. Although a lot of work will still need to be carried out in refining both the individual processes and their integration, it seems that this type of approach could be the future of both fuel and chemical production.

1.2 Extraction of Chemicals

There are many methods for the extraction of chemicals from macroalgae, including solvent, temperature and enzymatic methods. The choice of method depends on the chemical of interest and its various properties, as well as its position within the cell. However, in general, it is beneficial in the case of macroalgae to choose a method which utilises wet biomass, such as hydrothermal processing [130], as the harvested water content of the biomass is high, up to 80%, and the associated energy costs of drying significantly increase processing costs.

1.2.1 Conventional Chemical Extraction

For macroalgae, the traditional means of extracting desirable compounds is via chemical extraction. In general it relies on the different ways in which cell components interact in varying solvents and under varying conditions (for example, increased temperature). This commonly takes the form of dilute acid hydrolysis, using either H_2SO_4 or HCl at elevated temperatures to extract the carbohydrates [131]

The use of dilute acid to extract chemicals from macroalgae was first noted by Black et al., [132-134] who published a series of papers detailing the extraction of mannitol, laminarin and fucoidan from brown macroalgae. In each case, the seaweed sample was stirred with dilute HCl ranging in concentration from 0.09-0.17M depending on the desired chemical. Mannitol and laminarin were found to be readily extracted at room temperature and are separated by the addition of ethanol, which causes the laminarin to be precipitated from the mixture, leaving behind the mannitol. Fucoidan, however, requires 2 sequential extractions, heating to 70°C for 1 hour, to achieve good levels of extraction, which has also been noted by other authors [135]. The sequential extraction of chemicals in seaweed has been conducted by Rioux et al [136], who separates the biomass into 3 different fractions. After a pretreatment with 85% ethanol, laminarin and fucoidan is extracted with 2% $CaCl_2$ at 70°C, after which fucoidan, which is noted to have a different structure from that of the first fraction, is extracted with 0.01M HCl at 70°C. Finally, alginate is extracted with 3% $NaCO_3$, also at 70°C.

1.2.1.1 Conventional extraction of Fucoidan

Fucoidan is extracted from macroalgae in order to determine quantity and is performed via a lengthy extraction and purification process, resulting in dry fucoidan. In general this consists of four main steps: an initial purification to remove pigments and lipids, often using an alcohol; an extraction step, often repeated several times to ensure full extraction of fucoidan and most commonly using calcium chloride, dilute hydrochloric acid or water; further purification of the extract to remove alginate and other impurities before fucoidan is finally precipitated using ethanol [137-142]. One of the most prevalent of these was adapted from a method developed by Whyte [143] for the extraction of lipids from fish and involves the use of $CaCl_{2(aq)}$ to extract the fucoidan [18, 66, 69, 144] after pretreatment with a $MeOH:CHCl_3:H_2O$ mix. While effective, this technique is time and resource and time consuming, with some authors reporting 6 sequential, stirred extractions with $CaCl_2$ solution at 85°C for 24 hours [144]. The fucoidan is then precipitated out using a decyltrimethylammonium bromide solution. After this, a fairly lengthy purifying procedure is undertaken, which can involve fractionation using ion exchange chromatography [69] or dialysing against distilled water for several days [144].

A comparison of the three extraction solvents: distilled water, HCl and $CaCl_2$, was carried out by Ponce et al. [145]. The results indicated that distilled water and HCl extraction gave the highest and comparable yields of 10.8 and 9.6 wt% respectively, with the structure of each extract being very similar. Zhang and Row [146] further this, similarly comparing extraction solvents, but also identifying the best conditions for fucoidan extraction from *Laminaria japonica*. Their findings suggest an extraction time of 4 hours at 80°C and 0.1M HCl yields the best results, giving 17wt% fucoidan, although no analysis of the quality of the fucoidan has been made, so the results are based on the best yield alone. Overall, all of the methods described require long extraction and purification times in order to obtain a pure product and determine the accurate fucoidan content, taking up to 5 days depending on the number of extractions performed. Due to this lengthy procedure, novel extraction techniques are being proposed, which include enzyme-assisted [147], microwave-assisted [148] and ultrasound-assisted extraction [66] in order to reduce the process step and extraction time.

1.2.2 Microwave Assisted Extraction

Microwaves were first invented in World War II with the development of radar technology and were first used in a domestic setting in the 1950’s [149]. They were first used in a laboratory setting in the 1970’s for acid digestions for metal analysis [150]. It wasn’t until the 1980’s that they were applied to chemical extractions, with Ganzler et al [151] first using them for chemical extraction in preparation for analysis by liquid chromatography. Nowadays, microwaves hold a variety of uses throughout industry already, including in the food, plastics, animal feed and paper industries [152] and there is increased interest in their use within the biofuels industry. Applications such as pyrolysis [153] and transesterification [154] for the production of bio-oil are being investigated, but probably the application which has the most potential for seaweed is microwave-assisted extraction (MAE) and is defined by Eskilsson and Bjorklund [155] as a “process to heat solvents in contact with a sample in order to partition analytes from the sample matrix into the solvent”. Its ability to reduce extraction times and the amount of solvent required [155] make it a potential low cost alternative to traditional pretreatment techniques and it has also been proved that, in most cases, reproducibility and recovery of the species of interest is improved over conventional methods. In addition it is considered to be a more environmentally friendly process, requiring less energy and producing less waste [148].

Microwaves are being researched for a wide variety of extraction purposes. Early work using microwaves for chemical extraction, starting in the 1980’s and included the use of cotton and lupine seeds and other plant matter to efficiently remove chemicals of interest [149]. The trend for the use of microwaves to replace conventional heating for chemical extraction has continued since then, with more recent reports for their use relating to polysaccharide extraction from guava fruits [156] and tamarillo fruits [157]. A general trend in the literature suggests that the use of microwaves offers a more rapid alternative to conventional heating, while retaining polysaccharide structure and functionality.

1.2.2.1 Microwave Theory

Microwaves are electromagnetic waves with a frequency range of between 300MHz and 30GHz [158] however most research, medical and domestic equipment operates at a frequency of 2.45GHz so as not to interfere with other wave frequencies, such as radio waves [149]. They comprise of electric and magnetic components and therefore create electromagnetic energy [152]. The general principle behind microwave heating is via the rotating of dipoles, either permanent or induced, within a material. When a microwave field is applied, the dipoles arrange themselves in the direction of this field. The field is then removed and the dipoles return to their previous, disordered state, which causes heat to be emitted due to friction [152]. This means that microwaves heat the material evenly throughout the volume, unlike in conventional heating, where the material is heated from the outside, from where it travels via convection to the centre. Figure 1.11 shows a pictorial representation of this difference. There are several advantages to the use microwave heating of traditional methods, which include: non-contact; the transfer over microwave energy instead of heat; rapid, selective heating; the ability for high levels of automation and high levels of safety [159]. Microwaves present a safer alternative to conventional heating, as, rather than needing to heat an element, which in turn heats the medium of interest, the microwaves directly heats the medium via friction caused by the rotation of intermolecular forces, reducing the quantity of heated material. Microwaves are also usually applied in sealed vessels made of material “transparent” to microwave heating. This means that the heat is sealed within the vessel and any potential runaway reactions are well contained. Another potential advantage to this phenomenon is that, due to varying amounts and frequency at which dipoles are excited, materials are heated differently at different microwave powers. This gives the opportunity for sequential extraction based on the nature of dipoles present in the chemical [158].

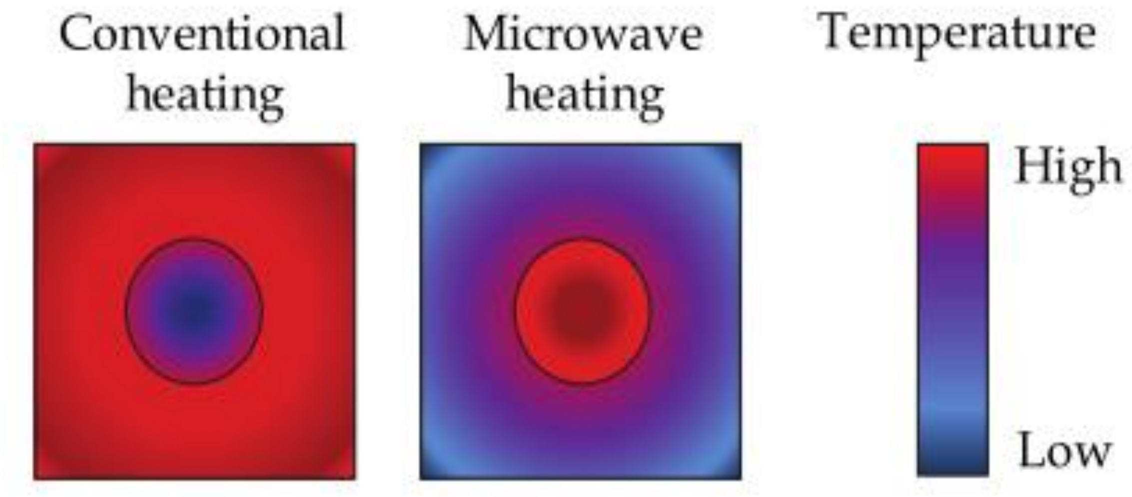


Figure 1.11: Differences between conventional and microwave heating.

Chan et al. [160] present a model for the cell rupture of plants during microwave heating. Their findings show that the microwaves interact with the moisture inside a cell, expanding as it is heated, stretching the cell wall and increasing the pressure inside the cell. As the pressure increases, the cell wall ruptures, releasing its compounds into the surrounding solvent. The time with which it takes for the cell to rupture is dependent on the tensile strength of the cell wall. As the moisture inside the cell can be heated directly, without the need to wait for convention from the outside in, as with conventional heating, the time it takes for cell walls to rupture is significantly reduced and, therefore, shorter processing times are required.

1.2.2.2 MAE for the Extraction of Macroalgae

Chhatbar et al. [161] detail the use of microwaves for the hydrolysis of sodium alginate. Conventionally, this requires sodium alginate to be subjected to strong acidic conditions for a long time, in the order of several hours. However, the use of microwave, mild conditions (0.15-0.25M) and short time frames (1-5 minutes) were able to give the same results with good reliability. This is of interest with regards to biofuels production as alginate is a major component in seaweeds and if this could be hydrolysed easily, then its possible conversion to ethanol may be facilitated.

The use of MAE has also be detailed by Rodriguez-Jasso et al. [148] for the extraction of fucoidan from the brown seaweed *Fucus vesiculosus*. These are traditionally extracted using large volumes of solvent and long extraction times, but optimum conditions using microwaves are reported to be 1g alga/25ml water for 1 minute: a significant saving in both costly use solvent and time. Yuan and Duncan [162] have designed microwave assisted algal biorefinery based on the conventional extraction method for fucoidan, described in section 1.2.1.1, creating a reduction in the extraction time required. The study, along with its associated study, detailing the quality of the fucoidan extracted in the process [163] gain good extraction of fucoidan and alginate, extracting 96% and 79% respectively of the total present in the biomass. This, however, seems to be achieved at the expense of quality of fucoidan, as it appears the quantity of sulphate and the MW of the polymer are reduced at the temperature required for optimum extraction, while the lower temperature required to keep the polymer intact significantly effects the amount extracted. This is likely due to the interaction of HCl with fucoidan, used for extraction, which has been shown in previous studies to have a degradative effect on fucoidan at elevated temperatures [164].

1.3 Conclusion

Overall, seaweed is clearly an important and underutilised resource which contains a range of both high value chemicals and potential precursors for fuel production. This includes a range of unique carbohydrates that have interesting applications for pharmaceutical development, ingredients for the food and cosmetics industry and building block molecules for use in fuel and bulk chemical production.

In order to make the most out of the resource, the development of a seaweed biorefinery, sequentially extracting high value chemicals while using the “waste” to produce fuel, offers an economical and sustainable option for fuel production. Furthermore, being able to process the seaweed in as energy efficient manner as possible is important for a world in which climate change is an important consideration. The use of microwaves offers a low energy alternative to traditional heating methods, with the potential to reduce both the temperature and time required for extraction. While microwaves are common place in the food industry, there is little literature to support their use in a biorefinery setting. More research into their application to seaweed extraction and their benefits over conventional heating is required.

An important consideration when considering seaweed as a feedstock for an industrial process is its seasonal variation. To avoid difficult and costly drying/storing of the biomass, year-round harvest would be preferred. However, the composition of the seaweed, as well as the structure of the high value carbohydrate, fucoidan, vary throughout the year. The literature is missing recent, in depth studies into the seasonal variation of three species of fucoid common to the coast of the UK; *Fucus serratus*, *Fucus vesiculosus* and *Ascophyllum nodosum*. These are good potential feedstocks to biorefinery due to their high fucoidan and carbohydrate content, coupled with a relatively low ash content. Furthermore, there is currently no literature detailing the structural variation of fucoidan. This is especially important for year-round harvest considerations, as the structure has a large effect on is bioactivity and, therefore, medical properties.

This thesis aims to cover these gaps in the literature, first detailing the seasonal variation in chemical content of *Fucus serratus*, *Fucus vesiculosus* and *Ascophyllum nodosum*. This will include caborhydrate content (fucoidan, mannitol, laminarin and alginate), as well as protein, ash and proximate and ultimate analysis. A detailed study into fucoidan is also presented, analysing its variation in molecular weight, fucose and sulphate content and structure. The information from these studies are then used to predict the best species and harvest month to take forward into the development of a microwave seaweed biorefinery.

The biorefinery development involves the sequential extraction of carbohydrates at increasing temperatures. Temperature, microwave residence time and seaweed to solvent ratio are varied to identify the optimum conditions for the extraction of each chemical. The residual “waste” biomass is assessed for its potential use for fuel production. Finally, the impact of seasonal variation on the final, proposed biorefinery schematic is assessed.

2 Methodology

2.1 Materials and Sample collection and preparation

All seaweed samples have been collected from Aberystwyth shore (Latitude: 52.41°N, Longitude: -4.08°W) at low tide. Seasonal variation samples of *Fucus serratus* (FS), *Fucus vesiculosus* (FV) and *Ascophyllum nodosum* (AN) were collected monthly between April 2010 – March 2011, freeze dried, ground and sieved to 500µm. Microwave extraction samples of FS were collected in June 2015, were air dried for 48 hours followed by oven drying at 50°C for 24 hours, ground and sieved to 500µm. All samples were dried within 24 hours of collection.

All chemicals and reagents have been supplied by Sigma Aldrich, VWR or Fluka and are of analytical grade.

2.2 CHNS

CHNS is used for the rapid determination of carbon, hydrogen, nitrogen and sulphur. The basic principle behind it involves 3 main steps: combustion, clean-up and detection. For the first stage, the samples, enclosed in tin capsules in the case of solids, are introduced into a high temperature (around 1000°C), oxygen rich combustion chamber, where the 4 elements of interest are converted into gasses [165]:

Carbon → Carbon Dioxide

Hydrogen → Water

Nitrogen → Nitrogen gas/nitrogen oxides

Sulphur → Sulphur dioxide

After combustion, the gasses go through a “clean-up” process. This involves, firstly, being passed over hot (around 600°C) high purity copper, which removes the oxygen that has not been consumed, as well as converting any nitrogen oxides to nitrogen gas. This is followed by a series of absorbent “traps” which collect a variety of unwanted products from combustion, such as HCl if chlorine is present [165]. This leaves only carbon dioxide, water, nitrogen gas and sulphur dioxide in the gas stream. Finally, these gasses are separated by gas chromatography, followed by quantification using thermal conductivity detection. This quantification requires calibration through the use of standards [165].

The carbon, nitrogen, hydrogen, oxygen and sulphur content of the biomass and extracted fucoidan has been analysed using a CHNS analyser (*CE instruments flash EA 1112 series*). 2.5±0.5mg of sample is weighed into a tin capsule, to which approximately 5mg of vanadium pentoxide is added as a combustion aid. This is required in order to achieve full combustion of sulphur compounds, giving a reliable sulphur reading. Capsules are loaded into the analyser, which is run at 900°C for 20 minutes. CHNS values are quoted, where possible, on a dry, ash free basis, meaning the oxygen value can be calculated by difference.

2.3 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) studies the change in mass of a sample as the temperature is varied [166]. Samples of known weight are placed on a sensitive balance in a heat proof crucible and subjected to controlled increases in temperature, over which the mass loss is monitored and recorded [166]. By controlling the atmosphere in which the sample is heated, it is possible to promote certain reactions over others. For example, it is possible to evolve the volatile components in a sample, without interference from combustion, if the sample is heated in an inert gas such as nitrogen [166].

In this case, proximate analysis of samples has be carried out via TGA. The program consists of ramp to 105°C at 10°C/min in nitrogen and held at this temperature for 10 minutes, giving the moisture fraction. The temperature is them ramped at 25°C/min to 900°C, where it is held for a further 10 minutes, driving off volatiles. Finally, the gas is switched to air, the temperature ramped to 815°C at 20°C/min and the temperature held at 815°C for 10 minutes, burning off any fixed carbon. Ash is then calculated by difference from these figures. Figure 2.1 gives an example TGA for FS and details each of these steps.

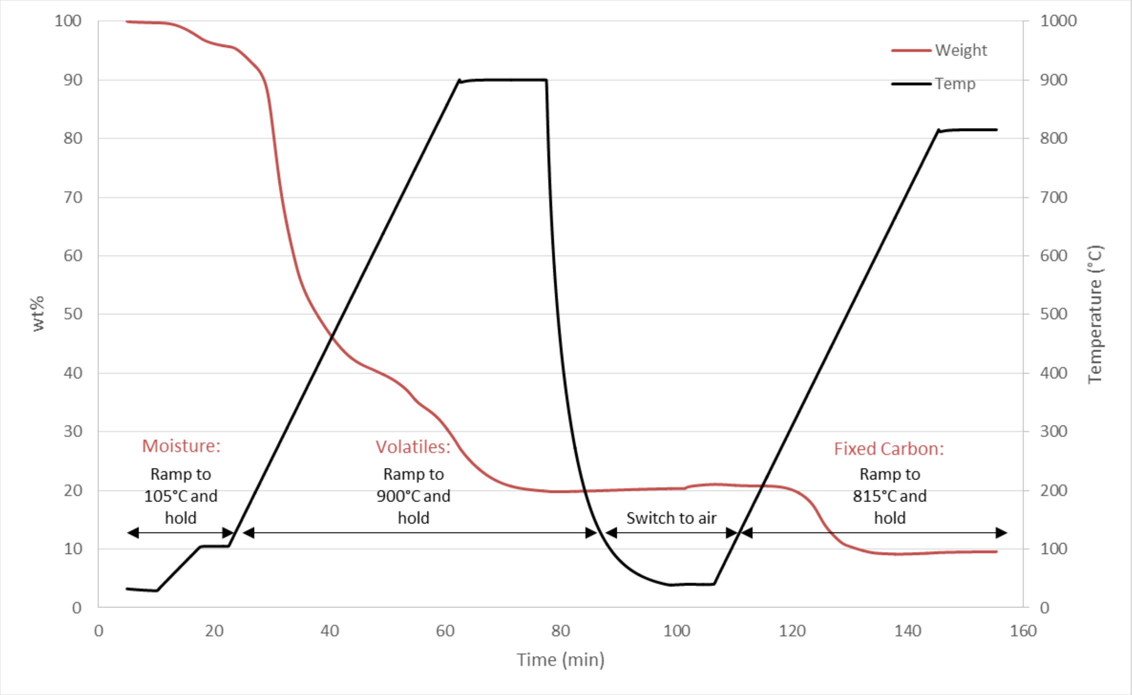


Figure 2.1: Example TGA curve, indicating each of the steps involved.

2.4 Sugar and Organic Acid Analysis

Sugar analysis is achieved via a two-step process: firstly, the samples are digested in acid, in order to hydrolyse the carbohydrates into their monomer units, followed by separation and detection of these monomers by HPLC. This method follows that of the NREL method of the determination of structural carbohydrates and lignin in biomass [167]. For acid digestion, 150±5mg of sample is weighed into a 50ml Pyrex tube, to which 1.5ml of 72% H_2SO_4 is added. A clean, PTFE stirring rod is added to each tube and mixed thoroughly. The tubes are then placed in a water bath at 30°C for 1 hours, stirring regularly throughout. The tubes are removed from the water bath and the concentration of H_2SO_4 diluted to 4% with water (approximately 42ml). The lids are screwed on tightly, inverted several times to mix and loosened by a quarter turn in order to prevent pressure build up in the autoclave. The tubes are sealed in an autoclave set to 121°C and held at that temperature for 1 hour. Once the tubes have cooled and the solid biomass has settled, a 5ml portion of the liquid is pipetted into a clean 50ml centrifuge tube. Calcium carbonate is added in small portions, swirling between additions, until the pH is 5-6. The tubes are centrifuged and the supernatant removed with a 2ml syringe. This is filtered through 0.2µm syringe filters into HPLC vials ready for analysis. See section 2.5 for details on HPLC set-up.

For this analysis, it is important that the ash content is below 10 wt%, to prevent interference by salts on the acid concentration, inhibiting hydrolysis. As seaweeds typically have a high ash content, well over 10wt%, this was overcome by washing the samples with ethanol prior to hydrolysis. 0.5±0.005g of biomass was mixed with 2x 20ml of 98% ethanol to remove surface salts, centrifuged, the ethanol poured off and the residue allowed to dry to a constant weight at room temperature. Ethanol is used as the carbohydrates present in seaweed are soluble in water, meaning an alternative solvent must be used. Although only a small amount of salts present in seaweed dissolve in ethanol [168], none of the carbohydrates dissolve and, with the high solvent to biomass ratio used here, enough of the lipids and salts are removed to stop their interference with the acid concentration for hydrolysis.

2.5 Liquid Chromatography

2.5.1 High-performance liquid chromatography (HPLC) and size exclusion chromatography (SEC)

High-performance liquid chromatography (HPLC) and size exclusion chromatography (SEC) both use high pressure to force eluent, carrying the sample with it, through a packed column containing small particles designed to specifically separate chemicals of interest [169]. Analytical scale equipment uses small columns, with diameters between 1 and 5mm and 5 to 30cm in length. Generally flow rates of 0.5-5ml/min with pressures between 50 and 400 bar [169]. Compounds within the sample are separated by the difference in affinities between stationary and mobile phase, which could be based on adsorption, size or charge [166]. SEC is primarily used to separate and identify large molecules, in this case carbohydrates, extracted from seaweed samples, which are separated based on size. HPLC has been used to determine sugars and organic acids, with the columns used for this separating based on the charge and number of bonding sites on the various sugars and acids.

Liquid samples for SEC or HPLC are prepared by filtering through a 0.2 µm syringe filter (VWR, 25mm) into glass HPLC vials sealed with pre-slit lids. The instrument used is a Dionex Ultimate 3000 fitted with a Shodex RI-101 refractive index detector or Ultimate 300 photodiode array (PDA) detector, depending on analysis. For sugar analysis, a de-ashing column (Micro-Guard De-Ashing cartridges and column, Bio-Rad Laboratories) has been added to the set-up, to remove any salts from the sample and the interference caused by these. SEC samples have been treated with an ion exchange resin (Dowex Marathon MR-3, Sigma) to similarly remove the interference in the chromatogram caused by the presence of salts. Samples are loaded into the racks and the analyser set with the appropriate program. The conditions and column type for each program used are detailed in Table 2.1. Each column is calibrated with the relevant standards. Figure 2.2 gives example standard calibrations for each HPLC set-up. SEC has been calibrated with a set of polyethylene glycol/polyethylene glycol standards (MW 200 to 1,015,000Da) (Fluka). All other standards have been purchased from Sigma.

Table 2.1: Key parameters for HPLC/SEC analysis

Parameter	**SEC**	**Sugars**	**Organic Acids**
Column Type	Ultrahydrogel 500 (Waters)	Pb 6% (Supelco)	C610H (Supelco)
Mobile Phase	Deionised water	Deionised water	0.1% phosphoric acid
Oven Temp (°C)	30	80	35
Run time (min)	30	45	30
Flow rate (ml/min)	0.5	0.5	0.5
Injection volume (μl)	10	10	10

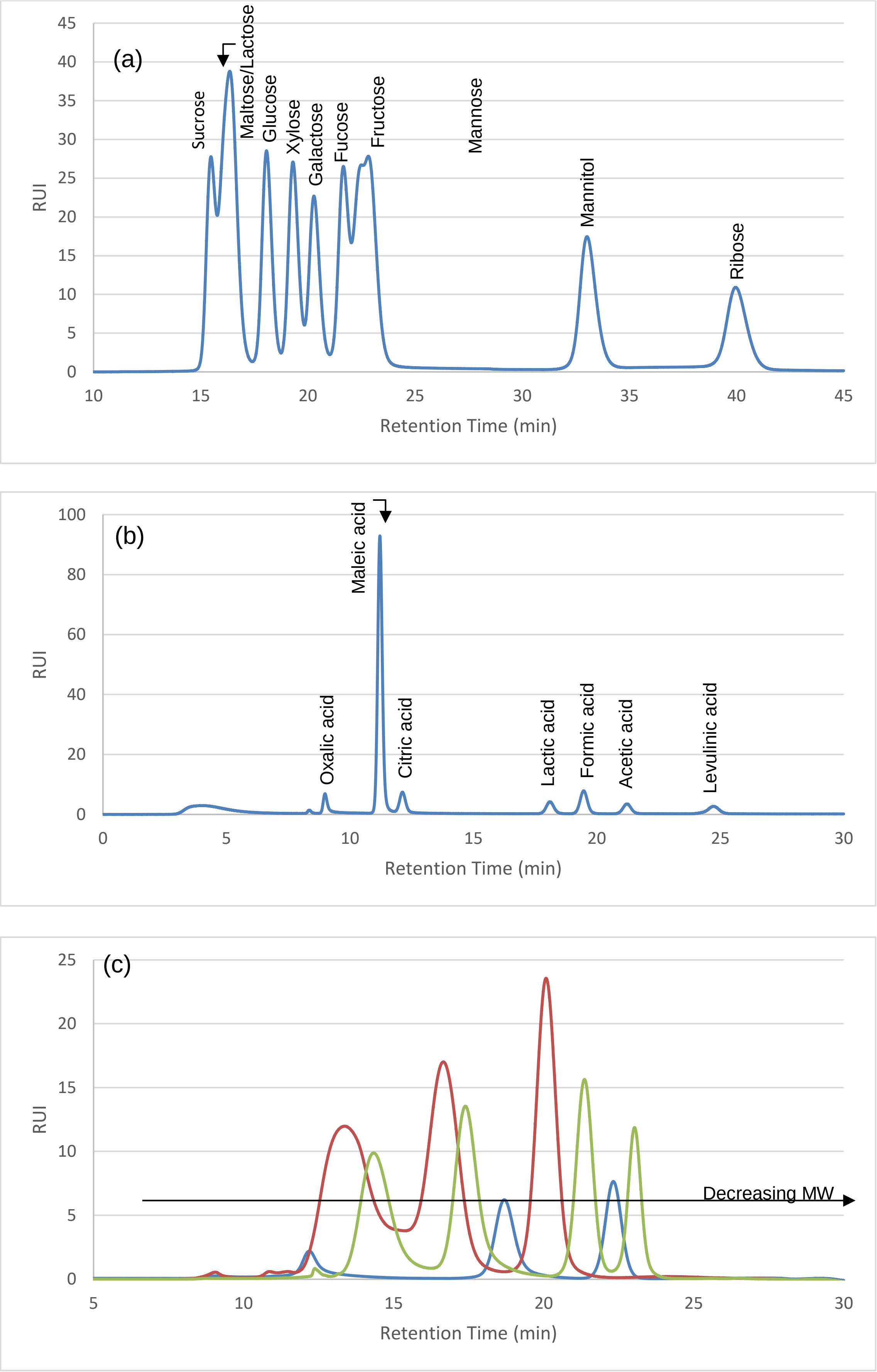


Figure 2.2: HPLC calibration chromatograms for (a) sugars, (b) organic acids and (c) SEC.

2.5.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS has been performed using an Aligent 1200 series HPLC and Brucker HCTultra MS. The samples have been run through the Waters Ultrahydrogel 500 used for SEC with the same conditions as in Table 2.1. The MS was operated in negative ion mode with a mass scan between 100 and 1300m/z. All samples have been filtered through 0.2µm syringe filters prior to running through the LC-MS.

2.6 Fucoidan Extraction

Fucoidan is extracted via a dilute acid method, as optimised by Zhang and Row [146]. An flowchart detailing an overview of the method is given in Figure 2.3. 0.5 ± 0.01g of sample is weighed into a 50ml centrifuge tube, to which 10ml of 85% ethanol is added and stirred for 4 hours at room temperature. The tubes are centrifuged for 5 minutes at 3500 rpm and the liquid decanted off. The solids are washed with 5ml of acetone before being allowed to dry to a constant weight at room temperature. 0.3g of the resulting biomass is weighed into a clean tube, to which 7.5ml 0.1M hydrochloric acid is added and stirred at 80°C for 4 hours. This is allowed to cool, centrifuged and the supernatant decanted into a clean tube. The residue is freeze dried. 1 volume (~6ml) of 1% $CaCl_2$ is added and left for at least 4 hours at 4°C to precipitate alginate. The tubes are centrifuged and the supernatant decanted to another clean tube to which ethanol is added to give a final concentration of 40% v/v to precipitate laminarin, left for at least 4 hours at 4°C, centrifuged and the supernatant decanted to a clean tube. Lastly, ethanol is added to give a final concentration of 70% v/v to precipitate fucoidan. This is left for at least 4 hours at 4°C to precipitate fucoidan, before being centrifuged and the supernatant is removed. The fucoidan fraction is washed with a small quantity of acetone and allowed to dry to a constant weight at room temperature, along with the laminarin fraction.

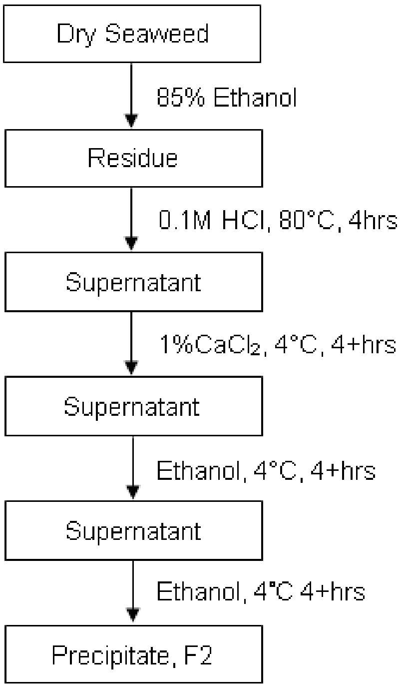


Figure 2.3: Schematic of the steps involved in fucoidan extraction

2.7 Spectrophotometric Fucoidan/Fucose Analysis

Fucose/fucoidan content is determined colourmetrically, via a method originally proposed by Dische and Shettles [135]. For fucoidan analysis from solid seaweed biomass, a full account of the development of this method is given in 4.2, including relevant calibration curves, so only an overview is given here. The method involves two main steps: extraction of fucoidan using dilute hydrochloric acid followed by hydrolysis of fucoidan with concentrated sulphuric acid. For fucose analysis, fucoidan, extracted as in section 2.6, is diluted to 1.25wt% and only the second, acid hydrolysis step is completed. A schematic for the two methods is given in Figure 2.4.

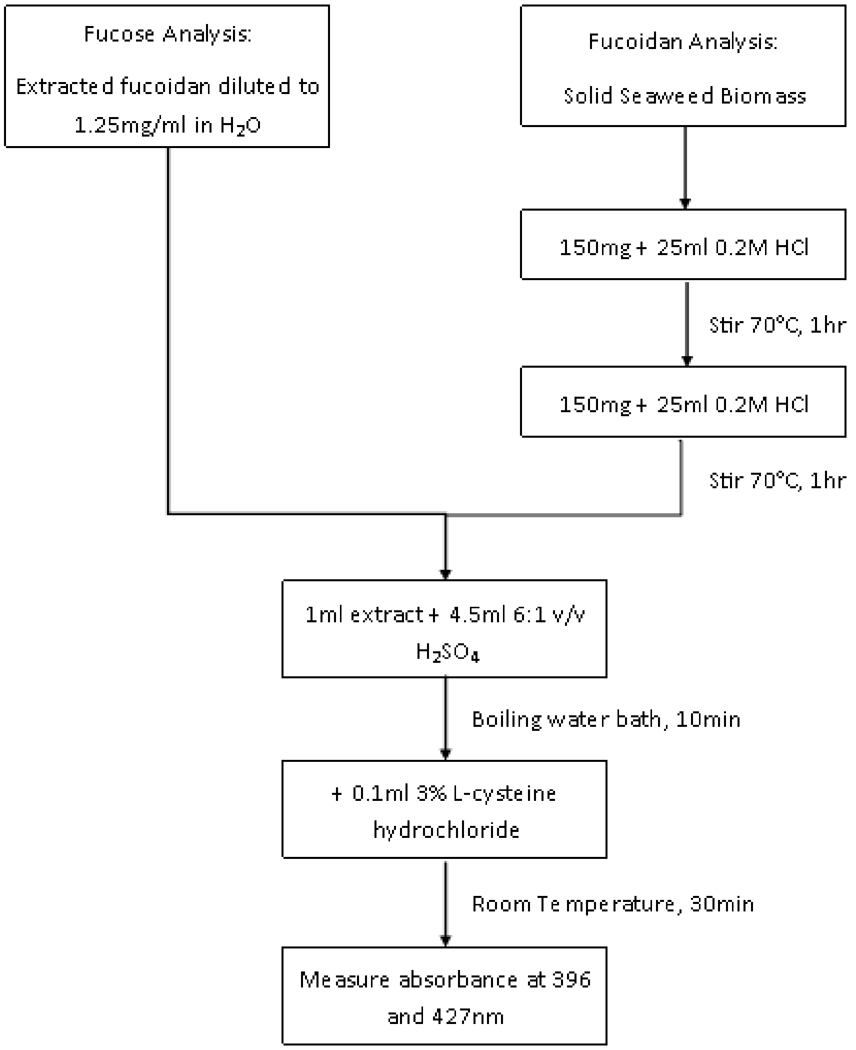


Figure 2.4: Schematic of the method for spectrophotometric determination of fucose and fucoidan

For acid extraction of fucoidan from solid seaweed, 150±5mg of sample is weighed into a 50ml centrifuge tube, to which 25ml of 0.2M HCl is added. This is stirred at 70°C for 1 hour, centrifuged and the supernatant decanted into another centrifuge tube. A further 25ml 0.2M HCl is added and the process repeated. The extracts are combined and the tube inverted several times to mix.

Concentrated acid hydrolysis is achieved in the following way: a set of aqueous fucose standards between 30 - 150mg l^{-1}, and relevant blanks are prepared. 1ml aliquots of sample, either diluted fucoidan extract for fucose analysis or acid extracted fucoidan (as above) for fucoidan analysis, standards and blank are pipetted into 15ml Pyrex tubes to which 4.5ml of 6:1 v/v H_2SO_4 is added. The tubes are capped, inverted several times to mix, left for approximately 5 minutes at room temperature and placed in a boiling water bath for 10 minutes to hydrolyse the fucoidan into fucose. The tubes are cooled under running water. 0.1ml of 3% aqueous L-cysteine hydrochloride is added to each tube, and inverted to mix. The reaction is left for 30 minutes, before the absorbance's at 396 and 427nm are recorded. The concentration of fucose (C_f), in mg/ml, is then calculate using Equation 1, where A_{396} and A_{427} are the absorbance readings at 396 and 427nm respectively, B is the blank reading and x is the slope of the calibration curve for fucose, derived experimentally:

$$C_f = \frac{(A_{396} - A_{427}) - B}{x} \qquad \text{Equation 1}$$

For fucoidan analysis via this method, correction factors for alginate and laminarin are required, the equation for which is given in Equation 2, where F_{adj} is the adjusted fucoidan, F_{int} is initial fucoidan and A is alginate, all in wt%. The method used to determine this adjustment factor is given in section 4.2.

$$F_{adj} = \frac{F_{int}}{1.2 + (0.007A - 0.09)} \qquad \text{Equation 2}$$

2.8 Sulphate Analysis

Sulphate analysis have been achieved via a kit supplied by Hach-Lange (LCK353). 1.25wt% solutions of extracted fucoidan are used. The method used in this kit is based on that originally described by Tabatabaia [170] and involves the reaction of barium chloride with weakly acidified samples to form barium sulphate. This is only sparingly soluble and the turbidity can be measured photometrically to give the concentration of sulphate in the original solution. 2ml of fucoidan solution is pipetted into the analysis vial, to which a measured portion (one scoop using the provided measure in the kit) of barium chloride is added. This is inverted for 1 minute to mix, followed by 30 seconds standing at room temperature. The vial is then inserted into the Hach-lange UV-vis, which is fitted with a barcode reader to determine the wavelength and calibration required for analysis.

2.9 Metal Analysis

2.9.1 Acid digestion

0.2±0.05g of sample is digested in 10ml of concentrated nitric acid using either microwave (Aston Parr, USA) enhanced digestion or on a hot plate. Samples are digested for approximately 1 hour at 200°C, after which the temperature is increased to 250°C and the samples evaporated to dryness. A further 5ml of concentrated nitric acid is added and warmed to dissolve the metals and samples are diluted to x500 for analysis. The metals in the digests have then been measured using ICP-MS (Perkin Elmer, USA). A schematic for hot plate digestion set-up is shown in Figure 2.5.

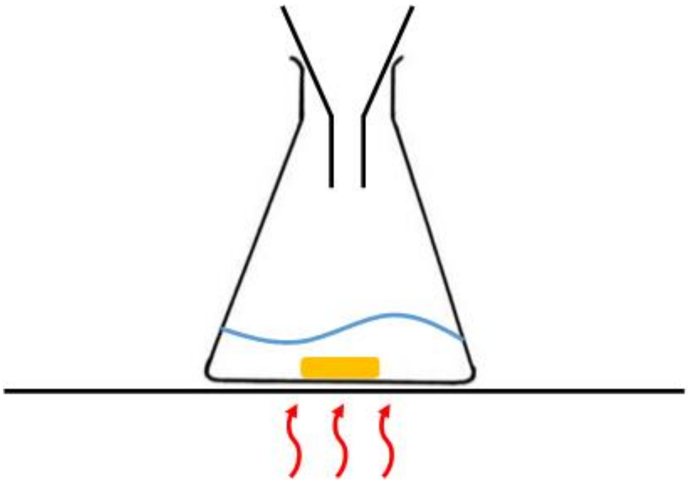


Figure 2.5: Schematic for the apparatus set-up for hot plate acid digestion for metal analysis.

2.9.2 Phosphorus Analysis

Acid digested samples have been reacted with an ammonium molybdavanadate solution to determine their phosphorus content. Ammonium molybdavanadate is made by adding 200ml of 0.313% solution of ammonium matavandate in 1:1 nitric acid to 200ml 12.5% solution of ammonium molybdate and making up to 500ml. In each case, a sample and sample blank are required, due to the interference of iron with the UV reading. These are prepared by pipetting 1ml of sample into 2ml Eppendorf tubes for both the sample and the sample blank. To the sample tubes, 0.4ml of ammonium molybdavanadate and 0.6ml of deionised water is added. 1ml of deionised water is added to the sample blanks. The colour reaction is left to develop for 1 hour, before the measurement in a spectrophotometer (Thermo Scientific, USA) at 430nm. For this, 250µl of each sample is pipetted into a 96-well plate compatible with the machine. A calibration curve between 5-25ppm is made in the same way with phosphate standard, with a sample and sample blank for each. The calibration curve used is given in Figure 2.6.

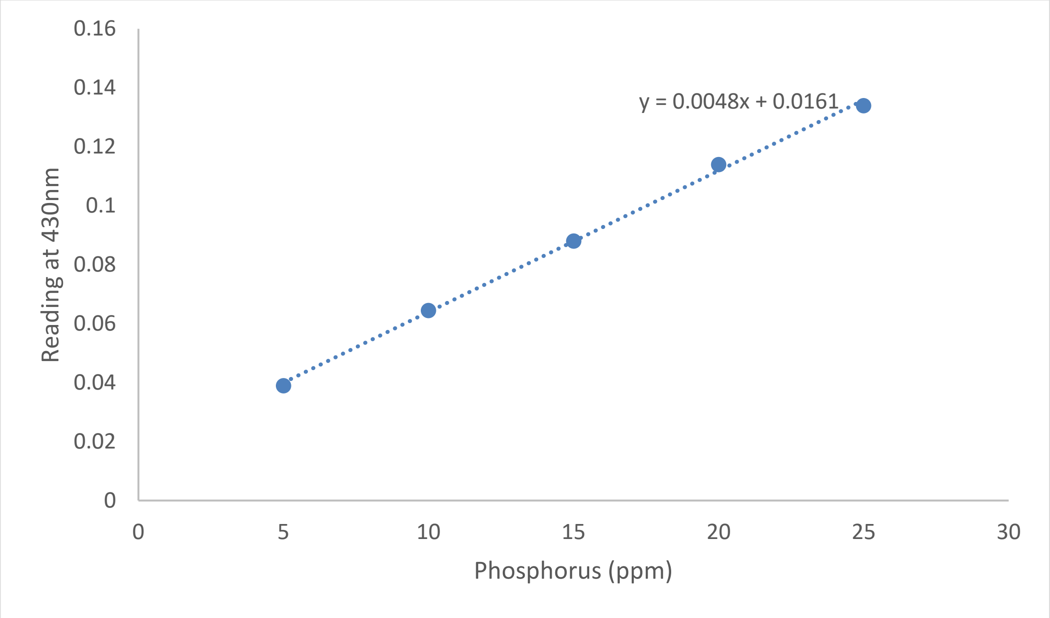


Figure 2.6: Calibration for phosphorus analysis

2.10 Hydrothermal Microwave Extraction of Seaweed

An overview of the process for hydrothermal microwave extraction of chemicals from seaweed is given in Figure 2.7. 1±0.01g of seaweed is weighed into a microwave reactor tube, to which 10 or 15ml of distilled water is added. The tube is sealed in a reactor tube and placed in the microwave (Milestone Start Synth Microwave Synthesis Labstation, with a reactor volume of 42L , using the Q20 closed vessel system, with a vessel volume of 45ml) in either the single or carousel configurations, as shown in Figure 2.8. On the whole, the single tube set-up was used, as this gives the best temperature control and reading, but, when a large volume of sample was required, the carousel was used. The microwave is then set to the desired program, which includes a ramp time of 5 minutes up to the desired temperature: between 50 and 200°C, and then held at that temperature for between 5 and 10 minutes, and is then cooled by a fan until the temperature is below 50°C. The tube is then removed from the microwave and the contents poured into a 50ml centrifuge tube. The microwave tube is rinsed thoroughly with distilled water, which is added to the centrifuge tube. This is centrifuged at 3500rpm for 5 minutes and the supernatant decanted into a clean tube. The residue is washed with ~10ml distilled water, centrifuged a second time and the supernatant combined with the previous. The supernatant is made up to 50ml and the residue and a portion of the supernatant are freeze dried for analysis.

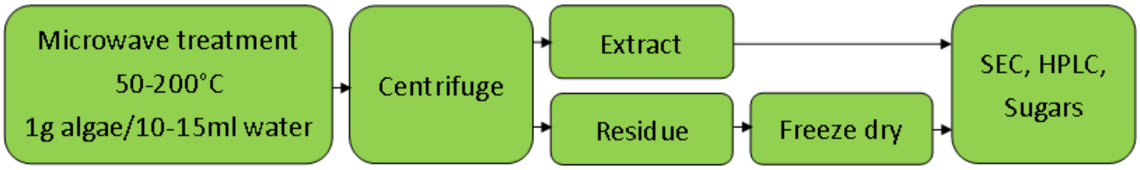


Figure 2.7: Schematic for hydrothermal microwave extraction of chemicals from seaweed.

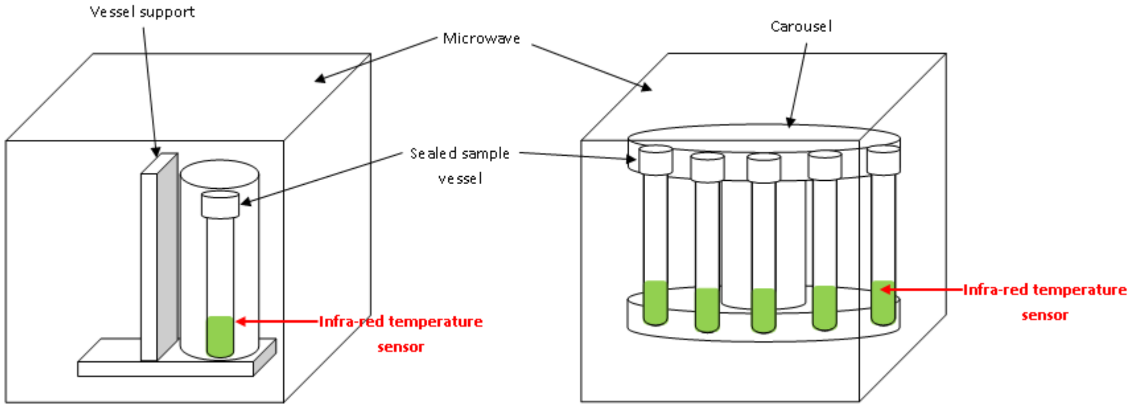


Figure 2.8: Microwave reactor set-up for single and carousel configurations.

2.11 Conventional Hydrothermal Extraction of Seaweed

Conventional hydrothermal extraction of seaweed is carried out in the same way as hydrothermal microwave extraction, with the microwave being replaced with a sand bath set at the desired temperature: between 50 and 200°C. A schematic of the main steps is given in Figure 2.9 and the reactor vessel shown in Figure 2.10, which has a volume of approximately 30ml. 1±0.01g of seaweed is added to a reactor tube, to which 10-15ml of distilled water is added and the reactor is tightly sealed. The reactor is placed in the sand bath and the timer started for the desired time when the contents of the reactor is up to temperature: this took around 5 minutes in each case. Once the desired time has been reached, the reactor is removed from the sand bath and plunged into cold water to cool. The contents of the reactor are treated in the same way as hydrothermal microwave extraction, with the supernatant being made to 50ml and the residue and portion of the supernatant being freeze dried.

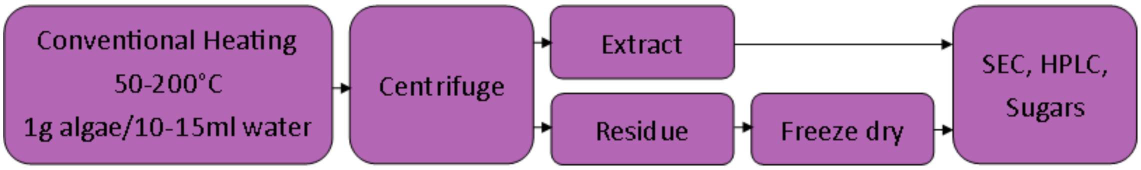


Figure 2.9: Schematic of conventional hydrothermal extraction of seaweed.

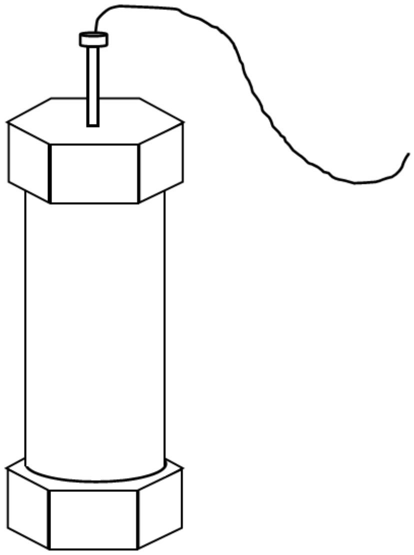


Figure 2.10: CHE reactor set-up.

2.12 Experimental Replication and Statistical Treatment

All analyses have been performed in a minimum of duplicate; it is indicated in the text were more than 2 samples have been analysed. The average values are reported along with the standard error in all tables and figures. For colourimetric analysis, such as fucose and phosphorus analysis, absorbance readings for each sample are taken in duplicate, of which the average is taken forward for further calculation to ensure a representative sample has been analysed.

3 Seasonal Variation of Three Species of Fucoid

3.1 Introduction

The seasonal variation in the chemical content of macroalgae is an important phenomenon for industrial consideration. Ensuring a consistent product and knowing the quantity of a chemical you are likely to extract is central for industry applications, where reliability is a key factor. Furthermore, different industry applications may wish to harvest at a different time of year, based on the chemicals in which they are interested. For example: the food industry may prefer a high protein content, whereas harvest for bioethanol production would benefit from a high carbohydrate content. In this chapter, the seasonal variation of three species of macroalgae: *Fucus serratus* (FS), *Fucus vesiculosus* (FV) and *Ascophyllum nodosum* (AN) harvested monthly off the coast of Aberystwyth has been explored. These seaweeds were chosen for their abundance around the UK coastline and their relatively high fucoidan and carbohydrate content, coupled with a comparatively lower ash content. Little research has been carried out on the seasonal variation of *Fucoids*, with the last published work found dating from the 1950's. Changes in water temperature and acidity due to climate change, levels of contamination due to industrial and anthropogenic activity and increased levels of eutrophication from farming activity are likely to affect seaweed composition. Recent interest in the extraction of high value, bioactive components from seaweeds is also growing and, along with the advancements in analysis equipment and techniques, support the necessity for an extensive, up to date survey of the seasonal variation of *Fucoids* found in the UK.

3.2 Selection of Species

The selection of a species for a macroalgal biorefinery is important to ensure good yields of the desired chemicals, as well as minimising any potential damage to equipment caused by high ash levels and be abundant around the coast of the UK for wild harvest or have good potential for cultivation as a farmed seaweed. The key points for the selection, based on the production of chemicals and fuel, are:

- A high fucoidan content, for extraction as a high value chemical.
- A high laminarin and/or mannitol content, which can be converted to fuel.
- Low ash and metal content, which can cause corrosion and slagging and fouling in equipment.
- An species which is abundant and fast growing around the coast of the UK.

Brown macroalgae are generally the largest in size and are also the most fast growing [33], so would be an ideal candidate for a macroalgal biorefinery in the UK. Most of the brown seaweeds fall into two main categories: kelps and wracks. Table 3.1 gives an overview of some common brown seaweed species and their chemical content, based on literature. All the species chosen for inclusion in this table are abundant around the coast of the UK, have fast growth times and have the possibility for offshore cultivation. Although data for fucoidan could not be found for all species, it is clear that there it is present in higher quantities in the wracks than in the kelps by a significant margin, with it accounting for up to 20 wt% in FV, when compared to a maximum of 4 wt% in L. digitata. Ash content of the wracks is also lower, with a range of 15-30 wt% across the species listed, compared to 15-45 wt% for the kelps. Although the laminarin content of the wracks is lower, with a highest value of 10 wt% reported compared to 29 wt% for the kelps, the mannitol and alginate content is fairly similar between both.

Table 3.1: Chemical content of seaweed species common to the UK coast [21-24, 64, 79, 81, 84, 136, 171-173].

Species	**Laminarin**	**Mannitol**	**Alginate**	**Fucoidan**	**Ash**
Wracks					
Fucus serratus	2-10	5-20	17-22	7	20-30
Fucus vesiculosus	2-5	8-16	14-17	4-20	15-20
Ascophyllum nodosum	2-7	7-11	25-28	3-12	15-25
Fucus Spiralis	2-10	6-12	13-17	-	15-25
Kelps					
Saccharina latissima	1-26	8-22	18-27	2.2	21-40
Laminaria digitata	0-25	5-32	13-31	2-4	14-42
Laminaria hyperborea	2-15	9-25	16-30	-	19-45
Laminaria cloustoni	0-29	4-18	14-25	-	22-43

Taking these factors into account, its seems the wracks offer the best option, with a low ash content coupled with a high fucoidan content. While the quantity of laminarin and mannitol is lower than in the kelps, the levels are still high enough to present a good option for fuel production. Furthermore, early seasonal variation data by Black [22, 24] suggests that the wrack species show less prominent seasonal variation in carbohydrates and ash than the kelps, which is also promising for industry, where a consistent product is important.

Once it was clear that wracks presented the best option for the desired application in this study, the three species presented in this study; FS, FV and AN were chosen primarily for their abundance at the harvest site in Aberystwyth, although they also present a good cross section *Fucoid* species and present desirable chemical contents.

3.3 Results and Discussion

The total chemical content of FS, FV and AN are given in Figure 3.1, with the error bar representing the cumulative standard error for all the constituents of the biomass (full numerical data for this is given in the Appendix in Figure A.1). In all cases, when taking into account the error bars, analysed components add up to around 100 wt%, giving a measure of validity to the accuracy of the results. As not all chemical components have been quantified: for example lipids and pigments, the components do not add up to 100% in all cases. From these graphs, some overall trends can be picked out: FV contains a smaller quantity of alginate throughout the year when compared to FS and FN, averaging 27.3 wt% compared to 34.9 wt% and 34.2 wt% respectively on a dry basis. Laminarin and Mannitol are generally higher in the *Fucus* species compared to AN. The ash content over all three species is relatively similar, averaging 14.2 wt%, 16.6 wt% and 16.1 wt% respectively for FS, FV and AN on a dry basis. FV has the highest fucoidan content, averaging 10.8 wt%, followed by AN with 8.4 wt% and FS has the lowest, with an average on 6.3 wt%.

Figure 3.1: Total chemical content of (a) FS, (b) FV and (c) AN

These overall trends paint a picture of the seasonal variation of the all the components in seaweed, with the composition across all the months being different. This highlights the difficulties which occur in their industrial use, where continuous production and year round harvesting would be desired. While the yearly trends in the variation have been shown to vary depending on the weather conditions and temperature in a particular year [21] and can vary by up to two months, an in depth understanding of seasonal variation is clearly key to the utilisation of this feedstock. Seaweed represents the world’s largest biomass resource [19], with only a small proportion of it being utilised, industrially or otherwise, currently. Alongside this, there is a large area of open sea in which it could be cultivated and harvested, adding to its potential market. It represents a wealth of useful chemicals, such as mannitol, an low-calorie sweetener and alginate, which has a wide range of uses, including as an thickener in the food industry and coating for tablets in the pharmaceutical industry [174] and also offers the opportunity for the production of building-block chemicals, such as succinic acid [128] and 5-hydroxymethylfurfural (HMF) [175]. Furthermore, with the advancement of seaweed fermentation [7, 51, 106, 176] and anaerobic digestion [118, 119, 122, 123], their use to produce fuels is also gaining interest. With all this in mind, understanding the seasonal variation of seaweeds and the challenges it presents is key to the full and effective use of this resource.

3.3.1 Proximate and Ultimate analysis

The proximate analysis, given in Figure 3.2, reveals a slight dip in the fixed carbon content in the summer months, but otherwise remain fairly constant, with average values of 10.5±1.4 wt%, 9.1±1.2 wt% and 10.9±0.9 wt% for FS, FV and AN respectively. Volatiles content also remain fairly constant throughout the year, with average values for FS, FV and AN of 69.4±2.2 wt%, 68.6±2.2 wt% and 67.0 ±1.6 wt% respectively. While the average volatile content is fairly similar for the three species, FS and FV show more variation over the year, while AN has a more constant value. The ash content has a clearer seasonal variation trend: decreasing through spring to a minimum at the end of summer, rising again over the winter months. The high ash content for these samples is due to the high metal content associated with sea water and is in line with previous studies into *fucoid* composition [22, 24]. The ash content in *fucoids* appears to be lower than for kelps, which have been reported to contain up to 45 wt% ash [79]. High ash is associated with increased slagging and fouling in industrial equipment during processing [81], so the lower ash content in fucoids makes them a more desirable macroalgal feedstock for processing. FS has a slightly lower ash content than the other two species, averaging 13.2±2 wt% compared to 15.4±2.2 wt% and 14.9±1.4 wt% on an as received basis for FS and AN respectively. However, the difference is not so great that it would be a deciding factor if other components are more favourable in a different species for the desired application.

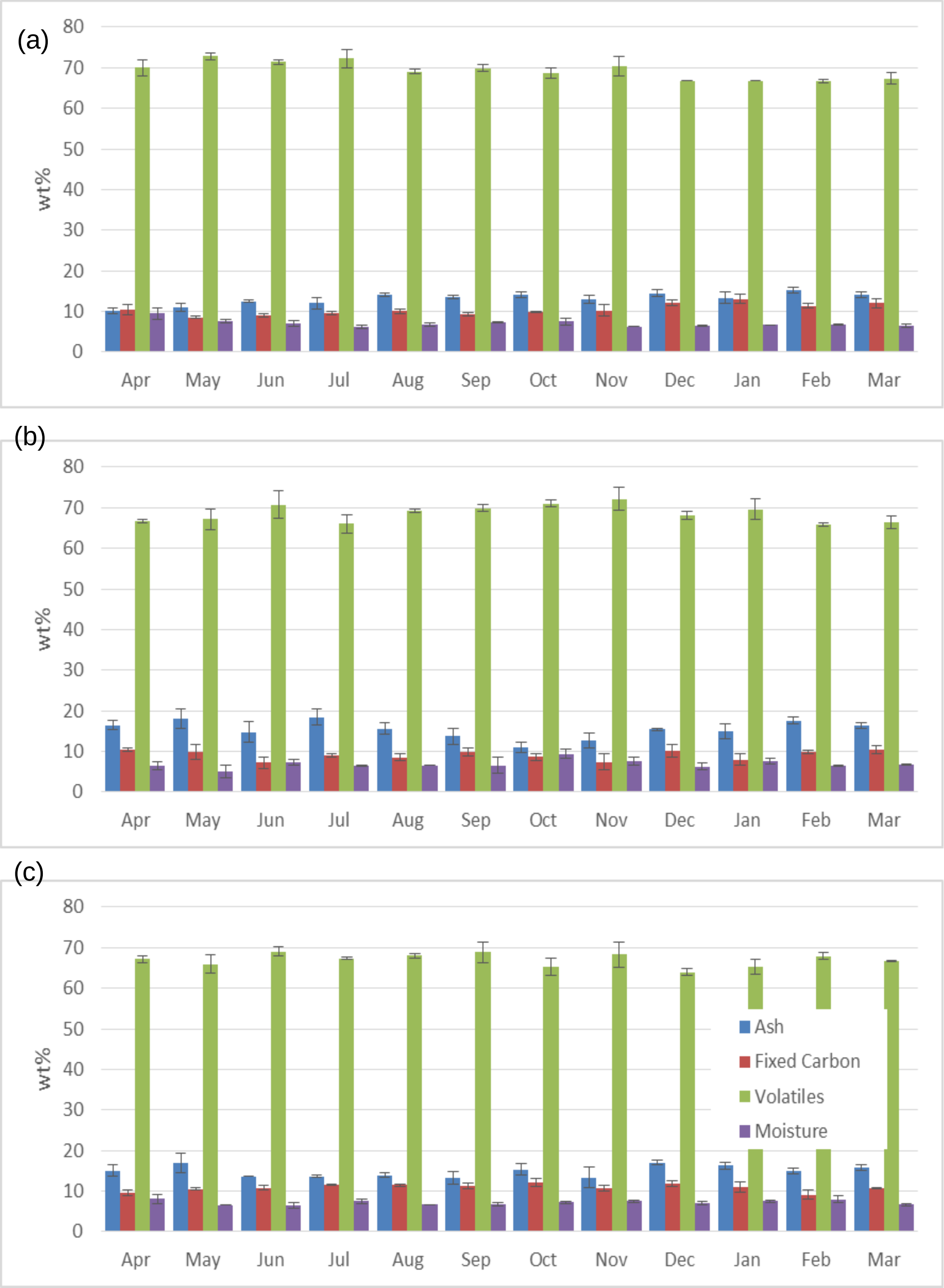


Figure 3.2: Proximate analysis for the seasonal variation of (a) FS, (b) FV and (c) AN

An overview from literature for ash content in *Fucoids* is given in Table 3.2. Ash values for FV and AN fall within those seen in previous literature. FS, however, is seen to have a significantly lower value: around half of that found in the literature. It is possible this is due to the natural and anthropogenic variation in metal content of seawater, depending on harvest location.

Table 3.2: A literature review of the typical chemical content of Fucoids.

Ref	Year	Species	Ash	Alginate	Mannitol	Laminarin	Protein
[177]	1968	*A. nodosum*	17-20%	20-26%	5-8%	2-5%	5-10%
[22]	1948	*A. nodosum*	17-24%	25-28%	7-11%	2-7%	5-10%
[24]	1949	*F. serratus*	20-31%	17-22%	6-17%	2-10%	6-15%
[24]	1949	*F. vesiculosus*	14-21%	14-17%	8-16%	2-5%	6-11%

The ultimate analysis of the 3 species of seaweed, given in Table 3.3, shows relatively constant quantities of C, H, S and O throughout the year; C and H show a slight increase in the summer months and S and O show a slight decrease. N shows an obvious decrease over the summer, rising again in the autumn, which relates to the protein content. Overall, FV contains more C than the other two species, with the average being 53.8 wt% compared to 42.4 and 44.2 wt% for FS and AN respectively on a dry ash free basis. All other elements are relatively similar, with the average being 2.6 wt%, 6.1 wt% and 2.1 wt% respectively for S, H and N. The reasonably high S content is indicates the presence of fucoidan in the samples, due to its sulphated fucose structure.

Comparing these results to published literature for the seasonal variation of kelps, it is clear that the C value is significantly lower in the kelps, with the average value presented by Adams et al. [81] for *L. digitata* of 31.3 wt% and by Sheiner et al. [79] for *L. digitata*, *L. hyperborea* and *S. latissima* of 29.2 wt%, 28.9 wt% and 26.6 wt% respectively. The N value is also significantly lower for kelps, averaging 1.0, 1.5 and 1.0 wt% for *L. digitata*, *L. hyperborea* and *S. latissima* respectively [79]. The sulphur value presented by Adams et al [81] is also significantly lower for *L. digitata*: 0.8 wt% compared to 1.8 wt%, 3.5 wt% and 2.6 wt% respectively for FS, FV and AN in this study. This indicates a lower quantity of fucoidan in the kelp species when compared to fucoids.

Table 3.3: Seasonal variation in the ultimate analysis

Species	Month	C	H	N	S	O
F. serratus	Apr	40.1±0.3	4.7±0.1	3.4±0.0	1.7±0.1	50.1±0.4
	May	43.6±1.9	5.3±0.1	2.9±0.1	1.5±0.0	46.6±2.1
	Jun	41.1±0.3	5.4±0.0	1.9±0.1	1.8±0.1	49.8±0.5
	Jul	44.2±0.3	6.1±0.0	1.4±0.1	1.6±0.0	46.8±0.4
	Aug	43.6±0.5	5.8±0.1	1.5±0.0	1.8±0.1	47.3±0.6
	Sep	42.3±0.0	5.6±0.0	1.3±0.0	1.8±0.0	49.0±0.1
	Oct	44.4±2.0	5.8±0.2	1.6±0.1	1.9±0.0	46.2±2.3
	Nov	42.3±0.8	5.6±0.1	1.8±0.0	1.8±0.1	48.4±0.1
	Dec	43.3±1.0	5.7±0.2	2.1±0.0	1.7±0.0	47.2±1.2
	Jan	42.5±0.0	5.5±0.0	2.6±0.1	1.8±0.1	47.5±0.1
	Feb	42.0±1.0	4.9±0.1	2.7±0.1	1.7±0.1	48.6±1.2
	Mar	39.2±2.5	5.2±0.4	3.2±0.0	1.9±0.1	50.5±2.9
F. vesiculosus	Apr	56.6±2.7	7.6±1.4	4.8±0.9	3.4±0.5	27.6±0.9
	May	50.5±3.4	6.8±0.5	2.9±0.4	2.8±0.1	37.0±1.8
	Jun	47.8±1.1	6.0±1.2	1.6±0.4	3.3±0.8	41.3±1.7
	Jul	56.7±3.8	7.6±0.9	2.2±0.0	4.6±0.4	28.9±2.1
	Aug	52.1±1.4	6.7±0.8	1.6±0.1	3.3±0.5	36.3±1.9
	Sep	51.9±0.5	6.7±1.5	1.3±0.2	2.8±0.5	37.3±2.7
	Oct	56.9±2.8	7.0±2.1	1.7±0.5	3.2±1.0	31.1±0.3
	Nov	52.9±2.3	6.7±0.9	1.7±0.3	3.3±0.3	35.3±3.7
	Dec	43.9±0.5	5.8±0.1	2.1±0.1	2.4±0.1	45.8±0.6
	Jan	50.2±2.2	6.3±0.3	2.8±0.0	3.8±0.3	36.8±2.6
	Feb	58.6±0.5	7.5±1.1	3.6±0.3	3.1±0.5	27.3±0.5
	Mar	57.3±1.6	9.0±1.1	5.1±0.3	5.5±0.5	23.1±1.8

A.nodosum	Apr	42.6±2.4	5.1±0.5	1.6±0.1	2.9±0.3	47.8±3.3
	May	39.1±0.8	5.3±0.0	1.6±0.1	3.0±0.0	51.0±0.8
	Jun	45.7±0.1	6.3±0.0	1.5±0.1	2.6±0.1	44.0±0.1
	Jul	45.7±0.1	6.0±0.0	1.2±00	2.2±0.0	44.9±0.1
	Aug	45.9±0.4	6.3±0.1	1.1±0.0	2.1±0.1	44.7±0.3
	Sep	45.1±0.6	6.1±0.0	1.2±0.0	2.5±0.0	45.2±0.6
	Oct	45.3±0.1	6.0±0.0	1.0±0.0	2.4±0.1	45.3±0.3
	Nov	45.3±0.8	5.9±0.1	1.2±0.0	2.5±0.0	45.0±0.9
	Dec	44.8±0.5	5.8±0.1	1.5±0.1	2.7±0.1	45.1±0.6
	Jan	45.9±0.0	6.0±0.0	1.8±0.0	3.0±0.0	43.3±0.0
	Feb	44.9±0.3	5.6±0.0	1.9±0.1	2.9±0.0	44.6±0.2
	Mar	39.7±2.8	5.4±0.3	2.7±0.2	2.5±0.1	49.7±2.8

The atomic ratios, calculated from CHNS values, are given in Figure 3.3. For all three species, the C:H ratio remains relatively constant throughout the year. The O:H values for FS and AN are, overall, lower in the autumn months and higher during the spring, while for FV they show more variation and little seasonal variation trends. Again, FV shows a more erratically variable variation for C:O, while FS and AN show an increase in the autumn months and decrease in the spring, although the variation is very small. These results suggest that, while seasonal variation in the carbohydrate, protein and other macro-components is seen, there is little variation in elemental ratios within these polymers. This trend was also seen for atomic ratios calculated by Adams et al [81] in their seasonal variation study of *L. digitata*.

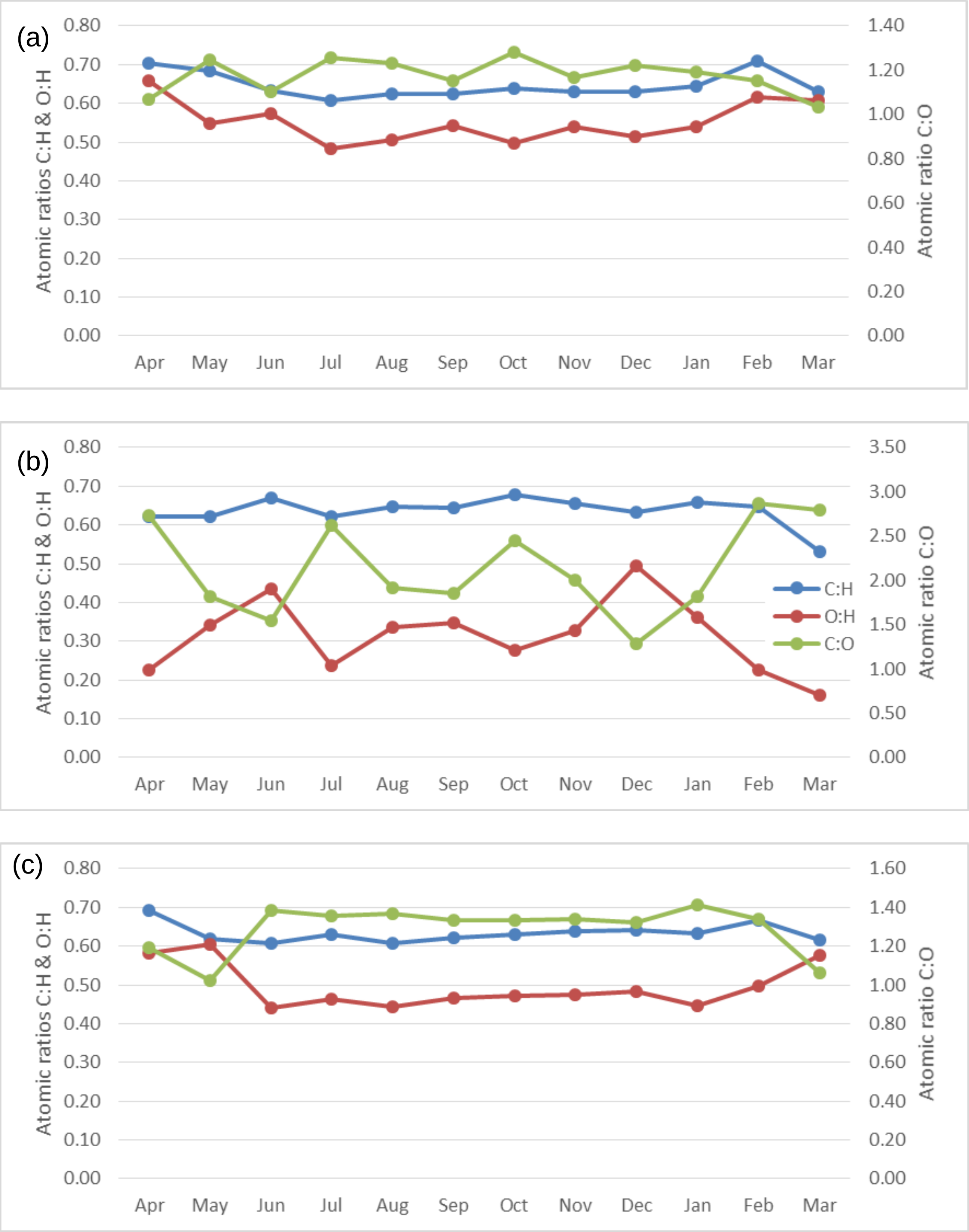


Figure 3.3: Atomic ratios calculated from CHNS on a d.a.f. basis analysis for (a) FS, (b) FV and (c) AN.

3.3.2 Carbohydrate Analysis

The seasonal variation of the 3 main carbohydrates; laminarin, mannitol and alginate, are given in Figure 3.4 As seen for previous studies, laminarin and mannitol are generally high during the summer and low in the winter, while alginate shows the reverse trend. FV shows the biggest variation in laminarin, with a difference between high and low values of 15.7wt%. This is followed by FS with 15.4 wt% and AN with 6.6 wt%. For all three species, the variation in mannitol is relatively similar, with the difference between high and low being 8.7, 8.3 and 7.8 wt% for FV, FS and AN respectively. Although alginate is generally higher in the winter, the seasonal trend seems to be less prominent and, unlike mannitol and laminarin where the quantity is seen to drop dramatically during the winter, remains at a fairly high value throughout the year. Minimum values for FS, FV and AN respectively are 29.5, 22.0 and 29.6 wt%. As alginate is a key structural component of the cell walls in macroalgae, it is likely that its level needs to remain relatively high throughout the year, to maintain the stability of the plant. Mannitol and Laminarin, however, increase over the spring and summer, when nutrients are abundant and there is more sunlight for photosynthesis. These are then seen to flatten off and begin to reduce in autumn as these storage carbohydrates are used up over winter through respiration [21].

Typical carbohydrate values for the main storage carbohydrates have been reported by several authors, which are shown in Table 3.2. In all three cases, the laminarin content of the analysed samples is higher than those values found in the literature. While the alginate content for AN is similar to the literature values, FS and FV also show higher values here too. This could be due to a differing harvest location, where the increase in storage carbohydrates is promoted due to environmental factors. The literature sited is also from a number of years ago, and anthropogenic factors, such as increased sea temperature and CO_2 concentration in seawater could be having an effect on the chemical composition of seaweeds. The values reported in this study for mannitol, however, fall within the values seen in previous literature.

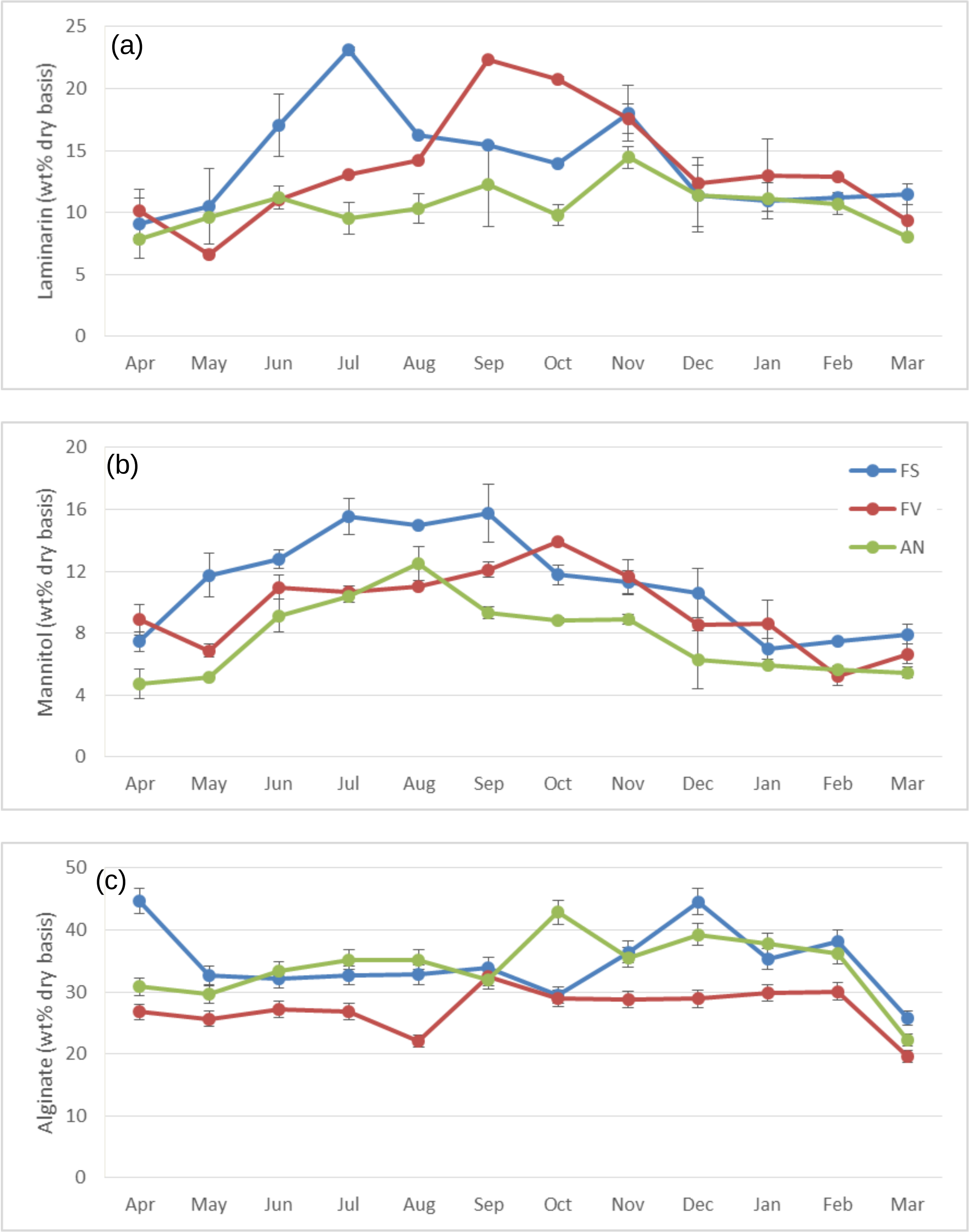


Figure 3.4: Seasonal variation of (a) laminarin, (b) mannitol and (c) alginate

3.3.3 Protein Analysis

The protein content of the 3 species shows a similar trend, with a clear seasonal variation pattern, shown in Figure 3.5. Protein is at a maximum in March/April, dropping over the summer to a minimum in September, before increasing again over the winter. The variation is quite wide, with the largest difference between the highest and lowest values being 19.3 wt% for FV. Although the trend is still evident, the difference in the variation is much less pronounced for AN, with the difference being only 8.5wt% and 11.8wt% for FS. These trends are comparable with previous literature for different species of brown macroalgae [178]. Proteins increase over the winter months as laminarin and mannitol, created over the summer via photosynthesis, are used to create amino acids in preparation for new growth in the spring, after which there is seen a rapid drop in protein content. In comparison with other reported literature for protein content, the amounts quantified here are relatively high for brown macroalgal species, which are reported to generally reach 14 wt% [179]. Makkar et al [179] report values of around 8 wt% for *Ascophyllum* species and 10 wt% for kelp species. However, the harvest month for these is not reported and the variation seen here includes months where these values are typical. Baardseth [177] quotes are variation of 5-10 wt% for protein for AN, for which the majority of values found in this study fall within. Peinado et al. [180], however, give the protein content of August harvest samples of FV and AN respectively to be 5.9 and 5.2 wt% respectively, which is in line with the protein quantity found in this study. Some other literature values for FS and FV are given in Table 3.2 and, again, the some values in this study are seen to be higher. It is important to note that it has been reported that the protein factor used here of 6.25 should be viewed with some caution, due to the levels of free nitrates present in brown seaweeds [181]. It may, therefore, be the case that the protein content is lower than reported here. Despite this, the seasonal variation trend shown will be the same, regardless of the protein factor used in calculation.

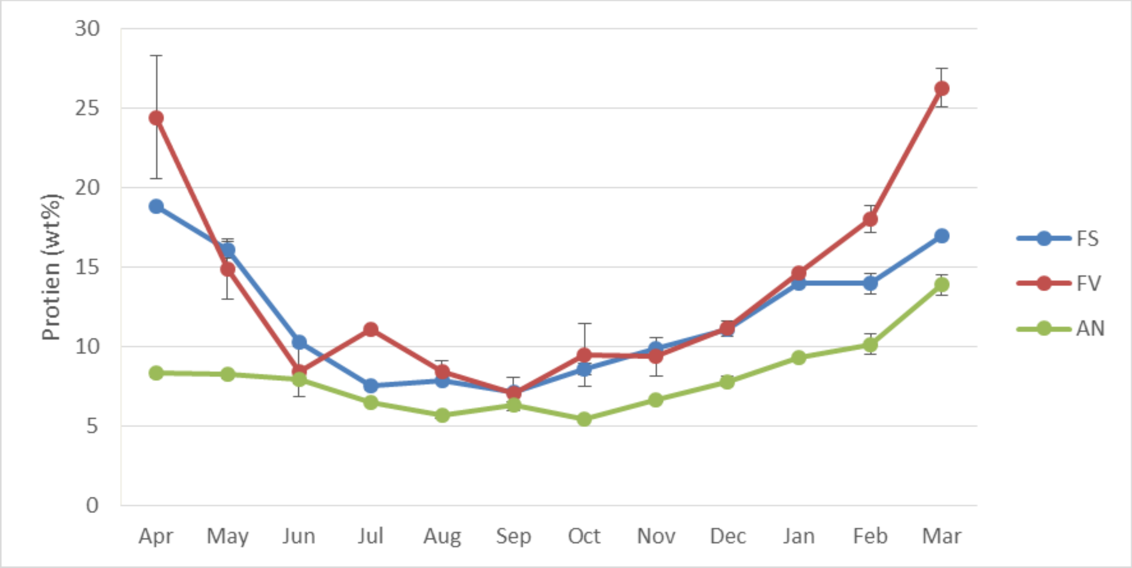


Figure 3.5: Seasonal variation of protein

3.3.4 Metal Analysis

The macro-metal content for the three species is given in Figure 3.6 (full numerical data is given in the Appendix as Figure A.2) and the micro-metal content in 3.7. The macro-metal content of the 3 species tended to be higher in May and June, although overall remained fairly constant throughout the year. The composition primarily consists of Na, K, Cl, Ca and Mg, which combined, make up 91.4%, 90.2% and 88.2% of FS, FV and AN respectively of the total metal content, with the remaining 10% consisting of mostly Ti, P and I. The high quantities of Cl, Na, Mg, Ca and K are due to their abundance in seawater; on average, salts account for 34 to 37 parts per thousand in seawater, of which over 90% can be accounted for by these 5 metals alone [182]. Previous studies have attributed the majority of the seasonal variation of the metals to potassium and phosphate, which are high in winter and low in summer. Sodium, calcium and magnesium were shown to remain relatively stable throughout the year [91]. A similar trend was seen in this study, with higher levels of potassium and phosphorus detected during the winter months. Unlike in Rosell and Srivastava's study, Ca was also seen to vary seasonally in the same way. All other metal components studied remain relatively stable over the year, with no distinguishable seasonal variation pattern.

On the whole, the quantity of each macro-metal is fairly similar over the three species, with the notable exception of Cl. This is much higher in FS than the other two seaweeds, with the average for FS being approximately 29,000 mg/kg, for FV approximately 15,000 mg/kg and for AN 9,000 mg/kg. It is likely, therefore, that FS accumulates Cl more readily than FV or AN. The metals present in June for FV, however, don't appear to align with the trend seen over the present of the months and species. There are several possible explanations for this: firstly, the quantity of metals present in a sample is largely dependant on the age of the plant at harvest. As seaweeds accumulate metals over their lifetime, older plants contain more than younger. As the age of plants at harvesting wasn't noted, it is possible that the June sample for FV was made up of more younger plants than the other samples. A second explanation for this decrease in metals is that the samples are unwashed, meaning that metals present in the seawater will have been dried into the sample. As the harvest locations is by a busy harbour, it is possible that fluctuations in the water due to harbour usage could account for some of the variation in metal content seen here.

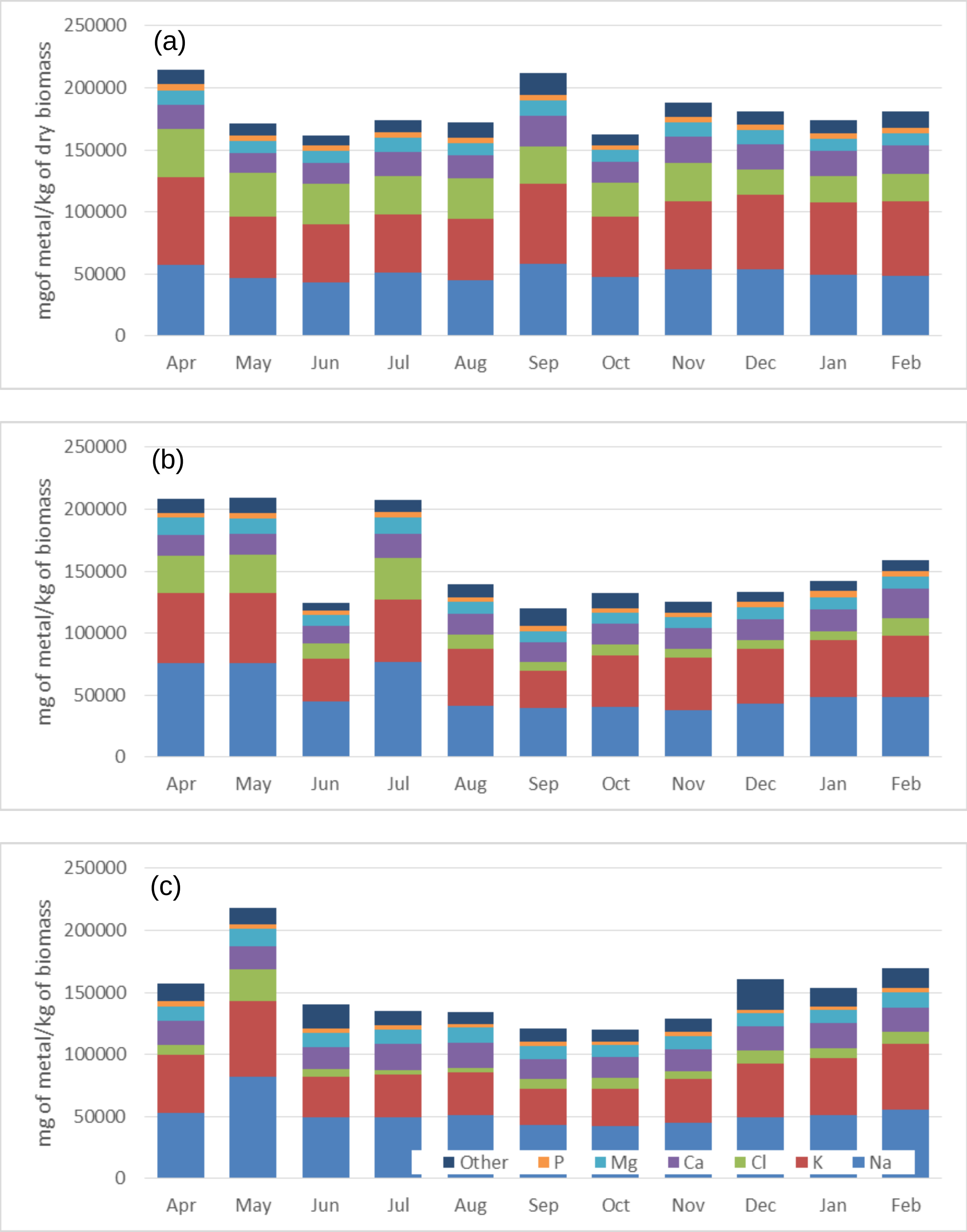


Figure 3.6: Seasonal variation of the macro-metals in the ash of (a) FS, (b) FV and (c) AN

The micro-metal content, shown in Figure 3.7 (for which full numerical data is given in the Appendix as Figure A.2), is also seen to be fairly constant over the year, with a similar variation pattern as seen for the macro-metals. The small “other” group in this case is mostly made up of As, Mn, Cu, Ni, Rb and Cr, all of which are present in quantities of less than 100 mg/kg. While some of these metals are known to be toxic to humans, the quantities present are so small as not to be a concern. On the whole, AN contains a lot less Ti and more Br and I. This suggests that Fucus species have more of an affinity to bio-accumulate Ti and AN to accumulate halides. As was also found by Adams et al. [81] in their seasonal variation study of *L. digitata*, Sr was found to be present in relatively high levels for all species: averaging 1560 mg/kg, 1450 mg/kg and 980 mg/kg respectively for FS, FV and AN.

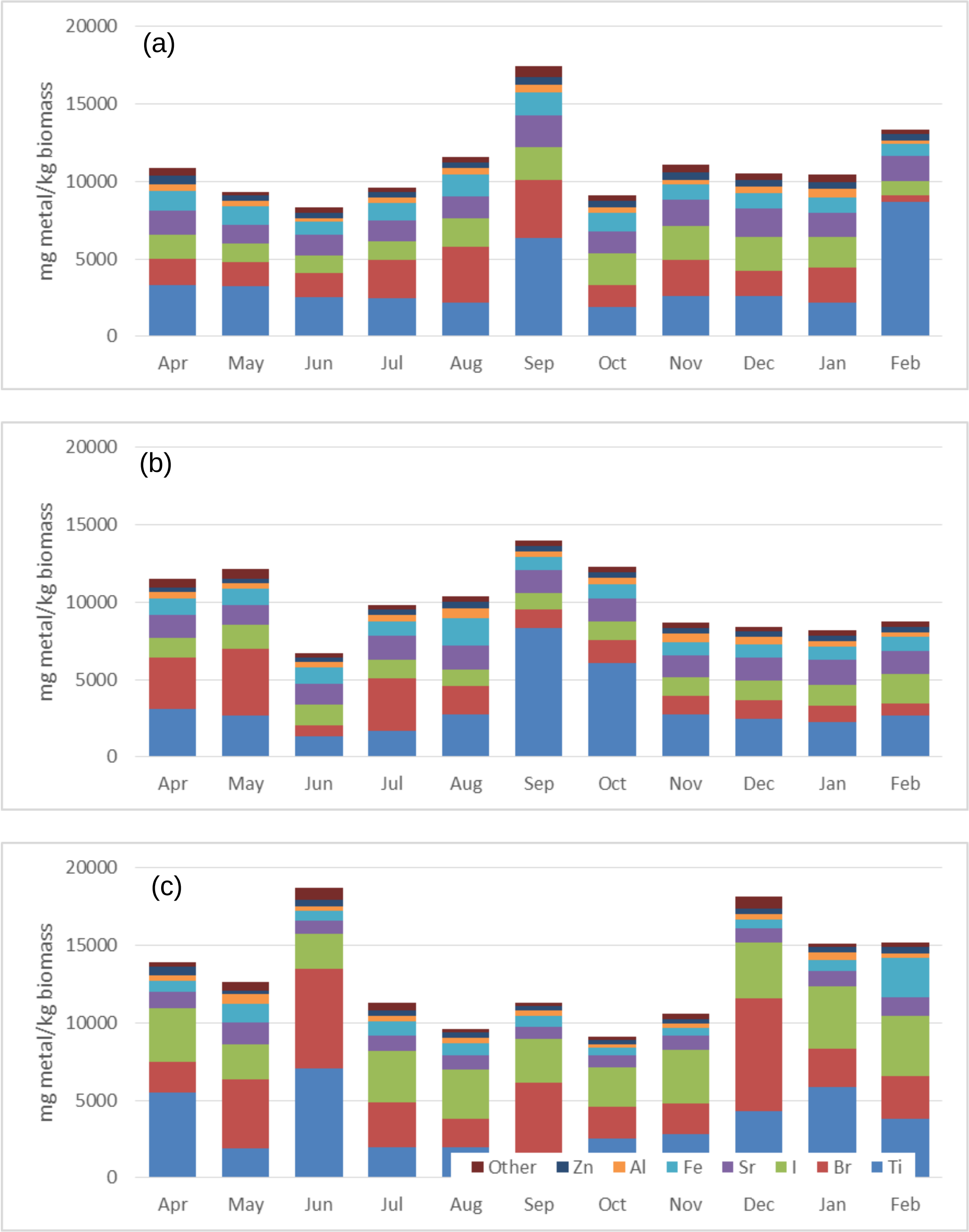


Figure 3.7: Seasonal variation of the micro-metals in in the ash for (a) FS, (b) FV and (c) AN

When analysing the metal content of seaweeds and their seasonal variation, it is important to note that they are unable to regulate their uptake of metals, meaning that their concentration will be dependant of that in the surrounding water [183, 184]. In fact, from the late 1970's, the trace metal content of brown seaweeds, particularly FS, FV and AN, have been used to determine the water quality in a particular area [85, 183, 185-187] and have been shown by Giusti [85] to vary significantly along a relatively short stretch of coastline. While this may have little impact on the seasonal variation pattern for the metal content of *fucoids*, it will have an impact on the total content throughout the year. High quantities of metals can be a problem for industrial applications, as they are associated with slagging and fouling in pipework, especially in an industrial setting, so consideration of the water pollution levels at a particular harvesting site may be an important consideration. However, although the metal content in all seaweeds is relatively high, the advantage of using *fucoids* over kelps is their apparent lower metal content. In this study, metal content has been shown to vary between 12 and 22 wt%. When compared to literature on the metal content in kelps, which range from 20-50 wt% [21, 23, 79, 81], it is clear that metals in *fucoids* are lower than for kelps. It has been shown in a study by Peinado et al. [180] that the NaCl content of FS and AN is roughly half that as compared to *L. digitata*, a kelp species, harvested in the same place at the same time. This indicated the kelps ability to accumulate more macro-metals when compared to fucoids.

Metals in these seaweed species make then a good source of minerals for both human and animal diets, especially the high levels and Ca and Fe, both of which are significantly higher than in other food sources [84]. Table 3.4 gives the recommended daily allowance (RDA) of metals for human consumption as part of a healthy diet and the amount of this which is fulfilled by 10g of FS, FV and AN, as an average over the year. 10g of seaweed provides 76%, 69% and 61% of the RDA of iron for FS, FV and AN respectively, and is significantly higher than most other food sources. For example, an equivalent weight of dark chocolate provides 5.5% of the RDA and an equivalent amount of beef only 1.5%, both of which are known for their high iron content [188]. Zinc also represents a high proportion of its RDA, giving 44%, 36% and 39% respectively for FS, FV and AN and offers a good vegetarian option for the mineral, which is otherwise commonly acquired from seafood and meat [189]. Other nutrients, such as copper, calcium, sodium and magnesium also give a high proportion of their RDA in 10g of seaweed, all well above 20%.

Table 3.4: RDA of metals for human consumption compared to the amount present in the three species.

Metal	RDA [190, 191]	FS	FV	AN
		Average across the year (% of RDA in 10g)		
Iron	14.8 mg	75.5	68.9	60.7
Zinc	9.5 mg	43.5	35.5	38.6
Calcium	800 mg	24.9	21.8	23.6
Phosphorus	800 mg	5.7	5.0	4.4
Magnesium	300 mg	34.5	34.9	37.7
Iodine	150 µg	11.1	8.8	21.1
Chromium	35 µg	0.6	0.5	0.4
Copper	900 µg	47.6	45.7	48.5
Potassium	4700 mg	11.8	9.6	8.7
Sodium	1500 mg	33.6	34.7	34.5

From an industrial processing viewpoint, high levels of metals and ash is undesirable, as they lead to corrosion and slagging and fouling of equipment [51], especially when high temperatures are involved and also presents a challenge for waste water treatment, where quantities of metals, especially heavy metals, allowed to be released are strictly regulated [192]. This point is discussed in detail in section 6.5.5. When considering the processing side, washing or other pretreatment to remove a portion of the metals would be reduce the impact they have of the rest of the process. Furthermore, reducing the severity of operating conditions, which is also conducive to chemical extraction without breaking down the carbohydrates, should also prolong the life of the equipment and reduce maintenance time and costs.

3.4 Discussion of Seasonal Variation and Conclusions

Overall, it is clear that there are seasonal trends in the chemical composition of the three species of fucoid studied here. It is likely that these changes are due to both external environmental factors, such as hours of sunlight and sea temperature, as well as due to the growth phases of the seaweed. Seasonal variation is an important consideration for industrial uses of seaweed, as harvest at the correct time for maximum yield of the desired product is key to making the most of the resource. For example, for the bioethanol industry, high carbohydrate content, in this case laminarin and mannitol, is desirable. To achieve this, harvesting in August/September would gain the best yield. However, if the seaweed is destined for the food market, for which all three species have been authorised for human consumption in the EU [171], high protein content may be more desirable, in which case harvest in March/April would be preferred. Seaweed destined for fucoidan extraction would also be best harvested in September. Seaweeds are also often used in the cosmetic industry, with extraction of “active ingredients” often being the aim [193]. Again fucoidan or polyphenols may be the desired product and, as the polyphenol content has been shown to be relatively stable over the year, a September harvest time may be appropriate.

Current EU legislation limits the harvesting of wild stock seaweed and the use of mechanical means is strictly regulated through most of Europe due to the adverse effect is can have on the marine environment [39] and wild stock harvesting in the UK requires a permit to be procured. This means that for a large scale seaweed industry for fucoids in the UK to become viable, open-sea cultivation on lines would be necessary to overcome the harvesting legislation. While the feasibility of these technique has been proven [194], the authors could find no literature comparing the difference in chemical composition and impact on seasonal variation open-sea cultivation has on fucoid species. Furthermore, other than the early studies completed by Black [21-24], which mainly focus on kelps, there is also little published data on the long term seasonal changes in seaweed composition. Black [21] noted that the maximum and minimum values for a particular component can vary by up to 2 months, but the author could find no published data which correlates this to weather conditions, sea temperature or other meta data. With a changing climate and increase in ocean acidification [195], being able to predict the best harvest time based on weather conditions is an important consideration for the industrial usage of macroalgae and ensuring the best harvest time is achieved.

3.5 Conclusion

In this study, the seasonal variation of carbohydrates, ash, metals, protein and polyphenols have been studied. The general trends for these components show the storage carbohydrates, mannitol, laminarin and fucoidan are highest in the late summer/early autumn, relating to higher photosynthesis due to increased sunlight. Ash, protein and alginate are highest in winter, as respiration dominates, due to decreased sunlight, and the plant makes and stores protein in preparation for new growth in the spring.

4 Seasonal Variation of the Chemical Composition of Fucoidan

4.1 The seasonal variation of fucoidan and its structure in three species of fucoid.

4.1.1 Introduction

The seasonal variation of fucoidan is a phenomenon widely stated in the literature, but of which there is little published experimental data. Differences in both quantity and composition of fucoidan have implications for industrial extraction, where a consistent product is key. By documenting the seasonal variation, optimum extraction times can be predicted, based on yield and desired chemical composition. This is important, as fucoidan functionality has been shown to be dependent on degree of sulphation, sulphation pattern and branching [196, 197]. Fucoidan composition is also dependent on harvest location, species and maturity [18], so it is important that these factors are taken into account when interpreting and analysing data.

This study attempts to review the seasonal variation in the quantity of fucoidan present in three species of macroalgae over a 12 month period between April 2010 and March 2011. Extracted fucoidan has been analysed for its fucose and sulphate content, as well as an attempt to gain insight into the structural differences through SEC and LC-MS.

4.1.2 Results and Discussion

4.1.2.1 Extraction of Fucoidan

The extraction of fucoidan is a five step process, described in section 2.6, extracting different components with each step to produce a relatively pure fucoidan product, an image of which is given in Figure 4.1. Figure 4.2 details the weights of the extracts and residues at each step, giving an overview of the total content seaweed and the extraction process (for which numeric data is given in the Appendix in Figure A.3). The step which shows the greatest variation over the year is defatting, which also includes the removal of pigments, and is achieved by mixing the raw, dried seaweed with ethanol. On the whole, this step extracted more matter in the summer than the winter months and indicates an increase in lipids and pigments during this time. There is also a marked variation in the 40% ethanol precipitate, which will be predominantly composed of laminarin. This is to be expected, due to the seasonal variation in laminarin content, which has been discussed in section 3.3.2. Interestingly, while soluble in 0.1M HCl, this fraction is only partly soluble in pure water once precipitated and dried. This is an indication that both the soluble and insoluble forms of laminarin have been extracted from the biomass. The residue weight remains relatively constant over the year, but there is a clear variation in the “remaining” quantities (which is calculated by difference). This is likely due to a variation in mannitol, protein and other constituents which are likely extracted into the liquid phase, but aren’t precipitated by the addition of ethanol and, therefore, are not accounted for in any of the other stages. Extracted fucoidan is pale yellow in colour, as shown in the image in Figure 4.1 and appears to form long strands when freeze dried, indicating its long-chain polymer structure.

Figure 4.1: Fucoidan which has been extracted via the conventional method and freeze dried for analysis.

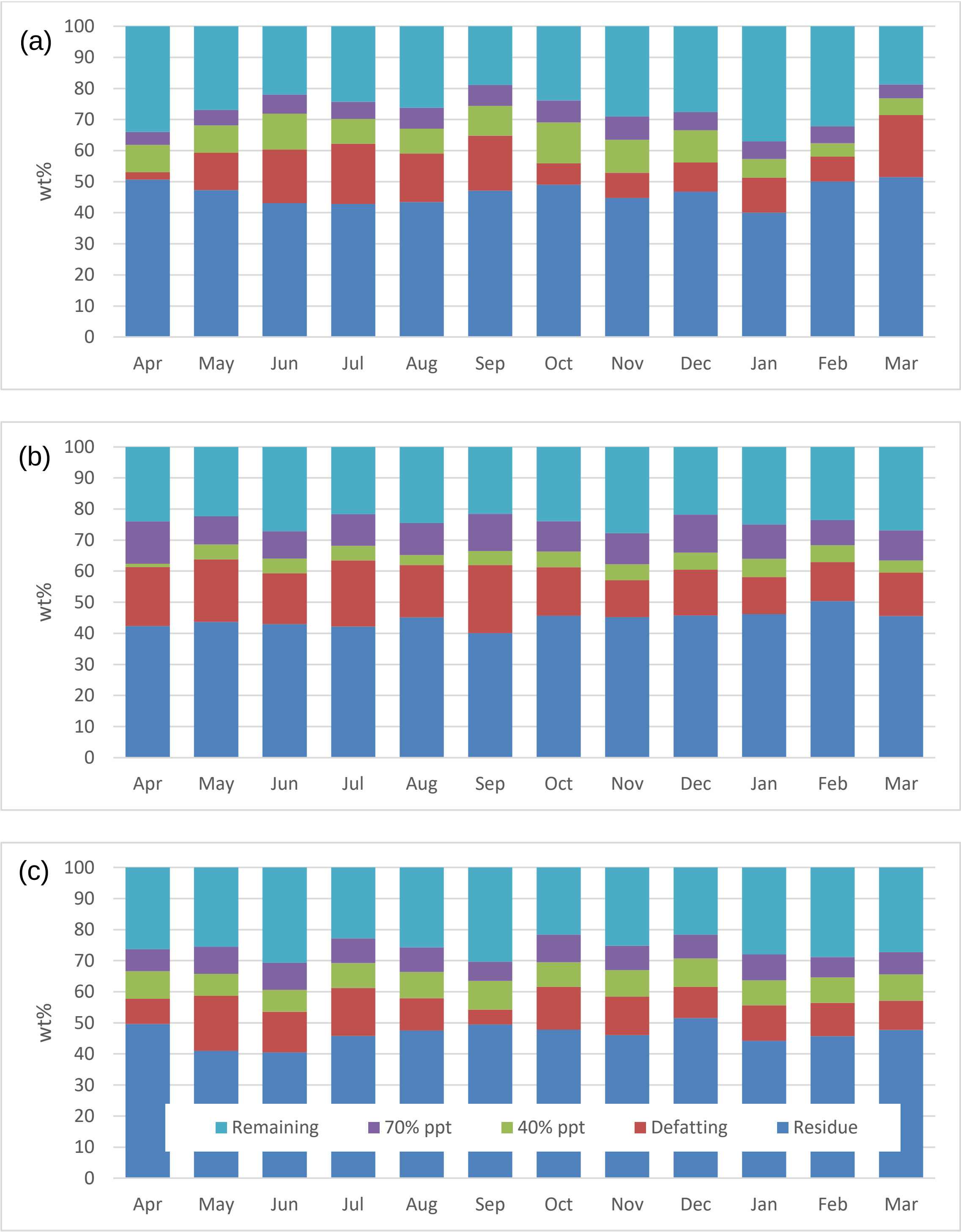


Figure 4.2: Extraction of fucoidan from monthly samples of (a) FS, (b) FV and (c) AN

4.1.2.2 Fucoidan Content

The fucoidan content of the 3 species varies throughout the year, as shown in Figure 4.3. In all three cases, the trend suggests lower fucoidan content in spring, rising to its maximum in early winter, before decreasing over the remaining winter months. FV has the highest content throughout the year, reaching a maximum of 12.2wt% in December. This is followed by AN, with a maximum of 8.9wt% in October, with FS reaching a high of 7.5wt% in November. Corresponding minima are 8.1wt% in February, 6.5wt% in February and 4.2wt% in April for FV, AN and FS respectively. This would suggest that the best time to harvest for maximum fucoidan content would be late autumn/early winter. However, the difference from maximum to minimum is 5.7wt%, 2.4wt% and 3.3wt% respectively for FV, AN and FS; a relatively small fluctuation suggesting a good yield could be obtained at any time of the year. This is particularly advantageous for industrial applications, removing the potential need for drying and/or storage. Fresh seaweed typically have a water content of approximately 80 wt% [79] and will decompose rapidly in a short period of time. If seaweed were only collected once a year, drying would be necessary in order to store and produce the pure fucoidan. Assuming functionality is prevalent throughout the year, the seaweeds could be harvested as needed and processed wet, reducing the energy consumption associated with drying.

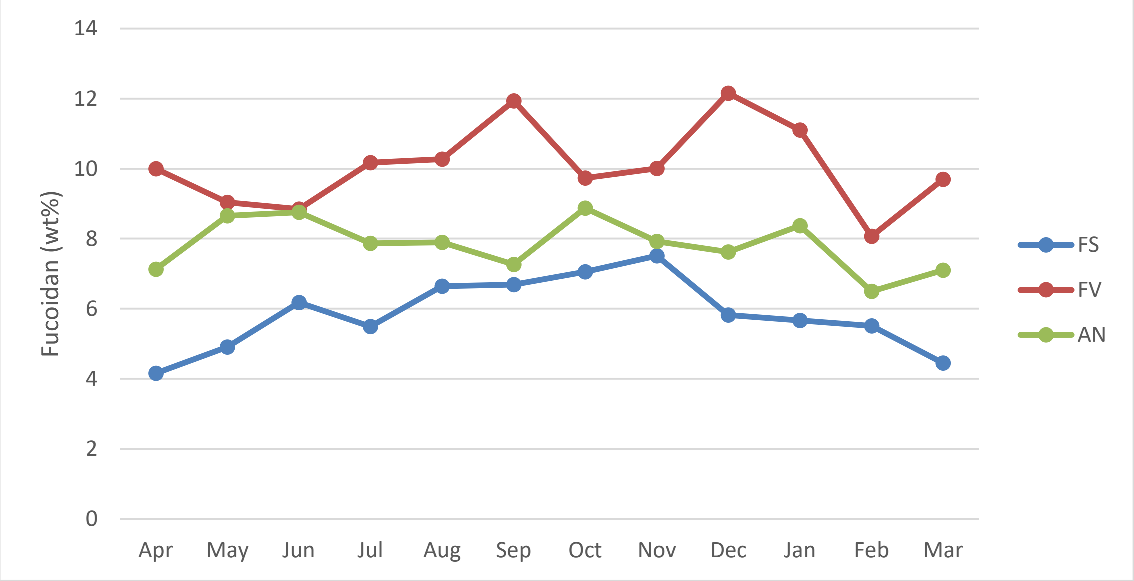


Figure 4.3: Seasonal variation of fucoidan

Table 4.1 gives an overview of the quantity of fucoidan extracted from the three species of interest in this study and found in the literature. From this, it is clear that there is a wide variation in fucoidan content, both between species as well as between harvest locations. On the whole, however, the results found in this study are in line with that shown in previous literature. The literature values for FS show the lowest fucoidan content, followed by AN, with FV having the most. Although there are no comparative seasonal variation studies from which to compare the data as a whole, the correlation between the single literature data points and the results found in this study give validity to these results.

Table 4.1: Literature review of fucoidan content in FS, FV and AN compared to the results of this study

Ref	**Species**	**Fucoidan (wt%)**
This study	*Fucus serratus*	4.2-7.5
[68]		0.42-7.16
This study	*Fucus vesiculosus*	8.1-12.2
[136]		4
[198]		16-20
[199]		3.4
This Study	*Ascophyllum nodosum*	6.5-8.9
[177]		10
[136]		3.3
[84]		11.6
[171]		4-10
[200]		1.75

4.1.2.3 Fucus and Sulphate content in Fucoidan

Fucus and sulphate content in fucoidan, shown in Figure 4.4, is seen to vary within all three species. Although there is no clear trend between the species, the fucose and sulphate levels vary proportionally to each other and inversely proportional to the total fucoidan content. The fucose content for FS, FV and AN range between 18–28 wt%, 26-39 wt% and 35-46 wt% respectively, while the sulphate content varies between 30-40 wt%, 9-35 wt% and 6-22 wt% respectively. Within each species there are distinguishable trend lines for fucose and sulphate: FS decreases in May and June, but is fairly constant over the rest of the year; FV increases throughout the year from a low point in April, reaching a maximum in November, before decreasing again and AN is low in September to October, but is again fairly constant over the rest of the year. Another notable point is that in FS, the fucose is lower than the sulphate content, however in FV and AN the reverse is true, with the sulphate content being higher. This indicates a higher degree of sulphation for each fucose residue in FS than for FV and AN. The variation in the sulphate content is especially important, as it has been reported that less than a 20% sulphate content leads to a complete loss of anti-proliferative and anticoagulant activity [73]. As the sulphate in FV and AN fall below this quantity during the summer months, it is an important consideration when harvesting these species for fucoidan extraction.

Previous studies have shown FS to have a sulphate to fucose ratio of between 0.9 and 1.5 [68] [201], while the average for this study is 0.73, which is comparable. The average ratios for FV and AN respectively are 2.0 and 2.7; significantly higher than for FS, but is comparable with literature values of 1.1 to 2.5 [201, 202] and 1.1 to 2.7 [201] [203] for FV and AN respectively.

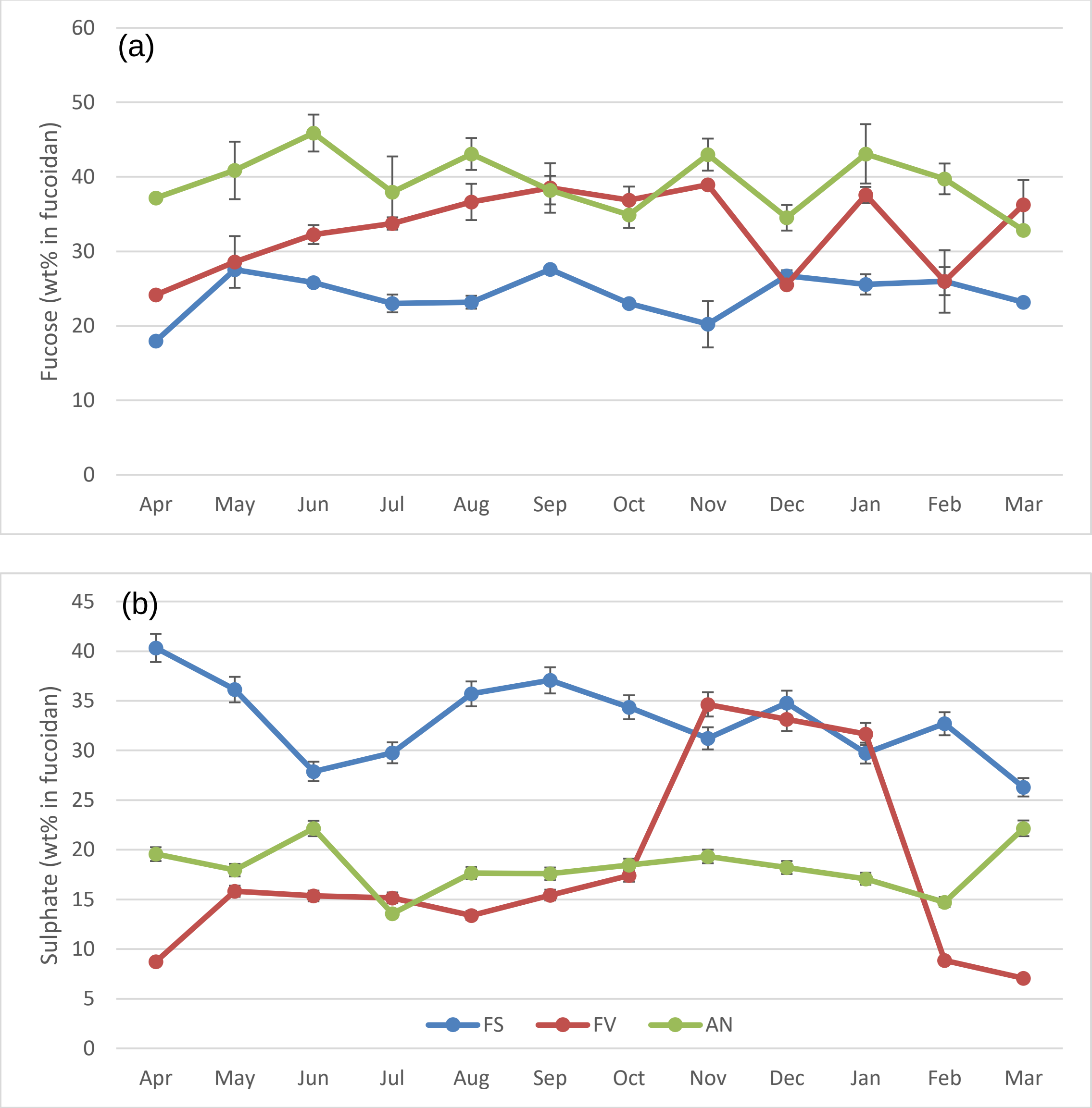


Figure 4.4: Seasonal variation of (a) fucose and (b) sulphate in fucoidan

Table 4.2 lists the fucose and sulphate content reported in the literature for extracted fucoidan from the *Fucoids* studied in this investigation. The quoted fucose and sulphate content of extracted fucoidan samples varies dramatically, with AN showing the widest quoted range; between 25 and 52.1 wt% for fucose content. The results presented in this paper correspond well with the range of values quoted previously in other research papers, with an average fucose ± one standard deviation of 24 ±3 .1%, 35 ± 4.4% and 40 ± 3.7% and average sulphate of 34 ± 3.7%, 19 ± 7.7% and 15 ± 4.5% for FS, FV and AN respectively.

The range of literature values quoted, as well as the variation in the presented results, shows clearly the need for a thorough understanding of the way in which fucoidan content varies in order to be able to make full use of the resource. It is important to note that the molecular weights shown in Table 4.2 have been measured in a similar way to the current study, with separation using SEC followed by detection with an RI detector., meaning comparisons can be drawn. As many papers have shown, the potential uses for fucoidan in pharmaceuticals are vast [16, 17, 71, 72]; however, each of these properties will be associated with a particular fucoidan, harvested in a particular place at a particular time of year. Without clear knowledge of all of these facts, the likelihood of being able to replicate the extracted fucoidan is reduced. This is also important from an industrial extraction standpoint, where economic viability will be based on being able to produce a sufficient quantity of an identical product with the desired properties.

Table 4.2: Review of analysis of extracted fucoidan found in the literature

Paper	Species	Month	MW	Fucose	Sulphate
current	*F. serratus*	Year average	1608kDa	24±3.1%	34±3.7%
[68]	*F. serratus*[1]	Aug	-	46.6%	31.8%[2]
[201]	*F. serratus*	-	-	24.8%	29.2%[2]
current	*F. vesiculosus*	Year average	1364kDa	35±4.4%	19±7.7%
[204]	*F. vesiculosus*[1]	Sept	-	48.1%	25.4%
[205]	*F. vesiculosus*	Commercial	-	33.3%	23.0%
[201]	*F. vesiculosus*	-	-	26.1%	23.6%[2]
[202]	*F. vesiculosus*	Commercial		13.8%	34.6%
current	*A. nodosum*	Year average	1374kDa	40±3.7%	15±4.5%
[204]	*A. nodosum*[1]	Sept	-	33.0%	20.9%
[201]	*A. nodosum*	-	-	26.6%	24.4%[2]
[203]	*A. nodosum*	Sept	420/47kDa	52.1%	19.0%
[206]	*A. nodosum*	Commercial	6.2kDa	25.0%	21.7%
[207]	*A. nodosum*	-	-	66mol%	31mol%

1 Values from the most abundant fucoidan fraction stated, 2 Sulphate content quoted as NaSO3.

4.1.2.4 Ultimate analysis of Extracted Fucoidan

Ultimate analysis of the fucoidan extract is displayed in Table 4.3. The average atomic ratio of C: H: S: O are very similar for the three species, being 1: 2.2: 0.1: 2.0; 1: 2.2: 0.2: 2.0 and 1: 2.3: 0.1: 2.0 for FS, FV and AN respectively. The nitrogen values are negligible and below the range for accurate detection by the instument. While the variation in the C: H values remain fairly constant over the year, the C: S values show a negative parabolic trend for all species. The variation of C is very similar for all species, with a minimum in April, rising to a maximum in September, decreasing again through the autumn and winter months. This same trend is seen for H, and the reverse is seen for S and Others, which are at a maximum in the winter months and low in the summer. These trends suggest that the extracted fucoidan contains a higher proportion of sulphate in the winter compared to the summer. As functionality is dependent on the degree of sulphation [196, 197], it is likely that the functionality of fucoidan varies over the year.

The large “other” value in the CHNS results, average 64.8, 62.9 and 62.9wt% respectively for FS, FV and AN, suggests a high oxygen content in the extracted fucoidan fraction. As the extraction process ensures a relatively pure product, many of the other possible contaminants such as salts will have been removed. Furthermore, high oxygen content would be expected due to the high sulphate content, where 4 oxygen atoms are associated with each sulphur atom and the high fucose content, which contains up to 5 oxygen atoms per monomer unit, depending on the degree of sulphation, which replace hydroxyl groups in the structure.

Table 4.3: Ultimate analysis of fucoidan extracts.

		Ultimate Analysis				Atomic ratio	
Species	Month	C	H	S	Other	C:H	C:S
Fucus serratus	Apr	21.8±1.0	4.0±0.0	6.9±0.2	66.8±0.8	2.2	1.15
	May	23.6±1.1	4.3±0.0	6.0±0.4	65.8±0.8	2.2	1.04
	Jun	24.2±0.6	4.3±0.1	5.7±0.3	65.5±0.6	2.1	1.01
	Jul	24.8±0.1	4.3±0.1	5.4±0.1	65.3±0.4	2.1	0.99
	Aug	25.5±0.3	4.5±0.4	5.7±0.6	64.1±1.3	2.1	0.94
	Sep	26.2±0.3	4.4±0.3	5.3±0.6	64.1±0.7	2.0	0.92
	Oct	23.4±0.5	4.2±0.3	6.7±0.3	65.6±1.0	2.1	1.05
	Nov	26.0±1.6	4.8±0.3	6.1±0.6	63.2±1.2	2.2	0.91

	Dec	24.2±0.1	4.6±0.1	7.4±0.5	63.7±0.5	2.3	0.99
	Jan	23.4±0.7	4.6±0.2	7.2±1.2	64.6±0.3	2.4	1.03
	Feb	23.5±0.4	4.3±0.3	6.6±0.1	65.2±0.6	2.2	1.04
	Mar	27.1±1.8	4.9±0.3	6.6±0.3	61.0±2.4	2.2	0.84
F. vesiculosus	Apr	25.4±3.1	4.7±0.7	9.1±0.2	60.2±3.7	2.2	0.89
	May	22.4±0.9	4.2±0.1	9.7±0.0	63.6±1.0	2.2	1.06
	Jun	24.0±0.0	4.3±0.0	10.1±0.1	61.5±0.1	2.1	0.96
	Jul	23.9±0.5	4.5±0.1	9. ±0.39	61.4±0.2	2.2	0.96
	Aug	25.0±1.2	4.5±0.3	8.1±0.1	62.2±1.4	2.2	0.93
	Sep	27.9±1.6	5.2±0.4	6.4±0.5	60.2±1.5	2.2	0.81
	Oct	23.6±0.9	4.2±0.1	9.4±0.4	62.4±0.7	2.1	0.99
	Nov	22.9±0.8	4.3±0.1	8.4±0.1	64.2±0.7	2.3	1.05
	Dec	26.6±1.0	4.8±0.0	7.7±0.1	60.7±1.0	2.2	0.86
	Jan	23.0±0.4	4.2±0.1	9.4±0.2	63.2±0.7	2.2	1.03
	Feb	24.4±0.2	4.4±0.1	10.1±0.1	60.9±0.4	2.2	0.93
	Mar	22.7±0.5	4.3±0.0	9.9±0.6	62.9±0.0	2.3	1.04
A. nodosum	Apr	20.3±0.2	4.2±0.0	12.1±0.2	63.1±0.4	2.5	1.16
	May	22.9±1.1	4.2±0.1	10.00.1	62.7±1.3	2.2	1.03
	Jun	25.5±0.5	4.7±0.0	7.6±0.5	61.8±0.2	2.2	0.91
	Jul	22.5±1.1	4.3±0.3	8.8±0.0	64.3±1.5	2.3	1.07
	Aug	23.8±0.1	4.6±0.0	8.7±0.1	62.7±0.1	2.3	0.99
	Sep	27.2±0.5	4.9±0.2	6.8±0.2	61.0±0.5	2.1	0.84
	Oct	23.2±.2	4.4±0.0	8.9±0.3	63.3±0.0	2.3	1.02
	Nov	24.0±0.4	4.6±0.1	8.2±0.1	63.0±0.4	2.3	0.98
	Dec	22.7±0.1	4.4±0.1	9.2±0.3	63.5±0.1	2.4	1.05
	Jan	22.2±1.2	4.2±0.3	9.5±0.6	63.8±2.0	2.3	1.08
	Feb	22.5±0.0	4.3±0.0	10.4±0.0	62.5±0.0	2.3	1.04
	Mar	23.7±0.2	4.6±0.0	9.9±0.3	61.5±0.4	2.4	0.97

4.1.2.5 Structural Analysis

The SEC curves of extracted fucoidan show differences between harvesting time and species. For comparison, chromatograms from extracted fucoidan harvested in April, July, October and January have been shown in Figure 4.5, representing samples from spring, summer, autumn and winter. The peaks for FS have a double peaks, which become less pronounced during the summer months. There is also some evidence of this for both FV and AN in spring, although it is much less distinct. The FV samples show similar peaks to that of the standard which is expected due to standard also being extracted from FV. AN shows increasingly broader peaks through the spring, reaching a maximum in summer and begin to narrow in autumn. The most significant differences between the three extracts are the two smaller, secondary peaks after the main fucoidan peak (retention time 14-16 minutes) which vary in size and width between species. For FS, these are broader and further apart, while for FV they are sharper and closer together. AN has a broad first peak, with a second sharp peak.

The main, double tipped peaks seen in the SEC chromatograms, especially evident in the FS samples, could be a sign of a more complex fucoidan structure. It is well known that FV gives the most simple form of fucoidan, with a linear chain of fucose [164]; this has also been shown for AN. FS, however, has been shown to have a more complex, branched structure [68]. The differences in the peaks shape and width suggests this more complex structure and variation in the chain length over the year period; a broad peak denotes high variation in MW of the macromolecule, while a double tipped peak indicates an increased abundance of two MW’s. Although the MW ranges found for the extracted fucoidan are quite high, they are in line with others in the literature for similar, crude extracts [46].

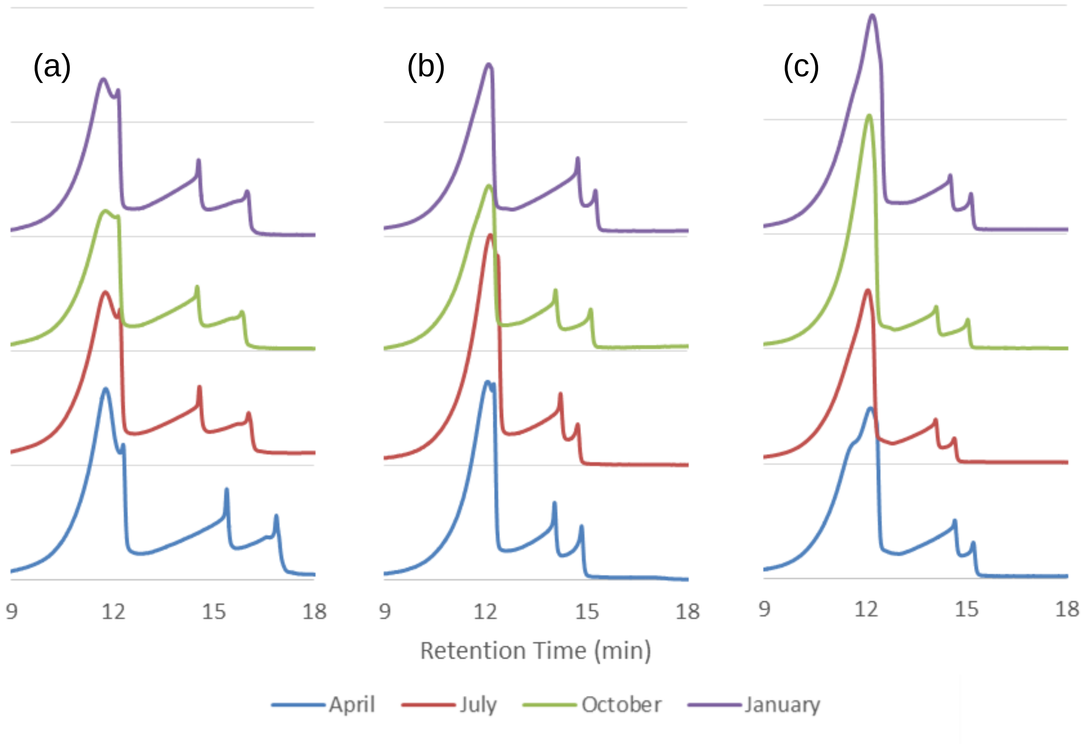


Figure 4.5: SEC chromatograms for fucoidan extracted from each season from (a) FS, (b) FV and (c) AN.

The MW of the main and secondary peaks of fucoidan is seen to vary over the year, as shown in Figure 4.6. The average MW for FS, FV and AN respectively are 1608, 1364 and 1374 kDa. The trend for the main peak shows a peak in MW in spring, around April/May, which gradually decreases over the year for FS and FV. AN shows a more steady MW over the year, with a difference between highest and lowest values of 178 kDa for AN, compared to 389 kDa and 357 kDa respectively for FS and FV. This implies less variation in its structure when compared to the *Fucus* species.

Other than for FS, where the MW of the secondary peaks appears to be higher in the September/October and lower in March/April, there doesn’t appear to be a clear seasonal variation pattern. This may be due to their presence being caused by the extraction process, rather than the structure of fucoidan itself. This could explain the more pronounced variation in FS, which is known to contain side chains which would be likely to more readily break off from the main body of the molecule and could vary in length throughout the year. FV and AN are known to have a simpler, linear structure, devoid of branching, which could explain the more erratic variation pattern seen. These peaks represent an average MW of 282/112 kDa, 347/187 kDa and 309/175 kDa for the first/second secondary peak for FS, FV and AN respectively. It is likely, as the LC-MS data presented in Figure 4.12 shows, that these secondary peaks are much smaller molecules than this but have been “pulled through” the column due to their association with the larger fucoidan macromolecule.

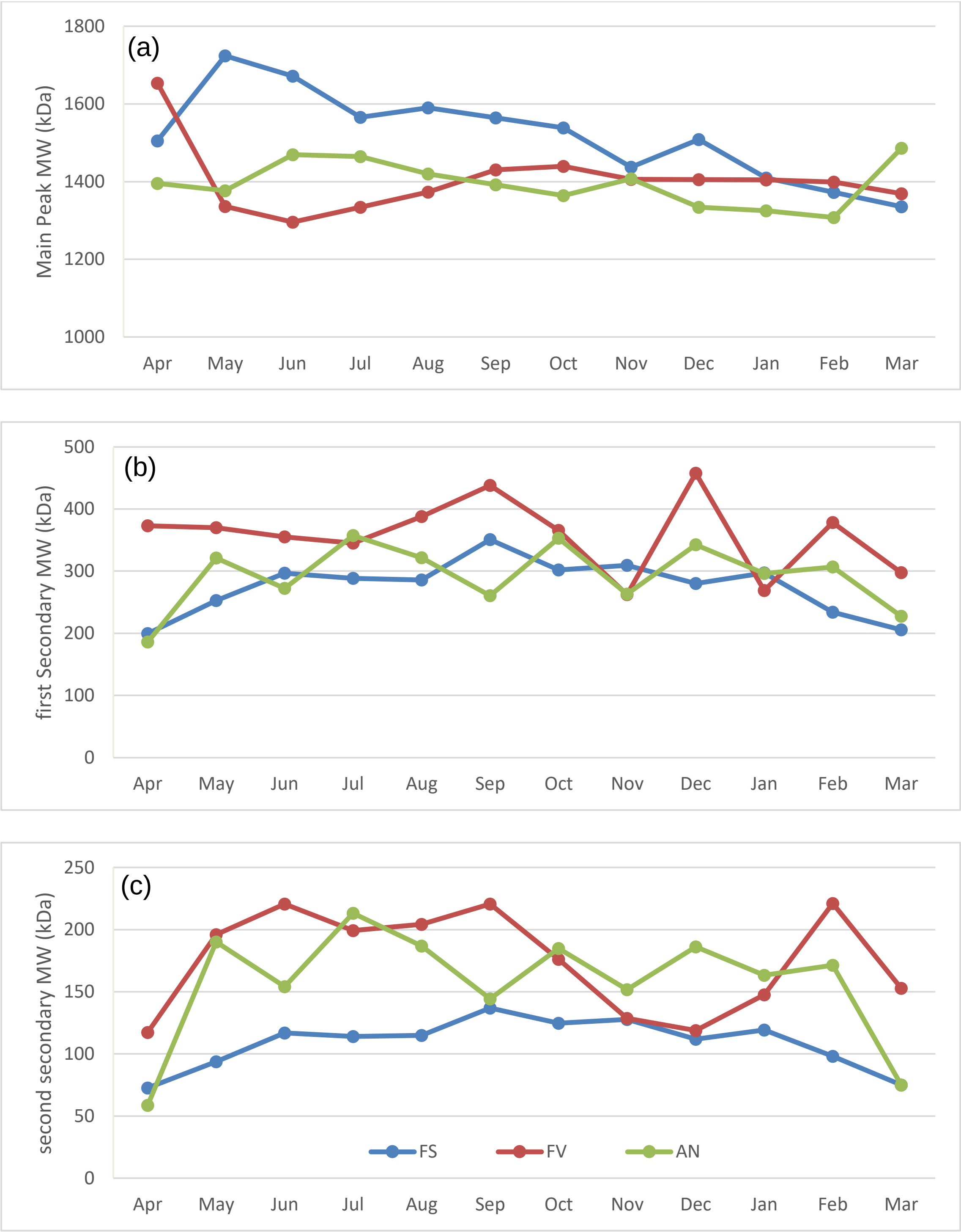


Figure 4.6: Variation in the MW of (a) the main peak, (b) the first secondary peak and (c) the second secondary peak of fucoidan extracts.

LC-MS analysis on the fucoidan extracts was undertaken in order to gain more understanding of the structural differences between the fucoidan samples and the secondary peaks identified in the SEC chromatogram. A comparison of the MS chromatogram for the main peaks of the three species and standard is shown in Figure 4.7. The overall shape of the peaks differ between species, indicating a difference in the structure of the polymer. FS has a roughly normally distributed curve, coming to a peak at around 500 Da and spread between ~200 to 800 Da, with a tail of high MW peaks above this. FV and AN have a somewhat negatively skewed distribution, with more higher MW fragments. There are also some notable differences between the most abundant peaks. The peak at 228 Da, denoting a fucose monomer with 1 sulphate group (see Figure 4.15), is significantly larger for FV and AN than for FS, while the peaks at 451 Da (a dimer with a sulphate group removed) is larger for FS and FV than for AN. The proportion of these two peaks is correlated to the amount of sulphate in each species. As shown in Figure 4.15, 228 Da is likely to be a monomer with 1 sulphate group and 293 Da is a monomer with 2 sulphate groups. The higher quantity of sulphate to fucose seen in FS is shown by a high quantity of 293 Da, which is more sulphate rich and a smaller quantity 228 Da, which is less sulphate rich. For FV and AN, where the sulphate content is less than fucose, the 228 Da peak, with only one associated sulphate per fucose monomer, is significantly more abundant that the 293 Da monomer. The biggest peaks for larger fragments differ in MW between species; another indicator for differing structures. Main peaks for a 4-chain of sulphated fucose (which would be expected at 1083 Da for an “ideal” structure), occur at 1064 Da, 1086 Da and 1088 Da for FS, FV and AN respectively, while a 3 chain, expected at 777/857 Da dependant on sulphation, show at 777/908 Da, 759/867 Da and 764/927 Da respectively. It is hypothesised that peak at 534 Da, representing a dimer chain containing 3 sulphate groups, relates to a side chain. This is due to its relative abundance in FS when compared to FV and AN, which has been shown to contain a higher degree of branching when compared to FV and AN, who have a more linear structure. Furthermore, the side chain proposed by Ale et al [164] is of this dimer structure.

LC-MS of standard fucoidan, shown in Figure 4.7(d) shows the same somewhat negative skewed distribution between ~200 and 800 Da as seen for FV and AN. However, there are a few more pronounced peaks than seen for any of the extracted samples. These occur at 228 Da, 293 Da, 371 Da, 451Da and 534 Da; fragments which are common and often giving the highest peaks across all samples. Differences between these fragments can be accounted for by a combination of a loss of hydroxide, methyl, sulphate or monomer units. Due to the structural information given by Ale et al [164], it is hypothesised that the peak at 534 Da is due to a branched-side chain. This is likely to readily fragment from the main body of the chain due and has also been shown to be more abundant in FS than the other two species, who have been shown previously to have a more linear structure [119].

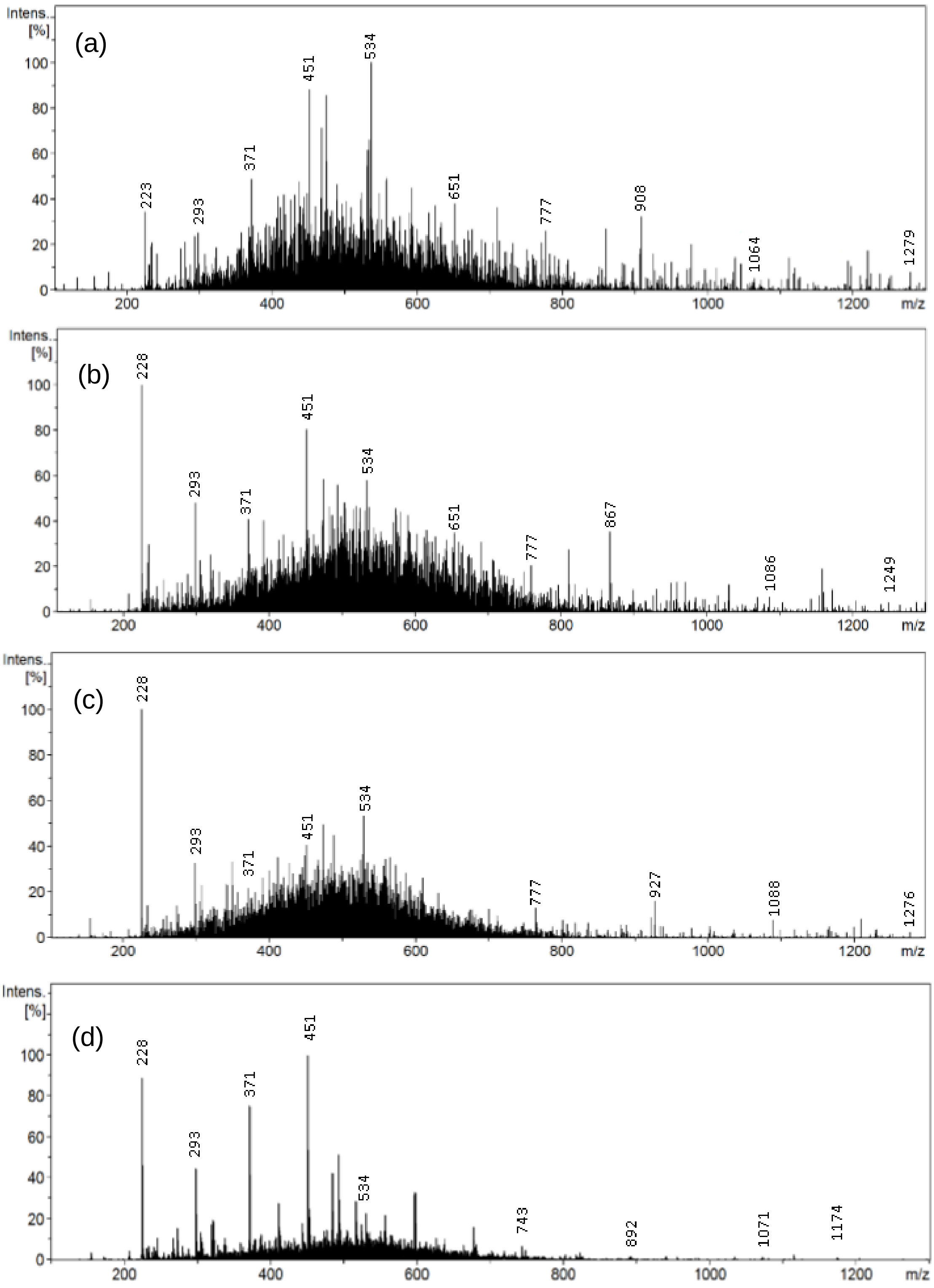


Figure 4.7: LC-MS data for the main peak of fucoidan for (a) FS, (b) FV, (c) AN, from representative samples from May, June and May respectively, and (d) standard fucoidan

Figure 4.8 shows chromatograms for fucoidan extracted from three samples of FS throughout the year. In all cases, the ratio of 228 Da to 293 Da monomer seems to remain fairly constant, although there is a slight increase in 293 Da in

September, when compared to the other months. The proportion of 451 Da and 534 Da fragments are higher in May and September than for December. The variation in 534 Da fragments suggests a change in the quantity of branching throughout the year, which is likely to have an effect on the functionality of the molecule. Higher molecular weight fragments (above ~800 Da) also differ between the months. This is likely due to differing levels of sulphate, monomer configuration and also differences in the additional sugars, other than fucose, present in the fucoidan structure.

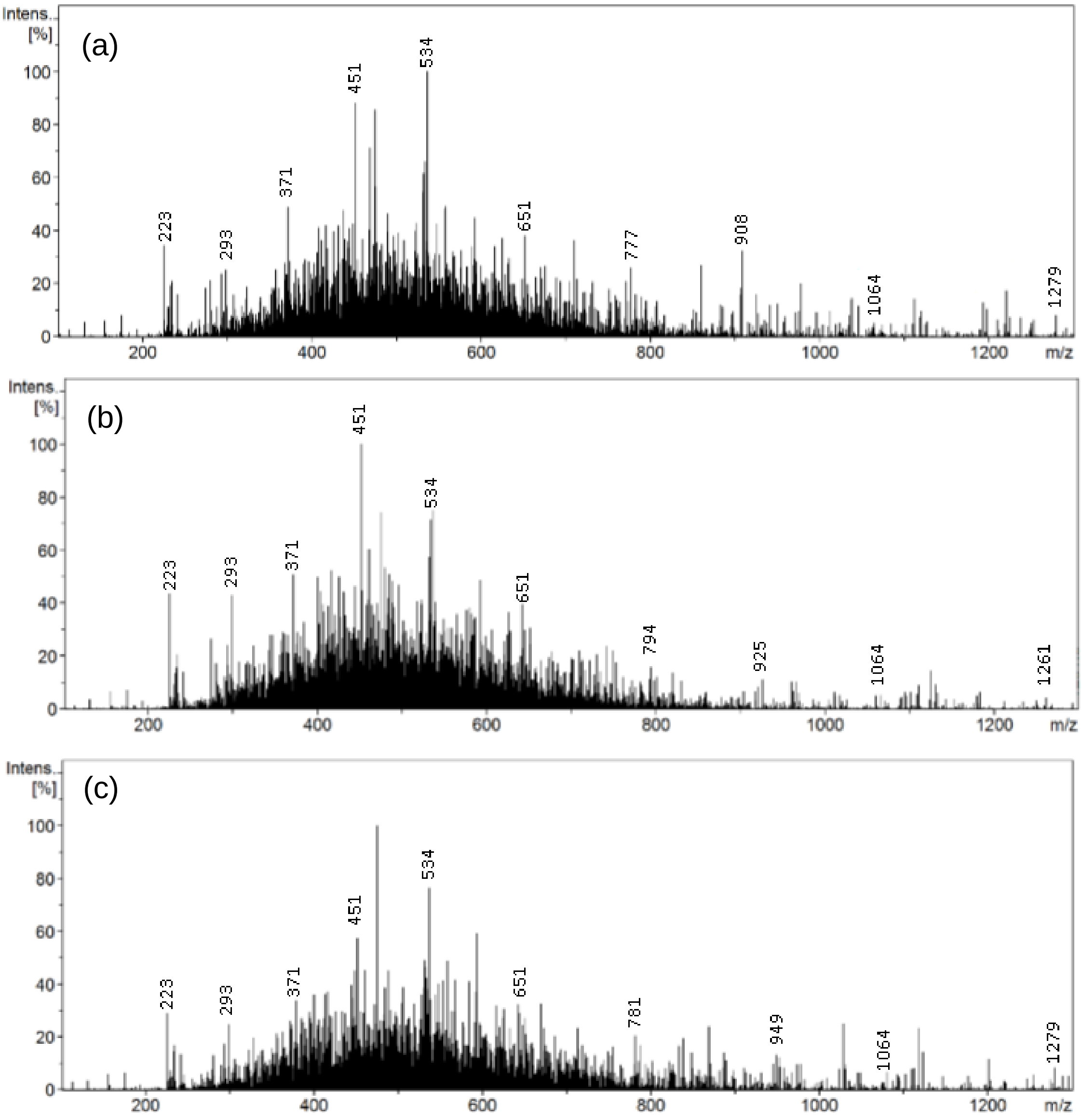


Figure 4.8: Comparison of LC-MS chromatograms of extracted fucoidan from FS for (a) May, (b) September and (c) December.

The seasonal variation in the LC-MS results for FV are shown in Figure 4.9. As noted previously, the 228 Da monomer for FV, shown in Figure 4.9, is significantly higher than for FS, shown in Figure 4.8, which is most likely due to the lower sulphate content in FV. The spread of fragments over the year appears to differ. The September and January chromatograms are more normally distributed, which the sample from June having a negatively skewed distribution. The abundance of the “main” fragments also differ, with 534 Da being significantly higher in January and 451 Da higher in June and September. This is likely due to a differing backbone structure, which creates different fragments on breakdown in the MS and could also be evidence of branching. Furthermore, FV also appears to have a higher proportion of larger (>800 Da) fragments when compared to FS and AN.

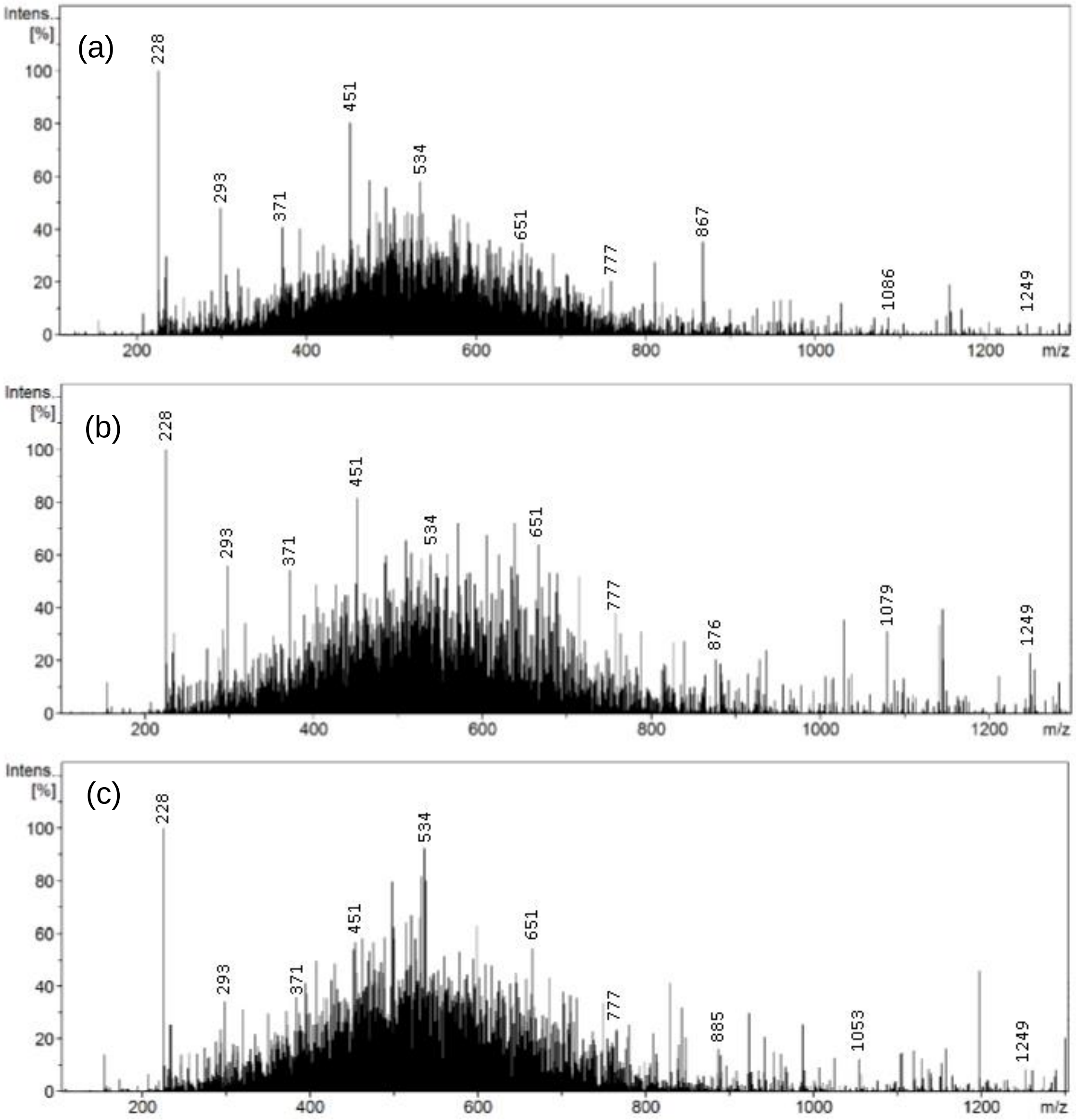


Figure 4.9: Comparison of LC-MS chromatograms of extracted fucoidan from FV for (a) June, (b) September and (c) January.

Comparison of three LC-MS chromatograms from May, August and September for fucoidan extracted from AN is shown in Figure 4.10. Again, the quantity of 228 Da monomer is significantly higher than for FS, due to a lower sulphate content. The spread of fragments below ~800 Da is relatively similar for May and August, showing a normal distribution, but is more negatively skewed in December. The quantities of these fragments, however, is significantly less across the year when compared to FS and FV. The quantity of 534 Da remains relatively low and similar throughout the samples analysed, indicating that AN sees little or no branching throughout the year with little variation in this.

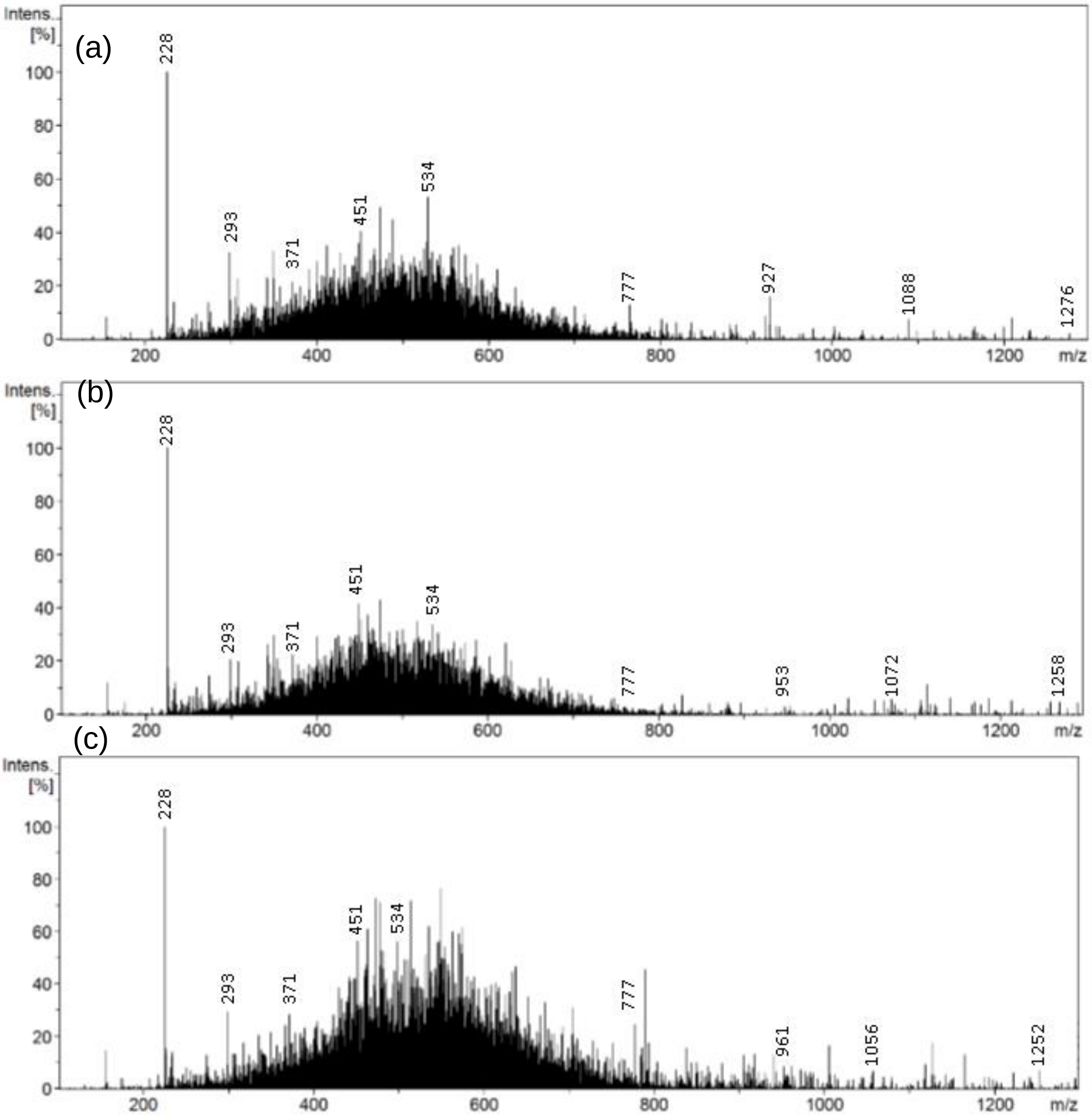


Figure 4.10: Comparison of LC-MS chromatograms for extracted fucoidan from AN for (a) May, (b) August and (c) December.

The variation in the ratio of the two monomer units which make up the fucose backbone of fucoidan, of molecular weight 228 Da and 293 Da are given for the main fucoidan peak in Figure 4.11.The trend shows an increase in less sulphated, 228 Da monomer in the summer and a higher proportion of the, more sulphated, 293 Da monomer in the winter. This variation also appears to correspond well with the variation in sulphate content. As expected, FS has the lowest 228:293 Da ratio, as it contains a higher proportion of the 293 Da monomer.

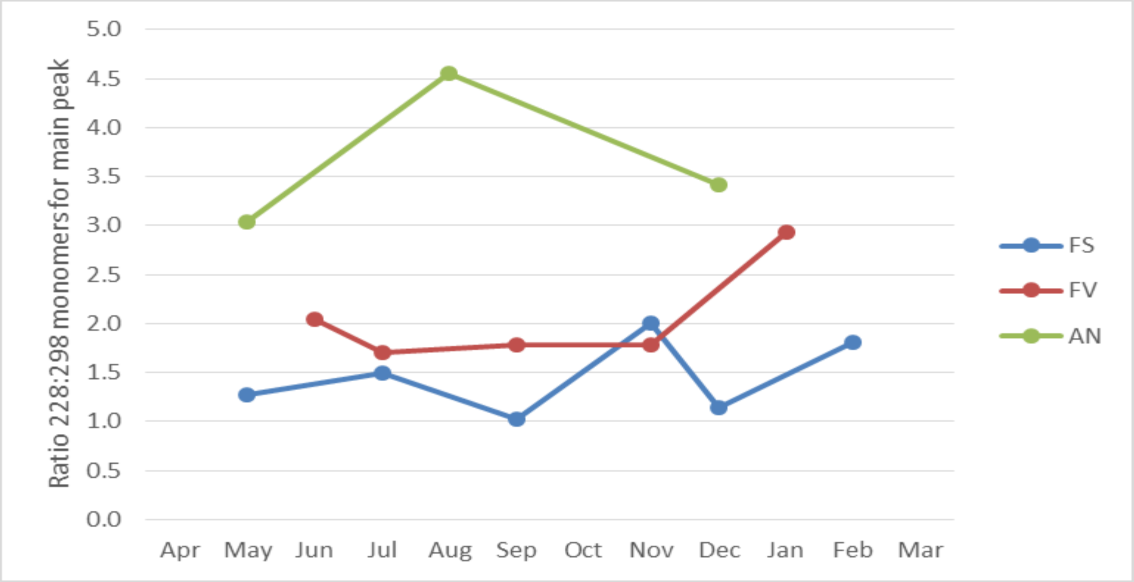


Figure 4.11: Ratio of 228:293 monomer units, calculated from LC-MS data.

Figure 4.12 shows the comparison LC-MS chromatograms for FS of the main and secondary peaks, where the most common mass fragments across all the species have been noted and for which possible structures have been given in Figure 4.15. In fact, these mass fragments were found to be common to all the samples analysed by LC-MS, for both the main and secondary peaks, indicating they are key building blocks in the structure of fucoidan in all species. In general, the difference between fragments can be attributed to the loss of a hydroxide, methyl or sulphate group, a monomer unit or a combination of these, although, in some cases, partial ring structures are required to form the mass indicated by LC-MS. For all main and secondary peak comparisons, the chromatogram has been shown only up to 800 Da, as there are no mass fragments for the secondary peaks above this value. It is likely, therefore, that they are fragments, which may have been produced during the extraction and purification process, or are simply naturally occurring, low mass fucoidan, which have been “pulled through” the column by interactions with the large molecular weight fucoidan.

The mass fragments seen observed for the secondary peaks appear to be in similar quantities throughout the year, with 228 Da monomer having the greatest quantity, followed by 293 Da, with 410 Da and 534 Da being present in the smallest, but relatively equal quantities. In comparison to the main peak, the proportion of 228 Da is significantly higher in the secondary peaks. This indicates a lower sulphate content in this fraction. It is possible that these four, large peaks correspond to branching on the fucoidan molecule, which are likely to be more readily broken off during extraction. The abundance of these peaks in the main fucoidan fraction, as was noted previously, also eludes to that fact that they are branches, which have been fractionated in the mass spectrometer. While other mass fraction peaks are still visible, they are small in comparison.

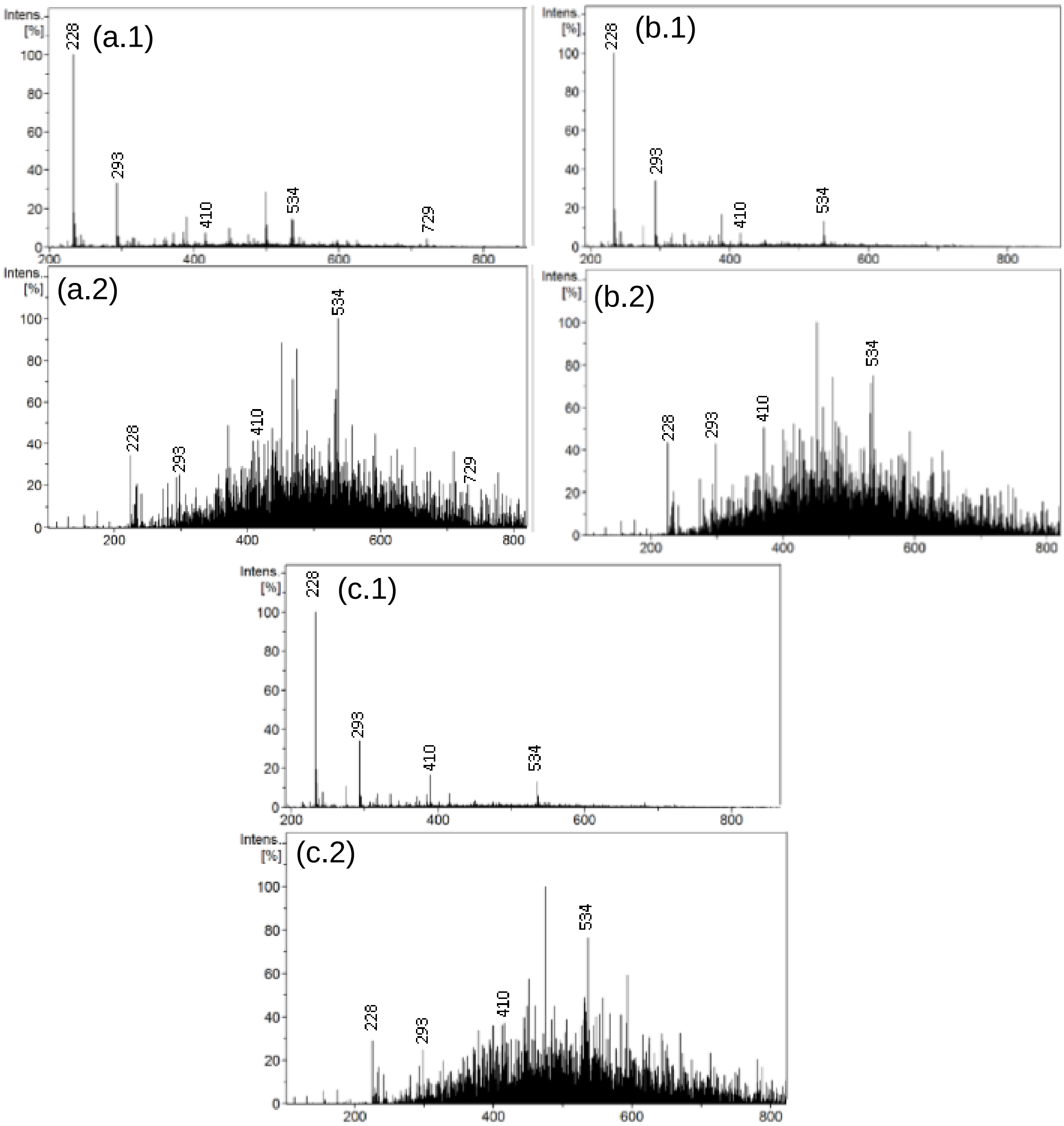


Figure 4.12: LC-MS chromatograms comparing (1) secondary peaks and (2) main peak fragments from extracted fucoidan of FS, where (a) is May, (b) is September and (c) is December.

The comparison of main and secondary peaks for FV are shown in Figure 4.13. In this case, the secondary peaks are seen to contain much more variation in the MW of the mass fragments. While the main four mass fragments, which have been noted on the figure, are still present, there are larger peaks at 275 Da, 451 Da and 592 Da, which are common to all the secondary peaks in FV, but aren't present in the FS samples. It is possible that this could be due to one of two different things: firstly, it could be down to a larger quantity of naturally occurring, low molecular weight fragments present in the seaweed, or secondly, due to an increased amount of breakdown during extraction.

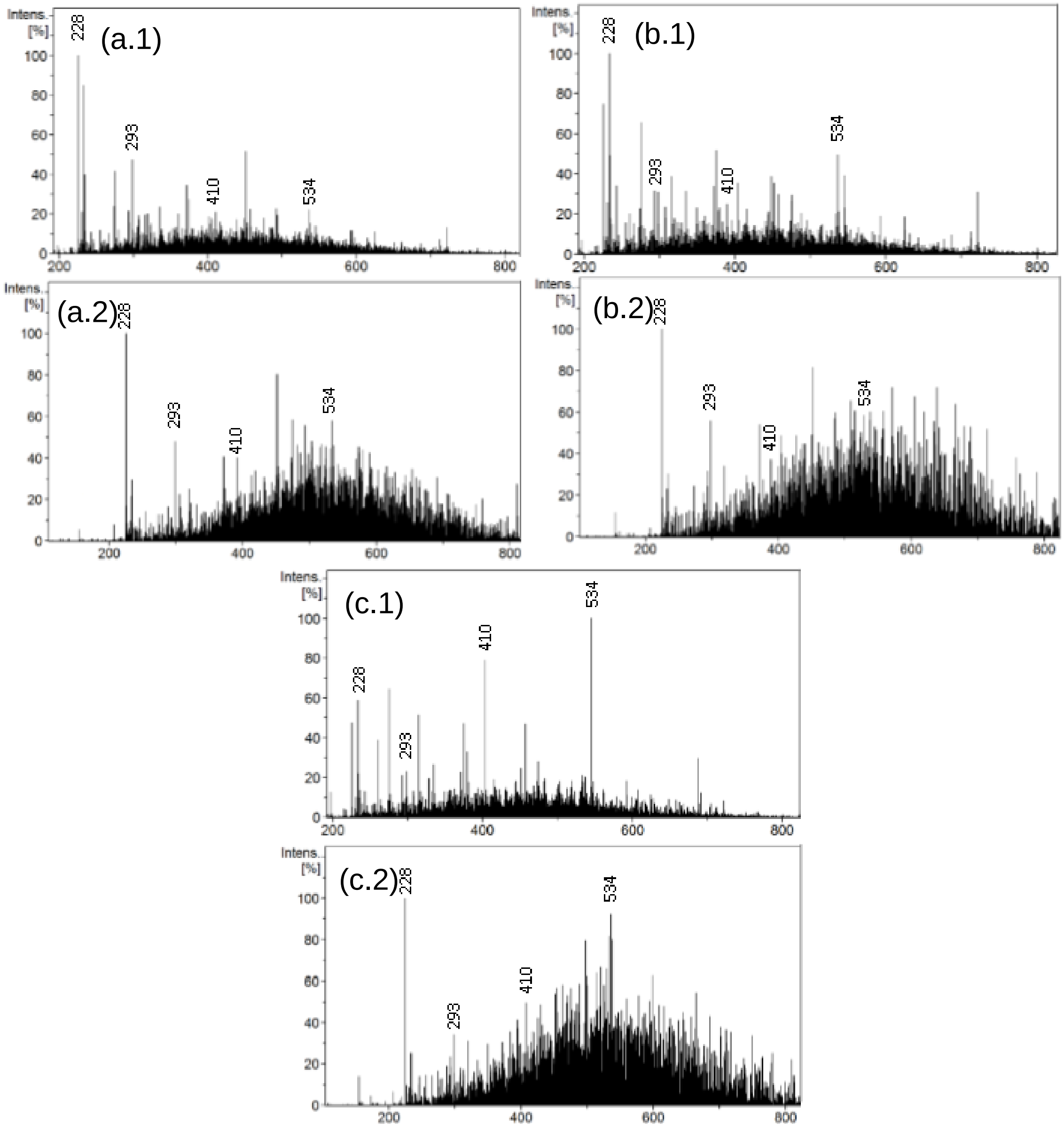


Figure 4.13: LC-MS chromatograms for FV, comparing (1) the secondary and (2) the main peaks in fucoidan extracted from (a) June, (b) September and (c) January samples.

Figure 4.14 shows the comparison of main and secondary peaks for AN. Again, the four common mass fragments are seen for all months, with most other peaks being relatively small in comparison. Notable additional peaks occur at 315 Da and 451 Da, which are present in all three months studied. While the quantity of different mass fragments is still greater than seen for FS, they are less than seen for FV. Interestingly, in May and August, the proportion of 228 Da, the less sulphated monomer, is less than 293 Da, containing two sulphate residues, for the secondary peaks, which is the opposite for what is seen in for the main peak.

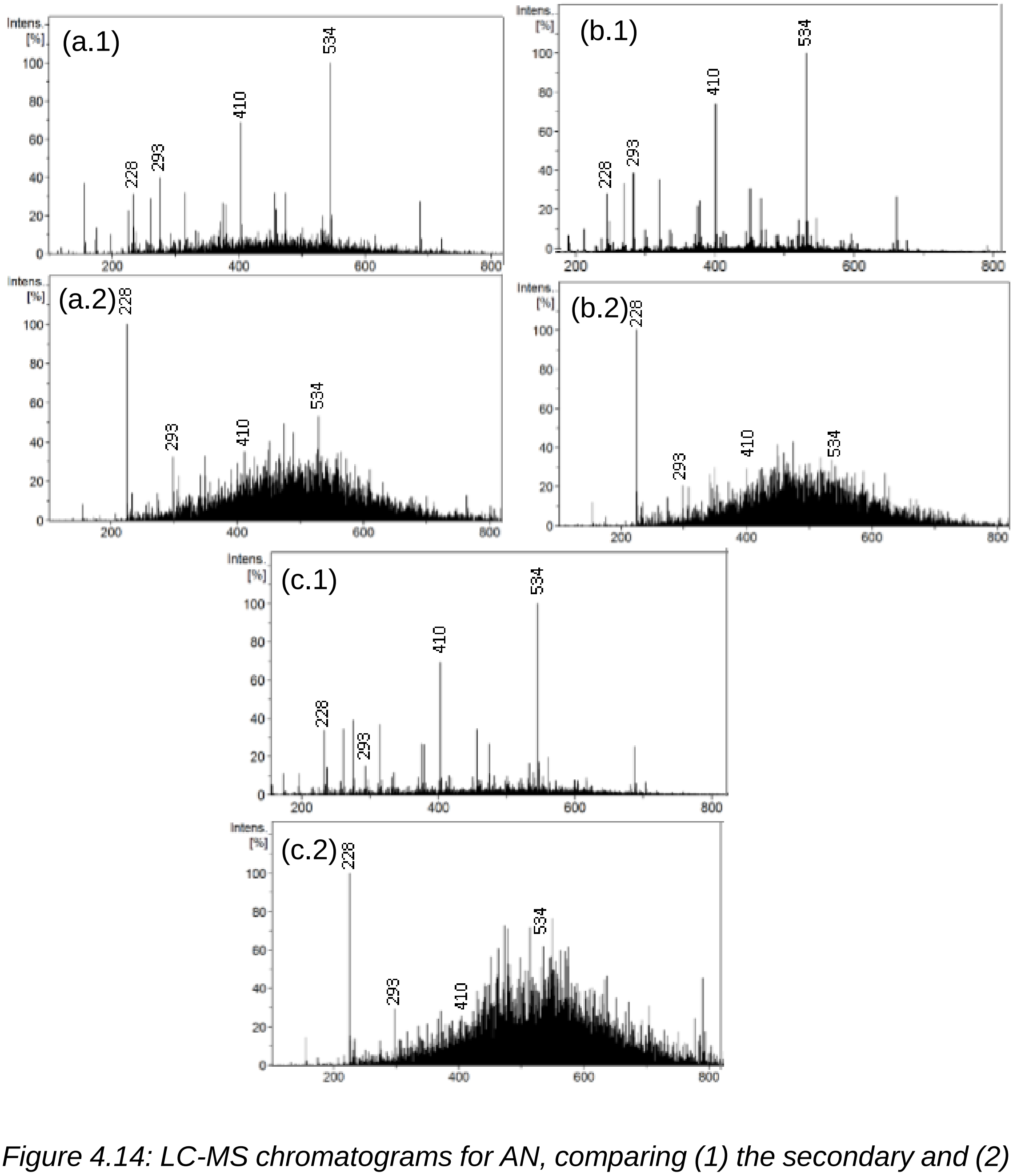


Figure 4.14: LC-MS chromatograms for AN, comparing (1) the secondary and (2) the main peaks in fucoidan extracted from (a) May, (b) August and (c) December samples.

In general, comparing the LC-MS chromatogram for the main and secondary peaks, it is obvious that the main peak contains a far wider range of fragments and with a wider ranges of masses. The maximum Da possible with this instrument is 1300 Da, but it is likely that there are fragments significantly larger than this, as indicated by the MW of fucoidan found by SEC. The two smaller peaks appear to either be fragments which have been created during the extraction process or oligomers, which have been pulled through the column by association with the larger fucoidan macromolecules. For either case, the presence of the fragments in both the main and secondary peaks infers they are from the same group of compounds and also associated with each other. The largest of these is 729 Da for FS, corresponding to a 3-fucose chain. For FV, the largest fraction in the secondary peaks is 1245; a 5-fucose chain and for AN, 829 Da; a 4-fucose chain. Possible structures for the most common fragments are given in Figure 4.15. These clearly show the loss of hydroxyl, methyl and sulphate groups due to fragmentation in the mass spectrometer.

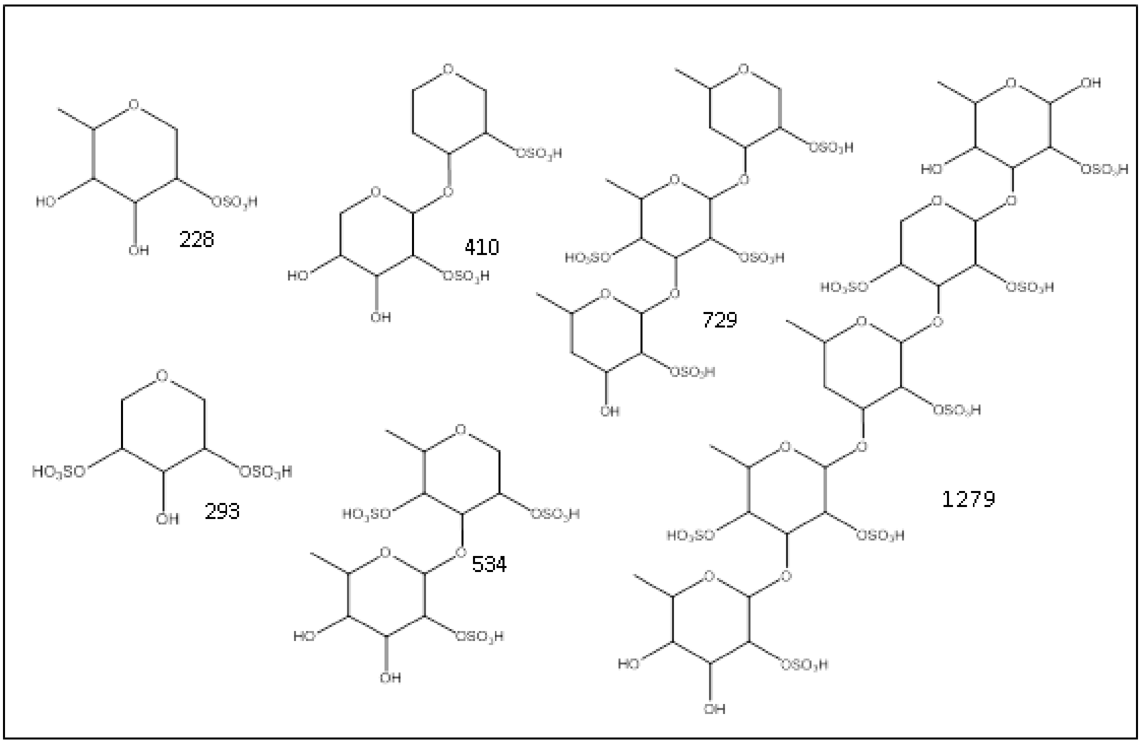


Figure 4.15: Potential molecular structures for fragments found in LC-MS

4.1.3 Discussion of the Implications of the Seasonal Variation of Fucoidan

The sulphate content of fucoidan is a key parameter in assessing its potential for biomedical properties. This was first shown by Nishino et al [208], who demonstrated that increased sulphation on fucoidan extracts from the brown seaweed *Ecklonia kurome* increased their ability to inhibit thrombin-fibrinogen reaction and amidolytic activity of thrombin. Later work conducted by Haroun-Bouhedja et al [73], also investigating the inhibition of thrombin using a low molecular weight fraction of fucoidan extracted from *A. nodosum*, demonstrated that fractions with less than 20% sulphate had little to no inhibitory effect. Comparing these results to those found in this seasonal variation study, it is clear that there are some months in which the sulphate content of the crude fucoidan falls beneath this minimum value, meaning the extracted fucoidan will have little to no bioactivity. By interpreting the data in Figure 4.4 and correlating it with the total quantity of extracted fucoidan each month, given in Figure 4.3, it is possible to predict both the best month for harvest in terms of maximum yield, but also in terms of the best potential for bioactivity. Figure 4.16 shows the fucoidan content plotted against the sulphate content, in order to determine the optimum harvest time for each species, where the dashed line at 20% sulphate shows the values below which will exhibit little bioactivity. This graph clearly shows that all FS samples fall above 20 wt% sulphate, so harvest throughout the year will yield good bioactivity. Optimum harvest time will then be dependent on the month in which the greatest fucoidan content occurs, which, in this case, is November. For FV and AN, only fucoidan extracted in a few months show a sulphate content above 20%. For FV, these occur in November, December and January. Incidentally, this peak in sulphate levels also corresponds to a peak in fucoidan quantity, with December having the greatest amount. This means that a December harvest of FV would yield both the highest quantity of fucoidan along with a good sulphate content. June and March samples of AN show sulphate contents over 20%, although there are several months whose error bars put them within the data range, including April and November samples. Again, higher sulphate content appears to correspond to a higher fucoidan content in most cases and a June harvest, having the second highest overall fucoidan content, will give the best balance between bioactivity and fucoidan extraction. The trend for increased sulphate content with higher yield of fucoidan was also noted by Mak et al. [93] in their study of *Undaria pinnatifida*, in which they investigated the fucoidan content and composition over 4 months.

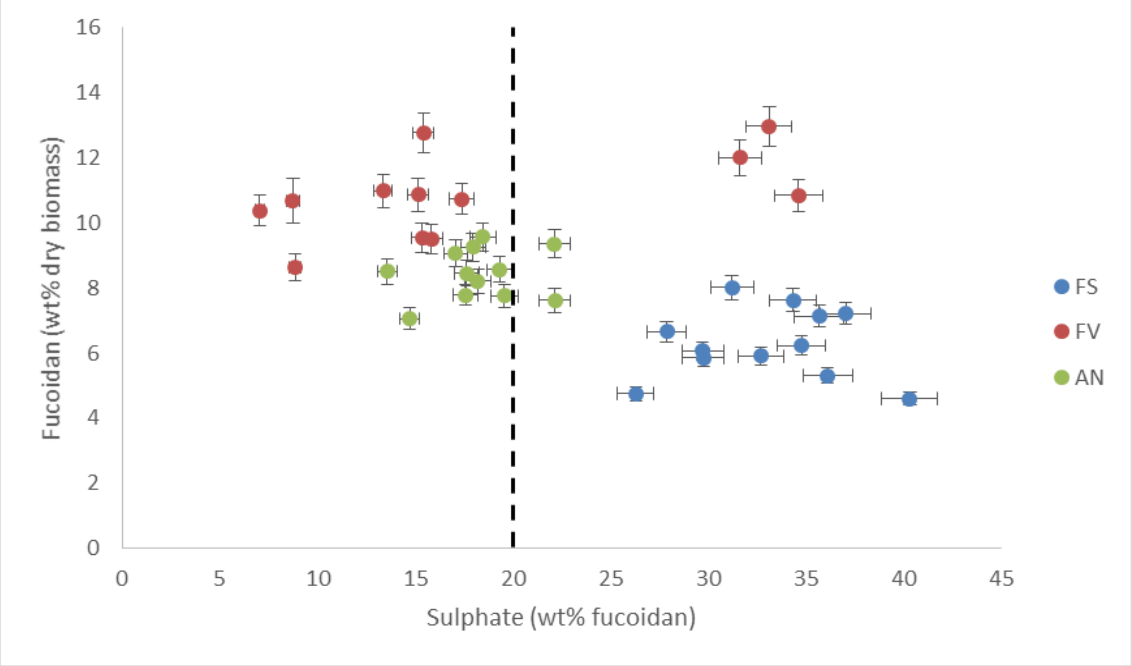


Figure 4.16: Fucoidan content in dry biomass against sulphate content of fucoidan.

Although optimum harvest months for the three species studied here can be chosen, for industrial extraction purposes, harvest throughout the year would be beneficial. This is due to the high energy costs associated with drying and storing seaweed biomass, which can contain up to 80% water at harvest weight. While the processing of wet biomass for fucoidan production has not been conducted in this study, nor by any other study the author could find, the ability to harvest and process wet would be very beneficial to industrial processes, and the ability to do so seems possible, if chemicals are added so their dilutions are achieved by the water present in seaweed. If this were the case, then FS would make the best feedstock for extraction, due to its high sulphate content year round.

As with the seasonal variation study of the chemical content of seaweed (Chapter 3), no long term studies on the effect of: anthropogenic changes, such as ocean acidification and climate change; weather conditions, including hours of sunlight or sea temperature; or locational variations in factors such as sea metal content have been conducted. It is likely that all these factors will have an effect on the quantity and composition of fucoidan and therefore a thorough review of these factors would be essential for the industrial extraction of fucoidan.

4.2 Development of Colourimetric Determination of Fucoidan and Comparison with Conventional Extraction

4.2.1 Introduction

Fucoidan is extracted from macroalgae in order to determine quantity and is performed via a lengthy extraction and purification process, resulting in dry fucoidan. In general this consists of four main steps: an initial purification to remove pigments and lipids, often using an alcohol; an extraction step, often repeated several times to ensure full extraction of fucoidan and most commonly using calcium chloride, dilute hydrochloric acid or water; further purification of the extract to remove alginate and other impurities before fucoidan is finally precipitated using ethanol [137-142] and is the method used the fucoidan seasonal variation study presented in section 4.1. A comparison of the three extraction solvents was carried out by Ponce et al. (2003) [145]. The results indicated that distilled water and HCl extraction gave the highest and comparable yields of 10.8 and 9.6 wt% respectively, with the structure of each extract being very similar. Zhang and Row (2015) further this, similarly comparing extraction solvents, but also identifying the best conditions for fucoidan extraction from *Laminaria japonica* [146]. Their findings suggest an extraction time of 4 hours at 80°C and 0.1M HCl yielding the best results, giving 17wt% fucoidan. Overall, all of the methods described require long extraction and purification times in order to obtain a pure product and determine the accurate fucoidan content, taking up to 5 days depending on the number of extractions performed.

An alternative option is spectrophotometric determination, using an acid hydrolysis of fucoidan into its monomer units, including fucose, followed by the reaction of the fucose with cysteine hydrochloride to produce a measurable colour based on concentration. This method was first described by Dische and Shettles [135], although has not been fully explored in more recent literature. Fucoidan is inferred, assuming fucose accounts for 50% of the fucoidan total mass, so the mass reading is doubled in order to obtain the total fucoidan.

A comparison of the two methods is examined in this section, to investigate whether the spectrophotometric technique is a viable, faster and less solvent intensive alternative to the traditional extraction of fucoidan for total quantity analysis. The spectrophotometric method has been fully developed in order to reduce errors and erroneously high results due to the presence of other sugar monomers in the extract. The possible benefits of this rapid method for industry has been assessed.

4.2.2 Development of the Spectrophotometric Method

The calibrations for the fucose and fucoidan standards are displayed in Figure 4.17. In each case, a clear linear trend line is observed, which passes through the origin. For this particular fucoidan standard, which is extracted from *Fucus vesiculosus* (Sigma Aldrich), the fucose content is 41.5%. This is in good agreement with previous reports using a similar standard fucoidan, where a fucose content of 44% was quoted [209]. It is worth noting that, due to the variation in fucoidan between species, the fucoidan calibration is only correct for *Fucus vesiculosus*. Calibrations for other species would need to be conducted to gain an accurate result. The original paper by Dische and Shettles [135] suggests that, on average, 50% of fucoidan is fucose, so the result based on the fucose calibration should be multiplied by 2 to give the fucoidan content. The initial results are based on this assumption, as, without full extraction and analysis of fucose content in the fucoidan, the actual value is unknown, which would be the case in an initial testing setting.

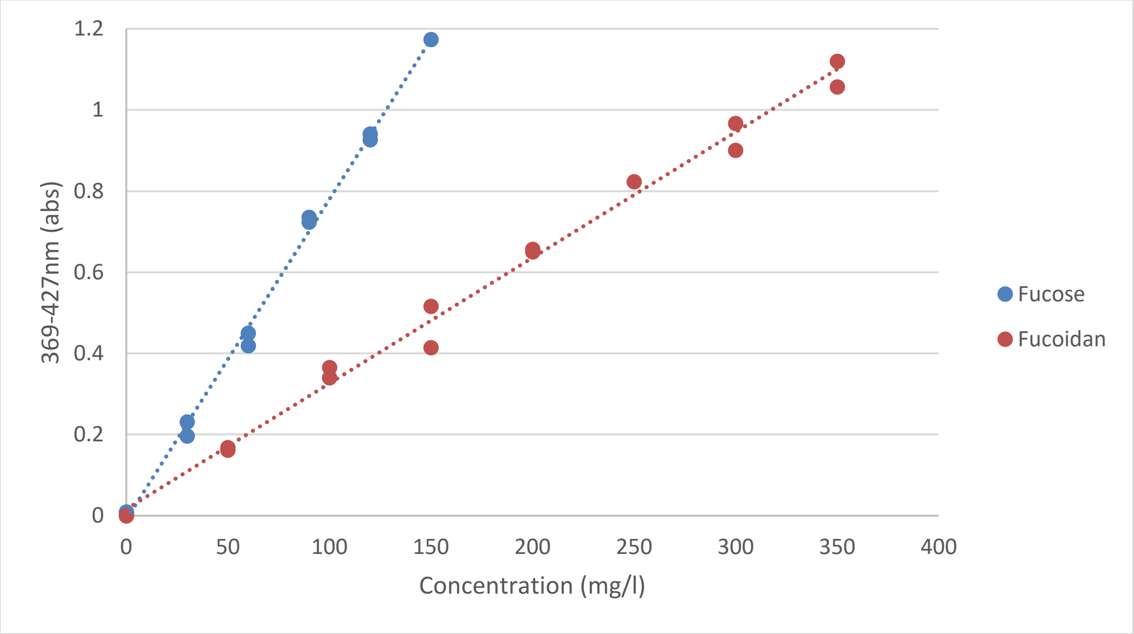


Figure 4.17: Calibration curve for fucose and fucoidan standards

It was noticed that a change in development time gave significant differences in readings while creating the fucose calibration. In order to understand this fully, the influence of reaction time was investigated and a set of fucose calibration standards were measured every 30 minutes over a 2 1/2 hour period, the results of which are given in Figure 4.18. Although a linear trend line is still evident for all development times, there is a doubling in the absorbance reading between 30 and 180 minutes for the highest concentration. A similar experiment was carried out for the fucoidan standard, although in this case the phenomenon was not observed (Figure 4.18). It is thought this is due to the difference between the L-fucose standard and sulphate fucose present in fucoidan.

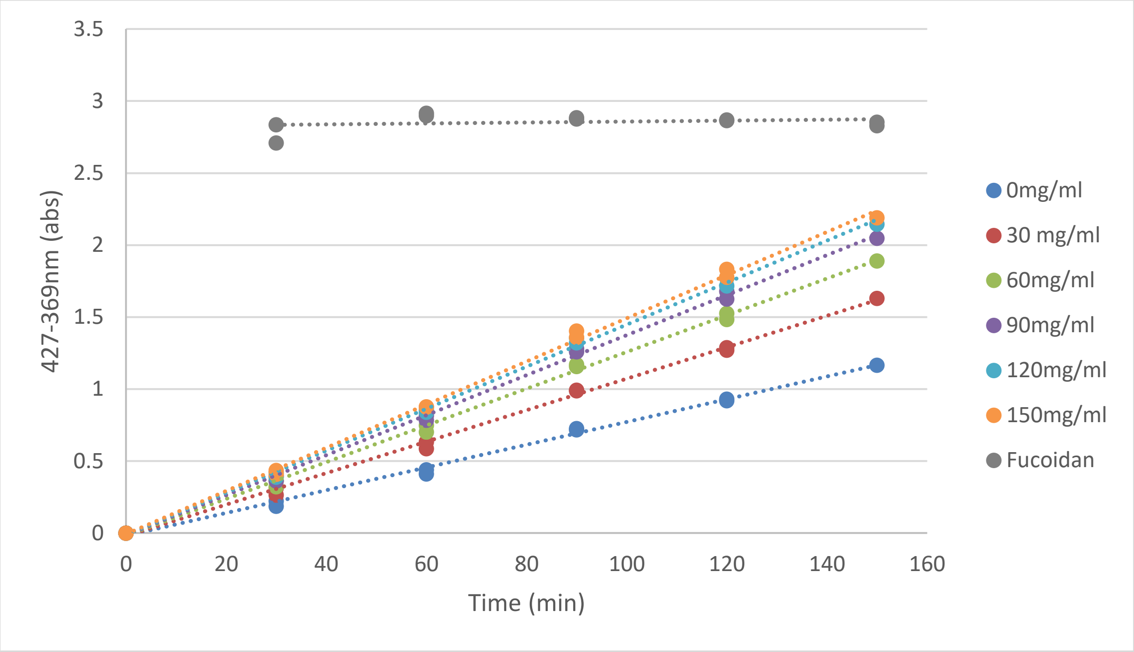


Figure 4.18: Calibration curve for fucose and fucoidan under differing colour development times

As shown in Figure 4.19, L-cysteine hydrochloride reacts with the OH group on the fucose, creating a colour change. While the standard has 4 possible bonding sites, sulphated fucose has less, commonly two, due to sulphate groups replace OH groups. This means that each l-fucose could create a colour change twice as intense as each sulphate fucose. However, after the first L-cysteine hydrochloride has bonded, each subsequent bond will be increasingly difficult to form, therefore giving the colour change over time seen for the L-fucose. Fucoidan, however, having less OH groups to bond with, achieves its full colour change in 30 minutes, after which there are no remaining OH groups for the L-cysteine hydrochloride to bond with. The number of OH groups for sulphated fucose could also be reduced by incomplete hydrolysis to monomers, as the fucose chain in fucoidan is linked by O-glycosidic bonds, which break into an OH group on each monomer. In order to achieve a consistent colour change for the standard, a 30 minute development time was strictly adhered to, which was also followed through subsequent experiments to eliminate any discrepancies which could be caused by this.

Another possible reaction to give the colour change seen is the N on the L-cysteine hydrochloride attacks the C to give an imine. However, as there is no literature available on the specific reaction, more studies would need to be undertaken to assess the details of this reaction.

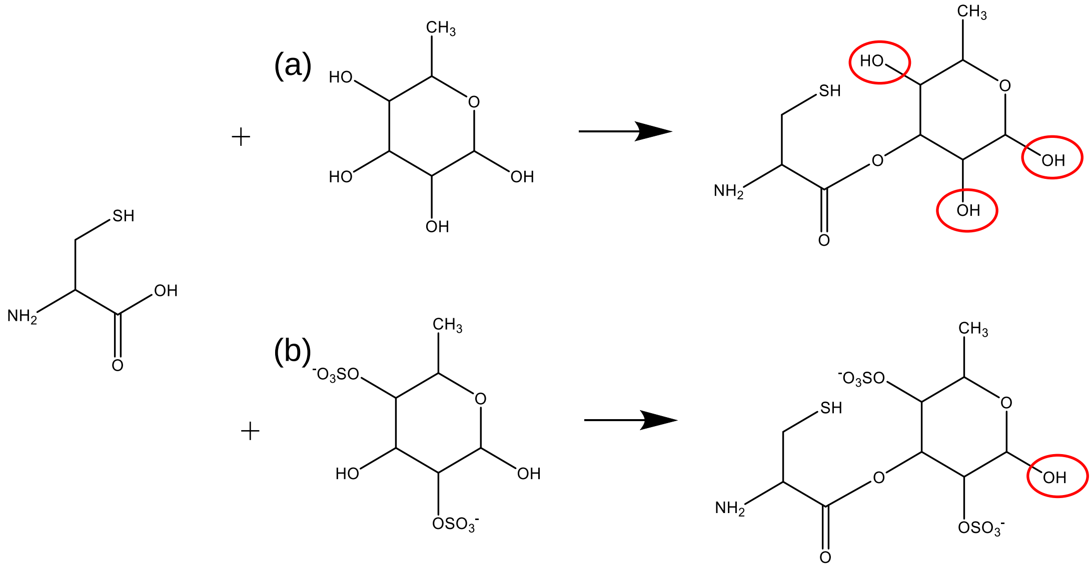


Figure 4.19: Reaction between L-cysteine hydrochloride and (a) L-fucose and (b) sulphated fucose. Remaining bonding sites are circled in red.

The method for hydrolysing the fucoidan, using concentrated sulphuric acid, is similar to the NREL method for the determination of sugars from biomass [210]. Although the method presented here calls for two absorbances to be measured, to eliminate colour interference caused by glucose present, it is unlikely that this alone will fully compensate for the other sugars present. This is due to there being several types of monomer unit present, resulting from the breakdown of different carbohydrates. Alginate, for example, will have been hydrolysed into guluronic and mannuronic acid [46], which will adsorb at a wavelength to glucose, for which the method is corrected.

In order to explore the method fully, the effect of glucose (the main constituent in laminarin) and alginate were determined. This was achieved by making solutions containing different concentrations of standard glucose and alginate, all containing the same amount of fucoidan standard; 0.3mg in the 1ml used for testing, which is equivalent to a sample containing 10wt% fucoidan. As the same fucoidan standard was used, in this case, the fucoidan calibration curve was used in calculation, with the intention that this would give the most accurate result. The results, presented in Figure 4.20, show that addition of glucose results in measurements 20% higher than the expected fucose concentration of 150mg/ml. Although alginate gives the expected fucose concentration at 300 mg/l, there is an increase of 27% over the expected reading for 150mg/l at 1600mg/l alginate concentration. As the trend lines are linear, it is possible to apply a correction factor to account for these inaccuracies, given as Equation 3, where F_{adj} is the adjusted fucoidan, F_{int} is initial fucoidan and A is alginate, all in wt%. Although this means measuring the alginate content of the sample, the method is relatively quick to determine by colourimetry [211] or by HPLC and the total analysis time is still far less than for the full extraction method.

$$F_{adj} = \frac{F_{int}}{1.2 + (0.007A - 0.09)} \qquad \text{Equation 3}$$

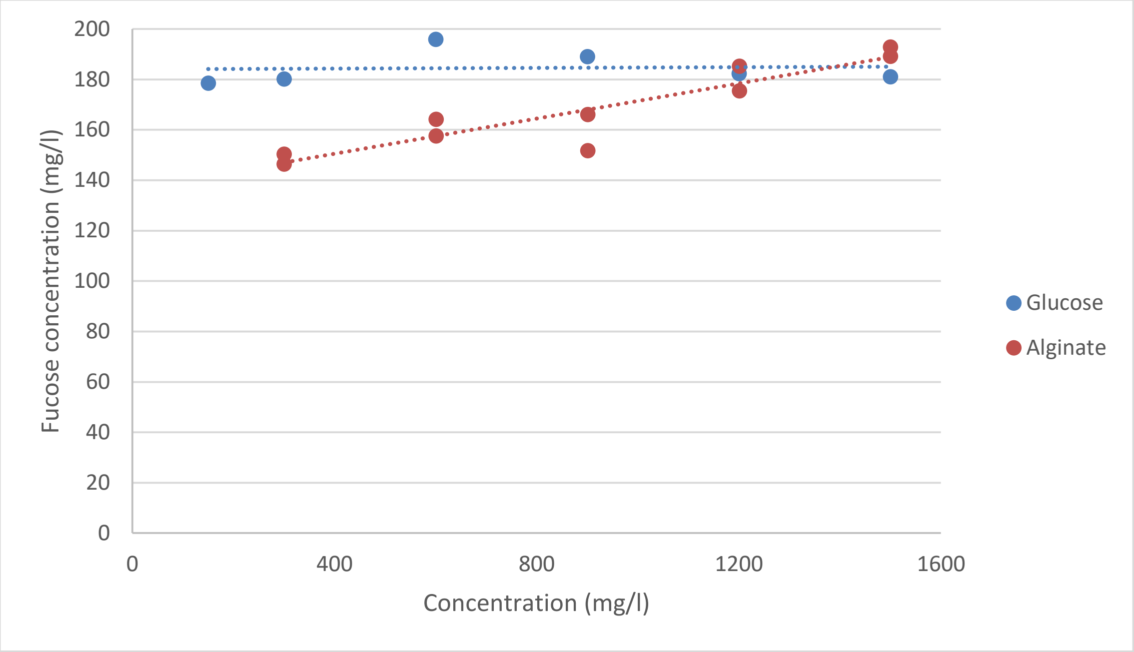


Figure 4.20: The effect of glucose and alginate on spectrophotometric determination of fucoidan

4.2.3 Comparison between Conventional and Spectrophotometric Extraction Methods

Nine samples from three species of macroalgae, one spring, one summer and one winter for each, have been tested using the spectrophotometric technique and compared to the results from conventional extraction. The results, shown in Figure 4.21, show little correlation between the conventional method and the un-adjusted spectrophotometric data for all three species. Once Equation 3 has been applied to the data, accounting for interference by laminarin and alginate, there is good agreement for FV, with all samples being within ±5% of the conventional method values.

FS and AN, however, still show little agreement. This is likely due to differing fucose content in fucoidan. Studies into fucoidan structure have shown that FV has the simplest form and regular structure, with a linear backbone of fucose, making the 50% fucose content assumption more or less accurate [164]. However, FS and AN have been shown to have different structures, including fucose side chains and differing quantities of sulphate, rendering the 50% assumption inaccurate [164]. By calculating the fucoidan content for FS and AN using a fucose content of 70 and 80% respectively gives much better agreement, as shown in Figure 4.21c. This is equivalent to multiplying the fucose value by 1.4, 2 and 1.25 respectively for FS, FV and AN. It is likely that these adjustments are required both for the differing fucose and sulphate content of the sample, and not solely on the fucose content alone. In this case, all values for all 3 species are within ±5% of the conventional method, with an R^2 of 0.93 and the slope of the trend line approaching 1. The good agreement between the three samples from each species suggests that, assuming the adjustment factor is applied, these fucose ratios could be applied to other samples with confidence, without the need to undertake the lengthy conventional extraction

It is likely that the intensity of the colour reaction, and therefore the initial spectrophotometric reading, will be dependent on both the differing fucose content between species, as well as the degree of sulphation in the fucoidan, due to the reaction shown in Figure 4.19. While the quantity of sulphate in fucoidan does vary throughout the year, the range of values is relatively small (see section 4.2.2), and an average value of 34, 19 and 15% was found for FS, FV and AN respectively.

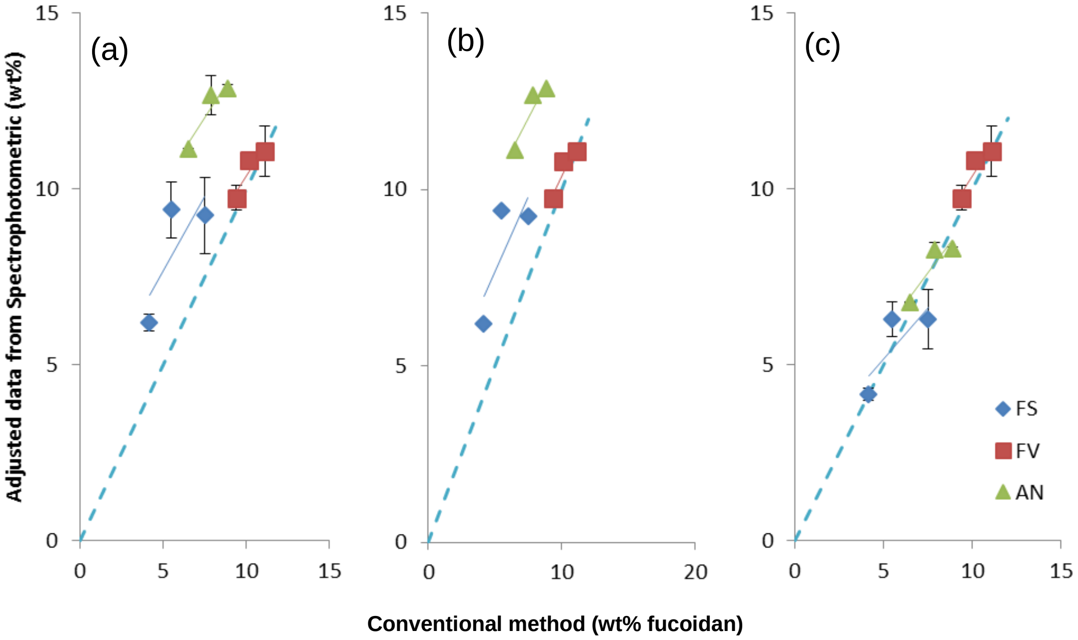


Figure 4.21: Correlation of the conventional method and (a) unadjusted spectrophotometric results, (b) adjusted photometric results based on 50% fucose in fucoidan and (c) adjusted photometric based on varying fucose content dependent on species. Ideal correlation of x=y is shown by the dashed line.

Overall, as long as calibration for the specific seaweed being analysed has been performed and compared with the conventional extraction method, the spectrophotometric determination of fucoidan presents a significantly faster method. From an industrial stand point, this method offers a good approximation of the fucoidan present in a sample, which, considering the variation in content throughout the year, would be important for an extraction process so ensure that sufficient product is made to fulfil requirements.

4.3 Conclusions

The seasonal variation of fucoidan and the variations in its chemical composition has been explored. Clear differences between the different species, as well as between months, has been seen. FS has been shown to be more abundant in fucoidan in the autumn months, with its highest value in November. It is also likely, form the SEC and LC-MS results, that FS has a higher degree of branching and a higher variation in MW than the other two species. FS also have the highest sulphate content, an important factor for bioactivity. FV has been shown to have its highest concentration of fucoidan in December. It’s MW has been shown to vary less than FS and also contains a lower sulphate content. AN has been shown to contain its maximum fucoidan content in October. The MW across the year is lower than that for FS, but slightly higher that for FV. Overall, AN has the lowest sulphate content of the three species.

The use of a spectrophotometric method for the determination of fucoidan in macroalgae samples is viable, as long as a correction factor for the laminarin and alginate content is applied. Although the correction factor for laminarin is constant, the method does require alginate content to be determined in order to gain an accurate result. However, the presented method for alginate content is sufficiently short that the time required is still less than for the full extraction method. While the original assumption of fucoidan containing 50% fucose only gave comparable results to the conventional method for FV, adjusting the assumed fucose content in fucoidan and multiplying the spectrophotometrically determined value by 1.4 for FS and 1.25 for AN gave good agreement to the conventional method. With this adjustment, all nine samples tested from the three species were within ±5% of the conventional value. The spectrophotometric method, if applied in an industrial setting, would prove useful as an initial testing for macroalgal feedstock for the extraction of fucoidan. This is due to its content varying due to a range of factors, affecting the quantity of fucoidan able to be extracted.

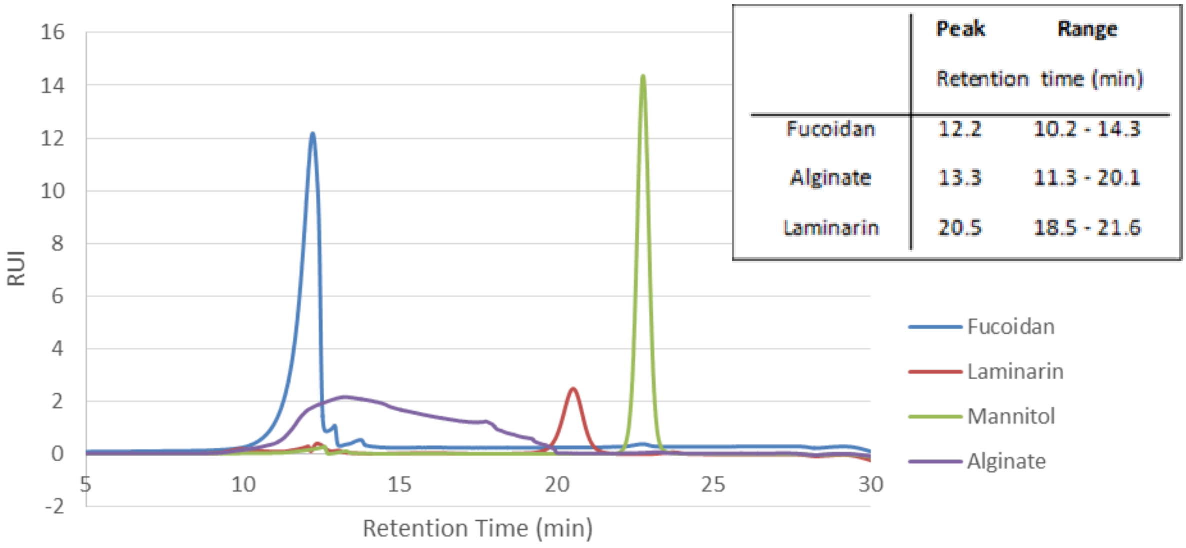
The overarching conclusion to this study is the need for a clear and thorough understanding of the seasonal variation of fucoidan and the impacts external factors play in its structure, including season, weather variation and anthropogenic influences. With so many factors playing a role in this, it is of great importance that these are thoroughly understood and quantified for the extraction of fucoidan to be viable at an industrial scale and for achieving a consistent, bioactive product. While this study has laid the ground works for this to be achieved, there is still a lot of work to be done in the area in order to fore fill the requirements.

5 Microwave extraction of Carbohydrates

5.1 Introduction

Microwaves have been used in the past for chemical extraction [149, 156, 157], with some references to their use with regards to seaweeds [162, 163] and are now widely used in industry for food production. Microwaves have been shown to have a lower heating requirement, higher controllability, more even heating and better repeatability than conventional heating [148], which makes them an ideal candidate for the extraction of chemicals from seaweed with a view to reducing the overall environmental impact of the system. The use of microwaves potentially offers more flexibility to operate more economically on a small scale. This is beneficial to the seaweed industry, as cultivation locations are often remote and seaweed decomposes rapidly after harvest [212]. Being able to harvest and process in close proximity to each other would negate the problem of transport of the biomass.

In this chapter, a comparison of extraction between microwave and conventional heating has been performed as an initial feasibility study for the development of a macroalgal biorefinery using microwaves. Size exclusion chromatography (SEC) has been used to determine the composition of various extracts, the standards for which, along with their average retention times, are given in Figure 5.1 for reference. It is worth noting that all standards have been analysed in the same concentration in this figure and the intensity of the signal varies depending on the compound which is being analysed. For example, mannitol and fucoidan give a much stronger signal when compared to laminarin. Sugar, organic acid and metal analysis has also been performed on both residues and extracts, in order to gain a full and quantified analysis of the composition of each fraction at each stage.



	Peak	Range
	Retention time (min)	
Fucoidan	12.2	10.2 - 14.3
Alginate	13.3	11.3 - 20.1
Laminarin	20.5	18.5 - 21.6

Figure 5.1: SEC chromatograms of standards of the main compounds found in seaweed with table of peak and range of retention times for each.

5.2 Characterisation of Feedstock

A sample of FS was collected from the coast of Aberystwyth on 16th June 2015. The seaweed was dried in an oven at 50°C for 24 hours, before being ground and sieved to 500µm for analysis. FS was chosen for this study as it was shown in the seasonal variation experiments to have the lowest ash content, which would minimise corrosion in an industrial setting [79], while maintaining high quantities of carbohydrates of interest, such as fucoidan, laminarin and alginate. In addition, while having a slightly lower fucoidan content, the composition of the fucoidan was shown to have a higher sulphate quantity, which relates to its bioactivity. A sulphate content of more than 20% is required for good bio-active properties [164] of which FS was the only seaweed studied to maintain throughout the year. Harvest in June was chosen also due to the high carbohydrate content and low ash content. The seasonal variation study in chapter 3 shows laminarin, mannitol and fucoidan to be higher in the summer, correlating to an equivalent low in the ash content.

Figure 5.2 shows the chemical composition of FS collected. Alginate is seen to make up the largest fraction of the biomass, accounting for 32.5±2.7wt% on a dry basis. The “other” fraction is likely to be made up of polyphenols, lipids and small quantities other metabolites and pigments, as shown in chapter 3. Comparison with this data from the June sample from the seasonal variation study (Chapter 3), shows that the alginate, protein, ash and fucoidan content are relatively similar. Laminarin and mannitol, however, are both significantly lower in the 2015 sample when compared to 2010: 6.1±0.4 wt% vs 17.0±2.5 wt% and 8.7±0.6 wt% vs 12.8±0.6 wt% respectively. However, as was noted by Black [21], that the seasonal pattern can vary by up to two months, dependant on the weather of the particular year. Here, the June 2015 sample of FS is more closely related to the April/May samples from 2010, where laminarin and mannitol were approximately 9 and 8 wt% respectively.

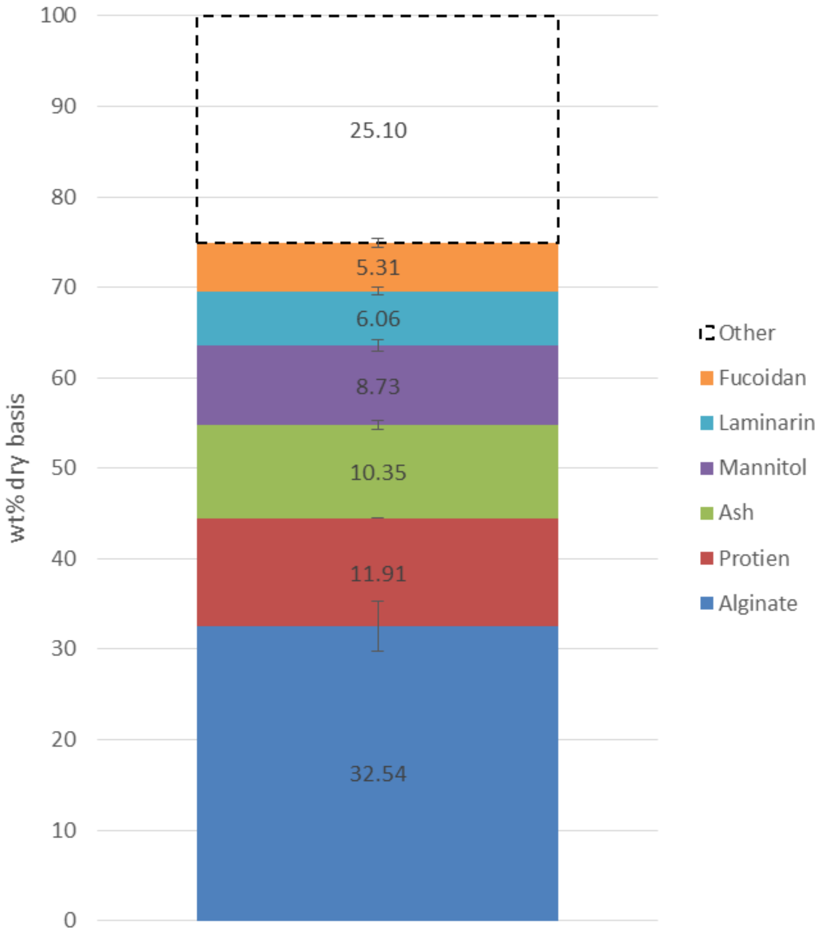


Figure 5.2: Chemical composition of FS on a dry basis

The composition of the ash from FS has been analysed, with the results shown in Figure 5.3. The majority of the metals are accounted for by sodium, potassium, calcium, magnesium and chlorine, who, combined, account for 97.1% of the total metals. As is common with brown seaweeds, FS contains a relatively high quantity of heavy metals, such as As and Zn. This is due to the bioaccumulation of heavy metals which they absorb from the seawater [213]. The small “other” bar in Figure 5.3(b) is mostly accounted for by iodine, boron, barium and manganese, with small quantities of rubidium, titanium and nickel present, all below 50 mg/kg. As was seen with the seasonal variation samples in chapter 3, there is a relatively high quantity of strontium.

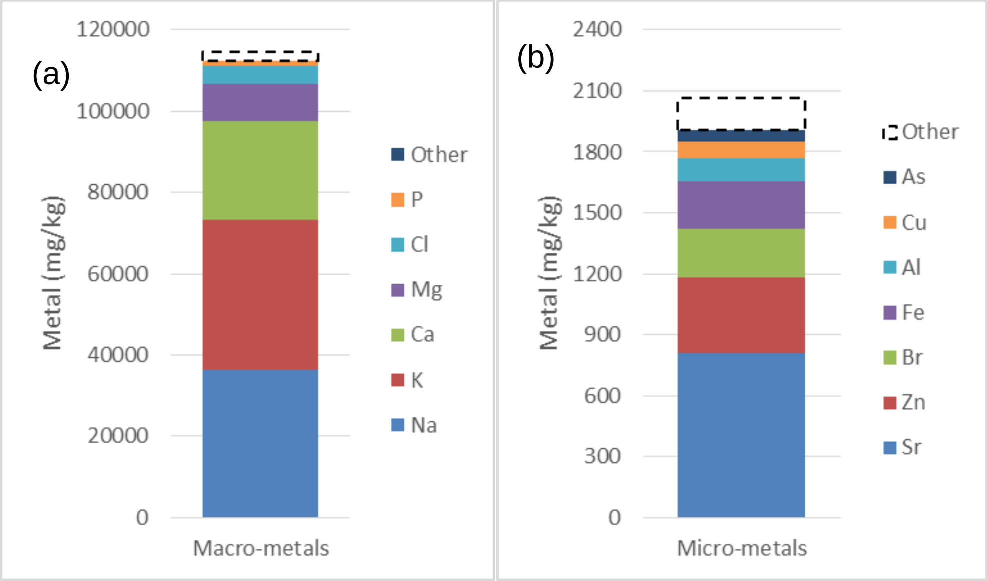


Figure 5.3: Metal content of FS on a dry basis, where (a) shoes the macro and (b) the micro metals.

5.3 Conventional vs Microwave: A comparison of Heating Methods and Effect of Temperature on Extraction

The effect of temperature on extraction of the whole seaweed biomass has been assessed, comparing conventional and microwave heating. Figure 5.4 shows the temperature profiles for conventional and microwave heating over the range of temperatures investigated. For microwave heating, the ramp rate was set to 5 minutes up to the desired temperature, which was then held for either 5 or 10 minutes. The long ramp rate was used in order to ensure good control of the heating: the microwave is calibrated to heat water [214], which is significantly less polar than the seaweed slurry used here. As the infra-red temperature sensor used has a relatively slow response time, shorter ramp rates were seen to give an overshoot in temperature. While it is not possible to calibrate the lab-scale microwave used in this study, it may be possible in an industry setting to reduce the ramp rate through a better temperature control system, using fibre-optic temperature sensors inside the reactor, which are transparent to microwave heating and calibrating the microwave energy input to correspond with the heating profile of the seaweed slurry.

In conventional heating, achieved by placing a metal reactor into a sand bath set to the desired temperature, the contents of the reactor was allowed to come to the desired temperature, measured using a thermocouple, before being left for the desired residence time. The ramp rate was found to be approximately 5 minutes in all cases, which gave a good comparison for its microwave heating equivalent. It is important to note that the thermocouple is placed in the centre of the reactor, meaning the slurry closest to the walls is likely to have reached the desired temperature before 5 minutes required to heat the centre.

The main difference in the temperature profiles, which can be seen between the two methods, is the oscillation in temperature seen for microwave heating, compared to the steady temperature seen for conventional heating. The microwave controls the temperature to within ±5% of the set temperature by applying energy only when the temperature has dipped below the lower value and heating to the higher [214]. For conventional heating, the aim is to get the reactor and contents in equilibrium with its surrounding temperature, this oscillation does not occur.

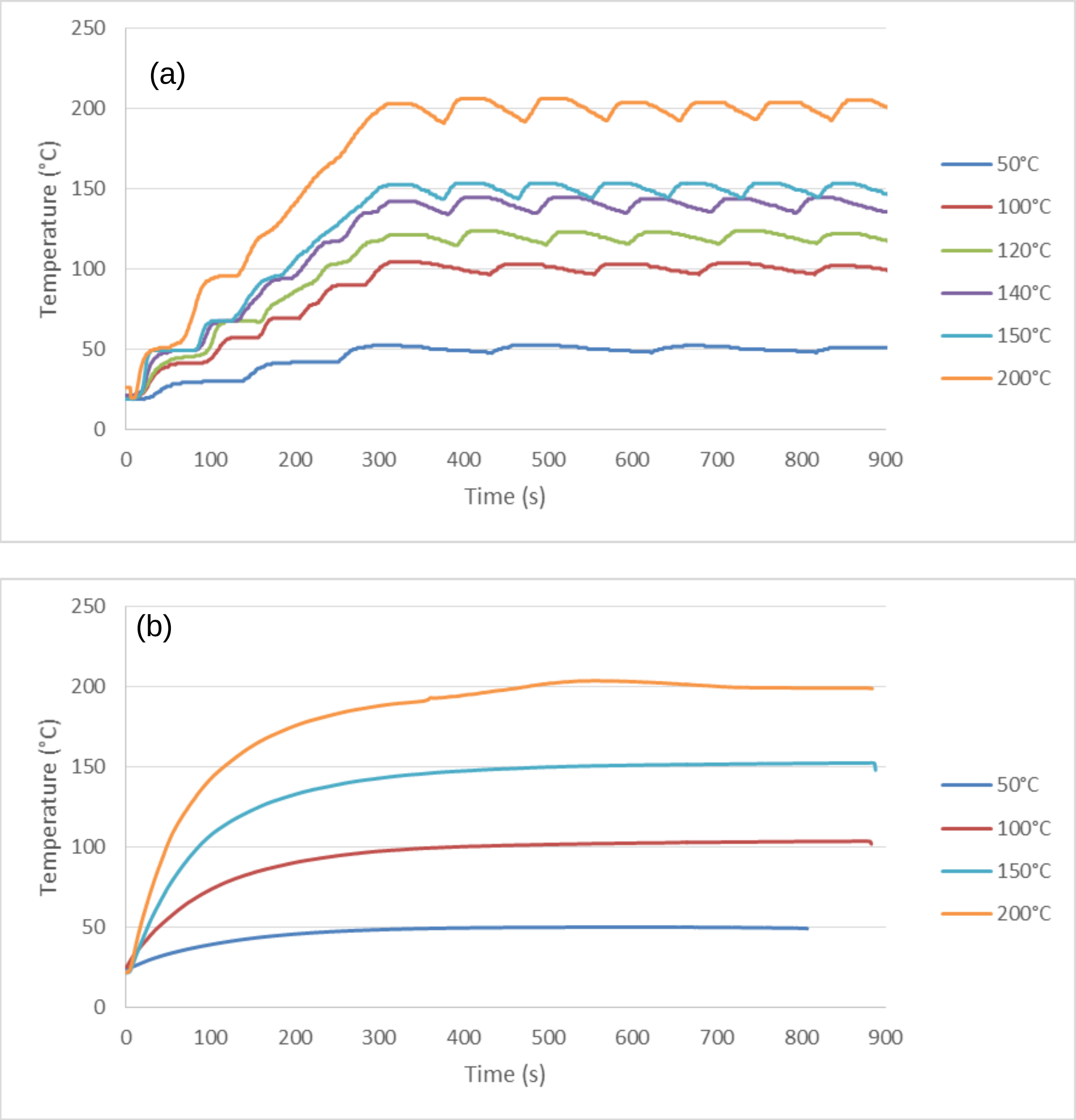


Figure 5.4: Temperature profiles for (a) microwave heating and (b) conventional heating for a 0 minute residence time.

Four scenarios for S:S ratio and residence time have been evaluated over a range of temperatures between 50 and 200°C, which are given in Table 5.1. The aim of varying these parameters is to ascertain if there is any significant difference seen for increased S:S and residence time, thereby fully optimising the process for best extraction. Each permutation of S:S and residence time have been performed over the full range of temperatures. S:S ratios of 1g:10ml and 1g:15ml and residence times of 5 and 10 minutes have been trialled. For both conventional and microwave heating, a ramp rate of 5 minutes up to the desired temperature was applied.

Table 5.1: Different scenarios for S:S ratio, residence time and temperature for conventional and microwave heating comparisons for the extraction of seaweed.

Scenario	**S:S ratio (g:ml)**	**Residence time (min)**	**Temperature (°C)**
5-10	1:10	5	50-200°C
5-15	1:15	5	50-200°C
10-10	1:10	10	50-200°C
10-15	1:10	10	50-200°C

The colour of the microwave extracts varies with temperature, as seen in Figure 5.5, with low temperature extractions for both microwave and conventional heating being very pale in colour, which increases in intensity with increasing temperature. This is likely due to both the extraction of pigments at higher temperatures, but also the beginnings of carbonisation, as sugars are “burnt” at higher temperatures. Although the viscosity was not measured, observation of the liquid extract showed a decrease in viscosity with increasing temperature, presumably due to the breakdown of long chain alginate into smaller units. Alginate is known to be a gelling agent [15], so its breakdown would decrease the viscosity of the extract. This phenomenon was also noted by Saravana et al. [215], who undertook conventional high-pressure water extraction of the brown seaweed, *Saccharina japonica* at 180-420°C. They noted both a decrease in viscosity and an increase pH with increasing temperature.

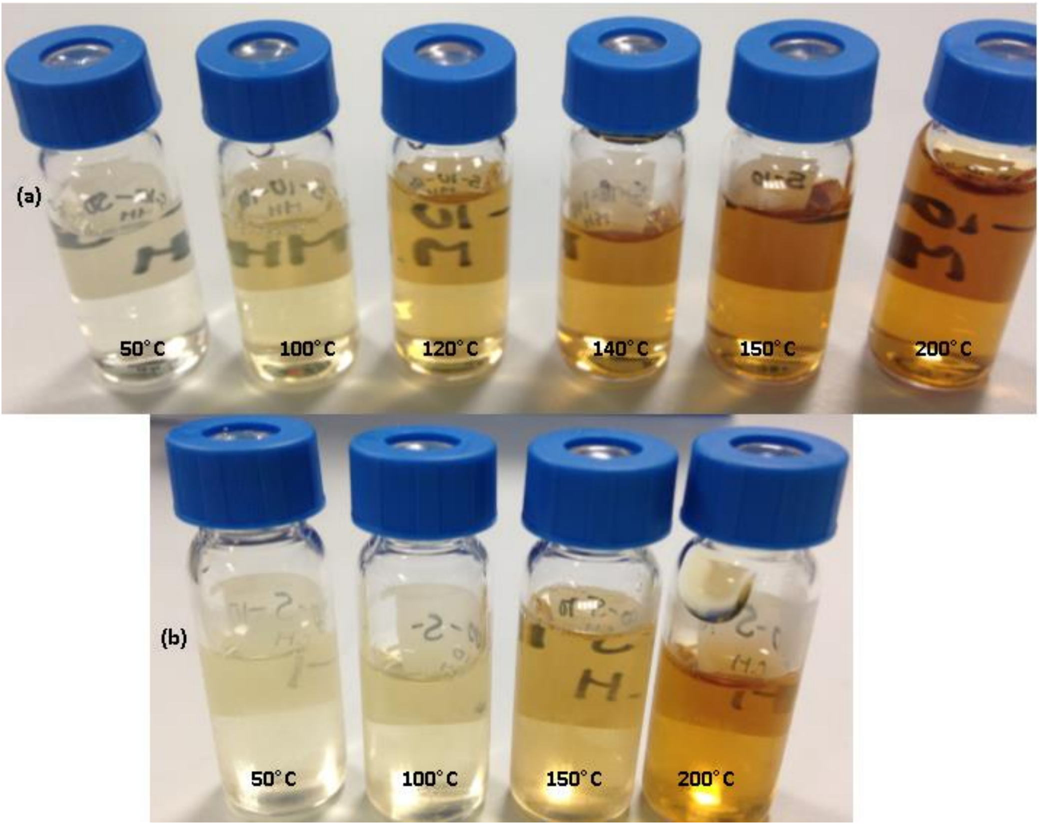


Figure 5.5: Images of liquid extracts from hydrothermal microwave extraction for 5-10 for (a) microwave and (b) conventional heating

The residues from conventional and microwave hydrothermal extraction also showed a distinct colour change with increasing temperature, which can be seen in Figure 5.6. 50°C extracts for both conventional and microwave heating are dark green in colour, which becomes paler up to 150°C, presumably due to the extraction of pigments which give seaweed its colour. At 200°C, the residue biomass becomes significantly darker and brown in colour. As was noted for the liquid extracts, this is likely due to the beginnings of carbonisation. The texture of the residues were also noted to change with increasing temperature, with higher temperatures having a denser, more granular feel when compared to the low temperature residues, which were more flake-like in texture.

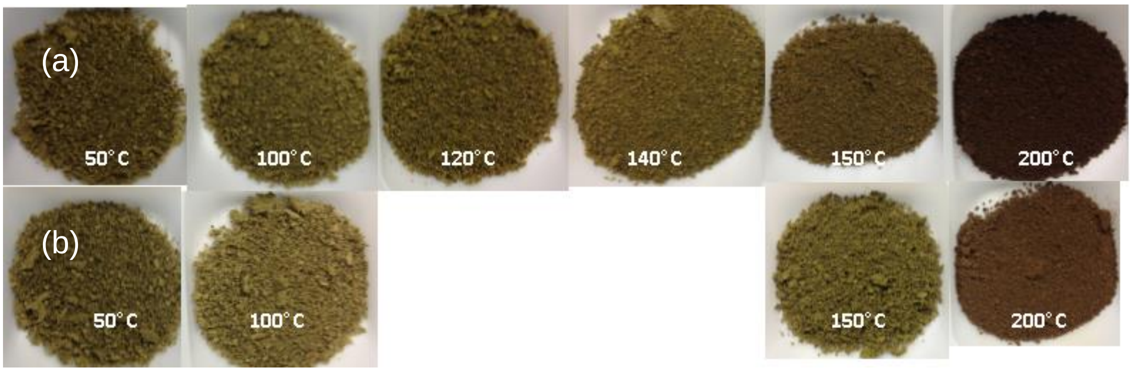


Figure 5.6: Images of 5-10 residues from hydrothermal microwave extraction for (a) microwave and (b) conventional heating

Figure 5.7 shows the weight of chemicals extracted from 1g of biomass for various temperatures, seaweed to solvent ratios (S:S) and residence times. The general trend in all cases is an increase in temperature leading to an increase in material extracted. From these figures, it is clear that there is little difference in extraction quantity between conventional and microwave heating, with the standard error between conventional and microwave heating at each point being less than 0.077 for all scenarios. Furthermore, there appears to be little difference between the various permutations of S:S and residence times across the four scenarios, with the standard error for each temperature being less than 0.025 across the four S:S and residence time scenarios.

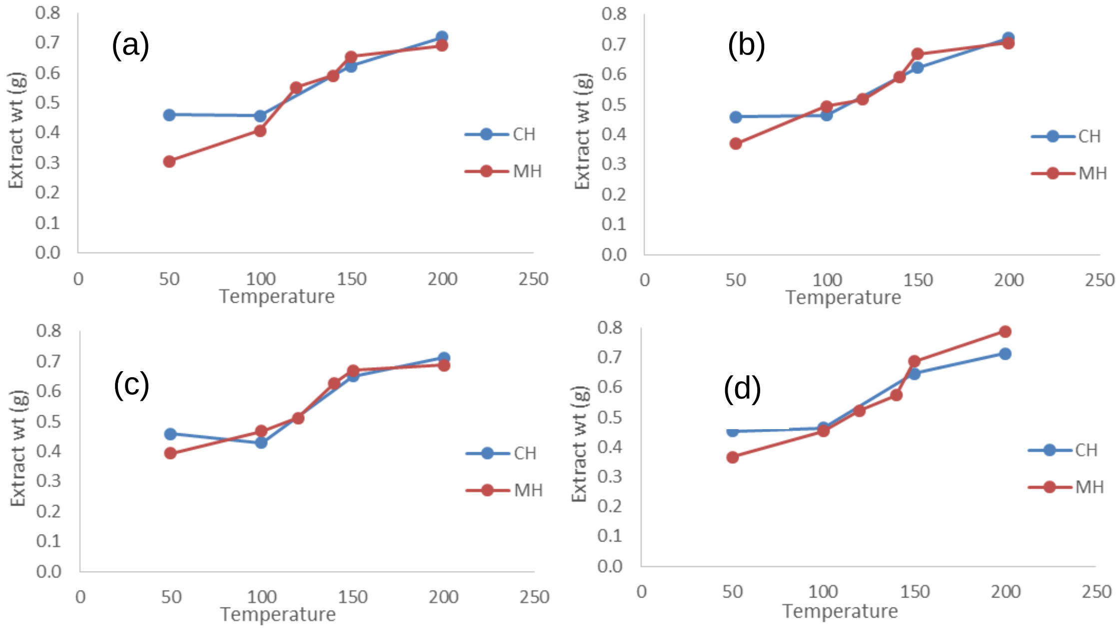


Figure 5.7: Extracted weight of microwave and conventional heating processing of FS for (a) 10ml water for 5 mins, (b) 10ml water for 15mins, (c) 15ml water for 10mins and (d) 15ml of water for 15mins.

While the quantity of material extracted is relatively similar for all conditions, differences occur in the MW profile and, therefore, the chemical constituents of the extract. Figure 5.8 compares the SEC chromatograms for each temperature for a residence time of 5 minutes and a S:S ratio of 1g:10ml, with the chromatogram for the fucoidan standard given for reference. Mannitol, whose peak elutes at 22.8 minutes, appears to be relatively consistent in height and therefore quantity at each temperature. The same is seen for laminarin, which elutes at 20.5 minutes, although it is relatively small. This is due to the lower quantity of laminarin present in the sample, only roughly 6 wt%, as well as the low intensity of the peak for laminarin by RI detection. The peak for fucoidan, at 12.2 minutes, shows the greatest variation with temperature and between the two heating methods. For microwave heating, only a small quantity of fucoidan is extracted at 100°C and below, while the MW profile at 150°C and above is significantly more spread out when compared to the standard, with extra peaks evident between 12 and 20 minutes, suggesting the breakdown of fucoidan and also alginate, which elutes at 13.3 minutes. For conventional heating, the trend at 100°C and below is similar, with only a small quantity of fucoidan being extracted. At 150°C, however, there is a large peak for fucoidan, which is of a similar width and shape of that for standard fucoidan, suggesting that fucoidan is extracted well at this temperature and is not being degraded. The chromatogram for conventional heating at 200°C, however, shows a much wider spread of MW fragments, particularly between 12 and 20 minutes similar to that seen for microwave heating, indicating the beginnings of breakdown for fucoidan at 150/200°C.

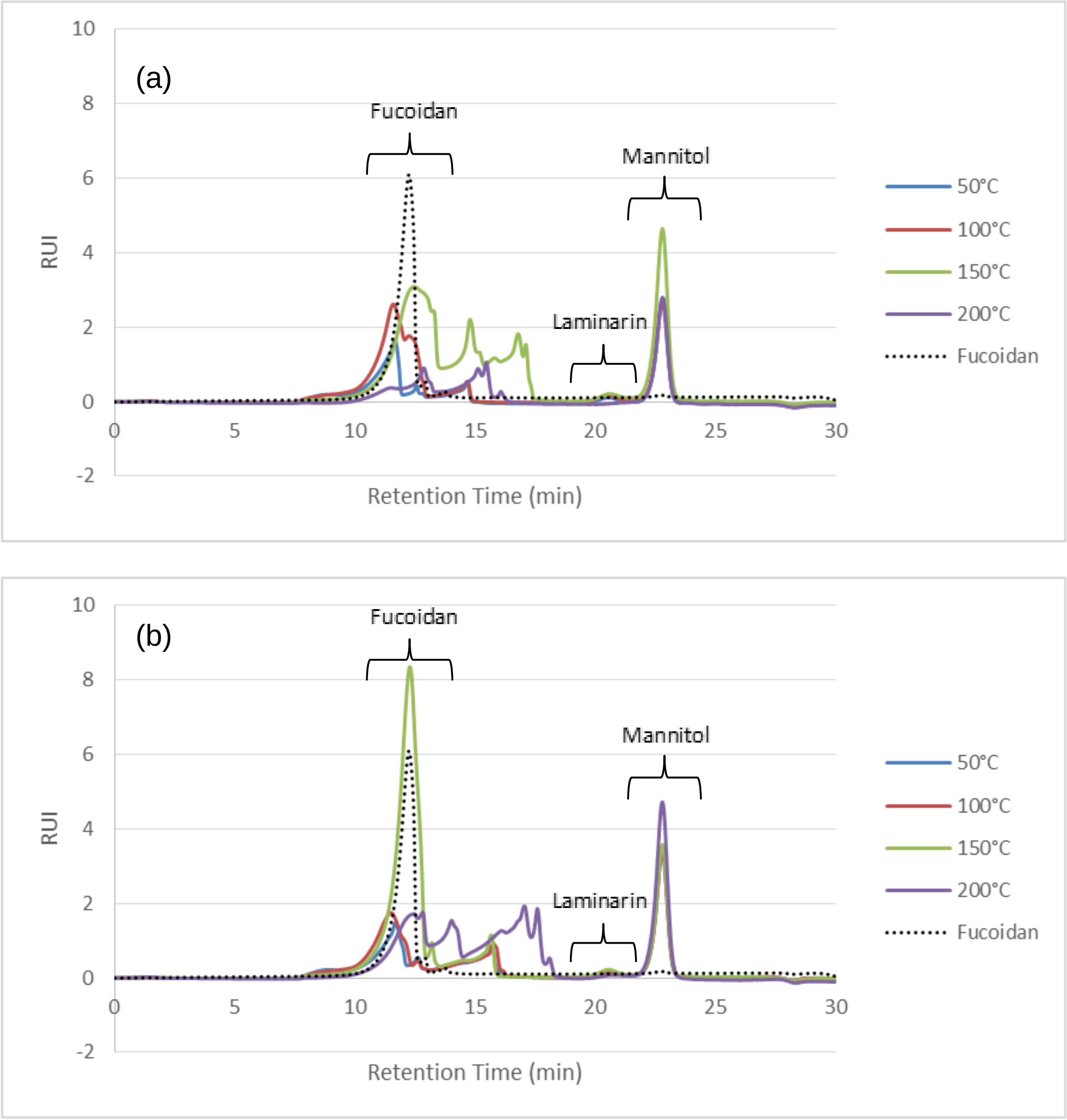


Figure 5.8: Comparison of SEC chromatograms from (a) microwave heating and (b) conventional heating extraction at 5 minutes residence time and S:S of 1g:10ml

In order to determine the optimum extraction temperature for fucoidan in the microwave, runs were have been conducted at 120°C and 140°C, the SEC chromatograms for which are shown in Figure 5.9 alongside the fucoidan standard for comparison. The results indicate good extraction of fucoidan at 120°C, which shows a similar shape and width of peak to the fucoidan standard. The peak for fucoidan at 140°C, however, is wider than for the standard, indicating an increase in the spread of MW fragments for fucoidan. This suggests that fucoidan is beginning to be degraded at this temperature. Extraction of laminarin and mannitol appears to be similar to the other temperatures investigated in this study, with an RUI of approximately 5.

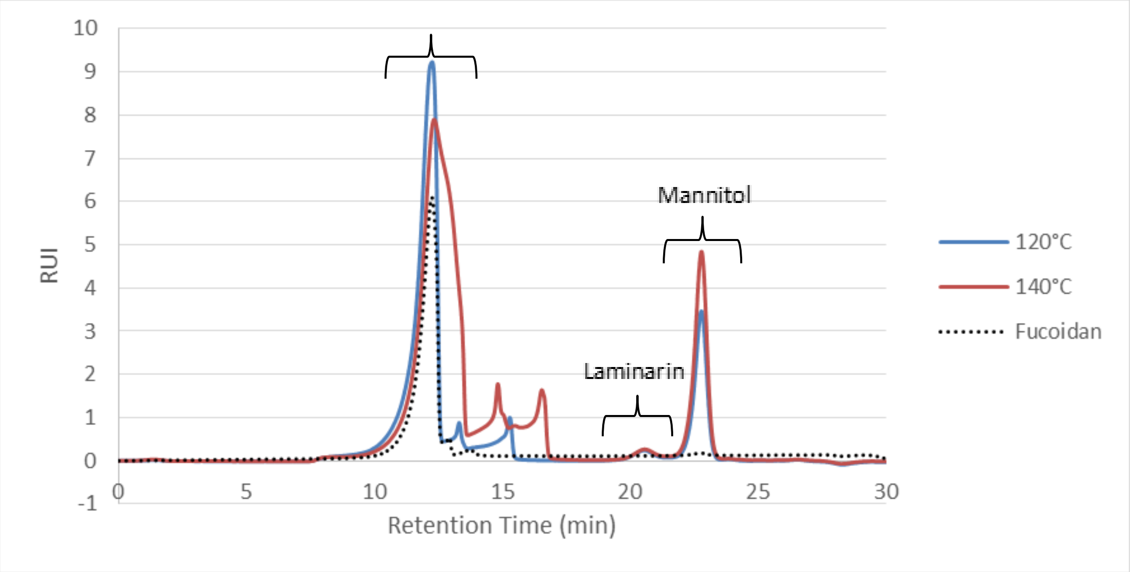


Figure 5.9: SEC chromatogram for microwave heating at 120°C and 140°C.

The variation in abundance of MW bands in the extracted seaweed has been calculated from the SEC chromatograms using standards, the results of which are given in Figure 5.10. Above 2500 kDa represents alginate, 2500 to 1000 kDa represent the main peak for fucoidan with 1000 to 5000 kDa representing both the secondary fucoidan peaks and any broadening in MW of the fucoidan main peak due to degradation. Less than 6.5 kDa represents laminarin and mannitol, as well as any small fragments or sugars created by the heating process and between 300 and 6.5 kDa represents only breakdown fragments made, as none of the standards are within this MW range. For microwave heating, shown in Figure 5.10(a), there is a clear increase in the 2500 to 1000 kDa range up to 120°C, after which it reduces. Components above 2500 kDa are also seen to reduce in MW with increasing temperature. The reduction in these high MW components correspond to an increase in lower MW fragments, with peaks between 500 and 6.5 kDa increasing significantly at temperatures of 140°C and above. There is also a distinct increase in material below 6.5 kDa, indicating an increase in small MW fragments and sugar monomer units. A very similar trend is seen for the conventional heating extracts in Figure 5.10(b), with increasing processing temperature leading to an increase in low MW material and a decrease in high MW material, however the shift is seen to occur at higher temperatures, with relatively similar amounts of low MW material below 150°C, only increasing above this temperature. The quantity and ratio of 2500 to 1000 kDa and 1000 to 500 kDa, for microwave heating at 120°C and conventional heating at 150°C are seen to be very similar, with approximately 3:5 rel. units for each. These have been shown in Figure 5.8 and Figure 5.9 to give SEC chromatograms closest to the standard and, therefore, give the best extraction of fucoidan. The similarities between the MW distribution of these two samples further confirms this analysis.

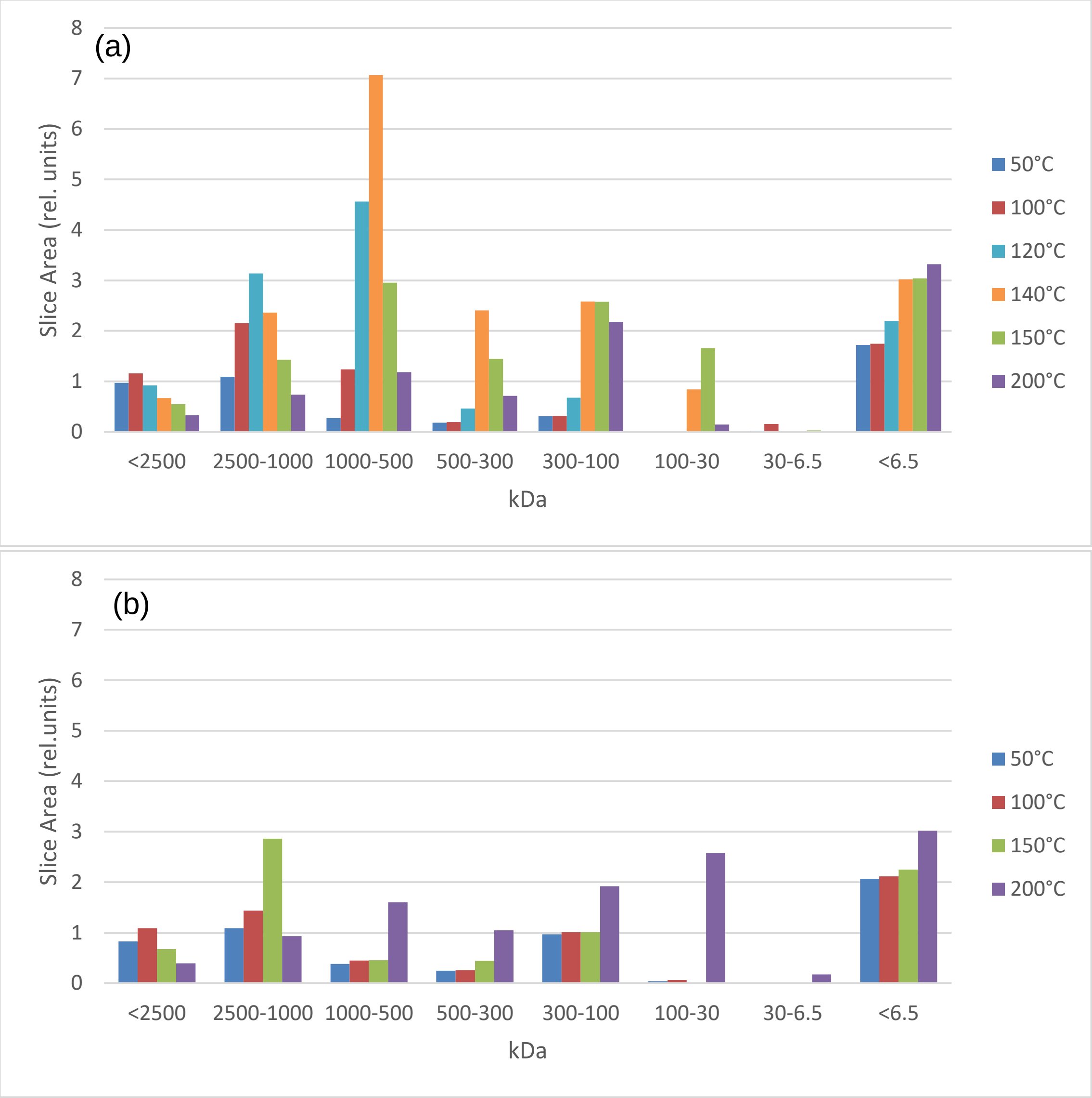


Figure 5.10:Breakdown of the products from extraction by MW in (a) microwave and (b) conventional heating.

Figure 5.11 compares microwave and conventional heating at 50°C for the four scenarios of S:S and residence time. The peak between 15 and 20 minutes is due to salts, which have not been fully removed from the extract before processing. Although this is an issue with SEC for analysis of the extracts, a method was later developed using a ion-trap column in the HPLC set-up and the salt peak doesn't obscure any of the peaks of interest, meaning analysis of the chromatograms is still possible. The quantity of mannitol and laminarin extracted in each case appears to be relatively similar, although this is explored in more detail later. The apparently low quantity of fucoidan and alginate extracted, coupled with the high extraction of mannitol means that 50°C would be a good temperature choice for pretreatment of the seaweed biomass for fucoidan and alginate extraction. It is also clear from these chromatograms that there is little difference between the different permutations of S:S and residence time at 50°C and also little difference between conventional and microwave heating. This means that the lower S:S of 1g in 10ml of water and a short residence time of 5 minutes could be used to gain full extraction, beneficial for reducing water and energy requirements of the system.

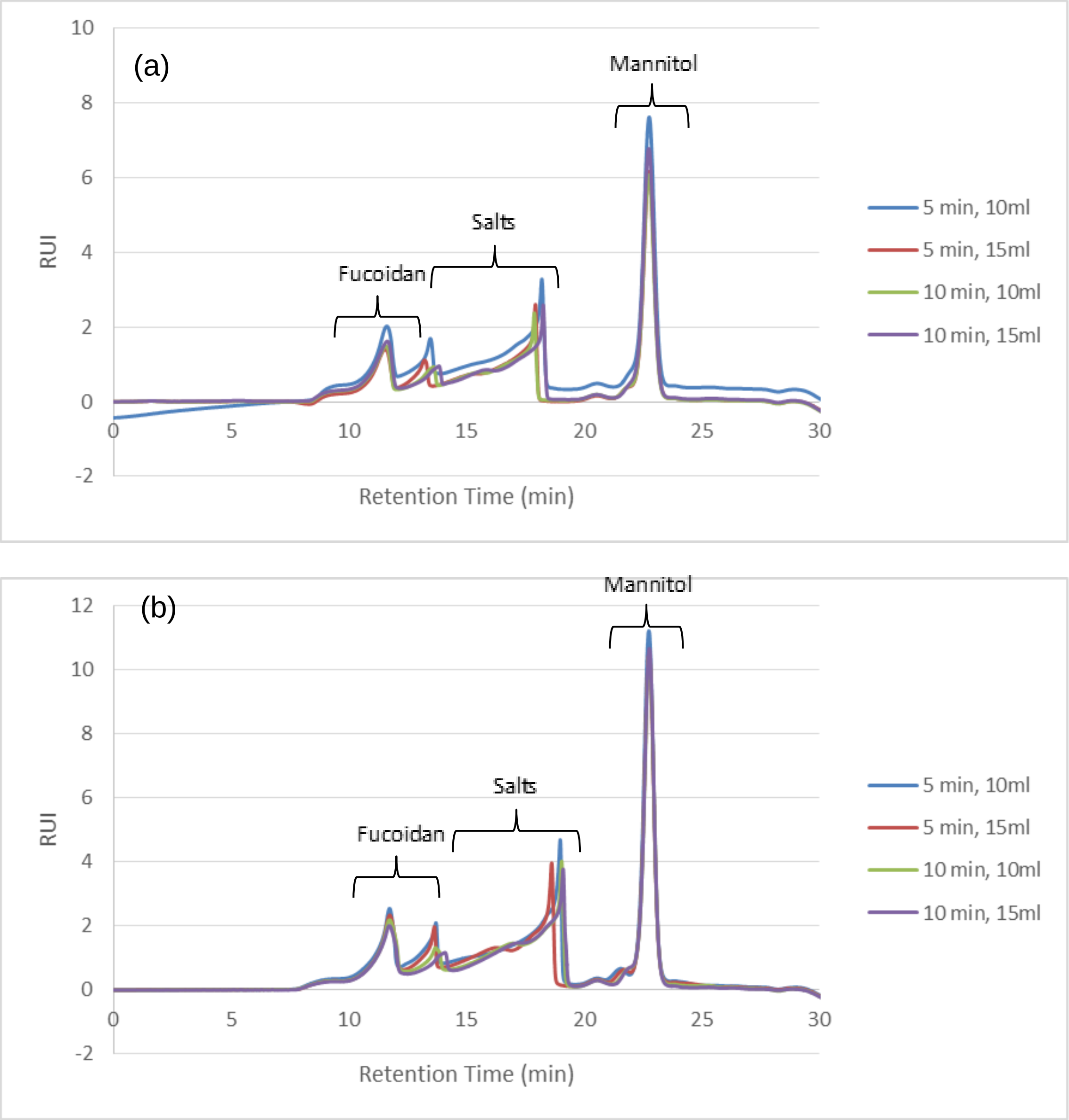


Figure 5.11: Comparison of different permutations of residence time and S:S ratio at 50°C for (a) Microwave and (b) conventional heating

Chromatograms comparing the four scenarios for S:S and residence time at 100°C are displayed in Figure 5.12. As with 50°C, there is only a small quantity of fucoidan and alginate extracted, with mannitol and laminarin extraction remaining similar throughout. There is also little variation between the different S:S and residence time scenarios.

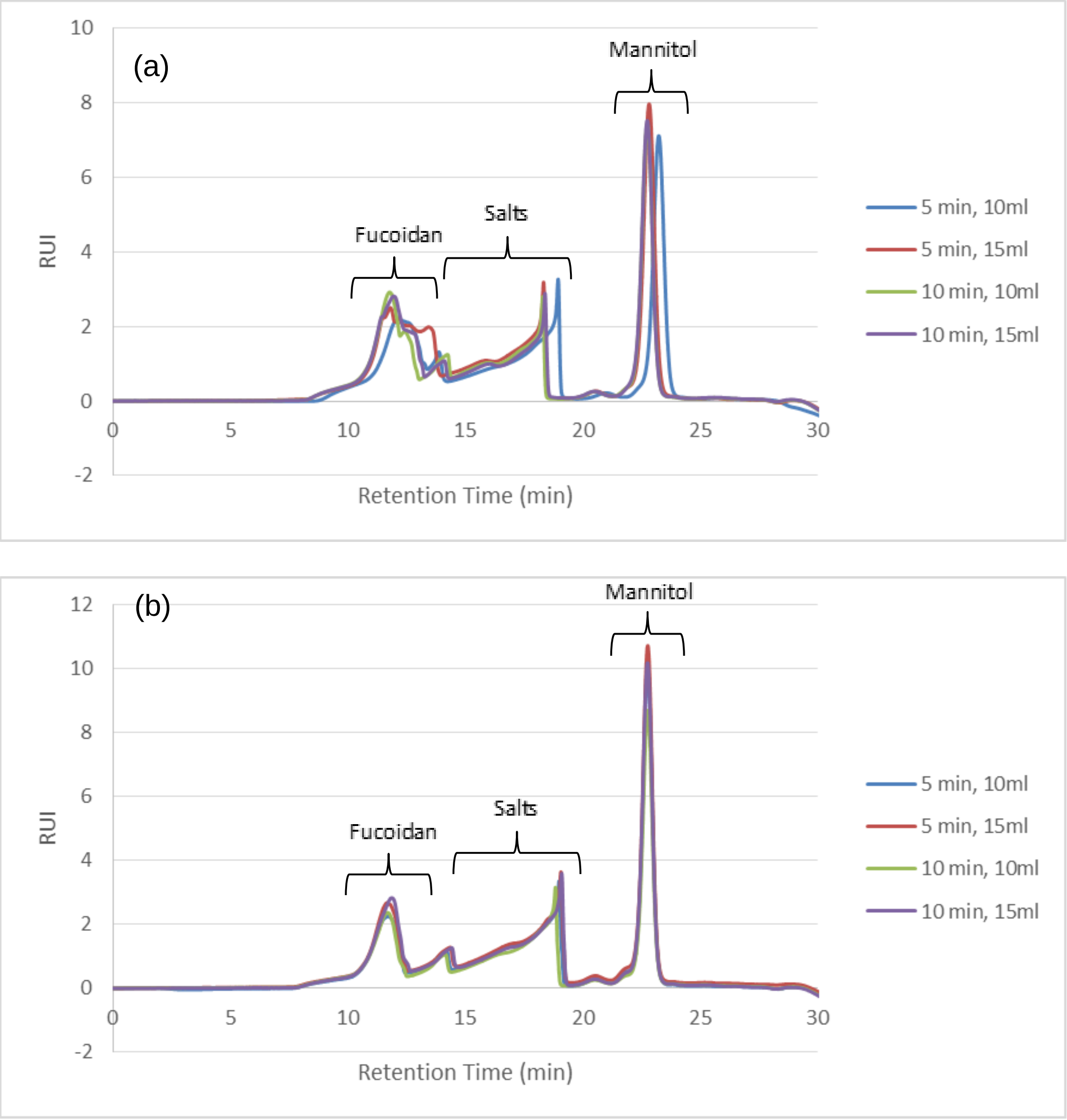


Figure 5.12: Comparison of different permutations of residence time and S:S ratio at 100°C for (a) microwave and (b) conventional heating

Figure 5.13 shows the comparison of conventional and microwave heating at 150°C for the 4 scenarios of S:S and residence time. The chromatograms for the four scenarios for conventional heating at this temperature appear to be relatively similar, with 5 minutes residence time having a slightly better extraction than 10 minutes, although S:S doesn’t seem to have an effect. This is likely due to an increase in residence time at this higher temperature causing fucoidan degradation to begin to occur.

In contrast to the 50°C and 100°C, the microwave heating chromatograms for 150°C do not closely match those of conventional heating. The peaks denoting fucoidan are significantly boarder and shorter for microwave heating, indicating an increase in the variation of MW and, therefore, breakdown of fucoidan. This is also seen to correspond with an increase in residence time and S:S ratio, with shorter, broader peaks occurring with an increase in these parameters. It is likely that this is due to the increase in microwave energy required to heat the extra solvent and hold at the temperature for longer causing more movement in the bonds in fucoidan and therefore more bond breakage. This variation in fucoidan peaks indicates that 150°C is the critical temperature for the assumed breakdown of fucoidan for microwave heating, while 150°C seems to be the optimum temperature for the extraction of fucoidan via conventional heating. To investigate the optimum extraction temperature for fucoidan extraction via microwave heating further, microwave heating runs at 120°C and 140°C have been carried out for all 4 scenarios.

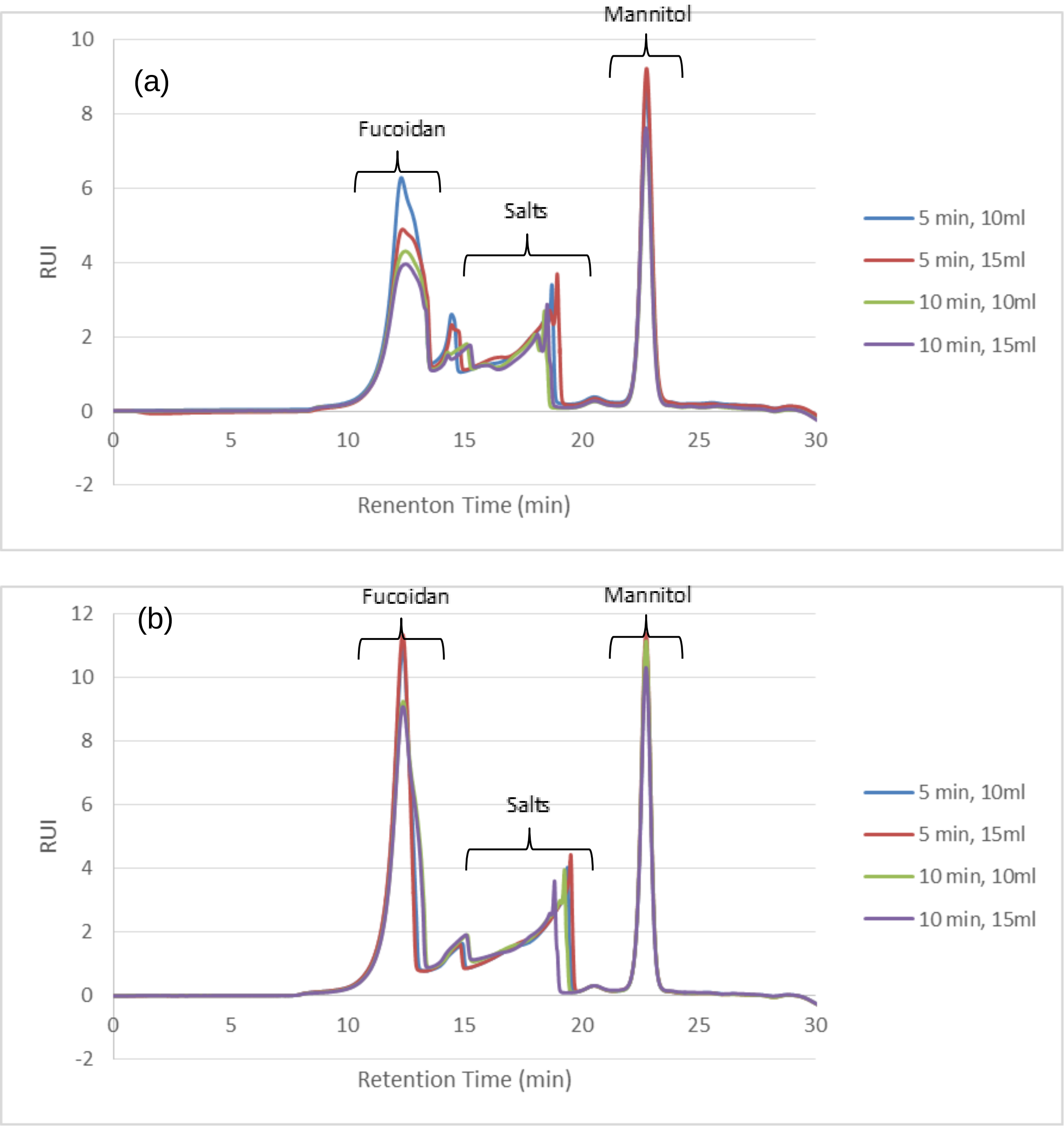


Figure 5.13: Comparison of different permutations of residence time and S:S ratio at 150°C for (a) microwave and (b) conventional heating

At 200°C, it is clear from the data in Figure 5.14 that there is a significant shift in MW of the extracted components. It is likely that this is due to breakdown of the carbohydrates into fragments and monomer units. This is explored further in section 5.3.2. However, the fucoidan peak seen at lower temperatures (at 12.2 minutes for the standard) is clearly missing for microwave heating and is significantly smaller for conventional heating. There is also extra peaks above this temperature, which are not present in the lower temperature extracts. These notably occur at 14, 18, 19 and 25 minutes and are likely to be accounted for by fucoidan and alginate fragments and monomer units. Temperatures above 200°C were not able to be performed, due to the limitations of the microwave vessels and their limit on pressure. However, it is likely that increasing the temperature would lead to an increase in hydrolysis of the long chain carbohydrates into their monomer units, resulting in an increase in low MW material and decrease in high MW material.

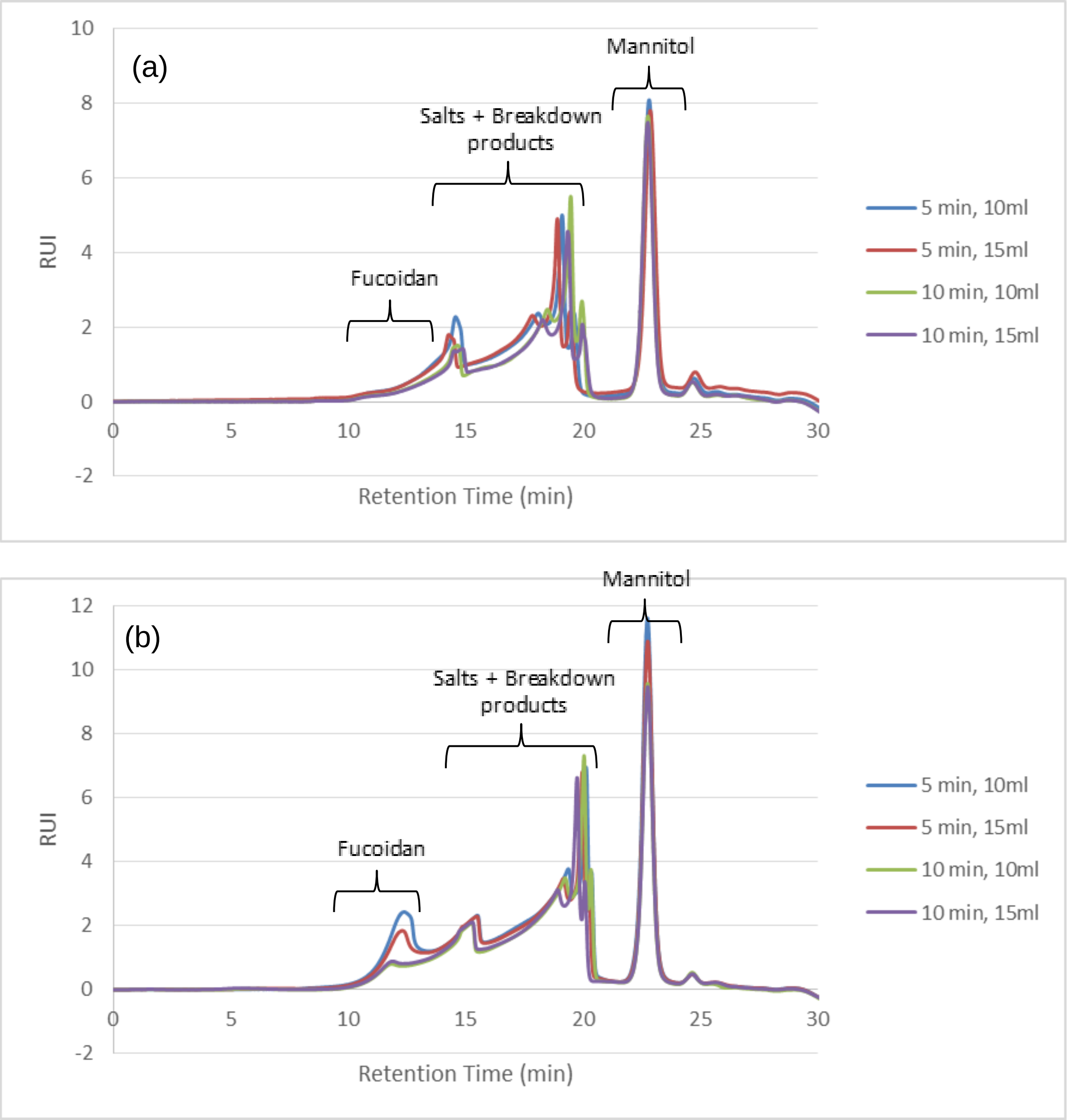


Figure 5.14: Comparison of different permutations of residence time and S:S ratio at 200°C for (a) microwave and (b) conventional heating

In order to determine the optimum extraction temperature for fucoidan from the raw biomass and to confirm the viability for good extraction of fucoidan from seaweed using microwave heating, further runs were performed at 120°C and 140°C. The results of these are shown in Figure 5.15. At 120°C (Figure 5.15(a)), there appears to be good extraction of fucoidan, giving a peak of similar width to the standard. In this case, a higher S:S ratio seems to give better extraction, with residence time seeming to have little effect on the quantity of fucoidan extracted. In all cases, mannitol and laminarin are seen to be sufficiently extracted and in a similar quantity consistent with that seen at other temperatures. As was seen with microwave extraction at 150°C (Figure 5.13(a)), there appears to be a broadening of the fucoidan peak, which increases with increasing residence time and S:S ratio at 140°C (Figure 5.15(b)). At 5 minutes residence time, both S:S ratios give relatively good extraction of fucoidan, with little broadening of the peak. However, an increase to 10 minutes residence time sees the beginning of the peak broadening, with 10 minutes residence time and 1g:15ml S:S ratio showing a significant broadening. This indicates that 140°C is the tipping point for breakdown of fucoidan for this sample of seaweed.

As the variation seems to depend more on 10 and 15ml of water, rather than residence time, so it was decided that this should be varied in continued experiments for the sequential extraction of seaweed, to ensure the best extraction yield is achieved.

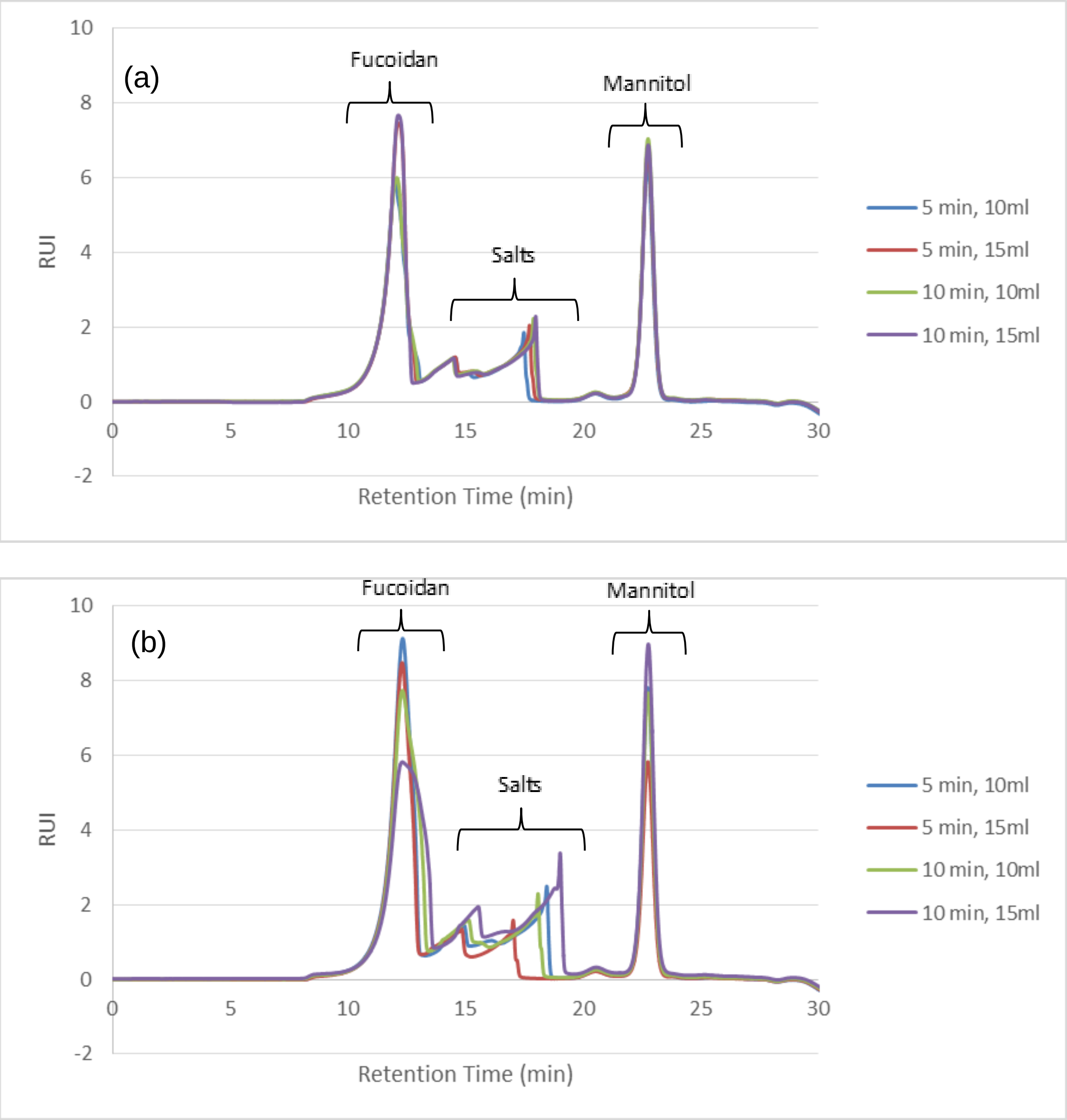


Figure 5.15: Comparison of different permutations of residence time and S:S for microwave heating at (a) 120 °C and (b) 140°C

5.3.1 Carbohydrate Analysis of the Microwave Extracts

The amount of fucoidan extracted for each scenario and temperature has been calculated from calibration of standards in SEC and the results are given in Figure 5.16. The overall trend for both microwave and conventional heating is an increase in the quantity extracted, up to a peak at 140°C and 150°C respectively, before dropping to zero at 200°C. As was seen in earlier figures, this drop at 200°C is likely due to fucoidan breakdown. The values above 100% are most likely due to the breakdown of fucoidan, causing a broader peak due to the wider range of MW. The distorted peaks do not match the shape of that for the standard and, as RI detectors detect the refracted light caused by the compound being eluted, more, smaller fractions of fucoidan will refract the light more than samples of lower polydispersity, causing an increase in the fucoidan detected, although the same mass is present. With this in mind and considering the results in this figure, it is likely that there is some breakdown of fucoidan at 150°C in conventional extraction, indicating that a slightly lower temperature may give better extraction of “whole” fucoidan.

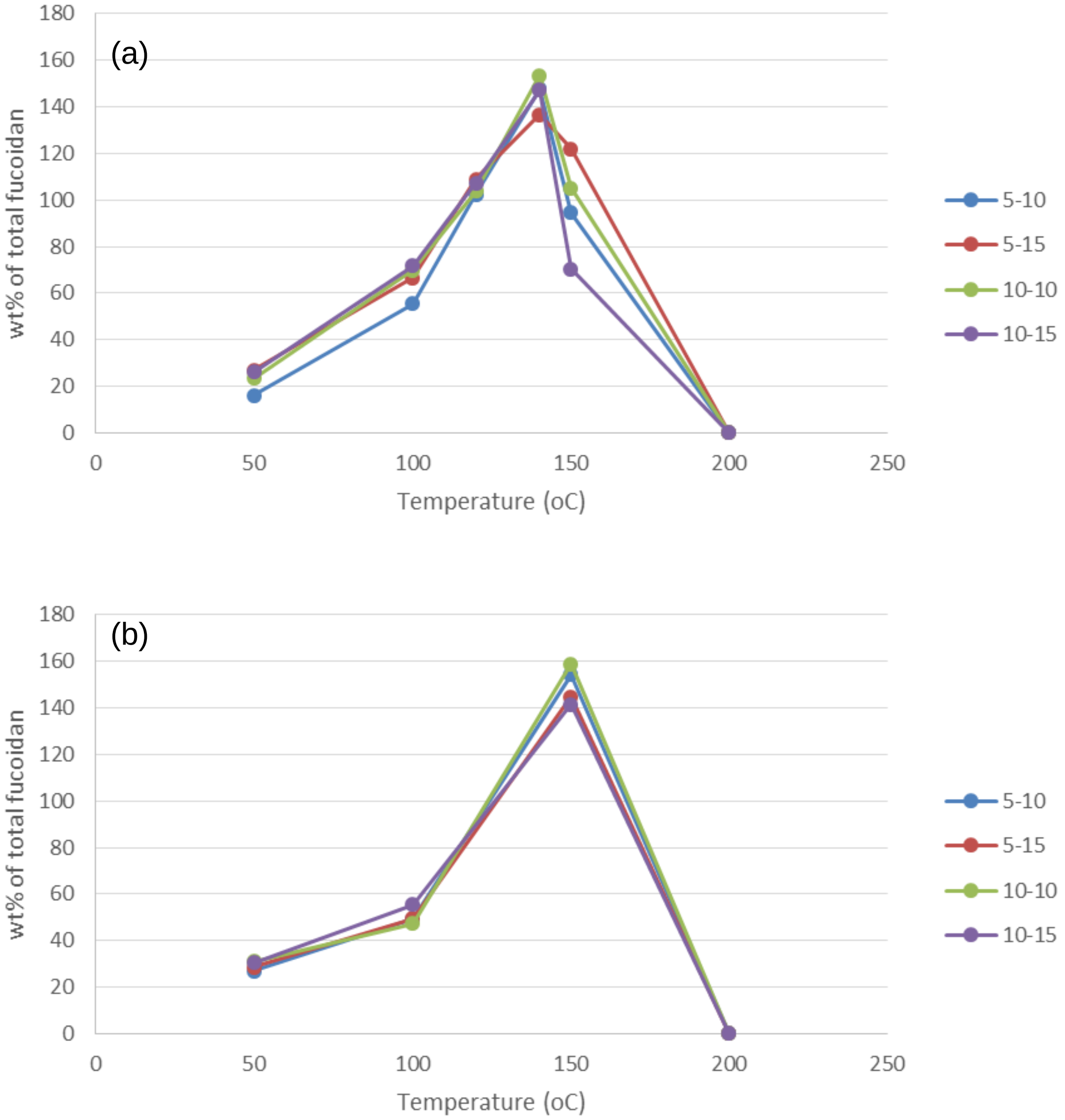


Figure 5.16: Amount of fucoidan extracted given as a percentage of the total fucoidan present in the sample. Where (a) is microwave heating and (b) conventional heating.

The quantity of laminarin extracted has also been calculated from the SEC chromatograms, with the results being shown in Figure 5.17. The general trend is for high extraction of laminarin at lower temperatures, which reduces with increasing temperature. Both conventional and microwave heating see similar trends and quantity of laminarin extracted at each temperature, although there appears to be more variation in the extraction quantity for microwave heating. As with fucoidan, the quantity of laminarin is seen to drop to almost zero at 200°C.

Again, this is likely to be due to the breakdown of laminarin, which would show a peak at a different MW, shifting the MW distribution.

Only a portion of the total laminarin is extracted, with a maximum of 6.9 wt% of total laminarin at 50°C for microwave heating and 6.4 wt% at 50°C for conventional heating across all temperatures. It has been shown previously [216] that laminarin is present in two forms in seaweed: soluble and insoluble. These results suggest that the insoluble form is responsible for a greater proportion of the total laminarin than the soluble form, which seems to be almost fully extracted at 50°C. It is likely that further extraction in water would not yield any further extraction of laminarin.

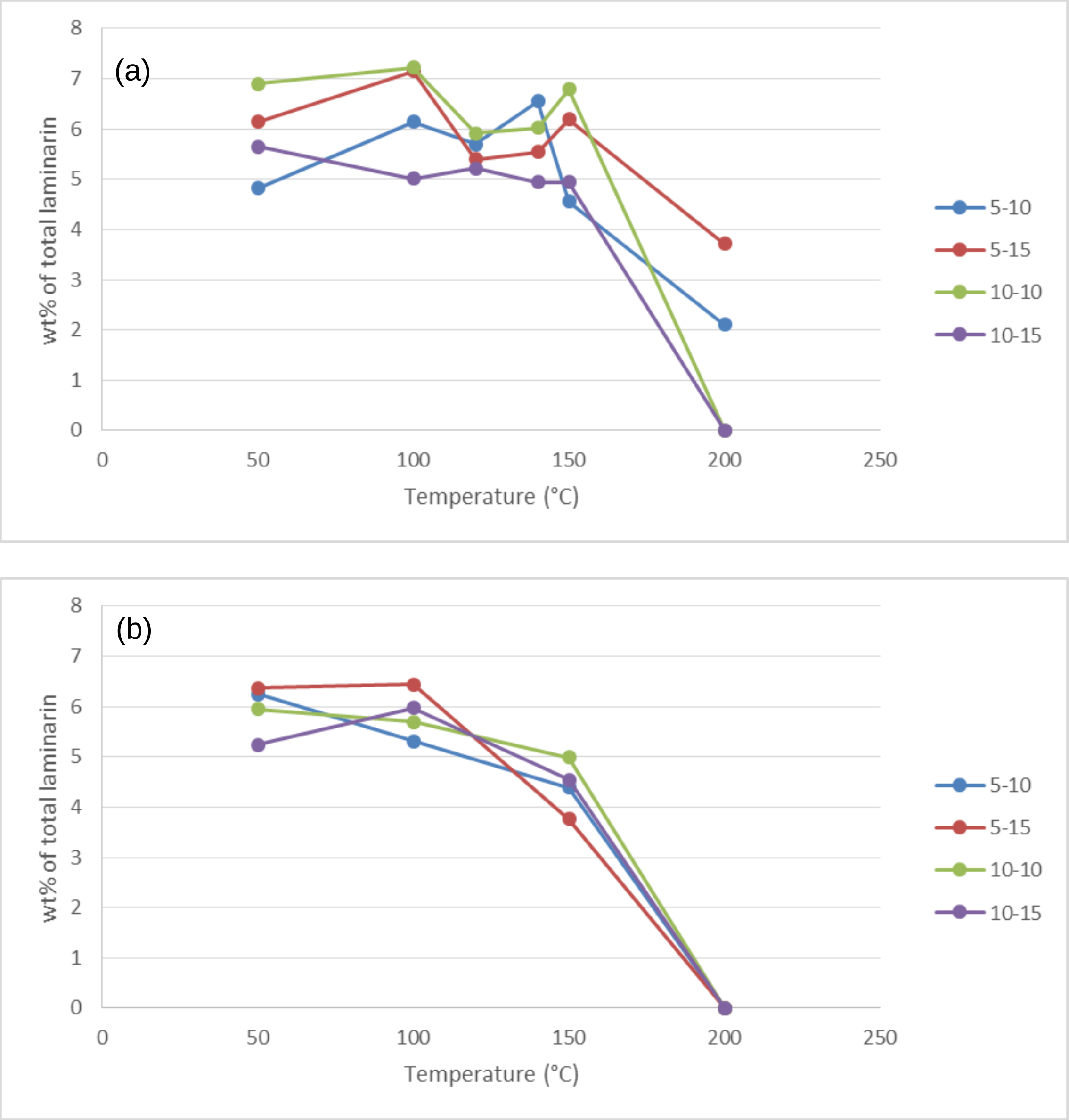


Figure 5.17: Amount of laminarin extracted in (a) microwave and (b) conventional heating over a range of temperatures, S:S ratios and residence times.

5.3.2 Sugar Analysis of the Microwave Extracts

The results for mannitol are shown in Figure 5.18 as a wt% of total mannitol. The extraction of mannitol is relatively consistent over the temperatures, residence times and S:S ratios performed, with the exception of 50°C for microwave heating. Under these conditions, it seems that 1g:15ml of water gives better extraction than the lower S:S ratio.

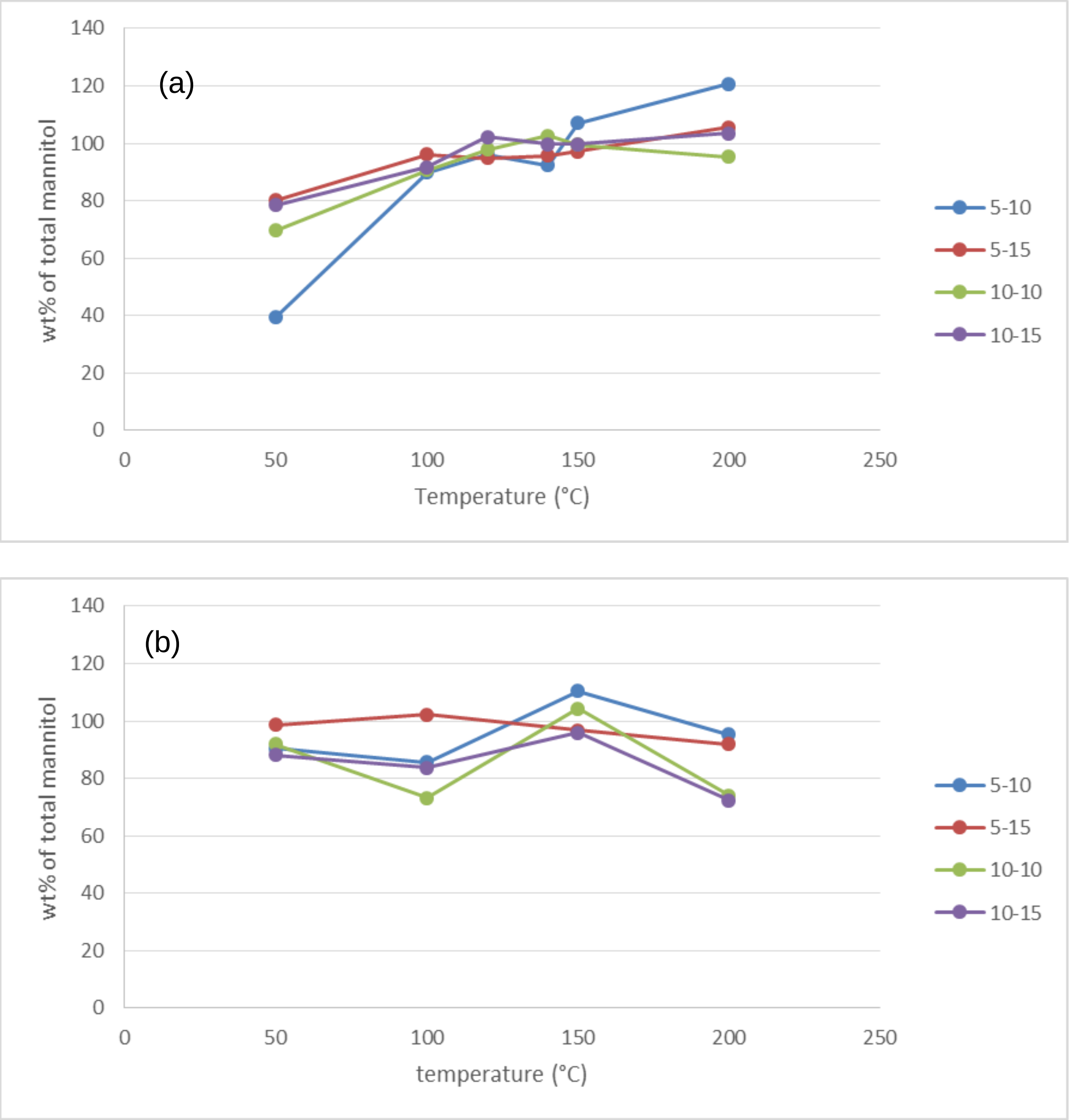


Figure 5.18: Amount of mannitol extracted in (a) microwave and (b) conventional heating for a range of temperatures S:S ratios and residence times

Figure 5.19 shows the glucose content in each of the extracts. For microwave heating, the quantity of glucose increases to a peak a 150°C, before dropping to zero at 200°C. for conventional heating the quantity of glucose increases throughout for 10 minutes residence time, but reaches a peak at 150°C and begins to reduce at 200°C for 5 minutes residence time. The small quantity of glucose present in the 50°C extracts for both conventional and microwave heating is most likely due to free glucose present in the seaweed. The increase is likely due to the breakdown of laminarin into its monomer units, and corresponds to the drop in laminarin extracted with temperature seen in Figure 5.16. The decrease after 150°C is likely to be due to the carbonisation of glucose, as higher temperatures “burn” the sugar, meaning it is no longer identifiable as glucose on the HPLC.

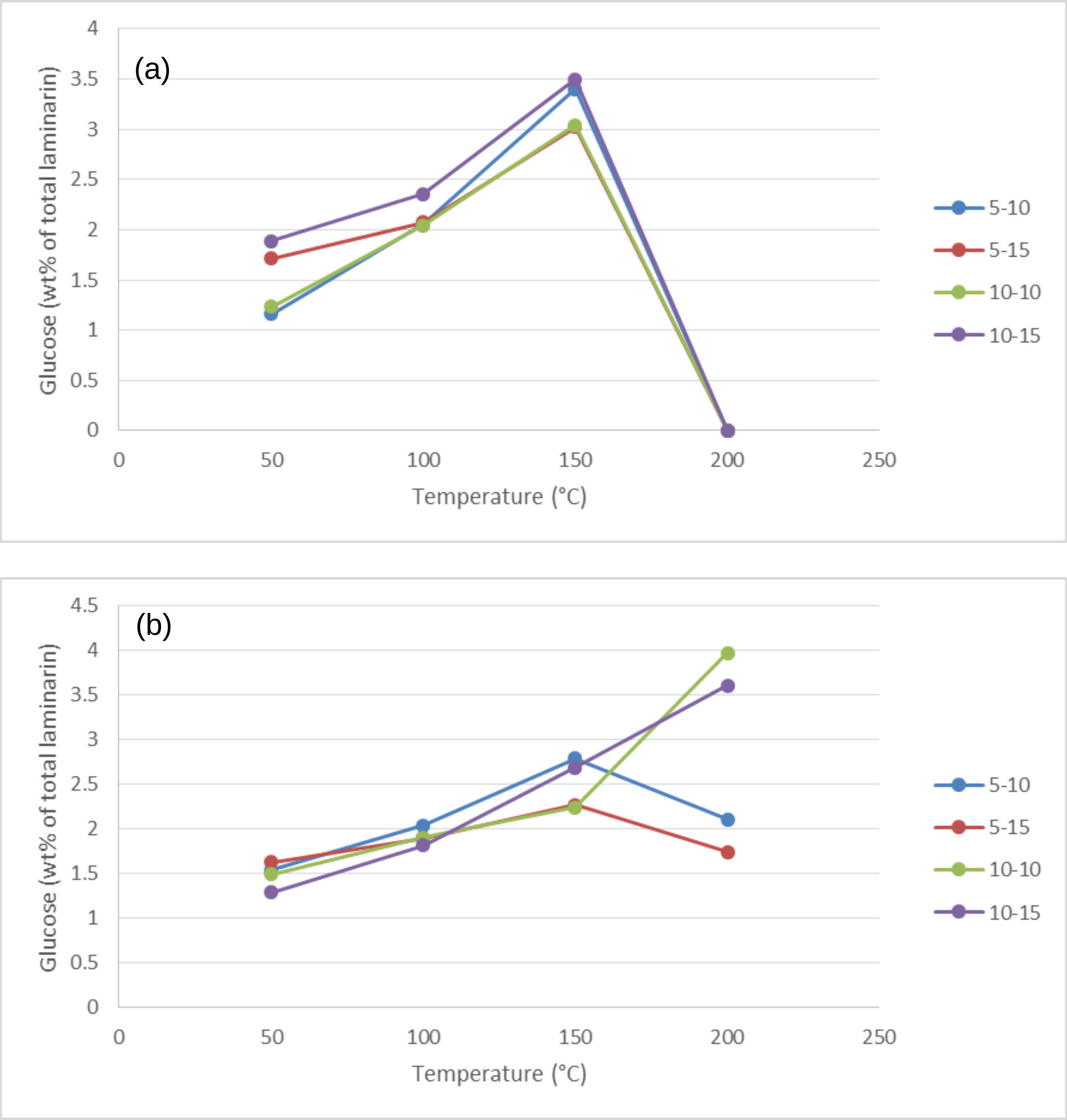


Figure 5.19: Amount of glucose in extracts from (a) microwave and (b) conventional heating for permutations of temperature, S:S ratio and residence time.

The difference between microwave and conventional heating is likely due to microwaves having more of an effect on the glycosidic bonds present in laminarin, meaning that breakdown occurs at a lower temperature, as more microwave energy is inputted. Where conventional heating adds energy to the system from an external source as a whole via convection, microwaves heat by direct action on the polar elements of the system, aligning and rotating charges as the waves are applied. As oxygen is strongly electronegative, it pulls electrons from the surrounding elements towards it, creating a slightly negative charge, as shown in Figure 5.20. As the microwaves act on polar regions, stress is directly applied to the bonds between glucose residues in laminarin, meaning they are likely to break at a lower temperature under microwave heating as opposed to conventional heating. For microwave heating, the tipping point for laminarin breakdown is between 150 and 200°C, whereas for conventional heating, the results presented here seem to suggest that the tipping point is 200°C.

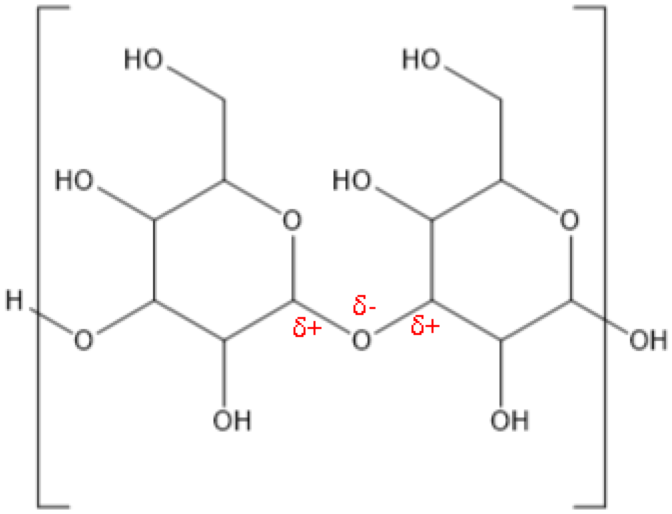


Figure 5.20: Structure of laminarin showing the charges associated with the glyosidic bonds.

The fucose content in the microwave samples is zero below 120°C, above which it increased up to a maximum at 150°C, before falling to zero again at 200°C, as seen in Figure 5.21(a). For conventional heating, fucose is zero up to 150°C for 5 minutes residence time, after which it increases up to 200°C. For 10 minutes residence time, fucose is zero up to 100°C, rising to a maximum at 150°C before falling to zero again at 200°C, as shown in Figure 5.21(b). These trends follow the fucoidan extraction pattern seen in Figure 5.16. This is likely due to fucoidan being broken down at higher temperatures: above 120°C for microwave and above 100/150°C for conventional heating, in part into its monomer units. It seems, as was seen with laminarin, that fucoidan begins to break down at a lower temperature in the microwave than for conventional heating. In both cases, an increase in residence time and S:S ratio appears to increase the degradation of fucoidan, with the highest S:S ratio coupled with the longest residence time giving the greatest amount of fucose in microwave heating, at 5wt% of the total fucoidan at 150°C. It is hypothesised that a greater quantity of fucose would be seen if processing were to be performed at temperatures between 150 and 200°C for microwave heating, at temperatures above 200°C for 5 minute residence time for conventional heating and between 150 and 200°C for 10 minutes residence time and conventional heating. As with laminarin, it is likely that the quantity of fucose drops to zero about 200°C due to the sugars being “burnt” in the reactor and, therefore, no longer react with the HPLC column in the same way and are eluted at a different time.

There is a relatively small amount of fucose in the extracts: a maximum of approximately 6wt% of the total fucose in the raw biomass in each case. It is likely that the majority of fucoidan degrades into fragments of lower MW, rather than into the monomer units, and is evidenced by the spread of molecular weight fragment peaks, which can be seen for the 200°C samples in Figure 5.14. Although the break down to monomer units is small, the trend seen is likely to be similar for the low molecular weight fractions and also is measureable evidence of the breakdown of fucoidan.

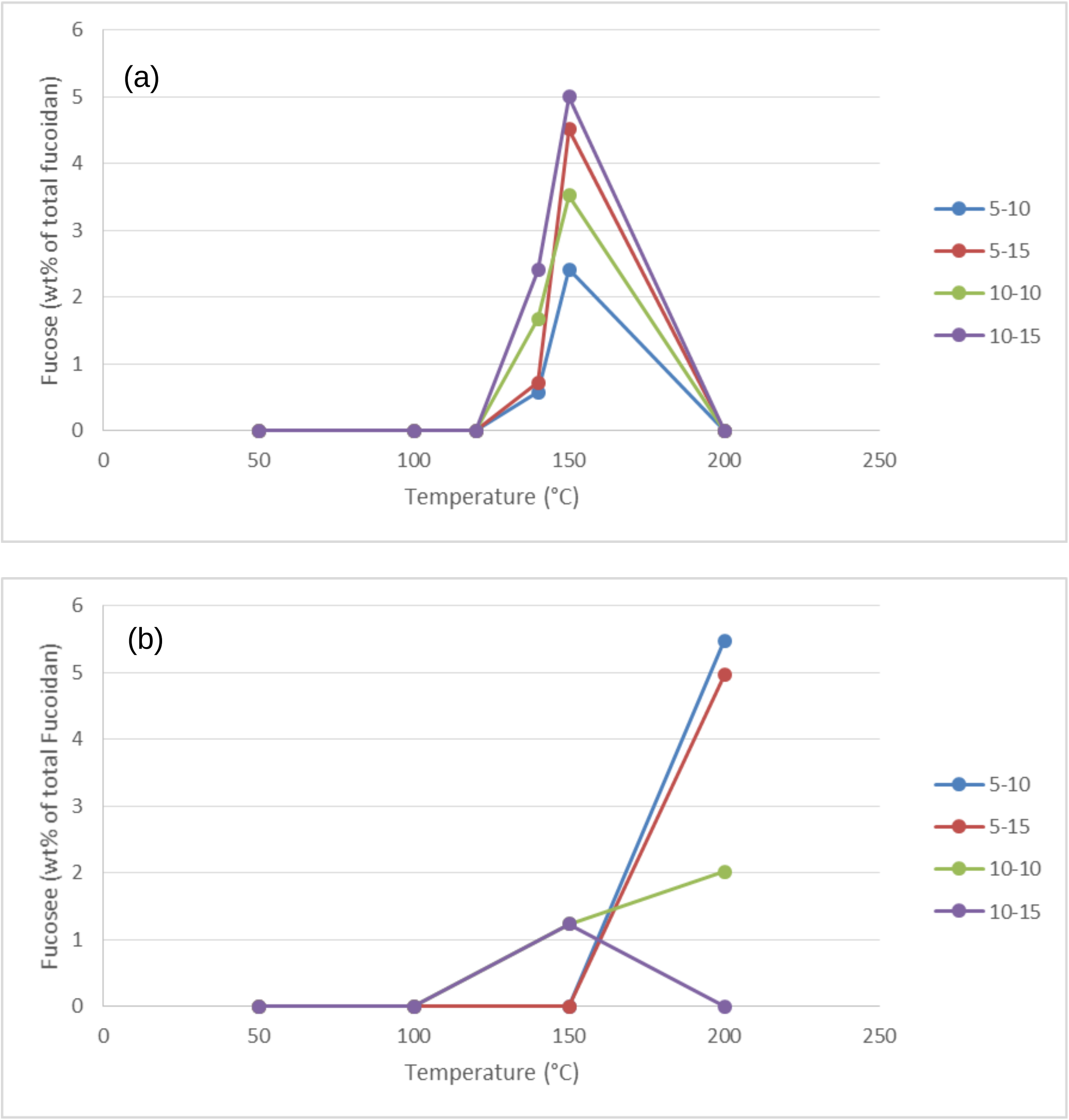


Figure 5.21: Amount of fucose in extracts from (a) microwave and (b) conventional heating for permutations of temperature, S:S ratio and residence time.

The quantity of uronic acids, which in this study is used as the term to denote guluronic and manuronic acids, the monomer units which makeup alginate, are seen increase at temperatures above 150°C for both microwave and conventional heating. However, the quantity is much higher at 200°C for the former: a maximum of 21.3wt% of the total alginate compared to 7.7wt% for conventional heating. This indicated significant breakdown of alginate above 150°C. As before, the more pronounced increase in uronic acids for microwave heating indicates that this method promotes hydrolysis of alginate at a lower temperature when compared to conventional heating.

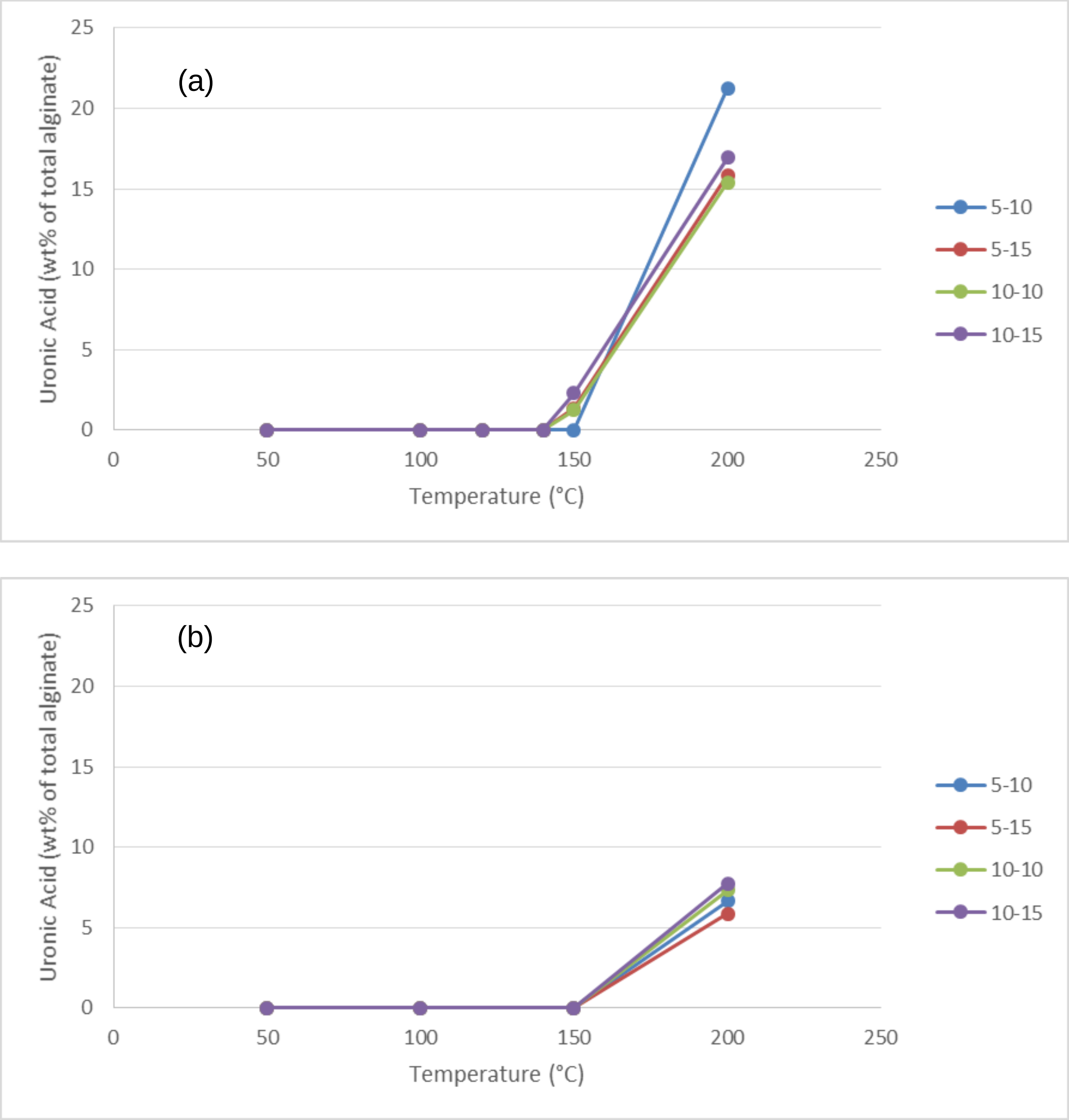


Figure 5.22: Amount of uronic acid in extracts from (a) microwave and (b) conventional heating for permutations of temperature, S:S ratio and residence time

5.4 Conclusion

From this study, it seems that extraction at 120°C for 5 minutes and 1g:15ml of water gives the best extraction of fucoidan for microwave heating, while for conventional heating, 150°C for 5 minutes and 1g:15ml of water are the optimum conditions. This shift in optimum temperature is likely to be due to the difference in how microwaves heat the biomass, as conventional heating uses convection from an external heat source to heat from the outside in, while the changing electromagnetic field created by microwaves aligns and then switches the dipoles in polar molecules present in the biomass, causing friction and, therefore, heat. Furthermore, as the sample of FS used contains a high quantity of salts, which have a high polarity, less energy is required to heat the sample. The result of this is that a lower temperature and less energy is required to extract the same quantity of fucoidan for microwave heating when compared to conventional.

Due to the reduction of salts in the pre-extraction step, the optimum temperature for extraction in a sequential extraction setting is likely to change, especially in the microwave, due to the high salt content of the sample. The quantity of salts extracted in the pretreatment steps and the effect this has on continued extraction is explored further in section 5.4, but, due to the likelihood that the second microwave step will require a different temperature to achieve the same extraction due to reduced salts, experiments to determine this temperature have been undertaken.

This study has also shown that the optimum conditions for pretreatment of seaweed for a biorefinery would be 50°C for 5 minutes with 1g:15ml of water. This gives a high extraction of mannitol and laminarin, while minimising the amount of fucoidan and alginate extracted.

6 Development of Sequential Extraction of Compounds on Seaweeds

6.1 Introduction

The use of macroalgal biomass as a feedstock for biofuel production has been widely cited in the literature, with bioethanol, bio-methane and bio-butanol among the end products that can be produced [120] [176]. However, for seaweed biomass be to a viable option for fuel production, which is a relatively low value product, co-extraction of high value chemicals within a biorefinery-type setting would be necessary. As well as improving the economics of the process, biorefineries also minimise waste and make use of the whole biomass resource, gaining maximum potential products from the cultivation area required. Extraction of several chemicals/products from one resource also reduces total GHG emissions, when compared to extracting only one product from the biomass. The use of microwaves has also been reported to reduce the energy and solvent requirements, further improving the environmental impact of the process. While a seaweed biorefinery is not a new concept [38, 125, 126, 129] and the use of microwaves in this setting has been cited once before in the literature [162], this is the first study with a view to minimising solvents, extraction times and liquid loading in the reactors to reduce the environmental and energy costs of the process. Moreover, the study presented by Yuan et al [162] uses HCl as an extraction solvent. This has been shown in previous studies to have a degradative effect on fucoidan, reducing its degree of sulphation and hydrolysing the polymer into smaller chained fragments [164]. This means that both the quality and the potential quantity of fucoidan extracted will be reduced, as lower extraction temperatures will be required to maintain the quality of the product. However, the lower temperature will potentially reduce the quantity of fucoidan which is able to be extracted. This has been shown to be the case in their connected study, which shows the quality of fucoidan is compromised under the extraction conditions optimum for full extraction [163].

The study in this chapter builds on the feasibility and comparison of microwave and conventional heating presented in chapter 5, developing biorefinery using microwave heating. The method proposed in this study attempts to utilise the seaweed biomass, specifically *Fucus serratus* (FS), to its full potential by extracting fucoidan and alginate as relatively pure products and explores the possibility of using the waste as a feedstock for bioethanol and anaerobic digestion (AD) production and for direct use as a fertiliser. As far as possible, the use of solvents and extraction chemicals has been minimised, with water being used for extraction as much as possible and the recycling of solvents required considered, when water was not found to be suitable. This would reduce both the cost of the process, but also the environmental impact associated with production of these chemicals. Furthermore, it is hoped that by using water as the extraction medium, the quality of the fucoidan can be maintained while extracting under the optimum conditions to give the best yield.

A sequential extraction of chemicals, based on the solubility of components with increasing microwave temperature, has been developed for a June sample of FS. Furthermore, the quality of the fucoidan extracted has been evaluated and compared to that extracted by the conventional method. Mass and energy balances for the system have been completed, comparing the results for if microwave or conventional heating were to be used to determine potential energy savings. Finally, utilisation of the waste has been considered, exploring its potential for bioethanol or bio-methane production and its use as a fertiliser.

6.2 Biorefinery Schematic

The feasibility of sequential extraction of seaweed using microwaves has been proven in Chapter 5, with optimum extraction conditions for raw seaweed biomass being assessed. In this Chapter, the full biorefinery schematic has been developed, building on the initial feasibility study of the previous section. For reference, Figure 6.1 gives an overview of the three main steps of the final biorefinery process, highlighting each stage and the conditions required for extraction. In brief, the process is composed of a pretreatment step at 50°C in water, removing mannitol, soluble laminarin and roughly half the metals. The second step, to extract fucoidan, is performed at 120°C with water as a solvent. The resulting extract contains both fucoidan and alginate, the latter of which is precipitated by the addition of $CaCl_2$ before the former is precipitated via the addition of ethanol, giving crude fucoidan in solid form. In the third step, the residue from the second step is treated further with NaCl at 120°C in the microwave. The addition of sodium ions turns insoluble Ca-alginate to its soluble, Na form, thereby dissolving it to form a relatively pure extract. The residue left from the third step combined with the first step extract is then considered for bioethanol and bio-methane production and for use as a fertiliser. The development and results leading to these steps are given in the following sections.

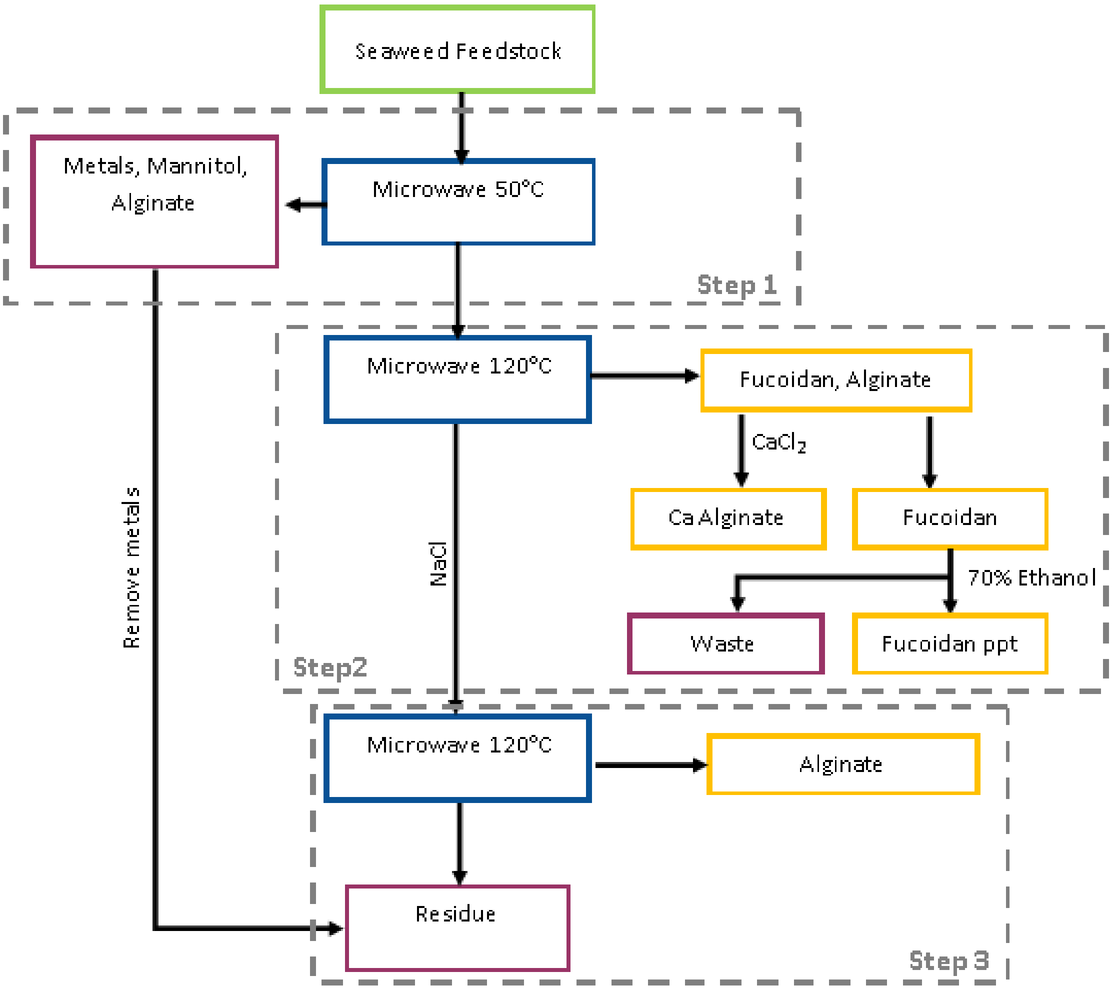


Figure 6.1: Proposed biorefinery schematic for the sequential, microwave extraction of chemicals from seaweed

6.2.1 First step – Pretreatment of the Seaweed Biomass

For the first step, the raw seaweed biomass is pre-treated, using the results from Chapter 5. 1g of seaweed is mixed with 15ml of distilled water and processed in the microwave at 50°C for 5 minutes. The SEC chromatogram for this step is shown in Figure 6.2. This clearly shows only a small peak for fucoidan at 12.2 minutes, while the mannitol and laminarin peaks show good extraction, with 90 wt% and 30 wt% of the total mannitol and laminarin present respectively being extracted in this initial step. It is likely that this represents the total extraction of the soluble form of laminarin present in the sample, as this is readily soluble at room temperature. The remaining laminarin is, therefore, likely to be mostly comprised of the soluble form.

The results from 50°C extraction have been compared to a room temperature extraction under the same conditions, in order to ascertain if the elevated temperature aided in extraction. As Figure 6.2 shows, a similar quantity of fucoidan, alginate and laminarin is extracted, but significantly less mannitol is removed. As the aim of this step is to remove both mannitol and metals, it is clear that processing at 50°C gives better pretreatment results that processing at room temperature.

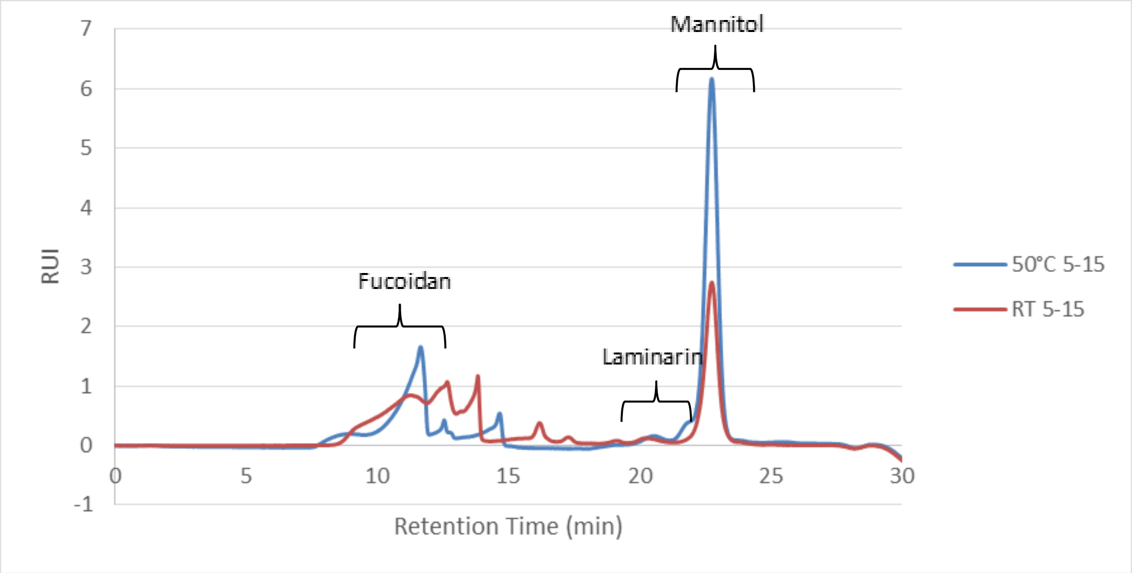


Figure 6.2: SEC chromatogram of the first step extraction at 50°C, 5 minutes residence time and S:S ratio of 1:15 compared with room temperature extraction.

The quantity of mannitol extracted under the 4 scenarios of S:S ratio and residence time used in Chapter 5 are given in Figure 6.3. As the main aim of this step is to remove mannitol and salts, 5 minutes residence time and a S:S ratio of 1g:15ml of water has been chosen, as this gives the best extraction at 80% of the total mannitol in the raw biomass. 1g:10ml results in a significantly reduced extraction of mannitol and, while giving a similar result to the chosen conditions, 10 minutes residence time and 1g:15ml S:S ratio was not chosen due to the higher energy requirements needed for longer processing time.

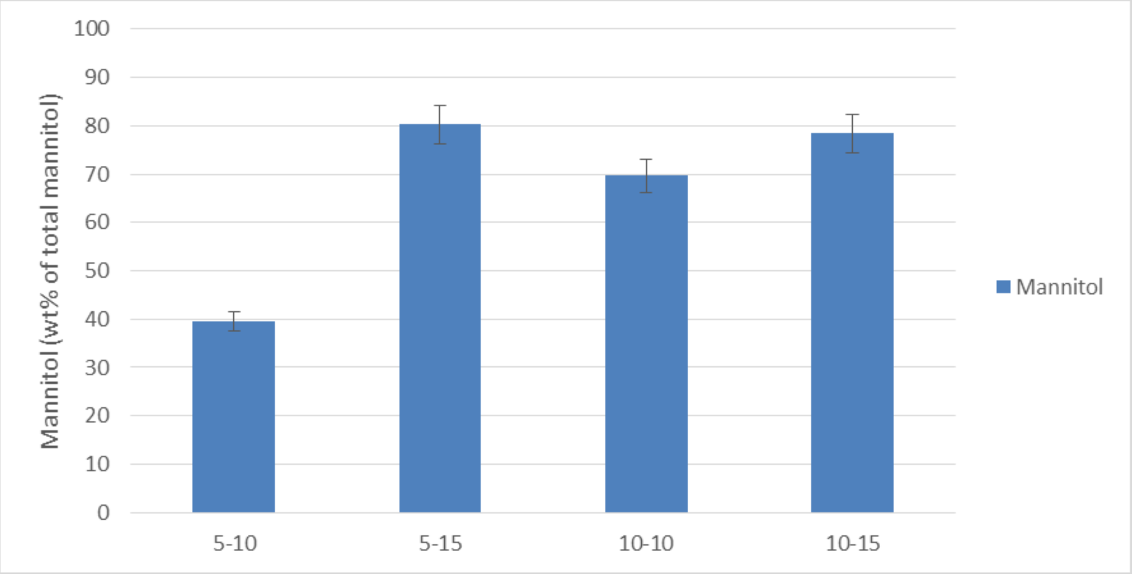


Figure 6.3: Mannitol extracted in the four scenarios of S:S and residence time for 50°C

The metal composition of the residue and extract has been assessed and the results are shown in Figure 6.4 (full numerical data is given in the Appendix in Figure A.4). Roughly half of all metals are extracted in this step, which are dominated by Na, K, Ca and Mg. Roughly half of Na and K are extracted into the liquid phase, with roughly two thirds of Ca, Mg, Cl and P from the feedstock remaining in the residue in each case. A significantly smaller portion of the “other” micro-metals, predominantly Sr, Br Fe and Al, are extracted into the liquid phase. Metals make up 21.8 wt% of the extract and 11.5 wt% of the residue. This means that, although a significant portion of the metals have been removed, their ratio in the residue for further processing is relatively similar to that of the raw seaweed feedstock, which contains 13.2 wt% metals. It is likely, therefore, that there will not be a large difference made in the processing temperature of the microwave in the second step processing, as the effect of metals on the heating will be similar. However, there may still be differences in the heating due to the difference in the composition of the biomass, as the carbohydrates and other compounds present contain polar elements.

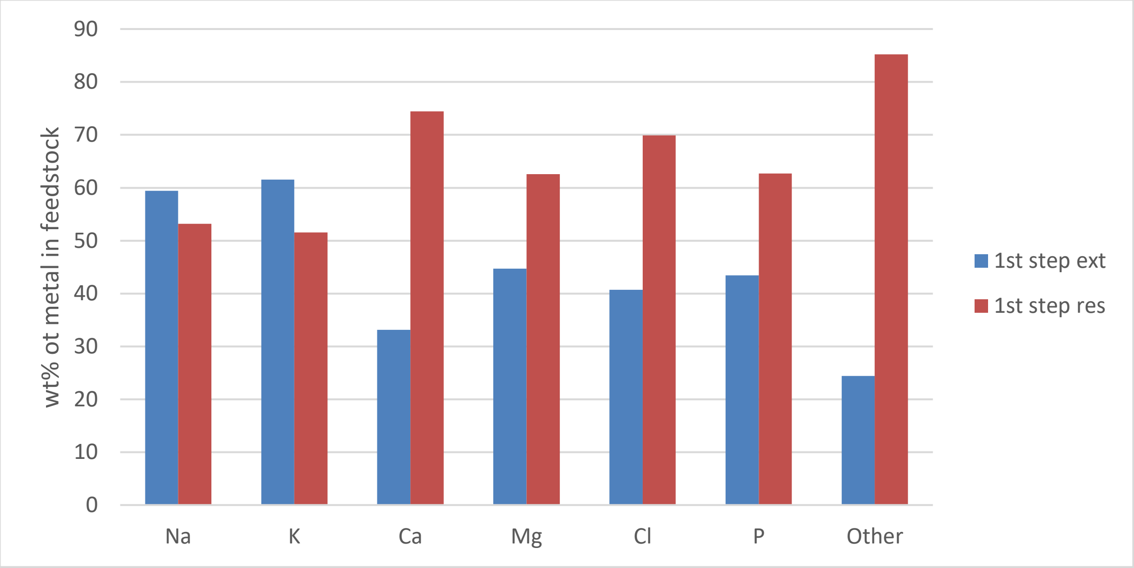


Figure 6.4: Metal composition of extract and residue from first step, 50°C microwave extraction.

Once the best conditions for pretreatment of the seaweed biomass had been found, a large volume was generated using the carousel in the microwave. This was tested fully for composition (see results in section 6.3.1) and was carried forward to the second step experiments.

6.2.2 Second step – Extraction of Fucoidan

The second extraction step is intended to remove fucoidan, although, based on the results in Chapter 5, it seems likely that alginate will also be extracted in this step. Conditions for water based extraction have been optimised, with further trials using $CaCl_2$ as a solvent, to ascertain if it would be possible to render soluble Na-alginate into its insoluble Ca form in the same step as extracting fucoidan, thereby eliminating the need to remove alginate from the extract. Figure 6.5 shows the composition of the feedstock for this step, which is the residue from first step processing at 50°C. Approximately 6 wt% of the feedstock to this step is laminarin, while 25 wt% is alginate. Very little of the “others” portion is extracted in the first step, meaning that there are still a large quantity of protein, pigments and lipids in the feedstock for this step.

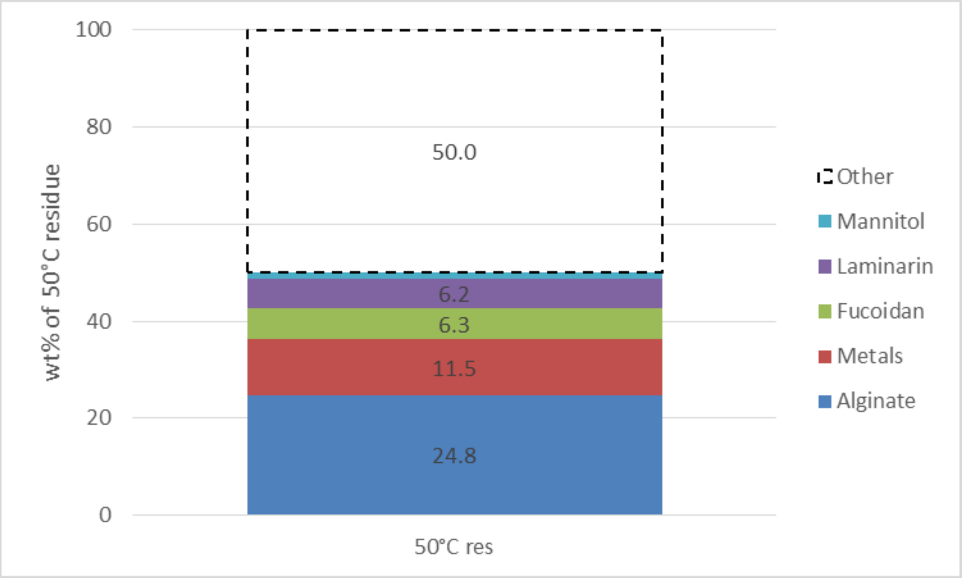


Figure 6.5: Composition of first step residue, processed at 50°C.

6.2.2.1 Microwave extraction with water

The extraction of fucoidan from the first step residue was performed first using water. A residence time of 5 minutes was used for all samples, as it was shown in Chapter 5 that longer residence times made little difference to extraction, and minimising residence time means less energy required. Temperatures between 60°C and 160°C were used and the S:S ratios of 1g:10ml and 1g:15ml were also tried. These temperatures were chosen to give a full overview of the extraction now a large portion of the salts and other compounds have been removed. The two S:S ratios were chosen as it was shown in Chapter 5 that differing the S:S ratio had an effect on the extraction of fucoidan, especially at critical temperatures, so in order to rule out any better extraction at lower temperature with a higher S:S ratio, both have been used in these experiments.

Images of the extracts and freeze dried residues from second step microwave processing with water are shown in Figure 6.6. As with the extracts and residues from processing of raw seaweed biomass in Chapter 5, there is in an appreciable colour change with varying processing temperature. For the extracts, increase in temperature leads to a darkening in colour, from pale yellow to dark brown, up to 140°C, after which the colour pales slightly to a pale brown. As was seen in the raw biomass extracts, the viscosity of the second step extracts was observed to decrease with increasing temperature. Residues, which start at a mid-green at 60°C, become progressively darker and more brown with increasing temperature. This is likely due to both the removal of pigments and also the beginnings of carbonisation occurring at the highest temperature of 160°C.

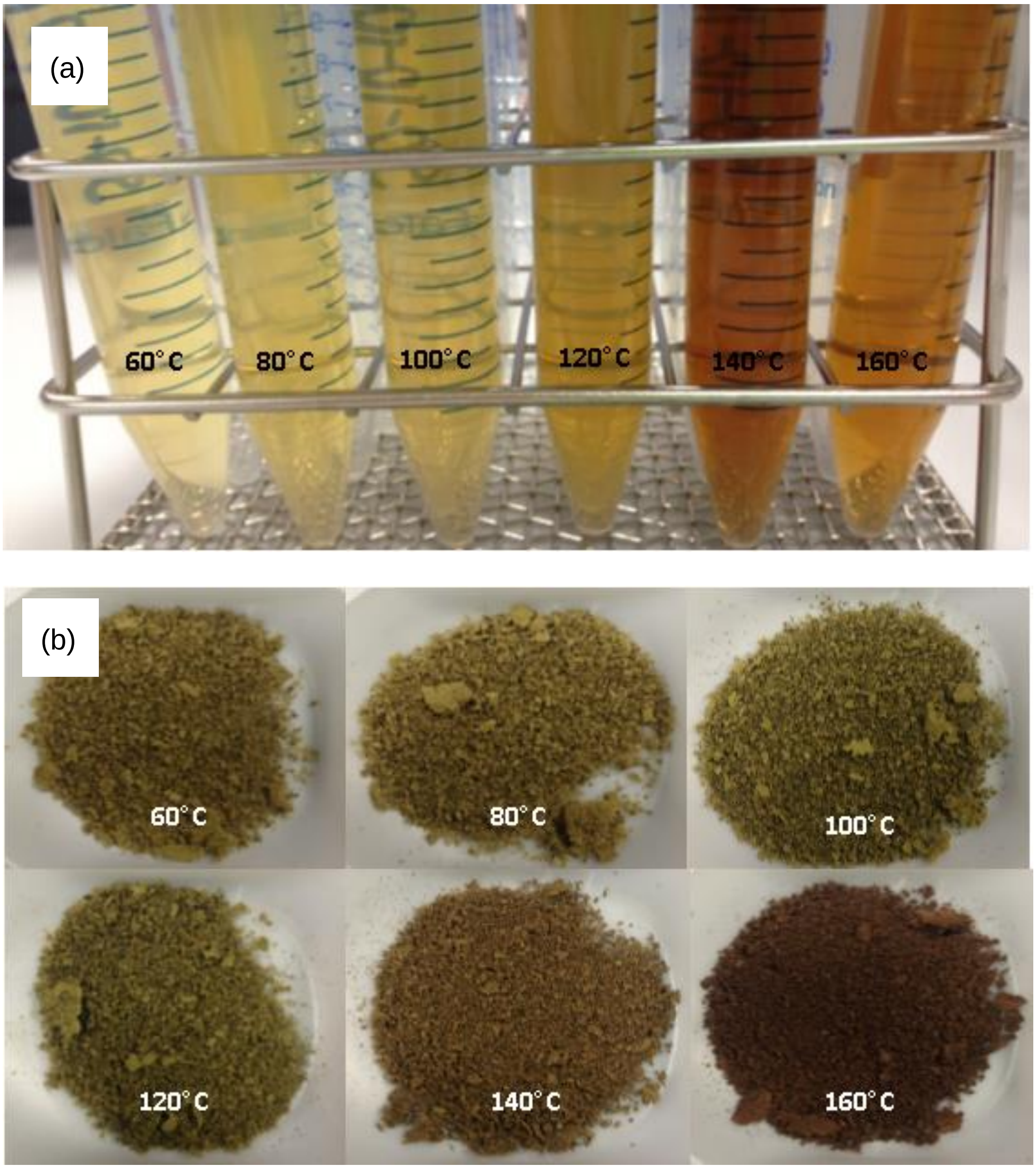


Figure 6.6: Images of (a) extracts and (b) residues from second step microwave extraction for S:S of 1g:10ml

The results for the comparison of SEC chromatograms of S:S 1g:10ml and 1g:15ml for 60°C, 80°C, 100°C and 160°C are shown in Figure 6.7. For these four temperatures, there is little difference between the two S:S ratios. Between 60 and 100°C, the quantity of fucoidan extracted increases with increasing temperature, with processing at 160°C showing a significant shift in the MW profile, suggesting the breakdown of fucoidan and/or alginate is occurring. These results follow a similar trend to those seen previously in Chapter 5.

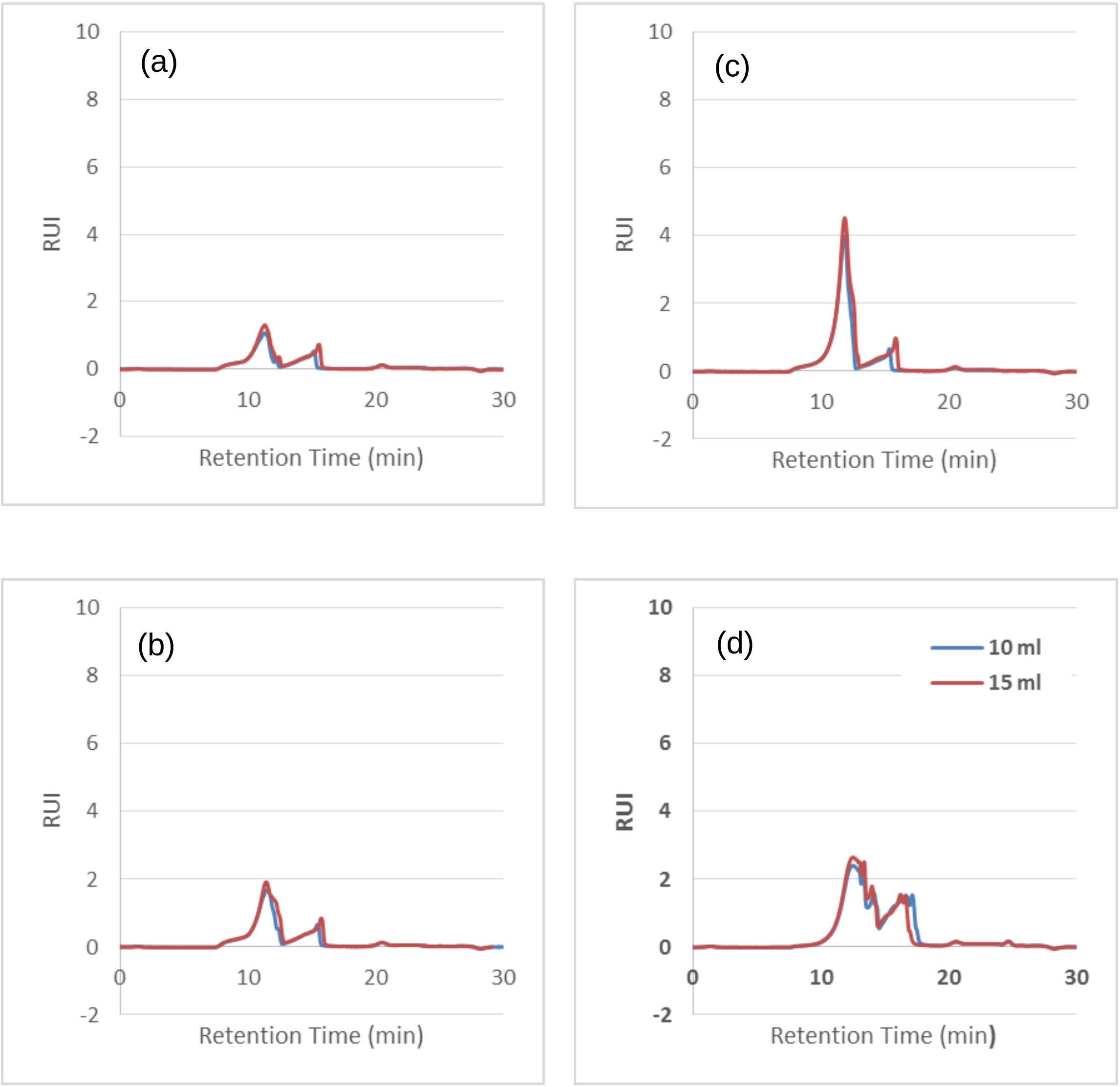


Figure 6.7: Comparison of microwave processing extracts by SEC of 1g seaweed in 10 and 15ml water for second step extraction for (a) 60°C, (b) 80°C, (c) 100°C and (d) 160°C

SEC chromatograms of the extracts processed at 120°C and 140°C are shown in Figure 6.8, along with the standard fucoidan chromatogram in each case for comparison. At 120°C, both 1g:10ml and 1g:15ml give a similar extraction of fucoidan, with 1g:10ml giving a slightly higher quantity. The shape of the curve for both closely resembles that of the standard, indicating that good extraction of the macro-polymer in its native form has been achieved. While the SEC chromatogram suggests more fucoidan has been extracted at 140°C (see Figure 6.8(b)), based on the area under the curve, the difference in peak shape from the standard shows significant broadening and therefore variation in MW. This indicates that the fucoidan is beginning to be broken down at this temperature. This broadening of the peak is more distinct for 1g:15ml than 1g:10ml, indicating the increase in S:S ratio enhances breakdown. This is in line with what was seen in the feasibility study in Chapter 5 and is likely due to the effect on bonds due to the increase in microwave energy associated with heating a higher volume of water.

From these figures, it is clear that processing at 120°C for 5 minutes with a S:S ratio of 1g:10ml of water gives the best result, with 95.1 wt% of the fucoidan from the input to this step being extracted. However, both fucoidan from 120°C and 140°C processing have been precipitated from the extract in order to fully evaluate the quality of the fucoidan present and to ascertain the effect microwave processing has on the chemical make-up and structure of the polymer, the results of which are presented in Section 6.4. 140°C was considered to gain some insight into the breakdown mechanics that processing at higher temperatures has on the fucoidan.

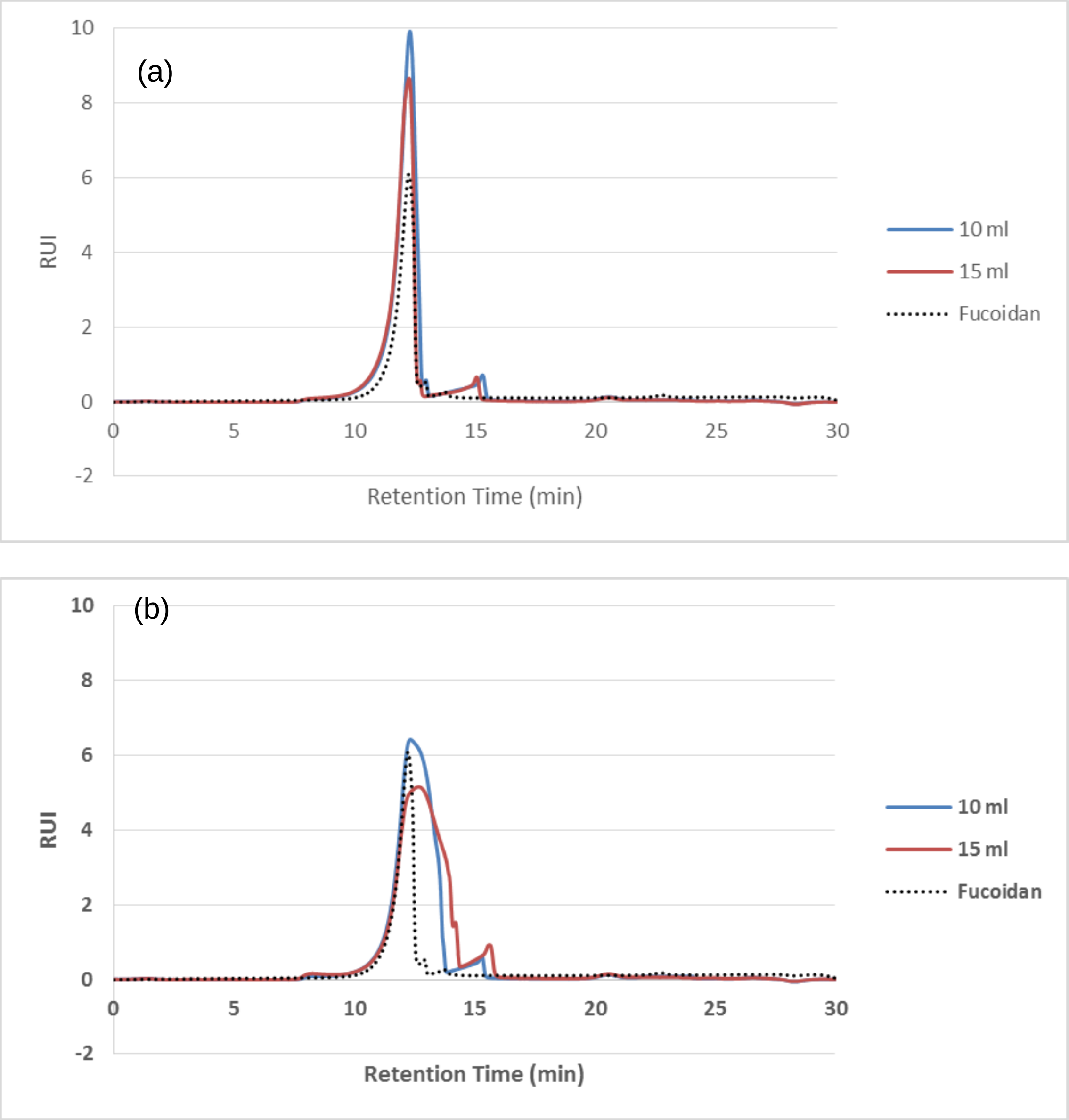


Figure 6.8: Comparison of microwave processing extracts by SEC of 1g seaweed in 10 and 15ml water for second step extraction for (a) 120°C and (b) 140°C.

The total quantity of fucoidan extracted, given as wt% of the fucoidan in the feedstock, is shown in Figure 6.9. The quantity of fucoidan extracted increases up to a peak at 140°C, before dropping dramatically at 160°C. However, processing at 140°C takes the total quantity over 100% and, as shown in Figure 6.8(b), there is evidence of the breakdown of fucoidan occurring. The over-estimation of the quantity of fucoidan, as mentioned previously, is likely due to the way in which the refractive index detector used for analysis “counts” the fucoidan molecules. The beginnings of breakdown are likely to be comprised of fucoidan polymers of shorter chain length, leading to a wider spread of molecular weight fragments, which was seen in Figure 6.8(b). The fragments will each get “counted” as their own molecule by the detector, thereby giving a falsely high fucoidan reading.

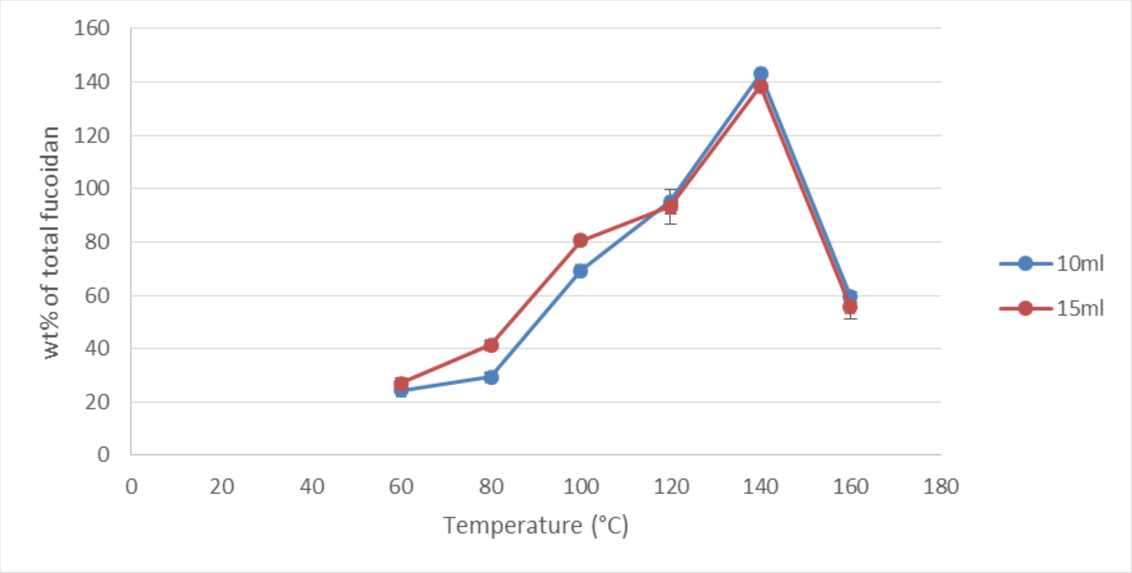


Figure 6.9: Fucoidan extracted at varying temperatures and S:S ratios from microwave extraction.

The MW distribution of the second step microwave extracts have been calculated from the SEC chromatograms, the results of which are given in Figure 6.9. Overall, the results for 1g: 10ml and 1g: 15ml S:S ratio are very similar, with only minimal changes in the amount between corresponding samples. Above 2500 kDa, the abundance of material is seen to decrease with increasing temperature. Between 2500 and 1000 kDa, the abundance of material is seen to increase up to 120°C, above which there is a decrease, with a similar trend for 1000 to 500 kDa, where the increase occurs up to 140°C, above which there is a decrease. These values correspond to the quantity and MW variation in fucoidan, with the primary peak falling between 2500 and 1000 kDa and the secondary peaks between 1000 and 500 kDa. The shift in ratio between 2500 to 1000 kDa and 1000 to 500 kDa of 1:0.7 to 1:2.7 for S:S of 1g: 10ml and 1:1.1 and 1:2.6 for 1g:

15ml for 120°C and 140°C respectively indicates a lowering in MW of fucoidan, as the smaller MW range becomes more abundant. This confirms the shift in MW seen between 120°C and 140°C in Figure 6.7 and Figure 6.8.

The lower MW material all shows a similar trend; below 500 kDa there is very little extracted material up to 140°C, with 160°C showing a distinct increase for all bands of MW. This indicates the breakdown of higher MW material into smaller fragments. The reduction in high MW material at this temperature also indicates this.

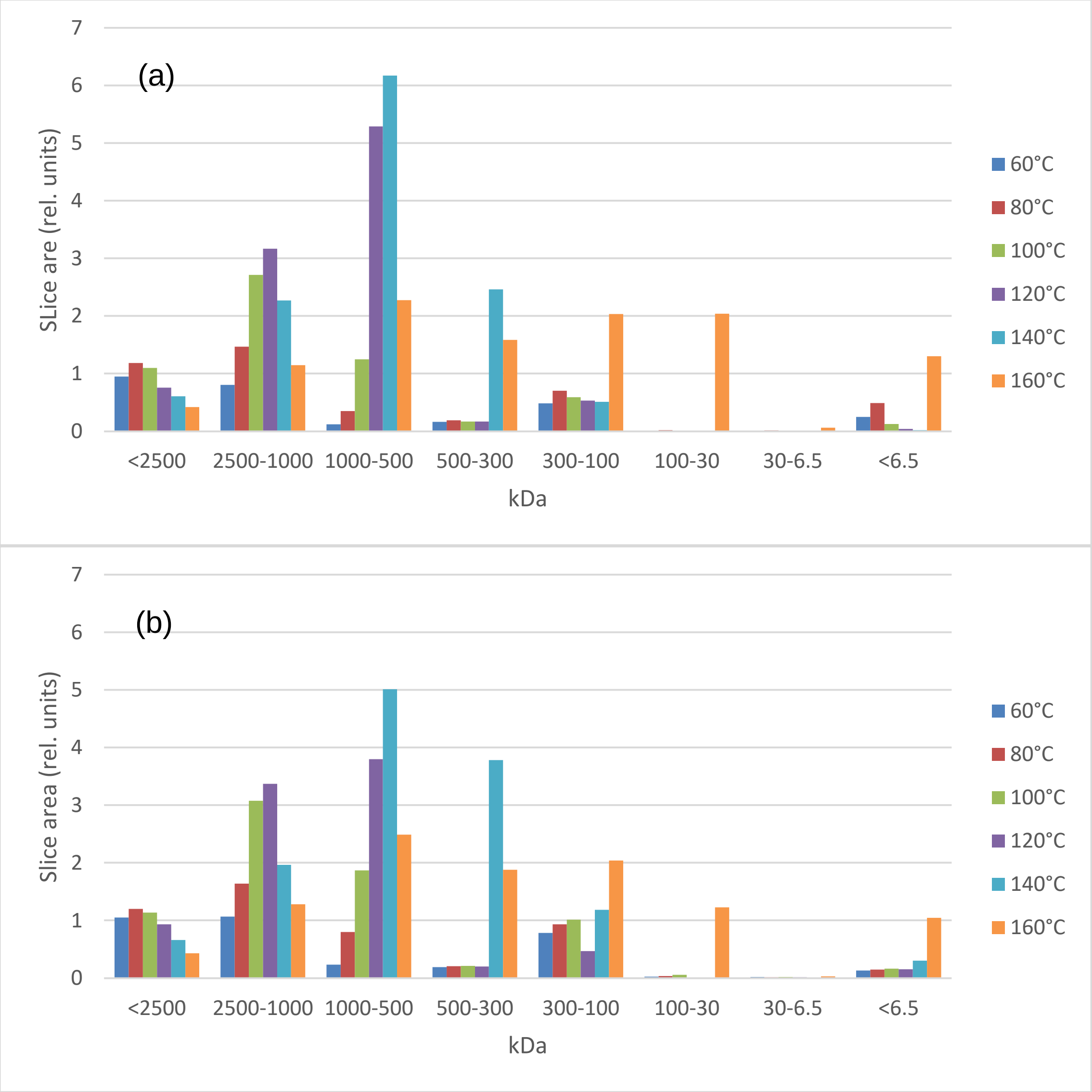


Figure 6.10: MW distribution for second step microwave extracts across a range of temperatures for a S:S ratio of (a) 1:10 and (b) 1:15.

The supposed breakdown of fucoidan is also evidenced by sugar analysis of the extracts, the results of which are given in Figure 6.11. Fucose, the predominate monomer unit of fucoidan, is seen to be zero up to 120°C, after which it begins to increase with increasing temperature. A similar trend is seen for glucose, the monomer unit in laminarin and uronic acids, which here are guluronic and manuronic acid, the monomer units from alginate. As with fucoidan, laminarin appears to begin to degrade at 140°C, with a small and constant amount of free glucose being extracted before this temperature. Alginate, on the other hand, seems to be more temperature stable than the other two carbohydrates, with evidence of degradation not occurring until 160°C. The quantity of uronic acids, however, is significantly more than for fucoidan and laminarin, with almost 40 wt% present in the extract. This suggests that alginate does not degrade into shorter polymer chains, as is suspected for laminarin and fucoidan, but, instead, largely breaks down to its monomer units at 160°C.

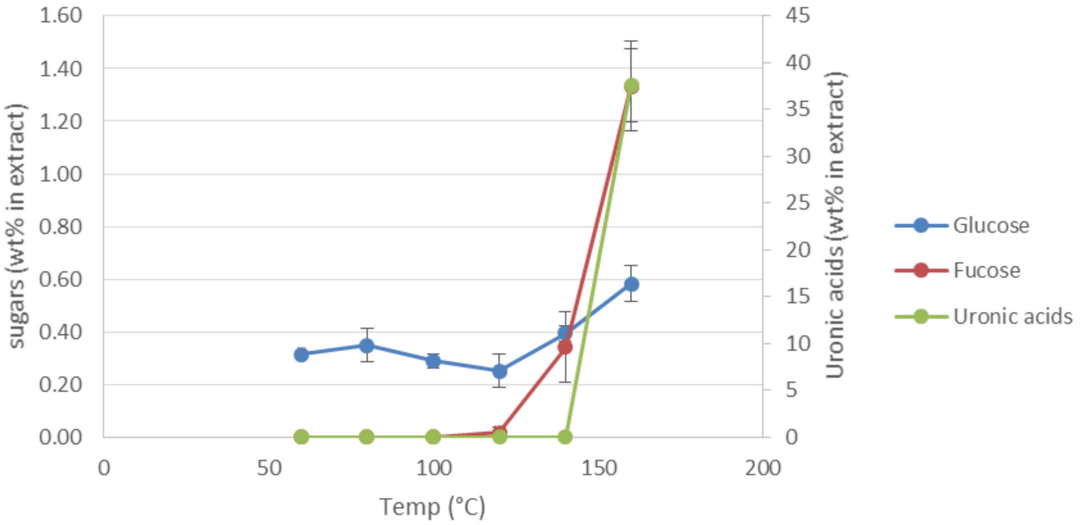


Figure 6.11: Amount of glucose, fucose and uronic acids in second step extracts

The sugars and organic acid content of the residue has been evaluated, achieved by the acid hydrolysis of the residues, the results of which are given in Figure 6.12. In this case, appreciable amounts of xylose and galactose has been identified below 120°C, which are likely to be associated with the structure of fucoidan. Fucose, xylose and galactose follow an opposite trend than for glucose in Figure 6.11 and total fucoidan extracted in Figure 6.9, with the quantity decreasing up to 120°C, above with temperature the amount remaining in the residue is close to zero. Alginate show a similar pattern, with a decrease in the quantity of uronic acids in the residue with increasing temperature. There is a more dramatic decrease between 140°C and 160°C, which corresponds to the increase in uronic acids between these temperatures see in the extract in Figure 6.11, as alginate begins to he hydrolysed into its monomer units. Laminarin, on the other hand, is seen to be relatively stable in quantity up until 120°C, after which the quantity of glucose in the hydrolysate is seen to increase. In reality, the weight of laminarin in the residue remains relatively constant across all temperatures at 11.9±0.5 mg. The increase is due to this weight accounting for a larger wt% of the residue, as more of the components are extracted into the liquid phase.

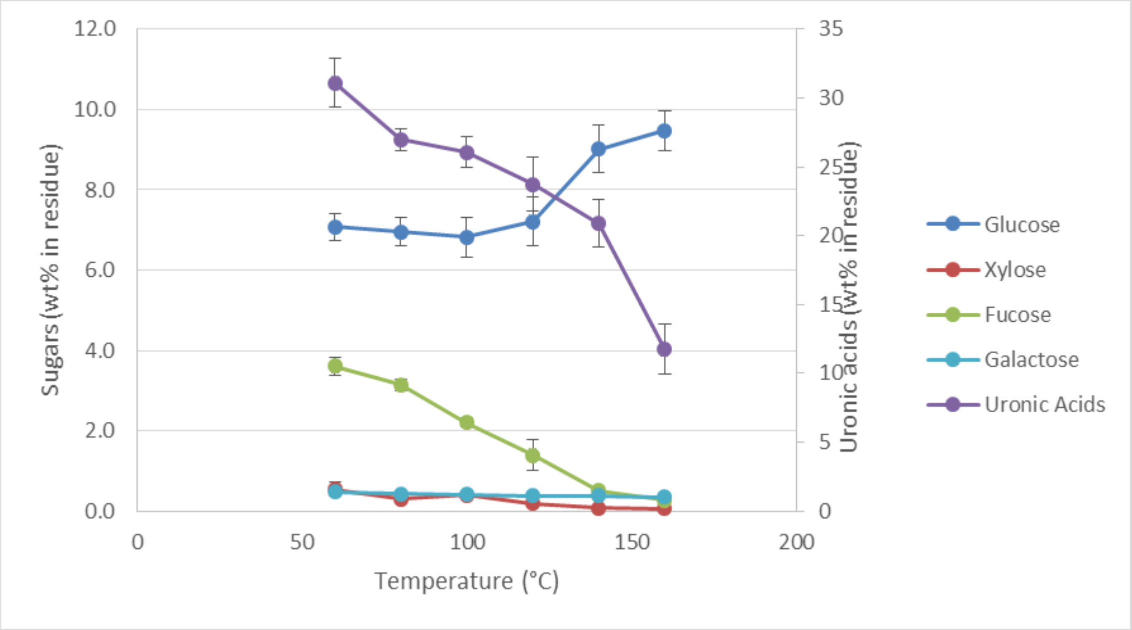


Figure 6.12: Sugars and uronic acids content in second step residues, determined via sugar analysis digestions.

The metal content and composition of each of the residues are given in Figure 6.13 (full numerical data is given in the Appendix in Figure A.4). The quantity of metals in the residue decreases with increasing temperature, with the result that roughly half of the total metals in the feedstock are extracted in each case. The quantity of metals extracted between 1g:10ml and 1g:15ml are relatively similar, indicating an increase in S:S ratio has little impact on the extraction of metals. In all cases, the predominant metals are Ca, Na, K, Mg and Cl, which are the main metals found in seawater [182]. There also significant quantities of P and Sr.

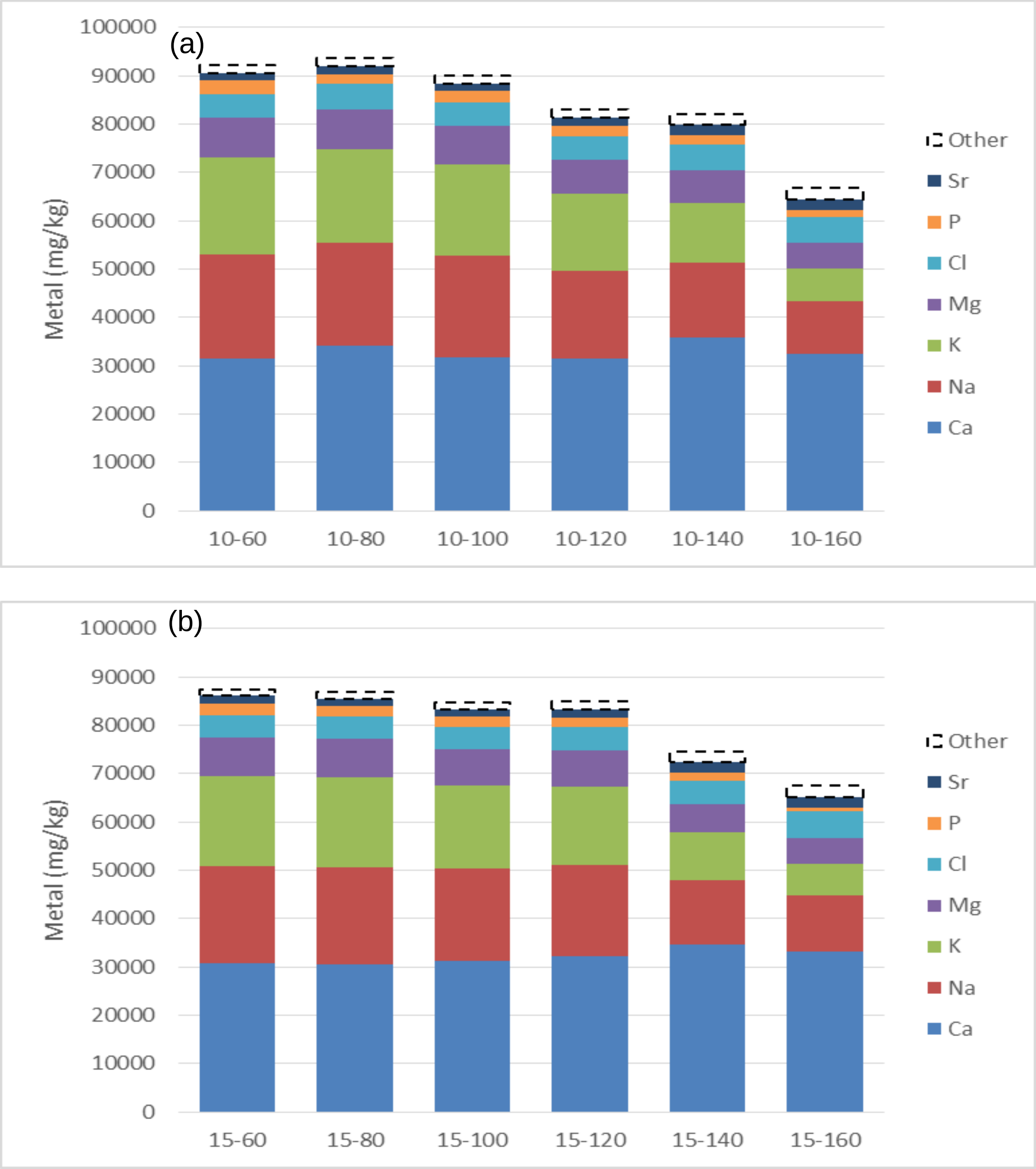


Figure 6.13: Metal analysis of the second step microwave residues for (a) 1g: 10ml S:S ratio and (b) 1g: 15ml S:S ratio.

6.2.2.2 Microwave extraction with $CaCl_2$

In order to ascertain if fucoidan extraction could be achieved alongside rendering soluble sodium alginate into insoluble calcium alginate in one step, microwave process with $CaCl_2$ was investigated. This was performed at 100°C and 120°C, as this is around the temperature fucoidan is extracted from previous investigations. The lower, 100°C was tried as salts increase the heating from microwaves, as explained previously. The results, given in Figure 6.14, indicate that processing in $CaCl_2$ inhibits the extraction of fucoidan. As there is a broadening of the fucoidan peak at 120°C, suggesting the beginnings of fucoidan degradation. From results in previous sections highlighting optimum extraction occurs at around 20°C below the point at which breakdown begins to occur, it is assumed that 100°C gives the best extraction under these conditions. This is also in line with optimum extraction temperatures seen for the other two salt concentrations from the first and second step. Although no literature could be found to explain why this occurs, it is possible that it is due to Ca ions attaching to the sulphate groups associated with fucoidan at elevated temperatures, also rendering it insoluble, as with the alginate.

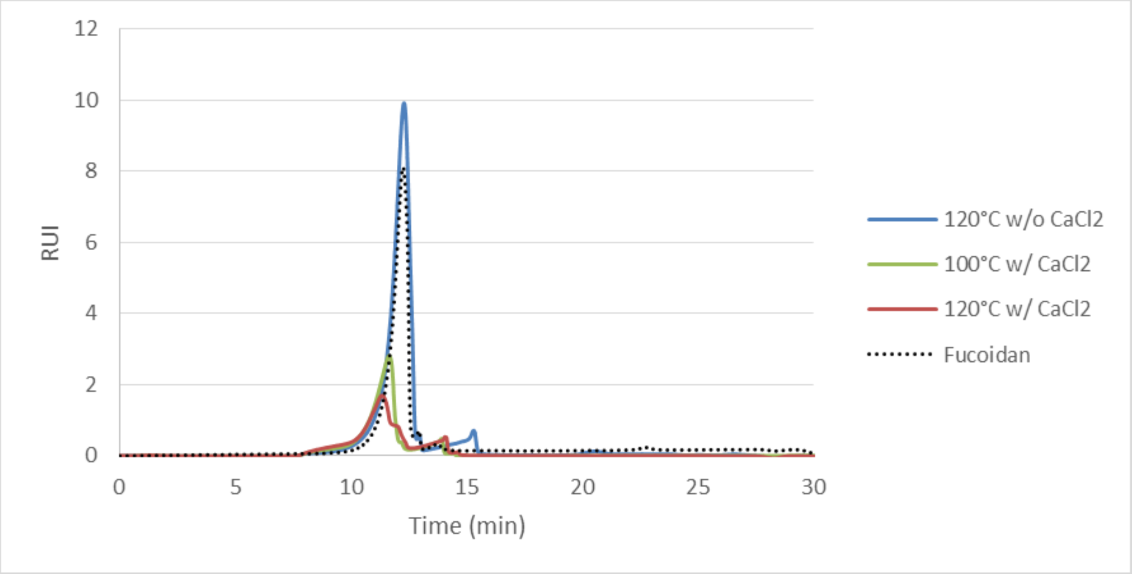


Figure 6.14: Comparison of second step microwave extracts by SEC for samples processed with and without $CaCl_2$.

The organic acid and sugar content of the residue, determined via acid hydrolysis before HPLC analysis, have been analysed and are shown in Figure 6.15. Extraction at both 100°C and 120°C give very similar results. The results show a high quantity of uronic acids, indicating alginate, which are similar in amount to that of alginate in the feedstock. The quantity of fucose in the hydrolysate, indicating fucoidan, is also very similar to that in the feedstock, which contains 6.3 wt%, at both temperatures. This confirms that fucoidan is not being extracted at all, rather than being extracted and then broken down and hence the lack of fucoidan in Figure 6.14. Overall, extraction with $CaCl_2$ was discounted as a possible method for the macroalgal biorefinery, due to low fucoidan extraction yields.

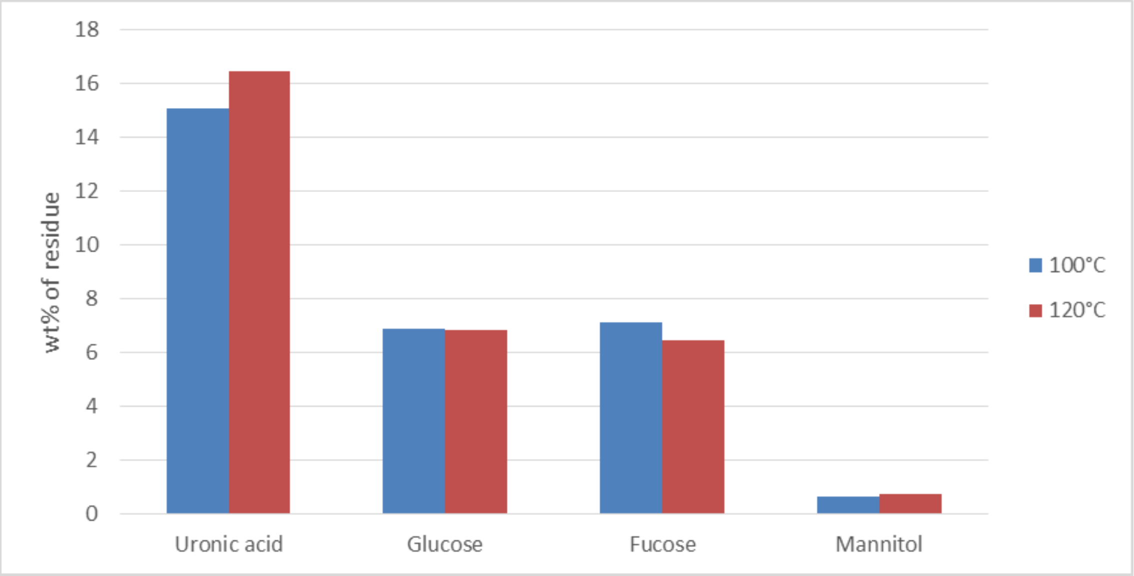


Figure 6.15: Organic acid and sugar analysis of the residue from $CaCl_2$ processing

In order to ascertain if the metals extracted had an influence on the extraction mechanism with $CaCl_2$, metal analysis of the residues from the $CaCl_2$ extractions were undertaken. The results of this are shown in Figure 6.16 (full numerical data is given in the Appendix in Figure A.4), along with the metal analysis of microwave processing at 120°C in water for comparison. The metal composition of both 100°C and 120°C processing with $CaCl_2$ is almost identical. When comparing the water and $CaCl_2$ processed samples, the most obvious differences are seen in the quantity of Ca, Na and K. The amount of Ca in the $CaCl_2$ extracts is roughly a third more than in its water counterpart. While this is expected, due to the addition of calcium to the process, it does indicate that precipitation of alginate into the residue has been successful, as it is likely that the majority of the Ca ions would remain in aqueous form if this were not the case. The reduction of Na and K is likely due to their replacement by Ca as the ion associated with alginate, and therefore they are associated with the liquid phase, as aqueous ions, rather than the solid. The quantity of all other metals, including those which make up the “other” portion, such as Sr, Zn and Br, all remain very similar, with negligible difference in their extraction between the water and $CaCl_2$ processing. While confirming alginate has been precipitated in its insoluble, Ca form into the residue, the lack of differences in other metals and explanations for the differences in Ca, Na and K related to alginate, it is difficult to draw any conclusions from this data about the effect metals are having in this case on the extraction of fucoidan.

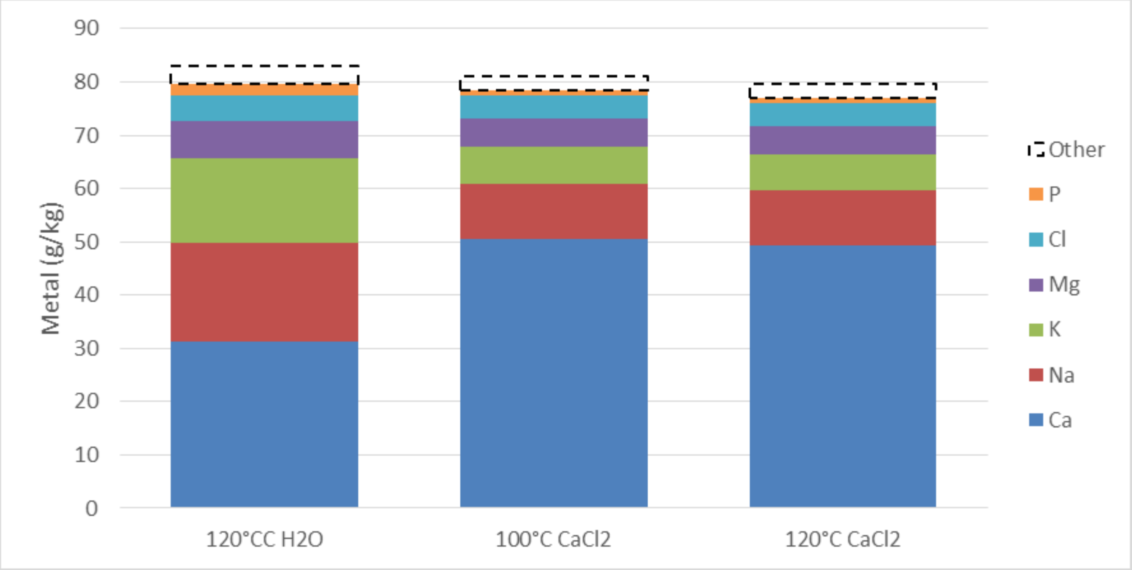


Figure 6.16: Metal analysis of $CaCl_2$ extracts compared with the water extract at 120°C

6.2.3 Third step – Extraction of Alginate

As there is still a relatively large quantity of alginate left in the second step residue, a third step, extracting with NaCl, was performed in order to attempt to extract alginate in its soluble, sodium form. Extraction was performed at 120°C, as alginate has been shown to begin to breakdown at 160°C, and it is possible that the increase in ions from adding NaCl would decrease the temperature at which breakdown occurs. Processing occurred at a S:S ratio of 1g:10ml and a residence time of 10 minutes. 1.5% NaCl used as this is 1.5x the number of Na required to replace all the Ca associated with alginate in the residue; a reasonable and fairly standard excess to ensure complete conversion. A mass balance for the extraction of alginate obtained is given in Figure 6.17. In 1g of second step residue there is 0.244g of alginate, of which 79% is extracted into the liquid phase. Although this extraction step has not been optimised at this point, the test shows the feasibility of this method as a way of extracting relatively pure alginate from the residue. The exact conditions need to be investigated to optimise this step. This would include: temperature, hold time in the microwave, residue to solvent ratio and the concentration of the NaCl solution. It may also be beneficial to test different sodium salts to see if better results can be obtained.

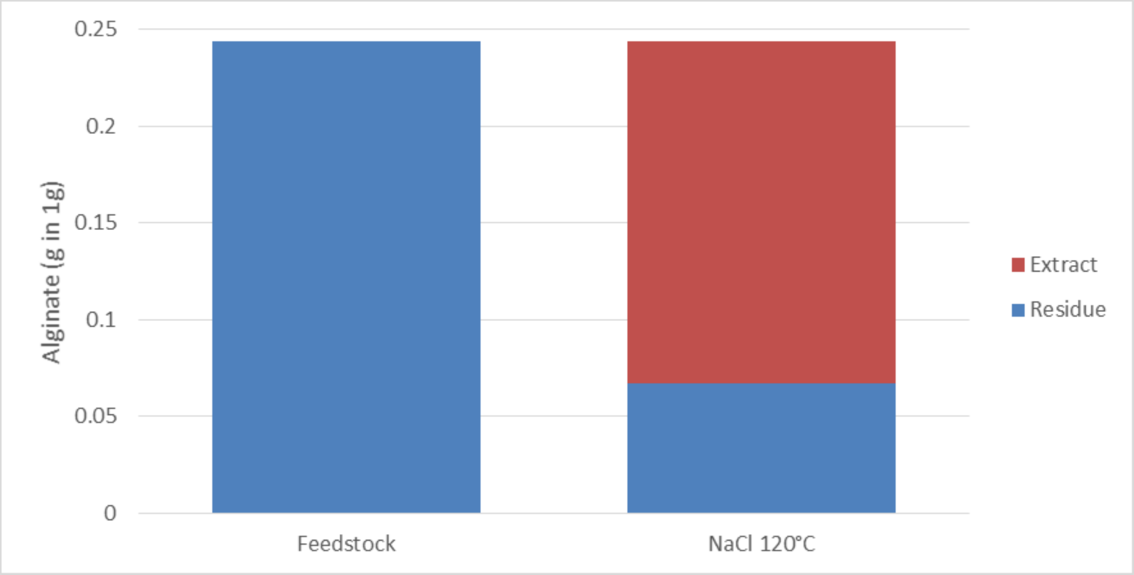


Figure 6.17: Mass balance for the extraction of alginate from the second step residue using NaCl at 120°C.

The residue was hydrolysed with concentrated acid in order to analyse the sugars and organic acids, the results of which are shown in Figure 6.18. The quantity of uronic acid corresponds with the amount of alginate observed in the extract, giving a good mass balance. Similarly, the quantities of glucose, fucose and mannitol correspond with the expected amounts at 9.9 wt%, 1.2 wt% and 0.4 wt% respectively.

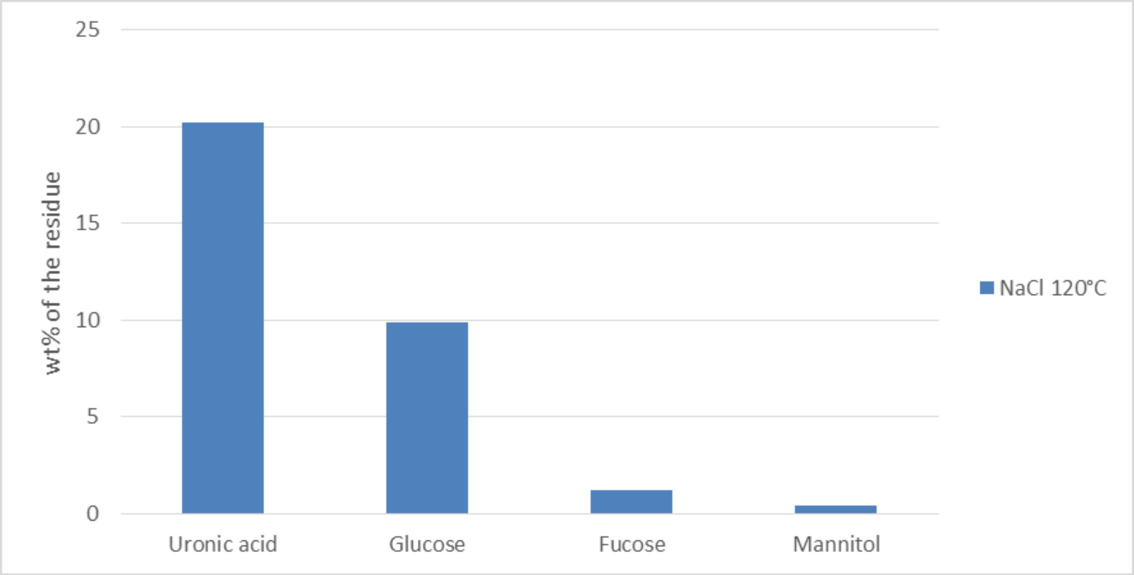


Figure 6.18: Organic acid and sugar analysis of third step residue, processed at 120°C with NaCl

The metal analysis of the third step extract and residue are given in Figure 6.19 (full numerical data is given in the Appendix in Figure A.4). The feedstock for this contains a very low amount of metals; 0.2 g/kg, while the extract and residue contain 5.4 and 1.6 g/kg respectively. The increase in metal content is due to the addition of NaCl as an extraction solvent. From Figure 6.19 it is clear that the majority of the Na are liquid phase in aqueous form, which is expected as they have displaced the Ca ions associated with alginate, rendering it soluble. The relatively large amount of Ca remaining in the residue indicates that Ca-alginate has not been fully extracted, which was also noted in the organic acid analysis in Figure 6.18. As stated before, it may be possible to extract more of this by increasing the concentration of NaCl, increasing the S:S ratio or processing for longer: all of which need to be explored further. The relatively large amounts of Ca and Mg are likely due to impurities in the NaCl used, which is quoted by the manufacturer to be only of 95% purity. The low metal content of the final residue from the biorefinery process is advantageous for use as a fertiliser, where regulations closely monitor the quantity of certain metals allowed to be applied to soil [217], but also for use as a feedstock for bioethanol or bio-methane production, where metals may inhibit microbes.

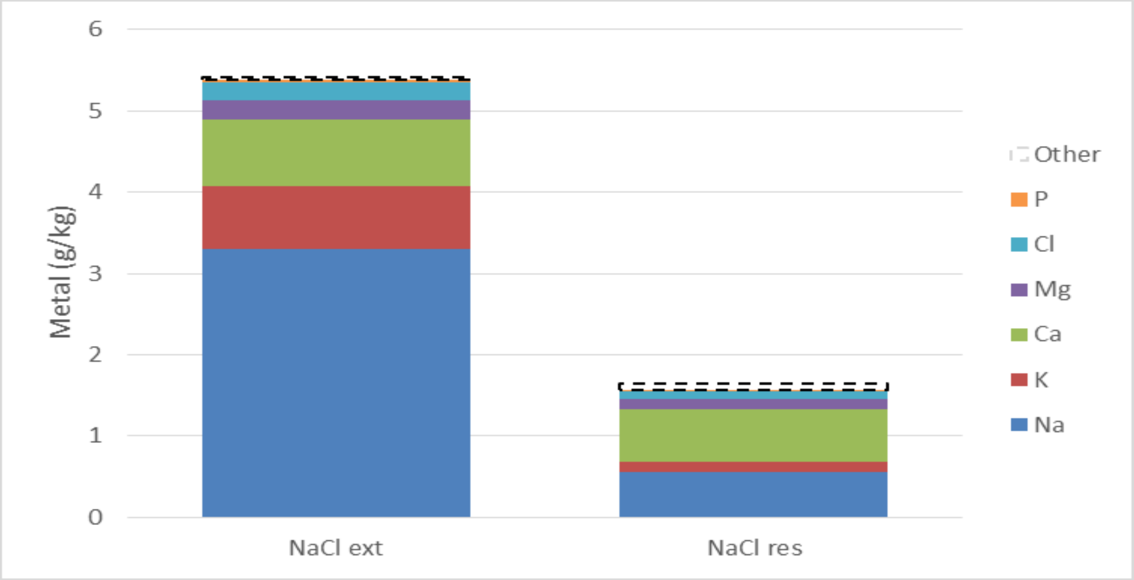


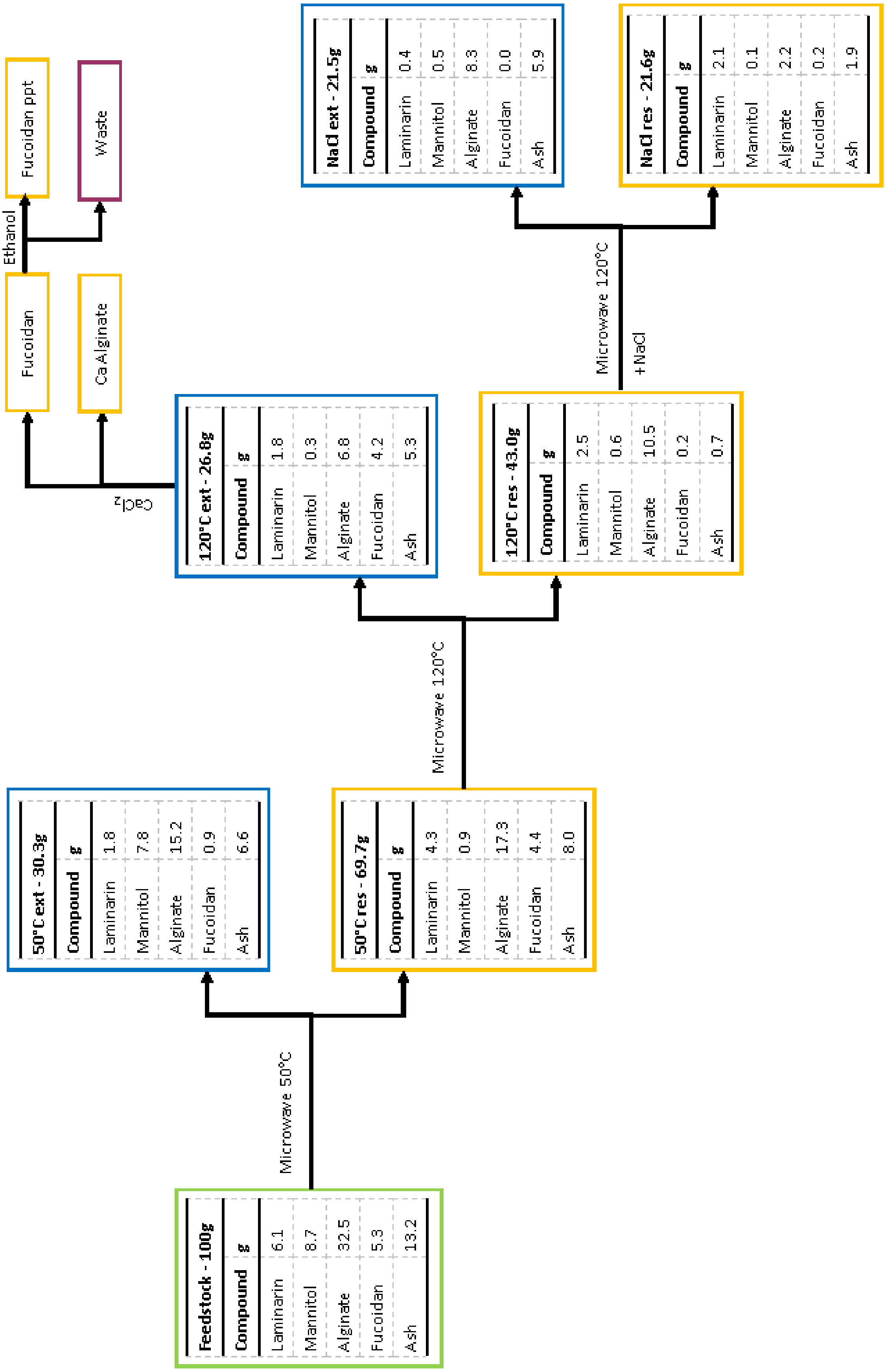
Figure 6.19: Metal analysis of third step NaCl extract and residue

6.3 Mass and Energy Balances for Microwave Assisted Sequential Extraction of Seaweed

The final biorefinery schematic, along with the mass balance for each step, based on 100g input, is given in Figure 6.20. As developed and discussed in Chapter 5, the extraction conditions for each step of the process are given in Table 6.1. There is seen to be a good recovery of fucoidan from the initial biomass, with 79.2% being extracted and purified in the second step. Depending on the commercial value of fucoidan, it would be possible to recover a further 17.0% of fucoidan, which has been extracted in the first step. Recovery of alginate at the first and second steps step via precipitation with $CaCl_2$, combined with the insoluble fraction recovered in the third step, would lead to a maximum of 93.2% recovery of alginate. Combination of the mannitol and laminarin from the first step extract and third step residue leads to a 90.8% and 63.9% recovery, respectively, from the initial biomass, which could be used for fermentation to bioethanol or anaerobic digestion (AD) to bio-methane. Overall, this process gives good chemical recovery of all components, while minimising the solvent required. Minimal solvents reduces the environmental impact of the process, as well as lowering the production costs, as additional chemicals are not required.

Table 6.1: Microwave conditions for each step of the microwave biorefinery

Step	**First**	**Second**	**Third**
Purpose	Remove mannitol, water soluble free alginate and metals	Extraction of fucoidan, soluble laminarin and soluble alginate	Extract water insoluble alginate
Conditions	50°C microwave	120°C microwave	120°C microwave
Residence time	5 minutes	5 minutes	5 minutes
Solvent	Water	Water	1.5% (w/v) NaCl
Seaweed : Solvent	15ml/1g dry seaweed	10ml/1g dry seaweed	10ml/1g dry seaweed



Feedstock - 100g	
Compound	**g**
Laminarin	6.1
Mannitol	8.7
Alginate	32.5
Fucoidan	5.3
Ash	13.2

50°C ext - 30.3g	
Compound	**g**
Laminarin	1.8
Mannitol	7.8
Alginate	15.2
Fucoidan	0.9
Ash	6.6

50°C res - 69.7g	
Compound	**g**
Laminarin	4.3
Mannitol	0.9
Alginate	17.3
Fucoidan	4.4
Ash	8.0

120°C ext - 26.8g	
Compound	**g**
Laminarin	1.8
Mannitol	0.3
Alginate	6.8
Fucoidan	4.2
Ash	5.3

120°C res - 43.0g	
Compound	**g**
Laminarin	2.5
Mannitol	0.6
Alginate	10.5
Fucoidan	0.2
Ash	0.7

NaCl ext - 21.5g	
Compound	**g**
Laminarin	0.4
Mannitol	0.5
Alginate	8.3
Fucoidan	0.0
Ash	5.9

NaCl res - 21.6g	
Compound	**g**
Laminarin	2.1
Mannitol	0.1
Alginate	2.2
Fucoidan	0.2
Ash	1.9

Figure 6.20: Proposed microwave biorefinery for the sequential extraction of chemicals from seaweed. Values given are based on 100g input of seaweed feedstock.

The overall metal balance of the biorefinery is shown in Figure 6.21 (full numerical data is given in the Appendix in Figure A.4). Metals account for 11.5 wt% of the raw biomass, which is relatively low for brown macroalgae. The majority of this is accounted for by sodium, potassium, calcium and magnesium. In each case, the small “others” bar in Figure 6.21(a) mostly consists of strontium, zinc, bromine, iron and aluminium. A more details account of these metals is given in Figure 6.21(b). In the first step, 46.6% of the total metals are extracted, with all metals being extracted roughly in proportion to this. The second step sees almost all of the remaining metals being extracted, with only 4.3% of the inputted metals remaining in the residue. The increase in metals in the third step is due to the addition of NaCl as an extraction aid. Potassium, calcium and magnesium are also increased in the third step. Calcium is due to the exchange of sodium ions for calcium in the extraction of alginate and it is likely that the small increase in potassium and magnesium are due to impurities in the NaCl used. The large amount of sodium remaining in both the third step extract and residue is an indication that a large excess of NaCl was added and that a similar extraction yield could be achieved with a lower concentration of NaCl. This is a point which should be investigated further for process optimisation.

It is hypothesised that the metals extracted in the first step are surface metals, which are easily washed from the surface at a relatively low extraction temperature. The second step, at 120°C, begins to break open the cells in the seaweed, meaning that any metals held inside are released to be extracted.

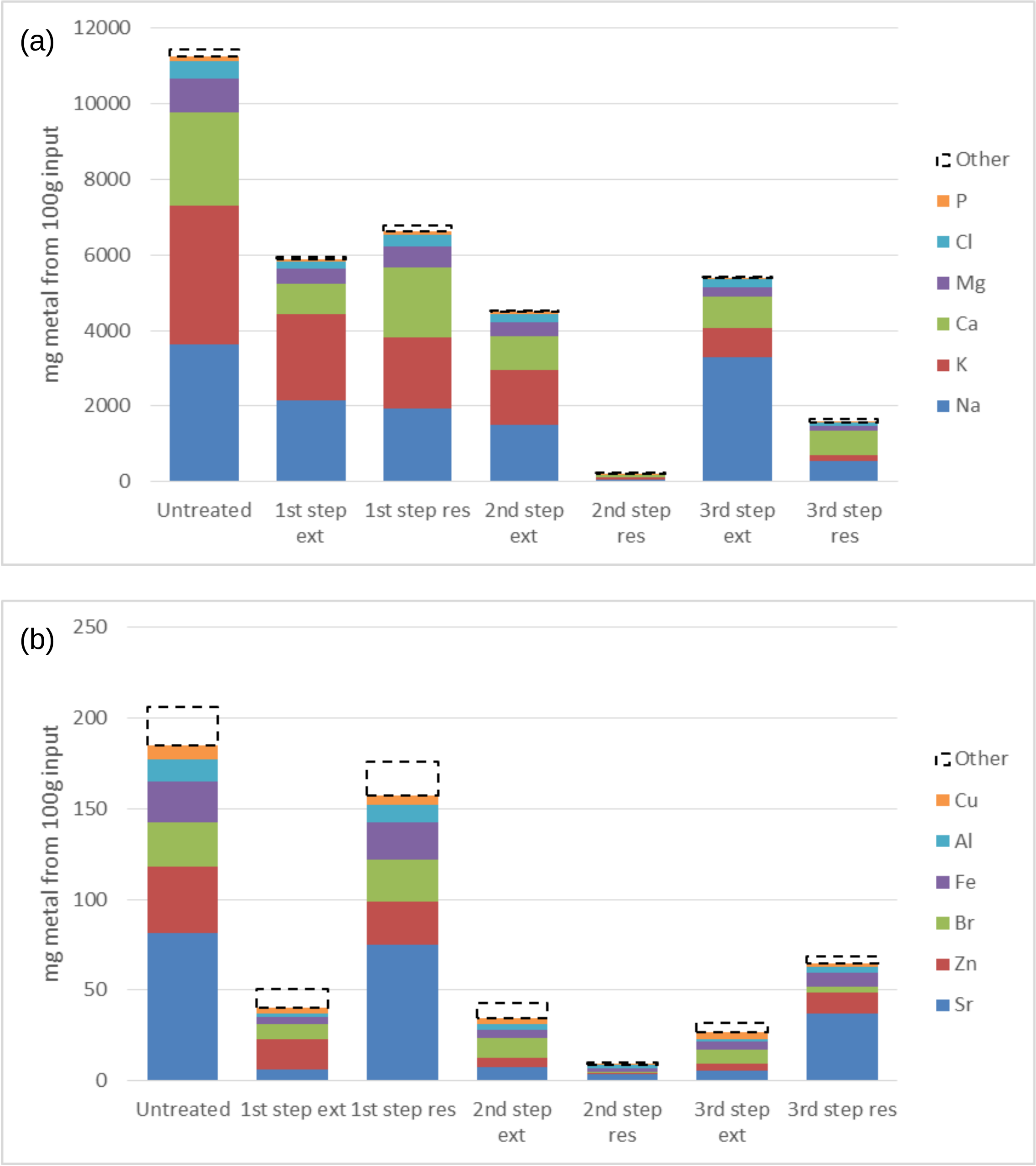


Figure 6.21: Metal balance from sequential microwave extraction of (a) macro-metals and (b) micro-metals, based on 100g input

6.3.1 Step 1 – 50°C Extraction

The first step of the biorefinery process is a washing step, removing any free, soluble metal ions, 89.7% of the total mannitol as well as water soluble alginate and laminarin. It is likely that the long chain carbohydrates, alginate and laminarin, removed in this step have been freed from their bonds within the cell wall during the drying and grinding processes. Furthermore, laminarin is commonly found in seaweed biomass in two distinct forms: one of which is soluble in water and the other insoluble in water [136]. It is likely, therefore, that the laminarin extracted in this first step is the soluble form, with the remainder remaining insoluble throughout the rest of the process. Figure 6.22 shows the mass balance for this step. The “other” section in each case will be primarily composed of protein, lipids, polyphenols and small amounts of other compounds such as pigments. As the majority of these components are not water soluble at low temperatures, in this step they have remained in the residue. Approximately half of the metals contained within the initial biomass are also removed during this step. The residue from this step is taken forward for further processing, while the extract could be treated in a number of ways. It could be fed directly into a bioethanol or AD system as it is, or alginate and/or fucoidan could be extracted using $CaCl_2$ and ethanol respectively, before the remainder being used for bioethanol or AD. The viability of each of these options would be dependent on the demand and market value of the chemicals. Furthermore, with the advancement in fermentation of seaweed [106, 113, 115], it may be possible to ferment alginate. In this case, the best option may be to produce a higher quantity of bioethanol, rather than extract the alginate.

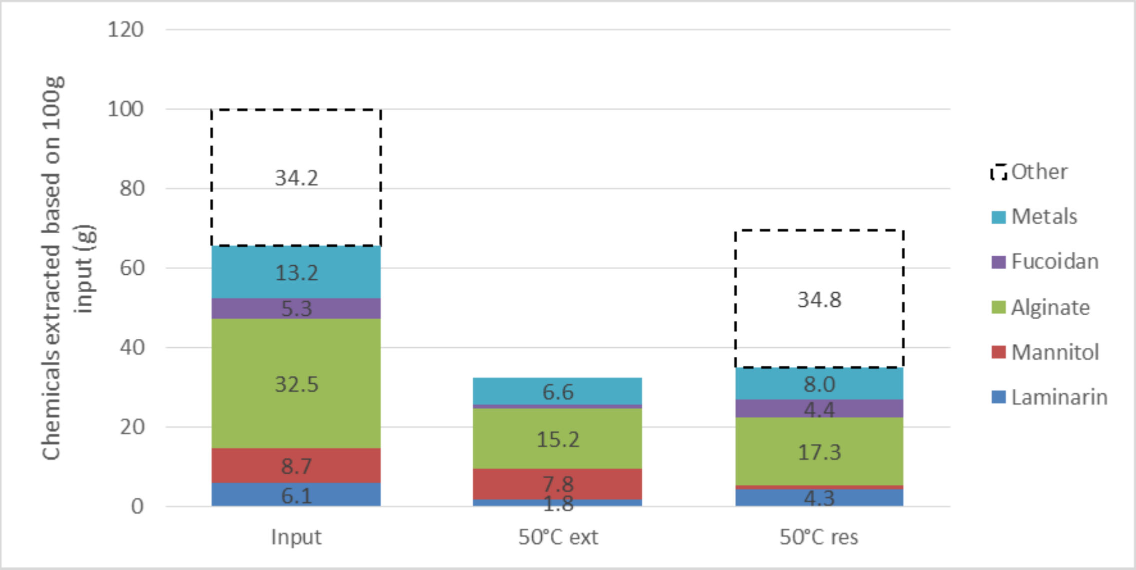


Figure 6.22: Mass balance for step 1 of the microwave biorefinery process, based on 100g input

6.3.2 Step 2 – 120°C Extraction

The residue from the first step is processed a second time at 120°C with water, in order to extract fucoidan, and a mass balance showing the composition of the extract and residue is shown in Figure 6.23. The fucoidan extracted is not degraded in any significant amount by this process, experimentation for which is given in section 6.4, which is contrary to what was found in a microwave study of fucoidan extraction by Yuan et al. [163] who processed with HCl. 79.2% of the total fucoidan present in the raw biomass has been extracted in this step, which is precipitated from the extract with ethanol, after alginate is removed with $CaCl_2$. Very little fucoidan is left in the remaining biomass after this extraction. Small quantities of laminarin, mannitol and alginate are also extracted in this step.

While the purification steps are relatively solvent intensive, it is possible that the solvents could be recycled. If bioethanol is produced as part of the biorefinery, it would be possible to purify the ethanol required for fucoidan precipitation using the equipment, such as distillation columns, required in bioethanol production. Furthermore, any additional ethanol required could be generated via this process.

With regards to $CaCl_2$, this is being produced in the third step, where NaCl is used to convert insoluble Ca-alginate into its soluble, Na-alginate form for extraction. Vice versa, NaCl is being produced in this step, where alginate is being precipitated in its insoluble form. Therefore, with some purification in between, it seems possible to cycle these two chemicals between the two steps.

The relatively large "other" portion being inputted into this step is only partly extracted at 120°C. Polyphenols and lipids, which make up a portion of this grouping, are typically insoluble in water, even at increased temperatures, so these are likely to remain in the residue. The portion of "other" in the extract is likely to be mostly comprised of protein. Proteins are intolerant to heat, degrading easily at the temperature used in this step, with the fragments likely to be water soluble [218].

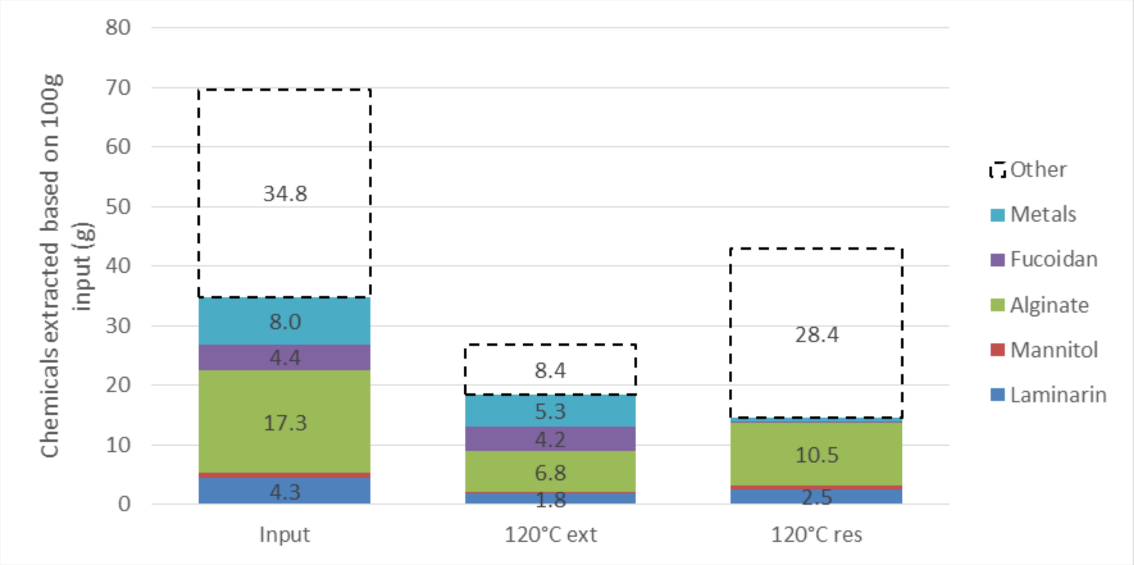


Figure 6.23: Mass balance for step 2 of the microwave biorefinery, based on 100g input

6.3.3 Step 3 – 120°C Extraction with NaCl

In the final step of the biorefinery process, insoluble Ca-alginate is converted into its soluble, Na-alginate form by processing at 120°C for 5 minutes in the presence of 1.5 w/v% NaCl. The mass balance for this step is shown in Figure 6.24.

Although, in theory, it would be possible to use any Na-salt for this process, NaCl was chosen for the potential to cycle NaCl and $CaCl_2$ through the biorefinery, as described in section 5.5.2. 79.0% of the alginate remaining in the biomass after the second step has been removed, although this may be able to be optimised further with different operating conditions, such as: temperature, residence time and solvent concentration. The residue at this point can be used for fermentation to bioethanol or for AN to bio-methane, while the extract contains almost solely alginate, with only small quantities of mannitol and laminarin.

The large "other" portion of the feedstock is seen to be split roughly equally between the extract and residue in this case. Polyphenols have been shown to be extracted with NaCl [219], so it is likely that this is the case here, with the "other" portion of the extract comprising mainly of polyphenols and the "other" portion of the residue being mostly lipids.

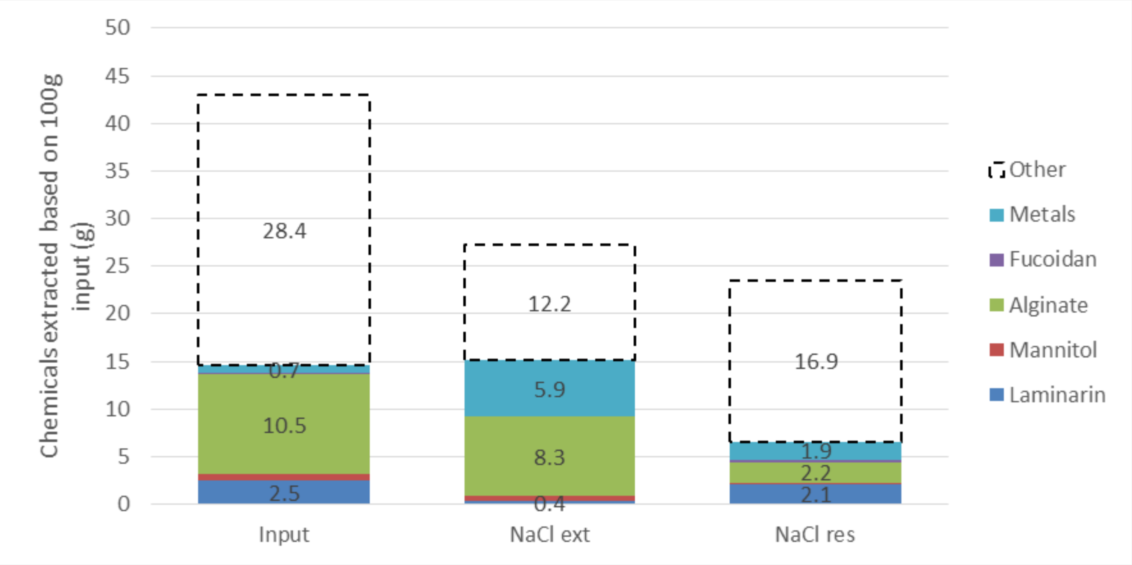


Figure 6.24: Mass balance for step 3 of the microwave biorefinery, based on 100g input

6.3.4 Energy Balance

The energy balance of the sequential extraction is an important consideration for industrial scale up, so a comparison of conventional (CH) and microwave (MH) heating has been calculated for the laboratory scale systems used throughout this study, in order to assess the potential energy saving via the use of microwaves. Although the laboratory scale equipment will, in all likelihood, incur different energy requirements and losses to industrial scale equipment, the results give a good indication of the potential and prove the feasibility and energy savings achieved as a result of converting to a microwave-based biorefinery system.

Figure 6.25 gives a comparison of the energy used to heat and hold a seaweed slurry of two different ratios: 1g: 10ml and 1g: 15ml of seaweed: water at residence times of 5 and 10 minutes over a range of temperatures between 50°C and 200°C. As the conventional heating value is based on the energy required to heat the sand bath, which is used to transfer heat to the slurry, and there was no significant difference seen for the temperature profiles through heating for 10 and 15ml of water, the energy requirements for this parameter have been assumed to be the same. In both cases, it is clear that there is a significant energy saving for microwave heating when compared to conventional heating. At 50°C, there is an average 2.5-fold increase in energy required for conventional heating over microwave heating. This drops to 1.9-fold increase at 200°C. Although industrial heating and loss profiles are likely to be different than on a lab scale, the large difference seen between the two heating mechanisms shows microwave heating can offer a large energy saving when compared to conventional heating.

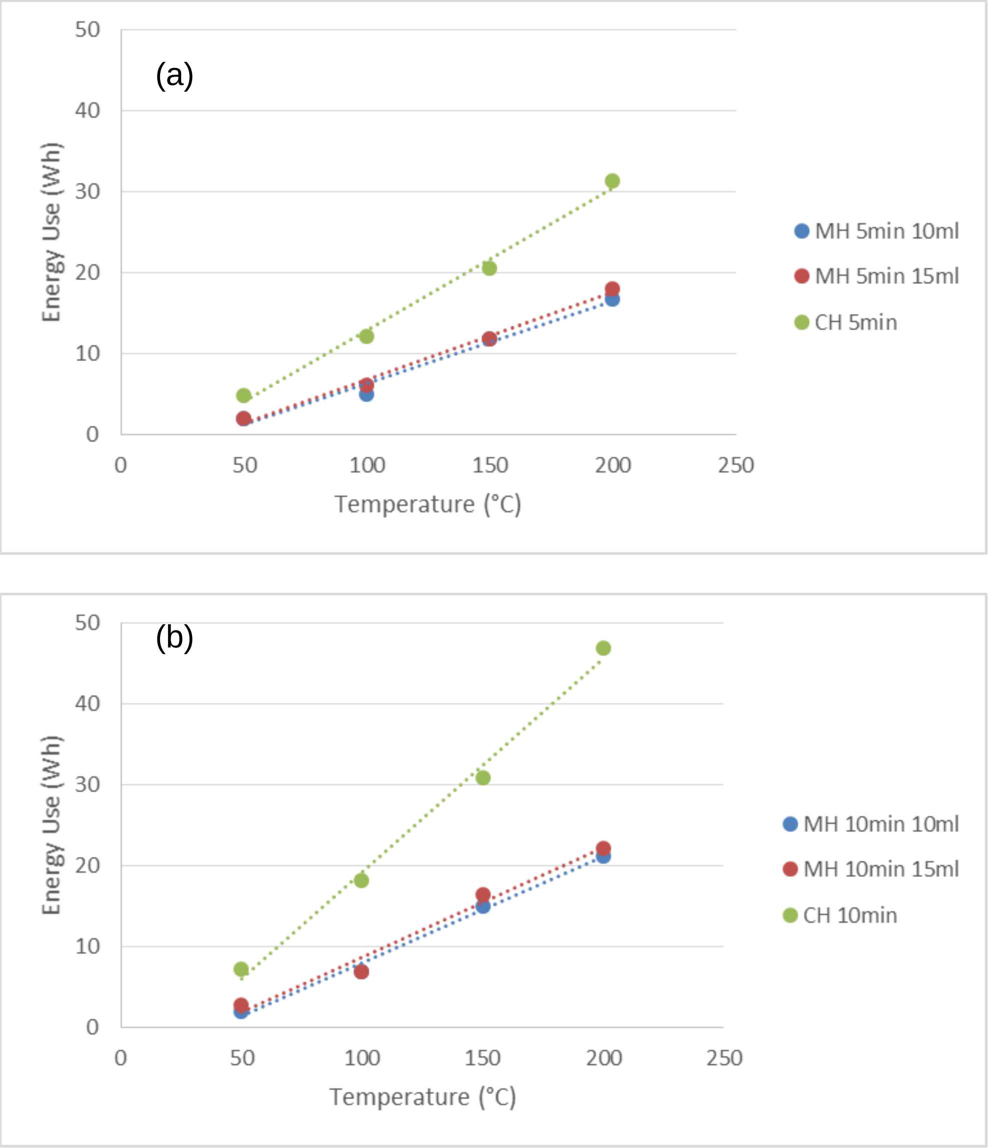


Figure 6.25: Energy use for conventional and microwave heating for residence times of (a) 5 minutes and (b) 10 minutes based on 1g of seaweed.

The energy required for each step of the sequential extraction biorefinery has been calculated and the results are shown in Figure 6.26. For the overall process, there is a 1.65-fold increase in the energy required for conventional heating over microwave heating, with a total for conventional heating of 2.24 Wh compared to 1.35 Wh for microwave heating. Over the energy savings shown here, microwave heating also has the advantage of heating the whole biomass evenly, rather than from the outside in. This should ensure a more reliable and even extraction of chemicals. Time at the desired temperature should be the same for all the material in microwave heating, whereas a gradient of temperature during the heating process will be evident for conventional heating. This will become more pronounced at a larger scale, where larger reactors/piping causes greater heating gradients from the wall to the centre of the pipe for conventional heating. For the extraction of fucoidan, which has been shown to breakdown at temperatures of around 140°C for both microwave heating and conventional heating, it is very important that there is even and reliable heating of the whole biomass to ensure a consistent product.

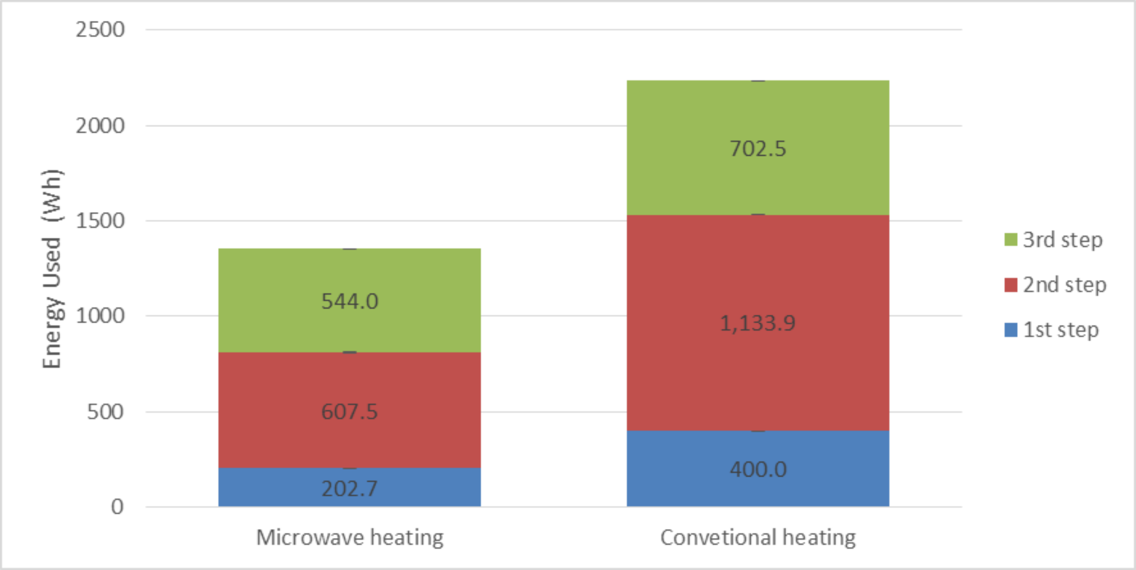


Figure 6.26: Comparison of energy used for sequential extraction in a microwave and conventional heating system based on 100g input

6.3.5 Potential Impact of Seasonal Variation

As noted in Chapter 3, the effect of seasonal variation is likely to have a big effect on the composition of the extracts from the process, owing to a change in the composition of the feedstock biomass. This will have a knock on impact for industry, where changes in the quantity of solvents required and the quantity of product produced will change over a year period. In an attempt to understand how this impact may affect the hydrothermal microwave biorefinery discussed in this study, the possible seasonal variation of the extracts from the process has been calculated, based on the values for FS found in Chapter 3 and the mass balance of the process, discussed in this section. While further study into the actual extract quantities would need to be completed through a microwave processing study, these results give an insight into the possible variation and highlight the potential challenges.

The potential seasonal variation in extraction for the first step extract is given in Figure 6.27. The sample of FS collected in 2015 for this study has a low laminarin and mannitol content compared to its counterpart collected in 2010, which has a large impact on the comparative composition of extracts. Thus, there is a much greater quantity of both of these components in the stream. There is a clear seasonal variation trend in these components, being higher in the summer months and lower in the winter. The quantity of alginate is relatively similar and, while having a slight increase in the winter months, is otherwise relatively stable over the year. The same is true for fucoidan, with only a very small quantity being extracted, that remains stable over the year. While this pretreatment step doesn’t have direct impact on the amount of fucoidan extracted, the additional storage carbohydrates in the summer make it a more attractive feedstock for bioethanol production than during the winter. This is explored further in section 6.5. If there were demand for the product, it would be possible to extract the alginate from this step via precipitation with $CaCl_2$.

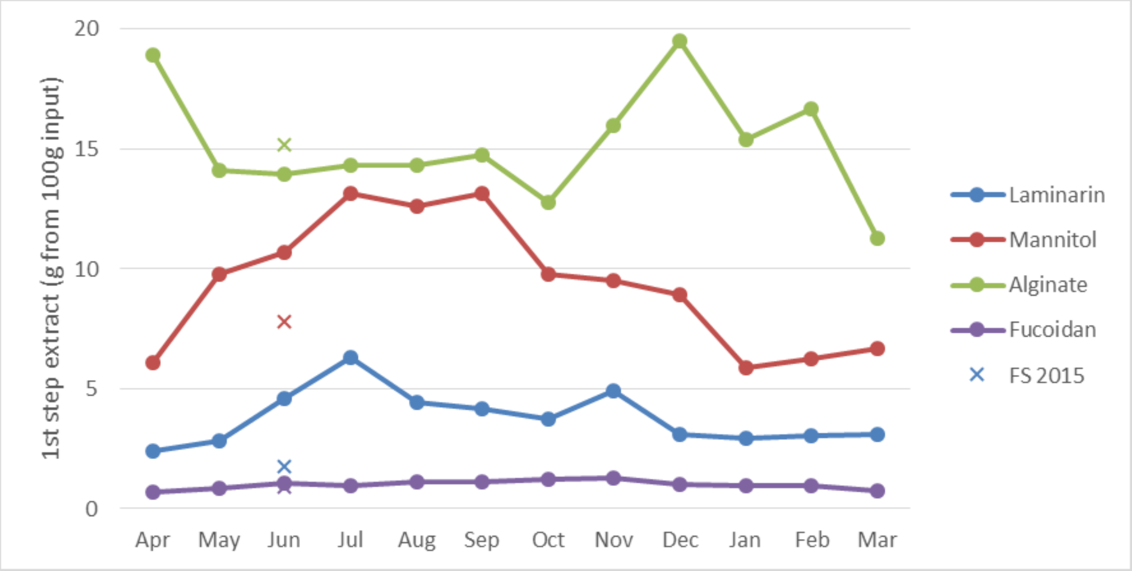


Figure 6.27: Potential seasonal variation in the composition of the first step extract, based on percentage extraction in this study.

There is a more noticeable difference in the composition of the second step extract, which is shown in Figure 6.29. While the mannitol content is low and relatively stable over the year, due to the majority being extracted in the first step, there is a marked increase in laminarin extracted over the 2015 sample. Again, this is due to the low carbohydrate content of the raw sample, but highlights the difficulties variation in a feedstock can represent. It is possible to precipitate the laminarin with ethanol at 40% v/v, with the new schematic shown in Figure 6.28. In the current schematic, shown in Figure 6.20, this step was excluded due to the low laminarin content in the extract, making it unnecessary. However, increased laminarin in the summer months will necessitate its removal to ensure the purity of fucoidan. This leads to the added benefit that relatively pure laminarin has been produced, which could be sold as a product in its own right, or could be added to the first step extract and used to increase the bioethanol yields in a fermentation process.

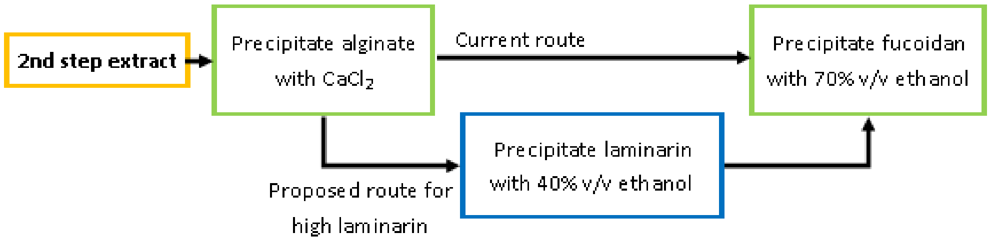


Figure 6.28: New schematic proposal for the extraction of laminarin from the second step extract

The quantity of alginate and fucoidan remain relatively similar over the year, with fucoidan showing only a slight increase in the autumn/winter months and alginate showing a slight increase in the winter months. This is good from an industrial viewpoint, as a consistent quantity of these chemicals will be extracted. Despite the increase in laminarin, as the volume of water it is extracted into will remain the same, the quantity of ethanol required will remain unchanged, even including the extra step. This is due to it being required on a volume basis, rather than being dependent on the quantity of the carbohydrates present in the extract. Any variation in alginate, however, will require an adjustment in the amount of $CaCl_2$ required to displace the Na ions and render it insoluble. However, as using a 1.5% excess is fairly standard, the variation seen in Figure 6.29 could be accounted for within this excess.

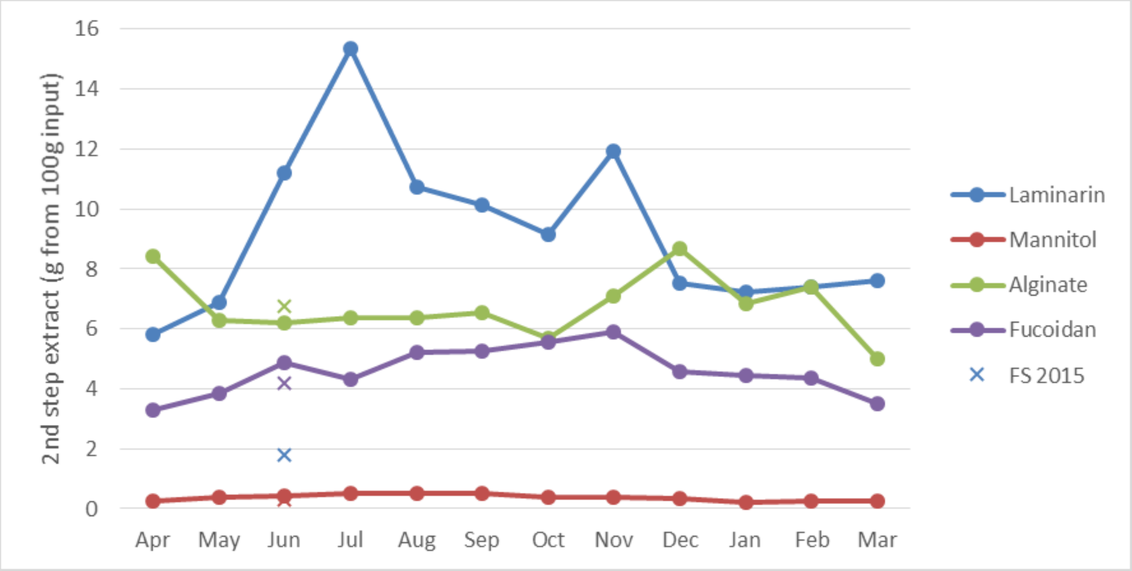


Figure 6.29: Potential seasonal variation in the composition of the 2st step extract, based on percentage extraction in this study.

By the third step of the process, almost all the fucoidan, laminarin and mannitol have been extracted, leaving only Ca-alginate remaining from the carbohydrates being studied. The results for seasonal variation can be seen in Figure 6.30. This clearly shows the very low values for mannitol and laminarin, with fucoidan being close to zero across the year, so has not been shown for clarity of the laminarin and mannitol values. Again, alginate shows the slight increase in extraction quantity in the winter months, but is otherwise fairly stable across the year, averaging 8.3g/100g input to the system. As with the second step, an excess of NaCl added to the process would cover these variations, allowing the inputs to the system to remain constant throughout the year.

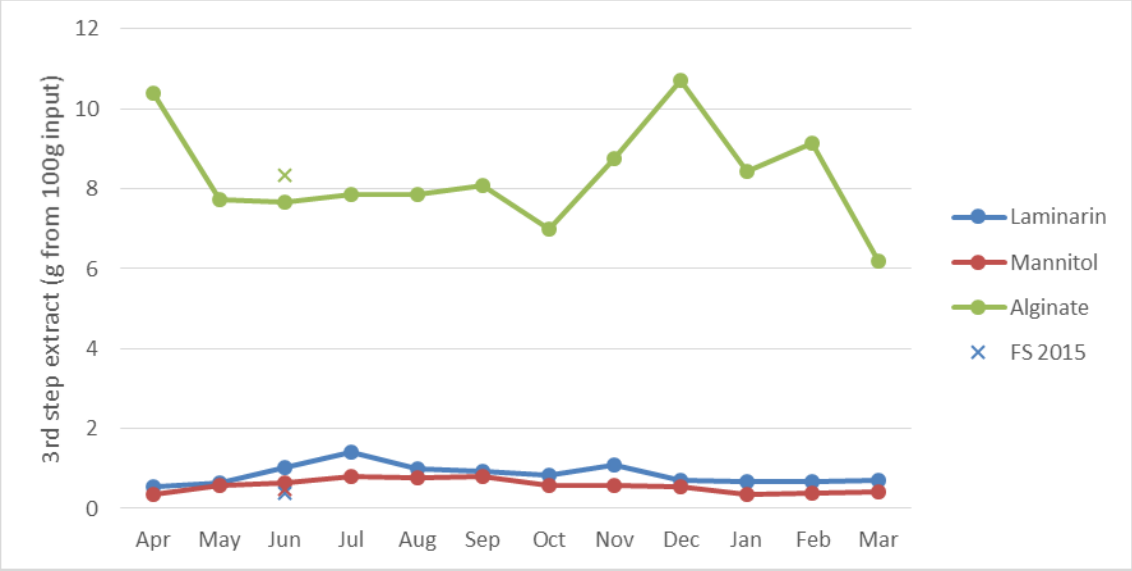


Figure 6.30: Potential seasonal variation in the composition of the third step extract, based on percentage extraction in this study.

6.4 Effect of Microwaves on the Structure of Fucoidan

While the feasibility of extracting fucoidan via sequential microwave processing has been validated, it is important that the quality of the fucoidan is assessed. This includes evaluating the purity of the crude product and the quantity of potential contaminants such as alginate, protein, laminarin and salts, as well as its fucose and sulphate content. Sulphate is of particular import, being reported to hold the key to the bioactivity of the carbohydrate [164]. As previous studies have shown processing at higher temperatures in HCl to reduce the sulphate content of the extracted fucoidan [163], assessing the sulphate content of the extracts is key. In order to achieve this, SEC, CHNS, LCMS and fucose and sulphate analysis has been carried out, in line with the study into the seasonal variation of fucoidan in Chapter 3. This gives a full picture into the quality of the fucoidan extracted and to gain some insight into the structure of the fucoidan from the unprocessed biomass, as well as any structural changes which have been caused by the microwave method. Although 120°C second step extract was shown to contain fucoidan closer in MW to that of the standard in section 6, the

140°C second step fucoidan was also analysed. It is important to understand how the fucoidan is degraded with microwave processing for industrial applications and to ensure the quality of product is maintained within variations in the process.

Fucoidan from each step of the sequential microwave biorefinery process has been extracted: from the untreated biomass and the first step residue via the long, conventional extraction procedure and from for the 120°C and 140°C extracts from the second step, fucoidan has been purified from the liquid phase using ethanol precipitation, before which alginate was removed with $CaCl_2$. The solid precipitates have been diluted to 2.5mg/ml for analysis.

The SEC chromatograms of the extracted fucoidan are shown in Figure 6.31. The fucoidan from the raw biomass, first step residue and 120°C extract give similar shaped main peaks, with little variation in the width, indicating minimal change in MW of the polymer. These peaks also have good agreement with the standard. Fucoidan form 140°C processing shows a distinct broadening of the main peak, indicating a change in the MW.

With regards to the secondary peaks, they show an increase in size and length with increased processing and temperature, which could indicate breakdown of fucoidan or perhaps a loss of sulphate groups. These will be associated with the fucoidan molecule, so will be “dragged through” the column, despite their smaller size. It is also possible that there are some salts remaining in the fucoidan extracts, which display similar shaped and sized peaks in SEC chromatograms, typically creating peaks with a long, shallow incline on the left, with a sudden drop to zero, spanning from around 15 to 20 minutes.

While fucoidan from first step processing has small amounts of laminarin, eluting at 20.2 minutes, and mannitol, eluting at 22.8 minutes, there are little impurities obvious from the SEC chromatogram for either of the second step extracts. This indicates that the extraction and purification process used here gives a relatively pure form of crude fucoidan. Depending on the intended final use of the extract, it is possible for further purification to be conducted, such as dialysis to remove remaining salts, in order to obtain a very high purity product.

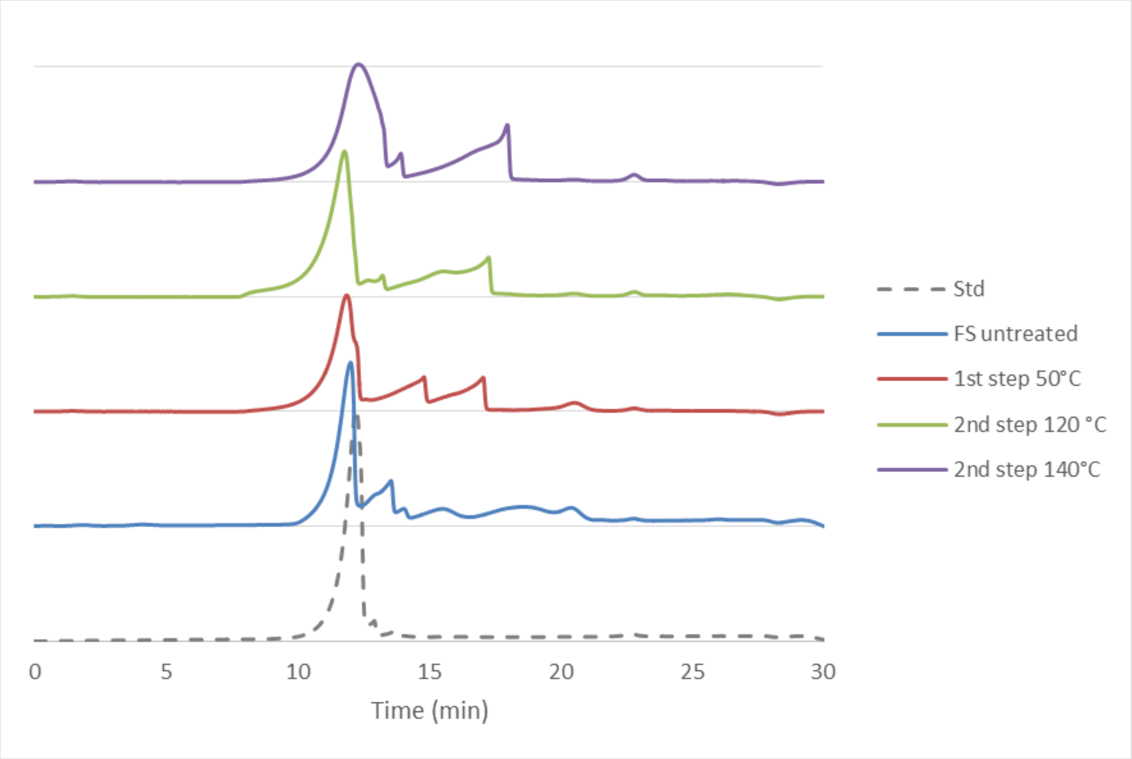


Figure 6.31: SEC chromatograms of fucoidan extracted from each step of sequential extraction.

The MW distributions of the SEC chromatograms in Figure 6.31 have been calculated, with the results shown in Figure 6.32. As expected, the abundance of each MW band for the fucoidan extracted from the untreated seaweed, from the first step residue and from the second step extract at 120°C are all similar, with only small variations between samples. The most notable of these are the bands below 250 kDa, although this is likely due to impurities in the samples such as laminarin and mannitol. For the sample from second step extract at 140°C, there is a marked decrease in the quantity of material between 2500 and 1000 kDa and a marked increase of material at 1000 to 500 kDa. This shift in MW is likely due to the beginnings of breakdown of fucoidan in to smaller MW fragments. This is in line with the broader fucoidan peak seen in the SEC chromatogram in Figure 6.31.

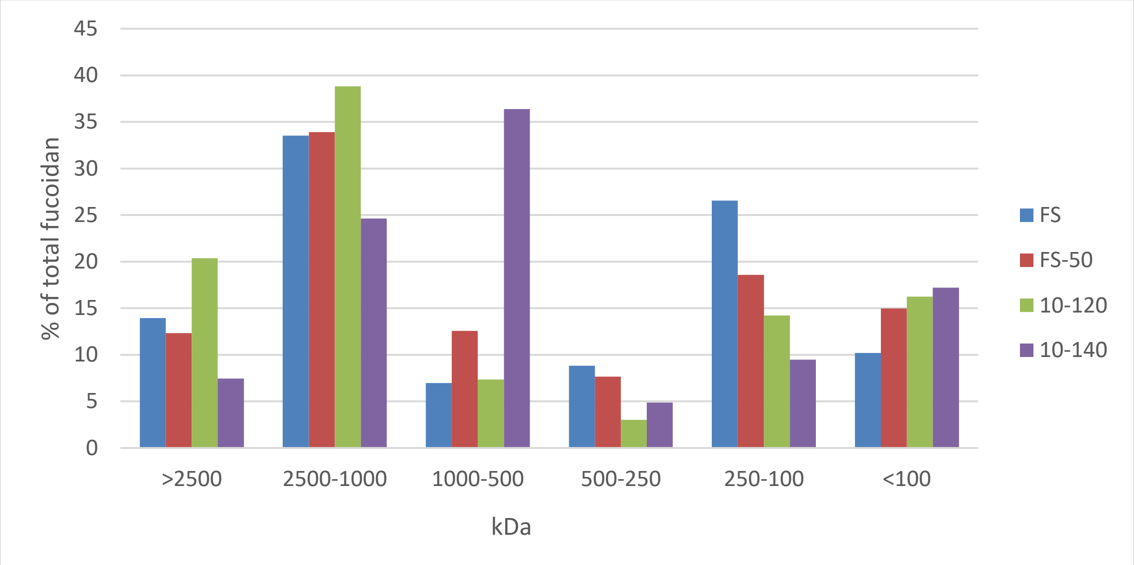


Figure 6.32: Molecular weight distribution of fucoidan extracted from raw seaweed and from the first step and second step of microwave processing.

The fucose and sulphate content of the fucoidans have been assessed, the results of which are given in Figure 5.51. The fucose content after 50°C and 120°C extraction are relatively close to that of the untreated fucoidan extract, while at 140°C, the amount is significantly reduced, presumably due to the breakdown of fucoidan. The sulphate content, arguably the more important of the two parameters due to its influence on bioactivity, shows some slight variation, with the first step extract containing 88% of the sulphate in the untreated sample and second step 120°C containing 86%. Second step 140°C contains only 55%; a significant reduction and is in line with the beginnings of break down seen in the SEC chromatograms in Figure 6.31. Untreated, 50°C and 120°C all contain more than 20% fucose, at 29.4 wt%, 26.0 wt% and 25.3 wt% respectively, which are all above the 20% stated by Ale et al. [164] for bioactivity in fucoidan. The 140°C processed sample, however, falls below this value, with only 16.1 wt% sulphate.

In a study conducted by Yuan et al. [163] into the quality of fucoidan extracted with microwaves with HCl as the extraction solvent, it was seen that the sulphate content and MW of the fucoidan extracted at the optimum yield was significantly lower than that of the conventionally extracted fucoidan: 14.7 wt% compared to 29.3 wt% for sulphate content and 40.2 kDa compared to 9.0 kDa for MW at the optimum temperature of 120°C compared to the conventionally extracted sample. While the molecular weight may have little effect on the bioactivity of fucoidan, the reduction to below 20wt% sulphate is likely to have a considerable effect, as shown by Ale et al. [164]. Extraction with water, presented here, proves to be a better extraction medium than HCl, maintaining a high level of sulphate and MW at the higher temperatures needed for good extraction.

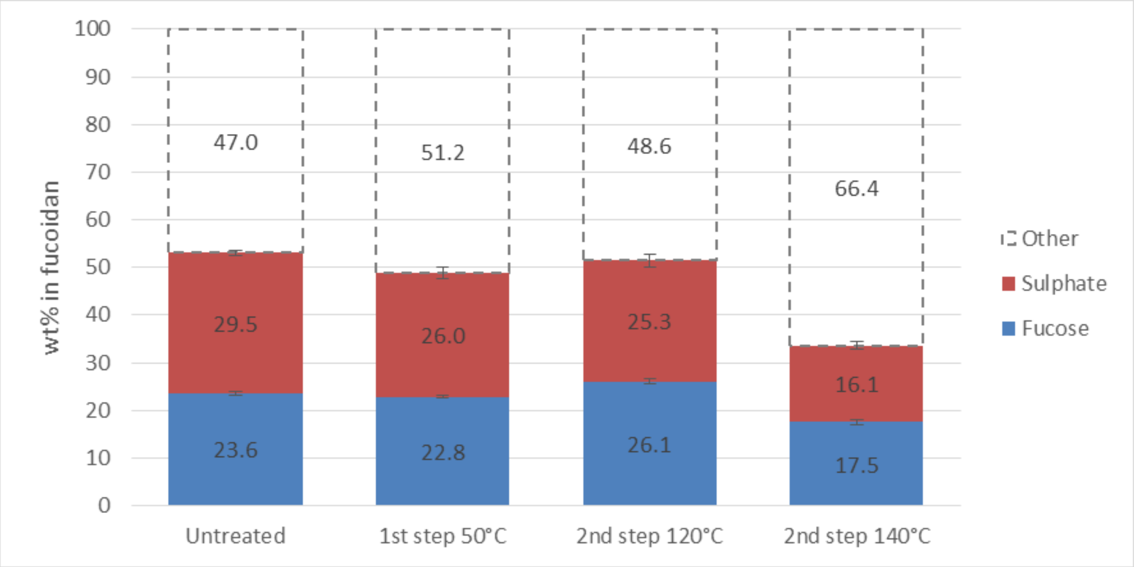


Figure 6.33: Fucose and sulphate content in fucoidan at each step of sequential extraction

Ultimate analysis of the fucoidans, shown in Figure 6.34, shows a similar trend in sulphur content as seen for sulphate in Figure 6.33, with sulphur decreasing slightly for first step 50°C and second step 120°C and a much more dramatic reduction for second step 140°C when compared to the untreated sample. The loss of sulphur is similar to that seen in sulphate, with 90, 85 and 60% of the total sulphur in the untreated sample being seen for first step, second step 120°C and second step 140°C respectively. The carbon in all samples is relatively similar, with its proportion increasing slightly as the quantity of sulphur decreases. Hydrogen and nitrogen are also constant across the four samples. The low nitrogen value, below 0.7 wt% in all cases, indicates low contamination from protein in the fucoidans.

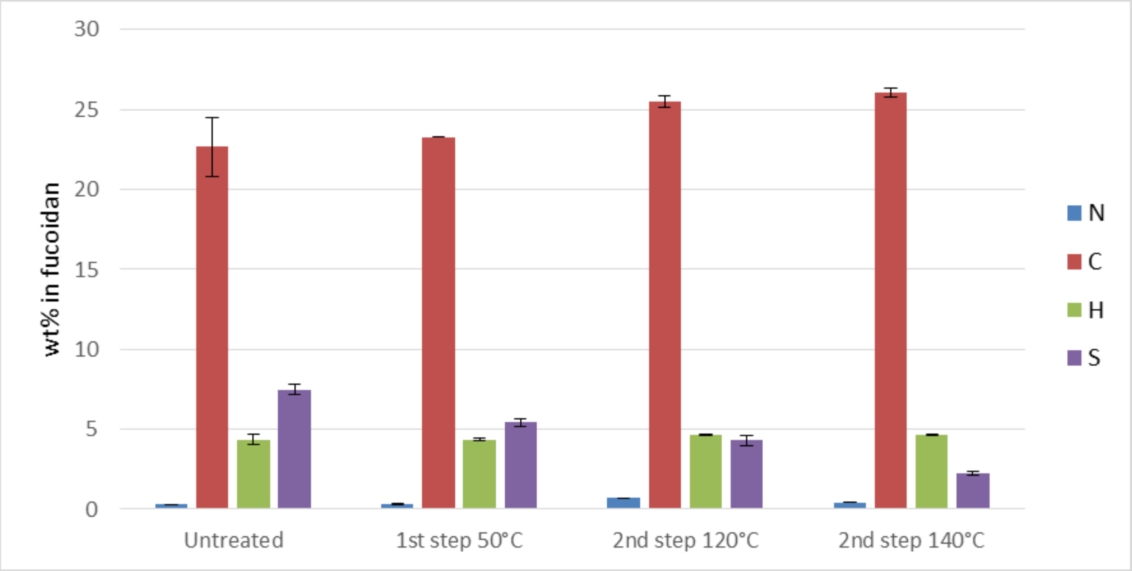


Figure 6.34: CHNS of fucoidan extracted from each step of sequential extraction.

In order to gain some insight into the structural changes in the fucoidan due to microwave processing, LC-MS analysis has been conducted, shown in Figure 6.35, with some suggested structures for the most abundant fragments given in Figure 6.36. The untreated and first step 50°C chromatograms appear to be relatively similar, with untreated having slightly more 535 Da and 50°C having slightly more 721 Da. The overall shape and distribution of the peaks are similar. Second step 120°C shows in increase in 493 Da and a slight decrease in 535 Da over the untreated fucoidan. This indicates structural fragments are being lost at increased temperature, and the difference between these two fragments can be accounted for by the loss of 2 methyl groups and a hydroxyl group. With the exception of the peak at 535 Da, there is little difference between the chromatograms for untreated, first step 50°C and second step 120°C. The overall shape and spread of MW fragments remains similar, with the dominant peaks in the smaller fragments at 174 Da, 228 Da, 294 Da and 373 Da remaining the very similar. With regard to the chromatogram for second step 140°C, there is clearly a shift in the fragments, suggesting the structure at this temperature has been altered. Peaks at 373 Da, 454 Da and 493 Da are significantly decreased, while there appears to be a higher degree of higher MW fragments. This could be due to the lower MW fragments which will be readily removed from the polymer being lost in the microwave extraction and have not been precipitated out into the fucoidan fraction due to their small size. This would mean the fragmentation in the MS is skewed toward the higher MW fragments, and hence the change in the overall shape of the chromatogram observed here.

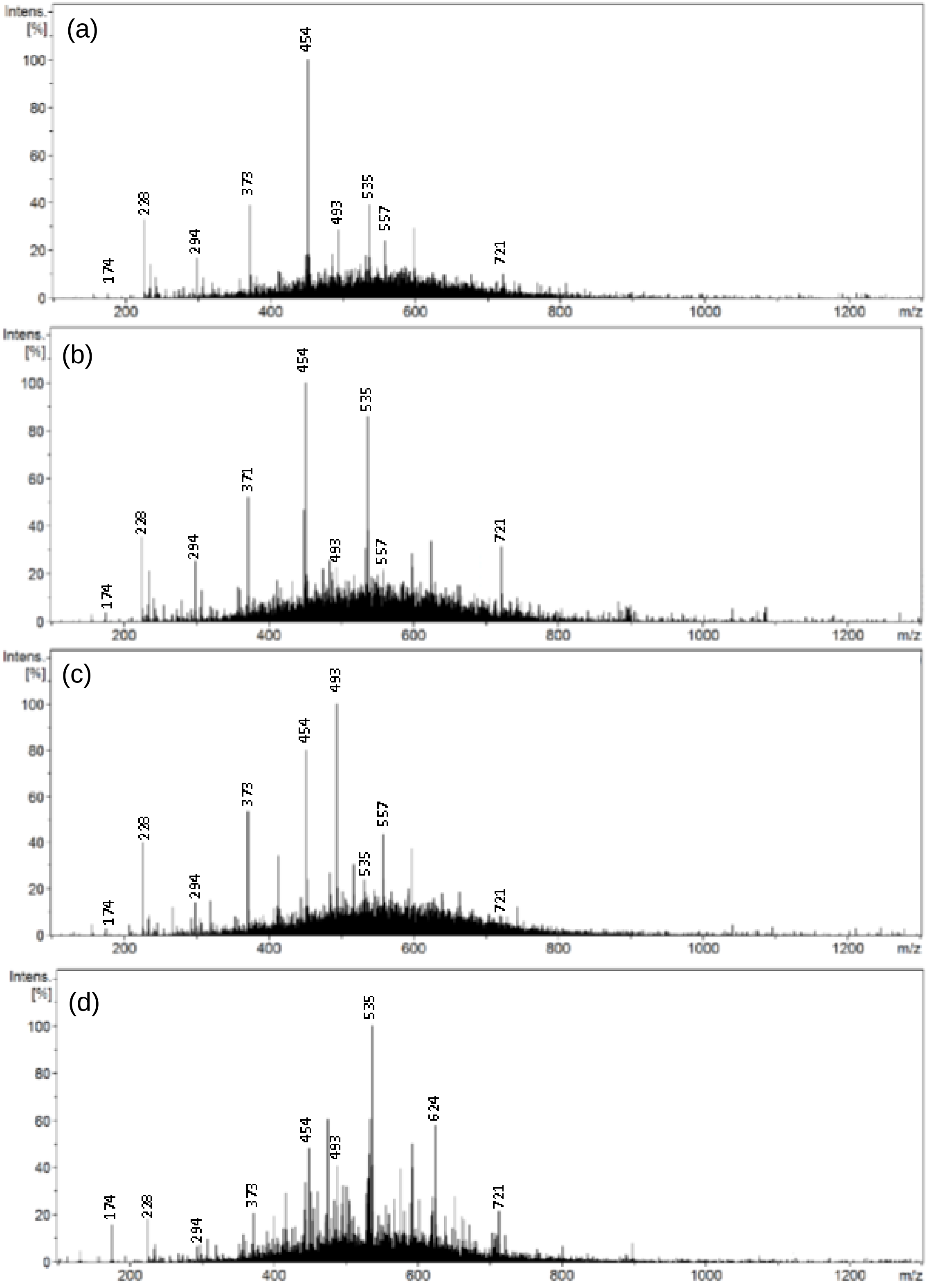


Figure 6.35: LC-MS chromatograms of fucoidan extracted from (a) FS untreated, (b) first step 50°C, (c) second step 120°C and (d) second step 140°C.

While little literature could be found on analysis of fucoidan by LC-MS, the fragments observed here are similar to those found by Thinh et al [220] from the brown seaweed *Sargassum mcclurei,* with the loss of sulphate, methyl and hydroxyl groups making up the majority of the differences between fragments. They also noted that, in some cases, the ring structure is broken during the fragmentation process, which is also likely to be the case for some of the fragments seen in this study.

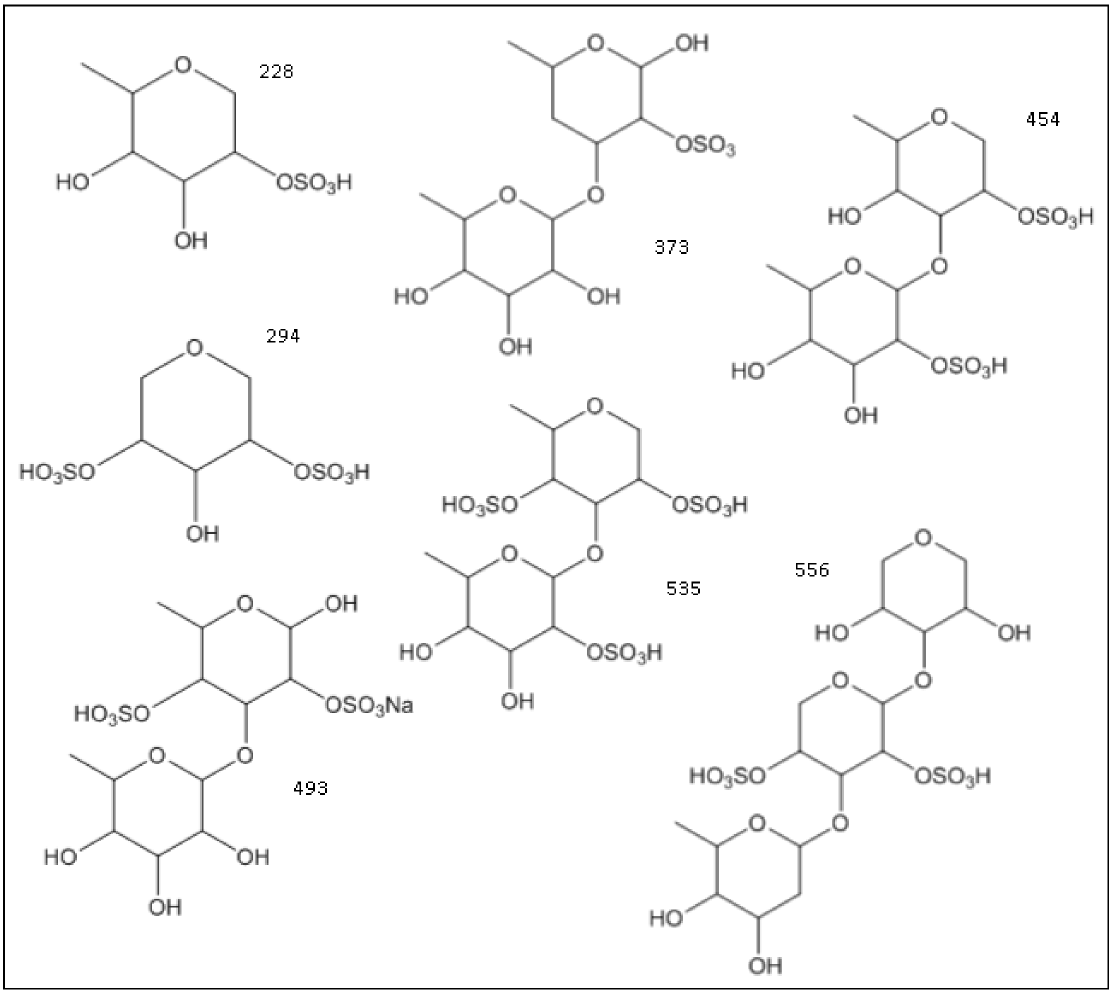


Figure 6.36: Suggested structures for the most common peaks from LC-MS analysis of fucoidan

6.5 Treatment of Residues

6.5.1 Introduction

The treatment of waste and environmental considerations are important for industrial processes, where strict limitations are applied to the quality of waste water leaving the plant and other waste chemicals must be paid for to be disposed of. In the case of this macroalgal microwave biorefinery, over the extraction of fucoidan and alginate for sale as high value chemicals, there are several possibilities for the utilisation of the remaining biomass in the first step extract and third step residue and for the purpose of this section, they will be referred to as the “waste” from the process. Figure 6.37 shows the composition of this stream, where the “other” section is likely to be primarily composed of lipids and proteins. Potential uses and upgrades to the biomass are considered, including phosphorus and nitrogen content for fertiliser, fermentation to bioethanol and AD to bio-methane. Links to the seasonal variation and potential yield of each of these components, based of year round harvest to supply the process, are also shown. This data allows good evaluation of the best harvest times for particular applications.

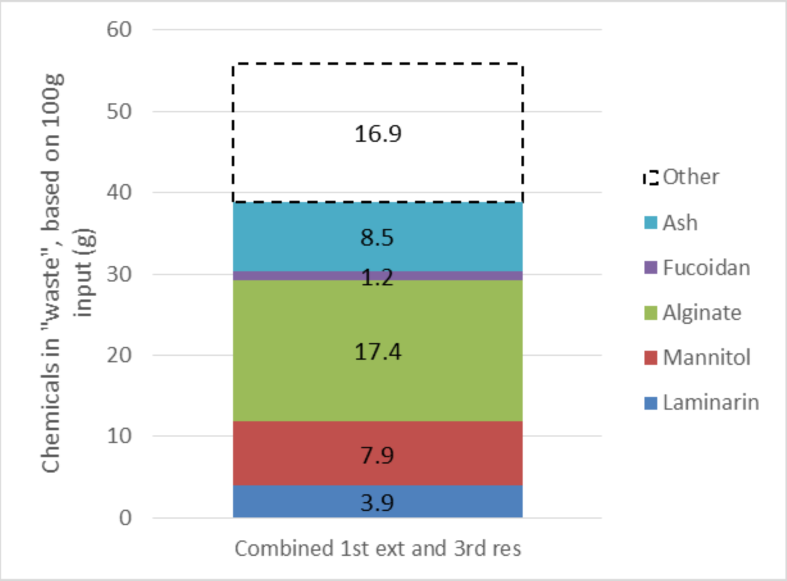


Figure 6.37: Composition of the "waste" stream, based on 100g input into the biorefinery

6.5.2 Bioethanol production from the “Waste” Stream

The bioethanol yield from FS 2015, analysed in this study and, assuming a similar extraction ratio in each step, for seasonal variation of FS, based on data in Chapter 3, has been calculated and is shown in Figure 6.38. These results are based on yield data from microwave extraction of 36.1% and 90.8% of the total input of laminarin and mannitol respectively in the combined 50°C extract and 120°C NaCl residue. From the 2010 samples, the best harvest time for maximum bioethanol yield would be July, with the minimum yield obtained between January and April. However, FS 2015 shows a lower value. As discussed before this is probably due to variation in chemical content for the time of year due to weather conditions. Presumably 2010 had a warmer spring, while 2015 a colder one. Further laminarin could be recovered from the fucoidan extraction in second step, which would increase the ethanol yield. As this needs to be removed for fucoidan purification, it would require little extra process steps to include this in the fermentation. In order to validate these results, samples from throughout the year would need to be processed to assess the extraction yields at different steps.

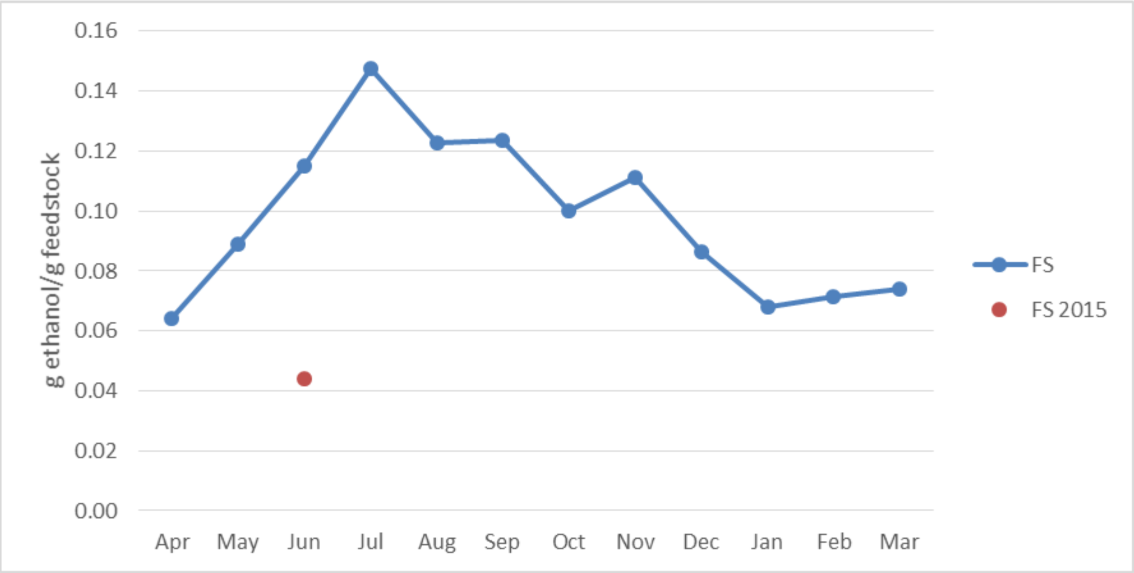


Figure 6.38: Theoretical maximum values of ethanol from fermentation of mannitol and laminarin in microwave residues from seasonal variation samples and FS 2015.

In a review conducted by Jiang et al [176], ethanol yields from unprocessed brown macroalgae are quoted to range between 0.3 and 2.9 g ethanol/ g biomass. While the theoretical ethanol yields in this study are shown to be significantly less than this, it is to be expected due to the extraction of some of the fermentable carbohydrates throughout the rest of the biorefinery process.

Furthermore, previous studies have focused on kelp species, which are known to have a higher mannitol and laminarin content that Fucoids. While fermentation of the “waste” streams would be possible, the low ethanol yields means that it is likely that a different use would be more favourable, both in terms of maximising profit from the biomass, but also for minimising waste as only a small portion of the material in the “waste” steam is utilised by fermentation.

An advantage of producing ethanol from the “waste” is the ease at which ethanol could be recovered from fucoidan precipitation in the second step, as the equipment needed, including distillation and some sort of de-watering equipment, such as molecular sieves, would also be required for the purification of the ethanol produced via fermentation. Similarly, the ethanol required for the production of fucoidan could be produced on site. Even is ethanol is not produced on site and the “waste” used for a different purpose, it would still be advantageous to recover the ethanol used in fucoidan precipitation, in order to decrease solvent usage and associated costs.

6.5.3 Bio-Methane Production from the “Waste” Stream

The theoretical methane (CH_4) and CO_2 yields from the “waste” stream have been calculated for FS 2015 and for the seasonal variation data, the results of which are displayed in Figure 6.39. These calculations are based on those detailed by Buswell [221] and Boyle [222]. Both the CH_4 and CO_2 values are relatively constant over the year, with an average value of 262.9 L/kg TS and 191.3 L/kg TS respectively. The values calculated for FS 2015 are significantly lower than those seen for the seasonal variation samples collected in 2010. This is due to the lower carbon content of the biomass due to the presences of less mannitol and laminarin present in the initial biomass.

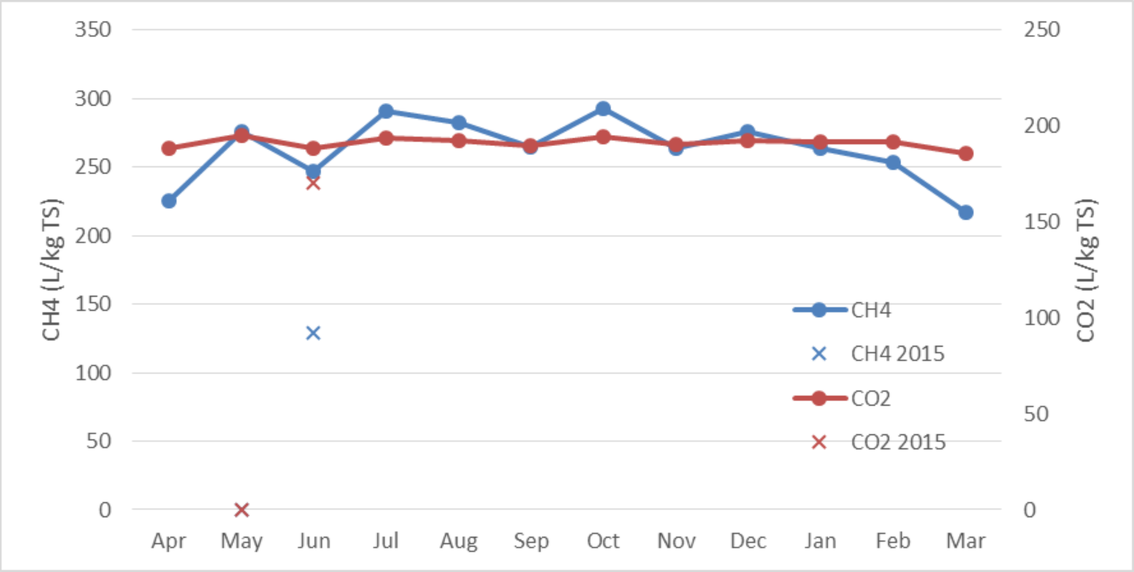


Figure 6.39: Theoretical maximum yield of methane and CO_2 *calculate using Buswell Boyle equation. TS = total solids*

Allen et al [118] note that the C:N ratio is very important in gaining the best yield of CH_4 from AN feedstocks, with a low ratio leading to inhibition of CH_4 production by ammonia. They quote the optimum values to be between 25:1 and 30:1. Figure 6.40 shows the C:N value of the “waste” stream for June 2015 and the seasonal variation in this value expected from the 2010 samples. The C:N value for untreated FS 2015 is 17.7:1, so there is a significant improvement in the “waste” C:N value of 26.5:1 compared to the untreated, putting it within the range of the optimum values. The seasonal variation shows an inverse trend for that seen for the nitrogen content (see Chapter 3 for data), with lowest values in March/April and peaking in the summer. From this, the optimum months for bioethanol production, according to Allen et al [118] would be June and between November and December. However, their data shows that higher C:N ratios also give a good CH_4 yield. According to the literature, on the whole, untreated seaweed performs poorly in AD, despite a good theoretical yield, due to a low C:N ratio [118, 119]. Using the “waste” from the proposed biorefinery appears to significantly improve this ratio and should, theoretically, improve the conversion to CH_4 seen in real life. Further experimentation would need to be undertaken to confirm this, but these initial calculations indicate a good possibility for this technology to be applied in this setting.

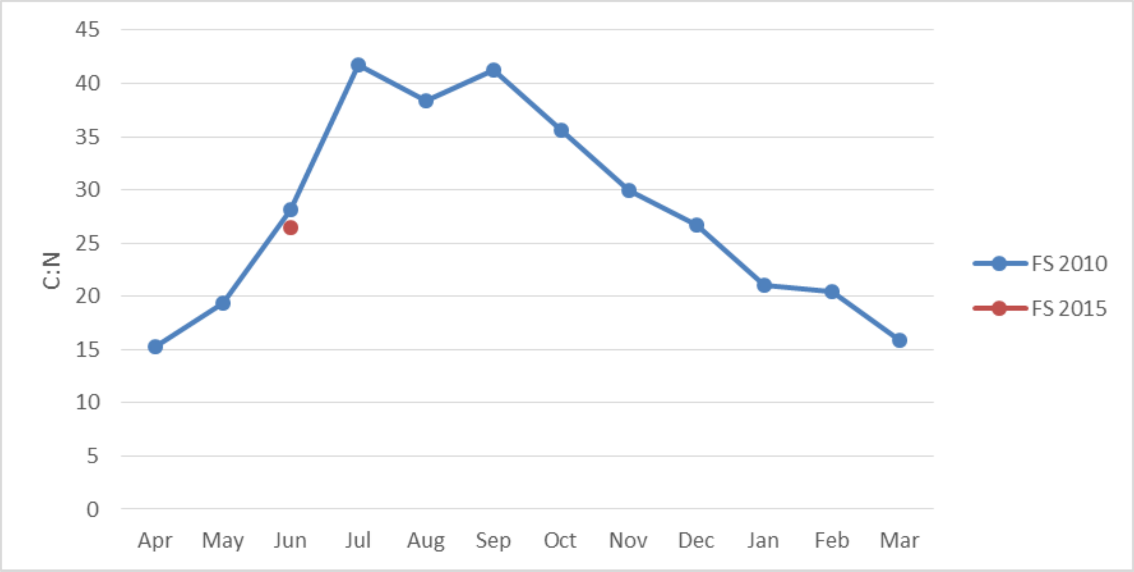


Figure 6.40: C:N ratio of the "waste" for FS for 2010 seasonal variation and June 2015

Tabassum et al [122] have studied the seasonal variation in bio-methane production from *Laminaria digitata*. They have found an average theoretical methane yield of 323.1 L/kg TS, utilising the whole biomass, which was found to be roughly halved to 191.9 L/kg TS when tested under laboratory conditions. They also found the seasonal variation in the CH_4 production to be strongly correlated with the total carbohydrate content, as expected. A review of AD of seaweed conducted by McKennedy and Sherlock [223] quotes the CH_4 production from a variety of seaweed and seaweed industry waste products to be between 80 and 425 L/kg TS. The theoretical CH_4 production from the seaweed “waste” seen here falls well within these boundaries and seems to be a good option for the biorefinery process. Furthermore, AD can also make use of the alginate, lipids and protien present in the remaining biomass, which bioethanol production currently is unable to do. This would mean that a greater proportion of the “waste” biomass could be converted into a useful fuel, increasing potential value.

6.5.4 Potential for use of “Waste” as a Fertiliser

Fertiliser requires a high quantities of N, P and K in order to feed plants with the nutrients they require for growth. The phosphorus balance, shown in Figure 6.41, shows only a small quantity in the initial biomass; an average of 0.37wt%. This figure is roughly halved with each step, with the final residue after the third step containing only 9.2wt% of the initial quantity. Combining the phosphorus in the residue and the first step extraction, which are the two “waste” streams from the extraction process, leads to a recovery of 52.3wt% of the initial phosphorus. The phosphorus value found by Ross et al [224] for FS is much higher than that seen here: 1.4wt% as compared to 0.37wt% in this study. This could be due to a differing harvest location (Cornish coast as opposed to Aberystwyth) and, therefore, quantity of phosphorus in the water. Furthermore, samples collected by Ross et. al were washed before analysis, which could leach some of the water soluble chemicals out, making the P account for a higher wt% overall. Samples were also collected in February, as opposed to June in this study, which could have an effect on the quantity of phosphorus present, as the total ash content is known to vary throughout the year and is generally higher in the winter months compared to the summer.

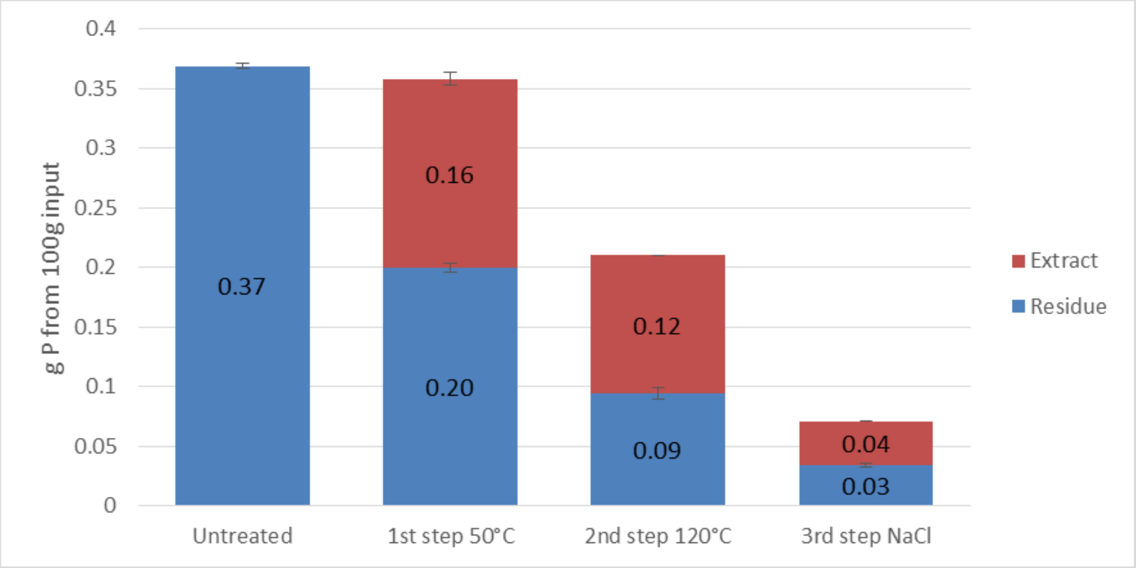


Figure 6.41: Phosphorus balance for sequential extraction, based on 100g input of FS.

To illustrate the variation in phosphorus, the potential quantity in the "waste" stream has been evaluated using the seasonal variation data presented in Chapter 3. The results of this are shown in Figure 6.42. Phosphorus is shown to remain fairly constant over the year, with a high of 2.7 g/kg of "waste" in the April and low of 1.7 g/kg of "waste" in October. The comparison between the June 2010 and June 2015 samples shows a relatively similar quantity of phosphorus.

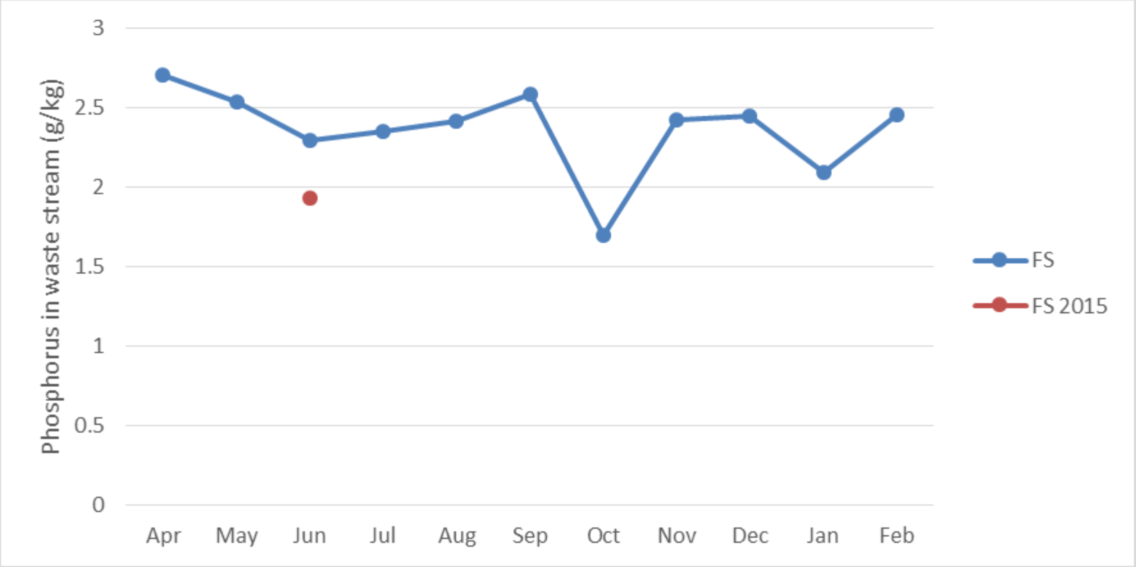


Figure 6.42: Seasonal variation of phosphorus in combined first step extract and third step residue

The nitrogen content of the biomass at each step has been analysed and the results are shown in Figure 6.43. There is a relatively small amount of nitrogen in the initial biomass, averaging 1.8wt%. 30.9wt% of this is extracted in the first microwave step, with no further extraction seen in the second step at 120°C. The greatest extraction of nitrogen is seen in the third step, where NaCl has been added. Here, 60.6wt% of the initial nitrogen is extracted. The combined total of the “waste” accounts for 57.6wt% of the total in the raw biomass.

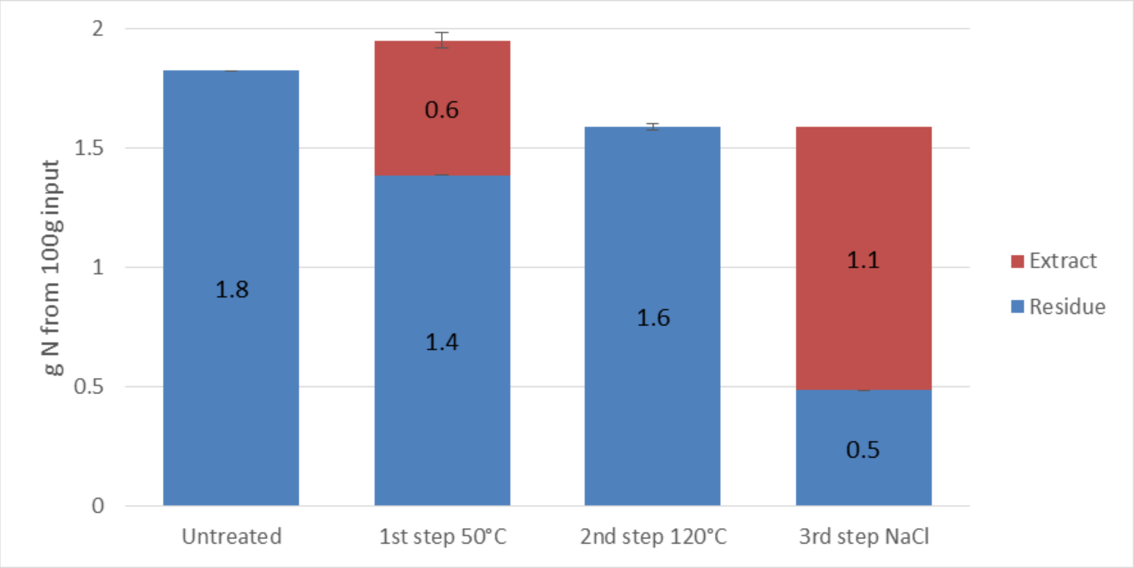


Figure 6.43: Nitrogen balance for sequential extraction, based on 100g input of FS.

The potential nitrogen variation in the “waste” stream over the year has been calculated and the results are shown in Figure 6.44. Nitrogen has a distinct seasonal variation pattern, so there is significantly less nitrogen remaining in the waste between June and November than the rest of the year. This could have potential impacts on the quality of fertiliser which could be produced at different times throughout the year from this process. If fertiliser was seen to be the best option for the “waste” stream, however, it would be possible to upgrade the fertiliser by adding nitrogen from a different source to ensure a consistent product. A comparison of the June 2015 and June 2010 samples shows similar quantities.

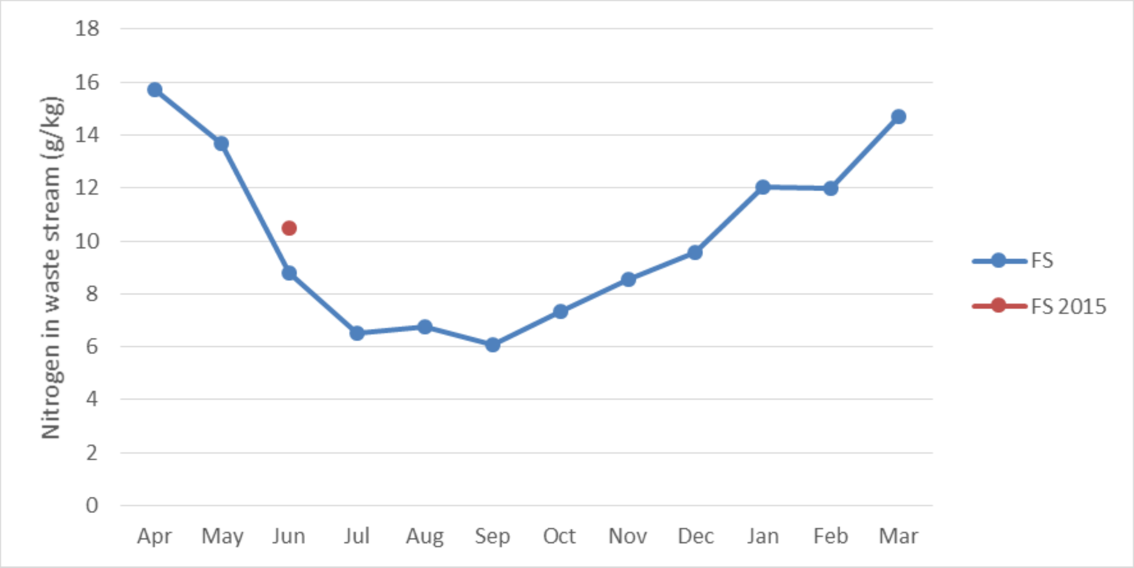


Figure 6.44 Seasonal variation of nitrogen in combined first step extract and third step residue

The potassium balance from the microwave macroalgal biorefinery is displayed in Figure 6.45. The initial potassium in the biomass is relatively high, averaging 3.7 wt%. Roughly half of this is removed in the initial step, with the majority of the remaining potassium in the first step residue being extracted in the second step. The increase in potassium in the third step is likely to be due to impurities in the NaCl added for processing.

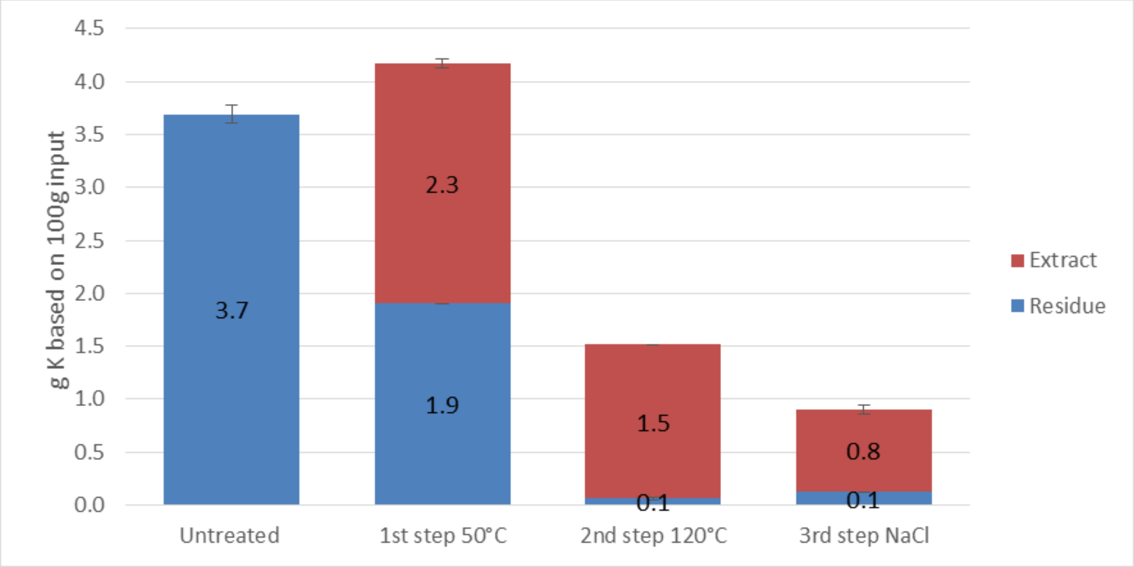


Figure 6.45: Potassium balance for sequential extraction, based on 100g input.

The potassium in the “waste” stream accounts for roughly 30% of that in the initial biomass. The potential seasonal variation of potassium, calculated based on this figure from the samples of FS collected in 2010, has been calculated and the results are shown in Figure 6.46. There appears to be little seasonal variation in the potassium content, which is advantageous for fertiliser production from the biorefinery “waste”, as year round harvest would lead to a relatively consistent product. Again, the comparative June samples from 2010 and 2015 are similar in potassium content.

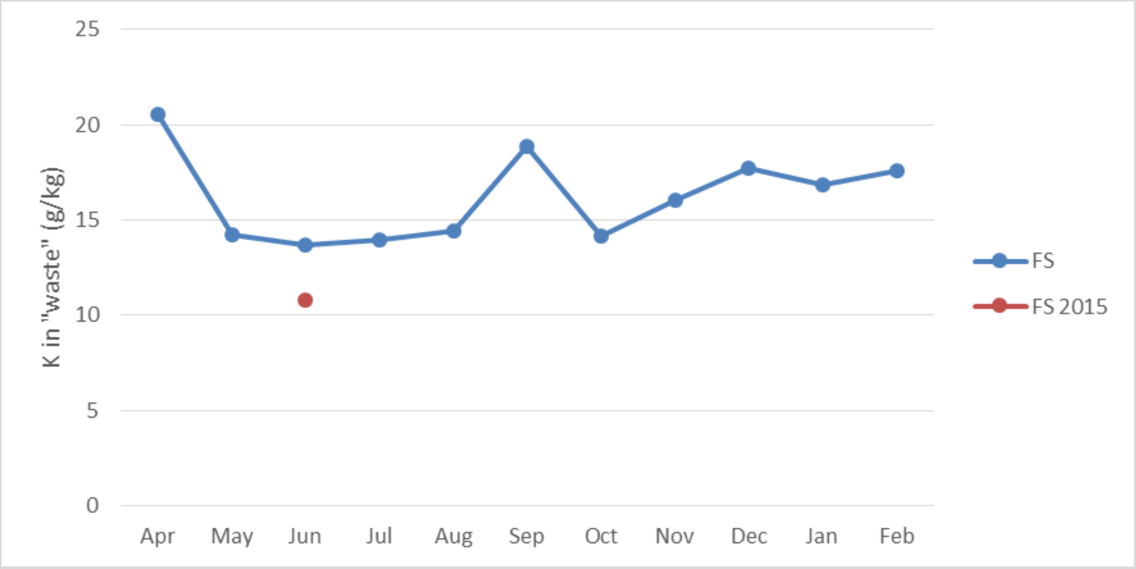


Figure 6.46: Seasonal variation of potassium in combined first step extract and third step residue

The NPK value of the “waste” biomass is 10.5:1.9:10.7 (2.31) from FS 2015, where the number in brackets represents the percent of the total biomass which is comprised of these nutrients and the values of N, P and K are ratio of these nutrients within that percent [225]. While the potassium value is low, depending on the application, the NPK ratio is relatively good. However, the quantity of these nutrients within the biomass is low, as typically fertilisers contain a high concentration of these nutrients. However, the high carbon content could make it a good “soil improver”, more like a compost or manure, rather than a fertiliser. It also has the added bonus of containing other nutrients necessary for plant growth, such as magnesium and calcium.

While potassium and phosphorus has been shown in this study to remain fairly constant over the year, meaning a relatively consistent fertiliser composition, nitrogen shows a clear seasonal variation trend, with greatest quantities in March/April and lowest in August/September. The lower nitrogen content in the summer months would have a negative effect on the quality of the biomass, as it will contain less nutrients. If year round harvest and processing of seaweed biomass, with the “waste” to be used as a fertiliser is to be used, a nitrogen rich improver will need to be added “waste” during the summer months in order to create a consistent product year round. This could be achieved via the addition of a nitrogen rich biomass source or by adding ammonia.

The heavy metal content of fertilisers is regulated by the EU [217] and is, therefore, an important consideration for the use of seaweed “waste” from the biorefinery process as a fertiliser. Heavy metals in seaweeds are typically high [213], as they bio-accumulate them throughout their lifetime from seawater. Figure 6.47 shows the heavy metal content of the “waste”, alongside the EU regulation limit (Cr and Cd are shown in µg/kg for clarity, all other metals are shown in mg/kg). Aside from As and Zn, all metals fall within the limits set for fertiliser use, although Cu is only a couple of mg less and variation in this metal could put it outside of the regulated value. This means that the seaweed “waste” would not be able to be applied as a fertiliser directly to crops and would instead either need to be further treated to reduce the Zn and As content or blended with another biomass source low in these metals, to reduce their overall concentration.

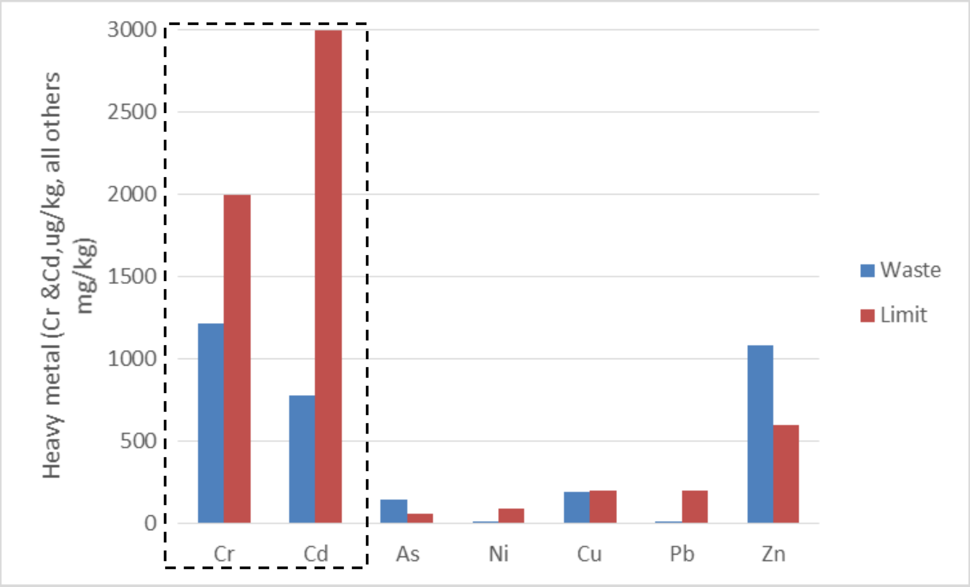


Figure 6.47: Regulated heavy metal content on combined first step extract and third step residue.

6.5.5 Other Potential Uses for the "Waste" Stream

Over bioethanol or bio-methane production of for direct use as a fertiliser, there are some other possible uses for the "waste" stream from the proposed biorefinery process. Smith et al [226] have used hydrothermal carbonisation (HTC) to produce bio-coal, bio-methane and fertiliser from seaweed and the process described may be a good option for utilisation of "waste" seaweed as well. The CHNS composition of the "waste" is similar to that of the three kelps, *L. digitata, L. hyperborea* and *A. esculenta*, presented in their study and the comparison of the two is presented in Figure 6.48 and they also have a similar ash content. Although further work would need to be carried out in order to confirm this, it is likely that similar results would be seen for the "waste" stream as were described by Smith et al [226], with a significant upgrade in the energy density of the residue. They quoted this to be from 10 MJ/kg to 25 MJ/kg in their study, a value similar to that of low grade coal, alongside the process water being a potential feedstock for bio-methane production.

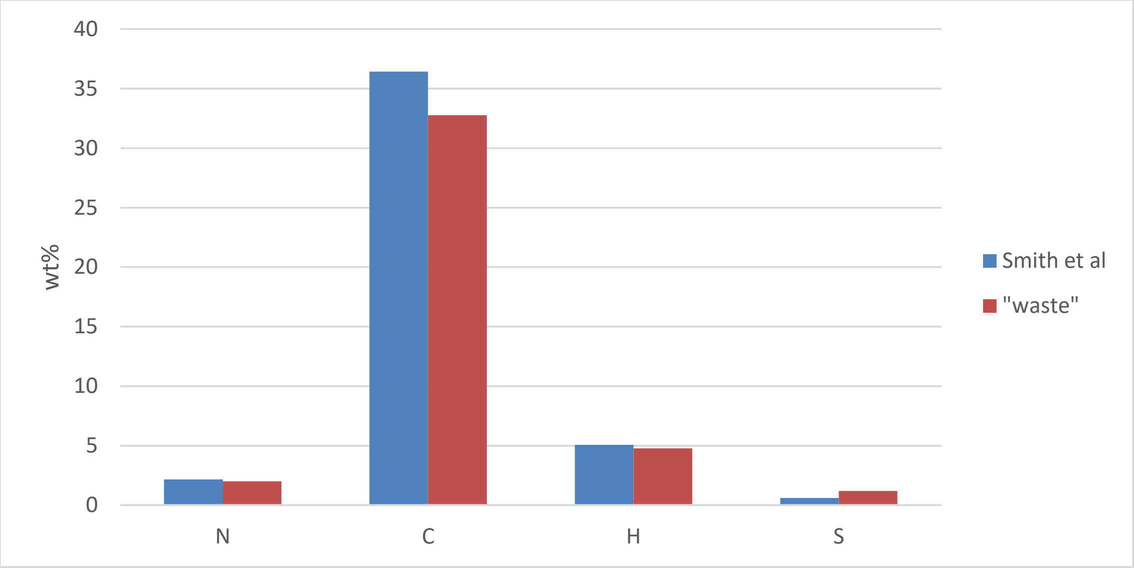


Figure 6.48: Comparison of CHNS values adapted from Smith et al [226] and the "waste" from the microwave biorefinery process.

It is possible to create a range of other building block chemicals from seaweed: succinic acid [128], 5-hydroxymethylfurfural (HMF) [175] and hydrogen and butyric acid [55] via fermentation; the use of sugars as a feedstock for polymer production [56, 227] and production of intermediates for detergents, fuel additives and plasticisers [57] are among the many possibilities for chemicals which could be produced. Based on product demand, the mechanisms and processes involved in creating these chemicals would require further research and development, but the idea offers a wealth of possibilities for a biorefinery, allowing adaptability to the process to fit requirements.

6.5.6 Waste Water Treatment

Waste water treatment is an important consideration for any industrial process, with strict limits on the quality of water released from the system which must be implemented. These limits are significant for environmental reasons, limiting the effects of eutrophication from a high nutrient content and damage to wildlife from heavy metals. In the case of seaweeds, the release of metals is the main concern, as seaweeds are famed for their accumulation of heavy metals and their high ash content [21-24, 51, 81]. Table 6.2 details heavy metal limits laid out in the EU directive on industrial emissions for waste water [192], alongside the total metals from the biomass in the waste water from the biorefinery process. On the whole, the amount present falls within the limits set out, with the exception of Al, Cu and Ni, highlighted in red. In these cases, the amount is clearly well above the limit, with Ni exceeding by over 28 times. The metals laid out in the EU directive are among the most hazardous, due to their increased solubility in aquatic environments, their ready adsorption by living organisms, their accumulation in living bodies and the serious health issues they can cause [228].

Table 6.2: EU limit on heavy metals in industrial waste water compared to the quantity in the waste water from the biorefinery process.

Metal	**Limit (mg/l) [192]**	**Amount in waste water (mg/l)**
Hg	0.03	0.00±0.0
Cd	0.05	0.01±0.0
Tl	0.05	0.00±0.0
Al	0.15	4.42±0.3
Pb	0.2	0.07±0.0
Cr	0.5	0.01±0.0
Cu	0.5	3.04±0.8
Ni	0.5	14.13±0.3
Zn	1.5	0.10±0.0

There are several options for the removal of heavy metals, a summary of which, including advantages, disadvantages and environmental considerations are laid out in Table 6.3. From this summary, it is clear that there are a lot of options, with the best one being dependent on the size and scale of the process. From an environmental point of view, the use of photocatalysis, which removes unwanted metal ions via a semiconductor charged by UV rays from the sun [228], presents the lowest energy option. However, with intermittent weather in the UK, this may not prove to be the most reliable option. The three most common options currently used in industry are chemical precipitation, electrochemical removal and adsorption [229]. While having the benefit of being well known to industry, they do not offer the best separation of heavy metals, especially at low concentrations. With the regulations on wastewater quality likely to only get stricter with time, it would be prudent to choose a technology which could withstand more stringent regulations without the need for modification. Thus, in this case, membrane filtration most likely represents the most promising option. Over the relatively simple technology, easy operation and compact, space saving design ideal for small plants [229], it is possible the set up sequential membranes to remove organic matter and heavy metals in one step. This means less equipment is required to treat the wastewater and, therefore, a reduction in energy demand [228].

Table 6.3: Options for the removal of heavy metals from waste water and their advantages and disadvantages.

Technology	Description	Advantages	Disadvantages	Environmental considerations
Chemical precipitation	Metal ions are removed via conversion to an insoluble form, often via the addition of hydroxides, before removal by filtration or sedimentation.[3]	• Most widely used[2] • Simple and inexpensive[2]	• Incomplete removal[1] • High energy requirements[1] • Production of toxic sludge[1]	• Requires the use of solvents. • Difficult to get good removal of metals due to mixed ions present.[3]
Electrochemical removal	The application of electricity can be used to,	• Scalable[4] • Simple and compact[4]	• Incomplete removal[1]	• High electricity requirement

	depending on the voltage applied, convert dissolved ions into their solid form [5]	- Widely used in industry[4]	- High energy requirements[1] - Production of toxic sludge[1]	
Adsorption	Metal ions are adsorbed onto a highly porous structure such as activated carbon, which is added to the waste water.[6]	- Flexibility in design and operation[2] - High quality treated effluent[2] - Adsorbents can be regenerated[2]	- Requires expensive adsorbents[7]	- Adsorbent regeneration can be energy intensive.[7]
Membrane filtration	Membranes are used to either filter out larger ions or via a difference in hydrostatic pressure by reverse osmosis.[2]	- Easy operation[2] - Space saving[2] - Removes heavy metals and larges organic matter[1]	- Possibility of membrane fouling requiring cleaning[8]	- May require less steps, meaning less energy usage[1]
Electrodialysis	The separation of ions over a membrane using an electric field as the driving force[2]	- Very good separation of ions[2] - Resistant to scaling and fouling: good for brackish water[9]	- Currently application specific due to interactions associated with various water chemistries[9]	- Energy intensive due to electricity demands[9]
Photocatalysis	Uses the UV rays from the sun to separate metal ions with a semiconductor. The UV rays charge the semiconductor, drawing the metal ions to it.[1]	- Fast and efficient[1] - Low energy requirements[1]	- Requires expensive semi-conductors as catalysts, such as titanium dioxide[1]	- Uses solar energy to power so low energy demand[1]
[1][228], [2][229], [3][230], [4][231], [5][232], [6][233], [7][234], [8][235], [9][236]				

6.6 Conclusions

During this study, the viability of FS in a hydrothermal microwave biorefinery has been proven. The sequential extraction of chemicals has been developed in a three stage process, with the waste biomass being assessed for its potential in bioethanol or bio-methane production or as a fertiliser. The microwave process involves first a low temperature pretreatment step, where raw, dried biomass is processed at 50°C to remove mannitol, metals and free, water soluble laminarin and alginate. The residue from this step is further processed in water at 120°C, extracting fucoidan, along with some soluble alginate and laminarin. The fucoidan is purified by precipitating the alginate with $CaCl_2$ and the laminarin with ethanol before fucoidan is also precipitated with ethanol, gaining a relatively pure, crude fucoidan fraction. In the third step, insoluble Ca-alginate is extracted by processing with NaCl at 120°C.

The process waste, namely the first step extract and third step residue, have been assessed for their potential for further usage. While fermentation would be viable, the yields are low due to the relatively low carbohydrates remaining after extraction and the lack of suitable organisms to ferment alginate. Use as a fertiliser or soil improver also appears to be a viable option, due to the high P, K and N content in the biomass, alongside other useful minerals. However, the high heavy metal content means it is unsuitable for direct application to soil, as Cr, Cd, Ni and Cu exceed the maximum EU limit for fertilisers.

The most promising use for the waste biomass, therefore, is anaerobic digestion to produce bio-methane. The theoretical yields is in line with those from the literature for untreated seaweed biomass, with the added benefit of an improved C:N ratio due to the removal of carbohydrates during the microwave extraction process. Furthermore, the flexibility of the AD process would allow mixed feedstocks, potentially including waste from other sources to be added to the mixture.

The results for the detailed study for the microwave extraction of chemicals in FS 2015 has been used to calculate the possible seasonal variation in the process, using the data gathered in chapter 3 and the ratio of components extracted in this study. While an in depth study would need to be conducted to confirm the results, they offer an insight into the potential problems the seasonal variation in seaweed would cause to a year-round process. The biggest variation is seen in the mannitol and laminarin content. The former, mostly extracted In the first step, only has major implications for the use of the waste. As AD is a fairly flexible process, the biomass can still be utilised for bio-methane production. While the variation in laminarin means extra purification of the fucoidan, its presence doesn't alter the quantity of ethanol needed for purification and extraction, as the precipitation is based on volume ratios of water to ethanol and not the quantity of either laminarin or fucoidan present in solution. This is beneficial for industry, as solvent requirement will remain the same despite fluctuations in product quantity. The amount of fucoidan generated, however, will remain fairly constant, as will the amount of alginate from the third step. Again, although there are slight fluctuations in the quantity produced, the excess if NaCl used for extraction covers the variation in quantity, meaning no changes to the process over the year are necessary.

The quality of the fucoidan extracted in the second step has been assessed and compared to that of the raw biomass and that present in the first step residue. Processing in water at 120°C appears to have little detrimental effect on the fucoidan, with only a slight decrease in sulphate content and no discernible effect on the fucose content. Comparison with the fucoidan extracted at 140°C shows a significant decrease in the sulphate content, indicating the degradation of the fucoidan at this temperature. LC-MS and SEC chromatograms back-up these findings, indicating comparable fucoidan between raw, 50°C and 120°C extractions and the beginnings of degradation at 140°C. While further studies to confirm its bioactivity would need to be conducted to give credibility to this method as a fucoidan extraction technique, the initial results and comparisons with literature are promising.

Finally, the treatment of the waste water has been considered. Due to the high ash content of seaweed, the need to remove heavy metals before release to the environment is important, with Al, Cu and Ni concentrations significantly exceeding EU limits. In this case, it is concluded that membrane filtration, with its simple, compact design would offer the best option, allowing for sequential membranes to remove both organic matter and metals in sequence. As small plants operated near the coastline would be beneficial to overcome harvest and storage issues, a simple, space saving design which can remove multiple contaminants in one system would offer the most economical solution.

7 Conclusions and Future Work

7.1 Conclusions

Macroalgae offers a novel feedstock for a biorefinery, being an underutilised biomass with huge potential for industrial use. It is an abundant resource with a wealth of room for expansion in cultivation. It also benefits over terrestrial biomass as there is little to no competition with food sources. The seaweed food market is already at saturation with plenty of unused seaweed available, while land space is growing more valuable as more food crops are required for a growing population. Brown seaweeds contain a range of unique chemicals, including laminarin, mannitol, alginate and fucoidan, the focus of this thesis. While mannitol and laminarin are potential feedstocks for fuel production and alginate already being extracted for used as a thickener in the food industry, fucoidan offers the most promising economic potential, due to its range of biomedical properties and potential in the pharmaceutical industry. In terms of a seaweed biorefinery, microwaves offer a good alternative to conventional heating due to their even heating of the biomass, reduces processing times, more targeted heating and reduced energy consumption. The milder operating conditions also offer benefits for carbohydrate extraction, helping to ensure the extraction of the whole, unmodified polysaccharide: especially important when trying to retain the functionality of fucoidan. The drawback to the use of seaweed in an industrial setting is its seasonal variation in chemical content, which impacts harvesting times and extraction yields. However, with a clear understanding on this phenomenon, the impact to industry should be minimal.

In this thesis, the seasonal variation of three species of *Fucoid* have been investigated. They were chosen for their high fucoidan content and relatively low ash content, when compared to kelps. Previous studies carried out in the 1950’s have also shown them to have less variation in their chemical content, although it is still prominent. With increased knowledge and technology in analytical techniques and in a changing climate, an updated seasonal variation study was deemed necessary for the industrial use of *Fucoids*. In general for the three species, the storage carbohydrates mannitol, laminarin and fucoidan were found to be highest in later summer/early autumn, with corresponding lows in winter. The reverse is seen for ash, protein and alginate, where highest values were found in the winter months, corresponding to lowest values in the summer. These results impact the optimum harvest time for different industrial applications. For example, a high carbohydrate content is best for bioethanol production, so harvest in August/September would give the best yields. This corresponds with the highest fucoidan content, which is beneficial for a biorefinery focused on these two products. For a food application, however, when a high protein content may be desired, harvest in March/April would be more advantageous. The results from this study are important for being about to tailor an industrial process to the desired application and also for understanding the challenges seasonal variation poses to the use of seaweeds in an in industrial setting, if year round harvest were to be employed.

With the extraction of fucoidan being an important part of the proposed biorefinery and with the knowledge that fucoidan make-up and structure varies with season, harvest location and maturity, a detailed seasonal variation study, including its composition and structure, is important. Furthermore, although the literature alludes to this phenomenon, there is very little published data to support this fact. The three species of *Fucoid* were used in this study, with fucoidan being extracted and purified for analysis. FS was shown to have the highest sulphate content throughout the year, with AN having the lowest. SEC and LC-MS suggests that fucoidan from FS has more branching than the other two species, who have more linear structures. The MW variation is also lower for FV than the other two species, indicating less variation in structure throughout the year. Overall, due to the higher sulphate content, indicating the greatest functionality, FS was deemed the best option for a macroalgal biorefinery.

A colourimetric method for the rapid determination of fucoidan in a seaweed sample has been developed and validated. The method relies on a reaction between fucose, the monomer unit in fucoidan, and L-cysteine hydrochloride, with the colour reaction seen measured in a UV-vis. In order to obtain accurate results, correction for glucose and alginate is required and the fucose ratio needs to be calibrated for each species. However, when this has been done, an accuracy of ±5% was achieved. This rapid fucoidan determination requiring minimal equipment offers a good alternative to the conventional fucoidan quantification method, which lengthy and full extraction and purification and also adds benefits for industry, where a fast estimate of fucoidan in a batch of seaweed would be required to ensure complete extraction in the process and solvent requirements may differ depending the chemical composition of the algae.

A feasibility study for a macroalgal biorefinery based on sequential extraction of chemicals has been conducted, comparing microwave and conventional heating. Overall, the results indicated that sequential chemical extraction based on temperature is possible, with increased extraction yields with increasing temperature. Furthermore, the comparison of microwave and conventional heating indicated a lower processing temperature for microwave heating for the same extraction yield of fucoidan: 120°C compared to 150°C. While reducing energy requirements, lower operating temperatures are also important for retaining the structure and functionality of the polysaccharide. SEC chromatograms and sugar analysis of the extracts indicates breakdown of carbohydrates at higher temperatures. This initial study has been used as a basis for the development of a three step biorefinery process using microwaves. In the first step, pretreatment at 50°C removes mannitol and a large portion of the metals. The second step extracts fucoidan and some alginate at 120°C. After precipitation of the alginate with $CaCl_2$, fucoidan is precipitated with ethanol to give a relatively pure, crude fucoidan. The third step extracts the remaining alginate from the residue using NaCl at 120°C. This leaves a residue which mainly consists of laminarin, protein, lipids and ash. The impact of microwave processing on the quality of the fucoidan has been considered, and it was shown that the conditions used for extraction had little effect on its chemical content or structure. The energy balance from this process has been calculated and compared to the that for conventional heating. This shows a significant energy saving for microwave processing when calculated for the laboratory scale process, indicating a promising saving for an industrial scale process.

The impact of seasonal variation on the process has been assessed, using the data collected in the study in Chapter 3. The results indicate that there will be minimal impact to the process, as the variation falls within the excess of solvents used in the process. Similar seasonal variation effects have been evaluated for the utilisation of the “waste”, which has been defined as the combination of the residue from the third step and extract from the first step. Theoretical yields of bioethanol and bio-methane have been calculated, indicating the potential as a feedstock for fermentation and AD. Its direct use as a fertiliser has also been assessed. AD appears to offer the best option, with good yields of bio-methane which are fairly stable across the year. This is due to the ability of the process to use any carbohydrate or proteins present in the biomass, and not only laminarin and mannitol, which is the case for fermentation. Furthermore, the C:N value of the "waste" is more favourable than that of the raw, untreated seaweed. This should lead to a better actual bio-methane yield compared to the theoretical. While direct application of the "waste" to terrestrial crops would be beneficial, due to its high organic matter and mineral content, the levels of N, P and K are not high enough not high enough for it to be considered a "fertiliser" and instead should be classed as a "soil improver".

Overall, the biorefinery processed developed shows good potential for the extraction of fucoidan and alginate coupled with the manufacture of a feedstock for energy production and microwaves have been shown to be an effective heating method, with considerable energy savings over conventional heating. The seasonal variation studies have highlighted the potential problems with using a macroalgal feedstock for industrial uses. While there are still barriers to the use of seaweed commercially, such as harvesting restrictions in Europe, advances in cultivation and harvesting technologies mean that seaweed is a very promising resource for the future.

7.2 Future Work

The work presented in this thesis has led to increased understanding of the seasonal variation of *Fucoids* and how this impacts on the industrial use of them as a feedstock. The development of a macroalgal biorefinery using microwaves has also been achieved, showing the feasibility and potential of the technology and the process. However, the work could be continued further, with improvements being made and more in depth knowledge being achieved.

While the seasonal variation studies carried out in Chapters 3 and 4 gives a good start to understanding how differences in the chemical components of seaweed would affect its use in industry, there are still some difficulties highlighted which would need to be overcome. These include: differences in composition of the same species harvested from the same location in different years, which is presumably due to changes in weather conditions such as water temperature, hours of sunlight and chemical composition of the sea water (eg quantity of salts and CO_2); differences between the same species in different harvest locations and the variation between species. To overcome these issues, in depth chemical analysis of different species of interest over a number of years, harvested from a number of locations would be required.

A long time period for data collection is required to gain full understanding as to how the weather affects seaweed composition. Weather data, such as ambient and sea temperature, seawater salinity and the amount of sunlight reaching the sea surface would all be beneficial data for assessing how chemical composition correlates to the external weather conditions and would aid in predictions of chemical content without the need for analysis. Furthermore, analysis of individual plants from the same location would give an indication of the variation within the seaweed community and would allow for differences in maturity to be accounted for. For this to be achieved, analysis of 3 or more individual plants from each species would be required. On this line, a study detailing the differences between wild harvest and cultivated seaweed would also be beneficial. This is due to all plants in cultivated harvest being of the same age, while wild harvest stock will include a mixture of plants of varying age.

The microwave assisted algal biorefinery, has been proven to have potential, extracting fucoidan and alginate with the remaining biomass being used for fuel production or as a fertiliser. There is, however, some further research which could be undertaken to improve the overall process. Firstly, it would be interesting to ascertain if it is possible to extract fucoidan using a Ca salt, in an attempt to extract a more purified form without alginate. Soluble Na-alginate would be rendered insoluble in its Ca-alginate form. By doing so, the next step of precipitating out Na-alginate would be obsolete, while maintaining the same solvent requirement. While this study has shown that $CaCl_2$ is an unsuitable salt for this purpose, it is possible that the Cl ions are interfering with fucoidan extraction. Therefore, a different Ca- salt may give a more positive result. In order to study this more fully, a full range of different Ca-salt should be tested, as well as varying the extraction temperature and time, in order to see if this has any effect on the results.

In the third step, successful trials for the extraction of alginate with NaCl were achieved, confirming the validity of this step. However, there was no optimisation done. To achieve this, variation in the temperature, salt concentration, seaweed to solvent (S:S) ratio and which Na- salt is used would all need to be investigated to determine the best possible conditions.

As was done in a macroalgal biorefinery study by Yuan et al [162], a fourth step to convert carbohydrates to monomer sugars, to aid in fermentation or AD, may be beneficial. While this study showed that partial hydrolysis was occurring at 200°C, higher temperatures would be needed to achieve full hydrolysis to monomer sugars. Unfortunately, the equipment available would not allow for higher processing temperatures, due to the limits pressure relief system, so this step was not able to be performed. However, if a different microwave system, capable of withstanding higher pressure were to be used, this could be achieved and would offer an interesting addition to the process.

A study using wet biomass would be advantageous. Due to the high levels of water present in seaweed biomass on harvesting, processing the biomass wet would significantly reduce the energy requirements of the process by negating the need for drying. Studies to discern if the microwaves conditions change due to processing wet would need to be undertaken. This is most likely to include changes to the S:S ratio, as the large volume of water may reduce the amount required to be added. In a changing climate, water is becoming a more valuable resource. Being able to reduce water consumption in a process is very advantageous. Pretreatment of the biomass, for example grinding or chopping, would also need to be investigated to find the differences this gives. Wet seaweed biomass is cartilaginous in texture and difficult to cut, so a suitable technique to break the biomass in to process-suitable sized pieces is important.

Finally, a continuous microwave system is important for an industrial setting. Further work in this area would include setting up a small scale extraction rig, such as the one described in Figure 7.1. Temperature and residence time experiments will need to be conducted to check that the conditions for continuous extraction are the same as for batch samples. For this system, the seaweed slurry would need to be of a pump-able viscosity to allow for it to be moved around the system. For wet biomass to be suitable for this system, again the pretreatment in terms of chopping the biomass is important, as large pieces would be difficult to pump and likely to clog the equipment.

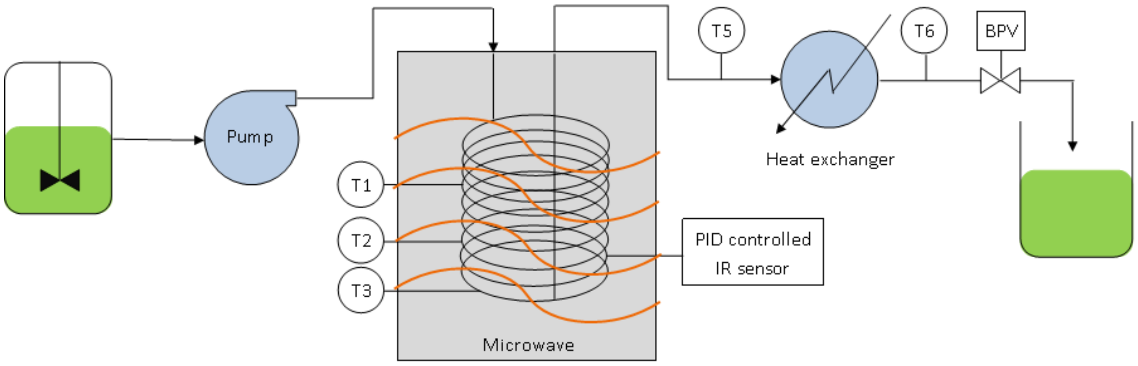


Figure 7.1: Proposed set-up for a small-scale continuous microwave reactor.

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A. Appendix

Month	Laminarin	Mannitol	Alginate	Moisture	Ash	Protein	Fucoidan	Polyphenol
				wt%				
Fucus serratus								
Apr	8.21	6.76	40.45	9.49	10.14	17.08	4.16	2.57
May	9.69	10.86	30.17	7.56	11.09	14.87	4.90	8.08
Jun	15.81	11.90	29.81	7.05	12.48	9.56	6.18	7.17
Jul	21.69	14.60	30.67	6.11	12.01	7.05	5.49	7.54
Aug	15.15	14.00	30.58	6.72	14.17	7.32	6.64	9.37
Sep	14.31	14.61	31.49	7.32	13.53	6.59	6.69	5.35
Oct	12.89	10.88	27.25	7.48	14.03	7.95	7.05	3.79
Nov	16.86	10.58	34.14	6.33	13.09	9.26	7.51	5.32
Dec	10.62	9.91	41.68	6.49	14.54	10.40	5.82	6.99
Jan	10.17	6.52	32.90	6.73	13.40	13.08	5.66	8.14
Feb	10.44	6.97	35.62	6.69	15.24	13.03	5.51	6.31
Mar	10.71	7.42	24.12	6.40	14.20	15.94	4.45	5.12
Fucus vesiculosus								
Apr	9.48	8.30	25.07	6.38	16.51	22.89	10.00	4.20
May	6.31	6.52	24.34	5.03	18.04	14.17	9.04	5.80
Jun	10.23	10.17	25.20	7.32	14.75	7.81	8.84	6.51
Jul	12.24	10.00	25.11	6.48	18.44	10.35	10.17	9.09
Aug	13.27	10.32	20.60	6.54	15.61	7.90	10.27	9.07
Sep	20.85	11.30	30.39	6.59	13.70	6.59	11.93	7.17
Oct	18.82	12.59	26.29	9.40	11.00	8.60	9.73	10.07
Nov	16.24	10.76	26.54	7.71	12.79	8.66	10.01	8.87
Dec	11.59	8.04	27.08	6.28	15.41	10.46	12.15	9.18
Jan	12.04	7.98	27.62	7.56	14.92	13.57	11.10	10.16
Feb	12.04	4.90	28.08	6.55	17.64	16.83	8.07	8.90
Mar	8.74	6.21	18.27	6.65	16.46	24.57	9.70	7.39
Ascophyllum nodosum								
Apr	7.23	4.35	28.36	8.13	15.10	7.64	7.13	9.18
May	8.98	4.81	27.66	6.57	17.00	7.69	8.65	5.65
Jun	10.49	8.54	31.18	6.49	13.62	7.46	8.75	13.79
Jul	8.84	9.64	32.56	7.48	13.58	6.00	7.87	16.45
Aug	9.63	11.67	32.88	6.63	13.95	5.28	7.90	14.07
Sep	11.41	8.71	29.88	6.68	13.27	5.93	7.27	14.93
Oct	9.08	8.18	39.77	7.23	15.34	5.07	8.87	12.83
Nov	13.35	8.24	32.89	7.55	13.36	6.13	7.92	16.38
Dec	10.62	5.84	36.49	7.11	17.06	7.27	7.62	14.19
Jan	10.24	5.50	34.87	7.60	16.25	8.60	8.37	13.63
Feb	9.86	5.17	33.24	7.99	14.94	9.35	6.49	14.50
Mar	7.52	5.11	20.76	6.69	15.86	12.99	7.10	12.09

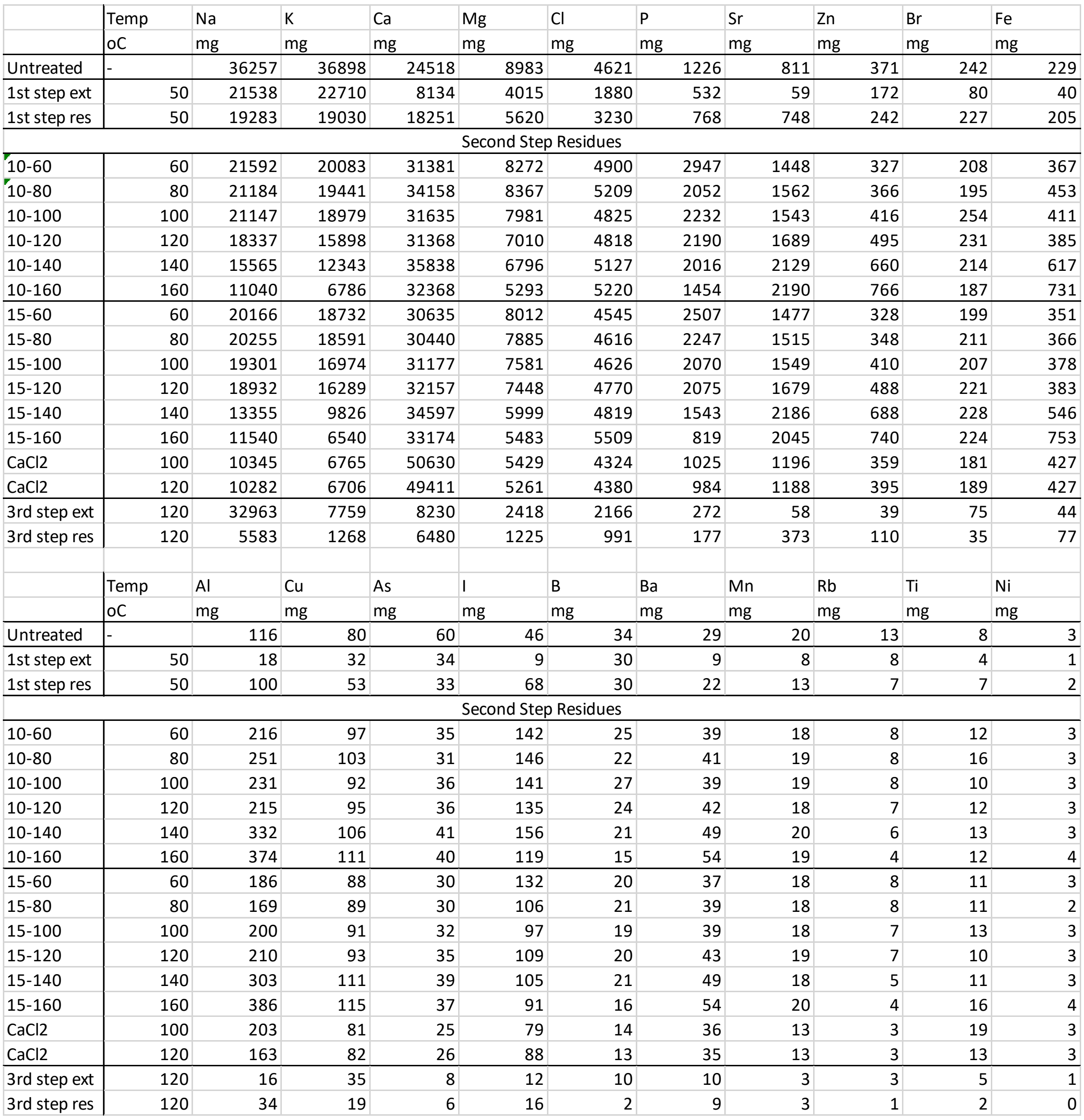
Figure A.1: Numerical data for the seasonal variation in chemical content of three brown macroalgae

Month	Na	K	Cl	Ca	Mg	P	Ti	Br	I	Sr	Fe	Al	Zn
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Fucus serratus													
Apr	57550	70287	38958	19936	11422	5180	3292	1707	1548	1599	1267	434	540
May	47133	48747	35589	16095	9348	4860	3215	1609	1192	1181	1224	356	304
Jun	43402	46888	32066	17494	9127	4394	2521	1584	1151	1307	825	226	370
Jul	50741	47603	30447	19431	11502	4505	2467	2488	1163	1401	1114	370	293
Aug	44763	49290	33283	18545	9655	4630	2168	3599	1869	1398	1426	391	390
Sep	58674	64495	29856	24528	12139	4942	6352	3752	2109	2039	1528	458	491
Oct	47571	48430	28023	16549	9420	3253	1892	1440	2044	1378	1252	358	397
Nov	53609	54752	31066	21497	11098	4640	2622	2320	2171	1723	950	313	461
Dec	53449	60528	19795	21167	10720	4680	2607	1651	2133	1905	989	360	441
Jan	49700	57629	21325	20836	9859	4013	2170	2294	1998	1549	981	540	423
Feb	48100	60154	22444	22856	9568	4693	8718	402	900	1645	735	232	437
Fucus vesiculosus													
Apr	75888	56216	30054	17170	13641	4076	3130	3267	1330	1441	1048	442	305
May	75574	56636	31279	16312	12609	4786	2705	4314	1522	1293	1064	318	315
Jun	44813	34865	12428	14050	8758	3232	1364	647	1411	1277	1069	368	317
Jul	76493	50897	33484	18876	13692	4454	1666	3393	1241	1514	939	452	326
Aug	41764	45538	11507	16974	9662	3797	2774	1827	1079	1517	1760	679	384
Sep	40012	30005	6740	16001	9162	3807	8321	1191	1104	1497	824	354	308
Oct	40442	41307	9235	16545	9268	3180	6098	1490	1162	1461	953	452	340
Nov	38286	42452	6238	17231	8974	3573	2743	1242	1193	1383	868	550	347
Dec	43203	44248	6670	17436	9645	4086	2489	1174	1266	1496	873	445	353
Jan	48121	46043	7102	17642	10317	4600	2236	1107	1338	1609	878	340	360
Feb	48465	49833	13896	23584	9485	4550	2683	788	1929	1459	938	280	357
Aschophylum nodosum													
Apr	52625	47523	7405	19201	12007	4383	5530	1946	3454	1087	701	322	608
May	82509	60104	26230	18692	13819	3836	1870	4497	2270	1373	1234	599	236
Jun	49616	32045	6591	18113	11376	3529	7089	6424	2208	885	664	280	397
Jul	49053	34786	3750	20686	11502	3664	1992	2875	3310	991	906	374	374
Aug	50747	34545	4071	20167	12002	3155	1972	1840	3151	973	783	330	360
Sep	43123	29668	7214	16467	9976	3494	1612	4556	2815	790	691	337	315
Oct	42247	30522	8194	16851	9737	2978	2531	2053	2581	740	467	237	319
Nov	44596	35934	5583	17982	10414	3927	2847	1944	3471	889	559	240	318
Dec	49420	43427	10155	19419	10853	2987	4340	7264	3584	899	620	292	357
Jan	50862	46138	8188	20088	10611	2981	5832	2470	4038	1031	691	466	380
Feb	55201	53046	9838	19769	12043	4158	3791	2744	3940	1158	2566	314	371

Figure A.2: Metal analysis for seasonal variation samples.

Month	Residue	Defatting	40% ppt	70% ppt	Remaining
Fucus serratus					
Apr	50.7	2.3	8.8	4.2	34.0
May	47.2	12.0	8.8	4.9	27.0
Jun	43.1	17.3	11.5	6.2	22.0
Jul	42.8	19.3	8.1	5.5	24.3
Aug	43.5	15.6	8.1	6.6	26.3
Sep	47.1	17.7	9.6	6.7	18.9
Oct	49.0	6.9	13.1	7.1	23.9
Nov	44.8	8.1	10.7	7.5	29.0
Dec	46.7	9.4	10.3	5.8	27.7
Jan	40.1	11.2	6.0	5.7	37.0
Feb	50.0	8.0	4.3	5.5	32.2
Mar	51.5	20.0	5.3	4.4	18.8
Fucus vesiculosus					
Apr	42.4	19.0	1.1	13.5	24.0
May	43.6	20.1	4.8	9.0	22.4
Jun	43.0	16.4	4.7	8.8	27.1
Jul	42.1	21.3	4.7	10.2	21.6
Aug	45.2	16.8	3.2	10.3	24.5
Sep	40.1	21.9	4.6	11.9	21.5
Oct	45.7	15.6	5.1	9.7	23.9
Nov	45.3	11.8	5.1	10.0	27.8
Dec	45.7	14.8	5.5	12.2	21.9
Jan	46.2	12.0	5.8	11.1	24.9
Feb	50.4	12.5	5.4	8.1	23.6
Mar	45.5	14.0	3.9	9.7	26.9
Ascophyllum nodosum					
Apr	49.7	8.0	8.9	7.1	26.3
May	41.0	17.7	7.1	8.7	25.6
Jun	40.5	13.0	7.1	8.8	30.7
Jul	45.8	15.5	8.0	7.9	22.9
Aug	47.5	10.4	8.5	7.9	25.7
Sep	49.5	4.8	9.2	6.3	30.3
Oct	47.8	13.8	8.0	8.9	21.6
Nov	46.1	12.4	8.5	7.9	25.1
Dec	51.5	10.1	9.1	7.6	21.6
Jan	44.3	11.4	8.1	8.4	27.9
Feb	45.7	10.8	8.2	6.5	28.9
Mar	47.7	9.4	8.5	7.1	27.3

Figure A.3: Numeric data from the steps involved in conventional fucoidan extraction



	Temp	Na	K	Ca	Mg	Cl	P	Sr	Zn	Br	Fe
	oC	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Untreated	-	36257	36898	24518	8983	4621	1226	811	371	242	229
1st step ext	50	21538	22710	8134	4015	1880	532	59	172	80	40
1st step res	50	19283	19030	18251	5620	3230	768	748	242	227	205
Second Step Residues											
10-60	60	21592	20083	31381	8272	4900	2947	1448	327	208	367
10-80	80	21184	19441	34158	8367	5209	2052	1562	366	195	453
10-100	100	21147	18979	31635	7981	4825	2232	1543	416	254	411
10-120	120	18337	15898	31368	7010	4818	2190	1689	495	231	385
10-140	140	15565	12343	35838	6796	5127	2016	2129	660	214	617
10-160	160	11040	6786	32368	5293	5220	1454	2190	766	187	731
15-60	60	20166	18732	30635	8012	4545	2507	1477	328	199	351
15-80	80	20255	18591	30440	7885	4616	2247	1515	348	211	366
15-100	100	19301	16974	31177	7581	4626	2070	1549	410	207	378
15-120	120	18932	16289	32157	7448	4770	2075	1679	488	221	383
15-140	140	13355	9826	34597	5999	4819	1543	2186	688	228	546
15-160	160	11540	6540	33174	5483	5509	819	2045	740	224	753
CaCl2	100	10345	6765	50630	5429	4324	1025	1196	359	181	427
CaCl2	120	10282	6706	49411	5261	4380	984	1188	395	189	427
3rd step ext	120	32963	7759	8230	2418	2166	272	58	39	75	44
3rd step res	120	5583	1268	6480	1225	991	177	373	110	35	77

	Temp	Al	Cu	As	I	B	Ba	Mn	Rb	Ti	Ni
	oC	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Untreated	-	116	80	60	46	34	29	20	13	8	3
1st step ext	50	18	32	34	9	30	9	8	8	4	1
1st step res	50	100	53	33	68	30	22	13	7	7	2
Second Step Residues											
10-60	60	216	97	35	142	25	39	18	8	12	3
10-80	80	251	103	31	146	22	41	19	8	16	3
10-100	100	231	92	36	141	27	39	19	8	10	3
10-120	120	215	95	36	135	24	42	18	7	12	3
10-140	140	332	106	41	156	21	49	20	6	13	3
10-160	160	374	111	40	119	15	54	19	4	12	4
15-60	60	186	88	30	132	20	37	18	8	11	3
15-80	80	169	89	30	106	21	39	18	8	11	2
15-100	100	200	91	32	97	19	39	18	7	13	3
15-120	120	210	93	35	109	20	43	19	7	10	3
15-140	140	303	111	39	105	21	49	18	5	11	3
15-160	160	386	115	37	91	16	54	20	4	16	4
CaCl2	100	203	81	25	79	14	36	13	3	19	3
CaCl2	120	163	82	26	88	13	35	13	3	13	3
3rd step ext	120	16	35	8	12	10	10	3	3	5	1
3rd step res	120	34	19	6	16	2	9	3	1	2	0

Figure A.4: Metal analysis for microwave processed samples