Impacts of Processing and Storage Methods on the Yield and Composition of Fucoidan from *Undaria pinnatifida*

by

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Impacts of Processing and Storage Methods on the Yield and Composition of Fucoidan from *Undaria pinnatifida*

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Signed: 

Date: 1 Nov 2014

Acknowledgements

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Abstract

Fucoidan is a water soluble sulphated polysaccharide, it is usually extracted from marine organisms. Increasing research is focusing on fucoidan and its bioactivities and health benefits. *Undaria pinnatifida* is regarded as an unwanted organism in New Zealand's ocean environment, because the strong invasion and proliferation ability. This research is to extract fucoidan from different treatment and storage conditions from *U. pinnatifida* samples, with the aim of turning this unwanted organism into commercial profit.

This study was designed to examine three main hypotheses around the impacts of processing and storage on the yield and composition of fucoidan from *Undaira pinnatifida*. First, potential differences if the seaweed was processed immediately as opposed to left for 24 hours. This mimics the potential for harvested seaweed to be left on a boat or wharf overnight following harvest. Second, to see if the fucoidan differs when the seaweed is processed fresh as opposed to freeze dried. This is important as drying the seaweed would add considerable cost to the processing. Finally, the difference between fucoidan extracted immediately following harvest was compared with the fuciodan from seaweed that was stored frozen for three months. This is an important comparison from an industrial point of view as the seaweed is an annual plant that can only be harvested for a few months of the year, so storing to set up a commercial scale extraction factory, it might be necessary to store the seaweed for some time before extract of the fucoidan.

For each of these treatments fucoidan was extracted, passed through a range of molecular filters (<3 kDa, 3-10kDa, 10-30kDa, 30-50kDa, 50-100kDa and >100kDa) and each of these fractions were collected separately and further tested for: fucose content, sulphate content, protein content, uronic acid content and antioxidant capability.

There was no significant effect of any of the treatments on total fucoidan yield, so it appears that in terms of total crude fucoidan, one can leave the seaweed for up to 24 hours before processing, can extract the fucoidan from fresh seaweed (without drying), and can store it for up to 3 months in a freezer, without a large difference in the amount of fucoidan recovered using the extraction method from this study.

Fucoidan quality however, did differ in some parameters and not in others. There was no difference between the treatments in terms of the distribution of molecular weight fractions, but there were significant differences in the distribution of these molecular fractions overall, with the majority (56.03% ± 4.02SD) being over 100kDa across all treatments and a considerable proportion (37.48% ± 4.22SD) being under 3kDa.

In terms of the composition of the fucoidan fractions, there were no significant differences between the three treatments tested, except more protein was found in the samples that were stored for three months. For fucose, sulphate, uronic acid and protein, there were significant differences in amount of these components in the various molecular size fractions. The 50-100 and over 100kDa fractions had the most fucose (20.29% ± 3.06SD and 20.66% ± 2.60SD respectively) and sulphate (21.91% ± 8.29SD and 21.33% ± 3.80 respectively), but little uronic acid (5.95% ± 3.40 and 7.86% ± 3.55 respectively. Protein was above 5% in all fractions except the less than 3kDa fraction.

The antioxidant properties of the fucoidan fractions were high and comparable to previous studies. There was evidence that the antioxidant activity was highest in samples stored for 24 hours (as opposed to those processed within 6 hours), and lower in freeze dried vs non-freeze dried and lower in samples stored for 3 months vs samples processed right away. There was an interaction between the freeze drying and the molecular size, with the 50-100 and >100 kDa treatments within the freeze dried treatments exhibiting lower antioxidant activity.

Overall this shows that the higher than 50 kDa fractions contain the most fucoidan, this fucoidan has a large protein fraction and havs the greatest antioxidant activity if it is left for processing for 24 hours, not freeze dried and not stored frozen for 3 months.

Given these results, to maximise fucoidan yield and bioactivity, the seaweed should be processed around one day after collection, and the fucoidan can be extracted without first drying the seaweed.

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Chapter 1. General Introduction

Fucoidan is a natural occurring polysaccharide first described by Kylin in 1913 (Kylin 1913). It has been isolated from species such as sea urchins, sea cucumbers, but is most commonly found in brown seaweeds such as *Fucus evanescens, Saccharina japonica, Ascophyllum nodosum* and *Undaria pinnatifida* (Cumashi *et al.*, 2007; Foley *et al.*, 2011; Vishchuk *et al.*, 2011).

Fucoidan has been shown to possess a wide range of health benefits for humans which will be outlined in detail below, but include: anti-oxidant, antiviral, immunomodulating, anticoagulant, heavy metal detoxification, anti-HIV effects and anticancer (Ale *et al.*, 2011b; Kwak *et al.*, 2014; Marudhupand *et al.*, 2014; Wang *et al.*, 2014a; Zhang *et al.*, 2014). Given its potential health benefits it is little wonder that fucoidan has become one of today's popular research topics in the development of marine drugs. The fucoidan product is sold at a very high price at present, in Korea, Japan, HongKong, USA and in New Zealand, the price per gram is over 10 NZD (White *et al.*, 2014a).

A common structural feature of fucoidan is that they contain a backbone of α-L-fucose residues and with sulphate groups and uronic acid, together with a minor amount of other monosaccharide residues including galactose, mannose, xylose, rhamnose, and glucuronic aicd (Vishchuk *et al.*, 2011). The composition of fucoidan differs in both the length and the width (size of the molecule and the composition, fucose amount, level of sulphation etc.), due to the species, geographic location, harvest season, age of population and extraction methods (Ale *et al.*, 2013).

Fucoidan products are sold in different ways, such as nutritional beverages, functional foods, tablets and capsules for drugs, but there are no industrial standard on the extraction and characterisation of fucoidan and give its variability, this makes it difficult to compare between products.

This first chapter of the thesis will introduce background of *Undaria* and fucoidan, as well as reviewing the methods of extraction, purification and determination of fucoidan. The other important part is the bioactivity and health benefit to human,

because the citations about fucoidan are nearly nine times more in 2013 than 2008 (White *et al.*, 2014a).

1.1 *Undaria pinnatifida*

Undaria pinnatifida is an annual brown alga that is cultivated widely in Korea, China and Japan where it is a commercially important food material (Athukorala *et al.*, 2006; Lee *et al.*, 2006). Because it has a subtly sweet flavour and is full of nutrients, it has been most often served in soups and salads especially in Asian countries. In China, *Undaria* annual production is over 1.75 million tons, Japan and Korea also have high-usage of *Undaria* (White *et al.*, 2014b), the potential products of this brown seaweed include wakama as a food product, fucoidan and fucoxanthin as bioactive, powdered and liquid fertilizer, animal feed supplement and biofuel (White *et al.*, 2014a), basically the whole plant can be used for manufacturing commercial products.

In 1987, *Undaria pinnatifida* was first found in Wellington Harbour, New Zealand (Parsons 1994). It was defined as harmful to natural and physical resources and human health (Biosecuruty 1993). Since its introduction, it has been subsequently found in Marlborough Sounds, Oamaru, Timaru and Otago Harbour of South Island, Golden Bay, Nelson, Chatham Islands and so on (White *et al.*, 2014a). In New Zealand, the government allowed to harvest the brown seaweed *Undaria* in 2010 (Mak *et al.*, 2013). In 1999, New Zealand government required a proposal of pest management on *Undaria* to control the spread (White *et al.*, 2014a). Later on, the policy was renewed to encourage commercial use of *Undaria*, and the new strategy turned this pest to profitable products. Meanwhile, more and more researches point out that *Undaria* seaweed is a good source of natural bioactive compounds such as complex and neutral lipids rich in essential ω -3 fatty acids, carotenoids (fucoxanthin), dietary fibre, proteins, vitamins, polyphenolic compounds, alginate, laminaran and sulphated polysaccharides (Billakanti *et al.*, 2012).

U. pinnatifida is still classed as an unwanted organism by Biosecurity New Zealand. *Undaria* is generally 1-2 meters long (Hay *et al.*, 1993), the body has four parts: blade, midrib, sporophyll and holdfast, spiral sporophyll and distinct midribs are key structures (White *et al.*, 2014a). It spreads in two ways: naturally, through the

millions of microscopic spores released by each fertile organism, the other is through the attachment to vessel hulls and marine farming equipment. It is a highly successful and fertile species, which makes it a serious invader. It can settle on mudstones, cobbles to shells of abalone, sea grasses, other seaweeds body and human-made structures (Parsons, 1994).

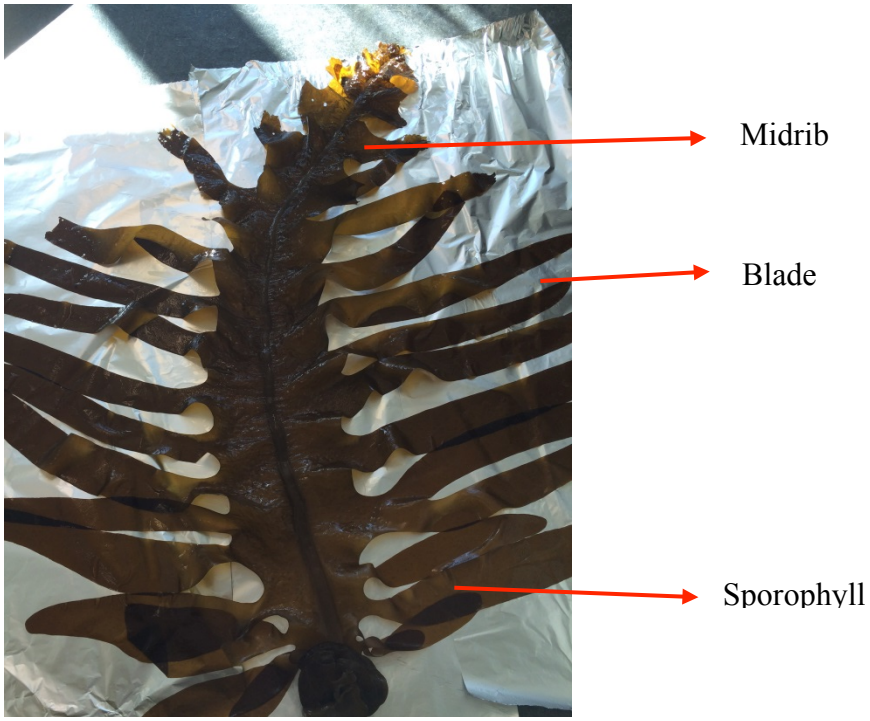


Figure 1. The appearance of *Undaria pinnatifida*

Figure 2. Living *Undaria pinnatifida*

In Figure 1, the *Undaria* plant is 68 cm in length, 55 cm in width, and the sporophyll is 8 cm long, and the weight is 246 g, it was harvested at Auckland City Central Harbour in July. When the alga is mature, the size can be up to 3 m in length. The most mature plants were found in 1m to 5 m deep sea (White *et al.*, 2014a). Figure 2 shows a plant lives on a human-made dock in Auckland, and Figure 3 is the sporophyll part of *Undaria*.

Figure 3. Sporophyll of *Undaria pinnatifida*

1.2 Fucoidan

1.2.1 Structure

Fucoidan is a type of polysaccharide that contains a substantial percentage of L-fucose and sulphate ester groups. Besides fucose and sulphate, fucoidan also may contain other monosaccharides (mannose, glucose, xylose, galactose etc.), proteins and uronic acids, and acetyl groups. Since fucoidan was first isolated, the structures of fucoidan from different brown seaweeds and other marine creatures have been investigated with structure and composition of fucoidan varying between species as well as with harvest time and the extraction methods.

The published structural data for algal fucoidan demonstrated that there was no consistent basic structure of fucoidan (Ale *et al*., 2013), with only a little regularity in

the structure (Rioux *et al.*, 2007). Unfortunately, it is difficult to carry out a detailed comparison due to non-standard extraction methodologies, which also may conflate the correlation between biological activity and fucoidan composition. For example, a study found fucoidan was very crude and differed in colour and components even between different batches, which lead to difficulty in developing new classes of drugs or nutraceuticals (Morya *et al.*, 2012).

Although the compositional and structural property of fucoidan varies greatly, the fucoidan extracted from brown seaweed has a primary structure of (1→3)-linked α-L-fucopyranosyl or alternating α(1→3) and α(1→4)-linked L-fucopyranosyls backbone structure (Figure 4). Depending on the seaweed species, the backbone is connected with various numbers of sulphate substitutions and/or have side branches containing fucopyranoses or other glycosyl units, e.g. glucuronic acid (Ale *et al.*, 2013). The backbones of fucoidan extracted from *Fucus serratus L* and *Ascophyllum nodosum* are linear, however some other structures show a side-chain on the backbones, such as *Chorda filum*, *Laminaria saccharina* and *cladosiphon okamuranus* (Figure 4).

In 1993, Pankter claimed that in fucoidan extracted from *F. vesiculosus*, the tri-0-methyl-L-fucose structure (pyranose and furanose forms) had a significant amount, which suggested a high degree of branching of fucoidan with terminal fucose. Thus revise the previous fucose 4-sulfate model and proposed a new one as shown in Figure 4, later studies on fucoidan structures are mostly based on Pankter's backbone (Patankar *et al.*, 1993).

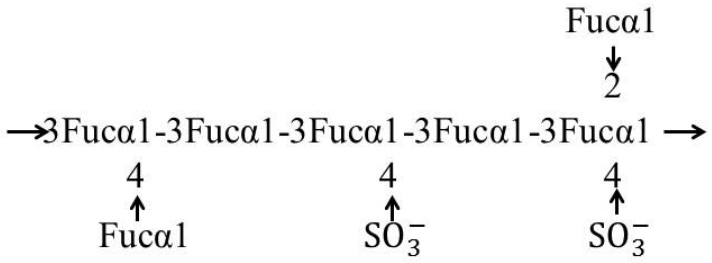
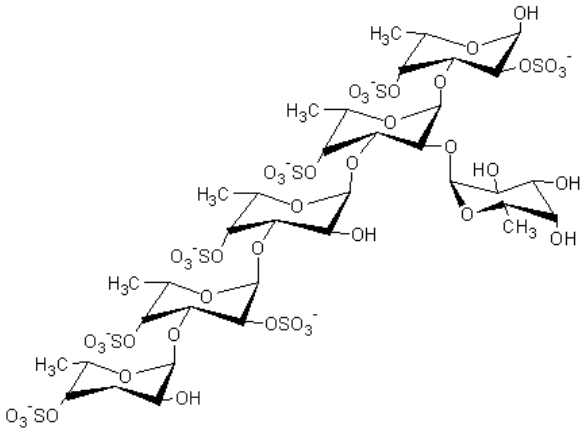
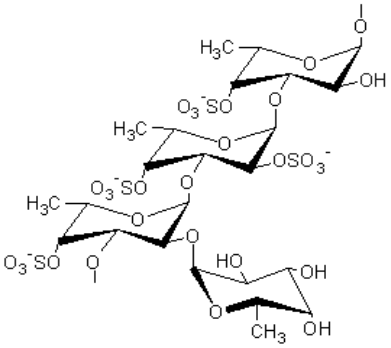


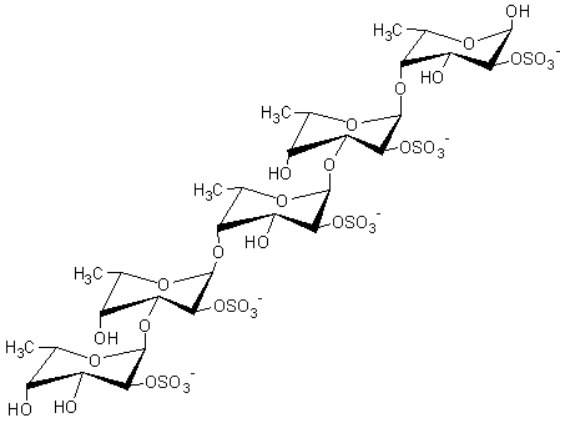
Figure 4. Pankter model for the average structure of fucoidan (Patankar *et al.*, 1993).



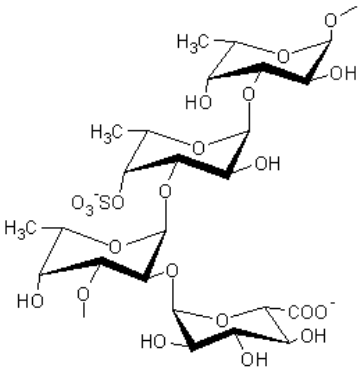
Chorda filum



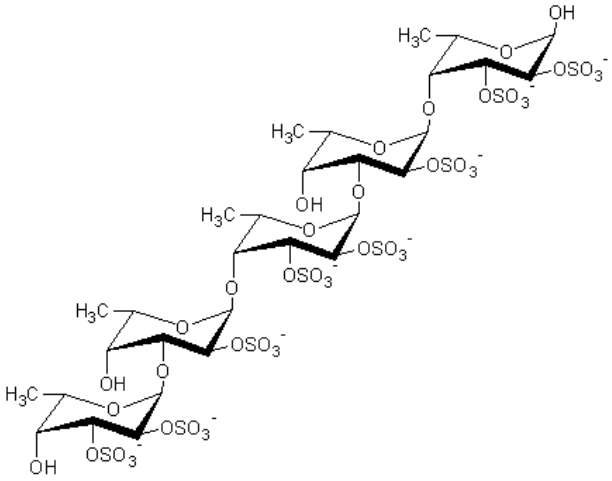
Laminaria saccharina



Fucus serratus L.



Cladosiphon okamuranus



Ascophyllum nodosum

Figure 5. Five typical structures of different species brown seaweed (Ale *et al*., 2013).

The position of sulphate ester groups influences the pharmaceutical activity of sulphated polysaccharides (Li *et al.*, 2008). Another study demonstrated the anticoagulant properties of fucoidan were mainly determined by the fucose-sulphated chains, especially by the disulphated fucosyl units (Duarte *et al.*, 2001). The anti-angiogenic and anticancer activities can be potentiated by increasing sulphate groups in fuoidan molecule (Koyanagi *et al.*, 2003). The positions of sulphate ester groups can be determined by IR spectroscopy, methylation analysis (GC-MS, GLC-MS etc), stability of sulphate esters to alkali, and desulphation (Li *et al.*, 2008). Apart from sulphate groups, the bioactivity of fucoidan is also related to the structural character, molecular weight and monosaccharide composition. An appropriate extraction method will help to preserve the biological properties and structural integrity of fucoidan (Ale *et al*., 2013).

By analysis of MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) and tandem ESI (electrosprayionization) mass spectrometry, it appeared that side chains were composed of D-glucuronate (*Cladosiphon okamuranus*) or fucose residues (*Chorda filum*), and were attached to the O-2 position of the main chain's fucose residues in a regular manner, with different side chain structures depending on the seaweed species (Anastyuk *et al.*, 2009). *L. vadosa* contained a polysaccharide made up of α-L-fucopyranosyl residues linked 1→3 mainly sulphated at position O-4 and partly sulphated at position O-2 (Chandía *et al.*, 2008). These monosaccharides may represent contamination with other polysaccharides or may in fact be genuine substitutions on the fucoidan molecular entities (Ale *et al*., 2013). In *C. filum* both the native and desulphated fucoidan had homofucan sulphate A-2 contained an (1→3)-linked poly-α-L-fucopyranoside backbone, and the degree of branching was rather high (Chizhov *et al.*, 1999).

The weaker acid treatment is better conserved for structural integrity of the polysaccharide (Hahn *et al.*, 2012). A concentration of 0.2 M HCl broke the integrity of the polysaccharide molecules resulting in a same effect as a hydrolysis of long-chain fucose backbone compound at elevated time and temperature (Hahn *et al.*, 2012). This proved that the higher acid levels might have caused a loosening of the cell wall matrix allowing local penetration of the acid into the fucoidan (Hahn *et al.*, 2012). The other paper demonstrated that a higher polysaccharide yield was produced

by a relative longer extraction time at higher temperatures, while the amounts of sulphate was lower, and proportion of glucuronic acid was higher (Ale *et al*., 2013). They also reported that there would be less fucose content within a longer extraction time, whereas sulphate decreased with time longer while glucuronic acid increasing, i.e., the sulphate content reached the peak when glucuronic acid was lowest (Ale *et al*., 2013).

Fucoidan is a mixture of several chemical compounds, fucose, xylose, galactose, glucose, rhamnose, sulphate, protein, sometimes mannose and lipid, and the ratio of monosaccharides varies from species of seaweeds, and the monosaccharide content is: fucose>galactose>glucose>manose>xylose>rhamnose, fucose ratio is significantly higher than the other monosaccharides (Guo *et al.*, 2013).

1.2.2 Extraction methods in existing literature

The yield and composition of crude fucoidan are strongly influenced by extraction method. Traditionally, extraction of crude fucoidan steps include removing lipids, protein and coloured pigments, extracting crude fucoidan from heating aqueous, adding calcium chloride to remove alginate acid, at last, precipitating crude fucoidan. Also, there are some innovated methods in pre-treating and extracting fucoidan, such as ultrasound, microwave assisted method, and enzyme assisted method (Hahn *et al.*, 2012).

1.2.2.1 Traditional extraction method

The first step of extracting fucoidan is by removing pigment with organic solvent such as methanol or ethanol. In addition of organic solvent, the pre-treatment also could be processed with a supercritical fluid. It achieved highly pure fucoidan fractions within the extraction by treating seaweed with pure CO_2 at a pressure of 550 bar and a modified fluid by adding ethanol (5%) at 60°C (Men'shova *et al.*, 2013).

Then the following is treating the seaweed with hot aqueous or acidic solutions at temperature ranging from 65 to 100℃. The brown seaweed *Adenocystis utricularis* was extracted separately in parallel with 800 mL of water, 2% $CaCl_2$, and HCl (diluted to pH 2) (Trinchero *et al*.,2009). In another previous study, the extraction was processed at both room temperature and 70℃, the extraction yields and characteristics of the products were similar after these three treatment, with only

minor differences (Ponce *et al.*, 2003). According to the Box-Behnken design which is a statistical design for parameter optimization, the extreme amount of sulphate is released at an extracting time of 13.7 h (Hahn *et al.*, 2012). They also put forward that extraction duration did not have a significant effect on the yield, it was suitable to use the lowest possible extraction time to extract the target fucoidan (Hahn *et al.*, 2012). Generally, the heating time range employed is 3–24 hours which is quite wide; a three-hour minimal timeframe is acceptable. It is agreed by another research that short the extraction time, there would be lower polysaccharide yield, but relatively higher fucose content, which is the bioactive structure of fucoidan (Ale *et al*., 2013).

The pH value of extraction aqueous ranges from 1-4. A research pointed that the optimum pH value was between 2.0 to 2.5 with a hydrochloric acid extraction (Pereira *et al.*, 1999). At pH 1, the amount of the sulphate esters was maximal, so an acidic pH was recommended (optimal pH was 2.7 according to the Box-Behnken design) (Hahn *et al.*, 2012). The pH value is adjusted while extraction experiment to increase the amount of crude fucoidan. Structurally, protons or hydroxide ions interfere with the hydrogen bonds among the different polysaccharides, releasing them into the solution resulting in a better yield. The acidic extraction is always repeated several times to reach the maximum yield, and the following neutralization is to prevent the degradation or hydrolysis of the target polysaccharide. Another advantage of hot acid extraction is the reaction of alginates becoming alginic acid simultaneously. Black (Black *et al.*, 1952) indicated that a maximal fucoidan yield was achieved by the optimal extraction procedure of 0.03 M HCl (pH=1.52), 90 ℃, 4 hours. However, in Thomas Hahn' s review, the extraction time didn't have a significant effect on the yield, although the maximum amount of sulphate was released at 13.7 h (Hahn *et al.*, 2012).

Alginate is a naturally anionic polymer normally obtained from brown seaweed; it is a polysaccharide comprising of 1,4-glycosidic linked with α-L-guluronate and β-D-mannuronate. Usually α-L-guluronates are connected forming a so-called GG block; so are the connected mannuronates, they form an MM block. The electrostatic repulsion between molecule decreases and the polymers associate via interchenar hydrogen bonds, because mannuronates and guluronates are protonated below their pKa values (acid dissociation constant) (Figure 6 and 7) (Ale *et al.*, 2011). However,

extraction with diluted acid leads to the partial cleavage of sulphate esters due to the hydrolysis. Since the bioactivity depends on the structure, the molecular size, the sulphate ratio and the monosaccharide composition, there must be cooperation among those factors (Hahn *et al.*, 2012).

Calcium chloride is often used as a method to precipitate the alginic acid comprised of C-5 epimers, guluronic and mannuronic acids, and alginic acids are regarded as a contamination in fucoidan products (Ale*, et al.*, 2011b). Based on the existence of the GG blocks, cavities are caused by the aggregation, which are the exact size and diameter to fit for Ca^{2+} ions. One Ca^{2+} ion coordinative attracts ten oxygen atoms (Lee *et al.*, 2006). So the alginic acid becomes lump sticky to seaweed, it could be discarded after filter or centrifuge.

The arrow points reacted position in the practical

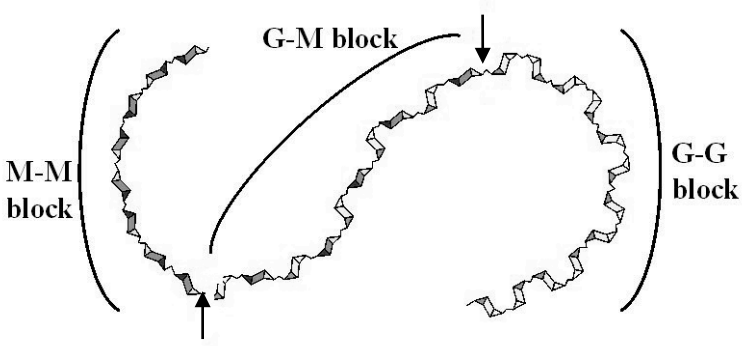


Figure 6. Explanation for the blocks

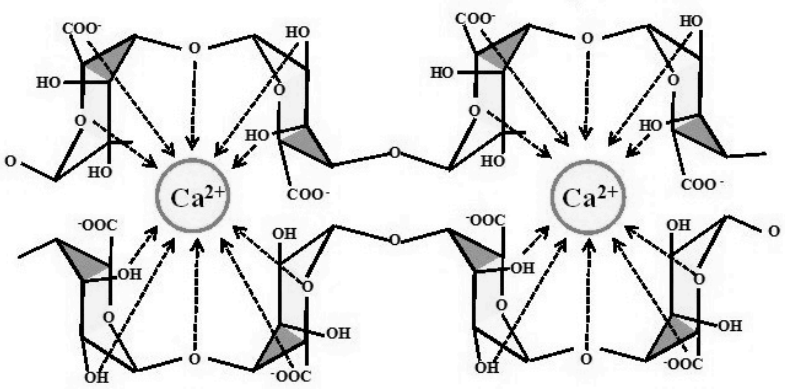


Figure 7. Ca^{2+} ion coordinative bound to ten oxygen atoms (Keita *et al.*, 2012)

For most traditional methods, it is straightaway following precipitation within organic solvents or tenside which interact with sulphated polysaccharides after the extraction (Li *et al.*, 2006; Maruyama *et al.*, 1984). For example, absolute ethanol is always used to remove the pigment, the salts and other small molecules from the extracted solution. The theory is that water has a high dielectric constant, so the oppositely charged groups are covered, and they are surrounded by hydration shells. While ethanol is present, the sulphated ester and positive ions can form ionic bonds due to a relatively lower dielectric constant of ethanol, thus to precipitate out the fucoidan (Hahn *et al.*, 2012).

Furthermore, tenside, such as the cationic surfactant cetyltrimethylammonium bromide (CTAB), or hexadecyltrimethylammonium bromide (Cetavlon) can be put on to the purification. CTAB forms capsular micelles in aqueous situations, which depend on the tenside properties and the critical micelle concentration. In the company of acidic polysaccharides, the CTAB molecules are adsorbed onto the ionic groups of the fucoidan. It is an interaction of the anionic polysaccharide with the charged quaternary ammonium of CTAB (Chen *et al.*, 2012b). When the lipophilic tail was exposed to the outside, there would be precipitation coming out due to the "inverted" micelles form high molecular weight aggregates (Ly *et al.*, 2005). The interaction between the polysaccharide and the detergent is so strong that it requires high salt concentrations to complete solubilisation cannot be achieved (Marudhupandi *et al.*, 2014). Additionally, the adsorption of the CTAB molecules to the anionic functional groups should provide improved chemical and biological properties for the resulting complex. The quaternary amine functions of the CTAB or Cetavlon and the anionic sulphate ester groups are involved by coulomb forces. The hydrophobic interactions within the alkyl chains of the detergent reject the water molecules, meanwhile link the fucoidan to form large molecule, which results in precipitation due to the conjugation of the molecules (Hahn *et al.*, 2012).

There are a few ways to make the traditional extraction methods more efficient, including increasing the water: seaweed ratio, the extraction time or repeating several times of extractions (Pereira *et al.*, 1999). However, it leads to a waste of time and materials.

The traditional methods to extract polysaccharides from brown seaweed are quite time-consuming and require a large amount of harsh chemicals and organic solvents to perform extraction and precipitation of the target polysaccharides. And it always results in environmental pollution. Hence, modern extraction and separation techniques for large-scale industrial production, such as supercritical extraction, ultrasound-aided extraction and membrane separation have recently been applied in polysaccharides preparation (Ye *et al.*, 2008).

1.2.2.2 Enzyme-assisted extraction

Enzyme-assisted extraction methods have been proved to accomplish high extraction yields for compounds including polysaccharides, flavours, medicinal compounds, natural pigments and oils extracted from plant or animal (Xu *et al.*, 2013). Enzymes catalyse the degradation of the cell walls during the isolation of fucoidan from seaweed and algae (Hahn *et al.*, 2012). In terms of the seaweed cell wall is more heterogeneous than the other cells both chemically and structurally, for instance plant cells, the application of a well-combined enzyme mixture is necessary for extraction. Enzymes assist the extraction to happen under moderate conditions to preserve the bioactivity of the fucoidan. Previous study indicated that enzymatic hydrolysis was better protecting sulphate group than the chemical hydrolysis (Morya *et al.*, 2012).

Various enzymes were tested to extract anti-oxidative fractions of the polysaccharide, including *Celluclast*, *Ultraflo*, *Viscozyme*, *AMG* and so on (Heo *et al.*, 2005a; Heo *et al.*, 2005b). These enzymes are used to separate polyphenols, which may bind fucoidan. The enzyme-assisted extraction method was a simple separation by using cross-flow-filtration or ultrafiltration membranes with an adequate molecular weight cut-off (1, 5, 10, 30 or 50 kDa), and fucoidan were retained on membranes (Athukorala *et al.*, 2006; Ye *et al.*, 2008). However the enzymes and the initial sulphated polysaccharides are retained on the membrane, smaller cell wall fragments, which might inhibit the enzymes, can be removed during cell lysis (Hahn *et al.*, 2012).

1.2.2.3 MAE

Microwave-assisted extraction (MAE) has been applied to isolate plant material from early time. The mechanism sequence of MAE is as following: energy of microwaves encourages the vibration of H_2O molecules in the plant; because of the vibrations, the

temperature of the intracellular liquids increases, so the water evaporates and exerts pressure on the seaweed cell walls. By way of the cell wall opening, the contents inside the cells are released into the medium. The aqueous microwave-assisted extraction methods of polysaccharides have been described only in a limited number of studies (Hahn *et al.*, 2012). The first time of microwave-aided extraction of fucoidan was reported by Rodriguez-Jasso in 2011 (Rodriguez-Jasso *et al.*, 2011), they demonstrated the yields of the target compound were high and extraction time was short; fucoidan represented 18.22 % of the *F. vesiculosus* dry mass using the microwave-assisted extraction method with conditions that pressure was 120 psi for 1 min, and the results were comparable to the amount of polysaccharide obtained by traditional multiple extractions at 70℃ (Rioux *et al.*, 2007; Rodriguez-Jasso *et al.*, 2011).

According the single-factor design and orthogonal array design, the best MAE conditions were as follows:

Table 1. Optimal microwave setup conditions for fucoidan extraction in literature

Ethanol	Solid/liquid	Temperature	Extraction time	Irradiation power	References
55%(w/w)	1:8	60°C	25 min	400 W	(He *et al.*, 2013)
80% (v/v)	1:31.1	nd	4.2 min	744.8 W	(Song *et al.*, 2009)
nd	1:25	70°C	1 min	nd	(Rodriguez-Jasso *et al.*, 2011)

The more water used in extraction step led to a larger volume ethanol cost while the precipitation. With a higher irradiation power and larger volume water, shorter extraction time is needed. A research compared two methods while extracting crude fucoidan, microwave-assisted extraction (MAE) and autohydrolysis (AH). They claimed that the yield of MAE is a little higher than AH, however, fucoidan product of AH method contained more L-fucose than MAE, they considered the reason could be the higher extraction time AH method processed (Rodriguez-Jasso *et al.*, 2014).

A previous study indicated MAE could produce polysaccharide with a shorter heating time than conventional extraction methods (He *et al.*, 2013), and another paper also

claimed that MAE was able for industrial producing polymers with high molecular weight (Song *et al.*, 2009). Considering complicated structural and chemical properties of sulphated polysaccharides, MAE was applied to extract fucoidan from brown seaweed material in the present study. It was expected that by using MAE method fucoidan could go through degradation. For MAE method, the controlling of microwave energy over the target material under pressure parameter is important, because the temperature measurements is one of the most common problems of heating by microwave fields, it is a complicated extraction by the presence of high intensity electromagnetic fields (Rodriguez-Jasso *et al.*, 2014). In conclusion, MAE is a potential method to recover fucoidan from brown seaweed, it takes shorter extraction time, environmentally friendly, and it can achieve a high yield.

1.2.2.4 Ultrasound

The most obvious advantage of ultrasound-assisted method is time saving. This method is derived from that sound waves could travel through medium inducing pressure variations. During this process, cavitation is engendered, then grows up and collapse, finally the sound waves are transformed into mechanical energy, which disrupts the cell and the cell wall. A study briefly described their experiment, seaweed cells were disrupted by ultrasound at first, then incubated by boiling water, added trichloroacetic acid to the supernatant after centrifuge. At last, the sample solution was pumped to pass different size of ultrafiltration membranes after adjusting pH value (Sheng *et al.*, 2007).

Generally, the ultrasound-assisted method is adding a certain quantity of seaweed powder to distilled water and mixed by stirring, according to diverse conditions (liquid to solid ratio, working power and time) in a water bath sonication, centrifuge, the supernatant was concentrated with 95% ethanol, then centrifuged to precipitate the crude fucoidan, washed with acetone and lyophilized (Ebringerová *et al.*, 2010).

Previous study indicated that the optimized solvent for ultrasound-extract was water, and the best conditions were following: and sonication time 20 min, sonic power 31.7 W, extraction temperature 45−53°C (Ebringerová *et al*., 2010). Even though ultrasound treatment is a short period, the yield of target polysaccharides, which belong to the group of pectin polysaccharides, is 20.2% which is more than the conventional extraction methods (Ebringerová *et al*., 2010; Lorimer *et al.*, 1995).

Ye performed the supercritical CO_2 extraction, ultrasound-aid extraction and advanced membrane separation technology onto *S. pallidum* seaweed to separate polysaccharides, in their research, the highest yield of polysaccharide fraction was the <50 kDa fraction (Ye *et al.*, 2008).

The advantages of using ultrasound are saving time, stable reaction, and less organic chemicals. Most papers recommended that promising devices for establishing a more durable and efficient extraction of fucoidan includes microwave-assisted and ultrasound-assisted extraction of the target polysaccharide. As well as the enzyme-assisted release of the sulphated polysaccharides, this is also a noble technique on manufacture.

Table 2. Literature of extraction methods and fucoidan composition differences

Seaweed species	Extraction method	Fucose content (%)	Sulphate content (SO_3Na)	Reference
Undaria Pinnatifida (New Zealand)	Seaweed powder treated with a MeOH-$CHCl_3$-water mixture (4:2:1), filtered powder was mechanically stirred with 2% aq $CaCl_2$, add 10% cetavlon to the supernatant to precipitate fucoidan.	16.4±0.29	34.6±0.61	(Mak *et al.*, 2013)
Saccharina japonica (Japan)	Fresh seaweed treated with ethanol, acetone, and chloroform sequentially, seaweeds (100 g) were extracted with 0.1 N HCl (2 L) for 1.5 h repeating three times.	26.3	23	(Vishchuk *et al.*, 2011)
Undaria pinnatifida (japan)	Fresh seaweed treated with ethanol, acetone, and chloroform sequentially, seaweeds (100 g) were extracted with 0.1 N HCl (2 L) for 1.5 h repeating three times.	29.7	29	(Vishchuk *et al.*, 2011)
Fucus evanescens (Kuril Islands)	Extraction of the dry defatted algal biomass with a dilute solution of calcium chloride, precipitation of acidic polysaccharides with Cetavlon.	58.7 (monosaccharide)	36.3	(Cumashi *et al.*, 2007)
F. vesiculosus (Portugal)	Microwave-assisted extraction. Milled seaweed was put into extraction vessel to be irradiated, after cooling and filter, 1% $CaCl_2$ solution was added, filter, add ethanol to precipitate fucoidan.	nd	35.5	(Rodriguez-Jasso *et al.*, 2011)
Sargassum (Japan)	Adding 100 g of dried ground seaweed to a 5 L flask containing 2 L of 0.03 M HCl at 90℃, filter	3.14	38.4	(Ale *et al.*, 2011a)

Seaweed species	Extraction method	Fucose content (%)	Sulphate content (SO_3Na)	Reference
	and add 60% ethanol to the supernatant to precipitate fucoidan,			
Ascophyllum nodosum (Ireland)	Seaweed was extracted with 80% ethanol at room temperature and 70 ℃ for 12 hours, and then extracted with milliQ water at room temperature and 70℃ for 12 hours.	43.2	35.3	(Foley *et al.*, 2011)
Ascophyllum nodosum (Kerean)	Milled seaweed was refluxed with ethanol at 75℃ for 1h, then dried biomass was extracted with 0.2 M HCl at 60℃, after filtering, add ethanol to the supernatant to precipitate fucoidan.	39.5	31.7	(You *et al.*, 2010)
Undaria pinnatifida (Kerean)	Dried sporophyll of seaweed was extracted with 0.1 M HCl for 24 h, then neutralized with 1 M NaOH, re-dissolved in water with 1 M HCl precipitated with $CaCl_2$, filter and add ethanol in supernatant to precipitate fucoidan.	50.8 (monosaccharide)	9.18 (S)	(Synytsya *et al.*, 2010)
Saccharina japonica (Japan)	Seaweeds were treated with ethanol, acetone, and chloroform sequentially. Samples of defatted, dried seaweeds (100 g) were extracted with 0.1 M HCl (2 L) at 60 ℃ for 1.5 h repeating three times.	47	33	(Vishchuk *et al.*, 2011)
Undaria pinnatifida (Japan)	Seaweeds were treated with ethanol, acetone, and chloroform sequentially. Samples of defatted, dried seaweeds (100 g) were extracted with 0.1 M HCl (2 L) at 60 ℃ for 1.5 h repeating three times.	53 (monosaccharide)	43	(Vishchuk *et al.*, 2011)
Sargassum (Vietnamese)	Crude fucoidan were extracted from the brown seaweed samples with an acidic solution 0.1M HCl with a solid/liquid ratio 1:10 in the presence of 2 wt % $CaCl_2$ for alginic acid separation, and partially purified by cetylpyridinum chloride and ethanol precipitation,	54 (monosaccharide)	23.5	(Ly *et al.*, 2005)
Undaria pinnatifida (Japan)	Air-dried algal tissue were ground and extracted at room temperature for 14 h with 0.1 M HCl (1:5 w/v). The solution was filtered and algal tissue residues were subjected to repeated extraction with water (1:5 w/v, 60°C, 5 h) and again filtered	29	52.38	(Skriptsova *et al.*, 2010)
Sargassum tenerrimum (India)	Seaweed powder was treated with ethanol, wash with acetone. Dried biomass was heated with water at	59.3±0.43	24.76±0.2	(Marudhupandi *et al.*, 2014)

Seaweed species	Extraction method	Fucose content (%)	Sulphate content (SO_3Na)	Reference
	65℃. Add calcium chloride to remove alginic acid. Then add ethanol to precipitate fucoidan.			
Ascophyllum nodosum (France)	Fresh seaweed was ground, at 70℃ with 0.01 NaCl containing 15 $CaCl_2$, repeated twice. Pool the extract and dialyze against distilled water. Crude fucoidan was precipitated with EtOH.	66	31	(Marais *et al.*, 2001)
S.latissima (Scotland)	The algal biomass was treated with a 4:2:1 MeOH-$CHCl_3$-H_2O mixture. 150 g of defatted material and 2% aqueous $CaCl_2$ solution (4*2 L) were mechanically stirred at 85℃ for 5 h. An aqueous hexadecyltrimethylammonium bromide solution (10%, 400 mL) was added to the combined extracts. Ethanol was used to precipitate.	30.8	28.8	(Bilan *et al.*, 2010)

Asia is the main place investigating and producing fucoidan. And seaweed species include *Sargassum, Undaria pinnatifida, Saccharina japonica, Ascophyllum nodosum* and so on. The fucose and sulphate content are significantly different among various species and via different menthods. It is a broad range of sulphate and fucose concentration, for example, in *Fucus evanescens* (Kuril Islands) and *Sargassum tenerrimum* (India), the fucose content is nearly 60%; meanwhile in *Undaria* from Japan, the sulphate content is over 52%, however in *Undaria* from Korea, there is only 9.18% of sulphate content in crude fucoidan extracted from seaweed. The data demonstrate the vulnerability of fucoidan structures to harsh extraction conditions and confirm that the extraction method significantly influences the yields and not least the composition of the extracted polysaccharides (Ale *et al*., 2013). Extraction method is usually within organic solvent and abundant ethanol for precipitation of crude fucoidan. The most general solvent used to remove pigment is MeOH-$CHCl_3$-H_2O mixture at a ratio of 4:2:1. And the extract procedure is heating in hot aqueous or acidic solutions, $CaCl_2$ is added to get rid of alginic acid. CTAB or ethanol is the common precipitated solvent to get fucoidan products; at last the fucoidan precipitate is lyophilised to get the final fucoidan powder.

The bioactivity of fucoidan mainly depends on nature and extent of sulphation, so it must be taken extra care while extraction. If the extraction method is harsh, the sulphation pattern may be interrupted and the bioactivity can thus be lost (Morya *et al.*, 2012). It is convinced that the extraction treatment affects the composition and structural features of the fucoidan and the lower molecular fractions.

In summary, time-consuming and potentially environmental pollution are the fundamentals people consider about. Most conventional extract methods are performed with replication, although the extraction time doesn't affect the fucoidan release ration significantly. Besides, the first step which is to remove pigment with organic chemicals always costs hours.

For the large-scale manufacture of fucoidan, the enzyme-based method acts to be advantageous, however the economic efficiency of the process depends on the supply of the required enzymes (Hahn *et al.*, 2012). MAE and ultrasound-assisted method can save time, but may cause fucoidan degradation (Ebringerová *et al*., 2010).

1.2.3 Purification methods in existing literature

The fucose backbone may be substituted with sulphate or acetate, sometimes with side branches containing fucopyranoses or glucuronic acid (Ale *et al*., 2013). Meanwhile, there are several other compounds in crude fucoidan, including lipid, protein and some monosaccharides (Ale *et al*., 2013). All these compounds are regarded as contamination to sulphate polysaccharide. A study indicated that fraction molecular weight between 5-30 kDa had a relatively stronger anti-cancer effect (You *et al.*, 2010). For different size fractions, the percentage of sulphate and fucose varies. The 5-30 kDa fraction had the most sulphate and fucose content (You *et al.*, 2010). That leads to a further purification of crude fucoidan.

The methods of hydrolysing the crude fucoidan include acid hydrolysis, radical hydrolysis, enzymatic hydrolysis, electro-dialysis, microbial method, and so on. Among them, acid hydrolysis with HCl is normally used, but this method causes the over-desulphation and acid waste problems (Guo *et al.*, 2013).

Following hydrolysis, anion-exchange chromatography and ion-exchange chromatography are widely adopted for further separation of crude fucoidan extracted

from brown seaweeds. Fucoidan shows high anionic charges even at a lower pH value, because the sulphate ester groups linking to the carbohydrate backbone are an important part of structure. DEAE-cellulose, QAE Sephadex A-25, DEAE Toyopearl 650 M and Mono-Q are the anion exchange resins used for this procedure (Béress *et al.*, 1996; Chizhov *et al.*, 1999; Nishino *et al.*, 1994; Wang *et al.*, 2007). The loading resins are functionalized with anionic exchanger groups, which are quaternary ammonium functions; therefore, effective coulomb attractive forces lead to the interactions. The elution of adsorbed fucoidan fraction is performed by applying a gradual or linear sodium chloride gradient (Kim *et al.*, 2003; Mabeau *et al.*, 1990). The strong interactions between the resin and the fucoidan are indicated by applying high molar salt concentrations (Rupérez *et al.*, 2002). Furthermore, ion exchange chromatography can be used to separate different fucoidan fractions that have distinct structural and chemical properties. This way, the following rule of elution can be applied: the lower the sulphate content, the lower the concentration of sodium chloride solution needed to perform an elution from a chromatography, thus to collect different layers as target fractions (Bilan *et al.*, 2010; Nishino *et al.*, 1989).

There are also other published purification methods. The application of gel permeation chromatography following anion exchange chromatography is typically used for the purification of highly valuable products. With this procedure, the salts used for the elution of the target compound from the anion exchange resin are removed. Millipore ultrafiltration membranes could also separate crude fucoidan into different size fractions. The previous article indicated that the main fraction could be separated by a 30 kDa Millipore membrane (Sheng *et al.*, 2007). This ultrafiltration was used for separating fucoidan fractions by molecular size. Ping Yu (Yu *et al.*, 2014b) performed High-speed countercurrent chromatography (HSCCC) to separate sulphated polysaccharide. The results of their study proved that HSCCC sequenced with DEAE-Sepharose F.F. anion-exchange chromatography was an effective method for the purification of the seaweed extracted fucoidan. In 1967, an enzyme called fucoidanase was isolated from abalone by Thanassi (Thanassi *et al.*, 1967), it was used for endo hydrolysis of fucoidan without release of sulphate. In the past 15 years, research on fucoidanase has increased, and people are still working on elucidating the genetic structure (Holtkamp *et al.*, 2009).

1.2.4 Determination method of fucoidan composition in existing literature

Since crude fucoidan is not a single compound, most literature discusses the ratio of fucose, sulphate, protein, and uronic acid which are proved as the bioactive substances showing great potential anticancer activities. The general monosaccharide compositions are fucose, galactose, mannose and xylose, mannose is underestimated due to incomplete hydrolysis of glucuronosidic linkages (Bilan *et al.*, 2010). The conventional composition test method for sulphate is by using $BaCl_2$ gelatin method. The other important content fucose is measured by Cysteine-H_2SO_4 method. Total carbohydrates content could be determined by phenol-sulphuric acid method. Traditional test methods for protein are BSA assay as well as Lowry and Bradford. All above traditional methods are performed on UV spectroscopy, and results are obtained by comparing readings to standards (Table 3).

For further test on partially methylated monosaccharides, HPLC-MS and GC-MS or altered methods were a high frequency adopted method. A previous study separated and quantified the monosaccharide in fucoidan by HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection) analysis (Ale *et al.*, 2011a). According to their results, hydrolysed fucoidan mainly consists of fucose, glucuronic acid and sulphate, with minor galactose, glucose, xylose, mannose, rhamnose and arabinose, and the ratio of these compound was various due to material species and extraction methods.

Besides, infrared spectroscopy is also adopted to analyse fucoidan composition. In Zhang's study (Zhang *et al.*, 2010), they performed five fucoidan extracted from different seaweeds. The results presented that, signals at 3420-3450 was correspond to O-H vibration, and peaks at 1220-1260 were caused by S-O stretching vibration, at 1640-1650 was C-O of uronic acids. The C-O-S bending vibration of sulphate in axial position gave out the peaks at 820-850. Similar results were presented by Synytsya (Synytsya *et al.*, 2010), the O-S-O stretching vibration of sulphate esters was at 1256 cm^{-1}. Vishchuk also regarded the bands at 836 and 823 as C-O-S bending vibration of sulphate content (Vishchuk *et al.*, 2011).

NMR is always performed for structure analysis. Alexey G. worked out 3 different fucoidan structure unit derived from 3 seaweed species by NMR spectroscopy

(Gerbst *et al.*, 2010). Because what people know about fucoidan structure is only the backbone rather than stable confirmed structure, a growing number of studies are focusing on NMR spectroscopy on a variety of fucoidan samples (Synytsya *et al.*, 2014; Wang *et al.*, 2014b; Yu *et al.*, 2014a).

Table 3. Summary of test methods for fucoidan

Methods	Content	References
$BaCl_2$ gelatin method	Sulphate	(You *et al.*, 2010) (Ly *et al.*, 2005) (Mak *et al.*, 2013) (Pereira *et al.*, 1999)
Phenol-sulphuric method	Total sugar	(You *et al.*, 2010) (Zhang *et al.*, 2010) (Qu *et al.*, 2014)
Cysteine-H_2SO_4, method	Fucose	(Mabeau *et al.*, 1990) (Pereira *et al.*, 1999)
Carbazole method	Uronic acids	(Ly *et al.*, 2005) (Mabeau *et al.*, 1990) (Mak *et al.*, 2013)
Lowry method	Protein	(You *et al.*, 2010)
Bradford method	Protein	(Berteau *et al.*, 2002) (Mak *et al.*, 2013)
HPLC	Monosaccharides	(Zhang *et al.*, 2010) (You *et al.*, 2010) (Skriptsova *et al.*, 2010) (Zhang *et al.*, 2009) (Berteau *et al.*, 2002)
Vibration spectroscopy	Structural analysis	(Pielesz *et al.*, 2011)
Raman spectroscopic analysis	Structural analysis	(Pielesz *et al.*, 2011)
Electrophoretic analysis	Structural analysis	(Pielesz et al., 2011)
HPAEC	Monosaccharides	(Foley et al., 2011) (Rioux et al., 2007)
HPAEC-PAD	Monosaccharides Sulphate	(Ale et al., 2011a) (Lee et al., 2006) (Ly et al., 2005)
FT-IR	Functional groups	(Zhang et al., 2010) (Skriptsova et al., 2010) (Synytsya et al., 2010) (Chen et al., 2012b)
NMR	Structural analysis	(Skriptsova et al., 2010) (Synytsya et al., 2010) (Ponce et al., 2003) (Bilan et al., 2002) (Ly et al., 2005)
GLC–MS	partially methylated monosaccharides	(Ponce et al., 2003) (Bilan et al., 2002)
GC-EIMS	partially methylated monosaccharides	(Marais et al., 2001) (Chizhov et al., 1999)
GC-MS	methylated sugars	(Pereira et al., 1999)

Basically, LC-MS and GC is used for analysing fucoidan fractions and monosaccharide compositions; for crude fucoidan, assay method is usually adopted to decide the quantity of fucose, sulphate and uronic acid content; NMR and IR method is for comprehending the structure of fucoidan extracted from different seaweed species.

1.2.5 Bioactivity and Relationship between structure and bioactivity

Table 4. Bioactivity summary of fucoidan

Bioactivity	References
Antioxidant	(Rodriguez-Jasso *et al.*, 2014) (Lim *et al.*, 2014) (Marudhupandi *et al.*, 2014)
Anticancer, antitumor, Anti-Metastasis	(Kwak, 2014) (Vishchuk *et al.*, 2013) (Thinh *et al.*, 2013) (Synytsya *et al.*, 2010) (Wang *et al.*, 2014a)
Anticoagulant, antiplatelet	(Jin *et al.*, 2013) (Zhao *et al.*, 2012) (Chen *et al.*, 2012a)
Antithrombotic	(Zhao *et al.*, 2012) (Min *et al.*, 2012)
Anti-inflammatory	(Lee *et al.*, 2013b) (Lee *et al.*, 2012) (Park *et al.*, 2011)
Anti-virus, antiretroviral	(Rabanal *et al.*, 2014) (Wang *et al.*, 2007) (Lee *et al.*, 2004) (Trinchero *et al.*, 2009)
Inhibitory effect on parasites	(Chen *et al.*, 2009) (Clark *et al.*, 1997)
Anti-depression	(Yende *et al.*, 2013)
Immunostimulatory	(Kim *et al.*, 2008) (Khil'chenko *et al.*, 2011) (Choi *et al.*, 2005)
Protective effects on the nervous system	(Mayer *et al.*, 2009)
Therapeutic effect in surgery and brain injury	(DeBow *et al.*, 2003)
Antiangiogenic	(Yu *et al.*, 2012) (Cumashi *et al*., 2007) (Dias *et al.*, 2005)

Fucoidan has been shown to have numerous benefits for human health (Table 4). It was a potential drug that could be used as natural antioxidant in treating diseases (Marudhupandi *et al.*, 2014). The bioactivity of fucoidan has been shown to vary based on its composition and size of molecule. The relationship between the molecular weight of oligosaccharides, sulphation and acetylation degree and their antioxidant action is not simply linear (Barahona *et al.*, 2011). The sulphate group could stimulate the hydrogen atom of the anomeric carbon, besides, the molar ratio of sulphate content to fucose could also influence the antioxidant activity (Wang *et al.*, 2010). The substitutions of the sulphate group, the di-substitute fucose units and esteric hindrance have effect on scavenging capacity (Ananthi *et al.*, 2010). It was apparent that the extracts showed strong proton-donating ability and could work as free-radical inhibitors or scavengers, acting possibly as primary antioxidants (Wang *et al.*, 2010). The low molecular weight sulphated polysaccharides could be readily incorporated into the cells. Despite their low molecular weight sulphated polysaccharides have shown more potent radical scavenging capacity and reducing power than high molecular weight (Choi *et al.*, 2009; Wang *et al.*, 2009; Zhang *et al.*, 2010; Zou *et al.*, 2008). In addition to this, position of branching point, composition and sequence of monosaccharides, configuration and position of glycosidic linkages are also determinant (Morya *et al.*, 2012).

Fucoidan exhibit anticoagulant activity as well, and they also demonstrated that anticoagulant activity did not depend on structural fucose, other neutral sugar and sulphate (Colliec *et al.*, 1991; Jin *et al.*, 2013; Ushakova *et al.*, 2009).

The biological functions are considered to be intimately associated with their characteristic sulphated fucose backbone, although there are a few different structures of the backbone. The extraction of the target sulphated fucose compound is one of the most important steps of the purification procedure. The correct adjustment of parameters, such as temperature, pH, and extraction time, apparently influence the yield, composition, and prevents the probable structural variation of the sulphated polysaccharides. In the meantime, alginate and alginic acid which is composed of guluronic and mannuronic acids, are contamination to fucoidan polysaccharides (Ale *et al*., 2013).

The relationship within the molecular weight of monosaccharides, sulphate groups and acetylation degree and their antioxidant activity is not simply linear (Barahona *et al.*, 2011). Hydrogen atom of the anomeric carbon could be stimulated by the sulphate group. Besides, the molar ratio of sulphate content to fucose could also affect the antioxidant activity (Wang *et al.*, 2010). According to other study, the substitutions of the sulphate group, the di-substitute fucose units and esteric interference also have effect on scavenging capacity (Ananthi *et al.*, 2010). Fucoidan acts possibly as primary antioxidants because the extracted fucoidan showed strong proton-donating ability and could work as free-radical inhibitors or scavengers, (Wang *et al.*, 2010). The lower molecular weight sulphated polysaccharides could be readily introduced into the cells.

1.3 Thesis aims and hypothesis

Given the interest from both New Zealand and abroad for the fucoidan production options and the lack of existing fucoidan extraction businesses, several questions needed to be addressed before moving forward.

First, how long the *Undaria* could be preserved on a harvest boat before processing extraction fucoidan. In order to see how the delay impact yield and composition of fucoidan, this study will test the yield and quality of fucoidan extracted from 6 hour and overnight seaweed after harvest.

Second, given that *U. pinnatifida* is an annual plant, growing mainly in spring and early summer, in order to process throughout the year, it is necessary to investigate the impacts of storage on fucoidan yield and composition, so this study will also do tests of fucoidan yield and quality on fresh seaweed and freezing for 3 months seaweed extraction.

Finally, there are several methods of fucoidan extraction, but many start by drying and milling the seaweed powder. The drying procedure must be done with freeze-dry method due to the oxidation and degradation happening during a high-temperature drying. This is an expensive step band so this study investigates the impact of yield and quality of fucoidan that is extracted from non-dried vs. freeze dried material.

Chapter 2. Methods

2.1 Sampling and experimental design

Seaweeds were collected from a mussel farm located in Man of War Bay on Waiheke Island in the Hauraki Gulf on Friday February 28 2014. The plants were transported to the laboratory and they were washed in fresh water to remove epibionts, dried with paper towels, and measured (width and length of blade, length sporophyll).

There were 8 treatments to determine whether there were impacts on yield and/or composition of fucoidan based on a) time to processing, b) extracting from freeze-dried seaweed or non-freeze-dried seaweed, c) extracting after 3 months freezing.

The 8 treatments were:

1. Left for less than 6 hours, extracted fresh ie. without drying (F6)
2. Left for less than 6 hours, freeze dried, then extracted (FD6)
3. Left for less than 6 hours, frozen for 3 months, extracted without drying (F6-3)
4. Left for less than 6 hours, frozen for 3 months, freeze dried, then extracted (FD6-3)
5. Left for 24 hours, extracted without drying (F24)
6. Left for 24 hours, freeze dried, then extracted (FD24)
7. Left for 24 hours, frozen for 3 months, extracted without drying (F24-3)
8. Left for 24 hours, frozen for 3 months, freeze-dried, then extracted (FD24-3)

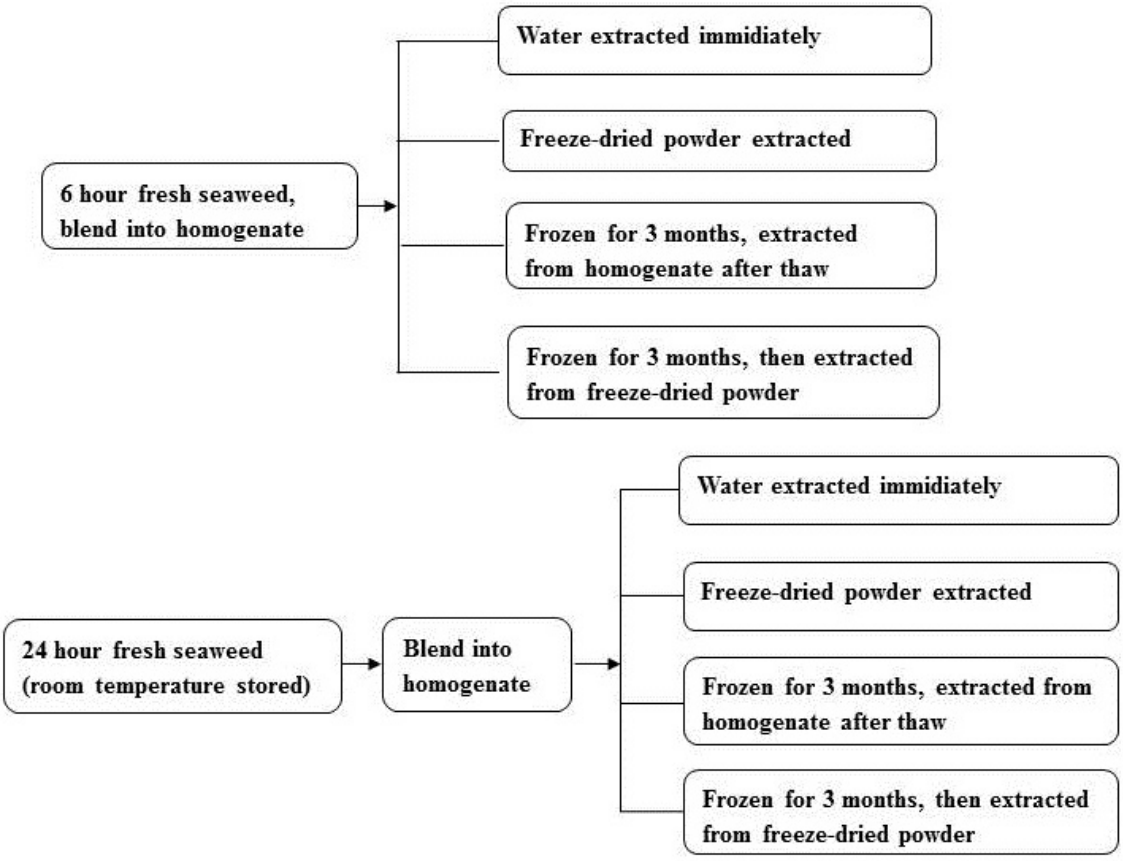


Figure 8. Sampling and treatment

For each of these treatments fucoidan was extracted (as described below). We then took each fucoidan sample and passed it through a range of molecular filters (<3 kilo Daltons (<3 kDa), 3-10 kDa, 10-30 kDa, 30-50 kDa, 50-100 kDa and >100 kDa) to determine the size range of the molecules. Each of these fractions were collected separately and further tested for

1. Protein content
2. Fucose content
3. Uronic acid content
4. Sulphate content
5. Antioxidant capability

For each of these tests, we have also measured the above in the crude (sometimes called total) fucoidan, e. g. the fucoidan before being passed through the filters.

To undertake the experiments on yield of fucoidan, 32 *Undaria pinnatifinda* plants were collected. Four replicate plants were assigned to each of the 8 treatments (outlined below). To undertake the experiments on the quality of fucoidan, a considerable amount of fucoidan was required, such that individual plants could not be used as replicates. Instead around 100 plants were collected and split into 8 pools, each pool assigned to a treatment and homogenised as below. Three replicate fucoidan extracts were used from each homogenate in the experiments.

The 6 hour samples were extracted within 6 hours, while the 24 hour samples were left in plastic bags at ambient temperature in the laboratory for 24 hours. Then each plant or pool of plants was homogenised in a blender in fresh water with a 1:1 ratio (wet weight seaweed:volume of H_2O) ready for either, immediate extraction (F6 and F24), freeze drying (FD6 and FD24), or stored for three months, when they were taken out and extracted immediately (F6-3 and F24-3) or freeze dried and then extracted (FD6-3 and FD24-3).

Figure 9. 32 *Undaria* Seaweed sample plants (collect date: 18/07/2014)

2.2 Extraction

The fucoidan was extracted based on a modified method of Black *et al.*, they extracted fucoidan with boiling water for 3 hours, and treated extracted liquid with ethanol to a final concentration of 60% (Black *et al.*, 1952).

In our study, neutral water was used as the hot aqueous for extracting water-soluble polysaccharide to preserve the native fucoidan structure. To extract the fucoidan, the seaweed homogenates were made up to a 1:1 ratio (wet weight seaweed:volume of H_2O). For homogenate material, the final extraction ratio of homogenate:extract water was 1.6:1, and the dry/wet percentage of freeze dry samples were calculated, the total water weight was kept the same as homogenate sample extractions. Then all the samples were heated for 4 hours at 85℃ with constant stirring. In the final 30 minutes 1.0 g calcium chloride was added with regular stirring to precipitate the alginic acid (the remaining seaweed residue also precipitated at this time) and the liquid became much clearer. The mixture was then filtered (Whatman® hardened ashless, Grade 541, 110mm) under vacuum (in the case of the large homogenates used for quantitative study). For yield experiment, extracted liquids were centrifuged for 10 minutes at a speed of 4000 rmp (Eppendorf Centrifuge 5810R) to remove the precipitate.

The supernatant was collected and mixed with 99% ethanol to make a final concentration of 70% ethanol. This was stored in refrigerator overnight at 4℃ to precipitate the fucoidan. The solution was filtered under vacuum (Whatman® hardened ashless, Grade 541, 110mm), and the precipitate collected and freeze-dried to get crude fucoidan powder. The freeze-dry seaweed and fucoidan of quantify experiment was proceed in Spring Brooks Ltd and Fresh As Ltd; seaweed and fucoidan for yield experiment was freeze-dried by AUT freeze dryer (Christ® Freeze-drying system Alpha 2-4 LD plus). The crude fucoidan samples were ground with a mortar and pestle and passed through a mesh (pore size: 106μm).

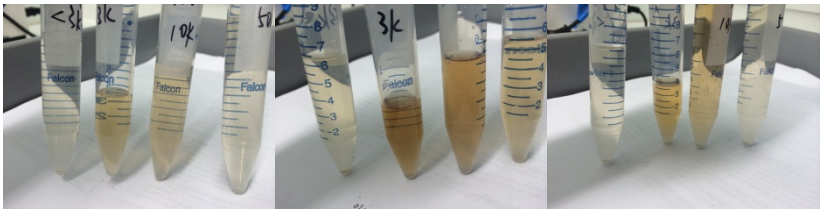


Figure 10. Recover solution of AA, AC &AE fucoidan fractions (<3 kDa, 3-10 kDa, 10-50 kDa, 50-100 kDa)

Figure 11. Millipore Ultrafiltration Membrane

2.3 Fractionation

To determine the molecular weight of the crude extracted fucoidan, the samples were re-solubilized in H_2O and passed through a series of molecular filters (Amicon® Ultra-15 Centrifugal Filter Devices from Merck Millipore Ltd, size: 3000, 10000, 30000, 50000, 100000 nominal molecular weight limit).

For each fucoidan sample, 1.0 g was dissolved in 45 mL hot deionized water (85 to 90 degree) and mixed. 14 mL of each fucoidan solution was added to a 100 kDa Millipore membrane tube, and centrifuged at 5 g, for 30 minutes at 35 degrees C. If, after this time, there was no solution remaining on the membranes, the filtered liquid was removed and placed into the next smallest sized filter and the process repeated. One hour was generally required for all filters smaller than 100 kDa. This filtration was performed 3 times on each sample to make sure there were no remaining small molecular weight fractions being blocked by larger molecules.

After centrifuging all of the sample fucoidan solutions through each sized membrane, the fractions on the membranes were recovered by resolubilising in deionized water in the centrifuge tubes containing the filters. These fractions were then freeze-dried and stored in a desiccator for later tests.

2.4 Composition tests

Because of electrostatic interactions and that the fucoidan fractions were weighed very little, all samples were dissolved into 8mg/mL as stock solutions. All the compositions tests and antioxidant experiments were based on this concentration.

2.4.1 L-Fucose

The fucose content of the fucoidan fractions was determined by the Cysteine-Sulphuric Acid method for methyl pentoses (Dische, 1948). First, each fucoidan fraction stock solution was diluted to a concentration of 680 μg/mL. The sulphuric acid solution was prepared by adding 420 mL concentrated sulphuric acid into 70 mL deionized water with regular stirring, degasing in a boiling water bath for 5 minutes. 3g L-cysteine hydrochloride monohydrate was dissolved in 100 mL deionized water to make a 3% CSOL (L-cysteine hydrochloride monohydrate) solution. From each sample fraction replicate, 357 μL was transferred to another 2 mL eppendorf tube, and 1607 μL of H_2SO_4 (6:1 v/v to water) and 36 μL of L-cysteine hydrochloride (CSOL) was added to make 2 mL total volume, the ratio of the three liquids was 10:45:1 (sample: H_2SO_4: CSOL). This was mixed and cooled to room temperature for 5 minutes. It was then transferred to a boiling water bath for another 5 minutes, and then cooled under running tap water. Duplicate 250 μL aliquots of all samples, blanks and standards were pipetted into a 96-well plate, and read (as soon as possible) at 396 nm and 430 nm in a Thermo Scientific Multiskan Go brand spectrophotometer. Absorbance values were calculated by subtracting A396 nm from A430 nm which corrects for the presence of hexoses (Dische *et al.*, 1951).

Standards and blanks were treated in the same way. The blank was made up of 357 μL of deionized water, 1607 μL of H_2SO_4, and 36 μL of CSOL. Standard solutions were prepared by dissolving 50 mg L-fucose in 50 mL of deionized water, and diluted to a series solution which concentration was from 80 to 400 μg/mL.

2.4.2 Sulphate

The measurement of sulphate content was based on Dodgson's Barium Chloride-Gelatin Assay (Dodgson, 1961). The gelatin reagent was prepared by adding 1 g of gelatin to 200 mL deionized water at 60-70 °C, and chilled at 4 °C overnight, the reagent remains stable for up to one week. 1 g of barium chloride was dissolved in 200 mL gelatin solution to make it $BaCl_2$-gelatin reagent, and let stand for 2-3 hours. Trichloroacetic acid (TCA) was made up with 30 g of TCA powder and 1 litre deionized water. From each standard solution, 40 µL will be transferred to another 2 mL eppendorf tube, mix with 760 µL of TCA (3% w/v) and 200 µL of $BaCl_2$ – gelatin reagent to make 1 mL total volume, stand at room temperature for 10 mins after vortex, then pipet duplicates of 250 µL of all treated standards into a 96-well plate. After mixing, the samples were allowed to stand for 10 mins.

125 µL of each fucoidan fraction, blank and stock solution were added to glass vials (size, manufacturer), 875 µL of 1.143 M HCl was added making a final concentration of HCl of 1M, then the vials were sealed under flame and placed into an oven for 16 hours hydrolysis at 105 °C. Each sample was assayed in triplicate. After cooling to room temperature and centrifuging (Alphatech Z326K) at 5000 rpm, 21 ℃ for 8 minutes, 40 µL from each vial was placed into a 2 mL eppendorf tube and 760 µL of TCA (3% w/v) and 200 µL of $BaCl_2$ – gelatin reagent was added to make 1 mL total volume. This was mixed and let to stand at room temperature for 10 minutes.

Duplicates of 250 µL of all samples, blanks and standards were pipetted into 96-well plates and read within an hour at 420 nm (Thermo Scientific Multiskan Go brand spectrophotometer) (Mak *et al.*, 2013) and 500 nm (Rupérez *et al.*, 2002).

The blank consisted of 40 µL deionised water, 760 µL of TCA (3% w/v), 200 µL of $BaCl_2$–gelatin solution. The SO_4^{2-} standard solution was prepared by adding 90.625mg K_2SO_4 to 50 mL of deionized water as a stock, SO_4^{2-} concentration was 1g/L, then diluted it to an assay of 5, 25, 50, 100, 150, 200, 250, 300, 350, 400 µg/mL.

2.4.3 Uronic acid

The test for uronic acid was based on a paper in 1962 (Bitter *et al.*, 1962), and it was modified by Hoogen (Van Den Hoogen *et al.*, 1998) and Cesaretti (Cesaretti *et al.*, 2003a). 1.016 g was dissolved into sodium tetraborate in 200 mL concentrated sulphuric acid solution and mechanically stirred overnight (this solution was called ST solution). Carbazole solution (which was called CS later on) was prepared by dissolving 50 mg carbazole in 40 mL ethanol, and stored in a brown glass bottle at 4 °C. A saturated benzoic acid deionized water solution was prepared by dissolving solid benzoic acid in water until a precipitate comes out. This was then filtered through filter paper to obtain the target solution. All samples were diluted to 1mg/mL by mixing 125μL sample solution and 875mL deionized water, then adding 1mL of benzoic acid saturated deionized water to make final volume of 2 mL, repeated in triplicate.

50 μL of each sample solution, blank and standards was added to a heatable 96-well plate in duplicate. 200 μL St solution was added using multiple channel pipette, mixed with three gentle in and out movements with the same pipet tips (Van Den Hoogen *et al.*, 1998). The plates were heated for 10 minutes at 100 °C in an oven (Cesaretti *et al.*, 2003b) and then cooled to room temperature for 15 minutes. 50 μL of 0.125% CS was added to each well with multiple channel pipet, and then put into the oven for another 10 minutes at 100 °C. The plates were then cool at room temperature for 15 minutes and absorbance measured at 550 nm on the spectrophotometer (Thermo Scientific Multiskan Go brand spectrophotometer).

The standards were prepared by dissolving 10 mg D-glucuronic acid in 1 mL deionized water in the glass vial, the standard range was 5, 10, 50, 100, 150, 200, 250, 300, 400μg/mL, also add in the same volume of benzoic acid solution as standard solution (standard:benzoic acid=1:1). The blank consisted of 250 μL deionized water and 250 μL of benzoic acid saturated deionized water.

2.4.4 Protein

The measurement of protein was based on Smith's method using bicinchoninic acid (BCA) (Smith *et al.*, 1985). The BCA assay kit was purchased from Sigma-Aldrich, and it comprised reagent A, bicinchoninic acid and reagent B, copper (II) sulphate

pentahydrate. The protein standard was also in the BCA kit, containing 1.0 mg/mL bovine albumin in 0.15M NaCl with 0.05% sodium azide as a preservative. The ratio of BCA working reagent was 50:1 of A:B, add 752.9 µL of reagent A to 37.64 mL reagent B and mix till colour is stable light green. The concentration range of BSA standard was from 25 to 1000µg/mL. Each 25 µL of standard was mixed with 200 µL of BCA working reagent. And the blank was prepared as a mixture of 25 µL deionized water and 200 µL BCA working reagent.

Each fucoidan sample solution was diluted to 3 mg/mL and 500 µL of reagent A was added, with samples prepared in triplicate. For each sample, blank and standard 3 replicates of 25 µL were pipetted to a 96-well plate. 200 µL of BCA working reagent was pipetted into each well, the plate mixed and incubated at 37 °C for 30 minutes. Absorbance was read at 562 nm (Thermo Scientific Multiskan Go brand spectrophotometer).

2.5 Anti-oxidant tests

DPPH assay was prepared by dissolving 24mg 2,2-diphenyl-1-picrylhydrazyl (DPPH) into 200 mL absolute methanol, in a glass bottle which was covered with foil. Fucoidan samples were prepared by diluting with deionized water to 0, 25, 100, 400, 1000, 2000, 3000, 4000 µg/mL. In triplicate, pipette 200µL fucoidan sample solution into a 96-well plate and add 40 µL methanolic DPPH solution to each well. .Each plate was left to stand for 30 minutes in the dark at room temperature, and the absorbance was then measured at 517 nm (Thermo Scientific Multiskan Go brand spectrophotometer).

The blank consisted of only the fucoidan sample (at each concentration) and was measured in duplicate. The positive control was 200 µL methanol and 40 µL DPPH treatment (also in duplicate. Ascorbic acid (Vitamin C) was used as the standard at the same concentrations as the samples (0, 10, 20, 25, 100, 400, 800, 1200, 1600, 2000, 3000, 4000µg/mL). To 4 replicate wells 200µL of standard solution was added. 40 µL methanolic DPPH was added to three of the wells (replicates), while 40 µL methanol added to the fourth well as a positive control.

The percentage of the inhibition of fucoidan sample was compared to ascorbic acid.

Data calculation: %inhibition=[1- $\frac{(\text{Absorbance of sample}-\text{absorbance of blank})}{(\text{Absorbance of control})}$]x 100

2.6 Statistical analysis

For the total fucoidan quantity and quality experiements the first analysis was of the three planned comparisons:

1. processing at 6 vs. 24 Hours, 6 hours (called “Delay”),
2. processing without freeze-drying (fresh) vs freeze dried samples (called “Method”) and
3. samples processed right away vs. stored for 3 months (called “Storage”).

For the fucoidan quantity experiments, the aggregated yield percentage was analysed using a linear multiple regression model which included five explanatory variables: Sporphyll, Weight, Delay, Method and Storage. Contrasts were tested using a likelihood-ratio test by leaving one variable at a time out of the multiple regression model. Thus, estimates of contrast effects were adjusted for variations in sporophyll and weight. Follwing this a one-way ANOVA was used to test for differences between the 8 treatments with a post-hoc Tukeys HSD to determine where any significant differences lay.

For the fucoidan quality experiments, for the planned comparisions, the two dependent variables were each analysed using multiple regression models:

1. composition - dry weight percentages for the fucose, sulphate, uronic acid and protein components, and
2. inhibition - percentage (antioxidant ability)

All sets of replicated measurements were found to be very precise (ICC = 0.997, 0.980, 0.999 for the three outcomes, respectively) explaining a negligible proportion of the overall variance, so these sets of replicates were aggregated by taking the mean in order to simplify the analysis. Molecular sizes and inhibition were analysed using a logit transformation to map the range 0-100% onto the whole real line, to avoid heteroscedasticity and numerical instability near the boundaries. This transformation

was not used with dry weight percentages as some observations were found to be negative as an artefact of the measurement process; these were not excluded as their exclusion would introduce bias.

For each dependent variable, an initial regression model was constructed to take into account variations by key explanatory variables other than the three planned comparisons outlined above (Delay, Method and Storage). The initial model of molecular sizes included only the mean response for each of the four bands of molecule size. The initial model of composition assessed variation by every combination of the four bands of molecule size and the four "parts" (fucose, sulphate, uronic acid and protein). The initial model of inhibition included a cubic curve of variation by dose (treated as ordinal) separately fitted for each band of molecule size. All initial models exhibited a balanced design across combinations of these covariates.

The main effect of each of the three binary contrast variables (Delay, Method and Storage) was assessed by adding that variable to the above initial model and tested using a Likelihood Ratio Test (LRT). Because of the incomplete design involving the contrast variables (two of the eight possible treatment combinations involving freeze-dried and three-month storage were not available – see Results for explanation), models of method included storage and vice versa.

Variations in the distribution of molecule size and the dry weight proportions of the four parts were then tested (separately) by adding the interaction term of that variable with each contrast variable of interest, using the LRT again to test for significant variation in its distribution between the two contrast levels.

Following the analysis of the planned comparisions, an examination of differences within each of the molecular size and compositon experiements were underatken to examine differences in the distribution of molecular weights for each parameter. One-way ANOVAs were used to test for differences between the sizes with a post-hoc Tukeys HSD to determine where any significant differences were.

Analyses were conducted in R version 3.1.2 (R Core Team, 2014) and a level of $p<0.05$ was used to indicate statistical significance.

Chapter 3. Results

3.1 Fucoidan quantity

Under normal circumstances the fucoidan yield would have been converted to a percentage of the dry weight of each individual within each treatment, but because some of the treatments required the plant to be homogenised without drying, determining a dry weight for these was not possible. Therefore we compared percentage fucoidan yield of wet weight for the statistical analysis, although for practical purposes we do estimate the percentage dry weight using the mean percentage water content of the plants that were measured.

There was a large significant impact of increasing fucoidan yield with sporophyll length, so this (and plant weight) were included in the model of the planned comparisons (Table 5). The planned comparisons indicated no significant differences between the main effects, although there was some increase in fucoidan yield after 3 months of storage ($p=0.06$). The one-way ANOVA confirmed that there was a difference when all treatments were compared (Figure 13, Table 6) ($p<0.045$), with Tukey's HSD indicating that the significant difference among all of the 28 possible pairwise comparisons was between FD24 and F24-3 (p-value 0.017), with the former being lower and the latter higher.

Table 5. Planned comparisons adjusted for sporophyll length and total plant weight

Contrast Variable	Model Term	Pvalue	Comments
Sporophyll	Main effect	2.10E-04	Strong evidence of increased yield with increasing sporophyll size
Weight	Main effect	0.53	No evidence of association
Delay	Main effect	0.86	No evidence of difference in means
Method	Main effect	0.10	No evidence of difference in means
Storage	Main effect	0.06	No evidence of difference in means

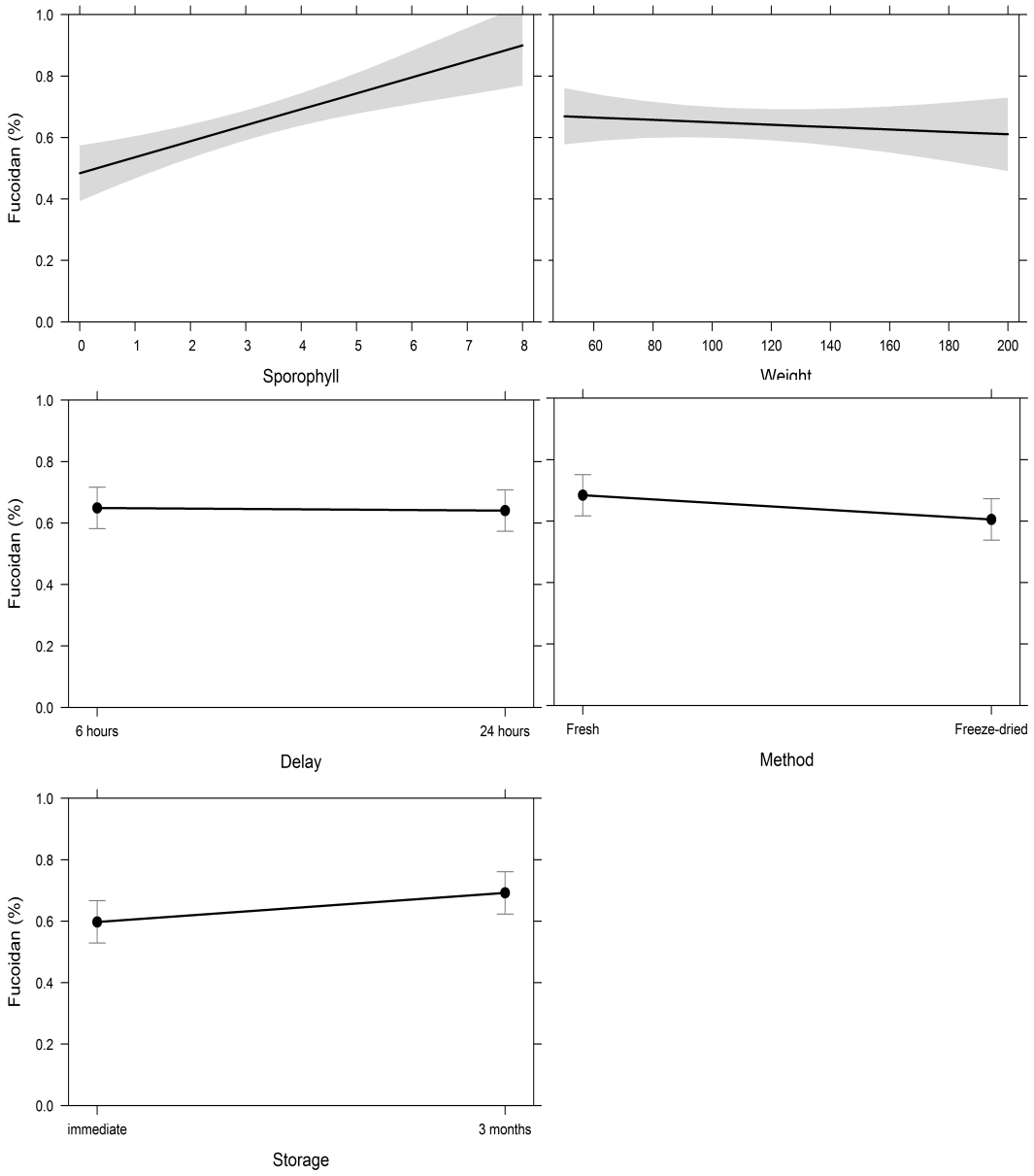


Figure 12. Planned comparison of crude fucoidan content.

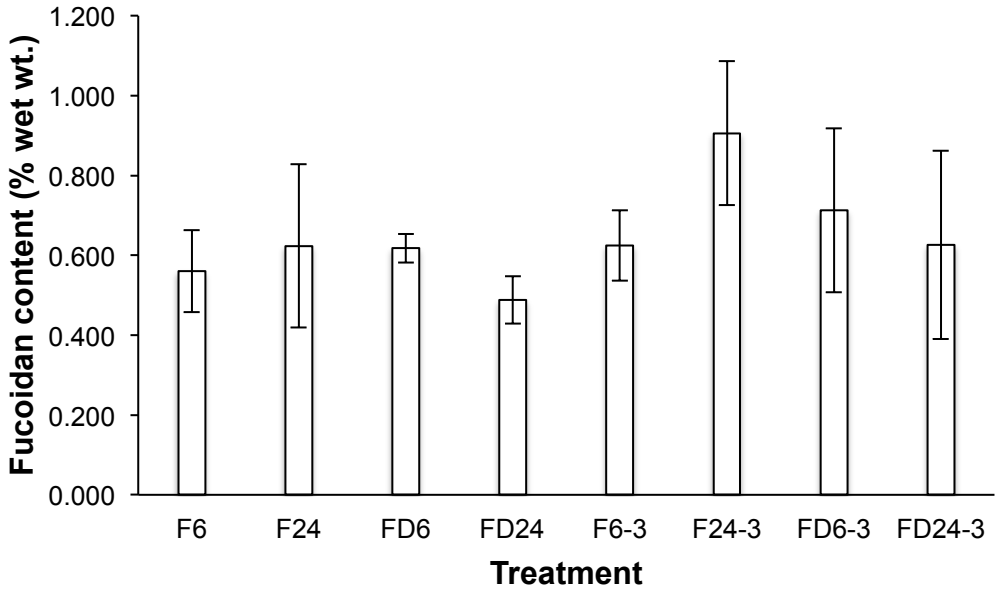


Figure 13. Crude fucoidan content (% wet weight) of each treatment

Table 6. One way ANOVA between treatments for the total crude fucoidan yield experiments.

	SS	df	MS	F	P-value
Between:	0.426	7	0.061	2.490	0.045
Within:	0.587	24	0.024		
Total:	1.013	31			

Table 7. Measurements (cm and g) of individual plants used in yield experiments.

Treatment	Replicate	Width	Length	Sporophyll length	Weight	% dry wt
F6	1	60	74	4	143.6	
	2	31	77	0	49.7	
	3	71	64	0	84	
	4	59	80	5	122.9	
FD6	1	56	66	5	74.6	8.92
	2	55	76	2	73.1	8.72
	3	78	70	0	137.4	8.62
	4	54	46	2	64.3	8.63
F6-3	1	50	26	5	82	
	2	59	79	0	111.7	
	3	44	65	0	57.4	
	4	80	70	2	160.2	
FD6-3	1	35	57	4	81.5	9.95
	2	68	55	8	246	10.69
	3	73	59	3	141.6	9.96
	4	75	65	3	130.9	8.80

Treatment	Replicate	Width	Length	Sporophyll length	Weight	% dry wt
F24	1	46	79	3	85.7	
	2	64	70	4	196.6	
	3	67	44	5	94	
	4	45	73	3	81.3	
FD24	1	47	63	2	78.4	8.62
	2	84	61	0	146.5	8.60
	3	61	49	5	74.3	9.81
	4	54	69	0	69.1	8.21
F24-3	1	60	52	4	93.2	
	2	69	77	4	130.5	
	3	56	47	6	143.2	
	4	52	48	2	69.9	
FD24-3	1	59	73	0	72.9	8.36
	2	65	89	5	157.9	9.33
	3	66	66	5	163.5	8.76
	4	95	43	8	181	10.95
					Mean	9.18
					Std. Dev.	0.84

Fractionation of these crude fucoidan samples was not undertaken (this was instead carried out on the pooled plants as described above), but from the composition tests carried out on the homogenised samples (see below), we determined that the average % of crude fucoidan less than 3 kDa was 37.48 ± 4.22 SD. For reasons that will become clear in the next section, this low molecular weight fraction is not considered to be fucoidan. Given this, we calculated the actual fucoidan yield from crude fucoidan by subtracting 37.48% from each sample. Following from this, using the actual dry weight data of the freeze-dry treatments and a dry weight estimate of 9.18%, the freeze-dry treatments for the treatments where dry weight was not determined (From Table 7), the mean percentage of fucoidan of the dry weight for each treatment was estimated (Table 8). The total mean fucoidan content (% dry weight) across all treatments was 3.74% ± 1.22SD.

Table 8. Estimated fucoidan content of *U. pinnatifida* following the 8 treatments

Treatment	% wet weight	% dry weight
F6	0.35 ± 0.06	3.21 ± 0.59
F24	0.39 ± 0.13	3.58 ± 1.18
FD6	0.39 ± 0.02	3.37 ± 0.25
FD24	0.30 ± 0.04	2.71 ± 0.56
F6-3	0.39 ± 0.05	3.58 ± 0.50
F24-3	0.57 ± 0.11	5.20 ± 1.03
FD6-3	0.45 ± 0.13	4.45 ± 1.60
FD24-3	0.39 ± 0.15	3.79 ± 1.93
Mean	**0.40 ± 0.11**	**3.74 ± 1.22**

3.2 Fucoidan quality

Due to circumstances out of our control, the seaweeds from two of the treatments FD6-3 and FD24-3) were destroyed and so the remaining analysis only takes 6 treatments into account. The number of observations included in statistical models for the three dependent variables were: molecular size (n=30), composition (n=96), and inhibition (n=168). Model assumptions regarding residuals (normality, homoscedasticity and independence) were tested and found to be adequately supported.

3.2.1 Molecular sizes

All of the treatments were pooled and analysed (one way ANOVA, Tukey's HSD) to examine differences in the distribution between the molecule sizes overall. From both the statistical analysis (Table 9) and the graphical representation (Figure 14), it is clear that there is a significant difference between the distribution of the crude extract from *U. pinnatifida*. Tukeys HSD indicates that the percentage of <3 kDa fractions differs from the rest, with the 3-10, 10-30, 30-50 and 50-100 kDa percentages not differing and finally the percentage of >100 kDa molecules is significantly different from the rest (Table10).

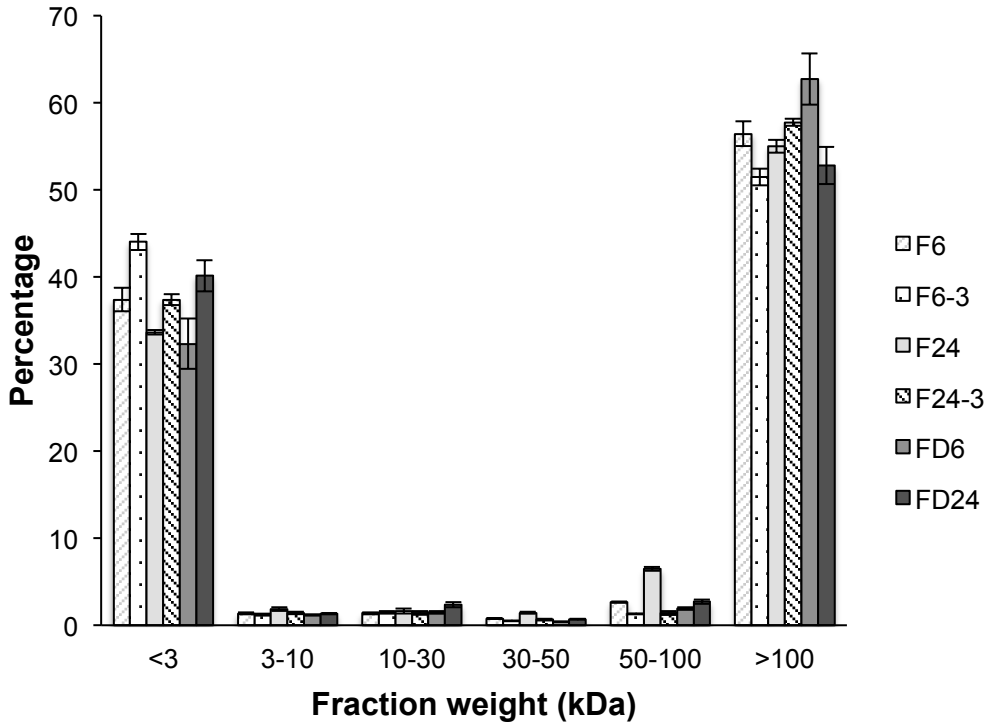


Figure 14. Proportion (%) of crude fucoidan collected on each of the molecular filters.

Table 9. One way ANOVA between percentages fractionated crude fucoidan at each molecular weight.

Source	SS	df	MS	F	P-value
Treatment	52,021	5	10,404.2	1,660.5	1.1102e-16
Error	639.	102	6.2		
Total	52,660	107			

Table 10. Tukeys HSD results of the pairwise comparison of percentage of fractionated crude fucoidan at each molecular weight.

Treatments pair	Tukey HSD Q statistic	p-value	Outcome
<3 vs. 3-10	61.1797	0.0010053	** p<0.01
<3 vs. 10-30	60.7928	0.0010053	** p<0.01
<3 vs. 30-50	62.2781	0.0010053	** p<0.01
<3 vs. 50-100	58.8934	0.0010053	** p<0.01
<3 vs. >100	31.4275	0.0010053	** p<0.01
3-10 vs. 10-30	0.3869	0.8999947	insignificant
3-10 vs. 30-50	1.0984	0.8999947	insignificant
3-10 vs. 50-100	2.2863	0.5775671	insignificant
3-10 vs. >100	92.6072	0.0010053	** p<0.01
10-30 vs. 30-50	1.4853	0.8999947	insignificant
10-30 vs. 50-100	1.8994	0.7333851	insignificant
10-30 vs. >100	92.2203	0.0010053	** p<0.01
30-50 vs. 50-100	3.3847	0.1684961	insignificant
30-50 vs. >100	93.7056	0.0010053	** p<0.01
50-100 vs. >100	90.3209	0.0010053	** p<0.01

3.2.2 Composition

For the statistical analysis of the composition of the fucoidan, the <3 kDa fraction was excluded on the basis that it is unlikely to be fucoidan given the near absence of fucose (Figure 17). Two of the fractions were combined for the composition tests (10-30 and 30-50 kDa), as there was not enough material in either fraction to carry out all of the composition tests.

The planned comparisons for composition (fucose, sulphate, uronic acid and protein), were:

1. processing at 6 vs. 24 Hours, 6 hours (called "Delay"),
2. processing without freeze-drying (fresh) vs. freeze-dried samples (called "Method") and
3. samples processed right away vs. stored for 3 months (called "Storage").

There were no significant differences between any of these comparisons except that there was evidence of more protein the treatments that were stored for 3 months (Table 11, Figures 15 and 16). Given that there was no evidence of a difference in distribution across the range of molecule sizes between treatments, all treatments were pooled and analysed (one way ANOVA, Tukey's HSD) to examine differences in the distribution between the molecule sizes for each fucoidan component. This analysis did not include the crude fucoidan. The results of each of these will be outlined in turn.

Table 11. Tests of planned comparisons for the composition of fractionated fucoidan. The component interaction is testing for an interaction between each comparison with each component (fucose, sulphate, uronic acic and protein), while the kDa interaction is testing for an interaction between each treatment comparison with each particular molecular size.

Contrast Variable	Model Term	Pvalue	Comments
Delay	Main effect	0.82	No evidence of difference in means
Method	Main effect	0.22	No evidence of difference in means
Storage	Main effect	0.15	No evidence of difference in means
Delay	Component interaction	0.19	No evidence of difference in distribution
Method	Component interaction	0.26	No evidence of difference in distribution
Storage	Component interaction	5.04E-05	More protein in 3-month stored samples
Delay	kDa interaction	0.68	No evidence of difference in distribution
Method	kDa interaction	0.24	No evidence of difference in distribution
Storage	kDa interaction	0.15	No evidence of difference in distribution

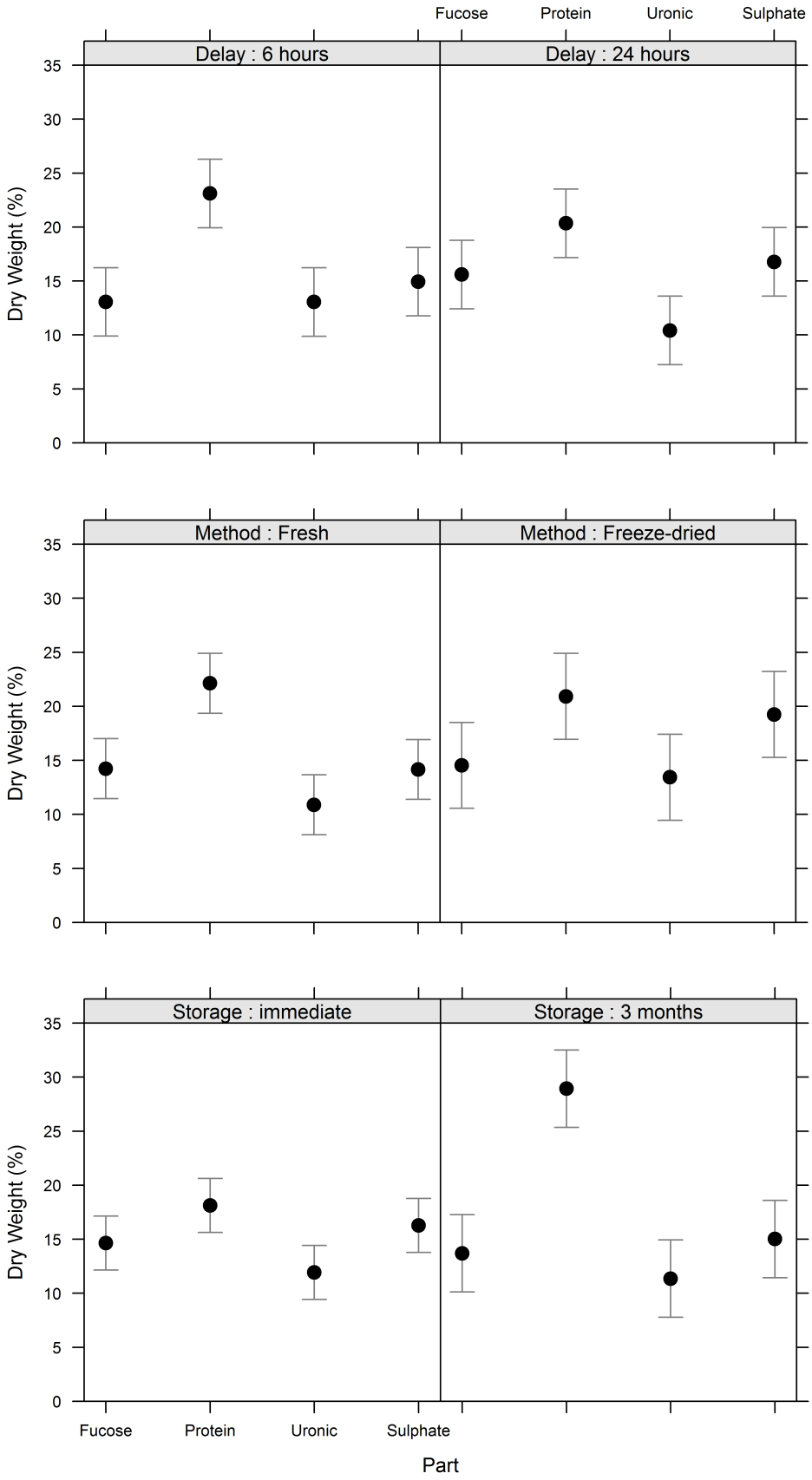


Figure 15. Planned comparisons of the composition of fucoidan following logit transformation. Error bars = Standard Error.

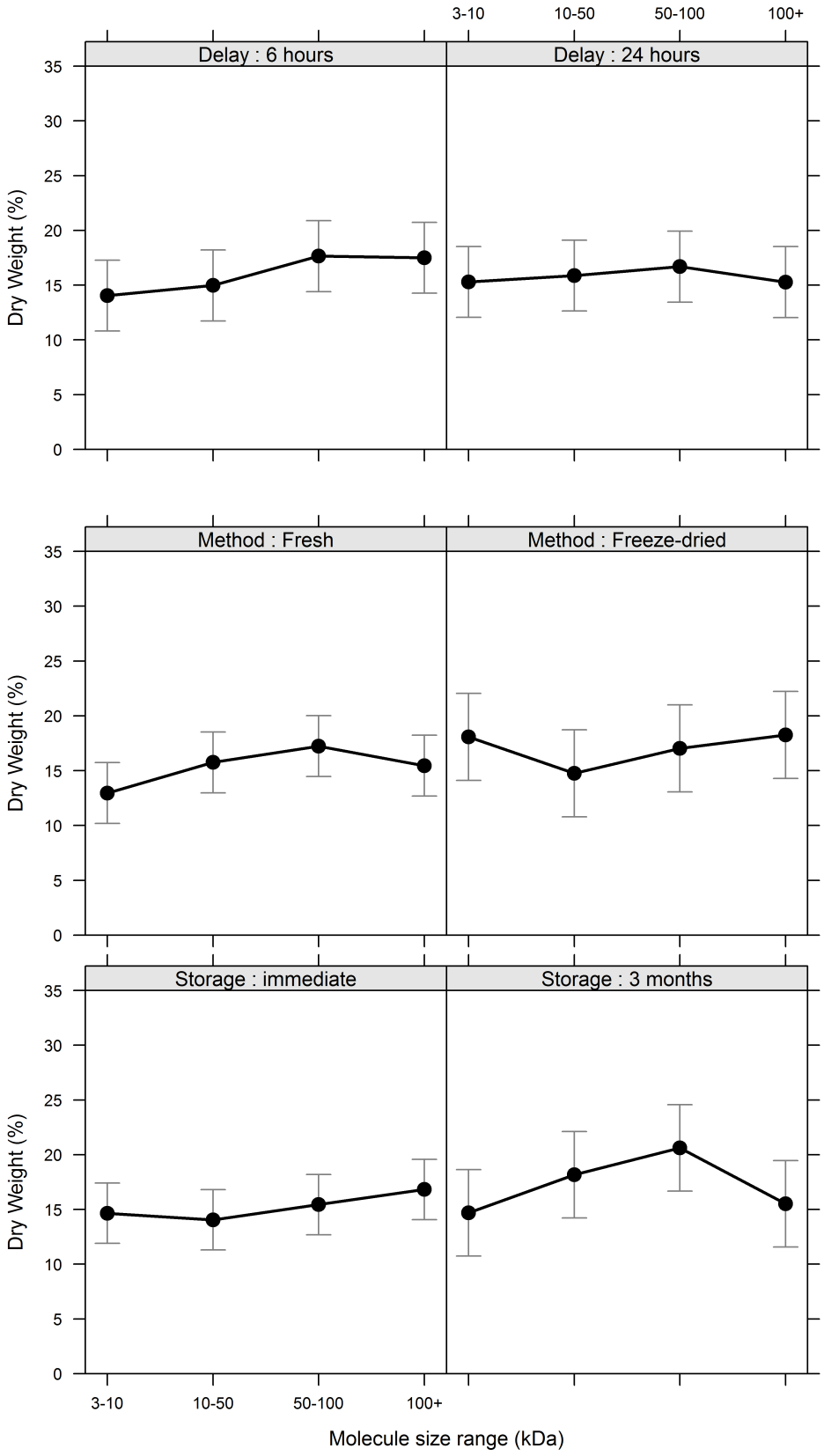


Figure 16. Planned comparisons of the combined composition of fucoidan at each molecular weight following logit transformation. Error bars = Standard Error.

3.2.2.1 Fucose

There was a significant difference in the distribution of molecule sizes in the fucose tests (Figure 17, Table 12). Tukeys HSD (Table 13), indicates that each of the <3, 3-10 and 10-50 kDa fractions are different, with the 50-100 and >100 kDa fractions not differing from each other and have the most fucose.

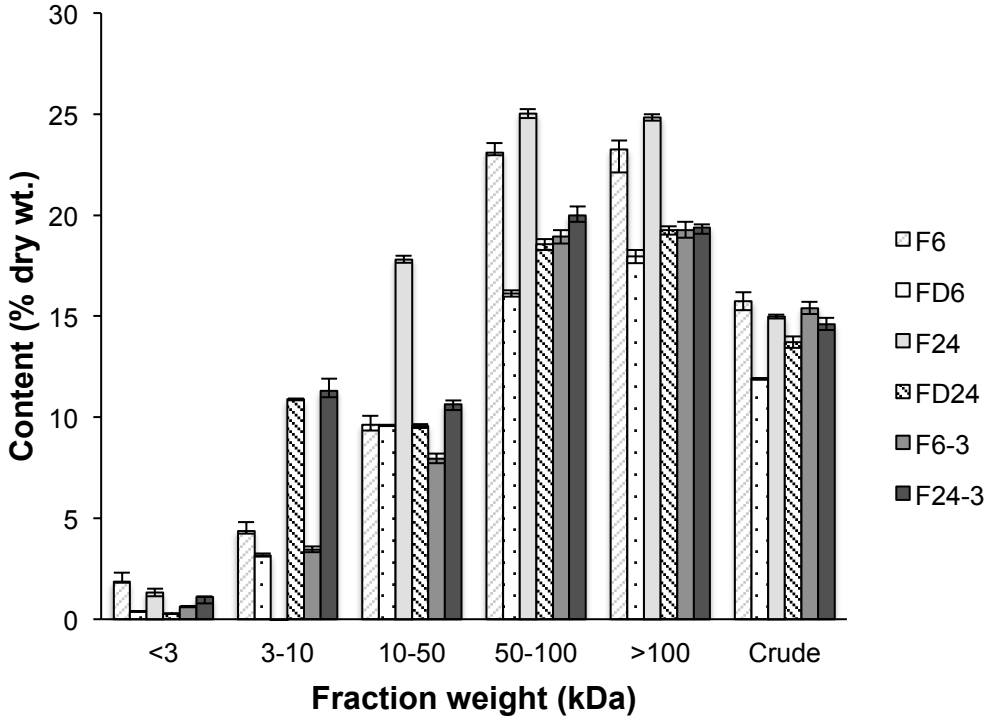


Figure 17. Fucose content of fractions of each molecular weight for each treatment. Error bars = Standard Deviation.

Table 12. One way ANOVA between the fucose content of the fucoidan at each molecular weight.

Source	SS	df	MS	F	P-value
Treatment	5,563	4	1,390.9	151.6	1.1102e-16
Error	779	85	9.1		
Total	6,343	89			

Table 13. Tukeys HSD results of the pairwise comparison of the fucose content of the fucoidan at each molecular weight.

Treatments pair	Tukey HSD Q statistic	P-value	Outcome
<3 vs. 3-10	6.4333	0.0010053	** p<0.01
<3 vs. 10-50	13.9191	0.0010053	** p<0.01
<3 vs. 50-100	27.1246	0.0010053	** p<0.01
<3 vs. >100	27.6439	0.0010053	** p<0.01
3-10 vs. 10-50	7.4858	0.0010053	** p<0.01
3-10 vs. 50-100	20.6913	0.0010053	** p<0.01
3-10 vs. >100	21.2106	0.0010053	** p<0.01
10-50 vs. 50-100	13.2055	0.0010053	** p<0.01
10-50 vs. >100	13.7248	0.0010053	** p<0.01
50-100 vs. >100	0.5193	0.8999947	insignificant

3.2.2.2 Sulphate

There was a significant difference in the distribution of molecule sizes in the sulphate tests (Figure 18, Table 14). Tukeys HSD (Table 15), indicates that the <3 and 3-10 kDa do not differ, the 3-10 and 10-50 kDa fractions do not differ, and, the same as for fucose, the 50-100 and >100 kDa fractions do not differ from each other and have the most sulphate.

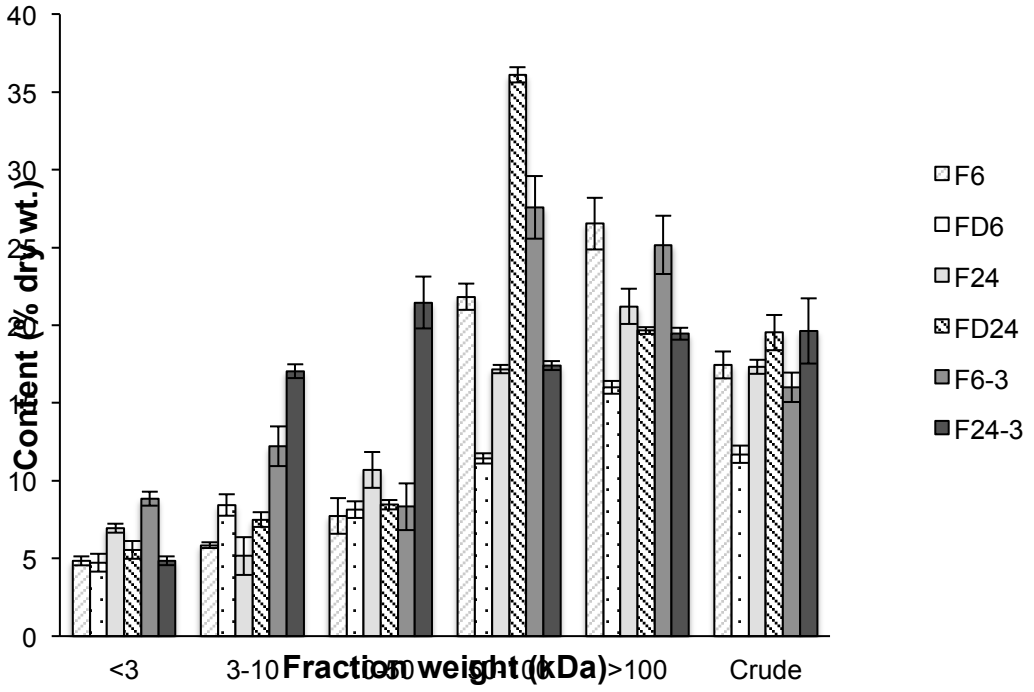


Figure 18. Sulphate content of fractions of each molecular weight for each treatment. Error bar = Standard Deviation. Treatment codes as in Methods Section.

Table 14. One way ANOVA between the sulphate content of the fucoidan at each molecular weight.

Source	SS	df	MS	F	P-value
Treatment	3,830	4	957.6	36.8	1.1102e-16
Error	2,209	85	25.9		
Total	6,040	89			

Table 15. Tukeys HSD results of the pairwise comparison of the sulphate content of the fucoidan at each molecular weight.

Treatments pair	Tukey HSD Q statistic	P-value	Outcome
<3 vs. 3-10	2.8352	0.2727122	insignificant
<3 vs. 10-50	4.0315	0.0424535	* p<0.05
<3 vs. 50-100	13.2811	0.0010053	** p<0.01
<3 vs. >100	12.7956	0.0010053	** p<0.01
3-10 vs. 10-50	1.1964	0.8999947	insignificant
3-10 vs. 50-100	10.4460	0.0010053	** p<0.01
3-10 vs. >100	9.9604	0.0010053	** p<0.01
10-50 vs. 50-100	9.2496	0.0010053	** p<0.01
10-50 vs. >100	8.7640	0.0010053	** p<0.01
50-100 vs. >100	0.4856	0.8999947	insignificant

3.2.2.3 Uronic Acid

There was a significant difference in the distribution of molecule sizes in the uronic acid tests (Figure 19, Table 16). Tukeys HSD (Table 17), indicates that the <3 and 3-10 kDa differ significantly from all of the other fractions, with the 3-10 kDa fraction being the highest overall. The 10-50 and the 50-100 kDa fractions do not differ from each other and neither do the 50-100 and >100 kDa fractions.

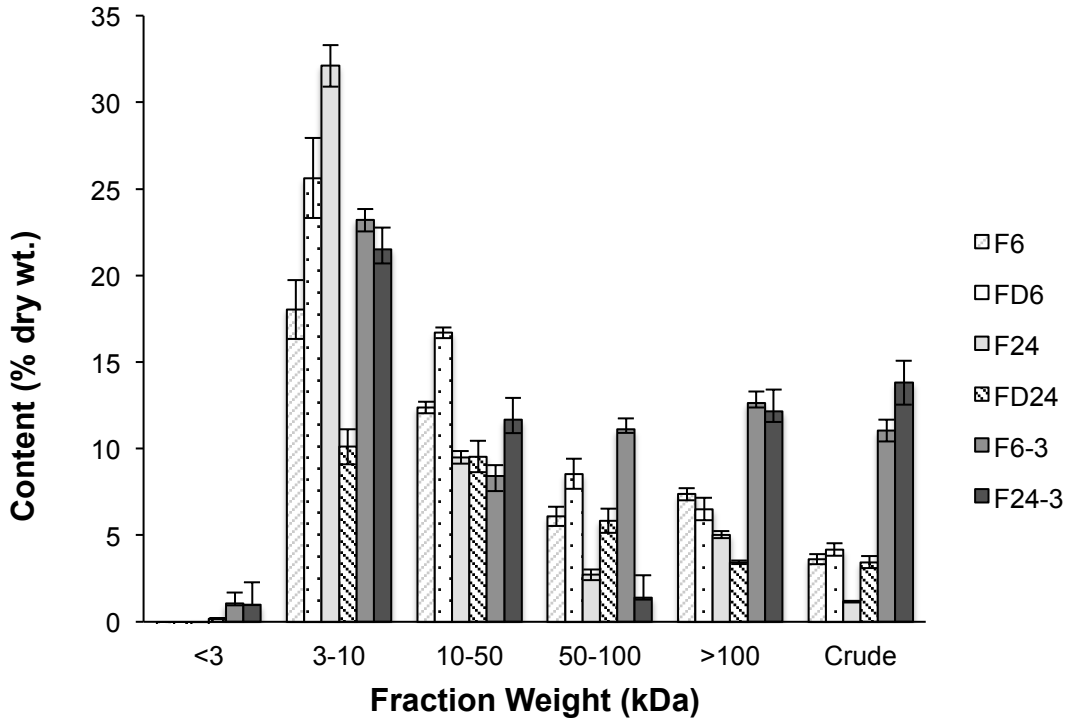


Figure 19. Uronic acid content of fractions of each molecular weight for each treatment. Error bar = Standard Deviation. Treatment codes as in Methods Section.

Table 16. One way ANOVA between the sulphate content of the uronic acid at each molecular weight.

Source	SS	df	MS	F	P-value
Treatment	4,983	4	1,245	72.5	1.1102e-16
Error	1,460	85	17.18		
Total	6,444	89			

Table 17. Tukeys HSD results of the pairwise comparison of the uronic acid content of the fucoidan at each molecular weight.

Treatments pair	Tukey HSD Q statistic	P-value	Outcome
<3 vs. 3-10	23.1911	0.0010053	** p<0.01
<3 vs. 10-50	12.5458	0.0010053	** p<0.01
<3 vs. 50-100	7.0023	0.0010053	** p<0.01
<3 vs. >100	8.9573	0.0010053	** p<0.01
3-10 vs. 10-50	10.6453	0.0010053	** p<0.01
3-10 vs. 50-100	16.1888	0.0010053	** p<0.01
3-10 vs. >100	14.2338	0.0010053	** p<0.01
10-50 vs. 50-100	5.5435	0.0016405	** p<0.01
10-50 vs. >100	3.5885	0.0918748	insignificant
50-100 vs. >100	1.9550	0.6229236	insignificant

3.2.2.4 Protein

There was a significant difference in the distribution of molecule sizes in the protein tests (Figure 20, Table 18). Tukeys HSD (Table 19), indicates that the <3 kDa fraction differs from the rest and has the least protein. The 10-50 kDa fraction differes from the rest and has the most protein. The 3-10 and 50-100 kDa do not differ from each other and neither do the 50-100 and >100 kDa fractions.

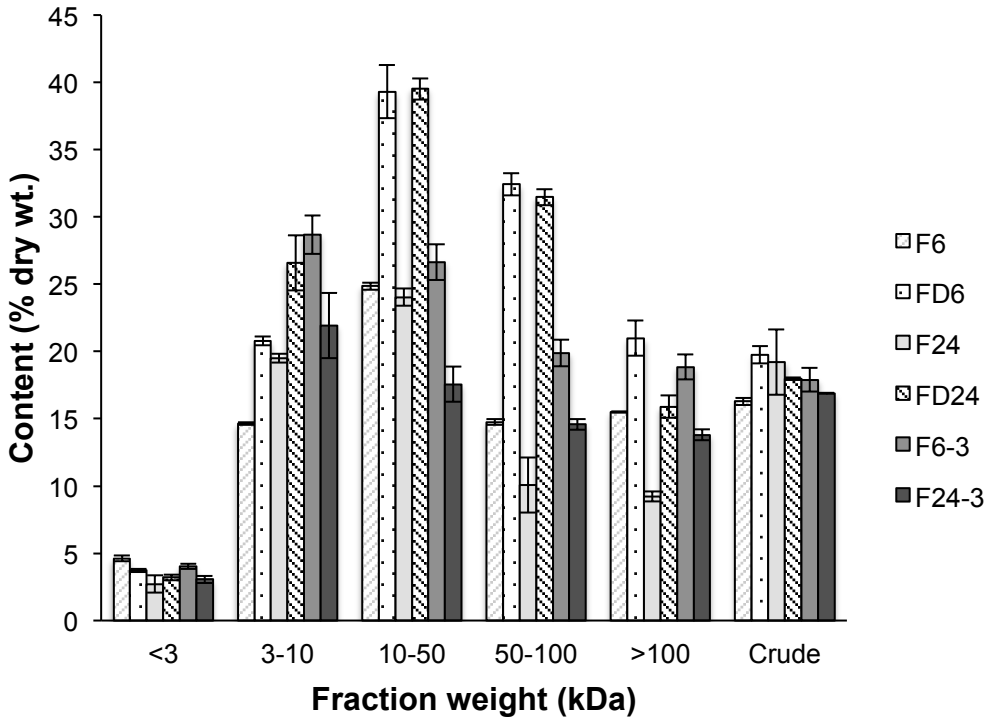


Figure 20. Protein content of fractions of each molecular weight for each treatment. Error bar = Standard Deviation. Treatment codes as in Methods Section.

Table 18. One way ANOVA between the sulphate content of the protein at each molecular weight.

Source	SS	df	MS	F	P-value
Treatment	6,297	4	1,574	41.7	1.1102e-16
Error	3,208	85	37.7		
Total	9,505	89			

Table 19. Tukeys HSD results of the pairwise comparison of the protein content of the fucoidan at each molecular weight.

Treatments pair	Tukey HSD Q statistic	P-value	Outcome
<3 vs. 3-10	12.7504	0.0010053	** p<0.01
<3 vs. 10-50	17.3313	0.0010053	** p<0.01
<3 vs. 50-100	11.7196	0.0010053	** p<0.01
<3 vs. >100	8.3949	0.0010053	** p<0.01
3-10 vs. 10-50	4.5810	0.0143923	* p<0.05
3-10 vs. 50-100	1.0308	0.8999947	insignificant
3-10 vs. >100	4.3554	0.0227806	* p<0.05
10-50 vs. 50-100	5.6117	0.0013891	** p<0.01
10-50 vs. >100	8.9364	0.0010053	** p<0.01
50-100 vs. >100	3.3246	0.1391362	insignificant

Table 20 summarises the grand mean of all replicates across all treatments for the total crude yield of fucoidan and the molecular size fractions. In terms of fucoidan quality, the key points are 1) the high amount of less than 3 kDa molecules, that, given the low fucose content, are unlikey to be fucoidan; 2) little of the remining crude fucoidan (less than 4%) is between 3 and 100 kDa in size, with the majority of the extract being over 100 kDa. 3); the fractions over 50 kDa contain the highest fucose and sulphate levels, indicating that this is the closest to pure fucoidan.

Table 20. Summary of composition experiments: Mean % dry weight ± stdev. of all replicates across all treatments (n=18).

	<3	3-10	10-30	30-50	50-100	>100
Yield	37.48± 4.22	1.39 ± 0.25	1.62 ± 0.39	0.74 ± 0.34	2.74 ± 1.81	56.03 ± 4.02
			10-50			
Fucose	0.92 ± 0.58	5.52 ± 4.30	10.86 ± 3.30		20.29 ± 3.06	20.66 ± 2.60
Sulphate	5.95 ± 1.58	9.36 ± 4.29	10.80 ± 5.09		21.91 ± 8.29	21.33 ± 3.80
Uronic Acid	-0.89 ± 1.95	21.77 ± 7.05	11.36 ± 2.87		5.95 ± 3.40	7.86 ± 3.55
Protein	3.56 ± 0.68	22.02 ± 4.88	28.65 ± 8.41		20.53 ± 8.83	15.71 ± 3.97

3.3 Antioxidant properties

As with the composition, the planned comparisons for antioxidant properties were:

1. processing at 6 vs. 24 Hours, 6 hours (called "Delay"),
2. processing without freeze-drying (fresh) vs freeze-dried samples (called "Method") and
3. samples processed right away vs. stored for 3 months (called "Storage").

Each of the main effects showed a statistically significant difference (Table 21, Figures 21-27). There was higher antioxidant activity in the 24-hour samples when compared to the samples that were processed within 6 hours. There was lower activity in the freeze-dried samples and lower activity in the samples that were stored for 3 months. There was no interaction in terms of molecule size for the delay or storage treatments, but there was lower activity in the 50-100 and >100 kDa fractions in the 3 months storage treatments.

Table 21. Tests of planned comparisons for the antioxidant activity at each molecular size. The kDa interaction is testing for an interaction between each comparison with each particular molecular size.

Contrast Variable	Model Term	Pvalue	Comments
Delay	Main effect	0.049	Higher inhibition at 24-hours
Method	Main effect	1.50E-18	Lower inhibition for freeze-dried
Storage	Main effect	4.08E-09	Lower inhibition for 3-month stored
Delay	kDa interaction	0.16	No evidence of difference
Method	kDa interaction	1.84E-10	Lower inhibition for FD 50-100 and >100kDa
Storage	kDa interaction	0.49	No evidence of difference

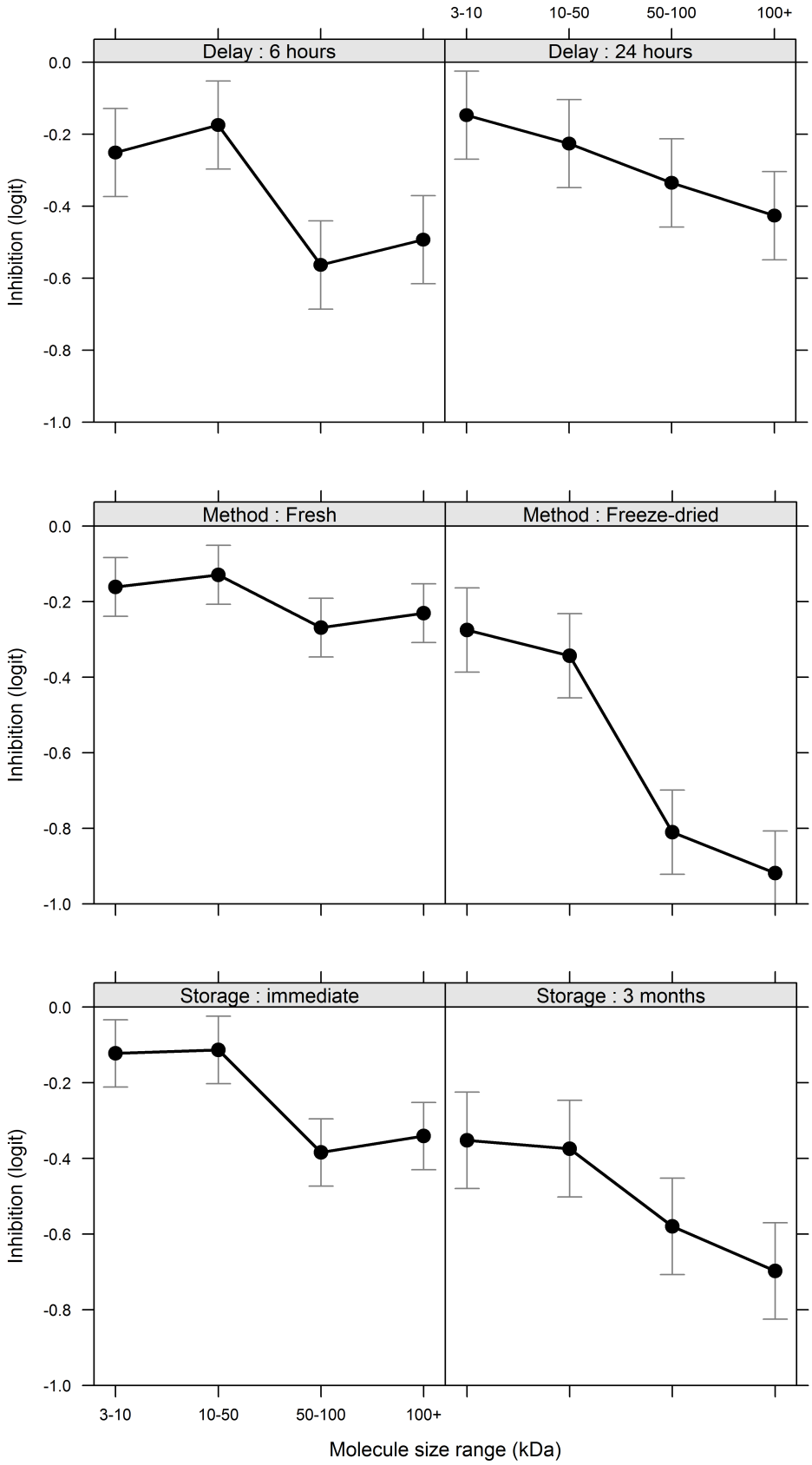


Figure 21. Planned comparisons of antioxidant activity of fucoidan at each molecular weight following logit transformation. Error bars = Standard Error.

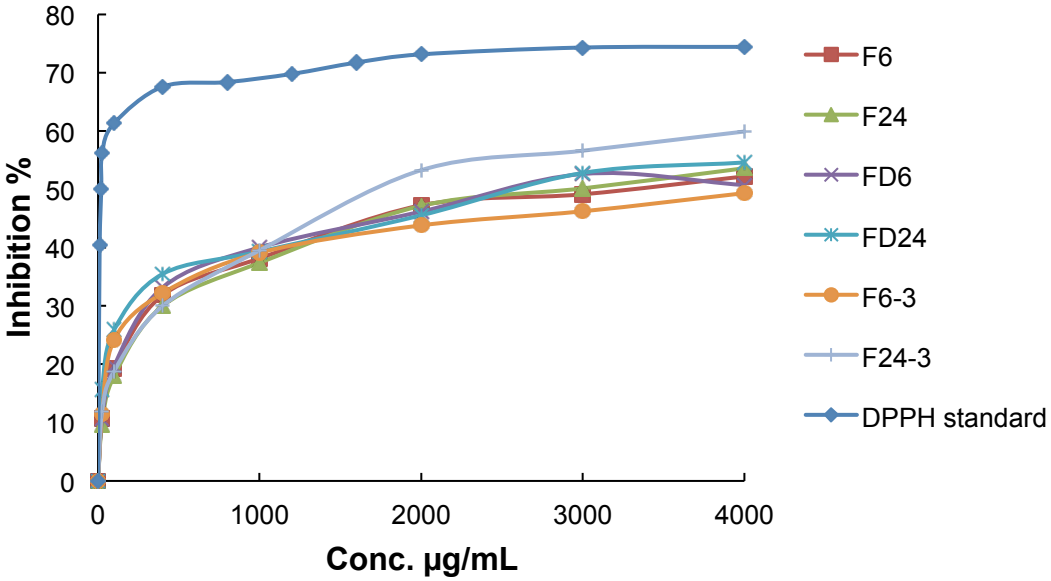


Figure 22. Antioxidant activity of crude fucoidan extract smaller than 3 kDa for each of 6 treatments

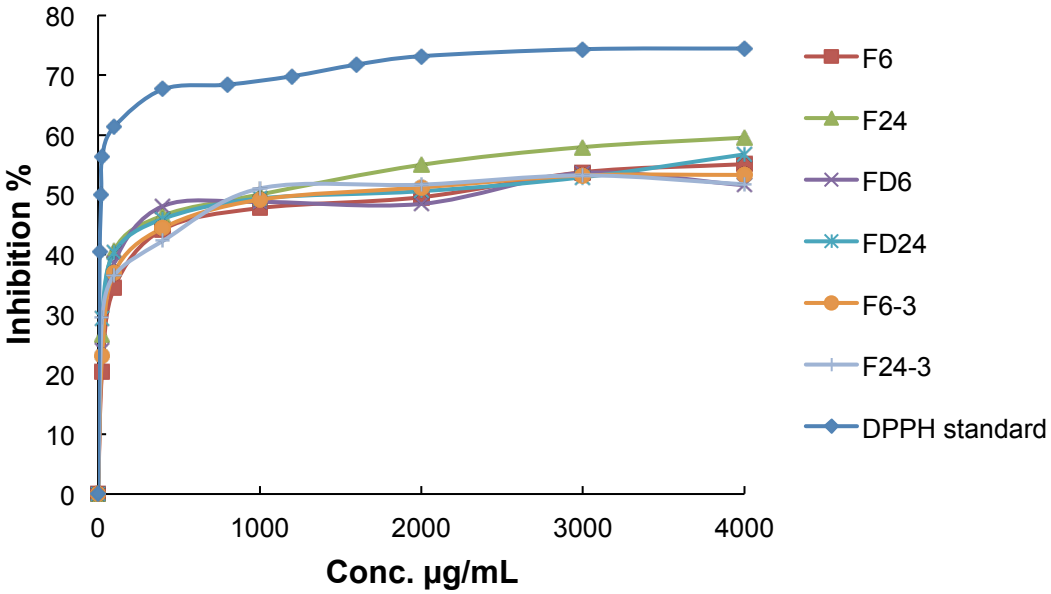


Figure 23. Antioxidant activity of crude fucoidan extract between 3 and 10 kDa for each of 6 treatments

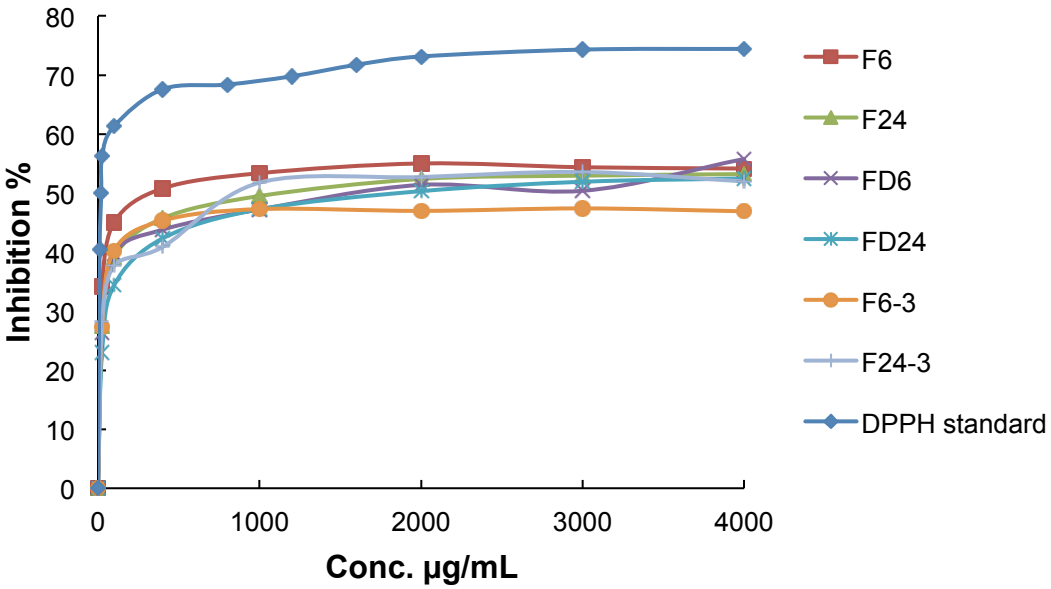


Figure 24. Antioxidant activity of crude fucoidan extract between 10 and 50 kDa for each of 6 treatments

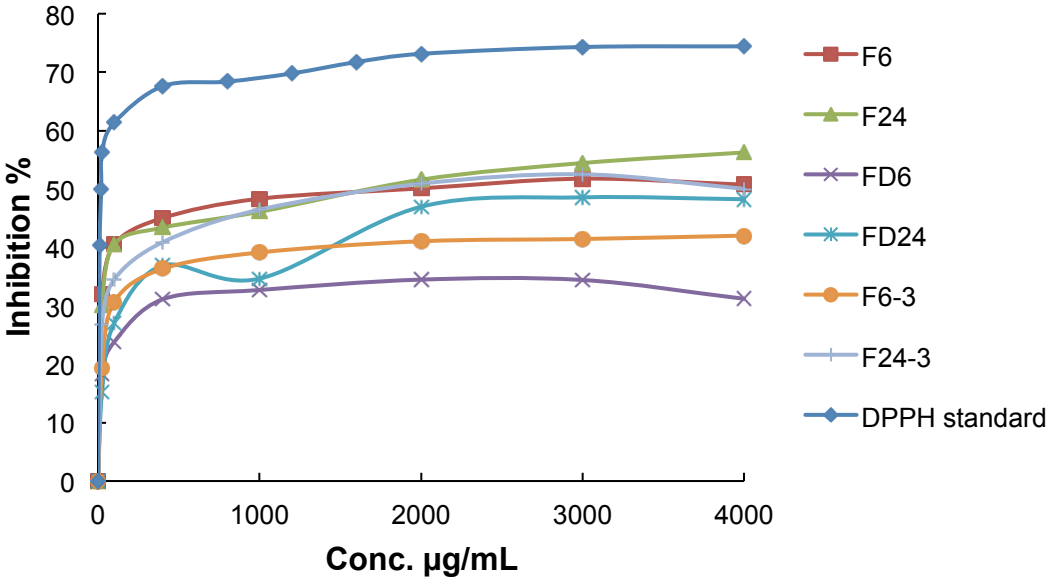


Figure 25. Antioxidant activity of crude fucoidan extract between 50 and 100 kDa for each of 6 treatments

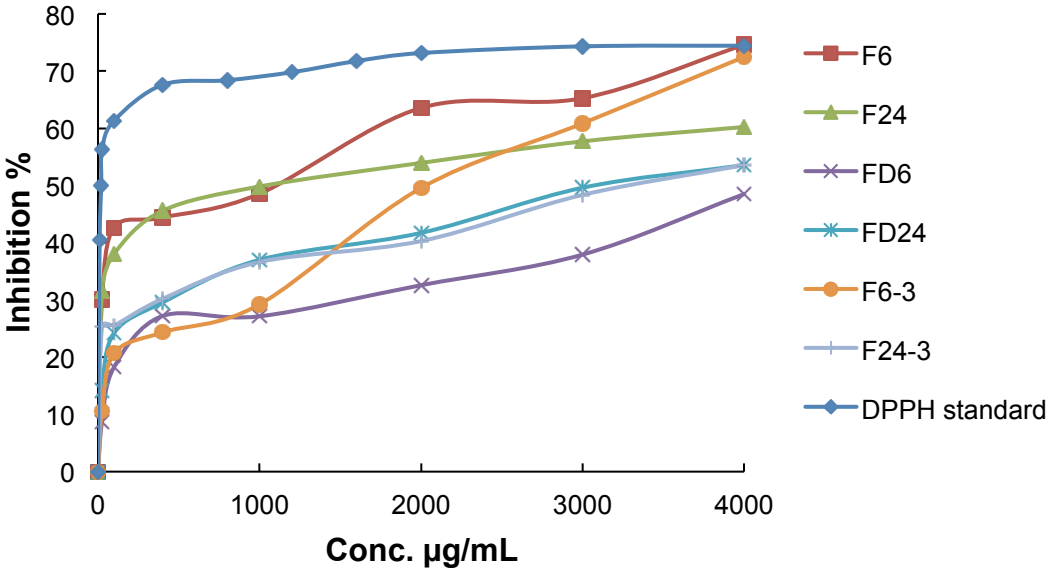


Figure 26. Antioxidant activity of crude fucoidan extract larger than 100 kDa for each of 6 treatments

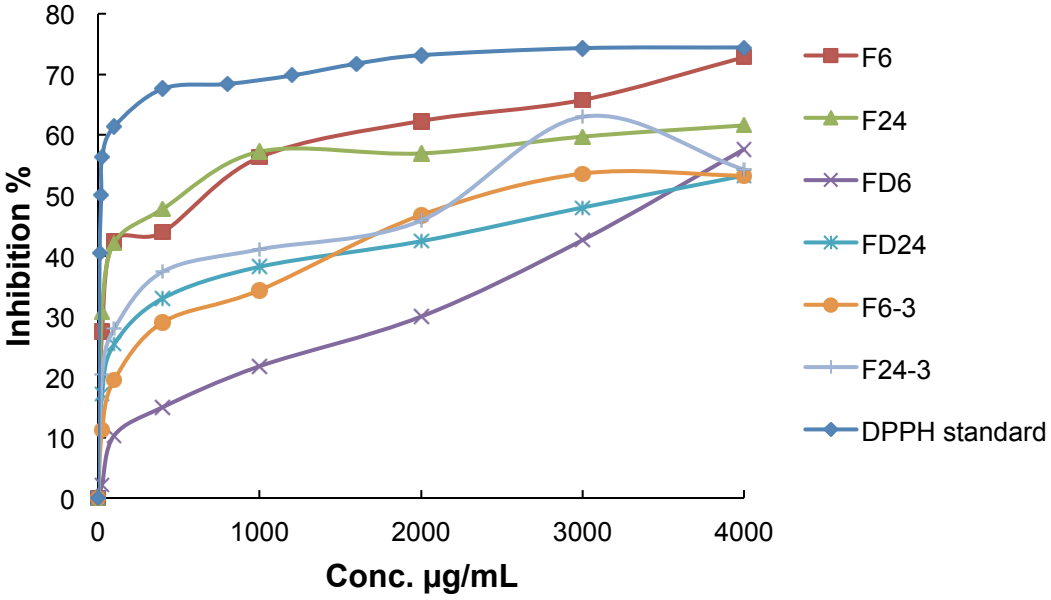


Figure 27. Antioxidant activity of crude fucoidan extract for each of 6 treatments

Chapter 4. Discussion

This study was designed to examine three main hypotheses around the impacts of processing and storage on the yield and composition of fucoidan from *Undaira pinnatifida*. First potential differences if the seaweed is processed immediately as opposed to left for 24 hours. This mimics the potential for harvested seaweed to be left on a boat or wharf overnight following harvest. Second, to see if the fucoidan differs when the seaweed is processed fresh as opposed to freeze dried. This is important as drying the seaweed would add considerable cost to the processing. Finally, the difference between fucoidan extracted immediately following harvest is compared with the fuciodan from seaweed that is stored frozen for three months. This is an important comparison from an industrial point of view as the seaweed is an annual plant that can only be harvested for a few months of the year, so storing to set up a commercial scale extraction factory, it might be necessary to store the seaweed for some time before extract of the fucoidan.

4.1 Quantity of fucoidan

The yield experiments that were carried out on 32 replicate plants with 4 randomly assigned to each of the 8 treatments, showed that, F6 and F24 had a relatively lower yield of crude fucoidan among all the eight treatments. There was only one significant difference: the yield of F24-3 was slightly higher than FD24. The mean of fucoidan yield was 3.74% (dry%).

In previous papers, most studies extracted fucoidan from dried and milled seaweed materials. Because, it turned out dry materials give out higher crude fucoidan yield within same conditions than extracting from fresh seaweed, also dried seaweed was much easier to preserve than fresh seaweed (Lee *et al.*, 2012; Marudhupandi *et al.*, 2014; Rabanal *et al.*, 2014; Thinh *et al.*, 2013), only a few paper chose raw seaweed as extraction materials (Sakai *et al.*, 2003; Teruya *et al.*, 2007).

The fucoidan out of dried seaweed (*Undaria pinnatifinda*) was 1.8% (Vishchuk *et al.*, 2011), 3-16% (Synytsya *et al.*, 2010), 4.2-15.4% (Lee *et al.*, 2004) and 1.0-3.8% (Lee *et al.*, 2006). Nearly 90% of fresh seaweed body is water, so the fucoidan in fresh seaweed is around 0.2% to 1.5%. Our crude fucoidan yield out of fresh seaweed was

around 0.488% to 0.905%, which was covered by the range of previous studies. Polysaccharides such as agar and fucoidan are functional compositions of seaweed cell walls (Christiaen *et al.*, 1987), and the freezing water turns to ice causing the volume of the seaweed cells increased, then inside change captures the cell walls, and ultimately fucoidan is released.

The extraction method and conditions also have effect on fucoidan yield. Higher temperature positively influenced fucoidan yield of aqueous extraction method (Ponce *et al.*, 2003). Traditional extraction method was using hot inorganic acid (HCl or H_2SO_4) to precede the fucoidan extraction (Ponce *et al.*, 2003; Vishchuk *et al.*, 2011), which was not a good choice for producing food grade fucoidan. Meanwhile, compared to water and $CaCl_2$ extraction method, acid extraction did not significantly lead to a higher yield (Mak *et al.*, 2013). In summary, water extraction with calcium chloride to precipitating alginic acid is an ideal producing method for industrial manufacture.

4.2 Quality of fucoidan

4.2.1 Molecular weight

To examine the differences in quality between the treatments, this study first fractionated the extracted crude fucoidan into molecular sizes: <3 kDa, 3-10 kDa, 10-30 kDa, 30-50 kDa, 50-100 kDa and >100 kDa, and then measured fucose, sulphate, uronic acid, protein content.

Fucoidan is large molecular weight, without hydrolysis, most fractions are over 100 kDa. The average molecular weight of crude fucoidan from the sporophylls was estimated to be 171 kDa by GPC according to former study on New Zealand *U. pinnatifida* (Mak *et al.*, 2013). After ion-exchange chromatography, there were always three fractions, the molecular weight ranged from 3 kDa to 94 kDa (Berteau *et al.*, 2002; Ponce *et al.*, 2003; Skriptsova *et al.*, 2010). By HPSEC, fucoidan fraction molecular weight decreased gradually from 5100-2200-490-390-260-30 kDa within heating time increasing (Yang *et al.*, 2008).

The composition of <3 kDa fraction of each sample fucoidan needs further study, this fraction had less of protein, fucose, sulphate and uronic acids than the other larger

fractions. The >100 kDa fraction had more sulphate-fucose structure than smaller molecular weight fractions without hydrolysis.

The average molecular weight of fucoidan was between 13 kDa (Daniel *et al.*, 2001) and 950 kDa (Li *et al.*, 2006). Based on previous studies, the average molecular weight was mostly between 100 kDa to 200 kDa (Rupérez *et al.*, 2002; Sakai *et al.*, 2003; Suppiramaniam *et al.*, 2006; Zhang *et al.*, 2005). Fractions were like brown cotton, and higher molecular weight fractions had a darker colour in this study.

The same devices were adopted in previous study, results showed >30 kDa fraction had the highest yield (43.3%), and the average molecular weight was 262 kDa by light scattering technique; 5-30k Da fraction was only 16.6% with an average molecular weight of 5.6 kDa in hydrolysed fucoidan; and the less than 5 kDa fraction was 39.9%, and its average molecular weight was 1.6 kDa (You *et al.*, 2010).

4.2.2 Composition of fucoidan

As outlined above, fucose makes up the backbone of fucoidan structure, and is routinely used as one of the measures of fucoidan (Table 2). In fucose experiment of crude fucoidan, F6, F24, FD6, FD24 were of a slightly higher percentage (15%) than 3 months sample. In crude fucoidan, the fucose content was from 11% to 16% (w/w). The 50-100 kDa fractions of F6 and F24 also had the highest fucose content compared to the others. There was more fucose content in bigger molecular size fucoidan. The commercial fucoidan extracted from *focus vesiculosus* is reported to be composed of 44.1% fucose, 26.3% sulphate, 31.1% ash and tiny aminoglucose, its $[\alpha]_D$ is -123° (Nishino *et al.*, 1994).

However, the 5-30 kDa hydrolysed fucoidan fraction had the highest fucose content according to a former study (You *et al.*, 2010), they used a similar fractionation method as us, and acidic extraction method. In Vishchuk's study, crude fucoidan from *Undaria* had 30% carbohydrate (w/w), and 47% of the carbohydrate was fucose (Vishchuk *et al.*, 2011). Summarize on the previous studies, the fucose content in crude fucoidan was from 3.14% (Ale *et al.*, 2011a) to 66% (Marais *et al*., 2001). Meanwhile diverse extraction methods and materials also cause the different composition of fucoidan (Table 2). For the other researches on *U. pinnatifidan*, after purified crude fucoidan with anion exchange chromatography on DEAE-Sephadex

A-25 column, the fucose was 58% (Skriptsova *et al.*, 2010), 53% (Vishchuk *et al.*, 2011) and 50.9% (Synytsya *et al.*, 2010) out of all monosaccharides content (HPLC); in Wilfred Mak's study, the fucose content in crude fucoidan extracted from New Zealand *Undaria* blade was 16.4% (dry%, Cysteine-H_2SO_4, method) after remove pigment and alginic acid (Mak *et al.*, 2013). The fucose in crude fucoidan of *P. canaliculata* was 13.1% tested on Cysteine-H_2SO_4, method (Mabeau *et al.*, 1990). In our study, impacts of processing and storage did barely effect on fucose content of fucoidan fractions, and the fucose content was within a general range.

The level of sulphation of fucoidan is one of the most important factors in the level of bioactivity of the molecule, as outlined in section of "bioactivity and relationship between structure and bioactivity". Sulphate content is different causing by various harvest position, season and species, as well as extract conditions. In this study, sulphate content of crude fucoidan was not significantly influenced by processing and storage conditions. The higher molecular fucoidan fractions showed a higher sulphated content.

Without hydrolysis, fucoidan is high molecular weight long chain sulphated polysaccharide, so in different fractions with different extraction treatment, the sulphate content could very dissimilar from each other. In previous studies on fucoidan extracted from *Undaria*, Peisheng Wang claimed sulphate content was 21% after DEAE-cellulose column chromatography (Wang *et al.*, 2014a), besides, Chung (Chung *et al.*, 2010) indicated the sulphate content was 7.4% in crude fucoidan (*Undaria*), and within Liu's experiment, the sulphate in fucoidan derived from *Undaria* was 21% after purify with ion-exchange chromatography (Liu *et al.*, 2012).

Sulphate content in different fractions of *Undaria* fucoidan also varies, generally, it is from 9.18% (Synytsya *et al.*, 2010), 10.4% (Lee *et al.*, 2004), up to 25% (Kim *et al.*, 2012) and 34.6% (Mak *et al.*, 2013).

Uronic acids are also a main composition of seaweed cell walls. They are a class of sugar acids with both carbonyl and carboxylic acid functional groups. Uronic acids are the main composition of cell wall and alginic acid. The unit of fucoidan extracted from *C. okamuranus* comprised a side chain of uronic acid structure, linked by C-O (Ale *et al*., 2013). In our study, fucoidan extracted from 6 hour seaweed have more

uronic acids than those of 24 hour for larger molecular weight fractions. The 3-10 kDa fractions of all fucoidan samples had the highest uronic acids content, and the less than 3 kDa fractions almost had no uronic aicds. Previous studies discovered that uronic acid in crude fucoidan extracted from *U. pinnatifida* was around 3% (Hemmingson *et al*., 2006; Mak *et al*., 2013). In another study, uronic acid was 10.89% by weight of crude fucoidan extracted by tryspin-enzymatic hydrolysis method (Yang *et al*., 2013). The uronic acid content could be up to 26% by an acid-$CaCl_2$ extraction method on *U. pinnatifida* (Foley et al., 2011), it also depends on extraction methods and materials.

Protein is an unwanted composition in fucoidan, it has less contribution to bioactivity of fucoidan. In our study of protein test, lower molecular weight fractions have higher protein content, it turned out there were higher protein content in 3 month samples. However, protein could be removed by using chloroform & methanol & water to pre-treat seaweed samples, it was less than 1% after removing pigment process (Mak *et al.*, 2013). In our study, protein content was more than 15% (w/w). The fucoidan extracted from *Adenocystis utricularis* by another study had 2% to 11% of protein by acidic extraction method (Ponce *et al.*, 2003). Mabeau indicated that protein content was usually lower than 15% (by dry weight) in most brown seaweeds industrially exploited (*L. digitata, A. nodosum*, *F. vesiculosus* and H. *elongata*), however, *U. pinnatifida* had a protein level between 11 and 24% (dry weight) (Mabeau *et al.*, 1993).

4.3 Antioxidant

Many research has focused on antioxidant activity of ocean plant extractions, such as carrageenan, fucoidan and fucoxanthin. Vitamin C and vitamin E are the general anti-oxidant health drugs widely known by people. Seaweeds have also caused an emerging interest in the biomedical area since it has anti-oxidant chemical compounds as well (Ye *et al.*, 2008). According to our study, the F6>100 kDa fraction and FD6>100 kDa fraction reached the same inhibition ability as ascorbic (Vitamin C) standard at a concentration of 4mg/mL. The fucoidan extracted from 6 hour and 24 hour fresh seaweed had a stronger inhibition activity compared to other samples which were all freeze to solid.

Some researches pointed out that the antioxidant activity property of fucoidan was depending on the sulphate content of the fraction, meanwhile the fucoidan has non toxicity effect to human body (Mabeau *et al*., 1993; Marudhupandi *et al.*, 2014), so an increasing number of researches have been focusing on antioxidant activity of seaweed extracts, and it is intended to replace synthetically drugs. In this experiment, high molecular weight fractions of fucoidan showed a stronger inhibition activity on DPPH assay. In Marudhpandi's research, the maximum scavenging effect happened on crude fucoidan at a concentration of 400μg/mL, rather than fucoidan fractions (Marudhupandi *et al.*, 2014). Fucoidan extracted from Malaysia brown seaweed *S. binderi and Padina sp.* both had antioxidant capacity in terms of superoxide anion and hydroxyl radical scavenging activities compared to those of the synthetic antioxidants (Lim *et al.*, 2014).

A review indicated that using one-dimensional methods to evaluate multifunctional food and biological antioxidants was a problem itself, since the result only means the inhibition activity within that method (Frankel *et al.*, 2000). Currently, there are several anti-oxidant capacity assays, including ORAC, TRAP, TEAC, FCR, DPPH, CUPRAC and FRAP (Huang *et al.*, 2005). Among them, DPPH is a general method that most papers adopted. However, polysaccharides were not soluble in methanol or ethanol, a study of mixing DPPH assay in DMSO solution found that sulphated polysaccharides from seaweed showed high DPPH radical scavenging capacity (Kim *et al.*, 2007). Another research performed hydroxyl radical scavenging activity (HRSA) and superoxide anion radical scavenging activity (SARSA) to evaluate the inhibition rate of fucoidan. Their results suggested low MW fucoidan had high scavenging activity (Qu *et al.*, 2014). However, our study showed that crude fucodian had a slightly higher inhibition activity than lower molecular weight fucoidan fractions.

Besides, natural products derived from marine algae protect cells by modulating the effects of oxidative stress. Because oxidative stress also plays important roles in inflammatory reactions and in carcinogenesis, marine algal products have the potential to be used as anticancer and anti-inflammatory products (Lee *et al.*, 2013a; Lee *et al.*, 2012; Park *et al.*, 2011).

In summary, < 3 kDa fractions of all samples had trace sulphate and fucose content, which means there was barely sulphated polysaccharides in smallest fractions. In >100 kDa fractions, the composition tests and DPPH antioxidant tests almost had the same results as crude fucoidan, however, lower molecular weight fractions was not of good quality, so there was no need to separate fucoidan into smaller size fractions.

The impacts of processing and storage methods did not have significant influence on fucoidan yield and quality. That means seaweed materials could stay on the harvest boat overnight. Since *U. pinnatifida* is an annual plant, the harvest season is only in spring and summer, however, harvested plants could be stored by in freezer for months without reducing fucoidan quality.

In conclusion, the delay processing and storage method did no change to fucoidan yield and composition quality, it solved the preservation problem after harvesting vast seaweed material. In following study, research could focus on extracting conditions on the yield and composition of fucoidan. Meanwhile different batch of fucoidan will have slightly distinction, it may cause functional difference in antioxidant activity or other bioactivity, so that's also an aspect needs further study.

References

Ale, M. T., Maruyama, H., Tamauchi, H., Mikkelsen, J. D., & Meyer, A. S. (2011a). Fucoidan from *Sargassum sp*. and *Fucus vesiculosu*s reduces cell viability of lung carcinoma and melanoma cells in vitro and activates natural killer cells in mice in vivo. *Int. J. Biol. Macromol., 49*(3), 331-336.

Ale, M. T., & Meyer, A. S. (2013). Fucoidans from brown seaweeds: an update on structures, extraction techniques and use of enzymes as tools for structural elucidation. *RSC Advances, 3*(22), 8131-8141. doi: 10.1039/C3RA23373A

Ale, M. T., Mikkelsen, J. D., & Meyer, A. S. (2011b). Important Determinants for Fucoidan Bioactivity: A Critical Review of Structure-Function Relations and Extraction Methods for Fucose-Containing Sulfated Polysaccharides from Brown Seaweeds. *Mar. Drugs, 9*(10), 2106-2130.

Ananthi, S., Raghavendran, H. R. B., Sunil, A. G., Gayathri, V., Ramakrishnan, G., & Vasanthi, H. R. (2010). In vitro antioxidant and in vivo anti-inflammatory potential of crude polysaccharide from *Turbinaria ornata* (Marine Brown Alga). *Food Chem. Toxicol., 48*(1), 187-192. doi: http://dx.doi.org/10.1016/j.fct.2009.09.036

Anastyuk, S. D., Shevchenko, N. M., Nazarenko, E. L., Dmitrenok, P. S., & Zvyagintseva, T. N. (2009). Structural analysis of a fucoidan from the brown alga *Fucus evanescens* by MALDI-TOF and tandem ESI mass spectrometry. *Carbohyd. Res., 344*(6), 779-787.

Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*. *Carbohyd. Polym., 66*(2), 184-191.

Barahona, T., Chandía, N. P., Encinas, M. V., Matsuhiro, B., & Zúñiga, E. A. (2011). Antioxidant capacity of sulfated polysaccharides from seaweeds. A kinetic approach. *Food Hydrocolloid., 25*(3), 529-535. doi: http://dx.doi.org/10.1016/j.foodhyd.2010.08.004

Béress, A., Wassermann, O., Bruhn, T., Béress, L., Kraiselburd, E. N., Gonazalez, L. V., Chavez, P. J. (1996). A New Procedure for the Isolation of Anti-HIV Compounds (Polysaccharides and Polyphenols) from the Marine Alga *Fucus vesiculosus*. *J. Nat. Prod., 59*(5), 552-552. doi: 10.1021/np960284u

Berteau, O., McCort, I., Goasdoué, N., Tissot, B., & Daniel, R. (2002). Characterization of a new α-L-fucosidase isolated from the marine mollusk Pecten maximus that catalyzes the hydrolysis of α-L-fucose from algal fucoidan (*Ascophyllum nodosum*). *Glycobiology, 12*(4), 273-282.

Bilan, M. I., Grachev, A. A., Shashkov, A. S., Kelly, M., Sanderson, C. J., Nifantiev, N. E., & Usov, A. I. (2010). Further studies on the composition and structure of a fucoidan preparation from the brown alga *Saccharina latissima*. *Carbohyd. Res., 345*(14), 2038-2047.

Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2002). Structure of a fucoidan from the brown seaweed Fucus evanescens C.Ag. *Carbohyd. Res., 337*(8), 719-730.

Billakanti, J. M., Catchpole, O., Fenton, T., & Mitchell, K. (2012). Extraction of Fucoxanthin from *Undaria Pinnatifida* using enzymatic pre-treatment followed by DME & EtoH co-solvent extraction. *Paper presented at the 10th International Symposium on Supercritical Fluids*.

Biosecuruty, A. (1993).

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Anal. Chem., 4*(4), 330-334.

Black, W. A. P., Dewar, E. T., & Woodward, F. N. (1952). Manufacture of algal chemicals. IV—Laboratory-scale isolation of fucoidin from brown marine algae. *J. Sci. Food Agr., 3*(3), 122-129. doi: 10.1002/jsfa.2740030305

Cesaretti, M., Luppi, E., Maccari, F., & Volpi, N. (2003a). A 96-well assay for uronic acid carbazole reaction. *Carbohyd. Polym., 54*(1), 59-61.

Chandía, N. P., & Matsuhiro, B. (2008). Characterization of a fucoidan from *Lessonia vadosa* (Phaeophyta) and its anticoagulant and elicitor properties. *Int. J. Biol. Macromol., 42*(3), 235-240.

Chen, A., Zhang, F., Shi, J., & Zhao, X. (2012a). Study on antithrombotic and antiplatelet activities of low molecular weight fucoidan from *Laminaria japonica*. *J. Ocean Uni. Chin., 11*(2), 236-240. doi: 10.1007/s11802-012-1874-1

Chen, J. H., Lim, J. D., Sohn, E. H., Choi, Y. S., & Han, E. T. (2009). Growth-inhibitory effect of a fucoidan from brown seaweed *Undaria pinnatifida* on Plasmodium parasites. *Parasitol. Res., 104*(2), 245-250.

Chen, X., Xing, R., Yu, H., Liu, S. Q., & Li, P. (2012b). A new extraction method of fucoidan from the soaked water of brown seaweed (Laminaria japonica). *Desalination and Water Treatment, 40*(1-3), 204-208.

Chizhov, A. O., Dell, A., Morris, H. R., Haslam, S. M., McDowell, R. A., Shashkov, A. S., Usov, A. I. (1999). A study of fucoidan from the brown seaweed *Chorda filum*. *Carbohyd. Res., 320*(1–2), 108-119.

Choi, E. Y., Hwang, H. J., Kim, I. H., & Nam, T. J. (2009). Protective effects of a polysaccharide from *Hizikia fusiformis* against ethanol toxicity in rats. *Food Chem. Toxicol., 47*(1), 134-139.

Choi, E. Y., Kim, A. J., Kim, Y. O., & Hwang, J. K. (2005). Immunomodulating activity of arabinogalactan and fucoidan in vitro. *J. Med. Food, 8*(4), 446-453.

Christiaen, D., Stadler, T., Ondarza, M., & Verdus, M. C. (1987). Structures and functions of the polysaccharides from the cell wall of *Gracilaria verrucosa* (Rhodophyceae, Gigartinales). In M. Ragan & C. Bird (Eds.), *Twelfth International Seaweed Symposium* (Vol. 41, pp. 139-146): Springer Netherlands.

Chung, H. J., Jeun, J., Houng, S. J., Jun, H. J., Kweon, D. K., & Lee, S. J. (2010). Toxicological evaluation of fucoidan from *Undaria pinnatifidain* vitro and in vivo. *Phytother. Res., 24*(7), 1078-1083. doi: 10.1002/ptr.3138

Clark, D., Su, S., & Davidson, E. (1997). Saccharide anions as inhibitors of the malaria parasite. *Glycoconjugate J., 14*(4), 473-479. doi: 10.1023/a:1018551518610

Colliec, S., Fischer, A. M., Tapon, B. J., Boisson, C., Durand, P., & Jozefonvicz, J. (1991). Anticoagulant properties of a fucoidan fraction. *Thromb. Res., 64*(2), 143-154. doi: http://dx.doi.org/10.1016/0049-3848(91)90114-C

Cumashi, A., Ushakova, N. A., Preobrazhenskaya, M. E., D'Incecco, A., Piccoli, A., Totani, L., Nifantiev, N. E. (2007). A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology, 17*(5), 541-552.

Daniel, R., Berteau, O., & Cardoso, M. A. (2001). Regioselective desulfation of sulfated l-fucopyranoside by a new sulfoesterase from the marine molluskPecten maximus.: Application to the structural study of algal fucoidan (*Ascophyllum nodosum*). *Eur. J. Biochem., 268*(21), 5617.

DeBow, S. B., Davies, M. L., Clarke, H. L., & Colbourne, F. (2003). Constraint-induced movement therapy and rehabilitation exercises lessen motor deficits and volume of brain injury after striatal hemorrhagic stroke in rats. *Stroke, 34*(4), 1021-1026.

Dias, P. F., Siqueira Jr, J. M., Vendruscolo, L. F., De Jesus Neiva, T., Gagliardi, A. R., Maraschin, M., & Ribeiro-do-Valle, R. M. (2005). Antiangiogenic and antitumoral properties of a polysaccharide isolated from the seaweed Sargassum stenophyllum. *Cancer Chemoth. Pharm., 56*(4), 436-446.

Dische, Z. (1948). A new specific color reaction of galacturonic acid. *Arch. Biochem., 16*(3), 409-414.

Dische, Z., & Shettles, L. B. (1951). A new spectrophotometric test for the detection of methylpentose. *J. Biol. Chem., 192*(2), 579-582.

Dodgson, K. S. (1961). Determination of inorganic sulphate in studies on the enzymic and non-enzymic hydrolysis of carbohydrate and other sulphate esters. *Biochem. J., 78*, 312-319.

Duarte, M. E. R., Cardoso, M. A., Noseda, M. D., & Cerezo, A. S. (2001). Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. *Carbohyd. Res., 333*(4), 281-293.

Ebringerová, A., & Hromádková, Z. (2010). An overview on the application of ultrasound in extraction, separation and purification of plant polysaccharides. *Cent. Eur. J. Chem., 8*(2), 243-257.

Foley, S. A., Mulloy, B., & Tuohy, M. G. (2011). An unfractionated fucoidan from *Ascophyllum nodosum*: Extraction, characterization, and apoptotic effects in vitro. *J. Nat. Prod., 74*(9), 1851-1861.

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agr., 80*(13), 1925-1941. doi: 10.1002/1097-0010(200010)80:13<1925::aid-jsfa714>3.0.co;2-4

Gerbst, A. G., Grachev, A. A., Ustuzhanina, N. E., Nifantiev, N. E., Vyboichtchik, A. A., Shashkov, A. S., & Usov, A. I. (2010). Application of Artificial Neural Networks for Analysis of 13C NMR Spectra of Fucoidans. *J. Carbohy. Chem., 29*(2), 92-102. doi: 10.1080/07328301003743657

Guo, H., Liu, F., Jia, G., Zhang, W., & Wu, F. (2013). Extraction optimization and analysis of monosaccharide composition of fucoidan from *Saccharina japonica* by capillary zone electrophoresis. *J. Appl. Phycol., 25*(6), 1903-1908. doi: 10.1007/s10811-013-0024-5

Hahn, T., Lang, S., Ulber, R., & Muffler, K. (2012). Novel procedures for the extraction of fucoidan from brown algae. *Process Biochem., 47*(12), 1691-1698.

Hay, C. H., & Villuota, E. (1993). Seasonality if the Adventure Asian Kelp *Undaria pinnatifida* in New Zealand. *Bot. Mar., 36*(5), 461-476.

He, Z., Chen, Y., Chen, Y., Liu, H., Yuan, G., Fan, Y., & Chen, K. (2013). Optimization of the microwave-assisted extraction of phlorotannins from *Saccharina japonica* Aresch and evaluation of the inhibitory effects of phlorotannin-containing extracts on HepG2 cancer cells. *Chin. J. Oceanol. Limn., 31*(5), 1045-1054. doi: 10.1007/s00343-013-2321-x

Hemmingson, J. A., Falshaw, R., Furneaux, R. H., & Thompson, K. (2006). Structure and Antiviral Activity of the Galactofucan Sulfates Extracted from Undaria Pinnatifida (Phaeophyta). *Journal of Applied Phycology, 18*(2), 185-193. doi: 10.1007/s10811-006-9096-9

Heo, S. J., Park, E. J., Lee, K. W., & Jeon, Y. J. (2005a). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technol., 96*(14), 1613-1623.

Heo, S. J., Park, P. J., Park, E. J., Kim, S. K., & Jeon, Y. J. (2005b). Antioxidant activity of enzymatic extracts from a brown seaweed *Ecklonia cava* by electron spin resonance spectrometry and comet assay. *Eur. Food Res. Technol., 221*(1-2), 41-47. doi: 10.1007/s00217-005-1187-3

Holtkamp, A. D., Kelly, S., Ulber, R., & Lang, S. (2009). Fucoidans and fucoidanases—focus on techniques for molecular structure elucidation and modification of marine polysaccharides. *Appl. Microbiol. Biot., 82*(1), 1-11. doi: 10.1007/s00253-008-1790-x

Huang, D., Boxin, O. U., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *J. Agr. Food Chem., 53*(6), 1841-1856.

Jin, W., Zhang, Q., Wang, J., & Zhang, W. (2013). A comparative study of the anticoagulant activities of eleven fucoidans. *Carbohy. Polym., 91*(1), 1-6. doi: http://dx.doi.org/10.1016/j.carbpol.2012.07.067

Keita, K., & Masanao, I. (2012). *Advanced Membrane Material from Marine Biological Polymer and Sensitive Molecular-Size Recognition for Promising Separation Technology*.

Khil'chenko, S. R., Zaporozhets, T. S., Shevchenko, N. M., Zvyagintseva, T. N., Vogel, U., Seeberger, P., & Lepenies, B. (2011). Immunostimulatory activity of fucoidan from the brown alga *Fucus evanescens*: Role of sulfates and acetates. *J. Carbohy. Chem., 30*(4-6), 291-305.

Kim, K. J., & Lee, B. Y. (2012). Fucoidan from the sporophyll of *Undaria pinnatifida* suppresses adipocyte differentiation by inhibition of inflammation-related cytokines in 3T3-L1 cells. *Nutr. Res., 32*(6), 439-447. doi: http://dx.doi.org/10.1016/j.nutres.2012.04.003

Kim, M. H., & Joo, H. G. (2008). Immunostimulatory effects of fucoidan on bone marrow-derived dendritic cells. *Immunol. Lett., 115*(2), 138-143.

Kim, M. Y., Varenne, A., Daniel, R., & Gareil, P. (2003). Capillary electrophoresis profiles of fucoidan and heparin fractions: Significance of mobility dispersity for their characterization. *J. Sep. Sci, 26*(12-13), 1154-1162.

Kim, S. H., Choi, D. S., Athukorala, Y., Jeon, Y. J., Senevirathne, M., & Rha, C. K. (2007). Antioxidant activity of sulfated polysaccharides isolated from *Sargassum fulvellum*. *J. Food Sci. Nutr., 12*(2), 65-73.

Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S., & Shimeno, H. (2003). Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochem. Pharmacol., 65*(2), 173-179.

Kwak, J. Y. (2014). Fucoidan as a Marine Anticancer Agent in Preclinical Development. *Mar. Drugs, 12*(2), 851-870.

Kylin, H. (1913). Biochemistry of sea algae. *Z. Physiol. Chem., 83*, 171-197.

Lee, J., Hou, M., Huang, H., Chang, F., Yeh, C., Tang, J., & Chang, H. (2013a). Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties. *Cancer Cell International*, 1-7.

Lee, J. B., Hayashi, K., Hashimoto, M., Nakano, T., & Hayashi, T. (2004). Novel antiviral fucoidan from sporophyll of *Undaria pinnatifida* (Mekabu). *Chem. Pharm. Bull., 52*(9), 1091-1094.

Lee, J. C., Hou, M. F., Huang, H. W., Chang, F. R., Yeh, C. C., Tang, J. Y., & Chang, H. W. (2013b). Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties. *Cancer Cell Int., 13*(1).

Lee, S. H., Ko, C. I., Ahn, G., You, S. G., Kim, J. S., Heu, M. S., . . . Jeon, Y. J. (2012). Molecular characteristics and anti-inflammatory activity of the fucoidan extracted from *Ecklonia cava*. *Carbohy. Polym., 89*(2), 599-606. doi: http://dx.doi.org/10.1016/j.carbpol.2012.03.056

Lee, Y. K., Lim, D., Lee, Y., & Park, Y. (2006). Variation in fucoidan contents and monosaccharide compositions of Korean *Undaria pinnatifida* (Harvey) *Suringar* (Phaeophyta). *Algea Inchon., 21*(1), 157.

Li, B., Lu, F., Wei, X., & Zhao, R. (2008). Fucoidan: Structure and bioactivity. *Molecules, 13*(8), 1671-1695.

Li, B., Wei, X. J., Sun, J. L., & Xu, S. Y. (2006). Structural investigation of a fucoidan containing a fucose-free core from the brown seaweed, *Hizikia fusiforme*. *Carbohyd. Res., 341*(9), 1135-1146. doi: http://dx.doi.org/10.1016/j.carres.2006.03.035

Lim, S. J., Wan Aida, W. M., Maskat, M. Y., Mamot, S., Ropien, J., & Mazita Mohd, D. (2014). Isolation and antioxidant capacity of fucoidan from selected Malaysian seaweeds. *Food Hydrocolloid.*(0). doi: http://dx.doi.org/10.1016/j.foodhyd.2014.03.007

Liu, F., Wang, J., Chang, A. K., Liu, B., Yang, L., Li, Q., . . . Zou, X. (2012). Fucoidan extract derived from *Undaria pinnatifida* inhibits angiogenesis by human umbilical vein endothelial cells. *Phytomedicine, 19*(8–9), 797-803. doi: http://dx.doi.org/10.1016/j.phymed.2012.03.015

Lorimer, J. P., Mason, T. J., Cuthbert, T. C., & Brookfield, E. A. (1995). Effect of ultrasound on the degradation of aqueous native dextran. *Ultrason. Sonochem., 2*(1), S55-S57.

Ly, B. M., Buu, N. Q., Nhut, N. D., Thinh, P. D., & Van, T. T. T. (2005). Studies on fucoidan and its production from Vietnamese brown seaweeds. *AJSTD, 22*(4), 371.

Mabeau, S., & Fleurence, J. (1993). Seaweed in food products: biochemical and nutritional aspects. *Trends Food Sci. Tech., 4*(4), 103-107. doi: http://dx.doi.org/10.1016/0924-2244(93)90091-N

Mabeau, S., Kloareg, B., & Joseleau, J. (1990). Fractionation and analysis of fucans from brown algae. *Phytochemistry, 29*(8), 2441-2445.

Mak, W., Hamid, N., Liu, T., Lu, J., & White, W. L. (2013). Fucoidan from New Zealand *Undaria pinnatifida*: Monthly variations and determination of antioxidant activities. *Carbohy. Polym., 95*(1), 606-614.

Marais, M.-F., & Joseleau, J.-P. (2001). A fucoidan fraction from *Ascophyllum nodosum*. *Carbohyd. Res., 336*(2), 155-159.

Marudhupandi, T., Kumar, T. T. A., Senthil, S. L., & Devi, K. N. (2014). In vitro Antioxidant Properties of Fucoidan Fractions From *Sargassum tenerrimum*. *Pak. J. Biol. Sci., 17*(3), 402-407.

Maruyama, H., & Yamamoto, I. (1984). *An antitumor fucoidan fraction from an edible brown seaweed, Laminaria religiosa.* Paper presented at the Eleventh International Seaweed Symposium.

Mayer, A., Rodríguez, A. D., Berlinck, R. G., & Hamann, M. T. (2009). Marine pharmacology in 2005–6: Marine compounds with anthelmintic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems, and other miscellaneous mechanisms of action. *BBA. General Subjects, 1790*(5), 283-308.

Men'shova, R. V., Lepeshkin, F. D., Ermakova, S. P., Pokrovskii, O. I., & Zvyagintseva, T. N. (2013). Effect of pretreatment conditions of brown algae by supercritical fluids on yield and structural characteristics of fucoidans. *Chem. Nat. Comp., 48*(6), 923-926. doi: 10.1007/s10600-013-0429-z

Min, S. K., Kwon, O. C., Lee, S. J., Park, K. H., & Kim, J. K. (2012). An Antithrombotic Fucoidan, Unlike Heparin, Does Not Prolong Bleeding Time in a Murine Arterial Thrombosis Model: A Comparative Study of *Undaria pinnatifida* sporophylls and Fucus vesiculosus. *Phytother. Res., 26*(5), 752-757. doi: 10.1002/ptr.3628

Morya, V. K., Kim, J., & Kim, E. K. (2012). Algal fucoidan: structural and size-dependent bioactivities and their perspectives. *Appl. Microbiol. Biot., 93*(1), 71-82. doi: 10.1007/s00253-011-3666-8

Nishino, T., Nishioka, C., Ura, H., & Nagumo, T. (1994). Isolation and partial characterization of a noval amino sugar-containing fucan sulfate from commercial *Fucus vesiculosus* fucoidan. *Carbohyd. Res., 255*, 213-224.

Nishino, T., Yokoyama, G., Dobashi, K., Fujihara, M., & Nagumo, T. (1989). Isolation, purification, and characterization of fucose-containing sulfated polysaccharides from the brown seaweed *Ecklonia kurome* and their blood-anticoagulant activities. *Carbohyd. Res., 186*(1), 119-129.

Park, H. Y., Han, M. H., Park, C., Jin, C. Y., Kim, G. Y., Choi, I. W., . . . Choi, Y. H. (2011). Anti-inflammatory effects of fucoidan through inhibition of NF-κB, MAPK and Akt activation in lipopolysaccharide-induced BV2 microglia cells. *Food Chem. Toxicol., 49*(8), 1745-1752. doi: http://dx.doi.org/10.1016/j.fct.2011.04.020

Parsons, M. J. (1994). Status of the introduced brown seaweed *Undaria* in New Zealand (D. o. Conservation, Trans.). Auckland, New Zealand.

Patankar, M. S., Oehninger, S., Barnett, T., Williams, R. L., & Clark, G. F. (1993). A revised structure for fucoidan may explain some of its biological activities. *J. Biol. Chem., 268*(29), 21770-21776.

Pereira, M. S., Mulloy, B., & Mourão, P. A. S. (1999). Structure and Anticoagulant Activity of Sulfated Fucans: Comparison between the Regular, Repetitive, and Linear Fucans from Echinoderms with the More Heterogeneous and Branched Polymers from Brown Algae. *J. Biol. Chem., 274*(12), 7656-7667.

Pielesz, A., Biniaś, W., & Paluch, J. (2011). Mild acid hydrolysis of fucoidan: Characterization by electrophoresis and FT-Raman spectroscopy. *Carbohy. Res., 346*(13), 1937-1944.

Ponce, N. M. A., Pujol, C. A., Damonte, E. B., Flores, M. L., & Stortz, C. A. (2003). Fucoidans from the brown seaweed *Adenocystis utricularis*: Extraction methods, antiviral activity and structural studies. *Carbohyd. Res., 338*(2), 153-165.

Qu, G., Liu, X., Wang, D., Yuan, Y., & Han, L. (2014). Isolation and characterization of fucoidans from five brown algae and evaluation of their antioxidant activity. *J. Ocean Uni. Chin., 13*(5), 851-856. doi: 10.1007/s11802-014-2260-y

Rabanal, M., Ponce, N. M. A., Navarro, D. A., Gómez, R. M., & Stortz, C. A. (2014). The system of fucoidans from the brown seaweed *Dictyota dichotoma*: Chemical analysis and antiviral activity. *Carbohy. Polym., 101*(0), 804-811. doi: http://dx.doi.org/10.1016/j.carbpol.2013.10.019

Rioux, L. E., Turgeon, S. L., & Beaulieu, M. (2007). Characterization of polysaccharides extracted from brown seaweeds. *Carbohyd. Polym., 69*(3), 530-537.

Rodriguez-Jasso, R. M., Mussatto, S., Pastrana, L., Aguilar, C. N., & Teixeira, J. A. (2014). Chemical composition and antioxidant activity of sulphated polysaccharides extracted from *Fucus vesiculosus* using different hydrothermal processes. *Chemical Papers, 68*(2), 203-209. doi: 10.2478/s11696-013-0430-9

Rodriguez-Jasso, R. M., Mussatto, S. I., Pastrana, L., Aguilar, C. N., & Teixeira, J. A. (2011). Microwave-assisted extraction of sulfated polysaccharides (fucoidan) from brown seaweed. *Carbohy. Polym., 86*(3), 1137-1144.

Rupérez, P., Ahrazem, O., & Leal, J. A. (2002). Potential Antioxidant Capacity of Sulfated Polysaccharides from the Edible Marine Brown Seaweed *Fucus vesiculosus*. *J. Agr. Food Chem., 50*(4), 840-845. doi: 10.1021/jf010908o

Sakai, T., Ishizuka, K., & Kato, I. (2003). Isolation and Characterization of a Fucoidan-Degrading Marine Bacterium. *Marine Biotechnology, 5*(5), 409-416. doi: 10.1007/s10126-002-0118-6

Sheng, J., Yu, F., Xin, Z., Zhao, L., Zhu, X., & Hu, Q. (2007). Preparation, identification and their antitumor activities in vitro of polysaccharides from Chlorella pyrenoidosa. *Food Chemistry, 105*(2), 533-539. doi: http://dx.doi.org/10.1016/j.foodchem.2007.04.018

Skriptsova, A. V., Shevchenko, N. M., Zvyagintseva, T. N., & Imbs, T. I. (2010). Monthly changes in the content and monosaccharide composition of fucoidan from *Undaria pinnatifida* (Laminariales, Phaeophyta). *J. Appl. Phycol., 22*(1), 79-86.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Chem., 150*(1), 76-85.

Song, J. F., Li, D. J., & Liu, C. Q. (2009). Response surface analysis of microwave-assisted extraction of polysaccharides from cultured *Cordyceps militaris*. *J. Chem. Technol. Biot., 84*(11), 1669-1673. doi: 10.1002/jctb.2227

Suppiramaniam, V., Vaithianathan, T., Manivannan, K., Dhanasekaran, M., Parameshwaran, K., & Bahr, B. A. (2006). Modulatory effects of dextran sulfate and fucoidan on binding and channel properties of AMPA receptors isolated from rat brain. *Synapse, 60*(6), 456-464. doi: 10.1002/syn.20319

Synytsya, A., Bleha, R., Synytsya, A., Pohl, R., Hayashi, K., Yoshinaga, K., . . . Hayashi, T. (2014). Mekabu fucoidan: Structural complexity and defensive effects against avian influenza A viruses. *Carbohy. Polym., 111*(0), 633-644. doi: http://dx.doi.org/10.1016/j.carbpol.2014.05.032

Synytsya, A., Kim, W. J., Kim, S. M., Pohl, R., Kvasnička, F., Čopíková, J., & Il Park, Y. (2010). Structure and antitumour activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida*. *Carbohyd. Polym., 81*(1), 41-48.

Teruya, T., Konishi, T., Uechi, S., Tamaki, H., & Tako, M. (2007). Anti-proliferative activity of oversulfated fucoidan from commercially cultured Cladosiphon okamuranus TOKIDA in U937 cells. *Int. J. Biol. Macromol., 41*(3), 221-226. doi: http://dx.doi.org/10.1016/j.ijbiomac.2007.02.010

Thanassi, N. M., & Nakada, H. I. (1967). Enzymic degradation of fucoidan by enzymes from the hepatopancreas of abalone, Haliotus species. *Archives of*

Biochemistry and Biophysics, 118(1), 172-177. doi: http://dx.doi.org/10.1016/0003-9861(67)90294-9

Thinh, P., Menshova, R., Ermakova, S., Anastyuk, S., Ly, B., & Zvyagintseva, T. (2013). Structural Characteristics and Anticancer Activity of Fucoidan from the Brown Alga *Sargassum mcclurei*. *Mar. Drugs, 11*(5), 1456-1476.

Trinchero, J., Ponce, N. M. A., Córdoba, O. L., Flores, M. L., Pampuro, S., Stortz, C. A., Turk, G. (2009). Antiretroviral activity of fucoidans extracted from the brown seaweed *Adenocystis utricularis*. *Phytother. Res., 23*(5), 707-712. doi: 10.1002/ptr.2723

Ushakova, N. A., Morozevich, G. E., Ustyuzhanina, N. E., Bilan, M. I., Usov, A. I., Nifantiev, N. E., & Preobrazhenskaya, M. E. (2009). Anticoagulant activity of fucoidans from brown algae. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 3*(1), 77-83. doi: 10.1134/S1990750809010119

Van Den Hoogen, B. M., Van Weeren, P. R., Lopes-Cardozo, M., Van Golde, L. M. G., Barneveld, A., & Van De Lest, C. H. A. (1998). A microtiter plate assay for the determination of uronic acids. *Anal. Biochem., 257*(2), 107-111.

Vishchuk, O. S., Ermakova, S. P., & Zvyagintseva, T. N. (2011). Sulfated polysaccharides from brown seaweeds *Saccharina japonica* and *Undaria pinnatifida*: Isolation, structural characteristics, and antitumor activity. *Carbohy. Res., 346*(17), 2769-2776.

Vishchuk, O. S., Ermakova, S. P., & Zvyagintseva, T. N. (2013). The fucoidans from brown algae of Far-Eastern seas: Anti-tumor activity and structure-function relationship. *Food Chem., 141*(2), 1211-1217.

Wang, H., Ooi, E. V., & Ang, P. O. J. (2007). Antiviral polysaccharides isolated from Hong Kong brown seaweed *Hydroclathrus clathratus*. *Science in China Series C : Life Sciences, 50*(5), 611-618. doi: http://dx.doi.org/10.1007/s11427-007-0086-1

Wang, J., Zhang, Q., Zhang, Z., Song, H., & Li, P. (2010). Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from *Laminaria japonica*. *Int. J. Biol. Macromol., 46*(1), 6-12.

Wang, P., Liu, Z., Liu, X., Teng, H., Zhang, C., Hou, L., & Zou, X. (2014a). Anti-Metastasis Effect of Fucoidan from *Undaria pinnatifida* Sporophylls in Mouse Hepatocarcinoma Hca-F Cells. *PLoS One, 9*(8).

Wang, Q., Song, Y., He, Y., Ren, D., Kow, F., Qiao, Z., Yu, X. (2014b). Structural characterisation of algae *Costaria costata* fucoidan and its effects on CCl4-induced liver injury. *Carbohy. Polym., 107*(0), 247-254. doi: http://dx.doi.org/10.1016/j.carbpol.2014.02.071

Wang, T., Jonsdottir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chem., 116*(1), 240-248.

White, L., Lu, J., & White, W. L. (2014a). Scoping assessment of the economic viability of harvesting *Undaria pinnatifida* from NZ mussel lines and potential uses of the collected material (pp. 76). Auckland: Institute for Applied Ecology New Zealand.

White, W. L., & Wilson, P. (2014b). World seaweed utilisation. *Seaweed Sustainability: Food and Non-Food Applications.*

Xu, M. S., Chen, S., Wang, W. Q., & Liu, S. Q. (2013). Employing bifunctional enzymes for enhanced extraction of bioactives from plants: Flavonoids as an example. *J. Agr. Food Chem., 61*(33), 7941-7948.

Yang, C., Chung, D., Shin, I. S., Lee, H., Kim, J., Lee, Y., & You, S. (2008). Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida*. *Int. J. Biol. Macromol., 43*(5), 433-437.

Yang, L., Wang, P., Wang, H., Li, Q., Teng, H., Liu, Z., Zou, X. (2013). Fucoidan derived from *Undaria pinnatifida* induces apoptosis in human hepatocellular carcinoma SMMC-7721 cells via the ROS-mediated mitochondrial pathway. *Mar. Drugs, 11*(6), 1961-1976.

Ye, H., Wang, K., Zhou, C., Liu, J., & Zeng, X. (2008). Purification, antitumor and antioxidant activities in vitro of polysaccharides from the brown seaweed *Sargassum pallidum*. *Food Chem., 111*(2), 428-432.

Yende, S. R., & Harle, U. N. (2013). Antidepressant-like Effect of *Sargassum ilicifolium* in Mice Model of Depression. *Ads. Phar. Toxicol., 14*(3), 7-13.

You, S., Yang, C., Lee, H., & Lee, B. Y. (2010). Molecular characteristics of partially hydrolyzed fucoidans from sporophyll of *Undaria pinnatifida* and their in vitro anticancer activity. *Food Chem., 119*(2), 554-559.

Yu, L., Ge, L., Xue, C., Chang, Y., Zhang, C., Xu, X., & Wang, Y. (2014a). Structural study of fucoidan from sea cucumber *Acaudina molpadioides*: A fucoidan containing novel tetrafucose repeating unit. *Food Chem., 142*(0), 197-200.

Yu, P., & Sun, H. (2014b). Purification of a fucoidan from kelp polysaccharide and its inhibitory kinetics for tyrosinase. *Carbohy. Polym., 99*(0), 278-283.

Yu, Q., Yan, J., Wang, S., Ji, L., Ding, K., Vella, C., Hu, Z. (2012). Antiangiogenic effects of GFP08, an agaran-type polysaccharide isolated from *Grateloupia filicina*. *Glycobiology, 22*(10), 1343-1352. doi: 10.1093/glycob/cws096

Zhang, J., Zhang, Q., Wang, J., Shi, X., & Zhang, Z. (2009). Analysis of the monosaccharide composition of fucoidan by precolumn derivation HPLC. *Chin. J. Oceanol. Limn., 27*(3), 578-582.

Zhang, Q., Li, N., Zhao, T., Qi, H., Xu, Z., & Li, Z. (2005). Fucoidan inhibits the development of proteinuria in active Heymann nephritis. *Phytother. Res., 19*(1), 50-53. doi: 10.1002/ptr.1623

Zhang, Z., Till, S., Jiang, C., Knappe, S., Reutterer, S., Scheiflinger, F., . . . Dockal, M. (2014). Structure-activity relationship of the pro-and anticoagulant effects of *Fucus vesiculosus* fucoidan. *Thromb. Haemost., 111*(3), 429-437.

Zhang, Z., Wang, F., Wang, X., Liu, X., Hou, Y., & Zhang, Q. (2010). Extraction of the polysaccharides from five algae and their potential antioxidant activity in vitro. *Carbohyd. Polym., 82*(1), 118-121. doi: http://dx.doi.org/10.1016/j.carbpol.2010.04.031

Zhao, X., Dong, S., Wang, J., Li, F., Chen, A., & Li, B. (2012). A comparative study of antithrombotic and antiplatelet activities of different fucoidans from *Laminaria japonica*. *Thromb. Res., 129*(6), 771-778. doi: http://dx.doi.org/10.1016/j.thromres.2011.07.041

Zou, Y., Qian, Z., Li, Y., Kim, M. M., Lee, S. H., & Kim, S. K. (2008). Antioxidant effects of phlorotannins isolated from *Ishige okamurae* in free radical mediated oxidative systems. *J. Agr. Food Chem., 56*(16), 7001-7009.